UNIVERSITY OF CRETE

The interplay between Inflammasome activation, mitochondrial oxidation, and bacterial burden in Interstitial Lung Diseases

Η αλληλεπίδραση του Φλεγμονοσώματος, της μιτοχονδριακής οξείδωσης και του βακτηριακού φορτίου στις Ινωτικές Διάμεσες Πνευμονοπάθειες

A thesis presented for the degree of Doctor of Philosophy by

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Declaration of originality

The studies described in this thesis were performed between February 2017 and July 2020 at the University of Crete and Imperial College London.

I confirm that the work presented in this thesis is my own. Where information has been derived from external sources or data obtained by others, I can confirm this has been referenced in the text.

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Abstract

Interstitial lung diseases (ILDs) in general, and idiopathic pulmonary fibrosis(IPF), in particular, are complex disorders with various disease behaviour profiles and different responses to treatment. In IPF epithelial dysfunction is considered major driver of the disease whilst in other ILDs inflammation pathways are considered as the original triggers. Evidence suggests that to some extend there are common pathobiologic pathways that drive tissue fibrosis and progression despite the specific underlying cause of fibrosis.

In all tissues, macrophages and monocytes are known regulators of the balance between tissue repair and fibrosis. In the lungs, resident alveolar macrophages (AMs) have anti-inflammatory and injury-resolution properties. However, after a fibrotic injury, bone-marrow derived monocytes are recruited in the lungs and differentiate into alveolar macrophages. Studies have linked this monocyte-derived AMs to initiation and promotion of fibrosis.

The inflammasome is one central cellular mechanism for chemokine release in macrophages. Inflammasomes are cytosolic multiprotein complexes that act as innate immune system sensors. Upon triggering, inflammasomes cleave IL-1β, a potent pro-inflammatory cytokine associated with acute lung injury and fibrosis. NLRP3 is activated by a variety of stimuli including ATP, nigericin and ROS. AIM2 inflammasome is activated by double stranded DNA (dsDNA), while NLRC4 by flagellin. Several inflammasome triggers have been identified as potential pathogenetic factors in lung fibrosis.

Mitochondrial dysfunction and subsequent mitochondrial oxidation is considered hallmark of IPF pathogenesis. Additionally, microbiota changes have been described in IPF and several bacterial genres have been associated with progression, exacerbation as well as specific immune cell cytokine release.

In this study we hypothesised that the inflammasome is overactivated in lung fibrosis-AMs as a result of mitochondrial oxidation and microbiota perturbation.

We prospectively recruited patients with lung fibrosis (IPF or other ILDs) and healthy controls. Subjects underwent bronchoscopy and bronchoalveolar lavage fluid (BALF) was obtained. From the BALF we isolated AMs which were subsequently cultured and stimulated to activate NLRP3, AIM2 and NLRC4. In parallel, we measured mitochondrial ROS by flowcytometry in unstimulated AMs and microbial burden in the BALF.

There was no notable difference in the baseline activation of inflammasomes in lung fibrosis. However, upon stimulation, NLRP3 could be overactivated in IPF and other ILDs compared to controls. NLRP3 overactivation was also associated with FVC decline in ILDs. AIM2 activation was also more inducible in IPF compared to controls, with similar trends observed in Non-IPF-ILDs. NLRC4 activation was similar across groups.

mtROS was significantly associated with heightened NLRP3 and AIM2 activation. NLRP3 activation coincided with a burst of mtROS and this could be abrogated by mitochondrial antioxidant. Similarly antioxidant therapy inhibited inflammasome activation. The microbial burden was measured in ILDs and it was linked to baseline IL-1β release from AMs and AIM2 and IL-18 relative expression independently of mtROS.

In conclusion, the above findings suggest 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.

Περίληψη

Οι Διάχυτες Διάμεσες Πνευμονοπάθειες(ΔΔΠ) και πιο συγκεκριμένα η Ιδιοπαθής Πνευμονική Ίνωση (ΙΠΙ) είναι νοσήματα με μεγάλη ετερογένεια όσον αφορά την πρόοδο νόσου καθώς και την ανταπόκριση στην θεραπεία. Στοιχεία πρόσφατων ερευνών έχουν αποκαλύψει κοινά παθογενετικά μονοπάτια στις διαφορετικές ινωτικές διάμεσες πνευμονοπάθειες που οδηγούν στην έναρξη και πρόοδο της ίνωση.

Τα μακροφάγα και τα μονοκύτταρα ελέγχουν την ισορροπία μεταξύ επούλωσης και ίνωσης μετά από κάποιο ερέθισμα στους ιστούς. Στους πνεύμονες τα κυψελιδικά μακροφάγα είναι κύτταρα εμβρυικής προελεύσεως με αντι-φλεγμονώδης και αντι-ινωτικες δράσεις και η λειτουργία τους αποσκοπεί στην διατήρηση της ισορροπίας. Μετά από ένα ινωτικό ή φλεγμονώδες ερέθισμα, μονοκύτταρα από τον μυελό τον οστών επιστρατεύονται στους πνεύμονες και παίρνουν χαρακτήρες κυψελιδικών μακροφάγων. Αυτού του είδους τα μονοκύτταρα/μακροφάγα είναι περισσότερο φλεγμονώδη και ινωτικά και έχουν συσχετισθεί με την παθογένεια της ίνωσης.

Το φλεγμονόσωμα είναι ενας από τους κεντρικούς μηχανισμούς με τους οποίους τα μακροφάγα παράγουν κυτταροκίνες. Πρόκειται για κυττοσολικά σύμπλοκα πρωτεϊνών που δρουν ως αισθητήρες του ανοσοποιητικού συστήματος. Ο ρόλος τους είναι να μετατρέπουν και να απελευθερώνουν την κυτταροκίνη IL-1β στην ενεργή της μορφή. Η IL-1β είναι μία ισχυρή προ-φλεγμονώδη κυτταροκίνη που έχει συσχετισθεί με οξεία πνευμονική βλάβη και ίνωση. Το NLRP3, το πιο γνωστό φλεγμονόσωμα, ενεργοποιείται από μια ποικιλία ερεθισμάτων συμπεριλαμβανομένων των ATP, nigericin και ROS. Το AIM2 φλεγμονοσωμα ενεργοποιείται από το ελεύθερο δίκλωνο DNA (dsDNA) και το NLRC4 από το μαστίγιο τον βακτηρίων. Αρκετοί παράγοντες ενεργοποίησης των φλεγμονοσωμάτων έχουν αναγνωριστεί ως πιθανοί παθογενετικοί παράγοντες στην ίνωση των πνευμόνων.

Η μιτοχονδριακή δυσλειτουργία και η επακόλουθη οξείδωση των μιτοχονδρίων θεωρείται ένα από τα βασικά παθογενετικά μονοπάτιατης ΙΠΙ. Επιπλέον, αλλαγές στο μικροβίωμα έχουν

συσχετισθεί με την παθογένεια της νόσου, καθώς η υπερανάπτυξη συγκεκριμένων μικροβιακών ειδών επηρεάζει την πρόοδο της ίνωσης και την απελευθέρωση συγκεκριμένων κυτταροκινών από τα κύτταρα του ανοσοποιητικού συστήματος. Σε αυτήν τη μελέτη υποθέσαμε ότι τα φλεγμονόσωματτα είναι υπερ-ενεργοποιημένα στα κυψελιδικά μακροφάγα ασθενών με ίνωση, ως αποτέλεσμα της μιτοχονδριακής οξείδωσης και της διαταραχής του μικροβιώματος.

Στην συγκεκριμένη προοπτική μελέτη συμπεριλήφθηκαν ασθενείς με ίνωση των πνευμόνων (τόσο ασθενείς με ΙΠΙ όσο και ασθενείς με ίνωση διαφορετικής της ΙΠΙ) και υγιείς μάρτυρες. Οι ασθενείς υπεβλήθησαν σε βρογχοσκόπηση και λάβαμε το βρογχοκυψελιδικό έκπλυμα (BALF). Από το έκπλυμα απομονώθηκαν τα κυψελιδικά μακροφάγα που στη συνέχεια καλλιεργήθηκαν με σκοπό την ενεργοποίηση των Φλεγμονοσωμάτων, NLRP3, AIM2 και NLRC4. Παράλληλα, μετρήθηκε το μιτοχονδριακό ROS στα μακροφάγα και μικροβιακό φορτίο στο κυψελιδικό έκπλυμα.

Δεν υπήρξε αξιοσημείωτη διαφορά στη βασική ενεργοποίηση των φλεγμονοσωμάτων στην πνευμονική ίνωση. Ωστόσο, μετά από ενεργοποίηση, το NLRP3 μπορούσε να υπερ-ενεργοποιηθεί στην ΙΠΙ και στα άλλα ΔΔΠ, σε σύγκριση με τους υγιείς μάρτυρες. Η ενεργοποίηση του AIM2 ήταν επίσης πιο επαγώγιμη στην ΙΠΙ σε σύγκριση με τους μάρτυρες, με παρόμοιες τάσεις να παρατηρούνται στα άλλα ΔΔΠ. Η ενεργοποίηση του NLRC4 ήταν παρόμοια μεταξύ των ομάδων. Η μιτοχονδριακή οξείδωση(mtROS) συσχετίστηκε σημαντικά με αυξημένη ενεργοποίηση τόσο του NLRP3 όσο και του AIM2 φλεγμονοσώματος. Η ενεργοποίηση του NLRP3 προκαλεί μια έκρηξη μιτοχονδιακού ROS, το οποίο μπορούσε να ανασταλεί έπειτα από μιτοχονδριακή αντιοξειδωτική θεραπεία. Παρομοίως, η αντιοξειδωτική θεραπεία αναστέλλει την ενεργοποίηση του φλεγμονώματος και την απελευθέρωση της IL-1β. Το μικροβιακό φορτίο μετρήθηκε στους ασθενείς με ΔΔΠ και ΙΠΙ και το επίπεδο του σχετιζόταν με την απελευθέρωση της IL-1β από τα καλλιεργούμενα μακροφάγα και επίσης με την έκφραση των AIM2 και IL-18 ανεξάρτητα από το mtROS.

Η μελέτη αυτή αποτελεί την πρώτη προσπάθεια χαρακτηρισμού της σχέσης μιτοχονδριακής οξείδωσης, μικροβιακού φορτίου και φλεγμονοσωμάτων σε ασθενείς με πνευμονική ίνωση. Τα

παραπάνω ευρήματα υποδηλώνουν ότι υπάρχει συσχέτιση υπερ-ενεργοποίσης των Φλεγμονοσωμάτων NLRP3 και AIM2 με αυξημένη μιτοχονδριακή οξείδωση. Επιπλέον το μικροβιακό φορτίο σχετίζεται και αυτό με την σειρά του με προ-ενεργοποίηση των φλεγμονοσωμάτων στους πνεύμονες ασθενών με πνευμονική ίνωση.

Chapter 1. Introduction

1.1 Introduction to Interstitial Lung diseases (ILDs)

1.1.1 Background and epidemiology

Interstitial Lung Disease (ILD) is a broad term used to describe multiple complex disorders with different pathogenetic pathways and variable disease behaviour[12]. ILDs are pulmonary fibrotic disorders that affect the alveoli of the lungs causing distraction of the lung parenchyma (figure 1) [13]. These disorders although they primarily affect the interstitium they also alter the peripheral airways and pulmonary vessels (figure 1). More than 300 different ILDs have been identified.

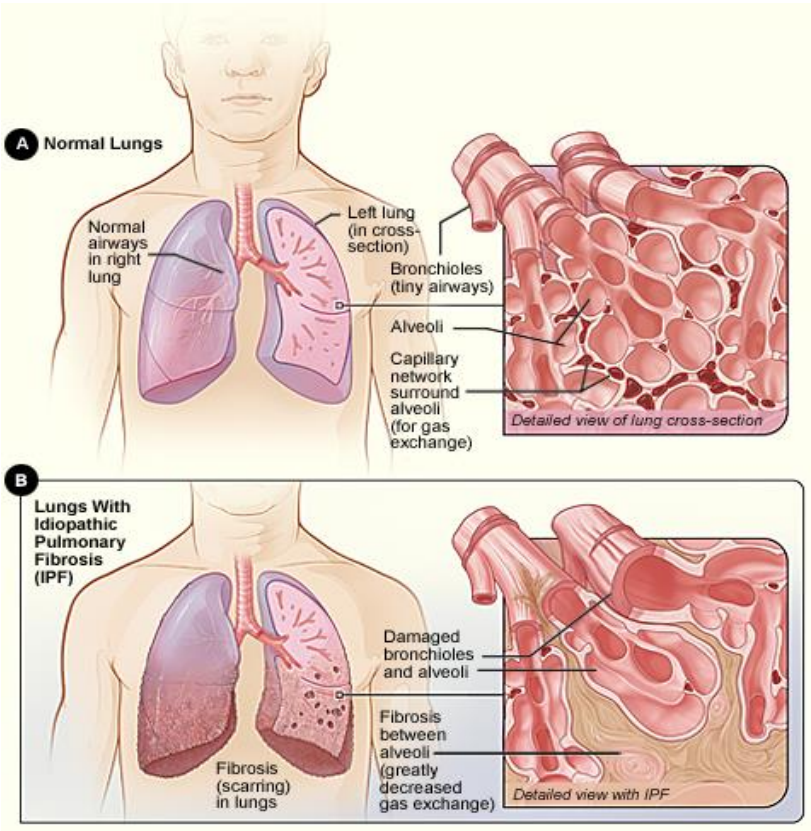


Figure 1: Representative illustration of healthy and fibrotic lungs (source: National Heart Lung and Blood Institute)

Patients generally present with breathlessness due to impaired gas exchange as a consequence of widespread fibrosis or inflammation of the alveolar walls. The aetiology can be diverse and only about one-third of the cases can be attributed to an underlying cause; most often connective tissue diseases (CTD-ILDs), drugs, environmental factors and radiation[14]. The majority of ILDs are Idiopathic, with no clear cause identified.

The classification of ILDs initially occurred around 20 years ago with a consensus statement by which ILDs were categorised in the following four categories: Diffuse Parenchymal Lung diseases (DPLDs), Idiopathic Interstitial Pneumonias (IIPs), Granulomatous DPLDs(sarcoidosis) and Other forms (figure 2)[6].

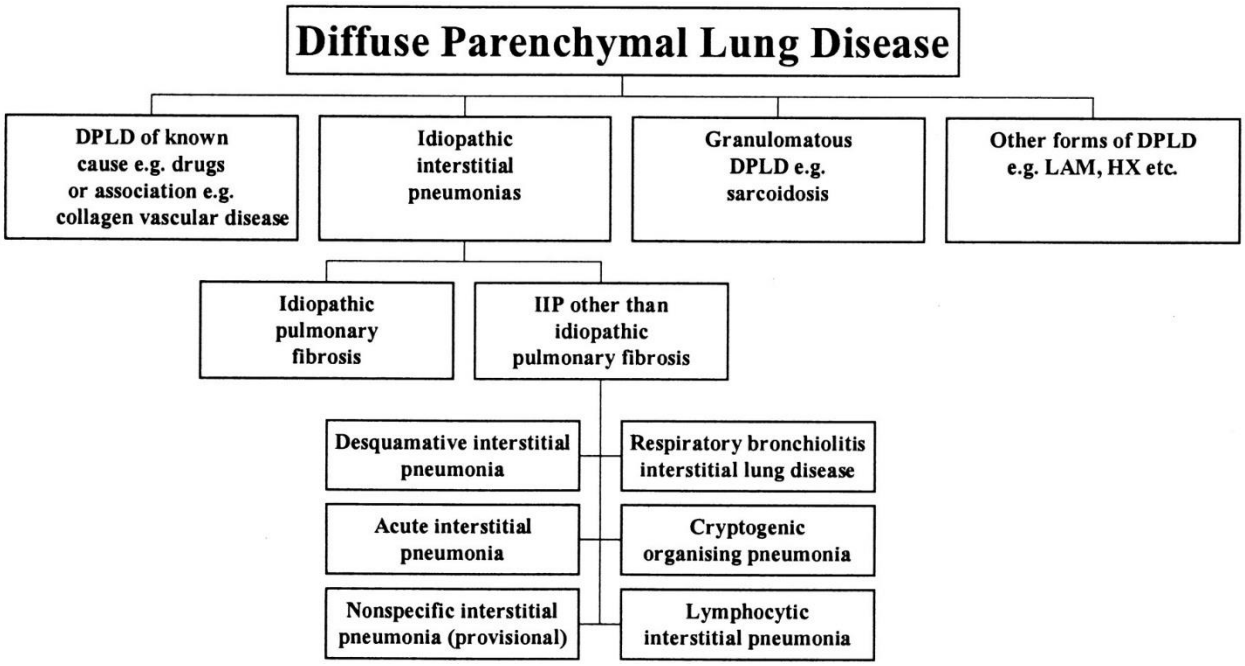


Figure 2: Classification of ILDs[6]

IIP is a group of disorders with unclear underlying cause. Due to the complicated categorisation and lack of experience in everyday practice several non-Idiopathic ILDs are misclassified as IIPs. The most well-known and devastating IIP is Idiopathic Pulmonary Fibrosis(IPF)[15].

IPF is the most progressive IIP with poor survival of 3-5 years despite treatment[16]. The natural history of the disease is variable and unpredictable and about 50-70% of the IPF patients will die from this disease. Only a small minority will have relative stability for years, with the great majority experiencing insidious decline or acute exacerbations, leading to respiratory failure[17].

