

## The Monocyte/Macrophage Lineage as a Therapeutic Target in Idiopathic Pulmonary Fibrosis

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### Abstract

Idiopathic pulmonary fibrosis (IPF), is a rare but increasingly prevalent lung disease, affecting approximately three millions people worldwide. With a median survival post diagnosis of two to four years, IPF is as devastating as some of the most aggressive cancers. Despite the recent approval by the FDA of two drugs able to slow down the disease progression, there is still no cure for IPF, and the mortality remains high in the populations affected. Due to the ineffectiveness of anti-inflammatory treatments (mainly corticosteroids and azathioprine) in IPF, the role of inflammation and immune cells in the disease pathogeny has become a controversial subject. Here we are reviewing data highlighting a role for the monocyte/macrophage lineage in IPF and experimental models of lung fibrosis. We will also examine recent publications shedding new lights on the mechanism of alveolar macrophage resistance to steroids and the effect of current and prospective IPF treatments on lung macrophages biology.

**Keywords:** *Idiopathic Pulmonary Fibrosis; Macrophages; Monocytes; Therapy*

### Abbreviations

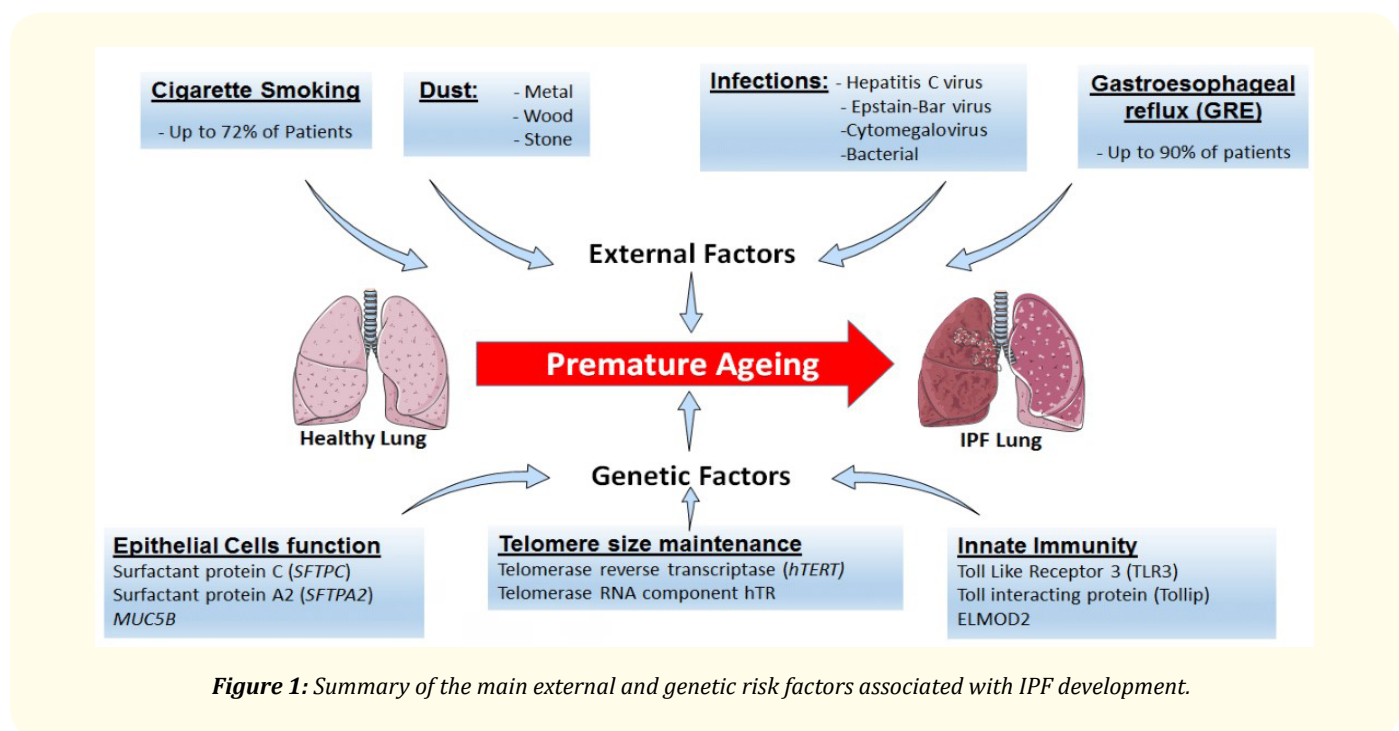
AAM: Alternatively Activated Macrophages; AE-IPF: Acute Exacerbation - Idiopathic Pulmonary Fibrosis; AM: Alveolar Macrophages; BALF: Bronchoalveolar Lavage Fluid; C/EBP $\beta$ : CCAAT/Enhancer Binding Protein  $\beta$ ; CCRX: C-C Chemokine Receptor Type X; CD: Cluster of Differentiation; CD: Cluster of Differentiation; CEACAMX: Carcinoembryonic Antigen-Related Cell Adhesion Molecule X; Clec10a: C-Type Lectin Domain Containing 10A; CXCL: C-X-C Motif Chemokine Ligand; CXCRX: C-X-C Chemokine Receptor type X; EMR1: EGF-Like Module-Containing Mucin-Like Hormone Receptor-Like 1; EMT: Epithelial to Mesenchymal Transition; ER stress: Endoplasmic Reticulum Stress; FCGR2A: Fc Gamma Receptor IIa; FF: Fibroblastic Focus; FGFR-X: Fibroblast Growth Factor Receptor-X; FVC: Forced Vital Capacity; GM-CSF: Granulocytes Macrophages-Colony Stimulating Factor; GM-CSFR: Granulocytes Macrophages-Colony Stimulating Factor Receptor; HC: Healthy Controls; HP: Hypersensitivity Pneumonitis; IFN- $\gamma$ : Interferon- $\gamma$ ; IL-17RB: Interleukin 17 Receptor Beta; IL-1Ra: Interleukin Receptor Antagonist; IL-23A: Interleukin-23 Subunit Alpha; IL-2RA: Interleukin-2 Receptor Alpha Chain; ILD: Interstitial Lung Diseases; IL-x: Interleukin-x; INF- $\gamma$ : Interferon- $\gamma$ ; IPF: Idiopathic Pulmonary Fibrosis; LCN2: Lipocalin-2; LPS: Lipopolysaccharides; LTF: Lactotransferrin; Ly6C: Lymphocyte Antigen 6 Complex, Locus C; MCP-1: Monocyte Chemoattractant Protein-1; M-CSF: Macrophage-Colony Stimulating Factor; MDM: Monocyte-Derived Macrophages; MIF: Macrophage Migration Inhibitory Factor; mL: Millilitre; MMP: Matrix Metalloproteinase; NTHi: Non-Typeable *Haemophilus influenzae*; OPN: Osteopontin; PaO<sub>2</sub>: Partial Pressure of O<sub>2</sub>; PARC: Pulmonary and Activation-Regulated Chemokine; PDGFA: Platelet-Derived Growth Factor Subunit A; PDGFR $\alpha$ : Platelet Derived Growth Factor Receptor Alpha; PGD2: Prostaglandins D2; PGE2: Prostaglandins E2; PIR-B: Paired Immunoglobulin-Like Receptor B; S100A9: S100 Calcium Binding Protein A9; SMA: Smooth Muscle Actin; TGF- $\beta$ : Transforming Growth Factor- $\beta$ ; Th1: T Helper 1; Th2: T Helper 2; TNF $\alpha$ : Tumor Necrosis Factor Alpha; UIP: Usual Interstitial Pneumonia; UPAR: uPA Receptor; VEGF: Vascular Endothelial Growth Factor; VEGFR-X: Vascular Endothelial Growth Factor Receptor-X

**Introduction**

Idiopathic pulmonary fibrosis (IPF) is a rare and progressive parenchymal/interstitial lung disease of unknown origin, whose incidence in the western world has risen in the past decades to reach 2.8 to 18 cases per 100 000 people per year [1]. Patients with IPF usually present with slowly progressive dyspnoea on exertion and dry cough. The disease mainly affects men, over 50 - 60 years of age, and for lack of effective treatments other than lung transplantation, the median survival time from diagnosis ranges between two and four years [1]. Although a progressive disease, 5 - 15% of IPF patients a year, can experience episodes of acute exacerbations of the disease (AE-IPF), characterised by a rapid deterioration of their lung function outside any known cause (e.g. infections) [2]. Despite treatments with high doses of corticosteroids and antibiotics, AE-IPF is fatal for approximately 50% of patients [2].

