UNIVERSITAT DE BARCELONA

Integrative approach to address the heterogeneity and progression of Idiopathic Pulmonary Fibrosis: role of the immune response

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INTEGRATIVE APPROACH TO ADDRESS THE HETEROGENEITY AND PROGRESSION OF IDIOPATHIC PULMONARY FIBROSIS: ROLE OF THE IMMUNE RESPONSE

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a mi abuela,

a los míos.

«Tal vez la felicidad sea esto: no sentir que debes estar en otro lado, haciendo otra cosa, siendo alguien más».

Isaac Asimov

Table of Contents

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Abbreviations

ACTB: Actin-beta

ADI: Alveolar differentiation intermediates

ALAT: Latin America Thoracic Association

AM: Alveolar Macrophage

APC: Antigen Presenting Cell

ATS: American Thoracic Society

AT1: Alveolar type I epithelial cells

AT2: Alveolar type II epithelial cells

BAFF: B cell activation factor

BAL(F): Broncho alveolar lavage (Fluid)

BMI: Body Mass Index

Breg: Regulatory B cell

BRM: Lung-resident Memory B cell

CD: Cluster of Differentiation

CRP: C-reactive protein

CT scan: Computed Tomography scan

C#: Cluster

DC: Dendritic cell

DAMP: Damage associated molecular pattern

DEG: Differentially Express Genes

DL_{CO}: Diffusing Capacity of the Lung for Carbon Monoxide

D#: Dataset

ECM: Extracellular matrix

EMT: Epithelial-Mesenchymal Transition

ERS: European Respiratory Society

ES: Enrichment Score

FACS: Fluorescence Activated Cell Sorting

FEV_1: Forced expiratory volume in 1 second

FF: Fibroblasts foci

FVC: Forced Vital Capacity

GEO: Gene expression Omnibus

GER: Gastroesophageal reflux

GGO: Ground Glass Opacities

GO: Gene Ontology

G-SCF: Granulocyte colony-stimulating factor

GSVA: The Gene Set Variation Analysis

HLDA: Human Leukocyte Differentiation Antigen

HRCT: High-Resolution Computed Tomography

iDC: Immature Dendritic cell

IF: Immunofluorescence technique

IL-1: Interleukin-1

IL-6: Interleukin-6

IL-10: Interleukin-10

ILA: Interstitial Lung Abnormality

ILD: Interstitial Lung Disease

INFγ: interferon-gamma

IPF: Idiopathic Pulmonary Fibrosis

LAA: Low attenuation areas

LDH: lactate dehydrogenase

LT: Lung tissue

LTRC: Lung Tissue Research Consortium

mAb: Monoclonal Antibody

MMP-7: matrix metallopeptidase 7

mRNA: messenger RiboNucleic Acid

miRNA: micro RiboNucleic Acid

NAC: N-acetylcysteine

NK cell: Natural killer cell

NKT-like cell: Natural killer T cell like

NLR: Nod-like Receptor

OSA: Obstructive Sleep Apnoea

PAR: Protease-activated Receptor

PB: Peripheral blood

PD1: Programmed cell death protein-1

PDL1: Programmed death ligand-1

PRR: Pattern Recognition Receptors

qPCR: Quantitative polymerase chain reaction

Rt-PCR: Reverse transcription polymerase chain reaction

SASP: Senescence-associated secretory phenotype

Sc-RNAseq: Single Cell RNA sequencing

SP-D: surfactant protein D

Tfh: Follicular helper T cells

TGD: Gamma delta T cells

TGFß: Transforming growth factor-beta

Th1: T-helper 1

Th2: T-helper 2

Th17: T-helper 17

TLR: Toll-like Receptor

TNFα: Tumour necrosis factor alpha

Treg: Regulatory T cells

TRG: Telomere-related Gene

UIP: Usual interstitial pneumonia

Publications Report

This thesis is presented in the form of a compendium of articles with two original articles published in 2023 that constitute the central theme. In these two central articles, the doctoral candidate is the first author of the first one and the second author of the second article, both of which have been published in scientific journals in the respiratory field in the first quartile (Q1).

Specifically:

1 **Blood Immunophenotypes of Idiopathic Pulmonary Fibrosis: Relationship with Disease Severity and Progression.**

Mendoza N, Casas-Recasens S, Olvera N, Hernandez-Gonzalez F, Cruz T, Albacar N, Alsina-Restoy X, Frino-Garcia A, López-Saiz G, Robres L, Rojas M, Agustí A, Sellarés J, Faner R. Int J Mol Sci. 2023 Sep 7;24(18):13832. doi: 10.3390/ijms241813832. PMID: 37762135; PMCID: PMC10531459. Impact factor: 4.56 JCR (Q1).

The doctoral candidate is the first author of this article and has performed the following tasks: participation in experimental design, sample processing, conducting experiments, statistical analysis of raw data, interpretation of results, extraction of conclusions, writing of the article and its revisions.

2 **Lung immune signatures define two groups of end-stage IPF patients.**

Cruz T, Mendoza N, Casas-Recasens S, Noell G, Hernandez-Gonzalez F, Frino-Garcia A, Alsina-Restoy X, Molina M, Rojas M, Agustí A, Sellares J, Faner R. Respir Res. 2023 Sep 28;24(1):236. doi: 10.1186/s12931-023-02546-8. PMID: 37770891; PMCID: PMC10540496. Impact factor: 3.92 (Q1).

The doctoral candidate is the second author of this article and has performed the following tasks: participation in experimental design, statistical analysis of raw data, interpretation of results, extraction of conclusions, writing of the article, and its revisions.

Likewise, the doctoral candidate formally declares that none of the co-authors of these two articles has used these works to carry out a doctoral thesis. In addition to these two articles, during her doctorate, the doctoral candidate collaborated on a total of nine articles (listed below), three of which have been used in other doctoral theses. All this work demonstrates the involvement of the doctoral candidate with the research group and the projects that stem from it.

OTHER PUBLICATIONS AS FIRST AUTHOR

The doctoral candidate has other publications as the first author in other fields of respiratory medicine, which have not been included as central publications in the thesis manuscript. However, in all these publications, the doctoral candidate has performed the following tasks: participation in the experimental design, sample collection and processing, experiments execution, statistical analysis of raw data, interpretation of results, extraction of conclusions, writing of the article, and its revisions.

Persistence of a SARS-CoV-2 T-cell response in patients with long COVID and lung sequelae after COVID-19.

Cruz T*, Mendoza N*, Lledó GM*, Perea L, Albacar N, Agustí A, Sellares J, Sibila O, Faner R. ERJ Open Res. 2023 May 9;9(3):00020-2023. doi: 10.1183/23120541.00020-2023. PMID: 37228290; PMCID: PMC10204814. Impact factor: 4.6 (Q1).

Not used in any thesis.

Liver epigenome changes in patients with hepatopulmonary syndrome: A pilot study.

Mendoza N, Rivas E, Rodriguez-Roisin R, Garcia T, Bruguera M, Agusti A, Faner R. PLoS One. 2021 Feb25;16(2): e0245046. doi: 10.1371/journal.pone.0245046. PMID: 33630849; PMCID: PMC7906328. Impact factor: 3.56 (Q1).

Not used in any thesis.

Peripheral immune cell profiling reveals distinct immune hallmarks in progressive pulmonary fibrosis.

Hernandez-Gonzalez F*, Mendoza N*, Casas-Recasens S, Cruz T, Albacar N, López-Saiz G, Alsina X, Rojas M, Agusti A, Sellarés J, Faner R. Arch Bronconeumol. 2023 Oct;59(10):681-684. English, Spanish. doi: 10.1016/j.arbres.2023.06.009. Epub 2023 Jul 4. PMID: 37468400. Impact factor: 8.0 (Q1).

Used in Fernanda Hernández-González's thesis (year 2023).

OTHER PUBLICATIONS AS CO-AUTHOR

Epigenome-wide association studies (EWAS) of COPD and lung function: A Systematic Review.

Sandra Casas-Recasens*, Raisa Cassim*, Núria Mendoza, Alvar Agusti, Haydn Walters, Caroline Lodge, David Martino, Shyamali Dharmage‡, Rosa Faner‡. Am J Respir Crit Care Med. 2024 Feb 29. doi: 10.1164/rccm.202302-0231OC. Epub ahead of print. PMID: 38422471. Impact factor: 24 (D1).

SARS-Cov-2 T-cell response in COVID-19 convalescent patients with and without SEQUELAE.

Tamara Cruz, Núria Mendoza, Lidia Perea, Núria Albacar, Azucena Gonzalez, Fernanda Hernandez-Gonzalez, Manel Juan, Alvar Agustí, Jacobo Sellares, Oriol Sibila, Rosa Faner. ERJ Open Research. ERJ open research, 8(1), 00706-2021. https://doi.org/10.1183/23120541.00706-2021. Impact factor: 2.98 (Q2).

Not used in any thesis.

Elevated plasma levels of epithelial and endothelial cell markers in COVID-19 survivors with reduced lung diffusing capacity six months after hospital discharge.

Oriol Sibilia, Lidia Perea, Núria Albacar, Jorge Moises, Tamara Cruz, Núria Mendoza, Belen Solarat, Gemma Lledó, Gerard Espinosa, Joan Albert Barberà, Joan Ramon Badia, Alvar Agustí, Jacobo Sellarés and Rosa Faner. Respir Res 23, 37 (2022). https://doi.org/10.1186/s12931-022-01955-5. Impact factor: 4.92(Q1).

Not used in any thesis.

Molecular Interactions of SARS-CoV-2 in Lung Tissue of Patients with Chronic Obstructive Pulmonary Disease.

Agusti A, Sibila O, Casas-Recasens S, Mendoza N, Perea L, Lopez-Giraldo A, Faner R. Ann Am Thorac Soc. 2021 Nov;18(11):1922-1924. doi: 10.1513/AnnalsATS.202006-619RL.PMID: 33950792; PMCID: PMC8641835. Impact factor: 6.831(Q1).

Not used in any thesis.

Telomere Length but Not Mitochondrial DNA Copy Number Is Altered in Both Young and Old COPD.

Casas-Recasens S, Mendoza N, López-Giraldo A, Garcia T, Cosio BG, Pascual-Guardia S, Acosta-Castro A, Borras-Santos A, Gea J, Garrabou G, Agusti A, Faner R. Front Med (Lausanne).2021 Nov 24;8:761767. doi: 10.3389/fmed.2021.761767. PMID: 34901077; PMCID: PMC8652089. Impact factor: 5.093 (Q1).

Used in Sandra Casas-Recasens' (year 2022).

Lung DNA Methylation in Chronic Obstructive Pulmonary Disease: Relationship with Smoking Status and Airflow Limitation Severity.

Casas-Recasens S, Noell G, Mendoza N, Lopez-Giraldo A, Garcia T, Guirao A, Agusti A, Faner R. Am J Respir Crit Care Med. 2021 Jan 1;203(1):129-134. doi:10.1164/rccm.201912-2420LE. PMID: 32822219 Impact factor: 17.45 (D1).

Used in Sandra Casas-Recasens' (year 2022).

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Resumen de la Tesis

Introducción: La fibrosis pulmonar idiopática (FPI) es una enfermedad pulmonar progresiva de etiología desconocida. Se cree que las células epiteliales pulmonares dañadas secretan mediadores profibróticos que conducen al depósito de matriz extracelular, destrucción pulmonar y reclutamiento de células inflamatorias. Sin embargo, en este contexto, el papel de la respuesta inmunitaria en la patogénesis y la progresión de la FPI sigue siendo controvertido.

Hipótesis y objetivos: La investigación de las poblaciones de células inmunitarias pulmonares y de sangre periférica en relación con diferentes fenotipos/endotipos puede aportar nuevos conocimientos sobre la patobiología y la progresión de la FPI. Los objetivos específicos son: 1) caracterizar el perfil de células inmunitarias periféricas y su asociación con la progresión de la FPI, y 2) explorar el nivel de infiltrado inmunitario en el tejido pulmonar y su asociación con la heterogeneidad en FPI.

Métodos: Se determinó mediante citometría de flujo en sangre total el perfil inmunológico en el momento del diagnóstico en 32 pacientes con FPI y en 32 controles sanos emparejados por edad y tabaquismo. 31 pacientes con FPI fueron sometidos a seguimiento durante un año y clasificados como *estables* o *progresivos* en función del deterioro de la función pulmonar y/o la muerte. A los 18-60 meses, se volvieron a caracterizar los inmunofenotipos. En relación con el segundo objetivo, se evaluaron las firmas inmunitarias con el *Gene Set Variation Analysis* (GSVA) y se aplicaron al transcriptoma pulmonar de 109 pacientes con FPI del *Lung Tissue Research Consortium* (LTRC), seguido de un análisis de *clustering* no sesgado del enriquecimiento inmunitario del GSVA. Los resultados se validaron experimentalmente en tejido pulmonar de 26 pacientes con FPI de la Universidad de Pittsburgh. Por último, mediante análisis de expresión génica diferencial se exploraron las diferencias no inmunitarias entre los *clusters*.

Resultados: Los pacientes con FPI mostraron alteraciones en el perfil inmunitario periférico en el momento del diagnóstico, en comparación con los controles, que se asociaron a anomalías en la función pulmonar. Los pacientes con FPI progresiva, pese al tratamiento antifibrótico, presentaban un inmunofenotipo "sobreactivado" y "agotado" en el diagnóstico, que se mantenía con el tiempo. En relación al segundo objetivo, se identificaron dos *clusters* de pacientes con FPI: C#1 (n=58) enriquecido en firmas inmunes (células T citotóxicas y de memoria), en comparación con C#2 (n=51). Estos resultados se validaron mediante citometría de flujo con una generación de *clusters* similar. A nivel de expresión génica, se identificaron diferencias en los genes de células ciliadas, epiteliales y secretoras, mostrando una correlación inversa con las firmas de respuesta inmunitaria. Curiosamente, ambos grupos mostraron características clínicas similares..

Conclusiones: Los pacientes con FPI mostraron alteraciones significativas en el perfil inmunológico periférico, especialmente aquellos con progresión de la FPI que presentaban un compartimento de células T $CD8^+$ desregulado, y una relación CD4/CD8 invertida. En el tejido pulmonar de pacientes con FPI terminal, se identificaron dos grupos con niveles muy diferentes de firmas inmunes y expresión génica, a pesar de mostrar características clínicas similares. Sin embargo, se desconoce si estos grupos inmunitarios pueden diferenciar diversas trayectorias de la enfermedad.

Thesis Summary

Background: Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease with unknown aetiology. It is believed that abnormally activated and/or damaged lung epithelial cells secrete a panel of profibrotic mediators that leads to extracellular matrix deposition, destruction of the lung architecture and recruitment of inflammatory cells. However, in this setting, the role of the immune response in the pathogenesis and progression of IPF remains controversial.

Hypothesis and Objectives: The investigation of peripheral blood and lung immune cell populations in relation to different phenotypes (and endotypes) may provide new insights into IPF pathobiology and progression. The two specific aims are: 1) to characterize the peripheral immune cell profile and its association with IPF progression, and 2) to explore the level of immune infiltrate in lung tissue and its association with IPF heterogeneity.

Methods: Whole blood immunological cell profile was determined by flow cytometry at diagnosis in 32 IPF patients, and 32 age- and smoking-matched healthy controls. Thirty-one IPF patients were followed up for one year and categorized as stable or progressors based on lung function deterioration and/or death. At 18–60 months, immunophenotypes were characterized again. Regarding aim two, immune signatures were assessed with Gene Set Variation Analysis (GSVA) and applied to the lung transcriptome of 109 IPF patients from the Lung Tissue Research Consortium (LTRC), followed by unbiased cluster analysis of GSVA immune-enrichment scores. Results were experimentally validated using flow cytometry analysis in the lung tissue of 26 IPF patients from the University of Pittsburgh. Finally, differential gene expression and hypergeometric test were used to explore non-immune differences between clusters.

Results: IPF patients showed alterations in the peripheral immune cell profile at diagnosis, compared to healthy controls, that were associated with lung function abnormalities. Patients with progressive IPF, despite antifibrotic therapy, presented an over-activated and exhausted immunophenotype at diagnosis, which was maintained over time. For the second aim, two clusters (C#1 and C#2) of IPF patients were identified: C#1 (n=58) presented an enrichment in GSVA immune signatures (mainly cytotoxic and memory T cells signatures), compared to C#2 (n=51). Those results were validated by flow cytometry with similar unbiased clustering generation. Differential gene expression between clusters identified differences in cilium, epithelial and secretory cell genes, showing an inverse correlation with the immune response signatures. Interestingly, both clusters showed similar clinical features.

Conclusions: Patients with IPF showed significant alterations in the peripheral blood immunological profile at diagnosis, especially those with IPF progression who presented a dysregulated T $CD8^+$ cells compartment, and an inverted CD4/CD8 ratio suggesting an over-activated, aged, and "exhausted" immune status potentially related to intracellular antigens. Moreover, two clusters of patients with very different levels of immune signatures and gene expression were identified in end-stage IPF lung tissue, despite showing similar clinical characteristics. However, whether these immune clusters can differentiate diverse disease trajectories remains unexplored.

Introduction

01

1.1. LUNG DEFENCE AND PRESERVATION

The lung architecture contains approximately 300 million alveoli, which fulfil about 20,000 breaths/day (1). Just because lungs are specialized in gas exchange, which takes place across a basement membrane that is only 0.3-0.5 µm thick, they represent one of the largest epithelial surfaces that are constantly in direct contact with the external environment. This large mucosa is a primary target for a wide variety of potential airborne pathogens, allergens, toxins and hazardous compounds, and accordingly, to preserve lung integrity, the immune system plays a crucial role. The respiratory mucosal immune system comprises a complex network of both circulating and nonrecirculating (resident) innate and adaptive immune cells, which orchestrate a delicate balance between immune defence, resolution, and tolerance to ensure lung health (1, 2).

The immune system has been categorized into two main compartments: 1) innate immunity, characterized by being an immediate defence but unspecific for a given antigen; and 2) adaptive immunity, characterized by its antigen specificity but a later response. In the lungs, both immunities are crucial and operate collaboratively, forming a dynamic defence network that protects the lungs against the assault of any pathogen (airborne bacteria, viruses, fungi), or foreign substances that can be a potential threat (3). The key players involved in lung defence will be briefly described below.

1.1.1. The first innate line of defence: The Airway Epithelium

The lung epithelium is the initial site of contact for all inspired substances which acts as both a physical and an immunological barrier. This mucosal surface is mainly composed of ciliated cells, mucous-producing cells and undifferentiated basal cells, and forms the interface between the lumen and the parenchyma from the nasal passage to the alveoli (4). Thus, all along the lung architecture, the mucociliary apparatus together with secreted antimicrobial substances, including enzymes and protease inhibitors, and tight junctions, forms de physical barrier that prevents the entrance of inhaled toxins, pathogens or any potentially harmful particles into the subepithelial tissue.

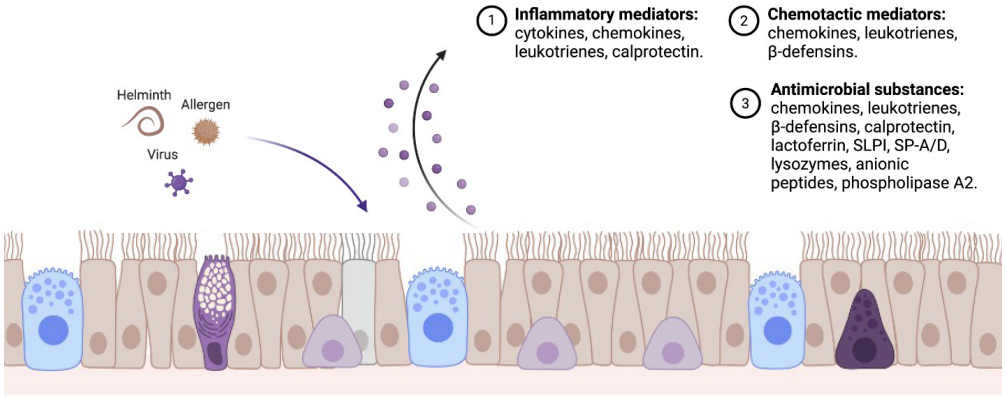

Figure 1. The role of the airway epithelium in the host's defence against infection: Overview of secreted molecules that play a role in inflammation and the host's defence. SLPI: Secretory Leukocyte protease inhibitor; SP: Surfactant protein. Adapted from R. Bals, et al. 2004 (5).

Epithelial cells have an active role in the innate defence, sensing pathogens via a variety of receptors, which include several families of pattern recognition receptors (PRR), such as Toll-like receptors (TLR), protease-activated receptors (PAR), Nod-like receptors (NLR), etc. The epithelium, in coordination with other innate immune cells described below, is the first line of defence coordinated by several components such as lysozymes, secretory leukoprotease inhibitors, proinflammatory cytokines, chemokines, antimicrobial secretory IgAs (sIgA) and defensins, among others (6).

The lung epithelium is key to maintaining homeostasis in the lung and therefore any disruption that may compromise the integrity of the epithelial barrier can affect the interaction of cytokines and growth factors, modulating the immunological response, and leading to the development of acute/chronic inflammation and respiratory diseases with the generation of DAMPs (Damage associated molecular patterns) (7, 8).

1.1.2. Innate immune cells

In the lungs, the innate immune system is mainly provided at the cellular level by resident cell populations, such as macrophages, innate lymphoid cells (ILCs) and dendritic cells (DCs), but also recruited cells, such as neutrophils, and monocytes, which respond rapidly to any inhaled materials.

Neutrophils are the most abundant subset of leukocytes in the blood circulation in search of potential injury stimuli, including pathogen- and damage-associated molecular pattern molecules (PAMPs and DAMPs), chemokines and cytokines, which will induce their rapid recruitment to the damaged site to fulfil their main role of pathogen killing and clearance (9). To do so, neutrophils undergo phenotypic and functional changes, leading to distinct fates that will orchestrate a complex network, which can be observed in blood and lung tissue under both homeostatic and pathological conditions. This phenotypic heterogeneity and high plasticity leads to different impacts on the innate and adaptive immune system, showing both pro- and anti-inflammatory actions (10).

Alveolar macrophages (AM), as its name claims, are mainly located in the alveolar space representing the predominant phagocytic and APC in the human respiratory tract. Under healthy conditions, alveolar macrophages are the most abundant cell type in BAL, while under acute or chronic inflammatory conditions, neutrophils or lymphocytes shift the ratio (10). AMs have shown to be very heterogeneous depending on the pulmonary compartment and present a high phenotypic, metabolic and functional plasticity (11), which involves the secretion of microbial-derived signals and both pro- or anti-inflammatory mediators (mainly IL-1, IL-6, IL-10 and TNFα) into the alveolar space, and crosstalk with the alveolar epithelium to induce immunosuppressive/-modulatory signals (12).

Innate lymphoid cells (ILCs) generally reside in mucosal surfaces and have a key role in both tissue homeostasis and anti-pathogenic functions (13). The most prototypical member of this family is the natural killer (NK) cell. NK cells can promote host defence and discern healthy cells from infected, senescent or tumorigenic cells, and cytotoxically eliminate the later ones via perforin and granzyme B production, independently of previous antigenic exposure (14). Previous studies have revealed different subpopulations of NK cells with diverse functions and characteristics, mostly determined by their local microenvironment. Here, two distinct phenotypes have been described: the cytotoxic $CD56^{dim}CD16^{+}$, or circulating NK cells, and

the proinflammatory $CD56^{bright}CD16^{-}$, also known as tissue-resident NK cells (15). Thus, in homeostasis, (lung) tissue-resident NK cells are hypofunctional and their cytotoxicity and IFN-γ production levels are lower than those of circulating NK cells (16).

The induction of an adaptive immune response begins when a potential pathogen is detected and ingested by an immature dendritic cell (iDC). On activation, the dendritic cells mature into antigen-presenting cells (APC) that will not only present the antigens to the pathogen-specific T lymphocytes but also secrete a variety of cytokines that will influence how the innate and adaptive immune systems are going to respond to that specific pathogen (17).

1.1.3. Adaptive immune cells

The adaptive immune system in the lungs mainly consists of cellular components including B and T lymphocytes. B cells are essential mediators of humoral immune responses in the airways through the production of antibodies and cytokines secretion (17). However, a subset of B cells characterized by their immunosuppressive role, called regulatory B cells (Bregs), are also critical for the maintenance of immune homeostasis in the lung (18). In addition, another subset of B cells, called lung-resident memory B cells (BRM cells) (very different in phenotype from the circulating memory B cells) have been demonstrated to have a key role in the immunity against viruses (19).

The lung was also found to have prominent memory T cell populations, which were first considered a migratory population from lymphoid organs. However, this population of lung T cells are known to be noncirculating tissue-resident memory T cells (TRMs). They are primed and activated and survive as memory T cells following pathogen clearance. Naïve T cells recognize antigens presented by DCs in the lymph nodes and recirculate to the lung. T cells are fully activated after receiving three signals: first signal TCR (specificity); second signal coestimulation CD28 and integrins such as LFA-1; and third signal, microenvironment, cytokines and chemokines induced by APCs and innate immune cells. These signals are going to induce different transcription factors (T-bet, GATA-3, RORg, Bcl-6, FoxP3) that in turn will drive the T cells effector linage expansion and differentiation (Th1, Th2, Th17 and Tregs). The differentiation of T cells into specific subtypes depends on both the specific antigen that triggered the response and the cytokines produced. For instance, Th0 cells undergo differentiation into Th2 when exposed to IL-5, particularly in response to large extracellular antigens, such as helminths. Thus, T cell lineage is delineated at the molecular level by the cell type-specific transcription factor network and epigenetic landscape (e.g. DNA methylation and histone modifications), both driving the main plasticity of the Th system.

On the contrary, lymphocytes that fail to receive any of these survival signals, undergo programmed cell death (apoptosis) or, in some cases, anergy (20) (tolerance mechanism in which the lymphocyte is in a hyporesponsive state after an antigen encounter). Note that in the T cell activation both programs, activation and inhibition are triggered at the same time by the antigen identification. It is the balance between the activatory and inhibitory signals that determine the final state.

In this line, altered immune cell phenotypes and/or abnormal interplay among the variety of cells conforming the lung immunity can lead to either a deficient or excessive immune response, that associates to lung disorders. In the former scenario, the immune system

may fail to mount an effective response against pathogens, leading to immunodeficiency and increased susceptibility to chronic infections. Whereas, the latter will lead to chronic inflammation and subsequent tissue damage within the lungs, which will contribute to the development and progression of diseases such as chronic obstructive pulmonary disease (COPD), asthma or interstitial lung diseases (i.e. Idiopathic Pulmonary Fibrosis (IPF)).