Since ILDs are such a diverse entity with more than 200 separate diagnosis included only few epidemiological studies devoted to the prevalence and incidence of all ILDs. Although the exact incidence of all ILDs is difficult to establish, it is estimated that they affect 20-30 patients per 100.000 per year[18]. A recent epidemiological study which included established that almost half

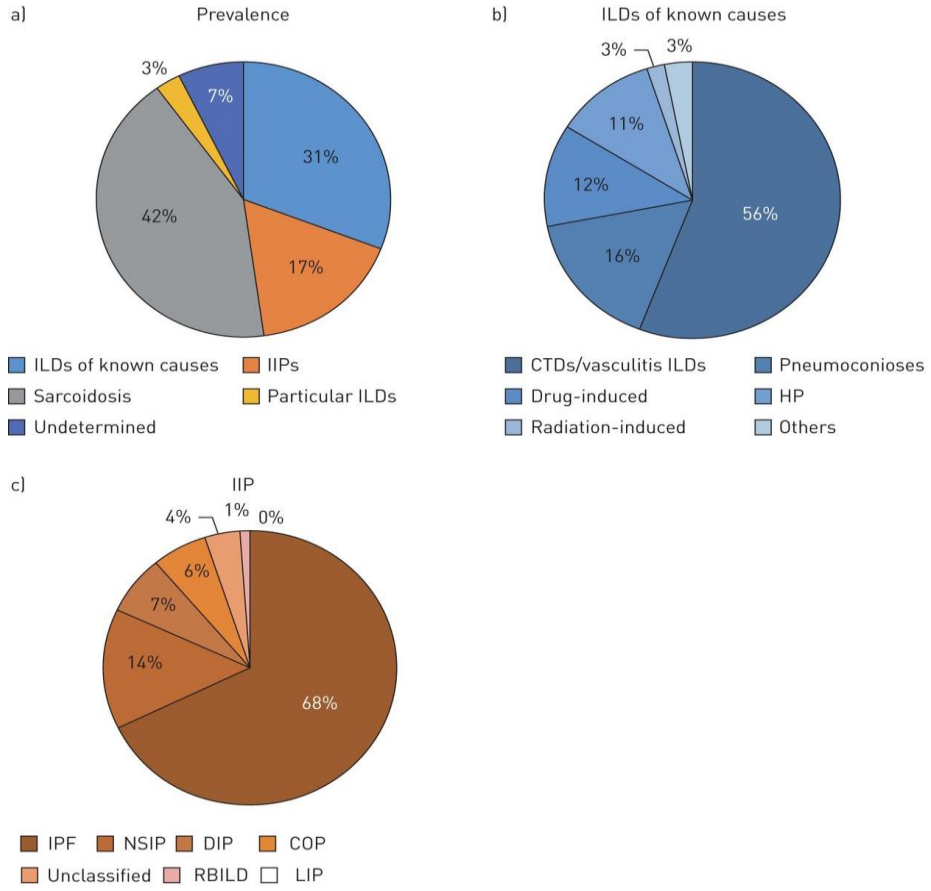


Figure 3: Prevalence of ILDs
IIPs: idiopathic interstitial pneumonias; CTDs: connective tissue diseases; HP: hypersensitivity pneumonitis; IPF: idiopathic pulmonary fibrosis; NSIP: nonspecific interstitial pneumonia; DIP: desquamative interstitial pneumonia; COP: cryptogenic organising pneumonia; RBILD: respiratory bronchiolitis with ILD; [11]

the ILD cases are attributable to sarcoidosis. When considering the fibrotic ILDs, one-third of those have IPF and the evidence suggest that the incidence of the disease is rising [19, 20]. Similarly, CTD-ILDs have marginally higher prevalence to IPF [11] and are more frequent in people from North Africa and in Afro-Caribbeans rather than in Europeans[11].

1.1.2 Clinical presentation and Diagnosis

In general, all clinically significant ILDs manifest primarily with respiratory symptoms. Dyspnoea is the most frequent symptom, but chronic cough, wheezing, haemoptysis, and chest pain can occur. Those symptoms are non-specific and can erroneously attributed to aging, obesity, deconditioning, or underlying cardiac diseases. Dyspnoea can be a sign of virtually every form of lung involvement, as such the diagnosis is commonly delayed by years. A study proposed that especially in IPF the symptoms initiation can proceed more than a year before the diagnosis for around 40% of the patients[21].

Progressive shortness of breath has significant implications in daily life with limitations in exercise capacity and ability to work. Several quality-of-life studies have established that health status is significantly impaired not only in physical function and independence but in all domains[22, 23]. Cough and depression are the major contributors for the diminished quality of life and especially in IPF the presence of significant comorbidities also contributes to the poor quality of life[23].

The physical examination may be completely normal. However in most cases, coarse inspiratory crackles are usually heard on auscultation and in the case of Hypersensitivity pneumonitis, high-pitched mid–end inspiratory wheeze known as “inspiratory squeaks” may be present[24].

The diagnosis and appropriate classification of each ILD is a dynamic process and often require extensive investigations. The diagnostic work-up includes a detailed medical history, High Resolution Computed Tomography (HRCT), Bronchoalveolar Lavage (BAL), autoimmune blood screen and occasionally open lung biopsy, or cryobiopsy[25]. Even despite the above investigations underlying diagnosis might be difficult to delineate. Due to the complexity of the

diagnostic process, the guidelines highlight the importance of the multidisciplinary discussion team (MDT) in the accurate diagnosis of ILDs [6].

HRCT is an integral part of the diagnostic algorithm and has revolutionized the diagnosis of ILDs. In particular, HRCT allows limiting over 300 different disorders into five diseases [9]. Of all ILDs, the recognition of a usual interstitial pneumonia (UIP) pattern is key, as it dictates the worst prognosis. UIP is characteristic of IPF but can be the observed in other ILDs as well[26]. A UIP HRCT pattern is characterised by a predominantly peripheral, basal and subpleural distribution of reticular abnormalities and clustered cystic airspaces known as 'honeycombing' with associated traction bronchiectasis and volume loss (figure 4). A UIP histological pattern is characterised by patchy, dense fibrosis with architectural distortion with or without honeycombing in the absence of alternative features such as prominent inflammation[27].

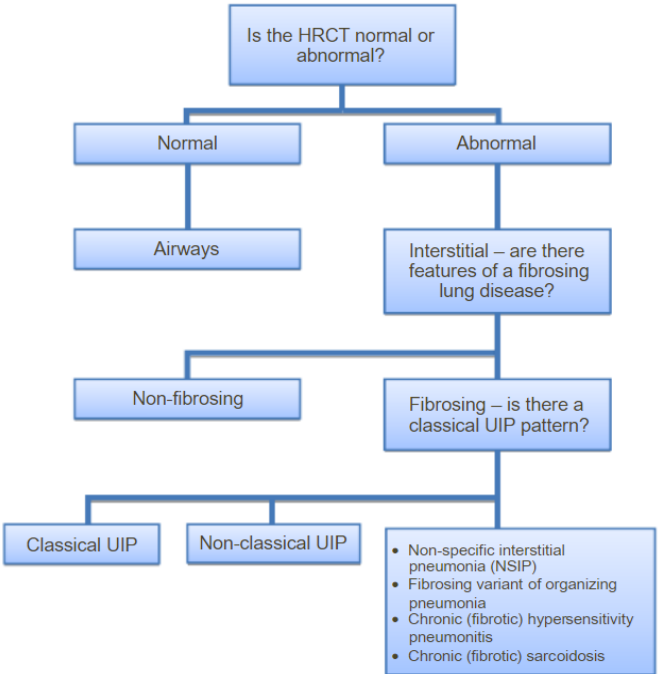


Figure 4: HRCT interpretation algorithm for ILDs UIP: Usual Interstitial Pneumonia, HRCT: High Resolution Computed Tomography [9]

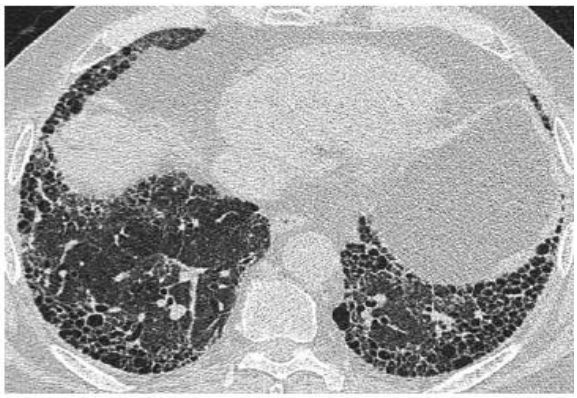


Figure 5: Representative HRCT image of classic usual interstitial pneumonia with traction bronchiectasis and honeycombing in the lung bases

Pulmonary lung function tests (PFTs) are required in the diagnosis and observation of interstitial lung diseases. The clinical evaluation is based on the most informative parameters: total lung capacity (TLC), vital capacity, FEV1 and DLCO. In most cases PFTs reveal a restrictive pattern and a decreased diffusing lung capacity (DLCO). DLCO carries prognostic information in all ILDs. But more specifically in CTD-ILDs, DLCO has been correlated with the extent of fibrosis in HRCT[28]. However, sole reduction in DLCO relative to FVC can be a marker of pulmonary hypertension or emphysema[29].

The accurate diagnosis is necessary for the appropriate management of ILDs especially since management varies depending on the specific underlying disease. Those ILDs that are considered to arise on a background of an inflammatory process, such as Hypersensitivity pneumonitis (HP) or CTD-ILDs are generally treated with immunosuppressive medication. To the other extend, IPF is considered a disease where inflammation is not an integral part of the progression of the disease.

Due to the difference in management among ILDs, for many years ILD experts focused on clearly identifying a specific diagnosis among the hundreds. To do so they proceeded to surgical lung biopsy in unclassifiable cases. However, surgical lung biopsy does not come without risks and limitations. Surgical lung biopsy is not an option for elderly patients with comorbidities, those with functionally severe disease or immunocompromised status, or with hypoxaemic respiratory failure. Additionally surgical biopsy comes with a non-negligible mortality of 4% [30].

Given the difficulties in obtaining a definite ILD diagnosis, a recent school of thinking suggests the amalgamation of different ILDs based on disease behaviour rather than underlying cause. This was the result of several studies and years of experience that have shown that ILDs other than IPF can also present with progressive disease [31]. As such disease behaviour rather than initial insult could be used to separate ILDs (figure 5). Using longitudinal lung function patients can be classified in five categories ranging from reversible, self-limited to progressive lethal disease. Patients presenting with progressive fibrosing ILDs despite available therapies clinically and

radiologically overlap with IPF and recently they were classified as 'PF-ILDs'(Progressive Fibrotic ILDs) [12].

Clinical Behavior	Treatment Goal	Monitoring Strategy
Reversible and self-limited (e.g., many cases of RB-ILD)	Remove possible cause	Short-term (3- to 6-mo) observation to confirm disease regression
Reversible disease with risk of progression (e.g., cellular NSIP and some fibrotic NSIP, DIP, COP)	Initially achieve response and then rationalize longer term therapy	Short-term observation to confirm treatment response. Long-term observation to ensure that gains are preserved
Stable with residual disease (e.g., some fibrotic NSIP)	Maintain status	Long-term observation to assess disease course
Progressive, irreversible disease with potential for stabilization (e.g., some fibrotic NSIP)	Stabilize	Long-term observation to assess disease course
Progressive, irreversible disease despite therapy (e.g., IPF, some fibrotic NSIP)	Slow progression	Long-term observation to assess disease course and need for transplant or effective palliation

Definition of abbreviations: COP = cryptogenic organizing pneumonia; DIP = desquamative interstitial pneumonia; HRCT = high-resolution computed tomography; IPF = idiopathic pulmonary fibrosis; NSIP = nonspecific interstitial pneumonia; RB-ILD = respiratory bronchiolitis–interstitial pneumonia.

* The distinctions in Table 3 are made by assimilating several factors: (*1*) A confident multidisciplinary diagnosis that often identifies the expected pattern of disease behavior (e.g., IPF). However, in other idiopathic interstitial pneumonias (e.g., NSIP) more than one pattern of behavior is possible; (*2*) disease severity, based on lung function and/or HRCT. In severe NSIP (154) a progressive irreversible course is frequent; (*3*) evaluation of potentially reversible and irreversible features based on review of the HRCT and biopsy if available; and (*4*) short-term disease behavior. Disease behavior classification must be refined over time in individual patients considering longitudinal changes in disease severity.

Figure 6: Disease behaviour classification in ILDs [1]

1.1.3 Management of Interstitial Lung Diseases

The management of ILDs is very much diverse depending on the underground condition. For drug induced ILD or hypersensitivity pneumonitis the removal of the agent might be enough to achieve remission or even resolution of the disease. CTD-ILDs have a more variable management depending on the extent of the lung involvement. In IPF or other detrimental ILDs such as Anti-synthetase ILD warrant timely diagnosis and early intervention.

Management of IPF has changed the last decade. Until recently IPF had no approved treatment and patients were treated with immunosuppression as with all other ILDs. However, the PANTHER study established that immunosuppression in IPF is not only ineffective but also detrimental [32]. Combination therapy with prednisone, azathioprine, and NAC carried a significantly higher risk of death and hospitalisation compared to placebo. The landscape for IPF management changed with the registration of two novel antifibrotic drugs; Pirfenidone and Nintedanib [33]. Both anti-fibrotics act differently but seem to partially inhibit fibrosis progression with diverse actions on different pathways. Pirfenidone is a non-selective, kinase inhibitor that exerts a multitude of anti-inflammatory, antifibrotic and antioxidant actions although its primary molecular mechanisms are not fully elucidated[34]. Overall pirfenidone has shown reduction in FVC% annual decline by 2.5% [35]. Nintedanib (BIBF 1120) is a potent intracellular tyrosine kinase inhibitor that exerts inhibitory effects on several profibrotic growth factor receptors. Similar to Pirfenidone, Nintedanib showed a reduction in annual FVC decline by around 120 mL, almost half compared to placebo[36]. Additionally evidence from real-world studies confirm the long term benefits of both antifibrotics in reducing mortality and progression free survival[37].

The management of other ILDs is even more challenging. Due to the very small number of patients, most of the 200 disorders are considered Orphan diseases and other than scleroderma, no controlled clinical trial has been performed to guide management decisions. The heterogeneity of diseases within this broad group and the scarcity of well-defined outcome measures contribute to the challenge. Additionally, not all ILD diagnosis warrants immediate treatment and the decision to intervene is mainly dependent on whether the disease is

considered clinically significant or if there is risk of progression. Assessment of the extent of fibrosis on CT and FVC reduction, as has been proposed for Scleroderma-ILD[38], might help to decide whether or not to treat. According to this staging system, involvement of more than 20% in HRCT or 10-20% and FVC below 70% was considered clinically significant disease[38].

When the decision to treat is taken management is guided by expert consensus, immunosuppressive therapy and steroids are the cornerstone. In most CTD-ILDs, corticosteroids coupled with a steroid-sparing agent are the first-line treatment. As a steroid-sparing agent, methotrexate, cyclosporine, azathioprine and mycophenolate mofetil can be used, although no official recommendation for a specific agent exists to date. Mycophenolate mofetil has a more favourable adverse-event profile and is commonly preferred over cyclophosphamide, which is mostly reserved for more refractory cases [39, 40]. Cyclophosphamide is one of the most potent steroids sparing immunosuppressive drugs used to treat autoimmune-mediated organ-threatening damage. It is cytotoxic to both resting and dividing lymphocytes and suppresses both humoral and cellular immune responses.

Hypersensitivity pneumonitis has similar management as CTD-ILDs. The most important intervention is the removal of the offending agent/antigen and depending on the extend of the lung involvement immunosuppression can be used. Systemic corticosteroids are the mainstay of pharmacology treatment in hypersensitivity pneumonitis. High doses of steroids(0.5mg/kg) are often used with the subsequent addition of a steroid sparing agent. Evidence has supported this use by Morisset and colleagues who showed that treatment with azathioprine and mycophenolate mofetil was associated with an improvement of gas exchange and reduction of prednisone [41].

Immunosuppressive medications in non-IPF ILDs seem to be more beneficial in patients with an NSIP pattern compared with those with UIP and it is considered that a UIP pattern in non-IPF ILDs dictates a similar to IPF phenotype [42]. New evidence also suggests that antifibrotics may have a place in the management of other Fibrotic ILDs. Nintedanib and Pirfenidone have shown positive results in preventing progression in PF-ILDs, Scleroderma, HP and Unclassifiable ILDs [43-

45]. This clinical evidence implies that to some extent the pathogenetic mechanisms underlying progressive fibrosis overlap in both IPF and Non-IPF Progressive fibrotic ILDs (PF-ILDs)[31].

Despite all the measures and available treatment options for those patients with progressive disease-in all ILD categories- the only curative approach to date is lung transplantation. However, lung transplant is available only to a minority of patients due to an established shortage of organ supply but also because patients are often ineligible for such interventions due to increased age and existence of multiple comorbidities. In IPF, when strict criteria are used, transplantation reduces the risk of death by 75% [30, 31]. In CTD-ILDs, lung transplant comes with even more challenges despite the younger age. Immune deregulation, high prevalence of pulmonary hypertension and the presence of extrapulmonary disease carry higher risk for allograft rejection and increased mortality after transplant [46, 47]. However, several studies have shed light on the role of lung transplantation in scleroderma- and non-scleroderma-associated ILD and the treatment can be offered in selected patients[48]. Especially in patients with severe secondary pulmonary hypertension, bilateral transplantation is a better option compared to single, as in the lateral case the majority of ventilation and perfusion are redirected to the other lung [49].

1.1.4 Prognostic Markers of Progression in ILDs

A plethora of molecular and clinical markers has been identified as predictors of outcomes in ILDs. Some of the most promising biomarkers of progressive disease are also markers of proposed pathogenetic mechanisms.

Epithelial dysfunction is considered central in the pathogenesis of IPF and is also considered to play a role in other ILDs. Markers of epithelial dysfunction, such as Krebs von den Lungen-6 (KL-6) and the mucin gene MUC5B encodes mucin 5B, have been proposed as proteinic and genetic biomarkers of the disease. Elevated serum KL-6 levels have been identified in several ILDs, including IPF, NSIP, hypersensitivity pneumonitis, CTD-ILD and a cut-off value of ≥1000 U·mL−1 was associated with poor prognosis[50]. On the other hand, a single nucleotide polymorphism

was identified in the promoter region of the MUC5B is associated with sporadic and familial IPF as well as RA-ILD and CHP [51-53]. However, the presence of this polymorphism is associated with improved outcomes in IPF[54].

Several extracellular matrix (ECM) turnover markers have also been described. Matrix metalloproteinases (MMPs), Lysyl oxidase-like 2 (LOXL2) which catalyses the cross-linking of collagen are key players in ECM remodelling and fibrogenesis. MMP-7 levels correlate with disease severity and can predict survival in IPF[55, 56]. Additionally, periostin produced by bronchial epithelial cells promotes extracellular matrix deposition, mesenchymal cell proliferation and fibrosis. Lung tissue and serum periostin levels are elevated in IPF and correlate with physiological progression[57, 58].

The immune system also plays a significant role in fibrosis progression. Markers of immune dysregulation have been associated with progressive disease. CCL18, CCL15 CXCL10 have been associated with progressive phenotype in several ILDs[59, 60]. Additionally, high blood monocyte counts (>0.95 K/μL) are strongly associated with progressive disease in all types of fibrosis[60]. Serum autoantibodies also associate with risk to develop ILD and worse disease in Scleroderma(anti-topoisomerase I antibodies, and small nuclear ribonucleoproteins-RNPs)[61] and Rheumatoid arthritis (anti-citrullinated protein antibodies)[62-64].

Despite all the advances in biomarker discovery the stronger markers of progressive disease remain clinical parameters. In many Non-IPF ILDs, the severity of traction bronchiectasis and the extent of honeycombing have been reported as predictors of mortality[65, 66]. Furthermore, progression of fibrosis in serial imaging is also associated with poorer survival[67]. Similarly spirometric values strongly associate with outcomes. Lower FVC or DLCO percentage is associated with dismal prognosis in all fibrotic ILDs[38, 68-70] and an annual decline in FVC > 10% predicted is another robust predictor of mortality[69, 71].

