MECHANISMS OF LUNG FIBROSIS: IS IMMUNITY BACK IN THE GAME?

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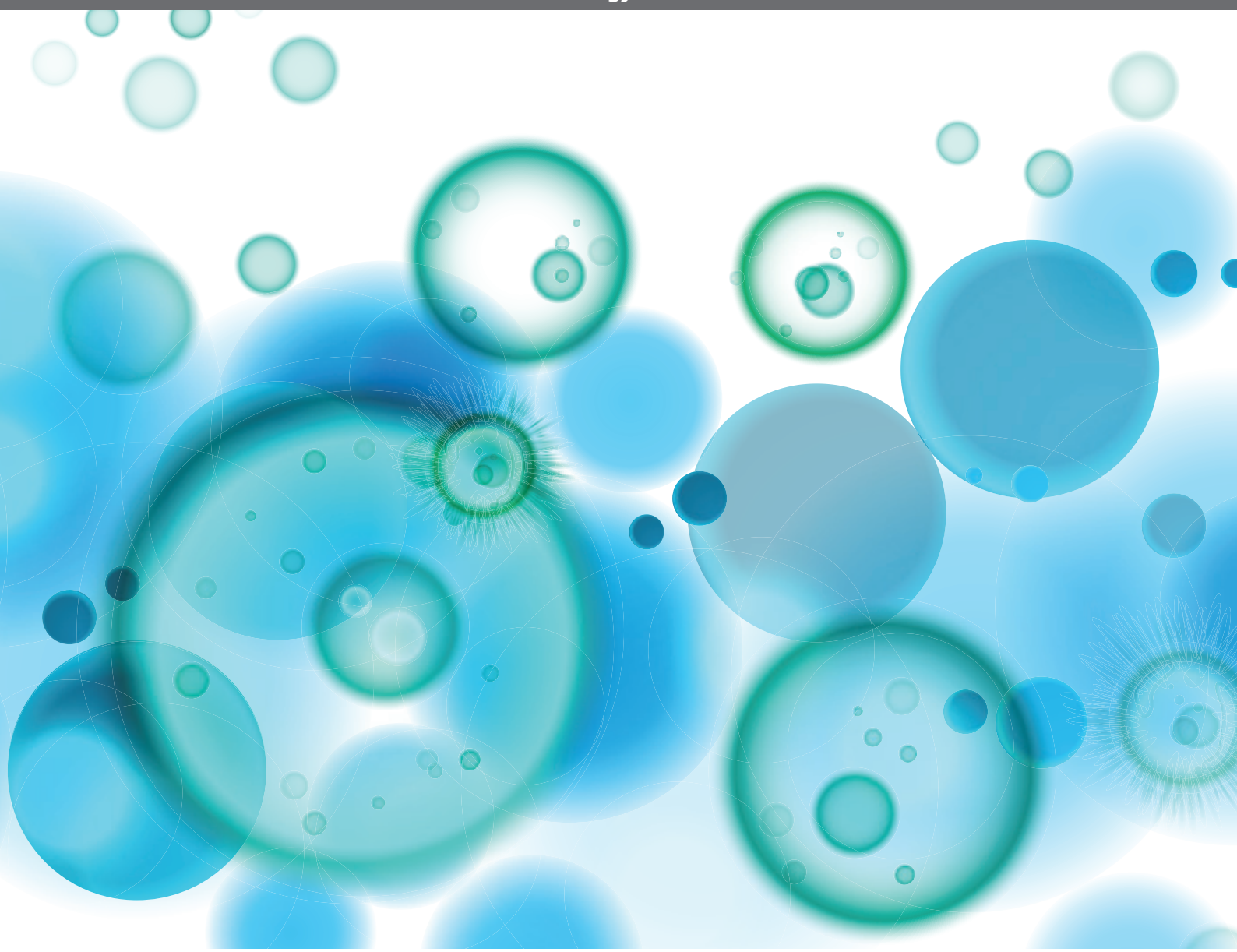
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88974-877-8
DOI 10.3389/978-2-88974-877-8

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MECHANISMS OF LUNG FIBROSIS: IS IMMUNITY BACK IN THE GAME?

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Citation: Conte, E., Antoniou, K., Bocchino, M., Froidure, A., eds. (2022). Mechanisms of Lung Fibrosis: Is Immunity Back in the Game? Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-877-8

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frontiers in Immunology

EDITORIAL
published: 17 March 2022
doi: 10.3389/fimmu.2022.882979

Editorial: Mechanisms of Lung Fibrosis: Is Immunity Back in the Game?

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Keywords: lung fibrosis, inflammation, immunity, inflammasome, fibrogenesis

Editorial on the Research Topic

Mechanisms of Lung Fibrosis: Is Immunity Back in the Game?

Pulmonary fibrosis is a common feature to many interstitial lung diseases (ILDs). Among these, idiopathic pulmonary fibrosis (IPF) represents the paradigm of a "pure fibrotic" condition characterized by an intrinsic aggressiveness with inexorable evolution. Nevertheless, inflammation-driven ILDs, including for example connective tissue disease-associated ILDs (CTD-ILDs), sarcoidosis, chronic hypersensitivity pneumonitis, desquamative interstitial pneumonitis (DIP), can acquire a fibrosing phenotype which may lead to an accelerated lung function decline and tissue distortion, similarly to IPF (1). This clinical scenario, along with the discovery of common pro-fibrotic pathways, has led to the most recent concept of progressive fibrosing ILDs (PF-ILDs) (2). These entities must be carefully intercepted due to their rapidly evolving clinical behavior and unresponsiveness to conventional anti-inflammatory and immune-suppressive drugs. Following the failure of the PANTHER trial (3), the role of the immune system in the pathogenesis of fibrotic lung diseases has been minimized. Interestingly, recent data reveal that the subtle interplay between immune dysregulation and fibrogenesis produces a converging effect that may ultimately lead to end-stage fibrosis. On top of that, there is wide evidence that the natural history of IPF, and potentially of any other ILD, may be diverted from the interoccurrence of unpredictable acute exacerbations (AEs) displaying a strong and uncontrolled inflammation (4). In the last years, the availability of two anti-fibrotic drugs, i.e. nintedanib and pirfenidone, has improved the clinical management of IPF patients slowing disease progression and preventing both AEs and hospitalization (5, 6). Their use also in patients affected by PF-ILDs appears as an increasingly concrete perspective in clinical practice (7, 8). Nevertheless, any effort should be made to tidy up the puzzle of lung fibrogenesis with the primary aim to open the way to more innovative and hopefully resolving treatment opportunities.

Immune mechanisms that may trigger, enhance, or modulate the intricate process of lung fibrosis are the focus of the current Frontiers Research Topic. Original research and review articles cover cumulating evidence of the role of macrophages, monocytes, lymphocytes, and other immune cell types such as innate lymphoid cells and dendritic cells in the process of lung fibrosis.

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *24 February 2022*
Accepted: *28 February 2022*
Published: *17 March 2022*

Citation:
Froidure A, Antoniou K, Bocchino M and Conte E (2022) Editorial: Mechanisms of Lung Fibrosis: Is Immunity Back in the Game? Front. Immunol. 13:882979. doi: 10.3389/fimmu.2022.882979

With specific reference to the Research Topic contents, Li et al. have shown that chronic hypoxia, a salient feature of pulmonary fibrosis, is able to trigger in IPF a specific immune response involving M1 and M0 macrophages, CD8 T cells, CD4 memory T cells, and mast cells. Very interestingly, the Authors have identified a hypoxia-immune-related gene signature that predicted disease prognosis. This signature, validated in 3 patient cohorts, is very promising in paving the way for future biomarkers. To demonstrate the close relationship between inflammation and fibrogenesis, Reynaud et al. have found that the loss of Club cells in the small airways of IPF patients was associated with aberrant regeneration processes. The data showed a not negligible relapse in clinical terms as lower SCGB1A1 expression, a Club cell marker, correlated with the extent of traction bronchiectasis on chest high-resolution computed tomography. Pro-fibrotic and pro-inflammatory profiles of airway macrophages (AMs) from IPF and PF-ILD patients were the focus of three additional original article contributions to the issue. Collagen-1a1 expression by AMs was described by Tsitoura et al. Profibrotic AMs co-expressing COL1A1 and OPN increased in the BAL of patients with IPF and other ILDs, while UIP pattern and a subsequent progressive phenotype were significantly associated with the higher BAL COL1A1 levels. Importantly, in non-IPF patients, higher COL1A1 levels were associated with a more than twofold increase in mortality. Additional data on the immune-inflammatory involvement in fibrogenesis comes from the observation that mitochondrial oxidation and alterations in bacterial burden in IPF and other ILDs may lead to augmented inflammasome activity in airway macrophages (AMs) (Jäger et al.). In this context, Trachalaki et al. have shown that NLRP3 was more inducible in IPF than in other ILDs in AMs, and that AIM2 inflammasome activation led to the expression of interleukin (IL)-1β, a key cytokine with both pro-inflammatory and pro-fibrotic properties. Whether this finding can be translated to explain the genesis of AEs remains elusive. Finally, inflammation-related pathways could constitute therapeutic targets outside "classical" immune-suppression. This is what was suggested by Steele et al. who showed that the TNF superfamily member 15 (TL1A) induced mucus production, inflammation and fibrosis through the expression of IL-13 by innate lymphoid cells. In a preclinical model of house dust mite-induced asthma, neutralization of TL1A by genetic deletion or antagonistic blockade of its receptor DR3 prevented all these events. This latter finding clearly illustrates how modulating inflammation could protect from fibrosis as in muco-secretory fibrotic diseases like severe asthma.

Additional contributions to this Frontiers Research Topic provide a quite extensive overview on current evidence of the involvement of frontline cell players like alveolar macrophages and epithelial cells in lung fibrogenesis. The interplay between the epithelium and immune cells is indeed able to significantly affect the lung microenvironment shaping a pro-fibrotic milieu. This picture may happen through the engagement of different pathways and mediators, as discussed in the papers by Plante-Bordeneuve and Froidure, and Kishore and Petrek, and is a prerequisite for the identification of promising therapeutic targets. Similarly, abnormal mucosal immunity along with the dysfunction and imbalance of dendritic cell subsets are intriguing and still poorly explored areas whose understanding will enrich our background of knowledge (Bocchino et al.). The role of regulatory immune cells was also reviewed (van Geffen et al.).

The commonalities of ARDS, COVID-19 and ILDs/IPF are also highlighted in this collection by Ntatsoulis et al. The authors emphasize on the increased levels of autotaxin (ATX) among the three syndromes and suggest that lysophosphatidic acid (LPA) signaling might be a shared pathogenetic pathway implicating amplified vascular damage, immune cell activation and promotion of fibrosis.

In conclusion, we believe that the characterization of the immune phenotype of IPF and fibrotic ILDs will help to discriminate endotypes with different clinical behavior thus allowing a personalized approach to the patient. In line with this expectation, Huaux proposes an interesting perspective on how we should interpret these findings and exploit them as attractive targets for future therapies. Our wish is that we may dispose in a not-too-distant time of increasingly effective treatments that can intercept and stop early aberrant fibrogenesis for the benefit of ILD patients.

AUTHOR CONTRIBUTIONS

All authors contributed to the redaction of the manuscript and approved its final version. AF and EC finalized redaction.

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frontiers in Immunology

ORIGINAL RESEARCH
published: 26 February 2021
doi: 10.3389/fimmu.2021.630096

Club Cell Loss as a Feature of Bronchiolization in ILD

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Background: Distal airway metaplasia may precede honeycombing in progressive fibrosing interstitial lung disease (ILD). The SCGB1A1$^+$ bronchiolar-specific club cell may play a role in this aberrant regenerative process.

Objective: To assess the presence of club cells in the small airways of patients suffering from ILD.

Methods: Small airways (internal diameter <2 mm) in lung samples [surgical lung biopsy (SLB) and/or transbronchial lung cryobiopsy (TBLC)] from 14 patients suffering from ILD and 10 controls were morphologically assessed and stained for SCGB1A1. SCGB1A1 was weighted by epithelial height as a marker of airway generation (SCGB1A1/EH). Correlations between clinical, functional, and high-resolution CT (HRCT) prognostic factors and histomorphometry were assessed.

Results: Small airways from samples with ILD patterns were significantly less dense in terms of SCGB1A1$^+$ cells [0.064 (0.020–0.172)] as compared to controls' sample's small airways [0.393 (0.082–0.698), $p < 0.0001$]. Usual interstitial pneumonia (UIP) patterns most frequently contained small airways with limited or absent SCGB1A1 expression (SCGB1A1/EH <0.025): UIP (18/33; 55%) as compared with non-UIP patterns (4/31; 13%) or controls (0/29; 0%): $p < 0.0001$. In addition, correlations with HRCT indicated a significant negative relationship between SCGB1A1 and bronchiectasis as a feature of bronchiolization (Rho −0.63, $p < 0.001$) and a positive relationship with both forced vital capacity (FVC) and Hounsfield unit (HU)-distribution pattern in kurtosis (Rho 0.38 and 0.50, respectively, both $p < 0.001$) as markers of fibrotic changes.

Conclusion: Compared with controls, the small airways of patients with ILD more often lack SCGB1A1, especially so in UIP. Low densities of SCGB1A1-marked cells correlate with bronchiectasis and fibrotic changes. Further research investigating SCGB1A1 staining as a pathological feature of the bronchiolization process is merited.

Keywords: club (clara) cell, idiopathic pulmonary fibrosis, metaplasia, SCGB1A1, bronchiolization, interstitial lung disease

OPEN ACCESS

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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *16 November 2020*
Accepted: *25 January 2021*
Published: *26 February 2021*

Citation:
Reynaud P, Ahmed E, Serre I, Knabe L, Bommart S, Suehs C, Vachier I, Berthet JP, Romagnoli M, Vernisse C, Mallet JP, Gamez AS and Bourdin A (2021) Club Cell Loss as a Feature of Bronchiolization in ILD. Front. Immunol. 12:630096. doi: 10.3389/fimmu.2021.630096

INTRODUCTION

Interstitial lung disease (ILD) are devastating affections nearly constantly leading to respiratory failure and death. The early phases of the disease are hallmarked by progressive fibrosis in the peripheral zones of the lung, which is associated with lung function decline (1). High-resolution CT (HRCT) has become the gold standard for diagnosing such fibrotic changes and characterizing their progression (2). Notably, the presence of traction bronchiectasis (the enlargement of small airways by traction) is now considered a red flag for subpleural fibrotic changes. Interestingly, histomorphological assessments of these fibrotic areas consistently report bronchiolar metaplasia of unclear origin (3). The latter "bronchiolization" may represent aberrant regeneration of the lung in a profibrotic environment, where distal airway precursors never reach their potential as functional alveoli (4). The result is the presence of proximal features in the distal compartment currently detectable only *via* histopathology (5).

Club cells are thought to be highly expressed at the small airway level (6), where they are supposed to play a protective role against noxious inhaled particles. Among the latter, cigarette smoke and occupational dust have also been implicated in the pathogenesis of idiopathic pulmonary fibrosis (IPF) (7, 8). Interestingly, club cells were also attributed an accessory stem cell role at the small airway level, but this role has mostly been evidenced in animal models at the bronchoalveolar duct junction, where they are preferentially located (9). Due to recent advances in single-cell biology, it is now clear that these cells are heterogeneous and not all are playing the same role. Currently, they are considered as differentiation of basal cells, and the transition from basal to club cells has been ontogenically evidenced. Additionally, club cells are situated at the crossroads of the epithelial phenotype. Their natural trend is to progressively acquire features of goblet cells, but some can also move toward ciliogenesis. Whether or not appropriate transition pathways are failing during the bronchiolisation process remain unclear.

New opportunities for lung tissue sampling are provided by the arrival of transbronchial lung cryobiopsies (TBLC). Beyond clinical and technical considerations, it seems that TBLC, as compared to traditional surgical lung biopsies (SLB), samples airway-centered, more proximal tissue, and this is likely to change not only the pathological patterns and eventually the final pathological diagnosis but also the representation of the different compartments. In particular, airways contained in TBLC samples are more likely to represent earlier generations than those taken in the more peripheral SLB samples (10). Intuitively, the small airways present in TBLC samples might then be less frequently affected by bronchiolization. Given the relationship between this process and the fibrotic changes affecting the lung in progressing ILD, these pathological changes may have some diagnostic and/or prognostic value identifiable by clinical correlations in a cross-sectional study.

The objective of this study was to assess the presence of club cells in the small airways of patients suffering from ILD by combining pathology, physiology, and HRCT data.

We hypothesized that "protective" club cells characterized by their specific protein SCGB1A1 might be deficient in fibrotic diseases and particularly at the site where bronchiolar metaplasia occurs.

MATERIALS AND METHODS

CryoPID [NCT02763540] was a prospective trial evaluating the diagnostic concordance between TBLC and SLB, which were performed sequentially in the same patients between January 2016 and March 2018 at the Montpellier University Hospital (11). The study included patients with ILD requiring an SLB as per a multidisciplinary approach decision, given an undefined HRCT pattern. For the current histology assessment, 14 CryoPID patients were compared with 10 control subjects randomly selected from the bio-bank of the Montpellier University Hospital. Pathological patterns reported from SLB and TBLC together by a pathologist involved in the present study were used. Controls had normal lung function, were free of chronic respiratory disease, and matched, as well as a possibility for smoking, in gender and age with the ILD population. They had undergone a lobar resection for a colic metastasis in the last 3 years. Lung parenchyma at a large distance from the metastasis was analyzed.

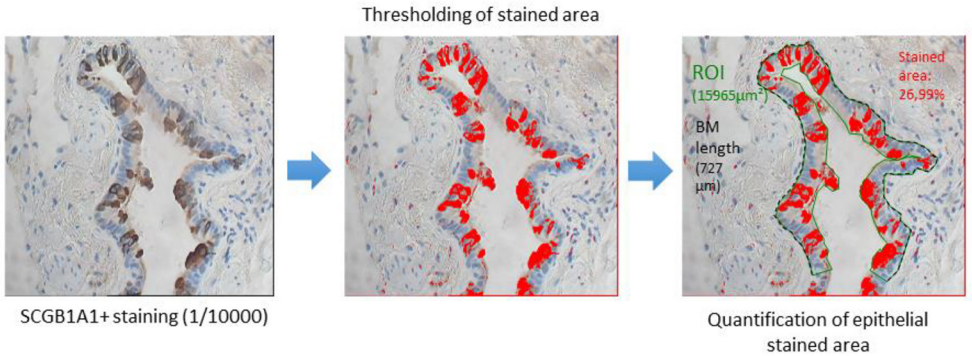

FIGURE 1 | After SCGB1A1 staining, small airway epithelia were isolated *via* thresholding, perimeters, thickness/height, and by the area quantified.

Briefly, lung samples were fixed in 4% formalin at pH 7.4 and embedded in paraffin. Three-millimeter-thick sections were stained by H&E. Lung samples were anonymized and blindly assessed by an expert pathologist (TVC). Serial sections were used for SCGB1A1 immunostaining (dilution 1/10,000; Biovendor, Czech Republic). For some slides, a Bleu Alcian staining could also be performed.

Small airways (identified by an internal diameter of 2 mm or less) were assessed in all samples. Morphometry was assessed using the Cell P software (Olympus, Tokyo, Japan). Bronchiolization was referred to small airways with evidence of proximal features, including the presence of ciliated and/or goblet cells, as per the previously published standards (12, 13). Epithelial area, epithelial height (EH), and the percentage of $SCGB1A1^+$ immune-stained area were determined for each small airway. The $SCGB1A1^+$/EH ratio was computed to adjust for variations known to affect club cell density through airway generation (**Figure 1**). The small airway density for each sample was based on a semi-quantitative scale ranging from 0 (absence of small airways) to 5 (highest small airway density). The absence or near-absence of club cells was defined by a SCGB1A1/EH ratio <0.025 by taking into account the background noise.

Prognostic factors were recorded and included clinical (age, gender, smoking history), biological/laboratory [arterial oxygen pressure (PaO_2), bronchoalveolar lavage cellularity (BAL)], and functional [forced vital capacity (FVC), diffusing capacity of the lungs for carbon monoxide (DLCO)] criteria.

A visual tomodensitometric analysis scored fibrotic changes (honeycombing and reticulations), intensity, and the spread of ground-glass opacity (GGO) (14), as well as the extent of bronchiectasis [ranking the severity of bronchiectasis by a lobe on a scale ranging from 0 to 3 (15) by acquiring consensus among two experienced thoracic senior radiologists (SB and GD)].

Automatic quantification *via* the Thoracic VCAR software (GE HealthCare, USA) was also performed, classifying pixels from the whole lung (WL) volume into four groups as proposed by Shin et al. (16) (<-950 HU: "emphysema," -950 to -700 HU "functional parenchyma," -700 to -500 HU "interstitial disease," >-500 HU "extra parenchymal"). Three prognostic variables were then derived: total lung capacity (TLC), percentage of normal lung (%NL), and mean lung attenuation (MLA) (17). Pulmonary attenuation distribution histograms were obtained for assessing skewness and kurtosis, previously reported as having the prognostic value (18).

TABLE 1 | Population characteristics.

	ILD [Median (IQR25–75)]	Controls [Median (IQR25–75)]	*p*-value
N	14	10	
Age (years)	63 [43.5–65]	64 [57.5–67.5]	0.21
Gender (W/M)	10 vs. 04	3 vs. 7	0.0987
Weight (kg)	65.5 [60.65–81.75]	65 [62.25–83.75]	0.49
Height (cm)	160 [158.5–170]	166.5 [160–170]	0.44
Smoking history (p.y)	2.5 [0–28.75]	19 [1.25–30]	0.77
Smoking status (current-former-never)	1/5/8	1/5/4	
Positive autoantibodies	0	0	
PaO_2 (mmHg)	84.2 [75.75–88.28]	88.65 [81.29–93.5]	0.15
FVC (l)	2.3 [1.74–3.31]	3.43 [2.75–4.07]	0.027*
FVC (% pred values)	84.5 [68.5–2.77]	100 [92.25–107.5]	0.004*
FEV1 (l)	2.1 [1.59–2.77]	2.39 [2.02–3.39]	0.077
FEV1 (% pred values)	84 [72.75–90]	95.5 [86–110.8]	0.010*
TLC (l)	3.69 [3.55–4.49]	6.23 [6.16–6.59]	0.001*
TLC (% pred values)	77.5 [66.25–84.75]	103 [101–106]	0.00005*
RV (l)	1.57 [1.31–1.79]	2.64 [2.58–3.12]	0.0002*
RV (% pred values)	89 [65–102]	116 [100–121]	0.0049*
TLCO (% pred values)	56 [40–60]	76 [70–80]	0.0097*
KCO	79 [73–96]	85 [78–103]	0.58
Kurtosis	6.33 [4.32–7.48]	14.77 [13.78–17.16]	0.0017*
Skewness	2.29 [2.02–2.53]	3.59 [3.39–3.85]	0.00014*
Whole lung (WL)	3.44 [2.79–4.12]	5.61 [3.54–6.17]	0.1095
% Normal lung (NL)	79.98 [71.14–82.04]	88.72 [88.2–90.25]	0.00045
Mean lung attenuation (Hounsfield)	−764 [−796 to −720]	−838 [−859 to −819]	0.0012*
Fibrotic score	15 [10–22.5]	0 [0–0]	0.00006*
Ground glass opacity	5 [5–5]	0 [0–0]	0.059
Bronchiectasis score	6 [5.25–8]	0 [0–0]	0.00003*

%NL, percentage of normal lung; BMI, body mass index; CT, computed tomography; DLCO, diffusing capacity of the lungs for carbon monoxide; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; ILD, interstial lung disease; IQR, interquartile range; KCO, transfer coefficient of the lung for carbon monoxide; MLA, mean lung attenuation; N, number; PaO_2, arterial oxygen pressure; RV, residual volume; SD, standard deviation; TLC, total lung capacity.

*$p < 0.05$.

Statistical Analysis

Parametric and non-parametric tests were used to describe and compare normally and non-normally distributed data as appropriate. Spearman's correlation coefficients were computed for testing variable relationships.

RESULTS

Population Description

Fourteen patients with ILD and 10 controls were studied (**Table 1**). As expected, patients with ILD had worse pulmonary function tests and higher HRCT semi-quantitative scores. Of note, as these patients were deemed eligible for SLB, most had a mild functional impairment, suggesting that many were at an early stage of the disease. As previously reported (11), discrepancies in the pathological diagnosis between SLB and TBLC existed, potentially leading to different final diagnoses during a second multidisciplinary assessment (**Table 2**). For subsequent classification as usual interstitial pneumonia (UIP) and non-UIP, only samples with a clear and confirmed (high level of confidence) UIP pattern were retained.

Among patients with ILD, the mean SLB sample size was 49.5 mm [41.5–60] compared to 7 mm (6–8) ($p < 0.00001$) for TBLC. A significant difference in the semi-quantitative scoring

of small airway density between SLB and TBLC sample was found (**Table 3**). No difference was found between TBLC and SLB regarding club cell representation in the small airways. Fewer airways positively stained for SCGB1A1 in ILD, in particular, for those with a pathological UIP pattern, and in general, this staining was less intense (**Figure 2A**). This decrease persisted after adjusting on epithelial height as a surrogate marker of the generation of the airway assessed. Hence, SCGB1A1/EH was statistically lower in the small airways of samples from patients with ILD vs. controls (**Figure 2B**; the Kruskal-Wallis test, $p = 7.598e\text{-}07$).

Moreover, sparsely stained airways (defined as SCGB1A1/EH <0.025) were significantly more represented in the UIP pathological patterns [UIP 18/33 vs. non-UIP 4/31 ($p = 0.0027$)] (**Table 2** and **Figure 3**).

The tested prognostic correlations are provided in **Figure 4**. Interestingly, statistically significant non-parametric correlations were found between SCGB1A1/EH and two surrogate markers of lung fibrosis. First, a correlation was found with the kurtosis value of the histogram distribution of lung attenuation assessed at HRCT (Rho = 0.50, $p < 0.001$). The less the lung is involved by fibrosis, the greater the club cell density found in the small airways, as the kurtosis value decreases with the extent of fibrosis in ILD (18). Second, SCGB1A1/EH also positively correlated with FVC (Rho = 0.38, $p < 0.001$), as expected.

The negative correlation found between SCGB1A1/EH and the bronchiectasis scores (Rho = −0.63, $p < 0.001$) is also represented in **Figure 4**. The latter indicates that fewer club cells were present in the small airways of patients with more intense bronchiectasis.

TABLE 2 | Blinded histology and multidisciplinary diagnostic results for each patient.

Patient	Histology diagnosis based on TBLC	Histology diagnosis based on SLB	Diagnosis based on MDA 1 (before histology)	Diagnosis based on MDA 2 (after histology)
1	UIP	UIP	Possible UIP	CHP
2	Non-diagnostic	PLCH	DIP	PLCH
3	UIP	CHP	NSIP	IPF
4	UIP	NSIP	No classification	NSIP
5	RB-ILD	RB-ILD	RB-ILD	RB-ILD
6	Non-diagnostic	UIP	NSIP	IPF
7	PLCH	UIP	Possible UIP	IPF
8	NSIP	NSIP	Fibrotic NSIP	NOS
9	UIP	UIP	NSIP	IPF
10	Non-diagnostic	ALI	NSIP	NOS
11	NSIP	LP	Sarcoidosis	CVID
12	UIP	NSIP	NSIP	NSIP
13	Non-diagnostic	UIP	CHP	IPF
14	UIP	UIP	Possible UIP	IPF

CHP, chronic hypersensitivity pneumonitis; CVID, common variable immune deficiency; DIP, desquamative interstitial pneumonia; HP, hypersensitivity pneumonitis; IPF, idiopathic pulmonary fibrosis; LP, lymphoid process; MDA1, a first multidisciplinary assessment; MDA2, the final multidisciplinary assessment; NOS, not otherwise specified; NSIP, non-specific interstitial pneumonia; OP, organizing pneumonia; PLCH, pulmonary Langerhans cell histiocytosis; RB-ILD, respiratory bronchiolitis-associated interstitial lung disease; SLB, surgical lung biopsy; TBLC, transbronchial lung cryobiopsy; UIP, usual interstitial pneumonia.

TABLE 3 | Samples characteristics.

	SLB (controls)	SLB (ILD)	TBLC (ILD)	*p*-value
N	10	14	14	
Number of small airways assessed	32	35	31	
Small airway density [semi quantitative score (0 = minimal; 5 = maximal)]	2.1 ± 0.1	2.4 ± 0.6	0.4 ± 0.3	0.04
Epithelial area (mean ± SD per bronchiole. μm^2)	20,818 ± 9,040	26,738 ± 11,733	19,638 ± 11,216	ns
Epithelial height (mean ± SD per bronchiole. μm)	37.8 ± 7.8	39.5 ± 11.9	35.8 ± 11.4	ns
SCGB1A1$^+$ stained area	19.7 (4.2–27.7)	3.6 (1.6–7.4)	0.9 (0.3–2.9)	<0.0001
SCGB1A1$^+$/EH	0.5 (0.1–0.7)	0.1 (0.0–0.2)	0.0 (0.0–0.1)	0.02
% airway without club cells[§]	0 (0/32)	14 (5/35)	55 (17/31)	0.01

[§] *Defined by SCGB1A1+/EH ratio < 0.025.*

DISCUSSION

With the aim of understanding the mechanisms of bronchiolization, we found that club cells are absent or nearly absent from these aberrant small airways in the most subpleural areas of fibrotic lungs. This was particularly true in ILD where a UIP pattern could be identified. Bronchiolized areas seem to fit with small airways saliently featuring proximal differentiation patterns very close to terminal airspaces. As expected, both TBLC and SLB could identify these patterns, even though the density of small airways in TBLC is largely lower. Interestingly, the correlation between these bronchiolization patterns (highlighted by the absence of club cells) and clinical prognostic markers (such as HRCT kurtosis and bronchiectasis), as well as physiology (FVC), was demonstrated.

Most of our results were driven by a particularly high number of unstained or sparsely stained airways in lung samples from patients with a UIP pattern. Most of these pauci-SCGB1A1 small airways displayed features of bronchiolar metaplasia. Interestingly, they were referred to as "bronchiolizing areas" in the routine pathology report.

Fukumoto et al. hypothesized that this phenomenon may indicate an impaired differentiation with aberrant proliferation (19). The latter hypothesis is supported by the morphologic appearance of these areas where proximal features are found where they should not be.

The underexpression of the club cell-specific protein SCGB1A1 may be directly linked to the bronchiolization process, as already supposed by Jensen-Taubman (20). A blockade in the

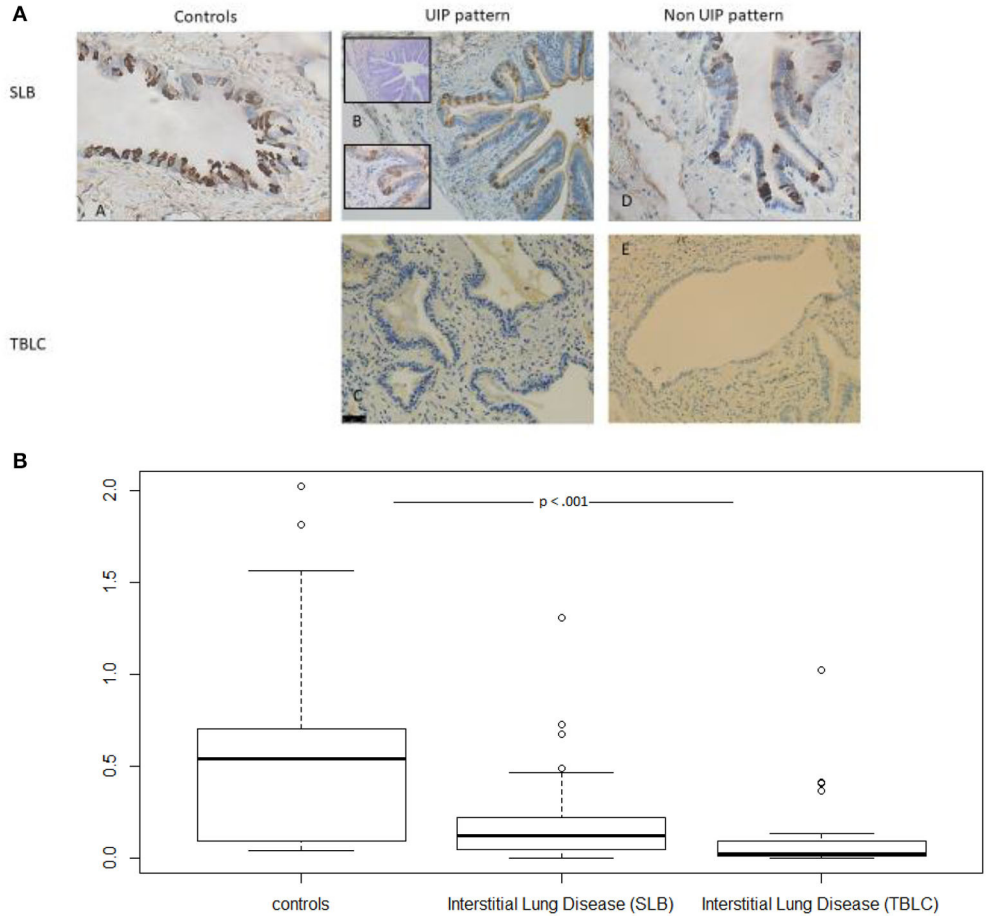

FIGURE 2 | (A) Small airways are immune-stained for SCGB1A1 in controls, UIP, and non-UIP patterns. Upper left: (A) SLB from a control (no TBLC were available in this group). Middle row: (B) Very limited staining in some (left lower part) but not all small airways sampled by SLB; Inserts are: Serial cut with a Blue Alcian staining suggestive of the presence of mucin-costaining and a higher magnification suggestive of goblet cell morphology for SCGB1A1 positively stained epithelial cells. Ectasic small airway from a TBLC UIP pattern with bronchiolar metaplasia nearly free of staining despite abundant mucostasis. Right: SCGB1A1 stained small airways obtained by SLB (D) and by TBLC (E) in non-UIP patterns. **(B)** Box plots demonstrating group differences (Control vs. ILD for SLB and TBLC) for SCGB1A1 staining variables. TBLC, transbronchial lung cryobiopsies; SLB, surgical lung biopsy; UIP, usual interstitial pneumonia; ILD, interstitial lung disease.

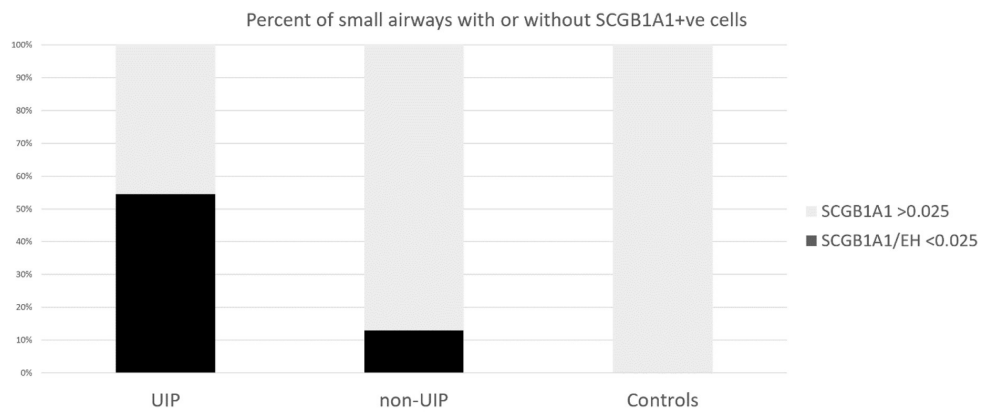

FIGURE 3 | The proportion of samples with SCGB1A1/EH <0.025 was used to define the absence of positive staining [UIP 18/33 vs. non-UIP 4/31 ($p = 0.0027$)].

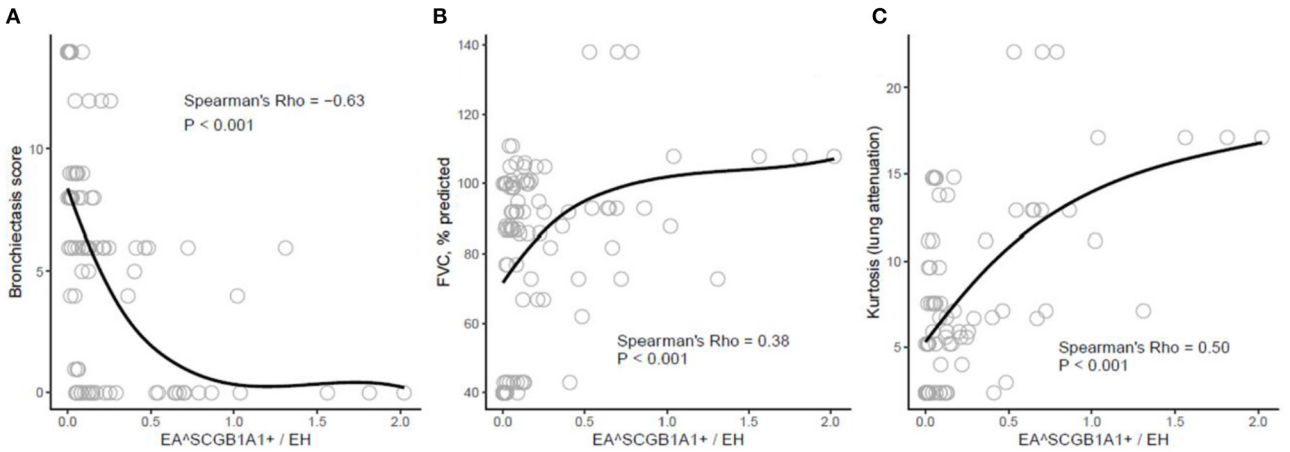

FIGURE 4 | Scatterplots demonstrating the relationships between selected pulmonary function variables [bronchiectasis score **(A)**, % predicted forced vital capacity (FVC, % predicted) **(B)**, and kurtosis of lung attenuation scores **(C)**] and % epithelial area of small airways that stains SCGB1B1^{+} weighted by epithelial height (EA$^{SCGB1B1+}$/EH). Smoothing is performed *via* a local loess estimator (in black). Spearman's correlation statistics (Rho and FDR-corrected *p*-value) are provided.

notch pathway might be involved in this ontogenically impaired process, as the notch is critically involved in basal to club cell transitioning (21). Wnt/β-catenin pathways were also shown to be involved.

This mechanism may help accelerate the paradigm shift that is already in motion, where instead of envisioning bronchiolization and bronchiectasis as a consequence of fibrosis, they are now understood to be an active part of the fibrotic process [as again recently suggested (4)].

Senescence may play a key role in the fibrotic process characteristic of UIP (22) possibly *via* this bronchiolization. Aberrantly proliferating cells can lead to both replicative and premature senescence (23), possibly leading to these poorly differentiated airways. The secretion of senescence-associated secretory protein (SASP), mainly interleukin-8, has already been shown to be involved (24).

Our study has several limits. Few patients were included due to the particular procedures used in the CryoPID study. Our patient group was so heterogeneous that artificially gathering patients from a "non-UIP" group was required. Moreover, staining for MUC5B, a validated bronchiolization marker, would have been of great interest to increase confidence in the mechanism and further characterize the phenotype of the remaining club cells. As shown by Best et al. (25), a higher proportion of SCGB1A1^{+}/MUC5B^{+} cells is observed in IPF, highly expressing genes related to mucins and chemoattractant cytokines for immune cells. Though we missed this opportunity, we nonetheless had attempted the Bleu Alcian staining in a few slides when some unused material was available; the latter suggested convergent findings and merits further investigation.

Of note, we decided to focus our analysis at the sample level (as opposed to the patient level) to take into account the regional heterogeneity of the fibrotic process where zones of normal appearance are classically neighboring the involved tissue (6). This was also justified by the aim of comparing TBLC and SLB in this regard. Correlations were tested at the individual level by expressing SCGB1A1 as a mean and our findings remained consistent.

There are a few steps left to definitively demonstrate that bronchiolization precedes the fibrotic process. Recently, by matching micro-CT imaging with histology, Verleden et al. showed that small airway involvement is the most striking finding in the early IPF stages, suggesting that the disease starts at this level (4).

Our study also supports that the bronchiolization process is key in the course of the disease as we could find correlations with two different HRCT-derived parameters. Kurtosis refers to the tailedness of the distribution histogram of lung attenuation, and this value was suggested to be a surrogate of interstitial changes (25). A significant negative relationship between the expression of the club cell marker SCGB1A1 and the bronchiectasis score was also found; an intuitively-expected finding enhancing the concept that bronchiectasis found at HRCT is related to the bronchiolization process hallmarked by the absence of club cells. Convincingly, a significant correlation was also found with FVC, a physiological marker more than relevant in interstitial lung diseases.

In summary, we demonstrated that bronchiolization, a typical characteristic of ILD and of UIP in particular, is also hallmarked by the absence of club cells. This suggests that an impaired transition process affected epithelial cell fate.

Pauci-SCGB1A1 staining status merits further study as a potential marker of bronchiolization in ILD.

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 CPP Ile de France. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AB elaborated the study, enrolled patients, performed biopsies, participated to the interpretation of the data, and the writing of the manuscript. PR participated to the study, enrolled patients, performed biopsies, acquisition of data, immunostaining, morphometry, HRCT analysis, and the writing of the manuscript. EA participated to the study, enrolled patients, performed biopsies, acquisition of data, and the writing of the manuscript. IS participated to the study, assessed biopsies, and contributed to the immunostaining. LK, CV, CS, and IV participated to the study, interpretation of the data, and the writing of the manuscript. SB participated to HRCT analysis. MR, JB, JM, and AG participated to the study, enrolled patients, and performed biopsies. 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.

frontiers in Immunology

REVIEW
published: 22 April 2021
doi: 10.3389/fimmu.2021.663203

Regulatory Immune Cells in Idiopathic Pulmonary Fibrosis: Friends or Foes?

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The immune system is receiving increasing attention for interstitial lung diseases, as knowledge on its role in fibrosis development and response to therapies is expanding. Uncontrolled immune responses and unbalanced injury-inflammation-repair processes drive the initiation and progression of idiopathic pulmonary fibrosis. The regulatory immune system plays important roles in controlling pathogenic immune responses, regulating inflammation and modulating the transition of inflammation to fibrosis. This review aims to summarize and critically discuss the current knowledge on the potential role of regulatory immune cells, including mesenchymal stromal/stem cells, regulatory T cells, regulatory B cells, macrophages, dendritic cells and myeloid-derived suppressor cells in idiopathic pulmonary fibrosis. Furthermore, we review the emerging role of regulatory immune cells in anti-fibrotic therapy and lung transplantation. A comprehensive understanding of immune regulation could pave the way towards new therapeutic or preventive approaches in idiopathic pulmonary fibrosis.

Keywords: idiopathic pulmonary fibrosis, mesenchymal stem/stromal cells, regulatory T cells, regulatory B cells, macrophages, myeloid-derived suppressor cells, pharmacotherapy, transplantation

OPEN ACCESS

Edited by:
Enrico Conte, University of Catania, Italy

Reviewed by:
Eliza Tsitoura, University of Crete, Greece
Adam Byrne, Imperial College London, United Kingdom

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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *02 February 2021*
Accepted: *22 March 2021*
Published: *22 April 2021*

Citation:
van Geffen C, Deißler A, Quante M, Renz H, Hartl D and Kolahian S (2021) Regulatory Immune Cells in Idiopathic Pulmonary Fibrosis: Friends or Foes?. Front. Immunol. 12:663203. doi: 10.3389/fimmu.2021.663203

INTRODUCTION

Pulmonary fibrosis (PF) is a chronic lung disease characterized by progressive fibrotic tissue remodeling and scarring of lung tissue (1). Various factors, such as smoking, chronic aspiration due to gastroesophageal reflux, infections, toxins, radiation, autoimmune reactions (e.g. rheumatoid arthritis, scleroderma, polymyositis, dermatomyositis, Sjögren's syndrome or systemic lupus erythematosus) and exposure to environmental pollutants can trigger chronic lung tissue damage resulting in fibrotic remodeling (2–6). However, PF can also occur without an identifiable underlying cause, known as idiopathic pulmonary fibrosis (IPF). IPF is an age-related interstitial lung disease, affecting mainly patients of 50 years and older with an incidence of 2.8–18 per 100,000

people and a prevalence of 1.25–27.9 per 100,000 people in Europe and North America (3). Unfortunately, IPF patients have a poor prognosis with a median survival of 2-4 years after diagnosis (3). A gain-of-function mutation in the *MUC5B* gene represents the highest genetic risk factor for the development of IPF (3). IPF is difficult to treat with pharmacological therapies and to date, the only effective curative therapy for IPF patients is lung transplantation (3).

Chronic alveolar-micro injuries presumably lead to a maintained and dysregulated wound healing process, which drives IPF (3). Fibrogenesis is marked by a massive accumulation of extracellular matrix (ECM) produced by myofibroblasts such as collagen, elastin, laminin, fibronectin, hyaluronan and glycoproteins, resulting in irreversible thickening of alveolar walls, compromising the exchange of oxygen and carbon dioxide between blood and alveolar air (7, 8). At the cellular level, repeating lung injuries mostly affect type I alveolar epithelial cells (AECs), which mainly form the alveolar surface. In response to this cell loss, type II AECs proliferate in a hyperplastic manner to mask the exposed basement membrane (2). Under healthy conditions, the cells would differentiate into type I AECs and hyperplastic type II AECs would undergo apoptosis (2). However, under the influence of transforming growth factor (TGF)-β, hyperplastic type II AECs remain at the alveolar surface resulting in alveolar collapse (2). Fibroblasts are the most frequent cell type in fibrotic tissues that produce ECM-producing cells which are recruited into the lung compartment (2). Fibroblasts differentiate into contractile myofibroblasts with massive ECM productive capacity (2). Cytokines and growth factors activating fibroblasts and myofibroblasts and inducing further fibrotic tissue remodeling include TGF-β, interleukin (IL)-1, IL-6, IL-13, IL-33, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), tumor necrosis factor (TNF)-α and leukotrienes (7). Activated fibroblasts produce TGF-β, IL-1β, IL-33, reactive oxygen species, C-X-C motif chemokines (CXC), C-C motif chemokines (CC) maintaining fibrogenesis and attracting immune cells to promote chronic inflammation, resulting in a positive feedback loop supporting fibrogenesis through differentiation of fibroblasts into myofibroblasts (7). TGF-β itself contributes to fibrosis progression *via* TGF-β/SMAD signaling by stimulation of ECM production, inhibition of ECM breakdown through matrix metalloproteinases (MMPs), and epithelial-mesenchymal transition (EMT) induction (9).

Cells of both the innate and adaptive immune system such as mesenchymal stem/stromal cells (MSCs), regulatory T cells (Tregs), regulatory B cells (Bregs), macrophages, dendritic cells (DCs) and myeloid-derived suppressor cells (MDSCs) have been linked to the pathogenesis of IPF, often with contradicting findings (**Figure 1**) (10–14). Immunomodulation by regulatory immune cells is crucial in dampening pathogenic immune responses and inhibiting the transition from inflammation to fibrosis. Identifying the role of regulatory immune cells in IPF is therefore key in understanding the imbalanced immune responses underlying IPF. In this review, we summarize and critically discuss the role of regulatory immune cells in IPF, and assess their interaction with current pharmacological therapies and lung transplantation in IPF.

REGULATORY IMMUNE CELLS IN IPF

Mesenchymal Stem/Stromal Cells

MSCs are multipotent stem cells that can differentiate into various cell types, including osteoblasts, chondrocytes, adipocytes, myocytes, fibroblasts and endothelial cells (15). Furthermore, MSCs play an important role in modulating immunity (16). MSC phenotype and function are dependent on environmental cues (17). Lung-resident (LR)-MSCs have been proposed to play a role in lung regeneration. The expression of high levels of telomerase by LR-MSCs provides self-renewal, survival and replication capacity which may promote lung regeneration by repopulating structural lung cells (17). LR-MSCs have also been suggested to play an important role in IPF. Several profibrotic factors, as mentioned above, have been found to induce myofibroblastic transition (MFT) of MSCs, which in turn promotes collagen production and ECM accumulation driving PF (17–21). Bleomycin-induced pulmonary fibrosis (BPF) in mice resulted in the loss of LR-MSCs, likely due to MFT induced by the upregulation of previously mentioned profibrotic factors (22). Inhibition of the underlying signals mediating MFT, such as Hedgehog, Wnt/β-catenin, Shh/Gli – Wnt or nuclear factor (NF) - κB signaling, were shown to reduce myofibroblast differentiation of LR-MSCs and attenuated BPF in mice (18, 23–25). Dysregulations in these pathways have been found to modulate TGF-β, MMPs, ECM production and EMT (9, 26). Manipulating the MFT of LR-MSCs by regulating microRNA-497 expression understated their detrimental effect in BPF (27). The expression and activity of collagen prolyl hydroxylase, an enzyme crucial for collagen synthesis, by fibrotic lung mesenchymal cells was observed to be one of the mechanisms underlying the TGF-β-mediated profibrotic effects (28). Suppression of collagen prolyl hydroxylase by pyridine-2,5-dicarboxylate attenuated TGF-β-mediated collagen production in both cultured fibroblasts as well as in BPF (28). Promotion of REVERBα, a transcriptional repressor that is upregulated in IPF, has been shown to inhibit both myofibroblast differentiation and collagen secretion in organotypic cultures from IPF patients (29).

In contrast, there is an increasing scientific interest in MSCs due to their immunomodulatory and anti-inflammatory effects (15) as well as an increasing amount of evidence showing anti-fibrotic effects of MSCs in rodents. MSC therapy in BPF models in rodents showed reductions in lung collagen deposition (22, 30–43), lung fibrosis (22, 32, 34, 35, 37, 38), TGF-β levels (22, 30–36, 38–41) and total and/or neutrophil cell count in the bronchoalveolar lavage fluid (BALF) (34, 36, 37, 39) and improved 14-day survival (22, 34, 36) after bleomycin administration. A large body of research is focused on MSC therapies in a variety of human diseases, including graft versus host disease (GVHD), autoimmune disorders, acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD) and asthma (44) as well as in novel diseases like COVID-19 (45, 46). Autologous or allogeneic MSCs are harvested from the peripheral blood, adipose tissue, bone marrow, umbilical cord, placenta, dental pulp, synovial fluid, endometrium, skin or muscle. Due to low numbers, harvested

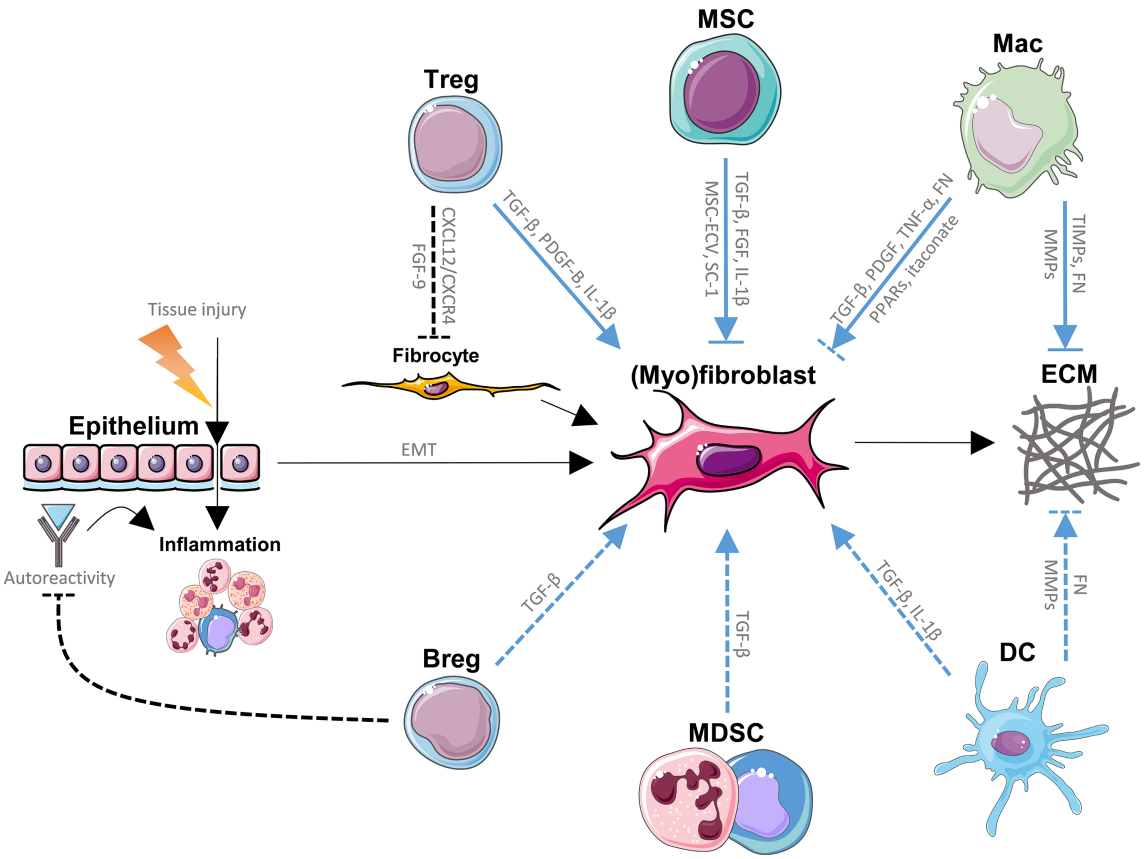

FIGURE 1 | Direct effects of regulatory immune cells on (myo)fibroblasts and extracellular matrix (ECM) in idiopathic pulmonary fibrosis (IPF). Repeated tissue injury triggering chronic tissue damage resulting in inflammation, epithelial mesenchymal transition (EMT) and ultimately in excessive production and buildup of ECM by fibrocytes and (myo)fibroblasts (fibrosis) in the lungs, represents the main paradigm involved in the pathology of IPF. Immune cells with regulatory functions modulate (myo)fibroblast generation, (myo)fibroblast function and ECM homeostasis through various signaling pathways, and known direct pathways are listed here. Mesenchymal stem/stromal cells (MSCs) promote (myo)fibroblasts through fibroblast growth factor (FGF), transforming growth factor (TGF)-β and interleukin (IL)-1β, while MSC-derived extracellular vesicles (MSC-ECV) and stanniocalcin (SC)-1 have opposite effects. MSCs are also prone to myofibroblastic transition. Tregs promote fibrogenesis through TGF-β, platelet-derived growth factor (PDGF)-B and IL-1β, while inhibiting the recruitment of fibrocytes by inhibition of the CXCL12/CXCR4 axis as well as FGF-9. Macrophages enhance fibrosis through TGF-β, PDGF, tumor necrosis factor (TNF)-α, fibronectin (FN) and inhibit fibroblasts *via* itaconate and peroxisome proliferator-activated receptor (PPAR) ligands. Myeloid-derived suppressor cells (MDSCs) and regulatory B cells (Bregs) have been suggested to activate lung fibroblasts, possibly through TGF-β. Bregs inhibit autoreactive immunoglobulins, which may deposit in lung tissue and promote inflammation and IPF. Dendritic cells (DCs) have been shown to produce pro-fibrotic TGF-β and IL-1β. Macrophages (Macs) as well as DCs break down the ECM *via* matrix metalloproteinases (MMPs), a process that is inhibited by tissue inhibitors of metalloproteinases (TIMPs) produced by other macrophage subtypes. Both Macs and DCs have been found to produce fibronectin (FN), another ECM component. Blue lines represent the direct effects of regulatory immune cells on (myo)fibroblasts and ECM in IPF. Dashed lines represent interactions that are not firmly established in IPF. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

MSCs are usually expanded *in vitro* in culture medium supplemented with fetal bovine serum, before being transferred to patients (44). The use of MSC-derived products, such as MSC-derived vesicles and exosomes were also reported (47).

At the time of writing, there are five clinical trials using MSC therapy to treat IPF, as registered on the National Institute of Health (NIH) clinical trial database (48)[1]. A phase Ib clinical trial, completed in 2015, studied the intravenous administration of 1 to $2*10^6$ MSC/kg allogeneic placenta-derived MSCs in patients with moderately severe IPF (NCT01385644) (49). The phase I AETHER trial, completed in 2016, studied the administration of a single intravenous infusion of 20, 100, or $200*10^6$ allogeneic bone marrow-derived MSCs in patients with mild-to-moderate IPF (NCT02013700) (50). Another phase I clinical trial, completed in 2018, studied the safety and feasibility of endobronchial administrated autologous bone marrow-derived MSCs in patients with mild-to-moderate IPF (NCT01919827). An uncompleted phase I/II clinical trial that planned to study adipose-derived MSCs in IPF has not recently been updated on its status (NCT02135380). Finally, a phase I/II clinical trial, completed in 2018, studied the intravenous administration of two doses of $2*10^8$ allogeneic bone marrow-derived MSCs every

[1] https://www.clinicaltrials.gov/

3 months for a total of $1.6*10^9$ MSCs in patients with moderate-to-severe IPF (NCT02594839) (51). Another phase Ib clinical trial studied the safety of $1.5*10^6$ autologous adipose-derived MSCs-stromal vascular fraction/kg infused into the lungs of patients with mild-to-moderate IPF at monthly intervals (52). Four of the completed clinical trials published their results showing that MSC therapy is a safe, feasible and promising method to treat IPF patients (49–52). However, more clinical trials are necessary to prove the efficacy of MSC therapy in IPF patients. Due to the urgency and severity of many diseases, including IPF, there are many reports of unapproved stem cell therapies being used, often due to a lack of regulation (53, 54). There remain key challenges in MSC therapy, such as identifying the most valuable source of MSCs, the ideal donors and recipients and the most effective handling and administration routes. Other challenges are in identifying during which stages of IPF patients are most susceptible to MSC therapy as well as possible interactions with pharmaceuticals.

Beneficial effects of MSC therapy in IPF have been suggested to be modulated by MSCs promoting alveolar repair by the secretion of growth factors (55), suppressing inflammation by the production of nitric oxide (NO) and indoleamine 2,3-dioxygenase (IDO) (56), promotion of Treg expansion (57), decreasing pro-inflammatory cytokines such as TNF-α, interferon (IFN)-γ and IL-2 by secreting IL-10 and soluble IL-1β receptor (56) and by protecting lung injury by restituting alveolar bioenergetics through mediating mitochondrial transfer (58). Other observed anti-fibrotic pathways were the production of stanniocalcin-1 which is shown to dampen BPF in mice (41), MSC-derived extracellular vesicles which were found to suppress TGF-β1-induced MFT (47, 59, 60), and MSCs ability to differentiate into type II lung cells expressing surfactant (60). MSCs are also immunoprivileged, lacking human leukocyte antigen (HLA) class II combined with low HLA class I expression, allowing the administration to immunocompetent patients without the need for further immunosuppression (61). However, transferred MSCs have been shown to secrete pro-fibrotic TGF-β1 and underwent MFT in BPF in mice (57). In contrast, MSCs secreting higher levels of TGF-β1 have been suggested to have optimal anti-fibrotic efficacy in BPF in mice (57).

Recent evidence suggests an increase in senescent bone marrow-derived (BM-) MSCs (expressing the senescence markers p21, $p16^{INK4A}$, p53 and senescence-associated β-galactosidase) in IPF patients contribute to disease progression (62, 63). Accumulation of DNA damage in senescent IPF BM-MSCs resulted in decreased mitochondrial and cellular function (62). These findings are in line with the observed decrease in migration capacity and reduced anti-fibrotic effects of BM-MSCs derived from IPF patients, which were adoptively transferred in BPF mice (62). Administrated MSCs derived from older mice were found to be less successful in ameliorating BPF compared to those derived from younger rodents, supporting the contribution of MSC senescence (64). DNA protein kinase catalytic subunits, involved in DNA repair mechanisms, were found to be reduced in IPF lung tissue, which was suggested to result in the expansion of stage-specific embryonic antigen-4^+ mesenchymal progenitors, and the senescence of mesenchymal progeny (63). The possible involvement of senescent MSCs in IPF disease progression may be correlated with age being the most important risk factor (62).

Taken together, literature suggests that an increase in MFT of LR-MSCs as well as an increase in MSC senescence are detrimental to IPF progression. However, the immune regulatory role of MSC has been linked with beneficial effects in IPF, and, as evidenced by the positive findings of studies investigating MSC therapy, seem to provide promising treatment strategies.

Regulatory T cells

Tregs have been found crucial in modulating immunity and maintaining immune tolerance, but their role in IPF remains contradicting and unclearly defined (65, 66). Activated $CD4^+$ T cells (67–69) as well as Tregs (70–73) were found to be upregulated in the peripheral blood of IPF patients and positively correlated with disease severity (67, 68, 70, 71, 73). Tregs were recruited to the lungs of murine models of PF (74, 75). In contrast, several studies observed reduced Treg numbers with reduced suppressive functions in the peripheral blood and BALF of IPF patients correlating to disease severity (76, 77).

Tregs have been suggested to be able to protect from TGF-β1-induced fibrosis *via* the release of IL-10, as Th1 cells exposed to TGF-β1 produce IL-10 (78). TGF-1β was shown to ameliorate BPF in wild-type mice, but not in IL-10-deficient mice (78). IL-10 also has been found to down-regulate type I collagen synthesis in human scar tissue-derived fibroblasts (79) and protect against BPF (80). However, TGF-β1 may also mediate the potential negative role that Tregs play in IPF (81). Tregs have been shown to increase collagen deposition through TGF-β1 in fibroblasts (82) and in lymphatic tissue (83) as well as in the lungs (74) of mice. TGF-β1 autocrine signaling in Tregs was also shown to induce secretion of pro-fibrotic PDGF-B in non-inflammatory conditions (74). Early depletion of Tregs indeed reduced TGF-β levels and lung fibrosis in BPF in mice (81). Semaphorin 7A is an immunomodulating protein involved in many processes such as monocyte chemotaxis (84) and DC migration (85). Adoptive transfer of semaphorin 7A-positive Tregs was found to exaggerate TGF-β1-induced lung fibrosis in TGF-β1-transgenic mice (70). However, the regulation of TGF-β1 levels is not limited to Tregs, confirmed by the finding that reduced Tregs and Foxp3 expression in mice lacking phosphatase and tensin homolog (an antagonist of phosphatidylinositol 3-kinases, which mediate myeloid effector function) was combined with a massive upregulation of TGF-β1 in the lungs (86).

The importance of the CXCL12/CXCR4 axis in IPF has previously been described, and is thought to contribute to fibrosis through recruitment and modulation of fibrocytes (77, 87, 88). Fibrocytes are cells expressing CD45, collagen-1 and CXCR4, exhibit pro-inflammatory and ECM-remodeling properties and share key features with fibroblasts (89, 90). Indeed, the recruitment of $CXCR4^+$ fibrocytes to the lungs of IPF patients was confirmed and blockage of CXCR4 ameliorated BPF in mice (87, 88). Tregs were found to decrease CXCL12 expression and may therefore play a key role in reducing

fibrocyte recruitment (91). Similarly, Tregs have been found to inhibit fibrocyte recruitment *via* suppression of FGF-9 (92).

Another pathway by which Tregs may influence IPF progression is by mediating the balance between T helper (Th) 1, Th2, and Th17 responses. Th1 cells were shown to exhibit anti-fibrotic effects through the production of IFN-γ and IL-12 (93), and IFN-γ production by T cells was found to be suppressed by Tregs (94). Th2 immune responses were shown to stimulate myofibroblasts and the progression of the disease through the production of IL-4, IL-5, IL-13 and IL-17A (95, 96). In radiation-induced lung disease in mice, an increase in Th17 was linked to enhanced lung fibrosis, while an increase in Th1 was found to have the opposite effect (97). The downregulation and depletion of Tregs as well as the neutralization of Treg-immunosuppressive activity has been shown to switch Th2-driven responses to Th1-driven responses (74, 98, 99) and attenuated PF progression in a silica-induced (99) as well as in an irradiation-induced murine model (100). Tregs were suggested to promote Th17 through TGF-β and IL-1β (100, 101). However, Th17 cells were found to be decreased in the peripheral blood (while Tregs were found upregulated) resulting in increased TGF-β/IL-17 ratio in IPF patients (72). Adoptive transfer of Tregs into recombination activating gene (Rag) 2 protein-deficient mice (lacking lymphocytes) was found to promote BPF progression and a loss of Foxp3 expression after transfer (10, 102). These findings suggest Tregs might lose their suppressor ability and adopt a Th2 phenotype through which they may promote disease progression (10, 102). In contrast, pro-inflammatory $CD4^{+}CD28^{null}$ helper T cells were found to be upregulated in IPF and were found to be unresponsive to Treg immunomodulation (103).

Treg expansion using IL-2 therapies was shown successful in treating diseases like vasculitis and GVHD (104, 105). Soluble IL-2 complexed to an IL-2 receptor neutralizing antibody allows for hyperstimulation of CD25 (IL-2 receptor alpha chain) by depleting the IL-2 receptor, which in turn promotes Treg expansion. Administration of IL-2 complex in a mouse model of BPF was found to exacerbate PF by increasing collagen deposition as well as inducing lung remodeling (102). The immune response was found to be switched from a Th1 to a Th2 response, while TGF-β production remained unaltered (102).

The adoptive transfer of Tregs did show positive effects in mice models of acute lung injury (91, 106). Adoptive transfer of Tregs was observed to reduce delayed lung recovery in $Rag1^{-/-}$ mice (lacking mature T and B cells) (106) as well as fibroproliferation (91) in lipopolysaccharide-induced acute lung injury. Interestingly, while early depletion of Tregs was found to reduce lung fibrosis and TGF-β1 expression in BPF in mice, late depletion of Tregs showed opposite effects (81). Late stage (after 21 days) increase of Tregs in BPF in CC receptor (CCR) 7 (receptor involved in B and T cell activation, T cell homing and DC maturation) deficient mice showed a positive suppressive effect of Tregs in IPF as well (107). These results suggest several translational limitations relating to animal models of PF that likely resulted in several contradicting results regarding the role of Tregs in IPF (10). Commonly used animal models, such as BPF, are not specific to IPF and rather poorly replicate the core characteristics of the disease, such as the slow development and its irreversible nature. Combined with the already existing gap between animals and humans, as well as the complexity of Tregs, it further complicates translation to the clinical setting.

Literature on the role of Tregs in IPF is often contradictory and the explanation to this phenomenon may be hiding in several factors (10). First of all, the phase of IPF progression appears to be of vital importance when assessing the role of Tregs. Second, there seems to be a high likelihood of discrepancies in the number of Tregs found in the periphery (i.e. the blood) versus the number of Tregs observed in the lungs. Third, there are translational limitations relating to animal models of IPF. Combined with the diverse role of Tregs, it is reasonable to assume these factors contributed to the contradicting observations regarding their role in IPF. All in all, Tregs appear to be both friend and foe in relation to IPF, which suggests that Treg modulation requires careful assessment of the phase of IPF disease state.