1.2. INTERSTITIAL LUNG DISEASES

To date, according to the American Lung Foundation, over 200 interstitial lung diseases (ILDs), from very rare to *relatively common*, are recognized worldwide. Most interstitial lung diseases are characterised by inflammation and/or fibrosis within the interstitial space, which change the lung structure, being gas exchange impairment the major consequence, which results in breathlessness, lower exercise tolerance, and decreased quality of life (21). In most cases, an accurate and early diagnosis can be challenging and the disease progression difficult to determine. The possible outcomes may vary considerably for each of the different interstitial lung diseases: in some, spontaneous reversibility or stabilization can occur, however, unfortunately, in most cases, especially in those with a progressive-fibrosing phenotype, such as in Idiopathic Pulmonary Fibrosis (IPF), respiratory failure and death are the most frequent outcomes (21).

1.3. IDIOPATHIC PULMONARY FIBROSIS

Idiopathic pulmonary fibrosis is a chronic progressive fibrosing interstitial lung disease of unknown origin that is associated with a radiological and histologic pattern of usual interstitial pneumonia (UIP) and it is mainly characterized by the accumulation of extracellular matrix (scar tissue) in the lung resulting in changes in the architecture of the parenchyma (22, 23). The incidence and prevalence of IPF are variable worldwide and present a high country heterogeneity, however, they have been reported as ranging from 0.09-1.30 and 0.33-4.51 per 10,000 persons, respectively (24, 25).

IPF is a disease associated with ageing, as it is generally diagnosed in 50-70-year-old adults, and it is clinically characterized by progressive worsening of dyspnoea and lung function, with a poor prognosis: without treatment, the average life expectancy is 3–5 years after diagnosis (23, 24). The survival time decreases with increasing age, male gender and the presence of comorbidities (i.e. ischaemic heart disease, COPD, lung cancer) (26, 27). However, survival has improved during the last two decades, up to 7-8 years, due to earlier diagnosis and improved treatment, including the reduction of immunosuppressive medications and the use of antifibrotic therapy. Nevertheless, even under treatment, the patient's course and the disease progression are often unpredictable and heterogeneous: some patients may remain stable for years, while other patients suffer from rapid clinical deterioration (28, 29).

Even though IPF is considered a rare disease, the physical, psychological, and socio-economic burden of IPF is currently a concern, and with the population ageing worldwide, the impact of IPF on patients and healthcare systems is expected to continue rising (25).

1.3.1. Risk factors in IPF

The research developed in the last years has identified different risk factors associated with the development of IPF, and other progressing fibrotic ILDs (see Figure 3). Some of them may independently increase susceptibility for IPF, act synergistically to contribute to an increased risk for disease development, or simply be an epiphenomenon of the disease (30). Including these risk factors in questionnaires within the diagnosis and management algorithm may help to define new phenotypes and endotypes of this disease and redirect towards a more personalized medicine (precision medicine) (31).

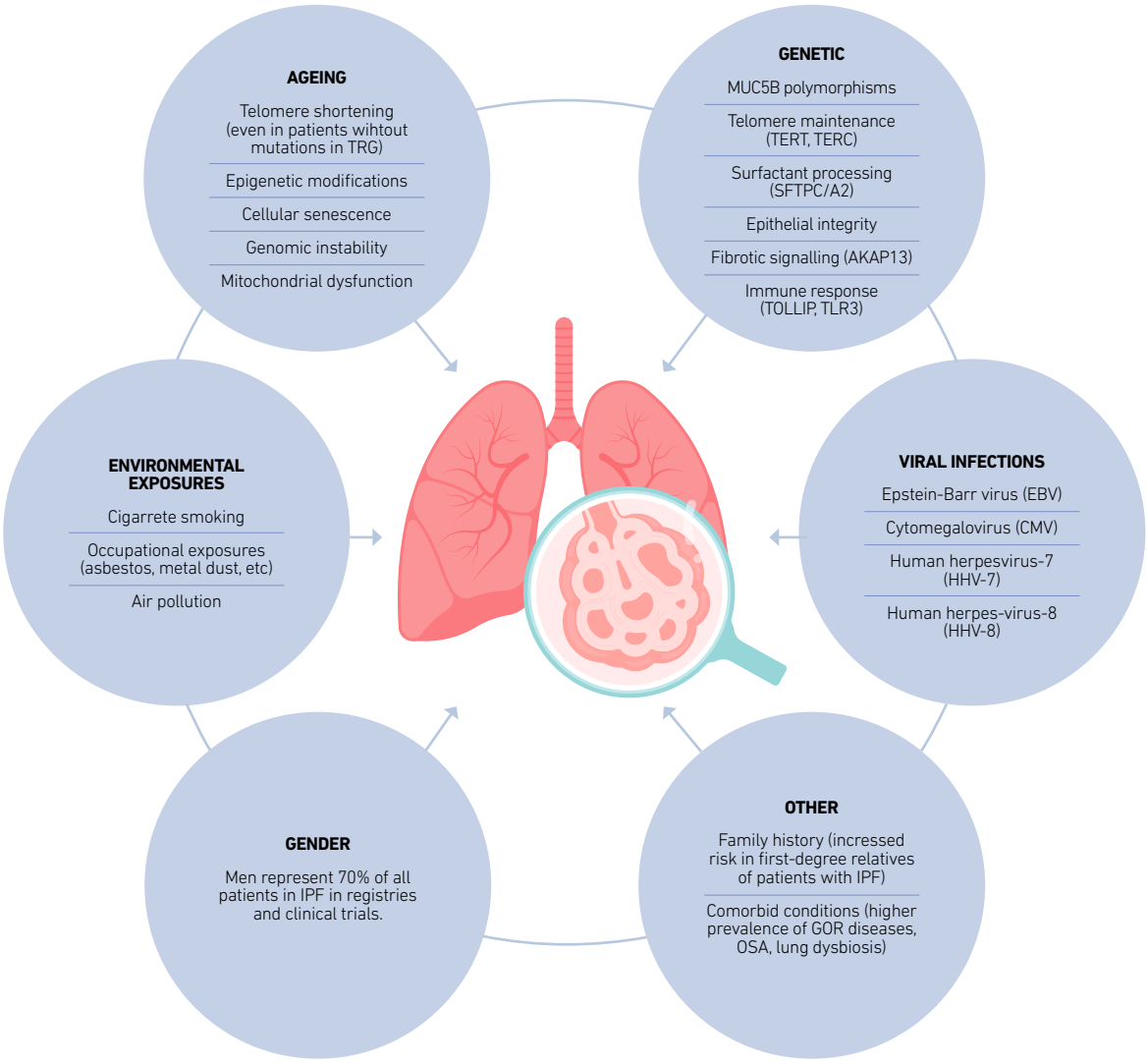

Figure 2. Summary of the risk factors for IPF. Adapted from Podolanczuk AJ et al. 2023 (32). TRG: Telomere-related genes; OSA: Obstructive Sleep Apnoea; GOR: gastro-oesophageal reflux.

1.3.1.1. Ageing

As previously mentioned, the incidence and prevalence of IPF increase with age and accordingly, cellular ageing and senescence are key factors. In this setting, accelerated ageing markers (i.e. loss of proteostasis, epigenetic alterations, mitochondrial dysfunction, genomic instability, telomere attrition), also known as hallmarks of ageing have been related to IPF (33). Of special relevance are telomere attrition and epigenetic modifications.

1.3.1.1.1. Telomere shortening

Telomeres protect chromosome ends from replicative shortening (i.e. loss) via a telomerase-dependent repeat expansion mechanism. They are very susceptible to age-related deterioration, and their premature exhaustion has been related to the development of several diseases (34). In this setting, telomere shortening has been reported in fibrotic areas, compared to non-fibrotic areas in IPF lungs. Also, shortened telomeres in peripheral blood leukocytes and lung tissue, compared to controls, have been reported even in those IPF patients without mutations in telomere-related genes (TRG), such as the telomerase reverse transcriptase (TERT) or telomerase RNA component (TERC), among others, that affect telomere homoeostasis (35). In addition to abnormal telomere shortening, the molecular basis underlying the susceptibility of the ageing lung towards a dysregulated response to repetitive lung microinjuries includes a senescent phenotype in both IPF lung fibroblasts and immune cell infiltrate, that may disrupt de balance between cell proliferation, wound healing and cell clearance, and thus perpetuate tissue remodelling and chronic inflammation (30, 36).

1.3.1.1.2. Epigenetic modifications

Epigenetics refers to "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence". The epigenetic modifications described in current literature generally comprise histone variants, posttranslational modifications of amino acids on the amino-terminal tail of histones, and covalent modifications of DNA bases (addition of methyl groups also known as DNA methylation) (37).

DNA methylation triggers conformational chromatin changes leading to different expression levels of genes, determining key biological processes/cell states. In gene promoters, hypomethylation should result in increased expression of the related genes, whereas hypermethylation results in a decrease in expression levels (38). These epigenetic changes can be influenced by environmental factors such as smoking, pollution and occupational exposures, and they are also a hallmark of ageing (39). In the context of IPF, researchers have been exploring the role of epigenetic changes in contributing to the development and progression of the disease (40). Aberrant DNA methylation and histone modification patterns have been observed in lung tissue from IPF patients affecting the expression of genes involved in the fibrotic process, modulation of immune cells and inflammation, and tissue repair (41).

Gene expression can also be controlled by microRNAs (miRNAs), which are non-coding RNAs composed of no more than 17-25 nucleotides that induce mRNA degradation and, thus, gene silencing. Several miRNAs have been related to pro- and antifibrotic

processes, and seen to be differently up- and down-regulated during different stages of IPF pathogenesis (40). Some of them, identified in IPF tissue and/or fibroblasts included members of let-7 family, miR-21, miR-9, miR-26a, miR-29a and miR-30 family, among others (42).

1.3.1.2. Environmental exposures

Determining the relevant exposures related to pulmonary fibrosis has been challenging; its unknown "pre-diagnosis" period before disease manifestation together with its diagnosis in elderly adults after a lifetime of mixed exposures, have made it difficult for both clinicians and researchers to isolate potential causes or risk factors. Nowadays, we know that repetitive inhalational exposures to noxious fumes and pollutants (in both countryside and urban areas), in and outside homes or workplaces, create a cycle of lung injury and tissue repair that may contribute to lung remodelling and fibrosis (43).

In this setting, long-term domestic, environmental and occupational exposures to pollution, toxins or hazardous materials, such as asbestos fibres, silica, coal or hard metal dust, and breathing in bird or animal droppings, among others, have been related to lung damage. In addition, radiation treatments for lung or breast cancer, or certain types of medications (chemotherapy, antibiotics or anti-inflammatory drugs) can also lead to pulmonary fibrosis.

A history of tobacco smoking, whether current or former, has also been overrepresented in IPF. The act of smoking has been linked to repetitive micro-injuries in the alveolar compartment, acceleration of telomere shortening and methylation changes leading to the overexpression of genes related to epithelial to mesenchymal transition and fibroblast-like phenotype (44). Several evidence also have highlighted the importance of ambient air pollution in both the incidence and progression of IPF. Pollutants not only conclusively cause epithelial damage, oxidative stress and airway inflammation but have also been observed to induce epigenetic changes in the lung (43). These alterations not only contribute to the existing damage but also intensify the pathogenicity of co-exposure to other antigens (45).

1.3.1.3. Gender

Even though, across the world, IPF is more prevalent in men (70% of the global cases), the exact foundation behind this gender bias remains unclear and controversial. Initially, the increased risk among men was mainly attributed to occupational and environmental exposures that were traditionally more common among men, such as tobacco smoking (46). However, the fact that nowadays these historical/social differences among men and women are fading away, and several recent sex-stratified studies have demonstrated that men are more likely to develop IPF than women, indicates that there is indeed an interaction between gender and IPF (47). In this scenario, sex hormones have been hypothesized to have a role in the gender-biased prevalence of IPF, where, in animal models, female hormones seem to have a protective role against pulmonary fibrosis (48). However, more human-based studies are needed to disentangle the interplay of gonadal hormones in gender-based differences in IPF disease development and prognosis.

1.3.1.4. Genetics

The role of genetic factors in determining susceptibility to IPF is well established and has been estimated to contribute to at least one-third of all IPF. Several studies have demonstrated that both common (polymorphisms), and rare or ultra-rare (mutations) genetic variants are important determinants of IPF risk. Genome-wide association studies (GWAS) have implicated more than 15 common genetic variants as risk factors for IPF (49). One of the most well-described is the variation in the *MUC5B* gene, which results in high mucus production in the respiratory bronchioles (50, 51). Others are associated with mutations in surfactant proteins (*SFTPA1*, *SFTPA2*, *SFTPC*), genes that maintain telomere integrity (*TERT, TERC*), as previously mentioned, genes related to host defence (TOLLIP), cell-to-cell adhesion (*DSP* and *DPP9*), and fibrotic signalling pathways (*AKAP13*) (52-54). Interestingly, these variants have been related to both sporadic and familial forms of IPF.

1.3.1.5. Viral Infections

Chronic viral infections have extensively been proposed as exacerbating agents or initiators of IPF by which the alveolar epithelial cells might undergo repetitive injuries and dysregulated immune and repair responses, that lead to the production of fibrotic factors. Here, the members of the Herpesviridae family are the most found in the evaluation of lung tissue and serum from patients with IPF compared to controls: Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus-7 (HHV-7) and human herpes-virus-8 (HHV-8). Moreover, in their meta-analysis study, Sheng et. al showed that the presence of persistent or chronic viral infections caused by the formerly mentioned viruses significantly increased the risk of developing IPF (55).

1.3.2. Natural History

IPF is considered the archetype of fibrosing interstitial lung disease with a progressive phenotype, which is mainly characterized by a decline in lung function, worsening of respiratory symptoms and eventual death, generally due to respiratory failure or any associated comorbidities. Even though is progressive behaviour, the patient's clinical course of such progression is heterogeneous and, in most cases, unpredictable (56, 57) (see Figure 3).

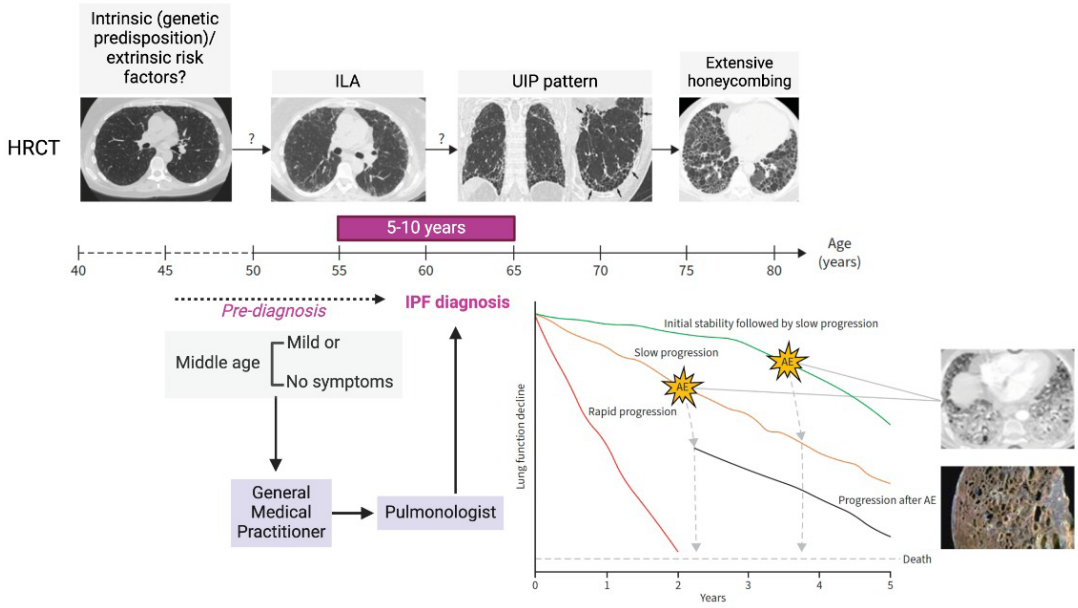

Figure 3. Proposed natural history of IPF. Adapted from Podolanczuk et al. 2023 (32). HRCT: High-resolution computed tomography; ILA: Interstitial lung abnormalities; UIP: usual interstitial pneumonia; IPF: Idiopathic pulmonary fibrosis; AE: Acute exacerbation. Of note that, although all patients start at their maximum lung function capacity, this is not 100% of all cases; some patients may have never reached their maximum lung function capacity (58). Here, understanding the different trajectories may help disentangle the heterogeneity in the disease progression and thus identify those patients with worse outcomes and new therapeutical targets.

Patients may have evidence of ILAs (radiologic abnormalities found incidentally on chest CT that are potentially related to interstitial lung diseases (59)) on HRCT several years before the diagnosis of IPF can be ascertained per the 2018 IPF guidelines (of note that not all ILAs will progress to IPF). During the years preceding the time of diagnosis of IPF, patients may be asymptomatic or have mild symptoms, which are often managed by a general medical practitioner. Here, the lack of specificity of those symptoms often leads to a substantial delay in the diagnosis. Once there is an ascertained diagnosis of IPF, patients generally follow one of three courses: 1) slow progression: slow decline over 3–5 years since the diagnosis (Figure 3, orange line); 2) rapid progression: a rapid decline in lung function over several months (Figure 3, red line); and 3) stable: others remain stable over several years before progressing (Figure 3, green line). In recent years, the rate of annual decline in forced vital capacity (FVC) has been slowed down in most patients treated with antifibrotics. However, some patients experience a rapid progression despite the use of antifibrotics.

In addition, acute exacerbations (AE) can occur at any time and may lead to accelerated loss of lung function or death (Figure 3, black line). Available data suggest that up to 46% of deaths in IPF are preceded by an acute exacerbation, and if not, the median survival of patients who experienced one is between 3-4 months (60-62). AE has been defined as an acute, clinically significant respiratory deterioration of unidentifiable cause (no alternative aetiology), mainly characterized by evidence of new widespread alveolar abnormality. AEs are more common in those patients with an advanced stage of the disease: low FVC, low DL_{CO}, low 6-minute walk distance (6MWD), increased dyspnoea or poor baseline oxygenation (63).

To date, there are no proven effective therapies for acute exacerbations, and the current care used to manage them remains controversial. Many patients are treated with systemic corticosteroids, however, still no clear evidence to support this approach, and even international guidelines on the management of IPF make a weak recommendation for their use. Current management leans on supportive care and supplemental oxygen (63, 64).

1.3.3. Diagnosis of IPF

Diagnosing IPF has been particularly challenging due to its non-straightforward nature: being more of a dynamic exclusion algorithm rather than a direct and clear-cut approach (see Figure 4). This comprehensive diagnostic process has required the continuous revision and update of the international guidelines. The diagnosis algorithm was updated in the most recent American Thoracic Society/European Respiratory Society/Latin America Thoracic Association/Japanese Respiratory Society (ATS/ERS/ALAT/JRS) guideline published in 2022, but it was first described in detail in the 2018 guideline (23, 65).

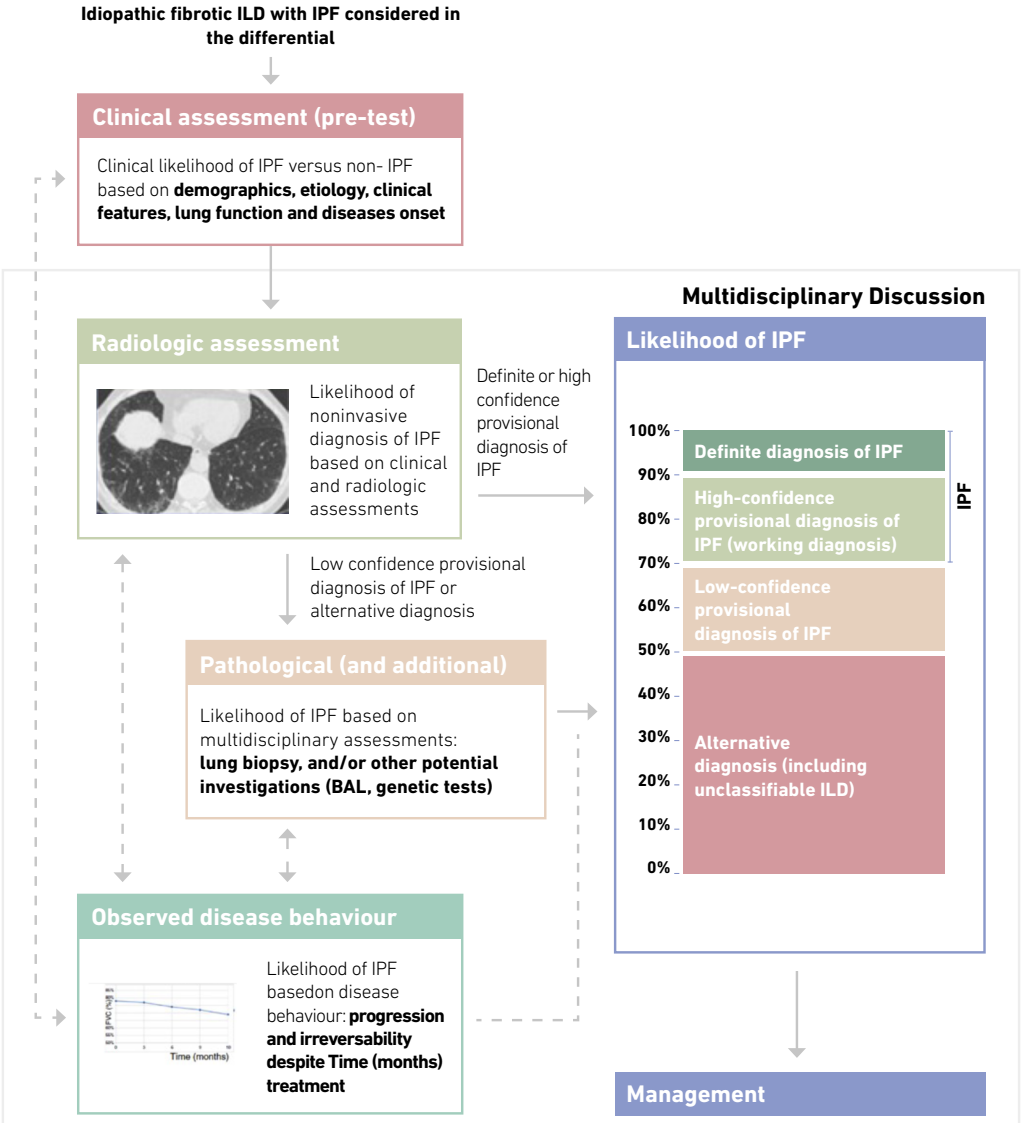

Figure 4. Summary of the steps of the diagnosis algorithm for IPF. ILD: Interstitial lung disease; IPF: idiopathic pulmonary fibrosis; BAL: Broncho-alveolar lavage; FVC: Forced vital capacity. Adapted from Cottin et al. 2022 (66).

First, the diagnosis of IPF requires the exclusion of other systemic conditions or exposures that are known to cause ILDs, including connective tissue disease, the use of certain medications, and/or exposure to environmental factors at work or home; as well as the exclusion of other ILD diagnosis. To do so, the guidelines suggest a thorough physical exam, serological testing, and general screening for autoimmunity and inflammatory markers, as well as a detailed (family) medical history record (23).

Thus, all patients suspected of IPF should undergo HRCT using the technical parameters established for image acquisition and reconstruction. A definite or high-confidence diagnosis of IPF can be established when the HRCT shows the presence of a UIP pattern. Here, the guidelines specify that lung fibrosis is recognized with the presence of traction bronchiectasis/bronchiolectasis (abnormal dilation of the bronchi/bronchioles) and/or honeycombing. Honeycombing is defined by clustered, thick-walled, cystic spaces of similar diameters, developed after the collapse of fibrotic alveolar septa and dilatation of terminal airways. Recent observations have shown that the remodelling process in IPF appears to be a continuum from traction bronchiectasis to honeycombing (23).

When HRCT is indeterminate for usual interstitial pneumonia (UIP) or suggestive of an alternative diagnosis, further diagnostic process may include cellular analysis of bronchoalveolar lavage fluid (BALF) or lung biopsy/cryo-biopsy, which should be only performed in patients with an acceptable level of risk for complications/exacerbations, and in case the findings are expected to affect the management of the disease (23).

Given the clinical, radiological and, in some cases, histological overlap between IPF and other progressive fibrotic ILDs, a definitive confirmation of IPF diagnosis cannot always be accomplished. That is why, the later algorithm includes levels of diagnostic certainty in the diagnosis of IPF that will guide further decision-making and management of the disease (see Figure 4) (66). The updated guidelines also highlight the importance of early detection and diagnosis re-evaluation over time (progression) (65). However, still, the applicability of some current diagnosis criteria has proved to be challenging in some cases: 10% of patients presenting a UIP pattern on HRCT scan cannot undergo surgical lung biopsy to complete the diagnosis due to age, advanced disease or poor clinical conditions. This reality leads to the need for more biomarkers to include in the diagnostic algorithm to facilitate early detection and/or intervention and also to predict the progression of the disease.

1.3.4. Disease Management and Treatment in IPF

Although the standard of care (SoC) for the treatment of IPF has evolved these past years, still no treatment that can cure IPF is available. Thus, the management is still focused on ameliorating symptoms, preserving lung function and improving the quality of life of these patients (see Figure 5).