Table 1: Biomarkers of progressive fibrosis

Biomarker	Significance
Alveolar epithelial cell dysfunction	
KL-6	Correlation with disease severity (imaging and PFTs); increased levels suggest worse prognosis; higher levels in AE-IPF compared with stable IPF
SP-A, SP-D	Strong predictors of early mortality
CC16	Correlation with baseline PFTs
CK18	Distinguish between IPF and other ILDs
Immune dysregulation and inflammation	
CCL18	Predictor of increased mortality
YKL-80	Predictor of worse outcome
CXCL2, CXCL4, CXCL13	Predictors of worse outcome
S100A8, S100A9, S100A12	Predictors of increased mortality
HSP70, HSP47	Correlation with baseline PFTs; predictors of worse outcome; HSP47 higher in AE-IPF compared with stable IPF
Regulatory T-cells	Association with progressive disease
α-Defensins	Higher in AE-IPF compared with stable IPF
IL-2, IL-12, IL-13, IL-16, IL-17, IL-23	Correlation with disease activity and baseline PFTs
Extracellular matrix remodeling and fibroproliferation	
MMP-3, MMP-7, MMP-9, MMP-12	Correlation with disease severity; predictors of worse outcome
Periostin	Correlation with physiological progression
LOXL2	Association with risk of progression and higher mortality

Biomarker	Significance
Collagen 1a1 expression in AMs	Association with risk of progression and higher mortality
Non-protein biomarkers	
Fibrocytes	Correlation with PFTs; increased level associated with worse survival
Microbial burden	Correlation with poor mortality in IPF
Raised blood monocytes	Correlation with disease progression in IPF
Raised BALF neutrophils	Correlation with disease progression in IPF

Adapted and modified from [72]. KL: Krebs von den Lungen; SP: surfactant protein; CCL: C-C chemokine ligand; YKL-80: secreted chitinase-like protein; CXCL: C-X-C chemokine ligand; HSP: heat shock protein; IL: interleukin; MMP: matrix metalloproteinase; LOXL: lysyl oxidase-like; PFT: pulmonary function test; AE: acute exacerbation; IPF: idiopathic pulmonary fibrosis; ILD: interstitial lung disease, BALF: Bronchoalveolar lavage fluid

1.2 Pathogenesis

1.2.1 Major Pathogenetic Pathways in Lung Fibrosis

Lung fibrosis is the end stage of several DPLDs and is characterised by excessive matrix deposition and destruction of the lung architecture, finally leading to respiratory insufficiency and death[73]. Most of the ILDs are sporadic diseases that arise from repeated injury which leads to epithelial injury and fibrosis on a background of genetic factors and changes in the environment. As described above only one third of ILDs have an identifiable cause. The fibrotic insult can be drugs, antigen exposure causing hypersensitivity pneumonitis, inhaled agents such as silica and asbestos, or a connective tissue disease.

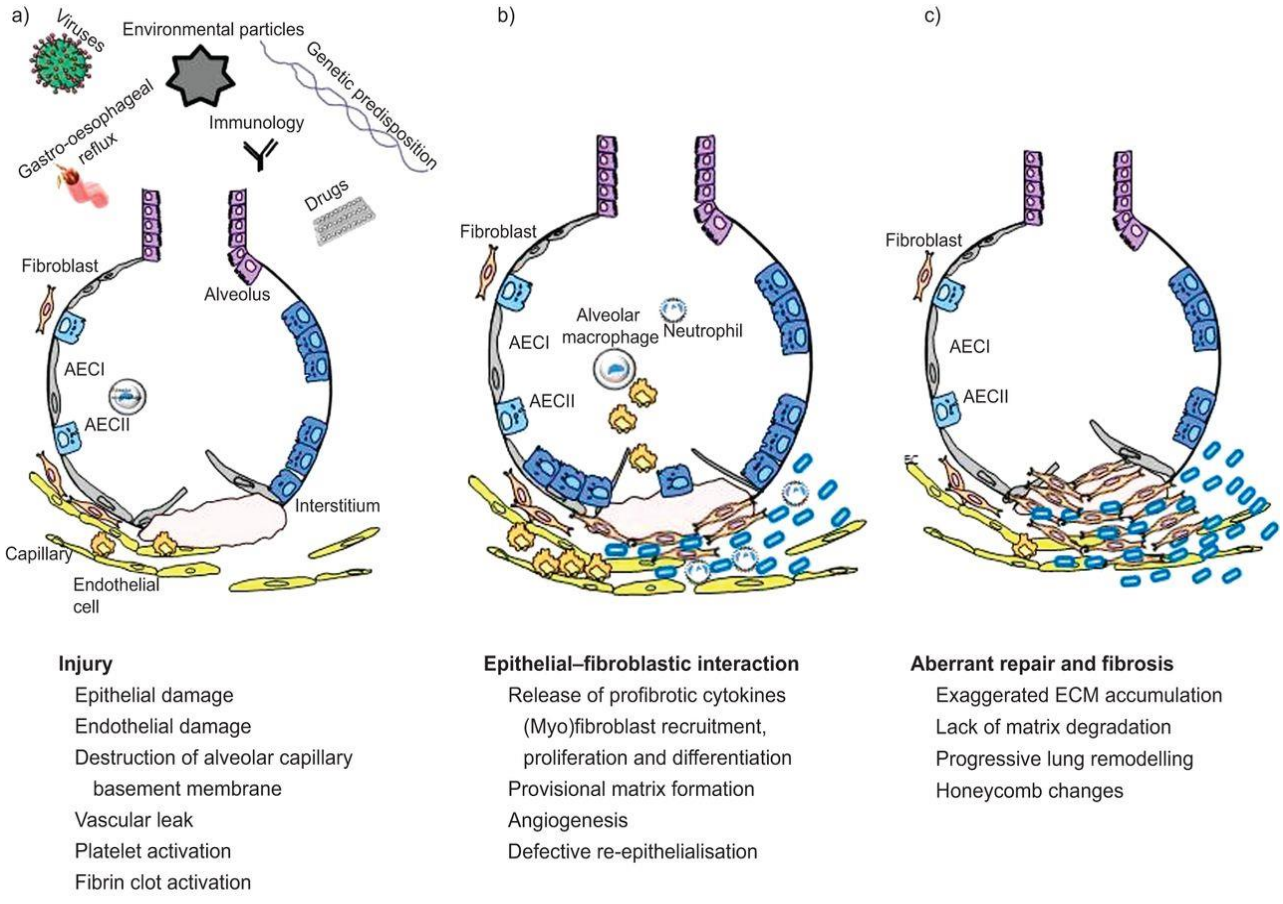


Figure 7: Processes leading to induction and progression of fibrosis[8]

Previously, it was widely believed that idiopathic fibrotic lung disease was the pathogenetic sequence of inflammation due to an unidentifiable injury. The “inflammatory fibrosis” hypothesis stated that chronic inflammation resulted in repetitive lung injury and fibrogenesis which led to the end-stage fibrotic scar [74]. IPF was thought of as a chronic inflammatory condition like other ILDs for example CTD-ILDs or hypersensitivity pneumonitis. In IPF however, inflammation is not a prominent histopathological finding, clinical measurements of inflammation fail to correlate with disease stage and experimental models have demonstrated that inflammation is not always required for the development of a fibrotic response[75].

The current paradigm of IPF pathogenesis is repetitive alveolar epithelial microinjury from ‘multiple unknown hits’ which leads to aberrant activation of Alveolar Epithelial Cells(AECs) This leads to fibroblast activation and differentiation into myofibroblasts with extracellular matrix disposition and abnormal lung remodelling[8].

Several genetic factors have been linked to the development of ILDs. A subtype of ILDs is clearly genetical and this involves about 5% of all IPF patients and familiar IPF has an autosomal dominant, vertical transmission pattern. Surfactant Protein C (SP-C) mutations have been identified in around 50% of children with severe idiopathic pneumonias. Other genetic mutations have been linked with IPF and other ILD pathogenesis with most important being mutations in hTERT, hTER and MUC5B. Genes linked to familiar disease fall into two major categories:

-Genes related to surfactant protein processing and trafficking (SP-C, SFTPC; SP- A2, SFTPA2; and ATP-binding cassette member A3, ABCA3)

-Genes linked to telomere function (telomerase reverse transcriptase, TERT; human telomerase RNA component, hTR; dyskerin, DKC1; telomere interacting factor 2, TINF2; and regulator of telomere elongation helicase, RTEL1[76].

Other genetic factors, MUC5B, TOLLIP and SNPs near the TERT genes have also been linked to a lesser extent with an increased risk for the development of ILDs. It is considered that those

genetic changes or other unknown lead to some susceptibility to the development of ILDs after an injurious insult, but no clear causal relationship has been established.

1.2.2 The role of Airway Macrophages

In all tissues, macrophages are a critical component of the primary innate immune response. In the lungs, macrophages are the most numerous immune cells present and are ideally positioned to coordinate the innate defence of the airways, remove cell debris and clear harmful bacteria [77].

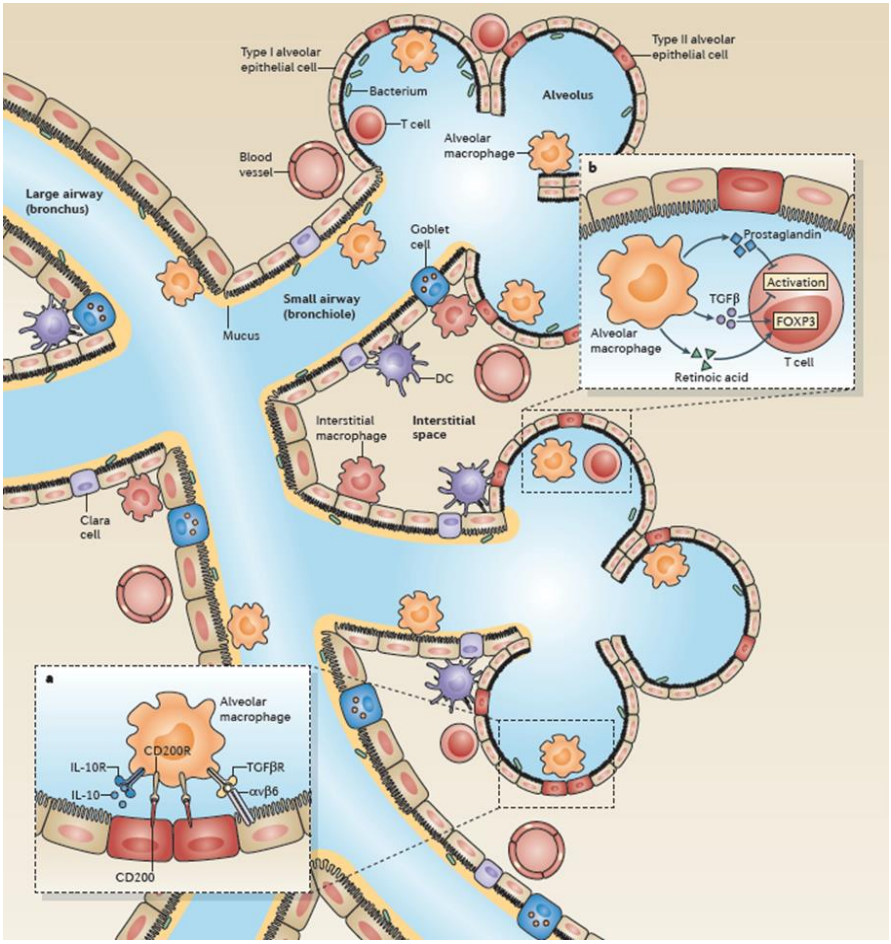


Figure 8: Macrophage populations in the healthy lung [2]

Pulmonary macrophage populations divide into alveolar/airway macrophages, which are positioned in the airways, and interstitial macrophages (IMs), located within the lung parenchyma. AMs and IMs may be distinguished by their unique surface marker expression. AMs express high levels of CD11c and low levels of CD11β. Conversely, IMs are within the lung parenchyma and express highly CD11β [2].

AMs are positioned at the interface between the pulmonary mucosa and the external environment, where they directly sense any immunological stimuli and perform a crucial role in

maintaining immune tolerance. AMs exert those regulatory effects via non-specific lines of defence, such as phagocytosis and secretion of antimicrobials such as nitric oxide, tumour necrosis factor (TNF)-α and interferon (IFN)-γ [2, 78].

Macrophages is a heterogeneous population of immune cells that exhibit remarkable plasticity and significant functional and phenotypical specialization to efficiently perform their specialised functions according to the signalling from their local micro-environment. Each distinct function requires plasticity within the macrophage population so that aberrant inflammation is constrained by simple actions such as clearance of tissue debris. The ability of macrophages to adapt and perform disparate functions led to their broad classification as either classically activated M1 macrophages or alternatively activated M2 macrophages [79].

Typically, M1-activated macrophages respond to IFN-γ, lipopolysaccharide (LPS) and TNFα to produce proinflammatory cytokines and directly phagocyte and destroy intracellular pathogens. M2-alternatively activated macrophages are a diverse phenotype, characterised by their participation in type 2 immune responses and drive parasite destruction, immunoregulation and

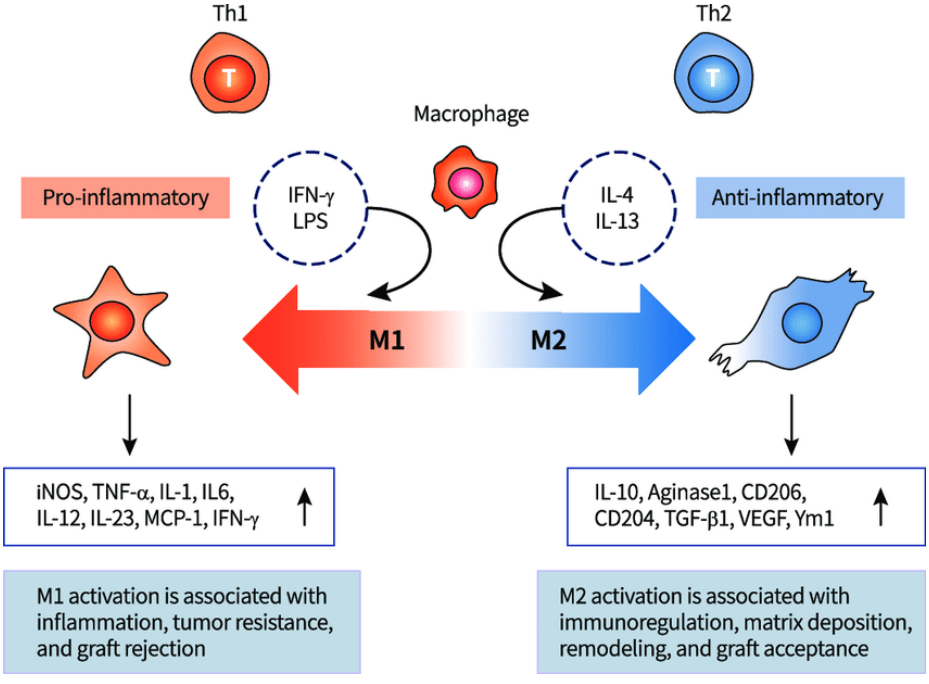


Figure 9: Diagram of the M1/M2 macrophage polarisation status showing stimulating factors and mediators released by each type of macrophage [4]

tissue remodelling[80]. In reality, the extreme M1-M2 phenotypes rarely exist given the rapid plasticity of macrophages [77].

More recently, the importance of macrophage function has focused on developmental origins rather than polarisation status. In the healthy lungs, alveolar macrophages originate from the yolk sac during embryogenesis and during adulthood there is in situ proliferation rather than independent replenishment from the bone marrow [81-83]. The intrinsic alveolar macrophages tent to adopt pro-inflammatory, pro-wound healing, pro-fibrotic, anti-inflammatory and anti-fibrotic properties. After a variety of insults, AMs exhibit only minimal transcriptional and functional changes, suggesting that tissue-resident, embryonic AMs are terminally differentiated, and not very plastic[84]. After any inflammatory insult, however, bone marrow derived monocytes are also recruited in the lungs to accelerate the immune response and differentiate into macrophages[85, 86].

A growing body of evidence supports a role of lung macrophages in the pathogenesis of pulmonary fibrosis. Macrophages numbers increase after several fibrotic stimuli and are often found in close proximity to fibrosis [87-90]. After injurious insults macrophages acquire a phenotype which promotes fibroproliferation, through secretion of matrix metalloproteases[91]. Ample studies support that not all macrophages are harmful and indeed some subpopulations might promote while others resolve fibrosis. Especially, embryonic tissue-resident macrophages are heavily involved in tissue repair, are anti-inflammatory and help maintain immune tolerance[92] [93].

In contrast to resident macrophages, monocyte-derived infiltrating macrophages have been shown to drive pulmonary fibrosis[94]. One study clearly implicated non-resident macrophages in lung fibrosis [95]. Ostelholzer el al. showed that the depletion of Ly6Chi circulating monocytes resulted in reduced fibrotic responses in mice, as well as a lower number of M2 macrophages[94]. The transfer of these cells into recipient mice with established, bleomycin-induced fibrosis exacerbated the disease[94, 96].

A more recent macrophage origin study showed that the macrophage population expansion in fibrosis is largely attributable to Monocyte-derived AMs (Mo-AMs) that substantially outnumber the resident macrophages (TR-AMs). Both types upregulated the expression of both M1 and M2 genes as such implying that the role of macrophage polarisation status is less important than the origin[97]. Of particular interest, studies in IPF patients have shown that the peripheral blood expansion of the monocyte, CD14+, population is biomarker of poor prognosis.[60]

Depending on their origin and phenotype macrophages can either resolve tissue injury or promote the development of fibrosis. Characteristically, pro-inflammatory macrophages phenotypes have been associated with initiation and progression of fibrosis. The different macrophage populations exert their roles by removing debris or producing matrix metalloproteases (MMPs) and other inflammatory mediators that drive the initial cellular response following injury [98] [10]. After an inflammatory stimulus, macrophages produce numerous growth factors, TGF-β1 like and vascular endothelial growth factor-α. They also

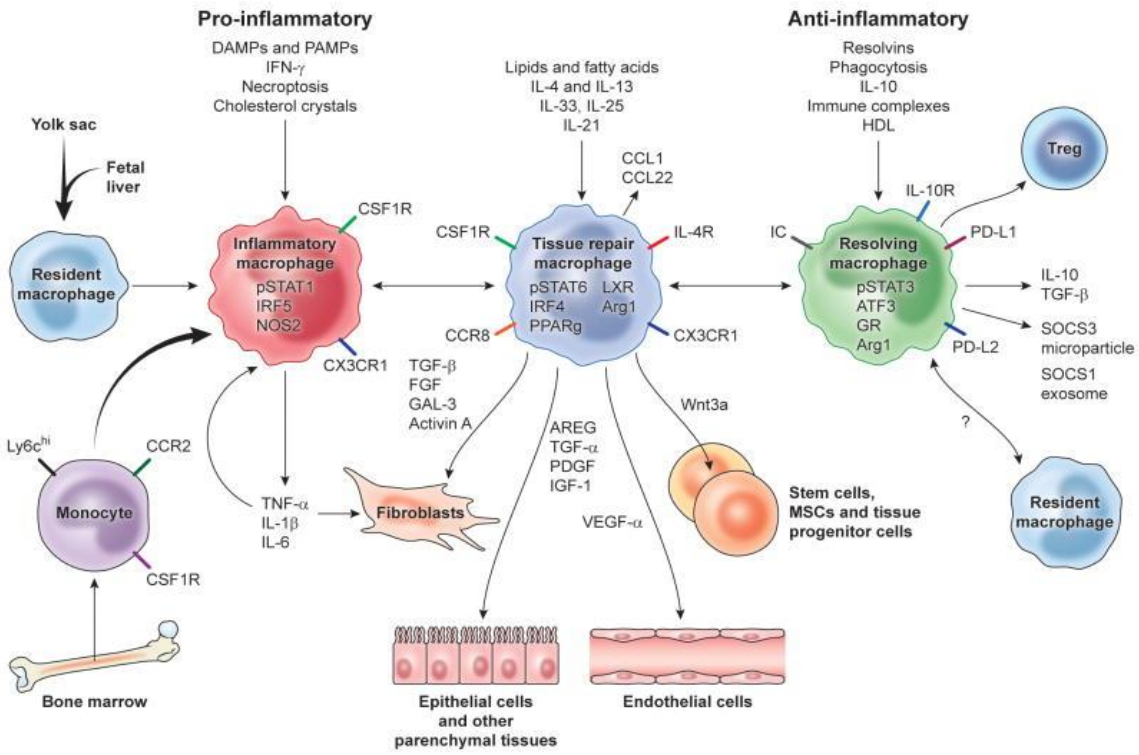


Figure 10. The role of macrophages in tissue regeneration and fibrosis [10]

produce soluble mediators that stimulate recruited fibroblasts to differentiate into myofibroblasts to promote wound healing through the synthesis of extracellular matrix (ECM). If there is disturbance of this process, then this would cause aberrant repair and development of the formation of pathological fibrosis[99].

