PULMONARY FIBROSIS: ONE MANIFESTATION, VARIOUS DISEASES

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PULMONARY FIBROSIS: ONE MANIFESTATION, VARIOUS DISEASES

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Editorial: Pulmonary fibrosis: One manifestation, various diseases

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KEYWORDS

pulmonary fibrosis (PF), idiopathic pulmonary fibrosis (IPF), interstitial lung disease (ILD), familial pulmonary fibrosis (FPF), autoimmune diseases

Editorial on the Research Topic
Pulmonary fibrosis: One manifestation, various diseases

This research topic collection entitled "**Pulmonary Fibrosis: one manifestation, various diseases**", involving authors from different countries, confirms that this disease is a hot topic (Confalonieri P et al.,2022, Orlandi M et al., 2022). There are over 200 different types of pulmonary fibrosis (PF), the most common is the idiopathic pulmonary fbrosis (IPF), called idiopathic because it has no known cause. Another rare form is familial PF, for which several studies reported correlation with few genes. An important group of PF are due to other diseases, for example, autoimmune diseases such as rheumatoid arthritis, systemic sclerosis or Sjogren's syndrome (Ruaro et al., 2022, Trombetta AC et al., 2017, Bernero Eet al., 2013). PF could correlate to viral infections (e.g. COVID-19), gastroesophageal reflux disease (GERD) (Baratella E et al, 2021, Ruaro et al., 2018), and the exposure to various materials (including naturally occurring such as bird or animal droppings, and occupational such as asbestos or silica). Furthermore, smoking, radiation treatments, and certain drugs can increase risk of developing PF. In the first article (Saketkoo et al.) of the collection, the authors evaluate the use of International Classification of Functioning, Disability, and Health (ICF) approved by World Health Organization (WHO) in patients affected by interstitial lung diseases (ILD). The results of the study supported the use of ICF in ILD, as ICF may help clinicians to collect data regarding the clinical status of their ILD patients.

The second article (Ma et al.) of the collection is an interesting and comprehensive review. The authors underlined the molecular mechanisms and pathogenic factors of IPF, which would be helpful in the diagnosis, development of new drugs and the improvement

of disease prognosis. In particular, the researchers underlined the novelties regarding multiple cell types, gene mutations, epigenetic and environmental factors.

The most important message reported in the third paper (Zhou et al.) is that the assessments by high-resolution computed tomography (HRCT) pattern and scores before transbronchial cryobiopsy (TBCB) were helpful for bronchoscopists to make a better patient selection and procedure planning. The authors also reported that the multivariate analysis supported radiological probable interstitial pneumonia (UIP) pattern as an independent risk factor for moderate bleeding.

The fourth article (Zhang et al.) is a case report. The authors performed a transbronchial cryobiopsy (TBCB) assisted by extracorporeal membrane oxygenation (ECMO) in a critical case of acute respiratory failure related to an organizing pneumonia (OP) pattern. In conclusion, the paper supported that when oxygenation cannot be maintained after endotracheal intubation and surgical lung biopsy is not feasible, TBCB supported by ECMO may be a good choice to obtain lung tissue for histopathological diagnosis in patients with acute lung injury of unknown etiology.

The fifth manuscript (Zhou et al.) is an interesting case report that evaluate the treatment by pirfenidone of PF secondary to ARDS-COVID-19. Over 96 weeks after pirfenidone, the score of the mMRC dyspnea scale, the 6 min walking test distance, total lung capacity, diffusion capacity for carbon monoxide and chest CT improved. In conclusion, this case demonstrated that pirfenidone might be a potential treatment option for the post-COVID-19 pulmonary fibrosis.

The sixth article (Wang et al.) is a retrospective study that evaluate 579 patients with fibrosing ILD, of which 227 (39%) met the criteria for progression. The authors observed that clubbing of fingers and a HRCT-documented UIP-like fibrotic pattern were more frequently associated with the progressive fibrosing.

The mortality was worse in patients with PF with hypoxemia, in those with baseline diffusion capacity of the lung for carbon monoxide (DLCO)% predicted <50%, or in those with UIP-like fibrotic pattern.

In the seventh paper (Ma et al.) the researchers provides an overview of different cytokines and growth factors involved in IPF.

The authors of the eighth article (Min et al.) demonstrated that lungs from mice with bleomycin (BLM)-induced PF were characterized by decreased expression of TNF receptor-associated factor 6 (TRAF6) in lung fibroblasts. Furthermore, the results indicate that reduced TRAF6 expression in fibroblasts is essential for the progression of PF, and therefore, genetically increasing TRAF6 expression or disrupting tribbles pseudokinase 3 (TRIB3)-TRAF6 interaction could be potential therapeutic strategies for fibroproliferative lung diseases.

In the ninth article (Xu et al.) the authors used human embryonic lung fibroblasts (HELFs) treated with different concentrations of vincristine (VCR) to study the molecular mechanism of VCR-induced PF and the possible involvement of the mitogen-activated protein kinase (MAPK) signaling pathway. In the conclusions, the researchers reported that VCR could promote the differentiation of fibroblasts into myofibroblasts by regulating the MAPK signal pathway.

In the penultimate article of the collection, the authors (Tanner et al.) used a series of *in vitro* and *in vivo* models to identify the therapeutic potential of bisphosphonate zoledronic acid (ZA) in the treatment of idiopathic pulmonary fibrosis (IPF). Furthermore, farnesyl diphosphate synthase (FDPS) was used as a potential antifibrotic target using a bleomycin mouse model. The results of the study reported that *in vitro* administration of ZA reduced myofibroblast transition and blocked NF-κB signaling in macrophages leading to impaired immune cell recruitment in a transwell assay. FDPS-targeting siRNA administration significantly attenuated profibrotic cytokine production and lung damage. In addition, ZA treatment of mice with bleomycin-induced lung damage displayed decreased cytokine levels in the BALF, plasma, and lung tissue, resulting in less histologically visible fibrotic scarring. Additionally, ZA polarized macrophages towards a less profibrotic phenotype contributing to decreased IPF pathogenesis.

The last research (Yu et al.) proved that catalpol (CAT) might work through Ang II/AT1/TGF-β/Smads pathway to improve lung pathological changes as well as suppress epithelial mesenchymal transition (EMT) in mice with PF. CAT may serve as a novel therapeutic candidate for the simultaneous blockade of Ang II and TGF-β pathway to attenuate PF.

In conclusion, this special issue pays particular attention to recently progress made on use of innovative tests and treatments, which is expected to provide new insights into research.

Author contributions

BR, MMC, FS and MH conducted the manuscript. MC and EB the final amendments and approved the final version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Vincristine Promotes Transdifferentiation of Fibroblasts Into Myofibroblasts *via* P38 and ERK Signal Pathways

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Background: Vincristine (VCR) is used in the clinic as an anti-tumor drug. VCR can cause pulmonary fibrosis (PF), leading to respiratory failure. The transformation of fibroblasts into myofibroblasts may play a key role in PF. The present study attempted to reveal the molecular mechanism of VCR-induced PF and the possible involvement of the mitogen-activated protein kinase (MAPK) signaling pathway.

Methods: Human embryonic lung fibroblasts (HELFs) were treated with different concentrations of VCR. Inhibitors of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAPK were added to HELFs. Cell proliferation state was assessed using cell counting kit-8 and by directly counting the number of cells. The expressions of vimentin and α-smooth muscle actin (α-SMA) were investigated using western blot and immunofluorescence analyses. Activation of ERK and P38 was estimated by the expression of phosphorylated p38 MAPK (p-p38), p38 MAPK, phosphorylated ERK1/2 (p-ERK1/2) and ERK1/2 using western blot analysis. Enzyme-linked immunosorbent assay was used to estimate the level of collagen I in cell culture supernatants.

Results: Results showed that VCR promoted cellular proliferation, secretion of collagen I and the expression of vimentin and α-SMA. High expression of p-p38 and p-ERK1/2 was associated with the activation of the MAPK signaling pathway. MAPK inhibitors SB203580 and PD98059 suppressed the expression of the above proteins.

Abbreviations: α-SMA, A-smooth muscle actin; CCK-8, Cell counting kit -8; DAPI, 4,6-diamidino-2-phenylindole; ECM, Extracellular matrix; ELISA, Enzyme-Linked Immunosorbent Assay; ERK1/2, Extracellular signal-regulated kinase 1/2; FB, Fibroblasts; FCS, Fetal calf serum; helfs, Human Embryonic Lung Fibroblasts; MAPK, Mitogen-activated protein kinase; MB, Myofibroblast; PBS, Phosphate-buffered saline; p-ERK1/2, Phosphorylated ERK1/2; PF, Pulmonary fibrosis; p-p38, Phosphorylated p38 MAPK; RIPA, Radioimmunoprecipitation assay; VCR, Vincristine.

Conclusion: Our study revealed that VCR could promote the differentiation of fibroblasts into myofibroblasts by regulating the MAPK signal pathway, which may be a promising way to treat VCR-induced PF.

Keywords: vincristine, pulmonary fibrosis, fibroblast, myofibroblast, MAPK

INTRODUCTION

Pulmonary fibrosis (PF) is a life-threatening pulmonary disease with multiple causes, including environmental factors (such as inhalation of particulates) (Biondini et al., 2020; Türkkan et al., 2021), various kinds of diseases ((such as the COVID-19 pandemic, Antisynthetase syndrome) (Baratella et al., 2021; Orlandi et al., 2021) and adverse effects of specific drugs (Wang et al., 2015; Wan et al., 2020; Weng et al., 2020). Some specific drugs, especially for carcinoma, can lead to PF, which is one of the major causes of death worldwide (Xu et al., 2021). Vincristine (VCR) is an alkaloid extracted from *Catharanthus roseus* of the Apocynaceae family. It is effective in treating acute lymphoblastic leukemia, Hodgkin's disease, lymphosarcoma and breast cancer (Okamoto et al., 2016; Yao et al., 2019). However, one of the most important adverse effects is PF. Although several studies have shown that VCR-induced PF may be associated with oxidative stress, inflammation or pulmonary epithelial cells, the underlying mechanism remains elusive (K. Zhang & Xu, 2020). Therefore, exploring the exact mechanism of VCR-induced PF will be of significant clinical importance.

Fibroblasts (FB) play a very essential role in the process of PF (H. Zhao et al., 2020). In pathological conditions, FBs are involved in the process of PF mainly through their abnormal proliferation and transformation and secretion of large amounts of extracellular matrix (ECM) (Chanda et al., 2019; Y.; Zhang et al., 2019). Moreover, several factors, especially the stimulation of a large number of cytokines, can cause FBs proliferation and transformation into myofibroblast (MB) during PF, leading to the secretion of a large amount of ECM. The MBs express α-smooth muscle actin (α-SMA), which is considered to be a crucial marker for MB (Xie et al., 2015; L.; Zhang et al., 2020).

Accumulating evidence has proved that MB differentiation is related to the pathogenesis of PF (Wei et al., 2019; Li N et al., 2020). MBs proliferate continuously, the survival time of single cells is greatly prolonged and a large number of collagen fibers are produced, which indicate MBs are important promoters of PF. Some studies showed that the mRNA expression of type I collagen increased in a-SMA positive cells, which proved that MBs were the key source of collagen gene expression. These pieces of evidence suggest that MBs are the primary cell types causing abnormal deposition of ECM. MBs can also accelerate the course of fibrosis by aggravating the epithelial injury and inflammatory reaction (Wei et al., 2019; Andugulapati et al., 2020).

Thus, inhibiting the transformation of lung FBs into MBs may be a promising strategy to treat VCR-induced PF. The signaling pathway of p38 and extracellular signal-regulated kinase (ERK) are considered to play an important role in cell proliferation and differentiation (Sun et al., 2015; Lavoie et al., 2020). Previous studies showed that p38 and ERK participated in the process of PF (Weng et al., 2019; Yu et al., 2019). Here, we attempted to reveal the molecular mechanism of VCR-induced PF and the potential effect of mitogen-activated protein kinase (MAPK) signaling pathway.

MATERIALS AND METHODS

Antibodies and Reagents

VCR injection was provided by Selleck Corporation (Beijing, China). Human embryonic lung fibroblasts (HELFs) were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Antibodies against vimentin, α-SMA, p-ERK, ERK, p-p38MAPK and p38MAPK were purchased from Cell Signaling Technologies (Boston, MASS, United States). Collagen I concentration was determined by the enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK). Cell counting kit -8 (CCK-8) was acquired from Abcam (Cambridge, UK). Antibodies to GAPDH were purchased from Proteintech (Wuhan, China).

Cell Culture and Treatment

HELFs were cultured in Eagles minimum essential medium (Corning Inc., Corning, NY, United States) supplemented with 10% fetal calf serum (FCS), streptomycin and penicillin (both 100 U/ml) under a 5% CO2-containing humidified atmosphere at 37°C. Cells were subcultured in T-flasks about every 5 days (Corning, United States). After washing twice with 10 ml of phosphate-buffered saline (PBS) and treating with trypsin (0.25%) and EDTA (0.02%) PBS, cells were removed from flasks after reaching 80–90% confluence. Cells were resuspended in a culture medium after incubating for 5 min at 37°C and seeded on a dish at a density of 4 × 106 cells/100-mm. HELFs were treated with different concentrations of VCR (0, 0.35, 0.7 and 1.4 μg/ml) for 24 h after overnight serum starvation.

Cell Proliferation Assay

HELFs (1 × 104 cells/well) were maintained in a complete culture medium in 96-well plates until they reached 60–70% confluence. Cells were incubated with CCK8 solution (10 μL) at 37°C for 3 h in the presence or absence of SB203580 or PD98059 (MedChemExpress, United States) after stimulation with VCR. The cell proliferation rate was then determined by measuring the absorbance at a wavelength of 450 nm using a microplate reader.

ELISA

As an indicator of PF, the amount of collagen I in cell culture supernatants was measured using commercial ELISA kits.

Supernatants were collected after centrifugation (2500 × g) at 4°C for 10 min. All of the samples were measured in triplicate, according to the manufacturer's instructions.

Western Blotting Analysis

Total cell protein was extracted by radioimmunoprecipitation assay (RIPA) buffer, and the protein concentration was detected using the Bicinchoninic Protein Assay kit (BCA, Beyotime Biotechnology, China). The cells were washed thrice in PBS and then re-suspended in cold RIPA buffer. The cell lysates were centrifuged at 12,000 × g at 4°C for 15 min, and supernatants were collected. Total proteins (5–10 μg) were exposed to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime Biotechnology, China) and then transferred to nitrocellulose membranes (Pall Corporation, United States). Next, the membranes were sealed for 2 h with 5% fat-free milk at room temperature, and then co-incubated overnight at 4°C with primary antibodies: anti-p-ERK1/2, anti-ERK1/2, anti-p-p38, anti-p38, anti-vimentin and anti-α-SMA. The membranes were washed and then incubated with secondary antibody goat anti-rabbit IgG at room temperature for 1 h. An anti-GAPDH antibody was used as the internal reference. Signals were detected using an enhanced chemiluminescence-detecting kit according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, United States). The band intensity was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, United States).

Immunofluorescence Analysis

Cells were fixed with 4% paraformaldehyde at room temperature for 20 min. Cells were then treated with 0.3% Triton X-100 for 6 min. Non-specific staining was prevented using 5% goat serum. Cells were incubated with combinations of primary antibodies: anti-vimentin or anti-α-SMA at 4°C overnight in a humidified chamber. Cells were washed thrice and then cultured with combinations of secondary antibodies for 1 h. After washing with PBS, cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The sections were cover-slipped and then examined using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

SPSS 19.0 statistical software (Armonk, New York: IBM Corp.) was used for data analysis. All data were presented as the mean ± SEM from at least three independent experiments. Comparisons between groups were performed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. $p < 0.05$ was considered statistically significant.

RESULTS

VCR Induces HELF Proliferation in a Concentration-Dependent Manner

As VCR was observed to be cytotoxic *in vitro* at high concentrations (J. E. Sun et al., 2016). The proliferation of HELFs treated with different concentrations of VCR (0, 0.35, 0.7 and 1.4 μg/ml) for 24 h was assessed using CCK-8 assay. The number of cells was also counted. Results showed that compared with the control group, VCR treatment significantly increased HELF proliferation in a concentration-dependent manner (F = 71.18, $p < 0.05$ and F = 62.98, $p < 0.05$ respectively) (**Figures 1A,C**). HELF proliferation was the most significant at a concentration of 1.4 μg/ml. Therefore, 1.4 μg/ml was used in subsequent experiments.

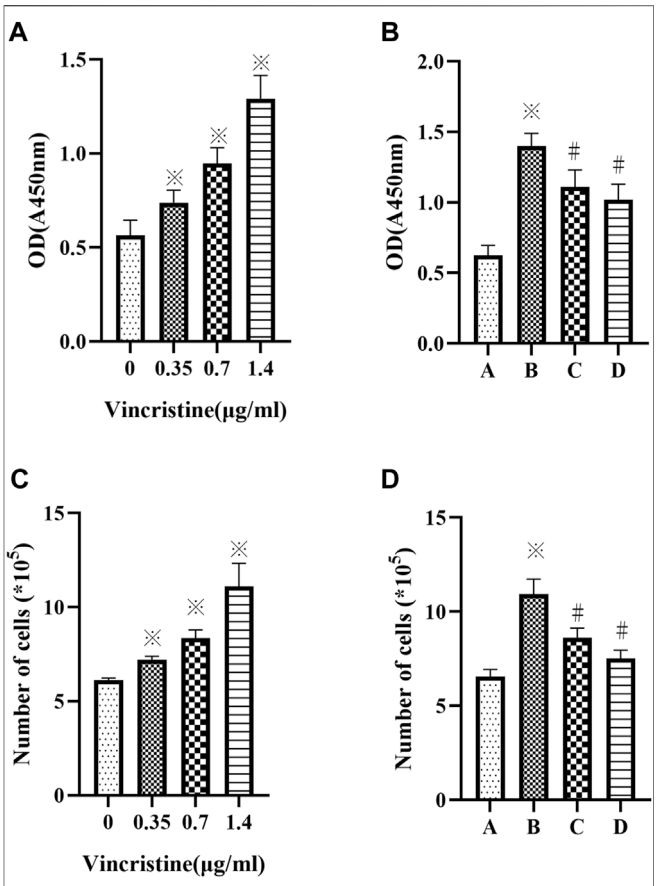

FIGURE 1 | (A,C): Vincristine (VCR) increases cell proliferation in HELFs. Cells were treated with different concentration of vincristine (0, 0.35, 0.7 and 1.4 μg/ml) for 24 h *$p < 0.05$ *vs.* the control group. **(B,D)**: VCR-induced cells proliferation was decreased by SB203580 and PD98059 in HELFs (cells were treated with 1.4 μg/ml VCR for 24 h). Cell proliferation was assessed by CCK8 assay. *$p < 0.05$ *vs*. the control group; #$p < 0.05$ *vs*. the VCR group. A: control group, B: VCR group, C: SB203580 group, D: PD98059 group.

HELFs were treated with VCR (1.4 μg/ml) for 24 h in the presence or absence of p38 MAPK inhibitor SB203580 or ERK1/2 inhibitor PD98059. It was found that HELF proliferation was markedly inhibited by SB203580 or PD98059. Similar results were obtained by counting the number of cells (F = 62.33, $p < 0.05$ and F = 70.35, $p < 0.05$, respectively) (**Figures 1B,D**).

VCR Induces Collagen I Secretion in a Concentration-Dependent Manner

Compared with the control group, treatment with VCR significantly increased the level of collagen I in a

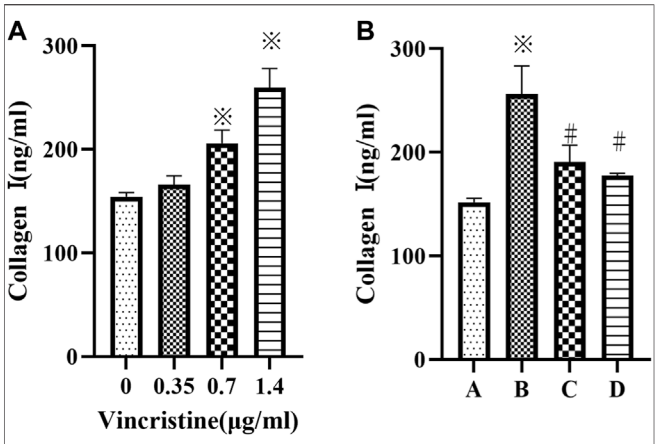

FIGURE 2 | (A): Cells were treated with different concentration of vincristine (0, 0.35, 0.7 and 1.4 μg/ml) for 24 h *$p < 0.05$ *vs*. the control group. **(B)**: VCR-induced collagen I production was decreased by SB203580 and PD98059 (cells were treated with 1.4 μg/ml VCR for 24 h). We measured the level of collagen I using ELISA assay. *$p < 0.05$ *vs*. the control group; #$p < 0.05$ *vs*. the VCR group. A: control group, B VCR group, C: SB203580 group, D: PD98059 group.

concentration-dependent manner (F = 92.98, $p < 0.05$) (**Figure 2A**). The most dramatic increase was detected at a concentration of 1.4 μg/ml. Furthermore, the level of collagen I decreased significantly in the presence of SB203580 or PD98059 incubation with 1.4 μg/ml VCR (F = 47.93, $p < 0.05$) (**Figure 2B**).

VCR Induces HELF Differentiation *via* p38 and ERK Signal Pathways

The expression of vimentin and α-SMA was detected using western blotting to confirm lung FB differentiation. The result showed that incubation of HELFs with different concentrations of VCR significantly increased the expression of vimentin and α-SMA in a dose-dependent manner. The most significant increase was detected at a concentration of 1.4 μg/ml. (F = 87.93, $p < 0.05$ and F = 78.05, $p < 0.05$, respectively) (**Figures 3A–C**).

In addition, the phosphorylation and total levels of p38 MAPK and ERK1/2 were detected using western blot analysis. The results revealed that compared with the control group, incubation with VCR significantly increased the amount of p-p38 MAPK, t-p38 MAPK, p-ERK1/2 and t-ERK1/2 in a dose-dependent manner. The most significant increase was detected at a concentration of 1.4 μg/ml. (F = 80.03, $p < 0.05$ and F = 78.20, $p < 0.05$, respectively) (**Figures 3A,D,E**). Collectively, these findings suggest that VCR induced MB differentiation *via* p38 MAPK and ERK signal pathways.

Inhibition of p38 MAPK and ERK Signaling Pathways Suppresses VCR-Induced PF

To further determine the mechanism of p38 MAPK and ERK signaling pathways in VCR-induced PF, SB203580 and PD98059 were used to inhibit p38 MAPK and ERK1/2 signaling pathways. Western blot and immunofluorescence assays were used to detect the level of α-SMA.

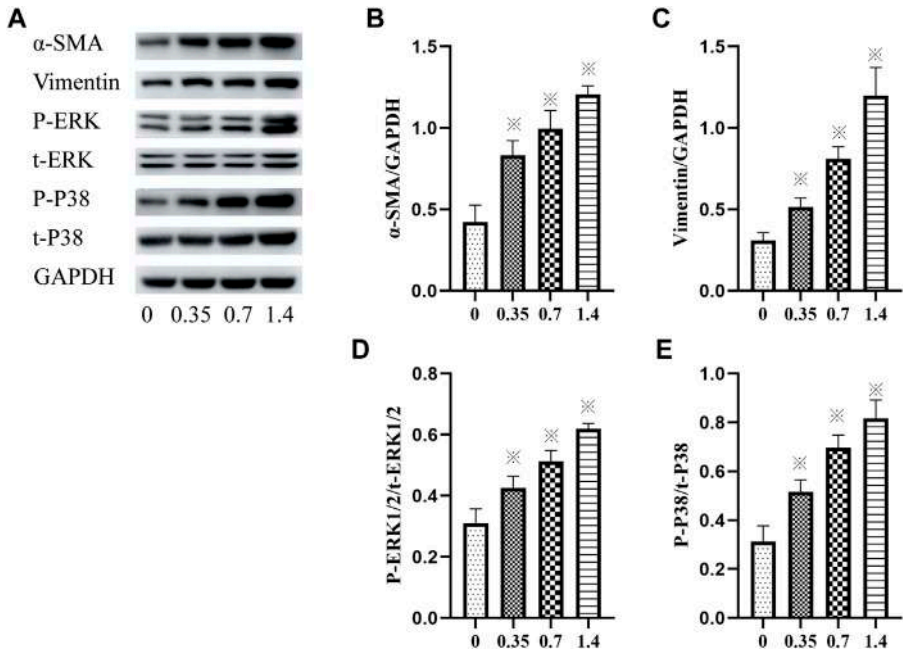

FIGURE 3 | (A–E): α-SMA, vimentin, p-ERK1/2, ERK1/2, p-p38 and p38 levels in HELFs were detected using Western blotting. Cells were incubated with different concentration of VCR (0, 0.35, 0.7 and 1.4 μg/ml) for 24 h. Data were presented as mean ± SEM from three independent experiments. *$p < 0.05$ *vs*. the control group. **(A–D)**: 0,0, 0.35,0.7 and 1.4 μg/ml VCR group.

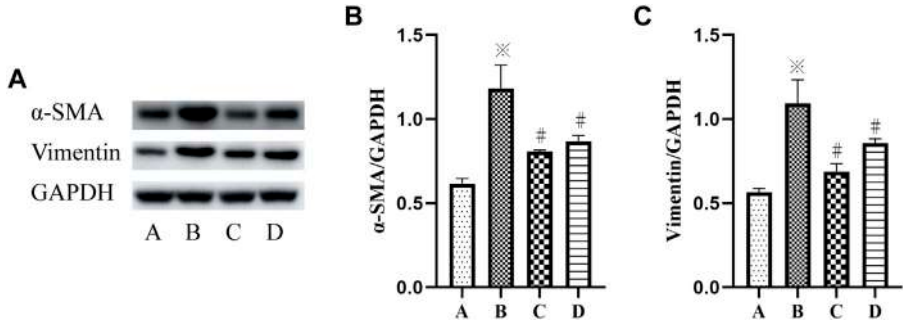

FIGURE 4 | (A–C): α-SMA and vimentin levels in HELFs were detected using Western blotting. Cells were which were stimulated with VCR (1.4 μg/ml) for 24 h in presence of SB203580 and PD98059. Data were presented as mean ± SEM from three independent experiments. *$p < 0.05$ *vs*. the control group; #$p < 0.05$ *vs*. the VCR group. A: control group, B: VCR group, C: SB203580 group, D: PD98059 group.

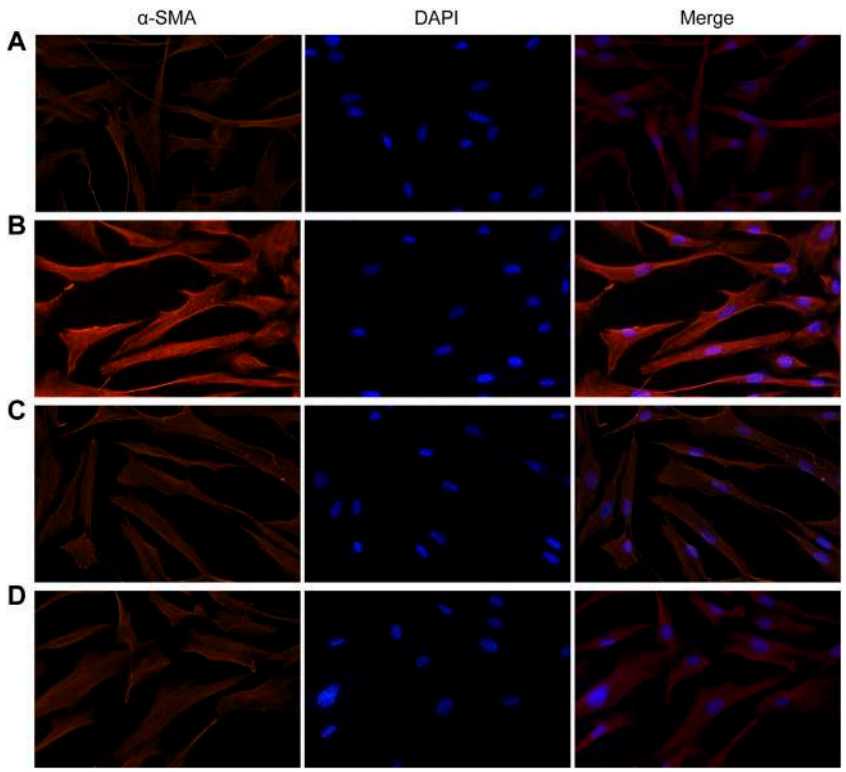

FIGURE 5 | Expression of α-SMA induced by VCR in HELFs (original magnification, ×400). HELFs were cultured with VCR (1.4 μg/ml) for 24 h. Immunocytochemical analysis showed that α-SMA expression was markedly increased in the VCR group compared with the control group, but was decreased in the two inhibitor groups. Red represents α-SMA; blue represents nuclei. **(A)**: control group, **(B)**: VCR group, **(C)**: SB203580 group, **(D)**: PD98059 group.

The results showed a significant increase in the protein expression of vimentin and α-SMA in the VCR group (1.4 μg/ml), and the protein expression level of vimentin and α-SMA were decreased after SB203580 and PD98059 treatment ($F = 55.95$, $p < 0.05$ and $F = 61.40$, $p < 0.05$) (**Figures 4A–C**).

Similarly, fluorescence analysis results showed that α-SMA expression was significantly increased in the VCR group (1.4 μg/ml) compared with the control group (**Figure 5**). α-SMA expression levels were dramatically reduced in groups pretreated with SB203580 and PD98059. Together, these results indicate that p38 MAPK and ERK are involved in the modulatory effects of VCR-induced PF.

DISCUSSION

While VCR is being widely in the clinic for the treatment of various conditions (such as breast cancer, bronchogenic

carcinoma, soft tissue sarcoma and neuroblastoma) (Chao et al., 2015), it may cause several adverse effects in the long term, for instance, lung toxicity, especially PF, which is life-threatening. Moreover, relatively little research has been conducted on molecular mechanisms of VCR-induced PF. Thus, revealing the underlying mechanism of VCR-induced lung injury may help to optimize the treatment strategy and reduce its adverse effect in clinical practice. Studies have shown that the proliferation of MBs is one of the main characteristic features of PF (Huang et al., 2017; J.; Li J et al., 2020). Our study shows that VCR could lead to the transformation of FBs into MBs, revealing the important role of FB proliferation in the pathogenesis of VCR-induced PF.

Pulmonary FBs are important effector cells in the occurrence and development of PF. HELFs have been widely used to study the transdifferentiation of fibroblasts into myofibroblasts and the mechanism of PF *in vitro* (Zhang et al., 2018; Gu et al., 2021). Therefore, we also regard HELFs as fibroblasts in this study. The expression of α-SMA is considered to be one of the markers of MBs and a phenotypic marker of FB differentiation (Shinde et al., 2017). In PF, there are a large number of FBs, mainly MBs, accumulating in lung connective tissues, which are speculated to be formed by the transformation of FBs in lung stroma (Pechkovsky et al., 2012; Lin et al., 2020). Dense bundles of α-SMA in MB cytoplasm have a contractile function and reduce lung compliance, which is closely related to its fibrogenic function. Vimentin is a member of the intermediate fiber family (Yang et al., 2021). It constitutes the main intermediate fiber in interstitial cells. Its primary function is to form the cell scaffold network with microtubule and microfilaments to maintain their integrity. It plays an important role in cell adhesion, migration, metastasis, proliferation and apoptosis. Vimentin is derived from MBs and endothelial cells. It is also a typical marker of mesenchymal cells. In the present study, we analyzed the specific protein levels of MBs. The expression of α-SMA and vimentin was assayed using western blot analysis. It was found that the protein expression of α-SMA and vimentin increased significantly after 12-h treatment with VCR. Hence, it was inferred that VCR promotes the development of PF by stimulating the transformation of FBs into MBs.

The mitogen-activated protein kinase (MAPK) is the most important growth regulatory protein. It is the key connection between the cytoplasm and nucleus. It influences the response of cells to changes in the external environment. It can regulate various inflammatory mediators and the expression of cytokines. It plays an important regulatory role in the physiological processes of cell growth, differentiation, development, neural function, immune function, etc. (P. Zhao et al., 2018). It is also reported to play a key role in MB differentiation and ECM deposition.

P38 MAPK, as an important member of the MAPK family, is mainly in the cytoplasm in the resting state. It can be phosphorylated and transferred to the nucleus after stimulation by hypoxia, ultraviolet and other factors. It plays a key role in the regulation of inflammatory response and wound healing by regulating the activity of transcription factors and the synthesis of cytokines (Deng et al., 2021).

The activation of the ERK signaling pathway is associated with many pro-fibrotic cytokines and kinases, such as transforming growth factor-β (TGF-β), platelet-derived growth factor, matrix metalloproteinases, etc. The expression of activated ERK1/2 protein was up-regulated in human or animal models of PF (Liu et al., 2021). The degree of PF was significantly reduced after the administration of inhibitors of ERK signaling pathway PD98059, indicating that this signal pathway is involved in the process of PF. Another study revealed that TGF-β-induced protein expression of human basic fibroblast growth factor (bFGF) could stimulate ERK phosphorylation, which can be inhibited by nonspecific inhibitors of the ERK signaling pathway PD98059. It showed that the ERK signaling pathway plays an important role in MB proliferation, apoptosis and type I collagen proliferation. Limiting the activity of the ERK signaling pathway can inhibit FB proliferation and reduce the production of ECM (Dong et al., 2017).

The current study showed that VCR induced increased protein and gene levels of p-p38 and p-ERK, and P38 and ERK signaling pathways were activated in the cell model of MBs. Our data showed that p38 MAPK and ERK are involved in the modulatory effects of VCR-induced PF.

In summary, for the first time, our study demonstrated that VCR could promote the transformation of FBs into MBs by inducing the phosphorylation of ERK and p38 MAPK. It means that the regulation of ERK and p38 MAPK may provide a target for clinical treatment of VCR-induced PF.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

CC and ZW were primarily responsible for making experiment plans and designed the experiments. HX and JY performed the experiments and took charge of paper writing. MT and JW analyzed the data and took charge of paper writing. MX and ZZ created figures. PZ and LW gave experimental technical support.

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TRAF6 Suppresses the Development of Pulmonary Fibrosis by Attenuating the Activation of Fibroblasts

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Pulmonary fibrosis (PF) has a high mortality rate, and its pathogenesis is unknown. TNF receptor-associated factor 6 (TRAF6), a signal transducer for inflammatory signaling, plays crucial roles in the pathogenesis of immune diseases. However, its function in PF remains unknown. Herein, we demonstrated that lungs from mice with bleomycin (BLM)-induced PF were characterized by decreased expression of TRAF6 in lung fibroblasts. Enhancing TRAF6 expression protected mice from BLM-induced PF coupled with a significant reduction in fibroblast differentiation. Furthermore, we demonstrated that overexpression of TRAF6 reversed the activation of myofibroblasts from PF mice by reducing the expression of Wnt3a and subsequently suppressing Wnt/β-catenin signaling. Additionally, the abundance of Tribbles pseudokinase 3 (TRIB3), a stress sensor, was negatively correlated with the abundance of TRAF6 in lung fibroblasts. TRIB3 overexpression decreased TRAF6 abundance by reducing TRAF6 stability in lung fibroblasts during PF. Mechanistic studies revealed that TRIB3 bound to TRAF6 and accelerated basal TRAF6 ubiquitination and degradation. Collectively, our data indicate that reduced TRAF6 expression in fibroblasts is essential for the progression of PF, and therefore, genetically increasing TRAF6 expression or disrupting the TRIB3-TRAF6 interaction could be potential therapeutic strategies for fibroproliferative lung diseases in clinical settings.

Keywords: pulmonary fibrosis, TRAF6, lung fibroblast, TRIB3, ubiquitination, protein degradation, Wnt3a

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INTRODUCTION

Pulmonary fibrosis (PF) is a type of chronic, progressive, and irreversible fibrotic lung disease characterized by an excess of extracellular matrix (ECM), destruction of normal lung architecture, and ultimately, respiratory failure (Sgalla et al., 2019; George et al., 2020). To date, only two therapies, pirfenidone and nintedanib, have been approved for use in PF. However, neither treatment is known to be curative. Lung transplantation remains the final and life-saving therapeutic option for PF. The lack of a successful treatment of PF is due to a lack of understanding of the underlying cellular and molecular mechanisms. Therefore, an in-depth investigation of the molecular pathogenesis of PF is urgently needed.

Although the cause of PF remains unclear, existing evidence suggests that lung fibroblasts play a crucial role in the pathogenesis of PF (Hinz and Lagares, 2020; Liu G. et al., 2021). Upon stimulation of fibrotic factors, such as chemokine (C-C motif) ligand 1 (CCL1), transforming growth factor-β1 (TGF-β1), and platelet-derived growth factor (PDGF), fibroblasts differentiate into myofibroblasts, which secrete excessive amounts of ECM proteins, including type I collagen, and fibronectin (Lo Re et al., 2011; Frangogiannis, 2020; Liu S. S. et al., 2021). Increased accumulation of ECM obliterates functional alveolar units, thereby decreasing gas exchange and reducing lung function. It is critically important to identify the key mediators contributing to the activation of myofibroblasts during PF development.

TRAF6 (TNF receptor-associated factor 6), a member of the TRAF protein family, plays a pivotal role in immune signal transduction pathways triggered by members of the TNF receptor superfamily and the IL-1 receptor superfamily (Cao et al., 1996; Walsh et al., 2015). By acting as a ubiquitin ligase (E3) catalyzing the formation of Lys63-linked polyubiquitin on itself and its substrates, TRAF6 participates in many biological processes, such as the operation of the innate immune system, lymph node organogenesis, and osteoclast formation (Naito et al., 1999; Wu and Arron, 2003; Yang et al., 2009). TRAF6 contains a RING-finger domain in the N-terminus, which is responsible for its E3 ligase activity, followed by several zinc-finger domains and a conserved C-terminal TRAF domain that enables interaction with receptors and adaptor proteins. Recent studies have revealed the role of

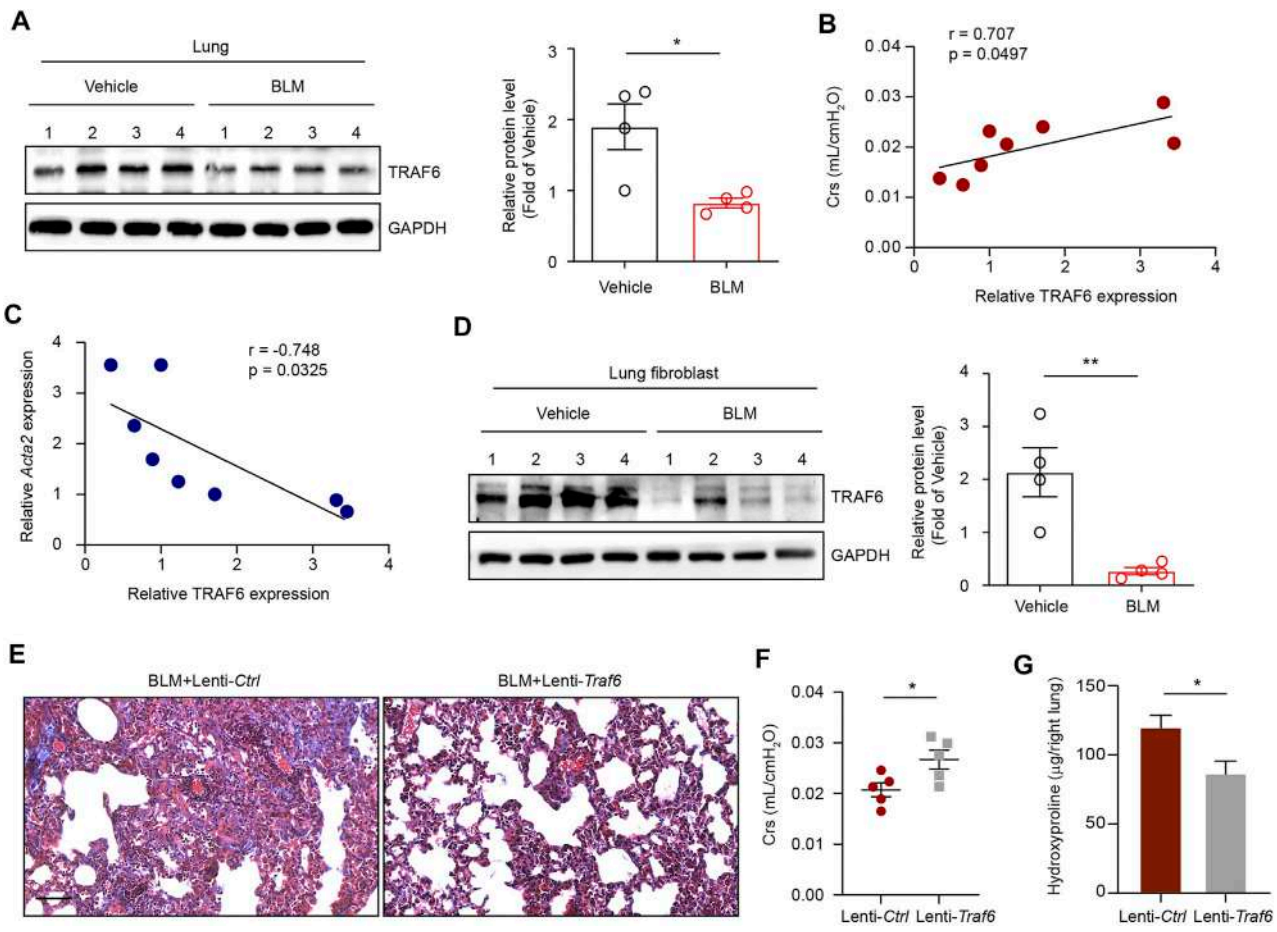

FIGURE 1 | TRAF6 expression in lung fibroblasts is negatively correlated with PF progression. **(A)** Western blot analysis of TRAF6 expression in the lungs of mice following BLM induction. **(B)** Correlation analysis between TRAF6 expression in the lungs of PF mice and respiratory system compliance (Crs) in mice. Each point represents the value of one mouse. Spearman's rank correlation test was employed to determine statistical significance. **(C)** Correlation analysis between TRAF6 expression and *Acta2* expression in the lungs of PF mice. Each point represents the value of one mouse. Spearman's rank correlation test was employed to determine statistical significance. **(D)** Western blot analysis of TRAF6 expression in lung fibroblasts isolated from the lungs of mice following BLM induction. **(E)** Masson staining was performed to evaluate fibrotic changes in the indicated mice (Group 1: BLM + Lenti-Ctrl, Group 2: BLM + Lenti-Traf6; n = 5 per group). Scale bars, 100 μm. Crs **(F)** and hydroxyproline content in the right lung **(G)** were assessed to evaluate pulmonary fibrosis and lung function (Group 1: BLM + Lenti-Ctrl, Group 2: BLM + Lenti-Traf6; *n* = 5 per group). Data are representative of 3 independent experiments. Data represent means ± SEM Statistical significance: $^{*}p < 0.05$ or $^{**}p < 0.01$.

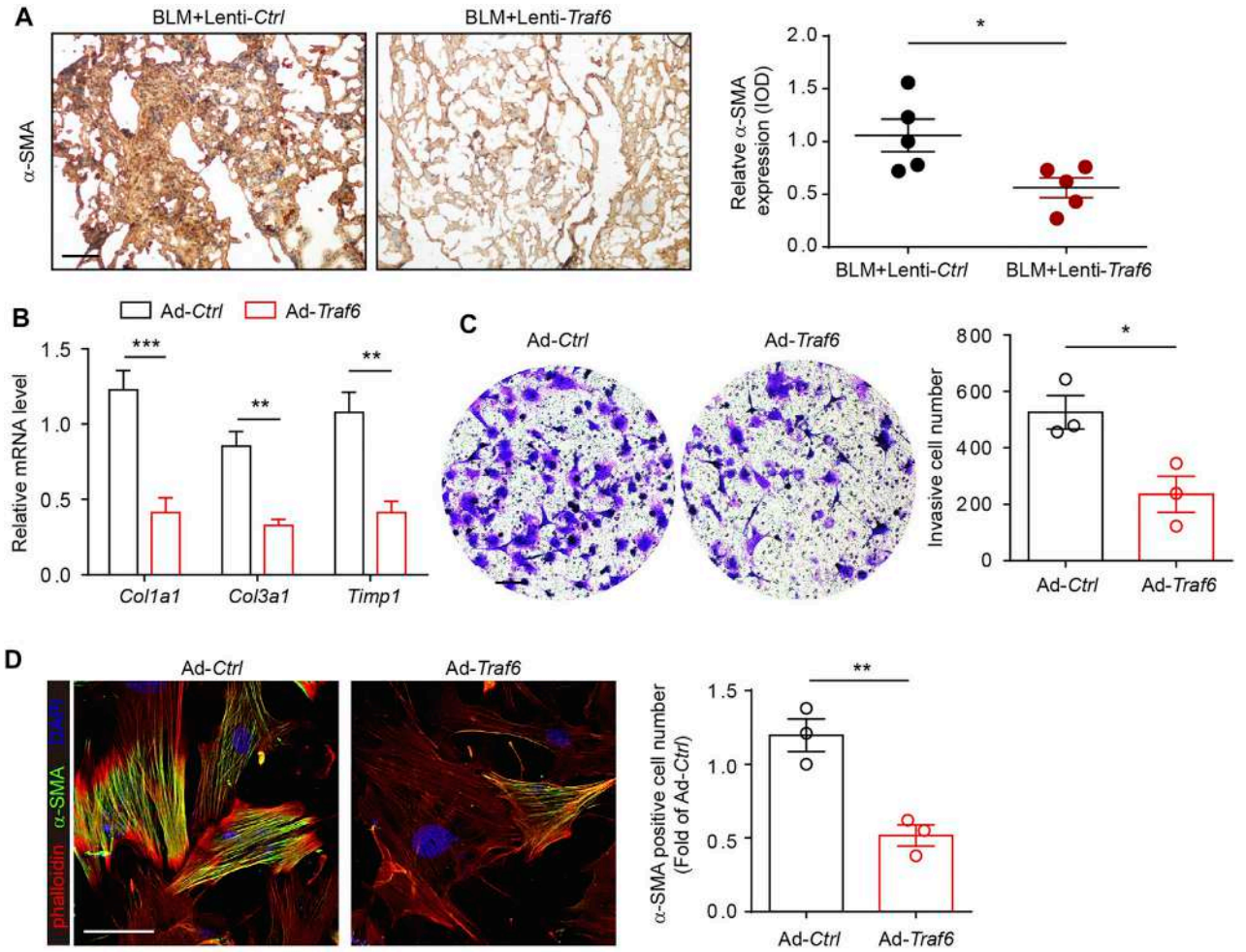

FIGURE 2 | Overexpression of TRAF6 suppresses fibroblast activation. **(A)** IHC staining showing the expression of α-SMA in lung tissues. Representative data and quantified results are shown (Group 1: BLM + Lenti-*Ctrl*, Group 2: BLM + Lenti-*Traf6*; *n* = 5 per group). Scale bar, 100 μm. **(B)** mRNA expression of fibrosis genes in mouse PF lung fibroblasts with or without TRAF6 overexpression. **(C)** Representative images and quantification of the invasion experiment results for mouse PF lung fibroblasts with or without TRAF6 overexpression. Scale bars, 100 μm. **(D)** Representative images and quantification of α-SMA immunostaining in mouse PF fibroblasts with or without TRAF6 overexpression. Scale bars, 50 μm. Data are representative of 3 independent experiments. Data represent means ± SEM Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$.

TRAF6 in fibrosis (Wang et al., 2020; Zhang et al., 2021). TRAF6 overexpression in hepatocytes promotes ubiquitination-dependent activation of ASK1, contributing to the pathogenesis of hepatic fibrosis during the progression of nonalcoholic steatohepatitis (NASH) (Wang et al., 2020). However, it remains unclear whether TRAF6 plays any important roles in the pathogenesis of PF.

In the present study, we observed reduced protein levels of TRAF6 in the lungs of PF mice, supporting a role for TRAF6 in PF development. Using the classical bleomycin (BLM)-induced PF mouse model, we found that overexpression of TRAF6 reduced BLM-induced PF changes. Using gain- and loss-of-function approaches *in vitro*, we demonstrated that the downregulation of TRAF6 expression is induced by the upregulation of expression of TRIB3 in lung fibroblasts during chronic lung injury. Furthermore, the loss of TRAF6 aggravated the progression of PF by acting as a novel negative regulator of Wnt3a, thereby activating lung fibroblasts into myofibroblasts. Collectively, our findings revealed a critical role for TRAF6 in suppressing the activation of lung fibroblasts and preventing the development of PF.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies against TRAF6 and TRIB3 were purchased from Abcam (Cambridge, MA, United States); antibodies against Axin 2, c-Myc, and cyclin D1 were obtained from Cell Signaling Technology (Danvers, MA, United States); an antibody against α-SMA was purchased from BOSTER (Wuhan, China); and antibodies against DDK, Myc and HA were obtained from MBL BIOTECH (Beijing, China). CoraLite® 594-phalloidin was obtained from Proteintech

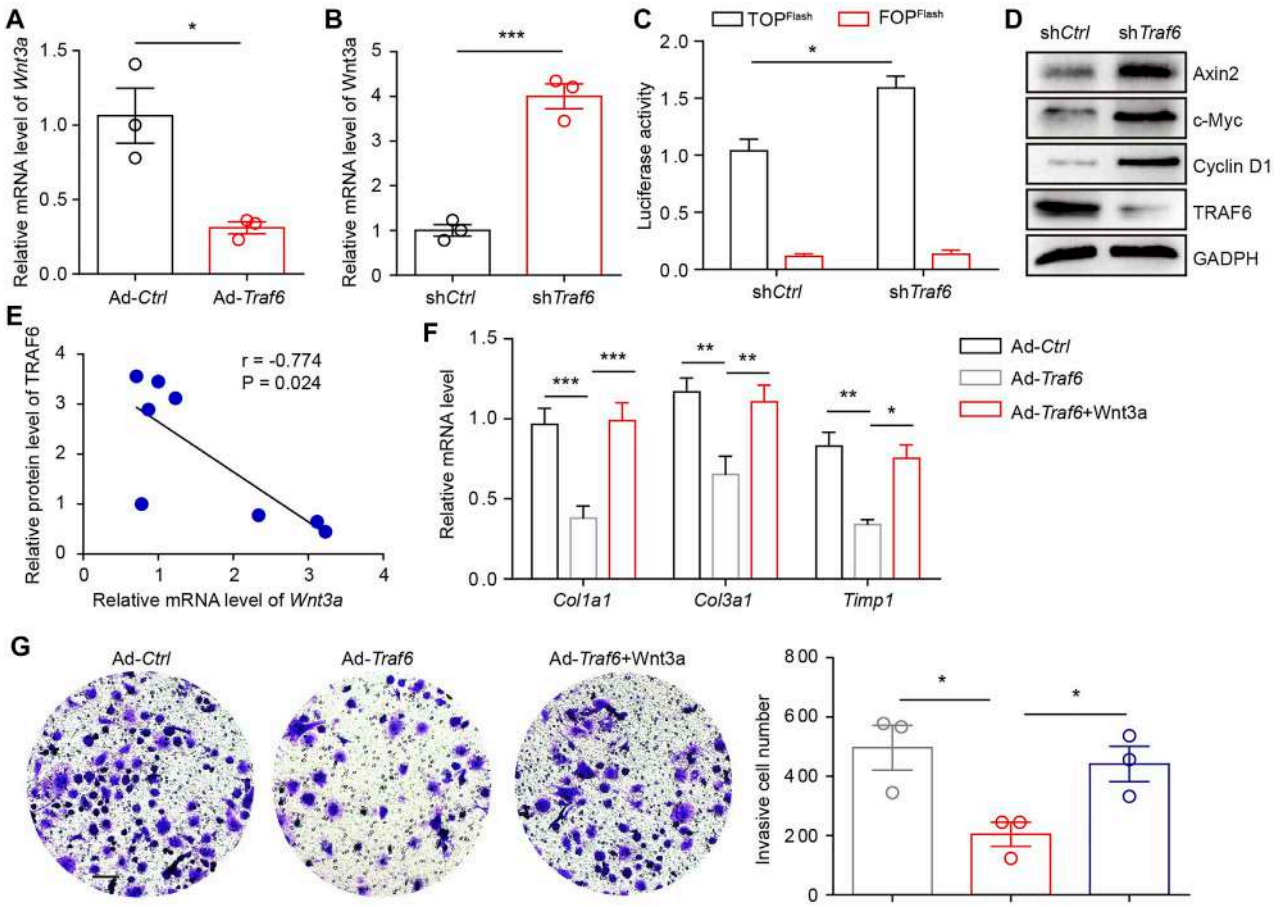

FIGURE 3 | TRAF6 suppresses fibroblast activation by reducing Wnt3a expression. **(A)** mRNA levels of *Wnt3a* as measured by qRT–PCR in TRAF6-overexpressing lung fibroblasts. **(B)** mRNA levels of *Wnt3a* as measured by qRT–PCR in TRAF6-depleted lung fibroblasts. **(C)** Wnt-reporter luciferase activity in PF lung fibroblasts with or without TRAF6 depletion. **(D)** Western blot analysis of β-catenin target gene expression in PF lung fibroblasts with or without TRAF6 depletion. **(E)** Correlation analysis of TRAF6 protein expression and *Wnt3a* mRNA levels in mouse PF lung fibroblasts. **(F)** qRT–PCR analysis of the expression of fibrosis genes in TRAF6-overexpressing mouse lung fibroblasts with or without Wnt3a stimulation. **(G)** Transwell assay for the evaluation of the invasion of TRAF6-overexpressing lung fibroblasts with or without Wnt3a stimulation. Scale bars, 100 μm. Data are representative of 3 independent experiments. Data represent means ± SEM Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$.

(Wuhan, China). CHX was purchased from Sigma (St Louis, MO, United States). Bleomycin (BLM), MG132, and 3-Methyladenine (3-MA) were obtained from MCE (Shanghai, China).

Bleomycin (BLM) Induction of Pulmonary Fibrosis

C57BL/6J male mice (8 weeks old) were purchased from SJA Laboratory Animal Co., Ltd. (Hunan, China). The mouse PF model was induced as previously reported (Liu et al., 2019). In brief, mice were anesthetized with 400 mg/kg avertin (Sigma–Aldrich) via intraperitoneal injection and then treated with 1 mg/kg BLM in 50 μl PBS via intratracheal injection. This was conducted 6 times, with 14 days between each challenge. Mice were sacrificed by excessive anesthesia at Day 40 after the last BLM challenge, and then lungs were obtained for immunoblotting and RT–PCR analysis.

Lentiviral Treatment in Vivo

For lentivirus administration, lentiviruses (5×10^7 I.U.) overexpressing *Traf6* in 50 μl of PBS were administered to mice via intratracheal instillation for a total of two treatments at 2-weeks intervals beginning on Day 10 after the last BLM administration.

Isolation of Lung Fibroblasts

Lung fibroblasts were obtained from mice as reported previously (Liu S. S. et al., 2021). The chests of mice sacrificed by excessive anesthesia were cut open, and the lungs were removed, rinsed with sterile PBS and cut into 1 mm^3 pieces in culture medium. After centrifugation at 1,500 rpm for 10 min, the tissue suspension was suspended in DMEM containing 15% FBS and spread evenly in a 10-cm dish. After 5–7 days of culture, the adherent fibroblasts were harvested for passage or for assays.

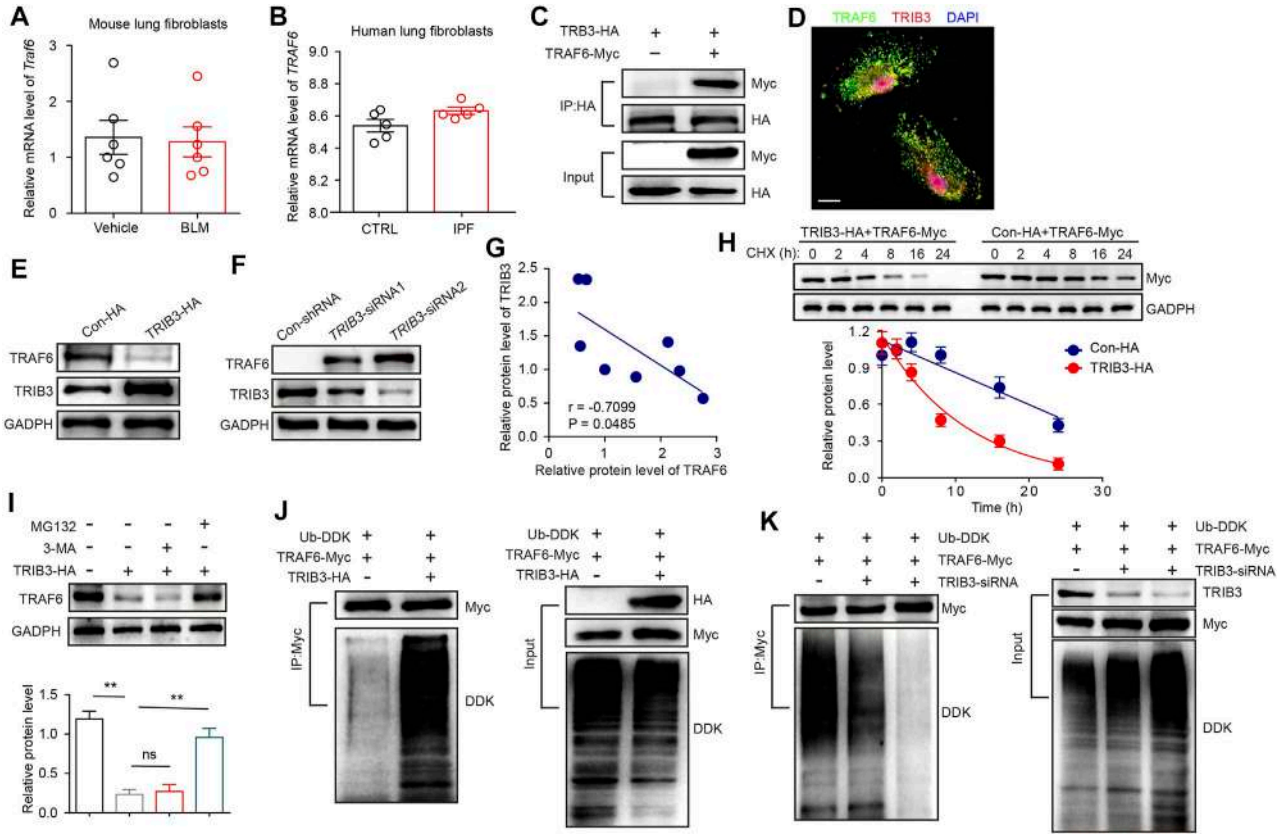

FIGURE 4 | TRIB3 decreases the expression of TRAF6 by promoting its degradation. **(A)** mRNA levels of *Traf6* as measured by qRT–PCR in lung fibroblasts from vehicle- and BLM-challenged mice. **(B)** *TRAF6* mRNA expression in lung fibroblasts of IPF patients compared with normal controls based on the reanalysis of a published dataset (GSE129164). **(C)** The interaction between TRAF6 and TRIB3 was evaluated by co-IP assays. HEK293T cell extracts were subjected to IP with an HA antibody and blotted with a Myc antibody. **(D)** Colocalization of TRAF6 and TRIB3 was evaluated in primary lung fibroblasts. Scale bars, 10 μm. **(E)** Sample immunoblot analyses of the expression of TRAF6 after TRIB3 overexpression in MRC5 cells. **(F)** Sample immunoblot analyses of the expression of TRAF6 after TRIB3 silencing in MRC5 cells. **(G)** Correlation analysis of TRAF6 protein expression and TRIB3 protein expression in mouse PF lung fibroblasts. **(H)** TRAF6 degradation in HEK293T cells with or without TRIB3 overexpression was determined by western blotting. **(I)** Analysis of the TRAF6 degradation pathway. Lung fibroblasts were incubated with CHX (20 μM), CHX plus 3-Methyladenine (3-MA, 5 mM), or MG132 (10 μM) for the indicated times. TRAF6 expression was detected by immunoblotting. **(J)** Immunoblots of ubiquitinated TRAF6 after TRIB3 overexpression. **(K)** Immunoblots of ubiquitinated TRAF6 after TRIB3 deletion. Data are representative of 3 independent experiments. Data represent means ± SEM Statistical significance: $^{**}p < 0.01$.

Cell Lines

MRC5 fibroblasts were cultured in minimal essential medium supplemented with 10% FCS, penicillin, streptomycin, and non-essential amino acid (NEAA) at 37°C in a humidified 5% CO_2 incubator.

Cell Transfection

Plasmids were transfected into cells with Lipofectamine 3,000 (Invitrogen) according to the manufacturer's instructions. siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Invasion Assays

Fibroblasts were cultured in serum-free DMEM for 24 h prior to cell invasion assays. Transwell invasion assays were performed using Transwell chambers with filter membranes of 8 μm pore size (Corning). The lower surface of the chambers was precoated with 10 μg ml^{-1} fibronectin, and the upper chambers were coated with BD Matrigel™ Basement Membrane Matrix (40 μl per well). Then, the chambers were inserted into 24-well culture plates. Single-cell suspensions were seeded into the upper chamber (1×10^4 cells per well in 0.4% FBS in DMEM), and the bottom chamber contained DMEM with 10% FBS. After 12 h, the medium was removed, and noninvasive cells were removed using cotton swabs. The invaded cells were fixed with 4% paraformaldehyde in PBS and stained with crystal violet staining solution. Invasive cells from 3 nonoverlapping fields of each membrane were imaged and counted using brightfield microscopy under 40x magnification.

RNA Extraction and Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells and tissues using the Eastep® Super Total RNA Extraction Kit (Promega) according to the manufacturer's instructions. RNA was quantified using a

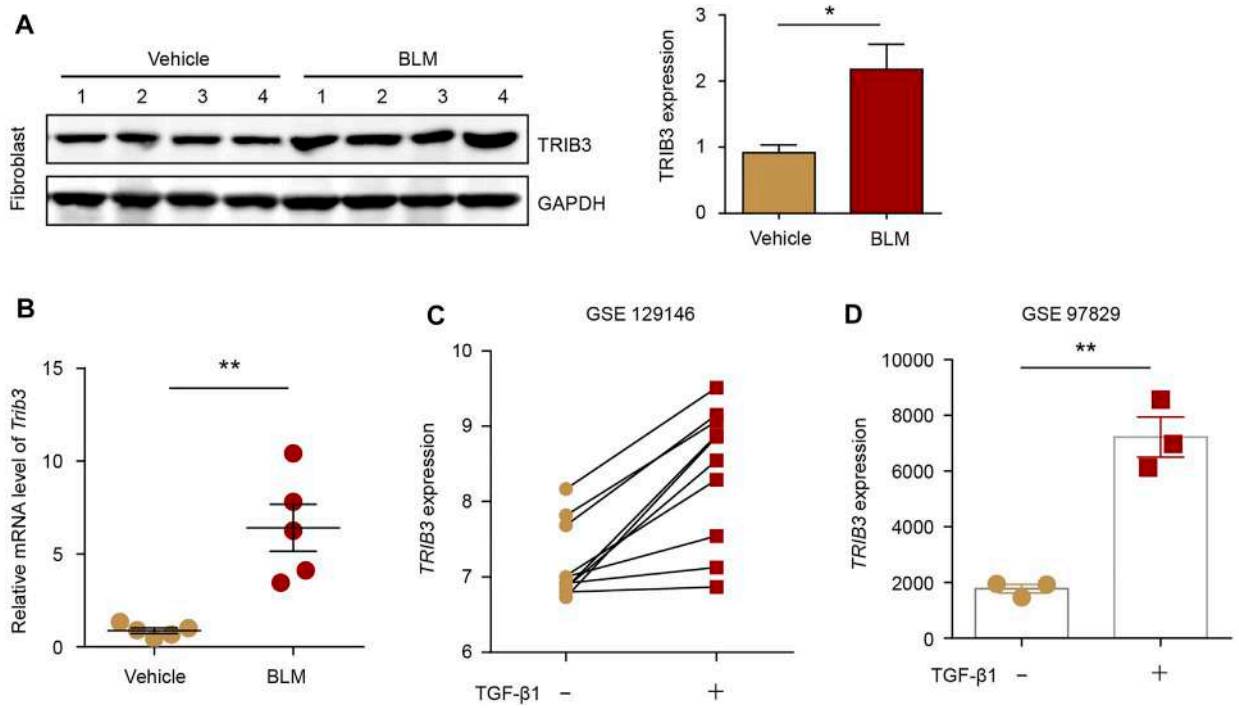

FIGURE 5 | TRIB3 expression is increased in PF lung fibroblasts. **(A)** Western blot analysis of TRIB3 expression in lung fibroblasts isolated from the lungs of mice following BLM induction. **(B)** mRNA levels of *Trib3* as measured by qRT–PCR in lung fibroblasts from vehicle- and BLM-challenged mice. **(C)** *TRIB3* mRNA expression in lung fibroblasts of IPF patients and normal controls treated with or without TGF-β1 based on the reanalysis of a published dataset (GSE129164). **(D)** *TRIB3* mRNA expression in lung fibroblasts treated with or without TGF- β1 based on the reanalysis of a published dataset (GSE97829). Data are representative of 3 independent experiments. Data represent means ± SEM Statistical significance: $^{*}p < 0.05$ or $^{**}p < 0.01$.

NanoDrop spectrophotometer. Reverse transcription was carried out with 1 mg of purified RNA using M-MLV reverse transcriptase (Transgen Biotech). Quantitative real-time PCR (qRT–PCR) was performed using the KAPA SYBR FAST RT–PCR Master Mix (2×) Kit (Kappa Biosystem, United States) and conducted by a LineGene 9,620 apparatus (Bioer). All primer details are listed in **Supplementary Table S1**.

Immunoblot

Tissues and cultured cells were lysed in RIPA buffer (Beyotime) containing protein inhibitors (Selleck). The protein concentration was determined by a BCA protein assay (Applygen Technologies Inc.). Proteins were mixed with 5× SDS sample loading buffer and incubated at 95°C for 10 min. Then, proteins were separated by SDS–PAGE gel, transferred to PVDF membranes (Millipore), and incubated with appropriate primary antibodies coupled with a horseradish peroxidase-conjugated secondary antibody. Images were visualized using ECL western blotting detection reagents (Tanon).

Coimmunoprecipitation (Co-IP)

Cells were washed twice in cold PBS and lysed in IP buffer (150 mmol/L NaCl, 25 mmol/L Tris-HCl (pH 7.4), 2.5 mmol/L $MgCl_2$, 0.5% NP-40, 0.5 mmol/L EDTA, and 5% glycerol) with protease inhibitors. Cell extracts were incubated with the indicated primary antibodies overnight at 4°C and then with Protein A/G Plus-Agarose (Santa Cruz, United States) at 4°C for 2 h. The beads were washed five times with wash buffer (IP buffer without NP-40), eluted in 2× SDS sample loading buffer, and then subjected to electrophoresis.

Immunostaining

For α-SMA immunostaining, cells cultured on coverslips were fixed with 4% (v/v) formaldehyde at room temperature for 10 min, permeabilized with 0.5% Triton X-100 at room temperature for 20 min, and blocked with 3% BSA at room temperature for 30 min. The slides were incubated with antibodies against α-SMA at 4°C overnight, followed by staining with Alexa Fluor 488-conjugated anti-mouse antibodies and CoraLite® 594-phalloidin for 2 h at room temperature. Finally, the slides were mounted with medium containing DAPI.

Luciferase Reporter Assay

For the luciferase reporter assay, MRC5 cells were cotransfected with TOP Flash or FOP Flash luciferase reporter. At 48 h post-transfection, the cells were collected and lysed with lysis buffer. The internal control used was pTK-Renilla. Firefly luciferase and Renilla luciferase activities were quantified using the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega, Madison, WI).

Statistical Analysis

Statistical significance was calculated using GraphPad Prism 6 software. Data are representative and/or the mean ± SEM of 3 assays. An unpaired two-sided Student's t test was used to compare two groups, and one-way ANOVA was used to compare multiple groups unless otherwise indicated. Correlations between groups were determined by Pearson's correlation test. All experiments were conducted with at least 3 biological replicates. In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

TRAF6 Expression Was Downregulated in Lung Fibroblasts From PF Mice and TRAF6 Overexpression Protected Mice Against BLM-Induced PF

To address the role of TRAF6 in PF, we first examined TRAF6 expression in the lungs of mice following BLM-induced PF. Western blot analysis showed decreased TRAF6 protein levels in the lungs of PF mice compared to those of controls (**Figure 1A**). Moreover,

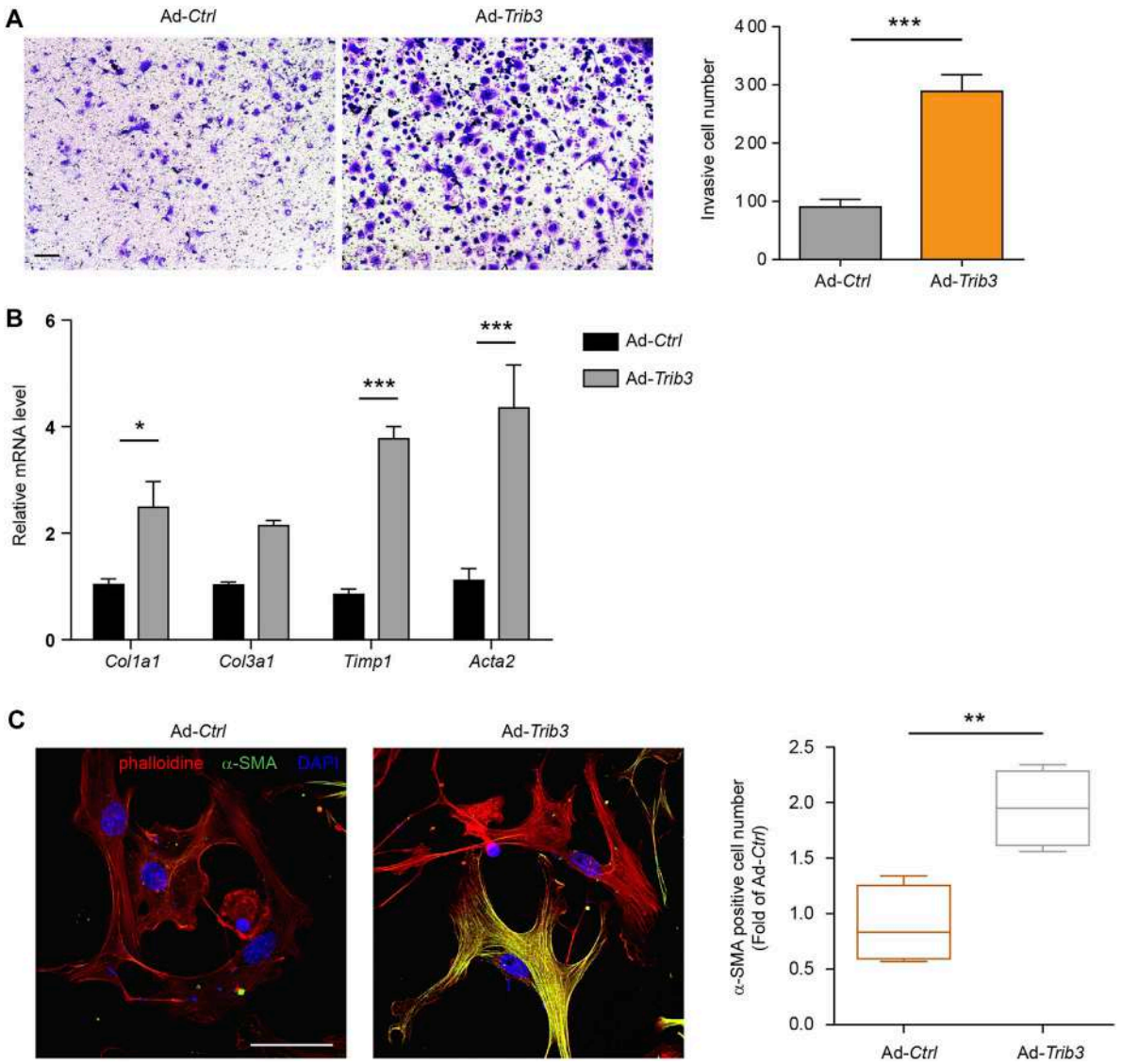

FIGURE 6 | Overexpression of TRIB3 activates lung fibroblasts. **(A)** Representative images and quantification of the invasion experiment results for mouse lung fibroblasts with or without TRIB3 overexpression. Scale bars, 100 μm. **(B)** mRNA expression of fibrosis genes in mouse lung fibroblasts with or without TRIB3 overexpression. **(C)** Representative images and quantification of α-SMA immunostaining in mouse lung fibroblasts with or without TRIB3 overexpression. Scale bars, 50 μm. Data are representative of 3 independent experiments. Data represent means ± SEM Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$.