Regulatory B Cells

B cells mediate the humoral immunity of the adaptive immune system by secreting antibodies (as plasma cells), and mediate immunity by functioning as antigen-presenting cells (APCs) and by producing cytokines (108). The amount of circulating B cells in the peripheral blood (109, 110) and B cells in the lungs (110–114) of IPF patients were found to be increased together with CXCL 13 (a critical chemokine for the homing of B cells) (115, 116), correlating with disease progression. Similarly, there was an increase in IL-6, IL-13 and B-cell-activating factor (BAFF) (all factors that promote B cells) found in IPF patients (110). $Rag1^{-/-}$ mice as well as gp130(757F);$uMT^{-/-}$ compound mutant mice (deprived of mature B cells) were protected from BPF, suggesting a negative role of B cells in IPF progression (117).

Bregs, however, have been shown to dampen T-cell driven immune responses, to support immunological tolerance, and have been shown to produce anti-inflammatory IL-10 and TGF-β (118). A loss of Bregs in both allergic and autoimmune diseases was found to exacerbate disease progression (119). Decreased numbers of Bregs were found in IPF patients, as well as an increase in the proliferation and activation of T follicular helper (Tfh) cells (12). Tfh cells are a specialized Th cell subset that assist B cells by the secretion of IL-4, IL-10 and IL-21 and CD40 ligand expression, which promotes the growth, differentiation and initiates class switching of B cells as well as induce antigen-specific antibody responses (120). Bregs were shown to suppress Tfh-cell maturation and inhibit Tfh cell-mediated antibody secretion (121). Increases of circulating antigen differentiated B cells (110), plasmablasts (110), autoreactive immunoglobulin (Ig) A in the lungs (109), Ig in BALF (122) and immune complexes were observed in the BALF and lungs of IPF patients (123–126). These findings are in line with the observed reduction of Bregs and observed increase of both Tfh and non-regulatory B cells in IPF. Autoreactive immune complexes are able to deposit in the lung and promote inflammation, while antibodies mediate antibody-dependent cellular cytotoxicity (mediated *via* complement activation or natural killer cells), processes that may drive IPF progression (127).

Antibodies, such as Rituximab, target the CD20 marker that is expressed on the surface of all B cells starting at the pre-B cell stage (128). These antibodies therefore inhibit $CD20^+$ B cells, which as described above were found to be positively correlated to IPF progression (109–117). Rituximab indeed may be a viable treatment option of IPF (129, 130), and is included in 6 NIH registered clinical trials treating IPF at the time of writing (48)[2]. Unfortunately, CD20 antibodies also target CD20 expressing Bregs, showing a downside to unspecific downregulation of B cells. However, levels of CD20 expression differs among different B cells populations, with Bregs generally expressing lower levels of CD20 (131). Depletion of most $CD20^+$ B cells by rituximab was found to enrich $CD20^{low}$ Bregs in mice and human cancer (131). The specific targeting of Bregs or non-regulatory B cells is limited by the lack of specific surface markers (132). However, Bregs might be targeted and upregulated *via* other pathways such as immunomodulation by MDSC (133). A study by Wu et al. found that PGE2 in the exosomes derived from polymorphonuclear (PMN)- MDSCs promote IL-10^+ B cells and ameliorates collagen-induced arthritis in mice (133).

Collectively, Bregs seem to be able to play a vital role in the suppression of IPF. Progress in the specific depletion of non-regulatory B cells or in the expansion of Bregs may provide promising clues for future treatment strategies.

Macrophages

Macrophages play an important role in mediating tissue homeostasis and inflammation as well as phagocytosing viral, bacterial and parasitic pathogens and inducing the adaptive immune response by functioning as an APC (134). The disturbed balance in wound healing processes underlying IPF is thought to be primarily mediated by macrophages (11). Furthermore, the interplay of MMPs and tissue inhibitors of metalloproteinase (TIMPs), secreted by macrophages, among other secreted factors, such as collagen, shape the ECM. Macrophages thereby contribute to IPF in either a pro- or anti-fibrotic manner (135).

Increased number of macrophages was observed in the lungs of IPF patients (136), yet their functional role remains a matter of active discussion. Lung macrophages have been shown to play a crucial role during the fibrotic phase of BPF in mice (136). Low numbers of macrophages during the resolution phase were found to reduce ECM degradation and exacerbate PF (137). Depletion of macrophages in an early phase of fibrosis was, however, shown protective in a murine model of liver fibrosis (137). Previously, the M1/M2 macrophage polarization nomenclature, inspired by the Th1/Th2 paradigm, was commonly used to classify macrophages (86, 96, 138–149). M1-like macrophages have been found to promote fibrosis by secreting pro-inflammatory IL-6 and TNF-α as well as promoting the Th17 and neutrophilic immune response (86, 139–141). M2-like macrophages have been linked to fibrotic processes through the promotion of CC ligand (CCL) 2 and 17, TIMPs, fibronectin, Th2 response through IL-4 production, fibroblasts and the MFT of LR-MSCs (96, 142–149). On the other hand, the production of arginase-1 and MMPs, as well as anti-inflammatory IL-10, TGF-β1 and heme oxygenase 1 by M2-like macrophages suggest a protective role in IPF (96, 142, 143, 149). Peroxisome proliferator-activated receptor (PPAR-) α and PPAR-γ activation has been found to inhibit inflammation by inducing M2-like macrophages, which in turn may inhibit pro-inflammatory cytokine production (150, 151). These findings suggest PPAR ligands may be interesting therapeutic targets in IPF, especially considering they have also been found to directly inhibit fibroblast activation induced by TGF-β1 (152). However, as the M1/M2 paradigm is based on findings from *ex vivo* cultured cells, it is unable to accurately recapitulate the complexity of macrophage phenotypes *in vivo*, and, especially those observed in the IPF lung (153).

Recent studies have begun profiling individual cells, including macrophages, in IPF in great detail through single-cell RNA sequencing (scRNA-seq) (154). Several research groups made their scRNA-seq findings on thousands of cells derived from the lungs of IPF and healthy control available (154). Analysis of such data sets enables the identification of many novel macrophage subsets and their specific ontogeny relating to different stages of IPF. Highly proliferative osteopontin-expressing macrophages were identified through scRNA-seq and were found to mediate myofibroblast activation in the lungs of IPF patients (155). Similarly, another expanded population of pro-fibrotic $CD36^+CD84^+$ macrophages in IPF were identified, which also showed increased expression of genes involved in ECM remodeling (156, PREPRINT).

On the other hand, itaconate has recently been described to mediate anti-fibrotic effects of macrophages in the lungs (157). Decreased levels of pulmonary itaconate as well as reduced itaconate-synthesizing cis-aconitate decarboxylase (*ACOD1*) expression (which mediates itaconate production) in lung macrophages were observed in IPF patients (157). *Acod1* deficient mice developed more severe BPF, while adoptive transfer of lung-derived monocytic macrophages from healthy donor mice alleviated the developed persistent fibrosis (157). Furthermore, itaconate was shown to decrease fibroblast proliferation and wound healing capacity of cultured fibroblasts (157).

The pentraxin-2 analogue, PRM-151, which inhibits the differentiation of monocytes into pro-inflammatory and pro-fibrotic macrophages (and fibrocytes), is therefore a promising therapy and is currently undergoing clinical trial for the treatment of IPF (158). However, several studies suggest possible anti-fibrotic and immunomodulating roles of macrophages, which may be suppressed in IPF, and therefore might provide interesting therapeutic targets. Furthermore, the recent unraveling of specialized macrophage subsets through tools such as scRNA-seq is rapidly increasing the understanding of the complex role they play in IPF and is expected to accelerate future research development substantially.

Dendritic Cells

DCs bridge the innate and adaptive immunity through their role as APCs (159). Besides their primary role as immune activators, DCs have also been described to play tolerogenic and regulatory roles (13). These tolerogenic DCs are crucial in the maintenance

[2]https://www.clinicaltrials.gov/

of central and peripheral tolerance by inducing clonal T cell deletion, T cell anergy, inhibition of memory and effector T cell responses and promoting Tregs (160).

DCs accumulate in the fibrotic lungs of BPF in mice (161, 162) as well as in the BALF and lungs of IPF patients (163–165), while circulating DCs were decreased (166). Fms-like tyrosine kinase-3 ligand (Flt3L), a cytokine that promotes differentiation and proliferation of DCs, was found to be upregulated in the peripheral blood and lungs together with an increase in DCs in the lungs of TGF-β1-induced murine model of PF as well as in IPF patients (167). The unspecific deprivation of DCs in mice lacking Flt3L resulted in more severe PF, while unspecific upregulation of DCs, by supplementing Flt3L, resulted in reduced PF progression in a TGF-β1-induced PF mouse model (167). Specific depletion of DCs by diphtheria toxin (DT) in a mouse strain expressing zinc-finger and BTB domain containing 46 (*Zbtb46*; transcription factor keeping DCs in a quiescent state)-DT receptors was found to result in severe PF in a TGF-β1-induced mouse model of PF (167). In contrast, depletion of DCs by the administration of DT to CD11c-DT receptor-transgenic mice attenuated BPF (168). However, in the latter study it is important to note that CD11c-expressing macrophages were depleted as well, which may explain the discrepancy between the two studies (168). Inactivating DCs using VAG539, a pro-drug of VAF347, which activates the transcription factor aryl hydrocarbon, attenuated BPF lung injury in mice (162). However, VAG539 modulates the cell physiology by binding the transcription factor aryl hydrocarbon receptor expressed in many other cell types besides DCs (169), and has been shown to promote the development of IL-22-secreting Th cells (170), making it questionable whether the attenuation can be attributed solely to DC inactivation. $CD11b^+$ DCs have been found to upregulate the expression of several MMPs, promoting collagen and ECM degradation (171). CXCL4 has recently been identified to be crucial in altering DC development into a pro-inflammatory and pro-fibrotic phenotype that induces ECM accumulation and MFT (172).

Taken together, these findings suggests that besides their tolerogenic role, DCs may also play a detrimental role in IPF disease progression. However, due to a lack of convincing evidence on immune regulation by DCs in IPF, their role remains unclear and needs to be studied in more detail.

Myeloid-Derived Suppressor Cells

MDSCs are immature myeloid-derived cells that potently suppress the immune response, and are mainly subdivided into PMN- and monocytic (M)- MDSCs (173). Due to MDSCs plasticity they have also been found to differentiate into M2-like macrophages and tolerogenic-like DCs (174). The role of MDSCs in cancer is well established, however, their role in many other diseases, such as IPF, remains incompletely defined (14). Increased numbers of PMN-MDSCs were found in the lungs of BPF (and clodronate-treated) mice, likely through increased CXCR 2 expression (40). PMN-MDSCs were also increased in the peripheral blood of patients with interstitial pulmonary disease, while M-MDSC numbers remained unaltered (175). Increased numbers of $CD33^+CD11b^+$ cells (suggestive of MDSCs, particularly M-MDSCs) were found in the peripheral blood in the lungs of IPF patients (69). Increased MDSC numbers were suggested to correlate to poor lung function and increased number of Tregs in IPF patients (69). Depletion of MDSCs was found to enhance fibrosis markers in both kidney and liver models of fibrosis in mice (176), while adoptively transferred MDSCs ameliorated renal fibrosis modeled in mice (177), suggesting a protective role. Increased number of PMN-MDSCs were suggested to be correlated to a decrease in parenchymal fibrosis and attenuation of BPF in mice (175). Another study reported a population of circulating MDSC-like fibrocytes in cancer (178). Furthermore, cells generated from $CD11b^+CD115^+Gr1^+$ MDSCs were shown to contribute to renal deposition of collagen type I in a murine model of renal fibrosis (179). As mentioned above, PMN-MDSCs have been found to promote Bregs, which was suggested to promote the potential protective role MDSCs play in IPF (133). Bone marrow-derived MSCs have been suggested to drive the differentiation of $Gr1^{high}CD11b^+$ cells (mainly PMN-MDSCs) towards a $Gr1^{low}CD11b^+$ phenotype (indicative of M-MDSCs), which was found to inhibit BPF progression in mice (180).

In summary, literature suggests that the immune regulatory functions of anti-inflammatory MDSCs may promote anti-fibrotic effects, making them potentially interesting cells to target in the treatment of IPF. However, there remains a lot of uncertainty on their role, partly attributed to MDSCs plasticity, and more research is needed to further clarify their significance in IPF.

INTERACTION OF PHARMACOLOGICAL THERAPY AND IMMUNE RESPONSES IN IPF

Previously, immune suppressive medications including glucocorticoids and azathioprine or cyclophosphamide were used in the treatment of IPF (181). In 2015, a new guideline for treatment of IPF formulated strong recommendations against the use of immunosuppressive drugs in IPF (182). This suggestion was primarily based on a single multicenter study, PANTHER-IPF, which was terminated prematurely due to the major safety concerns in patients receiving combination therapy of prednisone, azathioprine, and N-acetylcysteine compared with placebo (183). However, the question remained whether immunosuppressants should be tested once more in combination with anti-fibrotics like nintedanib and pirfenidone in the context of IPF. Further long-term studies are needed to determine the safety and efficacy of the combination of immunosuppressive and anti-fibrotic therapy in IPF.

Pirfenidone

Pirfenidone is an anti-fibrotic, anti-inflammatory, and antioxidant drug that has been found to interfere with fibroblast proliferation and MFT and the synthesis of ECM (184, 185). Three double blind randomized placebo-controlled

Phase III trials (CAPACITY 1 and 2 and ASCEND) approved the efficacy and safety of pirfenidone in PF (186, 187). These studies showed that pirfenidone reduced the rate of decline in forced vital capacity (FVC) over a period of 1 year, by approximately 50%, in IPF patients with mild, moderate and severe impairment in lung function (187, 188). Pirfenidone improved life expectancy in IPF patients, however, its use is unfortunately not associated with significant improvement in clinical symptoms like cough or shortness of breath (187). Pirfenidone has been shown to inhibit pro-inflammatory and pro-fibrotic cytokine production such as TGF-β, IL-4, IL-13, and TNF-α and promotes the production of anti-inflammatory IL-10 (189–194). The beneficial effect of pirfenidone in IPF may indeed be the result of the modulation of immune responses through modulating cytokines. However, the direct modulatory effects of pirfenidone on immune responses were not described for a long time. The direct immune modulatory properties of pirfenidone were first studied by Visner GA., et al., 2009, in a murine model of cardiac allograft transplantation (195). This study demonstrated the direct inhibitory role of pirfenidone on $CD4^+$ and $CD8^+$ cell proliferation index *in vivo*, whereas pirfenidone showed no effect on the immunosuppressive properties of Tregs (195). The same group of researchers studied the immunomodulatory role of pirfenidone in a murine model of lung allograft transplantation in 2012 (196). Here they showed that pirfenidone suppressed the activation of lung DCs *in vivo* (196). In this respect, *in vitro* treatment of DCs with pirfenidone reduced the expression of major histocompatibility complex (MHC) class II and costimulatory molecules and impaired DC's capacity to stimulate T cell activation (196). Considering the prolonged allograft survival and robust inhibitory effects of pirfenidone observed in *in vivo* models, as compared to *in vitro* conditions, it may be postulated that other regulatory immune mechanisms are involved in the immunosuppressive effect of pirfenidone besides its direct T cell inhibitory effects. Du et al. showed that pirfenidone reduced splenic germinal center B-cell and Tfh frequencies and reduced infiltration of M2-like macrophages into the lung as well as TGF-β production in a murine model of chronic GVHD (197). Recent studies attempted to investigate the efficacy of pirfenidone co-administered with standard chemotherapy, in cancer. A recent *in vitro* study suggested the inhibitory effect of pirfenidone on metastasis and immune suppressive capacity of cancer-associated fibroblasts (CAFs) in the tumor microenvironment (TME) through inhibition of expression of programmed death-ligand (PD-L) 1 on CAFs and cancer-promoting cytokines and chemokines secretion like TNF-β and CCL17 (198). The immunomodulatory role of pirfenidone needs to be studied in more detail in the TME of cancer models. Although the Food and Drug Administration (FDA) approved pirfenidone for the treatment of IPF since 2014, there remains a lack of robust evidence on the interaction of pirfenidone and the immune system in IPF. Considering the important role of immune responses in IPF, the interaction between pirfenidone and the immune system needs to be studied in more detail, especially in IPF patients receiving long-term pirfenidone treatment.

Nintedanib

Nintedanib is a triple tyrosine kinase inhibitor targeting PDGF, vascular endothelial growth factor and FGF receptors as well as the non-receptor members of the Src family (199, 200). Nintedanib is an anti-fibrotic and anti-inflammatory drug, and was found to reduce fibroblast proliferation, recruitment, and myofibroblast differentiation, and hinders the secretion of ECM in the lung (199). Two replicate Phase II randomized control studies (INPULSIS-1 and 2) confirmed the safety and tolerability profile of Nintedanib in PF (201). Nintedanib reduced the decline of lung function, improved life expectancy, and reduced the risk of acute exacerbations and mortality (201). Recently, the high affinity of nintedanib to FGF receptors and its favorable toxicity profile resulted in the successful application of nintedanib in combination with conventional chemotherapy in cancer patients, such as second-line therapy for rapidly progressing advanced non-small-cell lung cancer (NSCLC) (202–207). However, the direct impact of nintedanib on the host immune response in the clinical cancer setting as well as in IPF is not completely understood. A recent study showed that nintedanib in combination with paclitaxel, a widely used chemotherapy medication, reduced the number of leukocytes and MDSCs in peripheral blood, and reduced the number of $CD8^+$ and B cells in the tumors of Lewis lung carcinoma tumor bearing mice (208). However, nintedanib alone only reduced the mobilization of MDSCs into peripheral blood (208). Overed-Sayer et al., 2020, found that the number of lung mast cells is increased in IPF and was found to be negatively correlated with baseline lung function in humans (209). Additionally, they found that nintedanib, but not pirfenidone, inhibited human fibroblast mediated mast cell survival through stem cell factor receptors and reduced the recruitment of mast cells into the lungs of BPF in rats (209). Although there is a growing body of evidence supporting a dynamic interaction between nintedanib and immune cells, there remains limited understanding on its details, thus warranting further studies.

Novel Therapies for the Treatment of IPF

Future therapies in IPF would ideally target the phenotype and molecular endotype of IPF patients to further personalize treatments based on guided molecular testing. Completed or still ongoing clinical trials of other pharmacological therapies in IPF are listed (**Table 1**). Although some of the listed medications provided encouraging results on the safety and efficacy in IPF patients (227), several other medications failed to demonstrate benefits in treating patients with IPF in recent clinical trials (213–216). However, many of these new medications may potentially interact with the immune system and be beneficial in some endotype of the IPF patient population but both the exact effects of these medications on immune responses and the beneficial effects remain unclear. In recent years, novel revolutionary anti-cancer agents were also suggested to be used in IPF patients. A recent review article analyzed the available literature on the use of immune checkpoint inhibitors in IPF and suggested only a slight beneficial effect for PD-1/PD-L1 inhibitors (228). Cross talk between PD-1, expressed on $CD4^+$ T cells, and PD-L1,

TABLE 1 | Last completed and/or ongoing clinical trials in idiopathic pulmonary fibrosis.

Drug	Mechanism	Phase	NCT identifier (48)[3]	Ref.
Sirolimus (Rapamycin)	Phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) inhibitor	N/A	NCT01462006	–
Omipalisib (GSK2126458)	PI3K/mTOR inhibitor	I	NCT01725139*	(210)
HEC68498	PI3K/mTOR inhibitor	I	NCT03502902	–
Fresolimumab (GC1008)	Anti-TGF-ß monoclonal antibody	I	NCT00125385*	–
TD139	Galectin-3 inhibitor	II	NCT02257177*/NCT03832946	(211)
Tipelukast (MN-001)	Leukotriene antagonist	II	NCT02503657	–
Belumosudil (KD025)	Rho-associated coiled-coil kinase 2 (ROCK2) inhibitor	II	NCT02688647	(212)
CC-90001	Jun N-terminal kinase inhibitor	II	NCT03142191	–
Dactrekumab (QAX576)	Anti-IL-13 monoclonal antibody	II	NCT00532233*/NCT01266135#	–
Lebrikizumab	Anti-IL-13 monoclonal antibody	II	NCT01872689*	(213)
Tralokinumab (CAT354)	Anti-IL-13 monoclonal antibody	II	NCT02036580*/NCT01629667#	(214)
Romilkimab (SAR156597)	Anti-IgG4 monoclonal antibody that neutralizes IL-4 and IL-13	II	NCT02345070*	(215, 216)
BG00011 (STX-100)§	Anti-integrin (αvβ6) monoclonal antibody	II	NCT01371305*/NCT03573505#	–
Simtuzumab (GS-6624)§	Anti-lysyl oxidase like-2 (LOXL2) monoclonal antibody	II	NCT01769196#	(217)
Ianalumab (VAY-736)	Anti-B-cell activation factor (BAFF) receptor monoclonal antibody	II	NCT03287414	–
Rituximab	Anti-CD20 monoclonal antibody	II	NCT01969409/NCT03286556	–
Carlumab (CNTO 888)§	Anti-CCL2 monoclonal antibody	II	NCT00786201*	(218)
Fezagepras (PBI-4050)	Ligand of probable G-protein coupled receptor (GPR) 40 and 84	II	NCT02538536*	(219)
BMS-986020	Autotaxin-Lysophosphatidic acid pathway (ATX-LPA) inhibitor	II	NCT01766817*	(220)
GLPG1690	ATX-LPA inhibitor	III	NCT03711162/NCT03733444	(221)
PRM-151	Pentraxin-2 analogue	III	NCT04552899/NCT04594707	(158, 222)
ART-123	Recombinant thrombomodulin	III	NCT02739165*	(223)
Treprostinil	Prostacyclin	III	NCT04708782	–
Cotrimoxazole	Antimicrobial	III	NCT01777737#/NCT02759120#	(224)
Doxycycline	Antimicrobial	III	NCT02759120#	(224)
Sildenafil	Phosphodiesterase type 5 (PDE5) inhibitor	III	NCT00517933*/NCT02802345*	(225, 226)
Pamrevlumab (FG-3019)	Anti-connective tissue growth factor (CTGF) monoclonal antibody	III	NCT03955146/NCT04419558	(227)

Completed clinical trial.

#*Clinical trial withdrawn or terminated.*

§*Development in IPF discontinued.*

expressed on fibroblasts, was proposed to lead to IL17A and TGF-β production by $CD4^+$ T cells, which in turn promoted fibrogenesis (228). Recent research also highlighted promising novel drug targets, such as IL-11 in PF, that warrant further studies (229).

However, our incomplete understanding of IPF pathogenesis, particularly of the role of both structural cells and immune cells in the initiation and progression of IPF, and interaction of current as well as novel therapies with the immune system represent important challenges on the way to success for future precision medicine. To this end, considering the high inter-individual variation in IPF patients, responses to any medication will presumably result in similar variation, thus underscoring the importance of the identification of biomarkers predicting treatment response.

IMMUNOREGULATORY CELLS AND LUNG TRANSPLANTATION IN IPF

Progressive fibrosis in IPF can ultimately result in respiratory failure and death. Here, lung transplantation represents the only therapeutic option that has been linked with a survival benefit in patients suffering from IPF (230). In 2018, a total of 2562 lung transplants were performed in the US, with >60% for underlying disease of IPF (231). However, persisting donor organ scarcity is still limiting the availability of this life-saving therapeutic option. As a result, single-lung transplants (SLT) might represent an effective strategy to serve the increasing demand on donor organs. However, recent analysis of >9.000 patients undergoing lung transplantation for IPF within the United Network of Organ Sharing (UNOS) area demonstrated that double-lung transplantations (DLTs) have improved survival compared to SLTs in patients suffering from IPF (232). Still, five-year survival rates after lung transplantation for IPF are worse compared to other indications (233) with chronic lung allograft dysfunction (CLAD) affecting more than 50% of transplanted lungs after 5 years. Of note, fibrotic processes in the engrafted lung exhibit striking similarities to those in IPF (234).

Since the first report that Tregs are involved in preventing allograft rejection two decades ago (235), immunoregulatory cells have also been widely reported to play a significant role in the context of transplantation and graft tolerance. In more detail, graft acceptance could be linked to complex immunological cross-talk between many more cells, including Bregs, regulatory DCs and especially MDSCs. For example, MDSCs were shown to inhibit GVHD after cell transplantation *via* an

[3] https://www.clinicaltrials.gov/

arginase-1–dependent mechanism in an experimental model (236). In the clinical context, 50 patients with biopsy-proven acute T cell-mediated rejection (ATCMR) showed increased frequencies of MDSCs in peripheral blood mononuclear cells (PBMC) after renal transplantation, which were linked to improved allograft function compared with the MDSCs low group (237). In addition, MDSCs isolated from 29 adult kidney transplant recipients were demonstrated to expand Tregs *in vitro*, while their accumulation overtime after transplantation linearly correlated with an increase of Tregs *in vivo*, thus providing further evidence for an interactive cross-talk between regulatory cell types (238).

Immunoregulatory cells have also entered the stage in the context of both experimental and clinical lung transplantation. Experimental data demonstrated that long-term lung acceptance is associated with the induction of bronchus-associated lymphoid tissue (BALT), where Tregs accumulate and recipient T cells interact with $CD11c^+$ DCs, ultimately resulting in an immune quiescent state (239). Mechanistically, the depletion of Tregs from the BALT of tolerant lungs was shown to result in antibody-mediated rejection, which was characterized by the generation of donor-specific antibodies, complement deposition, and the destruction of airway epithelium. In contrast, the undepleted control group showed no evidence of rejection (240). Of note, a recent prospective human observational study could demonstrate that increased Treg frequencies after lung transplantations were reciprocal associated with chronic lung allograft dysfunction (241). Of particular interest, monocyte-derived DCs, isolated from the peripheral blood of lung transplant patients without bronchiolitis obliterans syndrome, were found to have higher IDO expression, implicating the involvement of MDSCs (242). A recent clinical study focused on MDSCs, assessing their phenotype and frequency in peripheral blood from 20 lung transplant recipients and its relationship to post-transplant complications and immunosuppression (243). In detail, MDSCs were isolated from PBMCs and their functionality was assessed *in vitro* by their capability to block $CD4^+$ and $CD8^+$ T cell proliferation. As a result, MDSCs were increased in stable lung transplant recipients (n=6) vs. non-transplant controls (n=4). Furthermore, patients with infection (n=5) or CLAD (n=9) had lower MDSCs compared to stable recipients. Of note, MDSCs tended to correlate with blood levels of immunosuppressive medication (i.e. cyclosporine A and tacrolimus).

In addition, Bregs have also been shown to interact during the immunological cross-talk subsequent to allotransplantation by mainly promoting the development of Tregs while at the same time suppressing effector $CD4^+$ and $CD8^+$ T cells, as demonstrated in experimental models (244). The first clinical study in 117 lung transplant recipients recently revealed that $CD19+CD24^{high}CD38^{high}$ Breg cells were associated with chronic rejection while no significant correlation with Tregs was found (245).

Interestingly, a recent publication could demonstrate that the application of MSC–based therapy during *ex vivo* lung perfusion (EVLP) before lung transplantation ameliorated ischemic injury in an experimental pig model. In detail, the MSC group showed significantly lower IL-18 and IFN-γ levels and a significantly higher IL-4 level in lung tissue at 12 hours of EVLP compared to the control group thus reflecting a shift of the inflammatory network towards protective conditions. Of critical relevance, the pathological acute lung injury score after transplantation was significantly lower in the MSC-treated group compared to the control group (246). These experimental data are intriguing and might already point towards future directions where immunoregulatory cells might represent promising candidates for cell therapies.

Thus, there is robust and growing evidence that immunoregulatory cells play a pivotal role in allograft acceptance from both experimental and clinical science (247). Coming back to MDSCs, current data attribute the immunoregulatory properties of these cells mainly to two mechanisms of action upstream of T cells to shift the immune response towards peripheral tolerance: First, to their capacity to inhibit the proliferation of allogeneic T cells (243); Second, to their capacity of Treg induction (248, 249). Interestingly, the impact of concomitant immunosuppressive therapy, to prevent allograft rejection, while at the same time acting on the immune cells in general, and MDSCs in particular, has also become a field of increasing research activities. Here, cyclosporine A, a widely used immunosuppressive drug from the group of calcineurin inhibitors, could be mechanistically linked to increased expression of IDO resulting in increased suppressive activities of MDSCs in a mouse skin transplant model (250).

Tacrolimus, another calcineurin inhibitor used to maintain immunosuppression, has recently been shown to increase the immunosuppressive capacity of MDSCs derived from human kidney allograft recipients *in vitro* (251). In the context of experimental trachea transplantation, the impact of Rapamycin combined with immature DCs (Rapa-imDC), isolated from bone marrow, was investigated in trachea recipient rats receiving Rapa-imDCs for 10 consecutive days after transplantation. Here, Rapa-imDC treatment induced T cell hyporesponsiveness by attenuating T cell differentiation into IFN-γ-producing Th1 cells while at the same time increasing Tregs. In addition, Rapa-imDC administration ameliorated airway obliteration symptoms and $CD4^+$ and $CD8^+$ T cell infiltration (252).

Taken together, immunoregulatory cells have been shown to play a central role in mediating the alloimmune response towards graft tolerance, thus becoming a promising target for new pharmacologic strategies in the clinical context of transplantation. However, our current understanding of how immunoregulatory cells are interacting in the clinical context of lung transplantation and IPF is still at its infancy, as particular data are missing thus far. Furthermore, we still have to foster our current understanding of how these cells are shaping the alloimmune response while interacting with current immunosuppressive drugs. Here, the clinical background of IPF patients undergoing lung transplantation with poor prognosis is calling for intensive research activities while the concept of tailored cell therapy is already arising from experimental science.

CONCLUSION

IPF is the most devastating interstitial lung disease, yet remains poorly characterized and understood. Lack of mechanistic understanding of the complex disease causality and the devastating chronic nature complicate research and development. There remains a lack of efficacy of pharmaceutical therapies and lung transplantation is currently still the only truly effective treatment IPF. Despite recent advances in our understanding of IPF, particularly the role of regulatory immune cells remains contradictory and poorly understood. Indeed most, if not all, of the regulatory immune cells involved exhibit both detrimental as well as beneficial effects in IPF. Substantial evidence underlines the negative role of macrophages in IPF, through the involvement in the dysregulated wound healing process and ECM build-up. On the other hand, the suppressed anti-fibrotic effects observed in lung macrophages hint to potential beneficial effects, resulting in a complex and controversially-discussed role for macrophages in IPF. There are several lines of evidence on the pro-fibrotic role of MSCs in IPF, mainly related to myofibroblast differentiation and cellular senescence. However, recent research on MSCs points to a beneficial role in IPF, mainly related to their anti-inflammatory characteristics. Tregs were also found to act beneficial or detrimental in IPF models, likely influenced by the state of the disease or the disease model used. The role of more recently discovered regulatory immune cells, namely Bregs, tolerogenic DCs, MDSCs and novel macrophage phenotypes in IPF remains unclear as well and needs to be studied in more detail. An increased understanding of the potential role of regulatory immune cells in IPF mouse models and, particularly, in human IPF will be of vital importance to design novel effective pharmaceutical treatments as well as improving the success of lung transplantation and to prevent related GVHD. This is especially true with regards to precision medicine that might define future healthcare frameworks. Therefore, further preclinical and human studies are crucial to better understand and define the role of immune regulatory cells in IPF pathogenesis.

AUTHOR CONTRIBUTIONS

SK conceptualized the review. CG, AD, MQ, and SK contributed to the original draft. CG, AD, MQ, HR, DH, and SK contributed to revising and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the fortüne program of the University of Tübingen to MQ (2461-0-0) and SK (2458-0-0 and # 2606-0-0). We acknowledge support by Open Access Publishing Fund of University of Tübingen.

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Conflict of Interest: Author DH was employed by the company Novartis.

The remaining 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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ORIGINAL RESEARCH
published: 23 April 2021
doi: 10.3389/fimmu.2021.642855

The NLRP3-Inflammasome-Caspase-1 Pathway Is Upregulated in Idiopathic Pulmonary Fibrosis and Acute Exacerbations and Is Inducible by Apoptotic A549 Cells

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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *16 December 2020*
Accepted: *06 April 2021*
Published: *23 April 2021*

Citation:
Jäger B, Seeliger B, Terwolbeck O, Warnecke G, Welte T, Müller M, Bode C and Prasse A (2021) The NLRP3-Inflammasome-Caspase-1 Pathway Is Upregulated in Idiopathic Pulmonary Fibrosis and Acute Exacerbations and Is Inducible by Apoptotic A549 Cells. Front. Immunol. 12:642855. doi: 10.3389/fimmu.2021.642855

Idiopathic pulmonary fibrosis (IPF) is a relentlessly progressive disease harboring significant morbidity and mortality despite recent advances in therapy. Regardless of disease severity acute exacerbations (IPF-AEs) may occur leading to considerable loss of function and are the leading cause of death in IPF. Histologic features of IPF-AE are very similar to acute respiratory distress syndrome (ARDS), but the underlying mechanisms are incompletely understood. We investigated the role of the NLRP3 inflammasome in IPF and IPF-AE. Bronchoalveolar lavage (BAL) cells were sampled from patients with IPF (n = 32), IPF-AE (n = 10), ARDS (n = 7) and healthy volunteers (HV, n = 37) and the NLRP3-inflammasome was stimulated *in-vitro*. We found the NLRP3 inflammasome to be hyper-inducible in IPF compared to HV with increased IL-1ß and pro-IL-1ß levels on ELISA upon stimulation as well as increased caspase-1 activity measured by caspase-1p20 immunoblotting. In IPF-AE, IL-1ß was massively elevated to an extent similar to ARDS. To evaluate potential mechanisms, we co-cultured BAL cells with radiated A549 cells (a model to simulate apoptotic alveolar epithelial cells), which led to increased NLRP3 mRNA expression and increased caspase-1 dependent IL-1ß production. In the presence of a reactive oxygen species (ROS) inhibitor (diphenyleneiodonium) and a cathepsin B inhibitor (E64D), NLRP3 expression was suppressed indicating that induction of NLRP3 activation following efferocytosis of apoptotic A549 cells is mediated *via* ROS and cathepsin-B. In summary, we present evidence of involvement of the NLRP3 inflammasome-caspase pathway in the pathogenesis of IPF-AE, similarly to ARDS, which may be mediated by efferocytosis of apoptotic alveolar epithelial cells in IPF.

Keywords: idiopathic pulmonary fibrosis, inflammasome, NLRP3, acute exacerbation, inflammation

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common of the idiopathic interstitial pneumonias and is characterized by its progressive nature and considerable mortality despite recent advances in antifibrotic therapy (1, 2). A severe complication of IPF may occur in the form of an acute exacerbation (IPF-AE), defined as unexplained worsening of the condition with new bilateral ground glass opacifications on chest computer tomography (CT) without evidence of pulmonary edema or lung embolism, overall sharing many features with acute respiratory distress syndrome (ARDS) (3, 4). Known triggers include lung injury due to thoracic surgery, chest trauma, invasive ventilation but also aspiration and infections (3). Notably, IPF-AE may occur irrespectively of disease severity and harbors a poor prognosis with in-hospital mortality of 50% (3). The most common histopathological pattern found in these patients is diffuse alveolar damage which is also found in ARDS (5, 6). The underlying pathomechanisms of IPF-AE are however, still poorly understood.

The NLRP3-inflammasome has been associated with various pulmonary diseases, including sarcoidosis (7), asbestosis and silicosis (8), rheumatoid arthritis associated interstitial lung disease and also IPF (9), but has not been studied in IPF-AE. Inflammasomes are multiprotein complexes which include a sensor, the adapter protein apoptosis-associated speck-like protein containing a CARD domain (ASC) and caspase-1 (10). Sensors include among others the nucleotide-binding oligomerization domain-like receptor (NLR) family, pyrin domain-containing 3 (NLRP3) (10). For the activation of caspase-1, two signals are necessary: signal 1 induces activation of pattern-recognition receptors (PRR) for example Toll-like receptors (TLRs) by pathogen associated molecular patterns (PAMPS) including lipopolysaccharides (LPS). This leads to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) regulated transcription of inflammasome components: pro-caspase-1, pro–IL-1β and NLRP3 (10). Signal 2 includes a magnitude of stimuli, including ATP and nigericin, which activate NLRP3 inducing inflammasome assembly *via* the oligomerization of ASC. This results in the activation of pro-caspase-1, which is spliced into its active forms caspase-1p10 and caspase 1p20 which in turn activate pro-IL-1β *via* proteolytic cleavage into the active cytokine IL-1β (10, 11). In ARDS, key cytokines include the caspase-1 dependent IL-1ß and IL-18 and the involvement of the NLRP3-inflammasome has been recently demonstrated (12, 13).

IPF pathogenesis is driven by dysfunctional repair mechanisms to microinjuries of the alveolar epithelium and especially of type II alveolar epithelial cells (AECs) (14). In the presence of persistent stress, the AEC-II dysfunctional response lead to cell apoptosis (15). These apoptotic cells are then ingested by alveolar macrophages (AMs), a process which is called efferocytosis (16), capable of providing a pro-fibrotic macrophage response which can induce pulmonary fibrosis (17). Dysregulated efferocytosis observed in IPF (18) can lead to increased production of reactive oxygen species (ROS). Interestingly also activation of the NLRP3 inflammasome by many trigger factors was shown to rely on ROS production (19–25) and cathepsin B leakage into the cytoplasm (26), suggesting a link between efferocytosis in IPF and NLRP3-inflammasome activation.

We aimed to examine the inducibility of the NLRP3-inflammasome in IPF, IPF-AE, and ARDS and the potential role of apoptotic epithelial cells in promoting NLRP3-inflammasome inducibility. We provide evidence of a hyper-inducible NLRP3-inflammasome-caspase 1 pathway which can be triggered by efferocytosis of apoptotic AECs.

MATERIAL AND METHODS

Patient Population and Bronchoalveolar Lavage Sample Preparation

BRONCHOALVEOLAR Lavage (BAL) sampling was performed in patients with acute respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis (IPF) with and without acute exacerbation (IPF-AE), and healthy volunteers (HV) at the University Medical Center in Freiburg im Breisgau (Germany) and at Hannover Medical School (Germany). Healthy volunteers were screened for pulmonary abnormalities by thorough medical history, physical examination, and pulmonary function testing. Diagnosis of ARDS was made in accordance with the Berlin 2012 definition (4), and a diagnosis of IPF was established per the practice guidelines issued by the American Thoracic Society and European Respiratory Society (1). The definition criteria of AE were: deterioration of dyspnoea >20% in <3 weeks, occurrence of new opacities, and absence of alternative cause (including infection, heart failure, or pulmonary embolism) (27). All IPF patients with suspicion of acute exacerbation received chest computed tomography.

All patients provided written informed consent, and collection of bio-samples was registered at the German Clinical Trials Register (DRKS00000017 and DRKS00000620). The respective institutional review boards approved of the bio-sampling (Freiburg 47/06 March 10th 2006, Hannover, #2923-2015 and #2516-2014, Nov 2nd 2015).

BAL was performed in the middle lobe or lingula. After BAL sampling, macro-impurities were removed by sample filtration through sterile gauze. Differential BAL total cell number was counted using May–Grunwald–Giemsa stain (Merck) on native sample. BAL samples were centrifuged at 500g for 10min at 4°C (28) and subsequently resuspended in Macrophage SFM Medium or DMEM medium. BALs were performed between 2010 and 2013 at the University Medical Center Freiburg and in 2017 and 2021 at Hannover Medical School.

Cell-Culture and NLRP3-Inflammasome Stimulation Protocol

The NLRP3 inflammasome was activated in accordance to our recently described protocol (7). In brief, 1×10^5 BAL cells derived from HV, IPF, and ARDS patients, were incubated with 100 µl of Macrophage SFM/Gibco Medium (Life Technologies, USA) with 1% penicillin and streptomycin (Biochrom, Germany) at 37°C and 5% CO_2 in a flat-bottom 96 well plate (**Figure 1A**). Two

steps are needed for the activation of inflammasome-dependent caspase-1 activation: a first signal 1 such as an Toll-like-receptor agonist (LPS) and a second tissue damage signal such as ATP or Nigericin (29). BAL-cells were primed with 1 µM LPS (Fluka Biochemika, Switzerland) initially for 4h. Thereafter, 1 mM ATP (Sigma Aldrich, USA) or 10 µM Nigericin (Sigma Aldrich, USA) was added with incubation of another 2h. Supernatants were sampled and stored at −80°C until analysis. Cells were lysed with lysis buffer [PBS (Life Technologies, USA) with 0.5% triton X (Sigma Aldrich, USA) and 10% FCS (Biochrom, Germany)] for pro-IL-1ß ELISA and with 200 µl Trizol (Thermo Fisher Scientific, USA) for RNA isolation and stored at −80°C until analysis.

Cell Culture and Stimulation With Co-Culturing BAL Cells With Radiated A549 Cells

Since chronic cellular stress has been demonstrated to induce AEC apoptosis and consequently efferocytosis by alveolar macrophages, which is believed to contribute to IPF pathogenesis, we examined the effects of co-culturing alveolar macrophages with apoptotic A549 cells on NLRP3-inflammasome activation (15, 16). A549 cells were cultured in DMEM/Gibco medium (Life Technologies, USA) with 10% FCS and 1% penicillin/streptomycin at 37°C/5% CO_2 and radiated with 10 Gy and then incubated for additional 72h (30–32). Harvesting after 72h resulted in highest rate of apoptotic cells on viability testing. Viability was assessed *via* trypan blue staining and the FACS annexin V apoptosis detection Kit (BD, USA) as per manufacturer's instructions. 1×10^6 BAL-cells were then co-cultured w/wo 1×10^5 radiated A549 cells in 500 µl of macrophage/Gibco medium (serum free) in a 24-well cell culture plate and incubated for 1h at 37°C/5% CO_2 to allow for efferocytosis and consequently stimulated as described above.

To evaluate the effects of efferocytosis on NLRP3 mRNA expression, we co-cultured radiated, apoptotic A459 with normal BAL-cells for 1h. Following that, we stimulated BAL cells with 1 µM LPS in a 24-well cell culture plate for 2h at 37°C. Supernatants were discarded, and cells were lysed with 200 µl Trizol. In some experiments, we assessed the effect of the NADPH-Oxidase inhibitor [ROS-inhibitor; DPI; 20 µM (Sigma Aldrich, USA)] and of the cathepsin B inhibitor E64D (10 µM) (MCE, USA). Both compounds were added with the radiated A549 cells at baseline, and DMSO 0.2% was added as vehicle (both E64D and DPI were suspended in DMSO). Cell lysates were again sampled in 200 µl Trizol following 2h incubation at 37°C/5% CO_2. In another set of experiments, the effects of the selective NLRP3 inhibitor (MCC950) (33) and caspase-1 inhibitor VX-765 (both Invivogen, France) on IL-1ß and TNF-α production following inflammasome stimulation were

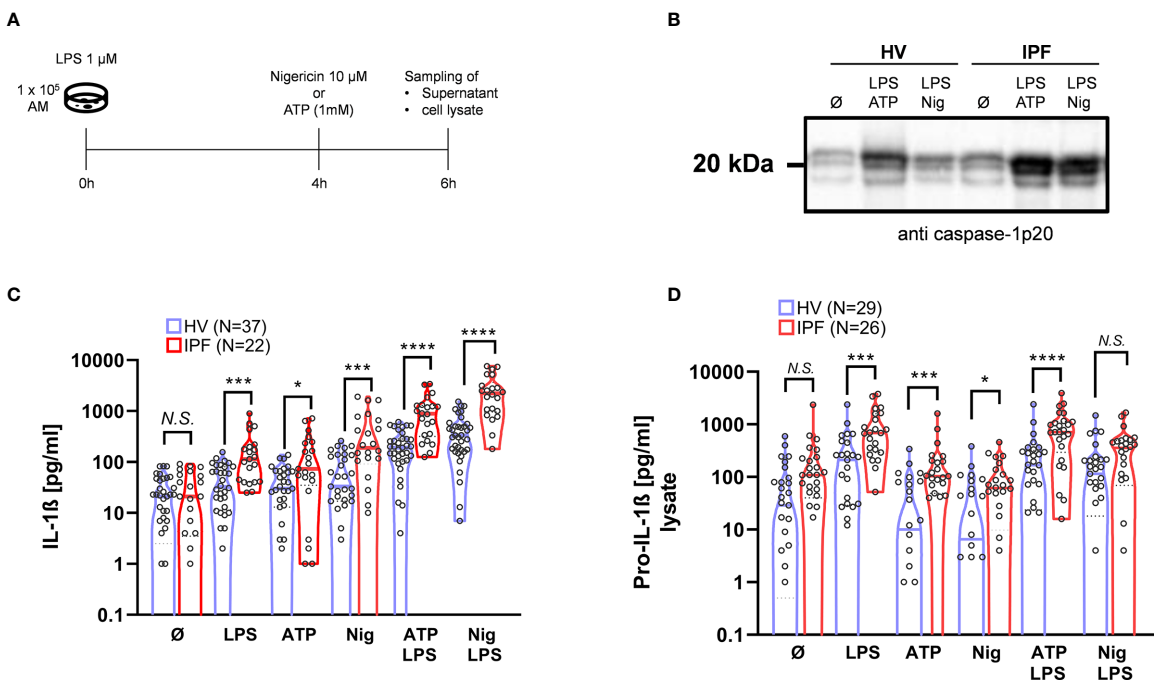

FIGURE 1 | The ability to activate the NLRP3 inflammasome is significantly increased in BAL cells from IPF patients. For NLRP3 stimulation, bronchoalveolar lavage (BAL) cells were incubated and stimulated with LPS at 0h, following Nigericin or ATP stimulation after 4h **(A)**. In both healthy volunteers (HVs) and idiopathic pulmonary fibrosis (IPF) there was a significant increase in IL-1ß production following NLRP3-inflammasome stimulation protocol **(B)**, with significant higher responses in IPF compared to HV. Caspase-1 activation is demonstrated by representative immunoblotting of cleaved caspase-1p20 (representative blot shown; total of n = 15 immunoblots performed). **(C)**. Pro-IL-1ß concentration in cell lysates was elevated in IPF patients compared to HV following stimulation with LPS, ATP, Nigericin, and LPS + ATP **(D)**. IL-1ß and Pro-IL-1ß values were compared using unpaired t-tests; *p < 0.05; ***p < 0.001; ****p < 0.0001, *N.S.* non-significant.

evaluated. Two different concentrations (1 + 10 μM and 25 + 75 μM, respectively) were added 30min before addition of radiated A549 cells.

Efferocytosis Assay

To demonstrate efferocytosis of radiated A549 cells by alveolar macrophages, 1×10^5 control BAL cells were incubated with 2 μM cytochalasin D (Sigma Aldrich #250255, USA), a known cytoskeletal disruptor of phagocytosis in macrophages, for 30min at 37°C. Radiated A549 cells were incubated with pHrodo (Red AM Intracellular pH Indicator, Thermo Fisher Scientific #P35372, USA) at a 1:100,000 dilution for 30min. Engulfment of A549 cells leads to a pH-shift in the alveolar macrophages inducing an increased pHrodo light emission (34). Afterwards 1×10^5 BAL cells were incubated with and without 3×10^5 pHrodo-labeled radiated A549 cells for 2h in a 96 well plate. All BAL cells with and without A549 cells were harvested with 100 μl PBS, and nuclear staining was performed with 1 μg/ml Hoechst-33342 dye solution (Thermo Fisher Scientific #H3570, USA) for 30min at RT. Immediately afterwards, a cytospin with 2×10^4 cells in 100 μl PBS was performed, and efferocytosis of macrophages was analyzed by fluorescence microscopy using Axio Observer Inverted microscope and ZEN navigation software (Zeiss, Germany). Percentage of $pHrodo^+$ and $Hoechst\text{-}33342^+$ cells was counted using ImageJ V.1.53e (NIH, USA) (35).

Immunoblot Analysis of Caspase-1p20 Activity

Supernatants collected after NLRP3-stimulation protocol were precipitated with methanol/chloroform. After initial centrifugation, the upper phase was discarded and methanol was added to the lower phase, followed by another centrifugation step. The supernatant was discarded, and the pellet was incubated at 55°C for 5min and resuspended in 20 μl Laemmli-buffer (Bio-Rad, USA). Samples were consequently cooked for 5min at 95°C. Samples were separated using 15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Cleaved caspase-1 (p20) was detected using primary antibody rabbit mAb cleaved caspase-1 (Asp297) (Cell Signaling Technology, USA) with the secondary antibody goat anti-rabbit (H + L) HRP conjugate (1:3,000) (Bio-Rad, USA). Enhanced chemiluminescence (Clarity™ Western ECL Substrate, Bio-Rad, USA) was used for detection with ChemiDoc™ MD Imaging System (BioRad, USA).

IL-1ß, pro-IL-1ß and TNF-α Measurement by ELISA

The concentration of both pro-IL-1ß and IL-1β was measured by ELISA (Human IL-1β/IL-1F2 DuoSet, R&D, USA) in the cell lysate and culture supernatant, respectively. (36). TNF-α concentration from culture supernatant was measured by ELISA (Human TNF-alpha DuoSet #DY210, R&D, USA).

RT-PCR for NLRP3

RNA was isolated *via* Trizol method (ThermoFisher Scientific, USA). 1×10^6 cells were lysed with 200 μl of Trizol. Extracted RNA was reverse-transcribed to cDNA using the iScript cDNA Synthesis kit (Bio-Rad, USA) as per manufacturer's instructions (7). The obtained cDNA was analyzed by Real-Time PCR (Light Cycler/Roche, Switzerland) with the following primers: huNLRP3 (5′-AGAATGCCTTGGGAGACTCA-3′ and 5′-CAGAATTCACCAACCCCAGT-3′), resulting in a 93 bp product, exon 6/7 overlapping; GAPDH (5′-ACAGTCAGCC GCATCTTCTT-3′ and 5′-GTTAAAAGCAGCCCTGGTGA-3′) as reference (7). The expression of huNLRP3 was normalized to GAPDH expression. A cycle threshold value was calculated and used to ascertain the relative level of huNLRP3 messenger RNA by the following formula: relative expression = [2 (cycle threshold of glyceraldehyde 3-phosphate dehydrogenase- ycle threshold of huNLRP3)] × 10,000 for each complementary DNA sample.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 Software (La Jolla, USA) and RStudio version 1.3.1093 (RStudio Inc., USA). Variables were compared by unpaired t-test. All data are expressed as mean + SD unless stated otherwise. A two-tailed p-value of <0.05 was considered to statistically significant.

RESULTS

Study Population

A total of 86 BALs were used in this study from patients with IPF (n = 32), IPF/AE (n = 10), ARDS (n = 7), and HV (n = 37) with demographics, pulmonary function tests (in IPF), and BAL cell counts shown in **Table 1**. All patients were naïve to antifibrotic therapy, since the majority of patients were recruited prior to the widespread introduction of antifibrotics. At the time of BAL, 41% of IPF and 60% of IPF-AE patients were receiving triple-therapy with n-acetylcystein, prednisolone, and azathioprine. In 2021, additional BALs in three IPF patients were performed (two female; mean age 71 years, mean forced vital capacity 70% of predicted, mean diffusion capacity for carbon monoxide 52% of predicted; mean alveolar macrophages in BAL 87%, mean neutrophils in BAL 4%).

The NLRP3 Inflammasome Activation Is Significantly Increased in AM From IPF Patients Compared to HV

In a first step, the IL-1ß production of normal BAL cells derived from HV was tested following NLRP3-inflammasome stimulation (**Figure 1A**). Normal BAL cells did not spontaneously produce IL-1ß, which production is tightly regulated (37). Mean basal IL-1ß production without stimulation was increased with LPS alone ($p = 0.002$), Nigericin alone ($p = 0.0002$), and with ATP alone ($p = 0.0005$) (**Figure 1B**). Most pronounced IL-1ß production was seen with co-stimulation of LPS + ATP ($p < 0.0001$) and LPS + Nigericin ($p < 0.0001$). Evidence of caspase-1 activation by NLRP3 inflammasome was seen by increased protein band intensity of the caspase-1p20 fragment by immunoblot with stimulation by LPS + ATP and LPS + Nigericin (**Figure 1C**).

TABLE 1 | Study population and BAL differential cell counts.

Characteristics	IPF (n = 32)	IPF/AE (n = 10)	ARDS (n = 7)	HV (n = 37)
Age, years (SD)	68 ± 10	68 ± 10	52 ± 15	29 ± 8
Sex (male), N (%)	30 (94)	9 (90)	6 (86)	20 (54)
Forced vital capacity, % of predicted, mean (SD)	64 ± 16	–	–	–
Diffusion capacity for carbon monoxide (single breath), % of predicted, mean (SD)	36 ± 20	–	–	–
Invasive ventilation, n (%)	0	2 (20)	7 (100)	0
Received n-acetylcysteine, prednisolone, and azathioprine	13 (41)	6 (60)	0	0
BAL cell count, $\times 10^6$/100ml (SD)	14.6 ± 7.8	18.0 ± 7.5	32.4 ± 30.7	7.0 ± 3.2
Alveolar macrophages, % (SD)	65.1 ± 21.2	59.1 ± 15.5	32.7 ± 19.5	86.0 ± 5.2
Lymphocytes, % (SD)	12.1 ± 12.8	4.0 ± 3.0	8.5 ± 3.6	10.5 ± 5.2
Neutrophils, % (SD)	18.9 ± 22.0	30.8 ± 17.3	56.2 ± 20.9	2.3 ± 1.8
Eosinophils, % (SD)	3.4 ± 4.7	6.3 ± 4.4	2.0 ± 3.9	0.3 ± 0.7

ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; HVs, healthy volunteers; IPF, idiopathic pulmonary fibrosis; IPF/AE, acute exacerbation of idiopathic pulmonary fibrosis.

Compared to BAL cells from IPF patients, basal (unstimulated) IL-1ß production was similar compared to HV. Of note, there was a consistently higher IL-1ß production in BAL cells from IPF patients after stimulation with LPS ($p = 0.0002$), Nigericin: ($p = 0.0001$), and ATP ($p = 0.0002$). With the combined stimulation with LPS + ATP and LPS + Nigericin, discrepancies were even more pronounced (both $p < 0.0001$).

In line with this, there was a significant increase in caspase-1 activation determined by caspase-1p20 protein-band intensity after co-stimulations in IPF patients compared to HV (**Figure 1C**).

Intracellular Pro-IL-1ß Protein Expression Following Stimulation Is Increased in BAL-Cells From IPF Patients Compared to HV, but Not Without Stimulation

In order to test whether the NLRP3 inflammasome is already primed (signal 1 activated) in BAL cells derived from IPF patients, we analyzed intracellular pro-IL-1ß levels. Notably, baseline (unstimulated) pro-IL-1ß levels were not statistically significantly elevated in IPF compared to HV, indicating that NLRP3 inflammasome is rather hyper-inducible than primed. As expected, intracellular pro-IL-1ß levels increased following priming with LPS; LPS + ATP and LPS + Nigericin, but not with ATP and Nigericin alone (**Figure 1D**). Compared to HV, pro-IL-1ß production was significantly increased following stimulation with LPS + ATP ($p < 0.0001$) and LPS alone ($p = 0.0003$), as well as ATP ($p = 0.03$) and Nigericin ($p = 0.005$) in IPF (**Figure 1D**).

NLRP3 Inflammasome Is Markedly Upregulated in Patients With Acute Exacerbation of IPF to Similar Extent as Patients With Primary ARDS

To see how the NLRP3 inflammasome contributes to IPF/AE, we stimulated BAL cells from patients with IPF/AE (n = 10) with the same protocol as described above. As shown in **Figure 2**, there was a markedly elevated IL-1ß production of BAL cells from patients with both IPF/AE and ARDS following singular and combined stimulation and without stimulation (compared to HV). With IPF/AE and ARDS, the fold-change for IL-1ß production compared to IPF (without AE) for the combined stimuli was 3–4 and 15–20 compared to HV (**Figure 2F**).

Co-Culturing BAL Cells From HV With Radiated A549 Cells Results in Efferocytosis and Increases NLRP3-Inflammasome Activation. Selective NLRP3 and Caspase-1 Inhibition Suppresses IL-1ß Production Following Co-Incubation

To further investigate potential mechanisms behind the increased NLRP3 inflammasome activation in IPF patients *vs* HV, AECs (A549) were radiated with 10 Gy and harvested 72h after radiation (**Figure 3A**). Radiation induced apoptosis in the majority of cells, while a small proportion was double positive for annexin V and propidium iodide (**Figure 3C**). To verify if efferocytosis was actually performed, we labeled radiated A549 cells with pHrodo before co-incubation with BAL cells. Following co-incubation with A549 cells, alveolar macrophages became pHrodo$^+$ in 83% of all Hoechst-33342$^+$ cells, indicating efferocytosis of A549 cells. Following pre-treatment with cytochalasin-D (2 μM), this effect was almost completely inhibited (9% pHrodo$^+$/H-33342$^+$ cells) (**Figure 4**).

BAL cells of HV were co-incubated with radiated A549 cells for 1h before running the NLRP3 stimulation protocol. There was a significant 2.2-fold increase in IL-1ß production in BAL cells co-cultured with radiated A549 at baseline (without stimulation; $p = 0.031$) and following stimulation with LPS + Nigericin (3.5-fold; $p = 0.025$) (**Figure 3B**). With the other stimulations, IL-1ß production was increased but did not reach statistical significance. Equally, on immunoblotting there was an increased protein band both with caspase 1-p20 with co-culturing of radiated A549 (**Figure 3D**). There was no difference in pro-IL-1ß concentration in the cell lysates with or without co-culturing of A549 (**Figure 3E**).

To evaluate if the IL-1ß response was mainly mediated *via* the NLRP3 inflammasome, we pre-treated BAL cells with a selective NLRP3-inhibitor (MCC-950) or caspase-1 inhibitor (VX-765) before co-culturing the A549 cells (**Figure 3A**). Both agents

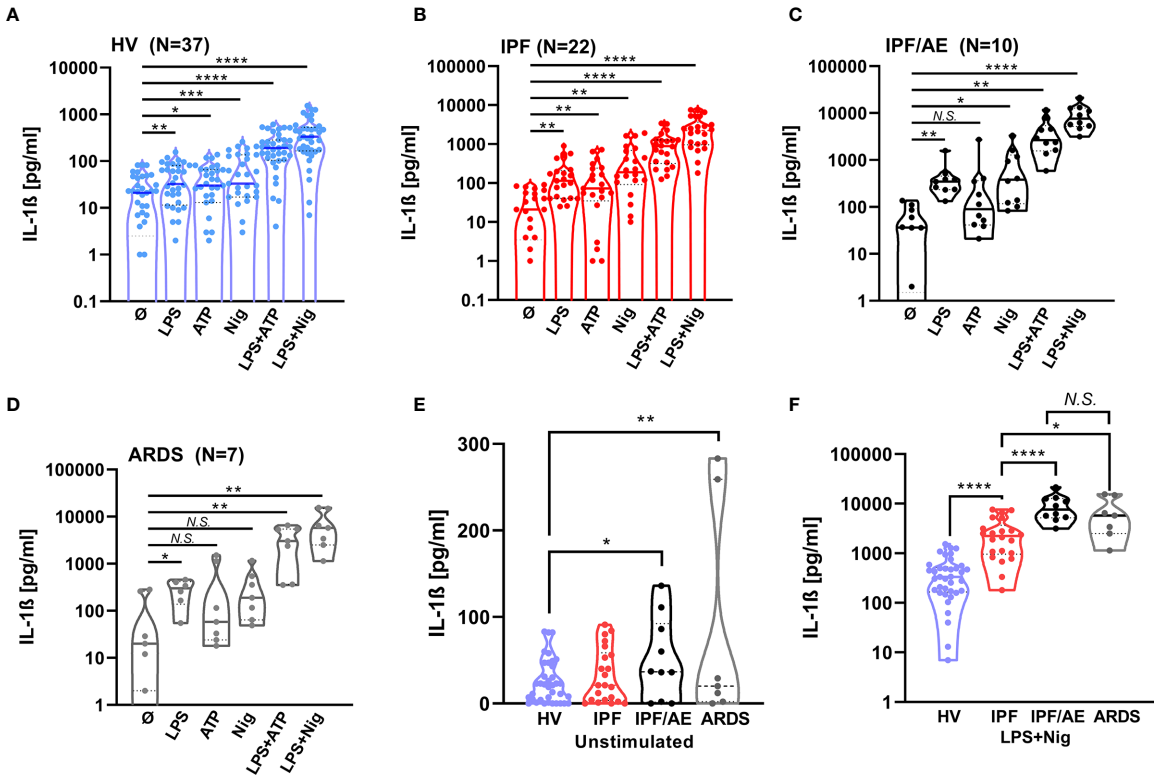

FIGURE 2 | IL-1β production after NLRP3 inflammasome stimulation is highest in BAL cells from patients with an acute exacerbation during IPF and BAL cells from patients with ARDS. IL-1ß production was detected by ELISA and is inducible by NLRP3 stimulation in alveolar macrophages of patients with acute exacerbation of idiopathic pulmonary fibrosis **(C)** and acute respiratory distress syndrome **(D)**. IL-1ß values for healthy volunteers (HVs) and IPF are shown for comparison **(A, B)**. IL-1ß production in these two cohorts is significantly increased compared to healthy volunteers (HVs) to a similar extent at baseline and following stimulation **(E, F)**. IL-1ß levels with stimulation compared to baseline and unstimulated IL-1ß levels between groups and LPS/Nigericin stimulation were compared using unpaired t-tests; *p < 0.05; **p < 0.01; *** p < 0.001; ****p < 0.0001, *N.S.* non-significant.

suppressed IL-1ß production >90% (**Figure 3H**), while TNF-α production remained largely (**Figure 3I**). These results indicate the increased IL-1ß response following efferocytosis of A549 cells is primarily mediated *via* the NLRP3/caspase-1 pathway.

NLRP3-Gene Expression Is Increased by Co-Culturing With Apoptotic A549 Cells and Attenuated by Inhibition of ROS and Cathepsin B

Acute lung injury and consecutive epithelial cell death trigger AE-IPF and ARDS. On this background we got interested in the role of efferocytosis in NLRP3 inflammasome activation. We therefore induced apoptosis of the AEC line A549 using radiation. Indeed, 72h after radiation 67% of A549 cells were apoptotic and only 14% necrotic (**Figure 3C**). These apoptotic A549 cells were then co-cultured with normal BAL cells for 1h and resulted in their phagocytosis by AM (**Figure 4**). Co-culturing with radiated A549 cells induced NLRP3 mRNA expression relative to GAPDH in a similar magnitude compared to stimulation with LPS and was significantly increased compared to baseline NLRP3 expression (**Figure 3F**). Combined stimulation with radiated A549 and LPS induced high NLRP3 gene expression. To evaluate possible mechanisms for the increased NLRP3 mRNA expression *via* radiated A549 cells, we tested a ROS and cathepsin B inhibitor. In the presence of a ROS inhibitor (DPI) or a cathepsin B inhibitor (E64D), the NRLP3 mRNA expression in response to co-culturing with radiated A549 cells was completely suppressed (**Figure 3G**), while GAPDH expression was maintained.

DISCUSSION

Although IPF-AE is the leading cause of death in IPF, underlying mechanisms are poorly understood (38). In this study we demonstrate that the NLRP3-inflammasome is hyper-inducible in BAL cells from IPF patients compared to HV. BAL cells harvested during IPF-AE produced extraordinarily high IL-1ß levels in a similar range as BAL cells of ARDS patients. We found that one potential mechanism driving NLRP3 hyperactivation in IPF-AE may be efferocytosis of apoptotic cells which led to increased NLRP3 expression and caspase-1 activation. The IL-1ß response could be almost completely suppressed by specific inhibition of NLRP3 and caspase-1-activity. Inhibition of ROS

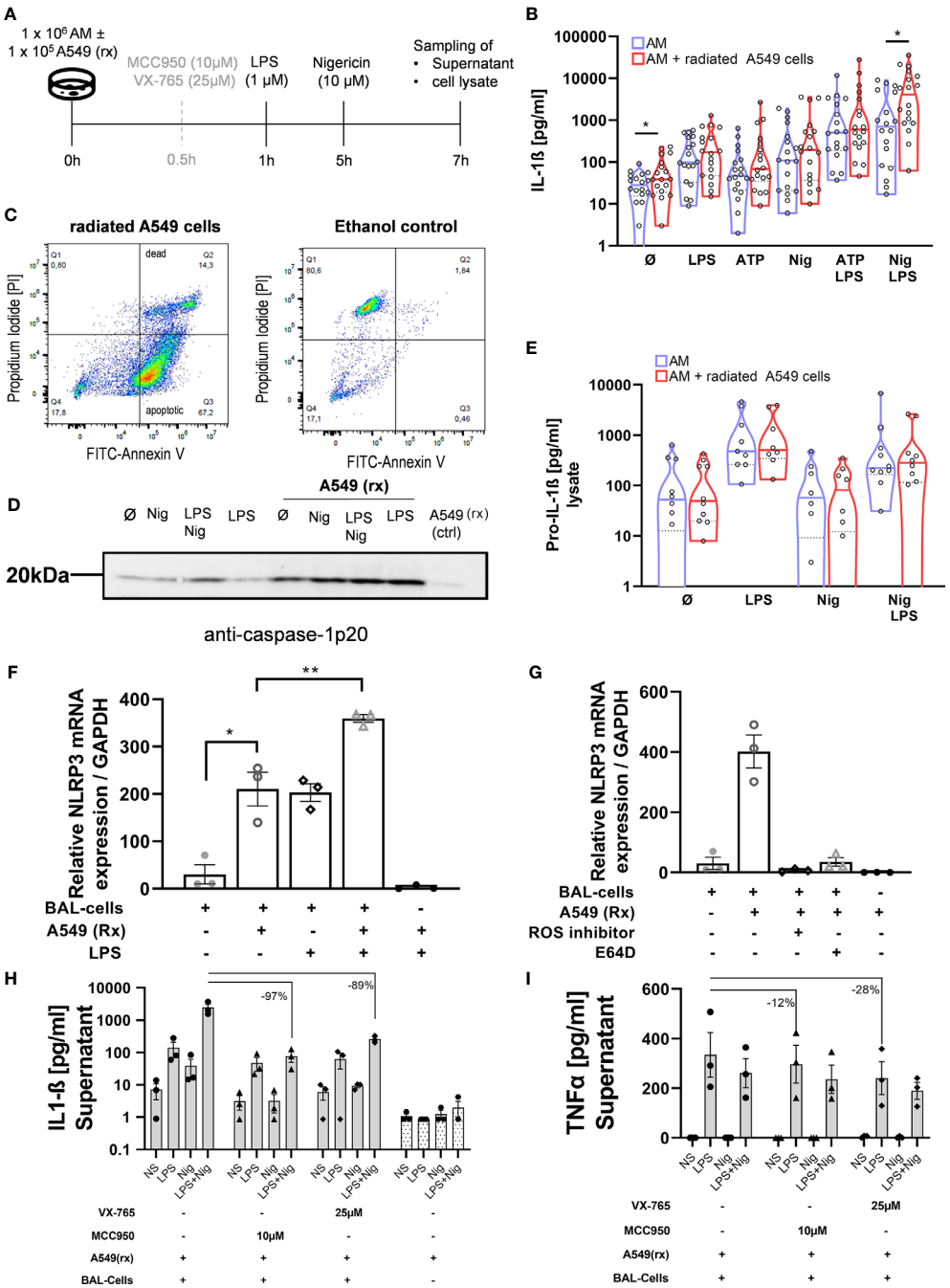

FIGURE 3 | Efferocytosis of apoptotic alveolar epithelial cells activates the NLRP3 inflammasome in AM. BAL cells were co-cultured with the radiated (rx) A549 cells, and the NLRP3 inflammasome was additionally stimulated **(A)**. A549 cells were radiated with 10 Gy and incubated for 72h, following which the majority of the cells were apoptotic demonstrated by Annexin-V staining **(C)**. There were increased IL-1ß levels with all stimulations, which were statistically significant at baseline and following LPS + Nigericin (Nig) stimulation **(B)**. Increased caspase-1 activation with A549(rx) co-culture was demonstrated by immunoblotting of cleaved caspase-1p20 subsegment (representative blot shown; total of n = 3 immunoblots performed) **(D)**. Pro-IL-1ß levels in cell lysate were not different between BAL cells and BAL cells co-cultured with A549(rx) cells **(E)**. NLRP3 mRNA expression was assessed after 2h of stimulation, w/wo the presence of A549(rx). BAL cells cocultured with A459(rx) expressed NLRP3-mRNA in a similar range as BAL cells stimulated with LPS alone **(F)**. Combined stimulation of LPS and A549(rx) resulted in a marked increase in NLRP3-mRNA expression. The effect on NRLP3-mRNA expression (relative to GAPDH) by efferocytosis was inhibited by either a NADPH-Oxidase inhibitor (ROS-inhibitor; DPI) or a cathepsin inhibitor (E64D) **(G)**. IL-1ß production could be inhibited by inhibition of NLRP3 (MCC950) and also caspase-1 (VX-765) **(H)**, while TNF-α levels were largely retained **(I)**. IL-1ß levels and NLRP3 mRNA levels were compared using unpaired t-tests; *p < 0.05; **p < 0.01; *N.S.* non-significant.

