

An investigation into the contribution of airway cells to the pathogenesis of idiopathic pulmonary fibrosis

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Declaration

The work presented in this thesis was conducted by me with the following exceptions:

Mass spectrometry and database searching to analyse the results of this work was carried out by Steven Lynham, Proteomics and Mass Spectrometry Department, King's College London.

Immunohistochemical staining of lung tissue sections for the bronchial epithelial cell markers KRT5, MUC5AC, SCGB1a1 and ACT was carried out by Alex Bowman, Histopathology Department, Respiratory Biomedical Research Unit, Royal Brompton Hospital.

Lung function testing was performed at the lung function laboratory, Royal Brompton Hospital.

Abstract

Idiopathic pulmonary fibrosis(IPF) is a debilitating lung disease leading to progressive destruction of the alveolar parenchyma. It is believed to be the result of an uncontrolled wound healing response triggered by repetitive injury to the alveolar epithelium and a self-propagating cycle of epithelial cell injury, fibroblast activation and collagen deposition. The hypothesis explored here is that airway macrophage and bronchial epithelial cell dysfunction contributes to the fibrotic process in the IPF lung, in part through the regulation of the IL-33/ST2 signalling axis. This hypothesis was investigated by examination of airway macrophage phenotype by flow cytometry, and characterisation of gene expression and *in vitro* function at baseline and in response to stimulation of cultured human bronchial epithelial cells (HBECs) and lung fibroblasts.

These data demonstrated that airway macrophages(AMs) in IPF and healthy control volunteers exhibit a complex spectrum of activation and do not conform to the bipolar M1/M2 paradigm of macrophage polarisation. AMs exhibit high levels of expression of both M1 and M2 markers and no difference was found when comparing marker expression between IPF and healthy control. Results of bronchial epithelial phenotyping were in accordance with a body of work suggesting the IPF lung to be a mucin rich environment, with an increase in the expression of MUC5AC expressing airway cells. This work also found an increase in SCGB1A1 expressing club cells in bronchioles in IPF when compared to healthy control lung tissue. This study did not find *in vitro* functional differences of IPF HBECs when examining proliferation, tight junction formation, barrier function, or direct effect on fibroblast extracellular matrix(ECM) regulation. Aside from an increase, in gene expression of a negative regulator of the IL-33 signalling axis (SIGIRR)in IPF HBECs compared to healthy control - this study not demonstrate any differences in IL-33/ST2 axis expression in airway, tissue or in the alveolar space in IPF. This work did not demonstrate evidence to support the hypothesis that AMs and HBECs contribute to the development of IPF and did not show any direct pro-fibrotic effects of the IL-33/ST2 signalling axis in IPF.

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Chapter 1.

Introduction

1.1 Interstitial Lung Disease

1.1.2 Background and Classifications

The interstitial lung diseases (ILDs), also known as diffuse parenchymal lung diseases (DPLDs), are a diverse and heterogeneous group of disorders which are characterised by thickening of the alveolar interstitium or alveolar filling. There are over 300 different types of ILD which can vary greatly in aetiology and prognosis but most share fibrosis as a common endpoint (ERS White Book, 2013).

These numerous diseases can be broadly categorised (figure 1.1) as;

- The idiopathic interstitial pneumonias
- ILDs of known aetiology - which includes commonly collagen vascular diseases and hypersensitivity pneumonitis
- Granulomatous ILD
- Other forms of ILD

Figure 1.1 Diffuse parenchymal lung disease

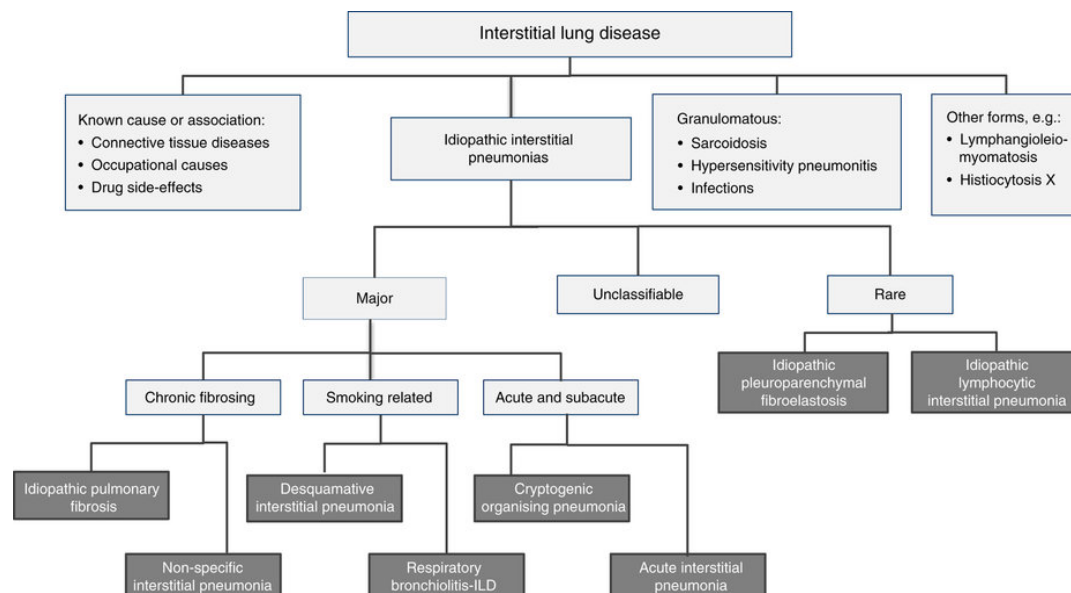


Figure 1.1 categorisation algorithm of the numerous different diseases which can affect the lung interstitium

Image courtesy of NPJ Primary Care Respiratory Medicine

The different ILDs exhibit varying responsiveness to anti-inflammatory and immunosuppressant treatment with some such as sarcoid and acute hypersensitivity pneumonitis responding well to corticosteroids, and at the other end of the spectrum, IPF, the archetypal and most common fibrotic lung disease which taking into account regional variations, accounts for 20-45% of all cases of ILD (ERS White Book 2013).

1.1.3 Idiopathic Pulmonary Fibrosis

1.1.3.1 Clinical Perspective

Idiopathic pulmonary fibrosis is a chronic fibrosing interstitial pneumonia of unknown cause, which is characterised by progressive fibrotic destruction of the lung parenchyma. This results in parenchymal architectural distortion and loss of elasticity of the lung tissue, leading to a restrictive pattern of lung function impairment, consequent deterioration in gas transfer and, inexorably, respiratory failure and death. It usually occurs in older adults (with a median age of diagnosis of 66) and shows a predilection towards those who have previously smoked (Plantier et al. 2018). The diagnosis is made based upon identification of a usual interstitial pneumonia pattern of radiological and/or histopathological findings (where pathology is available) in combination with the exclusion of other potential causes of fibrotic lung disease (Meyer et al. 2014). In clinical practice, when medical history and radiology alone are not sufficient to establish, with certainty, a diagnosis of IPF a bronchoalveolar lavage (BAL) is often performed prior to consideration of a lung biopsy - due to the high morbidity and mortality attached to lung biopsy in older individuals. BAL is a safe and well-tolerated procedure which has been in use for the study of human lung environment since the late 1960s/1970s (Reynolds et al, 1973). The technique allows sampling of secretions and cells coating the apical surfaces of alveolar and bronchial epithelium with a greater than 5% yield of lavage fluid being shown in one study to increase diagnostic yield (Baughman, Spencer et al. 2000) with the American Thoracic Society recommending a yield of >30% to be considered an optimal sample of the

alveolar space (Meyer et al, 2012). This technique is used to obtain an accurate sampling of the alveolar space and allow quantification of a representative cell differential count as well as to routine screen for concurrent infection. This is used primarily as a diagnostic tool to allow a more confident diagnosis in cases where the radiology and clinical picture are not completely typical - no BAL parameters have been validated for use as monitoring or prognostication.

The incidence of IPF has been reported as between 6.8 to 16.3 per 100,000 and this has been steadily rising with an estimated >5000 new cases diagnosed in the UK each year. It exhibits a male preponderance and is predominantly a disease of ageing, with the majority of those affected in their 6th or 7th decades of life at diagnosis and healthy control studies having shown the prevalence of areas of fibrosis in human lung in the absence of an underlying fibrosing disease (ERS White Book, 2013). It has a median survival time of 3.5 (Thickett et al. 2014) years from diagnosis and 5 year survival at approximately 20%.

IPF was previously referred to as cryptogenic fibrosing alveolitis. However in 2001 the American Thoracic Society/European Respiratory Society consensus classification of idiopathic interstitial pneumonias changed the nomenclature to remove the term 'alveolitis' reflecting a change in the prevailing hypothesis of pathogenesis away from a model of chronic inflammation (ATS/ERS Consensus Classification statement. 2001).

This re-shaping of our diagnostic categories has allowed the idiopathic interstitial pneumonias to be further stratified from a wide and heterogeneous group encompassing many different diagnoses to one with more clearly defined and delineated subtypes, including usual interstitial pneumonia, cryptogenic organising pneumonia, desquamative interstitial pneumonia, non-specific interstitial pneumonia and acute interstitial pneumonia (Meyer et al. 2014). NSIP is a pathological subset which can be found in the context of non idiopathic ILDs such as CTD-ILD, HP and a variety of exposures. Cases which would now be called NSIP had hitherto thought to be a variant presentation of IPF and had been diagnosed/labelled accordingly. Many of these cases demonstrated prominent

lymphoplasmacytic inflammation on surgical lung biopsy and often exhibited a distinct clinical course, showing a better long term prognosis and often a significant response to treatment with steroids (Kim et al. 2006). The fact that the study populations may, therefore, differ in the published literature pre-dating the classification change and studies published post this date should therefore be borne in mind.

It is now believed that, in affected individuals with a genetic predisposition, repetitive injury to the alveolar epithelium can trigger a cascade of inflammatory, coagulation and wound healing pathways which lead to uncontrolled extracellular matrix deposition and fibrosis. Putative initial triggers include industrial particulates, toxins, viruses, medical co-morbidities (in particular gastro-oesophageal reflux disease) and cigarette smoke (Maher et al, 2012).

1.1.3.2 Management of Idiopathic Pulmonary Fibrosis

The approach to treatment of IPF has undergone a radical change in the recent past. Prior to 2011, standard therapy for IPF consisted of low dose prednisolone, immunosuppression in the form of the purine analogue azathioprine and the anti-oxidant n-acetylcysteine (NAC). This practice was based on the weak evidence of a series of small studies showing a benefit in individuals treated with azathioprine in addition to corticosteroid, compared with corticosteroid alone (Winterbauer et al, 1978) (Raghu et al, 1991) and further work suggesting the addition of NAC to double therapy with NAC and corticosteroid to offer benefit over double therapy alone (Demedts et al, 2005).

However, no definitive large scale trial had yet been designed to assess the effectiveness of prednisolone, azathioprine and NAC therapy in IPF until 2012, which saw the publication of the PANTHER (Raghu et al, 1991) data. The PANTHER study was a randomized double blind placebo controlled trial with IPF individuals split into 3 treatment groups; triple therapy vs NAC alone vs

placebo, with FVC loss as the primary endpoint - a widely used surrogate endpoint in IPF clinical trials due to the impracticality of designing trials to examine mortality in patients with IPF .

An increased risk of death and hospitalisation in patients in the triple therapy treatment arm at interim analysis lead to its early closure with the trial continuing as a 2-drug study. The results of the continuing cohorts were published in 2014 demonstrating no benefit in FVC preservation in the NAC treatment group as compared with placebo (Martinez et al, 2014).

This followed the 2011 publication of the CAPACITY trial, which combined the results of 2 randomised controlled trials of the anti-fibrotic/anti-inflammatory compound Pirfenidone and demonstrated a slowing of deterioration of forced vital capacity (FVC) vs placebo (et al Noble, 2011).

This effect was subsequently confirmed by the ASCEND (King et al, 2014) data, leading to the licensing of pirfenidone in the USA, following on from its approval for use in the UK after the publication of the CAPACITY data.

In quick succession followed the results of the INPULSIS (Richeldi et al, 2014) trial. A randomised double blind placebo controlled trial of the tyrosine kinase inhibitor Nintedanib, this was the second compound to have demonstrated, in a large scale clinical trial, effectiveness in slowing FVC loss compared to placebo thus giving physicians a second and alternative potential treatment option for patients with IPF.

With, as of yet, no directly comparative evidence to suggest superiority for either treatment, choice of drug is currently based on tolerability and side effect profile and at the discretion of physician and patient.

1.2 Current Experimental Models of Fibrosis

Experimental models of fibrosis have provided many of the insights which have led to the recent advancements in the field, with much of the early evidence supporting the hypothesis of a pro-

fibrotic role for alternatively activated macrophages having been derived from mice modelling using bleomycin. However no one convincing model has been developed to mimic IPF and each comes with its own inherent limitations. Modelling systems include;

- intra-tracheal administration of various pro-fibrotic agents (including bleomycin, silica and asbestos),
- cytokine over-expression models (inc TGF- β , IL-13 IL-1 β), usually adenoviral vector delivery, limitation by single dosing and immune response generated by adenovirus
- targeted type II AEC induced fibrosis,
- radiation induced fibrosis, where a radiation injury is induced to the respiratory tract after whole body irradiation. induces inflammatory response, can cause systemic response if not shielded
- schistosoma model, where lung granulomata occur as a result of IV administration of schistosoma eggs and fibrosis subsequently in the liver and less so in the lung.

It is important to note the drawbacks of these models of fibrosis, in particular the bleomycin model as it has emerged as the most commonly used experimental model of fibrosis in rodents. This model, in common with many other above mentioned, replicates an acute lung injury characterised in the early stages exclusively by inflammation with no evident fibrosis, which evolves to a picture of admixed inflammation and fibrosis at 14-21 days post instillation (Moeller et al, 2008). As previously discussed, the development of IPF is believed to result from chronic micro-injury in the absence of significant inflammation. Therefore evidence arising from models which exhibit admixed inflammation and fibrosis inevitably lead to difficulty in discerning whether the pathways under investigation are involved in the fibrotic phase of the process or whether they are related to the inflammatory component. Moeller et al performed an illuminating analysis of the many publications of drug efficacy studies in the bleomycin model (Moeller et al, 2008). They found that 211 of those 221 studies examined used a preventative regime of drug administration less than 7 days after the

final dose of bleomycin. Only 10 Were therapeutic trials with the drug administered greater than 7 days after final bleomycin dose. This is not to say that no utility can be gleaned from data using bleomycin - it is a reliable and reproducible way of effecting lung fibrosis (most non-IPF fibrosing ILDs can develop along similar pathways of longstanding inflammation leading to fibrosis), and can provide insights into the development of fibrosis. The lessons learned from the clinic have shown us that IPF does not respond to broad anti-inflammatory therapy strategies, yet bleomycin studies continue to implicate inflammatory pathways in fibrosis and thus these must be interpreted with some scepticism when applied to IPF. It has more recently been proposed that, given that IPF is a disease of ageing and that many of the hallmarks of ageing - telomere shortening, genomic instability, cellular senescence and epigenetic alterations- are characteristic of the IPF lung, the use of aged mice in experimental models may yield new insights (Tashiro et al. 2017). This is a promising avenue to explore as it may, in combination with other models, take the insights a step beyond our current understanding and shine further light on the role that ageing and senescence play in the pathogenesis of fibrosis. However, the fact remains that judicious caution is required in translating the results wholesale from animal modelling to informing the direction of bench side research in human tissues as well as the future selection of compounds for clinical trials.

1.3 The Wound-healing Hypothesis of IPF

Although the evidence remained inconclusive, IPF had long been thought to be a process of unresolved chronic inflammation with fibrosis as an endpoint. This theory has recently come under scrutiny. Some early studies with small numbers seemed to back up the chronic inflammation hypothesis, with a suggestion of short term benefits with oral corticosteroid treatment, but no sustained long term benefits. However these trials were limited by small numbers and included individuals which, today, may not be classified as IPF, as demonstrated by the higher lymphocyte counts of the steroid responders (Rudd et al, 1981). Insights gleaned from clinical practice

combined with histological analysis of the IPF lung as well as animal models have led to a rethink. Inflammation is not a prominent histopathologic finding in the lungs of IPF individuals and when it is found, it is usually in areas of honeycomb change or collagen deposition (Katzenstein et al, 1998). Importantly, it is rarely seen in otherwise normal alveolar septa suggesting the absence of inflammation in the development of fibrosis. Furthermore In many fibrosing ILDs, the extent of ground glass opacity on HRCT has been shown to correlate with extent of alveolar inflammation, whereas, in IPF, ground glass - although not ruling out a diagnosis of IPF- does suggest an alternative diagnosis (Remy-Jardin et al, 1993) with areas of ground glass in IPF usually related to septal fibrosis and alveolar granulation tissue (Haslam et al, 1980).

The publication of the PANTHER data looking at the effect of combined treatment with steroid and immunosuppression has helped lend clarity to the issue, at least in clinical terms . There does exist some divergence of opinion over how the data should be interpreted, mainly centring around the fact that at the beginning of the trial patients in the treatment arm were given a high dose of corticosteroid than would be common in clinical practice (Wells et al, 2013). However, regardless of the subsequent debate on the true applicability of the mortality data to the clinical population, this landmark study does seem to have concluded the debate on the potential therapeutic role of anti-inflammatory therapy and, it is reasonable to infer, the prominence of inflammation in the fibrotic process in IPF.

This is in line with the recent shift in the consensus view of the prevailing hypothesis of IPF pathogenesis, away from an chronic inflammatory model towards a wound healing model, which proposes fibrosis as the result of dysregulated crosstalk between alveolar epithelial cells and fibroblasts following repetitive alveolar injury, in genetically susceptible individuals. Up to one in 20 cases of sporadic IPF have been found to cluster in families (Garcia-Sancho et al, 2011) and large scale GWAS have implicated polymorphisms in genes related to senescence, cell to cell adhesion, host defence and mucin production in determining an individual's susceptibility to developing IPF

(Alder et al, 2008) (Fingerlin et al, 2013) (Noth et al, 2013). Substantial evidence has accumulated suggesting that bi-directional communication between injured alveolar epithelial cells and activated fibroblasts, leads to extracellular matrix deposition and a propagation of this response by fibroblasts leading to a cycle of further alveolar epithelial damage and extracellular matrix deposition. This evolution of our understanding has been in part facilitated by the advent of HRCT scanning and refinement in radiological and histopathological classifications of ILD, leading to tighter and more refined diagnostic classifications as well as more detailed characterisation of the processes occurring in tissue during fibrosis.

1.3.2 Alveolar Injury and Fibroblast Dysfunction

Alveolar epithelial cells have been proposed as initiators of the fibrotic process, producing many of the factors that induce fibroblast activation/dysfunction in response to repetitive injury, with diseased fibroblasts able to propagate a positive feedback loop by secreting mediators that exacerbate alveolar injury. Although the injurious stimuli remain elusive -with cigarette smoke, micro-aspiration and viruses all proposed as potential triggers (Selman et al, 2006) (Doran et al, 2005) - the signposts of epithelial insult are seen in the form of increased epithelial cell death and phenotypic alteration of surviving cells, with increased numbers of apoptotic cells also found in IPF (Adamson et al, 1988) (Plataki et al, 2005). Evidence of this theory has been established from findings in animal models, predominantly using the bleomycin model, as well as insights gleaned from immunohistochemical analysis of IPF lung tissue.

The alveolar epithelium is an important source of numerous pro-fibrotic growth factors and cytokines, which regulate fibroblast activation, proliferation and migration. It is a significant source of the prominent pro-fibrotic cytokine TGF- β and many other factors such as platelet derived growth factor and insulin like growth factor as well as endothelin 1 and TNF- α (Kapanci et al, 1995)(Xu et al, 2003) (Bessa et al, 2014).

An important part of the wound healing process at the level of the alveolus is effective re-epithelialisation of the basement membrane after injury. It has been demonstrated in animal models that efficient alveolar re-epithelialisation suppresses fibroblast proliferation and ECM deposition (Adamson et al 1990) and conversely delayed re-epithelialisation enhances the fibrotic response of mice lungs after hyperoxic exposure (Adamson et al, 1988). In fibrosis, the capacity of ATII to repair damaged ATI is reduced, resulting in alveolar collapse and abnormalities in pulmonary surfactant (Kasper et al, 1996). In addition, the findings of proteomic data in IPF showing upregulation of stress induced genes involved in endoplasmic reticulum stress (Korfei et al, 2013), as well as the association between surfactant protein C mutations and familial interstitial pneumonia (Nogee et al, 2001) both lending further credence to the importance of epithelial cell injury.

Adamson et al showed that hyperoxic injury to the alveolar epithelium of mouse explant lung caused a decrease in AEC proliferation and increase of fibroblast proliferation as well as increased collagen deposition, showing the direct effect of AEC injury on fibroblast function. In addition, this was demonstrated in the absence of blood components, suggesting the capability for AEC injury to lead to fibrosis in the absence of inflammation (Munger et al, 1999) with the implication of this finding fitting cleanly with the view of IPF as a disease of the pulmonary micro-environment and not a manifestation of a systemic response. A line of evidence has accumulated to suggest that alveolar cell death is a crucial part of lung fibrosis and this may be due at least in part to increased apoptosis. Induction of alveolar epithelial cell death has been shown as sufficient to trigger a fibrotic response in mouse lung in several different models of acute injury, including transgenic over-expression of TGF- β , pulmonary delivery of anti-fas antibody and hyperoxic injury (Hagimoto et al, 1997) (Matute-Bello et al, 2007) (Lee et al, 2004). There is immunohistochemical evidence of increased expression of pro-apoptotic proteins in the alveolar epithelium of IPF patients (Adamson et al, 1988) (Plataki et al, 2005) and AEC apoptosis is detected adjacent to fibroblastic foci with the alveolar epithelium overlying areas of fibroblastic foci exhibiting abnormal morphology and altered gene expression patterns (Kasper et al, 1996). Furthermore it has been shown that this AEC apoptosis is directly

linked to the presence of α -smooth muscle actin (α SMA)-positive myofibroblasts, which co-localise in the IPF lung (Uhal et al, 1998). Kulkarni et al demonstrated, in co-culture experiments, that injured AECs are able to signal in a paracrine fashion to local fibroblasts and affect their behaviour by influencing type I and III collagen and inducing fibroblast to myofibroblast differentiation, as evinced by α -SMA expression, by stimulating the activation of TGF- β in the extracellular matrix (Kulkarni et al, 1993).

The primary sites of ongoing injury and repair are believed to be fibroblastic foci, which are areas of fibroblasts, myofibroblasts and collagen deposition and play a critical role in the progression of pulmonary fibrosis (Kuhn et al, 1991). Fibroblasts from IPF patients show increased apoptosis, reduced proliferation and a pro-secretory phenotype with an altered matrix metalloproteinase (MMP)/tissue inhibitor of metalloproteinase (TIMP) balance (Ramos et al, 2001), as well as exhibiting evidence of being able to contribute to the perpetuation of the cycle of fibroblast/epithelial cell dysfunction. Conditioned media from the supernatant of IPF fibroblasts induces apoptosis of cultured AECs (Uhal et al, 1995) with angiotensin II (ANGII) the soluble mediator responsible for this effect in this dataset. Various different mediators have been implicated in this process, with, prominent amongst them, ANGI and the reactive oxygen species (ROS) which are synthesised by hydrogen peroxide (H_2O_2) and have a pro-fibrotic effect upon AECs (Hecker et al, 2009). Co-culture experiments have demonstrated that endogenous generation of H_2O_2 by TGF- β stimulated IPF lung fibroblasts induces apoptosis of small airway epithelial cells in a paracrine manner (Waghray et al, 2005) with knockdown of fibroblast H_2O_2 expression suppressing fibrosis *in vivo* in both bleomycin- and FITC-induced mouse models of pulmonary fibrosis. In addition fibroblasts in the lung can both produce latent TGF- β and activate the latent form by the $\alpha v\beta 5$ integrin that they express (Zhou et al, 2010).

These findings taken together highlight the importance of the crosstalk between injured alveolar epithelium and activated fibroblasts in initiating the fibrotic response as well as ensuring that it is

propagated through mechanisms by which activated fibroblasts and myofibroblasts can contribute to further epithelial injury. This is as a result of local alveolar injury and seems to be a contained pulmonary phenomenon which can be initiated, propagated and sustained in the local micro-environment in the absence of a systemic and inflammatory response.

1.3.3 The Role of TGF- β in Fibrosis

Multiple studies have identified the pluripotent cytokine TGF- β as crucial in the fibrotic process, with TGF- β induced fibrosis an accepted and commonly used in vitro model of fibrosis.

TGF- β is a widely expressed cytokine that is found expressed in 3 forms (TGF- β 1, TGF- β 2, TGF- β 3), which exert their effects through binding to the cell surface receptor complex consisting of TGF- β R1 and TGF- β R2. TGF- β is important during embryonic development for tissue growth and morphogenesis, but subsequently plays an important role in homeostatic tissue maintenance thereafter, with TGF- β 1 null mice developing widespread lethal tissue inflammation and auto-immunity (Kulkarni et al, 1993), and over-exuberant TGF- β expression leading to induction of fibrosis, collagen formation and angiogenesis in vivo in mice (Roberts et al, 1986). It is produced by a variety of cell types in the lung, including macrophages, neutrophils, epithelial cells fibroblasts and myofibroblasts. TGF is secreted in the form of latent complexes, which are required to be activated before they can exert their biological effects. The activation of TGF- β is believed to be regulated by direct epithelial cell interaction, with the integrin family of cell adhesion molecules critical in this process.

In the alveoli, activation of latent TGF- β isoforms is controlled by the α V β 6 integrin, and this process seems central to the development of fibrosis, with blockade or deletion of β 6 ameliorating fibrosis in different mouse models of fibrosis (Hunninghake et al, 1981), secondary to the reduction of TGF- β signalling (Puthawala et al, 2008)(Horan et al, 2008).

When activated from its latent form it is a pleiotropic growth factor with numerous different roles dependent on site and context of release. Tissue injury leads to release of TGF- β and consequent fibroblast recruitment, proliferation, myofibroblast transformation and deposition of extracellular matrix with TGF- β over expression in the rodent lung having been shown to cause myofibroblast accumulation and fibrosis (Kenyon et al, 2003) (Bonniaud et al, 2005) (Gu et al, 2007) as well as AEC apoptosis (Plataki et al, 2005) (Lee et al, 2004) (Siegel et al, 2003). A study by Li et al showed that a reduction of TGF- β signalling by a cell specific knockdown of TGF- β r2 in AECIIs protected against bleomycin induced pulmonary fibrosis and resulted in a reduction in myofibroblast activation and induction of epithelial cell apoptosis (Li et al, 2011).

In turn the activated myofibroblast secretes TGF- β , which induces further AEC apoptosis, contributing to the perpetuation of the aberrant wound healing response. I see a common theme when drawing together these insights; of epithelial cell damage and apoptosis, pro-fibrotic mediator release and myofibroblast activation, leading to extracellular matrix deposition and further alveolar cell injury.

1.4 Immunological Pulmonary Homeostasis

As the primary site of damage and key initiator of the process in IPF, the conversation habitually centres around the alveolar epithelial cell. However it stands to reason that the epithelium as a continuum from large airway cell to alveolus, might reflect the damage from injurious stimuli in cells other than those most distal AECs. The respiratory tract has an approximate surface area of 70m² (Jahnsen et al, 2001) in adult humans and is uniquely constantly exposed to the external environment and the potentially harmful particulates therein, during respiration. It has thus been required to develop a finely counterbalanced system which allows efficient and tightly controlled inflammatory response to pathogenic antigens, whilst limiting response to the billions of potentially immunogenic pathogens and particulates to which it is daily exposed.

1.4.2 Airway Macrophages

Airway macrophages are mononuclear phagocytes that act as the sentinels of the lung's immune system and play an important role in bridging the innate and adaptive immune systems. They exhibit significant plasticity allowing them to fulfil a variety of different functions in the alveolar space, including response to infection, clearance of debris and resolution of inflammation.

1.4.3 Ontogeny

It has hitherto been widely believed that the majority of tissue macrophages are recruited from haematopoietic stem cell precursors which are released from bone marrow and extravasate through endothelium, where, under the influence of local microenvironment signals, they differentiate into mature tissue dependent macrophages (Gordon et al, 2003). However recent work in the murine model has suggested that rather than continual recruitment from adult HSCs, alveolar macrophage populations are, in fact, derived from yolk sac derived erythro-myeloid progenitors which migrate to the fetal liver and give rise to fetal tissue macrophages under the influence of nuclear receptor PPAR- γ induced by GM-CSF (Gomez Perdiguero et al, 2015). This local pool of tissue macrophages shows a remarkable capacity for self-renewal and independently self maintains with the main mechanism of cell renewal being in-situ expansion, which occurs in the absence of circulating monocytes (Hashimoto et al, 2013). Although in the case of acute depletion, it has been shown that HSC derived monocytes can be attracted to tissue and differentiate into mature macrophages.

The revelation that the airway macrophage is a long lived cell which in most circumstances renews locally from the in-situ potentially aged pool, has implications upon the nature of its possible role in fibrosis, given the context of IPF as a disease of ageing. It raises the possibility of a local macrophage population developing a fibrotic phenotype as they age and this exhausted pro-fibrotic pool self renewing and thus perpetuating the cycle of over exuberant wound healing.

1.4.4 The M1/M2 Paradigm

When wound healing responses become chronic, fibrosis and organ dysfunction can follow, and among the many cell types involved in the wound healing responses, the highly flexible nature of macrophage programming (Mosser et al, 2008) positions them to be uniquely and critically involved in the regulation of all stages of repair and fibrosis (Wynn et al, 2010). The specific plasticity of tissue macrophages allows them to dynamically adapt their specialised functional phenotypes according to the signalling of their local micro-environment. Similar to the Th1/Th2 paradigm, macrophages have been characterised as M1 (classically activated) or M2 (alternatively activated).

The concept of M1/M2 polarity was established in the pre-genomic era when a simplistic system of a few surface markers was used to establish macrophage responses to stimuli. The theory broadly posits that depending on the inducing stimuli macrophages can be directed towards 2 polarised states (Martinez et al, 2014) (figure 2);

- M1 which are pro-inflammatory and are strongly involved in the immunological response to infection, by generating reactive nitric oxide via inducible nitrate oxide synthase (iNOS).
- M2 which are involved in Th2 immune responses and are also believed to be pro-fibrotic, and can be induced by a variety of mediators, including IL-4, IL-13, TGF- β and IL-10 (Murray et al, 2014)

It has now been generally accepted that the M1/M2 concept is an over simplification of what is actually a complex activation spectrum, and pulmonary macrophages may co-express markers of M1/M2 activation (Anthony et al, 2014). Transcriptomic analysis of macrophages stimulated with diverse activation signals has revealed a wider sliding scale of activation than previously thought (Xue et al, 2014) and this has been reflected in recent suggested changes to our nomenclature which take into account ontogeny and the polarising stimuli to which the cells are exposed (Murray et al, 2014).

Figure 1.2 The M1/M2 macrophage polarisation model

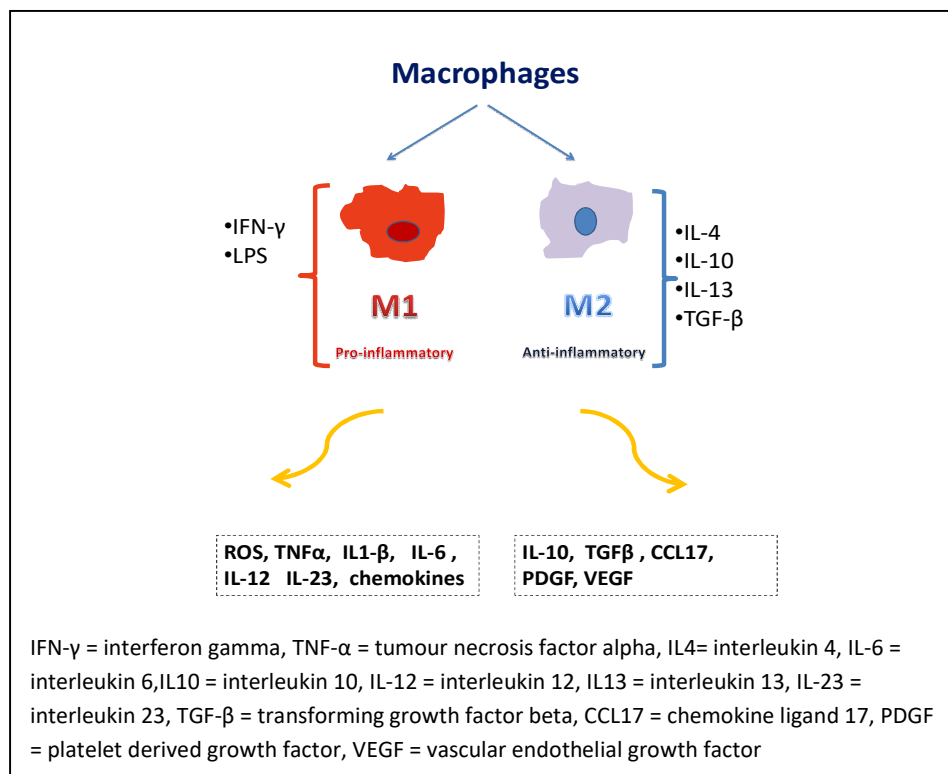


Figure 1.2 Diagram of the M1/M2 macrophage polarisation paradigm showing stimulating factors which induce macrophage differentiation and the range of mediators they induce them to release.

1.4.5 Role of Airway Macrophages in Fibrosis

Airway macrophages, in particular M2 like 'wound healing' AMs, have increasingly come under the microscope in fibrosis, with scope remaining for a clearer elucidation of their exact role in the process. Osterholzer et al demonstrated, using monocyte chemoattractant knockout mice (CCR2), that a type-II alveolar epithelial cell injury produces an macrophage laden inflammatory response (Osterholzer et al, 2013), with further work by Gibbons et al revealing Ly-6C^{Hi} monocytes (macrophage precursors) to facilitate the progression of pulmonary fibrosis (Gibbons et al, 2011). Their data showed that adoptive transfer of Ly-6C^{Hi} monocytes exacerbated bleomycin induced fibrosis and Ly-6^{Hi} monocyte depletion ameliorated fibrosis and decreased the this reduction in

fibrosis was associated with a reduction of alternately activated macrophages. In addition they have been shown to be involved in collagen uptake and secretion of matrix metalloproteinases, (MMPs) which are crucial players in ECM turnover (Dancer et al, 2011) (Atabai et al, 2009).

As well as being the predominant cell recruited by AEC injury, exacerbating bleomycin fibrosis and affecting ECM regulation, it has been suggested that airway macrophages could be the predominant source of the fibrosis inducing molecule TGF- β (Khalil et al, 1989). In addition the cytokine profile in the IPF lung skews towards Th2 responses (Wallace et al, 1995), which, as noted above, are believed to be involved in driving AMs towards an M2 phenotype.

In the mouse bleomycin model, interstitial macrophages acquire an M2 phenotype (as characterised by CD206 expression) during the fibrotic phase (Ji et al, 2014) (Misharin et al, 2013) with LY-6C^{HI} monocytes directing macrophages to an M2 phenotype with the expression of M2 marker CD163 shown to be increased in IPF tissue as compared to control (Gibbons et al, 2011). Song et al found that monocyte derived M2 macrophages enhance fibrogenesis by production of profibrotic factors (Song et al, 2000) and that IL-10 induces fibrosis in part by fibrocyte recruitment and M2 macrophage activation (Sun et al, 2011). In addition, alveolar macrophage dysregulation contributes to the pathogenesis of Hermansky-Pudlak syndrome Type 1, a rare form of hereditary pulmonary fibrosis (Rouhani et al, 2009).

However, given the remarkable plasticity shown by tissue macrophages and their ability to dynamically respond to changes in their environment, it is not surprising that the picture becomes more opaque when taking into account the evidence of the contradictory role that AMs can play in the setting of fibrosis. Fizz1 (upregulated on M2) deficiency in mice is associated with worsened fibrosis (Nair et al, 2009) and mice deficient for another M2 marker (Arg1) exhibiting worsened liver fibrosis and increased mortality in the schistosoma model.

1.5 Functions of Bronchial Epithelium

The bronchial epithelium forms a long continuous layer which acts as the first line of defence against pathogens. It is a dynamic tissue, which undergoes slow and constant renewal with resident cells as the source of the new cell population (Bowden et al, 1983). It is a ciliated pseudo-stratified columnar layer consisting of basal cells, ciliated cells, goblet cells and club cells allowing it to fulfil the roles of mediator release, mucociliary clearance and protection against inhaled noxious agents (figure 1.3). The integrity of the physical barrier protects the underlying tissue from the potentially harmful effects of the myriad of inhaled agents, whether noxious or benign. There are several mechanisms by which this barrier is maintained, with cell to cell adhesion mediated by the desmosome and intermediate junction, hemi-desmosomes anchoring epithelial cells to the basement membrane and the tight junction forming at the apical surface of the cell to cell contact (Wittekindt et al, 2017).

Figure 1.3 Cellular composition of the bronchial epithelium

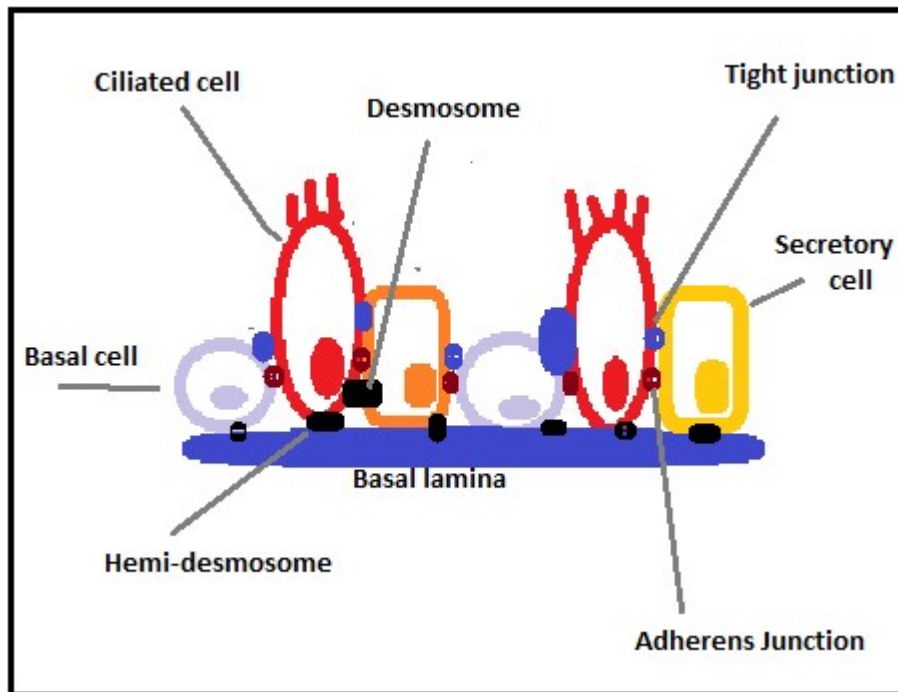


Figure 3 Schemata of the bronchial epithelium detailing the cell types and cell-cell interaction proteins of which it is composed

Apical junctional complexes consist of adherent junctions (AJs) and tight junctions (TJs). The predominant protein involved in the AJ is e-cadherin, which is linked to the actin cytoskeleton through its peripheral membrane proteins interacting with its cytoplasmic tail (Gonzalez-Mariscal et al, 2003).

This allows for stabilisation of the junction as well as regulated selective opening and closure of the junction. TJs form at the apical surface of the cell to cell contact region and act as a semi permeable gate which can selectively transport ions, solutes, water and cells paracellularly between the apical and basolateral surfaces of the membrane, which it acts predominantly to separate. They are composed of numerous different proteins with particular prominence assumed by zona occludens (ZO) proteins ZO-1 - a cytoplasmic plaque protein which interacts with transmembrane proteins forming a scaffold between them and the actin cytoskeleton- as well as claudins and occludens - transmembrane proteins that form intercellular adhesions (Gonzalez-Mariscal et al, 2003).

These tightly regulated cell to cell junctions provide stability, maintain tissue strength and selectively allow communication between neighbouring cells whilst blocking the passage of viruses, bacteria and inhaled pathogens to the basolateral membrane.

In addition to the physical barrier formed by the airway epithelium, a further line of homeostatic defence is formed by the co-ordinated effects of the goblet cells and cilia. Inhaled particles are trapped in the mucus produced by goblet cells and subsequently cleared by the co-ordinated and rhythmic beating of the cilia lining the epithelial surface. This mucociliary escalator is also aided by the production of surfactant, produced by ATII cells, which changes the surface charge properties making the particles less sticky. Mucociliary clearance depends on many factors, including amount and viscosity of mucus produced and ciliary beat frequency, which can be affected by cytokines such as TNF α , and IL-1, as well as bradykinin and histamine (Jain et al, 1995) (Burman et al, 1986) (Sanderson et al, 1989) (Khan et al, 1986) (Mussatto et al, 1988).

The epithelium also plays an active role in modulating the immune response by its ability to secrete pro and anti-inflammatory mediators which form an important part of the lung's response to insult. These mediators include;

- Chemokines, including RANTES, MCP1, IL-8, eotaxin (Becker et al, 1994) (Sousa et al, 1994) (Stellato et al, 1995) (Cromhill et al, 1992) (Lilly et al, 1997)
- Cytokines including GM-CSF, TNF α , IL-1 α , IL-1 β , IL-3, IL-6, IL-10, IL-11, IL-33 (Soloperto, 1991) (Woolley et al, 1996) (Devalia et al, 1993) (Noah et al, 1991) (Bellini et al, 1993) (Bonfield et al, 1995) (Haraldsen et al, 2009) (Elias et al, 1994)
- Growth factors (including EGF, TGF- β , IGF and PDGF (Nettesheim et al, 1992) (Sacco et al, 1992)

The diverse range of functions these mediators perform allows for initiation and perpetuation of inflammation by immune cell attraction and activation of the attracted leukocytes, as well as the down-regulation of the inflammatory response by the release of anti-inflammatory mediators, the

inactivation of pro-inflammatory mediators and the release of soluble receptors to bind and sequester their ligands (Takizawa et al, 1996) (Arnold et al, 1994).

1.5.2 Emerging Role of Bronchial Epithelium in Pulmonary Fibrosis

Given the importance of the alveolar epithelium as the key site of injury and the propagator of the fibrotic response, little attention has previously been focused on the function of the bronchial epithelium. The spotlight has recently fallen upon this hitherto largely ignored area, beginning with a genome wide linkage screen in 82 families which led to the discovery of a common variant in the promoter region of MUC5B which now stands as the strongest known genetic risk factor for development of pulmonary fibrosis (Noth et al, 2013). MUC5B encodes a member of the mucin family of proteins and is the main gel forming mucin in mucus. MUC5B has been demonstrated to be crucial for airway defence with its deficiency in mouse models leading to diminished mucociliary clearance, airway macrophage apoptosis and impaired phagocytosis, and conversely MUC5B over expression leading to improved macrophage function (Roy et al, 2014). The promoter polymorphism leads to an over expression of the protein in those with the at least one copy of the minor allele whether they are individuals with IPF or unaffected, with further immunohistochemical analysis showing the distal airways to be the predominant source of MUC5B expression in IPF (Seibold et al, 2011).

Stratification of the disease population by genotype has subsequently demonstrated that although conferring a greater risk of disease development, the MUC5B promoter polymorphism is also associated with a twofold improved survival, suggesting that this genotype actually identifies a unique clinical phenotype of the disease (Peljto et al, 2013). Although the findings of this study was contradicted by a smaller Chinese cohort, which found detection of minor T alleler mutation to lead to a decreased survival (Jiang et al, 2015), however this minor T allele was detected infrequently and the relatively small sample size in this study suggests that a larger cohort, powered to look at the

minor T allele, would be required to validate this finding. Furthermore, the association with IPF and the MUC5B polymorphism has been shown to be strongly dependent on racial/ethnic background. The variant has been found to be higher in non-hispanic white populations than hispanic and asian populations, and absent in African populations, which tallies with the clinical observation that populations of African descent are rarely diagnosed with IPF (Yang et al, 2015). This

This body of work presents a compelling case for a pathway by which IPF can develop as a consequence of defect upstream of the alveolar epithelium. It implicates dysfunction of mucociliary clearance as a potential trigger for IPF development.

Single cell RNA sequencing of bronchial epithelium in IPF the normal lung has also offered further insights into the potential contribution of bronchial epithelium to the fibrotic process. Xu et al revealed that epithelial cells co-expressed airway specific cell markers and AEC type I and AEC type II markers (Xu et al, 2016), suggesting indeterminate states of differentiation of HBECs not seen in the healthy state in the normal lung. Furthermore, Fukumoto et al identified a population of club cells which self renew and act as progenitor cells which can phenoconvert and form airway and alveolar cells *in vitro* in response to bleomycin but not under normal homeostatic conditions (Fukumoto et al, 2016). This data suggests that the unique micro-environment of the fibrotic lung predisposes to an abnormal program of differentiation in HBECs. This can result in loss of the distinction of proximal-peripheral patterns of cell differentiation as well as the abnormal migration and differentiation of club cells in response to fibrotic injury, lending further credence to the concept of abnormal bronchial epithelial responses in fibrosis and prompting questions as to whether the impairment of other areas of epithelial function might also be found to have pathogenic consequences in IPF.

Relatively little is known about the nature and regulation of tight junctions in the lung in both normal conditions and, particularly, in IPF. Some insights have been gleaned from studying the *in vitro* effect of the prominent fibrosis associated molecule TGF- β upon tight junctions in different cell types but the data in this area is sparse and does not build a comprehensive picture of . However, in

keeping with its established reputation as a pleiotropic molecule, the results of this work have shown TGF- β to have contradictory functions depending on the cell type and location. It decreases the permeability of colonic epithelial cells (Planchon et al, 1994) but conversely increases that of uterine epithelial cells. It has been shown to downregulate ZO-1 and e-cadherin in A549s (Chen et al, 2013), suggesting the possibility of a TGF- β mediated disruption of the epithelial barrier, but this effect has only been seen in cell lines and it has not been established in primary cells.

Taken together, these insights demonstrate evidence of a route by which a failure of normal bronchial epithelial homeostasis can precipitate fibrosis. It suggests a previously unseen function of airway defence and mucociliary clearance in IPF pathogenesis, directly implicating bronchial epithelial dysfunction as a possible predisposing factor to the alveolar micro-injury thought crucial to the development of fibrosis. However the other functions of the bronchial epithelium have not yet been exhaustively examined and it is not clear whether the epithelial contribution to the development of IPF rests with defects in mucociliary clearance or whether the other functions of these cells may be impaired or dysregulated in IPF, whether that may be through defective barrier function or potentially through an aberrant cell migration and regeneration. IPF consistently emerges as a disease characterised by dysfunctional cross talk between cell types, with imbalances in production of pro and anti-fibrotic mediators. Airway epithelial cells are known to be potential sources of many cytokines, chemokines and growth factors which have been widely implicated in the pathogenic fibrotic state. These have been largely studied in the alveolar epithelial cell but less so in the airway epithelium in the IPF lung.

1.6 IL-33 ST2 axis

IL-33 is one of the 11 members of the IL-1 superfamily of cytokines, the other being IL1- α , IL1- β , IL-18, IL-36 α , IL-36, IL-36 λ , IL-1Ra, IL-36Ra, IL-37 and IL-38. These cytokines induce a complex network of pro-inflammatory responses which can initiate and regulate inflammation.

Under homeostatic conditions, IL-33 is found in a variety of cells, primarily non-haematopoietic cells such as epithelial, endothelial and fibroblasts, as well as being inducible expression in some lymphoid cells including dendritic cells and macrophages. IL-33 appears to have dual functions, as a nuclear protein and an extracellular cytokine. IL-33 is found as a precursor or full length form, with an N-terminus required for nuclear localisation and at resting state, it localises to the nucleus of endothelial, epithelial and some lymphoid cells (Liew et al, 2016). With deletion of the N-terminus, IL-33 cannot translocate to the nucleus and in mice models, overwhelming eosinophilia and fatal multi-organ failure ensues (Choi et al, 2012). IL-33 has also been demonstrated to localise simultaneously to the nucleus as well as vesicles contained within the cytoplasm of primary cells in mice (Kakkar et al, 2012). When localised to the nucleus, IL-33 acts to negatively regulate inflammation - Nuclear IL-33 can regulate gene expression by different mechanisms. It can sequester NF- κ B thereby attenuating the pro-inflammatory signalling of this pathway (Carriere et al, 2007), it can modulate gene expression by binding to heterochromatin and modulating higher order chromatin structure and function (Roussel et al, 2008). It has also been proposed as a transcription factor which binds to the transcriptional repressor histone methyltransferase SUV39H1 (Carriere et al, 2007).