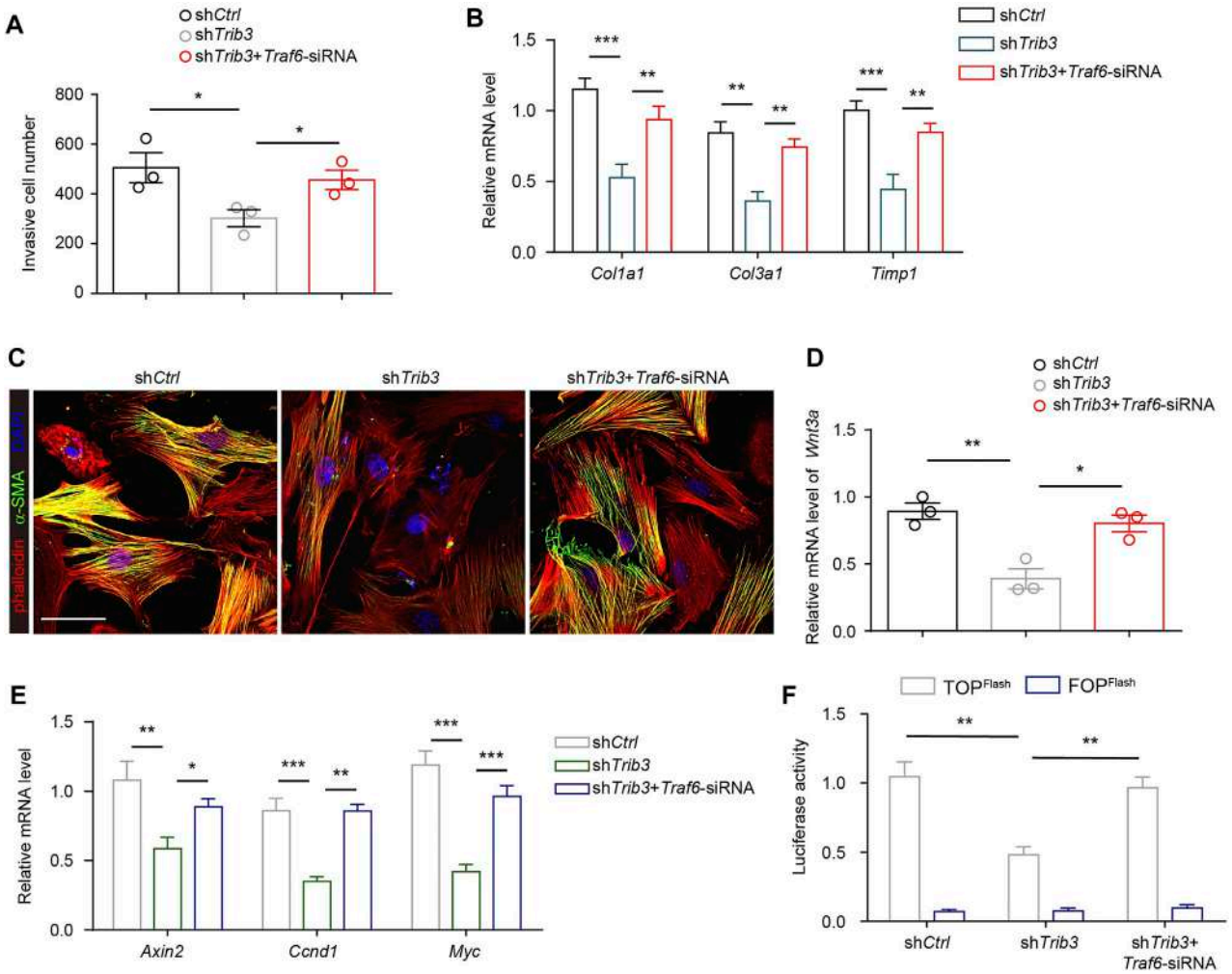

FIGURE 7 | TRIB3 activates lung fibroblasts by reducing TRAF6 expression. **(A)** Transwell assay for the evaluation of invasion of TRIB3-silenced PF lung fibroblasts with or without TRAF6 knockdown. **(B)** mRNA expression of fibrosis genes in TRIB3-silenced PF lung fibroblasts with or without TRAF6 knockdown. **(C)** Representative images of α-SMA immunostaining in TRIB3-silenced PF lung fibroblasts with or without TRAF6 knockdown. Scale bars, 50 μm. **(D)** mRNA expression of *Wnt3a* in TRIB3-silenced PF lung fibroblasts with or without TRAF6 knockdown. **(E)** mRNA expression of the β-catenin target genes in TRIB3-silenced PF lung fibroblasts with or without TRAF6 knockdown. **(F)** Wnt-reporter luciferase activity in TRIB3-silenced PF lung fibroblasts with or without TRAF6 knockdown. Data are representative of 3 independent experiments. Data represent means ± SEM Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$.

decreased TRAF6 in PF lungs was associated with reduced pulmonary function (**Figure 1B**). The protein expression of TRAF6 was negatively correlated with the mRNA expression of *Acta2* (a marker of myofibroblasts) in fibrotic lungs (**Figure 1C**). These results prompted us to detect TRAF6 expression in lung fibroblasts, the effector cells in PF progression. Indeed, lower expression of TRAF6 was observed in lung fibroblasts from PF mice than in those from controls (**Figure 1D**). These findings demonstrate that TRAF6 in lung fibroblasts is negatively correlated with PF development.

To further confirm whether TRAF6 loss contributes to PF development *in vivo*, we instilled TRAF6-overexpressing lentivirus into lung tissue 10 days after BLM challenge. Overexpression of TRAF6 in lung tissue reduced BLM-induced PF changes, as revealed by the reduction in collagen deposition (**Figure 1E**), the improvement in lung function (**Figure 1F**), and the decreased hydroxyproline levels (an indicator of collagen content) in BLM mice (**Figure 1G**). Thus, TRAF6 may play an indispensable protective role in the progression of PF.

TRAF6 Overexpression Attenuates the Activation of Lung Fibroblasts From PF Mice

We next explored the functional relevance of TRAF6 in fibroblasts during PF progression. IHC staining showed that the expression of α-SMA was reduced in lung tissues from BLM-challenged mice under conditions of TRAF6 overexpression (**Figure 2A**), suggesting that TRAF6 overexpression suppressed fibroblast activation. To confirm

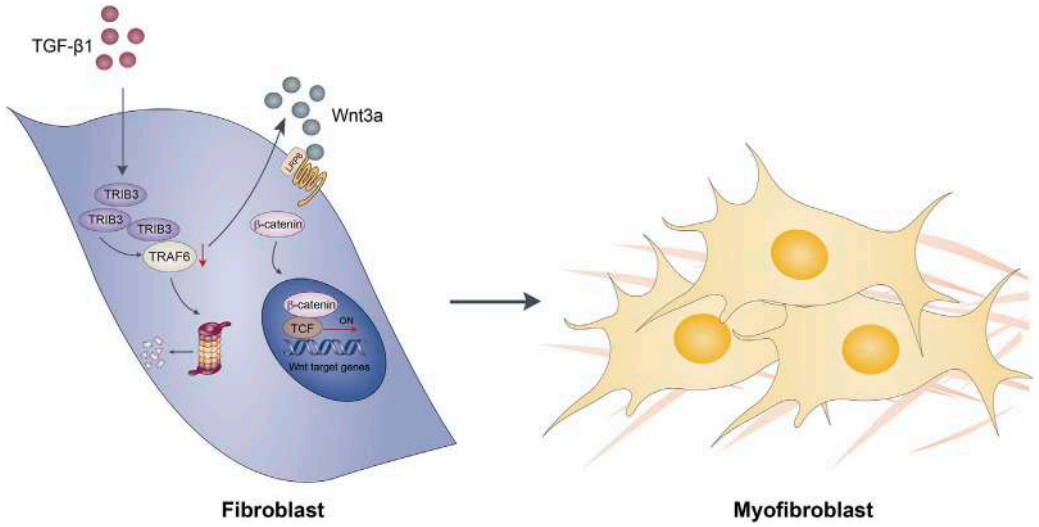

FIGURE 8 | Schematic diagram showing possible mechanisms by which TRAF6 involves in the development of pulmonary fibrosis.

this observation, primary mouse lung fibroblasts isolated from PF mice were transfected with a TRAF6-overexpressing adenovirus. PCR analysis revealed that the expression levels of several fibrosis-related genes and ECM proteins, including *Col1a1*, *Col3a1,* and *Timp1*, were reduced in fibroblasts overexpressing TRAF6 (**Figure 2B**). The matrigel invasion assay showed that the overexpression of TRAF6 decreased the invasive capacity of lung fibroblasts from PF mice (**Figure 2C**). In addition, the induction of TRAF6 overexpression in lung fibroblasts derived from PF mice decreased the percentages of α-SMA-positive cells (**Figure 2D**). Collectively, our data suggest that TRAF6 overexpression reverses the activation of myofibroblasts from PF mice and that TRAF6 deficiency during PF progression promotes the differentiation of lung fibroblasts into myofibroblasts.

TRAF6 Overexpression Decreases Wnt3a Expression

We explored how TRAF6 regulates the activation of lung fibroblasts. A previous study reported that TRAF6 overexpression in cardiac stem cells induced the downregulation of Wnt3a (Cao et al., 2015), which has been implicated in the pathogenesis of PF. We found that the overexpression of TRAF6 in lung fibroblasts from PF mice reduced the mRNA level of *Wnt3a* and that the depletion of TRAF6 in lung fibroblasts from control mice increased the mRNA level of *Wnt3a* (**Figures 3A,B**). TRAF6 depletion enhanced Wnt-reporter luciferase activity in PF lung fibroblasts (**Figure 3C**). In addition, western blot analysis demonstrated elevated expression of Wnt/β-catenin target genes in lung fibroblasts with TRAF6 depletion (**Figure 3D**). Moreover, the protein expression of TRAF6 was negatively correlated with the mRNA expression of *Wnt3a* in fibroblasts from PBS- and BLM-challenged mice (**Figure 3E**). The decreased expression of fibrosis-related genes caused by TRAF6 overexpression was reversed in lung fibroblasts treated with Wnt3a (**Figure 3F**). Furthermore, stimulation of lung fibroblasts with Wnt3a enhanced the invasive capacity that was suppressed by TRAF6 overexpression (**Figure 3G**). These data suggest that TRAF6 regulates fibroblast activation via Wnt3a.

TRIB3 Is Responsible for the Reduced Expression of TRAF6

We further investigated why TRAF6 expression is downregulated in fibroblasts during PF progression. PCR results showed that *Traf6* mRNA expression was comparable in lung fibroblasts from vehicle- and BLM-challenged mice (**Figure 4A**). We further analyzed TRAF6 gene expression in lung fibroblasts from IPF patients and healthy controls using a public microarray dataset (GSE129164). No difference in *TRAF6* mRNA expression was found between lung fibroblasts from IPF patients and healthy controls (**Figure 4B**). These data indicated that the decreased protein expression of TRAF6 in lung fibroblasts during PF progression might result from an alteration in its protein stability. Our group, along with others, has reported that TRIB3, a well-known stress sensor, is involved in regulating the stability of various proteins in the pathogenesis of chronic diseases (Lin et al., 2019; Yu J. M. et al., 2019; Liu S. et al., 2021). We then reasoned that TRIB3 might participate in regulating the altered protein expression of TRAF6. Indeed, an interaction between exogenous TRIB3 and TRAF6 was observed in HEK293T cells, and this was confirmed by the colocalization of TRIB3 and TRAF6 in primary mouse lung fibroblasts (**Figures 4C,D**). To map the interaction regions of TRAF6 and TRIB3, coIP assays were carried out using serial deletion mutants of either Myc-tagged TRAF6 or HA-tagged TRIB3. TRIB3 interacted with the ΔC domain but not with the ΔN domain

of TRAF6 (**Supplementary Figure S1A**), and TRAF6 interacted with the KD and ΔN domains of TRIB3 (**Supplementary Figure S1B**). These data indicate that the ΔC domain of TRAF6 and the KD and ΔN domains of TRIB3 are required for the interaction between TRAF6 and TRIB3.

Overexpression of TRIB3 decreased the protein level of TRAF6, and depletion of TRIB3 increased the protein level of TRAF6 (**Figures 4E,F**). Moreover, the expression of TRAF6 was positively correlated with that of TRIB3 in lung fibroblasts from PBS- and BLM-challenged mice (**Figure 4G**). Indeed, the overexpression of TRIB3 reduced the half-life of TRAF6 (**Figure 4H**). Intracellular proteins are degraded by lysosomal autophagy pathways or the ubiquitin–proteasome system (UPS). We found that the UPS inhibitor MG132, but not the autophagy inhibitor 3-MA, reversed the TRIB3 overexpression-induced reduction in TRAF6 expression (**Figure 4I**). Furthermore, the overexpression and depletion of TRIB3 increased and blocked the ubiquitination of TRAF6, respectively (**Figures 4J,K**). These data indicate that the protein expression of TRAF6 can be regulated by TRIB3.

We then detected the expression of TRIB3 in lung fibroblasts during PF progression. TRIB3 exhibited markedly higher protein and mRNA expression levels in lung fibroblasts from PF mice than in those from control mice (**Figures 5A,B**). Based on the reanalysis of a published dataset (GSE129164), we found that stimulation of lung fibroblasts of IPF patients and normal controls with TGF-β1 (the principal profibrotic cytokines in lung fibrosis) increased *TRIB3* mRNA expression (**Figure 5C**). Another dataset also revealed higher *TRIB3* mRNA levels in human lung fibroblasts treated with TGF-β1 than in untreated cells (**Figure 5D**). Collectively, these data indicate that the enhanced expression of TRIB3 in lung fibroblasts results in a reduction in TRAF6 expression by promoting its ubiquitination and subsequent degradation.

TRAF6 Is Required for the Role of TRIB3 in Regulating Fibroblast Activation

Since the reduction in TRAF6 expression caused by TRIB3 contributes to the differentiation of fibroblasts to myofibroblasts, the above data prompted us to analyze the role of TRIB3 in regulating fibroblast activation. TRIB3 overexpression enhanced the invasive capacity of primary mouse lung fibroblasts (**Figure 6A**). Much higher levels of fibrosis-related genes were detected in TRIB3-overexpressing fibroblasts than in control fibroblasts (**Figure 6B**). Immunostaining analysis also indicated that TRIB3 overexpression in lung fibroblasts significantly increased the number of α-SMA-positive cells (**Figure 6C**), suggesting that TRIB3 induced the differentiation of fibroblasts into myofibroblasts. Consistently, we found that TRIB3 deficiency reversed the activation of myofibroblasts, as indicated by decreased invasive capacity, reduced expression of fibrosis-related genes, and fewer α-SMA-positive cells (**Figures 7A–C**). However, depletion of TRIB3 failed to inhibit the activation of TRAF6-silenced fibroblasts (**Figures 7A–C**). In addition, TRIB3 knockdown decreased the expression of *Wnt3a* and Wnt/β-catenin target genes in wild-type PF lung fibroblasts but not in fibroblasts with TRAF6 depletion (**Figures 7D,E**). Furthermore, TRIB3 depletion failed to reduce Wnt-reporter luciferase activity in lung fibroblasts with TRIB3 knockdown (**Figure 7F**). These results collectively demonstrate a TRIB3-TRAF6-Wnt3a signaling axis in regulating fibroblast activation.

DISCUSSION

TRAF6, a member of the TRAF family, plays a crucial role in immune signal transduction by acting as an adaptor protein downstream of multiple receptor families, including the interleukin-1 receptor (IL-1R) superfamily, the Toll-like receptor (TLR) family, and the TNFR superfamily. In recent years, TRAF6 has been extensively investigated in tumors, immunity, neurodegenerative diseases, ischemic stroke and osteoporosis (Li et al., 2017; Dou et al., 2018; Muto et al., 2022). Recent studies have also reported that TRAF6 is involved in the pathogenesis of NASH (Wang et al., 2020). They found that depletion of TRAF6 attenuated liver fibrosis in diet-induced NASH models, supporting a role for TRAF6 in promoting liver fibrosis. However, the role of TRAF6 in lung fibrosis remains unknown. In this study, we found that TRAF6 was downregulated in fibroblasts within the lung during the course of fibrotic processes. TRAF6 overexpression in fibroblasts from PF mice suppressed the activation of these cells. Furthermore, TRAF6 overexpression protected mice from BLM-induced lung fibrosis, with a decrease in the number of activated fibroblasts, also called myofibroblasts. Our data indicate that the downregulation of TRAF6 expression in fibroblasts contributes to PF progression by inducing the activation of those cells (**Figure 8**). This study selectively focused on the role of TRAF6 in fibroblasts. Given that TRAF6 is an intracellular protein widely expressed in epithelial cells, endothelial cells, and macrophages, future studies are needed to investigate the expression and role of TRAF6 in those cells during pulmonary fibrosis.

TRIB3, a stress sensor, is involved in the pathogenesis of various diseases, including obesity, diabetes, and tumors (Du et al., 2003; Oberkofler et al., 2010; Lin et al., 2019). Accumulating evidence has widely demonstrated that TRIB3 plays a vital role in organ fibrogenesis (Wang et al., 2014; Tomcik et al., 2016; Zhang et al., 2020). Our previous study, along with others, reported the key role of TRIB3 in promoting PF (Yu W. et al., 2019; Liu S. et al., 2021). We found that TRIB3 was substantially upregulated in alveolar macrophages (AMs) from patients with PF, inducing the profibrotic phenotype of AMs. Moreover, genetic knockout of TRIB3 specifically in AMs suppressed BLM-induced fibrotic changes in the lung. Another study found a central regulatory role of TRIB3 in fibroblast activation by stimulating canonical TGF-β1/Smad signaling in systemic sclerosis (Tomcik et al., 2016). Here, we found that elevated TRIB3 expression in fibroblasts from PF mice contributes to PF progression by enhancing the expression of Wnt3a, a crucial factor in the regulation of fibroblast activation (Li X. et al., 2020; Liu T. et al., 2021). Collectively, these data support that TRIB3 exerts

profibrotic roles in different cell types during PF progression, demonstrating its potential value in the clinical treatment of PF.

As a member of the pseudokinase family, TRIB3 was found to regulate diverse processes in the progression of various diseases by interacting with functional proteins. For instance, the interaction of TRIB3 and the protein kinase GSK-3β interferes with the binding of GSK-3β to the E3 ligase UHRF1, thereby inhibiting the degradation of GSK-3β and leading to the profibrotic phenotype of Ams (Liu S. et al., 2021). TRIB3 interacted with MYC to suppress E3 ubiquitin ligase UBE3B-mediated MYC degradation, which induced the enhanced expression of MYC, causing the proliferation of lymphoma cells (Li K. et al., 2020). In the current study, the interaction of TRIB3 with TRAF6 in fibroblasts resulted in reduced TRAF6 expression. Intracellular proteins are degraded by the UPS or lysosomal autophagy pathway. We found that the UPS inhibitor MG132, but not the autophagy inhibitor 3-MA, reversed the TRIB3 overexpression-induced reduction in TRAF6 expression, indicating that the decrease in the protein level of TRAF6 caused by TRIB3 was related to its increased degradation through the UPS. This was consistent with previous studies showing that TRAF6 is degraded via the UPS (Li et al., 2014; Kim et al., 2017). Indeed, we observed increased ubiquitination and degradation of TRAF6 in TRIB3-overexpressing cells. We presumed that TRIB3, acting as a scaffolding protein, interacts with TRAF6 to recruit certain E3 ligases, thereby causing the ubiquitination and subsequent degradation of TRAF6. Obviously, further investigation of which kind of E3 ligase accounts for TRIB3 regulation of TRAF6 expression is warranted.

Increasing lines of evidence demonstrate that the aberrant activation of Wnt signaling is involved in the pathogenesis of PF (Morrisey, 2003; Skronska-Wasek et al., 2018). Attenuation of Wnt/β-catenin signaling can limit the development of fibrosis in mice. Wnt3a, the canonical ligand, is known to induce the activation of fibroblasts to myofibroblasts (Li X. et al., 2020). We found that depletion of TRAF6 in lung fibroblasts from control mice increased the expression of Wnt3a and Wnt/β-catenin target genes. A negative correlation was found between the expression of TRAF6 and Wnt3a in fibroblasts from PBS- and BLM-challenged mice. This was consistent with a prior study showing that TRAF6 overexpression induced the downregulation of Wnt3a in c-kit$^+$ cardiac stem cells. We also found that Wnt3a treatment reversed the suppressed activation of fibroblasts caused by TRAF6 overexpression. These results indicated that reduced expression of TRAF6 in lung fibroblasts induces the activation of fibroblasts by enhancing the expression of Wnt3a during PF progression. Thus, our work provides insight into the regulatory role of TRAF6 in Wnt3a expression in fibroblasts. Further investigation is necessary to determine the molecular mechanism by which TRAF6 controls Wnt3a expression.

Our findings revealed that the reduced expression of TRAF6 caused by TRIB3 overexpression in lung fibroblasts contributes to the progression of PF through upregulation of Wnt3a expression, which drives fibroblast differentiation into myofibroblasts. Accordingly, genetically enhancing TRAF6 expression or inhibiting the TRIB3-TRAF6 interaction may represent a novel therapeutic strategy for PF and other fibroproliferative lung diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The Second Xiangya Hospital of Central South University.

AUTHOR CONTRIBUTIONS

SL conceived the study. SL and JM designed the experiments. JM, QL, and MY performed the experiments. SL, QW, and YZ analyzed the data. BC and JY provided valuable suggestions and comments on the study design. JM and SL wrote and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.911945/full#supplementary-material

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Catalpol Attenuates Pulmonary Fibrosis by Inhibiting Ang II/AT_1 and TGF-β/Smad-Mediated Epithelial Mesenchymal Transition

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Background: Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating chronic lung condition affecting over 3 million people worldwide with a high mortality rate and there are no effective drugs. Angiotensin II (Ang II), as a major effector peptide of the renin angiotensin aldosterone system, has been shown to act in tandem with the transforming growth factor-β (TGF-β) signaling pathway to promote the infiltration of inflammatory cells, production of reactive oxygen species (ROS) and profibrotic factors after lung injury, and to participate in the process of epithelial mesenchymal transition (EMT). Catalpol (CAT) has been shown to have anti-inflammatory and antifibrotic effects. However, the effects and mechanisms of CAT on pulmonary fibrosis are not clear.

Purpose: To assess the effects and mechanisms of catalpol on bleomycin-induced pulmonary fibrosis in mice.

Methods: We used bleomycin-induced mouse model of pulmonary fibrosis to evaluate the alleviation effect of CAT at 7, 14, 28d, respectively. Next, enzyme-linked immunosorbent assay, hematoxylin-eosin staining, immunofluorescence, Masson trichrome staining and western blotting were used to study the underlying mechanism of CAT on bleomycin-induced pulmonary fibrosis.

Results: It's demonstrated that CAT exerted a potent anti-fibrotic function in BLM-induced mice pulmonary fibrosis via alleviating inflammatory, ameliorating collagen deposition, reducing the level of Ang II and HYP and alleviating the degree of EMT. Moreover, CAT attenuate BLM-induced fibrosis by targeting Ang II/AT_1 and TGF-β/Smad signaling *in vivo*.

Conclusion: CAT may serve as a novel therapeutic candidate for the simultaneous blockade of Ang II and TGF-β pathway to attenuate pulmonary fibrosis.

Keywords: idiopathic pulmonary fibrosis, Ang II, TGF-β/Smad signaling pathway, EMT, catalpol

HIGHLIGHTS

- Catalpol significantly ameliorates bleomycin-induced pulmonary fibrosis (PF) in mice.
- Catalpol attenuates pulmonary fibrosis via TGF-β/Smads signaling pathway.
- Catalpol inhibits bleomycin-induced epithelial mesenchymal transition.
- Catalpol decreases the expression of angiotensin II/type 1 receptor (AT_1) in PF mice.

INTRODUCTION

Pulmonary Fibrosis (PF) is a chronic interstitial lung disease characterized by massive proliferation of fibroblasts and accumulation of extracellular matrix (ECM). Pulmonary Fibrosis is accompanied by inflammatory injury, the epithelial-mesenchymal transition (EMT) and destruction of tissue structure (1). Pulmonary Fibrosis will cause lung function damage, scar of lung tissue obstructing the flow of oxygen from lungs into the bloodstream for distribution to other organs and patients will have dry cough, dyspnea and other symptoms. As the condition continues to deteriorate, the integrity of lung tissue structure is destroyed, respiratory function can be further affected, seriously endangering the patient's quality of life and even becoming life-threatening (2). Toxic insults, autoimmune injuries, drug-induced injuries, infectious injuries, traumatic injuries or other unknown factors may cause pulmonary fibrosis. Among them, idiopathic pulmonary fibrosis (IPF) is the most common and prevalent type of pulmonary fibrosis and is associated with risk factors such as genetic alterations, viral infections, lifestyle habits, environmental influences, occupational hazards (3). IPF prevalence has a correlation with age and sex. And the elderly is more prone to IPF and males have a higher incidence rate than females (4, 5). Pulmonary fibrosis is also a sequela in severe patients with the Coronavirus Disease 2019 (COVID-19) and severe acute respiratory syndrome (SARS).

The latest ATS/ERS/JRS/ALAT clinical practice guidelines recommend the conditional use of nintedanib and pirfenidone. In addition, glucocorticoids, immunosuppressive agents, and antioxidants are also used clinically in combination with western medicines to treat pulmonary fibrosis (6–9). The application of angiotensin inhibitor combined with glucocorticoid to treat pulmonary fibrosis also has been reported by many clinicians (10). However, due to the inability of existing drugs in reversing the course of pulmonary fibrosis and the lack of lung organ donors, as well as high treatment costs and so on, the median survival of patients with IPF after diagnosis is only between 3 and 5 years (11, 12). Therefore, the development of affordable and effective anti-fibrotic drugs is urgently needed.

Catalpol (CAT), an iridoid compound, exhibits anti-cancer, neuroprotective, anti-inflammatory, diuretic, hypoglycemic, anti-hepatitis virus effects and anti-fibrosis effect in multiple organs. It has been shown that CAT may inhibit bleomycin-induced pulmonary fibrosis in rats through downregulation of Wnt3a and GSK-3β, β-catenin and phosphorylation of Smad3 (13). CAT has become very popular for clinical medication candidate because of its good safety, fewer toxic side effects and convenient administration. In this context, the research and development of CAT have gradually become hot topics of interest. However, the mechanism by which CAT acts in the treatment of pulmonary fibrosis has not been thoroughly investigated. In this study, a mouse pulmonary fibrosis model was established to further explore the mechanism of action of CAT in the treatment of pulmonary fibrosis, in order to provide a reference basis for the pharmacological research and clinical application of this drug.

MATERIALS AND METHODS

Reagents

Catalpol (CAT, CAS: 2415-24-9, MW: 362.331, purity: 98%, dissolved in normal saline (NS) before use, Chengdu Purifa Technology Co., Ltd., Lot No.: PRF21111722,); Bleomycin for injection (chemical for lung-fibrosis modeling, BLM, Harbin Laibotong Pharmaceutical Co., Ltd., Lot No.: 20120727); Pirfenidone (positive control drug, PFD, Beijing Contini Pharmaceutical Co., Ltd., Lot No.: 20210511); Telmisartan (positive control drug, TEL, Shanghai Xinyi Tianping Pharmaceutical Co., Ltd. Lot No.: 87210302); Haematoxylin and eosin (H&E) (719033, Zhuhai Baso Biotechnology Co., Ltd., China); Masson Staining Kits (C210801, Zhuhai Baso Biotechnology Co., Ltd., China); HYP Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); Ang II Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); RIPA lysis buffer (Beyotime, China); Phosphorylated proteinase inhibitor (Beyotime, China); Phenyl methyl sulfonyl fluoride (PMSF, Beyotime, China); TGF-β1 (ab179695, abcom, USA); Anti-Phospho-Smad2 (18338T, CST, USA); Anti-Phospho-Smad3 (9520T, CST, USA); Anti-Smad2/3 (8685S, CST, USA); Anti-Snail (3879T, CST, USA); Anti-Angiotensin II Type 1 Receptor (ab124734, abcom, United States); Anti-MMP2(ab92536, abcom, USA); Anti-MMP9 (ab283575, abcom, USA); Anti-β-actin (AF7018, Affinity, USA); Horseradish peroxidase (HRP)-linked anti-mouse (A0286, Beyotime, China); Anti-rabbit (A0208, Beyotime, China); α-SMA (19245T, CST, USA); E-cadherin antibody (3195T, CST, USA), N-cadherin antibody (13116T, CST, USA); Goat anti rabbit IgG secondary antibody (A0468/A0423; Beyotime; Biotechnology Co. Ltd., Shanghai, China) and 4', 6-diamidino-2-phenylindole (DAPI; Beyotime; Biotechnology Co. Ltd., Shanghai, China).

Animal Care and Handling

Male C57BL/6 mice (18–20 g), an applicable animal model for BLM-induced PF model, were obtained from Shanghai Jihui Experimental Animal Breeding Co., LTD. The animals were housed in a specific pathogen-free environment and observed under a 12 h light-12 h dark cycle in a well-ventilated room at 23 ± 2°C.They were fed with standard pellet food and tap water *ad libitum*. Being approved by the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine, the study was performed in accordance with the guidelines for care

and handling of animals of the National Institute of Health (approval number PZSHUTCM220124010).

After a three-day adaptive feeding, the mice were randomized into six groups (n = 20 mice per group): (1) normal saline (NS) + vehicle control group; (2) BLM + vehicle control group, (3) BLM + PFD (300 mg/kg/day) group (14, 15); (4) BLM + TEL (10 mg/kg/day) group (16, 17); (5) BLM + High CAT (200 mg/kg/day) group; (6) BLM + Low CAT (100 mg/kg/day) group (18). On day 0, a single intratracheal instillation of BLM (5 mg/kg) was performed to induce pulmonary fibrosis in C57BL/6 mice (19). Mice in NS group (control group) received an equal volume of NS. One day after BLM induction, mice in PFD groups and TEL group were intragastrically administrated with PFD or TEL, CAT group was intragastrically administrated with high or low dose of CAT, NS group and BLM group were intragastrically administrated with equal volume of NS for 28 days. Body weight was measured every 3 days. On days 7, 14, and 28, 5, 5, and 10 mice were euthanized in each group, respectively, the mice anesthetized with pentobarbital sodium (50 mg/kg) intraperitoneally, lung tissues and blood were collected for subsequent studies.

Histological Examination and Evaluation

Lung tissues from mice were fixed with 4% paraformaldehyde (48 h) and blocked with paraffin, sectioned (5 μM thickness) after deparaffinization in xylene. Haematoxylin and eosin (H&E) and Masson Staining Kits were used for pathological evaluation. The tissues were morphologically analyzed using a StrataFAXS II image analysis system (StrataFAXS II, Vienna, Austria).

Western Blotting Assay

Proteins were extracted from lung tissue using RIPA lysis buffer with 2% phosphorylated proteinase inhibitor, 1% phenyl methyl sulfonyl fluoride, and 2% protease inhibitor, according to the manufacturer's protocol. 30–50 μg of protein from each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred to PVDF membranes. After blocking in 5% BSA solution for 2 h at room temperature, the membranes were incubated overnight at 4°C with anti-TGF-β1, anti-Phospho-Smad2, anti-Phospho-Smad3, anti-Smad2/3, anti-Snail, anti-Angiotensin II Type 1 Receptor, United States), anti-MMP2, anti-MMP9 or anti-β-actin.

After washing three times in TBS-T, the membranes were incubated in horseradish peroxidase (HRP)-linked anti-mouse or anti-rabbit for 2 h at room temperature. Detection of protein signal and analysis was performed using Tanon 4600SF (Tiangong Technology Co., Ltd., Shanghai, China) and ImageJ software (National Institute of Mental Health, Bethesda, MD, USA), respectively.

Immunofluorescence Assay

Immunofluorescence was used to verify E-cadherin, N-cadherin and α-SMA expression in lung tissue. Sections were incubated as previously described with anti-α-SMA or anti-E-cadherin antibody, anti-N-cadherin antibody at 4 °C overnight and then incubated with Goat anti rabbit IgG secondary antibody at 37 °C for 1.5 h. After washing, they were counterstained with 4', 6-diamidino-2-phenylindole for 10 min at room temperature in the dark. Images were captured with an immunofluorescence microscope (Leica SP8, Wetzlar, Germany).

Hydroxyproline Assay

Collagen content was assessed by measuring the hydroxyproline (a major component of collagen) content of the tissue as previously described by Woessner (20). After lung tissue was homogenized, a HYP Kit was used to detect the HYP content, following the manufacturer's instructions.

Ang II Assay

Angiotensin II (Ang II), a key vasoactive peptide of the RAAS, mediates pro-inflammatory and pro-fibrotic effects on the lung. After lung tissue was homogenized, an Ang II Kit was used to detect Ang II content following the manufacturer's instructions.

Statistical Analysis

Data are shown as mean ± Standard Error of Mean (SEM). Differences between the groups were evaluated using one-way analysis of variance (ANOVA with Dunnett's *post-hoc* analysis). $P < 0.05$ was considered statistically significant. Statistical analyses and figures were obtained using GraphPad Prism Version 8.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

CAT Attenuates BLM Induced Pulmonary Fibrosis in Mice

Intratracheal instillation of BLM in mice causes destruction of alveolar structure and the formation of fibrosis, and is a widely used model of pulmonary fibrosis. To explore the therapeutic effects of CAT on pulmonary fibrosis (21), we collected lung tissues from mice at 7, 14 and 28d of CAT treatment and performed relevant detection assessments (**Figures 1A,B**). H&E and Masson staining were used to examine the lung histopathological changes, which showed that after BLM modeling, the lung tissue injury was obvious, followed by an obvious interstitial inflammation and inflammatory cell infiltration, increasing with time, interstitial fibrosis was gradually aggravated, and the pathological morphological changes and collagen deposition in lungs were attenuated using CAT (**Figures 1C,D**). Some matrix metalloproteinases (MMPs) are overexpressed in pulmonary fibrosis and may be associated with more severe and advanced pathological stages in the lung (22, 23). In the present study, the protein expression levels of MMP2 and MMP9 in the lungs were evaluated by Western blot analysis at 7, 14, 28d. **Figures 1E–I** showed that compared with the Control group, the BLM-injured lung tissue showed distinct increases in the levels of MMP2 and MMP9 at 14th, 28th time point after modeling ($P < 0.01$), whereas CAT could down-regulate the protein levels of MMP2 and MMP9 in fibrotic lung tissue ($P < 0.05$, $P < 0.01$ or $P < 0.001$). To assess the effect of CAT on lung collagen and ECM, we assessed HYP (a major component of collagen) content in mouse

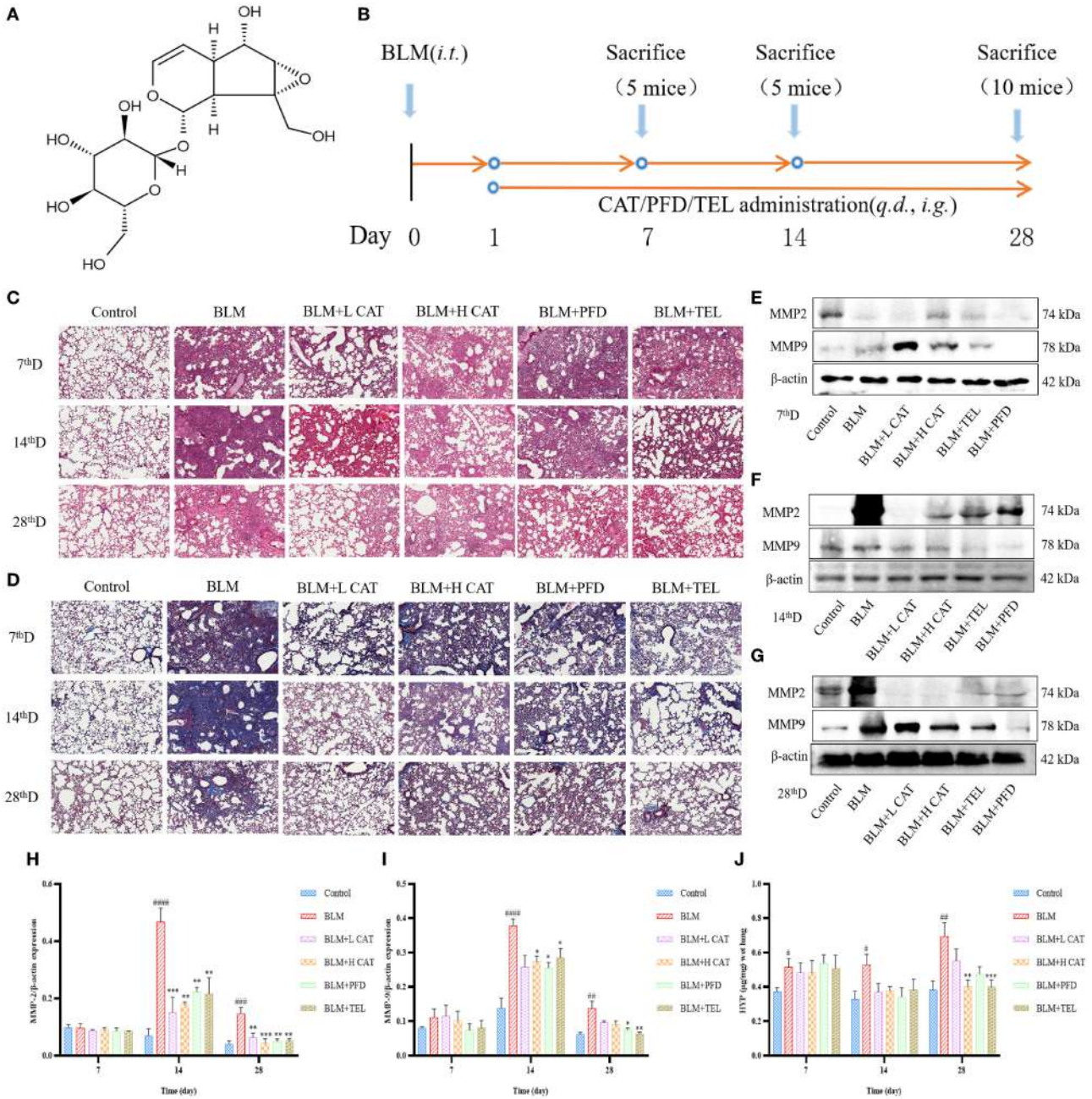

FIGURE 1 | **(A)** Structure of catalpol **(B)** Animal experiment flowchart:C57BL/6 mice were treated by oral gavage with the different doses of CAT (100, 200 mg/kg/day), telmisartan (TEL) (10 mg/kg/day) and pirfenidone (PFD) (300 mg/kg/day) for 28 days followed by intratracheal instillation with BLM (5 mg/kg) or an equal volume of sterile normal saline (NS, Control group), respectively. Control group and BLM group received equal amounts of NS by oral gavage each day (n = 5-10, the number of mice sacrificed per group was 5, 5 and 10 on Day 7, 14 and 28 respectively). From left to right: Samples were collected at 7, 14 and 28th day after BLM administration. **(C)** Representative images of HE staining. Scale bar: 100 μm. **(D)** Representative images of Masson staining. Scale bar: 100 μm. **(E–G)** Expression of MMP2 and MMP9 in the lung tissues at 7, 14, 28d as detected by Western blot. **(H,I)** The MMP2 and MMP9 protein levels at 7, 14, 28d were analyzed by ImageJ software. **(J)** HYP in mouse serum at 7, 14, 28d was measured. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ compared with the Control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the BLM group (ANOVA with Dunnett's *post-hoc* analysis).

lungs. As shown in **Figure 1J**, CAT obviously suppressed the expression of HYP compared with BLM group. It is known from the data that CAT could attenuate BLM induced pulmonary fibrosis progression.

CAT Inhibited BLM Induced EMT in Mice

Previous studies have shown that EMT plays an important role in fibrosis in multiple organs, such as promoting epithelial to fibroblast differentiation and participating in the constitution

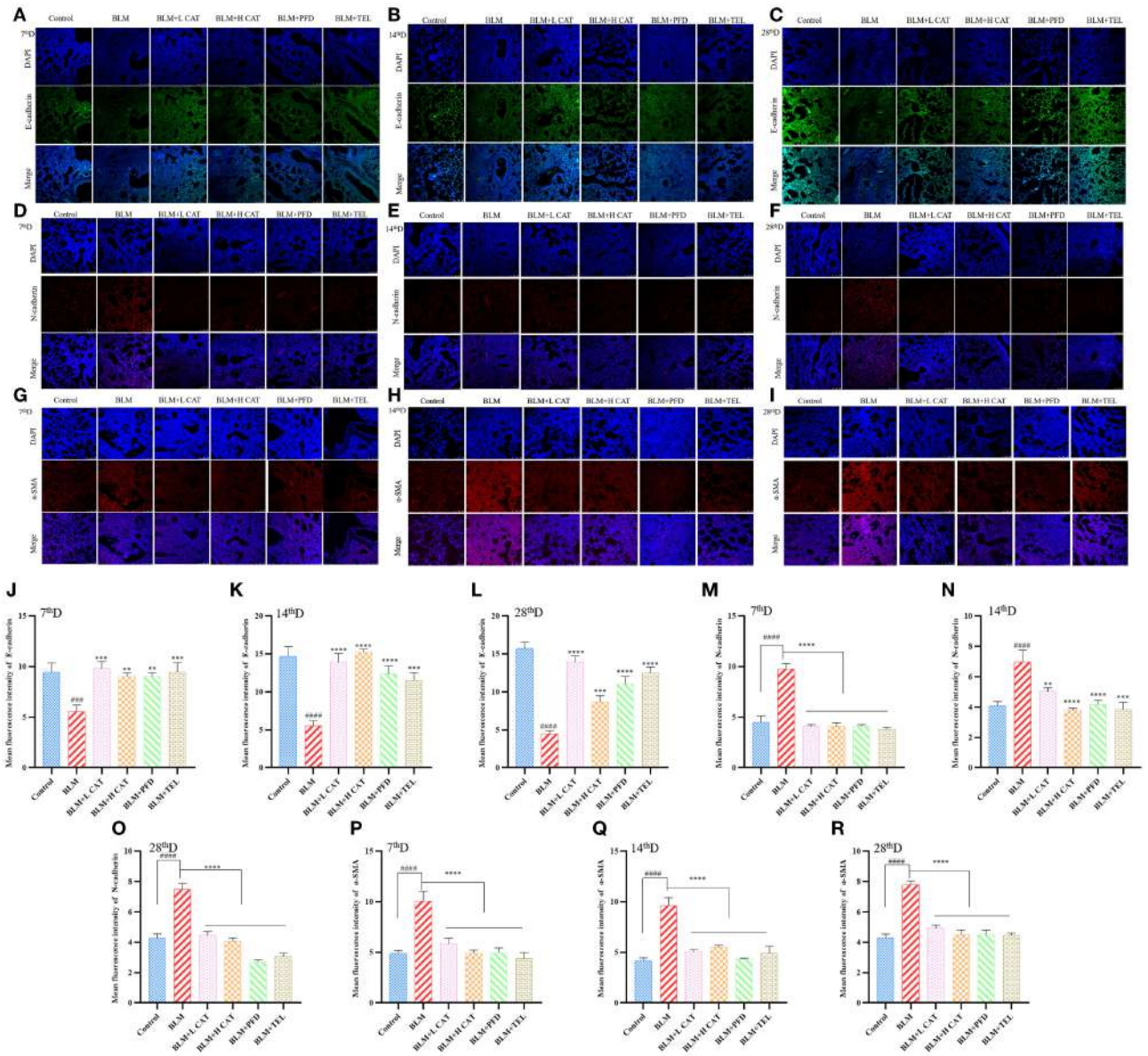

FIGURE 2 | Catalpol inhibits epithelial mesenchymal transition (EMT) in bleomycin induced pulmonary fibrosis. **(A–C,J–L)** Immunofluorescence analysis of E-cadherin in lung sections at 7, 14, 28d, E-cadherin (green), DAPI (blue), the fluorescence intensity of E-cadherin was analyzed by ImageJ software. **(D–F,M–O)** Immunofluorescence analysis of N-cadherin in lung sections at 7, 14, 28d, N-cadherin (red), DAPI (blue), the fluorescence intensity of N-cadherin was analyzed by ImageJ software. **(G–I,P–R)** Immunofluorescence analysis of α-SMA in lung sections at 7, 14, 28d, α-SMA (red), DAPI (blue), the fluorescence intensity of α-SMA was analyzed by ImageJ software. $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ compared with the Control group, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared with the BLM group (ANOVA with Dunnett's *post-hoc* analysis).

of fibroblast/myofibroblast foci (24, 25). We further evaluated the role of CAT on EMT progression during BLM induced pulmonary fibrosis in mice. Immunofluorescence analyses revealed that BLM administration in mouse lungs resulted in a significant decrease in E-cadherin expression along with an increase in N-cadherin and α smooth muscle actin (α-SMA), suggesting an epithelial mesenchymal phenotype transition and an increase in myofibroblasts during PF. After CAT administration, the expression of E-cadherin increased, while the expression of N-Cadherin and α-SMA decreased ($P < 0.01$, $P < 0.001$ or $P < 0.0001$) and EMT was inhibited in the lungs of mice compared with that in model group (**Figures 2A–R**).

CAT Attenuates Lung Fibrosis in Mice *via* the Ang II / AT_1 Axis

The physiological actions of Ang II are almost all mediated by type 1 angiotensin receptor (AT_1) on the cytoplasmic membrane representations, thereby promoting multiple signaling pathways

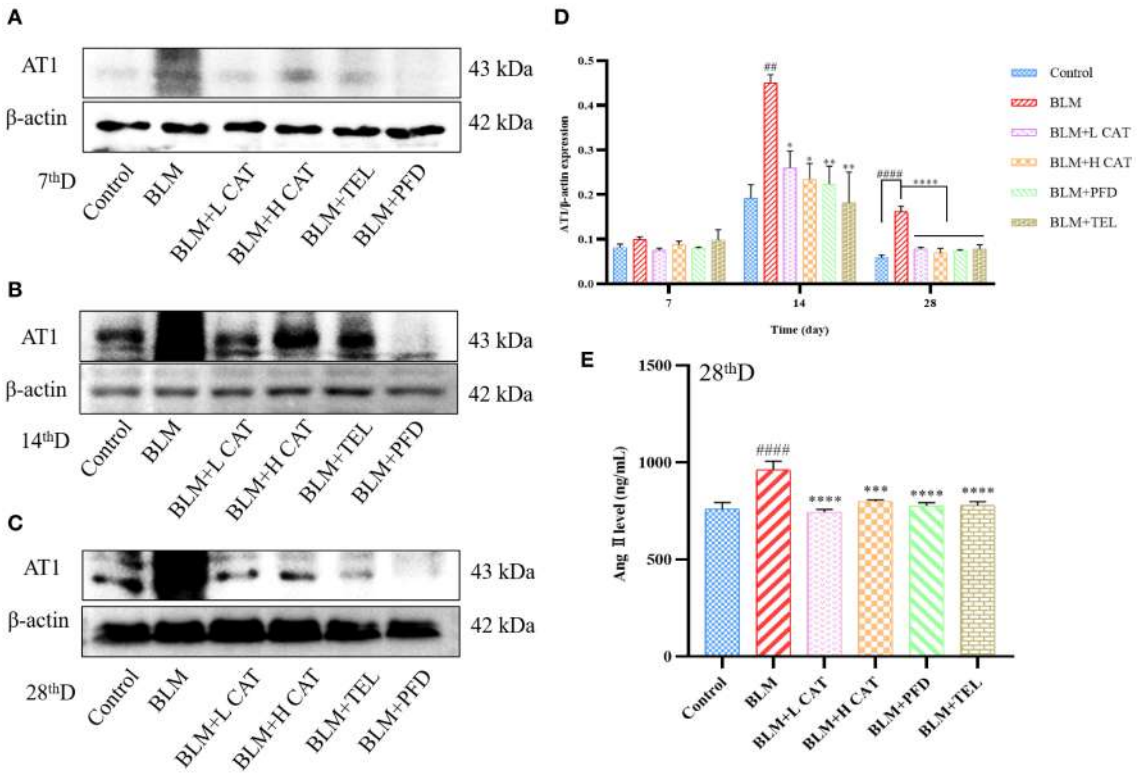

FIGURE 3 | Catalpol inhibits the up-regulation of Ang II and AT_1 in bleomycin-induced pulmonary fibrosis in mice. **(A–D)** Representative Western blots of AT_1 and gray scale quantified data of AT_1-to-β-actin expression ratio at 7, 14, 28d. **(E)** Ang II in mouse serum at 28 day was measured. $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ compared with the Control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ $^{****}p < 0.0001$ compared with the BLM group (ANOVA with Dunnett's *post-hoc* analysis).

that exert physiological functions. Study has found that not only Ang II has a promoting effect on lung fibroblast proliferation *in vitro*, but also AT_1 as well as TGF-β1 overexpression was found in the model of BLM induced lung fibrosis, accompanied by enhanced collagen synthesis with collagen deposition. Candessartan, an AT_1 receptor antagonist, could reduce TGF-β1 expression and the degree of fibrosis, indicating that Ang II might promote TGF-β1 expression and aggravate the degree of pulmonary fibrosis (26). **Figures 3A–E** showed that treatment with CAT significantly decreased the amount of Ang II in serum ($P < 0.001$ or $P < 0.0001$) and protein of AT_1 in lung tissue.

CAT Attenuates Pulmonary Fibrosis in Mice *via* TGF-β/Smads Signaling Pathway

Small molecules against decapentaplegic homologs (Smad) are classic mediators in the TGF-β signaling pathway and regulate the transcription of a variety of genes, such as AP-1 and snail, which promote pulmonary fibrosis. To determine whether CAT played an important role in the TGF-β signaling pathway in pulmonary fibrosis, we measured the protein expression levels of TGF-β1, Smad2/3, p-Smad2, p-Smad3, Smad7 as well as Snail. As shown in **Figures 4A–H**, TGF-β1, p-Smad2, p-Smad3 and Snail were significantly up-regulated in model group. After CAT treatment, the level of phospho-Smad2 was significantly decreased compared with that of model group ($P < 0.05$). CAT probably acts through TGF-β Signaling pathway to inhibit pulmonary fibrosis development.

Given all the above data, we conclude that catalpol attenuates pulmonary fibrosis by inhibiting Ang II/AT_1 and TGF-β/Smad-mediated epithelial mesenchymal transition (**Figure 5**).

DISCUSSION

Following damage to lung tissue, barrier integrity is generally repaired through the formation of a provisional matrix, transmigration of myofibroblasts and contraction of the wound. Then epithelial cells regenerate and remodel, remove debris and extracellular matrix (ECM) to achieve normal lung healing (27). However, excessive, persistent damage to the alveolar epithelium and/or abnormal wound healing contribute to a dysfunctional, often overexuberant, repair process. The lung develops an inflammatory response in which macrophages and immune cells infiltrate, and produce and release a large number of cytokines and growth factors. Subsequent transformation of alveolar epithelial cells to a mesenchymal phenotype, fibroblast and myofibroblast foci formation, and excessive accumulation of ECM and collagen result in scar formation and destruction of lung architecture, impairing lung function (10, 28).

Renin-angiotensin system (RAS) is the main endocrine regulatory system of the body. Renin (EC 3.4.23.15) is an

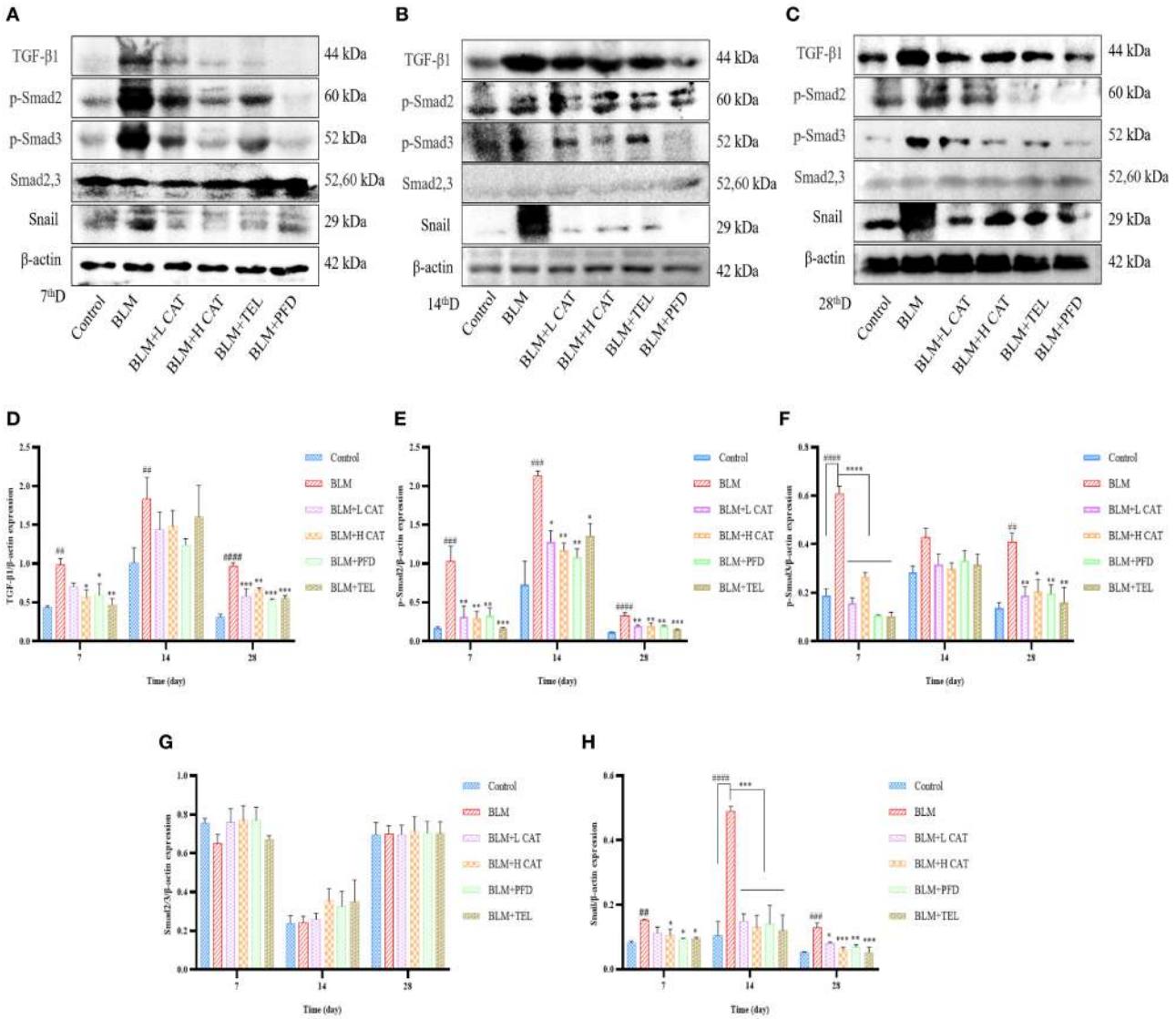

FIGURE 4 | Effect of catalpol on TGF-β/Smad2/3 signaling pathway. **(A–C)** Western blotting was conducted to detect the expression levels of TGF-β1, Smad2,3, p-Smad2, p-Smad3 and Snail in different lung tissue groups at 7, 14, 28d. **(D–H)** Densitometry data of TGF-β1-to-β-actin, p-Smad2-to-β-actin, p-Smad3-to-β-actin, Smad2/3-to-β-actin, Snail-to-β-actin expression ratio at 7, 14, 28d. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ compared with the Control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$,$^{****}p < 0.0001$ compared with the BLM group (ANOVA with Dunnett's *post-hoc* analysis).

aspartyl protease that can catalyze the specific cleavage of angiotensinogen into the decapeptide Ang I. Then, angiotensin converting enzyme (ACE) converts Ang I into the biologically active octapeptide Ang II, and regulate pressure as well as sodium and water homeostasis and so on (29). All major components of the RAS exhibit profibrotic activity, such as Angiotensin converting enzyme (ACE) and angiotensinogen may contribute to increased Ang II production, and Ang II plays important roles in the development of renal, hepatic, as well as pulmonary fibrosis (30, 31). Ang II is hydrolyzed from Ang I under the action of ACE, and the binding of Ang II with AT_1 may be involved in the process of pulmonary fibrosis as a pro-fibrosis mediating factor. Fibroblasts, which make up about 40% of lung cells, are responsible for the deposition of collagen and other ECM proteins. Myofibroblasts expressing α-SMA are extracellular in PF. It has been demonstrated *in vitro* that Ang II can activate the AT_1 to induce human lung fibroblast growth, proliferation, and to mediate the transition of fibroblast phenotype to myofibroblasts as well as procollagen production. And Ang II may also be one of several growth factors that induce and enhance TGF-β and connective tissue growth factor (CTGF) expression at the site of injury (26, 32, 33). Telmisartan, one of the positive controls used in this study, was an Ang II receptor antagonist.

BLM, an agent that induces experimental pulmonary fibrosis, has been described in a variety of experimental animal model

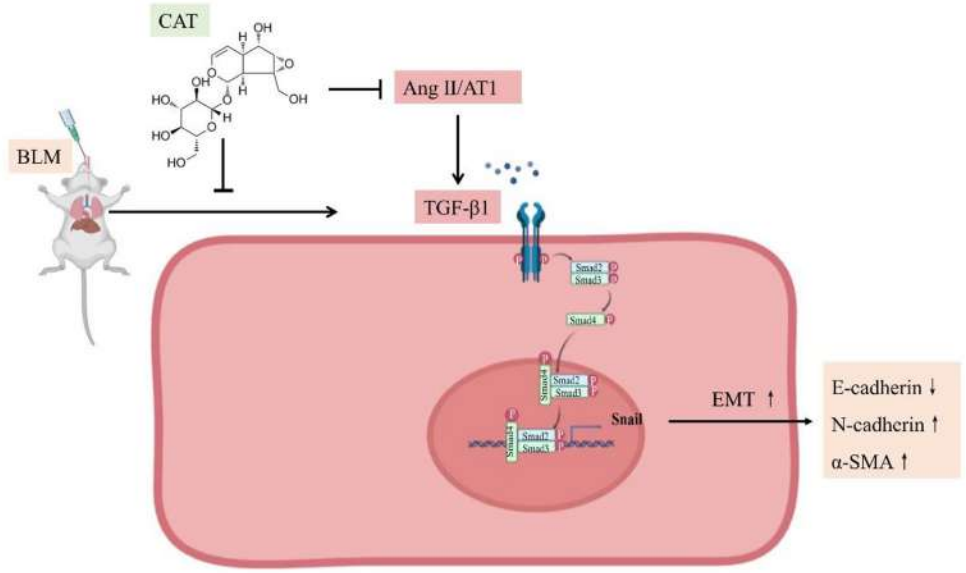

FIGURE 5 | Schematic representation of catalpol regulation of the Ang II/AT_1 and TGF-β/Smad pathway in pulmonary fibrosis.

and this BLM-induced model has the advantages of being simple to perform, widely available and reproducible (21). Intratracheal instillation of BLM in mice causes inflammatory and fibrotic responses within a short period of time. The first 7 days after modeling of BLM induced an inflammatory response characterized by increased levels of proinflammatory cytokines (interleukin-1 β, tumor necrosis factor-α, IL-6, interferon-γ). Inflammation subsequently resolves and fibrosis is detected, in which the expression of profibrotic markers (TGF-β1, Fibronectin, procollagen-1) increased and peaked around day 14. The stage of fibrosis can be maintained for 3–4 weeks after BLM modeling (34). In this experiment, three time points (7, 14, and 28d) were designed to observe the effect of CAT on pulmonary fibrosis at each time point. In this study, Ang II combined with AT_1 was up-regulated in BLM-induced pulmonary fibrosis mice model at 14 and 28 days, and in the CAT treated group, Ang II and AT_1 was down regulated significantly compared with that of the model group. CAT may attenuate pulmonary fibrosis through Ang II/AT_1 axis.

EMT is a crucial step in the process of lung development and many lung diseases (pulmonary fibrosis, chronic obstructive pulmonary disease (COPD), lung cancer). EMT is characterized by epithelial cells losing their characteristics of cell-cell adhesion and apical basal polarity and by gaining some mesenchymal characteristics for migration, invasion, and production of ECM components (35, 36). EMT in organ fibrosis is a major provider of pathogenic mesenchymal cell types with migratory and invasive behavior, such as myofibroblasts (37). Type II EMT is associated with wound healing, tissue regeneration and organ fibrosis, during lung fibrosis, it is generally defined by the detection of several biomarkers that reflect the loss of epithelial phenotype and the gain of mesenchymal phenotype. The changes of these biomarkers include decreased expression of the epithelial cell-cell adhesion molecule CDH1 (E-cadherin) and/or increased expression of mesenchymal markers (CDH2 (N-cadherin), VIM (vimentin), and/or α-SMA), as well as the change of ECM components (fibronectin and collagen) or degrading enzymes (MMP2 and MMP9) (38–40).

TGF-β, a key factor in fibrotic diseases and one of the most studied cytokines, it has various biological effects, such as increasing ECM deposition, recruiting inflammatory cells, contributing to fibrocyte differentiation and epithelial–mesenchymal transition (EMT) (41, 42). TGF-β induces EMT development and progression of lung fibrosis mainly through its canonical Smad dependent pathway. TGF-β family cytokines induce serine/threonine kinase type receptors on the cell membrane to form a functional complex that phosphorylates receptor regulated Smad2 and Smad3, and binds to Smad4, which in turn translocates into the nucleus and participates in transcriptional regulation of target genes (43). Among them, the transcription factors Snail1 and Snail2 downregulate E-cadherin expression and increase the expression of mesenchymal proteins such as N-cadherin, fibronectin and metalloproteinases (27).

The results of pulmonary TGF-β expression in this study may reflect the degree of lung inflammation/EMT in each experimental group. TGF-β and Smad2/3 were significantly upregulated in the model mice, indicating that TGF-β/Smad2/3 pathway is involved in BLM-induced pulmonary fibrosis. After treatment with CAT, the levels of TGF-β, phospho-Smad, as well as the expression of Snail were all decreased. Moreover, CAT can also up-regulate the epithelial cell marker E-cadherin and down-regulate the mesenchymal marker N-cadherin and α-SMA. Our study showed that CAT slowed down EMT progression and reduced myofibroblast proliferation in lung fibrosis. In addition, studies have shown that MMP2 and MMP9 can promote aberrant epithelial repair in fibrotic lungs, degrade ECM

and thereby promote aberrant epithelial cell migration as well as promote EMT (22). CAT significantly reduced the MMP2 and MMP9 levels in fibrotic lungs and inhibited the progression of pulmonary fibrosis. CAT may inhibit TGF-β/Smad signaling pathway to attenuate EMT progression of pulmonary fibrosis. Ang II and TGF-β have been proved to promote pulmonary fibrosis. Some studies have shown that there is signal crosstalk between them (44). Ang II binds to AT_1 on the cell membrane and phosphorylates Smad2/3 through the ERK/P38/MAPK pathway. It is then combined with Smad4 and is transferred into the nucleus as a transcription factor to regulate the transcription of TGF-β, procollagen I, procollagen III and fibronectin (33). In addition, Ang II-AT_1 could also directly activate TGF-β signal transduction and induce collagen synthesis in human fetal lung fibroblasts (27). Similarly, Accumulating evidence shows that Ang II stimulates macrophages to release AT_1-enriched exosomes, which promotes fibroblasts activation and lung fibrosis via transforming growth factor-β (TGF-β)/smad2/3 pathway (25, 45–47). In our study, treatment with CAT decreased Ang II and AT_1 as well as TGF-β/Smad2/3, indicating that CAT may attenuate the progression of BLM-induced lung fibrosis in mice partly by inhibiting phosphorylation of Smad2/3 via decreasing Ang II expression.

CONCLUSION

TGF-β1 and Ang II is a critical mediator of tissue fibrosis in disease. These pathways were disease-relevant because the levels of TGF-β and Ang II were increased and positively correlated with Smad2/3 in tissues from patients with idiopathic pulmonary fibrosis or scleroderma-associated interstitial lung disease. Chemicals capable of suppressing TGF-β1-induced production of collagen represent good candidates to treat IPF. Our study points out that the mechanism of CAT protecting against pulmonary fibrosis induced by BLM in mice is related to the regulation of TGF-β1, Smad2, Smad3, Ang II, AT_1, E-cadherin, N-cadherin and α-SMA as well as the phosphorylation of Smad2 and Smad3, also it is given orally which makes it easier to put into clinical practice. In summary, our study points out that catalpol is able to inhibit pulmonary fibrosis, and the mechanism may lie in down-regulating Ang II and AT_1 as well as inhibiting EMT progression through the TGF-β/Smad2/3 signaling pathway.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine.

AUTHOR CONTRIBUTIONS

Y-HS: conception and design, conceptualization, methodology, data curation, visualization, investigation, supervision, validation, writing-reviewing and editing, project administration, and funding acquisition. QY: software, methodology, data curation, data and image processing, visualization, and validation. D-WZ: investigation, conceptualization, methodology, data curation, visualization, validation, and writing-original draft preparation. YZ: investigation, data curation, data and image processing, and visualization. KW: designed and performed histological experiment. P-LR: carried out a portion of the animal experiments. All authors contributed to and have approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.878601/full#supplementary-material

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Zoledronic Acid Targeting of the Mevalonate Pathway Causes Reduced Cell Recruitment and Attenuates Pulmonary Fibrosis

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Background and aim: Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease causing irreparable scarring of lung tissue, with most patients succumbing rapidly after diagnosis. The mevalonate pathway, which is involved in the regulation of cell proliferation, survival, and motility, is targeted by the bisphosphonate zoledronic acid (ZA). The aim of this study was to assess the antifibrotic effects of ZA and to elucidate the mechanisms by which potential IPF treatment occurs.

Methods: A series of *in vitro* and *in vivo* models were employed to identify the therapeutic potential of ZA in treating IPF. *In vitro* transwell assays were used to assess the ability of ZA to reduce fibrotic-related immune cell recruitment. Farnesyl diphosphate synthase (FDPS) was screened as a potential antifibrotic target using a bleomycin mouse model. FDPS-targeting siRNA and ZA were administered to mice following the onset of experimentally-induced lung fibrosis. Downstream analyses were conducted on murine lung tissues and lung fluids including 23-plex cytokine array, flow cytometry, histology, Western blotting, immunofluorescent staining, and PCR analysis.

Results: *In vitro* administration of ZA reduced myofibroblast transition and blocked NF-κB signaling in macrophages leading to impaired immune cell recruitment in a transwell assay. FDPS-targeting siRNA administration significantly attenuated profibrotic cytokine production and lung damage in a murine lung fibrosis model. Furthermore, ZA treatment of mice with bleomycin-induced lung damage displayed decreased cytokine levels in the BALF, plasma, and lung tissue, resulting in less histologically visible fibrotic scarring. Bleomycin-induced upregulation of the ZA target, FDPS, was reduced in lung tissue and fibroblasts upon ZA treatment. Confirmatory increases in FDPS immunoreactivity was seen in human IPF resected lung samples compared to control tissue indicating potential translational value of the approach. Additionally, ZA polarized macrophages towards a less profibrotic phenotype contributing to decreased IPF pathogenesis.

Conclusion: This study highlights ZA as an expedient and efficacious treatment option against IPF in a clinical setting.

Keywords: IPF, Zoledronic Acid, mevalonate, Fibrosis, FDPS, drug repurposing

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a disorder characterized by progressive lung scarring with a median survival time of 3 years postdiagnosis (Raghu et al., 2011, 2019; Chang et al., 2020). IPF is associated with increasing cough and dyspnea, affecting approximately 3 million people worldwide (Martinez et al., 2017; Lederer and Martinez, 2018). Mechanistically, IPF is thought to be driven by chronic and repeated epithelial injury-repair responses leading to epithelial-mesenchymal transition and other resultant changes (Wynn and Vannella, 2016; Skibba et al., 2020; Baratella et al., 2021). Following repeated epithelial damage, fibroblasts and epithelial cells are triggered to transition, migrate, and proliferate resulting in excessive extracellular matrix (ECM) deposition (Hu and Phan, 2013; Cutolo et al., 2018; Piera-Velazquez and Jimenez, 2018; Zhang et al., 2019; Skibba et al., 2020).

Additionally, inflammatory cells such as monocytes and tissue-resident macrophages play crucial roles in tissue injury and wound healing responses, displaying diverse phenotypes to control a multitude of regulatory functions during IPF progression (Vannella and Wynn, 2017; Upagupta et al., 2018; Zhang et al., 2018; Chakarov et al., 2019; Guilliams et al., 2020; Henderson et al., 2020). Current IPF therapies focus on the inhibition of collagen deposition by blocking myofibroblast activation and proliferation, with limited success in achieving overall IPF resolution, necessitating the need for novel therapies (Lederer and Martinez, 2018). Drug repurposing allows for shortened preclinical and clinical trial periods from an estimated 10–12 years to 3–4 years, allowing patients to benefit from cheaper medications (Xue et al., 2018; Fetro and Scherman, 2020).

