ADAPTIVE IMMUNITY

in Interstitial Lung Diseases and Pulmonary Hypertension

Peter Heukels

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ISBN:	978-94-6416-894-5
Cover design and Lay-out:	Publiss | www.publiss.nl
Print:	Ridderprint | www.ridderprint.nl

Adaptive Immunity in Interstitial Lung Diseases and Pulmonary Hypertension

Adaptieve immuniteit in interstitiële longziekten en pulmonale hypertensie

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam op gezag van de
rector magnificus

Prof.dr. A.L. Bredenoord

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
dinsdag 23 november 2021 om 10:30 uur

door

Peter Heukels
geboren te Dordrecht

Erasmus University Rotterdam

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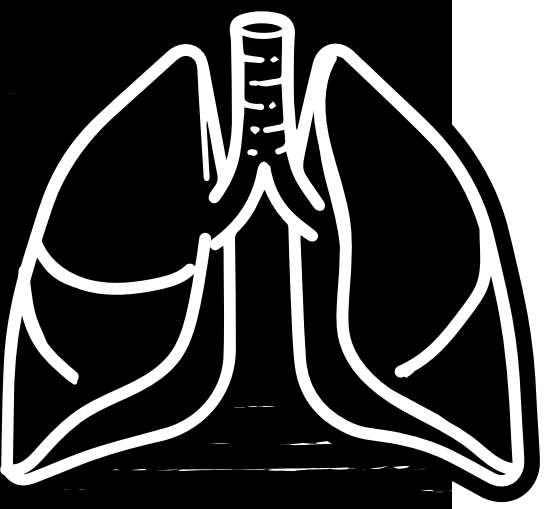

CHAPTER 1

General introduction and outline of thesis

Outline

IPF and IPAH in the clinic
Changing perspectives on the role of inflammation in IPF and IPAH
Involvement of B lymphocytes in IPF and IPAH pathogenesis
Aims of this thesis

IPF and IPAH in the clinic

Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic disease with a median survival of ~3 years when untreated.[1] IPF occurs predominantly in patients over 60 years and is more prevalent in males. The estimated prevalence in Europe is ~3-19 per 100.000 people, with a growing incidence due to better recognition as well as the aging population.[2, 3, 4] Cough, dyspnea and fatigue are the most common symptoms which increasingly impact quality of life for patients as the disease progresses. A diagnosis of IPF is made based on clinical features, imaging and exclusion of other diseases that may resemble IPF.[5] Figure 1. shows a HRCT-thorax mage from a patient with IPF, with an typical Usual Interstitial Pneumonia (UIP) pattern. In the past decade major advances have been made in the field. The introduction of diagnostic criteria allowed for structured clinical and translational research and has resulted in the first evidence- based disease-modifying treatments. Where a decade ago patients with IPF all received treatment regiments with high dose immunosuppression, we now know that this has detrimental effects in IPF. Currently treatment consists of "anti-fibrotic" medication (pirfenidone or nintedanib) that slow down disease progression and improve prognosis, but do not halt or reverse the decline in lung function.[6, 7] There is a need for better treatments as well as predictors for disease progression and response to therapy.

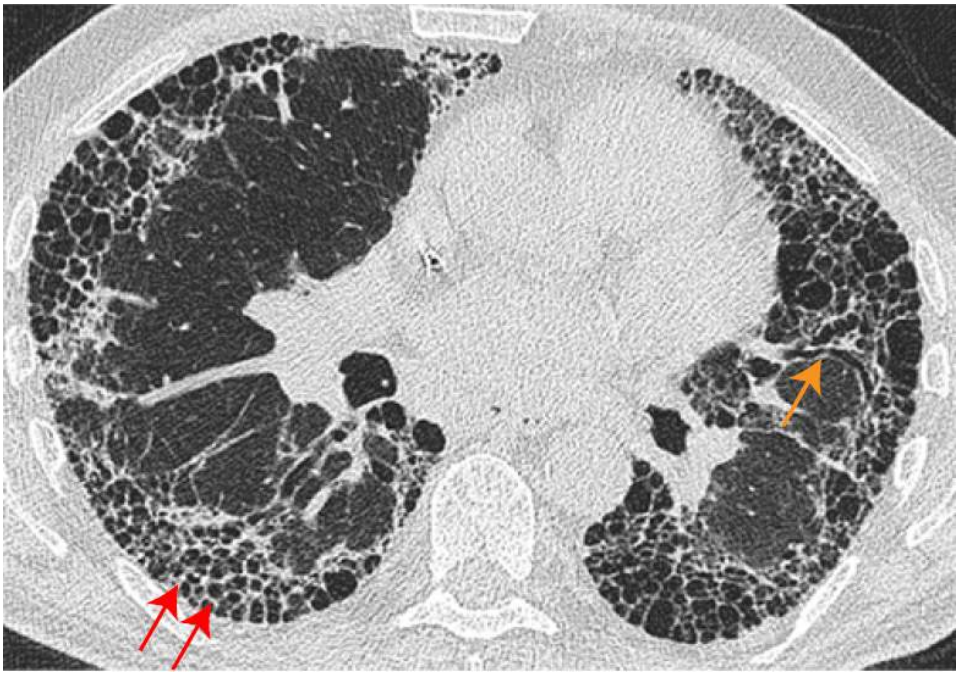

Figure 1. A representative CT-thorax image from a patient with IPF

Radiological usual interstitial pneumonia (UIP) pattern in a patient with IPF. CT scan showing advanced pulmonary fibrosis characterized by subpleural cystic changes, also called honeycombing (red arrows) and traction bronchiectasis (orange arrow). A UIP-pattern has a subpleural and posterior basal predominance.

Idiopathic pulmonary arterial hypertension

Idiopathic pulmonary arterial hypertension (IPAH), is a progressive disease characterized by progressive pulmonary vascular remodeling mediated by endothelial cell dysfunction, leading to increased pulmonary vascular pressure and right ventricle dysfunction. The annual incidence of IPAH is ~7-15 cases per million adults per year, and its prevalence ~50 cases per million adults.[8, 9] IPAH is a form of pulmonary hypertension (PH) in which no underlying disease or etiology is identified at time of diagnosis and belongs to group 1 of the World Health Organization (WHO) categorization of PH. Other disease in this group are PAH associated with connective tissue disease (CTD) and congenital heart disease (CHD) PAH. Development of PH can also occur in the context of sarcoidosis (WHO group 5). Sarcoidosis is a chronic inflammatory disease of unknown cause, which often affects the lungs and mediastinal lymph nodes.[10] Development of sarcoidosis-associated PH (SAPH) can lead to heart failure and is associated with a poor outcome.[11, 12] The etiology of SAPH is often multifactorial and poorly understood.

PH is defined as an increase in mean pulmonary arterial pressure (mPAP) ⩾25 mmHg at rest as assessed by right heart catheterization (RHC).[13] Patients with IPAH experience dyspnea, reduced exercise capacity, and premature death from right ventricular failure. The overall treatment goal in patients with IPAH is achieving a good exercise capacity, good quality of life, and better RV function.[13] The mainstays of current medical therapy, next to general supportive measures, are vascular-remodeling therapies, which aim to lower vascular pulmonary resistance and right ventricle dysfunction. Currently, a combination of PH-specific drugs are used to treat IPAH patients and include phosphodiesterase-5 inhibitors, endothelin receptor antagonists, soluble guanylate cyclase stimulators, and prostaglandins.[14, 15, 16, 17] However, even with PH-specific treatment strategies, survival remains poor with a mean 5-year survival of ~50-60% for IPAH.[18]

Changing perspectives on the role of inflammation in IPF and IPAH

Classic view: inflammation as an epiphenomenon

IPF and IPAH are clinically two distinct diseases with different treatment strategies. IPF is a chronic lung disease caused by progressive remodeling and scarring of the interstitial space, whereas IPAH is characterized by progressive vascular remodeling of the pulmonary arteries. In IPF and PAH the end-result is extensive deformation and scarring in the lung parenchyma and pulmonary arteries, respectively, leading to organ failure. Understanding of the mechanisms that initiate and perpetuate IPF and IPAH has improved over the last decades and it has become clear that multiple pathways drive disease pathobiology at different levels. Although the location of the primary injury of tissue and the remodeling process is different between IPF and IPAH, the basic mechanisms that underlie and modulate the diseases are overlapping and often stereotypical (**Figure 2**). The role of inflammation in these two disorders is still a topic of debate, however the overall scientific opinion changed over time and can be classified in two phases.

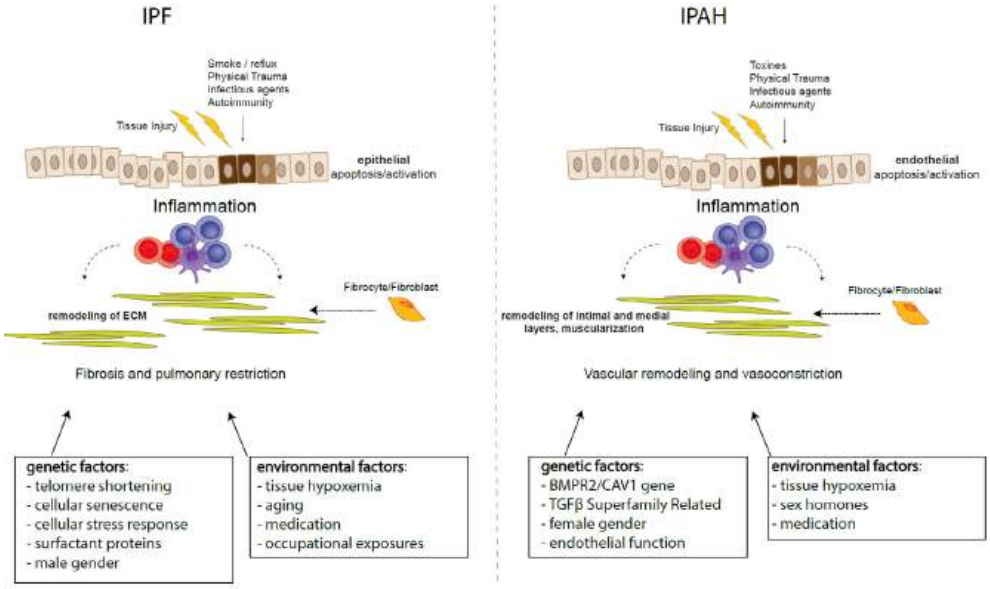

Figure 2. Schematic overview of fundamental similarities in IPF and IPAH pathobiology.

Tissue injury to respectively epithelium (IPF) or endothelium (IPAH) in susceptible individuals (predisposing genetic and environmental factors summarized in the boxes below) lead to a (chronic) inflammatory response. Ongoing and impaired resolution of inflammation can lead to abnormal wound repair and enhanced recruitment and activation of fibroblasts or fibrocytes and subsequently incite a cascade leading to fibrosis and tissue/vascular remodeling.

Traditionally, immunosuppressive therapy used to be the cornerstone of treatment for IPF. The overall opinion that inflammatory processes are critically involved in IPF pathogenesis and should be the main target for treatment shifted after Raghu and colleagues published their findings on the treatment combination of Prednisone, Azathioprine, and N-acetylcysteine in IPF in 2012.[19] They observed that the risk of death and hospitalization in IPF patients who were treated with this combination was increased, as compared with placebo-treated patients. The negative findings of the anti-inflammatory agents in this trail prompted the field to focus less on inflammation and more on the fibrotic process itself. Research shifted on elucidating mechanisms behind increased deposition of extracellular matrix, fibroblast/fibrocyte recruitment and proliferation and differentiation of fibroblast. This opinion was further fueled by several other studies aiming at modulated inflammation that failed to show favorable effects on primary end-points in clinical IPF trials (**Table 1**).[19, 20, 21, 22, 23, 24, 25]

Table 1. Landmark trials in IPF that intervened with inflammatory pathways.

Trials	Intervention	Main inflammatory target	Primary outcome
2003 Richeldi et al [20]	Prednisone	Broad acting: suppression of several innate and adaptive immune cells	meta-analysis
2008 Raghu et al [24]	Etanercept	Anti-TNF-α	FVC change
2009 King et al [21]	Interferon gamma-1b	T-cells, macrophages, cytokine production (IL-12, IL-18, TNF-α)	overall survival
2012 Raghu et al [19*]	Prednisone, Azathioprine, Acetylcysteine	Broad acting: suppression of several innate and adaptive immune cells	FVC change
2014 Martinez et al [25]	Acetylcysteine	Antioxidant, decreases oxidative stress and cytokine release (TNF-α, IL-1, and IL-8)	FVC change
2018 Raghu et al [26],, Parker et al [27], Maher et al [28]	Anti-IL-13/ (IL-4)[#]	Attenuation of Th-2 immune response	FVC change

** The independent data and safety monitoring board recommend terminating the combination-therapy group at a mean follow-up of 32 weeks. # The ESTAIR-trial used SAR156597 which is a humanized bispecific antibody binding and neutralizing IL-4 and IL-13. The other trials used Tralokinumab or Lebrikizumab which are humanized monoclonal IL-13 antibodies*

Changing perspectives

Why did the trials outlined in **Table 1** fail? An explanation could be that most of these trials used drugs with a broad acting range on several immune and structural cells. Prednisone has profound effects on almost all T-cells subsets. These include inhibition of IL-2 production, impaired release of cells from lymphoid tissues and induction of apoptosis.[29, 30] Prednisone also acts on local pulmonary cells. Alveolar cell death and increased alveolar replication contribute to enhanced fibroblast proliferation and fibrosis. Prednisone represses the replication of bronchial and alveolar epithelium during development and increases the incidence of apoptosis of epithelial cells.[31] Therefore, it is conceivable that not only "the bad guys" are targeted, but also cell subsets that directly attenuate fibrosis or have a regulatory impact on cells that exert pro-fibrotic functions. For example, adoptive transfer of regulatory T cells (Tregs), immune suppressive myeloid cells, and $CCR2^{+}CD4^{+}$ T-cells attenuated fibrosis development in murine models.[32, 33, 34] Also cytokines and chemokines produced by activated T cells of the TCRgd lineage, such as IL-17 and CXCL10, or by primary human T lymphocytes (e.g. prostaglandins) can ameliorate pulmonary fibrosis.[35 36, 37] Another important factor is that some pathways or signals that contribute to the remodeling process in IPF are inflammation-independent. These may include pathways involved in regulation of telomere length, inherited genetic alterations or environmental factors.[1]

In the last decade, understanding of the mechanisms underpinning innate and adaptive inflammatory abnormalities in both diseases regained a lot of attention and led to several review and opinion articles, teaching us that the role of inflammation should not be ignored (**Chapter 2**)[38, 39]. Inflammatory pathways are not always necessarily harmful but

often essential to deal with lung injury that is for example caused by infection or physical trauma. In this context, the pathogenesis of many respiratory diseases cannot simply be classified as inflammatory or non-inflammatory. This may especially be the case for chronic inflammatory diseases in the lungs, as immune cells are part of the normal anatomy and function of the lungs.[40] Most importantly, it is all about the proper balance between inflammation and subsequent resolution of inflammation. Impaired resolution may lead to a chronic inflammatory state which can ultimately result in fibrosis.[41] To make this even more complex, some immune cells have fibrotic properties while others harbor anti-fibrotic properties.[42] It is clear that a range of inflammatory processes are involved in IPF and PAH, but a 'one fits all' approach in anti-inflammatory treatments in IPF and IPAH does not apply.

Involvement of B lymphocytes in IPF and IPAH pathogenesis

Innate and Adaptive immunity

The immune response can be divided in two complementary pathways: innate and adaptive immunity. Innate immunity is rapid and occurs quickly after an injury or infection but is not specific for the encountered antigen. The adaptive immunity is mediated by antigen-specific B and T lymphocytes and equips the body with a memory function. This memory function enables a fast, more robust and antigen-specific immune response upon subsequent exposure to the same antigen. The hallmark of the adaptive immune system is clonal expansion of B and T lymphocytes that carry antigen-specific receptors, the B cell receptor (BCR) and the T cell receptor (TCR), respectively. This clonal expansion is characterized by a rapid increase of clones that originate from those T or B lymphocytes that specifically recognize the antigen. Given that each B or T cell can only recognize a single specificity, the immune system needs to generate vast numbers of these cells, referred to as clonal expansion, to be able to efficiently recognize the huge diversity of potential antigens.

Evidence for a role of the immune system in IPF and IPAH.

Over the past decades, the role of inflammation in IPF has been a topic for extensive research. This have led to new insights in IPF etiology. In **Chapter 2** we provide a broad overview how our innate and adaptive immune system is involved in the initiation and perpetuation of IPF pathobiology. A short overview of the role of inflammation in IPAH pathogenesis is provided below.

Over the years, evidence has accumulated pointing to a pathological role for the immune system in PAH, including IPAH. [38, 43, 44] Lungs of IPAH patients display an increased inflammatory mark consisting of T and B lymphocytes, mast cells, dendritic cells and macrophages.[45, 46] Furthermore, activation of B lymphocytes and circulating auto-antibodies were found in PAH patients. [47, 48, 49] A recent unbiased whole-blood transcriptome

analysis in PAH patients and healthy controls (HCs) [50] identified a signature of 507 PAH-associated genes, in which T cell signaling, phosphoinositide 3-kinase (PI3K) signaling in B lymphocytes and interleukin-6 (IL-6) signaling were among the top canonical pathways.

In cross-sectional studies of PAH patients, increased IL-6, IL-8 and IL-10 in serum correlated with reduced survival and quality of life. [51,52] Increased levels of circulating pro-inflammatory cytokines were also found in CHD-PAH and CTD-PAH. [53,54,55] In an unsupervised analysis of blood cytokine profiles of PAH patients, different immune phenotypes were distinguished with different clinical risk profiles, independent of WHO PH subgroups.[56] Accumulating evidence supports a major role for IL-6, considering that IL-6 receptor (IL-6R) expression and signalling is crucial for PAH development and progression [57] and that circulating IL-6 associates with specific clinical phenotypes and outcomes in various PAH subgroups [58]. Increased transforming growth factor (TGF)β receptor signalling and decreased bone morphogenetic protein receptor type 2 (BMPR2) signalling were shown to contribute to PAH pathogenesis.[59]

A typical feature in IPAH lungs is the presence of highly organized ectopic lymphoid follicles.[46, 60, 61] Whether the presence of ectopic lymphoid follicles around pulmonary arteries and chronic immune activation is cause or consequence of the primary remodeling process of pulmonary arteries remains a topic of debate. However, accumulating evidence suggests that vascular and lung injury could lead to impaired tolerance to self-antigens. Patients with IPAH have increased levels of circulating autoreactive plasmablasts and autoantibodies, which may be produced by plasma cells in TLOs in IPAH lungs. [62,47,63,64] These include antibodies that specifically target endothelial cell surface antigens.[65] Activation and apoptosis of endothelium by anti-endothelial cell autoantibodies contribute to vascular remodeling and subsequent PH development.[47] Moreover, PH can develop in a range of autoimmune diseases, including systemic lupus erythematosus and systemic sclerosis.[66]

Human B cell development

The generation of B cells with different BCRs occurs stochastically, which implies that the BCR repertoire also includes receptors that could potentially recognize self-antigens. To prevent autoimmunity, multiple checkpoints exist during B differentiation that aim to eliminate newly emerging autoreactive cells (**Figure 1**). After their development in the bone marrow, immature B cells migrate through the blood stream to the spleen where they are now termed transitional B cells. The transitional cells complete their maturation into two subtypes of mature B cells: naïve follicular and marginal zone (MZ) B cells. Naïve follicular B-cells have a more diverse BCR repertoire than MZ B cells and upon antigen recognition participate in T-cell dependent antibody response. MZ B cells engage rapidly in a T-cell independent response when they are activated by blood-borne pathogens.
Upon antigen-induced activation during a T-cell dependent response in lymph nodes or tertiary lymphoid tissue, B cells undergo a series of differentiation steps, which are collectively referred to as the germinal center (GC) reaction. The GC reaction include clonal expansion, immunoglobulin (Ig) heavy chain class switch recombination (shift from

expression of cell surface IgM or IgD to IgG or IgA), as well as affinity maturation through the induction of somatic mutations in the variable regions of the Ig heavy (H) and light (L) chains. During affinity maturation their cognate antigen is presented by follicular dendritic cells. The affinity of the interaction dictates whether B cells will undergo apoptosis (either low-affinity for the antigen, or highly autoreactive) or be selected for further differentiation (high affinity, but no autoreactivity). After completing differentiation, these GC B cells develop either into circulating long-lived memory B-cells or into plasma cells that are fully capable of producing high-affinity antibodies against foreign antigens (**Figure 3**).

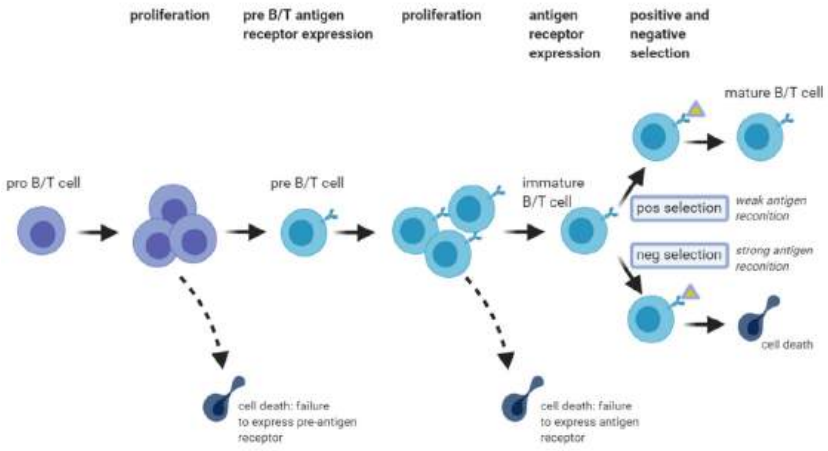

Figure 3. Checkpoints during B- and T-cell differentiation

Mechanisms of B-cell tolerance in bone marrow and periphery. In the bone marrow, development progresses through the pro-B-cell, pre-B-cell and immature-B-cell stages. During this differentiation, gene rearrangements at the immunoglobulin locus result in the generation and surface expression of the pre-B-cell receptor and finally a mature BCR. At this immature stage of development, B cells undergo a selection process to prevent any further development of self-reactive cells. Clonal deletion and anergy are mechanisms utilized in the primary and peripheral lymphoid organs. Strength of BCR signaling affects the fate of the B cells undergoing tolerance.[67] Although there are many parallels between B and T cell development, T cell development does not occur in the bone marrow but in the thymus, whereby positive and negative selection steps are confined to the thymus.

B cell receptor signaling

The BCR signaling pathway is crucial for adequate development, activation, proliferation and differentiation of B cells. These outcomes are strongly dependent on BCR specificity, BCR signal strength and the contributions of several B cell co-receptors. The BCR on the surface of mature B cells is an membrane-bound Ig in the form of a hetero-tetramer, consisting of two Ig H chains either of the μ or δ isotype (making IgM or IgD, respectively) complexed with two Ig L chains, either k or l.[68] Several membrane-bound receptors, including the BCR and CD19, together with co-stimulatory signals, such as CD40 binding to its ligand CD40L on activated T cells during a T cell-dependent B cell response, are needed to initiate B cell activation. **Figure 4** illustrates the BCR signaling pathways and the role of a critical downstream signaling molecule Bruton's tyrosine kinase (BTK).

Antigen engagement by the BCR initiates structural changes and aggregation of BCR components that lead to phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tails of the BCR-associated molecules CD79a and CD79b by Src-like kinases such as LYN. The phosphorylated ITAMs recruit the spleen tyrosine kinase (SYK) to the receptor, where it becomes activated by phosphorylation of tyrosines, and transmits the signal to downstream proteins. In parallel, LYN phosphorylates tyrosine residues in the cytoplasmatic trail of the BCR co-stimulator CD19, which allows binding of Phosphoinositide 3-kinases (PI3Ks) and proto-oncogene Vav. Together with activation of SYK, a signalosome is formed. This signalosome consists of several proteins including B-cell linker (BLNK; also known as SLP-65), BTK, phosphoinositide phospholipase C-gamma-2 (PLCγ2), PI3K, and Vav. Signals originating from this signalosome initiate and regulate downstream signaling pathways. Activation of these pathways result in the induction of several transcription factors, including Nuclear factor-κB (NF-κB), Nuclear factor of activated T-cells (NFAT), Ets-like protein-1 (ELK-1) and MYC. NF-kB regulates genes that are involved in cell cycle progression and anti-apoptotic pathways.[69, 70] Transcription factors MYC and ELK1 are essential for B cell proliferation and pre-B cell survival, respectively.[71, 72] NFAT is vital for early B cell differentiation and Ig class switching.[73, 74] Also, activation of cytoskeleton remodeling, which is crucial for antigen processing, is initiated upon activation via the BCR[75].

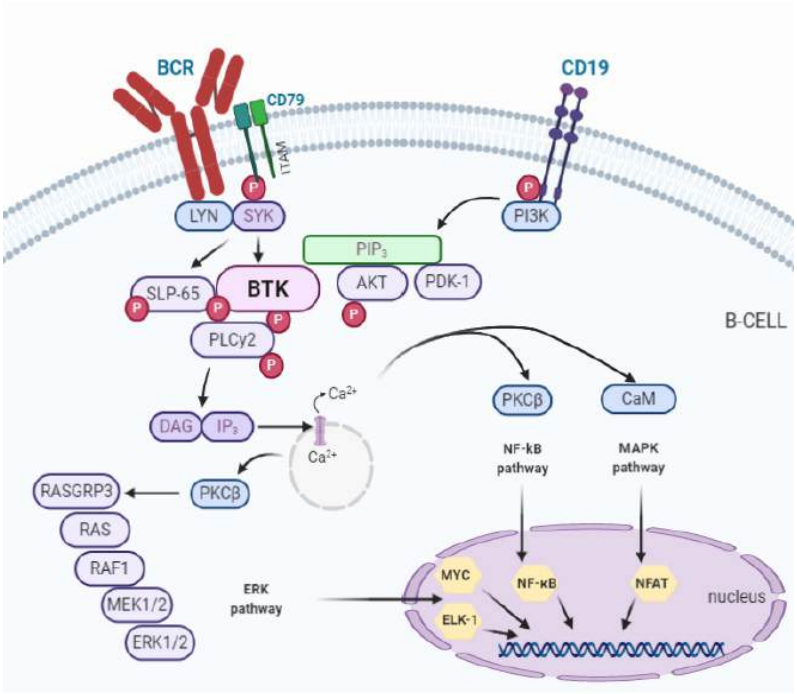

Figure 4. overview of BCR signaling pathways and the role of BTK

Simplified visualization of the BCR signaling pathway. After BCR is linked to the antigen, three main protein tyrosine kinases, LYN, SYK and BTK are activated. BTK subsequently phosphorylates and activates PLCγ2, which produces the second messengers IP3 and DAG, inducing the release of intracellular Ca 2+ and the activation of PKC enzymes, ultimately leading to activation of the NFAT, NF-κB, ELK-1 and MYC transcription factors. These transcription factors activate the expression of genes involved in B cell proliferation, differentiation, and survival.

Bruton's tyrosine kinase function in BCR signaling and other signaling pathways

BTK is a Tec family tyrosine kinase expressed in all cells of the hematopoietic lineage, except T cells, NK cells and plasma cells.[76, 77] BTK was first identified as the gene defective in the primary immunodeficiency X-linked agammaglobulinemia (XLA). Dysfunction of this protein results in a block in B cell development at the pre-B stage and an almost complete lack of peripheral B-cells and immunoglobulins.[78] For normal B-cell development, expression of appropriate levels of BTK is crucial.[79] Decreased levels or absence of BTK levels in B cells lead to a high rate of apoptosis[80], and loss of BTK functionality in B cells has been linked to compromised inflammatory responses. Conversely, increased BTK protein levels promote autoimmunity and autoantibody production in a transgenic mouse model.[76, 81, 82] An important signal that induces apoptosis in activated B cells is mediated via the FAS receptor. In vitro transgenic BTK-overexpressing B cells were shown to be resistant to FAS receptor signals.[79] After escaping cell death, BTK-overexpressing autoreactive B cells differentiate further into memory B cells or plasma cells that are able to produce autoantibodies.

Increased BTK expression in B-cells also activate T cells in CD19-hBTK transgenic mice. In these mice, increased BTK-mediated signaling in B cells involves a positive-feedback loop that establishes T cell-propagated autoimmune pathology and is accompanied by increased proportions of splenic Tfh cells.[81] Hereby, the increased surface expression of the co-stimulatory molecules CD80 and CD86 on CD19-hBTK transgenic B cells likely supports T-cell activation. Additionally, BTK overexpression was sufficient to induce spontaneous GC and plasma cell formation in the spleen.[79]

It is conceivable that in humans increased BTK levels in B cells also hinder adequate elimination of autoreactive B cells via unresponsiveness to apoptotic signals (**Figure 5**) and disrupting T cell homeostasis, which would contribute to autoimmune disease (see below).

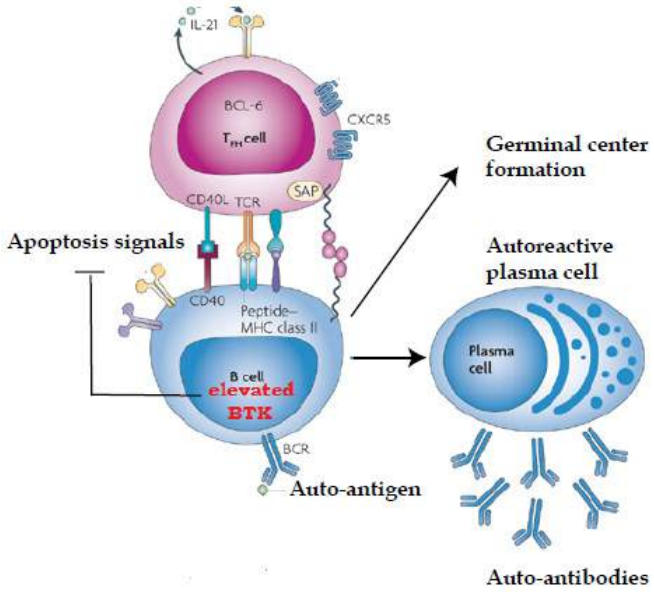

Figure 5. schematic overview how increased BTK levels promote the development autoimmunity
Upon antigen recognition, B cells can induce germinal center formation or enter an existing germinal center and undergo somatic mutation of the genes encoding their B cell receptor. Follicular T helper (Tfh) cells provide specialized help to these germinal center B cells through cognate T-B cell interactions via Tfh cell-derived co-stimulation. Under physiological conditions, this process leads to efficient selection of B cell clones that bind antigen with high affinity. Autoreactive B-cells, however, receive apoptotic signals from Tfh cells. Increased BTK and thus increased BCR signaling can rescue auto-reactive B cells from apoptosis. Auto-reactive B cells can further develop into autoreactive plasma cells producing high affinity auto-antibodies. Additionally, overexpression of BTK in B cells may also induce spontaneous GC formation. (Adapted from: Nature Reviews Immunology, 2009 [83])

It is important to realize that also other signaling pathways depend on BTK activity, including Toll-like receptor (TLR) and chemokine receptor pathways. TLR signaling in B cells provides a cell-intrinsic mechanism for innate signals (e.g. components of virus or bacteria, such as lipopolysaccharide) to induce adaptive immune responses.

Additionally, TLR signaling plays a variety of roles in B cell activation and differentiation, including cytokine secretion, antigen presentation capacity and Ig isotype switching, which is reviewed elsewere.[68, 84] Our group demonstrated that both in human and in mice BTK protein levels are upregulated when mature B cells are activated in vitro by various signals, including those initiated by TLR stimulation.[79]

Attracting B cells to lymph node follicles depends on chemokine gradients and chemokine receptor interactions[85]. For B cell trafficking and homeostasis, C-X-C chemokine receptor (CXCR) 4 and CXCR5 are expressed on B cells.[86] Upon binding of chemokines to the extracellular domain of its chemokine receptor, intracellular activation of PI3K results in activation of BTK.[87] This pathway downstream of the CXCR4 and CXCR5 receptor is crucial for the positioning of B cells within lymphoid tissue compartments.[88]

BTK and PLCg have been shown to mediate chemokine-controlled B cell migration and homing.[89]

Appropriate regulation and functional activity of BTK is crucial for adequate B cell activation, differentiation and prevention of autoimmunity. As mentioned above, BTK is also embedded in several other signaling pathways, which means that changes in BTK protein levels or phosphorylation status not always affect one single pathway. In this regard, parallel measurements of up- or downstream proteins of BTK (e.g. phosphorylation of PLCg) may be more reliable to draw unambiguous conclusions about the pathway of interest.

BTK and human autoimmune diseases

Increased BTK levels and altered BCR signaling are associated with active disease in several autoimmune disorders. In rheumatoid arthritis (RA), loss of B-cell tolerance to self-antigens (e.g. citrullinated proteins or collagen type II) is an important driver of disease.[90] In patients with RA, BCR signaling is already increased in naïve unstimulated B cells.[91] Additionally, these B cells harbor BCRs that recognize self-antigens.[92] Increased protein levels of BTK were found in both naïve and memory B cells from RA patients, when compared to healthy controls.[93] Interestingly, increased BTK protein levels correlated with the presence of antibodies against citrullinated proteins (ACPA), whereas ACPA-negative patients didn't show increased BTK levels. BTK protein levels are also increased in B cells in patients with other autoimmune diseases, including Sjögrens syndrome, granulomatosis with polyangiitis (GPA), and Systemic lupus erythematosus (SLE).[93, 94] In SLE patients, high frequencies of BTK^{high} B cells correlated with disease activity and some clinical indexes.[95]

Of note, BTK also plays a role in the maturation of neutrophils and macrophage signaling and activation.[96] Accumulation and activation of macrophages in inflamed tissue (e.g. synovial space) can modulate disease development in autoimmune diseases. This means that BTK activation can also contribute to disease development in a B cell-independent way. For example, in myeloid- and FcγR-dependent autoantibody-induced arthritis, BTK inhibition decreases cytokine levels within joints and ameliorates disease.[82, 97]

Aims of this thesis

The big question remains whether inflammation is a critical trigger for IPF/IPAH-onset and disease progression in susceptible patients or a consequence of established disease. Additionally, inflammatory pathways and immune disturbances are probably not the same from patient to patient. Inflammation phenotyping will be required to determine the best (treatment) strategy for each patient. Therefore, the aim of this thesis is to further unravel the immune-related pathogenesis of IPF (part 1) and IPAH (part 2). Novel insights may help determine how to fit inflammation in the primary process of fibrogenesis and vascular remodeling and give direction to new personalized treatment strategies that will hopefully advance patient-tailored care.

Part 1: adaptive immune responses in IPF and patient perspectives

In **chapter 2** we give an overview of the current knowledge on inflammation and immune cell activation in IPF pathogenesis and discuss whether immune cell activation is altered by current anti-fibrotic treatments. Finally, we provide an up-to-date overview of recent and upcoming therapeutic trials that target and modulate the immune system in patients with IPF. This review serves as a basis for new perspectives on how to proceed in further research addressing the role of inflammation and immunity in fibrogenesis and vascular remodeling in IPF (**Chapters 3 - 6**). This will help answering an important outstanding question: Is inflammation in IPF merely an epiphenomenon or a critical player?

Although Inflammatory processes can shape the course of IPF in different ways, individual patients' factors, should not be forgotten. In daily practice, disease behavior and response to therapy greatly vary among patients and a 'one fits all' approach for IPF treatment is not possible. To optimize personalized treatment in IPF, we give an overview how biology could be combined with patients' perspectives, such as comorbidities, lifestyle and preferences, and experiences with medication (**Chapter 3**).

In the next chapters (**Chapter 4-6**) we focus on the role of various immune cells and signaling pathways. In **Chapter 4** we zoom in on the role of circulating and tissue fibrocytes in IPF. Fibrocytes are bone marrow-derived cells, possibly implicated in IPF pathogenesis. However, it is unclear whether in diseased IPF lungs, fibrocytes are increased and whether circulating or cultured fibrocytes are a good reflection of lung fibrocytes. Therefore, we believe that identification and characterization of lung fibrocytes will aid our understanding of their role in tissue remodeling and fibrosis. Our objective is to develop a uniform strategy for fibrocyte identification for blood and tissue samples. By exploring fibrocyte biology in different types of tissue, we aim attribute to a uniform definition of fibrocytes and explore the potential of lung fibrocytes in IPF pathogenesis

In **Chapter 5** our goal is to provide a complete and clinically relevant overview of alterations in B-cell immunity. To this end, we assess B cells subsets and their activation status in explanted lungs and lymph nodes from IPF patients by detailed flow cytometry profiling, together with data obtained from circulating B cell subsets and (autoreactive)

immunoglobulins in IPF patients. In **Chapter** 6 we further explore the deviations in BCR signaling and the activity of several molecules downstream of the BCR in IPF patients. We also study the effect of anti-fibrotic medication on BCR signal responsiveness in IPF patients. The knowledge obtained in the studies described in **Chapters 5 and 6** may help to develop strategies to identify if there are subgroups of patients who may benefit from future immune or B cell-modulating (additional) therapies in IPF

Part 2: adaptive immune responses in PAH and SAPH

Given the similarities between IPF and PAH pathobiology and communalities of IPAH and autoimmune diseases, we hypothesized that loss of immune homeostasis contributes to the etiology of IPAH. In **Chapter 7** we studied if aberrant B-cell activation and T follicular helper cell activity are involved in the in IPAH development.
In **chapter 8** we study sarcoidosis associated pulmonary hypertenstion (SAPH). We set out to determine a wide variety of cytokines and growth factors that are involved in granuloma formation and vascular remodeling. We use unbiased principal component analysis to identify inflammatory profiles that discriminate between SAPH and chronic sarcoidosis. Given the low prevalence of SAPH in the population and limited biological knowledge, information obtained from this large worldwide biobank and cohort of SAPH patients may provide more insight in the fundamental inflammatory pathways of SAPH, which may help selecting patients at risk for subsequent PH development and provide insight in potential targets for treatment development.

Finally, in **chapter 9** the results of these studies will be evaluated in the context of current and newly emerged scientific literature. We will discuss future research directions and clinical implications.

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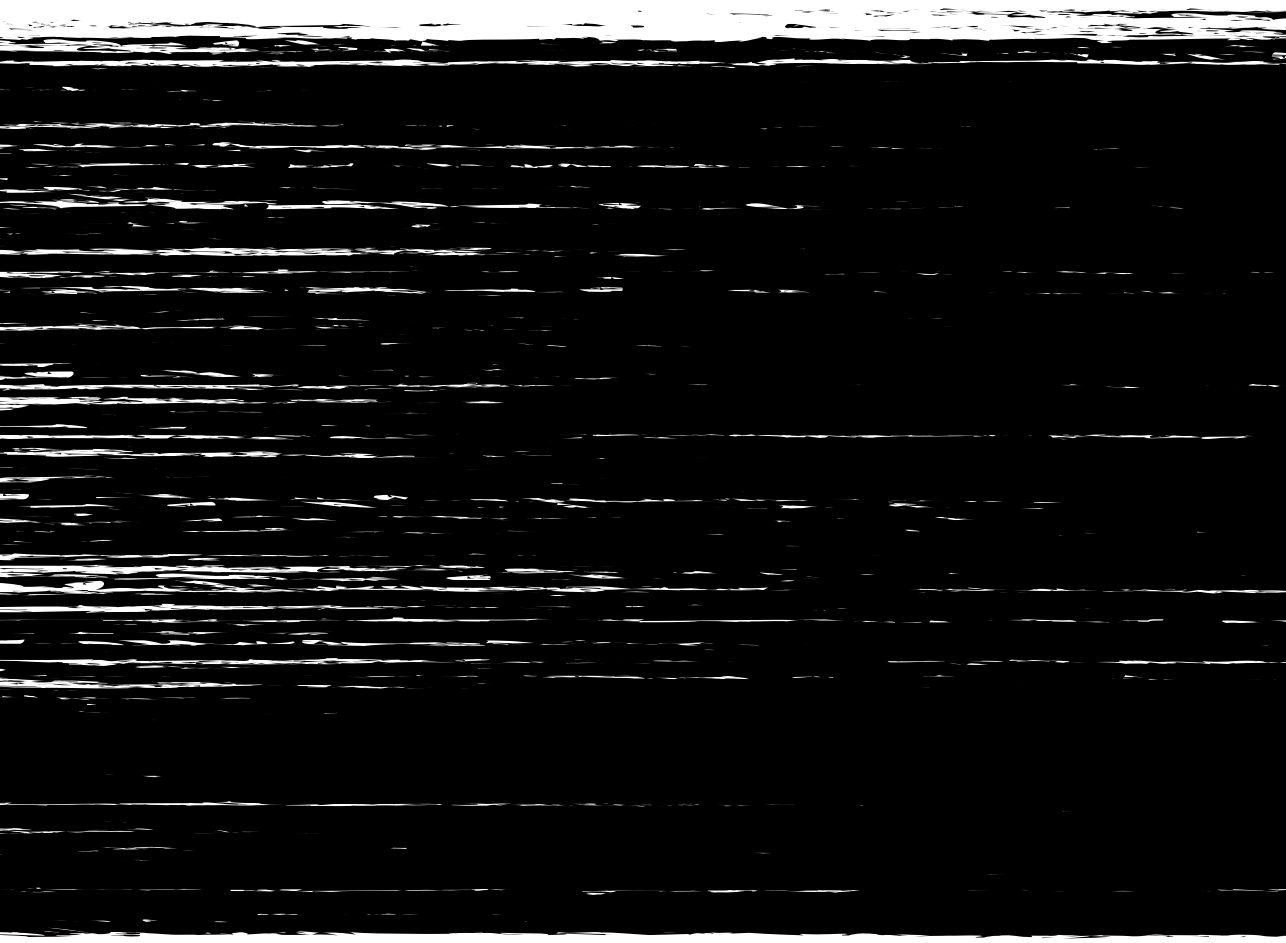
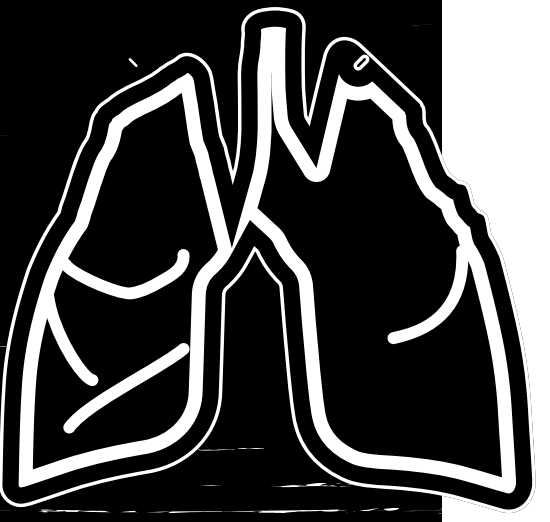


PART I

Adaptive immunity and personalized medicine in interstitial lung diseases

2



CHAPTER 2

Inflammation and Immunity in IPF Pathogenesis and Treatment

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive, and ultimately fatal, chronic interstitial lung disease characterized by enhanced extracellular matrix deposition. Repetitive alveolar epithelial injury triggers the early development of fibrosis. These injuries, in combination with dysregulated wound repair and fibroblast dysfunction, lead to ongoing tissue remodelling and fibrosis seen in end-stage pulmonary fibrosis. Although the exact etiology in IPF is unknown and probably diverse, all stages of fibrosis are accompanied by innate and adaptive immune responses. The role of inflammation as an important component in IPF etiology is controversial and sometimes seen as an epiphenomenon of fibrosis. This view is partly the result of negative multicenter trials of anti-inflammatory drugs for IPF treatment. However, new insights on the role of macrophages, the loss of T-cell and B-cell tolerance leading auto-immune responses in IPF, and the interaction of immune cells with (myo)fibroblasts have led to a slow change of this opinion. Clearly, more insight is needed to integrate basic immune mechanisms into translational research and finally new IPF therapies.

In this concise review, we will focus on the role of our innate and adaptive immune system in the initiation and perpetuation of IPF pathobiology. Next, we will discuss how immune responses are influenced by current anti-fibrotic treatments, such as pirfenidone and nintedanib and end with an overview of recent and upcoming therapeutic trials that target and modulate our immune system in patients with IPF.

Introduction

Idiopathic Pulmonary Fibrosis (IPF) is a progressive and ultimately fatal disorder, characterized by interstitial fibrosis of the lungs of unknown etiology.[1, 2] Although the natural history for patients with IPF varies (from rapid progressive to clinically relatively stable for years), the prognosis without treatment is 2 to 3 years.[3] In 2014, the Food and Drug Administration approved Nintedanib and Pirfenidone for the treatment of IPF after publication of two landmark studies, which both show that these new drugs were able to slow down disease progression.[4, 5] Although these new therapies have led to a better prognosis, IPF remains an incurable disease, which is partly the result of its complex and not well-understood etiology.[6, 7, 8]

The histologic hallmarks of IPF are excessive deposition of proteins of the extracellular matrix (ECM), the presence of fibroblast foci, and areas of fibrosis next to areas with normal lung parenchyma, so-called spatially heterogeneous fibrosis.[9, 10] Fibrosis is the end-result of exaggerated wound repair and tissue remodeling, which is believed to be caused by (repetitive) epithelial injury leading to chronic inflammation and finally fibrosis. Both our innate and adaptive immune system are involved during the development of fibrosis.[10] The basis for the excessive inflammatory response observed in IPF is thought to be multifactorial and one of the key-pathways involved is transforming growth factor-β (TGF-β) signaling.

Some connective tissue diseases (CTD) (e.g. systemic sclerosis and rheumatoid arthritis (RA)) have a high prevalence of interstitial lung disease (ILD), including pulmonary fibrosis.[11] Often a variable mixture of inflammation and fibrosis is seen, depending on the time in disease course and the underlying disease.

Similar mechanisms appear to be involved in the development of pulmonary fibrosis in IPF and RA-ILD patients.[12] Consequently, they both may benefit from the same treatment. Genome-wide association studies (GWAS) found multiple genetic variants linked to an increased risk for IPF development. Those genes are involved in either cell proliferation, host defense, or cell-cell adhesion (such as *FAM13A* (4q22), *DSP* (6p24), *OBFC1* (10q24))[13, 14, 15] Furthermore, several genes essential for lung development and response to environmental injury, like WNT, TGF, NOTCH and sonic hedgehog (SHH) are critically involved in the initiation of IPF.[10, 16, 17] Genes directly involved in inflammatory processes have also been linked to IPF. Gene variants in *TOLLIP (*Toll-interacting protein), the inhibitory protein of the Toll-like receptor (TLR) signaling pathway, lead to reduced protein expression, which possibly contributes to an increased pro-inflammatory response observed in IPF patients.[18] Furthermore, defective TLR3 function, caused by the *L412F* TLR3 SNP in IPF patients is associated with a more aggressive clinical phenotype.[19] Additionally, Fingerlin and colleagues found an association between HLA region and fibrotic idiopathic interstitial pneumonia. These putative risk alleles (*DRB1*15:01* and *DQB1*06:02*) are involved in immune and inflammatory response regulation.[20]

As IPF is a disease mainly affecting the elderly, immune senescence might also play an important role. Indeed, telomere maintenance dysfunction can lead to immune senescence[21], and diminished telomerase activity is found in familial IPF, due to mutations in telomerase reverse transcriptase (TERT) or telomerase RNA component (TERC).[22, 23]

Despite several lines of evidence that inflammation plays an important role in IPF pathogenesis, treatments that modulate inflammation (e.g. steroids) have failed or have even had deleterious effects on primary end-points in clinical IPF trials.[24, 25, 26, 27, 28, 29] This raises several issues. First, research should examine to what extent inflammation is a (co-)driver of disease or whether it is merely an epiphenomenon. To gain insight, we need to identify 'the good and bad cops' regarding inflammatory responses in IPF. As the natural history and clinical course greatly varies among IPF patients, this suggests several underlying (inflammatory) pathways could provoke disease. This also means different potential targets for treatment and possibly a subgroup of patients may benefit from additional/low dose anti-inflammatory therapies.

In this review we will focus on the role of our innate and adaptive immune system in the initiation and perpetuation of IPF pathobiology (**Figure 1 and 2**). We will also discuss the effect of currently used IPF treatments, e.g. pirfenidone and nintedanib on immune cells and why some anti-inflammatory drugs work in pre-clinical studies but not in humans. We end with current new IPF therapies that target basic immune mechanisms (**Figure 3**).

Involvement of the innate immune cells in IPF.

Neutrophils

Upon tissue damage or invading pathogens in human lungs, neutrophils are one of the first responders. Neutrophils migrate to damaged tissue in response to several chemokines and interleukins (IL), in which IL-8 (also called chemokine (C-X-C motif) ligand 8, CXCL8) is the predominant neutrophilic chemoattractant. Neutrophils can be activated by several receptors and proteins, including G-protein-coupled receptors, Fc-receptors, Toll-like receptors, adhesion molecules, and various cytokines (e.g. IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF)).[30] Next to phagocytosis and cytotoxic mechanisms, neutrophils influence subsequent immune response by their release of pro-inflammatory cytokines and production of reactive oxygen species (ROS)).[31] In patients with IPF, IL-8 and G-CSF are increased in broncho alveolar lavage fluid (BALF) and sputum, indicative of neutrophil attraction and activation. Neutrophil counts in BALF inversely correlate with FVC values in IPF patients and increased IL-8 concentrations in BALF are predictive for future exacerbations of IPF.[32, 33, 34] In mice, blocking neutrophil chemotaxis by inhibiting IL-8 function attenuate the development of bleomycin-induced lung fibrosis.[35] In IPF patients, no correlation was observed between BALF neutrophil percentages and prognostic parameters, thus neutrophilic inflammation may just be a marker of the extent of disease .[36] It is important to mention that, although the bleomycin model is the most frequently used model for lung fibrosis, it deviates from human IPF pathology as fibrosis development is not progressive and this model strongly depends on inflammation.[37]

One of the main products of neutrophils is neutrophil elastase (NE). NE has broad substrate specificity with both pro-fibrotic and anti-fibrotic properties. NE breaks down collagen-IV and elastin, thereby alleviating fibrosis. Conversely, NE promotes fibroblast proliferation, myofibroblast differentiation, and TGF-β activation, all contributing to enhance fibrosis.[38] Two recent studies using NE antagonist demonstrate attenuation of fibrosis in respectively an asbestos and bleomycin-induced fibrosis model, which may point toward a more pro-fibrotic role of NE in IPF.[38, 39]

In summary, neutrophils are key-players in the acute phase of inflammation and aggregated neutrophil accumulation in response to lung damage could lead to amplified tissue remodeling and fibrosis, possibly mediated via NE. Whether neutrophilic inflammation in IPF lungs has any prognostic value is unclear.

Macrophages

Macrophages play a central role in tissue repair and immunity. Macrophages are highly plastic cells and changes in environmental cues and molecular mediators can shift macrophages from an M1 into M2 phenotype and vice versa.[40, 41, 42] M1 macrophages are "classically" activated by LPS and IFNγ and produce pro-inflammatory cytokines such as TNFα, IL-1, and IL-6, thereby maintaining tissue inflammation.[43] M2 macrophages are "alternatively" activated after exposure to IL-4, IL-10, and IL-13 and harbor anti-inflammatory, pro-fibrotic properties. In chronic inflammatory conditions, pro-inflammatory M1 macrophages slowly convert into a more anti-inflammatory (pro-fibrotic) M2 phenotype secreting mediators that promote wound healing (**figure 1**). As expected, M2 macrophages are widely present in fibrotic lungs.[41, 42] M2 macrophages secrete several growth factors, including TGF-β, fibroblast growth factor (FGF), platelet-derived growth factor-α (PDGFα), insulin-like growth factor 1 (IGF1), and vascular endothelial growth factor (VEGF).[44] Especially VEGF serum concentrations correlate with clinical and biological parameters of disease severity in IPF patients.[45] M2 macrophages also contribute to the formation of extracellular matrix, specifically to collagen synthesis through up-regulation of the L-arginine metabolism. [43]

In IPF patients with an acute exacerbation (AE), enhanced concentrations of CCL18 in bronchoalveolar lavage fluid (BALF), most likely produced by M2 macrophages, are predictive of future exacerbations.[46] This suggests that macrophage-driven mechanisms are involved in AE of IPF.[46] Also in stable disease, CCL18 is increased in BALF.[46] CCL18 attracts T-cells to the lungs and activates fibroblasts.[47] Human lung fibroblasts and collagen can in turn stimulate macrophages leading to CCL18 production, suggesting a positive feedback loop might be involved in lung fibrosis.[48, 49] In humans, a subset of M2 macrophages (so-called regulatory macrophages) can even degrade components of the ECM through secretion of matrix metalloproteinases (MMPs), leading to reduced fibrosis.[50] The role of MMPs and tissue inhibitors of metalloproteinases (TIMPs) in fibrosis is comprehensively reviewed elsewhere.[51]

Concluding, macrophages may exhibit anti-fibrotic, pro-fibrotic and tissue-regenerating functions, which highly depend on the local cytokine environment. M2 macrophages appear to play a crucial role in regulation of fibrosis [43] and may even be involved in AE of IPF.[46]

Monocytes

Human monocytes have distinct roles in tissue homeostasis and immunity and are the circulating precursors of macrophages. One of the most important chemokines that regulate monocyte migration is CCL2 (also called monocyte chemoattractant protein-1, MCP1) via C-C chemokine receptor type 2 (CCR2). In IPF patients, increased serum CCL2 concentrations and enhanced CCL2 production by endothelial cells are found, suggesting increased homing of monocytes to the lungs of IPF patients.[52, 53, 54] A phase II trial investigating carlumab, a CCL2 inhibitor, in IPF patients did not show any treatment benefit and surprisingly, free CCL2 was still elevated in carlumab treated patients.[55]

Different monocyte subsets exist, which can be discriminated based on cell-surface expression of CD14 and CD16 into classical monocytes ($CD14^{hi}CD16^{low}$), intermediate monocytes ($CD14^{hi}CD16^{hi}$), and non-classical monocytes ($CD14^{low}CD16^{hi}$).[56, 57] Although the exact function of these three subsets is relatively unexplored in IPF pathogenesis, classical monocytes infiltrate the fibrotic lung, where they mature into macrophages in the early phases of fibrosis.[56, 57, 58] Increased proportions of circulating classical and intermediate monocytes correlate with disease progression and intermediate monocytes even predicted worse prognosis in IPF patients.[59] IPF intermediate monocytes produce increased amounts of pro-inflammatory cytokines, including IFN-α, MIB-α (CCL3) and MIP-1β (CCL4), which promote myofibroblast differentiation.[60] [61] .

After activation, CD14+ monocytes, including classical and intermediate monocytes, from patients with systemic sclerosis associated ILD (SSc-ILD) produce more CCL18 and IL-10.[62] In SSc-ILD patients with moderate to severe fibrosis, these circulating CD14+ monocytes show an activated phenotype indicated by increased expression of HLA-DR, IL-6, TNF-α, and TGF-β compared to SSc without ILD.[62] [63] This suggests that ongoing inflammation could skew monocytes into a pro-fibrotic phenotype.

The role of non-classical monocytes in IPF remains unclear. In patients with SSc-ILD, increased numbers of $CD16^{+}$ monocytes are associated with severity of pulmonary fibrosis[64]. The authors suggest that $CD16^{+}$ monocytes could be the main source of pro-fibrotic macrophages, however, they did not discriminate between non-classical and intermediate monocytes and did not discuss that local environmental cues can also influence macrophage fate.[64, 65]

In conclusion, monocytes function as critical cells during fibrogenesis; they serve as progenitor cells for (pro-fibrotic) macrophages and fibrocytes (see next paragraph), and are highly activated during fibrotic conditions, releasing (pro-fibrotic) inflammatory cytokines.

Fibrocytes

Fibrocytes are derived from monocytes and are precursors of fibroblasts. They were first described in an experimental skin wound model in mice as bone marrow-derived cells. Upon tissue injury, fibrocytes migrate to the affected organ in response to several ligands and chemotactic factors, where they differentiate into fibroblast-like cells and account for a small percentage of the total fibroblast pool.[66, 67, 68] Fibrocytes are characterized by expression of leukocyte common antigen CD45 and intracellular Collagen-1. In addition, chemokine receptors (e.g. CXCR4, CCR7, and CCR2) have been used to identify subsets of fibrocytes, possibly indicative of different activation stages. For instance, CCR7-possitive fibrocytes have been shown to produce increased amounts of TGF-β.[67, 69, 70, 71] To aid wound healing, several matrix proteins (collagen I, collagen III, vimentin, and fibronectin) are produced by fibrocytes.[72, 73, 74] Furthermore, fibrocytes are able to produce cytokines and growth factors like IL-8, IL-6, TGF-β, and PDGF, GM-CSF, VEGF, respectively (**figure 1**). These factors are known to induce fibroblast differentiation, neo-angiogenesis, and recruitment of inflammatory cells.[67, 75, 76] Fibrocytes also influence function of other fibroblasts as co-culture of fibrocytes with fibroblasts induced the secretion of type I collagen, matrix metalloprotease-1, and PDGF-β by fibroblasts.[77]

Fibrocyte migration and homing to the lung depends greatly on CXCL12, which is the ligand for CXCR4. Increased amounts of CXCL12 are found in BALF and serum of IPF patients and serum CXCL12 correlate with lung diffusing capacity.[72] Neutralizing CXCL12 attenuate pulmonary fibrosis and decrease the number of lung-fibrocytes in bleomycin-exposed mice. This suggest that CXCL12 is crucial for fibrocyte migration to the lung and contribute to lung fibrosis. [72, 78] In IPF patients, baseline circulating fibrocyte numbers correlate with disease progression and survival.[79] In this study, fibrocyte proportions above 5% of total blood leukocytes are an independent predictor of early mortality. The majority of the patients with >5% fibrocytes experience an AE of IPF.[79, 80]

An important area of concern is the lack of a uniform (gating) strategy to identify circulating fibrocytes and recently published papers suggest that circulating fibrocyte numbers are probably lower than previously reported.[81, 82, 83]

Altogether, in patients with IPF, fibrocytes contribute to fibroblast activation, ECM production, and display several paracrine functions, which all could lead to tissue remodeling and ongoing fibrosis.

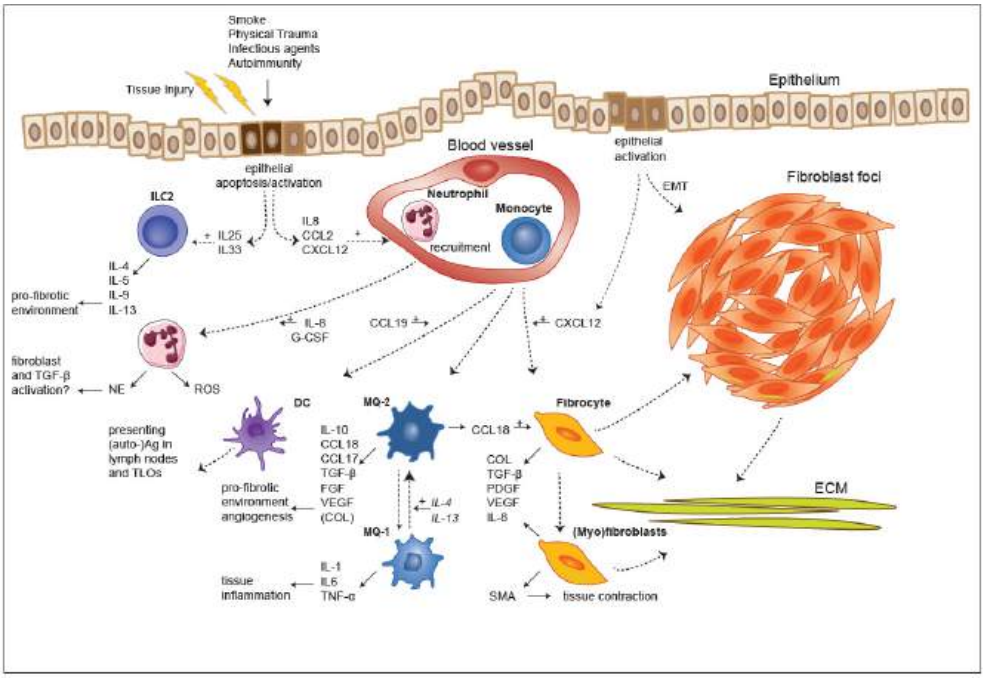

Figure 1 Schematic overview of the role of the innate immune system in IPF pathogenesis.
Abbreviations: EMT = epithelial–mesenchymal transition, ILC2 = type 2 innate lymphoid cells, DC = dendritic cell, MQ = macrophage, IL = interleukin, CCL = Chemokine (C-C motif) ligand, CXCL = C-X-C motif chemokine ligand, G-SCF = granulocyte-colony stimulating factor, NE = neutrophil elastase, ROS = reactive oxygen species, FGF = fibroblast growth factor, VEGF = vascular endothelial growth factor, COL = collagen, SMA = smooth muscle actin, ECM = extra cellular matrix, TNF = tumor necrosis factor, TGF = transforming growth factor

Type 2 Innate Lymphoid Cells and Mast Cells

It is well-established that the adaptive T-helper 2 (Th2) inflammatory response and type-2 cytokines play an important role in pulmonary fibrosis.[84, 85, 86, 87, 88, 89, 90, 91] Approximately 10 years ago, innate cells that produce type-2 signature cytokines (e.g. IL-4, IL-5, IL-9, IL-13) have been described, and named type 2 innate lymphoid cells (ILC2s)[92]. Cytokines responsible for attraction of ILC2s to the lung and their activation, such as IL-25 and IL-33 are produced by damaged epithelium and activated mast cells, eosinophils and macrophages. All these cells are thought to play an important role in the pathogenesis of IPF.[93, 94, 95, 96] Indeed, both proportion and number of ILC2s and IL-25 concentrations are increased in BALF of patients with IPF. [97] IL-13 production by ILC2s appears crucial to induce pulmonary fibrosis in experimental mice.[98] Activation of the IL13R leads to the activation of transcription factor STAT6, which is involved in regulating both collagen synthesis and type-2 immunity.[98, 99] Furthermore, IL-13 is a potent stimulator and activator of TGF-β.[100, 101] Recently, three independent trials blocking IL-13 (and IL-4) were performed in patients with IPF. All three trials failed to meet their primary endpoints.[102]

In SSc-ILD, ILC2 numbers in peripheral blood correlate with the extent of interstitial fibrosis.[103] IL-9 can activate and expand ILC2s and blocking IL-9 reduce airway inflammation and remodelling partly through decreased TGF-β production.[104] Next to the production of type-2 cytokines, ILC2s are also capable of producing amphiregulin.[105] Increased amphiregulin expression promotes fibroblast cell proliferation and collagen accumulation.[106] Although the exact role of other ILC subtypes in pulmonary fibrosis needs further research, cytokines produced by ILC1 (e.g. IFNγ and TNFα) and ILC3 (e.g. IL-17A and IL-22) exert important functions that influence pulmonary fibrosis pathogenesis in many different ways which have been comprehensively reviewed elsewhere.[95]

Mast cells are important players in type-2 immunity. They release a wide array of mediators, either from prestored granules or de-novo synthesized that induce tissue inflammation.[107] Mast cell granules can contain large amounts of pro-fibrotic type-2 cytokines (e.g. IL-4, IL-10 and IL-13) and growth factors (e.g. TGF-β and PDGF).[107] Increased numbers of (activated) mast cells in lung tissue and enhanced concentrations of mast cell products (e.g. tryptase, chymase and histamine) in BALF are found in IPF patients.[108, 109, 110] Histamine directly enhances fibroblast proliferation *in vitro* and tryptase stimulates the synthesis of type-I collagen by fibroblasts.[111, 112]

It is clear that type-2 cells, including ILC2 and mast cells are present in IPF lungs and contribute in orchestrating inflammatory responses and ECM homeostasis by releasing a wide array of mediators. However, most studies support the concept that ILC2 and mast cells are modulators of disease rather than critical players for disease pathogenesis.

Bridging innate and adaptive immunity

Dendritic cells and tertiary lymphoid structures

Dendritic cells (DCs) function as cells within both the innate and adaptive immune system. DCs are widely present throughout the normal lung where they repeatedly sample exogenous antigens.[113] After activation of pulmonary DCs with danger signals, for instance by Toll-Like-Receptor (TLR) ligands, DCs migrate from the inflamed or injured lung to the lung-draining mediastinal lymph nodes, where these activated DCs prime specific T-cell responses (**figure 2**).[114] In IPF lungs, DCs are widely present in both the periphery as well in tertiary lymphoid structures (TLOs) found in IPF lungs.[115, 116] TLOs have a structure similar to that of lymph nodes, including T-cell zones, B-cell follicles, and high endothelial venules (HEV) without encapsulation. Immature DCs are located in areas of fibrosis, whereas mature DCs are predominately found within TLOs.[115, 116] DCs play a crucial role in the maintenance of TLO structures.[117] TLOs arise in response to non-resolving ongoing inflammation and contribute to the (local) immune response.[116, 118, 119] Interestingly, TLOs persist and accumulate during disease progression in IPF patients.[120]

One of the chemokines crucial for DC attraction via CCR7 is CLL19.[121] CCL19 is expressed by epithelial cells and fibroblasts and increased amounts of CCL19 in BALF of IPF patients is observed together with an increase in predominately immature DCs in IPF lungs.[116, 122] Strikingly, fibroblasts can inhibit DC activation as co-culture of IPF fibroblasts or control

fibroblasts with DCs resulted in a decreased upregulation of activation markers.[123] However, this probably reflects the general immunosuppressive property of stromal cells, rather than IPF-specific decreased DC activation.

Lungs of mice treated with bleomycin show increased numbers of mature DCs and recently activated T-cells.[124] Hindering DC/T-cell communication with an immunomodulatory (VAG539) that inhibits DC activation, e.g. reduces CD86 and MHCII expression, attenuates lung fibrosis in bleomycin-treated mice.[124] These data suggest that DCs are able to sustain pulmonary inflammation and fibrosis in a bleomycin model.

Involvement of the Adaptive immune system in IPF

T-cells

T-cells are widely present in active-disease regions and TLOs in lungs of patients with IPF.[116, 119, 120] T-cells contribute to pulmonary fibrosis, as fibrosis, like ECM formation and fibroblast proliferation is reduced in mice lacking T-cells (a-thymic mice) after bleomycin exposure.[125] Historically, an imbalanced Th1/Th2 immune response has been thought to be central in IPF pathogenesis. Indeed, type-2 cytokines promote pro-fibrotic responses, whereas Th1 cytokines (IFNγ and IL-12) may be protective.[85, 86, 126, 127, 128, 129] However, the negative results of the INSPIRE trial (IFNγ treatment in IPF) have indicated that this view might be too simplistic.[26] In the past decade, there is emerging evidence that other Th-cells, such as Th17-cells, Th9-cells, and regulatory T-cells (Tregs) play a role in IPF pathogenesis. As Th2-cells and Th1-cell function in IPF have been discussed extensively elsewhere[86, 87, 88, 90, 130, 131], we will focus in this review on Th9-cells, Th17-cells, and Tregs (**figure 2**). A brief summary of Th2-cell and Th1-cell function in IPF is added in **table 1**.

Th9 cells

Th9-cells are a relatively new and less well-characterized T-helper subset, involved in anti-parasite and anti-tumor immunity, auto-immunity, and triggering allergic inflammation.[132] Environmental IL-4 and TGFβ, both cytokines linked to fibrosis and type-2 immunity, dictate Th9-cell differentiation.[132] The key-cytokine secreted by Th9-cells is IL-9.[133, 134] IL-9 is a pleiotropic cytokine involved in promoting T-cell growth, B-cell development, potentiating CCL11 and IL-8 release from smooth muscle cells, and activating and expanding ILC2s.[104, 135] IL-9 also harbors both anti-fibrotic and pro-fibrotic effects. Mice that constitutively express high levels of IL-9 display significantly less fibrosis in both silica and bleomycine models for lung fibrosis. [136] [137] This effect was associated with decreased type-2 immunity and reduced prostacyclin-dependent epithelial cell apoptosis.[137]

In contrast, neutralization of IL-9 in mice ameliorates hepatic fibrosis. This neutralization of IL-9 result in a decrease in Th17-cells, Th1-cells, and various cytokines (e.g. IL-17A, IFNγ, TGFβ, IL-6 and TNFα) in liver and plasma.[138] In a murine asthmatic airway remodeling-model (chronic exposure to *Alternaria alternata* extract), IL-9-overexpressing mice show increased airway fibrosis possibly via increased eosinophil recruitment and augmented expression of connective tissue growth factor (CTGF).[139]

In conclusion, opposite effects of Th9-cells and IL-9 have been described in different murine models of fibrosis, which makes it difficult to draw straightforward conclusions. It is worthwhile to investigate whether these cells are present in lungs of IPF patients.

Th17-cells

Th17-cells are effective inducers of tissue inflammation through their production of IL-17(A), IL-21, and IL-22. Th17-cells are involved in the pathogenesis of many autoimmune diseases and in host defense directed against extracellular bacterial and fungal infections.[140] Recently, a rare monogenetic autoimmune-mediated lung disease called COPA directly links Th17-cell immunity to pulmonary fibrosis.[141, 142] Th17-cells and IL-17 are found in and around inflammatory infiltrates in patients with IPF and it is clear that IL-17 plays a role in the development of fibrosis.[119] Importantly, IL-17 is not exclusively produced by Th17-cells. A variety of other immune cells, including macrophages, neutrophils, NK-cells, ILC3, and γδ-T cells also produce IL-17.[130]

Serum IL-17 concentrations positively correlate with lung inflammatory scores in bleomycin-treated mice.[143] IL-17 promotes pulmonary fibroblast proliferation *in vitro*, resulting in increased type I collagen synthesis, TGF-β, and IL-6 expression.[143] IL-17A antibody treatment alleviates pulmonary fibrosis in both bleomycin and radiation models.[143, 144] Furthermore, IL-17 activates epithelial cells to produce IL-8, leading to neutrophilic influx.[145] Th17-cells also maintain (local) inflammation by reducing susceptibility to suppression by autologous Tregs.[146] Indeed, IL-17A neutralization delays neutrophil accumulation and fibrosis, and also increase Treg accumulation. The increase in Tregs is most likely caused by a decrease in IL-6 and increase in IL-10. This increase in IL-10, together with TGF-β, favors Treg over Th17-cell development.[144, 147, 148] Additionally, shifting the balance between Tregs and Th17-cells by IL-27 administration, attenuates the development of pulmonary fibrosis in a bleomycin model.[149, 150] Thus the balance between anti-inflammatory Tregs and pro-inflammatory Th17-cells is crucial within the development of experimental fibrosis.

Interestingly, both Th17-cells and IL-17A are required for the early inflammation and IL-17A is crucial during end-stage lung fibrosis in an experimental silicosis model.[151] In humans with skin fibrosis, the IL-17 signaling pathway could be anti-fibrotic, shown by less skin sclerosis and increased serum IL-17A.[152]

The exact function of human Th17 cells and IL-17 in the development of IPF is not well understood and although most murine data suggest that Th17 cells and IL-17 exert pro-fibrotic functions by initiating and sustaining inflammation in the first-stage of tissue damage, it seems that its role in established (end-stage) disease may be less important.