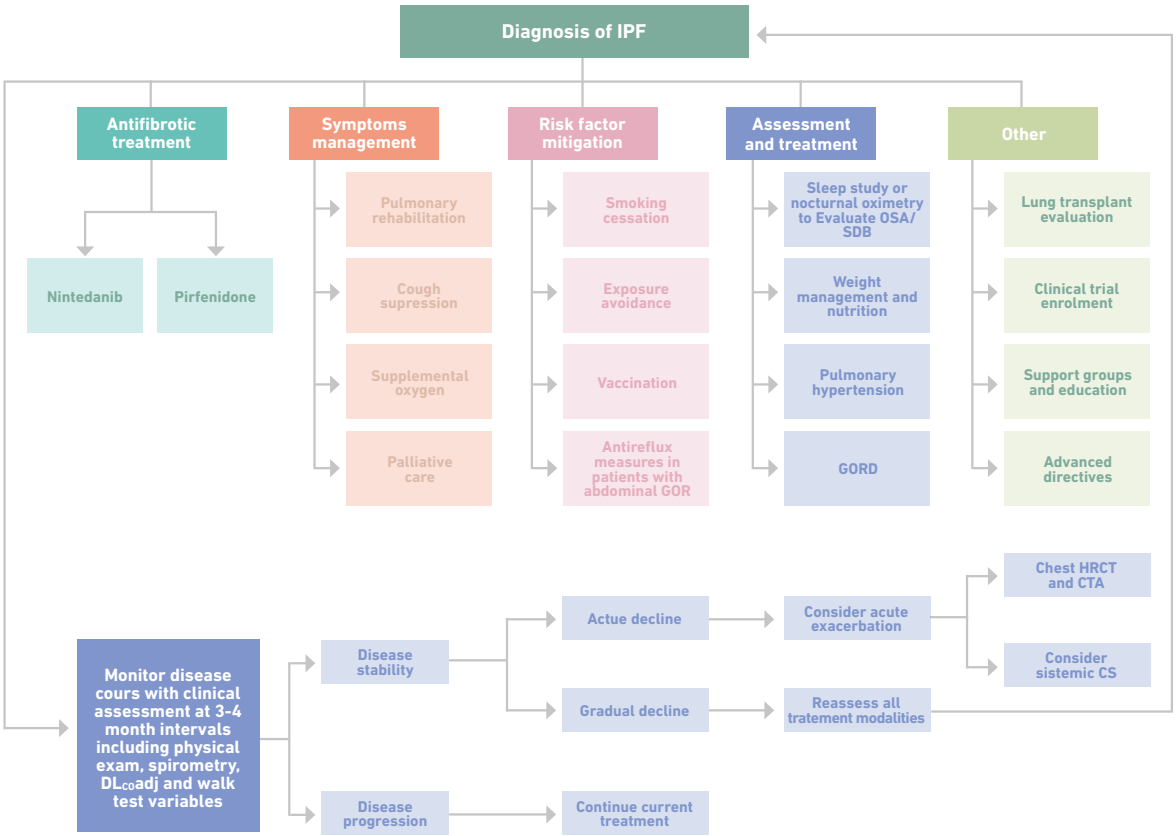

Figure 5. Suggested approach to the multimodal management of patients with IPF. Adapted from Podolanczuk et al. 2023 (32). OSA: Obstructive Sleep Apnoea; SDB: sleep-disordered breathing; HRCT: high-resolution computed tomography; CTA: Computed tomography angiography; CS: Corticosteroids; GORD: gastro-oesophageal reflux. Treatment considerations should include both pharmacological treatment (antifibrotics), and nonpharmacological treatments (supplemental oxygen, pulmonary rehabilitation, etc.) for symptom management, and existing comorbidities. Patients should be evaluated every 3-4 months interval for disease progression. Appropriate patients at high risk of mortality should be considered for lung transplantation.

1.3.4.1. Antifibrotic drugs

In 2014, two antifibrotic drugs, nintedanib and pirfenidone, were approved by the U.S Food and Drug Administration (FDA) and licensed for the treatment of IPF, as they both have been shown to reduce, not only ongoing fibrosis and delay its progression, but also reduce the risk of mortality and respiratory-related hospitalizations and/or exacerbations (67-69). However, neither antifibrotics have shown a positive effect on stabilizing lung function or quality of life, and they have shown tolerability issues in some patients (70).

1.3.4.1.1. Nintedanib

Nintedanib is a tyrosine kinase inhibitor (TKI) used to treat and manage IPF and other interstitial lung diseases with a progressive phenotype. Nintedanib directly hinders non-receptor tyrosine kinases, such as Src and Lck, preventing fibroblast proliferation, activation and migration (71). Two phase 3 trials consistently showed that nintedanib treatment, compared to placebo, was associated with a significantly reduced risk of disease progression and appears also to have a mortality benefit (72, 73). Nintedanib has been shown to present immunomodulatory effects by reducing cluster formation and T cell subset activation by inhibiting the release of IFNγ, IL-2, IL-4, IL-5 and IL-10 (74).

1.3.4.1.2. Pirfenidone

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a non-peptide synthetic molecule which was originally developed for its anti-pyretic and analgesic properties, and it was not until 1995 that its anti-fibrotic effects were discovered. Key actions of pirfenidone include not only altering the pleiotropic TGFβ pathway, and thus reducing fibroblast proliferation, but also directly affecting TGFβ-1 expression and collagen synthesis (75). Pirfenidone can also modulate immune responses: reduce T cell activation and stimulatory capacity, the secretion of proinflammatory cytokines, and impair the Th cell proliferation and polarization to Th2 (76).

1.3.4.2. Lung transplant

The percentage of total worldwide transplants for ILD has increased in the last decade. Lung transplantation is the only current intervention that has been shown to considerably increase life expectancy for patients with IPF, however, not all patients are suitable for this procedure (77, 78). International guidelines recommend evaluating lung transplantation as an option for individuals with advanced and/or progressively worsening fibrotic interstitial lung disease, especially those at significant risk of mortality within a two-year timeframe. This approach should take into account the current quality of life, anticipated survival, and, crucially, the preferences of the patient (79). Moreover, with disease progression despite the use of antifibrotics, there is a pressing need for refined approaches to assess prognosis in the different subtypes of IPF candidates and to identify those who are most in need of lung transplantation (72, 80).

1.3.4.3. Brief in novel approaches

The lack of a curative treatment together with the need for continuous basic, translational and clinical research activity in the pathogenesis of IPF. Recent multi-omics (i.e. epigenomic, transcriptomic, proteomic) data have helped to generate a single-cell atlas of IPF defining key molecular factors and pathways in the progression of fibrosis (81), which has led to the identification of several promising therapeutic targets and the development of novel strategies. However, the translation of these advances into truly efficacious drugs has proven extremely challenging and, so far, unsuccessful.

Over the past years, several prospective, double-blind, randomized clinical trials have been developed to find clinical benefits in IPF. Many of them used anti-inflammatory drugs (i.e. anti-TNFα, prednisone, rituximab), immunomodulatory agents (i.e. IFNγ, simtuzumab), or combined antifibrotic treatments, but none of them succeeded in meeting their primary endpoints: change in FVC time to disease progression or survival, or they even showed detrimental effects on those patients (82).

Thus, further research is required to better understand the mechanisms underlying the role of inflammation and immunomodulatory treatments in IPF, in order to stratify those patients who can benefit from them.

1.3.5. Pathogenesis of IPF: Our current understanding

Although the pathogenesis of IPF remains incompletely understood, currently, the most widely accepted hypothesis is that recurrent environmental and/or endogenous injury to alveolar epithelium leads to cell death, aberrant epithelial activation and repair, and secretion of a panel of pro-fibrotic mediators and growth factors. This promotes the recruitment, proliferation and differentiation of lung fibroblast into myofibroblast which, triggers the deposition of large amounts of extracellular matrix and scarring of the lung parenchyma that destroys the normal lung architecture and contributes to the recruitment of a wide range of inflammatory cells (see Figure 6) (83, 84).

The complexity of IPF biology has been demonstrated by numerous investigations on the diverse number of cell types and signalling pathways believed to be implicated in the disease pathogenesis, such as dysregulated epithelial repairs, cell senescence/apoptosis, immune and proliferative responses, etc (85). In addition, the spatial heterogeneity within the lung (different structures and affectations across the lung, or differences between lower and upper lobes) exists, which complicates the study of its pathobiology. Some authors postulate that these different regions of the lung may reflect different stages of the same process or reveal distinct endotypes (86).

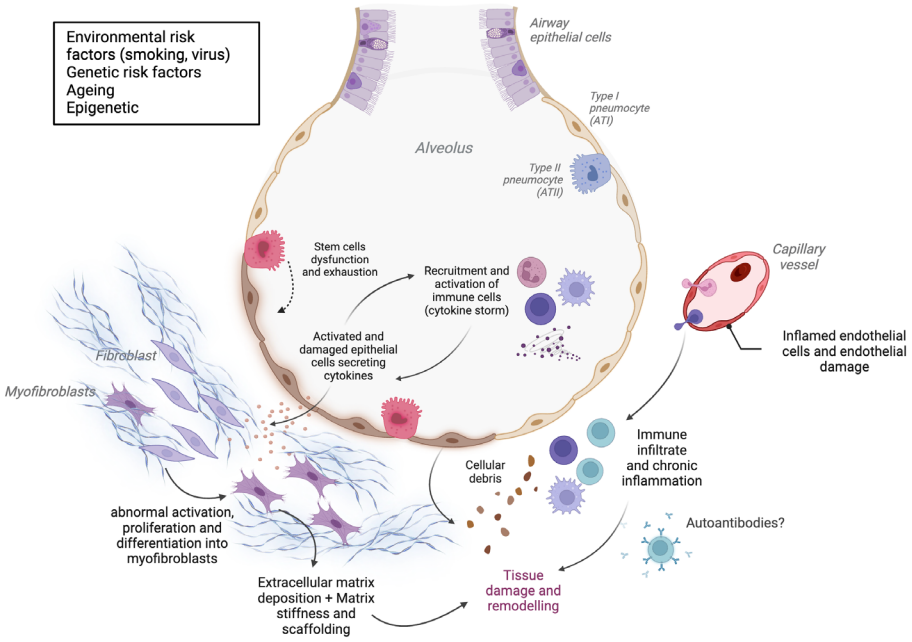

Figure 6. Summary of the pathogenesis of idiopathic pulmonary fibrosis (IPF). Elaborated by the doctoral student using BioRender. Activated alveolar epithelial cells and recruited inflammatory cells release potent pro-fibrotic growth factors (i.e., TGFβ) which perpetuate lung damage leading to apoptosis of alveolar epithelial cells, chronic inflamamtion and the induction of activation and invasion of fibroblasts, and extracellular matrix (ECM) deposition.

1.3.5.1. Alveolar Epithelial Cells in IPF: From Type II to Type I

One of the main contributors described in the pathogenesis of IPF is the alteration of the intracellular homeostasis of alveolar epithelial cells, mainly composed of alveolar type I epithelial cells (AT1), alveolar type II epithelial cells (AT2) and basaloid cells (87). These alterations result in aberrant epithelial activation and repair, which will further promote fibroblast/myofibroblast activation and increased extracellular matrix (ECM) deposition (88).

Covering more of the 95% of the surface area of the lungs, AT1 cells are responsible for the gas exchange, relying on their intrinsic ion and fluid transport functions (via aquaporins), which can be compromised when exposed to environmental insults (89). They also act as regulators of inflammation, either promoting fibrosis or repressing the progression of lung fibrosis and expressing pro-inflammatory receptors (TLR4 or RAGE) involved in innate immunity (90). Increasing evidence is showing that AT1 cells might be more involved in the progression of fibrosis than once believed and have also a role in alveologenesis and alveolar regeneration (91).

On the other hand, AT2 cells are defined as alveolar stem cells, having the ability of self-renewal and differentiation into AT1 cells after alveolar epithelial damage, promoting alveolar regeneration. They are also essential for the production of pulmonary surfactant, reduction of the alveolar epithelial surface tension and the prevention of alveolar collapse, all necessary for maintaining alveolar homeostasis. Several studies have shown that disruption of protein homeostasis, telomere damage, mitochondrial dysfunction, increased endoplasmic reticulum stress and epigenetic changes in AT2 cells, lead to cell dysfunction, apoptosis, senescence and pro-fibrotic signalling (92). In recent years, studies using single-cell sequencing and lineage tracing technology have identified a previously undiscovered population termed alveolar differentiation intermediates (ADI), also known as basaloid cells. This population emerges in the differentiation process from AT2 cells to AT1 cells. In IPF, abnormal basaloid cells have been significantly increased and highly enriched in areas of severe fibrosis (86).

In summary, dysfunction of the alveolar epithelium has been proposed to be a pivotal step in the initiation of IPF disease. However, still, the factors that contribute to the persistent epithelial damage and progression of fibrosis remain poorly understood.

1.3.5.2. Mesenchymal cells, Fibroblasts and Myofibroblasts

It is hypothesized that, after repetitive microinjuries, aberrant activation of the alveolar epithelial cells leads to the proliferation and activation of mesenchymal stem cells (MSC) and fibroblasts (93). This activation can induce the formation of focal lesions of active fibrogenesis, known as fibroblast foci (FF), that will promote an excessive ECM deposition, leading to scarring and destruction of the alveolar architecture (94).

MSCs, identified as self-renewal cells located in the perivascular niche, play a crucial role in regulating tissue repair and immune responses through the secretion of anti-inflammatory cytokines Additionally, they inhibit epithelial apoptosis and the fibrotic process, and promote epithelial repair and regeneration (85). Previous studies have shown that in IPF, MSCs undergo ageing, and present DNA damage, mitochondrial dysfunction and impaired paracrine functions, which increase pro-inflammatory responses and are related to disease severity (95).

Fibroblasts, as connective tissue cells that derive from embryonic mesenchymal cells, participate in tissue repair and also modulate local immune responses. In the context of IPF, fibroblasts undergo abnormal activation, proliferation and differentiation into myofibroblasts, which mainly involves the activation of TGF and related pathways (i.e. JAK2-STAT3, pSmad2/3, and P38-MAPK) (96, 97). In addition, mitochondrial dysfunction in these cells leads to increased reactive oxygen species, senescence-associated secretory phenotype (SASP) formation and cell senescence (98).

This whole scenario contributes to the excessive overproduction and deposition of ECM proteins (i.e. collagen and fibronectin), leading to the development of fibrosis in the lung tissue. In this setting, scRNA-seq studies have revealed the presence of cuboidal epithelial cells overlaying FF, which exhibit characteristics of aberrant basaloid cells, expressing a combination of basal epithelial, mesenchymal, senescence markers together with a sub-population of macrophages with pro-fibrotic features (86). In addition, within FF, AECs and fibroblast/myofibroblasts have shown disruption of several pathways (i.e. Wnt/beta-catenin) and multi-functional tumour suppressors, premature senescence, and overexpression of pro-inflammatory and pro-fibrotic factors, including TGFß (99, 100). Thus, understanding the role of FF and its cellular components is crucial for unravelling the mechanisms underlying the initiation of IPF and its progression, and they may serve as key targets for research aiming to develop therapeutic interventions that potentially slow down or reverse the progression of fibrosis.

1.3.5.3. Involvement of the immune cells in IPF: *Friends or foes?*

To date, the role of the immune response in the pathogenesis of IPF remains controversial, leaving it unclear if it is a cause, an effect, or just a mere epiphenomenon of the fibrotic process. On the one hand, the study PANTHER-IPF, where the safety and efficacy of immunosuppressive treatment In IPF (prednisone, azathioprine and N-acetylcysteine) was assessed, showed that IPF patients treated with these three-drug regimen immunosuppressors were at increased risk of death and hospitalization (101). However, on the other hand, abnormal innate and adaptive immune responses have been identified in lung tissue explant of patients with end-stage IPF, including changes in the amount of the infiltrate and/or functionality of neutrophils, macrophages and natural killer cells (NKs) (83). Likewise, in peripheral blood decreased expression of T cell regulatory genes, downregulation of the T cell costimulatory molecule CD28 expression (a marker of lymphocyte exhaustion) and increased expression of BAFF (B cell activation factor) and CXCL13 (a B cell lymphoid follicle homing cytokine) in serum (and lung), have also been described in patients with IPF, and some inversely correlated with survival (102-105). This evidence supports the idea that the immune system has a major role in the heterogeneity and progression of IPF (see Figure 6), however, it is still controversial if it is beneficial or detrimental. We will briefly review some of the major players described so far.

Neutrophils have a key role in the acute phase of inflammation, however, if accumulated, they can lead to tissue remodelling and fibrosis. Patients with IPF present an increase in IL-8 and G-SCF (Granulocyte colony-stimulating factor) in BAL, indicating activation and migration of neutrophils towards lung injury (106, 107). Other studies have also related the neutrophil count in BAL with a decrease in FVC, and the amount of IL-8 with future exacerbations (108).

The role of alveolar macrophages in IPF remains controversial due to their dual role in lung fibrosis: their release of profibrotic factors (e.g. TGF-ß1 and PDGF), may drive disease progression; but, on the other hand, their ability to release proteases (MMP) that can digest the extracellular matrix may have antifibrotic/fibrinolytic activities. In this context, human lung fibroblasts and components of the ECM can stimulate macrophages towards a "profibrotic" phenotype (109). An increase in macrophages in BAL with a "profibrotic" M2 phenotype and CCL18 have both been related to IPF, especially in acute exacerbations (AE) (110).

In IPF, the number and activity of lung NK cells are seriously compromised, correlating with a more severe pathology, while in peripheral blood the circulating NK cells were increased, suggesting an accumulation of NK cells in the blood, maybe due to poor recruitment, and an altered toward a senescent phenotype in the lung NK cells (15).

An increased number of B cells have been found in the lungs of IPF patients and several studies have described specific gene signatures of inflammation containing genes related to B cell markers and specific chemokines (i.e. CXCL13, CXCR5, CCR6 and CCR7), which are increased in lung explants and serum from IPF patients, and some correlated with disease progression and survival (i.e. CXCL13 and BAFF) (111-114). Although, by definition, IPF is not considered an autoimmune disease, auto-reactive B cells, and thus, auto-antibodies have been described in the patients with IPF, in most cases recognizing epithelial proteins

(e.g. annexin-1, cytokeratin-18, etc.) (115, 116). These auto-antibody-driven immune responses may play an important role in repeated epithelial and endothelial damage and impartment in wound repair and have been related to clinical outcomes in IPF (i.e. acute exacerbations) (116, 117).

T cells have also been widely related to active disease regions in the fibrotic lungs in IPF (111). Previous studies showed a significant increase in $CD8^+$ T cells in the lung tissue infiltrate and BAL of patients with IPF, and their relation with the disease severity (grade of dyspnoea and functional parameters). In addition, evidence showed that $CD8^+$ T cells accelerate lung damage when they are recruited and react to viral infection. Other studies, where a more detailed characterization of the T cells phenotype and function was done, showed a significant increase in $CD8^+$ cytotoxic T cells lacking the coestimulatory molecule CD28 ($CD28^-D8^+$ T cells) in explanted lung tissue and also expressing CD45RA or CD45RO (118). Also, RNA-seq data analysis of those IPF lung explants shows enrichment of T cell activation, inflammatory mediators (i.e. TNF, IL1-β, IL6 and IFN-γ), microbial sensors (i.e. TLR2, TLR3, TLR4, TLR7 and TLR9) and proinflammatory pathways (i.e. PDGF-BB, TGF-β1, IL18, and IL13).

Moreover, several studies focused their interest on the imbalance of Th1/Th2 as a key process in the pathogenesis of IPF, where increased type-2 cytokines promoted pro-fibrotic responses whereas type-1 responses (mainly IFNγ) were thought to have a protective role (119, 120). However, the negative results of the INSPIRE trial, where the patients were treated with INFγ, indicated that the scenario was more complex than once thought (121). Th17 are known for their effective induction of tissue inflammation (IL-17, IL-21) and their involvement in the pathogenesis of many autoimmune diseases (122). In IPF, Th17 cells and IL-17 are found in the lung infiltrate, which can indicate that certain autoimmune reactivity is present in the disease as suggested by others (117, 123).

Traditionally, it has been believed that Tregs had a protective role against fibrosis development and IPF, by reducing fibrocyte accumulation and inflammatory responses. However, recent data showed that their function may vary during the different stages of IPF (from early to end-stage) and that in some stages they may trigger profibrotic processes: Tregs might be harmful in early stages but protective in late stages (124).

Table 1. Summary of the contribution of each cell type described in the pathogenesis of IPF. Adapted from Zhang, Y. et.al. 2023 and Heukels et.al. 2019 (83, 85).

CELLS	FUNCTION AND MECHANISMS	EFFECTS IN IPF
AT1	Gas exchange, Ion and liquid transport, congenital immunity.	Impaired barrier, Abnormal activation and Apoptosis. Involved in inflammation.
AT2	Self-renewal and differentition, Damage repair.	Impaired stem cell function, Pro-fibrotic signalling, ER stress, Telomere attrition, Mitochondrial dysfunction.
Basaloid cells	Transdifferentiation	Abnormal phenotype, Pro-fibrotic signalling.
MSC	Self-renewal and differentiation, Damage repair, Immunoregulatory functions.	Exhibit immunomodulatory properties, which can help regulate the inflammatory responses associated with IPF.
Fibroblasts	Tissue repair.	ECM deposition, Differentiate into myoblasts, Telomere shortening, Mitochondrial damage, Resistance to apoptosis, Cellular senescence.
Alveolar Macrophages	Phagocytosis and pathogens clearance, double sided effect reducing ECM deposition via MMPs, but also protoing fibrosis dependint on the microenvironment.	Polarize into M2-type macrophages, Secrete growth factors and chemokines (TGF-β), Accumulate in fibrotic niches and promote fibrosis.
Monocytes	Role in tissue homeostasis and immunity. Divided in 3 traditional subsets: classical (CD14hiCD-16low), intermediate (CD14hiCD16hi) and non-classical monocytes (CD14lowC16hi).	Progenitor cells for pro-fibrotic macrophages and fibrocytes (release pro-fibrotic cytokines). Intermediate monocytes areassociated with worse prognosis in IPF.
Neutrophils	First line responders upon acute inflammation. Antimicrobial functions releasing proteases, oxidative stress enzymes and cytokines, Participate in tissue remodeling.	NE damage alveolar epithelium and capillary endothelial cells, Increased levels of MMP-9, Chronic elevation of NETs that promote fobroblasts differentiation.
Eosinophils	Involved in the immune response against parasitic infections and certain types of allergies, Tissue repair and remodelling.	Active eosinophils aggregate in the lung tissue and induce fibroblasts proliferation and differentiation.
Lung DC	Concentrated in the alveolar epithelium and interstitium, Mainly APC but their function depends on their microenvironment.	Involved in the chronic inflammation and porgression of fibrosis.
NK cells	Early defense against infections, Stimulate DC, B cell maturation and support T cell polarization and activation.	Important regulatory role in pulmonary fibrosis due to their different phenotypes.
NK T cells	Subset of T cells with characteristics of NK cells (antigen recognition via TCR and rapid response).	IFN-γ-producing NKTs may play a novel anti-fibrotic role in pulmonary fibrosis by regulating the production of TGF-β1.
Th1 cells	Mediate cellular immune response producing cytokines that include IL-2, IFNg, TNF.	Th1/Th2 imbalance may play a role in pulmonary fibrosis, Th2 cytokines can promote the activation and proliferation of fibroblasts, increase collagen synsthesis and inhibit its degradation.
Th2 cells	Involved in chronic diseases and tissue repair. Mediate immune response producing cytokines that include IL-4, IL-5, IL-6, IL-10, IL-13.	
Th17 cells	Production of interleukin-17 (IL-17), Defense against extracellular pathogens such as bacteria and fungi, Implicated en autoimmune diseases.	Increased in IPF and correlates with the severity of the disease, IL-17 can stimulate the secretion of G-CSF and M-CSF in fibroblasts and respiratory epithelial cells, and the aggregation of neutrophils in the lesion causing damage to the epithelial cells.
Cytotoxic T cells	(CD8+ T cells) Anti-virus and anti-tumor immunity.	Increased CD8+ T cells in the BALF of IPF patients, Activated CD8+ is related to tissue damage and ongoing fibrosis.
B cells	Primary component of the humoral immunity (antibody production), Immune surveillance, Coordination of immune responses through the interaction with T cells.	Abnormal activation of B cells related to the formation and progression of lung fibrosis, Increased CD20+ B cells in the lung tissue of IPF patients, Elevated BAFF in BAL of IPF patients.
Treg cells	Generally involved in maintaining host tolerance and prevention of autoimmunity (imunosupressive functions). Secrete IL-10 and TGF-β.	Treg cell deficiency results in the promotion of autoimmunity and tissue damage, but also, Treg cells can contribute to the progression of pulmonary fibrosis by secreting TGF-β and other related factors, and by promoting the EMT.

IPF: Idiopathic pulmonary fibrosis; AT1/2: alveolar type I/II epithelial cells; MSC: Mesenchymal stem cell; ECM: Extracellular matrix; MMP: Metalloproteases; NET: neutrophil extracellular traps, NE: Neutrophil elastase; DC: Dendritic cell APC: Antigen-presenting cell; NK cell: Natural killer cell; IFNγ: interferon-gamma; Th1: T helper type 1; Th2: T helper type 2; Th17: T helper type 17; IL: Interleukin; G/M-CSF: granulocyte-macrophage colony-stimulating factor; EMT: Epithelial-mesenchymal transition; Treg: Regulatory T cells; BAL: bronchoalveolar lavage.

1.3.6. Novel biomarkers in IPF: Impact of transcriptomics

The analyses of the levels of mRNA, known as transcriptomics, provides a comprehensive view of the status of the cells of a tissue. From gene expression microarrays to the development of RNA-seq and single-cell RNA-seq, for deeper sequencing and greater sample size analysis, transcriptomic technologies have led to the identification of novel genes and key biological pathways in ILD, and IPF. This approach has not only provided some conceptual insights into its pathogenesis, but also has had an impact on disease classification, and the discovery of endotypes and potential biomarkers (125). To conduct these analysis, consortia collaborating in the collection of large numbers of lung tissue, blood and clinical data, have been instrumental. Examples of that are the Spanish CIBERES pulmonary biobank, or the American Lung Tissue Research Consortium (LTRC) (NCT02988388), as well as the public availability of data through the Lung Genomics Research Consortium (LGRC) (1RC2HL101715). Transcriptomic studies revealed novel molecules and pathways implicated in the IPF pathogenesis (125), such as: 1) Pro-fibrotic signatures/programs, previously not characterized as the effector functions of fibroblasts/myofibroblasts and inflammation: extracellular matrix (ECM) deposition and WNT/b-catenin pathways, both aberrantly activated in alveolar epithelial cells adjacent to myofibroblast foci and fibroblasts (126-128); 2) matrix metalloproteases (MMP) (i.e. MMP1, MMP7), which contribute to the profibrotic environment, were increased in IPF lung (129); 3) TGF-β pathways (i.e. TGF-β/Smad) and ECM related genes (i.e. collagens), increased also in myofibroblasts (130, 131); 4) alterations in apoptosis, metabolic and mitochondrial pathways, showing impaired mitophagy and changes in glucose, fatty acid and citric acid metabolism in IPF (132, 133); and 5) immune response/inflammatory-related pathways, also altered in the IPF lung (134). In another study, microarray gene expression analysis on RNA isolated from PBMCs distinguished two groups of IPF patients with significant differences in transplant-free survival. Interestingly, increased mortality after transplant was associated with a decrease in the T cell co-stimulatory molecules (i.e. CD28, ICOS, LCK, and ITK). These results highlight the role of aberrations in the T cell compartment, supporting the concept of "immunosenescence" in IPF (135). In this line, a more recent study identified peripheral blood gene expression signatures capable of distinguishing three groups of IPF patients with differences in clinical outcomes (i.e. survival over time and DL_{CO} % predicted), supporting the idea of multiple endotypes in IPF. In this study, gene enrichment analysis showed that: cluster 1 was significantly enriched for biological mechanisms related to metabolic changes and cellular respiration; cluster 2 was enriched for biological processes related to DNA repair, cell cycle and apoptosis, while cluster 3 was enriched for biological processes related to the immune response (136). Single cell transcriptomic studies, have provided a variety of new transitional cell types associated to fibrosis, including alterations in mesenchymal progenitor cells, epithelial cells and fibroblasts. Beyond transcriptomics, other approaches have characterized the proteins associated with disease subtypes and disease progression. Table 2 summarizes the biomarkers that have been proposed for the diagnosis and prognosis of the disease. However, to date, none of them have been integrated into the diagnostic algorithm or clinical decision-making. In this setting, recent studies showed that IPF patients with progressive disease present higher levels of surfactant protein D (SP-D), matrix metallopeptidase 7 (MMP-7) and intercellular adhesion molecule 1 (ICAM-1), among others, and that these biomarkers (individually or as a composite) may have value in predicting risk of disease progression and mortality (137-139). In addition, telomere length has been also considered a prognostic biomarker with potential utility in assessing treatment response.