In this study we repurposed zoledronic acid (ZA), a drug commonly used for the treatment of post-menopausal onset of osteoporosis and hypercalcemia associated with multiple myeloma (Watts and Diab, 2010), in *in vitro* and *in vivo* models of pulmonary fibrosis. In our study, a bleomycin-challenged murine model was used to assess ZA's anti-fibrotic efficacy. Bleomycin-induced fibrosis reproduces several phenotypic features of human IPF, including peripheral alveolar septal thickening, dysregulated cytokine production, and immune cell influx (Tashiro et al., 2017; Tanner and Single, 2019).

The primary target of bisphosphonates is farnesyl diphosphate synthase (FDPS), a key enzyme in the mevalonate pathway (Dunford et al., 2001). Isoprenoid intermediates derived from the mevalonate pathway are utilized in post-translational modifications of several proteins, including Rho GTPases, regulating important cellular functions including modulation of the actin cytoskeleton (Tsou et al., 2014; Larson-Casey et al., 2019). Taken together, increased mevalonate pathway intermediates facilitate the differentiation and movement of pro-fibrotic cell-types and an increased production of reactive oxygen species, potentially leading to IPF onset (Zeng et al., 2003; Chen et al., 2007; Larson-Casey et al., 2016).

We show herein, that FDPS is decreased in control patient lung samples versus IPF lung tissue. Subsequent administration of FDPS-targeting siRNA directly to the lung, reduced bleomycin induced fibrotic lung damage. Similarly, bleomycin administered mice displayed increased *FDPS* expression versus ZA-administered animals. In an IPF context, increased profibrotic macrophage populations have been found in the blood of IPF patients as well as in the lungs of bleomycin-treated mice (Zhou et al., 2014). We show that ZA administration confers beneficial macrophage repolarization and modulation of the fibrotic response, highlighting FDPS as a tractable therapeutic target. ZA is particularly interesting in a clinical context due to its FDA approval and acceptable safety profile, with transient flu-like symptoms and less-commonly occurring osteonecrosis of the jaw the main reported side effects (Rosen et al., 2003).

The aim of this study was to assess the antifibrotic effects of ZA and to delineate any antifibrotic mechanisms associated with FDPS-targeting. The repurposing of bisphosphonates for use in pulmonary fibrosis-related disease is a unique concept that is not currently described in the literature. If successfully applied in the clinic, the novel treatment strategies described in this study would decrease the rate of disease progression associated with IPF.

MATERIALS AND METHODS

Study Design

Further details regarding the materials and methods used can be found in the **Supplementary Material**. *In vivo* murine studies using intratracheally-administered bleomycin were chosen as well-established and relevant models of experimental lung fibrosis (Tashiro et al., 2017; Tanner and Single, 2019). Sample sizes were calculated by power analysis based on previous experience, feasibility, and to conform to the ARRIVE guidelines (arriveguidelines.org). For lung fibrosis experiments testing, $n \geq 8$ to 15 mice per group were used to achieve statistical significance as calculated using G Power Software (ANOVA, with fixed effects, omnibus, one-way). For siRNA experiments, demonstrating FDPS as a target for IPF treatment, $n = 5$ mice per group were used. Mice were randomly assigned to treatment groups. Downstream analyses were conducted with the investigator blinded to the treatment groups, and no animals were excluded as outliers from the reported dataset. All *in vitro* and *in vivo* experiments were performed in 2–4 technical replicates. Macroscopically normal, tumor-free lung tissue samples (control tissue) were obtained during transplantation or resection from patients undergoing cancer surgery or from

patients with IPF diagnosed by techniques supplemented by spirometry (**Supplementary Table S2**). Patients undergoing lung biopsies with confirmed distal lung fibrosis (by histological analysis), or high-resolution computerized tomography (HRCT) scans with characteristic IPF features (e.g., opaque fibrosis-like areas and 'honeycombing' in the lung parenchyma) were included in this study (n = 4 per group).

Ethical Approval

All animal experiments in this study were approved by the Malmö-Lund Animal Care Ethics Committee (M17009-18). Lung tissue used for immunoreactivity assays was obtained after written informed consent, approval by the Regional Ethical Review Board in Lund (approval no. LU412-03), and performed in accordance with the Declaration of Helsinki as well as relevant guidelines and regulations.

Fibroblast Transwell Experiment

Human lung fibroblasts (HFL-1; ATCC, Manassas, VA, United States) were cultured until confluent in complete growth medium supplemented with L-glutamine, 100 U/mL penicillin, 100 μg/ml streptomycin, and 10% FBS. Fibroblast chemotaxis was measured using 24-well Nunc (8-μm pore size) Transwell inserts (Thermo Fisher, Waltham, MA, United States). Following confluence, cells were seeded (5×10^5 cells/ml) into the upper chamber in FBS-free medium, whilst the lower chamber contained complete medium with additional 10% FBS as a chemoattractant. Experiments were carried out in experimental triplicate, with each experiment containing 4 biological replicates. Medium containing transforming growth factor beta 1 (TGF-β1; 2 ng/ml) followed by the addition of medium containing zoledronic acid (ZA; 2 μM), medium only, or vehicle only (dimethyl sulfoxide; DMSO 2% and phosphate-buffered saline (PBS) pH 7.4). Following 48 h, medium was removed and cells in the lower chamber were stained (crystal violet) and imaged using a Nikon Eclipse 80i Compound Fluorescent Microscope and analyzed using Nikon NIS Elements F4.60.00.

MTT Assay

Mosmann's MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to determine cell viability in both RAW264.7 and THP-1 cells. After the addition of the compounds at a starting concentration of 1000 μM (serial diluted to 0.1 μM), and incubation for 48 h, each well received MTT at a concentration of 5 mg/ml in phosphate-buffered saline (PBS), with blank samples receiving only medium and MTT. Each compound concentration tested in this study was completed in triplicate. DMSO was added to each well, followed by plate shaking for 5 min to dissolve the formazan crystals. The absorbance of the formazan salt was measured at 540 nm by a VICTOR 1420 Multilabel plate reader. The following formula **Eq. 1** was used to calculate the cell viability:

$$\begin{aligned} \textit{Percentage viability} = {} & \textit{sample absorbance} \\ & - \textit{blank absorbance}/\textit{control absorbance} \\ & - \textit{blank absorbance } X100 \end{aligned} \quad (1)$$

Nonlinear dose response curves were constructed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, United States) and Microsoft Excel.

Immunostaining of Embryonic Mouse Fibroblasts

Murine C57BL/6 embryonic fibroblasts (MEFs) were seeded (1×10^4 cells/mL) into 24-well plates containing rounded glass cover slips. TGF-β1 (2 ng/ml) was added to each well, followed by the addition of medium containing ZA or vehicle only. Following 48 h of treatment, cells were washed and fixed with ice cold methanol, containing 0.5% Triton-X100. Slides were blocked using Dako Protein Block (Agilent, Santa Clara, CA, United States) for 1 h at room temperature and then stained with primary antibodies (overnight), rabbit anti-COL1AI, rabbit anti-αSMA, and rabbit anti-fibronectin (Abcam, Cambridge, United Kingdom). Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen, Carlsbad, CA, United States) was used as secondary antibody (2 h). Glass cover slips were removed and mounted onto glass slides, with nuclei counterstained using DAPI containing fluoroshield (Abcam). Images were visualized using a Nikon Eclipse 80i Compound Fluorescent Microscope and analyzed using Nikon NIS Elements F4.60.00.

Ex vivo Cell Recruitment Assay

BAL was performed on C57BL/6 mice (n = 10) with a total volume of 1 ml PBS containing 100 μM EDTA. BALF was collected in Eppendorf tubes on ice and immediately separated using the Anti-F4/80 MicroBeads UltraPure (mouse; 130-110-443), dead cell removal kit (130-090-101), MS columns (130-042-201), and QuadroMACS™ Separator (130-090-976; Miltenyi Biotec, Bergisch Gladbach, Germany). Purified murine macrophages were counted and run on a BD Accuri C6 flow cytometer to determine cell purity. Concurrently, murine blood was isolated *via* heart puncture and collected in EDTA-containing tubes. Murine PBMCs were isolated using Ficoll-Paque PREMIUM 1.084 (Sigma-Aldrich, Saint Louis, MO, United States) density centrifugation (400 x g for 40 min) and subsequently counted. In the transwell plate, murine macrophages were added to the bottom well (5×10^5 cells/well), with murine PBMCs (6×10^6 cells/well) added to the top chamber. Following 24 h of RANKL (10 ng/ml) macrophage stimulation, cells were harvested and stained for flow cytometry analysis carried out using a BD Accuri C6 (BD, Franklin Lakes, NJ, United States). Samples were separated into 3 aliquots, 1) alveolar macrophages as identified by F4/80 (BM8; 11-4321-42), CD11b (BD553312), and I-A/I-E (BD557000); 2) B and T cells as identified by CD45 (BD553079), CD3 Molecular Complex (BD 555276), CD19 (BD561736); 3) macrophage subtypes and neutrophils as identified by CD11b (BD553312), CD11c (BD558079), Ly-6G (BD551461).

Mouse Macrophage (M1/M2) *Ex Vivo* Assay

BAL was performed on C57BL/6 mice (n = 10) with a total volume of 1 ml PBS containing 100 μM EDTA. BALF was collected in Eppendorf tubes on ice and immediately separated

using the Anti-F4/80 MicroBeads UltraPure and QuadroMACS™ Separator as indicated above. Isolated murine macrophages were added to 12-well plates at 5×10^8 cells/well. Cells were stimulated with interleukin-4 (IL-4) and IL-13 (20 ng/ml, PeproTech, NJ, United States) for 48 h followed by flow cytometry analysis (BD Accuri C6) to assess M1/M2 phenotype. Cells were gated using the following antibodies: CD206 (BioLegend, 141705), CD11b (BD553312), CD11c (BD558079), and F4/80 (BD 565635).

Animals

10-12-week-old male C57BL/6 mice (Janvier, Le Genest-Saint-Isle, France) were housed at least 2 weeks in the animal facility at the Biomedical Service Division at Lund University before initiating experiments and were provided with food and water *ad libitum* throughout the study. Mice were randomly allocated into five groups (n = 4-15 animals per group): intratracheally (i.t.)-administered bleomycin (2.5 U/kg) + vehicle (i.p./i.t.), bleomycin (i.t.) +ZA (0.1 mg/kg; i. p./i.t.), saline (i.t.) + vehicle (i.p./i.t.), and saline (i.t.) + ZA (0.1 mg/kg; i. p./i.t.).

siRNA Administration

For lung fibrosis experiments testing the viability of *Fdps* as a drug target in siRNA experiments, $n \geq 4$ to 5 mice per group were used to achieve statistical significance. Mice were randomly assigned to treatment groups. For the siRNA experiments, lung fibrosis was induced by intratracheal introduction of bleomycin (2.5 U/kg) or saline as control. Following 14 days, siRNA targeting mouse *Fdps* (L-059452-01–0020) or nontargeting control (D-001810-01-50) mRNA (Horizon Discovery, Water Beach, United Kingdom) was administered intratracheally at either 25 μg per mouse (*Fdps* siRNA) or 25 μg of nontargeting siRNA. On day 21, mice were euthanized followed by collection of blood, lung tissue, and BALF. Downstream analyses were conducted with the investigator blinded to the treatment groups, and no animals were excluded as outliers from the reported dataset. All *in vitro* and *in vivo* experiments were performed in two to four technical replicates. Human resected lung sections were obtained with informed consent, with a statistically significant n = 4 samples used for disease and control samples.

Blood Collection

Blood was collected in 0.5 M EDTA tubes by cardiac puncture and centrifuged at 1,000 x *g* for 10 min. Plasma supernatant was used for the analysis of inflammatory mediators using a multiplex assay (Bio-plex assay; Bio-Rad, Hercules, CA, United States).

Collection of Lung Tissue

Right lungs were collected in Eppendorf tubes on dry ice and stored at -80°C. The snap-frozen lungs were thawed and homogenized in tissue protein extraction reagent (T-PER) solution (Thermo Fisher Scientific) containing protease inhibitor (Pefabloc SC; Sigma-Aldrich) at a final concentration of 1 mM. Lung homogenates were centrifuged at 9,000 x *g* for 10 min at 4°C, and the supernatants were collected for multiplex analysis. Left lungs were collected in Histofix (Histolab, Göteborg, Sweden) and submerged in 4% buffered paraformaldehyde solution.

Bronchoalveolar Lavage Fluid (BALF) Collection

BAL was performed with a total volume of 1 ml PBS containing 100 μM EDTA. BALF was collected in Eppendorf tubes on ice, with aliquots made for flow cytometry, cytospin differential counts, and an aliquot transferred to -80°C for multiplex cytokine analysis. Cytospin preparations of cells were stained with modified Wright-Giemsa stain (Sigma-Aldrich).

SDS-PAGE and Western Blotting

Lung homogenate lysates were analyzed for total protein concentration (Pierce BCA Protein Assay Kit, Thermoscientific). Samples were run on Mini-PROTEAN® Precast Mini polyacrylamide gel electrophoresis (PAGE) Gels (BioRad). Samples were transferred to Trans-Blot Turbo Mini 0.2 μm; polyvinylidene difluoride (PVDF) Transfer Packs with blots incubated overnight with primary antibodies (1:1000) in blocking buffer, collagen 1A1 (COL1A1; ab88147), arginase 1 (ARG1; ab239731), cluster of differentiation 206 (CD206; ab64693), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ab8245), farnesyl diphosphate synthase (FDPS; ab153805, Abcam), alpha smooth muscle actin (α-SMA; 19245, cell signaling). Blots were washed with PBS-Tween, before 1 h incubation with Alexa Fluor 488-conjugated goat anti-rabbit/mouse secondary antibodies (Invitrogen, Carlsbad, CA, United States). Blots were imaged using a BioRad ChemiDoc system. Quantification was performed *via* densitometry and normalized using GAPDH.

Flow Cytometry

Flow cytometry was carried out using a BD Accuri C6 (BD). The washed cells were incubated with Fixable Viability Stain 510 (FVS510) (BD564406) to differentiate live and dead cells. Cells were washed with Stain buffer 1x (BD554656) and incubated with Lyse Fix 1x (BD558049 (5x)). Fixed cells were washed with stain buffer and aliquoted into two parts. One was incubated with CD11b (BD553312), CD11c (BD558079), Ly-6G (BD551461) antibodies and the other aliquot was incubated with CD11c, MHC (BD558593), SiglecF (BD562680) antibodies.

Bioplex Cytokine Analysis

For the detection of multiple cytokines in BALF, plasma, and lung homogenate, the Bio-Plex Pro mouse cytokine assay (23-Plex Group I; Bio-Rad) was used on a Luminex-xMAP/Bio-Plex 200 System with Bio-Plex Manager 6.2 software (Bio-Rad). A cytometric magnetic bead-based assay was used to measure cytokine levels, according to the manufacturer's instructions. The detection limits were as follows: Eotaxin (21372.02–1.15 pg/ml), GCSF (124018.4–6.97 pg/ml), GMCSF (1161.99–3.73), IFN-γ (14994.64–0.72 pg/ml), IL-1α (10337.5–0.63 pg/ml), IL-1β (28913.54–1.57 pg/ml), IL-2 (22304.34–1.21 pg/ml), IL-3 (7639.21–0.47 pg/ml), IL-4 (6334.86–0.36 pg/ml), IL-5 (12950.39–0.76 pg/ml), IL-6 (11370.16–0.66 pg/ml), IL-9 (2580.93–2.46 pg/ml), IL-10 (76949.87–4.09 pg/ml), IL-12p40 (323094.58–17.38 pg/ml), IL-12p70 (79308.46–19.51 pg/ml), IL-13 (257172.3–53.85 pg/ml),

IL-17 (8355.61–0.5 pg/ml), KC (23377.88–1.3 pg/ml), MCP-1 (223776.6–45.04 pg/ml), MIP-1α (14038.07–0.58 pg/ml), MIP-1β (928.18–2.39 pg/ml), RANTES (4721.74–4.42 pg/ml), and TNF-α (73020.1–4.61 pg/ml). Cytokine measurements for samples were corrected for protein concentration, measured using a Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific).

Hydroxyproline Assay

Hydroxyproline levels were determined using the QuickZyme Hydroxyproline Assay kit (Quickzyme Biosciences, Leiden, Netherlands). Hydroxylation of proline groups within the collagen molecule result in the formation of hydroxyproline, with the modified amino acid stabilizing the helical structure in mature collagen (Berg and Prockop, 1973). Hydroxyproline is highly specific for collagen, allowing hydroxyproline measurements to accurately reflect the amount of collagen in fibrotic lung tissue (de Jong et al., 2012). Lung tissues were homogenized as described above. Homogenates were diluted (1:1 vol:vol) with 12 N HCl and hydrolyzed at 95°C for 20 h. After centrifugation at 13,000 × *g* for 10 min, 200 µl from the supernatant was taken and diluted 1:2 with 4 N HCl. Hydroxyproline standard (6.25–300 µM) was prepared in 4 N HCl and transferred to the microtiter plate. Following addition of a chloramine T-containing assay buffer, samples were oxidized for 20 min at RT. Detection reagent containing p-dimethylaminobenzaldehyde was added and after incubation at 60°C for 1 h, absorbance was read at 570 nm with a VICTOR 1420 Multilabel plate reader (PerkinElmer, Waltham, MA, United States). The hydroxyproline content in lung tissue is given as hydroxyproline (µg) per mg lung tissue, corrected using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

H&E and Picrosirius Red Staining of Lung Tissue

A segment of the left lung was fixed in Histofix (Histolab) and embedded into paraffin and sectioned (3 µm) with a microtome. The tissue sections were placed on slides (Superfrost Plus; Fisher Scientific) and deparaffinized in serial baths of xylene and ethanol followed by staining using Mayer hematoxylin and 0.2% eosin (Histolab) or picrosirius red staining kit (Abcam, Cambridge, United Kingdom). The stained slides were imaged using an Olympus BX60F microscope with an SC50 camera.

Immunostaining of Murine Lung Sections

Lung tissue sections were fixed as reported above. Lung samples underwent antigen retrieval (pH 9 buffer) using a Dako PT Link pre-treatment module (Agilent). Samples were washed and blocked for 10 min (Dako protein block; Agilent, Santa Clara, CA) before being treated with primary antibodies overnight. Mouse anti-COL1A1, ARG1, and rabbit anti-FDPS, CD206 (Abcam, Cambridge, United Kingdom) antibodies were used. Alexa Fluor 488-conjugated goat/anti-mouse and Alexa Fluor 647 goat/anti-rabbit (Invitrogen, Waltham, CA, United States) were used as secondary antibodies. Glass cover slips were placed onto slides and mounted with DAPI-containing fluoroshield (Abcam). Microscopy was performed on a Widefield Epifluorescence Ti2 microscope equipped with a Nikon DS-Qi2 camera and fluorescence was quantified using ImageJ software.

Real-Time PCR

Extraction of total mRNA from frozen lung tissue and MEF cells was performed using RNeasy Mini Kit (Qiagen, Valencia, CA, United States), in accordance with manufacturer's instructions. RNA concentrations were determined using a NanoDrop ND1000 (Saveen Werner AB, Malmö, Sweden). RNA to cDNA conversion was achieved using an iScript Advanced cDNA Synthesis Kit (Bio-Rad). Gene expression was measured using TaqMan™ Fast Advanced Master Mix together with TaqMan™ probes listed in **Supplementary Table S1**. RT-PCR reactions were analyzed using a QuantStudio™ 7 Flex system (Thermo Fisher Scientific) in 384-well plates. Data was analyzed with QuantStudio™ Real-Time PCR Software v1.3. The obtained Ct-values were normalized to succinate dehydrogenase complex subunit A (*Sdha*) expression, generating ΔCt-values. ΔΔCt-values were obtained by normalizing values to ΔCt-of the PBS only group.

Immunohistochemistry of Human Lung Samples

Macroscopically normal, tumor-free lung tissue samples were obtained during transplantation or resection from patients undergoing cancer surgery, with IPF patients diagnosed/identified by techniques supplemented by spirometry. Patients undergoing lung biopsies with confirmed distal lung fibrosis (by histological analysis), or high-resolution computerized tomography (HRCT) scans with characteristic IPF features (e.g., opaque fibrosis-like areas and "honeycombing" in the lung parenchyma) were included in this study, with clinical data reported in **Supplementary Table S2**. Immediately after collection, samples were placed in 4% buffered formaldehyde. Following dehydration and embedding in paraffin, sections (3 µm) were produced. A single staining protocol (EnVision™ Detection system, K5007, Dako, Glostrup, Denmark) was used for visualization of FDPS. Briefly, after antigen retrieval, FDPS was detected using rabbit anti-FDPS (Abcam) antibody (1:1000) and visualized using secondary goat anti-rabbit antibodies conjugated with peroxidase polymers (Dako). Sections were counter-stained with Mayer's hematoxylin for visualization of background tissue, dehydrated in alcohol/xylene, and mounted on Pertex (Histolab, Göteborg, Sweden). The stained slides were imaged using an Aperio CS2 image capture device. Positive staining was quantified manually (number of cells/area of tissue) or as positivity (positive brown pixels divided by all stained pixels) using computerized image analysis on blinded sections using ImageScope (Aperio, Leica Biosystems, Wetzlar, Germany).

Statistical Analysis

To assess the effects of ZA in murine models, results were compared using one-way analysis of variance (ANOVA) with

Dunnett's post hoc test. In experiments using two groups, results were compared using an unpaired *t*-test with Welch's correction. Results in this study are displayed throughout as mean ± standard error of the mean (SEM). Statistical testing was carried out using GraphPad Prism 9.1.1 with statistical significance defined as $p < 0.05$.

RESULTS

Zoledronic Acid Reduces Fibroblast to Myofibroblast Transition and Production of Fibrotic Proteins

To determine ZA involvement in fibroblast to myofibroblast transition (FMT), human lung fibroblasts (HFL-1) were utilized in a transwell assay (**Figure 1A**). Following stimulation with TGF-β1 for 48 h, cell transition through the transwell membrane was measured. ZA treatment (2 μM) significantly reduced myofibroblast transition ($p < 0.001$). Additionally, staining of mouse embryonic fibroblast cells (MEF) treated with ZA revealed the production of less collagen, fibronectin, and α-smooth muscle actin than in the TGF-β1 control, as measured by immunofluorescence (**Figure 1B**).

Reduced NF-κB Macrophage Signaling Moderates Immune Cell Recruitment

To demonstrate the role of ZA in reducing NF-κB signaling, monocytic (THP1-Blue™ NF-κB) cells were stimulated with RANK ligand (RANKL) for 48 h (**Figure 1C**). ZA significantly reduced RANKL-induced NF-κB signaling at all tested concentrations with ZA not displaying cytotoxicity in either murine macrophages (RAW264.7) or THP1 cells at levels greater than 3 μM (**Figure 1D**). Following reductions in NF-κB, we examined macrophage-mediated immune cell recruitment from murine peripheral blood inhibition by ZA treatment (**Figure 1E**). A wide variety of immune cells were measured using flow cytometry and revealed significant decreases between the RANKL and ZA/RANKL treated samples in recruitment of T cells, neutrophils, inflammatory macrophages, and alveolar macrophages (**Figure 1F**).

Zoledronic Acid Attenuates Murine Weight Loss and Fibrotic Lung Damage

Given the *in vitro* effect of ZA treatment in reducing fibrotic-related damage, a murine model of bleomycin-induced lung damage was employed, with ZA dosed either intratracheally (i.t.) or intraperitoneally (i.p.; 0.1 mg/kg) at day 15 (**Figure 2A**). Murine weight loss was dramatically mitigated following i. p. ZA treatment, whilst i. t. ZA treatment offered less protection (**Figure 2B**). The probability of survival was significantly increased following ZA i. p. administration ($p = 0.0329$), whereas i. t. administration showed no statistically significant difference (**Figure 2C**). Murine lung weight and hydroxyproline levels provide proxies for generalized lung damage and collagen content, with i. p. administration of ZA reducing both lung weight ($p < 0.0001$) and hydroxyproline levels ($p = 0.0143$) compared to vehicle/bleomycin mice (**Figures 2D,E**). Additionally, western blot analysis on lung tissue showed a significantly reduced production of the fibrotic proteins COL1A1 and α-SMA after administration of ZA (**Figure 2F** and **Supplementary Figure S8**). Images of murine lungs revealed decreased areas of fibrosis after i. p. treatment with ZA (**Figure 2G**).

Murine Immune Cell Recruitment to the Lung Is Impaired by Zoledronic Acid

Administration of ZA effectively reduces regulatory T cell involvement in immune cell recruitment in cancer and fibrosis (Ichikawa et al., 2019; Liu et al., 2019), with a large proportion of these recruited cells being macrophages and neutrophils. Investigation of ZA treatment effects on immune cell infiltration into the airways was performed by flow cytometry on BALF obtained from *in vivo* bleomycin studies at day 21. Decreases in neutrophil count and inflammatory macrophages were seen following both i. p. ($p < 0.0001$) and i. t. (neutrophils: $p = 0.0417$; inflammatory macrophages: $p = 0.0213$) ZA administration compared to vehicle/bleomycin samples (**Figures 3A–C**). Giemsa-Wright-stained BALF samples showed bleomycin-treated macrophages were enlarged and present in greater number, a phenomenon not seen in mice treated with ZA (i.p.; **Figure 3D**).

Murine Lung Structure Is Maintained Following Intraperitoneal ZA Administration

To determine whether murine histological damage was reduced more efficaciously by a single route of administration, both i. p. and i. t. lung samples from mice treated with ZA were assessed using H&E and picrosirius red staining (**Figures 4A,B**; **Supplementary Figure S1 and 2**). ZA administered i. p. quantitatively reduced bleomycin-induced closure of the alveolar structures ($p < 0.0001$), with significant decreases in collagen deposition ($p < 0.0001$) seen in these samples compared to the vehicle/bleomycin samples. Conversely, intratracheally-administered ZA did not confer protection from lung damage as seen in both the H&E ($p = 0.7009$) and picrosirius red staining ($p = 0.0839$).

Cytokine Production Elucidates Decreased Immune Cell Recruitment Following ZA Treatment

The effect of i. p. ZA treatment on inflammatory cytokine levels was investigated in murine samples from the *in vivo* study using a 23-cytokine inflammation multiplex assay (**Supplementary Figures S3–S5**). The majority of cytokines were reduced in the BALF, plasma, and lung tissue following ZA treatment. In particular, lung homogenate decreases were seen for IL-4 ($p = 0.0289$), IL-6 ($p = 0.0007$), GM-CSF ($p < 0.0001$), KC ($p < 0.0001$), and MCP-1 ($p = 0.0389$) compared to the vehicle/bleomycin

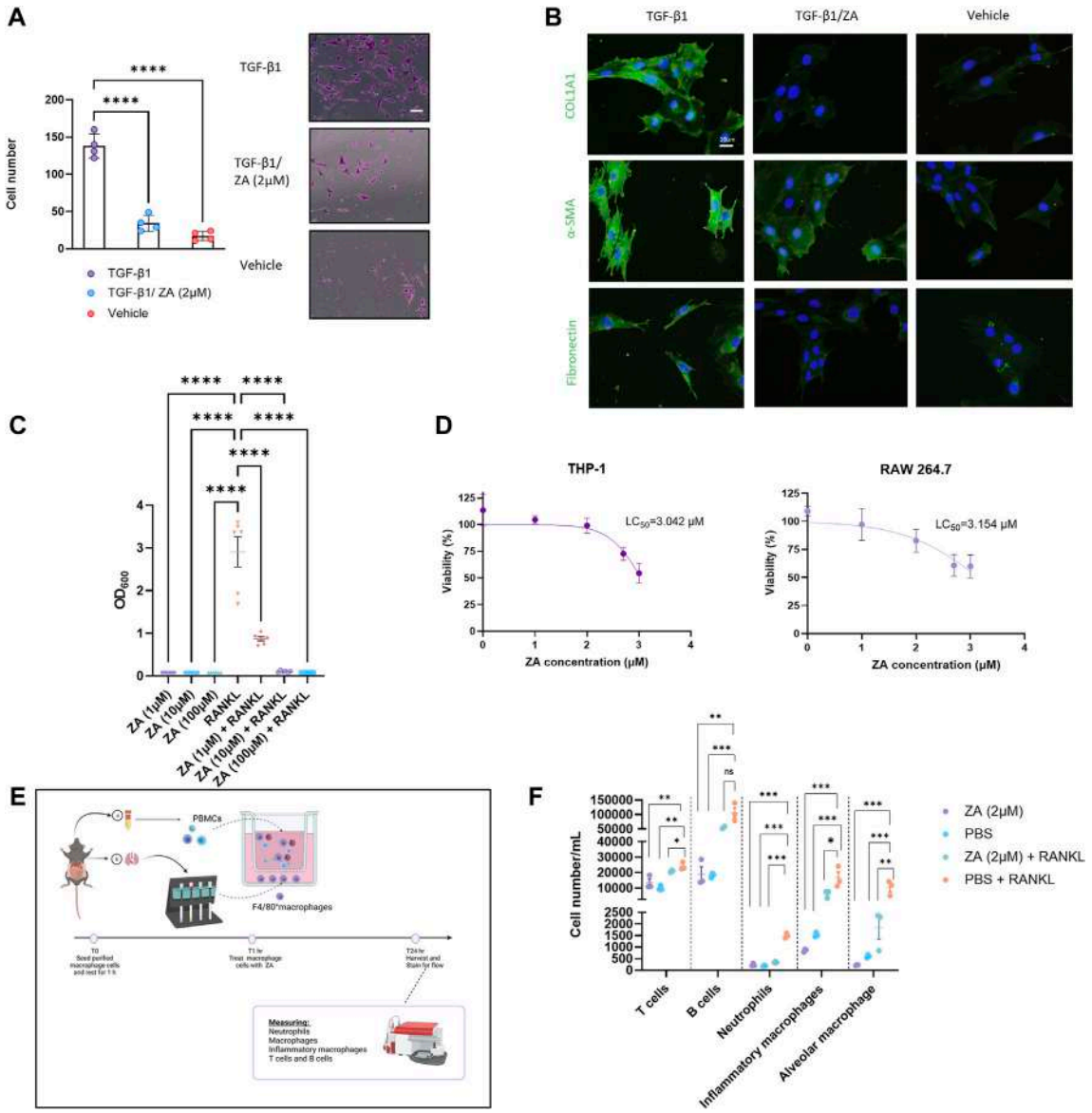

FIGURE 1 | Zoledronic acid (ZA) inhibits *in vitro* transwell migration and fibrotic marker expression, whilst simultaneously reducing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling and immune cell recruitment. **(A)** ZA reduced myofibroblast migration and proliferation in a transwell assay compared to vehicle treatment and no treatment controls (Student's *t*-test, $p < 0.005$ (***) and $p < 0.0001$ (****), with representative myofibroblast cells in lower chamber of transwell insert following 48 h shown. **(B)** Immunostaining of fibroblast cells following 48 h of TGF-β1 treatment, with ZA treatment displaying visually reduced levels of collagen, fibronectin and alpha smooth muscle actin (α-SMA) compared to no treatment control. **(C)** RAW264.7 and THP1-Blue™ NF-κB cells were subjected to receptor activator of nuclear factor kappa beta (RANKL) treatment for 48h, with NF-κB signaling significantly reduced by ZA ($p < 0.0001$) and **(D)** low levels of cytotoxicity reported for ZA against both THP-1 and RAW 264.7 cells. **(E,F)** Isolated murine macrophage cells (bottom well) and peripheral blood mononuclear cells (PBMCs) (top well) were exposed to RANKL in a transwell system, with significant reductions in T cells, neutrophils, and macrophage cells following ZA treatment (drug-treated samples were compared to untreated control samples using a one-way ANOVA followed by a Dunnett's post-hoc test; $N = 3$ technical replicates, from 3 biological replicates per measurement).

group (**Figures 5A–C**). Decreased immune cell recruitment following ZA treatment in murine BALF is supported by reduced murine cytokine levels in murine BALF and plasma. Shared decreases were seen in both fluids for IL-6, GM-CSF, KC, MCP-1, and other cytokines indicative of reduced immune cell recruitment following ZA (i.p.) treatment (**Figures 5A,B**; **Supplementary Figure S3–S5**).

Bleomycin-Induced FDPS Production in Murine Lung Tissue Is Decreased by Zoledronic Acid Treatment

Increased flux through the mevalonate pathway enhances posttranslational modification of Rac1, promoting macrophage/fibroblast signaling (Larson-Casey et al., 2014,

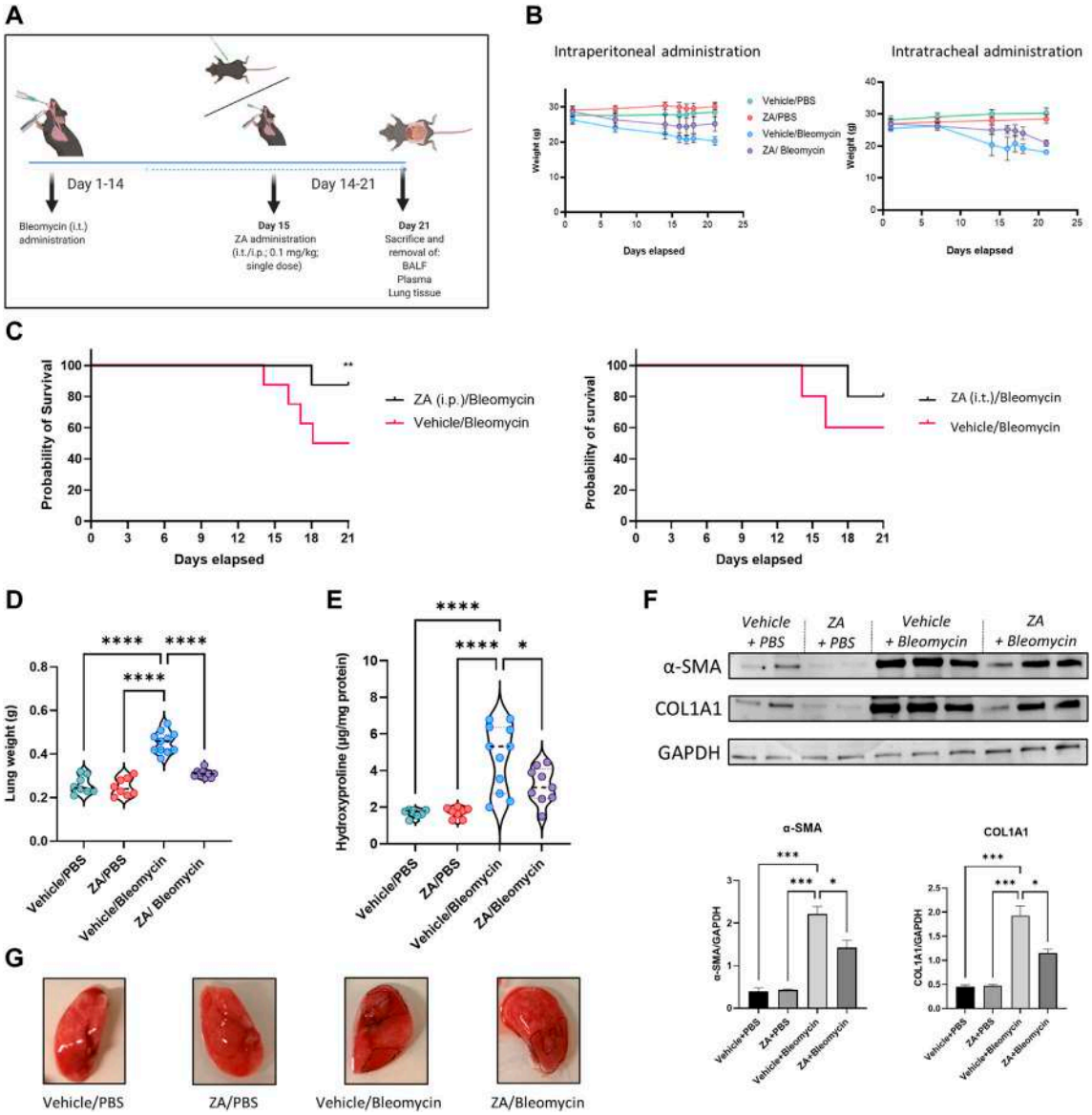

FIGURE 2 | ZA administration, murine weights, survival curve, and lung readouts. **(A)** ZA was administered intraperitoneally (i.p.) and intratracheally (i.t). on day 15 post-bleomycin introduction, with **(B)** murine total weight remaining stable in the ZA (i.p.) group for the duration of 21 days, with both bleomycin groups displaying significant weight loss after 14 days. **(C)** Resultant weight loss in the bleomycin/vehicle group caused significant mortality, with mice in the ZA/Bleo (i.p. and i. t.) groups seemingly protected. **(D)** Murine lung weight showed significant reductions in the ZA treated mice compared to the vehicle/bleomycin group (drug-treated samples were compared to untreated control samples using a one-way ANOVA followed by a Dunnett's post-hoc test: $^{****}p < 0.0001$). **(E)** Hydroxyproline collagen readout displayed significant reduction following ZA (i.p.) treatment (drug-treated samples were compared to untreated control samples using a one-way ANOVA followed by a Dunnett's post-hoc test: $^{****}p < 0.0001$). **(F)** Immunoblotting of ZA-treated murine lung lysates displayed significant reductions in both α-SMA and COL1A1 compared to bleomycin only control lysates **(G)** Representative murine images show bleomycin induced lung damage (collagenous deposition highlighted in dotted areas). Mice were allocated into groups as follows: Vehicle (i.p.)/phosphate buffered saline (PBS; i. t.); zoledronic acid (ZA; i. p.)/PBS (i.t.); Vehicle (i.p.)/Bleomycin (i.t.); ZA (i.p.)/Bleomycin (i.t.).

2016; Politiek and Waterham, 2021). Further elucidation of this mechanism was conducted by probing the downstream signaling pathway (**Figure 6A**) by assessing farnesyl diphosphate synthase (FDPS). Bleomycin-induced FDPS production was significantly decreased following ZA administration, with ZA/bleomycin samples displaying significantly reduced fluorescence ($p = 0.0077$) and immunoreactivity compared to the vehicle/bleomycin group (**Figures 6B,C**). Western blot analysis revealed reduced levels of FDPS in lung homogenates in the ZA/bleomycin group compared to the vehicle/bleomycin group (**Figure 6D**). Furthermore, RT-PCR analysis on lung tissues showed a

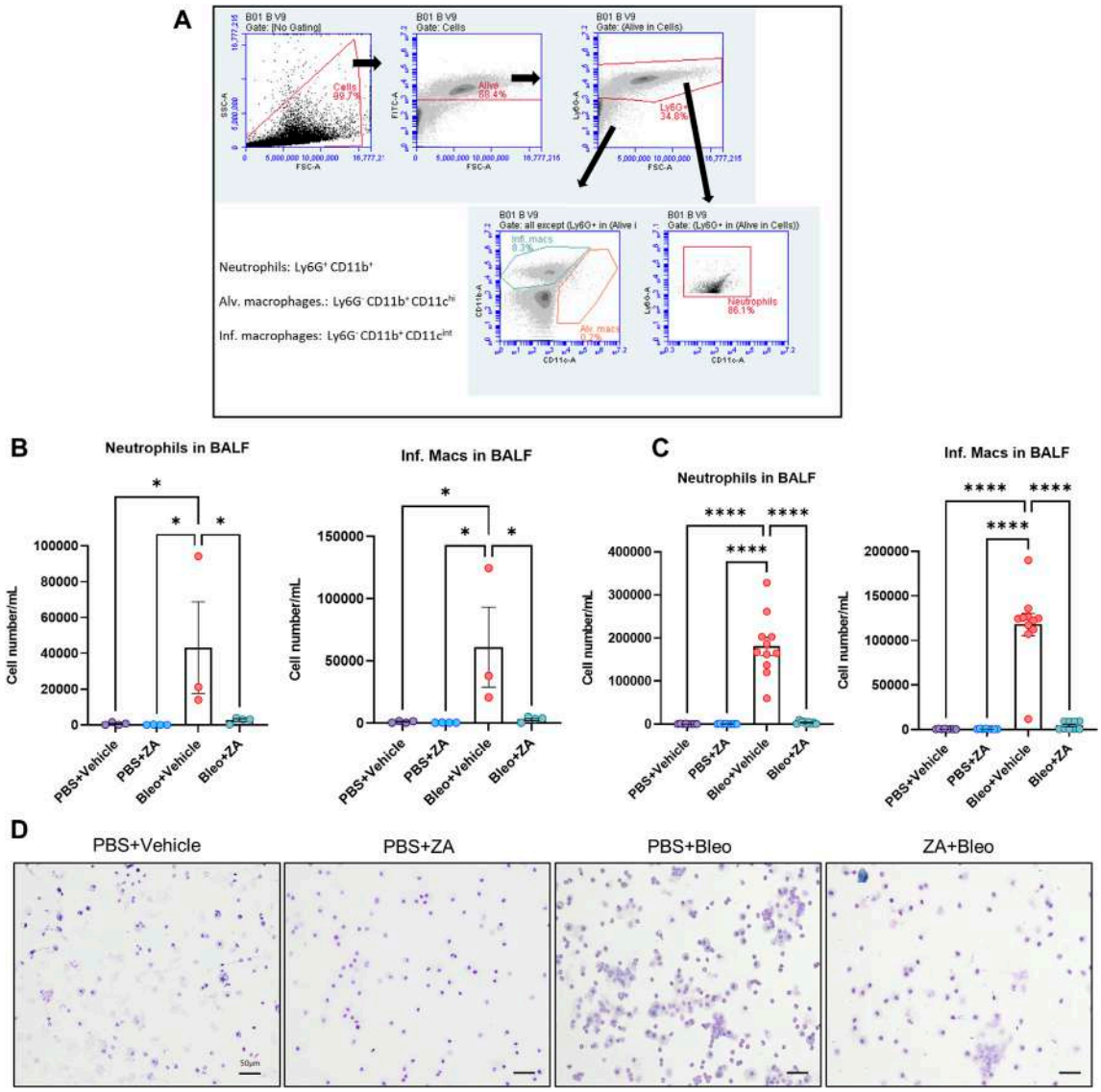

FIGURE 3 | Inflammatory cell influx measured in murine bronchoalveolar lavage fluid (BALF). Murine BALF was assessed for neutrophils and inflammatory macrophages **(B,C)** using flow cytometry, with the representative gating strategy depicted inset **(A)**. Decreased numbers of neutrophils and inflammatory macrophages were detected in response to ZA (i.t.) treatment, with increased significant differences reported between the neutrophils and inflammatory macrophages of mice treated with ZA (i.p.). No significant differences were reported for alveolar macrophage numbers (not shown). Inflammatory cell numbers were compared to the vehicle/bleomycin group using a one-way ANOVA followed by a Dunnett's post-hoc test (*$p < 0.05$; ****$p < 0.0001$). **(D)** Giemsa-Wright stained cytospin slides showing PBS/bleomycin BALF samples containing enlarged inflammatory macrophages, with ZA (i.p.) treatment reducing the presence of inflammatory macrophages. Scale bar = 20 μm.

significantly increased expression of the downstream enzyme *Fdft1* in the vehicle/bleomycin group compared to the ZA/bleomycin group (**Figure 6E**). Trends towards a decreased expression of *Fdps* and the upstream enzyme *Hmgcs1*, after ZA treatment, were also found. No statistical difference in gene expression was seen for the downstream enzyme *Dhdds,* indicative of preferential targeting of the steroid synthesis pathway by ZA. RT-PCR analysis of *Fdps* expression in MEF cells after TGF-β1 exposure showed an increase in FDPS, which was reduced upon ZA treatment (**Figure 6F**). Immunofluorescence staining of the MEF cells revealed a similar trend with enhanced FDPS staining after exposure to TGF-β1, and a decreased staining following ZA treatment (**Figure 6G**).

The role of human IPF and control samples in this study was to demonstrate the potential translational utility of targeting FDPS in IPF patients. Lung samples were stained for FDPS expression using 3,3′-diaminobenzidine (DAB) chromogenic

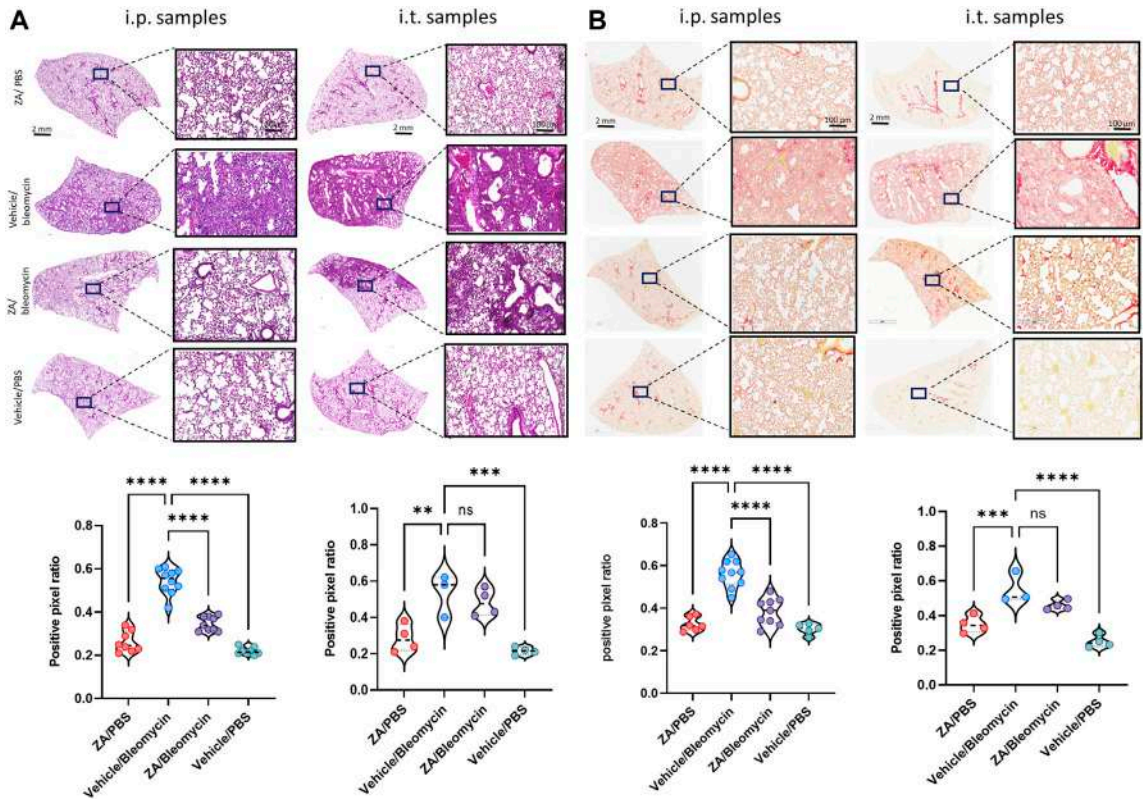

FIGURE 4 | Murine lung staining displays reduced levels of fibrotic-related lung damage following ZA (i.p.) treatment compared to bleomycin/vehicle samples **(A)** ZA (i.p.) significantly decreased lung damage in bleomycin-treated mice (H&E) and **(B)** collagen deposition (picrosirius red) in both macroscopic and microscopic structures compared to vehicle/bleomycin lungs and was confirmed by positive pixel analysis of whole-lung scanned images (scale bar of microscopic image = 100 µm; scale bar of whole lung scan = 2 mm). Statistical analyses were conducted using a one-way ANOVA followed by a Dunnett's post-hoc test (**$p < 0.01$; ***$p < 0.005$; *$p < 0.0001$).

staining. Human IPF resected lung samples displayed significantly increased FDPS immunoreactivity compared to controls, indicative of a role for FDPS in IPF (**Figure 6H** and **Supplementary Figure S6**).

Intratracheal Administration of siRNA Confirms FDPS as a Tractable Target for Idiopathic Pulmonary Fibrosis

To verify that reduced FDPS levels in the lungs explains ZA's ability to reduce bleomycin-induced lung damage, intratracheal administration of *FDPS* siRNA was used (**Figure 7A**). Measurement of FDPS protein levels in lung homogenates using western blot confirmed a significant reduction of FDPS levels after siRNA addition (**Figure 7B**). Similar to the ZA treatment, administration of *FDPS*-targeting siRNA resulted in a reduced murine weight loss (**Figure 7C**). Lung weights and hydroxyproline levels were significantly decreased after *FDPS* siRNA administration compared to the control siRNA/bleomycin group (**Figures 7D,E**). Measurements on inflammatory cytokine levels in the BALF after FDPS siRNA administration revealed a very similar pattern to the ZA treatment, whereby a reduction was seen in almost all investigated cytokines compared to the control siRNA/bleomycin group (**Figure 7F** and **Supplementary Figure S7**). Histological evaluation of lung sections from control siRNA/bleomycin treated mice revealed more closure of alveolar structures and increased collagen deposition, whereas lung sections from mice treated with *FDPS*-targeting siRNA were comparable to the control mice (**Figure 7G**). Furthermore, western blot analysis on synthesis of the fibrotic proteins COL1A1 and α-SMA showed a significant decrease in FDPS siRNA treated compared to control siRNA/bleomycin treated mice (**Figure 7H**). Finally, immunofluorescence staining of FDPS in lung sections was amplified in mice treated with control siRNA/bleomycin, which was reduced to control levels upon FDPS siRNA administration (**Figure 7I**).

Farnesyl Diphosphate Synthase Targeting Results in Anti-fibrotic Macrophage Reprogramming

Previous studies have implicated M2 macrophages in IPF pathogenesis, with pro-fibrotic M2 macrophages characterized by expression of CD206 as well as increased metabolism of arginine into ornithine and urea by arginase (Ruaro et al.,

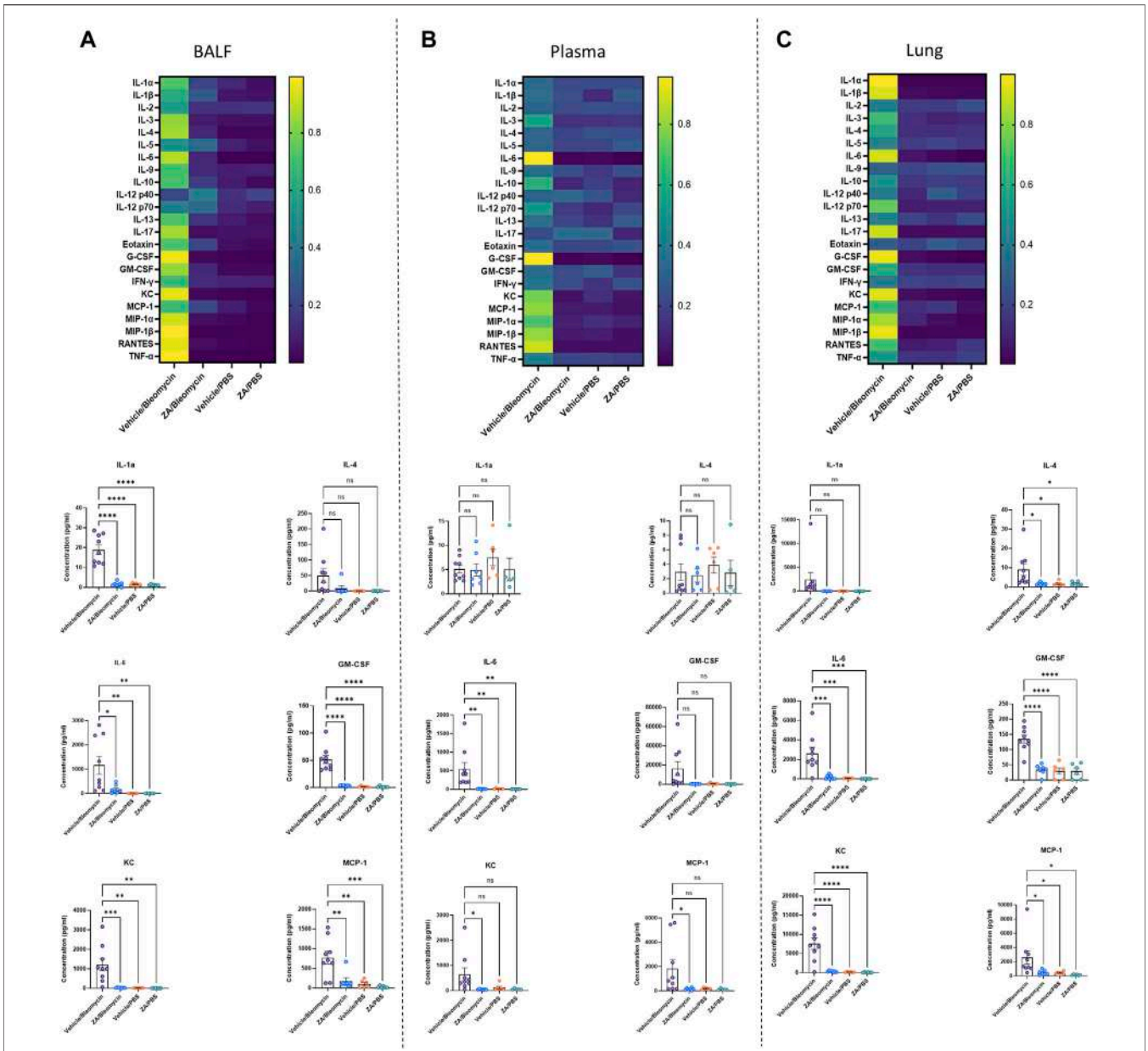

FIGURE 5 | Significantly decreased murine cytokines following intraperitoneal ZA administration. Heatmaps **(A–C)** showing the differences in cytokine levels, as measured by multiplex assay, in murine BALF, plasma, and lung homogenate (yellow indicating high value; blue indicating low value) with selected corresponding significantly decreased or increased cytokine levels below. Cytokine values were compared to the vehicle/bleomycin group using a one-way ANOVA followed by a Dunnett's post-hoc test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ****$p < 0.001$).

2019; Papadimitriou et al., 2022). Stimulation of macrophages with profibrotic cytokines IL-4 and IL-13 lead to an increased number of M2 macrophages and fewer M1 macrophages indicative of a pro-fibrotic polarization. Addition of ZA to murine *ex vivo* macrophages stimulated with IL-4 and IL-13 resulted in retention of M1 macrophages and a reduction of M2 macrophages (**Figure 8A**). Protein levels of Arg1 and CD206 in the lung, measured by western blot, were found to be significantly increased in the bleomycin/vehicle group, whereas treatment with ZA resulted in a significant decrease of both proteins (**Figure 8B**). This was supported by a trend towards an increased expression of Arg1 seen in the bleomycin/vehicle animals, which was reduced after treatment with ZA (**Figure 8C**). No differences in expression of *CD206* were found between the groups. Immunofluorescence staining of lung sections revealed a bleomycin induced increase in fluorescence from both ARG1 and CD206 compared to the controls. The fluorescent signal for both proteins was almost undetectable in the lung sections from ZA treated mice, and a faint signal was seen in the FDPS siRNA treated mouse lungs (**Figures 8D,E**).

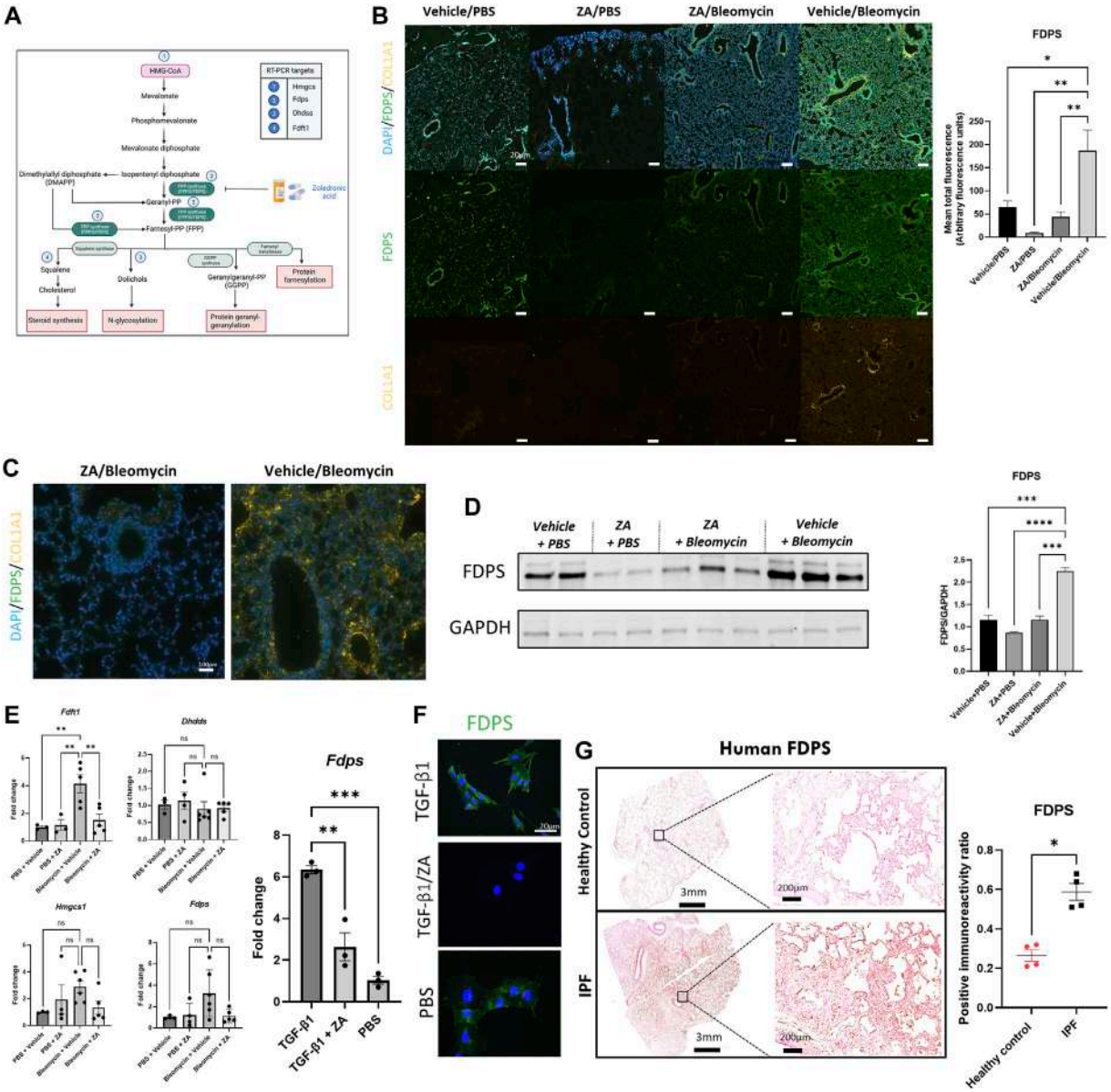

FIGURE 6 | Lower production of FDPS and COL1A1 in murine lungs upon ZA treatment. (A) Mevalonate pathway with key enzymes and intermediates highlighted inset and numbered points indicating PCR measurements (Hmgcs: hydroxymethylglutaryl-CoA synthase; Fdps: farnesyl diphosphate synthase; Dhdds: dehydrodolichol diphosphate synthase; Fdft1: farnesyl-diphosphate farnesyl transferase). **(B)** Murine lung immunofluorescence analysis showed decreased levels of immunoreactivity for FDPS and collagen 1A1 (COL1A1) in ZA (i.p.)-treated mice compared to bleomycin (Bleo) controls (scale bar = 20 μm), with quantification of green fluorescence. ZA-treated and control samples were compared to bleomycin-treated samples using a one-way ANOVA followed by a Dunnett's post-hoc test (*p < 0.05; **p < 0.01) and enlarged images of murine airways displaying co-staining of FDPS and COL1A1 **(C)**. **(D)** Mouse lung homogenate samples analyzed by SDS-PAGE, followed by immunoblotting using rabbit antisera specific to FDPS revealed decreased protein levels within ZA-administered mice. **(E)** RT-PCR analysis of murine lung lysates reveals decreased levels of *Fdft1* and *Fdps* in ZA-treated samples compared to bleomycin controls. Murine embryonic fibroblast cells stimulated with TGF-β1 and treated with ZA/PBS for 48 h displayed significantly reduced *Fdps* levels compared to TGF-β1 control as confirmed by **(F)** immunofluorescent staining of TGF-β1 treated MEF cells (scale bar = 20 μm). **(G)** FDPS immunoreactivity was measured in human IPF and control resected lung tissues, with IPF tissues showing significantly more immunoreactivity than control tissue (*p = 0.0286) as compared using an unpaired t test with Welch's correction. Scale bar = 3 mm and 200 μm for inset images.

DISCUSSION

With current drug targets and therapies predominantly directed toward fibroblast-mediated collagen deposition and fibrotic intercession, our focus turned to indirect intervention into immune cell-mediated inhibitory activities through mevalonate intermediate targeting. The mevalonate pathway has shown utility in multiple diseases, with evidence supporting mevalonate regulation of cell proliferation and energy homeostasis (Clendening et al.,

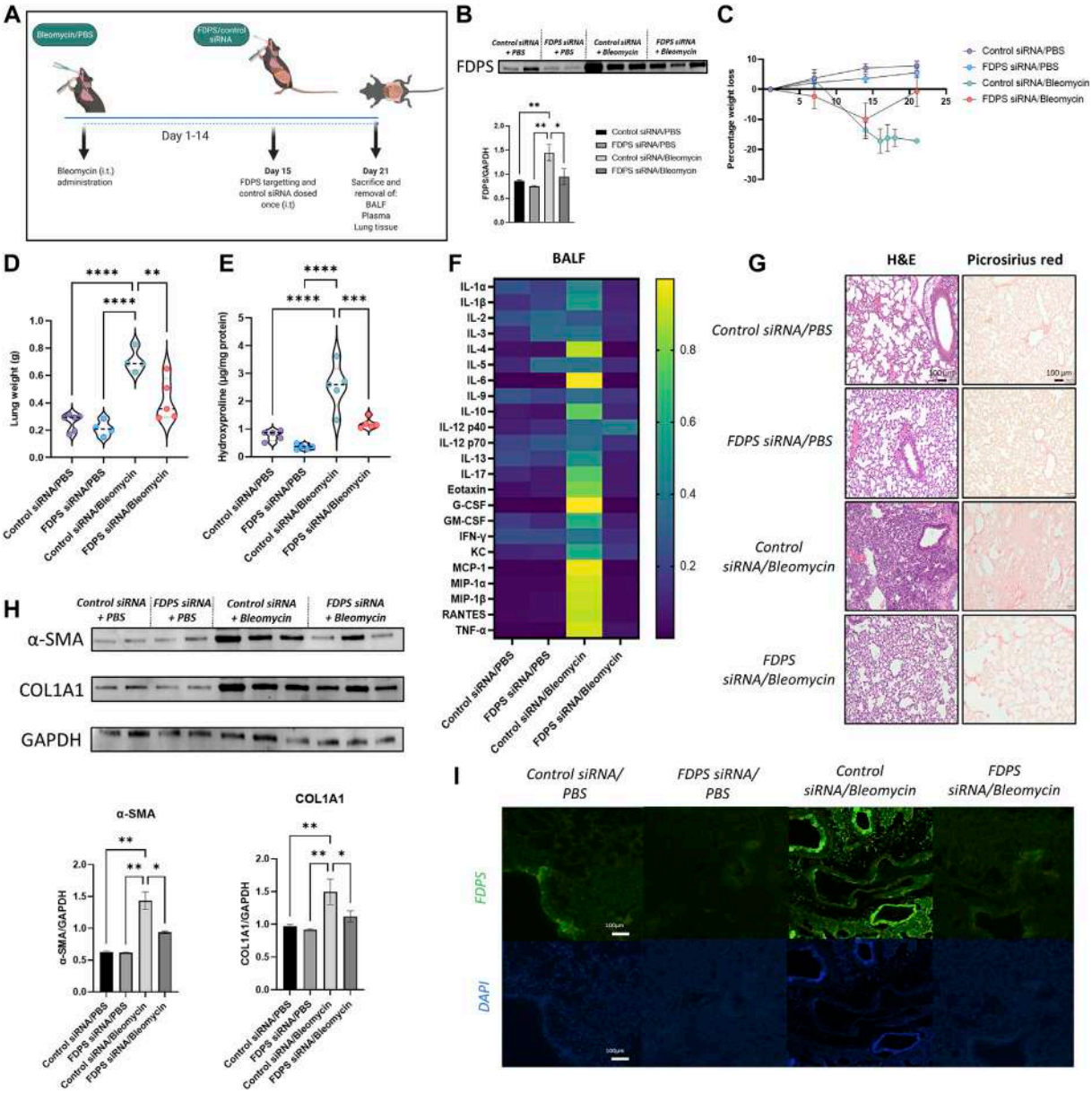

FIGURE 7 | Intratracheal administration of FDPS siRNA confers protection to bleomycin-challenged mice. **(A)** Mice were subjected to bleomycin treatment at day 0, administrated FDPS or control siRNA intratracheally on day 15 and sacrificed on day 21. **(B)** SDS-PAGE followed by western blot analysis using antisera specific for FDPS in mouse lung homogenates confirms significantly decreased levels of FDPS after siRNA administration. Samples were compared using a one-way ANOVA followed by a Dunnett's post-hoc test (*$p < 0.05$, **$p < 0.01$). **(C)** Measurement of murine weight loss following bleomycin exposure shows a protective effect of FDPS siRNA compared to control siRNA. **(D)** Bleomycin induced increases in murine lung weights were significantly reduced after FDPS siRNA treatments compared to control siRNA. Comparison between groups were performed using a one-way ANOVA followed by a Dunnett's post-hoc test (**$p < 0.01$, ****$p < 0.0001$). **(E)** Hydroxyproline levels, which is a measurement of collagen content, was significantly increased in mice treated with bleomycin/control siRNA compared to the FDPS siRNA treated mice. Differences between groups were calculated with a one-way ANOVA followed by a Dunnett's post-hoc test (***$p < 0.001$, ****$p < 0.0001$). **(F)** Heatmap showing the difference in inflammatory cytokine levels in BALF between treatment groups (blue indicating low value; yellow indicating high value). **(G)** Bleomycin/control siRNA mice showed increased lung damage, determined by H&E, and more intense collagen staining (picrosirius red). Lungs from mice receiving FDPS siRNA were resembling lungs from control mice regarding both H&E and collagen. **(H)** Detection and quantification of the fibrotic proteins α-smooth muscle actin (α-SMA) and collagen (COL1A1) using SDS-PAGE followed by western blot revealed a significant increase in both proteins in lungs from bleomycin/control siRNA mice, which was reduced significantly with FDPS siRNA treatment. **(I)** Immunofluorescence staining of lung sections with anti FDPS antibodies displaying an increase immunoreactivity in bleomycin/control siRNA mice, whereas lungs from mice administered FDPS siRNA were similar to lungs from control mice.

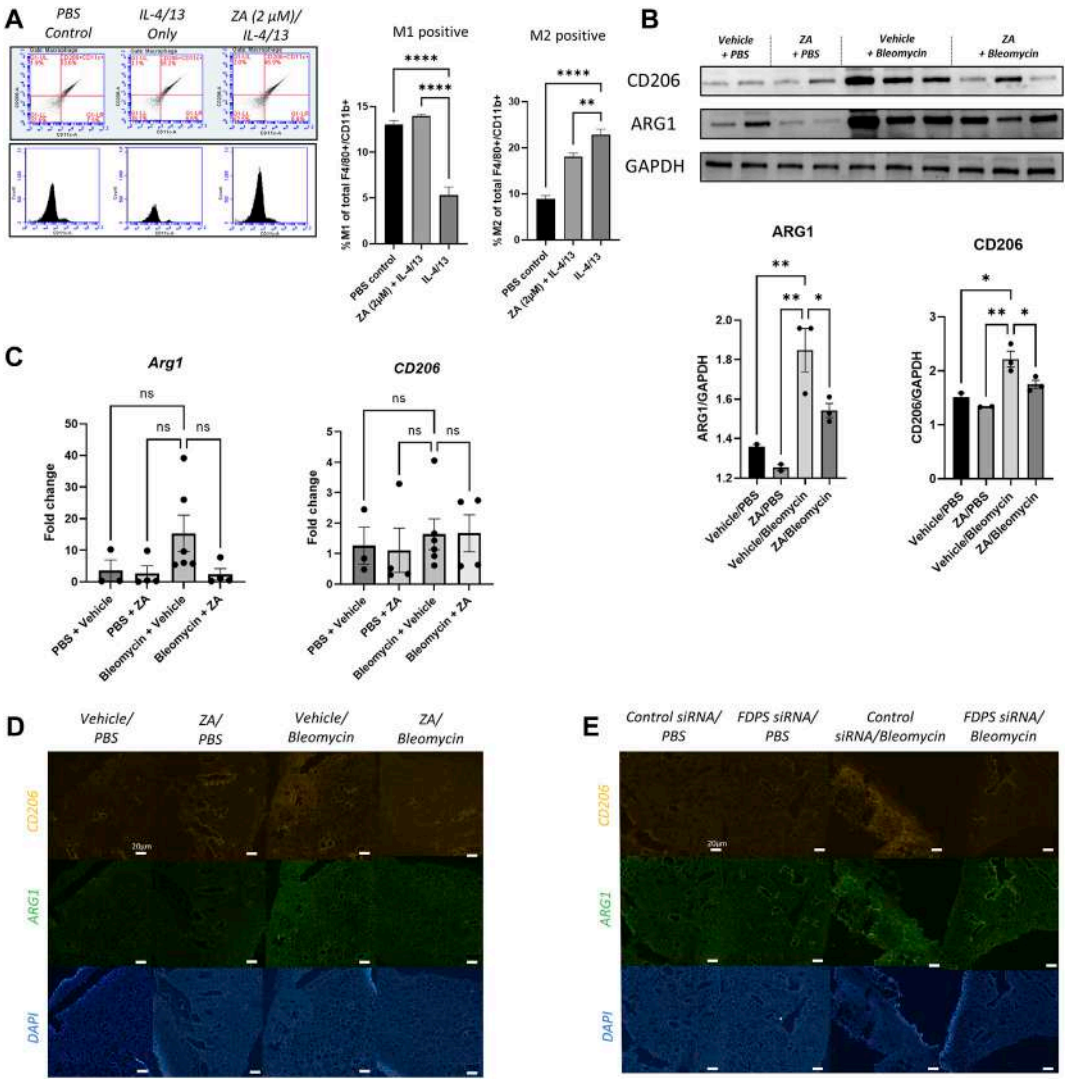

FIGURE 8 | Decreased profibrotic macrophage markers following ZA or FDPS siRNA treatment **(A)** *Ex-vivo* macrophages were analyzed for M1/M2 using flow cytometry, with the gating strategy shown. Stimulation with IL-4/13 for 48 h shifted the macrophages from M1 to M2, which was in part blocked after ZA administration. M1 positive ($CD206^-/CD11c^+/F4/80^+/CD11b^+$) and M2 positive ($CD206^+/CD11c^-/F4/80^+/CD11b^+$) macrophages graphed as percentages. Statistical comparison between groups were performed using a one-way ANOVA with Dunnett's post-hoc test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). **(B)** SDS-PAGE followed by western blot with antibodies for arginase 1 (ARG1) and cluster of differentiation 206 (CD206). Intensity quantification, normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), of the bands showed decreased protein levels of ARG1 and CD206 after treatment with ZA. Statistical comparison between groups were performed using a one-way ANOVA with Dunnett's post-hoc test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). **(C)** Real-time quantitative PCR (RT-qPCR) analysis of Arg1 and *CD206* in lung after bleomycin exposure and treatment with ZA. The mRNA expression was normalized against succinate dehydrogenase complex subunit A (*Sdha*) and fold change was calculated by difference between all treatment groups compared to PBS/vehicle. Statistical differences between groups were calculated using a one-way ANOVA with Dunnett's post-hoc test **(D,E)** Immunofluorescence staining of ARG1 and CD206 in lung sections revealed an increased signal intensity after bleomycin exposure, which was reduced with administration of ZA **(D)** or FDPS siRNA **(E)**.