IPF is characterised by a histological pattern of usual interstitial pneumonia (UIP), which distinguishes it from other interstitial lung diseases (ILD), such as, chronic hypersensitivity pneumonitis (HP), which is characterised by an airway-centered pattern of fibrosis [3]. The UIP pattern presents three main features: (1) A patchy pattern of fibrosis mainly in the subpleural/paraseptal areas which alternate abruptly with histologically normal alveolar areas. (2) Enlarged alveolar spaces, lined by bronchiolar metaplastic epithelium, filled with mucus and inflammatory cells, called Honeycomb. (3) The presence of dome-shaped areas, formed by actively proliferating fibroblasts/myofibroblasts, embedded in a pale myxoid matrix, and covered by hyperplastic pneumocytes called fibroblastic focus (FF) [3].

Although of unknown aetiology many external and genetic risk factors have been associated with IPF development (Figure 1). Thus epidemiologic studies have highlighted a history of cigarette smoking in 73% of IPF patients, whereas other studies have incriminated exposure to metal, wood, stone and silica dust, often in a professional environment [4]. Apart from inhaled particles, gastroesophageal reflux is also believed to contribute to the external aggression of the lung and is found in as much as 90% of patients suffering from IPF [5]. Finally, bacterial and viral infections especially herpes viruses, Epstein-Barr and hepatitis C virus infections have been observed at a higher rate in IPF patients and could participate in disease initiation and progression [6]. In addition to these external factors, the recent advances in genomics have led to numerous genome-wide association studies in patients with sporadic as well as familial forms of IPF. These studies led to the identification of genetic variants positively associated with IPF, in genes related to lung epithelial cells function (e.g. MUC5B, SFTPC, SFTPA2), telomere size maintenance (e.g. TERT, hTERT) and innate immunity (e.g. TLR3, Tollip, ELMOD2) (Figure 1) [7]. All these data suggest that IPF emerges from a lifetime chronic aggression of the lung by various external factors in individuals whose genetics predispose to premature ageing, Endoplasmic Reticulum stress (ER stress) and abnormal innate immune response.



**Figure 1:** Summary of the main external and genetic risk factors associated with IPF development.

From a mechanistic perspective, IPF has long been perceived as a fibrotic lung disease driven by an underlying chronic inflammatory response of the lung parenchyma, in response to repeated micro injuries of unknown origin. However, the inefficiency of anti-inflammatory treatments (e.g. corticosteroids) together with the mild inflammatory findings in histological specimens compared to others lung interstitial diseases, prompted the scientific community to reassess its view of IPF pathogeny [1]. The current predominant concept involves repetitive micro-injury of the lung epithelium leading to aberrant epithelial-fibroblast communication and fibrosis development [1]. Several key cellular and molecular processes involved in epithelium/mesenchyme interactions, such as epithelial to mesenchymal transition (EMT), ER stress, TGF- $\beta$  pathway activation, have been shown to be active in IPF [8]. However changes in, inflammatory cells proportions, the concentration of cytokines and chemokines, in the blood, lung and Bronchoalveolar Lavage Fluid (BALF) of IPF patients also support a role for the immune system and in particular the monocyte/macrophage lineage in this disease. Here we are presenting a review of data supporting the involvement of the monocyte/macrophage lineage in IPF and experimental lung fibrosis while highlighting the effect of past, current and prospective IPF treatments on their function.

### Origin, diversity and function of monocytes and pulmonary macrophages

Macrophages are leucocytes, first described in 1883 by Ilya Metchnikoff for their phagocytic abilities [9]. They are present in virtually all the tissues of our organism, where their phagocytosis of apoptotic cells and others debris, make them active participants in tissue homeostasis and defence against pathogens. The latter function explains, in particular, their presence in different organs during the early steps of embryogenesis which is associated with intense proliferation, apoptosis and morphogenesis [9]. Whereas most tissue resident leucocytes arise from haematopoietic stem cells found in the bone marrow, macrophages from many mammalian organs such as the lung and the liver find their origin in the yolk sac and the foetal liver. Consequently, these cells are referred to as resident macrophages (RM) in adult tissues [10]. On the other hand, the intestine is mainly populated by macrophages derived from the bone marrow haematopoietic stem cells niche with the monocytes as immediate precursors. Therefore, these cells are referred to as monocyte-derived macrophages (MDM) [11]. Resident macrophages have been shown to be, long living and self-renewing, thereby maintaining themselves in adult tissues throughout life [10]. In most adult tissues, there is a co-existence, between both macrophage populations, with a more or less important proportion of MDM, depending on tissues and age. However, during episodes of infections, and other tissues damages, MDM are massively recruited to most organs and actively contribute with resident macrophages to the immune response [12].

Unlike macrophages, monocytes are found primarily in the blood, and secondary lymphoid organs such as the spleen and are produced in the bone marrow from the differentiation of hematopoietic stem cells [13,14]. Although resident monocytes, has been described in some tissue such as the lung, the scientific consensus is that after migration into the tissues, monocytes tend to differentiate into macrophages, dendritic cells (DCs) and fibrocytes [15]. Three different populations of monocytes have been identified in human blood according to their expression of the cluster of differentiation (CD) 14 and 16. Thus the classical monocytes are described as CD14<sup>++</sup>/CD16<sup>-</sup>; the intermediate monocytes as CD14<sup>++</sup>/CD16<sup>+</sup> and the non-classical monocytes as CD14<sup>+</sup>/CD16<sup>++</sup> [15,16]. Recently taking advantage of the accuracy and sensitivity of single-cell transcriptomic Villani, *et al.* identified four human monocytes subtypes, adding thereby more depth to our initial understanding of monocytes diversity [17].

In the lung, there is three main type of macrophages named according to their location. Most pulmonary macrophages are found in the bronchiolar and alveolar space and are called respectively bronchiolar and alveolar macrophages (AM), whereas those present within the pulmonary interstitium are referred to as interstitial macrophages [18,19]. AM play a critical role in pulmonary homeostasis by preventing the accumulation of surfactant proteins, mucus and dust particles that could impair lung function and damage the epithelium [19]. They also contribute to a tolerogenic response to innocuous inhaled particles to avoid excessive inflammation and tissues damages [20]. However, despite sharing common origins, tissue-resident macrophages are undertaking very different, tissues specific functions, (e.g. clearance of amyloid beta plaques in the brain, recycling of iron in the spleen and liver), that require the expression of particular sets of proteins [21]. A recent article by Lavin, *et al.* has highlighted the marked plasticity of macrophages, and the critical role of environmental

factors in shaping their transcriptional landscape through epigenetic remodelling of their chromatin [22]. This capacity to adapt the set of protein they express to the environmental context defined different phenotype/polarisation state [23]. One of the specificities of pulmonary macrophages compare to other populations is their dependency upon the Granulocytes Macrophages-Colony stimulating Factor (GM-CSF), whereas most macrophage populations are heavily dependent upon Macrophage-Colony Stimulating Factor (M-CSF) [24,25]. Thereby patients with anti-GM-CSF autoantibodies or genetic mutations affecting the GM-CSF/GM-CSF receptor pathway present impair pulmonary macrophage function, leading to surfactant protein accumulation in the alveolar space and subsequently to a condition named pulmonary alveolar proteinosis [26].