However, no current biomarkers have proved their ability to predict the treatment response of patients with IPF to antifibrotic agents. Thus, to determine their clinical utility and consider their inclusion in the diagnostic process, these biomarkers need prospective validation.

Table 2. Summary of some relevant peripheral blood and molecular biomarkers in IPF. Adapted from Somogyi et al. 2019 (140).

		MECHANISMS OF ACTION	OUTCOMES AND EFFECTS ON IPF
BLOOD BIOMARKERS	**CCL18**	Alternative macrophage activation Upregulation of collagen production by lung fibroblasts.	High serum concentrations correlated with higher indicende of disease progression and mortality.
	ICAM-1	Adhesion molecule/ Marker of oxidative stress in the lungs.	Predicts poor transplant-free and progression free survival.
	KL-6/MUC1	High molecular weight glycoprotein expressed at ECM surface of type II pneumocytes.	Predicts mortality in IPF.
	SPA-A/B/D	Surfactant proteins produced by type II pneumocytes.	High levels in IPF; associated with the time to death or lung transplantation.
	MMP1/7	MMP1: the most highly expressed interstitial collagenase degrading fibrillar collagens. MMP7: the smallest member capable of degrading multiple components of ECM.	Distinguish between IPF and HP, higher levels in IPF. Related to FVC decline and to higher all-cause mortality.
	VEGF	Growth factor regulating angiogenesis enhancing vascular permeability.	Positive correlation with HRCT interstitial score, influence on monthly FVC decline.
	CD28	CD28 co-stimulatory molecule providing signal for activation of naive T lymphocytes.	Correlated with decreased FVC and freedom from major adverse events (death or lung transplantation).
	HSP70 IgG antibodies	HSP70 antibody working against HSP70 autoantigene and activating IL-8 production of monocytes.	Associated with decreased FVC and 1-year survival.
	Periostin	Fibroblast activating matrix proteins.	Negative correlation with monthly changes in VC, DL_{CO}. Increase of honeycombing score on HRCT, predictor of shortened overall survival, time-to-event.
	Circulating fibrocytes	Produce ECM components, mesenchymal markers.	High levels correlated with poor survival regardless to preservation of lung function, counts increased further during AE-IPF.
	CXCL13	Chemokine playing a role in autoimmune processes, mediating B-cell homing to inflammatory foci.	High levels correlated with poor FVC and poor major event-free survival (i.e. transplant-free survival).
	EGFR	Epidermal growth factor required for TGF-β1-induced epithelial-mesenchymal transition Crucial in signalling in bronchial epithelium.	Lower levels in IPF.
	Clusterin	Known as apolipoprotein J Glycoprotein upregulated by cytotoxic stimuli, maintaining epithelium viability during lung repair.	Lower levels in IPF.
	CRPM	C reactive, acute-phase protein degrading by matrix metalloprotease.	Higher levels in IPF, could discriminate between stable and progressive subjects and indicated poor overall survival.
	CA-125/19-9	Tumor markers, mucous associated carbohydrate antigens increasing inmetaplastic epithelium in fibrotic lesions.	High levels highly predictive of progressive fibrosis.
MOLECULAR BIOMARKERS	**MUC5B**	Mucin associated with the development of both familial interstitial pneumonia and sporadic IPF.	MUC5B promoter gene polymorphism associated with survival independent of clinical factors.
	TERT	Reverse transcriptase maintaining telomere integrity.	Mutation associated with familial interstitial pneumonias and sporadic, adult-onset IPF.
	Telomere length	Length of nucleoprotein structures that protect chromosomal ends.	Shorter telomere length associated with progression-free survival of IPF.
	TLR3	Receptor mediating innate immune response to tissue injury, inflammation and viral infection.	Polymorphism associated with early lung function decline and death.
	a-Defensin	Antimicrobial peptides presenting in granules of neutrophils inhibiting activation of the classical complement pathway.	Increased α-defensins localised in the epithelium of the lungs and apoptosis of epithelium in AE-IPF.

CCL18: CC chemokine ligand 18; ICAM-1: intercellular adhesion molecule 1; KL: Krebs von den Lungen; MUC: mucin; SP: surfactant protein; MMP: matrix metallopeptidase; VEGF: vascular endothelial growth factor; HSP: heat shock protein; Ig: immunoglobulin; CXCL: C-X-C motif chemokine ligand; EGFR: epidermal growth factor receptor; CRPM: C-reactive protein degraded by metalloproteinase-1/8; CA: cancer antigen; TERT: telomerase reverse transcriptase; TLR: toll-like receptor; ECM: extracellular matrix; ILD: interstitial lung disease; UIP: usual interstitial pneumonia; HRCT: high-resolution computed tomography; HP: hypersensitivity pneumonitis; FVC: forced vital capacity; WHO: World Health Organization; VC: vital capacity; DL_{CO}: diffusing capacity of the lung for carbon monoxide; PH: pulmonary hypertension; AE-IPF: acute exacerbation of idiopathic pulmonary fibrosis; TGF: transforming growth factor.

1.3.6.1. Biomarker unmet needs in ILDs

Despite the recent advancements in the understanding and management of the IPF, several unmet needs in this field, including:

- Precise and personalized treatment approaches.
- Biomarkers of early diagnoses, with potential use for screening.
- Biomarkers to predict disease progression. Still, there are no biomarkers available to accurately identify those patients at diagnosis who will experience a rapid progression of the disease. Thus, these immunological features could be useful in clinical practice for the early identification of patients susceptible to progression.
- A better understanding of the disease heterogeneity. Among the wide variety of cell types that contribute to the pathobiology of IPF, in addition to the pivotal role of alveolar cells and myofibroblasts/fibroblasts, initiating the fibrogenic cascade, the immune system continues constituting one controversial enigma. As stated before, recent studies agree on its key role in the development and persistence of lung fibrosis, and even consider it as a possible explanation for the heterogeneous progression of the disease.

Hypothesis

02

The **general hypothesis** of this Doctoral Thesis is that in clinically and biologically heterogeneous chronic respiratory diseases, such as IPF, the investigation of peripheral blood and tissue (lung) immune cell populations may provide new insights into its pathobiology, progression and management of the disease.

This general hypothesis can be divided into the following **specific** ones:

1 **The type of the immune response differs between specific subgroups of the disease and is associated with different IPF outcomes. The type of immune response can be identified due to this association, in peripheral blood even in the early stages of the disease.**

2 **The investigation of the level and type of immune infiltration in the lung tissue of patients with well-established IPF might identify different biological and clinical phenotypes.**

Objectives

03

The **general objective** of this PhD is to explore the associations of the peripheral blood and lung immune cell populations with the IPF heterogeneity, severity and progression.

To do so, the following **specific aims** have been addressed:

1

Characterization of the peripheral immune cell profile and its association with IPF heterogeneity, specific aims:

- To determine the immune cell populations in the blood of recently diagnosed IPF patients.
- To investigate in IPF the relationship between peripheral immune cells and the severity of airflow limitation.
- To assess the ability of the baseline immune cell populations in the classification of progressor and stable patients.
- To evaluate the stability of the peripheral blood profile over time.

2

Characterization of the lung immune infiltrate and its association with the clinical and biological heterogeneity in IPF, specific aims:

- To explore the level of immune infiltrate in lung tissue of patients with IPF.
- To correlate the levels of infiltrate with the clinical features.
- To validate the results in an independent cohort using flow cytometry analysis.
- To investigate the association between the level of infiltrate with the differentially expressed genes (DEG) and molecular processes.

Results

04

The **core results** of this PhD Thesis have been recently published in the form of two original papers in high-impact factor international journals.

Original Paper I:

Blood Immunophenotypes of Idiopathic Pulmonary Fibrosis: Relationship with Disease Severity and Progression.

Mendoza N, Casas-Recasens S, Olvera N, Hernandez-Gonzalez F, Cruz T, Albacar N, Alsina-Restoy X, Frino-Garcia A, López-Saiz G, Robres L, Rojas M, Agustí A, Sellarés J, Faner R. Int J Mol Sci. 2023 Sep 7;24(18):13832. doi: 10.3390/ijms241813832. PMID: 37762135; PMCID: PMC10531459. Impact factor: 4.56 (Q1).

The doctoral candidate is the first author of this article and has performed the following tasks: participation in experimental design, sample processing, conducting experiments, statistical analysis of raw data, interpretation of results, extraction of conclusions, writing of the article and its revisions.

Not used in any thesis.

Original Paper II:

Lung immune signatures define two groups of end-stage IPF patients.

Cruz T, Mendoza N, Casas-Recasens S, Noell G, Hernandez-Gonzalez F, Frino-Garcia A, Alsina-Restoy X, Molina M, Rojas M, Agustí A, Sellares J, Faner R. Respir Res. 2023 Sep 28;24(1):236. doi: 10.1186/s12931-023-02546-8. PMID: 37770891; PMCID: PMC10540496. Impact factor: 3.92 (Q1).

The doctoral candidate is the second author of this article and has performed the following tasks: participation in experimental design, statistical analysis of raw data, interpretation of results, extraction of conclusions, writing of the article, and its revisions.

Not used in any thesis.

Thesis Co-directors:

Dra. Rosa Faner Canet Dr. Jacobo Sellarés Torres

4.1. METHODOLOGICAL CONSIDERATIONS FOR AIM 1 (PAPER I):

Circulating Immune Cells Monitoring Using Flow Cytometry

Multicolour flow cytometry is a useful tool that allows simultaneous analysis and quantification of multiple cellular characteristics (extracellular and/or intracellular) as they are transported in a fluid and incident by different beams of light. The flow cytometer measures cell size and granularity, as well as relative cell fluorescence. These characteristics are determined using an optical system coupled to an electronic procedure that records how the cell scatters the light beams and emits fluorescence (141).

Human leukocyte differentiation antigens (HLDA), more commonly known as CD (cluster of differentiation) have been used in the study of molecular composition and function of cells of the immune system. The cell surface is a site where many important biological processes take place, which are involved in the recognition and interaction between different cell types and their microenvironment, as well as cell proliferation, differentiation or death.

So, the presence or absence of particular molecules on the membrane can be used to identify different phenotypes of cell populations. To do so, the approach used to target these molecules are specific antibodies, most often monoclonal antibodies (mAb) (142).

Relating these concepts to the flow cytometry analysis, the selected antibodies, chosen for their ability to specifically bind to the molecules of interest, will be combined with specific fluorochromes. The resulting fluorescence signals emitted by these fluorochrome-conjugated antibodies are then detected by the flow cytometer's detectors, enabling the generation of high-dimensional data. This meticulous pairing of antibodies with fluorochromes enhances the specificity and sensitivity of the flow cytometry analysis, allowing the discrimination between different cell populations based on the expression levels of these specific antigens (143).

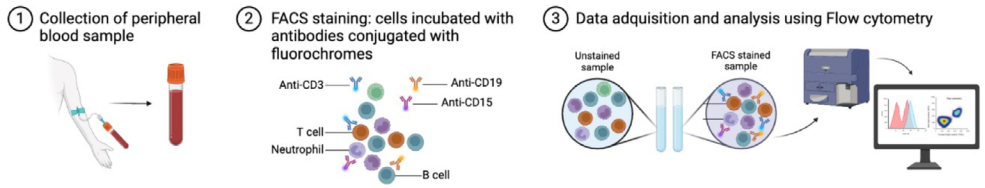

Figure 1. Schematical Flow cytometry analysis workflow. Created by the author using BioRender.

4.2. SUMMARY OF THE MAIN RESULTS FROM SPECIFIC OBJECTIVE I

Regarding the **first aim**, the main and **novel findings** of this work (Paper I) showed that:

1 at baseline, patients with a recent diagnosis of IPF presented an abnormal peripheral immune cell profile compared to age- and smoking-matched healthy controls, characterized by a higher percentage of circulating neutrophils, Th1, $CD8^+HLA\text{-}DR^+$ T cells (i.e. cytotoxic activated cells) and $CD8^+CD28^-$ T cells (i.e. "exhausted" cytotoxic cells), and a lower percentage of B cells, Th17 and naïve $CD8^+$ T cells;

2 in IPF patients, baseline FVC was positively related to naïve $CD4^+$ T cells, but negatively related to central memory $CD4^+$ T cells, and DL_{CO} was positively related to lymphocytes and eosinophils, but negatively related to neutrophils, neutrophil- and monocyte-lymphocyte ratios (NLR, MLR);

3 at baseline, the immune cell profile in IPF progressors was different from that of stable IPF, the former being characterized by an increased percentage of circulating NKT-like cells, $CD8^+$ T cells, effector memory $CD4^+$ and $CD8^+CD28^-$ T cells, and a decrease in naïve T cells and central memory $CD8^+$ T cells, and a lower CD4/CD8 ratio;

4 our machine learning method showed that the combination of NKT-like cells, $CD8^+CD28^-$, memory effector $CD4^+$ T cells and CD4/CD8 ratio offered the best classification model of progressor vs. stable patients; and

5 the immune profile was assessed again in alive patients between 18 to 60 months of follow-up, and the immune populations included in the machine learning classification model remained stable.

4.3. ORIGINAL PAPER I:

Blood Immunophenotypes of Idiopathic Pulmonary Fibrosis: Relationship with Disease Severity and Progression.

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(Supplementary material is enclosed in Appendix I).

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Blood Immunophenotypes of Idiopathic Pulmonary Fibrosis: Relationship with Disease Severity and Progression

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Abstract: (1) The role of the immune response in the pathogenesis of idiopathic pulmonary fibrosis (IPF) remains controversial. We hypothesized that peripheral blood immune phenotypes will be different in IPF patients and may relate to the disease severity and progression. (2) Whole blood flow cytometry staining was performed at diagnosis in 32 IPF patients, and in 32 age- and smoking-matched healthy controls. Thirty-one IPF patients were followed up for one year and categorized as stable or progressors based on lung function, deterioration and/or death. At 18–60 months, immunophenotypes were characterized again. (3) The main results showed that: (1) compared to matched controls, at diagnosis, patients with IPF showed more neutrophils, $CD8^+HLA\text{-}DR^+$ and $CD8^+CD28^-$ T cells, and fewer B lymphocytes and naïve T cells; (2) in IPF, circulating neutrophils, eosinophils and naïve T cells were associated with lung function abnormalities; (3) patients whose disease progressed during the 12 months of follow-up showed evidence of cytotoxic dysregulation, with increased $CD8^+CD28^-$ T cells, decreased naïve T cells and an inverted CD4/CD8 ratio at baseline; and (4) blood cell alterations were stable over time in survivors. (4) IPF is associated with abnormalities in circulating immune cells, particularly in the cytotoxic cell domain. Patients with progressive IPF, despite antifibrotic therapy, present an over-activated and exhausted immunophenotype at diagnosis, which is maintained over time.

Keywords: interstitial lung diseases; antifibrotic therapy; immunity and inflammation

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease characterized by the accumulation of scar tissue, subepithelial fibroblast foci and microscopic honeycombing [1]. Although the pathogenesis of IPF remains incompletely understood, it is thought that abnormally activated and/or damaged lung epithelial cells secrete a panel of mediators that differentiate fibroblasts to myofibroblasts which, in turn, deposit large amounts of extracellular matrix that destroys the normal lung architecture and contribute to the recruitment of inflammatory cells [2,3]. The original triggers causing the abnormal activation of lung epithelial cells are varied and include cigarette smoking, chronic viral infections,

accelerated aging and genetic predisposition [4]. It is generally believed that these repeated micro injuries, in combination with exaggerated wound repair and dysregulated tissue remodelling, lead to IPF [5] with impaired lung function, breathlessness and, eventually, death [6,7].

The role of the immune response in the pathogenesis of IPF remains controversial. On the one hand, abnormal innate and adaptive immune responses have been identified in the lung tissue of patients with end stage IPF [2], including changes in the amount of the infiltrate and/or functionality of neutrophils, macrophages and natural killer cells (NKs) [2], and in this setting they are considered as an epiphenomenon of the fibrotic process. Likewise, in peripheral blood, the decreased expression of T cell regulatory genes [8], downregulation of the T cell costimulatory molecule CD28 expression (a marker of lymphocyte exhaustion) [9,10] and increased expression of CXCL13 (a B cell lymphoid follicle homing cytokine) [11] have also been described in patients with IPF. On the other hand, however, IPF patients treated with immunosuppressors are at increased risk of death and hospitalization [12]. We reasoned that a detailed characterization of the blood immune cell phenotype of IPF patients at diagnosis can provide new insights into the pathogenesis and progression of IPF over time. To explore this hypothesis, this study sought to: (1) compare the immune-phenotypes in the peripheral blood of patients with IPF at the time of diagnosis vs. healthy controls; (2) explore their potential associations with the severity of the disease at baseline and with disease progression during a 1-year follow-up; and (3) explore if these associations were maintained long-term, during 18–60 months of follow-up in survivors.

2. Results

2.1. Participant Characteristics

Table 1 presents the main demographic and clinical characteristics of patients and controls at recruitment and in patients during a 1-year follow-up. By design (matching procedure), at recruitment, age (about 71 years) and smoking history were similar. As expected, lung function was abnormal in patients and normal in controls. At recruitment, 12 patients (37.5%) were receiving antifibrotic treatment and 20 (62%) were not (Table 1). A total of 31 of the 32 patients profiled at recruitment (96%) were followed-up for 1 year (Table 1, Figure S1). Disease progression, defined as above (annual FVC decline $\geq$ 10%, DLCO decline $\geq$ 15% and/or death within the first-year of follow-up), occurred in 18 patients (58%), despite the fact that 16 of them (88.9%) received antifibrotic treatment (pirfenidone or nintedanib) during follow-up. At recruitment, age, gender, and smoking status was similar between stable and progressor IPF patients (Table 1). Lung function was different in both groups during the 1-year follow-up (Table 1). Seven progressors died during the follow-up (38.9%).

2.2. Blood Immunophenotype Differences in IPF Patients vs. Healthy Individuals at Baseline

We found significant differences in both the innate and adaptive immunophenotypes in the blood of IPF patients vs. healthy controls at baseline (Figure 1 and Table S3). Specifically, the percentage of circulating neutrophils was higher, and that of B cells was lower, in IPF patients (Figure 1a). Also, the proportion of $CD8^+HLA\text{-}DR^+$ (i.e., cytotoxic activated cells) and $CD8^+CD28^-$ T cells (i.e., cytotoxic exhausted, mainly of effector and memory effector phenotype) was higher in IPF patients than in controls, whereas that of naive $CD8^+$ T cells was lower (Figure 1b and Table S3). Given that the later alterations resemble the immunophenotype in aged individuals, we performed a two-way ANOVA to evaluate the effect of age, and the differences in $CD8^+CD28^-$ and naïve $CD8^+$ T cells remained statistically significant ($p = 0.027$ and $p = 0.007$, respectively). No differences were observed in the percentage of PD-1^+ T cells. Th17 lymphocytes were also decreased in IPF while Th1 and Tregs (trend, $p = 0.072$) were increased, and, accordingly, the Th1/Th17 and Th17/Tregs ratios were different between patients and controls. (Figure 1c,d and Figure 2 and Table S3).

Table 1. Characteristics (*n* (%) or mean ± SD) of participants at study entry and during 12-month follow-up.

	At Study Entry			IPF Patients during 1-Year Follow-Up		
	Control (*n* = 32)	**IPF (*n* = 32)**	***p*-Value**	**Progressor (*n* = 18)**	**Stable (*n* = 13)**	***p*-Value**
Age	71.1 ± 5.17	71.6 ± 7.01	0.344	71.8 ± 5.99	70.7 ± 8.39	0.679
Males, *n* (%)	13 (40.6%)	23 (71.9%)	0.023	14 (77.8%)	9 (69.2%)	0.689
Smoking status			0.445			0.784
Former smoker	21 (65.6%)	25 (78.1%)		15 (83.3%)	10 (76.9%)	
Never smoker	6 (18.8%)	5 (15.6%)		3 (16.7%)	2 (15.4%)	
Current smoker	5 (15.6%)	2 (6.25%)		0 (0.00%)	1 (7.69%)	
BMI, Kg/m^2	25.3 (3.5)	29.2 (8.72)	0.037	29.7 (10.0)	28.5 (6.81)	0.721
FVC, % ref.	106 (20.9)	69.8 (18.3)	<0.001	60.3 (10.8)	79.5 (16.8)	0.002
FEV1, % ref.	96.4 (17.2)	77.0 (16.7)	<0.001	69.3 (11.7)	85.3 (16.8)	0.008
FEV1/FVC, %	98.0 (8.27)	81.1 (5.80)	<0.001	83.1 (5.38)	78.9 (5.63)	0.055
DLCO, % ref.	NA	46.9 (16.8)		41.9 (14.7)	53.9 (17.9)	0.063
Antifibrotic before *, *n* (%)			<0.001			1.000
Yes	0 (0%)	12 (37.5%)		7 (38.9%)	5 (38.5%)	
Antifibrotic after *, *n* (%)			<0.001			0.497
Yes	0 (0%)	30 (93.8%)		16 (88.9%)	13 (100%)	
Antifibrotic drug, *n* (%)			<0.001			0.348
Nintedanib	0 (0%)	22 (73.3%)		13 (72.2%)	8 (61.5%)	
Pirfenidone	0 (0%)	8 (26.7%)		3 (16.7%)	5 (38.5%)	
Death, *n* (%)			1.000			0.025
Yes	0 (0%)	0 (0%)		7 (38.9%)	0 (0%)	

NA = Not available information; BMI = Body mass index; FVC = Forced Vital Capacity; FEV1 = Forced expiratory capacity 1 s; DLCO = Single breath carbon monoxide diffusing capacity; Antifibrotic before * = Antifibrotic treatment before recruitment; Antifibrotic after * = Antifibrotic treatment after recruitment.

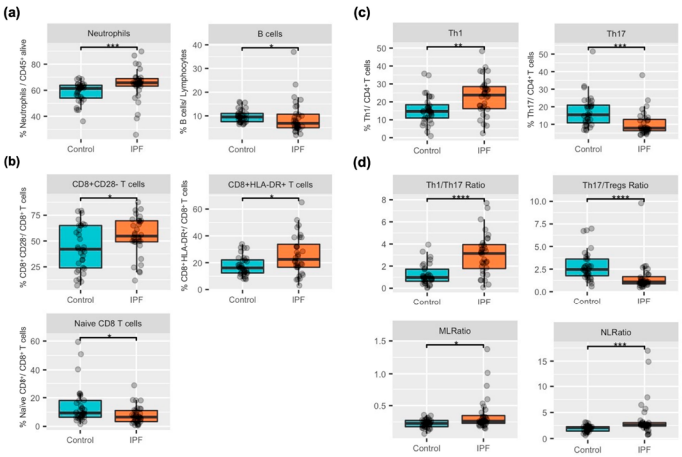

Figure 1. Characterization of the innate and adaptive compartment in IPF patients (orange box) and controls (blue box) (median [IQR]) (**a**) Comparison of % neutrophils and B lymphocytes. (**b**) Comparison of % $CD8^+CD28^-$, HLADR+, and naive $CD8^+$ T cells from $CD8^+$ pool. (**c**) Comparison of % Th1 and Th17 from $CD4^+$ pool. (**d**) Comparison of Th1/Th17, Th17/Tregs, Neutrophil-to-lymphocyte (NRL), and Monocyte-to-lymphocyte (MLR) ratios. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

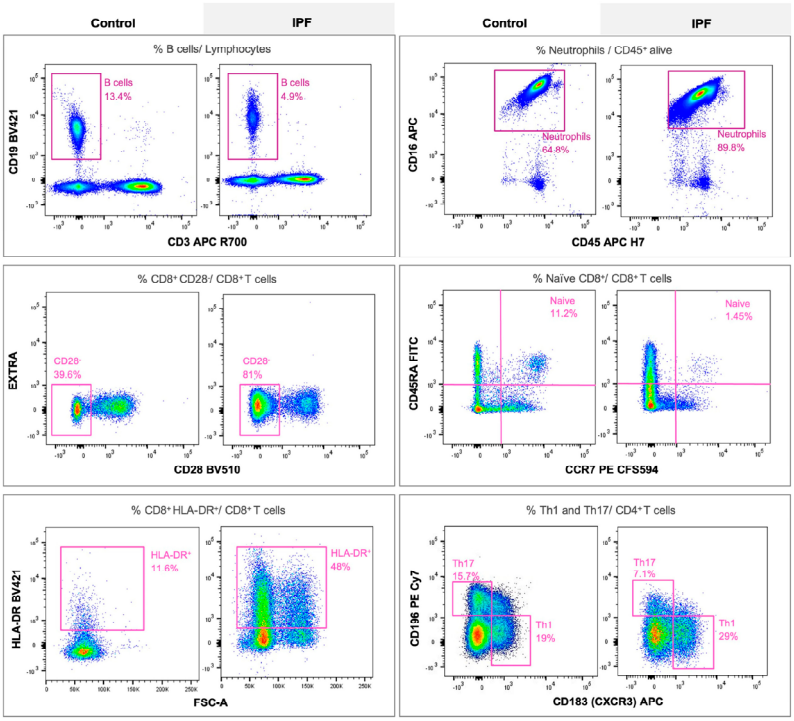

Figure 2. Representative flow cytometry gatings of controls and IPF patients of the peripheral immune populations that were statistically significant between the study groups: comparison of neutrophils and B lymphocytes, $CD8^+CD28^-$, $CD8^+HLADR^+$ and naive $CD8^+$ T cells from $CD8^+$ pool, and Th1 and Th17 from $CD4^+$ pool.

2.3. Association between Blood Immunophenotype and Severity of IPF at Baseline

Figure 3a presents the correlation between the immune cell types and lung function at baseline in IPF patients. FVC was positively related to naïve $CD4^+$ T cells and negatively related to central memory $CD4^+$ T cells. DLCO was positively related to the proportion of circulating eosinophils and lymphocytes, and negatively related to neutrophils, the neutrophile-to-lymphocyte Ratio (NLR) and the monocyte-to-lymphocyte Ratio (MLR) (Table S4).