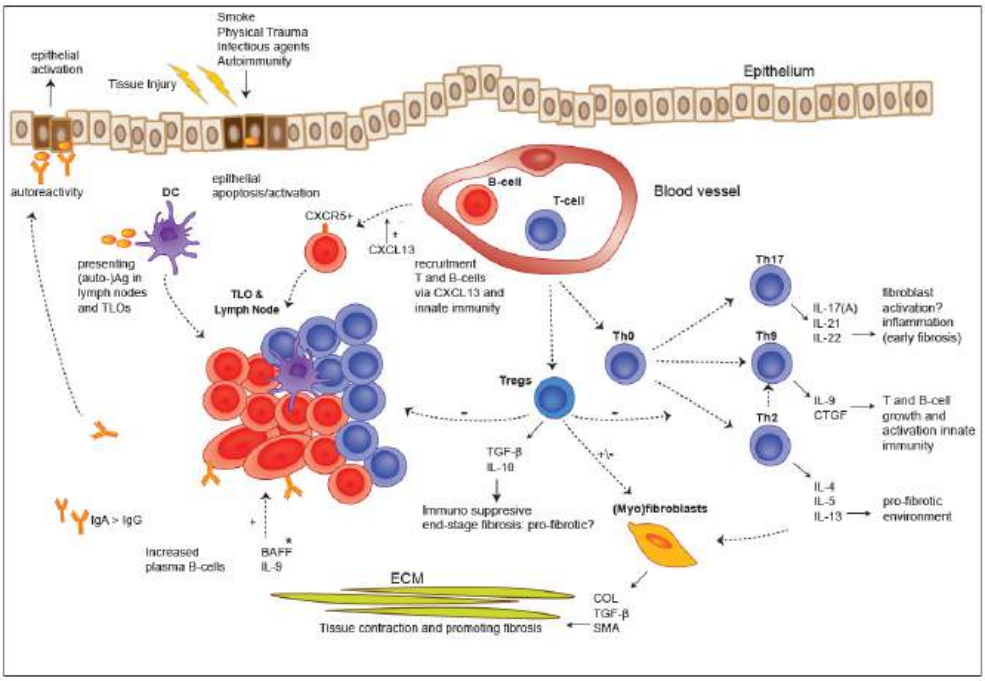

Figure 2 Schematic overview of the role of the adaptive immune system in IPF pathogenesis.

* also known as B lymphocyte stimulator (BLyS)

Abbreviations: DC = dendritic cell, CXCR = C-X-C motif chemokine receptor, CXCL = C-X-C motif chemokine ligand, TLO = tertiary lymphoid structure, IL = interleukin, Th = T-helper cell, Tregs = regulatory T-cell, SMA = smooth muscle actin, ECM = extra cellular matrix, TGF = transforming growth factor, COL = collagen, BAFF = B-cell-activation factor, CTGF = connective tissue growth factor

Regulatory T-cells

Tregs are crucial for maintaining host tolerance and the prevention of autoimmunity.[153] Tregs also produce TGF-β, leading to increased PDGF-β production and fibroblast proliferation.[154] The suppressive function of Tregs on inflammatory processes governing IPF has been widely studied. One of the first reports of Tregs in IPF patients show that Tregs are decreased in number in BALF and peripheral blood and have a decreased suppressor potential.[155] Decreased Treg proliferation also correlates with decreased FVC and diffusion capacity.[155] Mice with accumulated Tregs in the lung (e.g. CCR7$^{-/-}$ mice) show attenuation of fibrosis.[70] These initial reports suggest that Tregs have an anti-fibrotic effect. However, as for many other cells, also pro-fibrotic effects have been observed. Depletion of Tregs in a transgenic lung-specific TGF-β1 overexpression murine model causes enhanced intrapulmonary accumulation of fibrocytes and augmented fibrosis.[156] Unexpectedly, Treg administration in mice lacking lymphocytes (Rag1$^{-/-}$mice) exacerbated bleomycin-induced pulmonary fibrosis.[157] The authors hypothesized that expanding the Treg population may be unfavorable due to bleomycin-induced plasticity of Tregs (e.g. down-regulation of CD25 and Foxp3) resulting in more acute injury and augmentation

of the type-2 immune response.[157] Recently, it has been shown that especially activated Tregs are increased in IPF patients.[158] This could, in part, explain previous findings that the total Treg-pool is unchanged or decreased.[155]

The phase (e.g. early vs late) of fibrosis might be important for Treg function. Tregs might be harmful in early stages, but protective in late stages of pulmonary fibrosis in mice.[159] Additionally, Tregs with increased expression of membrane protein semaphorin-7a, which is involved in the production in pro-inflammatory cytokines, are less immunosuppressive and are associated with a more progressive clinical course in IPF patients.[160]

Historically, it has been thought that Tregs have a protective role in IPF as they decrease fibrocyte accumulation and dampen inflammatory responses. However, more recent data show that their role may differ during the different stages of fibrosis and that Tregs also possess pro-fibrotic functions by secreting pro-fibrotic cytokines.

B-cells

As B-cells are present within TLOs, it is logical that increased numbers of B-cells are found in the lung of IPF patients. [116,120] B-cells present within TLOs are most likely responsible for the local production of (auto-) antibodies.[161] Specific gene clusters of inflammation, which also comprise genes related to B-cell markers and specific chemokines (e.g. CXCL13, CXCR5, CCR6, and CCR7) are significantly increased in explanted lung tissue of IPF patients.[162] Chemotaxis of B-cells to B-cell follicles within TLOs of IPF lungs primarily depends on CXCL13 and its receptor on B-cells (CXCR5). CXCL13 is produced by follicular DCs and follicular helper T-cells (Tfh) in B-cell follicles/TLOs.[163] Increased CXCL13 concentrations are found in lungs and serum of patients with IPF. [162, 164] CXCL13 concentrations inversely correlate with survival of IPF patients.[162, 164]

B-cell-activation factor (BAFF, also known as BLyS) regulates B-cell survival, development, function, and plays a critical pathogenic role in autoimmune diseases.[165] In IPF patients, BAFF plasma concentrations correlate with disease progression.[166] A possible mode of action of BAFF in IPF patients might be promoting survival of auto-reactive B cells.[167] By definition, IPF patients do not fulfill the (clinical) criteria for an underlying auto-immune disease, however, self-reactive immunoglobulins can be found in the majority of patients.[168] Auto-antibodies produced by auto-reactive B-cells target self-antigens, which in IPF patients in most cases recognize epithelial antigens. This auto-antibody-induced inflammation plays an important role in repeated lung injury leading to aberrant wound repair (**figure 2**). Over the past two decades several specific auto-antibodies to self-antigens have been identified in IPF.[168, 169, 170, 171, 172, 173, 174, 175] The majority of the identified self-antigens (e.g. annexin-1, cytokeratin-18, periplakin, and vimentin) are intracellular epithelial proteins. Most of these auto-antibodies concentrations correlate with outcome measures in IPF, including the development of acute exacerbations.[170, 171, 172, 173]

The concentration of total immunoglobulins also correlate with outcome. For instance, IPF Patients with increased circulating total IgA levels at baseline (> 2·85 g/l) have a worse prognosis.[176] This may not be surprising since IgA production and class-switching are regulated by TGF-β and elevated TGF-β is found in IPF lungs.[176, 177, 178] In addition, the majority of IPF patients with auto-antibodies against periplakin, a desmosomal protein expressed by alveolar epithelial cells, are from the IgA subclass.[172] Using an unbiased proteomic analysis, it was shown that MZB1^{+}CD38^{+}CD138^{+} plasma B-cells are widely present in TLOs in human fibrotic lung tissue and had a high antibody secretory activity.[161]

Collectively, these data indicate that B-cells accumulate in IPF lungs and that humoral autoimmunity against epithelial (auto-)antigens can promote and maintain ongoing inflammation in IPF lungs.

Table 1 Summary of each cell-type and its role in IPF pathogenesis.

Celltypes	Description	Role in IPF	Ref.
Innate immune system			
Neutrophils	First-line responders upon acute inflammation and tissue damage. Important for removing microorganisms and damaged cells, and generation of inflammatory agents.	BALF Neutrophil counts inversely correlated with FVC values. Neutrophil elastase may boost tissue remodeling and fibrosis.	30-34, 38,34
Macrophages	Play a central role in tissue repair and immunity. Two subsets exist; activated (M1) macrophages and alternatively activated (M2) macrophages. Function strongly depends on local environmental state.	M2 macrophages have anti-inflammatory, pro-fibrotic, and tissue-regenerating properties. Increased number of macrophages in BALF in IPF patients are predictive for future exacerbations.	40-44,46
Monocytes	Role in tissue homeostasis and immunity. Divided in 3 subsets: classical monocytes (CD14^{hi}CD16^{low}), intermediate monocytes (CD14^{hi}CD16^{hi}) and non-classical monocytes (CD14^{low}CD16^{hi}).	Progenitor cells for pro-fibrotic macrophages and fibrocytes, release (pro-fibrotic) inflammatory cytokines and activate our adaptive immune system. Intermediate monocytes are associated with worse prognosis in IPF	50-61
Fibrocytes	Precursors of fibroblast derived from the common monocyte lineage, characterized by expression of leukocyte common antigen CD45 and intracellular collagen-1.	Fibrocytes contribute to fibroblast activation and ECM production. Circulating fibrocyte numbers correlate with disease progression and survival.	66-68,72-76,78-80
Mast cells	Tissue bound and fully differentiated cells. Upon activation, release a wide array of mediators (either from their granules or de novo synthesized) which induce tissue inflammation and recruitment of other cell types	Probably modulators of disease rather than critical players for disease pathogenesis.	107-112
Type 2 innate lymphoid cells	Innate immune cell that lack a specific antigen receptor. Producer of type Th2 effector cytokines and amphiregulin. Functions in tissue remodeling and repair (especially at barrier surfaces).	Increased ILC2 numbers are present in BALF of IPF patients Contribute to Type-2 environment seen in IPF lungs. IL-13 produced by ILC2 is involved in tissue remodeling and ECM production.	92-101

Celltypes	Description	Role in IPF	Ref.
Dendritic cells	Bridging innate and adaptive immunity. Shape the adaptive immune response by sampling antigens and subsequently migrate to TLOs or lymph nodes to present these antigens to (specific) T-cells.	Immature DCs are located in areas of fibrosis and are increased in IPF lungs. Mature DCs are predominately found within TLOs. Possibly involved in ongoing inflammation in IPF lungs.	113-116 120
Adaptive immune system			
Th2 cells	Involved in chronic inflammatory diseases and tissue repair. Secrete large amounts of IL-4, IL-5, IL-9, IL-13.	IL-4, IL-5, and IL-13 are linked to the development of fibrosis. IL-4 and IL-13 promote (myo)fibroblast activation and proliferation, and skew macrophages towards a pro-fibrotic phenotype	129-129 85-88,90 130,131
Th1 cells	Involved in phagocyte dependent inflammation and cell-mediated immunity. Secrete large amounts of IL-2 and IFN-γ	IFN-γ is associated with attenuation of fibrosis	85-88,90 130,131
Th9 cells	Involved in involved in anti-parasite and anti-tumor immunity, auto-immunity, and triggering allergic inflammation. Secrete IL-9.	The role in IPF etiology is unclear as opposite effects of Th9 cells and IL-9 have been described in the development of fibrosis	132-139
Th17 cells	Involved in the pathogenesis of several autoimmune diseases and in host defense directed against extracellular bacterial and fungal infections. Secrete IL-17(A), IL-21, and IL-22	IL-17 induces fibroblast proliferation and collagen secretion, however the role in human IPF disease not well understood. Murine data suggest a pro-fibrotic role for Th17 cells and IL-17.	140 143-152
Regulatory T-cells	Generally involved in maintaining host tolerance and prevention of autoimmunity. Secrete IL-10 and TGF-β.	Most likely a protective role in IPF: decrease fibrocyte accumulation and dampen inflammatory (auto-immune) responses. Possibly pro-fibrotic in end-stage disease.	70 153-160
B-cells	Humoral component of the adaptive immune response by secreting antibodies. B cells can mature into plasma cells that produce the antibodies to fight infections	Increased numbers of plasma cells in human fibrotic lung tissue, which can produce auto-antibodies against self-antigens (mainly epithelial antigens). Increased concentration of BAFF and CXCL13 found in serum of IPF patients. The concentration of IgA correlates with disease outcome.	116-120 161-164 166-178

Abbreviations: BALF = Broncho alveolar lavage fluid, ECM = extra cellular matrix, ILC2 = type 2 innate lymphoid cells, TLO = tertiary lymphoid structure

Influence of pirfenidone and nintedanib on inflammation

In 2014 pirfenidone and nintedanib were approved by the U.S. Food and Drug Administration and recommended for the treatment of IPF, as both drugs are effective in slowing down disease progression.[4, 5] In the past years advances have been made towards a better understanding of the role of both drugs on inflammatory processes, which is outlined below.[179]

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. It was not until 1995 that Lyer and colleagues demonstrated the anti-fibrotic effects of this drug in murine models.[180, 181]

One of the most important actions of pirfenidone is altering the pleiotropic TGF-β pathway.[182, 183, 184] Pirfenidone decreases Smad3, p38 and Akt phosphorylation, which are downstream intracellular proteins of the TGF-β pathway, reducing fibroblast proliferation.[183] Pirfenidone also directly affects TGF-β1 expression as it interacts with the hedgehog signaling pathway by destabilizing the glioma-associated oncogene homolog (GLI)2 protein, which is a transcription factor for TGF-β1 expression.[185]

Collagen synthesis is also influenced by pirfenidone (and also nintedanib), as both drugs inhibit collagen secretion in lung fibroblasts and hampered extracellular collagen fibril formation.[186]

Pirfenidone can modulate cross-talk between DCs and T-cells. Although antigen uptake was preserved, DCs treated with pirfenidone were less activated and their T-cell stimulatory capacity was impaired, together with a reduced production of pro-inflammatory cytokines *in vitro*.[187] Pirfenidone also directly decreases Th-cell proliferation and impairs Th2-cell polarization by down-regulation of Th2 master transcription factor GATA-3.[188, 189] The numbers of Th2-cells and Th2 cytokines in the peritoneal cavity are reduced after pirfenidone treatment in a rat model for intraperitoneal adhesion formation after surgery .[190]

In addition to the well-known anti-fibrotic effects, which are primarily driven by inhibition of the TGF-β pathway, pirfenidone can also attenuate adaptive (Th2) immune responses in a DC-dependent and independent way.

Nintedanib

Nintedanib is a small molecule that inhibits multiple receptor tyrosine kinases (TKI) including platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR) and vascular endothelial growth factor receptor (VEGFR) and therefore acts downstream of PDGF, FGF and VEGF, all of which are key growth factors in the pathogenesis of IPF.[179, 191] The anti-inflammatory properties of nintedanib are less well-known. In both bleomycin and silica-induced pulmonary fibrosis, nintedanib dose-dependently reduces total lung neutrophilic and monocytic inflammation especially when nintedanib is given in a prophylactic manner.[179, 191] The anti-inflammatory activity of nintedanib is less when given in a therapeutic setting (e.g. 10 or more days after silica or bleomycin exposure).[191] In two non-pulmonary fibrosis models, nintedanib decreased perivascular inflammatory cell infiltration (chronic allergic airway remodeling model) and liver inflammation (CCl4-induced liver fibrosis model).[192, 193] However, is it unclear whether changes observed in inflammation are the result of anti-inflammatory properties of nintedanib itself or indirect via tyrosine kinase inhibition.

Anti-inflammatory trials in IPF

Over the past three decades several therapeutic prospective, double-blind, randomized clinical trials have been executed to find clinical benefit in IPF.[194] Several of these landmark trials used anti-inflammatory drugs (anti-TNF-α, prednisone) or immunomodulatory agents (IFNγ, simtuzumab) and all these trials failed to meet their primary endpoints (change in FVC, time to disease progression or survival) or even had detrimental effects.[25, 26, 27, 28, 29, 195] Some important issues may help explain the failures in the development of (anti-inflammatory) drugs in IPF. First, preclinical trials often utilize mouse models of bleomycin induced pulmonary fibrosis, which is based on inflammation.[196] The second reason of failure of broad acting anti-inflammatory molecules is that they also interact with potential anti-fibrotic properties of immune cells.[197, 198, 199 200 201, 202]Finally, emerging evidence suggest that the etiology of acute exacerbation of IPF (AE-IPF) is different than that of slowly progressing IPF.[203, 204, 205] This means that (new) drugs that might be effective for relatively stable IPF patients may not be effective in AE-IPF or the other way around.

Despite all concerns, new and more specific drugs that alter inflammatory processes are currently being tested in phase 2 and 3 trials (**Figure 3**) and some of these studies are discussed below.

PRM-151, also known as recombinant human pentraxin-2, inhibits differentiation of monocytes into pro-inflammatory and pro-fibrotic macrophages and fibrocytes.[206] Recently, a phase 2 trial of PRM-151 (10 mg/kg every 4 weeks) in subjects with IPF resulted in a small but significant decrease in change in FVC percentage and change in 6-minute walk distance from baseline.[207]

Targeting anti-inflammatory cytokine IL-13 with antibodies tralokinumab and lebrikizumab have been tested in phase 2 trials in patients with IPF. Both studies did not meet the primary endpoint (change FVC from baseline).[208, 209, 210, 211] A bi-specific antibody against IL-4/IL-13 (SAR156597) failed to demonstrate benefit in treating patients with IPF. The primary (FVC change at 52 weeks) and important secondary end-points (disease progression and all-cause mortality) were not met.[211] Interestingly, the data from the anti-IL-13 trials show some positive effects on AE-IPF rates.[102, 211] This suggests that type-2 inflammation might be involved in AE-IPF, however it does not seem to control disease progression.

Interfering with the action of connective tissue growth factor (CTGF) using pamrevlumab, led to a reduction of pro-inflammatory factors (e.g. IL-1β and CCL3) and a decrease in ECM accumulation.[212] A phase 2 open-label trial of pamrevlumab in patients with IPF demonstrated a good safety profile and promising results in change of pulmonary function as 30% of patients showed an increase of FVC after 48 weeks of treatment.[213] A phase 3 clinical randomized trial of pamrevlumab in IPF patients was recently published and showed promise as a safe and effective treatment for idiopathic pulmonary fibrosis (NCT01890265).

Phospholipid lysophosphatidic acid (LPA) signaling governs several pro-fibrotic pathways and may play a role in proliferative and inflammatory responses to injury.[214] Decreasing LPA via autotaxin inhibition alleviates pulmonary fibrosis in mouse model.[215] Recently, an exploratory phase II study showed that autotaxin inhibition in patients with IPF resulted in an absence of lung function (FVC) decline over 12 weeks of treatment.[216] A larger randomized, double-blind, placebo-controlled trial with a LPA-1 antagonist (BMS-987020) also met its primary end point (change in FVC from baseline), however was terminated prematurely because of safety issues.[217]

Rituximab, an antibody against CD20 which destroys (auto-reactive) CD20$^+$ B cells, is effective in the treatment of several auto-immune diseases.[218] Compared to a historical cohort, autoantibody-targeted therapy with rituximab, plasma exchange and intravenous immune globulin (IVIG) in 11 patients with an acute exacerbation of IPF resulted in an improvement of gas exchange and clinical outcome.[219] A phase 2 double-blind, prospective clinical trial investigating rituximab in stable IPF patients with detectable auto-antibodies has been initiated. Its primary endpoint is reduction of anti-Hep-2 autoantibodies titers and secondary outcome measures are changes in FVC, survival and exacerbation rate (NCT01969409).

VAY736 is an antibody that targets the BAFF-receptor and hinders B-cell survival and development. This drug is currently under investigation in a phase 2 double-blind, prospective clinical. The results of this trial are expected late 2022 (NCT03287414).

Conclusion

IPF is chronic progressive fibrosing interstitial lung disease in which innate and adaptive inflammatory processes are involved (summarized in **table 1)**. The results of previous negative landmark trials altering inflammation may suggest that inflammatory changes seen in IPF occur independently of the primary fibrotic remodeling process. However, current treatments (e.g. pirfenidone and nintedanib) or new drugs that are effective in Phase-II trials for IPF (e.g. PMR151) also modulate inflammatory processes.

It is important to realize that inflammation is often necessary to deal with lung injury (e.g. infections, physical trauma). This issue may be especially true for inflammatory diseases in the respiratory system as immune cells are part of the normal anatomy and function of human lungs.[220] More importantly, it is all about the right balance between inflammation and adequate resolution of inflammation. Impaired resolution may lead to a chronic inflammatory state which can result in fibrosis.[89] Interestingly, some immune cells have fibrotic properties while others harbor anti-fibrotic properties.[88] It obvious that a 'one fits all' approach about the role of inflammation or immunomodulatory treatments in IPF does not apply.

Therefore, there is a strong need for stratified medicine based on genomics, biomarkers, but also inflammatory profiles to select patients with IPF who may be eligible for co-treatment with anti-inflammatory/immunomodulating therapies besides the now

considered standard of care with "anti-fibrotic" medication. This precision medicine should lead to a precise health advice, diagnosis, and a personalized treatment plan for patients with IPF.[221]

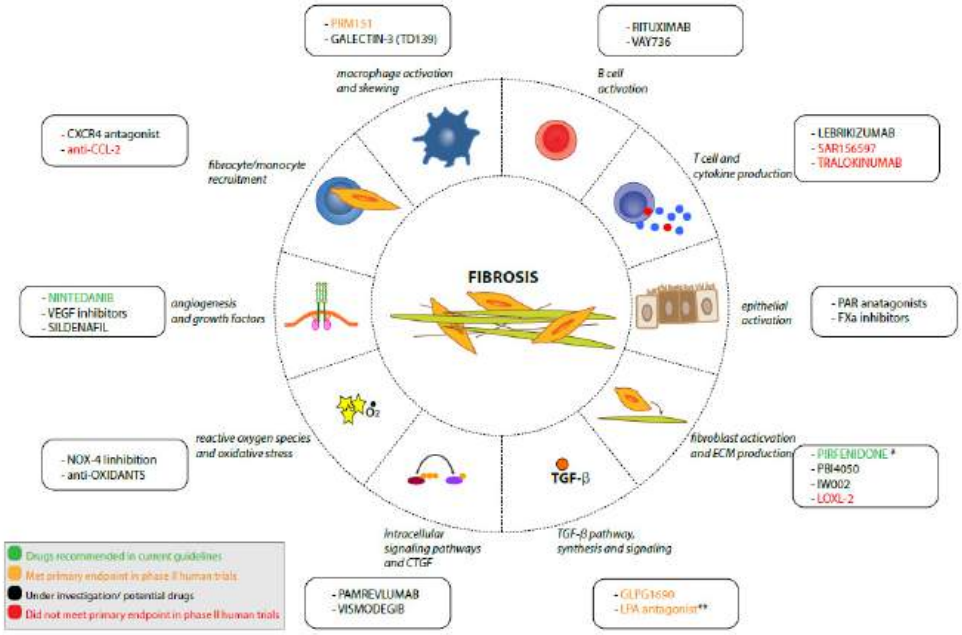

Figure 3 Overview of the most recent anti-inflammatory drugs and their target of different features of IPF disease pathogenesis. Depicted in green are drugs that have been proven effective in recent human phase II or III trials. In green drugs that are recommended in current guidelines. In orange drugs that met their primary endpoint in phase II human trials. In black are potential drugs that are currently being tested in human or pre-clinical studies. In red drugs that did not meet their primary end-point in human phase II clinical trials.

*Other targets of pirfenidone include altering the TGF-β pathway, inhibition of collagen synthesis, and hampering pro-fibrotic Th2 development.

** Study terminated early because of safety issues (three cases of drug-related cholecystitis)

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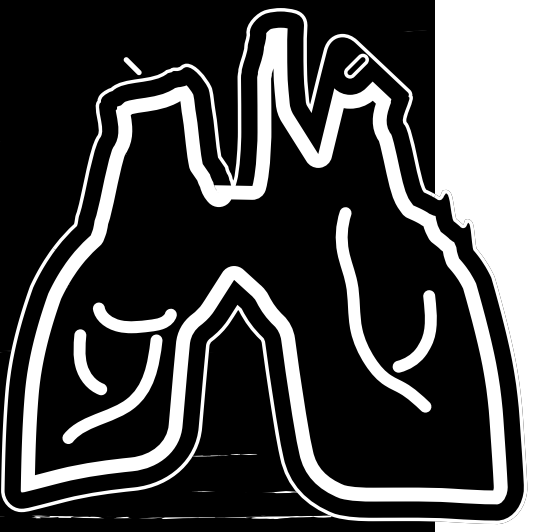

CHAPTER 3

Integrating patient perspectives into personalized medicine in Idiopathic Pulmonary Fibrosis

C.C. Moor, P. Heukels, M. Kool, M.S. Wijsenbeek

Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive and ultimately fatal disease which has a major impact on patients' quality of life. Except for lung transplantation, there is no curative treatment option. Fortunately, two disease-modifying drugs that slow down disease decline were recently approved. Though this is a major step forward, these drugs do not halt or reverse the disease, nor convincingly improve health-related quality of life. In daily practice, disease behavior and response to therapy greatly vary among patients. It is assumed that this is related to the multiple biological pathways and complex interactions between genetic, molecular and environmental factors that are involved in the pathogenesis of IPF. Recently, research in IPF has therefore started to focus on developing targeted therapy through identifying genetic risk factors and biomarkers. In this rapidly evolving field of personalized medicine, patient factors such as lifestyle, comorbidities, preferences, and experiences with medication should not be overlooked. This review describes recent insights and methods on how to integrate patient perspectives into personalized medicine. Furthermore, it provides an overview of the most used patient-reported outcome measures in IPF, to facilitate choices for both researchers and clinicians when incorporating the patient voice in their research and care. To enhance truly personalized treatment in IPF, biology should be combined with patient perspectives.

Introduction

"Give different ones [therapeutic drinks] to different patients, for the sweet ones do not benefit everyone, nor do the astringent ones, nor are all patients able to drink the same things" - ***Hippocrates*** (1)

Idiopathic pulmonary fibrosis (IPF) is the most common idiopathic interstitial pneumonia (IIP) (2). IPF is characterized by progressive decline of lung function, with a median survival of only 3-5 years (3). Common symptoms as breathlessness, cough and fatigue have a major impact on the quality of life (QOL) of patients (4). IPF occurs more often in men than women and usually affects elderly patients, aged 50 years and above (3). There are two approved anti-fibrotic drugs that slow down disease decline, but these drugs do not halt or reverse the disease, and ultimately IPF remains a fatal disease (5, 6). The heterogeneity in disease behavior and response to therapy in IPF has (further) stimulated research to identify possible distinct underlying genetic, molecular and environmental factors associated with IPF (7, 8).

The potential to enhance personalized treatment has prompted excitement also in the IPF field (7). Until now, the focus of personalized medicine has been on physiology and the use of this biological information to predict response to treatment and to develop targeted therapy (9). In this process, patient factors should not be overlooked. For real personalized treatment patient perceptions and preferences should also be taken into account. In this article, we describe recent insights and methods on how to integrate patient perspectives into personalized medicine.

Impact of disease

IPF is a heterogeneous disease, with a highly variable disease course (10, 11). Additionally, different phenotypes of IPF exist. Most patients have a slow disease progression, while some patients display relative stable periods followed by acute exacerbations and a small group of patients experiences a rapid decline in lung function (12). Uncertainty about the disease course and prognosis can cause emotional distress and anxiety, and, as a result, IPF has a major impact on most patients' health-related quality of life (HRQOL). HRQOL can be defined as a patient's perceived well-being affected by disease and treatment of the disease (13). IPF affects patients in almost every domain of life; hence, the burden of the disease is high, not just for patients but also for their partners and families. Patients often struggle with loss of independence because of functional limitations and deteriorating symptoms. Not only can breathlessness, cough and fatigue diminish quality of life, but also other symptoms such as sleep disorders, loss of appetite, and psychological problems can (14-18).

Most clinical trials in IPF that have been performed so far, have shown no convincing improvement of patient HRQOL (5, 6, 19). To date, the main focus in research has been to stabilize or improve physiological outcomes rather than HRQOL. Physiological parameters, such as lung function, do not correlate well with HRQOL measurements (20, 21). To our knowledge for parameters as imaging and biomarkers, relationships with HRQOL have

not yet been established. Thus, decline in lung function does not adequately reflect the perceived impact of the disease on patients' lives.

Every person has a different lifestyle, personal circumstances, and coping strategies. These factors can play an important role in how a disease manifests itself; hence, the same disease affects each person in a different way (16, 22, 23). Medication may show promising results at group level in randomized controlled trials, but still in some individual patients, treatment may fail (22). For example, the side effects of medication may outweigh the positive effects of medication in daily practice, or the burden of treatment might be too high for patients. To improve and personalize treatment of IPF, we should also include patient perspectives and quality of life.

Personomics

Personalized, stratified or precision medicine is a broad term which can be referred to as "delivering the right treatment to the right patient at the right time" (24). Personalized medicine has gained increasing attention during the past decade (22, 25). However, the concept is not new; Hippocrates already mentioned the importance of a personalized approach to diagnosis and treatment in the 5th century BC, stating that "individuality of human beings affects predisposition to disease and response to treatment", and also noting that "not all patients are able to drink the same therapeutic drinks" (1, 26). His concepts already include the notion that experiences with treatment differ among patients. This idea is also acknowledged by Britten and colleagues, who suggest that because individuals are more than their genetic profile, the main concept of stratified medicine is too limited at the moment (22). Personalized treatment comprises not only "biology", but should also focus on patient perspectives, needs, experiences, personality, environment, lifestyle and other personal circumstances (Figure 1) (9, 22). Accordingly, the term "personomics" has been introduced to capture a patient's life circumstances that may alter disease behavior and response to treatment (23).

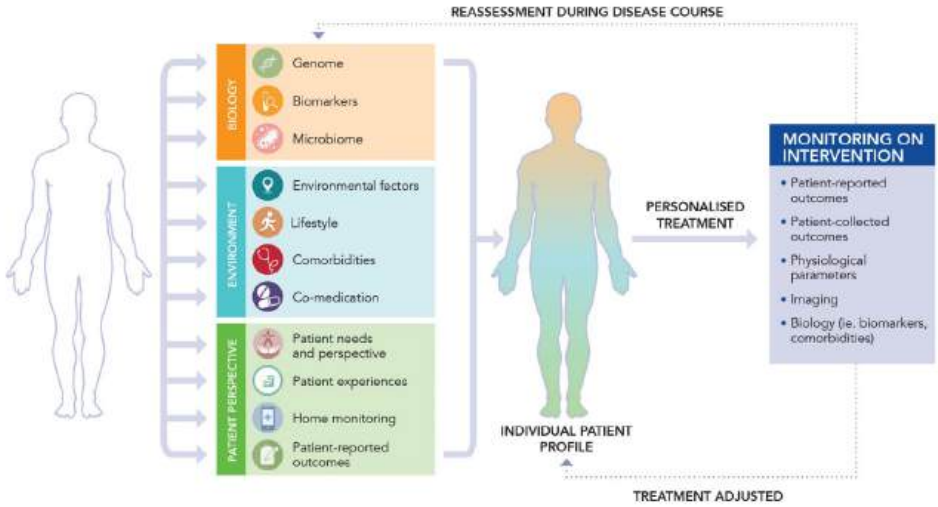

Figure 1. To enhance tailored treatment in IPF, "biology" should be combined with patient factors to generate an individual patient profile. Close monitoring, timely reassessment and treatment adjustment during the disease course are required to optimize personalized care.

Current view of personalized medicine in IPF

In other fields, especially oncology, personalized medicine has dramatically changed clinical practice during the last few years. Biomarkers have been used to develop targeted therapy and allocate patients to individual treatment plans (27-29).

Currently, the diagnosis of IPF is based on clinical, radiological and pathological findings (3). The exact etiology of IPF is however incompletely understood. One of the proposed hypotheses is the concept of dysfunctional wound healing: repeated epithelial injury and dysfunctional regeneration possibly in combination with a dysregulated immune system normally facilitating wound healing leads to fibrogenesis and, as a consequence, excessive scarring of the lung tissue (11, 30). Epithelial injury might be caused by risk factors such as cigarette smoking, micro-aspiration of gastric content, and lead to development of IPF in susceptible individuals (11). At present, it is assumed that multiple biological pathways and complex interactions between genetic, molecular and environmental factors are involved in the pathogenesis of IPF. Improved understanding of the pathogenesis of IPF has led to the identification of potential molecular biomarkers (7, 11, 31-33). Genome-wide association studies found genetic mutations that correlate with disease risk and possibly also disease progression (34-37); subsequently, the first examples of drug-gene interactions in IPF were found (38). To date, the value of biomarkers in IPF has not been fully clarified, and, therefore biomarkers or genetic endotyping are not yet used in clinical practice (7, 33).

Novel studies in IPF suggest that the 'respiratory microbiome' is also involved in IPF pathogenesis, disease progression and mortality (39-41). Patients with IPF have a higher bacterial burden and abundance of specific pathogens in the lung microbiome than the normal population. Furthermore, interactions have been found between specific gene expression and an altered lung microbiome in IPF, which is the first evidence for host-environmental interactions in IPF (42, 43). The lung microbiome may serve as a prognostic factor in the future, and clinical trials aimed at altering the microbiome of patients with IPF have already started (44).

A detailed description of (molecular) biology and its current role and potential in the IPF field is beyond the scope of this review.

How to integrate personomics into personalized medicine

Patient needs and perspectives in IPF care

The importance of engaging patients in IPF care has gained increasing attention during the last several years (45). Recent qualitative studies have reported a need for better education about IPF, information about specific treatment options and palliative care, and access to specialist centers and specialist nurses. Additionally, more support for caregivers is warranted (16, 17, 46-48). These recommendations underscore the idea that not only pharmacological treatment but also non-pharmacological treatment options such as oxygen therapy, pulmonary rehabilitation, psychological support and palliative care, are an important part of personalized management. With regard to pharmacological treatment, it is important to assess the needs and perspectives of patients before starting treatment, thereby enhancing shared decision-making. For instance, some side effects of disease-modifying drugs might have a devastating impact on one patient, but be far less bothersome to other patients (22). At the moment, over-use and under-use of medication, compliance problems and waste of medication are not unusual in IPF (22, 49, 50). Non-adherence to medication could therefore be prevented when patients' preferences and lifestyle are taken into account (9). Since patient preferences and needs may change because of disease progression or personal circumstances, an important aspect of disease management is iterative evaluation of the situation of individual patients (16, 46, 51). Only in this way can "holistic" personalized care be given in IPF.

Comorbidities and co-medication

Holistic care also means looking further than the lungs. IPF is associated with a number of pulmonary and extra-pulmonary comorbidities, such as pulmonary hypertension, respiratory infection, cardiovascular disease, emphysema, lung cancer, diabetes mellitus, venous thromboembolism and gastroesophageal reflux (52-56). Comorbidities are more prevalent in patients with IPF than in the normal population and have a negative influence on QOL and survival (54, 56-58). Hence, early identification and treatment of comorbid conditions have the potential to improve QOL, functional outcomes, and survival for patients with IPF (53). Kreuter et al. (54) proposed the "IPF comorbidome", which visually

displays prevalence of comorbidities and their strength of association with mortality in patients with IPF. This comorbidome could be used to predict prognosis for individual patients with IPF, and thus enhance personalized treatment.

Moreover, extra attention should be paid to the frail, elderly patients who have multiple comorbidities and functional impairment (55). As a consequence, these patients might have a higher risk of harmful side effects of disease-modifying medication and should be closely monitored during treatment. Besides, polypharmacy may play an important role in this group of patients. It is generally known that polypharmacy decreases medication compliance, increases risk of adverse drug events, and might lead to impaired functional status and cognitive impairment in elderly patients (59). Furthermore, co-medication can also interfere with disease-modifying medication, and subsequently increase side effects or reduce treatment efficacy (60). Accordingly, co-medication could play an important role in the choice of pharmacological treatment in IPF. Expected risk-benefit ratio, comorbidities, and co-medication should be taken into account before pharmacological treatment is started in individual patients.

Measuring quality of life and monitoring treatment response

It remains challenging how to measure patients' disease burden, experiences and response to treatment in IPF. For this purpose, it is important to receive structured patient input throughout the whole disease course, starting already when the diagnosis is established. At present, digital solutions can facilitate more collaboration with patients in monitoring disease behavior, their experiences, and response to therapy (Figure 2).

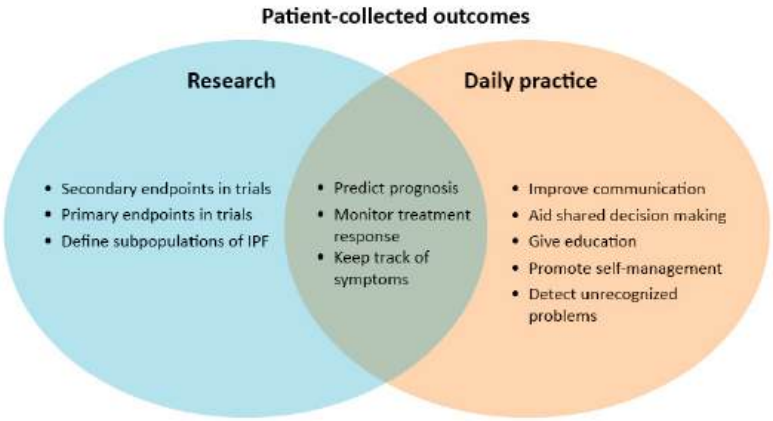

Figure 2. Patient-reported and recorded outcomes can be used to enhance personalized treatment

Patient-reported outcome measures in IPF

A patient-reported outcome (PRO) is defined as "any report of the status of a patient's health condition that comes directly from the patient, without interpretation of the patient's response by a clinician or anyone else" (61). Patient-reported outcome measures (PROMs) can be used to measure (HR)QOL, assess symptoms and evaluate disease progression. There is a difference between generic and disease-specific PROMs. Disease-specific PROMs are developed to assess symptoms and (HR)QOL in a specific disease, whereas generic PROMs address more general questions and can be used in the whole population (62). One of the most commonly used generic PROMs in IPF trials are the Short-Form 36 (SF-36) and the Euroqol-5D (EQ5D), which is also a widely accepted instrument for economic evaluation in healthcare (63, 64). An overview of the most widely used PROMs in IPF is given in Table 1.

Disease-specific PROMs

Although PROMs can play an important role to improve care for IPF, only a few well-validated, disease-specific questionnaires have been developed (19). Until a few years ago, most questionnaires used in clinical trials in IPF were originally intended for other chronic diseases (64-66). The validity of these questionnaires, such as the Saint George Respiratory Questionnaire (SGRQ) and COPD Assessment Test (CAT), has been confirmed in patients with IPF (66, 67). For the SGRQ, even an adapted version, the SGRQ-I, has been developed (68). This revised PROM consists of questions from the original SGRQ that were most relevant for patients with IPF. The reliability and validity of the SGRQ-I are comparable to the SGRQ. However, PROMs which are developed in a target population from the start, are thought to be more precise in capturing changes in HRQOL for this group of patients (58). One of the first questionnaires specifically developed in a population of patients with interstitial lung diseases (ILDs), among whom patients with IPF, is the Kings' Brief Interstitial Lung Disease health status questionnaire (K-BILD) (21). This is a brief, valid questionnaire that is increasingly used in IPF and other ILD clinical trials. One of the emerging PROMs in IPF is the 'Living with Idiopathic Pulmonary Fibrosis (L-IPF) questionnaire, which is a revised, electronic version of the ATAQ-IPF (a Tool to Assess Quality of Life in IPF). The L-IPF was adapted from the ATAQ-IPF following feedback from patients, and a validation study is underway at the moment (69). Another questionnaire which is currently being developed with the help of a multidisciplinary group of patients and carers is the IPF-PROM. (70).

Domain-specific PROMs

Additionally, domain-specific PROMs, which are questionnaires related to a specific symptom or organ, can be used to capture and objectify different aspects of disease. A few measures to evaluate breathlessness, such as the University of California San Diego Shortness of Breath Questionnaire (UCSD), the modified Medical Research Council (mMRC) scale, the Baseline and Transition Dyspnea Indexes (BDI-TDI) and the Borg scale, are commonly used in IPF, although none were originally developed for IPF (71-

74). Even though cough is a major problem in IPF, no specific cough questionnaires for IPF exist. However, the Leicester Cough Questionnaire (LCQ) and the Cough Quality of Life Questionnaire (CQLQ) are currently used instead (75, 76). A widely-known PROM to assess anxiety and depression is the Hospital Anxiety and Depression Scale (HADS), which is increasingly used in IPF (77). No specific fatigue questionnaires for IPF exist; however, the Fatigue Assessment Scale (FAS), originally developed for sarcoidosis, is used and might be adapted for IPF in the future (78).

Table 1 Overview of most used patient-reported outcomes in IPF

Patient-reported outcome measure	Description	Validation studies and MCID	Advantages	Disadvantages
Disease-specific				
SGRQ (65)	50-item questionnaire with 3 domains assessing HRQOL in chronic respiratory diseases	Validated in IPF MCID in IPF: 5-8 points (67)	Used in many clinical trials in IPF	Originally developed for COPD and asthma; Lengthy, difficult questionnaire
SGRQ-I (68)	IPF-specific version of original SGRQ; Contains 34 items	Validity comparable to SGRQ	Questions more relevant for IPF than SGRQ	Responsiveness and MCID not known yet; Limited experience
CAT (66)	Composed of 8 symptom items on a 0-5 response scale	Validated in IPF	Simple and quick instrument	Originally developed for COPD; Limited experience in IPF
K-BILD (21)	15-item health status questionnaire in ILD with 3 domains	Validated in IPF MCID in IPF: 5 points (79)	Brief Developed in ILD including IPF patients	Limited experience in clinical trials, though increasingly used
L-IPF (69) (revised version ATAQ-IPF)	Contains 2 modules with different domains	Currently in validation process	Adapted with feedback from patients	Not available yet
IPF-PROM (70)	Concise questionnaire to asses QOL in IPF	Study is ongoing	Developed with patients and caregivers	Not available yet
PESaM (80)	Generic and disease specific module; evaluates patients' expectations, experiences and satisfaction with disease modifying drugs	Currently in validation process	Developed together with IPF patients.	Not validated yet; Responsiveness unknown
IPF-PREM (81)	Questionnaire to assess experiences with care delivery	Study is ongoing	Measures experiences of patients	Not available yet
Domain-specific				
UCSD (74)	Contains 24 items on a 0-5 response scale assessing dyspnea in the last week	Validated in IPF; MCID in IPF: 8 points	Already used in different IPF trials; Valid to assess change in dyspnea in IPF	Takes considerably more time compared to other dyspnea measures; Not originally developed in IPF
mMRC (72)	Consists of one question with five grades for the level of dyspnea	Not validated in IPF	Quick, easy tool for use in daily practice; Relates to disease progression	Responsiveness in IPF unclear; Not originally developed in IPF
BDI-TDI (73)	BDI scores 3 components of dyspnea on baseline; TDI measures changes compared to baseline	Not validated in IPF; MCID in COPD: 1 point (73)	Measures both baseline and change over time	Only interview-administered or computerized version; Not originally developed in IPF

Patient-reported outcome measure	Description	Validation studies and MCID	Advantages	Disadvantages
Borg Scale (71)	Level of dyspnea scored on a scale from 0-10	Not validated in IPF; MCID in COPD: 1 point (82)	Useful during 6-min walk test in daily practice	Only measures dyspnea during exertion, does not measure dyspnea over time; Not originally developed in IPF
HADS (77)	Consist of 14 items in the subscales anxiety and depression	Not validated in IPF; MCID in COPD: 1.5 points (77)	Reliable screening tool for anxiety and depression	Should not be used as diagnostic test; Not originally developed in IPF
CQLQ (76)	Consists of 28 cough-specific questions in 6 domains	Validated in IPF; MCID in IPF: 5 points	Comprehensive; Responsive outcome measure	Good validity for total score in IPF, but not for all domains; Limited experience in IPF; Not originally developed in IPF
LCQ (83)	Chronic cough quality of life questionnaire with 19 items in 3 domains	Not validated in IPF; MCID in chronic cough: 1.3 points(84)	High reliability; Ability to detect a response to change	Limited experience in IPF; Not originally developed in IPF

IPF: Idiopathic Pulmonary Fibrosis; MCID: minimal clinically important difference; ILD: interstitial lung disease; HRQOL: Health-related quality of life; SGRQ: Saint George Respiratory Questionnaire; K-BILD; Kings' Brief Interstitial Lung Disease health status questionnaire; L-IPF: Living with Idiopathic Pulmonary Fibrosis; ATAQ-IPF: a Tool to Assess Quality of Life in IPF; IPF-PROM: Idiopathic Pulmonary Fibrosis-Patient-reported outcome measure; PESaM: Patient Experiences and Satisfaction with Medication; IPF-PREM: Idiopathic Pulmonary Fibrosis – Patient-reported experience measure; UCSD: University of California San Diego shortness of breath; mMRC: modified Medical Research Council; BDI-TDI: Baseline and Transition Dyspnea Indexes; HADS: Hospital Anxiety and Depression Scale; CQLQ: Cough Quality of Life Questionnaire; LCQ: Leicester Cough Questionnaire

Patient-reported outcomes in research and daily practice

PROs could be very helpful to enhance personalized treatment in IPF (Figure 2). Until now, PROMs have been mainly used for research purposes, as a secondary endpoint in clinical trials. The most used primary endpoint in IPF trials is forced vital capacity (FVC), which is accepted as a surrogate measure for mortality (85). One study showed that HRQOL, assessed with the SGRQ, is also an independent prognostic factor for mortality in IPF (86). PROMs probably reflect another dimension of disease compared with traditional physiological parameters (86). In the future, PROMs could possibly be used to predict treatment success in IPF.

PROM use in daily practice can allow healthcare providers and patients to gain more insight into the individual disease and patient behavior. In a study of Sampson et al. (46), most patients were uncertain about their own disease course and progression and had difficulties interpreting objective hospital-based parameters. PROMs could allow both patients and healthcare providers to keep track of symptoms and disease progression easily. PRO results can even be used as a simple tool to communicate with patients, educate them, promote self-management and aid shared decision making during the course of the disease (19, 87). A systematic review in oncology has shown strong evidence that routine collecting of PROs improved patient-centered care, patient satisfaction, and detection of unrecognized problems (88).

Patient-reported experience measures in IPF

Optimal treatment requires close monitoring of the balance between the effects and side effects of disease-modifying drugs. Nonetheless, to our knowledge, a reliable measure to assess patient experiences with medication in IPF is not yet available in clinical practice. For this reason, a consortium of doctors, scientists and patient representatives has joined forces to develop the Patient Experiences and Satisfaction with Medications (PESaM) questionnaire, which has a generic module and a disease-specific part for IPF (78). The PESaM questionnaire focuses on perceived effectiveness, side effects and ease of use of medication and its impact on patients' lives. This patient reported experience measure (PREM) could not only be used in future clinical trials, but also in clinical practice to help with better detection of side effects and adjustment of medication. Moreover, Russell and colleagues, together with patients, are currently developing the 'IPF-PREM'. This is a measure to assess patient experiences with healthcare and can possibly be used to improve the quality of care for patients (79).

Home monitoring

Ideally, for a better tailored treatment, frequent monitoring with a low burden for the patient is needed. In the last decade, the use of e-health in chronic diseases has been growing, and shows mostly promising results (89-91). E-health involves the exchange of data between a patient and a healthcare provider using information and communication technologies (ICT) (92). By using e-health tools, patients may better understand their health condition and become actively involved in the management of their own disease. It allows frequent monitoring in between regular visits and collection of PROs at home (93). Recently, a study showed that daily home spirometry in a population of patients with IPF was highly feasible and informative (94). Home-based spirometry predicts disease decline and mortality better than hospital-based measurements. Routine home spirometry could be very helpful to identify patients with rapid decline in lung function and to evaluate response to treatment. The authors suggest that daily home spirometry will allow for more individualized patient care. The feasibility of home-based spirometry in IPF was confirmed by Johannson et al. (95), who additionally showed that home spirometry might reduce sample size as well as the length of future clinical trials. Another promising example of home monitoring in IPF is the longitudinal follow-up of physical activity with activity trackers worn by patients at home (96). Decline in physical activity can provide reliable, objective data on disease progression and could be integrated into a home monitoring program. A comprehensive home monitoring program, consisting of an e-health tool combined with home spirometry and online collecting of PROs, has the potential to enhance trial design, stimulate self-management, allow for early treatment adaption to minimize side effects, prevent hospital admissions, and subsequently improve personalized management and quality of life for patients with IPF.

Conclusion

The potential to enhance personalized treatment has prompted excitement also in the IPF field. In the future, patients' genetic, biomarker and microbiome profiles may guide clinical trial design and treatment decisions. In this process, patient perspectives should not be overlooked. Only by integrating biological information with patient-reported and patient-collected information, will we be able to realize truly personalized treatment.

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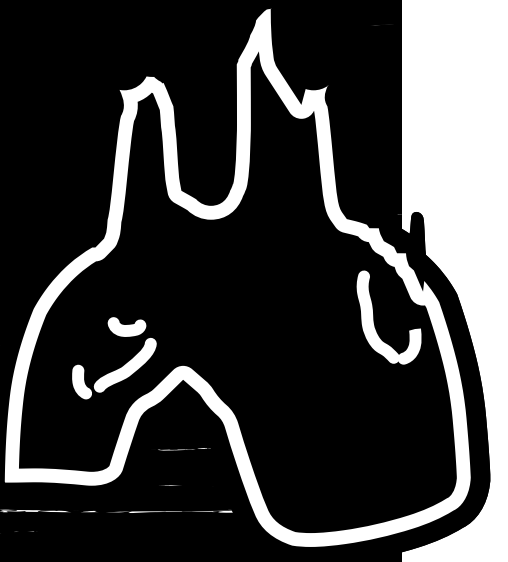

CHAPTER 4

Fibrocytes are increased in lung and peripheral blood of patients with Idiopathic Pulmonary Fibrosis.

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Abstract

Rationale: Fibrocytes are implicated in Idiopathic Pulmonary Fibrosis (IPF) pathogenesis and increased proportions in the circulation are associated with poor prognosis. Upon tissue injury, fibrocytes migrate to the affected organ. In IPF patients, circulating fibrocytes are increased especially during exacerbations, however fibrocytes in the lungs have not been examined. Therefore, we sought to evaluate if fibrocytes can be detected in IPF lungs and we compare percentages and phenotypic characteristics of lung fibrocytes with circulating fibrocytes in IPF.

Methods: first we optimized flow cytometric detection circulating fibrocytes using a unique combination of intra- and extra-cellular markers to establish a solid gating strategy. Next we analyzed lung fibrocytes in single cell suspensions of explanted IPF and control lungs and compared characteristics and numbers with circulating fibrocytes of IPF.

Results: Using a gating strategy for both circulating and lung fibrocytes, which excludes potentially contaminating cell populations (e.g. neutrophils and different leukocyte subsets), we show that patients with IPF have increased proportions of fibrocytes, not only in the circulation, but also in explanted end-stage IPF lungs. These lung fibrocytes have increased surface expression of HLA-DR, increased intracellular collagen-1 expression, and also altered forward and side scatter characteristics compared with their circulating counterparts.

Conclusions: These findings demonstrate that lung fibrocytes in IPF patients can be quantified and characterized by flow cytometry. Lung fibrocytes have different characteristics than circulating fibrocytes and represent an intermediate cell population between circulating fibrocytes and lung fibroblast. Therefore, more insight in their phenotype might lead to specific therapeutic targeting in fibrotic lung diseases.

Introduction:

Fibrocytes are thought to be the precursors of fibroblasts and were first described in an experimental skin wound model in mice as bone marrow-derived cells producing extracellular matrix proteins aiding wound healing[1]. Fibrocytes are derived from a common monocyte lineage[2] and upon tissue injury, they migrate to the affected organ in response to chemotactic factors, where they differentiate into fibroblast-like cells. Next to differentiation into (myo)fibrobalsts, fibrocytes are thought to display several paracrine functions, including fibroblast activation, alternative macrophage-dependent and -independent inflammatory processes, which all could lead to tissue remodeling and fibrosis[3-7]. The contribution of fibrocytes to the pathogenesis of fibrotic diseases and their potential use as a biomarker in fibrotic lung diseases and pulmonary hypertension (PH) has investigated, as they correlate to disease progression and survival [8-11].

However, a uniform (gating) strategy to identify fibrocytes is lacking. It is generally accepted that the minimally needed markers are CD45 (hematopoietic marker) and intracellular collagen-1[12-14]. Discrepancies in opinion how to further accurately characterize fibrocytes may originate from two possible problems. First, it is unclear whether other extracellular markers are really needed, and if so, which ones would then be the most optimal. Most often CD34 (hemopoietic stem cell marker) and CXCR4 (C-X-C chemokine receptor 4), which are expressed on almost all circulating fibrocytes, are used. Secondly, there is no consensus whether circulating fibrocytes are cells with high side scatter (SSC) characteristics, a measure of cell granularity or internal complexity. Most studies have shown that $CD45^{+}$/collagen-1^{+} fibrocytes represent a heterogeneous cell population primarily found in the polymorphonuclear (PMN) cell fraction with high SSC [8, 9, 13-15]. In contrast, others have demonstrated that circulating fibrocytes share side scatter characteristics comparable with blood mononuclear cell fraction based on cell sorting experiments[16, 17]. As a consequence, it is unclear whether differences in SSC represent different subpopulations, different stages of development or whether this reflect methodological issues.

lung fibrocytes may hold promise in a better understanding of fibrocyte biology, as they have become fully differentiated effector cells and their paracrine and inflammatory function have taken shape.

Increased $CD45^{+}$/collagen-1^{+} fibrocytes have been found in bronchoalveolar lavage (BAL) of IPF patients[18]. However, whether lung fibrocytes can be detected in IPF lung tissue homogenates using flow cytometry is currently unknown.
In our study, we propose a gating strategy and phenotypic staining for a more specific selection of circulating and lung fibrocytes and compare proportions and phenotypical characteristics of lung fibrocytes in IPF lung tissue with circulating fibrocytes in patients with IPF. Reliably identification of circulating and lung fibrocytes could be of great value of developing new therapies that target circulating and lung fibrocytes in IPF.

Materials and methods

Study design and subjects

Human lung tissue was collected from patients with end-stage IPF undergoing lung transplantation. As a control, lung tissue was obtained from long volume reduction procedures during lung transplantation upon size mismatch of oversized donor lungs or residual material obtained during lung surgery for pulmonary tumors. Healthy residual tissue was obtained at least >3cm from the tumor and only patients with a normal pulmonary function test (PFT) or mild airflow obstruction (Gold 1) were selected. All patient and healthy subject characteristics are shown in **Additional file 1**.

The Medical Ethical Committee of the Erasmus MC Rotterdam approved this study (*METC 2012-512*). Informed consent was obtained from every participant and healthy control (HC) before collection of blood samples. The diagnosis of pulmonary hypertension or pulmonary fibrosis was conform the current diagnostic guidelines of the ATS/ERS[19-21].

Blood processing

Blood samples were collected in EDTA tubes (BD Vacutainer K2E). Peripheral blood mononuclear cells (PBMC) and total white blood cells were obtained according to standard protocols. In short, PBMC were obtained using the Ficoll separation technique and whole white blood cells with the simple Pasteur pipette tube technique after spinning samples at 1000 x g as previously described[22]. Red blood cells were lysed using osmotic lysis buffer (8.3% NH_4CL, 1% $KHCO_3$, and 0.04% NA_2EDTA in Milli-Q). Upon isolation, PBMC and total white blood cells were resuspended in 0.1% BSA + 2mM EDTA in PBS and immediately processed for flow cytometry and fluorescence-activated cell sorting (FACS). Isolated PBMC or total white blood cells, not used for direct flow cytometry or FACS, were aliquoted and cryopreserved in complete RPMI (RPMI medium 1640 + glutaMax, Life Technologies) with 10 % DMSO (Sigma), 40% Fetal calf Serum (FCS) and stored at -196˚C until thawing.

Lung Tissue Processing

Fresh lung tissue was stored in cold PBS and processed within 24 hours following lung transplantation or resection. Lung tissue was processed for isolation of single cell suspensions described in section *"preparation of single cell suspensions"*. Furthermore, peripheral lung tissue was frozen and stored at -80˚C until further use.

Preparation of single cell suspensions

Lung resection specimens were rinsed with PBS to remove residual blood. After mincing the lung, specimens were enzymatically digested in digestion medium (20ml HBSS (Life Technologies, 14170-088) with 10ug Liberase (Roche, Liberase™, research grade) and 40 Units of DNA-se (Roche, DNase I recombinant, RNase-free)) for 30 minutes in a humidified incubator at 37˚C while gently shaking the samples. The remaining cell debris was removed by passing the cells through a 100µm-diameter disposable cell mesh filter.

The cells were washed in RPMI with 5% FCS and centrifuged for 10 min at a speed of 400 × g. Samples were subjected to RBC lysis, washed and counted. Finally, samples were aliquoted and cryopreserved in complete RPMI with 10 % DMSO (Sigma), and 40% Fetal Calf Serum (FCS) and stored at -196˚C.

Human fibrocyte and human fibroblast culture

Human fibrocytes were cultured from peripheral blood as previously described[7], with some modifications. Briefly, following Ficoll density centrifugation, 2 × 10^5 PBMCs were plated into culture-slides (sigma, C7182, 0.8 cm^2/well) in complete culture medium (Dulbecco's modified Eagle's medium) (DMEM) supplemented with 20% fetal calf serum, 2mM L-glutamine, 100 U/mL of penicillin, 100 mg/mL of streptomycin) (Life Technologies, Grand Island, NY) at 37˚C and 5% CO2. After 3 days, non-adherent cells were aspirated and discarded and fresh medium was applied. Following 10–14 days of culture, slides were washed three times with ice-cold PBS and the chambers were removed from the glass. Normal human lung fibroblasts (NHLF) were also cultured on culture slides (50,000 NHLF/well) in complete culture medium. NHLF were donated from the Laboratory of the University of Virginia (School of Medicine, Charlottesville, VA, USA.) After 2-3 days non-adherent NHLF were aspirated and discarded. Subsequently, the cultured NHLF were washed with ice-cold PBS, dried and and processed similarly to the cultured human fibrocytes.

Flow cytometry and FACS staining

Freshly isolated PBMCs and total white blood cells were stained for extra- and intracellular markers using the following antibodies: CD45-V450 (HI30), CD56-Af488 (B159 RUO), CD15-PE (HI98), CD16-PerCP-Cy5.5 (3G8), HLA-DR-BV711 (G46-6), CXCR4(CD184)-Pe-Cy7 (12G-5) (BD Biosciences), strep-APC-eF780, CD3-FITC (UCHT1), CD19-FITC (HIB19) (eBiosciences), Collagen-1-Biotin conjugated Bio (Rockland, 600-406-103), CD14-PE-Texas Red (Tuk4) (Invitrogen). To control for non-specific labeling Rabbit IgG-Biotin conjugated (Rockland, 011-0602) was used.For the cell sorting experiments, the same extra-cellular antibodies were used, except for HLA-DR: HLA-DR-APC (G46-6) (BD Biosciences).

Since macrophages in lung single cell suspensions have high auto fluorescence, we did not use fluorochromes FITC and Alexa Fluor 488 in the staining and used the following antibodies: CD3-BV711 (UCHT1), CD19-BV786 (J25C1), CD56-BV605 (NCAM16.2) (BD Biosciences). Fixable Viability Dye eFluor 506 (eBiosciences) was applied as a live-dead marker for flow cytometry experiments and 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Molecular Probes) was used as live-dead marker for the cell sorting experiments. In short, cells were incubated in FACS buffer (PBS, 0.25% BSA, 0.5mM EDTA, 0.05% NaN3 sodium azide) with fluorescent antibodies for 30 minutes at 4˚C using methods recommended by the manufacturers. Of note, extra-cellular CXCR4-Pe-Cy7 staining was performed separately in MACS buffer (0.5% BSA + 2mM EDTA in PBS). After fixation and permeabilization (BD Cytofix/Cytoperm kit, 554714), cells were incubated with the biotinylated Collagen-1 antibody or Isotype control in permeabilization buffer for 30 minutes at 4˚C. Biotinylated antibodies were visualized with streptavidin-APC-eF780.

Cells were measured on a either a LSRII or a FACS Aria™ IIu Flow cytometer (both BD Biosciences). We analyzed a minimum of 200,000 alive cells for blood samples and 100,000 alive cells for the lung tissue samples for cytometric analysis. Data was analyzed by FACS Flow-Jo software.

Cytospin

Sorted cells were washed in PBS. Cytospins were made using a cytocentrifuge and 50,000 cells were added per spot. Slides were air-dried and stored at -80˚C in a watertight box until further use. Immunocytochemistry was performed within 4 weeks after storing at -80˚C.

Immunocytochemistry

Cytospin-slides were fixed in 100% acetone at room temperature for 15 minutes. For the collagen-1 staining, slides were pre-incubated with 10% normal goat serum (Sigma, G9023) in block buffer (1% Blocking Reagent, Roche, in PBS according to the manufacturer's protocol) for 30 minutes. After rinsing with PBS, slides were incubated for 60 minutes with mouse anti-human collagen-1 (Abcam, ab6308, 1:2000) or Isotype control in block buffer. As second (goat anti-mouse antibody biotin-labeled) and third antibody (streptavidin**,** alkaline phosphatase (AP) conjugate) we used the Link-Label kit from Biogenex (link: HK-325-UM, label HK321-UK) according to the manufacturer's protocol. To detect the collagen-1 positive cells we used New Fuchsin Alkaline Phosphatase Substrate Solution (0.01% New Fuchsin, 0.02% Sodium Nitrite, 0.03% Naphthol AS-BI Phosphate, 1mM Levamisole, in 0.2M Tris-HCl, pH 8.5). Cells were counterstained with hematoxylin (Sigma, Gill No. 3), dried and mounted in Vecta Mount (Vector, Burlingame, CA, USA). For CD15 detection we used the same protocol, but with different antibodies; slides were pre-incubated with normal rabbit serum (Sigma, R9133) and subsequently stained with mouse anti-human CD15-FITC (BD Biosciences, HI98) or isotype control and rat anti-FITC AP conjugate (Sigma, A4843).

Statistics

Statistical analysis was performed using IBM SPSS Statistics 21 and GraphPad Prism 6 software. When evaluating differences in continuous variables between multiple independent groups, the Kruskal-Wallis test was used. For calculating the level of significance of differences between groups we used the Mann-Whitney U test. Correlation coefficients were calculated using Spearman's rank method. P values < 0.05 were considered significant. Flow cytometry data is either represented as percentage population or as mean fluorescence intensity (MFI).

Results

Circulating CD45+/Col-1+ fibrocytes may be contaminated with polymorphonuclear leukocytes

Since discrepancies have been reported about fibrocytes concerning their granularity and/or internal complexity, we first evaluated SSC characteristics of fibrocytes identified based on CD45 and collagen-1 (Col-1) expression. Fibrocytes were detected using the gating strategy shown in **figure 1A**. Col-1 expression was based on the control isotype staining. Circulating $CD45^+/Col\text{-}1^+$ fibrocytes represented a heterogeneous cell population based on SSC and have predominantly a high SCC (**figure 1B)** Because SSC-high cells contain polymorphonuclear cells, such as neutrophils, we examined the adhesion molecule CD15, which is expressed on circulating neutrophils[23]. The $CD45^+/Col1^+$ cells showed a high extracellular expression level of CD15 (**figure 1C**). To investigate whether this population could be contaminated with neutrophils, we isolated circulating $CD45^+/Col\text{-}1^+$ cells based on extracellular markers (sort strategy shown in **Additional file 2)** and analyzed these cells with immunocytochemistry (**figure 1D**). Almost all cells (98,6%, 95% CI 97,9–99,2) in the flowcymetric enriched $CD45^+/Col\text{-}1^+$ population were negative for collagen-1 and positive for CD15 with immunocytochemistry, whereas cultured fibrocytes (**Figure 1D**) and fibroblasts (**Additional file 3**) were positively stained for collagen-1 and negative for CD15. Additionally, all cells in the enriched $CD45^+/Col\text{-}1^+$ group had a multi-lobulated shaped nucleus. We also found a significant correlation between circulating $CD45^+/Col\text{-}1^+$ cells and neutrophils (R=0.39, p= 0.006)(**Additional file 4**)

In conclusion, our data show that PMN-leukocytes and especially neutrophils contaminate fibrocyte identification when using only CD45 and collagen-1 as identification markers. Consequently percentages of fibrocytes in the circulation are most likely lower than previously reported.

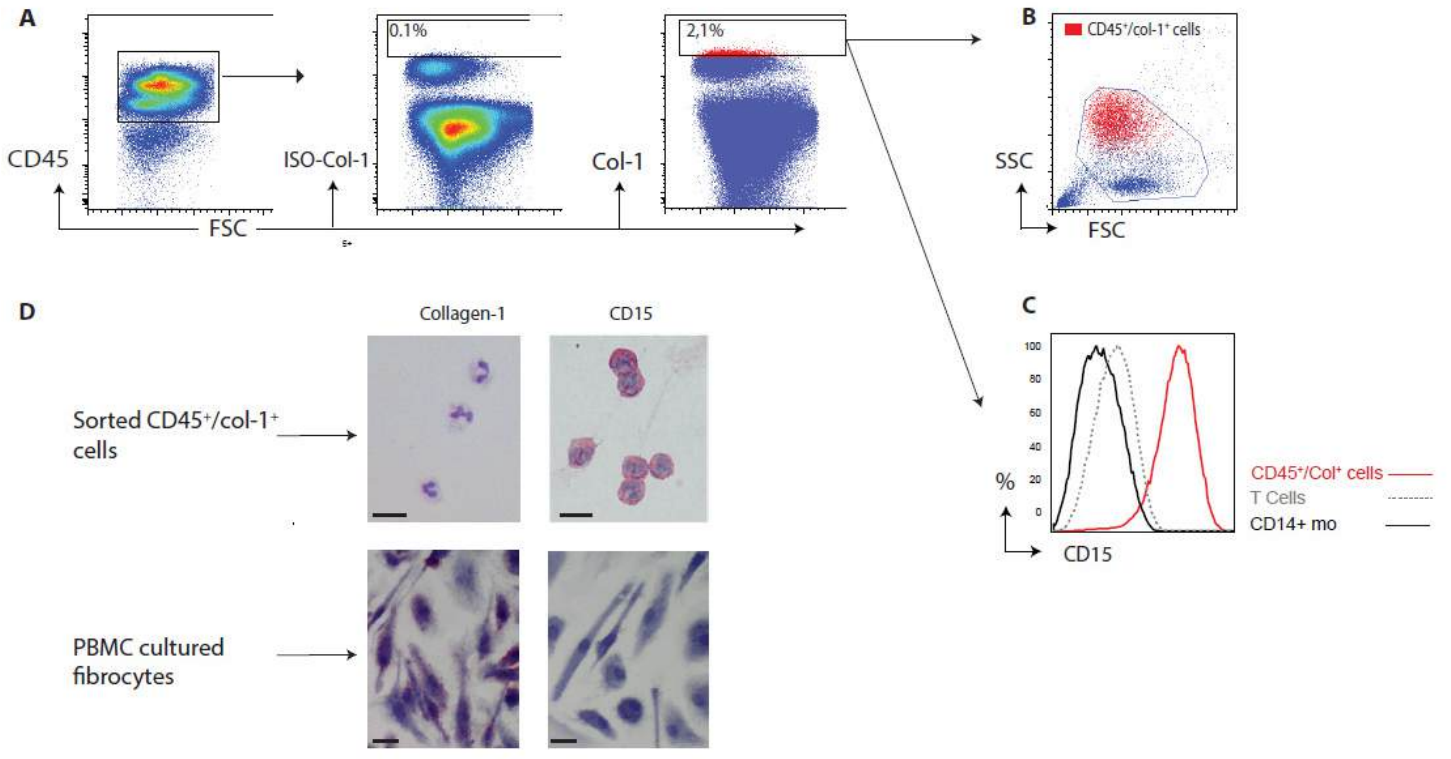

Figure 1. Circulating CD45$^+$/Col-1$^+$ fibrocytes are contaminated with polymorphonuclear leukocytes.

(A) Representative gating strategy for identification of circulating CD45$^+$/Collagen-1$^+$ fibrocytes from PBMCs. Isotype control for collagen-1 (Col-1) was used to set the gate for Col-1+ cells within alive CD45$^+$ cells. Red cells are CD45$^+$Col-1$^+$. (B) FSC and SSC characteristics of CD45$^+$/Col-1$^+$ cells (in red) compared to all alive cells (blue) showing that most CD45$^+$/Col-1$^+$ cells are found in the polymorphonuclear leukocytes fraction. (C) Histogram overlay showing surface expression of CD15 assessed by flow cytometry on CD45$^+$/Col-1$^+$ cells (red), CD14+ monocytes (black) and T cells (gray). (D) CD45$^+$/Col-1$^+$ cell enriched fraction and PBMC cultured fibrocytes were analyzed with immunocytochemistry (ICC) for CD15 and collagen-1 expression. Magnification for all ICC figures was 200x and sections were counterstained with hematoxylin. This is representative of 7 experiments. CD14+ Mo = CD14+ monocytes, PBMC = peripheral blood mononuclear cells, FSC = forward scatter, SSC = side scatter

Identification and characterization of lung fibrocytes in IPF lungs.

As neutrophils hamper the identification of fibrocytes in peripheral blood, we developed a strategy to selectively identify fibrocytes. Since circulating fibrocytes are a putative source for fibroblastic foci, a hallmark of IPF, we used IPF lungs to test our gating strategy. Lung fibrocytes in IPF lungs initially maintain CD45 expression and their presence has been previously confirmed with immunofluorescence[11, 24]. We obtained single-cell suspensions of explanted IPF lungs (n=3). As a control we used healthy lung tissue from volume reduction procedures during lung transplantation or residual material of patients who underwent a lobectomy for lung cancer, hereafter called control lungs (n=4).

All explanted lungs of IPF patients used in this study were reviewed by a pathologist and fulfilled the criteria for an usual interstitial pneumonia (UIP) pattern (**Figure 2A**). We next evaluated whether we could selectively identify fibrocytes after exclusion of neutrophils, T cells, NK cells, and B cells. Using this strategy, we could distinguish a fibrocyte population expressing CD45, CXCR4 and intracellular collagen-1 in both IPF lungs and control lungs (**figure 2B**).

Fibrocytes express CD45, CXCR4 and CD34 and intracellular collagen-1 and are generally believed to mature from a subpopulation of CD14+ mononuclear cells[25]. **Figure 2C** shows the expression level of these markers and HLA-DR, CD14, CD16 on lung fibrocytes ($CD45^+CD3^-CD19^-CD56^-CD15^-CXCR4^+Col\text{-}1^+$). CD45 expression on lung fibrocytes was slightly lower than on T-cells and CD14+ monocytes. As expected, collagen-1 and CXCR4 were clearly expressed by lung fibrocytes compared to CD14+ monocytes and T cells. CD34, a commonly used progenitor cell marker, is expressed on fibrocytes when they have entered the lungs. HLA-DR expression on lung tissue-resident fibrocytes is similar to CD14+ monocytes and higher compared to T cells of the same donor. Tissue-resident fibrocytes showed an intermediate expression of CD14 (expression level between CD14+ monocytes and T cells, which are CD14 negative) and relatively low expression of CD16.
In conclusion, lung fibrocytes can be detected in lung single cell suspension after exclusion of neutrophils, T cells, NK cells and B cells. Lung fibrocytes express known fibrocyte surface markers such as CD34, CD45, and CXCR4, and intracellular marker Col-1, suggesting that they have just entered the lung tissue and have not differentiated into myofibroblasts yet. The high expression by HLA-DR of lung fibrocytes suggests that they have a potential role in antigen presentation.