### The M1/M2 paradigm: Implication for the monocyte/macrophage lineage in IPF

Advances in the fields of lymphocytes biology have led to the characterisation of different inflammatory responses to intracellular and extracellular pathogens [27]. Thus intracellular pathogens are classically associated with the release of interferon-gamma (IFN- $\gamma$ ) by T helper lymphocytes and define a Th1 polarisation, whereas parasitic pathogens trigger the release of Interleukin-4 (IL-4) determining a Th2 response [27]. Because macrophages are an active participant in these responses, *in vitro* experiments, have been carried out using stimuli triggering or released during Th1 and Th2 responses to determine their effects on macrophage phenotype. These experiments led to the establishment of the classically activated macrophages or M1 macrophages phenotype and the alternatively activated macrophages (AAM) or M2 macrophages phenotype [28]. The M1 phenotype is induced by stimuli such as GM-CSF, IFN- $\gamma$ , lipopolysaccharides (LPS) and Tumour Necrosis Factor alpha (TNF $\alpha$ ) whereas, the M2 phenotype is induced by stimuli such as M-CSF, Interleukin-4 (IL-4), -13 (IL-13), -10 (IL-10) and TGF- $\beta$ . Further analysis of the M2 phenotype led to the establishment of subtypes (M2a-c) depending on the mediators used for their generation and the profiles of mediators they secrete [28]. *In vitro* experiments suggest that those phenotypes are not exclusive of each other's and are reversible. Indeed Smith, *et al.* recently demonstrated that M1 macrophages could switch to an M2 phenotype when stimulated for an extended period with M2 inducers. Moreover, co-exposition of macrophage to M1 and M2 inducers (i.e. IFN- $\gamma$ , LPS, IL-4 and IL-13) lead to a mixed phenotype with co-expression of M1 and M2 markers [29]. Interestingly the authors showed that M1 polarisation is associated with an enhanced response to M2 inducers whereas, M2 polarisation tends to inhibit the sensitivity to M1 inducers [29]. *In vivo*, macrophage phenotype/polarisation results from the integration of multiple and sometimes conflicting stimulations based on the M1/M2 nomenclature. Thereby, the *in vivo* phenotype of macrophages in IPF and other diseases is undoubtedly more complex than suggested by the M1/M2 classification.

### The Monocytes Macrophage activation in Idiopathic pulmonary fibrosis

Unlike sarcoidosis and other connective tissue diseases associated lung fibrosis, which develop in the lung in response to systemic factors, IPF is a lung centered disease most likely driven by inhaled particle and with discrete systemic repercussion [3]. Throughout the years several reports have reported changes in term of inflammatory cells proportion, cytokines, and chemokines levels in the blood, and BALF of IPF patients with some report directly pointing to changes in monocytes or macrophages function.

### AM in the Bronchoalveolar lavage fluid of IPF patients

Bronchoalveolar lavage is a medical procedure often used in the diagnostic of lung diseases and consisting in injecting and recovering a saline solution in one lobe of the lung using a bronchoscope. The collected BALF allow the analysis of the inflammatory state of the lung. Thus many clinical studies conducted on patients suffering from interstitial lung diseases have been investigating inflammatory cell and mediator changes in their BALF. However one of the limitations of BALF analysis is due to the fact, that it doesn't allow an appreciation of the localisation of the harvested cells. In diseases with a uniform pattern of fibrosis such as connective tissues disease, this is not a hurdle to the interpretation of the results. However, due to the irregular pattern of fibrosis in IPF, the results from BALF cellularity might mask essential disparities between the number and phenotype of cells in active and healthy areas of the lung.

Keeping this in mind, table 1 presents the summary of BALF cellularity analysis of six independent studies [30-35]. The results show that the BALF of normal individuals contains in average  $158 \times 10^3$  cells/mL with AM representing around 90% of these cells, followed by the lymphocytes 8%, the neutrophils 1.43%, and the eosinophils 0.3%. In IPF patients, on the other hand, the BALF contain in average  $288 \times 10^3$  cells/mL representing an 80% increase in cellularity mainly due to the recruitment and/or proliferation of leucocytes. Thus, in IPF the BALF contain in average 80.4% AM, 7.6% lymphocytes, 8.6% neutrophils, and 3.6% eosinophils (See Table 1). Whereas the absolute number of all these populations are increased in the BALF of IPF patients the number of neutrophils and eosinophils is increased by 11 and 26 fold respectively, the neutrophils even outnumbering the lymphocytes ( $26.5$  vs  $19.6 \times 10^3$  cells/mL) on average. Interestingly some studies comparing these trends with the changes in other lung interstitial diseases showed that sarcoidosis and HP have a different pattern of inflammation. Indeed in sarcoidosis, there is a sharp increase in the proportion of lymphocytes and minor changes in neutrophils and eosinophils counts [32,33], whereas, in HP, both lymphocytes and granulocytes were significantly increased [33,35]. These differences indicate that unlike other ILD the BALF inflammatory signature of IPF is marked by the activation of the innate harm of the immune system. Despite a slight increase in the absolute number of lymphocytes in the BALF of IPF patients compare to the healthy controls (HC) ( $20$  vs  $11 \times 10^3$  cells/mL), the CD4/CD8 ratio remains similar to that of the HC. In the BALF of sarcoidosis patients, on the other hand, the CD4/CD8 ratio increases by 2.77 fold [32,36], signing the activation of a T helper response. Despite having a CD4/CD8 ratio similar to HC, IPF lymphocytes present a decreased expression of the receptor CCR5. Since CCR5 is a marker of Th1 lymphocytes, this suggests a possible Th2 polarisation of lymphocytes in IPF [30].

	Average HC	Average IPF	Delta IPF-HC	FC IPF vs HC
Cells. mL <sup>-1</sup> x 10 <sup>3</sup>	158 (91 - 240)	288 (120 - 400)	130.00	1.82
Macrophages mL <sup>-1</sup> x 10 <sup>3</sup>	143.6 (82 - 230)	231.7 (89 - 363)	88.07	1.61
Lymphocytes mL <sup>-1</sup> x 10 <sup>3</sup>	11.0 (6.3 - 17.3)	19.6 (9.6 - 36.4)	8.67	1.79
Neutrophils mL <sup>-1</sup> x 10 <sup>3</sup>	2.3 (0.73 - 6.5)	26.5 (6.2 - 61.6)	24.16	11.52
Eosinophils mL <sup>-1</sup> x 10 <sup>3</sup>	0.41 (0 - 1.2)	10.6 (3.1 - 24)	10.21	25.67
Macrophages %	90 (79 - 96)	80.4 (74 - 84)		
Lymphocytes %	8 (2.9 - 16.2)	7.6 (3.6 - 11.6)		
Neutrophils %	1.43 (0.6 - 2.97)	8.6 (3.0 - 13)		
Eosinophils %	0.3 (0 - 0.55)	3.6 (1.8 - 5.0)		

**Table 1:** Summary of BALF cells counts in Healthy Control (HC) and IPF patients across six study.

Definition of abbreviations: HC: Healthy Controls; IPF: Idiopathic Pulmonary Fibrosis; FC: Fold Change.

It is not always clear in those study whether the patients were undergoing treatments before the BALF sampling. Therefore the question of the impact of those treatments on the cell count can be raised. In a 2013 article Abdel Khalik, *et al.* analysed the effects of some conventional IPF treatments (i.e. Corticosteroids, azathioprine, acetylcysteine) on BALF cellularity. The authors found that BALF neutrophils count was positively correlated with disease severity (e.g. FVC%, PaO2) and that none of the treatments significantly influenced BALF composition [37]. These results suggest that the inflammatory response in IPF is resistant to standard immunosuppressants and anti-inflammatory drugs, a topic that we will later develop in this review.

One of the primary function of AM is to prevent unnecessary inflammation by phagocytising dying/dead cells in a process called efferocytosis. The analysis of AM efferocytosis in patients suffering from different ILD and HC showed a marked decrease of efferocytosis in IPF patients compared to other ILD and HC. Thereby defective efferocytosis may be a hallmark of AM dysfunction in IPF and offer potential insight into their contribution to IPF pathogeny [38]. Interestingly enough to be mentioned the phagocytosis of apoptotic cells by macrophages play a crucial role in their production and secretion of prostaglandins (i.e. PGE2, PGD2) and hepatocytes growth factor, which have been proven essential to the prevention by macrophages of EMT a process favouring fibrosis and observed in IPF [39].