1.2.3 Inflammasomes and their implication in Lung Fibrosis

The inflammasome is one central cellular mechanism for chemokine release in macrophages[100]. These multiprotein complexes contain a pattern recognition receptor, typically a nucleotide-binding oligomerization domain-like receptor (NLR) or an absent in melanoma 2 (AIM2)-like receptor, which upon activation by pathogen-associated molecular patterns (PAMPS) or damage-associated molecular patterns (DAMPS), oligomerize and recruit the adaptor protein ASC and caspase which ultimately cleaves active IL-1β and IL-18.

Among the different inflammasomes previously identified, NLRP3, AIM2, and NLRC4 are the best characterised (figure 11). NLRP3, the most studied inflammasome, is activated by a variety of stimuli including ATP, nigericin, crystals and ROS[101]. Other inflammasomes are more specific and activated by merely one stimulus; NLRC4 by cytosolic Flagellin and AIM2 by cytosolic double stranded DNA [102].

The current dogma for the Inflammasome activation suggests that a two-step model is required for the release of IL-1β for macrophages (Classic activation). The first signal (Signal-1) mediates transcriptional and post-translational priming of several inflammasome components including NLRP3, AIM2, NLRC4, IL-1β and IL-18. Only after signal-1, macrophages become sensitive to signal-2 dependent inflammasome activation. For NLRP3, a plethora of signal-2 agonists have been reported, but none directly activates the NLRP3. Instead, most signal-2 agonists cause an efflux of potassium which in turn activates the NLRP3 [103, 104].

After this second signal, NLR sensor molecule (NLRP3, NLRC4 or AIM2) assemble with the adapter molecule ASC forming a high-molecular weight complex, the pyroptosome. Caspase-1 is also recruited to the pyroptosome, which allows the maturation of active caspase-1 from induced proteolytical auto-activation. Active Caspase-1 then cleave the precursor pro-IL-1β and pro-IL-18

to their mature active forms (Canonical activation). Besides this caspase-1 activates gasdermin D to induce pore opening which causes an inflammatory type of cell death called pyroptosis which also releases IL-1 β (Non-canonical activation) [105] [102] [106, 107].

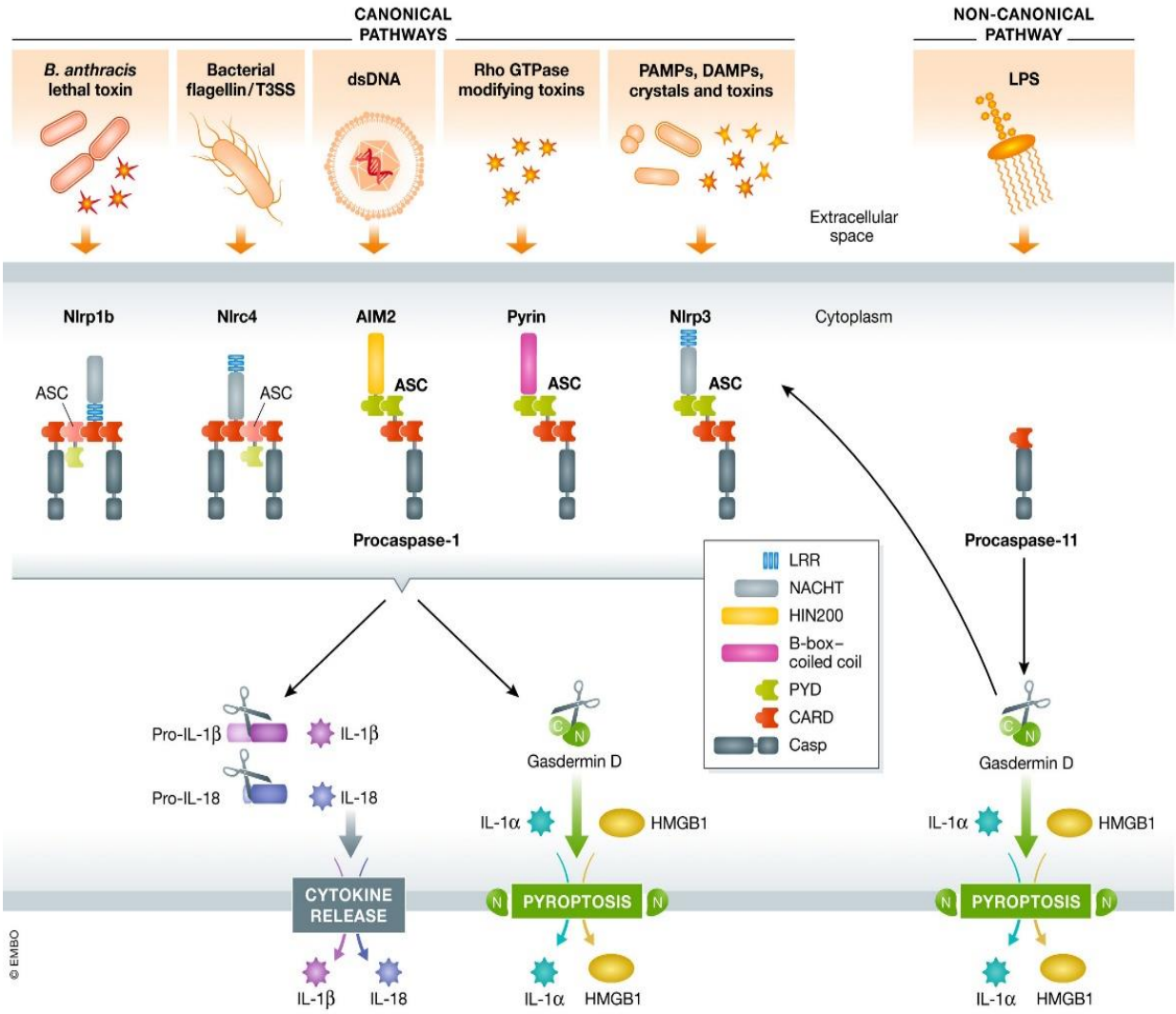


Figure 11: Representative illustration of Inflammasome signalling pathways; canonical and non-canonical [5]

In human compared to murine monocytes, an alternative NLRP3 activation process has been established that does not involve pyroptosis. In human peripheral blood mononuclear cells (PBMCs), TLR4 signalling (Signal-1) alone, induces inflammasome activation that involves the interconnection of ASC, NLRP3 and caspace-1 but is independent of the pyroptosome formation.

This pathway allows the cell to respond with a gradual release of IL-1β without committing to non-reversible cell death (Alternative activation) [108].

The activation of AIM2 is also different in murine and human immune cells. AIM2 inflammasome is known to recognise non-self, nucleic acids(ds-DNA), for example viral DNA. The cytosolic DNA can also be recognized by the cGAS-STING signaling axis that drives antiviral immunity by inducing type I interferons[109]. There is evidence to suggest that in human compared to mice monocytes ds-DNA triggers the NLRP3 through the cGAS-STING, whilst AIM2 is non-functional. This is thought to be driven by lysosomal mediated cell death [110].

Several studies have analysed the involvement of inflammasomes in the pathogenesis of fibrosis[111-113]. IL-1β as a potent pro-inflammatory cytokine has been associated with acute lung injury and fibrosis[114-116] and characteristically has been linked to TGF-β production, which stimulates proliferation and trans-differentiation of epithelial cells into collagen-producing myofibroblasts. Furthermore, it has been observed that silica or asbestos crystals, known inducers of fibrosis, engulfed by resident macrophages activate the NLRP3, leading to excessive IL-1β production. Mice deficient in NLRP3 show impaired inflammation and are protected from fibrosis after exposure to silica or asbestos[113]. Similarly, aged, bleomycin-exposed mice lacking NLRP3-/- also displayed reduced fibrosis [112]. Furthermore, Arlett et al demonstrated that scleroderma dermal and lung fibroblasts display inflammasome activation while inhibition of caspase-1 leads to decreased α-Smooth muscle actin (aSMA) expression and reduced thickness of fibers in myofibroblasts[117].

Specifically, in IPF, our group showed an impairment of the NLRP3 pathway in IPF AMs [118], while increased basal protein levels of IL-1β in the BALF were noticed. A recent transcriptomic study in fibroblasts revealed a depression of inflammation and immunity pathways[119]. In this study AIM2 inflammasome and IL-1Receptor were downregulated. Conversely, in IPF blood leukocytes, the mRNA of NLRC4 inflammasome was significantly elevated and was strongly inversely correlated with survival[120]. AIM2 inflammasome was also recently linked to the pathogenesis of lung fibrosis. Notably, AIM2 is overexpressed in IPF-AMs and this is related to

increased Drosha ribonuclease III (DROSHA), a class 2 ribonuclease III enzyme expression[121]. 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[122]. Of particular interest, GLUT-1 dependent glycolysis promotes exacerbation of lung fibrosis during Streptococcus pneumoniae infection via AIM2 activation[123] and several studies have suggested a relative abundance of Streptococcus genera in IPF[124-126].

Inflammasomes triggers are known to be present in the lung microenvironment in lung fibrosis. are generally activated by a variety of stimuli in the lungs. Elevated reactive oxygen species (ROS), inhibited autophagy and microbiota dysbiosis have been linked to the pathogenesis of IPF and known inducers of inflammasomes.

1.2.4 The role of mitochondria in lung fibrosis pathogenesis

Mitochondria are organelles that exist in almost all eukaryotic cells and orchestrate several vital cellular functions such as metabolism, bioenergetics, programmed cell death and innate immune responses. Mitochondria are the ‘’powerhouse of the cell’ that drive ATP production through oxidative respiratory Phosphorylation (OXPHOS) and they are the major generator of reactive oxygen species[127].

Dysregulation of their life cycle can lead to mitochondrial dysfunction which is characterised by an increase in the generation of ROS and altered bioenergetics. Mitochondrial dysfunction refers to the damage of the mitochondrial structure, respiratory chain defects, reduction of mitochondria number and biogenesis. This may lead to maladaptation to cellular stress, aberrant apoptosis and senescence, a state of cell growth arrest[128]. Mitochondrial dysfunction can lead to several disease pathologies, ranging from neurodegenerative to metabolic disorders and is a recognized as a hallmark of aging[129].

Meanwhile, mitochondria have multiple mechanisms of self-repair and renewal. With the mitophagy process dysfunctional mitochondria are removed by fusion with the lysosomes[130]. ROS can induce mitophagy by activating the phosphatase and tensin homology deleted on

chromosome 10 (PTEN) induced putative kinase 1 (PINK1)/Parkin pathway[131]. PINK1 recognizes and aggregates on the surface of damaged mitochondrial membrane, activates phosphorylation, and recruits Parkin. Mitochondria are then encapsulated to form mitophagosomes, which are fused with lysosomes and reduced by hydrolases[132].

In immune cells the cellular metabolism shapes immune responses. In homeostasis, decreased mitochondrial ROS diminishes multiple TLR-initiated pathways and bactericidal activity of macrophages[133]. Studies have established that mitochondria are central in fighting bacteria and mitochondrial dysfunction impairs the capacity to of macrophages to respond to infections. Aside from the bactericidal impairment, mitochondrial dysfunction and oxidation leads to age-related systemic inflammation, often termed ‘’Inflammaging ’’[134].

NLR signaling pathway is also depend on mitochondrial ROS. Pharmacological inhibition of ROS production decrease NLRP3 inflammasome activation[135]. Similarly, blockade of autophagy, triggers the accumulation of damaged, ROS-generating mitochondria due to impaired mitophagy and augments the activation of NLRP3 inflammasome [135-138]. Aside from ROS, the release of mitochondrial DNA (mtDNA) enhances NLRP3 activation[139]. Notably, high ROS production in aged mice is associated with higher levels of NLRP3 inflammasome activation and caspase-1-dependent IL-1β and IL-18 production[112]. Furthermore, in a mouse model of NLRP3-mediated Streptococcus pneumoniae infection, the deficiency of NOX4 reduced the mitochondrial fatty acid oxidation, inhibited NLRP3 activation and improved survival[140].

Several studies have implicated mitochondrial dysfunction in lung fibrosis pathogenesis. In IPF the primary fibrotic signal is considered to arise from repetitive AECs injury and apoptosis. Mitochondrial abnormalities and mitochondria-mediated apoptosis in AECs have been linked to pulmonary fibrosis initiation. In IPF-lung tissues mitochondrial respiration and oxygen consumption is reduced [141]. In AECII the electron transport chain complex exhibit reduced activity[141]. Similarly, in IPF fibroblasts mitochondrial ROS is increased, while ATP production and oxygen consumption are reduced[142, 143]. In AMs the expression of mitochondria encoded OXPHOS genes is decreased and the production of mtROS is significantly increased[144, 145].

1.2.5 The role of bacteria and infection in the pathogenesis of ILDs

The lungs are constantly under the exposure to the outside world through the inhalation of around 7000 litres of air per day. Yet, for centuries scientists though that the lower respiratory tract was sterile given the inability to culture bacteria under normal methods. The development of new sequencing techniques allowed the identification of bacteria through 16S rRNA gene sequencing, a highly conserved gene within the bacterial genome. This allowed the identification of several bacterial communities within the human lung[7, 146].

The microbiome has been defined as the "community of commensal, symbiotic and pathogenic organisms that share our body"[147]. In the lungs specifically the bacterial biomass is smaller compared to the gut, but our growing understanding of their interaction with the immune system has implicated microbiota alterations to alveolar immunity and clinical outcomes in several lung disease[125, 148, 149].

Especially in IPF, several studies have been conducted and have implicated microbiota dysbiosis in the pathogenesis and progression of the disease. IPF patients compared to healthy individuals, have higher predominance of Haemophilus, Neisseria, Streptococcus, and Veillonella species in there airways and the bacterial diversity is reduced [125]. Similarly, Han et al reported an association between an abundance of airway Staphylococcus and Streptococcus operational taxonomic units (OTU) and disease progression in patients with IPF[124]. Furthermore, there was an association of the microbiota changes and gene signatures of host defence in the peripheral blood of the patients, suggesting that lung microbiome alters peripheral immune cell phenotypes[150].

In parallel, not only certain communities are more prevalent in IPF lungs but also the level of the airway bacterial biomass is raised and has clearly been associated with disease progression. Increased bacterial burden in the bronchoalveolar lavage has been independently validated to associated with progression of the disease[125]. Another study in whole lung explants has shown an abundance of microbiota proximal in the airways rather than the lung peripheries and this

carried increased risk of acute exacerbation and poor mortality[151]. Similarly, in Chronic Hypersensitivity Pneumonitis (CHP), bacterial burden is higher than healthy controls, albeit at lower levels compared to IPF[126].

The true implication of microbiota alterations in Lung fibrosis mainly stems from their contribution in infections and exacerbations. It is well documented that the course of disease in lung fibrosis can be very variable with a subset of patients living for years with gradual progression, while others experience sharp deterioration which often leads to death, as a result of acute exacerbations (AE) of the disease. AE are defined as acute, clinically significant respiratory deterioration with no identifiable cause and are characterised by new, widespread alveolar abnormalities[152].

One study investigated the change in microbiota in stable disease and AE-IPF and found marked changes in the lung microbiome with an increase in Campylobacter and Stenotrophomonas species[153]. A sputum culture study from a large cohort also showed that in AE-IPF Klebsiella pneumonia, Mycobacterium tuberculosis, and Acinetobacter baumannii (10%) dominate in the lungs[154]. Another study proved that germ-free mice with bleomycin induced fibrosis had less mortality rates compared to conventional mice[155].

Microbiota signalling has been shown to activate the inflammasome [156-158]. As described above the microbiota composition is altered in lung fibrosis [125, 159]. These alterations have been linked to host immune response transcriptional changes [160] and variable cytokine secretion [149]. Reduced bacterial diversity was found to associate with increased concentrations of proinflammatory (including IL-1β), profibrotic cytokines and growth factors in the bronchoalveolar lavage[149]. Streptococcus has been established by independent studies to associate with worse disease progression in IPF [124, 125]. Of note, streptococcal infection leads to acute exacerbation of lung fibrosis in mice through AIM2 inflammasome activation [123] while inflammasome activation was dysregulated in BAL cells from ILD patients[118].