IL-33 exerts its functional effect by binding to a heterodimeric complex of ST2 and IL1 receptor accessory protein (IRAP), inducing TH2 responses. This effect is antagonised by the membrane bound negative regulator SIGIRR, and, when released extracellularly, by soluble ST2 which is a negative regulator exerting its effect by sequestering free IL-33 and thus negating its signalling ability (Schmitz et al, 2005) (Chackerian et al, 2007).

On activation of the receptor complex, MyD88 and downstream IRAK1/4 activation lead to TRAF6 mediated liberation of NF κ B from its kinase complex (IKK). ST2 contains 3 extracellular IgG domains with a single trans-membrane domain and an intracellular TIR domain, the latter of which are lacking in the soluble form. It is expressed on numerous cell types primarily T helper cell type 2

(TH2), ILC2 and mast cells as well as macrophages, eosinophils and fibroblasts (Xu et al, 1998) (Lohning et al, 1998). The ST2 gene encodes at least 3 splice variants, a membrane bound form, a soluble form and a variant form are produced (Iwahana, 1999).

Figure 1.4

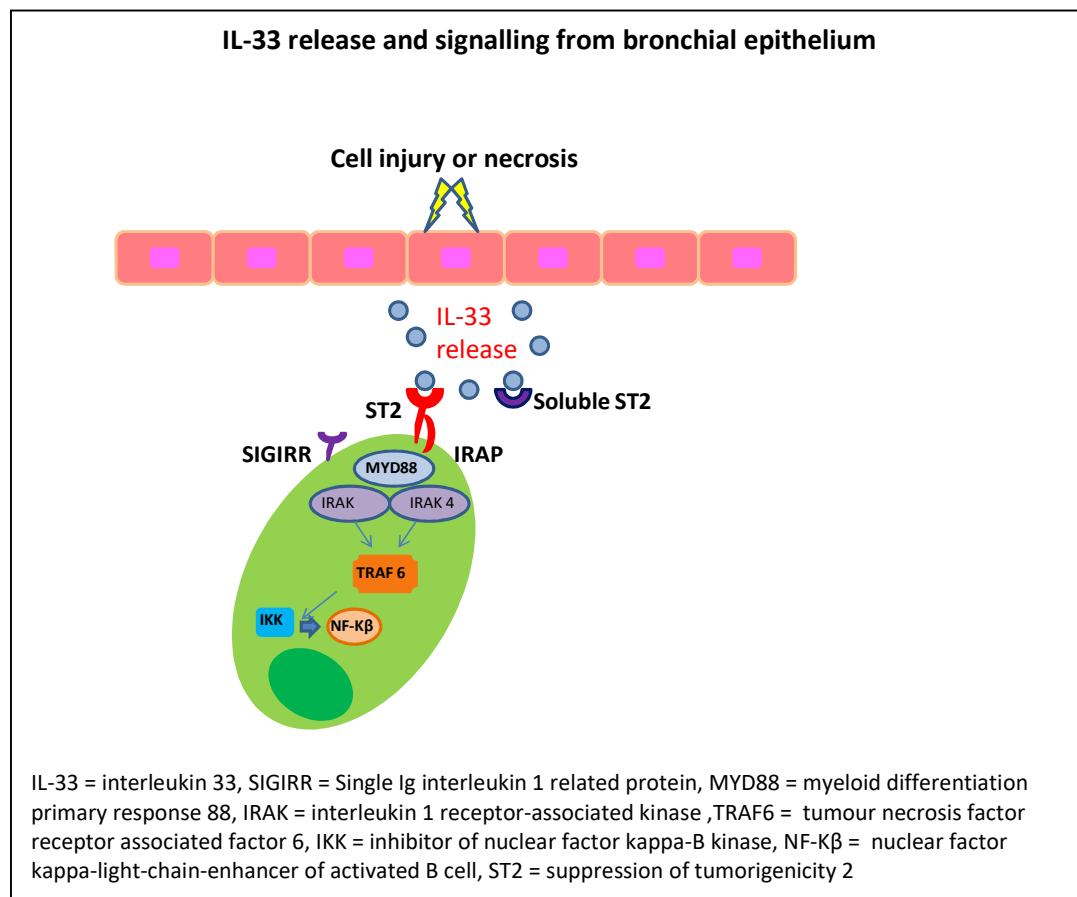


Figure 4 The IL-33/ST2 signalling axis - IL-33 release from damaged cells and signalling pathway in ST2 bearing cells

Upon death or necrosis of intact barrier cells, IL-33 can be released into the extracellular space where it can act as a so-called 'alarmin' (Haraldsen et al, 2009), and endogenous danger signal which can trigger an immunologic response. Full length released IL-33 remains biologically active, however it can subsequently be processed into mature forms the biological activity of which differs depending upon the nature of the processing. IL-33 is inactivated by caspase-3 and caspase-7 during apoptosis (Luthi et al, 2009) but proteolytic cleavage by neutrophil, macrophage and mast cell

derived proteases produces an N-terminally truncated form of the cytokine which increase the activity of IL-33 by up to 30 times (Lefrancais et al, 2014). Furthermore, it has recently been reported that IL-33 can undergo rapid oxidization resulting from the formation of 2 disulfide bridges, which can completely abolish its ability to bind the receptor complex (Cohen et al, 2015).

Further insights from Gordon et al have revealed that in human airway cells, alternative splicing of the IL-33 transcript is consistently present, with cytoplasmic localisation facilitated by the deletion of exons 3 and 4 (Δ exon 3,4) , resulting in extracellular secretion of a biologically active form with retained signalling ability. Furthermore, they found Δ exon 3,4 to be strongly associated with type 2 inflammation in stable asthmatics whereas full length IL-33 was not (Gordon et al, 2016).

1.6.2 IL-33 in pulmonary fibrosis

Given the nature of the release of IL-33 in response to cell injury and necrosis, it is unsurprising that this area has become a focus of attention in pulmonary fibrosis. As previously documented (Schmitz et al, 2005), IL-33 has been shown to exhibit potent effects in promoting the induction and maintenance of Th2 signals (Murakami-Satsutani et al, 2014) with the innate stimulation of IL-13 by ILC2s dependent on IL-33 (Guo et al, 2015). There is a great deal of evidence in animal models to suggest Th2 cells to be important in the induction of chronic inflammation and fibrosis (Wynn et al, 2004). Over-expression of IL-4 and IL-13 in transgenic mice confirms a direct effect of Th2 cytokines on the activation of myofibroblasts (Rankin et al, 1996) (Zhu et al, 1999) with IPF fibroblasts exhibiting hyper-responsiveness to IL-13 and elevated levels of IL-13 found in IPF BAL.

In both the human ex-vivo setting and animal models of fibrosis levels of IL-33 expression have been found to be elevated. Immunostaining of lung tissue from IPF individuals showed an increased intensity IL-33 of staining over control, particularly within inflammatory infiltrates and in the vicinity of fibroblastic foci, although with the caveat that the staining was assessed semi-quantitatively

(Luzina et al, 2012). Serum levels of IL-33 have also been found to be elevated in individuals with systemic sclerosis, and this was found to correlate with the development of restrictive lung spirometry and radiological evidence of pulmonary fibrosis, again implicating IL-33 in the fibrotic response in the lung (Yanaba et al, 2011).

This was further corroborated by the findings of Lee et al (Lee et al, 2017), who examined BAL in individuals with a range of ILDs and found levels of secreted IL-33 to be higher than both normal controls and other ILDs (hypersensitivity pneumonitis, NSIP and sarcoid), however the findings in these studies were measured using the IL-33 ELISA and should be interpreted with caution given the previously documented limitations of this low sensitivity assay (Lee et al, 2017) (Nygaard et al, 2016) (Ketelaar et al, 2016) (Riviere et al, 2016).

The finding that these levels were higher in IPF than other ILDs - which are known to be highly inflammatory in nature - hints at the possibility that any potential role in fibrosis could be independent of the known pro-inflammatory actions of IL-33 and raised the possibility of direct pro-fibrotic activity.

Upon lung injury, epithelial cells release IL-33, IL25 and TSLP, facilitating the development of pro-fibrotic Th2 responses. Type 2 Innate lymphoid cells (ILC2) are present in the lungs of IPF individuals and absence of ILC2 impairs the development of fibrosis and furthermore IL-13 release from ILC2 has been shown sufficient to drive collagen deposition in challenged mice in schistosoma-induced fibrosis (Hams et al, 2014). Further findings using this model suggested that combinatorial targeting of IL-33, IL-35 and TSLP suppressed type 2-cytokine driven inflammation and fibrosis in the liver (Vannella et al, 2016). Interestingly, this group found no effect when each of these mediators was ablated individually, and the reduction in hepatic fibrosis and IL-13 producing ILCs was seen only when all 3 were targeted simultaneously.

The evidence of a functional role of IL-33 in the pathogenesis of fibrosis is not confined to the schistosoma model, with similar findings replicated in the bleomycin model. Separate studies have

shown that bleomycin administration in mice increases IL-33 expression (Luzina et al, 2013) with a peak 7 days after administration and a gradual decrease thereafter, along with a concomitant upregulation in its classical signalling pathway- with a detected increase in ST2, MyD88 and TRAF 6 (Hams et al, 2014). Li et al (Li et al, 2014) also examined the effect of bleomycin upon this signalling axis, demonstrating that bleomycin increases the production of mature IL-33 in lung tissue and induces its production in macrophages. Additionally they found that in addition IL-33 polarises macrophages to an IL-13 and TGF producing M2 phenotype.

IL-33 over expression in combination with bleomycin treatment increases collagen deposition, expression of TGF- β , IL-6 and MCP-1 as well as several heat shock proteins that have been shown to be associated with ILD (Luzina et al, 2013). However, it is interesting to note that this same study also found that deficiency of ST2 did not affect bleomycin injury and fibrosis. This would suggest the pro-fibrotic effects of IL-33 in this model to be independent of the traditional ST2 pathway.

Taken together these data present a case for the involvement of IL-33 in the fibrotic response, with its role as a damage response molecule and the distribution of receptor positive effector cells positing a plausible mechanism of Th2 cytokine production driven by ILC2 in response to IL-33 release from damaged cells. However, the finding of an increase in IL-33 in IPF over classically inflammatory ILDs as well as the lack of effect on fibrosis of ST2 blockade suggests that this effect could be exerted separate to the conventional IL-33 signalling pathway.

1.7 Hypothesis

Airway cells in individuals with IPF contribute towards the fibrotic process via a skew towards a putative 'wound healing' M2 phenotype of airway macrophages and pro-fibrotic function of bronchial epithelial cells, in part through dysfunction of the release of IL-33

1.8 Aims

1. To characterise the activation status of airway macrophages within the M2/M2 paradigm in individuals with ILD using cell surface phenotyping markers by multicolour flow cytometry
2. To investigate the phenotype and functional capacity of bronchial epithelial cells in IPF, by immunohistochemical staining of cell surface markers, and a number of *in vitro* assays examining their proliferation and barrier function, as well as their contribution to the regulation of extracellular matrix deposition by determining their effect on the gene expression of human lung fibroblasts.
3. To determine, by immunohistochemical staining of tissue and ELISA measurement of soluble mediators, the locality and expression of IL-33 and its receptor in IPF compared to healthy control and assess its direct non-immune cell mediated effects upon the gene expression of human lung fibroblasts stimulated *in vitro*.

Chapter 2.
Materials and Methods.

2.1 Subjects

BALF and endobronchial brushings were taken at the time of clinically indicated bronchoscopy from individuals under investigation for interstitial lung disease, and healthy volunteers with no interstitial lung disease. All study participants supplied written informed consent approved by an ethics committee at the Royal Brompton Hospital (Ethics number 10/HO720/12).

2.2 IPF diagnosis

Diagnosis of idiopathic pulmonary fibrosis was made according to consensus ATS/ERS classification guidelines and agreed by multidisciplinary team composed of a thoracic radiologist, specialist histopathologist and specialist ILD clinician , as per NICE guidance.

2.3 Control volunteers

These were adult volunteers with no relevant past medical history or signs suggestive of respiratory disease on physical examination who had given informed consent to undergo a research bronchoscopy which was approved by a Royal Brompton Hospital ethics committee (10/HO720/12). Spirometric values were obtained prior to bronchoscopy.

2.4 Clinical Information

Demographic details, smoking status, treatment data, and serial pulmonary function tests (PFT) measures were extracted from hospital records retrospectively upon all subjects in whom this complete dataset was available. Lung function values at bronchoscopy were taken from the period

of up to 3 months before and up to 3 months after bronchoscopy. Longitudinal data was taken up to 18 months post bronchoscopy.

Current smokers were defined as smoking or having stopped smoking less than 6 months from the time of investigations. Patients were considered smokers if they had smoked more than one cigarette per day for more than one year. All else were considered non-smokers.

2.5 Bronchoscopy

All bronchoscopies were performed under light sedation with topical anaesthesia (2% lignocaine), as per routine clinical practice at the Royal Brompton Hospital. Intubation was via the oropharyngeal route. To obtain bronchoalveolar lavage (BAL) fluid, 4 aliquots of 60mls of warmed saline were instilled separately into the right middle lobe, or the area with the greatest radiographic degree of abnormality in cases where disease was not diffuse. The instilled saline was gently aspirated and all 4 fractions pooled. 40 mls was decanted and sent for differential cell count at the histopathology lab at the RBH and 5mls sent for bacterial culture. The remaining BAL fluid returned was kept on ice until transport to the laboratory, within 3 hours, for processing. If BAL in consented individuals did not return more than 45mls of fluid, none was taken for research purposes and the individual was removed from this study.

Bronchial epithelium was obtained by the insertion of a disposable pulmonary brush with covered sheath (MODEL BC-202D-3010 Olympus) via the bronchoscope into the sub-segmental bronchi of the right lower lobe. 3 or 4 brushings (depending on patient tolerance and extent of brush site bleeding) were performed in different places with gentle upward and downward strokes and the brushes were immediately transferred to a 50ml conical tube (Falcon) containing bronchial epithelial growth media (LONZA) and anti-bacterial/anti-fungal solution (Primocin, Invivogen) by shaking the brush in the media.

2.6 Lung function

Spirometry was performed using the Vitalograph 2120 (Vitalograph Ltd, Buckinghamshire) according to standard criteria using a pneumotachograph with the integration of flow to derive volume (Miller et al. 2005). The device was calibrated daily with a 1L syringe with a view to ensuring an accuracy of within 3% of the calibration volume. Subjects were asked to take a deep inspiration, completely filling the lungs and were then asked to perform a maximal forced expiration through a disposable mouthpiece attached to a filter. This was repeated until 3 attempts within 5% of each other were obtained, with the best FEV1 and FVC recorded.

Gas transfer was determined by inhaling a known concentration of carbon monoxide (CO) and measuring the end expiratory CO concentration after a single breath hold and adjusting for alveolar volume (Compact Master lab system, Jaeger, Germany). All tests were performed and interpreted in accordance with ATS/ERS guidelines (Macintyre et al. 2005) (Wanger et al. 2005). Calibrations on all equipment were undertaken regularly. Predictive values were expressed in accordance with the British Thoracic Society guidelines (British Thoracic Society 1997).

Lung function values were obtained up to 3 months and 3 months post the bronchoscopy and follow up values taken up to 18 months post bronchoscopy.

2.7 BAL fluid processing

BAL fluid was immediately placed on ice and transported for processing within 3 hours of collection and stained on the same day. Following bronchoscopy and lavage, mucus was filtered out using a 70 μ m filter. The remaining fluid was spun down at 400 x g, 4°C for 5 minutes, break 9 (Hettich Centrifuge, Rotina 420R). BAL supernatant was collected and aliquoted to 1.5ml microcentrifuge tubes (Eppendorf) and stored at -80°C for future analysis. Cell pellets were re-suspended in red cell lysis buffer ((155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.3)) for 10 minutes. The cells and

RCC lysis solution are then centrifuged for 5 minutes at 400 x g with supernatant discarded and cell pellet re-suspended in complete RPMI (RPMI + 10% FCS, 100u/ml penicillin streptomycin). 10 µl of re-suspended cells were added to 90 µl of Trypan Blue and live unstained cells counted using a haemocytometer. Cell count was determined by multiplying the mean of 3 separately counted 4 x 4 squares by the sample dilution (i.e. 10 as a 1:10 dilution of sample to stain) x 10^4 = cells/ml

2.8 Reagents

Chemicals, solvents and tissue culture materials; All chemicals were of analytical grade or above and were obtained from Sigma Aldrich (Poole, UK) unless otherwise stated. Sterile tissue culture flasks and plates were all obtained from Corning.

Bronchial epithelial growth medium was obtained from Lonza (containing bovine pituitary extract, human epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, gentamicin/amphotericin, primocin). DMEM and heat inactivated fetal bovine serum were obtained from Gibco.

All deionised water or phosphate buffered saline used for the preparation of buffers were obtained from Sigma Aldrich (Poole, UK)

Cytokines; Recombinant proteins were used for cell stimulations.

Recombinant human IL-33 was obtained from Biolegend (581804)

Recombinant human TGF-β1 was obtained from R & D systems (240-B-002)

2.9 Bronchial epithelial cells

2.9.1 Bronchial epithelial brushings

Cells obtained by brushing were pelleted by centrifugation (400 x g, 5 minutes) and then re-suspended in red cell lysis buffer (ebioscience) for 5 minutes. The re-suspended solution was then centrifuged (400 x g 5 minutes) with supernatant discarded and cells re-suspended in BEGM. 10 ul of re-suspended cells are added to 90 ul of Trypan Blue and counted using a haemocytometer.

Epithelial cells were then transferred to a T25 or T75 collagen coated culture flask and incubated T 37°C in a humidified atmosphere containing 5% CO₂ in bronchial epithelial growth media (BEGM) ((Lonza, cat# CC-3170 with gentamicin removed from supplements and primocin (invivogen, cat# ant-pm-1) at 50 mg/ 500 mL media substituted). Experiments were performed using cells between passages 1-3.

Control epithelial cells were either grown as per the method above from healthy control volunteers, or bought from Lonza.

2.9.2 Bronchial Epithelial cell culture

Bronchial epithelial cells were seeded into collagen coated 12 Well culture plates in BEGM and incubated at 37°C, with media change every 2 days. When the cells had reached 90% confluence, media was replaced with basal medium minus supplements BEGM bullet kit supplements to serum starve overnight. Basal medium was replaced with basal BEGM containing recombinant protein used for stimulation.

2.10 Fibroblasts

2.10.1 Fibroblast isolation

IPF Fibroblast cell lines were established as described (Akers et al, 2000) previously from surgical lung biopsy of individuals with IPF. Control fibroblasts were bought from Lonza, or established from individuals undergoing resection of localised lung tumours. In brief histologically normal lung distal to the resected tumour was cut into pieces of $<1\text{mm}^3$ and placed onto a 10cm diameter petri dish (Corning costar, Cambridge, MA) with 1 ml of DMEM containing 10% FCS, penicillin (100U/ml), streptomycin (100ug/ml) and primocin and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 3 hours. Once adhered, a further 9mls of media was added and incubated as before. The medium was changed every 7 days and when fibroblasts (identified by morphological appearances) had grown to confluence out of the explant tissue, the cells were trypsinised and re-seeded at 500,000 cells into 75-cm² flasks (Falcon, Marathon, London, UK) in DMEM as above. Cell dissociation was performed by aspirating the cell culture medium and covering the cells in a layer of cell dissociation re-agent (TrypLE™ Thermo Fisher Scientific) for 5-10 minutes until approximately 90% of the cells were detached, and the flask was gently tapped to release the majority of the cells from the culture surface. Trypsin was then neutralised by addition of complete medium containing 10% FCS. Experiments were performed with cells between passages 2 and 5.

2.10.2 Fibroblast cell culture

Fibroblasts were seeded into culture plates (Corning) in complete DMEM and incubated at 37°C and media was changed every 2 days. When the cells had reached 90% confluence (confirmed visually by direct microscopy) media was replaced to DMEM with penicillin/streptomycin to serum starve overnight. Serum starvation media was gently aspirated and replaced with stimulation media (detailed below) and incubated at 37°C.

2.11 Cell Freezing

To freeze cells for later use, 10% FCS and 10% DMSO was added to either complete DMEM medium (for fibroblasts) or BEGM (for epithelial cells) and placed on ice. Cells were then resuspended at 5×10^5 /mL in the above cell freezing mix. 1 mL of cells in freeze mix was added to 1.8 mL cryovials and placed in liquid nitrogen insert for at least 2 hours before placing cells in liquid nitrogen for long term storage (1 x T75 at 100% confluence is approximately 6 vials).

2.12 Lung tissue histopathology

Lung tissue sections were fixed in 4% paraformaldehyde for 24 hours and then kept in 70% ethanol until paraffin embedding. Paraffin embedded lung tissue sections (3 μ m) were counterstained with haematoxylin and eosin and then stored at 20°C. Paraffin embedding and sectioning were performed by Lorraine Lawrence in the leukocyte biology department of Inflammation Repair and Development at Imperial College London

2.13 Lung tissue immunohistochemistry (DAB)

Paraffin embedded lung sections were used for immunolocalisation of IL-33, ST2, MUC5AC, ACT, SCGB1a1, and CK5. Sections were acquired from individuals with IPF who had undergone surgical lung biopsy and from histologically normal lung distal to lung cancer resections. Sections were treated with citrate buffer, pH 6, for 30 minutes for antigen retrieval. Sections were then blocked with serum free protein block (DAKO, X0909) and then incubated with primary antibodies diluted in 1% BSA in PBS with 0.05% tlen. This was then followed by a horseradish peroxidase conjugated secondary detection antibody and DAB substrate (Vector laboratories) prior to washing in dH2O to quench endogenous peroxidase activity and counterstaining with haemotoxylin.

2.14 Immunocytochemistry (fluorescence)

Passaged HBEs were seeded onto collagen coated 8 Well chamber slides and grown to confluence over 6-8 days. 48-72 hours after reaching 100% confluence, cells were fixed in 4% PFA for 5 minutes. Cells were permeabilised for 15 minutes in permeabilisation buffer (0.5% triton-X BSA PBS) and then incubated with primary antibody in permeabilisation buffer overnight at 4⁰. Cells were then washed 3x in wash buffer(PBS with 0.05% Tlen) and fluorescent secondary antibodies added for 2 hours at room temperature. Nuclei staining was performed using a Hoechst nuclear staining dye (Thermo Fisher Scientific, H3570) and cells imaged using confocal microscopy.

Table 2.1 Antibodies used for immunohistochemistry

Antibody	Clone	Dilution	Supplier
Cytokeratin 5 (CK5)	Mono / XM26	1/50	Abcam
Acetylated Tubulin (ACT)	Mono/6-11B-1	1/3000	Sigma-Aldrich
Mucin 5 AC (MUC5AC)	Mono / 45M1	1/400	Sigma-Aldrich
Secretoglobulin (SCGB1A1)	Poly	1/8000	Sigma-Aldrich
IL-33	Mono/ Nessy-1	1/100	Enzo life sciences
Anti-IL1RL1/ST2	Poly	1/100	Sigma-Aldrich

Table 1 antibodies used for immunohistochemistry, clone, supplier and dilution

2.15 Reverse Transcriptase Polymerase Chain Reaction

Real time PCR was used to quantify expression of a number of genes involved in IL-33 and TGF- β signalling and extracellular matrix regulation in human bronchial epithelial cells and fibroblasts.

2.15.1 RNA extraction

Cells were lysed directly in Wells of 12 Well culture plate in 300ul Trizol (thermofisher scientific) and stored in eppendorfs at -80°C until analysis. Phase separation was performed by addition of 1:5 volume of chloroform and centrifugation for 15 minutes at 12,000 g and 4°C for 15 minutes to separate the mixture into a lower phenol contaminated layer, an interphase and a colourless aqueous phase. The upper phase aqueous layer was mixed in a 1:1 ratio with 70% RNA grade and 700µl of RW1 buffer was added to the column then spun at 8000 x g for 30 seconds ethanol and the flow through transferred to RNeasy plus micro columns (Qiagen, Crawley, UK) and spun at 8000 x g for 30 seconds. The flow through was discarded. Lyophilized DNASE 1 (Qiagen, Crawley, UK) is reconstituted in RNase free water and then 80µl of reconstituted DNASE1/Buffer RDD solution (1:7) added directly to the column and incubated for 10 mins at room temperature. 700µl of RW1 buffer was added to the column then spun at 8000 x g for 30 seconds with flow through discarded. Then 500 µl RPE buffer added to the column and spun as above with flow through discarded and this step repeated. The column was then spun at 8000 x g for 2 minutes to dry the membrane. The collection tube was changed to an RNase free 1.5ml eppendorf and 30 µl of RNase free water added directly to the membrane and the column was then spun at 8000 x g for 1 minute to elute the RNA.

The concentration of RNA was then measured using a NanoDrop 1000 Spectrophotometer with V3.7 software to analyse the data - To ensure RNA quality and accuracy of NanoDrop measurements, RIN value and quantity of RNA was determined on a selection of the samples using RNA screentape and screentape buffer reagents (Agilent Technologies, Santa Clara, California) and analysed using Agilent 2200 TapeStation System (Agilent Technologies, Santa Clara, California). The Agilent bioanalyser is an automated device which uses microfluidics technology to perform electrophoretic separations of RNA samples according to their molecular weight. This allows generation of an electropherogram showing representations of the amount of RNA of a given size as detected by laser-induced fluorescence detection. This also allows for the automated and reproducible generation of a

numerical value for the integrity of the RNA, the RNA Integrity Number (RIN) as well as quantity of RNA.

2.15.2 RNA reverse transcription

RNA was reverse transcribed to complementary DNA (cDNA) using high capacity reverse transcription kit (ThermoFisher, Cat# 4368813) according to manufacturer's instructions. A 20 μ l reaction is composed of 2 μ L of 10X RT buffer, 0.8 μ L of 25x dNTP Mix (100 mM), 2 μ L of 10X random primers, 1 μ L of MultiScribe™ Reverse Transcriptase and 4.2 μ L of nuclease free H₂O (total volume 10 μ L) and up to 10 μ L of RNA. Total volume was made up to 20 μ l with nuclease free H₂O in PCR 0.2 mL tube on ice (ThermoFisher, A30588) and sealed. The tubes were placed in a PCR thermocycler (Veriti® 96-Well Fast Thermal Cycler, Applied Biosystems®) and run to the following protocol;

Table 2.2 Immunohistochemistry thermal cycling conditions

Step 1	25°C for 10 minutes
Step 2	37°C for 120 minutes
Step 3	85°C for 5 minutes
Step 4	4°C until storage

Table 2.2 thermal cycling conditions used for reverse transcription to cDNA

Samples were held at 4°C if being used within 24 hours or stored at -20°C for later use.

2.15.3 Gene expression analysis

Gene expression was measured using TaqMan primers for specific genes. Reactions were carried out in duplicate in 384 Well plates (MicroAmp® Optical 384-Well Reaction Plate, ThermoFisher, Applied Biosystems, cat# 4309849). For each reaction 2.4µl of cDNA was added to 3.6 µl of a master mix composed of; 0.3 µl nuclease free H₂O, 0.3 µl TaqMan Primer of interest, 3 µl TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, cat # 4444557)

The plate was sealed with adhesive tape (MicroAmp® Optical Adhesive Film, ThermoFisher, Applied Biosystems cat# 4313663), centrifuged for 1 minute at 400 x g at 4°C and 6 µl real time PCR reactions were carried out with Taqman Fast Advanced Master Mix (Lifetechnologies, cat# 4368813) and performed on Vii7 system (Applied Biosystems, Warrington, UK).

The housekeeping gene GAPDH was used as a reference molecule for PCR amplifications.

Target gene amplification was performed under the following thermal cycling conditions (table 2.3)

Table 2.3 cDNA Target gene amplification conditions

parameter	UNG	Polymerase activation	PCR (40 cycles)	
	incubation			
	<i>hold</i>	<i>Hold</i>	<i>denature</i>	<i>anneal/extend</i>
temp°	50	95	95	60
time	02:00	00:20	00:01	20:00

Table 2.3 conditions used for target gene amplification during qPCR

Table 2.4 Primers used to quantify gene expression

Gene	Supplier	Assay ID	Cat #
MUC5B	ThermoFisher	Hs00861595_m1	4331182
MUC5AC	ThermoFisher	Hs01365616_m1	4331182
KRT5	ThermoFisher	Hs00361185_m1	4331182
TUBB4A	ThermoFisher	Hs00760066_m1	4331182
SCGB1A1	ThermoFisher	Hs00171092_m1	4331182
IL-33	ThermoFisher	Hs04931857_m1	4331182
IL1RL1	ThermoFisher	Hs00249384_m1	4331182
IL1RAP	ThermoFisher	Hs00895050_m1	4331182
SIGIRR	ThermoFisher	Hs00222347_m1	4331182
COL1A1	ThermoFisher	Hs00164004_m1	4331182
TGF- β 1	ThermoFisher	Hs00998133_m1	4331182
TGF- β r1	ThermoFisher	Hs00610320_m1	4331182
α -SMA	ThermoFisher	Hs00426835_g1	4331182
GAPDH	ThermoFisher	Hs02786624_g1	4331182

Table 2.4 primers, assay ID, catalogue number and supplier of primers used for qPCR

2.15.4 Reverse transcription for PCR array

Examination of a selection of genes was performed using a human fibrosis RT² Profiler PCR Array (SABiosciences, PAHS-120Z) according to manufacturer's instructions. Reverse transcription was performed using all components from the RT² First Strand Kit (Qiagen, cat# 330401). RNA concentration was determined as previously described and 400ng of RNA used in the reaction. Genomic DNA elimination was performed by incubation of samples at 42°C for 5 minutes with genomic DNA elimination mix (buffer GE and RNase free H₂O) after which the samples were immediately placed on ice. 4 µL of 5x buffer BC3, 1 µL of control P2, 2 µL of RE3 reverse transcriptase mix and 3 µL of RNase-free water was added to each reaction tube and this was then incubated for exactly 15 minutes at 42°C then stopped by incubating at 95°C for 5 minutes. RNase free H₂O was added to the desired volume and the RT PCR protocol performed.

2.16 Fibrosis RT-PCR array

The fibrosis array was then performed according to manufacturer's guidance. The reaction mix was of 2x RT2 SYBR Green Mastermix (Qiagen, cat# 330520), 102 µL of cDNA synthesis and 1248 µL of RNase-free water were combined and 25 µL of this PCR reaction mix was added to each of the 96 Wells on the PCR array plate (SABiosciences, cat# PAHS-120Z). The plate was tightly sealed with the optical adhesive film provided with the kit. The plate was then centrifuged for 1 minute at 1000 x g. The settings applied to the qPCR 96 Well fast-block (Applied Biosystems, Viia 7) were 1 cycle at 10 minutes 95°C to activate the HotStart DNA Taq Polymerase, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C to collected the fluorescence data for gene analysis. All gene expression analysis was performed using RT² Profiler PCR Array Data Analysis version 3.5.

2.17 Protein quantification

Protein content was determined by bicinchoninic acid colorimetric (BCA) assay according to manufacturer's protocol. 25µl of samples and a protein standard (BSA at 2 mg/mL in 0.9% saline and 0.05% sodium azide) in 7 serial dilutions were added to a 96 Well plate (Corning, cat #3590) in duplicate. BCA reagent A and BCA reagent B were combined in a ratio of 1:50 and 200 µl added to the samples. After 30 minute incubation at 37°C the plate was allowed to cool to room temperature and the optical density (OD) measured at 562nm wavelength using Sunrise Tecan plate reader (F039300). Analysis was performed on Magellan V5.0 software. The average absorbance of the blank standard was subtracted from the samples and protein standards and unknown protein concentrations interpolated out using a line of best fit from the standard curve on GraphPad Prism V6. A standard curve was generated separately for each experiment.

2.18 General ELISA protocol

Duoset ELISAs were performed according to R&D manufacturer's instructions. In brief, enhanced protein binding 96 Well plates (Corning, cat #3590) were coated with the capture antibody in PBS at room temperature overnight. Plates were washed 3 times with wash buffer (0.05% Tlen in PBS) and then incubated with blocking buffer (1% BSA in PBS) to block non-specific binding. Plates were washed 3x as before. A 7 point curve was generated in duplicate by addition of 50µl of recombinant protein diluted in reagent diluent (0.1% BSA, 0.05% Tlen in PBS) as well as 50 µl of samples. Plates were washed x3 as before after 2 hour incubation and detection antibody in reagent diluent added for a further 2 hour incubation. Bound antibody was detected by addition of streptavidin-HRP(horse radish peroxidase, R&D systems) for 20 minutes at room temperature. Plates were washed as before and TMB (3,3',5,5'-Tetramethylbenzidine) Substrate Reagent (Thermo Fisher Scientific, cat # N301) was added for 15-20 minutes to develop a graded blue appearance and

allow for quantification. The reaction was stopped with stop solution (2N H₂SO₄). Optical density was measured at 450 nm with 540 nm as a reference wavelength using Sunrise remote Tecan reader (F039300) with analysis on Magellan V5.0 software.

For BAL fluid analysis, protein concentration was determined by BCA assay (as described above) and ELISA values expressed per µg protein.

Table 2.5 DuoSet ELISA details

ELISA	Cat#	Supplier
soluble ST2	DY523B-05	R&D systems
IL-33	DY3625-05	R&D systems
IL-8	DY208-05	R&D systems
IL-6	DY206-05	R&D systems
MCP-1	DY279	R&D systems

Table 2.5 ELISA antibody, catalogue number and supplier

2.18 Flow cytometry

2.18.1 Extracellular staining protocol for flow cytometry

BAL cells were plated in a round bottomed 96 Well plate at a concentration between 2-5x10⁶ cells/Well in 100-200µl PBS. The plate was spun for 4 minutes at 500 x g at 4°C (Hettich Centrifuge, Rotina 420R). The supernatant was removed and cells were re-suspended 1:1000 live/dead stain (Zombie UV, Biolegend, cat# 423107) and incubated in the dark for 20 minutes at 4°C. The plate was spun as above and the supernatant removed. Cells were then incubated with Fc Block (Biolegend, cat# 422301) in filtered FACS buffer at a ratio of 1:100 for 10 minutes prior to staining to prevent non-specific antibody binding. The plate was spun, as above, and the supernatant discarded and the cells then washed in 200 µl of filtered FACS buffer. Cells were then re-suspended in 50 µl

fluorescently conjugated antibodies (table 2.6) in filtered FACS buffer for 30 minutes at 4°C, washed x3 as above and fixed for 10 minutes IC fix (ebioscience, cat# 00-8222-49) at 4°C, in the dark. They were then spun and resuspended in FACS buffer. The plate was then kept in refrigerator in the dark and Flow cytometry was performed within 24 hours using 5 laser BD LSRFortessa™ and analysed using Flowjo7 software (Treestar US).

Table 2.6 FACS antibodies and details

Molecule	Manufacturer	Conjugated dye	Dilution	Clone
Live/dead	Biolegend	Zombie/UV	1:1000	Dye
CD45	Biolegend	PeCY5	1:50	HI30
CD11c	Biolegend	PeCY5	1:50	3.9
CD206	Biolegend	AF 647	1:50	15-2
CD163	Biolegend	BV421	1:50	GHI/61
CD86	Biolegend	BV605	1:50	IT2.2
HLA-DR	Biolegend	APC/Cy7	1:50	L243

Table 2.6 antibodies, fluorescent conjugate, dilution, manufacture and clone used for BAL cell phenotyping by flow cytometry

Figure 2.1

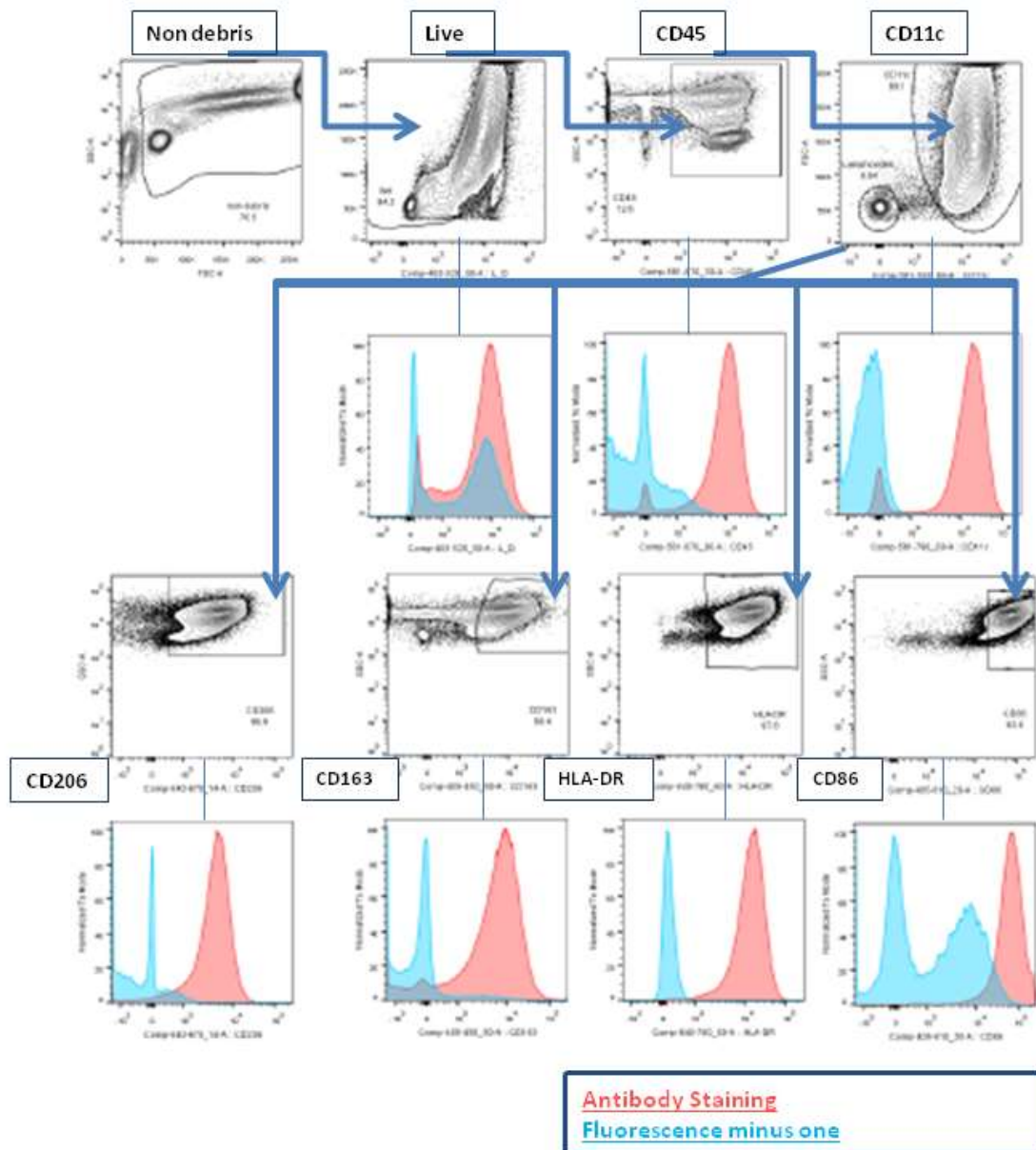


Figure 2.1 FACS gating strategy. Schematic of gating strategy for flow cytometry of BAL cells. Figure demonstrates gating of macrophages and lymphocytes from population of live, non debris, CD45^{Hi} cells, with histograms showing antibody stained sample and sample fluorescence minus one control sample.

2.18.2 Flow cytometry set up and sample acquisition

Compensation beads were used to set up single colour compensation. Compensation was calculated automatically using Fortessa software. To ensure the accuracy of the gating, fluorescence minus one (FMOs) were used - for each antibody the stain was examined with all of the fluorescent antibodies in the panel minus each antibody individually. Samples were stained and analysed in polypropylene coated plates to minimise the adherence of cells to plates.

2.19 Western Blot

Protein was quantified using BCA protein assay kit and sample volumes are adjusted to normalise protein. Pierce 4X LDS sample buffer (Thermo Fisher Scientific, cat# 84788) including reducing agent B-mercaptoethanol was added to samples and the volume adjusted to 15ul or 45 ul with demineralised water, depending on size of pre-cast gel. Samples were then heated to 95°C for 5 minutes to denature the proteins. Samples were cooled and transferred to pre-cast gels (Bio-Rad, criterion XT pre-cast gels 10% Bis-Tris cat# 3450123) with 5ul of protein ladder (Precision plus protein dual color standard, Bio-Rad cat# 1610374). Novex Bolt 20X running buffer(Thermo Fisher Scientific, cat# BT00061) was diluted 20 fold in demineralised H₂O added to the tank and gel was run at 120V until the ladder arrived at the end of the gel. A PVDF membrane was cut to the size of the gel, activated using methanol and transferred onto the gel.

Proteins were transferred to the membrane using Pierce Polr Blotter(Thermo Fisher Scientific) and this was then incubated in block buffer (PBS with 5% non-fat dry milk and 0.05% Tlen) at room temperature on a roller for 1 hour. The membrane was washed in wash buffer (PBS with 0.05% Tlen) prior to addition of antibodies at working concentration in block buffer overnight at 4°C. The membrane was then washed in wash buffer and incubated with appropriate secondary antibodies for 1 hour at room temperature. The membranes were then washed in wash buffer and developed

in ECL plus Western blotting detection system prior to imaging on the myECL Imager (Thermo Fisher Scientific)

Table 2.7

Primary antibody				HRP linked secondary antibody		
<i>Protein</i>	<i>Clone</i>	<i>Cat #</i>	<i>Supplier</i>	<i>Species</i>	<i>Cat #</i>	<i>Supplier</i>
IL-33	Polyclonal Goat IgG		R&D	Anti rat IgG	7077	Cell signalling technology
IL-33	Nessy-1 - monoclonal mouse IgG		Enzo Life Sciences	Anti mouse IgG	7076	Cell signalling technology

Table 2.7 Antibodies for Western blot. Primary and secondary antibodies used for Western blot experiments, the clone, category number and suppliers

2.20.1 Mass spectrometry

Gel sample was processed in the sterile fume hood to avoid contamination. 4x LDS sample buffer including reducing agent B-mercaptoethanol was added to samples and adjusted to a volume of 45 µl with demineralised water, to fit the pre-cast gel Well. Samples were then heated to 95°C for 5 minutes to denature the proteins. Samples were cooled and transferred to pre-cast gels (bio-rad, criterion XT pre-cast gels 10% Bis-Tris, cat# 3450123) with 5ul of protein ladder (precision plus standards). 1X running buffer was added to the tank and gel was run at 120V until the ladder arrived at the end of the gel.

Peptides were extracted from the gel pieces by a series of acetonitrile and aqueous washes. The extract was pooled with the initial supernatant and lyophilised. Each sample was then resuspended in 10mL of 50mM ammonium bicarbonate and analysed by LC/MS/MS. Chromatographic separations were performed using an EASY NanoLC system (ThermoFisherScientific, UK). Peptides were resolved by reversed phase chromatography on a 75 mm C18 column using a three step linear

gradient of acetonitrile in 0.1% formic acid. The gradient was delivered to elute the peptides at a flow rate of 300 nL/min over 60 min. The eluate was ionised by electrospray ionisation using an Orbitrap Velos Pro (Thermo Fisher Scientific) operating under Xcalibur v2.2. The instrument was programmed to acquire in automated data-dependent switching mode, selecting precursor ions based on their intensity for sequencing by collision-induced fragmentation using a Top20 CID method. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the mass-to-charge ratio (m/z) and the charge state of the peptide.

2.20.1 Database Searching

Raw mass spectrometry data were processed into peak list files using Proteome Discoverer (Thermo Fisher Scientific; v1.4).

Processed raw data was searched using the Mascot search algorithm (www.matrixscience.com) against All Taxonomy in the Uniprot database. The data was specifically filtered for the Human species within the All Taxonomy output after searching

<https://www2.chemistry.msu.edu/faculty/reusch/virttxtjml/spectrpy/massspec/masspec1.htm>

This was performed by the Steven Lynham, at the Proteomics Department, Kings College Hospital.

2.21 Real time cell imaging

Proliferating cells were imaged over time using a real time cell imaging system; Incucyte Zoom Live-Cell analysis system (Essen Bioscience) allowing capture of multiple images across different time intervals, generation of time-lapse movies and cell proliferation quantified by analysis of the

occupied area of cell images over time. Quantification was performed using Incucyte analysis software (Essen Bioscience).

2.22 Cell Proliferation

Epithelial cells were seeded into 96 well plate at a density of 5000 cells/Well and left to adhere for 1 hour. Plating media was then aspirated and replaced with 200 µl stimulation media and the plates placed in real time cell imaging systems. Cells were left for 44 hours with regular imaging of a pre-determined area of each Well. Each image was then quantified using the imaging system compatible analysis software, and growth curves generated from the confluence data of each image over time.

2.23 Wound Healing assay

Cells were seeded into 96 Well plates at a density of 5000 cells/Well in triplicate and left to grow to confluence over 3-5 days. A wound was then made in the centre of the Well using a 96 pin woundmaking tool - a 96-pin mechanical device designed to create homogeneous, 700-800 micron wide scratch wounds in cell monolayers on IncuCyte® ImageLock 96-Well microplates (Essen Bioscience). Wells were all washed 2x in PBS and then 200µl stimulation mix was added and cells incubated at 37°C, with imaging of pre-determined areas of each Well at regular time points using real time live cell imaging technology (Essen Bioscience). Each image was then quantified using the imaging system compatible analysis software, and wound confluence data over time generated.

2.24 Sircol assay

Sircol is a dye binding method which measures acid-soluble and pepsin-soluble collagens. Cell supernatants were added in duplicate to Sircol dye reagent (sirius red in picric acid) to saturate the

collagen within the sample. A standard curve was generated in duplicate using 7 serial dilutions of a collagen standard 1000µg/ml . They were then inverted gently for 30 minutes to form a collagen-dye complex, centrifuged for 10 minutes at 11269 x g and the unbound soluble complex aspirated off. The complex was then re-suspended in 750µl of Acid Salt wash, centrifuged for 10 minutes as above and the unbound fraction carefully removed. The precipitate was dissolved in Alkali Reagent and transferred to a 96 Well flat bottomed plate and Optical density (OD) was measured at 555nm.

2.25 MMP9 zymography

Bioactive MMP9 was analysed by measurement of the proteolytic digestion of gelatin embedded in a polyacrylamide gel. The samples were diluted in non-reducing sample buffer, and 25µl of sample was added to each lane of a precast gel (Invitrogen). All samples were electrophoresed at 45 V for 5 h at 4°C. Following electrophoresis, gels were washed in 2.5% Triton X-100 for 30 minutes at 25°C, and then incubated in 50 mM Tris and 5.0 mM CaCl (pH = 8.0) for 16 h at 37°C. Gels were stained in 0.05% Coomassie blue for 30 min and subsequently de-stained in acetic acid and methanol for optimal exposure. Regions of protease activity appear as clear bands against a dark blue background where the protease has digested the substrate.

Higher molecular weight bands on gelatin zymograms likely represented MMP-9/NGAL complexes

2.26.1 TGF-β secreted embryonic alkaline phosphatase (SEAP) reporter assay principle

The chemiluminescent substrate CSPD is dephosphorylated by alkaline phosphatase (AP), resulting in an unstable dioxetane anion that decomposes and emits light. The light emission has maximal activity at wavelength 477nm. The light signal is proportional to the concentration of alkaline phosphatase.

Secreted alkaline phosphatase (SEAP) gene is cloned into vectors behind the promoter under investigation, then transfected into eukaryotic cells. The translated SEAP gene product is then secreted from cells

Embryonic fibroblasts from *Tgfb1*^{-/-} mice were stably transfected with a reporter plasmid consisting of TGF-β responsive Smad-binding elements coupled to a secreted alkaline phosphatase reporter gene (SBE-SEAP). The cells were treated with BAL fluid supernatant and bioactivity measured according to manufacturer's instructions (Clontech)

2.26.2 TGF-β reporter assay

MFB-F11 cells were cultured in D10 medium (containing 15μg/ml hygromycin B) and then plated into a flat bottomed 96 Well plate at a density of 4x10⁴ cells/Well.

50μl of BAL samples were then added to duplicate Wells and incubated for 24 hours. A 7 point standard curve was generated in duplicate using serial dilutions of recombinant TGF-β1.