AM from IPF patients have also been shown to be resistant to apoptosis [40], and present an exaggerated expression of TNF $\alpha$  and IL-10 in response to LPS stimulation [41]. This last point is of particular interest. Indeed as monocytes transit from the blood to the tissue and differentiates into macrophages their expression of CD14 which play a crucial role in LPS response and TLR signalling is decreased [42]. The rationale for such a change is the fact that as the cell is leaving the bloodstream for mucosal/peripheral areas the level of tolerance toward pathogens must increase to prevent unnecessary inflammation. However, several studies have highlighted a more monocytic-like phenotype of AM in IPF reflecting either an alteration of the monocyte to macrophage differentiation process or the active and rapid recruitment of MDM. Thus Kiemle-Kallee, *et al.* reported a monocytic-like phenotype of AM in IPF characterised by the retention of monocytes markers and an increase in the release of free oxygen radicals [43]. In another study evaluating AM phenotype in ILD, the monocytic-like phenotype of AM in IPF was confirmed and demonstrated as well, in other ILD such as sarcoidosis and HP [44]. More recently Yu, *et al.* reported in the BALF of IPF patients the presence of a population of AM with high expression of the monocyte marker CD14 (CD206+/CD169+/CD14+ cells) that was absent in HC individuals whether smoker or not [45]. Overall these data are supportive of an alteration of AM phenotype and function in IPF patients that may contribute to disease progression.

### Peripheral blood monocytes phenotype in IPF

In addition to changes in BALF composition, changes in peripheral blood leucocytes of IPF patients have also been observed, although less drastic than in other ILD. Indeed analysis of blood leucocytes from, 35 IPF patients showed that increases in the circulating CD14<sup>hi</sup>/CD16<sup>hi</sup> subset of monocytes (i.e. > 0.5% of total blood mononuclear cells) measured at baseline correlated with disease progression and poorer outcome. However longitudinal monitoring of these patients showed considerable variation in the level of circulating monocytes over time, suggesting a disease progression made up of, short inflammatory episodes [46]. These results indicate that a shift in the proportions of the different monocyte subsets in IPF could favour disease progression. In a 2014 article, Fahim, *et al.* observed an increase in platelet binding to monocyte of IPF patients compared to other interstitial lung disease and HC, suggesting an increase platelet activation in these patients [47]. Indeed an earlier publication has shown that platelet from IPF patients had increase reactivity due to an unidentified plasma factor [48]. Interestingly it has been demonstrated that platelet aggregation to monocyte trigger the acquisition of a pro-inflammatory phenotype by these cells that may favour IPF development [49].

In another study involving the phenotyping of blood leucocyte from 18 IPF patients and 20 HC, the authors observed a shift in the expression of chemokines receptors by monocytes in IPF with an increase and a decrease in the number and percentage of IL-17RB and CXCR4 positive monocytes respectively [50]. Further analysis of the gene expression profile of the peripheral blood mononuclear cells of these patients revealed the differential expression of 18 genes involved in inflammation and signalling. Of these, EMR1, CCR3, UPAR, FCGR2A, OPN, CEACAM3, CD16a, CD18, CD11b, LTF, and LCN2 were up-regulated, whereas IL-17RB, IL-10, PDGFA, CD301/Clec10a, CD25/IL-2RA, IL-23A, and IL-15 were down-regulated in IPF [50]. Interestingly some of the upregulated genes such as osteopontin (OPN) have been shown to strongly regulate monocyte/macrophage development and function [51]. Moreover, OPN has been found to be highly expressed by AM located in fibrotic areas in the bleomycin model of lung fibrosis and was found to promote the migration, proliferation, and adhesion of fibroblasts, all participating to the development of lung fibrosis [52].

Lastly, another study conducted on 13 IPF patients and 12 age-matched HC showed an increase in the number of monocytes expressing the M2 marker CD163 [53]. Furthermore, the number of CD163 expressing monocytes was significantly increased in patients with progressive IPF compare to the stable ones [53]. This skewed M2 phenotype of monocytes in IPF may favour the recruitment to the lung of AAM with a “repair” rather than “anti-inflammatory” phenotype that will contribute to the disease progression. To get more clue in the molecular mechanisms driving these changes in IPF, researchers also measured the level of cytokines and chemokines in the BALF and serum of IPF patients.

### Monocyte/Macrophage related mediators in the BALF and serum of IPF patients

Cytokines and chemokines are inflammatory mediators released by immune and non-immune cells and playing an essential role in the polarisation of the immune response. The monocyte/macrophage lineage is all at once, a great source of many chemokines and cytokines, as well as a target of many of them. In Table 2, we summarise the changes of mediators relevant to the monocyte/macrophage lineage, observed in the BALF and serum of IPF patients. Since the recruitment of neutrophils is one of the leading discovery of IPF patients BALF analysis, we will focus in this sections on the mediators likely to be involved in this process. In a study investigating the level of the key inflammatory cytokines, IL-1 $\beta$  and TNF $\alpha$  in the BALF of IPF patients, Losa-Garcia, *et al.* observed a modest but significant increase in their concentration [33]. To distinguish the respective contribution of BALF cells and epithelial cells to the cytokine and chemokine content of the BALF, Schupp, *et al.* measured their level *in vitro* in the supernatant of BAL cells from IPF patients and HC. They found that BAL cells from IPF patients secrete higher levels of, IL-1 $\beta$  and its antagonist IL-1Ra, but no change in TNF $\alpha$  production was observed [31]. However, another study reported higher production of both IL-1 $\beta$  and TNF $\alpha$  by AM from IPF patients compare to HC [54]. Moreover, the authors found a positive correlation between TNF $\alpha$  production by AM, and neutrophil count in BALF [54], suggesting a role for TNF $\alpha$  in neutrophils recruitment in IPF. In addition to TNF $\alpha$ , the potent neutrophils chemoattractant IL-8/CXCL8 has been found to be increased in IPF patients and HP patients by 2.2 and 2.5 fold respectively whereas its concentration in the BALF of sarcoidosis patients drop by 70% [33]. Interestingly the authors observed that the expression pattern of IL-8/CXCL8 and other cytokines such as IL-1 $\beta$ , TNF $\alpha$  and IL-6 in the BALF of IPF patients was similar to the profile found in the supernatant of the AM isolated from those BALF. Thus, suggesting that AM are the primary source of BALF cytokines and chemokines in IPF [33]. Consistent with an increase AM-derived IL-8/CXCL8 in IPF, another study has shown that AM from IPF patients express higher quantities of this chemokine which correlates with neutrophils count and disease severity [55]. The same way an earlier study showed an increased ability of AM from IPF patients to attract neutrophils *in vitro* and showed that this could be due to the stimulation of AM IgG receptors by immune complexes present in the BALF of these patients [56]. In another study, it has been shown that the BALF concentration of the CC chemokines CCL2, 3 and 4 were significantly increased in IPF patients compared with HC [30]. Moreover, the authors showed that a positive correlation existed between the concentration of CCL3 in the BALF and the neutrophil count, whereas a negative correlation was found between the concentration of the potent monocyte chemoattractant CCL2/MCP-1 and the partial pressure of O<sub>2</sub> (PaO<sub>2</sub>) [30].

Another study, analysing the secretory activity of BALF cells from IPF patients, showed that they secrete higher amount of CCL2, 17, 18, 22, IL-8, IL-1 $\beta$  and IL-1Ra then those from HC individuals [31,57]. Interestingly in one of these study, the authors showed that AE-IPF was associated with further increases in the concentration of the same mediators and the recruitment of the same cells rather than with a change in the inflammatory cells landscape [31]. Among those mediators the chemokines CCL18/PARC has attracted much attention. Indeed its level has been found to be increased in the BALF, lung tissue and serum of patients with many ILD including IPF [58]. Moreover, AM have been shown to be the main source of this chemokines in many of these ILD including IPF, sarcoidosis and systemic sclerosis [57,59,60]. In another study, involving 72 IPF patients, a significantly higher mortality rate was associated with serum CCL18 concentration above 150 mg/ml [61]. Interestingly, *in vitro* experiments showed that AM production of CCL18 was stimulated by, fibroblast co-culture, Th2 cytokines (i.e. Here IL-13 and IL-4), and IL-10 [57]. Moreover, the authors showed that binding to collagen of AM stimulated with Th2 cytokines further increases their expression of CCL18. More importantly, supernatants of AM from IPF patients was able to stimulate the production of collagens by lung fibroblasts partly through CCL18 stimulation [57]. In line with the idea of a Th2 polarisation of the immune system in IPF, the Th2 cytokines IL-4 and IL-13 were both increased in the BALF of IPF patients [34]. Those of the Th1 cytokines (i.e. IL-12p40, INF- $\gamma$ ) and Th17 (i.e. IL-23 and IL-17A) on the other hand showed no change compared to HC [34,35]. Moreover IL-13 concentration inversely correlated with FVC% ( $r = -0.47, P = 0.043$ ) and DLCO% ( $r = -0.58, P = 0.014$ ) in IPF patients [34]. Immunostaining of both IL-13 and IL-13R $\alpha$ 1/2 on IPF lungs sections revealed strong expression by AM, endothelial cells, smooth muscle cells, and the bronchiolar epithelium [34].