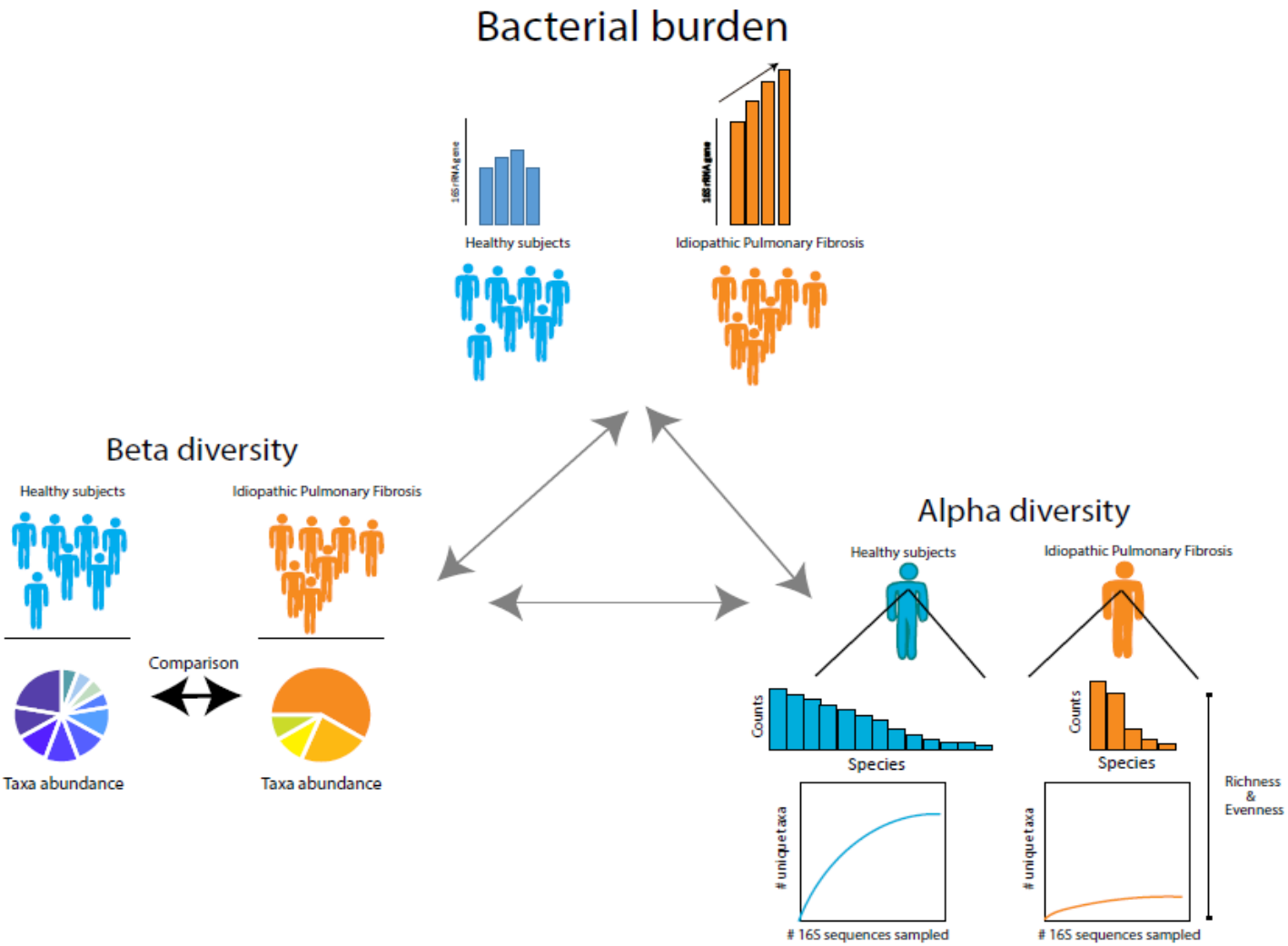


Figure 12: Lung microbiome in health and lung fibrosis. Diversity is measure of the evenness and richness of a bacterial community; within a biological sample (alpha diversity) or between samples (beta diversity).[7]

Chapter 2. Aims and Methodology

2.1 Aims and objectives

Our group has established that mitochondria in IPF and other ILD-AMs are more oxidized compared to health [3, 144] and inflammasome activation is deregulated in IPF BAL cells[118].

We hypothesised that alterations in the lung environment including microbiome changes and mitochondrial oxidation could cause functional AM changes that drive excessive inflammasome activation. This could have possible implications in the pathogenesis of disease exacerbations.

The objective of this study was to measure the baseline Inflammasomes activation and the potent activation capacity in relationship with the mitochondrial ROS and the bacterial burden in AMs from patients with lung fibrosis and healthy individuals.

2.2 Ethics approval

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.

2.3 Study Population

We prospectively enrolled thirty-five (35) patients in the Respiratory Medicine Department at the University Hospital of Heraklion, Crete, Greece between June 2017 to June 2019.

Twelve (12) healthy controls, and twenty-three (23) fibrotic ILD patients. Fourteen (14) patients had IPF and twelve (12) non-IPF ILDs. Patients with a recent infection (1 month prior to bronchoscopy) were excluded from the study. Subjects underwent bronchoscopy and Bronchoalveolar Lavage Fluid (BALF) was obtained as part of the diagnostic algorithm. All ILD patients were treatment naïve.

Patient Groups:

1. IPF patients

   The diagnosis of IPF was based on ATS/ERS criteria or on the Fleischer Society criteria after Multidisciplinary discussion [161].

2. Non-IPF patients

   We included patients with fibrotic Interstitial Lung Diseases as assessed by the presence of reticulation and traction bronchiectasis on High-Resolution Computed Tomography, with extend >10%.

   This category included eight (8) patients with fibrotic chronic hypersensitivity pneumonitis (CHP), one (1) asbestosis related ILD, one (1) Idiopathic pneumonia with autoimmune features (IPAF) and two (2) unclassifiable ILD. We excluded patients with autoimmune disease that were treated with immunosuppressive agents as immunomodulatory treatment could be difficult to interpret.

3. Control group

   Control subjects were 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 summarized in Table 2. All patients were evaluated within one month from bronchoscopy, with Pulmonary Lung Function tests. Since controls were healthy no Pulmonary Function Tests were performed.

Pulmonary Lung Function tests:

Lung volumes (forced expiratory volume in one second – FEV1, forced vital capacity – FVC), and diffusion capacity (DLco, corrected for haemoglobin) were measured using the computerized

system (Jaeger 2.12; MasterLab, Würzburg, Germany). Predicted values were obtained from the standardized lung function testing, European Coal and Steel Community, Luxembourg (1993).

Table 2. Clinicopathological characteristics of the subjects included

	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	89±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

2.4 BALF cell isolation and determination of cellular composition

For the BAL, 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 a total of 180 ml of normal saline in 60-mL aliquots, each retrieved by low suction.

The BALF samples were subsequently kept on ice and were processed within 2 hours of collection. Samples were filtered through sterile 70nm cell strainers (BD) and centrifuged at 1500rpm for 5 minutes at 4^{o}C. Cell pellets were washed and re-suspended with cold PBS. Total cell count and cell viability were subsequently assessed using Trypan blue (ICN). Differential cell population count was analysed following May-Grunewald-Giemsa staining.

2.5 Airway Macrophages isolation

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 ^{o}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.

2.6 Development of assay of Inflammasomes activation

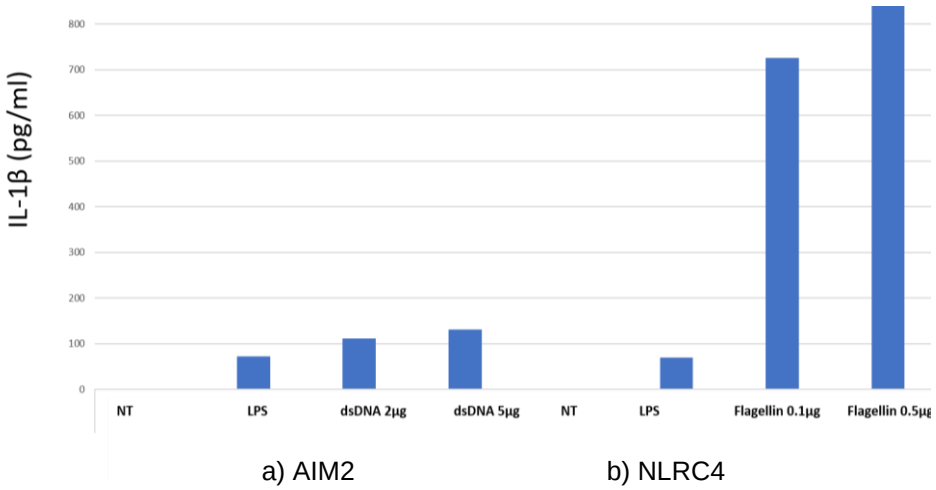
Human macrophages are known to require a two-step mechanism to activate NLRP3; an evolutionary process preventing uncontrolled NLRP3 activation and IL-1β release. The primary TLR-mediated signal activates NF-kappaB (NF-κB) to drive transcription of NLRP3, ASC, pro-IL-1β

and pro-IL-18 and the second signal results in proteolytic cleavage of IL-1β and IL-18 by caspase-1 [162].

To establish the inflammasome activation protocol Peripheral blood leukocytes (PBMCs) were used. PBMCs from healthy individuals were collected through Ficoll gradient centrifuge. Cells were seeded and primed with LPS (to upregulate the inflammasome components).

For the activation of NLRC4 and AIM2 we tested different concentrations and timelines to identify the most suitable conditions. Cells were primed for 1 hour with LPS(10ng/ml) and then treated with different concentrations and at different timepoint (2 hours, 4 hours, 24 hours) of each stimulus. For NLRC4 cells were transfected with flagellin at a concentration of 0.5µg or 0.1µg/ml and lipofectamine 2000. For AIM2 activation, cells were transfected with Poly (dA-dT) at a concentration of 2µg or 5µg/ml and lipofectamine.

We observed a dose (figure 13 a-b) and time dependent IL-1β (figure 14 a-b) release, by ELISA, suggesting successful activation of each inflammasome. The most pronounced activation was seen after overnight experimentation, however there was a significant IL-1β post LPS alone as well. Previous experimentation has also established that after overnight culture there is increasing apoptosis of AMs which could account for the observed released post LPS. As such we opted to choose the 4-hour experimental procedure for the experiments going forward.



a) AIM2 b) NLRC4

Figure 13: Dose dependent activation of AIM2 and NLRC4 Inflammasome
AMs were primed for 1 hour with LPS followed by transfection with a) 2µg or 5 µg dsDNA or b) 0.1 or 0.5 µg of flagellin for 4 hours
Data presented as median

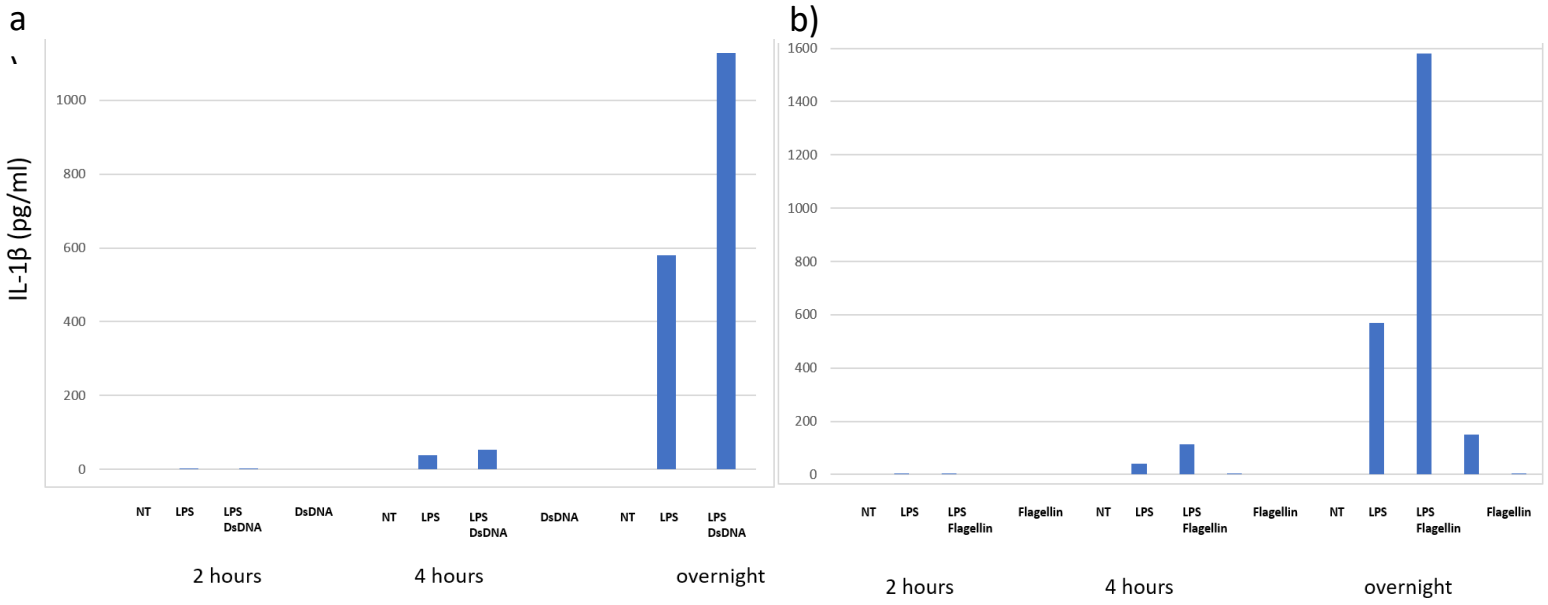


Figure 14: Time dependent a) AIM2 activation and b) NLRC4 activation
AMs were primed for 1 hour with LPS followed by transfection with a) 2 µg dsDNA or b) 0.1 µg flagellin for either 2, 4 hours or left overnight
Data presented as median

Subsequently, we also tested the LPS priming by western blot. In non-treated cells, we did not identify any pro-IL-1β in their cytoplasm. LPS priming resulted in increased in Pro and mature IL-1β protein translation. (figure 15). Inflammasome activation resulted in decreased pro-IL-1β, compatible with the increased secretion noticed by ELISA.

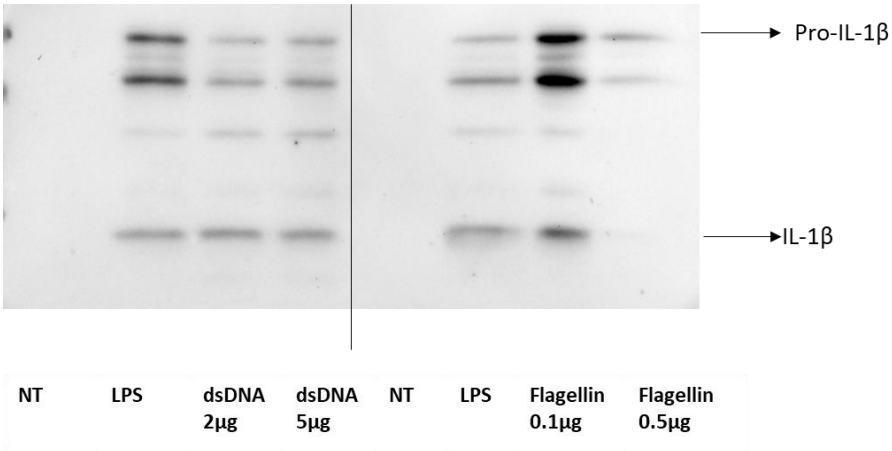


Figure 15: Representative western blot of IL-1β

PBMCs were either left untreated or primed for 1 hour with LPS(10ng/ml) followed by 4 hours transfection with 2µg or 5 µg dsDNA (for AIM2 activation) or 0.1 or 0.5 µg of flagellin (for NLRC4 activation)

Following these observations, we concluded that the most suitable conditions for the activation of NLRC4 and AIM2 in our samples were 1-hour priming with 4-hour stimulus, 0.1 µg/ml flagellin and 2µg Poly(dA-dT).

2.7 Protocol for Inflammasome activation in AMs

For our final AMs experiments, AMs were primed with 10ng/ml LPS to upregulate inflammasome related genes.

For the NLRP3 activation, cells were primed with LPS for 2 hours and then stimulated with 5 mM ATP for 30 minutes. For the activation of the NLRC4 and AIM2 inflammasomes cells were primed for 1h and then transfected with 2 μg/ml dsDNA and 0.1ug/ml Ultrapure Flagellin from S. Typhimirium, respectively for 4 hours (Figure16). For the transfection Lipofectamine 2000 was used.

Inflammasome-specific activation was confirmed using 1μM of the selective NLRP3 inhibitor MCC950, or 10uM of the caspase-1 inhibitor for the NLRC4 and AIM2 inflammasome (non-selective global inflammasome inhibitor), 1h prior to the addition of stimuli.

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

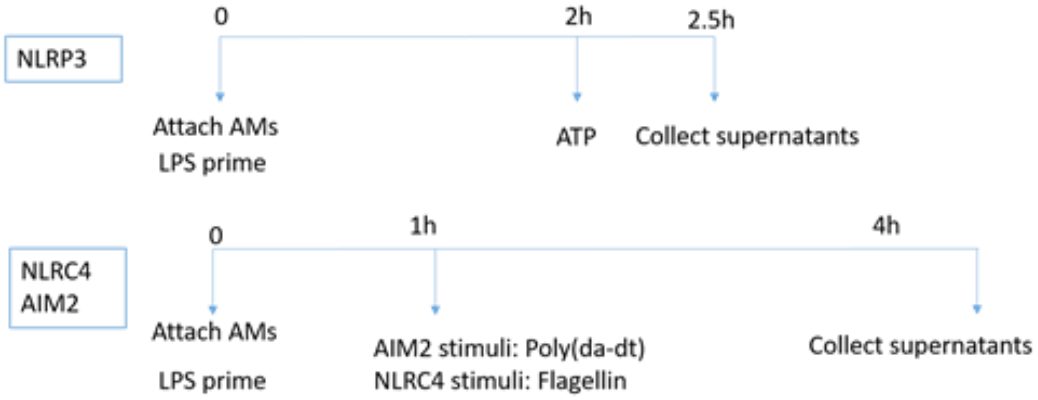


Figure 16: Representative stimulation timeline

2.8 Flow cytometry and mtROS experiments

Mitochondrial ROS was measured by MitoSOXTMRed staining. MitoSOXTMred is targeted to mitochondria in live cells and is readily oxidized by superoxide reactive oxygen species.

0.5 million 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 37oC. For MitoSOX staining quantification, AMs populations were selected according to high forward and side scatter and CD45 (FSChighSSChighCD45+) as previously (27). 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).

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

2.9 Bacterial DNA extraction and 16sRNA qPCR

Bacterial extraction and 16SRNA qPCR was performed for quantification of bacterial burden [163]. This part of the experiments was conducted in collaboration with Imperial College London at the National Heart and Lung Institute, London, UK.

Unfiltered BALF samples (1 mL) were centrifuged at 21 000×g for 30 min to pellet cell debris and bacteria. Pellets were resuspended in 100 μL of supernatant and added to lysing matrix E tubes (MP Biomedicals, Solon, OH,USA) containing 500 μL cetyl trimethylammonium bromide (CTAB) buffer (10% w/v CTAB in 0.5 M phosphate buffered NaCl) and 500 μL phenol:chloroform:isoamyl

alcohol (25:24:1), and shaken in a FastPrep Instrument (MP Biomedicals) at 5.5 ms−1 for 60 s. Following bead-beating, samples were extracted with an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated with 2 volumes of precipitation solution (30% w/v PEG6000 in NaCl) and, following ethanol washing, DNA was resuspended in 100 µL Tris-EDTA. The quality and quantity of the isolated DNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Hemel Hempstead, UK) and the DNA was stored at −80°C until further use.

For the 16S rRNA gene quantitative PCR we used triplicates of 10 µL quantitative PCRs (qPCR), containing 1 µL of bacterial DNA and 9 µL of Femto bacterial qPCR premix (Cambridge bioscience, Cambridge, UK). Each run contained a 10-fold dilution series of the Vibrio natriegens DSM 759 gene cloned into a plasmid of known size and a non-template control. For data acquisition, the following cycling parameters were used: 1 cycle of 95°C for 10 min; 40 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min; and 1 cycle of 72°C for 7 min.

2.10 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^{o}C. Total RNA extraction, cDNA synthesis and real-time PCR were performed. GAPDH levels were used as endogenous control for the normalization of mRNA expression levels in BAL samples.