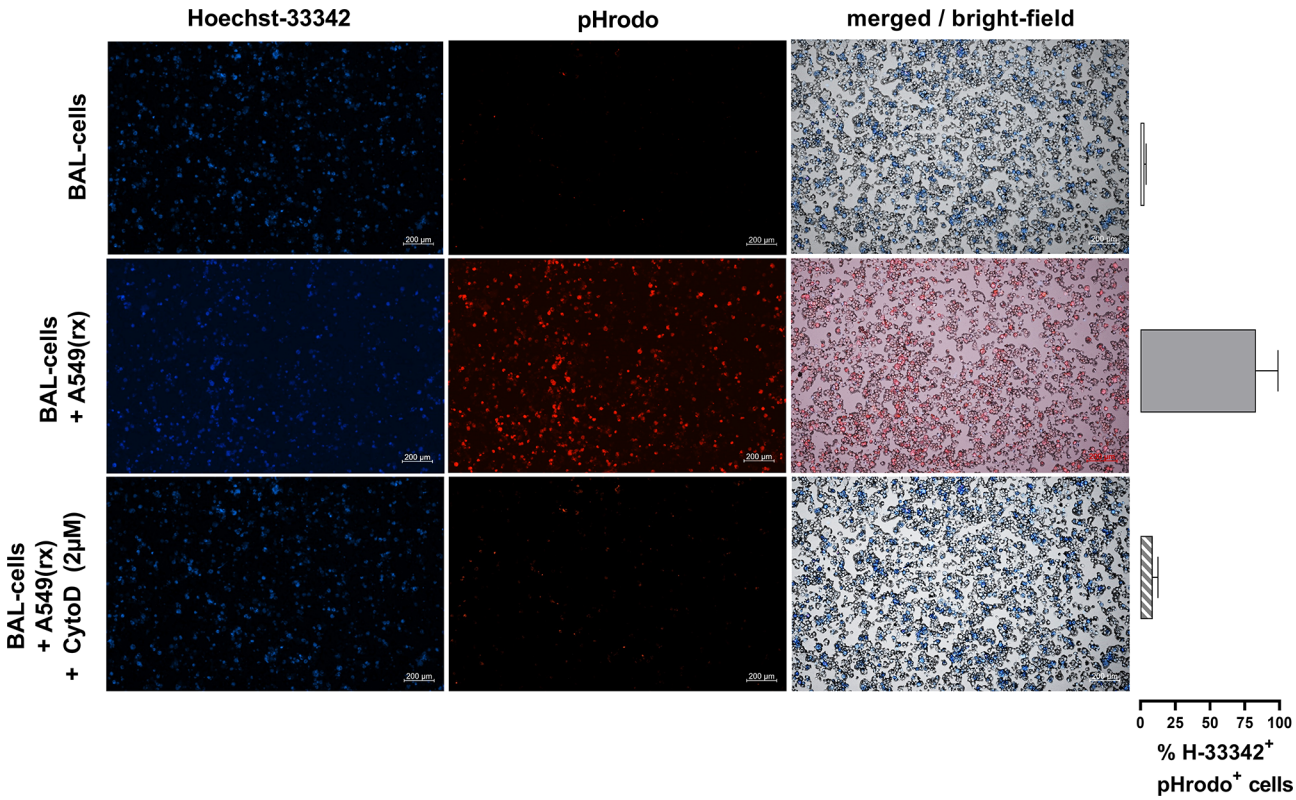

FIGURE 4 | Co-incubation of alveolar macrophages with pHrodo-labeled radiated A549 cells leads to efferocytosis of A549 cells. Nucleolar staining with Hoechst-33342 of BAL cells was performed. Following co-incubation with pHrodo labeled radiated A549 cells, pHrodo positive alveolar macrophages were detected, indicating efferocytosis of A549 cells. Following pre-treatment with cytochalasin-D (2 µM), this effect was almost completely inhibited. Exemplary fluorescence microscopy images (5× magnification) of BAL cells with and without co-incubation and pre-treatment of cytochalasin D are shown, and the percentages of Hoechst$^+$/pHrodo$^+$ cells of all Hoechst$^+$ cells are shown in the right hand panel. Bronchoalveolar lavages from three patients were used and measured in triplicates for each condition.

or cathepsin B signaling blocked the effect of co-culturing with radiated A549 cells on NLRP3-mRNA transcription in BAL cells.

Our data indicate that the NLRP3 inflammasome is hyper-inducible in IPF BAL cells and especially during acute exacerbation. BAL cells harvested at the time of IPF-AE produced tremendously high levels of IL-1ß and in a similar range as AMs of patients with ARDS. While the role and significant involvement of the NLRP3-inflammasome in ARDS are well documented, activation of the NLRP3 inflammasome pathway in IPF-AE is a novel finding. Our observations in BAL cells from IPF patients are in line with previous observations of elevated IL-1ß and IL-1ß mRNA in IPF (10, 35–37). Activation of the NLRP3-inflammasome pathway has been demonstrated to induce IL-1ß and IL-18 production of BAL cells and is tightly regulated (11). Although we have not purified macrophages from BAL prior to inflammasome stimulation protocol and therefore cannot exclude contribution of other cell types, we think, in line with the literature, that our experiments mainly reflect inflammasome activation of alveolar macrophages. In addition, the relatively short incubation time of 6h used in this study render cell–cell contact dependent mechanisms unlikely as the primary driver for increased NLRP3 activation and IL-1ß production. Furthermore, the neutrophil count between IPF patients with and without AE was relatively small owing to inclusion of IPF patients with advanced disease (39), while the changes in NLRP3-inflammasome inducibility between these groups were considerable.

Several factors have been shown to activate the NLRP3 inflammasome such as phagocytosis of crystalline substances such as silicates or cholesterol but is also triggered by viral and bacterial infection releasing LPS and other TLR ligands as well as DNA and RNA (12, 40). Of note, mechanical ventilation and ventilation induced lung injury also activated the NLRP3-inflammasome in murine models (41, 42). These triggers are also commonly reported to precede acute exacerbation in IPF (3, 27), and it is thus conceivable that elevated inflammasome inducibility in IPF patients (such as the increased pro-IL-1ß an IL-1ß production shown herein upon signal 1 or signal 2 stimulation) predisposes them to increased risk of acute exacerbation when exposed to these stimuli. Notably, in a multicentric BAL gene expression analysis in IPF, a gene set was identified to carry poor prognosis, among which the IL-1ß gene and the NLRP3 gene were included (43). Thus, our data indicate a role of NLRP3 inflammasome signaling in acute exacerbation of IPF.

The NLRP3 inflammasome and IL-1ß signaling are closely linked to neutrophil influx and represent a key pathway in response to cell injury (44–46). IL-1ß and the NLRP3 inflammasome activation also are part of an acute injury response and promote fibrosis and pro-fibrotic pathways such as transforming growth factor (TGF)-*ß* signaling in animal models (47–50). Inflammation caused by bleomycin induced lung injury is triggered by NLRP3 inflammasome activation, while the resulting inflammation and fibrosis were completely prevented by NLRP3 inhibitors (48). Moreover, asbestos and silica, which are known inducers of pulmonary fibrosis, are capable of activating the NLRP3 inflammasome with subsequent IL-1ß production (51). IL-1ß itself can promote TGF-*ß* responses, considered as a key driver in pulmonary fibrosis *via* fibroblast activation (52). Interestingly, both approved antifibrotic agents for the treatment of IPF (nintedanib and pirfenidone) have been shown to reduce IL-1ß expression in lung tissues (53, 54), which might be a mechanism for the reduced risk of acute exacerbation in patients receiving antifibrotic treatment (55, 56).

Another key finding of our study is that co-incubation of BAL cells with apoptotic AEC (A549) and their phagocytosis increase inflammasome-dependent caspase-1 activation. Our model for inducing apoptosis in A549 AECs is well accepted (30–32), and we demonstrated apoptosis in the majority of cells *via* annexin-V expression, and we also demonstrate their efferocytosis by alveolar macrophages. Phagocytosis and generation of phagolysosomes are linked to ROS production and lysosomal stress. Interestingly also activation of the NLRP3 inflammasome by many trigger factors was shown to rely on ROS production (19–25) and cathepsin B leakage into the cytoplasm (26). Based on these findings we tested the effects of ROS or cathepsin B inhibition and found that both of them completely attenuated NLRP3 expression in the context of efferocytosis (16). Studies suggest that ROS, among other direct effects on inflammasome assembly, also upregulates NLRP3 gene expression *via* TLR-4 signaling (57), providing a potential mechanisms for the reduction in NLRP3-mRNA expression observed in this study. A recent murine model showed that apoptotic AEC-II induce a pro-fibrotic gene expression signature in AM following efferocytosis, which on repeated exposure can induce pulmonary fibrosis (17), a process which may be equally important in IPF. Previous animal studies have also implicated that impaired autophagy mechanisms in IPF and aging result in deranged mitochondrial turnover resulting in mitochondria-generated reactive oxygen species (ROS) leading to caspase-1 activation (19–25, 50). Equally, recent reports demonstrated a dependency on cathepsin B (which is also induced by nigericin used in our model) for NLRP3-inflammasome assembly (58, 59). Cathepsin B is released into the cytoplasm upon lysosomal membrane permeabilization (26). Thus, efferocytosis triggers NLRP3 inflammasome activation in alveolar macrophages. While ROS inhibitors convey multifactorial effects, the possibility to suppress the observed IL-1ß response by the specific NLRP3-inhibitor MCC950 (33) carries potential treatment options for IPF-AE and other forms of acute respiratory failure such as COVID-19 (60), which remain to be clinically studied.

Our study has significant limitations to consider: Our data derived from human samples demonstrates evidence for an increased activation of the NLRP3 inflammasome, but given a limited number of measured inflammasome components, we cannot exclude that additional inflammasome subtypes may have equally been activated. Age, comorbidities, and respiratory failure in patients with IPF-AE often preclude these patients from undergoing bronchoscopy which primary function lies in exclusion of infection, rendering these bio samples available only in few cases recruited in the era before antifibrotic treatment was available. The considerable younger age of the recruited healthy volunteers also limits comparability to IPF patients since inflammasome activation may also occur with aging alone (61). Although the differences observed in the present study appear unlikely to be explained solely by age, at least a contributory effect can be assumed. Another important limitation of this study is that we did not purify macrophages prior to the described *in vitro* experiments. On one side this experimental protocol leaves cells untouched since methods for cell purification have been shown to activate macrophages. On the other hand, because we studied BAL cells in complete, we cannot exclude a major contribution of other cell types such as neutrophils (62). Neutrophils have been recently shown to activate NLRP3 inflammasome upon respiratory infections (63) which can be achieved *via* production of neutrophil extracellular traps (44, 45). Compared with macrophages, neutrophils are however short-lived and difficult to study *in vitro* because most of the neutrophils are lost within 2h of cell culture.

In conclusion, our study demonstrates in human BAL cells the hyper-inducibility of the NLRP3-inflammasome in IPF and particularly at acute exacerbation. We identified efferocytosis of apoptotic cells caused by lung injury as one responsible mechanism. The activated NLRP3 inflammasome in IPF-AE may be of potential therapeutic value, and compounds blocking NLRP3-inflammasome activation might be of potential benefit for this fatal condition (53). Future research is needed to explore the clinical role of inflammasome inhibitors on the course of pulmonary fibrosis and acute exacerbation in particular.

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 the Ethics committee of The University of Freiburg im Breisgau. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BJ and BS wrote the first draft of the manuscript, conducted the experiments, contributed to the study design, and interpreted the data. OT conducted the experiments, provided technical support, and revised the manuscript. GW, TW, and CB interpreted the data, critically revised the manuscript, and supervised the project. MM conducted the experiments, provided technical support, and interpreted the data. AP designed the study, supervised the project, interpreted the data, and critically revised the manuscript. All authors contributed to the article and approved the submitted version. All authors take responsibility for the integrity of the data.

FUNDING

BS is supported by PRACTIS–Clinician Scientist Programme of Hannover Medical School, funded by the Deutsche Forschungsgemeinschaft (DFG, ME 3696/3-1) and the German Center for Lung Research (DZL). BS, TW, and AP are supported by KFO311. AP is supported by E-RARE IPF-AE.

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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.

frontiers in Immunology

MINI REVIEW
published: 30 April 2021
doi: 10.3389/fimmu.2021.664109

Dendritic Cells Are the Intriguing Players in the Puzzle of Idiopathic Pulmonary Fibrosis Pathogenesis

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Idiopathic pulmonary fibrosis (IPF) is the most devastating progressive interstitial lung disease that remains refractory to treatment. Pathogenesis of IPF relies on the aberrant cross-talk between injured alveolar cells and myofibroblasts, which ultimately leads to an aberrant fibrous reaction. The contribution of the immune system to IPF remains not fully explored. Recent evidence suggests that both innate and adaptive immune responses may participate in the fibrotic process. Dendritic cells (DCs) are the most potent professional antigen-presenting cells that bridge innate and adaptive immunity. Also, they exert a crucial role in the immune surveillance of the lung, where they are strategically placed in the airway epithelium and interstitium. Immature DCs accumulate in the IPF lung close to areas of epithelial hyperplasia and fibrosis. Conversely, mature DCs are concentrated in well-organized lymphoid follicles along with T and B cells and bronchoalveolar lavage of IPF patients. We have recently shown that all sub-types of peripheral blood DCs (including conventional and plasmacytoid DCs) are severely depleted in therapy naïve IPF patients. Also, the low frequency of conventional $CD1c^+$ DCs is predictive of a worse prognosis. The purpose of this mini-review is to focus on the main evidence on DC involvement in IPF pathogenesis. Unanswered questions and opportunities for future research ranging from a better understanding of their contribution to diagnosis and prognosis to personalized DC-based therapies will be explored.

Keywords: idiopathic pulmonary fibrosis, dendritic cells, immunity, cancer, immunotherapy

OPEN ACCESS

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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *04 February 2021*
Accepted: *15 April 2021*
Published: *30 April 2021*

Citation:
Bocchino M, Zanotta S, Capitelli L and Galati D (2021) Dendritic Cells Are the Intriguing Players in the Puzzle of Idiopathic Pulmonary Fibrosis Pathogenesis. Front. Immunol. 12:664109. doi: 10.3389/fimmu.2021.664109

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating fatal lung disease that usually remains refractory to treatment (1–3), with an estimated median survival of 2 to 5 years from the first diagnosis. In the last two decades, disease incidence has steadily increased, varying from 2.8 to 19 cases per 100.000 people per year in Europe and North America, respectively (1). Disease behavior is also highly variable, with associated comorbidities potentially exerting a detrimental impact on prognosis (4, 5). The current availability of anti-fibrotic drugs (i.e., nintedanib and pirfenidone) has improved patients' short-term life expectancy through the slowdown of the lung function decline and the reduction of hospitalization rate and episodes of acute exacerbation (6).

Despite many efforts, the pathogenesis of IPF has not yet been elucidated. No longer considered just an inflammatory disorder (7), IPF pathogenesis likely relies on the aberrant cross-talk between injured alveolar cells and myofibroblasts. This interaction ultimately promotes a pro-fibrotic microenvironment through the engagement of a vicious circle supported, among others, by oxidative stress (8–10). The immune system's contribution to IPF remains poorly understood, with several pieces of emerging evidence suggesting that both innate and adaptive responses can orchestrate the fibrotic process (11–13). In this scenario, dendritic cells (DCs) may play a significant role because of their involvement in the lungs' immune surveillance, where they are strategically placed within the airway epithelium and interstitium (14).

Notably, DCs encompass a heterogeneous family of bone marrow-derived cells recognized as the most specialized and potent antigen-presenting cells (APCs) of the immune system (15, 16). DCs are located in almost all tissues, where they detect and process Ags for presentation to T lymphocytes, thus establishing a tailored link between innate and adaptive immune responses. Besides, DCs are pivotal in regulating the delicate interplay between immunity and tolerance (17–19) as they promote the deletion of clonal autoreactive immature T cells in the thymus. Conversely, DCs interact in the periphery with T cells to achieve immune tolerance by inducing T-cell anergy, T cell deletion, and amplification and stimulation of regulatory T cell (Treg) subsets (18, 19). Due to their pleiotropic functions and properties within the immune system, DCs have been broadly studied in different experimental and internal medicine areas, including transplantation, allergy, autoimmunity, infectious diseases, cancer (20), and, more recently, fibrosis. Significant efforts have explored the fibrogenesis of different organs, including the liver, the kidney, and the heart (21–24).

The present review aims to offer an overview including the most relevant contributions in the field of IPF to focus on the emerging evidence addressing the role of DCs in disease pathogenesis and clinical behavior and potentially in immune-targeted therapy development.

DEVELOPMENT OF DENDRITIC CELLS

DCs originate from bone marrow progenitors through hematopoiesis, a finely regulated development process that involves several cellular and molecular events. Recent studies have identified a common DC precursor, the human granulocyte-monocyte-DC progenitor (GMDP), which supports the development of all the three major human DC subtypes (25). The GMDPs, through an intermediate maturation state into monocyte-dendritic progenitors (MDPs), differentiate into the common DC progenitors (CDPs). CDPs are restricted to the bone marrow, where they give rise to plasmacytoid DCs (pDCs) and conventional DC precursors (pre-cDCs). Frequencies of pre-cDCs increase in response to circulating FMS-like tyrosine kinase-3 Ligand (Flt3L) and then terminally differentiate into conventional DC (cDC) subsets in the periphery (25, 26). Accordingly, colony-stimulating factor-1 (CSF-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are major cytokines required for human DC differentiation. In particular, Flt3L is a crucial regulator of DC commitment to both cDCs and pDCs (27–29). Additional transcription factors such as Ikaros, PU.1, growth factor independent 1 transcriptional repressor (GFi1), interferon regulatory factor 8 (IRF8), basic leucine zipper ATF-like transcription factor 3 (BATF3), and inhibitor of DNA binding 2 (ID2) synergistically regulate DC development and subset specification through the engagement of different signaling pathways (30–35), as illustrated in **Figure 1**.

CLASSIFICATION AND FUNCTION OF DENDRITIC CELL SUBTYPES

In humans, blood DC subtypes include $CD11c^+$cDCs, that are $CD1c^+$ or $CD141^+$ cells, and $CD11c^-$ pDCs, including $CD123^+$ or $CD303^+$ cells. Conventional DCs, previously termed type-1 ($CD1c^+$) and type-2 ($CD141^+$) myeloid DCs (mDCs), have recently reclassified as cDC2 and cDC1, respectively (36–38) (**Figure 1**). Conventional DCs exert a key function ranging from pathogen detection to cancer immunity as they are critical, through antigen presentation, to initiate specific T-cell responses. On the other, pDCs display high anti-viral activities due to their ability to produce type I interferon and are thought to be involved in immune tolerance (39, 40).

Finally, a new DC subtype is represented by the so-called monocyte-derived DCs (mo-DCs). Evidence shows that mo-DCs arise from monocytes recruited to the inflammatory site and express CD11c, CD1c, CD1a, FcεR1, IRF4, and ZBTB46. It is thought that mo-DCs promote $CD4^+$ T cell polarization within inflammatory contexts (41). A synoptical view of the previous and actual classification of DCs is reported in **Table 1**.

DENDRITIC CELL ACTIVATION AND FUNCTIONAL MATURATION

Mature DCs display phenotypic and functional profiles distinct from their naïve (immature) counterparts. Immature DCs express low levels of major histocompatibility complex (MHC) and co-stimulatory molecules and are usually found in peripheral tissues where they play as sentinels for immune monitoring. These cells can endocytose and process antigens but are poorly effective in generating peptide-MHC complexes to ensure optimal antigen presentation and efficient T-cell activation (42–45). Tissue damage, inflammatory processes, microorganisms, and tumor-derived products may promote the maturation of DCs. After that, these cells lose endocytic activity, increase MHC-peptide complexes, up-regulate co-stimulatory molecules, and secrete inflammatory cytokines essential for the activation of T-cell responses (46, 47). Lastly, following maturation, DCs acquire an increased migratory potential that allows them to move into different compartments, such as non-lymphoid and lymphoid tissues and blood (48, 49).

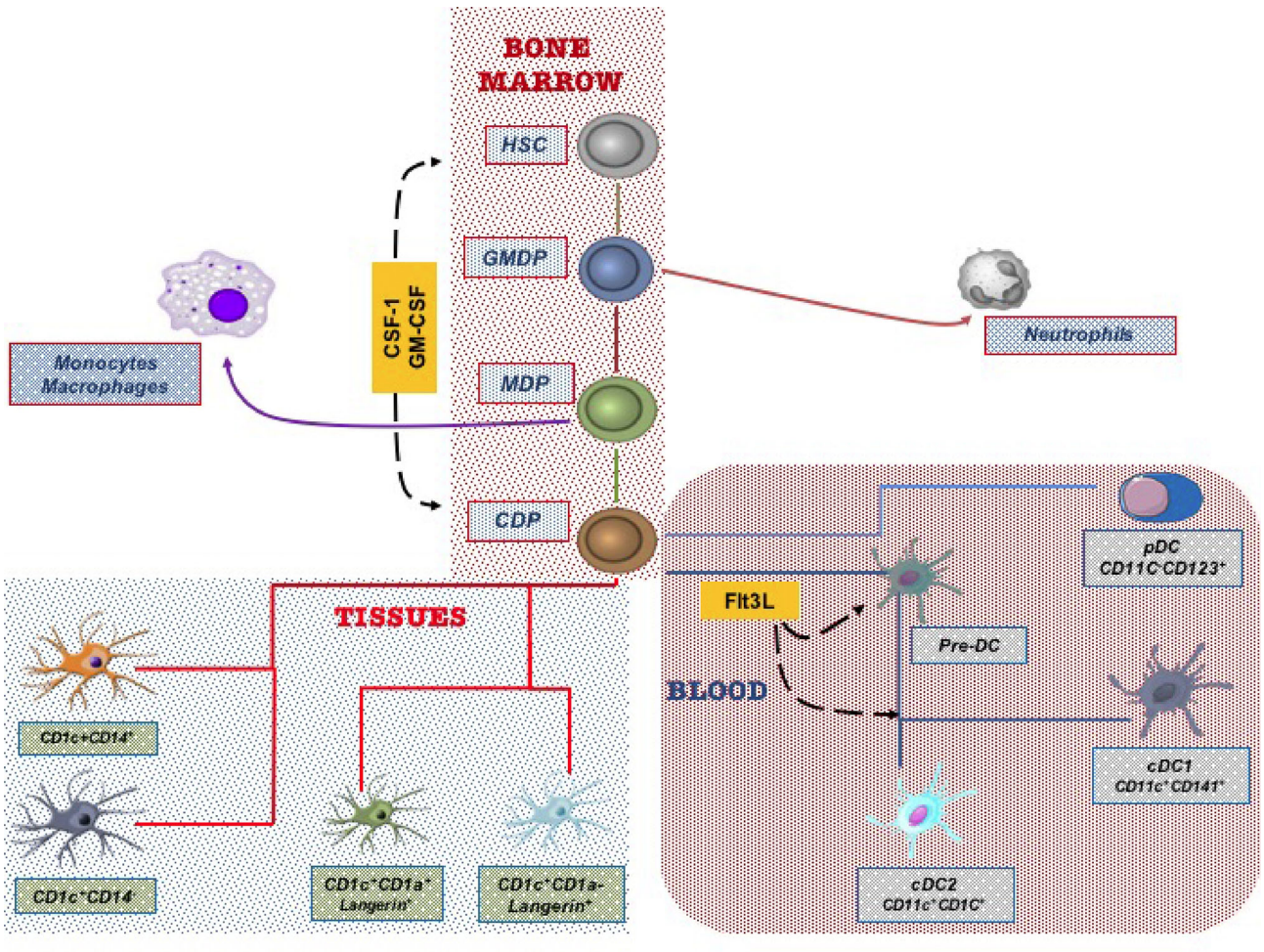

FIGURE 1 | Dendritic cells (DCs) derive from hematopoietic stem cells in the bone marrow. Progenitor cells give rise in the final step to Common Derived Progenitors (CDPs) that differentiate in the blood circulating DC subtypes and in lung tissue DC subsets.

DENDRITIC CELL SUBSETS IN THE HUMAN LUNG MICROENVIRONMENT

Due to their anatomy and function, the lungs are vital organs constantly exposed to the external environment. Consequently, inhaled particles of different nature and origin and potential pathogens need to be efficiently counteracted by a finely adjusted immune response to preserve lung health (50). The activity of lung DCs mainly depends on their organ distribution. For instance, DCs located in the alveolar septa have many dendritic projections able to continuously sample, while those located in the conducting airways seem to do so most rarely (51). Usually, DCs exist in an immature state in the lung periphery, skilled to take up inhaled particulate and soluble antigens. Upon activation, lung DCs, as previously described, become qualified to (52) induce a tailor-made immune response by T-cells (T-helper cell (Th) type 1, Th2, or Th17, depending on the type of pathogen) and B-cells (53, 54).

The lack of validated markers and technical difficulties in obtaining human lung tissues for investigation has significantly limited human lung DC subsets' characterization and functional studies. Since the first observations by Demetds et al., who initially identified human lung DC subsets through the BDCA markers previously applied to characterize blood DCs (55), understanding pulmonary DC subtypes has improved chiefly only in the last few years. In particular, both genomic and functional studies have shown that human epithelial-associated DCs can be divided into four major subpopulations: pDCs, cDC2 $CD1c^{+}$, cDC1 $CD141^{+,}$ and mo-DCs (36–38, 41). More recently, lung DCs have been reclassified into five subtypes based on the differential expression of Langerin, CD1c, and CD14 (56). Interestingly, transcriptome analysis performed in bronchoalveolar lavage (BAL) samples has revealed in the human lower respiratory tract the existence of $Langerin^{+}$, $CD14^{+,}$ and $CD14^{-}$ subsets of CD1c DCs functionally related with alveolar macrophages. Noteworthy, the higher mRNA expression levels of several dendritic cell-associated genes,

TABLE 1 | Synoptical classification of dendritic cell subsets.

Dendritic cell (DC) subtypes based on CD11c expression		Specific DC markers	Old classification	New classification
Myeloid / Conventional	$CD11c^+$ DC	**CD1c / BDCA-1**	Type-1 Myeloid DC (mDC1)	**Conventional DC2 (cDC2)**
Myeloid / Conventional	$CD11c^+$ DC	**CD141 / BDCA-3**	Type-2 Myeloid DC (mDC2)	**Conventional DC1 (cDC1)**
Plasmacytoid	$CD11c^-$ DC	**CD123** **CD303 / BDCA-2**	Plasmacytoid DC (pDC)	**Plasmacytoid DC (pDC)**
Monocyte-derived DC	$CD11c^+$ DC	**CD1c** **CD1a** **FcεR1** **CD206**	–	**Monocyte-derived DC (mo-DC)**

DC, dendritic cell; mDC, myeloid DC; cDC, conventional DC; Mo-DC, monocyte-derived DC; pDC, plasmacytoid DC; BDCA, Blood Dendritic Cell Antigen; FcεR1, Fc Fragment of IgE Receptor 1.

including CD1, FLT3, CX3CR1, and CCR6, have disclosed a specific gene signature of DCs distinct from that of monocytes/macrophages (56). **Figure 1** synthetically depicts the DC subtype differentiation in the lung.

THE ROLE OF DCs IN IPF PATHOGENESIS

The involvement of DCs in the pathogenesis of IPF is a challenging field of relatively recent interest, with only a few reports available in humans.

In 2006 it was first reported that fully mature DCs expressing CD40, CD83, CD86, and DC-lysosome-associated membrane protein, along with non-proliferating B and T lymphocytes, contribute to the creation of ectopic organized lymphoid structures in the lung of IPF patients (57). Conversely, immature DC subsets seem to heavily infiltrate the IPF lungs, specifically in areas of epithelial hyperplasia and fibrosis, and to be present in the BAL fluid (58–60). It is thought that fibroblastic foci of IPF patients can orchestrate blood immature DC recruitment through chemokines' expression (CCL19, CXCL12, and CCL21) (58, 61). This effect may maintain a condition of chronic inflammation by maturing DCs *in situ* within ectopic lymphoid follicles. Two physiologically relevant models showed that both human and mouse lung fibroblasts are critically involved in DC trafficking by secreting chemokines that play a crucial role in fibrosis and inflammation (62). Accordingly, co-cultures of DCs with lung fibroblasts from control subjects and IPF patients further confirmed the *in vitro* ability of lung fibroblasts to modulate the activation and maturation of DCs. These findings suggest that IPF fibroblasts might sustain chronic inflammation and immune responses by locally maintaining a pool of immature DCs (63). In a clinical trial published in 2015, the DC-specific growth factor Flt3L was found to increase cDC1 and cDC2 cell populations' precursors in bone marrow biopsies and peripheral blood samples from healthy volunteers (64). Following this finding, Flt3L has further been shown to be up-regulated in the serum and lung tissue of IPF patients, likely contributing to the accumulation of lung DCs during pulmonary fibrogenesis (65).

We previously showed that quantitative reduction of blood DCs was a feature shared by other respiratory diseases, including chronic obstructive pulmonary disease (COPD) and obstructive sleep apnea (66–68). We have recently also investigated the distribution of peripheral DCs subtypes in a prospective cohort of therapy naïve IPF patients. All blood DC subsets were severely depleted in the context of a pro-inflammatory milieu characterized by high expression levels of reactive oxygen species (ROS) and interleukin (IL)-6. In agreement with data previously reported, we likely attributed such a depletion, at least in part, to an increased cell turnover and recruitment at the lung level. Noteworthy, IL-6 levels and perturbations of the cDC2 subset were not influenced by anti-fibrotic therapies but were associated with reduced survival. Of note, low frequencies of cDC2 were an independent predictive biomarker of worse prognosis (69). **Figure 2** shows the role of DC subtypes undergoing the maturation process in the fibrotic lung tissue. Certainly, as mentioned, DCs involvement is not exclusive to IPF as it may also affect other respiratory diseases. In this context, it is worthy of note the report by Naessens T et al. The Authors have shown that cDC2 are potent inducers of T follicular helper cells and contribute to tertiary lymphoid tissue formation in the lung of COPD patients (70).

THE WAY FORWARD: SIMILARITIES WITH CANCER BIOLOGY AND RATIONALE FOR IMMUNE-TARGETED THERAPIES

In the light of the above evidence, DCs appear to play a role in the fibrotic process and, more specifically, in IPF pathogenesis. IPF notably shares many similarities with lung cancer, ranging from genetics to clinical behavior (71). It is also estimated that the overall cancer incidence in IPF patients is 29 cases per 1000 persons-years, with lung neoplasms being the most frequent ones (72). DC alterations have been widely studied and characterized in solid and blood malignancies (73). Like the liver fibrosis model leading to tumorigenesis (22), DC imbalance and functional impairment may represent a pathogenic bridge between IPF and cancer. This aspect merits further investigation for its prevention and therapeutic repercussions (13, 69). In this regard, DC-based treatments represent emerging alternatives to conventional chemotherapy in cancer patients (74), while such an approach is conceptually missing in fibrosis-related diseases. The lack of animal models that faithfully reproduce IPF pathogenesis is undoubtedly a significant limit in this setting. Despite this, the bleomycin model of inflammation-driven pulmonary fibrosis has still helped explore different purposes over time. In this regard, it has been shown that the immune-mediator VAG539 was able to attenuate the hallmarks of bleomycin-induced lung injury

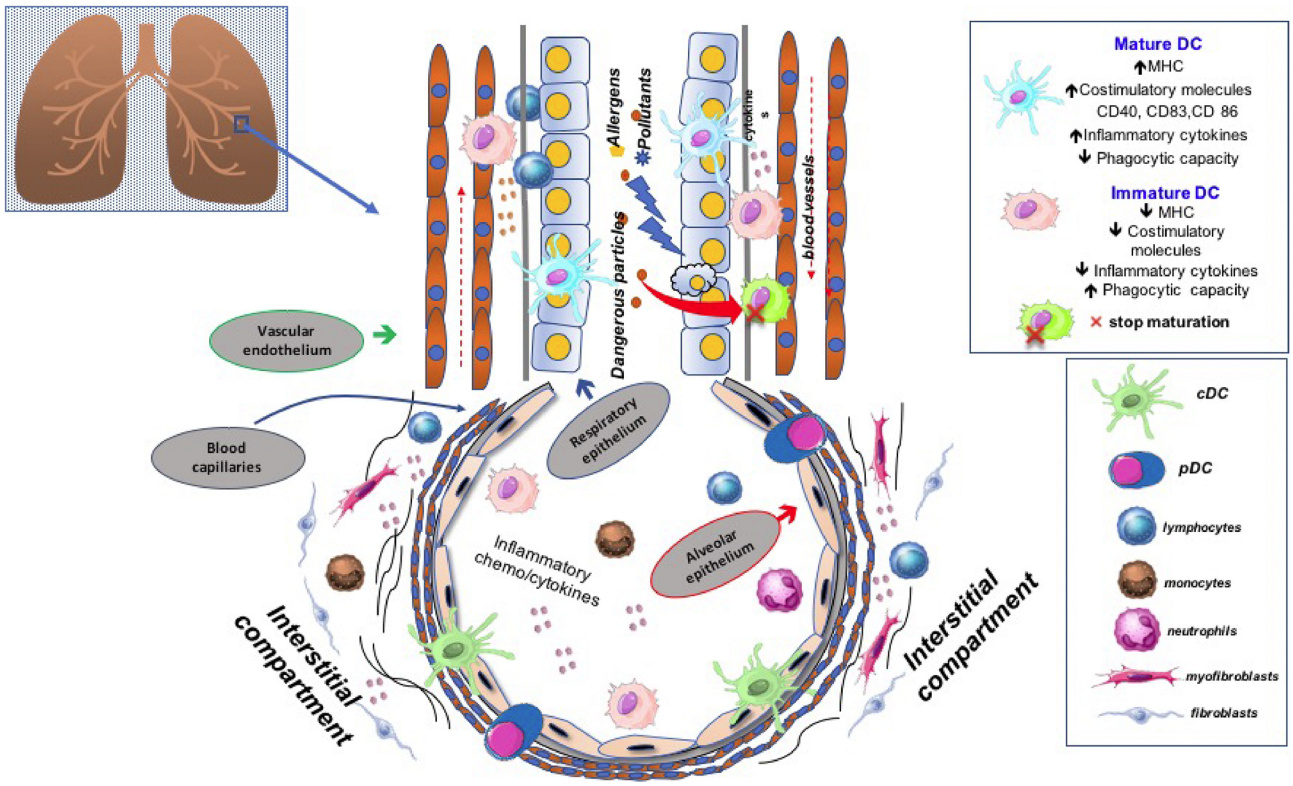

FIGURE 2 | Dendritic cells (DC) are located in the lung interstitium and alveoli, where they act as sentinel cells. Any imbalance of their frequency distribution and functional status may have significant consequences in disease pathogenesis. Emerging evidence suggests that plenty of local factors along with different arrays of chemo-cytokines can modulate DCs maturation in the lung of patients affected by idiopathic pulmonary fibrosis, thus affecting their tolerogenic or immunogenic properties.

through the inactivation of DCs, suggesting a crucial role of these cells across the modulation of both inflammation and fibrosis (75). Likewise, infusion of CD11c-diphtheria toxin (DT) receptor (DTR) in bleomycin-treated mice prompted DCs depletion, thus mitigating lung fibrosis (76). Indeed, both studies have some limitations. First, the expression of aryl hydrocarbon receptor as the key molecular target of VAG539 is not restricted to DCs (77), and, second, the infusion of DT to CD11c-DTR mice depletes not only DCs but also pulmonary macrophages as CD11c is highly expressed on both cell types (78). Even with the awareness of these limitations, we believe that this area of interest deserves wider attention. Accordingly, recent clinical trials have explored the safety and efficacy of recombinant human Flt3L in healthy volunteers and cancer therapy to trigger DC expansion in humans (79–81). Interestingly, recombinant Flt3L increased the numbers of $CD11b^{+}$ DCs, reducing lung fibrosis in wild-type (WT) mice exposed to AdTGF-beta1 (65).

IPF remains, for the most part, an unexplored field due to the non-recognition of the trigger cause. Perturbations of the lung microbiome and viral infections have been hypothesized to have a potential link with the development of IPF (82–86). Therefore, it is not negligible that any dysregulation of DCs, as major APCs and anti-viral effectors, may actively contribute to the puzzle of IPF pathogenesis through a wider involvement at different levels. Overall, accumulated evidence and related considerations further strengthen the concept that participation of DCs in the fibrotic process could be a driving force for future deepening.

CONCLUSION

Interpreting the involvement of the immune response in the pathogenesis of IPF has become a prosperous field of investigation only in recent years. New reports reveal expanding potential pathogenic roles for DCs in lung fibrosis. These findings promise to open new scenarios to understand better the cause and the biological mechanisms underlying the disease. Further efforts and challenges will be to evaluate their potential in terms of easy to perform biomarkers predictive of clinical behavior and targets of immune-based treatments. In analogy with cancer, combination therapy strategies with anti-fibrotic drugs could optimistically represent a milestone shortly.

AUTHOR CONTRIBUTIONS

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.

frontiers in Immunology

REVIEW
published: 19 May 2021
doi: 10.3389/fimmu.2021.631235

The Epithelial-Immune Crosstalk in Pulmonary Fibrosis

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Interactions between the lung epithelium and the immune system involve a tight regulation to prevent inappropriate reactions and have been connected to several pulmonary diseases. Although the distal lung epithelium and local immunity have been implicated in the pathogenesis and disease course of idiopathic pulmonary fibrosis (IPF), consequences of their abnormal interplay remain less well known. Recent data suggests a two-way process, as illustrated by the influence of epithelial-derived periplakin on the immune landscape or the effect of macrophage-derived IL-17B on epithelial cells. Additionally, damage associated molecular patterns (DAMPs), released by damaged or dying (epithelial) cells, are augmented in IPF. Next to "sterile inflammation", pathogen-associated molecular patterns (PAMPs) are increased in IPF and have been linked with lung fibrosis, while outer membrane vesicles from bacteria are able to influence epithelial-macrophage crosstalk. Finally, the advent of high-throughput technologies such as microbiome-sequencing has allowed for the identification of a disease-specific microbial environment. In this review, we propose to discuss how the interplays between the altered distal airway and alveolar epithelium, the lung microbiome and immune cells may shape a pro-fibrotic environment. More specifically, it will highlight DAMPs-PAMPs pathways and the specificities of the IPF lung microbiome while discussing recent elements suggesting abnormal mucosal immunity in pulmonary fibrosis.

Keywords: lung fibrosis, mucosal immunity, epithelium, mucus, innate immunity

OPEN ACCESS

Edited by:
Mats Bemark,
University of Gothenburg, Sweden

Reviewed by:
Francesco Bonella,
Essen University Hospital, Germany
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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *19 November 2020*
Accepted: *04 May 2021*
Published: *19 May 2021*

Citation:
Planté-Bordeneuve T, Pilette C and Froidure A (2021) The Epithelial-Immune Crosstalk in Pulmonary Fibrosis. Front. Immunol. 12:631235. doi: 10.3389/fimmu.2021.631235

INTRODUCTION

The role of the immune system in the development and disease course of idiopathic pulmonary fibrosis (IPF) has been a matter of heated debate over the last decades. Initial observations of increased neutrophil counts in the broncho-alveolar lavage (BAL) (1, 2) alongside the histologic presence of neutrophils, lymphocytes and macrophages in the proximity of fibrotic areas (1) led to the hypothesis that IPF starts as an inflammatory alveolitis and progresses to alveolar septal fibrosis over time. These observations formed the basis for the use of immunosuppressive therapies, in particular corticosteroids, in IPF. Although randomized controlled trials evaluating the role of steroids were missing (3, 4), observational data suggested a heterogeneous response in patients (5). In the early 2000s, the influence of immunity and immunomodulatory medication in IPF began to be questioned, with the emergence of alveolar epithelial dysfunction as one of the main contributors to pathogenesis (6) and the observations that, with further refinement of disease classification criteria (7), better

characterized patients with a usual interstitial pneumonia pattern (UIP) displayed only mild inflammation (8). Finally, a milestone study assessing the effect of N-acetylcysteine, azathioprine, and prednisone in IPF reported a deleterious effect of this combination therapy (9) further weakening the "inflammatory hypothesis" in IPF. The emergence of high-throughput technologies, such as single-cell RNA sequencing, have allowed for the discovery of fibrosis-specific cell populations and fueled a renewed interest for the immune system in this disease. Thus, the place of immunity and inflammation in the course of this pathology has evolved, from causal to modulating (10) and unravelling the subtleties underlying this influence could help discover new targets and understand why immunosuppressive interventions have failed in the past.

The distal lung epithelium forms a continuous layer of cells responsible for gas transport and exchange as well as host defense. A complete overview of pulmonary cell composition can be found in (11, 12). Briefly, whereas in proximal conducting airways, it is principally composed of ciliated, secretory and basal stem cells, monostratified type-1 and type-2 alveolar epithelial cells (AEC) are present in the alveoli (11) (**Figure 1**). As the lung lays at the interface between host and environment, constantly exposed to external stimulation, a tight regulation of inflammatory mechanisms is required to preclude inadequate immune reactions. Lung epithelial cells participate in this equilibrium through several mechanisms. While the contribution of myeloid cells to lung immune mechanisms and secondary fibrosis in IPF has been extensively studied, the participation of the epithelium remains to be fully determined. Although *ex vivo* epithelial cultures are a tedious process, notably hampered by the rapid dedifferentiation of, for example, monocultured alveolar type-2 epithelial cells (AEC2) (13), both *in vivo* and *in vitro* evidence point towards the implication of the epithelium in the aforementioned processes. In this review, we will summarize how epithelial cells' biology and their crosstalk with immune cells and microbes may, under some circumstances, conduct to aberrant, pro-fibrotic signaling in the lung. We will discuss how epithelial cells form a physical barrier through their secretion and removal of mucus, while forming a continuous cell layer, and how alterations in these mechanisms can fuel pro-fibrotic mechanisms. Furthermore, we will review the data regarding their ability to sense and react to danger and pathogen associated molecules and the existing links between alterations in those mechanisms and lung fibrosis. Finally, we will address the epithelial capacity to modulate lung immune responses, notably through the secretion of several soluble mediators (14, 15), and to trigger the recruitment, polarization and activation of pro-fibrotic myeloid cells.

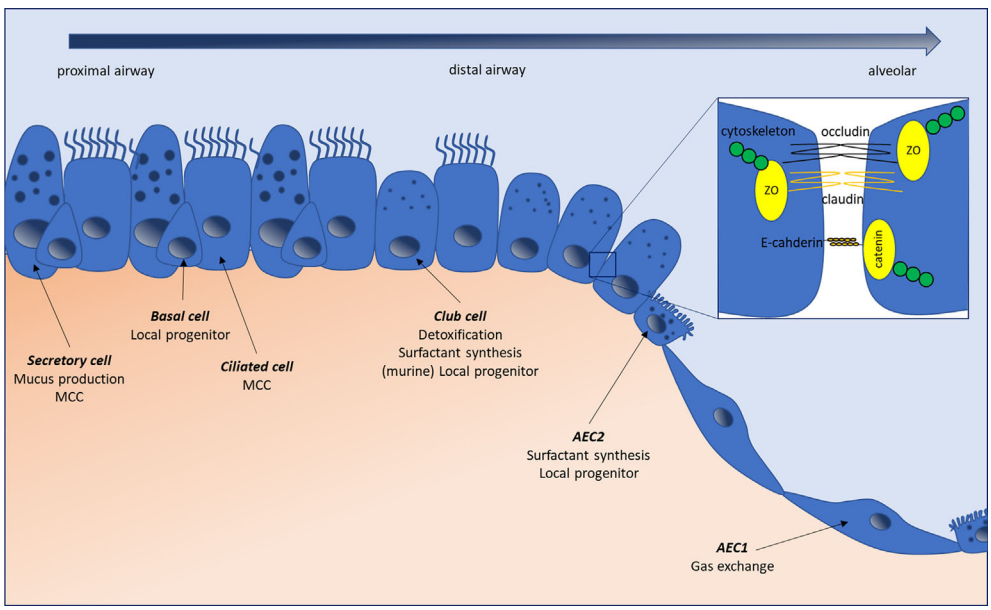

FIGURE 1 | The normal lung epithelium composition changes along the respiratory tree from proximal airways to alveolar areas. Secretory cells produce the mucus lining the airways, which is moved upstream by the ciliated beats originating from ciliated cells. Basal cells have a local progenitor function, possessing the ability to differentiate into several cell types, including secretory and ciliated cells. In small airways, basal and secretory cells are progressively replaced by club (ex-Clara) cells, which can serve as local facultative progenitors (besides basal cells), secrete components of the bronchiolar lining fluid, and play a detoxifying role through their expression of cytochrome p450. In the alveoli, alveolar type-1 epithelial cells (AEC1) are responsible for gas exchange, while alveolar type-2 epithelial cells (AEC2) produce surfactant and serve as local progenitors. Epithelial cells are connected by tight- and adherens junctions, forming a continuous layer separating the intra-luminal content from the submucosal environment and regulating intercellular permeability. Tight junctions are composed of integral membrane proteins like claudins and occludins, which are linked to the cytoskeleton through cytosolic protein complexes such as Zonula Occludens (ZO). Adherens junctions, formed by E-cadherin proteins, linked to the cytoskeleton by catenins are responsible for the maintenance of cell-cell adhesion while being involved in many intracellular signaling and transcriptional pathways. MCC, mucociliary clearance.

THE EPITHELIUM AS A PHYSICAL BARRIER

Mucins and Mucociliary Clearance

The mucus layer covering the respiratory tract epithelium is able to trap and remove noxious stimuli thanks to mucociliary clearance and cough, forming the lung's first line of defense in the airways (16). Mucins are glycosylated proteins that help constitute this visco-elastic layer, isolating the underlying structures from the outer world. The human lung expresses 16 different types of mucins, which can be separated into two families, namely secreted (predominantly MUC5AC and MUC5B) and membrane-bound mucins (mainly MUC1, MUC4 and MUC16) (17). Mucins fulfill multiple roles, forming a mesh hampering epithelial access to noxious stimuli, acting as lubricant as well as (decoy) receptors for pathogens, associating with several cytokines and growth factors, and, for membrane bound mucins, influencing intracellular signaling pathways such as NFκB or β-catenin (18–22). Mucin expression is regulated by numerous signals, including cytokines such as TNF-α, IL-1β, IL-6, IL-13 or IL-17, growth factors like EGF, Damage-Associated Molecular Patterns, bacterial and viral products or proteases (23–28). Of note, membrane-bound mucins consist of 2 non-covalently linked α- and β-chains, which, when exposed to physical stress, inflammatory mediators or changes in their ionic environment, can separate, causing the release of the α-chain (29).

Mucins seem to play a favoring role in the development of lung fibrosis and its subsequent course. Indeed, the most important genetic risk factor associated with IPF is the single nucleotide polymorphism (SNP) rs35705950 in the promoter region of *MUC5B* (30). This common allelic variant, present in 38% of IPF patients and 9% of controls (30), is both predictive and prognostic in lung fibrosis (31), as it is associated with a significant increase in the risk of having pulmonary fibrosis in the Framingham Heart Study population (32) and decreased mortality in 2 IPF cohorts (33). This polymorphism is linked with an increased expression of *MUC5B* (30) and its homonymous mucin protein (34). Furthermore, independently of their genetic background, IPF patients display increased levels of MUC5B in the distal airways (35, 36) and MUC5B is the main mucin present in honeycomb cysts (36). How MUC5B accumulation influences lung fibrosis is still not completely determined but could involve decreased mucociliary clearance with local inflammation or abnormal epithelialization. Supporting the former, a recent link between C3, a component of the complement cascade, the MUC5B polymorphism and IPF has been described (37). Additionally, distal overexpression of MUC5B in mice leads to a thickened mucus layer, impaired mucociliary clearance, augmented honeycomb cyst size and increased fibrosis after bleomycin challenge (38, 39). *In vivo* data indicates a crucial role for MUC5B in the maintenance of healthy interactions between the host and bacteria, as *Muc5b*$^{-/-}$ but not *Muc5ac*$^{-/-}$ animals display impaired survival related to respiratory infections (40). Impaired mucociliary clearance, present in both *Muc5b*$^{-/-}$ and Muc5b overexpressing animals could result in suboptimal clearance of organisms and increased epithelial-bacterial contact. Besides, although currently no causal relationship can be established, IPF subjects with increased bacterial loads display worse survival (41) while the presence of the rs35705950 SNP is associated with lower bacterial burden (41) and improved outcomes (33). Much less is known about the potential implication of MUC5AC in IPF. Recently, a single nucleotide variant in *MUC5AC* was described (42), but the exact effects on protein expression and clinical outcome remain to be determined. Independently from this observation and similarly to MUC5B, MUC5AC expression is increased in the distal IPF lung (36) and is expressed within HC, albeit at a much lower level (35, 36). Similarly to their secreted counterparts, the expression of MUC1 and MUC4 is increased in IPF lungs (43, 44). These mucins are involved in lung fibrosis through their α- and β-chain. In fact, the MUC1 and MUC16 extracellular domains contain the KL-6 and CA125 epitopes respectively, which have been linked with disease progression (45, 46). Furthermore, KL-6 can promote fibroblast proliferation and migration while exerting anti-apoptotic activities (47, 48) and was implicated in an *in vivo* experimental model of lung fibrosis (49). Finally, implication of the cytoplasmic tails of both MUC1 and MUC4 is suggested by the fact that their genetic and pharmacologic modulation is sufficient to protect bleomycin treated mice and by their role in TGF-β1-induced EMT or myofibroblast differentiation (43, 44).

Intercellular Junctions

Tight junctions (TJ) and adherens junctions (AJ) act as apical junctional complexes, connecting adjacent cells, regulating the transport of solutes, allowing cell polarity and permitting the separation of the airway lumen and the underlying mucosa through a physical barrier (50, 51). Briefly, TJ are composed of integral membrane proteins, such as claudins and occludins and cytosolic protein complexes comprising Zonula Occludens proteins (ZO-1, 2, 3) (52) linked to actin binding proteins and the cytoskeleton (**Figure 1**) (51, 53). Claudin expression varies in function of the tissue (54) and these proteins can be divided in two groups based on their permeability properties, with claudins-2, -7, -10, -15 and -16 promoting paracellular flux, while claudins-1, -4, -5, -8, -11, -14 and -18 have a sealing function (55, 56). Within the human lung, claudin expression is variable, the main bronchiolar claudins being claudin-1, -2, -3, -4, -5 and -7, while alveolar cells are positive for claudin-3, -4, -7 and -18 (57–59), suggesting tailored expression in function of the localization. AJ are especially important for the maintenance of cell-cell adhesion but are also involved in many intracellular signaling and transcriptional pathways. In the alveolar epithelium, the hallmark structure of AJ consists of a complex formed by the E-cadherin cell adhesion molecules linked to the actin cytoskeleton thanks to catenins (**Figure 1**) (51). β-catenin, in particular, serves important signaling functions, linking structural junctions with the Wnt pathway. At last, desmosomes, specialized membrane complexes, help maintain the mechanical integrity of tissues and are particularly represented in tissues undergoing high mechanical stress, such

as the lungs (60). They are composed by desmosomal cadherins, Armadillo proteins and plakins, and are present throughout the bronchial and alveolar epithelium (61). Lungs of patients affected by IPF present several signs of epithelial integrity disruption, with basement membrane denudation (62) and downregulation of several junctional proteins, suggesting that alterations in one, or several, of these structures are present.

Tight junctions are altered in IPF, with immunohistochemical observations showing an increased expression of occludin, claudin-1, -2, -3 and -7 and a downregulation of claudin-18 within regions of abnormal epithelialization (57–59). Discrepant results exist for claudin-4, with reports of increased (58, 59) or decreased expression (57) but this can at least partly be explained by differences in epithelial classification between studies, since alveolar and bronchiolar zones were not always separated. Measures of lung epithelial permeability through 99m-labelled diethylenetriamine penta-acetic acid (^{99m}Tc-DTPA) measurement, although quite non-specific, shows that patients have faster clearance than control subjects, suggesting increased epithelial permeability (63). Similarly, intraperitoneal bleomycin injections, resulting in lung fibrosis, lead to decreased pulmonary expression of claudin-5 and -18 as well as occludins (64) while claudin-4 is upregulated after experimental acute lung injury (65). The mechanisms underlying these alterations are unclear; however, TGF-β1, one of the main profibrotic cytokines involved in IPF, is capable of inducing TJ disassembly (64), increases claudin-4 (66) and decreases claudin-18 expression (67). Interestingly, genetic deletion of *cldn18* results in (pathologic) epithelial regeneration efforts with alveolar enlargement, impaired barrier function, alveolar type-1 epithelial cell (AEC1) injury, AEC2 expansion and YAP activation, a proliferation/differentiation protein activated in IPF alveolar cells (68–70). Furthermore, preserved epithelial barrier integrity and polarization permit modulation of the interaction between growth factors or cytokines and their receptors, further implicating TJ in innate immune processes and epithelialization. For instance, expression of heregulin, a Human Epidermal growth Receptor (HER) ligand, is normally restricted to the apical surface of the lung epithelium, separated from its coreceptor HER2/3 at the basal level by intact TJ (71). Upon disruption of TJ integrity, the ligand is able to gain access to its receptor, prompting downstream signaling implicated in experimental pulmonary fibrosis (72). Although these lines of evidence point towards a role for TJ dysfunction in lung fibrosis, it is still uncertain whether TJ alterations can directly influence this process or are mere bystanders of abnormal epithelialization, necessitating further mechanistic studies before definitive conclusions can be drawn.

Loss of E-cadherin and gain of N-cadherin is a salient feature of epithelial-mesenchymal transition (EMT), a process by which epithelial cells gain mesenchymal characteristics, as observed in IPF. Accordingly, the IPF lung epithelium displays alterations in the expression of these AJ proteins, with decreased basal cell expression of E-cadherin and co-expression of E-cadherin and N-cadherin in hyperplasic pneumocytes (73). Additionally, treatment with bleomycin, either in experimental models of lung fibrosis or on an alveolar epithelial cell-line reduces E-cadherin expression (74, 75). Similarly to TJ, TGF-β1 seems to be one of the main mediators of AJ alteration, as it has the ability to downregulate E-cadherin (76, 77). A complete overview of the role of EMT in IPF is proposed by Salton et al. (78). Finally, lung-specific deletion of E-cadherin in mice results in loss of airway epithelial cells, epithelial denudation, and increased presence of α-smooth muscle actin (α-SMA) expressing cells alongside increased alveolar diameters (79).

Periplakin and desmoplakin, two plakins linking the desmosomal plaque with intermediate filaments have also been implicated in lung fibrosis. Recently, variants of *DSP*, the gene coding for desmoplakin, were associated with IPF while mRNA levels are elevated in diseased lungs (80). Periplakin was initially identified as a potential contributor to pulmonary fibrosis due to the presence of anti-periplakin antibodies in the serum of 40% of IPF patients, and alterations in its alveolar expression (61). Further mechanistic insights show that these antibodies impact epithelial migration and wound closure while BAL of IPF patients downregulates *Ppl* mRNA in murine alveolar cells (61, 81). Furthermore, *Ppl*$^{-/-}$ animals are protected from experimental lung fibrosis, display altered downstream signaling in pro-fibrotic pathway synchronously to an anti-inflammatory alveolar environment and decreased, pro-fibrotic, alternatively activated macrophages (81). No alterations of other cell junctional components could be observed, arguing against a loss of epithelial integrity and for a direct role of periplakin as modulator of its immune milieu and downstream profibrotic signals.

THE LUNG EPITHELIUM SENSES AND REACTS TO DANGER SIGNALS

Aside from disrupting the physical barrier separating the basal membrane and submucosal tissue from the luminal content, epithelial injury also leads to the release of danger signals, so called Damage-Associated Molecular Patterns (DAMPs). This results in the activation of inflammatory pathways and the promotion of damaged structures clearance in a process of "sterile inflammation" (82). A wide variety of proteins can act as DAMPs, sharing the feature of being either mislocalized or altered. High Motility Group Box 1 (HMGB1) is the first described DAMP following the "danger theory" (83) and is normally spatially restricted to the nucleus, where it regulates DNA organization and transcription, but can act as a strong pro-inflammatory stimulus when passively released in the surrounding milieu by necro(pto)tic cells (83). Next to passive release, HMGB1 can also be actively secreted by non-necrotic cells of the immune system and intestinal epithelial cells after immune stimulation (84, 85). Similarly, the production of hyaluronan fragments from extracellular matrix high-molecular weight (HMW) hyaluronan can trigger inflammatory pathways (86). Furthermore, disruption of physical defense mechanisms will also lead to increased contact with bacterial and viral products named Pathogen-Associated

Molecular Patterns (PAMPs), such as lipopolysaccharides, ds/ssRNA or unmethylated CpG DNA (87). Both DAMPs and PAMPs downstream signaling is mediated through Pattern Recognition Receptors (PRR), intracytoplasmic and membrane receptors consisting of 4 classes, Toll-Like Receptors (TLR), NOD-Like Receptors (NLR), C-type Lectin Receptors (CLR) and RIG-I-Like receptors (RLR) (88). These receptors are present on cells from the immune system, but also expressed by lung epithelial cells (15) and can trigger a wide array of effects, resulting in activation of NFκB, MAPK and interferon pathways.

The Epithelium as Source and Target of DAMPs in IPF

Although DAMPs primarily serve an inflammatory function, they are increased in IPF and, based on experimental results, seem to be involved in fibrogenesis. As stated previously, DAMPs can originate from necro(pto)tic cells, and increased levels of RIPK3, a regulator of necroptosis have been observed in IPF lungs and in experimental, bleomycin-induced pulmonary fibrosis, particularly within alveolar epithelial cells (89). Further implication comes from the observation that HMGB1, uric acid or extracellular ATP (eATP), all recognized DAMPs, are increased in both human BAL as well as *in vivo* and *in vitro* experimental conditions (89–93). Although the origin of these signals is multiple, distal lung epithelial cells contribute to this altered environment as they show staining for HMGB1 and bleomycin-stimulated alveolar cells produce high levels of HMGB1 and eATP (90, 93). Additionally, inhibition of HMGB1 by a neutralizing antibody, of uric acid levels by a xanthine-oxidase inhibitor and interference with eATP signaling all decrease bleomycin-induced lung fibrosis (90, 92, 93). The exact mechanisms implicating DAMPs in fibrosis are currently incompletely elucidated but include direct interactions with fibroblasts as well as epithelial cells and promotion of IL-1β production, a cytokine involved in lung fibrosis (94). Indeed, addition of HMGB1 to fibroblasts promotes cell viability and myofibroblast differentiation (90, 95) while decreased IL-1β levels are observed in bleomycin instilled mice treated with anti-HMGB1 antibodies (90). HMGB1 also influences epithelial behavior, as it enhances scratch-wound closure by AECs through the production of IL-1β and activation of TGF-β1 (96), potentially fueling frustrated repair mechanisms in the alveoli, and promotes epithelial-mesenchymal transition in bronchial cells (97). Finally, HMGB1 can shape the immune contribution to fibrosis as it prompts macrophages to produce high levels of IL-1β, which could influence collagen deposition (98) and triggers the release of chemokines such as MCP-1/CCL2 by lung epithelial cells (99), a molecule known to enhance fibrocyte recruitment (100). Additionally, epithelial cells exposed to this molecule produce higher levels of TNF-α, which has been linked with TJ disassembly (101), fibroblast apoptosis (102) and EMT (103, 104). The latter pro- and anti-fibrotic effect are mirrored by *in vivo* data, reporting both protective and promoting roles of this cytokine in lung fibrosis (105, 106). Likewise, extracellular application of ATP is able to provoke an upregulation of TGF-β1, collagen and fibronectin mRNA in cultured fibroblasts (107), increases fibroblast migration and proliferation (108) while $P2X_7^{-/-}$ mice, a knock-out model for a receptor of eATP, are protected from fibrosis and show lower IL-1β levels than control animals (93).

PAMPs and the Lung Epithelium

PAMPs are similarly capable of influencing cell behavior and ultimately fibrosis. Lipopolysaccharides (LPS), membrane components of gram-negative bacteria, recognized by the membrane receptor TLR4, have been involved in experimental lung fibrosis (109, 110), are capable of promoting fibroblast proliferation *in vitro* (111) and induce the early secretion of IL-1β, MCP-1/CCL2 or IL-8 by AEC2 (112, 113). Additionally, bacterial and viral DNA contains hypomethylated CpG zones, and treatment of UIP lung fibroblasts and healthy peripheral monocytes with CpG oligodeoxyribonucleotides (ODN), results in increased myofibroblast as well as fibrocyte differentiation respectively (114, 115). Moreover, fibroblasts from rapidly progressive IPF patients show an enhanced susceptibility to CpG stimulation, probably due to an increased expression of its cytosolic receptor TLR9 in these subjects (115). Epithelial cells are also capable of sensing and responding to CpG, with most experiments linking CpG, lung epithelium and fibrosis conducted in the alveolar A549 cell-line. Both TLR9-dependent and -independent mechanisms could be implicated. Indeed, the induction of EMT observed after CpG treatment of alveolar cells is absent after TLR9 silencing (115) but their upregulation of CCN1, a matricellular protein with pleiotropic functions implicated in IPF and experimental lung fibrosis (116), is predominantly linked to CpG-induced endoplasmic reticulum-(ER) stress (117). Interestingly, integrin αVβ6, an epithelial cell surface receptor implicated in the activation of latent TGF-β, is simultaneously upregulated, potentially linking this with increased TGF-β1 signaling. Conversely, experiments in mice showed that addition of CpG after bleomycin instillations reduced fibrosis (118), possibly reflecting immunological species differences.

Implication of TLR in Lung Fibrosis

Additional evidence implicating these pathways and epithelial cells arise from clinical, *in vivo*, and *in vitro* studies showing alterations of PRR in lung fibrosis. Firstly, polymorphisms affecting PRR, more specifically, TLR have been associated with IPF. In 2013, the L412F *TLR3* polymorphism was linked with respiratory decline and mortality in IPF and shown to influence fibroblast proliferation (119). The same year, SNPs in the *TOLLIP* genetic locus, resulting in lower *TOLLIP* expression levels, were associated with IPF susceptibility and for one of them disease course and mortality (120). *TOLLIP* codes for the Toll-interacting protein (TOLLIP), an inhibitory adaptor protein of downstream TLR2/4 signaling, hampering NF-κB activation (121). In addition, epithelial expression of TOLLIP is associated with resistance to *in vitro* bleomycin induced apoptosis and is locally increased in an aberrant basaloid cell population in IPF (122). Furthermore, TLR2/4 expression is increased at the epithelial level in patients with an UIP pattern

(123). Secondly, evidence from knock-out experiments show differential effects in experimental lung fibrosis. TLR2 and -4 are membrane PRR recognizing DAMPs (for example HMGB1 or hyaluronan fragments) and PAMPs from Gram-positive (lipoproteins) and -negative bacteria (LPS) respectively (124). They have seemingly paradoxical effects as TLR2 alteration exerts a protective, and TLR4 alteration a promoting effect on lung fibrosis. In fact, TLR2 deficiency is associated with improved survival and decreased fibrosis in an experimental model, attenuating the pro-fibrotic T_H2 environment (125), altering immune cell recruitment (125, 126) and diminishing IL-17 production through epithelial IL-27 production (126). TLR2 is expressed by both epithelial and immune cells, and further involvement of lung epithelial cells was shown by chimeric experiments revealing that epithelial TLR2 expression is probably the main contributor to these findings (126). Conversely, $TLR4^{-/-}$ animals show augmented deposition of collagen in the lungs when challenged with different fibrotic stimuli (127), and display a shift towards a T_H2 immune milieu as well as decreased autophagy, potentially impacting collagen degradation (127). Illustrating the complexity and the interplay between these different mechanisms, hyaluronan low molecular weight fragments are responsible for the production of chemokine by macrophages and redundantly signal through TLR2 and 4 whereas high molecular weight hyaluronan only need TLR4 to promote AEC regeneration and renewal (128, 129). Contradictory results, suggesting a protective role of TLR4 inhibition have also been published (110) and further studies are sorely needed to evaluate the exact contribution of each component of these complex systems.

MODIFIED LUNG BACTERIAL LANDSCAPE COULD INFLUENCE EPITHELIAL BIOLOGY

Fueled by negative results of culture-based assessments, the lungs were until recently considered as a sterile environment. The advent of high throughput bacterial sequencing has allowed the identification of a diversified bacterial flora in healthy human lungs which showed modifications in chronic respiratory diseases (130). Current techniques are based on the sequencing of highly conserved genes, such as the 16S ribosomal RNA gene, to identify and quantify bacterial communities and cluster them into operational taxonomic units (OTU) (131, 132). In the healthy lung, bacterial composition resembles the oropharyngeal flora and its structure is regulated through three mechanisms, namely the amount of bacterial immigration, the rate of elimination and the reproduction rate of local bacteria (133). Architectural changes and disruption of these homeostatic pathways in disease cause the genesis of niches permitting the emergence of select bacterial populations, resulting in changes in composition and diversity of airway bacteria (134–137). The epithelium from the respiratory tract can influence the two last determinants through mucus production, mucociliary clearance, secretion of inflammatory mediators as well as alterations of the local micro-environment (133, 138).