Beyond the cytokines and chemokines mentioned above many macrophage-related mediators potentially involved in IPF pathogeny have been identified including members of the matrix metalloproteinases (MMPs), as well as several angiogenic [62] and growth factors [63,64]. In particular, AM have been shown to be a great source of many MMPs (i.e. MMP-1,-7,-8-, -10) in IPF lung. Moreover, increases of these MMPs in the serum or BALF of IPF patients have often been negatively correlated with disease progression and survival. For review see [60,65]. In addition to MMPs, two matricellular proteins (i.e. Osteopontin and Periostin) released by and, influencing the function of monocytes and macrophages, have also been shown to be increased in the serum and/or BALF of IPF patients. Moreover, indications from experimental models suggest their direct contribution to lung fibrosis development [52,60,66]. Macrophages have such an important impact on IPF physiopathology that some of their mediators are candidate biomarkers in IPF. Those include CCL18, IL-8/CXCL8, CCL2, S100A9, and MIF [67]. Table 2 summarises the variations in BALF and serum concentration of many mediators related to the monocytes/macrophages lineage in patients with IPF. In conclusion, it is clear that macrophages and in a lesser extent monocytes contributes greatly to the changes in mediators observed in the BALF and serum of patients with IPF. Whether they have a positive or negative impact on IPF development is the topic of the next chapter.

Mediators	BALF		Serum	
	Changes	References	Changes	References
IL-1β	↑↑	[33,68]	↑	[68]
TNFα	↑	[33]	→	[69]
INF-γ	→	[34]	↓	[70]
IL-6	↑	[33]	↑	[69]
IL-4	↑	[34]	→	[70]
IL-10	↓	[71]	↑↓	[69,70]
IL-12p40	→	[35]	↑	[70]
IL-13	↑↑↑	[34,72,73]	↑↑↑	[72-74]
IL-23	→	[35]	-	-
TGF-β1	↑§	[75]	↑→	[69,76]
M-CSF	↑	[77]	-	-
GM-CSF	-	-	↑	[78]
IL-1Ra	↑↑	[53,68]	↓	[68]
CCL2/MCP-1	↑↑↑↑	[30,35,53,79,80]	↑↑	[77,79]
CCL3	↑↑	[30,80]	-	-
CCL4	↑	[30]	-	-
CCL5/RANTES	→	[35]	-	-
CCL18/PARC	↑↑→	[35,58,61]	↑	[58,61]
CCL22/MDC	↑↑	[35,80]	-	-
CXCL8/IL-8	↑↑	[33,35]	↑↑	[78] [70]
CXCL10/IP10	→	[81]	→	[81]
CXCL5/ENA-78	↑	[81]	→	[81]
sICAM1	-	-	↑↑↑	[82-84]
MMP-1	↑→	[85,86]	↑↑	[85,87]
MMP-2	↑	[88]	↑	[87]
MMP-3	↑	[88]	→	[87]
MMP-7	↑↑↑	[85,88,89]	↑↑	[87,90]
MMP8	↑↑↑↑	[35,65,86,88]	↑	[65,87]
MMP9	↑↑↑	[35,86,88]	→	[87]
MMP10	-	-	↑	[87]
MMP12	-	-	↑	[87]
TIMP-1	↑→	[86,88]	-	-
TIMP-2	↑	[88]	-	-
VEGF	↓↑*↑	[35,88,91]	→	[78]
PAI-1	↑↑↑	[35,92,93]	→	[94]
Angiopietin-2	→	[95]	→	[78]
S100A9	↑	[96]	→	[96]
YKL-40	↑↑	[97,98]	↑↑	[97,98]
Osteopontin	↑	[99]	↑	[50]

**Table 2:** Variation of monocyte/macrophage-related mediator's in the BALF and serum of IPF patients.

\*: Rapidly progressive IPF; §: Active TGF-β1; ↑: upregulated; →: Stable; ↓: Downregulated. Each arrow represents one reference.



### The role of the monocyte/macrophage lineage in experimental lung fibrosis models

Despite observation of changes in immune cells numbers and phenotype in IPF patients, it is difficult to conclude as for their contribution to the disease progression. The use of animal models especially rodents is a way to bypass this disadvantage by allowing the depletion of specific cell population or gene during experimental lung fibrosis. One of the most common model, of lung fibrosis, is a model induced by a single intratracheal administration of the chemotherapeutic agent bleomycin, often in rodents [100]. However, this model is criticised, because it is characterised by a very acute and transitory inflammatory phase, followed by a repair phase which is almost entirely resolved after a few weeks. IPF, on the other hand, is a chronic disease with low/mild inflammation in which the fibrotic process is developing inexorably probably over decades. Modified versions of the bleomycin model, involving repetitive intra-tracheal instillation of bleomycin [101] or the use of aged male mice [102], have been shown to mimic more closely IPF development. More recently a few models relying on targeted type II epithelial injury, fibroblastic autonomous effects, and targeted genetic defects, have been developed [103]. However, by nature the cause of IPF is unknown, and many suspect it to be multifactorial. Therefore it is almost impossible to create a single model recapitulating all the aspect of IPF pathophysiology. Moreover, the recent marketing of new IPF treatments (i.e. Pirfenidone, Nintedanib) discovered using the bleomycin model support its relevance to the disease.

Using the bleomycin model, Wei, *et al.* have observed an increase in circulating Ly6<sup>Chi</sup> monocytes from day one to twenty-one after bleomycin instillation. This increase was later followed, by an increase in M2 alveolar and interstitial macrophages at day 21 during the fibrotic phase. During the same period, they observed a concomitant decrease in M1 macrophages [104]. Consistent with a role for the monocytes/macrophage lineage in lung fibrosis and IPF, mice deficient for the monocyte/macrophages chemoattractant CCL2 and M-CSF, are protected against bleomycin-induced lung fibrosis [77]. The respective contribution of interstitial and alveolar macrophages to lung fibrosis progression is not yet fully understood. In a recent article, it has been shown that administration of the mineralocorticoid receptor antagonist spironolactone inhibits lung fibrosis development in the mouse bleomycin model. Further analysis showed that mineralocorticoid administration was associated with a decrease in blood Ly6<sup>Chi</sup> monocytes expansion and the number of alternatively activated macrophages (AAM) (F4/80+/Cd11c+/CD206+ cells) in alveoli, whereas the IM (F4/80+/Cd11c<sup>-</sup> cells) remained unchanged [105].

Whereas monocytes derived interstitial macrophages doesn't seem to play a key role in experimental lung fibrosis, recent evidence suggested that CCR2<sup>+</sup> tissue monocytes also known as monocytic myeloid-derived suppressor cells (M-MDSCs) are a source of TGF- $\beta$ 1 in a mouse model of silica induced-lung fibrosis [106]. The authors also showed that co-culture of those lung M-MDSCs with fibroblasts resulted in the release by the later of collagens and the metalloproteinase inhibitor TIMP1. Finally using mice deficient for CCR2 in the monocytes/macrophages lineage (i.e. LysMCreCCR2loxP/loxP mice) the authors showed that the loss of M-MDSCs was associated with decrease TGF- $\beta$ 1, TIMP1 and collagen expression in the lung of mice treated with silica [106]. Overall these data highlight the role of the M-MDSCs in the creation of a non-degrading collagen microenvironment in experimental lung fibrosis.