2010; Gruenbacher and Thurnher, 2017; Larson-Casey et al., 2019).

HMG-CoA reductase inhibitors such as statins, are reversible competitive inhibitors of HMG-CoA reductase, a molecule that catalyzes the de-acylation of HMG-CoA to mevalonate in cholesterol biosynthesis (Hampton et al., 1996; John R; Burnett and Vasikaran, 2002). Statins have predominantly been used to probe the mevalonate pathway in interstitial lung diseases, offering mixed results. Whilst lung function decline and hospitalizations in IPF patients from the CAPACITY and ASCEND trials was diminished by statin use (Alexeeff et al., 2007; Kreuter et al., 2017), the post-hoc analysis of the INPULSIS trials did not establish beneficial effects of statin use in IPF patients (Kreuter et al., 2018). Furthermore, an FDA report associated statin use with onset of interstitial lung disease (Fernández et al., 2008), with statins shown to increase lung fibrosis in a murine bleomycin model (Xu et al., 2012).

The approach reported in this study utilized a third-generation bisphosphonate, ZA, already employed for the treatment of osteoporosis, through potent effects against the resorptive activity of osteoclasts (Dunstan et al., 2007). This activity is mediated by inhibiting farnesyl diphosphate synthase (FDPS), an enzyme responsible for catalysing the conversion of isopentenyl pyrophosphate and dimethylallyl pyrophosphate to their geranylated and farnesylated states, respectively (Kim et al., 2018). Herein, we report several arguments supporting FDPS targeting by ZA as an appealing and clinically relevant treatment for IPF.

Reported data indicate that increased geranylgeranylation substrates increase Rac1 activity in macrophages, with resultant polarization to a profibrotic phenotype (Larson-Casey et al., 2019). Rac1 also mediates ROS generation (Osborn-Heaford et al., 2012), with IPF-derived fibroblasts and macrophages generating higher levels of ROS (Waghray et al., 2005). Furthermore, IPF-derived BAL cells displayed increased apoptotic resistance and increased TGF-β1 production (Larson-Casey et al., 2016). Our study found that TGF-β1-stimulated HFL-1 cells treated with ZA were less prone to myofibroblast transition and produced decreased levels of fibrotic proteins. RANKL induces phenotypic switching in macrophages from the initial state to M1-like cells, whereas IL-4, IL-10, IL-13, and other cytokines secreted by T helper cells are involved in M2 activation, skewing macrophages toward a profibrotic lineage (Huang et al., 2017). RANK additionally provokes biochemical signaling *via* the recruitment of intracellular TNF receptor-associated factors (TRAFs) after ligand binding and receptor oligomerization. We showed that ZA-treated monocytic cells stimulated with RANKL displayed reduced NF-κB signaling, a key regulator of proinflammatory responses in immune cells. In addition, *ex vivo* macrophage cells from murine BALF exposed to RANKL stimulation and ZA treatment displayed reduced cell recruitment efficiency.

Importantly, uncontrolled lung injury and immune cell recruitment is a hallmark of IPF initiation and progression, resulting in pro-inflammatory and pro-fibrotic cytokine release driving further fibrosis-related immune cell influx and ECM remodeling (Wynn and Ramalingam, 2012; Spagnolo et al., 2018; Upagupta et al., 2018; Baratella et al., 2021). Whilst recent efforts have focused on removal of senescent or damaged fibroblasts (David et al., 2017; Schafer et al., 2017), our approach advantageously suppresses pro-fibrotic mediators and immune cell recruitment with reduced cytotoxicity.

To elucidate the mechanism by which these effects were mediated, we examined murine lung samples. Whilst no direct targeting of FDPS has, to our knowledge, been reported in the airways, FDPS has shown involvement in the progression of cardiac remodelling, with *FDPS*-targeting siRNA attenuating murine cardiac fibrosis (Zhao et al., 2016). This is further supported in our study, with *FDPS*-targeting siRNA reducing fibrotic lung damage. FDPS has displayed involvement in cardiac remodelling, with FDPS-targeting siRNA attenuating murine cardiac fibrosis (Zhao et al., 2016). Furthermore, ZA's ability to decrease FDPS production was demonstrated in human lung tissue, murine lung tissue, and directly in fibroblast cells, correlating with subsequent decreases in collagen deposition. In addition, our study examined the most appropriate route of ZA administration, a key factor in any drug discovery program. Surprisingly, intraperitoneal administration of ZA produced the most effective alleviation of lung fibrosis. We hypothesize that intratracheal administration of ZA to the murine lung may produce cytotoxic damage or result in elimination of the macrophage population entirely [44]. Conversely, intraperitoneal introduction of ZA displayed the ability to repolarize murine macrophages to an anti-fibrotic phenotype, highlighting further advantages of ZA administration (Kaneko et al., 2018).

Important limitations addressed in this study include whether treatment using ZA can display utility in human IPF pathologies. Whilst the translational aspect of this study is suggested using murine lung sections, it is important to demonstrate that ZA treatment successfully reduces FDPS levels and subsequent IPF lung damage in human clinical trials. Transition to such clinical trials is eased by the pre-existing FDA approval of ZA (i.v.) for osteoporosis, multiple myeloma, and bone metastases in cancer.

Together, our findings demonstrate that ZA possesses a mechanism of action targeting FDPS to suppress pulmonary fibrosis, which is distinct from currently employed therapeutic interventions. This study highlights the downstream effects of FDPS inhibition, including decreasing myofibroblast transition, decreasing inflammatory cell recruitment, reducing pro-fibrotic macrophage populations, and eventual inhibition of fibrotic-related lung remodelling. These data support the use of ZA in clinical trials for the treatment of IPF.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Ethical Review Board in Lund (approval no. LU412-03), and performed in accordance with the Declaration of Helsinki as well as relevant guidelines and regulations. The patients/participants provided their written informed consent to participate in this study. All animal experiments were approved by the Malmö-Lund Animal Care Ethics Committee (M17009-18).

AUTHOR CONTRIBUTIONS

Conceptualization: AE, LT, ABS, and JB; Methodology: LT, JB, ABS, RKVB, and JSE; Investigation: LT, JB, and RKVB, JSE; Funding acquisition: AE, ABS, and LT; Project administration: AE, LT Supervision: AE, LT; Writing—original draft: LT, JB; Writing—review and editing: LT, JB, ABS, AE, RKVB, and JSE.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.899469/full#supplementary-material

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REVIEW
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Targeting Growth Factor and Cytokine Pathways to Treat Idiopathic Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease of unknown origin that usually results in death from secondary respiratory failure within 2–5 years of diagnosis. Recent studies have identified key roles of cytokine and growth factor pathways in the pathogenesis of IPF. Although there have been numerous clinical trials of drugs investigating their efficacy in the treatment of IPF, only Pirfenidone and Nintedanib have been approved by the FDA. However, they have some major limitations, such as insufficient efficacy, undesired side effects and poor pharmacokinetic properties. To give more insights into the discovery of potential targets for the treatment of IPF, this review provides an overview of cytokines, growth factors and their signaling pathways in IPF, which have important implications for fully exploiting the therapeutic potential of targeting cytokine and growth factor pathways. Advances in the field of cytokine and growth factor pathways will help slow disease progression, prolong life, and improve the quality of life for IPF patients in the future.

Keywords: cytokine, growth factor, signaling pathway, clinical trials, idiopathic pulmonary fibrosis, emerging pharmacotherapy

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1 INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive fibrotic interstitial lung disease of unknown etiology that usually results in death from secondary respiratory failure within 2–5 years of diagnosis (Meltzer and Noble, 2008). It is a rare familial and sporadic disease. CT imaging of IPF usually shows a typical usual interstitial pneumonia (UIP) pattern, characterized by irregular reticular opacities with obligatory honeycombing, associated with traction bronchiectasis. IPF also exhibits histological features of UIP/IPF pattern characterized by dense fibrosis causing architecture remodeling with frequent honeycombing, patchy lung involvement by fibrosis, subpleural and/or paraseptal distribution, fibroblast foci at the edge of dense scars (Spagnolo et al., 2018; Baratella et al., 2021). With extensive basic and clinical research on the pathogenesis of IPF in recent years, some potential therapeutic targets have been discovered (Wang et al., 2021). A large number of these targets are growth factors, cytokines, and their signaling pathways, including TGF-β, CTGF, IL-13, CCL-2, leukotriene receptor, lipid proinflammatory mediators, and their downstream signaling. In addition, targeting pentraxin 2, galectin-3, oxidative stress, and B cell-mediated autoimmunity showed the potential to treat IPF. Lots of investigational drugs have entered clinical trials to test their efficacies in IPF therapy. However, there are only two currently approved IPF drugs, Pirfenidone and Nintedanib. They

both slow the progression of IPF but are not able to reverse lung fibrosis (Chu et al., 2020). Lung transplantation is the only option for patients with end-stage IPF, but the donor organ shortages are an intractable problem worldwide, which means only a minority of patients have the opportunity to undergo lung transplantation (Spagnolo et al., 2018). In addition, lung transplantation is expensive, and the 10-year survival rate after surgery is only 33%–55% (Lederer and Martinez, 2018; Villavicencio et al., 2018). Therefore, there is still an urgent need to develop new drugs to treat IPF.

Although the pathophysiological mechanism of IPF remains unknown, significant progress in understanding the pathogenesis of IPF has been made in the last decade. The current paradigm assumes that recurrent alveolar epithelial cell injury and the crosstalk between dysregulated epithelial cells and mesenchymal, immune, endothelial cells can trigger abnormal wound healing responses and pulmonary fibrosis *via* multiple signaling pathways- (Mei et al., 2021; Moss et al., 2022). The pathogenesis of IPF is believed to be mediated by various cytokines, chemokines, and growth factors (Kelly et al., 2003). Cytokines and growth factors regulate the phenotypic switch of fibroblasts and alveolar epithelial cells (AECs), the recruitment and proliferation of mesenchymal cells, and the deposition and degradation of matrix through multiple mechanisms. Uncoordinated expression of several cytokines may be responsible for the severe matrix remodeling and epithelial-mesenchymal crosstalk in the lung microenvironment of IPF. Although the efficacy of previous anti-inflammatory treatments (e.g., TNF-α neutralization, immunosuppressants) and immunomodulatory treatments (e.g., interferon-γ) in clinical trials for the treatment of IPF has been unsatisfactory, targeting these pathways remains promising. Pirfenidone and Nintedanib, two small-molecule drugs that block multiple cytokine and growth factor signaling pathways, have been approved to slow the progression of pulmonary fibrosis. However, they have obvious defects, such as poor specificity caused by multiple targets and large doses, which lead to undesired side effects. Therefore, it is still necessary to explore the mechanism of the cytokine/growth factor pathway in IPF to find promising targets and develop targeted drugs.

Due to the pivotal role of the cytokine/growth factor pathway in the pathogenesis of IPF, this review comprehensively introduces the association of various growth factors, chemokines, interleukins, lipid proinflammatory mediators and their related signaling pathways with IPF. Related signaling pathways are also attractive therapeutic targets, including RTK and non-RTK pathways, Hedgehog pathway, Wnt pathway and Notch pathway, PI3K/Akt/mTOR pathway, MAPK pathway, and Hippo YAP/TAZ pathway. A large number of drugs, including many small molecules and biologics targeting cytokine/growth factor signaling pathways, have entered clinical trials to determine their efficacy against IPF, as described in this review. Taken together, the aim of this review is to provide an overview of cytokines, growth factors, and the related signaling pathways in IPF, thus providing a basis for the development of novel treatment options to alleviate or even reverse IPF.

2 THE PATHOLOGICAL PROCESS OF IDIOPATHIC PULMONARY FIBROSIS

The complexity of the pathological process of pulmonary fibrosis lies in how crosstalk between epithelial-mesenchymal cells and multiple imbalanced cytokines contribute to the disease. First, alveolar epithelial cells (AECs) are damaged, and the continuity of the basement membrane is interrupted due to a variety of external stimuli, such as radiation, the microbiome, allergens, environmental particles, autoimmunity, antineoplastic drugs (Wilson and Wynn, 2009), and SARS-CoV2 (George et al., 2020). Then, many cytokines and growth factors are released by AECs to recruit and activate inflammatory cells and fibroblasts. Inflammatory cells and some coagulation factors [e.g., tissue factors (TF) and plasminogen activation inhibitors (PAI-1)] are jointly involved in the formation of wound clots (King et al., 2011b; Betensley et al., 2016). Activated AECs and endothelial cells participate in (myo)fibroblast migration, proliferation, and differentiation.

Under normal physiological conditions, the repair process of local lesions is controlled, but in IPF, epithelial-mesenchymal transition (EMT) and differentiation of fibroblasts into myofibroblasts occur, promoting the expansion of myofibroblast population which is the main source of extracellular matrix (ECM) (Yagihashi et al., 2016; Skibba et al., 2020). Notably, EMT may be an indirect mechanism of IPF, because it does not directly contribute to the expansion of the myofibroblast population *via* the epithelial-to-myofibroblasts transition (Rock et al., 2011; Salton et al., 2020). In contrast, EMT is indirectly involved in the pathological process of IPF through the paracrine of fibroblast activating factor (Hill et al., 2019). It has also been reported that EMT is the result of aberrant mechanical forces and signaling pathways in IPF (Qian et al., 2020; Saito et al., 2020; Salton et al., 2020; Su et al., 2020; Wu et al., 2020). Due to the presence of excess fibrin in the ECM, the lung elasticity of IPF patients decreases, which manifests as alveolar collapse and the cystic dilation of residual bronchioles/alveoli. This alveolar collapse leads to a decrease in the effective volume of the alveoli used for gas exchange. Moreover, matrix stiffness forms a positive feedback pathway through the mechanosensor transient receptor potential vanilloid 4 (TRPV4) and α6 integrin, which continuously aggravates pulmonary fibrosis (Rahaman et al., 2014; Chen et al., 2016), causing the loss of pulmonary function (respiratory ventilation) in IPF patients. In addition, excessive ECM encapsulates pulmonary capillaries, resulting in a decrease in the diffusion coefficients of oxygen and carbon dioxide and the loss of gas exchange function in the alveoli, eventually leading to the death of IPF patients from respiratory failure or related syndromes. If injury factors persist, AECs are continuously damaged, often manifesting as AEC death, an increase in the proportion of type II AECs (AT II), and impaired reepithelization (Liu et al., 2017). Therefore, different from normal repair, the repair processes involved in IPF are uncontrolled, continuous, and abnormal.

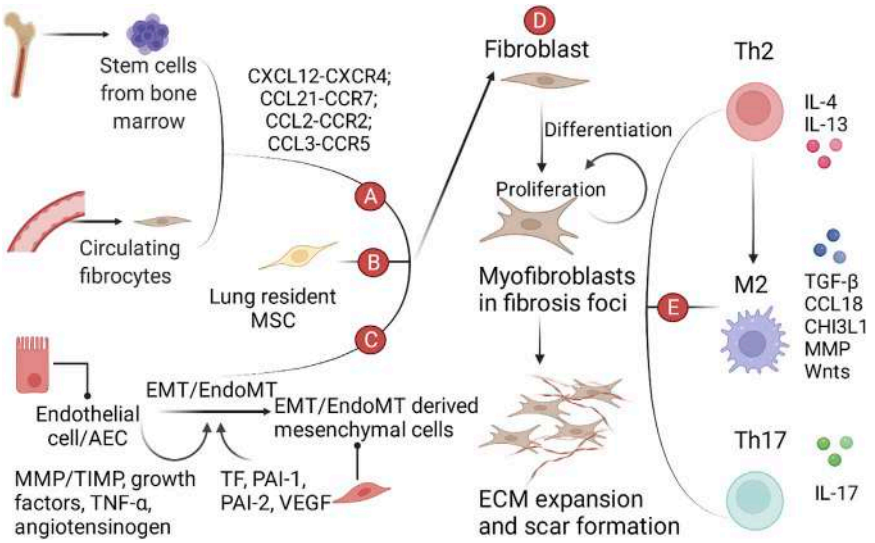

FIGURE 1 | A schematic view of the role of various cells, growth factors and cytokines in IPF. **(A)** Bone marrow-derived mesenchymal progenitor cells and circulating fibrocytes in the peripheral circulation are recruited to the lung by the chemokine-chemokine receptor axis. **(B)** Lung resident MSCs. **(C)** EMT/EndoMT-derived mesenchymal cells. **(D)** Cells from the above three sources aggregate, proliferate, and differentiate into myofibroblasts in the lung, ultimately leading to ECM expansion and scarring. **(E)** Immune cells and cytokines involved in IPF.

A large number of cytokines and their signaling pathways are directly involved in the accumulation of fibroblasts in lung fibrosis foci (**Figure 1**). Fibroblasts are the culprits directly involved in pulmonary fibrosis, and their sources include stem cells recruited from the bone marrow, fibrocytes recruited from the peripheral circulation, mesenchymal stem cells (MSCs) residing in the lung, and EMT/EndoMT-derived interstitial cells. Plasma cells, etc. The CXCL12-CXCR4 axis (Phillips et al., 2004), CCL2-CCR2 axis (Chong et al., 2019), CCL3-CCR5 axis (Ishida et al., 2007; Besnard et al., 2013), and CCL21-CCR7 axis (Ziegenhagen et al., 1998; Habiel and Hogaboam, 2014) are directly involved in the recruitment of fibroblasts. EMT/EndoMT of epithelial and endothelial cells is affected by the coagulation cascade and angiogenesis-related cytokines (TF, PAI-1, and VEGF) and secretion released by AECs (TGF-β, growth factors, TNF-α, MMP/TIMP, and angiotensinogen), which are implicated in multiple signaling pathways (TGF-β, Wnt, SHH, Notch, and ER stress/UPR pathways) (King et al., 2011b; Selman and Pardo, 2014; Betensley et al., 2016).

Growth factors and cytokines are an integral part of the fibrotic microenvironment, which leads to differences in the phenotype of immune cells in the alveoli between patients with pulmonary fibrosis and healthy individuals. Th1/Th2 imbalance and M1-M2 polarization are hallmarks of pulmonary fibrosis. Th2 polarization is characterized by increased secretion of IL-4 and IL-13 and decreased secretion of IFN-γ. M2 polarization can be induced by the microenvironment shaped by Th2 polarization and promotes pulmonary fibrosis through the production of TGF-β, CCL18, chitinase 3-like 1 (CHI3L1), MMPs, and activation of the Wnt/β-catenin pathway (Shenderov et al., 2021). Th17 cells can promote fibroblast proliferation and ECM secretion by secreting IL-17 (Zhang et al., 2019a). In recent years, polymorphisms in immune-related genes have also been reported to be involved in the process of pulmonary fibrosis. For example, an increased risk or severity of IPF is associated with polymorphisms in the TLR3, Toll-interacting protein (TOLLIP), and interleukin-1 receptor antagonist (IL-1RA) genes (Whyte et al., 2000; Korthagen et al., 2012; Noth et al., 2013; O'Dwyer et al., 2013). In addition, activation of TLR2 and TLR9 has been reported to show profibrotic effects, whereas TLR3 has antifibrotic effects (Karampitsakos et al., 2017).

3 THE KEY ROLE OF THE TGF-β SIGNALING PATHWAY IN IDIOPATHIC PULMONARY FIBROSIS

Transforming growth factor-β (TGF-β) is a potent profibrogenic cytokine that plays a central role in the development of pulmonary fibrosis by promoting fibroblast proliferation and phenotype modulation, stimulating the deposition of ECM, and participating in crosstalk with other cytokines and signaling pathways (Ong et al., 2021).

3.1 A Brief Introduction to TGF-β Signalling Pathway

The activation of TGF-β pathway includes five steps, including i) synthesis of TGF-β, ii) activation of latent TGF-β, iii) interaction between TGF-β and TGF-β receptor (TBR), iv) activation of classical and non-classical pathways, and v) regulation of nuclear transcription factors and cell

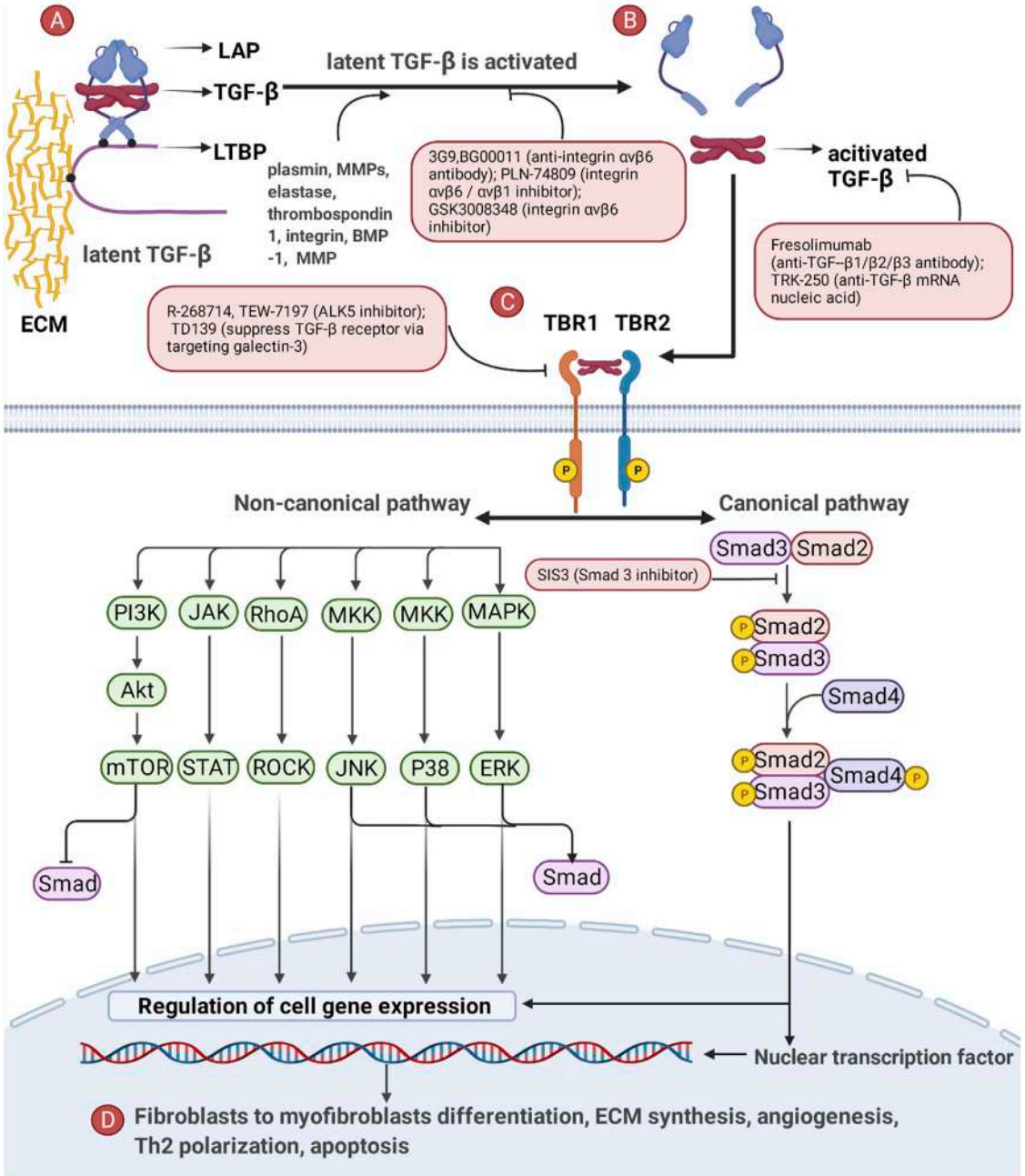

FIGURE 2 | The critical role of the TGF-β pathway and drugs targeting the TGF-β pathway in IPF. **(A)** Latent TGF-β (TGF-β trapped in LAP) binds to ECM through LTBP. **(B)** TGF-β is detached from LAP and activated only in the presence of specific stimuli. **(C)** Activated TGF-β activates the downstream Smad-dependent pathway (canonical pathway) and Smad-independent pathway (non-canonical pathway) by combining with TBR. **(D)** The TGF-β pathway ultimately causes phenotypic reprogramming of AECs, fibroblasts and immune cells and influences fibroblast-to-myofibroblast differentiation, ECM synthesis, angiogenesis, Th2 polarization, and apoptosis.

phenotypes. A brief introduction to the TGF-β pathway is as follows and shown in **Figure 2**.

i) In the endoplasmic reticulum (ER), TGF-β precursors are assembled into dimers wrapped with latency-associated peptides (LAPs). TGF-β-LAPs bind to latent TGF-β binding protein (LTBP) and undergo intracellular proteolytic cleavage by the endopeptidase furin. Then, TGF-β-LAP-LTBPs are secreted into the extracellular medium and eventually bind to ECM (e.g., fibrillin and fibronectin) through LTBP. At this time, latent TGF-β cannot exert its biological function (Derynck and Zhang, 2003).

ii) Latent TGF-β is activated under special conditions [e.g., plasmin (Coutts et al., 2001), thrombospondin 1 (Murphy-Ullrich and Suto, 2018), elastase, integrin, BMP-1 and MMP 2 (Tzavlaki and Moustakas, 2020)] and binds to TGF-β receptor 2 (TBR2).
iii) TBR2 can phosphorylate TGF-β receptor 1 (TBR1) and activate the downstream classical and non-classical pathways.
iv) In classical pathways, Smad2/Smad3 activated by TBR1 binds to Smad4 to form Smad2/Smad3/Smad4, which enter the nucleus and participate in regulating transcription factors (Derynck and Zhang, 2003). Classical pathways also involve Smad7, ubiquitin, coactivator P100, PPAR-γ, Ski/SnoN, and other signal-regulating molecules (Tzavlaki and Moustakas, 2020). In non-classical pathways, TGF-β can activate many signaling pathways, such as the Ras-Raf-Mek1/2-ERK1/2 pathway, TAK1-MKK3/6-P38 pathway, TAK1-MKK4-JNK pathway, and PI3K-Akt-mTOR pathway (Derynck and Zhang, 2003; Ong et al., 2021), and participate in regulating nuclear transcription factors.
v) Some transcription factors of fibroblasts, alveolar epithelial cells, endothelial cells, and Th cells are changed, causing the phenotypic transformation of cells involved in pulmonary fibrosis, which eventually leads to the differentiation of myofibroblasts, the deposition of ECM, the abnormal function of AT II, and an imbalance of immune cells in IPF.

The TGF-β pathway is interrelated with the Wnt/β-catenin pathway, PI3K/Akt pathway, and pathways of other growth factors (Yan et al., 2014). Furthermore, in terms of the pathogenesis of IPF, TGF-β has been implicated in redox imbalance, mitochondrial dysfunction, EMT, MMP/TIMP imbalance, and fibrinolytic system imbalance (Chu et al., 2020).

3.2 Strategies to Treat IPF by Targeting TGF-β Pathway

Strategies for the treatment of IPF by targeting the TGF-β pathway include blocking TGF-β synthesis, preventing activation of latent TGF-β, neutralizing TGF-β ligands/receptors, and blocking canonical and non-canonical pathways (Saito et al., 2018; Hamanaka and Mutlu, 2021; Ong et al., 2021). These strategies are summarized in **Figure 2**.

Integrins mediate the mechanotransduction positive feedback to ECM stiffness in a TGF-β-dependent or TGF-β-independent manner, which is an important therapeutic target. TGF-β-dependent fibrosis is activated by integrins releasing TGF-β through tensile forces generated by actin-cytoskeleton interactions. In the TGF-β-independent pathway, after binding specific ECM ligands to the ectodomain of integrins, integrins bind to the cytoskeleton and various signaling proteins through their cytoplasmic tails, translating the mechanical force of cytoskeleton contraction and ECM stiffness into biochemical signals. Then, F-actin activates the downstream Rho/ROCK-YAP/TAZ signaling pathway *via* FAK phosphorylation, ultimately leading to fibroblast phenotype reprogramming.

There are corresponding preclinical candidate drugs for IPF that target different steps of the TGF-β pathways, including the synthesis, receptor binding, and downstream signal transduction (Ong et al., 2021), but no drug specifically for the TGF-β pathway has been approved due to the side effects of anti-TGF-β treatment (systemic autoimmune, cardiac valve problems, and carcinogenesis) (Henderson et al., 2020). Notably, to avoid systemic autoimmune disease induced by persistent systemic inhibition of TGF-β, it may be necessary to choose the correct dose or duration of treatment, coadminister anti-inflammatory drugs, selectively block TGF-β in targeted organs.

4 THE ROLE AND UNDERLYING MECHANISM OF CYTOKINES AND GROWTH FACTORS IN IDIOPATHIC PULMONARY FIBROSIS

As described above, cytokines, growth factors, and related signaling pathways are intensively involved in the pathogenesis of IPF. Therefore, they might be promising targets to develop novel treatment options for IPF. In this section, we describe the roles of growth factors, chemokines, interleukins, lipid pro-inflammatory mediators, and their signaling pathways in IPF. Many drugs targeting these pathways are in development, and we summarize those that have entered clinical trials to treat patients with IPF. In recent years, therapeutics targeting these pathways have shown many limitations in clinical trials. Therefore, we propose possible approaches to overcome these limitations, aiming to provide insights into the development of therapies with fewer side effects and better efficacy.

4.1 Growth Factors

Growth factors can participate in the development and progression of IPF in TGF-β-dependent or TGF-β-independent ways. These growth factors comprise platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), connective tissue growth factor (CTGF), and insulin-like growth factor (IGF). Due to the successful marketing of Nintedanib (an antagonist of PDGFR/VEGFR/FGFR), many studies have focused on growth factors and their corresponding receptors. However, there remains considerable controversy regarding the roles of many growth factors in promoting fibrosis and resisting fibrosis in IPF. One of the possible reasons for the controversy is that there are many subtypes of these growth factors and their receptors, and the functions of different subtypes differ. Although pharmacological analysis of these subtypes of growth factors is difficult, it is a vital step toward precise treatment and personalized treatment, which is of substantial significance. Next, we introduce the crucial growth factors PDGF, FGF, VEGF, EGF, and CTGF in detail.

4.1.1 Platelet-Derived Growth Factor

Platelet-derived growth factor (PDGF) is a key growth factor that stimulates the proliferation and migration of fibroblasts. In a mouse model of bleomycin-induced IPF, RT–PCR (Maeda et al., 1996), PDGF antibody neutralization (Walsh et al., 1993), and Northern

blotting (Zhuo et al., 2004) all demonstrated increased protein or mRNA expression of multiple subtypes of PDGF. However, the main subtypes of PDGF found in these experiments differed slightly, and the specific mechanism requires further research. A clinical study also observed that the high expression of PDGF was correlated with a low overall survival rate (Zhu et al., 2017).

Single strands encoded by the PDGF-A, B, C, and D genes can be combined in pairs to form five types of dimers, AA/BB/AB/CC/DD (Heldin and Westermark, 1999; Li and Eriksson, 2003; Günther et al., 2012). PDGFRs (PDGF receptors) are also homo/heterodimerically formed by combinations of single-chain PDGF-α/PDGF-β. After PDGF binds to the PDGF receptor, PDGFR dimerization can be induced; subsequently, the two PDGFRs after autophosphorylation can couple various downstream signal transduction pathways (Heldin and Westermark, 1999), such as Ras-mitogen-activated protein kinase (MAPK) through Grb2 and Shc adaptor proteins and PI3K and phospholipase C-γ (PLCγ) (Nishioka et al., 2013). PDGFR also participates in the migration and chemotaxis of fibroblasts through the integrin-FAK pathway. PDGF, as the main mitogen, can strongly promote the proliferation of fibroblasts and stimulate collagen synthesis (Heldin and Westermark, 1999). PDGF also participates in cell migration through Ca^{2+} influx and cytoskeleton rearrangement (Nishioka et al., 2013).

4.1.2 Fibroblast Growth Factor

FGFs have been divided into seven subgroups encoded by 22 mammalian genomes. Among the 22 FGFs encoded by genomes, 4 FGFs are FGFR-independent, and the remaining 18 extracellular FGFs bind to 7 FGFR subtypes (FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, FGFR3c, and FGFRR4). An FGF can bind to multiple FGFRs. FGFs rely on heparan sulfate proteoglycan (HSPG) and Klotho-type coreceptors to improve their binding to FGFRs (Ornitz and Itoh, 2022). After FGF-FGFR binding, through the autophosphorylation of FGFRs, with the help of FGFR substrate 2 and PLCγ, signals are transferred to the RAS-ERK, PI3K/AKT, PKC, and JAK-STAT signaling cascades, which mediate the survival, proliferation, differentiation, or migration of cells (Inomata et al., 2015; Katoh, 2018).

Subtypes of FGFs have functional differences and play different roles in IPF (Yang et al., 2021). FGF1 (acid FGF or aFGF) has an anti-fibrotic function. The serum FGF1 level of patients with IPF was found to be higher than that of the control group (Shimbori et al., 2016). In rat models induced by TGF-β1, FGF1 could relieve IPF by inhibiting the TGF-β1 signaling pathway and promoting the proliferation of AECs (Shimbori et al., 2016). Further research found that "FGF1 + heparin treatment" could reverse EMT through the MAPK/ERK kinase pathway, leading to phosphorylation of ERK-1 and dephosphorylation of Smad2 (Ramos et al., 2010). FGF2 (basic FGF or bFGF) has a proliferative effect on lung fibroblasts (Hetzel et al., 2005; Khalil et al., 2005), and soluble FGFR2c significantly reduces TGF-β-induced IPF in mice (Ju et al., 2012). However, it has also been reported that FGF2 is antifibrotic in part through decreased collagen expression and fibroblast to myofibroblast differentiation (Koo et al., 2018). FGF9 and FGF18 promote the survival and migration of HLFs and inhibit myofibroblast differentiation *in vitro* (Joannes et al., 2016). More FGF9 was expressed in lung tissue myofibroblasts in patients with IPF than in healthy individuals, and it promoted epithelial cell growth and expansion of pulmonary interstitial through the Wnt7B/β-catenin signaling pathway (Yin et al., 2008). FGF10 plays an antifibrotic role *via* autocrine and paracrine signaling. During autocrine signaling, by activating peroxisome proliferator-activated receptor γ (PPAR γ), FGF10 blocks the lipofibroblast-to-myofibroblast transformation induced by TGF-β1 and promotes the transformation from myofibroblasts to lipofibroblasts. In addition, the FGF10 paracrine signal was considered crucial to the differentiation of alveolar epithelial progenitor cells during development and the maintenance of AT II in a steady state (Wu et al., 2018). FGF21 attenuates the TGF-β pathway *via* decreased oxidative stress in bleomycin-induced pulmonary fibrosis in mice (Zhang et al., 2018). Coadministration of FGF23 and its coreceptor α-Klotho led to a significant reduction in fibrosis and inflammation (Barnes et al., 2019).

In summary, FGF1, FGF10, FGF21, and FGF23 are anti-fibrotic, while FGF2, FGF9, and FGF18 exhibit contradictory functions.

4.1.3 Vascular Endothelial Growth Factor

The VEGF family has seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor, and snake venom vascular endothelial growth factors (Inomata et al., 2015). Most studies have focused on the correlation between VEGF-A and IPF; thus, our description mainly focuses on VEGF-A. The most studied and dominant VEGF-A165 can be divided into VEGF-A165a and VEGF-A165b by the splice site. VEGF-A165a can promote angiogenesis, and VEGF-A165b can inhibit angiogenesis.

VEGF-A can bind to VEGFR1 (Flt-1), VEGFR2 (KDR or Flk1), and their coreceptors neuropilin-1 and neuropilin-2. Among them, VEGFR1 binds to circulating VEGF-α and reduces its bioavailability to VEGFR2. The coreceptors neuropilin-1 and neuropilin-2 assist in the signal transduction of VEGFR1 and VEGFR2. After binding to VEGFR2, VEGF-A165 can activate the downstream Akt pathway, Src signaling pathway, NCK and the p38 MAPK pathway, and integrin/FAK pathway (Fruttiger, 2008; Barratt et al., 2018). VEGF can stimulate the growth of alveolar epithelial type II cells and the production of alveolar surfactant, form new blood vessels, and help epithelial cells and endothelial cells resist apoptosis (Gerber et al., 1998a; Gerber et al., 1998b; Brown et al., 2001; Compernolle et al., 2002; Alavi et al., 2003; Mura et al., 2006; Roberts et al., 2007; Kuhn et al., 2010; Varet et al., 2010; Barratt et al., 2018).

Many contradictory effects of VEGF-A on IPF have been observed in animal models and clinical trials. The possible reasons for these differences are as follows: different animal models, different sampling sites, heterogeneity within and between individuals, and different VEGF-A subtypes caused by diverse splice sites. Different subtypes of VEGF-A may have mutually coordinated pathophysiological relationships. In

in vitro experiments, VEGF-A165a has been proven to promote the proliferation of AT II and fibroblasts, increase the expansion of ECM, and play a role in promoting fibrosis, and VEGF-A165b could counteract this effect (Varet et al., 2010; Barratt et al., 2017). Thus, VEGF-A165b may be a compensatory protective mechanism.

4.1.4 Epidermal Growth Factor

The EGF receptor (EGFR, also known as ErbB1 or HER1) belongs to the ErbB family receptor tyrosine kinases. EGFR has seven corresponding ligands: EGF, transforming growth factor-α (TGF-α), amphiregulin, betacellulin (BTC), epiregulin, epigen, and heparin-binding EGF-like growth factor (HB-EGF). After ligands bind to EGFR, the autophosphorylation of EGFR can activate the downstream MAPK, Akt, and JNK pathways and promote cell proliferation (Iwamoto and Mekada, 2012).

The relationship between the ErbB family (HER) and IPF remains unclear and thus requires further research. After blocking HER *in vivo*, studies have found that collagen deposition decreases and lung morphology improves, which indirectly indicates that the ErbB family plays a role in fibrosis (Rice et al., 1999; Faress et al., 2007). In addition, in patients with IPF, the mRNA level of EGFR was upregulated in proliferative alveolar epithelial cells around fibrosis, and the mRNA level of EGFR was positively correlated with the mRNA level of type I collagen and negatively correlated with the clinical prognosis (Tzouvelekis et al., 2013).

4.1.5 Connective Tissue Growth Factor

CTGF, also known as CNN2, HCS24, or IGFBP8, belongs to the CNN family. CTGF can interact with a wide range of ECM components, but this also means that the biological action of CTGF is highly dependent on the local microenvironment. CTGF directly binds to other growth factors (e.g., TGF-β, BMPs, and VEGF), which influence signal emission/transduction (Choi, 2012). CTGF can be secreted by interstitial cells, such as proliferating AT II and activated fibroblasts (Pan et al., 2001). The peak of CTGF content mainly appears at the early stage of IPF, and the peak of CTGF appears earlier than the deposition of collagen in the lungs (Wang et al., 2011), which indicates that CTGF may be involved in the early-stage repair of lung tissue injury.

As an auxiliary regulator of TGF-β in IPF in the local microenvironment, CTGF can participate in abnormal tissue repair processes, such as ECM generation and the mobilization of fibroblasts, by assisting TGF-β (Wang et al., 2011). There was an interaction between TGF-β and CTGF in IPF animal models induced by TGF-β/bleomycin, and IPF could be alleviated by the CTGF antibody (FG-3019, pamrevlumab) (Wang et al., 2011). CTGF-deficient transgenic mice had the ability to resist IPF induced by bleomycin (Liu et al., 2011). Therefore, a general belief is that CTGF, as a fibrosis-promoting medium, is a possible target for the research and development of anti-IPF drugs. Pamrevlumab, a human recombinant mAb of CTGF, is the only antibody drug that has shown activity in a phase II clinical trial against IPF. In a recent randomized, placebo-controlled phase II trial for patients with IPF, pamrevlumab showed good safety and certain therapeutic effects, such as slowing the decline of pulmonary function and delaying the progression of fibrosis by HR-CT, which is a milestone of single-target therapy (Lipson et al., 2012; Raghu et al., 2016). The phase III clinical trial of pamrevlumab for IPF is at the recruitment stage (NCT04419558/NCT03955146).

4.2 Chemokines and Chemokine Receptors

Chemokines are small molecule proteins with chemotactic effects on specific cells, and there are four conserved cysteine residues. According to the differences in cysteine residues, chemokines have been divided into four main subfamilies: CXC, CC, CX3C, and XC. Although the role of some chemokines in IPF remains unknown, many studies have demonstrated that chemokines can promote fibrosis or resist fibrosis. It has been reported that pulmonary fibrosis in animal models can be attenuated by knocking out genes encoding chemokines or neutralizing chemokines with antibodies [CCL2-CCR2 (Moore et al., 2001; Murray et al., 2008; Phan et al., 2021), CCL11-CCR3 (Huaux et al., 2005), CCL17 (Agostini and Gurrieri, 2006), CCL21-CCR7 (Habiel and Hogaboam, 2014), CCL22 (Strieter, 2005), CXCL6 (Besnard et al., 2013), CXCL12 (Phillips et al., 2004), CXCL14 (Li et al., 2019), CX3CL1-CX3CR1 (Rivas-Fuentes et al., 2020), CCR5 (Ishida et al., 2007)]. The CXCL11-CXCR3 axis has anti-pulmonary fibrosis effects in a mouse model of bleomycin-induced pulmonary fibrosis (Strieter, 2005).

Chemokines play a crucial role in the pathological process of IPF (**Figure 3**). CXCL12-CXCR4 (Phillips et al., 2004), CCL2-CCR2 (Chong et al., 2019) CCL3-CCR5 (Ishida et al., 2007; Besnard et al., 2013), CCL11-CCR3 (Puxeddu et al., 2006), CCL21-CCR7 (Ziegenhagen et al., 1998; Habiel and Hogaboam, 2014), CCL26 (Kohan et al., 2010) promote the migration of fibrocytes to the lung, whereas CXCL10 (Agostini and Gurrieri, 2006) inhibits the migration of fibrocytes to the lung. CCL2-CCR2 (Hambly et al., 2015), CCL11 (Puxeddu et al., 2006), CCL21-CCR7 (Ziegenhagen et al., 1998; Habiel and Hogaboam, 2014), CCL24 (Kohan et al., 2010) promote fibroblast proliferation. CCL18 (Hambly et al., 2015) and CCL21-CCR7 (Ziegenhagen et al., 1998; Habiel and Hogaboam, 2014) participate in the differentiation of fibroblasts to myofibroblasts and stimulate collagen synthesis. CCL21-CCR7 is involved in the survival of fibroblasts (Ziegenhagen et al., 1998; Habiel and Hogaboam, 2014). CCL2-CCR2 (Agostini and Gurrieri, 2006; Besnard et al., 2013; Hambly et al., 2015), CCL3-CCR5 (Ishida et al., 2007), CCL17-CCR4 (Karman et al., 2021), CCL22-CCR4 (Yogo et al., 2009), CX3CL1-CX3CR1 (Rivas-Fuentes et al., 2020) promote migration of monocytes and macrophages. CCL2 (Rose et al., 2003), CCL17 (Besnard et al., 2013), CCL22 (Besnard et al., 2013), CCR3 (Fulkerson et al., 2006), CCR4 (Yoshinouchi et al., 2007) are involved in type 2 immunity, whereas CCR5 (Loetscher et al., 1998) is involved in type 1 immunity. CXCR3 counteracts the profibrotic effect of IL-13 by assisting IL-13 receptor α2 gene expression (Pignatti et al., 2006; Yoshinouchi et al., 2007; Barnes et al., 2015). A phase II clinical trial of CNTO 888 (CCL2 mAb) failed to provide benefit to FVC in IPF patients (NCT00786201).

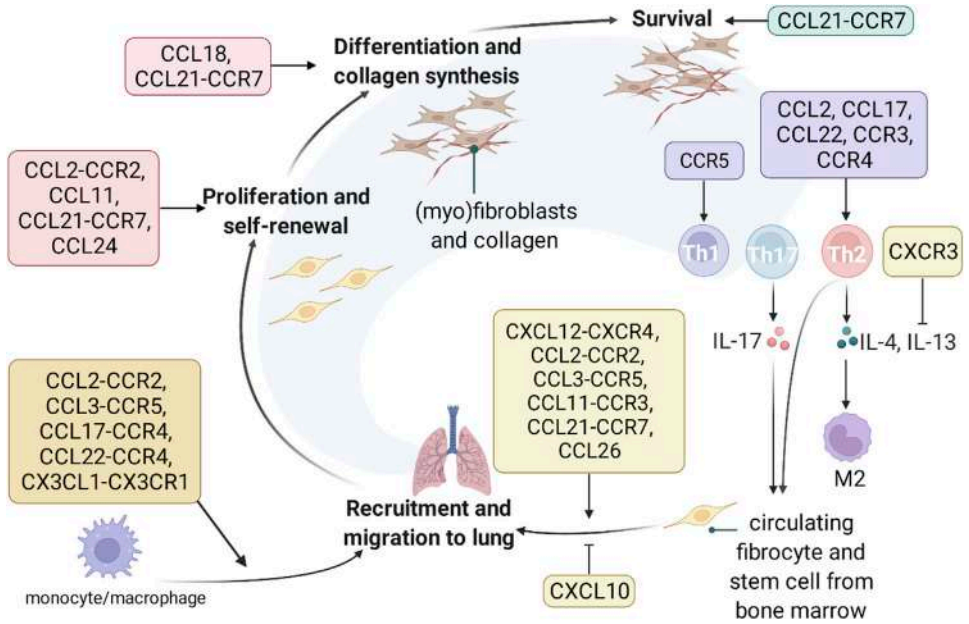

FIGURE 3 | The role and underlying mechanisms of chemokines in IPF. Chemokines are involved in recruitment, proliferation, differentiation, collagen synthesis, survival of fibroblasts, regulation of type 1 and 2 immune balance, and recruitment of monocytes and macrophages.

Nonetheless, the role of chemokines in IPF remains to be further investigated.

4.3 Interleukins

Interleukins are a class of cytokines generated by lymphocytes, monocytes, and other non-monocytes. In addition to affecting fibrosis by modulating the Th1/Th2 balance, many studies have shown that interleukins can also directly affect fibroblasts and epithelial cells (**Table 1**). Thus, interleukins are expected to be developed as drugs and biomarkers for treating IPF.

However, clinical trials of IPF treatments targeting IL-4 and IL-13 have not gone well. Several drugs targeting IL-13 have entered clinical trials for IPF, but none of them have shown protective effects on lung function. Although clinical trials of IPF antibody drugs have mostly failed in phase II, there is still hope for interleukin therapy. Inhibition of IL-11 blocks TGF-β1, PDGF, FGF2, IL-13, OSM (Oncostatin M), and endothelin 1-mediated fibroblast activation (Ng et al., 2019).

Notably, interleukin supplementation alone may not reverse the profibrotic phenotype of cells, and the regulation of cell–cell interactions or phenotypic transformation may be more promising. In addition, anti-inflammatory interleukins should be used with caution in clinical trials, and attention should be given to their side effects on patient immune function. In addition, rational design of interleukin dosage forms is very important because the concentration of the drug in the lungs can explain some of the differences in the study results.

4.4 Lipid Proinflammatory Mediators

Various lipids and their metabolic derivatives play vital roles in IPF. Although glucocorticoids (PLA2 inhibitor) have not shown positive effects in the treatment of IPF in clinical trials, these findings do not indicate that all lipid metabolism pathways have no significance as therapeutic targets of IPF. In contrast, metabolomic studies on fibroblasts have received increasing attention in recent years.

LT and PG are implicated in the pathogenesis of IPF. In arachidonic acid metabolic pathways, phospholipids produce arachidonic acid under the catalysis of phospholipase A2 (PLA2). Then, arachidonic acid produced leukotriene (LT) and prostaglandin (PG) under the catalysis of 5-lipoxygenase (5-LO) and cyclooxygenase (COX), respectively. Among them, PGF2a and LTs promote fibrosis, and PGE2 resists fibrosis (Suryadevara et al., 2020). A phase II trial (NCT02503657) on the safety and tolerability of Tipelukast/MN-001 (LT receptor inhibitor, 5-LO inhibitor and PDE inhibitor) in patients with IPF is ongoing.

The SPHK1/S1P/S1PR axis is involved in pulmonary fibrosis. In sphingolipid metabolism, sphingosine kinase 1 (SPHK1) phosphorylates sphingosine to produce sphingosine-1-phosphate (S1P). Then, the binding of S1P to the S1P receptor can lead to mitochondrial reactive oxygen species (mtROS) and promote YAP1 entry into cell nuclei, affecting the differentiation of myofibroblasts and matrix remodeling (Huang et al., 2020). Targeting the SPHK1/S1P/S1PR axis, PF543 (SPHK1 inhibitor), Mito TEMPO (mitochondria-targeted superoxide dismutase, which can reduce mtROS) and verteporfin (YAP inhibitor) have been reported, but these drugs have not entered clinical trials on IPF treatment.

TABLE 1 | The role and underlying mechanism of interleukins in IPF.

Interleukins	Pro/anti-fibrotic	Mechanism of action	References
IL-1β	Pro-fibrotic	1) IL-1β-driven pulmonary fibrosis is dependent on IL-17A. 2) Gene polymorphism of IL-1β is associated with risk of IPF	Wilson et al. (2010); Korthagen et al. (2012)
IL-4	Pro-fibrotic	1) IL-4 can promote the synthesis of collagen I/III, fibronectin, and other ECM in fibroblasts. 2) IL-4 is a chemokine for fibroblasts. 3) IL-4-induced macrophage-derived IGF-I protects myofibroblasts from apoptosis	Wynes et al. (2004); Antoniou et al. (2007)
IL-6	Pro-fibrotic	1) The IL-6/STAT3/Smad3 axis has profibrotic effects. 2) IL-6 inhibits apoptosis of IPF-derived fibroblasts and promotes apoptosis of normal fibroblasts. 3) High levels of IL-6 and IL-8 are early features of AE-IPFs and are associated with worse outcome	Moodley et al. (2003); Papiris et al. (2018); Epstein Shochet et al. (2020)
IL-8	Pro-fibrotic	1) IL-8 promotes self-renewal, proliferation, and migration of mesenchymal progenitor cells in an autocrine manner. 2) IL-8 stimulates the recruitment and activation of macrophages in a paracrine manner	Yang et al. (2018)
IL-10	Anti-fibrotic	1) IL-10 has powerful anti-inflammatory effects. 2) IL-10 inhibits collagen I synthesis, although the antifibrotic effect of IL-10 is controversial	Arai et al. (2000); Barbarin et al. (2005); Nakagome et al. (2006); García-Prieto et al. (2010); Li et al. (2011)
IL-11	Pro-fibrotic	IL-11 promotes fibrosis *via* JAK/STAT pathway, Ras/Raf/MEK/ERK1/2 pathway, and PI3K/Akt/mTOR pathway	Ng et al. (2019); Kortekaas et al. (2021)
IL-13	Pro-fibrotic	IL-13 stimulates fibroblast proliferation, and induces TGF-β, PDGF, CTGF, collagen I, and fibronectin production	Murray et al. (2008); Passalacqua et al. (2017)
IL-17	Pro-fibrotic	1) IL-17A promotes cell proliferation, ECM deposition, and myofibroblast differentiation through NF-κb and JAK2 signaling. 2) IL-17B is also involved in dysbiosis of lung microbiota. IL-17 cooperates with TGF-β1-mediated Smad2/3 and ERK1/2 to induce EMT in human pulmonary alveolar epithelial cells	Wilson et al. (2010); Wang et al. (2017); Zhang et al. (2019a); Yang et al. (2019)
IL-18	Pro-fibrotic	IL-18 promotes senescence and SASP in pulmonary fibroblasts by blocking the Klotho pathway	Zhang et al. (2019b)
IL-22	Anti-fibrotic	IL-22 inhibits TGF-β-induced signaling pathways and reduces EMT and myofibroblast differentiation	Gu et al. (2021)
IL-24	Pro-fibrotic	IL-24 cooperates with IL-4 to promote macrophage M2 polarization	Rao et al. (2021)
IL-25	Pro-fibrotic	1) IL-25/IL-33/TSLP$^+$ AECs-IL-25R/IL-33R/TSLPR$^+$ (myo)fibroblasts axis is involved in epithelial-mesenchymal crosstalk. 2) Autocrine IL-25/IL-33/TSLP (thymic stromal lymphopoietin) from alveolar epithelial cells can cause damage and phenotypic changes in alveolar epithelial cells	Xu et al. (2020)
IL-31	Pro-fibrotic	1) IL-31 regulates the transcription of ECM and AECs-related genes. 2) IL-31 can cause collagen deposition and decreased lung function	Yombo et al. (2021)
IL-37	Anti-fibrotic	1) IL-37 resulted in enhanced autophagy and attenuated TGF-β1 of IPF fibroblasts. 2) IL-37 inhibits oxidative stress-induced death of AECs	Kim et al. (2019)

The ATX/LPA/LPAR axis plays a potent role in pulmonary fibrosis. Lysophosphatidic acid (LPA) has been proven to activate G protein-mediated signal transduction pathways by binding to its receptors (LPAR1 and LPAR2), which leads to different reactions from lung cells, including the promotion of the apoptosis of epithelial cells, regulation of endothelial permeability, activation of αvβ6 integrin-mediated TGF-β signaling, secretion of IL-8, and recruitment and survival of fibroblasts (Tager et al., 2008; Ninou et al., 2018). LPA can promote the apoptosis of epithelial cells (Suryadevara et al., 2020). LPA is produced by many metabolic pathways *in vivo*, among which ATX/LPA/LPAR is the main pathway. Phosphatidylcholine (PC) generates LPC (lysophosphatidylcholine) under the action of phospholipase A1/phospholipase A2 (PLA1/PLA2), and LPC is hydrolyzed under the action of autotaxin (ATX)/lysoPLD to generate LPA. LPA exerts biological effects by binding to LPAR (Suryadevara et al., 2020). Drugs targeting different positions of the ATX/LPA/LPAR axis have entered clinical trials. The ATX inhibitors BBT-877 and GLPG1690 entered phase I clinical trials (completed) and phase III clinical trials (terminated) on their anti-IPF treatment, respectively. The LPA1 receptor antagonists BMS-986278 (recruiting) and BMS-986020 (completed) have also entered phase II clinical trials.

5 GROWTH FACTOR AND CYTOKINE SIGNALING CASCADES IN IDIOPATHIC PULMONARY FIBROSIS

5.1 Receptor-Type Tyrosine Kinases and Non-Receptor-Type Tyrosine Kinases Signaling Cascades

Due to the central role of growth factors in IPF, the signaling cascade of growth factor receptors is an indispensable topic. Growth factor receptors (GFRs) are receptor-type tyrosine kinases (RTKs), and their counterparts are non-receptor-type tyrosine kinases (non-RTKs) free in the cytoplasm. Src family kinases (SFKs) are a non-RTK family with eleven members, among which Src, Yes and Fyn are ubiquitously reported (Li et al., 2020). RTK and non-RTK signaling crosstalk with

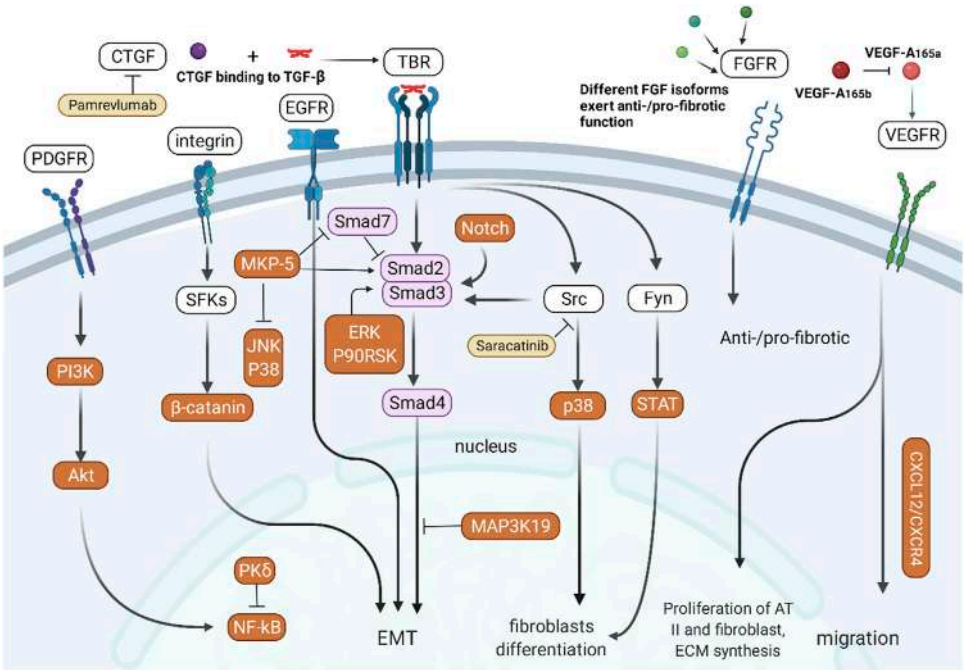

FIGURE 4 | Crosstalk between RTK/non-RTK and other signaling pathways. PDGFR activates NF-κB signaling through the PI3K/Akt pathway. Integrins participate in EMT of epithelial cells through SFK/β-catenin-mediated signaling. TGF-β/Smad signaling interacts with the CTGF, Src, Fyn, and Notch pathways to participate in EMT in epithelial cells. Different subtypes of FGF and VEGF have different pro- or antifibrotic effects.

numerous other pathways and contribute to fibrosis *via* phenotype modulation of fibroblasts and AECs (**Figure 4**).

The combination of TGF-β and TBR can not only initiate the TGF-β/Smad signaling pathway but also indirectly participate in the differentiation of fibroblasts by activating Src/p38 (Pechkovsky et al., 2008) and Fyn/STAT (Xu et al., 2019). Integrins can activate SFKs to activate β-catenin and participate in EMT of epithelial cells (Ulsamer et al., 2012). CTGF participates in abnormal tissue repair by assisting TGF-β. The Notch pathway can also promote TGF-β-mediated fibroblast differentiation through activation of Smads (Aoyagi-Ikeda et al., 2011). MAP3K19 regulates nucleocytoplasmic shuttling of the activated R-Smads, which promotes TGF-β-mediated fibrosis (Boehme et al., 2016). MKP-5, a tyrosine phosphatase negatively regulating the p38 and JNK pathways, inhibits Smad7 activity but promotes Smad3 phosphorylation and the expression of fibrogenic genes (Xylourgidis et al., 2019).

PDGFR activates NF-κB *via* the Src/PI3K/Akt pathway (Cheng et al., 2014). PKCδ attenuates pulmonary fibrosis by enhancing the stability and activity of A20, an inhibitory protein of NF-κB signaling. The PDGFR/mTOR signaling pathway is associated with PINK1/PARP2 dysregulation-induced mitophagy deficiency, leading to myofibroblast differentiation and proliferation (Tsubouchi et al., 2018). Different isoforms of FGF participate in the process of fibrosis through FGFR; for example, FGF1 (Ramos et al., 2010) and FGF10 (Cheng et al., 2014) are anti-fibrotic, while FGF2 is pro-fibrotic (Ju et al., 2012). VEGF-A165a has been proven to promote the proliferation of AT II cells and fibroblasts and ECM expansion, and VEGF-A165b could counteract this effect (Varet et al., 2010; Barratt et al., 2017). VEGFR1 induces pulmonary fibrosis by promoting the migration of VEGFR1+ cells, which is dependent on the SDF-1/CXCR4 axis (Amano et al., 2021). In addition to the above GFR, Gas6/TAM receptor-type tyrosine kinases have also been reported to promote pulmonary fibrosis (Espindola et al., 2018).

5.2 Developmental Pathways: Hedgehog, Wnt, and Notch

Developmental pathways play an important role in the developmental stages of the lung. In adults, some developmental pathways enter a dormant state but are activated again when abnormal lung damage occurs. These signaling pathways include the TGF-β, FGF, Hedgehog, Wnt, and Notch signaling pathways (Chanda et al., 2019) (**Figure 5**). Among them, TGF-β and FGF have been mentioned in the previous section and will not be repeated here.

The Hedgehog pathway starts with the Hedgehog ligand (Hh) and its receptor ptch1 and then activates downstream pathways in a Gli transcription factor-dependent (classical) or independent (non-classical) manner. In the canonical pathway, the binding of Hh to ptch1 relieves the inhibitory effect of ptch1 on Smo. Then, Smo is transferred to the plasma membrane and participates in the regulation of genes in the nucleus by activating full-length Gli to become Gli1/2/3 (active form), thereby inducing AEC

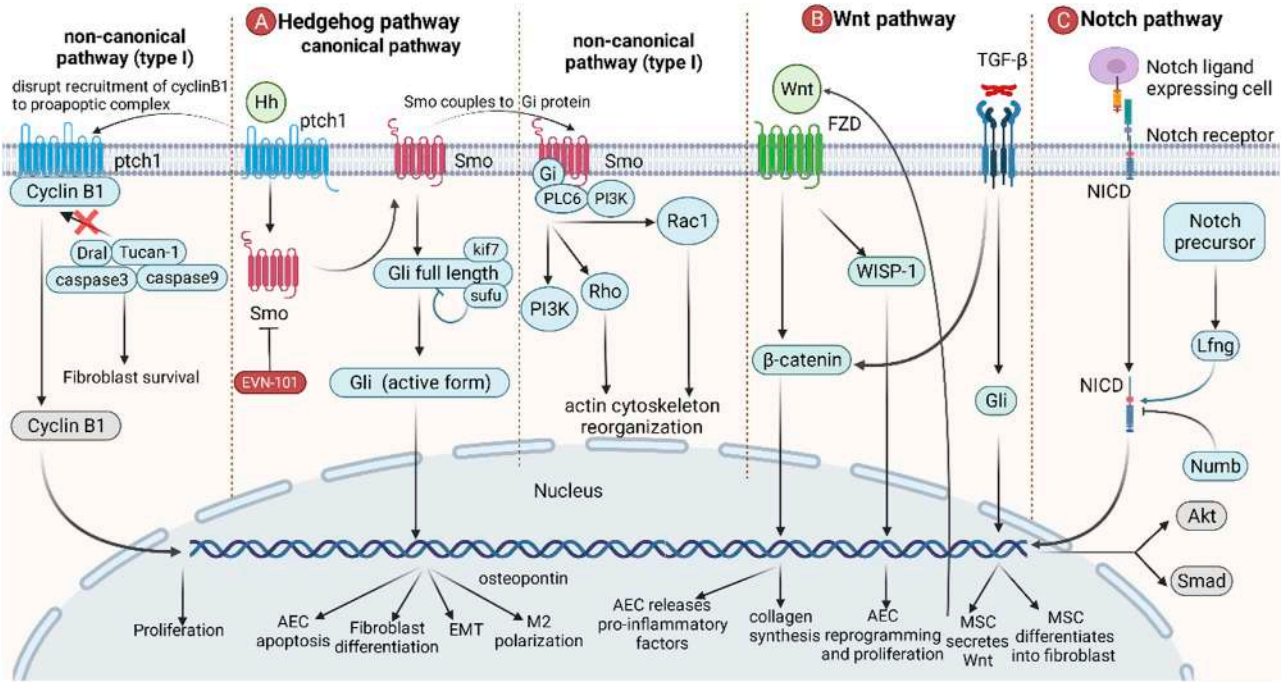

FIGURE 5 | The role of developmental pathways in IPF. **(A)** Canonical hedgehog pathways start from the binding of Hh to ptch1, and Smo then migrates to the plasma membrane, releasing and activating full-length Gli, which leads to AEC apoptosis, fibroblast differentiation, EMT, and M2 polarization. In non-canonical hedgehog pathways (type I), binding of Hh to ptch1 blocks the recruitment of the pro-apoptotic complex by cyclin B1, resulting in anti-apoptosis and promotion of cell proliferation. In non-canonical hedgehog pathways (type II), Smo is coupled to Gi protein, activating downstream PI3K, Rho, and Rac1, resulting in an increase in intracellular calcium concentration and a rearrangement of the cytoskeleton. **(B)** Wnt promotes the accumulation of β-catenin by binding to FZD, thereby stimulating AECs to release proinflammatory factors and promote collagen synthesis in fibroblasts. Wnt also directly induces AEC reprogramming and proliferation *via* WISP-1. TGF-β can induce MSCs to secrete Wnt and promote MSC differentiation. **(C)** The Notch ligand binds to the Notch receptor, causing the Notch intracellular domain (NICD) of the Notch receptor to break and enter the nucleus, leading to gene transcription. Lfng positively regulates this process, while Numb negatively regulates this process. Wnt is also involved in the regulation of Akt and Smad pathways.

apoptosis, fibroblast differentiation, EMT, and M2 polarization (Effendi and Nagano, 2021). In the non-canonical pathway (Type I), the binding of Hh to ptch1 prevents cyclin B1 from recruiting proapoptotic complexes, thereby failing to activate caspase-mediated apoptosis. After entering the nucleus, cyclin B1 can also regulate cell cycle progression by promoting cell proliferation (Robbins et al., 2012; Effendi and Nagano, 2021). In the non-canonical pathway (Type II), the coupling of Smo to Gi protein activates downstream PI3K, Rho, and Rac1, leading to an increase in intracellular calcium concentration and cytoskeleton rearrangement (Robbins et al., 2012; Effendi and Nagano, 2021).

The Wnt signaling pathway is an important signaling pathway in response to postnatal injury and regeneration. Binding of Wnt3a to Frizzled (FZD), followed by β-catenin accumulation, induces AECs to release proinflammatory factors (IL-6, IL-1β) and promotes fibroblast collagen synthesis. In addition, Wnt-inducible signaling protein-1 (WISP-1) is also involved in AEC reprogramming and proliferation (Königshoff et al., 2009). TGF-β promotes the differentiation of mesenchymal stem cells (MSCs) into fibroblasts by activating Gli to secrete Wnt5a, Wnt10a, and Wnt7b, thereby initiating the Wnt pathway (Chen et al., 2018; Martin-Medina et al., 2018). Since Wnt signaling is associated with a large number of normal physiological functions, there are currently no anti-IPF drugs targeting this pathway.

The Notch pathway is a highly conserved signaling pathway that mediates short-range signaling in neighboring cells. The Notch ligand-expressing cell binds to the Notch receptor, causing the Notch intracellular domain (NICD) of the Notch receptor to break and enter the nucleus to lead to gene transcription. Lfng positively regulates this process, while Numb negatively regulates this process (Kiyokawa and Morimoto, 2020). Notch is also involved in the TGF-β/Smad3-mediated transformation of fibroblasts to myofibroblasts (Aoyagi-Ikeda et al., 2011). Notch also induces phosphorylation of Akt by stimulating the expression of insulin-like growth factor-1R (IGF1R), which in turn promotes pulmonary fibrosis by hypoxia-inducible factor-1 (HIF-1) (Eliasz et al., 2010).

5.3 Other Critical Signaling Cascades in Idiopathic Pulmonary Fibrosis: JAK/STAT, PI3K/Akt/mTOR, MAPK, and Hippo-YAP/TAZ

In recent years, research on the molecular mechanism of fibrosis has become increasingly in depth, and the JAK/STAT, PI3K/Akt/

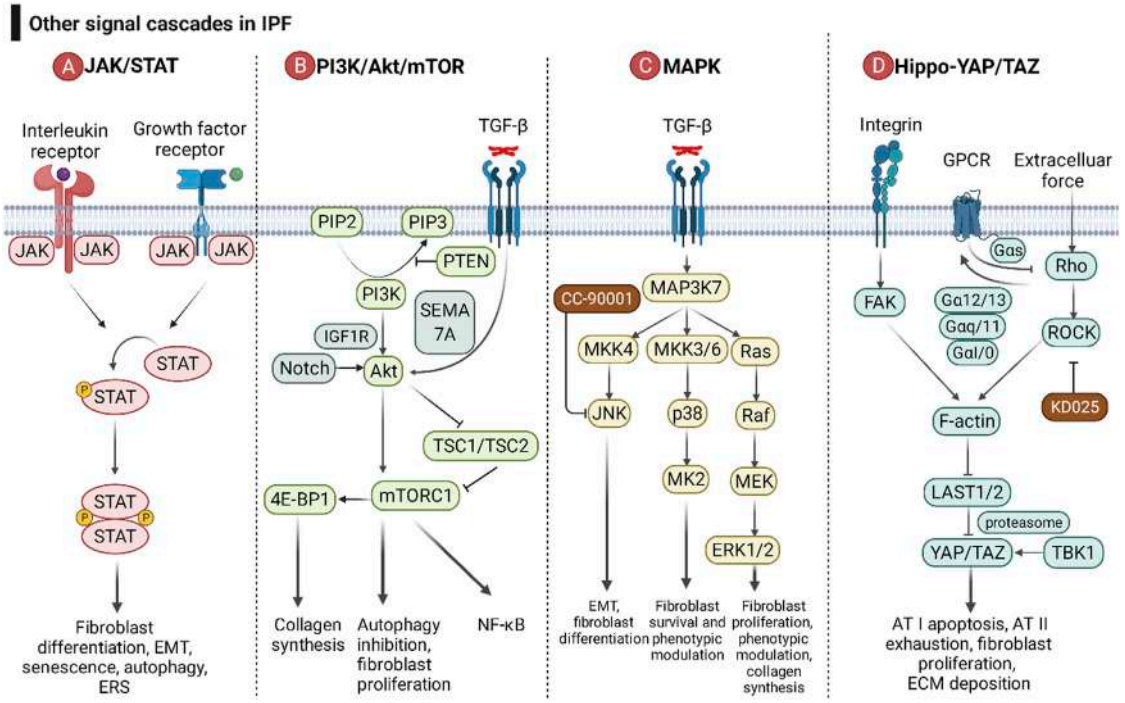

FIGURE 6 | The roles of the JAK/STAT pathway, PI3K/Akt/mTOR pathway, MAPK pathway, and Hippo-YAP/TAZ pathway in IPF. **(A)** After interleukins and growth factors bind to the corresponding receptors, they can activate the JAKs coupled to the receptors, and then JAKs can phosphorylate STAT. Homodimerized STAT enters the nucleus and affects gene transcription. **(B)** Growth factors and cytokines can activate PI3K by binding to receptor-type tyrosine kinases, and then PI3K catalyzes the phosphorylation of PIP2 to PIP3. PIP3 can activate Akt and its downstream mTOR complex (mTORC). PTEN is a negative regulator of the PI3K/Akt/mTOR pathway. TGF-β and Notch are positive regulators of Akt. **(C)** The MAPK pathway consists of three downstream cascades, the JNK, p38, and ERK pathways. JNK and p38 signaling are activated through a pattern of MAP3K-MAP2K-MAPK signaling cascades, while ERK is activated through the MAP3K-Ras-Raf-MEK-ERK cascade. MK2 is a downstream signaling molecule of p38. Growth factors can activate ERK5, and ERK5 and p90RSK can acetylate Smad, thereby promoting the TGF-β/Smad pathway. **(D)** The Hippo-YAP/TAZ pathway is involved in pulmonary fibrosis through the integrin pathway, G protein-coupled receptor pathway, and mechanotransduction pathway. ECM force initiates the Hippo-YAP/TAZ pathway with activation of FAK and Rho/ROCK. FAK and Rho/ROCK relieve the inhibition of the transcription factor YAP/TAZ by large tumor suppressor kinase 1 and 2 (LAST1/2) by promoting the growth, stability, and contractibility of F-actin. Different GPCR signaling pathways regulate LAST1/2 activity through the Rho/ROCK/F-actin pathway.

mTOR, MAPK, and Hippo-YAP/TAZ signaling cascades have received extensive attention (**Figure 6**). Drugs targeting these signaling pathways are already in clinical trials.