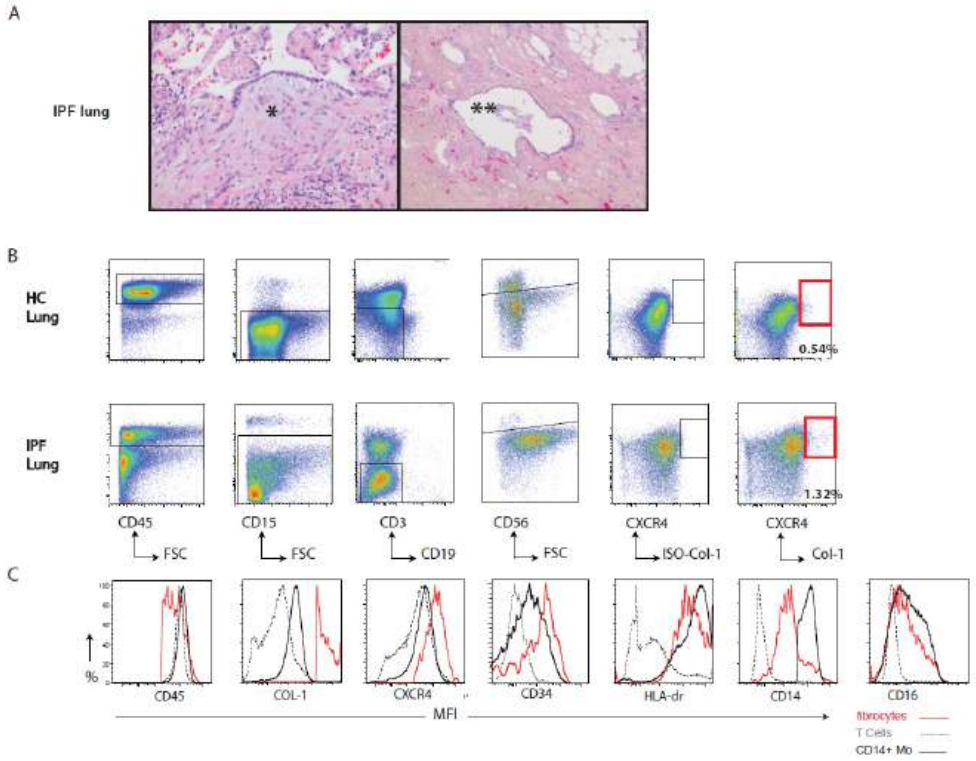

Figure 2. **Identification and characterization of lung fibrocytes in IPF lungs.**
(A) Representative histological picture of an explanted end-stage IPF lung showing (left): a usual interstitial pneumonia (UIP) pattern with (*) a fibroblast focus with overlying reactive epithelium and (right): (**) area of completely fibrotic remodeled lung tissue with cyst formation and bronchiolisation, amounting to honeycombing. Magnification 20x (left) and 5x (right). (B) Representative gating strategy for lung fibrocytes ($CD45^+CD15^-CD3^-CD19^-CD56^-CXCR4^+Col\text{-}1^+$-cells) in explanted IPF lungs (n=3 bottom) and control lungs (HC, top) (n=4). Single cell suspensions were thawed and alive viable cells were further analyzed. Lung fibrocytes are present in the $CD45^+$ cell population. Contaminating and unwanted $CD15^+$ neutrophils, $CD3^+$ T cells, $CD19^+$ B cells and $CD56^+$ NK-cells were sequentially excluded. Isotype control for Col-1 was used to set the gate for Col-1+ cells. (C) Representative histogram overlay showing expression levels depicted as MFI of indicated markers assessed with flow cytometry of lung fibrocytes (red), T cells (gray) and CD14+ monocytes (black).
CD14+ Mo = CD14+ monocytes, MFI = mean fluorescence intensity

Detailed identification of circulating fibrocytes

Having shown that fibrocytes can be reliably detected in lung single-cell suspensions after exclusion of neutrophils and lymphocytes, we hypothesized that this would also be applicable for the detection of circulating fibrocytes. Next to IPF patients, we investigated fibrocytes in pulmonary hypertension (PH) patients, as elevated number in the periphery have been observed before[10]. We examined fresh PBMC of IPF patients (n=5), PH patients (n=4) and healthy controls (HC)(n=4). Patient characteristics are detailed in **Additional table 1**.

A representative dot-plot of the gating strategy to identify circulating fibrocytes ($CD45^+lin^-CD15^-CXCR4^+Col\text{-}1^+$-cells) is shown in **figure 3A**. (Lineage mix contains: CD3, CD19, and CD56) A well-defined population of circulating fibrocytes was identified in PBMC fractions of HC, IPF and PH patients.

Next, we investigated the expression profile of circulating fibrocytes. Circulating fibrocytes expressed high levels of collagen-1, CXCR4 and CD34 and lower levels of CD45 compared with CD14+ classical monocytes and T cells (**figure 3B)**. HLA-DR expression on circulating fibrocytes was comparable to HLA-DR expression on T cells, whereas CD14+ classical monocytes contained cells with high, low, and intermediate HLA-DR expression. Interestingly, the majority of circulating fibrocytes have a low expression of CD14 and only a small proportion (11,3% 95% CI 9,7-13,0%) expressed CD14 comparable to classical monocytes. CD16 expression on circulating fibrocytes was lower compared to classical monocytes and slightly higher than T cells of the same donor.

In conclusion, these data show that circulating fibrocytes can be detected after exclusion of neutrophils, T, B and NK cells, and express markers in common with lung fibrocytes. HLA-DR and CD16 expression are low on circulating fibrocytes and only a small fraction expresses high levels of CD14.

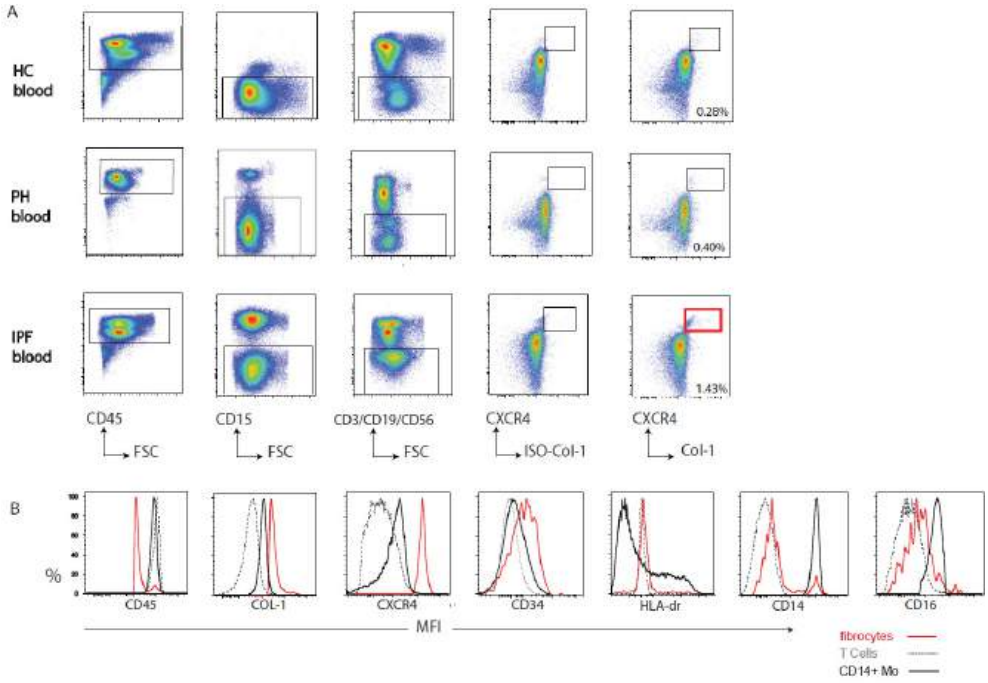

Figure 3. Characterization of circulating fibrocytes in IPF and PH patients.
(A) Representative gating strategy for the detection of circulating fibrocytes ($CD45^+CD15^-Lin^-CXCR4^+Col\text{-}1^+$-cells) in freshly analyzed PBMCs of heathy controls (n=5), PH patients (n=5) and IPF patients (n=4). The lineage mix includes markers CD3, CD19 and CD56 to exclude T-cells, B-cells and NK-cells. (B) Representative histogram overlay showing expression levels depicted as MFI of indicated markers assessed with flow cytometry of circulating fibrocytes (red), T cells (gray) and CD14+ monocytes (black).

Quantification of fibrocyte numbers is independent of leukocyte isolation strategy used

Since the presence of neutrophils hampers the detection of fibrocytes and to confirm the specificity of our staining, we compared two common leukocyte isolation techniques. We compared the Ficoll separation technique to isolate PBMCs to remove PMN-leukocytes, and the simple Pasteur pipette tube technique to isolated all white blood cells. We analyzed paired total white blood cells and PBMCs on the same day as blood withdrawal of 9 patients (4 IPF patients and 5 PH patients) and 5 healthy controls. The absolute number of circulating fibrocytes (CD45+Lin-CD15-CXCR4+Col-1+) per milliliter (ml) blood was not different between the two leukocyte isolation techniques (2,5 $x10^3$ (95% CI -0,4$x10^3$-9,0$x10^3$)(Pasteur pipette technique) versus 1,8$x10^3$ (95% CI 1,1$x10^3$-1,7$x10^3$)(Ficoll separation technique)) (**figure 4A)**. As expected, the proportions of circulating fibrocytes from CD45+ cells was relatively higher in PBMC samples than in whole blood samples, because the Ficoll technique eliminated most PMN-leukocytes (**figure 4B**). The lack of difference in fibrocytes percentages in this figure between groups of patients and controls is probably the result of the low number of patients used for this experiment.

In conclusion, our strategy to identify fibrocytes is reliable, irrespective of which leukocyte isolation technique is used, for the quantification of absolute numbers of circulating fibrocytes.

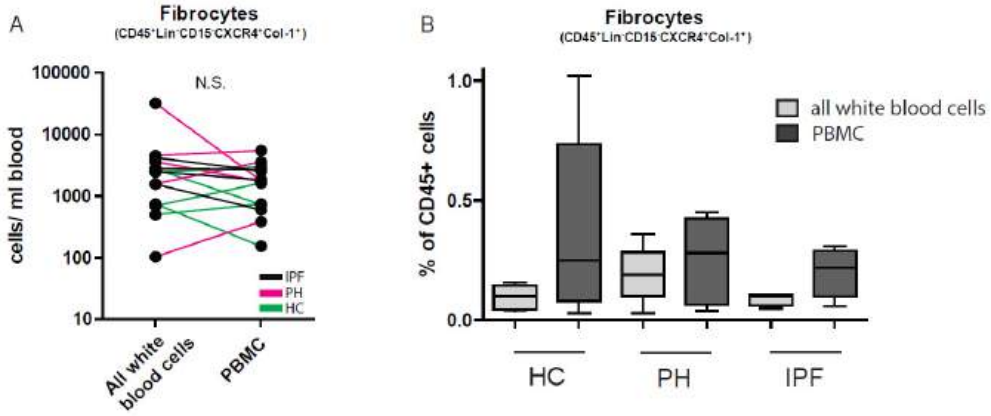

Figure 4. **The total number of circulating fibrocytes is unaffected by the leukocyte isolation strategy used.** (A) Total number of circulating fibrocytes (defined as CD45$^+$lin$^-$CD15$^-$CXCR4$^+$Col-1$^+$ cells) per ml blood using two common leukocyte isolation techniques, e.g. the simple Pasteur pipette tube technique to isolate all white blood cells and Ficoll separation technique to isolate PBMCs. For this experiment we analyzed paired total white blood cells and PBMCs on the same day as blood withdrawal of 9 patients (4 IPF patients (black) and 5 PH patients (purple)) and 5 healthy controls (green). (B) Percentage of fibrocytes (of CD45$^+$ cells) in the same patients after isolating all white blood cells (gray) and PBMCs (black). Data are depicted as median and interquartile.

Comparison of circulating fibrocytes with lung fibrocytes

Upon tissue injury, fibrocytes migrate to target organs and mature in (myo)fibroblasts and participate in tissue remodelling and fibrosis. It is unclear if circulating fibrocytes become more activated, or modulate surface markers (e.g. CD45, CD34 or CXCR4), or upregulate intracellular collagen-1 when entering the lung. Therefore we simultaneously compared the expression levels of these markers between circulating and tissue-resident fibrocytes. Collagen-1 expression in fibrocytes did not differ between IPF/(IPAH) patients and (healthy) controls in either lung cell suspensions or PBMCs (**figure 5A, left**). The collagen-1 expression in lung fibrocytes was significantly higher compared with circulating fibrocytes in patients with IPF, IPAH and controls.

Expression of HLA-DR was similar between IPF/IPAH patients and controls (**figure 5A, right**). Tissue-resident fibrocytes showed a significantly increased expression of HLA-DR compared to circulating fibrocytes. CD34 and CXCR4 expression between circulating and tissue-resident fibrocytes did not differ (data not shown).

To examine the size and complexity of circulating and lung fibrocytes, we examined SSC and FSC of the fibrocytes selected as shown in figure 2B and 3A. Circulating fibrocytes had FSC and SSC characteristics comparable to monocytes. In the lung, both FSC and

SSC of lung fibrocytes were increased compared to circulating fibrocytes (**Figure 5B**). The variation in FSC and SSC characteristics of lung fibrocytes could be the result of differences in granularity, activation status and collagen content.

In conclusion, these data show that lung tissue-resident fibrocytes have increased expression levels of HLA-DR and collagen-1 and also gain size and internal complexity compared with their circulating counterpart. No differences were observed within a compartment between controls or patients with IPF or IPAH, which may suggest an important role of the local environment in lungs on fibrocyte development.

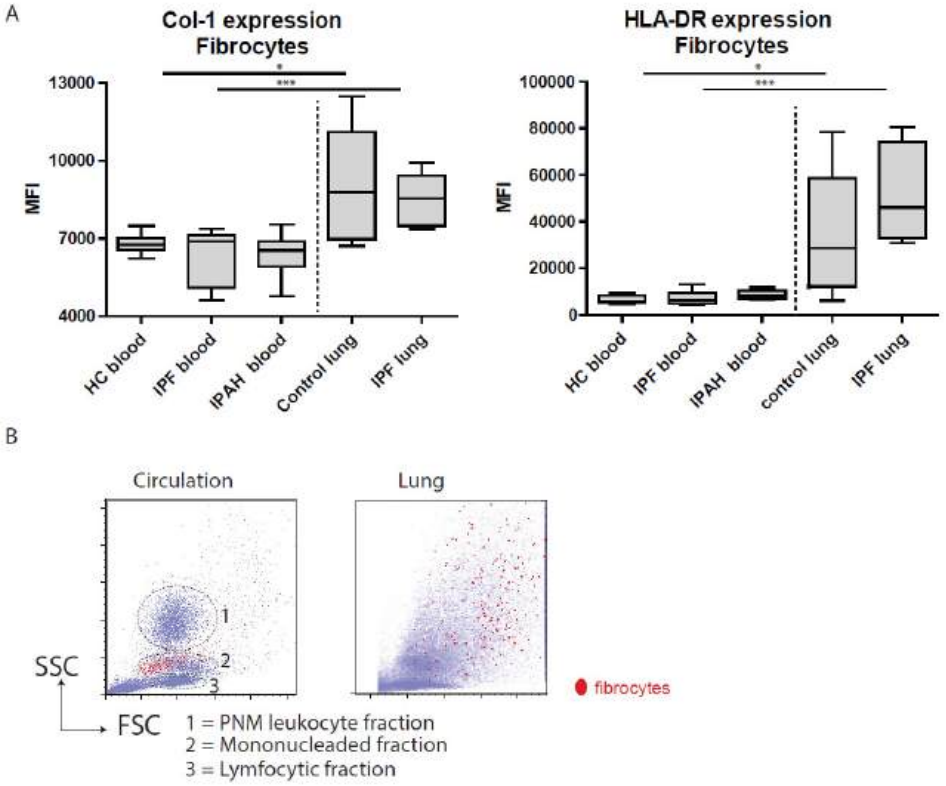

Figure 5. Comparison of circulating fibrocytes with lung fibrocytes

(A) Intracellular Collagen-1 and surface HLA-DR expression analyzed with flow cytometry and depicted as MFI. For this experiment, we used frozen PBMC of 10 HC, 10 IPF patients and 10 IPAH patients and frozen single cells suspensions of 5 control lungs and 5 explanted IPF lungs.

(B) representative dot-plot of the FCS and SSC characteristics assessed with flow cytometry of circulating and lung fibrocytes (red dots) in the circulation (left panel) and explanted IPF-lung (right panel). Nonparametric two-tailed Mann-Whitney test was used. Data are depicted as median and interquartile. * $P < 0.05$ *** $P < 0.001$

IPAH = idiopathic pulmonary arterial hypertension, PMN = polymorphonuclear

Increased proportions of fibrocytes in the lungs and circulation of patients with IPF

Having shown that our gating strategy to detect fibrocytes is reliable and specific, we wanted to apply this technique in a larger, clinically relevant, cohort of PH and IPF patients, in which increased percentages of circulating fibrocytes have been described before [8, 10, 12]. To minimize variability in fibrocytes due to heterogeneity in etiology of PH, only patients with idiopathic arterial pulmonary hypertension (IPAH) were examined. We thus determined fibrocyte percentages in PBMCs of patients with IPF (n=14), IPAH (n=10), and HC (n=10).

Circulating fibrocytes are significantly increased in patients with IPF compared with control samples ($p < 0.01$)(**figure 6A and additional file 5**). The average percentage of circulating fibrocytes in IPF patients was 0.25% (95% CI 0.17-0.33) of all CD45+ cells, compared with 0.10% in HC (95% CI 0.03-0.17). The percentage of circulating fibrocytes in IPAH patient was 0.18% (95% CI 0.08-0.22) and not increased compared with HC (p=0.14).

In line with previous literature, we have shown that circulating fibrocytes are increased in patients with IPF. We next evaluated whether lung fibrocytes would also be increased in lungs of patients with IPF (n=8) and compared them to control lungs (n=9)(**figure 6B**). Clinical characteristics of these patients are shown in **additional file 1.**

Indeed, the percentage of lung fibrocytes in IPF lungs was 2,6% (95% CI -0.8-5.9) of all CD45+ cells, which was increased (p=0.002) compared with control lungs (0.7%, (95% CI 0.02-1,3%)).

Collectively, these data show that patients with IPF have increased proportions of fibrocytes, not only in the circulation, but also in the lungs at end-stage disease.

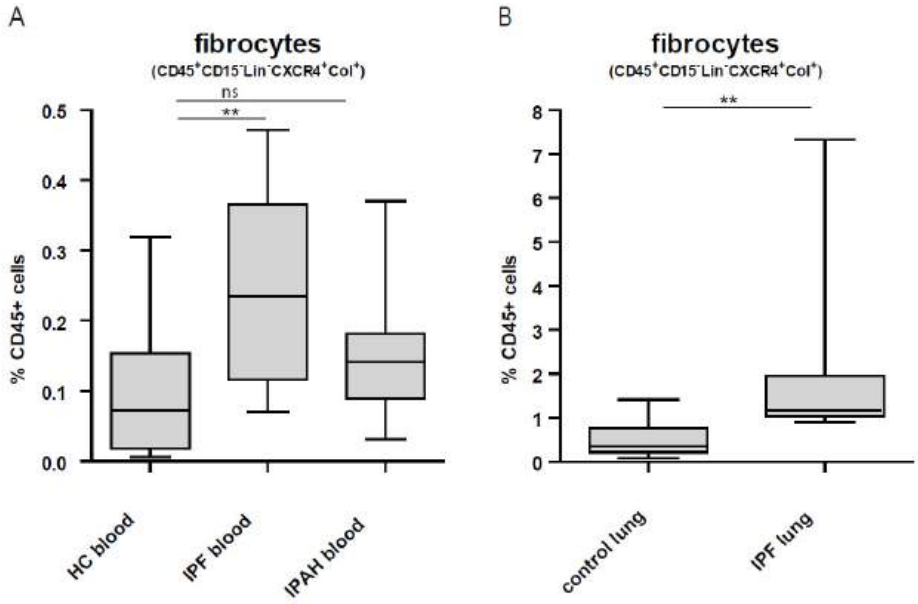

Figure 6. Circulating and Lung fibrocyte numbers in patients with IPF and idiopathic pulmonary hypertension (IPAH) (A) Percentage of circulating fibrocytes of CD45+ cells in frozen PBMC of HC, patients with IPF and patients with IPAH. (B) Percentage of lung fibrocytes of CD45+ cells in frozen single cell suspensions of control lungs (n=9) or end-stage explanted IPF lungs (n=8). Nonparametric two-tailed Mann-Whitney test was used. Data are depicted as median and interquartile. ** $P < 0.01$

Discussion

To our knowledge, this is the first time that lung fibrocytes in IPF lung tissue homogenates is assessed by flow cytometry. We have shown that lung fibrocytes are elevated in IPF lungs and express higher levels of HLA-DR and collagen-1 and gain size and internal complexity compared with circulating fibrocytes. We have used a reliable gating strategy, which excludes disruptive cell populations, and especially granulocytes, for a specific identification of circulating and lung fibrocytes

Identifying circulating fibrocytes using flow cytometry is challenging since no uniform gating strategy exists, leading to a high variability in circulating fibrocyte numbers in various reports. Circulating fibrocytes are bone marrow-derived monocyte-like cells capable of producing components of the extra-cellular matrix, and therefore at least CD45 and intracellular Collagen-1 should be used to identify circulating fibrocytes. In addition, CD34 and a variety of chemokine receptors have been used to identify subtypes of circulating fibrocytes, most notably CXCR4, CCR7, and CCR2. In the present study, we have shown that CXCR4 is equally expressed on lung and circulating fibrocytes irrespective of underlying disease. Most circulating fibrocytes also express CD34[5, 16, 26]**.** We have shown that lung fibrocytes also expresses CD34. CD34 binds to L-selectin and in L-selectin KO-mice fibrosis induction is hampered in the bleomycin exposure model of lung fibrosis[27]. This

suggest that CD34 might be needed for circulating fibrocytes to enter the lung tissue via L-selectin expressed on activated endothelial cells. It is unclear if or at what point CD34 may be downregulated *in vivo, a*s Andersson-Sjoland and colleagues have shown by demonstrating that mature alfa-SMA positive fibrocytes in IPF lungs still express CD34[24]. *In vitro,* CCR7 expression correlates with increased migration of circulating fibrocytes and TGF-β production[28]. The CCR2-CCL2 axis promotes fibrocyte recruitment and induces their differentiation into (myo)fibroblasts[29, 30].Therefore, it is believed that these chemokines receptor-bearing fibrocytes may represent a more activated subtype, however expression levels of these markers varies between different diseases and the majority of fibrocytes do not express CCR2 and CCR7[6, 7, 28, 31, 32]. The gating strategy described here could be used for reliable determination of these homing receptors and other markers of interest.

Our study has shown that the circulating fibrocyte pool is contaminated with granulocytes when employing a gating strategy based on CD45 and collagen-1 alone. Contamination of granulocytes is most likely the result of non-specific binding of collagen-1 antibody to granulocytes. Neutrophils, the most prevalent granulocytes, express a variety of collagen receptors and play an important role in collagen breakdown. However, to our knowledge there are no reports of collagen-containing neutrophils, suggesting that antibody binding is non-specific[33]. It is well known that Fc Receptors on granulocytes can cause nonspecific, false-positive antibody staining, even in the presence of specific blocking reagents[34]. Presumably, in very ill patients granulocytes could express more Fc-receptors compared with healthy subjects[35, 36]. Additionally, we observed that circulating fibrocytes had similar FSC and SSC characteristics compared to monocytes. Therefore, we strongly advocate the use of markers that exclude granulocytes in current gating protocols to identify fibrocytes.

In line with previous reports[8, 12], we found increased percentages of circulating fibrocytes in patients with IPF, but not in patients with IPAH. Yeager and colleagues described increased percentages of circulating fibrocytes in children with idiopathic or hereditable PH[10]. It is conceivable that in our relative small sample size, the extent of tissue remodeling or fibrosis in IPAH patients is relatively too low to pick up differences in circulating fibrocytes. Therefore, a larger cohort of patients is probably needed to investigate if circulating fibrocytes are also elevated in adults with IPAH.

The proportions of circulating fibrocytes we describe after eliminating disruptive cell populations is much lower than previously reported. Like macrophages, fibrocytes are monocyte-derived and their development into an effector cell is primarily established outside the circulation and depends on local tissue environment[37]. It is though that the monocyte-fibrocyte pathway may be similar and that the majority of fibrocytes is present in target organs. Indeed, we have shown that tissue-resident fibrocytes can be identified in IPF lungs using flow cytometry and that they make up a greater proportion of the $CD45^+$ cell compartment compared with their circulating counterpart. These lung fibrocytes express high levels of collagen-1 compared to the circulating fibrocytes, which makes the

collagen-1 staining more robust. HLA-DR expression is also upregulated in lung fibrocytes compared to circulating fibrocytes. As HLA-DR molecules can provoke or suppress T-(helper)-cell responses and are upregulated in response to signaling, the increased expression may point toward antigen-presentation or cross talk with local T cells *in vivo*. It has also been shown that cultured fibrocytes express all the necessary costimulatory molecules for antigen-presentation, that they are potent stimulators of naive T-cells and induce a Th2 cytokine response *in vitro[38, 39].*

Collectively, this gating strategy holds great potential in investigating lung fibrocytes in more detail. This method could replace experiments on artificially cultured fibrocytes and allow for direct investigation of the role of lung fibrocytes in tissue remodeling and fibrosis in its target organ.

Conclusion

Using a gating strategy that excludes possible contaminating cell populations, we show that lung fibrocytes in IPF lungs can be assessed by flow cytometry and that their phenotype differs from circulating fibrocytes. This new approach could be interesting for scientists investigating the fascinating role of fibrocytes in disease pathogenesis or their potential as a biomarker and therapeutic target.

List of abbreviations

CCL = C-C motif ligand
CCR = C-C chemokine receptor
CXCL = C-X-C motif ligand
CXCR = C-X-C chemokine receptor
Col-1 = collagen-1
FSC = forward scatter
HC = healthy control
IPAH = idiopathic pulmonary arterial hypertension
IPF = idiopathic pulmonary fibrosis
PBMC = peripheral blood mononuclear cell
PF = pulmonary fibrosis
PH = pulmonary hypertension
PNM = polymorphonuclear
SSC = side scatter

Funding

This study was partially supported by a grant from Actelion Pharmaceuticals

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Additional file 1. Characteristics of patients and the healthy subjects used for experiments.

		BLOOD				LUNG		Pt for SORT	
		HC (n=15)	IPF (n=14)	IPAH (n=10)	PH (non IPAH)* (n=5)	Control lung+ (n=9)	IPF lung (n=8)	HC (n=11)	PF# (n=11)
General	age (years)	43±10	69±7	48±17	51±10	61±6	63±2	33±6	69±8
	male/female (n)(% male)	6/9 (40)	11/3 (79)	1/10 (10)	2/3 (40)	5/4 (56)	4/7 (36)	4/7 (36)	3/8 (27)
Functional	FVC (% predicted		66±16			105±17	50±11		68±18
	TLCO (% predicted)		37±14			80±21	35±8		35±8
	FEV1 (% predicted)					105±15	56±12		
	PH secondary to PF yes/no/nd (n)**		1/0/9				3/4/1		3/6/2
	Mean PAP (mmHg)			55±14	44±11				
	RAP (mmHg)			12±6	8±1				
	Cardiac index (L/min/m2)			2,1±0,9	2,9±0,8				
	SvO2 (%)			62±7	67±7				
	NYHA-class 2/3 (n)			3/7	2/3				
medication	Steroids		2	0	0	0	3		3
	Pirfenidone		4	0	0	0	0		5
	Nintedanib		1	0	0	0	2		0
	No treatment		7	0	0	0	4		3
	PDE5i		0	2	1	0	0		0
	PDE5i + ERA		0	6	3	0	0		0
	PDE5i +ERA + Pros-A (n)		0	2	1	0	0		0

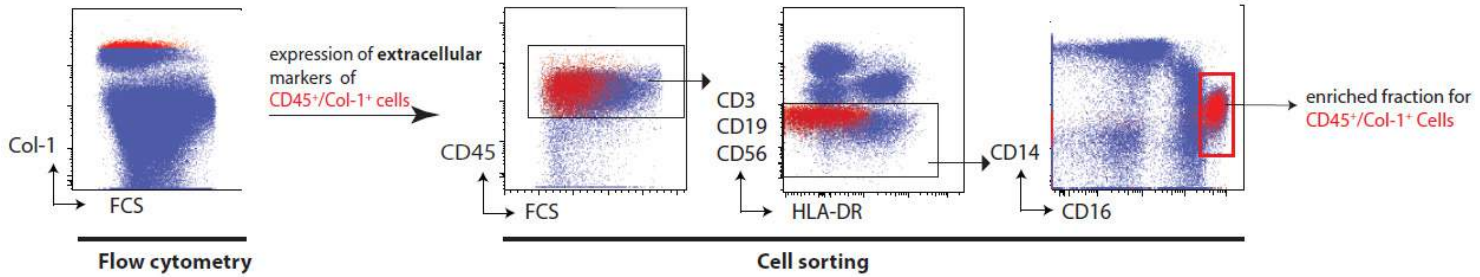
[a] 1 patient with chronic thromboembolic PH and 4 patients with PH secondary to an auto-immune disease

[b] 6 patients with IPF, 3 patients with UIP pattern secondary to extrinsic allergic alveolitis, 2 patients with an non-specific interstitial pneumonia and 1 patient with anti-synthetase syndrome

[c] Mean FVC/FEV1 ratio = 0.71 (2 out of 9 had an obstructive pulmonary function test, both classified as GOLD A)

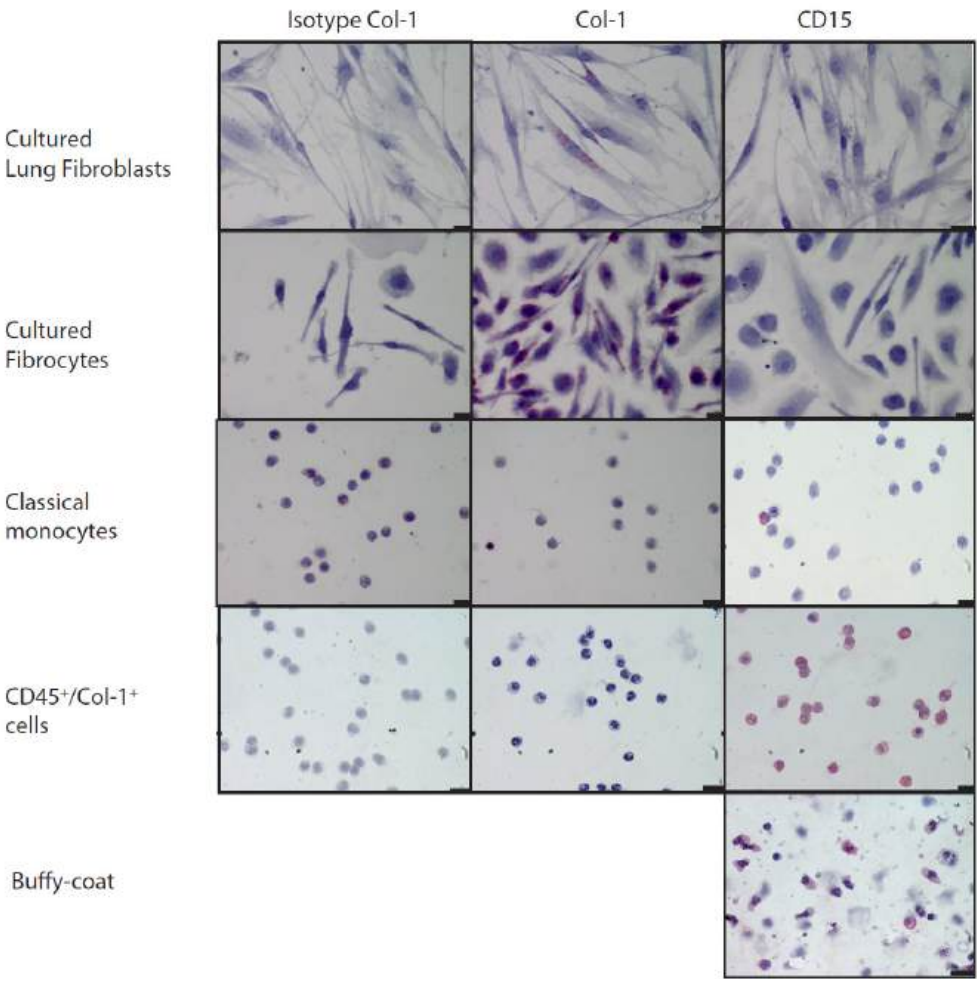
[d] Assessed by right heart catheterization or suspected with echocardiography

IPF = idiopathic pulmonary fibrosis, IPAH = idiopathic pulmonary arterial hypertension, FVC = forced vital capacity, TLCO = diffusing capacity for carbon monoxide, PAP= pulmonary arterial pressure, RAP = right atrium pressure, Svo2 = mixed venous saturation, PDE5i = phosphodiesterase type 5 inhibitor, ERA = endothelin receptor antagonist, Pros-A = prostacyclin agonist

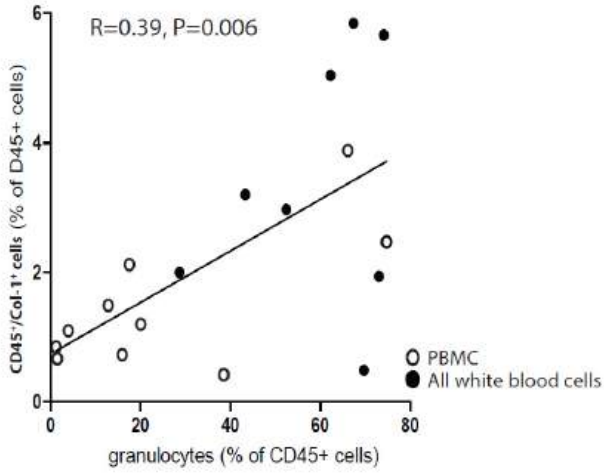


Additional file 2. Sort strategy for CD45⁺/Col-1⁺ cells.

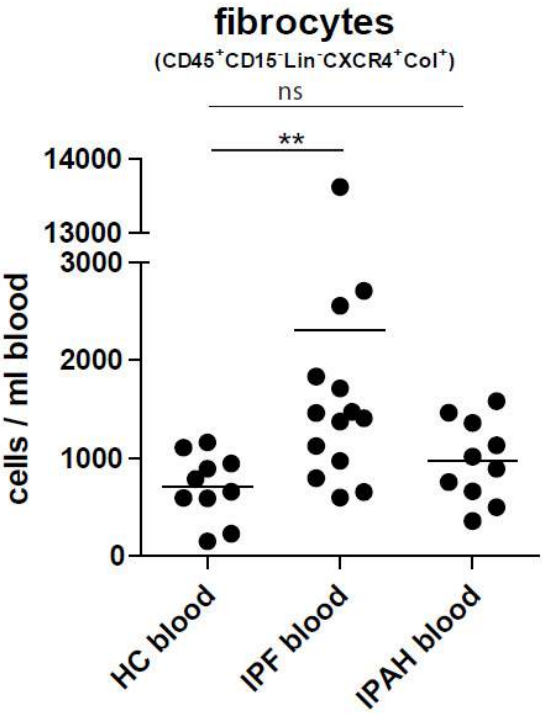
(A) The conventional strategy with the CD45⁺/Col-1⁺ cells in red. (B) Position of the same fibrocytes when employing a gating strategy based on additional extracellular markers. The sort strategy is based on extracellular markers and with some modifications previously published [16] (of note: for our research question we did not exclude SSC^{hi} cells). CD45+ cells were analyzed for HLA-DR expression and lineage markers to exclude B-cells (CD19), NK cells (CD56) and T-cells (CD3). Lineage negative cells were plotted as CD14 versus CD16 to create a distinct group of cells enriched for CD45⁺/Col-1⁺ cells (red box).



Additional file 3. Complete overview collagen-1 and CD15 staining on CD45$^+$/Col-1$^+$ cells and controls
Immunocytochemical images of cultured fibroblasts, cultured fibrocytes, sorted classical monocytes and sorted CD45$^+$/Col-1$^+$ cells. Indicated cells were stained with Collagen-1 or isotype control (rabbit IgG) and CD15. As a control for CD15 we used a buffy coat, nicely showing positive granulocytes next to negative lymphocytes. Magnification for all images was 100x.



Additional file 4. Correlation circulating CD45⁺/Col-1⁺ fibrocytes and granulocytes
For this experiment we analyzed paired total white blood cells (open dots) and PBMCs (black dots) on the same day as blood withdrawal of 9 patients (4 IPF patients and 5 PH patients). Correlation coefficients were calculated using Spearman's rank method.



Additional file 5. Circulating fibrocyte numbers in patients with IPF and idiopathic pulmonary hypertension (IPAH). Absolute numbers of circulating fibrocytes per ml blood in frozen PBMC of HC, patients with IPF and patients with IPAH. ** $P < 0.01$

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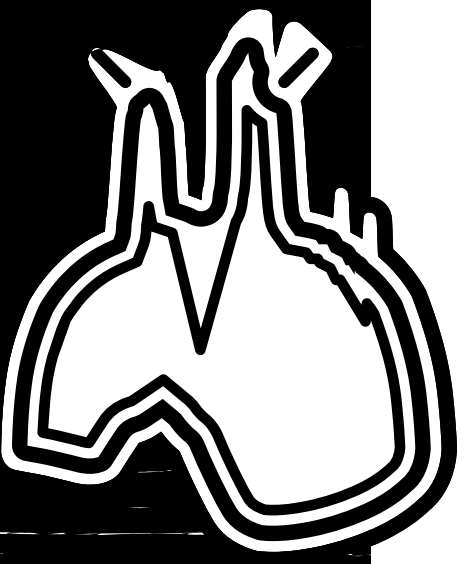

CHAPTER 5

Enhanced Bruton's Tyrosine Kinase in B-cells and autoreactive IgA in patients with Idiopathic Pulmonary Fibrosis

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Abstract

Rationale:
Idiopathic Pulmonary Fibrosis (IPF) is thought to be triggered by repeated alveolar epithelial cell injury. Current evidence suggests that aberrant immune activation may contribute. However, the role of B-cell activation remains unclear. We determined the phenotype and activation status of B-cell subsets and evaluated the contribution of activated B-cells to the development of lung fibrosis both in humans and in mice.

Methods:
B-cells in blood, mediastinal lymph node, and lung single-cell suspensions of IPF patients and healthy controls (HC) were characterized using 14-color flow cytometry. Mice were exposed to bleomycin to provoke pulmonary fibrosis.

Results:
More IgA$^+$ memory B-cells and plasmablasts were found in blood (n=27) and lungs (n=11) of IPF patients compared to HC (n=21) and control lungs (n=9). IPF patients had higher levels of autoreactive IgA in plasma, which correlated with an enhanced decline of forced vital capacity ($p=0.002$, $r= -0.50$). Bruton's tyrosine kinase expression was higher in circulating IPF B-cells compared to HC, indicating enhanced B-cell activation. Bleomycin-exposed mice had increased pulmonary IgA$^+$ germinal center and plasma cell proportions compared to control mice. The degree of lung fibrosis correlated with pulmonary germinal center B-cell proportions ($p=0.010$, $r= 0.88$).

Conclusion:
Our study demonstrates that IPF patients have more circulating activated B-cells and autoreactive IgA, which correlate with disease progression. These B-cell alterations were also observed in the widely used mouse model of experimental pulmonary fibrosis. Autoreactive IgA could be useful as a biomarker for disease progression in IPF.

Background

Idiopathic Pulmonary Fibrosis (IPF) is a progressive and ultimately fatal disorder.[1, 2] Although the natural history of patients with IPF varies from rapid progressive to episodes of relative stability for years, the median survival is 3–5 years following diagnosis.[3] Fibrosis in IPF patients is considered the end-result of exaggerated wound repair due to repetitive subclinical epithelial injury, leading to (myo-)fibroblast activation and uncontrolled matrix deposition.[4] Current disease modifying therapy consists of two drugs that slow down progression of fibrosis.[5, 6] Despite all evidence that immunity is also involved in IPF pathogenesis, treatments that modulate inflammation have failed or even had deleterious effects on primary end-points in clinical IPF trials.[7] It is a topic of debate to what extent immunity is a (co-)driver of disease or whether it is merely an epiphenomenon which can correlate with disease severity.

Recently, several lines of evidence suggest a role for B-cell immunity.[8, 9] Firstly, tertiary lymphoid organs (TLOs) have been found in lungs of IPF patients, which persist and accumulate during disease progression.[10, 11] TLOs harbor a distinct T-cell and B-cell zone, and attraction of B-cells into TLOs depends on CXCL13.[12] CXCL13 was reported to be elevated in lungs and serum of patients with IPF.[12, 13] Secondly, over the past two decades several specific auto-antibodies have been identified in IPF[8], likely involving local production of (auto)-antibodies in lung TLOs. Autoreactive antibodies, recognizing pulmonary proteins, may also contribute to repeated lung injury.[8] Thirdly, elevated serum B-cell activating factor (BAFF) was indicative for worse disease outcome in IPF patients.[14] A role for B-cell immunity in IPF may be especially relevant because new therapies that (only) target B-cells or decrease antibody production are effective in other B-cell-mediated interstitial lung diseases and possibly in acute exacerbations of IPF.[15, 16, 17] Whether dual treatment of available anti-fibrotic drugs with anti-inflammatory therapies targeting B-cell activation is beneficial in IPF is currently unknown. Hence, we need a better understanding of B-cell profiles in blood and especially in lungs of IPF patients.

Therefore, we performed an in-depth analysis of B-cell phenotype and activation status in blood, mediastinal lymph nodes (LN), and lungs of IPF patients and controls. We analyzed total and/or autoreactive antibodies in plasma and lungs of IPF patients and identified immunological biomarkers for disease progression. Finally, we investigated B-cell subsets in a bleomycin-induced pulmonary fibrosis mouse model.

Methods

Study design and subjects

Human lung and Lymph Node (LN) tissue were collected from patients with IPF undergoing lung transplantation. IPF was diagnosed according to current guidelines of the ATS/ERS/JRS/ALAT.[1, 18] All explanted lungs fulfilled pathological criteria for an usual interstitial pneumonia (UIP). As a control, lung tissue was obtained from volume reduction procedures during transplantation upon size mismatch of oversized donor lungs or residual material

obtained during surgery for pulmonary tumors. Control residual lung tissue was taken at least >3cm from the tumor. For this, only patients with a normal pulmonary function test or mild airflow obstruction (GOLD 1) without emphysema were selected. Control LN were collected from lung transplantation donors. Blood samples were obtained after patient's informed consent. All patient and healthy subject characteristics are shown in **Table 1**. The Medical Ethical Committee of the Erasmus MC Rotterdam approved this study (METC 2012-512) and gave consent for collection of residual material of explanted lungs and LN.

Human tissue and blood processing

Fresh lung and LN tissues were stored in cold phosphate buffered saline (PBS) and processed within 24 hours following transplantation or resection.

Lung resection specimens were rinsed with PBS to remove residual blood. After mincing the lung, specimens were enzymatically digested in digestion medium (Life Technologies) with 10ug Liberase (Roche, LiberaseTM) and 40 Units of DNA-se (Roche, DNase I recombinant, RNase-free) for 30 minutes in a humidified incubator at 37˚C while gently shaking the samples. The remaining cell debris was removed by passing the cells through a 100μm-diameter disposable cell mesh filter. Fresh LN were separately past through a 100μm-diameter disposable cell mesh filter. Single cell suspensions specimens were washed in Roswell Park Memorial Institute medium (RPMI) with 5% fetal calf serum and centrifuged for 10 minutes at a speed of 400 × g. Lung single cell suspensions were also subjected to red blood cell lysis, washed and counted. Finally, all samples were aliquoted and cryopreserved .

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes (BD Vacutainer K2E). Peripheral blood mononuclear cells (PBMCs) and plasma were obtained, processed and stored according to standard protocols.[19]

PBMCs and single-cell suspensions were stained for extra- and intracellular markers described in **additional table 1.** PBMCs and single cell suspensions were stained with antibodies for extra- and intracellular markers described in additional table 1. To control for non-specific labeling Fc-block (Anti-Mouse CD16/CD32 Fc Block) was used. Fixable Viability Dye eFluor 506 (eBiosciences) was applied as a live-dead marker. In short, cells for the B-cell staining were incubated in FACS buffer (PBS, 0.25% BSA, 0.5mM EDTA, 0.05% NaN3 sodium azide) and for the T-cell staining in MACS buffer (0.5% BSA + 2mM EDTA in PBS) with fluorescent antibodies for 60 minutes at 4˚C using methods recommended by the manufacturers. Cells for the T-cell staining underwent a second extracellular incubation step for antibodies with Brillaint Viotet (BV) conjungates in BV-bufferin Brilliant Stain-buffer(BD Biosciences, cat#563794). After fixation and permeabilization (2% paraformaldehyde solution and 0,5% saponin (Quillaja Bark, Sigma cat#S7900) cells were incubated with the Bruton's tyrosine kinase (BTK)-antibody or Isotype control in Saponine buffer for 60 minutes at 4˚C (only B-cell staining). Biotinylated antibodies were visualized with streptavidin-BV650. Cells were measured on a LSRII Flow cytometer (BD Biosciences).

We analyzed a minimum of 200,000 alive cells for blood samples and 100,000 alive cells for the lung tissue samples for cytometric analysis. Data was analyzed by FACS Flow-Jo software. To compare mean fluorescence intensity (MFI) values between experiments, identical control samples (n=3) were measured in each (subsequent) experiment for standardization.

Immunohistochemistry

Immunohistochemical analyses were performed according to standard procedures.[19] Antibodies used are listed in **Additional table 1**

Self-reactive and total immunoglobulin (Ig)G, IgM and IgA

HEp-2 analyses and enzyme-linked immunosorbent assay (ELISA) on plasma samples were performed according to standard procedures. Serum samples (1/50 diluted) of IPF patients and healthy controls (HC) were incubated for 1 hour on Kallestad human epithelial cell (HEp-2) slides (Bio-Rad Laboratories). As detection antibodies Ig F(ab')2 fragments were applied to the HEp2 slides (**Additional table 1**). The fluorescence intensity of HEp2 slides was evaluated using a LSM 311 META confocal fluorescence microscope (Zeiss) and LSM Image Browser Version 4.2.0.12 software (Zeiss) in an automated and thus independent manner. The fluorescence intensity was corrected for number of cells per slide. A positive HEp-2 result was set on 2SD above the mean of the HC for each immunoglobulin subtype.

Mice

Mice were bred and kept under specified pathogen-free conditions in the Erasmus MC experimental animal facility. All experimental protocols have been reviewed and approved by the Erasmus Medical Center Committee of animal experiments. Procedures of mouse experiments are described in **Additional Methods 1**.

Statistics

Statistical analysis was performed using IBM SPSS Statistics 21 and GraphPad Prism 6 software. For calculating the level of significance of differences between groups we used the Mann-Whitney U test. Correlation coefficients were calculated using Spearman's rank method. P values <0.05 were considered significant. Flow cytometry data is either represented as percentage population or as MFI.

Table 1 Patient and healthy subject characteristics.

	Blood		**Lung**		**MLN**	
	HC (n=21)	**IPF** (n=27)	**Control** (n=9)	**IPF** (n=11)	**Control** (n=13)	**IPF** (n=10)
Age, years (95% CI)	56 (52-61)	70 (67-73)	50 (56-66)	59 (55-63)	59 (56-61)	58 (53-61)
Gender: M/F (n, (%))	12 (57%) /9	19 (70%)/8	5 (55%)/4	9 (81%)/2	8 (62%)/5	9 (90%)/1
Diagnosis						
-MDD: IPF/Prob IPF/Pos IPF(n, (%))		22 (81%)/5/0		11 (100%)/0/0		10 (100%)/0/0
-PA obtained (%)		22		100		100
History						
-Cardiovascular disease (n, (%))*	0	14 (51%)	3 (33%)	2 (18%)		2 (20%)
-Auto-immune disease (n, (%))#	0	1 (3%)	1 (11%)	0 (0%)		0 (0%)
PFT, percentage predicted (95% CI)						
-TLCO		47 (42-53)	86 (76-95)	33 (27-39)		34 (27-40)
-FVC		77 (72-83)	108 (95-121)	53 (45-60)		52 (44-60)
-FEV1/FVC			69 (63-73)			
COPD (n) (stage)		0	3 (all GOLD1)	0		0
Smoking, n (%)						
-never		5 (19%)	2 (22%)	2 (18%)		2 (20%)
-former		22 (81%)	7 (78%)	9 (82%)		8 (80%)
-current		0 (0%)	0 (0%)	0 (0%)		0 (0%)
Medication						
-prednisone use: (n, (%))		8 (30%)	0 (0%)	4 (36%)		3 (30%)
dose (mg/day)						
>10mg/d		0 (0%)		1 (9%)		0 (0%)
</= 10mg/d		8 (30%)		3 (27%)		3 (30%)
-Other immunosuppressive (n, (%))		0 (0%)	0 (0%)	0 (0%)		0 (0%)
-Pirfenidone (n, (%))		0 (0%)		2 (18%)		2 (20%)
-Nintedanib (n, (%))		1 (3%)		3 (27%)		3 (30%)

* *IPF patients (blood)*: Hypertension (n=9), ischemic cardiac disease (n=5), or transient ischemic attack (n=2), *Control lung*: Hypertension (n=3), Cerebro Vasculair Accident (n=2) *IPF lung and LN*: Hypertension and transient ischemic attack (n=1), Hypertension (n=1) # *IPF patients (blood)*: colitis ulcerosa (not active and no immunomodulating medication) (n=1), *Control lung*: Rheumatoid arthritis (not active and no immunomodulating medication) without lung involvement or anti-CCP antibodies (n=1)

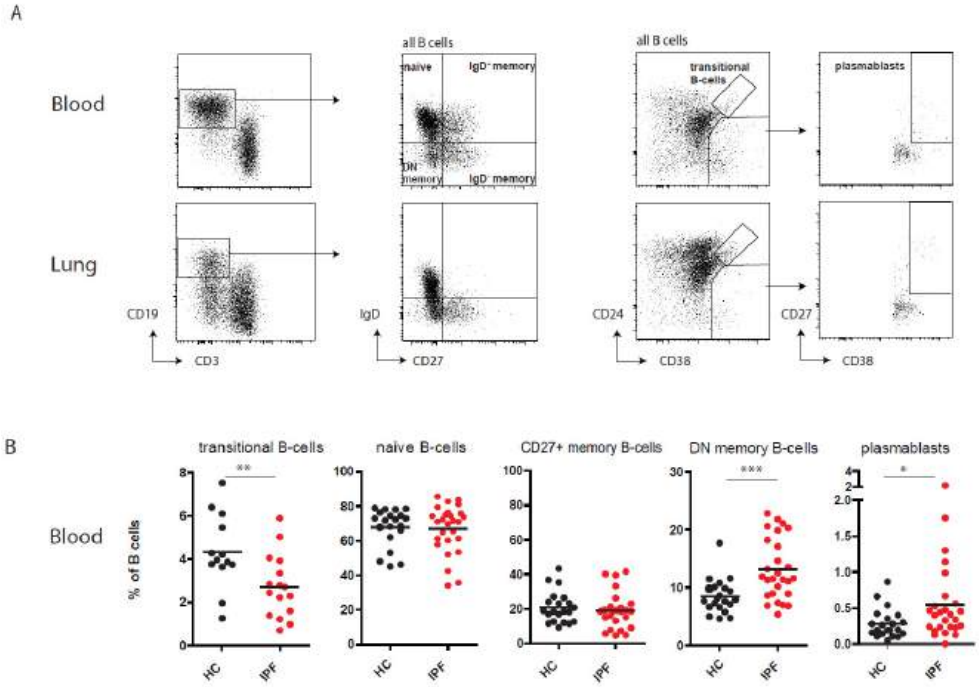
Abbreviations: MLN = mediastinal lymph node, MDD = multidisciplinary diagnosis, PFT = pulmonary function test, TLCO = carbon monoxide transfer factor, FVC = forced vital capacity, FEV1 = forced expiratory volume in one 1 second

Results

Alterations in B-cell subsets in blood, LN and lungs of IPF patients

We first evaluated different B-cell subsets in blood, explanted lungs, and LN of IPF patients. We quantified naïve B-cells (IgD$^+$ CD27$^-$), IgD$^+$ and IgD$^-$ memory CD27$^+$ B-cells, double negative (DN) B-cells (IgD$^-$CD27$^-$), transitional B-cells (CD19$^+$CD24$^+$CD38$^+$), and plasmablasts (CD19$^+$CD38$^+$CD27$^+$), following the gating strategy in **Figure 1A**.

The numbers and percentages of total B-cells in blood, LN, and lung were similar between IPF patients and controls (A**dditional figure 1**). The proportions of naïve B-cells were higher in blood than in lung, but did not differ between control and IPF (**Figure 1B/C**). In the circulation, IPF patients had a lower frequency of transitional B-cells, but significantly higher proportions of DN memory B-cells and plasmablasts compared with HC (**Figure 1B**). IPF lungs harbored higher proportions of transitional B-cells and plasmablasts compared to control lungs (**Figure 1C**). No alterations in B-cell subsets were observed between IPF LN and control LN (**Additional figure 2**).



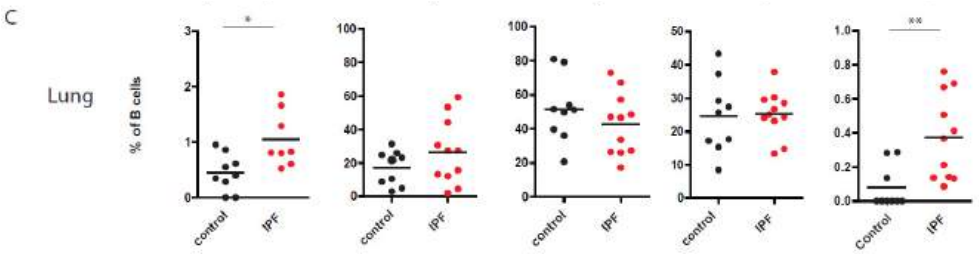


Figure 1 Alterations in B-cell subsets in blood, LN and lungs of IPF patients. (A) Representative gating strategy for identification of B-cell subsets in blood and single cell suspensions of lungs. Naïve B-cells (CD19$^+$IgD$^+$CD27-) IgD$^+$ and IgD$^-$ memory CD27$^+$ B-cells, double negative (DN) B-cells (CD19$^+$IgD$^-$CD27$^-$), transitional B-cells (CD19$^+$CD24$^+$CD38$^+$), and plasmablast (CD19$^+$CD38$^+$CD27$^+$) were identified. (B) c Percentage of circulating B-cell subsets of total B cells in HC (n=21) and IPF patients (n=27). (C) Proportion of B-cell subsets of total B-cells in single cell suspensions of control lungs (n=9) and explanted IPF lungs (n=11). Non-parametric two-tailed Mann-Whitney test was used. Data are expressed as mean and dots represent individual patient values. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Augmented proportions of IgA-expressing memory B-cell subsets

We next evaluated surface immunoglobulin (Ig) expression on memory B-cell subsets in blood and single-cell suspensions of explanted IPF lungs. Surface IgM and IgG expression was evaluated on IgD$^-$ memory B-cells and DN-memory B-cells (**Figure 2A**) as depicted in a representative blood and lung sample from an IPF patient and a healthy control in **Figure 2B**. The IgD$^-$IgM$^-$IgG$^-$ memory and IgM$^-$IgG$^-$ DN-memory B-cells were enriched for IgA surface expression (**Additional figure 3-4**). These populations are hereafter called IgA$^+$ memory B-cells.

The proportion of IgA$^+$ cells within the CD27$^+$ memory B-cell fraction was significantly higher in IPF patients in blood (~44%, compared to ~30% in controls) and in the lung (~60% versus ~40%, respectively) (**figure 2C**). Likewise, within in the DN-memory B-cells, the IgA$^+$ population was significantly higher in blood of IPF patients compared to controls (F**igure 2D**). Within both memory B-cell populations, this increase of IgA$^+$ B-cells was at the expense of IgM$^+$ B-cells in blood and at the expense of IgG$^+$ B-cells in lungs (both significant) (**Figure 2C, 2D; Additional figure 3-4**). No differences in surface Ig expression on memory B-cell subsets were observed between explanted IPF and control LN (**Additional figure 3-4**).

Summarizing, within the two memory populations in blood and lung, IgA-expressing B-cells were increased in IPF patients and dominant over B-cells expressing other Ig subclasses.

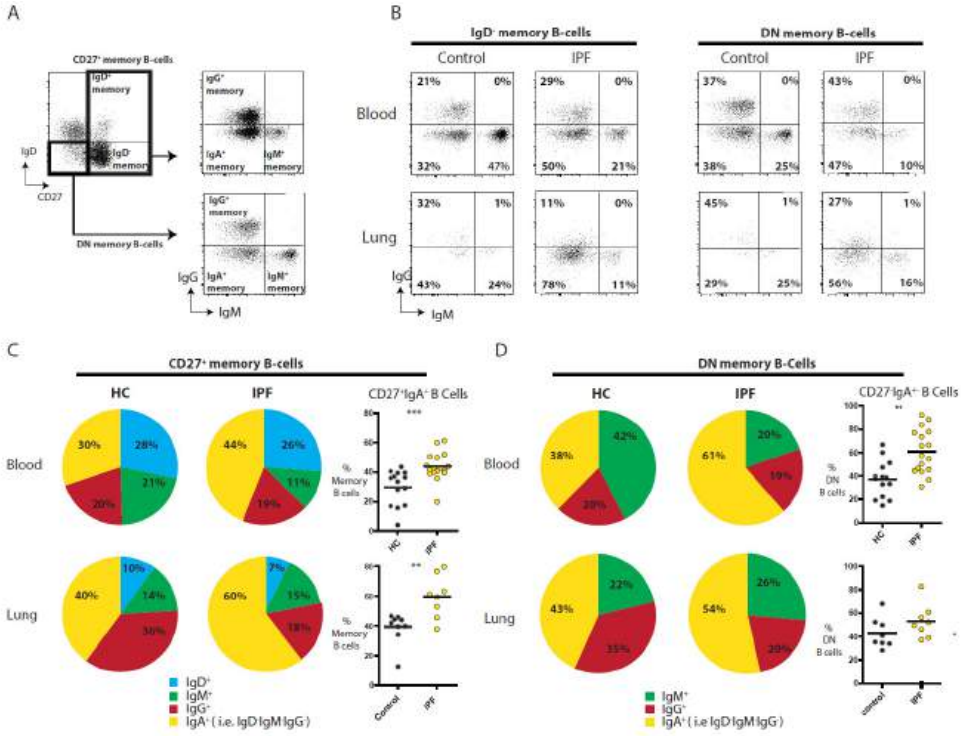


Figure 2 Augmented proportions of IgA-expressing memory B-cell subsets. (A) Representative gating strategy for the identification of immunoglobulin surface expression (IgM or IgG) on IgD$^-$ memory B-cells CD19$^+$CD27$^+$IgD$^-$) or double negative (DN) B-cells (CD19$^+$CD27$^-$IgD$^-$). (B) Gating strategy as described in panel A for a representative blood and lung sample for a control and IPF patient. (C; left) Pie chart showing the distribution of surface expression of IgD, IgM, IgG, and IgA on CD27$^+$ memory B-cells depicted with mean percentage ; (C: right) Proportions of IgA$^+$ memory B-cells of total CD27$^+$ memory B-cells in blood and lung samples. (D; left) Distribution of immunoglobulin expression of IgM, IgG and IgA on DN memory B-cells depicted in a pie-chart with mean percentage. (D; right) Proportions of IgA$^+$ DN memory B cells of total DN B-cells in blood and lung samples.
Non-parametric two-tailed Mann-Whitney test was used. Data are expressed as mean and dots represent individual patient values. ** $P < 0.01$ *** $P < 0.001$

IgA+ B cells are present within TLO structures of IPF lungs

We next examined whether IgA expressing B-cells are present within TLOs found in IPF lungs[11]. In the lungs of our IPF patient cohort numerous TLOs could be detected (**Figure 3A**). We next confirmed that IPF TLOs had a specific organization with segregated T and B-cell zones (**Figure 3B**). Within the B-cell area, IgG staining was weak, however IgA staining was strong (**Figure 3C**). IgA could also be detected outside TLOs, likely reflecting secreted IgA. Thus, IgA$^+$ B-cells were present in TLO in IPF lungs and probably contribute to the local IgA production.

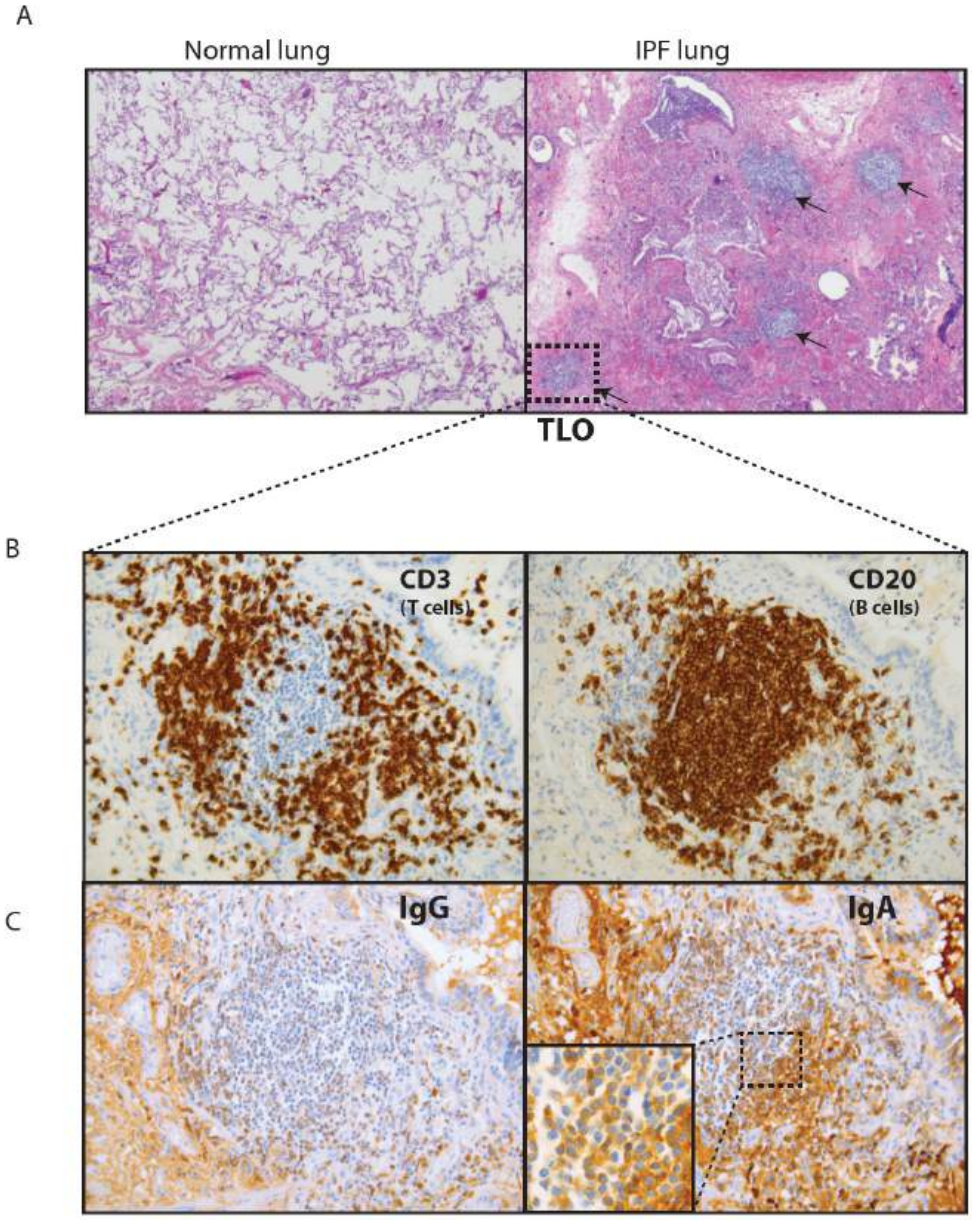


Figure 3 IgA⁺ B cells are present within TLO structures of IPF lungs. (A) Hematoxylin and eosin (H&E) staining of a control lung and IPF lung showing numerous TLOs (black arrows). (B) Pulmonary TLOs of IPF lung stained with anti-CD3 (T cells) and anti-CD20 (B-cells). (C) Representative images of staining with anti-IgG and anti-IgA. Magnification: 10x (A) and 40x (B and C)

More activated follicular helper T-cells (Tfh) in IPF lungs

Active TLOs contain germinal centers (GCs) in which follicular T helper (Tfh) cells provide essential help to B-cells to promote their activation. Tfh-cells, identified as $CD3^+CD4^+CXCR5^+CD45RA^-$ cells, were evaluated for the expression of PD-1, which is a reliable marker for activated Tfh-cells (**Figure 4A**).[20] The proportions of Tfh-cells as percentage of $CD4^+$ T-cells did not differ between HC and IPF patients (blood and lung, data not shown). PD-1 expression was low in IPF and control blood, but was higher in IPF lung tissue (**Figure 4A, 4B**). Hereby the proportions of activated $PD\text{-}1^{high}$ Tfh-cells in IPF lungs was significantly higher compared to control lungs (**Figure 4C)**, consistent with the presence of active GCs in pulmonary TLOs.

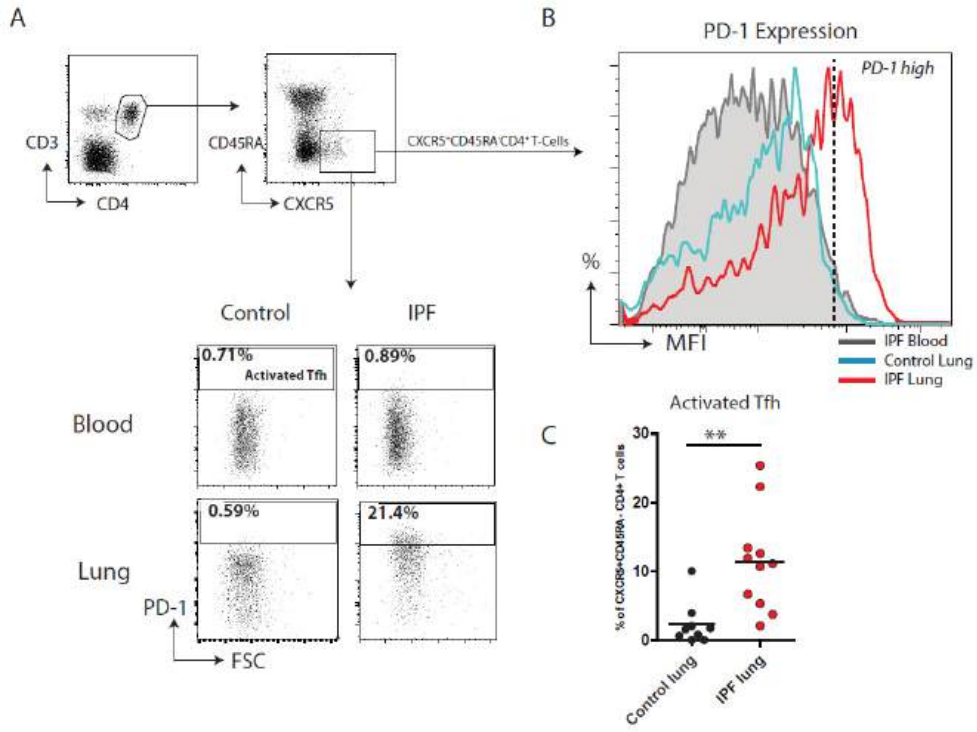


Figure 4 More activated follicular helper T-cells (Tfh) in IPF lungs. (A) Representative gating strategy for identification of Tfh ($CXCR5^+CD45RA^-CD4^+$) and activated Tfh ($CXCR5^+CD45RA^-CD4^+PD\text{-}1^{HI}$) as depicted for a representative blood and lung single cell suspension for a control and IPF patient. (B) Representative histogram overlay showing PD-1 expression levels of Tfh-cells depicted as MFI for a blood sample of an IPF patient (gray), control lung (blue) and IPF lung (red). (C) Activated Tfh-cells as percentage of total Tfh-cells in single cell suspensions of control lungs (n=8) and IPF lungs (n=11). Non-parametric two-tailed Mann-Whitney test was used. Data are expressed as mean and dots represent individual patient values. ** $P < 0.01$

Autoreactive IgA and IgG levels are higher in IPF and autoreactive IgA correlates with disease progression.

Total IgG and IgA – but not IgM – was significantly higher in plasma of patients with IPF compared with HC (A**dditional figure 5**). To explore the presence of auto-antibodies in plasma from IPF patients, we evaluated HEp-2 staining patterns and their fluorescence intensity for IgM, IgG and IgA. The staining patterns revealed the presence of autoreactive IgG and IgA, mainly recognizing nuclear antigens and showing a homogeneous staining across the nucleoplasm. Quantification of the autoreactive IgG and IgA fluorescence intensities, reflecting their plasma concentrations, revealed a significant increase in plasma of IPF patients compared to HC (F**igure 5B**). Also, the proportion of IPF patients harboring autoreactive IgM, IgG, or IgA was increased compared to HC (**Figure 5A, 5B**). There was no correlation between total and autoreactive IgG/IgA levels (A**dditional figure 5**). In ~48% of the IPF patients detectable autoantibodies were present versus in 18% of the HC, being IgM, IgG, or IgA **(Figure 5C**). Interestingly, within the IPF patients that had autoreactive antibodies, a majority (~62%) had autoantibodies of multiple isotypes.

We next evaluated whether levels of circulating auto-antibodies correlated with measures of disease progression. No correlation was observed between auto-reactive IgG or IgM with 1-year forced vital capacity (FVC) change (**Additional figure 6**). However, higher amounts of autoreactive IgA correlated with an increased decline of FVC (**Figure 5D**).

In summary, about half of the IPF patients analyzed had circulating IgM, IgG or IgA anti-nuclear auto-antibodies (ANAs), whereby autoreactive IgA correlated with disease progression.