Airway bacterial composition is altered in IPF, with patients displaying increased bacterial loads and decreased diversity (41, 139). Additionally, patients with the highest bacterial burden have a markedly worse prognosis than those with lower loads, further supporting a link between bacteria and IPF (41, 140). Of note, this correlation could not be found in a recent study evaluating chronic hypersensitivity pneumonitis patients, suggesting disease specific features (139). Prior observations had identified *Streptococcus* sp., *Prevotella* sp., and *Veillonella* sp. as the most identified bacteria in IPF lungs, questioning the association between bacterial composition and disease. Several studies have suggested a relation between certain genera and OTUs, host defense pathways (140, 141), fibroblast behavior (141) or clinical outcomes (139, 142, 143). Although descriptive, these data suggest that changes in the local bacterial landscape could lead to epithelial injury as well as influence the fibrotic and immune response. Further implication of the bacterial landscape in lung fibrosis development can be gathered from animal studies in which the flora can be controlled to express no or selected bacteria. Indeed, germ-free animals instilled with bleomycin display lower mortality (140, 144) and indices of fibrosis (144). Although this data suggests a potential role of bacteria in the development of fibrosis, studies demonstrating a causal link are scarce. In one study, macrophages exposed to outer membrane vesicles from gram-negative bacteria released IL-17B through TLR2/4 sensing, subsequently inducing the secretion of chemokines and growth factor by alveolar epithelial cells, resulting in the development of pulmonary fibrosis (144). Next to influencing immune-epithelial crosstalk, certain bacteria could directly harm the epithelium by secreting cytotoxic compounds. Indeed, streptolysin (a pore-forming cytotoxin) producing *Streptococcus* and corisin (a recently discovered cytotoxic compound) secreting *Staphylococcus* had direct effects on experimental lung fibrosis, increasing AEC2 apoptosis (145) and hampering anti-fibrotic mechanisms (146). The interactions between the microbiome, the epithelium and the immune system have just started to be unraveled and form an exciting prospect for research in the coming years. Understanding the mechanisms underlying these interactions could help to identify prognostic or therapeutic targets, especially in patients developing acute exacerbations of the disease.

THE EPITHELIUM AS A MODULATOR OF LUNG IMMUNITY

Epithelial Injury can Promote a T_H2 Polarized Environment

T-helper 2 (T_H2) lymphocytes, type 2 innate lymphoid cells (ILC2) and alternatively active macrophages (M2) shape a type 2 immune landscape and form the basis of complex crosstalk networks between epithelial, mesenchymal, innate, and adaptive immunity cells. Studies conducted in typical type 2 pathology such as asthma, have revealed a major role for the airway epithelium in the genesis and maintenance of this immune milieu (147), through the recruitment, polarization and activation of myeloid cells. This

environment has been involved in mechanisms of tissue repair through TGF-β1-dependent and -independent pathways. Furthermore, studies initially conducted in *S. mansoni* infected mice allowed to show that the development of fibrosis was linked with a T_H2 environment, involving cytokines like IL-4 and IL-13 (148). These cytokines are mainly produced by T_H2 lymphocytes, ILC2 and macrophages. In IPF, IL-4 as well as IL-13, are elevated in the BAL of patients (149), suggesting a role in lung fibrotic processes. Congruently, overexpression of GATA3, a transcription factor implicated in T_H2 differentiation leads to augmented lung collagen deposition (150) while animals in which IL-4 and IL-13 has been modulated, are protected from bleomycin-induced lung fibrosis (151, 152). Nonetheless, IL-13 seems to be the main fibrotic driver as on the one hand overexpression of IL-13 but not IL-4 induces spontaneous lung fibrosis (153, 154) and on the other hand *IL-13*$^{-/-}$ mice but not *IL4*$^{-/-}$ are protected from FITC-related fibrosis (155). Furthermore, IL-13 promotes fibrosis by enhancing TGF-β production by macrophages and epithelial cells, influencing TGF-β activation (154), and directly impacting myofibroblast differentiation (156). Although the bases of epithelial cell implication in type 2 immunity have been extensively studied in asthma, several links can also be established in the distal lung with regards to IPF and lung fibrosis.

First of all, epithelial cells can recruit immune cells partaking in type 2 immunity and by extension IL-13 secretion. Indeed, they can secrete chemokines such as CCL17 and CCL22, acting on T_H2 cells and ILC2, next to the eotaxins CCL11, CCL24 and CCL26 (147). Both CCL17 and CCL22 are increased in the BAL of IPF patients as well as bleomycin treated mice and are expressed by hyperplasic (alveolar) epithelial cells (157–159). Intriguingly, CCL17 but not CCL22 inhibition leads to decreased lung collagen deposition even though they both share the same receptor, CCR4 (159). The implication of eotaxins in lung fibrosis are poorly understood, nonetheless, CCL11 is increased in experimental lung fibrosis while CCL11 deficient mice are protected and both CCL11, CCL24 and CCL26 are able to influence fibroblast behavior (160–162).

Secondly, the epithelium can influence the behavior of surrounding immune cells through the secretion of IL-25, Thymic Stromal Lymphopoietin (TSLP) or IL-33, several type-2 promoting components. IL-25 can be released by different cell types, including AEC and bronchial epithelial cells (163, 164). T-cells and ILC2, are some of the targets of this cytokine and respond by expansion and secretion of type 2 cytokines like IL-4 and IL-13 (163, 165). Its potential role in disease is suggested by the fact that IPF subjects have higher IL-25 levels in their BAL compared to controls (166). This cytokine can be involved in fibrosis by both its direct effects on fibroblasts as well as its indirect influence on IL-13-dependent fibrosis. Indeed, *in vitro* data shows a direct influence on fibroblast differentiation, cytokine and growth factor secretion (167, 168). Moreover, IL-25 overexpression is associated with perivascular fibrosis in an IL-4 and IL-13 dependent manner (169) and *IL-25*$^{-/-}$ animals are protected from *S. Mansoni* and bleomycin-induced lung fibrosis due to ILC2 related IL-13 production (166), emphasizing its upstream role in type 2 immunity mediated fibrosis.

Similarly, TSLP can be produced by a wide range of cells, including epithelial and mesenchymal cells, similarly promoting a pro-T_H2 environment (170, 171). Staining for TSLP in IPF lungs reveals the presence of this protein in alveolar epithelial cells and fibroblasts within fibroblastic foci (172). Additionally, its concentration in the BAL of patients is significantly elevated, showing an upregulation in this disease (173). Bleomycin instillation induces the expression of TSLP in bronchial and alveolar epithelial cells, but contradictory results have been published regarding the protective character of TSLP deletion in mice (174, 175). Furthermore, stimulation of primary human fibroblasts with this cytokine results in the secretion of CCL2 and chemotaxis of monocytes to the site of injury (172) while AEC undergo EMT (176). The role of TSLP thus seems complex with seemingly contradictory *in vivo* observation and further studies are needed to evaluate its exact role in the fibrotic cascade.

After injury or necrosis, epithelial full-length IL-33 (flIL-33) will be released from the cell nucleus in the surrounding environment, where neutrophil and mast cell proteases will cleave it to its modified form (mIL-33) (177). mIL-33 binds to cells expressing its receptor, ST2, such as ILC2, T_H2 lymphocytes, macrophages, dendritic cells or mast cells, and promotes a pro-T_H2 environment (178). Similarly to IL-25 or TSLP, IL-33 can be found in increased concentrations in the BAL and lung tissue of IPF patients (173, 179) and is upregulated in experimental lung fibrosis (179). Both full-length and the modified form seem to be involved as addition of either recombinant protein enhances collagen deposition after bleomycin challenge (179, 180). The processes underlying this effect are ill-defined but seem to be both ST2 dependent and independent. On the one hand, flIL-33 affects lung fibrosis by modulating the innate immune landscape, directly or indirectly increasing the presence of MCP-1/CCL2, IL-6, TGF-β1 and DAMPs such as HSP70, independently of ST2, IL4 or IL-13 (179). On the other hand, mIL-33 provokes the polarization of lung macrophages, ILC2 expansion and subsequent IL-13 secretion, relying on ST2 to do so (180). Interestingly, peripheral recruitment of ST2 positive cells by IL-33 seems to be one of the prevalent factors driving this observation, as selective bone-marrow ST2 deficiency was sufficient to protect mice from bleomycin lung fibrosis (181).

Next to these cytokines, other DAMPs like HMGB1 or uric acid can promote the formation of a T_H2 driven environment. Indeed, addition of HMGB1 enhances the expression of GATA3 by T_H2 cells and increases the levels of IL-4 and IL-13 (182) and uric acid is implicated in the release of IL-33 and TSLP by airway epithelial cells and the production of IL-13 after respiratory syncytial virus infection (183).

Finally, a T_H2 environment can in turn affect epithelial cell biology. Indeed, continuous exposure of bronchial cells to IL-13 results in an increase in MUC5AC production and induces collagen deposition by fibroblasts in a co-culture model (184). Additionally, IL-13 alters the integrity of the bronchial epithelial barrier by downregulating TJ (185). In the distal lung, AEC2 serve a progenitor function in the alveolar epithelium and are capable of renewing AEC1. Exposure of these cells to IL-13

results in impaired AEC1 differentiation and development of a bronchiolar transcriptomic phenotype (186) aside from increased *in vitro* apoptosis (187), potentially affecting the development of lung fibrosis.

This suggests that the lung epithelium is capable of actively and passively altering its immune environment towards a type-2 polarization and thus exert a pro-fibrotic influence through an additional mechanism. Despite the fact that overwhelming evidence exists regarding the role of type 2 immunity in lung fibrosis, these findings should be contrasted with the disappointing results of therapeutic trials of IL-13 and dual IL-4/IL-13 inhibition in IPF, which both failed to meet their therapeutic endpoints (188, 189). Arguably, these results could be explained by the fact that IL-4/IL-13 are mediators of an upstream fibrotic process of which type 2 inflammation is only one of the (redundant) aspects, resulting in the observed lack of efficacy. This is illustrated by the fact that pirfenidone, one of the two currently validated treatments of IPF with broad anti-fibrotic effects, decreases IL-4 and IL-13 concentrations in the BAL of ovalbumin challenged mice (190).

Epithelial Cells Are Implicated in Alveolar Homeostasis and Pathologic Monocyte/Macrophage Recruitment

Alveolar macrophages (AM) are a self-renewing population of the distal lung, maintaining lung homeostasis through their role in surfactant recycling, repair following injury and tightly controlled inflammatory processes (191). To exert their many functions, macrophages can notably polarize into different subsets, namely classically activated macrophages (M1) and alternatively activated macrophages (M2). Although historically, they have been divided into two subtypes, macrophage polarization should be approached as a reversible continuum rather than a definitive dichotomic classification. Briefly, M1 macrophages are induced by LPS, IFN-γ and TNF-α, produce pro-inflammatory cytokines such as IL-1β, TNF-α, IL-12, IL-23 and promote a T_H1 response, displaying enhanced pathogenicidal properties. M2 macrophages are promoted by TGF-β, IL-4, IL-13 and secrete pro-fibrotic chemo- or cytokines like TGF-β, PDGF, or CCL18, promoting tissue repair and immunomodulation (192, 193). Damaged AEC can release a range of signals promoting the recruitment and activation of macrophages to the site of injury, fueling a pro-inflammatory environment. In a normal response, this phase would be subsequently followed by a self-limited anti-inflammatory repair stage, characterized by M2 polarization and the production of TGF-β1 or PDGF (194). Pathologic perpetuation of these processes leads to an aberrant wound response with excessive collagen deposition and ultimately organ function impairment. AEC2 dysfunction is one of the hallmark features of IPF and *in vivo* experimental data has shown that AEC2 injury is sufficient to trigger lung fibrosis (195). Furthermore, this triggers the influx of monocyte-derived macrophages (Mo-MA) possessing a pro-fibrotic phenotype *via* an interaction with CCR2, the MCP-1 receptor (196). Accordingly, *in vivo* models have subsequently demonstrated the importance of alveolar epithelial cells MCP-1/CCL2 secretion in lung fibrosis (197, 198). MCP-1/CCL2 is a chemotactic factor for myeloid cells such as monocytes, macrophages and fibrocytes (198, 199), which can also influence fibrocyte as well as fibroblast migration, proliferation, and differentiation *in vitro* (200–202). The exact link between epithelial injury and CCL2 secretion are not fully determined, but stimulation with TGF-β1 or tunicamycin (mimicking ER-stress), 2 components implicated in AEC2 dysfunction in IPF, directly upregulate CCL2 secretion by isolated AEC2 (197). Mo-MAs can replace the native AM after depletion of this compartment, for example after bleomycin administration (203), and are one of the drivers of experimental lung fibrosis (203). In line with their monocytic origin, they express high levels of *Ccr2* mRNA (204), suggesting that CCL2 (partly) mediates the recruitment of these cells. Evidence reinforcing this interaction comes from a model in which AEC-specific deletion of CCL12 (the murine equivalent of CCL2) was able to ablate the recruitment of these cells after bleomycin challenge (197). It is unclear if this mechanism similarly mediates the recruitment of a recently discovered macrophage subpopulation in IPF (205). Of note, monocytic myeloid-derived suppressor cells (M-MDSC), a population of immunosuppressive, pro-fibrotic cells also express CCR2 (206) and emerging evidence points towards their implication in IPF (207). Furthermore, IPF patients display increased concentrations of CCL2 in their BAL (208) and immunostainings have shown a partly epithelial origin for this chemokine (209). Based on overwhelming evidence implicating CCL2/CCR2 in (experimental) pulmonary fibrosis, a trial with carlumab, an anti-CCL2 antibody was conducted in IPF. Unfortunately, no effect of this treatment could be observed, and the study was halted prematurely (210). Of note, free CCL2 levels rose in the treatment, but not the placebo group (210), suggesting the activation of compensatory mechanisms.

CONCLUDING REMARKS

Alveolar epithelial dysfunction due to repetitive injury in susceptible/ageing lungs forms the current paradigm of IPF pathogenesis. Experimental evidence supports the involvement of the immune system in (pathologic) repair attempts and collagen deposition. The pulmonary epithelium, laying at the forefront of mucosal immunity plays a crucial role in lung homeostasis, inflammation, and subsequent repair mechanisms. It is thus capable of sensing and reacting to danger stimuli to ultimately regulate lung responses at the level of both structural and immune (myeloid) cells (**Figure 2** and **Table 1**). Aberrant alveolar epithelial biology represents a hallmark of IPF, also potentially impacting immune mechanisms. Determining the exact contribution of these mechanisms remains a challenge, as they are at the cross-point of multiple regulatory networks also involving myeloid and mesenchymal cells. For example, whether differential expression of co-stimulatory molecules such as B7 complex (including PD-L1) may interfere with the crosstalk between epithelium and immune cells remains elusive. Importantly, trials evaluating immunosuppressive medications have yielded disappointing results until now, questioning our understanding of the mechanisms at stake. Nonetheless, in-depth understanding of the epithelial contribution to the immune-fibrotic paradigm should

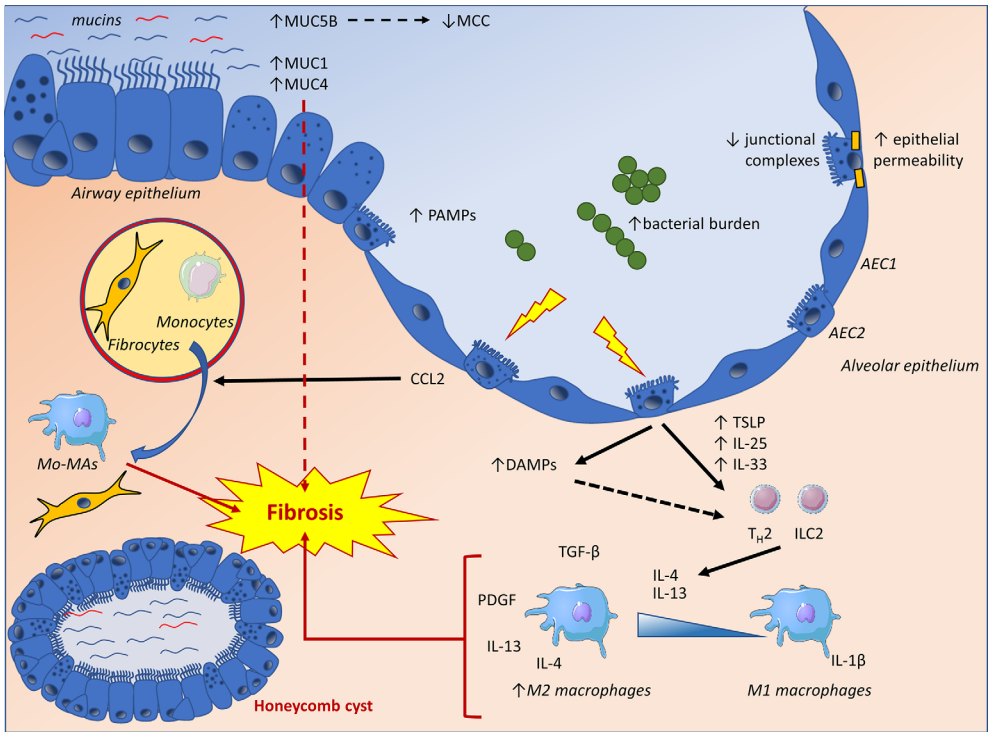

FIGURE 2 | The IPF lung epithelium displays increased concentrations of secreted and membrane-bound mucins, as well as altered junctional complexes, potentially influencing local barrier mechanisms and fibrosis through impaired mucociliary clearance (MCC), promotion of epithelial to mesenchymal transition (EMT) and increased epithelial permeability. Lung epithelial cells are also confronted to an increased bacterial burden and pathogen-associated molecular patterns (PAMPs). Furthermore, epithelial damage will result in the production of damage-associated molecular patterns (DAMPs), triggering pro-inflammatory pathways and T_H2 polarizing cytokines. These cytokines exert a pro-fibrotic influence by directly affecting mesenchymal cells and polarizing macrophages towards an alternatively activated phenotype (M2). Finally, epithelial dysfunction will result in the release of CCL2, a chemokine directly affecting fibroblasts as well as fibrocyte recruitment and differentiation while mediating the recruitment of monocytes to the site of injury. The latter will differentiate into monocyte-derived macrophages (Mo-MA), which have been implicated in lung fibrosis. AEC1, alveolar type-1 epithelial cell; AEC2, alveolar type-2 epithelial cell; Mo-MA, monocyte-derived macrophage; MCC, mucociliary clearance; ILC2, type 2 innate lymphoid cell; T_H2, type 2 helper T-cell.

TABLE 1 | Summary of the epithelial-immune interactions in IPF.

	Observations in IPF	Putative mechanisms (experimental data)
Physical barrier properties		
- **Mucus production**	↑MUC5B (35, 36) ↑MUC1 (43) ↑MUC4 (44)	MUC5B: ↓MCC (38) MUC1/4: TGF-β1 signaling, fibroblast differentiation, EMT (43, 44)
- **Intercellular junctions**	↑claudin-1, -2, -3, -7 (57–59) ↓claudin-18 (57) ↓E-cadherin (73)	↑epithelial dysfunction (69, 70) ↑polarized receptor-ligand interactions (71) ↑epithelial denudation (79)
Environmental sensing		
- **PRR**	↑TLR2 ↑TLR4 ↑TLR9 (123, 211)	$TLR2^{-/-}$: ↓fibrosis, ↓T_H2 environment, altered immune cell recruitment, ↓IL-17 production (125, 126) $TLR4^{-/-}$: ↓↑fibrosis, ↑T_H2 environment, ↓autophagy ↓AEC2 proliferation (110, 127, 128) TLR9: ↑EMT ↑myofibroblast differentiation (114, 115)
- **DAMPs**	↑HMGB1 ↑eATP ↑uric acid ↑HA (90–93, 212)	↑EMT ↑IL-1β ↑CCL2 ↑fibroblast proliferation and differentiation (90, 95, 97–99, 108)
- **Bacterial PAMPs**	↑Bacterial load ↓diversity (41)	LPS: ↑fibroblast proliferation (111) ↑IL-1β ↑CCL2 (112)
Modulation of the immune environment		
- **T_H2 environment promotion**	↑CCL17 ↑CCL22 ↑IL25 ↑TSLP ↑IL-33 (158, 166, 173)	↑Recruitment of T_H2 and ILC2, ↑fibroblast proliferation, differentiation and collagen synthesis, ↑EMT (167, 176, 180)
- **Recruitment of myeloid cells**	↑CCL2 (208)	↑Recruitment of Mo-AM (197)

MCC, mucociliary clearance; EMT, epithelial-mesenchymal transition; eATP, extracellular ATP; HA, hyaluronan; LPS, lipopolysaccharide; TSLP, Thymic Stromal Lymphopoietin; Mo-AM, monocyte-derived alveolar macrophages.

help to appreciate the reasons underlying these clinical failures and design more targeted and effective therapies.

AUTHOR CONTRIBUTIONS

TP-B designed and wrote the manuscript. CP wrote and revised the manuscript. AF designed, wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

TP-B is the recipient of a Fonds de la Recherche Scientifique grant (FNRS grant n°ASP/DM A814). CP is postdoctoral specialist of the Fonds de la Recherche Scientifique (FNRS grants 1.R016.16 and 1.R016.18). AF is supported by the Fonds de Recherche Clinique, Cliniques universitaires Saint-Luc, as well as by the Fonds de la Recherche Scientifique (FNRS CDR grant n° J007820).

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 14 June 2021
doi: 10.3389/fimmu.2021.629854

Investigation of a Hypoxia-Immune-Related Microenvironment Gene Signature and Prediction Model for Idiopathic Pulmonary Fibrosis

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: 16 November 2020
Accepted: 25 May 2021
Published: 14 June 2021

Citation:
Li X, Cai H, Cai Y, Zhang Q, Ding Y and Zhuang Q (2021) Investigation of a Hypoxia-Immune-Related Microenvironment Gene Signature and Prediction Model for Idiopathic Pulmonary Fibrosis. Front. Immunol. 12:629854. doi: 10.3389/fimmu.2021.629854

Background: There is growing evidence found that the role of hypoxia and immune status in idiopathic pulmonary fibrosis (IPF). However, there are few studies about the role of hypoxia and immune status in the lung milieu in the prognosis of IPF. This study aimed to develop a hypoxia-immune-related prediction model for the prognosis of IPF.

Methods: Hypoxia and immune status were estimated with microarray data of a discovery cohort from the GEO database using UMAP and ESTIMATE algorithms respectively. The Cox regression model with the LASSO method was used for identifying prognostic genes and developing hypoxia-immune-related genes. Cibersort was used to evaluate the difference of 22 kinds of immune cell infiltration. Three independent validation cohorts from GEO database were used for external validation. Peripheral blood mononuclear cell (PBMC) and bronchoalveolar lavage fluid (BALF) were collected to be tested by Quantitative reverse transcriptase-PCR (qRT-PCR) and flow cytometry from 22 clinical samples, including 13 healthy controls, six patients with non-fibrotic pneumonia and three patients with pulmonary fibrosis.

Results: Hypoxia and immune status were significantly associated with the prognosis of IPF patients. High hypoxia and high immune status were identified as risk factors for overall survival. CD8+ T cell, activated CD4+ memory T cell, NK cell, activated mast cell, M1 and M0 macrophages were identified as key immune cells in hypoxia-immune-related microenvironment. A prediction model for IPF prognosis was established based on the hypoxia-immune-related one protective and nine risk DEGs. In the independent validation cohorts, the prognostic prediction model performed the significant applicability in peripheral whole blood, peripheral blood mononuclear cell, and lung tissue of IPF patients. The preliminary clinical specimen validation suggested the reliability of most conclusions.

Conclusions: The hypoxia-immune-based prediction model for the prognosis of IPF provides a new idea for prognosis and treatment.

Keywords: idiopathic pulmonary fibrosis, microenvironment, hypoxia, immune, prognosis

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease. The prognosis of patients with IPF is poor, with a median survival of 3 to 5 years (1). Several prognostic staging systems for IPF have been established by clinical and physiologic variables (2–5). Biomarkers in peripheral blood are also evaluated as a tool for prognosis (6–8). A recent study also revealed the role of genetic variability in the survival of IPF (9). In addition, molecular markers of IPF patients could also be identified by bronchoalveolar lavage (BAL) cells, and the collection of BAL cells is non-invasive compared with lung biopsy (10). However, very little is known whether molecular events in the lung milieu are predictive of outcome in IPF. Considering accurate diagnosis and personalized treatment, there is still a critical need for a way to predict the progression of IPF.

As a chronic lung disorder, the central processes in IPF are inflammation and fibrosis (11). Immune dysregulation has been considered as a promoting factor in the development of IPF, including several biomarkers associated with the prognosis of IPF (12). Inflammatory cytokines released by immune cells may activate fibroblasts, connective tissue cell proliferation, angiogenesis (11). Furthermore, hypoxia is common in the process of fibrosis in many diseases (13, 14). Excessive collagen synthesized by fibroblasts deteriorate oxygen supply and accelerate the pathological process. Studies showed the relationship between immune response and hypoxia and lung function (15). However, the underlying mechanisms have not been discussed.

With a series of genetic and bioinformatics analyses, we associated immune status with hypoxia and explore its value for the prognosis of patients with IPF. Here, we developed a hypoxia-immune-related prediction model for the prognosis of IPF, intended to provide novel ideas for accurate diagnosis and treatment at the gene level. Better knowledge of the oxygen balance control and the immune regulation involved is important to advance the development of IPF.

MATERIALS AND METHODS

Patient Cohort and Data Preparation

The discovery cohort of the study contained 176 IPF patients from the Gene Expression Omnibus (GEO, available at: https://www.ncbi.nlm.nih.gov/geo/) database (GSE70866). The tissue source of sequencing samples is the patients' BAL cells. The microarray data of GSE70866 was based on GPL14550 Platform (Agilent-028004 SurePrint G3 Human GE 8x60K Microarray, Agilent Technologies) and GPL17077 Platform (Agilent-039494 SurePrint G3 Human GE v2 8 × 60K Microarray, Agilent Technologies), including 176 IPF patients' BAL cells. Three validation cohorts were used for external validations (GSE93606, GSE28221, and GSE32537) to examine the predictive effect of the prediction method. The microarray data of GSE93606 was based on GPL11532 Platforms (Affymetrix Human Gene 1.1 ST Array, Affymetrix, Santa Clara, CA, USA), including 57 IPF patients' peripheral whole blood. The microarray data of GSE28221 was based on GPL5175 Platforms (Affymetrix Human Exon 1.0 ST Array, Affymetrix, Santa Clara, CA, USA) and GPL6480 Platforms (Agilent-014850 Whole Human Genome Microarray 4x44K G4112F, Agilent Technologies), including 120 IPF patients' peripheral blood mononuclear cell. The microarray data of GSE32537 based on GPL6244 (Affymetrix Human Gene 1.0 ST Array, Affymetrix, Santa Clara, CA, USA) included 119 lung tissues with IPF. The batch effect was eliminated by sva package, which contains functions for identifying and building surrogate variables for high-dimensional data sets.

All procedures of this study complied with the protocol. For analyses of data from a public database, approval and informed consent from the local ethics committee were not required.

Identification of Hypoxia Status and Hypoxia-Related DEGs

To identify the hypoxia status, a non-linear dimensionality reduction algorithm of Uniform Manifold Approximation and Projection (UMAP) was applied, which could divide or condense a group of patients into a series of distinct clusters, according to the given hallmarks or signatures. The hallmark gene sets of hypoxia include 200 genes and were downloaded from the Molecular Signatures Database (MSigDB version 6.0). Based on the clusters, two groups including "hypoxia^{low}" and "hypoxia^{high}" were identified to identify the hypoxia status. The limma algorithm was applied to identify differentially expressed genes (DEGs) between the two groups (16). Genes with a false discovery rate (FDR) adjusted p-value <0.0001 and an absolute value of log2 (fold change) >1 were considered as hypoxia-related DEGs.

Identification of Immune Status and Immune-Related DEGs

To identify the immune status, the Estimation of Stromal and Immune cells in MAlignant Tumours using Expression data (ESTIMATE) algorithm was applied to identity the infiltration degree of immune cells and predict the immune status (17). Based on the immune status, patients were classified into two groups. Maximally selected rank statistics was applied by using an R package "survival", and "survminer" to identify the optimal cutting point to divide patients. Based on the optimal cutting

point, patients with high immune scores were attributed to "immune^{high}" group and "immune^{low}" group. The limma algorithm was applied to identify DEGs between the two groups. Genes with a FDR adjusted p-value <0.0001 and an absolute value of log2 (fold change) >1 were considered as immune-related DEGs.

To further identify the abundance of 22 immune cells, CIBERSORT is a deconvolution algorithm based on the gene expression data to resolve immune cell composition (18). Those with p <0.05 were included.

Identification of Hypoxia-Immune-Related Prognostic DEGs

According to the above hypoxia and immune grouping, patients were divided into three groups, *i.e.*, hypoxia^{high}/immune^{high}, hypoxia^{low}/immune^{low}, and mix groups. The limma algorithm was applied to identify DEGs between "hypoxia^{high}/immune^{high} group and hypoxia^{low}/immune^{low} group. Genes with a FDR adjusted p-value <0.0001 and an absolute value of log2 (fold change) >1 were considered as hypoxia-immune-related DEGs. DEGs were then divided into protective and risk DEGs. The risk DEGs contained all EDGs highly expressed in hypoxia^{high}/immune^{high} group and the rest were protective DEGs. To obtain hypoxia-immune-related prognostic DEGs, univariate Cox analyses were further performed to screen all protective and risk DEGs. Those with a p <0.001 were considered as significant.

Prognosis Prediction Model of IPF Based On Hypoxia-Immune-Related DEGs

The Least Absolute Shrinkage and Selection Operator (LASSO) is a kind of linear regression using shrinkage, which is applied to survival analysis with high-dimensional data (19). In this study, the LASSO Cox regression model was applied to select the optimal variables from all identified hypoxia-immune-related prognostic DEGs in the discovery cohort. Three-fold cross-validation and 1,000 iterations were conducted to reduce the potential instability of the results. The optimal tuning parameter λ was identified *via* 1-SE (standard error) criterion. Then we create the prognosis prediction model of IPF using the selected prognostic gene signature. For each patient, the risk score was the sum of the expression of the characteristic DEGs and the corresponding coefficients derived from the multivariate Cox regression model. According to the risk scores, the optimal cutting point was identified using the maximally selected rank method, and the prognosis prediction model of IPF was formed.

Functional and Pathway Enrichment Analysis

Database for Annotation, Visualization and Integrated Discovery was used for Gene Ontology (GO) enrichment analysis (20). The risk DEGs of IPF patients were screened for functional enrichment. GO analysis was used to evaluate the degree of enrichment of the DEGs in biological processes, cellular components, and molecular functions. Those with p-value <0.05 and count (the number of enriched genes) ≥3 were considered as the cutoff criterion.

Preliminary Validation of Clinical Specimens

Peripheral blood mononuclear cell (PBMC) and bronchoalveolar lavage fluid (BALF) were collected from 22 clinical samples, including 13 healthy controls, six with non-fibrotic pneumonia and three with pulmonary fibrosis. The clinical information such as age, gender, alveolar-arterial oxygen gradient (A-aDO2), and hospital day was shown in **Supplementary Materials**. The study was reviewed and approved by the institutional review board (Ethics Committee) of the 3rd Xiangya Hospital, Central South University (No. 21028).

Quantitative reverse transcriptase-PCR (qRT-PCR) was used to quantitative expression of key DEGs. Total RNA was extracted from the tissues using TRIzol Reagent (Thermo Fisher Scientific). PCR was performed using an Thermo Scientific PikoReal PCR cycler. The cycle threshold (CT) data were determined, and the mean CT was determined from triplicate PCRs. Relative gene expression was calculated with the equation $2^{-\Delta CT}$.

Flow cytometry analysis was used to determine the proportion of immune cells. The cell suspension was counted and mixed with ACK Lysis Buffer (Thermo Fisher Scientific) to remove red blood cells. Then 1×10^6 cells were resuspended in 100 µl staining buffer and incubated with monoclonal antibodies in dark for 15 min at 4°C. Our flow cytometric staining strategy consisted of the following fluorochrome-conjugated monoclonal antibodies: anti-CD3-Alexa-Flour700 (Biolegend), anti-CD4-eFlour450 (eBioscience), anti-CD45RA-APC-eFluor 780 (eBioscience), anti-CCR7-PerCP-eFluor 710 (eBioscience), anti-CD16-eFluor506 (eBioscience), anti-CD56-PE (eBioscience), anti-CD206- PE-Cyanine7 (eBioscience), anti-CD68-FITC (eBioscience), anti-CD107a-eFluor660 (eBioscience). CD3 and CD4 were used to identify T cells. $CD3^+CD4^+CD45RA^-CCR7^-$ cells were defined as activated $CD4^+$ memory T cells. CD56 and CD16 were used to identify NK cells. $CD107a^+$ NK cells were defined as activated NK cells. $CD68^+CD16^-CD206^-$ cells were defined as M1-like macrophages and $CD68^+CD16^-CD206^+$ cells were defined as M0-like macrophages. After washing and resuspending, samples were detected using BD FACSDiva software and performed using BD FACSCanto II.

Statistical Analysis

All analyses were performed with R version 4.0.2 (www.r-project.org/) and the corresponding packages. UMAP algorithm was performed by using R package "umapr" for non-linear dimensionality reduction. Immune score was performed by using R package "estimate". The Lasso Cox regression model was performed by using R package "glmnet". Data were analyzed with standard statistical tests as appropriate. Multiple testing was adjusted by the FDR method. Multivariate Cox regression analysis was performed to identify optimal signatures. Flowjo V 10.62 was used to analyzed flow cytometric data. Original data from PCR and flow cytometry were presented as the mean ± standard deviation (SD) and were compared using Student's t-test, Welch's t-test or the Mann–Whitney U test, where appropriate. GraphPad Prism 7.0 (GraphPad Software Inc., La

Jolla, CA, USA) was used to perform the statistical analyses. Values of p <0.05 were considered statistically significant.

The general idea and methodologies used in this study were drawn as a flow chart (**Figure 1**).

RESULTS

Hypoxia Status and Hypoxia-Related DEGs in IPF

The discovery cohort contained 176 IPF patients from the GEO databases. The batch effect was eliminated by sva package (**Figures 2A, B**). Patient clinical information is shown in **Table 1**. With the expression matrix constructed by 200 hypoxia marker genes from MSigDB, the non-linear dimensionality reduction algorithm UMAP was used to determine two clusters, and each patient is assigned to the nearest clusters (**Figure 2C**). Cluster 1 and Cluster 2 contained 95 and 81 patients respectively. Expression profiles were compared between the two clusters, and 239 DEGs related to hypoxia were obtained. Enrichment analysis showed overexpressed genes in Cluster 2 were enriched in "oxygen transport (GO:0015671)" and "response to hypoxia (GO:0001666)" (**Figure 2D**). This indicated that the level of hypoxia in Cluster2 was at high status. Thereby, the patients in Cluster1 and Cluster2 were determined as hypoxia^{low} and hypoxia^{high} groups. In addition, overexpressed genes in Cluster2 were also enriched in positive regulation of GTPase activity and cell adhesion. Patients' clinical information of each cluster is shown in **Table 2**. The survival status of patients in different groups was further analyzed (**Figure 2E**). There was a significant difference in survival between two clusters (log rank test, $p < 0.0001$), and the prognosis of patients with a high level of hypoxia is worse. Among 239 DEGs, 232 DEGs were overexpressed in the hypoxia^{high} cluster, which were regarded as hypoxia-associated risk DEGs. The other seven genes overexpressed in the hypoxia^{low} cluster, which are regarded as hypoxia-associated protective DEGs. In a word, most of the hypoxia-related DEGs are regarded as risk factors.

Immune Status and Immune-Related DEGs in IPF

The immune score was calculated by ESTIMATE to identity the infiltration degree of immune cells. The optimal cutting point "2,959.22" was determined based on maximally selected rank statistics (**Figures 2F, G**). Then the immune^{high} group and immune^{low} group were divided, containing 108 and 68 patients respectively. Patients' clinical information of each cluster is shown in **Table 3**. Further survival analysis showed a significant difference between two groups (log rank test, $p < 0.05$), and the survival of patients with a high level of immune infiltration is worse (**Figure 2I**). Therefore, high immune infiltration is also a risk factor for bad prognosis. This conclusion is also supported by the highly enriched immune-related pathways in the hypoxia^{high} group with a poor prognosis. Expression profiles were compared between the two groups, and 196 DEGs related to immune status were obtained (**Figure 2H**). Among them, 191 genes were overexpressed in the immune^{high}

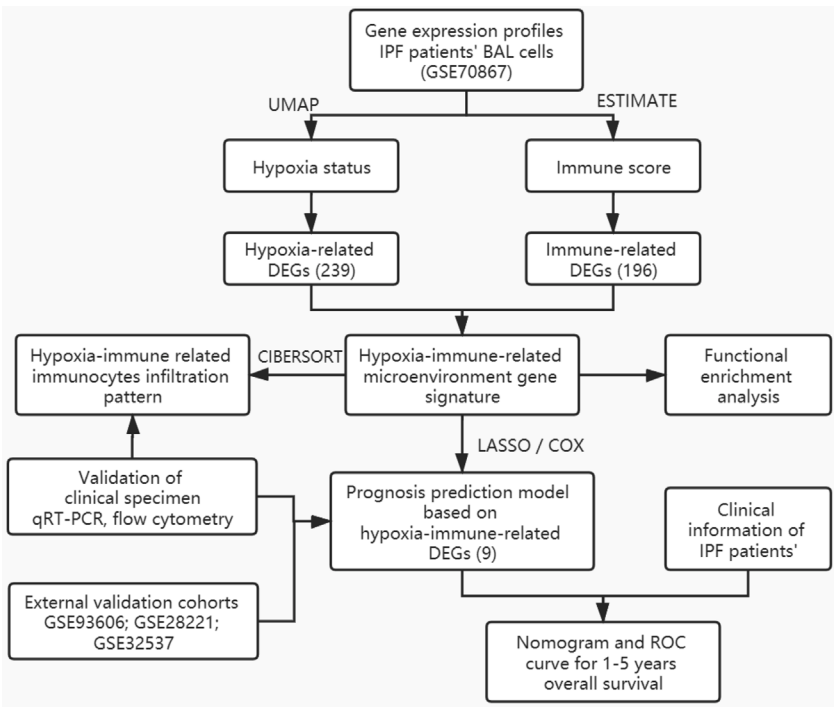

FIGURE 1 | Flow chart of methodologies used in this study.

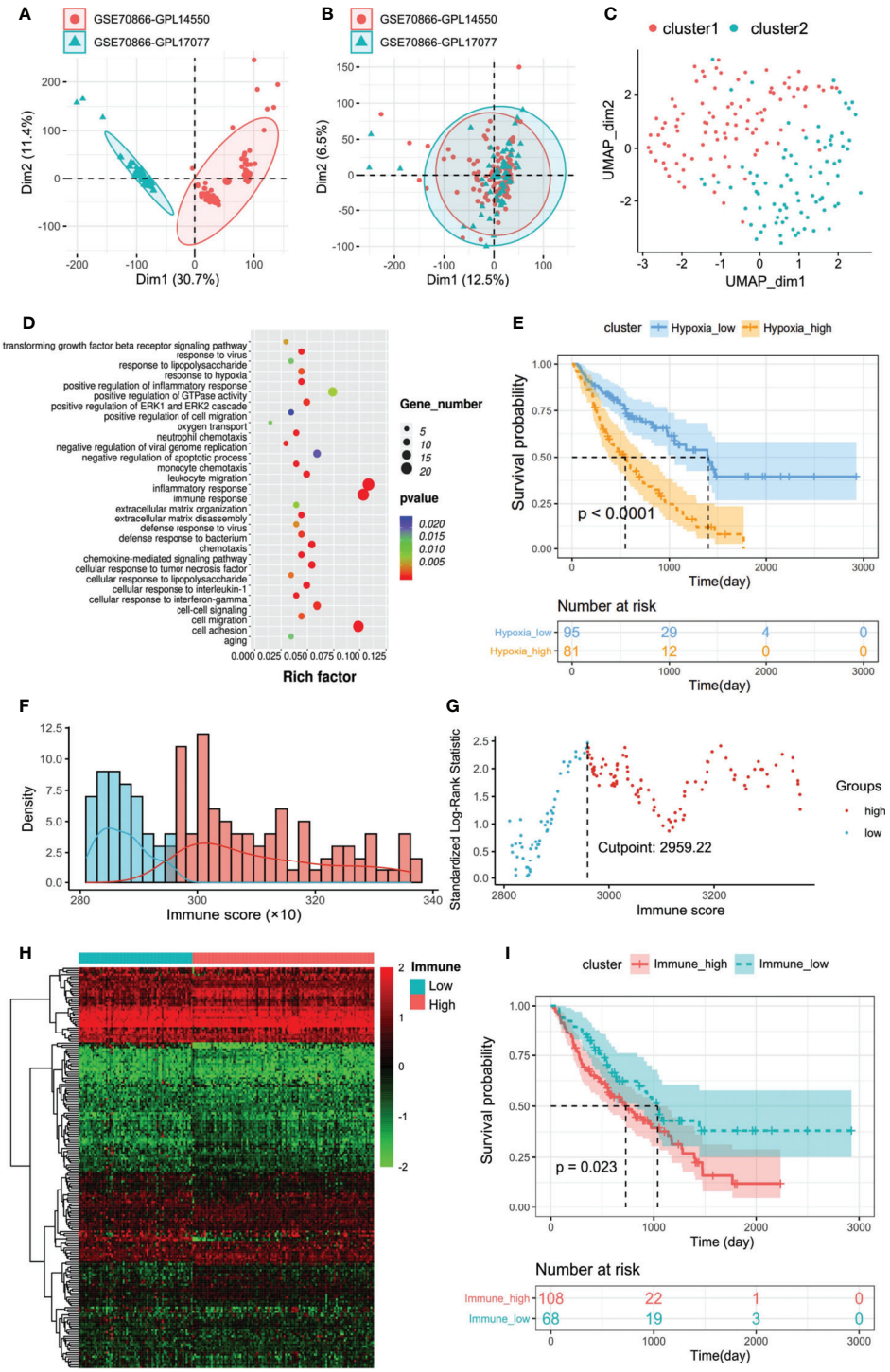

FIGURE 2 | **(A, B)** Eliminating the batch effect between different sequencing platforms. **(A)** The PCA plot before elimination of batch effect, and **(B)** is the PCA plot after elimination. The distance of sample point clusters indicates that they come from different batches and sequencing platforms. While in B, after eliminating the batch effect, the difference in distance between batches is reduced. **(C)** UMAP clustering plot based on marker gene set of hypoxia. **(D)** Biological process functional enrichment analysis of differentially expressed genes between hypoxia^{high} and hypoxia^{low} groups. **(E)** Kaplan–Meier plot of overall survival in two clusters. **(F)** Histogram based on maximally selected rank grouping. **(G)** The cut-off point with the maximum standard log-rank statistic was marked with a vertical dashed line. **(H)** The differential gene expression profiles between hypoxia^{high} and hypoxia^{low} groups were visualized in heatmap. **(I)** Kaplan–Meier plot of overall survival between immune^{high} and immune^{low} groups.

TABLE 1 | Basic information of IPF patients in discovery cohort.

Characteristics	Whole cohort (176)	Low risk (55)	High risk (121)
Gender			
Male	117 (0.665)	31 (0.564)	86 (0.711)
Female	59 (0.335)	24 (0.436)	35 (0.289)
Age			
≥65 years	86 (0.489)	30 (0.545)	56 (0.463)
<65 years	90 (0.511)	25 (0.455)	65 (0.537)
UMAP clustering			
Cluster1	95 (0.540)	47(0.855)	48 (0.397)
Cluster2	81 (0.460)	8 (0.145)	73 (0.603)
Hypoxia status			
High	81 (0.460)	8 (0.145)	73 (0.603)
Low	95 (0.540)	47(0.855)	48 (0.397)
Immune status			
High	108 (0.614)	21 (0.382)	87 (0.719)
Low	68 (0.386)	34 (0.618)	34 (0.281)
Risk group			
High	121 (0.688)	0	121 (1.000)
Low	55 (0.312)	55 (1.000)	0

TABLE 2 | Basic information of IPF patients in different hypoxia-based clusters.

Characteristics	Whole cohort (176)	$hypoxia^{low}$ (95)	$hypoxia^{high}$ (81)
Gender			
Male	117 (0.665)	58 (0.610)	59 (0.728)
Female	59 (0.335)	37 (0.390)	22 (0.272)
Age			
≥65 years	86 (0.489)	48 (0.505)	38 (0.470)
<65 years	90 (0.511)	47 (0.495)	43 (0.530)

TABLE 3 | Basic information of IPF patients in different immune-based clusters.

Characteristics	Whole cohort (176)	$immune^{low}$ (68)	$immune^{high}$ (108)
Gender			
Male	117 (0.665)	44 (0.647)	72 (0.667)
Female	59 (0.335)	24 (0.353)	36 (0.333)
Age			
≥65 years	86 (0.489)	38 (0.559)	48 (0.445)
<65 years	90 (0.511)	30 (0.441)	60 (0.555)

cluster, which were regarded as immune-associated risk DEGs. The other five genes overexpressed in the $immune^{low}$ cluster, which are regarded as immune-associated protective DEGs.

Hypoxia–Immune-Related DEGs in IPF

According to the above hypoxia and immune grouping, we further combined to form three groups: $hypoxia^{high}$/$immune^{high}$, $hypoxia^{low}$/$immune^{low}$, and mix groups. The survival status of patients in different groups was further analyzed (**Figure 3A**). There was a significant difference in survival among three groups (log rank test, $p < 0.0001$). Survival in mix group is at an intermediate level. As we expected, a high level of hypoxia and immune activity is the most dangerous factor while patients in group $hypoxia^{low}$/$immune^{low}$ have the best prognosis.

The differential gene expression profiles between $hypoxia^{high}$/$immune^{high}$ and $hypoxia^{low}$/$immune^{low}$ groups were visualized in heatmap (**Figure 3B**). We further intersect the hypoxia-related DEGs and the immune-related DEGs to identify the hypoxia–immune-related DEGs in IPF. We obtained a total of 62 DEGs, of which 61 were highly expressed in $hypoxia^{high}$ and $immune^{high}$ groups, so they were defined as hypoxia-immune-related risk DEGs (**Figure 3C**). Correspondingly, the remaining DEG is defined as hypoxia-immune-related protective DEG (**Figure 3D**). The GO enrichment analysis showed that "immune response", "inflammatory response", and "positive regulation of ERK1/2 cascade" are main biological process (**Figure 3E**).

Prognosis Prediction Model of IPF Based on Hypoxia-Immune-Related DEGs

To further determine the DEGs significantly related to the prognosis, we used univariate Cox analysis for screening and 29 DEGs with $p < 0.001$ were retained (**Figure 4A**). Among them, one protective and 28 risk DEGs were included.

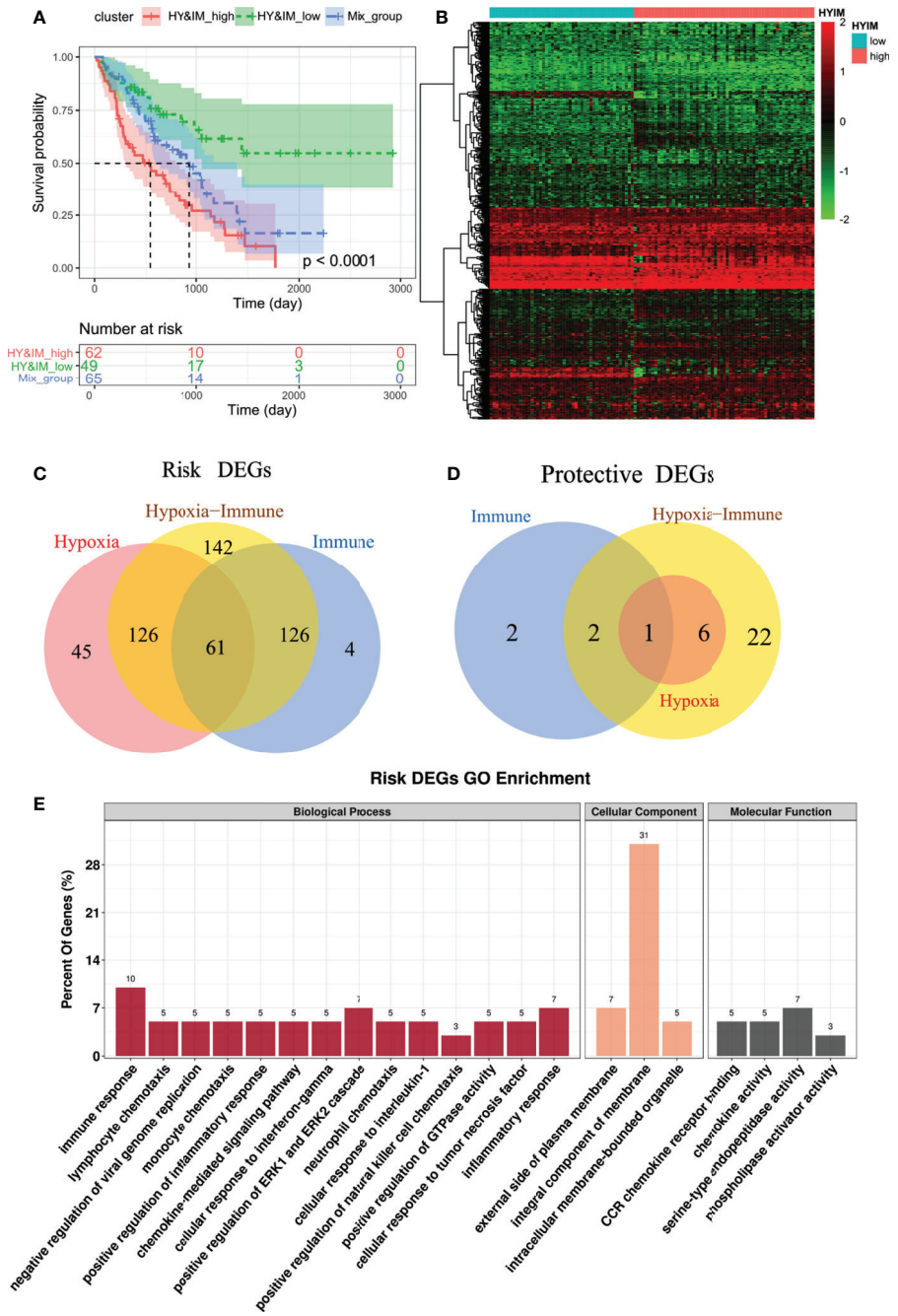

FIGURE 3 | **(A)** Kaplan–Meier plot of overall survival between hypoxia^{high}/immune^{high}, hypoxia^{low}/immune^{low}, and mix groups. **(B)** Heatmap shows the differential gene expression profiles between hypoxia^{high}/immune^{high} and hypoxia^{low}/immune^{low} groups **(C,D)** Venn diagrams show the hypoxia-immune related risk DEGs (61) and protective DEG (1). € GO enrichment analysis of risk DEGs.

Using lasso regression method, nine optimal variables were obtained from the above 29 hypoxia-immune-prognostic-related DEGs (**Figures 4B, C**). Then we use the expression levels of nine characteristic DEGs and the corresponding coefficients derived from the multivariate Cox regression model to estimate the risk score for each patient: risk score = −0.13307 × expression of NALCN + 0.09893 × expression of IL1R2 + 0.06226 × expression of S100A12 + 0.06890 × expression of PROK2 + 0.04883 × expression of CCL8 + 0.05654 × expression of RAB15 + 0.10671 × expression of MARCKSL1 + 0.09986 × expression of TPCN1 + 0.05696 × expression of HS3ST3B1.

By calculating the risk score of each patient, we divided the patients into two groups through the maximally selected rank method: high-risk group and low-risk group (**Figure 4D**). The

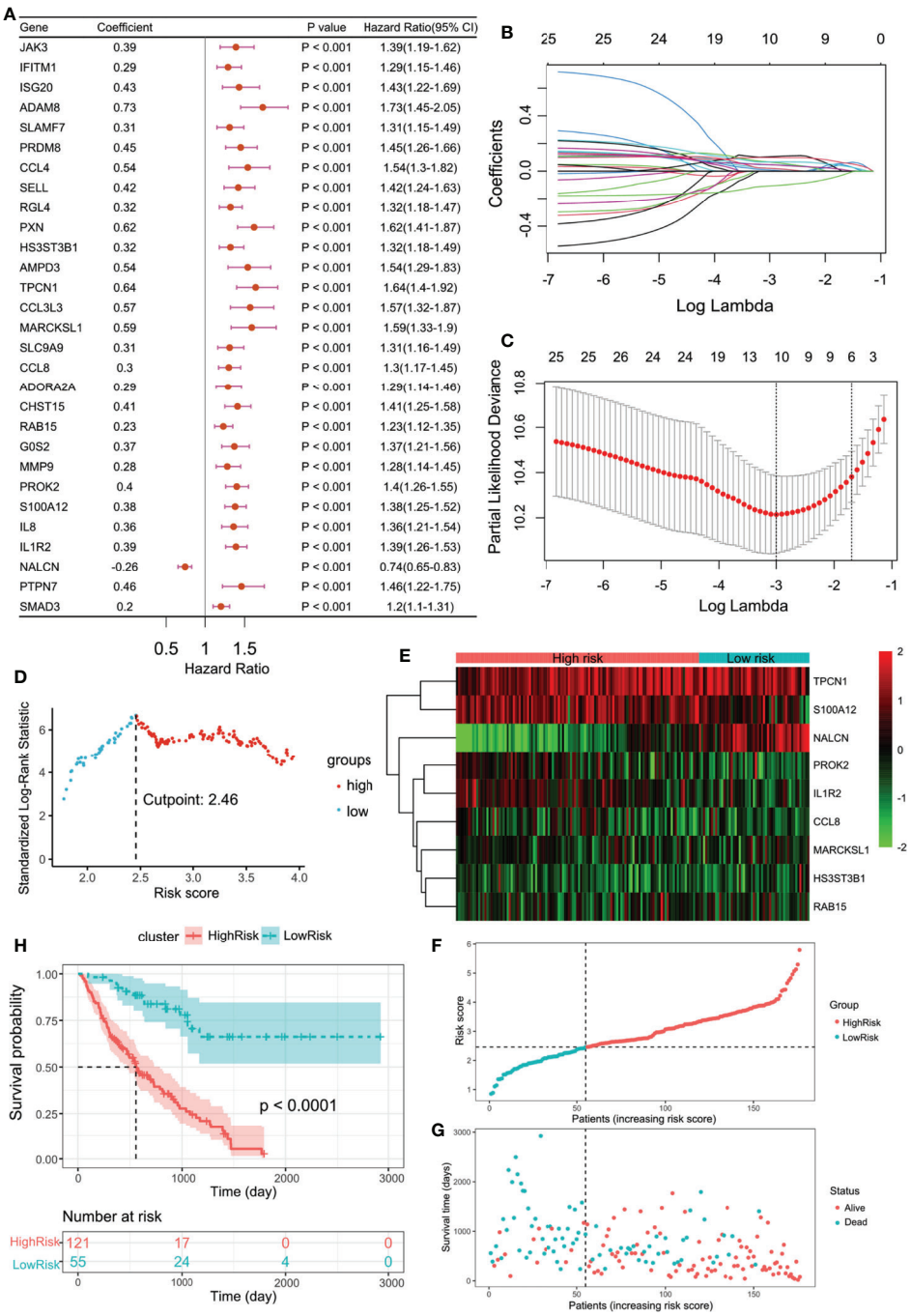

FIGURE 4 | **(A)** Forest plot of 29 DEGs with P <0.001 by univariate Cox regression. **(B)** LASSO coefficient profiles of 29 screened DEGs. **(C)** Three-fold cross-validation of lasso analysis. Error bars represented the SE. The dotted vertical lines showed the optimal values. **(D)** The cut-off point with the maximum standard log-rank statistic was marked with a vertical dashed lines. **(E)** The expression profiles of DEGs involved in multivariate Cox regression model. **(F)** The distribution of patients with increased risk score in two groups. **(G)** Scatter plot showed the survival of patients with increased risk score. **(H)** Kaplan–Meier plot of overall survival between high-risk and low-risk groups.

nine optimal DEG expression profiles between high-risk and low-risk groups were visualized in heatmap (**Figure 4E**). Survival analysis showed that there was a significant difference between the two groups (log rank test, $p < 0.0001$). Compared with the low-risk group, the prognosis of the high-risk group is significantly worse (**Figures 4F–H**).

Hypoxia-Immune Related Immunocyte Infiltration Pattern

In addition, CIBERSORT was used to estimate the infiltration of 22 kinds of immune cells in the samples. Correlation analysis showed a general association in different immune cells (**Figure 5A**). Among them, the infiltration of six specific immune cells was significantly different in hypoxia^{high}/immune^{high} and hypoxia^{low}/immune^{low} group, that is, CD8+ T cell, activated CD4+ memory T cell, activated natural killer (NK) cell, activated mast cell, M0 macrophage, M1 macrophage (**Figure 5B**). Further correlation analysis showed the relationship between six specific immune cells and risk score. Among them, most infiltration degree is positively correlated with risk DEGs expression and risk score, but the infiltration of M0 cells was negatively correlated with the risk DEGs expression and risk score (**Figure 5C**). Among them, M0 macrophages and NK cells had the most significant correlation with key DEGs and risk score.

Validation of the Prognostic Prediction Model in External Independent Cohorts

The ROC curve showed that the AUCs within 1–5 years were all greater than 0.75 in discovery cohort (**Figure 6A**). This suggested the evaluation model had a good predictive value for the prognosis of IPF patients. We also developed a nomogram for 1–5 years overall survival prediction based on Cox model (**Figure 6B**).

We further verify the above prediction method in external data sets" GSE93606", "GSE28221", and "GSE32537". Patient clinical information is shown in **Table 4**. In each independent validation cohort, we divided the IPF patients into high-risk and low-risk groups based on the risk score. In GSE28221 and GSE93606 IPF cohorts, survival comparison showed that low-risk group had significantly better prognosis outcomes than high-risk group (**Figures 6C, D**). In addition, we focused on the clinicopathologic features of IPF in GSE32537 cohort. The patients in low-risk group generally had higher forced vital capacity (FVC) and carbon monoxide diffusing capacity

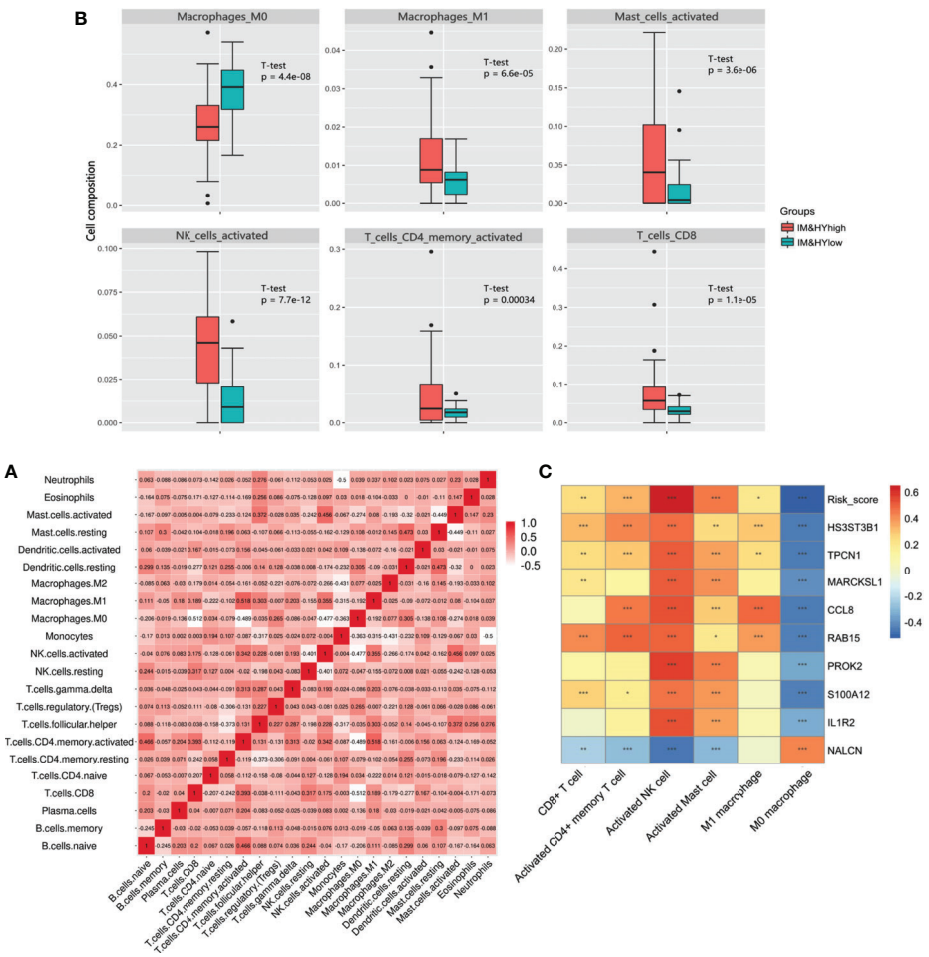

FIGURE 5 | **(A)** Heatmap showed the correlation coefficient between different immune cells. **(B)** The box plot showed the significant difference of immune cells' infiltration between two groups. **(C)** Heatmap showed the correlation coefficient between immune cells and DEGs involved in Cox model (*** means P < 0.01, ** means P < 0.05, and * means P < 0.1).

(DLCO) ($P < 0.05$), which meant better lung function (**Figures 6E, F**). While the patients in high-risk group had higher St. George's total score ($P < 0.05$), which suggested worse lung function and quality of life (**Figure 6G**).

In a word, these results suggest that our prognostic prediction model is also of great significance based on peripheral whole blood, peripheral blood mononuclear cell, and lung tissue.

Preliminary Validation of Clinical Specimens

The results of qRT-PCR in PBMC suggested that DEGs of CCL8, IL1R2, NALCN, S100A12, and PROK2 were significantly different between the patients and healthy controls (**Figure 7A**). Among the patients, CCL8, IL1R2, and PROK2 were significantly up-expressed in fibrotic samples than non-

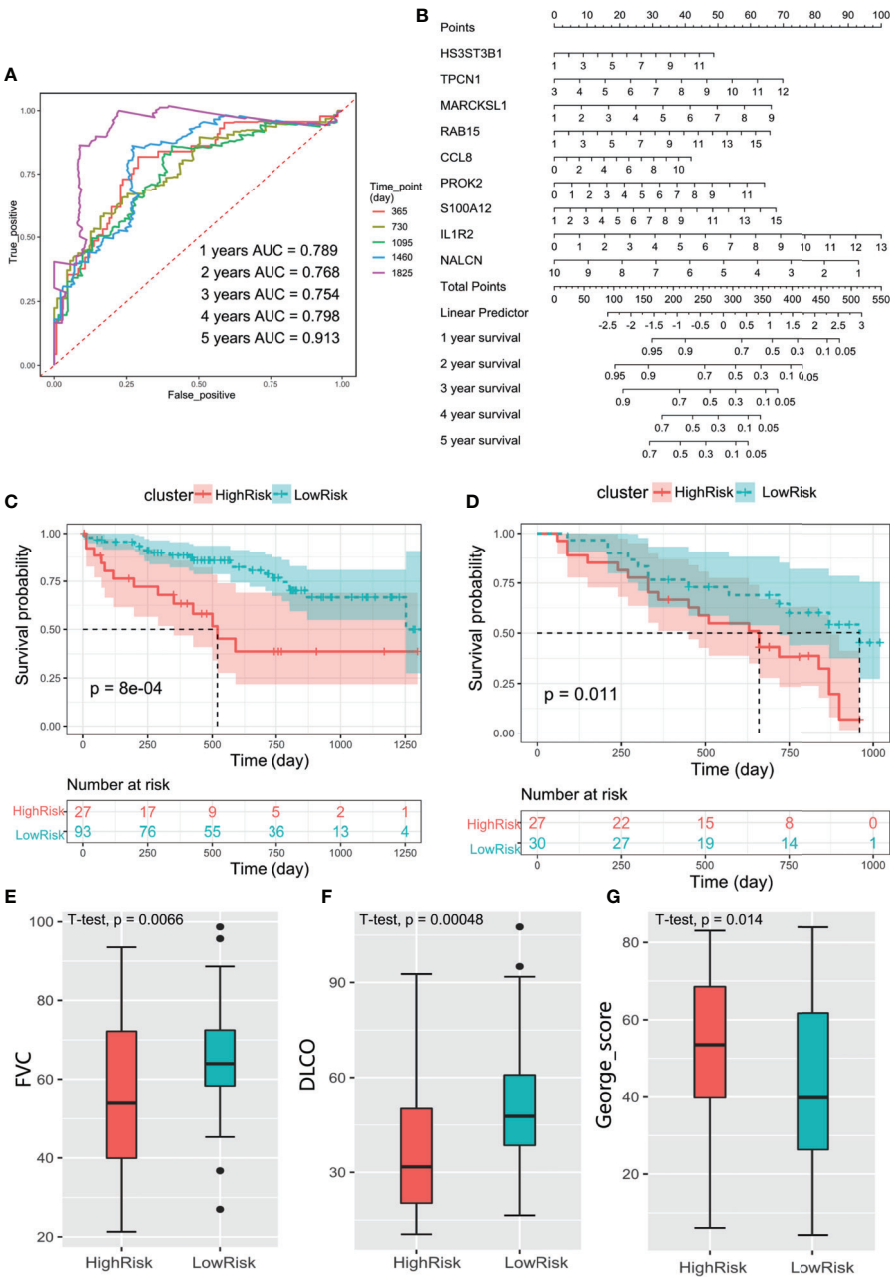

FIGURE 6 | **(A)** ROC curve evaluated the predictive value of the model for the prognosis of patients in discovery cohort within 1–5 years. **(B)** The nomogram for 1–5 years overall survival based on Cox model. **(C)** Kaplan–Meier plot of overall survival between high-risk and low-risk groups in GSE28221 validation cohort. **(D)** Kaplan–Meier plot of overall survival between high-risk and low-risk groups in GSE93606 validation cohort. **(E–G)** The box plot showed the difference of FVC, DLCO, and George's score between low-risk group and high-risk groups in GSE32537 validation cohort.

TABLE 4 | Basic information of IPF patients in validation cohort.