2.4. Relationships between Baseline Blood Immunophenotype and Disease Progression

Figure 3b (and Table S5) shows that, compared to stable patients, at baseline the percentage of circulating NKT-like cells, $CD8^+$ T cells, effector memory $CD4^+$, and $CD8^+CD28^-$ T cells was higher, and that of naive $CD4^+$ and $CD8^+$ T cells and central memory $CD8^+$ T cells lower, in progressors. Accordingly, the CD4/CD8 ratio was lower in progressors, suggesting that a predominant T cell response to intracellular antigens ($CD8^+$) [13] is associated with worse prognosis despite the use of antifibrotic treatment. Again, no statistically significant differences in the percentage of PD-1+ T cells were observed between those who were stable and progressors; a two-way ANOVA was used to evaluate the effect of age on $CD8^+CD28^-$ T cells and naïve $CD4^+$ and $CD8^+$ T cells, which was still significant ($p = 6.67 \times 10^{-5}$, $p = 0.003$, $p = 0.017$, respectively). Table S6 provides the differences between the three groups: controls, those who were stable, and progressors, showing the latter having one the greatest differences.

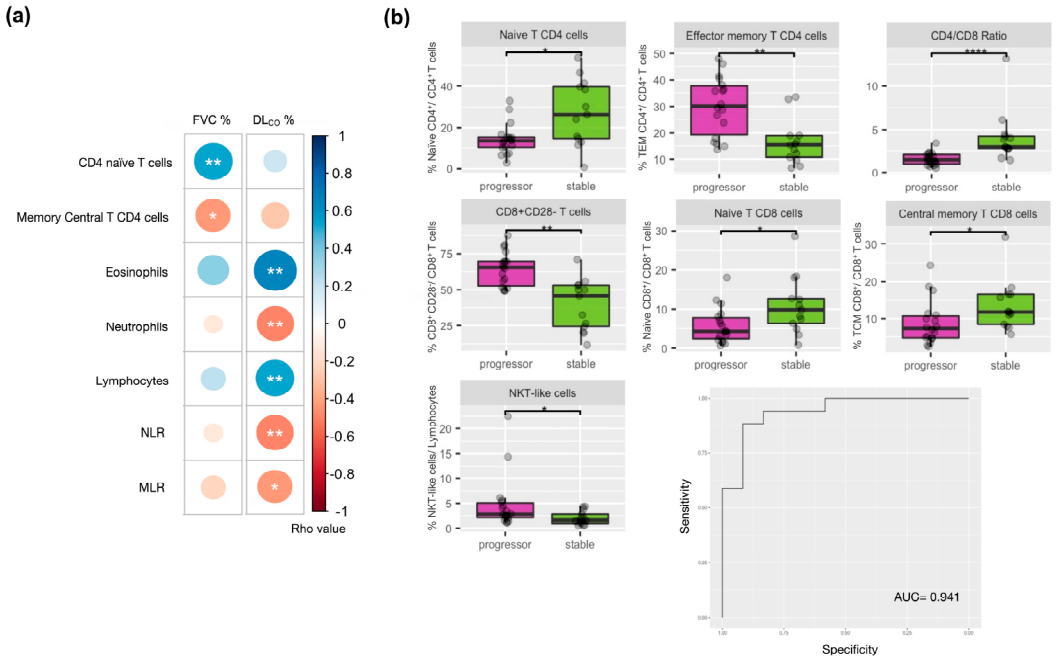

Figure 3. (**a**) Significant Spearman correlations between blood immune populations/ratios and lung function at recruitment in IPF patients. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$. (**b**) Differential distribution of the baseline immune population in IPF progressors (pink box) and stable (green box); (median [IQR]): Comparison of % NKT-like cells; $CD8^+CD28^-$ from $CD8^+$ pool, different $CD8^+$ and $CD4^+$ T memory cells and CD4/CD8 ratio; Receiving Operating Characteristics (ROC) curve of the Elastic Net multivariate analysis identifying the immune cell populations associated with disease progression.

2.5. Machine Learning Classification

We used a machine learning method (Elastic Net [14]) to investigate what combination of immune cells, determined at baseline, maximized the classification between progressors and stable patients. The constructed model showed that the combination of T $CD8^+CD28^-$, memory effector $CD4^+$ T cells, CD4/CD8 ratio, and NKT-like cells offered the best classification model of stable vs. progressor patients (Figure 3b, AUC of 0.94 and accuracy of 0.86).

2.6. Reproducibility of IPF Immunophenotypes at Long-Term

To assess the stability and reproducibility of the immune differences observed at baseline between stable and progressors, we determined the immune profile again in alive patients (stable or progressor) during 18 to 60 months of follow-up. Figure 4a,b presents the paired analysis between the baseline and the long term assessment, and shows that the populations included in the machine learning classification model, $CD8^+CD28^-$, memory effector $CD4^+$ T cells, CD4/CD8 ratio, and NKT-like cells, were stable in the long-term.

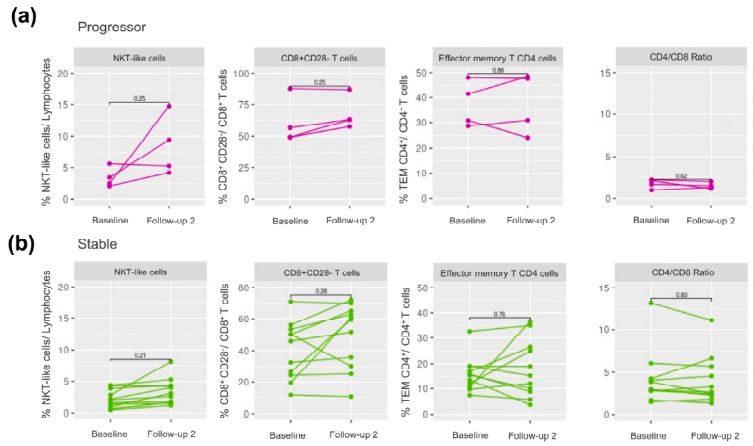

Figure 4. Paired analysis comparing the stability of the immune cell populations included in the Elastic Net, at baseline and second follow-up (18–60 months) in (**a**) progressors and (**b**) stable patients.

3. Discussion

The three main novel observations of this study are that: (1) abnormalities in both the innate and adaptive immune response can be detected in the circulating blood of patients with IPF at diagnosis; (2) some of these abnormalities relate to the severity of lung function at diagnosis; and, (3) a specific and longitudinally stable immune phenotype characterized by increased NKT-like cells, $CD8^+$ T cells with an exhausted phenotype, and less naïve T cells, with an impaired CD4/CD8 ratio, is associated with IPF progression over time (AUC 0.94), despite the use of antifibrotic treatment. Collectively, these observations contribute to a better understanding of the role of the immune response in IPF, provide new prognostic biomarkers of potential utility in clinical practice, and pinpoint novel potential therapeutic targets that deserve further research.

3.1. Previous Studies and Interpretation of the Findings

Neutrophils are key players in the acute phase of inflammation but, chronically, they can lead to tissue remodelling and fibrosis [15]. Previous studies in IPF have shown that neutrophil attraction and activation markers, such as IL-8 (interleukin-8) and G-CSF (granulocyte colony-stimulating factor), are increased in broncho-alveolar lavage fluid (BALF) and sputum, and that they may predict future exacerbations in IPF [16]. In blood, it has been seen that neutrophils and NLR can be an indicator of disease progression in IPF and other fibrosing interstitial lung diseases [17,18]. Our results are in keeping with these previous observations, adding, however, that they can already be observed in peripheral blood at the time of IPF diagnosis, particularly in patients with lower DLCO.

Some previous publications have suggested that BALF eosinophilia can be a marker of disease progression in IPF [19,20]. Others showed that higher peripheral eosinophil counts were associated with reduced lung function (FVC and DLCO), although they were not associated with disease progression, exacerbations or antifibrotic discontinuation [21]. In our study, we found that circulating eosinophils are positively correlated with DLCO% ref. at recruitment (Figure 3a, Table S4) but did not correlate with disease progression during follow-up (Table S5).

Previous studies reported that peripheral blood T cells in patients with well-established IPF present a surface signature characterized by the loss of co-stimulatory molecules, specifically CD28 [10]; our results at diagnosis (hence, the earlier stages of disease) agree with these previous findings. T $CD28^-$ cells are antigen-experienced memory T cells that accumulate in multiple diseases [22]. Considered "exhausted" cells, they have short

telomeres, express markers of senescence, and secrete high levels of perforin, granzymes, IFNγ, and TNFα [10]. Previous reports have shown an abundance of $CD8^+CD28^-$ T cells in explanted IPF lung tissues (end stage disease) and reported that they predict poor prognosis [9,10,22]. Our results complement these findings, showing that at diagnosis in blood, the percentage of $CD8^+CD28^-$ T cells is associated with a poor prognosis and lung function decline (both FVC and DLCO). On the other hand, it is well-known that the balance between activating and survival markers is critical for the homeostatic maintenance of T cell responses [23]. In this setting, an increase in T $CD8^+CD28^-$ might reflect a continuous increase in the effector T cell subset, causing a state of activated but inefficient immunological responses that alters the distribution of the memory $CD8^+$ T cell population which, in turn, may contribute to the ongoing fibrotic scenario by a deficient clearance of intracellular antigens [24]. This hypothesis is in keeping with the clinical observation that the pharmacological inhibition of the immune response is associated with worse prognosis, and our observation that progressors present abnormalities in the CD4/CD8 ratio at recruitment that are maintained at follow-up. The normal ratio of CD4/CD8 is $\geq$1.8 [25], and an inverted ratio (i.e., <1) has traditionally been associated with immune senescence, myelodysplasia, and persistent viral infections such as HIV (Human immunodeficiency virus), HCMV (Human cytomegalovirus), and EBV (Epstein–Barr virus) [25–27]. Some previous studies have reported a higher prevalence of seropositive EBV, HCV (Hepatitis C virus), and HCMV in patients with IPF, suggesting that viral infections may play a role, either as agents that predispose and/or aggravate lung fibrosis [28]. Further studies are required to unveil if the altered CD4/CD8 ratio that we report in progressive IPF is associated with persistent viral infections.

Concomitant with the CD8 decrease, we observed an increase in NKT-like cells in progressors. These cells are instrumental in the response to infections, tumours, and autoimmune diseases [29]. Interestingly, although this population slightly increased over time in stable patients, this increase was smaller than that observed in progressors.

Still, little is known about the T cells phenotype in circulating blood in patients with IPF at diagnosis and its evolution with time. Here, we observed that, at diagnosis, IPF patients have reduced Th17 lymphocytes and increased Th1, leading to disrupted Th1/Th17 and Th17/Tregs ratios. A depletion of circulating Th17 cells, along with a non-compromised regulatory T cells (Treg) compartment (similarly to what is observed in cancer) has been previously described by Galati et al. in the blood of IPF patients [30]. Here, we observed a tendency towards increased Treg in IPF, approaching statistical significance ($p = 0.07$).

Finally, a reduction in the proportion of naive $CD4^+$/$CD8^+$ T cells and T cell repertoire have also been reported in patients with IPF in relation to immune-senescence which, in turn, relates to impaired virus-specific T cell responses [31]. In this setting, we found a positive correlation between circulating naive $CD4^+$ T cells and baseline FVC, and reduced levels of both circulating naive $CD4^+$ and $CD8^+$ T cells in IPF progressors at both recruitment and a trend in the follow-up, despite the use of anti-fibrotic treatment.

In light of our findings, we hypothesize that an inefficient immune response may allow the disease to progress. However, additional studies are needed to explore if the immunophenotype is already altered in patients with minimal interstitial changes before the disease onset, if the immunophenotype might be different in progressors with or without acute exacerbations, and if our AI model could be of use in this setting to predict such outcomes.

3.2. Clinical Implications

The advent of new anti-fibrotic treatments improved the prognosis of patients with IPF [32] but, for reasons still unclear, some patients progress despite their use [33]. Our results here show that an exhausted cytotoxic blood immune profile is associated with disease progression despite the use of anti-fibrotic treatment (Figure 3b). These observations have two important clinical implications. First, they highlight potential targets for new studies and therapies directed to this exhausted phenotype. And second, though it needs

to be confirmed in larger studies, they provide potentially relevant prognostic information for the practicing physician.

3.3. Strengths and Limitations

The main strength of our study is that it characterizes in detail a wide range of immune cell types in circulating blood, an easily accessible tissue in clinical practice or clinical research, at the time of IPF diagnosis. Likewise, the fact that these patients were followed up over time allowed us to investigate the relationship of these baseline immune-phenotypes with disease progression. Among the potential limitations, we acknowledge that our sample size is relatively small, and that there were more females among the controls, but were matched by two major confounders in cell populations, age and smoking [34,35]. Likewise, our results need validation in larger, likely multicentre, cohorts to confirm the predictive power of our classifying model.

4. Materials and Methods

4.1. Study Design and Ethics

This was a prospective, observational, and controlled study that enrolled 32 consecutive patients who were diagnosed with IPF in the Pneumology Service of Clinic Barcelona between July 2016 and July 2021, and 32 age- and smoking-matched healthy individuals. Most IPF patients $n = 26$ (81.3%) were recruited at the time of diagnosis (some were referred from other centres where the diagnosis of IPF was already established and/or treatment initiated). Thirty one of them (96%) were followed up for 60 months (Figure S1). A first clinical visit at 12 months was used to determine disease stability or progression (see below). Survivors ($n = 15$) were visited again at 18–60 months. Biological samples were collected at recruitment and at 18–60 months. The diagnosis of IPF was established according to current international recommendations (ATS/ERS/JRS/ALAT) by a multidisciplinary committee [36]. All patients were treated according to international guidelines [37]. The Ethics Committee of our institution approved the study (HCB/2017/0901, 23 November 2017; HCB/2019/0687, 11 July 2019) and all participants signed their informed consent.

4.2. Measurements

4.2.1. Lung Function and Disease Progression

Forced spirometry and the single breath carbon monoxide diffusing capacity of the lungs (DLCO) were measured according to international standards. Reference values were those of Roca et al. [38,39]. Disease progression was defined as an annual decline of relative forced vital capacity (FVC) $\geq$ 10%, DLCO $\geq$ 15% and/or death in the first year.

4.2.2. Fluorescence-Activated Cell Staining Analysis (FACS)

We used FACS to profile B cells, T cells (and subpopulations), NK cells, NKT cells, monocytes, neutrophils, and eosinophils (Table S1). Briefly, 120 µL of blood was incubated with 30 µL of the antibody mix (Table S2) for 30 min at 4 °C. Then, erythrocytes were lysed (BD FACS Lysing Solution, San Jose, CA, USA) and cells were incubated with the Fixable Viability Stain (440UV, BD 566332) for 15 min at room temperature in the dark, washed, and fixed using PFA 4%. Fixed samples data were acquired with a LSRFortessa SORP (BD, San Jose, CA, USA). FlowJo version 10 software (FlowJo LL, Ashland, OR, USA) was used for analysis. The gating strategy to determine the populations is described in Figures S2 and S3.

4.3. Data Analysis

Results are presented as number, percentage, mean $\pm$ SD or median [95% CI]. The normality of the distribution of immune cell populations was tested with the Shapiro–Wilkinson test, and groups were compared via *t*-test or Mann–Whitney test, as appropriate. Correlations between immune cell populations and clinical parameters were tested using

the Spearman test and considered significant if Rho > |0.3| and the p value < 0.05. All statistics were computed with R version 3.6.2 (12 December 2019) using custom scripts.

5. Conclusions

Patients with IPF show significant alterations in the peripheral blood immunological profile at diagnosis, both in their innate and adaptive immune responses, particularly in the cytotoxic compartment. Moreover, IPF progression is associated with T $CD8^+$ cells dysregulation, and an inverted CD4/CD8 ratio suggesting an over-activated, aged, and "exhausted" immune status potentially related to intracellular antigens.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms241813832/s1.

Author Contributions: Study conceptualization and design, R.F., J.S. and A.A.; methodology and data acquisition, N.M., F.H.-G., S.C.-R., T.C., N.A., G.L.-S., L.R., M.R., X.A.-R. and A.F.-G.; data analysis: N.M., S.C.-R., N.O., F.H.-G., R.F., J.S. and A.A.; writing—original draft preparation, R.F., A.A., N.M. and J.S.; writing—review and editing, all.; funding acquisition, R.F. and J.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data generated or analysed during this study are included in this published article [and its Supplementary Information files].

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4.4. METHODOLOGICAL CONSIDERATIONS FOR AIM 2 (PAPER II):

Transcriptomics and immune cell deconvolution

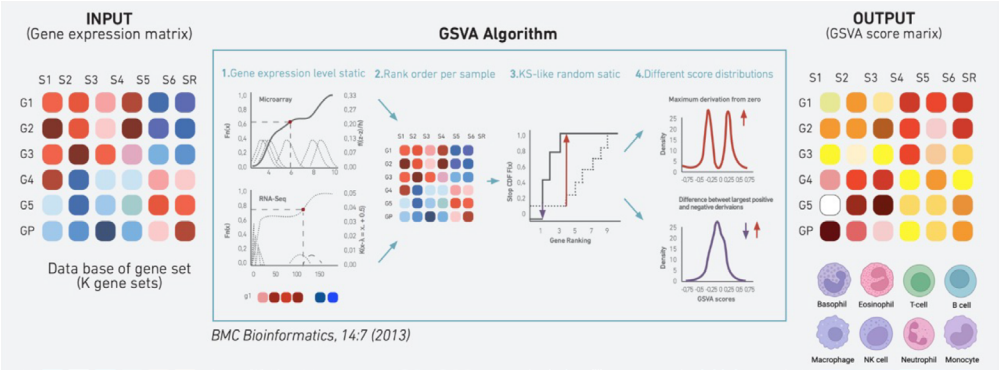

Figure 1. GSVA pipeline outline. The input for the GSVA algorithm is a gene expression matrix in the form of microarray expression values or RNA-seq counts and a database of gene sets. The output of the algorithm is a matrix containing the enrichment scores for each gene set analysed in each sample. Adapted from BMC *Bioinformatics* 14, 7 (2013).

Whole genome expression by microarrays was introduced in 1995, since then this technology has been widely used in all fields, and specifically in biomedical research as a tool to assess the underlying pathobiology and heterogeneity of human diseases. Currently advances in transcriptomics moved towards RNA-seq and single-cell RNA-seq. Gene set enrichment (GSE) analysis is a popular framework for condensing information from gene expression profiles into a pathway or signature summary. The strengths of this approach over single gene analysis include noise and dimension reduction, as well as greater biological interpretability.

Gene Set Variation Analysis (GSVA), is a GSE method that estimates the variation of activity of signatures (sets of genes of interest) over a sample population in an unsupervised manner (144). Since 2009 several works have described the transcriptomic profile of different isolated immune cell types (145, 146). This information, in combination with GSE methods, has been used by several groups, mainly in cancer (145) to infer the immune cell signature composition in tissues of interest. Generally, this computational approach is termed "cell deconvolution" and, coupled with immune histochemistry (IHC) validation approaches, has been proved to represent well the immune composition of a tissue (147). Even though the main information is not from isolated lung resident cells, these methods can be seen as the first approach to studying the immune composition of complex tissues such as the lung.

4.5. SUMMARY OF THE MAIN RESULTS FROM SPECIFIC OBJECTIVE II:

Concerning the **second aim**, the main and **novel findings** of this work (Paper II) showed that:

1 the unbiased cluster analysis of enriched immune signatures in the LTRC differentiated two clusters in the IPF continuum in dataset 1 (D#1), C#1 and C#2, the former with higher immune expression than the later one;

2 main differences in the immune infiltrate were those related to cytotoxic cells and memory T cells;

3 those clusters identified differed also in some clinical parameters: C#1 (higher immune expression) included slightly younger individuals, with more symptoms (short of breath, cough, etc) and less low attenuation areas by CT scan;

4 a sensitivity analysis showed that there were no differences in the immune signatures' enrichment between upper and lower lobes, only differences in the CT scan parameters with increased extent of fibrotic areas in the lower lobes;

5 all results were replicated in the validation dataset (D#2);

6 unbiased clustering of flow cytometry data from IPF explanted lung homogenates from another cohort confirmed also the existence of two clusters that differed in the percentage of $CD4^+$ and $CD8^+$ T cells, B cells, NK, NKT-like cells and macrophages;

7 a hypergeometric test comparing differentially express genes (DEG), their related ontologies, and biological gene signatures already reported in IPF, between the two clusters showed that C#1 presented ontologies related to activation of the immune response, viral response, and immune infiltrate, while C#2 presented ontologies related to ciliary function and altered epithelial lineage.

4.6. ORIGINAL PAPER II:

Lung immune signatures define two groups of end-stage IPF patients.

Cruz T, Mendoza N, Casas-Recasens S, Noell G, Hernandez-Gonzalez F, Frino-Garcia A, Alsina-Restoy X, Molina M, Rojas M, Agustí A, Sellares J, Faner R. Respir Res. 2023 Sep 28;24(1):236. doi: 10.1186/s12931-023-02546-8. PMID: 37770891; PMCID: PMC10540496. Impact factor: 7.16 (Q1).

(Supplementary material is enclosed in Appendix II).

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Lung immune signatures define two groups of end-stage IPF patients

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Abstract

Background The role of the immune system in the pathobiology of Idiopathic Pulmonary Fibrosis (IPF) is controversial.

Methods To investigate it, we calculated immune signatures with Gene Set Variation Analysis (GSVA) and applied them to the lung transcriptome followed by unbiased cluster analysis of GSVA immune-enrichment scores, in 109 IPF patients from the Lung Tissue Research Consortium (LTRC). Results were validated experimentally using cell-based methods (flow cytometry) in lung tissue of IPF patients from the University of Pittsburgh (n = 26). Finally, differential gene expression and hypergeometric test were used to explore non-immune differences between clusters.

Results We identified two clusters (C#1 and C#2) of IPF patients of similar size in the LTRC dataset. C#1 included 58 patients (53%) with enrichment in GSVA immune signatures, particularly cytotoxic and memory T cells signatures, whereas C#2 included 51 patients (47%) with an overall lower expression of GSVA immune signatures (results were validated by flow cytometry with similar unbiased clustering generation). Differential gene expression between clusters identified differences in cilium, epithelial and secretory cell genes, all of them showing an inverse correlation with the immune response signatures. Notably, both clusters showed distinct features despite clinical similarities.

Conclusions In end-stage IPF lung tissue, we identified two clusters of patients with very different levels of immune signatures and gene expression but with similar clinical characteristics. Weather these immune clusters differentiate diverse disease trajectories remains unexplored.

Keywords Immune-signatures, Transcriptome, Idiopathic pulmonary fibrosis, Flow cytometry

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Introduction

Idiopathic Pulmonary Fibrosis (IPF) is an interstitial lung disease of unknown origin characterized by progressive lung fibrosis [1]. The pathogenesis of IPF is complex and still unclear. Previous studies of whole genome transcriptomics have described alterations in different molecular pathways in end-stage IPF lungs, including aberrant activation of epithelial cells that promote fibroblast to myofibroblast differentiation [2, 3], excessive production of extracellular matrix proteins, such as matrix metalloproteases (MMPs), collagen and fibronectin [4, 5], aberrant activation of lung developmental pathways [6, 7], mitochondrial abnormalities [8, 9] and oxidative stress [9, 10, and type II epithelial cells and fibroblasts senescence [2, 11, 12]. The combination of all these pathogenic mechanisms leads to a highly heterogeneous disease, in which the identification of disease endotypes is an important unmet clinical need to move toward precision treatment [13].

In this setting, the role of the immune system is unclear. Some studies have proposed a role of immune pathways such as CD3+and CD20+lymphocytes in the development of fibrosis [14, 15] through the promotion of epithelial to mesenchymal transition (EMT) [4, 7, 15–17]. Further, the progression of IPF and the occurrence of exacerbations was associated with B cell responses [18, 19] through their capacity to modify the pro or antifibrotic lung micro-environment, thus influencing fibroblasts activity [20]. However, other findings challenge the role of the immune response in IPF [21]. First, clinical trials with immune-suppressive agents showed increased mortality and fibrosis in treated patients [22]. Second, the expression of markers of lung T lymphocytes exhaustion (such as PD-1, ICOS and CD28) is associated with enhanced TGF-β production and poor survival in IPF [23, 24]. Finally, the proportion of NK cells with impaired activity is reduced in IPF lungs [25] and their functionality is profoundly compromised by the lung microenvironment [26].

We therefore hypothesized that it is likely to be significant immune-related molecular heterogeneity in patients with IPF. To test this hypothesis, we used gene set variation analysis (GSVA) in lung tissue samples of patients with IPF, instead of previous studies using conventional analysis of single-gene expression. GSVA is a statistical technique that enables the discovery of inflammatory and leukocyte lineage gene signatures by comparing combined enrichment scores (ESs) of established and predefined gene sets, especially in heterogeneous samples [27, 28]. Specifically: (1) we first applied GSVA to lung transcriptomic data of 109 severe IPF patients (explanted lungs) available at the Lung Tissue Research Consortium (LTRC) to estimate the proportion of immune cells in their lungs; (2) we then used unbiased cluster analysis to identify distinct groups of IPF patients with overall distinct level of immune signatures; and, finally, (3) we explored differential gene expression between observed clusters, both for newly identified signatures as well as for previously stablished IPF related pathways.

Methods

Availability of data and materials

The datasets supporting the conclusions of this article are available in the NIH public repository Lung Tissue Research Consortium (LTRC), https://www.nhlbi.nih.gov/science/lung-tissue-research-consortium-ltrc. Tables with the full results of the analysis performed to support the conclusions are available in the online supplement.

Study design, patients and ethics

Transcriptomic data of IPF explanted lungs (n=109) was obtained from the LTRC following established procedures. Experimental validation using cell-based (not mRNA) methods (flow cytometry) was performed in lung tissue samples of IPF patients undergoing bilateral lung transplant at the University of Pittsburgh (USA). The Institutional Review Board and the Committee for Oversight of Research and Clinical Training Involved Decedents of the University of Pittsburgh, approved the study and the sample transfer respectively. In all cases, a signed informed consent form was collected before organ procurement.

Clinical characterization of IPF patients

Available clinical data in LTRC include age, sex, body mass index, Forced Expiratory Volum (FEV1), Forced Volum Capacity (FVC), carbon monoxide diffusing capacity (DLCO), quantify Computed Tomography (CT) of the thorax by an adapted version of the CALIPER software and daily activity and health questionaries. All procedures were realized following LTRC protocols, the diagnosis of IPF was performed by a specialist evaluating the medical record, CT scan report and the post-transplant pathology report.

GSVA, immune-signatures enrichment and unbiased cluster analysis

We analyzed the transcriptomic data set GSE47460 from the LTRC [29]. This data set was split in two, GPL14550 was used as a discovery data set (D#1, n=109) whereas GPL6480 was used for validation (D#2, n=34). For the current analysis we used the normalized matrix downloaded from GEO, selecting only patients with a diagnosis of IPF. Gene set variation analysis (GSVA) was used to determine patient-wise enrichment scores (ES) that indicate the relative collective expression of genes within

the gene signatures for patients relative to the rest of the cohort of patients in a given transcriptomic dataset [30]. Sets of the immune signatures used were based on available gene expression publications (n = 31, Additional file 2: Table S1) [27, 31]. Unbiased clustering of the GSVA immune signatures were identified using the dendextend R package in R [32]. To maximize the differences in the GSVA scores, the number of clusters was set at 2, the distance metric was calculated with the minkowski method and the hierarchical clustering method was ward. D2 [32].