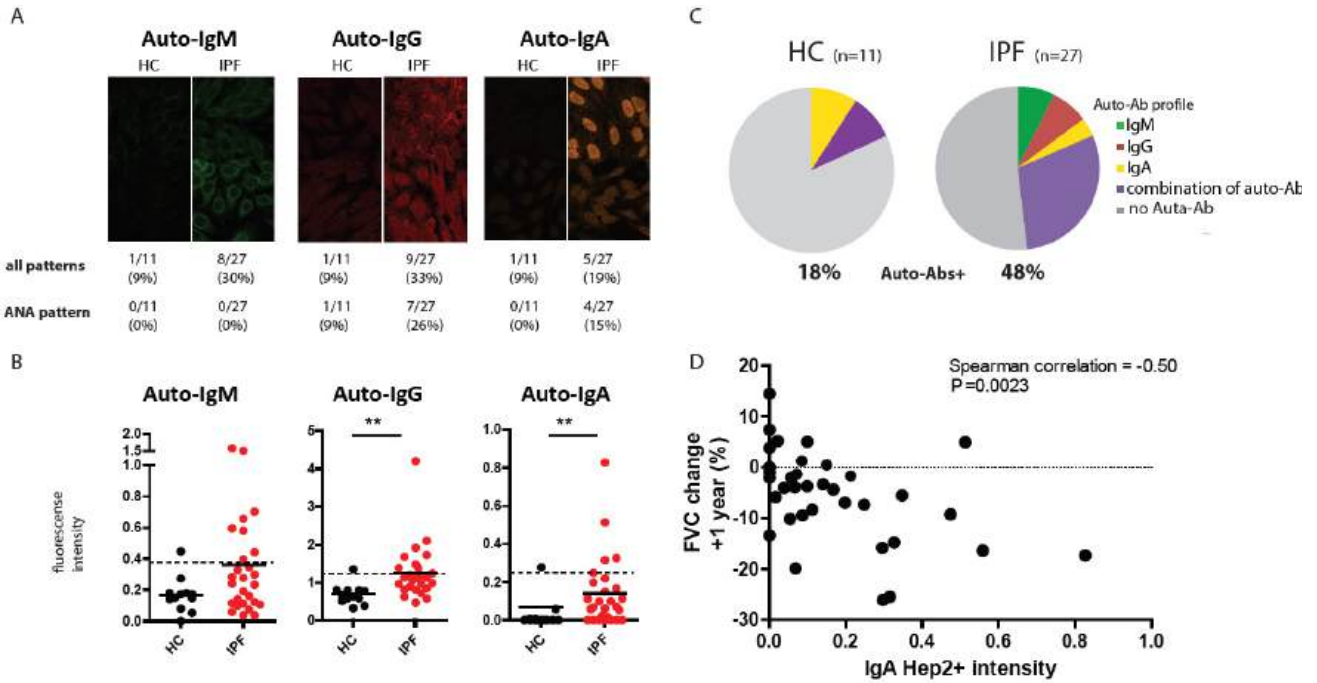


Figure 5 Autoreactive IgA and IgG levels are higher in IPF and autoreactive IgA correlates with disease progression. (A) Total IgM, IgG, and IgA in plasma of IPF patients and HCs. (B) Representative staining pattern of human epithelial cells (HEp)-2 slides with plasma of HC or IPF patients. First row describes the number and percentage of patients with a positive staining and second row number and percentage of patients with a specific antinuclear antibody (ANA) staining pattern. (C) Indirect quantification of auto-reactive immunoglobulins levels depicted as fluorescence intensity for autoreactove-IgM, IgG and IgA assessed with HEp-2 staining. The fluorescence intensity of HEp2 slides was evaluated in an automated and thus independent manner. The fluorescence intensity was corrected for number of HEp-2 cells per slide. A positive HEp-2 result was set on 2x standard deviation above the mean of the HC for each immunoglobulin subtype (see also additional methods 1) (D) Pie-chart of percentage of HC or IPF patients with detectable autoreactive antibodies and subclass (IgM, IgG and IgA (or combination)). (E) Increased fluorescence intensity for plasma autoreactive IgA correlate with decline in forced vital capacity (FVC) over 1-year period. Data of 12 IPF patients (with multidisciplinary diagnosis (MDD) of definitive or probable IPF) were used from our original cohort of 27 patients. 3 patients in the original cohort died within one year and could not be used for this analysis. Correlation coefficients were calculated using Spearman's rank method. Non-parametric two-tailed Mann-Whitney test was used. Data are expressed as mean and dots represent individual patient values. * $P < 0.05$ ** $P < 0.01$

Increased Bruton's tyrosine kinase expression levels in B cells of IPF patients

Given the reported increased protein expression of the signaling molecule Bruton's tyrosine kinase (BTK) in B-cells from patients with various autoimmune disorders, such as rheumatoid arthritis, Sjogren's Syndrome and vasculitis[21], we next evaluated BTK expression in circulating B-cells by intracellular flow cytometry. We observed higher BTK protein levels in IPF compared to HC (**Figure 6A**). Specifically, naïve B-cells showed the most pronounced enhanced BTK expression in IPF patients (**Figure 6B**). We found no difference in BTK levels between treatment-naïve patients and patients using low-dose prednisone (data not shown). In lungs and LN of IPF patients, BTK expression in B-cell subsets was similar to controls (**Figure 6C**).

Taken together, our data demonstrate that in blood of IPF patients, BTK expression in B-cells was higher than in HC.

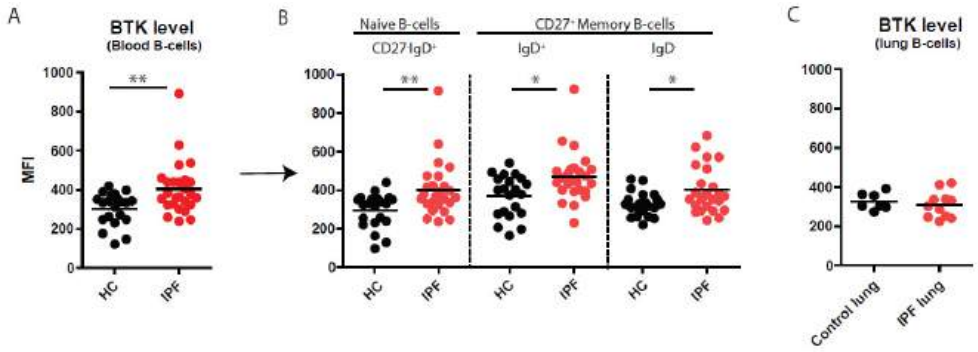


Figure 6 Increased Bruton's tyrosine kinase (BTK) expression levels in B cells of IPF patients. (A) Mean fluorescence intensity (MFI) of BTK in all circulating B-cells ($CD19^+$) and (B) naïve B-cells ($CD27^-IgD^+$), IgD^+ memory B-cells ($CD27^+IgD^+$) and IgD^- memory B-cells ($CD27^+IgD^-$) of HCs and IPF patients. (C) BTK expression (MFI) in pulmonary B-cells of control lungs and IPF lungs. Non-parametric two-tailed Mann-Whitney test was used. Data are expressed as mean and dots represent individual patient values. * $P < 0.05$ ** $P < 0.01$

Bleomycin-induced IgA+ induction and GC B-cells correlate with fibrosis scores in a mouse model

To explore if changes seen in human B-cell subsets would also occur in a well-established mouse model for fibrosis, we subjected C57Bl/6 mice to bleomycin or saline (control group). Histological analysis revealed that pulmonary fibrosis was present in mice 21 days after bleomycin exposure (**Figure 7A**). Lungs of bleomycin-exposed mice contained inflammatory aggregates with a core of GL-7+ GC B-cells and a ring of IgD^+ naïve B cells (F**igure 7A**). Blinded histopathology scoring confirmed that the total fibrosis score (TFS) was elevated in the bleomycin-exposed group (**Figure 7B**). Next, we evaluated B-cell subsets in lungs by flow cytometry (**figure 7C and additional figure 7**). The frequency of pulmonary GC B-cells and plasma cells of alive cells were significantly higher in mice

exposed to bleomycin compared to saline-exposed mice. Within GC B-cells or plasma cells respectively, the proportions of pulmonary IgA$^+$ GC B-cells and IgA$^+$ plasma cells were increased (**Additional figure 8**). In bleomycin-exposed mice, the TFS and the frequency of bronchoalveolar lavage fluid (BALF) GC B-cells showed a positive correlation (**figure 7D**). In summary, mice subjected to bleomycin-induced pulmonary fibrosis showed similar pathobiological changes as in human IPF. The extent of fibrosis correlated with the proportion of BALF GC B-cells.

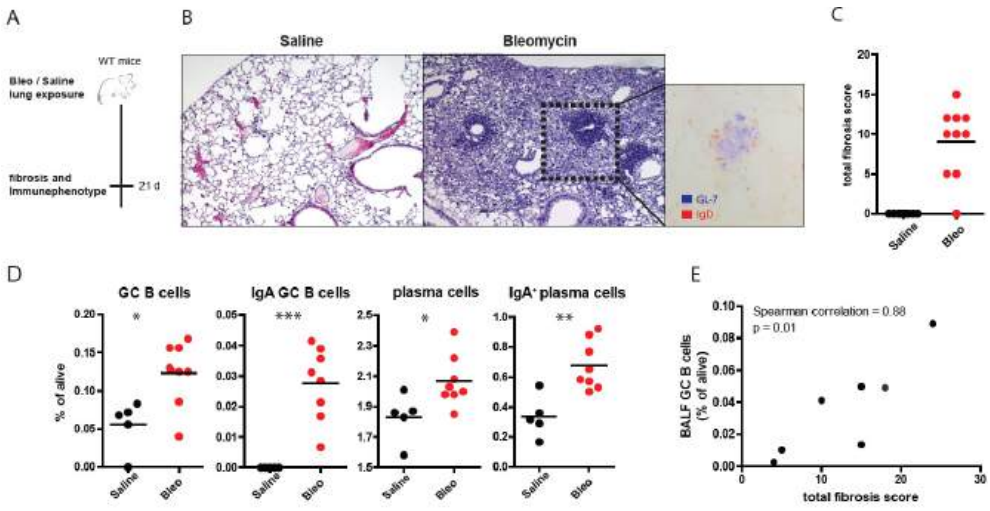


Figure 7 Bleomycin-induced fibrosis promotes germinal center IgA$^+$ B-cells and IgA$^+$ plasma cells. (A) Mice were sacrificed 21 days after saline or bleomycin exposure and analyzed for fibrosis indices and inflammation. (B) Representative hematoxylin and eosin (H&E) staining of cryo-sections of lung tissue after PBS or bleomycin exposure. Histological analysis revealed that pulmonary fibrosis was present in mice 21 days after bleomycin exposure as shown by typical characteristics including thickening of alveolar walls with or without obvious damage and formation of fibrous bands. The dashed square shows a probable TLO structure, which showed a center GL-7-positive cells (present on GC B-cells) surrounded by IgD- positive naïve B-cells . (C) Total fibrosis score (TFS). The TFS is the product of the Ashcroft scale and level of lung involvement (see additional methods 1). ((D) Proportion of GC B-cells (CD19$^+$CD95$^+$IgD^{low}), IgA GC B-cells (CD19$^+$CD95$^+$IgD^{low}IgA$^+$), plasma cells (CD19^{low}CD138$^+$), and IgA$^+$ plasma cells (CD19^{low}CD138$^+$IgA$^+$) of alive cells in lungs. (E) Correlation of the proportion of GC B cells of alive cells in bronchoalveolar lavage fluid (BALF) and TFS. Non-parametric two-tailed Mann-Whitney test was used. Correlation coefficients were calculated using Spearman's rank method. Data are expressed as mean and dots represent individual values of 4-7 mice per group and representative of 2 or more independent experiments. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Discussion

Our study assessed B-cell subsets and their activation status in IPF lungs and LN by 14-color flow cytometry. We observed that IPF patients had increased plasmablasts and IgA$^+$ memory B-cells in blood, intense IgA staining in lung TLOs, and more serum IgA antibodies recognizing nuclear self-antigens. Higher autoreactive IgA levels correlated with an increased decline of FVC. Furthermore, we showed that circulating IPF B-cells had elevated BTK expression, which could contribute to a loss of immune tolerance and development of autoimmunity in IPF. In a bleomycin pulmonary fibrosis mouse model, we observed the induction of IgA$^+$ GC B-cells and IgA plasma cells, indicating similarities with human pathobiology. The bleomycin model also showed that proportions of BALF GC B-cells correlated with the extent of fibrosis.

The increased numbers of TLOs in lungs of IPF patients, together with elevated serum CXCL13 and BAFF, indicate involvement of B-cells in IPF.[11, 12, 13, 14] The decrease in circulating transitional B-cells, together with an increase of B cells in the lungs of IPF patients, suggests migration from the blood towards the lungs. Transitional B-cells, which are immature B cells that have recently emigrated from the bone marrow, are possibly attracted to pulmonary TLO in IPF patients by CXCL13. We confirmed that plasmablasts are increased in blood[14], and importantly, also in IPF lungs. Furthermore, DN-memory B-cells are increased blood of IPF patients. This is particularly interesting as DN-memory B-cells activate telomerase when stimulated and are thought to be age-related exhausted memory B-cells.[22, 23] As both telomerase dysfunction and immune exhaustion have been implicated in IPF pathogenesis,[24, 25, 26] this B-cell subset would be an interesting topic for further research.

Repetitive alveolar epithelial injury exposes our immune system to self-antigens, increasing the risk of induction of autoreactive antibodies. Transforming growth factor-β, a key profibrotic cytokine, promotes IgA class-switch recombination of B-cells.[27,28] Serum IgA concentrations at the time of IPF diagnosis have been shown to be a predictor of survival.[29] In our patient cohort, plasma levels of total IgA and autoreactive IgA (mostly recognizing ANA) were also enhanced and specifically autoreactive IgA correlated with disease progression. Previous studies showed that autoantibodies found in IPF patients are often of the IgA subclass.[8, 30] Although (autoreactive) IgG antibodies are also implicated in IPF pathogenesis, we did not find a correlation with lung function decline.[8, 9] Autoreactive IgA may promote lung fibrosis through fibroblast proliferation and extracellular matrix protein production. [31, 32] This leads to a detrimental feedforward loop as the profibrotic milieu in IPF lungs promotes IgA class switching in B-cells.

BTK is a key molecule in BCR signaling and increased BTK expression in B-cells promotes the development of autoreactive B-cells.[21] Small-molecule BTK inhibitors have been proven effective in the treatment of autoimmune diseases in preclinical studies.[21, 33] In IPF patients, BTK expression was increased in circulating B-cells, especially in naïve B-cells. This might either reflect global B-cell activation due to pro-inflammatory micro-environments or pathogenic B-cell activation that directly contributes to autoimmune pathology. Activated Tfh-cells, which are present in IPF lungs, have the capacity to

engage with B-cells in the GCs of pulmonary TLOs and might promote autoreactive B-cell development.[34] Interestingly, mice with B-cell specific overexpression of BTK develop an autoimmune phenotype, together with increased Tfh-cell proportions.[35] These Tfh-cells might be induces by increased CD80 and CD86 expression on B-cells, which provide costimulatory signals for T-cell activation.[35] Consequently, our findings in IPF patients that (i) increased proportions of circulating plasmablasts and (IgA-)memory B-cells with (ii) enhanced BTK expression, (iii) augmented pulmonary Tfh-cell activation, and (iv) enhanced plasma autoreactive IgA are present indicate an important role for B-cells in IPF pathogenesis.

To investigate whether the same B-cell alterations would occur in a well-established model for pulmonary fibrosis , mice were exposed to bleomycin. Indeed, bleomycin exposure increases lung IgA^+ GC B-cells and IgA^+ plasma cells. The degree of pulmonary fibrosis correlated with the frequencies of GC B-cells in BAL fluid. This suggests that TLO formation with an active GC and the presence of IgA^+ GC B-cells reflects the degree of pulmonary fibrosis. The local pro-fibrotic environment together with the pulmonary mucosal environment is most likely responsible for the IgA class-switch recombination.
The bleomycin model deviates from human IPF pathology as fibrosis development is not progressive and it depends on inflammation.[36] Nevertheless, our data show similarities with human B-cell pathobiology. A previous study explored the effects of the irreversible BTK-inhibitor ibrutinib on fibrosis development and unexpectedly exacerbated fibrosis was found[37]. However, the interpretation of this finding is complicated, because ibrutinib is shown to have off-target effects and BTK is also expressed in myeloid lineages.[37, 38]
At this moment, it remains unclear if changes in B-cell activation or subsets are the primary culprit for IPF disease onset and/or progression. Nevertheless, changes in B-cell immunology may be a valuable biomarker for disease progression and/or survival, as high BAFF and CXCL13 plasma concentrations, involved in B-cell activation and homing, are predictive for poor survival in IPF patients.[13, 14]

Rituximab, an antibody against CD20 which destroys $CD20^+$ B cells, is effective in the treatment of several auto-immune diseases.[39] Compared to a historical cohort, an improvement of gas exchange and clinical outcome was observed in IPF patients with an acute exacerbation treated with rituximab, together plasma exchange and intravenous immune globulin.[15] The same experimental therapy is now under investigation in patients with acute exacerbations of IPF in a phase 2 trial (NCT03286556). If rituximab alone could be beneficial in the era of anti-fibrotic drugs is also currently examined and the results of this trial are expected in 2020 (NCT01969409). We believe that rituximab or other B-cell modulating therapies could be of value in the treatment of IPF patients next to 'anti-fibrotic' medication. Large landmark trials in the past using broad acting anti-inflammatory molecules in unselected IPF patients have learned that "one size fits all" approach does not always work.[7] Therefore, in our opinion, selection of IPF patients eligible for B-cell modulating therapies should be based on inflammatory biomarkers, preferably those that reflect B-cell (auto-) reactivity or activations status.

Our study has some limitations: First, lungs of diseased patients who underwent surgery, were used as controls. Three of these controls had mild COPD. We cannot rule out that inflammation profiles are affected, as recent literature show an increase of IgA expression and accumulation of B-cells in lung TLO in patients with severe COPD.[40, 41] However, subgroup-analysis showed that the COPD lungs had similar outcomes in B-cell subsets and activation markers compared with other controls (data not shown). Using disease controls could also contribute similar BTK expression in pulmonary B-cells in IPF patient and controls, as the majority of pulmonary B-cells have been activated. Second, no differences in B-cell subsets were observed in LN in our human data. The IPF LN examined in this study came from transplanted IPF patients, indicating end-stage IPF, which could be different in T- and B-cell proportions compared to LNs in early IPF disease. TLOs persist and accumulate during disease progression in IPF lungs, which may suggest that local immune activation becomes more important in end-stage disease.[11] Furthermore, LN enlargement does not necessarily change the proportion of B-cell subsets. Third, the relatively small sample size of human IPF lungs and LN used in our experiments. To the best of our knowledge, our study is the first that assessed B-cell subsets and activation in explanted IPF lungs and LN by detailed flow cytometry profiling and significant differences could be observed. Fourth, potential confounding by subject drop-out in the 1-year FVC change correlation analysis, as 4 patients died within the first year. Finally, two specimens (one IPF blood sample and one control lung) were obtained from subjects with a history of auto-immune disease. These samples were not excluded as (i) both subjects did not use immunomodulating medication, (ii) showed no clinically active disease at the time of sample collection, and (iii) the obtained B-cell data were within the range of the other samples.

Conclusion

We provide evidence that (autoreactive) B-cells, especially IgA-memory B-cells, are increased in IPF patients, possibly driven by increased intracellular BTK expression. Additionally, autoreactive IgA could be a predictor for FVC decline, however larger validation studies are needed to investigate the potential of autoreactive IgA as a new biomarker in IPF.

There is a need for stratified medicine based on inflammatory biomarkers and profiles to select patients with IPF who may be eligible for co-treatment with anti-inflammatory or immunomodulating therapies next to 'anti-fibrotic' medication. Our study provides a rationale for B-cell modulating therapies in selected IPF patients.

List of abbreviations:

ANA = antinuclear antibody
BAFF = B-cell activating factor
BALF = Broncho alveolar lavage fluid
BCR = B-cell receptor
CXCL = CXC chemokine ligand
DN = double negative
EDTA = ethylenediaminetetraacetic acid
FVC = forced vital capacity
HC = healthy control
HEp-2 = human epithelial cells
IPF = idiopathic pulmonary fibrosis
LN = lymph Node
MFI = mean fluorescence intensity
PBS = phosphate buffered saline
RPMI = Roswell Park Memorial Institute
Tfh = follicular helper T-cells
TFS = total fibrosis score
TLO = tertiary lymphoid organ

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Supplementary data

Aditional Methods 1

Inducing pulmonary fibrosis (mice)

To induce pulmonary fibrosis, bleomycin-hydrochloride was administered intratracheally in 8-10 week old mice (0,04U/80 μl saline) or saline as a control.[1] Mice were sacrificed 21 days after bleomycin exposure.

Flow cytometic procedures (mice)

Preparations of single-cell suspensions using standard procedures. Monoclonal antibodies are listed in additional table 1. For intracellular staining, cells were fixed in Cytofix/Cytoperm and permeabilized, and then stained in Perm/Wash buffer (BD Bioscience). All measurements were performed on a LSRII flow cytometer (BD Bioscience), and results were analyzed using FlowJo software.

Immunohistochemistry (mice)

Immunohistochemical analyses and staining were performed according to standard procedures.[2] Used antibodies are listed in additional table 1. After staining, tissue sections were embedded in Kaiser glycerol gelatin (Merck). Micrographs were made using a DM LB light microscope (Leica), a DFC500 camera (Leica), and Imaging for Windows Version 1.0 software (Kodak). A pathologist (blinded for treatment) scored the Ashcroft scale (grade 1-8)[3] and the percentage of lung involvement (grade 1-5; 1 =0-10% to 6 = 75-100% of total lung involvement). The Total Fibrosis score (TFS) is the product of Ashcroft scale and lung involvement and was previously described.[4]

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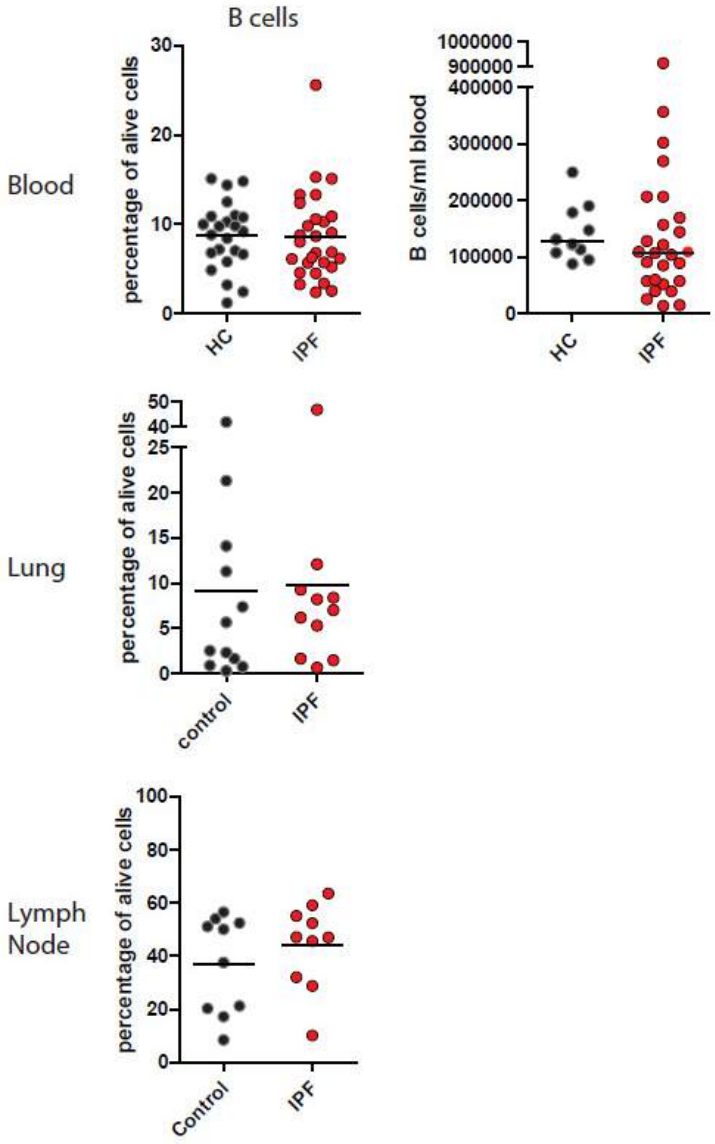
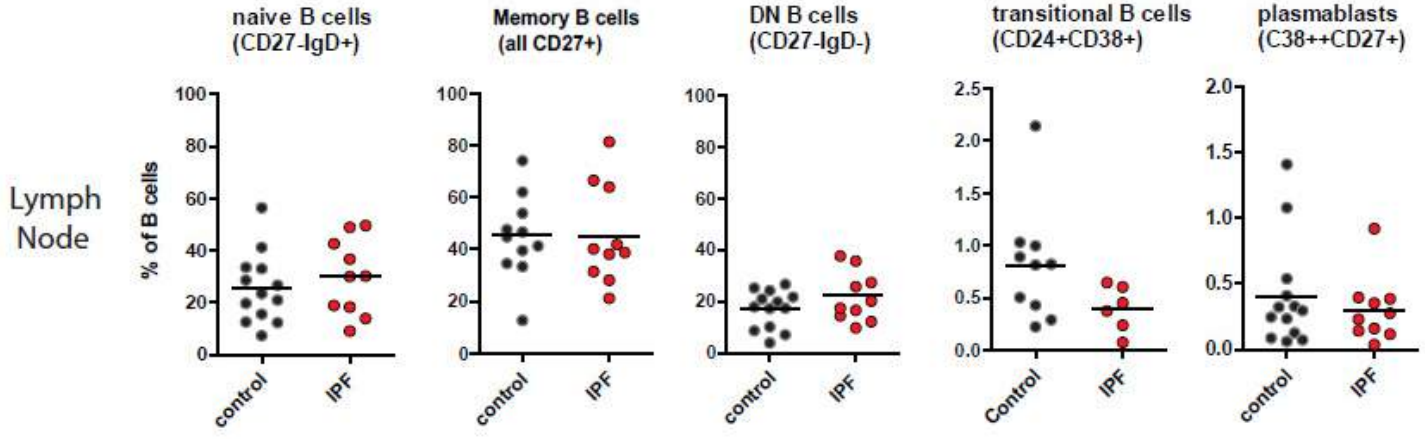
Additional Table 1

marker	conjugate	company	Cat#	intra/extracellular	dilution
HUMAN					
antibodies used for flow cytometry					
IgG	FITC	BD	555786	extracellular	1:20
Btk	PE	BD	611117	intracellular	1:5
IgM	Bio	BD	555781	extracellular	1:20
CD19	PerCP-Cy5.5	BD	332780	extracellular	1:400
CD38	APC	BD	560980	extracellular	1:10
IgD	APC-H7	BD	561305	extracellular	1:10
CD27	BV421	BD	562513	extracellular	1:80
CD24	BV711	BD	563401	extracellular	1:40
CD3	AF700	eBioscience	56-0038-42	extracellular	1:40
CD33	PeCy7	eBioscience	E10580-351	extracellular	1:30
IgA	PE	BD	555935	extracellular	1:10
CXCR5	PerCP5.5	BD	562781	extracellular	1:20
CD3	APC ef780	eBioscience	47-0038-42	extracellular	1:100
CD4	AF700	eBioscience	E08948-1631	extracellular	1:100
CD45RA	BV650	BD	563963	extracellular	1:40
PD1	BV786	BD	563789	extracellular	1:20
FoxP3	PE	eBioscience	12-4777-42	intracellular	1:20
Immunohisto-chemistry Lung					
CD3		Ventana	Clone: 2GV6		ready to use
CD20		Ventana	Clone: L26		ready to use
CD79		Ventana	Clone: SP18		ready to use
IgA		Cell Marque	Rabbit Polyclonal		ready to use
IgG		DAKO	Rabbit Polyclonal		1:24000
HEp-2 antibodies					
IgG	Cy3	Jackson IR	109-166-003		
IgM	af488	Jackson IR	109-546-129		
IgA	af647	Jackson IR	109-606-011		

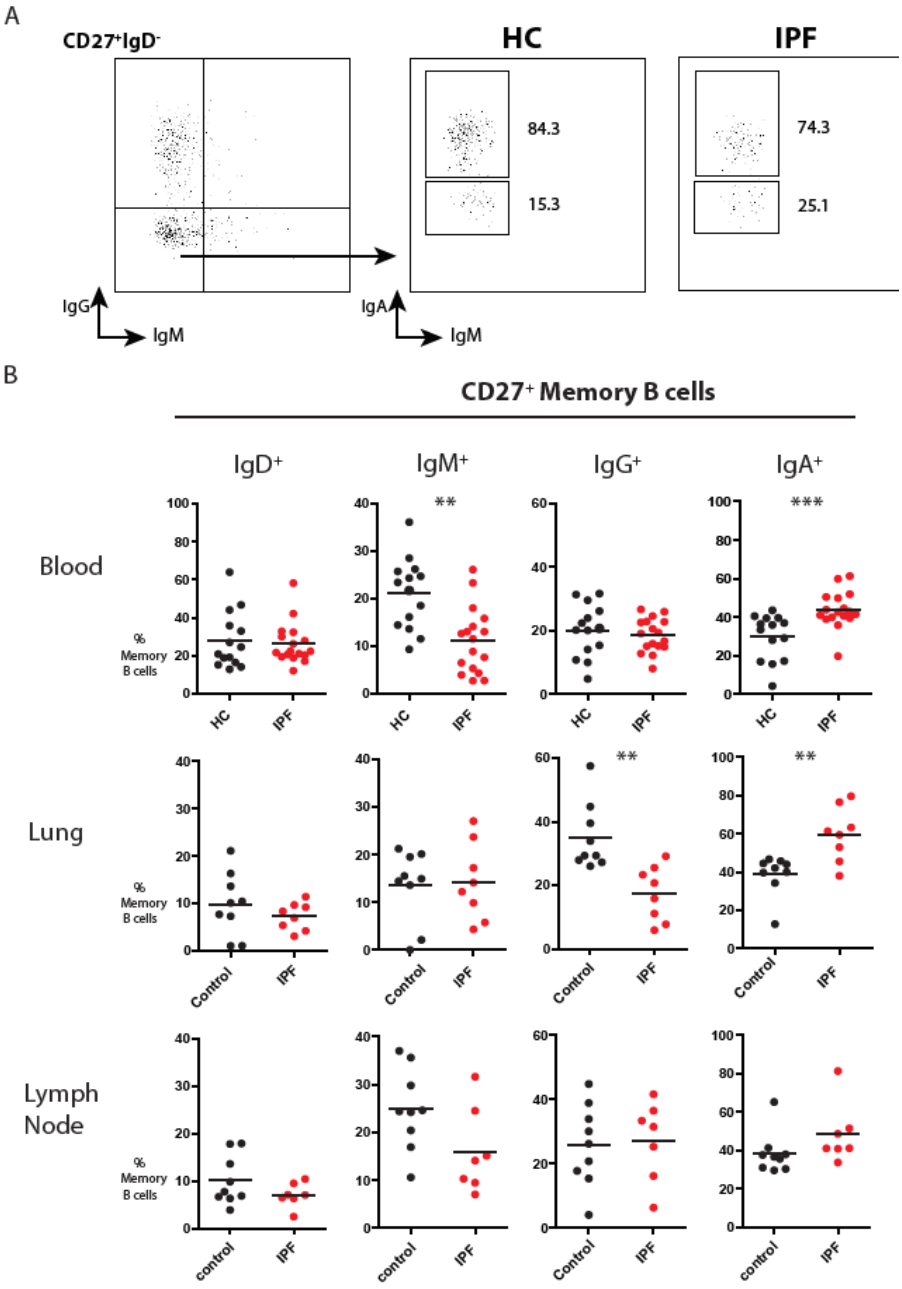
marker	conjugate	company	Cat#	intra/extracellular	dilution
MOUSE					
antibodies used for flow cytometry					
GL7	FITC	BD	553666	extracellular	1:2000
CD95	PE-TxR	BD	562499	extracellular	1:400
IgM	Pe-Cy7	eBioscience	25-5790-82	extracellular/ intracellular	1:500
IgD	APC	eBioscience	17-5993-82	extracellular/ intracellular	1:1280
CD19	Af700	eBioscience	56-0193-82	extracellular	1:50
CD138	BV605	BD	563147	extracellular	1:400
CD3	PE-CF594	BD	562286	extracellular	1:100
CD4	Af700	eBioscience	56-0041-82	extracellular	1:200
CD4	Af700	eBioscience	56-0041-82	extracellular	1:400
IgA	FITC	BD	559354	intracellular	1:200
Immunohisto-chemistry Lung					
GL-7	FITS	BD	3036700		1:50
IgD	PE	eBioscience	E02008-1634		1:50



Additional file 1. No changes of proportions of total B-cells between controls and IPF patients.
Flow cytometric quantification of total B-cells (CD19$^+$) in blood, lungs and lymph nodes (LN) as percentage of alive cells. For blood samples data also depicted as absolute number of B-cells per ml blood. Data are expressed as mean and dots represent individual values.

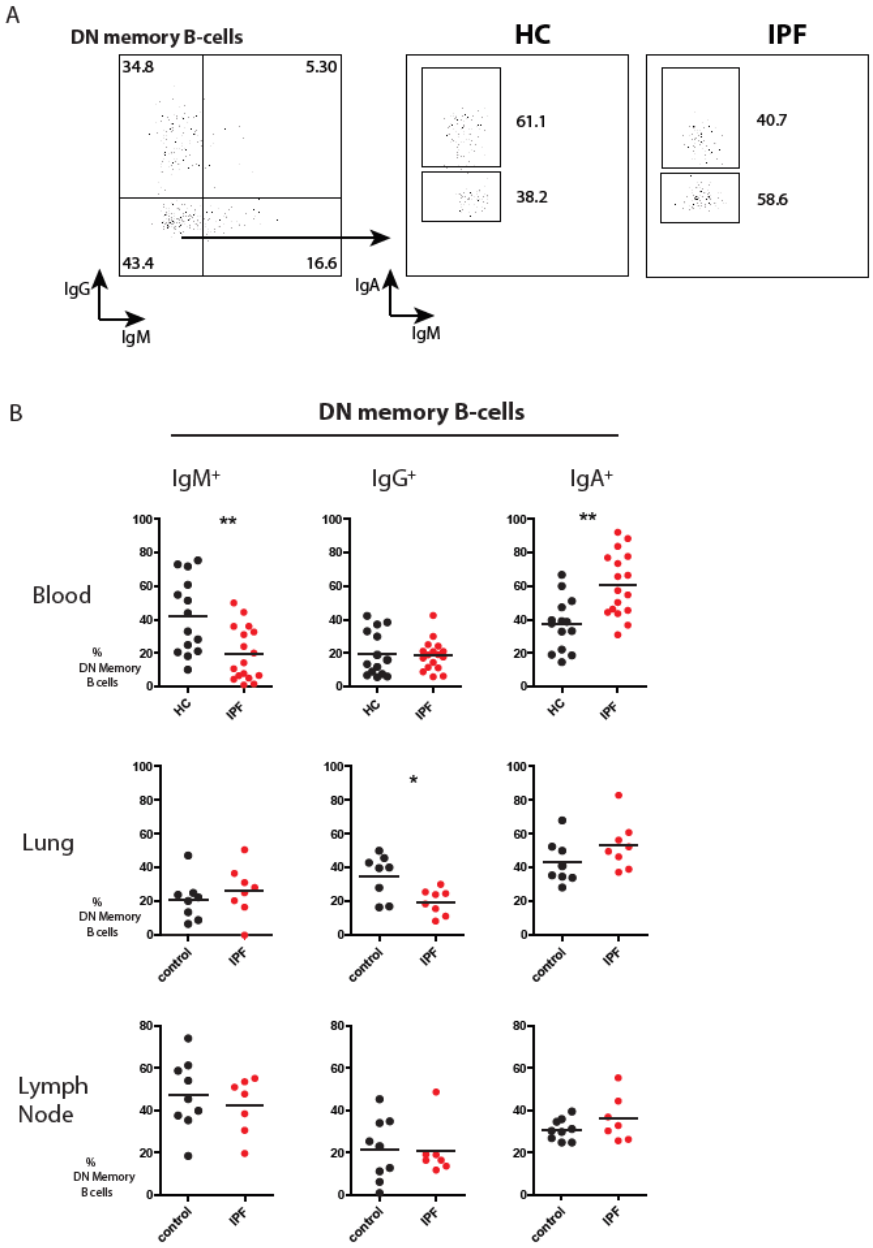


Additional file 2. No alterations in B-cell subsets between control lymph nodes (LN) and IPF LN.
Flow cytometric quantification of naïve B-cells ($CD19^+IgD^+CD27^-$) IgD^+ and IgD^- memory $CD27^+$ B-cells, double negative (DN) B-cells ($CD19^+IgD^-CD27^-$), transitional B-cells ($CD19^+CD24^+CD38^+$), and plasmablast ($CD19^+CD38^+CD27^+$) as percentage of alive cells. Data are expressed as mean and dots represent individual values.

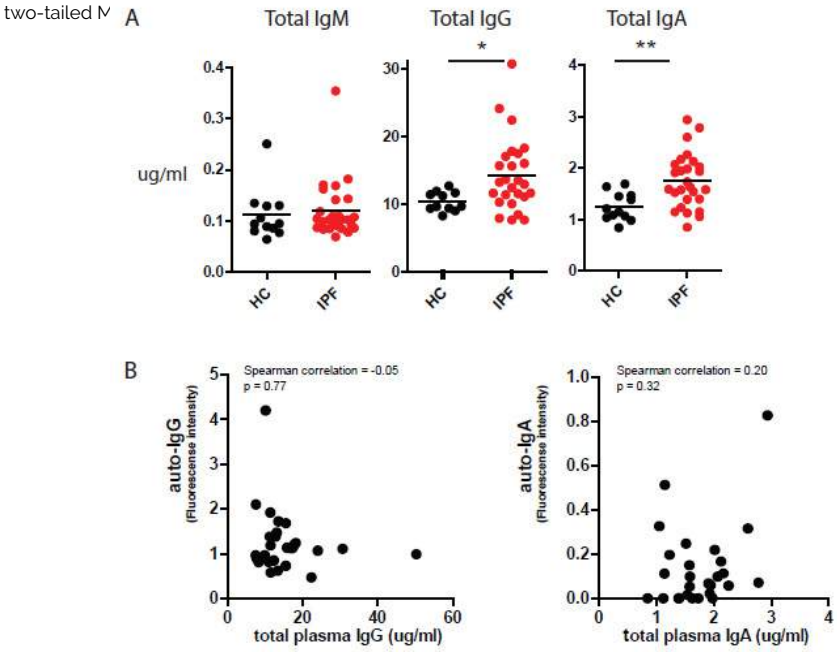


Additional file 3. Augmented proportions of IgA-expressing CD27⁺ memory B-cell subsets.
(A) Representative gating strategy for the identification of surface IgA expression on $CD27^{+}IgD^{-}$ memory B-cells. The $CD27^{+}IgD^{-}IgM^{-}IgG^{-}$ memory cells are enriched for IgA^{+} surface expression as depicted for a healthy control (HC) and IPF patient. (B) Flow cytometric analysis of the distribution of surface expression of IgD, IgM, IgG and IgA on $CD27^{+}$ memory B-cells for blood, lungs and lymph nodes for controls and IPF patients. Non-parametric two-tailed Mann-

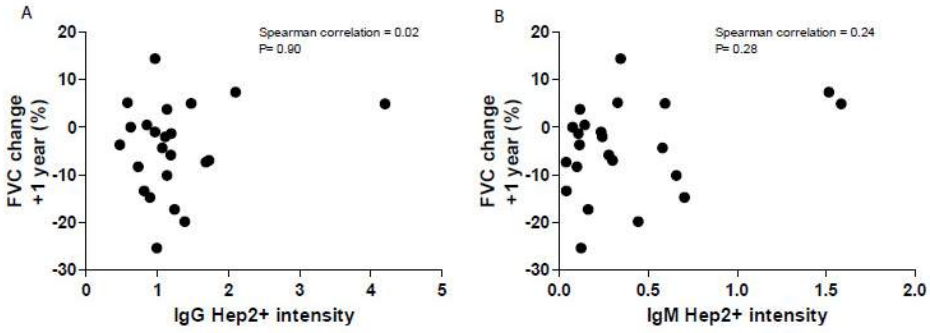
Whitney test was used. ** P < 0.01 *** P < 0.001



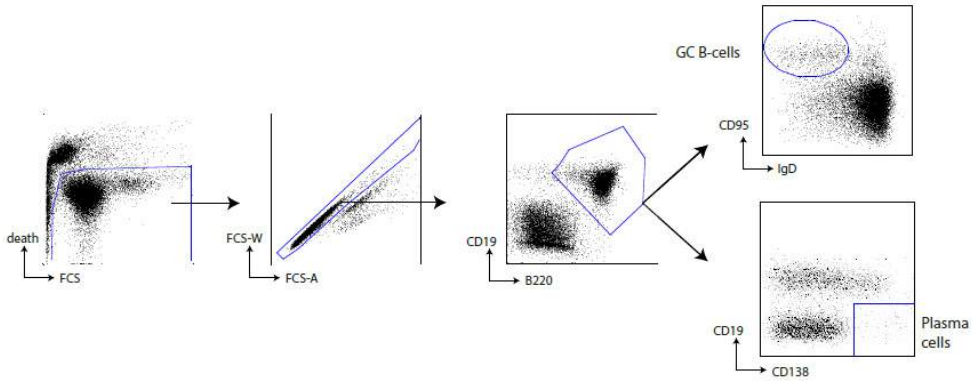
Additional file 4. Augmented proportions of IgA-expressing DN memory B-cell subsets.
(A) Representative gating strategy for the identification of surface IgA expression on double negative (DN) memory B-cells ($CD19^+CD27^+IgD^-$). The IgM^-IgG^- DN memory cells are enriched for IgA^+ surface expression as depicted for a healthy control (HC) and IPF patient. (B) Flow cytometric analysis of the distribution of surface expression of IgM, IgG and IgA on DN memory B-cells for blood, lungs and lymph nodes for controls and IPF patients. Non-parametric



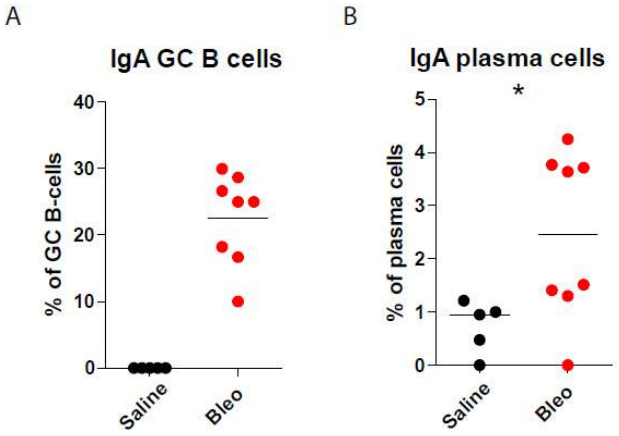
Additional file 5. Increased total IgG and IgA in plasma of IPF patients
(A) total plasma IgM, IgG and IgA levels (μg/ml) for HC and IPF patients. (B) Correlation between total IgG or IgA (μg/ml) and autoreactive IgG or IgA. Indirect quantification of auto-reactive immunoglobulin levels depicted as fluorescence intensity measured on HEp-2 slides. Non-parametric two-tailed Mann-Whitney test was used. Correlation coefficients were calculated using Spearman's rank method. Data are expressed as mean and dots represent individual patient values. * $P < 0.05$ ** $P < 0.01$



Additional file 6. Autoreactive IgG and IgM does not correlate with disease progression.
fluorescence intensity for plasma autoreactive IgG (A) and IgM (B) does not correlate with decline in forced vital capacity (FVC) over 1-year period in IPF patients. Correlation coefficients were calculated using Spearman's rank method.



Additional file 7. Gating strategy for B-cell subsets in mice.
Representative gating strategy used for mice experiments for the identification of GC B-cells ($CD19^+CD95^+IgD^{low}$), IgA GC B-cells ($CD19^+CD95^+IgD^{low}IgA^+$), plasma cells ($CD19^{low}CD138^+$) and IgA$^+$ plasma cells ($CD19^{low}CD138^+IgA^+$).



Additional file 8. Increased proportions of IgA$^+$ GC B-cells and IgA$^+$ plasma cells in lungs in mice exposed to saline and bleomycin.
(A) Flow cytometric quantification of IgA$^+$ GC B-cells ($CD19^+CD95^+IgD^{low}IgA^+$) as percentage of GC B-cells and (B) IgA$^+$ plasma cells ($CD19^{low}CD138^+IgA^+$) as percentage of plasma cells in lungs of mice exposed to saline or bleomycin. Data are expressed as mean and dots represent individual patient values. Nonparametric two-tailed Mann-Whitney test was used. * $P < 0.05$

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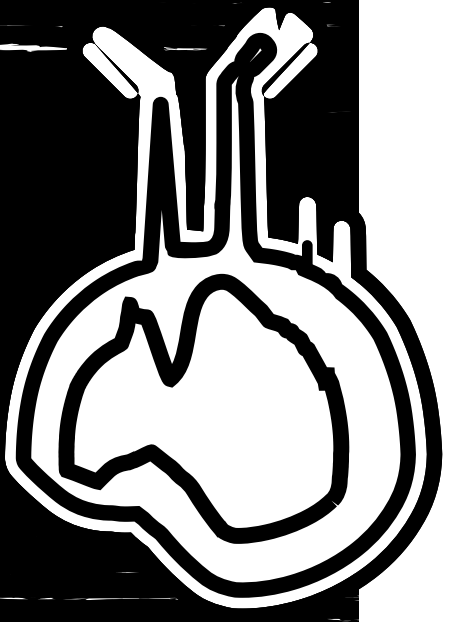

CHAPTER 6

Aberrant B Cell Receptor Signaling in Naïve B Cells from Patients with Idiopathic Pulmonary Fibrosis

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** Shared senior authorship

Abstract:

Idiopathic pulmonary fibrosis (IPF) is a chronic and ultimately fatal disease in which an impaired healing response to recurrent micro-injuries is thought to lead to fibrosis. Recent findings hint at a role for B cells and autoimmunity in IPF pathogenesis. We previously reported that circulating B cells from a fraction of patients, compared with healthy controls, express increased levels of the signaling molecule Bruton's tyrosine kinase (BTK). However, it remains unclear whether B cell receptor (BCR) signaling is altered in IPF. Here, we show that the response to BCR stimulation is enhanced in peripheral blood B cells from treatment-naïve IPF patients. We observed increased anti-immunoglobulin-induced phosphorylation of BTK and its substrate phospholipase Cg2 (PLCg2) in naïve but not in memory B cells of patients with IPF. In naïve B cells of IPF patients enhanced BCR signaling correlated with surface expression of transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) but not B cell activating factor receptor (BAFFR), both of which provide pro-survival signals. Interestingly, treatment of IPF patients with nintedanib, a tyrosine kinase inhibitor with anti-fibrotic and anti-inflammatory activity, induced substantial changes in BCR signaling. These findings support the involvement of B cells in IPF pathogenesis and suggest that targeting BCR signaling has potential value as a treatment option.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and irreversible interstitial lung disease (ILD) of unknown cause, characterized by an imaging and pathological pattern of interstitial pneumonia [1]. IPF has been associated with various risk factors, including cigarette smoking, pulmonary microbiome, and genetic predisposition. The disease is thought to develop slowly with a dysregulation in the response to subclinical recurrent pulmonary micro-injuries, leading to abnormal tissue remodeling [2]. IPF has a poor median survival of 3–5 years following diagnosis. Because effective therapies are limited, unraveling of the disease pathogenesis is required to explore novel treatment options that slow disease progression or that may even prevent disease development.

Currently, two treatment options for IPF patients are recommended, nintedanib and pirfenidone, both of which modulate fibrosis. Nintedanib is a tyrosine kinase inhibitor that inhibits signaling downstream of pro-fibrotic receptors, such as fibroblastic and vascular endothelial growth factor receptors (FGFR and VEGFR) [3]. The exact mechanism by which pirfenidone exerts its clinical effect is not completely understood, but it is known to have the capacity to modulate both pro-fibrotic and pro-inflammatory factors, such as transforming growth factor (TGF)-b, tumor necrosis factor (TNF)-a, and interleukin (IL)-6 [4,5]. Although these therapies delay the decrease in lung function and improve survival, better therapies that control disease progression are in high demand.

An increasing body of evidence suggests that B cells and autoimmunity play a role in the pathogenesis of IPF [6]. The lungs of IPF patients often contain lymphocytic aggregates that co-localize with fibrotic areas [7–9]. Levels of the B cell chemoattractant C-X-C motif ligand 13 (CXCL13) are increased both locally in the lungs and systemically in the serum of IPF patients compared with healthy controls (HCs), and are predictive of survival [10,11]. Similarly, B cell activating factor (BAFF) and immunoglobulin (Ig)A levels are increased in the serum of IPF patients and are similarly predictive of survival [12,13]. Several studies have shown the presence of autoreactive antibodies of various specificities in the serum of IPF patients, especially during acute exacerbations (AE) of the disease [14]. Concordantly, beneficial effects of B cell-targeting therapies in AE-IPF patients have been suggested, such as plasma exchange in combination with rituximab or intravenous Ig [15,16]. The involvement of B cells in IPF pathogenesis is further supported by the observation of a significant increase in activated follicular T helper (Tfh) cells both in the circulation and locally in the lungs of IPF patients [17,18].

Bruton's tyrosine kinase (BTK) is best known for its role in signal transduction downstream of the B cell receptor (BCR) [19]. We recently identified increased levels of BTK in naïve B cells as an overarching characteristic of systemic autoimmune diseases, including rheumatoid arthritis (RA), primary Sjögren's syndrome (pSS), and granulomatosis with polyangiitis (GPA) [20,21]. In addition, we showed that B cell-specific overexpression of human BTK under the control of the CD19 promotor in CD19-hBtk transgenic mice leads to the development of a T cell-dependent systemic autoimmune phenotype [22,23]. Moreover, proteome profiling of IPF peripheral blood indicated highly increased BTK expression compared with HCs [24].

In line with these results, we observed that in a major fraction of IPF patients, BTK protein expression in resting peripheral blood B cells was increased compared with HCs [18]. Given that BTK is also phosphorylated downstream of many other receptors in B cells [19], it remains to be determined whether the increased BTK protein expression in IPF is linked to a general enhancement of BCR signaling. It is then conceivable that BTK activity is not only enhanced in resting B cells, but also upon BCR engagement. This may be a reflection of an altered activity of various upstream molecules, including spleen tyrosine kinase (SYK), which upon activation can subsequently phosphorylate and activate BTK. Moreover, it is unknown whether increased BTK protein expression in resting B cells translates into increased downstream signaling, involving phosphorylation of its primary substrate, phospholipase Cg2 (PLCg2), or activation of the phosphoinositide-3-kinase (PI3K)-AKT-mTOR (mammalian target of rapamycin) pathway.

It has been shown that survival of naïve B cells critically depends on low-level—so-called "tonic"—BCR signals in the absence of antigen engagement and pro-survival signals from the BAFF receptor (BAFFR). Hereby, the latter was demonstrated to transduce survival signals by crosstalk with the BCR, involving SYK and BTK [25,26]. It is therefore possible that the increase in BAFF levels in IPF patients is linked to aberrant BTK levels and activity, and hence BCR signaling, in naïve B cells. To address these questions, in this report we aim to investigate BCR signaling, particularly focusing on SYK, BTK, and PLCg2 activity, in peripheral blood B cells from untreated and nintedanib-treated IPF patients, as well as the connection with circulating BAFF.

Materials and Methods

Patient Characteristics and Study Design

IPF was multidisciplinarily diagnosed and managed based on current ALAT/ATS/ERS/JRS guidelines [27,28]. Cohort 1 consisted of 16 treatment-naïve IPF patients (Table 1), who were compared with 14 HCs (age: 67.1 ± 5.2; m/f: 6/8; for flow cytometry analysis of circulating B cells), and of which 12 patients were compared with 7 HCs (age: 54.2 ± 4.8; m/f: 3/4; for analysis of circulating cytokines in plasma). Peripheral blood samples from these treatment-naïve patients were collected at the time of diagnosis. In a separate cohort (cohort 2), consisting of 12 IPF patients who started nintedanib treatment, peripheral blood samples were collected at baseline (in this case, not at the time of diagnosis) and at follow-up (~1.7 ± 1.0 months) (Table 1). Ten of these twelve patients had a history of therapy prior to the start of nintedanib treatment. Sixteen HCs (age: 59.8 ± 8.0; m/f: 10/6) were used as a control group for cohort 2. HCs were selected as individuals without a history of any systemic, immunological, or pulmonary condition.

Table 1. IPF patient characteristics.

	IPF Cohort 1	IPF Cohort 2
subjects, n	16	12
age (years), mean (±SD)	68.5 (±7.2)	69.1 (±7.7)
male sex, n	10 (63%)	9 (75%)
time to diagnosis (years), mean (min–max)	3.1 (0.1–12.0)	1.8 (0.2–7.0)
CT diagnosis, definite UIP/probable UIP/inconsistent *	8/6/2	5/6/1
smoking, active/previous/never	0/15/1	1/11/0
PY (years), mean (min–max) *	23.0 (0–80)	24.8 (1–60)
FVC (L), mean (±SD) *	2.8 (±0.8)	2.5 (±1.0)
FVC % predicted, mean (±SD)	80.1 (±14.1)	75.3 (±22.9)
Tiffeneau index, mean (±SD)	82.9 (±5.4)	77.3 (±11.2)
prednisone, n	0	0
nintedanib, n	0	0
pirfenidone, n	0	10 (83%)
time between last intake of medication and starting nintedanib (weeks), mean (±SD)	-	2.3 (±2.1)

* CT, computed tomography; UIP, usual interstitial pneumonia; PY, pack years; FVC, forced vital capacity.

Peripheral blood was drawn by venous phlebotomy and collected in EDTA microtubes (BD Biosciences, San Jose, CA, USA) after informed consent. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated using Ficoll-Paque™ (GE Healthcare, Chicago, IL, USA) density gradient centrifugation and stored at –196 ˚C and –80 ˚C, respectively, according to standard protocols. This study was approved by the medical ethics committee of the Erasmus Medical Center Rotterdam (METC 2012/512) and complies with the Declaration of Helsinki.

Flow Cytometric Analysis

Flow Cytometry for B Cell Surface Markers and Intracellular BTK

Frozen PBMCs were thawed and resuspended in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 5% fetal calf serum (Gibco; referred to as medium). A total of 5.0×10^5 cells were plated in 96-well round-bottom plates and washed with MACS buffer (0.5% BSA, 2 mM EDTA in PBS). Binding of antibodies to Fc-receptors was prevented by prior incubation with TruStain FcX (Biolegend, San Diego, CA, USA) for 5 min at 4 ˚C. Cells were subsequently stained for surface markers with a combination of conjugated antibodies in MACS buffer for 60 min at 4 ˚C. After washing, cells were stained with Fixable Viability Dye (Invitrogen, Thermo Fisher Scientific) as live/dead marker, and fluorescently labeled streptavidin for 20 min at 4 ˚C to detect biotinylated antibodies. Cells were fixed and permeabilized with 2% paraformaldehyde and 0.5% saponin (Sigma-Aldrich, St. Louis, MO, USA), respectively, or using Cytofix/Cytoperm (BD Biosciences). Subsequent

intracellular BTK staining was performed for 60 min at 4 °C, as previously described [22]. The complete staining panel is provided in Table S1. Cells were resuspended in MACS buffer and measured on an LSR II flow cytometer (BD Biosciences).

BCR Signaling Measurement by Phosphoflow Cytometry

Analysis of phosphorylated signaling proteins was performed by intracellular phosphoflow cytometry (phosphoflow) using an optimized protocol that allows for analysis on cryopreserved materials, essentially as described previously [29,30]. For both cohorts, samples from IPF patients and HCs were measured in single experiments to prevent inter-experimental variation. Briefly, PBMCs were thawed in medium; 3.0×10^5 cells were plated per well in a 96-well round bottom plate and were brought to 37 °C. Fixable Viability Dye (Invitrogen) was added 10 min before fixation as a live/dead marker. Cells were either stimulated with 20 µg/mL goat $F(ab')_2$ anti-human Ig (a-Ig) (Southern Biotech, Birmingham, AL, USA) in medium or left unstimulated by adding plain medium. For optimal detection of phosphorylation, cells were stimulated either for 5 min (phosphorylated (p) BTK and pPLCg2), for 2 min (pSYK), or for 1 min (pPI3K p85) at 37 °C. The stimulation was terminated by fixation with the eBioscienceä Foxp3/Transcription Factor Staining Fixation/ Permeabilization Buffer (Invitrogen) for 10 min at 37 °C. Cells were then stained intracellularly for markers to define B cell subpopulations, for 30 min at 4 °C in Permeabilization Buffer (Invitrogen). pSYK Y348-PE, pBTK Y223-AF647, and pPLCg2 Y759-AF647 were stained in Permeabilization Buffer for 30 min at room temperature (RT). pPI3K p85 Y458 was stained in Permeabilization Buffer for 15 min at RT, after which cells were washed and incubated with donkey anti-rabbit-PE (Jackson ImmunoResearch, Ely, UK; for complete staining panel, see Table S2). Cells were resuspended in MACS buffer and measured on an LSR II flow cytometer. Flow cytometric data were analyzed with FlowJo v10 (BD Biosciences), and the geometric mean fluorescent intensity (gMFI) was determined for quantification of signal intensities.

Cytokine Detection by Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed to determine plasma levels of BAFF (R&D Systems, Minneapolis, MN, USA) and transforming growth factor (TGF)-b1 (Invitrogen) according to the manufacturer's instructions. Optical density (OD) values were measured at 450 nm and subtracted from 570 nm background, using a SpectraMax plate reader (Molecular Devices, San Jose, CA, USA). Samples were measured in duplicate, blanked against untreated wells, and concentrations were calculated by averaging the duplicate OD values and subsequent interpolation from the standard curve.

Statistical and Computational Analysis

Graphs were created and statistics were calculated using GraphPad Prism 9 software (GraphPad Prism Inc, San Diego, CA, USA.). A Shapiro–Wilk test was performed to test for Gaussian distribution within each group. If the compared groups showed a Gaussian distribution, an unpaired or paired Student's *t*-test was performed. In the case of a

nonparametric distribution, a Mann–Whitney *U* test was performed. For correlation analysis, a Pearson correlation coefficient (*r*; in the case of a normal distribution) or a Spearman's rank correlation coefficient (r; in the case of a nonparametric distribution) was calculated. Survival Kaplan–Meier curves were analyzed using a log rank test for statistical significance. A p-value < 0.05 was considered statistically significant.

Principal component (PC) analysis (PCA) was performed using R version 3.6.1 and RStudio version 1.2.5001. PCA plots were built and the relative contribution of each variable to the PCs was calculated using the *FactoMineR* and *factoextra* package [31].

Results

Naïve B Cells from IPF Patients Show Aberrant BCR Signaling upon Stimulation

To study BCR responsiveness, total PBMCs from HCs and treatment-naïve IPF patients were left unstimulated or were stimulated with a-Ig. Using phosphoflow cytometry, the phosphorylation status of several signaling molecules was determined in different B cell subsets (gating strategy in Figure S1A). In these experiments, B cells from both IPF patients and HCs showed a robust response to BCR stimulation, as for all phosphorylated proteins tested, we found a significant difference in signal between unstimulated and a-Ig stimulated B cells (data not shown). Given the previously found increased BTK expression in B cells in a major fraction of IPF patients [18], we first determined BTK phosphorylation status. In unstimulated B cells, pBTK (Y223) levels were similar between HCs and IPF patients (Figure 1A). However, following stimulation of the BCR, B cells from IPF patients showed enhanced BTK phosphorylation compared with HCs. When this was analyzed separately for different B cell subpopulations, only a-Ig-stimulated naïve—and not memory—B cells showed increased BTK phosphorylation compared with a-Ig-stimulated naïve and memory B cells from HCs (Figure 1A). Notably, the proportion of naïve B cells unresponsive to BCR stimulation, as inferred by the pBTK-low population following a-Ig stimulation, was significantly lower in IPF patients than in HCs (~20.2% versus ~40.7%, respectively) (Figure 1A). Within the pBTK-high population, the signal intensity in naïve B cells was also greater in IPF patients than in HCs (Figure S1B). The enhanced pBTK signal in a-Ig-stimulated naïve B cells from IPF patients, as compared with HCs, can therefore be attributed to both a substantial reduction in the fraction of unresponsive B cells and enhanced phosphorylation within the responding naïve B cell population.

Next, phosphorylation of BCR signaling molecules upstream of BTK, SYK (Y348), and directly downstream of BTK, PLCg2 (Y759), was measured. pSYK (Figure 1B) and pPLCg2 (Figure 1C) levels were comparable in unstimulated B cells from IPF patients and HCs. Like BTK, phosphorylation of SYK and PLCg2 was increased in naïve but not in memory IPF B cells compared with HCs upon a-Ig stimulation, although significance was only reached for pPLCg2. The proportion of pPLCg2-low naïve B cells after stimulation was lower in IPF patients than in HCs (~26.2% versus ~41.9%, respectively) (Figure 1C). When selecting for

pSYK- and pPLCg2-high naïve B cells, no difference was found in signal intensity between IPF patients and HCs (Figure S1C, D). Thus, for pSYK and pPLCg2, the decreased fraction of unresponsive naïve B cells was responsible for the increase in overall phosphorylation signals in IPF patients.

The enhanced BCR responsiveness was not due to an increased expression of BCRs, as surface IgD and IgM expression was similar between HC and IPF naïve B cells (Figure S1E). No differences were found between IPF patients and HCs in a-Ig-induced phosphorylation of BTK, SYK, or PLCg2 in the individual memory B cell subsets, such as IgD$^+$, IgM$^+$, or class-switched cells (data not shown).

Together, these data indicate that, similar to patients with a systemic autoimmune disease [21], naïve B cells from IPF patients display aberrant BCR signaling following stimulation.

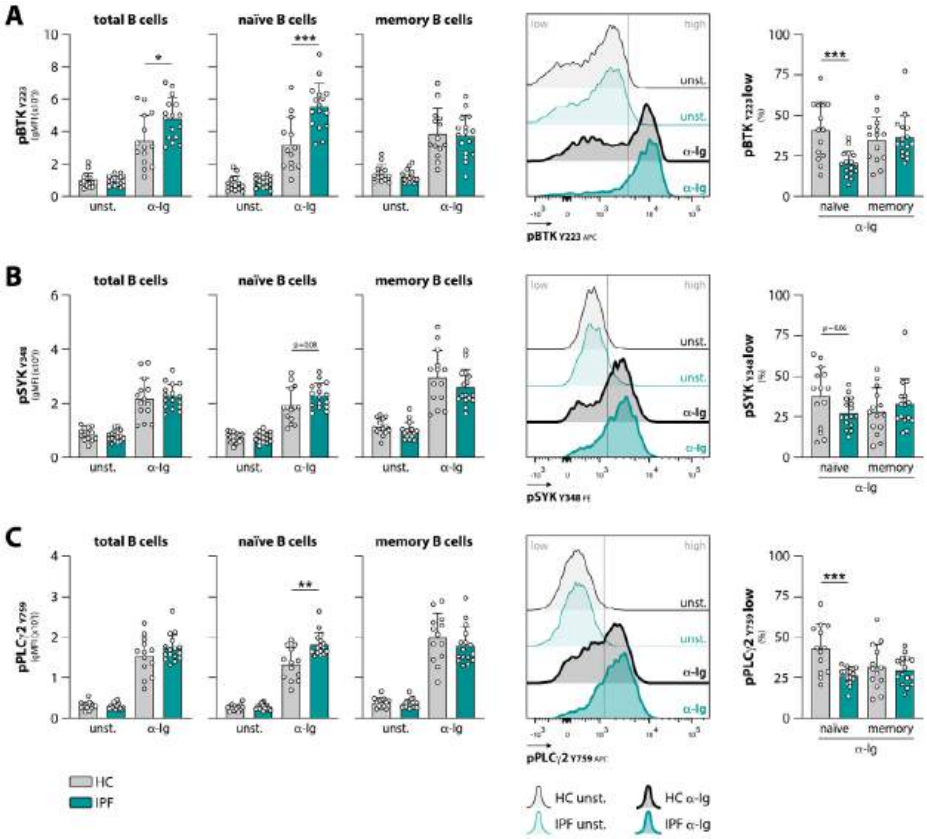

Figure 1. Phosphoflow analysis vertical line indicating the gate for selecting the population low/high in phosphorylation following α-Ig stimulation (*middle panel*). Proportions of (**A**) pBTK-low, (**B**) pSYK-low, and (**C**) pPLCγ2-low cells, expressed as percentage of total naïve or memory B cell fraction (*right panel*). Subjects are indicated by individual data points, and bars indicate mean values + SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in (**A**, **B**, and **C** (right panel)) by an unpaired two-tailed Student's *t*-test or (in **C** (left panel)) by a Mann–Whitney *U*.

Enhanced BCR Signaling in Naïve B Cells from IPF Patients Correlates with TACI Expression

To assess whether the increase in BCR signaling found in naïve B cells from IPF patients was associated with an altered B cell phenotype, we measured intracellular BTK protein, surface activation marker CD86, BAFFR, and transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) expression in different B cell subsets (gating strategy in Figure S1F). We did not detect an altered expression of these markers in IPF patient compared with HC B cells (Figure S2). Principal component analysis (PCA) of the BCR signaling molecules and these B cell markers measured in naïve B cells, however, indicated that IPF patients and HCs significantly separated on principal component 1 (PC1) (Figure 2A). This component predominantly comprised the phosphorylation status of BCR signaling molecules and the associated proportions of phosphorylation-low B cells following stimulation (Figure 2B). Hereby, the contribution of signaling molecule phosphorylation of unstimulated and a-Ig-stimulated naïve B cells to PC1 appeared to be similar. Expression levels of BTK and CD86, the most important contributors to PC2, did not significantly differentiate between HCs and IPF patients.

Next, we created a correlation matrix for the phosphorylation status of BCR signaling molecules measured in vitro in unstimulated and stimulated conditions, and the expression levels of B cell surface and intracellular markers ex vivo. We found that in HCs and IPF patients, phosphorylation of the signaling molecules within the BCR signalosome (BTK, SYK, and PLCg2) strongly correlated with one another, in unstimulated conditions and following BCR stimulation in naïve B cells (Figure 2C, D), as well as in unstimulated memory B cells (Figure S3). For both IPF patients and HCs, the correlation between pBTK and pPLCg2 is shown as an example (Figure 2E, F). A positive correlation was found for unstimulated and stimulated naïve B cells from HCs between BCR signalosome phosphorylation and ex vivo BAFFR surface expression levels (Figure 2C, E; pPLCg2 used as an example). This reached significance for pSYK and pPLCg2 in stimulated cells. In contrast, in both unstimulated and stimulated naïve B cells from IPF patients, this correlation between BCR signalosome phosphorylation and ex vivo BAFFR surface levels was not found but had apparently shifted towards TACI surface expression (Figure 2D, F; pPLCg2 used as an example). In the memory B cell compartment of HCs and IPF patients, we observed a similar phenomenon (Figure S3). Despite the absence of detectable differences in the mean BTK protein expression levels in B cells from HCs and the IPF patients within this cohort (Figure S2A), the BCR signalosome of unstimulated IPF B cells strongly correlated with BTK expression, while this correlation was absent in HCs (Figure 2C, D).

Taken together, these data show that in naïve B cells from IPF patients, BCR signaling is dysregulated and, in contrast to HCs, does not correlate with BAFFR but instead with TACI surface expression levels.

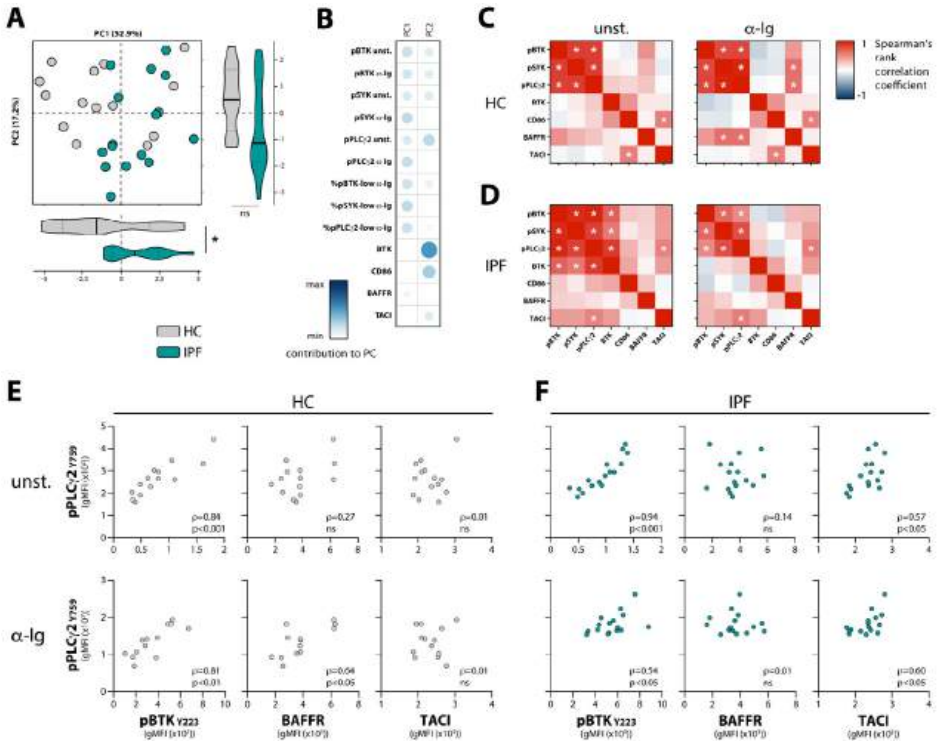

Figure 2. Correlation analysis of ex vivo measured B cell markers and in vitro measured phosphorylation of BCR signalosome molecules in naïve ($CD3^-CD19^+CD38^{lo}CD27^-IgD^+$) B cells from HCs and IPF patients (cohort 1). (**A**) Principal component analysis (PCA) of BCR signalosome molecules and B cell activation markers and (**B**) the individual contribution of each variable to PC1 and PC2 from IPF and HC naïve B cells. (**C**, **D**) Spearman's rank correlation matrix for (**C**) HCs and (**D**) IPF patients for the indicated markers measured ex vivo and phosphorylated proteins of unstimulated (unst.) and BCR-stimulated (α-Ig) naïve B cells. (**E**, **F**) Spearman's rank correlation analysis of (**E**) HCs and (**F**) IPF patients for the correlation of pPLCγ2 with pBTK, BAFFR, and TACI in unstimulated (unst.) and BCR-stimulated (α-Ig) naïve B cells. Subjects are indicated by individual data points. * $p < 0.05$ in (**A**) by an unpaired two-tailed Student's *t*-test; ns, not significant.

Negative Correlations between Circulating BAFF and BAFFR Expression, and between Circulating TGF-b and Phosphorylation of BCR Signalosome Molecules Following Stimulation

As a pleiotropic cytokine, TGF-b plays an important role in stimulating fibrosis, though it is also known for its regulatory effects on lymphocytes [32]. We observed a trend towards increased circulating levels of TGF-b in IPF patients compared with HCs (Figure 3A). Circulating TGF-b levels showed a negative correlation with phosphorylation of BTK following BCR stimulation, specifically in naïve B cells (Figure S4A). The same negative trend was observed for the other BCR signalosome molecules, SYK and PLCg2, but these were not significant (data not shown).

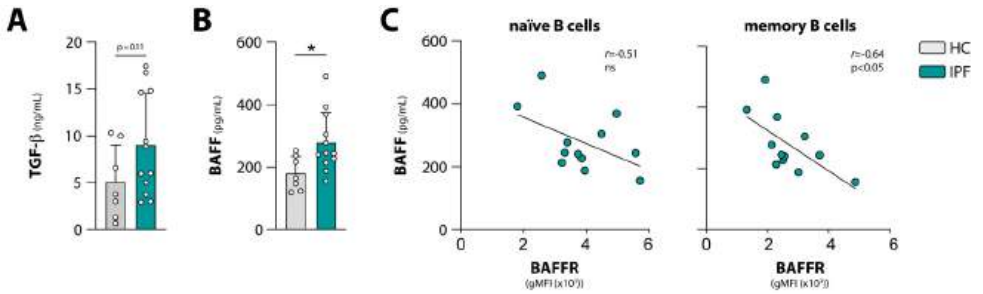

Figure 3. Analysis of circulating BAFF and TGF-β levels in HC and IPF patients (subset of cohort 1). (**A**) TGF-β levels in plasma of HCs and IPF patients measured by ELISA. (**B**) BAFF levels in plasma of HCs and IPF patients measured by ELISA. (**C**) Pearson correlation analysis between BAFF levels in plasma and BAFFR surface expression on naïve ($CD3^-CD19^+CD38^{lo}CD27^-IgD^+$) and memory ($CD3^-CD19^+CD38^{lo}CD27^+$) B cells in IPF patients. Subjects are indicated by individual data points, and bars indicate mean values + SD. * $p < 0.05$ in (**B**) by an unpaired two-tailed Student's *t*-test.