10 μl of medium was transferred to white luminometer plates and incubated for 30 minutes at 65⁰C prior to addition of substrate solution. Unknown concentrations were extrapolated from a standard curve.

2.27 Dextran Perfusion assay

The permeability of cultured HBEC monolayers *in vitro* was investigated by dextran perfusion assay.

HBEs were seeded into collagen coated 0.4μm transWell inserts (corning) at a density of 10,000 cells/insert and grown to confluence over 5-7 days - non confluent chambers were discarded.

Inserts were placed in 12 well plates with 250μl BEGM in the inner transwell chamber and 1ml BEGM in the outer chamber of the Well. 50μl Dextran fluorescein isothiocyanate (FITC) (sigma) was added to

each tranwell insert inner chamber and incubated for 3 hours at 37°C with 25µl removed from the outer chamber of the 12 Well plate (Corning) at pre-determined time points. Optical density (OD) of the diffused solute was measured at 520nm wavelength using Sunrise Tecan plate reader.

2.28 Trans epithelial electrical resistance measurement

Transepithelial electrical resistance (TEER) is the measurement of electrical resistance across a cellular monolayer and is a method to used confirm the integrity and permeability of a confluent cell monolayer. TEER is a widely accepted quantitative functional measure of the integrity of tight junctions, basic on measuring the trans epithelial impedance of barrier forming cells. Trans epithelial electrical resistance of cultured IPF and control HBECs was measured using the The RTCA iCELLigence™ (ACEA biosciences). The RTCA iCELLigence™ instrument uses noninvasive electrical impedance monitoring to quantify cell proliferation, morphology change, and attachment quality in a real-time manner. HBEs were seeded in triplicate for each stimulation condition into 8 Well plates with micro-electrodes allowing real time measurement of impedance across the electrodes (ACEA Biosciences) and grown to confluence at 37°C. When electrical impedance reached a plateau and cells were confirmed to be confluent by direct visualisation by microscopy, media was removed and replaced with stimulation media. Electrical impedance was measured at regular timepoints over 24 hours and data analysed using the RTCA iCELLigence Software.

2.29 Cell stimulations

Fibroblasts were seeded into 12 Well plates (Corning) at a density of $1-2 \times 10^6$ and grown to 90% confluence at 37°C. Once 90% confluence was confirmed by direct microscopy, cell media was then replaced with serum free media overnight prior to media with stimulation 1.5 ml of stimulation

media (complete with recombinant proteins). Media was then removed at pre-determined timepoints and stored at -80°C for future analysis.

Figure 2.2 Schemata of HBEC conditioned supernatant fibroblast stimulation

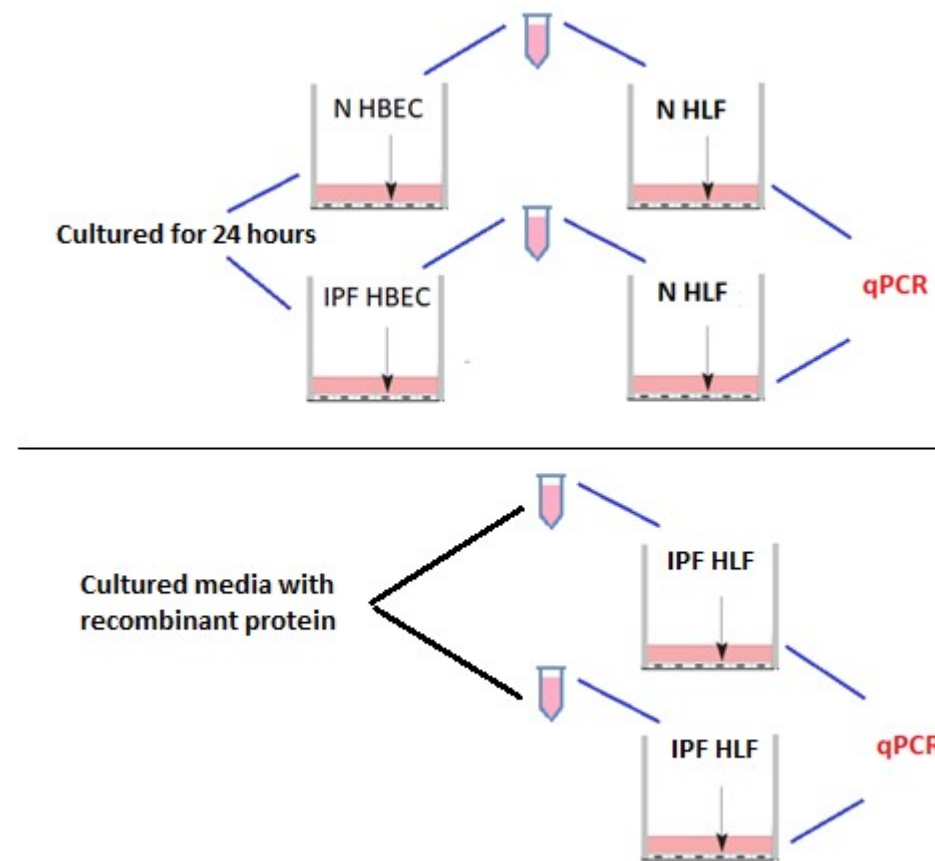


Figure 2.2 Representative picture of process of stimulating normal human lung fibroblasts (NHLF) with supernatant conditioned by normal and IPF HBEC (human bronchial epithelial cells) for 24 hours, and stimulation of IPF HLF with recombinant protein.

2.30 Data analysis

Gaussian distribution was examined by D'Agostino Pearson omnibus normality test. Statistical significance was examined using Mann Whitney U test for unpaired non-parametric comparisons.

For unpaired parametric data, unpaired t-test was used. For multiple comparisons of non-parametric data a one way ANOVA test was performed with Dunn's multiple comparison test.

Correlations were performed using 2 tailed Spearman rank correlation.

Lung tissue histology was analysed using Aperio ImageScope technology - the software was used to determine the number of positive pixels per selected tissue or airway area. Analysis of parenchymal tissue was performed by defining the mean number of positive pixels of staining in 3 randomly selected areas per tissue section. Airway protein expression was analysed as a mean of the number of positive pixels detected in 3 measured airways per tissue section, and expressed per μm of measured airway circumference to control for length of airway measured.

Statistical differences required confidence level of 95%, a probability value (p value) of less than 0.05. Significant differences are denoted using asterisks; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and calculated using GraphPad Prism V6. All statistical analysis was carried out using GraphPad Prism 6 software.

Chapter 3.

**An investigation into the immunophenotype of airway
macrophages in interstitial lung disease.**

3.1 Introduction

This chapter describes the immunophenotype of airway macrophages from individuals with a variety of different interstitial lung diseases compared with healthy aged matched controls.

Interstitial lung disease is the name given to a broad and diverse group of diseases that vary in aetiology and clinical course. They exhibit a wide spectrum of pathophysiologies encompassing inflammation resulting from inhaled pathogens (such as chronic HP), to vascular inflammation such (as granulomatosis with polyangiitis), and purely fibrotic non inflammatory wound healing over-exuberance which is believed to be the case in IPF - the most prevalent interstitial lung disease. AMs act as sentinels which patrol the alveolar space and are critical to the maintenance of pulmonary homeostasis. Their ability to acquire different phenotypes depending on their surrounding environment, allows them to act as a bridge between the innate and acquired immune systems, acting to both initiate and limit inflammation as well as performing their suggested critical role in directing wound healing responses. Macrophages have hitherto been classified as M1 (pro-inflammatory) or M2 (pro-fibrotic) (Gordon et al, 2003). Historically, studies have suggested that pro-inflammatory cytokines, produced by AMs during aberrant wound-healing, contribute to fibrotic processes in the lung (Byrne et al. 2015) and that a M2 contribute to the fibrotic process by a number of processes, including effects on matrix metalloproteinases (Dancer et al. 2011) and collagen uptake . (Atabai, Jame et al. 2009) with a skew towards M2 found in the IPF lung (Gibbons et al. 2011). This data suggests that pulmonary macrophages are intricately involved at each of the key stages of the fibrotic process but their relation to clinical phenotypes has not yet been established.

3.1.1 Hypothesis

Airway macrophages from individuals with fibrosing lung disease exhibit distinct activation states compared to healthy controls and skew towards an M2 phenotype.

3.1.2 Aims

- 1) To characterise the activation status of airway macrophages within the M2/M2 paradigm in individuals with ILD using cell surface phenotyping markers by multicolour flow cytometry
- 2) To analyse the relationship between expression of airway macrophage M1/M2 markers in ILD to disease severity and disease progression

3.1.3 Methods

3.1.4 Study subjects

BAL was collected from a total of 90 patients, with a range of ILDs, who were undergoing diagnostic bronchoalveolar lavage as part of routine clinical protocol and compared with 5 recruited healthy volunteers. This was a pilot study and therefore no power calculation was performed.

After routine clinically indicated bronchoscopy and lavage of 90 individuals with ILD, 45mls of BAL fluid was sent for clinical analysis and the volume of remaining BAL fluid was taken for this study. 5 healthy control volunteers were recruited with the full yield of BAL fluid (performed under the same conditions as above) taken for analysis. Immunophenotype was determined using flow cytometry to detect cell surface markers. The level of cell surface marker expression was correlated with clinical parameters and compared against healthy controls.

Lung function measurements (forced vital capacity and transfer factor of carbon monoxide) were used as surrogate markers of disease severity and progression.

Diagnosis was made according to ATS/ERS consensus classification criteria (2002), by an experienced ILD physician, with input from a specialist ILD MDT, including a thoracic radiologist and thoracic histopathologist.

Patients were stratified according to diagnosis based on a combination of disease history and radiological and histopathological factors and separated into disease groups. For the purposes of analysis individuals were then stratified into 3 groups - IPF, non-IPF ILD and non-IPF ILD with no radiological evidence of established fibrosis. IPF is a disease predominantly of fibrosis in the absence of conventional inflammation. The common non-IPF ILDs in our dataset (chronic HP, sarcoid, drug induced ILDs) characteristically result from chronic inflammation which can progress to fibrosis and therefore this group had radiological evidence of fibrosis as well as pathogenetic inflammatory involvement. The final group did not have any radiological evidence of fibrosis, as evidenced by the absence of reticular shadowing, traction bronchiectasis and honeycombing on HRCT. This allowed a comparison across the spectrum of disease from fibrotic to fibro-inflammatory to non-fibrotic.

3.1.5 Clinical Information and patient demographics

As shown in table 3.1, there was a preponderance of males and ex-smokers -particularly amongst the IPF cohort- and a wide range of diagnoses. There was a higher number of male controls and control group were significantly younger than the disease groups due to the difficulty – in practice – of recruiting age matched healthy volunteers. The IPF cohort was significantly ($p=0.038$) older than the non-IPF cohort but the groups did not exhibit significant differences in lung function parameters at baseline.

3.1.5.1 Lung function parameters

As fibrosis in the lung advances there is a manifest reduction of transfer factor of the lung for carbon monoxide (TLCO) as well as a continuing reduction in dynamic lung volumes as ventilation becomes more restricted by increasing burden of scar tissue. Disease progression is clinically monitored by serial lung function measurements over time with TLCO being used as a marker of severity and TLCO as well as lung capacity - measured by forced vital capacity (FVC)- used as a surrogate marker of stability or decline. A TLCO of <40% of predicted is indicative of advanced disease and a fall of $\geq 10\%$ in FVC or a fall of $\geq 15\%$ in TLCO in the first 6-12 months identifies patients with a higher mortality. In this group of patients, change in FVC (Schwartz et al, 1994) has emerged as the lung function measurement most predictive of mortality. TLCO decline has also been found to have a mortality predictive value (Schwartz et al, 1994), however due to the difficulties of standardisation of this measurement, these findings can be more variable and thus the need for a greater % decline before a change is deemed to be significant (Bradley et al, 2008). In practice this means that follow up of patients with IPF centres around measurement of lung function at minimum 6 monthly intervals, with disease progression largely defined by lung function trend over time. For the significant subset of individuals with IPF who undergo bronchoscopy, the BAL findings are used to confirm diagnosis with some studies suggesting higher eosinophil counts to be indicative of a more aggressive clinical course (Boonars et al, 1995).

Functional decline during follow-up was assessed by serial lung function tests, with lung function assessed between a period of up to 6 months pre bronchoscopy to up to 18 months post bronchoscopy. Mortality was examined at up to 18 months post bronchoscopy.

Table 3.1

	IPF	UCTD	HP	Sarcoid	Other	Control
n	41	11	13	6	19	5
Age	70 (66-75)	69 (60-74)	69 (58.5-73)	47 (40.5-56)	62 (53-68.5)	49 (29-64)
% male	68.3	27.3	38.5	50	52.6	80
Smoking status	ex=21, ns=14, u=6	ex=5, ns=5, u=1	ex=10, n=3	ex=1, ns=2, u=3	ex=7, ns=8, c=2, u=2	c=2, u=2
<i>Pulmonary function</i>						
FEV1 % predicted	79.3 (72.2-95)	76.2 (70.5-92.2)	76 (59-100.5)	70.55 (67.5-83.9)	63 (55.3-83.9)	86 (82-112)
FVC % predicted	73 (61.3-80.7)	83.2 (69.7-87.4)	71.2 (73.4-91.7)	79 (73.4-91.7)	79 (65.2-102.1)	97 (89-115.5)
TLCO % predicted	40 (31.8-53)	38 (30.5-64.6)	33.9 (30.2-50.8)	40.1 (31.7-75)	51.2 (37.9-62.9)	

Table 3.1 Demographic and clinical data in the study population; age, gender, smoking status and lung function parameters at time of bronchoscopy of study population, median and interquartile ranges in parentheses. For some study subjects and all controls, full lung function data was not performed with only spirometric data available.

Numbers shown as median and interquartile range (IQR)

FEV1 = forced expiratory volume in 1 second, FVC = forced vital capacity, TLCO = transfer factor of the lung for carbon monoxide, ns= never smoker, u=unknown, c=current

3.1.6 Collection of BAL samples

Bronchoalveolar lavage is widely accepted as a key diagnostic tool in interstitial lung disease and as such is performed in patients with a variety of different ILDs. Many factors can influence the amount of fluid and/or the cellular composition of the lavage fluid returned with, predominant amongst them, the underlying disease process/co-existing pathologies and whether or not they lead to airway obstruction. Airway obstruction leads to the mechanical problem of aspirating fluid through narrowed airways and can adversely influence the return of BAL fluid. BAL was obtained at clinically indicated bronchoscopy in the work up of individuals with a range of ILDs - consequently a

40 ml volume of the BAL returned at the procedure was taken for the cell differential count, 5 mls for microbiological culture and the volume over and above 45mls was available to be collected for research purposes.

All patients and control subjects provided written, informed consent to participate in the study, which was approved by a Royal Brompton Hospital ethics committee (10/HO720/12).

Bronchoscopies were performed under a light sedation with midazolam in combination with local anaesthesia with lidocaine. Four 60 mL aliquots of warmed sterile saline were instilled in to the right middle lung lobe and aspirated by syringe and lavage aliquots collected after each instillation were pooled for each patient. Volume and BAL appearance was recorded for all samples.

3.1.7 BAL processing

BAL samples were processed and stained on the day of sample collection. Whole BAL was strained through a 70 μ M sterile strainer and subsequently centrifuged (700 X g, 5 min, 4°C), and pellets subjected to red blood cell lysis (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.3) for 10 minutes before washing and resuspension in complete media (RPMI + 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin). Cells were counted blind to diagnosis and pelleted onto glass slides by cytocentrifugation (5 ×10⁴ cells/slide). Differential cell counts were performed on Wright-Giemsa–stained cytospins. Percentages of eosinophils, lymphocyte/mononuclear cells, neutrophils, and macrophages were determined from a total of 400 cells. To obtain absolute numbers of each leukocyte subtype, these percentages were multiplied by the total number of cells obtained in the lavage fluid.

3.1.8 Flow cytometry

Cells were washed and incubated with near-IR fixable live/dead stain (life technologies inc.), as per the manufacturer's instructions. Cells were washed prior to incubation with human Fc Block (BD pharmingen, inc.) and surface staining was performed with the following antibodies (clones in brackets) purchased from Biolegend, inc.; CD45 (HI30) CD11c (3.9), CD163 (GHI/61), CD206 (15-2), CD86 (IT2.2), HLA-DR (L243) (table 3.3). Surface staining was followed by fixation. Labelled cells were acquired on a BD fluorescence-activated cell sorting LSR Fortessa II (BD Bioscience) and further analysed by using FlowJo (Treestar).

Gating was performed on a number of parameters to isolate the population of airway macrophages from the analysed BAL cell pellet and allow quantification of marker expression on these cells only (gating strategy, figure 3.1).

BAL was analysed by forward scatter and side scatter to organise by size and granularity. All events registering lower FSC and SSC than lymphocytes were excluded as debris, as lymphocytes are the smallest cells present in BAL (figure 3.2). Dead cells were excluded from analysis by binding to an amine binding dye, and leucocytes were identified by high expression of CD45 (CD45^{hi}). Our study was primarily focused on looking at airway macrophage phenotypes which were gated by expression of CD45, a pan leucocyte marker and CD11c, a transmembrane protein abundantly expressed on monocytes and macrophages. Macrophages were gated on the basis of CD11c^{hi} expression, allowing exclusion of CD11c^{lo} monocytes and neutrophils. Data on lymphocytes was gathered by gating on the population of CD45^{hi} cells which were identifiable by FSC/SSC parameters. Airway macrophages were gated by high expression of CD45 and CD11c (CD45^{hi} CD11c^{hi}) and lymphocytes by FSC/SSC and CD45 expression (CD45^{hi}) (figure 3.1).

3.1.8.1 Flow cytometry surface marker choice

CD45, alternatively known as leucocyte common antigen, is a transmembrane glycoprotein which is expressed on all haematopoietic stem cells bar mature erythrocytes. Its expression remains stable in mature cells and is widely used as a leucocyte marker. CD11c is an active complement receptor, a type 1 transmembrane protein which is expressed on monocytes, granulocytes dendritic cells and macrophages. It is expressed at high levels on airway macrophages in both mouse and human lung and expressed at lower level on granulocytes and monocytes as previously demonstrated, allowing separation of these cell by gating on high CD11c expression, as previously described (Gordon and Taylor 2005, Misharin, Morales-Nebreda et al. 2013)

Multiple markers are used to denote M1 and M2 in a variety of human and mouse studies and some of these markers vary between mouse and human cells, with, for example no human homologs of mouse markers Ym1 Fizz1 and arginase-1. Given the wide variety of putative M1 and M2 markers, I chose to investigate these populations by examining two reputed M1 and two reputed M2 markers and comparing across the expression of all markers.

The surface markers CD86 and HLA-DR were used to identify M1 macrophages markers and CD206 and CD163 to identify M2 macrophages. These markers have been derived predominantly from monocyte polarised macrophages with the markers used in this study are widely published in a multiplicity of studies in human lung macrophages (Bowden et al, 1983. Ma et al. 2010, Gibbons et al. 2011, Kaku et al. 2014, Bazzan et al. 2017, De Souza et al. 2017, Dewhurst et al. 2017, Raggi et al. 2017)

I sought to ensure the robustness of our analysis by using two separate markers to denote M1 and 2 separate markers to denote M2.

CD86 and HLA-DR were used to denote M1, with M2 phenotyped by expression of CD163 and CD206.

CD86 is a membrane protein expressed on the surface of antigen presenting cells and is one of the two co-stimulatory molecules which bind to CD28 and activate T-cells. Macrophages represent a large proportion of the antigen presenting cells (APCs) which express CD86 and it is widely published as a marker of M1.

The second marker I used for M1 was HLA-DR- an MHC class II surface receptor, the primary function of which is in antigen presenting to T-helper cells and is also widely present on APCs, with expression found on 'inflammatory' M1.

CD206 also known as the mannose receptor, is a transmembrane glycoprotein which binds and internalises glycoproteins and collagen ligands. It is highly expressed on several types of tissue resident macrophages and is seen highly expressed on anti-inflammatory M2 type macrophages.

CD163 is a scavenger receptor which acts as a receptor for haemoglobin-haptoglobin complexes as and has been suggested to play a role in the resolution of inflammation which is highly expressed on M2

Recent work in 2017 validated the use of CD206 to discriminate between monocyte derived M1 and M2 with more equivocal findings on the utility of CD163 as a discriminator, with the same study validating the use of CD86 in M1 identification (Bertani et al. 2017).

Table 3.3

Molecule	Conjugated dye	Function	Clone/Dye	Source
Live/dead stain	Zombie aqua	dead cell marker	Cat# 423101	Biolegend
CD45	PeCy5	Pan leucocyte marker	HI30	Biolegend
CD11c	Pe-Cy7	Pan macrophage marker	3.9	Biolegend
HLA-DR	APC-Cy7	M1 marker	L243	Biolegend
CD 86	BV 605	M1 marker	IT2.2	Biolegend
CD206	AF 488	M2 marker	15-2	Biolegend
CD163	AF 647	M2 marker	GHI/61	Biolegend

Table 3.3 FACS antibodies; molecule, conjugated dye, clone and source of FACS antibodies used in flow cytometry experiments

Flow cytometry data was collected across the study cohort (table 3.1) but reliable data was not able to be collected on all study subjects due to antibody availability and early optimisation of staining in some flow cytometry colour channels. Details of the number of subjects in whom data was collected for each parameter is shown in table 3.2.

Table 3.2

n numbers	IPF	UCTD	HP	Sarcoid	Other	Control
Live cells	41	11	13	6	19	5
CD45	41	11	13	6	19	5
Lymphocytes	41	11	13	6	19	5
CD11c	41	11	13	6	19	5
CD163	25	7	9	5	15	5
CD206	41	11	13	6	19	5
CD86	25	8	12	4	18	5
HLA-DR	31	11	12	6	17	5

Table 3.2 Data on individual staining parameters; numbers of subjects with available data for each parameter of interest

Figure 3.1

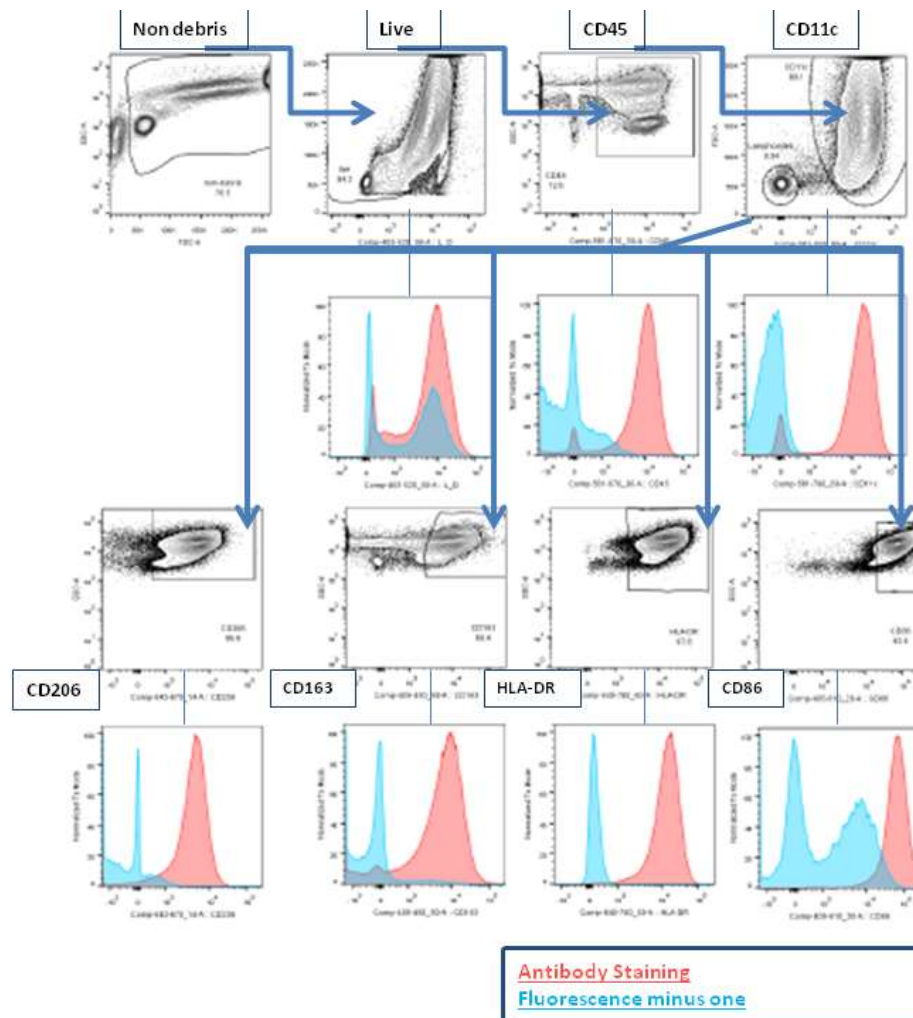


Figure 3.1 Gating strategy for airway macrophages by flow cytometric analysis, with FMOs shown

Prepared BAL cells were incubated with antibodies for CD45, CD11c, CD206, CD 163, HLA-DR, CD86 and an amine binding live/dead stain. Live/CD45^{hi}/CD11c^{hi} cells were determined as above. This cell population was gated to determine expression of CD206, CD163, HLA-Dr and CD86 as shown above. Representative FACS plots shown. As the smallest cells identifiable in BAL, lymphocytes were identified by size using the forward scatter and side scatter parameters of flow cytometric analysis to gate on size and granularity of CD45 positive cells (figure 3.2), as previously published (Nicholson, 1996).

Figure 3.2

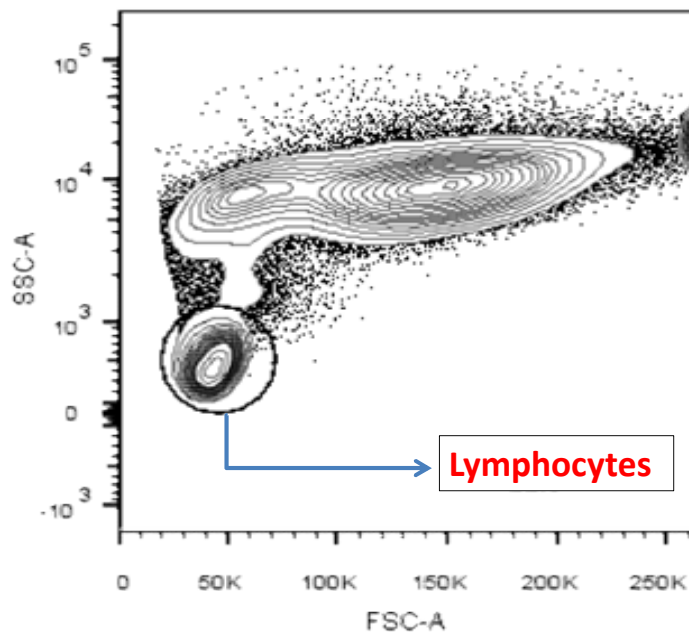


Figure 3.2 Gating strategy for lymphocytes by flow cytometric analysis ;representative FACS plot image of gating strategy used to identify lymphocytes from BAL based on FSC/SSC properties of non-debris/live/CD45^{HI} cells. This population was subsequently confirmed to be a population of pure lymphocytes by CD45 and CD3 positivity.

3.1.9 Statistics

Differences between non-continuous groups were compared using a Mann-Whitney's U test. To explore associations between pairs of continuous variables, Spearman's rank correlation was used. Analysis was performed using GraphPad Prism V6 software. All tests were 2-tailed, and $P < 0.05$ was considered to be statistically significant.

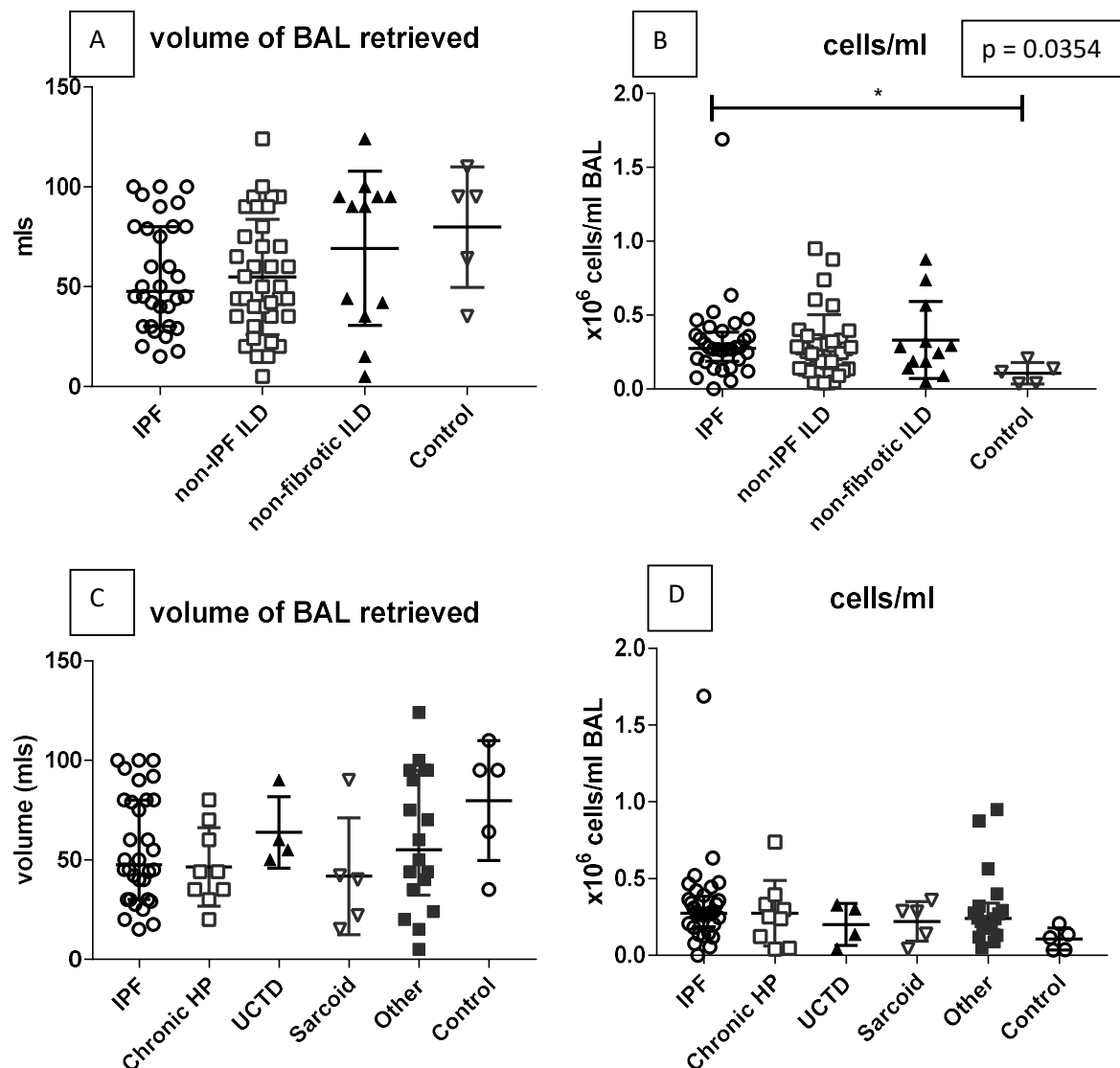
3.2 Results

3.2.1 Characterisation of BAL yields and cell counts in ILD and control

I obtained BAL fluid from a group of individuals presenting with a spectrum of different ILD diagnoses and stratified them to specific ILDs as well as comparing between groups which showed radiological evidence of fibrosis vs those with no established fibrosis on CT.

The volume of excess discard BAL retrieved and collected for analysis was measured and total cell numbers were counted manually - post red blood cell lysis- and shown per ml of BAL fluid obtained. This analysis (figure 3.3) revealed there to be no statistically significant difference between the volume of BAL volume yielded between ILD groups or healthy control but did show that there was a statistically significant increase in the absolute number of cells retrieved from BAL of individuals with IPF when compared to control($p=0.0354$). No difference was detected between IPF and non-IPF ILDs or when stratified to separate non-IPF ILD diagnoses.

Figure 3.3



HP= hypersensitivity pneumonitis, UCTD= undifferentiated connective tissue disease

Figure 3.3 BAL fluid yields and cells counts across spectrum of ILD and healthy control; Volume of BAL retrieved was measured, minus the volume sent for clinical evaluation. Cell counts were determined and expressed per ml of BAL. These data did not demonstrate any statistically significant difference when comparing means of volume of BAL in IPF(n=32) vs non-IPF ILD(n=37), non-fibrotic ILD(n=12) and control(n=5) **A)** number of cells/ml comparing ILDs with disease groups stratified to IPF vs non-IPF ILD, non-fibrotic ILD and control **B)** number of cells/ml in IPF(n=32) stratified to specific ILD diagnoses; chronic HP(n=9), UCTD(n=4), sarcoid(n=5), other(n=18), control (n=5) **C)** number of cells/ml comparing between ILDs stratified to specific

ILD diagnoses **D**). Data presented as median +/- interquartile range, 1 way ANOVA with Dunn's multiple comparisons

These data suggest the alveolar space in the fibrotic lung to be a cell rich environment when compared with healthy control, but little has traditionally been known about how individual cellular components found in the alveolar space impact upon prognosis and disease progression.

3.2.2 Flow cytometric analysis of BAL cells in ILD and control

The characterisation of the BAL samples included in this study was begun by identifying and excluding dead cells from our analysis, via detection of their conjugation to an amine binding dye. No difference was found in the percentage of live cells from BAL of individuals with IPF vs non-IPF ILDs, non-fibrotic ILDS or control, or across different ILD diagnoses (figure 3.4), showing that our analyses were not skewed by difference in the viability of the cells across our different comparison groups.

Figure 3.4

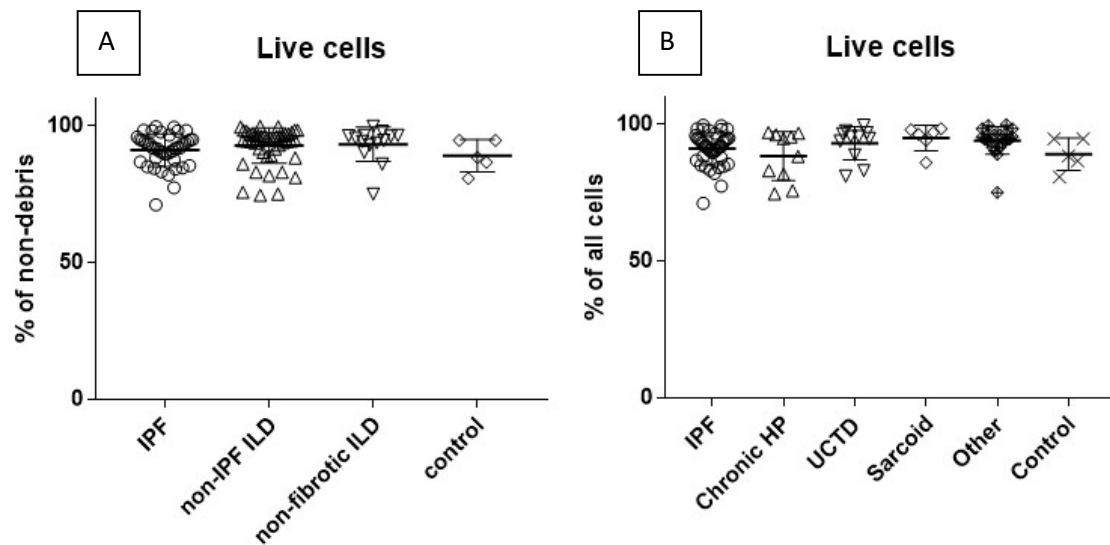


Figure 3.4 Percentage of live cells from BAL in ILD vs healthy control measured by flow cytometry; % of live cells comparing IPF(n=41) vs non-IPF ILD(n=51), non-fibrotic ILD(n=14) and control(n=5) A) % of live cells comparing between specific ILDs; IPF(n=41), HP(n=11), UCTD (n=12), sarcoid (n=6), other (n=20) and control (n=5) B). Results showed no difference in the number of live viable cells which were obtained from lavage and survived to analysis between the different groups.

Values expressed as median and interquartile range

The percentage of live CD45^{Hi} cells from BAL showed no statistically significant difference between IPF and non-IPF ILD, non-fibrotic ILD or control. The majority of the cells retrieved from bronchoalveolar lavage were found to be leucocytes and the population was equivalent across the studied groups, with no differences detected between individual ILDs or between examined ILD groups and healthy control (figure 3.5). Within the CD45^{Hi} leucocyte population I sought to further characterise the lymphocyte and macrophage populations. When comparing IPF vs specific ILD diagnoses there was found to be a significant difference between the medians of the groups, but no significant differences between the individual groups tested. Lymphocytes were found in BAL in IPF,

non-IPF ILD and healthy control, with no differences detected in the proportions across the comparison groups.

Macrophage populations were identified as live, CD45⁺, SSC^{hi}, CD11c^{hi} cells. . Macrophages were the most abundant cell retrieved from the bronchoalveolar space in all sampled groups. I did not find any differences in the proportion of macrophages between IPF and either non-fibrotic ILDs, different ILD diagnoses or healthy control (figure 3.7).

Figure 3.5 Percentage CD45^{hi} cells in BAL

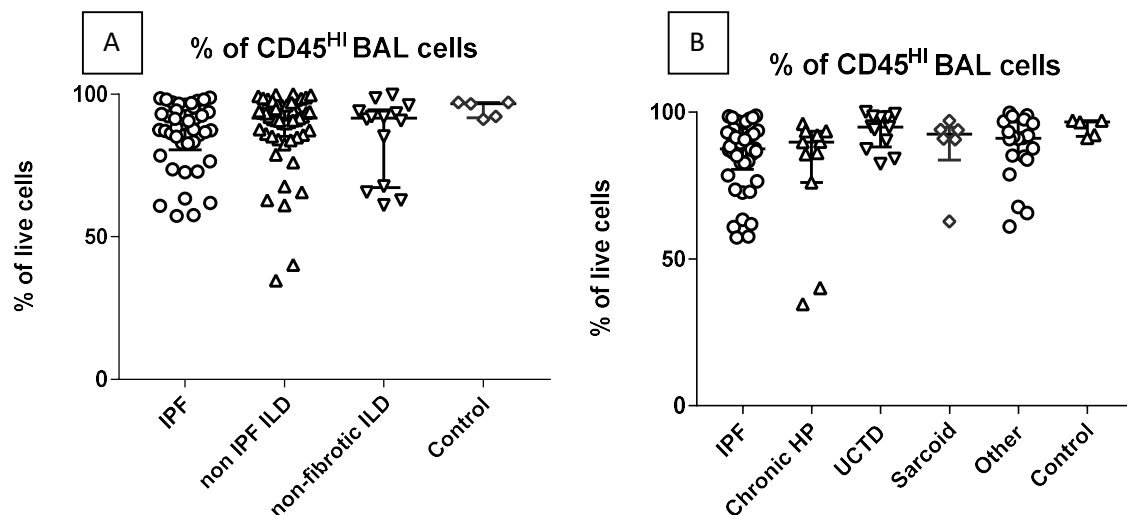


Figure 3.5 Number of CD45^{hi} cells expressed as a percentage of all live counted BAL cells. Figure shows % of CD45^{hi} cells of all live cells comparing IPF(n=41) vs non-IPF ILD(n=51), non-fibrotic ILD(n=14) and control(n=5) **A**) % of CD45^{hi} cells of all live cells comparing between specific ILDs; IPF(n=41), HP(n=11), UCTD (n=12), sarcoid (n=6), other (n=20) and control (n=5) **B**). There was found to be a significant difference between the medians of the groups, using a 1 way anova test (p=0.0071), but a Dunn's multiple comparison test did not reveal any significant differences between the individual groups tested.

Values expressed as median and interquartile range

Figure 3.6

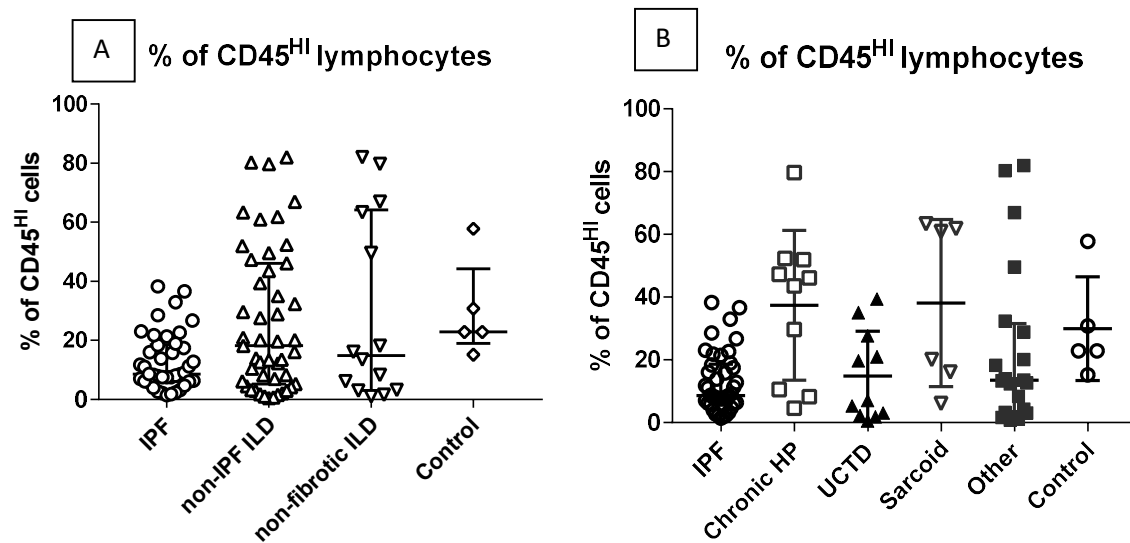


Figure 3.6 Percentage CD45^{HI} lymphocytes in BAL; Number of CD45^{HI} lymphocytes cells expressed as a percentage of all live counted BAL cells. Figure shows % of CD45^{HI} lymphocyte cells of all live cells comparing IPF(n=41) vs non-IPF ILD(n=47), non-fibrotic ILDs(n=14) and control(n=5) **A)** % of CD45^{HI} lymphocytes comparing between specific ILD diagnoses; IPF(n=41), HP(n=1), UCTD (n=10), sarcoid (n=6), other (n=20) and control (n=5) **B).** Lymphocyte numbers did not exhibit significant differences across the comparison groups.

Values expressed as median and interquartile range

Figure 3.7

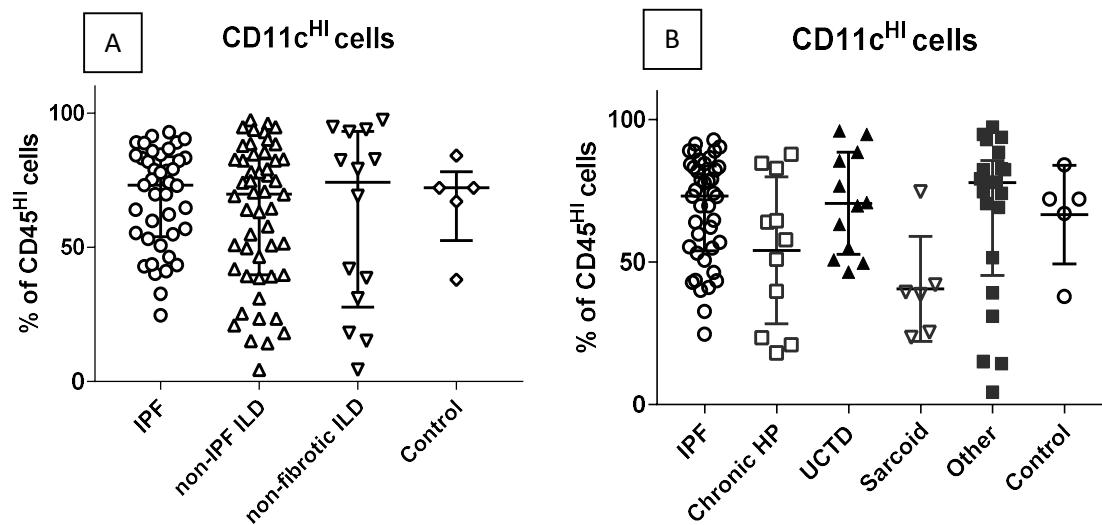


Figure 3.7 Percentage CD45^{hi} CD11c^{hi} macrophages in BAL; Number of CD11c^{hi} airway macrophages cells expressed as a percentage of all live CD45^{hi} cells. Figure shows % of CD11c^{hi} airway macrophages of all live CD45 cells comparing IPF(n=41) vs non-IPF ILD(n=51), non-fibrotic ILDs(n=14) and control (n=5) **A)** % of CD11c^{hi} airway macrophages of all live CD45 cells comparing between specific ILDs; IPF(n=41), HP(n=11), UCTD (n=12), sarcoid (n=6), other (n=20) and control (n=5) **B).**

Values expressed as median and interquartile range

3.2.3 Characterisation of macrophage phenotypes in ILD

Macrophage sub-populations were further stratified by expression of multiple putative M1/M2 macrophage cell surface markers.

CD45^{hi} CD11c^{hi} cells were analysed for their expression of our chosen M1/M2 phenotyping markers and results revealed that airway macrophages show high expression of both M1 and M2 surface markers across the spectrum of interstitial lung diseases with no statistically significant difference between IPF, non-IPF ILD, non-fibrotic ILD and healthy control (figure 3.8). When re-analysed to investigate the possibility of differential M1/M2 expression between ILDs stratified to separate ILD

diagnoses our data again showed no statistically significant differences between % airway macrophage expression of CD163, CD206, CD86 and HLA-DR (figure 3.9).

These data do not present any evidence of a skew towards M2 in the alveolar space in the lung in ILD or in the healthy state.