For the extraction and quantifications of mitochondrial DNA (mtDNA) we used 5µl of clarified BAL fluid directly for real time-PCR amplification of the mt-ND1 specific sequences.

Primer sequences are shown in table 3.

Table 3. The sequences of primers used for qPCR

Gene name	Primer sequences
NLRP3	F: CACCTGTTGTGCAATCTGAAG
	R: GCAAGATCCTGACAACATGC
AIM2	F: TGGTTTGTTTGTAGTCCAGAAGG
	R: CCTCGTTTCTAACCCCCAGT
NLRC4	F: CCTGAGCAGCCTGTTGAAA
	R: AAGTTTTTCAGAGGGTTCTTTCC
GAPDH	F: AGCCACATCGCTCAGACAC
	R: GCCCAATACGACCAAATCC
For MT-DNA quantification	
MT-ND1 (MRC complex I)	F: AACCTCTCCACCCTTATCACAA
	R: TCATATTATGGCCAAGGGTCA

2.11 Western blot analysis

1-1.5 million cells were centrifuged and cell pellets were homogenised in RIPA buffer (Invitrogen) containing protease and phosphatase inhibitors, Pierce), followed by storage at -80oC. 40-60 µg. Total protein lysates of BAL samples were separated in 12% SDS-PAGE, transferred to 0.45nm nitrocellulose membrane (Biorad), followed by detection of IL-1β (anti-IL-1β Cell Signaling technologies). Appropriate HRP conjugated secondary antibody (Chemicon) was used and immunodetection was performed with enhanced chemiluminescence reagent LuminataTM

(Millipore). Bands were visualised with the ChemiDocXRS+ system (Biorad) and densitometry analyses were performed using Image Lab TM software (Biorad).

2.12 Reagents

Ultrapure LPS (E. coli O111:B4), dsDNA, naked Poly (dA:dT), purified S. typhimurium Flagellin, MitoSOXTMRed was from Invivogen, ATP and mitoTEMPO was from Sigma-Aldrich. MCC950 was purchased from Cayman chemical and caspase-1 inhibitor from Calbiochem

For the immunoblot analysis anti-mouse IL-1β (12426S, Cell Signaling Technologies) was used. Inflammasomes activation was assessed by the IL-1β release, using ELISA (ThermoFischer)

2.13 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 *t*-test, as appropriate. All data are expressed as mean + interquartile range unless stated otherwise. Receiver operating characteristics (ROC) curve analysis was used to select an optimal cut point for Mitochondrial ROS. 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).

Chapter 3. Results

3.1 BALF baseline IL-1β and inflammasome components gene expression

Initially, we measured the baseline IL-1β levels in the BALF to examine whether there was inflammasome pre-activation and subsequent IL-1β excess in the lung microenvironment.

Median basal IL-1β concentration in the BALF did not differ significantly between ILDs and healthy patients, while some sporadic IL-1β release was detectable in all three groups (Figure 17a). Similarly, IL-1β secretion by unstimulated freshly isolated AMs was also measured and no differences were observed among the groups (Figure 17b).

We then assessed the mRNA expression of genes encoding core inflammasome components *NLRP3*, *NLRC4*, and *AIM2* as well as *IL-18* in BALF cells (Figure 17c-17e). *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.

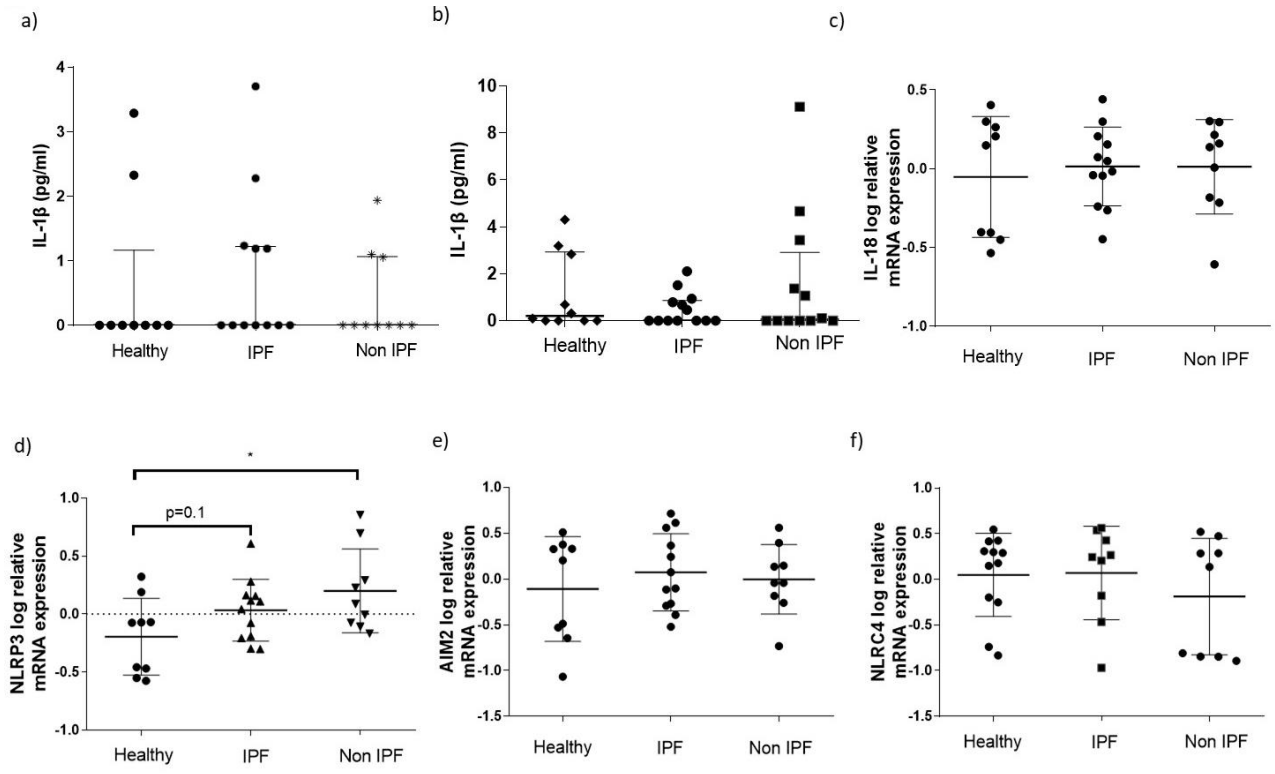


Figure 17: Baseline characterization of the Inflammasome in BALF cells.

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) IL-18 d) NLRP3, e) AIM2, f) NLRC4. Data represented as mean ± SD, t-test.

3.2 NLRP3 inflammasome activation in Airway Macrophages

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 an non-canonical way in human myeloid cells [108, 164].

For NLRP3 inflammasome activation, AMs were primed with LPS for 2 hours followed by ATP stimulation for 30 minutes. Treatment with LPS resulted in a significant IL-1β release by AMs from all groups, indicating Inflammasome activation in all groups. No significant differences were noted after LPS treatment (Figure 18a).

The addition of ATP resulted in excessive IL-1β release in IPF, and non-IPF-AMs compared to controls (p=0.0004 and 0.007 respectively, Figure 18b). Between IPF and Non-IPF patients, NLRP3 activation was similar. This effect was NLRP3 specific, as it was abrogated by MCC950 treatment (Figure 18b), a novel specific NLRP3 inhibitor [165].

To determine whether ILD-AMs were pre-stimulated by an NF-κB related stimuli in the lungs, we also treated cells with ATP (with no proceeding signal- 1). ATP alone resulted in muted responses compared to LPS and ATP across all groups. However, in IPF the addition of ATP was sufficient to generate IL-1β release, compared to healthy controls (p=0.04, Figure 18c). Similar trends were noted in non-IPF-AMs (p=0.1, Figure 18c). When all ILDs were combined, ATP resulted in increased NLRP3 activation compared to controls (p=0.04).

In a subset of patients with available serial PFTs we then examined whether inflammasome activation was associated with progression, as defined by an FVC decline of 10%. Those patients that progressed has significantly higher NLRP3 activation without LPS pre-stimulation compared to stable patients (p=0.04) (figure 18d).

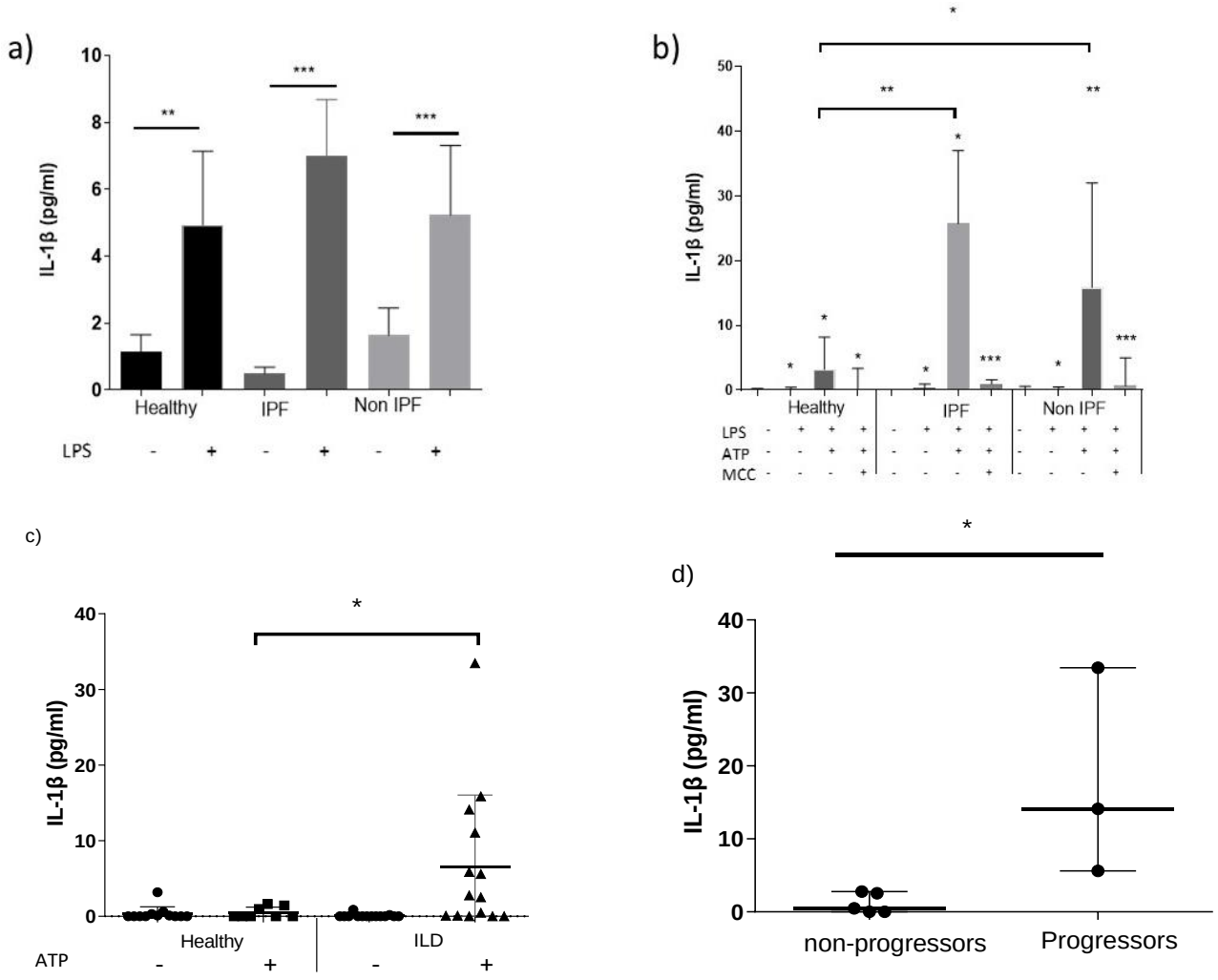


Figure 18: NLRP3 activation in Airway Macrophages

a) NLRP3 activation by LPS alone (5 hours treatment)

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) Difference in NLRP3 activation without LPS priming in ILD patients that progressed compared to ILD patients that remained stable

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

3.3 AIM2 inflammasome activation in Airway Macrophages

For AIM2 activation, cells were primed with LPS for 1 hour followed by transfection with dsDNA for 4 hours. In all three groups the addition of dsDNA resulted in significant IL-1β release, indicating that Inflammasome activation was achieved in all groups (Figure 19a). IL-1β release 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 19a).

It was recently suggested that in human myeloid cells, in contrast to mice, dsDNA activates the NLRP3 inflammasome through the cGAS-STING pathway, whilst AIM2 is non-functional[110]. Activation of the cGAS-STING pathway potentiates lysosomal damage and subsequent cell death which activates the NLRP3 rather than the AIM2 inflammasome[166]. To address this possibility, cells were treated with dsDNA in the presence of MCC950, the NLRP3 specific inhibitor. A significant inhibition of IL-1β release was observed in the presence of MCC950. 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 treatment inhibited dsDNA-mediated inflammasome activation as well (Figure 19b).

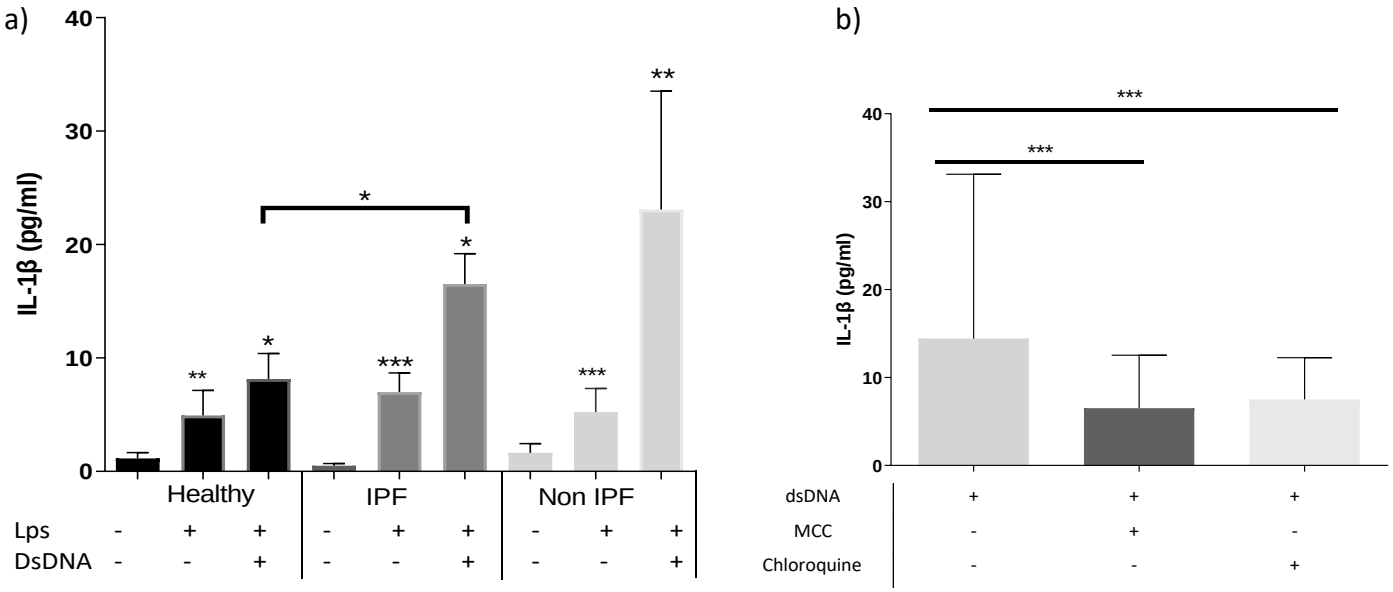


Figure 19. AIM2 activation in Airway Macrophages (AMs)

a) For the activation of AIM2 inflammasome AMs were treated for 1 hour with LPS priming followed by 4 hours dsDNA transfection

b) AMs from the same ILD patients were pre-stimulated with LPS and then treated with dsDNA, or pretreated with LPS and MCC950 or Chloroquine before being treated with dsDNA

MCC: MCC950, selective NLRP3 inhibitor, dsDNA: double stranded DNA. 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

3.4 NLRC4 inflammasome activation in Airway Macrophages

For the activation of NLRC4, cells were primed with LPS for 1 hour, followed by flagellin transfection for 4 hours. The addition of flagellin was linked to significant IL-1β release in all groups, although the activation of NLRC4 inflammasome was similar across groups (Figure 20a).

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 with the addition of MCC950 did not influence IL-1β release, whilst the addition of caspace-1 inhibitor significantly abrogated NLRP4 activation thus suggesting that IL-1β release post NLRC4 stimulation is caspase-1 dependent and NLRP3-independent (Figure 20b).

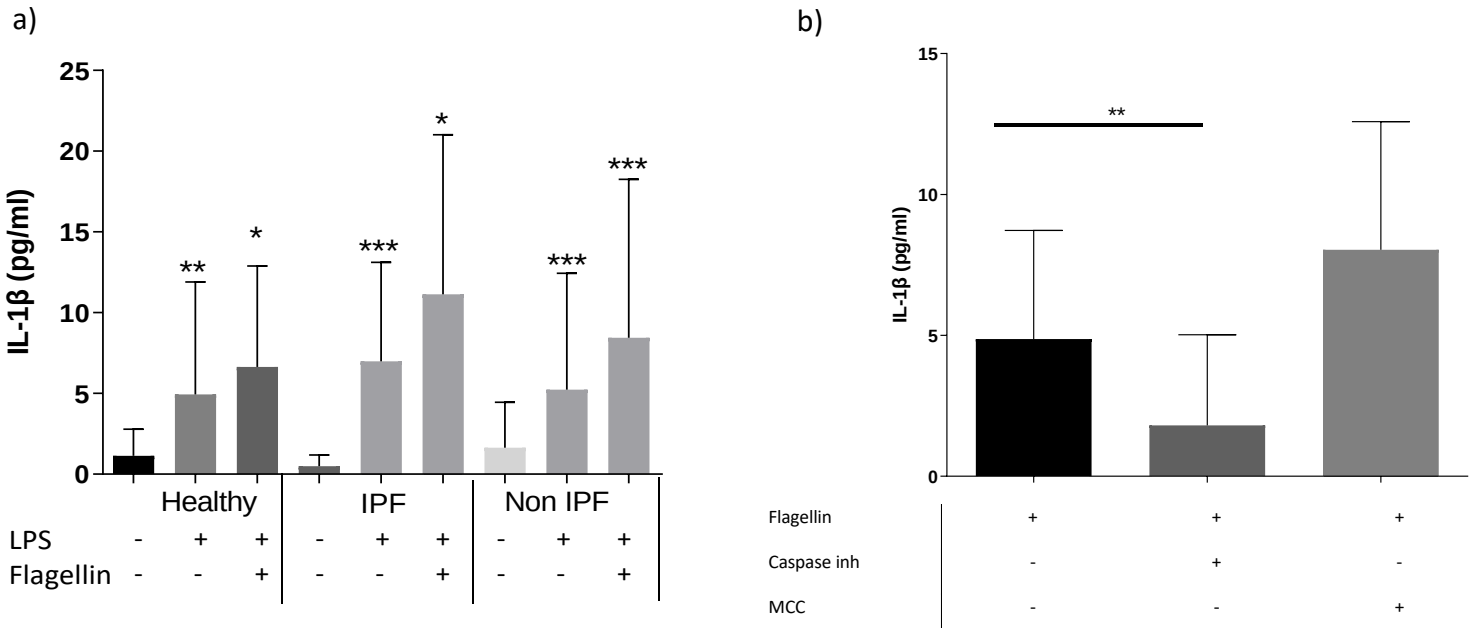


Figure 20: NLRC4 inflammasome activation

a) AMs were treated for 1 hour with LPS priming followed by 4 hours flagellin transfection
b) AMs from the same ILD patients were pre-stimulated with LPS and then transfected with flagellin, or pretreated with MCC950 or Caspase-1 inhibitor 1 hour before the addition of flagellin

MCC: MCC950 selective NLRP3 inhibitor, Caspase inh: non-selective Caspace-1 inhibitor. For within 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

3.5 Inflammasome activation and mitochondrial oxidation

MtROS is widely recognized as an inducer of inflammasome activation, and it was previously shown that mtROS is elevated in IPF and Non-IPF AMs[167, 168].