As a ligand for BAFFR and TACI, BAFF plays a crucial rule in B cell survival and thus in peripheral selection of autoreactive B cells and the maintenance of the mature B cell pool [33]. Because the BAFF–BAFFR axis and BCR signaling are intertwined and positively regulate one another [25,34], we hypothesized that in IPF patients, BAFFR and TACI expression on B cells and the altered BCR signaling were associated with increased circulating BAFF levels. In line with reported findings [12], circulating BAFF levels were significantly increased in IPF patients compared with HCs (Figure 3B). A negative correlation was observed between circulating BAFF levels and BAFFR surface expression on B cells, which reached significance for the memory B cell compartment (Figure 3C). We could not detect significant correlations between circulating BAFF levels and BTK expression, TACI expression, or phosphorylation of BCR signaling molecules in B cells from IPF patients (Figure S4B, and data not shown).

We observed reduced survival in patients with high BAFF levels, compared with patients with low BAFF levels, as previously reported [12] (Figure S5A). Conversely, patients with low BAFFR expression on naïve B cells tended to have reduced survival compared with patients with high BAFFR expression (Figure S5B). No association was observed between BTK expression, TACI expression, or the phosphorylation of the BCR signalosome molecules, and survival (data not shown).

In summary, our data indicate that in IPF patients, increased circulating BAFF levels correlate with reduced BAFFR expression and that increased circulating TGF-b levels are associated with decreased BCR signaling.

Nintedanib Treatment Induces Substantial Changes in BCR Signaling in Naïve and Memory B Cells

Nintedanib is a receptor tyrosine kinase inhibitor which suppresses downstream signaling of FGFR and VEGFR, thereby dampening pro-fibrotic processes in IPF patients [35]. However, non-receptor tyrosine kinases are also predicted targets of nintedanib, such as the Src family of kinases including lymphocyte-specific protein tyrosine kinase (LCK), tyrosine-protein kinase (LYN), and proto-oncogene c-Src (SRC) [36]. More recently, BTK was also added to that list of predicted targets [37]. Because these molecules are crucial in BCR signaling, we hypothesized that BCR signaling in B cells from IPF patients may be affected by nintedanib treatment. To that end, we investigated BCR signaling in a second cohort of IPF patients. We analyzed peripheral blood samples from patients just before and on average 1.7 months after the start of nintedanib treatment. In this cohort, the same BCR signalosome molecules were investigated as in the first cohort, with the addition of PI3K p85 (Y458). We found that after the start of nintedanib treatment, mean pBTK levels significantly increased in a-Ig-stimulated naïve B cells—but not in a-Ig-stimulated memory B cells—from IPF patients (Figure 4A). The average levels of upstream pSYK and pPI3K p85, and downstream pPLCg2 in both unstimulated and a-Ig stimulated conditions were not significantly altered following treatment (Figure 4B-D). When these IPF patients, analyzed before and after the start of treatment, were compared with a HC cohort, no significant differences in the phosphorylation of these four signaling molecules were observed (data not shown).

Interestingly, however, a considerable trend was observed between paired samples following a-Ig stimulation: patients that showed high phosphorylation of the BCR signalosome molecules before treatment, showed low phosphorylation after the start of nintedanib treatment, and vice versa. This phenomenon was observed in both naïve and memory B cells and was reflected by strong negative correlations when comparing B cells from patients before and after the start of nintedanib treatment (shown for pSYK and pPLCg2 in naïve B cells in Figure 4E). However, pBTK did not follow this pattern (Figure 4F). Taken together, these results indicate that in IPF patients, BCR signaling in peripheral blood naïve and memory B cells is markedly affected by nintedanib treatment.

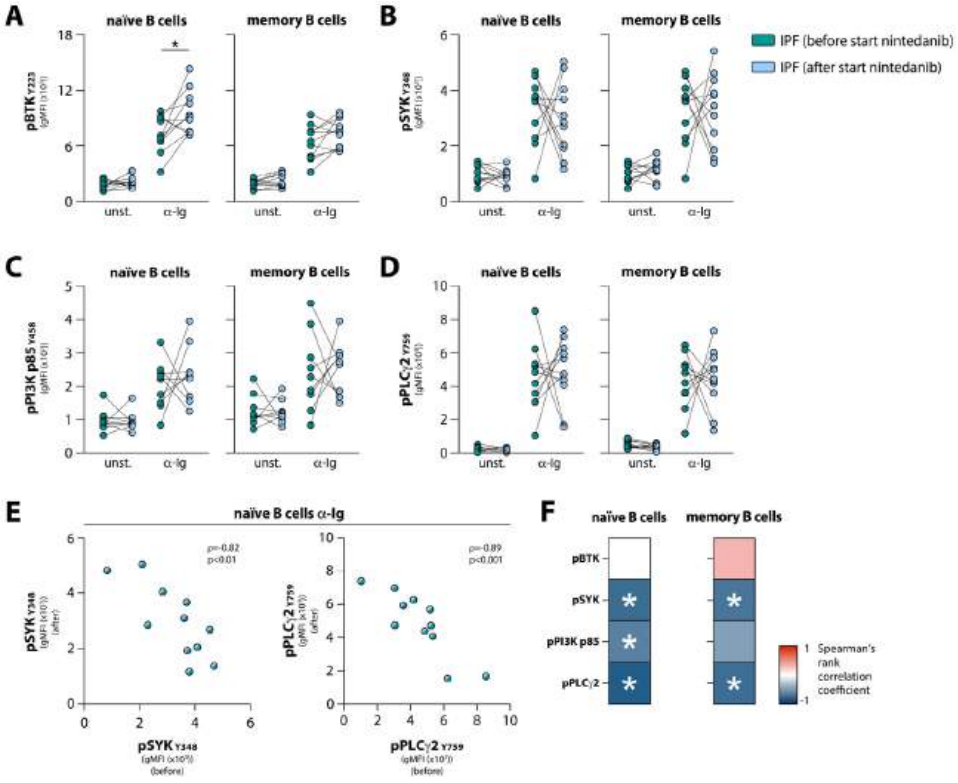

Figure 4. Phosphoflow analysis of BCR downstream signaling in nintedanib-treated IPF patients' peripheral blood B cells (cohort 2). (**A**–**D**) Phosphorylation levels in unstimulated (unst.) conditions and after α-Ig stimulation for (**A**) pBTK $_{Y223}$, (**B**) pSYK $_{Y348}$, (**C**) pPI3K p85 $_{Y458}$, and (**D**) pPLCγ2 $_{Y759}$ in naïve ($CD3^-CD19^+CD38^{lo}CD27^-IgD^+$) and memory ($CD3^-CD19^+CD38^{lo}CD27^+$) B cells from IPF patients before and after the start of nintedanib treatment. (**E**, **F**) Spearman's rank correlation analysis of the phosphorylation levels for (**E**) pSYK (*left*) and pPLCγ2 (*right*) in naïve B cells following BCR stimulation, before versus after the start of nintedanib treatment. (**F**) Spearman's rank correlation matrix for phosphorylation of BCR signalosome molecules before and after the start of nintedanib treatment. Subjects are indicated by individual data points. * $p < 0.05$ in (**A**) by a paired two-tailed Student's *t*-test.

Discussion

Evidence has been accumulating that B cells and autoimmunity are involved in the pathogenesis of IPF. In this study, we used phosphoflow cytometry to distinguish different B cell subsets and to simultaneously analyze phosphorylation of several critical downstream BCR signaling molecules in peripheral blood B cells from two cohorts of IPF patients and healthy controls. We found that naïve B cells, but not memory B cells, from treatment-naïve IPF patients displayed increased phosphorylation of the BCR signaling molecules SYK, BTK, and PLCg2 following BCR stimulation in vitro. This enhanced BCR signaling correlated with surface expression levels of TACI but not with BAFFR, both of which bind BAFF and therefore provide pro-survival signals to B cells. Remarkably, treatment of IPF

patients with the anti-fibrotic tyrosine kinase inhibitor nintedanib induced major changes in BCR signaling. Taken together, our findings do not only support the involvement of B cells in the pathogenesis of IPF, but also suggest that targeting BCR signaling might contribute to the therapeutic effect of nintedanib.

Developing B cells undergo selection on the basis of BCR reactivity and signaling strength, preventing the generation and activation of autoreactive B cells [33,38]. Within the circulating naïve B cell pool, autoreactive naïve B cells are kept in check by a decreased or even lack of response to BCR stimulation, referred to as B cell anergy [39]. In this context, it is notable that our phosphoflow analysis showed that the enhanced overall phosphorylation signals following BCR stimulation in the naïve B cell population from IPF patients was due to a reduced percentage of naïve B cells unresponsive to BCR stimulation. Therefore, it is tempting to speculate that the induction or maintenance of an anergic state in a—possibly autoreactive—fraction of naïve B cells is defective in IPF patients. This would also be in line with the absence of aberrant BCR signaling within the memory B cell population of IPF patients.

BAFF plays an important role during B cell development and the negative selection of autoreactive B cells, as increased levels can lead to dysfunctional negative selection and systemic autoimmunity [40–42]. Since IPF is associated with repetitive alveolar epithelial injury, it is conceivable that locally infiltrated B cells in close contact with local debris gain access to autoantigens. Increased BAFF levels could help to overcome negative selection of activated B cells in the absence of T cell help [33,43,44]. Indeed, studies using animal models have shown the importance of BAFF in pulmonary fibrosis [43,44]. Next to the BAFFR, BAFF can bind to TACI, and evidence has been provided that enhanced BAFF levels, at least partially, exert their pathogenicity in autoimmunity through TACI [45,46]. Though incompletely understood, TACI has both stimulatory and regulatory roles in B cell responses, and a balanced expression is crucial [47]. Because we observed that phosphorylation of BCR signaling molecules in naïve B cells correlated in HCs with BAFFR expression, but in IPF patients with TACI expression, defective fine-tuning of BAFF responses may contribute to immunopathology in IPF.

TGF-b is well-known for its pathogenic pro-fibrotic properties, though it also functions as an anti-inflammatory cytokine. We observed a trend towards increased TGF-b levels in the circulation of IPF patients, which showed a negative correlation with a-Ig-induced phosphorylation of BCR signalosome molecules. This is in line with previously reported suppressive effects of TGF-b on BCR signaling and immunoglobulin production in vitro [48,49]. However, it remains unclear how the observed correlation connects to disease severity or stage, because increased levels in circulation are not consistently found [50–53]. IPF therapies are largely focused on specific inhibition of pathogenic TGF-b signaling [54]. Because of the known immunoregulatory effects of TGF-b, including induction of regulatory T cells [55], it is important to investigate whether inhibition of TGF-b may be accompanied by increased BCR signaling or inflammatory responses.

We detected alterations in BCR signaling after the start of nintedanib treatment, as BCR signaling characteristics of patient B cells seemed inverted. Nintedanib is thought to suppress pro-fibrotic pathways by inhibiting VEGFR, FGFR, and platelet-derived growth factor receptor signaling. However, various molecules involved in BCR signaling, including LYN, SRC, and BTK, are also predicted targets of nintedanib with expected IC_{50} within the physiological treatment range [36,37,56]. Moreover, nintedanib has the capacity to activate SH2 domain-containing phosphatase-1 (SHP-1), a negative regulator of BCR signaling [57]. Therefore, our findings could be driven by B cell intrinsic effects of nintedanib. Alternatively, nintedanib treatment may affect other signaling pathways in other cell types such as T cells or affect the cytokine milieu, which would subsequently affect BCR signaling [58–61]. Irrespective of the mechanisms involved, it can be concluded that part of the beneficial effects of nintedanib treatment might be due to inhibitory effects on signaling pathways in lymphocytes.

It is currently disputed whether B cell-targeting therapies are beneficial for IPF patients [15,16,62], and a large trial with rituximab in CTD-ILD is currently ongoing (NCT01862926) [63]. In mouse models, BTK inhibition shows divergent effects [64,65]. Nevertheless, clinical studies targeting B cells in IPF are currently ongoing or have recently been performed (NCT03287414, NCT01969409), and BTK targeting in IPF is still of interest [65].

Our study has some limitations. In both cohorts, we analyzed small numbers of patients. Since IPF is a heterogenous disease, our study did not allow for the identification of correlations with clinical parameters or survival of these patients, which would require larger numbers of treatment-naïve patients. Ideally, future studies should include functional analyses of BAFF receptor and TACI signaling. Furthermore, possibly due to the low number of patients analyzed, we were not able to replicate our previous findings of increased BTK levels in B cells in a subset of IPF patients. Nevertheless, clear differences in phosphorylation of BTK, its upstream kinase SYK, and its downstream substrate PLCg2 were observed following BCR stimulation. Our assay on BTK phosphorylation, therefore, seems more sensitive and physiologically more relevant, as it reflects BTK enzymatic activity instead of only protein levels. In addition, we were able to identify remarkable correlations, e.g., between the phosphorylation of BCR signaling molecules, and surface BAFFR and TACI expression, as well as the effects of nintedanib on BCR signaling.

Therefore, our results contribute to the evidence for a role for B cells in IPF pathogenesis and, most interestingly, point to abnormalities in naïve B cells. Further studies building on these findings and involving larger patient cohorts should clarify whether targeting BCR signaling, for example with currently available specific small molecule inhibitors [66], has potential value as a treatment option for IPF patients.

Funding

This research was funded by an internal grant from the Erasmus MC (MRace) and the Target2B consortium.

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Supplementary data

Table S1. Antibodies used for surface and intracellular staining

Marker	Label	Clone	Company
Human TruStain FcX	-	-	Biolegend
CD19	FITC	HIB19	BD Biosciences
CD19	AF700	HIB19	Invitrogen
CD3	AF700	UCHT1	Invitrogen
BTK	PE	REA367	Miltenyi
CD86	BV650	FUN-1	BD Biosciences
CD38	BV785	HIT2	Biolegend
IgM	Biotin	G20-127	BD Biosciences
IgD	PE-Cy7	IA6-2	BD Biosciences
BAFFR	PerCP-Cy5.5	11C1	Biolegend
CD27	BV421	M-T271	BD Biosciences
Fixable Viability Dye	eFluor506	-	Invitrogen
TACI	APC	1A1	Biolegend
Streptavidin	APC-ef780	-	Invitrogen

Table S2. Antibodies used for phosphoflow cytometry

Marker	Label	Clone	Company
Human TruStain FcX	-	-	Biolegend
CD19	FITC	HIB19	BD Biosciences
CD3	AF700	UCHT1	Invitrogen
CD38	BV785	HIT2	Biolegend
CD27	BV421	M-T271	BD Biosciences
IgD	BV605	IA6-2	BD Biosciences
IgM	Biotin	G20-127	BD Biosciences
Fixable Viability Dye	eFluor506	-	eBioscience
Streptavidin	APC-ef780	-	Invitrogen
pBTK Y223	AF647	N35-86	BD phosflow
pSYK Y348	PE	I120-722	BD phosflow
pPLCγ2 Y759	AF647	K86-689.37	BD phosflow
pPI3K p85 Y458	-	E3UI1H	Cell Signaling Technologies
Donkey-anti-rabbit	PE	-	Jackson ImmunoResearch

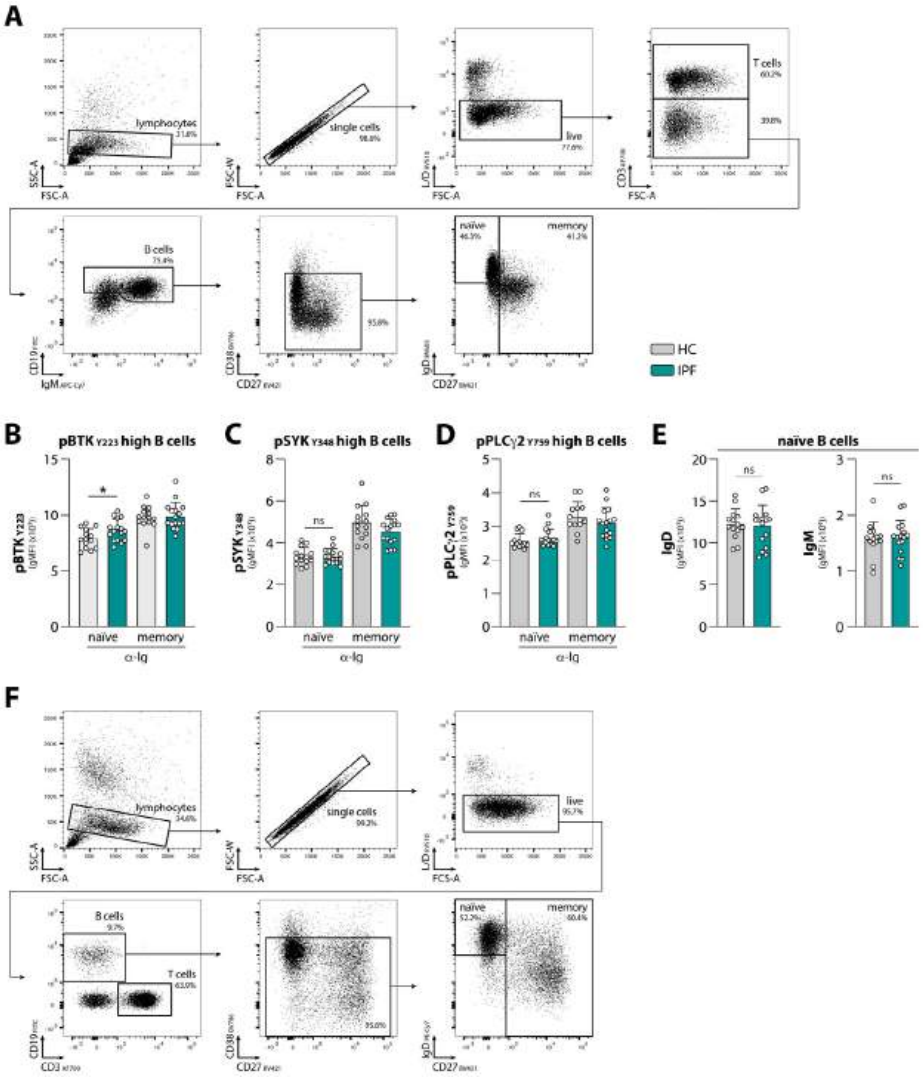


Figure S1. Flow cytometric gating strategies and expression profiles. (**A**) Strategy used for phosphoflow cytometry analysis of the indicated subpopulations. (**B**–**D**) gMFI of pBTK-, pSYK-, and pPLCγ2-high naïve ($CD3^-CD19^+$ $CD38^{lo}CD27^-IgD^+$) and memory ($CD3^-CD19^+CD38^{lo}CD27^+$) stimulated B cells from HCs and IPF patients (cohort 1). * $p < 0.05$ by an unpaired two-tailed Student's *t*-tests. (**E**) BCR expression on naïve B cells, showing IgD (*left*) and IgM (*right*) surface expression, in HCs and IPF patients (cohort 1). Subjects are indicated by individual data points and bars indicate mean values + SD. (**F**) Strategy used for the analysis of surface and intracellular markers of the indicated subpopulations. SSC-A, side scatter area; FSC-W, forward scatter width; FSC-A, forward scatter area; gMFI, geometric mean fluorescence intensity; L/D, live-dead marker; α-Ig, anti-immunoglobulin; ns, not significant.

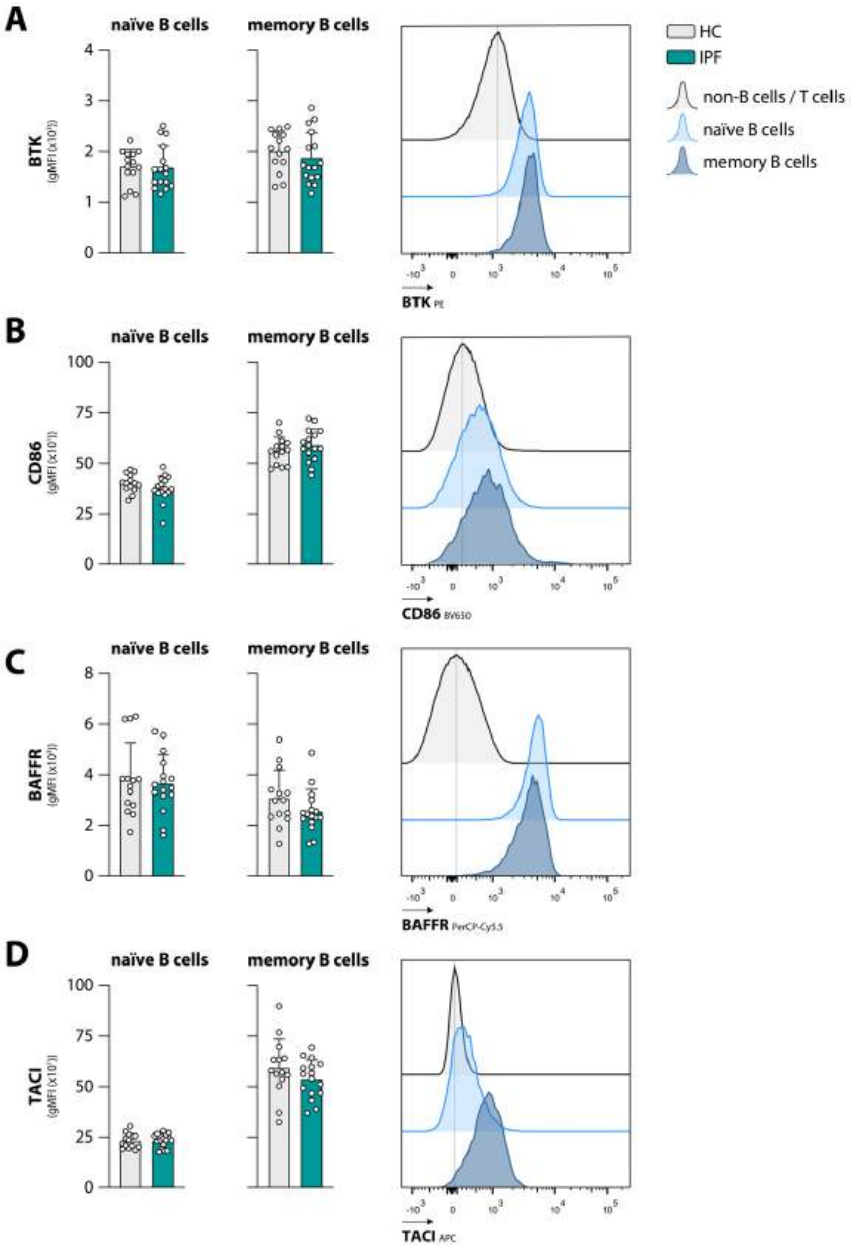


Figure S2. Expression of BTK, CD86, BAFFR and TACI in HC and IPF B cells. Intracellular expression of (**A**) BTK, and surface expression of (**B**) CD86, (**C**), BAFFR, and (**D**) TACI in HC and IPF (cohort 1) naïve and memory B cells (*left*). A representative histogram for non-B cell / T cell, naïve ($CD3^-CD19^+CD38^{lo}CD27^-IgD^+$), and memory ($CD3^-CD19^+CD38^{lo}CD27^+$) B cell populations from a HC is shown (*right*). Subjects are indicated by individual data points and bars indicate mean values + SD. Differences between IPF patients and HCs were not significant by an unpaired two-tailed Student's *t*-test or Mann-Whitney *U*.

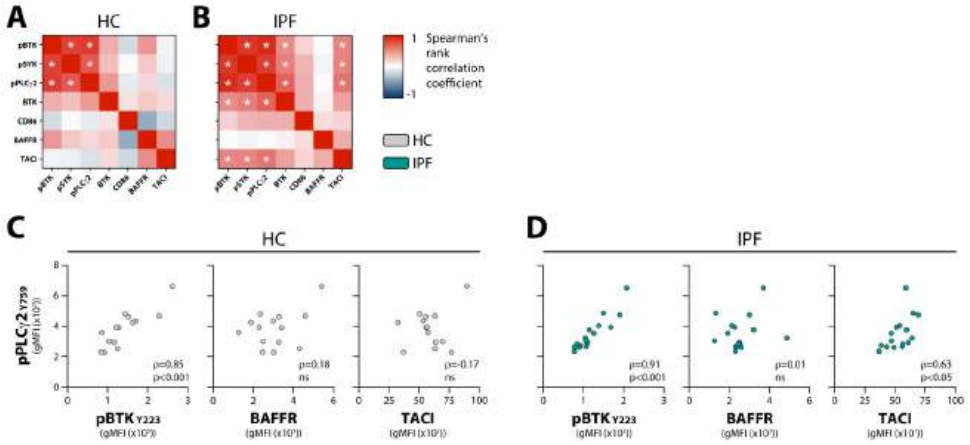


Figure S3. Correlation analysis of ex vivo measured B cell markers and in vitro measured phosphorylation of BCR signalosome molecules in unstimulated memory ($CD3^-CD19^+CD38^{lo}CD27^+$) B cells from HCs and IPF patients (cohort 1). (**A**, **B**) Spearman's rank correlation matrix for (**A**) HCs and (**B**) IPF patients for the indicated markers measured ex vivo and phosphorylated proteins of unstimulated memory B cells. (**C**, **D**) Spearman's rank correlation analysis of (**C**) HCs and (**D**) IPF patients for the correlation of pPLCγ2 with pBTK, BAFFR, and TACI expression in unstimulated memory B cells. Subjects are indicated by individual data points. ns, not significant.

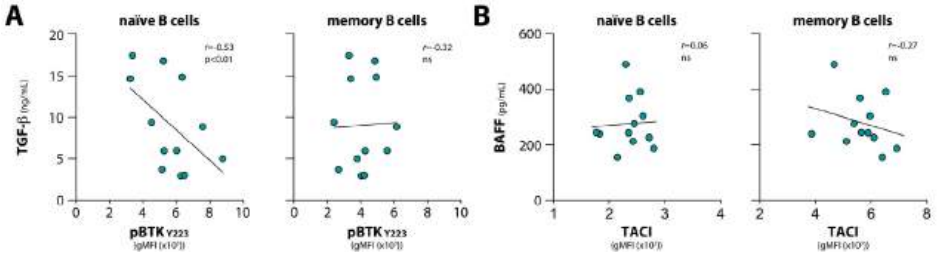


Figure S4. Correlations of circulating cytokines in IPF patients (cohort 1). (**A**) Correlation of circulating TGF-β levels and phosphorylation of BTK following α-Ig stimulation in naïve ($CD3^-CD19^+CD38^{lo}CD27^-IgD^+$) and memory ($CD3^-CD19^+CD38^{lo}CD27^+$) B cells from IPF patients. (**B**) Correlation between circulating BAFF levels and TACI expression on naïve or memory B cells from IPF patients. Subjects are indicated by individual data points. ns, not significant.

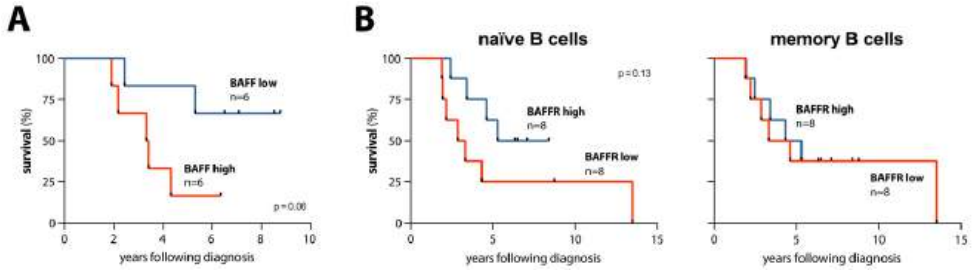
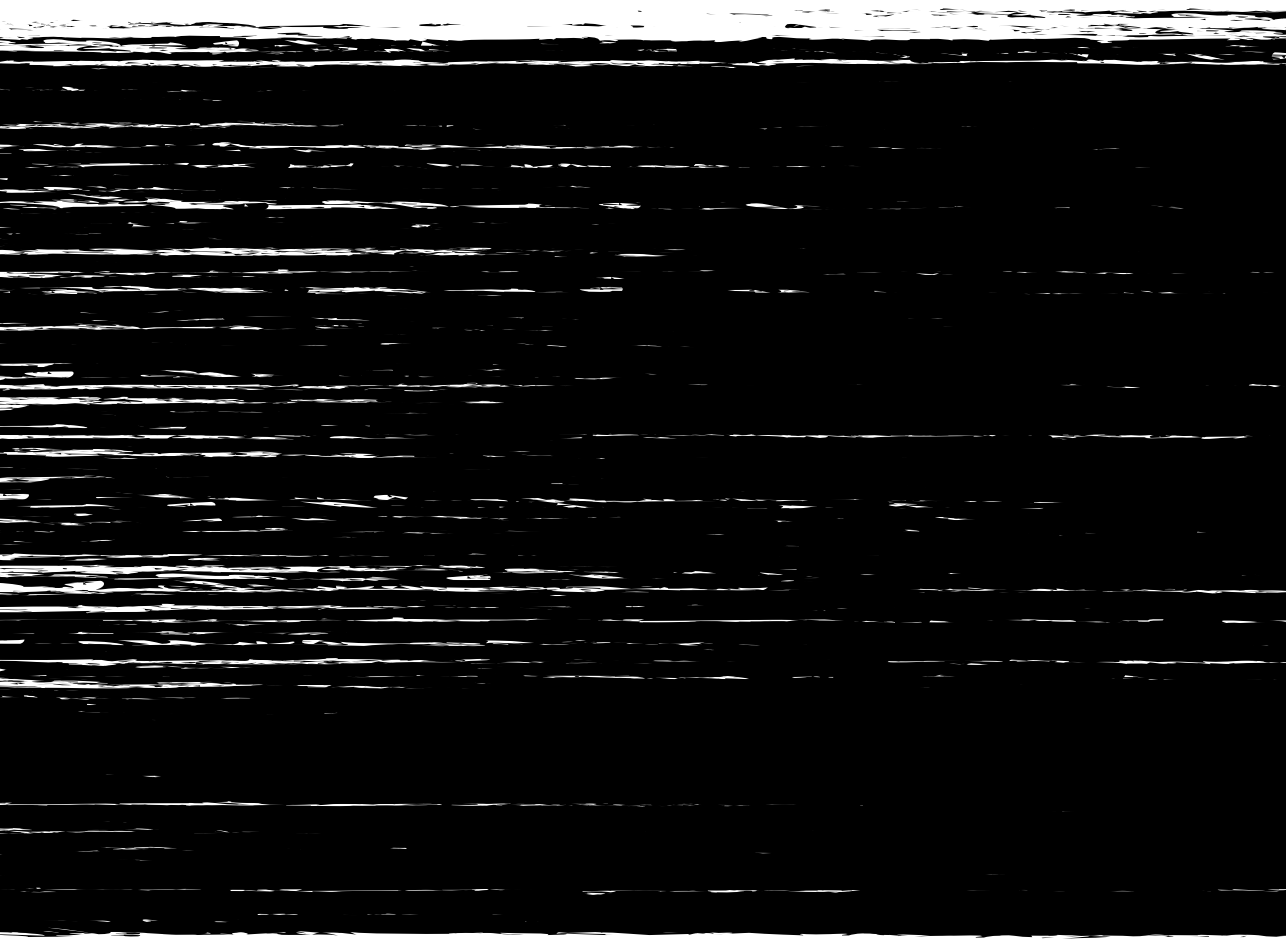


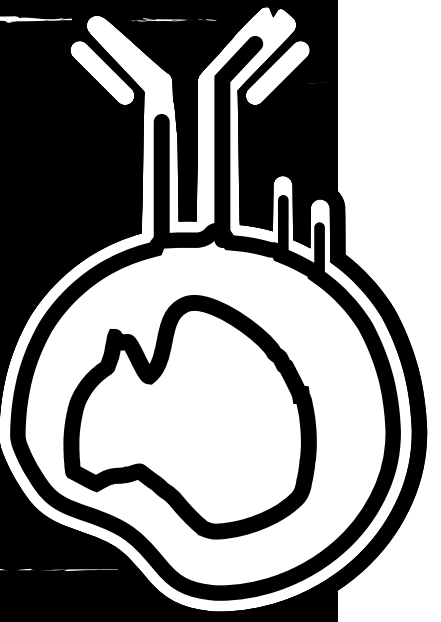
Figure S5. Survival analysis for IPF patients based on circulating BAFF levels and BAFFR surface expression. IPF patients (cohort 1) were ranked from highest to lowest BAFF level or BAFFR surface expression and subsequently split into two equal groups. Survival Kaplan-Meier curve for (**A**) circulating BAFF levels and (**B**) naïve ($CD3^-CD19^+CD38^{lo}CD27^-IgD^+$) and memory ($CD3^-CD19^+CD38^{lo}CD27^+$) B cell surface BAFFR expression. Data were analyzed using a log rank test for statistical significance.



PART II

Adaptive immunity in pulmonary hypertension

7



CHAPTER 7

Loss of Immune Homeostasis in Patients with Idiopathic Pulmonary Arterial Hypertension

Peter Heukels, Odilia B.J. Corneth, Denise van Uden, Jennifer A.C. van Hulst, Leon M. van den Toorn, Annemien E. van den Bosch, Marlies S. Wijsenbeek, Karin A. Boomars, Mirjam Kool* and Rudi W. Hendriks*

** Shared senior authorship*

Abstract

Introduction

Autoreactivity against pulmonary vascular structures is thought to be involved in idiopathic pulmonary arterial hypertension (IPAH), but the underlying mechanisms remain poorly understood. We hypothesized that aberrant B-cell activation contributes to IPAH etiology.

Methods

Mice with enhanced B-cell activation due to B-cell-specific overexpression of the B-cell receptor (BCR) signaling molecule Bruton's tyrosine kinase (BTK) were subjected to lung injury and examined for several PH indices. Peripheral blood lymphocytes from patients with IPAH (n=13), connective tissue disease associated PAH (CTD-PAH, n=9), congenital heart disease PAH (n=7), interstitial lung disease associated PH (n=17), and healthy controls (n=19) were characterized by 14-color flow cytometry.

Results

Following pulmonary injury, BTK-overexpressing mice showed prolonged activation of B cells and CXCR5$^+$ follicular T-helper (Tfh) cells, as well as features of PH development. CTD-PAH and CHD-PAH patients displayed reduced proportions of circulating non-switched-memory B cells (p=0.03, p=0.02, respectively). Interestingly, we observed increased BTK protein expression in naïve (p=0.007) and memory B-cell subsets of IPAH and CTD-PAH patients. BTK was particularly high in IPAH patients with circulating autoantibodies (p=0.045). IPAH patients had low frequencies of circulating CXCR5$^+$ Tfh cells (p=0.005). Hereby, the increased BTK protein expression in B cells was associated with high proportions of Tfh17 (p=0.018) and Tfh17.1 (p=0.007) cells within the circulating Tfh population.

Conclusions

Our study shows that pulmonary injury in combination with enhanced B-cell activation is sufficient to induce PH symptoms in mice. In parallel, immune homeostasis in patients with IPAH is compromised, as evidenced by increased BCR signaling and cTfh17 polarization, indicating that adaptive immune activation contributes to IPAH disease induction or progression.

Background

Pulmonary arterial hypertension (PAH) is a fatal disease characterized by progressive pulmonary vascular remodeling mediated by endothelial cell dysfunction leading to increased pulmonary vascular pressure and right ventricle dysfunction.[1, 2, 3]

Idiopathic PAH (IPAH) is a form of pulmonary hypertension (PH) in which no underlying disease or etiology is identified at time of diagnosis. The presence of various immune cells in and around hypertensive pulmonary vascular lesions indicates their contribution to disease.[4, 5] Several lines of evidence point towards a role for increased adaptive immune responses and cytokines involved in vascular remodeling.[2, 6] It is currently unclear whether chronic immune activation is cause or consequence of the primary remodeling process.[7] Nevertheless, accumulating evidence suggests that vascular and lung injury could lead to impaired tolerance to self-antigens, which may contribute to vascular remodeling and subsequent PH development.[2, 8] A considerable fraction of patients with IPAH ƒhas increased levels of circulating autoreactive plasmablasts and autoantibodies, which may often be produced by plasma cells in tertiary lymphoid structures in the lung.[9, 10]

Well-regulated B-cell receptor (BCR) signaling is crucial to maintain self-tolerance.[11] A key signaling protein downstream of the BCR is Bruton's tyrosine kinase (BTK).[12] Increased BTK levels are associated with active disease in several autoimmune disorders in humans.[14] Moreover, B-cell specific BTK overexpression in CD19-hBTK transgenic mice induced spontaneous germinal center (GC) formation in the spleen, production of anti-nuclear autoantibodies and development of an autoimmune phenotype.[13, 14] Another crucial step for adequate B-cell activation and selection is T cell help from follicular T-helper (Tfh) cells.[15] Tfh cells are antigen-experienced $CD4^+$ T cells that express the B-cell follicle homing receptor CXCR5 and are therefore present in B-cell follicles in lymph nodes, spleen, Peyer's patches and TLOs. A breakdown of self-tolerance could result from aberrant Tfh cell activation in GCs.[16 17] Interestingly, BTK overexpression in CD19-hBTK transgenic mice is associated with an increase in Tfh cells in the spleen and drives systemic autoimmunity by disrupting T-cell homeostasis, providing a detrimental feedforward loop.[18] Tfh cells in TLOs and GCs are not easily accessible in patients. However, circulating $CXCR5^+CD4^+$ T cells, which are often referred to as circulating Tfh (cTfh) cells, share functional properties with Tfh cells.[19] These cells are thought to represent a circulating pool of memory Tfh cells and alterations in Tfh cells are associated with autoimmunity in humans.[19, 20]

Given the parallels of IPAH and autoimmune diseases, it is conceivable that aberrant B-cell activation and Tfh activity contribute to the etiology of IPAH. Similar to our observation of robust induction of autoantibodies in the lung following influenza infection in CD19-hBTK mice[13, 14], we hypothesized that autoantibodies reactive against pulmonary vascular structures may arise in these mice upon induction of pulmonary injury. Although bleomycin exposure is often used to induce lung fibrosis in mice, several groups have applied the bleomycin mouse model to investigate experimental PH.[21, 22] Therefore, we investigated whether CD19-hBTK mice developed enhanced PH symptoms upon bleomycin exposure. Next, we used 14-color flowcytometry to investigate B cells and circulating Tfh cell subsets

and their activation status in peripheral blood of patients with IPAH. In these analyses, we also included blood samples from patients with connective tissue disease associated PAH (CTD-PAH), congenital heart disease PAH (CHD-PAH), interstitial lung disease related PH (ILD-PH) and healthy controls.

Table 1. Patient characteristics.

	IPAH (n=13)*	CTD-PAH (n=9)#	CHD-PAH (n=7)^
Gender (male)	3 (23%)	1 (11%)	5 (71%)
Age (years)	50 (33-60)	71 (50-74)	41 (26-40)
Time to diagnosis (months)	40 (12-80)	6 (3-15)	1 (1-12)
Right heart catheterization			
mPAP (mmHg)	58 (47-78)	37 (31-52)	43 (38-61)
RAP (mmHg)	13 (8-19)	8 (6-11)	8 (6-9)
PAWP (mmHg)	13 (11-17)	9 (5-13)	12 (10-16)
CI (L/min/m^2)	2.4 (1.6-2.8)	3.1 (2.4-3.6)	3.3 (2.9-3.5)
CO (L/min)	4.2 (2.7-5.1)	5.6 (4.8-6.9)	6.0 (4.1-7.4)
PVR (dynes s/cm^5)	1025 (628-1430)	371 (189-830)	426 (293-577)
SvO2 (%)	60 (56-73)	63 (61-75)	70 (60-79)
Functional parameters			
NYHA functional class 1 (n)	0 (0%)	0 (0%)	1 (14%)
NYHA functional class 2 (n)	5 (38%)	4 (44%)	2 (28%)
NYHA functional class 3 (n)	7 (54%)	4 (44%)	4 (57%)
NYHA functional class 4 (n)	1 (8%)	1 (11%)	0 (0%)
6MWD (m)	320 (226-365)	289 (267-432)	452 (263-592)
NT proBNP (umol/l)	69 (32-203)	69 (15-79)	34 (11-43)
Vasoactive medication (n)$	10 (77%)	5 (56%)	0 (0%)

* Genetic testing revealed that 2 patients had a *BMPR2* gene mutation.
# 7 Patients with rheumatoid arthritis, 1 patient with systemic sclerosis, 1 patients with systemic lupus erythematosus.
^ 5 Patients with atrial septal defect, 1 patient with Eisenmenger's syndrome and ventricular septal defect, 1 patient with major aortopulmonary collateral arteries.
$ Vasoactive medication at time blood sample taken.
Healthy subject characteristics: n=19, gender (male) = 6 (32%), age (years) = 48 (30-52). Continuous variables are presented as median and IQR in parentheses and categorical variables as counts and percentagse in parentheses.
Abbreviations: IPAH = idiopathic pulmonary arterial hypertension, CTD-PAH = connective tissue disease associated pulmonary arterial hypertension, CHD-PAH = congenital heart disease associated pulmonary arterial hypertension, mPAP = mean pulmonary arterial pressure, RAP = right atrial pressure, PAWP = pulmonary arterial wedge pressure, CI = cardiac index, CO = cardiac output, PVR = pulmonary vascular resistance, SvO2 = central mixed venous oxygen saturation, NYHA = New York Heart Association, 6MWD = 6 minute walk distance.

Methods

Subjects

Twenty-nine PAH patients who entered the 'Biomarker activity in adults with pulmonary hypertension' (*BioPulse*) study or were diagnosed in the Erasmus MC (See **Table 1** for patient characteristics) were included in the study. Patients were diagnosed according to the current ESC/ERS guidelines and therefore all patients underwent a right heart catheterization.[23] Healthy controls (HCs; n=19) were age- and sex-matched. All patients and controls gave written informed consent. This study adheres to the Declaration of Helsinki and the Medical Ethical Committee of the Erasmus MC approved this study (MEC-2011-392 and 2012-512) and gave consent for collection of blood samples.

Flow cytometry procedures

Procedures of flow cytometry experiments are described in **supplementary methods.**

Self-reactive immunoglobulin (Ig)G

Plasma samples (1/50 diluted) of patients with PH and HCs were incubated for 1 hour on Kallestad HEp-2 slides (Bio-Rad Laboratories), using various Ig F(ab')2 fragments as detection antibodies (**Supplementary Table 1**). Fluorescence intensities of HEp2 slides signals were evaluated using an LSM 311 META confocal fluorescence microscope (Zeiss) and LSM Image Browser Version 4.2.0.12 software (Zeiss).

Mice

CD19-hBtk transgenic mice[14] were bred on the C57BL/6J background and kept under specified pathogen-free conditions in the Erasmus MC experimental animal facility. Experimental protocols were reviewed and approved by the Erasmus Medical Center MC Committee of animal experiments. Procedures of mouse experiments are described in **Supplementary Methods**.

Principal component analysis

Principal component analyses (PCA) were performed using R and RStudio, and the packages FactoMineR and Factoextra. Prior to PCA, data were log10-transformed to better fit a normal distribution and scaled. Contribution of the variables to the dimensions was determined in percentages by (squared cosine of the variable*100) / (total squared cosine of the principal component).

Statistical analyses

For calculating the significance of differences between >2 groups we used the Kruskal-Wallis test combined with a Dunn's multiple comparison test. Mann-Whitney U test was used for comparison of two groups. The variability explained by the PCA was tested for statistical significance by inertia of the first two dimension using the R package

FactoInvestigate. Correlation coefficients were calculated using Spearman's rank method. Statistical analyses were performed using IBM SPSS Statistics 21 and GraphPad Prism 6 software. P values <0.05 were considered significant.

Results

CD19-hBTK transgenic mice develop PH and local autoreactivity upon bleomycin exposure

We have previously shown that aged CD19-hBTK transgenic mice spontaneously develop autoimmune pathology, accompanied by inflammatory infiltrates around pulmonary vascular structures with segregated T- and B-cell zones (**Figure 1A**).[14] To provoke lung injury, we subjected these mice to intratracheal bleomycin (or saline as a control). Because the bleomycin model is generally employed as a model for lung fibrosis, we first investigated whether the presence of the CD19-hBTK transgene would lead to augmented fibrosis at 3 and 10 weeks after bleomycin exposure (**Supplementary Figure 1**). The hydroxyproline content, lung tissue elastance and total fibrosis score were similar between hBTK-mice and control littermates at 3 weeks and a similar degree of resolution of fibrosis occurred at 10 weeks, as previously reported for WT mice.[24]

PH symptoms have been reported at early timepoints (2-4 weeks) after bleomycin exposure in mice.[21, 22] At 10 weeks after exposure, we still observed a significant bleomycin-induced increase of right ventricular systolic pressure (RVSP) (p=0.015) and signs of RV hypertrophy (Fulton Index) (p=0.030) in CD19-hBTK mice, but not in WT mice (**Figure 1B,C**). In both mouse strains the numbers of pulmonary vessels with perivascular and endothelial cells expressing α-smooth muscle actin (α-SMA) and the proportions of α-SMA-positive areas of the total artery did not significantly change at 10 weeks after bleomycin exposure (**Supplementary Figure 2**).

To examine whether lymphocyte activation upon bleomycin exposure was enhanced in CD19-hBTK mice, we analyzed the B-cell compartment in mediastinal lymph nodes (MedLNs) using the gating strategy in **Supplementary Figure 3.** The numbers of memory B-cells ($CD19^+B220^+CD80^+PDL2^+$), GC B-cells ($CD19^+B220^+IgD^-CD95^+$), plasmablasts ($CD19^+CD138^+$), and plasma cells ($CD19^{low}CD138^+$) in MedLNs of CD19-hBTK mice exposed to bleomycin were significantly increased (p=0.017, p=0.015, p=0.019, p=0.031, respectively), compared with bleomycin-exposed WT mice (**Figure 1D**). Likewise, MedLN from bleomycin-exposed CD19-hBTK mice showed a significant increase (p=0.028) of Tfh-cells (**Figure 1E**), which displayed enhanced surface expression of inducible costimulator (ICOS) (p=0.022), a marker for T-cell activation (**Figure 1F**).

Next, we evaluated whether increased B-cell activation via BTK upregulation also resulted in increased autoantibody production. At 21 days post exposure ~86% of bleomycin-treated CD19-hBTK mice – and none of the control littermates - had serum anti-nuclear IgG autoantibodies, as shown by HEp2 reactivity (**Figure 1G**), but at day 70 these

autoantibodies were no longer detectable. At 70 days post exposure, serum of a fraction of bleomycin-treated CD19-hBTK mice – and not any of the other mice – was positive for autoreactive IgM (~50%) and IgG (~33%) recognizing endothelial antigens, as detected in cryo-sections of lung tissue of *Rag1*$^{-/-}$ mice (**Figure 1H**).

In conclusion, CD19-hBTK mice developed hemodynamic and cardiac signs of PH after pulmonary injury with bleomycin, which was not seen in WT mice. Additionally, bleomycin-induced pulmonary injury in CD19-hBTK mice resulted in enhanced B-cell activation and production of autoantibodies, including antibodies recognizing vascular structures in the lung. Although we did not observe profound vascular remodeling in this model, these findings were hypothesis-generating and suggested that increased B-cell activation may enhance the susceptibility to PH development.

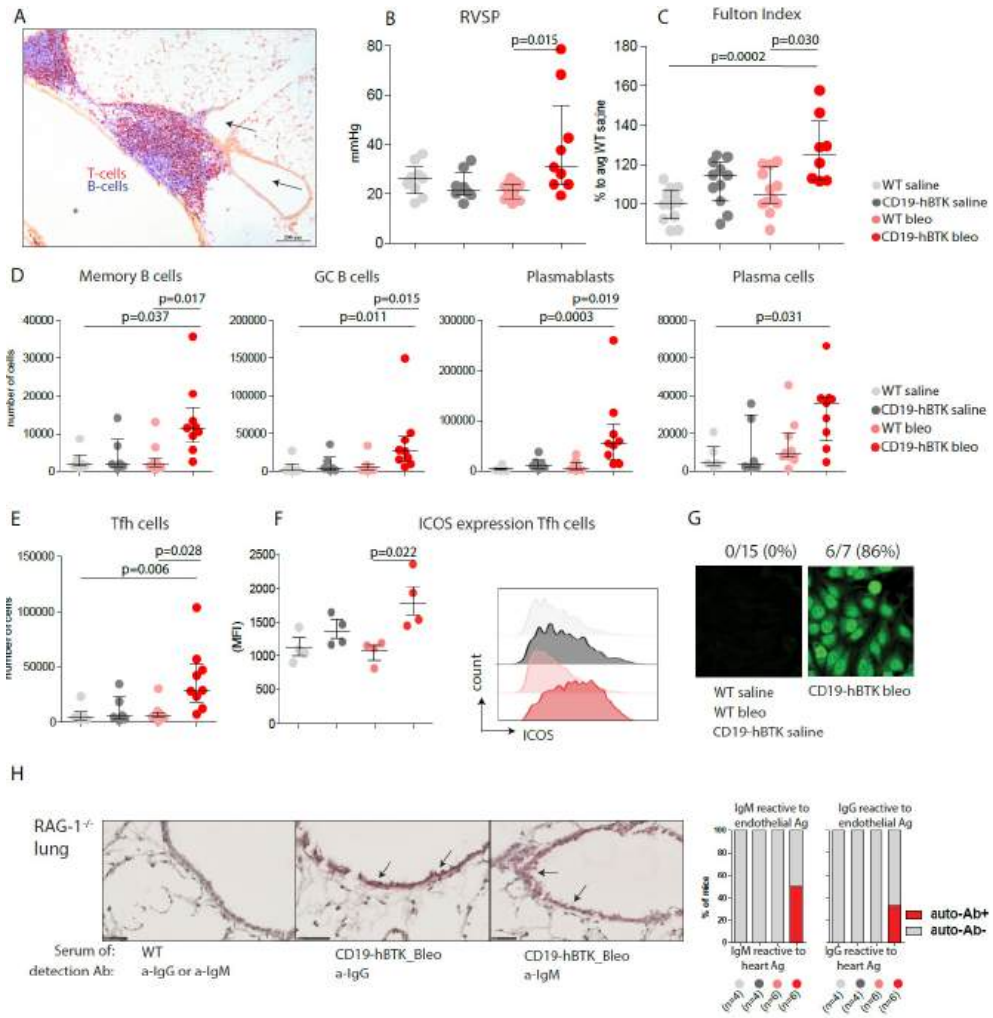


Figure 1. CD19-hBTK transgenic mice develop PH and local autoreactivity upon bleomycin exposure.
(A) Pulmonary inflammatory infiltrate in lung tissue (cryo-section) in a 40-week-old CD19-hBTK mouse stained with anti-CD3 (T cells, *red*) and anti-B220 (B-cells, *blue*). This inflammatory infiltrate is located next to a pulmonary vessel (*arrows*) and airway (*asterisk*). (B–C) Pulmonary hypertension indices in WT and CD19-hBTK mice 10 weeks after saline or bleomycin exposure: right ventricular systolic pressure (RVSP) (B) and Fulton index (ratio of right ventricular to left ventricular and septal weight) (C) in the indicated four mouse groups. (D) Quantification of total numbers of B-cell subpopulations in wild-type (WT) and CD19-hBTK transgenic mice 10 weeks after bleomycin or saline exposure: memory B-cells ($CD19^+B220^+CD80^+PDL2^+$), germinal center (GC) B-cells ($CD19^+B220^+IgD^-CD95^+$), plasmablasts ($CD19^+CD138^+$) and plasma cells ($CD19^{low}CD138^+$). (E) Total numbers of Follicular T helper (Tfh) cells ($CD3^+CD4^+CXCR5^+PD1^+$) in MedLN. (F) Mean fluorescence intensity (MFI) values of inducible co-stimulator (ICOS) expression on gated MedLN Tfh cells (*left*) and representative histogram overlays of ICOS expression on gated Tfh cells (*right*) (G) Representative staining patterns of human epithelial cells (HEp)-2 slides with serum of the indicated mice WT and CD19-hBTK mice, at 3 weeks after bleomycin or saline exposure. (H) Cryo-sections of lung tissue of *Rag1*$^{-/-}$ mice incubated with serum of WT or CD19-hBTK mice, 10 weeks after bleomycin or saline exposure, as indicated. Anti-IgM or anti-IgG was used for visualization of antibodies reactive to endothelial antigens (*arrows*) *(left)*.

Proportions of mice in which antibodies reactive to lung antigens were detected in the serum (*right*). The survival rates for the four mouse groups at ~10 weeks were: 100% for saline treated mice, 83% for bleomycin/WT and 65% for bleomycin/CD19-hBTK mice. The results are shown as median (IQR), p exact values were obtained following a Kruskal-Wallis test (B-F). Dots represent individual mice.

Decreased proportions of circulating non-switched memory B-cells in patients with PAH.

Given the link between BTK expression, B-cell activation and PH development in mice, we evaluated B-cell subsets and BTK expression in peripheral blood of patients with IPAH (n=13) and compared these with HCs (n=19) and patients with CTD-PAH (n=9) or CHD-PAH (n=7). Increased B-cell activation and autoantibody reactivity against vascular structures contribute to the development of CTD-PAH and therefore this subgroup served as a "positive" disease control.[25] CHD-PAH is mainly driven by (congenital) structural cardiac changes and served as a "negative" disease control. Patient and HC characteristics are shown in **Table 1.**

We used flow cytometry to quantify total peripheral blood $CD19^+$ B cells, as well as $CD38^+CD27^-$ transitional, IgD^+CD27^- naïve, IgD^+CD27^+ and $IgD^-IgM^+CD27^+$ non-switched memory (NSM), $IgD^-IgM^-CD27^+$ switched memory B cells and $CD38^+CD27^+$ plasmablasts, following the gating strategy in **Figure 2A.** The total proportions of B cells did not differ between patients with IPAH and control groups (**Figure 2B**). Within the B-cell population, NSM B cells were decreased in all three PAH subgroups compared with HC, reaching significance for patients with CTD-PAH (p=0.03) and patients with CHD-PAH (p=0.02), paralleling published findings in rheumatoid arthritis and systemic sclerosis.[26, 27]

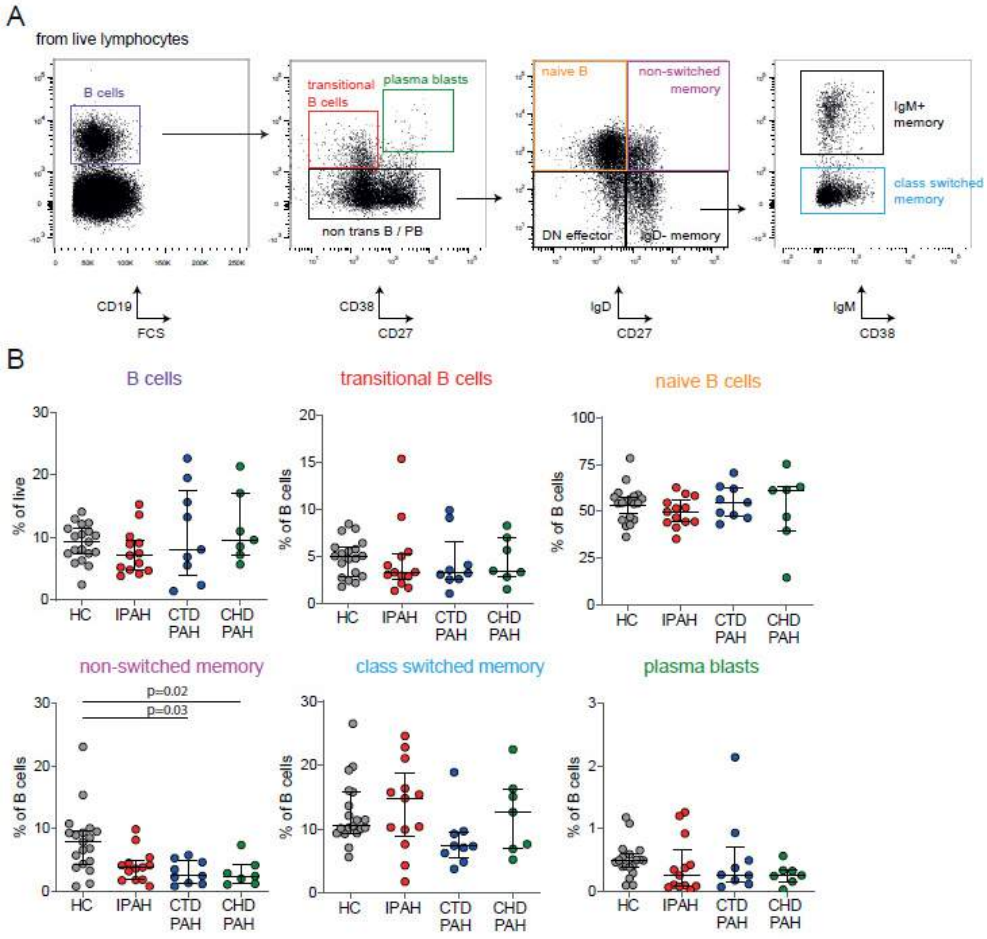


Figure 2. Decreased proportions of circulating non-switched memory B-cells in patients with PAH.
(A) Representative gating strategy for the identification of B-cell subsets in peripheral blood. Starting with live single cells, total B cells ($CD19^+$), transitional B cells ($CD19^+CD38^+CD27^-$), plasmablasts ($CD19^+CD38^{hi}CD27^+$), naïve B cells ($CD19^+IgD^+CD27^-$), non-switched memory B cells ($CD19^+IgD^+CD27^+$), IgD^- memory B cells ($CD19^+IgD^-CD27^+$), IgM^+ memory B cells ($CD19^+CD27^+IgD^-IgM^+$) and switched memory B cells ($CD19^+CD27^+IgD^-IgM^-$) were identified. FCS, forward scatter. (B) Proportions of circulating total B cells and the indicated B cell subpopulations in healthy controls (HC) (n=19) and patients with IPAH (n=13), connective tissue disease-associated pulmonary arterial hypertension (CTD-PAH) (n=9) and congenital heart disease (CHD)-PAH (n=7). The results are shown as median (IQR), p exact values were obtained following Kruskal-Wallis test. Dots represent values in individual patients or HC.

Increased BTK protein expression and phosphorylation in circulating B-cells of patients with IPAH.

Because increased BTK levels in B cells were associated with enhanced PH development in mice, we next evaluated BTK expression and BCR signaling in circulating B cells in the three groups of patients with PAH and HCs.
Representative histogram overlays showing BTK protein expression in total B cells and B-cell subsets, as determined by intracellular flow cytometry, are depicted in **Figure 3A**. We observed higher BTK protein levels in all B-cell subsets, except for class-switched memory B cells, in patients with IPAH and CTD-PAH compared with HCs and patients with CHD-PAH (**Figure 3A;** quantified in **Figure 3B**).

To link BTK protein levels to its activity, we measured BTK phosphorylation at Y551 (pBTK) by phosphoflow analysis in B-cells from patients *ex vivo* (without *in vitro* stimulation). pBTK expression was significantly increased (p=0.041) in B-cells from patients with IPAH compared with HCs (**Figure 3C**) and correlated significantly (p=0.009) with BTK protein levels (**Figure 3D**).

To obtain a more comprehensive overview of the B-cell profiles across the three patient groups and HCs, we performed a principal component analysis (PCA) of 16 parameters of B cell subsets and BTK expression levels. HCs and patients with IPAH were significantly separated by dimension 1 (Dim1), to which particularly BTK levels across B-cell subsets contributed (p=0.008; **Figure 3E**) and not proportions of B cell subpopulations, which dominated Dim 2.

Our finding that bleomycin as a second trigger induced PH in the CD19-hBTK mouse model, raised the question if BTK expression is also increased in ILD-PH. Analysis of a cohort of patients with ILD-PH (see for patient characteristics: **supplementary Figure 4A**) did not reveal alterations in B cell subsets, compared with HCs (**supplementary Figure 4B**). We even observed decreased BTK protein levels in B cells in patients with ILD-PH, compared to HC, which reached significance in patients receiving treatment (p=0.001; **supplementary Figure 4C**). This analysis suggests that enhanced B-cell activation is not a crucial driver of ILD-PH.

Taken together, our multivariate data analysis demonstrates that BTK protein expression was increased in circulating B cells of patients with IPAH. This increase in BTK protein also reflected augmented BCR signaling.

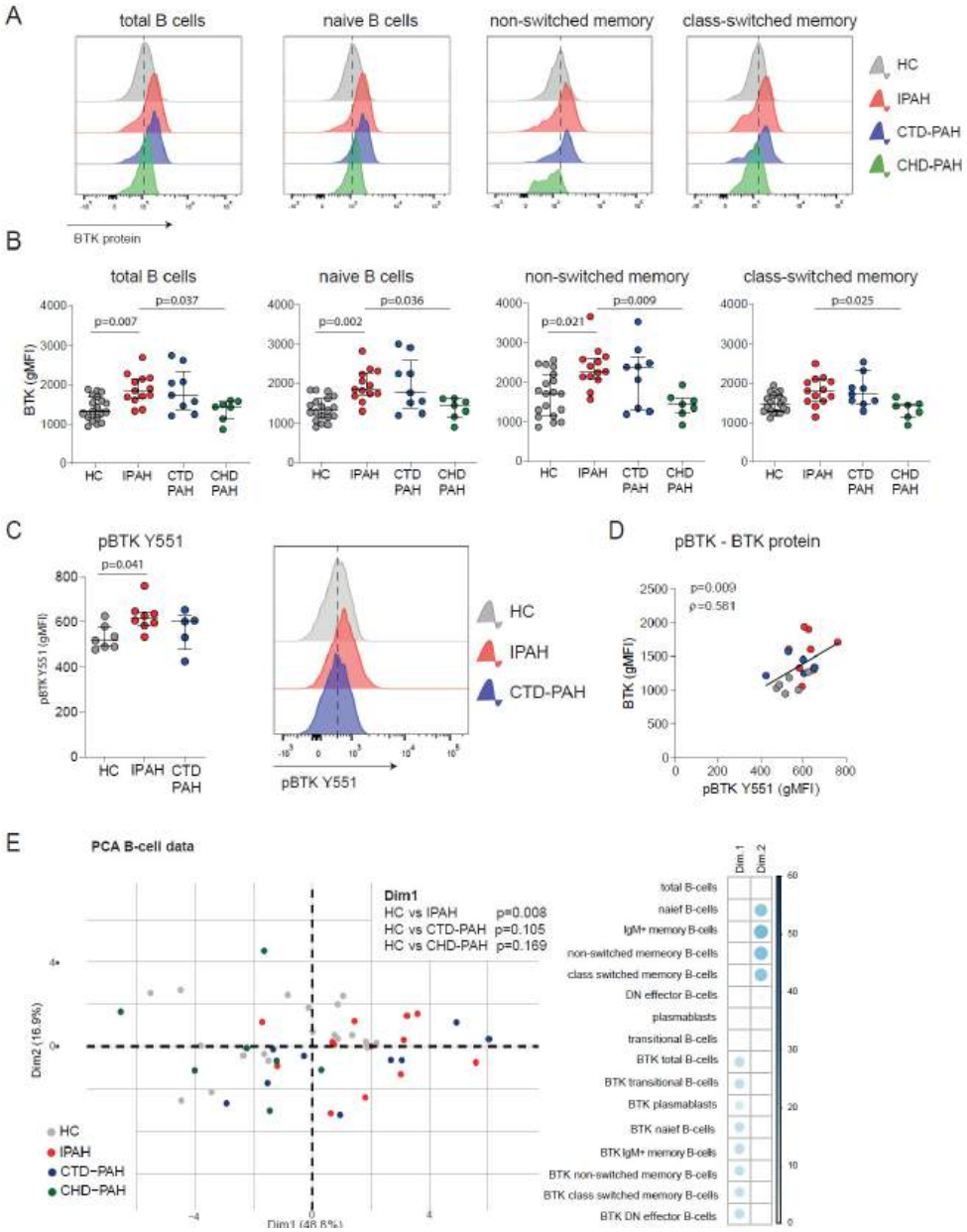


Figure 3. Increased BTK protein expression and BCR signaling in circulating B cells of patients with IPAH.
(A) Representative histogram overlays of BTK geometric mean fluorescence intensity (gMFI) values assessed by intracellular flow cytometry analysis of the indicated B cell subpopulations of healthy controls (HCs) and patients with IPAH, CTD-PAH and CHD-PAH, as indicated. (B) Quantification of BTK protein expression levels, shown as gMFI values of intracellular flow cytometry analysis of the indicated B-cell subpopulations in HCs and the three patient groups. (C) Quantification of phosphorylated BTK protein (pBTK-Y551) levels, shown as gMFI values of intracellular flow cytometry analysis (*left*) and representative histogram overlays of pBTK-Y551 expression (*right*). (D) Correlation of pBTK

and BTK protein expression in HCs and patients with IPAH and CTD-PAH. Correlation coefficient was calculated using Spearman's rank method. (E) Principal component analyses (PCA) of B-cell subsets and Btk levels in PH subgroups and HC. Contribution of variables on the first dimension (Dim1) and second dimension (Dim2) of the PCA. The PCA of the B-cell subsets and Btk levels showed a non-random distribution over Dim1 and Dim2, which was not due to gender (p=0.083 (dim1) and p=0.378 (dim2)) or age (p=0.069 (dim1) and p=0.790 (dim2)). PCAs were on log10-transformed and scaled values. The results are shown as median (IQR; B-D), p exact values were obtained following Kruskal-Wallis test (>2 groups) or Mann-Whitney U test. Dots represent values in individual patients or HC (B-E).

Autoreactive IgG antibodies in plasma of patients with IPAH correlate with BTK levels

To explore the presence of autoantibodies in plasma of patients with PH and controls, we screened HEp-2 slides (**Figure 4A**). Six out of eleven (~55%) patients with IPAH had detectable auto-reactive IgG, versus one of 11 HCs and 1 of 7 patients with CHD-PAH. As expected, all patients with CTD-PAH had detectable IgG autoantibodies. Interestingly, the HEp2-positive patients with IPAH had higher intracellular BTK protein levels compared with the HEp2-negative patients with IPAH, p=0.045 (**Figure 4B**).

This finding indicates that increased intracellular BTK protein expression in peripheral blood B-cells of patients with IPAH was associated with the presence of circulating autoantibodies.

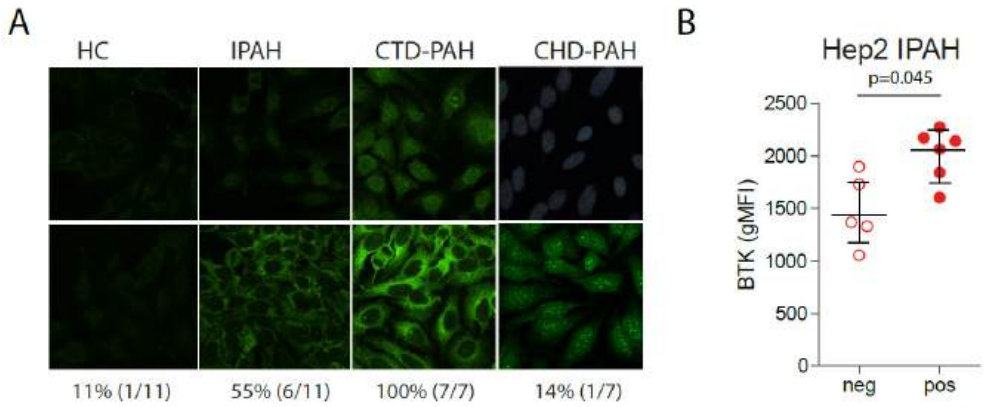


Figure 4. **Autoreactive IgG antibodies in plasma of patients with IPAH correlate with BTK levels**
(A) Staining pattern of human epithelial cells (Hep)-2 slides incubated with plasma of HC and patients with IPAH, CTD-PAH and CHD-PAH. As detection antibodies IgG F(ab')2 fragments were applied. For each group two representative slides are depicted. (B) Quantification of BTK protein expression levels (gMFI values) in total circulating B-cells from Hep2-positive and Hep2-negative IPAH patients. The results are shown as median (IQR), p exact values were obtained following Mann-Whitney U test. Dots represent values in individual patients.

Increased proportions of cTfh-17 cells in patients with IPAH.

As we found BTK overexpression in B-cells from patients with IPAH, we focused on patients with IPAH for the evaluation of cTfh cells, which can be induced by BTK overexpression in B-cells[18]. Based on surface C-C chemokine receptor 6 (CCR6) and C-X-C motif chemokine receptor 3 (CXCR3) expression, different cTfh subsets can be discriminated with cytokine profile characteristics of Th1, Th2, and Th17 cells[19] (**Figure 5A**).

Patients with IPAH exhibited significantly reduced proportions of $CXCR5^+$ memory T-cells in the circulation ($p=0.005$; **Figure 5B**). Within this cell-population we noticed an altered cTfh subset distribution. The fraction of cTfh17 cells was significantly increased ($p=0.014$) compared with HCs (**Figure 5C)**. Interestingly, in HCs, and patients with IPAH increased BTK protein levels in B cells correlated with increased proportions of circulating cTfh17 ($p=0.018$) and cTfh17.1 cells ($p=0.007$). BTK protein levels in B cells showed a negative correlation with proportions of cTfh2 cells ($p=0.014$; **Figure 5D**) and did not correlate with proportions of cTfh1 cells (**Figure 5D**) or total cTfh cells (**Supplementary Figure 5**).

In conclusion, patients with IPAH displayed an imbalance in cTfh-cell subset distribution, with increased cTfh17 cells, whereby increased cTfh17 and cTfh 17.1 proportions correlate with enhanced B-cell activation.

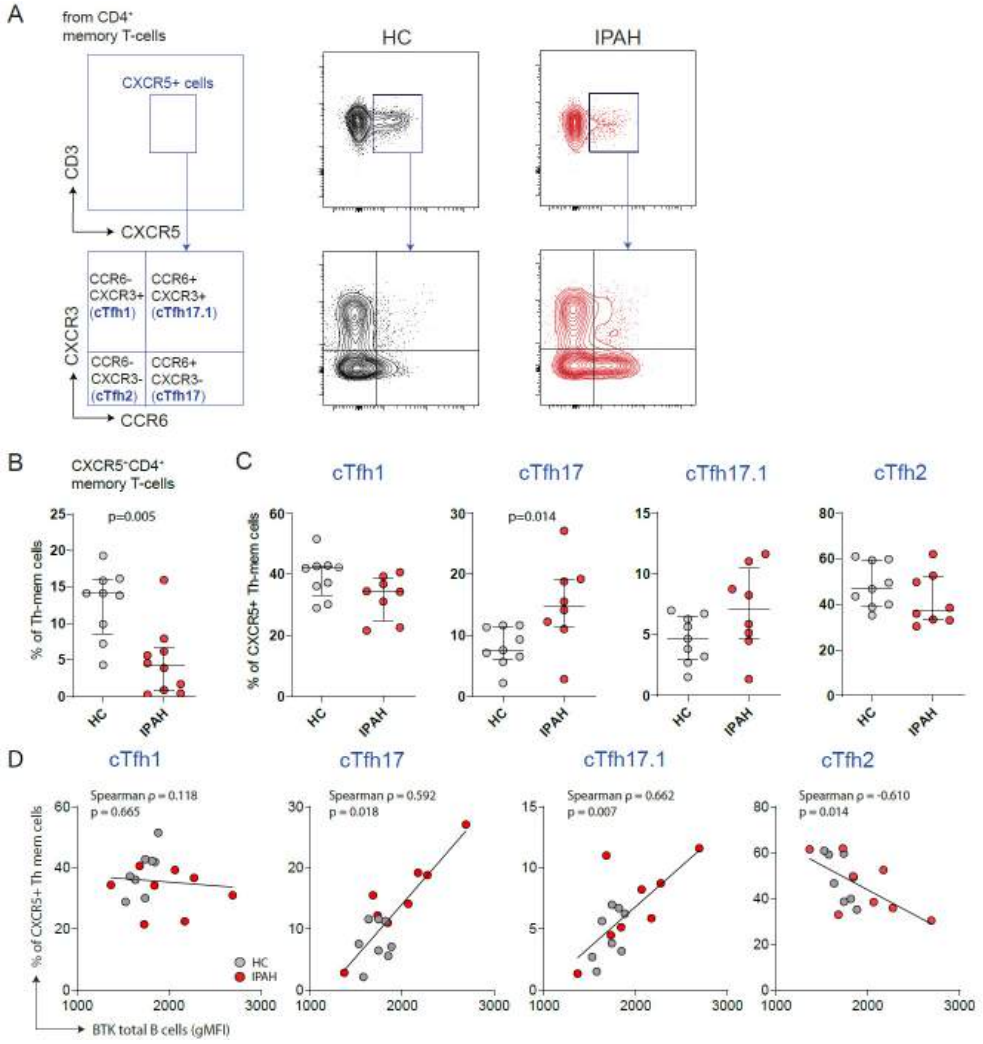


Figure 5. Increased proportions of cTfh-17 cells in patients with IPAH

(A) Representative gating strategy for the identification of CXCR5$^+$ circulating T follicular T helper (cTfh) cells, starting from non-regulatory (CD25$^+$CD127^{low}) CD4$^+$CD45RA$^-$ memory T-cells, and further discrimination of cTfh subsets based on surface CCR6 and CXCR3 expression: cTfh-1 (CCR6$^-$CXCR3$^+$), cTfh17.1 (CCR6$^+$CXCR3$^+$), cTfh17 (CCR6$^+$CXCR3$^-$) and cTfh2 (CCR6$^-$CXCR3$^-$), as depicted for a HC and patient with IPAH. (B) Proportions of CXCR5$^+$ memory T cells as percentages of total memory CD4$^+$ T cells. (C) Proportions of the indicated cTfh subsets as percentages of CXCR5$^+$ memory CD4$^+$ T cells. (D) Correlations of cTfh-subsets and BTK protein in total B-cells. The results are shown as median (IQR), p exact values were obtained following Mann-Whitney U test. Correlation coefficient was calculated using Spearman's rank method. Dots represent values in individual patients.