Figure 3.8

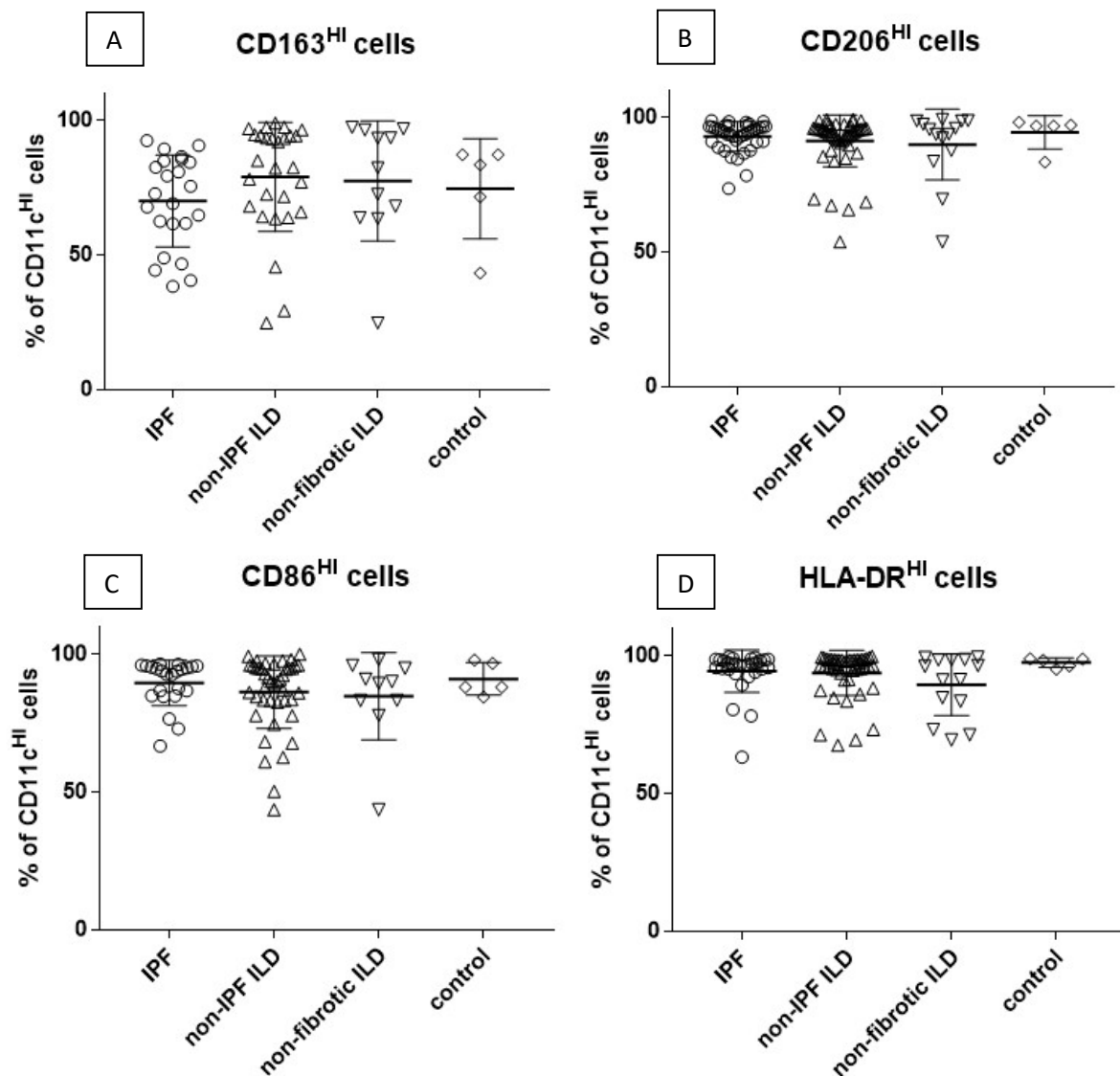


Figure 3.8 Percentage M1/M2 markers of CD45^{hi} CD11c^{hi} BAL cells in IPF vs fibrotic ILD, non-fibrotic ILD and healthy control; M1/M2 cell surface marker expression of CD11c^{hi} BAL cells measured by flow cytometry, presented as IPF vs non-IPF ILD, non-fibrotic ILD and control. Figure shows mean % of CD11c^{hi} cells expressing M2 marker CD163 in IPF(n=25), non-IPF ILD(n=36) non-fibrotic ILD(n=11), control(n=5) **A) CD11c^{hi} cells expressing M2 marker CD206 in IPF(n=41), non-IPF ILD(n=49), non-fibrotic ILD(n=15), control(n=5) **B**) CD11c^{hi} cells expressing M1 marker CD86 in IPF(n=25), non-IPF ILD(n=42), non-fibrotic ILD(n=10), control(n=5) **C**) of CD11c^{hi} cells expressing M1 marker HLA-DR in IPF(n=31), non-IPF ILD(n=46), non-fibrotic ILD(n=14), control(n=5) **D**).**

Values expressed as median and interquartile range

Figure 3.9

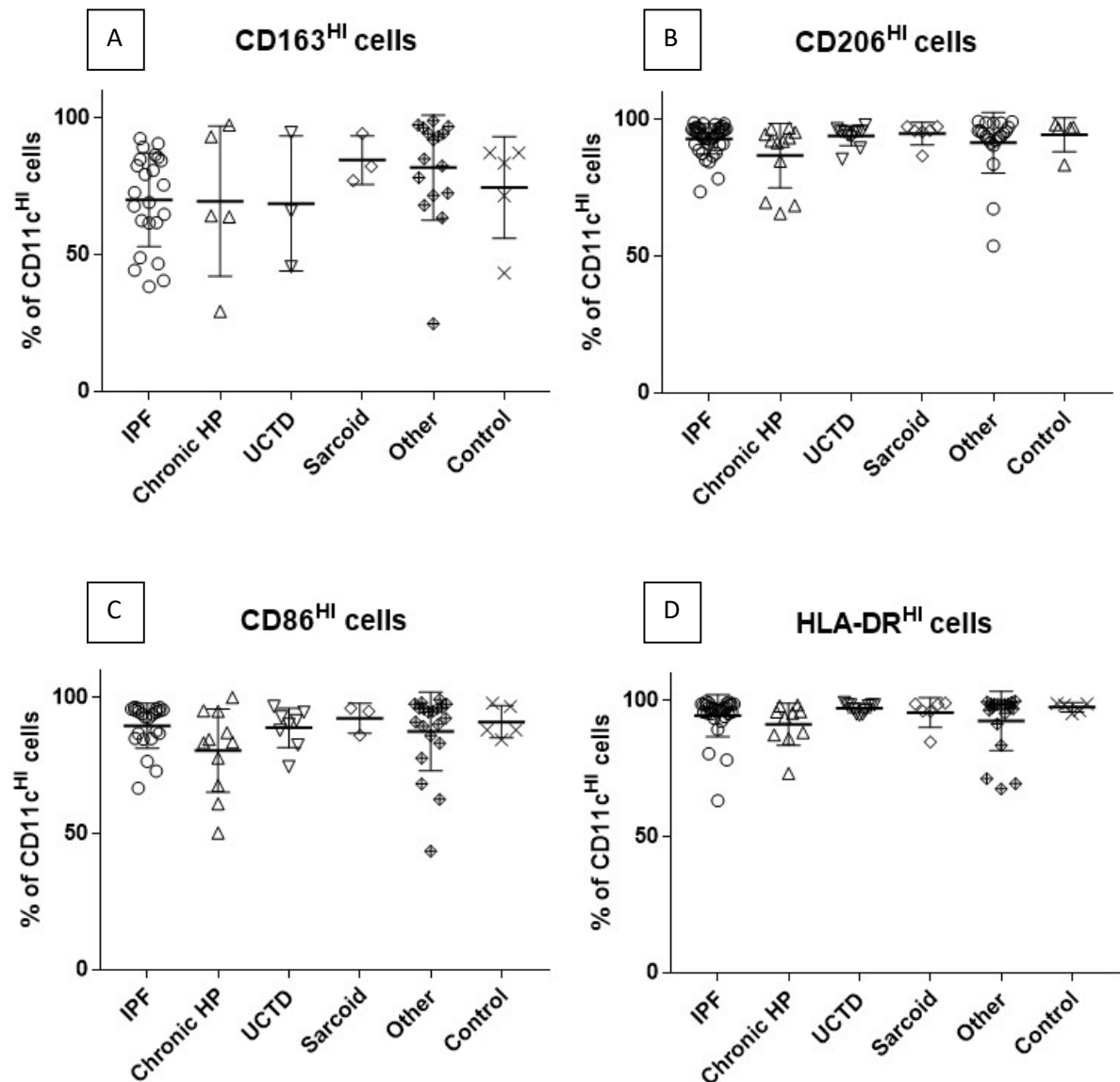


Figure 3.9 Percentage of M1/M2 markers of CD45^{HI} CD11c^{HI} BAL cells in IPF vs other ILDs and healthy control; M1/M2 cell surface marker expression of CD11c^{HI} BAL cells measured by flow cytometry, presented as IPF vs individual ILD diagnoses and control. Figure shows mean % of **A)** CD11c^{hi} cells expressing M2 marker CD163 **B)** CD11c^{hi} cells expressing M2 marker CD206 **C)** M1 marker CD11c^{hi} cells expressing CD86 **D)** of CD11c^{hi} cells expressing M1 marker HLA-DR.

Values expressed as median and interquartile range

I subsequently hypothesised that one possible reason for the discrepant results found between these data and previously published studies - some of which have found increased expression of M2 markers in the human lung (Gibbons, 2011) (Pechkovsky, 2010) and some work having found a decrease in M2 IPF (Yamashita, 2018) - could be differential expression of markers which are reputed to delineate the same populations. i.e. that there would be a difference found between the airway macrophage expression of between the M1 markers or between the M2 markers.

Our data demonstrated that the different M1 and M2 markers included in this study did not exhibit the same levels of expression on airway macrophages (figure 3.10). These airway macrophages exhibited differential expression between our chosen M1 and M2 markers with alveolar macrophages in both IPF and non-IPF ILD showing significant differences in expression between M1 markers HLA-DR and CD86 (HLA-DR n=24, CD86 n=17, p=0.036), and M2 markers CD206 and CD163 (CD206 n=32, CD163 n=23, p=0.007). I found that when re-analysed to compare single M1/M2 markers, a significant difference was found between HLA-DR and CD163 (HLA-DR n=24, CD163 n=23, p=0.0001). This pattern was the same in IPF and non-IPF ILD.

Figure 3.10

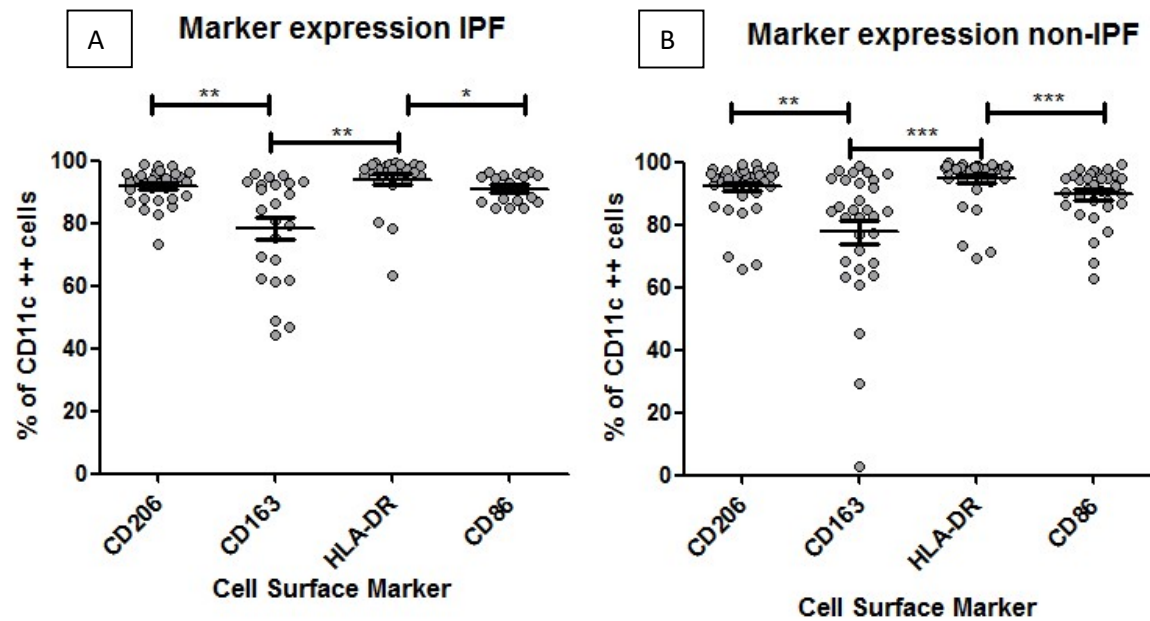


Figure 3.10 Comparison of percentage expression of individual M1/M2 markers in IPF and non-IPF

ILD mean % of individual cell surface markers of CD11c^{HI} cells in IPF; CD206(n=41), CD163(n=25), HLA-DR(n=31), CD86(n=25) **A**) individual cell surface markers of CD11c^{HI} cells in non-IPF ILD; CD206(n=49), CD163(n=36), HLA-DR(n=46), CD86(n=42) **B**).

Values expressed as median and interquartile range

3.2.4 Clinical correlation of BAL cell characteristics

These data were correlated against lung function parameters at time of bronchoscopy and over the subsequent 12 months-18 months to establish the relationship between macrophage marker expression and disease severity and rates of disease progression. Full lung function tests (including both FVC and TLCO) at the time of bronchoscopy and over the subsequent 12 months was not available for all of the participants in our study. Data on the available lung function at baseline and over time are detailed in Table 3.4.

Table 3.4

	CD11c	Lymphocytes	CD163	CD206	CD86	HLA-DR
IPF						
<i>FVC</i>	32	32	18	32	18	25
<i>TLCO</i>	31	29	16	31	17	25
<i>Longitudinal FVC</i>	24	24	12	24	13	18
<i>Longitudinal TLCO</i>	23	23	11	23	12	17
Non-IPF ILD						
<i>FVC</i>	37	37	24	37	32	34
<i>TLCO</i>	38	38	21	38	31	33
<i>Longitudinal FVC</i>	28	28	14	28	24	26
<i>Longitudinal TLCO</i>	27	27	15	27	25	25

Table 3.4 numbers of each staining parameter for which longitudinal lung function was available;

number of subjects for whom data was collected on each flow cytometry analysis parameter and longitudinal lung function measurements.

3.2.5 Correlation of lymphocyte and macrophage proportions with clinical parameters

To assess the possibility of a correlation between lymphocyte or macrophage proportions and disease severity, I examined these parameters against FVC and TLCO at the time of bronchoscopy . These data did not reveal a statistically significant correlation between forced vital capacity (FVC) or transfer factor of the lung for carbon monoxide (TLCO) at time of bronchoscopy and CD11c^{hi} airway macrophages or lymphocytes in individuals with IPF (figure 3.11) or in non-IPF ILD (figure 3.12). This did not demonstrate a correlation between BAL macrophage or lymphocyte proportions and disease severity as measured by FVC and TLCO % predicted at the time of bronchoscopy. These data do not suggest that BAL macrophage or lymphocyte numbers can be used to identify or predict clinical disease phenotypes.

Figure 3.11

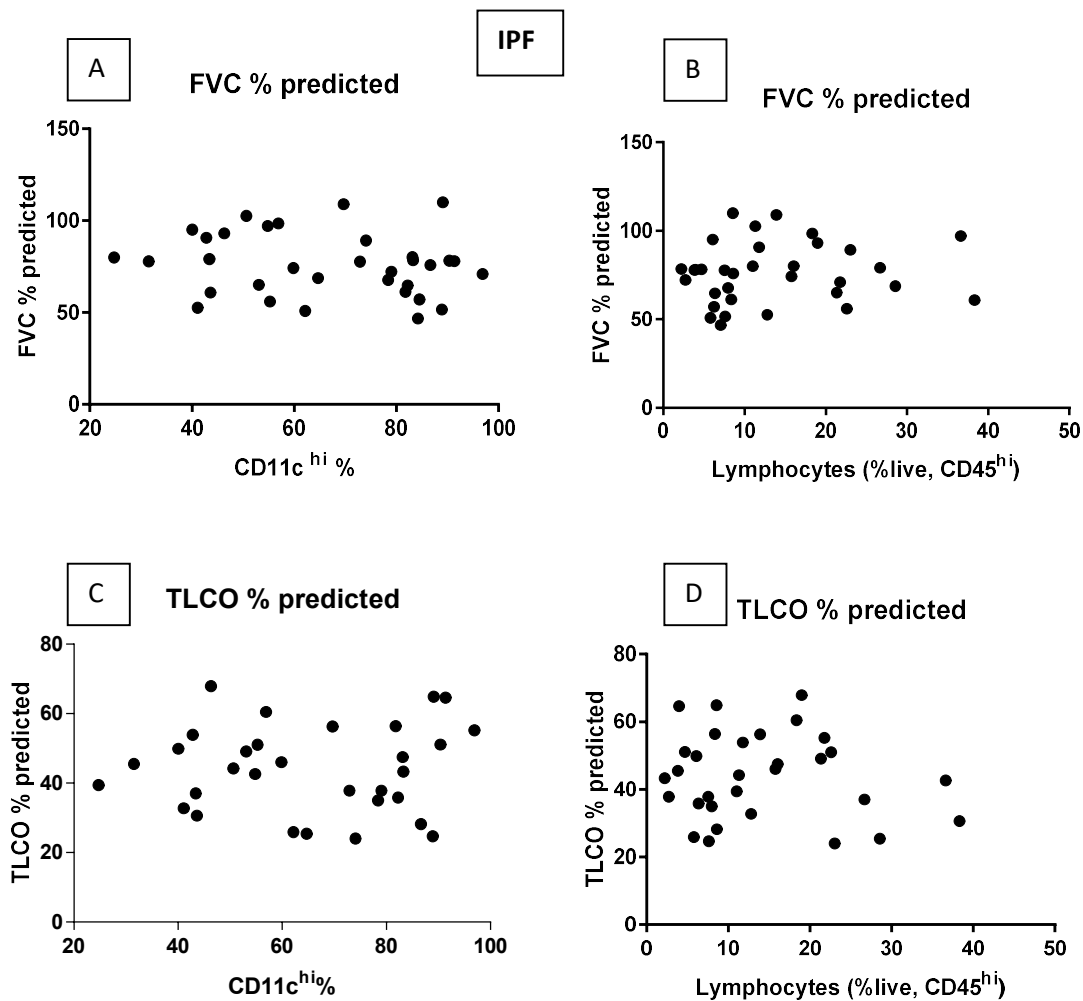


Figure 3.11 Lymphocyte and CD11c^{hi} macrophage numbers compared to FVC (% of predicted) and TLCO (% predicted) at time of diagnosis in IPF; lung function parameters correlated to lymphocyte and macrophage percentages in BAL in IPF. FVC values as a percentage of predicted at the time of bronchoscopy correlated to CD11c^{hi} CD45^{hi} BAL cells ($r=-0.1701$, $n=33$, $p=0.3438$) **A) FVC values correlated to CD45^{hi} BAL lymphocytes ($r=0.09859$, $n=33$, $p=0.5852$) **B**) TLCO values as a percentage of predicted at the time of bronchoscopy correlated to CD11c^{hi} CD45^{hi} BAL cells ($r=0.04524$, $n=31$, $p=0.8090$) and **C**) TLCO values correlated to CD45^{hi} BAL lymphocytes ($r=-0.1202$, $n=31$, $p=0.5195$) **D**).**

Figure 3.12

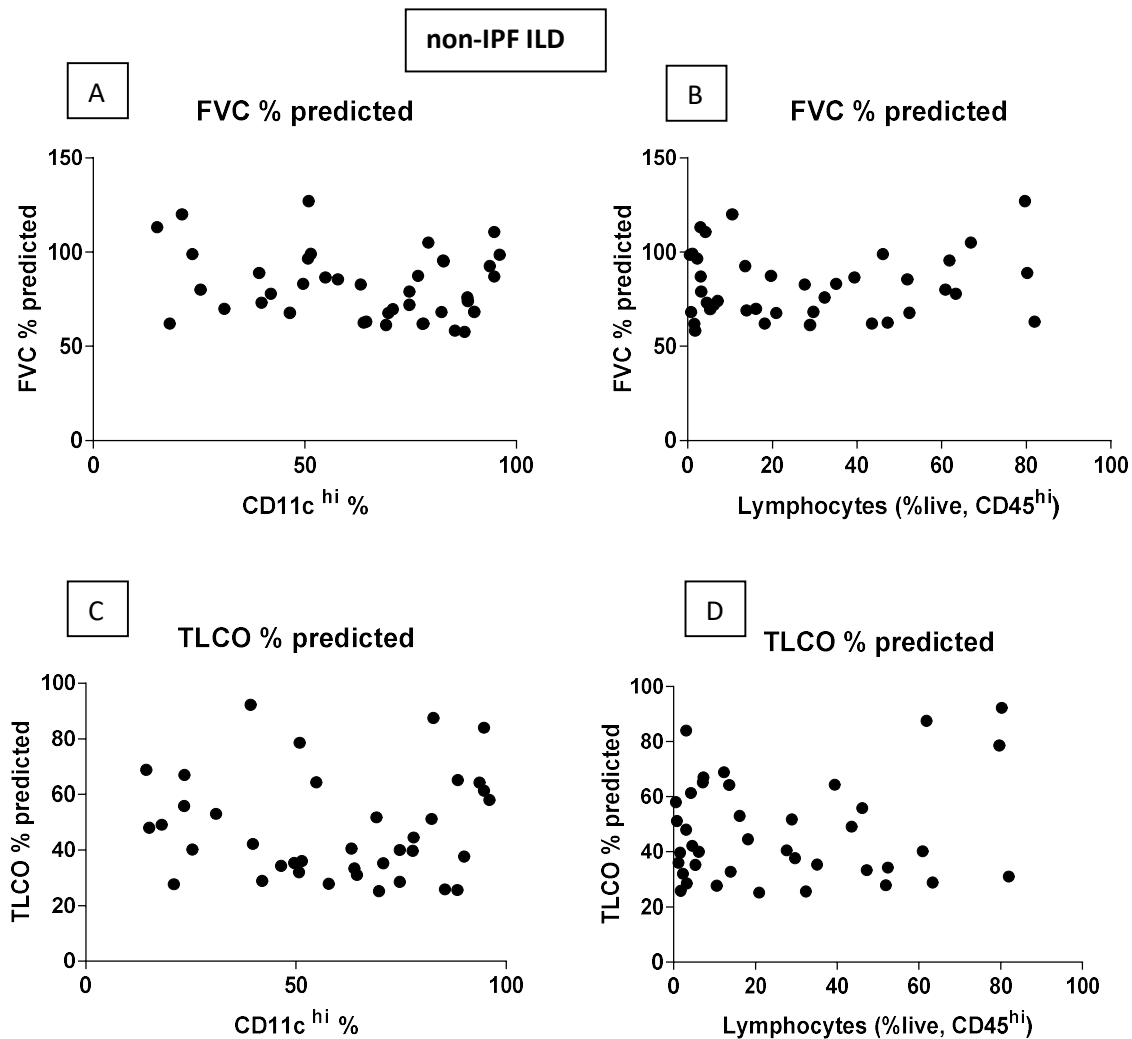
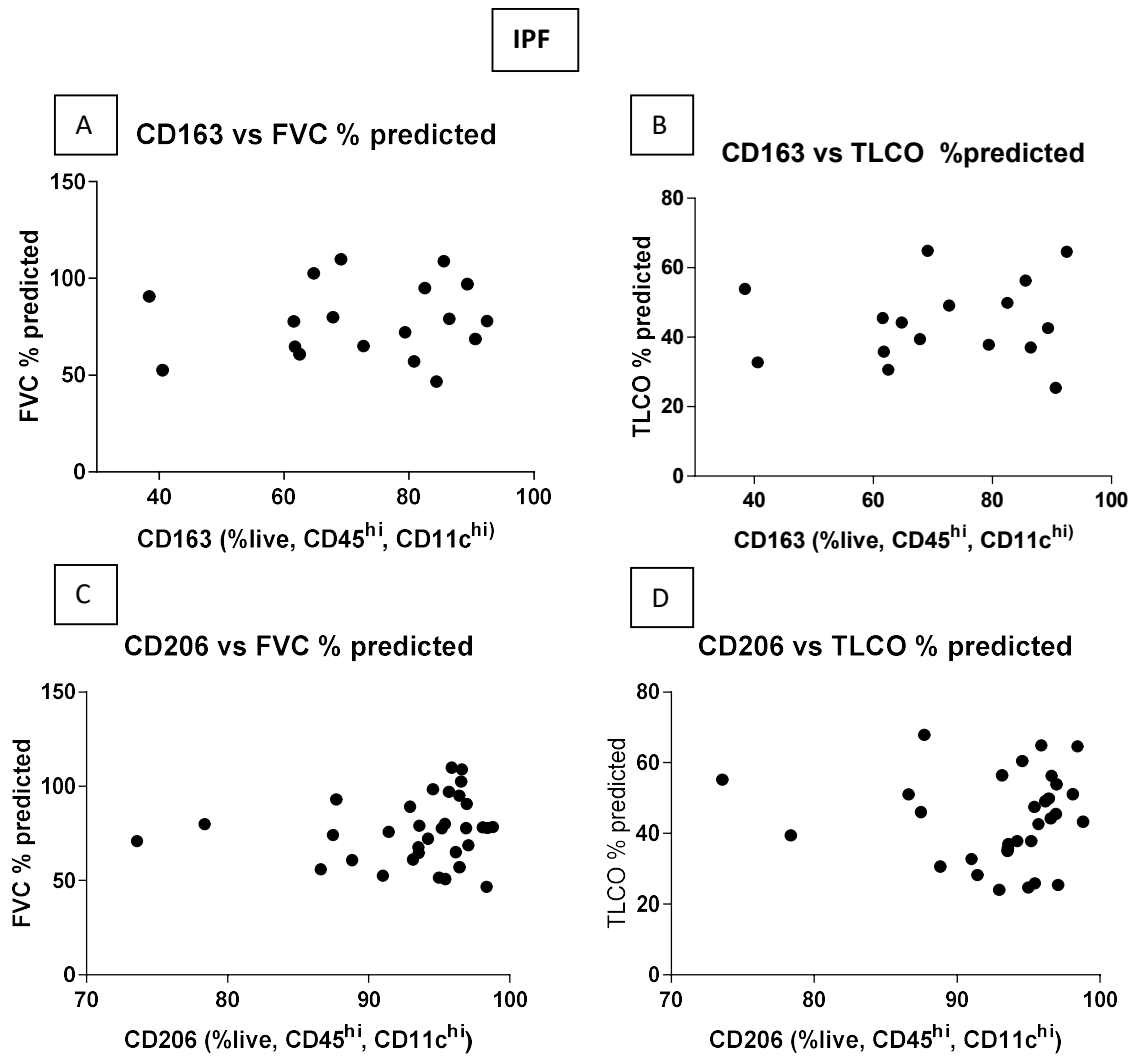


Figure 3.12 Lymphocyte and CD11c^{HI} macrophage percentage compared to FVC (% of predicted) and TLCO (% predicted) at time of diagnosis in non-IPF ILD; FVC values as a percentage of predicted at the time of bronchoscopy correlated to CD11c^{HI} CD45^{HI} BAL cells ($r=-0.2035$, $n=40$, $p=0.2078$) **A)** FVC values correlated to CD45^{HI} BAL lymphocytes ($r=0.08829$, $n=39$, $p=0.5930$) **B)** TLCO values as a percentage of predicted at the time of bronchoscopy correlated to CD11c^{HI} CD45^{HI} BAL cells ($r=0.01118$, $n=38$, $p=0.9469$) **C)** TLCO values correlated to CD45^{HI} BAL lymphocytes ($r=0.1591$, $n=39$, $p=0.3332$) **D).**

The data previously presented showed that there was no preponderance of M2 type macrophages in IPF BAL. The clinical relevance of putative M1/M2 marker expression was further interrogated by looking at all macrophage phenotyping markers correlated against lung function markers of disease

severity at the time of bronchoscopy. This analysis showed that there was no statistically significant correlation between level of expression of our 4 examined M1/M2 markers and disease severity by surrogate marker of lung function values in IPF (figure 3.13) or non-IPF ILD (figure 3.14). This study encompassed and is reflective of subjects with a wide range of disease severity as reflected by lung function impairment and the findings do not support the hypothesis of a skew towards M2 macrophage polarisation in the pathogenesis of IPF.

Figure 3.13



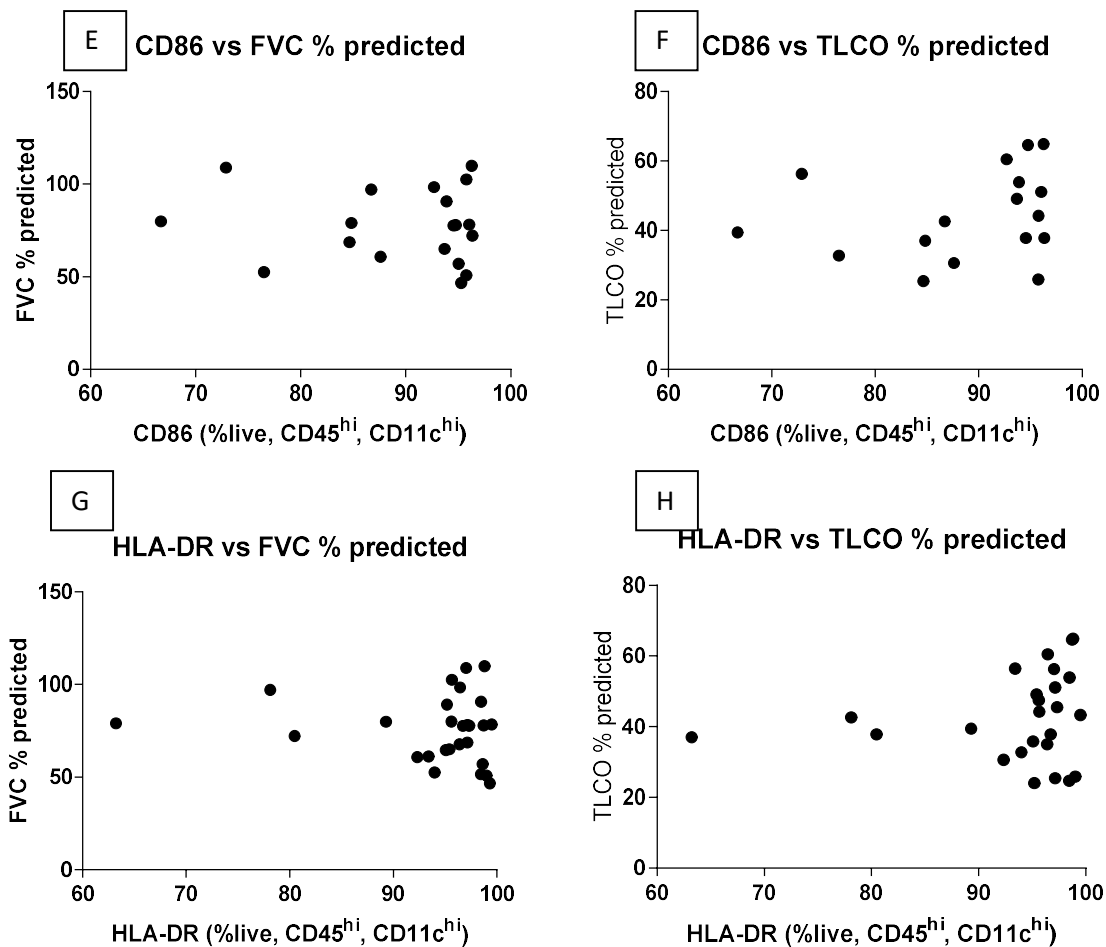
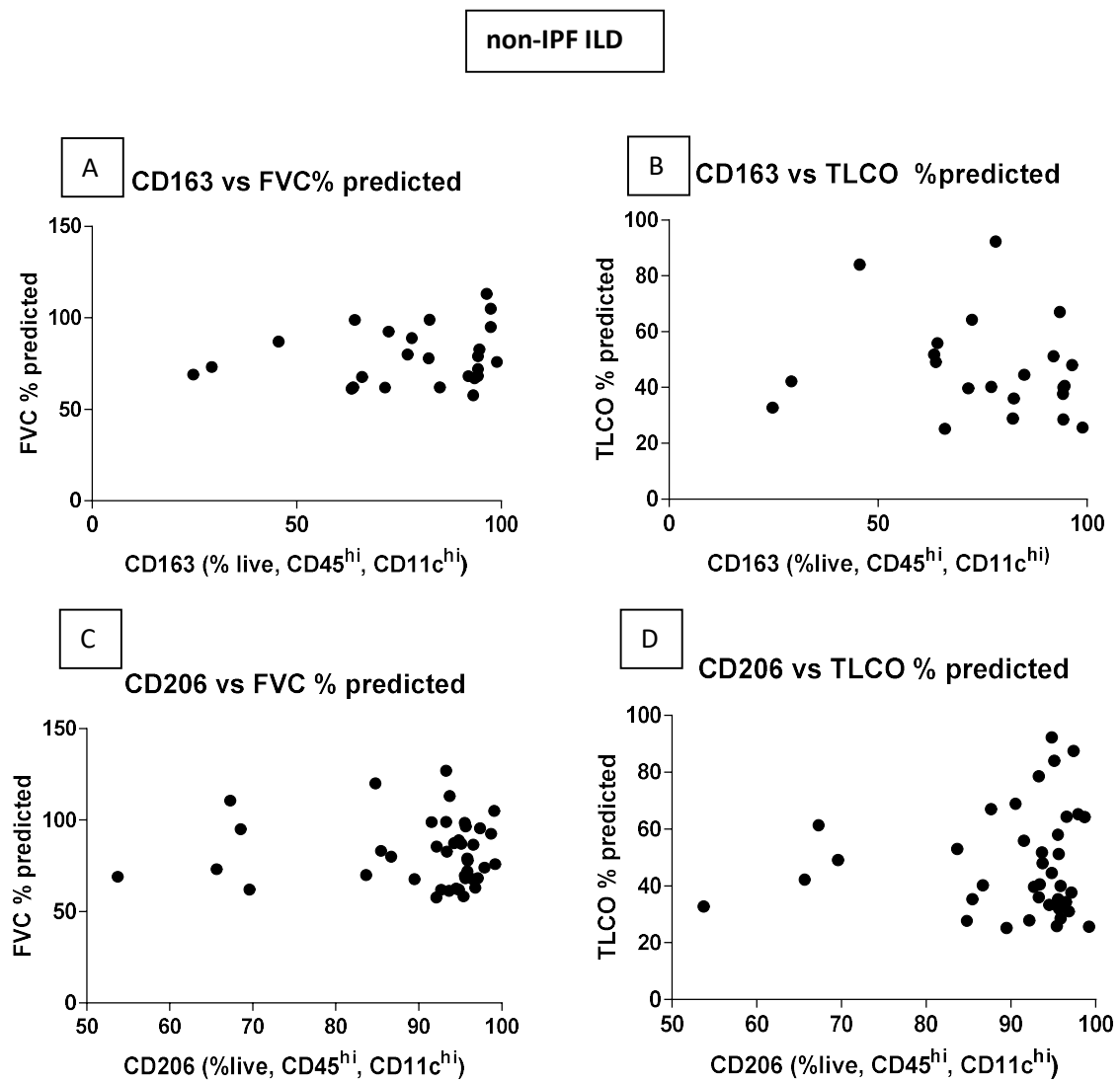


Figure 3.13 M1/M2 markers proportions compared to FVC (% of predicted) and TLCO (% predicted) at time of diagnosis in IPF; correlation between lung function parameters (% predicted) taken at time of diagnosis and macrophage phenotyping markers in IPF, expressed as a percentage of total macrophage number in BAL. FVC at time of diagnosis and CD163 ($r=0.1029$, $n=18$, $p=0.6884$) **A)** TLCO at time of diagnosis and CD163 ($r=0.1085$, $n=16$, $p=0.6892$) **B)** FVC at time of diagnosis and CD206 ($r=0.1225$, $n=33$, $p=0.4970$) **C)** TLCO at time of diagnosis and CD206 ($r=-0.02764$, $n=31$, $p=0.8827$) **D)** FVC at time of diagnosis and CD86 ($r=-0.08867$, $n=19$, $p=0.7181$) **E)** TLCO at time of diagnosis and CD86 ($r=0.2325$, $n=17$, $p=0.3692$) **F)** FVC at time of diagnosis and HLA-DR ($r=-0.1138$, $n=27$, $p=0.5719$) **G)** TLCO at time of diagnosis and HLA-DR ($r=0.1628$, $n=25$, $p=0.4368$) **H).**

Figure 3.14



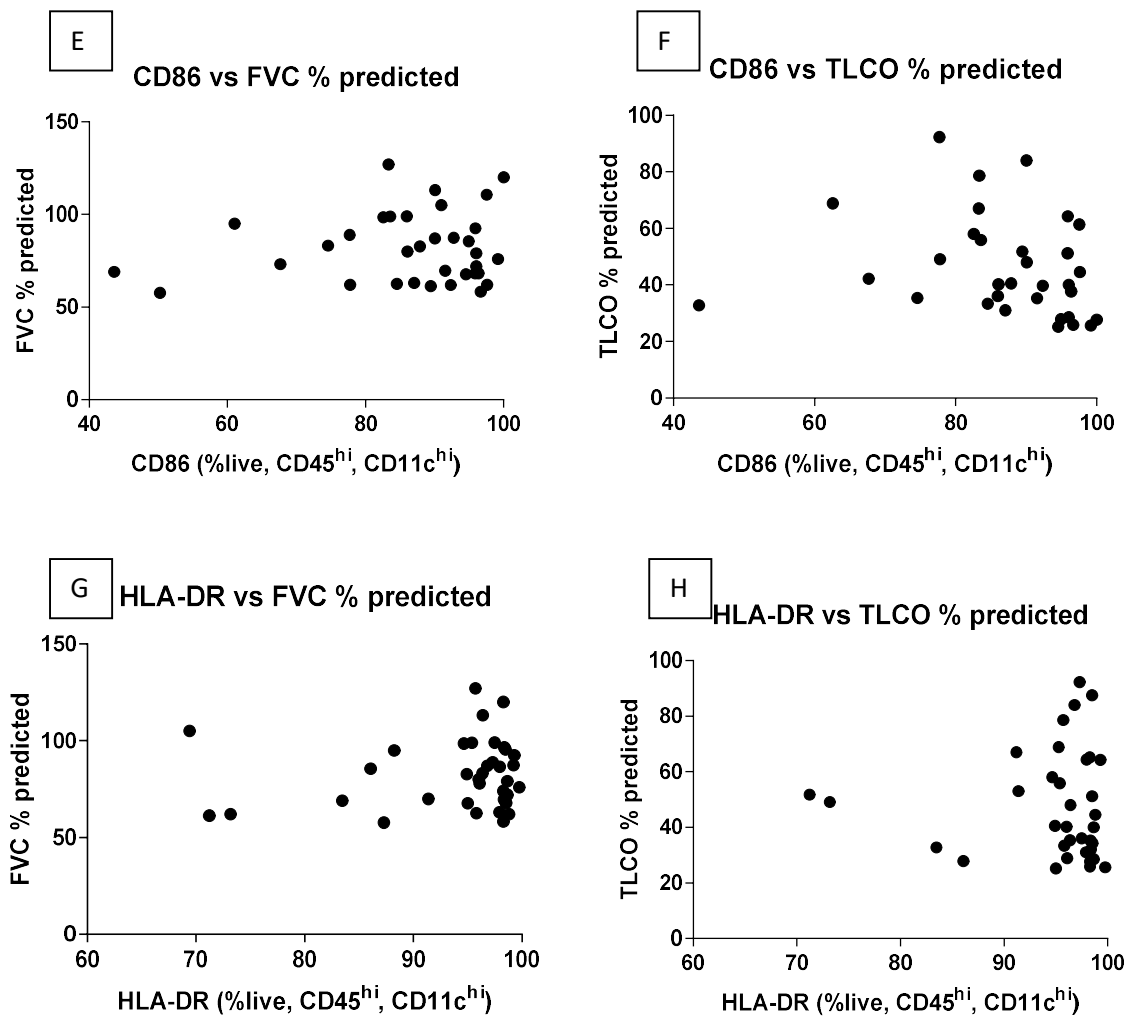
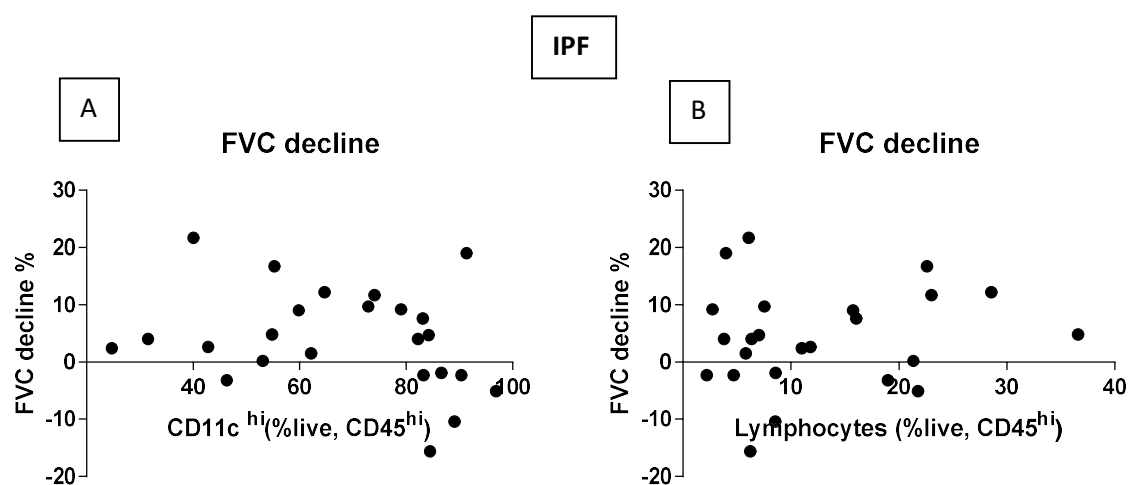


Figure 3.14 M1/M2 markers proportions compared to FVC (% of predicted) and TLCO (% predicted) at time of diagnosis in non-IPF ILD ; correlation between lung function parameters (% predicted) taken at time of diagnosis and macrophage phenotyping markers in non-IPF ILD, expressed as a percentage of total macrophage number in BAL. FVC at time of diagnosis and CD163 ($r = 0.1845$, $n = 25$, $p = 0.3773$) **A) TLCO at time of diagnosis and CD163 ($r = -0.1426$, $n = 22$, $p = 0.5266$) **B**) FVC at time of diagnosis and CD206 ($r = -0.01146$, $n = 41$, $p = 0.9433$) **C**) TLCO at time of diagnosis and CD206 ($r = 0.07745$, $n = 39$, $p = 0.6393$) **D**) FVC at time of diagnosis and CD86 ($r = 0.1291$, $n = 34$, $p = 0.4669$) **E**) TLCO at time of diagnosis and CD86 ($r = -0.2028$, $n = 32$, $p = 0.2656$) **F**) FVC at time of diagnosis and HLA-DR ($r = 0.117$, $n = 37$, $p = 0.4904$) **G**) TLCO at time of diagnosis and HLA-DR ($r = 0.005253$, $n = 35$, $p = 0.9761$) **H**).**

A number of the study subjects remained under clinical follow-up which allowed lung function measurements to be recorded over time and the following data includes those who had lung function repeated between 12 and 18 months post the date of their bronchoscopy, with the change in lung function between bronchoscopy and repeat measurement at 12-18 months correlated with lymphocyte and macrophage proportions and macrophage phenotyping markers. Lung function parameters are presented as a percentage of expected predicted value. The numbers in our dataset with full longitudinal lung function were reduced as some patients were lost to further follow up.

Our dataset did not demonstrate any statistically significant correlation between macrophage or lymphocyte proportions and lung function decline over time in IPF (figure 3.15) or non-IPF ILD (figure 3.16). Taken together with our earlier data, this is further evidence that single timepoint measurements of lymphocyte and macrophage proportions in BAL are not predictive of clinical phenotypes or disease course in ILD. They do not correlate with either disease severity at time of presentation or subsequent disease progression.

Figure 3.15



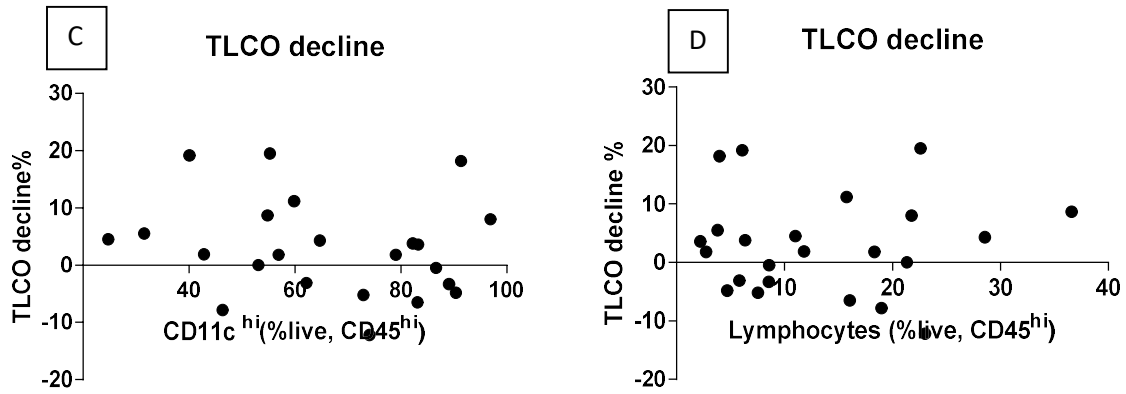


Figure 3.15 Correlation of lymphocyte and macrophage proportions in BAL against decline in FVC and TLCO over time in individuals with IPF; correlation between decline in lung function (expressed in % of predicted values) and macrophage and lymphocyte proportions in IPF BAL with figure showing **A)** FVC decline against % CD11c^{HI} CD45^{HI} cells ($r=-0.2604$, $n=24$, $p=0.2191$) **B)** FVC decline against % CD45^{HI} lymphocytes ($r=0.1271$, $n=24$, $p=0.5539$) **C)** TLCO decline against % CD11c^{HI} CD45^{HI} cells ($r= -0.1967$, $n=23$,) **D)** TLCO decline against % CD45^{HI} lymphocytes ($r=0.01492$, $n=23$, $p=0.9461$)

Figure 3.16

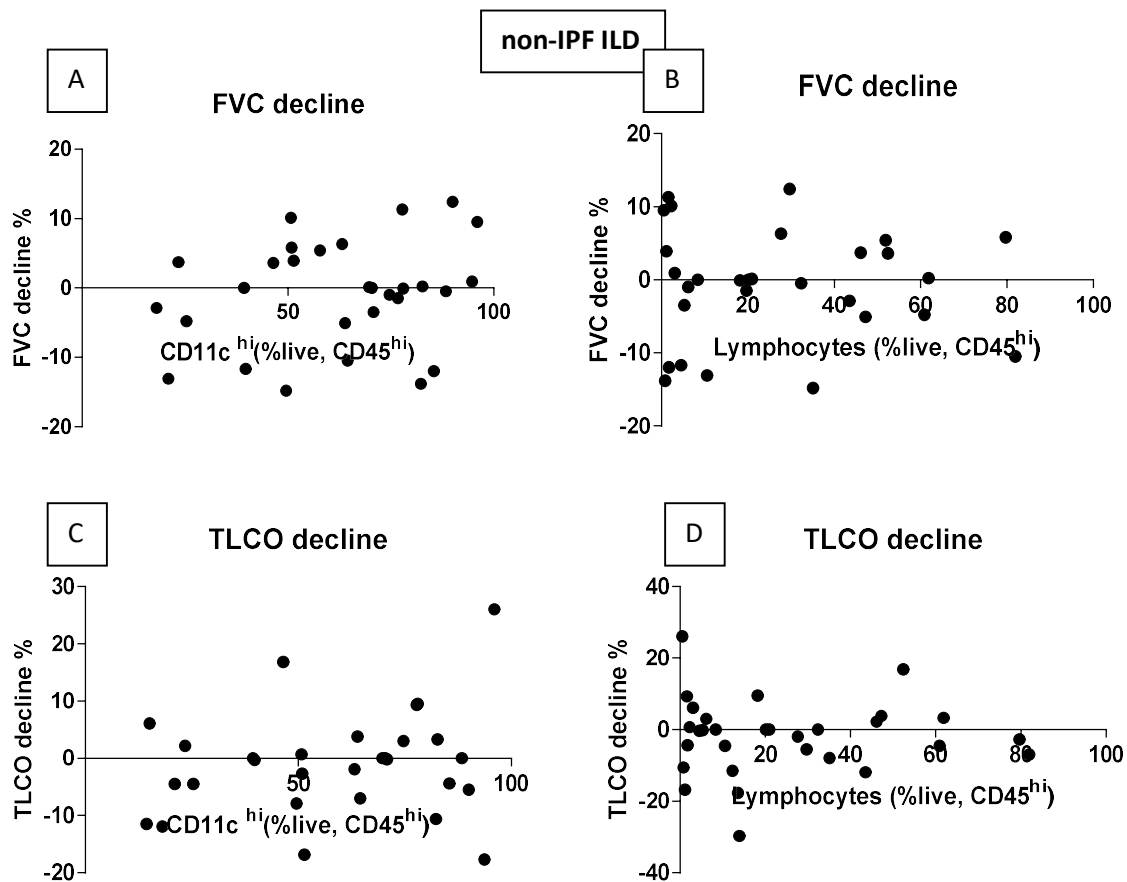


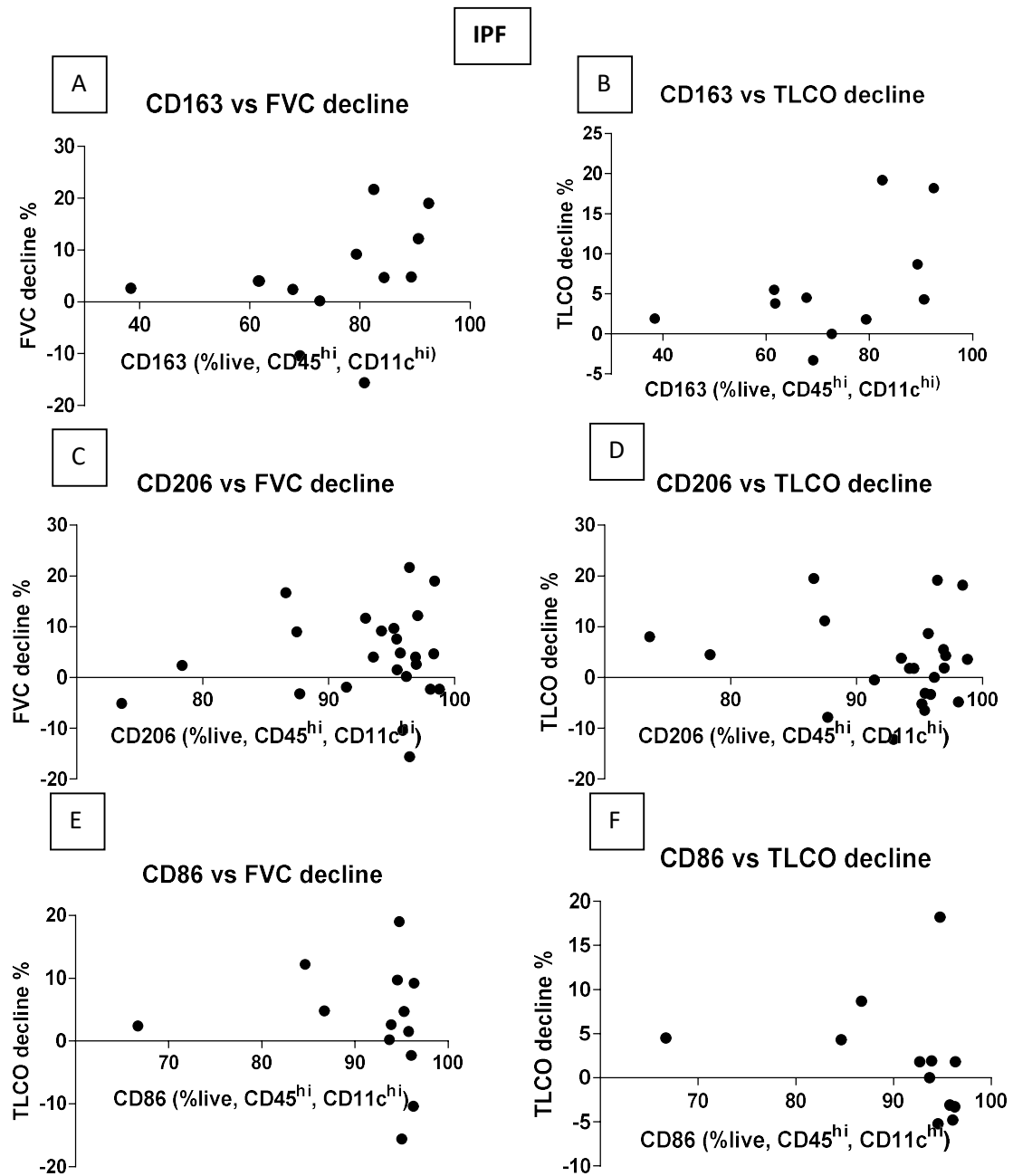
Figure 3.16 correlation between decline in lung function (expressed in % of predicted values) and macrophage and lymphocyte proportions in non-IPF ILD BAL; FVC decline against % CD11c^{hi} CD45^{hi} cells (r=0.2176, n=29, p=0.2567) **A) FVC decline against % CD45^{hi} lymphocytes (r=-0.005773, n=29, p=0.9763) **B**) TLCO decline against % CD11c^{hi} CD45^{hi} cells (r=0.1883, n=29, p= 0.3279) **C**) TLCO decline against % CD45^{hi} lymphocytes (r=0.006662, n=30, p=0.9721) **D**)**

To complete this characterisation of the clinical application of the M1/M2 phenotype in ILD, I analysed the expression of these airway macrophage phenotyping markers correlated against the change in lung function parameters over time. This did not show there to be a correlation between

CD163, CD206, CD186 and HLA-DR expression and lung function decline over time in individuals with IPF (figure 3.17) and non-IPF ILD (figure 3.18).