Differential gene expression between clusters was investigated using limma [33]. To build the correlation network with the clinical parameters and to further understand the relationship between the immune and epithelial cells in these patients, the gene sets included in our GSVA analysis were extended, while preserving the already obtained immune-based unbiased clustering, to include epithelial lineage cell signatures (skipping genes already included in the immune cell signatures) (Additional file 2: Table S2).

Experimental validation of LTRC results in fresh lung tissue samples by flow cytometry

To validate results from the GSVA immune enrichment in the LTRC, we used flow cytometry, a non-mRNA related method. Fresh lung tissue samples of IPF patients undergoing bilateral lung transplant at the University of Pittsburgh (USA) were washed with PBS and enzymatically digested as previously described [34]. Lung homogenates included multiple areas of the same lung lobe, ensuring the representability of the sample to address patient's heterogeneity. Lung tissue homogenates (10^6 cells) were then stained 5 min with the viability staining (Fixable viability-Alexa600, BD, USA) and 30 min at 4°C in the dark with the following conjugated monoclonal antibodies CD3-PECy5.5, CD45-Alexa700, CD16-BV412, CD56-FITC, CD8-V500, CD4-APC-Cy7, CD19-BV650 (BD, USA) and CD14-PE (BioLegend, USA). A minimum of 5×10^5 cells per sample were acquired in a FACS LSRII (BD Biosciences, USA), and data was analyzed using FlowJo v10 (FlowJo LLC, USA). Immune cell populations were determined using the gating strategy depicted in Additional file 1: Fig. S1.

Biologic pathway analysis

To evaluate the enrichment of biological signatures in the observed clusters, gene ontology (GO) enrichment and hypergeometric tests were used [35]. The gene signatures for the hypergeometric test were selected from previously published sc-RNAseq studies: epithelial cells signatures [36–39] and fibroblast related signatures [37–41]; or from the Gene Ontology (GO) extracellular matrix (GO:0031012), oxidative stress (GO:0000302), mitochondrial transport (GO:0006839), mitochondrial respiratory chain (GO:0005746) and response to stress (GO:0006950). Additional file 2: Table S3 shows the complete list of gene signatures investigated here.

Statistical analysis

Quantitative and qualitative data is presented as mean, or n and proportion, respectively. Results were compared using the ANOVA or Fisher tests, as appropriated. Differences in the distribution of the GSVA calculated signatures between clusters were assessed with the ANOVA test too. Correlations between immune cell signatures and clinical features were assessed using the Spearman correlation test, which was considered statistically significant if its r value was $>|0.5|$ and the p value < 0.05. To explore correlations between biological and clinical features, we used network analysis, where each node was the variable of interest, its size was proportional to its mean value in each cluster, and links (edges) represent the Spearman Rho between linked variables, with results being plotted using Cytoscape [42]. All statistics were computed with R 4.2.2, using custom scripts.

Results

Cluster analysis of enriched immune-signatures in the LTRC

The main demographic and clinical characteristics of IPF patients included in D#1 and D#2 were similar (Additional file 2: Table S4). Briefly, the studied population presented the clinical characteristics of end-stage IPF disease, a severe impairment of the DLCO and FVC, and fibrotic features in the CT scan, presence of honeycombing, ground grass opacity, reticular densities and vessels. As shown in Fig. 1, in both data-sets (panels A and B) k-means unbiased clustering of GSVA enriched immune signatures identified two clusters of IPF patients (C#1 and C#2) with different levels of immune expression. Additional file 2: Table S5 shows the mean ES in each cluster and the p-value for the comparison of both clusters. C#1 had a higher ES than C#2 in all analyzed immune signatures except for three of them where no significant differences were observed between clusters. The biggest differences were found in cytotoxic cells (both adaptive CD8 + T cells and innate NK lymphocytes) and memory T cells.

Table 1 compares the main clinical differences between IPF patients included in the two clusters (C#1 and C#2) identified in D#1. Briefly, C#1 (high immune expression) included slightly younger individuals, with more symptoms, and less low attenuation area by CT scan. These differences were reproduced in the two clusters determined in D#2 (Fig. 1B and Additional file 2: Table S6).

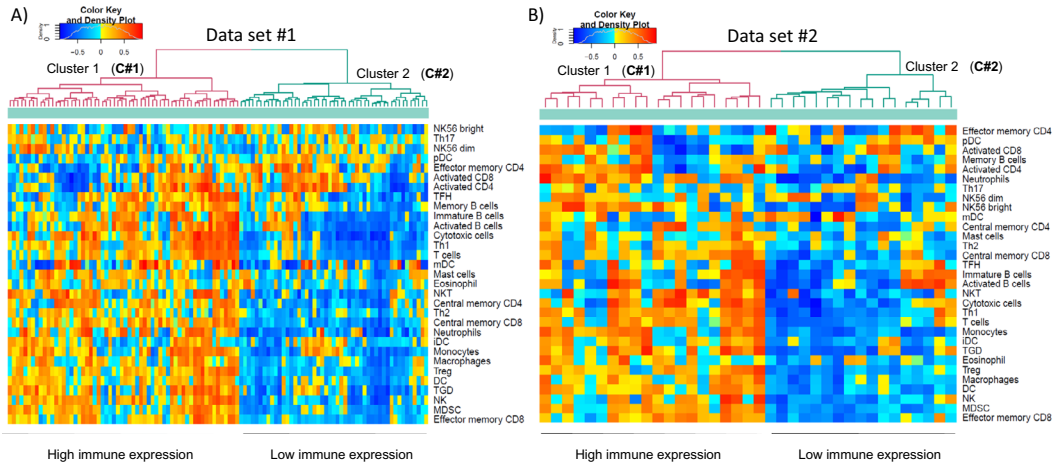

Fig. 1 Unbiased clustering of obtained GSVA-immune enrichment scores, in the IPF LTRC samples. **A** Data-set 1 and **B** Data-set 2. The density color keys at the top left of each figure define the scoring for each gene signature ranging from – 1 in blue to 1 in red

Table 1 Main clinical characteristics of the two IPF GSVA clusters in D#1. Statistically significant results are highlighted in bold

	Cluster 1			Cluster 2			p-value
	n	mean	SD	n	mean	SD	
Age	54	63.67	8.73	46	66.91	7.24	**0.048**
Weight (kg)	55	88.36	17.31	47	91.73	18.05	0.338
BMI	55	30.48	4.97	47	30.67	5.82	0.852
Smoking (pack/year)	39	28.45	24.3	27	23.02	18.25	0.329
Quit smoking (years)	39	21.11	13.47	27	24.25	13.92	0.361
GAP score (1–7)	60	4.64	1.23	49	4.94	1.16	0.207
Lung function test							
Predicted DLCO	55	29.37	5.69	47	30.35	4.9	0.357
Predicted FEV1	55	69.56	16.54	47	73.77	18.74	0.232
Predicted FVC	55	63.15	14.87	47	66.57	18.43	0.301
FEV1/FVC	55	0.83	0.06	47	0.83	0.06	0.973
TAC							
Total Segmented Volume with density less than -950 HU (cm^2)	29	155.27	140.98	21	244.15	164.3	**0.044**
Lower Attenuation areas (cm^2)	29	4.38	3.42	21	6.08	3.22	0.080
Ground Glass Opacity (cm^2)	29	17.15	14.38	21	11.22	11.11	0.119
Honeycombing (cm^2)	29	1.2	1.95	21	1.27	1.24	0.893
Normal (cm^2)	29	52.86	13.69	21	46.99	16.22	0.169
Reticular densities (cm^2)	29	4.14	3.03	21	4.47	3.56	0.719
Vessels (cm^2)	29	5.62	2	21	5.14	1.88	0.398
Categorical variables							
Short of breath when talk, n (%)	60	34 (56.7)		49	16 (32.6)		**0.023**
Cough disturbs sleep, n (%)	60	37 (45)		49	10 (20.4)		**0.014**
Long time to wash or dress, n (%)	60	22 (36.7)		49	6 (2.2)		**0.007**
Chronic bronchitis, n (%)	60	7 (12.1)		49	0 (0.0)		**0.044**

A sensitivity analysis, done using only the data of upper lobes or lower lobes showed that there were no differences in the immune signatures enrichment in upper *vs.* lower lung lobes (Additional file 1: Figure S2 and Additional file 2: Table S7, S8). Likewise, the direct comparison between different lobes did not identify differences in the immune-signatures ES, although we found the expected differences in CT scan parameters with increased extend of the fibrosis related parameters in the lower lobes (Additional file 2: Table S9).

Validation of results by flow cytometry in fresh lung tissue

To validate the above discussed results, we used flow cytometry in fresh lung tissue samples harvested from IPF explanted lungs (Additional file 2: Table S10). Further, to exclude the possibility that the two clusters identified above may actually correspond to pathology heterogeneity within the sampled lung lobe rather than differences between patients, for flow cytometry measurements we used lung homogenates from multiple areas to ensure proper representation of the whole pulmonary lobe. By doing so, unbiased clustering of flow cytometry data confirmed the existence of two clusters of patients that differed in the proportion of T-cells, CD4, CD8, B-cells, NK cells, NKT-like cells and macrophages (Fig. 2A).

Biological pathways

To gain insight into the biological process altered in the two clusters of IPF patients identified from the immune signatures enrichment in the LTRC, we investigated differentially express genes (DEG) in C#1 and C#2. Using an adjusted p value < 0.05 and log Fold Change (LgFC) $> |0.65|$ we found 777 DEG; 153 (19.7%) of them were upregulated in C#1 and 624 (80.3%) in C#2 (Additional file 2: Table S11).

Additional file 2: Table S12 lists the biological ontologies associated with these DEG. Of note, C#1 showed activation of immune response ontologies whereas C#2 included ontologies related to ciliary function. To contrast these results with pathways previously reported in IPF, we performed a hypergeometric test on DEG with specific IPF related signatures, including epithelial lineage, cell cycle, senescence, extracellular matrix, myofibroblast activation, response to pirfenidone treatment, oxidative stress, endoplasmic reticulum stress, mitochondrial related genes and immune lineage (Additional file 2:

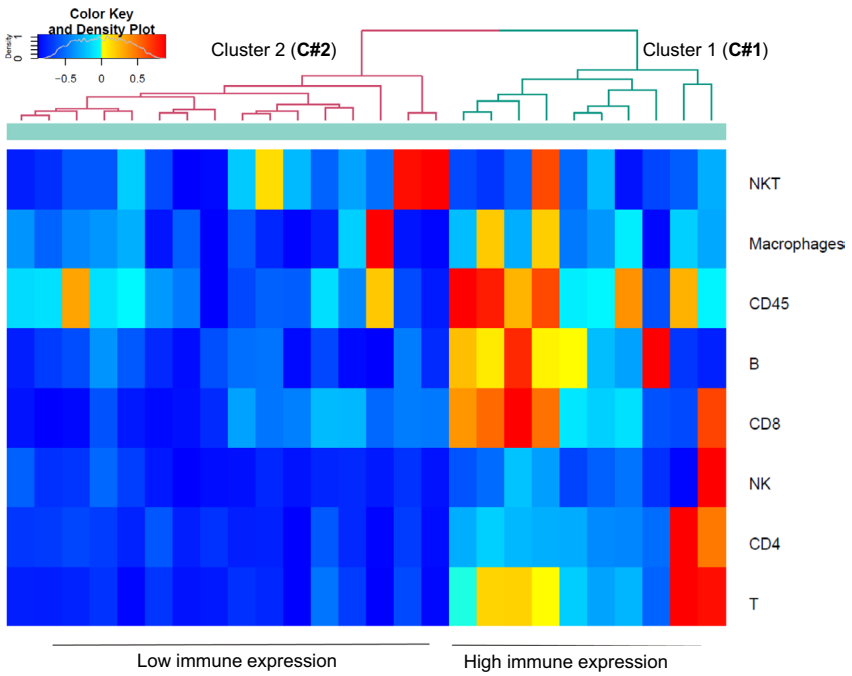

Fig. 2 Unbiased clustering of the flow cytometry data generated for the validation. Flow cytometry determination of the main lung immune populations followed by unbiased clustering showed the presence of two clusters of IPF patients based on their level of immune infiltrate in fresh lung samples. The density color key at the top left define the scoring for each gene signature ranging from (– 1) in blue to (1) in red

Table S3). We observed that C#1 showed increased viral response and immune infiltrate gene signature, thus supporting GSVA unbiased clustering results. By contrast, C#2 was characterized by altered epithelial cell lineage (Fig. 3 and Table 2), particularly upregulation of genes related to EMT, secretory and ciliated cells. Interestingly, there were no differences between C#1 and C#2 in fibrosis associated gene signatures (Fig. 3 and Table 2).

Network analysis

Finally, to further understand the relationship between the immune and epithelial cells in these IPF patients, the GSVA analysis was extended by adding the epithelial lineage cell signatures (Additional file 2: Table S2) and correlation networks were built considering the transcriptomic immune and epithelial signatures enrichment and the clinical characteristics of the patients. Of note, to analyze the relationship between CT findings and cell signatures we used only CT measures in the profiled pulmonary lobe. Additional file 2: Figure S3 shows a first neighbor correlation network of the clinical parameters and epithelial signatures in C#1 and C#2. In both clusters we observed a negative correlation between epithelial and immune cells (dashed edges (links)). Specifically, epithelial, ciliated, and secretory cell signatures were negatively correlated with central memory CD8 + T cells, Th2 T cells and immature B cells. Interestingly, only in C#2 we identified a correlation between the transcriptomic signatures and disease severity: a positive correlation with fibrosis associated CT parameters and a negative correlation with the FVC value.

Discussion

The main and novel observation of this study are that, by using unbiased cluster analysis of lung immune signatures in a large cohort of patients with IPF (n = 109),

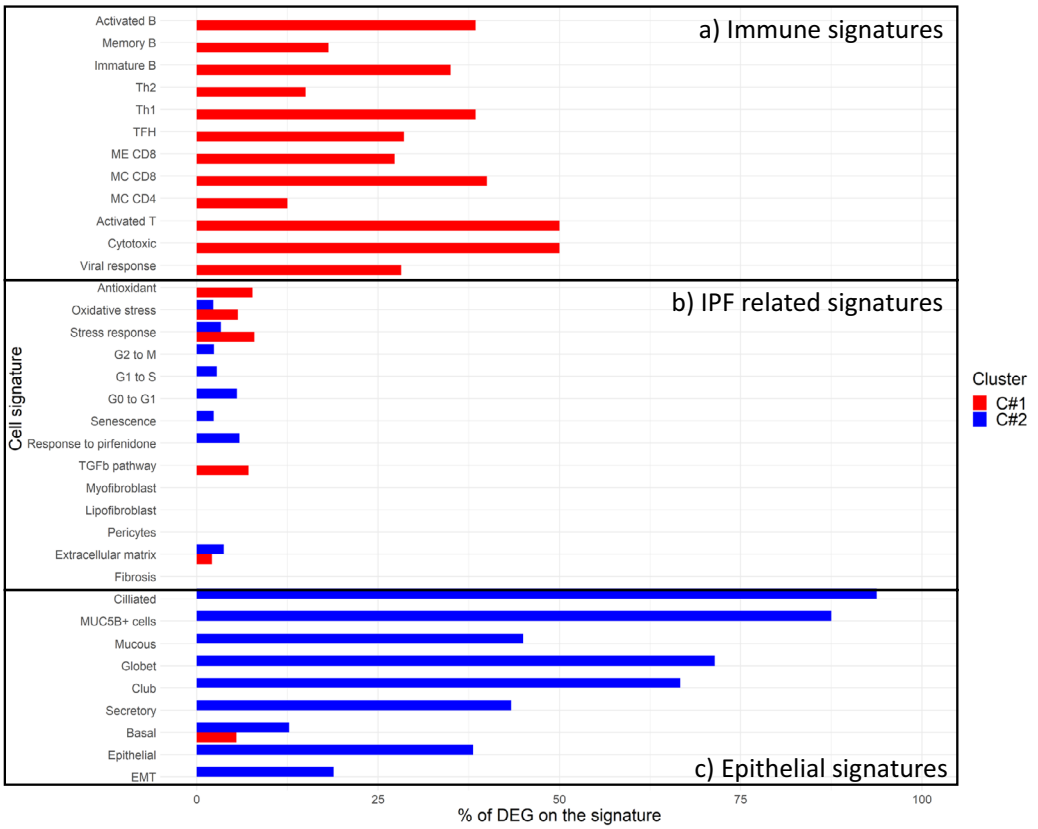

Fig. 3 Hypergeometric test of the percentage of cluster differentially express genes in the studied biological gene signatures. **A** Immune signatures, **B** IPF related signatures and **C** epithelial signatures

Table 2 Hypergeometric test comparing the percentage of differentially express genes between clusters that belong to the following specific signatures. Statistically significant results are highlighted in bold

	Cluster 1			Cluster 2		
	OR	**Matched genes (%)**	**p-value**	**OR**	**Matched genes (%)**	**p-value**
Immune signatures						
Activated T cells	59.30	50.00	**1.62E−03**	0	0	1
Activated B cells	37.55	38.46	**1.43E−06**	0	0	1
Activated CD4	0	0	1	0	0	1
Activated CD8	0	0	1	0	0	1
Central memory CD4	8.48	12.50	**0.03**	0	0	1
Central memory CD8	40.20	40.00	**8.92E−08**	0	0	1
Cytotoxic cells	59.78	50.00	**5.00E−06**	0	0	1
Effector memory CD4	0	0	1	1.85	8.33	0.44
Effector memory CD8	22.88	27.27	**2.34E−09**	0	0	1
Immature B cells	32.60	35.00	**2.12E−08**	0	0	1
Memory B cells	13.20	18.18	**0.01**	0	0	1
NK cells	4.55	7.14	0.21	0	0	1
NK CD56 bright	0	0	1	0	0	1
NK CD56 dim	0	0	1	0	0	1
NKT cells	9.86	14.29	0.11	0	0	1
T follicular helper	23.75	28.57	**5.50E−03**	0	0	1
TGD	5.76	8.82	**0.02**	0	0	1
Th1	38.30	38.46	**5.82E−12**	0	0	1
Th17	0	0	1	1.07	5.00	0.62
Th2	11	15	0	0.00	0.00	1.00
Viral response	24.15	28.20	**2.46E−11**	0	0	1
Epithelial signatures						
EMT	0	0	1	4.79	18.87	**1.49E−04**
Epithelial	0	0	1	12.65	38.10	**2.63E−06**
Basal cells	3.43	5.45	0.06	2.99	12.73	**0.01**
Activated basals	5.38	8.33	0.18	1.85	8.33	0.44
Aberrant basaloid	0	0	1	0	0	1
Primed basals	6.58	10.00	0.15	8.75	30.00	**0.01**
Proliferative basals	0	0	1.00	0	0	1
Multipotent basal	3.29	5.26	0.27	3.83	15.79	0.06
Secretory cells	0	0	1	15.82	43.33	**2.68E−10**
Club cells	0	0	1	40.89	66.67	**6.64E−05**
Globet cells	0	0	1	51.53	71.43	**4.03E−11**
Mucous cells	0	0	1	16.85	45.00	**1.09E−07**
Serous cells	0	0	1	7.98	28.00	**1.10E−04**
MUC5Bpos	0	0	1	143.70	87.50	**3.69E−09**
Cilliated cells	0	0	1	4.26	17.24	**0.01**
Cilliated cells type 1	0	0	1	82.57	80.00	**4.07E−14**
Cilliated cells type 2	0	0	1	102.45	83.33	**1.28E−06**
Differentiated cilliated	0	0	1	309.85	93.75	**1.52E−19**
IPF cilium associated signatures [11]						
Pattern A	0	0	1	2139.52	98.92	**1.23E−123**
Pattern B	1.00	1.67	0.64	54.66	71.67	**3.64E−44**
Fibrosis associated signatures						
Fibrosis	0	0	1	0	0	1

Table 2 (continued)

	Cluster 1			Cluster 2		
	OR	Matched genes (%)	p-value	OR	Matched genes (%)	p-value
Fibroblast activation	0	0	1	0	0	1
Lipofibroblast	0	0	1	0	0	1
Myofibroblast	0	0	1	0	0	1
Smooth muscle cells	0	0	1	0	0	1
Myofibroblast	0	0	1	0	0	1
Pericytes	0	0	1	0	0	1
Extracellular matrix	1.2	2.12	0.36	0.47	3.72	0.99
Matrix features	0	0	1	0	0	1
Response to pirfenidone	0	0	1	1.27	5.88	0.56
TGFb signaling	4.23	7.14	0.22	0	0	1
Senescence	0	0	1	0.48	2.33	0.87
G0 to early G1	0	0	1	1.20	5.56	0.58
G1 to S cycle	0	0	1	0.57	2.76	0.91
G2 to M cycle	0	0	1	0.49	2.37	0.96
Mitochondrial transport	1.21	2.17	0.57	0.28	2.17	0.96
Mitochondrial respiratory chain	0	0	1	0	0	1
Response to stress	5.25	7.94	**4.50E−08**	0.43	3.34	0.99
Oxidative stress	3.37	5.68	**0.02**	0.29	2.27	0.99
Pro-oxidant	5.82	9.52	**0.05**	0	0	1
Oxidative response	5.5	9.09	0.18	0	0	1
Antioxidant	4.61	7.69	0.07	0	0	1

we identified two clusters (C#1 and C#2) of similar size with different immune-related characteristics and differentially expressed genes: C#1 (n = 55, 53%) was characterized by a higher expression of immune signatures, particularly cytotoxic and memory T cells, whereas C#2 (n = 49, 47%) was characterized by an upregulated expression of cilium associated genes, epithelial and secretory cells (structural cell cluster). Interestingly, though, the clinical presentation of these two clusters was remarkably similar, indicating that at the end-stage of the disease the identified molecular heterogeneity does not translate directly into a different clinical phenotype. However, further research is need to understand whether these clusters are already present in earlier phases of the disease and/or associated with the disease progression.

Previous studies

A few previous studies used transcriptomic data to identify clusters of IPF patients. Using lung transcriptomics, Yang et al. identified a cilium associated subtype and a fatty acid metabolism one [43], but the expression of immune related genes or the associated cell types was not reported. Using blood transcriptomics, Kraven et al. described three clusters of IPF patients, one of them enriched in immune response genes [44]. Additionally, Herazo-Maya JD et al. identified a 52 gene signature on PBMCs that stratified patients with different disease outcomes [45, 46], and an increase of peripheral blood monocytes has been associated with poor prognosis [47]. Finally, De Sadeleer et al. used transcriptomic results of bronchoalveolar lavage fluid analysis identified 6 clusters in IPF patients, one of them again enriched in immune signatures [48]. Collectively, these studies support our observation of immune heterogeneity in IPF. To our knowledge, however, no previous study has used unbiased cluster analysis of IPF lung immune signatures enrichment. Importantly, results were validated experimentally in independent lung tissue samples using non-mRNA related method (flow cytometry).

Interpretation of novel findings

The application of this cutting-edge methodology to IPF lung tissue allowed us to identify two clusters of IPF patients (C#1 and C#2) with marked biological differences: while C#1 was an "*immune-cell*" cluster, particularly enriched in cytotoxic and memory T cells, C#2 was a "*structural cell*" cluster, with marked upregulation of cilium, epithelial and secretory cells genes. Because in the study mentioned above Yang et al. also identified a cilium associated IPF subtype using lung transcriptomics

[11], we explored the degree of overlap between their results and our identified clusters. The hypergeometric test showed that our C#2 shared a 99% and 72% of their described genes, indicating that our unbiased clustering of immune signature enrichment generates a similar grouping of IPF patients than the more traditional transcriptomic hierarchical clustering.

From the clinical viewpoint, it is of note that these two very different biologic clusters of patients with IPF show remarkably similar clinical characteristics (Table 1). We think that this may likely be due to the fact that lungs were harvested at transplantation, this is at an end-stage course of the disease. It is possible that at an earlier stage, clinical differences may have been more evident or that these two clusters represent different disease trajectories, varying in either rate of progression, frequency of infections or exacerbations and/or the response to treatment. All these possibilities require and deserve future research. This is the main limitation of the study, the lack of longitudinal information to understand the disease evolution, progression and a record of infections and exacerbations that could have a direct impact in the lung immunological state.

Conclusions

The use of unbiased clustering of the transcriptomic enrichment in immune signatures in lung tissue of patients with end-stage IPF identified two distinct clusters, an immune-cell one and a structural-cell one, with a negative correlation between the expression of immune and epithelial related signatures. These very different biological clusters are not related with clinical characteristics but whether they are present at an earlier stage and/or there is an association with disease phenotypes or progression should be further studied.

Abbreviations

IPF	Idiopathic Pulmonary Fibrosis
GSVA	Gene Set Variation Analysis
LTRC	Lung Tissue Research Consortium
C#	Cluster
NIH	National Institute of Health
mRNA	Messenger Ribonucleic Acid
FEV1	Force Expiratory Volume in 1 s
FVC	Force Vital Capacity
DLCO	Diffusing Capacity of Carbon Monoxide
CT	Computerized Tomography
ES	Enrichment Score
CD	Cluster of Differentiation
FACS	Fluorescence Activated Cell Sorting
sc-RNAseq	Single Cell RNA Sequencing
GO	Gene Ontology
ANNOVA	Analysis of Variance
NK	Natural Killer
D#	Dataset
NKT-like	Natural Killer T-cell like
DEG	Differentially Express Genes
LgFC	Logarithmic Fold Change
EMT	Epithelial Mesenchymal Transition
Th	T-cell Helper response

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-023-02546-8.

Additional file 1: Figure S1. Flow cytometry gating strategy to identify the main immune populations in the lung**.** Cell debris is excluded and single cells are selected using FSC VS SSC. Live cells are selected using a cell viability marker and the hematopoietic cells are selected as CD45+. Macrophages/monocytes are selected by gating the CD14 population. For lymphocyte determinations complex cells are excluded based on the SSC to reduce the lung autofluorescence. From this lymphocyte population, B lymphocytes are selected as CD19+, NK cells are selected as CD3-CD56+and T cells are CD3+. CD4+and CD8+T lymphocytes are selected from the CD3+population. **Figure S2**. GSVA unbiased clustering of the IPF samples dividing by the profiled lung lobe**. A** Upper lobe **B** Lower lobe. **Figure S3.** First neighbor correlation networks. **A** Cluster 1 **B** Cluster 2. Nodes represent the FC of the median between the 2 clusters. Lung clinical parameters are represented in octagons (CT scan are light green and pulmonary function test parameters are dark green), GSVA cell types in circles (lung cell types are turquois, cytotoxic cells are yellow, T cells are blue, B cells are pink and innate cells are light purple) and the biological pathways are denoted in rhombus. The width of the edges represents the correlation coefficient, negative correlations are marked with dotted lines and positive correlations are indicated with solid gray. $R>|0.5|$ and $p<0.05$.