Characteristics	Whole cohort (296)	Low risk (182)	High risk (114)
GSE93606	Whole cohort (57)	Low risk (30)	High risk (27)
Gender			
Male	38 (0.667)	18 (0.600)	20 (0.741)
Female	19 (0.333)	12 (0.400)	7 (0.259)
Age			
≥65 years	35 (0.614)	18 (0.600)	17 (0.630)
<65 years	22 (0.386)	12 (0.400)	10 (0.370)
GSE32537	Whole cohort (119)	Low risk (59)	High risk (60)
Gender			
Male	42 (0.353)	25 (0.424)	17 (0.283)
Female	77 (0.647)	34 (0.576)	43 (0.717)
Age			
≥65 years	53 (0.445)	31 (0.525)	22 (0.367)
<65 years	66 (0.555)	28 (0.475)	38 (0.633)
GSE28221	Whole cohort (120)	Low risk (93)	High risk (27)
Gender			
Male	92 (0.767)	71 (0.763)	21 (0.778)
Female	28 (0.233)	22 (0.237)	6 (0.222)
Age			
≥65 years	76 (0.633)	55 (0.591)	21 (0.778)
<65 years	44 (0.367)	38 (0.409)	6 (0.222)

fibrotic samples (**Figure 7B**). The PBMC results of flow cytometry showed that the proportion of NK cells was increased significantly in the patients than healthy controls (**Figure 7C**), and among the patients' BALF, the proportion of NK cells was also increased significantly in fibrotic samples than in non-fibrotic ones (**Figure 7D**). The proportion of activated NK cells in BALF samples was significantly higher than those in PBMC samples, and it had the following characteristics: the increasing trend of peripheral blood to pulmonary bronchus (**Figure 7E**). The proportion of NK or activated NK cells' infiltration in BALF was positively correlated with patients A-aDO2 and hospital day, suggesting that high NK infiltration is a risk factor for poor prognosis (**Figures 7F, G**). In addition, the flow cytometry showed that the increasing CD4+ T cells in peripheral blood might promote the macrophage infiltration in pulmonary bronchus and promoted their polarization to M1-like phenotype (**Figure 7H**). The proportion of CD4+ T, activated CD4+ memory T cells, and M1-like macrophages infiltration in BALF or PBMC was positively correlated with patients' A-aDO2 (**Figures 7I–K**). **Figure 7L** showed the relationship between the results of BALF and PBMC. The detailed data of sample information and experimental results of BALF/PBMC were shown in the **Supplementary Materials**.

DISCUSSION

Since the prognosis of patients with IPF is poor, the importance of building a prognostic staging system for personalized treatment is self-evident. The prognostic staging system could divide the patients into several groups according to the given markers. In this study, we used the transcriptional profiles of the bronchoalveolar lavage fluid (BALF) to analyze the relationship between the level of biomarkers and the prognosis of patients. We found that both hypoxia and immune status were related to the survival and even respiratory function of patients with IPF. Furthermore, we established a new prognostic classifier including nine-gene signature for patients with IPF. It is effective in the prognosis of patients with IPF in the discovery cohort and three independent validation cohorts. These findings provide a new insight to the relationship between biomarkers in the lung milieu and the prognosis and stratification of patients with IPF.

Several articles reported the role of immune and hypoxia microenvironment in lung diseases (15). On one hand, immune dysregulation and inflammation are regarded as the basis of chronic lung diseases, including IPF (11). Bioinformatics analysis of RNA network and immune infiltration showed that immune cells were associated with the severity of IPF (21). Both innate and adaptive immunity were activated in fibrogenesis (22). On the other hand, hypoxia is common in lung disease. Hypoxia-inducible factor-1α (HIF-1α) is a key regulating factor in cell response to hypoxia, which has been found to participate in many lung diseases (23–25). In hypoxia, the activation of HIF-1α mediates glycolysis modification, angiogenesis, and other adaptive mechanism (26, 27). Hypoxia promoted the epithelia–mesenchymal transition (EMT) of alveolar epithelial cells (AECs) in IPF, and transforming growth factor β (TGF-β) also promoted EMT with increased lactic acid produced by metabolic modification (24, 28). Also, HIF-1α was found to be active in fibroblasts from IPF patients and induced myofibroblast differentiation with the existence of TGF-β (24, 26, 29, 30). It is worth mentioning that hypoxia facilitated proliferation and the secretion of proinflammatory cytokines in mast cells, and thus influenced fibrogenesis (31). In IPF, alveolar macrophages showed a perturbation of mitochondria homeostasis, including increased mitochondria reactive oxygen species (mtROS), in which HIF-1α may have participated (32). These findings were in accordance with the results. The result of infiltration of different

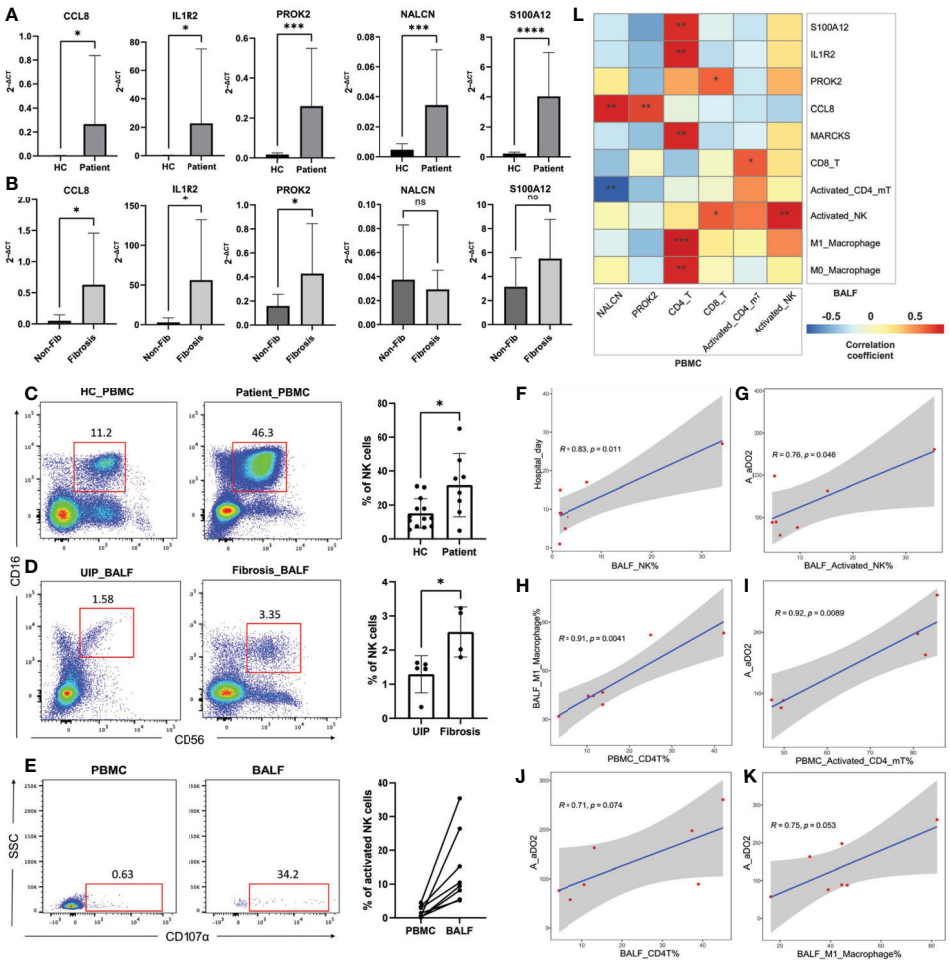

FIGURE 7 | **(A, B)** The plots showed the results of qRT-PCR. Plot A showed the difference of DEGs expression in PBMC between healthy controls (HC) and patients. Plot B showed the difference of DEGs expression in PBMC between non-fibrotic pneumonia group (Non-Fib) and pulmonary fibrosis group (Fibrosis). **(C–E)** CD56 and CD16 were used to identify NK cells in flow cytometry. $CD56^{+}CD16^{+}CD107a^{+}$ cells were defined as activated NK cells. Plot C showed the difference of NK% in PBMC between healthy controls (HC) and patients. Plot D showed the difference of NK% in BALF between usual interstitial pneumonitis (UIP, from Non-Fib group) and pulmonary fibrosis group (Fibrosis). **(F–K)** Pearson analysis showed the correlation between cell proportion and clinical features. **(L)** Pearson analysis showed the correlation between the results of BALF and PBMC. *, **, ***, **** respectively represent P values of t-test < 0.05, < 0.01, < 0.001, < 0.0001.

types of immune cells showed that both innate immunity and adaptive immunity were activated in $hypoxia^{high}/immune^{high}$ group, presenting poorer prognosis. In a word, hypoxia, as the inducement of immune activation, mediates chronic airway inflammation and leads to fibrosis. Then, cell and organ dysfunction caused by fibrosis aggravates the formation of hypoxic inflammatory microenvironment, which forms a feedback loop.

The result of CIBERSORT showed that the infiltration of M0 macrophage in $hypoxia^{low}/immune^{low}$ group was higher than $hypoxia^{high}/immune^{high}$ group. As inactive and naive macrophages, the low proportion of M0 macrophages in the low-risk group suggested a lower level of inflammatory activation (33). At the same time, the infiltration of CD8+ T cell, activated CD4+ memory T cell, activated NK cell, activated mast cell, and M1 macrophage in $hypoxia^{high}/immune^{high}$ group was higher than that in $hypoxia^{low}/immune^{low}$ group, presenting a higher level of inflammation. As was discussed above, the role of CD4+ T cell, mast cell, and M1 macrophage in fibrosis was widely reported. It is worth noting that M0 macrophage and NK cell had the most significant correlation with key hypoxia-immune DEGs and risk score. However, the role of CD8+ T cell, NK cell, and M0 macrophage in fibrosis has not been fully verified. These may provide a new idea to understand the characteristic immune cells in fibrotic infiltration.

Also, we found that hypoxia-immune DEGs were mainly risk DEGs, taking part in the integral component of membrane and immune response, and the protective DEG was a gene encoding voltage-independent, non-selective cation channel. The roles of these predictive signature genes have been reported previously in

lung diseases. Interleukin-1 receptor 2 (IL-1R2) is an anti-inflammatory cytokine. The increase level of IL-1R2 has been reported to be associated with poor prognosis in lung cancer (34, 35), and the increase level of IL-1 was associated with the development of chronic obstructive pulmonary disease (COPD) (36, 37). Another risk gene S100A12 is a novel inflammatory disease biomarker in acute respiratory distress syndrome (ARDS) (38), interstitial lung disease (ILD) (39), and COPD (40). Moreover, C-C Motif Chemokine Ligand 8 (CCL8) is a kind of monocyte chemoattractant, regulating group 2 ILCs in lung inflammation (41). These results were in accordance with the results in this article that the overexpression of IL-1R2, S100A12, and ILC2s may be predictive for poor diagnosis of IPF patients. NALCN gene encodes a voltage-independent, non-selective cation channel. Other signature genes are rarely reported in lung diseases. In this study, combing hypoxia and immune status, we identified these signature genes to provide new insights into the prognosis of IPF.

In particular, in the independent external validations of this study, the prognostic prediction model was performed in peripheral whole blood, peripheral blood mononuclear cell, and lung tissue of IPF patients, and the outcomes were significant. The preliminary clinical specimen validation suggested the reliability of most conclusions, but there are still limitations, such as insufficient sample size. And because of the individual differences and other confounding factors, the results based on the existing database must have some deviation from the reality. Although these results provided more possibilities and a wider application of this predictive model in clinical setting, a well-designed and multi-center study is needed for further exploration.

CONCLUSIONS

The immune and hypoxia status in alveolar molecular environment is associated with the prognosis of patients with IPF. The prognostic model based on several signature genes raised a new way to predict the progression and prognosis of IPF.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Gene Expression Omnibus (GEO, available at: https://www.ncbi.nlm.nih.gov/geo/) database (GSE70866).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board (Ethics Committee) of the 3rd Xiangya Hospital, Central South University (No. 21028). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The study was conceived and designed by QZhu. Statistical analyses were performed by XL and YC. Software package was prepared by QYZ and YD. Flow cytometry was performed by XL and HC. Manuscript was written by XL, HC, and QZhu. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants from the National Natural Science Foundation of China (81700658) and the Hunan Provincial Natural Science Foundation-Outstanding Youth Foundation (2020JJ3058).

ACKNOWLEDGMENTS

We thanked Ms. Hanying Liu from Department of Pulmonary Disease. the 3rd Xiangya Hospital of Central South University for supplying clinical samples.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.629854/full#supplementary-material

Supplementary Figure 1 | Kaplan–Meier plot of overall survival between hypoxia^{high}/immune^{high}, hypoxia^{low}/immune^{low}, hypoxia^{high}/immune^{low} and hypoxia^{low}/immune^{high} groups.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 15 June 2021
doi: 10.3389/fimmu.2021.661811

Enhanced IL-1β Release Following NLRP3 and AIM2 Inflammasome Stimulation Is Linked to mtROS in Airway Macrophages in Pulmonary Fibrosis

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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *31 January 2021*
Accepted: *21 May 2021*
Published: *15 June 2021*

Citation:
Trachalaki A, Tsitoura E, Mastrodimou S, Invernizzi R, Vasarmidi E, Bibaki E, Tzanakis N, Molyneaux PL, Maher TM and Antoniou K (2021) Enhanced IL-1β Release Following NLRP3 and AIM2 Inflammasome Stimulation Is Linked to mtROS in Airway Macrophages in Pulmonary Fibrosis. Front. Immunol. 12:661811. doi: 10.3389/fimmu.2021.661811

Fibrotic Interstitial lung diseases (ILDs) are complex disorders of variable clinical behaviour. The majority of them cause significant morbidity, whilst Idiopathic Pulmonary Fibrosis (IPF) is recognised as the most relentless. NLRP3, AIM2, and NLRC4 inflammasomes are multiprotein complexes driving IL-1β release; a proinflammatory and profibrotic cytokine. Several pathogenetic factors associated with IPF are identified as inflammasome activators, including increases in mtROS and bacterial burden. Mitochondrial oxidation and alterations in bacterial burden in IPF and other ILDs may lead to augmented inflammasome activity in airway macrophages (AMs). IPF (n=14), non-IPF-ILDs (n=12) patients and healthy subjects (n=12) were prospectively recruited and AMs were isolated from bronchoalveolar lavage. IL-1β release resulting from NLRP3, AIM2 and NLRC4 inflammasomes stimulation in AMs were determined and baseline levels of mitochondrial ROS and microbial burden were also measured. Our results showed that NLRP3 was more inducible in IPF and other ILDs compared to controls. Additionally, following AIM2 activation IL-1β release was significantly higher in IPF compared to controls, whereas similar trends were observed in Non-IPF-ILDs. NLRC4 activation was similar across groups. mtROS was significantly associated with heightened NLRP3 and AIM2 activation, and mitochondrial antioxidant treatment limited inflammasome activation. Importantly, microbial burden was linked to baseline IL-1β release and *AIM2* and *IL-18* relative expression independently of mtROS. In conclusion, the above findings suggested a link between the overactivation of NLRP3 and AIM2 inflammasomes, driven by mitochondrial oxidation, in the pathogenesis of lung fibrosis while changes in the microbiota may prime the inflammasome in the lungs.

Keywords: IPF – idiopathic pulmonary fibrosis, ILD, NLRP3, AIM2, NLRC4, mtROS, mitochondrial reactive oxygen species, microbiome

INTRODUCTION

Interstitial Lung Disease (ILD) is a broad term used to describe multiple complex disorders with different aetiologies and disease behaviours. ILDs are pulmonary fibrotic disorders that affect the lung alveoli causing distraction of the lung parenchyma. The classic paradigm is Idiopathic Pulmonary Fibrosis (IPF), a devastating chronic lung disease of unknown aetiology (1). An interplay of age dependent deregulation of host defence pathways, immune cell homeostasis and microbiome balance is currently speculated to be the underlying cause of the disease (2). Other ILDs are considered to be driven mostly through inflammatory pathways (2).

In the last decade, the landscape of the IPF behaviour has changed with the discovery of two novel antifibrotic drugs (3–5). Of note, the two antifibrotic treatments are similarly effective in other progressive ILDs (6–8). This suggests progressive fibrosis arises from common underlying mechanisms, despite the initial cause, but the exact pathways remain to be identified (9).

Macrophages and monocytes are recognised as drivers of the balance between tissue repair and fibrosis (10). Recent studies in ILD patients have characterised Airway Macrophages (AM) subpopulations which may contribute to different subtypes of pulmonary fibrosis (11). Macrophages, as a source of chemokines and other inflammatory mediators, are responsible for the initial cellular response to injury (12) and can initiate or exacerbate fibrosis (10).

The inflammasome is one central cellular mechanism for chemokine release in macrophages (13). Inflammasomes are cytosolic multiprotein complexes that act as innate immune system sensors. NLRP3, the most studied inflammasome, is activated by a variety of stimuli including ATP, nigericin and ROS (14). Other inflammasomes are more specific and activated by merely one stimulus; NLRC4 by cytosolic Flagellin and AIM2 by cytosolic double stranded DNA (dsDNA) (15). Upon triggering, Inflammasomes cleave pro-IL-1β and pro-IL-18 to their active forms mainly through caspase-1 (15). IL-1β is a potent pro-inflammatory cytokine that can initiate and amplify lung inflammation and has been associated with acute lung injury and fibrosis (16–18). Inflammasome activation has been associated with silicosis, asbestosis and pulmonary fibrosis, mostly in mice models (19–21).

Mitochondria as drivers of metabolic cascades are increasingly recognised as controllers of macrophage activation status (22–25). Mitochondrial reactive oxygen species (mtROS) and oxidised mitochondrial DNA (mtDNA) are both critical for NLRP3 activation (25, 26). It is established that mitochondria homeostasis is defective in aged and IPF individuals (27, 28) and their dysfunction contribute directly to fibrosis in mouse models (29). Our group and others have recently demonstrated that mitochondria in AMs are more oxidised in IPF and other ILDs compared to healthy individuals (30–32).

Microbiota signalling has been shown to alter mitochondrial metabolism and activate the inflammasome (33–35). In IPF, the microbial burden is increased, and its composition is altered (36, 37). Similarly in Chronic Hypersensitivity Pneumonitis (CHP), bacterial burden is higher, albeit at lower levels compared to IPF (38). These alterations have been linked to host immune response transcriptional changes (39) and variable cytokine secretion (40). Of note, streptococcal infection leads to acute exacerbation of lung fibrosis in mice through AIM2 inflammasome activation (41) while inflammasome activation was dysregulated in BAL cells from ILD patients (42).

In this study we hypothesised that alterations in the lung microenvironment including microbiome changes and mitochondrial dysfunction in AMs could drive excessive inflammasome activation with possible implications in the pathogenesis of ILD disease. As such, we sought to determine whether increased mtROS and microbial burden were associated with inflammasome activity in AMs from ILDs, including IPF.

MATERIALS AND METHODS

Patients and Inclusion Criteria

Thirty-six (34) patients were prospectively enrolled at the Respiratory Medicine Department at the University Hospital of Heraklion, Crete, between June 2017 to June 2019.

Twelve (12) healthy controls, and twenty-four (24) ILD patients [fourteen (14) patients with IPF and twelve (12) non-IPF patients] were included. Patients with a recent infection (1 month prior to bronchoscopy) were excluded from the study. ILD patients were treatment naïve. All patients underwent bronchoscopy and Bronchoalveolar Lavage Fluid (BALF) was obtained as part of the diagnostic algorithm.

IPF patients: The diagnosis of IPF was based on either ATS/ERS criteria or the Fleischer Society criteria after Multidisciplinary discussion (22).

Non-IPF patients: Patients with fibrotic Interstitial Lung Diseases as assessed by the presence of reticulation and traction bronchiectasis on High-Resolution Computed Tomography were included. This category included eight (8) patients with fibrotic chronic hypersensitivity pneumonitis (CHP), one (1) with asbestosis related ILD, one (1) with Idiopathic pneumonia with autoimmune features (IPAF) and two (2) with unclassifiable ILD. Any patients with autoimmune diseases treated with immunosuppressive agents were excluded as immunomodulatory treatment could affect inflammatory responses. All patients were evaluated within one month from bronchoscopy, with Pulmonary Lung Function tests.

Control group: Control subjects were either patients undergoing bronchoscopy for the investigation of haemoptysis, without any overt pulmonary comorbidities, with normal bronchoscopy findings and cytology results or healthy volunteers.

Patient demographics, smocking status, and pulmonary function tests (PFTs) were prospectively collected and are summarised in **Table 1**. Since controls were healthy Pulmonary Function Test (PFTs) were not performed.

Abbreviations: AECIIs, alveolar epithelial cells; AIM2, Absent in melanoma 2; AMs, Airway Macrophages; ARDS, Acute Respiratory Distress Syndrome; CHP, chronic hypersensitivity pneumonitis; BALF, Bronchoalveolar Lavage Fluid; dsDNA, double stranded DNA; IPF, Idiopathic Pulmonary Fibrosis; LPS, lipopolysaccharide; NLRC4, NLR Family CARD Domain Containing 4; NLRP3, NOD-, leucine rich region- and pyrin domain-containing-3; NLR, NOD-Like receptor; mtROS, Mitochondrial Reactive Oxygen Species; mtDNA, mitochondrial DNA; ROS, Reactive Oxygen Species.

TABLE 1 | Patient characteristics.

	Control	IPF	Non-IPF	
n	12	14	12	
Age	56.9 ± 14	74.9 ± 6	69 ± 11	P<0001
Male/Female	8/4	12/2	8/4	P ns
Smoking status				P ns
Never smokers	1	2	4	
Smokers	11	13	8	
Pack years	38.1 ± .33.8	40.2 ± 17.4	37.5 ± 19.5	P ns
FVC%		87 ± 21	90 ± 26	P ns
FEV1%		95 ± 19	95 ± 27	P ns
DLCO%		65 ± 21	58 ± 22	P ns
TLC%		81 ± 17	82.8 ± 26	P ns
Macrophages%	93 ± 5	88 ± 5	s89 ± 8	P ns
Lymphocytes%	6 ± 4	6 ± 5	4 ± 3	P ns
Neutrophils%	2 ± 1.5	3 ± 2	4 ± 4	P ns
Eosinophils%	0.4 ± 0.2	1.4 ± 1.3	1.4 ± 1.8	P ns

ns, non significant.

PFTs. Lung volumes (forced expiratory volume in one second – FEV1, forced vital capacity – FVC), and diffusion capacity (DLco, corrected for haemoglobin) were measured using the computerised system (Jaeger 2.12; MasterLab, Würzburg, Germany). Predicted values were obtained from the standardised lung function testing of the European Coal and Steel Community, Luxembourg (1993).

AM Isolation and Culture

Freshly isolated BALF cells were obtained as previously described (30). BALF contains a diverse population of primarily macrophages and variable levels of neutrophils, lymphocytes and eosinophils. To allow macrophage enrichment, BAL cells were cultured for 1 hour prior to experimentation. For each experimental condition $0.5x10^6$ BALF cells were allowed to attach for 1 hour in 24 well plates in DMEM(Biosera) growth media supplemented with 2% FCS (Biosera) and 1x concentration of penicillin-streptomycin (from 100x concentrated solution, Biosera) in a humidified incubator at 37°C containing 5% CO2 at a concentration of 10^6 cells/ml, with subsequent washes to remove non-adherent cells. The remaining attached cell population comprised mainly of macrophages and monocytes from the alveolar space.

Inflammasomes Activation

Human macrophages are known to require a two-step mechanism to activate NLRP3; an evolutionary process preventing uncontrolled NLRP3 activation and IL-1β release. The primary TLR-mediated signal activates NF-kappaB (NF-κB) which results in NLRP3, ASC, pro-IL-1β and pro-IL-18 transcription. The second signal results in proteolytic cleavage of IL-1β and IL-18 by caspase-1 (43).

Here, AMs were primed with 10ng/ml LPS (Liposacccharide E.Coli 0111:B4 strain, Invivogen) to upregulate inflammasome related genes. For NLRP3 activation, cells were primed with LPS for 2 hours and subsequently stimulated with 5 mM ATP(Sigma) for 30 minutes. For the activation of AIM2 and NLRC4 inflammasomes, cells were primed with LPS for 1 hour, followed by transfection with 2 μg/ml dsDNA(naked Poly(dA:dT), InvivoGen) or 0.1ug/ml Ultrapure Flagellin from S. Typhimirium, (InvivoGen) respectively, using Lipofectamine 2000 (Life Technologies) for 4 hours. Inflammasome-specific activation was confirmed using the selective NLRP3 inhibitor MCC950(Cayman) at 1μM, or the caspase-1 inhibitor for the NLRC4 and AIM2 inflammasome (non-selective global inflammasome inhibitor) at 10uM final concentrations, for 1 hour prior to stimulation. The simulation protocol is summarised in **Supplementary Figure 1**.

For the study of mtROS effect on NLRP3 activation, cells were treated with the selective mitochondrial antioxidant agent MitoTempo (Sigma) at a final concentration of 100μM for 1 hour prior to the addition of ATP.

Supernatants and cell lysates were collected after the appropriate stimulation time. ELISA immunoblot was used to quantify IL-1β, as a surrogate marker of inflammasome activation.

Flow Cytometry and mtROS Determination

Mitochondrial ROS was measured by MitoSOX^{TM}Red (Invitrogen) staining. $0.5x10^6$ freshly isolated BALF cells resuspended in RPMI-1640, supplemented with 2% FCS, were stained with MitoSOX Red at a final concentration of 5 μM and CD45-FITC for 10 minutes at 37°C. For MitoSOX staining quantification, AMs populations were selected according to high forward and side scatter and CD45 ($FSC^{high}SSC^{high}CD45^+$) as previously described (30). Identical cell samples were independently stained with Propidium Iodide (PI) at a final concentration of 1ng/ml, for 5 minutes immediately before flow cytometry analysis, for the detection of necrotic/apoptotic cells. The percentage of MitoSOX positive cells was determined by the percent of cells showing FL-2 fluorescence higher than the unstained control, followed by subtraction of the PI positive percentage of cells. Relative mean fluorescence intensity (MFI) was calculated by normalizing the MFI of the FL-2 channel/MitoSOX positive cells by the MFI of the FL-2 channel of the unstained cells since patient samples displayed wide ranges of autofluorescence. (Data were acquired from Beckman Coulter flow cytometer and analysed with FlowJo 8.7).

Bacterial DNA Extraction and 16S rRNA Gene qPCR

Bacterial DNA extraction and 16S rRNA gene qPCR was performed for quantification of bacterial burden as previously described (36).

Total RNA Extraction and mRNA Expression

1-1.5x10^6 BALF cells were centrifuged and cell pellets were homogenised in TriReagentTM (MBL) for total RNA extraction, followed by storage at -80°C. Total RNA extraction, cDNA synthesis and real-time PCR were performed as previously described (24). GAPDH levels were used as endogenous control for the normalization of mRNA expression levels in BAL samples. Primer sequences are shown in **Supplementary Table 1**.

Statistical Analysis

Data were analysed using SPSS 25 (IBM) software and graphs were produced using GraphPad Prism 8. Comparisons were made with paired or unpaired student's *t*-test, when appropriate. All data were expressed as means with interquartile range unless stated otherwise. Receiver operating characteristics (ROC) curve analysis was used to select an optimal cut point for mtROS. Spearman's correlation coefficient (*r*) analysis measured the association between two variables. A *p* value less than 0.05 was considered statistically significant (*p<0.05, **p<0.01, ***p<0.001).

Ethics Approval and Patient Consent

The study was approved by the Ethics Committees of the University Hospital of Heraklion (IRB number: 5889). Approval for the study in the UK was obtained from the local research ethics committee (15/SC/0101 and 15-LO-1399). All patients provided written informed consent.

RESULTS

BALF Baseline IL-1β and Inflammasome Components Gene Expression Is Similar in ILDs and Healthy Subjects

Initially, the baseline IL-1β levels were measured in the BAL Fluid (BALF), as a marker of inflammasome pre-activation in the lung microenvironment. Median basal IL-1β concentration in the BALF did not differ significantly between the ILDs and control groups, while some sporadic IL-1β release was detectable in all three groups (**Figure 1A**). IL-1β secretion by unstimulated freshly isolated AMs was also measured and no differences were observed among the groups (**Figure 1B**). Furthermore, we assessed the mRNA expression of genes encoding core inflammasome components *NLRP3*, *NLRC4*, and *AIM2* as well as the gene encoding *IL-18* in BALF cells (**Figures 1C–F**). *NLRP3* relative mRNA expression was significantly elevated in Non-IPF ILDs (p=0.03) and tended to be elevated in IPF as well (p=0.1). *AIM2*, *NLCR4* and *IL-18* expression was similar between groups.

AMs in ILDs Exhibit Enhanced NLRP3 Inflammasome Activation

Typically, monocytes/macrophages require two independent simulation signals to secrete IL-1β *in vitro*; an NF-κB priming step (signal 1), such as LPS or other TLR4 agonists, which leads to the upregulation of inflammasome pathway related genes, followed by a stimulation with a DAMP or PAMP which acts as a second stimulus (signal 2) and leads to robust IL-1β release. Signal 1 alone is known to activate the NLRP3 in a non-canonical way in human myeloid cells (44, 45).

In this study, for NLRP3 inflammasome activation, AMs were primed with LPS followed by ATP stimulation. Treatment with LPS resulted in a significant release of IL-1β by AMs from all groups (**Figure 2A**), although no differences between groups were noted (**Figure 2A**). Upon NLRP3 activation, IPF and non-IPF AMs produced excessive IL-1β compared to controls (p=0.0004 and 0.007 respectively, **Figure 2B**). Between IPF and Non-IPF patients, NLRP3 activity was similar. This effect was NLRP3 specific, as it was abrogated by MCC950 treatment (**Figure 2B**), a novel specific NLRP3 inhibitor (46).

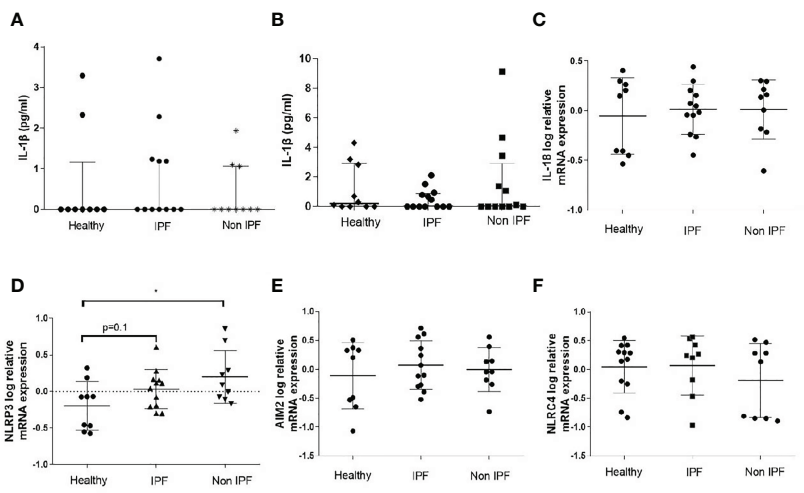

FIGURE 1 | Baseline characterization of the Inflammasome in BALF cells. **(A)** IL-1β protein concentration in BAL fluid as measured by ELISA and **(B)** IL-1β protein release by unstimulated AMs (5-hour culture). Data represented as median with interquartile range Mann-Whitney test. Relative log mRNA expression in unstimulated BALF cells **(C)** *IL18* **(D)** *NLRP3*, **(E)** *AIM2*, **(F)** *NLRC4*. Data represented as mean ± SD, t-test. *p<0.05.

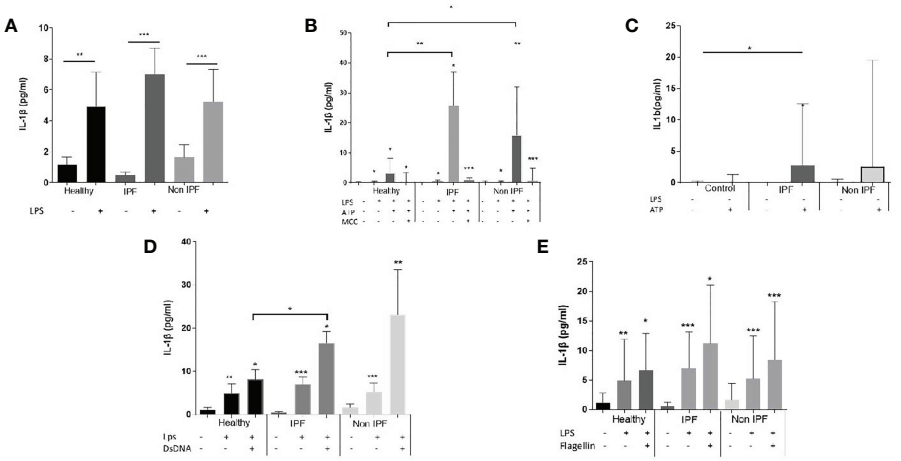

FIGURE 2 | Assay of inflammasomes activation in Airway Macrophages (AMs). **(A)** AMs treated for 5 hours with LPS **(B)** Activation of NLRP3 upon priming with LPS for 2 hours and ATP for 30minutes. Cells were also treated in presence of the Selective NLRP3 inhibitor MCC950 to show specificity of the NLRP3 activation, **(C)** AMs cultured for 2 hours in serum and treated for 30 minutes with ATP without priming with LPS **(D)** AIM2 inflammasome activation: 1 hour of LPS priming followed by 4 hours dsDNA transfection, **(E)** NLRC4 inflammasome activation: 1 hour of LPS priming followed by 4 hours flagellin transfection. For with-in group analysis one-sided paired Wilcoxin test was used. For between group analysis Mann-Whitney test was used. Data presented as median with interquartile range, *p<0.05, **p<0.005, ***p<0.0001.

To determine whether ILD derived AMs were pre-primed by an NF-κB related stimuli in the lungs, we also treated cells with ATP (with no proceeding signal 1). Generally, ATP alone resulted in muted responses compared to LPS/ATP. However, in IPF the addition of ATP was sufficient to generate IL-1β release, compared to healthy controls (p=0.04, **Figure 2C**). Similar trends were noted in non-IPF ILDs (p=0.1, **Figure 2C**). When all ILDs were combined, ATP resulted in increased NLRP3 activation compared to controls (p=0.04).

AIM2 and NLRC4 Activation

For AIM2 activation, cells were primed with LPS followed by transfection with dsDNA. In all three groups the addition of dsDNA resulted in significant IL-1β release (**Figure 2D**). IL-1β production following dsDNA stimulation was significantly elevated in IPF compared to healthy controls (p=0.04) whilst a similar trend was observed for the Non-IPF patients (p=0.09) (**Figure 2D**).

It was recently suggested that in human myeloid cells, in contrast to mice, dsDNA activates the cGAS-STING pathway. This action potentiates lysosomal damage and in turn activates the NLRP3 rather than the AIM2 inflammasome (47). To address this possibility, cells were treated with dsDNA in the presence of MCC950 and a significant inhibition of IL-1β release was observed (**Supplementary Figure 2A**). To test further the above hypothesis of STING-mediated lysosomal damage, cells were treated with chloroquine, a lysosomal acidification inhibitor that blocks lysosomal induced cell death. Chloroquine inhibited dsDNA-mediated inflammasome activation (**Supplementary Figure 1A**).

For the activation of NLRC4, cells were primed with LPS, followed by flagellin transfection. The activation of NLRC4 inflammasome was similar across groups (**Figure 2E**). To confirm the specificity of NLRC4 activation in AMs, both the pan-inflammasome Caspase-1 inhibitor and the NLRP3 specific inhibitor-MCC950 were tested. NLRC4 stimulation resulted in IL-1β release in a caspase-1 dependent and NLRP3-independent manner (**Supplementary Figure 2B**).

NLRP3 and AIM2 Activation Is Associated With Mitochondrial Oxidation and Can Be Inhibited by Antioxidant Treatment

MtROS is widely recognised as an inducer of inflammasome activation and it was previously shown that mtROS is elevated in IPF and Non-IPF AMs (30, 32). It was therefore hypothesised that elevated mtROS could be associated with higher NLRP3 inflammasome activity in ILDs. The levels of mtROS in fresh untreated AMs was measured by flow cytometry using MitoSOX^{TM}red. MtROS was higher in ILD-AMs (IPF and Non-IPF) compared to controls (p=0.03, **Figure 3A**).

Using ROC curves the optimal cut-off value for mtROS level was determined and ILD-AMs samples were stratified according to high or low mtROS. AMs exhibiting high mtROS showed enhanced NLRP3 activation in the absence of LPS priming (p=0.004, **Figure 3B**) and there was also a trend for greater NLRP3 activation in LPS-primed AMs (p=0.08) (**Figure 3C**). AIM2 activation was likewise significantly heightened in AMs with high mtROS (p= 0.02) (**Figure 3D**).

Interestingly, *in vitro* stimulation of AMs with LPS and ATP coincided with a burst of mtROS (**Figures 4A, B**). We subsequently sought to determine whether antioxidant treatment could inhibit IL-1β release by AMs. Treatment with mitoTempo a mitochondria-targeting antioxidant significantly inhibited mtROS accumulation (**Figures 4A, B**) and blocked NLRP3 activation, as assessed by IL-1β release (**Figure 4C**).

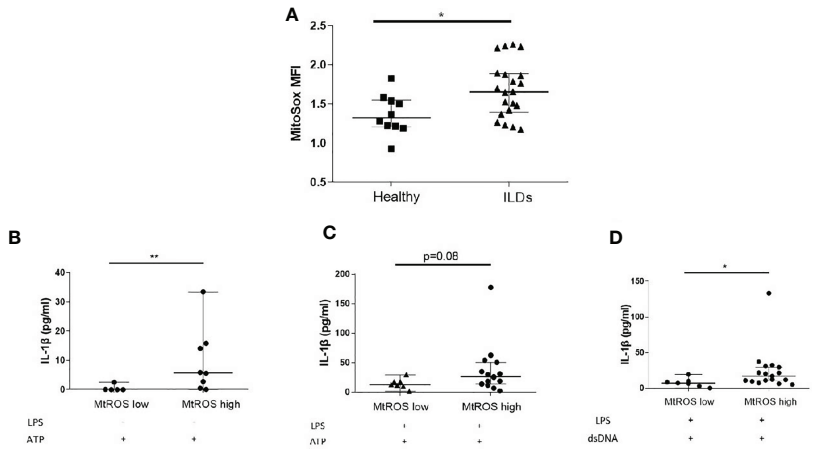

FIGURE 3 | Mitochondrial Oxidation status and the inflammasome. **(A)** MtROS Mean Florescence Intensity Index (MFI) as assessed by flowcytometry in healthy and ILD patients. ILD Patients were categorised according to MtROS MFI using the cut-off value of 1.5(generated by ROC curve). Comparisons on inflammasome activation were made in MtROS high and low groups **(B)** NLRP3 activation without LPS priming, **(C)** NLRP3 activation with LPS priming, **(D)** AIM2 activation (LPS priming followed by dsDNA transfection). Data presented as median with interquartile range, *p<0.05, **p<0.005, Mann-Whitney test.

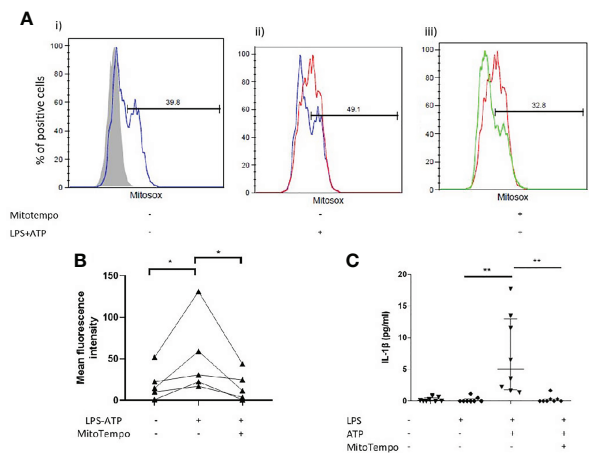

FIGURE 4 | Mitochondrial antioxidant treatment effect on inflammasome activation. AMs were treated with MitoTempo (100μM), a mitochondrial antioxidant for 1 hour prior to the addition of ATP. Cells were subsequently treated with ATP for 30minutes. **(A)** Representative histograms of freshly isolated AMs from ILD subjects, labelled with CD45-FITC and MitoSOX Red analysed by flowcytometry. AMs were selected and percentage of MitoSOX positive cells and mean fluorescence intensities were analysed relative to CD45-FITC labelled populations (grey histograms). AMs were either left untreated(i), treated with (ii) LPS/ATP for NLRP3 activation or (iii) MitoTempo and LPS/ATP. **(B)** MitoSox (assessed by flow-cytometry) and **(C)** IL-1β release measured at baseline, after NLRP3 activation and after NLRP3 activation in the presence of MitoTempo (100μM), Data presented as median with interquartile range, *p<0.05 **p<0.005, Paired Wilcoxon test.

Microbial Burden Is Associated With Baseline *AIM2* and *IL-18* Gene Expression and Il-1β Release

In IPF, it is established that microbial burden is increased and this correlates with disease progression (36, 37). In CHP, microbial burden is also elevated compared to health but at lower levels compared to IPF (38). We hypothesised that microbiota fluctuations in the lung microenvironment could be priming the inflammasomes. BALF microbial burden, as assessed by 16S rRNA gene copies, increased in ILDs relative to healthy individuals (p=0.03) (**Figure 5A**). Furthermore, 16S rRNA gene copies significantly correlated with IL-1β secretion in unstimulated AMs (R^2:0.53; p=0.02)(**Figure 5B**) and *AIM2* (R^2: 0.68 p=0.004, **Figure 5C**) and *IL-18* (R^2: 0.59 p=0.015, **Figure 5D**) relative mRNA expression. There was no association between bacterial burden and mtROS.

DISCUSSION

In this study we demonstrated that AMs in pulmonary fibrosis secrete abundantly IL-1β following stimulation of the NLRP3 and AIM2 inflammasomes compared to healthy subjects. Several factors that could be involved in inflammasomes stimulation have been previously identified in IPF, including increased mtROS and increased microbiome burden. This study showed that higher mtROS was associated with pronounced NLRP3 and AIM2 activation which could be inhibited by antioxidant therapy. Furthermore, BALF microbial burden correlated with baseline IL-1β production, *AIM2* and *IL-18* relative mRNA expression.

The overactivation of the inflammasome has multiple implications in health and disease. Several animal model studies have suggested that NLRP3 activation and subsequent IL-1β release can induce acute lung injury and fibrosis (48–50). In mice models the NLRP3/IL1-b axis is required for the development of bleomycin induced fibrosis (49). In silicosis there is a clear link between NLRP3 and the development of the disease (51). Furthermore, in mice age-dependent mitochondrial dysfunction results in enhanced NLRP3 activation and lung fibrosis (20). In this

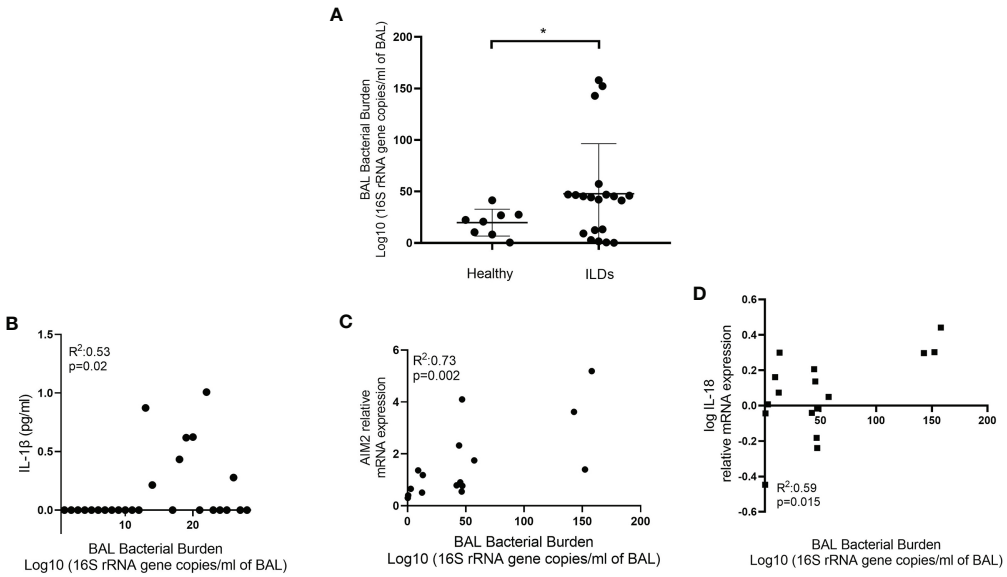

FIGURE 5 | Bacterial burden and the inflammasome. **(A)** Bacterial burden in bronchoalveolar lavage (BAL) of healthy and ILD subjects. Bacterial burden calculated by qPCR and expressed as log10 (16S rRNA gene copies/ml of BAL). Mann-Whitney test. Data are presented as median and interquartile range, *p<0.05. Illustrating correlation between bacterial burden and **(B)** IL-1β release from AMs (Spearman correlation) and **(C)** *AIM2* and **(D)** *IL-18* log relative mRNA expression (Pearson Correlation).

study we provide evidence of an implication of NLRP3 and AIM2 inflammasome in patients with lung fibrosis.

ROS generation by the mitochondria is a well-known trigger of the NLRP3 with mitochondrial death and mitochondrial DNA acting as activators (25). Mitochondrial dysfunction has emerged as a driver of IPF pathogenesis. Mitochondria in IPF AMs have morphological defects and are oxidised (30). Similarly, mtROS is elevated in other ILDs (32). To the best of our knowledge this is the first study to show that in ILD-AMs NLRP3 and AIM2 inflammasome are hyper-inducible and this is associated with mitochondrial oxidation, a hallmark of IPF pathogenesis. In agreement with previous studies (26), we showed that mtROS is crucial for NLRP3 inflammasome activation in patient derived-AMs, since antioxidant treatment inhibits IL-1β release.

It is established that a priming step is required for the activation of the inflammasome which results in the overexpression of several inflammasome components (43). The second signal results in the release of active IL-1β. ROS can act as a direct NLRP3 activator (52), while other studies have suggested that ROS could also exert its role at the priming step (24). Of note, treatment with ATP without LPS pre-stimulation resulted in pronounced IL-1β release in ILDs compared to controls. Furthermore, inflammasome was more inducible in patients with higher mtROS levels, in the absence of LPS priming. This implies that mitochondrial oxidation may be priming the inflammasome in lung fibrosis.

Mitochondria are in the core of metabolic switches in macrophages fighting bacterial infections (53) and a burst of mtROS is crucial for their antibacterial responses (51). By contrast, mitochondrial damage results in deregulated and diminished antioxidant responses to bacteria (54). It is recognised that microbiota are altered in IPF (55, 56). In this study, microbial burden, which is known to be increased in ILDs, was not associated with mtROS, indicating that microbiota changes were not likely the cause of the observed mitochondrial oxidation. The microbial burden was however associated with baseline IL-1β release as well as *AIM2* and *IL-18* mRNA expression in lung fibrosis.

AIM2 inflammasome was recently linked to the pathogenesis of lung fibrosis and progression. Notably, AIM2 is overexpressed in IPF-AMs and this is related to increased Drosha ribonuclease III (DROSHA), a class 2 ribonuclease III enzyme expression (57). Interestingly, a previous study showed that in IPF, peripheral mononuclear cells stimulated to activate the AIM2 released high concentrations of pro-fibrotic mediators and most importantly IL- 1α (58). Of particular interest, GLUT-1 dependent glycolysis promotes exacerbation of lung fibrosis during S. pneumoniae infection *via* AIM2 activation (41) and several studies have suggested a relative abundance of Streptococcus genera in IPF (37, 38, 56). A novel finding of our study is that AIM2 activation is increased in IPF and tended to be higher in other fibrotic ILDs. AIM2 activation was enhanced in patients with higher mtROS and an increase in the bacterial burden was associated with baseline *AIM2* expression

For years, AIM2 was recognised as the central DNA-responding inflammasome. Recent evidence suggests that AIM2 might not be functional in human immune cells in contrast to murine models (47). Researchers showed that cytosolic DNA causes lysosomal damage and activation of the NLRP3 inflammasome through the STING mediated cell death pathway. In our experiments, addition of dsDNA resulted in significant release of IL-1β in all groups. Inhibition of NLRP3 with MCC950,

a specific NLRP3 inhibitor, resulted in partial reduction of IL-1β release, as such suggesting that dsDNA activation is to a degree NLRP3-dependent. We also showed that higher mtROS was associated with higher IL-1β production following dsDNA treatment, a result which could be driven by NLRP3 activation rather than AIM2.

In contrast to NLRP3 and AIM2, NLRC4 activation was similar across groups. NLRC4 activation was caspase-1 dependent and NLRP3 independent. A previous transcriptional study in IPF, showed that NLRC4 expression is increased in the peripheral blood and highly associated with increased microbial burden in the lungs (39). Here we focused on AMs and failed to notice overexpression or overactivation of the NLRC4 inflammasome either at baseline or upon stimulation.

Our study sheds light into the pathogenesis of acute exacerbations, a well-recognised complication of IPF and other ILDs (59, 60), characterised by rapid deterioration and death. The pathogenesis of acute exacerbations is not well characterised but is thought to resemble acute lung injury in response to infections or sterile insults (60, 61). Microbiota changes have been implicated in the pathogenesis of IPF exacerbations (37, 56, 62). It is well established that dysregulated NLRP3 inflammasome activity results in uncontrolled inflammation and can cause acute lung injury and fibrosis (18, 50). Intriguingly, a transcriptomic study identified NLRP3 and IL-1β among the top up-regulated genes in AE-IPF (63).

This study indicates that in ILDs NLRP3 and AIM2 can be overactivated. Basal *AIM2* and *IL-18* mRNA expression and IL-1β production was correlated with the bacterial burden in the BALF. Although, it is difficult to prove a direct causal relationship between microbial burden and inflammasome activation, we speculated that microbiota changes prime the inflammasome in the lungs. It is established that microbiota dysbiosis influence systematic immune responses (64) and gut-microbiota changes shape cytokine release from leukocytes (65). More specifically in IPF, disruption of the lung microbiome was associated with variable cytokine production leading to lung inflammation and fibrosis progression (40). Targeting NLRP3, AIM2 or their ultimate effector, IL-1β and IL-18 (66) may prove a novel treatment for ILD exacerbations, which are still considered lethal in most cases.

The main limitation of this study is related to the small number of patients recruited, as differences among different types of lung fibrosis were not established. However, even in this small cohort, we were able to identify changes in lung fibrosis compared to health. Furthermore, different macrophage/monocyte populations exist in the lungs especially following a fibrotic insult (67). It is likely that one or more subpopulations drive the observed excessive inflammasome activation. As such, separation of the different AM populations and subsequent stimulations might be informative of the role of each subpopulation in the disease pathogenesis. One further limitation of our study is the measurement of IL-1β alone and not IL-18 as a marker of inflammasome stimulation. We opted to measure only IL-1β as it is considered a robust marker of inflammasome activation and has generally been associated with initiation and progression of fibrosis. Importantly, IL-1β is not constitutively expressed under homeostasis (68) and has detrimental effects on epithelial cells if over-released (69). Finally, although inflammasome activation in lung fibrosis was increased, we cannot determine whether this plays a causal role in disease progression, nor can we exclude that other factors may relate to the increased inflammasome activation observed here.

In conclusion, this is the first study to show that NLRP3 and AIM2 activation is heightened in ILD-AMs and this is linked to mitochondrial oxidation. Microbial burden is associated to some extent with pre-activation of the inflammasome. Our findings provide evidence of excessive AM-inflammatory response upon activation which could contribute to the progression of fibrosis. Targeting the inflammasome pathway may prove a novel strategy for ILD treatment or a rescue therapy during acute exacerbations of the disease.

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 study was approved by the Ethics Committees of the University Hospital of Heraklion (IRB number: 5889). Approval for the study in the UK was obtained from the local research ethics committee (15/SC/0101 and 15-LO-1399). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception and design: AT, ET, KA. Analysis interpretation and drafting the manuscript: AT, ET, KA. TM, PM. Technical support and interpretation of the data: AT, ET, SM, RI, EV, EB. All authors critically revised the manuscript and take responsibility for the data presented. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a Hellenic Thoracic Society Research Award (AT, KA HTS 2017). AT is the recipient of an ERS fellowship STFR (STRTF201904-00633).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.661811/full#supplementary-material

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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

frontiers in Immunology

ORIGINAL RESEARCH
published: 08 July 2021
doi: 10.3389/fimmu.2021.692127

Targeting TL1A/DR3 Signaling Offers a Therapeutic Advantage to Neutralizing IL13/IL4Rα in Muco-Secretory Fibrotic Disorders

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Mucus secretion is an important feature of asthma that highly correlates with morbidity. Current therapies, including administration of mucolytics and anti-inflammatory drugs, show limited effectiveness and durability, underscoring the need for novel effective and longer lasting therapeutic approaches. Here we show that mucus production in the lungs is regulated by the TNF superfamily member 15 (TL1A) acting through the mucus–inducing cytokine IL-13. TL1A induces IL13 expression by innate lymphoid cells leading to mucus production, in addition to promoting airway inflammation and fibrosis. Reciprocally, neutralization of IL13 signaling through its receptor (IL4Rα), completely reverses TL1A-induced mucus secretion, while maintaining airway inflammation and fibrosis. Importance of TL1A is further demonstrated using a preclinical asthma model induced by chronic house dust mite exposure where TL1A neutralization by genetic deletion or antagonistic blockade of its receptor DR3 protected against mucus production and fibrosis. Thus, TL1A presents a promising therapeutic target that out benefits IL13 in reversing mucus production, airway inflammation and fibrosis, cardinal features of severe asthma in humans.

Keywords: mucus, asthma, TL1A, IL13, fibrosis

OPEN ACCESS

Edited by:
Marialuisa Bocchino, University of Naples Federico II, Italy

Reviewed by:
Adam Byrne, Imperial College London, United Kingdom
Stewart J. Levine, National Heart, Lung, and Blood Institute (NHLBI), United States

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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *07 April 2021*
Accepted: *17 June 2021*
Published: *08 July 2021*

Citation:
Steele H, Sachen K, McKnight AJ, Soloff R and Herro R (2021) Targeting TL1A/DR3 Signaling Offers a Therapeutic Advantage to Neutralizing IL13/IL4Rα in Muco-Secretory Fibrotic Disorders. Front. Immunol. 12:692127. doi: 10.3389/fimmu.2021.692127

INTRODUCTION

Many fibro-proliferative disorders of the lung such as asthma and chronic obstructive pulmonary disease (COPD) exhibit increased morbidity associated with mucus hypersecretion (1, 2). Existing therapies are available, but these have varying effectiveness. Bronchodilators do not specifically target mucus hypersecretion (3, 4). Moreover, severe asthmatics become gradually resistant to corticosteroids (5–7). Therefore, there is an absolute necessity to develop novel therapies to limit mucus hypersecretion and improve patient outcomes.

Interleukin-13 (IL13) has recently emerged as a primary target for asthma therapy, with ongoing clinical trials targeting IL13 or approved therapeutics targeting its receptor interleukin-4 receptor α

Abbreviations: IPF, Idiopathic pulmonary fibrosis; TNF, Tumor necrosis factor; TL1A, TNF-like ligand 1A; DR3, Death Receptor 3; ILC, Innate lymphoid cells; COPD, Chronic obstructive pulmonary disease; IL13, Interleukin 13; IL4Rα: Interleukin 4 receptor α; TNFSF15, Tumor necrosis factor superfamily member 15; ClcA1, Chloride channel regulator, Calcium-Activated-1; PAS, Periodic Acid-Schiff; H&E, Hematoxylin and Eosin; i.t., Intratracheal; i.n., Intranasal; i.p., Intraperitoneal.

(IL4Rα) (8, 9). It is thought that IL13 is central for mucus hypersecretion *via* direct effects on bronchial epithelial cells, in addition to promoting smooth muscle contractility, inflammation and fibrosis (10, 11). *In vitro*, lung epithelial cells that were differentiated in an air-liquid interphase and stimulated with IL13, increased their expression of ClcA1, a goblet cell-derived calcium-activated chloride channel tightly linked to mucus production, in addition to the mucin MUC5AC (10). Silencing ClcA1 inhibited IL13-driven mucin production by these cells, indicating that IL13 is dependent on ClcA1 to induce mucus production (10). IL4 another Th2 cytokine that binds to the IL4Rα, is unable to promote MUC5AC production by differentiated goblet cells, as compared to ones treated with IL13 (12). Despite their common receptor, IL4 and IL13 have non redundant activities in asthma. Whereas IL4 acts predominantly in the early phase of asthma development, IL13 is thought to be active in the late phase of allergic reactions. IL4 is involved in regulating T cell proliferation and survival, and IgE synthesis. In contrast, IL-13 is predominantly involved in airway remodeling and mucus hypersecretion (12–14). Several lines of evidence support the contention that IL13, and not IL4, controls mucus production in asthma. In fact, the *in vivo* blockade of IL13 alone, by genetic deletion (15) or antagonistic administration of the IL13Ra2-Ig both prevents and reverses established mucus cell changes (16). Anti-IL4 administration did not reduce the numbers of mucin-secreting cells in the bronchial epithelium of mice induced with ovalbumin (15). Moreover, the *in vivo* administration of recombinant IL13 in the airways enhanced mucus secretion, in IL4-deficient mice (17). Additionally, IL4-deficient Th2 cells adoptively transferred to mice receiving inhaled ovalbumin failed to enhance mucus production by their bronchial epithelium (18). Furthermore, conjugate vaccines against IL4 failed to reduce mucus production in murine model of asthma induced by HDM, as opposed to ones directed against IL13 that abrogated mucus production in the lung bronchial epithelium (14). Collectively this data indicates IL4 is unable to mount a muco-secretory phenotype independently of IL13.

Other soluble molecules could possess similar activities on goblet cells. We have recently discovered that TL1A is able to induce lung fibrosis and tissue remodeling associated with eosinophilic asthma and idiopathic pulmonary fibrosis (IPF) (19). TL1A (aka TNFSF15) is a member of the TNF superfamily that signals through death receptor 3 (DR3) (20, 21). We demonstrated that interrupting TL1A/DR3 signaling, by genetic deletion of the receptor or antagonistic blocking, decreases collagen deposition and smooth muscle accumulation in the lungs in two different models: the allergen-induced eosinophilic asthma model (Th2 asthma) and the bleomycin-induced IPF model (19). Airway resistance in response to methacholine was significantly decreased when active TL1A signaling was lacking (19). Moreover, we showed that the airway administration of recombinant TL1A in isolation is sufficient to promote collagen deposition and smooth muscle hypertrophy. We demonstrated that aside from its pro-inflammatory role, TL1A can exert direct activity on stromal cells (fibroblasts and epithelial cells) to induce fibrosis (19). This was an unprecedented discovery linking TL1A to tissue remodeling and lung fibrosis. Here, we investigated the role of TL1A in mucus production. This is interesting, as mucus plugs and airway obstruction lead to respiratory failure and constitute the main causes of death in asthma and COPD (22, 23).

In this work, we demonstrate that the intra-tracheal administration of TL1A into the airways induces mucus production (aside from fibrosis), and conversely, when blocking TL1A signaling through DR3, mucus production was abrogated in murine eosinophilic asthma. Additionally, we show that TL1A muco-secretory activity is indirect on goblet cells. TL1A induces IL13 production by group 2 innate lymphoid cells (ILC2), which subsequently promotes mucus production. TL1A represents a promising therapeutic target for muco-secretory diseases. This work has tremendous potential significance in human asthma, COPD and cystic fibrosis therapies.

MATERIALS AND METHODS

Mice

Six- to eight-week-old male and female DR3-deficient mice and WT littermates (house on the C57BL/6 x 129 background), derived by Taconic Biosciences (#TF3529; Rensselaer, NY), were bred in-house. Male and female WT C57BL/6 or BALB/c and IL4Rα-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME). RAG2-deficient mice bred on the BALB/c x 129 background were a generous gift of Dr. Nunzio Bottini (UCSD), and RAG2γc-deficient mice on the BALB/c x 129 background were purchased from Jackson Laboratories (#014593). In all studies, both male and female mice were used. All studies and protocols were approved by and in compliance with the regulations of the Institutional Animal Care and Use Committees of Cincinnati Children's Hospital Medical Center.

Experimental Protocols

Activity of recombinant protein: WT C57BL/6 mice, RAG2-deficient mice, and RAG2γc-deficient mice, were given 10 μg of recombinant mouse TL1A (R&D Systems, Minneapolis, MN) or PBS intratracheally on days 1 and 2 and sacrificed for analyses one day later on day 3. Ocean Ridge Biosciences (Deerfield Beach, FL) performed the RNAseq transcriptomic screen and analysis on the lungs of mice induced with TL1A, *via* Illumina HiSeq 2000 (NCBI SRA database BioProject # PRJNA735223).

Asthma model: WT littermates and DR3-deficient mice on the C57BL/6 x 129 background, from both sexes, were sensitized i.n. on day 0, 7, and 14 with 200 and 100 μg house dust mite extract protein (HDM; GREER Labs Inc, Lenoir, North Carolina; endotoxin levels at 930EU/vial) in PBS, followed by chronic i.n. challenges of 50 μg of HDM protein administered twice a week for the following 4 weeks as previously described (24). Analyses were performed 24 hours after the last challenge. For neutralization of TL1A-DR3 interactions, mouse DR3-Fc (generous gift of Dr. Soloff) or isotype control IgG (BioXCell,

Lebanon, NH) were administered intraperitoneal (i.p.) to WT C57BL/6 mice after the initial sensitization period starting at day 14 and were given every three days until the end of the experiment (100 μg/injection/mouse).

Immunofluorescence Staining of ClcA1, MUC5AC, and α-Smooth Muscle Actin

Paraffin-embedded tissues are sliced 4μm thick each section. Deparaffinization is performed by successive incubation in xylene, followed by 100% ethanol and 75% ethanol for 5 min each time. Sections are washed in PBS before being treated with antigen retrieval solution (Citrate buffer pH 6) for 20 min in the microwave (Power 3) and rested for an additional 20 min at room temperature (RT). The sections are then washed in PBS and incubated in blocking solution containing 10% Donkey Serum and Fc block for 1h at RT. Primary antibodies for ClcA1 (clone EPR12254; Abcam, Cambridge, MA) and MUC5AC (clone 45M1; Abcam) are used at 1:100 dilution according to the manufacturer recommendations and incubation is performed overnight at 4°C. Sections are washed in PBS three times before staining with secondary antibodies Donkey anti-mouse and Donkey anti-rabbit (ImmunoJackson Research, West Grove, PA) at 1:500 dilution for 3h at RT. Sections are then washed in PBS three times and nuclear staining is performed using DAPI at 1:10000 dilution for 5 min at RT. Sections are mounted in ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA) and imaged on Nikon Inverted immunofluorescence microscope.

Immunohistochemistry Staining

PAS, Hematoxylin and Eosin (H&E) and Trichrome stains are performed according to the manufacturers' recommendations (Poly Scientific R&D, Bay Shore, NY and Thermo Fisher Scientific). Sections are scanned using the Zeiss Axioscanner ×20 objective and analyzed using Image-Pro Premier software.

Type 2 Innate Lymphoid Cells Sorting and DR3 Stain

Lung ILC2 are sorted on the following markers $CD45.2^+Lin^-$ $Thy1.2^+$ $ST2^+Sca1^+$All antibodies are purchased from Biolegend (San Diego, CA) with exception to ST2 (101001PE, MD Biosciences). DR3 stain is performed using the clone 4C12 from Biolegend.

ILC2 Culture and Stimulation With TL1A

ILC2 are cultured for five days in the presence of 50ng/ml IL2, IL7, and IL33 (Peprotech, Rocky Hill, NJ and R&D Systems) followed by two days culture with 10ng/ml IL2, IL7, and IL33 and 100 ng/ml TL1A (R&D Systems# 1896 TL). Measurements of IL13 in the culture supernatants is performed using mouse IL13 DuoSet ELISA (R&D Systems).

Flow Cytometry

BAL and lung cells were treated with RBC lysing buffer (Sigma). Lungs were dissociated using a Lung Dissociation Kit (Miltenyi Biotec) and Gentle MACS (Miltenyi Biotec). LIVE/DEAD cells were stained with Fixable Aqua Dead Cell Staining Kit (Thermo Fisher Scientific), and after Fc block with the 2.4G2 mAb (eBioscience), cells were stained with the following Abs: DR3-PE (4C12; BioLegend), SiglecF (E50-2440; BD Biosciences), CD11b (M1/70; BD Biosciences), CD11c (HL3; BD Biosciences), Ly6G (1A8; BioLegend), ST2-PE (101001PE, MD), CD90.2 (30-H12; BioLegend). For lineage markers for ILC2 staining, the following Abs were used: CD3-FITC (145-2C11; eBioscience), CD4-FITC (GK1.5; eBioscience), CD8- FITC (5H-10-1; BioLegend), CD19-FITC (1D3; BioLegend), NK1.1-FITC (PK136; BioLegend), CD11b-FITC (M1/70; BioLegend), CD11c-FITC (HL3; BD Biosciences), and Gr1-FITC (RB6-8C5; BioLegend). Flow cytometry analysis was performed on a Fortessa (BD Biosciences), and data were analyzed using FlowJo Software (version 10; FlowJo, Ashland, OR). Live CD45+ lung immune cells were separated into T cells (CD3+, CD90.2+), macrophages (CD11b+, CD11c+ SiglecF+), DCs (CD11c+ MHC class II+), neutrophils (GR1+, CD11b+ SigF-), eosinophils (Ly6C+, SigF+, CD11c-) and ILC2 (Lin^- $Thy1.2^+$ $ST2^+Sca1^+$).

Statistical Analyses

Statistical analysis was performed using GraphPad Prism software. One way ANOVA or non-parametric Mann-Whitney U test was used where indicated. When One way ANOVA was used, multiple comparison was employed. A P value < 0.05 was considered statistically significant. All data are representative of at least three independent experiments with multiple mice as indicated or different donor cell populations.