Our dataset demonstrated a statistically significant correlation between decline in TLCO and CD206 expression ($r=0.4656$, $n=30$, $p=0.0095$), and also a statistically significant correlation between decline in TLCO and CD86 ($r=0.4245$, $n=27$, $p=0.0273$) in individuals with non-IPF ILD (figure 3.15). However these data showed no statistically significant correlation between any of our phenotyping macrophage markers and lung function markers of disease progression in IPF.

Figure 3.17



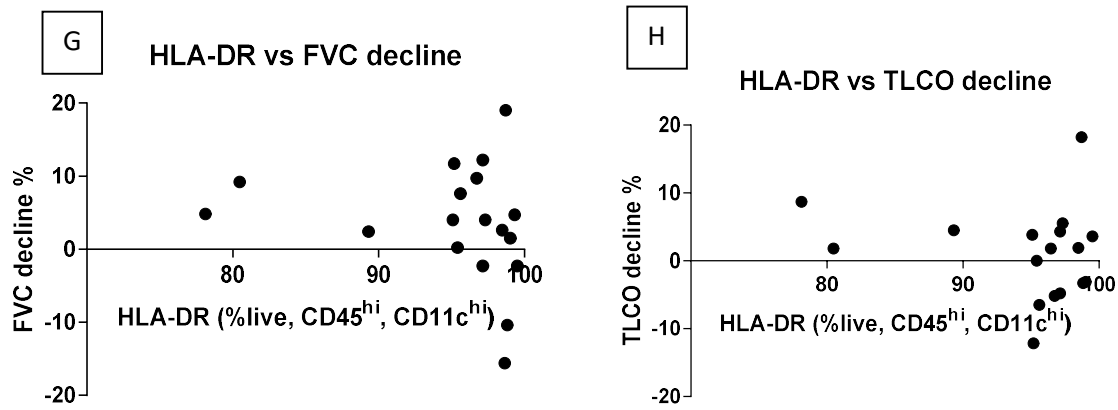
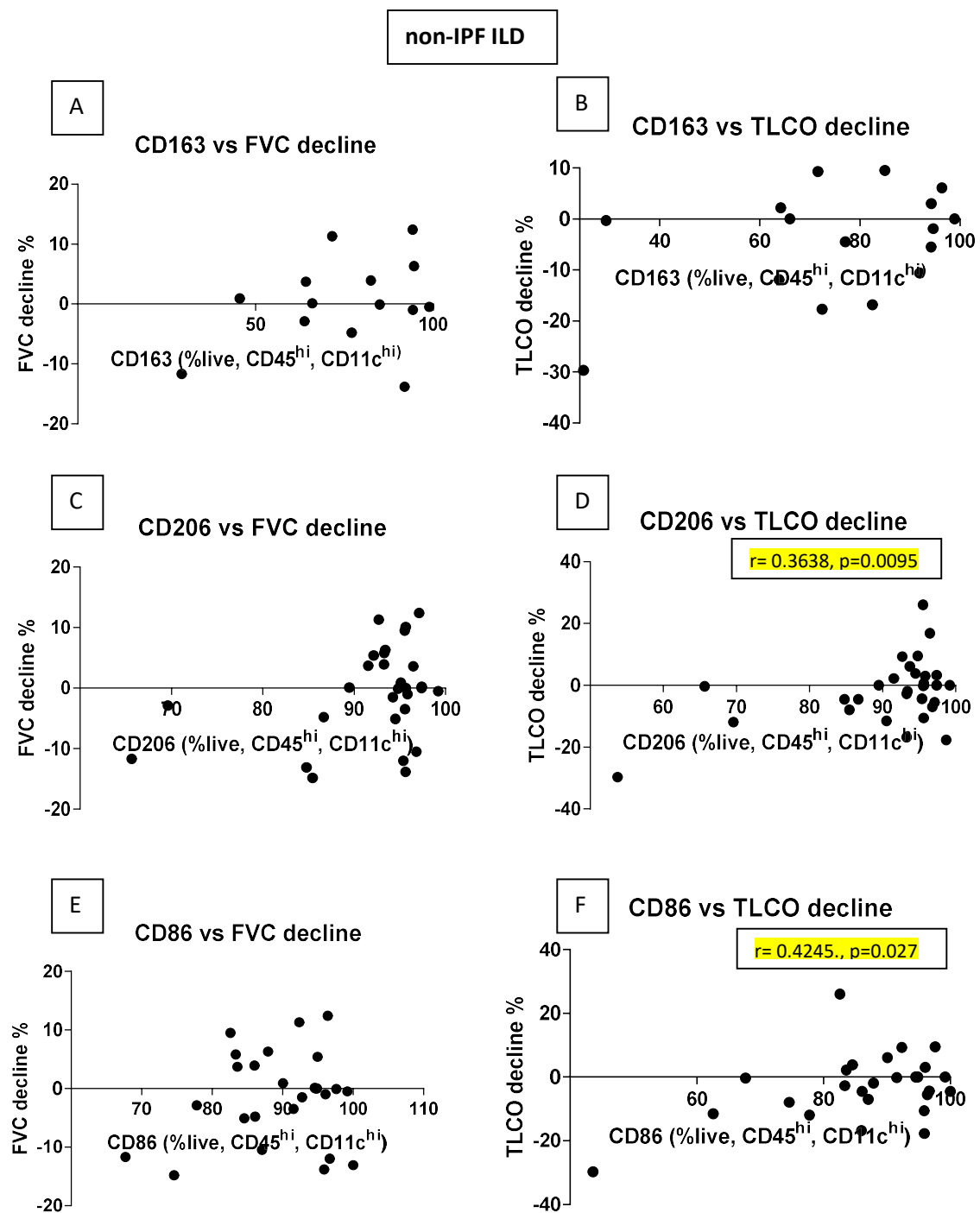


Figure 3.17 M1/M2 markers correlated against change in FVC and TLCO over time in individuals

with IPF; change in FVC (% predicted) and CD163 expression ($r=0.3301$, $n=13$, $p=0.2706$) **A)** change in TLCO (% predicted) and CD163 expression ($r=0.502$, $n=11$, $p=0.1157$) **B)** change in FVC and CD206 expression ($r=0.1125$, $n=24$, $p=0.6008$) **C)** change in TLCO and CD206 expression ($r=-0.1397$, $n=23$, $p=0.5249$) **E)** change in FVC and CD86 expression ($r=-0.1269$, $n=13$, $p=0.6796$) **D)** change in TLCO and CD86 expression ($r=0.2325$, $n=17$, $p=0.4074$) **F)** change in FVC and HLA-DR expression ($r=-0.2044$, $n=18$, $p=0.4158$) **G)** change in TLCO and HLA-DR expression ($r=-0.1847$, $n=17$, $p=0.4778$) **H).**

Figure 3.18



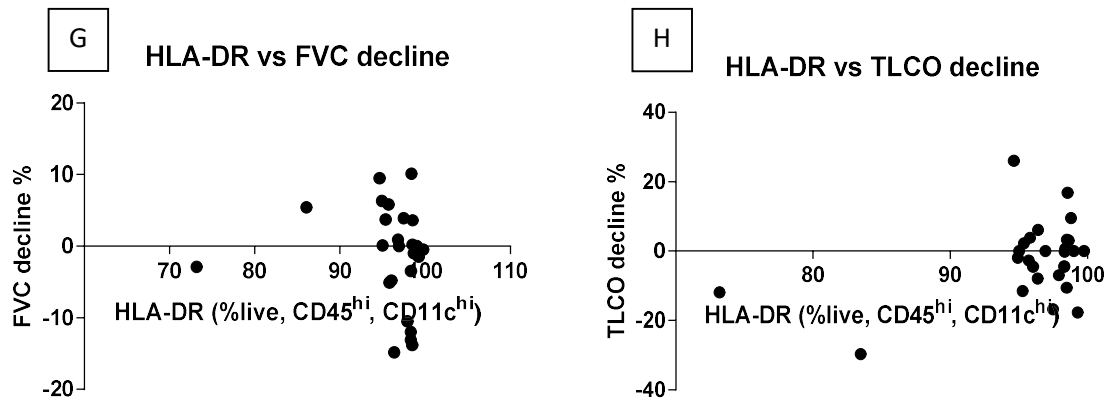


Figure 3.18 M1/M2 markers correlated against change in FVC and TLCO over time in individuals

with non-IPF ILD; no significant correlation was found between change in FVC and CD163 expression ($r=0.2924$, $n=14$, $p=0.3103$) **A)** change in TLCO and CD163 expression ($r=0.3517$, $n=27$, $p=0.1130$) **B)** change in FVC and CD206 expression ($r=0.3638$, $n=29$, $p=0.0524$) **C)** change in TLCO and CD 206 expression ($r=0.4656$, $n=30$, $p=0.0095$) **D)** change in FVC and CD86 expression ($r=0.1575$, $n=26$, $p=0.4421$) **E)** change in TLCO and CD86 expression ($r=0.4245$, $n=27$, $p=0.0273$) **F)** change in FVC and HLA-DR expression ($r=-0.1128$, $n=26$, $p=0.5832$) **G)** change in TLCO and HLA-DR expression ($r=0.3598$, $n=27$, $p=0.0653$) **H).**

3.4 Discussion

The primary aim of this chapter was to characterise the immunophenotype of airway macrophages obtained by bronchoalveolar lavage from across a spectrum of ILDs, with the focus primarily on IPF, to ascertain if AMs in fibrotic lung diseases skew towards a putatively fibrotic 'M2' phenotype. The prognostic significance of and relationship to baseline severity of macrophage phenotype in IPF, as a comparison to non-IPF ILDs, was also examined in order to establish if BAL macrophage counts and phenotyping could be used to detect subsets of IPF with different clinical characteristics and outcomes. This study did not find evidence to support the hypothesis that airway macrophages polarise to discrete activation phenotypes in IPF and did not find evidence of an M2 rich environment in the IPF lung. Rather these data demonstrated that they exhibit a complex spectrum of macrophage activation phenotypes, which did not show any significant correlation to lung function parameters in IPF.

3.4.3 Summary of Findings

The results of this study lead us to conclude that the M1/M2 paradigm of macrophage activation is an oversimplification of a complex spectrum based model and that, in the lung, airway macrophages show high expression of putative M1 and M2 markers rather than separating to form discrete separate M1/M2 populations. I did not find any difference between IPF and non-IPF ILDs or healthy controls. Our work suggested that in macrophage phenotyping studies, marker choice is of paramount importance. Using single markers in this study could have produced results suggesting higher proportions of M1 over M2 in the fibrotic lung, with significant differences found between expression of HLA-DR when compared with CD163, which is not reproduced when comparing HLA-DR to CD206, or CD86 to either M2 marker.

These data did not suggest a role for BAL cell macrophage phenotyping as a marker of IPF severity or as an indicator of disease progression in interstitial lung disease, and did not support the hypothesis that this method could be used as a surrogate marker of disease activity.

As in previously published studies (Ohshimo et al, 2009) our dataset showed that the absolute number of cells retrieved and the cells retrieved/ml of BAL fluid aspirated did not differ between IPF and non-IPF ILDs. I did however show a significant increase in BAL cellular yield in IPF when compared with cellular yield from healthy control volunteers. The yield of BAL fluid did not differ between the disease groups and controls and thus the increase in BAL cells does not appear due to yield differences and the proportion of the BAL cells retrieved that were dead was unchanged between the disease and control groups and I therefore concluded that the cells surviving to analysis across disease groups and therefore the cell populations being analysed, were equivalent.

BAL fluid analysis in IPF typically shows an increase in total cell counts which are predominantly neutrophils and eosinophils (Costabel et al, 2001) and our data accords with previously published studies, which have noted increased cellularity of the sampled alveolar space. There does remain some ongoing dubiety about the relationship between individual cell numbers to survival. Boomars et al (Boomars et al, 1995) showed a negative correlation between eosinophil numbers and survival in IPF and no increase in the absolute number of macrophages in a cohort of 49 individuals with IPF. It should be noted however that these individuals were diagnosed based on a chest radiograph and biopsy rather than HRCT, raising the possibility of inclusion of disease that would not today be labelled as IPF. Further studies (Drent et al. 1993) examining BAL cell profile in ILD did not find a relationship between BAL findings and survival in IPF patients and this finding was re-produced in this work.

These data did demonstrate a significant difference in lymphocyte counts between IPF and control and IPF and non-IPF ILD. However on further sub-group analysis, statistical significance was not reached on comparison between the groups. This finding would be expected given that a BAL

lymphocytosis of >20% points towards a diagnosis alternate to IPF, with sarcoid and chronic HP amongst the differentials of ILDs which show high lymphocyte counts in BAL (Meyer et al, 2012). One might expect there to be a significantly higher lymphocyte populations in certain disease, such as sarcoid and hypersensitivity pneumonitis which are characterised by lymphocytic inflammation which is absent in IPF, however as I was primarily recruiting individuals with IPF, the numbers in these other diagnosis arms were small and this may explain why I did not detect this effect. Furthermore, this lymphocytic BAL effect can be lost in more fibrotic advanced disease in otherwise inflammatory ILDs and this may also, in part, explain our findings.

When stratified to IPF vs all non-IPF ILDs or fibrotic versus non-fibrotic lung diseases there is no difference demonstrated in airway macrophages expressed as a percentage of CD45^{hi} cells. Previously published work has shown some differences in macrophage and lymphocyte counts between IPF and some non-IPF ILDs when stratified in to separate disease groups (Ohshimo et al. 2009) such as HP and sarcoid but not in other types of fibrotic lung disease such as NSIP (Veeraraghavan et al. 2003). I found there to be no correlation between macrophage and lymphocyte proportions and lung function decline in our group of individuals, either with IPF or non-IPF ILD and this may be accounted for by the small numbers of individual non-IPF ILD diagnoses included in our study. Given the wide potential differences in pathogenesis and the expected BAL cell differential of the various ILDs, the diagnoses included in the non-IPF ILD group is of critical importance to the expected respective cell counts. When analysing data in the context of previously published work, it is also important to note the fact that the advent of HRCT lead to a revield international multidisciplinary consensus classification of the idiopathic interstitial pneumonias (2002) which created the new disease entity know as non-specific interstitial pneumonitis (NSIP). Prior to this distinction, NSIP would have been diagnosed as IPF, and therefore some older published data on IPF may be contain datasets with individuals with a combination of IPF and what I would now call NSIP. This means that the earlier published work on IPF potentially encompasses a more

heterogeneous disease group and when compared to the post HRCT IPF classification, some differences may be expected.

The question of the relationship between individual BAL cell numbers and survival has not yet been categorically answered - possible in part due to recruiting sufficient patients and following up long enough to show a survival effect - however, numerous smaller studies have repeatedly not identified an increase in airway macrophages in IPF. It therefore does seem clear that, although the predominant cell in the alveolus in IPF, the airway macrophage pool does not seem to be expanded in IPF.

The M1/M2 classification system has increasingly been accepted as an oversimplification which does not fully recognise and embrace the context specific heterogeneity of macrophage activation status, with M1 and M2 more likely representing the opposing ends of a continuum of intermediate cells (Mosser et al, 2008). Recent evidence has shown that macrophage plasticity allows for numerous different activation phenotypes to which macrophage precursors can be directed depending on the inducing stimuli. In 2014, Xue et al published a large transcriptomic data set of human macrophage activation which extended the current model of macrophage activation from the polarisation model to a spectrum activation model (Xue et al. 2014). These data suggested macrophages to have a much broader spectrum of transcriptional responses to their environmental milieu than previously thought. A new nomenclature has been proposed to describe macrophage activation based upon macrophage source, activators and agreed markers (Murray, Allen et al. 2014). The role of 'wound healing' M2 as crucial in pulmonary fibrosis has been largely established in mouse models of fibrosis. The M1/M2 paradigm emerged from murine macrophages which were noted to differentially activate depending on whether they were exposed to IFN γ and/or LPS or IL-4 (Stein et al. 1992). Alveolar injury in mice prompts a macrophage laden infiltrate, composed predominantly of M2 (Osterholzer et al. 2013) and in the bleomycin model, the macrophage pool skews to a predominantly M2 phenotype (Gibbons et al. 2011). Although published work in humans

has suggested a skew towards alternate activation, with CD163 expression (as a marker of M2) on both AMs and circulating monocytes increased in IPF as compared with control (Murray, Rosada et al. 2010) (Gibbons et al. 2011) no one single parameter has been validated for, or accepted as denoting, M1 or M2 in humans. Authors have used different parameters to delineate M2s including CD163 (Gibbons et al. 2011) and CD206 surface expression as well as macrophage production of TGF- β , IL-10 (Larson-Casey et al. 2016) CCL2, CCL 18 (Stahl et al. 2013).

The BAL samples in this study were collected from individuals with a variety of different ILDs which differ greatly in pathogenesis, encompassing non-inflammatory purely fibrotic processes such as IPF as well as inflammation predominant diseases such as hypersensitivity pneumonitis and sarcoidosis and pathologies exhibiting no evidence of any fibrotic disease. Furthermore the BALs were performed on individuals at differing stages of disease with varying degrees of fibrosis, from mild to severe impairment, as demonstrated by the range of TLCO at time of bronchoscopy. This dataset therefore reflects a wide variety of pathologies from moderate to severe disease which has been compared against a healthy control group. No significant differences were detected when comparing putative M1 and M2 marker levels between different disease group and when compared against healthy controls. No correlation was detected between cell surface markers and extent of fibrosis or subsequent clinical course, as determined by lung function parameters in IPF. However these data did demonstrate a statistically significant correlation between CD206 and TLCO decline, and CD86 and TLCO decline in non-IPF ILD. It is not clear why this finding was present in non-IPF ILD and is not replicated in our IPF cohort, but the correlations found were not strong ($r = 0.4245$ and $r = 0.3638$ respectively), and when viewing the plotted data points, may have been skewed by a low expression outlier which was present in both groups. The diagnoses included in the non-IPF arm were diverse and therefore this group was not as clearly defined a patient population as in the IPF cohort. If this correlation was a significant feature of the fibrotic process then the findings would have been expected to be robustly re-produced in our IPF arm. Furthermore the conflicting nature of a correlation between both expression of an M1 marker and TLCO decline and an M2 marker and

TLCO decline, suggests that - if real- this effect is not M1/M2 specific but a function of increased macrophage numbers, and this is not borne out by a similar correlation to expression of pan macrophage marker CD11c.

Our data demonstrated that a high percentage of these AMs expressed both M1 and M2 markers and did not find any differences between IPF, control and non-IPF ILDs. I subsequently re-stratified our disease groups to allow identification of a sub set of ILD patients who did not have any evident radiologically established fibrosis in order to ascertain if there were differences in macrophage phenotype based on the presence of a fibrotic process rather than the pathological process of the different ILDs. This analysis did not reveal any differences in macrophage markers owing to the presence of a fibrosing interstitial process.

This work found that significant differences could be found between the level of expression of the different putative M1 markers and the different M2 markers that I included, suggesting that even widely accepted M1/M2 markers are not expressed at the same level. Taken together these data suggest that airway macrophages in interstitial lung disease exhibit a spectrum of activation phenotypes which do not conform to a bipolar M1/M2 phenotype and therefore cannot be delineated by simple surface marker phenotyping. These data are in accordance with the evolving understanding of complex macrophage activation as a spectrum based model.

These data also suggest that marker choice is of critical importance in the analysis of M1/M2 phenotyping studies and may explain the differences in M2 expression that have been found in previous studies which have not been replicated in our study.

However, given the range of reported M1/M2 markers, a potential limitation of the study is the reliability of those I have chosen. To ensure the robustness of our phenotyping, I collected data on 2 different markers for both M1 and M2 which have both been widely used in the published literature.

These data do include a comparison with healthy controls, however it was not possible to age match entirely and our control group evinces a wider age range than those of the disease comparison group. I did however compare with both a non-IPF ILD disease comparison group and a non-fibrotic disease group which do not exhibit differences in CD163 expression levels. This gives us an insight into M1/M2 marker status in the IPF lung compared with normal homeostasis, as well as when compared with a variety of interstitial lung diseases which show varying degrees of fibrosis.

This work does not offer evidence to support the hypothesis that airway macrophages polarise along M1/M2 lines to form discrete activated macrophage phenotypes, and consequently did not find evidence of a skew towards M2 in the IPF lung. This work does not confirm BAL airway macrophage proportions or M1/M2 phenotypes to have any correlation to clinical parameters and therefore does not support the possibility of their use in disease stratification or prognostication. The majority of the work which implicates M2 comes from murine models which conform more closely to the M1/M2 model and are not representative of the environment in the fibrotic lung, and from blood derived monocytes which are polarised very specifically along M1/M2 lines in tightly controlled in-vitro culture conditions which are not replicated in vivo.

3.4.2 Strengths and Weaknesses of study

The nature of our dataset is that all individuals undergoing bronchoscopy were those whose disease was not classifiable on the basis of radiology or history alone. Therefore this is a cohort of individuals who do not have completely typical disease patterns as those who do would have no clinical indication for bronchoscopy. This should be borne in mind when contextualising the results. Furthermore the numbers of controls recruited in our study were small compared to the IPF cohort - due to our good access to individuals with ILD and the relative difficulty of recruiting healthy control

volunteers for non-indicated bronchoscopy- and the controls were younger than the IPF cohort.

Given the nature of IPF as a disease of ageing, this is a further limitation of the data I present here.

The strengths of this study lie in the size of the dataset of individuals with ILD on whom I have been able to correlate a comprehensive phenotyping FACS approach to clinically relevant clinical parameters.

3.4.3 Future work

Future work in this area should focus more on classifying macrophage subtypes by origin and function than M1/M2 polarisation status, as this study does not provide evidence to support the M1/M2 polarisation model in IPF AMs. To confirm the findings of this pilot study would require a power calculation and a sample size powered to detect differences.

In order to examine AMs in IPF beyond phenotypic classification, a 3D co-culture model with AECs and fibroblasts would offer fresh insights into how airway macrophages fit into the complex interaction between AEC and fibroblast and how they can affect ECM deposition and turnover in disease and healthy states. This may bring new insights into the wound healing role of the airway macrophage in fibrotic lung disease.

Chapter 4.

**An investigation into the immunophenotype and function of
bronchial epithelial cells in idiopathic pulmonary fibrosis.**

4.1 Introduction

In the previous chapter, the data presented demonstrated that airway macrophages do not conform to the M1/M2 paradigm of macrophage activation and there demonstrated no significant difference between proportions of M2 macrophages in BAL fluid comparing IPF with other ILDs and healthy controls. M1/M2 phenotypes have been well reported in animal modelling but these data suggest that airway macrophages exist upon a spectrum of activation and do not polarise to discrete activation phenotypes. I did not demonstrate any correlation between clinical markers of severity or disease progression in IPF and putative classically activated macrophage or alternately activated macrophage cell surface markers.

Given the absence in our dataset of strong evidence suggesting a correlation between airway macrophage phenotype and clinical parameters, I switched focus on to examining the role of bronchial epithelial cells in the pathogenesis of IPF. The alveolar epithelial cell has been widely studied as the key effector cell in the development of IPF (Hagimoto et al, 1997) (Matute-Bello et al, 2007) (Lee et al, 2004) (Adamson et al, 1988)(Plataki et al, 2005) (Kasper et al, 1996) (Uhal et al, 1998), but the bronchial epithelium has begun to come under more recent scrutiny. This has been prompted by the discovery of a link between MUC5B over expression and the development of IPF (Fingerlin et al, 2013), which for the first time directly implicates dysfunctional bronchial epithelial defence mechanisms in the development of fibrosis.

The aim of this study was to investigate the phenotype and *in vitro* functions of bronchial epithelial cells from individuals with idiopathic pulmonary fibrosis as compared with healthy controls.

This was carried out by characterising the phenotype and *in vitro* behaviour of IPF HBEs with particular respect to characterisation of the cellular composition of the bronchial epithelium, gene expression of classically pro-fibrotic genes, and the functional parameters of cell proliferation and barrier function.

IPF HBECs were obtained from bronchial brushings and cultured prior to analysis, with comparison made to healthy control HBECs, which were either obtained from bronchial brushings of healthy volunteers or from normal donors purchased from Lonza.

4.1.1 Hypothesis

The phenotype of the bronchial epithelium is altered from the healthy state in idiopathic pulmonary fibrosis, and disruptions are seen in bronchial epithelial proliferation and barrier function when compared to healthy control.

4.1.2 Aims

- 1) To characterise the phenotype of epithelial cells in lung tissue sections using histological staining methods comparing IPF to healthy control.
- 2) To investigate the proliferative capacity and barrier function of IPF HBECs, by *in vitro* functional assays of cultured IPF HECs compared to healthy control
- 3) To assess the contribution of IPF HBECs to the pro-fibrotic environment present in the IPF lung, by gene expression analysis of cytokines, chemokines and growth factors traditionally thought to be involved in the fibrotic process, as well as assessment of direct effects of IPF HBECs upon normal human lung fibroblasts.

4.1.3 Methods

4.1.3.1 Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded paraformaldehyde fixed lung tissue obtained from biopsy of individuals with IPF, or from distal and histologically normal sections of tissue from lung cancer resections.

For all study subjects, diagnosis was made according to ATS/ERS consensus classification criteria, by an experienced ILD physician, with input from a specialist ILD MDT, including a thoracic radiologist and thoracic histopathologist

The study explored 27 lung tissue blocks from 16 IPF patients and 12 from controls, obtained at diagnostic open lung biopsy. The IPF tissue was obtained during open lung biopsy from individuals with radiologically unclassifiable disease. The control tissue was obtained during lung lobectomy due to suspected lung cancer from otherwise healthy individuals. Only patients with solid (and typically centrally located) tumours were included and the tissue for the present analysis was collected from peripheral lung regions, as far from the tumour as possible. Sequential sections were immunolabeled with Cytokeratin 5 (CK5), Mucin 5AC (MUC5AC) SCGB1A1 and acetylated tubulin (ACT) specific antibodies (table 1) to identify expression of basal, club, ciliated and mucin producing epithelial cell. Distal airways were defined as airways with no cartilage support or glandular elements, surrounded by smooth muscle bands and characterized by an undulating epithelium.

Table 4.1

	Control	IPF
n	11 (2 with unknown demographic details)	16

Age	67 (59.5-74)	64 (54.8-69)
% male	27.2	93.8

Table 4.1 - Immunohistochemistry subject demographic details

Age shown as median (interquartile range)

Table 4.2

Protein	Function
Cytokeratin 5 (KRT5)	Basal cell marker
Muc5AC (MUC5AC)	Mucus component
Acetylated Tubulin (ACT)	Ciliated cell marker
Secretoglobin 1A1(SCGB1A1)	Club cell marker

Table 4.2 Epithelial cell protein markers used for epithelial phenotyping

Lung tissue sections were imaged using Leica DM2500 microscope and positive staining was quantified using Aperio ImageScope technology and expressed per μm of measured airway length. The software was used to determine the number of positive pixels per total tissue area (excluding airspaces) using a positive pixel algorithm (figure 4.1) and data is expressed as positive pixel area per μm of airway length. A mean of 3 quantified airways were analysed for each sample and comparison between IPF and control was performed. Only airways that were cross sectioned were quantified.

Quantification was separated to analyse both total pixel positivity in the imaged section of airway and strong positivity measured in the section of airway.

Figure 4.1

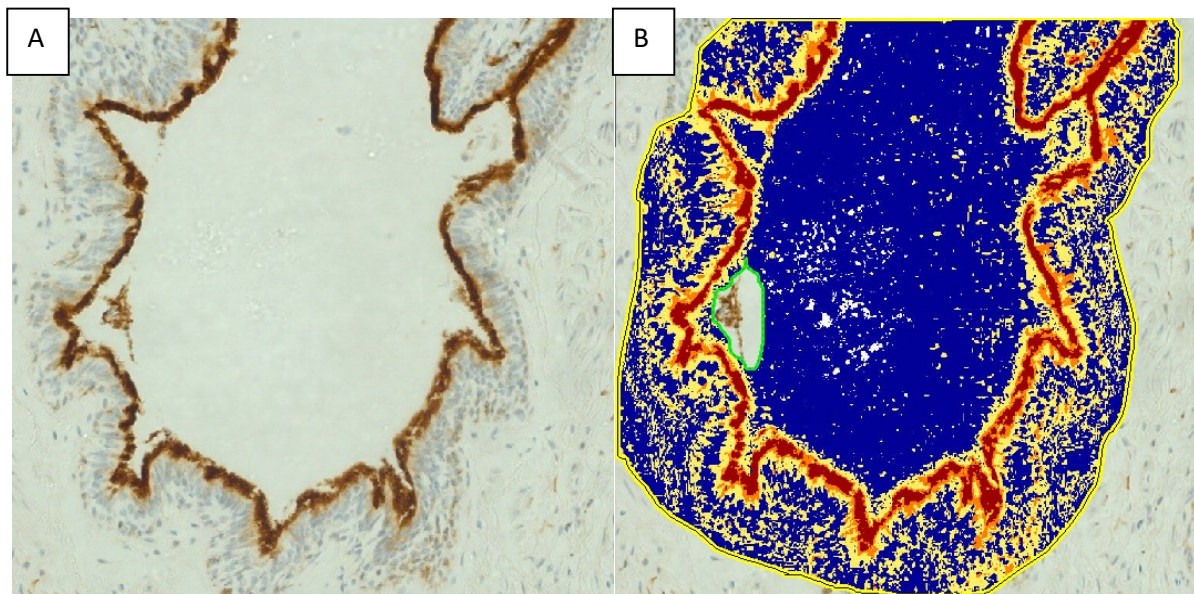


Figure 4.1 Representative picture of Aperio ImageScope staining quantification showing an airway stained for ciliated cell marker ACT **A)**, and the stained airway post quantification of staining using ImageScope positive pixel algorithm, with a small area of staining detected intraluminal staining excluded from the analysis **B)**. Strong positive staining is indicated by red areas, moderate positive staining by orange areas.

4.1.3.2 Isolation and culture of HBECs from bronchial brushings

HBECs were isolated from brushings of patients undergoing clinically indicated bronchoscopy and bronchoalveolar lavage to confirm the diagnosis of IPF. Bronchial epithelial cells were obtained from

these individuals (n=10) by brushing of sub-segmental bronchi under direct bronchoscopic visualisation following collection of BAL. Healthy control HBECs were either cultured from bronchoscopic brushing of healthy control volunteers as above (n=2), or bought from Lonza (n=3). Demographics of subjects and controls detailed in table 4.3.

HBECs were expanded in culture and used between passage 1 and 3. HBECs were obtained from bronchial brushings of large airways and mRNA expression of HBE phenotypic markers was determined using reverse transcriptase polymerase chain reaction (RT-PCR) of RNA from cultured bronchial epithelial cells at passage 1. Cells were examined for gene expression of 5 phenotypic cell markers (table 4.4).

Table 4.3

	Control	IPF
n number	5	10
Age	62 (32.5-67)	74 (69.75-80)
% male	100	100

Table 4.3 Demographics of study subjects from whom bronchial epithelial cells were obtained

Table 4.4

Marker	Gene	Function
Cytokeratin 5	CK5	Basal cell marker
Mucin 5AC	MUC5AC	Mucin component
Mucin 5B	MUC5B	Mucin component
β –Tubulin 4A	TUB β 4A	Ciliated cell marker
Secretoglobin 1A1	SCGB1A1	Club cell marker

Table 4.4 Gene expression analysed in cultured HBECs; protein, name and protein function of genes analysed in cultured HBECs.

4.1.3.3 HBEC gene expression by RT-PCR

Baseline mRNA expression of IL-33 and its receptor complex elements was determined using RT-PCR of RNA from passage 1 HBECs from IPF (n=13) and healthy control (n=4).

4.1.3.4 HBEC qPCR array

HBECs were cultured and RNA extracted at passage 1 from individuals with IPF (n=4) and healthy control (4) and a panel of 80 fibrosis associated genes examined by PCR array (full list of genes examined detailed in supplementary table S.1).

All gene expression analysis was performed using RT² Profiler PCR Array Data Analysis version 3.5. The software calculated the average delta CT (Δ CT) using the following equation; Δ CT= Average CT GOI (gene of interest) – Average CT HKG (housekeeping gene), which was followed by calculation of

delta delta CT e.g. $\Delta\Delta CT = \Delta CT \text{ (IPF cells)} - \Delta CT \text{ (Healthy cells)}$. Fold change was then calculated from the $\Delta\Delta CT$ (Expression Fold change = $2^{-\Delta\Delta CT}$). Statistical analysis was performed using RT² Profiler PCR Array Data Analysis version 3.5, determining significant differences comparing fold change using an unpaired T test with p values less than 0.05 being classified as significantly different.

4.1.3.5 HBEC proliferation

Proliferation was examined by real time cell imaging using the Incucyte ZoomTM, a live-cell imaging and analysis platform that enables automated quantification of cell behaviour over time. IPF and healthy control HBECs were seeded in triplicate onto cell culture plates and imaged at regular intervals over a period of 44 hours to create a time lapse video of HBEC proliferation. The resultant images were analysed using Incucyte analysis software to quantify the area of cell wells occupied by HBECs over the time period. Despite normalising the number of cells seeded per well, there was significant variation in the confluence of the cells in each well at time point 0, and therefore the rate of proliferation was analysed and expressed as a fold change from confluence at seeding point to control for this variable.

4.1.3.6 Immunofluorescent staining of HBECs

Protein expression of cultured HBECs between passage and 2-3 was examined by immunofluorescent staining. IPF (n=2) and healthy control (n=2) HBECs were seeded onto collagen coated chamber slides and grown to confluence. When confluence was confirmed by microscopy, cells were stained with primary antibody. Quantification was not deemed to be reliable due to the

variability of staining evident in cells which had been cultured at confluence for differing lengths of time

4.1.3.7 Transepithelial electrical resistance

HBECs were seeded in triplicate into collagen coated TEER measurement plates and incubated at 37°C in a humidified atmosphere containing 5% CO₂, with media replaced every 48 hours. When impedance plateaued, plates were removed and confluence confirmed by microscopy. TEER measurement across the layer at plateau was measured and IPF was compared to control.

To assess TEER in response to stimulation, confluent HBECs were stimulated with IL-33 (100ng/ml), TGF- β (5ng/ml), IL-33 (100ng/ml) and TGF- β (5ng/ml) and with cell culture media alone and monitored over 12 hours post-stimulation. The concentration of IL-33 used was determined based on the range of previously published (1-100ng/ml) *in vitro* IL-33 treatment doses (Sanada, 2007) (Xu, 2008) (Zhu, 2012) with the higher dose chosen in light of early work (n=1) which suggested soluble collagen secretion to be increased at dose of 100ng/ml. The effects of TGF- β have been previously demonstrated to be dose dependent (Chen, 2012) (Lal, 2003) with maximal effects seen between 1 and 10ng/ml and the dose of TGF- β used for stimulation compares with other published literature, which have widely used a range between and 10ng/ml (Treutlein, 2014) (Du, 2015) (Vaughan, 2015).

4.1.3.8 Dextran perfusion assay

Tight junction permeability was assessed by measuring perfusion of a fluorescently labelled dextran substrate across a monolayer of HBECs grown to confluence on a permeable transWell insert.

HBECs were seeded into transWell inserts in triplicate and the cells were grown to a confluent monolayer and placed into 12 Well plates. Fluorescent FITC labelled dextran was added to the apical

layer of the insert and incubated for 3 hours, with samples removed from the basal compartment for colorimetric wavelength analysis at 1, 2 and 3 hours to assess the amount of diffused dextran.

Experiments were conducted in triplicate with mean of 3 Wells taken.

4.1.3.9 Stimulation of healthy fibroblasts with HBEC conditioned media

Supernatant of cultured IPF HBECs (n=6) and normal HBECs (n=4) was removed at 24 hours and added in triplicate to confluent Wells of confluent serum starved healthy control HLFs (n=1) at passage 5. HLF RNA was extracted at 6 and 24 hours and a selection of genes involved in regulating ECM turnover were examined by RT-PCR. The mean $\Delta\Delta CT$ of triplicate Wells was used for analysis.

4.1.3.10 Statistics

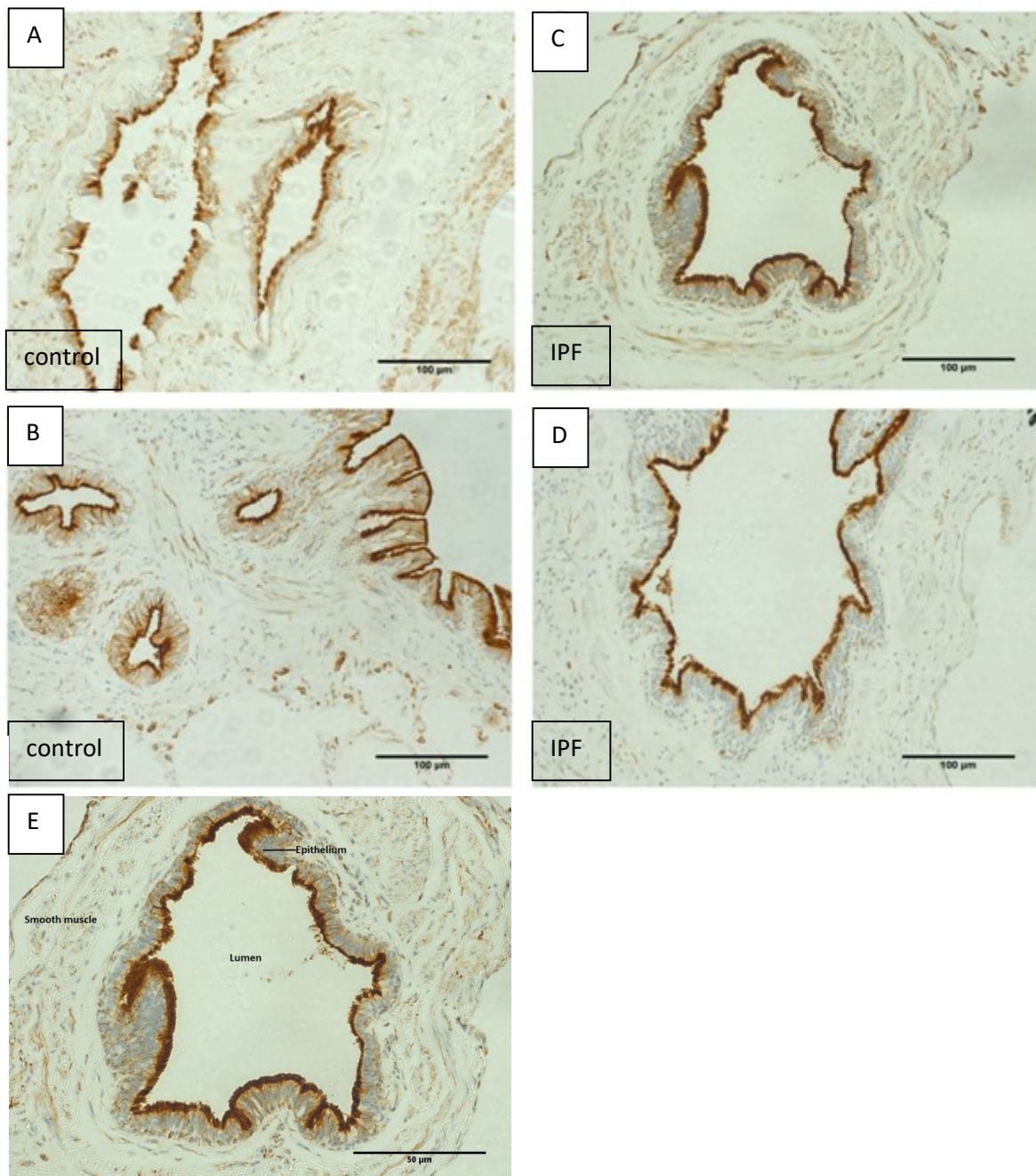
Sample sizes Were not large enough to prove Gaussian distribution of our comparator groups and this was confirmed using D'Agostino and Pearson normality tests. Values are presented as median and interquartile range and differences between non-continuous groups were compared using a Mann-Whitney U test.

4.2 Results

4.2.1 Characterisation of bronchial epithelial phenotype

The staining pattern in these samples revealed heavy staining of ACT confined to ciliated cells on the luminal aspect of the airways in both IPF and healthy control (figure 4.2). CK5, a basal cell marker, was as expected expressed on the basolateral layer within the airway epithelium (figure 4.3). Neither ACT nor KRT5 exhibited any difference in expression between IPF and healthy control but SCGB1a1 evinced strong staining of cubical non-ciliated secretory cells scattered within the epithelial layer of bronchioles (figure 4.4), with a statistically significant increase in IPF (n=16) when compared with control (n=11) (p=0.0071 all positive pixels of SCGB1a1 staining, p=0.0087 strong positive pixels of SCGB1a1 staining). MUC5AC was found in the lumen and scattered in goblet cells in IPF tissue, however only small amounts were detected in healthy control tissue and no staining was found in cells in the epithelial layer in healthy control (figure 4.5). There was a statistically significant increase in epithelial but not luminal MUC5AC detected (control n=11, IPF n=16; epithelial MUC5AC p=0.0218) with the length of the airways measured in the study significantly longer in the IPF group (n=16) than the control group (n=11) p=0.0049). Values are shown as median and interquartile range unless otherwise stated.

Figure 4.2



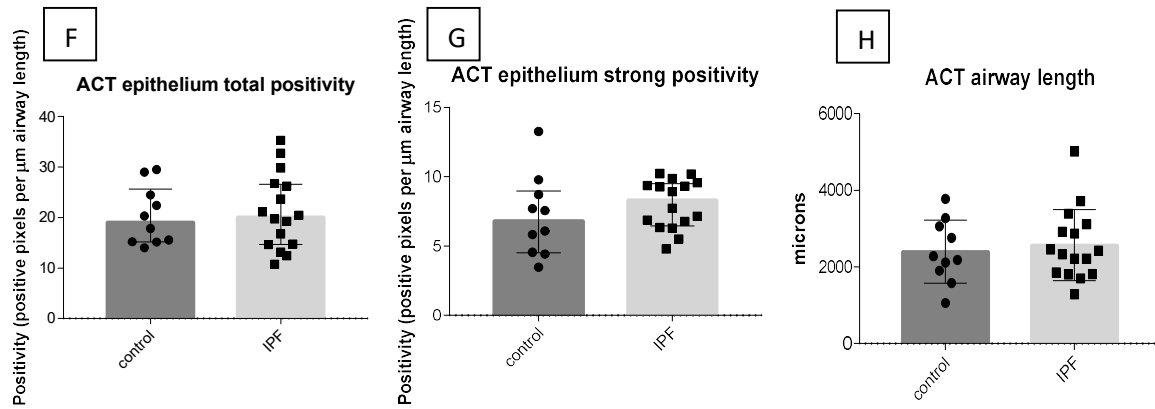
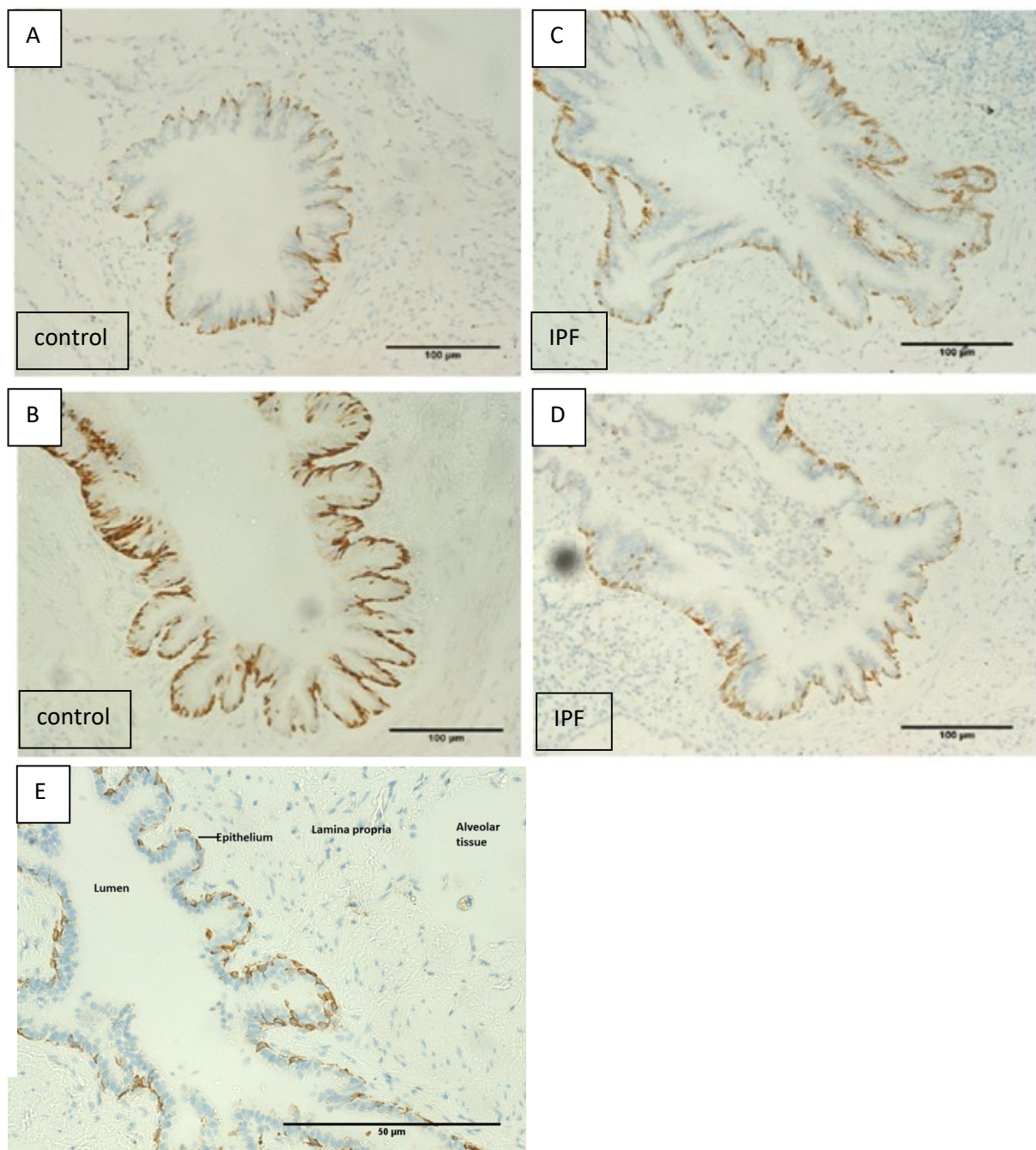


Figure 4.2. Representative image of staining and quantification of ACT in IPF and control; In all sections, strong staining was confined to the apical region of the airway epithelium Healthy control **A-B**) and IPF **C-D**). Representative image with marked features of interest **E**). There was no difference detected between the IPF (n=16) or control groups (n=7) when analysed for total positive staining of epithelial ACT **F**) strong positive staining of ACT or **G**) length of airways measured **H**).