Additional file 2: Table S1. Immune gene set signatures used in the GSVA pipeline to performed the unbiased clustering. **Table S2.** Extended gene set signatures with the epithelial and fibrosis associated signatures used in the GSVA to generate the correlation networks. **Table S3.** Hypergeometric test gene signatures for immune, epithelial and fibrosis related signatures. **Table S4.** Main clinical characteristics of D#1 y D#2. **Table S5.** Differences in immune cell signatures across clusters on D#1. **Table S6.** Differences in clinical characteristics and immune cell signatures across clusters on D#2. **Table S7.** Differences in clinical characteristics and immune cell signatures across clusters on D#1 filtering by upper lobes. **Table S8.** Differences in clinical characteristics and immune cell signatures across clusters on D#1 filtering by lower lobes. **Table S9.** Differences in clinical characteristics and immune cell signatures between upper and lower lobe. Results are expressed as median [95% coefficient interval] or mean (SD) as appropriate. **Table S10.** Main clinical characteristics of the validation cohort. **Table S11.** Statistically significant (adjusted p-value < 0.05) genes on the limma test of cluster 2 over cluster 1. **Table S12.** Gene ontologies enrichment of differentially express genes between the 2 clusters. On the left side the gene ontologies of the cluster 1 are represented (negative values of LgFC Cluster 2/Cluster1), on the right side the gene ontologies of the cluster 2 are represented (positive values of LgFC Cluster 2/Cluster1).

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Author contributions

Study conception and design: (RF, TC, AG, JS), data acquisition: (TC, SCR, NM, FH), data analysis: (TC, GN, SCR, NM, JS), manuscript preparation: (TC, RF, AA, JS), manuscript revision: All.

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Availability of data and materials
The datasets supporting the conclusions of this article are available in the NIH public repository Lung Tissue Research Consortium (LTRC) 45. Tables with the full results of the analysis performed to support the conclusions are available in the online supplement.

Declarations

Ethics approval and consent to participate
The Institutional Review Board and the Committee for Oversight of Research and Clinical Training Involved Decedents of the University of Pittsburgh, approved the study and the sample transfer respectively. In all cases, a signed informed consent form was collected before organ procurement.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Discussion

05

An extended discussion of each of the two main objectives of the present thesis isincluded in the published articles. However, here I will specifically **discuss and integrate the main findings of each objective of both studies**.

5.1. MAIN NOVEL OBSERVATIONS IN PAPER I: PERIPHERAL IMMUNE CELL PROFILE

The main novel observations of this study are that: **1)** abnormalities in both the innate and adaptive immune response can be detected in the circulating blood of patients with IPF at diagnosis; **2)** some of these abnormalities relate to the severity of lung function at diagnosis; and, **3)** a specific immune phenotype characterized by increased NKT-like cells, $CD8^+$ T cells with an exhausted phenotype, and less naïve T cells, with an impaired CD4/CD8 ratio is associated with IPF progression over time (AUC 0.94), despite the use of antifibrotic treatment; and **4)** the progressors immune phenotype is longitudinally stable. Collectively, these observations contribute to a better understanding of the role of the immune response in IPF, provide new prognostic biomarkers of potential utility in clinical practice, and pinpoint novel therapeutic targets that deserve further research.

5.2. MAIN NOVEL OBSERVATIONS IN PAPER II: LUNG IMMUNE ENDOTYPES

The main novel observation in this study are the following ones: **1)** two clusters (C#1 and C#2) were identified in the IPF continuum dataset D#1 after the unbiased cluster analysis used in the LTRC transcriptomic data, presenting the former a higher enrichment of immune signatures than the later one; **2)** main differences in the immune infiltrate were those related to cytotoxic cells and memory T cells; **3)** the two clusters differed also in some clinical parameters: C#1 (higher immune expression) included slightly younger individuals, with more symptomatology and less LAA by CT scan; **4)** no differences were found between upper and lower lobes, except a higher extend of fibrotic areas in the CT in the lower lobes; **5)** all results were validated in dataset (D#2); **6)** unbiased clustering in flow cytometry data from IPF explanted lung homogenates from an independent cohort also confirmed the existence of two clusters; **7)** a hypergeometric test comparing DEG, ontologies, and biological gene signatures between the two clusters showed that C#1 presented ontologies related to activation of the immune response, viral response, and immune infiltrate, while C#2 presented ontologies related to ciliary function and altered epithelial lineage.

5.3. GENERAL DISCUSSION

To date, the role of the immune system in the development and progression of IPF is still an area of active research and controversy. IPF was traditionally considered a primarily fibrotic disorder, but emerging evidence in the past years suggests that immune system dysregulation and chronic inflammation may contribute to its pathogenesis and may even determine its clinical evolution. The controversy arises from the question of whether the immune response is a primary driver of fibrosis, if it is a secondary response to tissue damage, or if it is simply an epiphenomenon. Some studies propose that chronic inflammation in the lungs, involving immune cells and well-known inflammatory pathways, contributes to the activation of fibroblasts and the deposition of extracellular matrix. However, the exact mechanisms and the nature of the immune response in IPF remain not fully understood.

In blood, it has been seen that neutrophils (and so, neutrophil-to-lymphocyte ratio (NLR)) can be an indicator of disease progression in IPF and other fibrosing interstitial lung diseases, as, when chronically active, they can lead to tissue remodelling and fibrosis (148, 149). Our results in the first study are in keeping with these previous observations, adding, however, that they can already be observed in peripheral blood at IPF diagnosis, and, particularly, in patients with lower DL_{CO} (% ref.), suggesting an association with the presence of PAMPs and/or DAMPs in the lung.

In the context of IPF, eosinophils were not considered a prominent feature. However, some studies showed that higher peripheral eosinophil counts were associated with reduced lung function (both FVC and DL_{CO}, % ref.), although they were not associated with disease progression, exacerbations or antifibrotic discontinuation (150). In our study, we found that circulating eosinophils were positively correlated with DL_{CO} (% ref.) at recruitment but did not correlate with disease progression during follow-up. Further studies are required to elucidate the role of eosinophils in the development of pulmonary fibrosis.

Previous studies reported that peripheral blood T cells in patients with well-established IPF present a surface signature characterized by the loss of co-stimulatory molecules, specifically CD28 (118); our results at diagnosis agree with these previous findings. Accumulation of T $CD28^-$ cells (antigen-experienced, highly differentiated and aged T cells) has been reported in multiple inflammatory disorders, such as autoimmune diseases, atherosclerosis and chronic viral infections (151). Considered "exhausted" cells, they are characterized by having short telomeres, expressing markers of senescence, apoptosis resistance and secreting high levels of perforin, granzymes, IFNγ, TNFα and other mediators that can damage tissues and amplify inflammatory pathways (151). Differential gene expression analysis from a previous study also showed that genes related to immune responses were upregulated in $CD28^-$ T cells, especially those related to the regulation of migration and recruitment of other cell types. In this same study, KEGG analysis showed that DEGs were enriched for biological pathways related to autoimmune diseases, infections or allograft rejection (152).

Previous works have shown the abundance of $CD8^+CD28^-$ T cells in explanted IPF lung tissues (end-stage disease) and reported that they predict poor prognosis (103, 118, 151). Our results complement these findings, showing that at diagnosis in blood, the percentage of $CD8^+CD28^-$ T cells is associated with a poor prognosis and lung function decline (both FVC and DL_{CO}, % ref.). In this setting, an increase in T $CD8^+CD28^-$ might reflect a continuous increase in the

effector T cell subset, causing a state of activated but inefficient immunological responses that alters the distribution of the memory $CD8^+$ T cell population which, in turn, may contribute to the ongoing fibrotic scenario by a deficient clearance of intracellular antigens, whatever these might be (153).

In addition, an inverted ratio between the proportions of CD4/CD8 in the blood (i.e., <1) has been associated with immune senescence, myelodysplasia, and persistent viral infections, such as HIV (Human immunodeficiency virus), HCMV (Human cytomegalovirus), and EBV (Epstein–Barr virus) (154-156). Some previous studies have reported a higher prevalence of seropositive EBV, HCV (Hepatitis C virus), and HCMV in patients with IPF, suggesting that viral infections may play a role, either as agents that predispose and/or aggravate lung fibrosis (157). In this setting, although our observation that IPF progressors presented abnormalities in the CD4/CD8 ratio at recruitment, which are maintained during follow-up, is in keeping with the stated above, further studies are required to unveil whether the altered CD4/CD8 ratio that we reported in progressive IPF is associated with persistent viral infections (and if so, the nature of these infection), or to whether it is associated to a T $CD8^+$ response to senescent cells.

Concomitant with the "exhausted" $CD8^+$ T cell increase, we also observed an increase in NKT-like cells in the progressors groups. These cells are instrumental in the response to infections, abnormal cells (including cancer cells and senescent cells), and autoimmune diseases because of their well-known cytotoxic activity, antiviral defence and immunoregulatory functions (158). Interestingly, although this population slightly increased over time in stable patients, this increase was smaller than that observed in progressors.

Still, little is known about the T cell phenotype in circulating blood in patients with IPF at diagnosis and its evolution with time. In our study, we observed that, at diagnosis, IPF patients have reduced Th17 lymphocytes and increased Th1, leading to disrupted Th1/Th17 and Th17/Treg ratios. Depletion of circulating Th17 cells, along with a non-compromised regulatory T cells (Treg) compartment (similar to what is observed in cancer) has been previously described by Galati et al. in the blood of IPF patients. This may hightlight the potential pro-fibrotic role of Treg cells during the early stages of the disease (159).

Finally, a reduction in the proportion of naive $CD4^+$/$CD8^+$ T cells and T cell repertoire has also been reported in patients with IPF concerning immune-senescence which, in turn, relates to impaired virus-specific T cell responses (160). In this setting, we found a positive correlation between circulating naive $CD4^+$ T cells and baseline FVC (% ref.), and reduced levels of both circulating naive $CD4^+$ and $CD8^+$ T cells in IPF progressors at both recruitment and a trend in the follow-up, despite the use of anti-fibrotic treatment. In this context, no data are available concerning the interplay between antifibrotics and genetic predisposition, epigenetics, proteomics (and other omics), as well as the impact of intrinsic and environmental factors associated with IPF that could be considered important covariates influencing both response to treatment and progression of the disease.

Moreover, the results of our second study showed that at the end stages, patients with IPF present a heterogeneous lung immune infiltrate. This lung infiltrate consisted of, at least, two well-differentiated subtypes (clusters), which matched previous studies (Yang, et al.). In our study, the IPF continuum C#1 (cluster 1) showed a higher lung immune infiltration, basically characterized by: 1) an increased proportion of $CD8^+$ T cells, innate NK lymphocytes

and memory T cells; and 2) also an increase in T and B activated cells, Th1 cells and viral response signatures in the hypergeometric test. Interestingly, these latter populations in the lung infiltrate in C#1 were similar to the immune phenotype that we observed in peripheral blood in the progressors group.

On the contrary, in C#2 (cluster 2), several basal, secretory and cilium-associated signatures were found, showing that in this subtype of IPF, not only the dysregulation of epithelial cells but also of other cell types in the distal airway might have an important contribution in the pathogenesis of IPF. This fact also matches the overexpression of MUC5B in C#2, which has been associated with the failure of the reparative mechanisms of the alveolar epithelium, mucociliary dysfunction and pollutants retention, and the basis for the development of honeycombing cysts (161). Previous studies have related the excessive release of mucin 5B with exacerbations in IPF, but, unfortunately, no data regarding exacerbations were registered in the LTRC that we could include in the analysis (50).

Interestingly, no clinical differences were found in the two clusters, probably because lungs were harvested at transplantation, representing an end-stage IPF. Unfortunately, no information about the progression of the disease was available in the LTRC (lung function decline, CT scan changes, exacerbations or number of days from diagnosis to lung transplant) that let us relate the lung immune cell signatures with clinical evolution before lung transplant.

Based on our discoveries, we propose that an ineffective immune response might facilitate the advancement of the disease. Nevertheless, further investigations are needed to determine whether the blood immunophenotype is altered in individuals with minimal interstitial changes even before the onset of the disease. Additionally, further analysis of the immunophenotype among progressors should be conducted, so more longitudinal data could be included in our AI model, such as acute exacerbations, antifibrotic discontinuation, CT scan progression, or comorbidities, to better define possible disease progression trajectories in IPF.

5.4. STRENGTH AND LIMITATIONS

One of the main strengths of our first study is that, while other studies have focused their investigations generally on lung tissue or BAL at end-stages of the disease, ours characterizes in detail a wide range of immune cell types in circulating blood, an easily accessible tissue in clinical practice or clinical research, at the time of IPF diagnosis. Moreover, the fact that these patients were followed up over time allowed us to investigate the relationship of these baseline immune cell phenotypes with disease progression, and their maintenance over time. Among the potential limitations, we acknowledge that our flow cytometry study at diagnosis is monocentric, with no validation cohort and that the sample size is relatively small. In addition, we acknowledge that there were more females among the control group, however, study groups were matched by two major confounders in cell populations, age and smoking status, and all analyses were adjusted by age, gender and smoking status.

Regarding the second study, all the transcriptomic, functional, structural and clinical data used for the analysis were obtained from the LTRC/LGRC. This whole dataset was divided into two: a discovery (D#1) and a validation dataset (D#2), where the main findings were replicated. Here, no temporal (longitudinal follow-up) heterogeneity was assessed due to the cross-sectional character of the study and the use of transcriptomic profiles of microarray data from whole lung (flash frozen) homogenates, which do not capture this complexity. Transcriptomic data at different stages of the disease would be key to determining the evolution of those endotypes, their characteristics over time, and their relation to clinical outcomes.

Results were validated by flow cytometry in a second cohort (Pittsburgh, USA) using flow cytometry analysis. Unfortunately, no additional longitudinal data was registered that allowed us to relate our two molecular clusters with the progression of the disease.

Conclusions

06

The **main conclusions** of this thesis are the following:

1

Peripheral immune cell profile:

- Patients with IPF show significant alterations in the peripheral blood immunological profile at diagnosis, both in their innate and adaptive immune responses, particularly in the cytotoxic compartment.
- In IPF, the severity of airflow limitation is associated with a set of immune cell populations in blood.
- IPF progression is associated with NKT-like and $CD8^+$ T cell dysregulation, and an inverted CD4/CD8 ratio at baseline, which suggests an over-activated, aged, and "exhausted" immune status potentially related to intracellular antigens.
- The former immune cell populations have shown the ability to discriminate the classification of progressors and stable patients and they are maintained over time, showing their potential role as biomarkers of disease progression.

2

Lung immune endotypes:

- An unbiased clustering of the transcriptomic enrichment in immune cell signatures in lung tissue of patients with end-stage IPF revealed two clusters that differed in the proportion of immune cell infiltration.
- Although profound biological differences between the two clusters, no significant clinical differences were found.
- The lung tissue flow cytometry analysis performed in an independent tissue cohort replicated the main results showing two different clusters that were characterized mainly by the infiltration of the cytotoxic compartment.
- The differential gene expression analysis between the two clusters showed that C#1 was an "immune cell" cluster mainly enriched in cytotoxic and memory T cells, whereas de C#2 was a "structural cell" cluster with upregulation of cilium, epithelial and secretory cells genes.

Future lines of research

07

To **continue disentangling the heterogeneity in IPF**, from this Doctoral Thesis the following lines of **future research** emerge:

- Detailed cell phenotyping and cytokine response assays: to assess whether there is reduced cytokine responsiveness in patients with IPF compared to age-smoking-matched healthy controls, especially in those patients categorized as progressors. These assays will be performed on frozen PBMCs from patients with IPF (progressors and stable patients) collected at the study recruitment, and two years follow-up.

- Measurement of circulating cytokines/inflammatory mediators (in serum) (samples also collected at recruitment and two-year follow-up) to explore the senescence-associated secretory phenotype (SASP) in progressors vs. stable patients.

- Total RNA isolation (Maxwell® RSC simplyRNA Blood Kit) from blood samples collected from IPF patients and healthy controls for RNA sequencing. Quantification of expression levels of genes and identification of differentially expressed genes (DEGs) and biological pathway analysis between progressors, stable individuals and healthy controls. Coupled with single-cell RNA-seq of specific patient profiles.

- Validation of the selected genes or pathways identified through RNA-seq using other experimental techniques, such as quantitative real-time PCR (qPCR) or immunohistochemistry.

Appendices

08

Below are the **supplementary materials** corresponding to the two Idiopathic Pulmonary Fibrosis articles that comprise this thesis.

8.1. APPENDIX I - SUPPLEMENTARY MATERIAL FROM PAPER I:

Blood Immunophenotypes of Idiopathic Pulmonary Fibrosis: Relationship with Disease Severity and Progression.

Blood Immunophenotypes of Idiopathic Pulmonary Fibrosis: Relationship with Disease Severity and Progression

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Online supplement: 314 words; 5 Tables; 3 Figures.

Supplementary tables

Table S1. Gating immunophenotypes explored in the flow cytometry analysis.

Immune populations	Gating Phenotype (after $CD45^+$ cells/Single/Live)	% Reference population
Lymphocyte panel		
Lymphocytes $CD3^+$	$CD45^+$ $CD3^+$	$CD45^+$ alive
$CD8^+$ T cells	$CD45^+$ $CD3^+$ $CD8^+$	$CD45^+$ $CD3^+$
$CD4^+$ T cells	$CD45^+$ $CD3^+$ $CD4^+$	$CD45^+$ $CD3^+$
Effector T cells	$CD45^+$ $CD3^+$ $CD45RA^+$ $CD197^-$	$CD4^+$/$CD8^+$ T cells
Effector memory T cells	$CD45^+$ $CD3^+$ $CD45RA^-$ $CD197^-$	$CD4^+$/$CD8^+$ T cells
Central memory T cells	$CD45^+$ $CD3^+$ $CD45RA^-$ $CD19^+$	$CD4^+$/$CD8^+$ T cells
Naive T cells	$CD45^+$ $CD3^+$$CD45RA^+$$CD197^+$	$CD4^+$/$CD8^+$ T cells
Th1 cells	$CD45^+$ $CD3^+$ $CD4^+$ $CD183^+$ $CD196^-$	$CD4^+$ T cells
Th17 cells	$CD45^+$ $CD3^+$ $CD4^+$ $CD183^-$ $CD196^+$	$CD4^+$ T cells
Regulatory T cells (Tregs)	$CD45^+$ $CD3^+$ $CD4^+$ $CD25^+$ $CD127^-$	$CD4^+$ T cells
$HLA\text{-}DR^+$ T cells	$CD45^+$ $CD3^+$ $HLA\text{-}DR^+$	$CD4^+$/$CD8^+$ T cells
Antigen experienced T cells	$CD45^+$ $CD3^+$ $CD28^-$	$CD4^+$/$CD8^+$ T cells
$PD\text{-}1^+$ cells	$CD45^+$ $CD3^+$ $PD\text{-}1^+$	$CD45^+$ $CD3^+$
NKT cells	$CD45^+$ $CD3^+$ $CD56^+$	Lymphocytes
B cells	$CD45^+$ $CD3^-$ $CD19^+$	Lymphocytes
NK cells	$CD45^+$ $CD3^-$ $CD56^+$	Lymphocytes
$CD56^{bright}$ $CD16^-$ NK cells	$CD45^+$ $CD3^-$ $CD56^{++}$ $CD16^-$	NK cells
$CD56^{low}$ $CD16^-$ NK cells	$CD45^+$ $CD3^-$ $CD56^+$ $CD16^-$	NK cells
$CD56^{low}$ $CD16^+$ NK cells	$CD45^+$ $CD3^-$ $CD56^+$ $CD16^+$	NK cells

Myeloid Panel		
Neutrophils	$CD45^+$ $CD16^+$	$CD45^+$ alive
Neutrophils $CD16^+$ $CD15^{low}$	$CD45^+$ $CD16^+$ $CD15^{low}$	Neutrophils
Neutrophils $CD16^+$ $CD15^+$	$CD45^+$ $CD16^+$ $CD15^+$	Neutrophils
Eosinophils	$CD45^+$ $CD16^-$ Siglec-8^+	$CD45^+$ alive
Atypical monocytes	SSC/FSC	$CD45^+$ alive
Monocytes	SSC/CD14+	$CD45^+$ alive
$CD14^+$ $CD16^-$ monocytes	$CD45^+$ $CD14^+$ $CD16^-$	Monocytes
$CD14^+$ $CD16^+$ monocytes	$CD45^+$ $CD14^+$ $CD16^+$	Monocytes
$CD14^{low}$ $CD16^+$ monocytes	$CD45^+$ $CD14^{low}CD16^+$	Monocytes

Table S2. Flow cytometry panels used to evaluate the study groups

Antibody cocktail	Fluorophore	Company and Reference number	Volume per test (uL)
#1: Neutrophils, Eosinophils, monocytes, B, NK, NKT lymphocytes			
CD45	APC-H7	BD. 560178	1.25
CD16	APC	Palex. 302012	0.63
CD15	BV510	BD. 563141	1.25
Siglec-8	BV711	BD. 747870	1.25
CD3	APC-R700	BD. 565119	0.63
CD19	BV421	BD. 562440	0.63
CD56	BV785	Palex. 362549	0.31
CD14	FITC	BD. 555397	10
CD163	PE-Cy7	Biolegend. 333614	2.5
#2: T lymphocytes subpopulations			
CD45	APC-H7	BD. 560178	1.25
CD3	APC-R700	BD. 565119	0.63
CD4	BV711	BD. 563028	2.5
CD8	BV650	BD. 563821	1.25
CD45RA	FITC	BD. 555488	1.25
CD197 (CCR7)	PE-CFS594	BD. 562381	2.5
CD196	PE-Cy7	BD. 560620	2.5
CD183 (CXCR3)	APC	BD. 550967	5
CD28	BV510	BD. 563075	1.25
CD25	PE	BD. 555432	10
CD127	BV786	BD. 563324	0.63
HLA-DR	BV421	BD. 562804	1.25
PD-1	PerCpCy5.5	Biolegend 329914	2.5

Table S3. Differential distribution of the immune populations assessed between age-smoking matched controls and IPF. A Saphiro test was performed for each variable and the appropriate statistic test was selected accordingly to their distribution using "compareGroups" R package. Data is shown as mean ± SD or median [IQR] accordingly.

Baseline immune populations	Control (n=32)	IPF (n=32)	p-value
Innate immune cells			
Eosinophils	0.77 [0.43;1.42]	1.56 [0.42;2.39]	0.260
Neutrophils	61.4 [54.0;63.9]	65.8 [63.2;69.0]	***0.001***
Monocytes	7.14 [6.19;7.81]	6.92 [5.99;7.44]	0.612
$CD14^+$ $CD16^-$ monocytes	87.7 [83.3;88.9]	88.1 [85.4;90.3]	0.349
$CD14^+$ $CD16^+$ monocytes	6.65 [5.80;9.31]	6.96 [5.28;9.74]	0.773

$CD14^{low}$ $CD16^{+}$ monocytes	3.09 [2.23;3.81]	2.74 [1.81;3.71]	0.272
NK cells	11.3 [7.21;15.6]	11.0 [6.95;16.4]	0.978
$CD56^{bright}$ $CD16^{-}$ NK cells	3.96 [2.55;6.84]	2.75 [1.88;4.89]	0.143
$CD56^{dim}$ $CD16^{+}$ NK cells	90.0 [84.6;91.8]	90.3 [84.1;94.5]	0.486
$CD56^{dim}$ $CD16^{-}$ NK cells	4.79 [3.92;8.02]	4.61 [2.05;8.23]	0.220
NKT cells	2.34 [1.40;5.54]	2.31 [1.46;4.08]	0.490
Adaptive immune cells			
Lymphocytes	29.6 [27.4;37.0]	25.1 [22.0;27.2]	***<0.001***
B cells	9.51 [7.51;11.0]	6.84 [4.98;10.7]	***0.025***
$CD8^{+}$ T cells	31.4 (9.64)	32.8 (12.8)	0.644
$CD8^{+}$ $HLA\text{-}DR^{+}$ T cells	16.1 [12.4;22.2]	22.5 [16.6;33.7]	***0.023***
$CD8^{+}$ $CD28^{-}$ T cells	42.0 [23.9;65.0]	54.8 [49.2;69.6]	***0.040***
Effector $CD8^{+}$ T cells	27.8 [17.3;38.8]	35.9 [15.9;46.2]	0.324
Central memory $CD8^{+}$ T cells	15.4 [7.86;21.8]	8.87 [5.53;12.9]	***0.016***
Effector memory $CD8^{+}$ T cells	39.8 (14.2)	46.9 (14.9)	0.055
Naive $CD8^{+}$ T cells	9.34 [6.38;18.1]	6.48 [3.34;11.0]	***0.010***
$CD4^{+}$ T cells	61.9 (11.1)	61.0 (14.2)	0.779
$CD4^{+}$ $HLA\text{-}DR^{+}$ T cells	8.77 [6.31;10.2]	9.08 [6.23;14.4]	0.229
$CD4^{+}$ $CD28^{-}$ T cells	3.58 [0.67;9.69]	3.46 [1.47;10.0]	0.814
Effector $CD4^{+}$ T cells	0.32 [0.14;1.35]	0.53 [0.13;1.84]	0.643
Central memory $CD4^{+}$ T cells	57.9 [50.5;63.6]	53.7 [42.7;65.8]	0.330
Effector memory $CD4^{+}$ T cells	23.6 [16.2;30.7]	21.5 [15.2;34.0]	0.989
Naive $CD4^{+}$ T cells	17.9 [12.3;20.7]	15.1 [11.4;27.3]	0.973
Th1 cells	15.5 (7.87)	23.1 (10.4)	***0.002***
Th17 cells	15.4 [10.8;20.9]	7.83 [6.32;12.8]	***<0.001***
Th1Th17 cells	6.42 [4.17;8.46]	3.96 [2.69;6.88]	***0.021***
Regulatory T cells (Tregs)	6.04 [5.25;7.87]	7.20 [5.85;9.03]	0.072
$PD\text{-}1^{+}$ cells	1.96 [1.63;3.62]	2.82 [1.30;6.32]	0.679
Ratios			
NLR(Neutrophil-to-lymphocyte Ratio)	2.09 [1.45;2.27]	2.61 [2.40;3.05]	<0.001
MLR(Monocyte-to-lymphocyte Ratio)	0.23 [0.18;0.27]	0.26 [0.23;0.35]	0.012
CD4/CD8 Ratio	1.95 [1.42;2.80]	1.97 [1.27;2.92]	0.809
Th1/Th17 Ratio	0.97 [0.63;1.72]	3.14 [1.78;3.94]	<0.001
Th17/Tregs Ratio	2.46 [1.76;3.62]	1.09 [0.87;1.66]	***<0.001***

Table S4. Spearman correlations between baseline blood immune populations and inflammation ratios, and lung function at recruitment in IPF patients.