It was therefore hypothesised that elevated mtROS could be associated with higher NLRP3 inflammasome activity in ILD-AMs. The levels of mtROS in fresh untreated AMs was measured by flow cytometry using MitoSOX^{TM}red. MtROS is higher in IPF and mitochondria are oxidised as previously published (figure 21a) [3]. When all ILDs were combined mtROS was higher in ILD-AMs (IPF and Non-IPF) compared to controls (p=0.03, Figure 21b).

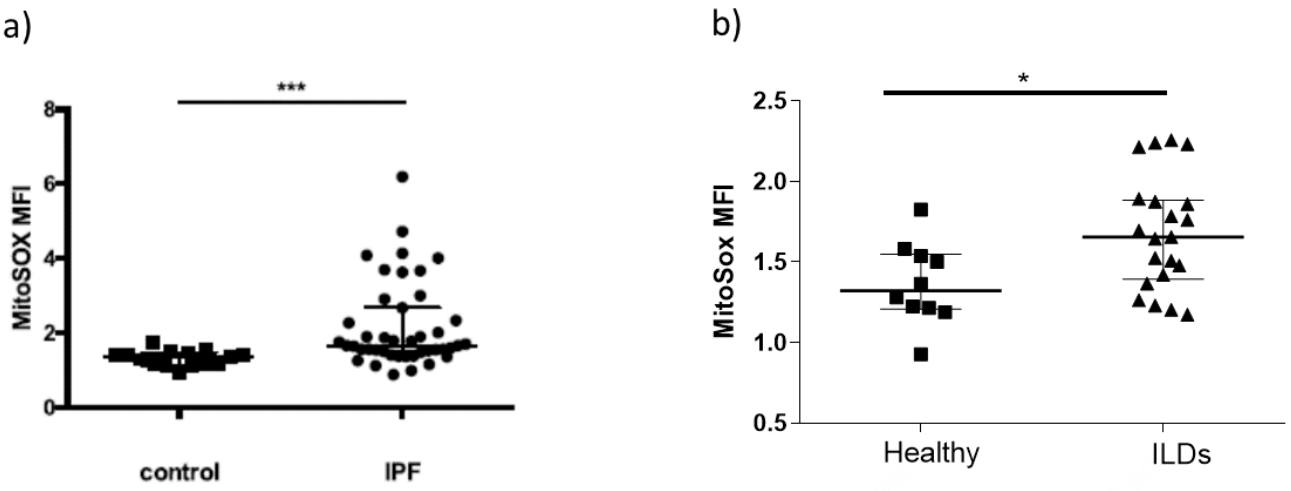


Figure 21: Mitochondrial oxidation status in ILDs
mtROS Mean Florescence Intensity Index (MFI) as assessed by flowcytometry in a) IPF compared to controls[3] and b) healthy compared to ILD patients(IPF and other ILDs combined)

Data presented as median with interquartile range, *p<0.05, **p<0.005, ***p<0.0001, Mann-Whitney test.

Using ROC curves, we determined the optimal cut-off value for mtROS level in AMs. ILD-AM samples were then stratified according to high or low mtROS levels. AMs exhibiting high mtROS showed enhanced NLRP3 activation in the absence of LPS priming (p=0.004, Figure 22a). There was also a trend for greater NLRP3 activation in LPS-primed AMs as well (p=0.08) (Figure 22b). AIM2 activation was likewise significantly heightened in AMs with high mtROS (p= 0.02) (Figure 22c).

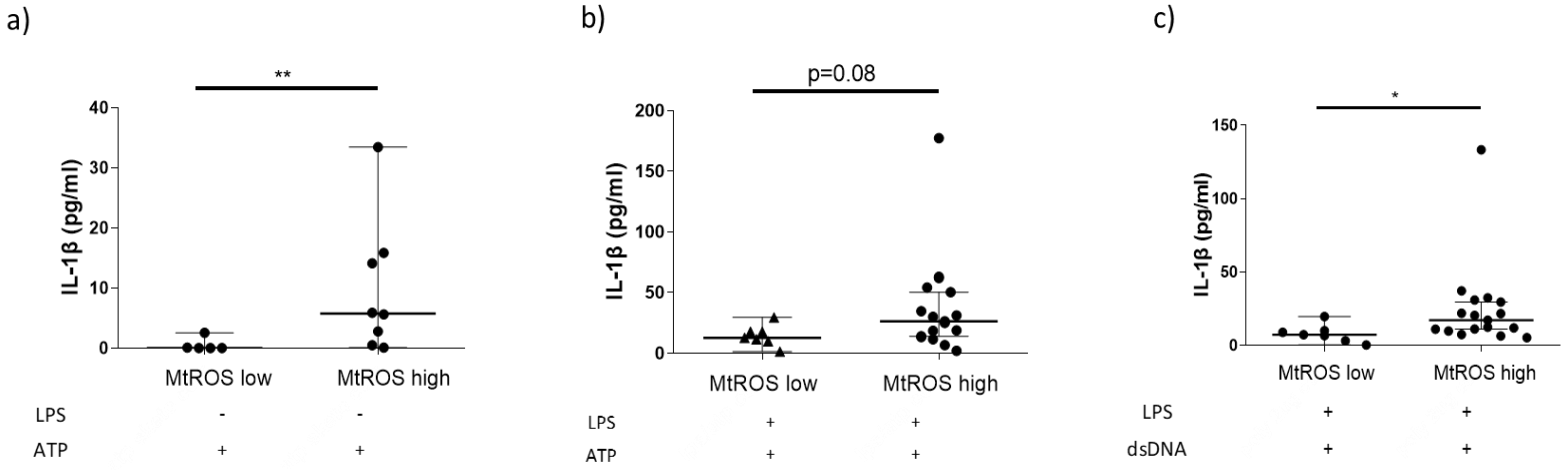


Figure 22: Mitochondrial Oxidation status and the inflammasome
Comparisons on inflammasome activation were made in MtROS high and low groups a) NLRP3 activation without LPS priming, b) NLRP3 activation with LPS priming, c) AIM2 activation (LPS priming followed by dsDNA transfection)
Data presented as median with interquartile range, *p<0.05, **p<0.005, Mann-Whitney test.

We further tested this by Flow cytometry. Stimulation of AMs with LPS and ATP coincided with a burst of mtROS which was reduced with mitochondrial antioxidant treatment (Figure 23a (i-iii) and 23b). We subsequently sought to determine whether antioxidant treatment could inhibit IL-1β release in AMs. Treatment with mitoTempo a mitochondria-targeting antioxidant significantly inhibited mtROS accumulation (Figure 23a (i-iii) and 21b) and blocked NLRP3 activation, as assessed by IL-1β release (Figure 23c).

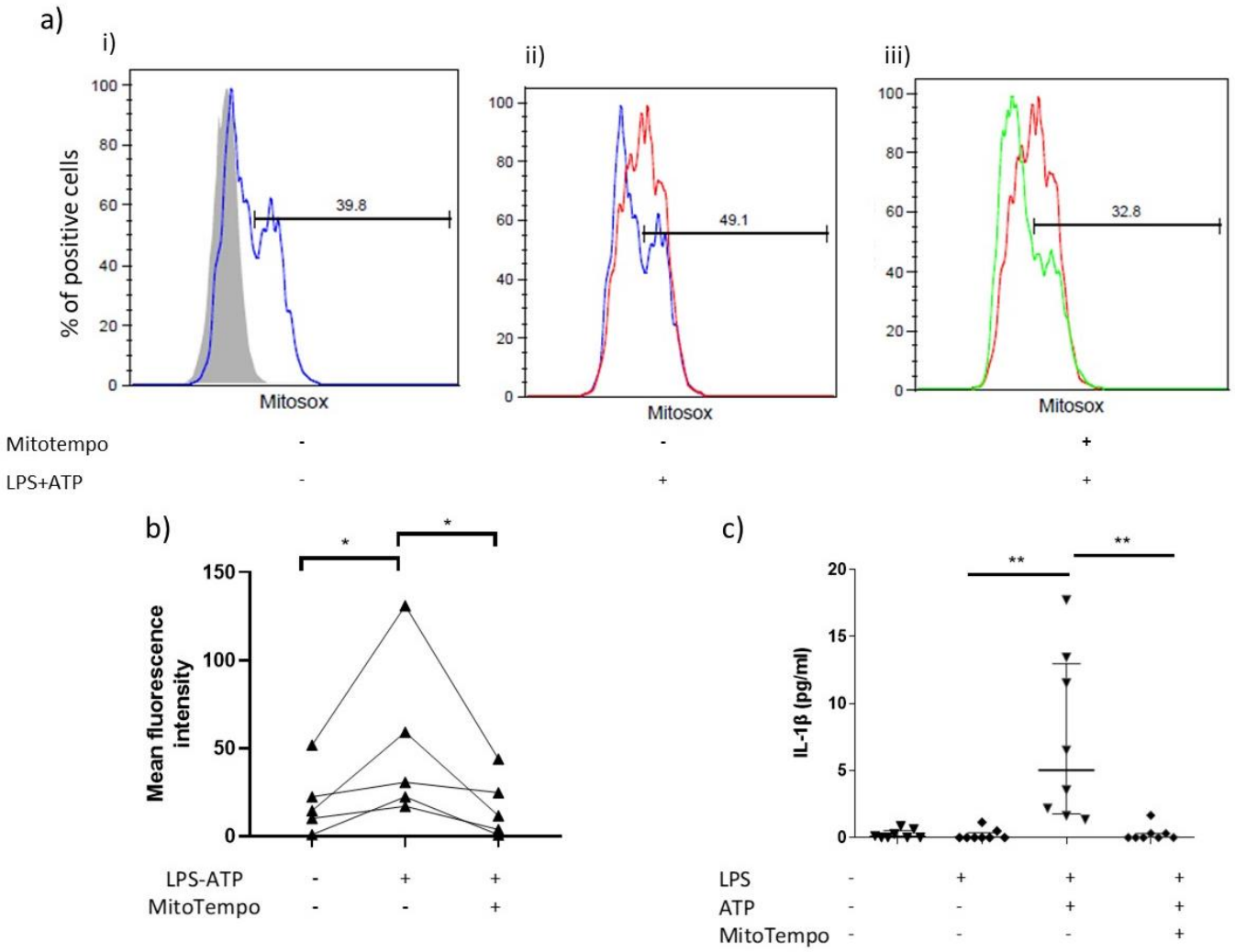


Figure 23: Mitochondrial antioxidant treatment effect on inflammasome activation

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

3.6 Free Mitochondrial DNA quantification in the BALF

Free mtDNA in the BAL was previously suggested to be raised in IPF as a marker of fibroblast damage [169] and has been reported to promote NLRP3 activation and acute lung injury[170]. We measured the ND1 expression in cell free BALF as a marker of mtDNA.

ND1 expression was similar in healthy and ILD-AMs (figure 24). Additionally, there was no association between the free mtDNA and any inflammasome component or the bacterial burden.

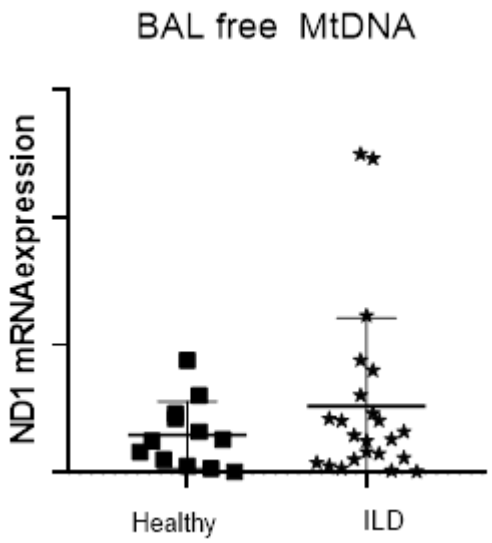


Figure 24: Free mtDNA in the BALF
Free mtDNA measured by PCR in the BALF
Data presented as median with interquartile range, Mann-Whitney test; mtDNA: mitochondrial DNA, BALF: Bronchoalveolar lavage fluid

3.7 Inflammasome activation and lung microbial burden

In IPF, it is established that microbial burden is increased and this correlates with disease progression [125, 159]. Similarly, in CHP, microbial burden is also elevated compared to health albeit to a lesser extend compared to IPF [126]. We hypothesised that microbiota fluctuations in the lung microenvironment could be priming the inflammasomes in AMs through their constant interaction.

BALF microbial burden was through qPCR of 16S rRNA gene copies present in 1ml of BAL. Microbial burden was increased in ILDs (IPF and non-IPF combined) relative to healthy individuals ($p=0.03$) (Figure 25a). Furthermore, 16S rRNA gene copies significantly correlated with IL-1β secretion in unstimulated AMs (R^2:0.53; $p=0.02$) (Figure 25b) and *AIM2* (R^2: 0.68 $p=0.004$, Figure 25c) and *IL-18* (R^2: 0.59 $p=0.015$, Figure 25d) relative mRNA expression. There was no association between bacterial burden and mtROS.

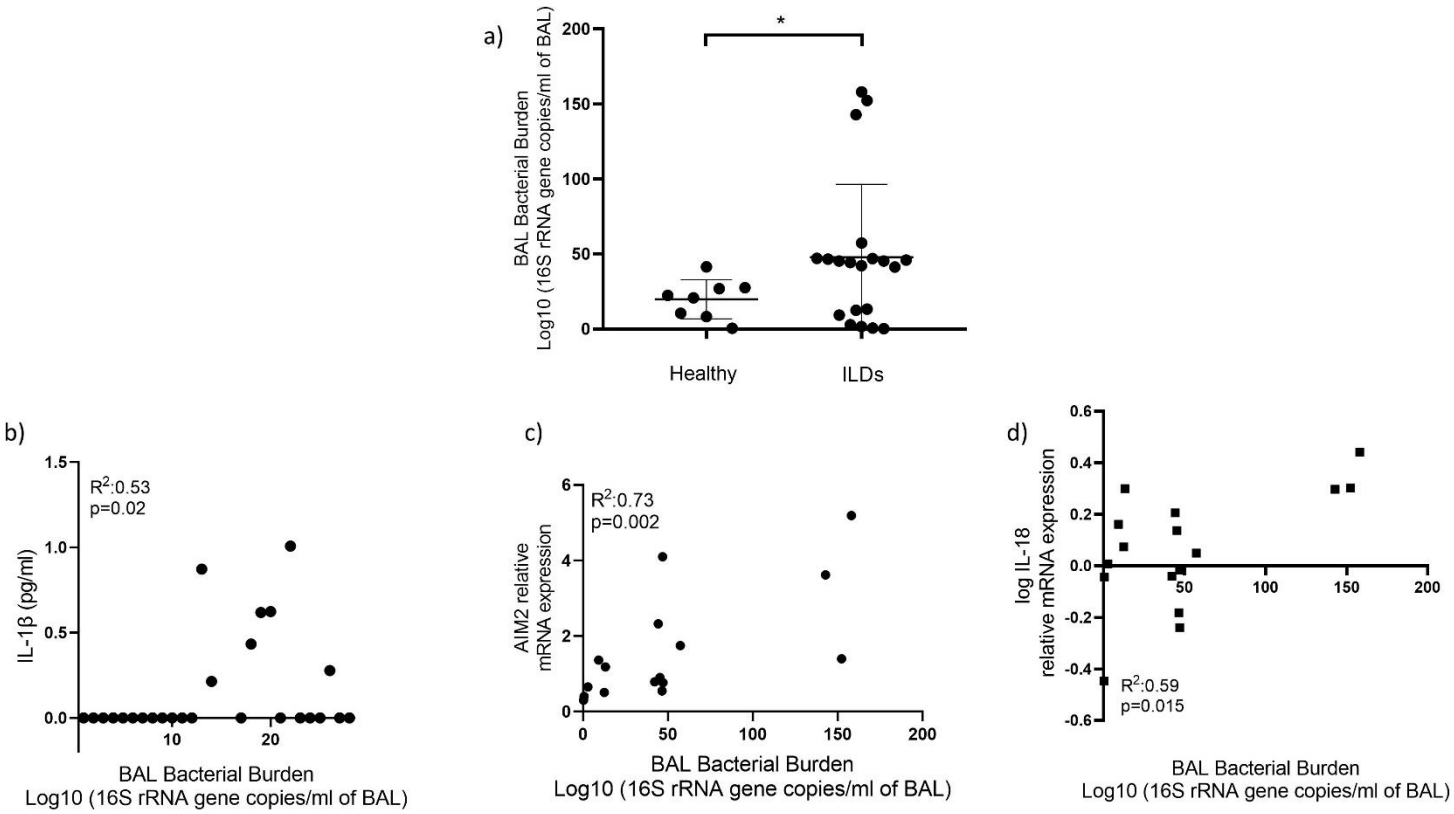


Figure 25: Bacterial burden and the inflammasome

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)

3.8 Inflammasome activation in PBMCs

For the PBMC experiments we included ten (10) Healthy individuals, ten (10) IPF and 10 CHP patients.

The addition of ATP, dsDNA and flagellin resulted in NLRP3, AIM2 and NLRC4 activation in all groups in PBMCs. Baseline and after LPS treatment, IL-1β release did not differ significantly in

fibrosis or control groups (figure 26 a-b). The activation of inflammasome post NLRP3, AIM2 and NLRC4 was similar between all groups (figure 26c-e). Of note there was a trend towards higher IL-1β from untreated cells from CHP patients, but this did not reach significance threshold.