Figure 4.3



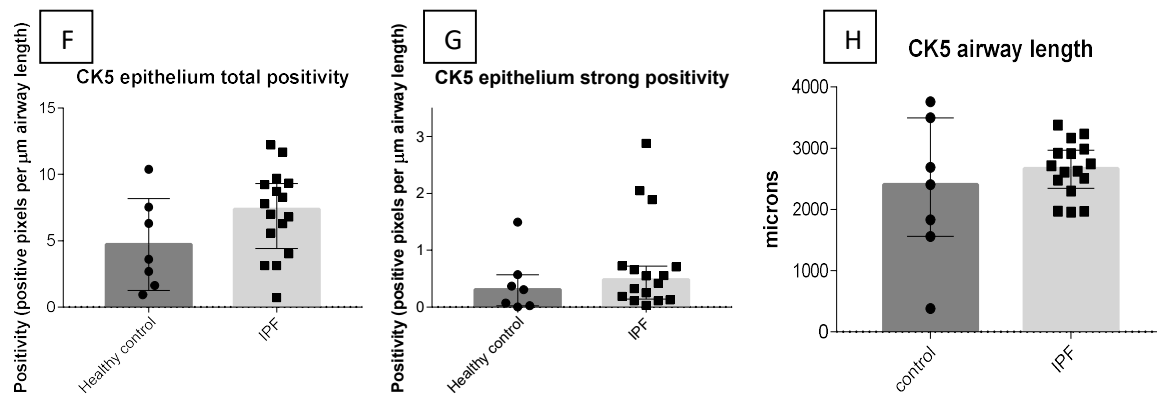
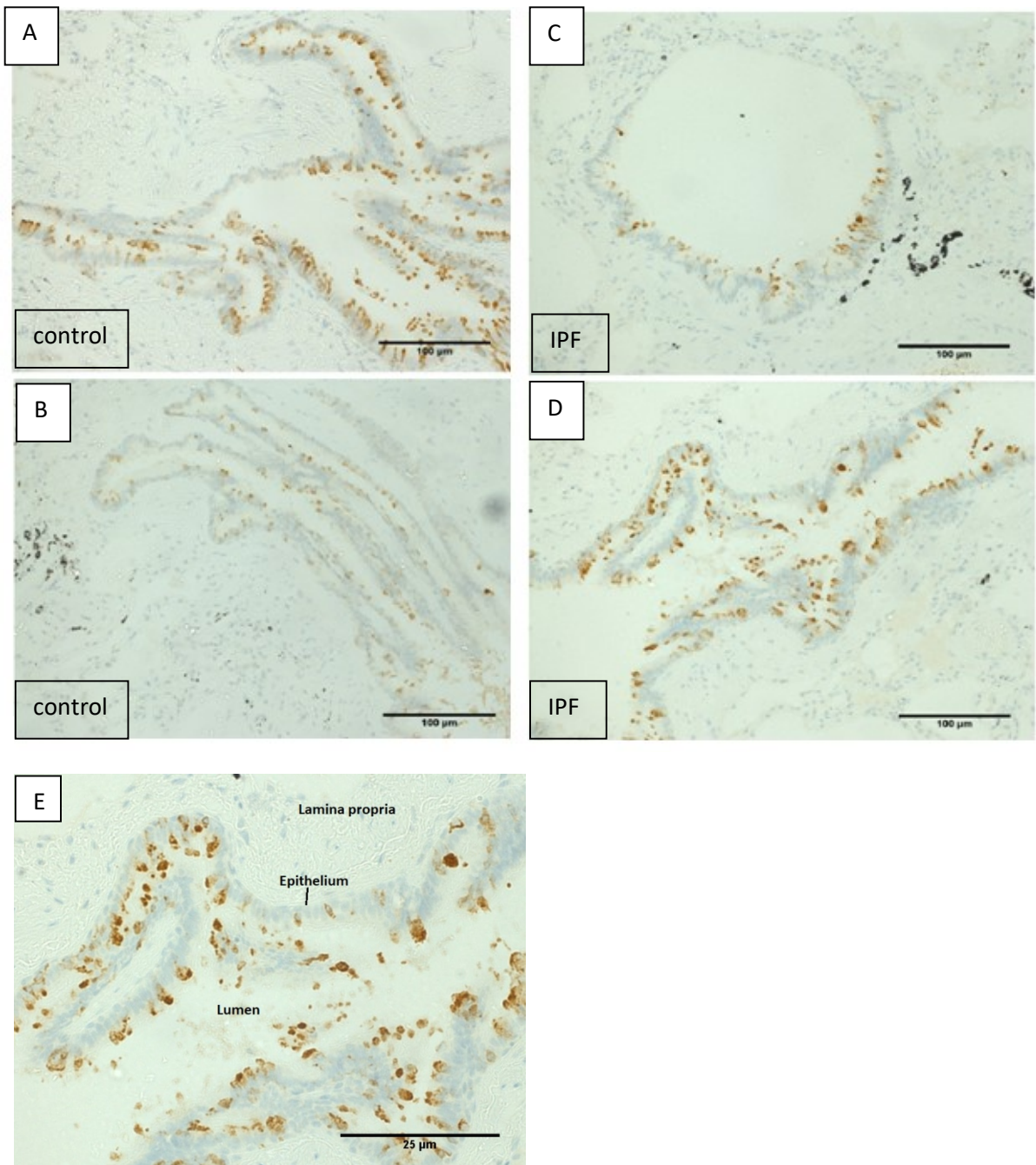


Figure 4.3 Representative images and quantification of cytokeratin 5 (CK5) staining in IPF vs control airways; KRT5 positively labelled basal cells displaying a strong staining within the airway epithelium of healthy control **A-B)** and IPF **C-D)** with no staining detected in other regions of the stained tissue. Staining was seen in a monolayer of cells in the basolateral later of the airway epithelium. Representative image with labelled features of interest **E)** There was no difference detected between the IPF (n=16) or control groups (n=7) when analysed for total positive staining of epithelial CK5 **F)** strong positive staining of CK5 **G)** or length of airways measured **H)**.

Figure 4.4



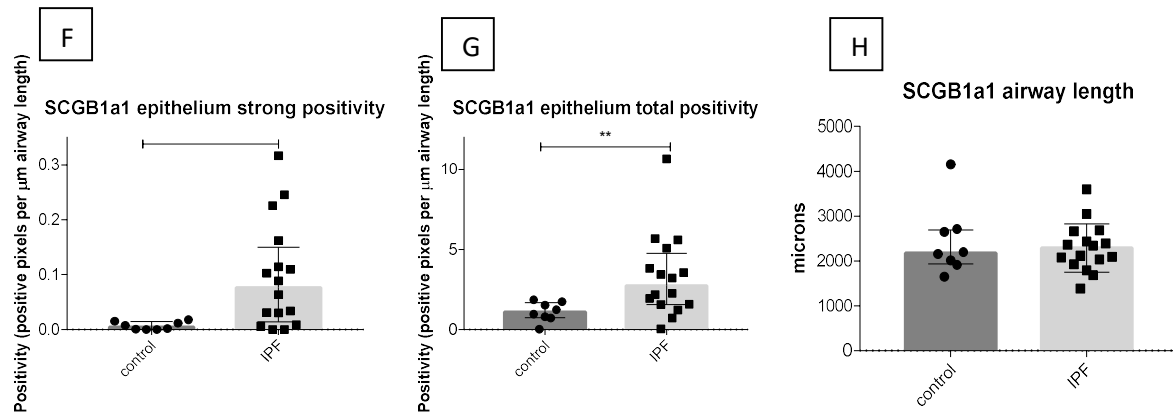
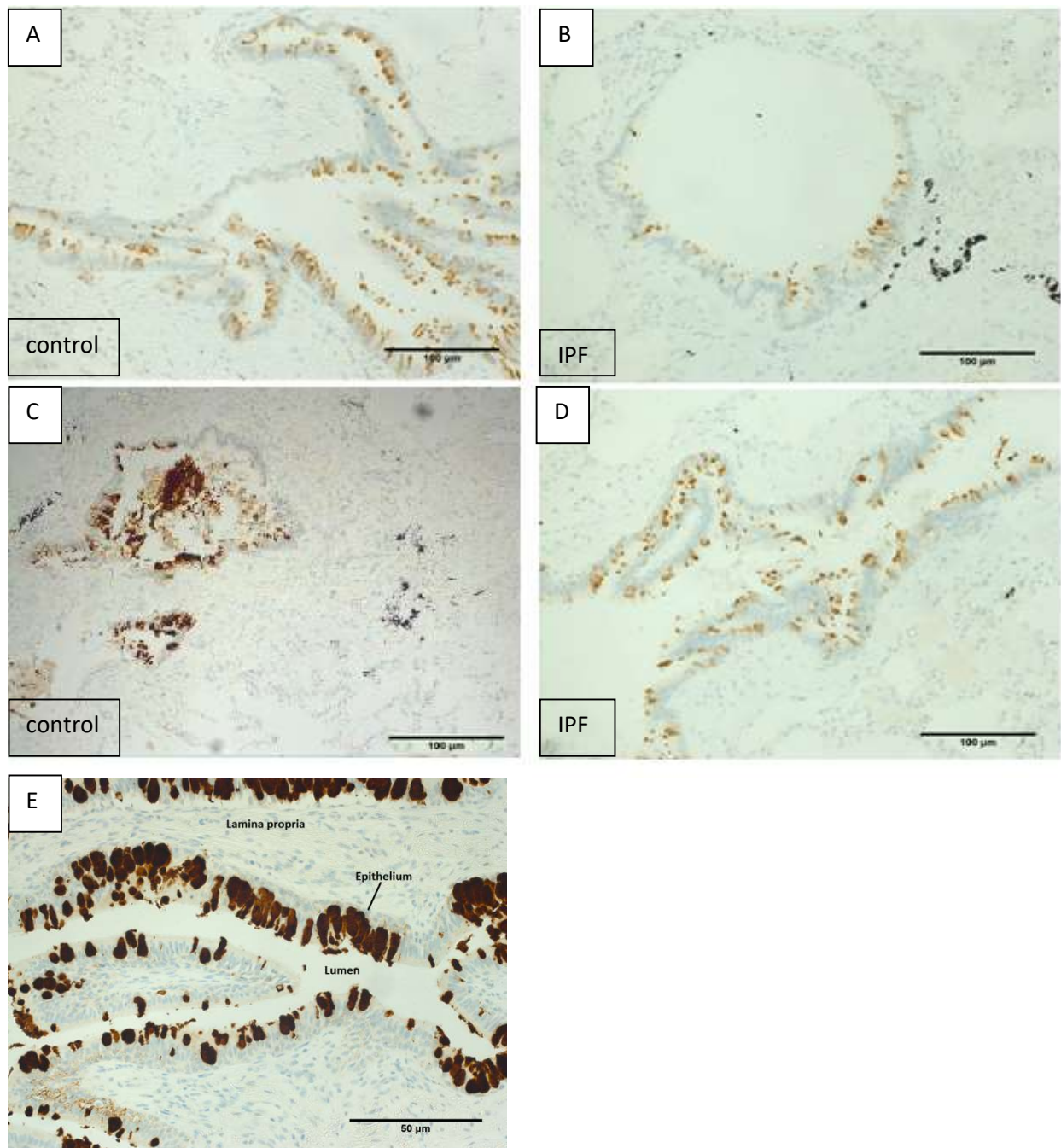


Figure 4.4 Representative images and quantification of club cell marker (SCGB1A1) staining in IPF vs control airway; healthy control **A-B)** and IPF **C-D)** showing a pattern of staining restricted to airway epithelium, with positive staining found in columnar to cuboidal cells, consistent with the morphology of club cells. Representative image with labelled features of interest **E)**. A significant increase was detected in IPF (n=16) over healthy control (n=8) for both total positive pixels of SCGB1A1 staining (p=0.0071) **F)** and strong positive SCGB1A1 staining (p=0.0087), when expressed per μm of measure airway length **G)**. There was no significant difference in the length of the airways measured between the groups **H)**.

Figure 4.5



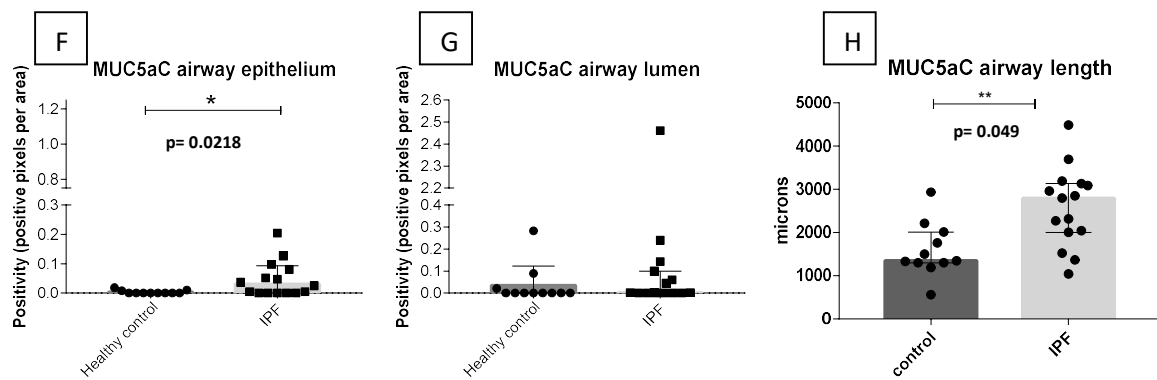


Figure 4.5 Representative images of MUC5AC staining in IPF vs control airway; Mucus positive goblet cells were found scattered within the airway epithelium in control patients **A-B)** and IPF **C-D)**. Representative image with labelled features of interest **E)**. A significant increase was detected in IPF (n=16) over healthy control (n=11) total positive pixels of MUC5AC staining in the epithelium **F)** but there was no significant difference detected in airway lumen **G)**. The airways in the IPF cohort were significantly longer than in the control group (p=0.0049; unpaired t-test, data shown as mean and SD) **H)**.

4.2.2 Characterisation of cells from bronchial brushings

This characterisation of airway epithelium demonstrated phenotypic differences between IPF and healthy control. Taken together with previously published work, these data offer additional supportive evidence of mucin dysregulation in the IPF lung, as well as an increase in club cells in IPF epithelium. The next aim was to investigate the functional capacity of bronchial epithelium in IPF and to assess its possible direct contribution to the fibrotic process through direct effects on genes involved in previously described fibrogenic signalling pathways and direct effects on regulation of extracellular matrix deposition.

Cultured bronchial epithelial cells were isolated from brushings of IPF (n=10) individuals and healthy controls (n=5) for *in vitro* investigation of function. Gene expression of cells at passage 1 demonstrated these to be a purified population of basal cells which highly expressed basal cell

marker protein KRT 5 (figure 4.6)- a protein coding for a type II cytokeratin which forms the intermediate filaments of basal epithelial cells. Low expression of secretoglobin family 1A member 1 (SCGB1A1)(figure 4.6), a protein specifically expressed by club cells was detected in 3 of 15 samples, and low levels of TUB β 4A – a key component of the tubulin cytoskeleton ubiquitously expressed in ciliated cells – was detected in 4 of the 15 samples (figure 4.7). The cultured cells did not express MUC5A or MUC5B mRNA . No differences were detected between the expression of these genes in the HBECs obtained from IPF and healthy control.

Figure 4.6

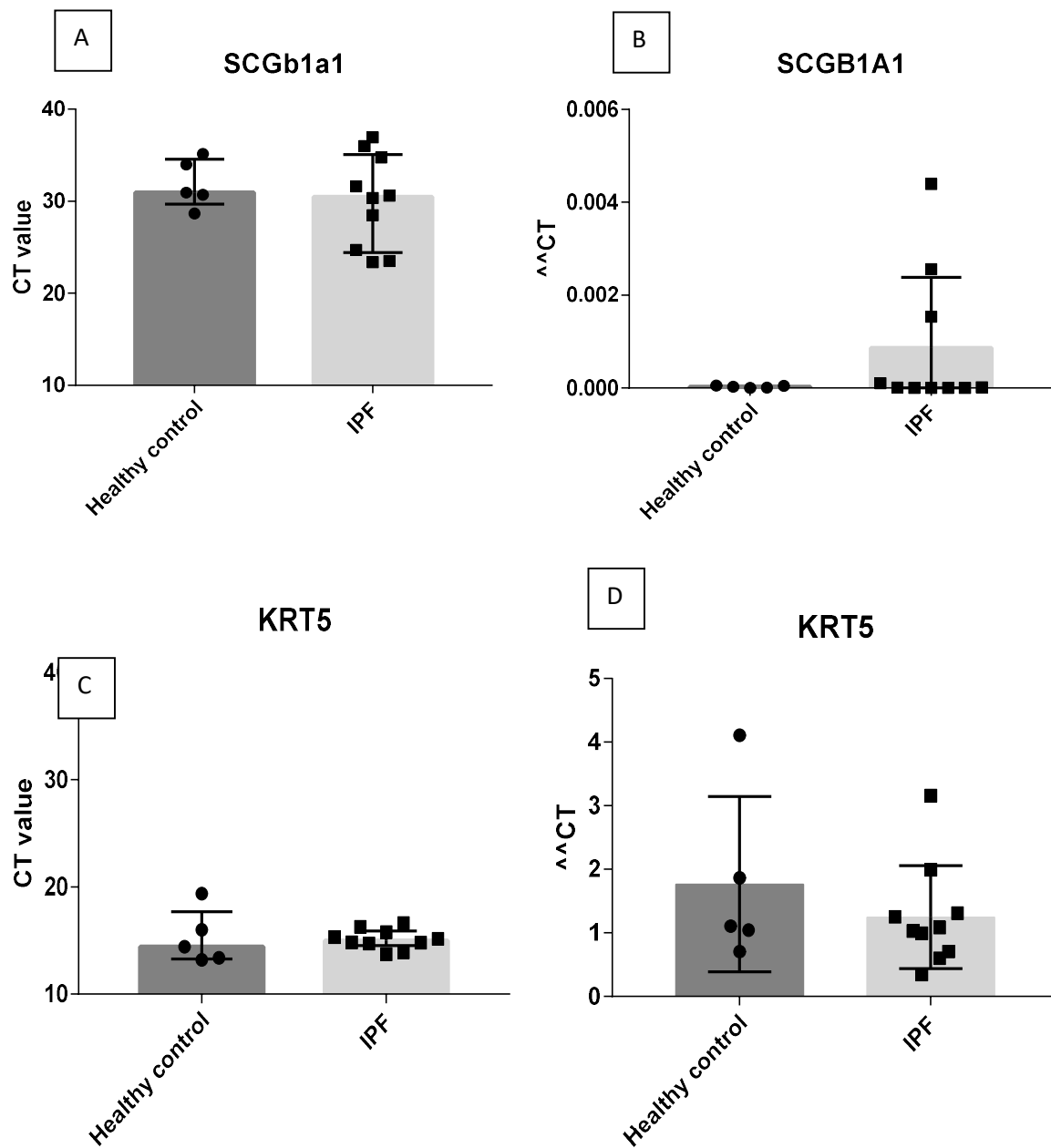


Table 4.6 RNA expression of SCGB1A1 and KRT5 of cultured HBECS from IPF and control; CT and $\Delta\Delta CT$ values of gene expression in IPF (n=10) and healthy control (n=5) HBECS; SCGB1A1 CT values by qPCR in IPF and control HBECS **A)** and $\Delta\Delta CT$ of SCGB1A1 expression in IPF and control HBECS **B)** HBECS KRT5 CT values by qPCR in IPF and control HBECS **C)** and $\Delta\Delta CT$ values of KRT5 expression in IPF and control HBECS **D)**

Values shown as median and interquartile range

Figure 4.7

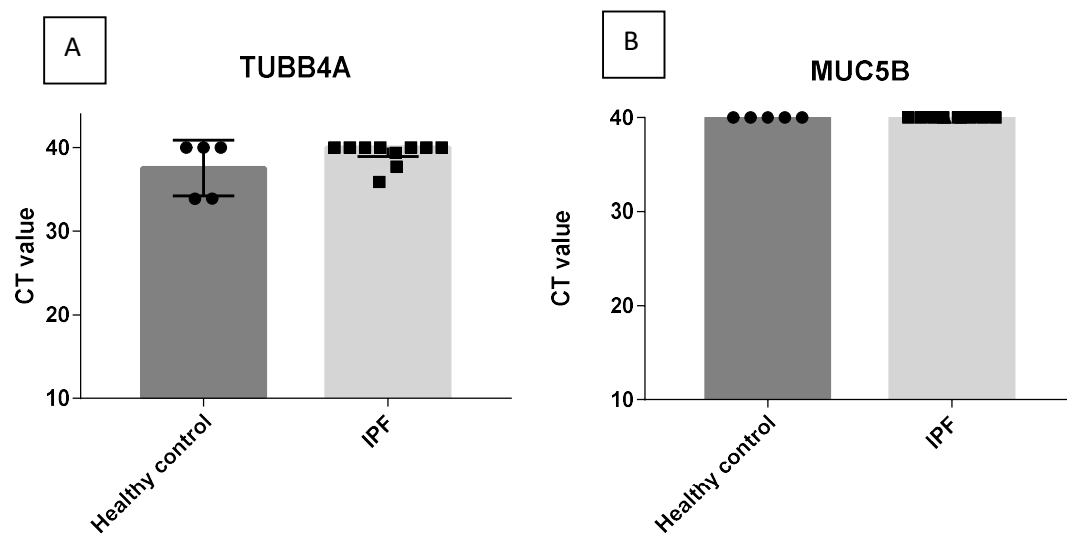


Figure 4.7 Gene expression of cultured healthy control (n=5) and IPF (n=10) HBECs obtained from bronchial brushings, at passage 1; qPCR data presented as CT values showing TUBB4A expression A) and MUC5B expression B) in IPF vs control.

Values shown as median and interquartile range

4.2.3 HBEC gene expression

Idiopathic pulmonary fibrosis is widely thought to be a disease characterised by abnormal cross-talk between injured alveolar epithelial cells, with aberrant cell signalling responses leading to a propagation of the fibrotic process. There is a strong body of evidence detailing the differences in the transcriptome of IPF alveolar epithelium, but comparably little available evidence detailing the regulation of the fibrosis associated gene pathways in airway epithelium. Having characterised the cells obtained from bronchial brushings and established them to be a population of basal epithelial cells, I next sought to investigate the regulation of a panel of genes traditionally associated with the fibrotic process. 4 of the 80 genes showed a statistically significant upregulation (table 4.5, figure 4.8); Thrombospondin (THBS1) ($p=0.03407$, fold change= +1.648), Chemokine receptor ligand 4

(CXCR4) ($p=0.03579$, fold change= +1.582), endoglin ENG) ($p=0.04141$, fold change= +1.910) and integrin α 1 (TGA1) ($p=0.01941$, fold change= + 2.109), with only ITGA1 evincing an above 2-fold up-regulation.

No genes were statistically significant down regulated in the IPF cohort as compared with control.

Figure 4.8 PCR array of pro-fibrotic genes in IPF vs control

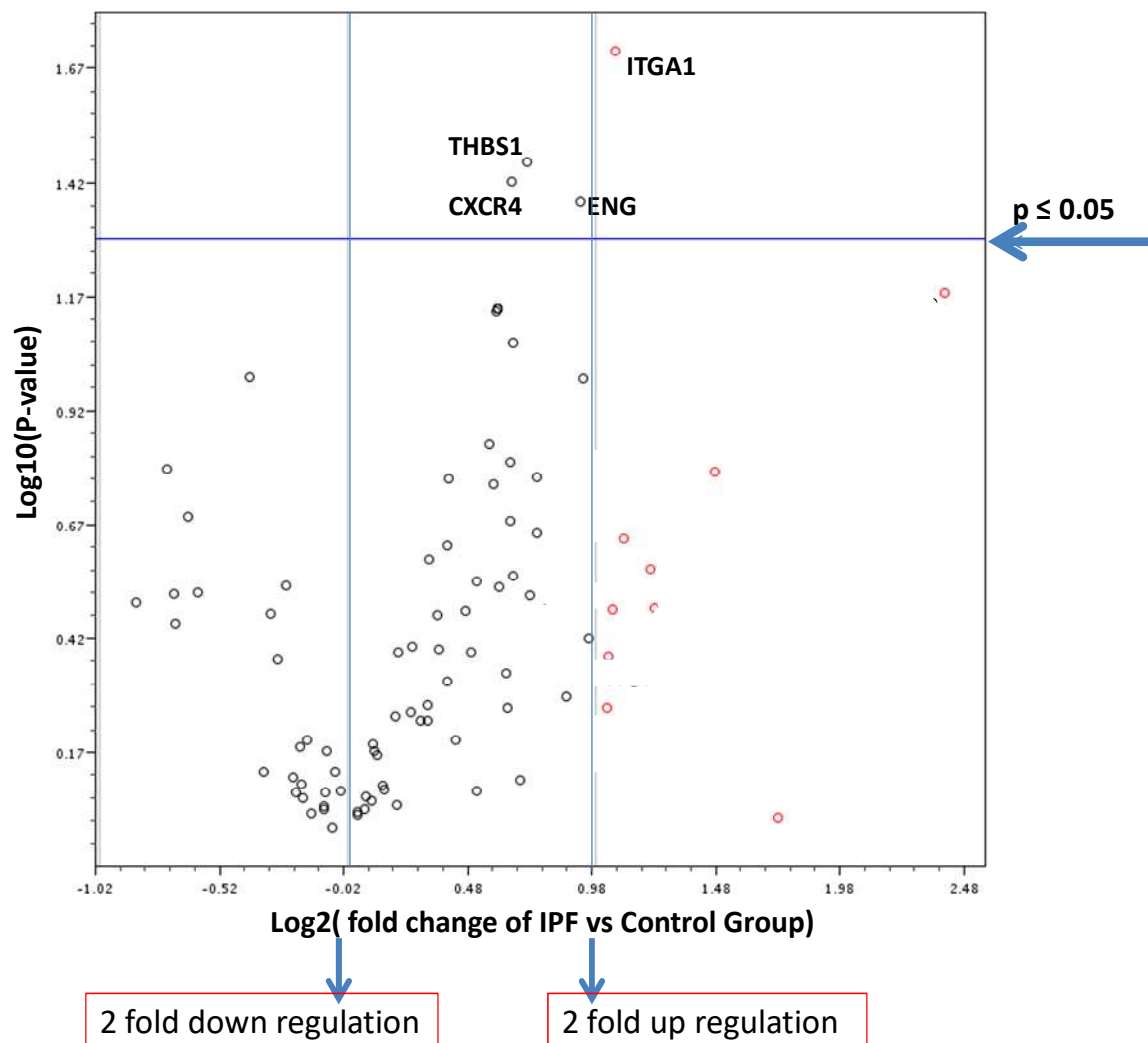


Figure 4.8 shows volcano plot of 84 genes included in fibrosis array. Plot shows the fold regulation of IPF ($n=7$) genes compared against control ($n=4$). Each individual plot on the graph corresponds to a single gene. Genes from IPF samples exhibiting significant ($p < 0.05$) fold change from control are

represented above the horizontal line. ITGA1, THBS1, CXCR4 and ENG genes were significantly up-regulated in IPF HBECs as compared with control. No genes were significantly down-regulated.

ITGA1=integrin α 1, THBS1=thrombospondin1, CXCR4= chemokine receptor ligand 4, ENG= endoglin

Table 4.5

	Mean CT			
	Healthy control	IPF	Fold-regulation	p-value
ITGA1	28.82	28.35	+ 2.1091	0.01941
CXCR4	35	34.94	+1.5816	0.03759
THBS1	21.28	21.16	+1.6481	0.03407
ENG	28.66	28.33	+1.9095	0.04141

Table 4.5 Summarising qPCR array data of statistically significantly up regulated genes;

CT values, p-values and fold regulation of genes found to be significantly upregulated by PCR array.

No genes were statistically significantly down regulated.

ITGA1= integrin α 1, ENG=endoglin, THBS1=thrombospondin1, CXCR4=chemokine receptor 4

Our examination did not reveal a skew towards differential expression of many traditionally fibrotic genes leading us to conclude that any effect that may be exerted by the bronchial epithelium in IPF may be through novel pathways not conventionally heavily associated with fibrosis. This lead us to examine the regulation of a the IL-33/ST2 signalling axis - consisting of IL-33, its receptor ST2, the membrane bound negative regulator SIGIRR and the soluble decoy receptor soluble ST2..

Gene expression results by qPCR revealed a statistically significant up-regulation of SIGIRR in the IPF group and a trend towards an increase in IL-33, ST2 and IRAP in the IPF group which did not reach statistical significance. The expression of IL-33 and ST2 both showed low level expression in 1 cluster of HBECs, with a second cluster of samples with higher expression (figure 4.9). The numbers in this study were small but I found no clinical parameters clearly associating with either our higher or lower expression clusters of expression of IL-33 or ST2. This dataset included information on age, gender, pre bronchoscopy lung function and presence of infection at time of bronchoscopy.

Figure 4.9

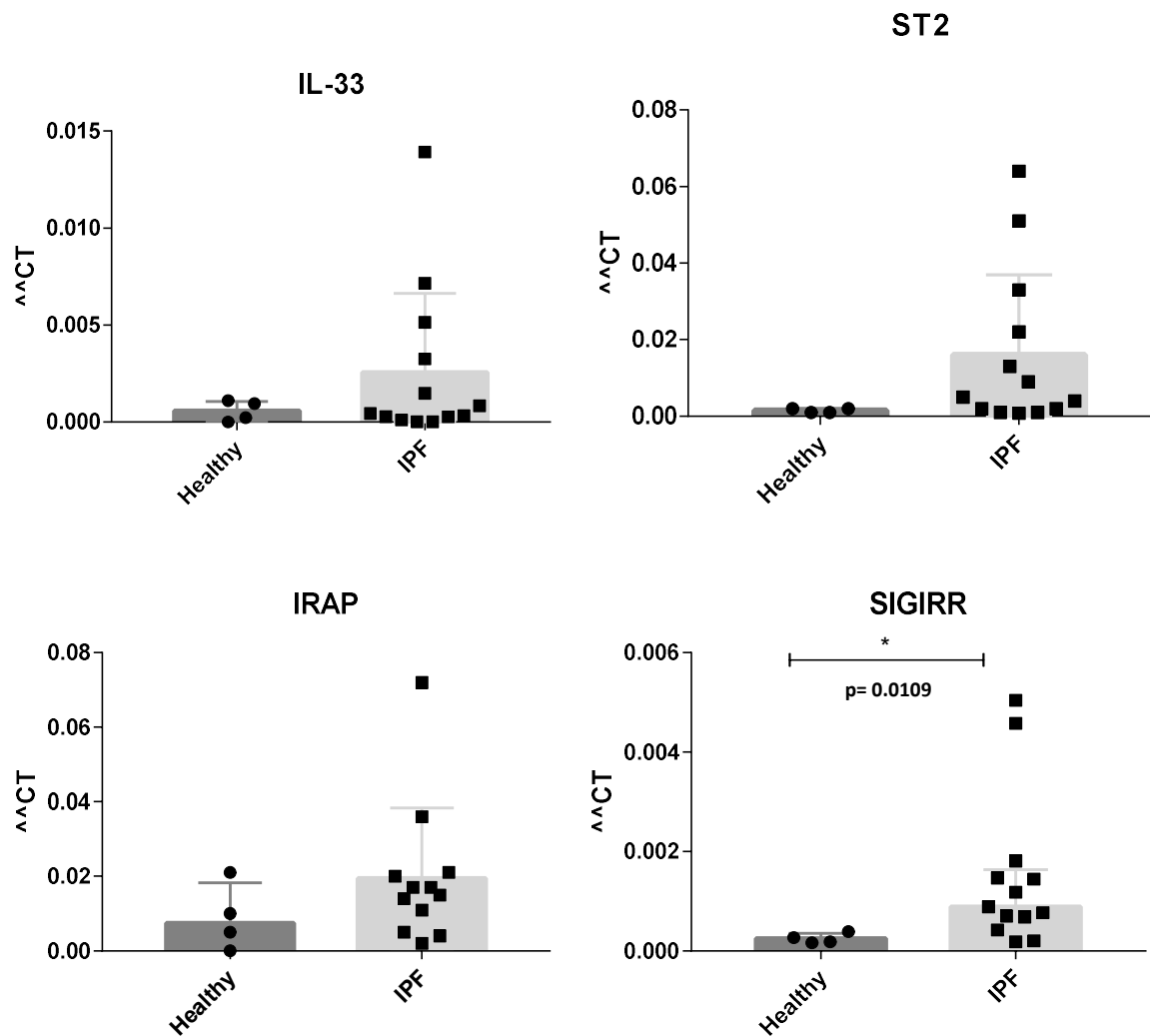


Figure 4.9 IL-33 receptor axis gene expression in IPF and healthy control HBECS; mRNA expression of control (n=4) and IPF HBECS (n=13) by qPCR from cultured cells at passage 1. Results showed a trend towards higher expression of IL-33 **A)** ST2 **B)** and IRAP **C)** in IPF HBECS as compared to control but no statistically significant difference was detected between the groups. These data did demonstrate a statistically significant increase in SIGIRR expression in IPF over control ($p=0.0109$, Mann Whitney U test) **D)**

4.2.4 In vitro bronchial epithelial cell functions

4.2.4.1 Proliferation

Having determined that I had isolated and cultured a purified population of basal epithelial cells with high expression of basal cell marker KRT5 and sporadic or absent expression of ciliated, mucin producing or club cell marker proteins, functionality was then investigated by *in vitro* investigation of HBEC proliferation and barrier function.

Proliferation was examined by real time cell imaging and these data did not reveal any statistically significant differences between the rates of proliferation in cultured IPF HBECs (n=4) when compared to those of healthy controls (n=4) (figure 4.10). Data was analysed by Mann whitney U test comparing IPF v control at each individual timepoint.

Figure 4.10

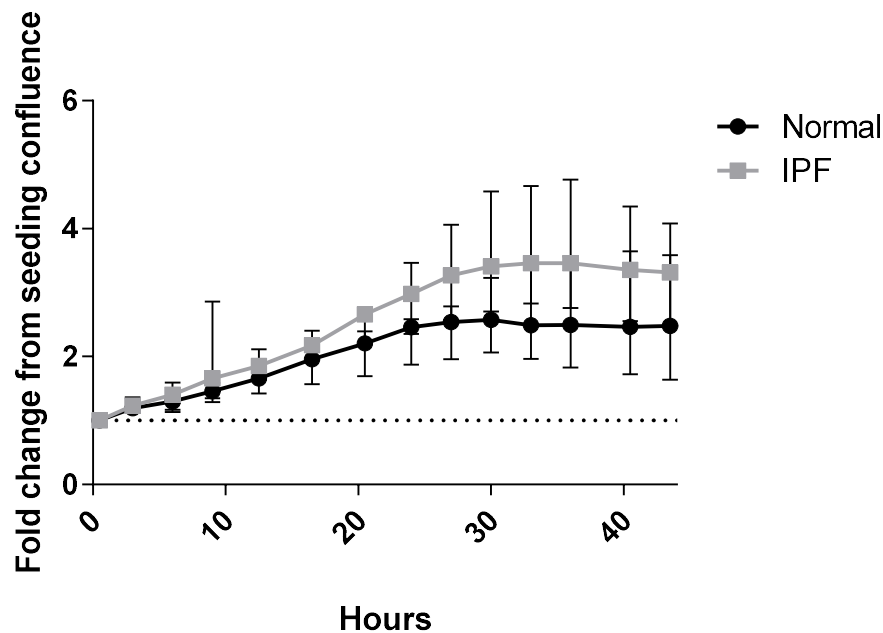


Figure 4.10 Proliferation rates in IPF and healthy control HBECs; IPF (n=4) and healthy control (n=4)

HBEC proliferation rates over 44 hour time period. HBECs were seeded in triplicate into culture Wells (at the 0 hour time point) and live real time cell images taken at regular intervals over the subsequent 44 hours. Values expressed as fold change from the initial seeding density, to normalise for the density of cells initially seeded.

4.2.4.2 Tight junction formation

Having not demonstrated any differences in the proliferative capacity of basal HBECs, I moved on to examine the formation and function of tight junctions in bronchial epithelium.

Immunohistochemical staining showed that in cultured confluent human bronchial epithelial cells zona occludens 1 (ZO-1) expression was detected in confluent IPF and control HBEC but did not demonstrate expression of e-cadherin or claudin. ZO-1 was expressed in confluent areas of densely cultured cells in both groups and did not form in sparse areas of cellular coverage with no cell to cell

adhesion (figure 4.11). This did not suggest any clear evidence of disruption of the ability to form intact tight junction in basal epithelial cells in IPF (figure 4.11).

To investigate this further I aimed to establish if there was any functional difference between the nature of the *in vitro* barrier formed by IPF cells.

Figure 4.11

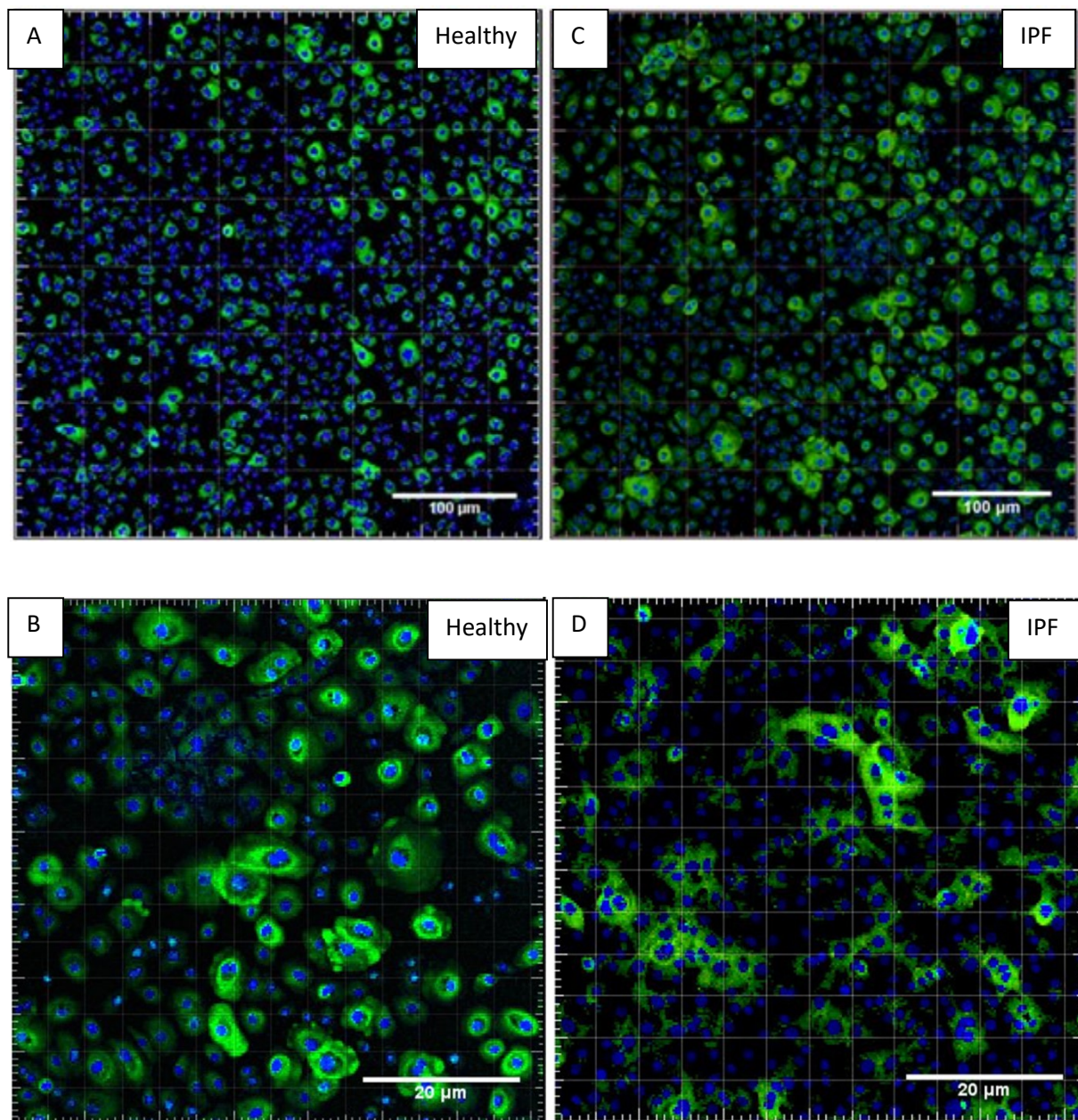


Figure 4.11 ZO-1 expression in HBE tight junctions; ZO-1 staining in control HBEs A-B) IPF HBEs C-D). IPF and control HBECs were seeded into chamber slides and grown to confluence and fixed for staining. Primary ZO-1 antibody was conjugated to a FITC secondary antibody, with ZO-1 therefore staining green, and cell nucleus staining blue. Staining was detected in both IPF and control with staining predominantly found circumferentially between cells in close contact, forming tight

junctions. . Representative images from observations from cultured HBECs from 4 individuals with IPF and 4 from healthy control

4.2.4.3 Transepithelial electrical resistance

Our work revealed normal formation and expression of ZO-1 expression in IPF HBECs in sub-merged culture. I subsequently examined tight junction function by measuring the trans-epithelial electrical resistance (TEER) of cultured monolayers of IPF and healthy control HBECs. This revealed a trend to an increase in transepithelial electrical resistance across confluent monolayers of IPF HBECs when compared to control but the n numbers were small (figure 4.12).

Figure 4.12

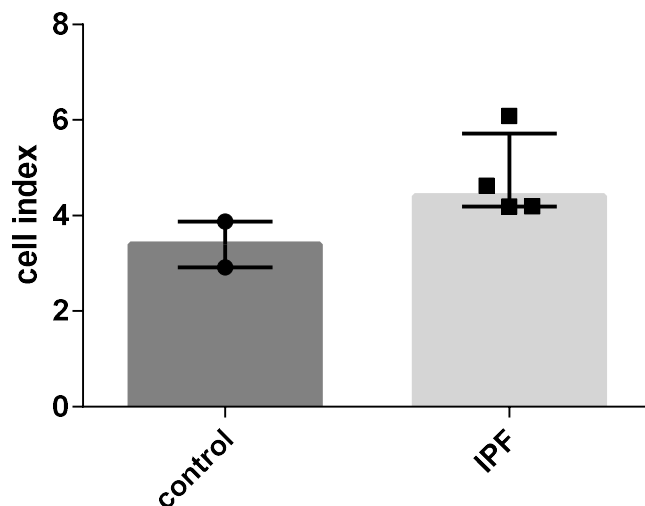


Figure 4.12 transepithelial resistance in IPF and healthy control HBECs; median and interquartile range of peak TEER measurements of confluent monolayer of IPF (n=4) and healthy control (n=2) HBECs. HBECs were seeded in triplicate into TEER measurement plates and grown until confluent.

The mean of the peak electrical impedance (measured as cell index) of triplicate Wells was taken and IPF compared to control.

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4.2.4.4 Tight junction permeability

To further interrogate the nature of the epithelial barrier function in IPF, the ability to regulate flow from the apical to the basal compartments was examined by measurement of the diffusion of soluble solute across a cultured monolayer of IPF and healthy control HBECs.

These results showed no difference in the diffusion of dextran across IPF and healthy control HBECs across a 3-hour time course. This work revealed the barriers formed in vitro by IPF HBECs to have equivalent permeability to those of healthy control HBECs (figure 4.13) and -taken together with the insights gleaned from our immunohistochemical analysis of tight junction proteins- does not support the hypothesis that the barrier formed by the airway epithelium in IPF is dysfunctional.

Figure 4.13

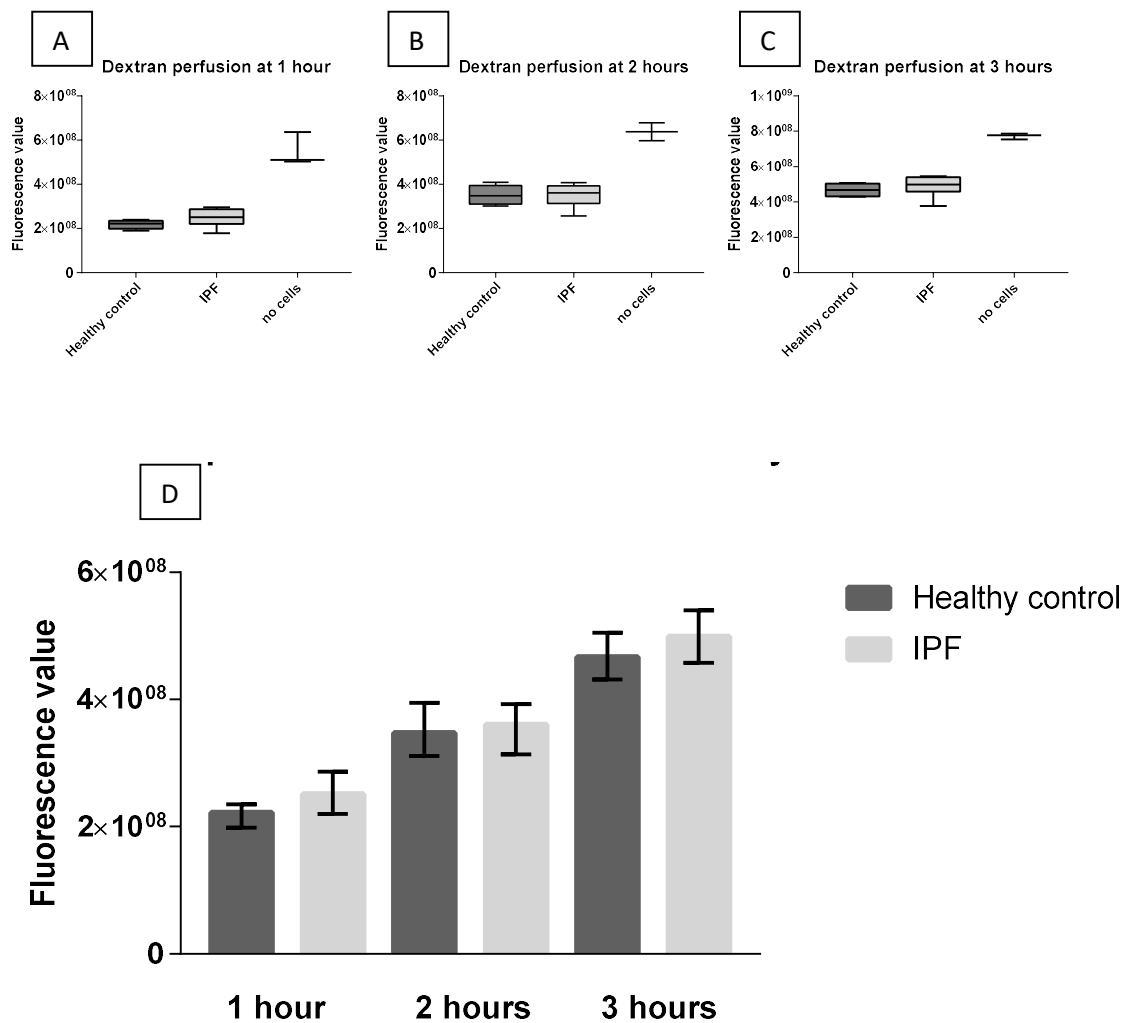


Figure 4.13 Perfusion of fluorescently labelled solute across confluent HBE monolayer; dextran perfusion across HBEC monolayer across a 3 hour time course. HBECs were seeded onto transwell inserts and grown to confluence. When confluent, fluorescently labelled soluble solute was added to the apical segment of the insert and the optical density of solute measured at 1, 2 and 3 hours. No difference was detected between IPF (n=6) and healthy control (n=4) at 1 hour **A**) 2 hours **B**) or 3 hours **C**) Dextran perfusion across the time course collated **D**).