Recently Lin, *et al.* highlighted the role of PAR-1 in the recruitment of macrophage in response to bleomycin instillation [107]. They showed that the macrophage-dependent induction of fibroblasts migration, differentiation and secretion of collagen require PAR-1 activation at the surface of the fibroblast by macrophage-derived FXa [107].

In a recent article Li, *et al.* showed that similar to that observed in the BALF and lung tissue of IPF patients [108], bleomycin-induced lung fibrosis in mice is associated with a rise of IL-33 expression. They showed that IL-33 expression was induced in AM and induces an M2 polarisation characterised by the release of IL-13, TGF- $\beta$  and the expansion of type 2 innate lymphoid cells [109]. Conversely blockage of IL-33 signalling or depletion of macrophage impaired fibrosis development in this model [109]. Recently the IL-1R-associated kinase-M (IRAK-M), a MyD88-dependent inhibitor has been shown to be necessary for the development of AAM during bleomycin-induced lung fibrosis. Indeed macrophages from fibrotic mice deficient in IRAK-M had impaired IL-13 secretion reducing their ability to stimulate fibroblast expression of alpha-SMA and collagens [110]. Another regulator of M2 macrophage, the MMP28, has been shown to promote AAM

dependant lung fibrosis in the bleomycin model [111]. Interestingly, the macrophage suppressor Paired Immunoglobulin-like Receptor B (PIR-B) has been shown to negatively regulate AAM differentiation in response to IL-4 *in vitro* [112], and mice deficient for PIR-B present increase macrophage expression of the alternatively activated markers OPN, MMP12 and Relm- $\alpha$  [112]. The central contribution of AAM to the development of bleomycin-induced lung fibrosis has been suggested by TNF $\alpha$  therapy a well-known M1 macrophage activator. TNF $\alpha$  therapy was associated with decreased AAM, and reduced fibrosis [113]. These results indicate that activator of the M1 macrophage program could represent a therapeutic target in lung fibrosis and probably most fibrotic diseases.

The contribution of monocyte to lung fibrosis is not limited to the bleomycin model. Indeed in a model of lung fibrosis induced by type II epithelial cells targeted injury, Osterholzer, *et al.* observed an increase in circulating Ly6<sup>Chi</sup> monocytes and non-resident exudate macrophages characterised by cd11b expression. They further showed that CCR2 deficient mice presented a reduced accumulation of AAM and fibrosis in this model [114].

Many other articles published over the years have involved lung macrophages in experimental lung fibrosis. However, for the sake of concision, I will end this chapter by focusing on three critical recent publications.

In early 2016 Cao, *et al.* used an improved model of IPF based on repetitive instillation of bleomycin in the mice. Using this model they showed the critical role played by a hematopoietic-vascular niche in alveolar repair and lung fibrosis. Indeed they showed that repeated lung injury by bleomycin instillation was associated with, sustained suppression of CXCR7 expression by the pulmonary capillary endothelial cells (PCECs), and the recruitment of VEGFR-1 positive perivascular macrophages [115]. The interaction between the PCECs and the VEGFR-1 positive macrophage resulted in the upregulation of the Notch ligand Jagged1 (encoded by Jag1) in PCECs in a Wnt/ $\beta$ -catenin dependent fashion, leading to increase Notch signalling in perivascular fibroblasts and increase fibrosis. Conditional deletion of the VEGFR-1 in the monocyte/macrophage lineage using genetically engineered mice (i.e. Vegfr1 $\Delta$ LysM/ $\Delta$ LysM mice) or the adoptive transfer of VEGFR-1 deficient monocytes in wild-type mice led in both cases to a significant reduction of lung fibrosis compared to control animals [115]. However, whether such a mechanism exists in humans and is involved in IPF pathogeny remain to be demonstrated. Flow Cytometry analysis or immunohistochemistry staining of alveolar macrophages from IPF tissue or sections should help answer this question.

Earlier this year, in an effort to identify a monocyte population specifically recruited to the lung during the fibrotic phase of the bleomycin-induced lung fibrosis model, Satoh, *et al.* described an atypical monocyte playing a critical role in experimental lung and liver fibrosis and named segregated-nucleus-containing atypical monocytes (SatM) s [116]. This new cell type has been phenotypically characterized as Ceacam1<sup>+</sup>Msr1<sup>+</sup>Ly6C<sup>-</sup>F4/80<sup>-</sup>Mac1<sup>+</sup> monocytes. The authors showed that the SatM express granulocytic markers and is dependent for its development upon the transcription factor CCAAT/enhancer binding protein  $\beta$  (C/EBP $\beta$ ) (Satoh, T; Nakagawa, k; 2017). Interestingly the conditional deletion of C/EBP $\beta$  in the haematopoietic compartment was associated with a lack of SatM and the inhibition of bleomycin-induced fibrosis but not inflammation. However, the adoptive transfer of WT SatM into these mice restored the fibrotic development. The SatM have been found to be derived from a new Ly6C<sup>-</sup>Fc $\epsilon$ RI<sup>+</sup> granulocyte/macrophage progenitor in which the expression of C/EBP $\beta$  is required to produce both the immediate SatM progenitor and the fully differentiated SatM [116]. It is not clear whether there is a human equivalent of the SatM. Using microarray analysis of the SatM transcriptome, the authors characterised their molecular signature. Recently Villani, *et al.* using single cells RNAseq discovered new subsets of human monocytes. The search for human orthologue of genes associated with the SatM in single cells RNA sequence data from peripheral blood CD14<sup>+</sup> cells of IPF patients could potentially lead to the discovery of a human equivalent of the SatM. This article also suggests that C/EBP $\beta$  pharmacological inhibitors could be of interest in preventing experimental lung and liver fibrosis and on the long-term in the treatment of IPF and other fibrotic diseases.

As discussed in the introduction, lung macrophages are a heterogeneous population, due to differences in localisation, and origin. Whereas the critical impact of environmental factors and therefore macrophage localisation on their phenotype and function has recently been demonstrated, the effect of macrophage origin (i.e. resident/foetal derived or Exudate/Monocyte-derived), on their phenotype and

function in health and disease is not well understood. In a recent study, published in the *Journal of Experimental Medicine*, Misharin, *et al.* investigated the respective contribution of resident and monocyte-derived macrophages, to lung fibrosis induced by intratracheal instillation of bleomycin in the mouse. Using a combination of engineered mouse strain and bone marrow transplantation they have been able to generate mice devoid of either population. They found that monocyte-derived but not resident alveolar macrophages contribute to lung fibrosis in this model [117]. Using transcriptomic analysis of flow-sorted cells, they observed distinct expression profile of profibrotic genes between both populations during lung fibrosis development. Interestingly they noted that MDM persist in the lung up to one year after resolution of fibrosis and are becoming increasingly similar to resident macrophages. Indeed comparison of their respective transcriptome ten months after initiation of lung fibrosis revealed only 330 differentially expressed genes [117]. It is not clear however whether the initial phenotypic distinction was due to the ongoing process of monocyte to macrophages differentiation or whether the reduced differences at ten months was due to the lack of pathological signal in this model at this time point. In other words, it will be interesting to determine whether there is a persistent epigenetic signature in macrophage linked to their origins, and dictating their responses to the pathological lung milieu found in experimental fibrosis and IPF. An analysis of the resident, and monocytes-derived macrophages transcriptome shortly after re-challenge with bleomycin of mouse initially challenged ten months earlier could offer the beginning of an answer to this question. Last but not least, some of the profibrotic genes identified in MDM was also up-regulated in human AM from ILD lungs including some IPF samples [117]. In conclusion, this article supports the targeting of MDM in the treatment of ILD including IPF [117]. Consequently, the research of drugs targeting the monocyte to macrophage differentiation process while preserving resident macrophage and monocytes should be encouraged as well as their testing in experimental models of lung fibrosis.