Cells respond to inflammatory factors rapidly through JAK/STAT signaling. After interleukins (IL-4, IL-6, IL-11, IL-13, and IL-31) and growth factors (TGF-β, PDGF, VEGF, EGF, and FGF) bind to the corresponding receptors, they can activate and interact with the receptors. Receptor-coupled JAKs (JAK1, JAK2, JAK3, and TYK2) phosphorylate STATs (STAT1, STAT2, STAT3, STAT5, and STAT6) (Montero et al., 2021). Homodimerized STAT enters the nucleus to affect gene transcription, leading to fibroblast differentiation, EMT, senescence, autophagy, and ERS (Montero et al., 2021). Despite numerous isoforms of JAK and STAT, JAK2/STAT3 plays a dominant role in pulmonary fibrosis (Milara et al., 2018). Jaktinib dihydrochloride monohydrate and CC-90001, as JAK1/2 inhibitors, have entered phase II clinical trials (NCT04312594 and NCT03142191, respectively). Since different subtypes of STAT share downstream pathways, blocking only one subtype of STAT may lead to ineffective treatment due to the compensatory effect of other subtypes of STAT. Therefore, no therapeutic drugs targeting STAT have entered clinical trials.

PI3K/Akt/mTOR signaling is involved in autophagy and has caused a huge hit in the field of tumor therapy. A genome-wide association study revealed an association of mTOR with susceptibility to fibrosis (Allen et al., 2020). Growth factors and cytokines activate PI3K by binding to receptor-type tyrosine kinases, which in turn catalyze the phosphorylation of PIP2 to PIP3. PIP3 activates Akt and its downstream mTOR complex (mTORC), which promotes collagen synthesis, proliferation in fibroblasts, and EMT in epithelial cells (Lawrence and Nho, 2018; Fang et al., 2020). PTEN can inhibit the phosphorylation of PIP2, thereby inhibiting the signaling pathway downstream of PI3K and reducing the senescence of AECs (Qiu et al., 2019). Akt relieves the inhibition of mTORC1 by TSC1/TSC2 by phosphorylating TSC2 (Huang and Manning, 2009). mTOR also intersects with NF-κB signaling (Guo et al., 2013). TGF-β signaling can activate Akt in a SEMA 7A-dependent manner, which in turn activates downstream mTOR signaling and promotes lung fibrosis (Reilkoff et al., 2013). mTOR can activate collagen synthesis

via 4E-BP1 (Woodcock et al., 2019). RPS6KB2 (ribosomal protein S6 kinase B 2) is reported to be involved in the process of aging and IPF due to its activation by growth factors and regulation of the protein kinase mTOR signaling pathway (Hao et al., 2017).

Mitogen-activated protein kinase (MAPK) mainly consists of three downstream cascades, the JNK, p38 and ERK pathways. JNK/p38 is activated *via* a MAP3K-MAP2K-MAPK signaling cascade, whereas ERK is activated *via* a MAP3K-Ras-Raf-MEK-ERK cascade (Woodcock et al., 2019). JNK and p38 signaling participates in apoptosis, necroptosis, and EMT of AEC; fibroblast-to-myofibroblast differentiation; and maintenance of the myofibroblast phenotype (Kasuya et al., 2021). MK2, downstream of the p38 pathway, has a role in fibroblast invasion and fibrosis (Liang et al., 2019). Growth factors can activate ERK5, and then ERK5 and p90RSK can acetylate Smad, thereby promoting the TGF-β/Smad pathway (Kim et al., 2020). The ERK1/2-calpain pathway has been reported to be associated with pulmonary fibrosis *in vivo* and *in vitro* (Zou et al., 2020).

The Hippo-YAP/TAZ pathway is involved in pulmonary fibrosis through the integrin pathway, G protein-coupled receptor pathway, and mechanotransduction pathway. Integrins promote the growth, stability, and contractibility of F-actin by activating FAK, thereby relieving the inhibitory effect of large tumor suppressor kinase 1 and 2 (LAST1/2) on YAP/TAZ. After entering the nucleus, YAP/TAZ regulates AT I apoptosis and AT II exhaustion, fibroblast proliferation and ECM expansion (Sun et al., 2021). The mechanical force of ECM can also activate F-actin *via* Rho/ROCK signaling and promote pulmonary fibrosis (Zhou et al., 2013). Different GPCR signaling pathways (Gαs, Gα12/13, Gαq, and Gαi/o) regulate LAST1/2 activity through the Rho/ROCK/F-actin pathway (Haak et al., 2020). Tank binding kinase 1 (TBK1) activates YAP/TAZ in a proteasomal machinery-dependent but LAST-independent manner (Aravamudhan et al., 2020).

6 ADVANCES IN THE DEVELOPMENT OF ANTI-IPF DRUGS TARGETING CYTOKINE AND GROWTH FACTOR PATHWAYS

6.1 Pirfenidone and Nintedanib, Two FDA-Approved Drugs for Patients With Idiopathic Pulmonary Fibrosis

Antifibrotic therapy provides survival benefit and protection against all-cause and respiratory-related hospitalization for IPF patients (Mooney et al., 2021). FDA approved antifibrotic drugs for treating IPF include Pirfenidone and Nintedanib, and their clinical efficacy is similar though there are no high-quality clinical trials directly comparing the efficacy of the them (Marijic et al., 2021). Regarding their mechanism of action, both of them target multiple growth factor and cytokine-related signaling pathways. Now that the pathogenesis of IPF remains unclear, targeting multiple pathways may be an effective treatment strategy for IPF. In addition, since the mechanisms of Pirfenidone and Nintedanib are not the same, a combination treatment may have synergistic effects. The combination of Pirfenidone and Nintedanib has been shown to be well tolerated and safe, which encourages further research into combination therapy (Flaherty et al., 2018; Vancheri et al., 2018). How to relieve the side effects caused by the off-target effects of Pirfenidone and Nintedanib is also an important topic.

6.1.1 Pirfenidone

Pirfenidone is a pyridine compound approved for marketing in Japan in 2008. It was originally developed as an anti-inflammatory drug. Interestingly, studies in animal models demonstrated that it had the effect of resisting fibrosis of various organs, and it was later approved for IPF treatment. The anti-fibrotic mechanism of Pirfenidone has not been fully elucidated. Published studies indicated that it could inhibit fibrosis mainly by blocking TNF-α and TGF-β/Smad pathways (Oku et al., 2002; Schaefer et al., 2011; Conte et al., 2014).

Three large-scaled RCTs demonstrated that Pirfenidone could delay FVC decline, improve progression-free survival, increase exercise tolerance, and reduce all-cause or IPF related mortality (Taniguchi et al., 2010; Noble et al., 2011; Noble et al., 2012; King et al., 2014). Recently, a systematic review and meta-analysis of RCTs also indicated that Pirfenidone treatment was associated with a longer progression-free-survival and a lower incidence of acute exacerbation (Wu et al., 2021). In the real-world practice, Pirfenidone also provided beneficial effects on survival and pulmonary function decline (Lee et al., 2021). Moreover, A *post hoc* analysis of ASCEND and CAPACITY program suggested a clinically relevant benefit of Pirfenidone in IPF patients with more advanced lung function impairment (Nathan et al., 2019).

However, the use of Pirfenidone has some limitations, such as a short half-life (2.5 h) and a high daily dose (2,403 mg/day). Although generally well tolerated, a minority of patients discontinued treatment due to gastrointestinal and skin-related adverse events. These side effects can be mitigated or prevented by taking it with/after meals, avoiding Sun exposure, wearing protective clothing, and using broad-spectrum sunscreens (Costabel et al., 2014). In addition, caution is recommended prior to Pirfenidone use in IPF patients with severe hepatic and renal insufficiency.

6.1.2 Nintedanib

Nintedanib is a small molecule tyrosinase inhibitor approved for marketing in 2014. Initially, researchers intended to screen drugs that selectively inhibit VEGFR-2 for anticancer treatment. However, in subsequent research, Nintedanib had good antifibrotic and anti-inflammatory effects and was approved for IPF treatment. Nintedanib mainly inhibits tyrosine kinase receptors, such as PDGFR, FGFR, and VEGFR, and non-receptor tyrosine kinases, such as Src and Flt-3 (Hilberg et al., 2008).

The INPULSIS-1 and INPULSIS-2 programs demonstrated that Nintedanib could delay FVC decline and reduce the risk of disease progression (Richeldi et al., 2014; Keating, 2015). However, the parameter of time to first AE and HRQL (Health-related Quality of Life) was inconsistent between

TABLE 2 | Emerging drugs targeting cytokine and growth factor pathways for IPF treatment in clinical trials.

Targets	Drugs	Clinical trial information
TGF-β	GC1008/fresolimumab (TGF-β antibody)	Phase 1 (completed, NCT00125385)
TGF-β	PLN-74809 (integrin αvβ6/αvβ1 inhibitor)	Phase 2 (recruiting, NCT04072315)
		Phase 2 (recruiting, NCT04396756)
TGF-β	TRK-250 (anti-TGF-β mRNA nucleic acid)	Phase 1 (Active, not recruiting, NCT03727802)
TGF-β	TD139 (suppress TGF-β receptor by targeting galectin-3)	Phase 1/2 (completed, NCT02257177)
		Phase 2 (recruiting, NCT03832946)
TGF-β1, CTGF, IL-β23p19, IL-6	PBI-4050 (inhibitor of TGF-β1, CTGF, IL-β23p19, IL-6)	Phase 2 (completed, NCT02538536)
CTGF	Pamrevlumab (CTGF mAb)	Phase 3 (recruiting, NCT03955146); Phase 3 (recruiting, NCT04419558)
IL-13	QAX576 (IL-13 mAb)	Phase 2 (terminated, NCT01266135)
	Tralokinumab (IL-13 mAb)	Phase 2 (terminated, NCT01629667)
	Lebrikizumab (IL-13 mAb)	Phase 2 (completed, NCT01872689)
IL-4, IL-13	SAR156597 (IL-4 and IL-13 Antibody)	Phase 2 (completed, NCT02345070)
CCL-2	CNTO 888 (CCL2 mAb)	Phase 2 (terminated, NCT00786201)
JNK	Jaktinib Dihydrochloride Monohydrate (JNK1/2 inhibitor)	Phase 2 (recruiting, NCT04312594)
	CC-90001 (JNK1/2 inhibitor)	Phase 2 (active, not recruiting, NCT03142191)
Src	Saracatinib (Src kinase inhibitor)	Phase 1/2 (recruiting, NCT04598919)
Hedgehog pathways	taladegib/ENV-101 (Smo receptor inhibitor)	Phase 2 (not yet recruiting, NCT04968574)
ROCK	KD025 (ROCK2 inhibitor)	Phase 2 (completed, NCT02688647)
Leukotrienes	MN-001/Tipelukast (leukotriene receptor antagonist)	Phase 2 (active, not recruiting, NCT02503657)
LPC-ATX-LPA	BMS-986278 (LPA1R antagonist)	Phase 2 (recruiting, NCT04308681)

INPULSIS-1 and INPULSIS-2 (Keating, 2015). Interestingly, despite similar efficacy of Pirfenidone and Nintedanib, there was a higher rate of discontinuation of Nintedanib due to adverse effects (Takehara et al., 2022). Gastrointestinal adverse events (diarrhea, nausea, and vomiting) were reported most commonly (Keating, 2015). Patients treated with Nintedanib who experience diarrhea should maintain hydration and take antidiarrheal therapy as soon as symptoms occur. Dose reduction can also be taken into consideration (Corte et al., 2015).

The timing of antifibrotic therapy may influence the outcome of IPF treatment. A retrospective study indicated that patients who initiated Nintedanib immediately after IPF diagnosis might have reduced hospitalization risk and medical costs compared with those who start treatment later (Singer et al., 2022). A *post hoc* analysis of INPULSIS program suggested that probable UIP with traction bronchiectasis in high-resolution CT might be sufficient for patients to benefit from Nintedanib, implying the importance of early antifibrotic therapy (Lobo, 2016).

Although high-quality clinical trial data are currently lacking to directly demonstrate that Nintedanib is effective in end-stage IPF, the latest *post hoc* analyses of the INPULSIS and INSTAGE programs suggest that it has some effect in IPF patients with more severely impaired gas exchange (Richeldi et al., 2020).

6.1.3 Implications of Pirfenidone and Nintedanib for Anti-Idiopathic Pulmonary Fibrosis Drug Discovery

With the launch of Pirfenidone and Nintedanib in multiple countries, the number of IPF patients not receiving anti-fibrotic therapy will decrease in the future, and it will become increasingly difficult to observe the natural progression of IPF. For ethical reasons and feasibility of patient recruitment, more and more clinical trials of new drugs will use Pirfenidone and Nintedanib as controls. This sets a higher standard for the efficacy of new drugs. In addition, there will be a trend to combine new drugs with existing anti-fibrotic therapies in clinical trials, and whether they can bring additional benefits will be the determining factor. Furthermore, whether a single drug is sufficient to effectively delay the progression of IPF is an important pharmacoeconomic proposition. Since IPF is a rapidly progressive and irreversible disease, whether anti-fibrotic therapy is effective in different stages of IPF, especially in end-stage IPF and suspected IPF, also deserves further study. There are currently no high-quality clinical trials that directly demonstrate the efficacy of antifibrotic therapy in end-stage IPF. In addition, whether patients with suspected IPF on unbiopsied CT images without biopsy require prophylactic antifibrotic therapy remains to be further investigated.

6.2 Anti-Idiopathic Pulmonary Fibrosis Drugs Targeting Cytokine and Growth Factor Pathways in Clinical Trials

With the continuous exploration of the pathophysiological process of IPF and the continuous clarification of the cytokine and growth factor pathways, the targets of IPF treatment have also become diversified. Many targets and drugs developed based on them have entered clinical trials (**Table 2**). Although most of them fail to enter phase III clinical trials, a few drugs show promise in the treatment of IPF. Currently, pamrevlumab (CTGF mAb) and PRM-151 (recombinant pentraxin 2) have successfully entered Phase III clinical trials.

6.3 Information About Clinical Trials That Target Complications and Comorbidities of Idiopathic Pulmonary Fibrosis

Complications and comorbidities are of substantial significance to the disease progression and quality of life in IPF patients.

Cough affects up to 80% of patients with IPF, is frequently disabling, and lacks effective therapy. A 24-week, double-blind, two-treatment, 24 patients enrolled, two-period crossover trial showed that thalidomide improved cough and respiratory quality of life in patients with IPF (Horton et al., 2012).

Patients with IPF are also at risk of developing pulmonary hypertension as the underlying condition worsens (Tanaka et al., 2017). The BUILD-3 program showed no benefit of Bosentan in the life quality of IPF patients (King et al., 2011a). However, a prospective study showed that Bosentan was associated with a trend toward decreased adverse events and improved respiratory status (Tanaka et al., 2017). Sildenafil is an FDA-approved drug for pulmonary arterial hypertension. A double-blind randomized clinical trial showed that the combination of Sildenafil and Nintedanib significantly reduced BNP (B-type natriuretic peptide, an indicator of heart failure) in IPF patients with right ventricular dysfunction compared with Nintedanib alone, but did not improve HRQL (Behr et al., 2019; Suarez-Cuartin and Molina-Molina, 2019). More recently, a systematic review and meta-analysis of RCTs reported that Sildenafil probably reduced all-cause mortality in IPF patients (Pitre et al., 2022), although more studies are needed to confirm this. The 2015 ATS/ERS/JRS/ALAT clinical practice guideline conditionally recommends against the use of Bosentan and Sildenafil, while the 2011 version strongly recommends against the use of them.

Increased bacterial load and loss of microbial diversity have been reported in IPF (Han et al., 2014; Molyneaux et al., 2014), which are associated with disease progression and immune response (Huang et al., 2017; Molyneaux et al., 2017). However, the role of several antibiotics in IPF appears to be controversial. A retrospective study of 85 AE-IPF patients showed that azithromycin may improve survival in patients with AE-IPF compared with a fluoroquinolone-based regimen (Kawamura et al., 2017). On the contrary, a randomized controlled crossover study of 25 IPF patients does not support the use of low-dose azithromycin for chronic cough in IPF (Guler et al., 2021). In addition, a pragmatic randomized unblinded clinical study of 513 IPF patients didn't support the additional use of co-trimoxazole or doxycycline (Martinez et al., 2021). Thus, the selection of antibiotics and the effect of them on immune cells need to be further explored.

Gastroesophageal reflux occurs in a high proportion of IPF patients (Raghu et al., 2015), and chronic microaspiration secondary to gastroesophageal reflux is thought to play a role in disease pathogenesis and progression (Savarino et al., 2013). The 2015 ATS/ERS/JRS/ALAT clinical practice guideline conditionally recommends anti-acid therapy. However, whether anti-acid therapy is effective in IPF patients remains highly controversial (Lee et al., 2011; Lee et al., 2013; Kreuter et al., 2016; Raghu et al., 2018; Tran et al., 2021). In addition, whether anti-acid treatment should choose anti-acid drugs or radical therapy (such as Nissen fundoplication) requires further study (Johannson et al., 2017). Therefore, to investigate the efficacy of anti-acid therapy in slowing the progression of IPF, a phase III, randomized, placebo-controlled, double-blind, multicenter clinical trial using lansoprazole is currently enrolling patients with IPF (NCT04965298).

7 CHALLENGE AND OUTLOOK

Research on the pathogenesis of IPF has made considerable progress. After years of preclinical research and clinical studies, the pathogenesis of IPF has changed from simple inflammation to abnormal epithelial-mesenchymal crosstalk and other pathogenic mechanisms. Based on the emerging pathological mechanism, many studies have systematically studied the key roles of cytokine and growth factor pathways in the pathogenesis of IPF. The approval of Pirfenidone and Nintedanib shows that therapy targeting these pathways is very promising. Although investigational drugs targeting some cytokines such as IFN-γ, TNF-α, and IL-13 have not shown satisfactory efficacy, the development of anti-IPF drugs targeting cytokine and growth factor pathways is still promising. Currently, there are numerous clinical trials investigating the efficacy of these drugs in the treatment of IPF and more drugs may be approved based on the results of these trials.

Advances in the field of cytokine and growth factor pathways would help slow disease progression, prolong lives, and improve the quality of life of IPF patients in the future. Therefore, this review tried to provide an overview of cytokines, growth factors, and their signaling pathways in IPF. Furthermore, key information about emerging drugs targeting these pathways for IPF treatment in clinical trials is provided.

Based on the findings of this review, the following points need to be considered in future studies to make strategies targeting these signaling pathways more promising for the treatment of IPF patients. Although upregulating or antagonizing certain cytokine or growth factor signaling pathways alone may not reverse differentiated cells, promoting fibrotic phenotype transition is more promising. Since cytokine and growth factor signaling pathways also play vital roles in other physiological and pathological processes, including damage repair, defense against infection, autoimmune disease, and antitumor immunity, long-term non-selective targeting of these pathways may lead to undesired side effects. Therefore, the identification of more specific targets and the development of drugs having high selectivity on them, the optimization of drug delivery to increase drug concentrations at the target site, and the identification of biomarkers to help select patients who might benefit more from drugs are all worth exploring. In addition, combination therapy with Pirfenidone and Nintedanib might bring better clinical benefits for IPF patients than single drug administration.

One of the novelties of this review is giving an extensive description of the multiple roles of cytokine and growth factor pathways in pulmonary fibrosis, which provide new insights into therapeutic strategies for IPF. These pathways form a complex network in the pathological process of pulmonary fibrosis, and exploring the crosstalk between different signaling cascades may help discover more potential targets. Clinical trials are a critical stage that all approved drugs must go through. Some of the drugs in clinical trials for

the treatment of IPF target cytokines and growth factors. Therefore, we summarize key information from these clinical trials and hope to give insights for the development of more effective drug.

Although IPF is idiopathic by definition, it may become less mysterious with progress in the understanding of the pathogenesis of IPF. This review showed that the number of therapeutic targets from cytokine or growth factor signaling pathways and new targeted drugs entering clinical trials is increasing. These advances show that slowing disease progression, prolonging prognosis, and improving quality of life might be possible in the future for patients with IPF.

In conclusion, cytokine and growth factor signaling pathways are indispensable in the pathogenesis of IPF, and this review hopes to provide theoretical support for the development of novel anti-pulmonary fibrosis drugs targeting cytokine and growth factor pathways.

AUTHOR CONTRIBUTIONS

HBM contributed to the conception of this review and preparation of the manuscript, tables, and figures. SML collected information about the clinical trials. SRL revised the manuscript and helped design the figures; YX contributed to the conception, supervision, and revision of the manuscript. All authors have approved the final article and are included in the disclosure.

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GLOSSARY

AECs alveolar epithelial cells

AE-IPF acute exacerbation of idiopathic pulmonary fibrosis

AT I/AEC I type I AECs

AT II/AEC II type II AECs

ATX autotaxin

BALF bronchoalveolar lavage fluid

BLM bleomycin

COVID-19 corona virus disease 2019

COX cyclooxygenase

CTGF connective tissue growth factor factors

DC dendritic cell

Th helper T cell

DLco/VA diffusion capacity for carbon monoxide per liter of alveolar volume

ECM extracellular matrix

EGF epidermal growth factor

EMT epithelial-mesenchymal transition

EndoMT endothelial-to-mesenchymal transition

ER endoplasmic reticulum

ERK extracellular signal-regulated kinase

ERS endoplasmic reticulum stress

FAK focal adhesion kinase

FEV1 forced expiratory volume in one second

FGF fibroblast growth factor

FVC forced vital capacity

GF growth factor

Hh Hedgehog

HLF-1 human lung fibroblast-1

HRQL health-related quality of life

IGF insulin-like growth factor

IL interleukin

IPF idiopathic pulmonary fibrosis

JAK/STAT Janus kinase/signal transducer and activator of transcription

JNK c-Jun N-terminal kinase

LAP Latency-associated peptide

LPA Lysophosphatidic acid

LT Leukotriene

LTBP Latent TGF-β binding protein

miR micro-RNA

MAPK mitogen-activated protein kinase

MKK Mitogen-activated protein kinase kinase

MMP/TIMP matrix metalloproteinase/tissue inhibitor of metalloproteinase

mAb monoclonal antibody

MSC Mesenchymal stem cell

mtROS mitochondrial reactive oxygen species

PAI plasminogen activation inhibitors

PDGF platelet-derived growth factor

PI3K/Akt/mTOR phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin

PLA2 phospholipase A2

PPAR-γ peroxisome proliferators-activated receptors γ

Rho/ROCK Rho/Rho-associated coiled-coil kinase

RCT randomized controlled trial

ROS reactive oxygen species

SFK Src family kinases

SPHK1/S1P/S1PR sphingosine kinase/sphingosine 1 phosphate/sphingosine 1 phosphate receptor

TBR TGF-β receptor

TF tissue factor

TGF-β transforming growth factor-β

TIMPs tissue inhibitors of metalloproteinases

UPR unfolded protein response

VEGF vascular endothelial growth factor

Wnt Wingless/Integrated

α-SMA α-smooth muscle actin

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CASE REPORT
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Case Report: Pirfenidone in the Treatment of Post-COVID-19 Pulmonary Fibrosis

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Background: Pulmonary fibrosis is one of the sequelae of the COVID-19, which seriously affects the quality of life of survivors. Currently, there are no optimal evidence based guidelines targeting this population.

Case Presentation: We report a 66-year-old female patient without underlying comorbidities admitted to Changsha Public Health Center because of COVID-19. During hospitalization, she developed co-bacterial infection and acute respiratory distress syndrome, and received broad-spectrum antibacterial therapy, invasive mechanical ventilation and extracorporeal membrane oxygenation. After the acute phase, she developed post-COVID-19 pulmonary fibrosis subsequently treated with pirfenidone. Over 96 weeks after pirfenidone treatment, her modified Medical Research Council Dyspnea level improved to 2 from 4 at discharge. Her 6 minutes walk test distance, total lung capacity, and diffusion capacity for carbon monoxide all increased. Chest CT performed on 2 years after illness onset showed regressing fibrosis. The Hospital Anxiety and Depression Scale, Athens Insomnia Scale, and 36-Item Short Form Health Survey questionnaire all improved.

Conclusion: Post-COVID-19 pulmonary fibrosis is a challenging consequence of COVID-19, and our case suggests that pirfenidone may be an effective treatment option.

Keywords: pulmonary fibrosis, post-COVID-19, pirfenidone, long COVID, sequelae

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INTRODUCTION

Since the first report of the Coronavirus disease 2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in Wuhan city on December 2019, the COVID-19 pandemic has lasted more than 2 years. As of March 29, 2022, more than 480 million confirmed cases and 6 million deaths had been reported worldwide according to the World Health Organization (WHO) (1). The clinical manifestations, pathophysiology, and treatment during the acute phase of COVID-19 are well described (2, 3). Several long-term longitudinal follow-up cohorts of patients recovered from COVID-19 have shown that although most patients recover well, up to 57.7–100% of patients still have persistent lung structural abnormalities and pulmonary function impairment (4–6). The common pattern of pulmonary interstitial lesions, also known as post-COVID-19 pulmonary fibrosis or COVID-19 pulmonary fibrosis-like (7), were ground glass opacities (GGOs), ground-glass attenuation and reticular abnormalities (8). At the same time,

chest imaging showed bilateral diffuse interstitial lung disease among patients who complicated acute respiratory distress syndrome (ARDS) during acute phase (9). Relatedly, these survivals suffered from more serious 'Long COVID' (10, 11), a condition described by signs and symptoms of COVID-19 that last longer than 12 weeks and are not explained by an alternative diagnosis, with the most common symptoms of dyspnea, fatigue and 'brain fog'. However, there was no definite long-term medical advice or treatment to reverse pulmonary fibrosis for this population.

Pirfenidone, one of only two drugs approved for the treatment of idiopathic pulmonary fibrosis (IPF) in the world, is a class of pleiotropic pyridine compounds with anti-inflammatory, anti-fibrotic and antioxidant properties. Another drug is nintedanib, which can significantly reduce the absolute value of the drop in forced vital capacity (FVC) among IPF patients (12). The results of a multi-center, double-blind, randomized controlled phase 3 clinical study (ASCEND study) suggest that pirfenidone significantly delayed the decline in FVC in patients with IPF at 52 weeks compared with placebo (−235 ml vs. −428 ml, $P < 0.001$) (13). Besides, at 52 weeks, pirfenidone can reduce all-cause mortality in patients with IPF according to a pooled analyse and meta-analyses (14). To our knowledge, the effectiveness of pirfenidone in the treatment of post-COVID-19 pulmonary fibrosis is unclear. Here, we report a case of post-COVID-19 pulmonary fibrosis treated with pirfenidone over 96 weeks. During the 2-year follow-up period, the patient's symptoms, diffusing function, and interstitial lesions showed continuous improvement.

CASE DESCRIPTION

A previously healthy, 66-year-old female patient presented to the emergency department of Xiangya Hospital on February 2, 2020 (day 2 of illness onset) with a 1-day history of dizziness, nausea and diarrhea. There were no background of pulmonary problems and impaired physical capacity. She underwent a quantitative Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) test for SARS-CoV-2 and a chest computed tomography (CT) examination because of a travel history from Wuhan City within 14 days. The results showed positive to SARS-CoV-2 and multiple GGOs in the right lower lobe (**Figure 1**). Consequently, she was referred to the Changsha Public Health Center for isolation and hospitalization (day 3).

On admission, physical examination showed a body temperature of 37.3°C, a level at 98% of resting finger oxygen saturation without additional oxygen supplement, overweight with a body mass index (BMI) of 25.3 kg/m^2, and moist rales in bilateral lower lungs. The laboratory examination results were as follows: white blood cell (WBC) count was 2.79×10^9/L, with a lymphocyte percentage of 22.2%, the procalcitonin concentration was 0.054 ng/mL, the level of C-reactive protein was 65.7 mg/L, the erythrocyte sedimentation rate was 106 mm/h, the ferritin level was 505.5 ng/mL. The serum β-(1,3)-glucan, galactomannan, RT-PCR results for influenza A and B viruses were normal. A reviewed chest high resolution CT taken on February 3 (day3) presented new and progressive, diffuse, peripheral GGOs in the right upper lobe, right middle lobe and left lower lobe, and consolidation in the right lower lobe (**Figure 1**), typical of acute COVID-19. She was diagnosed with COVID-19 (common type) according to the Chinese Clinical Guidance for COVID-19 Pneumonia Diagnosis and Treatment (4th edition) (15), and received oral antiviral drugs Arbidol and low dose intravenous methylprednisolone hemisuccinate. From day 9, she developed progressive hypoxemia due to suspected co-bacterial infection and COVID-19 aggravation. She was treated with empirical broad-spectrum antibiotics (moxifloxacin, piperacillin tazobactam, meropenem), intravenous immunoglobulin convalescent serum and supported by non-invasive high concentration oxygen. On day 31, she became critically ill and invasive mechanical ventilation (IMV) was conducted because of refractory hypoxemia and progressive lung shadows. Immediately, she was commenced on extracorporeal membrane oxygenation (ECMO) on the next day after IMV. The antibiotics were adjusted to polymyxin B sulfate combined with linezolid, based on a report of bronchoalveolar lavage fluid metagenomic next-generation sequencing which detected 2,55,418 sequences mapped to *Actinomyces odontolyticus* and 3,897 sequences mapped to *Staphylococcus aureus*. Her hypoxia was improved on day 37. She was weaned off ECMO support, meanwhile, de-escalation of antibiotic therapy was conducted. On day 89, she was extubated and freed from IMV.

Chest CT scanned on day 61 showed diffuse interstitial changes, with extensive areas of subpleural and peribronchovascular septal thickening, subpleural reticular abnormalities, traction bronchiectasis, and honeycomb-like changes under the subpleura in both lung fields, which manifested as interstitial lung disease (ILD) (**Figure 2**), and she started taking pirfenidone (Initially 600 mg per day, after 1 week maintain 1,800 mg per day). After 2 months of therapy, her dyspnea and pulmonary fibrosis improved (**Figure 2**), and subsequently, the patient was discharged on day 130, with a level of modified Medical Research Council Dyspnea Scale (mMRC) at 4 (**Table 1**). The patient maintained long-term pirfenidone therapy at home without any liver function damage and hematological complications, and she was followed up on the 7th (1 year after onset) and 20th (2 year after onset) month after discharge. The patient's symptoms and physical capacity had improved with the mMRC scores improved from 4 to 2 and 6-min walking test distance (6-MWD) recovered to 309 meters from 188 meters during treatment. Similarly, the Hospital Anxiety and Depression Scale (HADS), Athens Insomnia Scale (AIS), and Chinese vision of the 36-Item Short Form Health Survey questionnaire (SF-36) all improved on 2 year after initial insult. Pulmonary function tests showed improvement in restrictive ventilatory and diffusing function, with FVC improved from 1.98 liters to 2.30 liters, total lung capacity (TLC) improved from 61.7% of predict to 72.3% and diffusion capacity for carbon monoxide (DLCO) improved from 30.3% of predict to 47.9%. Reticular abnormalities, interlobular septal thickening and honeycomb-like changes were reduced in bilateral lung fields on chest CT screened on day 758 (**Figure 2**).

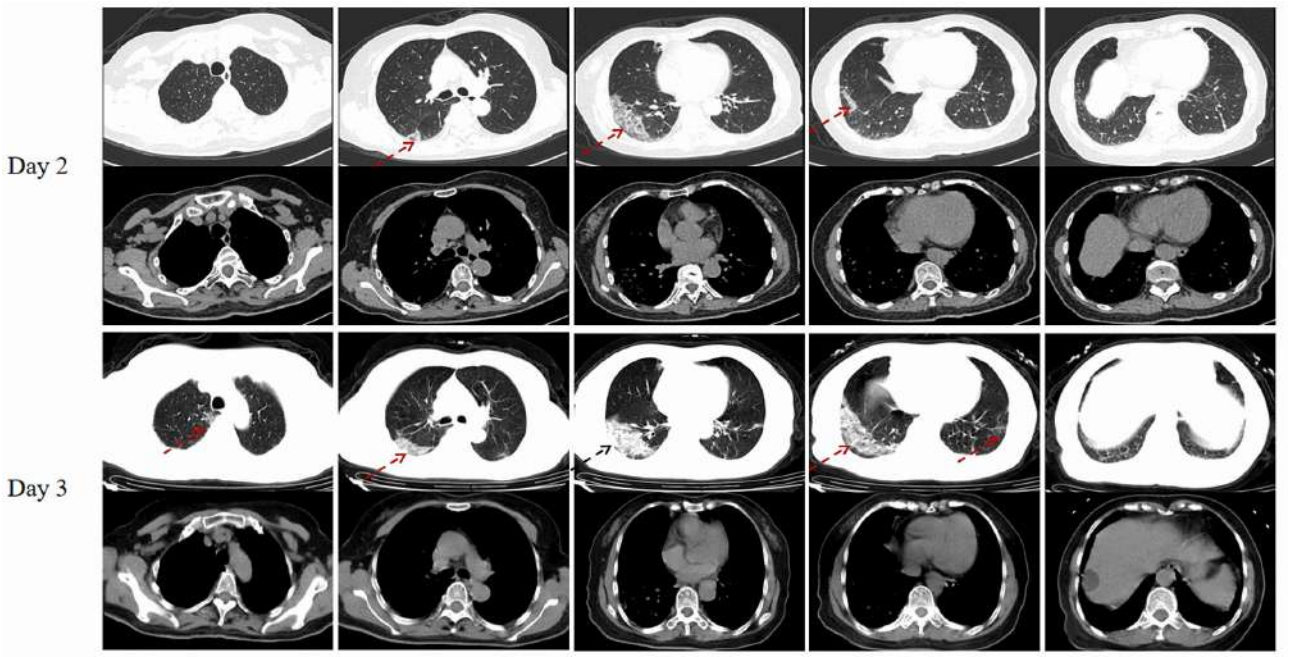

FIGURE 1 | Chest CT of the patient on day 2 and day 3 (On admission) after illness onset. Red dotted arrow: multiple ground glass opacities (GGOs); Black dotted arrow: consolidation.

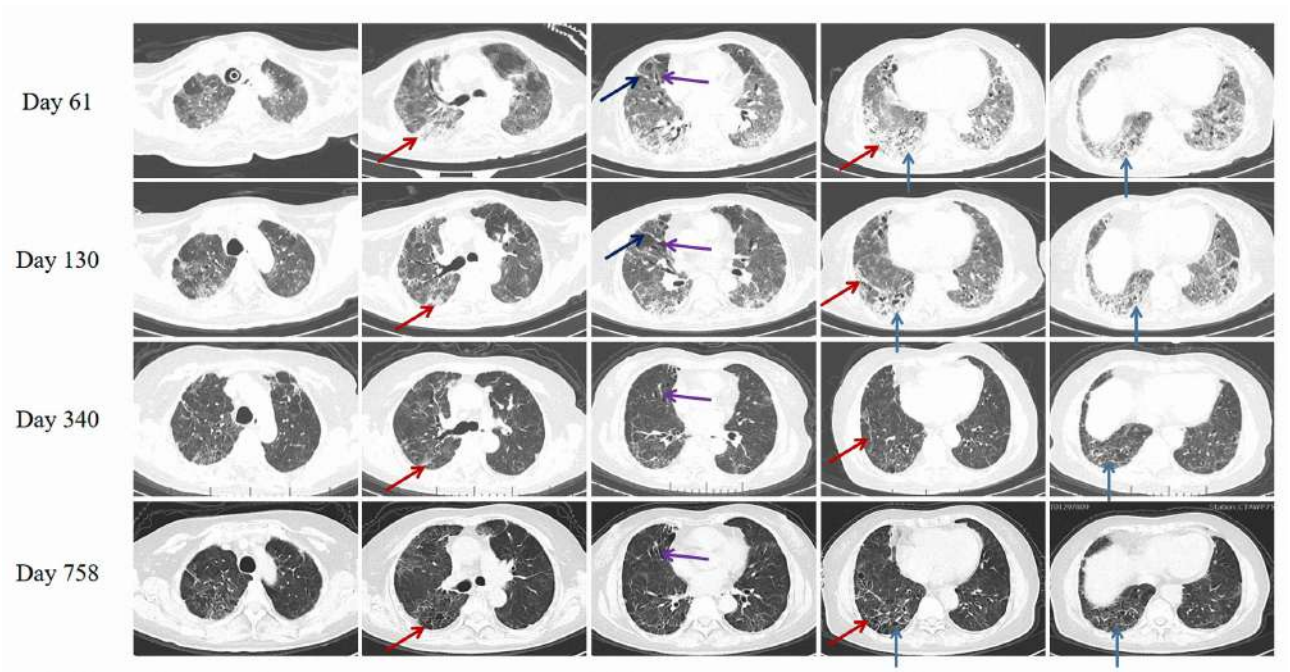

FIGURE 2 | Chest CT on day 61 (Starting taking pirfenidone), day 130 (At discharge), day 340 and day 758 after illness onset. Red solid arrows: subpleural reticular abnormalities and fibrous cords. Black solid arrow: interlobular septal thickening. Purple solid arrow: traction bronchiectasis. Blue solid arrow: honeycomb-like change under the subpleura.

TABLE 1 | Evolution of clinical features from illness onset and taking pirfenidone (2 months after onset).

Clinical features	Before COVID-19	Acute stage	At discharge (4 months after onset)	1 year after onset	2 years after onset
Symptoms					
Fever		√			
Cough		√	√	√	√
Dyspnoea		√	√	√	√
Headache		√			
Fatigue		√	√	√	√
Myalgia		√	√	√	
Nausea/Vomiting		√			
Diarrhea		√			
Chest pain		√	√	√	√
Anosmia		√	√		
Dysgeusia		√	√		
Other		√	√		
Pulmonary rale	/	Moist rale	Velcro rale	Velcro rale	Velcro rale
mMRC, level	0	/	4	3	2
6-MWD, m	/	/	/*	188	309
HADS score					
Anxiety	/	/	/	1	0
Depression	/	/	/	0	0
AIS Score	/	/	/	10	2
SF-36 score					
Physical functioning	/	/	/	25	85
Role-physical	/	/	/	0	100
Bodily pain	/	/	/	72	74
General health	/	/	/	35	55
Vitality	/	/	/	55	85
Social functioning	/	/	/	37.5	87.5
Role-emotional	/	/	/	100	100
Mental health	/	/	/	84	92
Health transition	/	/	/	75	75
Pulmonary function					
FVC, L	/	/	/*	1.98	2.30
FEV1, L	/	/	/*	1.64	1.80
FEV1/FVC	/	/	/*	0.82	0.83
TLC, % of predict	/	/	/*	61.7	72.3
DLCO, % of predict	/	/	/*	30.3	47.9

**The patient can't tolerate it. mMRC, modified Medical Research Council Dyspnea Scale; 6-MWD, 6 Minutes Walk Test Distance; HADS, Hospital Anxiety and Depression Scale; AIS, Athens Insomnia Scale; SF-36, Chinese vision of the 36-Item Short Form Health Survey questionnaire; FVC, Forced Vital Capacity; FEV1, Forced Expiratory Volume in 1 s; TLC, Total Lung Capacity; DLCO, Diffusion Capacity for Carbon monoxide.*

The patient gradually returned to activities of daily living, with self-care on the 7th month and physical activity on the 20th month after discharge.

DISCUSSION

The developing and toughest challenge is how to manage COVID-19 sequelae, in view of our greater understanding of the consequences of post-COVID-19 infection (11). A cohort study of 2,73,168 discharged patients from the United Kingdom showed that 36.5% of patients recorded one or more symptoms between 3 and 6 months after discharge (16).

Post-COVID-19 pulmonary fibrosis is a heterogeneous disease with different imaging performance. The researchers found that residual changes after acute stage vary over time. A retrospective study shown that (17), 3 months after acute phase, pulmonary fibrosis was clinically confirmed in 56% and 71% of the patients with moderate and severe COVID-19 symptoms respectively. In a prospective study (4), Wu et al. found that 78% of patients (65/83) had residual changes on CT at 3 months after discharge and the most common CT features were GGO (78%), interlobular septal thickening (34%), reticular opacity (33%), and subpleural curvilinear opacity (11%). In the same study, 24% of patients (20/83) had persistent imaging abnormalities

TABLE 2 | Clinical trials[a] of pirfenidone and deupirfenidone[b] for the treatment of post-COVID-19 pulmonary fibrosis.

Identifier		Start time	Study design	Phase	Objects	Number enrolled and distribution	Intervention	Primary outcome
NCT04607928 (26)		29-Oct-20	Multicenter, randomized, placebo-controlled	II	Patients with fibrotic lung sequelae after recovery from acute phase of severe COVID pneumonia	148 2:1 (Pirfenidone: Placebo)	Pirfenidone: 2403 mg/day for 24weeks; Controlled: placebo for 24 weeks;	Change from baseline in % in forced vital capacity (FVC) and % fibrosis in high resolution computed tomography of the lung
NCT04856111 (27)		22-Apr-21	Singlecenter, randomized	IV	Patients with fibrotic lung disease after COVID-19	48 1:1 (Pirfenidone: Nintedanib)	Pirfenidone: 2,400 mg/day for 24 weeks; Nintedanib: 300 mg/day for 24 weeks;	Change in the forced vital capacity (FVC)
NCT04652518 (28)	Part A	3-Dec-20	Multicenter, randomized, double-blind, placebo-controlled	II	Adults with post-acute COVID-19 respiratory complications	168 1:1 (Deupirfenidone: Placebo)	Deupirfenidone: 500 mg for 91 days; Controlled: placebo for 91 days	Change in distance walked on the 6-minutes walk test (6 MWT)
	Part B	-	Open label extension study	II	Eligible patients who completed part A	-	Deupirfenidone: an additional 91 days	Assess the longer-term safety, tolerability, and efficacy of Deupirfenidone through up to 182 days of treatment; (One of the secondary objective: Inflammatory, fibrosis and other biomarkers;)
ChiCTR2000030892 (29)		16-Mar-20	Parallel	-	Patients with severe post-COVID-19 fibrosis	40 1:1 (Pirfenidone: blank control)	-	Change in HRCT pulmonary fibrosis score

[a] *Data retrieved from ClinicalTrials.gov and chictr.org;* [b] *Deupirfenidone: a selectively deuterated form of pirfenidone.*

during the 1-year follow-up, without progressive interstitial changes and definite intervention. However, another prospective study presented higher proportion. According to Huang and his colleagues (6), the rate of persisting imaging abnormalities at 12 months after discharged was 76% among patients with severity scale 5 to 6. The phenomenon was similar to severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) survivors. In a retrospective study (18), 21.5% (67/311) of SARS patients had radiographic pulmonary fibrosis at discharge and 40 patients exhibited improvement of fibrosis at one-year follow-up. Analogously, a study of MERS (19) survivors revealed 33% of patients presented chest radiological abnormalities at 80 days after discharge.

In our case, the patient presented with marked restrictive ventilatory dysfunction and impaired diffusing function, which is consistent with previous studies. Mo et al. (20) reported abnormal diffusion function and decreased TLC in 47.2 and 25.0% of patients at discharge. Although this percentage was undoubtedly underestimated, disease severity in the acute phase was inextricably linked to pulmonary function at discharge. Significant difference in impaired diffusing capacity among the different groups of severity, which accounted for 30.4% in mild and 84.2% in severe illness, respectively (20). Meanwhile, the proportion of anomalies gradually decreased over time. Follow-up studies revealed that the proportion of survivors with abnormal pulmonary diffusing function decreased to 35–55% and 21–33% at 3 and 12 months after discharge respectively (4, 6, 21). In a 12-month longitudinal follow-up study, GGO and irregular lines were positively associated with risk of lung diffusion impairment at 12 months (6). This suggested that further reductions in pulmonary fibrosis could improve their lung function and quality of life.

The treatment strategy, oral pirfenidone, 1,800 mg per day (22), for post-COVID-19 pulmonary fibrosis, was bold, novel and aggressive, given there was no reliable evidence of effectiveness of pirfenidone in this population. This protocol was developed because her chest imaging changes were consistent with ILD, including common IPF. In the CAPACITY-004 study (23), pirfenidone reduced decline in FVC with a mean FVC change at −8.0% in the pirfenidone (2,403 mg/day) group and −12.4% in the placebo group at week 72. However, in another concurrent RCT study (CAPACITY-006), the difference between groups in FVC change at week 72 was not significant. More attention should be paid to the application of pirfenidone in interstitial lung diseases other than IPF. A multi-center, double-blind, randomized controlled phase 2 clinical study (uILD study) (24) reported that, for unclassifiable interstitial pneumonia, the use of pirfenidone (2,403 mg/d) for 24 weeks significantly delayed the decline in FVC (−17.8 vs. −113 ml, $P = 0.002$). Although evidence shown that pirfenidone could alleviate inflammatory responses in hospitalized adult patients with severe COVID-19 (25), its effectiveness in post-COVID-19 pulmonary fibrosis is still unknown.

Surprisingly, encouraging effectiveness was observed in our patient. Fibrotic lesions gradually subsided, lung diffusing function improved and she returned to normal daily life after pirfenidone therapy. However, this study has some limitations. First, there is reporting bias and it was unclear whether her improvement was benefited from pirfenidone or a natural regression of the disease course. Second, this study is not general, and the effect of pirfenidone in patients with other lung diseases is still unknown. Ongoing clinical trials (26–29) about pirfenidone and deupirfenidone in the treatment of post-COVID-19 pulmonary fibrosis may be able to provide a clear answer (**Table 2**).

CONCLUSION

In summary, our study reported a case of post-COVID-19 pulmonary fibrosis treated with long-term oral pirfenidone and the patient recovered well in terms of symptoms, pulmonary function and chest CT images. Pirfenidone may be an effective therapeutic strategy for post-COVID-19 pulmonary fibrosis. RCTs are needed for validation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Review Board of the Second Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XZ and DY collected the data and wrote the manuscript. XK, CW, SQ, LW, YL, and ZY analyzed, interpreted the data, and performed the clinical assessment. ZZ and HL designed the study and performed the clinical assessment. All authors reviewed, edited, and approved the final manuscript.

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Prognostic Predictive Characteristics in Patients With Fibrosing Interstitial Lung Disease: A Retrospective Cohort Study

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Background: Limited data are available regarding the entire spectrum of interstitial lung disease with a progressive fibrosing feature. We investigated the prevalence and prognostic predictive characteristics in patients with PF-ILD.

Methods: This retrospective cohort study included patients with fibrosing ILD who were investigated between 1 January 2015 and 30 April 2021. We recorded clinical features and outcomes to identify the possible risk factors for fibrosing progression as well as mortality.

Results: Of the 579 patients with fibrosing ILD, 227 (39.21%) met the criteria for progression. Clubbing of fingers [odds ratio (OR) 1.52, 95% confidence interval (CI) 1.03 to 2.24, p = 0.035] and a high-resolution computed tomography (HRCT)-documented usual interstitial pneumonia (UIP)-like fibrotic pattern (OR 1.95, 95% CI 1.33 to 2.86, p = 0.001) were risk factors for fibrosis progression. The mortality was worse in patients with PF with hypoxemia [hazard ratio (HR) 2.08, 95% CI 1.31 to 3.32, p = 0.002], in those with baseline diffusion capacity of the lung for carbon monoxide (DLCO) % predicted <50% (HR 2.25, 95% CI 1.45 to 3.50, $p < 0.001$), or in those with UIP-like fibrotic pattern (HR 1.68, 95% CI 1.04 to 2.71, $p < 0.001$).

Conclusion: Clubbing of fingers and an HRCT-documented UIP-like fibrotic pattern were more likely to be associated with progressive fibrosing with varied prevalence based on the specific diagnosis. Among patients with progressive fibrosing, those with hypoxemia, lower baseline DLCO% predicted, or UIP-like fibrotic pattern showed poor mortality.

Keywords: progressive fibrosing interstitial lung disease, prognosis, lung function test, blood count values, anti-fibrotic treatment

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Abbreviations: AISI, aggregate index of systemic inflammation; BMI, body mass index; CI, confidence interval; CTD, connective tissue disease; DLCO, diffusion capacity of the lung for carbon monoxide; FHP, fibrotic hypersensitivity pneumonitis; FVC, forced vital capacity; HR, hazard ratio; HRCT, high-resolution computed tomography; iNSIP, idiopathic non-specific interstitial pneumonia; IIP, idiopathic interstitial pneumonia; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; IQR, interquartile range; MLR, monocyte-to-lymphocyte ratio; NLR, neutrophil-to-lymphocyte ratio; OR, hazard ratio; PF, progressive fibrosing; PFT, pulmonary function testing; PLR, platelet-to-lymphocyte ratio; pSS-ILD, primary Sjögren syndrome-associated ILD; SD, standard deviation; RDW: red cell distribution width; SIRI, systemic inflammatory response index; SSc-ILD, sclerosis-associated ILD; UIP, usual interstitial pneumonia; WBC, white blood cell.

INTRODUCTION

The disease course in fibrosing interstitial lung disease (ILD) is highly heterogeneous and difficult to predict. Some patients experience a stable course or slowly functional decline while other patients may experience a rapidly progressive change (Cottin et al., 2018). Progressive fibrosing interstitial lung disease (PF-ILD) is a term referring to a group of diseases characterized by a high-resolution computed tomography (HRCT)-documented increase in the extent of pulmonary fibrosis, a decline in lung function, worsening respiratory symptoms and quality of life, and a high risk of early mortality despite available treatments, with a clinical course similar to that of idiopathic pulmonary fibrosis (IPF) (Cottin et al., 2018). PF-ILDs include a heterogeneous group of disorders associated with multifactorial etiology, including IPF, connective tissue disease-associated ILD (CTD-ILD), fibrotic hypersensitivity pneumonitis (FHP), unclassifiable idiopathic interstitial pneumonia (uIIP), and idiopathic non-specific interstitial pneumonia (Cottin et al., 2019). Reportedly, PF-ILDs may develop in approximately 33.0% of patients with fibrosing ILDs other than IPF (Olson et al., 2018; Nasser et al., 2021a; Ruaro et al., 2021; Lee et al., 2022). The estimated incidence of PF-ILD ranged from 4.0 to 4.7/100,000 person-years and the estimated prevalence from 6.6 to 19.4/100,000 individuals (Nasser et al., 2021a; Nasser et al., 2021b).

Several clinical trials have encompassed patients with progressive fibrosing, with the eligibility criteria for these studies helping to guide a standardized diagnosis of PF-ILD (Wongkarnjana et al., 2020). Although no uniform criteria were established, guidelines recommended monitoring of fibrosis progression based on evaluation of multiple components, such as a decline in lung function, increase in chest imaging-documented fibrosis, symptomatic worsening, and composite measures of these variables (George et al., 2020; Wongkarnjana et al., 2020; Raghu et al., 2022).

In view of the clinical and underlying physiological similarities between IPF and other PF-ILDs, anti-fibrotic agents, such as nintedanib or pirfenidone, are considered to be effective against progressive fibrosis (Flaherty et al., 2019; Gibson et al., 2020). However, watchful observation until the onset of HRCT-documented lung function decline and extensive fibrosis delays initiation of early anti-fibrotic therapy, and patients invariably present with clinically significant and irreversible injury. Several studies have investigated the clinical features and likely predictors in patients at high risk of PF-ILD at baseline (Kolb and Vašáková, 2019). Certain conditions, such as FHP (which is not associated with any specific antigen) and increasing age predispose to PF-ILD (Sharma et al., 2021; Churg, 2022). Smoking status was a risk factor for rheumatoid arthritis-ILD (RA-ILD) or primary Sjögren syndrome associated with ILD (Johnson, 2017; Wang et al., 2018). Male sex and a high baseline modified Rodnan skin score were strong predictors of forced vital capacity (FVC) decline in patients with systemic sclerosis-ILD (SSc-ILD) (Hoffmann-Vold et al., 2021). Risk factors for mortality have also been identified. Regardless of a specific diagnosis of ILD, patients with an HRCT-documented usual interstitial pneumonia (UIP) pattern or extensive traction bronchiectasis, FVC decline, or older age showed the highest mortality risk (Walsh et al., 2018; Yang et al., 2022). In addition, peripheral blood monocyte counts have been investigated in IPF and other ILDs as a predictor of prognosis (Misharin et al., 2017; Barratt et al., 2021). Although studies have reported findings for individual types of PF-ILDs (for example, SSc-ILD, RA-ILD, other CTD-ILDs, and FHP), limited data are available regarding the PF-ILDs as a whole group (Khanna et al., 2020; Skibba et al., 2020; Orlandi et al., 2022). Early accurate diagnosis of most ILDs was difficult. Studying clinical courses for the heterogeneous group of fibrosing ILDs as a whole group was essential to identify patients with the most rapidly progressive feature or poor prognosis and initiate early anti-fibrotic treatment.

The aims of this study were: 1) to investigate the prevalence and clinical features of the PF-ILDs and 2) to explore the potential factors associated with progression or mortality.

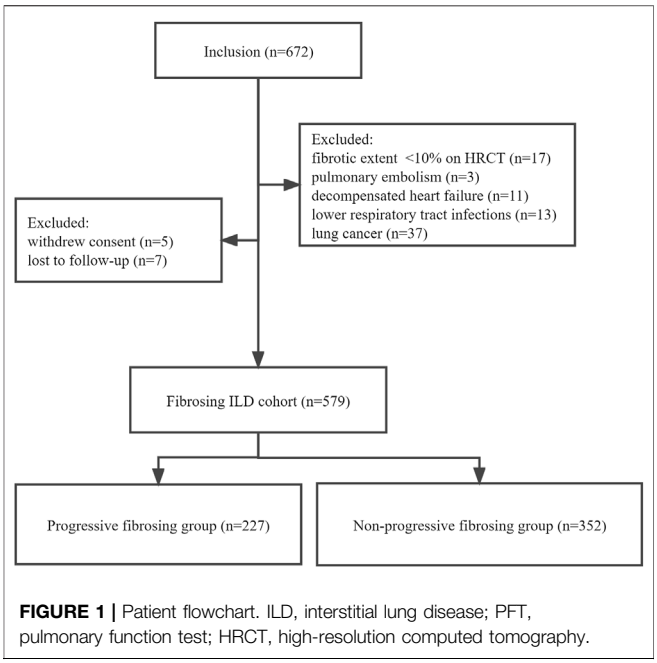

FIGURE 1 | Patient flowchart. ILD, interstitial lung disease; PFT, pulmonary function test; HRCT, high-resolution computed tomography.

MATERIALS AND METHODS

Study Design and Participants

This observational retrospective cohort study was performed at Beijing Chao-Yang Hospital, China, a regional tertiary referral center specialized in the management of ILDs. We retrospectively screened all patients aged ≥18 years with a multidisciplinary diagnosis of fibrosing ILDs between 1 January 2015 and 30 April 2021. Multidisciplinary diagnoses were conducted between pulmonologists (QY, NW, and YW), radiologists (YF and JW), a rheumatologist (ZG), and a pathologist (RM) experienced in the diagnosis of ILD based on clinical characteristics, HRCT, and lung biopsy if appropriate. Patients whose results of baseline pulmonary function tests (PFTs) and

HRCT were available were enrolled in the study. Following were the exclusion criteria: 1) <10% fibrosis documented on baseline HRCT, 2) diagnosis of pulmonary embolism, decompensated heart failure, or lower respiratory tract infections associated with disease progression, 3) lung cancer at baseline, 4) withdrawal of consent for study participation, and 5) loss to follow-up (**Figure 1**). The study was registered at www.chictr.org.cn ChiCTR2100049247 and was approved by the Ethics Committee of Beijing Chao-Yang Hospital (2020-KY-437). All procedures were performed in accordance with the principles of the Declaration of Helsinki.

Data Collection

We reviewed patients' medical records to uniformly extract clinical data at the first clinical visit, including demographic information, physical examination, and routine clinical laboratory test results. Clinical and survival data were obtained from medical records, outpatient follow-up records (usually every 6–12 months), hospitalization details, and telephone interviews. Hypoxemia was defined as the partial pressure of oxygen in the arterial blood (PaO2) of less than 80 mm Hg obtained from the arterial blood gas (ABG) test at rest. The derivative blood cell count inflammation indexes included the neutrophil-to-lymphocyte ratio (NLR: neutrophils/lymphocytes), monocyte-to-lymphocyte ratio (MLR: monocytes/lymphocytes), platelet-to-lymphocyte ratio (PLR: platelet/lymphocyte), systemic inflammatory index (SII: neutrophils × platelets/lymphocytes), systemic inflammatory response index (SIRI: neutrophils × monocytes/lymphocytes), and aggregate index of systemic inflammation (AISI: neutrophils × platelets × monocytes/lymphocytes). The HRCT-documented UIP-like pattern included definitive UIP or a probable UIP pattern according to the Clinical Practice Guideline of IPF (Raghu et al., 2011; Raghu et al., 2018). Two thoracic radiologists (YF and JW) blinded to the clinical data independently determined whether the fibrosis involved >10% of the total lung and reviewed the HRCT scans by visual assessment. The disagreements were resolved *via* consensus. The kappa value for the interobserver correlation was 0.84.

The follow-up period ended on 30 April 2021. The primary outcome was progressive fibrosing. Patients who fulfilled any of the following criteria within 24 months despite administration of standard treatment were considered experiencing progressive fibrosis (Cottin et al., 2018; Flaherty et al., 2019): 1) a relative decline of ≥10% in FVC, 2) relative decline of ≥15% in diffusion capacity of the lung for carbon monoxide (DLCO), and 3) worsening symptoms and/or worsening radiological findings accompanied by a ≥5% to <10% relative decrease in FVC. The secondary outcome was all-cause mortality during the follow-up period. Survival was calculated from the time of the first visit to the outcome or the end of follow-up.

Sample Size Calculation

PASS software, version 11.0 (NCSS, LLC. Kaysville, Utah, United States) was used to calculate the sample size. The sample allocation ratio was 1: 2 (PF: non-PF), given the estimated prevalence of PF-ILD among patients with fibrosing ILDs was 33.0% (Nasser et al., 2021a). We used a two-sided log-rank test for the 6-year all-cause mortality with 1-year accrual periods and an estimated 10.0% proportion of dropping out for both groups. An overall sample size of 260 patients (174 in the non-PF-ILD group and 86 in the PF-ILD group) was deemed sufficient to achieve >90% power at a 0.05% significance level to detect a hazard ratio (HR) of death with the 6-year mortality rate considered to be 40.0% for the PF group and 18.0% for the non-PF group (Adegunsoye et al., 2018). However, we attempted to enroll as many patients as possible within the study period. The final cohort included 579 patients, of which 227 patients were diagnosed with PF-ILD (85 deaths), fulfilling the 10-events-per-variable criterion of multivariable logistic regression and Cox analysis.

Statistical Analysis

Statistical analyses were performed using SPSS Statistics software, version 26 (IBM, Inc., Chicago, IL, United States). Missing data were imputed using mean/median or removed for a complete case analysis. Data were expressed as means (SD) or medians (range), depending on the distribution. The Mann–Whitney U or *t*-test was used for continuous variables for comparison of intergroup differences and the chi-squared test or Fisher's exact test for categorical variables. Linear mixed-effects analysis was applied to analyze disease behavior over time, with random terms for intercept and slope (for time-from-diagnosis). The covariance for the repeated measures was left autoregressive 1: heterogeneous, which yielded the best fit. Multicollinearity diagnostic tests were performed. Logistic regression analyses were used to investigate risk factors for the PF-ILD, and continuous variables were converted into dichotomous variables mainly through the median cut-off. The cut-off value was 70.0% for FVC% predicted and 50.0% for DLCO% predicted (Abe et al., 2021; Lacedonia et al., 2022). Survival curves were obtained using the Kaplan–Meier method, and Cox proportional analyses were performed to identify prognostic factors for mortality. Proportional hazards in the Cox analysis were checked using the Schoenfeld residual test. A p-value < 0.05 was considered statistically significant.

RESULTS

Demographic and Clinical Characteristics

In this study, 277 (39.21%) out of 579 ILD patients met the criteria for PF-ILD; 64, 88, and 108 patients met criteria 1, 2, and 3, respectively; 28 patients met both the criteria 1 and 2, and 16 patients met both the criteria 2 and 3 (**Supplementary Figure S1**). Statistically significant baseline differences were observed in age (p = 0.001), underlying diagnosis (p < 0.001), clubbing of fingers (p = 0.005), and HRCT-documented UIP-like fibrotic pattern (p < 0.001) between the PF-ILD and non-PF-ILD groups (**Table 1**). Baseline blood count values did not differ significantly between patients with PF and non-PF. Within the PF-ILD group, gender, age, smoking status, UIP-like fibrotic pattern, FVC, GAP stage distribution, and anti-fibrotic treatment were statistically significant between IPF and non-IPF patients (**Table 2**).

TABLE 1 | Demographics and clinical characteristics of the cohort.

	All	PF-ILD group	Non-PF-ILD group	*p*-value
Number	579	227 (39.21)	352 (60.79)	
Male, *n* (%)	296 (51.12)	121 (53.30)	175 (49.72)	0.399
Age, years (IQR)	62 (55–68)	64 (57–70)	62 (54–68)	0.001
BMI, kg/m^2 (SD)	25.90 (3.31)	25.92 (3.50)	25.89 (3.19)	0.907
Ever smoker, *n* (%)	233 (40.24)	98 (43.17)	135 (38.40)	0.248
Diagnosis, *n* (%)				<0.001
IPF	145 (25.04)	77 (33.92)	68 (19.32)	
CTD-ILD	297 (51.30)	109 (48.02)	188 (53.41)	
FHP	56 (9.67)	16 (7.05)	40 (11.36)	
uIIP	81 (13.99)	25 (11.01)	56 (15.91)	
Signs, *n* (%)				
Velcro crackles	531 (91.71)	214 (94.27)	317 (90.06)	0.072
Clubbing of fingers	154 (26.60)	75 (33.04)	79 (22.44)	0.005
Blood count values				
WBC (*n* * 10^9/L)	7.60 (5.98–8.53)	7.40 (5.69–8.63)	7.77 (6.28–8.52)	0.132
Neutrophils (*n* * 10^9/L)	4.87 (3.46–8.50)	4.54 (3.23–5.97)	5.06 (3.68–5.66)	0.156
Lymphocytes (*n* * 10^9/L)	1.91 (1.42–2.30)	1.91 (1.35–2.29)	1.91 (1.46–2.30)	0.622
Monocyte (*n* * 10^9/L)	0.47 (0.37–0.54)	0.47 (0.35–0.55)	0.48 (0.38–0.54)	0.293
RDW	13.20 (12.60–13.99)	13.20 (12.70–13.99)	13.20 (12.52–13.99)	0.570
LMR	3.90 (3.22–5.26)	3.90 (3.14–5.07)	3.90 (3.27–5.27)	0.393
NLR	2.68 (1.84–3.42)	2.60 (1.74–3.46)	2.68 (1.88–3.40)	0.914
PLR	121.68 (92.16–159.01)	121.68 (90.36–157.86)	121.68 (94.10–163.73)	0.214
SIRI	1.31 (0.76–1.65)	1.25 (0.73–1.85)	1.31 (0.77–1.55)	0.265
AISI	305.26 (145.40–396.00)	286.60 (139.56–433.36)	305.26 (156.84–377.23)	0.947
Pulmonary function (SD)				
FVC (L)	2.64 (0.80)	2.64 (0.79)	2.64 (0.82)	0.932
FVC% pred	85.45 (19.72)	85.94 (19.53)	85.15 (19.86)	0.633
DLCO% pred	60.26 (18.61)	58.94 (18.82)	61.11 (18.46)	0.171
UIP-like fibrotic pattern on HRCT, n (%)	163 (28.15)	86 (37.89)	77 (21.88)	<0.001
Hypoxemia, *n* (%)	119 (20.55)	54 (23.80)	65 (18.50)	0.122
Hospitalization at baseline, n (%)	162 (27.98)	70 (30.84)	92 (26.14)	0.219
Hospitalization frequency, n (IQR)	0 (0.1)	1 (0.1)	0 (0.1)	<0.001
Hospitalization frequency, *n* (%)				0.002
0	298 (51.47)	101 (44.49)	197 (55.97)	
1–2	236 (40.76)	99 (43.61)	137 (38.92)	
≥3	45 (7.77)	27 (11.89)	18 (5.11)	
Death, *n* (%)	128 (22.11)	85 (37.45)	43 (12.22)	<0.001
Median duration of follow-up, years (IQR)	3 (2.4)	3 (2.5.4)	3 (2.4)	0.054

Data were presented as mean ± SD or n *(%) or median (IQR).*

AISI, aggregate index of systemic inflammation; BMI, body mass index; CTD, connective tissue disease; DLCO, diffusion capacity of the lung for carbon monoxide; FHP, fibrotic hypersensitivity pneumonitis; FVC, forced vital capacity; HRCT, high-resolution computed tomography; iNSIP, idiopathic non-specific interstitial pneumonia; IPF, idiopathic pulmonary fibrosis; MLR, monocyte-to-lymphocyte ratio; NLR, neutrophil-to-lymphocyte ratio; PF, progressive fibrosing; PLR, platelet-to-lymphocyte ratio; RDW: red cell distribution width; SIRI, systemic inflammatory response index; uIIP, unclassifiable idiopathic interstitial pneumonia; UIP, usual interstitial pneumonia; WBC, white blood cell.

Comorbidity information for the whole cohort is shown in **Supplementary Figure S2**. The prevalence for pulmonary hypertension was 8.81% in the PF group and 5.11% in the non-PF group.

Prevalence of Progressive Fibrosing Interstitial Lung Disease

The most frequent diagnoses included CTD-ILD (*n* = 297%, 51.30%), IPF (*n* = 145%, 25.04%), uIIP (*n* = 81%, 13.99%), and FHP (*n* = 56%, 9.67%) (**Table 1**). The prevalence of PF-ILD in patients with ILDs, including those with IPF (*n* = 77), CTD-ILD (*n* = 109), FHP (*n* = 16), and uIIP (*n* = 25) was 53.10%, 36.70%, 28.57%, and 30.86%, respectively (**Supplementary Figure S3**).

Treatments and Hospitalization

In the entire cohort, 74.96% of patients (*n* = 434) received at least one of the following treatments: administration of glucocorticoids, immunosuppressive agents, anti-fibrotic treatment, and oxygen therapy. Anti-fibrotic treatment was administered to 32.16% of patients with PF-ILD (*n* = 73) (**Supplementary Table S1**). In the PF group, 42 (54.50%) of IPF patients and 31 (20.70%) of non-IPF patients (29 CTD-ILD, 1 FHP, and 1 uIIP) received anti-fibrotic treatment (**Table 2**). Patients hospitalized within 1 month of the first visit were considered hospitalized at baseline. We observed that 298 (51.47%) patients underwent hospitalization at least once during follow-up, and 27 (11.89%) of 227 patients in the PF-ILD group vs. 18 (5.11%) of the 352 patients in the non-PF group underwent hospitalization at least thrice (**Table 1**).

TABLE 2 | Demographics and clinical characteristics of patients within the PF group.

	IPF	Non-IPF	*p*-value
Number	77	150	
Male, *n* (%)	60 (77.92)	61 (40.67)	<0.001
Age, years (IQR)	66 (60–74)	63 (55–68)	<0.001
BMI, kg/m^2 (SD)	26.29 (3.19)	25.73 (3.65)	0.255
Ever smoker, *n* (%)	55 (71.43)	43 (28.67)	<0.001
Signs, *n* (%)			
Velcro crackles	29 (37.70)	46 (30.67)	0.337
Clubbing of fingers	71 (92.20)	143 (95.33)	0.289
Pulmonary function (SD)			
FVC (L)	2.80 (0.66)	2.45 (0.81)	0.001
FVC% pred	82.96 (16.86)	87.46 (20.67)	0.100
DLCO% pred	56.61 (19.47)	60.14 (18.43)	0.180
UIP-like fibrotic pattern on HRCT, n (%)	65 (84.42)	21 (14.00)[#]	<0.001
Hypoxemia, *n* (%)	19 (24.68)	35 (23.33)	0.822
GAP score	3 (1–2)	2 (1–3)	
Stage I (%)	55 (71.43)	123 (82.00)	
Stage II (%)	19 (24.68)	26 (17.33)	
Stage III (%)	3 (3.90)	1 (0.67)	0.077
Anti-fibrotic treatment (%)	42 (54.55)	31 (20.67)	<0.001

Data were presented as mean ± SD or n *(%) or median (IQR).*

#: 29 patients with CTD-ILD, 1 patient with FHP, and 1 patient with uIIP were included.

BMI, body mass index; DLCO, diffusion capacity of the lung for carbon monoxide; FVC, forced vital capacity; HRCT, high-resolution computed tomography; IPF, idiopathic pulmonary fibrosis; PF, progressive fibrosing; UIP, usual interstitial pneumonia.

Lung Function Changes

Figure 2 showed the mean lung function measures across different time intervals. The slope of FVC differed between the PF-ILD (−0.07 L/6 months, 95% CI −0.09 to −0.05) and non-PF-ILD (0.01 L/6 months, 95% CI 0.01–0.05) subgroups (p = 0.001) (**Figure 2D**). The slope of FVC% predicted in the PF-ILD group was −1.42%/6 months (95% CI −1.99 to −0.86), whereas that in the non-PF-ILD group was 2.80%/6 months (95% CI 2.12–3.49) (p = 0.04) (**Figure 2E**). The slope of DLCO% predicted also differed between subgroups (−1.84%/6 months, 95% CI −2.43 to −1.25 in the PF-ILD group vs. 1.93%/6 months, 95% CI 1.35 to 2.51 in the non-PF-ILD group, p = 0.003) (**Figure 2F**).

Predictive Characteristics Associated With Progressive Fibrosing

Table 3 shows the results of logistic regression analysis for predictors of progressive fibrosing. Multivariable analysis was performed on significant factors obtained from univariate analysis with a p-value < 0.2. Although the NLR (OR 0.69, 95% CI 0.55 to 1.08, p = 0.039) was significantly associated with PF, the OR included the value 1. Patients with clubbing of fingers (OR 1.52, 95% CI 1.03 to 2.24, p = 0.035) and an HRCT-documented UIP-like fibrotic pattern (OR 1.95, 95% CI 1.33 to 2.86, p = 0.001) showed a high risk of fibrosis progression.