RESULTS

Intratracheal TL1A Induces a Muco-Secretory Signature in the Lung

We conducted a transcriptomic RNAseq analysis on murine lungs induced with recombinant TL1A, challenged on two successive days and euthanized 24h after the last injection (**Figure 1A**). We discovered that the highest differentially expressed gene in response to TL1A was chloride channel accessory 1 encoding the goblet cell protein ClcA1 (109.700 fold increase), which is involved in mucin synthesis (**Figure 1B**). ClcA1 is thought to signal through TMEM213 to promote MUC5AC production by bronchial epithelial cells stimulated with IL13 (10). In addition to ClcA1, many muco-secretory genes were upregulated in response to TL1A stimulation. These include genes encoding for IL13 (4.166 fold increase), the mucins MUC5AC (5.590 fold increase) and MUC5B (2.268 fold increase), TNFα (4.130 fold increase), TFF2 (3.530 fold increase), TMEM213 (2.776 fold increase) and AREG (2.632 fold increase) (**Figure 1B**). IL4 transcripts were not increased in the lungs of TL1A-induced mice. The intratracheal administration of TL1A in isolation to the airways induces a muco-secretory signature in the lung. Additionally, airway inflammation was increased in TL1A-induced mice compared to PBS (**Supplementary Figures 1A, B**). Flow cytometry analyses of immune cells performed on lung tissue showed a significant increase in T cells, eosinophils, neutrophils, macrophages and

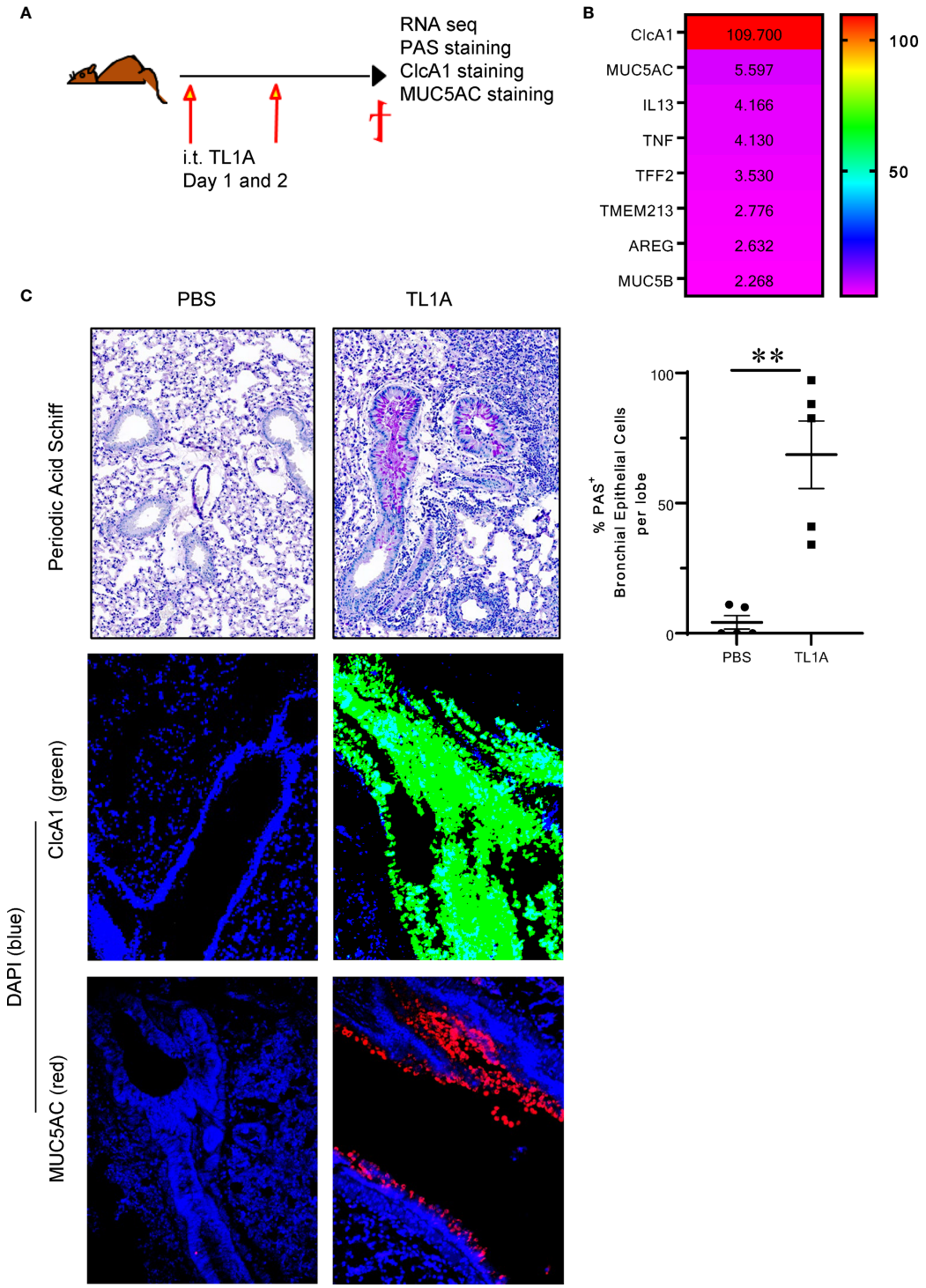

FIGURE 1 | TL1A induces mucus production when administered in isolation into the airways. **(A)** Schematic representation of the protocol used. Briefly, C57BL/6 mice were induced with 10μg of recombinant TL1A i.t. on two successive days. Mice were euthanized 24h after the last i.t. injection, and lungs were harvested for RNAseq analysis, PAS, ClcA1 and MUC5AC stains. **(B)** Heatmap of the top differentially regulated genes involved in mucus production, upregulated in the lungs of TL1A-induced mice. **(C)** Top panel: PAS stain of mucus produced in the lungs and quantified using Image Pro Premier and graphed as %PAS+ bronchial epithelial cells per lobe (right panel). Mid and Lower panels: Immunofluorescence stains of CLcA1 (green), MUC5AC (red) and nuclear DAPI (blue). All results representative of three experiments with four to six mice per group. **$p < 0.005$.

innate lymphoid cells (**Supplementary Figure 1A**). Consistent with the flow analysis, H&E scoring of lung biopsies demonstrated a significant increase in airway inflammation (**Supplementary Figure 1B**). We have additionally monitored fibrosis by scoring trichrome stain of collagen in lung biopsies and showed TL1A-induced mice increased airway fibrosis [**Supplementary Figure 1B** and (19)]. TL1A was able to induce changes in airway resistance and lung function [**Supplementary Figure 1C** and (19)]. Lastly, we monitored levels of IL4, IL5, IL13, TSLP and Periostin transcripts in the lungs of TL1A-induced mice by qPCR (**Supplementary Figure 1D**) and demonstrated mRNA transcripts of IL13 are the most highly increased in TL1A-induced mice (**Supplementary Figure 1D**). IL4 was not enhanced after the intratracheal instillation of TL1A in the airways (**Supplementary Figure 1D**).

After injecting recombinant TL1A directly into the airways, we assessed mucus production by Periodic Acid-Schiff (PAS) stain as well as ClcA1 and MUC5AC expression by immunofluorescence. We observed that the airway administration of 10μg TL1A in isolation into the airways on two successive days, was sufficient to promote mucus production 24h after the last injection (**Figure 1C**).

Interrupting TL1A/DR3 Signaling Decreases Mucus Production in Allergen-Induced Asthma

Chronic challenges with house dust mite in the airways after a sensitization phase, generates an asthma endotype (19, 24) with strong airway eosinophilia (24), Th2 (25) and type 2 innate lymphoid cells infiltrating the lung (26). This model involves type 2 cytokines like IL4, IL5, IL13 and periostin (19), IgE and alarmins like TSLP, IL25 and IL33 (27). We chronically induced wildtype and $DR3^{-/-}$ mice with house dust mite (HDM from Greer Laboratories) for six weeks by first sensitizing the mice using 200μg HDM intranasal (i.n.) at day 0, 100μg HDM i.n. at day 7, and 50μg HDM i.n. at day 14. We then challenged the mice every other day with 50μg i.n. HDM for four weeks and monitored mucus production 24h after the last HDM dose (**Figure 2A**). Strikingly, mucus production assessed by PAS was significantly dampened in mice lacking an active TL1A/DR3 signal. Moreover, we treated wildtype mice induced with chronic HDM, with 100μg DR3-Fc, an antagonist reagent to TL1A, during the challenge phase (starting day 14) twice a week until the end of the experiment. Another group received similar doses of an isotype control to DR3-Fc. We showed that DR3-Fc significantly decreases mucus production, post-disease onset (**Figure 2B**). Additionally, inflammation and fibrosis, assessed by H&E, Trichrome and smooth muscle actin stains respectively, were significantly decreased when TL1A signal was removed by genetic deletion of its receptor DR3 or antagonistic neutralization (DR3-Fc treatment) (**Figure 2C**). Baseline trichrome and H&E scores were comparable between wild type and DR3-deficient mice (**Supplementary Figure 2A**). Flow cytometry analysis on bronchial lavage revealed strong airway eosinophilia compared to neutrophils which were modestly recruited. Additionally, cellular infiltrates were decreased in the bronchial lavage when TL1A signal was interrupted by genetic mutation of its receptor or antagonistic blockade, albeit not significantly (**Supplementary Figure 2B**). Strikingly, Lung ILC2 gated on $CD45.2^{+}Lin^{-}$ $Thy1.2^{+}$ $ST2^{+}Sca1^{+}$ (**Supplementary Figure 2D**), were significantly decreased when TL1A signal was absent (**Supplementary Figure 2C**). Taken together, these data demonstrate that characteristic traits of asthma, including both mucus production and airway inflammation and remodeling, were drastically reversed post-TL1A neutralization.

TL1A Muco-Secretory Activity Is Dependent on IL13/IL4Rα Signaling

IL13 is thought to play a central role in promoting mucus production associated with asthma and COPD, making IL13 neutralizing biologics the focus of both an approved therapeutic, dupilumab, and multiple other ongoing clinical trials (28–31). We questioned whether TL1A muco-secretory activity was dependent on IL13 signaling through its receptor IL4Rα, expressed on goblet cells. To answer this, we injected IL4Rα-deficient mice with recombinant TL1A on two successive days and monitored mucus production at day 3 (**Figure 3A**). Surprisingly, IL4Rα-deficient mice were completely mucus free as compared to wildtype BALB/c mice that induced mucus production after TL1A instillation (**Figure 3B**). TL1A muco-secretory activity is therefore completely dependent on IL13 signaling through its IL4Rα. Thus, we hypothesized that TL1A acts indirectly on goblet cells to promote mucus production by upregulating IL13. Indeed, IL13 transcripts were increased 21.3 folds in wildtype mice induced with TL1A and 17.7 folds in IL4Rα-deficient mice that can't signal through IL13 despite the increase in its transcript expression (**Supplementary Figure 3B**). IL4 transcripts were not increased in neither the wildtype nor the IL4Rα-deficient mice (**Supplementary Figure 3B**). Most importantly, while mucus production was completely abrogated in IL4Rα-deficient mice, inflammation persisted (**Figure 3C**, top). Collagen deposition was lessened in IL4Rα-deficient mice (**Figure 3C**, middle), whereas smooth muscle hypertrophy remained unchanged (**Figure 3C**, bottom).

TL1A Acts on Innate Lymphoid Cells to Promote IL13 Expression Necessary for Mucus Production, Independently of Adaptive Immunity

The two predominant cellular sources of IL13 are T cells and innate lymphoid cells. We first questioned whether TL1A requires adaptive immunity, specifically, whether IL13-expressing T cells drive mucus production. For this purpose, we injected RAG-deficient mice with recombinant TL1A on two successive days and monitored mucus production (**Figure 4A**). Surprisingly, TL1A maintained its capacity to promote mucus production in mice lacking lymphocytes (**Figure 4B**). We therefore concluded that TL1A muco-secretory activity is independent of adaptive immunity.

Alternative to T cells, type 2 innate lymphoid cells (ILC2) are major cellular sources of IL13 and are increased in allergen-induced asthma (32, 33), but not IL4 (13, 34). We therefore questioned whether TL1A induces IL13 production by innate

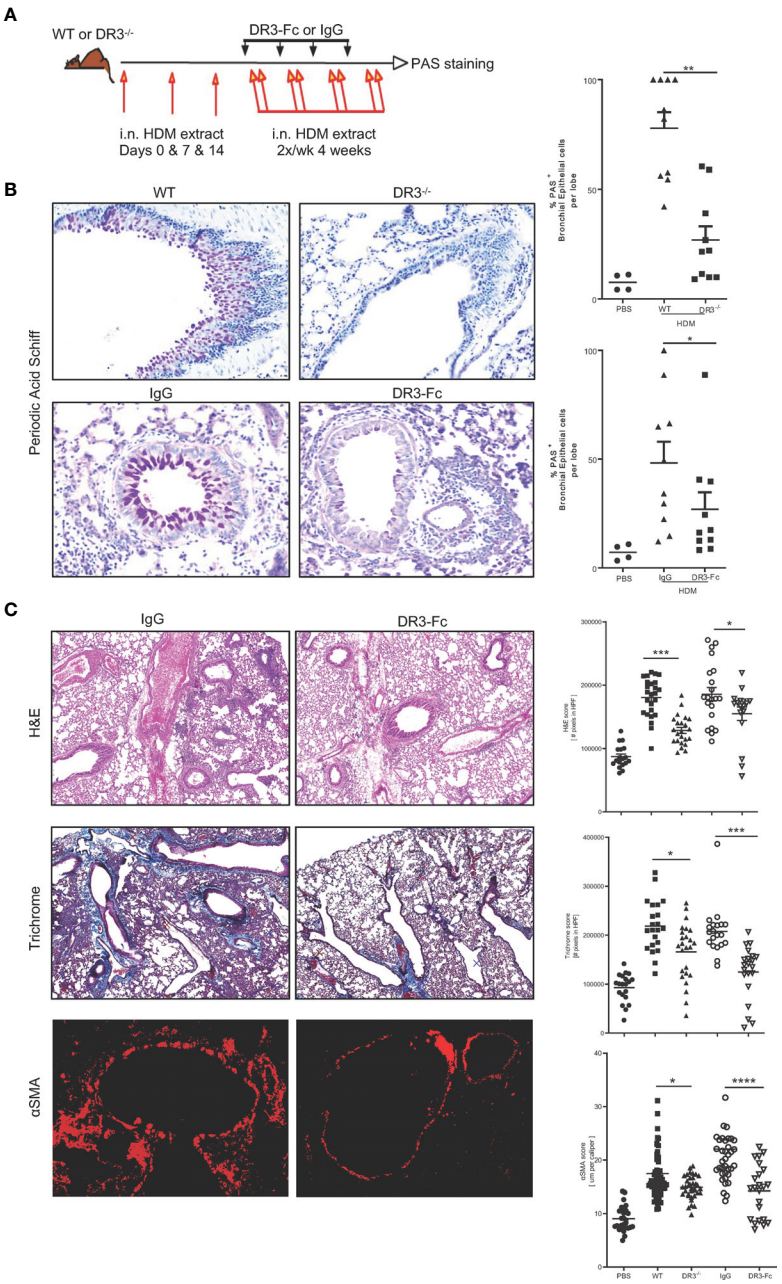

FIGURE 2 | Interrupting TL1A/DR3 signaling decreases mucus production in allergen-induced asthma. **(A)** Schematic representation of protocol used. Briefly, WT littermates and DR3-deficient mice on the C57BL/6 x 129 background were sensitized i.n. on day 0, 7, and 14 with 200 and 100 μg house dust mite extract protein in PBS, followed by chronic i.n. challenges of 50 μg of HDM protein administered twice a week for the following four weeks. Analyses were performed 24 hours after the last challenge. For neutralization of TL1A-DR3 interactions, mouse DR3-Fc or isotype control IgG were administered i.p. to WT C57BL/6 mice after the initial sensitization period starting at day 14 and were given every three days until the end of the experiment (100 μg/injection/mouse). **(B)** PAS stain of mucus produced in the lungs and quantified using Image Pro Premier. **(C)** Inflammation assessed by H&E stain (top panels), collagen deposition assessed by trichrome stain (middle panels), and smooth muscle hypertrophy assessed by αSMA immunofluorescence stain (red) (bottom panels) on lung biopsies of WT C57BL/6 mice after treatment with either IgG or DR3-Fc. Quantifications for each parameter, completed using Image Pro Premier, are shown to the right, including untreated WT C57BL/6 and DR3$^{-/-}$ mice (images not shown). All results representative of three experiments with five mice per group. *p < 0.05, ** < 0.005, ***p < 0.0005, ****p < 0.00005.

lymphoid cells to control mucus production. We injected 10μg of recombinant TL1A on two successive days into RAGγc-deficient mice that lack ILC in addition to lymphocytes (**Figure 4A**). TL1A was unable to induce mucus production in the RAGγc-deficient mice as compared to RAG-deficient mice (**Figure 4B**). Moreover, TL1A increased 14.8 folds IL13 transcripts in the lung

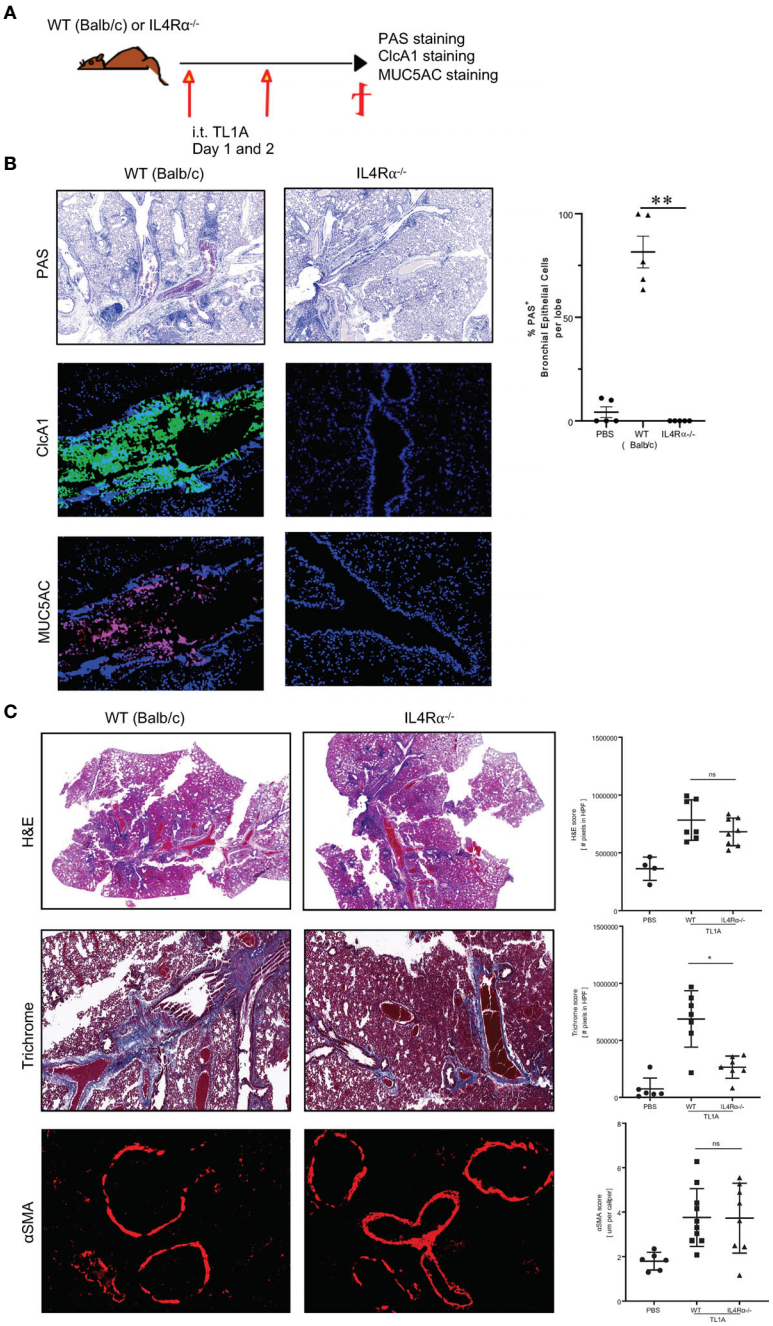

FIGURE 3 | TL1A muco-secretory activity is dependent on IL13/IL4Rα signaling. **(A)** Schematic representation of the protocol used, as previously described in **Figure 1**. **(B)** Top panel: PAS stain of mucus produced in the lungs and quantified using Image Pro Premier (right panel). Mid and Lower panels: Immunofluorescence stains of CLcA1 (green), MUC5AC (red) and nuclear DAPI (blue). **(C)** Inflammation assessed by H&E stain (top panels), collagen deposition assessed by trichrome stain (middle panels), and smooth muscle hypertrophy assessed by αSMA immunofluorescence stain (red) (bottom panels) on lung biopsies of BALB/c and IL4Rα-deficient mice induced with PBS or TL1A. Quantification for each parameter is done using Image Pro Premier. All results representative of three experiments with four to six mice per group. ns, not significant, *p < 0.05, ** < 0.005.

of RAG-deficient mice, whereas TL1A-induced RAGγc-deficient failed to enhance IL13 expression (**Supplementary Figure 3A**). IL4 levels remained unchanged in both RAG and RAGγc-deficient mice, challenged with TL1A (**Supplementary Figure 3A**). This data suggests TL1A acts on ILC to promote IL13 production. To determine whether TL1A exerts a direct effect on ILC, we first verified they express its receptor DR3. We sorted lung ILC2 cells from the lungs of RAG-deficient mice

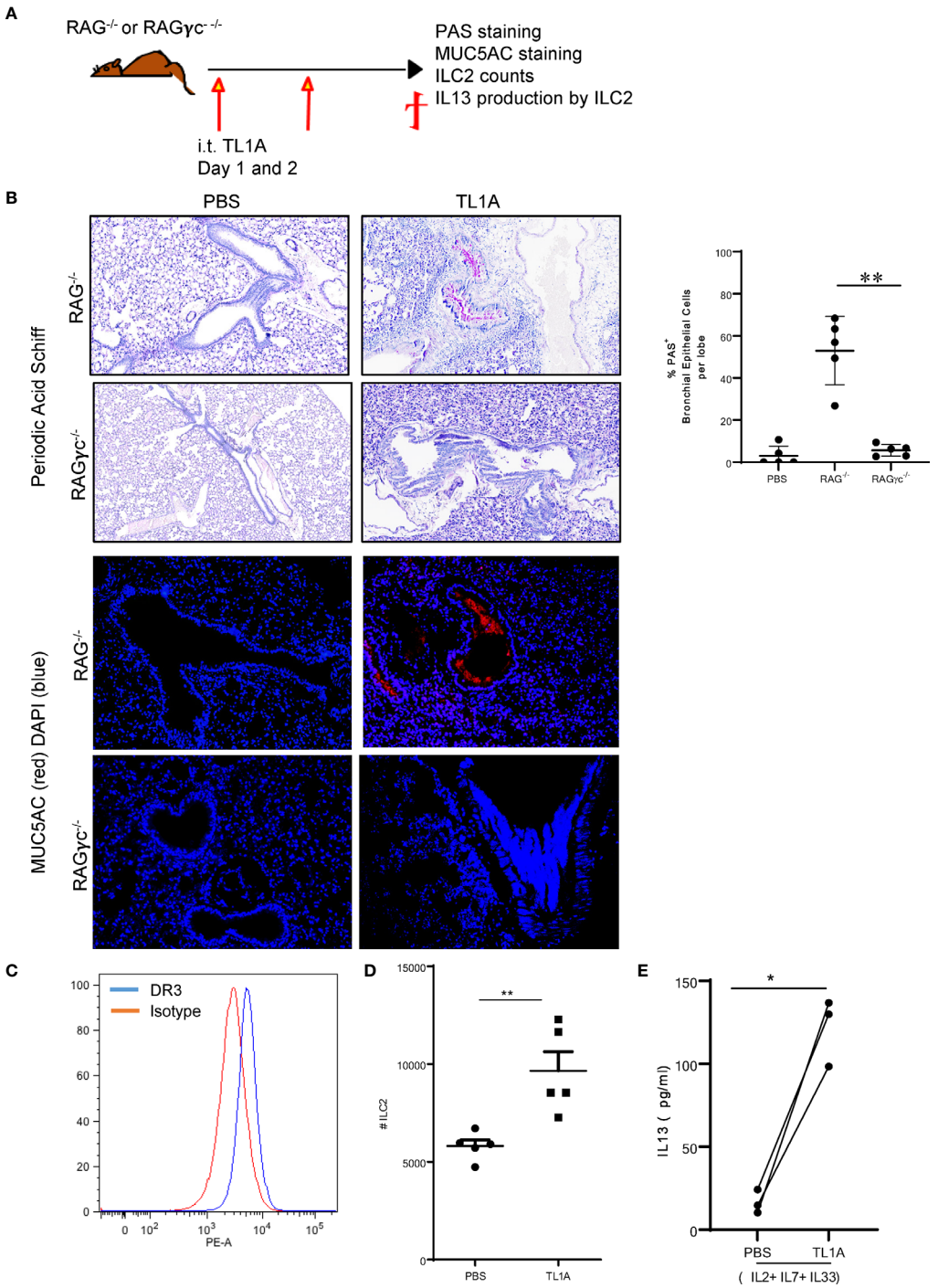

FIGURE 4 | TL1A induces mucus production independently of adaptive immunity, bystander to IL13 production by ILC2. **(A)** Schematic representation of the protocol used, as previously described in **Figure 1**. **(B)** Top panel: PAS stain of mucus produced in the lungs of RAG$^{-/-}$ and RAGγc$^{-/-}$ mice induced with PBS or TL1A and quantified using Image Pro Premier (right graph). Lower panels: Immunofluorescence stains of MUC5AC (red) and nuclear DAPI (blue). **(C)** DR3 staining on ILC2 assessed by flow cytometry (DR3 in blue and Isotype in red). **(D)** Number of lung ILC2 in the left lobe of RAG$^{-/-}$ mice induced with TL1A. **(E)** IL13 levels assessed by ELISA, in the supernatants of ILC2 cultured in the presence of IL2, IL7, and IL33 and stimulated with or without TL1A. All results representative of three experiments with four to six mice per group, or three individual replicate cultures. *$p < 0.05$, **$p < 0.005$.

induced with TL1A and examined DR3 expression. Lung ILC2 sorted on CD45.2$^+$Lin$^-$ Thy1.2$^+$ ST2$^+$Sca1$^+$ expressed DR3 on their surface (**Figure 4C**). *In vivo*, TL1A induced proliferation and activation of lung ILC in RAG-deficient mice (**Figure 4D**). We observed an increase in the numbers of lung ILC2 (**Figure 4D**) as well as upregulation of IL13 production by these cells (**Figure 4E**). ILC2 cultured for five days in the presence of 50ng/ml IL2, IL7, and IL33 followed by two days culture with 10ng/ml IL2, IL7, and/IL33 and 100 ng/ml TL1A, increased IL13 secretion assessed by ELISA in the culture supernatant (**Figure 4E**). We concluded TL1A promotes IL13 production by ILC2, which is necessary for mucus secretion.

DISCUSSION

Mucus plugs are the major cause of death in asthma, COPD and cystic fibrosis. Current therapies involve the administration of mucolytics; however, these aren't always effective at improving patient outcomes and there seems to be an increase in mortality associated with hypersecretory disorders. In this work, we describe, for the first time, a novel activity of TL1A as a driver of mucus production in the lung. To date, the most characterized cytokine involved in mucus production is IL13. It has been shown to drive mucus production by goblet cells differentiated *in vitro*, in Air-Liquid-Interface (ALI) (10). Additionally, silencing IL13 signaling through MAPK inhibition abrogated mucus production in murine models of asthma (35, 36). We have evidence that TL1A drives IL13 production by ILC2, which are increased in asthma. This is consistent with prior findings, showing that TL1A can promote allergic immunopathology through ILC2s (37, 38). Moreover, we show that TL1A can sustain a muco-secretory activity in the absence of T and B cells; however, ILC2 cells were necessary for this activity (**Figure 5**). ILC2 cells stimulated with TL1A, in the presence of the homeostatic cytokines IL2, IL7 and IL33, promoted IL13 production by these innate cells. We demonstrate that blocking TL1A signaling through its receptor DR3, by genetic deletion or antagonist blocking, abrogated mucus and IL13 production. Taken together with our data, which shows that blocking TL1A reduces collagen deposition and smooth muscle hypertrophy [(**Figure 2C** and (19)], targeting TL1A appears to be a novel approach to treat all features of airway inflammation and remodeling associated with asthma. TL1A acts upstream of IL13, driving mucus production through direct activity on ILC2. TL1A can act directly on structural

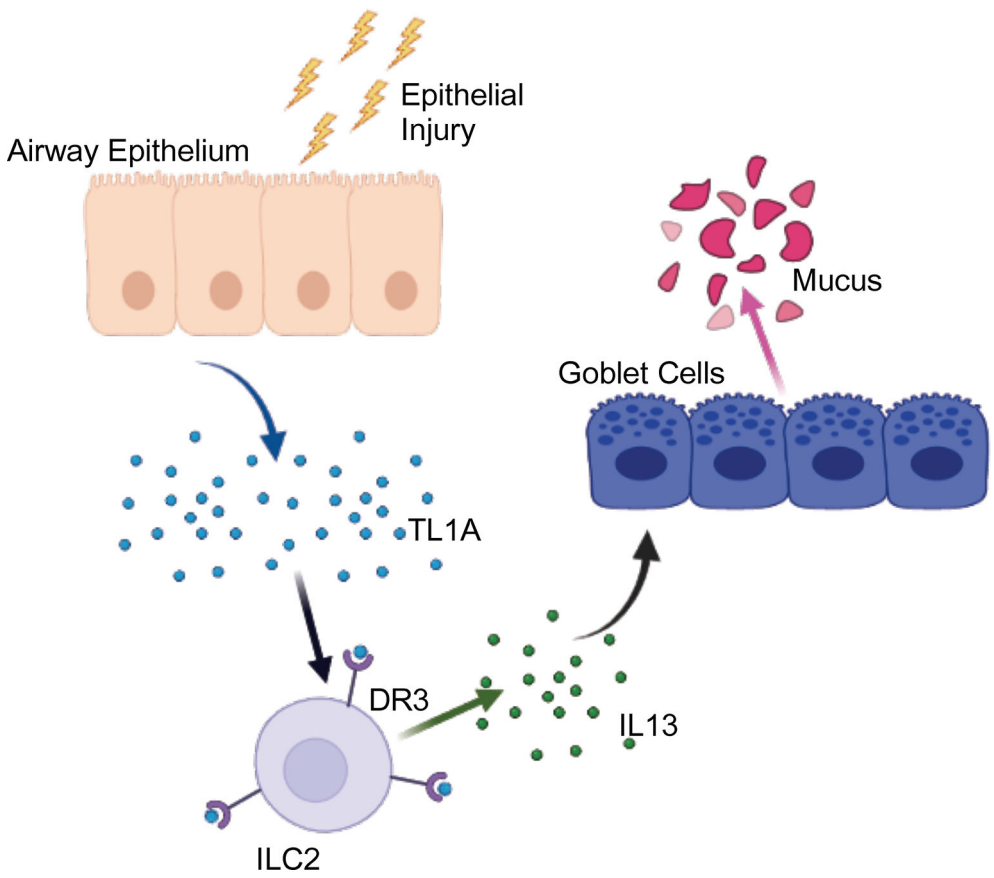

FIGURE 5 | Graphical Abstract. Upon epithelial injury, such as exposure to allergens, TL1A is produced and acts on ILC2 *via* DR3 to promote IL13 secretion, leading to mucus production. Created with BioRender.com.

cells of the lung, namely fibroblasts and epithelial cells, to promote airway remodeling and fibrosis.

Targeting TL1A shows a competitive advantage to neutralizing IL13, as this work shows that IL4Rα-deficient mice sustained an inflammatory response and smooth muscle hypertrophy, despite the absence of mucus and subsequent to the lack of an active IL13 signal. In humans, eight clinical trials failed to demonstrate benefit in neutralizing IL13 in severe asthmatic patients (28, 39). Three monoclonal antibodies directed against IL13 (tralokinumab, lebrikizumab and anrukinzumab) were used to interrogate the role of this cytokine in the pathophysiology of severe asthma and responses to treatment. The primary end points included FEV_1 measurements, Mean ACQ6 scores, and asthma exacerbations recorded by the intake of systemic glucocorticoids and number of hospitalizations (29). All three antibodies showed a pattern of improvement without reaching significant decrease in the endpoints measured. Alternative to anti-IL13 antibodies, IL4Rα antagonists (dupilumab) have been recently FDA approved as add-on-maintenance therapy to treat moderate-to-severe asthma (31). Add-on therapy with dupilumab significantly reduced the oral glucocorticoid dose while simultaneously reducing the rate of severe exacerbations and improving lung function (FEV_1) in patients with glucocorticoid-dependent severe asthma (31). More recently, in the "Liberty Asthma Quest Trial," dupilumab treatment lowered significantly the rates of severe asthma exacerbation and improved lung function as compared to placebo treated controls. Greater benefits were seen in asthmatic patients with higher eosinophilia (31). Nevertheless, the effect of dupilumab on mixed granulocytic or neutrophilic asthma patients that are resistant to steroid treatment remain unanswered. It is becoming evident that neutralizing IL13 is not sufficient to reverse certain features of airway inflammation and fibrosis once established. Consistent with this, our study in the IL4Rα-deficient mice induced with TL1A demonstrated a complete abrogation in mucus production and reduced collagen deposition, while inflammation and fibrosis persisted. IL4 was not increased after TL1A airway challenge whereas IL13 was significantly induced. Additionally, several lines of evidence have refuted the idea that IL4 drives mucus production by bronchial epithelial cells. Interrupting IL13 in isolation by genetic deletion or antagonistic blockade reversed established mucus secretion (15, 16), whereas anti-IL4 blockade did not reduce mucin-secreting goblet cells in a murine model of asthma (15). Vice versa, IL13 administration in IL4-defiicent cells enhanced mucus production (17), supporting a unique role for IL13 in regulating mucus secretion. Collectively, this indicates that IL13, not IL4, controls mucus secretion through IL4Rα signaling.

Recently we showed soluble TL1A can be detected in the bronchial lavage of mice. TL1A is expressed on the surface of alveolar macrophages, dendritic cells, innate lymphoid type 2 cells, and subpopulations of lung structural cells. DR3 was found on CD4 T cells, innate lymphoid type 2 cells, macrophages, fibroblasts, and some epithelial cells and is expressed primarily by alveolar macrophages (19). In asthmatic patients presenting with the eosinophilic endotype, DR3 is upregulated in sputum ILC2 after allergen challenge or by IL2, IL33, and TSLP stimulation *in vitro*. $DR3^+$ILC2 significantly increased IL5 expression upon TL1A stimulation (40). Combined with our data showing TL1A is upstream of IL13, this indicates that targeting TL1A signaling offers an advantage over targeting IL13/IL4Rα or IL5 signaling in isolation to treat mucus secretion associated with asthma.

Taken together, we demonstrate that interrupting TL1A/DR3 signaling abrogates mucus production associated with asthma, post-disease onset. We further show that TL1A acts on ILC2 to promote IL13 secretion leading to mucus production. Aside from its muco-secretory activity, TL1A acts directly on stromal and immune cells, perpetuating fibrosis and airway inflammation. While mucus secretion was dependent on IL13/IL4Rα signaling, in this work, we demonstrate that interrupting IL4Rα was not sufficient to decrease smooth muscle hypertrophy, fibrosis and airway inflammation. Targeting TL1A is therefore, more beneficial than targeting IL13/IL4Rα signaling in asthma and muco-secretory disorders.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in NCBI SRA database BioProject # PRJNA735223.

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee, Cincinnati Children's Hospital Medical Center.

AUTHOR CONTRIBUTIONS

HS and RH performed experiments and analyzed data. KS, AM, and RS contributed reagents and expertise. RH directed the study and wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by funds from NIH grant U01 AI095542-08 MIST (Mucosal Immunology Study Team) to RH and Kyowa Kirin Pharmaceutical Research, Inc. (KKR).

ACKNOWLEDGMENTS

We acknowledge Drs. Chandrashekhar Pasare, Sing Sing Way, and Michael Croft for fruitful discussions. We also thank the microscopy core, flow core, and division of veterinary services at Cincinnati Children's Hospital Medical Center for their help.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.692127/full#supplementary-material

Supplementary Figure 1 | TL1A induces airway inflammation and remodeling. **(A)** Flow cytometry analyses of cellular infiltration in the lungs of TL1A-induced mice as previously described. Total numbers of leucocytes, T cells, eosinophils, neutrophils, macrophages, and dendritic cells are shown. **(B)** Immunohistochemistry analyses of cellular infiltration assessed by H&E and collagen deposition assessed by Masson Trichrome. Quantifications are shown on the right panel. **(C)** Airway hyperresponsiveness (AHR) to increasing concentrations of methacholine of TL1A versus PBSinduced mice is shown. **(D)** Fold change in mRNA levels of IL4, IL5, IL13, TSLP and periostin in the lungs of TL1A over PBS-induced mice are shown. All results are representative of 5 to 10 mice per group. ****p < 0.00005.

Supplementary Figure 2 | TL1A inhibition decreases the infiltration of ILC2 in the lungs and airway fibrosis. **(A)** Quantification of Masson Trichrome and H&E scores in the lungs of HDM-induced mice are shown. **(B)** Flow cytometry analysis of cellular infiltration in the bronchial lavage of mice induced with HDM as previously described. The grey dotted line indicates baseline infiltration. **(C)** Numbers of ILC2 in the left lobe of the lungs of mice induced with HDM. The grey dotted line indicates baseline infiltration. **(D)** Gating strategy of ILC2 cells used during the sorting. All results are representative of 10 mice per group. One-way Anova using a multi-comparison to WT HDM is used to calculate the p values. *p < 0.05, ***p < 0.0005.

Supplementary Figure 3 | IL13 and IL4 expression in the lungs of TL1A -induced mice. **(A)** Relative expression of IL4 and IL13 in the lungs of RAG and RAGγc-deficient mice induced with TL1A as previously described. **(B)** Relative expression of IL4 and IL13 in the lungs of WT (BALBc) and IL4Rα- deficient mice induced with TL1A as previously described. All results are representative of 4 mice per group. *p < 0.05.

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Conflict of Interest: Authors KS, AM, and RS were employed by Kyowa Kirin Pharmaceutical Research, Inc. The authors declare that this study received funding from Kyowa Kirin Pharmaceutical Research, Inc. Authors KS, AM, and RS of Kyowa Kirin Pharmaceutical Research, Inc participated in discussing results and editing the manuscript.

The remaining 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.

frontiers in Immunology

MINI REVIEW
published: 13 August 2021
doi: 10.3389/fimmu.2021.678457

Roles of Macrophage Polarization and Macrophage-Derived miRNAs in Pulmonary Fibrosis

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This mini-review summarizes the current evidence for the role of macrophage activation and polarization in inflammation and immune response pertinent to interstitial lung disease, specifically pulmonary fibrosis. In the fibrosing lung, the production and function of inflammatory and fibrogenic mediators involved in the disease development have been reported to be regulated by the effects of polarized M1/M2 macrophage populations. The M1 and M2 macrophage phenotypes were suggested to correspond with the pro-inflammatory and pro-fibrogenic signatures, respectively. These responses towards tissue injury followed by the development and progression of lung fibrosis are further regulated by macrophage-derived microRNAs (miRNAs). Besides cellular miRNAs, extracellular exosomal-miRNAs derived from M2 macrophages have also been proposed to promote the progression of pulmonary fibrosis. In a future perspective, harnessing the noncoding miRNAs with a key role in the macrophage polarization is, therefore, suggested as a promising therapeutic strategy for this debilitating disease.

Keywords: macrophage plasticity, M1/M2 polarization, MicroRNAs, exosomes, pulmonary fibrosis

OPEN ACCESS

Edited by:
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Reviewed by:
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Eirini Vasarmidi,
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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *09 March 2021*
Accepted: *29 July 2021*
Published: *13 August 2021*

Citation:
Kishore A and Petrek M (2021) Roles of Macrophage Polarization and Macrophage-Derived miRNAs in Pulmonary Fibrosis. Front. Immunol. 12:678457. doi: 10.3389/fimmu.2021.678457

INTRODUCTION

Pulmonary fibrosis (PF) is a progressive, irreversible and lethal lung disease and has remained a challenge for clinicians and researchers. The tissue injury accompanied by cellular inflammation in the lungs drives fibrotic response and thus, plays a crucial role in the pathogenesis of fibrosis. Inflammatory cells release TGF-β, the key regulator of several profibrotic cytokines/chemokines, their receptors/subunits, and growth factors inducing epithelial-mesenchymal transition (EMT) (1–3). The pro-inflammatory and profibrotic cytokines involved in PF promote inflammation and irreversible damage to lung architecture with the loss of alveolar-capillary barrier basal membrane leading to persistent fibrosis (4). These pathogenic factors for PF have further been reported as associated with genetic factors including gene variants and non-coding regulatory microRNAs (3–7).

The tissue-resident macrophages (M0) are versatile cells that exhibit a high degree of plasticity represented by classically activated M1 (pro-inflammatory) or alternatively activated M2 (anti-inflammatory/pro-fibrotic) macrophages (8). The macrophage polarization is extremely variable and switching of one "activation-type" to another, stimulated by appropriate factors or tissue

microenvironment, is a rapid and reversible process. The cross-talk between macrophages and the microenvironment regulates tissue regeneration, flagged with key surface markers in both pro-regenerative and profibrotic environments (9).

Thus, besides the development of tissue homeostasis, these cells are suggested with sequential roles in both induction and resolution of inflammation. The M0 macrophages can polarize to M1 or M2 (M2a, M2b, M2c, and M2d) in response to different activators, such as LPS/IFNγ and IL4/IL13, respectively (10). The M1 macrophage phenotype can also be stimulated without the presence of lymphocytes, for example, by inflammatory cytokines and microorganism-derived molecules (10–13). The anti-inflammatory or immune-compromised state associated with the M2 macrophage phenotype is also supported with suppressed multiple interferon-associated pathways as one of the most prominent signals common among all M2-polarizing stimuli (14). The M1 macrophage can readily repolarize to M2a (stimulated with reduced IL10 and TNFα, and increased Ym1 level) and M2b (increased IL10 and reduced TNFα) subpopulations (13). Similarly, macrophage M2b can convert to other M2 subtypes in response to different stimuli (15). The different approaches to macrophage polarization have been associated with both the demerits and benefits influencing their utility for specific tissues. Where possible, future therapeutic approaches are suggested to consider tailoring of strategy towards the formation of a specific tissue-microenvironment, as well as promotion of specific disease-associated cell subsets, to improve efficacy and minimize off-target effect (11).

In deciphering pathomechanisms of lung fibrosis, pulmonary macrophages have been implicated with a key role in the fibrogenic process. The PF has been proposed to be regulated by macrophage plasticity (M1/M2 polarization) with an immunogenic signature network of chemokines such as MCP-1, MIP-1α, CCL18, and cytokines such as TNFα, TGFβ1, and their respective signaling pathways (16–18). The pro-inflammatory M1 macrophage polarization with overexpression of iNOS, TNFα, IL1, IL6, IL12, IL23, MCP-1, and IFNγis associated with inflammation, antitumoral functions and graft rejection (**Table 1**). The anti-inflammatory M2 macrophage polarization, characterised by overexpression of signature proteins such as TGFβ1, IL10, Arginase1, CD204, CD206, VEGF, Ym1, PDGF, MMPs, and IL4Ralpha, was associated with immune regulation, matrix deposition, tissue remodelling, protumoral functions, and graft acceptance (17, 19). The antitumoral and protumoral role of M1 and M2 macrophages, respectively, are further supported with longer survival outcomes among patients with a high M1/M2 ratio in cancers such as ovarian (20, 21) and breast (22) cancer.

ROLE OF LUNG MACROPHAGE POLARIZATION IN PULMONARY FIBROSIS

Macrophages are innate immune cells with antimicrobial phagocytic activity and also play a key role in the pathogenesis of fibrotic disease of pulmonary interstitium. Macrophages are involved at all stages of lung injury and repair, and can promote as well as inhibit fibrosis (16, 23). Airway lumen-based Alveolar macrophages (AM) with surface markers $CD11b^{low}$ $CD11c^{++}$

TABLE 1 | Macrophage subtypes, its activators and implication of cytokines and chemokines in functional response that is also regulated by microRNAs-mediated macrophage plasticity.

M1 and M2 subtypes	Polarization activators	Cytokines and chemokines	Functional response	MicroRNAs expression and its role in M1/M2 macrophage polarization
M1	LPS, IFNγ, TNFα and GM-CSF	Cytokines: TNFα, IL1β, IL6, IL8/CXCL8, IL12, IL23 Chemokines: CCL2, CCL3, CCL4, CCL5, CCL8, CCL9, CCL10, CCL11, CXCL1	Th1 response to infection; produces pro-inflammatory molecules, including TNFα and IL1, IL6, IL12, IL23	↑: miR-21 (M1⊣; M2→), -33 (M1→; M2⊣), -34a (M1→; M2⊣), -101 (M1→; M2⊣), -125b-5p (M1→; M2⊣), -146b (M1⊣), -155 (M1→; M2⊣), -342-5p (M1→) ↓: miR-125b-5p (M1⊣), let-7e (M1⊣)
M2a	IL4, IL13	Cytokines: IL10, TGFβ, IL1Rα Chemokines: CCL17, CCL22, CCL24	Th2 cells, eosinophils, basophils, and macrophages produce IL4. Facilitation of parasite encapsulation	↑: miR-124 (M1⊣; M2→), -125a-5p (M1→), -135b (M2↑), -146a (M1⊣; M2→), let-7c (M1⊣; M2→), -511-3p (M2→), -378-3p (M2→), -223 (M1⊣; M2→), ↓: miR-140 (M2→)
M2b	Immune complexes plus TLR or IL1R ligands	Cytokines: TNF, IL1β, IL6, IL10 Chemokines: CCL1	Immunoregulation with up-regulated IL10 and antigen presentation (MHC II, CD86), and down-regulated IL12	
M2c	IL10, TGFβ1 and glucocorticoids	Cytokines: IL10, TGFβ	Tissue remodelling and extracellular matrix production	
M2d	IL6 and adenosine	–	Tumour-associated immune regulation	

TNF-α, Tumour necrosis factor-α; IFN-γ, Interferon-γ, LPS, lipopolysaccharide; GM-CSF, granulocyte-macrophage colony stimulation factor; IL, interleukin; miR, microRNA.
Symbols for MicroRNA expression level: ↑, up-regulation; ↓, down-regulation.
Symbols for M1/M2 macrophage polarization: →, progression; ⊣, inhibition.

$CD169^+$, and lung parenchymal interstitial macrophages (IM) with $CD11b^+$ $CD11c^{lo}$ $CD169^-$ are the two major distinct macrophage populations contributing to lung homeostasis (24). During the development processes of tissue injury and inflammatory reaction and their subsequent progression to PF, AM and IM are polarized to different cell phenotypes - M1 and M2 macrophages, respectively (16, 25). During tissue damage and the early inflammatory phase, activation of M1 macrophages clears the pathogenic microorganisms and promotes inflammation through extracellular matrix degrading matrix metalloproteases (MMPs) and pro-inflammatory cytokines. The active cytokine milieu, including elevated Th1 cytokines, IL2, IFNγ, and TNFα, is believed to drive the classical pro-inflammatory (M1) macrophage activation, while a proportion of anti-inflammatory M2 macrophages tends to be higher in other types of interstitial lung diseases (ILDs), including idiopathic pulmonary fibrosis (IPF) (26).

The enhanced M2 macrophage polarization has been suggested to inhibit the inflammatory reaction and/or directly regulate the development and progression of fibrotic lung diseases through the production of chemokines, MMPs, tissue inhibitor of metalloproteinases (TIMPs), and fibronectin as well as, the capability of M2 to differentiate into fibrocyte-like cells that express collagen (27–30). Among ILDs, an increased proportion of M2 macrophages has been observed in granulomas of patients with sarcoidosis as compared with tuberculous granulomas (31). It still needs to be established if a higher proportion of M2 macrophages identifies a profibrotic mechanism inherent to the pathogenesis of sarcoidosis rather than as a part of a generalized wound-healing mechanism to lung inflammation and injury (30, 31). Further, activated macrophages secrete cytokines that attract and stimulate proliferation, promote survival and migration of fibroblast mediated by platelet-derived growth factor (PDGF) (32). In a recent study, inhibition of M2 macrophage polarization has been shown to inhibit bleomycin-induced IPF in rats (33). Similarly, Wang et al. reported that treatment with microcystin-leucine arginine ameliorates PF through suppressed $CD206^+$ M2-like macrophage polarization by blocking EMT and fibroblast-myofibroblast transition (FMT), and also substantial reduction of TGFβ1/Smad signaling in rat pulmonary tissues (34). Thus, pro-fibrotic processes such as EMT, FMT, and TGFβ1/Smad signaling represent potential targets in mitigating the development and/or progression of PF (**Figure 1**). Supporting the profibrotic role of M2, a recent study showed attenuation of M2 macrophage infiltration in the lung to significantly protect mice against bleomycin-induced lung injury and fibrosis through suppression of Sart1 by small interfering RNA-loaded liposomes (35).

The macrophage-based pathways implicated in PF majorly include signaling pathways such as TGFβ/Smad (36–39), Wnt/beta-catenin (37, 40–42) and interleukin signaling (43–45). Other signaling pathways reported in a limited number of studies include Lrp5/beta-Catenin (46), MAPK (23), Notch (47), PI3K-AKT-mTOR (48, 49), STAT1 and NF-kappaB (50), IGF-1 receptor (51), 4-1BB (52), NRG-1/ErbB4 (53) and M-CSF/M-CSFR (54). Deciphering the molecular mechanisms of macrophage involved in the development of PF, M2 macrophage was shown to promote EMT through the TGFβ1/Smad2 pathway in bleomycin-induced PF mouse model (39). The PF has been alleviated by pirfenidone through suppressed Wnt/GSK-3beta/beta-catenin and TGFβ1/Smad2/3 signaling pathways (37), and by neohesperidin through TGFβ1/Smad3 inhibition (36); whereas, multiwall carbon nanotubes has been reported to mediate macrophage activation and PF progression through induced TGF-beta/Smad signaling pathway (38).

Macrophage M2 promotes myofibroblast differentiation and is associated with pulmonary fibrogenesis. This process is mitigated by suppressed Wnt/beta-catenin signaling through pirfenidone (37), salinomycin administration (40), and targeted inhibition by PRI-724 (41) and ICG-001 (42). The IL signaling is implicated in PF by IL-4–mediated M2 polarization with elevated Gab 1/2 docking proteins (43), by IL-4Ralpha pathway through crystalline silica exposure (45), or by IL-13 pathway in macrophages induced through sphingosine-1-phosphate receptor-2 (44). Also, activation of p38 MAPK signaling pathway mediated through loss of a transcription factor Forkhead box M1 (FOXM1) in macrophages was shown to promote PF. Regarding its molecular substance, activation of p38 MAPK pathway in macrophages was reported with the production of pro-fibrotic mediators IL1β, IL6, and TNFα that stimulated fibroblast activation and survival, thus, exacerbating PF (23). Thus, the interplay between M1/M2 macrophage phenotypes has been suggested to play a key role in the development and progression of lung fibrosis (**Figure 1**).

MICRORNA-BASED REGULATION OF MACROPHAGE POLARIZATION IN IMMUNE RESPONSE, INFLAMMATION, AND FIBROSIS

miRNAs and Their Regulatory Role Towards Macrophage Phenotypes

MicroRNAs (miRNAs) are transcriptional regulators that participate in lung inflammatory responses (5, 55) and are also shown to mediate macrophage polarization. The macrophage subtypes release a various spectrum of cytokines and chemokines that are either pro-inflammatory (M1 phenotypes) and sometimes pro-inflammatory with enhanced tissue destruction, or wound healing and tissue repair (M2 phenotypes), both of which are also regulated through miRNAs (**Table 1**). As an example, miRNA-regulated macrophage polarization is strongly related to miRNA-124, miRNA-155, and miRNA-223. Briefly, higher expression of miRNA-124 attenuates M1 macrophage, whereas miRNA-155 promotes M1 and miRNA-223 depletion also produces M1 polarization (56) (**Supplementary Table S1**). The **Supplementary Table S1** lists a wide spectrum of miRNAs involved in macrophage polarization along with their target proteins and their plausible roles in regulating lung fibrosis.

The regulatory roles of microRNA-mediated macrophage activation and polarization in immune response and inflammation have been extensively reviewed (57–59).

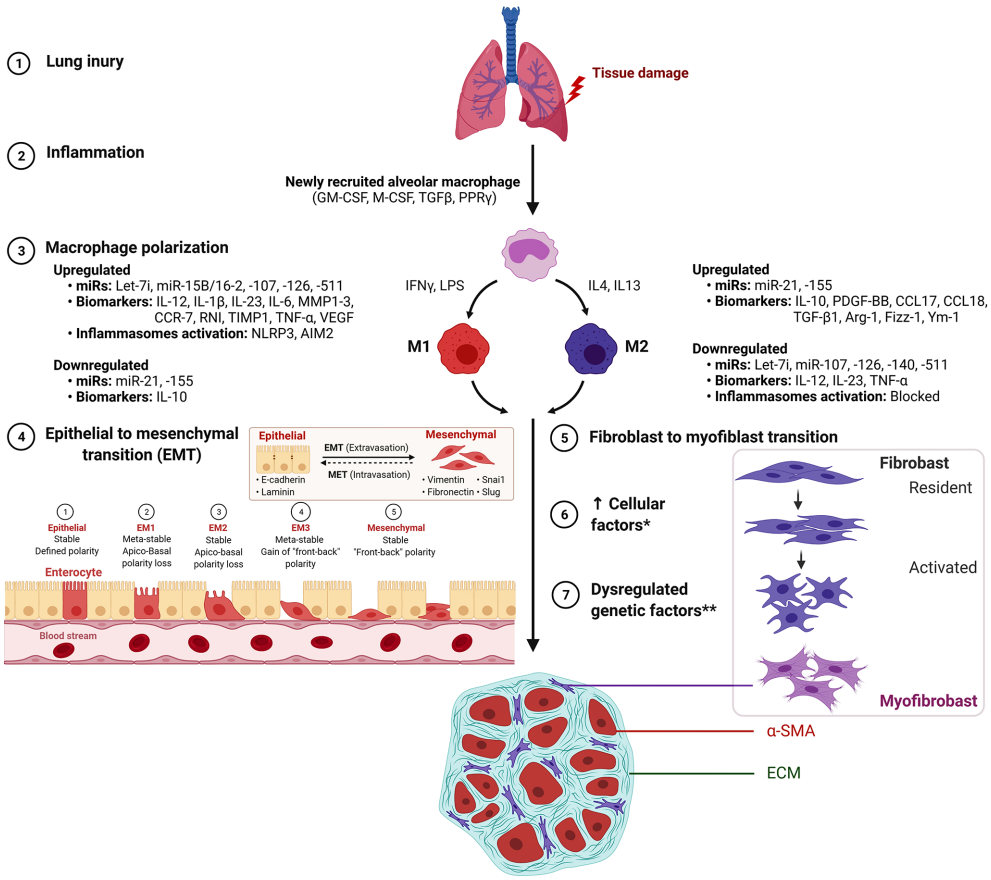

FIGURE 1 | M1 macrophage and M2 macrophage polarization during the development of pulmonary fibrosis. *Increased cellular factors include proliferation, α-smooth muscle actin (α-SMA), matrix factors, collagen, growth factors and cytokines. **Dysregulated genetic factors include upregulated miR-21 and miR-155 and down-regulated Let-7i, miR-107, mir-126, miR-140 and miR-511. Figure created with BioRender. *AIM2*, absent in melanoma 2; *ARG1*, Arginase 1; *Fizz1/RETNLB*, resistin like beta; *Ym1/Chil3*, chitinase-like 3.

This and the following section, therefore, updates the findings of miRNA-mediated macrophage polarization and modulation of pro-inflammatory M1 and/or pro-fibrotic M2 phenotypes in lung disease, in particular, lung fibrosis.

MicroRNA-17, miR-20a, and miR-106a (miR-17/20a/106a) have been shown to effectively regulate alveolar macrophage inflammatory responses such as macrophage infiltration, phagocytosis, and proinflammatory cytokine secretion through targeting leukocyte signal-regulatory protein-α (SIRPα) in both *in-vitro* and *in-vivo* assays (60). The up-regulation of miR-33 in alveolar macrophages exhibited the M1 phenotype with elevated pro-inflammatory cytokines and was demonstrated to promote granuloma formation in a murine model of chronic granulomatous disease, resembling human sarcoidosis pathology, through the suppression of anti-inflammatory lipid transporters (61). An over-expression of macrophage miR-34a has been demonstrated to favour pro-inflammatory M1 phenotype and inhibition of M2 polarization in lipopolysaccharides (LPS) induced acute lung injury (ALI) in mice (62). Similarly, miR-155 was shown to be induced during the macrophage inflammatory response and it orchestrated inflammatory cytokine production in tumour-associated macrophages (TAM). The pro-inflammatory effect of miR-155 has been indicated to promote fibrosis mediated by cross-signaling between macrophages and fibroblasts that governed upregulation of collagen synthesis through TGFβ1 signaling (63). Jaiswal et al., (64) reported overexpression of Let-7c and miR-99a miRNAs in murine bone marrow-derived macrophages (BMDMs) to mitigate Angiotensin-II-induced M1 phenotype activation and to promote M2 phenotype. This inhibition of miR-99a was further shown to reduce ovalbumin-induced Th2 dominance and alleviate allergic airways inflammation (64).

There have also been reports of miR-124 acting to attenuate M1 macrophages as a universal regulator of macrophage into the M2 subtype by decreasing NFκB activity in various subsets of monocytic cells and tissue-resident macrophages including lung macrophages (65, 66). The dysregulation of miR-142-5p and miR-130a-3p was characterised as an important factor governing the polarization of macrophages with higher levels of M2-like phenotypic markers and was associated with airway remodelling

in ovalbumin-sensitized mice (67). Another miRNA, miR-146a has been reported to modulate macrophage polarization by inhibiting Notch pathways in RAQ264.7 macrophage cell lines (68). In this context, we observed an elevated level of miR-146a in pulmonary sarcoidosis inflammation (69). Concerning miRNAs involvement in TAM polarization miR-146a-5p, miR-324-5p, miR-223-3p, miR-223-5p, miR-21, miR-125a, miR-130a, and miR-155a were characterized as oncogenic miRNA, while, miR-1207 and miR-320a as a tumour suppressor miRNA in lung cancer including non-small-cell lung carcinoma (NSCLC) (70). These reports thus emphasize the important role of miRNAs in regulating M1/M2 macrophage polarization in lung diseases in general.

Potential Role of Macrophage-Derived microRNAs in Pulmonary Fibrosis

The microRNA crosstalk influences epithelial-to-mesenchymal and fibroblast-to-myofibroblast transitions implicated in process of macrophage polarization. However, to date, only limited studies have explored the miRNA-based genetic regulation of macrophage polarization and its role in lung fibrosis. An overexpression of let-7c was reported in alveolar macrophages from fibrotic lungs in a bleomycin-induced mouse model as compared with normal lungs, and thus, indicated upregulation of let-7c in macrophages to mitigate M1 phenotype while promoting M2 phenotype polarization (71). Duru et al., (72) reviewed the miRNA-based regulation of macrophage polarization with M2 predominant population in radiation-induced lung fibrosis (RILF) and characterized miR-21 and miR-155 as pro-fibrotic, while let-7i, miR-107, mir-126, miR-140, and miR-511 as anti-fibrotic (72). The IL4 and IL13 induced increased expression of miR-142-5p and downregulated miR-130a-3p transcripts were reported to regulate macrophage profibrogenic expression in tissue samples of patients with IPF (73). The macrophage miR-155 was reported to promote lipopolysaccharide-induced ALI in mice and rats (74). Similarly, miR-155$^{-/-}$ in murine lung macrophages and fibroblasts, and in human IPF lung fibroblasts was implicated in exacerbated pathogenic PF (75). In another study, miR-140 was reported with a key protective role against RILF by inhibiting myofibroblast differentiation and inflammation, and its loss was suggested to induce lung fibrosis through reprogramming fibroblasts and M2 macrophages (76). The role of non-coding RNAs in modulating macrophage phenotypic plasticity and functional heterogeneity among different fibrotic diseases has been recently reviewed (77). These reports further highlight the plausible role of macrophage-derived microRNAs in PF.

Macrophage-Derived Exosomal miRNAs Mediate Pulmonary Fibrosis

Exosomes are cell-derived vesicles produced by several cell types that function in signaling between cells. Exosomes carry a variety of different biomolecules, such as cytokines and microRNAs, and their content may vary from progenitor or target cells. Exosomal miRNAs have also been implicated in interstitial lung diseases including pulmonary sarcoidosis (55) and IPF (78). Recently, interest has also been gained to decipher the role of macrophage-derived exosomal microRNA (miRNA) in lung fibrosis. Exosomal miRNA-328 from M2 macrophages was shown to enhance pulmonary interstitial fibroblast proliferation and promote the progression of PF in a rat model (78). Besides, macrophage-derived exosomes have been recently suggested to mitigate PF progression *via* delivery of antifibrotic miR-142-3p to alveolar epithelial cells and lung fibroblasts by repressing transforming growth factor β receptor 1 (TGFβ-R1) (79). Another study demonstrated that miRNAs contained in alveolar epithelial type-I cells derived-EVs are actively delivered into alveolar macrophages, subsequently promoting inflammasome activation, neutrophil recruitment, and M1-macrophage polarization and thus endorse pro-inflammatory responses in bacterial lung infection (80). In ALI, young mesenchymal stem cells-derived extracellular vesicles (MSC-EVs) showed higher expression of miR-223-5p and lower levels of miR-127-3p and miR-125b-5p compared with aging MSC-EVs. Further, inhibition of miR-127-3p and miR-125b-5p in BMDMs was reported to downregulate M1 and thus, supported their role in M1 macrophage polarization (81). Besides, MSC-EVs were reported to mitigate ALI at least partially through the transfer of miR-27a-3p to alveolar macrophages and promoted M2 macrophage polarization (34). MiR-27a-3p was also shown to target NFKB1 and thus, was suggested as a key regulator of M2 macrophage polarization (34). Recently, a study investigated the potential connections between arsenic and epigenetic changes that mediate M2 macrophage polarization in the development of PF and reported arsenite, elevated LncRNA H19, c-Myc, and Arg1 along with decreased let-7a to be associated with PF in mice (82). Another recent study in a mouse model reported MSC –derived exosomal miR–135b to promote M2 polarization of synovial macrophage by targeting MAPK6, thus mitigating cartilage injury (83). Thus, evidence supports the role of M2 macrophage-derived exosomal miRNA in pulmonary interstitial fibroblast proliferation and in promoting the progression of lung fibrosis. This is further supported by MSC–EVs-derived miRNAs that are suggested to mediate M2 macrophage polarization in the development of PF (34). In ALI, young and aging MSC-EVs harbours differentially expressed miRNAs associated with M1/M2 macrophage polarization (81).

CONCLUSION

The present minireview summarizes major findings on the role of macrophage polarization in diseases, in particular, PF. The non-coding regulatory miRNAs are also discussed in the context of their modulation of M1/M2 macrophage phenotypes in the development and progression of IPF. Further, exosomal miRNA from M2 macrophages favouring pulmonary interstitial fibroblast proliferation and promoting the progression of PF are also described.

In summary, the regulation of macrophage polarization by miRNA is suggested to represent one of the key pathogenetic factors in the development and progression of PF. Further research focused on distinct levels of these processes will

undoubtedly provide updated information. Apart from detailing our current theoretical knowledge, it could be translated into future diagnostic approaches and/or designing novel therapeutic strategies helping to combat IPF, which despite the advancements still constitutes a major debilitating disease.

AUTHOR CONTRIBUTIONS

AK and MP contributed to the conception and writing of this review. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded in part from Palacky University and Czech government (IGA PU 2021_014, RVO00098892 and CZ.02.1.01/0.0/0.0/16_019/0000868, ENOCH).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.678457/full#supplementary-material

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frontiers in Immunology

MINI REVIEW
published: 20 August 2021
doi: 10.3389/fimmu.2021.690375

Interpreting Immunoregulation in Lung Fibrosis: A New Branch of the Immune Model

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Immunostimulation is recognized as an important contribution in lung fibrosis in some animal models and patient subsets. With this review, we illustrate an additional scenario covering the possible implication of immunoregulation during fibrogenesis. Available animal and human data indicate that pulmonary fibrosis also includes diverse and discrete immunoregulating populations comprising regulatory lymphocytes (T and B regs) and myeloid cells (immunosuppressive macrophages and myeloid-derived suppressive cells; MDSC). They are initially recruited to limit the establishment of deleterious inflammation but participate in the development of lung fibrosis by producing immunoregulatory mediators (mainly TGF-β1 and IL-10) that directly or indirectly stimulate fibroblasts and matrix protein deposition. The existence of this silent immunoregulatory environment sustains an alternative mechanism of fibrosis that explains why in some conditions neither pro-inflammatory cytokine deficiency nor steroid and immunosuppressive therapies limit lung fibrosis. Therefore, the persistent presence of immunoregulation is an important parameter to consider for refining therapeutical strategies in lung fibrotic disorders under non-immunostimulatory conditions.

Keywords: immunosuppression, inflammation, regulatory lymphocytes and myeloid cells, carbon nanotubes, silica and asbestos

OPEN ACCESS

Edited by:
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University of Catania, Italy

Reviewed by:
Athol Wells,
Royal Brompton Hospital, United Kingdom
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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *02 April 2021*
Accepted: *06 July 2021*
Published: *20 August 2021*

Citation:
Huaux F (2021) Interpreting Immunoregulation in Lung Fibrosis: A New Branch of the Immune Model. Front. Immunol. 12:690375. doi: 10.3389/fimmu.2021.690375

DIVERSE LUNG FIBROTIC DISEASES WITH DIVERSE MECHANISMS

Repair of damaged tissue is a fundamental biological process that allows the ordered replacement of dead or injured cells. However, although initially beneficial, the healing process becomes pathogenic when it is not controlled appropriately, leading to considerable tissue remodeling and the formation of permanent scar tissue and fibrosis (1). The major site of histopathological lung fibrosis is the interstitium, which consists of alveolar epithelium, pulmonary capillary endothelium, basement membrane, and perivascular and perilymphatic tissues. There are now almost 300 distinct injurious or inflammatory causes of interstitial lung disease that can result in progressive lung scarring. Many others are referred as idiopathic (i.e. idiopathic pulmonary fibrosis, or IPF) when it arises for no obvious reason (2). Silicosis is one the oldest recorded interstitial lung disease characterized by alveolitis and progressive nodular fibrosis (3). This fibroproliferative disorder can be traced back to ancient Egypt, where it was caused by inhalation of crystalline silica. It is also well known that long-

term asbestos fiber inhalation causes asbestosis also comprising persistent nodular fibrosis (4). Although the incidence of silicosis and asbestosis has diminished, it continues to be a major cause of occupational lung disease in exposed workers, particularly in developing nations (5). More recently, carbon nanotubes (CNT), which present some of the physical characteristics of asbestos (long and rigid), also induce granulomatous lung disorder characterized by persistent immune responses, culminating in the development of lung fibrosis (6). Additionally, lung fibrogenic reaction may arise from other exogenous environmental stimuli such as organic dusts (bacterial, fungal and avian antigens) (7).