Values presented as median and interquartile range, mean ranks compared using Mann Whitney U test.

To corroborate this finding *In vitro* barrier function in IPF HBECs was further investigated by monitoring TEER values over time in response to stimulation. TGF- β is a pluripotent cytokine, the prominence of which in fibrosis is well established and widely reported. Its effect on TEER of confluent HBECs was studied in comparison to and in combination with IL-33, the signalling axis which previously presented data suggested may exhibit dysregulated control in IPF HBECs.

This work did not show any effect of IL-33 stimulation upon either IPF or healthy control HBECs, but did show a variable response to TGF- β stimulation in IPF HBECs, but an increase in cell index as a marker of epithelial integrity in healthy control HBECs

Figure 4.14

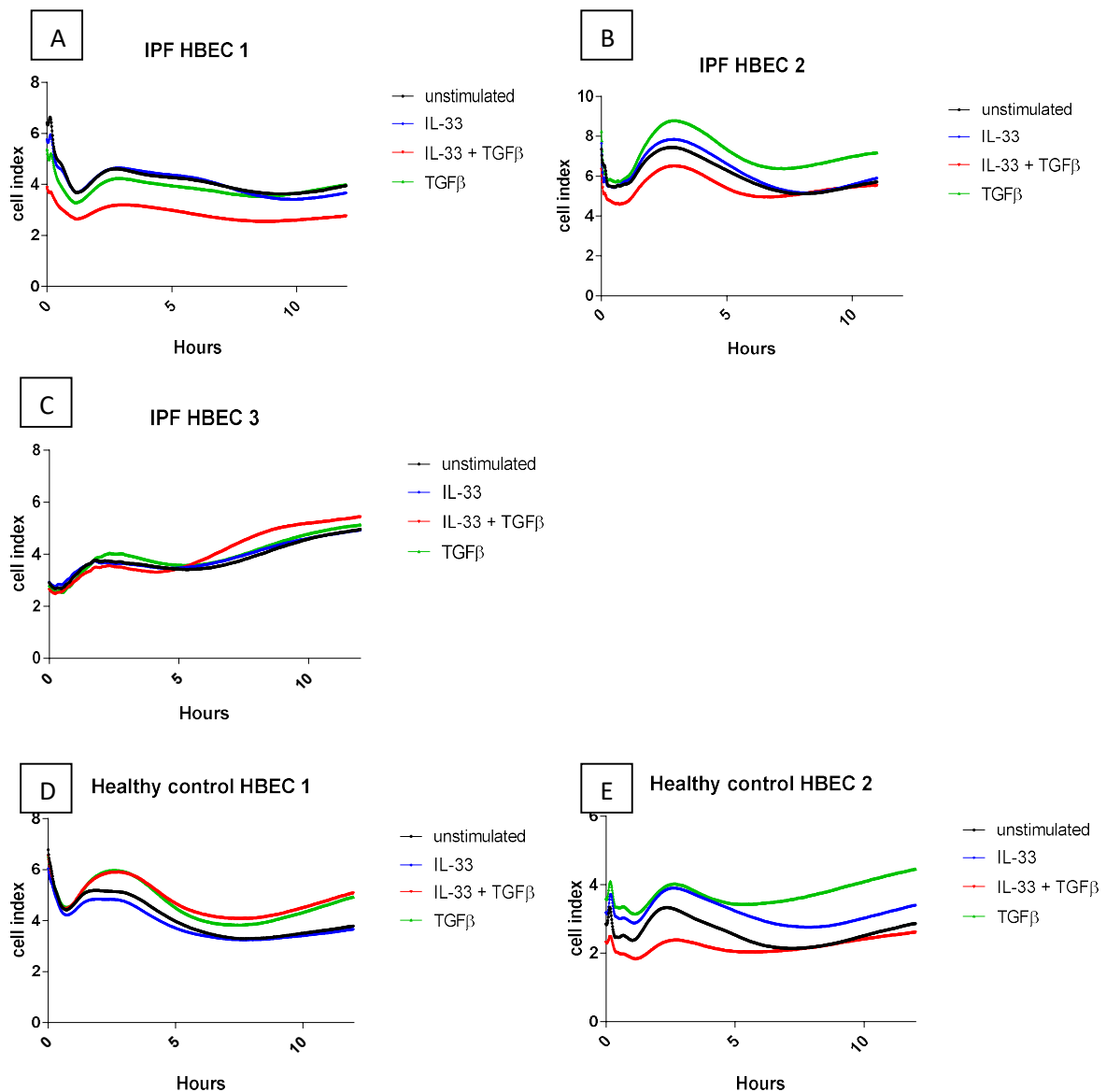


Figure 4.14 TEER derived cell index in stimulated IPF and healthy control HBECs; IPF (n=3) and healthy control (n=2) HBECs were grown to confluence and then stimulated (at hour zero) with mean of triplicate values over the subsequent 12 hours presented. 3 IPF HBEC lines **A-C) showed no effect on stimulation with IL-33 and a variable response to TGF- β treatment. 2 Healthy control HBEC lines **E-F**) showed no effect with IL-33 stimulation but showed an trend towards an increased integrity of the HBEC tight junction above upon stimulation with TGF- β when compared with the unstimulated group.**

4.2.5 Effect of HBEC supernatant on human lung fibroblasts

The data presented in this chapter so far has shown the cellular composition of the bronchial epithelium to differ from that of healthy control, with some minor differences in fibrosis associated gene expression but did not demonstrate any disruption of the proliferative capacity or barrier function of IPF HBECs. This body of work was designed to test the function of epithelial cells in isolation, to assess the indirect effects they may be having on the development of fibrosis. To investigate the direct non-immune mediated effects of IPF epithelium on the regulation of extracellular matrix deposition, I looked to the effect that HBECs have on the key fibrosis effector cell, the lung fibroblast.

I found that the supernatant of cultured IPF HBECs did not induce a pro-fibrotic response in normal human lung fibroblasts (HLFs), as measured by their effect on COL1A1, COL3A1 and α -SMA expression at 6 and 24 hours (figure 4.15).

Figure 4.15 COL1A1, COL3A1 and α -SMA expression in healthy control HLFs stimulated with supernatant from IPF and healthy control HBECs

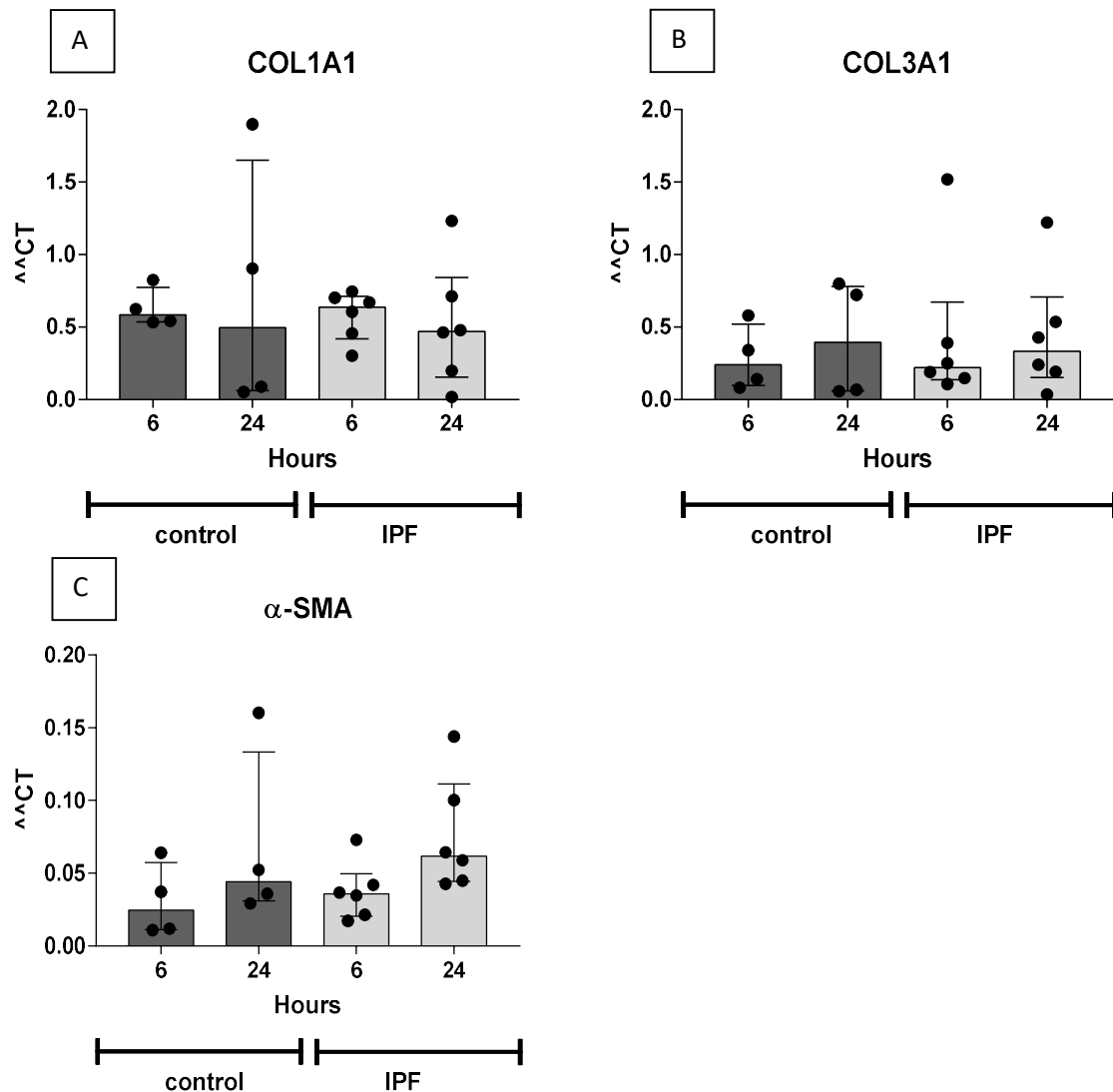


Figure 4.15 showing gene expression by qPCR of) COL1A1 **A**) COL3A1 **B**) and α -SMA **C**) in control HLFs stimulated by supernatant conditioned by IPF HBECs (n=6) and healthy control HBECs (n=4) for 24 hours. In both IPF and healthy control there was a trend towards an increase in COL3A1 and α -SMA from 6 to 24 hours but this effect was small. IPF HBEC supernatant did not result in a change in COL1A1, COL3A1 or α -SMA when compared to control (Mann Whitney U test).

Values presented as median and interquartile range of $\Delta\Delta CT$.

Having established that mediators secreted into supernatant of cultured IPF HBEs did not show any clear effect on collagen or α -SMA expression of normal HLFs, I subsequently looked to establish if these cells might be contributing to fibrosis indirectly by their effect on key pro-fibrotic pathways. I measured the expression of TGF- β 1 and its receptor TGF- β r1 and connective tissue growth factor (CTGF) - a secreted matricellular protein believed to be an important regulator of the fibrotic process (Yue et al, 2010)- and found no differences between HLFs simulated by HBE supernatant from IPF and healthy control. These results do not support the hypothesis of a direct effect of the bronchial epithelium on extracellular matrix regulation in the IPF lung.

Figure 4.16

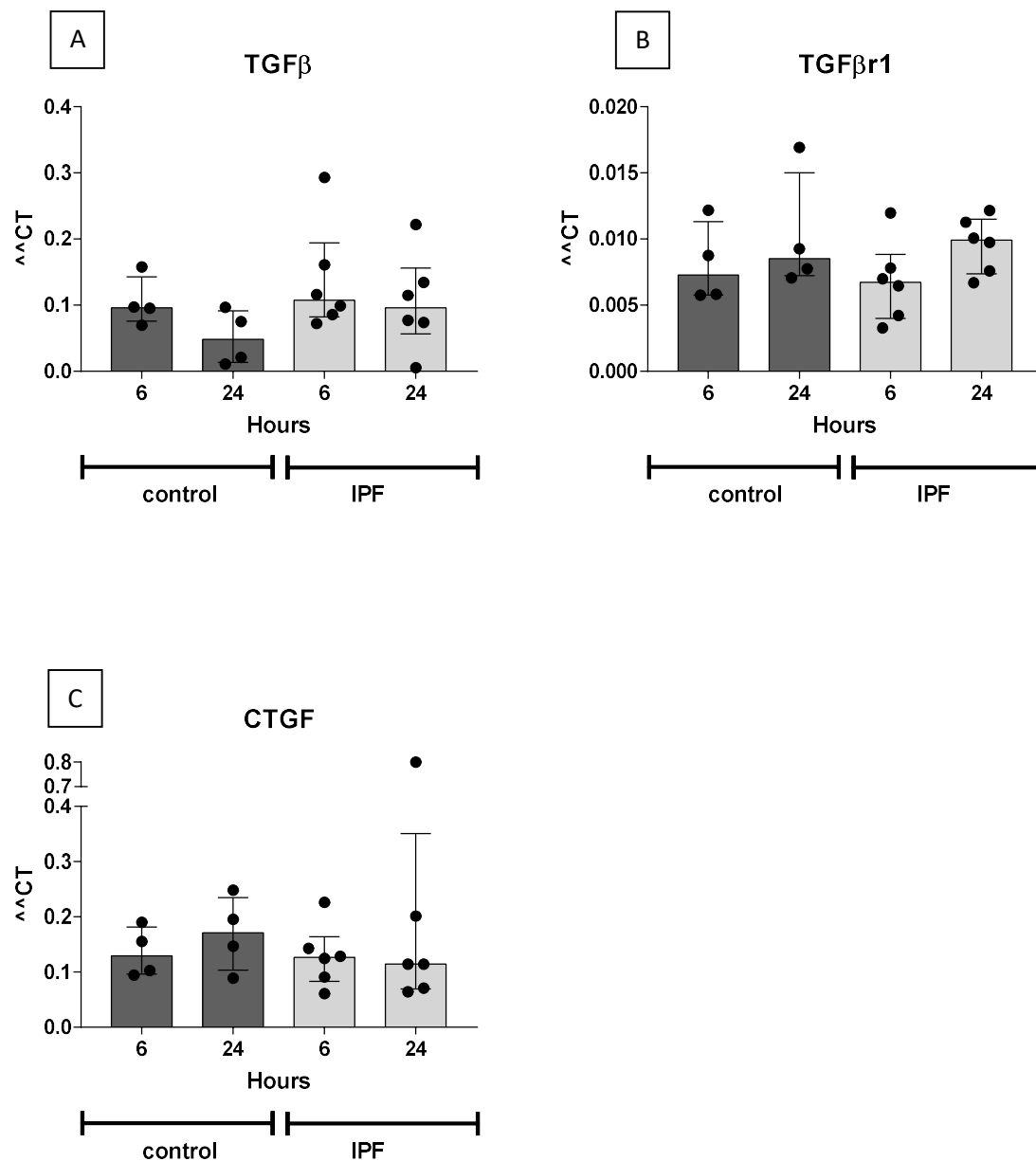


Figure 4.16 TGF-β1, TGF-βr1, and CTGF expression in healthy control HLFs stimulated with supernatant from IPF and healthy control HBECs; gene expression by qPCR of TGF-β1 A) TGF-βr1 B) or CTGF C) expression in control HLFs stimulated by supernatant conditioned by IPF (n=6) HBECs and healthy control (n=4) for 24 hours. No differences were seen in the effect of IPF and normal HBEC supernatant on COL1A1, COL3A1 or α-SMA at 6 or 24 hours (mann whitney U test).

Values presented as median and interquartile range

4.3 Discussion

4.3.1 Summary of results

In this chapter I described the phenotype of the epithelium in distal airways sections and investigated the nature of the bronchial epithelial cells obtained from brushings of large airways in the IPF lung and healthy control.

The brushed large airway cells were used in submerged culture experiments to investigate epithelial barrier function and proliferative capacity, and gene expression analysis was performed to assess the regulation of a host of genes which are traditionally thought to be prominent in the fibrotic process. Media conditioned by cultured epithelial cell mediators was used to assess the cellular signalling functions of the bronchial epithelium with regards its effect upon normal lung fibroblasts. Taken together, these data suggest that IPF airway epithelium does show phenotypic differences in cellular composition when compared to healthy control. Our work backs up previously published studies suggesting an epithelial over-expression of mucin as a risk factor for IPF, but also gives the strongest evidence yet of a proliferation of SCGB1A1 cells in IPF, whose previously suggested capacity for phenoconversion and migration may point to a future avenue to explore in order to more completely understand the role of the bronchial epithelium. This work did not find any evidence of a direct pro-fibrotic effect of HBECs on extracellular matrix regulation *in vitro* or of dysfunctional barrier function or proliferation in cultured basal cells.

4.3.2 Bronchial epithelial phenotypic differences

This phenotypic characterisation of distal airways in lung tissue in IPF control demonstrated a statistically significant increase in mucin producing cells and in SCGB1A1 (club cell secretory protein) expressing cells. The observation of increased mucin expression is not novel - the findings I present here are in accordance with a body of data which, collectively, are believed to indicate mucin

overexpression to be a factor predisposing to the development of IPF. A strong association has been reported between the promoter polymorphism rs35705950 of MUC5B and the development of familial/sporadic IPF (Seibold et al, 2011) (Wang et al, 2014) (Zhang et al, 2011) (Peljto et al, 2015) (Borie et al, 2013) (Horimasu et al, 2015) (Noth et al, 2013) (Li et al, 2014), with its presence seeming to indicate a more benign disease course (Stock et al, 2013) (Peljto et al, 2013) and seeming to be specific to IPF, with the same pattern not detected in fibrotic NSIPs (Conti et al, 2016) . This observation has been further investigated with overexpression of the main mucins, MUC5B and MUC5AC having been described in IPF lungs (Seibold et al, 2013) (Plantier et al, 2011) and this phenomenon is re-produced in our data.

The observation of an increased proportion of SCGB1A1 positive club cells in IPF has, to our knowledge, not been published before. SCGB1A1 is a member of the secretoglobin family of small secreted proteins mainly produced by club cells localized in bronchioles including terminal ones. CC16, a protein secreted by club cells, has been shown to be elevated in individuals with IPF compared to other ILDs (Buendia-Roldan et al, 2016) and in 2005 Kim et al identified a population of cells in mice which co-express SCGB1A1 and surfactant protein C. They self renew and act as progenitors to form both airway and alveolar cells *in vitro* (Fukumoto et al, 2016). Subsequent studies have shown these cells not to contribute to the alveolar epithelial cell population in normal homeostasis or after hyperoxic injury (Rock et al, 2011) but that in the case of bleomycin induced fibrosis they give rise to large numbers of type I and type II AECs (Conti et al, 2016). This nascent area of research, although not yet definitive, does point to the possibility of club cells - following certain types of injury -, having the potential to phenoconvert and migrate, to act as a reservoir of progenitor cells which participate pathogenically in the process of IPF. This would appear to be in keeping with the interesting findings of Xu et al whose single cell analysis of bronchial epithelium showed that in IPF these cells can undergo an abnormal process in which I see disruption of the proximal to distal patterns of epithelial cell differentiation with individual cells displaying

characteristics of both airway conducting cells and alveolar cells (Xu et al, 2016) - a phenomenon not seen in embryogenesis or the mature lung (Treutlein et al, 2014) (Du et al, 2015).

I analysed both the total positivity of staining intensity and then only the strong positivity to ensure the robustness of the analysis. I did not find any discrepancy in the results by the different analysis methods. In addition, airway length was measured for all analysed airways, with no difference found in airway size between our studied groups (control n=11, IPF n=16), confirming the equivalence of the sample airways included in this study. n number of stained control sections varies between analyses as some tissue sections were found to not contain any airways, post staining.

4.3.3 Basal cell proliferation and barrier function

Our results showed that the cultured cells obtained from large airway bronchial brushings predominantly expressed KRT5, in keeping with the conventional wisdom that submerged culture monolayers do not differentiate into mucociliary cells *in vitro* but proliferate as a population of basal cells. The subsequent cell culture work therefore focused exclusively on the functions of the basal cell subset of the bronchial epithelium.

As multipotent stem cells of the respiratory epithelium, basal cells are believed to drive homeostasis and play a vital role in re-generation after injury (Rock et al, 2010). Recent studies support the hypothesis that after chronic and severe injury, lineage negative progenitor basal cells can be activated and migrate to the site of injury in the alveoli (Vaughan et al, 2015) (Zuo et al, 2015). It has been established that alveolar epithelial cells (AECs) drive fibroblast proliferation in IPF (Sakai et al, 2013) and AECs themselves demonstrate accelerated proliferation in honeycomb lesions after injury (Selman et al, 2006) however, to our knowledge, there is no comparative data on HBEC proliferation in pulmonary fibrosis.

I posited that that given their involvement in replenishment of cell populations post damage, IPF bronchial epithelial basal cells would also display dysregulated proliferation when compared to healthy epithelial basal cells. However our data did not show any difference in the rates of proliferation of HBECs between IPF and healthy control. The work I present here could, taken with other studies into this area, support the hypothesis that club cells in IPF could act as a progenitor cell population which respond to injury by phenoconversion then proliferation and migration, but does not seem to suggest a similar role for basal cells, which do not exhibit dysregulated proliferation when compared to healthy control.

The IPF HBECs formed confluent monolayers in culture which exhibited similar properties to the healthy control HBECs with expression of the crucial ZO-1 proteins detected in both and no clear differences were found in either transepithelial electrical resistance or barrier function as measured by the perfusion of fluorescently labelled solute. Previous work has shown that ZO-1 expression is down-regulated by TGF- β (Wellis et al, 2005) with evidence of loss of epithelial markers such as ZO-1 expression forming a central plank in the epithelial-mesenchymal transition hypothesis and lending credible evidence to the theory that the multitude of pro-fibrotic factors involved in the fibrotic process also lead to a disruption of the epithelial barrier (Milara et al, 2012.) This effect was not confirmed in the data I present here, as the *in vitro* observations I made did not present any evidence of a 'leaky' damaged epithelial barrier in IPF. However, the observation in this study is derived from observations made *in vitro*. The increase in albumin and other circulatory proteins found in the alveolar compartment in IPF found *in vivo* (Ward et al, 1997) does present evidence of a loss of alveolar epithelial integrity, but this effect has not been replicated in the bronchial epithelium in the data presented here. Data on the integrity of tight junctions in IPF is sparse, but some insights have been gleaned from studying the in-vitro effect of the prominent fibrosis associated molecule TGF- β upon tight junctions in different cell types. It decreases the permeability of colonic epithelial cells (Planchon et al, 1994) but conversely increases that of uterine epithelial cells. It has been shown to downregulate ZO-1 and e-cadherin in A549s (Chen et al, 2013),

suggesting the possibility of a TGF- β mediated disruption of the epithelial barrier, but this effect has only been seen in cell lines and it has not been established in primary cells. The work presented here is not sufficiently sensitive to detect differences between the IPF and healthy control groups that I looked at, but it did show that ,at confluence *in vitro*, IPF HBECs are able to form tight junctions between cells and express ZO-1 as in healthy control. It is unclear how these observations fit into the current literature on ZO-1 expression and barrier function in IPF but this work does not offer supporting evidence of barrier dysfunction or reduced integrity of tight junctions in IPF airway epithelium.

4.3.4 Direct pro-fibrotic signalling

I wished to ascertain if HBECs in IPF might exert a more traditionally pro-fibrotic effect in the lung as well as the indirect effects I had hitherto investigated. Our work looking at gene expression profiles in IPF and control HBECs found similar regulation of a number of pathways which are known to be intimately involved in fibrosis, including growth factors, ECM components remodelling enzymes and inflammatory cytokines and chemokines. PCR array of 80 fibrosis associated genes demonstrated statistically significant up-regulation in 4 genes; integrin $\alpha 1$ (ITGA1), chemokine receptor type 4 (CXCR4), endoglin (ENG) and thrombospondin1 (THBS1), with only ITGA1 showing a greater than 2 fold up-regulation and with CXCR4 found to be expressed at a very low level. The integrin $\alpha 1$ (ITGA1) gene encodes for the $\alpha 1$ subunit of integrin receptors. It heterodimerises with the $\beta 1$ subunit to form a cell-surface receptor for collagen and laminin (Henderson et al, 2012). It is involved in cell to cell adhesion. Thrombospondin (THBS1) encodes a protein which is an adhesive glycoprotein which mediates cell to cell and cell to matrix interactions. It has interactions with ECM components, and can bind to laminin, type V collagen, fibronectin, fibrinogen and laminin. It has roles in platelet aggregation, angiogenesis and tumorigenesis (Sweetwyne et al, 2013). Endoglin (ENG) encodes a major glycoprotein of the vascular endothelium. It acts as a TGF- β co-receptor and is involved in

TGF- β /BMP signalling cascade - it is known to modulate TGF- β signalling, having been demonstrated to be able to enhance or inhibit the effects of TGF- β signalling (Maring et al, 2012). C-X-C motif chemokine receptor 4 (CXCR4) encodes a cell surface chemokine receptor for stromal-derived-factor 1, a molecule with chemotactic activity for lymphocytes. It has been found to play a role in recruiting fibrocytes from bone marrow to fibrotic lung tissue (Griffiths et al, 2018)

Our data suggests, that of the 80 genes examined, the only one to exhibit a greater than 2 fold change from control was, which I found to be up-regulated in epithelial cells in IPF as compared to healthy control, and this could be as a possible consequence of the effect of increased TGF- β signalling found in the fibrotic lung. Integrins have been shown to recruit scaffolding and signalling molecules and drive numerous pathways involved in cancer progression (Boudjadi et al, 2016) with the ITGA1 forming a cell surface receptor for collagen and laminin and activating the Ras/MEK/ERK pathway regulating cell proliferation and differentiation (Hynes et al, 1992). The Ras/MEK/ERK pathway has been implicated in TGF- β induced fibrosis (Chen et al, 2005). Mice modelling has shown inhibition of this pathway to attenuate fibrosis in the TGF- β fibrosis model and increasingly interest has been focused on the integrin α V β 6, which plays a critical role in the activation of latent constitutively expressed TGF- β (Abe et al, 1994). However, with the number of genes examined in this array and a p value of <0.05 , the finding of only 4 statistically significant gene expression changes (with only 1 greater than 2 fold) does not present compelling evidence of widespread changes in the transcriptome of IPF HBECs. The genes which were upregulated do not share common pathways and, the lack of widespread changes in gene expression across a number of examined pro-fibrotic genes is, seems to in fact to refute rather than support the hypothesis that IPF HBECs contribute to the fibrotic milieu in IPF.

Having found no clear evidence of a wide dysregulation of the numerous pathways conventionally believed to be crucial in the development of fibrosis, I decided to cast the net wider and look for disruption to pathways which have not hitherto been thought intimately involved. This led us to

examining the expression of the IL-33/ST2 axis in HBECs, as this epithelial-expressed damage related axis has been increasingly a focus of interest in fibrosis research. Previously demonstrated to be crucial in regulating Th2 responses in asthma, the finding of elevated secreted IL-33 in BAL in IPF when compared to both normal controls and other ILD has led to more focus in this area. Recent work has shown an increase in IL-33 expression which leads to M2 polarisation and worsening of consequent fibrosis in fibrosis models in mice and the soluble receptor sST2 has been found to be elevated in BAL of individuals with IPF who were undergoing an acute exacerbation.

This work found that the IL-33 axis shows minor evidence of dysregulation in IPF HBECs with a significant up-regulation of the membrane bound negative regulator SIGIRR and a trend towards increased expression of the receptor ST2. This suggests that the IL-33 axis in IPF may evince an upregulation compared with normal healthy controls, however the evident increase in both mediator and negative regulators does not lend clarity to the question of whether IL-33/ST2 is pro-fibrotic or involved in the initiation of fibrosis, or involved in the resolution process. This is further examined in chapter 5.

Taken together, these data do not suggest that HBECs in IPF differ from in health in the parameters that I have primarily examined. The bulk of the evidence which has implicated bronchial epithelium has focused on mucin production and the effects of its over-production. With the role of mucociliary dysfunction having become recently elucidated in the body of work from Schwarz et al (2015), our data in this chapter has sought to characterise the other principal functions of epithelium in IPF. I have not found evidence to support the hypothesis that IPF HBECs show abnormal cellular composition, barrier function or proliferation when compared to control. Fibroblast stimulations did not suggest a direct pro-fibrotic effect of HBECs upon fibroblast function, however I did find changes in the regulation of the IL-33/ST2 signalling which will be further explored in the next chapter.

In summary, these data presented here found evidence to support the hypothesis that the phenotype of the bronchial epithelium in IPF is altered from the healthy state, but did not find

evidence to support the hypothesis that bronchial epithelial proliferation or barrier function are dysregulated nor that alterations in bronchial epithelium had direct pro-fibrotic effects in the IPF lung.

4.3.5 Limitations of this study

A variety of methods can be used to model the human bronchial epithelium *in vitro* including immortalised cancer cell lines, and the use of primary cell lines from human donors. In order to assess the pseudostratified phenotype observed *in vivo*, HBECs must be cultured at air liquid interface, where the apical surface is exposed to air and the basal surface in contact with culture medium. Submerged culture of primary HBECs can give insights into certain aspects of epithelial function however they do not show mucociliary differentiation meaning that this method does not allow for any interrogation of mucociliary function.

Our characterisation of the cells I isolated show these to be a population of basal cells and, therefore, any conclusions I draw from the *in vitro* work presented here, are confined to the role of basal epithelial cells and do not comment on club, goblet or ciliated cells.

N numbers in individual *in vitro* experiments were small to allow multiple functional assays to be performed, looking for large differences between IPF and control.

4.3.6 Future work

Following on from the findings of this chapter, I proceed to examine the expression and the regulation of IL-33 and its signalling axis in the IPF lung. Future work investigating the role of bronchial epithelium should be directed towards developing an air liquid interface culture model, which would allow a more robust assessment of HBEC functions *in vitro*, continuing on from the work solely examining basal cells presented here. The finding of an increase in club cells in IPF

epithelium could be further explored by examining the ontogeny and functional role of club cells in IPF. Given the difficulty of obtaining a purified population of these cells, mice modelling could offer further insights into detailing the stimuli which induce this population to expand and the mechanisms by which they contribute to dysfunctional re-epithelialisation of the injured alveolus.

Chapter 5.

An investigation into the role of the IL-33/ST2 axis in fibrotic lung disease

5.1 Introduction

The previous chapter described the phenotype and function of cultured HBECs in IPF. The data presented did not find evidence of a direct pro-fibrotic effect of HBECs upon normal human lung fibroblasts but this work did align with the extensive body of evidence published by Seibold et al, documenting an increase in expression of MUC5B in the lungs of individuals with IPF and an increase in the levels of mucin produced in the IPF lung (Seibold et al, 2011). Our data demonstrated an increase in mucin as well as an increase in SCGB1A1 positive club cells in IPF epithelium. I did not demonstrate any difference in proliferation rates or barrier function when compared with healthy control.

In the previous chapter, I sought to characterise the gene expression profile of IPF HBECs by analysing a panel of genes involved in extracellular matrix regulation, pro and anti-fibrotic pathways growth factors and cytokines/chemokines. This investigation found only ITGA1, THBS1, ENG and CXCR4 showing significant up-regulation, of which only ITGA1 was up-regulated greater than 2 fold. These data also demonstrated evidence of altered regulation of the IL-33 cell signalling axis. There was a statistically significant difference in the expression of SIGIRR - the membrane bound negative regulator of IL-33, with a trend towards increased expression of the membrane receptor ST2. .

This chapter further investigates the regulation of the IL-33/ST2 axis in human fibrotic lung disease. Preliminary data from animal modelling has suggested the fibrotic milieu induces IL-33 expression and amplifies the fibrotic effect of bleomycin (Luzina et al, 2013) (Li et al, 2014). Early work has suggested increased IL-33 expression in the IPF lung (Luzina et al, 2012), with semi-quantitative analysis of immunohistochemistry suggesting increased IL-33 staining in the vicinity of fibroblastic foci and inflammatory infiltrates in the IPF lung when compared with control. The purpose of this study was to comprehensively characterise the expression of the cytokine IL-33 and its receptor complex in the IPF lung to ascertain if the upregulation seen in animal models translates to human subjects, and to explore its functional effect in vitro on human fibroblast function. This was carried

out by characterising the distribution and intensity of IL-33 staining found in tissue sections of IPF lung and secreted into the alveolar space in the IPF lung, and by detailing the functional effects of IL-33 stimulation upon lung fibroblasts from control and IPF donors.

5.1.1 Hypothesis

Direct tissue specific effects of the IL33/ST2 axis contribute to the development of lung fibrosis through non-immune cell mediated mechanisms

5.1.2 Aims

- 1) To determine if IL-33 and its receptor complex are expressed in the IPF lung and to describe the distribution of the cells in which it is found, through immunohistochemical staining of IPF and healthy control lung tissue.
- 2) To compare the levels of IL-33 expression in the IPF lung vs healthy control, through characterisation of the expression in tissue and measurement in bronchoalveolar lavage.
- 3) To investigate the direct effects of IL-33 on the fibrotic process by measuring the effect of IL-33 stimulation upon a variety of fibroblast functions, including wound healing, proliferation, pro-fibrotic gene expression and extracellular matrix regulation.

5.1.3 Methods

5.1.3.1 Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded paraformaldehyde fixed lung tissue obtained from biopsy of individuals with IPF, or from distal and histologically normal sections of

tissue from lung cancer resections. The study explored 21 large lung tissue blocks from 10 IPF patients, diagnosed based on established criteria and confirmed by open lung biopsy. The 11 blocks of control tissue were obtained during lung lobectomy or pneumonectomy due to suspected lung cancer from otherwise healthy individuals. Only patients with solid (and typically centrally located) tumours were included and the tissue for the present analysis was collected from peripheral lung regions, as far from the tumour as possible. The demographics of the immunohistochemistry subjects are presented below (table 5.1).

All sections were stained with the same reagents at the same time. Quantification of the intensity of staining was performed by using image analysis software (Aperio imagescope). Intensity of staining was measured in 3 airways per individual and the mean intensity of staining expressed per μm of airway circumference and IPF compared with control. Staining in lung parenchyma was performed by analysing the intensity of staining in 3 randomly selected areas of parenchyma with the mean value presented.

Table 5.1 Immunohistochemistry subject demographic details

	control	IPF
N	11	10
Age	72 (68.5-74.5)	64 (54.5 68.3)
% male	40	91.7

Table 5.1 showing demographic details of immunohistochemistry subjects. Age shown as median (interquartile range). There was no significant difference between the ages of the participants in IPF and control arm] but the percentage of males was significantly higher in the IPF group (Mann Whitney U test)

The staining was analysed using Imagescope Aperio software and intensity of staining was measured in airways in IPF and healthy control lung. The analysis of airway IL-33 was performed by taking the mean of 2-3 randomly selected airways and quantified by analysing the number of positive pixels of the mean of 2-3 imaged airways, expressed per μm of measured airway length, to ensure the analysis was not skewed by length of airway measure. IL-33 in tissue was measured by assessing the total positivity of staining in 3 randomly selected areas of parenchyma which did not include an airway, and presenting this value as Well as the total positivity divided by the total number of positive pixels in the analysis field - i.e. the analysed amount of tissue in the analysis window. This allowed me to control for any effect that could be introduced by analysing sections of tissue with different volumes of lung tissue.

5.1.3.2 Immunofluorescence staining

Bronchial epithelial cells were isolated from the lungs of individuals with IPF and healthy controls as described in the previous chapter. These cultured HBECs were then examined to determine the cellular localisation of IL-33. Given the difference in intensity of staining exhibited between repeat experiments, reliable quantification was not possible so I did not attempt to make a comparison between IPF and control, but simply to describe the location of IL-33 within the bronchial epithelial cell.

5.1.3.3 Mass spectrometry of BAL fluid

To investigate the bands found on Western blot analysis, proteins from BAL samples were electrophoretically separated by molecular light onto precast gels. The bands corresponding to the molecular light of the bands revealed on IL-33 binding (~ 30 kda and 50kda) were cut and peptides extracted, with samples analysed by LC.MS/MS.

Processed raw data was searched using the Mascot search algorithm (www.matrixscience.com) against All Taxonomy in the Uniprot database. The data was specifically filtered for the Human species within the All Taxonomy output after searching.

5.1.3.4 Human lung fibroblast culture

Fibroblasts were grown from lung tissue of IPF individuals who had undergone surgical lung biopsy (n=6) and healthy control (n=8) as previously described , or bought from Lonza. They were expanded and used between passage 3 and 5.

5.1.3.5 Human lung fibroblast wound healing assay

Wound healing was measured by real time cell imaging (Incucyte Zoom[®]) of scratch assays. A uniform wound was made in triplicate Wells of a 96 Well plate using the Incucyte WoundMaker[®] and closure of the wound measured every 4 hours over a 48 hour time course. The zero point was taken as the first image taken immediately post administration of the wound to the confluent fibroblasts. Wound healing was measured by relative wound density, a metric which analyses the spatial density in the wound area relative to the spatial cell density outside of the wound, which self normalized for changes outside the wound (representative image 5.1).

All cell stimulations were conducted with following concentrations of recombinant protein DMEM media, DMEM media + IL-33 100ng/ml, TGF- β 5ng/ml, IL-33 100ng/ml + TGF- β 5ng/ml.

Figure 5.1

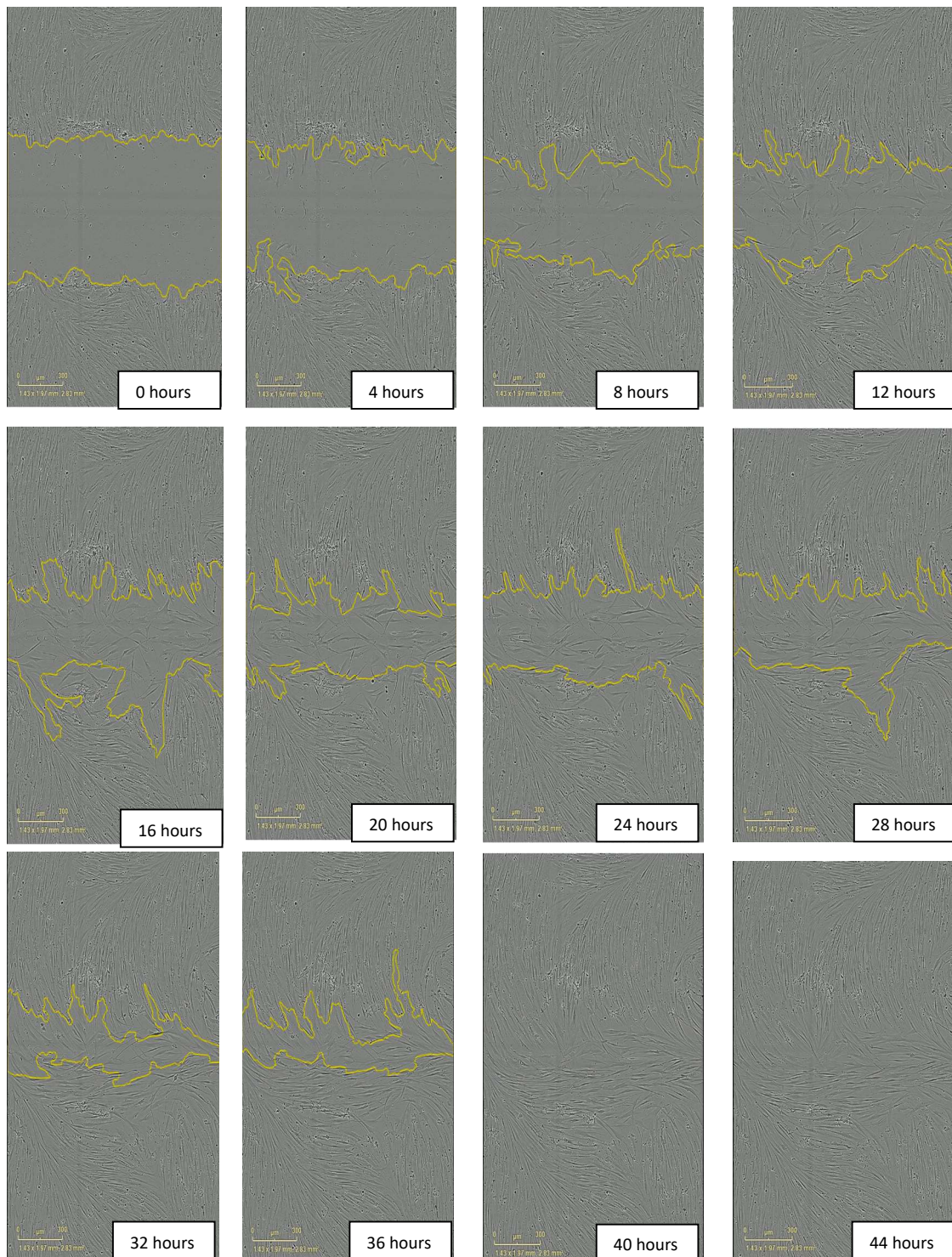


Figure 5.1 Representative images of scratch assay real time cell imaging; wound healing analysis metric across a 44 hour time course at 4 hour intervals. Yellow line on each image represents the analysis software's detection of the healing edge of scratch in the fibroblast monolayer.

5.1.3.6 Human lung fibroblast stimulations

For cell stimulation experiments fibroblasts were seeded into 12 Well plates, grown to 90% confluence, serum starved overnight and then stimulated with recombinant protein. Cells were harvested at 3, 6, 12, 24, 48 and 72 hours with cells lysed for RNA and supernatant stored for further analysis (figure 5.2).

All experiments were conducted with primary IPF HLFs (n=3) at passage 3-5, with gene expression quantified by RT-PCR at 3, 6, 12 and 24 hours.

I analysed the expression of COL1A1, α -SMA, TGF- β , TGF- β 1, IL-33 and ST2.

Figure 5.2

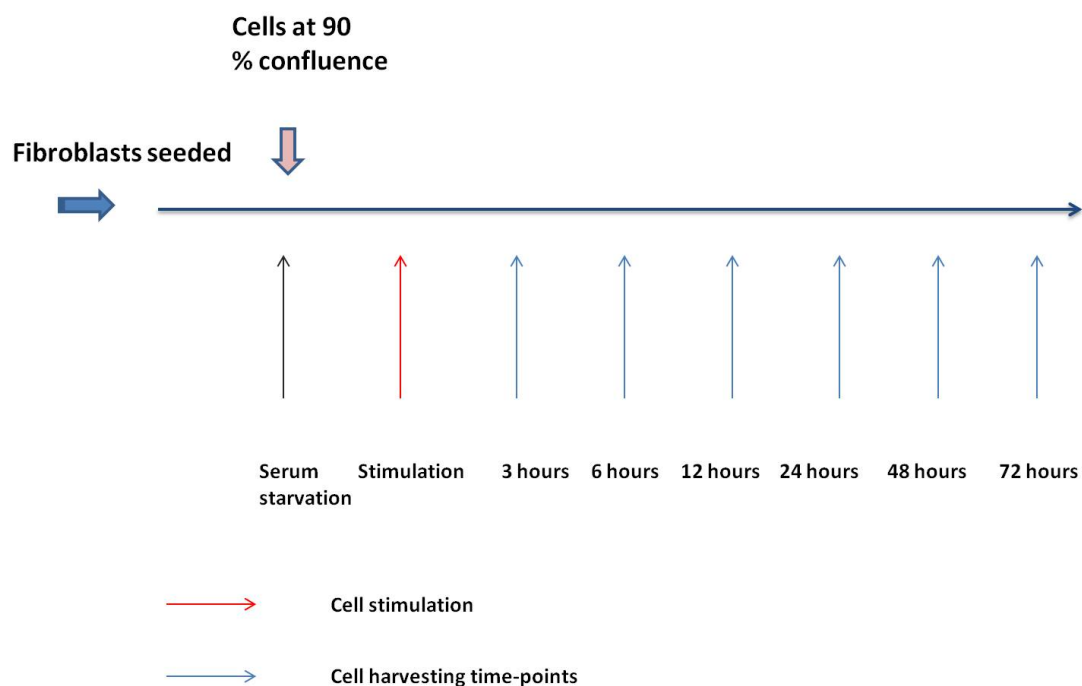


Figure 5.2 Experimental protocol of fibroblast stimulation experiment

5.1.3.7 MMP9 quantification

MMPs and their inhibitors, tissue inhibitors of MMPs (TIMPs), have been implicated in the pathogenesis of IPF based upon the results of clinical studies reporting elevated levels of MMPs (including MMP-1, MMP-7, MMP-8, and MMP-9) in IPF blood and/or lung samples. MMP9 induction has been linked to IL-33 activity (Ariyoshi et al, 2017).

Released bioactive MMP9 was measured in cell supernatant of IPF (n =3) and healthy control (n =2) fibroblasts stimulated with IL-33 and TGF- β by gelatine zymogram, with the gelatinolytic activity quantified by measuring the integrated density of the bands, visualised by coomassie blue staining

5.2 Results

5.2.1. IL-33 staining in lung tissue

IL-33 is present in human lung tissue - both IPF and healthy control- and is found predominantly in basal cells in bronchial epithelium. IL-33 was also detected in cells morphologically identified as endothelium and tissue macrophages with staining also detected in lung fibroblasts (figure 5.3).

The histological areas which are associated with the strongest staining are lymphoid aggregates with the staining predominantly detected in lymphoid cells in these regions. Low intensity staining was found in fibroblastic foci - the characteristic lesion found in IPF lung tissue (figure 5.4) .

This pattern of staining is in keeping with previously published data on IL-33 localisation in the lung - it is known to be constitutively expressed in the nucleus of basal bronchial epithelial cells, with lower expression in endothelial cells and fibroblasts and expression inducible in some lymphoid cells. The finding of intense staining in lymphoid aggregates with much lower expression in fibroblastic foci is a notable observation. The role of the IL-33/ST2 axis in the inflammatory response is well documented and this is reflected in the intensity of staining found in lymphoid cells in lymphoid

aggregates. These samples did not exhibit high level of staining in fibroblastic foci, however IL-33 is a soluble mediator, the significance of this finding remains uncertain.