Discussion

To investigate the involvement of activated B-cells in the pathogenesis of IPAH, we studied an autoimmune-prone mouse model with enhanced BCR signaling, as well as circulating B- and T-cells in three groups of patients with PAH.

We observed that CD19-hBTK transgenic mice with increased protein levels of the BCR-associated kinase BTK specifically in B cells developed hemodynamic and cardiac signs of PH, following induction of pulmonary injury with bleomycin. In this hypothesis-generating two-hit model the MedLNs contained active GCs with prolonged B-cell activation and increased proportions of activated $ICOS^{high}$ Tfh-cells, while autoantibodies with reactivity against vascular antigens were present in the serum. In parallel, peripheral blood B-cells from patients with IPAH displayed increased BTK protein expression, already in naïve B-cells. The increase in BTK levels was associated with enhanced BTK phosphorylation in B-cells, the presence of IgG autoantibodies in plasma, as well as higher proportions of circulating Tfh17-cells. Taken together, our study provides evidence that loss of immune homeostasis - characterized by altered BCR signaling in (naïve) B-cells, activation of B-cells in GCs and an unbalanced Tfh-cell subset distribution - can contribute to PH development.

Our findings are in line with RNA expression studies on peripheral blood total B-cell samples that suggest that B-cells are activated in patients with IPAH, compared with HCs.[28] Several checkpoints exist in the bone marrow and in the periphery that prevent the development of autoreactive B-cells and their inadvertent activation. However, enhanced BCR signaling due to BTK overexpression in mice is sufficient to induce resistance to Fas-mediated apoptosis and development of B-cell mediated autoimmunity[14]. Parallel to our recent observation of increased BTK expression and phosphorylation in patients with anti-citrullinated protein antibody-positive rheumatoid arthritis, Sjogren's syndrome and autoimmune vasculitis[13, 29], we found that BTK expression was also increased in naïve B-cells in patients with IPAH. Since such B-cells have not encountered antigen, a possible explanation of these findings is that signals from the micro-environment, such as cytokine and chemokine levels or presence of Toll-Like receptor ligands, induce changes in the epigenome, transcriptome or proteome of naive B-cells.[30] It is therefore attractive to speculate that the threshold for activation of naive B-cells by self-antigens is reduced, which would then contribute to engagement of naive B-cells in IPAH pathology. Alternatively, it is possible that in patients with IPAH the proportions of circulating autoreactive naïve B-cells is increased as a result of defective self-tolerance in developing B-cells, as was also shown in the autoimmune disease systemic lupus erythematosus (SLE).[31] In any case, we found that 6 out of 11 patients with IPAH had circulating autoantibodies as well as high BTK levels in their peripheral blood B-cells. We therefore conclude that in this regard the phenotype of patients with IPAH is most probably heterogeneous, whereby a substantial fraction of patients with IPAH displays striking immunological similarities to patients with CTD-PAH.[32]

We observed that BTK levels were not increased in untreated patients with ILD-PH and were decreased in patients receiving treatment with anti-fibrotics. Although the pathogenesis of ILD-PH is multifactorial, sustained hypoxia is believed to be one of the most frequent

inducers of PH in this group.[33, 34] Chronic hypoxia may lead to decreased nitric oxide, increased hydrogen peroxide (H^2O^2), and alterations in voltage-gated K^+ channels.[33, 34] It is conceivable that these mechanisms leading to hypoxic-induced PH operate in an inflammation-independent way and do not involve enhanced B-cell activation. Because BCR signaling is sensitive to treatment or disease remission[13, 29] , anti-fibrotic treatment of ILD-PH patients might have influenced BTK protein expression.

We observed that in patients with PAH the proportions of non-switched memory B-cells in the circulation were decreased, which was also observed in various auto-immune diseases.[26, 27, 35] This is particularly interesting because self-reactive B cells are removed from the repertoire at the transition from $CD27^-$ naïve B-cell to non-switched $CD27^+$ memory B-cell, thus at a stage that precedes the induction of somatic hypermutation.[36] These NSM B cells have the capacity to interact with T cells in secondary and tertiary lymphoid organs in patients with IPAH, whereby their increased BTK expression levels may disrupt T cell homeostasis. This would be supported by our finding that increased BTK-mediated signaling in B cells involves a positive-feedback loop that establishes T cell-propagated autoimmune pathology and is accompanied by increased proportions of splenic Tfh cells.[18] Hereby, the increased surface expression of the co-stimulatory molecules CD80 and CD86 on CD19-hBTK transgenic B cells likely supports T-cell activation. Interestingly, BTK levels in B cells correlated with ICOS expression on cTfh cells in rheumatoid arthritis patients and with parotid gland T cell infiltration in Sjögren syndrome patients.[13] In this context, it is of note that we found that proportions of cTfh17 cells were increased in patients with IPAH, similar to findings in systemic sclerosis[20] and correlated with BTK expression levels in circulating B-cells. In general, IL-17 immune polarization is a feature of several chronic inflammatory and autoimmune conditions.[37] Moreover, mounting evidence suggest that Th17 immune polarization is also a feature of PAH. $CD4^+$ T cells isolated from patients with PAH contain higher levels of IL-17A.[38, 39] Additionally, the numbers of Th17 cells are increased and these cells are often localized in perivascular regions.[38, 39, 40] Also adoptive transfers of Th17 cells to *Rag1*$^{-/-}$ mice, lacking mature T and B-cells, induced pulmonary hypertension symptoms independent of chronic hypoxia.[38] However, whether the cTfh17 or cTfh17.1 cells represent a functional and clinical relevant T cell subtype is currently unknown and is a topic for further research.

The use of a bleomycin mouse model for PH research raises some concerns, since bleomycin exposure induces extensive lung damage, fibrotic changes and PH symptoms at 3 weeks.[21, 22, 41] It is unclear if PH indices persist at later time points, but resolution of fibrosis occurs before ~10 weeks after bleomycin exposure.[24] Although endothelial damage or pulmonary injury associated with viral infections are thought to be involved in PAH development[42], the extent and dynamics of lung damage in the bleomycin mouse model generally does not reflect lung injury seen in patients. Moreover, the bleomycin mouse model depends on inflammation and therefore we cannot formally rule out that additional inflammatory processes critically contribute to PH development in our mouse model. It is conceivable that PH development in CD19-hBTK mice may be promoted by chronic B-cell activation and autoantibodies. This would be supported by our observation that

that influenza virus infection provoked rapid autoantibody formation in the lungs of CD19-hBTK mice.[14] Nevertheless, it would be interesting to explore if enhanced BCR signaling in other PAH models, such as the BMPR2-targeted mice, would lead to progressive PAH. Although our mouse model is primarily hypothesis-generating, we nevertheless observed interesting similarities with human B-cell pathobiology.

Together with the described anomalies in the B-cell compartment, suggestive for a sustained immune response against self-antigens in patients with PAH.[9, 10, 28, 32, 43], our findings indicate that B-cell modulating therapies may hold potential for the treatment of PAH. Accordingly, B-cell depletion with anti-CD20 antibodies attenuated PH development in rats exposed to SU5416 and ovalbumin immunization.[44] Very recently, a double-blinded, randomized placebo-controlled clinical trial including 57 patients with systemic sclerosis (SSc)-PAH indicated that B cell depletion therapy with anti-CD20 antibody rituximab is a potentially effective and safe adjuvant treatment for SSc-PAH.[45] Whether anti-B-cell therapies could be effective in patients with IPAH needs to be further evaluated. Crucial will be the selection of patients with IPAH eligible for B-cell modulating therapies next to the current standard of care with vasoactive medication. Selecting patients should be based on inflammatory biomarkers, preferably those that reflect B-cell (auto-)reactivity or activation status. From our perspective, BTK levels could be a candidate biomarker, however its usefulness should be tested in prospective trials.

A limitation of our study is that we investigated only a relatively small number of patients within each PAH WHO-subclass, which precluded subgroup analyses and identification of correlations with clinical parameters or survival. Further research with larger sample sizes is needed to confirm our findings.

In conclusion, our findings provide evidence that enhanced BCR signaling in B-cells and increased circulating Tfh17 cell polarization – together with lung damage – contribute to autoimmune-mediated vascular remodeling and disease pathogenesis in patients with IPAH. This new perspective on the etiology of IPAH may prompt future translational studies on immune cells and inflammatory mediators as a potential diagnostic or prognostic marker in IPAH. Moreover, the identification of loss of immune homeostasis in patients with IPAH points to potential for new therapeutic anti-inflammatory targets in selected patients, next to existing vasoactive therapies.

Acknowledgments

We would like to thank Jasper Rip, Laurens Kil, Marjolein de Bruijn, Ingrid Bergen and the EDC Animal Facility staff (Erasmus MC Rotterdam) for their contribution to this project.

Abbreviations list

BCR = B-cell receptor
BTK = Bruton's tyrosine kinase
CD = cluster of differentiation
CD19-hBTK mice = transgenic mice with increased BTK protein levels in B-cells
CHD-PAH = congenital heart disease Pulmonary arterial hypertension
CTD-PAH = connective tissue disease associated Pulmonary arterial hypertension
CCR = C-C chemokine receptor
CXCR = C-X-C motif chemokine receptor
EDTA = ethylenediaminetetraacetic acid
GC = germinal center
HC = healthy control
HEp2 = human epithelial cells
IgG = immunoglobulin G
ICOS = inducible costimulator
IPAH = idiopathic pulmonary arterial hypertension
LN = Lymph node
MedLN = mediastinal lymph node
NSM= non-switched memory
PBS = phosphate-buffered saline
PBMC = Peripheral blood mononuclear cell
PH = pulmonary hypertension
PAH = Pulmonary arterial hypertension (PAH)
RVSP = right ventricular systolic pressure
SLE = systemic lupus erythematosus
Tfh = follicular T helper
TLO = tertiary lymphoid organ
WT = wild type

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Supplementary data

Supplementary methods

Human flow cytometry procedures

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes (BD Vacutainer K2E). Peripheral blood mononuclear cells (PBMCs) and plasma were obtained, processed and stored according to standard protocols.[1]

PBMCs were stained for extra- and intracellular markers (**supplementary table 1**). To prevent non-specific labeling Fc-block (Anti-Mouse CD16/CD32 Fc-Block) was used. Fixable Viability Dye eFluor 506 (eBiosciences) was applied as a live-dead marker. Flow cytometry procedures for B-cell staining have been described previously[2]. Cells for the T-cell staining were first incubated in MACS buffer (0.5% BSA + 2mM EDTA in PBS) with fluorescent antibodies against chemokine receptors for 60 minutes at 4˚C. A second extracellular incubation step was performed for antibodies with Brilliant Violet (BV) conjugates in Brilliant Stain-buffer (BD Biosciences, cat#563794). After fixation and permeabilization, cells were incubated with a forkhead box P3 (FOXP3)-specific antibody in permeabilization buffer for 60 minutes at 4˚C. Live cells (>200,000) were acquired and data were analyzed by FACS Flow-Jo software.

Mouse experiments and procedures

Genotyping and inducing pulmonary injury

CD19-hBtk[3] on a mixed background (Fvb × 129/Sv × C57BL/6J) were backcrossed on C57BL/6J for > 10 generations. Genotyping was performed by polymerase chain reaction (PCR), as previously described.[4] Wild-type mice used for the experiments are non-transgenic littermates. Mice were bred and kept at specified pathogen-free conditions in the Erasmus MC experimental animal facility. All experimental protocols have been reviewed and approved by the Erasmus MC Committee of animal experiments.
To induce pulmonary injury, bleomycin-hydrochloride was administered intra tracheally in 8-10 week old mice (0,04U/80 µl saline) or saline as a control as previously described.[5] Mice were sacrificed 21 days and 70 days after bleomycin exposure.

Right ventricular systolic pressure (RVSP) and lung tissue elastance

Mice were anaesthetized with isoflurane and right ventricular pressures were recorded using right heart catheterizations (mikro-tip catheter 1,4F , Millar instruments, model SPR-671) and analyzed with WinDaq Data acquisition software. Lung tissue elastance was measured with a flexiVent FX system as previously described.[6] Data was analyzed with flexiWare 7 software.

Flow cytometric procedures

Preparations of single-cell suspensions of MLN using standard procedures. Monoclonal antibodies are listed in **supplementary table 1**. For intracellular staining, cells were fixed in Cytofix/Cytoperm and permeabilized, and then stained in Perm/Wash buffer (BD Bioscience). All measurements were performed on a LSRII flow cytometer (BD Bioscience), and results were analyzed using FlowJo software.

Immunohistochemistry

Immunohistochemical analyses and staining were performed according to standard procedures.[7] Used antibodies are listed in **supplementary table 1**. After staining, tissue sections were embedded in Kaiser glycerol gelatin (Merck). Pulmonary vascular remodeling was studied by quantification of intraacinar pulmonary vessels containing a-smooth muscle actin (α-SMA)-positive cells in their walls. Vessels between and 15 and 50um external diameter and located in normal lung tissue were assessed.
To assess the presence of self-reactive antigens in serum of CD19-hBTK and control mice, serum was stored and frozen until further use. Serum was subjected to cryo-sections of lungs of $RAG^{-/-}$ mice (lacking mature B and T lymphocytes). Detection Antibodies against IgG and IgM are listed in **supplementary table 1.** Micrographs were made using a DM LB light microscope (Leica), a DFC500 camera (Leica), and Imaging for Windows Version 1.0 software (Kodak).

The Fulton index

Hearts were excised and dissected to determine the ratio of right ventricular to left ventricular and septal weight [RV/(LV+S)].

Hydroxy proline assay

Whole left lung homogenates were analyzed by quantitative hydroxyproline assay. The left lung homogenate was hydrolyzed in 6M HCl at 95˚C for 20 hours. Hydroxyproline was oxidized with chloramine t, and visualized with Erlich's reagent (4-DMAB, isopropanol and perchloric acid) measured at a microplate reader at 560nm.

HEp-2

To assess the presence of self-reactive antigens in serum of CD19-hBTK and control mice, serum was stored and frozen until further use. Serum samples (1/50 diluted) were incubated for 1 hour on Kallestad human epithelial cell (HEp-2) slides (Bio-Rad Laboratories). As detection antibodies Ig F(ab')2 fragments were applied to the HEp2 slides (**supplementary table 1**). The fluorescence intensity of HEp2 slides was evaluated using a LSM 311 META confocal fluorescence microscope (Zeiss) and LSM Image Browser Version 4.2.0.12 software (Zeiss)

α-SMA area to total artery area

Percentage α-SMA area to total artery area was evaluated using NanoZoomer 2.0-HT slide scanner (Hamamatsu) and NDP.view 2.7.25 (Hamamatsu). Photos were analysed using Adobe Photoshop 2021 in an automated and thus independent manner.

Total fibrosis score

A pathologist (blinded for treatment) scored the Ashcroft scale (grade 1-8)[8] and the percentage of lung involvement (grade 1-5; 1 =0-10% to 6 = 75-100% of total lung involvement). The Total Fibrosis score (TFS) is the product of Ashcroft scale and lung involvement and was previously described. [9]

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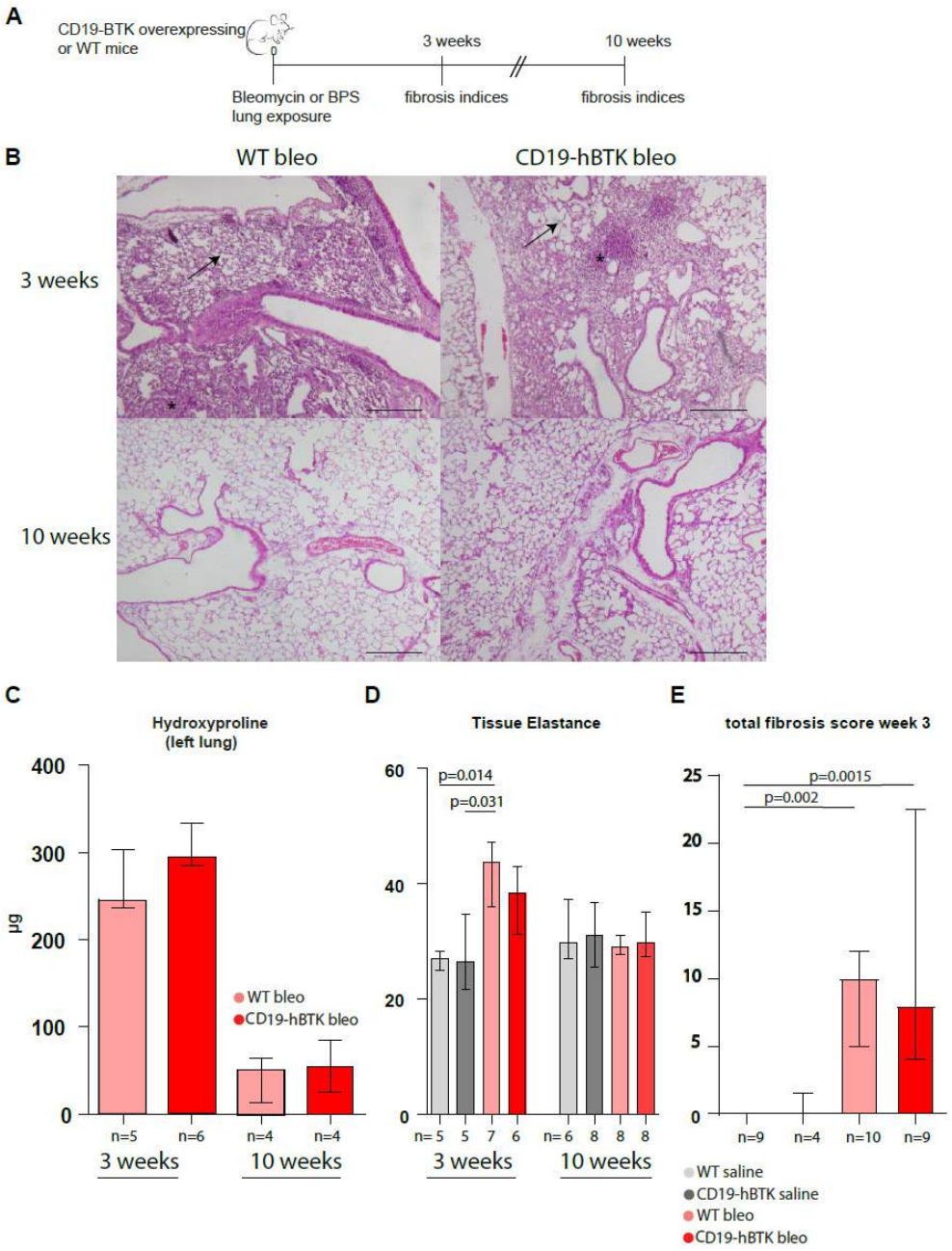
Supplementary Table 1 Overview of extra- and intracellular antibodies use for human and mouse experiments.

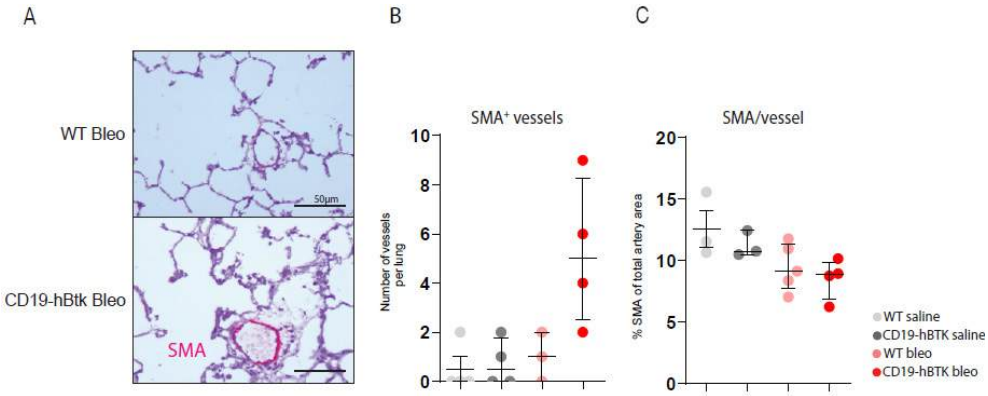
marker	conjugate	company	Cat#	intra/extracellular	dilution
HUMAN					
antibodies used for flow cytometry					
IgG	FITC	BD	555786	extracellular	1:20
Btk	PE	BD	611117	intracellular	1:5
IgM	Bio	BD	555781	extracellular	1:20
CD19	PerCP-Cy5.5	BD	332780	extracellular	1:400
CD38	APC	BD	560980	extracellular	1:10
IgD	APC-H7	BD	561305	extracellular	1:10
CD27	BV421	BD	562513	extracellular	1:80
CD24	BV711	BD	563401	extracellular	1:40
CD3	AF700	eBioscience	56-0038-42	extracellular	1:40
CXCR5	PerCP5.5	BD	562781	extracellular	1:20
CD3	APC ef780	eBioscience	47-0038-42	extracellular	1:100
CD4	AF700	eBioscience	E08948-1631	extracellular	1:100
CD45RA	BV650	BD	563963	extracellular	1:40
PD1	BV786	BD	563789	extracellular	1:20
FoxP3	PE	eBioscience	12-4777-42	intracellular	1:20
CXCR3	BV711	BD	563156	extracellular	1:20
CCR4	FITC	BD	FAB1567F	extracellular	1:20
CCR6	APC	BD	560619	extracellular	1:5
CXCR5	PerCP5.5	BD	**562781**	extracellular	1:20
HEp-2 antibodies					
Anti-Mouse F(ab') IgG	Cy3	Jackson IR	115-166-003		
MOUSE					
antibodies used for flow cytometry					
GL7	FITC	BD	553666	extracellular	1:2000
CD95	PE-TxR	BD	562499	extracellular	1:400
IgM	Pe-Cy7	eBioscience	25-5790-82	extracellular/ intracellular	1:500

marker	conjugate	company	Cat#	intra/extracellular	dilution
IgD	APC	eBioscience	17-5993-82	extracellular/ intracellular	1:1280
CD19	Af700	eBioscience	56-0193-82	extracellular	1:50
CD138	BV605	BD	563147	extracellular	1:400
CD3	PE-CF594	BD	562286	extracellular	1:100
CD4	Af700	eBioscience	56-0041-82	extracellular	1:200
MHC II	FITC	BD	553565	extracellular	1:200
PD-1	PE	BD	551892	extracellular	1:100
CD3	PE-CF594	BD	562286	extracellular	1:100
CD40L (CD154)	PerCP-eFl710	eBioscience	46-1541-82	extracellular	1:100
CXCR5	biotin	BD	551960	extracellular	1:50
ICOS	APC	eBioscience	17-9949-82	extracellular	1:1600
CD4	AF700	eBioscience	56-0041-82	extracellular	1:400
CD11c	APC-eFl750	eBioscience	47-0114-82	extracellular	1:200
CD11b	eFl450	eBioscience	48-0112-82	extracellular	1:200
PD-L1	BV711	BD	563369	extracellular	1:100
HEp-2 antibodies					
Anti-Human F(ab') IgG	Cy3	Jackson IR	109-166-003		
Immunohisto-chemistry Lung					
Anti-hαSMA	PE	R&D	IC1420P		
Anti-PE	AP	Rockland	600-105-387		
Anti-B220	Unlabeled	Bioceros			
Anti-Rat	AP	Sigma	A8438 – 1ML		
Anti-hCD3	Unlabeled	DAKO	A0452		
Anti-Rabbit	Biotin	Biogenex	HK326-UR		
Anti-Goat	AP	Sigma	A4187 – 1ML		
Anti-IgG	Biotin	S. Biotech	1030-08		
Anti-IgM	Biotin	S. Biotech	1020-08		
Streptavidin	AP	Biogenex	HK321-UK		
Stretavidin	PO	Biogenex	HK320-UK		

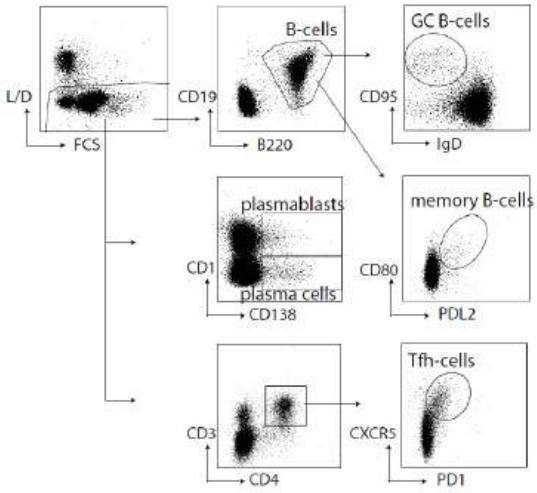


Supplementary Figure 1. Similar fibrosis indices in WT and CD19-hBTK mice upon bleomycin exposure. (A) Mice were sacrificed 3 and 10 weeks after saline or bleomycin exposure and analyzed for fibrosis indices. (B) Representative hematoxylin/eosin (H&E) staining of cryo-sections of lung tissue of a WT or CD19-hBTK mouse 3 and 10 weeks after bleomycin exposure. Inflammatory exudate and obvious damage to lung architecture (asterisk) and diffuse fibrous thickening of alveolar septa (arrow). Resolution of fibrosis at 10 weeks. Magnification 40x. (C) Hydroxyproline content

(µg per left lung) in WT or CD19-hBTK mice 3 and 10 weeks post bleomycin exposure (D) Tissue elastance assessed with a flexiVent FX system in WT or CD19-hBTK mice 21 days and 70 days post saline or bleomycin exposure. (E) Total fibrosis score. A pathologist (blinded for treatment) scored the Ashcroft scale (grade 1-8) and the percentage of lung involvement (grade 1-5; 1 =0-10% to 6 = 75-100% of total lung involvement). The total fibrosis score is the product of Ashcroft scale and lung involvement. The results (C-E) are shown as median (IQR), p exact values were obtained following a Kruskal-Wallis test. Number of mice used for each experiment are depicted below the graph.



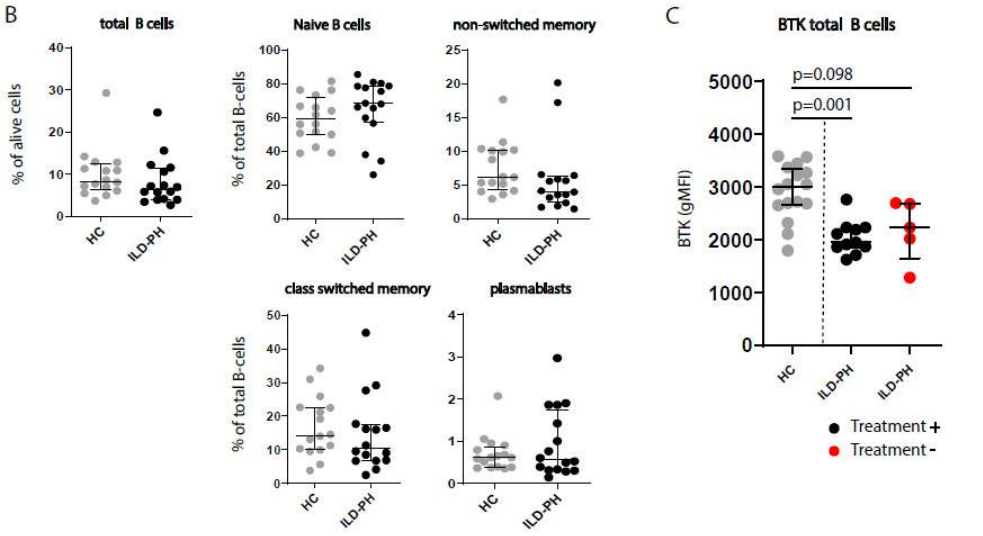
Supplementary Figure 2. α-SMA **in WT or CD19-hBTK mice 10 weeks post bleomycin exposure.**
(A) Representative hematoxylin/eosin and **α**-smooth muscle actin (**α**-SMA) staining of cryo-sections of the indicated lung tissue. (B) Number of SMA-positive vessels per lung. Vessels between 15 and 50µm external diameter and located in normal lung tissue were assessed. (C) Percentage **α**-SMA area to total artery area of the assessed vessels. The results are shown as median (IQR), p exact values were obtained following Kruskal-Wallis test. Dots represent individual mice.



Supplementary figure 3. Representative gating strategy for identification of B-cell subsets and follicular T helper (Tfh)-cells in mediastinal lymph nodes (MedLN) in mice, starting with live single cells. L/D, life-death marker; FCS, forward scatter. From the total CD19^{+}B220^{+} B-cell population, memory B-cells (CD19^{+}B220^{+}CD80^{+}PDL2^{+}), germinal center (GC) B-cells (CD19^{+}B220^{+}IgD^{-}CD95^{+}), plasmablasts (CD19^{+}CD138^{+}) and plasma cells (CD19^{low}CD138^{+}) were identified. From the CD3^{+}CD4^{+} population, total numbers of Tfh-cells (CD3^{+}CD4^{+}CXCR5^{+}PD1^{+}) were identified.

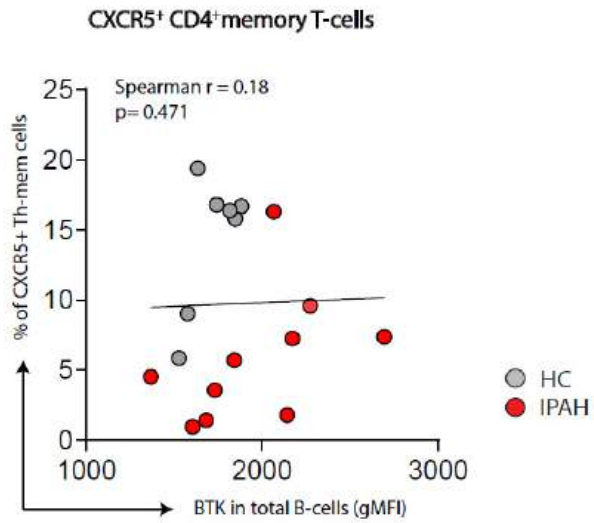
A

	gender (male)	age (years)	mPAP (mmHg)[A]	Immunomodulating therapies [B]	Anti fibrotics[C]
HC (N=16)	10 (63%)	59 (54-64.5)	-	-	-
ILD-PH (N=17)[D]	5 (29%)	63 (57.5-76)	35 (27-40)	5 (29%)	7 (41%)

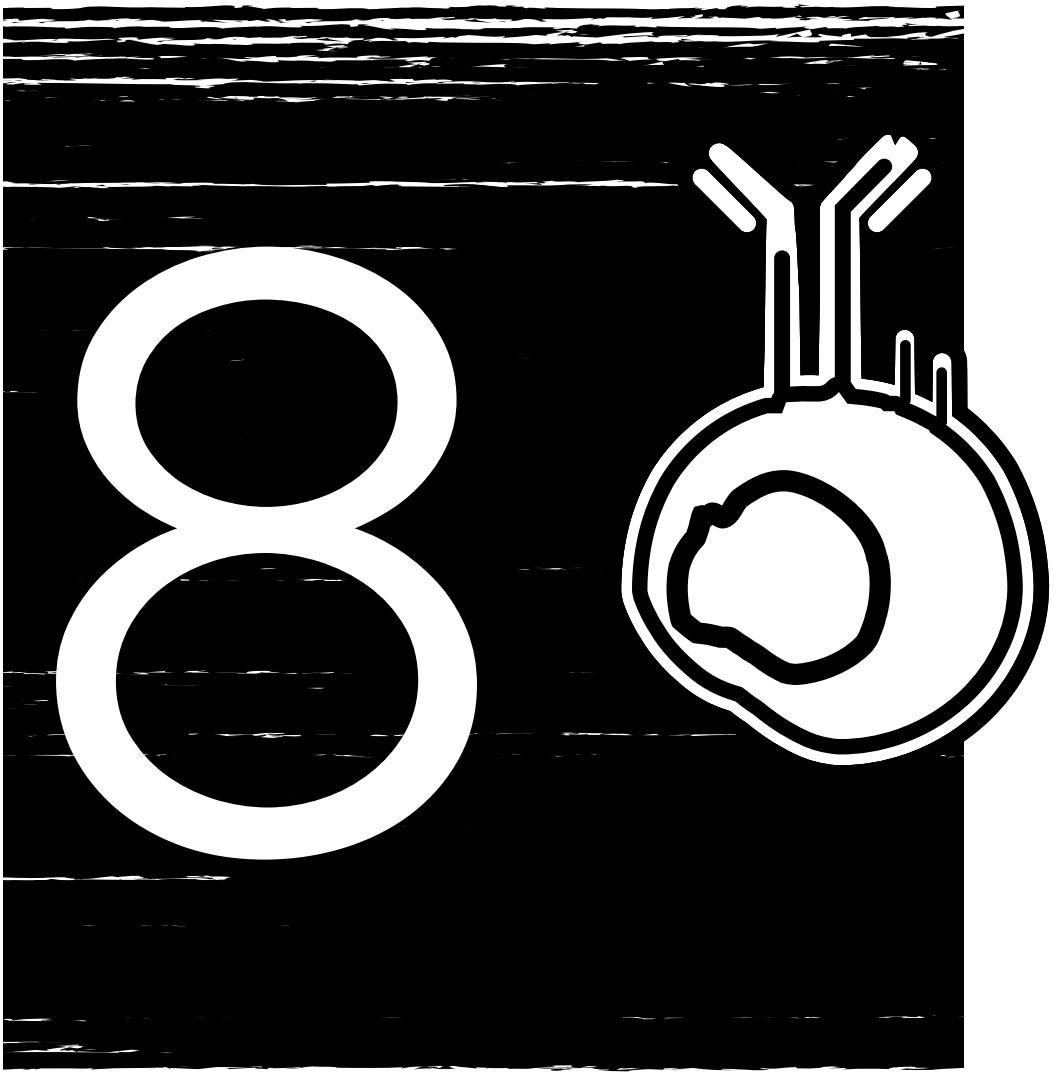


Supplementary Figure 4: B-cell subsets and BTK expression in ILD-PH . (A) Table showing the characteristics of patients with interstitial lung disease-related PH (ILD-PH) and healthy subject (HC). mPAP; mean pulmonary artery pressure. [A] In 6 patients only echocardiographic measurements available, showing estimated RVSP> 35mmHg and signs of RV dysfunction and no signs of left heart disease or left sided heart failure. [B] Four patients used prednisone >10mg/day and one patient was on azathioprine. [C] Nintedanib (n=3) or pirfenidone (n=4). [D] Ten patients with IPF, 3 patients with non-specific interstitial pneumonia, 1 patient with respiratory bronchiolitis interstitial lung disease, 1 patient with extrinsic allergic alveolitis, 1 patient with combined pulmonary fibrosis and emphysema, and 1 patient with interstitial pneumonia with autoimmune features. Continuous variables are presented as median and IQR in parentheses and categorical variable as count and percentages in parentheses.

(B) Proportions of circulating total B-cells and B-cell subpopulations (naïve B-cells ($CD19^+IgD^+CD27^-$), non-switched memory B-cells ($CD19^+IgD^+CD27^+$), and class switched memory B-cells ($CD19^+CD27^+IgD^-IgM^-$), and plasmablasts ($CD19^+CD38^{hi}CD27^+$)) in HC and patients with ILD-PH. (C) Quantification of BTK protein expression levels, shown as gMFI values of intracellular flow cytometry analysis of total B cells in HCs and ILD-PH patients receiving immunomodulatory or anti-fibrotic treatment (*black dots*) or no treatment (*red dots*). p exact values were obtained by a Mann-Whitney U test or Kruskal-Wallis test (>2 groups). Dots represent individual values in patients.



Supplementary Figure 5: No correlation of cTfh cells and BTK protein level in total B-cells. No correlation of circulating follicular T helper (cTfh) cells ($CD4^+CD45RA^-FoxP3^{low}CXCR5^+$) and BTK protein in total B cells in HC and patients with IPAH. Correlation coefficient was calculated using Spearman's rank method.


8

CHAPTER 8

Inflammatory mediators in Patients with Sarcoidosis Associated Pulmonary Hypertension: The Registry of Sarcoidosis Associated Pulmonary Hypertension (ReSAPH)

MANUSCRIPT IN PREPARATION

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Abstract

Introduction

Pulmonary hypertension (PH) is a significant cause of morbidity and mortality in sarcoidosis, however little is known about the underlying pathophysiology and inflammatory mediators that govern PH development. We analyzed disease-related inflammatory mediators in a multi-national registry of sarcoidosis-associated PH (SAPH) patients.

Methods

Inflammatory mediators in serum of patients with SAPH (n=66), advanced sarcoidosis (n=33), idiopathic pulmonary arterial hypertension (n=16) and healthy controls (n=20) were analyzed with multiplex cytokine and enzyme-linked immunosorbent assays.

Results

We found elevated serum concentrations of C-X-C chemokine ligand (CXCL) 13, CXCL9, Interleukin (IL)-7, C-C motif ligand (CCL) 4, IL-8, tumor necrosis factor alpha (TNF-α), Vascular endothelial growth factor (VEGF) in patients with SAPH, compared with healthy controls. The CXCL13 and CXCL9 chemokines were increased in patients with SAPH but not in patients with advanced sarcoidosis without PH, nor in patients with IPAH. Additionally, we found a correlation between serum VEGF levels and increased mean pulmonary arterial pressure. CCL4 correlated with cardiac index, but also separated sarcoidosis patients from SAPH patients in unbiased principal component analyses (PCA). The PCA also revealed that IL-7, CXCL13 and CXCL9 are most relevant to discriminate between SAPH and other patient groups or healthy controls.

Conclusions

Our study identified possible new underlying pathways and candidate biomarkers in a large multicenter SAPH cohort. Particularly alterations in CXCL13 and CXCL9 suggest that underlying autoimmune pathways are involved or co-occurring in SAPH and sarcoidosis. Additionally, VEGF and CCL4 correlate with hemodynamic parameters, indicating their involvement in vascular remodeling. Our findings may boost new prospective translational studies that investigate (mechanistic) biomarkers and therapeutic targets in SAPH patients.

Background

Sarcoidosis is a chronic inflammatory disease of unknown cause[1]. It mainly affects the lungs and mediastinal lymph nodes, but can affect any organ of the body. About a quarter of patients develop chronic or progressive disease, which leads to a high degree of morbidity and health-care use[1]. An important complication is the development of pulmonary hypertension (PH) with an incidence of ~3 to 20% in sarcoidosis patients.[2, 3, 4, 5] Sarcoidosis-associated PH (SAPH) can lead to heart failure and is associated with a poor outcome.[2, 6] The etiology of SAPH is often multifactorial and includes chronic hypoxia, mechanical obstruction of the lymphatic and venous system, as well as inflammation and fibrosis in the pulmonary vasculature.[7] On a microscopic level, granuloma formation in the vasculature and heart contribute to SAPH development.[7, 8]

Granuloma formation is the result of immunological changes that lead to an exaggerated antigen-driven adaptive immune response and the creation of a conglomerate of epithelioid- and multinucleated-giant cells encircled by T cells and B cells.[9, 10, 11] T and B cell-related inflammatory markers including cytokines, chemokines and growth factors, are needed for the formation of granulomas and recruitment of T and B cells.[10] Various inflammatory mediators are evaluated in sarcoidosis.[10, 11, 12] Serum of patients with sarcoidosis contain increased levels of C-X-C chemokine ligand (CXCL) 9 and correlate with systemic organ involvement.[13] Likewise, elevated C-C motif ligand (CCL) 4 levels in bronchoalveolar lavage fluid (BALF) were observed across all sarcoidosis scadding stages.[14] Both of these chemokines are involved in the differentiation of interferon gamma (IFNγ) producing T-helper 1 (Th1) cells and contribute to the Th1 inflammatory response commonly seen in sarcoidosis.[1] Also CXCL13, which is a chemoattractant for B cells, is overexpressed in lungs of patient with sarcoidosis.[15] Interleukins (IL) are also altered in sarcoidosis.[10] For example, IL-7 has a critical role in lymphocyte regulation, homing and homeostasis and polymorphisms in the IL7-receptor contribute to the genetic risk in sarcoidosis.[16] Vascular endothelial growth factor (VEGF), implicated in vascular remodeling, is increased in BALF and serum of patients with sarcoidosis[17] and could be an interesting link to SAPH development.

Despite the growing evidence of the involvement of inflammatory mediators in sarcoidosis, serum inflammatory mediators that predict patients with sarcoidosis who are at risk for PH are still lacking. Additionally, gaining more knowledge on the underlying pathobiology and better understanding which inflammatory mediators are involved in granuloma formation and vascular remodeling in SAPH may also help selecting new targets for future therapies.[18] To gather more clinical and immunological knowledge on SAPH, an observational Registry for Sarcoidosis Associated Pulmonary Hypertension (ReSAPH) was established in which blood samples were prospectively collected from patients with incident or prevalent SAPH. The registry was also designed to collect information regarding the initial presentation and subsequent clinical course of SAPH patients from sarcoidosis centers across the world. Clinical data have recently been published elsewhere.[19] We compared inflammatory mediators in SAPH patients, patients with advanced sarcoidosis without PH (SRC), patients with idiopathic pulmonary arterial hypertension (IPAH) as well as healthy

controls (HC). We identified relationships between clinical, functional and hemodynamic parameters, and performed an unbiased principal component analysis (PCA) to evaluate which inflammatory mediator is most relevant to discriminate SAPH patients.

Methods

Study design and subjects

Patients with a diagnosis of sarcoidosis based on the ATS/ERS/WASOG criteria and hemodynamic diagnosis of PH were enrolled in an eleven-center observational registry for ReSAPH.[19] The ReSAPH was initiated in October 2011. All included patients had at least one right heart catheterization (RHC) demonstrating a mean pulmonary artery pressure (mPAP) ≥25mmHg and a pulmonary artery wedge pressure (PAWP) of 15 mmHg or less. The study is registered at ClinTrials.gov under number NCT01467791.

Information about SAPH patients who entered the ReSAPH registry was recorded in a secure web-based electronic database (REDCap).[20] Each investigator had obtained local institutional review board approval prior to entering patient information into the database. For each SAPH patient, the first RHC identifying PH was recorded. At time of entry into the study, patients had their medical history taken and were subjected to a focused physical examination. An overview of all values recorded has been described elsewhere.[19] Our study included serum samples of SAPH patients (total n=66) collected at the University of Cincinnati Medical Center (Cincinnati, OH, USA) (n=35) and the Cleveland Clinic (Cleveland, OH, USA) (n=21). To expand this SAPH cohort, we also included baseline serum samples (n=10) of patients who were recruited for the BoSAPH trial at the University of Cincinnati Medical Center (Bosentan for sarcoidosis-associated pulmonary hypertension), NCT00581607.[21]

As a control, we included serum samples of SRC patients (n=33) without clinical signs of PH, IPAH patients (n=16), as well as aged and sex-matched healthy controls (n=20). Serum of sarcoidosis patients was collected at the University of Cincinnati Medical Center (Cincinnati, OH, USA). Serum of IPAH patients was collected from patients diagnosed with PH who entered the 'Biomarker activity in adults with pulmonary hypertenssion' (BioPulse) Registry in Erasmus MC (Rotterdam, Netherlands). IPAH was diagnosed according to current ESC/ERS Guidelines for the diagnosis and treatment of PH and all patients underwent a right heart catheterization.[22] Serum was collected at the time of diagnosis. The Medical Ethical Committee of the Erasmus MC approved this registry (MEC-2011-392).

All samples were obtained after informed consent and each investigator had obtained local institutional review board approval prior to collection of blood samples.

Serum processing and inflammatory mediator levels

Peripheral venous blood samples were collected (BD Vacutainer Serum tubes, cat# 367812), kept on ice, and centrifuged within 60 minutes. Serum samples were stored at -80˚C until analyzed. After thawing serum samples, inflammatory mediators were measured using a 12-plex cytokine assay (BioPlex Multiplex human cytokine multiplex panel; Bio-Rad, Hercules, CA, USA)(Lot# 4042555) for IL-8, tumor necrosis factor alpha (TNFα), and vascular endothelial growth factor (VEGF), according to the manufacturer's instructions. A Bio-Plex 200 instrument (Bio-Rad) and its software (Bio-Plex 6.1) were used for the analysis. For all samples, two rounds of experiments were performed.
For the detection of IL-7(Catalog # DY207), CXCL9(DY392), , CXCL4(DY795), CCL4(DY271), and CXCL13(DY801) an enzyme-linked immunosorbent assay (Human DuoSet ELISA) was performed according to standard procedures (R&D Systems, Inc, UK). Assays were read at 490/450 nm using an OpsysMR microplate reader (Dynex Technologies Ltd, UK).

Principal component analysis

Principal component analyses (PCA) were performed using R and RStudio, and the packages FactoMineR and Factoextra. Missing data were determined using the package MissMDA.[23, 24, 25] Prior to PCA, data were log10-transformed to better fit a normal distribution and scaled. Contribution of the variables to the dimensions was determined in percentages by (squared cosine of the variable*100) / (total squared cosine of the principal component).

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 21 and GraphPad Prism 6 software. For calculating the significance of differences multiple groups and the PCA, we used the Kruskal Wallis test combined with a Dunn's multiple comparison test. The variability explained by the PCA was tested for statistical significance by inertia of the first three dimension using the R package FactoInvestigate. Correlation coefficients were calculated using Spearman's rank method. P values <0.05 were considered significant.

Table 1. Overview of demographic and clinical characteristics of healthy subjects and patients used for this study.

	HC (n= 20)	IPAH (n=16)	SRC (n=33)	SAPH (n=66)
Females n (%)	10 (50)	10 (63)	18 (55)	28 (42)
Age (years ± SD)	54 ± 5	47 ± 14	52 ± 12	59 ± 10
Right heart catheterization (± SD)				
RAP (mmHg)		11.9 ± 6.4		7.2 ± 4.6
mPAP (mmHg)		58.6 ± 16.8		34.9 ± 8.0
PVR (wood units)		12.4 ± 5.5		5.0 ± 2.6
Carciac Index (L/min/m2)		2.2 ± 0.4		2.8 ± 0.7
Pulmonary function test (± SD)				
FEV1 (%pred)		74.6 ± 17.7	70.1 ± 23.9	59.5 ± 23.3
FVC (%pred)		85.5 ± 16.6	74.6 ± 17.0	65.5 ± 21.1
DLCO (%pred)		59.8 ± 17.3	..	45.7 ± 17.7
Exercise capacity (± SD)				
6MWD (m)		321 ± 152	332 ± 124	367 ± 126
Scadding stage 4 (% of pt)		0%	73%	66%

Results

Serum levels of inflammatory mediators in patients with SAPH

We analyzed serum samples of four groups; 66 patients with SAPH, 33 patients with advanced sarcoidosis without clinical sign of PH (SRC), 16 patients with IPAH, and 20 HC. **Table 1** summarizes the demographic features of all patients and HC in this study. Scadding stage, six minute walking distance and age/gender were similar across patients with SRC and SAPH.

Using a multiplex cytokine assay and ELISA, we determined serum concentrations of eight inflammatory mediators in HC and the three patient groups (**Figure 1A; Suppl. Table 1)**. The serum concentrations of the chemokines CXCL13 and CXCL9 and the cytokines IL-7 and IL-8 and the chemokine CCL4 were increased in both SRC and SAPH patients compared with patients with HC and IPAH. However, we did not observe significant differences for these four inflammatory mediators between SRC and SAPH patients. The levels of IL-8 were significantly higher in patients with SAPH than in HC and patients with IPAH or SRC.

We evaluated additional inflammatory mediators in HC, SAPH patients and patients with SRC (TNFa and CXCL4) and patients with IPAH (VEGF) (**Figure 1B; Suppl. Table 1)**. Compared with HC, serum TNFa concentrations were higher in patients with SRC and SAPH, but was not different between the two patient groups. CXCL4 levels were lower in SAPH patients compared with SRC patients, but in the same range as in HC. Finally, VEGF concentrations were significantly higher in SAPH patients compared to HC and IPAH patients.

In conclusion, patients with SAPH displayed a unique inflammatory profile that differed significantly from the patients with IPAH, and to a lesser extent also from patients with SRC.

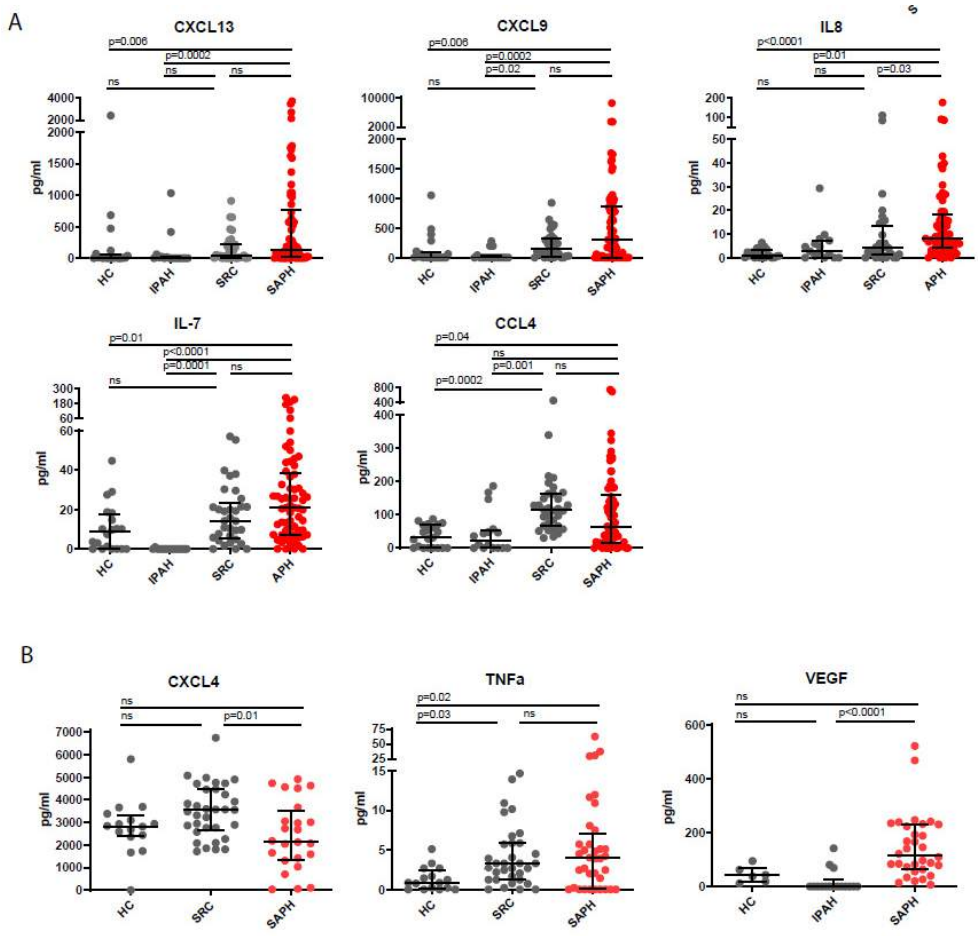

Figure 1. Serum levels of inflammatory mediators in SAPH

Serum concentrations of inflammatory mediators in healthy subject and IPAH, SRC and SAPH patients. The results are shown as median (IQR), p exact values were obtained following Kruskal-Wallis test. Dots represent values in individual patients or HC. Abbreviation: ns = not significant

Correlations of inflammatory mediators with hemodynamic parameters in SAPH patients

Having shown that serum inflammatory mediators were altered in SAPH patients, we sought to determine whether the levels of inflammatory mediators correlated with hemodynamic parameters (mPAP, right atrial pressure (RAP), cardiac index (CI), pulmonary vascular resistance (PVR), 6min walk distance, or pulmonary function tests (forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC) and diffusing capacity for carbon monoxide (DLCO)) in SAPH patients.

We observed a significant positive correlation between serum VEGF levels and mPAP (r=0.53, p=0.0017) (**Figure 2A**) and a significant negative correlation between serum CCL4 and CI (r=-0.41, p=0.0031) (**Figure 2B**). This suggest that within SAPH, VEGF and CCL4 may be involved in the process of vascular remodeling.

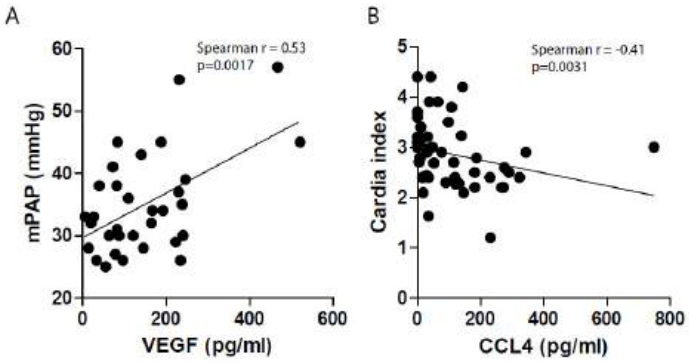

Figure 2. Correlations of inflammatory mediators with hemodynamic parameters in SAPH patients
Correlation of serum VEGF and CCL4 level with hemodynamic parameters. Correlation coefficients were calculated using Spearman's rank method.

Principal component analyses of inflammatory mediators

To obtain a more comprehensive overview of the inflammatory mediator profiles across the three patient groups and HCs, we performed PCA, which reduced the dimensionality of the data set. The PCA was performed on those five inflammatory mediators that were measured in all three patient groups (**Figure 1A**). The PCA of the inflammatory markers IL8, CCL4, XCXL9, CXCL13 and IL7, showed a non-random distribution over Dim1, Dim2 and Dim3. (**Figure 3A**)**.** Dim1 revealed a significant separation of SAPH from IPAH patients and HC (**Figure 3A and 3D**), whereby the impact of CXCL9, CXCL13, and IL7 was dominant. Dim2 only revealed significant separation of HC with the three patient groups which was mainly determined by IL-8 (**Figure 3A and 3E)**. Interestingly, in Dim3 we found significant separation of SAPH from SRC patients, whereby the impact of CCL4 was dominant (**figure 3B and 3F**)

Taken together, this multivariate data analysis of inflammatory mediators shows that CCL4 separated SRC patients from SAPH patients and that IL-7, CXCL13 and CXCL9 are most relevant to discriminate between SAPH and other patient groups and HC.

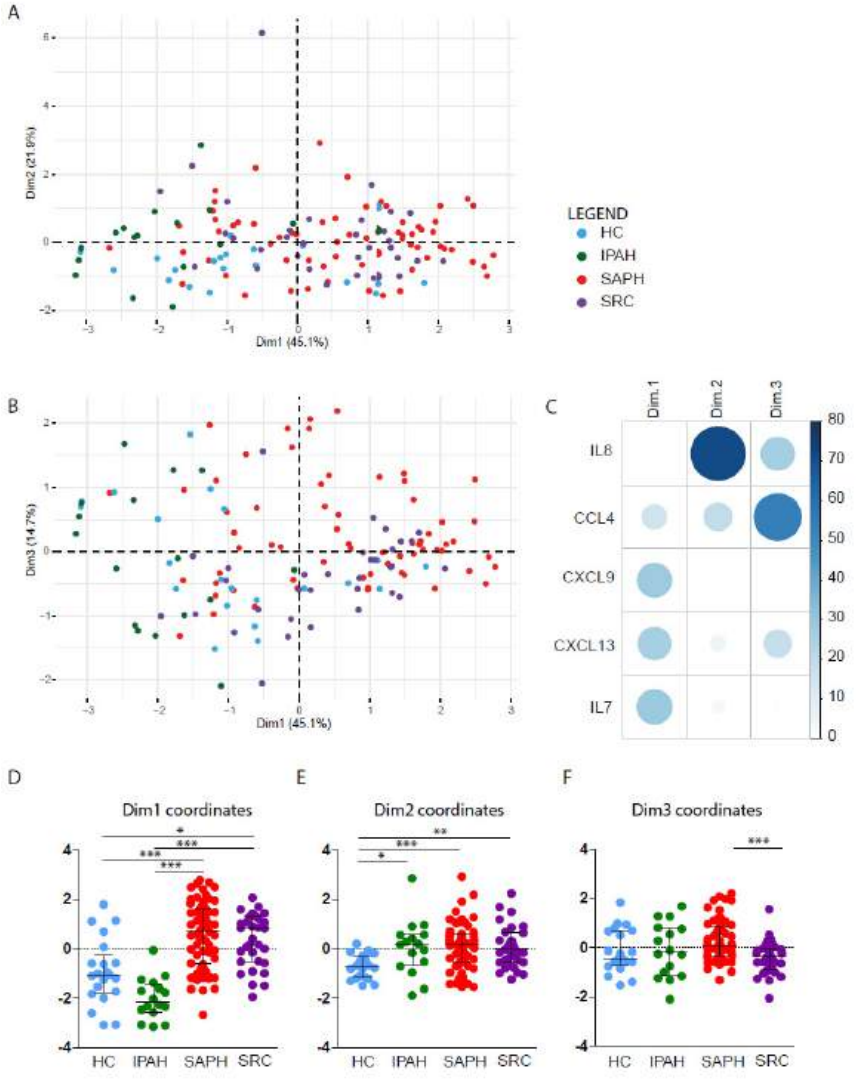

Figure 3. Principal component analyses of inflammatory mediators

(A-B) Principal component analyses of inflammatory mediators in HC, IPAH, SAPH and SRC patients for dimension (Dim) 1,2 and 3. (C) Contribution of each inflammatory mediator on the first dimension (Dim1), second dimension (Dim2) and third dimension (Dim3) of the PCA. (D-F) statistical analyses of the indicated dimension coordinates between HC and patient groups.

PCAs were on log10-transformed and scaled values. The results are shown as median and p exact values were obtained following Kruskal-Wallis test. Dots represent values of individual patients or HCs. Abbreviations: HC = healthy control, IPAH = idiopathic arterial pulmonary hypertension, SAPH = sarcoidosis associated pulmonary hypertension, SRC= sarcoidosis patients, Dim = Dimension

Discussion

Pulmonary hypertension is an important complication of sarcoidosis.[2, 6] Large studies that explore inflammatory profiles and biomarkers in this scarce subgroup of sarcoidosis patients are limited and therefore we analyzed inflammatory mediators that are known to be involved in sarcoidosis and chronic inflammation [ref]. The present study identifies elevated serum concentrations of CXCL13, CXCL9, IL-7, CCL4, IL-8, TNFa and VEGF in a cohort of SAPH. Strikingly, CXCL13 and CXCL9 were significantly increased in SAPH patients but not in patients with advanced sarcoidosis without PH, nor in IPAH patients. Using unbiased PCA, we identified that CXCL13, CXCL9 and IL-7 are most relevant to discriminate between SAPH and other patients groups or HC. In these PCA, the third dimension which was dominated by CCL4 significantly separated SRC patients from SAPH patients, although no significance was reached in an individual analysis of this chemokine. To our knowledge, this is the first time that such large real-life multi-center cohort of SAPH patients was analyzed for inflammatory profiles and identified potential new underlying mechanisms for SAPH development.

Using unbiased PCA on cytokines and chemokines that are known to be involved in sarcoidosis and chronic inflammation[10], we found that especially IL-7 and the chemokines CXCL9 and CXCL13 are most relevant to discriminate between SAPH and sarcoidosis patients from IPAH and HC. Especially in sarcoidosis patients with fibrotic pulmonary disease increased levels of IL-7 were observed, suggesting a role in the fibrotic response or ongoing inflammation which may indirectly lead to PH development.[26]

Cytokines CXCL9 and CXCL13 provide an interesting link to chronic inflammation and possibly autoimmunity. The majority of patients who entered the ReSAPH registry also had extra-pulmonary disease, which could explain the observed increase of CXCL9 levels in SAPH patients and may reflect a higher inflammatory burden and/or more extensive disease in sarcoidosis patients.[19] The receptor for CXCL9 is CXCR3, which is differentially expressed across Th1, Th17 and Th17.1 cells. CXCL9 is required for optimal IFN-γ production by Th1 and Th17.1 cells *in vivo* and especially IFN-γ-producing Th17.1-cells appear to be more pathogenic and possibly linked to disease progression in sarcoidosis.[11, 27, 28, 29, 30, 31] Additionally, these IL-17/IFNγ double-producing cells are pathogenic drivers in several autoimmune diseases.[28] Although the activating antigens have been a mystery - or holy grail - of sarcoidosis research, recent work showed that autoantibodies, possibly against misfolded proteins, are present in a subgroup of sarcoidosis patients.[32, 33] A possible role for autoimmunity and B cells in SAPH and sarcoidosis is further supported by increased levels of CXCL13, which is a chemoattractant for B-cells, we observed in our cohort of SAPH. Together with increased BAFF levels[34, 35], CXCL13 is important for the local formation of the B-cell conglomerates that are found around granulomas.[36, 37] It could be that these B cell conglomerates are producers of pathogenic antibodies, because IL-17 polarization in coordination with IL-21 and BAFF is sufficient to promote germinal center formation in experimental models.[38] Additionally, elevated serum CXCL13 and increased numbers of tertiary lymphoid follicles (TLOs) have been found in Idiopathic pulmonary fibrosis implicating that CXCL13 (and B cells) could play a role in the fibrotic process,[39] which is also often seen in SAPH patients.[19] Whether TLOs

are present in the lungs of SAPH patients and how local pathogenic antibodies may lead to pulmonary hypertension in patients with SAPH needs further research. Nevertheless, our work raises the suspicion that underlying autoimmune pathways or humoral immunity are involved or co-occuring in SAPH and sarcoidosis.

Although CCL4 levels did not differ between SRC and SAPH patients, our PCA showed that CCL4 (perhaps together with IL-8) was most relevant to discriminate between SAPH and sarcoidosis patients. Moreover, CCL4 levels correlated with a lower cardiac index in SAPH patients. Therefore, CCL4 could be interesting from a pathophysiological point of view. CCL4, also known as macrophage inflammatory protein-1β (MIP-1β) is a chemoattractant for several immune cells, including monocytes and T-cells.[40, 41] CCL4 also induces the release of pro-inflammatory cytokines such as TNF, IL-1, and IL-6 from (alveolar) macrophages.[41] In patients with extensive sarcoidosis, CCL4 levels were significantly reduced after treatment with infliximab and were associated with greater improvements in FVC and 6MWD.[42]. Only a very small fraction of SAPH patients in our cohort received infliximab. It would be interesting to see if SAPH patients would also benefit from infliximab treatment and if CCL4 could be a biomarkers of treatment response in SAPH.

We observed a correlation between increased serum VEGF levels and increased mPAP. VEGF stimulates the formation and remodeling of blood vessels and is therefore potentially interesting in SAPH. The role of VEGF in PH has been extensively explored and reviewed.[43] VEGF levels are elevated in patients with severe PAH and correlate with clinical worsening in pediatric PAH patients.[44, 45] Conversely, several experimental PH models that targeted the VEGF pathway or receptor point toward a protective role for VEGF.[46, 47] Whether or how VEGF is involved in the development of PH is still topic of debate.[43] Tuteta and colleagues found increased levels of VEGF and especially in scadding stage III patients, suggestive for a possible role in disease progression.[48] However, this could also be a reflection of augmented fibrosis as VEGF may facilitate fibrogenesis and increased levels correlated with higher radiologic fibrosis scores in idiopathic interstitial pneumonias.[49, 50] In our study we did not measure VEGF serum levels in SRC patients. However, VEGF levels in SAPH patients were increased compared with IPAH patients and higher VEGF levels correlated with increased mPAP in SAPH patients. This may suggest that VEGF is actively involved in vascular remodeling in SAPH patients. In any case, VEGF may be an interesting candidate biomarker for SAPH development and its usefulness should be tested in prospective trials. Our study has some limitations. Firstly, available clinical information such as medication, DLCO, disease duration, of the SCR patients who entered that study was rather limited. However, we found no differences in 6MWD and demographic features between SAPH and SRC patients. Secondly, a large proportion of SAPH patients received PH-directed therapy next to background sarcoidosis treatment at the time of study entry and therefore we cannot rule out that this may affect levels of inflammatory mediators.

In conclusion, our study provides an overview of inflammatory mediators in a large multicenter cohort of patients with SAPH. Patient with SAPH display a unique inflammatory profile, identifying possible new underlying pathways. These inflammatory mediators,

especially CXCL9, CXCL13, IL-7, CCL4 and VEGF, may be boost new translational studies that investigate their potential as (mechanistic) biomarker and therapeutic target in certain SAPH subgroups.

All source(s) of support in the form of grants:
Funding from Gilead Sciences Inc., which provided an unrestricted grant to support the registry. The National Institutes of Health (NIH) Clinical and Translational Science Award (CTSA) program funds the research database at the University of Cincinnati, grant 1UL1TR001425-01. The CTSA program is led by the NIH's National Center for Advancing Translational Sciences (NCATS).

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Additional data

Supplementary Table 1. Serum concentration of each inflammatory mediator and the number of samples analyzed.

	HC		IPAH		SRC		SAPH	
Cytokine (mean with 95% CI)								
CXCL13 (pg/ml)	193 (-65-453)	(20)	97 (-46-241)	(16)	164 (84-244)	(33)	544 (342-748)	(66)
CXCL9 (pg/ml)	124 (3-245)	(20)	43 (-4-94)	(16)	219 (137-301)	(33)	644 (366-962)	(66)
IL-7 (pg/ml)	10 (5-16)	(20)	0	(16)	17 (12-23)	(33)	34 (22-46)	(66)
CCL4 (pg/ml)	36 (21-51)	(20)	47 (13-81)	(16)	129 (98-160)	(33)	114 (79-149)	(66)
IL-8 (pg/ml)	1.7 (0.8-1.6)	(20)	5.0 (0.9-9.2)	(15)	11.6 (2.1-20.1)	(32)	16.2 (9.8-22.6)	(65)
TNFa (pg/ml)	1.3 (0.5-2.1)	(16)	na*		4.2 (2.8-5.6)	(33)	7.6 (3.3-12.0)	(36)
VEGF (pg/ml)	45 (13-76)	(6)	18 (-4-40)	(16)	na		147 (104-190)	(32)
CXCL4 (ng/ml)	2791 (2153-3433)	(16)	na		3499 (3079-3918)	(33)	2414 (1769-2059)	(24)

*na = not valuated

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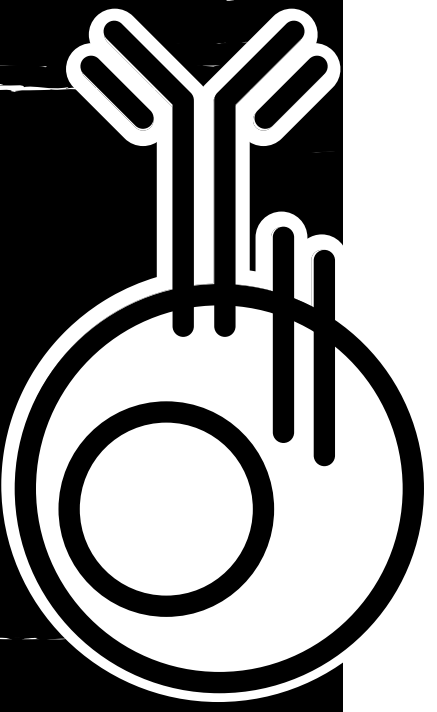

CHAPTER 9

General discussion of the thesis

General discussion of the thesis

In this thesis, we addressed how adaptive immune responses influence the development of two important lung diseases: interstitial lung diseases (ILD), and idiopathic pulmonary fibrosis (IPF) in particular (**Part 1**) and pulmonary arterial hypertension (PAH) (**Part 2**). In **Part 1** we first gave a broad overview on the role of our innate and adaptive immune system in the initiation and perpetuation of IPF pathobiology and describe upcoming therapeutic trials that target and modulate our immune system in patients with IPF (**Chapter 2**). Next, we explored how to integrate patient perspectives and individual disease features, such as genetics, biomarkers and environmental factors, in personalized IPF treatments and gave an overview of patient-reported outcome measures in IPF (**Chapter 3**). In **Chapter 4** we showed that fibrocytes in IPF lungs can be assessed by flow cytometry and that their phenotype differs from circulating fibrocytes. We ended **Part 1** with demonstrating that autoreactive B-cells, especially IgA-memory B-cells, are increased in IPF patients, possibly driven by increased intracellular expression of the signaling molecule Bruton's tyrosine kinase (**Chapter 5**). In **Chapter 6** we further highlighted the role of B cell receptor (BCR) signaling in IPF patients with and without anti-fibrotic treatment. In **Part 2** we demonstrated that enhanced BCR signaling in B cells and increased circulating follicular T helper 17 (Tfh17) cell polarization contributes to autoimmune-mediated vascular remodeling and disease pathogenesis in idiopathic PAH patients (**Chapter 7**). Finally, we identified inflammatory phenotypes that discriminate between sarcoidosis-associated pulmonary hypertension (SAPH) and other groups, including chronic sarcoidosis (**Chapter 8**).

In this chapter, results of the thesis will be put into perspective in the context of directions for future research and clinical implications. This chapter will be centered around 5 main topics:

- Circulating fibrocytes in lung diseases: hype or hoax?
- B-cell activation and BCR signaling in IPF and IPAH.
- How to fit inflammation in the primary process of fibrogenesis and vascular remodeling.
- Treatment in IPF and IPAH: B-cell modulation and personalized medicine.
- Novel insights in the pathogenesis of sarcoidosis-associated pulmonary hypertension

Circulating fibrocytes in lung diseases: hype or hoax?

Bucala and colleagues introduced the fibrocyte in 1994 as a bone morrow derived cell with fibroblast-like tissue remodeling properties.[1] Upon tissue damage these cells home to affected areas and aid to wound repair and tissue remodeling.[1] The idea that non-residual cells are actively recruited to sites of tissue injury or chronic inflammation is appealing as this may open opportunities for therapeutic interventions, especially in chronic disorders in which tissue remodeling or fibrosis fulfill a central role. The role and function of circulating and tissue fibrocytes have been extensively examined in several chronic lung diseases. Most of these studies utilize flow cytometry for fibrocyte identification. **Table 1** summarizes the most important studies involving fibrocytes in pulmonary diseases and markers used for their identification (in bold studies involving pulmonary hypertension or pulmonary fibrosis).