Suppression of chronic inflammation by immunosuppressive therapy turn off pulmonary fibrogenesis in some sub-groups of patients (e.g. non-specific interstitial pneumonia). These findings argue that inflammation represents a major pathological pathway in lung fibrosis (8, 9). A pathogenesis paradigm that did not require inflammation and immunostimulation was, however, proposed since there is little evidence of inflammation in the histopathological samples obtained from susceptible ageing individuals developing IPF undergoing surgical lung biopsy. Treatment with anti-inflammatory agents, such as steroids or anti-cytokines, seemed to have no effect on disease progression and outcome (10, 11). These last clinical observations strongly suggested that inflammation represents an important but dispensable event and that other mechanisms than immunostimulation exist or co-exist in pulmonary fibrosis (12, 13).

THE RELEVANCE OF RELIABLE ANIMAL MODELS OF LUNG FIBROSIS

It is unanimously recognized that animal models currently available are particularly useful to discover new pro-fibrotic mediators and pathological avenues related or unrelated to the inflammatory concept. The development of experimental models producing long-lasting lesions akin to those seen in human fibrosis and defined by progressive and irreversible matrix deposition has already been the subject of many studies (14). Presently, chemical insults such as those caused by bleomycin are widely used to induce fibrotic disease. Many research studies have focused on changes in inflammatory phenomena after a single instillation of bleomycin and have yielded similar findings: the lung injuries caused by bleomycin induce acute recruitment and activation of inflammatory leukocytes which produce mediators (cytokines, chemokines, growth factors, and prostaglandins) activating fibroblast and driving fibrotic disease. While bleomycin represents the preferred molecule in this context, the lung fibrosis obtained by an unique dose of this drug is not systematically progressive and resolves itself over time after bleomycin metabolization (15).

This discrepancy led researchers to explore other models and discover new pathological avenues not apparent in the acute bleomycin-induced fibrosis model, which mainly (if not exclusively) supports that inflammation drives fibrosis. Multiple instillation of bleomycin has previously been shown to recapitulate the epithelial remodeling and fibrosis progression in the lungs of patients with IPF (16). Indeed, a repetitive alveolar epithelial injury caused by repetitive intratracheal injections of bleomycin is now proposed as the initial and major event that triggers a series of repair pathways that are in some way aberrant, leading to inappropriate fibrosis. Repeated injuries prevent epithelial cell regeneration, re-epithelialization and epithelial structure restauration and lead to a sustained disruption of alveolar epithelial morphology and fibrogenesis in injured alveoli. This experimental approach thus represents an ultimate model to address mechanistic questions on epithelial remodeling (17).

Murine models of univocal chronic lung responses and progressive fibrotic lesions using inorganic particles (crystalline silica, asbestos and CNT) are well known to cover severe fibrotic respiratory disorders in exposed human. Instillation of mineral particles into mouse and rat lungs results in the development of fibrotic nodules that resemble lesions which develop in humans. Rodents exposed to silica particles present alveolar fibrotic nodules, increased pulmonary lymphoid tissue and enhanced numbers of macrophages in the broncho-alveolar lavage fluid. Interestingly, silica, asbestos and CNT are retained in the lung and the response is characterized by a persistent and progressive fibrosis (18). Based on findings on particle-induced pulmonary fibrosis in mice, it has been recently proposed a new hypothesis that the fibrotic response may result from an exaggerated and persistent immunoregulating responses instead of or along with inflammation. This new concept have been confirmed in human silicosis but also in other animal models and patients. These results, summarized hereafter, may explain how lung fibrosis can develop in absence of immunostimulation.

HISTORY OF IMMUNOREGULATING SURVEILLANCE

The quality, magnitude, and persistence of immune reactions results from the balance between immunostimulating and immunoregulating responses. The basic mechanism of immunoregulation comprises an interconnecting system involving diverse anti-inflammatory and immunosuppressive cytokines, lymphocytes and myeloid cells. There is now evidence that this sophisticated immunoregulatory systems is crucial in maintaining immune homeostasis and resolving persistent inflammation. Immunoregulatory alterations are often implicated in the pathogenesis of several inflammatory diseases such as infection, allergy, and autoimmune disorders. Conversely, uncontrolled and exacerbated immunoregulation is strategically exploited by cancer cells to survive, proliferate and escape detection by anti-tumor effector T lymphocytes (19). A well-knowledge of immunoregulation now serves for future strategies of diagnosis and treatment. The following sections are devoted to describe (i) historical aspects of immunoregulatory mechanisms (**Figure 1**) and (ii) matching evidence supporting a deranged immunoregulation in pulmonary fibrogenesis (**Figure 2**).

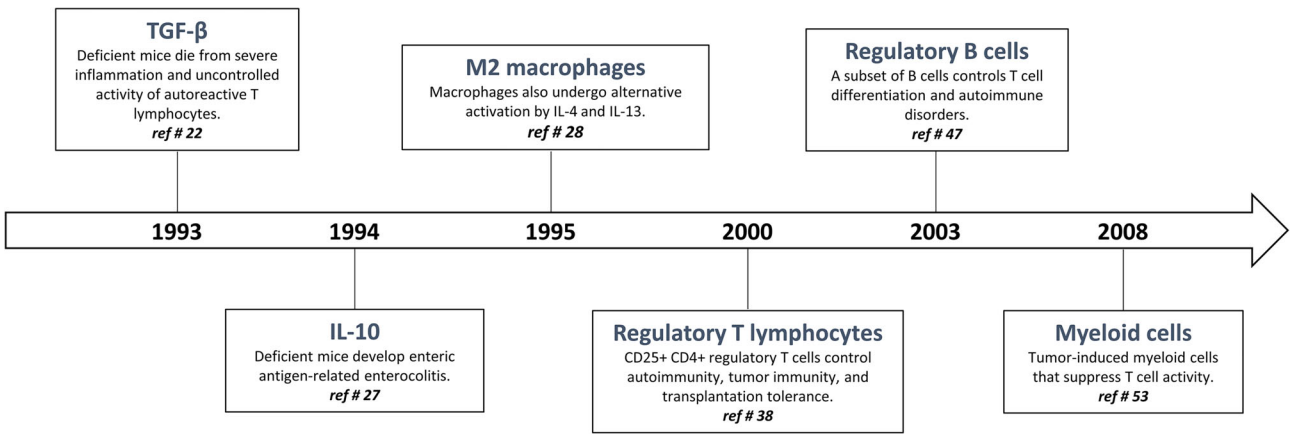

FIGURE 1 | Timeline: historical progression of immunoregulation in the literature.

TGF-β1 and IL-10 as Master Immunoregulating Cytokines

The most striking and non-redundant function of TGF-β1 is to regulate the immunostimulatory and inflammatory responses by orchestrating immunoregulation (20, 21). Indeed, in 1993, Kulkarni and Karlsson demonstrated that mice deficient for the gene encoding TGF-β1 die rapidly from a multi-systemic inflammatory syndrome related to deleterious T cell autoreactivity (22) (**Figure 1**). To become active, mature TGF-β1 must be released from the LAP (latency-associated peptide), a process referred to as TGF-β1 activation (23). The best-characterized mechanism of TGF-β1 activation imply conformational changes in the latent TGF-β1 molecule involving interaction of latent TGF-β1 with integrin αVβ8 and GARP (glycoprotein A repeats predominant protein or LRRC32) (21). The binding of the active TGF-β1 peptide to its receptors leads to a series of signaling events that are mainly mediated by SMAD2/3/4 complex that binds SMAD response elements located in the promoter regions of many genes involved in the immunoregulatory responses (21).

The cytokine interleukin-10 (IL-10) was originally described as a 'cytokine synthesis inhibitory factor' (CSIF), which was produced by mouse T helper 2 (Th2) clones and inhibited cytokine production by activated Th1 clones (24). IL-10 reportedly inhibits nuclear factor-κB (NF-κB) activation and expression of the most inducible cytokines and chemokines that are involved in inflammation. The anti-inflammatory activities of IL-10 also include induction of IL-1 receptor antagonist (IL-1ra) (24) and soluble p55 and p75 TNF receptor production, indicating that IL-10 induces a shift from production of pro-inflammatory to anti-inflammatory mediators (25). IL-10 strongly inhibits cytokine production and proliferation of CD4+ effector T cells *via* its down-regulatory effects on APC function. IL-10 also induce the differentiation of naive Th cells in a subset of regulatory T cells which are defined by their

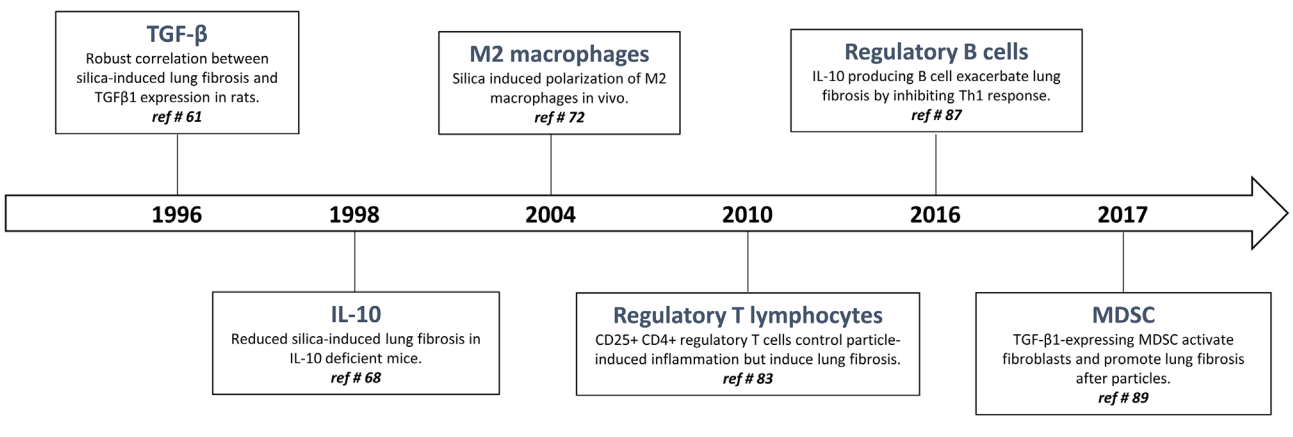

FIGURE 2 | Timeline: historical progression of immunoregulation in particle-induced lung fibrosis.

ability to produce high levels of IL-10 and TGF-β1 (26). The IL-10-related potent immunosuppressive functions and its proximity to TGF-β1 are perfectly illustrated by the work of Kuhn and colleagues demonstrating that IL-10 deficient mice suffer from chronic enterocolitis characterized by extensive inflammatory reactions and aberrant expression of major histocompatibility complex class II molecules (27) (**Figure 1**).

M2/Immunoregulatory Macrophages and Type 2 Like Immune Response

During the 1990s, Gordon and colleagues demonstrated that IL-4 and IL-13 induced an "alternative" form of activation in macrophages beside the "classically" polarized macrophages (28, 29) (**Figure 1**). In 2000, Mills and colleagues identified the fundamental M1/M2 polarization axis of macrophages and proposed that M1/inflammatory macrophages are necessary to eliminate infectious organisms and tumor cells while M2/immunoregulatory macrophages are implicated in repair process and wounds. Importantly, the uncontrolled M1 and M2 activation is associated with tissue injury and cancer progression, respectively (30, 31).

The so-called M2 phenotype express anti-inflammatory and immunosuppressive mediators such as IL-10 and TGF-β1, specific chemokines (CCL17 and CCL22, C-C motif), the subclass B scavenger receptor (CD 163), and the mannose C receptor type 1 (CD206 or MRC1) (32). The TGF-β1- and IL-10 producing M2 macrophages are thus intimately involved in the regulation of immune responses. Transcription factors such as IRF4 and STAT6 are required for M2 macrophage activation and differentiation. These different stimuli are likely to initiate during Th2-like responses (33). Th2 responses and cytokines (mainly IL-4 and IL-13) induce the differentiation of M2 macrophages.

The model categorizing macrophages (or T helper cells) in only two divergent groups M1 vs M2 (or Th1 and Th2) does not cover the intrinsic heterogeneity of macrophages (and T lymphocytes) now identified and described in diseases. A consensus now refers to M1-like or M2 like-subpopulations for avoiding oversimplified categories and highlighting the diversity and versatility of macrophages (34). M2-like macrophages possessing various functions, characteristics and deleterious activities are well known to be crucial in asthma, interstitial lung diseases and cancer (35, 36).

Regulatory T Cells

Regulatory T cells were initially described by Gershon et al. in the early 1970s and were called suppressive T cells because they practiced immune suppression (37). Unfortunately, despite the importance of these studies there was extensive skepticism in the immunological field about the existence of these cells, and suppressive T cells left the centre stage of immunology for decades. However, in 1996, Sakaguchi and colleagues rehabilitated the concept of "suppressive T cells", now renamed "regulatory T cells" (38) (**Figure 1**). CD4+ Foxp3+ regulatory T cells (T regs) constitute a thymus-derived sub-population of CD4+ T lymphocytes that constitutively express the transcription factor Foxp3 (forkhead box P3), required for their development and their anti-inflammatory and immunosuppressive function (39, 40). T regs are developed in the thymus (naturally occurring T regs; nT regs) or are differentiated from naive T cells in the presence of TGF-β1 following T-cell receptor stimulation (induced T regs; iT regs) (41). These lymphocytes are crucial to maintain tolerance by downregulating undesired immune responses to self and non-self-antigens (42, 43). Absence or defective function of regulatory T cells is correlated with the development of immuno-pathologies such as auto-immune diseases (e.g. psoriasis and rheumatoid arthritis) and asthma. In contrast, their accumulation is associated with tolerance and implicated, for instance, in the development of cancer (44). Several mechanisms have been proposed to explain immunosuppressive functions of T regs. These include secretion by T regs of immunosuppressive cytokines, cell-contact-dependent suppression and functional modulation. Most *in vivo* studies indicate that T regs mediate immunosuppression by producing IL-10 and TGF-β1 (45). The strong immunosuppressive activity T regs is also related to their capacity to regulate the polarization and function of effector T lymphocytes (T eff, i.e. Th1, Th2, and Th17).

B Regs

The historical description of the new B-cell subtype named regulatory B-cells (B regs) dates back to 1974. In a mouse model of eczema, adoptive transfer of total splenocytes had a suppressive effect, whereas adoptive transfer of splenocytes from which B-lymphocytes were removed had no effect (46). In 2003, the regulatory role of B cells was shown in a mouse model of experimental autoimmune disease and this regulatory role was attributed to the ability of B cells to produce IL-10 (47) (**Figure 1**). B cells have been shown to restore homeostasis, possess important immunosuppressive functions and play a key role in disease control and immune tolerance (48, 49). IL-10-producing B cells produce anti-inflammatory IgG4 and activate Treg cells. By promoting M2-like macrophage polarization, B regs cells also reduce auto-reactive Th1 and Th17 cell responses and limit damaging inflammatory responses. In contrast, B regs impair cytotoxic T-cell and NK cell responses to tumor cells and thereby promote progression of cancer (50). Beside IL-10, the exact mechanism by which B regs act *in vivo* remains unclear. A close contact between B-lymphocytes and the T lymphocytes, notably through the CD40-CD40L pathway are required (51, 52).

Myeloid Derived Suppressive Cells

MDSC were first identified by Van Ginderachter and his team in 2008. They detected a discrete myeloid-derived suppressor cell subpopulations with T cell-suppressive activity invading tumors. Thus, it was suggested that this MDSC population is responsible for down-regulating immune responses related to tumor progression and metastasis (53) (**Figure 1**). MDSC are now defined as heterogeneous and immature myeloid cells generated from the bone marrow and active in cancer development and inflammation regulation (54). Under normal physiological conditions, MDSC are rapidly differentiated into mature granulocytes, macrophages and dendritic cells. However, the differentiation of these cells into mature myeloid cells is blocked in chronic pathologies such as cancer (54). Two categories of these cells can be observed, one with granulocyte

morphology and the other with monocyte morphology (55). MDSC suppress CD8, CD4 T cell activities and NK cell activity by inducing a suppressive environment (56). Several different mechanisms of action have been identified to explain the strong immunosuppressive activity of MDSC involving direct cell contacts and/or the production of several released mediators (54). MDSCs have the ability to induce differentiation and expansion of regulatory T cells by producing cytokines such as IL-10 and TGF-β1 or *via* CD40 (57). These cells can also deprive T cells of amino acids essential for their activity such as arginine, which is necessary for lymphocyte activation. Arginase, highly expressed by MDSCs, metabolizes arginine to urea and ornithine. Indoleamine-2.3-dioxygenase (IDO), an enzyme that catalyzes tryptophan, an essential amino acid for T cells, might also be involved in the immunosuppressive mechanisms of MDSC (54).

THE DISCOVERY OF IMMUNOREGULATION IN PARTICLE-INDUCED LUNG FIBROSIS

Inorganic particle-induced lung fibrosis consists of an uncontrolled inflammation of the respiratory system tissues characterized by a chronic macrophage and neutrophil infiltration that ultimately causes fibrosis (58). Based on the widespread study of Piguet and colleagues published in 1990 in Nature, it is accepted that silica particles activate intracellular signaling pathways that culminate in the production of the pro-inflammatory mediator TNF-α, which is crucial in driving alveolitis and lung fibrosis (59). Additionally, a recent and pivotal study demonstrated that silica activate caspase-1 in a NALP3 inflammasome-dependent manner leading to the processing and secretion of the pro-inflammatory cytokine IL-1β. Evidence demonstrate that IL-1β initiates a cascade of reactions leading to inflammation and uncontrolled fibrosis (60). These observations support the concept that inflammation and sustained expression of inflammatory cell-secreted pro-fibrotic cytokines participate in the etiology of fibroproliferative diseases associated to inorganic particles.

The interconnection between inflammation and fibrosis was, however, questioned when active TGF-β1 was pointed as a key pro-fibrotic factor in response to particles. In silicosis and asbestosis, TGF-β1 expression has been found to be increased in lung tissues from patients with accelerated fibrotic disease progression (7) (**Figure 2**). The crucial activity of TGF-β1 during fibrogenesis has been unanimously recognized in the experimental studies using silica-, asbestos- and CNT-treated mice (6, 61–63). TGF-β1 is a major profibrogenic cytokine by delaying epithelial wound healing and expanding mesenchymal compartment (64). While the activity of TGF-β1 on fibroblasts is undisputed and not anymore debated, these observations intrigued immunologists because the presence of this powerful immunosuppressive cytokine (see above) was not in line with the IL-1/TNF-related immunostimulation axis.

Additional data challenged the inflammatory concept. IL-10, another potent immunoregulatory cytokine, has also fibrogenic activities in responses to fibrogenic particles. To examine the immune responses over the whole course of the pathological process induced by fibers and particles, validated experimental models in mice and rats with contrasting sensitivities were developed (65). By comparing these models and using deficient mice, it was newly discovered that IL-10 produced by particle-activated macrophages limit neutrophilic inflammation but is a key mediator implicated in the fibrotic lung response to silica by controlling the balance between pro- and anti-fibrotic mediator production (respectively TGF-β1 and prostaglandin E2) (65–68) (**Figure 2**). IL-10 is also a prevailing inducer of type-2 immune responses in particular M2-like and Th2-like pro-fibrotic cells (69, 70). The fibrogenic activity of macrophage-derived IL-10 and M2-like macrophages is not limited to silicosis (71, 72). Indeed, these macrophages are also implicated in the fibrotic lung response induced by asbestos and CNT (6, 73, 74) (**Figure 2**).

The little evidence of inflammation in the histopathological samples obtained from silicotic patients undergoing surgical lung biopsy (75) consolidate the pathogenesis paradigm that did not require inflammation. In contrast, high levels of IL-10 in BAL or serum were detected in patients with silicosis and asbestosis in the absence of clear inflammatory reaction (73, 76). Treatment with anti-inflammatory agents, such as corticosteroids, seemed to have no effect on outcome of these fibrotic diseases (75, 77, 78). These human observation are in accordance with several animal data indicating that steroid and numerous anti-inflammatory strategies reduce particle-induced lung inflammation but not IL-10-TGF-β1 expression and lung fibrosis in sensitive animals (79, 80). At this time, it was thus suggested that these two immunoregulatory cytokines are the major event that triggers a series of repair pathways that are aberrant and lead to inappropriate fibrosis under non-inflammatory conditions.

According to the concept that inflammation is responsible for fibrogenesis and given their ability to dampen inflammatory responses, CD4+ regulatory T lymphocytes (T regs) were first supposed to slow the progression of fibrosis (81). Indeed, it is well recognized that T regs, by restraining effector T cell responses and inducing tolerance through the production of TGF-β1 and/or IL-10, control lung inflammatory disorders (82). However, CD4+ Foxp3+ regulatory T cells are persistently recruited during long-term responses to particles (69, 83) (**Figure 2**). T regs purified from the lung of silica-treated mice highly express fibrogenic mediators, stimulate fibroblast proliferation *in vitro* and increase lung collagen deposition upon transfer into naive mice. Interestingly, the effects of T regs on fibroblast proliferation recapitulate the main function of PDGF as a primary mitogen for fibroblasts during lung fibrosis. The stimulatory effect of T regs on fibroblasts *in vitro* and *in vivo* was completely abolished by a PDGF receptor inhibitor (imatinib mesylate). It is thus likely that the role of T regs is to increase tissue fibroblast numbers, and consequently, amplify the subsequent fibroblast activation and collagen deposition (83). The pro-fibrogenic functions of T regs also comprise a stimulatory activity on Th2-like pro-fibrotic cells

(69). In contrast to what it is thought, T regs thus participate in the fibrogenesis and are able to aggravate lung fibrosis induced by fibrogenic particles (i.e. silica and asbestos) in absence of inflammation (74). Finally, Xin and colleagues also observed a clear balance between inflammatory Th effector cells (Th1 and Th17) and T regs in mice treated with silica (84, 85). Neutralization of T regs-immunosuppressive activity resulted in enhanced lung inflammation and Th17 accumulation further demonstrating that T regs are initially recruited to control inflammatory responses (85, 86).

Based on the immunosuppressive profile of silica-treated mice, other immunosuppressive populations among lymphocytes have been investigated. Regulatory B lymphocytes (B regs), another immunosuppressive cell population (see above), also accumulate and participate in granuloma formation and fibrosis development by producing lung IL-10 in mice treated with silica. A heightened accumulation of inflammatory T effector cells (Th1 and Th17) but limited pulmonary fibrosis were observed in B reg-depleted mice treated with silica (87) (**Figure 2**). IL-10-producing B regs were also noted in silicotic patients in absence of inflammatory reaction (76). B regs exacerbate fibrogenesis by stimulating T regs functions and polarization *via* the release of IL-10 (88). These findings indicated that the accumulation and polarization of immunoregulatory lymphocytes is a central event during particle-induced pulmonary fibrosis with limited immunostimulation.

Finally, M-MDSC are also progressively and specifically accumulated during the development of pulmonary fibrosis. Indeed, a close relationship between the accumulation of MDSC, pulmonary immunosuppression and lung fibrosis was clearly found in mice treated with silica or CNT (89, 90) (**Figure 2**). Beside M2-like macrophages and T regs, immunosuppressive MDSC also expressed TGF-β1 conferring to these myeloid cells the capacity to down regulate T effector cell activity (91). In order to define their role in fibrosis, lung MDSC were purified from silica-treated mice and co-cultured with naive lung fibroblasts. MDSC stimulates lung fibroblasts to release tissue inhibitor of metalloproteinase and collagenolytic activity by expressing TGF-β1. They contribute to lung fibrogenesis by inducing a non-degrading collagen microenvironment (89).

The persistent accumulation of immunoregulatory macrophages, lymphocytes and myeloid cells in the lung during the progressive establishment of experimental silicosis is consistent with studies on tuberculosis (92) and lung cancer (93) that often affect the silicotic patients. Indeed, these cells control neutrophilic inflammation and anti-tumor T effector lymphocytes dedicated to microorganism and tumoral cell elimination. However, authors found that human silicosis is accompanied by a reduced number of blood regulatory T cells and speculated that the absence of these regulatory cells may explain the occurrence of autoimmune diseases (e.g. systemic scleroderma, rheumatoid arthritis and systemic lupus erythematosus) (94, 95). These conflicting results highlight the possible limitations of the mouse models. Injection of silica in mice does not fulfill all conditions encountered in patients with silicosis (infection, cancer and autoimmune diseases). Moreover, experimental models used to study the effects of silica are relatively short compared to human silicosis. These contradictory results also suggest that the fibrogenic activity of immunoregulation is only effective in a non-inflammatory environment.

INCLUSIVE IMPLICATION OF IMMUNOREGULATORY MEDIATORS AND CELLS IN LUNG FIBROTIC DISEASES

Immunoregulation is operative in different fibrotic context and not specifically concomitant to particle-induced fibrogenesis. Indeed, recent investigations using complementary mouse models of lung fibrosis also reported that inflammation is not an absolute prerequisite for fibrogenesis and that the fibrotic pathological process can develop through immunoregulation.

IL-10 induce lung collagen deposition and fibrosis when overexpressed in transgenic mice (96, 97). These observations correspond to those noted when TGF-β1 is overexpressed in murine lungs (98). Intratracheal transfer of adenoviral recombinant IL-10 or TGF-β1 to murine lung has been shown to dramatically increase fibroblast accumulation and expression of type I and type III collagen around airways as well as in the pulmonary interstitium (70, 99, 100). The pro-fibrotic function of IL-10 is associated to infiltration of fibrocytes and M2-like macrophages (97). In addition; IL-10 and TGF-β1 contribute to lung injury and fibrosis by sensitizing epithelial cells to apoptosis (98, 101). TIM-3+ M2-like macrophages that possess strong immunoregulatory functions is now considered as an important pro-fibrotic population by being a key source of TGF-β1 and IL-10. Adoptive transfer of this immunoregulatory population promoted bleomycin-induced lung fibrosis by highly secreting TGF-β1 and IL-10 (102). Recent data at single cell level suggest that pro-fibrotic macrophages did not demonstrate a shaped and clear M2-like polarization but resemble to alveolar macrophages deriving from monocytes (103). Altogether, these studies indicated that the long-term overexpression of M2 like-related immunoregulating cytokines that suppress inflammation such as TGF-β1 and IL-10 are also profibrotic factors in the lung.

In vivo expansion of lung CD4+CD25+Foxp3+ T regs cells during bleomycin-induced lung fibrosis unexpectedly led to an increase of fibrogenesis. More important, this pro-fibrotic effect was a lymphocyte-dependent process. A marked down-regulation of type 1 and an increase of type 2 immune responses in the lungs were proposed to explain T reg fibrogenic activity (104, 105). These observations were corroborated by Chakraborthy and colleagues. Depletion of T regs ameliorate bleomycin-induced acute lung fibrosis by modulating Th effector cell balance. In addition, adoptive transfer of Sema 7a1 T regs induces fibrosis in the TGF-β1–exposed murine lung by altering the production of T-cell mediators (106).

Accumulation of MDSC with functional immunosuppressive activity was also noted in bleomycin-induced experimental pulmonary fibrosis and their potential role in fibroblast activation investigated (107, 108). Purified MDSC differentiate into lung fibroblasts as manifested by significantly elevated α-smooth muscle actin and TGF-β1 expression. Differentiation of MDSC

into fibrocytes could also be possible during tissue repair processes. Indeed, there is some evidence that peripheral monocytes and MDSCs differentiate into fibrocytes (109, 110). These cells have the ability to promote fibroblast proliferation, migration, and collagen production but also differentiate into myofibroblasts (111, 112). Altogether, these previous experimental studies are well in accordance with the immunoregulation concept elaborated from findings reported after particle exposure and supporting that persitent immunoregulatory environment is profibrotic in absence of immunostimulation (**Figure 3**).

The disconnection between fibrosis and inflammation is not limited to patients developing silicosis or asbestosis. Clinical measurements of inflammation in IPF patients developing fibrosis fail to correlate scar formation with inflammation and immunostimulation. Corticosteroids have never conclusively been shown to significantly alter the course of pulmonary fibrosis in patients and have, at best, limited efficacy in the treatment of scarring disease (113).

Interestingly, the expression TGF-β1 and IL-10 mainly by macrophages was increased in lung biopsies from patients with IPF compared with controls, suggesting the presence of M2-like macrophages (114, 115). Single-cell multi-omics approaches characterizing macrophage populations in health and lung fibrotic disease at high resolution tempered a clear accumulation of M2-like polarized macrophages in fibrotic tissue. Fibrotic macrophages in IPF patients are now identified as proliferating SSP1-positive macrophages (116, 117). However, it has been suggested that Th2-related cytokines such IL-4 and IL-3 as well as M-CSF activate this subset of proliferating macrophages (117). Clinical reports showed increased number and function of CD4+CD25+FoxP3+ T regs in the

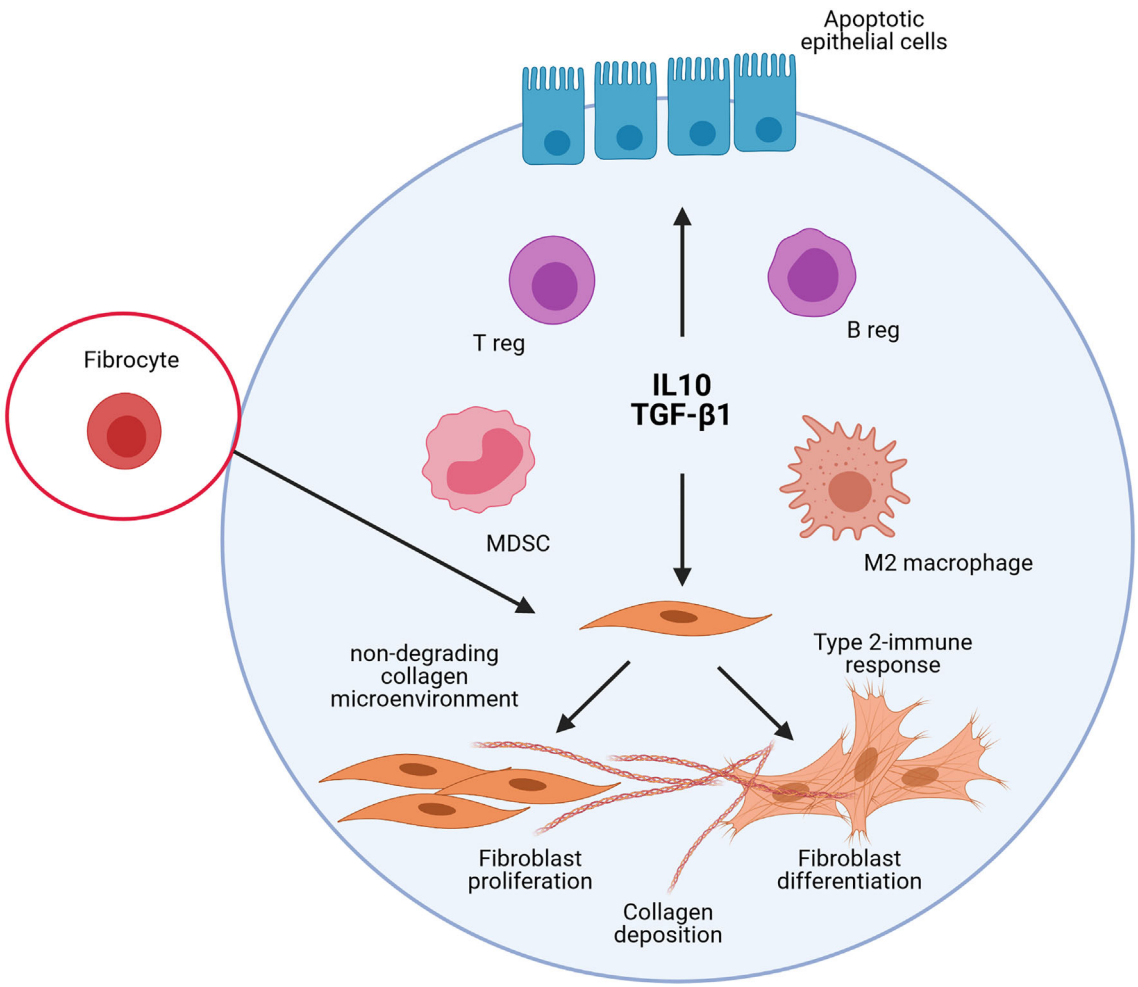

FIGURE 3 | Schematic representation of the implication of the different immunoregulatory cells and mediators in lung fibrosis. The studies on experimental lung fibrosis have highlighted a new pathological pathway, which suggests that pulmonary fibrosis is orchestrated by an immunoregulatory response characterized by a persistent accumulation of pulmonary immunoregulatory cells (regulatory T and B lymphocytes, i.e. T and B regs; regulatory myeloid cells, i.e. M2/immunosuppressive macrophages and Myeloid Derived Suppressive Cells, MDSC) and a sustained production of IL-10 and TGF-β1. The persistent accumulation of these elements to control immunostimulatory responses in the lungs contribute, however, to pulmonary fibrosis. This sustains the view that immunoregulation is important pro-fibrotic environment that could markedly explain the development of the lung fibrotic response under non-inflammatory conditions.

lungs and blood of patients with IPF associated with a more progressive clinical course (106, 116, 118–120). More recently, the importance of MDSC was also suggested by the observation of MDSC accumulation in IPF lungs (108, 121).

In conclusion, the presence of immunoregulatory microenvironment may be relevant to human pathology. Based on these recent findings, it is important to consider the possibility that regulatory lymphocytes and myeloid cells may also drive fibroproliferative wound healing. Consequently, these cells and their cytokine products could become therapeutic targets in patients developing fibrotic diseases. Particularly, these studies identified as potentially important targets the production of TGF-β1 and IL-10 by immunoregulatory cells in non-immunostimulatory conditions. The clinical separation of patients reaches from immunoregulatory or immunostimulatory scar formation could offer novel markers for the pathological assessment as well as novel regulators and drug targets to treat pulmonary fibroproliferative diseases.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

This work was funded by the Actions de Recherche Concertées, Fédération Wallonie-Bruxelles (ARC 19/24-098, CYTAID), Fondation Contre le Cancer (2019-219), Fonds de la Recherche Scientifique (FNRS, PDR T.0119.13), ANSES (Agence nationale française de sécurité sanitaire de l'alimentation, de l'environnement et du travail, MacFibOsis) and European Commission under H2020 project (Contract no. 874707, Eximious). FH is a Senior Research Associate with the FNRS, Belgium.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 04 October 2021
doi: 10.3389/fimmu.2021.687397

Commonalities Between ARDS, Pulmonary Fibrosis and COVID-19: The Potential of Autotaxin as a Therapeutic Target

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *29 March 2021*
Accepted: *13 August 2021*
Published: *04 October 2021*

Citation:
Ntatsoulis K, Karampitsakos T, Tsitoura E, Stylianaki E-A, Matralis AN, Tzouvelekis A, Antoniou K and Aidinis V (2021) Commonalities Between ARDS, Pulmonary Fibrosis and COVID-19: The Potential of Autotaxin as a Therapeutic Target. Front. Immunol. 12:687397. doi: 10.3389/fimmu.2021.687397

Severe COVID-19 is characterized by acute respiratory distress syndrome (ARDS)-like hyperinflammation and endothelial dysfunction, that can lead to respiratory and multi organ failure and death. Interstitial lung diseases (ILD) and pulmonary fibrosis confer an increased risk for severe disease, while a subset of COVID-19-related ARDS surviving patients will develop a fibroproliferative response that can persist post hospitalization. Autotaxin (ATX) is a secreted lysophospholipase D, largely responsible for the extracellular production of lysophosphatidic acid (LPA), a pleiotropic signaling lysophospholipid with multiple effects in pulmonary and immune cells. In this review, we discuss the similarities of COVID-19, ARDS and ILDs, and suggest ATX as a possible pathologic link and a potential common therapeutic target.

Keywords: COVID-19, ARDS, pulmonary fibrosis, Autotaxin, lysophosphatidic acid

COVID-19

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection leads to the development of Coronavirus Disease 2019 (COVID-19), proclaimed pandemic on March 11, 2020 (1, 2). Upon airborne, mainly, CoV-2 transmission, the viral spike (S) glycoprotein mediates viral entry *via* binding to angiotensin-converting enzyme 2 (ACE2), supported by the transmembrane protease serine protease 2 (TMPRSS2) that proteolytically processes the S protein (3, 4). Infected cells in the lung, as detected with immunocytochemistry, include the upper airway bronchiolar epithelium and submucosal gland epithelium, as well as alveolar epithelial cells and macrophages (1). ACE2 is distributed mainly in the intestine, heart, kidney, as well as the lung, where alveolar epithelial type II cells are the major expressing cells. TMPRSS2 is highly expressed in several tissues; in the lung is co- expressed with ACE2 in nasal epithelial cells and alveolar epithelial type II cells, which might partially explain the tissue tropism of CoV-2 infection (3, 4).

CoV-2 infection is either asymptomatic or causes only mild respiratory diseases (non-pneumonia or mild pneumonia) in most individuals. However, a significant number of elderly individuals, frequently with comorbidities (such as cardiovascular diseases, diabetes, obesity), will

develop a more severe form of the disease and will require hospital care (1, 2). COVID-19 most common clinical manifestations include fever, fatigue and dry cough, and dyspnea in severe cases (5). Severe COVID-19 associated histopathological changes are found mainly in the lungs, characterized by diffused alveolar damage (DAD), hyaline membranes and fibrin deposits, as well as severe endothelial injury, capillary microthrombi and exudative inflammation (6–12). A systematic review of published case reports and studies identified three main COVID-19 histological patterns: epithelial (85%), vascular (59%) and fibrotic (22%), with a frequent overlap (60%), whereas the epithelial and vascular patterns were present in all stages of severe COVID-19 (13).

COVID-19, ARDS AND PULMONARY FIBROSIS

The rapid replication of SARS- CoV-2 and the associated epithelial cell death may, depending on the underlying genetic, inflammatory or metabolic context, trigger alveolar macrophages to produce excessive amounts of cytokines (such as TNF, IL-1b, IL-6, MIP1, IFN-γ and VEGF), a "cytokine storm", associated with systemic infections such as sepsis or immunotherapies aftermath (14). The highly divert cytokine profile of COVID-19 hyperinflammation resembles, in some cases, other cytokine release syndromes, such as macrophage activation syndrome (15), although it is more heterogeneous and less robust, both quantitively (levels) and qualitatively (number of elevated cytokines). Noteworthy, IL-6 was found to be the most consistently upregulated cytokine and among the most overall predictive biomarkers (16, 17). In turn, the excessive production of cytokines further induces lung injury and Acute Respiratory Distress syndrome (ARDS), leading frequently to respiratory and multi organ failure and death (18).

ARDS develops most commonly in the setting of bacterial and viral pneumonias, or non-pulmonary sepsis, and is characterized by focal epithelial damage and excessive alveolocapillary permeability, leading to interstitial and alveolar edema and hypoxemia in the acute phase (18). Many severe COVID-19 patients will develop ARDS with impaired gas exchange and characteristic CT findings; however, the combination of multiple pathogenetic stimuli in COVID-19-induced ARDS results in a highly heterogeneous, "atypical" clinical appearance that has stimulated considerable controversy (2, 19–24). Nevertheless, excessive inflammation and endothelial dysfunction are among the top candidate pathologic events linking ARDS and COVID-19 (21, 25, 26) and markers of endothelial dysfunction have been recently correlated with COVID-19 mortality (27). Moreover, endothelial dysfunction is also a major characteristic of the most common comorbidities of COVID-19 that are associated with worse prognosis, hypertension, diabetes and obesity (21, 25).

The initial acute exudative inflammatory phase of ARDS is followed by a proliferative phase characterized by alveolar epithelial cell hyperplasia (18). A subset of acute ARDS survivors will further develop a fibroproliferative response, including fibroblast accumulation, deposition of collagen and other lung extracellular matrix (ECM) components (28), the magnitude of which was associated with ARDS duration (29). Moreover, and although invasive mechanical ventilation has revolutionized the management of ARDS, ventilator associated lung injury is considered as an additional contributor to pulmonary fibrosis in ventilated ARDS patients (30). Accordingly, a literature review of published histopathological analyses of COVID-19 lungs *postmortem* indicated, beyond DAD and hyaline membranes, the frequent presence of pulmonary fibrosis (31), while abnormal pulmonary architecture and functions have been reported in many recovering COVID-19 patients (32–34), suggesting persisting fibrotic abnormalities, pending large-scale and long-term follow up studies. Finally, CoV-2 infection *per se* has been reported to induce the expression of different pro-fibrotic factors including TGFβ (35). On the other hand, patients with interstitial lung diseases (ILD) had increased odds for ARDS development and severe COVID-19 (12, 36–39), while COVID-19-related acute exacerbation of ILDs had worse prognosis than non-COVID ILD acute exacerbations (40), thus suggesting pulmonary fibrosis both as a disease risk and a possible complication of COVID-19.

AUTOTAXIN (ATX; *ENPP2*) AND COVID-19

ATX is a secreted glycoprotein that can be detected in most biological fluids, including blood and bronchoalveolar lavage fluid (BALF) (41). A large percentage (~40%) of serum ATX is thought to originate from the adipose tissue (42), while the damaged liver has been suggested as an additional possible source of serum ATX (43). High ATX expression has been reported from endothelial cells in high endothelial venules (HEVs) (44, 45), however their expected relative contribution to circulating levels should be low. Inflammatory macrophages have been also reported to express ATX upon inflammation (46–48), thus contributing to BALF ATX levels (46).

ATX is a constitutively active lysophospholipase D, that catalyzes the extracellular hydrolysis of lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA) (49). LPC is synthesized from fatty acids or membrane phosphatidylcholine (PC) by phospholipase A_2 (PLA_2) enzymes and is highly abundant in the plasma, associated with oxidized low-density lipoprotein (oxLDL) and albumin (50, 51). LPA is a growth factor-like signaling phospholipid with numerous effects in most cell types through its G-protein coupled receptors (LPAR1-6) (51–53). ATX has been suggested to bind to cell surface integrins (54–56), thus avoiding clearance, as well as localizing LPA production to its adjacent receptors, that exhibit widespread distribution and overlapping functions (51, 57).

Viral infections have been shown to increase systemic ATX levels, including HCV, HIV and HBV (43, 58, 59), while LPA has been also shown to directly affect HCV viral infection and replication (43, 60). Increased *ENPP2* mRNA expression was detected in nasopharyngeal swabs from COVID-19 patients,

likely from immune cells (61), suggesting that ATX/LPA might stimulate viral infections, that could include SARS-CoV-2.

Increased serum/plasma ATX levels have been also reported in different diseases, including different forms of cancer, liver diseases, as well as respiratory diseases such as asthma and idiopathic pulmonary fibrosis (IPF) (**Table 1**) (41, 51). A variety of methods has been utilized, while reported levels exhibit remarkable heterogeneity, with no apparent consensus on healthy levels (**Table 1**). Increased ATX serum levels were recently reported in non-surviving ARDS patients, where ATX serum levels were shown to be an independent prognostic factor for 28 day mortality, outperforming the established SOFA/APACHE scores (62). Plasma ATX levels correlated with mortality also in a cohort of patients with severe sepsis (63), suggesting a role for ATX/LPA in systemic hyperinflammation. ATX serum levels in ARDS correlated with the increased IL-6/IL-8 serum levels (62), further supporting an interplay of ATX/LPA with inflammation, as previously suggested in breast cancer (64). ATX serum levels also correlated with the severity of lung injury (62), while increased ATX BALF levels upon endotoxin-induced acute lung injury (ALI) (65, 66), and ventilator-induced lung injury (VILI) in mice (67) have been reported. Moreover, ATX BALF levels in ARDS patients were positively associated with inflammatory and fibrotic mediators (IL-6, IL-8, TNF-α, MMP-7, fibronectin, OSM, and SPARC), suggesting that ATX may also have a role in the fibrotic component of ARDS (62). In line with the above, increased ATX levels have been detected in IPF patients and fibrotic animal models (46, 68), where results from genetic and pharmacologic studies have established a pro-fibrotic role for ATX (46, 69–72). Increased serum ATX levels were very recently detected also in COVID-19 patients hospitalized in the intensive care unit (ICU) as compared with less severe patients hospitalized in the clinic (61), thus adding ATX expression to the commonalities of COVID-19, ARDS and pulmonary fibrosis, and suggesting ATX as a possible pathologic link.

ATX levels in severe COVID-19 patients correlated with the increased IL-6 serum levels (61), as recently also shown in ARDS (62), as well in acute-on-chronic liver failure (ACLF) patients (73), suggesting interdependent regulation of expression. Accordingly, IL-6 has been reported to stimulate ATX expression from adipocytes (73) and human dermal fibroblasts (74). *Vice versa*, LPA has been reported to stimulate the expression of IL-6 from synovial fibroblasts (75, 76) and dermal fibroblasts (74), suggesting an ATX/LPA/IL-6 expression loop. Among the different components of the cytokine storm, IL-6 is the most predictive biomarker in COVID-19 (16, 17), correlating with respiratory failure and the need for mechanical ventilation (77), as well as with mortality risk (78).

Beyond hyperinflammation, and/or as its consequence, endothelial dysfunction is a major characteristic of COVID-19/ARDS (21, 25, 26). The increased ATX levels that were detected in severe COVID-19 patients correlated with markers of endothelial dysfunction (sP-sel, sICAM) (61) that have been independently correlated in the same samples with COVID-19 mortality (27). Similarly, ATX correlated with angiopoietin-2 levels and mortality in severe septic patients (63). In support for a major role of ATX/LPA on vascular homeostasis, ATX expression and LPA signaling have been shown necessary for the embryonic development of the vascular (and neural) system in mice (79–81). In adult mice, in studies unraveling the molecular mechanisms of SARS-CoV and MERS-CoV pathogenesis in the Collaborative Cross mice, *Enpp2*, the gene encoding ATX, has been reported to be a high priority candidate gene for pulmonary hemorrhage (82, 83). More importantly, LPAR1 null mice were reported to be protected from bleomycin (BLM)-induced pulmonary fibrosis, attributed to fibroblast accumulation and reduced vascular leak (68), as well as from *Candida albicans* water-soluble fraction (CAWS)-induced vasculitis, attributed to reduced CXCL1/IL-8-mediated neutrophil infiltration (84). Noteworthy, the stability of LPAR1 in the context of acute lung injury in mice has been proposed to be regulated by ubiquitination (84).

LPA SIGNALING IN PULMONARY AND IMMUNE CELLS

Overall, any ATX effect will rely on its local levels (locally produced and/or extravasated) and its possible cell surface attachment, the local availability of LPC, the cell-specific expression profile of LPA receptors, as well as of the expression of the transmembrane lipid phosphate phosphatases (PLPP1-3; PPA2 A-C) that catabolize LPA (41, 85, 86). In this context, the possible effects of increased ATX levels can be deduced from the corresponding effects of LPA in the relative cells in the tissue microenvironment in question.

A plethora of LPA effects on pulmonary non-immune cells *in vitro* have been reported, as previously reviewed (87–89) and as summarized at **Table 2**. These include the promotion of apoptosis and the secretion of chemotactic signals (IL-8, MCP-1, CXCL1) from epithelial cells, the integrin-mediated activation of TGFβ on epithelial and smooth muscle cells, the modulation of permeability, leukocyte adhesion and cytokine secretion from endothelial cells, and the chemoattraction and accumulation of myofibroblasts (**Table 2**). LPAR1 has been reported as the main receptor mediating these effects, involving different well-known G-protein mediated pathways (**Table 2**). Moreover, LPA has been reported to transactivate different growth factors including TGFβ, PDGF and EGF that activate similar signal transduction pathways, while LPA was reported to signal also *via* RAGE (**Table 2**), further increasing the pleiotropic complexity of LPA signaling in the lung.

The effects of ATX and LPA signaling on the regulation of immune cells have been previously reviewed in detail (87–92). Briefly, high ATX expression from ECs in HEVs in lymph nodes has been reported (44, 45), where ATX has been suggested to facilitate lymphocyte homing *via* the promotion of the adhesion (44), transmigration and motility of lymphocytes (45, 93, 94). Intriguingly, LPA signaling has been proposed to intersect with sphingosine phosphate (S1P) signaling (95), a closely related

TABLE 1 | Autotaxin serum levels in patients of different inflammatory diseases and cancer.

Disease classification	PMID	Disease	Samples (M/F/M+F)	ATX[1]	Method
Viral hepatitis	33102751	Chronic hepatitis C	28	1.1 ± 0.8	Two-site enzyme immunoassay
		Non-alcoholic steatohepatitis	19	1.4 ± 0.4 *	
		Alcoholic steatohepatitis	15	1.2 ± 0.4 *	
		vs.	*vs.*	*vs.*	
		Chronic hepatitis B	38	0.9 ± 0.3	
	21419756	Chronic Hepatitis C (histologically proven fibrosis)	74	2.40 ± 0.96	Two-site enzyme immunoassay
		Chronic Hepatitis C (FibroScan proven fibrosis)	134	2.20 ± 1.22	
	27981605	Chronic viral hepatitis	14	0.19 (0.13 - 0.35) *	ELISA
			21	0.17 (0.04 - 0.13)	
		vs.	*vs.*	*vs.*	
		Healthy controls	8	0.13 (0.02 - 0.20)	
			12	0.18 (0.09 - 0.35)	
	28425454	Chronic hepatitis C	292	1.16 (0.85 - 1.68) *, #	Two-site enzyme immunoassay
			301	1.64 (1.19 - 2.20) *	
			593	1.39 (1.01 - 1.99) *	
		vs.	*vs.*	*vs.*	
		Healthy controls	80	0.76 #	
			80	0.82	
			160	0.76	
	31933517	Liver cirrhosis	240	1.58 ± 0.68 #	Two-site enzyme immunoassay
		(multiple aetiologies)	160	1.99 ± 0.73	
		Chronic hepatitis B	33	1.36 ± 0.62 #, ~	
			17	1.82 ± 0.5	
		Chronic hepatitis C	64	1.62 ± 0.67 #, $	
			66	2.09 ± 0.71	
		Non viral hepatitis	143	1.49 ± 0.71 #	
			77	1.96 ± 0.79	
	29114991	Chronic hepatitis B	62	1.10 (0.85-1.24)	Two-site enzyme immunoassay
			39	1.36 (1.23-1.64)	
			101	1.22 (0.95-1.42)	
Non-viral liver disorders	25062038	Liver cirrhosis	181	0.77 ± 0.41 *, #	ELISA
			89	0.86 ± 0.43 *	
			270	0.81 ± 0.42 *	
		vs.	*vs.*	*vs.*	
		Healthy controls	35	0.18 ± 0.04 #	
			50	0.35 ± 0.47	
			85	0.26 ± 0.40	
	29568204	Non-alcoholic fatty liver disease	186	0.86 *	Two-site enzyme immunoassay
		vs.	*vs.*	*vs.*	
		Healthy controls	160	0.76	
	30905718	Liver cirrhosis	50	0.44 ± 0.22 *	ELISA
		vs.	*vs.*	*vs.*	
		Healthy controls	20	0.19 ± 0.06	
	31144415	Non-alcoholic fatty liver disease	173	0.67 ± 0.21 #	Two-site enzyme immunoassay
			134	0.97 ± 0.36	
			307	0.81 ± 0.32	
Bile duct disorders	31186435	Primary sclerosing cholangitis	193	6.3 ± 3.0 #, *	Homovanillic acid assay
			59	8.6 ± 4.9 *	
			252	6.8 ± 3.7	
		vs.	*vs.*	*vs.*	
		Healthy controls	57	2.5 ± 0.7 #	
			142	3.2 ± 1.5	
	31651244	Primary biliary cholangitis – Severe	25	1.25 (0.72 - 4.31)	Two-site enzyme immunoassay
		vs.	*vs.*	*vs.*	
		Primary biliary cholangitis – Moderate	94	1.08 (0.58 - 3.12)	
	27506882	Primary biliary cholangitis	118	10.2 ± 4.4	Homovanillic acid assay
		Primary sclerosing cholangitis	115	7.3 ± 3.4	
		vs.	*vs.*	*vs.*	
		Healthy controls	Undisclosed	3.1 ± 1.7	

(Continued)

TABLE 1 | Continued

Disease classification	PMID	Disease	Samples (M/F/M+F)	ATX[1]	Method
			Undisclosed	2.5 ± 0.7	
			109	2.8 ± 1.4	
	29802350	Primary biliary cholangitis	20	1.00 (0.82 - 1.13) *,#	Two-site enzyme immunoassay
			108	0.78 (0.66 - 0.98) *	
			128	0.97 (0.79 - 1.11) *	
		vs.	*vs.*	*vs.*	
		Healthy controls	80	0.76 #	
			80	0.82	
			160	0.76	
	25450205	Preeclampsia / HELLP syndrome	17	16.8 ± 8.9	Homovanillic acid assay
		Pruritic disorders of pregnancy	33	16.8 ± 6.7	
		Intrahepatic cholestasis of pregnancy	55	43.5 ± 18.2 *, †	
		vs.	*vs.*	*vs.*	
		Normal pregnancy	44	19.6 ± 5.4 *	
		vs.	*vs.*	*vs.*	
		Healthy controls	57	2.5 ± 0.7 #	
			142	3.2 ± 1.5	
Malignancies	2464234	Hepatocellular carcinoma	105	1.94 ± 1.01 #	Two-site enzyme immunoassay
			43	2.87 ± 0.76	
			148	2.21 ± 1.03	
	18710386	Acute myeloid leukemia	26	0.86 ± 0.29	ELISA
		Chronic lymphocytic leukemia	14	0.93 ± 0.30 *	
		Follicular lymphoma	25	1.47 ± 0.69 *	
		Diffuse large B-cell lymphoma	28	0.94 ± 0.39 *	
		vs.	*vs.*	*vs.*	
		Healthy controls	74	0.66 ± 0.12 #	
			46	0.85 ± 0.18	
			120	0.73 ± 0.18	
	27583415	Hepatocellular carcinoma	58	1.07 (0.84 - 1.37) *	Two-site enzyme immunoassay
		vs.	*vs.*	*vs.*	
		Healthy controls	74	0.68 ± 0.12 #	
			46	0.97 ± 0.17	
			120	0.73 ± 0.18	
	29724718[a]	Non-small cell lung cancer	19	0.124 *	TOOS assay
		vs.	*vs.*	*vs.*	
		Healthy controls	49	0.088	
	30921203	Breast cancer	112	0.29 ± 0.04 *	ELISA
		vs.	*vs.*	*vs.*	
		Healthy controls	50	0.25 ± 0.02	
Metabolic disorders	26727116	Obese – overweight people >60 yo	20	0.17 ± 0.01 #	ELISA
			40	0.29 ± 0.02	
			60	0.25 ± 0.11	
	26831013	Diabetic nephropathy	38	0.75 ± 0.27	ELISA
Autoimmune disorders	22493518[b]	Rheumatoid arthritis	10	0.87 ± 0.83 *	ELISA
			16	1.12 ± 1.08 *	
			26	1.03 ± 0.98 *	
		vs.	*vs.*	*vs.*	
		Osteoarthritis	11	0.27 ± 0.19	
			15	0.32 ± 0.19	
			26	0.30 ± 0.19	
	24984830	Multiple sclerosis	20	12.11 ± 1.42 *	TOOS assay
		vs.	*vs.*	*vs.*	
		Other neurological disorders	20	7.05 ± 1.51	
Various disorders	26083365	Chronic liver diseases	18	1.37 ± 0.77 *	Two-site enzyme immunoassay
			17	1.46 ± 0.67 *	
		Follicular lymphoma	10	0.95 ± 0.27 *	
			15	1.28 ± 0.47 *	
		vs.	*vs.*	*vs.*	
		Healthy controls	76	0.98 ± 0.58 #	
			98	1.49 ± 0.98	

(Continued)

TABLE 1 | Continued

Disease classification	PMID	Disease	Samples (M/F/M+F)	ATX[1]	Method
	32826822	Sepsis	84	443.6 (285.8 - 632.2)	TOOS assay
	33568105	Pancreatic diseases Benign pancreatic diseases *vs.* Healthy controls	114 94 *vs.* 120	0.39 * 0.27 *vs.* 0.26	ELISA
	34130757	Acute respiratory distress syndrome (survivors) Acute respiratory distress syndrome (non-survivors)	31 21	39.01 ± 13.89 44.79 ± 13.38	Human Magnetic Luminex Assay

Only publications analyzing more than 10 samples are included.
[1]All reported values were converted to mg/L and presented as in the original publication as means ± SD, or as median (interquartile range). Individual values represent medians unless stated otherwise.
**: Compared to the same sex group of the controls; $p < 0.05$.*
[#]: Compared to within-the-group opposite sex; $p < 0.05$.
[†]: Compared to females with normal pregnancy.
[~]: Compared to non-viral hepatitis.
[$]: Compared to hepatitis B.
[a]: ATX activity mean values are indicated.
[b]: ATX concentration in the serum was calculated anew by utilizing the supplementary data of this publication.

phospholipid (96), that has been shown to affect lymphocyte egress from the lymph nodes (97).

Non-withstanding the effects of ATX/LPA on lymphocyte homeostasis, highly pertinent for both ILD/IPF and COVID-19, a role for ATX/LPA on the homeostasis of the monocyte phagocyte system is emerging. Macrophages are central players in the pathogenesis of both IPF (98, 99) and COVID-19 (15, 100, 101), exhibiting remarkable heterogeneity and spatiotemporal plasticity. LPA has been suggested to stimulate the expression of macrophage chemotactic factors from ECs, such as monocyte chemoattractant protein-1 (MCP-1) (102) and CXCL1 (103), thus promoting both monocyte migration as well as adhesion to ECs (102–104). Beyond LPA-induced macrophage chemoattraction and adhesion to ECs, inflammatory macrophages *per se* have been reported to express ATX upon BLM-induced pulmonary inflammation and fibrosis, while IPF macrophages have been shown to stain for ATX (46). scRNAseq analysis of BALF cells from COVID-19 patients indicated a predominance of macrophages (100, 101), where *ENPP2* mRNA expression was detected in monocyte-derived alveolar macrophages (Mo-AMs) (61), that have been shown to drive the development of BLM-induced pulmonary fibrosis in mice (105). In turn, accumulating evidence indicates that LPA co-stimulate macrophage maturation and/or activation (47, 106–109), suggesting an autocrine role of ATX/LPA in macrophage pathologic responses. Moreover, LPA has been suggested to stimulate oxLDL uptake and foam cell formation (110, 111), linking macrophages and ATX/LPA with hyperlipidaemia and cardiovascular diseases (112), major comorbidities of COVID-19.

While LPA promotes bone marrow derived monocyte ($CD11b^+$) activation (F4/80 expression) *in vitro* as potently as M-CSF (106), LPA has been also reported to co-stimulate the

TABLE 2 | Reported Lysophosphatidic acid (LPA) effects on pulmonary, non-immune, cells.

Cell type	LPA effect	Receptor	Pathway	PMID
Epithelial cells				
Human bronchial epithelial cells	induction of anchorage dependent apoptosis	LPAR1	-	22021336
	induction of TSLP & CCL20	–	-	18757306
	activation of TGF-β	LPAR2	integrin $\alpha_v\beta_6$	19147812
	induction of soluble ST2 expression	LPAR1, 3	-	21871564
	transactivation of EGFR & secretion of IL-8	-	-	16687414, 16197369
	induction of IL-13Ra2	–	$G_{\alpha i}$	17287216
	enhancement of epithelial barrier integrity	LPAR1, 3	-	19586906, 17359381
	decrease of EGFR-EGF binding	–	-	17640953
	induction of COX-2 expression & PGE2 secretion		$G_{\alpha i}$	18294142
	transactivation of PDGFR-β	–	–	12890682
	redistribution of c-Met on the membrane	-	-	17689924, 23624790

(Continued)

TABLE 2 | Continued

Cell type	LPA effect	Receptor	Pathway	PMID
Human bronchial epithelial cells (BEAS-2B)	transactivation of EGFR	LPAR1	–	17640953
	inhibition of IFN/TNF-induced CCL5/RANTES expression	LPAR1	G_i/PI3K	20861350
	decrease of EGFR-EGF binding	–	–	17640953
Human alveolar epithelial carcinoma cells (A549)	decrease of p53 abundance	-	-	18025263
	increase of cell migration	LPARs	PKCδ, cortactin	21696367
	promotion of EMT, proliferation and migration	RAGE	PKB	33109194
Human basal cells	induced signaling by CREB	–	ERK1/2	33794877
Mouse alveolar and bronchial epithelial cells	induction of apoptosis	LPAR1	-	22021336
	induction of apoptosis	LPAR2	–	23808384
Mouse lung epithelial cells (MLE12)	induction of migration	LPAR1	TrkA	26597701
	induction of KC secretion	LPAR1	ERK, p38	27448760
Endothelial cells				
Human microvascular endothelial cells	increase of the endothelial layer permeability	LPAR2, 6	-	23084965
Human pulmonary artery endothelial cels	increased adhesive properties	LPAR1, 3	–	25621161
Human aortic endothelial cells	induction of VCAM, E-selectin	-	G_i	10595650
	induction of E-selectin, MCP-1, monocytic migration and adhesion	–	ROCK2, NF-κB	30884801
	induction of VCAM, ICAM	-	ROCK2, NF-κB	20164172
Human endothelial cells	CXCL1 secretion, monocyte adhesion	LPAR1, 3	–	21531341
Human airway epithelial cells	inhibition of the attachment to the ECM	LPAR1	Rho-kinase	27500235
Mouse endothelial cells	vascular leak/extravasation	LPAR1	–	18066075
Bovine pulmonary artery endothelial cells	migration, chemotaxis	-	ERK, Hic-5	17337598
		-	-	15333043
Fibroblasts				
Human fibroblasts	chemoattraction, accumulation	LPAR1	–	18066075
	proliferation, EGFR ectodomain shedding	-	ERK1/2	21362091
	differentiation, profibrotic gene expression (TGFβ, col1a2, FN, SMA)	LPAR2	ERK1/2, Smad 3, Akt, p38	23808384
Mouse lung fibroblasts	lamelipodia formation, motility	LPAR1	-	14744855
Mouse fibroblasts (NIH 3T3)	migration, protection from apoptosis, proliferation	–	–	16219296
	protection from apoptosis, proliferation		G_i	11062066
Rat Rat1/c-Myc fibroblasts	protection from apoptosis	–	Rac1	11062066
Mesenchymal cells derived from fibrotic lung allografts	promotion of NFAT1 nuclear translocation	LPAR1	β-catenin	28240604
Smooth muscle cells				
Human smooth muscle cells	proliferation, stimulation of EGFR signaling	-	-	9252534, 11741820
	activation of TGF-β	–	integrin $\alpha_v\beta_5$	22025551
Rabbit smooth muscle cells	contraction	-	-	9338431
Stem cells				
Human mesenchymal stem cells	migration	LPAR1	β-catenin	22782863
	migration, differentiation into myofibroblasts	LPAR1	–	24251962

GM-CSF/IL-4-induced conversion of monocytes to DCs (113, 114). Moreover, LPA has been also reported to modulate the activity of TCF4 (115), a decisive transcription factor in plasmacytoid dendritic cells (pDCs) development and homeostasis (116). Increased *ENPP2* expression was detected in COVID-19 DCs and pDCs, correlating with markers of immature DCs (61), while an anti-inflammatory role of LPA has been previously proposed for DCs *via* LPAR2 (117), suggesting that ATX/LPA could be also involved in suppression of DC responses in COVID-19.

Therefore, increases of ATX levels and LPA local production in ARDS, ILD/IPF and COVID-19 can exacerbate numerous

TABLE 3 | Representative small molecules targeting ATX in late-stage preclinical and clinical development.

Compound (Company)	*In vitro* properties			Pre-clinical data			Clinical trials
	IC50(assay)	Mode of Binding (Ki)ATX inhibitor type (PDB structure entry)	ADMET	Pharmacokinetics	LPA inhibition	Disease targeting (Dose, route)	
GLPG-1690 Ziritaxestat (Galapagos)	131 ± 12 nM (hATX, TOOS assay)[1] 418 nM (mouse plasma, 18:2 LPA, LC-MS/MS)[1] 542 nM (rat plasma, 18:2 LPA, LC-MS/MS)[1] 242 nM (human plasma, 18:2 LPA, LC-MS/MS)[1]	Competitive (15 nM, hATX), type IV inhibitor1 (5MHP)	hERG IC_{50}: = 15 μM[1] CYP3A4 TDI: negative[1]	**iv clearance (L/h·kg)**: 0.23 (mouse), 0.69 (rat), 0.12 (dog)[1] **C_{max}** (mouse, 30 mg/kg per os): 21.367 μg/mL[1] **t_{max}** (mouse, 30 mg/kg per os): 1 h[1] **$t_{1/2}$** (mouse, 30 mg/kg per os): 3.8 h[1] per os bioavailability (F%): 29 (mouse), 37 (rat), 63 (dog)[1]	95% (maximum) (30 mg/kg per os)[1]	**Pulmonary fibrosis** (3, 10 or 30mg/kg per os)[1]	Phase I NCT03143712 NCT02179502 IPF Phase II NCT02738801 Phase III NCT03711162 NCT03733444 Scleroderma Phase II NCT03798366 NCT03976648
BLD-0409 Cudetaxestat (Blade Therapeutics)	≤ 0.5 μM (LPC assay)[2]	–	–	–	–	**Metabolic disorders** (15mg/kg)[2]	Phase I NCT04146805 NCT04814472 NCT04814498 NCT04939467
ONO-8430506 (Ono Pharmaceuticals)	5.1 nM (recomb. ATX, FS-3 assay)[3] 10.2 nM (hATX, LPC assay)[3, 4] 6.4 nM (mouse plasma, LPC assay)[3] 19 nM (rat plasma, LPC assay)[3] 5.5 nM (human plasma)[3, 4]	type II inhibitor	Protein plasma binding: rat (95.1%), human (99%)[4] High selectivity to ATX[3]	**iv clearance (mL min-1 kg-1):** 8.2 (mouse), 4.7 (rat), 5.8 (dog)[4] **Vdss (L/kg)**: 1.5 (mouse), 1.9 (rat), 2.3 (dog)[4] **Cmax (1 mg/kg per os)**: 124 ng/mL (mouse), 261 ng/mL (rat), 1670 ng/mL (dog)[4] **t1/2** (1 mg/kg per os): 5.4 h (mouse), 2.5 h (rat), 5.9 h (dog)[4] **per os Bioavailability (F)**: 51.6% (rat), 71.1% (dog), 30.8% (monkey)[4]	96% (18:2 LPA, 3 mg/kg)[3] 93% (20:4 LPA, 3 mg/kg)[3] >99% (18:2 & 20:4 LPA, 30 mg/kg)[3]	**Prostatic hyperplasia** (0.3-10mg/kg, id)[3] **Breast cancer** (10mg/kg, per os)[5] **Thyroid cancer** (2mg/kg, per os)[6]	Preclinical evaluation
PF-8380 (Pfizer)	2.8 nM (hATX, FS-3 substrate)[7] 1.7 nM (hATX, LPC substrate)[7] 1.16 nM (mATX, FS-3 substrate)[7] 1.15 nM (fetal fibroblast cell line, LPC substrate)[7] 101 nM (human whole blood)	Competitive (0.02-0.04 nM), type I inhibitor[7]	Solubility (pH = 6.8) = 0.011 mg/mL[8] Poor solubility at physiological pH (7.4)[8] IC50 hERG (cardiotoxicity) = 2.7 μM[8] Permeability (PAMPA assay) = 81%[8]	**rat iv clearance (mL min-1 kg-1)** = 31[7] **Vdss (L/kg)** = 3.2[7] t1/2 = 1.2 h[7] **Cmax** (10 mg/kg per os) = 2.55 μM[7] **tmax** (10 mg/kg per os) = 0.67 h[7] **rat per os F** (10 mg/kg) = 83%[7]	EC50 = 54.7 nM (16:0 LPA)[7] EC50 = 84.6 nM (18:0 LPA)[7] EC50 = 51.7 nM (20:0 LPA)[7]	**Arthritis Hyperalgesia** (30mg/kg, po)[7] **Glioblastoma** (10mg/kg, ip)[9] **Liver fibrosis** (30mg/kg, ip)[10] **Lung allograft fibrosis** (30mg/kg, per os)[11]	Preclinical evaluation

[1-11] refer to the following hyperlinked publications (PubMed ID): ***1****:* *28414242*, ***2****:* *33342311*, ***3****:* *24747415*, ***4****:* *32551021*, ***5****:* *24599971*, ***6****:* *25398768*, ***7****:* *20392816*, ***8****:* *29798825*, ***9****:* *24062988*, ***10****:* *27981605*, ***11****:* *28240604*.

pathogenic responses in the lung, likely in co-ordination with other pathologic inflammatory and fibrotic factors.