### Effects of past, present and prospective IPF treatments on the monocyte/macrophage biology Corticosteroids

A few years ago the standard treatment for IPF included a tritherapy of, prednisone (corticosteroid immunosuppressant), azathioprine (Immunosuppressant), and N-acetyl cysteine (Mucolytic agent) that was classically and with some success used in the treatment of non-specific interstitial pneumonia [3,118]. However, a clinical trial assessing the effectiveness of this treatment showed it was associated with an increased risk of death or hospitalisation [119]. More recently an analysis of the beneficence of steroids treatment in acute exacerbation of IPF (AE-IPF) also concludes to a higher risk of mortality in the treated group [120]. These studies had a profound impact on the scientific community perception of IPF pathogeny, and IPF is no longer considered as a chronic inflammatory disease [121]. This conclusion, however, is based on a truncated view of inflammation, reducing it to its acute phase. Many fibrotic conditions linked to inflammation such as atherosclerosis evolve silently over decades in the absence of acute tissue damages or inflammation. Whereas anti-inflammatory steroids are very efficient at blocking the production of acute inflammatory mediators such as IL-1 $\beta$ , TNF $\alpha$ , INF $\gamma$  and IL-6, they failed to prevent the expression of others. Indeed in a recent article Khalaf, *et al.* demonstrated that AM from COPD patients stimulated with the bacteria Non-typeable *Haemophilus influenza* (NTHi) was resistant to the immunosuppression induced by the corticosteroid dexamethasone. The authors found that whereas dexamethasone treatment of AM inhibits TNF $\alpha$ , IL-6 and IL-10 production induced by NTHi it fails to block that of the chemokines CXCL8 [122]. This resistance of CXCL8 expression to dexamethasone treatment was due to the activation by NTHi of the p38 MAPK pathway. However, whereas dexamethasone or the p38MAPK inhibitor BIRB-796 did not affect CXCL8 expression induced by NTHi in AM, a combination of both drugs successfully decreased it [122]. Interestingly the potent neutrophil chemoattractant IL-8/CXCL8 has been shown to be upregulated in IPF but not sarcoidosis in several studies [35,78]. Interestingly an old report by Carre, *et al.* established a positive and negative correlation between IL-8/CXCL8 expression by AM, and respectively the number of neutrophils in the BAL of IPF patients and their partial pressure of O<sub>2</sub> (PaO<sub>2</sub>) [55]. These results suggest that corticosteroids in IPF treatment might fail to limit CXCL8 release by AM and neutrophils recruitment to the lung. Beyond CXCL8 it is probable that other inflammatory mediators released by alveolar macrophage in IPF might share the same type of regulation and shows corticosteroid resistance as well. Indeed an old article by Lacroix, *et al.* showed that AM from IPF patients, although expressing glucocorticoid receptors, failed to decrease their secretion of fibronectin and other AM derived growth factors [123]. The resistance of IPF macrophages to corticosteroid-induced apoptosis has also been reported and might contribute to the increase BAL AM seen in IPF [40]. Moreover, another study comparing the BALF

cellularity of IPF patients treated or not with corticosteroids failed to identify any changes in macrophages and neutrophils number in the treated group confirming their resistance to this treatment [37]. The same way, the administration of high doses of the corticosteroid methylprednisolone in a rat model of bleomycin-induced lung fibrosis failed to block the increased expression of the profibrotic cytokine TGF- $\beta$  in AM [124].

Not only steroids fail to inhibit some part of the inflammatory response of AM, but recent data also suggest that they might promote the generation of AAM or M2 macrophage. Indeed in a mouse model of acute lung injury induced by intravenous administration of lipopolysaccharide (LPS, 5 mg/kg), administration of the glucocorticoids methylprednisolone (5 mg/kg) was found to attenuates acute lung injury presumably by promoting type 2 macrophage expansion and their release of IL-10 and TGF- $\beta$  [125]. Moreover using the mouse model of bleomycin-induced lung fibrosis researchers showed that dexamethasone does not alter the severity of lung fibrosis in this model despite reducing the number of tissue-resident macrophages, lymphocytes and eosinophils [126]. Interestingly they found that dexamethasone treatment has no impact on monocyte-derived alveolar macrophages supporting data from Misharin, *et al.* suggesting their implication in lung fibrosis and IPF. In conclusion, the failure of anti-inflammatory steroids to stop IPF progression cannot be used to rule out a contribution of these cells to IPF pathogeny. The articles presented above suggest instead: 1) a failure of these treatments in suppressing, AM release of critical mediators (e.g. CXCL8, fibronectin, Growth Factors), and the recruitment of MDM. 2) their promotion of a pro-fibrotic AAM phenotype.

### Pirfenidone

Pirfenidone (5-methyl-1-phenyl-2- (1H)-pyridone) is a small molecule known for its anti-fibrotic properties (i.e. inhibition of procollagen I and II, PDGF isoforms, TGF- $\beta$ , FGF, IL-13 in the mouse model of bleomycin-induced lung fibrosis), but whose exact mechanism of action remain elusive [127].

Based on its observed anti-fibrotic action in experimental lung fibrosis several clinical trials have been conducted evaluating its safety and effectiveness in IPF treatment). Most of these clinical trials concluded to a significant benefit of Pirfenidone treatment due to a significant reduction in forced vital capacity (FVC) decline and a significant increase in progression-free survival (improved survival in the treated group) [128]. These results prompted its marketing for the treatment of patients suffering from IPF in Japan, Europe and USA respectively in 2008, 2011 and 2014.

Interestingly some data suggest that Pirfenidone might slowdown IPF progression and lung fibrosis by targeting the monocyte/macrophage lineage. Indeed it has been recently demonstrated in a murine model of chronic, graft versus host disease-induced bronchiolitis obliterans, that Pirfenidone administration inhibits disease progression by limiting macrophage infiltration and TGF- $\beta$  production [129]. The authors concluded that the macrophages were a major source of TGF- $\beta$  in this model and that the reduction observed in Pirfenidone treated animals was due to a decrease in macrophage number and capacity to secrete TGF- $\beta$  [129]. Moreover, Pirfenidone has also been shown to decrease macrophage infiltration in nephrectomized rats [130].

In the mouse model of bleomycin-induced lung fibrosis, Pirfenidone treatment is associated with a reduction of CCL2/MCP-1 production offering a molecular basis for its inhibition of the monocyte/macrophage lineage in lung fibrosis [131]. This result was confirmed in another study, published in 2014, and showing that Pirfenidone treatment inhibits CCL2/MCP-1 expression and reduces BALF macrophage count in the same model [132]. Direct *in vitro* evaluation of Pirfenidone effect on macrophage (i.e. J774 macrophages) migration showed its ability to significantly block the chemoattraction induced by IL-17A and MCP-1/CCL2 [129]. One hypothesis to explain such effects could be the inhibition by Pirfenidone of the expression of the CCL2/MCP-1 receptor CCR2. Indeed it has been shown *in vitro* that Pirfenidone reduces fibrocytes migration toward CCL2 by lowering CCR2 expression in these cells [132].

Altogether these data suggest that Pirfenidone inhibit experimental lung fibrosis and other fibrotic disorders in part through inhibition of the monocyte/macrophage lineage function implying that such mechanism could account for its effect on IPF progression.

### Nintedanib

Nintedanib is a small molecule that was originally designed as an ATP-competitive inhibitor of the proangiogenic and receptor tyrosine kinase, Fibroblast Growth Factor Receptor-1 (FGFR-1) and vascular endothelial growth factor receptor-2 (VEGFR-2) to serve as an anticancer drug [133]. It was later found to also inhibit the receptor for Platelet-derived Growth factors alpha and beta (PDGFR $\alpha$  and  $\beta$ ) prompting its assessment as a potential treatment in IPF [133]. Besides, Nintedanib has been shown to also inhibit VEGFR-1 and 3 as well as FGFR-3 [134].

The potential benefit of Nintedanib treatment in IPF has been assessed in the “To Improve Pulmonary Fibrosis with BIBF 1120” (TOMORROW) clinical trial [135], and the INPULSIS-1, and INPULSIS-2 trials [136]. In all these studies, Nintedanib treatment showed its ability to improve FVC% against the placebo treatment over a one year period [128].