Predictive Characteristics Associated With Survival

Patients were followed up for a median duration of 3 (2–4) years with 128 (22.1%) deaths during follow-up, and the median overall survival (OS) time for whole patients with fibrosing ILDs was 6 years. In the non-PF group, the median overall survival was not reached, while in the PF-ILD group, 85 of 227 (37.45%) patients died, with a median OS of 5 years (**Supplementary Figure S4**). The median survival time was shorter in patients with hypoxemia (OS: 4 years), baseline DLCO% predicted <50% (OS: 4 years), and UIP-like pattern (OS: 4.5 years). Subgroup analysis based on anti-fibrotic treatment using the log-rank test showed no significant intergroup difference (p = 0.435) (**Figure 3**). Within patients in the PF group who received anti-fibrotic treatment, IPF patients showed worse OS (OS: 4.5 years) than non-IPF patients (OS: 6 years) (log-rank test, p = 0.003, **Supplementary Figure S5**). **Table 4** shows the results of univariate and multivariate Cox analyses of predictors for all-cause mortality. By multivariate Cox analysis, lower DLCO% predicted (HR 2.25, 95% CI 1.45 to 3.50, p < 0.001), hypoxemia (HR 2.08, 95% CI 1.31 to 3.32, p = 0.002), and UIP-like fibrosis pattern on HRCT (HR 1.68, 95% CI 1.04 to 2.71, p = 0.034) were associated with an increased risk of mortality.

DISCUSSION

We provided a clinical cohort profile of patients with fibrosing ILD in China. A significant percentage of patients with fibrosing ILD tend to develop progressive fibrosing, which is associated with accelerated decline in lung function secondary to progressive fibrosis and symptomatic worsening (Nasser et al., 2021b). Prevalence of non-IPF PF-ILD varied from 28.57% to 39.07% owing to various diagnosis subgroups in our data, consistent with the findings reported by previous studies (approximately 33.0%) (Flaherty et al., 2019; Nasser et al., 2021b). A large-scale survey estimated that 18.0%–32.0% of patients diagnosed with non-IPF ILDs developed progressive fibrosis with overall survival of 61–80 months in the US, France, Germany, Italy, Spain, the

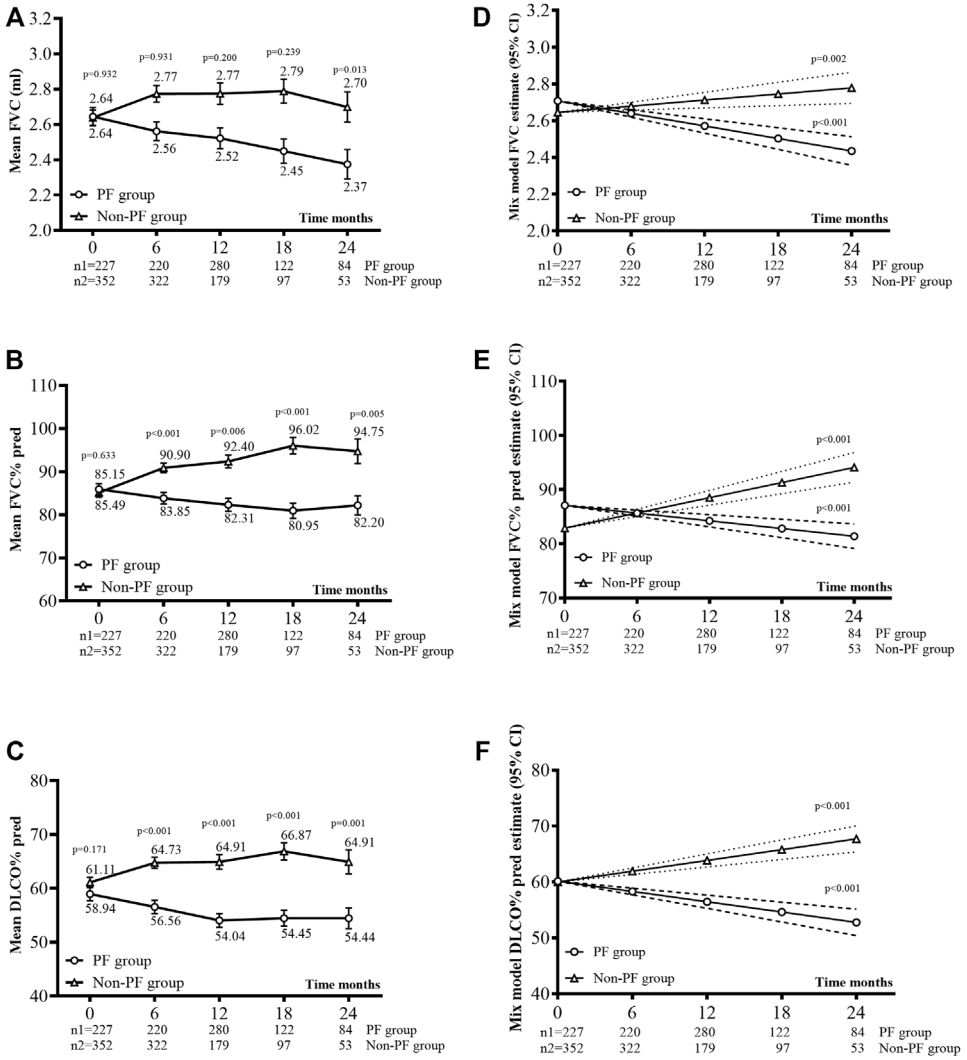

FIGURE 2 | Lung function changes. **(A)**, **(B)**, and **(C)** Mean FVC ml (±SE), mean FVC% predicted (±SE), and mean DLCO% predicted (±SE) during observation. **(D)**, **(E)**, and **(F)** Disease behavior (FVC, FVC% pred, and DLCO% pred) over time was analyzed using a linear mixed model for each group, with a random effect for the intercept and slope. The covariance for the repeated measures was left AR (autoregressive)1: heterogeneous, as this yielded the best fit.

United Kingdom, and Japan (Wijsenbeek et al., 2019). PF-ILD was identified in 135 of 396 (34.1%) patients with non-IPF ILDs in a cohort of Korean patients (Kwon et al., 2021). In another Chinese single-center cohort of 608 patients, 132 patients (21.7%) with ILD met the criteria for PF-ILD (Chen et al., 2021). The difference in findings between previous studies and our study may be attributable to the older age, poorer DLCO% predicted, or various disease classifications used for non-IPF ILDs in our patients. In our study, half of the patients with IPF showed rapid disease progression within 24 months. The clinical course of IPF is variable and difficult to predict, with a median survival time of 2–3 years after diagnosis without anti-fibrotic therapy. However, approximately 25.0% of patients survive over 5 years after the initial diagnosis (Kärkkäinen et al., 2019). Therefore, it is possible that lung function in patients with IPF remained stable or declined only slightly during the first 24 months but eventually led to poor outcomes.

Healthcare utilization is expected to be higher in patients with PF-ILD (Holtze et al., 2018; Wuyts et al., 2020). Hospitalization frequency was higher in the PF-ILD group than in the non-PF-ILD group during follow-up. Patients with PF-ILD received glucocorticoids for primary therapy, which emphasizes the need for alternative treatment. Many clinical trials have confirmed the efficacy of anti-fibrotic drugs to slow disease progression in patients with PF-ILD (Behr et al., 2021; Nambiar et al., 2021). Our data showed a trend of better survival in patients with PF-ILD, though not statistically significant, who received anti-fibrotic treatment; nintedanib and pirfenidone are known to prolong life expectancy in

TABLE 3 | Factors associated with progressive fibrosing.

Covariate	Univariable analysis		Multivariable analysis	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Age	1.43 (1.02–2.00)	0.039	1.21 (0.85–1.73)	0.294
Male	1.16 (0.83–1.61)	0.400	—	—
BMI	0.85 (0.60–1.20)	0.351	—	—
Smoking	1.22 (0.87–1.71)	0.249	—	—
FVC% pred	0.69 (0.46–1.06)	0.090	0.72 (0.46–1.11)	0.134
DLCO% pred	1.24 (0.87–1.76)	0.237	—	—
Velcro	1.15 (0.82–1.61)	0.406	—	—
Clubbing of fingers	1.71 (1.17–2.48)	0.005	1.52 (1.03–2.24)	0.035
Hypoxemia	1.38 (0.92–2.07)	0.123	1.38 (0.90–2.11)	0.135
Hospitalization at baseline	1.26 (0.87–1.82)	0.219	—	—
UIP-like pattern on HRCT	2.18 (1.51–3.15)	<0.001	1.95 (1.33–2.86)	0.001
WBC	0.82 (0.59–1.15)	0.249	—	—
Neutrophils	0.75 (0.53–1.04)	0.086	0.76 (0.54–1.08)	0.122
Lymphocytes	0.82 (0.59–1.14)	0.237	—	—
Monocyte	0.88 (0.63–1.23)	0.464	—	—
RDW	0.98 (0.70–1.37)	0.913	—	—
LMR	1.08 (0.77–1.51)	0.656	—	—
NLR	0.69 (0.49–0.96)	0.028	0.69 (0.55–1.08)	0.039
PLR	0.94 (0.67–1.32)	0.712	—	—
SIRI	0.85 (0.61–1.19)	0.354	—	—
AISI	1.03 (0.73–1.46)	0.856	—	—

AISI: aggregate index of systemic inflammation; BMI, body mass index; CTD, connective tissue disease; DLCO, diffusion capacity of the lung for carbon monoxide; FVC: forced vital capacity; HRCT, high-resolution computed tomography; MLR, monocyte-to-lymphocyte ratio; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; RDW: red cell distribution width; SIRI, systemic inflammatory response index; UIP, usual interstitial pneumonia; WBC, white blood cell.

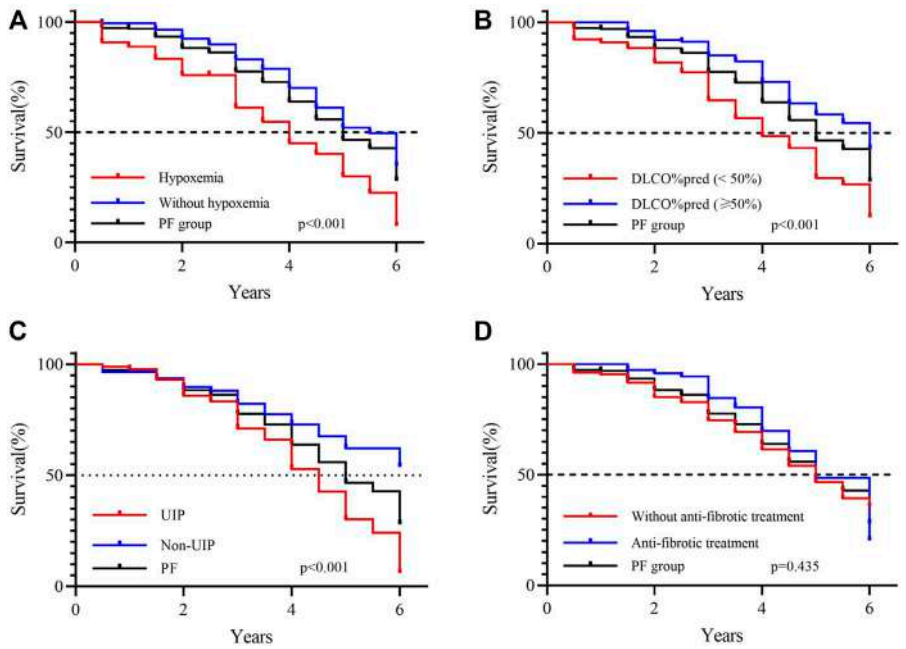

FIGURE 3 | Survival in patients with progressive fibrosing ILD. **(A)** Survival according to with or without hypoxemia at baseline (log-rank test, $p < 0.001$); **(B)** survival according to DLCO% pred at baseline with a 50% threshold (log-rank test, $p < 0.001$); **(C)** survival according to the UIP-like fibrotic pattern on HRCT (log-rank test, $p < 0.001$); **(D)** survival according to with or without anti-fibrotic treatment during observation (log-rank test, $p = 0.435$).

patients with IPF (Costabel et al., 2017; Lancaster et al., 2019). In the INBUILD trial, the annual rate of decline in the FVC in patients with PF-ILD was significantly lower among patients who received nintedanib (−80.8 ml/year) than among those who received a placebo (−187.8 ml/year) (Flaherty et al., 2019). However, only 32.16% of patients receive anti-fibrotic

TABLE 4 | Factors associated with 6-year all-cause mortality in the PF-group.

Covariate	Univariable analysis		Multivariable analysis	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age	1.57 (0.99–2.47)	0.054	1.1 (0.67–1.82)	0.698
Male	1.06 (0.69–1.64)	0.777	—	—
BMI	1.02 (0.65–1.58)	0.944	—	—
Smoking	1.23 (0.80–1.88)	0.347	—	—
FVC% pred	1.13 (0.66–1.95)	0.66	—	—
DLCO% pred	2.30 (1.50–3.45)	<0.001	2.25 (1.45–3.50)	<0.001
Velcro	0.97 (0.63–1.49)	0.88	—	—
Clubbing of fingers	0.77 (0.49–1.22)	0.269	—	—
Hypoxemia	2.24 (1.43–3.50)	<0.001	2.08 (1.31–3.32)	0.002
Hospitalization at baseline	0.90 (0.50–1.61)	0.719	—	—
UIP-like pattern on HRCT	0.23 (1.45–3.45)	<0.001	1.68 (1.04–2.71)	0.034
WBC	1.19 (0.78–1.83)	0.419	—	—
Neutrophils	1.09 (0.71–1.67)	0.697	—	—
Lymphocytes	1.24 (0.81–1.89)	0.33	—	—
Monocyte	1.19 (0.76–1.82)	0.429	—	—
RDW	1.15 (0.75–1.77)	0.516	—	—
LMR	0.86 (0.56–1.32)	0.482	—	—
NLR	1.16 (0.76–1.78)	0.495	—	—
PLR	0.78 (0.50–1.21)	0.268	—	—
SIRI	1.39 (0.91–2.13)	0.132	1.17 (0.76–1.82)	0.480
AISI	1.08 (0.69–1.67)	0.746	—	—
Anti-fibrotic treatment	0.84 (0.54–1.32)	0.450		

AISI: aggregate index of systemic inflammation; BMI: body mass index; CTD, connective tissue disease; DLCO, diffusion capacity of the lung for carbon monoxide; FVC, forced vital capacity; HRCT, high-resolution computed tomography; MLR, monocyte-to-lymphocyte ratio; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; RDW: red cell distribution width; SIRI, systemic inflammatory response index; UIP, usual interstitial pneumonia; WBC, white blood cell.

treatment in our real-world clinical settings, which indicates the possible financial burden associated with these drugs.

Overall, the mean lung function measures remained stable during follow-up in the non-PF-ILD group but showed a decline in the PF-ILD group. In the PF-ILD group, the change in the estimated mean annual FVC was −140 ml. In our study, the decline in the FVC in patients with PF-ILD was similar to that reported by the PROGRESS clinical cohort after 52 weeks (−136 ml) (Nasser et al., 2021b). Another study reported that the mean annual FVC change was −69.9 ml in a United States cohort and −50.0 ml in a United Kingdom cohort of patients with PF-ILD (Oldham et al., 2021). These differences may be attributable to the heterogeneous lung function trajectory across the ILD subgroups. Analysis of diagnosis subgroups showed a mean annual FVC change of −37.2 ml in the CTD-ILD, −92.0 ml in the FHP, and −69.5 ml in the IIP groups (Oldham et al., 2021). We did not perform diagnosis stratification owing to the limited sample size. A greater number of studies that perform the stratification are warranted to investigate the lung function trajectory.

We observed that the HRCT-documented UIP-like fibrotic pattern in patients with fibrosing ILDs was associated with more rapid disease progression, which suggests that morphological patterns may be prognostically significant; our findings are consistent with those reported by previous studies (Nasser et al., 2021b). Moreover, our study showed that clubbing of fingers (enlargement of the ends of the finger accompanied by a downward sloping of the nails) significantly predicted PF-ILD. A previous study reported that the prevalence of clubbing of fingers in cases of fibrosing ILDs ranged from 7.0% to 42.0% (van Manen et al., 2017). Clubbing of fingers is considered an essential clinical finding in patients with fibrosing ILDs and represents decreased oxygen levels and poor prognosis (Kanematsu et al., 1994; van Manen et al., 2017).

Hypoxemia, low DLCO% predicted, and HRCT-documented UIP pattern were associated with worse mortality in patients with PF. Reportedly, male sex, older age, lower FVC or DLCO at baseline, decline in lung function compared with the baseline, subgroups based on diagnosis, HRCT-documented UIP-like fibrotic pattern, or honeycombing were indicators of poor prognosis (Wongkarnjana et al., 2020). Cluster analysis was used to classify patients with ILD into clinical subgroups based on their clinical courses. Patients with the most rapid decline in lung function or fibrosis progression and poor survival were usually clustered together in ILDs or IP with myositis-specific autoantibodies (Adegunsoye et al., 2018; Lia et al., 2022). These findings suggest that evidence of progressive pulmonary fibrosis may be useful to define a group of patients with fibrosing ILD of multifactorial etiologies and poor prognoses. Although accurate and prompt diagnosis is important to enable initiation of optimal management, it is possible to identify patients at high risk of progression through observation of disease behavior despite the specific diagnosis. Validation cohorts are required to confirm mortality predictors.

Given the possible relationship between prognosis and biomarkers obtained by the routinely provided blood cell count test in IPF, we sought to explore the prognostic capacity of the blood cell count and the combined indexes in PF-ILDs. A pooled retrospective analysis of 2,067 IPF patients derived from the clinical trials showed that patients with higher monocyte

count had a higher 1-year risk of progression, all-cause mortality, and all-cause hospitalization (Kreuter et al., 2021). On the contrary, we did not observe the relationship between disease progression or mortality and blood cell count and the combined index. According to a multicenter retrospective study, higher baseline NLR and absolute monocyte counts predict worse survival in IPF but not in fHP (Barratt et al., 2021), highlighting the potential divergence in the underlying mechanisms of these diseases. However, our sample size is smaller than the previous reports for the prognostic role of blood cell counts. Second, single peripheral blood cell measures may be influenced by many short-term factors. Further multicenter studies with a larger population would be informative about the applicability of blood cell counts in predicting prognosis.

We provided data on longitudinal clinical outcomes in patients with fibrosing ILD from China. Patients evaluated in a tertiary referral center through a multidisciplinary discussion were routinely followed up every 6–12 months with pulmonary function tests, HRCT, and visits. Unlike previous studies for FP-ILDs (Flaherty et al., 2019; Wijsenbeek et al., 2019; Nasser et al., 2021b) which generally emphasized non-IPF patients, our cohort included both IPF and non-IPF patients as a whole group and also provided separate data for both groups. In this way, the exploration between clinical characteristics and prognosis of patients with PF-ILD is of more clinically utility because it is hard to make specific diagnosis for some ILDs at an early stage, especially for IPF. In addition, we tried to study the relationship between the patient's blood count values and prognosis, which was not explored in PF-ILD patients as a whole group before. The negative results were supported by some previous research studies while opposed by others studying specific diagnosis group of diseases, highlighting cautious use of routine blood test values as prognosis predictors.

Several limitations should be considered: 1) the retrospective and monocentric design of this study may have led to a selection bias. 2) This was a small-scale study in which we observed a reduction in available data on lung function over time, which resulted in an inaccurate estimation of the lung function trajectory. 3) Acute exacerbations, as one of the leading causes of death in patients with IPF (annual incidence of up to 20.0%) (Richeldi, 2015; Kolb et al., 2018; Ruaro et al., 2021; Yoon et al., 2021), were not investigated because some exacerbations were not recorded in the medical system of our hospital. The annual incidence of acute exacerbations in patients with non-IPF PF-ILD was 13.9% (Nasser et al., 2021b; Petnak et al., 2021; Yoon et al., 2021). Further studies are warranted to thoroughly investigate the effects of acute exacerbations. 4) We used all-cause mortality rather than ILD-related mortality as the outcome because all deaths did not occur at the hospital, and some causes of death were not reported reliably.

In summary, we presented a hospital population-based cohort profile of patients with PF-ILDs in China. We reported that 28.57%–53.10% of patients with fibrosing ILD developed a progressive fibrosing feature. Patients with clubbing of fingers or an HRCT-documented UIP pattern showed a high potential risk for PF-ILD. The risk factors associated with mortality in patients with PF-ILD included hypoxemia, low DLCO% predicted, or an HRCT-documented UIP pattern. The relationship between prognosis and blood cell count and the combined index was not observed, highlighting the divergence in the underlying mechanisms of PF-ILDs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Beijing Chao-Yang Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The authors were responsible for all content of the manuscript. YW performed data collection, analyzed the data, contributed to interpretation, and wrote the manuscript. ZG, RM, JW, and YF were responsible for recruiting the patients and collecting the data. NW helped in the drafting and revision. QY contributed as primary investigator and was responsible for designing the study, recruiting the patients, analyzing and interpreting the data, and writing the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.924754/full#supplementary-material

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CASE REPORT
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Extracorporeal Membrane Oxygenation Supported Transbronchial Cryobiopsy in the Diagnosis of Severe Organizing Pneumonia: A Case Report

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This case report describes a 58-year-old, never-smoking housewife with chief complaints of progressively worsening cough, dyspnea, and intermittent fever, who was initially misdiagnosed with community-acquired pneumonia (CAP). However, her pulse oximetry oxygen saturation continued to decline, and eventually, she underwent an endotracheal intubation. Fortunately, transbronchial cryobiopsy (TBCB) assisted by extracorporeal membrane oxygenation (ECMO) was performed in the most critical situation, and it revealed an organizing pneumonia (OP) pattern. OP describes a histological pattern of acute or subacute pulmonary damage, which may be idiopathic or associated with a known or unknown underlying disease. A definitive diagnosis of OP usually obtained from pathology, and surgical lung biopsy with large lung tissue is recommended. However, since the surgical lung biopsy was not convenient for this patient after mechanical ventilation, bedside TBCB supported by ECMO was selected. To our knowledge, we are the first to report the pathological diagnosis of ECMO assisted TBCB in acute respiratory failure. When oxygenation cannot be maintained after endotracheal intubation and surgical lung biopsy is not feasible, ECMO-supported TBCB may be a good choice to obtain lung tissue for histopathological diagnosis in patients with acute lung injury of unknown etiology.

Keywords: organizing pneumonia, extracorporeal membrane oxygenation, mechanical ventilation, transbronchial cryobiopsy, case report

INTRODUCTION

Organizing pneumonia (OP) describes a histological pattern of acute or subacute pulmonary damage. The first description of OP can be traced back to Lange in 1901 (1). It is a clinical entity (1–3) associated with non-specific clinical manifestations, and is associated with a known or unknown underlying disease, leading to delay in diagnosis, or even misdiagnosis. A definitive

diagnosis is usually obtained from lung biopsy because of the unavailability of other specific detection methodologies (4). Corticosteroid treatment represents the standard therapy in OP (5).

In this report, we present the case of a patient who was initially misdiagnosed with community-acquired pneumonia (CAP), and her respiratory failure progressed rapidly. Fortunately, transbronchial cryobiopsy (TBCB), assisted by extracorporeal membrane oxygenation (ECMO), was performed in the most critical situation, and it revealed an OP pattern. TBCB supported by ECMO has improved the pathological diagnosis rate of unexplained acute respiratory failure to a certain extent, which is significant in guiding precise treatment and disease prognosis. This study aims to suggest an alternative approach to the diagnosis of OP in the scenario of an acute respiratory failure.

CASE PRESENTATION

A 58-year-old, never-smoking housewife presented to the Department of Pulmonary and Critical Care Medicine of China-Japan Friendship Hospital with the chief complaints of cough, dyspnea, and intermittent fever. She had a history of leukopenia of unknown origin that was untreated. Chest computed tomography (CT) revealed scattered solid nodules in both lungs, with a fibrous striped shadow in the medial segment of the middle lobe of the right lung and slight enlargement in the left hilum (**Figure 1A**). Though broad-spectrum antibiotics were administered, the patient's clinical status worsened, developing a severe pneumonia with type I respiratory failure and her pulse oximetry oxygen saturation (SpO_2) decreased to 70% while on oxygen delivered at the rate of 5 L/min through a nasal cannulae. The patient was transferred to the intensive care unit (ICU) for close monitoring.

A general physical examination was performed. The patient, who was conscious, displayed the following vital signs: temperature, 38.6°C; pulse rate, 105 bpm; respiratory rate, 35 breaths/min; blood pressure, 118/77 mmHg; oxygen saturation, 93% while on oxygen delivered through a nasal cannulae at a rate of 5 L/min. Chest auscultation revealed raw breathing sounds and wet rales in both lungs. Pitting edema in both lower extremities was found as well. Results of laboratory tests were: white blood cell count 8.54*10^9/L with neutrophil predominance (89.7%); reactive protein > 200mg/L (reference value <10 mg/L); serum procalcitonin 1.67 ng/mL, alanine transaminase 48 U/L, aspartate transaminase 68 U/L. Autoimmune tests including anti-neutrophil antibody (ANA), anti-neutrophil cytoplasmic antibody (ANCA), and rheumatoid factor (RF) were negative. Results of blood culture, 1-3-β-D-glucan (BDG) assay, galactomannan (GM) tests, T-spot, cryptococcal antigen nucleic acid, blood EB virus DNA, cytomegalovirus DNA were negative. Fiberoptic bronchoscopy with bronchoalveolar lavage (BALF) revealed extensive hyperemia and edema in the bronchial mucosa, and no other abnormalities were observed. Fungal cultures were positive for *Candida albicans*. Cultures for bacteria and mycobacteria were negative. All virus, *Mycoplasma pneumoniae*, and Chlamydia nucleic acid amplification tests were negative. No pneumocystis infection was identified and tumor cytology was negative. Contrast-enhanced chest CT revealed patchy areas of ground-glass opacities (GGO) and consolidation in both lungs scattered all over and with slightly enlarged mediastinal lymph nodes (**Figure 1B**).

On ICU day 2, her respiratory status continued to decline, and bedside chest radiographs (**Figure 2A**) showed progression of bilateral infiltrates. Mechanical ventilation (PC mode, FiO_2 100%, F 35/min, PC 18 cmH_2O, PEEP 10 cmH_2O), and venovenous extracorporeal membrane oxygenation (V-V ECMO) were performed to maintain adequate oxyhemoglobin saturation. We delivered an almost constant flow of 6 L/min/m^2 *via* ECMO. A bedside TBCB was performed on the same day to obtain a definitive diagnosis. Three specimens were obtained from the outer and posterior basal segments of the lower lobe of the left lung, and histological examination revealed fibroblasts intermixed with loose connective tissue and collagen in the alveoli and the distal bronchioles (**Figures 3A,B**). These findings were typical of OP (5). Thus, an OP diagnosis was established.

The following day, the patient's treatment was changed to intravenous methylprednisolone in continuous infusion, 240 mg for 3 days, followed by 80 mg for 5 days, and then 40 mg as the maintenance dose. The fever subsided the day after the steroid was given, and her general condition improved significantly. The patient was extubated 4 days after endotracheal intubation and tracheotomized for 24 days. She remained on VV-ECMO for 15 days. Systemic heparinization is required during ECMO treatment. On the one hand, thrombus formation can be seen in front of the ECMO membrane and behind the membrane, and thrombosis can be seen around the PICC tube. On the other hand, the patient developed hemoptysis several times, hematuria, hematoma of abdominal muscle successively. For hemoptysis, we did a bronchoscopy to stop the bleeding. And we adjusted the heparin pump rate according to the APTT target value to prevent massive bleeding. After the patient was withdrawn from ECMO, heparin was stopped, and the hemoptysis was gradually relieved. There was no recurrence of hematuria and no increase in abdominal hematoma. No thrombosis was found in the deep veins of the extremities.

Bedside chest radiographs showed consolidation of both lungs decreased significantly on ICU day 10 (**Figure 2B**). On day 21 after steroid use, her chest radiograph (**Figure 1C**) showed that the consolidation was less than the previous. On day 41 after steroid use, her symptoms and chest radiograph (**Figure 1D**) were indicative of a significant improvement, with complete resolution of pulmonary symptoms.

DISCUSSION

To our knowledge, ECMO-assisted TBCB has never been reported in the pathological diagnosis of acute respiratory failure.

Clinically, patients with OP may have acute or subacute disease progression that may include constitutional symptoms with flu-like illness, including cough, fever, malaise, weight loss, and dyspnea (1, 6). OP may be idiopathic or associated with a known or unknown underlying disease, leading to

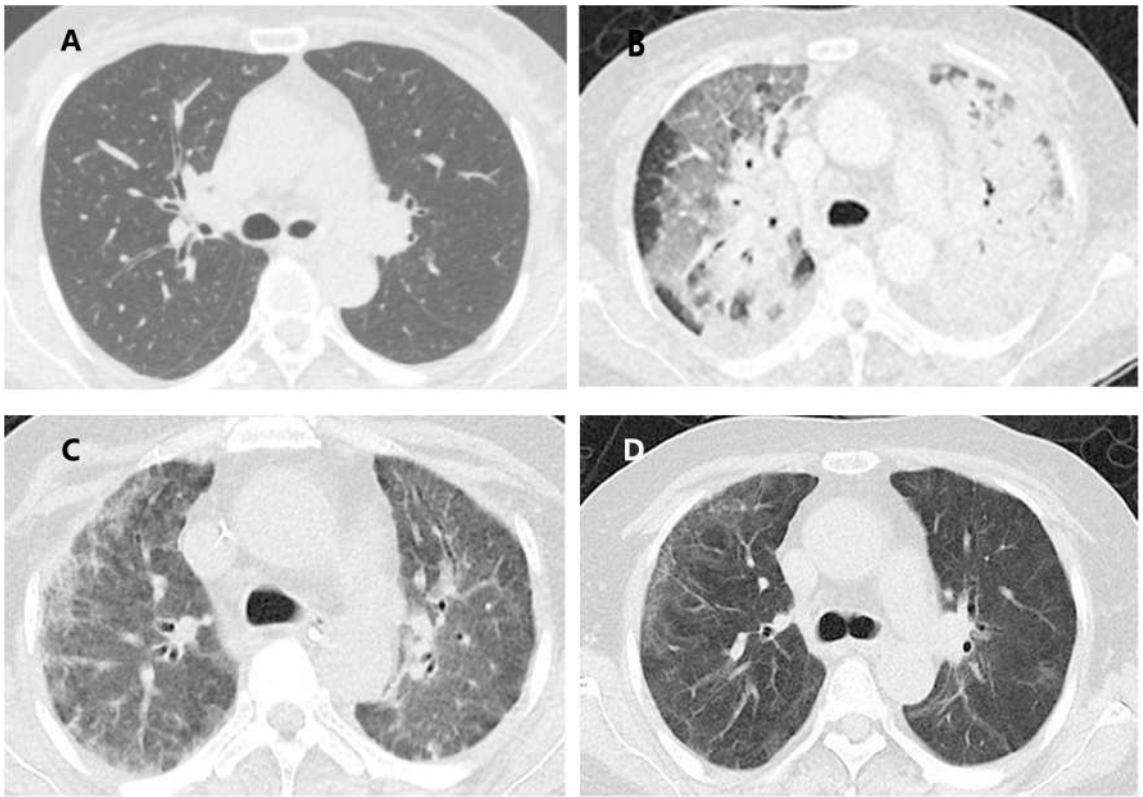

FIGURE 1 | Evolution of the computed tomography (CT) findings of the patient. **(A)** Scattered solid nodules in both lungs, with fibrous stripe shadow in the medial middle lobe of the right lung and slight enlargement in the left hilum; **(B)** patchy areas of ground glass opacities (GGO), consolidation in the double lungs scattered all over both lungs and slightly enlarged mediastinal lymph nodes; **(C)** consolidation and GGO in both lungs decreased compared with the previous one; **(D)** only a small amount of GGO and fibrous stripe shadow.

delay in diagnosis or even misdiagnosis. Similarly, our patient was diagnosed with infectious pneumonia initially and was treated with antibiotics, which may have delayed her early steroid use and led to the eventual progression of her illness. Fortunately, the patient in our case recovered, which may be because ECMO-supported TBCB was decisively performed on this patient, enabling us to develop a pathological diagnosis and use glucocorticoids at the right time.

Obtaining lung tissue for histopathological diagnosis in patients with acute lung injury of unknown etiology is helpful for precision therapy. This is because of the atypical symptoms, non-specific laboratory findings, and variable CT findings associated with OP (7). The patient had undergone ultrasound-guided percutaneous transthoracic needle biopsy in the general ward, but no valuable results were obtained. The failure of the aforementioned procedure was mainly due to the respiratory distress, which made impossible for the patient to hold her breath, resulting in unsatisfactory lung tissue sampling. A study (8) pointed out that it is difficult to diagnose OP based on bronchoscopic biopsy specimens because of its patchy distribution. Methods of obtaining large specimens, such as surgical lung biopsy, are better options for the disease. However, in our case, oxygenation could not be maintained after endotracheal intubation, making surgical lung biopsy even more challenging to perform. In our case, ECMO rendered possible a safe bedside TBCB in an otherwise extremely unstable patient.

Currently, VV-ECMO has been described for managing different lung diseases in the ICU for severe cardiorespiratory failure. However, ECMO-assisted TBCB is rarely reported in the pathological diagnosis of acute respiratory failure. Our patient's deteriorating clinical condition was a major limitation of lung biopsy. Thus, she underwent TBCB with VV-ECMO support as a bridge to diagnosis and treatment. This may show that ECMO-assisted TBCB is feasible for the histopathological diagnosis of acute respiratory failure of unknown cause. ECMO enables biopsy in critically ill patients to find the exact pathological evidence in acute lung injury management.

OP is a unique model of intraalveolar fibrotic inflammation that is fully reversible with treatment (5). However, the disease continued to progress in this case despite receiving 40 mg/day of methylprednisolone outside our hospital for 9 days, perhaps due to the inadequate treatment of steroids. The day after the patient was diagnosed, we administered pulse corticosteroid treatment of methylprednisolone 240 mg/day in continuous infusion for 3 consecutive days and then reduced it to 80 mg/day. We believe that the patient's improvement is largely dependent on appropriate steroid therapy. The standard treatment for

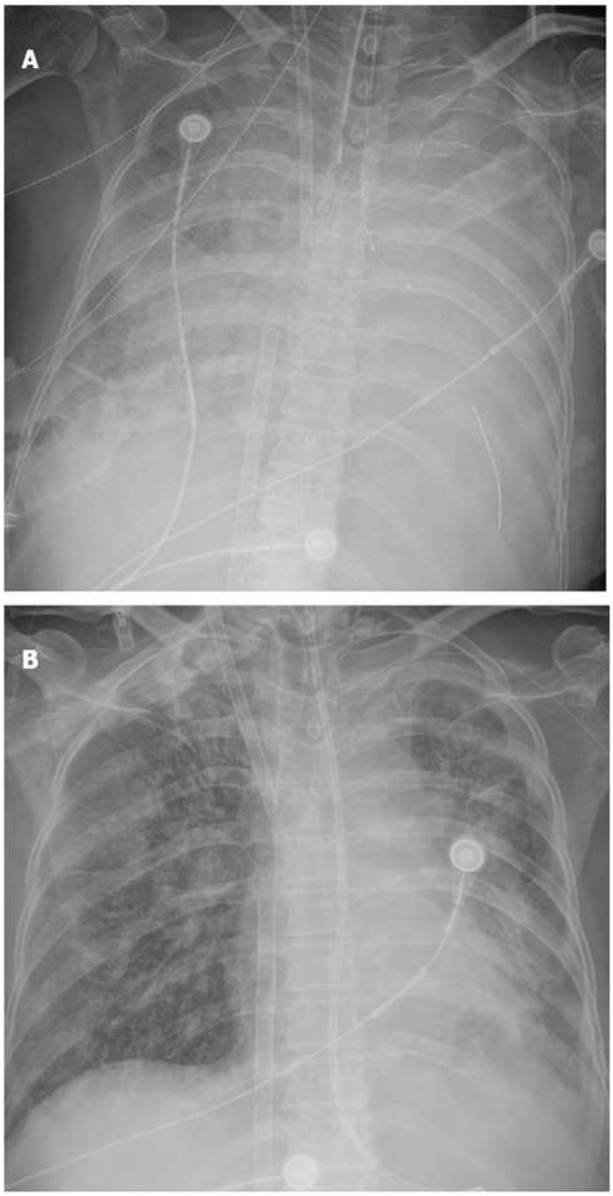

FIGURE 2 | Evolution of the chest X-ray of the patient. **(A)** Extensive consolidation of both lungs; **(B)** consolidation of both lungs decreased significantly.

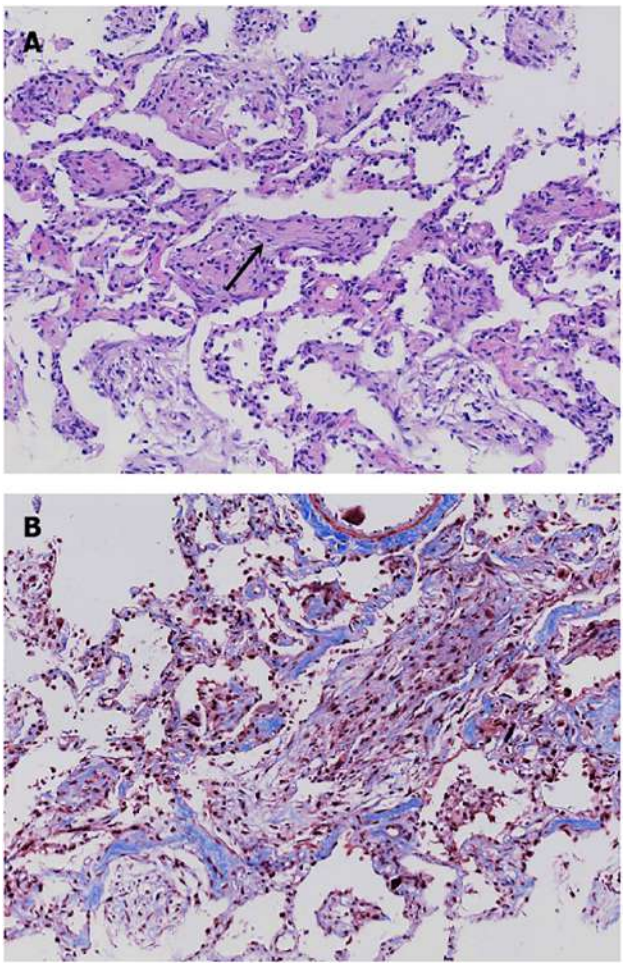

FIGURE 3 | Histological patterns of the patient's lung tissue. **(A)** Hematoxylin-eosin stain revealed the presence of fibroblasts intermixed with loose connective tissue and collagen in the alveoli and the distal bronchioles. **(B)** Masson stain showed a mass of blue collagen fibers stained with aniline blue.

OP has not yet been determined (9–11). For patients with cryptogenic OP, the recommended initial prednisone dose is 0.75 mg/kg/day for 2–4 weeks (12). However, physicians still need to adjust the dose of prednisone based on disease severity and response to therapy. In severe OP, intravenous prednisolone can be administered for 3 consecutive days (13–15) and immunosuppressive therapy with cyclophosphamide, azathioprine, or cyclosporin A can be used (16, 17), especially for acutely ill patients.

In addition, it is important to find the right opportunity to use ECMO and avoid the associated complications. Bleeding and thrombosis are serious complications of ECMO. Dealing with possible complications requires continuous management from relevant experts. One reason for the successful treatment of this patient is that we used ECMO at the right time and cleverly managed its complications.

CONCLUSION

OP is an unusual cause of acute lung injury. Diagnosis is mostly biopsy-proven. When oxygenation cannot be maintained after endotracheal intubation and surgical lung biopsy is not feasible, ECMO-supported TBCB may be a good choice to obtain lung tissue for histopathological diagnosis in patients with acute lung injury of unknown etiology, because ECMO can provide a relatively safe bedside TBCB for extremely unstable patients to avoid delayed diagnosis or even misdiagnosis. However, care should be taken to avoid ECMO-related complications.

DATA AVAILABILITY STATEMENT

All data discussed in this manuscript are included within this published article.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. The patient provided her written informed consent to participate in this study. Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YW, XZ, YF, and LZ produced the first draft of the manuscript. YZ, HY, BX, WG, and TS were major contributors in analyzed and interpreted the patient's data. YT, XZ, and QZ were major contributors in writing the manuscript. All authors read, approved the final manuscript, reviewed, edited, and approved the final versions of the submitted manuscript.

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REVIEW
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Research Progress in the Molecular Mechanisms, Therapeutic Targets, and Drug Development of Idiopathic Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a fatal interstitial lung disease. Recent studies have identified the key role of crosstalk between dysregulated epithelial cells, mesenchymal, immune, and endothelial cells in IPF. In addition, genetic mutations and environmental factors (e.g., smoking) have also been associated with the development of IPF. With the recent development of sequencing technology, epigenetics, as an intermediate link between gene expression and environmental impacts, has also been reported to be implicated in pulmonary fibrosis. Although the etiology of IPF is unknown, many novel therapeutic targets and agents have emerged from clinical trials for IPF treatment in the past years, and the successful launch of pirfenidone and nintedanib has demonstrated the promising future of anti-IPF therapy. Therefore, we aimed to gain an in-depth understanding of the underlying molecular mechanisms and pathogenic factors of IPF, which would be helpful for the diagnosis of IPF, the development of anti-fibrotic drugs, and improving the prognosis of patients with IPF. In this study, we summarized the pathogenic mechanism, therapeutic targets and clinical trials from the perspective of multiple cell types, gene mutations, epigenetic and environmental factors.

Keywords: idiopathic pulmonary fibrosis, cells crosstalk, gene mutations, epigenetics, emerging drugs

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1 INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive, life-threatening, interstitial lung disease of unknown pathogenesis. IPF has a familial and sporadic onset with a poor prognosis, and death usually occurs within 2–5 years of diagnosis due to secondary respiratory failure (Noble et al., 2012). CT imaging of IPF usually shows a typical usual interstitial pneumonia (UIP) pattern, characterized by irregular reticular opacities with obligatory honeycombing, associated with traction bronchiectasis. IPF also exhibits histological features of UIP/IPF pattern characterized by architecture remodeling due to dense fibrosis with frequent honeycombing, patchy lung involvement by fibrosis, subpleural and/or paraseptal distribution, fibroblast foci at the edge of dense scars (Spagnolo et al., 2018; Baratella et al., 2021). Although the etiology of IPF is unknown, various imbalances centered on alveolar epithelial cell/fibroblast apoptosis imbalance has been shown to play an important role in the pathogenesis of IPF (Wang et al., 2021). Therefore, it is necessary to understand the respective roles and interactions of alveolar epithelial cells, fibroblasts,

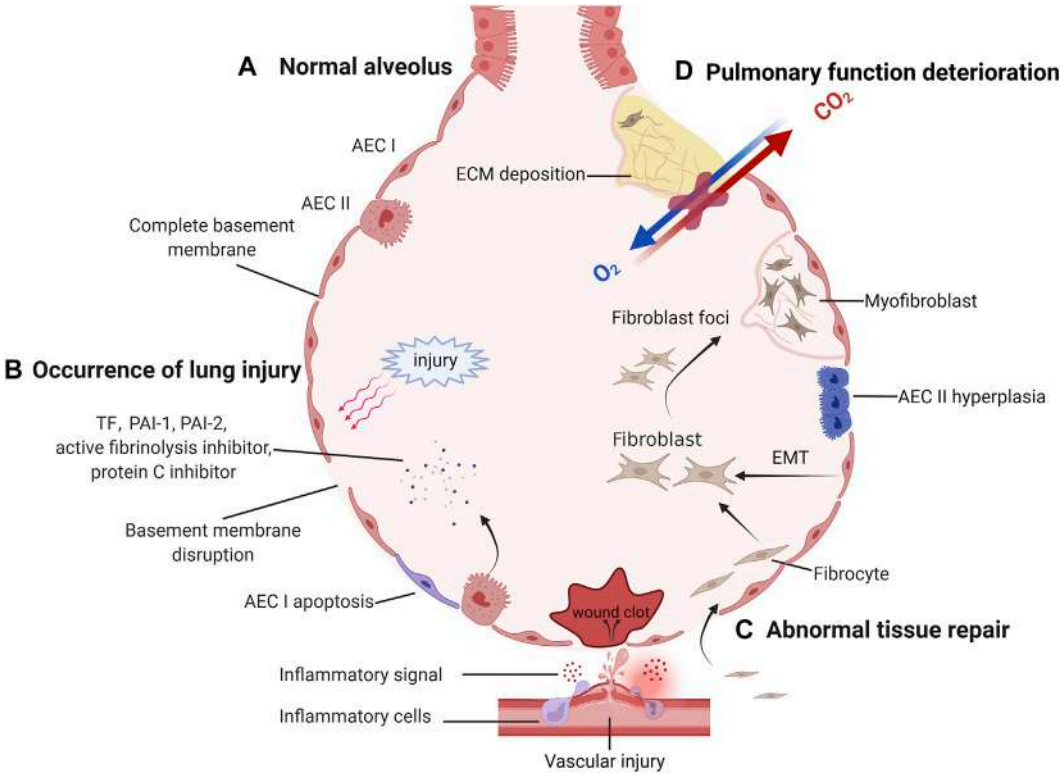

FIGURE 1 | The pathological process of idiopathic pulmonary fibrosis. **(A)** After normal alveoli are damaged and abnormally repaired, irreversible lung function deterioration occurs. Normal alveolus has a complete basement membrane and gas exchange function. **(B)** When the basement membrane continuity is disrupted by external injury, the damaged capillaries and activated AECs release inflammatory signals and coagulation factors, forming a local inflammatory microenvironment. **(C)** If the damage persists, abnormal repair will be initiated. Lung mesenchymal progenitors, fibrocytes recruited to the lung, and endothelial cells undergoing EMT can aggregate to form fibroblasts foci and differentiate into matrix-secreting myofibroblasts. To compensate for the local blood supply to the alveoli, new blood vessels are gradually formed. **(D)** As fibroblast foci increased, more ECM was deposited and cross-linked together, triggering a deterioration in lung compliance and gas exchange function.

immune cells, and extracellular matrix (ECM) in the complex crosstalk. In addition, we discuss potential factors affecting these pro-fibrotic cells, including genetic mutations, epigenetic alterations, environmental factors and aging, with the aim of finding the underlying cause of the disease. The currently approved IPF treatment drugs are pirfenidone and nintedanib, both of which can slow the progression of IPF, but there is no evidence that they can reverse IPF-related pulmonary fibrosis (Chu et al., 2020). Lung transplantation is the only option for patients with end-stage IPF (Lederer and Martinez, 2018; Villavicencio et al., 2018). Therefore, there is a necessity to develop novel agents for the treatment of IPF. This article reviews the roles of various cells and extracellular matrix associated with pathogenic mechanisms, potential pathogenic factors, and the latest information on clinical trials of IPF.

2 THE PATHOLOGICAL PROCESS OF IDIOPATHIC PULMONARY FIBROSIS

The current paradigm suggests that IPF occurs as a result of epithelial injury and dysregulation of the epithelial/mesenchymal crosstalk, which continuously activates multiple interconnected downstream profibrotic pathways, ultimately leading to an abnormal repair response and decreased lung function (Spagnolo et al., 2021).

In the next sections, we provide a brief overview of the pathogenic mechanisms of IPF (**Figure 1**). Damage to alveolar epithelial cells in response to external stimuli leads to disruption of the basement membrane and release of large amounts of cytokines. Many cytokines (interleukins, chemokines, and growth factors) are released by alveolar epithelial cells (AEC) to recruit and activate inflammatory cells and fibroblasts (Phan et al., 2021; She et al., 2021). Coagulation factors (tissue factor (TF), plasminogen activation inhibitors (PAI 1 and PAI 2), fibrinolysis inhibitors and protein C inhibitors) induce a microenvironment that promotes coagulation and inhibits fiber degradation (King et al., 2011; Selman and Pardo, 2014; Betensley et al., 2016). In addition to microenvironmental changes in the lung, cellular processes (apoptosis, senescence, epithelial-mesenchymal transition, endothelial-mesenchymal transition, and epithelial cell migration) have been shown to play a key role in IPF-associated tissue

remodeling (Phan et al., 2021). In addition, fibroblasts differentiate into myofibroblasts and secrete large amounts of extracellular matrix, which eventually leads to the formation of fibroblast foci and the development of pulmonary fibrosis (Yagihashi et al., 2016). In conclusion, following injury to the AEC, the lung microenvironment and cellular processes are altered, which initiates abnormal repair and ultimately leads to the development of IPF and pulmonary function deterioration.

3 IMPORTANT CELLS AND EXTRACELLULAR MATRIX INVOLVED IN THE PATHOGENESIS OF IPF

There are AECs, alveolar capillary endothelial cells, immune cells, fibroblasts, and mesenchymal progenitor cells near the alveoli. These cells maintain the homeostasis of the alveolar environment under normal physiological conditions. However, in the pathophysiological process of IPF, the intercellular crosstalk

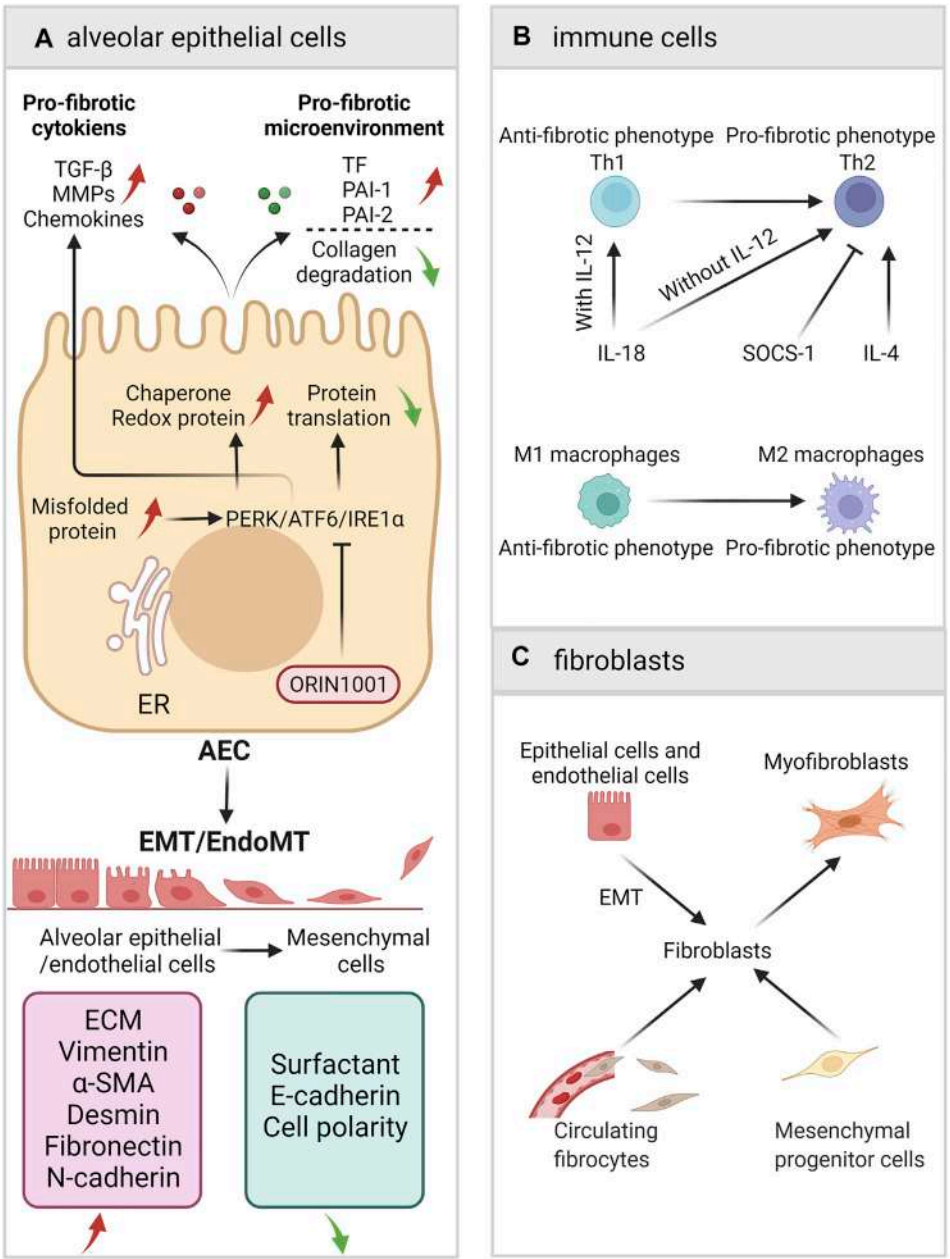

FIGURE 2 | A schematic view of the roles of AECs, immune cells and fibroblasts. **(A)** Alveolar epithelial cells/endothelial cells participate in IPF via ER stress/UPR, EMT, coagulation cascade, and the secretion of a variety of signaling factors. **(B)** The balance of Th1/Th2 and M1/M2. **(C)** The sources of fibroblasts and the fibroblast-to-myofibroblasts differentiation.

leads to reprogramming of cell phenotype. AECs, endothelial cells, and immune cells work together through multiple signaling pathways to regulate fibroblast phenotype. Then, fibroblasts recruitment, proliferation, differentiation, and secretion of extracellular matrix directly lead to fibrosis and exhaustion of pulmonary function. therefore, it is necessary to clarify the roles of various cells and extracellular matrix in the development of IPF.

3.1 Alveolar Epithelial Cells and Endothelial Cells

Alveolar epithelial cells/endothelial cells participate in IPF via various ways, including unfolded protein response (UPR), Epithelial-mesenchymal transition (EMT), coagulation cascade, angiogenesis, and the secretion of a variety of signaling factors (such as TGF-β) (Margaritopoulos et al., 2017; Upagupta et al., 2018; Chanda et al., 2019; Hill et al., 2019; Salton et al., 2019; Selman and Pardo, 2020). In this section, we show how AECs are involved in pulmonary fibrosis (**Figure 2A**).

3.1.1 Unfolded Protein Response

The UPR of AEC is one of the underlying mechanisms for the development of IPF. As an important site for cellular protein synthesis, the ER (endoplasmic reticulum) must maintain relative homeostasis. Under pathological conditions [e.g., viral infection, smoking, asbestos exposure, ROS, hypoxia, senescence, mechanical stretching, proteasome dysfunction, and autophagy disorders (Burman et al., 2018)], misfolded proteins accumulate in the ER of AECs, resulting in ER stress. To restore protein metabolism homeostasis, PERK/ATF6/IRE1α (Protein kinase R-like endoplasmic reticulum kinase/activating transcription factor 6/inositol-requiring enzyme 1α) receptors will be activated, followed by the activation of the UPR to reduce overall protein translation and increase the expression of chaperone and redox proteins. The UPR/ER stress regulates AEC apoptosis and EMT (Burman et al., 2018). However, UPR can also participate in pulmonary fibrosis by increasing the expression of profibrotic mediators, such as TGF-β1, platelet-derived growth factor (PDGF), CXCL12, and CCL2 (Zolak and de Andrade, 2012; Wolters et al., 2014). Because the upstream targets of the UPR/ER stress are of great significance for the maintenance of cell survival and organ development, pathways targeting upstream molecules may cause severe cytotoxicity. Therefore, targeting downstream molecular pathways or chaperones seems to be a better choice. (Burman et al., 2018). Currently, ORIN1001, which targets inositol-requiring transmembrane kinase endoribonuclease-1α (IRE1α), is undergoing a phase I clinical trial for the treatment of IPF(NCT04643769). The crosstalk between autophagy and endoplasmic reticulum stress is important in pulmonary fibrosis (Ghavami et al., 2018; Maciel et al., 2018). Therapeutic approaches targeting autophagy have been shown to have great potential in cancer and aging (Xia et al., 2021; Cassidy and Narita, 2022). Interestingly, studies have found that autophagy also plays an important role in IPF. TGF-β can inhibit autophagy in fibroblasts, while rapamycin and Tubastatin can promote autophagy and inhibit bleomycin-mediated pulmonary fibrosis (Patel et al., 2012; Saito et al., 2017).

3.1.2 Epithelial-Mesenchymal Transition

EMT refers to a process in which AECs gradually lose their epithelial characteristics under specific stimuli and conditions, with the subsequent appearance of interstitial cells characteristics. During this process, AECs are reprogrammed with changes in secretory phenotype, cytoskeletal proteins, intercellular junctions, and cell polarity (Salton et al., 2019). The conditions that induce EMT in AECs include alveolar epithelial cell injury and abnormal apoptosis, the UPR/ER stress, mechanical stress, smoking, and infection (Salton et al., 2019). Under the repeated stimuli, AECs are severely damaged and cannot complete repair processes and re-epithelialization normally, resulting in reprogramming and manifesting as abnormal repair processes (Salton et al., 2020). TGF-β, epidermal growth factor (EGF), fibroblast growth factor (FGF), IL-1, connective tissue growth factor factors (CTGF), insulin-like growth factor-2 (IGF-2), nuclear factor-kb (NF-kB) and Wnt can activate the transcription factors SNAIL, TWIST1 and ZEB through a variety of cytokine pathways to directly initiate EMT (Salton et al., 2019). FGFR1-3 inhibitor SKLB-YTH-60 ameliorates EMT and fibrosis in bleomycin-induced lung fibrosis mouse models (Liu et al., 2021).

However, in recent years, the central role of EMT in the pathogenesis of IPF has been questioned. The localization of type 2 epithelial cells by markers showed that the conversion of epithelial cells into myofibroblasts was incomplete (Gjorevski et al., 2012) and that the marker protein of myofibroblasts, α-SMA, and EMT epithelial cells could not be colocalized, indicating that epithelial cells may not completely convert to fibroblasts (Rock et al., 2011); additionally, mesenchymal AECs have a very limited ability to secrete ECM (Yao et al., 2019). Studies have shown that EMT indirectly promotes the formation of a profibrotic microenvironment through the dysregulation of paracrine signals between epithelial cells and mesenchymal cells (Hill et al., 2019; Yao et al., 2019). Therefore, although there is a large amount of evidence that EMT does exist in IPF, due to the latest lineage tracing results, EMT is more regarded as an indirect process. Nevertheless, the profibrotic microenvironment that regulates the occurrence of EMT is still quite promising in the inhibition of IPF (Hill et al., 2019).

3.1.3 Coagulation Cascade and Angiogenesis

Under pathological conditions, the coagulation cascade and angiogenesis are important driving forces for the promotion of pulmonary fibrosis. Because tissue factor can activate PAI 1, PAI 2, fibrinolysis inhibitors and protein C inhibitors through the coagulation cascade, resulting in a local pro-coagulation microenvironment, it inhibits the degradation of the ECM in this microenvironment and promotes the differentiation of fibrocytes (King et al., 2011; Selman and Pardo, 2014; Betensley et al., 2016). IPF patients have a relatively lower number of endothelial progenitor cells, which may potentially contribute to suppressed repair of the damaged pulmonary endothelium and thereby may drive the sequence of events in profibrogenic direction (Malli et al., 2013). Besides, it is reported

that compensatory pro-angiogenic VEGF increases, which is a pro-fibrotic mediator (Malli et al., 2013).

3.1.4 Pro-Fibrotic Secretory Phenotype of AEC

In addition to the above pathways, AECs also participate in pulmonary fibrosis through the secretion of a variety of mediators, including growth factor (TGF-β, PDGF/CTGF/IGF-I/insulin-like growth factor binding proteins 3 and 5), matrix metalloproteinases (MMP1/MMP2/MMP7), chemokines (CCL17/CCL2/CXCL12), pigment epithelium-derived factor, autotaxin, sphingosine-1-phosphate, neuregulin (NRG) 1α, growth and differentiation factor 15 (GDF15), transmembrane protease serine 4 (TMPRSS4), tumor necrosis factor-alpha (TNF-α), osteopontin, and angiotensinogen. Mareike Lehmann et al. summarized the possible roles of these cytokines in detail in the occurrence and development of IPF (Selman and Pardo, 2020).

3.2 Immune Cells

A large number of studies have demonstrated that immune cells play a role in IPF. However, anti-inflammatory therapies, i.e., TNF-α monoclonal antibodies (Utz et al., 2003; Raghu et al., 2008) and glucocorticoids (Raghu et al., 2012), fail to achieve primary outcomes in clinical trials. Neither of these promising conventional treatments prevent a decline in forced vital capacity (FVC) or the progression of pulmonary fibrosis or benefit survival. In addition, a phase 3, randomized, double-blind, placebo-controlled study including 826 participants (NCT00075998) showed that the subcutaneous injection of IFNγ-1b neither improved the FVC of patients nor prolonged the survival time of patients. These clinical trials are important milestones for clinical treatment strategies for IPF, causing a shift from traditional anti-inflammatory treatment to simultaneous interventions for multiple pathogenic links. Past treatments generally treated inflammation as a whole while ignoring the dual profibrotic/antifibrotic roles of different inflammatory factors and different inflammatory cells. Perhaps by further distinguishing the roles of different inflammatory factors and pathways involved in different inflammatory cells, more precise therapeutic targets can be found. Therefore, inflammation, as one of the potential pathogeneses of IPF, remains an important focus of research.

With the deepening of research in the field of immunization, the role of immune factors in IPF has received increasing attention. Although the diagnostic criteria for IPF require the exclusion of autoimmune diseases as the underlying pathogenesis, many IPF patients still have unexplained elevated autoantibodies, and some autoantibodies are associated with acute exacerbations of IPF (AE-IPF) (Ogushi et al., 2001; Kurosu et al., 2008; Taillé et al., 2011; Kahloon et al., 2013). To inhibit the function of B cells, Ianalumab (B-cell activating factor receptor mAb) and Rituximab (CD20 mAb) have entered phase II clinical trials.

Heukels conducted a detailed review on the role of each immune cell in IPF and the correlation of each cell type with the development of fibrosis (Heukels et al., 2019). Nevertheless, the mechanism of action of some immune cells in the pathogenesis of IPF is still not very clear. The following subsections introduce the roles of the Th1/Th2 balance and the M1/M2 balance in IPF, although these paradigms simplify the role of immune cells (**Figure 2B**).

3.2.1 Th1/Th2 Balance

Th1 (helper T cell type 1) cells are helper T cells produced by $CD4^+$ cells under IFN-γ/IL-12 induction. CXC chemokine receptor 3 expressed by Th1 cells can recognize interferon-inducible T cell a chemoattractant (I-TAC), interferon g-inducible protein of 10 kD (IP-10), and monokine induced by interferon gamma (Mig) (Sumida et al., 2008). The main function of Th1 is to secrete IFN-γ. IFN-γ is considered to be antifibrotic and can reduce the production of ECM (Bouros et al., 2006; Smaldone, 2018).

Th2 (helper T cell type 2) are helper T cells produced by $CD4^+$ cells under IL-4 induction. Th2 cells that express CC chemokine receptor 4 can recognize thymus- and activation-regulated chemokines (TARCs) and macrophage-derived chemokines (MDCs) (Sumida et al., 2008). The main function of Th2 cells is to secrete IL-4/IL-5/IL-13; these interleukins are considered to promote fibrosis (Romagnani, 2000).

Th2 polarization has been observed in IPF. In the BALF of IPF patients and in systemic circulation, the levels of Th1 cells and their secretion of IFN-γ are relatively low, and the levels of Th2 cells and their secretion of IL-4/IL-5/IL-13 are relatively high (Hams et al., 2014). In addition, in a bleomycin-induced mouse model, an increase in IFN-γ levels and a reduction in pulmonary fibrosis were observed after the administration of IL-12, an inducer of Th1 cells; in contrast, an increase in fibroblast proliferation and fibrosis was found with the use of IL-4, an inducer of Th2 cells (Hams et al., 2014; Passalacqua et al., 2017).

The inhibition of Th2 polarization is a possible direction for the treatment of IPF. Many factors can cause Th2 polarization. Galectin-1 and prostaglandin E2 promote Th2 polarization by inducing Th1 apoptosis and reducing the synthesis of Th1 inducers (Kaliński et al., 1997; Cedeno-Laurent and Dimitroff, 2012; Corapi et al., 2018), and suppressor of cytokine signaling-1 (SOCS-1) inhibits the expression of Th2 inducers to prevent excessive Th2 cell accumulation (Bao et al., 2014). Among the interleukins that regulate Th1 and Th2 differentiation, IL-18 is notable; the polarization direction mediated by IL-18 is regulated by IL-12. Under the synergistic effect of IL-12, IL-18 induces Th1 cells to produce IFN-γ, IL-12, and GM-CSF and upregulate the expression of IL-2Rα to promote an inflammatory response. In contrast, in the absence of IL-12, IL-18 induces the production of Th2-related cytokines, such as IL-13/IL-4, by T cells, NK cells, basophil cells, and mast cells and promotes the differentiation of Th2 cells (Wawrocki et al., 2016). IL-4 is another important interleukin that promotes Th2 differentiation and is an important marker of type 2 immunity. Studies have shown that significant polymorphisms are found in the IL-4 promoter of IPF patients and that these polymorphisms are strongly associated with IPF (Vasakova et al., 2006). In addition to cytokine therapy, a study used the serum IFN-γ/IL-4 ratio to represent the Th1/Th2 balance to predict the development of IPF and found that the IFN-γ/IL-4 ratio was associated with symptoms, imaging

changes, FEV1 (forced expiratory volume in one second), FVC (Forced vital capacity), TLC (total lung capacity), and 6-min walking distance in IPF patients and can predict IPF progression (Peng et al., 2013).

3.2.2 M1/M2 Balance

In addition to the Th1/Th2 balance, the balance of macrophage subpopulations also plays an important role in the pathogenesis of IPF. Because cytokines secreted by Th1/Th2 cells greatly affect the differentiation of M1/M2 macrophages, Th1/Th2 cells and M1/M2 macrophages interact with each other to jointly shape the type 1 and type 2 immune microenvironments (Wang et al., 2021).

The main function of M1 (type I macrophages) is to respond to lipopolysaccharide (LPS), IL-1, and IL-6. They can secrete type 1 immune factors such as IL-12, induced nitric oxide synthase (iNOS), TNF-α, IL-1β, IL-23, IL-6, and CXCL10 (Liu G. et al., 2019), thereby playing a role in the early stage of inflammation. The main function of M2 (type II macrophages) is to respond to type 2 immune factors (IL-4, IL-10, and IL-13), glucocorticoids, and immune complexes. They can secrete cytokines that promote tissue repair, limit inflammation (Martinez et al., 2008; Zhang et al., 2018; Liu G. et al., 2019), participate in immune regulation, suppress immune responses, and remodel tissue. M2 macrophages can also be divided into M2a, M2b, and M2c macrophages based on induction conditions and functions (Martinez et al., 2008).

M2 polarization is one of the important links in the occurrence and development of pulmonary fibrosis. The M2-mediated type 2 immune response is an important component of pulmonary fibrosis. M2 macrophages provide an important microenvironment for pulmonary fibrosis by secreting profibrotic substances such as CCL18, IL-10, TIMP1 (tissue inhibitors of metalloproteinases 1), TGF-β, FGF, PDGFα, IGF1, and VEGF (Prasse et al., 2006; Heukels et al., 2019; Wang et al., 2021). Macrophages can not only secrete chemokines to recruit other cells (like CCL18), but also are attracted by chemokines themselves. CCL2 and CCL3 are important signaling molecules involved in monocyte/macrophage recruitment and help macrophages migrate to the lungs. Therefore, the antagonism of CCL2 and CCL3 may have an antifibrotic effect (Iyonaga et al., 1994; Suga et al., 1999; Deshmane et al., 2009).

3.3 Fibroblasts and Myofibroblasts

Fibrocytes are monocyte progenitor cells with differentiation potential derived from bone marrow; these cells can differentiate into adipocyte cells, chondrocytes, osteoblasts, and fibroblasts under the action of different tissue environments and humoral factors. In the *in vitro* environment, CD45+/CD34+ primary bone marrow fibrocytes were stimulated by the ECM of IPF patients; then the hematopoietic surface antigens CD45 and CD34 rapidly disappeared, and mesenchymal markers, i.e., α-SMA, rapidly increased. The fibrocytes transformed into fibroblasts with a contractility like that of smooth muscle cells and a strong ability to synthesize ECM (Mori et al., 2005; Lama and Phan, 2006; Kage and Borok, 2012; Chong et al., 2019). In addition to the ECM of IPF patients, TGF-β, endothelin, CTGF, interleukins (IL-3 and IL-4), serum response factors (Shi-Wen et al., 2004) and microRNA (miRNA-21, miRNA-22, miRNA-29, miRNA-125b, miRNA-126, miRNA-130a and miRNA-132, miRNA-142a) (Pandit et al., 2011; Wang et al., 2012; Cushing et al., 2015; Kuse et al., 2020) have been shown to be associated with the differentiation of fibrocytes into fibroblasts. After getting activated, fibroblasts proliferate, differentiate, resist apoptosis and can directly lead to IPF through the secretion of profibrotic factors and the remodeling of the ECM (Selman et al., 2000). Under normal circumstances, the secretion and degradation of the ECM by fibroblasts are in a dynamic balance. However, when exposed to inflammation and environmental stress, fibroblasts are reprogrammed and continue to be active and resist apoptosis (Filer and Buckley, 2013), ultimately leading to an increase in the relative rate of ECM synthesis. In patients, the number of fibrocytes and fibroblasts in the lung is positively correlated with collagen deposition and the progression of pulmonary fibrosis. When fibroblast foci start to cross-link with each other, patient lung function may decrease substantially (Snijder et al., 2019).

Although fibroblast is the chief culprit in pulmonary fibrosis, its source is still unclear. Possible sources include peripheral recruitment, mesenchymal progenitor cells in lung tissues, and EMT (**Figure 2C**). When local lung injury occurs, epithelial cells and endothelial cells are activated and release chemokines. Through chemokine ligand–receptor pathways (including CXCL12/SDF1-CXCR4, CCL21-CCR7 and CCL2-CCR2) (Chong et al., 2019), many circulating fibrocytes and mesenchymal progenitor cells are recruited into local tissues and undergo phenotypic transformation to become fibroblasts. In the lungs of IPF patients, there is a high level of CXCL12/CXCR4 (Mehrad et al., 2007), which is conducive to the recruitment of circulating fibrocytes to the lungs along the concentration gradient of chemokines. In addition, AECs that cannot re-epithelialize can convert into interstitial AECs through EMT, leading to a decrease in intercellular junctions and epithelial features. However, the contribution of epithelial EMT in pulmonary fibrosis is still controversial (Salton et al., 2020). In addition, local fibrocytes and fibroblasts can self-proliferate rapidly. Under the influence of cytokines, growth factors and TIMPs, local fibroblasts in IPF can resist apoptosis and continue to proliferate (Andersson-Sjöland et al., 2011).

3.4 Extracellular Matrix

In IPF, ECM remodeling and collagen deposition are the classical pathological features of the disease. ECM is mainly produced by fibroblasts/myofibroblasts, epithelial cells, inflammatory cells, and mesenchymal progenitor cells.