Baseline immune populations	Baseline FVC%		Baseline DLCO%	
	Rho	p-value	Rho	p-value
Innate immune cells				
Eosinophils	0,349	0,186	0,662	***0,010***
Neutrophils	-0,124	0,498	-0,496	***0,005***
Monocytes	0,074	0,688	0,112	0,557
$CD14^{+}$ $CD16^{-}$ monocytes	-0,041	0,822	-0,144	0,447
$CD14^{+}$ $CD16^{+}$ monocytes	0,136	0,458	0,028	0,884
$CD14^{low}$ $CD16^{+}$ monocytes	0,088	0,634	0,294	0,114
NK cells	0,037	0,840	-0,271	0,147
$CD56^{bright}$ $CD16^{-}$ NK cells	-0,102	0,578	0,138	0,467
$CD56^{dim}$ $CD16^{+}$ NK cells	-0,063	0,731	-0,152	0,422
$CD56^{dim}$ $CD16^{-}$ NK cells	0,141	0,443	0,175	0,354
NKT cells	-0,141	0,440	-0,123	0,517
Adaptive immune cells				
Lymphocytes	0,220	0,226	0,470	***0,009***
B cells	0,001	0,997	-0,091	0,631
$CD8^{+}$ T cells	-0,227	0,212	-0,213	0,259
$CD8^{+}$ HLA-DR^{+} T cells	-0,142	0,437	-0,005	0,981
$CD8^{+}$ $CD28^{-}$ T cells	-0,273	0,130	-0,193	0,306
Effector $CD8^{+}$ T cells	0,009	0,959	0,085	0,654
Central memory $CD8^{+}$ T cells	-0,132	0,473	-0,044	0,817
Effector memory $CD8^{+}$ T cells	-0,063	0,733	-0,186	0,326
Naive $CD8^{+}$ T cells	0,268	0,138	0,203	0,282
$CD4^{+}$ T cells	0,235	0,195	0,252	0,180
$CD4^{+}$ HLA-DR^{+} T cells	0,033	0,860	-0,064	0,738
$CD4^{+}$ $CD28^{-}$ T cells	-0,043	0,814	-0,149	0,431
Effector $CD4^{+}$ T cells	-0,063	0,733	0,049	0,798
Central memory $CD4^{+}$ T cells	-0,410	0,020	-0,272	0,146
Effector memory $CD4^{+}$ T cells	-0,184	0,312	-0,067	0,727
Naive $CD4^{+}$ T cells	0,533	***0,002***	0,192	0,309
Th1 cells	-0,116	0,528	-0,058	0,761
Th17 cells	-0,290	0,107	-0,215	0,255
Th1Th17 cells	-0,142	0,439	-0,056	0,770
Regulatory T cells (Tregs)	0,101	0,584	-0,176	0,353
PD-1^{+} cells	-0,121	0,510	-0,015	0,936
Ratios				
NLR(Neutrophil-to-lymphocyte Ratio)	-0,129	0,482	-0,467	***0,009***
MLR(Monocyte-to-lymphocyte Ratio)	-0,216	0,234	-0,434	***0,016***
CD4/CD8 Ratio	0,232	0,201	0,237	0,207
Th1/Th17 Ratio	0,205	0,260	0,137	0,472
Th17/Tregs Ratio	-0,293	0,103	-0,040	0,832

Table S5. Differential distribution of the baseline immune populations assessed between the two subgroups of IPF. Data is shown as mean ± SD or median [IQR]. A Saphiro test was performed for each variable and the appropriate statistic test was selected accordingly to their distribution.

Baseline immune populations	Progressor (n=18)	Stable (n=13)	p-value
Innate immune cells			
Eosinophils	0.98 [0.40;1.92]	1.96 [1.54;3.31]	0.278
Neutrophils	65.6 [63.4;66.2]	66.5 [55.8;69.9]	0.471
Monocytes	7.01 [6.04;7.94]	6.47 [5.61;7.12]	0.317
$CD14^+$ $CD16^-$ monocytes	87.5 [85.2;90.3]	88.2 [86.8;90.3]	0.734
$CD14^+$ $CD16^+$ monocytes	6.52 [5.11;11.4]	7.50 [5.68;9.20]	0.857
$CD14^{low}$ $CD16^+$ monocytes	2.74 [2.25;3.89]	2.86 [1.77;3.20]	1.000
NK cells	11.0 [6.07;16.0]	8.39 [7.16;16.3]	0.826
$CD56^{bright}$ $CD16^-$ NK cells	3.43 [1.92;5.60]	2.16 [1.99;3.87]	0.447
$CD56^{dim}$ $CD16^+$ NK cells	86.9 [82.4;94.4]	92.6 [87.9;94.5]	0.562
$CD56^{dim}$ $CD16^-$ NK cells	6.30 [2.10;10.4]	3.04 [1.95;4.94]	0.347
NKT cells	2.79 [2.06;5.07]	1.54 [0.92;2.79]	***0.031***
Adaptive immune cells			
Lymphocytes	25.4 [24.0;27.2]	24.9 [18.9;27.1]	0.734
B cells	6.49 [4.74;8.99]	9.13 [5.55;14.2]	0.186
$CD8^+$ T cells	39.4 (10.6)	22.4 (8.20)	***<0.001***
$CD8^+$ $HLA\text{-}DR^+$ T cells	29.2 (15.3)	21.4 (13.5)	0.145
$CD8^+$ $CD28^-$ T cells	64.4 (12.0)	39.6 (18.0)	***<0.001***
Effector $CD8^+$ T cells	40.6 [25.9;58.0]	22.5 [14.3;42.1]	0.109
Central memory $CD8^+$ T cells	7.28 [4.68;10.7]	11.8 [8.42;16.5]	***0.020***
Effector memory $CD8^+$ T cells	44.9 [31.0;60.1]	42.1 [37.2;59.2]	0.435
Naive $CD8^+$ T cells	4.22 [2.30;7.79]	9.84 [6.33;12.6]	***0.024***
$CD4^+$ T cells	53.6 (11.2)	72.5 (9.80)	***<0.001***
$CD4^+$ $HLA\text{-}DR^+$ T cells	11.6 [8.63;16.4]	7.58 [6.08;13.5]	0.128
$CD4^+$ $CD28^-$ T cells	4.31 [1.98;15.8]	1.62 [0.54;5.59]	***0.050***
Effector $CD4^+$ T cells	0.69 [0.18;2.17]	0.51 [0.11;1.61]	0.548
Central memory $CD4^+$ T cells	52.3 (14.7)	54.5 (13.8)	0.675
Effector memory $CD4^+$ T cells	30.0 (11.1)	16.3 (8.40)	***0.001***
Naive $CD4^+$ T cells	13.5 [10.3;15.3]	26.2 [14.7;39.6]	***0.013***
Th1 cells	26.1 (10.4)	19.0 (9.58)	0.061
Th17 cells	7.63 [5.03;12.1]	7.93 [6.90;14.7]	0.298
Th1Th17 cells	4.87 [2.13;7.29]	3.71 [3.07;5.00]	0.749
Regulatory T cells (Tregs)	7.50 (2.71)	7.40 (1.76)	0.902
PD-1^+ cells	2.40 [1.31;3.94]	1.27 [0.94;2.15]	0.230
Ratios			
NLR(Neutrophil-to-lymphocyte Ratio)	2.51 [2.43;2.75]	2.67 [2.06;3.47]	0.575
MLR(Monocyte-to-lymphocyte Ratio)	0.27 [0.24;0.33]	0.24 [0.20;0.39]	0.496
CD4/CD8 Ratio	1.46 [0.99;2.03]	2.94 [2.76;4.22]	***<0.001***
Th1/Th17 Ratio	3.49 (1.99)	2.41 (1.70)	0.115
Th17/Tregs Ratio	1.04 [0.84;1.76]	1.11 [0.97;1.58]	0.401

Table S6: Differential distribution of the significant immune cell populations across groups (Controls, Stable and Progressors). Differences in the distribution were assessed using Kruskal-Wallis and post-hoc Mann-Whitney.

	Mean±SD			Kruskal-Wallis	Mann-whitney Post-hoc		
	Control	Stable	Progressor	p-value	Control vs Stable	Control vs Progressor	Progressor vs Stable
Innate immune cells							
Neutrophils	58,41±7,88	65,05±15,1	63,78±11,47	0,006	***0,048***	***0,010***	0,483
Adaptive immune cells							
Lymphocytes	30,14±7,31	24,54±12,83	24,51±7,64	0,010	***0,032***	***0,030***	0,904
B cells	9,96±2,78	11,4±8,74	7,98±5,5	0,036	0,615	***0,024***	0,294
$CD8^+$ T cells	31,44±9,64	22,44±8,2	39,42±10,6	0,000	***0,007***	***0,018***	***0,000***
$CD8^+$ HLA-DR^+ T cells	17,75±7,41	21,44±13,45	29,21±15,25	0,018	0,540	***0,014***	0,164
$CD8^+$ $CD28^-$ T cells	43,39±22,01	39,59±17,98	64,4±11,98	0,001	0,647	***0,002***	***0,002***
Central memory $CD8^+$ T cells	15,5±8,59	13,42±6,81	8,81±5,99	0,010	0,540	***0,012***	***0,032***
Naive $CD8^+$ T cells	14,47±13,32	10,9±7,44	5,74±4,6	0,003	0,647	***0,002***	***0,037***
$CD4^+$ T cells	61,91±11,09	72,52±9,79	53,58±11,2	0,000	***0,004***	***0,028***	***0,000***
Effector memory $CD4^+$ T cells	24,63±11,97	16,32±8,4	29,97±11,06	0,002	***0,019***	0,070	***0,005***
Naive $CD4^+$ T cells	18,94±11,95	27,33±15,86	14,17±7,53	0,023	0,088	0,088	***0,041***
Th1 cells	15,49±7,87	19±9,58	26,08±10,44	0,001	0,220	***0,001***	0,085
Th17 cells	17,29±9,24	12,32±9,13	9,29±5,24	0,001	***0,024***	***0,001***	0,312
NKT cells	4,23±3,91	2,03±1,38	4,75±5,38	0,072	0,087	0,562	0,087
Ratios							
NLR(Neutrophil-to-lymphocyte Ratio)	1,92±0,61	4,61±5,19	3,01±1,74	0,002	***0,031***	***0,001***	0,594
MLR(Monocyte-to-lymphocyte Ratio)	0,22±0,06	0,37±0,32	0,35±0,22	0,045	0,251	***0,047***	0,514
CD4/CD8 Ratio	2,35±1,52	4,07±2,99	1,53±0,72	0,000	***0,008***	***0,020***	***0,000***
Th1/Th17 Ratio	1,23±0,94	2,41±1,7	3,49±1,99	9,88E+09	***0,038***	***2,69E+09***	0,183
Th17/Tregs Ratio	2,83±1,64	2±2,43	1,29±0,67	0,000	***0,006***	***0,000***	0,417

Supplementary Figures

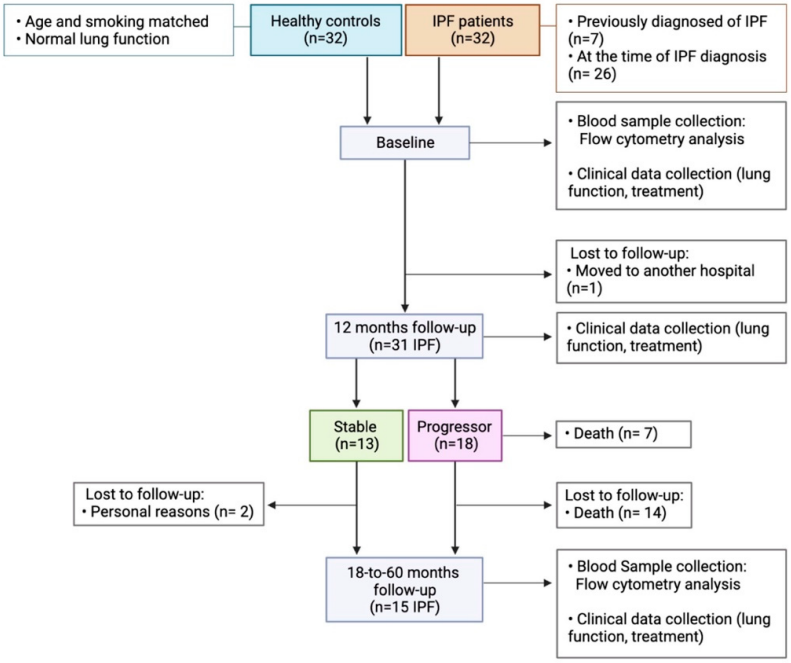

Figure S1. CONSORT flow diagram showing participant flow through each stage of the study (Created with BioRender.com).

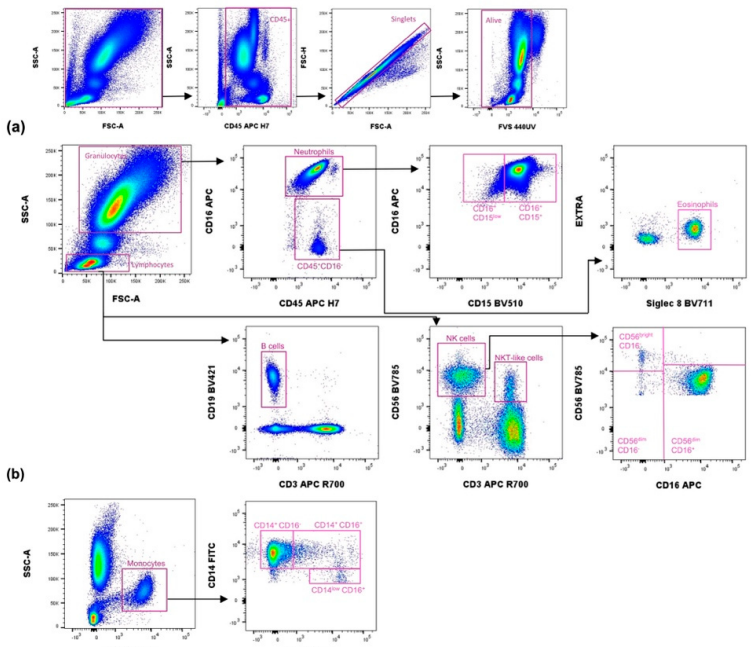

Figure S2. Representative gating strategy 1 for the evaluation of immune parameters in peripheral blood from IPF patients or healthy individuals. Flow cytometry staining was performed as described in *Methods.* The gating strategy outlined in A) shows immune panel used to identify the following populations from a sample after excluding debris and gating on $CD45^+$ single live cells: neutrophils, eosinophils, B cells, NK and NKT cell populations. Gating strategy B) shows monocytes and their subcategorization into classical ($CD14^+CD16^-$). intermediate ($CD14^+CD16^+$) and non-classical ($CD14^{low}CD16^+$).

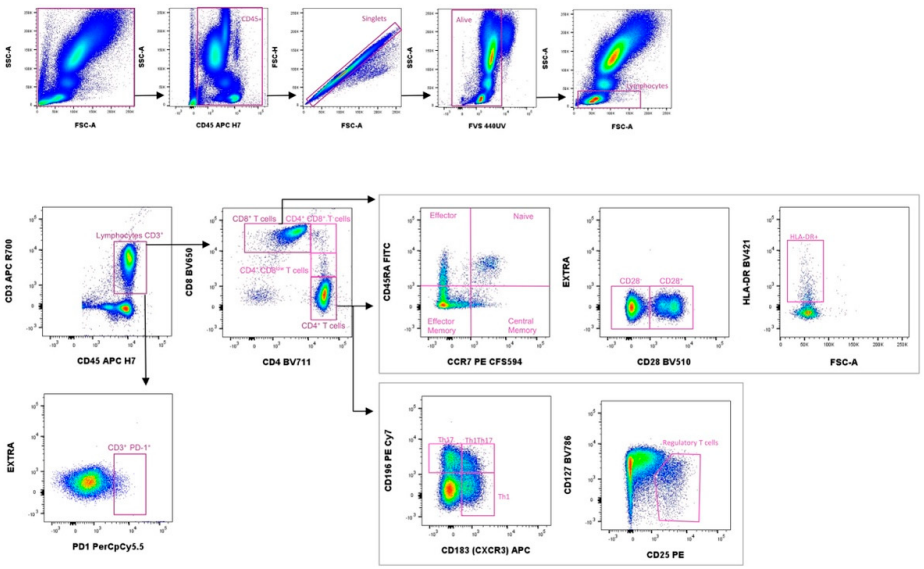

Figure S3. Representative gating strategy 2 for the evaluation of immune parameters in peripheral blood from IPF patients or healthy individuals. Flow cytometric staining was performed as described in *Methods.* The gating strategy shows immune panel used to identify the following T cell populations from a sample after excluding debris and gating on $CD45^+$ single live cells: $CD8^+$, $CD4^+$, Th1, Th17, Treg cells, Effector, Naive, Effector memory and Central memory T cells. Populations of $CD8^+$ and $CD4^+$ were also evaluated for the expression of CD28, PD-1 and HLA-DR activation and/or exhaustion markers.

8.2. APPENDIX II - SUPPLEMENTARY MATERIAL FROM PAPER II:

Lung immune signatures define two groups of end-stage IPF patients.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Flow cytometry gating strategy to identify the main immune populations in the lung**.** Cell debris is excluded and single cells are selected using FSC VS SSC. Live cells are selected using a cell viability marker and the hematopoietic cells are selected as CD45+. Macrophages/monocytes are selected by gating the CD14 population. For lymphocyte determinations complex cells are excluded based on the SSC to reduce the lung autofluorescence. From this lymphocyte population, B lymphocytes are selected as CD19+, NK cells are selected as CD3-CD56+ and T cells are CD3+. CD4+ and CD8+ T lymphocytes are selected from the CD3+ population.

Supplementary Figure 2. GSVA unbiased clustering of the IPF samples divided by the profiled lung lobe**. A)** Upper lobe **B)** Lower lobe.

Supplementary Figure 3. First neighbour correlation networks. **A)** Cluster 1 **B)** Cluster 2. Nodes represent the FC of the median between the 2 clusters. Lung clinical parameters are represented in octagons (CT scans are light green and pulmonary function test parameters are dark green), GSVA cell types in circles (lung cell types are turquoise, cytotoxic cells are yellow, T cells are blue, B cells are pink and innate cells are light purple) and the biological pathways are denoted in rhombus. The width of the edges represents the correlation coefficient, negative correlations are marked with dotted lines and positive correlations are indicated with solid gray. R>|0.5| and p<0.05.

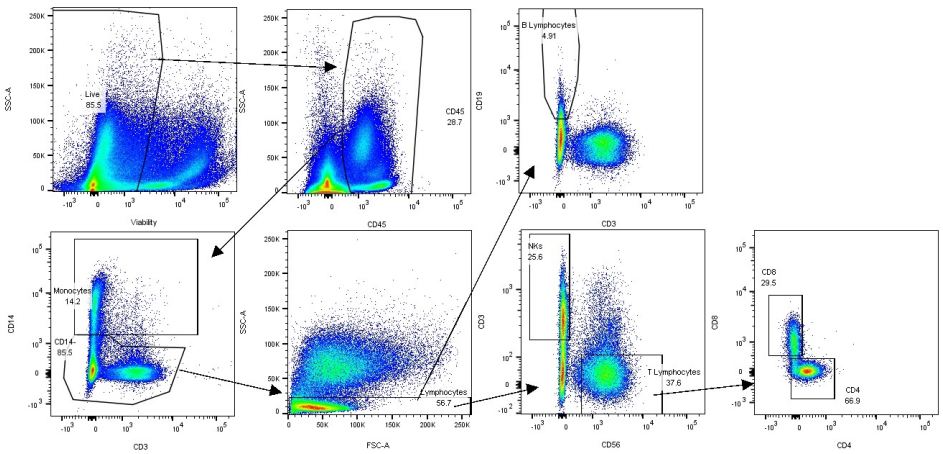

Figure S1

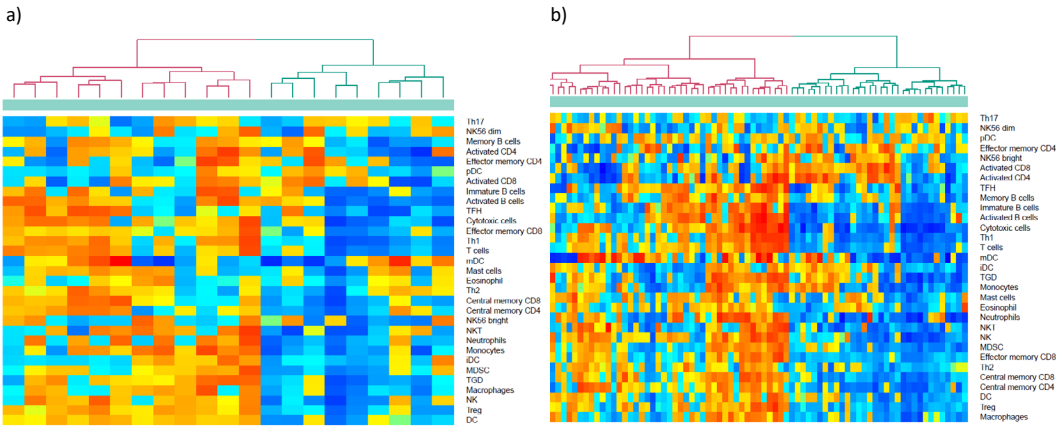

Figure S2

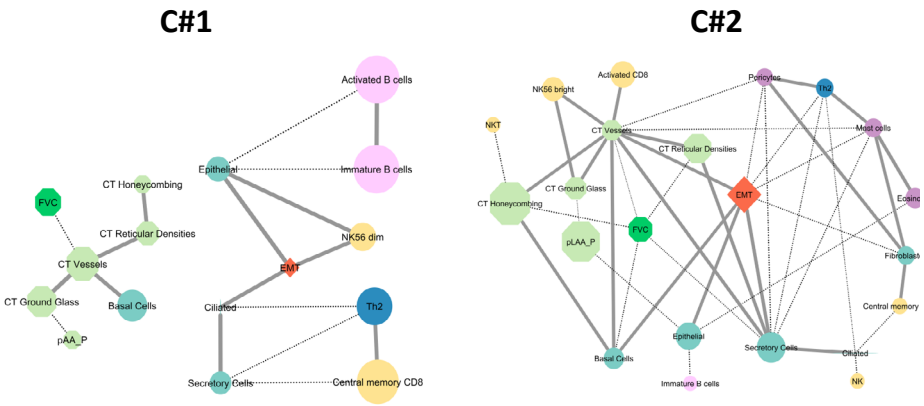

Figure S3

Link:

Additional file 1: Figure S1. Flow cytometry gating strategy to identify the main immune populations in the lung. Cell debris is excluded and single cells are selected using FSC VS SSC. Live cells are selected using a cell viability marker and the hematopoietic cells are selected as CD45+. Macrophages/monocytes are selected by gating the CD14 population. For lymphocyte determinations complex cells are excluded based on the SSC to reduce the lung autofluorescence. From this lymphocyte population, B lymphocytes are selected as CD19+, NK cells are selected as CD3-CD56+and T cells are CD3+. CD4+and CD8+T lymphocytes are selected from the CD3+population. **Figure S2.** GSVA unbiased clustering of the IPF samples dividing by the profiled lung lobe. **A** Upper lobe **B** Lower lobe. **Figure S3.** First neighbor correlation networks. **A** Cluster 1 **B** Cluster 2. Nodes represent the FC of the median between the 2 clusters. Lung clinical parameters are represented in octagons (CT scan are light green and pulmonary function test parameters are dark green), GSVA cell types in circles (lung cell types are turquois, cytotoxic cells are yellow, T cells are blue, B cells are pink and innate cells are light purple) and the biological pathways are denoted in rhombus. The width of the edges represents the correlation coefficient, negative correlations are marked with dotted lines and positive correlations are indicated with solid gray. $R>|0.5|$ and $p<0.05$.

SUPPLEMENTARY TABLE LEGENDS

Supplementary Table 1. Immune gene set signatures used in the GSVA pipeline to performed the unbiased clustering.

Supplementary Table 2. Extended gene set signatures with the epithelial and fibrosis associated signatures used in the GSVA to generate the correlation networks.

Supplementary Table 3. Hypergeometric test gene signatures for immune, epithelial and fibrosis related signatures.

Supplementary Table 4. Main clinical characteristics of D#1 y D#2.

Supplementary Table 5. Differences in immune cell signatures across clusters on D#1.

Supplementary Table 6. Differences in clinical characteristics and immune cell signatures across clusters on D#2.

Supplementary Table 7. Differences in clinical characteristics and immune cell signatures across clusters on D#1 filtering by upper lobes.

Supplementary Table 8. Differences in clinical characteristics and immune cell signatures across clusters on D#1 filtering by lower lobes.

Supplementary Table 9. Differences in clinical characteristics and immune cell signatures between upper and lower lobe. Results are expressed as median [95% coefficient interval] or mean (SD) as appropriate.

Supplementary Table 10. Main clinical characteristics of the validation cohort

Supplementary Table 11. Statistically significant (adjusted p-value < 0.05) genes on the limma test of cluster 2 over cluster 1.

Supplementary Table 12. Gene ontology enrichment of differentially expressed genes between the 2 clusters. On the left side the gene ontologies of the cluster 1 are represented (negative values of LgFC Cluster 2/Cluster1), on the right side the gene ontologies of the cluster 2 are represented (positive values of LgFC Cluster 2/Cluster1).

Additional file 2: Table S1. Immune gene set signatures used in the GSVA pipeline to performed the unbiased clustering. **Table S2.** Extended gene set signatures with the epithelial and fibrosis associated signatures used in the GSVA to generate the correlation networks. **Table S3.** Hypergeometric test gene signatures for immune, epithelial and fibrosis related signatures. **Table S4.** Main clinical characteristics of D#1 y D#2. **Table S5.** Differences in immune cell signatures across clusters on D#1. **Table S6.** Differences in clinical characteristics and immune cell signatures across clusters on D#2. **Table S7.** Differences in clinical characteristics and immune cell signatures across clusters on D#1 filtering by upper lobes. **Table S8.** Differences in clinical characteristics and immune cell signatures across clusters on D#1 filtering by lower lobes. **Table S9.** Differences in clinical characteristics and immune cell signatures between upper and lower lobe. Results are expressed as median [95% coefficient interval] or mean (SD) as appropriate. **Table S10.** Main clinical characteristics of the validation cohort. **Table S11.** Statistically significant (adjusted p-value<0.05) genes on the limma test of cluster 2 over cluster 1. **Table S12.** Gene ontologies enrichment of differentially express genes between the 2 clusters. On the left side the gene ontologies of the cluster 1 are represented (negative values of LgFC Cluster 2/Cluster1), on the right side the gene ontologies of the cluster 2 are represented (positive values of LgFC Cluster 2/Cluster1).

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