Several lines of evidence suggest that Nintedanib beneficence in IPF could be linked to the inhibition of the monocyte/macrophage lineage function in the lung. Indeed it has been shown that macrophage and epithelial cells are the primary sources of PDGF in the lung [137,138]. Moreover AM from IPF patient releases around four times more PDGF than those from control donors [139]. In addition, lung macrophages are also a significant source of ligands (i.e. FGF-2, VEGF) for the other targets of Nintedanib the FGF2R [140], and the VEGFR-1 [133,141]. In light of the publication by Cao, *et al.* presented above, involving VEGF-R1<sup>+</sup> macrophages in experimental lung fibrosis, one might think that if such mechanism was present in IPF, its inhibition by Nintedanib could contribute to its effectiveness in IPF.

Earlier this year Huang, *et al.* using the FRA2 mouse model of pulmonary hypertension associated with systemic sclerosis, showed that Nintedanib treatment resulted in the normalisation of VEGF serum level, the impaired M2 polarisation of monocytes, and reduced numbers of M2 macrophages in the lung [142].

Moreover in an abstract, recently presented at the American Thoracic Society (ATS) conference 2017, Tandon, *et al.* studied the effect of Nintedanib on macrophage polarisation *in vitro* and *in vivo* in the mouse model of bleomycin. The authors showed that Nintedanib inhibits the M2 polarisation of both murine bone marrow-derived macrophage and the human monocytic cell line THP-1 in response to IL-4/IL-13 or IL-4/IL-13/IL-6 stimulation as assessed by the measurement of arginase activity and CCL18 production respectively. The authors further demonstrated the ability of Nintedanib to inhibit CSF1 receptor tyrosine phosphorylation at a concentration of 0.3  $\mu$ M. *In vivo*, Nintedanib inhibition of bleomycin-induced lung fibrosis was associated with a decreasing number of macrophages expressing the AAM markers YM1 and arginase [143].

Pirfenidone has also been shown to inhibit the expression of the cytokine IL-1 $\beta$  in the lung of bleomycin-treated mice [144]. Interestingly, IL-1 $\beta$  is a cytokine well known to drive the development of fibrosis in many organs including the lung [145]. Furthermore AM from IPF patients have been shown to release an increased amount of IL-1 $\beta$  compared to those from HC [54]. Moreover, BALF macrophages of IPF patients have been shown to present an imbalance in the IL-1Ra/IL-1 $\beta$  ratio, resulting in increased IL-1 $\beta$  activity [146]. Therefore the inhibition of IL-1 $\beta$  signalling by Nintedanib is another potential mechanism by which it can inhibit macrophage contribution to lung fibrosis.

The same way interstitial macrophages from IPF patients have been shown to express higher levels of the MMP inhibitor TIMP-1 whose expression is also inhibited by Nintedanib in experimental lung fibrosis [144,147]. However, unlike what observed with Pirfenidone, Nintedanib treatment in mouse models of lung fibrosis induced by bleomycin or silica particles instillation failed to reduce the number of BAL macrophages significantly [144].

Further studies are required to determine the impact of Nintedanib on the monocyte/macrophage lineage recruitment and polarisation in the lung with regard to tissular localisation (interstitial vs alveolar) and origin (Resident or Foetal liver-derived vs Monocyte-Derived).

### The PI3K/mTOR pathway inhibitor GSK2126458

Based on some similarities between IPF and lung cancers pathogeny, Mercer et al., recently evaluated the effectiveness in IPF of a novel, potent PI3K/mammalian target of rapamycin (mTOR) inhibitor named GSK2126458 currently in clinical trial for application in oncology [148]. The study showed by immunofluorescence on tissues sections from IPF patients that, AKT phosphorylation on serine 473 and threonine 308 was present in fibroblast of the fibroblastic foci, alveolar macrophages and bronchiolar epithelium of IPF sections. Moreover, the phosphorylation of AKT was confirmed in the BALF cells from IPF patients (mainly AM) and was inhibited in these cells by treatment with GSK2126458 [148]. Interestingly recent data showed that monomeric collagen type I binding to AMs from IPF patients through CD204 induces PI3K signalling supporting an M2 polarisation characterised by the release of CCL18, IL-1RA and CCL2 [149]. Therefore PI3K/mTOR inhibitors could represent a novel therapeutic avenue in IPF involving the targeting of the monocyte/macrophage lineage function. However, as discussed above lung macrophages are dependent for their development upon the GM-CSF/GM-CSFR pathway, and inhibition of this pathway by an autoreactive antibody or genetic defect leads to the development of pulmonary alveolar proteinosis [26]. Unfortunately, GM-CSF triggers three central pathways in macrophages the JAK/Stat, the Ras/mitogen-activated protein kinase, and the PI-3 kinase (PI-3K)-Akt pathways [150]. Therefore studies should be conducted to assess the risk of pulmonary alveolar proteinosis associated with this treatment and the best administration route to ensure preferential targeting of the MDM.

### The Mitogen-activated protein kinase Kinase Kinase19 (MAP3K19)

MAP3K19 is a newly describe MAP 3-kinase that has been shown to be expressed mainly in the lung and trachea, and more specifically in the bronchial/alveolar epithelium (type II epithelial cells), interstitial/alveolar macrophages and neutrophils of both human and mouse lung [151]. In an early publication, MAP3K19 has been shown to be upregulated in rice cultivated under high salt condition [152]. Thereafter, MAP3K19 have been shown to regulate the nuclear translocation of activated smad2/3 following TGF- $\beta$  stimulation in the monocytic cell line THP-1 and the type II epithelial cell line A549 [153]. Beyond TGF- $\beta$  signalling, MAP3K19 was also found to be activated in numerous cell types in response to oxidative stress, making it a probable molecular mediator between environmental stressor and the development of lung fibrosis [151]. MAP3K19 has been shown to be overexpressed in AM from both IPF, and COPD patients compare to HC [151,153]. MAP3K19 was found to be upregulated in bleomycin-induced lung fibrosis, and its pharmacological inhibition (Molecule: US patent number 60/096308) was able to diminish the development of lung fibrosis in the mouse bleomycin model [153]. The authors also showed that MAP3K19 synergise with Pirfenidone and Nintedanib in the repression of pro-fibrotic genes expression at the transcriptional and translational level [153]. Further study should determine the role of MAP3K19 in the differentiation of the AAM induced by TGF- $\beta$  and other inducers of AAM.

### Concluding Remarks

IPF is a devastating disease affecting a growing number of individuals worldwide, and whose prognostic remains poor despite the recent marketing of Pirfenidone and Nintedanib as treatments [121]. Due to the ineffectiveness of anti-inflammatory therapies in IPF, the concept of dysregulated epithelial-mesenchymal interaction has replaced the initial idea of chronic inflammation as the driving force of fibrosis in this disease [154]. Therefore, in the past few years, the research effort has been mainly concentrated on the targeting of epithelial-mesenchymal interaction. In this review, we presented convergent data highlighting the activation, of the monocytes/macrophage lineage in IPF and their critical contribution to the changes in BALF mediator concentration, and the recruitment of neutrophils. Moreover, several aspects of their function such has, efferocytosis have been found to be impaired in IPF patients. A body of evidence suggests a monocyte-like phenotype of these cells in the BALF of IPF patients that could sign the active recruitment of MDM in this disease or a defective monocyte to macrophage differentiation. Furthermore, recent publications highlighted a detrimental contribution of the monocyte/macrophages lineage in experimental lung fibrosis supporting their possible contribution to IPF pathogeny. Finally, we showed evidence that corticosteroids treatments in IPF failed to inhibit the activation of the monocyte/macrophages lineage consistently and could favour their differentiation toward the profibrotic AAM phenotype. Therefore, the failure of anti-inflammatory treatments in IPF cannot be used

to rule out their involvement in the disease pathogeny. Interestingly several lines of evidence suggest that current IPF treatments (i.e. Pirfenidone and Nintedanib) and new prospective drugs target some aspect of the monocyte/macrophage function related to fibrogenesis. However broad targeting of the monocyte/macrophage lineage in human have been associated with significant side effects especially in the gut [117]. In recent years nano- and micro-based inhaled drug delivery systems allowing specific targeting of alveolar macrophages have been developed and could be used to bypass such side effects [155]. Efforts are also required to identify mechanisms specific to monocyte recruitment to the lung that could serve as therapeutic targets in IPF and other lung inflammatory diseases. Overall the data presented in this review support the targeting of the lung monocyte/macrophage lineage as a promising strategy in IPF treatment.

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