ECM has complex functions in IPF (**Figure 3**). ECM has a certain degree of mechanical stiffness, which plays an important role in the sclerosis of lung tissues (Elowsson Rendin et al., 2019). Besides, ECM also serves as a pool for variety of growth factors (bFGF, VEGF), stimulating factors (GM-CSF, M-CSF), and interleukins (IL-1, IL-8), allowing signal exchange with different cells (Andersson-Sjöland et al., 2011). The mechanical force of the ECM itself can also directly participate

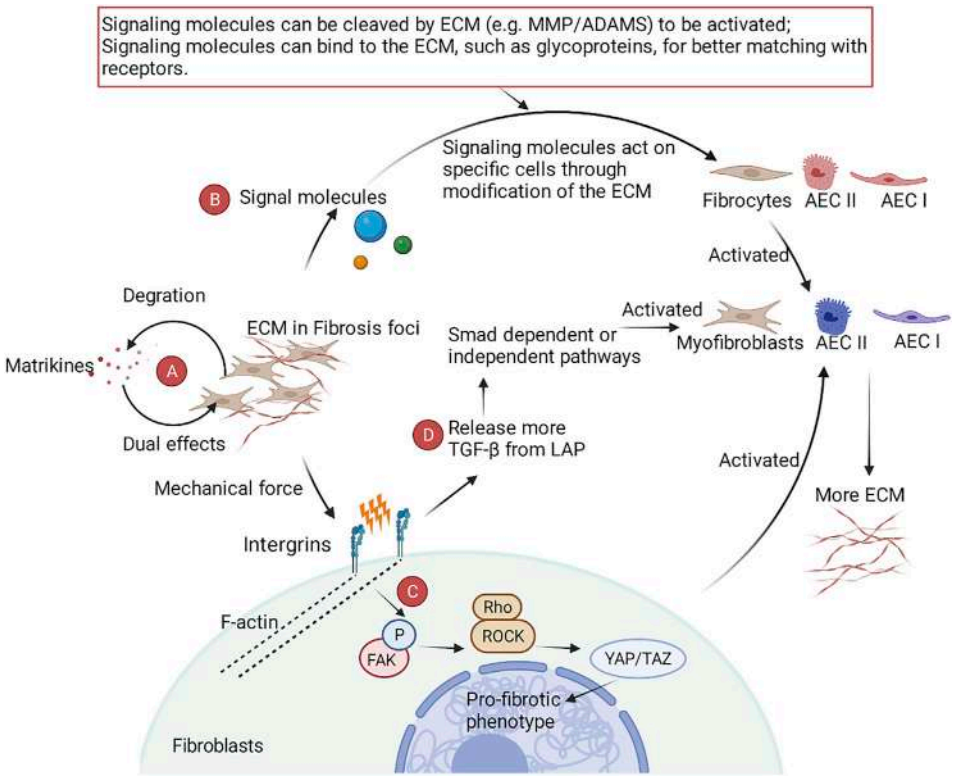

FIGURE 3 | A schematic view of the role of ECM in IPF. **(A)** Matrkines, which contain multiple degradation products of the ECM, exert dual pro- and anti-fibrotic roles in IPF. **(B)** The ECM is involved in the phenotypic reprogramming of fibroblasts and AECs by modifying key signaling molecules. **(C)** After binding specific ECM ligands to the ectodomain of integrins, integrins bind to the cytoskeleton and various signaling proteins through their cytoplasmic tails, translating the mechanical force of cytoskeleton contraction and ECM stiffness into biochemical signals. **(D)** Integrins activate TGF-β-dependent fibrosis by releasing TGF-β through stretch force generated by actin-cytoskeleton interactions.

in pulmonary fibrosis through mechanoreceptors and some cellular pathways. Under the action of mechanical force, α6 integrin (Rahaman et al., 2014) and the transient receptor potential vanilloid 4 (TRPV4) channel (Chen et al., 2016) act as sensors to detect mechanical stimulation signals and transfer the signals into myofibroblasts. Subsequently, F-actin contracts, resulting in the phosphorylation of focal adhesion kinase (FAK). Phosphorylated FAK activates Rho kinase (ROCK) by binding Rho to ROCK. This step activates yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ), which ultimately activates the transcription, translation, and expression of profibrotic genes in the nucleus. In addition to the FAK-ROCK-YAP/TAZ axis, mechanical force can also release TGF-β bound by latency-associated peptide (LAP), thus activating the TGF-β-Smad dependent/independent pathway in fibroblasts and AECs and directly participating in myofibroblast differentiation (Upagupta et al., 2018).

Matrikines, the degradation product of the ECM, also have special biological activities in lung diseases (Burgess and Weckmann, 2012). Matrikines are biologically active fragments derived from the degradation of ECM. These biologically active fragments exert dual biological properties by binding to integrins, heparan sulfate proteoglycans, and growth factor receptors. For example, endotrophin (the degradation product of collagen VI) promotes fibrosis, while endostatin (the degradation product of collagen XVIII) has antifibrosis effects (Ricard-Blum and Salza, 2014).

ECM includes the core matrisome and associated matrisomes. Core matrisomes include collages, ECM glycoproteins, and ECM proteoglycans. Associated matrisomes include ECM regulators, ECM-affiliated proteins, and secreted factors (Kreus et al., 2021). In the ECM of IPF patients, laminins and collagen IV associated with the basement membrane decreased, while the vast majority of core matrisomes showed an increasing trend (Elowsson Rendin et al., 2019). Although there have been numerous studies, very little is known about the role of some mediators in IPF. The various substances in the ECM are described in detail below.

3.4.1 Collagen

Collagen is the main component of ECM. Collagen is derived from activated (myo)fibroblasts, fibrocytes, epithelial cells (which can transform into mesenchymal cells through EMT), mesenchymal stromal cells, and pericytes. The vast majority of interstitial and fibrillar collagens (mainly collagen I and collagen III) are secreted by (myo)fibroblasts. These collagens constitute the main frame of the ECM and

TABLE 1 | The potential role of MMPs/TIMPs in IPF.

MMPs	Cellular Sources	Pro/ Antifibrotic	Mechanism	Ref
MMP1	AEC, macrophage	Profibrotic	MMP1 Induces lung alveolar epithelial cell migration and proliferation, protects from apoptosis, and represses mitochondrial oxygen consumption by activation of HIF-1α	Herrera et al. (2013)
MMP2	BECs, AECs, fibroblasts, fibrocytes	Profibrotic	MMP2 damages the integrity of alveolar walls, regulates EMT, and involves in activation of TGF-β	Nguyen et al. (2001); Wu et al. (2007); Lam et al. (2014)
MMP3	BECs, AECs, macrophage, fibroblasts	Profibrotic	MMP3 activates the β-catenin and TGF-β pathway, induces EMT, releases endothelin	Wu et al. (2007); Richter et al. (2009); Yamashita et al. (2011)
MMP7	Lung epithelial cell, fibroblast	Pro/ Antifibrotic	MMP7 regulates osteopontin, mediates E-cadherin ectodomain shedding, and regulates anti-inflammatory and antifibrotic pulmonary dendritic cells	McGuire et al. (2003); Pardo et al. (2005); Manicone et al. (2009)
MMP8	blood monocytes, AM, BECs, AECs, fibrocytes	Profibrotic	MMP8 reduces the levels of IL-10, IP-10 and MIP-1a, affects the migration of fibrocytes mediated by PDGF-B or stromal cell-derived factor-1α	García-Prieto et al. (2010); Craig et al. (2013)
MMP9	AECs, neutrophils, AM, fibrocytes, fibroblasts	Profibrotic	MMP9 production is related to TGF-β1. MMP9 cleaves SP-D; MMP9 involves in macrophage-induced fibroblast migration and TGF-β1/Smad2-dependent EMT.	Murthy et al. (2010); Bratcher et al. (2012); Li et al. (2019); Zhang et al. (2019)
MMP10	AM, BECs, AECs	Antifibrotic	MMP10 involves in the migration of macrophages and macrophage-mediated collagenase degradation	Murray et al. (2013); Rohani et al. (2015)
MMP11	furin-like proconvertase enzyme	Profibrotic	MMP11 activates Notch pathway and promotes fibroblasts to myofibroblasts differentiation	Aoyagi-Ikeda et al. (2011)
MMP14	AECs, AM, and endothelial cells	Antifibrotic	MMP14 involves in activation of TGF-β, fibroblast-dependent collagenolysis and invasion	Sabeh et al. (2009); Xiong et al. (2017)
MMP19	monocytes, AM, fibrocyte, AEC	Antifibrotic	MMP19 promotes wound healing and cell migration by inducing PTSG2 (prostaglandin endoperoxide synthase 2); MMP19 regulates ECM formation, migration, proliferation, and autophagy of fibroblasts	Yu et al. (2012); Jara et al. (2015)
MMP28	Macrophages	Profibrotic	MMP28 promotes M2 polarization and TGF-β-dependent EMT.	Jara et al. (2015)

increase the mechanical stiffness of fibrotic tissue (Snijder et al., 2019). Currently, it is believed that type III collagen plays a role in the early disease stage and that type I collagen is associated with the deterioration of lung function in the late disease stage due to its involvement in collagen crosslinking mediated by lysyl oxidase (LOX) family enzymes (Rock et al., 2011). In view of the important contribution of collagen to the mechanical stiffness of pulmonary fibrosis, collagen turnover has naturally become an important means for monitoring disease progression and predicting patient prognosis. Increased concentrations of the collagen degradation markers type 1/3/6 collagen, and C-reactive protein are associated with an increased risk of overall mortality, and elevated levels of the collagen synthesis markers Pro-C3 and Pro-C6 are associated with IPF progression (Organ et al., 2019; Jessen et al., 2021).

3.4.2 Glycoproteins and Proteoglycans

There are relatively few studies on ECM glycoproteins and proteoglycans. In a radiation-induced pulmonary fibrosis model, the glycoprotein glectin-3 produced by type I AECs increased dramatically (Kasper and Hughes, 1996). In recent years, studies on proteoglycans have shown that the proteoglycan decorin reduces pulmonary fibrosis by antagonizing TGF-β and antagonizing CTGF-mediated collagen deposition (Nikaido et al., 2018). In addition, the proteoglycan lumican directly acts in the differentiate of monocytes into fibroblasts through an integrin-dependent pathway (Pilling et al., 2015). Chondroitin sulfate type E (CS-E), another proteoglycan, inhibits the expression of α-SMA, CTGF, LOXL2, and CCL2/MCP-1 by silencing the genes of CS-E and the related enzyme carbohydrate sulfotransferase 15 (CHST15) through miRNA (Kai et al., 2017). Syndecan-4 is a heparan sulfate proteoglycan. Silencing of syndecan-4 can reduce SMA-α and collagen deposition (Tanino et al., 2019). In addition, proteoglycans assist other factors in producing biological effects. For example, FXIIa must bind to the proteoglycan heparan sulfate to stimulate the migration of human lung fibroblasts (Wujak et al., 2015).

3.4.3 ECM Regulators

ECM regulators include serine protease inhibitors, cystatins, TIMPs, MMPs (Kreus et al., 2021), a disintegrin and metalloproteinases (ADAMs), and crosslinking enzymes (Ricard-Blum and Salza, 2014; Liu et al., 2016); these molecules are mainly involved in the regulation of ECM decomposition. MMPs/TIMPs play an important role in

TABLE 2 | Genetic mutations associated with IPF.

Targets	Physiological function of the target site	Potential pathogenic mechanisms of gene mutations	Clinical significance	Ref
TERT and TERC	TERT and TERC are important components of the telomerase complex	Telomere shortening may affect the turnover and healing of AEC.	TERT (rs2736100) and TERC (6793295) mutations are associated with IPF susceptibility	Armanios et al. (2007); Borie et al. (2016)
DKC1	DKC1, a pseudouridine synthase, is involved in the synthesis of non-coding ribonucleic acids	Mutations in DKC1 can shorten telomeres in alveolar epithelial cells and affect the stability of telomerase RNA.	DKC1 mutations cause dyskeratosis congenita and pulmonary fibrosis	Kropski et al. (2017); Gaysinskaya et al. (2020)
TIN2	TIN2 is an important component of the shelterin complex	Mutations in TIN2 can shorten telomeres	Heterozygous mutations in TINF2 causes IPF	Fukuhara et al. (2013)
PARN	PARN, a 3′exoribonuclease, is responsible for telomere maturation	PARN mutations lead to shortened telomeres	PARN mutations and telomere shortening are associated with leukopenia	Stuart et al. (2015)
RTEL	RTEL is a DNA helicase crucial for unwinding the T-loop structure	Loss of functional RTEL1 leads to cleavage of the telomeric end proximal to the T-loop by endonuclease SLX4, leading to release of T-loops and shortened telomere	RTEL and telomere shortening are associated with leukopenia	Cogan et al. (2015); Stuart et al. (2015)
NAF1	NAF1, a box H/ACA RNA biogenesis factor, is required for stability and assembly into a mature telomerase holoenzyme complex	NAF1 mutations can reduce telomerase RNA levels, resulting in shorter telomeres	Pulmonary fibrosis-emphysema in NAF1 mutation patients is telomere-mediated	Stanley et al. (2016a)
OBFC1	OBFC1 associates with TPP1 and is implicated in telomere length regulation	N.A.	rs11191865 was associated with a lower risk of IPF.	Fingerlin et al. (2013)
MUC5B	Mucin 5B is involved in mucosal clearance along with surfactant protein C and ciliates	Excess Mucin may increase the retention of harmful particles in the lung and interfere with the normal developmental pathway and alveolar epithelial repair	rs35705950 was the strongest genetic risk factor for IPF, but was associated with lower mortality. MUC5B and MUC5AC expression was increased in patients with IPF.	Peljto et al. (2013); Conti et al. (2016); Evans et al. (2016)
SFTPC	SFTPC regulates alveolar surface tension	SFTPC mutations may promote lung fibrosis by inducing endoplasmic reticulum stress and apoptotic cell death in AEC II.	SFPTC mutations are associated with familial and sporadic IPF onsets	Ono et al. (2011); Venosa et al. (2017)
SFTPA2	SFTPA is involved in the intrinsic immunity of the lung	SFTPA mutant mouse models exhibit intracellular retention of SFTPA and enhanced ER stress	Mutations in SFTPA2 leads to the trafficking of several proteins and causes the development of IPF.	Yongyu Wang et al. (2009); Maitra et al. (2010); Guenther et al. (2019)
ABCA3	A type of phospholipid carrier, involved in the secretion and transport of surface-active substances in AEC II.	ABCA3 mutations may induce ER stress and proteostasis failure through misfolded alveolar surface-active substances	Heterozygous variants of the ABCA3 gene are associated with IPF susceptibility. pG1205R, an ABCA3 gene allele, is more frequently expressed in patients with IPF and ILDs	Zhou et al. (2017); Manali et al. (2019)
ATP11A	ATP11A encodes ABCA1, a transmembrane protein with general transport function	N.A.	rs1278769 was associated with a lower risk of IPF.	Fingerlin et al. (2013)
IL1RN	IL-1RN is a competitive antagonist of IL-1R receptor	MSC exerts anti-inflammatory and anti-fibrotic effects via IL-1RN.	The proportion of IL-1RN gene polymorphisms in patients with fibrosing alveolitis was more	Whyte et al. (2000); Ortiz et al. (2007)
IL-4	IL-4 is associated with type 2 immunity	IL-4 gene polymorphisms may promote a Th2 cytokine environment with exaggerated fibroproliferative healing	Higher percentage of IL-4 gene polymorphisms in IPF patients	Vasakova et al. (2013)
IL-8	IL-8 is a chemokine secreted by macrophages and is involved in the recruitment of neutrophils	IL-8 increases the fibrogenicity of mesenchymal progenitor cells and is involved in the proliferation, activation, and recruitment of mesenchymal cells	IL-8 gene diversity is associated with lung alveolitis and lung function decline	Ziegenhagen et al. (1998); Yang et al. (2018)
TLR3	TLR3 is known as one of the innate immunity receptors, which mediate	Defective TLR3 L412F gene activates abnormal inflammation and promotes fibroplasia in IPF, which may be	rs3775291 increase the risk for IPF patients and also reduces forced volume capacity (FVC)	O'Dwyer et al. (2013); O'Dwyer et al. (2015); Evans et al. (2016)

(Continued on following page)

TABLE 2 | (*Continued*) Genetic mutations associated with IPF.

Targets	Physiological function of the target site	Potential pathogenic mechanisms of gene mutations	Clinical significance	Ref
	inflammation, tissue injury and viral infection	associated with dysregulation of fibroblast proliferation mediated by a sluggish IFN-β response		
TOOLIP	TOLLIP is involved in the signaling pathway of TGF-β, TLR and ILs	rs3750920 may lead to unregulated TLR signaling pathway	rs5743890 was associated with a lower susceptibility to IPF, whereas rs5743894 was associated with a higher susceptibility to IPF. The rs3750920 polymorphism was associated with the efficacy of NAC. rs5743890 was associated with increased IPF morbidity and mortality	Noth et al. (2013); Oldham et al. (2015)
HLA-DRB1	HLA gene encodes major histocompatibility complex (MHC)	N.A.	HLA-DRB1*1501 is related to greater differences in gas exchanges and immunogenic process	Xue et al. (2011); Zhang et al. (2015)
MDGA2	MDGA2 encodes a paralogue for ICAM, which has been shown to be a potential biomarker of IPF disease activity	N.A.	rs7144383 was associated with a higher risk of IPF.	Noth et al. (2013)
DSP	DSP, a desmosomal protein, is mainly expressed in the airway epithelium and is involved in cell adhesion	rs2076304 might influence the binding of RHOXF1	rs2076304 and rs2076295 increased the IPF risk and rs2744371 decreased the IPF sub-risk	Mathai et al. (2016); Wang et al. (2018)
DPP9	DPP9 is a serine protease that belongs to a member of the S9B family. DPP9 is expressed in epithelial cells and is involved in cell adhesion, cell migration and apoptosis	N.A.	rs12610495 is associated with IPF susceptibility	Fingerlin et al. (2013); Zhou and Wang, (2016)
SPPL2C	SPPL2C is a transmembrane GxGD type of cleavage proteases	N.A.	rs17690703 was also known to reduce FVC in IPF. A low survival rate and mortality were reported in people with greater gene SPPL2C expression	Wu et al. (2016); Lorenzo-Salazar et al. (2019)
AKAP13	AKAP13 is a Rho guanine nucleotide exchange factor regulating activation of RhoA	AKAP13 mutations may affect the RhoA/ROCK signaling pathway	rs62025270 was associated with increased production of AKAP13, but no correlation with survival was observed	Allen et al. (2017)
FAM13A	FAM13A contains a protein domain called Rho GTPase activating protein (Rho GAP)	FAM13A mutation may affect the RhoA/ROCK signaling pathway	The rs2609255 was associated with higher mortality rate. The FAM13A allele was associated with worse disease and lower DLCO.	Hirano et al. (2017); van Moorsel, (2018); Ruffin et al. (2020)
MAPT	MAPT encodes Tau protein, a microtubule-associated protein	N.A.	rs1981997 is associated with a lower risk of IPF.	Fingerlin et al. (2013); van Moorsel, (2018)

IPF and are directly involved in ECM remodeling. MMPs belong to zinc-dependent endopeptidases of the M10A subfamily, and 24 gene subtypes are expressed in the human body. As a crucial component of ECM, MMPs participate in the formation and progression of IPF via many pathways, especially TGF-β signaling pathway. Many studies have focused on MMPs and explored their potential value in resisting IPF (**Table 1**). Although the pathological processes of MMPs and IPF have been verified, there are no MMP inhibitors for the treatment of IPF in the clinic (Yue et al., 2021).

ECM contains a variety of signal molecules secreted by cells; therefore, the ECM is a transfer station for signal exchange. In IPF, the signaling molecules from different cells constitute a complex signaling network through the bridge function of the ECM. Upstream signals are modified and regulated by positive feedback in the ECM; this feedback, e.g., the TGF-β pathway and Rho/ROCK signaling, can affect the transcription and translation of ECM-related genes and eventually lead to pulmonary fibrosis (Snijder et al., 2019).

Although there have been many studies on active fragments, the roles of these active products in IPF are still not clear. In recent years, the ECM has shown extraordinary clinical and scientific value in the fields of anti-infection, anti-angiogenesis, and wound healing (Ricard-Blum and Salza, 2014; Ricard-Blum and Vallet, 2019). Therefore, research on

active fragments and their derivatives as targets for antifibrosis therapies is innovative and has great potential.

4 UNDERLYING FACTORS ASSOCIATED WITH THE PATHOGENESIS OF IPF

4.1 Genetic Susceptibility and Epigenetic Alterations in the Development of IPF

A growing number of studies have shown that genetic mutations are associated with susceptibility, diagnosis, progression, prognosis, and adverse effects of treatment in IPF. The proportion of patients carrying genetic mutations in IPF may be underestimated. A cohort study showed that up to 36% of patients with IPF have familial genetic mutations (Barros et al., 2019). In addition, IPF mutations are present in nearly 40% of sporadic pulmonary fibrosis cases (Wolters et al., 2014). These common and rare genetic mutations mainly include mutations in telomeres, alveolar surfactant and muco-ciliary transportation system, immune and cytokine-related genes, cell adhesion and cell integrity-related genes (**Table 2**). MUC5B and telomere related gene mutations are more common in IPF patients, which has inspired a very large number of studies to explore the pathogenic mechanisms of gene mutations (Evans et al., 2016; Zhang et al., 2021). Besides, genetic variants have been associated with the imaging presentation of IPF. Patients with TERT mutations were more likely to exhibit the classical UIP pattern compared to patients without mutations with two-year-follow up (Baratella et al., 2021).

Notably, the majority of existing studies still remain to verify the correlation between mutations and IPF susceptibility, and there are only a few studies related to the causality and pathogenesis of IPF. Moreover, further research needs to focus on how to cross the gap between genetic testing and clinical treatment. Drugs targeting histone modifications and DNA modifications have also shown potential in preclinical studies, so we will also cover the role of epigenetics in IPF in the next sections.

4.1.1 Genetic Mutations in Telomeres

Telomeres are a TTAGGG repeat sequence at the end of human chromosomes, whose function is to stabilize the chromosome, prevent fusion of chromosome ends, protect structure of chromosomes, and determine cell life span, but telomerase activity in adult human cells is extremely low (Lister-Shimauchi et al., 2022).

Telomeres are protected by the shelterin complex, which consists of the protein components TRF1, TRF2, RAP1, TIN2, TPP1, and POT1. The ends of telomeres are bonded with telomerase, which is comprised of the catalytic subunit of TERT, the telomerase RNA component of TERC, and accessory proteins. The telomerase exerts its effects by adding telomeric sequences to the telomeric ends (Zhang et al., 2021). Telomere shortening is one of the important phenotypes of IPF. Telomere length of half of IPF patients rank in the lowest 1% of their age group (Stanley et al., 2016b). And telomeres are shorter in IPF patients than in other interstitial pneumonia (Snetselaar et al., 2015). However, it is questionable whether the cause of telomere shortening in IPF patients necessarily stems from telomere gene mutations. In patients with sporadic and familial pulmonary fibrosis, telomere shortening is present regardless of the presence of known telomere related gene mutations (Snetselaar et al., 2015). A possible reason for this is the presence of other unknown genetic mutations that can shorten telomere length.

The causal relationship between mutation-mediated telomere shortening and the development of IPF is inconclusive because not all individuals carrying a known telomere defective gene develop telomere shortening and IPF (Hoffman et al., 2018). Firstly, telomere gene defects in different cells may lead to different outcomes. systematically TERT knock-out does not induce spontaneous pulmonary fibrosis in mice, but can increase susceptibility to bleomycin (Degryse et al., 2012), while mesenchymal-specific TERT knock-out can alleviate bleomycin-induced pulmonary fibrosis (Liu et al., 2015); and AEC II-specific TERT defect reduces AEC II proliferation and induces AEC II cell senescence (Liu T. et al., 2019). Secondly, telomere gene defect-mediated DNA damage may be slow, which requires multiple generations of mice to become apparent (Blasco et al., 1997). Thirdly, due to the heterogeneity of the clinical manifestations, even the same genetic mutations may have different phenotypes, leading to the appearance of extra-pulmonary pathological features (rather than interstitial lung disease) (Borie et al., 2016). Fourthly, telomere length may differ in peripheral blood leukocytes and lung tissue (Snetselaar et al., 2017). Therefore, before telomere length measurements can be applied to clinical decisions, there is a need to clarify the effect of telomere length on different cells, the optimal sample type, and the corresponding intrapulmonary/extrapulmonary clinical manifestations.

The mechanism by which telomerase gene mutations lead to IPF is unclear. Available data suggest that cellular senescence or death of alveolar stem cells induced by telomere dysfunction is implicated in pulmonary fibrosis (Zhang et al., 2021). Knockdown of telomerase related genes leads to lung stem cell regenerative capacity and the senescence/death of AEC II (Jackson et al., 2011; Alder et al., 2015; Snetselaar et al., 2017; Wang et al., 2020). In addition, knockdown of telomerase related genes leads to elevated SASP (including TGF-β) levels and lung inflammation mediated by intrinsic immune cell infiltration (Chen et al., 2015; Povedano et al., 2015; Naikawadi et al., 2016; Liu et al., 2018).

Telomere length and specific telomere gene defects are instructive for susceptibility, diagnosis, progression, prognosis, and early warning of adverse effects in IPF. Shorter telomere length as an independent predictor is associated with worse survival and more pronounced imaging changes in IPF patients (Stuart et al., 2014; Newton et al., 2016; Ley et al., 2017). Pirfenidone treatment did not improve FVC and DLCO in IPF patients carrying TERT/TERC mutations (Justet et al., 2018). The relationship between TERT/TERC mutations and resistance to drug therapy needs to be further validated. Patients carrying telomere-related mutations may be at higher risk for complications after lung transplantation, such as death,

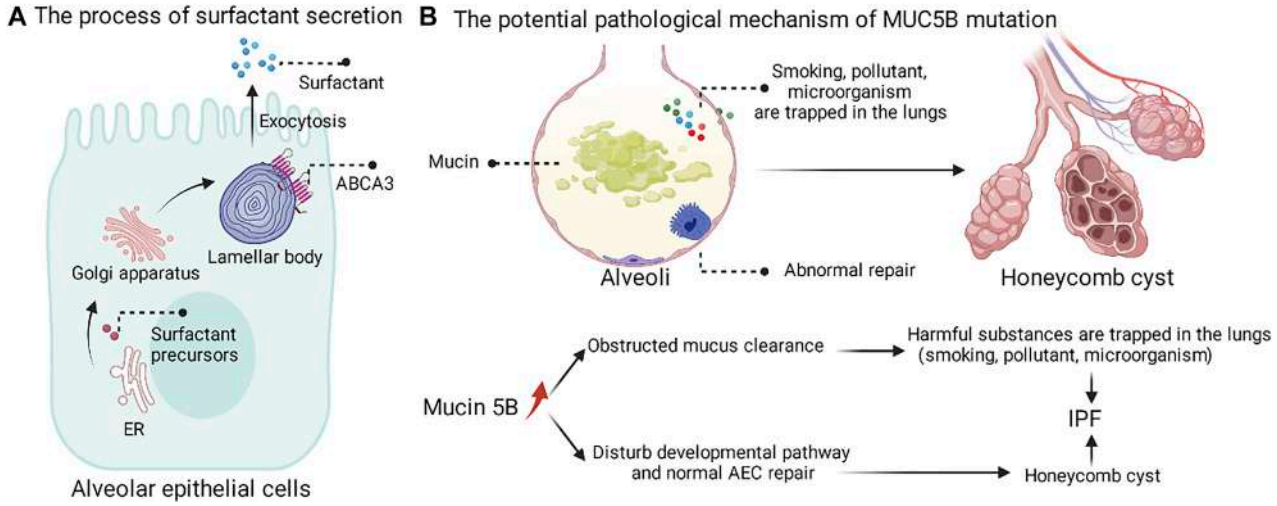

FIGURE 4 | The process of surfactant secretion and the potential pathological mechanism of MUC5B mutation. **(A)** The surfactant precursors are synthesized on the endoplasmic reticulum, followed by Golgi modifications, and finally secreted into the periphery by cytosolic exocytosis of the lamellar body. **(B)** MUC5B mutation may promote the retention of harmful substances in the lung and interfere with the normal repair of AEC.

chronic lung allograft dysfunction, renal disease, CMV complications (Popescu et al., 2019; Swaminathan et al., 2019), hematological complications (Silhan et al., 2014; Borie et al., 2015), anemia, leukopenia, recurrent lower respiratory tract infections (Tokman et al., 2015).

Drugs that target telomeres have also shown therapeutic potential. GRN510, a telomerase agonist, reduces inflammatory infiltration and collagen deposition in a mouse model of bleomycin-induced pulmonary fibrosis (Le Saux et al., 2013). PAP-associated domain-containing protein 5 (PAPD5) is involved in the degradation of TERC RNA. BCH001 (a PAPD5 inhibitor) and RG7834 (a PAPD5/7 inhibitor) rescue the TERC levels and telomerase activity in the cellular model of dyskeratosis congenital (Nagpal et al., 2020; Shukla et al., 2020). One study reported that hormones may regulate telomerase activity through an imperfect oestrogen response element within the TERT promoter (Bayne and Liu, 2005), which has inspired a series of subsequent clinical trials of hormones for IPF. Danazol, a synthetic androgen, was shown to increase telomere length and stabilize DLCO and FVC in a small-scale clinical trial (Townsley et al., 2016a; Townsley et al., 2016b). Danazol is currently in phase II clinical trials for plumonary fibrosis (NCT04638517). However, hepatotoxicity and worsening pulmonary fibrosis associated with long-term use of Danazol has been reported after danazol initiation and withdrawal (Chambers et al., 2020). Therefore, lower doses of danazol (200 mg) are being used in clinical trials to reduce hepatotoxicity. Nandrolone Decanoate, an anabolic androgenic steroid, is undergoing phase I/II clinical trials (NCT02055456). Telomerase reactivation therapy still has a long way to go before it becomes a therapeutic option, especially in terms of the serious adverse effects associated with hepatotoxicity. Whether peripheral blood telomere length can be used as a substitute outcome for pulmonary telomere length and how effective is telomerase reactivation therapy for IPF are still awaiting the results of current clinical trials.

In summary, details remain unclear in the complex field of the role telomeres play in the pathogenesis of ILDs and no clinical trials employing gene therapy have been initiated in patients with telomeropathies. Hopefully, adeno-associated virus-9 (AAV9)-mediated gene therapy has been reported to be successful in mouse models (Bär et al., 2016), and perhaps a telomerase-directed therapeutic strategy may be used for the treatment of IPF in the future.

4.1.2 Genetic Mutations in Alveolar Surfactant/Mucin

Phospholipids and four surfactant proteins (SFTPA, B, C, D) can be secreted by AEC II. The process of surfactant secretion is shown (**Figure 4A**). ATP-binding cassette subfamily A member 3 (ABCA3) is essential for the intracellular synthesis of alveolar surface-active substances (Mulugeta et al., 2015). Among four surfactant, genetic mutations of SFTPA (Wang Y. et al., 2009; Guenther et al., 2019), SFTPC (Venosa et al., 2017) and ABCA3(Zhou et al., 2017; Manali et al., 2019) have been identified in IPF patients. Although SFTPA is not directly involved in the formation of alveolar surface-active tension, it plays an important role in intrinsic lung immunity. Mouse models with SFTPA mutations exhibit intracellular retention of SFTPA and enhanced ER stress (Wang Y. et al., 2009; Maitra et al., 2010). Molecular signatures of UPR signaling and apoptotic activation associated with SFTPC have been reported in patients with IPF (Korfei et al., 2008; Lawson et al., 2008). Patients with pure mutations in ABCA3 and ABCA3-deficient mice exhibit a complete lack of alveolar surface-active substance, deformation of lamellar bodies, and death from respiratory distress shortly after birth (Shulenin et al., 2004; Cheong et al., 2007; Fitzgerald et al., 2007; Wambach et al., 2014). In lung disease, there are also reports

of ABCA3 heterozygous mutations that appear to interact with SFTPC mutations (Bullard and Nogee, 2007; Crossno et al., 2010). ABCA3 mutations may induce ER stress and proteostasis failure through misfolded alveolar surface-active substances (Cheong et al., 2006; Matsumura et al., 2006; Weichert et al., 2011).

The MUC5B promoter variant, rs35705950, as the strongest genetic risk factor and common variation, was found in up to 30% of IPF (Evans et al., 2016). The rs35705950 mutation leads to increased Muc5B expression, but a causal relationship between mucus and progression of pulmonary fibrosis disease has not been established. Christopher M. Evans proposed two hypotheses for the pathogenic mechanism of MUC5B mutations (**Figure 4B**) (Evans et al., 2016). The first possible mechanism is that overexpression of Muc5B at the bronchoalveolar junction impairs mucociliary clearance, which in turn promotes prolonged retention of harmful particles (e.g., air pollutants, cigarette smoke, microorganisms, etc.) in the lung and induces fibrosis in the lung tissue. The second possible mechanism is that overexpression of Muc5B may lead to fibrosis and cellulitic cyst formation by interfering with the normal developmental pathway and alveolar epithelial repair. In summary, the overexpression of MUC5B at the bronchoalveolar junction in IPF patients led us to recognize that the peripheral airway also seems to influence interstitial fibrosis.

4.1.3 Genetic Mutations in Inflammatory Regulators

Genetic mutations in cytokines have also been reported in patients with IPF. IL1RN gene polymorphisms may be associated with susceptibility to fibrosing alveolitis, a disease characterized by interstitial lung fibrosis (Whyte et al., 2000). Mesenchymal stem cells (MSC) can exert anti-inflammatory and anti-fibrotic effects via IL-1RN (Ortiz et al., 2007). Association between genetic polymorphisms of IL-4 and IPF has been reported (Riha et al., 2004). IL-4 may be pro-fibrotic by promoting type 2 immunity. IL-8 gene diversity is associated with lung alveolitis and lung function decline (Ziegenhagen et al., 1998). IL-8 increases the fibrogenicity of mesenchymal progenitor cells and is involved in the proliferation, activation, and recruitment of mesenchymal cells (Yang et al., 2018). TLR3 (Toll-like receptor 3) mutation rs3775291 increase the risk for IPF patients and also reduces forced volume capacity (FVC) (Evans et al., 2016). Defectiveness of the gene TLR3 L412F causes aberrant inflammation and fibroblasts proliferation in IPF, which may be related to dysregulation of fibroblast proliferation mediated by a sluggish IFN-β response (O'Dwyer et al., 2013). The different mutations of TOOLIP (TOLL interacting protein) may be associated with different IPF susceptibility. The rs5743890 was associated with lower IPF susceptibility, while the rs5743894 was associated with higher IPF susceptibility (Oldham et al., 2015). In addition, rs5743890 was associated with increased morbidity and mortality in IPF (Noth et al., 2013). rs3750920 polymorphism is associated with the efficacy of N-Acetyl-L-cysteine (NAC) (Oldham et al., 2015). It is important to note that different studies with different populations and specimens may reach different conclusions. In a study conducted in China, except for the polymorphisms of HLA haplotype, none of the other cytokines showed an association for IPF susceptibility (Zhang et al., 2015). Therefore, the study of genetic polymorphisms of cytokines should pay more attention to the reproducibility of experimental results.

In recent years, the launch of pirfenidone and nintedanib has witnessed breakthroughs in the signaling pathways of cytokines and growth factors in the treatment of IPF, however, studies focusing on cytokine and immune-related gene polymorphisms need to be further developed.

4.1.4 Other Potential Genetic Mutations Associated With IPF

In recent years, with the availability of next-generation sequencing technologies, more and more novel mutations have been identified in IPF patients. The mutations of the gene DSP (Mathai et al., 2016; Wang et al., 2018) and DPP9 (associated with cell adhesion) (Fingerlin et al., 2013; Zhou and Wang, 2016), AKAP13 166] and FAM13A (associated with RhoA/ROCK signaling pathway) (Hirano et al., 2017; van Moorsel, 2018), and SPPL2C (associated with intramembrane proteases) (Wu et al., 2016; Lorenzo-Salazar et al., 2019) were reported to be a risk of IPF susceptivity, progression and prognosis. However, the pathogenesis of these mutations remains unclear, and current studies are limited to correlation analysis (**Table 2**).

4.1.5 DNA Methylation

DNA methylation is a chemical modification process in which a specific base on the DNA sequence acquires a methyl group by covalent bonding with S-adenosyl methionine (SAM) as the methyl donor. DNA methylation is catalyzed by DNA methyltransferase (DNMT). In humans, DNA methylation occurs mainly at the fifth carbon atom of cytosine in CpG dinucleotides. It is generally believed that DNA methylation can inhibit the transcription and expression of the gene.

Global methylation and methylation of specific gene have been shown to be involved in the pathogenesis of IPF. Global DNA methylation analysis based on whole lung tissue showed that DNMT expression was increased in IPF, and DNA methylation of 16 genes (including CLDN5, ZNF467, TP53INP1 and DDAH1) was associated with decreased mRNA expression (Sanders et al., 2012). In order to clarify the contribution of specific cell types to IPF, single-cell-based or cell-type-specific sequencing is necessary. The global DNA methylation analysis based on fibroblasts showed that cells derived from IPF patients differ in the methylation of multiple CpG sites (including CDKN2B, CARD10, and MGMT) (Huang et al., 2014). Hypermethylation of specific genes not only can suppress the expression of some genes [Thy-1, COX-2, PTGER2, $p14^{ARF}$, IP-10 (Zhou et al., 2020), SFRP1/4, CDKN2B (Xue et al., 2021)], but also can promote the expression of some genes [α-SMA, MeCP2, KCNMB1 (Xue et al., 2021)], which eventually leads to the development of IPF (**Figure 5A**). Decitabine, a DNMT inhibitor, reduced fibrotic gene and DNMT-1 expression, enhanced miR-17–92 cluster expression (Dakhlallah et al., 2013). PGE2 has the capacity to limit fibrosis through its inhibition of numerous functions of these fibroblasts, and

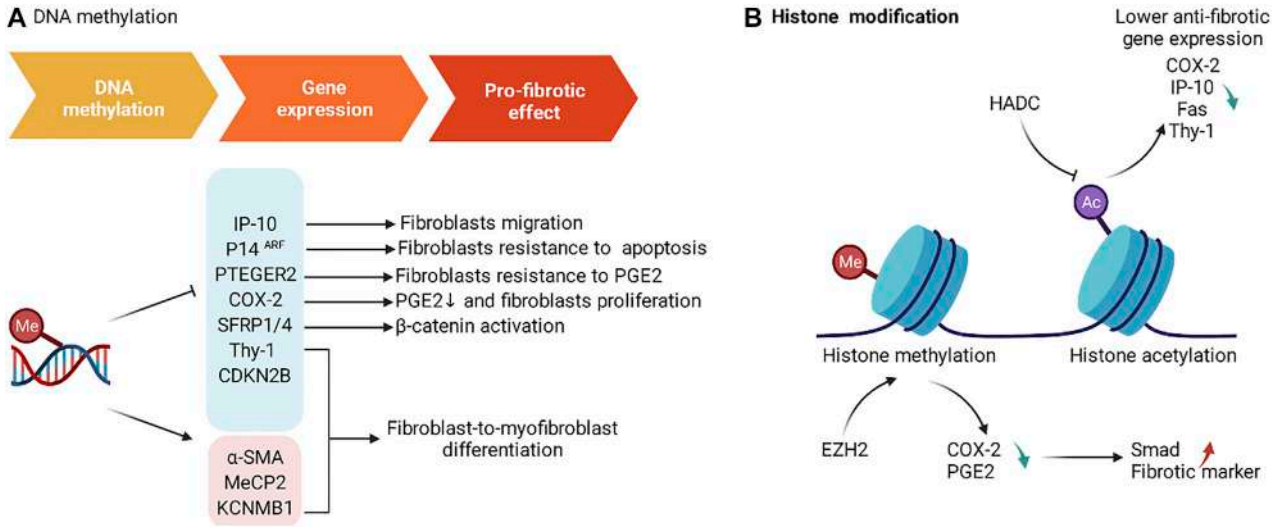

FIGURE 5 | The roles of DNA methylation and histone modification in IPF. **(A)** DNA methylation affects the expression of gene associated with fibrosis and produces a pro-fibrotic effect. **(B)** EZH2 and HADC induce changes in gene expression associated with fibrosis by affecting histone modifications.

TABLE 3 | Drugs targeting histone modification in IPF.

Drugs	Targets	Cell or animal model	Mechanism of action	Ref
3-DZNeP	EZH2/G9a inhibitor	BLM mice and LL29 cell	Reduce p-Smad2/3 nuclear translocations *in vitro* and downregulate α-SMA, COL1A1 and COL3A1 expression *in vivo*. Restore COX-2 expression and PGE2 production	Coward et al. (2014); Xiao et al. (2016)
A6	p300i	BLM mice; lung fibroblast cells	Decrease histone acetylation and pro-fibrotic gene expression *in vitro*, and reduce collagen deposition *in vivo*	Hwang et al. (2020)
Ac-SDKP	α-TAT1	MRC5/A549 cell	Promote apoptosis	Shifeng et al. (2019)
Romidepsin	HDACi	BLM mice	Inhibit LOX expression	Conforti et al. (2017)
LBH589	pan-HDACi	Primary IPF	Reduce expression of genes associated with ECM synthesis, proliferation and cell survival and suppress HDAC7 level	Korfei et al. (2015)
CG-745	HDACi	BLM/PHMG mice	Inhibit collagen production, inflammatory cell accumulation, and cytokines release	Kim et al. (2019)
Entinostat and vorinostat	HDACi	HFL-1 cell	Upregulate XPLN mRNA expression and reverse TGF-β-induced SPARC expression	Kamio et al. (2017)
TSA	pan-HDACi	NHLF cell	Reduce p-Akt level to inhibit TGF-β-mediated α-SMA expression	Guo et al. (2009)
SAHA	pan-HDACi	HLF cell	Promote the differentiation of fibroblasts into myofibroblasts and collagen deposition	Z Wang et al. (2009)
SpA	HDACi	Primary IPF	Inhibit the proliferation of IPF fibroblasts by increasing p21 expression	Davies et al. (2012)
JQ1	Bromodomain protein inhibitor	IPF fibroblasts	Bromodomain protein is the "Reader" of acetylated lysine in histone, and it is the only protein domain that can recognize and bind acetylated lysine in histone	Filippakopoulos et al. (2010)

Decitabine restored PGE2 responsiveness in fibrotic fibroblasts (Huang et al., 2010).

4.1.6 Histone Modification

Nucleosome, the basic unit of chromatin, is comprised of 146–147 base pairs of DNA and a histone octamer with one H2A-H2B tetramer and two H3-H4 dimers (Jenuwein and Allis, 2001).

The N- and C-terminal tails of H3 and H4 are the most frequently modified regions. The types of modifications include methylation, acetylation, phosphorylation, SUMOylation, and ubiquitination. The histone mark is named according to the histone, the modification site of these tails, and the type of modification. Histone modifications can change the electronic charge and structure of DNA-bound histone tails, thus altering chromatin state and gene expression (Kouzarides, 2007). The effect of histone acetylation and methylation in IPF has been extensively studied, and a large number of agents have been designed and validated in preclinical studies (**Figure 5B**).

Histone methylation is catalyzed by the histone methyltransferase (HMT), which uses S-adenosyl methionine (SAM) as the substrate to transfer methyl groups onto the

lysine residues of histones. Methylation of H3K4, H3K36, and H3K79 is thought to be associated with gene activation, while H3K9, H3K27, and H4K20 are thought to be associated with gene inactivation (Black et al., 2012). Inhibition of EZH2, an H3K27me-specific methyltransferase, by 3-DZNeP and BIX01294 increased the expression of COX-2 and PGE2, and suppressed the expression of Smad signaling and fibrosis markers (Coward et al., 2014; Xiao et al., 2016).

Histone acetyltransferases (HATs) promote histone acetylation, while histone deacetylases (HDACs) reduce histone acetylation. Histone acetylation is thought to be associated with gene activation (Clayton et al., 1993). In addition, sirtuins (SIRTs) are a family of deacetylases. SIRT1 inhibits the expression of aging-related secretions by deacetylating the SASP gene (Hayakawa et al., 2015). Inhibition of SIRT3 expression promotes fibrosis in human lung fibroblasts (Sosulski et al., 2017). Histone deacetylation is associated with the repression of anti-fibrotic genes, which includes COX-2 (Coward et al., 2009), IP-10 (Coward et al., 2010), Fas (Huang et al., 2013), Thy-1 (Sanders et al., 2011).

Inhibition of histone deacetylation may have anti-pulmonary fibrosis effects. HDACi consists of a zinc-binding-group, a hydrophobic group for protein recognition and interaction, and a linker connecting both of them (Wang and Dymock, 2009). A large number of agents targeting histone modification have already demonstrated antifibrotic effects in preclinical trials or in cell levels (**Table 3**).

4.2 Environment Factors

Environmental factors such as asbestos, metal dust, stone dust, wood dust, chemotherapy and allergen exposure are important causes of IPF (Taskar and Coultas, 2006). Epidemiological surveys have shown that 38% of IPF patients were exposed to high-risk environments; however, it is uncertain whether these factors directly lead to the occurrence of IPF (Behr et al., 2015; Wuyts et al., 2018).

Smoking is a relatively special environmental factor. Both direct smoking and indirect smoking are independent exposure factors, and the correlation between the direct/indirect smoking and IPF increase with the increase of smoking in dose (Bellou et al., 2021). Interestingly, according to the German S2K guidelines, although 60%–70% of patients had a history of smoking, less than 7% of patients were active smokers when they developed IPF symptoms. In other words, most patients developed the disease several years after quitting smoking; the average time interval was 21 years (Behr et al., 2021). This indicates that across such a long period of time, the injury caused by smoking may continue to progress or transform into IPF.

4.3 Body Aging and Cell Senescence

According to epidemiological surveys, advanced age is one of the most relevant demographic factors for IPF (Sgalla et al., 2018). The incidence of IPF in elderly men over 50 years of age is significantly higher than that in younger men (Raghu et al., 2018). However, the mechanism of how senescence leads to IPF is still unclear. In a mouse model of fibrosis, researchers observed a series of signs of cellular senescence, including elevated senescence-associated β-galactosidase (SA-β-Gal) in lysosomes and elevated reactive oxygen species (ROS) in mitochondria, an increase in cell cycle arrest proteins such as P53/P21/P16, an increase in the apoptosis-related protein BCL-2/Bax ratio, and an increase in DNA damage in the nucleus (Blokland et al., 2020). In addition, a variety of senescence-associated secretory phenotypes (SASPs) have been observed in animal models of pulmonary fibrosis, and these SASP components are known to include powerful profibrotic molecules such as TGF-β (Cho and Stout-Delgado, 2020; Rana et al., 2020). Similarly, studies have found that the lung fibroblasts of elderly IPF patients exhibit the above characteristics (Álvarez et al., 2017). Therefore, although there is a lack of human research evidence that senescence directly causes IPF, epidemiological data from both animal experiments and cell experiments suggest that IPF is a senescence-related disease.

The main manifestations of lung senescence are decreased lung tissue elasticity, decreased effective lung volume, the thickening of intercellular substances, and decreased lung function. Cell senescence mainly manifests as cell morphology and structure disorder, cell dysfunction, and cell growth/proliferation arrest (Rana et al., 2020).

The mechanism of pulmonary fibrosis caused by aging is not very clear, but there are quite a few theories to explain the mechanism of senescence in IPF. Cell senescence can disrupt of a variety of processes and generate imbalances (for example, shortened telomeres and DNA damage, oncogene activation, redox imbalances, mitochondrial dysfunction, lysosomal and proteasome dysfunction, ER stress), resulting in increased SASP, myofibroblast resistance to apoptosis, and stem cell depletion, ultimately manifesting as abnormal repair at the lung injury site (e.g., failure of the alveolar epithelium to regenerate properly and ECM deposition) (Sgalla et al., 2018; Glass et al., 2020; Liu and Liu, 2020; Merkt et al., 2020). Aging has been reported to reprogram the hematopoietic-vascular niche to impede regeneration and promote fibrosis (Chen et al., 2021).

Because senescent cells that resist apoptosis can continuously produce profibrotic cytokines, which in turn aggravate pulmonary fibrosis, the induction of apoptosis in senescent cells (e.g., dasatinib/quercetin, ABT-263, and NOX4 inhibitors) or selective antagonism of profibrotic senescence-related factors (e.g., IL-6, TGF-β or leukotrienes) may help to alleviate IPF (Merkt et al., 2020). Many antiaging drugs have passed preclinical animal studies and entered clinical trials. For example, dasatinib (D) and quercetin (Q) can promote the apoptosis of senescent cells. The DQ combination regimen has been investigated in a phase I clinical trial (NCT02874989). A total of 14 patients were enrolled in the trial; except for one patient who had severe adverse reactions (bacterial pneumonia), the remaining patients tolerated the treatment well, and improvements in parameters such as 6-min walking distance, 4-m gait speed, and chair stand time were reported (Schafer et al., 2017; Justice et al., 2019). Interestingly, Nifurtimox (NIF), originally used to treat diarrhea, has been reported to improve pulmonary fibrosis by blocking the production of myofibroblasts (Gan et al., 2022).

TABLE 4 | Summary of emerging therapeutic targets for IPF in drug discovery.

Therapeutic targets	Mechanism	Ref
TGF-β	TGF-β is a powerful pro-fibrotic mediator	Ong et al. (2021)
αvβ6 Integrin	Integrin αvβ6 binds to an arginine-glycine-aspartic (RGD) sequence on LAP to activate latent form TGF-β1	Munger et al. (1999)
CTGF/CNN2	As a co-regulator of TGF-β in the pulmonary fibrosis microenvironment, CTGF can cooperate with TGF-β to participate in abnormal tissue repair processes including ECM production, fibroblast activation and differentiation	Lipson et al. (2012)
Galectin-3	Modulate macrophage phenotype/Gal-3 expression and fibroblast activation, reduce the effects of key profibrotic growth factors that act on myofibroblasts, and inhibiting EMT.	Humphries et al. (2021)
Leukotrienes	Leukotrienes have profibrotic effects by inducing fibroblast migration, proliferation, and matrix protein synthesis	Antoniou et al. (2007)
ATX-LPA-LPAR	The binding of LPA-to-LPAR can promote apoptosis of epithelial cells, regulation of endothelial permeability, activation of αvβ6 integrin-mediated TGF-β signaling, secretion of IL-8, recruitment and survival of fibroblasts. ATX is the key enzyme for LPA synthesis	Tager et al. (2008); Ninou et al. (2018); Suryadevara et al. (2020)
SPHK1-S1P-S1PR	The binding of S1P-to-S1PR can lead to mitochondrial reactive oxygen species (mtROS) and promote YAP1 to enter cell nuclei, affecting the differentiation of myofibroblasts and matrix remodeling. SPHK1 is the key enzyme for S1P synthesis	Huang et al. (2020)
PTX-2/SAP	As a ligand for the Fcγ receptor, PTX-2 downregulates monocyte and macrophage activity (especially M2)	Castaño et al. (2009)
JAK	JAK/STAT is a downstream pathway of IL-6, IL-11, IL-13, PDGF, TGF-β1 and FGF. The effect of JAK/STAT phosphorylation on cellular fibrotic processes includes proliferation, senescence, autophagy, endoplasmic reticulum stress, or epithelial/fibroblast to mesenchymal transition	Montero et al. (2021)
Src	Src is a group of nonreceptor tyrosine kinases, which participate in the TGF-β pathway by activating FAK.	Hu et al. (2014)
PI3K/Akt/mTOR	PI3K/Akt/mTOR plays a critical role in cell survival, growth, proliferation, protein synthesis, and EMT. *In vitro*, mTOR inhibitors can reduce TGF-β-induced fibroblast proliferation and type I collagen synthesis	Hennessy et al. (2005); Mercer et al. (2016); Lawrence and Nho, (2018)
Smo receptor	Smo is an important mediator of hedgehog signaling which is reactivated in adulthood within IPF	Effendi and Nagano, (2021)
Nitric oxide synthase	Activated macrophages, contributing to the cellular injury mediated by ROS, produce both nitric oxide (NO) and peroxynitrite	Giri, (2003)
GPR40/GPR84	GPR40 and GPR84 are G protein coupled receptors with free fatty acid ligands and are associated with metabolic and inflammatory disorders. PR40 agonist and GPR84 antagonists act on cells that involved in fibrotic pathways: macrophages, fibroblasts, and epithelial cells, and finally reduce inflammation	Gagnon et al. (2018)
LOX and LOXL	Lysyl oxidase (LOX) and LOX-like (LOXL) are enzymes involved in collagen cross-linking	Chen et al. (2019)

5 RECENT PROGRESS IN THERAPEUTIC TARGETS AND NEW DRUG DEVELOPMENT FOR IPF

Pirfenidone and nintedanib are two drugs approved by the FDA for the treatment of IPF. They can delay lung function deterioration and prolong patient survival in IPF patients. Although the launch of Pirfenidone and nintedanib has demonstrated the feasibility of drug treatment for IPF, they do not reverse pulmonary fibrosis and their efficacy in patients with end-stage IPF is not fully clear. Therefore, there is a need to develop additional anti-pulmonary fibrosis drugs. The development of drugs for some specific targets is currently underway, which will be described in the following sections. In addition, drug combinations for antifibrotic therapy are worth investigating. Immune checkpoint inhibitors (ICIs), either as single agents or in combination regimens, have shown great success in clinical treatment (Wu et al., 2022). A case reports indicates that the addition of nintedanib to ICIs therapy might prevent drug-induced pneumonitis or acute exacerbation of IPF (Yamakawa et al., 2019).

5.1 Summary of Emerging Therapeutic Targets for IPF in Drug Discovery

In this section we briefly describe the current therapeutic targets in clinical trials and the latest clinical trial developments. We summarize the emerging therapeutic targets and representative mechanisms in the development of IPF (**Table 4**).

TGF-β plays an important role in the pathogenesis of IPF by stimulating the activation and proliferation of fibroblasts (Ong et al., 2021). Both anti-TGF-β mAb and anti-TGF-β mRNA nucleic acid have entered clinical trials (NCT00125385 and NCT03727802, respectively). Monoclonal antibodies against integrins that activate latent TGF-β have also entered phase II clinical trials (NCT04072315 and NCT04396756). Tyrosine kinase inhibitors targeting the TGF-β receptor have also entered phase II clinical trials (NCT03832946). Other growth factors (PDGF, VEGF, FGF, EGF, CTGF) are also involved in the process of pulmonary fibrosis. The mAb targeting CTGF, Pamrevlumab, demonstrated clinical benefit in phase II clinical trials and is now in phase III clinical trials (NCT03955146 and NCT04419558).

Chemokines and interleukins are also involved in recruitment of pro-fibrotic cells and formation of pro-fibrotic microenvironment, as we discussed in our previous paper (Ma et al., 2022). However, CCL2, a key chemokine for monocyte/macrophage migration and infiltration, failed to demonstrate clinical benefit in a phase II clinical trial (NCT00786201). Anti-IL-13 therapy didn't contribute to lung function in patients with IPF (NCT02345070 and NCT01872689) or was terminated due to Lack of evidence of efficacy (NCT01266135 and NCT01266135).

Leukotrienes have profibrotic effects by inducing fibroblast migration, proliferation, and matrix protein synthesis (Antoniou et al., 2007). MN-001/Tipelukast, leukotriene receptor antagonist, is undergoing phase II clinical trial (NCT02503657).

As lipid proinflammatory mediators, The ATX-LPA-LPAR axis and SPHK1-S1P-S1PR axis have also been reported as targets of IPF. LPA and S1P have been demonstrated to promote the development of fibrosis (Tager et al., 2008; Ninou et al., 2018; Huang et al., 2020; Suryadevara et al., 2020). BMS-986278 (LPA1R antagonist) has entered phase II clinical trials (NCT04308681).

PTX-2, a ligand for the Fcγ receptor, can downregulate monocyte and macrophage activity (especially M2) (Castaño et al., 2009). PRM-151, a recombinant human pentraxin-2, has entered phase II clinical trials (NCT04594707 and NCT04552899).

Multiple cellular signaling pathways play a crucial role in the development of IPF. JAK/STAT signaling (Montero et al., 2021), receptor-type tyrosine kinase/non-receptor-type tyrosine kinase signaling (e.g., Src) (Hu et al., 2014), PI3K/Akt/mTOR signaling (Hennessy et al., 2005; Mercer et al., 2016; Lawrence and Nho, 2018), and Hedgehog signaling (Effendi and Nagano, 2021) comprise complex fibrosis regulatory signaling pathway. In our previous reviews, we have discussed the aforementioned signaling pathways (Ma et al., 2022).

There are also drugs in clinical trials targeting oxidative stress (Giri, 2003), GPR40/GPR84 (Gagnon et al., 2018), and collagen cross-linking enzymes (Chen et al., 2019).

5.2 Summary of Ongoing Clinical Trials for IPF

We searched clinical trials in recent years and summarized the anti-IPF drugs undergoing clinical trials and their mechanisms of action (**Table 5**). We also update the latest clinical trial progress for these drugs.

TABLE 5 | Ongoing clinical trials about some investigational compounds for IPF.

Targets	Drugs	Clinical trial information
LPC-ATX-LPA	BMS-986278 (LPA1R antagonist)	Phase 2 (recruiting, NCT04308681)
PTX-2/SAP	PRM-151(Intravenous recombinant human pentraxin-2)	Phase 3 (recruiting, NCT04594707, NCT04552899)
CTGF/CNN2	FG-3019/Pamrevlumab (CTGF mAb)	Phase 3 (recruiting, NCT04419558, NCT03955146)
Galectin 3	TD139 (small-molecule antagonist of Galectin-3)	Phase 2 (recruiting, NCT03832946)
Oxidative stress	Niacin (nicotinic acid)	Phase 2 (recruiting, NCT0386592)
	Setanaxib/GKT137831(NOS1/4 inhibitor)	Phase 2 (recruiting, NCT03865927)
JNK	Jaktinib Dihydrochloride Monohydrate (JNK1/2 inhibitor)	Phase 2 (recruiting, NCT04312594)
	CC-90001 (JNK1/2 inhibitor)	Phase 2 (active, not recruiting, NCT03142191)
Src	Saracatinib (Src kinase inhibitor)	Phase 1/2 (recruiting, NCT04598919)
Hedgehog pathways	taladegib/ENV-101(Smo receptor inhibitor)	Phase 2 (not yet recruiting, NCT04968574)
Leukotrienes	MN-001/Tipelukast (leukotriene receptor antagonist)	Phase 2(Active, not recruiting, NCT02503657)
LOXL2	EGCG (irreversible inhibitor of both LOXL2 and TGF-β receptors 1 and 2 kinase)	Early Phase 1 (recruiting, NCT03928847)
IRE1	ORIN1001(IRE1 inhibitor)	Phase 1 (recruiting, NCT04643769)
PDE4b	BI 1015550(PDE4b inhibitor)	Phase 2 (active, not recruiting, NCT04419506)
NDMA	NP-120/Ifenprodil (N-methyl-d-aspartate (NDMA) receptor glutamate receptor antagonist)	Phase 2 (recruiting, NCT04318704)
B cell	Ianalumab/VAY736(B-cell activating factor receptor mAb)	Phase 2 (active, not recruiting, NCT03287414)
	Rituximab (CD20 chimeric mAb)	Phase 2 (active, not recruiting, NCT01969409); Phase 2 (recruiting, NCT03584802); Phase 2 (recruiting, NCT03286556); Phase2 (recruiting, NCT03500731)
Traditional medicine	Jin-shui Huan-xian granule	Not Applicable (recruiting, NCT04187690)
	Fuzheng Huayu tablet	Phase 2 (recruiting, NCT04279197)

6 CONCLUSION AND OUTLOOK

The research on the pathogenesis of IPF has made considerable progress. After years of basic and clinical studies, the pathogenesis of IPF has changed from simple inflammation to abnormal epithelial-mesenchymal crosstalk and other common pathogenic mechanisms. Based on this pathological mechanism, many studies have systematically studied the key roles of fibrocytes/(myo)fibroblasts, epithelial/endothelial cells, and ECM in the pathogenesis of IPF. With the progresses in understanding the key role of genetics and epigenetics in IPF, researchers have found that an increasing number of genetic loci and their apparent modifications are related to the maintenance of lung homeostasis although the effects of gene mutations and epigenetics on IPF still need further study.

The advent of pirfenidone and nintedanib is undoubtedly a sensational event in the field of IPF treatment, but they also have some major limitations, such as insufficient curative effect and poor pharmacokinetic properties. In addition, combination therapy based on them needs to be further studied. The number of clinical trials for therapies targeting cytokines, growth factors, and their signaling pathways is increasing rapidly. Furthermore, drugs targeting MMPs, telomerase activity, and epigenetic modifications need to be further developed.

Although IPF is idiopathic by definition, this deadly disease may become less mysterious with the progress in the understanding of the pathogenesis of IPF. The number of observed therapeutic targets is increasing, as well as the number of new drugs entering clinical trials. For patients with IPF, these advances described in this review show that slowing the IPF progression, prolonging their lives, and improving their quality of life might be possible in the future.

AUTHOR CONTRIBUTIONS

HM contributed to the conception of this review and preparation of the manuscript, tables, and figures; XW collected information about the clinical trials; YL revised the tables and figures; YX contributed to the conception, supervision, and revision of the manuscript. All authors have approved the final article and are included in the disclosure.

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The associations of radiological features of high-resolution computed tomography with the outcomes of transbronchial cryobiopsy in interstitial lung diseases: A cohort study

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Background: Transbronchial cryobiopsy (TBCB) is a critical procedure in the diagnosis of interstitial lung diseases (ILD). The associations between high-resolution computed tomography (HRCT) features and outcomes of TBCB were unknown.

Methods: This study was conducted as a single-center prospective cohort study between September 2018 and January 2020 (NCT04047667). HRCT was obtained before performing TBCB. The clinical and radiological characteristics, complications, pathological and multidisciplinary discussion (MDD) diagnoses were recorded. The relationships between HRCT features and outcomes of TBCB were analyzed.

Results: TBCB was performed on 216 ILD patients. The radiological features usually interstitial pneumonia (UIP) or probable UIP, indeterminate for UIP, ground-glass opacities (GGO) and cysts were found in 55 (25.5%), 38 (17.6%), 84 (38.9%) and 9 (4.2%) patients, respectively. And 118 (54.6%) patients had high HRCT score (involved lung proportion ≥50%) in the biopsied lobe. Multivariate analysis suggested radiological probable UIP pattern may be an independent risk factor for moderate bleeding (OR = 4.304; 95% CI: 1.383–13.393; $P = 0.012$), while GGO may be a protective factor from moderate bleeding (OR = 0.173, 95% CI: 0.043–0.687; $P = 0.013$). The pathological diagnostic yield in patients presenting cysts on HRCT was significantly lower than others (44.4 vs. 87.9%; $P = 0.009$). While performing TBCB in the lobe with high HRCT score increased pathological diagnostic yield (91.5 vs. 79.6%; $P = 0.022$). Neither pneumothorax nor MDD diagnostic yields were found to be associated with HRCT features.

Conclusions: HRCT features were associated with moderate bleeding and pathological diagnosis. Pre-TBCB assessments of HRCT pattern and scores were helpful for bronchoscopists to make a better patient selection and procedure planning.

KEYWORDS

cryobiopsy, interstitial lung diseases, HRCT, complications, diagnostic yields

Highlights

- HRCT features were associated with transbronchial cryobiopsy-related moderate bleeding and pathological diagnosis.
- Pre-procedure assessments of HRCT features were helpful for bronchoscopists to make a better patient selection and procedure planning.

Introduction

Transbronchial cryobiopsy (TBCB) is a critical procedure in the assessment of patients with suspected interstitial lung diseases (ILD) when an accurate diagnosis cannot be made solely based on clinical and radiological assessments. Recent reports have suggested that the diagnostic value of TBCB approaches that of surgical lung biopsy (SLB) (1–4). However, the complication rates (such as pneumothorax and significant bleeding) and diagnostic yields reported by different investigations varied. Pneumothorax rate varied from 1.9 to 19.2%, moderate to severe bleeding rate varied from 4.0 to 56.4%, histopathological diagnostic yield varied from 40.0 to 95.1% (5, 6). Several procedural factors have been thought to be associated with the safety profile and diagnostic efficacy of TBCB, such as the cryoprobe-pleura distance, cryoprobe type, guidance method, and the number of cryobiopsies (6–8). However, there are few studies investigating the associations between HRCT features and the outcomes of TBCB. Ravaglia's study found pneumothorax was much more frequent in patients with evaluated grading of the distribution of reticular abnormalities, traction bronchiectasis and honeycombing on HRCT, but no other correlation was found (6). In our preliminary studies, we found that the complication rates were significantly associated with high-resolution computed tomography (HRCT) pattern but not with cryoprobe type (9, 10). The risk of moderate-to-severe bleeding was obviously higher in patients with fibrotic HRCT pattern than those with non-fibrotic pattern.

In order to furtherly demonstrate the potential value of HRCT features on predicting the outcomes of TBCB, we analyze it based on a prospective cohort study in which cryoprobe placement was guided by three dimensional (3D) images acquired by cone beam computed tomography (CBCT) during TBCB. We present the following article in accordance with the STROBE reporting checklist.

Methods

Patients

This study was conducted as an updated single-center prospective cohort study concerning on the CBCT guided TBCB for ILD. The trial was conducted in accordance with the Declaration of Helsinki and the Harmonized Tripartite Guideline for Good Clinical Practice from the International Conference on Harmonization. This study was reviewed and approved by the Ethics Committee of China-Japan Friendship Hospital and was registered at clinicaltrial.gov (NCT04047667). Written informed consent was obtained from all patients enrolled. Participant registration was carried out between September 2018 to January 2020.

All patients diagnosed with ILD who met the following eligibility criteria were recommended to undergo TBCB under guidance by CBCT (Figure 1): aged $>$ 18 years old with evidence of diffuse parenchymal lung disease; a diagnosis of ILD that could not be established after integration of clinical data, laboratory tests, and HRCT features; forced vital capacity (FVC) $>$ 50%; and diffusing capacity of the lung for CO (DLCO) $>$ 35%. Patients who met any of the following criteria were excluded from this study: acute exacerbation in the previous 30 days; bleeding diathesis; anticoagulant therapy; current use of antiplatelet drugs; pulmonary hypertension; respiratory failure; liver or kidney dysfunction; cardiac insufficiency; or platelet count $< 50 \times 10^9$/L. Eventually, 216 patients were enrolled.

HRCT protocol and assessment

CT scans were obtained using a second-generation, dual-source CT system (SOMATOM Definition Flash, Siemens Healthineers, Forchheim, Germany) with the following parameters before biopsy: 120 kV reference tube voltage, 110 quality reference mAs tube current with automatic exposure

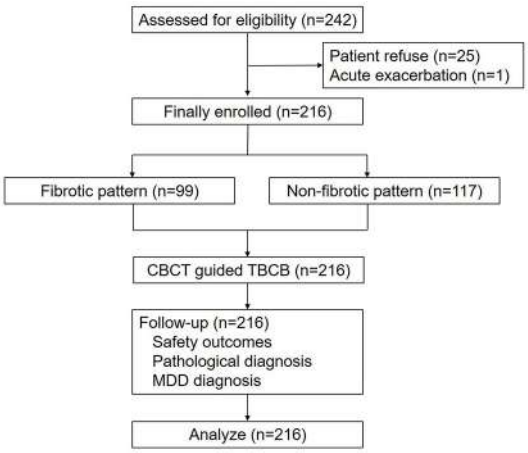

FIGURE 1
Flow diagram of the enrolment and follow-up processes for the patients who underwent transbronchial cryobiopsy (TBCB).

control (CarekV,CareDose4D), and 0.5 seconds scanning time, and 128×0.6-mm detector configuration, pitch 1.2. Images were reconstructed with slice thickness of 1mm, and reconstruction increment of 1.0 mm. Images were displayed using lung window [width: 1,600 Hounsfield units (HU); level: −600 HU] and mediastinal window settings (width: 350 HU; level: 50 HU). No contrast was administered. Two experienced physicians (G. Zhou and Y. Ren) independently reviewed each patient's HRCT features. When disagreements between them occurred, or their results did not accord with the HRCT reports from the department of radiology, they consulted the radiological expert (M. Liu) during multidisciplinary discussions (MDD).

Fibrotic pattern included the following HRCT features (11, 12): honeycombing or reticulation with traction bronchiectasis (UIP or probable UIP), reticulation predominant (indeterminate for UIP), and ground-glass opacities with traction bronchiectasis or honeycombing (others).

Non-fibrotic pattern included the following HRCT features: ground-glass opacities predominant without traction bronchiectasis or honeycombing, military nodules, consolidation, cystic manifestation, and others such as mosaic attenuation.

HRCT scores were assessed according to the protocol described by Kazerooni et al. (13). Both observers initially scored the limited CT images, with the three images taken at the level of the aortic arch, the carina, and 1 cm above the diaphragm and later scored the CT images of the entire lungs. Each lobe of the lung was scored on a scale of 0–5 depending on the percentage of each lobe involved: 0, no lung involved; 1, manifestations involving ≤5% of the lobe; 2, manifestations involving 5–25% of the lobe; 3, manifestations involving 25–49%; 4, manifestations involving 50–75% of the lobe; 5, manifestations involving >75% of the lobe. The scores for each lobe were averaged for both readers for data analysis. The score of the entire lungs were averaged for all five lobes. Scale more than 3 was defined as high HRCT score.

TBCB procedure

TBCB was conducted following the protocol of procedure as previously described (9, 10). Routine post-procedure CBCT or X-ray imaging was used to screen for acute pneumothorax. Bleeding severity was graded according to the following scale (14): no bleeding (traces of blood not requiring suctioning), mild bleeding (requiring suction to clear but no other endoscopic procedures), moderate bleeding (requiring endoscopic procedures such as bronchial occlusion-collapse and/or instillation of ice-cold saline), and severe bleeding (causing hemodynamic or respiratory instability, requiring tamponade or other surgical interventions, transfusions, or admission to the intensive care unit).

Follow-up

For each patient, electrocardiogram, blood pressure, and oxygen saturation were monitored for 24 h after bronchoscopy. Chest X-rays were performed for patients who exhibited discomfort or disease progression without any other reason.

Histopathological diagnosis was conducted by an experienced pathologist. Multidisciplinary discussions (MDD) based on clinical presentations, radiological findings, and TBCB histopathological features were conducted for each patient. All patients underwent a 30-day follow-up for intensive care unit admission, disease progression, and death after TBCB.

Endpoints and sample size

The primary endpoints were defined as the incidences of pneumothorax and moderate-severe bleeding. The second endpoints included diagnostic yields, specimen quality, cryoprobe re-position rates after CBCT guidance, and procedure duration. The estimated sample size was designed to have ∼80% power for detecting a 6% decrease in the pneumothorax rate (from 9 to 3%), or a 10% decrease in the moderate-severe bleeding rate (from 20 to 10%) after CBCT guidance.