PHARMACOLOGIC TARGETING OF ATX AS AN ADDITIONAL THERAPEUTIC OPTION IN COVID-19

Dexamethasone (Dex), the first line of defense against systemic inflammation, has been proven effective in COVID-19 patients requiring oxygen or ventilation (118, 119), the only approved single therapy against severe COVID-19. Remarkably, Dex treatment of ventilated COVID-19 patients attenuated serum ATX levels, suggesting that the therapeutic effects of Dex include the suppression of ATX expression (61) and that ATX can be druggable.

The exacerbated production of IL-6 and other storm cytokines, where present, is considered among the leading causes of COVID-19/ARDS-related mortality, and therefore many clinical trials have been conducted targeting storm cytokines or their receptors, with inconsistent results, spurring controversial opinions on the use of systemic anti-inflammatory drugs (120). ATX and IL-6 levels were shown to correlate in ACLF (73), ARDS (62) and COVID-19 (61) patients, suggesting that simultaneous inhibition of both IL-6 and ATX may be an effective therapeutic strategy for COVID-19, as previously suggested in systemic sclerosis (74).

The antifibrotic compounds pirfenidone and nintedanib, approved for IPF, have shown efficacy in fibrotic lung diseases other than IPF (121–125). Therefore, since COVID-19 and IPF share disease severity risk factors, such as sex/age and comorbidities, existing and developing anti-fibrotic compounds have been suggested as additional therapeutic options in COVID-19 (126–128). Among them, GLPG1690 (**Table 3** and **Figure 1**) targets ATX and, together with the standard of care treatment (pirfenidone or nintedanib), has entered phase III international clinical trials (ISABELA 1 and 2, NCT03711162 and NCT03733444) (129). Given the above, the same or a similar regime might also prove effective in COVID-19.

The crystal structure of ATX has been solved (55, 130, 131), allowing a deep understanding of its structure and function relationship (132) (**Figure 1**), and thus promoting rational drug design. Given the establishment of ATX as a therapeutic target in IPF, as well as the promising results from the initial clinical trials, a plethora of ATX inhibitors have been developed (133, 134); the ones at late-stage development as shown in **Table 3** and their mode in binding at **Figure 1**.

Inducible genetic deletion of ATX in adult life, resulting in 70-80% decreases in serum ATX levels and mRNA expression levels in different tissues, did not have any appreciable effects in gross pathophysiology of major organs (135), suggesting that the bulk of ATX activity in mice is dispensable for adult life. Moreover, potent (IC50 2 nM), long term (3 weeks) pharmacological

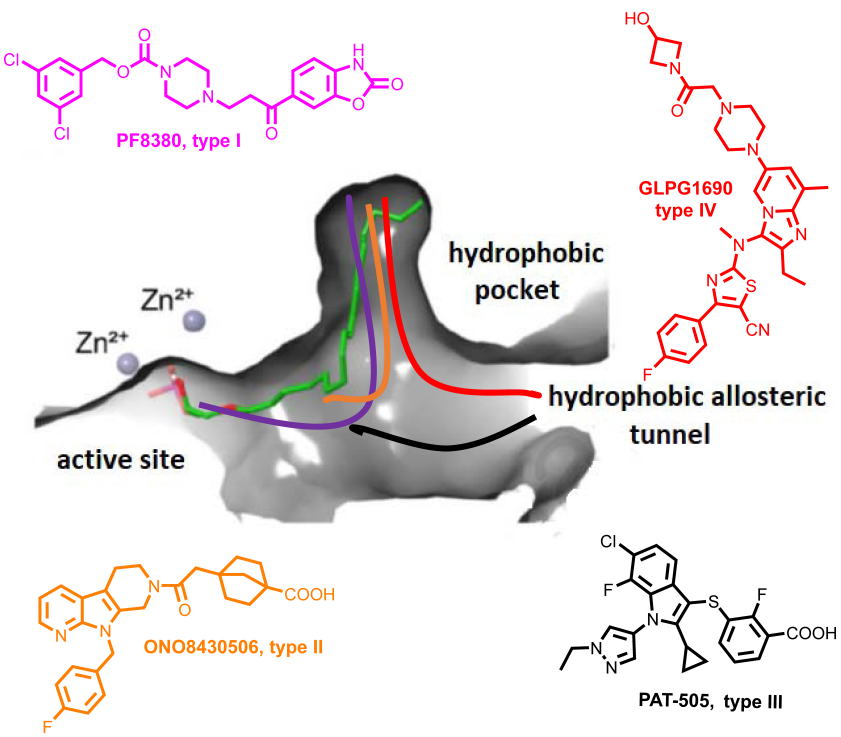

FIGURE 1 | Schematic representation of the mode of binding (color coded) to ATX by the 4 different types of ATX inhibitors (I-IV). The mode of binding of LPA is also displayed (in green).

inhibition of ATX with PF-8380 (120 mg/Kg - 4 times the effective concentration; PO; bid) had no effects in survival or gross pathology of major organs (135), suggesting that ATX pharmaceutical targeting is safe and well tolerated in mice. In humans, GLPG1690 was reported to be well tolerated in a phase 1 randomized clinical trial (NCT02179502), safe and efficacious in a phase 2a randomized placebo-controlled clinical trial (NCT02738801), supporting ATX inhibition as a novel treatment for IPF (136, 137). In addition, administration of BBT-877, another orally available small molecule inhibitor targeting ATX (IC50 ~6.7 nM), to healthy volunteers in a phase I clinical trial (NCT03830125), did not reveal severe adverse events (138, 139). However, the GLPG1690 phase III clinical trial was recently discontinued on account of "low benefit to risk ratio". Likewise, BBT-877's scheduled phase II clinical trial was also postponed due to "toxicity concerns". Since the relative results are not announced yet, it is not known if the toxicity was imposed from the compounds themselves or their target. Nevertheless, several new candidates are emerging, while possible compound toxicity can be eliminated with targeted modifications or bypassed *via* inhaled administration.

CONCLUSIONS

The increased levels of ATX in ILDs/IPF, ARDS and COVID-19 add yet another commonality between them and suggest that LPA signaling is involved in their pathogenesis, including the amplification of vascular damage, the regulation of the immune system and the promotion of fibrosis. Therefore, the therapeutic targeting of ATX in IPF and fibrotic diseases could be also applied in COVID-19, alone or together with approved anti-fibrotic, anti-rheumatic and anti-viral drugs, especially given its predicted short-term administration, as well as the emergency nature and unmet medical need for the treatment of COVID-19 severe cases.

AUTHOR CONTRIBUTIONS

KN, TK, ET, ES and AM drafted the paper. AT and KA critically commented on the draft version. VA finalized the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work has been co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call Research – Create – Innovate (project code: T1EDK-0049. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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frontiers in Immunology

ORIGINAL RESEARCH
published: 17 November 2021
doi: 10.3389/fimmu.2021.645548

Collagen 1a1 Expression by Airway Macrophages Increases In Fibrotic ILDs and Is Associated With FVC Decline and Increased Mortality

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Within the Interstitial Lung Diseases (ILD), patients with idiopathic pulmonary fibrosis (IPF) and a subset of those with non-IPF fibrotic ILD have a distinct clinical phenotype of progression despite management. This group of patients has been collectively termed the progressive fibrotic phenotype (PFP). Their early recognition may facilitate access to antifibrotic therapies to prevent or slow progression. Macrophages/monocytes within the lung orchestrate the progression and maintenance of fibrosis. A novel role for monocyte-derived macrophages during tissue damage and wound healing is the expression of collagens. We examined Collagen 1a1 expression in airway macrophages from ILD patients at diagnosis. COL1A1 mRNA levels from BAL cells were elevated in IPF and Non-IPF patients. The presence of a UIP pattern and a subsequent progressive phenotype were significantly associated with the higher BAL COL1A1 levels. In Non-IPF patients, higher COL1A1 levels were associated with a more than twofold increase in mortality. The intracellular localisation of COL1A1 in airway macrophages was demonstrated by confocal microscopy in CD45 and CD163 co-staining assays. Additionally, airway macrophages co-expressed COL1A1 with the profibrotic SPP1 gene product osteopontin. The levels of SPP1 mRNA and OPN in the BAL were significantly higher in IPF and Non-IPF patients relative to healthy. Our results suggest that profibrotic airway macrophages are increased in the BAL of patients with IPF and other ILDs and co-express COL1A1 and OPN. Importantly, COL1A1 expression by pro-fibrotic airway macrophages could be a marker of disease progression and poor survival in ILDs.

Keywords: IPF, RA-ILD, NSIP, airway macrophages, SPP1, osteopontin, collagen 1A1, PF-ILD

OPEN ACCESS

Edited by:
Paul W. Bland,
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Reviewed by:
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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *23 December 2020*
Accepted: *13 October 2021*
Published: *17 November 2021*

Citation:
Tsitoura E, Trachalaki A, Vasarmidi E, Mastrodemou S, Margaritopoulos GA, Kokosi M, Fanidis D, Galaris A, Aidinis V, Renzoni E, Tzanakis N, Wells AU and Antoniou KM (2021) Collagen 1a1 Expression by Airway Macrophages Increases In Fibrotic ILDs and Is Associated With FVC Decline and Increased Mortality. Front. Immunol. 12:645548. doi: 10.3389/fimmu.2021.645548

INTRODUCTION

Interstitial Lung Disease (ILD) is a broad term currently used to include more than 200 different disease entities (1). Connective tissue associated ILD (CTD-ILD) is the most common subtype, whilst idiopathic ILDs lie within the orphan characterization of rare lung diseases. Amongst idiopathic interstitial pneumonias, the most characterized and well-studied is IPF, a lethal chronic disease (1). IPF is distinguished by a clinical phenotype of inexorable progression and a median survival of 3 years, prior to the advent of anti-fibrotic agents (2). Amongst other ILDs, disease behaviour is strikingly diverse, ranging from self-limited, reversible to progressive, IPF-like disease (1–3). A subset of non-IPF ILD patients exhibit a more progressive disease course despite usual management, similar to IPF (PF-ILD) (3–5).

Commonalities in clinical behaviour in IPF and non-IPF ILDs with the PFP suggest common underlying pathogenetic pathways that drive progression, irrespective of the initial trigger (6, 7). Given the strong links between progression and subsequent mortality in non-IPF ILDs, it is vital that algorithms be developed to identify the PFP at presentation. It is known that a pattern of usual interstitial pneumonia (UIP), on biopsy or as judged by CT (UIP or "probable UIP") indicates a higher likelihood of disease progression (8, 9). Molecular biomarkers have also been identified as candidate markers of progression in individual ILDs, including KL-6, PDGF, FGF, VEGF and M-CSF (7, 10).

The role of the innate and adaptive immune systems in the pathogenesis of IPF is not clearly defined (11) and immune cells such as macrophages, monocytes and fibrocytes are subject to intense investigation as novel therapeutic options in IPF effectively alter their activation (12, 13). Macrophages, the most abundant immune cells in the lung, play important roles in tissue remodelling during pulmonary fibrosis (14). Following a fibrotic insult such as bleomycin in murine models, the pool of tissue resident alveolar macrophages is replaced by monocyte-derived macrophages that have distinct phenotypes including the upregulation of pro-fibrotic genes (15). Macrophages produce profibrotic mediators such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF), directly orchestrating the activation of fibroblast functions and implicated in the aberrant wound-healing cascade during fibrosis (16). Fibrocytes, a minor fraction of circulating leukocytes, portray a dual phenotype of monocytes (CD34, CD45, CD11b) and fibroblasts (collagens I and III, and fibronectin (17) that migrate to the lungs following tissue damage (18). Single-cell RNAseq analyses of the airway cell population from human lungs reveal a plethora of cell entities with monocyte and macrophage markers. This approach is reshaping the field of macrophage/monocyte biology, with the observation that lung macrophage/monocyte populations shift during lung fibrosis towards distinct entities with yet uncharacterised functions (19).

Macrophages were long believed not to produce collagens. However there is cumulative evidence that they express almost all known collagen mRNAs (20, 21). Furthermore, recent evidence from a mouse model of fibrotic scar formation in the heart shows that recruited monocyte-derived macrophages directly contribute to collagen-I fibril formation, suggesting that cell-autonomous production of collagen is a component of the pro-fibrotic monocyte/macrophage response (22). The role of collagen expression by alveolar macrophages in IPF is unknown; however, a recent study showed that Collagen VI is upregulated in macrophages overexpressing Fra-2 in mouse models of pulmonary fibrosis and in IPF tissue macrophages while Col-VI knockout bone marrow chimeras were protected from bleomycin-induced lung fibrosis (23). We have previously observed that mRNA levels of COL1A1 are detectable in BAL cells and are elevated in IPF patients relative to controls (24, 25).

The results of the lung fibrosis cell atlas suggest that macrophage populations in the lungs are distinct in lung fibrosis compared to healthy subjects. Independent studies have identified an increase of alveolar macrophages expressing the *SPP1* gene that encodes for osteopontin (OPN) in fibrotic lungs (15, 19, 26). OPN-expressing macrophages of monocyte origin also accumulate in murine models of hepatosteatosis (27). OPN dramatically increased in the BAL and serum of fibrotic patients and was initially explored as a potential IPF serum biomarker (28, 29). However, results from later studies established a wider pattern of upregulation of OPN in fibrotic disease suggesting a dominant role in the pathogenesis of lung fibrosis (30). OPN may act as a cytokine and a transcription factor and is directly involved in extracellular matrix regulation and collagen expression in fibrotic disorders of the liver, kidneys and heart (31, 32).

In this study, we established that collagen1a1 expression occurs in airway macrophages and increases in pulmonary fibrosis both in mice and humans. We compared the COL1A1 mRNA levels between IPF and non-IPF fibrotic ILDs at diagnosis in order to establish a possible link between disease progression and collagen expression in myeloid cells. In addition, in view of the involvement of OPN in collagen expression, we also examined a possible association of SPP1 and COL1A1 levels in BAL cells.

MATERIALS AND METHODS

Patients

134 patients were prospectively recruited. 69 subjects (ILD, n=50; controls, n=19) were recruited in the Department of Respiratory Medicine, University Hospital of Heraklion, Crete, Greece from May 2012 to December 2018. 65 subjects all with ILD, were recruited in the Royal Brompton Hospital from March 2003 to October 2009.

Patient characteristics are summarised in **Table 1** and **Supplementary Table 1**. Diagnostic sub-groups comprised IPF (n=53) and non-IPF ILDs (n=52). All subjects underwent

Abbreviations: AMs, Airway macrophages; COL1A1, Collagen 1a1; CTD-ILD, Connective tissue associated ILD; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HP, Hypersensitivity Pneumonitis; ILD, Interstitial Lung Diseases; IPF, Idiopathic Pulmonary Fibrosis; NSIP, Non-Specific Pneumonia; OPN, osteopontin; OP, organising pneumonia; RA-ILD, Rheumatoid Arthritis ILD; SSc-ILD, Scleroderma ILD; SLE, Systemic Lupus erythematosus; SPP1, secreted phosphoprotein 1; UIP, Usual Interstitial Pattern.

TABLE 1 | Patient characteristics.

	Control Group (n = 19)	IPF (n = 53)	Non-IPF (n = 62)	P value
Age	54.1 ± 13.5	68.1 ± 10	59 ± 14	P<0.0001 Control *vs* IPF p=0.001 IPF *vs* Non-IPF p<0.001 Control *vs* Non-IPF ns
gender (female/male)	6/13	8/45	41/21	P<0.0001 *
Smoking History				P<0.0001*
Never	2 (10.5%)	16 (30.8%)	35 (57.4%)	
Ex-smoker	3 (15.8%)	31(59.6%)	6 (32.8%)	
Smoker	14 (73.7%)	5 (9.6%)	20 (9.8%)	
Macrophages	87.3 ± 9	79.1 ± 15.3	79 ± 12.6	P ns
Lymphocytes	9.9 ± 7.6	11 ± 15.2	11 ± 9	P ns
Neutrophils	4.2 ± 1.6	7.3 ± 6.3	6.5 ± 6.2	P=0.006 Control *vs* IPF p<0.001 Control *vs* Non-IPF p<0.001
Eosinophils	0.4 ± 0.5	2 ± 3.3	2.3 ± 2.8	P=0.087 Control *vs* IPF p=0.007 Control *vs* Non-IPF p<0.0001
FVC		78.5 ± 19.7	79.4 ± 25.2	P ns
DLco		52.3 ± 18.5	51 ± 16.5	P ns
CPI		44 ± 15	43 ± 14.7	P ns

**Stands for Chi-square test; "ns" stands for non significant.*

bronchoalveolar lavage (BAL) and chest high resolution computed tomography (HRCT). For the ILD patients BAL and HRCT were part of the initial diagnostic evaluation (33–36).

IPF Group

The diagnosis of IPF was based on ATS/ERS criteria or on multidisciplinary discussion according to the Fleischer criteria (34, 37). Patients were anti-fibrotic naïve.

Non-IPF Group

This category of patients included patients with a known or new diagnosis of Connective tissue disease (CTD-ILD); namely Systemic Sclerosis (SSc), Rheumatoid arthritis (RA), Systemic Lupus erythematous (SLE) or Undifferentiated CTD. Patients with CTD-ILD were enrolled at the initial stages of there ILD diagnosis. Additionally, included patients with Idiopathic Non-Specific Pneumonia (NSIP), Hypersensitivity Pneumonitis (HP) and organizing pneumonia/NSIP overlap (OP/NSIP).

Control Group

Control subjects were undergoing bronchoscopy for the investigation of haemoptysis, without any overt pulmonary comorbidities and with normal bronchoscopy findings and cytology results. Since controls were healthy subjects, no PFTs were performed.

All patients were evaluated with complete pulmonary function tests (PFTs) within one month of bronchoscopy. Lung volumes were measured using body plethysmography and the diffusion capacity (DLco, corrected for haemoglobin) was measured using the single breath technique. The computerized system (Jaeger 2.12; MasterLab, Würzburg, Germany) was used and predicted values were obtained from the standardized lung function testing of the European Coal and Steel Community, Luxembourg (1993).

Patients were classified as non-smokers, current smokers or former smokers (defined as having smoked a minimum of one cigarette a day for a minimum of 1 year, stopping at least 6 months before presentation).

All patients provided written informed consent. The study was approved by the Ethics Committees of the University Hospital of Heraklion (IRB number: 1045 and 17030) and the Royal Brompton Hospital (REC reference 13/LO/0857).

BAL Cell Isolation and Determination of Cellular Composition

BAL fluid was obtained at room temperature. A flexible bronchoscope was wedged into a sub-segmental bronchus of a predetermined region of interest based on radiographical findings. A BAL technique was performed by instilling 180 ml of normal saline in 60-mL aliquots, retrieved by low suction. BAL samples were kept on ice and processed within two hours of collection. Samples were filtered through sterile 70nm cell strainers (BD) and centrifuged at 500g for 5 minutes at 4°C. Cell pellets were re-suspended with cold PBS. Total cell count and cell viability were assessed using Trypan blue (ICN). Differential cell population count was analysed following May-Grunewald-Giemsa staining (25).

Mice

Mice were bred under SPF conditions at the local animal facility at "20–22°C, 55 ± 5% humidity, and a 12-h light/dark cycle; water and food were given ad libitum". All experimentation in mice, in line with the ARRIVE guidelines, was approved by the Veterinary service and Fishery Department of the local governmental prefecture (#2816), following the positive

opinion of the Institutional Protocol Evaluation Committee of BSRC Alexander Fleming.

Pulmonary fibrosis was induced through the administration of 0.8U/Kg of bleomycin (Nippon Kayaku) to anesthetized mice (IP ketamine/xylazine/atropine, 100/10/0.05 mg/kg, respectively) *via* the oropharyngeal (OA) route and bronchoalveolar Lavage Fluid (BALF) was collected as previously described (38). In brief, anaesthetized mice were stabilized on a plastic wall. The tongue was carefully pulled out in order to get a clear view of the trachea and at the same time, the nares were blocked to force bleomycin inhalation. The appropriate volume of bleomycin diluted in normal saline (~50μL for each mouse) was directly delivered in the oropharyngeal cavity using a conventional pipette tip. At day 14 after bleomycin administration mice were euthanized and bronchoalveolar fluid was obtained by lavaging the airways with 3mL of normal saline using a cannula through the trachea (three times, 1mL each). Then, BALF cells were collected by a 15 min centrifugation at 1.200rpm/4°C. Cell pellets were resuspended in NucleoZol (Macherey-Nagel, 740404.200) for isolation of total RNA as specified by the manufacturer. SuperScript ™ IV VILO™ (Invitrogen, 11766050) was used following the manufacturers guidelines for reverse transcription.

Quantitative real-time polymerase chain reaction (QRT-PCR) was performed using SoFAst EvaGreen Supermix on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Ltd, CA, USA).Values were normalized to β2-microglobulin (B2M).

RNA Extraction and mRNA Expression

1-1.5 million cells were centrifuged and cell pellets were homogenised in TriReagentTM(MBL) for total RNA, followed by storage at -80°C. Total RNA was isolated as previously described (25). Oligos for RT-PCR amplifications were retrieved from http://www.universalprobelibrary.com. COL1A1: Fwd:5'GGGATTCCCTGGACCTAAAG 3', Rev: 5' GGAACACCTCGCTCTCCA 3', SPP1: Fwd: 5' GGGCT TGGTTGTCAGCAG 3', Rev: 5'TGCAATTCTCATGGTAG TGAGTTT 3', GAPDH: Fwd: 5'AGCCACATCGCTCA GACAC3', Rev: 5'GCCCAATACGACCAAATCC 3'. Primer pairs correspond to gene specific assays #67, 63, and 60 respectively. GAPDH levels were used as an endogenous control for the normalization of mRNA expression levels in BAL samples. Gene expression analysis was performed following incorporation of relative expression values in average (duplicates) normalized by GAPDH. Relative expression values for the patient cohort were calculated by $2^{-\Delta\Delta Ct}$ method, where ΔΔCt =(sample Ct GOI-sample Ct GAPDH)-(Calibrator Ct GOI- Calibrator Ct GAPDH), GOI= gene of interest, calibrator= mean of all Cts. Relative gene expressions were log transformed.

Mouse Q-Real-time PCR was performed on a BioRad CFX96 TouchReal-Time PCR Detection System (Bio-Rad Laboratories Ltd, CA, USA). For the detection of collagen 1α1 transcript we used the following primers: 5'-CTACTACCGGGC CGATGATG-3' (F) & 5'-CGATCCAGTACTCTCCGCTC-3' (R). Values were normalized to the expression of b-2 microglobulin [B2M, primers: 5'-TTCTGGTGCTTGTC TCACTGA-3' (F) & 5'-CAGTATGTTCGGCTTCCCATTC-3' (R)].

Airway Macrophage Cell Culture

0.5 million freshly isolated BAL cells were cultured in DMEM, high glucose, with stable glutamine and sodium pyruvate (Biosera) supplemented with 2% Fetal Calf Serum (FCS) (Biosera) and 1x concentration of penicillin-streptomycin (from 100x concentrated solution, Biosera) in a humidified incubator at 37°C containing 5% CO_2 for 30min, with washes to remove non-adherent cells, such as lymphocytes and eosinophils. In order to avoid changes in gene expression caused by the contact of cells with rigid surfaces that may lead to detectable altered protein levels of COL1A1 or OPN, cells were cultured for a short period of thirty minutes. Cells were fixed with 4% formaldehyde (FA) for 20 min at RT and were stored overnight in 1% FA.

Immunofluorescence

FA fixed cells were washed three time with PBS and permeabilization buffer (0.5%FBS, 0.2%Triton in TBS) was added for 10 minutes followed by antibody blocking buffer (0.5%FCS, 0.1%Triton, 2mg/ml BSA in TBS) for 10 minutes at RT. Primary antibody incubations were carried out for 60 minutes at room temperature in antibody blocking buffer followed by washing with TBS. Secondary antibody incubations were performed for 30 minutes followed by washing with TBS. ToPro-633 or DAPI was added for nuclear staining. Finally, TrueBlack (Biotium) was added for 30 seconds to eliminate autofluorescence signal. COL1A1 was detected with rabbit anti-Col1a1 antibody (TA506380, Boster biologicals) at final concentration 5μg/ml, CD45 with mouse anti-CD45 (M0701, Dako) diluted 1/100 times, CD163 with mouse anti-CD163 (TA506380, Origene) at final concentration 3.5μg/ml, osteopontin was detected with mouse anti-OPN (MAB1433, RnD systems) at final concentration 3μg/ml. Secondary antibodies were: for CD45 and CD163 staining, anti-Mouse Cy3 (M 30010, Thermo Scientific), for OPN staining of fresh BAL cultures Alexa-Anti-mouse 633 (A-21126 Thermo Scientific) or Alexa anti-mouse 488(A11001, Thermo Scientific) and for COL1A1, Alexa-anti-rabbit 488 (A11008, Thermo Scientific). All secondary antibodies were used in 1/500 dilution. Secondary antibody controls were also performed with no background fluorescence. Images were taken by Sp2 Leica confocal microscopy with 63x Plan-Apochromat oil lens. Mean intensity of protein levels/cell were calculated with ImageJ v2.0.0-rc-69/1.52i as follows, photomicrographs, with identical acquisition settings, were adjusted for background, AMs were contoured and mean pixel signal intensity was measured for each cell. 5-8 photomicrographs, depending on cell density and an average of 100 cells per patient sample were measured.

Statistical Analysis

Data were analysed using Prism 8 (Graph Pad) software. Gene expression comparisons between two groups of a normal distribution were performed with Welch's T test, when sample numbers between the populations were unequal or a t-test when

population numbers were similar When populations were of a not normal distributions Mann-Whitney test was performed. Gene expression comparisons between more than two groups were performed with one-way Anova and multiple comparisons were performed with Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli recommended in Prism 8 software. Comparisons of intracellular protein levels/cell obtained by immunofluorescence between the groups were performed with Kruskal-Wallis test and multiple comparisons were performed with Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli recommended in Prism 8 software.

Survival was analysed using SPSS 25 (IBM) software. Survival analysis was performed using Cox proportional hazard analysis. We used receiver operating characteristics (ROC) curve analysis to select a cut point for COL1A1 that predicted progression and generated hazard ratios (HRs) to compare patients assigned to groups based on the optimal cut-point. A *p* value less than 0.05 was considered statistically significant (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

RESULTS

COL1A1 mRNA Expression Was Elevated in the BAL Cells From IPF Patients and Experimental Fibrosis in Mice

We have previously observed that mRNA levels of COL1A1 are detectable in BAL cells and are elevated in IPF patients relative to controls (24, 25). Whole BAL cell analyses by RT-PCR in the current cohort confirmed the significant upregulation of COL1A1 mRNA in IPF compared to controls (p=0.0036) (**Figure 1A**). Similar results could be obtained by an alternative approach, using microarray transcriptomic data from BAL cell samples of IPF patients (39). Re-analysis of the above dataset showed that COL1A1 was among the significantly upregulated genes that increased more than 2 fold relative to healthy individuals (0.41 log2FC; 0.0007 p value; 0.008 FDR corrected p value) (**Supplementary Figure 1A**).

We also measured COL1A1 expression by RT-PCR in BAL cells from bleomycin treated mice and found that COL1A1 mRNA was induced by the bleomycin treatment (**Supplementary Figure 1B**). In agreement with our results, re-analysis of RNAseq data from alveolar macrophages isolated from mice fourteen days following bleomycin instillation (40), showed significant upregulation of co1a1 relative to saline treated mice (3.41 log2FC; 1.38e-06 value; 4.4e-05 FDR corrected p value) (**Supplementary Figure 1C**).

COL1A1 mRNA Expression Was Elevated in the BAL Cells From Non-IPF ILD Patients and in Particular Those With UIP Pattern on CT

COL1A1 expression in the BAL was upregulated during fibrosis in both humans and animal models. We therefore sought to examine whether COL1A1 upregulation was specific to IPF or was a characteristic of Non-IPF ILD patients as well. Whole BAL cell mRNA analysis showed that COL1A1 mRNA levels were higher not only in IPF but showed a similar trend in other ILD diagnostic subgroups, collectively compared to controls (one-way Anova test: p=0.03 and Welch's t-test pairwise comparisons, IPF relative to healthy: p=0.036, N-IPF relative to healthy: p=0.051) (**Figure 1A**). Within the non-IPF ILD subgroup, significant differences were observed between the groups, (One-way Anova test: p= 0.04) and in particular RA-ILD and NSIP, showed significantly increased COL1A1 mRNA expression relative to healthy individuals following pairwise comparisons (t-test, p=0.035 and p=0.024 respectively) (**Figure 1B**).

Next, we examined the expression of COL1A1 according to fibrosis pattern on CT, and found that in the whole ILD cohort a UIP pattern was associated with higher COL1A1 levels compared to all other patterns combined (Welch's t-test, p=0.04) (**Figure 1B**). Importantly, within the Non-IPF cohort, patients with a UIP pattern of fibrosis had significantly higher levels of COL1A1 expression (t-test, p=0.04) whilst, in the IPF group, no significant difference was observed between patients with UIP when compared to probable UIP (**Figure 1C**).

Airway Macrophages Express Intracellular COL1A1 in Fibrotic ILDs

The cellular source of COL1A1 in the BAL was examined by immunofluorescence in freshly isolated AMs from BAL of IPF and non IPF f-ILD patients. COL1A1 staining demonstrated a characteristic ER localization, illustrated by a membranous web in the cells (**Figure 2**). Some extent of phagocytosis could not be excluded as COL1A1 positive cytoplasmic vacuoles were also detectable. Furthermore, to confirm that COL1A1 positive cells were of myeloid origin and to exclude the possibility that undetected fibroblasts were responsible for the COL1A1 mRNA measured, cells were co-stained with anti-CD45 a pan-leukocyte marker. As shown in **Figure 2B**, CD45 positive cells showed high COL1A1 expression confirming the expression of COL1A1 by leukocytes. Airway macrophage identity of COL1A1 stained cells was subsequently demonstrated by the co expression of macrophage scavenger receptor CD163 on their surface (**Figure 2C**), which we have previously shown to be elevated in IPF AMs (24).

Intracellular COL1A1 Is Elevated in AMs From f-ILDs

Furthermore, COL1A1 protein levels were semi-quantified by immunofluorescence in formalin fixed whole BAL cell cytospins from samples of IPF, N-IPF and healthy subjects. As shown in **Figure 3A**, COL1A1 expression was heterogeneous among the BAL cells, and was strongly elevated in cells displaying airway macrophage morphology in f-ILD patients. The mean expression of COL1A1/cell calculated from all samples tested per group showed that IPF and N-IPF derived BAL cells had significantly higher COL1A1 expression than healthy subjects (**Figure 3B**). The mean expression of COL1A1/cell for each patient tested, as well as within non-IPF diagnostic subgroups is shown in **Supplementary Figure 2**.

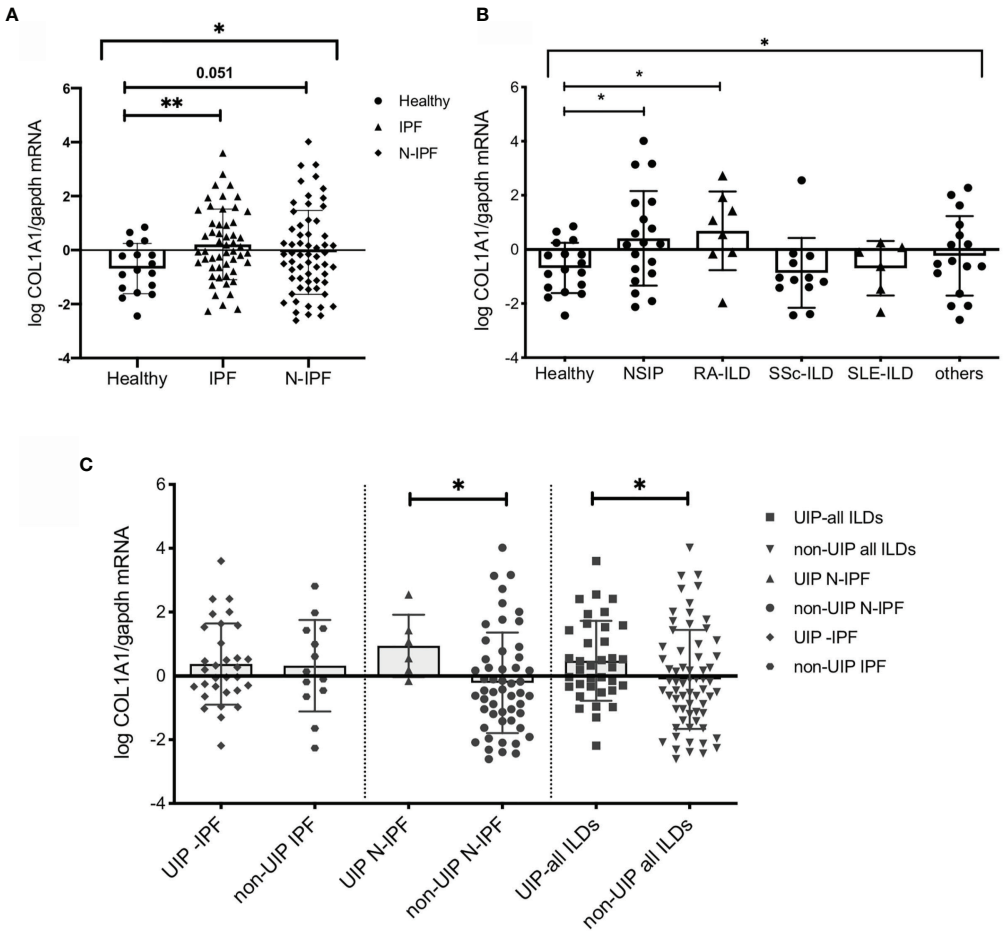

FIGURE 1 | COL1A1 mRNA expression is upregulated in BAL cells from fibrotic ILDs and is higher in ILD patients with UIP *versus* non-UIP patterns of interstitial pneumonia. COL1A1 relative mRNA levels in: **(A)** IPF and N-IPF relative to healthy subjects, (One way Anova test and pairwise t tests), **(B)** COL1A1 expression in whole BAL cells in the ILD diagnostic subgroups relative to heathy. One-way Anova, and pairwise Welch's T tests, p = 0.024), and H *vs* RA p = 0.031 (or Welch's T test p = 0.035). **(C)** Definitive UIP *versus* all other non-UIP patterns in IPF, N-IPF and all ILD patients included in the study. (*p < 0.05, **p < 0.01).

SPP1/OPN Is Elevated in BAL Cells From F-ILDs and Co-Expressed With COL1A1 in AMs

The *SPP1* gene encodes for the cytokine OPN that regulates COL1A1 expression and independent recent studies have shown an increase in a population of pulmonary macrophages expressing SPP1 mRNA in IPF and other ILDs (26). As such, we tested if COL1A1 expression was associated with SPP1 expression in BAL cells. Our results confirmed the increase of SPP1 mRNA in whole BAL cell extracts in IPF relative to healthy subjects however, we found that SPP1 was also significantly elevated in N-IPF relative to both healthy and IPF (Kruskal-Wallis test: p<0.0001, pairwise comparisons IPF relative to healthy: p=0.02, N-IPF relative to healthy: p<0.0001 and N-IPF relative to IPF p: <0.0001) (**Figure 4A**). Within the fibrotic ILDs diagnostic subgroups, NSIP, RA-ILD and SLE-ILD BAL cells had significantly higher SPP1 levels relative to healthy (**Supplementary Figure 3**).

The expression of OPN was evaluated by immunofluorescence in whole BAL cytospins. AMs in N-IPF patients showed particularly high levels of OPN expression when compared to controls (**Figure 4B**), as were NSIP and RA-ILD (**Supplementary Figure 4**). OPN was detected mainly in the outer membrane of AMs, although high levels of cytoplasmic expression were also observed in a fraction of cells in the fibrotic patients (**Figure 4C**). Neutrophils also stained positive for OPN in f-ILDs (**Supplementary Figure 5**).

Finally, we tested if AMs co-expressed OPN and COL1A1. COL1A1 mRNA levels were moderately associated with higher SPP1 mRNA in f-ILDs other than IPF (Pearson's R=0.36, p=0.006). In IPF, SPP1 levels although elevated, did not correlate with COL1A1. However, co-expression of OPN and COL1A1 could be observed in AMs in IPF and N-IPF BAL cultures as shown in **Figure 5**.

Elevated COL1A1 mRNA in BAL Cells Is Associated With Worse Survival and ILD Progression

We subsequently tested possible associations of increased COL1A1 and SPP1 expression with disease severity in IPF and

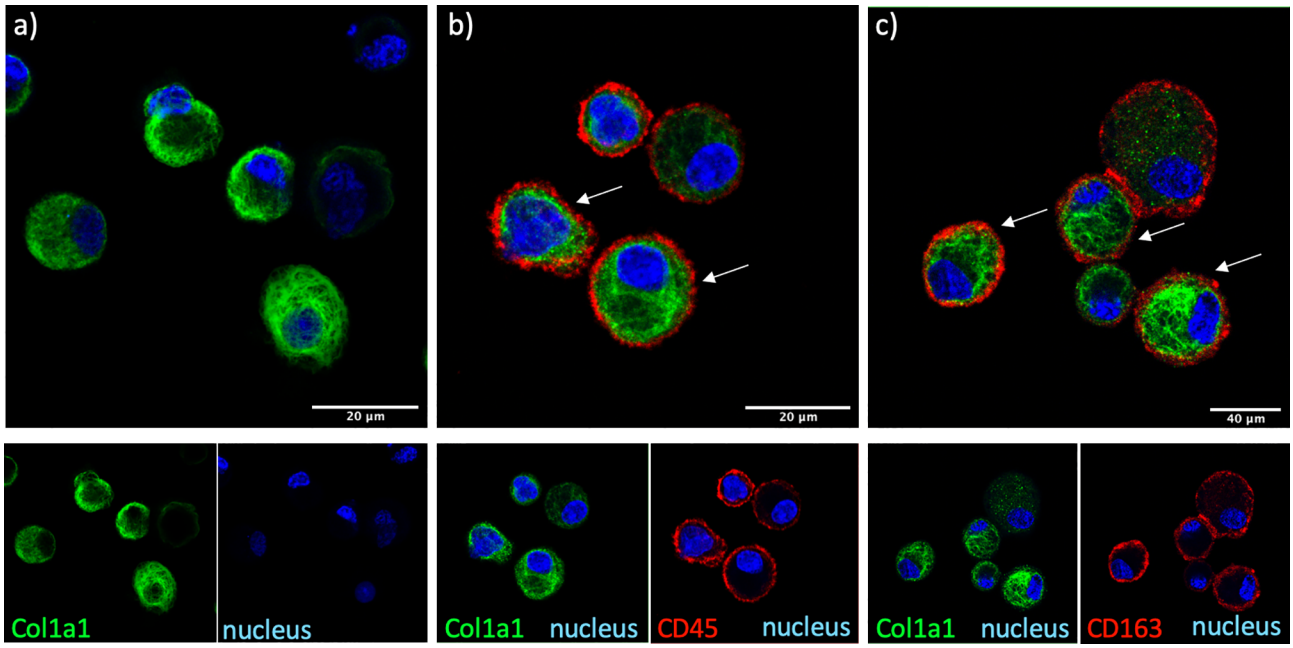

FIGURE 2 | COL1A1 is expressed by cells of myeloid origin and AMs. COL1A1 expression in fresh BAL cultures from a Non-IPF patient with CTD-ILD, stained with rabbit anti-human-COL1A1 and ToPro-633 nuclear stain and **(A)** mouse isotype control, **(B)** mouse anti-CD45 and **(C)** mouse anti-CD163 antibodies. Arrows indicate CD45 and CD163 positive cells expressing COL1A1.

non-IPF patients. In the subgroup of patients with available serial PFTs (n=66), higher COL1A1 relative mRNA expression was associated with 12 month-FVC trends in the whole ILD population (R=-0.38, p=0.001, Pearson correlation). Subgroup analysis revealed that FVC decline was largely associated with higher COL1A1 mRNA in non-IPF patients (n=36) (R=-0.56, p=0.004, Spearman correlation), but not in IPF (n=33) (R=-0.084, p=NS, Spearman correlation) (**Figures 6A–C**).When ILD patients were dichotomized in two categories, stable and progressive ILD, as defined by FVC decline of 10% or more, progressive ILD patients had higher mean COL1A1 mRNA levels compared to stable ILDs (**Figures 6D–F**).

COL1A1 as a continuous variable was linked to higher mortality risk in ILDs (HR 1.3 CI 1.1-1.6, p=0.007), independently of age or disease severity at baseline, CPI (marker of severity) and UIP (independent predictor of worse survival) (HR 1.3; CI 1-1.7, p=0.02). Subgroup analysis of the IPF and N-IPF groups showed that COL1A1 was mostly associated with mortality in the non-IPF cohort with a HR ratio of 1.4 (CI: 1.1-1.8, p=0.01). After adjustment for age, gender, CPI and UIP pattern, COL1A1 remained independently associated with worse survival (HR: 1.6; CI 1.1-2.2 p=0.008) in Non-IPF patients. This was not observed in IPF (HR 1; CI 0.7-1.6, p=NS).

ROC curve-defined cut-off showed that a relative mRNA expression of log 0.5382 was associated with 1.8 times worse risk of mortality in all ILDs (HR 1.8; CI 1.06-3, p=0.03). This remained significant after adjusting for age (HR 1.8; CI 1.2-3.1, p=0.02) or CPI (HR 1.9; 1.1-3.3, p=0.02), but not UIP (HR 1.5 CI 0.8-2.7, p=0.6). Subgroup analysis revealed that the COL1A1 expression above this cut-off was associated with 2.2 times increased risk of death in the non-IPF ILD cohort (HR 2.2; CI 1-4.8, p=0.04) but not in IPF (HR 1.55; CI 0.7-3.2, p=NS). In the Non-IPF group this significant association was independent of age, CPI and UIP (HR 3.8; CI 1.4-10.2 p=0.01). Kaplan Meier curves for the patients with collagen expression above and below the cut-off are shown in **Figures 6G–I**.

SPP1 mRNA expression by airway macrophages in unadjusted analysis was not associated with survival in either group and after adjustment for age, CPI and UIP pattern, SPP1 associate with better outcomes in IPF (HR 0.6; CI 0.4-0.97 p=0.04) but not in the Non-IPF group (HR 0.9, CI 0.7-1.2, p=NS).

DISCUSSION

In this study, we show that alveolar macrophages express collagen 1a1 and this is associated with worse outcomes in ILDs. We examined a cohort of 115 ILD patients and found that the expression of COL1A1 increased in IPF and other progressive ILDs. A UIP pattern of fibrosis was characterised by higher COL1A1 expression in all ILDs and the higher expression was largely associated with worse survival in Non-IPF ILDs. COL1A1 and OPN were abundantly co-expressed in airway macrophages from all ILDs and were significantly higher relative to healthy individuals.

For years, IPF has been considered a distinct disease, of unknown cause, characterised by a UIP pattern of fibrosis and

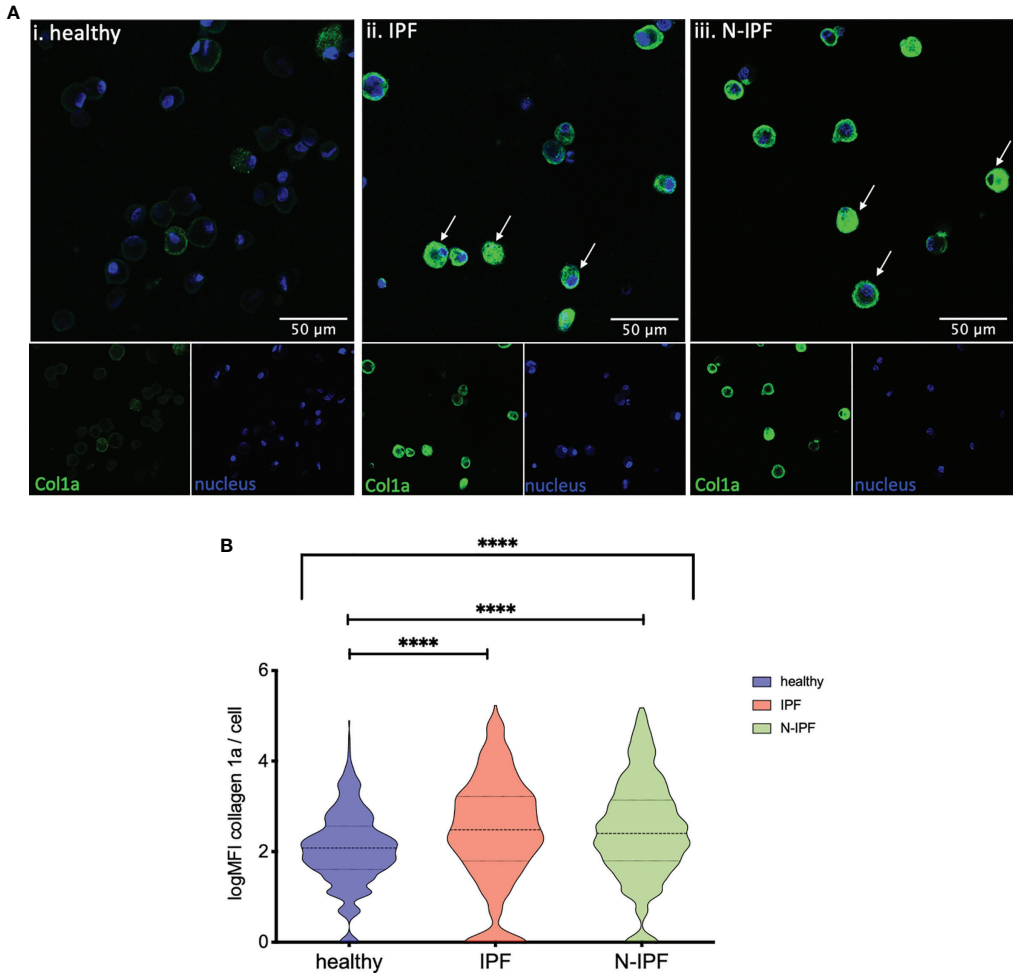

FIGURE 3 | Increased expression of COL1A1 in AMs from fibrotic ILDs. **(A)** Typical images of BAL cytospins stained with anti-human COL1A1 and ToPro-633 nuclear stain from (i) healthy, (ii) IPF, and (iii) a representative N-IPF (RA-ILD) sample. Arrows indicate prominent COL1A1 expression in BAL cells. **(B)** Violin plots of mean log COL1A1 expression/cell per group. (Brown-Forsythe Anova, with pairwise comparisons, ****$p < 0.0001$).

progressive fibrosis leading to dismal prognosis (41). New evidence suggests that some Non-IPF ILD patients may exhibit similar progression despite therapy and it is well known that Non-IPF ILDs can present with a UIP pattern of fibrosis. While the lumping of ILDs is controversial, it has been supported by recent evidence (3). Treatment with Nintedanib, a novel antifibrotic that changed the landscape in IPF treatment (42, 43), has similar efficacy in other PF-ILDs (44, 45), as well as in SSc-ILD (46). Similarly, Pirfenidone, another antifibrotic used in IPF (47), reduces FVC decline in unclassifiable ILDs (48). Based on the commonalities in clinical behaviour of IPF and PF-ILDs with the progressive fibrotic phenotype, it is speculated that common underlying pathogenetic pathways exist and drive fibrosis progression, despite variability of the initial trigger (6, 7).

Biomarkers that could predict progression in fibrotic ILD patients include UIP pattern on CT across different entities such as CTD-ILD (8, 49), hypersensitivity pneumonitis (9) and IPAF (50), severity of the disease at baseline such as DLCO, and/or CPI (51) and genetic or molecular biomarkers including MUC5b polymorphisms (52). Here we show that fibrotic ILD patients with a more progressive phenotype and in particular RA-ILD and NSIP patients, had higher COL1A1 expression than healthy individuals, much similar to IPF. Of particular interest, we showed that within the Non-IPF-ILD cohort higher COL1A1 expression in the BAL at the time of diagnosis was associated with more than two-fold risk of mortality, independently of a UIP pattern, disease severity and age. Similarly, we showed that patients with a UIP pattern of fibrosis, a classic imaging marker of progressive disease, have higher COL1A1 expression. BAL is a largely safe, minimally invasive procedure, used in most ILD centers worldwide for the diagnosis of ILDs (33). The discovery of this novel association between COL1A1 expression and survival could have implications in the management of ILDs, as BAL and COL1A1 measurement could act as a biomarker of progression at the initial stages of the ILD diagnosis. This would be a useful personalised medicine tool to stratify early, patients at

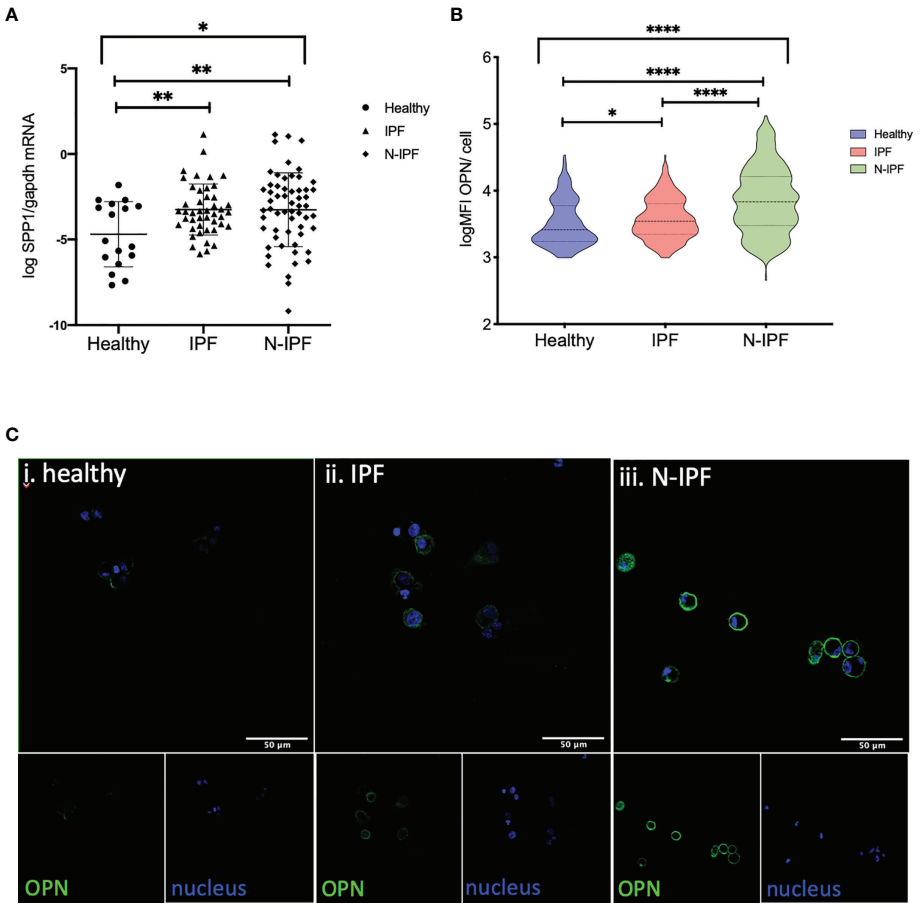

FIGURE 4 | SPP1 and OPN expression in BAL cells. **(A)** SSP1 relative mRNA levels in IPF and N-IPF relative to healthy (Brown-Forsythe Anova, with individual comparisons), **(B)** Violin plots of mean OPN expression/cell per disease group (Kruskal-Wallis test and individual comparisons) and **(C)** Typical Images of BAL cytospins stained with anti-human OPN antibody and ToPro-633 nuclear stain in (i) healthy, (ii) IPF and (iii) N-IPF samples (*p < 0.05, **P < 0.01, ****p < 0.0001).

risk of progression that would benefit from access to antifibrotic therapy before the detrimental progression occurs.

Although the expression of collagens by macrophages may be considered controversial it has been previously demonstrated in animal models. The COL1A1 expression atlas of fibrotic mouse lungs demonstrated that cells with hematopoietic marker CTPRC (encoding for CD45 antigen), CCR2 and CD68 macrophage markers expressed COL1A1 (53). Additionally, our re-analysis of alveolar macrophages isolated from mice fourteen days following bleomycin instillation (40), showed that COL1A1 was significantly upregulated relative to controls. We previously observed that mRNA levels of COL1A1 were detectable in whole BAL cell mRNA and were elevated in IPF patients relative to controls (24, 25). We also performed meta analyses of previously published BAL transcriptomic data from IPF patients (39) showing upregulation of COL1A1 in IPF BAL cells. In that study COL1A1 expression was associated with increased mortality, similarly to our study.

In order to exclude that this higher COL1A1 expression in the BAL might represent the presence of fibroblasts, we showed that COL1A1 was intracellularly expressed in airway macrophages with immunofluorescence and confocal microscopy. Cells with macrophage morphology isolated from BAL and positively stained for CD45 or CD163, were shown to contain high levels of COL1A1. CD163 positive macrophages accumulate in the tissue of fibrotic patients as previously shown (54) and in the BAL of IPF patients (24). According to previous reports that aimed at the identification of markers that would discriminate macrophages and fibrocytes from fibroblasts (21) CD45 was clearly not expressed on fibroblasts. In the same study, similar levels of COL1A1, CD45 and CD163 could be detected on macrophages and fibrocytes. Further characterisation is therefore needed in order to clarify the relative abundance of COL1A1, CD45 and CD163 positive macrophages and fibrocytes in the BAL of ILD patients.

An important finding of our study was that osteopontin and COL1A1 expression were both elevated in airway macrophages. Independent studies of lung macrophages using Single-cell RNAseq analyses have demonstrated the striking upregulation of SPP1 positive macrophages in IPF. Although the molecular

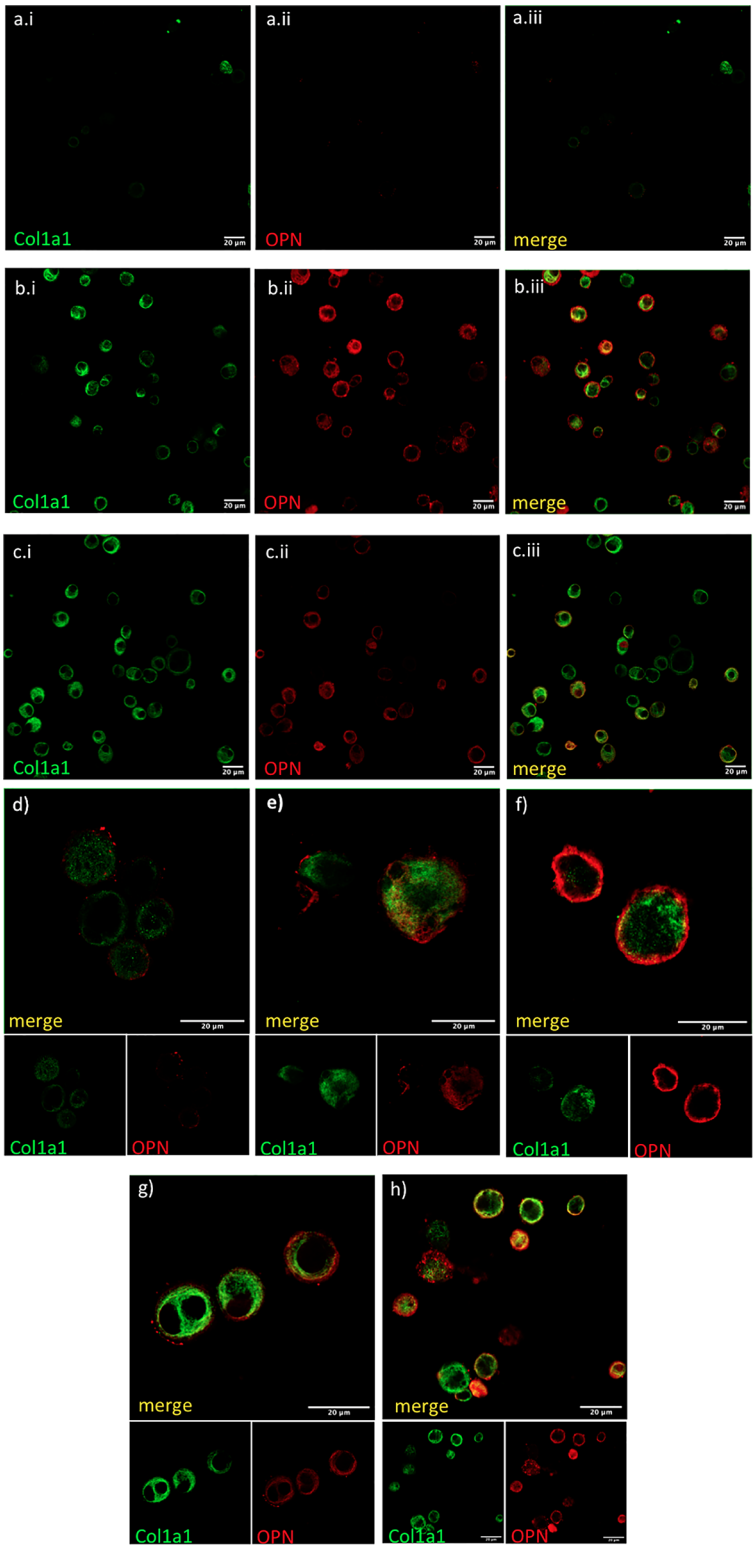

FIGURE 5 | COL1A1 and OPN are coexpressed in AMs from fibrotic ILDs. Fresh BAL cultures were stained with rabbit anti-human-COL1A1and anti-human OPN in healthy **(A, D)**, IPF **(B, E, F)**, and RA-ILD **(C, G, H)**. Panels **(A.i, B.i, C.i)** show COL1A1 stained cells, panels **(A.ii, B.ii, C.ii)** show OPN stained cells and panels **(A.iii, B.iii,C.iii)** are the corresponding merged images. Panels **(D–H)** show merged COL1A1 and OPN images with corresponding single stainings as smaller insets below.

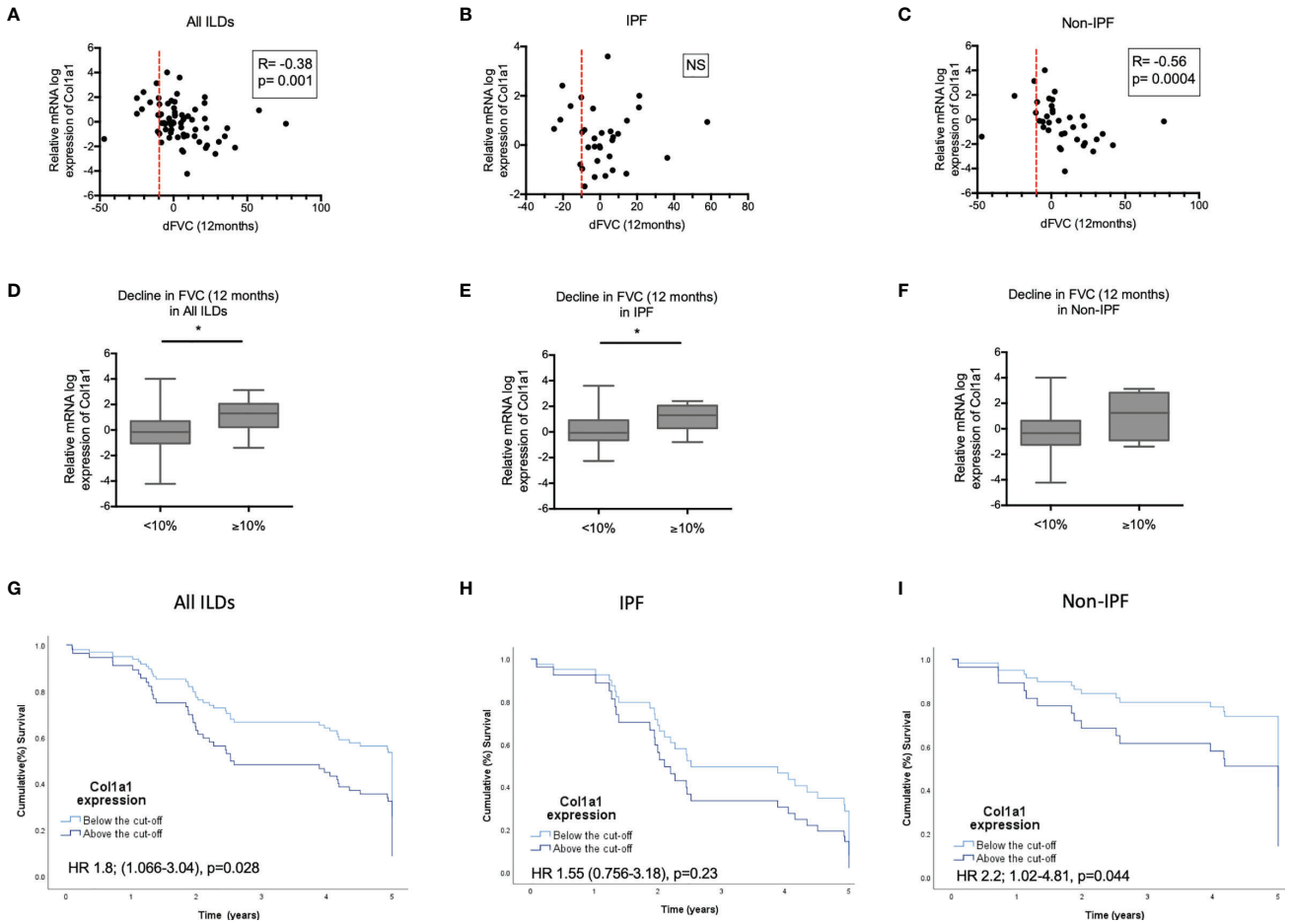

FIGURE 6 | COL1A1 expression in relation to progression and mortality. Spearman correlation coefficient between relative mRNA expression of COL1A1 and FVC decline in 12 months in: **(A)** all ILDs combined (IPF and other ILDs), **(B)** IPF and **(C)** ILDs other than IPF. Comparison of COL1A1 relative mRNA expression in progressors (≥10% FVC decline at 12 months) and non progressors (<10% FVC decline at 12 months in **(D)** all ILDs, **(E)** IPF and **(F)** ILDs other than IPF unpaired T-test or Mann-Whitney tests. Kaplan Mayer Survival curves of patients with high (above the ROC curve-defined cut-off) and low expression of COL1A1 mRNA expression in: **(G)** all ILDs combined, **(H)** IPF and **(I)** ILDs other than IPF. (*p < 0.05). HR, Hazard ratio).

mechanism of the induction of collagens by OPN is not thoroughly characterised, recent reports suggest that OPN acts through integrin $\alpha_v\beta_3$ engagement and activation of the phosphoinositide 3-kinase/phosphorylated Akt/nuclear factor kappa B (PI3K/pAkt/NFκB)–signalling pathway at least in hepatic stellate cells (Urtasun et al. Hepatology 2012). Interestingly SPP1 levels correlated with COL1A1 expression in the Non-IPF ILDs but not within the IPF group suggesting a possible uncoupling of collagen expression from OPN in the IPF macrophages. Additionally, we observed that SPP1 mRNA was elevated in the BAL of most ILDs tested, irrespective of disease severity and CT patterns. Intriguingly, increased levels of SPP1 were associated with more favorable outcomes in IPF, in contrast to the rest of the ILDs. Circulating OPN was recently associated with immune complexes-driven profibrotic macrophage activity in SSc-ILD (55) and it could be speculated that OPN plays a more significant role in autoimmune-driven fibrosis as compared to IPF. Furthermore, recent studies with models of tissue repair and scar formation following heart injury showed that osteopontin expressing recruited macrophage populations play both a pro-fibrotic and pro-resolving role (56).

Our work also infers to a less characterised role of airway macrophages in pulmonary fibrosis, as it demonstrates a link between the macrophage derived expression of COL1A1 and fibrotic disease pathogenesis. There are few reports describing a direct role of macrophages in ECM production during fibrosis. A recent mouse model of fibrotic scar formation in the heart shows that recruited monocyte-derived macrophages to the injured site directly contribute to collagen-I fibril formation, suggesting, that collagen production is a component of the pro-fibrotic monocyte/macrophage response (22). Additionally, tumour associated macrophages of monocyte origin were shown to upregulate COL1A1 directly, affecting collagenous matrix organisation in the tumour microenvironment (57). In IPF,

monocyte derived macrophages are also believed to play a detrimental role in the disease although the exact mechanism is not understood.

The main limitation of our study is the non-prospective validation of COL1A1 as a biomarker of ILD progression. This was mainly due to the small number of patients with non-IPF ILD and the small number of control patients. Additionally, further work is required for the precise elucidation of the macrophage populations that express COL1A1 as well as, their function. In conclusion our findings suggest that COL1A1 expression by macrophages may be a novel pathogenetic pathway in fibrotic ILDs that is related to worse outcomes. Our observations are exciting as COL1A1 expression could be further evaluated as a biomarker of Progressive ILD and raises hope for the early identification of patients that will develop a Progressive Fibrotic Phenotype.

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 Ethics Committee of the University General Hospital of Heraklion and the Ethics Committee of Royal Brompton Hospital, London, UK.

AUTHOR CONTRIBUTIONS

ET and AT designed study, performed experiments, analyses and wrote the manuscript. EV, GM, and MK collected patient information and critically read the manuscript, SM performed experiments. DF performed meta-analyses from whole transcriptome data. AG performed mouse experiments. VA designed mouse experiments and critically read the manuscript. ER and NT recruited patients and critically read the manuscript. AW and KA designed study, recruited patients, and wrote manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a Hellenic Thoracic Society (HTS) research award 2018 to ET and KA.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.645548/full#supplementary-material

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