Table 1. Overview of studies on fibrocytes in pulmonary diseases

study	Main finding	Markers used to identify fibrocytes [*type of tissue*]
Schmidt et al, 2003	Allergen exposure induces the accumulation of fibrocytes in the bronchial mucosa of patients with allergic asthma	CD34, Col-1, aSMA[1)] [*lungs*]
Andersson-Sjoland et al, 2008	Positive correlation between the abundance of fibroblastic foci and the number of lung fibrocytes in IPF lungs	CD34, CD45, and pro-Col-1 or aSMA or CXCR4 [*lungs*]
Moeller et al, 2009	Increased circulating fibrocytes in acute exacerbations of IPF. Increased fibrocytes were a predictor of mortality	CD45, Col-1 [*blood*]
LaPar et al, 2011	Increased circulating fibrocyte levels correlate with the development of bronchiolitis obliterans syndrome after lung transplantation	CD45, Col-1 [*blood*]
Fujiwara et al, 2012	Circulating fibrocytes were significantly increased in all patients with ILD and correlated with pulmonary function test	CD45, Col-1 [*blood*]
Yeager et al, 2012	Higher fibrocytes correlated to increasing mean pulmonary artery pressure in children and young adults with PH	CD45, Col-1 [blood]
Borie et al, 2013	Fibrocytes can be detected in BALF of patients with IPF/ILD	CD45, Col-1 [*BALF*]
Trimble et al, 2014	Fibrocyte correlated with outcome of ILD in subjects with Hermansky-Pudlak syndrome	CD45, Col-1, and CXCR4 and/or CCR2 and/or CCR7 [*blood*]
Alhamad et al, 2014	Using a whole blood lysis method fibrocytes were increased in blood of IPF patients	CD45, Col-1, CD34 [*blood*]
Shipe et al, 2016	Increased circulating fibrocytes correlated with asthma severity and expressed higher cell-surface chemokines	CD45, Col-1, CXCR4 or CCL7 or CCL2 [*blood*]
Dupin et al, 2016	Increased circulating fibrocytes in patients with COPD during an exacerbation and associated with an increased risk of death	CD45, Col-1 [*blood*]
Just et al, 2017	Patients with RA with or without ILD had increased levels of circulating fibrocytes. Fibrocytes correlated with diffusion capacity.	Negative lineage mix*, CD45, CD34, CD11b [*blood*]
Heukels et al, 2018 (*Chapter 4*)	Increased fibrocytes in blood and lungs of IPF patients. Optimizing fibrocyte identification with flow cytometry	Negative lineage mix*, CD45, Col-1, CXCR4 [*blood and lungs*]

Abbreviations used: aSMA= alpha smooth muscle actine; BALF= bronchoalveolar lavage fluid; Col-1= Collagen-1; COPD= chronic obstructive pulmonary disease; IPF= idiopathic pulmonary fibrosis; ILD= interstitial lung disease; RA= rheumatoid arthritis

Although scientific progress in our understanding of the biological and clinical significance of fibrocytes has been made over the last years, the studies did not lead to prodigious changes in clinical decision making or treatment choices in pulmonary fibrosis or pulmonary hypertension. Why is that? Moreover, should we change our opinion on the role of fibrocytes in disorders that involve chronic remodeling, such as pulmonary fibrosis and pulmonary hypertension? An important issue is a lack of a clear definition and robust detection methods of fibrocytes. Several intra- and extracellular (membrane bound) proteins have been characterized on fibrocytes, however most of these markers are not specific.[2] Hence, no uniform gating strategy exists to identify fibrocytes, which leads to a high variability and low reproducibility between reports on for example the number of circulating fibrocytes. Secondly, circulating fibrocytes are not directly isolated from human blood. If fibrocytes could be reliably isolated directly from the circulation, this would open opportunities to conduct meaningful functional or pharmacological *in vitro* studies. Up until now, almost all studies that aimed to isolate fibrocytes, utilized culture systems to 'generate' fibrocytes from mononuclear cells. In this artificial *in vitro* process, mononuclear cells transform in the presence of human serum and occasionally TGF-β, IL-4 and/or IL-13, in spindle-shaped fibroblastic cells.[3] Although T helper-2 (Th2) cytokines and TGF-β are important in mediating fibrosis in general, it is unclear whether these cytokines are pivotal or that other local signals are required for fibrocyte development *in vivo.*

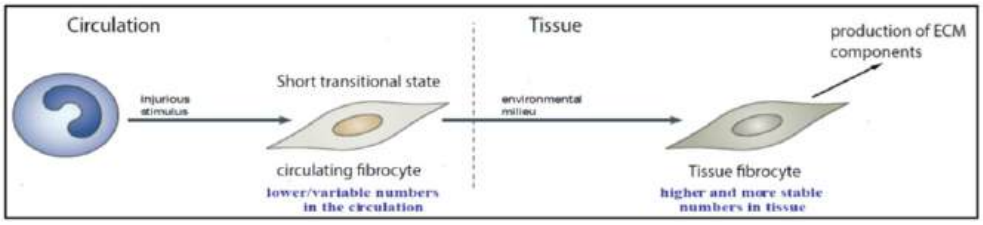

Figure 1. Overview of the development of tissue fibrocytes.
During development into a cell with fibroblast-like properties, several phases can be discriminated. Upon tissue damage, monocytes transform into cells that have features of fibroblasts and home to affected areas and aid to wound healing and tissue remodeling. This intermediate state in which fibrocytes can be detected in the circulation probably represents a short phase. Circulating fibrocytes rapidly enter sites of tissue damage, where local environmental triggers are important for further transformation into a fibroblast-like cell. Adapted from Reilkoff et al, 2011.[4]

Our study (**Chapter 4**) acknowledged this problem and therefore we optimized an identification method to select circulating fibrocytes and tissue fibrocytes from IPF lungs. In concordance with two recent studies, we utilized a negative lineage mix to eliminate contaminating cell populations with flow cytometry.[5, 6] Of note, our method for fibrocyte identification could be applied for both blood and tissue samples. We observed much lower levels of circulating fibrocytes in the circulation than previously reported and these results were also seen in the two other reports.[5, 6] Tissue fibrocytes in IPF lungs comprise ~1-3% of CD45-positive cells (**Chapter 4**). In this perspective, we feel that focusing on tissue fibrocytes holds more potential as these cells directly contribute to the remodeling process itself.

How should we appreciate the circulating fibrocyte in fibrotic disorders? Although the monocyte/fibrocyte/fibroblast-axis, plays an important role in attracting bone marrow-derived fibroblastic cells to the site of disease, circulating fibrocytes represent probably a short-lived transitional state on the move to its target organ (**Figure 1**). We should regard tissue fibrocytes as one of a number of cell types that can differentiate from monocytes, including macrophages, osteoclasts and dendritic cells.[7] Accordingly, we believe that the development of fibrocytes into effector cells is primarily established outside the circulation and depends on local tissue environments. **Table 2** summarizes the most important similarities and differences between monocytes, fibrocytes and fibroblasts. Therefore, circulating fibrocytes may provide information about recruitment rate, rather than the primary remodeling process itself. For example, patients with acute exacerbation of IPF (AE-IPF), which is a sudden acceleration of disease, have higher proportions of circulating fibrocytes.[8] However, attempts to interfere with fibrocyte recruitment via inhibition of the CXCL12-CXCR4 chemokine signaling with an CXCR4 antagonist, AMD070, did not slow down disease progression.[9] A plausible explanation of higher numbers of detected fibrocytes in the circulation during AE-IPF might be non-specific binding of Collagen-1 antibodies to proteins expressed on granulocytes (**Chapter 4**)**.** Increased numbers of circulating and activated granulocyte are present during AE-IPF, because of enhanced recruitment of granulocytes to IPF lungs as result of simultaneous (secondary) infections, as well as general immune activation.

Table 2. similarities and differences between monocytes, fibrocytes and fibroblasts[2, 4, 10]

	Monocyte	Fibrocyte	Fibroblasts
Compartment	Circulation	Circulation/organ	Organ
Originate from	Bone marrow	Monocytes, possibly skewing from macrophages	Resident cells, small proportion originate from fibrocytes or epithelium (EMT)[1)]
Cell functionality	Progenitor cells, rapid deployment and recruitment to sites of injury and inflammation	ECM production (+), antigen presentation, tissue destruction, neo-angiogenesis, recruitment of T-cells	Wound contraction and ECM production (++)
Paracrine mediators		TGFβ, PDGF, FGF, VEGF-A, MMPs	TGF-b, VEGF, FGF, CTGF, IGFBP-3 and -5, IGF-II, MMPs
Exclusive cell surface molecules	CD64	-	CD90
Other cell surface molecules	CD45, CD34, CD11a, CXCR4, CCR7, CCR2, CD14, CD64, CD80, CD86	CD45, CD34, CD11a, CXCR4, CCR7, CD10, CD13, CD80, CD86	CD90, no chemokine receptors
Cytokine release	IL-1, IL-6, IL-8, IL-12	IL-6, IL-8, IL-10	IL-6, IL-13, IL-33, IL-1β
ECM proteins	-	Col-1, Col-2, Col3, aSMA (low levels) Vimentin	Col-1, Col-2, Col3, aSMA (high levels), fibronectin and vimentin

Abbreviations used: ECM= extracellular matrix; EMT= epithelial to mesenchymal transition; TGFb= transforming growth factor beta; PDGF= platelet-derived growth factor; FGF= fibroblast growth factor; VEGF= vascular endothelial growth factor; MMP= matrix metalloproteinases; CTGF= connective tissue growth factor; IGF= insulin-like growth factor; IGFBP= insulin-like growth factor-binding protein; Col= Collagen; aSMA= alpha-smooth muscle actin.

Targeting tissue fibrocytes however, may be more effective in the treatment of pulmonary fibrosis. Pirfenidone and nintedanib, which are the only approved drugs known to decelerate disease progression, influence tissue fibrocyte function. Pirfenidone inhibits tissue fibrocyte accumulation in the lungs in bleomycin-induced murine pulmonary fibrosis[11] and the receptor kinase inhibitor nintedanib inhibits the response of tissue fibrocytes to growth factors such as PDGF, FGF and VEGF-A.[12] Another compound, serum amyloid P (SAP) gained a lot of interest after showing clinical effectiveness in a Phase 2 clinical trial in IPF.[13] SAP inhibits the differentiation of monocytes into fibrocytes and promotes the differentiation into immunoregulatory macrophages.[14, 15] Although we cannot rule out that the skewing process of monocytes into a less fibrotic effector cell already starts in the circulation, it is plausible that its differentiation is largely tissue-dependent.[16]

Our observations in **Chapter 4** indicate that especially tissue fibrocytes are involved in the etiology of fibrotic lung diseases and hold potential as therapeutic target. However, to maximize the significance of investigations into tissue fibrocytes and to facilitate the interpretation of obtained results, future research should (1) use a uniform and reliable gating strategy and (2) study tissue fibrocytes derived from patients, preferably from affected tissue. In this regard, our strategy for direct identification of fibrocytes with flow cytometry could be used as a basis for new cell sort protocols and thereby boost fibrocyte research.

B-cell activation and BCR signaling in IPF and IPAH

Our experimental model of IPF and PH (Chapters 5 and 7)

Our group generated transgenic mice that overexpress human BTK (hBTK), which is an essential signaling molecule downstream of various receptors including the BCR[17]. The BTK transgene is expressed under the control of the CD19 promoter region (CD19-hBTK), which implies that only B cells overexpress BTK. B cells from these mice show increased survival, increased cytokine production, and have the capacity to engage T cells in spontaneous germinal center (GC) formation.[17] Over time, and without further interventions, CD19-hBtk transgenic mice spontaneously develop autoimmune pathology, characterized by lymphocyte infiltrates in several tissues and production of anti-nuclear autoantibodies (ANAs). This pathology was observed from the age of ~25 weeks onwards. When these mice were subjected to pulmonary injury by intratracheal administration of bleomycin, we observed a disease phenotype with PH characteristics (**Chapter 7**). These PH characteristics included increased right ventricle systolic pressure (RVSP) and increased right ventricle weight and were observed after ~10 weeks after exposure of bleomycin. Originally, this experiment was designed to investigate whether overexpression of BTK in B cells would lead to augmented fibrosis after ~3 weeks of bleomycin exposure. Although we found increased B-cell activation, fibrosis indices including hydroxyproline content and tissue elastance were similar between CD19-hBTK mice and control littermates (**Chapter 7**). In a follow-up experiment after ~10 weeks of exposure, we found similar results on fibrosis indices, but surprisingly observed signs of PH development (**Chapter 7**). Although PH signs have been found in the bleomycin model at ~3 weeks,[18] it is unclear if PH indices persist at later time points. Given that the bleomycin model is based on transient inflammation with resolution of fibrosis before ~10 weeks after bleomycin exposure, it is conceivable that resolution should also occur with PH indices. Therefore, we feel that PH development in CD19-hBTK mice is probably the result of enhanced and chronic B-cell activation and is possibly driven by autoantibodies. This notion is strengthened by the previous observation that ~75% of CD19-hBTK mice that were infected with influenza virus showed detectible autoantibodies after 3 weeks in the broncho-alveolar lavage fluid, while serum influenza-specific antibody titers were unchanged.[17] Although this pulmonary insult is of lesser magnitude compared to bleomycin, we conclude that a CD19-hBTK background in combination with lung injury is sufficient to induce autoimmune features at a substantially earlier stage, likely involving auto-antibodies against pulmonary epitopes. Therefore, after ~10 weeks post bleomycin exposure (and not ~3 weeks), this mouse model is probably a better reflection of human group 1 PH (connective-tissue disease pulmonary arterial hypertension; CTD-PAH), rather than group 3 PH (hypoxemia/ILD related). In particular, because (i) aging CD19-hBTK mice spontaneously develop a systemic autoimmune disease that resembles human systemic lupus erythematosus (SLE) and Sjögren syndrome, and BTK expression levels are increased in patients with anti-citrullinated protein antibody-positive (ACPA+) rheumatoid arthritis (RA), Sjögren syndrome[19] and systemic sclerosis (SSc) (O. Corneth, unpublished findings).

As mentioned above, CD19-hBTK mice did not show augmented fibrosis development at ~3 weeks after bleomycin exposure compared to control littermates (**Chapter 7**). These observations seem to contradict our findings in **Chapter 5**, where we describe increased BTK levels in circulating B cells of IPF patients compared to healthy subjects. This raises an important issue; how should we interpret data obtained from the bleomycin mouse model in relation to human IPF pathobiology? And could this discrepancy be one of the reasons why numerous drugs show efficacy in these animal models but only a minority of these drugs have replicated such beneficial effects in clinical trials?[20] Several points need to be addressed to answer these questions. Most importantly, the bleomycin model deviates from human IPF pathology as in this model fibrosis development is not progressive and it strongly depends on inflammation.[21] Fibrosis starts at around day 7 and peaking between day 14 and 21 after bleomycin instillation. Furthermore, the bleomycin model is in general more suitable for interventions or drugs that aim to attenuate fibrosis rather than for the investigation of genetic factors that may promote or augment fibrosis. The reason behind this phenomenon is that the bleomycin dosage that is generally used causes extensive damage, which is difficult to exceed. Lastly, timing of the intervention or drug delivery is crucial and changes the outcome. Drugs can be delivered within the first 7 days after bleomycin exposure (=preventive stage) or after 7 days (= therapeutic stage).[22]

Regardless of these concerns, the bleomycin model does help to identify targets for intervention and or pathways that may be involved in human IPF pathobiology. Nevertheless, results obtained with the bleomycin model should be interpreted with some caution and novel experimental mouse models that closely resemble human IPF are warranted. Promising is the recent observation that a deletion of the ubiquitin ligase NEDD4-2 (also known as NEDD4L) in lung epithelial cells in adult mice produces chronic lung disease sharing key features with human IPF, including progressive fibrosis and honeycombing.[23] The NEDD4-2 protein is implicated in the regulation of TGFβ signaling and of the epithelial Na^+ channel critical for proper airway surface hydration and mucus clearance, pointing to a role for mucociliary dysfunction in fibrosis development.

From our perspective, the CD19-hBTK fibrosis model helped us to generate new perspectives on the role of autoimmunity and B-cell activation in pulmonary hypertension. Serendipity eventually led to new insights in human PAH pathobiology (**Chapter 7**). Although we couldn't enhance fibrosis utilizing a hBTK-CD19 background, it would still be interesting to test whether an intervention with a specific BTK inhibitor in the therapeutic stage of the bleomycin model would lead to attenuation of fibrosis. Additionally, given the spontaneous development of autoimmune pathology over time in hBTK-CD19 mice, this model may be more suitable to study connective tissue disease-associated ILD.

B-cell activation and BCR signaling in IPF and IPAH (Chapters 5, 6 and 7)

From an immunological point of view, IPF and IPAH etiology share several features. It is commonly accepted that in both IPF and IPAH not one single pathway or primary insult can fully establish disease. This is referred to as the multiple hit theory, whereby a number

of potential injuries in susceptible individuals may lead to abnormal wound repair and subsequently enflame a cascade of fibroses and tissue remodeling (see also **Figure 1**).[24] In IPF, smoking, infections, physical trauma or autoantibodies against epithelium may disrupt epithelial integrity and lead to edema, recruitment of different inflammatory cells and angiogenesis.[25] In IPAH, activation and apoptosis of endothelial cells by autoantibodies, toxins or viral infections are believed to initiate inflammation and remodeling in and around pulmonary arteries.[26] In physiological conditions, resolution of inflammation through apoptotic and phagocytic pathways restores normal tissue architecture.[25] In case of ongoing or chronic inflammation, and defects in pathways that are crucial for the clearance of cellular debris, our immune system will persistently or recurrently be exposed to self-antigens. In **Figure 2** we describe how alterations in adaptive immune responses, described in **Chapters 5, 6 and 7**, contribute to ongoing inflammation. The subsequent development of autoreactive antibodies to epithelial and endothelial epitopes further exacerbates local inflammation, which may create a detrimental feed-forward loop.

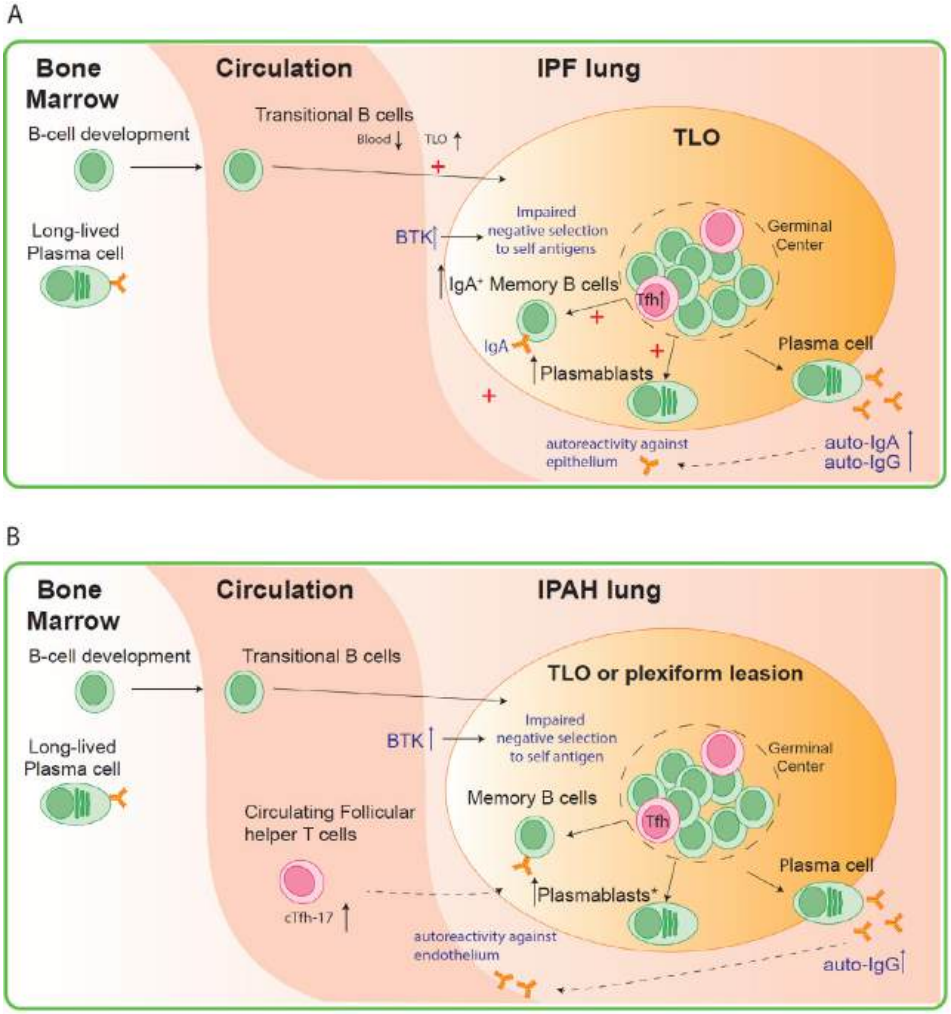

Figure 2. Graphic summary integrating our key findings involving B-cell and T helper subset alterations in IPF and IPAH. *(A) Immature B cells leave the bone marrow as transitional B cells for further differentiation. Decrease in circulating transitional B cells together with their increase in IPF lungs suggest homing towards pulmonary tertiary lymphoid organs (TLO). Increased BTK levels in (immature) B-cells hinder adequate elimination of autoreactive B cells recognizing (pulmonary) self-antigens and might contribute to the development of autoreactive B cells in pulmonary TLOs after epithelial injury. Furthermore, activated (PD-1 high) follicular T helper (Tfh) cells are present in IPF lungs and engage with B cells in the germinal centers (GC) of pulmonary TLOs, possibly further promoting differentiation of autoreactive B cells. Cytokines produced by Tfh-cells and the local environment in IPF lungs induces predominantly IgA class-switch recombination. Upon activation, IgA memory B-cells and plasmablasts leave pulmonary TLOs, leading to an increase of IgA memory B-cells, plasmablasts and autoreactive IgA in blood of IPF patients. (B) In our cohort of IPAH patients, B-cell subsets were unchanged. Also, in IPAH patients, increased BTK levels in B cells hinder adequate elimination of autoreactive B cells. Increased autoreactive IgG directed against pulmonary endothelial antigens could contribute to the primary remodeling process of pulmonary arteries which may lead to PH. The imbalance of circulating Tfh cells, with increased circulating Tfh17 (cTfh17) cells, may contribute to Th-17 immune polarization, which could be favorable for PH development. *Blum and colleagues found increased proportions of circulating plasmablasts in IPAH patients.*[26]

Although IPF and IPAH are considered "idiopathic" and thus diseases with no clear underlying cause, in a subgroup of patients we found striking similarities with autoimmune diseases (**Chapters 5, 6 and 7**). Increased B-cell activation and BCR signaling are commonly seen in autoimmune disorders.[27, 28] Parallel to our observation of increased BTK expression in B cells (**Chapters 5 and 7**) and enhanced phosphorylation of signaling proteins downstream of the BCR (**Chapters 6 and 7**), similar results have been found in patients with ACPA+ RA, Sjogren's syndrome and autoimmune vasculitis.[19, 29] We found increased BTK levels already in naïve B cells. Since such B cells have not encountered antigen, a possible explanation of these findings is that other signals from the micro-environment, such as cytokine levels or presence of Toll-Like receptor ligands, induce changes in the epigenome, transcriptome or proteome of naive B-cells.[30] The proportions of circulating autoreactive naïve B cells in IPF and IPAH appeared to be increased, which might be related to defective self-tolerance in developing B cells as was observed in SLE.[31] Diminished clearance of apoptotic cell debris due to perpetuating insults to the vasculature or lung tissue likely results in increased exposure to self-antigens. In combination with an increased number of autoreactive naïve B cells, this may further exacerbate local autoreactivity against pulmonary and vascular antigens.

Pulmonary fibrosis and PH can develop in a range of autoimmune diseases, such as SLE, SSc and RA.[32] For instance, patients with ACPA+ RA are more likely to develop pulmonary fibrosis, especially when they are active smokers.[33, 34] A plausible explanation is that smoke inhalation enhances local tissue damage and upregulation of citrullinated proteins in lungs, initiating the development of auto-antibody mediated fibrosis.[33] Interestingly, the radiological appearance of pulmonary fibrosis seen in RA (usual interstitial pneumonia pattern) is often undistinguishable of that of IPF.

An interesting finding in **Chapter 5** is that higher autoreactive IgA levels correlated with an increased decline of forced expiratory volume (FVC). Other reports also highlight the role of (autoreactive) IgA in IPF and PAH.[35] **Table 3** summarizes the most notable auto-antibodies identified in IPF and IPAH. In IPAH for instance, endothelial-specific IgA can promote cytokine production and the upregulation of adhesion molecules by endothelial cells.[36] Bronchus-associated lymphoid tissue in IPAH lungs could be the source of these antibodies.[37] Autoreactive IgA may promote fibrosis through fibroblast stimulation [38] and may promote the production of the key profibrotic cytokine TGF-β by type II epithelial cells.[39] Conversely, IgA class switch recombination of B cells is also promoted by TGF-β.[40] In conclusion, it is conceivable that enhanced BCR signaling, next to local stimuli in IPF and IPAH lungs, contribute to production of specific autoreactive IgA antibodies against endothelial and epithelial antigens.

Table 3. overview of autoantigens found in IPF and (I)PAH.

IPF Autoantigen	Location	Function
Annexin-1[41]	lung cells	Ca^{2+}-dependent phospholipid-binding protein
BP1FB1[42]	product of goblet cells	bactericidal
Collagen-V[43]	lung interstitium	barrier function
Cytokeratin-8[44]	type-2 alveolar and epithelial cells	component of intermediate filaments
Cytokeratin-18[45]	glandular epithelial cells	component of intermediate filaments
Heat shock protein 70[46]	all cells	cellular chaperones that serve a number of vital maintenance and repair functions
Interleukin-1a[47]	epithelial cells, endothelial cells, macrophages	pro-inflammatory cytokine
Periplakin[48]	keratinocyte	component of desmosomes and the epidermal cornified envelope
Vimentin[49]	mesenchymal cells	protein important in the dynamic organization of the cytoskeleton
(I)PAH		
Anti-endothelial cell[26, 50]	endothelial cells	specific protein unknown
Tropomyosin-1[51]	smooth muscle cells	involved in the contractile system
Heat shock protein 27,70[51]	all cells	cellular chaperones that serve a number of vital maintenance and repair functions
Vimentin[51]	mesenchymal cells	protein important in the dynamic organization of the cytoskeleton

Having shown that loss of immune homeostasis can contribute to IPF (**Chapter 5**) and IPAH (**Chapters 6 and 7**) development, we also examined the role of Tfh cells. Tfh-cells are crucial for priming B cells to initiate antibody responses including autoreactive IgA and are crucial for affinity maturation and maintenance of humoral memory.[52] Circulating Tfh (cTfh) are thought to represent a circulating pool of memory Tfh cells and alterations in Tfh cells are associated with autoimmunity in humans.[53, 54] In IPAH we observed that proportions of cTfh-17 cells are increased. This provides an additional link with autoimmunity, because similar findings have been observed in SSc patients.[55] In IPF, on the other hand, a recent paper showed that the proportions of the cTfh17 subset was lower than that in healthy subjects.[56] In patients with PH secondary to ILD or chronic hypoxia (WHO group 3 PH) we also found decreased proportions of cTfh17 cells (**Figure 3**, P.H. unpublished data). How do we interpret this discrepancy?

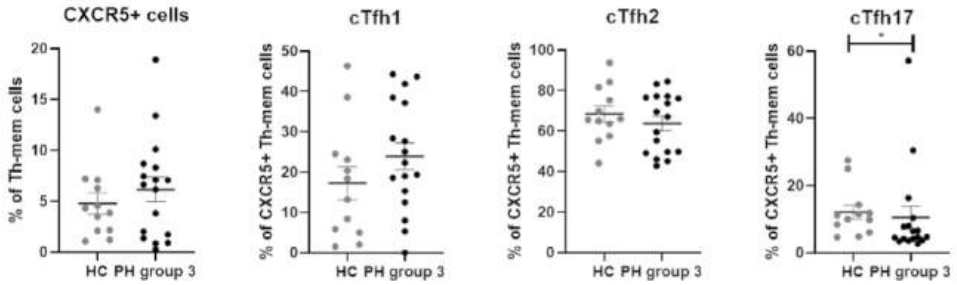

Figure 3. Proportions of cTfh cell populations in patients with group 3 PH.

*Proportions of CXCR5⁺ memory T cells (cTFh) as percentages of total memory CD4⁺ T cells and proportions of the indicated cTfh subsets as percentages of CXCR5⁺ memory CD4⁺ T cells. Mann–Whitney U test was used. Data are expressed as mean values and the error bars represent the standard error of the mean. Dots represent individual patient values. * $P < 0.05$*

In IPF, imbalanced Th1/Th2 immune response, favoring Th2, is central in IPF pathogenesis.[57] The overall burden of fibrosis, and thus Th2 immune polarization, is much higher in IPF compared with IPAH. Indeed, type-2 cytokines promote pro-fibrotic responses and differentiation of cTfh cells with a cTfh2 signature.[58] Additionally (myo) fibroblast and pro-fibrotic macrophages have paracrine functions that favor the formation of a Th2 environment.[59, 60] On the other end, growing evidence suggests that Th17 immune polarization is a feature of PAH (**Chapter 7**). It is therefore conceivable that Th17 immune polarization is more important in IPAH than in IPF. Despite these differences, B-cell responses and alterations in B-cell signaling in IPF and IPAH are highly comparable (**Figure 2**) and may thus partly occur independently from the type of Th polarization. For example, co-culture of naïve B cells with either blood cTfh2 or cTfh17 cells still resulted in the secretion of IgA[53]. This suggests that increased levels of autoreactive IgA in IPF and IPAH, which we and others[35] found, may occur irrespective of cTfh immune polarization.

Finally, is it fair to discuss the same immunological pathways in two totally different clinical diseases? In our opinion it is. It is true that the location of disease and genetic and environmental triggers - which are different in IPF and IPAH - to a great extent determine the clinical presentation and outcome. However, we show that basic immunological mechanisms that contribute to autoimmune-mediated tissue or vascular remodeling in IPF and IPAH can be the same. Additionally, we showed that both IPF and PAH patients have increased BCR signaling (**Chapter 5-7**).

Lessons learned from IPF pathobiology turned out to be helpful in exploring IPAH. Nevertheless, we have to keep in mind that the contribution of autoimmune-mediated tissue remodeling as modulator or driver of disease varies between patients.

How to fit inflammation in the primary process of fibrogenesis and vascular remodeling

In case of tissue damage, the human body will naturally respond with an inflammatory response. A response is needed to cope with unwanted intruders, local trauma or injuries. After the initial innate immune response, an adaptive immune response will provide further help with antigen-specific T and B cells. Late-stage inflammation involving phagocytosis of damage can even have anti-fibrotic properties and is likely required for successful resolution of inflammation and for wound-healing responses.[25] After eliminating the initial injury with successful resolution, the inflammatory response subsides, restoring normal homeostasis.

Because repetitive injury and tissue damage to epithelium or endothelium precede the process of fibrogenesis and vascular remodeling in IPF and IPAH, respectively, it would be unexpected not to see some changes in adaptive responses. In this regard, some degree of immune activation, and probably even some degree of autoimmune-mediated responses, is thought to be part of a normal physiological response. Yet, the key question remains, whether the alterations seen in B and T cell function or presence of autoantibodies we observed are part of disease etiology or even critical drivers of disease. This leaves us with two important questions how to fit inflammation in the primary process of fibrogenesis and vascular remodeling:

(1) Is it merely an epiphenomenon or critical player?
(2) Can we integrate this in our treatment plan?

Inflammation in fibrogenesis and vascular remodeling: epiphenomenon or critical player?

As already emphasized above, inflammation is part of the IPF and IPAH pathology and in a subgroup of patients' immunological alterations are critical for disease onset and progression. The spectrum between fibrogenesis, with limited immune alterations to fibrosis and PH secondary to a 'classic' autoimmune disease is wide. Most patients diagnosed with IPF and IPAH fall in the category where inflammatory alterations are probably secondary to the primary fibrotic or remodeling process (**Figure 4**). It should be noted that all previous landmark trials that tested immunosuppressive therapies for IPF did not show efficacy or turned out be detrimental.[61, 62, 63, 64, 65, 66, 67] Conversely, adaptive immune responses are probably critical in disease onset and progression in ILD or PH in the setting of an autoimmune disorder. However, even in the setting of an autoimmune disorder, fibrogenesis and vascular remodeling will eventually be the dominant factor in end-stage lung or vascular disease. Likewise, it is conceivable that during the natural course of IPF and IPAH, the contribution of inflammatory processes gradually changes and diminishes.

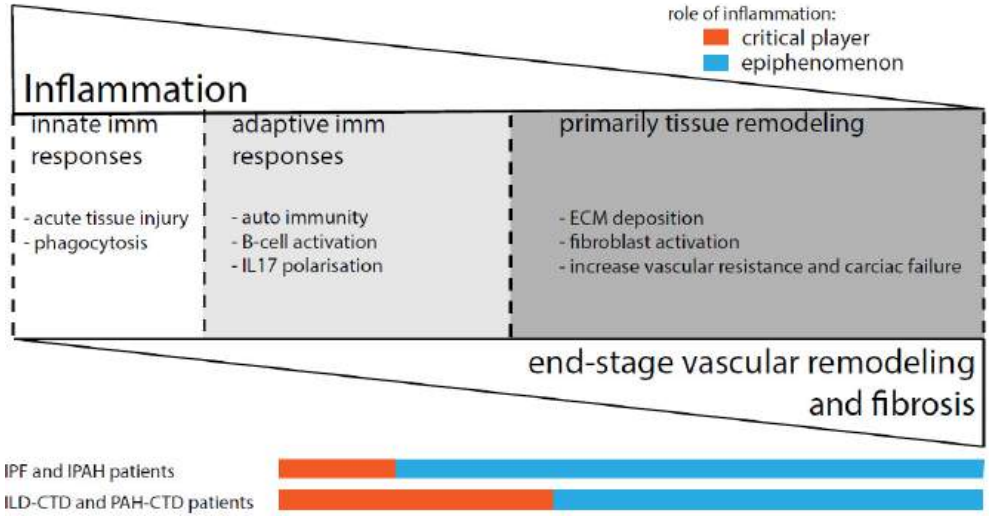

Figure 4. Schematic representation of the contribution of inflammatory pathways in IPF and IPAH.
In a small proportion of IPF and IPAH patients' inflammatory alterations are probably critically involved IPF/(I)PAH-onset and disease progression. This proportion is obviously much higher in patients with underling auto-immune diseases. Also timing of diagnosis is important as in progressive disease the contribution of inflammatory processes gradually diminishes, which is probably also the case in end-stage ILD-CTD and PAH-CTD. CTD = connective tissue disease. ILD = interstitial lung disease.

The key task is to find those patients with IPF and IPAH where inflammation and immunity are the main drivers of disease. Over the last years progress has been made in selecting patients. There is increasing evidence that patients with clinical or biochemical evidence for immune activation may benefit from immune-modulating therapies. For instance, a substantial group of patients with ILD have autoimmune manifestations not classifiable as a 'classic' connective tissue disease (CTD) and are designated as interstitial pneumonia with autoimmune features (IPAF).[68] Though IPAF is not accepted as an official diagnosis, it is often used in clinical practice as well as research. The criteria for IPAF are organized around three central domains. One of the domains is a serologic domain where specific circulating autoantibodies are present. A recent pilot study showed that the majority of patients with IPAF treated with the B-cell deleting antibody rituximab showed clinical improvement or remained stable.[69] A case series of patients with steroid refractory unclassifiable idiopathic interstitial pneumonia treated with cyclophosphamide demonstrated an increase of forced vital capacity (FVC), especially those patients meeting the IPAF criteria.[70] This could indicate that in a small subgroup of patients with IPF - especially those with clinical and serological signs of autoimmunity - adaptive immune responses are the primary culprit for disease onset and/or progression.

In a subgroup of PAH patients changes in immune activation are important in disease progression and initiation. In this regard, we may learn from patients with CTD-PAH. Patients with SLE or mixed CTD-PAH may benefit from an immunosuppressive therapy combining

glucocorticoids and cyclophosphamide.[71] Although the role of immunosuppression in SSc-PAH is probably limited, a subgroup of SSc-PAH patients with anti-U1RNP antibodies showed a trend towards decreased mortality upon treatment with immunosuppression.[72] It is therefore possible that some SSc-PAH patients may benefit from immunosuppressants, especially those with circulating autoantibodies. In a recent landmark paper, proteomic analysis of PAH patients revealed that four distinct immune clusters can be identified.[73] Each cluster had a distinct circulating cytokine profile and prognosis. One of these clusters displayed an adaptive immune phenotype, harboring both IPAH and PAH-CTD patients. These findings suggest that the underlying inflammatory profile could directly define phenotypes that are related to clinical characteristics and possibly responders to (anti-) inflammatory treatments.

In conclusion, in a subgroup of patients where the disease seems 'idiopathic' at first glance, underlying adaptive immune responses may well be detrimental for disease progression. This thesis identifies possible cellular markers and proteins, including BTK levels in B cells, enhanced BCR signaling and increased numbers of cTfh17 cells, which could help identify those patients.

Inflammation in fibrogenesis and vascular remodeling: how to fit these features into our current treatment plan?

In chronic fibrotic lung diseases, including IPF, several phases can be identified during the natural course of disease. In the early phase, innate immune responses are probably involved in controlling acute tissue injury. Over time, ongoing and impaired resolution of inflammation lead to abnormal wound repair and alterations in adaptive immune responses. In a subgroup of patients these alterations are critical for disease progression. In a later phase additional factors become more important, including enhanced recruitment and activation of fibroblasts or fibrocytes. Eventually, this will incite a cascade leading to irreversible fibrosis and tissue remodeling. Finally, cumulative fibrosis and tissue remodeling become the dominant factors in burden of disease. The initial (inflammatory) pathways responsible for fibrosis development may become less important over time as pathologic signaling loops may incite self-perpetuating fibrosis.[74] Therefore, the optimal treatment plan does not only depend on type of the diagnosed disease, but also on (1) the stage of disease and (2) specific patient characteristics, to select those that might be eligible for additional immune modulating therapies. In early stages of disease and when favorable inflammatory biomarkers are present, patients may be treated with specific immune-modulating therapies, before or next to drugs that primarily target the fibrotic remodeling process (i.e. anti-fibrotics). During each phase of disease, patient perspectives and treatment of comorbidities should be integrated into the medical treatment plan. In the end-stage of disease, timely consultation on decisions on well-being and end-of-life decisions should be made in accordance with personal values in the context of advanced care planning. **Figure 5** summarizes the different phases of fibrotic lung diseases and proposes a strategy how to fit anti-inflammatory therapies into standard treatment regimes.

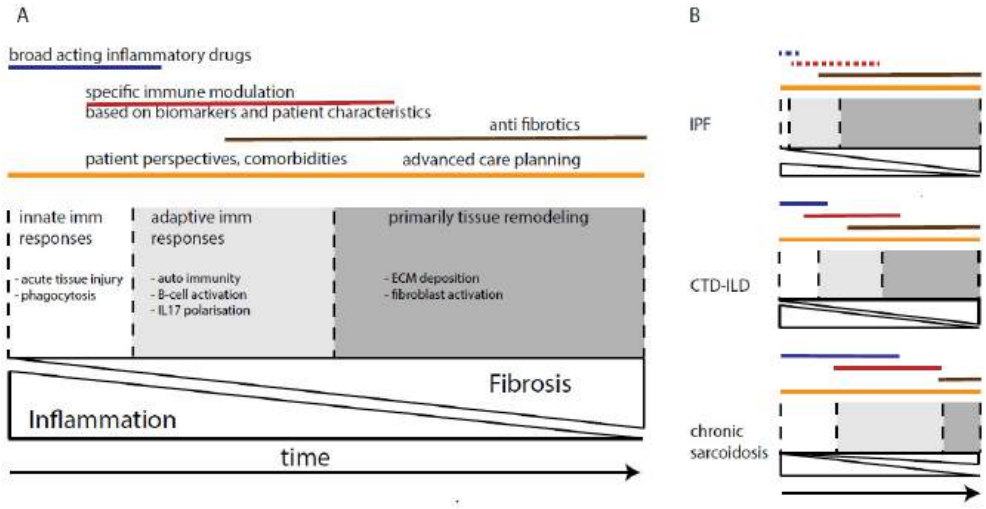

Figure 5. Overview and proposal when and how to treat fibrotic lung diseases.

(A) The optimal treatment choice depends on the phase of disease. The degree of inflammation and amount of extracellular matrix deposition changes over time. During early inflammation (acute injury), influx of innate immune cells such as neutrophils, eosinophils, lymphocytes, and macrophages, can be stopped using broad acting inflammatory drugs including steroids. Over time, adaptive immune responses and finally tissue remodeling and fibrosis are the main drivers of disease and require a different personalized treatment approach. Patient-specific biomarkers and characteristics may identify patients who are eligible for specific immunomodulatory treatment next to anti-fibrotic drugs. During all phases of disease and treatment, patient perspectives and treatment of comorbidities should be integrated into the treatment plan. In the end stage of disease, timely consultation on decisions on well-being and end-of-life decisions should be made in accordance with personal values in the context of advanced care planning. (B) Examples how and when to treat IPF, CTD-ILD and chronic sarcoidosis. The contribution of innate and adaptive immune responses may change over time, depending on type of fibrotic lung disease and individual patient course. Additionally, in some patients or diseases, not all phases will be present. For example, whether the innate immune phase in IPF is present (or clinically relevant) is unclear. Likewise, not all patients with chronic sarcoidosis will develop pulmonary fibrosis. Abbreviations: ECM= extracellular matrix; CTD-ILD= connective tissue disease-associated interstitial lung disease

While substantial progress has been made in long-term management of IPF, management of acute exacerbations of IPF (AE-IPF) is still ad its infancy. AE-IPF, defined as an acute, clinically significant, respiratory deterioration, is associated with a rapid decline in lung function and a high mortality rate.[75, 76] The etiology of AE-IPF is largely unknown and probably different from slowly progressing IPF. Despite the presence of excessive inflammation during the first phase of AE-IPF, an effective medication does not exist.[77] This implies that the role of the innate immune phase in AE-IPF is probably limited and the fast deterioration seen probably depends on other signaling pathways. This implies that novel immune-modulating drugs that might be effective for relatively stable IPF patients may not be effective in AE-IPF patients, or the other way around. In this regard it is notable that patients treated with SAR156597, which is a bi-specific antibody against IL-4 and IL-13, showed a decline in AE-IPF.[78] Although the number of events was low, this suggest that Th-2 driven pathways are involved in AE-IPF.

In PAH, the role of immune-modulating therapies can be roughly positioned in the same way as in fibrotic lung diseases (**figure 6**). Patients with specific biomarkers or characteristics, including antibodies against endothelial cells, increased BCR signaling and Th17 immune polarization or genetic characteristics, may be eligible for specific immune-modulatory treatment next to vasoactive medication. The diagnosis of PAH is primarily centered around hemodynamics and patient characteristics. This means that increased vascular resistance and thus increased pulmonary pressure may already point towards advanced vascular remodeling. In this stage, the role of immune-modulating therapies may be limited. Therefore, diagnosing PAH at an earlier stage or at lower mean pulmonary arterial pressure, may provide better opportunities for specific immune-modulation.

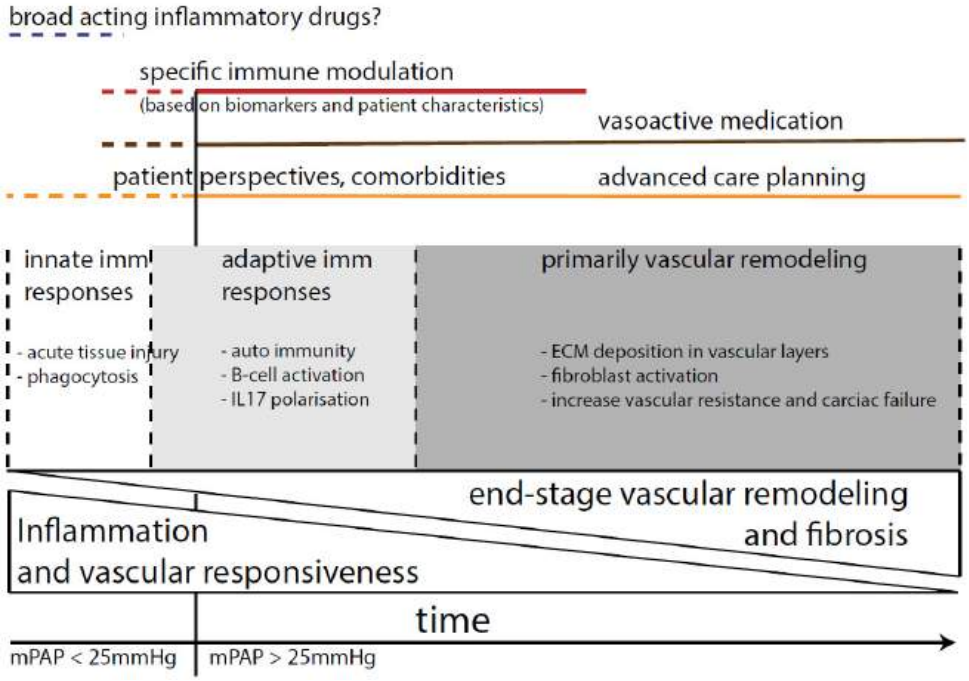

Figure 6. Proposal how to integrate treatment modalities in different stages of (I)PAH.

Choice of treatment may depend on the phase of disease. The degree of inflammation, vascular responsiveness, and amount of extracellular matrix deposition in the vascular layer's changes over time. Adaptive immune responses and end-stage vascular remodeling and cardiac failure are the main drivers of disease and require a different personalized treatment approach. Patient-specific biomarkers and characteristics may identify patients who are eligible for specific immune-modulatory treatment next to vasoactive medication. Dashed lines represent the phase were the mPAP is below 25mmHg. In this stage, current guidelines do no advocate treatment, however the 6th World Symposia on Pulmonary Hypertension (WSPH) proposed to start treatment of pre-capillary PH associated with mPAP >20 mmHg.[79] It could be that in early PH (mPAP 20-25mmHg) the contribution of innate or adaptive immunity is more prominent. Abbreviations: ECM= extracellular matrix; mPAP= mean pulmonary artery pressure

In summary, timely diagnosis of IPF and IPAH is crucial, because in later stages of disease end-stage fibrosis or end-stage vascular remodeling seems to be the dominate factor, which are less likely to responds to immune modulating therapies. It could be speculated that with better biomarkers of inflammation, immune-modulating therapies could also play a role in the treatment of IPF/IPAH. However, we have to keep in mind that in a proportion of patients, adaptive immune responses are not the driver of disease in IPF and PAH and probably secondary to the primary remodeling process. In these patients we need to keep looking for other causal links, such as prematurely shortened telomere length, inherited genetic causes, or environmental factors, and accordingly other treatment options.

Treatment in IPF and IPAH: B-cell modulation and personalized medicine

B-cell modulation

Therapies targeting immune activity ameliorate pulmonary fibrosis and pulmonary hypertension in pre-clinical studies and are currently the focus of several clinical trials (**Chapters 1 and 2**). The B-cell specific surface protein CD19 plays a crucial role in the development of bleomycininduced pulmonary fibrosis.[36, 80] Trials in pulmonary fibrosis and PAH that target B cell signaling are scarce, however, over the past years this field is slowly changing. Rituximab, an antibody against CD20, which eliminates all B cells irrespective of their specificity, is widely used in the treatment of auto-immune disease.[81] Rituximab, plasma exchange and intravenous immune globulin (IVIG) in patients with an acute exacerbation of IPF seemed to have a positive effect on an improvement of gas exchange and clinical outcome.[82] Currently, rituximab is tested in selected IPF and PAH patients with signs of autoimmunity (**Chapter 1 and 2** and NCT01086540). B-cell activating factor (BAFF), which enhances B-cell survival and activation via the BAFF receptor (BAFFR), is increased in patients with IPF and correlates with survival.[83] Blocking this pathway could be appealing, because in physiological conditions self-reactive B cells have reduced responsiveness to BAFF and die when limiting levels of BAFF are available.[84]. We found decreased BAFFR expression on B cells in IPF patients (**Chapter 7**), which may help to reduce responsiveness of self-reactive B cells to increased BAFF levels. It is possible that increased serum BAFF concentrations provoke intrinsic negative feedback mechanisms or increased internalization of BAFFR upon activation. It is therefore interesting to see whether blocking the BAFFR in IPF patients with VAY736 (NCT03287414) will be effective by elimination of self-reactive B cells that remain responsive to BAFF.

Several BTK inhibitors, including ibrutinib and acalabrutinib, have been approved for the treatment of various B-cell malignancies, while additional inhibitors are currently in clinical development.[85] Whereas ibrutinib irreversibly inhibits both BTK and its family member TEC and the second-generation BTK inhibitor acalabrutinib is more specific, they both inhibit BTK by covalent modification of the cysteine residue C481 in the ATP-binding region of the kinase domain.[86] Because acalabrutinib exhibits a higher specificity towards BTK, it has less off-target toxicity. BTK inhibitors have shown good inhibition in biological activity

in animal models of RA and SLE, however specific data on pulmonary fibrosis and PAH in these situation are not yet available.[87] With the knowledge that BTK inhibitors are generally well-tolerated, it is appealing to target BTK in human systemic autoimmune diseases. To date, several BTK inhibitors in are being tested in pre-clinical or early phase clinical studies (BMS-986142[88], RN486[89], GDC-0853[90]). Moreover, in a phase 2 clinical trial involving 267 patients with multiple sclerosis BTK inhibition was shown to significantly interfere with pathological B-cell activity in this central nervous system demyelinating disease.[91, 92]

Unexpectedly, we might learn interesting lessons from the Covid-19 pandemic with regards to BTK inhibition and fibrosis. It is notable that the group in whom SARS-CoV-2 infection is most lethal overlaps with individuals that are most at risk to develop IPF, characterized by male gender, older age and similar comorbidities.[93] Indeed, a small proportion of patients with severe Covid-19 develop pulmonary fibrosis.[94] Although BTK inhibition in acute severe COVID-19 did not meet the primary endpoint of increasing the proportion of patients who remained alive and free of respiratory failure [*https://www.astrazeneca.com/media-centre/press-releases/2020/update-on-calavi-phase-ii-trials-for-calquence-in-patients-hospitalised-with-respiratory-symptoms-of-covid-19.html*], it may still be interesting to see if BTK inhibition in these patients alters long-term outcome measures, also because it is thought that the immunological effects of acalabrutinib in Covid-19 is dependent on its capacity to inhibit IL-6 production by myeloid cells.[95]

Therefore, this raises the question whether BTK inhibition may also be used in future treatment regimens of IPF or PAH. To date, one study explored the effects of ibrutinib on bleomycin-induced fibrosis in mice and unexpectedly found augmented fibrosis.[96] However, the interpretation of this finding is complicated, because (i) ibrutinib is shown to have off-target effects and (ii) BTK is also expressed in myeloid lineages. Moreover, ibrutinib was administered in the phase where BTK inhibition may diminish resolution of inflammation by macrophages. Targeting experimental fibroses with more selective BTK inhibitors, preferably in an early phase of the disease, is warranted, given the findings in our experimental models for IPF and PH. Accordingly, results from **Chapters 5-7** provide a rationale to test BTK inhibitors in carefully selected patients with IPF and IPAH with autoimmune features or biological signs of increased B-cell autoreactivity. BTK inhibitors could be used next to existing therapies, possibly creating a synergistic two-edged sword. One side may limit the primary autoimmune-mediated damage and the other side may attenuate the concomitant or secondary fibrotic or vascular remodeling process.

Nevertheless, we should keep in mind that targeting fibrogenesis and immunological pathways at the same time should be studied in well-defined patients. Combination therapy may not work for all patients, which was also observed in a recent study which combined pirfenidone with inhaled N-acetylcysteine for the treatment of IPF.[97] Therefore, (preclinical) research is required to determine which patients or biomarkers would be best in selecting candidates eligible for additional B-cell modulation in clinical trials. Inferred from the findings in this thesis, patients with enhanced BCR signaling, increased autoreactive IgA$^+$ memory B-cells or the presence of specific anti-endothelial or anti-epithelial autoantibodies would be potential candidates.

Personalized medicine

Personalized medicine is the tailoring of pharmacologic and non-pharmacologic medical treatment to the individual characteristics of each patient at the right time (**Chapter 3**). Personalized medicine combines biological pathways and complex interactions between genetic, molecular and environmental factors that are involved in the pathogenesis and disease progression. Evaluating all these factors opens the opportunity to identify patients with a distinct biological and clinical profile. This enables the design of a patient-specific therapeutic treatment plan. Additionally, obtaining insight on patients' perspectives on how and when this treatment plan should be executed is crucial. This decision-making also involves consultation about lifestyle, comorbidities, preferences and experiences with medication in a two-way fashion between health-care professional and patient to reach shared decision making. Finally, consultation on decisions on well-being and end-of-life decisions in accordance with personal values, referred to as advanced care planning, should not be postponed until end-stage disease. In this regard important lessons can be learned from the lung oncology field where several meaningful biomarkers such as PDL-1 and activating driver mutations changed clinical practice, resulting in distinct personized treatment plans.[98]

Although personalized medicine, or precision medicine, is still in its infancy in ILD and PAH, the first steps have been made. In IPF, for instance, a study with N-Acetylcysteine showed that response to therapy was linked to a single-nucleotide polymorphism (SNP) in the *TOLLIP* locus.[99] Patients with a *TOLLIP TT*-genotype showed a favorable response, where as in patient with the *TOLLIP CC*-genotype experienced a detrimental effect. Similar results have been found in patients with a polymorphism (rs35705950) in the promoter region of the *MUC5B* gene. Retrospective analyses of large clinical trial data revealed that IPF patients with the minor allele (GT/TT) at rs35705950 in MUC5B had an improved survival when compared with wild-type (GG) subjects of the same cohort.[100] This has led to a first treatment study which includes IPF patient on the basis of their genetic profile. (NCT04300920). Also, telomere length is a predictive biomarker for response on therapy in fibrotic ILD. Patients with IPF and a telomere length less than the 10th percentile experienced more adverse outcomes when treated with immunosuppressive medication compared with patients with a telomere length more than the 10th percentile.[101] Similar results were observed in patients with chronic hypersensitivity pneumonitis. Short telomeres in this study were associated with a lack of improved survival or lung function when treated with mycophenolate mofetil.[102]

Over the last three decades numerous biomarkers have been identified in IPF[103]. For instance, high BAFF and CXCL13 plasma concentrations, involved in B cell activation and homing, are predictive for survival of IPF patients.[83, 104] However, single biomarker detection is unlikely to have a transformative effect on clinical practice. Advances have been made in biomarker discovery and panels of biomarkers to predict disease course for the individual patient[105], unfortunately this same biomarker panel failed to predict response to therapy.[106] Therefore, a combination of biological and clinical biomarkers should be considered to improve the accuracy of prognosis and treatment plan for each patient. Additionally, new

techniques of multi-variate statistics, such as unsupervised principal component analysis (PCA), could be of value of interpreting large amounts of variables in patient cohorts in order to select meaningful IPF or PAH subgroups.

Also, on the patients' side, advances are being made in personalizing care. Several projects are now being incorporated and tested that rely on home-monitoring or home-spirometry as outcome, potentially allowing for more tailored treatment.[107] In a randomized control trial with IPF it was shown that patients that used the home-monitoring application had more medication adaptations, suggesting better tailoring of care.[108] Also, the patients' needs and perceptions of care are increasingly collected. A study with a simple supportive care decision aid tool, resulted in more tailored care to patient needs.[109]

Furthermore, trials could be designed differently to gain better insight into the effect of interventions on patients' well-being. Most (pharmacological) trials reflect on a single physiological endpoint, such as change in FVC or 6-minute walking distance (6MWD). However, previous work showed that lung function does not correlate well with scores in health-related quality of life questionnaires.[110] Therefore, better and possibly more meaningful endpoints should be explored. For instance, a composite endpoint on clinical worsening could be used that embraces several clinical and biological features. From our perspective, symptom burden or health related quality of life questionnaires could be part of such a composite endpoint.

Although there are small steps forward, increasing awareness and new developments in these fields will hopefully advance patient tailored care.

Novel insights in the pathogenesis of sarcoidosis-associated pulmonary hypertension

Recognizing sarcoidosis-associated pulmonary hypertension (SAPH) from (end-stage) sarcoidosis is often challenging.[111] Some radiological and functional signs can be helpful in directing clinicians to SAPH. For instance, SAPH patients have a reduction in 6 minute walking distance compared with other sarcoidosis patients.[112, 113] Decreased diffusing capacity of the lungs for carbon monoxide (DLCO) and FVC values in pulmonary function tests are observed in SAPH patients.[114] Also radiological signs can be used as predictors of PH. Pulmonary artery diameter on chest CT indexed to body surface area (BSA) is a predictor of PH in patients with pulmonary sarcoidosis [115] and the presence of pulmonary fibrosis on chest X-ray can be indicative of concomitant PH.[116] On the other hand, the ReSAPH registry showed that only half the patients had scadding stage 4 chest x-ray findings.[113] This means that a large group of patients who develop PH do not have fibrotic changes, which is also observed in other studies.[114, 117] Therefore, there is a need for more knowledge on the underlying pathobiology of SAPH and this will help us gaining more insight how to discriminate SAPH from sarcoidosis.

Using unbiased PCA on cytokines that are known to be involved in sarcoidosis and chronic inflammation[111], we found that especially chemokines CXCL9 and CXCL13 are most relevant to discriminate between SAPH and sarcoidosis patients (**Chapter 8**). These cytokines provide an interesting link to chronic inflammation and possibly autoimmunity. The receptor for CXCL9 is CXCR3 is differentially expressed on Th1, Th17 and Th17.1 cells. CXCL9 is required for optimal IFN-γ production by Th1 and Th17.1 cells *in vivo* and especially IFNγ-producing Th17.1-cells appear to be more pathogenic and possibly linked to disease progression in sarcoidosis.[118, 119, 120, 121, 122, 123] Additionally, these IL-17/IFNγ double-producing cells are pathogenic drivers in several autoimmune diseases.[119] Although the activating antigens have been a mystery (or holy grail) of sarcoidosis research, recent work showed that autoantibodies, possibly against misfolded proteins, are present in a subgroup of sarcoidosis patients.[124, 125] A possible role for autoimmunity and B cells in SAPH is further supported by increased levels of CXCL13, which is a chemoattractant for B cells (**Chapter 8**). Together with increased BAFF levels, CXCL13 is important for the local formation of the B-cell conglomerates that are found around granulomas.[126, 127] It could be that these B-cell conglomerates are producers of pathogenic antibodies, because IL-17 polarization in coordination with IL-21 and BAFF is sufficient to promote germinal center formation in experimental models.[128] If TLOs are present and how local pathogenic antibodies may lead to pulmonary hypertension in SAPH needs further research. However, our work raises the suspicion that underlying autoimmune pathways are involved or co-occuring in SAPH.
Our unbiased PCA (**Chapter 8)** also highlighted other cytokines that discriminate SAPH from other groups. **Figure 8** summarizes these cytokines and proposes an integration of these findings in SAPH pathogenesis.

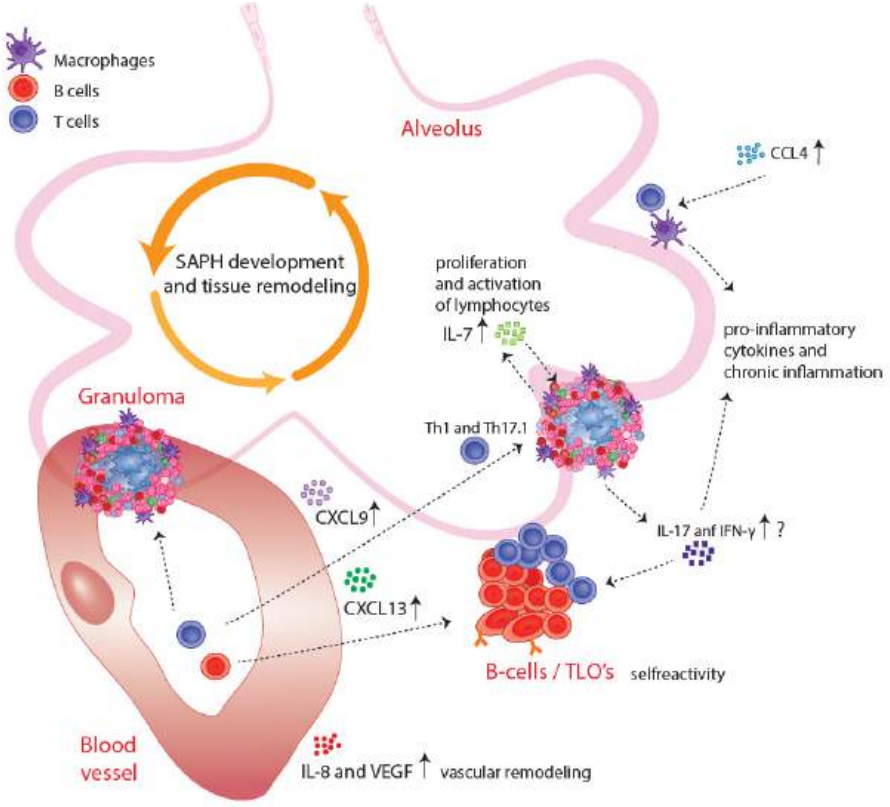

Figure 7. Model of underlying immunologic mechanisms in SAPH development.
Several cytokines discriminate SAPH from other groups, including patients with chronic sarcoidosis, idiopathic pulmonary hypertensions and healthy controls. CXCL9 and CXCL13 link SAPH to selfreactivity to local proteins and IL-17 polarization, which may lead to chronic inflammation. Induction of Th17.1 cells lead to progression of pulmonary sarcoidosis and possibly SAPH. IL-7 and CCL4 are actively involved in the recruitment and activation of various immune cells, including macrophages and T lymphocytes. Upon activation, these cells produce a variety of pro-inflammatory cytokines, aiding to local inflammation and tissue remodeling. Chemotaxis of T cells and granuloma formation in and around the vascular wall in combination with IL-8 and VEGF may promote vascular remodeling and subsequent PH development. TLO= tertiary lymphoid organ.

Taken together, the underlying inflammatory profile of SAPH is different from that of chronic sarcoidosis and is characterized by immune pathways involved in chronic inflammation, vascular remodeling and possibly autoimmunity. These new insights may eventually lead to new therapeutic approaches in SAPH. For instance, blocking the IL-23 receptor, which is essential for conversion of Th17 cells towards pathogenic Th17.1 cells, with risankizumab was affective in psoriasis and Crohn's disease.[129, 130] In refractory pulmonary sarcoidosis, a phase 2 trial with rituximab treatment improved respiratory function in a subset of patients, however the sample size for subgroup analysis was too small.[131] Together with our observation that CXCL13 is a dominant cytokine in discriminating SAPH from chronic sarcoidosis, rituximab may also be an interesting drug for future trials.

Final remarks

Findings described in this thesis provide a new perspective on how dysregulated immune homeostasis influences tissue and vascular remodeling in ILD and PH. In this thesis, the main focus was on adaptive immune responses, and B cells and BCR signaling in particular. However, tissue and vascular remodeling are the result of a complex interplay between tissue-resident cells, including (myo)fibroblasts, tissue-resident macrophages, epithelial cells and bone marrow-derived cells, such as monocytes and T cells. Therefore, the findings described in this thesis should be integrated in the complex biology of other cellular mechanisms underlying tissue remodeling and fibrosis. To find key inflammatory and non-inflammatory pathways that drive disease new approaches are currently being employed. Especially single-cell genomics methodologies, including single-cell RNA-sequencing together with multiple-omics readouts (i.e. genomes and epigenomes) are changing our understanding of mechanisms that drive ILD and PH pathogenesis.[60] It is clear that these underlying mechanisms differ between patients, explaining why patients with ILD and PH are clinically and biologically heterogeneous. To further improve the yield of basic and translational research we recommend, (i) the development of experimental (animal) models that better resemble human pathology, (ii) to subdivide patients who take part in (drug) trials or translational research on a mechanistic basis rather than organ basis, and (iii) to explore patient tailored multi-drug regimens as multiple pathways collaborate in tissue remodeling and fibrosis. Combining these new approaches and methodologies is necessary to optimize personalized treatment of patients with ILD and PH.

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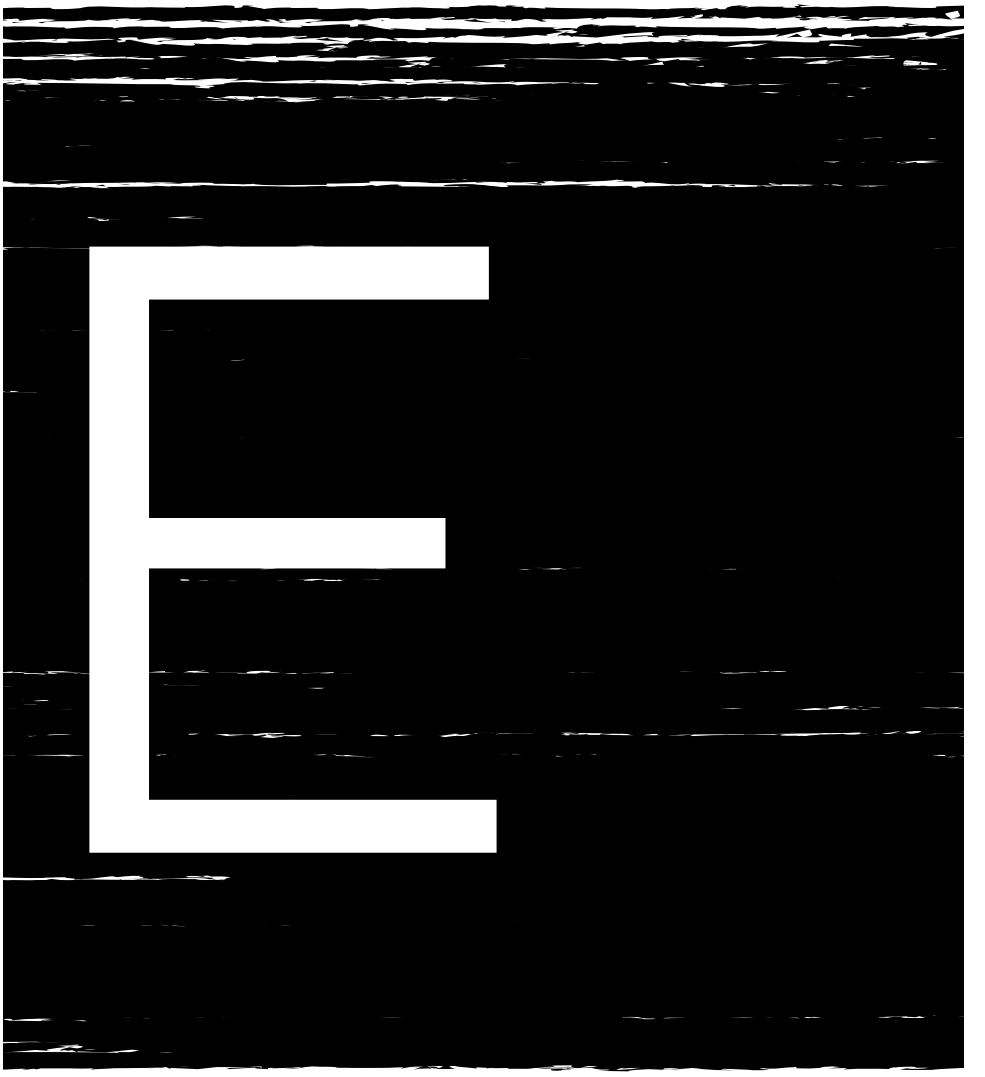

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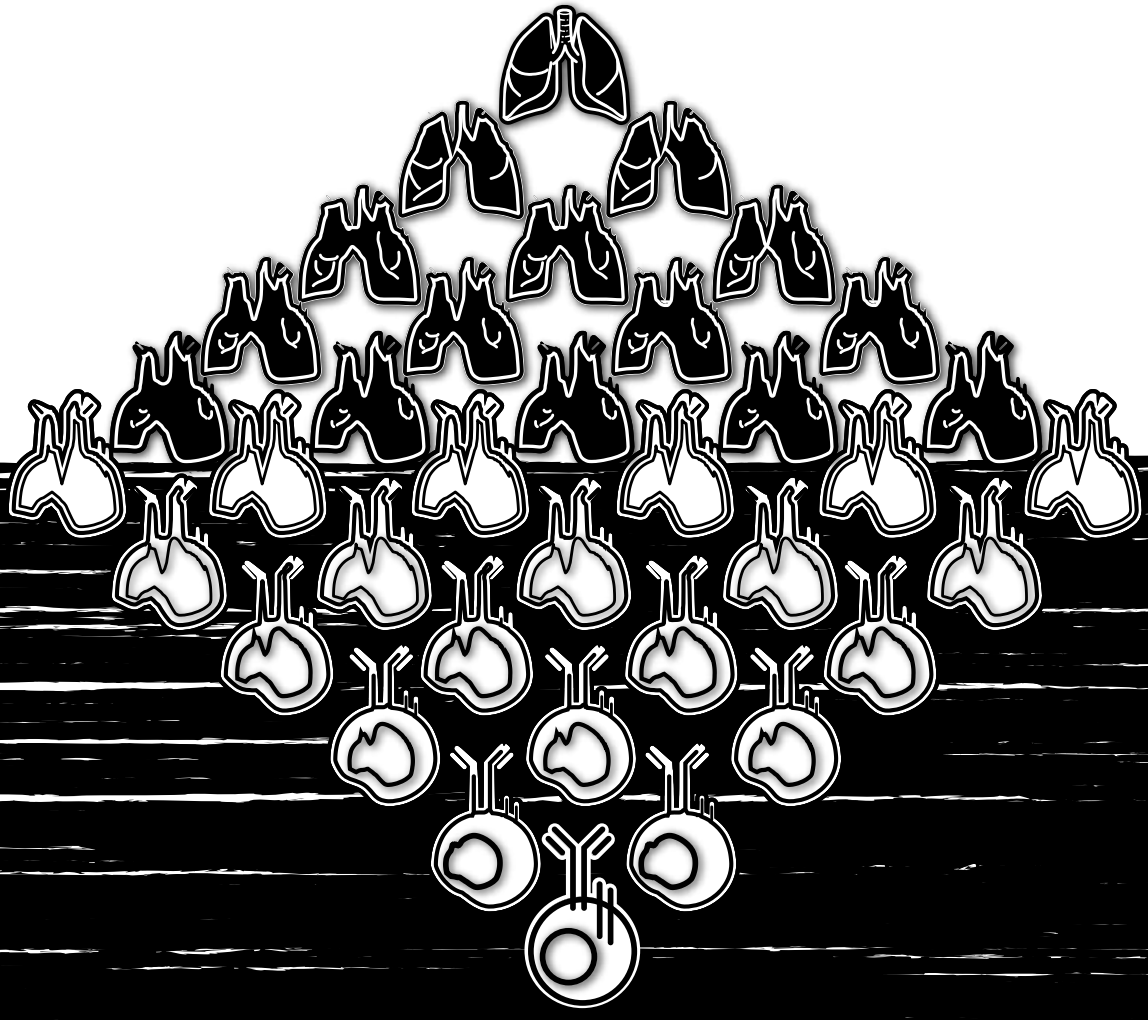


ENGLISH SUMMARY

Gas exchange is one of the most important functions of the lung. During gas exchange oxygen diffuses from the lungs into the bloodstream and carbon dioxide passes from the blood into the lungs. Gas exchanges occurs between the alveoli and a fine network of blood vessels. In between the alveoli and blood vessels lies a thin layer called the pulmonary interstitium, which is a collection of supporting tissues. Several diseases can affect the interstitial space (interstitial lung diseases (ILD)) or pulmonary vasculature. Loss of integrity of the interstitial space or surrounding vasculature may result in poor gas exchange, leading to disabling symptoms, including shortness of breath or a decreased exercise tolerance.