Statistical analysis

All patients competed 30-day follow-up and were included in this analysis. Anthropometric and lung function data were expressed as mean ± SD. All remaining results were presented

as descriptive statistics, absolute numbers, and percentages. Continuous data were tested for normality using the Shapiro-Wilk test. Differences in complication rates were analyzed for statistical significance using the Fisher exact test. Multivariate logistic regression analyses were used to analyze the associations between safety outcomes and HRCT features, as well as between diagnostic yields and HRCT features. Statistical analyses were performed using STATA software (version 14, StataCorp, College Station, TX, USA), and a p value < 0.05 was considered statistically significant.

Results

Patients characteristics

Of 242 patients eligible for enrolment in the study, 26 were excluded due to patient refusal ($n = 25$) or acute exacerbation ($n = 1$) prior to the procedure. Consequently, 216 patients (male-to-female ratio 1.4, 126/90) were enrolled and underwent TBCB (Figure 1), with a mean age of 55.2 ± 11.8 years; FVC was 89.4 ± 21.2% and DLCO was 67.7 ± 18.5% (Table 1). Fibrotic pattern on HRCT were found in 99 (45.8%) patients: reticulation predominant in 38 (17.6%) patients; honeycombing or reticulation with traction bronchiectasis in 55 (25.5%) patients; GGO with traction bronchiectasis or honeycombing in 6 (2.8%) patients. Non-fibrotic pattern on HRCT were found in 117 (54.2%) patients: GGO predominant in 84 (38.9%) patients; military nodules in 11 (5.1%) patients; consolidation in 11 (5.1%) patients; cystic lesion in 9 (4.2%) patients and others in 2 (0.9%) patients. A total of 80 (37.0%) patients had HRCT scores of more than 3 for all lobes of the lung, while 118 (54.6%) patients had HRCT scores of more than 3 for the biopsied lobe. The risk of moderate bleeding (no severe bleeding occurred) and pneumothorax were 9.7% (21/216) and 2.8% (6/216), respectively. The pathological and MDD diagnostic yields were 86.1% (186/216) and 91.2% (197/216).

Multivariate analysis (Table 2) indicated that FVC % predictive less than 70%, number of specimen <3, and HRCT fibrotic pattern were associated with increased risk of moderate bleeding while environmental history was associated with decreased risk of moderate bleeding. Age more 65 year-old was associated with increased risk of pneumothorax. Gender of male was associated with lower MDD diagnostic yields while smoking status was associated with higher MDD diagnostic yields.

TABLE 1 Clinical characteristics in interstitial lung diseases (ILD) patients undergoing transbronchial cryobiopsy (TBCB).

Patients	***N* = 216**
Median age (SD), year-old	55.2 (11.8)
Male-to-female ratio	126/90 (1.4)
Smokers	101 (46.8%)
Environmental or occupational history	67 (31.0%)
Mean FVC (SD), %pred	89.4 (21.2)
Mean DLCO (SD), %pred	67.7 (18.5)
Fibrotic pattern	99 (45.8%)
UIP or Probable UIP pattern	55 (25.5%)
Indeterminate for UIP pattern	38 (17.6%)
Other fibrotic pattern	6 (2.8%)
Non-fibrotic pattern	117 (54.2%)
GGO	84 (38.9%)
Profuse micronodules	11 (5.1%)
Consolidation	11 (5.1%)
Cysts	9 (4.2%)
Others	2 (0.9%)
HRCT score	
≤3	136 (63.0%)
>3	80 (37.0%)
HRCT score of the target lobe	
≤3	98 (45.4%)
>3	118 (54.6%)
Cryoprobe size	
1.9 mm probe	52 (24.1%)
2.4 mm probe	164 (75.9%)
Biopsy site	
Single segment	79 (36.6%)
Multiple segments	137 (63.4%)
Number of specimen (SD)	3.5 (1.0)
Procedure duration (SD), min	37.4 (13.9)
Outcomes	
Moderate-to-severe bleeding	21 (9.7%)
Pneumothorax	6 (2.8%)
Pathological diagnosis	186 (86.1%)
MDD diagnosis	197 (91.2%)

SD, standard deviation; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; DLCO, diffusing capacity of the lung for CO; HRCT, high-resolution computed tomography; CBCT, cone beam computed tomography; RET, reticulation; TB, traction bronchiectasis; HC, honeycombing; GGO, ground-glass opacities.

HRCT features and safety profile

In general, the risk of moderate bleeding in patients with HRCT fibrotic patterns was significantly higher than those with HRCT non-fibrotic patterns (15.2 vs. 5.1%, $P = 0.013$; Table 3). The moderate bleeding rates in the subgroup of UIP or probable UIP pattern, indeterminate for UIP pattern and other fibrotic patterns were 14.5, 15.8, and 16.7% respectively. The moderate bleeding rate in the patients with GGO was 4.8%, which was significantly lower than others ($P = 0.05$). In two out of nine patients with cysts on HRCT

TABLE 2 Multivariate analysis for the associations between clinical characteristics and the outcomes of transbronchial cryobiopsy in ILD patients.

Characteristics	Moderate bleeding		Pneumothorax		Pathological diagnosis		MDD diagnosis	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Age > 65 year-old	1.102 (0.321, 3.790)	0.877	**6.973 (1.080, 45.029)**	**0.041**	0.780 (0.258, 2.361)	0.661	0.322 (0.064, 1.624)	0.170
Male	0.580 (0.107, 3.147)	0.527	0.356 (0.013, 10.077)	0.545	0	0.991	**0.041 (0.002, 0.792)**	**0.035**
Smoker	1.425 (0.253, 8.041)	0.688	3.368 (0.138, 82.333)	0.457	0	0.991	**30.788 (1.567, 604.767)**	**0.024**
Environmental or occupational history	**0.159 (0.031, 0.821)**	**0.028**	1.323 (0.173, 10.141)	0.788	0.654 (0.233, 1.835)	0.420	0.625 (0.191, 2.052)	0.439
FVC %pred <70%	**6.156 (1.407, 26.935)**	**0.016**	1.169 (0.086, 15.923)	0.907	0.342 (0.067, 1.749)	0.198	2.079 (0.565, 7.645)	0.271
DLCO %pred <50%	0.153 (0.018, 1.333)	0.089	2.314 (0.215, 24.754)	0.488	1.292 (0.285, 5.857)	0.740	1.605 (0.359, 7.184)	0.536
2.4-mm cryoprobe	0.631 (0.170, 2.335)	0.490	N/A		2.098 (0.616, 7.144)	0.236	1.097 (0.303, 3.970)	0.888
Multiple biopsy sites	0.679 (0.191, 2.410)	0.549	0.985 (0.124, 7.787)	0.988	0.896 (0.318, 2.526)	0.835	0.950 (0.306, 2.954)	0.930
Number of specimen <3	**2.681 (1.154, 6.232)**	**0.022**	0.697 (0.158, 3.073)	0.634	0.745 (0.360, 1.543)	0.429	0.652 (0.277, 1.535)	0.328
Fibrotic pattern	**8.660 (2.515, 29.821)**	**0.001**	0.790 (0.113, 5.540)	0.812	0.703 (0.263, 1.876)	0.481	0.769 (0.250, 2.364)	0.647

MDD, multidisciplinary discussion.
Bold values indicate significant p-value < 0.05.

occurred moderate bleeding (22.2%, $P = 0.214$). Multivariate analysis suggested that honeycombing or reticulation with bronchiectasis on HRCT may be an independent risk factor for moderate bleeding (OR = 4.304; 95% CI: 1.383–13.393; $P = 0.012$; Table 4), while GGO predominant on HRCT may be a protective factor from moderate bleeding (OR = 0.173, 95% CI: 0.043–0.687; $P = 0.013$; Table 4). No relationship between HRCT score and moderate bleeding was found.

The risk of pneumothorax had no significant differences between fibrotic and non-fibrotic patterns (3.0 vs. 2.6%). There was a trend for the increased incidence of pneumothorax in the patients who underwent TBCB in the lobe with high HRCT score, but without no significant statistical difference (4.2 vs. 1.0%, $P = 0.309$). The associations between HRCT features and pneumothorax were not found by multivariate analysis in this study (Tables 3, 4).

HRCT features and diagnostic yields

The pathological diagnostic yield in the patients presenting cysts on HRCT was significantly lower than others (44.4 vs. 87.9%, $P = 0.003$; Table 3). When TBCB was performed in the lobe with high HRCT score, the pathological diagnostic yield would significantly increase (91.5 vs. 79.6%, $P = 0.012$; Table 3). Multivariate analysis also demonstrated that cysts on HRCT predicted failure of pathological diagnosis (OR = 0.111, 95% CI: 0.022–0.573; $P = 0.009$; Table 4), while performing TBCB in the lobe with high HRCT score may improve pathological diagnostic yields (OR = 7.172, 95% CI: 1.335–38.532; $P = 0.022$; Table 4).

The associations between HRCT features and MDD diagnostic yields were not found in this study (Tables 3, 4).

Discussion

This is the first prospective study to investigate the association between HRCT features and the outcomes of TBCB in ILD patients. Our results offer new insights into the relationship between radiological manifestations and TBCB outcomes, it may be helpful for bronchoscopists to make a better patient selection in order to improve procedure safety and diagnostic yields. Prophylactical placement of bronchial blocker should be routinely used if high risk of significant bleeding is suspected and TBCB should be conducted in the lobe with high HRCT score.

Bleeding and pneumothorax are the most frequent complications of TBCB (15, 16). Previous studies suggested the complications were related with the cryoprobe-to-pleura distance and procedure methods (7, 8). Casoni et al. (17) found that the risk of pneumothorax was increased when fragments of pleura were present in the biopsy. Ravaglia et al. indicated pneumothorax was much more frequent in patients who underwent TBCB by using 2.4-mm probe (6). However, in our study TBCB was conducted under the guidance of 3D CBCT images which were reviewed in axial, coronal, and sagittal planes to accurately assess the cryoprobe-to-pleura distance. Few patients presented fragments of pleura and the pneumothorax rate was much lower than the average incidence by pooled previously reported studies in which TBCB were mostly performed under fluoroscopic guidance (2.8 vs. 9.4%). The varied risks of pneumothorax may be due to the difference of guiding accuracies between 3D CBCT and traditional fluoroscopy. The relationships between safety profile and HRCT features were analyzed in our study without the influence of guiding accuracy, and the results may be more convincible. Our study did not found any associations between pneumothorax and HRCT features, besides procedure

TABLE 3 The safety profile and diagnostic efficacy of transbronchial cryobiopsy (TBCB) in patients with different HRCT features.

Characteristics	Moderate bleeding		Pneumothorax		Pathological diagnosis		MDD diagnosis	
	N (%)	*P*	*N* (%)	*P*	*N* (%)	*P*	*N* (%)	*P*
HRCT manifestations								
Fibrotic pattern ($n = 99$)	15 (15.2%)	**0.013**	3 (3.0%)	0.835	86 (86.7%)	0.767	90 (90.9%)	0.888
UIP or Probable UIP ($n = 55$)	8 (14.5%)	0.162	2 (3.6%)	0.979	45 (81.8%)	0.286	51 (92.7%)	0.852
Indeterminate for UIP ($n = 38$)	6 (15.8%)	0.164	0 (0.0%)	0.546	35 (92.1%)	0.239	33 (86.8%)	0.296
Other fibrotic pattern ($n = 6$)	1 (16.7%)	0.463	1 (16.7%)	0.157	6 (100.0%)	1.000	6 (100.0%)	1.000
Non-fibrotic pattern ($n = 117$)	6 (5.1%)		3 (2.6%)		100 (85.5%)		107 (91.5%)	
GGO ($n = 84$)	4 (4.8%)	**0.050**	3 (3.6%)	0.887	75 (89.3%)	0.282	78 (92.9%)	0.494
Profuse micronodules ($n = 11$)	0 (0.0%)	1.000	0 (0.0%)	1.000	10 (90.9%)	1.000	9 (81.8%)	0.250
Consolidation ($n = 11$)	0 (0.0%)	1.000	0 (0.0%)	1.000	10 (90.9%)	1.000	11 (100.0%)	1.000
Cysts ($n = 9$)	2 (22.2%)	0.214	0 (0.0%)	1.000	4 (44.4%)	**0.003**	7 (77.8%)	0.182
Others ($n = 2$)	0 (0.0%)	1.000	0 (0.0%)	1.000	1 (50.0%)	0.259	2 (100.0%)	1.000
HRCT score in whole		0.072		0.812		0.803		0.606
≤3 ($n = 136$)	17 (12.5%)		3 (2.2%)		116 (85.3%)		123 (90.4%)	
>3 ($n = 80$)	4 (5.0%)		3 (3.8%)		70 (87.5%)		74 (92.5%)	
HRCT score of target lobe		0.254		0.309		**0.012**		0.251
≤3 ($n = 98$)	12 (12.2%)		1 (1.0%)		78 (79.6%)		87 (88.8%)	
>3 ($n = 118$)	9 (7.6%)		5 (4.2%)		108 (91.5%)		110 (93.2%)	

HRCT, high-resolution computed tomography; MDD, multidisciplinary discussion. RET, reticulation; TB, traction bronchiectasis; HC, honeycombing; GGO, ground-glass opacities. Bold values indicate significant p-value < 0.05.

TABLE 4 Multivariate analysis for the associations between HRCT features and the outcomes of transbronchial cryobiopsy in ILD patients.

Characteristics	Moderate bleeding		Pneumothorax		Pathological diagnosis		MDD diagnosis	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Fibrotic pattern	**8.660 (2.515, 29.821)**	**0.001**	0.790 (0.113, 5.540)	0.812	0.703 (0.263, 1.876)	0.481	0.769 (0.250, 2.364)	0.647
UIP or probable UIP	**4.304 (1.383, 13.393)**	**0.012**	1.954 (0.274, 13.950)	0.504	0.450 (0.165, 1.227)	0.119	1.277 (0.345, 4.735)	0.714
Indeterminate for UIP	2.675 (0.787, 9.089)	0.115	N/A		1.649 (0.408, 6.655)	0.482	0.392 (0.108, 1.419)	0.153
Other fibrotic pattern	2.465 (0.181, 33.595)	0.498	5.906 (0.219, 159.522)	0.291	N/A		N/A	
Non-fibrotic pattern								
GGO	**0.173 (0.043, 0.687)**	**0.013**	2.108 (0.308, 14.444)	0.448	0.424 (0.152, 1.182)	0.101	0.645 (0.211, 1.972)	0.442
Profuse micronodules	N/A		N/A		1.530 (0.166, 14.080)	0707	0.257 (0.040, 1.659)	0.153
Consolidation	N/A		N/A		2.489 (0.258, 24.060)	0.431	N/A	
Cysts	2.878 (0.462, 17.918)	0.257	N/A		**0.111 (0.022, 0.573)**	**0.009**	0.376 (0.054, 2.639)	0.325
Others	N/A		N/A		0.221 (0.010, 4.743)	0.335	N/A	
HRCT score in whole	0.208 (0.042, 1.032)	0.055	0.443 (0.053, 3.704)	0.453	0.273 (0.046, 1.619)	0.153	1.177 (0.234, 5.933)	0.843
HRCT score of target lobe	1.000 (0.277, 3.612)	1.000	5.904 (0.354, 98.577)	0.216	**7.172 (1.335, 38.532)**	**0.022**	1.751 (0.406, 7.543)	0.452

HRCT, high-resolution computed tomography; MDD, multidisciplinary discussion. RET, reticulation; TB, traction bronchiectasis; HC, honeycombing; GGO, ground-glass opacities. Bold values indicate significant p-value < 0.05.

methods. On the other hand, we found the risk of moderate bleeding could be predicted by pre-procedure HRCT pattern: radiological UIP or probable UIP pattern was associated with higher risk of moderate bleeding while GGO was associated with lower risk.

As we known, surgical lung biopsy (SLB) was not recommended in the patients having HRCT images presenting a typical UIP pattern, because of the relatively high complication rates and mortality in this part of patients (12). Compared to SLB, TBCB had lower morbidity and it was suggested that sometimes TBCB could be proposed in patients with a typical radiological UIP pattern (7). In our study, we found honeycombing or reticulation with bronchiectasis manifestations on HRCT, which was the

typical radiological abnormality of UIP or probable UIP pattern, was significantly associated with TBCB related moderate bleeding (OR = 4.304, 95% CI: 1.383–13.393). Furthermore, Ravaglia et al. found that pneumothorax was much more frequent in patients with evaluated grading of the distribution of reticulation, traction bronchiectasis and honeycombing (6). As a result, TBCB should be performed with all the proper precautions and prophylactical placement of bronchial blocker should be routinely used in this kind of patients. As for the typical radiological UIP pattern, which presented obvious honeycombing with or without bronchiectasis manifestations, broadening indication of TBCB to this part of patients should be carefully assessed by investigations.

For the patients with cystic manifestation on HRCT, transbronchial lung biopsy was one of the valuable methods for pathological diagnosis. Previous study reported that Langerhans cell histiocytosis (LCH) and lymphangioleiomyomatosis (LAM) could be successfully diagnosed by TBCB (18–20). However, our study showed the pathological diagnostic yield for the patients with cysts on HRCT was low (44.4%, $P = 0.003$). And multivariate analysis demonstrated cystic manifestation predicted failure of pathological diagnosis. The low diagnostic yield may be explained by the small scale of lesion. The HRCT score of cysts in the biopsied lobe of lung was less than 3 in most patients [8/9]. Our study suggested the pathological diagnostic yield in patients with HRCT score in the target lobe less than 3 was significantly lower than those patients with HRCT score more than 3. On the other hand, our study showed a relatively high incidence of TBCB related moderate bleeding (2/9, 22.2%). As a result, the safety and diagnostic value of TBCB for cystic manifestation needed to be proved by more investigations with larger sample size.

Conclusions

The HRCT features were associated with moderate bleeding and pathological diagnostic yields. Radiological UIP or probable UIP pattern was associated with higher risk of moderate bleeding while GGO was associated with low risk. Cystic manifestation predicted failure of pathological diagnosis while performing TBCB in the lobe with high HRCT score may increase pathological diagnosis. Pre-procedure assessment of HRCT pattern was helpful for bronchoscopists to make a better patient selection and procedure planning, in order to improve procedure safety and diagnostic yields.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of China-Japan Friendship Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conception and design: HD, GZ, YR, and CW. Administrative: HD. Provision of study materials or patients: GZ, YR, JL, TY, NS, LZ, SW, DW, YL, ZT, and RL. Collection and assembly of data, data analysis and interpretation, and manuscript writing: GZ and YR. Revision of manuscript and final approval of manuscript: all authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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World Health Organization (WHO) International Classification of Functioning, Disability and Health (ICF) Core Set Development for Interstitial Lung Disease

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Abbreviations: CM, Center for Medicare and Medicaid Services, CTD, Connective Tissue Disease; DLCO, Diffusion Capacity of Lung for Carbon Monoxide; FVC, Forced Vital Capacity; HRCT, High Resolution Computed Tomography; ICD, International Classification of Diseases; ICF, International Classification of Functioning Disability and Health; IIP, Interstitial Idiopathic Pneumonitis; IPF, Idiopathic Pulmonary Fibrosis; PF/IPF-PROM, Pulmonary Fibrosis/Idiopathic Pulmonary Fibrosis Patient Reported Outcome Measure; ILD, Interstitial Lung Disease; K-BILD, King's Brief Interstitial Lung Disease Questionnaire; L-IPF, Living with IPF; PSFS, Patient Specific Functional Scale; PROM, Patient-Reported Outcome Measures; PF, Pulmonary Fibrosis; pf, Personal factors; SF-36, Short Form 36; UCSD-SBQ, University of California-Shortness of Breath Questionnaire; WHO, World Health Organization.

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Background: The World Health Organization (WHO) introduced the International Classification of Functioning, Disability, and Health (ICF) as a scientific method of disability data collection comprised of >1,200 categories describing the spectrum of impairment types (functional, symptoms-based and anatomical) under the bio-psycho-social model with consideration of *environmental* and *personal factors* (pf). ICF Core Sets and ICF Checklists are streamlined disease-specific resources for clinical use, service provision, and for use in health economics and health policy. ICF can disclose strengths and weaknesses across multiple patient-reported outcome measures (PROMs) and help consolidate best-fitting question-items from multiple PROMs. Interstitial lung diseases (ILDs), are generally progressive, with restrictive physiology sometimes occurring in the context of multi-organ autoimmunity/inflammatory conditions such as connective tissue diseases (CTDs). In spite of significant associated morbidity and potential disability, ILD has yet to be linked to the ICF.

Methods: Each instrument and their question-items within the consensus-recommended core sets for clinical trials in ILD were deconstructed to single concept units, and then linked per updated ICF linkage rules. Inter-linker agreement was established. Three additional subsequently validated measures were also included.

Results: One-hundred-eleven ICF categories were identified for ten PROMs and three traditional objective measures that were amenable to ICF linkage. The proportion of agreement ranged from 0.79 (95% CI: 0.62, 0.91) to 0.93 (0.76, 0.99) with the overall proportion of inter-linker agreement being very high 0.86 (0.82, 0.89) for the initial instruments, with 94–100% for the three additional PROMs. Thirty-four new 'Personal Factors' emerged to capture disease-specific qualities not elsewhere described in ICF, e.g. 'pf_embarrassed by cough' or 'pf_panic/afraid when can't get a breath'.

Conclusion: This first known effort in ICF linkage of ILD has provided important revelations on the current utility of the ICF in lung disease. Results have indicated areas for meaningful assessment of ICF descriptors for lung impairment. The mapping across PROMs provides insight into possibilities of developing more streamline and precise instrumentation. Finally, familiarity with the ICF in ILD may enable clinicians to experience a smoother transition with the imminent harmonization of ICD and ICF, ICD-11.

KEYWORDS

fibrosis, ICD-11, patient-reported outcomes, connective tissue, cough, CTD-ILD

Introduction

Interstitial lung diseases (ILD) are a heterogeneous group of predominately restrictive lung diseases imparting significant morbidity and mortality (Travis et al., 2013; Wijsenbeek and Cottin, 2020). Idiopathic pulmonary fibrosis (IPF) is a progressive pauci-immune fibrotic process predominantly involving the lungs and with no known cure (Spagnolo et al., 2018). Connective tissue diseases (CTD) such as systemic sclerosis, inflammatory myopathy, Sjogren's syndrome and rheumatoid arthritis (RA) affect the lungs in the setting of other systemic/multi-organ autoimmunity with radiographic and pathological patterns varying in degrees of inflammatory and/or fibrotic progression (Wijsenbeek and Cottin, 2020; Ruaro et al., 2021). Through iterative medical expert and patient consensus methodology (Saketkoo et al., 2014a; Saketkoo et al., 2014b), recent efforts identified a minimum set of instruments for inclusion in clinical trials and longitudinal observational studies in IPF and CTD-ILD, respectively.

The World Health Organization's (WHO) International Classification of Functioning, Disability and Health (ICF) is an alphanumeric classification system integrating the bio-psychosocial model of health and was officially adopted by the World Health Assembly in 2001 (Figure 1 and online supplement.).

As with the WHO's other classification system, the International Classification of Diseases (ICD), a system originally developed to quantify burden of specific health conditions (diagnoses), the ICF can be used on global, national, regional, local, and institutional levels. The ICF was originally devised as a standardized means to scientifically assess the global burden of disability and chronic disease (World Health Organization, 2001; World Health Organization Website, 2015), thus providing guidance for reimbursement, infrastructural support, allotment of research and service funding, and, importantly, policymaking. Through a hierarchical mechanism of over 1,200 "categories" (or codes) that depict functioning and disability (World Health Organization International Classification of Functioning Disability and Health, 2001), the ICF attempts to describe the spectrum of impairment type (symptomatic and anatomical) and to quantify the burden of disability of a population, as well as capturing "environmental factors" that either improve function and mitigate disability (such as ramps, assistive technology, medications, supportive family, etc), or that worsen impairment (staired entries, lack of accessible transport, unaffordable assistive aids, etc.).

Beyond epidemiologic and health economics use, the ICF has emerged to be highly versatile and multi-purpose in its utility including clinical assessment of specified health conditions, It initially focused on rehabilitative care (e.g. traumatic brain injury, stroke etc.) and subsequently expanded to other chronic health conditions (e.g. RA, diabetes, or obesity). Clinical applications of the ICF gave rise to the development of ICF Core Sets (collections of ICF categories relevant to a disease) along with ICF Checklists (clinical forms with selected categories from the ICF Core Set), abbreviated collections of ICF categories/codes for specific health conditions that facilitate assessment of symptom burden, impairment, treatment response, side effects and service needs.

ICF Core Sets and Checklists can be administered online or on paper, and are patient or clinician reported. ICF is multi-purpose and can be used along with the Patient Specific Functional Scale St (PSFS) (Stratford et al., 1995; Fairbairn et al., 2012; Patient specific functional scale, 2021), MACTAR (Alemo Munters et al., 2014) or Canadian Occupational Performance Measure (COPM) (Stamm et al., 2004) to help identify and prioritize unique preferences in functional achievements. Similarly, ICF can help disclose strengths and weaknesses across multiple patient-reported outcome measures (PROMs) and consolidate best-fitting question items.

The ICD and ICF classification systems complement each other, and the current WHO intention is to harmonize these two classification systems for ICD-11 (Escorpizo et al., 2013). The Center for Medicare and Medicaid Services (CMS) in the United States (Iezzoni and Greenberg, 2003; Health and Human Services/Centers for Medicare and Medicaid Services, 2000; SocialSecurity Administration, 2013; Escorpizo and Stucki, 2013) has adopted the 11th revision of the ICD, which will integrate the ICD-10 and ICF classifications by simultaneously conveying the diagnosis with the type and degree of impairment (Kohler et al., 2012; Escorpizo et al., 2013; Selb et al., 2015a; Selb et al., 2015b). Our goal in linking ILD to the ICF is the development of disease-specific ICF Core Sets to aid pulmonary-focused clinicians in this transition (Saketkoo et al., 2012a; Selb et al., 2015a). The results provide significant utility beyond our original goals, including ICF language enhancements and mapped variations of current PROMs that may inform improved patient-reported instrumentation.

Methods

The goals of the study are to produce a feasible approach to ICF for clinicians and researchers working in interstitial lung disease, and to foster confidence and familiarity with the ICF during the ICD-11 transition (Escorpizo et al., 2013).

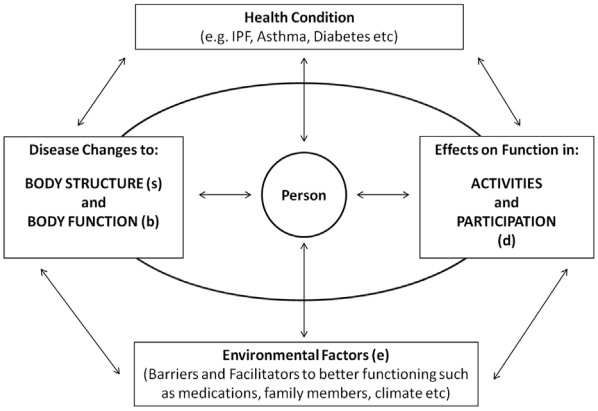

FIGURE 1
Interactions between domains of ICF in relation to the health condition. *Courtesy of LA Saketkoo, with permission, rights reserved.*

Additionally, the study interrogates for needed modifications and updates to advance the current ICF content in pulmonary disease.

Structure of the ICF classification

The ICF Classification consists of two over-arching parts with independent components:

1. *Functioning and Disability,* the predominant operational part of the classification consists of:

a) *"Body Structure"* ("**s**" terms) are *abnormalities of anatomical structure*, such as that of lung parenchyma (s4301)
b) *"Body Function"* ("**b**" terms) are physiologic functions including *symptomatic experience* of physical, mental and emotional functions e.g. energy/fatigue (b1300), dyspnea (b460), cough (b450) or chest pain (b28011)
c) *"Activities and Participation"* ("**d**" terms) are defined under "activities" e.g. lifting (d4300), bathing (d510), cooking (d630), or moving between locations (d460); and under "participation" such as life situations with work (d850) or family (d760).

2. *Contextual Factors* are divided into:

a) external or *"Environmental Factors"* ("**e**" terms) that either positively (e.g., personal assistive devices, e1151) or negatively (e.g., inaccessible transportation, e120) impact functioning
b) internal or *"Personal Factors"*, a developing ICF area, are individualized factors that potentially influence disability e.g., gender, age, coping styles, behavior and psychological characteristics.

Categories are tiered into "levels" of specificity (e.g., b5 digestive system, b510 ingestion, b5105 swallowing, b51052 esophageal swallowing).

Linking the CTD-ILD and IPF consensus instruments to the ICF

A diverse expert team of clinicians, patients and researchers provided responsive feedback regarding instrument selection, analysis and interpretation. These consensus measures included seven vetted PROMS and four "objective" measures considered for use in clinical trials for IPF and for CTD-ILD (Saketkoo et al., 2014b) (Table 1) as well as three other subsequently validated PROMs (Patel et al., 2012; Russell et al., 2017; Russell, 2018; Swigris et al., 2020) that were included because of anticipated high utilization. Each of these measures was attempted to be linked to ICF categories (Table 2) by two independent investigators (RE,LAS) according to updated ICF linking rules (Cieza et al., 2005). (As the *Short Form Health Survey (SF-36)* was previously linked by ICF scholars, these linkages were used.) To accomplish the linking, each of the measures were deconstructed to its most basic single-concept units, which required PROM question-items to each be deconstructed. For example, an item querying "mowing lawn makes me breathless" is comprised of two discrete concepts linked individually to ICF, "mowing lawn" and "breathless". Each concept-item, however, may evoke more than one linkage, such as "mowing lawn" suggests "pushing an object" and "caring for home". If any item analysis identified discordance, these were resolved between the linkers. This was done by each linker stating their position of support for the items they had chosen, then each stating if they had any positions of disagreement for the discordant items chosen by the other. This was then followed by discussion of

TABLE 1 Consensus on minimal core sets of instruments and measures for IPF and CTD-ILD trials (Saketkoo et al., 2014b)

Domain	Instruments/Measures	CTD-ILD	IPF
Dyspnea (Breathlessness)	Medical Research Council Chronic Dyspnea Scale	+	+
	Dyspnea 12	+	+
	UCSD-Shortness of Breath Questionnaire	-	+
Cough	Leicester Cough Questionnaire	+	+
HRQoL	Medical Outcomes Study Short Form-36	+	+
	St. Georges Respiratory Questionnaire	+	+
	VAS Patient Global Assessment of Disease Activity (VAS-PtGA)	+	–
Lung Imaging	Overall Extent of ILD on HRCT	+	+
Lung Physiology	Forced Vital Capacity (FVC)	+	+
	Diffusion Capacity of Lung (DLCO)	+	+
Survival	All Cause Mortality	+	+
Additional PROMs Validated After Consensus Project with Anticipated High Utility			
	Pulmonary Fibrosis PROM (PF-PROM)^	^	^
	King's Brief ILD Questionnaire (K-BILD)^	^	^
	Living with IPF Questionnaire (LIPF)^	^	^

"+" signifies "*part*" and "–"signifies "*not part*" of minimal core set of consensus instruments for IPF, or CTD-ILD, clinical trials,^signifies validated subsequent to consensus. (Travis et al., 2013).

TABLE 2 Distribution of ICF categories and instrument occurrence per domain with example linkages. (*Courtesy of LA Saketkoo, with permission, rights reserved*)

ICF domain	Description	Instruments linked	No. of ICF categories linked	Examples from CTD-ILD and IPF core sets
Body Structure	Relates to involvement of anatomical structures	HRCT	1	s4301, Structure of lungs
Body Function	Relates to physical, mental and emotional functions including symptoms	D-12, DLCO, FVC, LCQ, MRC-DS, PF/IPF-PROM, K-BILD, LIPF, SF-36, SGRQ, UCSD-SOBQ	28	b1300, Energy level b134, Sleep functions b4402, Depth of respiration b455, Exercise tolerance b28011, Pain in chest
Activities and Participation	Execution of task or action; involvement in daily and overall life situation	D-12, LCQ, MRC-DS, PF/IPF-PROM, K-BILD, LIPF, SF-36, SGRQ, UCSD-SOBQ	71	d330, Speaking d430, Lifting, carrying objects, d4600, Moving around house d510, Washing oneself d8451, Maintaining a job
Environmental Factors	**Positive** (e.g. family, medications, assistive devices, oxygen, lifts) or **Negative** (e.g. stairs, lack of income, cold climate, distance from services) influences on performance	LCQ, K-BILD, LIPF, SGRQ	11	Products and technology for personal use in daily living e115_oxygen supplementation, Financial assets e1650, Tangible assets e1651, e260, Air quality e460, Societal attitudes e2100, Land forms, such as mountains, hills, valleys and plains
Total			**111**	

each item to keep or dismiss. Irresolvable disagreement between linkers on an item would be decided by at least one person also trained in the ICF (AMR,HA,HP,OD).

Pre-resolution inter-linker agreement was analyzed (KJK) for each instrument with the estimates of the proportion of agreement and confidence intervals according to the exact

TABLE 3 Instruments from the previously published CTD-ILD and IPF minimum core sets for clinical trials with instrument comparison and inter-reviewer agreement. (*Courtesy of LA Saketkoo, with permission, rights reserved*)

Consensus instruments for CTD-ILD and IPF (Saketkoo et al., 2014b)	Number of concept-items linked	Number of categories identified	Agreement (%)	Agreement 95% confidence interval
Medical Research Council (MRC) Dyspnea Scale	27	34	79%	(62, 91)
Dyspnea 12 (D-12)	25	27	93%	(76, 99)
University of California San Diego –Shortness of Breath Questionnaire (UCSD-SBQ)	68	83	82%	(72, 90)
Leicester Cough Questionnaire	44	56	79%	(66, 88)
St Georges Respiratory Questionnaire	126	138	91%	(85, 95)
Medical Outcomes Study Short Form 36 (SF-36)		26[a]		Previously linked version
Visual Analogue Scale-Patient Global Disease Activity		Not defined by ICF (too broad)		
Overall Initial Agreement on Consensus Questionnaires	**290**	**338 (+26[a])**	**86%**	**(82, 89)**
		Traditional Measures		
Forced Vital Capacity on Spirometry	2	2[a]	100%	N/A
Diffusion Capacity of the Lung for Carbon Monoxide (DLCO)	1	1[a]	100%	N/A
Overall Extent of ILD on High Resolution CT (HRCT)	1	1[a]	100%	N/A
All Cause Mortality		Not defined by ICF (too broad)		
		Linker Agreement of Additional PROM Instruments		
PF/IPF-PROM	—	—	100%	Not done
King's Brief Interstitial Lung Disease Questionnaire (K-BILD)	—	—	100%	Not done
Living with IPF (LIPF) Questionnaire	—	—	94.3%	Not done

[a]Linked to an objective instrument and not from a questionnaire.

binomial test using release 3.1.0 of the R statistical software package (Core Team, 2014).

Results

One-hundred and eleven ICF categories were identified under the four ICF components ("*Body Structure*", "*Body Function*", "*Activities and Participation*", and "*Environmental Factors*") for nine patient-reported questionnaires and three traditional objective measures (Table 2).

Linking agreement

Agreement between linkers was high (Table 3). The pre-resolution proportion of agreement ranged from 0.79 (95% CI: 0.62, 0.91) to 0.93 (0.76, 0.99) for the five-remaining consensus PROMs (Saketkoo et al., 2014b) (as no linking occurred for *Visual Analogue Scale-Patient Global Assessment of Disease Activity* (*VAS-PtGA*) and *SF-36*) with the overall proportion of inter-linker agreement 0.86 (0.82, 0.89). There was 100% agreement between the linkers for pulmonary function measures of *forced vital capacity* (*FVC*) and *diffusion capacity* (*DLCO*), and the *Overall Extent of ILD on HRCT*. There was 94–100% linking agreement for the three additional PROMs (*PF/IPF-PROM*, *King's Brief Interstitial Lung Disease Questionnaire* (*K-BILD*), and *Living with IPF* (*LIPF*) *Questionnaire*). Initial linking discrepancies were resolved to 100% between linkers without need for arbitration.

Linkages

Of the combined total 111 linkages identified, 28 fell under "*Body Function*", one under "*Body Structure*" (lung), 71 under "*Activities and Participation*" and 11 under "*Environmental Factors*". *All-Cause Mortality* and the *VAS-PtGA* were not definable in ICF terms. *Extent of ILD on HRCT* was the only measure demonstrating linkage under "*Body Structure*" representing a single category, *s4301*, "*Structure of Lungs*".

The ICF contained no direct and specific linkages for the pervasive ILD symptoms of *breathlessness* and *cough*. To address this, many new linkages created to temporarily accommodate the concepts held in the PROMs. However, we propose these enduring ICF additions:

- two new ICF categories under "*additional respiratory functions*": "*cough*" *(b4501)* and "*phlegm production*" *(b4502)*,
- one new ICF category under "*respiratory functions*": "*respiratory flow* including airflow interrupted by inspiratory cough"(*b4403*), and
- three under "*sensations associated with cardiovascular and respiratory functions*": "*sensation of breathlessness*"

TABLE 4 Categories according to instrument (*Courtesy of LA Saketkoo, with permission, rights reserved*)

WHO ICF category	WHO ICF descriptor	MRC	D-12	UCSD-SBQ`	LCQ	SF-36	SGRQ	PF/IPF-PROM	K-BILD	LIPF	HRCT	FVC	DLCO
Body Structure													
s4301	Structure of lungs	—	—	—	—	—	—	—	—	—	HRCT	—	—
Body Functions													
b1263	Psychic stability	—	—	—	—	SF-36	—	—	—	—	—	—	—
b1300[a]	Energy level	—	—	—	LCQ	SF-36	—	PF/IPF-PROM	—	LIPF	—	—	—
b134	Sleep functions	—	—	—	LCQ		SGRQ	—	—	LIPF	—	—	—
b152	Emotional functions	—	—	—		SF-36	—	—	—		—	—	—
b280	Sensation of pain	—	—	—	—	—	SGRQ	—	—	—	—	—	—
b28011	Pain in chest	—	D-12	—	LCQ	—	—	—	—	—	—	—	—
b28012	Pain in stomach or abdomen	—	—	—	LCQ	—	—	—	—	—	—	—	—
b3101	Quality of voice	—	—	—	LCQ	—	—	—	—	—	—	—	—
b440[a]	Respiration functions: Functions of inhalation, gas exchange, and exhalation	—	—	—	—	—	—	—	K-BILD	—	—	FVC	DLCO
b4402	Depth of respiration	—	D-12	—	—	—	—	—	—	LIPF	—	—	—
b4408_sputum_phlegem production	Respiration Functions Other specified	—	—	—	LCQ	—	SGRQ	—	—	—	—	—	—
b4408_cough with deep inspiration	—	—	—	—	—	—	—	—	—	LIPF	—	—	—
b450[a]	Additional respiratory functions: Additional functions related to breathing, such as coughing, sneezing and yawning	MRC	—	—	LCQ	—	SGRQ	PF/IPF-PROM	—	LIPF	—	FVC	—
b450_cough/ing[a]	Additional respiratory functions	—	—	—	LCQ	—	SGRQ	—	—	LIPF	—	—	—
b455[a]	Exercise tolerance functions	MRC	D-12	UCSD-SBQ`	LCQ		SGRQ	PF/IPF-PROM	K-BILD	LIPF	—	—	—
b455_physical exertion	—	—	—	—	—	—	—	—	—	LIPF	—	—	—
b455_stamina	—	—	—	—	—	—	—	—	—	LIPF	—	—	—
b4550	General physical endurance	—	—	—	LCQ	—	—	—	—	—	—	—	—
b4552[a]	Fatigability	—	D-12	—	LCQ	—	SGRQ	PF/IPF-PROM	—	—	—	—	—
b460[a]	Sensations associated with cardiovascular and respiratory functions: such as skipped heart beat, palpitation and shortness of breath	MRC	D-12	UCSD-SBQ`	—	—	SGRQ	Pf/IPF-PROM	K-BILD	LIPF	—	—	—
b460_air hunger/gasp	Ibid	—	—	—	—	—	—	—	K-BILD	—	—	—	—
b460_chest tightness	Ibid	—	—	—	—	—	—	—	K-BILD	—	—	—	—

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TABLE 4 (*Continued*) Categories according to instrument (*Courtesy of LA Saketkoo, with permission, rights reserved*)

WHO ICF category	WHO ICF descriptor	MRC	D-12	UCSD-SBQ`	LCQ	SF-36	SGRQ	PF/IPF-PROM	K-BILD	LIPF	HRCT	FVC	DLCO
b460_cough/ing[a]	Ibid	—	—	—	LCQ		SGRQ	—	—	LIPF	—	—	—
b460_wheeze/whistling sound	ibid	—	—	—	—	—	—	—	K-BILD	—	—	—	—
b469_at rest	Additional functions and sensations of the cardiovascular and respiratory systems, other specified and unspecified	—	—	—	—	—	—	—	—	LIPF	—	—	—
b469_bouts of coughing	ibid	—	—	—	LCQ	—	—	—	—	—	—	—	—
b469_cough/ing	Ibid	—	—	—	LCQ	—	—	—	—	LIPF	—	—	—
b469_cough/ing with deep inspiration	Ibid	—	—	—		—	—	—	—	LIPF	—	—	—
b469cough/ing with over-exertion	Ibid	—	—	—	—	—	—	—	—	LIPF	—	—	—
Activity and Participation													
d2	General tasks and demands	—	—	—	—	—	—	—	K-BILD	LIPF	—	—	—
d210	Undertaking a single task	—	—	—	—	—	—	—	—	LIPF	—	—	—
d2100	Undertaking a simple task	—	—	—	—	—	—	—	—	LIPF	—	—	—
d2102	Undertaking a single task independently	—	—	—	—	—	—	—	—	LIPF	—	—	—
d220	Undertaking multiple tasks	—	—	—	—	—	—	—	—	LIPF	—	—	—
d2202	Undertaking multiple tasks independently	—	—	—	—	—	—	—	—	LIPF	—	—	—
d230[a]	Carrying out daily routine	—	—	—	LCQ	SF-36	—	PF/IPF-PROM	K-BILD	LIPF	—	—	—
d299	General tasks and demands, unspecified	—	—	—	—	—	—	—	—	LIPF	—	—	—
d3	Communication	—	—	—	—	—	—	—	K-BILD	LIPF	—	—	—
d330	Speaking	—	—	—	LCQ	—	—	—	—	—	—	—	—
d350	Conversation	—	—	—	LCQ	—	—	—	—	LIPF	—	—	—
d3600	Using telecommunication devices	—	—	—	LCQ	—	—	—	—	—	—	—	—
d4	**Mobility**	—	—	—	—	—	—	—	—	LIPF	—	—	—
d4102	Kneeling	—	—	—	—	SF-36	—	—	—	—	—	—	—
d4103	Sitting	—	—	UCSD-SBQ`	—		—	—	—	—	—	—	—
d4104	Standing	—	—	UCSD-SBQ`	—		—	—	—	—	—	—	—
d4105[a]	Bending	—	—	UCSD-SBQ`	—	SF-36	SGRQ	—	—	—	—	—	—
d430[a]	Lifting and carrying objects	—	—	—	—	SF-36	SGRQ	—	K-BILD	LIPF	—	—	—
d4300	Lifting	—	—	—	—	SF-36	—	—	—	LIPF	—	—	—

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TABLE 4 (*Continued*) Categories according to instrument (*Courtesy of LA Saketkoo, with permission, rights reserved*)

WHO ICF category	WHO ICF descriptor	MRC	D-12	UCSD-SBQ`	LCQ	SF-36	SGRQ	PF/IPF-PROM	K-BILD	LIPF	HRCT	FVC	DLCO
d4301	Carrying in the hands	—	—	—	—		—	—	—	LIPF	—	—	—
d435	Moving objects with lower extremities	—	—	—	—	SF-36	—	—	—	—	—	—	—
d440	Fine hand use	—	—	—	—	SF-36	—	—	—	—	—	—	—
d445	Hand and arm use	—	—	—	—	SF-36	—	—	—	—	—	—	—
d4451	Pushing	—	—	UCSD-SBQ`	—	SF-36	—	—	—	—	—	—	—
d449	Carrying, moving and handling objects, other specified and unspecified	—	—	—	—	SF-36	—	—	—	—	—	—	—
d450[a]	Walking	MRC	—	UCSD-SBQ`	—	SF-36	SGRQ	PF/IPF-PROM	—	LIPF	—	—	—
d4500[a]	Walking short distances	MRC	—	—	—	SF-36	—	—	—	LIPF	—	—	—
d4501	Walking long distances	MRC	—	—	—	SF-36	—	—	—	—	—	—	—
d4502[a]	Walking on different surfaces	MRC	—	UCSD-SBQ`	—	—	SGRQ		K-BILD	—	—	—	—
d4508_walknig for periods[a]	Walking, other specified	MRC	—	UCSD-SBQ`	—	—	—	PF/IPF-PROM	—	—	—	—	—
d4508_walking pace	Walking, other specified	MRC	—	—	—	—	—	PF/IPF-PROM	—	—	—	—	—
d455	Moving around	—	—	—	—	—	SGRQ	—	—	—	—	—	—
d4551[a]	Climbing	—	—	UCSD-SBQ`	—	SF-36	SGRQ	—	K-BILD	LIPF	—	—	—
d4552	Running	—	—	—	—	SF-36	SGRQ	—	—	—	—	—	—
d4554	Swimming	—	—	—	—	—	SGRQ	—	—	—	—	—	—
d460	Moving around in different locations	—	—	—	—	—	—	—	—	LIPF	—	—	—
d4600	Moving around within the home	—	—	—	—	—	SGRQ	—	—	LIPF	—	—	—
d4601	Moving around within buildings other than home	—	—	UCSD-SBQ`	—	—	—	—	—	—	—	—	—
d4602	Moving around outside the home and other buildings	—	—	—	—	—	SGRQ	—	—	LIPF	—	—	—
d5	General self-care	—	—	—	—	—	—	—	K-BILD	LIPF	—	—	—
d510	Washing oneself	—	—	—	—	—	SGRQ	—	—	LIPF	—	—	—
d5101	Washing whole body	—	—	—	—	SF-36	—	—	—	—	—	—	—
d5109	Washing oneself, unspecified	—	—	UCSD-SBQ`	—	—	—	—	—	—	—	—	—
d520	Caring for body parts	—	—	—	—	—	—	—	—	LIPF	—	—	—
d5201	Caring for teeth	—	—	UCSD-SBQ`	—	—	—	—	—	LIPF	—	—	—

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TABLE 4 (*Continued*) Categories according to instrument (*Courtesy of LA Saketkoo, with permission, rights reserved*)

WHO ICF category	WHO ICF descriptor	MRC	D-12	UCSD-SBQ`	LCQ	SF-36	SGRQ	PF/IPF-PROM	K-BILD	LIPF	HRCT	FVC	DLCO
d5202	Caring for hair	—	—	UCSD-SBQ`	—	—	—	—	—	LIPF	—	—	—
d540[a]	Dressing	—	—	UCSD-SBQ`	—	SF-36	—	—	—	LIPF	—	—	—
d550	Eating	—	—	UCSD-SBQ`	—	—	—	—	—	—	—	—	—
d570[a]	Looking after one's health	—	—	UCSD-SBQ`	—	—	SGRQ	—	—	LIPF	—	—	—
d6	Domestic Life	—	—	—	—	—	—	—	K-BILD	LIPF	—	—	—
d6200	Shopping	—	—	UCSD-SBQ`	—	—	SGRQ	—	—	—	—	—	—
d640[a]	Doing housework	—	—	UCSD-SBQ`	—	SF-36	SGRQ	—	—	—	—	—	—
d6400	Washing and drying clothes and garments	—	—	UCSD-SBQ`	—	—	—	—	—	—	—	—	—
d6402	Cleaning living area	MRC	—	UCSD-SBQ`	—	—	—	—	—	—	—	—	—
d6403	Using household appliances	—	—	UCSD-SBQ[a]	—	—	—	—	—	—	—	—	—
d6408_mowing lawn	Doing housework, other specified	—	—	UCSD-SBQ`	—	—	—	—	—	—	—	—	—
d6408_shovel snow	Doing housework, other specified	—	—	—	—	—	SGRQ	—	—	—	—	—	—
d6408_watering lawn	Doing housework, other specified	—	—	UCSD-SBQ`	—	—	—	—	—	—	—	—	—
d6503	Maintaining vehicles	—	—	UCSD-SBQ`	—	—	—	—	—	—	—	—	—
d6505	Taking care of plants, indoors and outdoors	—	—	UCSD-SBQ`	—	—	SGRQ	—	—	—	—	—	—
d7702	Sexual relationships	—	—	UCSD-SBQ`	—	—	—	—	—	—	—	—	—
d845	Acquiring, keeping and terminating a job	—	—	—	LCQ	—	—	—	—	—	—	—	—
d8451	Maintaining a job	—	—	—	LCQ	—	—	—	K-BILD	—	—	—	—
d850[a]	Remunerative employment	—	—	—	LCQ	SF-36	—	—	K-BILD	—	—	—	—
d855	Non-remunerative employment	—	—	—	LCQ	—	—	—	—	—	—	—	—
d9	Community, social and civic life	—	—	—	—	—	—	—	K-BILD	LIPF	—	—	—
d920	Recreation and leisure	—	—	—	—	—	SGRQ	—	—	—	—	—	—
d9200	Play	—	—	—	—	—	SGRQ	—	—	—	—	—	—

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TABLE 4 (*Continued*) Categories according to instrument (*Courtesy of LA Saketkoo, with permission, rights reserved*)

WHO ICF category	WHO ICF descriptor	MRC	D-12	UCSD-SBQ`	LCQ	SF-36	SGRQ	PF/IPF-PROM	K-BILD	LIPF	HRCT	FVC	DLCO
d9201	Sports	—	—	—	—	SF-36	SGRQ	—	—	—	—	—	—
d9202	Arts and culture	—	—	—	—	—	SGRQ	—	—	—	—	—	—
d9205	Socializing	—	—	—	—	SF-36	—	—	—	—	—	—	—
Environmental Factors													
e115_supplemental_oxygen	Products and technology for personal use in daily living	—	—	—	—	—	—	—	—	LIPF	—	—	—
e1650	Financial assets	—	—	—	—	—	—	—	K-BILD	—	—	—	—
e1651	Tangible assets	—	—	—	—	—	—	—	K-BILD	—	—	—	—
e2100	Land forms: Features of land forms, such as mountains, hills, valleys and plains	—	—	—	—	—	SGRQ	—	—	—	—	—	—
e2450	Day/night cycles	—	—	—	—	—	SGRQ	—	—	—	—	—	—
e260	Air quality	—	—	—	LCQ	—	—	—	—	—	—	—	—
e340	Personal care providers and personal assistants	—	—	—	—	—	—	—	—	LIPF	—	—	—
e410	Individual attitudes of immediate family members	—	—	—	LCQ	—	SGRQ	—	—	—	—	—	—
e415	Individual attitudes of extended family members	—	—	—	—	—	SGRQ	—	—	—	—	—	—
e420	Individual attitudes of friends	—	—	—	LCQ	—	SGRQ	—	—	—	—	—	—
e425	Individual attitudes of acquaintances, peers, colleagues, neighbours and community members	—	—	—	—	—	SGRQ	—	—	—	—	—	—
e460	Societal attitudes	—	—	—	LCQ	—	—	—	—	—	—	—	—

MRC: medical research council dyspnea scale; D-12: Dyspnea-12, UCSD-SBQ: University of California Shortness of Breath Questionnaire, LCQ: Leicester Cough Questionnaire, SF-36: IPF-PROM: Pulmonary Fibrosis-Patient-Reported Outcome Measure; K-BILD: King's Brief ILD, questionnaire, LIPF: Living with IPF Questionnaire, Medical Outcomes Study Short-Form 36, VAS-PG: visual analogue scale patient global assessment of disease activity; HRCT: extent of interstitial disease on high resolution computed tomography; FVC: forced vital capacity; DLCO: diffusion capacity of lung for carbon monoxide. All instruments apply to both IPF, and CTD-ILD, unless where indicated.

[a]Indicates an ICF, category with linkages to 3 or more instruments. Indicates the consensus instrument was for IPF, only.

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(b4600), "sensation of air hunger" (b4601), "wheezing" (b4602).

Regarding concepts of high ILD relevance (Swigris et al., 2005; Saketkoo et al., 2014a; Saketkoo et al., 2014b) cited by people living with ILD, there appeared to variation of frequency across PROMs.

Exercise tolerance (*b455*), as did *respiratory symptoms* (*b440-b460*) demonstrated linkage in all PROMS except *SF-36*. Depth of respiration (*b4402*) was queried in two PROMs, the *Dyspnea-12* (*D-12*) and *LIPF*. "*Coughing*" in any form was noted in only three PROMs: *LCQ*, *SGRQ*, and *LIPF*. *"Cough with deep inspiration"* and "*coughing with over-exertion" were queried* in *LIPF* and *"bouts of coughing"* in *LCQ*. Linkages relating to voice quality (*b3101*), speaking (*d330*) and conversation (*d350,d3600*) were represented by the *LCQ* and *LIPF*.

Of nine PROMs, *Energy level, (b1300)* and *fatiguability (b4552)* was queried in six of the nine included PROMs (*D-12, LCQ, SF-36, SGRQ, PF/IPF-PROM, LIPF*). While *sleep (b134)* was queried in three PROMs (*LCQ, SGRQ, LIPF*). Ability to "*carry out daily routine" (d230)* demonstrated linkage in five PROMs (*LCQ, SF-36, PF/IPF-PROM, K-BILD, LIPF*); but only the LIPF queried complexity of task performance and did so in multiple dimensions (*d210, d2100, d2102, d220, d2202*) as well as self-pacing (*d2309_pace self*). While the *University of California San Diego-Shortness of Breath Questionnaire (UCSD-SBQ)*, accounted for unique linkages (Table 4, 5) all of which occurred under "*Activities and Participation*" and related to ability to sit, stand, to perform domestic care and move around outside the house; while both the *UCSD-SBQ* and the *LIPF* demonstrated linking to more highly detailed levels of self-care activities such dental care, washing and grooming.

The *UCSD-SBQ* is the only included PROM to query self-nourishment (*d550*) and sexual activity (*d7702*). Concepts of financial solvency, such as maintaining remunerative employment and assets were only queried in the *K-BILD* and *LCQ*.

Personal and environmental factors

Thirty-four "*Personal Factors*" (Table 6) reflected disease-specific qualities not described elsewhere in the ICF, e.g. "pf_embarrassed by cough" or "pf_panic/afraid when can't get a breath". "*Personal Factors*" mainly captured the emotional impact of living with ILD ranging from episodic feelings such as panic, fright, distress, frustration and embarrassment; to those of more goading nature such as fear, worry, agitation; and more chronic undercurrents of emotion such as anxiety, coping with uncertainty, fear of symptoms and thinking about death. "*Personal Factors*" also described perceptions of health status such as quality of life and frailty, "*Environmental Factors"* related to the attitudes of others (family *e410/e415*, friends *e420*, acquaintances *e425*, societal *e460*) potentially impacting impairment were predominantly represented by the *LCQ* and *SGRQ*. *SGRQ* uniquely queried terrain (*e2100*) and circadian timing of symptoms (*e2450*). *LCQ*, *K-BILD* uniquely queried air quality (*e260*) and assets (*e1650*, *e1651*), respectively. *LIPF* uniquely queried about products/technology for personal use (*e111_supplemental oxygen*) and personal care providers (*e340*).

Discussion

Herein, we provide a reference of 111 ICF categories describing impairment in ILD for use in the clinical setting with potential transferability for clinical trial use, especially with regard to optimization of PROMs. The importance of ICF Core Sets is heightened in rare or commonly misunderstood diseases and their manifestations, as they are intended to provide an assembly of biophysical and psychosocial features relevant and important to a health condition. In so doing, they can provide a clinical focus for patient experiences of disease that may often go under-recognized; and can potentially be teaching tools to familiarize clinicians with the patient experience of rarer diseases.

Disability is the impact of a health condition on a person's global functioning characterized by body-level impairments, society-level participation limitations and impact on psychological well-being. "*Activities and Participation*", representing >70% of the linkages identified in our study, is possibly the most relevant ICF component to a patient's experience of disability. This was demonstrated in previous studies (Saketkoo et al., 2014a; Saketkoo et al., 2014b) where patients with ILD almost exclusively discuss their condition in terms of activity and life participation aspects.

Considering real-world examples of an ICF Core Set or Checklist can help illustrate utility. An initial evaluation of a person with ILD in pulmonary rehabilitation, for example, elicits the top three concerns of living with ILD of which "*coughing spells*" is stated by the patient as the most pressing priority. The therapist advises that there are several strategies that can be taught and practiced in pulmonary rehabilitation to help manage and recover from coughing spells. The therapist might then work with the patient using the Core Set in ILD to identify the relevant categories that reflect life activities that they feel are most impaired by their experience of cough. Each of these patient-indicated categories can then be monitored over time via use of a numeric rating scale (NRS). The presentation of the Core Set helps support patient discussion in developing a personalized tool to gauge patient-perceived progress in areas that are of high priority for the patient. During the therapist's query the patient expresses *urinary incontinence* during coughing spells has become a major issue. Though *urinary incontinence* may not be a part of the Core Set for

TABLE 5 Categorical concepts unique to a single instrument (*Courtesy LA Saketkoo, with permission, rights reserved.*)

ICF descriptor	ICF category	Instrument
Structure of lungs	s4301	HRCT
Psychic Stability	b1263	SF-36
Emotional Functions	b152	SF-36
Pain in stomach or abdomen	b28012	LCQ
Quality of voice	b310	LIPF
	b3101	LCQ
Respiration functions not specified: cough with deep inspiration	b4408_cough with deep inspiration	LIPF
Sensations associated with cardiovascular and respiratory functions	b460_air hunger/gasp	K-BILD
	b460_chest tightness	K-BILD
	b460_wheeze/whistling sound	K-BILD
Additional sensations of the cardiovascular/respiratory system specified	b469_at rest	LIPF
	b469_bouts of coughing	LCQ
	b469_cough/ing with deep inspiration	LIPF
	b469_cough/ing with over-exertion	LIPF
Pace Self Throughout Day	d2309_pace self	LIPF
Speaking	d330	LCQ
Using telecommunication devices	d3600	LCQ
Kneeling	d4102	SF-36
Fine hand use	d440	SF-36
Changing position between sitting and standing	d4103, d4104	UCSD-SBQ
Hand/arm use	d445	SF-36
Moving around outside the home	d4601	UCSD-SBQ
Eating	d550	UCSD-SBQ
Sexual Relationships	d7702	UCSD-SBQ

ILD, it will be added to the patient's list as a complication of cough that will be monitored over time.

The ICF linkage process, however, is limited in its transferability. For example in the case of PROM, ICF linkage demands that each question from a PROM be dissected into its single-concept units, with each linkage reflecting one concept of a question-item. Although inferences from an entire question-item can be made, the process does not accommodate joining of concept units to reflect the entirety of a question-item's meaning.

ICF categories are dual-edged in that they are specific yet also generic. Each ICF category is sufficiently generic and able to be linked to many different health conditions (e.g. "*ability to concentrate*", *b140*, may apply to diabetes, heart failure, traumatic brain injury, etc.), thus providing a comprehensive yet feasible system for tracking a type of disability. Whereas, the specific nature of the categories has potential to accurately capture the nature of symptomatic impairment. For example, *b4402*, "*depth of inspiration*" is a highly granular descriptor of a respiratory function (*b440*), and further still the following categories describing respiratory muscle function (*b445*) alone or in combination distinctively characterize respiratory muscle weakness: b4450, "*thoracic*" versus *b4451*, "*diaphragmatic*" versus *b4452*, "*accessory*" respiratory muscle function.

Some areas of ICF categories, can also be abstract, and devoid of contextual life circumstances. Such bare generic descriptions confer feasibility for clinical documentation purposes but pose limitations on accuracy and meaningfulness of patient query. In contrast, a single question from a PROM often contains several converging concepts reflective of a patient's experience of that impairment, and thus an isolated ICF category may not hold strong patient-reported relevance a potential vulnerability in the ICD-11 implementation. The ICF is an evolving system that can tolerate expansion to include ICF categories that more closely reflect patient experiences of specific impairments and, thus, improve accuracy of patient responses.

Most included PROMs were developed with careful qualitative methods but have yielded significant conceptual variation. For example, the *Dyspnea-12 (D-12)* and *L-IPF* provide an ICF category, *b4402*, "*depth of respiration*", which is a ubiquitous concern of patients with ILD (Saketkoo et al., 2014a; Saketkoo et al., 2014b) yet appears only in these two PROMs. This is also true of *d7702*, "*sexual relationships*", which is meaningful to patients but only found in the *USCD-SBQ*. This

TABLE 6 Additional considerations for ICF ILD linkages from instruments and domains (*Courtesy LA Saketkoo, with permission, rights reserved*)

ICF components	Newly proposed descriptors	Instrument
Health Condition	General Health	SF-36
	HC_chest condition	SGRQ, K-BILD
	HC_chest problem	SGRQ
	HC_IPF	LIPF
	HC_lung complaint	K-BILD
	HC_lung disease	K-BILD
	HC_pulmonary fibrosis	PF/IPF-PROM
Not Defined	Mortality_nd	MORTALITY
	VAS-PG_nd	VAS-PG
	nd_getting worse	K-BILD
	nd_how much of the time	K-BILD
	nd_a problem	LIPF
	nd_day to day life	LIPF
	nd_hassle	LIPF
	nd_need to rest	LIPF
	nd_physical activity	LIPF
	nd_tickle in throat	LIPF
Personal Factors	pf_afraid/panic when can't get breath	SGRQ
	pf_agitated	D-12
	pf_annoying	LIPF
	pf_anxious	LCQ, K-BILD
	pf_avoid	K-BILD
	pf_cope with uncertainty	PF/IPF-PROM
	pf_cough caused worry about illness	LCQ
	pf_cough interfered with joy of life	LCQ
	pf_depressed	D-12, K-BILD
	pf_distressing	D-12
	pf_embarassing/ed	SGRQ, LIPF
	pf_embarrassed by cough	LCQ
	pf_exercise not safe	SGRQ
	pf_expected/anticipated	K-BILD
	pf_fear	LIPF
	pf_fear of hurting self by overexertion	UCSD-SBQ*
	pf_fear of shortness of breath	UCSD-SBQ*, PF/IPF-PROM
	pf_fed up	LCQ, K-BILD
	pf_felt in control	K-BILD
	pf_feel in control of cough	LCQ
	pf_frail/invalid	SGRQ
	pf_frightening	LIPF
	pf_frustrated	PF/IPF-PROM, LIPF
	pf_frustrated by cough	LCQ
	pf_frustrated by being tired	PF/IPF-PROM
	pf_get sick easier than others	SF-36
	pf_irritating	D-12, K-BILD
	pf_miserable	D-12
	pf_not in control of chest problem	SGRQ, K-BILD
	pf_quality of life	LIPF
	pf_think about death	K-BILD
	pf_worry	PF/IPF-PROM
	pf_worried about serious illness	K-BILD

lack of capture occurs in other health conditions, such as myositis, where *intimacy* and *sexual relations* are of high patient-reported importance in more private data-collection circumstances, such as surveys or semi-structured interviews. Patients may be more reticent to supply ultra-sensitive information in larger focus groups (Alexanderson et al., 2002; Alemo Munters et al., 2011).

In contrast, single-occurring linkages may also demonstrate weak relevance to the specific disease. In this study, *d440*, "*fine hand use*" from the *SF-36*, does not provide information relevant to ILD. However, the reason for continued use of a generic measure like the *SF-36* lies in its global validation across diseases, making it an essential anchor and comparator.

The utility of an ICF Core Set in ILD also enables application to a CTD-ILD. Using the example of RA for which an ICF Core Set already exists, the current recommendation for RA-ILD would be to combine two separate ICF Core Sets, one for ILD and the RA (Stucki and Cieza, 2004). The development of ICF Core Sets in more multi-organ system predominant conditions like systemic sclerosis (SSc), idiopathic inflammatory myopathies (IIMs) or sarcoidosis, are likely to incorporate an ILD ICF Core Set into their frameworks (Saketkoo et al., 2012b; Saketkoo et al., 2012c).

An important example highlighting the influence of analytic approach on patient-relevant concepts arose during *K-BILD* development. Academic curiosity spurred applications to the original dataset using Rasch analysis (resulting in the currently circulating *K-BILD-R*) and also item response theory resulting in *K-BILD-I*. Remarkably, only *K-BILD-R* retained the questions regarding financial solvency; while only *K-BILD-I* retained items on fatigue, a predominant concept in other ILD PROMs and both are crucial concepts for people living with ILD. This finding led to group discussion regarding "fatigue" persistently being relegated to a function of HRQoL in ILD. While it was agreed that there is value in re-examining "fatigue" in ILD as a complex and multi-dimensional core symptom domain (De Vries et al., 2000; De Vries et al., 2001; De Vries and Drent, 2006; Saketkoo et al., 2014a; Hendriks et al., 2018; Kølner-Augustson et al., 2020).

The ICF linkage provides an expansive view of the great wealth of these PROMs that may together be harmonized into a streamlined instrument incorporating the optimal aspects of each. Such an effort would entail wide global engagement of patients and patient partners in tightly iterative applications of consensus methodology and testing (Stucki and Cieza, 2004).

Recommendations for lung disease in the WHO ICF classification system

The ICF was designed to be broadly comprehensive rather than detail exhaustive. The ICF structure was intended to be responsive to modification and development over time, exemplified by the ICF evolution of "*Personal Factors*". Our instrument linkage demonstrated essential symptoms related to chronic pulmonary and cardiovascular diseases that require consideration for inclusion in future ICF updates. Three of these essential areas are described below.

Biophysiologic mechanisms discussion

Cough is an intrinsic experience creating significant impairment in pulmonary disease. In ILD a restrictive physiology, inspiratory and predominantly dry cough creates significant interference in life activities. Phlegm production, though not as frequent or troubling in ILD as in COPD, is an essential descriptor of cough. Temporary placeholder categories were created through this investigation (e.g. b450_xx, b460_ xx or b469_xx) to capture the intrinsic in ILD and lung disease that currently lack sufficient representation in ICF language. Enduring additions to ICF were proposed to directly designate cough, inspiratory cough, dyspnea, wheeze, air hunger and phlegm production.

Psychosocial function discussion

Though not a direct bio-physical manifestation of pulmonary disease, patients perceive psychosocial impact as an intrinsic experience of ILD (De Vries et al., 2000; Swigris et al., 2005; Saketkoo et al., 2014a; Saketkoo et al., 2014b), especially when biophysical symptoms are present with rest or slight exertion, resulting in disabling breathlessness or cough and which appear to be deeply entwined with embarrassment, frustration, fear, safety with exertion, and loss of control. Capturing and describing the disease-related psychological impact of a non-psychiatric health condition is a current challenge within the ICF.

Several instruments contain question-items that measure degrees of frustration, disgust, embarrassment, distress, fear or sense of safety with exertion. Embarrassment, one such frequent descriptor, as an example, greatly impacts psychological functioning and coping with cough which is a ubiquitous experience drawing both visual and auditory unwanted attention in ILDs (De Vries et al., 2000; Swigris et al., 2005; Key et al., 2010; Jones et al., 2011; Theodore et al., 2012; Saketkoo et al., 2014a; Saketkoo et al., 2014b) and other lung conditions. These concepts are strongly echoed in ILD patient qualitative data (De Vries et al., 2000; Swigris et al., 2005; Saketkoo et al., 2014a; Saketkoo et al., 2014b) and perceived to influence level of functioning. These would be important dynamics for an ILD ICF Core Set but are yet awaiting evolution within ICF under the "personal factors" component.

Physical function discussion

The ICF's generic nature can impede accurate symptom query and may warrant added contextualization of ICF descriptors. Activity-descriptors common in respiratory-related PROMs and patient-reported visit history that require significant cardiopulmonary exertion, such as mowing lawn and

shoveling snow, are not sufficiently defined by current ICF categories. The closest combined categories for "mowing lawn" are d4551 "pushing" which is intended to describe upper extremity function buts lacks sufficient discrimination between cardiopulmonary, muscular or joint capability and d6505 "taking care of outdoor plants'. Elements of cardiopulmonary recovery (stopping, resting mid-activity), pace (e.g. performing more slowly; potentially related to recovery); punctuation of disease behavior (such as "attack", "flaring") as well as frequency of symptoms are temporal and dynamic associations essential to pulmonary disease requiring future ICF updates.

A proposal for future ICF revisions, is the addition of a discrete category or an additional component to the ICF that describes the global perception of disease burden from the patient's perspective. The *VAS-PtG* is widely validated across many diseases as a reliable marker, sensitive to change and correlative with objective measures of disease activity (Singh et al., 2011; Bartlett et al., 2012). Its inclusion into the ICF could enhance report of perceived function and burden of disease as well as the incremental impact of modification of environmental factors.

Future steps

This study's identification of 111 ICF categories, 34 "*personal factors*" and multiple further descriptors under "*health condition*" (*hc*) and "*not defined*" (*nd*) will develop into a manageable ICF Core Set over time (Kostanjsek et al., 2011; Finger et al., 2014). Our continuance of iterative multi-disciplinary methodological applications that include patient research partners as essential team members will refine and identify the most relevant and important concepts of both the somatic and psychosocial realms experienced by people living with ILD. Pursuant to this is working with the WHO ICF to address the expansion of descriptors to accurately reflect functional impairment intrinsic to living with ILD. Discussions amongst the authors presented creating central pathways to assess side effects of disease-related treatment beyond being under "*environmental factors*" but rather under "b" and "d" categories (Proesmans et al., 2019). Finally, the dedicated efforts in the development of past PROMs have provided a wealth of information that can result in a potentially streamlined exquisitely responsive instrument.

Conclusion

This is the first effort to examine ICF Core Sets in ILD. This investigation provided an important and useful step to facilitating clinician preparation for ICD-11 and other performance quality assessments that will require ICF use (Iezzoni and Greenberg, 2003; Health and Human Services/Centers for Medicare and Medicaid Services, 2000; SocialSecurity Administration, 2013; Escorpizo and Stucki, 2013). The utility of disease-specific ICF Core Sets is multi-factorial on individual, regional and global levels offering value to epidemiologic, health economics, clinical assessment, PROM development and comparison for fair representation in policy, service provision and research funding assessments as well as the potential development of concise PROMs. Future steps may build on harmonizing these PROMs to widely validate concepts, context and language in ILD. Our investigation identifies new ICF categories, for general pulmonary disease to be considered in the future ICF revisions.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

Conception and design: LAS, RE, JV, KJK, KF, HA, HP, JP, MR, CV, SSB, DL, CS, MBS, MRL, OD, and AMR. Analysis and interpretation: LAS, RE, JV, KJK, KF, SSB, DL, CS, OKB, OD, LS, ERV, EB, TF, ONO, MD, KCP, and AMR. Manuscript drafting for important intellectual content: LAS, RE, JV, KJK, HAC, SSB, HA, HP, JP, MR, JL, LS, ER, JV, ML, MBS, OKB, OD, ERV, EJB, TF, CV, ONO, MD, KCP, and AMR.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.979788/full#supplementary-material

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