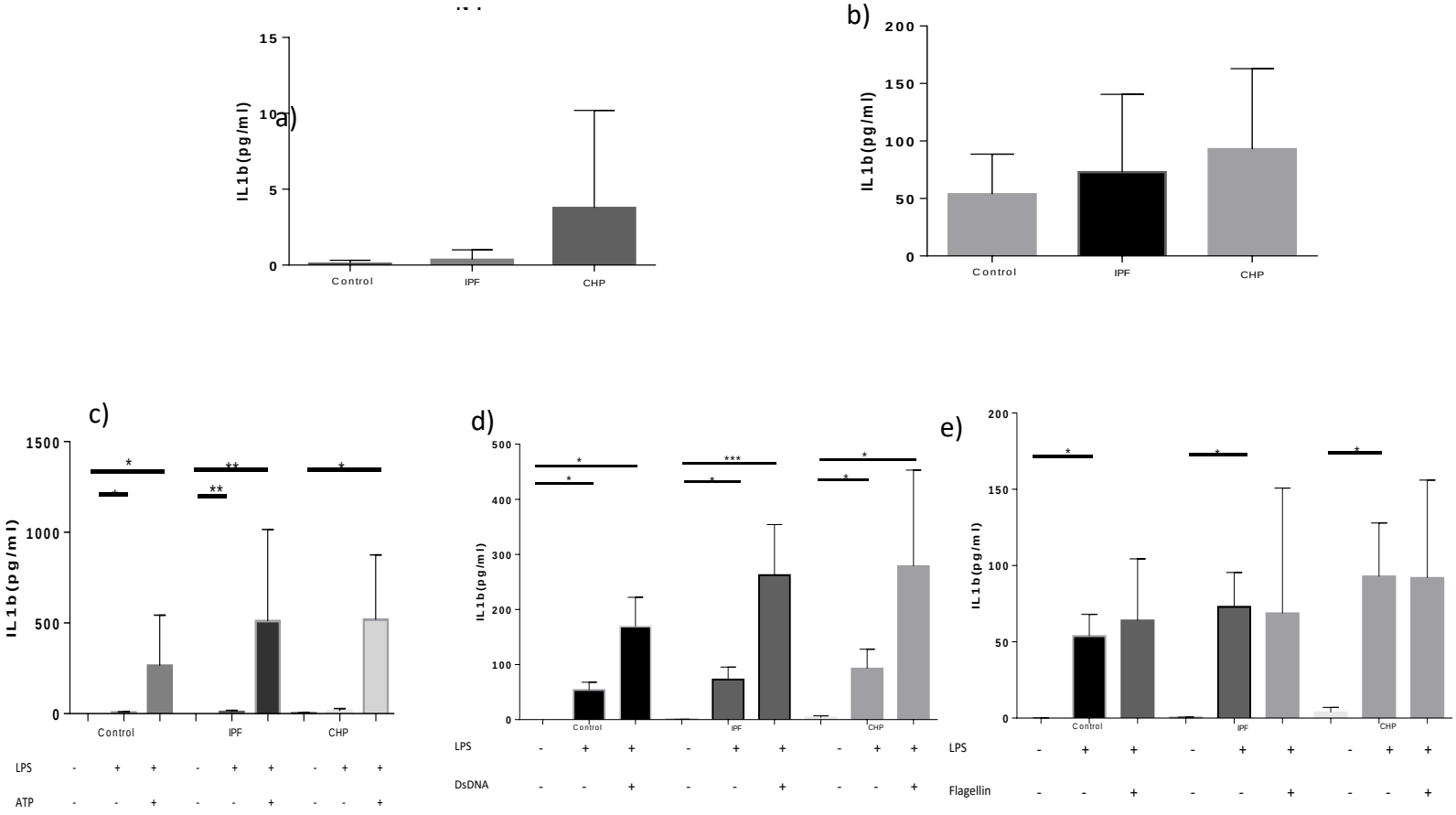


Figure 26: Activation of NLRP3, AIM2 and NLRC4 activation in PBMCs
PBMCs were treated with LPS for 2 hours for the NLRP3 and 30 minutes for AIM2 and NLRC4 activation. Then treated for 30 minutes for NLRP3 or transfected for 4 hours with ds-DNA (AIM2 activation) or Flagellin (NLRC4 activation)
For with-in 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; PBMCs: Peripheral mononuclear cells

Chapter 4: Discussion

4.1 Summary of key findings

The overall hypothesis tested in this thesis is that inflammasomes are activated in ILD-AMs as a consequence of mitochondrial oxidation and alterations in the lung microbiome. The main findings of this thesis are summarised below:

a) NLRP3 and AIM2 inflammasomes are hyper-inducible in alveolar macrophages in lung fibrosis

b) Higher mtROS is associated with enhanced NLRP3 and AIM2 activation

c) Microbial burden is associated with baseline inflammasome activation and the expression of several inflammasome components.

4.2 General Discussion

Inflammasomes are signaling platforms that are activated by a variety of signals, such as reactive oxygen species (ROS) and bacteria, to produce and release IL-1β and IL-18. The inflammasome can be activated in immune cells to cause lung injury[171] and a variety of inflammasome stimuli are present in the fibrotic lungs.

Mitochondrial dysfunction in immune and non-immune cells is a hallmark of IPF pathogenesis. Mitochondria are not only the "powerhouses" of our cells but also shape immune responses. Immune cells transform from a catabolic to an anabolic state through their mitochondria, and this drive change their polarization and function. In IPF and other ILD, AMs are more oxidized compared to health [3, 144]. Furthermore, lung microbiome is altered in IPF and bacterial biomass is increased [125]. We therefore hypothesised that alterations in the lung environment including microbiome changes and mitochondrial oxidation could cause functional AM changes that drive excessive inflammasome activation, with possible implications in the pathogenesis of

disease exacerbations. To address this hypothesis, lung fibrosis patients and healthy controls were recruited, and AMs were isolated and stimulated to activate the inflammasomes. In parallel, mitochondrial oxidation and microbial burden were measured.

The experimental model used to test this hypothesis produced several observations. NLRP3 and AIM2 inflammasomes, but not NLRC4 are hyper-inducible in IPF and other ILDs. The activation of NLRP3 without pre-stimulation with ATP was also heightened in ILDs. The overactivation of the inflammasome was found to be associated with increased mitochondrial ROS and could be inhibited by mitochondria anti-oxidant therapy. The bacterial burden was associated with baseline IL-1β and the expression of several inflammasome components.

IL-1β is a potent pro-inflammatory cytokine which can initiate and amplify lung inflammation[172]. IL 1 cytokine family is involved in various disorders of the lung and has been firmly implicated in the pathogenesis of acute lung injury as well as ventilation induced lung injury[116, 171]. Uric acid and mitochondrial ROS production have both been linked to IL-1β induced lung inflammation and injury[114, 173, 174]. There is also increasing evidence that IL-1β has potent profibrotic actions. Transient IL-1β overexpression induces acute alveolar damage with subsequent induction of TGF-β expression and fibrogenesis[116]. Furthermore, pharmacological inhibition of the IL-1 cytokines, can reduce or cure fibrosis induced by bleomycin or silica induced fibrosis [175].

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 [176-178]. The NLRP3/IL-1β axis has been implicated to lung fibrosis mainly in mice models as it is required for the development of bleomycin induced fibrosis[177]. Furthermore, in mice, age-dependent mitochondrial dysfunction results in enhanced NLRP3 activation and lung fibrosis [112]. In humans, only silica-driven fibrosis is clearly linked with the NLRP3 inflammasome[179]. In IPF, or other ILDs, the inflammasome is not firmly established to play a role in the disease. In this study we provide evidence of a novel implication of NLRP3 and AIM2 inflammasome in patients with lung fibrosis.

Using our model we showed that NLRP3 is indeed hyperinducible in lung fibrosis, IPF and non-IPF, although baseline activation was similar to controls. Similar to our findings, a recent study showed that NLRP3 inflammasome can be overactivated in IPF and IPF-AE at similar levels as ARDS. The authors also proposed a possible mechanism of acute exacerbations that is guided by NLRP3 as radiated/damaged A549 (a model to simulate apoptotic alveolar epithelial cells) can cause increased NLRP3 expression[180]

In these experiments AIM2 inflammasome was also found to be more inducible in ILDs. AIM2 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[121]. Of particular interest, GLUT-1 dependent glycolysis promotes exacerbation of lung fibrosis during S. pneumoniae infection via AIM2 activation[123] and several studies have suggested a relative abundance of Streptococcus genera in IPF[124-126].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[122]. 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 recognized as the central DNA-responding inflammasome. Recent evidence suggests that AIM2 might not be functional in human immune cells in contrast to murine models[166]. Researchers showed that cytosolic DNA causes lysosomal damage and activation of the NLRP3 inflammasome through a STING mediated cell death pathway. In our experiments, the 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 [160]. Here we focused on AMs and failed to notice overexpression or overactivation of the NLRC4 inflammasome either at baseline or upon stimulation.

Several factors that could be involved in inflammasomes stimulation have been previously identified in IPF, including increased mtROS, cell free mtDNA and increased microbiome burden. Mitochondrial dysfunction and oxidation have emerged as a driver in IPF pathogenesis. Mitochondria in IPF-AMs have morphological defects and are oxidized [168]. Similarly, mtROS is elevated in other ILDs [167].To establish a link between mitochondrial dysfunction and cytokine production in lung fibrosis the mtROS was measured at baseline in AMs. mtROS measured in this work was also high in ILDs. The experimental model used showed that in ILD-AMs, NLRP3 and AIM2 inflammasome are hyper-inducible and this is associated with mitochondrial oxidation, a hallmark of IPF pathogenesis. Similarly, to previous studies, our experimental process confirmed that mtROS is crucial for the 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. [162]. The second signal results in the release of active IL-1β. ROS generation by the mitochondria is a well-known trigger of the NLRP3 with mitochondrial death and mitochondrial DNA acting as activators [181]. Although ROS can act as a direct NLRP3 activator [182], studies suggest that ROS could also exert its role at the priming step [183]. An interesting observation is that treatment with ATP without LPS pre-stimulation resulted in pronounced IL-1β release in ILDs compared to controls. This observation implies that AMs in ILDs are pre-stimulated by a signal-1 effector in the lung microenvironment and the addition of ATP resulted in an abundant IL-1β release. Inflammasome activation with ATP without LPS pre-stimulation was also higher in those ILD patients with higher mtROS. This observation supports the original hypothesis that mitochondrial dysfunction and oxidation are priming the inflammasome in lung fibrosis.

One further hypothesis was that free mitochondrial DNA in the lungs could be activating the Inflammasome. Cellular disruption has been shown to release mitochondrial DAMPs which have similar effects as bacterial PAMPs[184]. Mitochondria carry their own genome which closely resembles bacterial DNA. mtDNA has been found in several inflammatory conditions including in joint fluids in rheumatoid arthritis and is known to induce inflammation [185]. Extracellular DNA is an activator of NLRP3. Additionally, free mtDNA is increased in the BALF and is thought to be generated by fibroblasts[169]. In this small cohort the measured mtDNA was similar in controls and ILD patients. There was no association between extracellular mtDNA and the observed overactivation of the inflammasome.

Mitochondria are in the core of metabolic switches in macrophages to promote tissue clearance or antibacterial responses[186] and a burst of mtROS is crucial for their bactericidal properties [187]. By contrast, mitochondrial damage results in deregulated and diminished antioxidant responses to bacteria [188]. It is recognized that microbiota composition is altered in IPF[124, 189]. The bacterial burden was established and verified that it is increased in ILDs compared to controls. The microbial biomass was not associated with mtROS, indicating that microbiota changes were unlikely to be the cause of the observed mitochondrial oxidation.

The bacterial burden in the BALF of ILD patients was found to associated with basal *AIM2* and *IL-18* mRNA expression as well as IL-1β production by AMs at baseline. 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 [190] and gut-microbiota changes shape cytokine release from leukocytes [191]. More specifically in IPF, disruption of the lung microbiome was associated with variable cytokine production leading to lung inflammation and fibrosis progression [155].

The observations presented here shed light into the pathogenesis of acute exacerbations (AEs), a well-recognized complication of IPF and other ILDs [192, 193]. Although it is an even mostly established in IPF, with an annual incidence of 5-19%, AEs can happen in all ILDs and are more

prevalent in progressive ILDs. Exacerbations are detrimental in the prognosis with a mortality of more than 60% and a median survival of 2.2 months post the event[194]. The pathogenesis of acute exacerbations is not well characterized but is thought to resemble acute lung injury in response to infections or sterile insults such as procedures or gastroesophageal reflux [193, 195]. Among the top overexpressed genes in BAL cells in IPF-AEs were NLRP3 and IL-1β[196]. Additionally, in IPF-AE NLRP3 activation is heightened to similar extend to ARDS[180].

Infections either bacterial or viral have been implicated in the pathogenesis of acute exacerbations. Microbiota changes have been reported in exacerbations[124, 125, 197]. It is well established that dysregulated NLRP3 inflammasome activity results in uncontrolled inflammation and can cause acute lung injury and fibrosis [116, 198]. Intriguingly, a transcriptomic study identified NLRP3 and IL-1β among the top up-regulated genes in AE-IPF[199].

Our results have possible therapeutic implications. Targeting NLRP3, AIM2 or their ultimate effector, IL-1β and IL-18[200] may prove a novel treatment for ILD exacerbations, which are still considered lethal in most cases. Additionally, therapeutic manipulation of macrophage population has also been proposed for the management of ILDs. Therapeutic strategies that reduce the accumulation of tissue-destructive infiltrating blood monocytes might hold promise for the future.

4.3 Limitations

The main limitation of our 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[97]. 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.

Finally, although microbial burden was associated with inflammasome activation in lung fibrosis we did not associate this with disease progression, nor did we establish potential associations with specific microbiota species and the overactivation of the Inflammasome.

4.4 Future implications and directions

Several observations from the work presented in this thesis deserve further study. The main observation of this study is that mitochondrial oxidation is linked to increased NLRP3 and AIM2 activation in vitro. Although this is a very interesting finding, we did not establish the mechanism of action nor did we examined the cause of mitochondrial oxidation. It is plausible that this is the result of mitochondrial dysfunction as previously shown by our group and as such this arises in the context of defective autophagy/mitophagy and Inflammaging. However, many causes for mitochondrial oxidation exist. It is known that the activation of macrophages associates with elevation of their ROS production[201] and it is possible that in lung fibrosis macrophages are more activated that in health. Furthermore, ROS production has been clearly associated with hypoxia, which is a clinical feature of severe lung fibrosis[202, 203].

Additionally, although we showed that AIM2 and NLRP3 are overactivated in lung fibrosis, we did not provide evidence of causality with the disease pathogenesis and progression. However, evidence of causality has arisen from mice studies in bleomycin induced lung fibrosis. More specifically NLRP3 has been associated with worsening lung fibrosis in aged mice, due to mitochondrial oxidation[112]. Sadly, though the bleomycin model is fibrotic model that arise from inflammation and as such is not considered the exact replication of what happens in IPF.

Finally, it is known that different airway macrophage populations exist in the lungs especially in lung fibrosis, with different phenotypes and actions. The main characterisation of M1 and M2 macrophage phenotype seem to be out of date and a new focus has arisen towards the understanding of the role of tissue resident and monocyte derived macrophages. Moving forward from this study it would be important to identify different macrophage populations that drive the

observed aberrant inflammasome activation. We have previously found that CD163 positive AMs are increased in the lungs, a marker of alternatively activated macrophages[204]. Similarly, CD14 is a marker of monocytes. Future studies would involve isolating deferent airway macrophages by CD163 and CD14 and establishing inflammasome activation by comparison among these different categories. Therapeutic deletion of the involved macrophages population might be a therapeutic approach to be pursuit in the future.

4.5 Conclusion

Overall, the work in this thesis provides novel insights on the role of inflammasomes in the pathogenesis of lung fibrosis. The original hypothesis of this thesis was that there is increased inflammasome activation in the fibrotic lungs due to several pre-existing stimuli in the lung microenvironment, such as mitochondrial oxidation and bacterial alterations. The main findings of this thesis are that NLRP3 and AIM2 activation upon stimulation 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 in AMs.

Appendix I

List of Figures

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List of abbreviations

AE	Acute exacerbations
AECs	Alveolar epithelial cells
AIM2	Absent in melanoma 2
AMs	Alveolar macrophages
BALF	Bronchoalveolar lavage fluid
CHP	Chronic Hypersensitivity Pneumonitis
CTDs	Connective tissue diseases
DAMPs	Damage-associated molecular patterns
DLco	diffusion capacity
DPLDs	Diffuse Parenchymal Lung diseases
Ds-DNA	Double stranded DNA
FEV1	forced expiratory volume in one second
FVC	forced vital capacity
HRCT	High Resolution Computed Tomography
IFN	Interferon
IIPs	Idiopathic Interstitial Pneumonias
ILDs	Interstitial lung diseases
IMs	Interstitial macrophages
IPAF	Idiopathic pneumonia with autoimmune features
IPF	Idiopathic Pulmonary Fibrosis
LPS	Lipopolysaccharide
MDT	Multidisciplinary discussion team
MFI	Mean fluorescence intensity
MMPs	Metalloproteases

Mo-AMs	Monocyte-derived Alveolar Macrophages
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial reactive oxygen species
NLR	Nucleotide-binding oligomerization domain-like receptor
OTU	Operational taxonomic units
OXPHOS	Oxidative respiratory Phosphorylation
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral mononuclear cells
PF-ILDs	Progressive fibrotic ILDs
PFTs	Pulmonary function tests
PINK1	Phosphatase and tensin homology deleted on chromosome 10 induced putative kinase 1
qPCR	Quantitative PCRs
ROS	Reactive oxygen species
SP-C	Surfactant Protein C
TNF	Tumour necrosis factor
UIP	Usual interstitial pneumonia

Appendix II

Awards arising from this thesis

- July 2019: ERS Short-term Fellowship, "Lung microbiome and cytokine production capacity in fibrotic ILDs: A proof of concept study"

- November 2017: Research Grant awarded by Hellenic Thoracic Society for the implementation of the study "Lung Microbiome and Innate responses in Idiopathic Pulmonary Fibrosis" (€15,000)

- December 2018: Travel Award and participation at a mentorship programme in the Lung Science Conference, provided by ERS

- December 2018: Award for best abstract submitted in the area of Interstitial Lung Diseases in Hellenic Thoracic Society Conference 2018. A study to evaluate inflammasomes' activity in the peripheral blood and in alveolar macrophages in fibrotic lung diseases. Awarded by Hellenic Thoracic Society

Publications arising from this thesis

- **Trachalaki A,** TsitouraE , Mastrodimou S, Invernizzi R, Vasarmidi E, Bibaki E, Tzanakis N, Molyneaux P.L , Maher TM and Antoniou KM
  Enhanced IL-1β Release Following NLRP3 and AIM2 Inflammasome Stimulation Is Linked to mtROS in Airway Macrophages in Pulmonary Fibrosis. Frontiers in Immunology 2021

- Tsitoura E*, Vasarmidi E*, Bibaki E, **Trachalaki A,** Koutoulaki C, Papastratigakis G, Papadgiannaki S, Chalepakis G, Tzanakis N, Antoniou KM.
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  Clinical experience with antifibrotics in fibrotic hypersensitivity pneumonitis: a 3-year real-life observational study ERJ Open Res 2020

- Margaritopoulos GA*, **Trachalaki A*,** Wells AU, Vasarmidi E, Bibaki E, Papastratigakis G, Detorakis S, Tzanakis N, Antoniou KM. *equal first author
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Enhanced IL-1β Release Following NLRP3 and AIM2 Inflammasome Stimulation Is Linked to mtROS in Airway Macrophages in Pulmonary Fibrosis

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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

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