Pulmonary fibrosis (scarring of the lung tissue) can be one of the features of ILD. Fibrosis is the end-result of exaggerated wound repair and tissue remodeling and is characterized by enhanced extracellular matrix deposition in the interstitial space. Idiopathic pulmonary fibrosis (IPF) is a form of pulmonary fibrosis with an unknown etiology and a median survival of 3 years when untreated.

Pulmonary hypertension (PH) is a disease that affects the pulmonary arteries and is characterized by progressive pulmonary vascular remodeling mediated by endothelial cell dysfunction, leading to increased pulmonary vascular pressure and right ventricle dysfunction. Idiopathic pulmonary arterial hypertension (IPAH) is a form of PH in which no underlying disease or etiology is identified at time of diagnosis.

Understanding of the mechanisms that initiate and perpetuate ILD and PH has improved over the last decades and several lines of evidence suggest a role for imbalanced immune responses. The immune response can be divided in two complementary pathways: innate and adaptive immunity. Innate immunity is rapid and occurs quickly after an injury or infection, but is not specific for the encountered antigen. The adaptive immunity is mediated by antigen-specific B and T lymphocytes and equips the body with a memory function. This memory function enables a fast, more robust and antigen-specific immune response upon subsequent exposure to the same antigen. Additionally, innate and adaptive immune responses are crucial for adequate restoration of tissue damage. Impaired resolution of inflammation or an imbalanced immune response may lead to a chronic inflammatory state which can result in tissue fibrosis and vascular remodeling.

One of the hallmarks of the adaptive immune system is clonal expansion of B cells that carry antigen-specific receptors, the B cell receptor (BCR). This clonal expansion is characterized by a rapid increase of clones that originate from those B cells that specifically recognize the antigen. Additionally, B cell-derived cytokines guide the development of lymphoid tissues and promote or regulate effector and memory T cell responses. To exert all these functions, B cells depend on antigen recognition by the BCR. Upon binding of a specific antigen, the intracellular domain of the BCR complex will activate downstream signaling proteins. One of the proteins in this BCR signalosome is Bruton's tyrosine kinase (BTK).

Generation of B cells with different BCRs occurs stochastically, which implies that the BCR repertoire also includes receptors that could potentially recognize self-antigens. To prevent autoimmunity, multiple checkpoints during B differentiation exist to eliminate newly emerging autoreactive B cells. For normal B-cell development and elimination of B cells that recognize self-antigens, expression of appropriate levels of BTK is crucial. Increased BTK protein levels can rescue auto-reactive B cells from apoptosis. These auto-reactive B cells may further develop into autoreactive plasma cells producing high affinity auto-antibodies.

How and if auto-reactive B cells and, more generally speaking, adaptive immune responses contribute to the development of IPF and IPAH is unclear. The big question remains whether inflammation is a critical trigger for IPF/IPAH-onset and disease progression in susceptible patients or a consequence of established disease. Additionally, inflammatory pathways and immune disturbances are probably not the same from patient to patient. Therefore, the aim of this thesis is to further unravel the immune-related pathogenesis of ILD and PH.

In **Chapter 2** we give an overview of the current knowledge on inflammation and immune cell activation in IPF pathogenesis and discuss whether immune cell activation is altered by current anti-fibrotic treatments. Additionally, we discuss recent and upcoming therapeutic trials that target and modulate the immune system in patients with IPF. **Chapter 2** serves as a basis for new perspectives on how to proceed in further research addressing the role of inflammation in fibrogenesis and vascular remodeling in IPF.

Although Inflammatory processes can shape the course of IPF in different ways, individual patients' factors, should not be forgotten. In daily practice, disease behavior and response to therapy vary among patients. **Chapter 3** describes how biology could be combined with patients' perspectives, such as comorbidities, lifestyle, and experiences with medication to optimize personalized treatment in IPF.

In **Chapter 4** we studied the role of fibrocytes in IPF. Fibrocytes are bone marrow-derived cells with fibroblast-like tissue remodeling properties. Upon lung damage, circulating fibrocytes home to the affected tissue, migrate out of the circulation and aid to local wound repair and tissue remodeling. The now called lung fibrocytes produce components of the extracellular matrix and pro-fibrotic cytokines. We optimized flow cytometric detection of circulating and lung fibrocytes using a unique combination of intra- and extra-cellular markers to establish a solid gating strategy. With this strategy, we analyzed lung fibrocytes in single-cell suspensions of explanted IPF and control lungs and compared characteristics and numbers with circulating fibrocytes in patients with IPF. We show that patients with IPF have increased proportions of fibrocytes, not only in the circulation, but also in explanted end-stage IPF lungs. These lung fibrocytes have increased surface expression of Human Leukocyte Antigen - DR isotype (HLA-DR), increased intracellular collagen-1 expression, and also altered forward and side scatter characteristics compared with their circulating counterparts. Our gating strategy may boost fibrocyte research and targeting fibrocytes may hold potential as a therapeutic target for fibrotic lung diseases.

In **Chapter 5 and 6** we studied the role of B cells and BCR signaling in IPF. In **Chapter 5** we assessed B-cell subsets and their activation status in lungs, lymph nodes and blood samples of IPF patients and healthy controls (HC) by flow cytometry. We observed that IPF patients had increased plasmablasts and IgA$^+$ memory B-cells in blood, intense IgA staining in lung tertiary lymphoid organs (TLO), and more serum IgA antibodies recognizing nuclear self-antigens. Higher autoreactive IgA levels correlated with an increased decline of forced vital capacity. Furthermore, we showed that in patients with IPF circulating B cells had elevated BTK expression. This increase in BTK expression was already present in naïve B cells, which have not encountered an antigen yet. This might either reflect global B-cell activation due to pro-inflammatory micro-environments or pathogenic B-cell activation that directly contributes to autoimmune pathology. Finally, in a bleomycin pulmonary fibrosis mouse model, we observed the induction of IgA$^+$ Germinal Center (GC) B-cells and IgA$^+$ plasma cells, indicating similarities with human pathobiology. The bleomycin model also showed that proportions of GC B cells in bronchoalveolar lavage fluid correlated with the extent of fibrosis.

Although our finding of increased BTK expression in B cells from IPF patients suggested altered BCR signaling in IPF, we analyzed in **Chapter 6** the BCR signaling pathway in more detail. This was particularly essential, because also other signaling pathways in B cells depend on BTK activity, including toll-like receptor and chemokine receptor pathways. We studied spleen tyrosine kinase (SYK), which upon activation phosphorylates and activates BTK. Activation of BTK leads to phosphorylation of its primary substrate, phospholipase Cy2 (PLCy2). This signaling cascade eventually regulates genes that are involved in cell cycle progression and anti-apoptotic pathways. Studying proteins and their activation status up- or downstream of BTK may clarify if increased BTK activation in IPF can be linked to a general enhancement of BCR signaling. We found that naïve B cells, but not memory B cells, from treatment-naïve IPF patients displayed increased phosphorylation of the BCR signaling molecules SYK, BTK and PLCy2 following BCR stimulation in vitro. Additionally, we sought to determine the effect of the anti-fibrotic tyrosine kinase inhibitor nintetanib on BCR signaling. Interestingly, treatment of IPF patients with nintedanib induced major changes in BCR signaling. Patients that showed high phosphorylation of the BCR signalosome molecules before treatment, showed low phosphorylation after the start of nintedanib treatment, and vice versa.

Taken together, the findings in **Chapter 5 and 6** support that in a subgroup of patients, B cells and alterations in BCR signaling are involved in the pathogenesis of IPF. These changes may contribute to a loss of immune tolerance and development of auto-reactivity against pulmonary tissue, contributing to ongoing tissue damage and subsequent remodeling. Prospective trials are needed to test whether B-cell modulating therapies, next to anti-fibrotic treatment, could be beneficial as treatment in selected IPF patients.

In **Chapter 7** we investigated the involvement of activated B cells in the pathogenesis of IPAH. We studied an autoimmune-prone mouse model with enhanced BCR signaling, as well as circulating B cells and T cells in three groups of patients with PAH. We observed

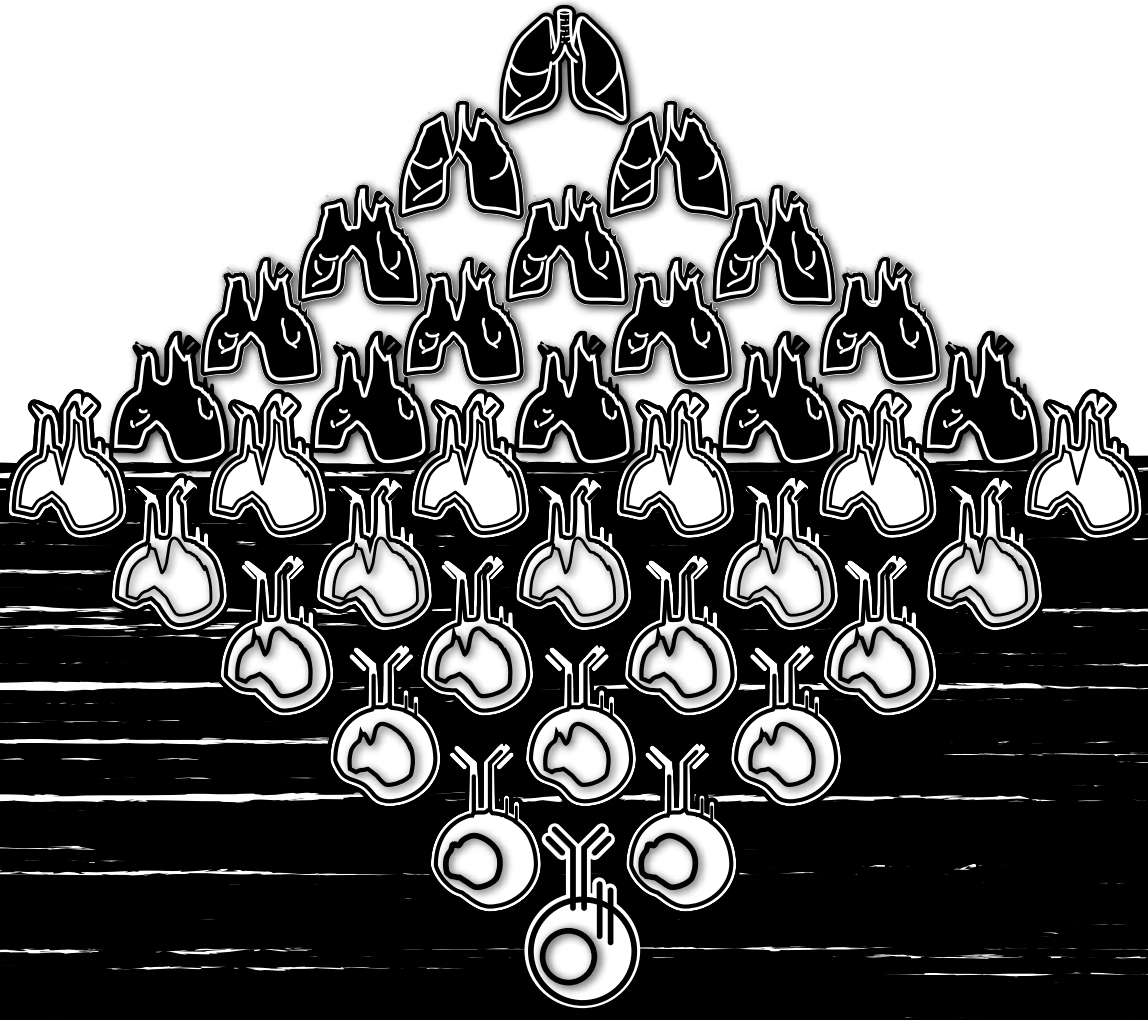
that transgenic mice with increased protein levels of the BTK specifically in B cells developed haemodynamic and cardiac signs of PH, following induction of pulmonary injury with bleomycin. In this hypothesis-generating two-hit model, the mediastinal lymph nodes contained active GCs with prolonged B-cell activation and increased proportions of activated inducible T-cell costimulator ($ICOS^{high}$) follicular T-helper cells, while autoantibodies with reactivity against vascular antigens were present in the serum. In parallel, peripheral blood B cells from patients with IPAH displayed increased BTK protein expression, already in naive B cells. The increase in BTK levels was associated with enhanced BTK phosphorylation in B cells, the presence of IgG autoantibodies in plasma, as well as higher proportions of from follicular T-helper 17 (Tfh17) cells. In conclusion, the findings presented in **Chapter 7** provides evidence that loss of immune homeostasis can contribute to PH development.

In **Chapter 8** we studied a subtype of PH that can be seen in patients with sarcoidosis. Sarcoidosis is a chronic inflammatory disease of unknown cause, which often affects the lungs and mediastinal lymph nodes. Sarcoidosis is characterized by granuloma formation, which are conglomerates of epithelioid- and multinucleated-giant cells encircled by T cells and B cells. An important complication of sarcoidosis is the development of PH. Sarcoidosis-associated PH (SAPH) can lead to heart failure and is associated with a poor outcome. The etiology of SAPH is often multifactorial and poorly understood. To gain better insight into the pathogenesis of SAPH, we compared inflammatory mediators (cytokines and growth factors) in SAPH patients with patients with advanced sarcoidosis without PH (SRC), patients with IPAH, and HC. We identified relationships between functional and hemodynamic parameters, and performed an unbiased principal component analysis (PCA) to evaluate which inflammatory mediator is most relevant to discriminate SAPH patients.

Our analyses showed that chemokine (C-X-C motif) ligand (CXCL)13 and CXCL9 were increased in SAPH patients but not in patients with advanced sarcoidosis without PH, nor in IPAH patients. The PCA revealed that CXCL13 and CXCL9 are most relevant to discriminate between SAPH and other patient groups or HC. Additionally, we found a correlation between increased serum levels of vascular endothelial growth factor (VEGF) and C-C motif chemokine (CCL)4 and increased mean pulmonary arterial pressure (mPAP) and decreased cardiac index, respectively. Follow up research is underway to test if inflammatory mediators correlate with clinical outcomes, such as survival.

In **Chapter 9**, the results presented in this thesis is put into perspective of the latest insights and we discuss how our findings could guide future research and clinical practice. In this thesis, we show that adaptive immune responses are involved in the etiology and disease progression of ILD and PH. Importantly, upon tissue damage, it's all about the right balance between inflammation and adequate resolution of inflammation. Impaired resolution may lead to a chronic inflammatory state and local auto-reactivity, which may contribute to tissue remodeling and fibrosis. It is appreciated that the contribution of immune activation to the pathogenesis of ILD and PH varies considerably between diseases and even within patients with the same disease. In a relatively small fraction of patients, the alterations

seen in adaptive immune responses will be the primary culprit or driver of disease. Therefore, it will be crucial to select only those patients that are sensitive for co-treatment with immunomodulating therapies, including drugs that interfere with B-cell activation. In general, more effort should be undertaken to identify which underlying mechanisms (e.g. genetic, environmental, inflammatory) is likely to contribute most to disease pathogenesis. Combining knowledge on key immunological mechanisms involved with disease behavior, response to therapy and patients' perspectives should result in a personalized treatment plan. This approach will advance patient-tailored care and it obvious that a 'one fits all' approach in ILD and PH does not apply anymore.



NEDERLANDSE SAMENVATTING

De longen hebben als functie zo efficiënt mogelijk de uitwisseling van zuurstof en koolzuur mogelijk te maken. Dit proces wordt ook wel gasuitwisseling genoemd. De longen bestaan uit longblaasjes met daaromheen een netwerk van bloedvaten. Tussen de longblaasjes en bloedvaten zit een ruimte die het interstitium wordt genoemd. In normale omstandigheden is dit interstitium een dun vlies, waardoor de gasuitwisseling zonder problemen verloopt. Als door een ziekte het interstitium of de wand van de bloedvaten dikker wordt, raakt de gasuitwisseling verstoord en ontwikkelen patiënten (invaliderende) klachten van kortademigheid en een verminderd inspanningsvermogen. Er zijn meerdere aandoeningen die het interstitium aantasten, deze worden overkoepelend interstitiële longziekten genoemd.

In het geval van longfibrose is het interstitium dikker, waarbij rondom de longblaasjes fibrose (littekenweefsel) wordt gevormd. De long wordt hierdoor stugger en er ontstaat onherstelbare schade. In het geval van idiopathische pulmonale fibrose (IPF) is de oorzaak van het ontstaan van fibrose onbekend (idiopathisch = zonder bekende oorzaak). IPF is een chronische en een ongeneeslijke ziekte.

In het geval van pulmonale hypertensie (PH) zijn juist de bloedvaten in de longen aangedaan. Bij deze aandoening is de vaatwand verdikt, waardoor het lumen van het bloedvat nauwer is. Daarnaast is ook het aantal bloedvaten in de longen verminderd. Dit heeft als gevolg dat de bloeddruk in de longvaten toeneemt. Omdat het hart het bloed door de longen moet pompen, leidt deze verhoogde druk in de longvaten ertoe dat er op termijn hartfalen kan ontstaan. Ook binnen de verschillende vormen van PH is er een groep waarbij de oorzaak onbekend is. Deze groep wordt idiopathisch pulmonale arteriële hypertensie (IPAH) genoemd.

Zowel bij interstitiële longziekte als bij PH zijn er aanwijzingen dat ons afweersysteem een rol kan spelen bij de ontstaanswijze van deze ziekten. Het afweersysteem of immuunsysteem is het verdedigingssysteem van het menselijk lichaam tegen ziekteverwekkers en lichaamsvreemde stoffen. De aangeboren afweer reageert snel, maar is aspecifiek. De verworven (adaptieve) afweer daarentegen, wordt langzaam geactiveerd ter bestrijding van een specifieke ziekteverwekker. Hierna zal dit deel van ons afweersysteem een geheugenfunctie tegen deze specifieke ziekteverwekker opbouwen, waardoor er bij een tweede infectie een snellere en effectievere eliminatie van deze verwekker is. Daarnaast speelt zowel de aangeboren als verworven afweer een belangrijke rol bij het herstellen van schade in het lichaam. In het geval van aanhoudende prikkels die schade veroorzaken waardoor het herstellen hiervan niet goed lukt of in het geval van een niet goed functionerend immuunsysteem kan er littekenweefsel ontstaan. Zowel bij IPF als IPAH zijn er aanwijzingen dat het herstel van schade niet goed lukt met overmatige productie van littekenweefsel van het interstitium (bij IPF) en de vaatwand (bij IPAH) als gevolg. De laatste jaren zijn er steeds meer aanwijzingen dat B cellen, welke een belangrijk onderdeel vormen van onze specifieke afweer, betrokken zijn bij fibrosevorming.

B cellen (B lymfocyten) maken gerichte en specifieke antistoffen tegen ziekteverwekkers, presenteren antigenen (stuk eiwit afkomstig van de ziekteverwekker) aan andere cellen van het afweersysteem en produceren signaalstoffen (cytokines) om andere cellen te activeren of af te remmen. Om deze functies goed uit te voeren hebben B cellen een B-cel

receptor (BCR) op hun celoppervlak. Op het moment dat de BCR een antigeen specifiek herkent, zullen er in de cel verschillende eiwitten geactiveerd worden. Deze eiwitten worden BCR-signaleringsmoleculen genoemd. Een goede balans in de hoeveelheid en mate van activiteit van deze eiwitten is cruciaal voor de juiste werking van B cellen. Een van die BCR-signaleringsmoleculen is Bruton's tyrosine kinase (BTK).

In ons lichaam worden ook continu B cellen gevormd waarvan de antistoffen die ze produceren lichaamseigen eiwitten kunnen herkennen. Tijdens de ontwikkeling van B cellen zijn er bepaalde fases, die autoreactiviteit checkpoints worden genoemd, waarbij potentiële auto-reactieve B-cellen geëlimineerd worden. Eerder onderzoek heeft aangetoond dat verhoogde activiteit van het BTK eiwit in B cellen kan bijdragen aan verminderde eliminatie van auto-reactieve B-cellen. Wanneer deze B cellen niet voortijdig geëlimineerd worden, kan er een auto-immuunziekte ontstaan, waarbij er ontstekingsreacties tegen lichaamseigen cellen en weefsels optreden. In welke mate auto-reactieve B cellen en andere cellen van de verworven afweer bijdragen aan de ontstaanswijzen van IPF en IPAH is onduidelijk. Een belangrijke vraag die we in dit proefschrift proberen te beantwoorden is, of de veranderingen in de functie en activatie van het verworven afweersysteem actief bijdragen aan het ontstaan en verergering van IPF en IPAH. Omdat het ziekteverloop van beide aandoeningen grillig kan zijn en vaak verschilt tussen patiënten, kan het ook zo zijn dat veranderingen in de verworven afweer alleen bij een deel van patiënten bijdraagt aan de ziekte. Deze kennis kan bijdragen aan patiënt-specifieke behandelingen.

In **Hoofdstuk 2** geven wij een uitgebreid overzicht van de huidige kennis van de rol van zowel de aangeboren als de verworven afweer in de ontstaanswijze en het ziektebeloop van IPF. Op dit moment zijn er 2 geregistreerde medicijnen voor de behandeling van IPF, namelijk pirfenidone en nintedanib. Deze medicamenten kunnen de ziekte afremmen, maar niet genezen. Wij beschrijven welke invloed deze anti-fibrose medicijnen uitoefenen op ons afweersysteem en hoe dit mogelijk bijdraagt aan de remmende werking op fibrose vorming. Tot slot beschrijven we welke (nieuwe) medicijnen voor de behandeling van IPF er in ontwikkeling zijn en hun werkingsmechanismen.

Inmiddels is het duidelijk dat de ene IPF patiënt de andere niet is. Het beloop van de ziekte verschilt, waarbij er patiënten zijn die relatief langzaam progressief zijn, terwijl er ook patiënten zijn bij wie het ziektebeloop veel sneller verloopt. Daarnaast is de reactie op behandeling ook verschillend. Dit heeft te maken met grote verschillen in onderliggende factoren die bijdragen aan het beloop en ontstaanswijze van de ziekte. Hierbij moeten we denken aan patiënt-specifieke genetische, moleculaire en omgevingsfactoren, die van persoon tot persoon verschillend zijn.

In **Hoofdstuk 3** beschrijven wij hoe wij deze patiëntgebonden factoren kunnen gebruiken om voor een individuele patiënt een op maat gemaakte behandeling te kunnen maken. Bij het opstellen van een gepersonaliseerde behandeling is het belangrijk dat de wensen en het verwachtingspatroon van de patiënt meegenomen worden.

In **Hoofdstuk 4** onderzoeken wij wat de rol van fibrocyten is bij IPF en hoe fibrocyten betrouwbaar kunnen worden gemeten in bloed en longen van IPF patiënten en gezonde proefpersonen. Fibrocyten zijn cellen die hun oorsprong vinden in het beenmerg en differentiëren uit een voorloper cel die een monocyt heet. Als fibrocyten nog in de bloedbaan zitten, worden ze bloed fibrocyten genoemd. In het geval van weefselschade in de longen migreren bloed fibrocyten naar de longen toe. In de longen aangekomen maken fibrocyten, nu long fibrocyten genoemd, componenten van het littekenweefsel. Een van de bindweefseleiwitten die door fibrocyten worden gemaakt is collageen en daarnaast produceren fibrocyten cytokines die bijdragen aan de fibrosevorming. Wij presenteren een nieuwe methode om zowel long als bloed fibrocyten te identificeren en te kwantificeren. Met deze methode tonen wij aan dat de aantallen van long fibrocyten in IPF longen hoger zijn in vergelijken met niet-IPF longen. Daarnaast zijn deze aantallen in bloed ook hoger in IPF patiënten dan in gezonde controles. Deze long fibrocyten bevatten ook meer collageen en zijn meer geactiveerd in vergelijking met bloed fibrocyten. Het op de juiste manier remmen van de ontwikkeling of het deactiveren van fibrocyten zou een toekomstige behandeling kunnen zijn van fibrotische longziekten.

In **Hoofdstuk 5 en 6** gaan wij dieper in op de rol van B-cellen en BCR signalering in IPF. In **Hoofdstuk 5** bestuderen wij eerst welke subtype B cellen er voorkomen in bloed, lymfklieren en longen van patiënten met IPF en vergelijken dit beeld met bloed van gezonde proefpersonen en overgebleven gezond longweefsel van patiënten die een longresectie hebben ondergaan. Daarnaast bestuderen wij de eigenschappen van deze B cellen en welk type antistoffen ze produceren. We laten eerst zien dat er in het bloed van IPF patiënten meer plasmablasten en IgA-memory B cellen circuleren. Deze IgA memory cellen zijn ook verhoogd aanwezig in de longen van IPF patiënten. Wij vinden aanwijzingen dat deze IgA antistoffen vaker gericht zijn tegen lichaamseigen eiwitten. Dit worden autoantistoffen genoemd. In patiënten met IPF correleert verhoogde concentraties van IgA autoantistoffen in het bloed met versnelde achteruitgang van longfunctie. Daarbij heeft een deel van de IPF patiënten verhoogde intracellulaire BTK eiwitlevels in circulerende B cellen. Opvallend is dat deze verhoging van BTK al reeds aanwezig is in de zgn. naïeve B cellen, die nog niet eerder door antigeen geactiveerd zijn. Dit suggereert dat er mogelijk omgevingsfactoren zijn die zorgen voor deze verhoging of dat deze B cellen al intrinsiek gevoeliger zijn om autoantistoffen te (gaan) maken. Tot slot tonen wij in een experimenteel longfibrose muismodel (bleomycine model) aan dat ook fibrose longen van muizen meer IgA-geactiveerde B cellen bevatten.

Weliswaar suggereerde de gevonden verhoging van BTK eiwit in IPF patiënten een afwijkende BCR signalering, maar er zijn echter ook andere signaleringscascades in B cellen die BTK kunnen beïnvloeden verhoogde intracellulaire BTK eiwitlevels in circulerende B cellen. Daarom gaan wij in **Hoofdstuk 6** dieper in op eiwitten die betrokken zijn bij de BCR-signalering in IPF patiënten. We onderzoeken naast het eiwit BTK ook de eiwitten spleen tyrosine kinase (SYK) en phospholipase Cy2 (PLCy2), die respectievelijk voor en na het BTK eiwit in de signaleringscascade zitten. Activatie van SYK zal normaliter leiden tot activatie van BTK, wat op zijn beurt onder andere PLCy2 activeert. Uiteindelijk leidt het activeren van deze gehele cascade tot activatie en celdeling van B cellen. Door het bepalen van de activatie status van deze verschillende eiwitten in de BCR cascade,

krijgen wij een beter inzicht op welke wijze BTK activatie veroorzaakt wordt in IPF patiënten. Daarnaast onderzoeken we welk effect het anti-fibrose medicijn, nintedanib, op de BCR signalering heeft. Eerst laten we zien dat een groter deel van naïeve B cellen van IPF patiënten geactiveerd raakyt na stimulatie, ten opzichte van B cellen van gezonde mensen. Daarnaast zijn zowel de SYK en PLCγ2 in naïeve B-cellen meer geactiveerd in IPF patiënten en correleert dit met het BTK eiwit niveau. Dit verschil wordt niet meer waargenomen in B cellen die al verder in ontwikkeling zijn (memory B cellen). Tot slot tonen wij aan dat BCR signalering door nintedanib beïnvloed wordt. We laten namelijk zien dat geactiveerd BTK in naïeve B cellen na het starten van nintedanib verhoogd is in vergelijking met voor start van nintedanib in dezelfde patiënten. Opvallend is dat nintedanib behandeling de activatie van SYK en PLCγ2 weer anders lijkt te beïnvloeden. Patiënten bij wie voor de start de activatiestatus hoog was van SYK en PLCγ2 observeren we een daling van deze activatie status na de start met nintedanib. Opvallend is dat bij patiënten bij wie de activatie status juist verlaagd is voor de start met nintedanib de activatie omhoog gaat na starten. Hoe nintedanib deze eiwitten mechanistisch direct of indirect beïnvloed is nog onduidelijk.

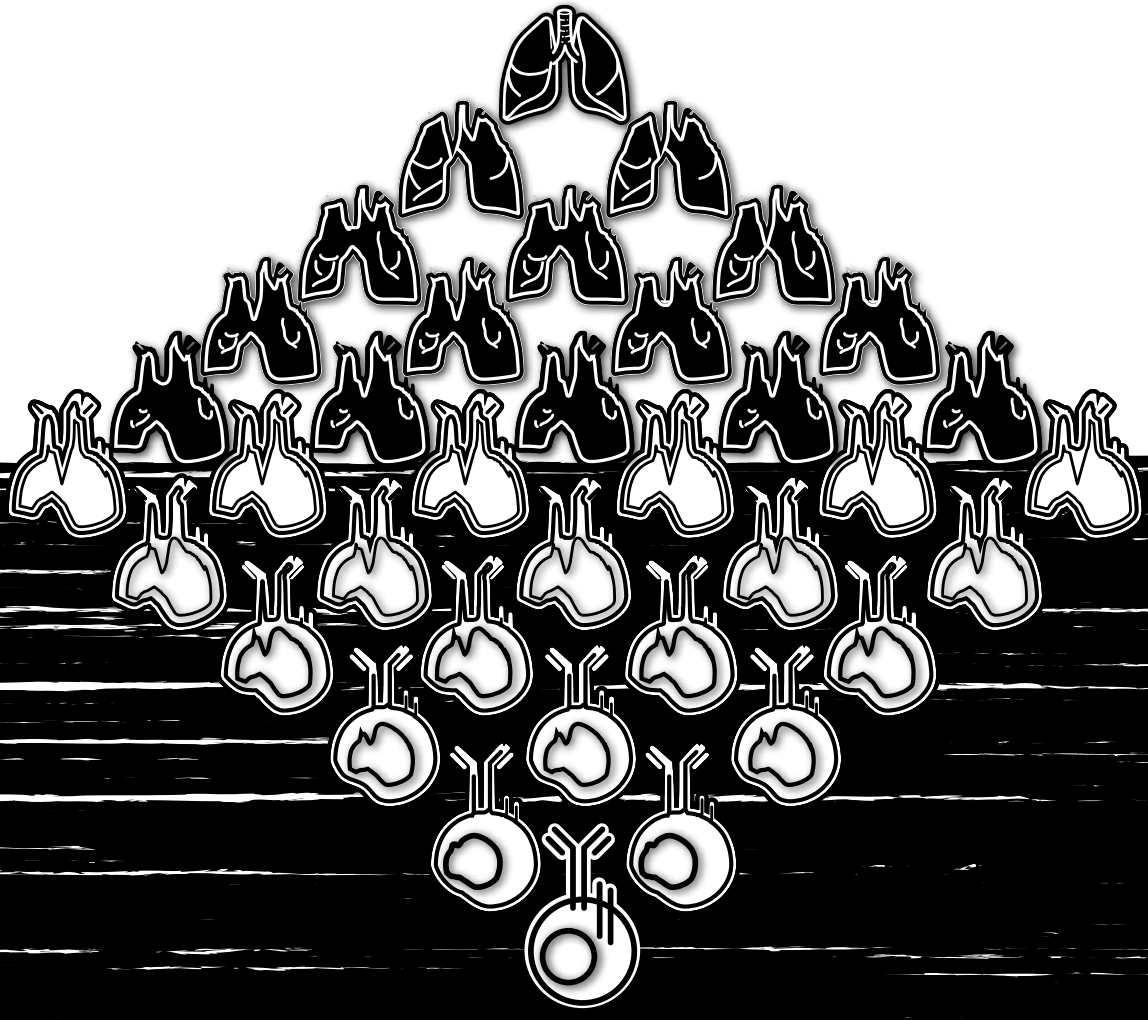
De bevindingen in **Hoofstuk 5 en 6** tonen aan dat B cellen en BCR signalering bij een deel van de patiënten betrokken zijn bij de ontstaanswijze of het beloop van IPF. Toekomstig onderzoek moet uitwijzen of het beïnvloeden van B cellen of BCR signalering met medicatie een onderdeel zou kunnen vormen van de behandeling van IPF patiënten.

In **Hoofdstuk 7** onderzoeken wij of veranderingen in B cel activatie ook betrokken zijn in verschillende vormen van PH. In IPAH zijn er aanwijzingen dat er bij een deel van patiënten autoantistoffen aanwezig zijn die gericht zijn tegen de vaatwand en dat veranderingen in het verworven immuunsysteem een rol spelen bij het ontstaan van IPAH. In dit hoofdstuk gebruiken wij een experimenteel muismodel waarbij een deel van de muizen versterkte BCR signalering heeft door (transgene) overproductie van het BTK eiwit in B cellen. Wij laten zien dat de combinatie van overproductie van BTK in B cellen en het induceren van schade aan longen door bleomycine leidt tot de ontwikkeling van PH. Dit is niet het geval bij het induceren van longschade alleen of bij muizen zonder verhoogd BTK in B cellen. In patiënten met IPAH en PH als gevolg van een auto-immuunziekte vinden we ook verhoogde BTK eiwit levels en BTK eiwit activatie in B cellen. Ook hier was de stijging het meest uitgesproken in naïeve B cellen en bij patiënten waarbij er autoantistoffen in het bloed detecteerbaar zijn. Tot slot onderzoeken wij T cellen die specifiek betrokken zijn bij de activatie van (auto reactieve) B-cellen in lymfeklieren. Deze T cellen worden folliculaire T (Tfh) cellen genoemd. Hierbij vinden wij dat de bepaalde subtypes van Tfh cellen, namelijk Tfh-17 en Tfh-17.1, correleert met B cel activatie. Dit hoofdstuk toont aan dat veranderingen in verworven immuunsysteem die normaal zorgen voor de (juiste) balans en het voorkomen van autoreactiviteit, een rol kunnen spelen bij het ontwikkelen van PH.

In **Hoofdstuk 8** onderzoek wij een andere vorm van PH, namelijk een vorm van PH die kan ontstaan bij patiënten met sarcoïdose. Sarcoïdose is een ziekte die wordt gekarakteriseerd door granulomen die ontstaan in verschillende organen en weefsels in het lichaam. Granulomen zijn ophopingen van ontstekingscellen, waaronder T cellen en cellen van de aangeboren afweer. Bij een groot deel van de patiënten zitten deze granulomen in

de longen. Bij patenten bij wie de ziekte chronisch is, kan er PH ontstaan. Dit wordt ook wel sarcoïdose geassocieerde PH (SAPH) genoemd. In een cohort van SAPH patiënten bij wie PH net is vastgesteld, hebben we in het bloed gekeken naar de concentraties van verschillende cytokines. Cytokines zijn ontstekingsmediatoren die verschillende cellen van de aangeboren en verworven afweer aantrekken en/of stimuleren, maar soms ook kunnen afremmen. Door een beeld te krijgen welke cytokines betrokken zijn bij SAPH, krijgen we meer inzicht in de etiologie van SAPH. Hierbij hebben we ons op cytokines gericht, waarbij al vaststaat dat ze een rol spelen bij sarcoïdose of PH alleen. Met behulp van een multivariate analyse, namelijk principale component analyse, hebben we tevens onderzocht welke cytokine het best in staat is om patiënten met SAPH te onderscheiden van sarcoïdose patiënten zonder PH, patiënten met IPAH en gezonde proefpersonen. Uit deze analyse komt naar voren dat met name chemokines CXCL13 en CCL9 verhoogd zijn in patiënten met SAPH. Deze chemokines zijn stoffen die een gericht migratie kunnen induceren van immuuncellen naar plaatsen van ontsteking en de gemeten waarden in het bloed blijken goed SAPH te kunnen onderscheiden van sarcoïdose patiënten zonder PH en IPAH patiënten. Tevens blijken de concentraties van vasculaire endotheliale groeifactor (VEGF) en het CCL4 chemokine te correleren met respectievelijk de hoogte van de druk in de bloedvaten bij diagnose en de pompkracht van het hart. In een vervolgstudie wordt bekeken of cytokines voorspellend kunnen zijn voor de overleving en de mate van klinische achteruitgang van patiënten met SAPH.

In **Hoofdstuk 9** plaatsen wij de resultaten van dit proefschrift in de context van reeds gepubliceerd onderzoek en beschrijven wij hoe onze nieuwe bevindingen kunnen bijdragen aan de klinische praktijk en richting kunnen geven aan vervolgonderzoek. Dit proefschrift toont aan dat de verworven afweer een rol speelt bij het ontstaan en onderhouden van interstitiële longziekten en pulmonale hypertensie. Een juiste balans tussen, enerzijds het activeren van ons immuunsysteem bij schade en anderzijds het voldoende en op tijd afremmen, om auto- reactiviteit te voorkomen, is cruciaal. Wanneer deze balans verstoord raakt kan dit bijdragen aan blijvende schade aan longen en bloedvaten met weefselverlittekening van longen en bloedvaten als gevolg. Dit proefschrift leert ons ook dat de immunologische heterogeniteit van interstitiële longziekten en PH erg hoog is. Bij een deel van de patiënten zullen stoornissen in de verworven afweer primair verantwoordelijk zijn voor het ontstaan en/of onderhouden van de ziekte. Het is dan ook cruciaal zijn om juist deze patiënten te selecteren bij wie het moduleren van de verworven afweer, met bijvoorbeeld medicatie die B cel activatie afremt, zinnig kan zijn. Bij een patiënt met een interstitiële longziekte of vorm van PH zal het steeds belangrijker worden om te onderzoeken welk onderliggend mechanismen (genetisch, omgevingsfactoren en inflammatoir) de dominante spelers zijn in het ziekteproces. Tezamen met het beloop van de ziekte (wel of niet progressief) en de wensen en verwachtingen van de patiënt, kan er dan een op maat gemaakte behandeling volgen. Alleen met deze gepersonaliseerde aanpak en begeleiding kunnen we betere en voor de patiënt waardevollere zorg blijven leveren. Hierbij geldt dus "*one size doesn't fit all*"!



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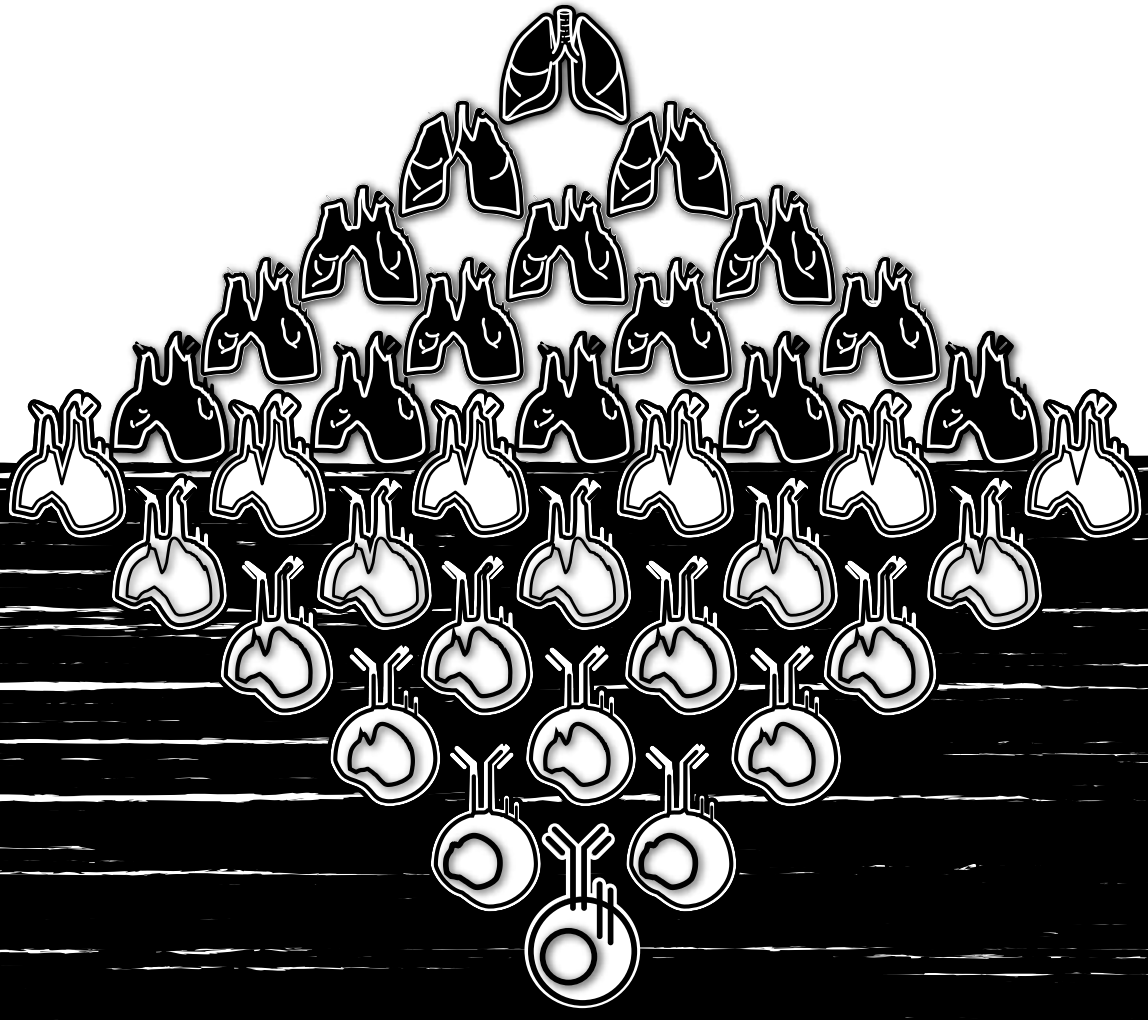

PHD PORTFILIO

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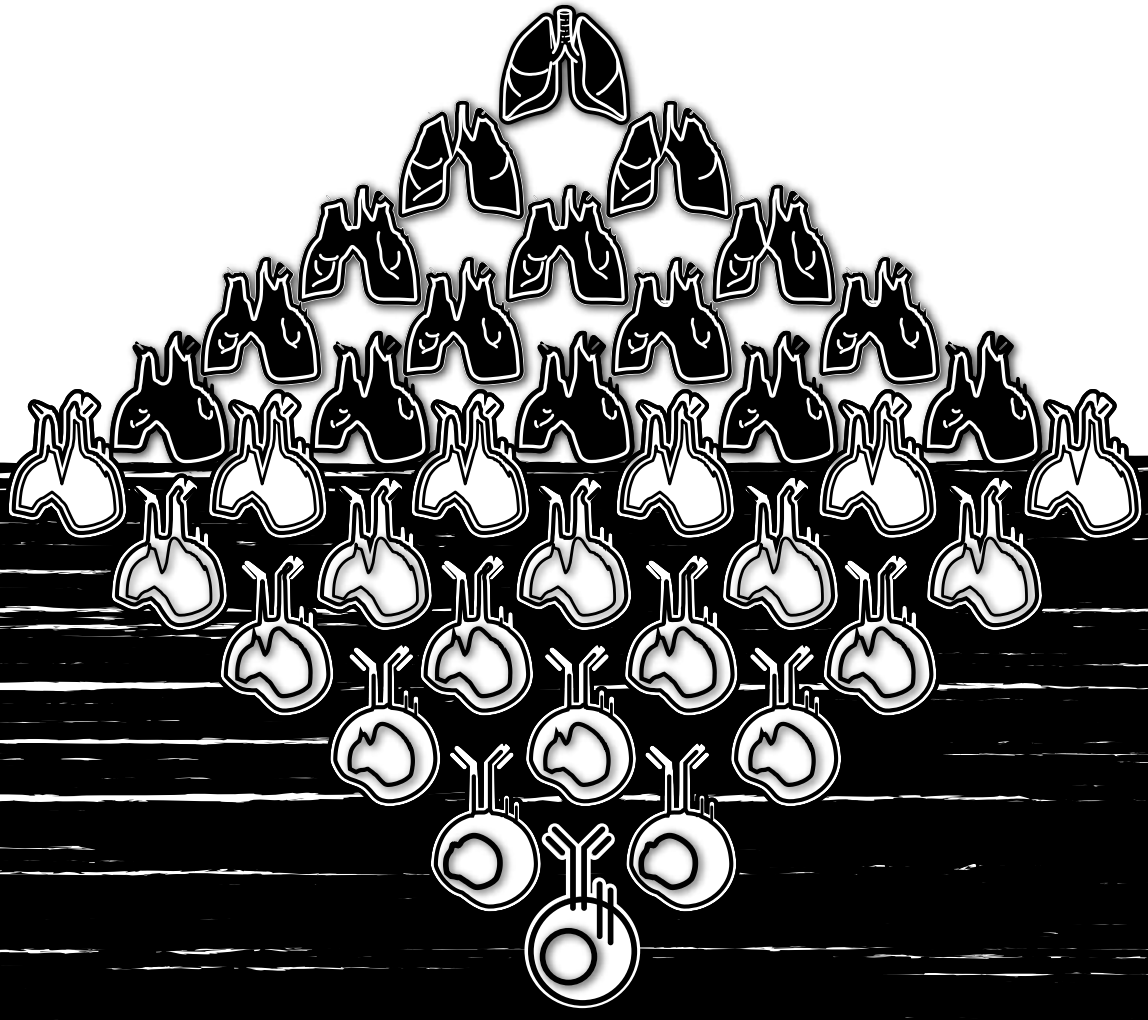
2016 Erasmus MC - Good Clinical Practice
2015 Erasmus MC - Biomedical English Writing
2014 Erasmus MC - Open Clinica: management and building a clinical database
2013 Erasmus MC - Follow-up Photoshop and Illustrator CC
2012 Erasmus MC - Basic Introduction Course on SPSS

(International) scientific presentation

2019 American Thoracic Society, Dallas, USA (poster presentation)
2017 Winter ILD school, Davos, Switserland (oral presentation)
2016 American Thoracic Society, San Francisco, USA (poster)
2015 3rd Scientific seminar on PAH, Lund, Sweden (oral presentation)
2015 European Respiratory Society, Amsterdam (poster)
2015 American Thoracic Society, Denver, USA (poster presentation)
2014 NVALT ledenvergadering, Utrecht (oral presentation)
2014 Fibrosis from bench to bedside, Keystone symposium, Keystone, USA (poster presentation)
2013 6th World Association of Sarcoidosis and Other Granulomatous disorders (WASOG) meeting, Paris (poster presentation)
2013 ILD course, Groote Schuur Hospital, Kaapstad, Zuid-Afrika (oral presentation)

Teaching

2014 Erasmus MC - Teaching and Supervising of a medical research student
2012 Erasmus MC - Teaching medical doctors/residents and nurses



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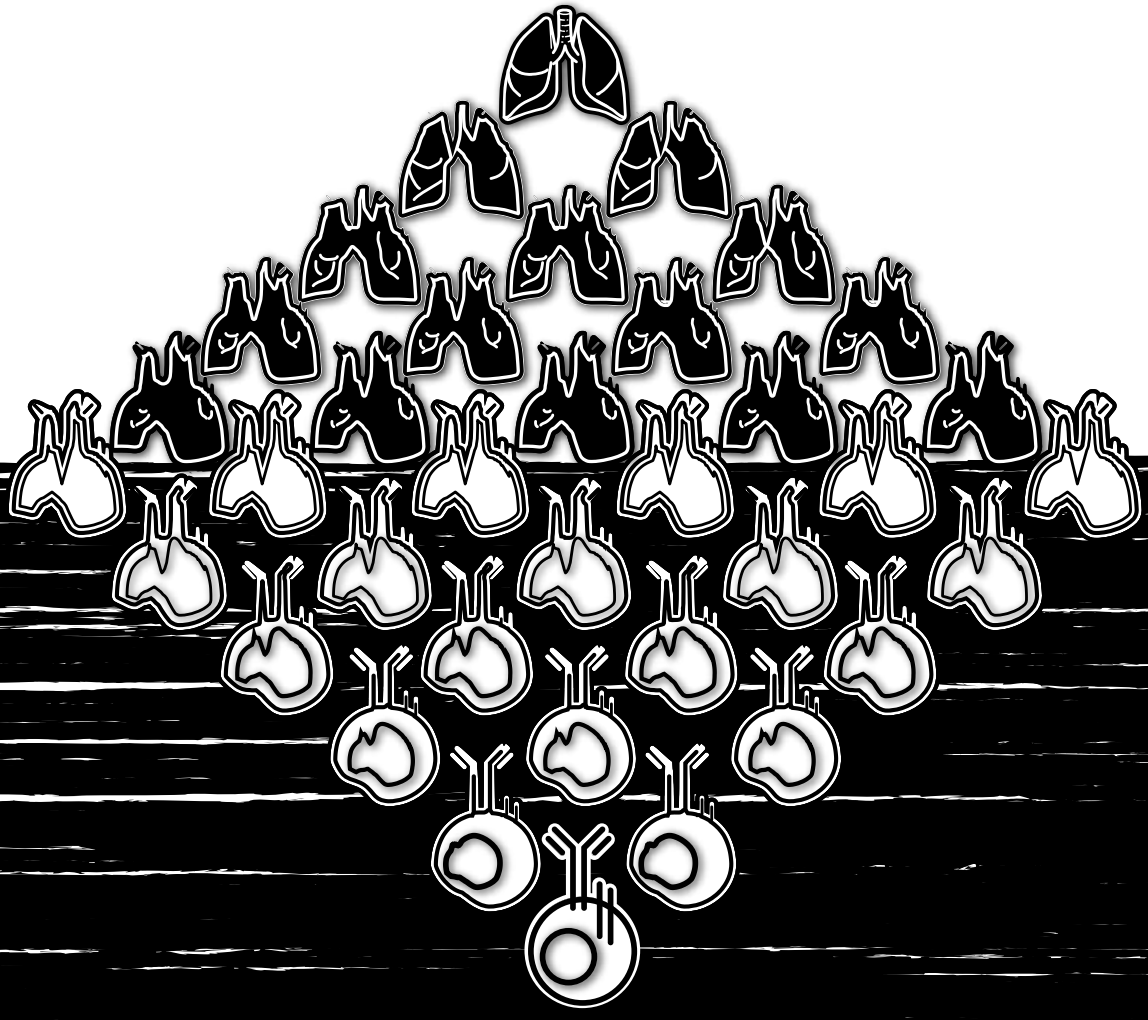


OVER DE AUTEUR

Dat onze zoon Peter een nieuwgierig is bewees hij al door op 12 december 1980, in Dordrecht, twee maanden te vroeg uit de buik van zijn moeder te komen om de wereld te aanschouwen. Nieuwsgierig is hij altijd gebleven. Al tijdens zijn basisschool tijd wist hij door geknutsel met zijn 1000 chemische proefjesdoos de meest fantastische knallen en vuurwerkachtig spektakel te fabriceren. En dat allemaal zonder het bijbehorende boek met de uitleg over op een veilige en verantwoorde manier de proefjes te doen een blik waardig te gunnen, of zijn ouders daarover te informeren.

Op de middelbare school werd het allemaal wat serieuzer en tegen het eind van deze schooltijd wist hij voor zichzelf wat hij wilde worden als hij groot was “dokter”.

Niet alleen werd de school serieus aangepakt ook op sportief gebied bleek hij niet zonder talent. Peter liep bijvoorbeeld regelmatig als junior mee in senioren politie hardloopwedstrijden en wist met grote regelmaat prijzen voor de neus van volwassen politieagenten weg te slepen. Kortom een veelzijdige en snelle puber.

Na de middelbare school werd de studie geneeskunde aan de Erasmus Universiteit te Rotterdam zijn nieuwe uitdaging. Lekker dichtbij huis zagen wij Peter veranderen van puber naar jongvolwassene die na zijn start op de universiteit vanuit de thuissituatie op zoek ging in de studentenwereld. Hij verruilde de thuissituatie voor een studentenkamer in Rotterdam en vond na enige amoureuze omzwervingen zijn huidige vriendin Lillian.

Gedurende de studie die Peter voor een deel met zijn boezemvriend Shandrich volbracht bleef hij nieuwsgierig en zo belande zij voor hun afstudeerstage een half jaar in Suriname om onderzoek te doen naar malariaresistentie in het diepe donkere oerwoud van Suriname.

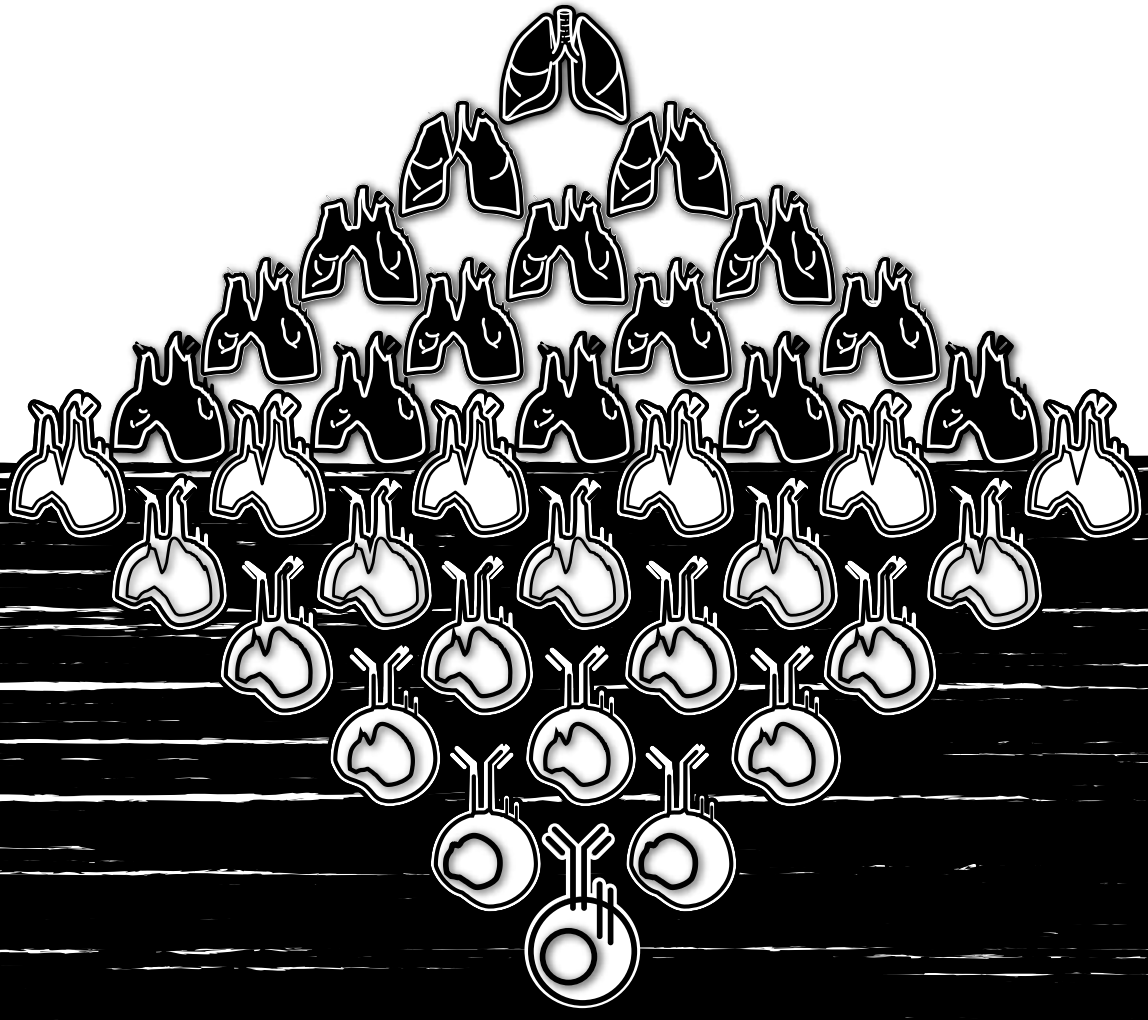
Later is Peter tijdens zijn coschap nog 3 maanden in Tanzania geweest en heeft daar de geneeskunde in zijn volle breedte mogen ervaren. (Zie foto) Later tijdens zijn specialisatie tot Longarts heeft hij een half jaar gewerkt in het Grote Schuurziekenhuis in Kaapstad Zuid Afrika.

Al tijdens zijn specialisatie tot Longarts startte Peter, na daarvoor gevraagd te zijn, met het promotieonderzoek dat nu voor u ligt. Samen met een team van laboratorium-medewerkers, promotors en tal van andere vormen van ondersteuning, niet in de laatste plaats met de steun van Lillian is dit proefschrift tot stand gekomen. Dit onderzoeksverslag

dat aanvankelijk startte met een andere onderzoeksopdracht maar uiteindelijk uitmondde in deze studie naar heel veel over iets ontzettend kleins.

Tegelijkertijd bouwde Peter met zijn vriendin Lillian aan een gezin en settelde zich uiteindelijk weer in Schiedam. Nu met Lillian, drie kinderen, drukke banen en uitermate trotse ouders gaan wij ervan uit dat nu de periode van "relatieve" rust aanbreekt met meer tijd zijn voor gezin.

Paula Heukels Persijn & Cor Heukels
Ouders van Peter.



DANKWOORD

In 2012 startte mijn avontuur met dit promotietraject. Ik zei in het verleden wel eens gekscherend; "een promotietraject is een ontgroening van vier jaar". Nu achteraf bezien is het geen ontgroening geweest maar een zegen. Het was een bijzonder interessante periode, waarin ik veel van mijzelf en anderen geleerd heb. Dit varieerde van omgaan met tegenslag, projecten opzetten, omdenken en uiteraard kritisch onderzoek doen. Daarnaast is mij duidelijk geworden dat onderzoek doen echt een teamsport is. De hulp en ondersteuning van heel veel mensen is cruciaal gebleken voor het tot stand komen van dit proefschrift. Daarom zeer veel dank aan:

Het begeleidingsteam & paranimfen.

Beste **Rudi**, als promotor heb jij een enorm belangrijke rol gespeeld. Jouw vermogen om uit de aanwezige - en vaak niet altijd eenduidige - onderzoeksresultaten een duidelijk onderzoekslijn en passende vervolgstappen te bedenken, deed mij verstelt staan. Het enthousiasme en de kennis van de basale immunologie werkt enorm aanstekelijk en heeft mij, ook als arts, geholpen mij verder te ontwikkelen. Daarnaast vind ik het bewonderenswaardig hoe jij je de afgelopen jaren ontfermd hebt over de PhD kandidaten in de *inflammation and remodeling (I&R)* groep. En niet zonder succes, getuige de recente en aankomende promoties binnen deze groep.

Beste **Marlies**, de laatste jaren ben jij mijn copromotor geweest. De samenwerking gaat natuurlijk veel verder terug en jij bent er medeverantwoordelijk voor dat ik überhaupt longarts ben, vanwege jouw zitting in de sollicitatiecommissie van 2007. Naast het opleiden tot longarts heb jij mij geholpen door altijd oog te houden voor de juiste vertaling van (basale) wetenschap naar de klinische praktijk. Deze "bench to bedsite" visie kan jij als geen ander overbrengen. Dat dit ook de rest van de wereld niet is ontgaan, blijkt uit het rijtje mastodonten waar je inmiddels toe behoort (en dan bedoel ik mondiale experts) op het gebied van ILD en idiopathische pulmonale fibrose.

Beste **Mirjam** en **Bernt**, jullie waren mijn copromotoren van het eerste uur. Inmiddels hebben jullie beiden een andere weg ingeslagen. Of het niveau van mijn eerste manuscripten hier (in)direct voor verantwoordelijk is geweest, weet ik niet, maar aan jullie input heeft het niet gelegen. In deze periode hebben de adviezen die jullie mij gegeven hebben, geholpen mij verder te ontwikkelen als onderzoeker. Ik wens jullie veel succes met jullie huidige werkzaamheden.

Beste **Jennifer**, Ik - arts niet gehinderd door enige laboratoriumkennis - en jij - net klaar met de hoge laboratorium opleiding - werden in 2012 bij de start van dit traject gekoppeld. Jij hebt mij wegwijsgemaakt in het bijhouden van labjournaals, pipeteren en de wereld van flowcytometrie. Onze samenwerking en bijbehorende experimenten hebben door de tijd heen een enorme vlucht genomen, van het zoveelste (mislukte) collageen expiriment tot belangrijke sleutel-experimenten voor een figuur. Ik ben je hier erg dankbaar voor, niet in het minst om je geduld en ik ben dan ook erg blij dat je mijn paranimf bent.

Mijn andere paranimf, **Thomas**. Dank dat je begon als student binnen mijn project. Het figuur waaraan jij destijds 2 maanden hebt gewerkt, is nooit gepubliceerd, maar dat heeft ons niet van een mooie samenwerking afgehouden. Daarnaast heb jij je inmiddels tot een volleerd pulmonale hypertensie onderzoeker weten te ontwikkelen. Het is dan ook geweldig dat jij binnenkort ook jouw proefschrift zal verdedigen en je opleiding tot longarts zal afronden. De gezellige borrels, jouw droge humor en het relativeren van het lab- en artsenleven bleken perfecte ingrediënten om een lab-dag door te komen.

Tot slot, wil ik graag de leescommissie, te weten Harm-Jan Bogaard, Antje Prasse en Joachim Aerts bedanken voor de beoordeling van dit proefschrift.

Het lab longziekten

Het afgelopen decennium heb ik veel mede (kandidaat) PhD, laboranten en Postdocs ontmoet binnen het lab longziekten. Wij (**Caroline, Heleen, Tridib en ik**) begonnen als PhD kandidaten tussen 2012 en 2013 binnen de I&R-groep aan onze researchlijnen en dit heeft tot evenzoveel mooie promoties geleid. Het samen ontmaskeren en begrijpen van het PhD-leven was bijzonder prettig. Zonder jullie hulp had ik waarschijnlijk nog steeds niet begrepen hoe SPSS, Graphad (of überhaupt mijn computer, als die weer eens niet deed wat ik wilde) werkte.

Nadien parttime een kamer gedeeld met **Denise, Stefan** en **Jasper**. Ik wil jullie bedanken voor de samenwerking en de fraaie gezamenlijke projecten.

Projecten en experimenten bedenken is een ding, het (goed) uitvoeren is toch echt iets anders. **Jennifer, Menno, Odilia, Koen, Ingrid en Marjolein**, ik wil jullie graag bedanken voor de ondersteuning en het veelal uitvoeren van deze experimenten. Dankzij jullie zijn termen als "Sorten, MilliQ, BD en "kijk uit, je snijdt je vinger eraf" voor mij gemeengoed geworden. Odilia, jouw revisies op de B cel georiënteerde stukken en het 'oppimpen' van enkele figuren in dit boekje waren zeer waardevol.

Gedurende mijn onderzoeksperiode zijn er fraaie samenwerkingsverbanden ontstaan waar wij als lab longziekten nog steeds de vruchten van plukken. Het uitwisselen en verzamelen van transplantatie materiaal en delen van data tussen het Erasmus MC Rotterdam en UMC Groningen is een groot succes gebleken en heeft geleid tot enkele fraaie publicaties. Mijn dank voor de fijne samenwerking gaat dan ook uit naar **Barbro Melgert** en **Carian Boorsma**.

Beste **Jan von der Thüsen**, ik wil jou en de afdeling pathologie van het Erasmus MC bedanken. Niet alleen heeft de samenwerking geleid tot mooie immunohistochemische figuren in mijn proefschrift, ook het meedenken op cellulair niveau en het kwantificeren van fibrose bij muis en mens was erg waardevol.

Dat landgrenzen geen beperking hoeven te zijn voor goed onderzoek blijkt uit de samenwerkingen met **Robert Baughman** (Cincinnati Medical Center, USA) en **Daniel Culver** (Cleveland Clinic, USA). *Dear **Bob** and **Daniel**, I would like to thank you for the opportunity to collaborate and your contribution to our work on sarcoidosis associated pulmonary hypertension.*

Beste **Harm-Jan**, hartelijk dank voor de samenwerking op het gebied van pulmonale arteriële hypertensie. Dankzij jouw passie voor onderzoek naar dit zeldzame ziektebeeld en de samenwerking tussen het Erasmus MC Rotterdam en Amsterdam UMC worden er goede stappen gezet om pulmonale hypertensie beter te begrijpen en te behandelen.

Zonder plezier, geen focus. Naast werken was er gelukkig ook ruimte voor het nodige vertier. **Menno, Thomas** en **Floris** (a.k.a. *De Puristen*), dank voor alle goede en inhoudelijke gesprekken waarin we vaak met een (niet altijd) nuchtere blik ons leven in en rond het lab analyseerde en nogal eens bekritiseerde met de nodige dosis humor. Daarnaast ben ik jullie erg dankbaar voor alle vrolijke belevenissen de we samen meegemaakt hebben. **Menno**, ik hoop de termen *pot-odds*, *quads*, en "waarom fold je in godsnaam met aas-koning" nog vaak uit jouw mond te horen.

Daarnaast wil ik **Koen en Tridib** bedanken. Jullie creatieve geest heeft geleid tot enkele memorabele uitstapjes. Onze "surprise-trip" naar Wroclaw (Polen) zal ik niet snel vergeten.

Opleiding & Amphia Ziekenhuis

Gedurende de eerste 4 jaar van mijn promotietraject heb ik met veel plezier mijn opleiding tot longarts volbracht in het Erasmus MC met **Henk Hoogsteden** als afdelingshoofd en **Leon van der Toorn** als opleider. **Karin Boomars**, dank voor de ondersteuning (o.a. via *Actelion*) en begeleiding bij het opzetten van de *BioPulse* studie en het hierbij behorende "biobanken" van patiëntmateriaal. De informatie verkregen uit deze databases, zowel klinisch als immunologisch, is cruciaal voor een aanzienlijk deel van de hoofdstukken in dit boek. Daarnaast ben ik alle longartsen in het EMC en oud-opleidingsassistenten erg dankbaar voor de leuke en leerzame (opleidings)tijd, waarin mij de tijd en ruimte is gegund mijn research kwaliteiten te ontwikkelen. Het is mooi om te zien dat alle opleidingsassistenten goed terecht zijn gekomen en dat er onderling nog steeds contact is met elkaar.

In 2017 bewandelde het huidig hoofd van de afdeling longziekten van het ErasmusMC, **Joachim Aerts**, de weg van het Amphia ziekenhuis in Breda naar Rotterdam. Dit bood mij vervolgens weer de kans om te solliciteren in Noord-Brabant. Deze en andere kruisbestuivingen tussen beide ziekenhuizen heeft er mede toe geleid dat er op verschillende aandachtgebieden nu een brede samenwerking bestaat waar we trots op mogen zijn. Beste **Joachim**, ik vind het dan ook een eer dat je deel uitmaakt van de leescommissie.

In 2017 ben ik gestart als longarts in het Amphia Ziekenhuis in Breda en tot op de dag van vandaag ben ik enorm trots dat ik deel uitmaak van deze maatschap. **Maten**, wij vinden onszelf de beste vakgroep van het ziekenhuis. Ondanks dat er in deze uitspraak mogelijk een hoog "*wij van wc-eend vinden*"-gehalte heeft, zit hier een grote kern van waarheid in

(al zeg ik het zelf). Naast de zeer goede onderlinge sfeer en flexibiliteit, mogen we trots zijn op de opleiding tot arts voor longziekten en tuberculose en de bruisende groep arts-assistenten die we begeleiden. Daarnaast is er altijd de ruimte om onszelf op andere (aandachts)gebieden te ontplooien en de tijd die ik heb gekregen voor het voltooien van dit proefschrift is daar een treffend voorbeeld van.

Sport & Ontspanning

Iedereen die mij een beetje kent, weet dat ik lastig stil kan zitten. Lichamelijke beweging is dan ook een belangrijke tool voor mij om mijn hoofd leeg te maken en mijzelf weer op te laden. Na gevoetbald te hebben bij SDV, waarbij wij voornamelijk verdienstelijk waren in de *derde* helft, heb ik mij weer op een oude liefde, het hardlopen gestort. Sinds 2014 ben ik lid van de B-groep van Rotterdam Atletiek, ook wel bekend onder de naam **Geen Getrut**. Dank loopmaatjes voor de leuke loopjes, vermakelijk gesprekken en het altijd *extensief* trainen.

"Een goede buur is beter dan een verre vriend". Deze uitspraak is zeker op ons blokje buren van toepassing. Dank lieve **buren** voor de behulpzaamheid, gezelligheid en (voortuin)borrels.

Daarnaast kan ik op een groep vrienden rekenen die mij al heel lang kennen. Lieve **Tim, Inge, Shandrich** en **Remko**, ook al zijn jullie uitgewaaid over het land en zien wel elkaar niet zo vaak meer als vroeger, als ik jullie spreek, voelt het nog wel altijd als vroeger. Het maakt niet uit of het nu gaat over wie de beste ploeg is van de eredivisie (Ajax natuurlijk), mijmeren over onze tijd in Suriname, of domweg slap ouwehoeren, het voelt altijd vertrouwd.

Het allerbelangrijkste…

Ik prijs mij enorm gelukkig met mijn (schoon)familie. Zonder de vele uurtjes oppas waardoor ik in mijn vrije tijd aan dit proefschrift kon werken of mij toch even kon uitleven op de atletiekbaan was dit promotietraject niet mogelijk geweest. Jullie vormen een rotsvaste basis waar ik en mijn gezin altijd op kunnen terugvallen.

Schoonfamilie kun je niet uitkiezen, maar mocht ik dit wel kunnen, dan zou ik nog steeds uitkomen bij **Jeannette, Bart en Babette**. Het is fijn dat wij altijd op jullie kunnen rekenen en geniet met volle tuigen van de gezellige dynamiek tussen ons. Bart en Babette, ik kijk enorm uit naar jullie aankomende gezinsuitbreiding.

Lieve **pa & ma**, dank voor jullie onvoorwaardelijke steun, liefde en tijd die jullie voor ons vrijmaken. Het is daarnaast onvoorstelbaar om te zien hoe jullie, ook na het pensioen, je blijven inzetten voor de maatschappij. Het belangeloos inzetten voor Slachtofferhulp, SooS Blauwhuis, Fietsmaatje, of vrijwilligerswerk in een hospice of Vlietland ziekenhuis is hier een kleine greep uit. Deze eigenschap is iets wat ik mijn kinderen (en eigenlijk ook mijzelf) wil meegeven. Daarnaast zijn jullie een geweldige opa en oma voor onze kinderen. Lieve zus, **Suzanne**, qua karakter zijn we best verschillend, maar wat wij gemeen hebben is een groot portie doorzettingsvermogen. De laatste periode was soms best lastig voor

je, maar hoe jij je hier doorheen hebt geslagen is bewonderenswaardig. Het is mooi om te zien hoe jij, samen met 2 mooie kids, je staande hebt weten te houden en je eigen pad hebt gekozen. Ben trots op je!

Dan de allerbelangrijkste persoon. Lieve **Lillian**, ik ben zo blij met jou in mijn leven en dat je dit gehele traject, misschien wel meer dan je lief is, hebt meegemaakt. We zijn samen een mooi team en zonder jou was dit niet gelukt. Ik bewonder je zorgzaamheid, inlevingsvermogen en doorzettingskracht. We vullen elkaar aan waar het moet en geven elkaar de ruimte waar het kan. Onze symbiose is recent bekroond met de geboorte van onze derde spruit **Isa** en samen met **Siem** en **Lauren**, met nu wat meer vrije tijd, gaan we nog heel veel mooie herinneringen maken!

The lungs are constantly exposed to inhaled agents that may induce an inflammatory response. The inflammatory response encompasses several stages, starting with the influx of activated inflammatory cells, and ending with tissue repair and restoration of tissue function. When the delicate balance between inflammation and resolution becomes dysregulated, ongoing inflammation can lead to tissue remodeling or fibrosis.

In interstitial lung diseases and pulmonary hypertension, the big question is whether the observed changes in adaptive immunity are a critical trigger for disease onset and progression in susceptible patients or a consequence of established disease. The findings described in this thesis contribute to our knowledge on the immune-related pathogenesis of interstitial lung diseases and pulmonary hypertension. Combining the immunological mechanisms with disease behavior, response to therapy and patients' perspectives will help to advance patient-tailored care.