Figure 5.3

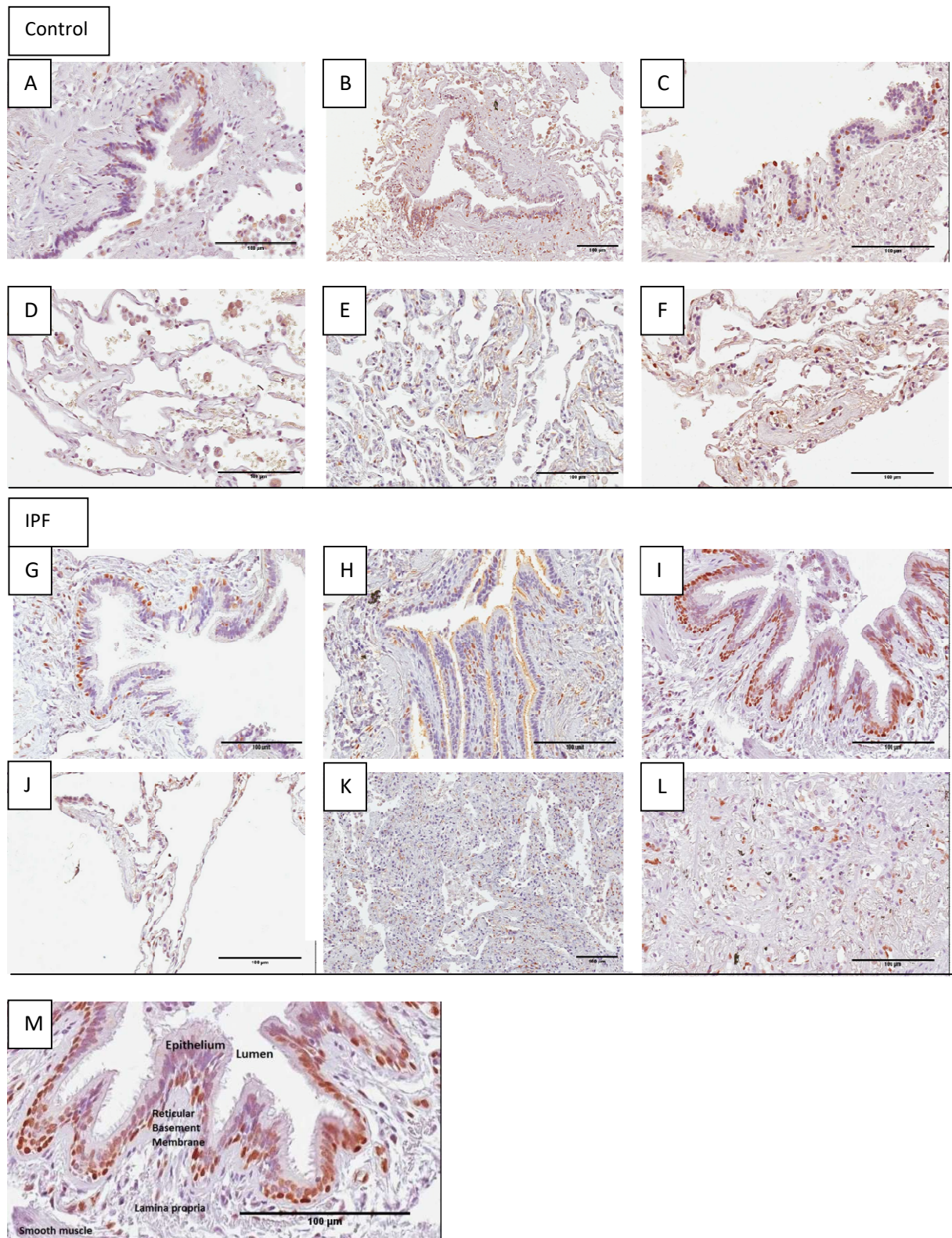


Figure 5.3 IL-33 staining in healthy control and IPF lung tissue; representative images of IL-33 in healthy bronchial epithelium A-C) with healthy control parenchyma D-F) IPF bronchial epithelium G-

I) IPF parenchyma J-L). Staining was predominantly found in basal cells of airways in both IPF and healthy control. In parenchyma in both healthy control and IPF, IL-33 staining was detected in cells morphologically consistent with endothelium, tissue macrophages and fibroblasts. Representative image of IL-33 airway staining with labelled features of interest **M).**

Figure 5.4

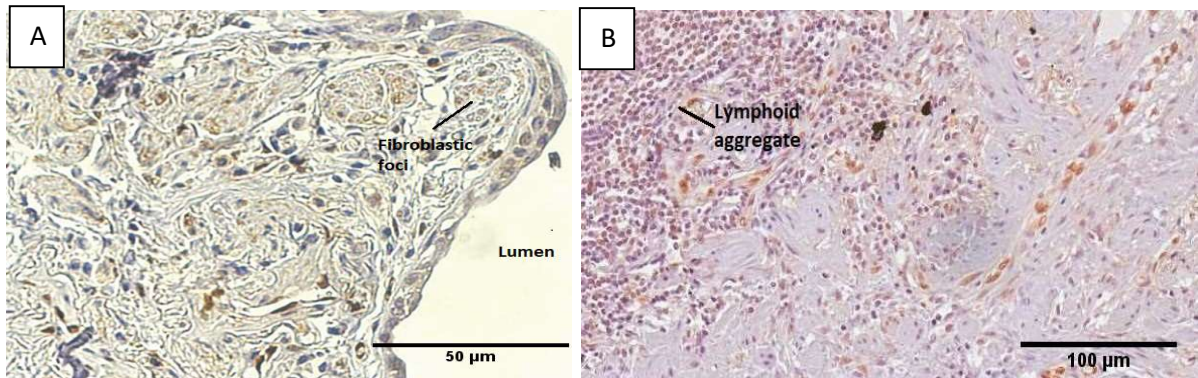
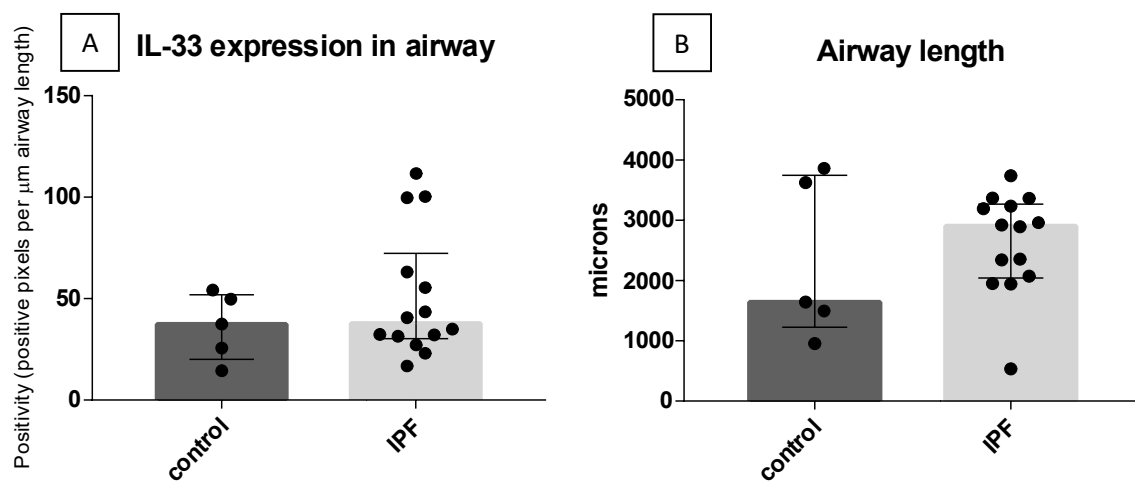


Figure 5.4 IL-33 immunoreactivity in fibroblastic foci and lymphoid aggregates;

immunohistochemical analysis showed IL-33 immunoreactivity in fibroblastic foci **A)** and in lymphoid aggregate in IPF lung tissue **B)**.

Figure 5.5



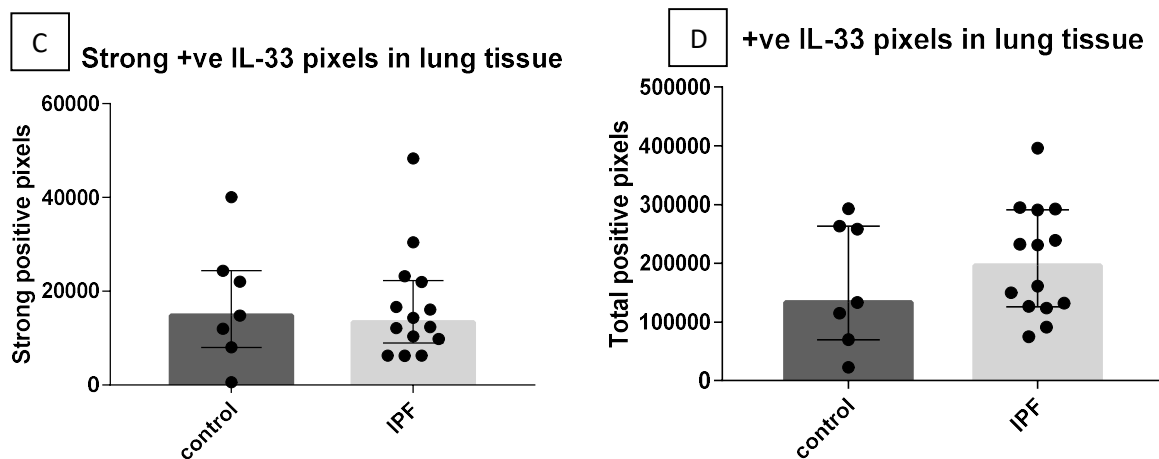


Figure 5.5 Quantitative analysis of immunohistochemical staining of IL-33 in IPF and control lung tissue. No difference is detected between IL-33 expression in IPF(n=14) and control airways (n=5) expressed per μm of airway length **A)** length of IPF and control airways measured in analysed tissue sections **B)** number of strong positive pixels of IL-33 staining in lung tissue in IPF (n=14) vs control (n=7) **C)** Total number of positive pixels of IL-33 staining in randomly selected area of tissue section in IPF (n=14) vs control (n=7) **D)** (Mann Whitney U test).

This analysis did not reveal any significant differences between IL-33 expression in tissue or in airway between the groups, either as an absolute value or when expressed in absolute numbers or per airway length.

Strong positive staining was found in lymphoid aggregates in IPF tissue. There was no difference between the size of IPF or control airways included in the study (figure 5.5).

5.2.2 ST2 staining in IPF and healthy control lung tissue

In both control and IPF tissue ST2 expression was detected on bronchial and alveolar epithelium, and cells morphologically consistent with lymphocytes and macrophages. ST2 staining was demonstrated lining the luminal surface of the bronchiolar epithelium rather than being found to be intracellular

(figure 5.6). This distribution of staining is consistent with binding to the secreted soluble form of ST2 coating the cilia in the luminal surface of the airway. Lymphoid aggregates exhibited intense staining in lymphoid cells, with much lower intensity staining found in fibroblastic foci (figure 5.7). Furthermore, no significant differences were found between quantified expression levels of IL-33 or ST2 (figure 5.8) in IPF tissue as compared with control by this method, which is in contrast to animal modelling work which has found an IL-33 upregulation in fibrosis precipitated by bleomycin injury.

Figure 5.6

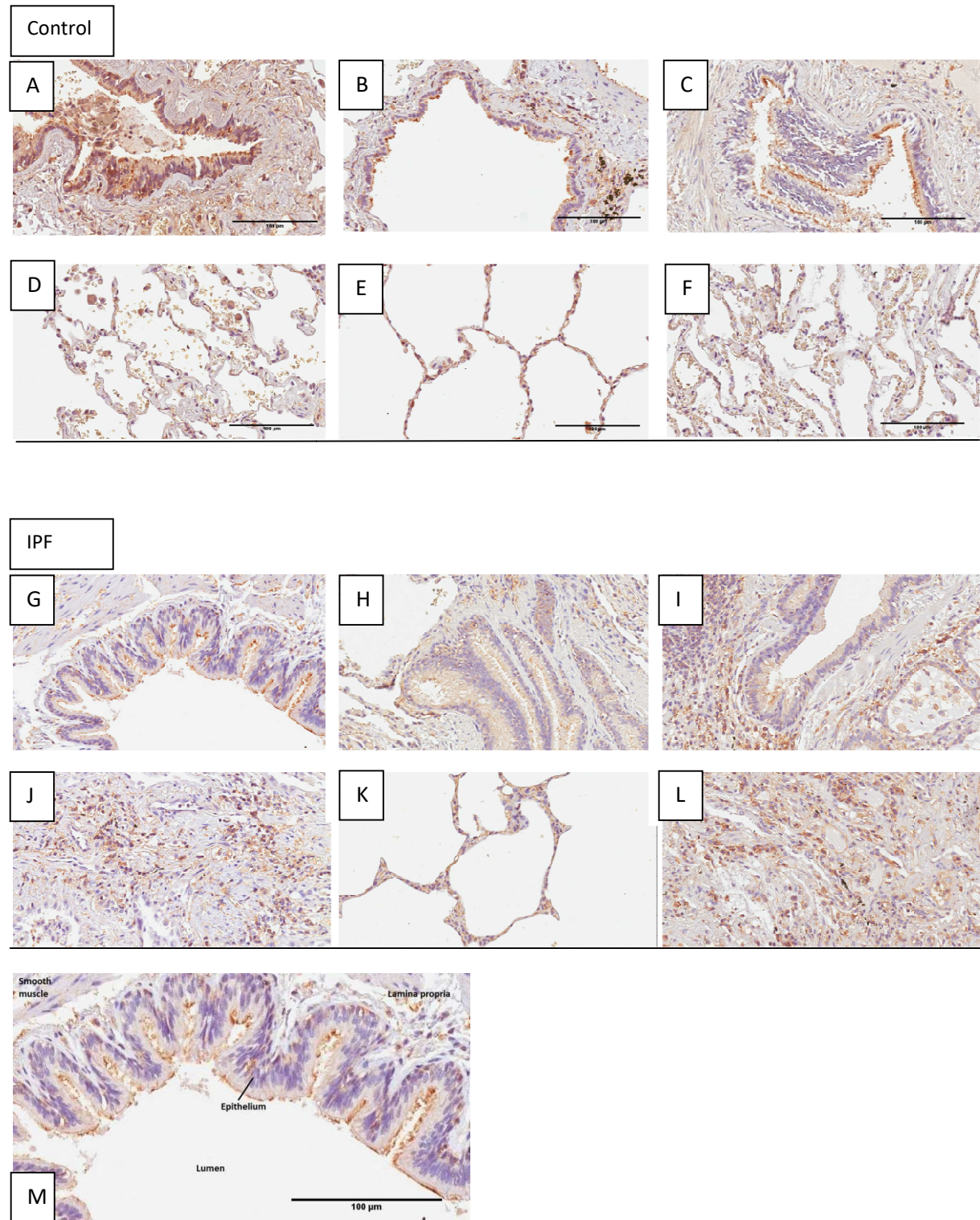


Figure 5.6 Representative micrographs of ST2 immunoreactivity in lung tissue; healthy control
bronchioles **A-C)** healthy control parenchyma **D-F)** IPF bronchioles **G-I)** IPF parenchyma **J-L).**

Representative image of ST2 staining with labelled features of interest.

Staining was predominantly seen lining the ciliary cells of distal bronchioles, the distribution of which was suggestive of the soluble form of ST2 having been revealed trapped in the cilia of the airway.

Low level staining was also detected on bronchial epithelium. Analysis of ST2 staining in parenchyma revealed ST2 expression detected of cells morphologically consistent with macrophages and lymphocytes.

Intensity of ST2 expression was quantified and IPF compared to healthy control. This did not reveal any significant differences between the groups (figure 5.8).

Figure 5.7

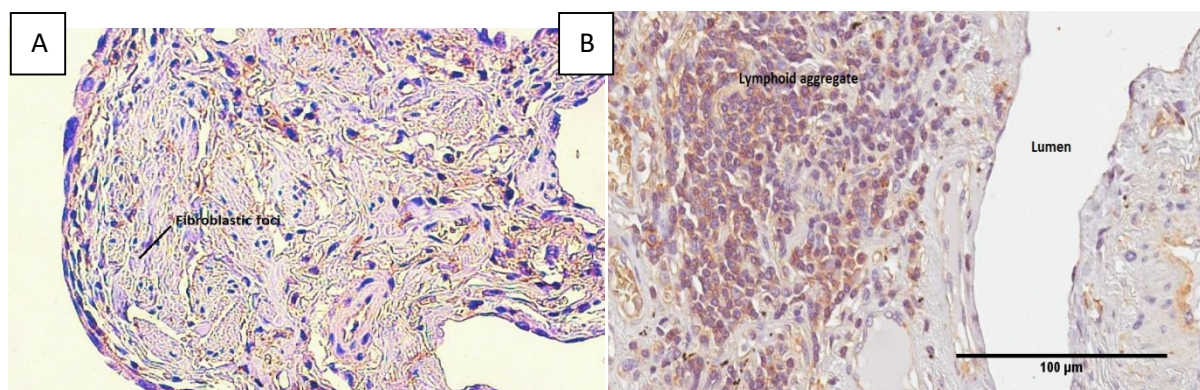


Figure 5.7 Representative micrographs of ST2 immunoreactivity in IPF lung tissue; fibroblastic foci
A) and in lymphoid aggregates in IPF lung tissue B)

Figure 5.8

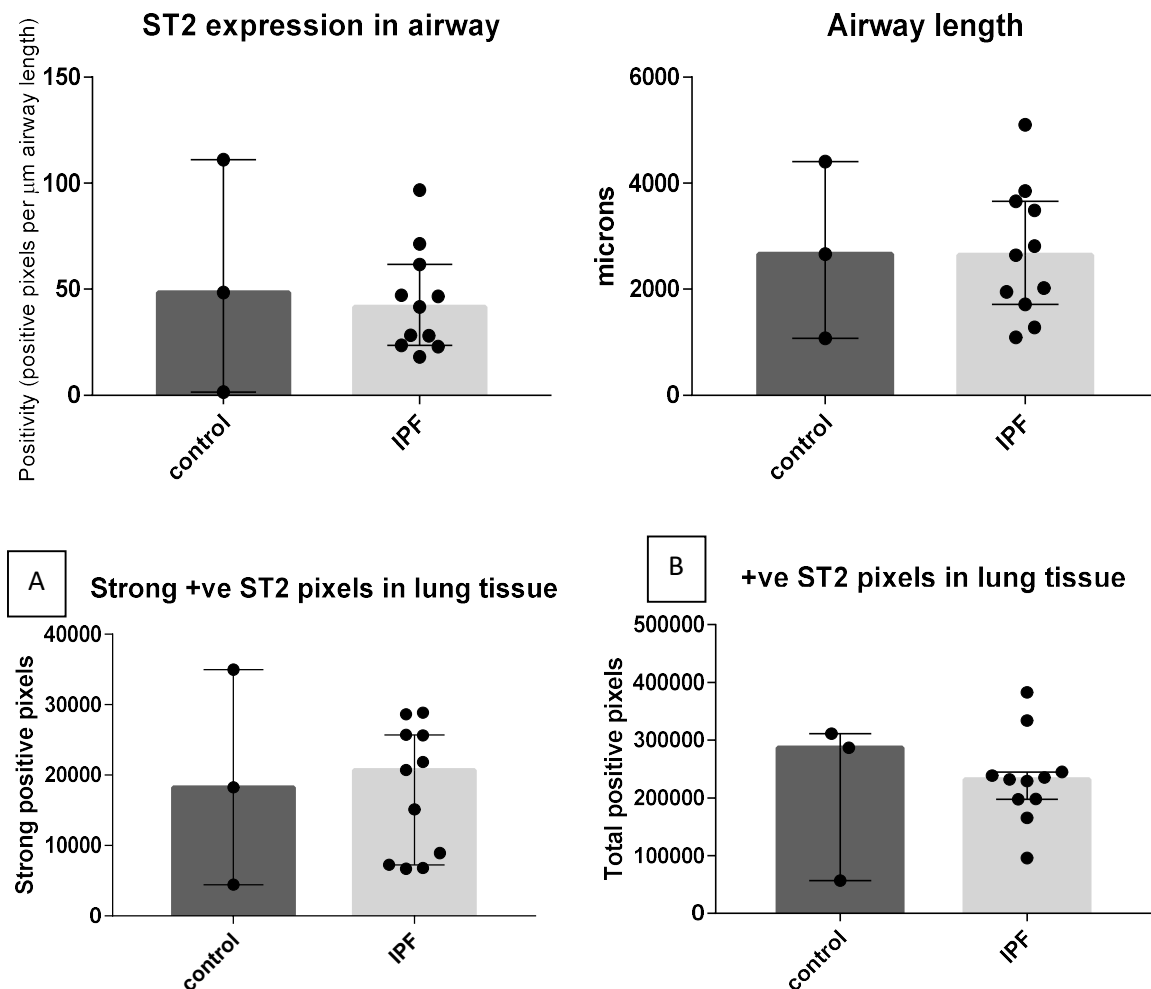


Figure 5.8 Quantitative analysis of immunohistochemical staining of ST2 in IPF and control lung

tissue; No difference was detected between ST2 expression in IPF (n=11) and control airways (n=3) expressed per μm of airway length **A)** length of IPF and control airways measured in analysed tissue sections **B)** Total number of strong positive pixels of ST2 staining in randomly selected section of lung tissue in IPF (n=11) vs control (n=3) **C)** Total number of positive pixels of IL-33 staining in randomly selected area of tissue section in IPF (n=11) vs control (n=3) **D)** (Mann Whitney U test)

5.2.3 IL-33 localisation in primary HBECs

IL-33 is constitutively expressed at resting state in the nucleus of cells where it can act, via different mechanisms, to limit inflammation. IL-33 has been shown to also localise simultaneously to cytoplasmic vesicles as well as nuclear euchromatin and, upon cell damage or necrosis, is secreted into the extracellular space where it exerts its pro-inflammatory effects (Liew et al, 2016).

Having localised the distribution of staining of IL-33 morphologically to cell type in IPF and healthy control lung sections, I proceeded to examine the intracellular location of IL-33 in primary bronchial epithelial cells and primary lung fibroblasts.

IL-33 staining was demonstrated both in the nucleus and the cytoplasm of healthy control and IPF HBECs (figure 5.9).

Figure 5.9

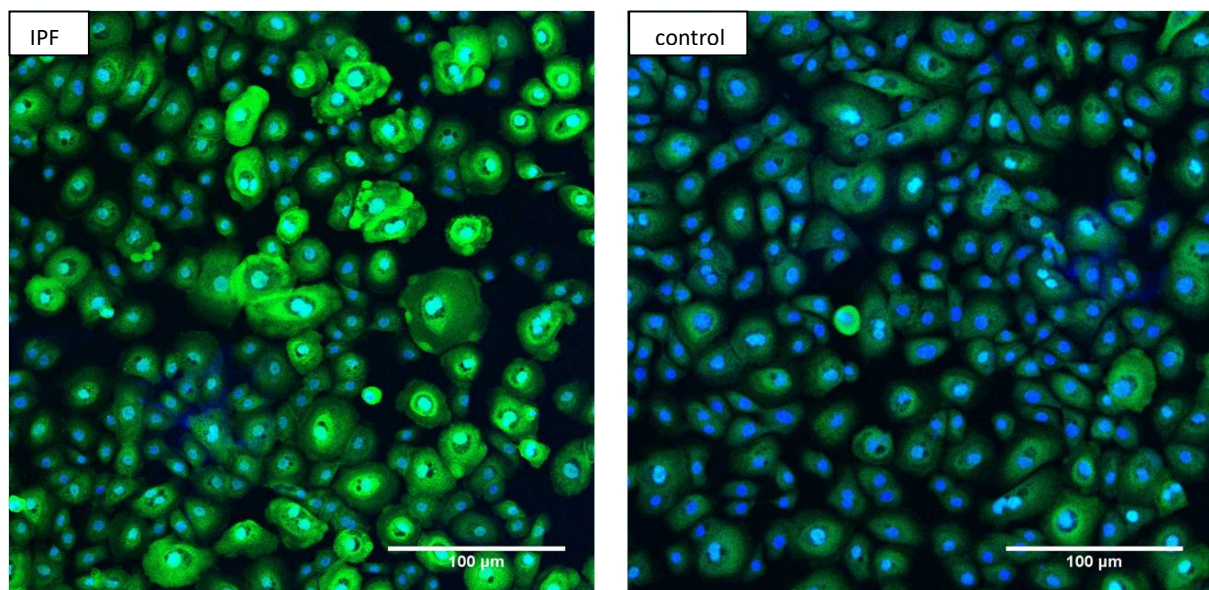


Figure 5.9 Localised immunofluorescent staining of IL-33 in cultured primary HBECs; Staining was found in nucleus and cytoplasm of IPF HBECs **A)** healthy control HBECs **B)**. Images representative of staining in IPF HBECs (n=2) and healthy control (n=2).

5.2.4 Characterisation of soluble secreted IL-33 and ST2 in the alveolar space

Having examined the expression of IL-33 in tissue of IPF and control lung, I proceeded to ascertain if levels of secreted IL-33 could be detected in bronchoalveolar lavage from individuals with pulmonary fibrosis. BAL was collected from individuals with IPF and healthy control as described in Chapter 3 and secreted IL-33 measured by ELISA of BAL fluid.

5.2.4.1 IL-33 levels in the alveolar space

Secreted IL-33 was detectable predominantly at low levels in BAL fluid of IPF and healthy control samples. Although the number of healthy controls in our study is small there was no significant differences detected between the groups when comparing absolute values or when normalised to the amount of protein per sample (figure 5.10). Of our study subjects, IL-33 was detected above the limit of detection in 1 out of 6 healthy controls and 14 out of 26 IPF samples. This is in contrast to previously published work with a large study published in 2017 (Lee, 2017) finding a statistically significant elevation in IL-33 in human BAL fluid in IPF when compared to control which was not replicated here. The 2017 study had higher n numbers, with 40 controls and 100 IPF samples and, by ELISA, found IL-33 expressed at similarly low levels but with their findings reaching statistical significance. In order to interrogate this discrepancy, further work was directed towards investigating our findings via a different quantification method, due to the previously reported possibility of false positive measurements of IL-33 via ELISA (Lee et al, 2017) (Nygaard et al, 2016).

BAL fluid samples were subsequently analysed by Western blot, to establish if IL-33 is detectable with more sensitivity by this method. The BAL fluid of individuals with IPF and a range of inflammatory were examined non-IPF ILDs which might be expected to have high levels of IL-33 given the predominance of inflammation in their pathogenesis. Western blot of BAL samples performed using anti-human IL-33 (R&D systems) revealed bands of positive staining at ~30kD and ~50kD in IPF and non-IPF ILD. IL-33 can undergo post transcriptional modifications allowing for multiple splice variants, whose molecular weights are between 18-20kda for proteolytically cleaved IL-33, or ~30kda for the full length isoform (figure 5.11). The bands found on Western blot were analysed by mass spectrometry.

These results revealed that of the 436 protein identified in the analysed bands, human IL-33 was not detected in either band.

The implications of this are twofold. Firstly, there was no full length IL-33 detectable by proteomic analysis in the BAL of our study subjects. The molecular light region of the band that was sent for analysis corresponds to the molecular light of full length IL-33 and thus if this were present in these samples, it would be expected to be detected by this method. Secondly it suggests that the binding found by this antibody by Western blot was non-specific and did not correlate to genuinely detected IL-33. This experiment was repeated with a separate IL-33 antibody (Enzo Lifesciences) and did not show any bands of binding to IL-33.

Figure 5.10

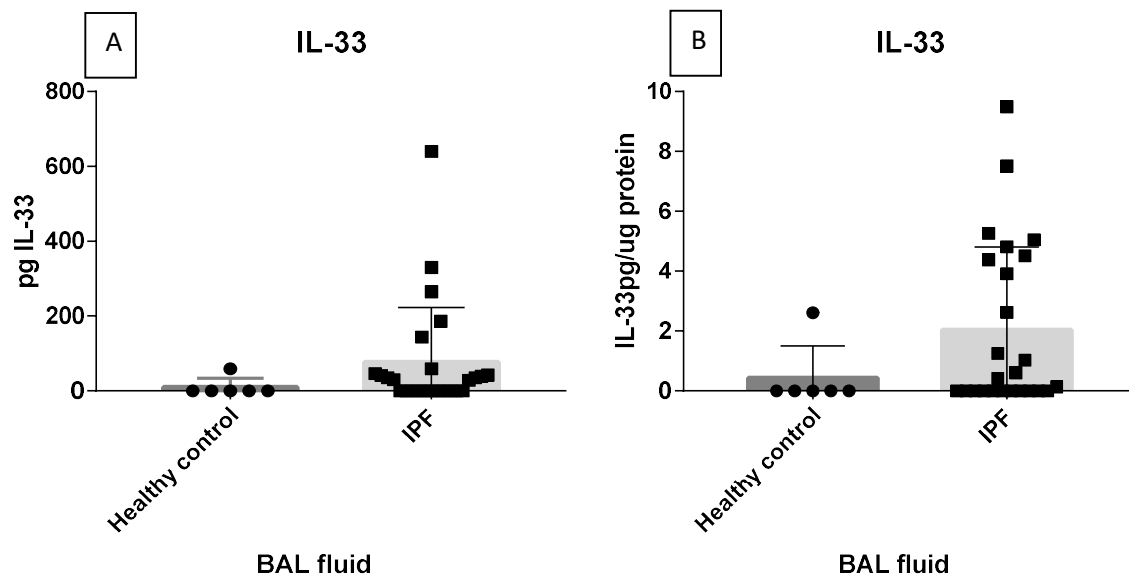


Figure 5.10 IL-33 in BAL fluid detected by ELISA; No significant differences were detected between the groups ($p=0.1452$ Mann Whitney test) when measured as IL-33 in BAL fluid expressed per μg protein (protein measured by Bradford Colorimetric Assay) with healthy control compared ($n=6$) to IPF ($n=25$) or **(A)** total IL-33 in BAL fluid with healthy control compared to IPF **(B)**.

Values expressed as median and interquartile range

Figure 5.11

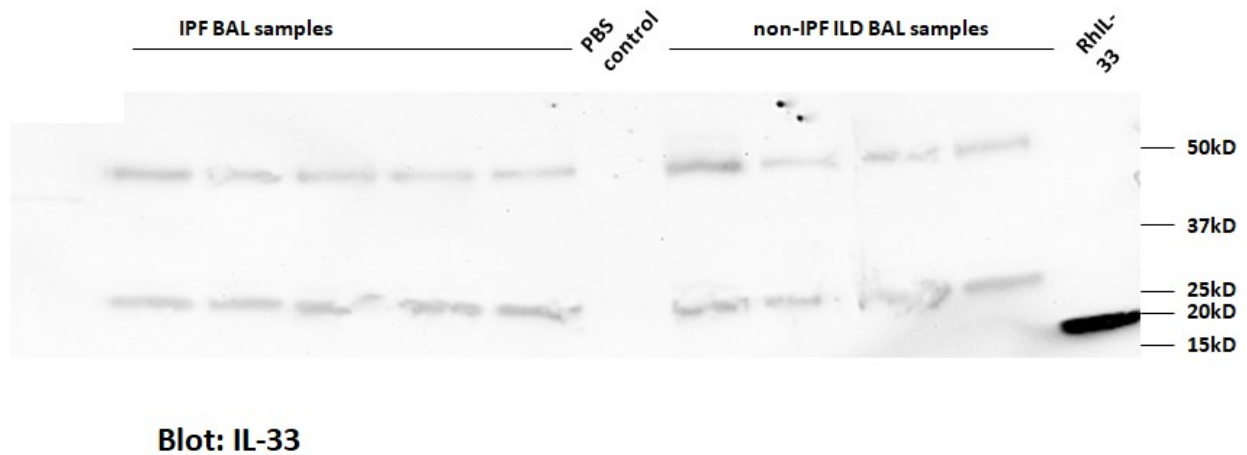


Figure 5.11 Western blot of IL-33 in BAL fluid; IL-33 staining in IPF BAL fluid by Western blot with a polyclonal anti-hIL-33 antibody (RD). Molecular mass markers are indicated on the right. Samples run include a PBS negative control and a sample of PBS spiked with recombinant human IL-33 (RhIL-33) in addition to IPF and non-IPF ILD BAL. 2 bands of staining were detected in the BAL samples, at around 30kD and 50kD with Rh-IL-33 detected at 18kD.

5.2.4.2 Soluble ST2 in the alveolar space

These data, presented herein, did not demonstrate any clear upregulation of IL-33 or ST2 in the IPF lung, however the regulation of its signalling effects is not dependent on these molecules alone. The effects of IL-33 can be attenuated by the presence of the soluble negative regulator soluble ST2, or the membrane bound SIGIRR which can sequester IL-33 and dampen its biological activity. Having found an upregulation of SIGIRR gene expression in IPF HBECS, I sought to establish if this trend

towards increased negative regulation was replicated in the levels of soluble ST2 found in the alveolar space. Levels of soluble ST2 in BAL fluid from the same cohort of individuals with IPF and healthy controls as were examined for levels of IL-33 were analysed by ELISA. This found that Soluble ST2 was detected but predominantly at low levels in IPF and healthy control BAL fluid. No significant differences were detected between IPF and control (Mann Whitney U test) when either comparing the absolute values or when normalised per μg of protein in the BAL samples (figure 5.12). These data are in concurrence with previously published which found that - although it is elevated in acute exacerbation of IPF - at baseline there is no difference between soluble ST2 levels in serum of individuals with IPF over control.

Figure 5.12

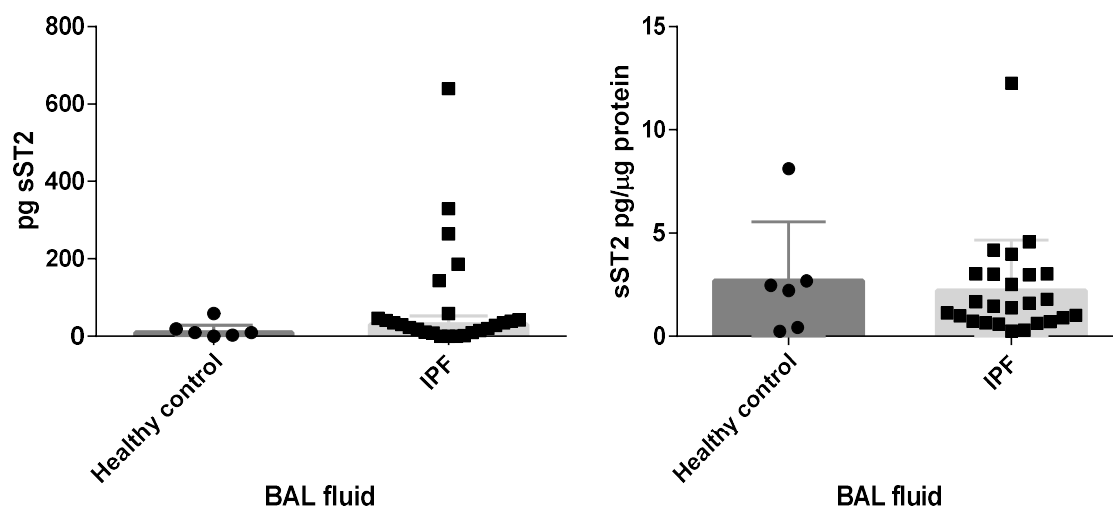


Figure 5.12 Soluble ST2 in BAL fluid in IPF and healthy control; comparison of soluble ST2 levels by ELISA in BAL fluid in IPF (n=25) and healthy control volunteers (n=6) by ELISA, Data are represented as **A)** pg of soluble ST2 detected **B)** pg of soluble ST2 per μg of protein, as measured by BCA assay.

Values expressed as median and interquartile range

5.2.5 Fibroblast function

Having investigated IL-33 expression in lung tissue, primary cells and BAL fluid I further wished to characterise the role of IL-33 in fibrosis by assessing its direct effect upon functional parameters, and this work concentrated upon the effect upon the primary fibrosis effector cell, the lung fibroblast.

This work examined the effect upon a number of key parameters in IPF and control lung fibroblasts of stimulation with recombinant IL-33. I examined wound healing, soluble collagen deposition and pro-fibrotic gene regulation.

5.2.5.1 Wound healing

These data suggest that IPF (n=5) fibroblasts showed a trend towards supra-normal wound healing post injury, compared to healthy control (n=4) (figure 5.13). This trend did not however reach statistical significance. Fibroblast to myofibroblast transformation is a critical part of the fibrotic process in the IPF lung with activated myofibroblasts not usually seen in healthy lung but present in IPF and key in the dysregulated wound healing response. Fibroblasts have previously been shown to exhibit an increased rate of proliferation over healthy control (Ramos et al, 2001) and this increased proliferative rate has been reported to be unaffected by TGF- β stimulation (Raghu, 1989), however data on fibroblast proliferation should be viewed in the context of the fact that proliferative rates have been found to be dependent on the degree of fibrosis present in the area of lung tissue the fibroblasts have been grown from (Raghu et al, 1988).

Fibroblast wound healing was further investigated by studying the effect of stimulation with IL-33 and the pro-fibrotic molecule TGF- β upon fibroblast wound healing using the method detailed above. In IPF lung fibroblasts (n=5) these data show a trend towards a reduction in the rate of wound healing with TGF- β treatment, which did not reach statistical significance with no effect of IL-33 noted on the rate of wound closure (figure 5.14). Results revealed that TGF- β and IL-33

stimulation did not exert an effect on wound closure of scratched normal HLFs (n=4) over this time course (figure 5.15).

Figure 5.13

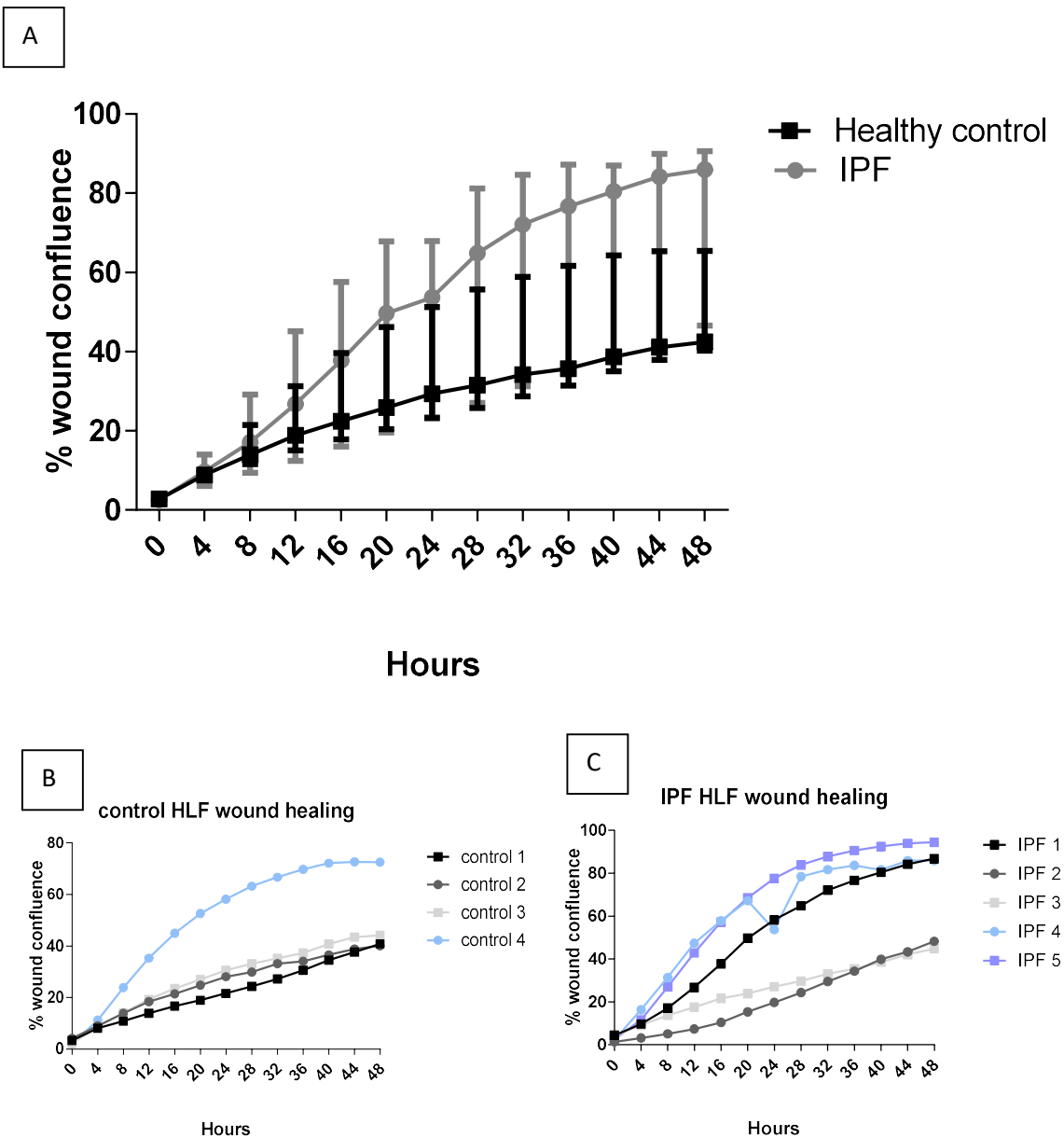


Figure 5.13 Wound confluence of HLF scratch assay; confluence of scratch wound assay over 48

hour time course comparing IPF (n=5) with control (n=4) **A)** wound confluence of individual control HLF lines **B)** wound confluence of individual IPF HLF lines **C).**

Data presented as median and error, confluence at each time point compared using Mann Whitney U test.

These data showed a trend towards an increase in rate of wound healing of IPF HLFs but this did not reach statistical significance.

Figure 5.14

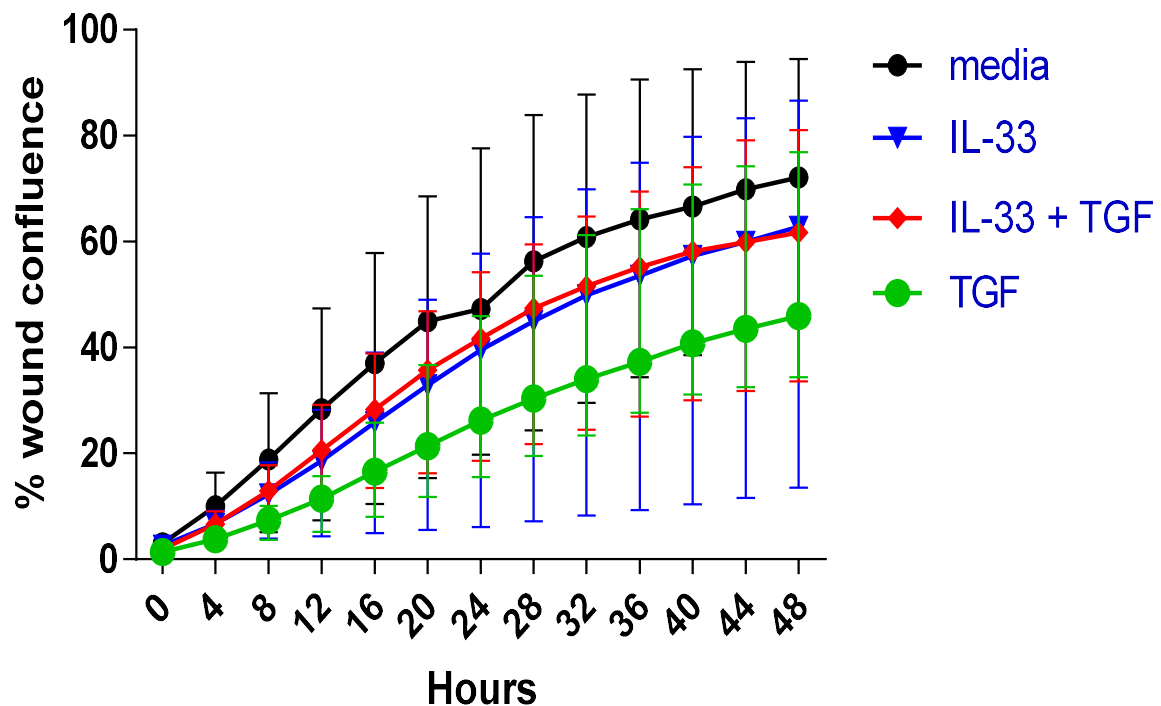


Figure 5.14 Wound healing of IPF HLFs in response to stimulation; effect of IL-33 and TGF- β
stimulation upon wound healing of scratch assay in IPF HLFs (n=5) over 48 hour time course on stimulation with DMEM media, DMEM media + IL-33 100ng/ml, TGF- β 5ng/ml, IL-33 100ng/ml + TGF- β 5ng/ml. Data shown as median and range. Statistical analysis performed using one way ANOVA

Figure 5.15

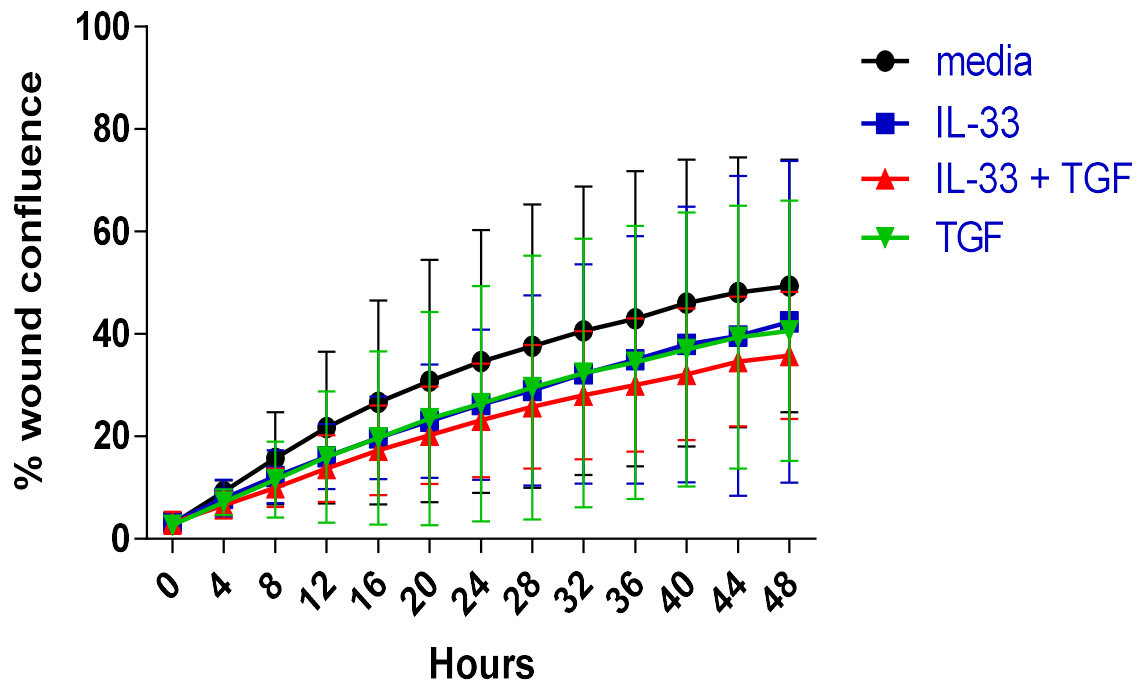


Figure 5.15 Wound healing of healthy control HLFs in response to stimulation; Wound healing of primary fibroblasts from healthy control (n=4) determined, by real time imaging of scratch assay over 48 hour time course, on stimulation with stimulation conditions DMEM media, DMEM media + IL-33 100ng/ml, TGF- β 5ng/ml, IL-33 100ng/ml + TGF- β 5ng/ml. Data is shown as median and range. Statistical analysis performed using one way ANOVA

5.2.5.2 Fibroblast function in response to stimulation

I did not find any evidence of a direct effect of IL-33 upon fibroblasts (either IPF or healthy) when I looked at the parameter of wound healing. I then broadened the scope of our stimulations to

investigate the hypothesis that IL-33 exerts a direct effect upon the extracellular matrix deposition regulated by human lung fibroblasts.

All experiments were conducted with primary IPF HLFs (n=3) at passage 3-5, with gene expression quantified by RT-PCR at 3, 6, 12 and 24 hours. I analysed the expression of COL1A1, α -SMA, TGF- β , TGF- β 1, IL-33 and ST2.

The results of gene expression analysis showed that on stimulation with IL-33, IPF lung fibroblasts (n=3) did not show any upregulation of gene expression of COL1 α 1 (figure 5.16) or α -SMA (figure 5.17) - a marker of fibroblast to myofibroblast differentiation - across a 24 hour time course. This lack of effect with IL-33 treatment was consistent when $\Delta\Delta$ CT values were normalised to the earliest available untreated timepoint. I did not find evidence of a co-stimulatory effect when TGF- β was added to IL-33 stimulation. Highest α -SMA expression was found in all treatment arms at the 3 hour time point and normalised values did not exhibit any clear fold change from control in the IL-33 or TGF- β stimulated treatment groups (figure 5.16).

In these samples, upregulation of TGF- β (figure 5.18) and TGF- β 1 (figure 5.19) were seen in TGF- β stimulated cells but this effect was not evident with IL-33 stimulation alone. These effects were time dependent with TGF- β 1 upregulation highest at the 3-6 hour time point and TGF- β at the 6-12 hour timepoints.

TGF- β stimulation resulted in up-regulation of the IL-33/ST2 axis, with an increase in expression of both ST2 (figure 5.20) and IL-33 (figure 5.21) in a time dependent manner. These data show a time dependent increase in ST2 expression post treatment, with the greatest change seen in cells stimulated with IL-33 and TGF- β combined. Results also revealed a time dependent increase in IL-33 expression post treatment with the greatest change seen in TGF- β treatment group and an increase also seen with IL-33 stimulation. This effect peaked at 12 hours. Combining IL-33 and TGF- β in stimulation did not augment the ST2 response. The effect of IL-33 gene expression upon

stimulation with IL-33 was large, however the data for both ST2 and IL-33 expression did evince significant variability between replicates and this requires further validation in more cell lines. Statistical analysis was not performed on these experiments due to the small numbers involved, with analysis limited to observing trends for large effects. Further work would be needed, with larger numbers, to confirm this effect.

Figure 5.16

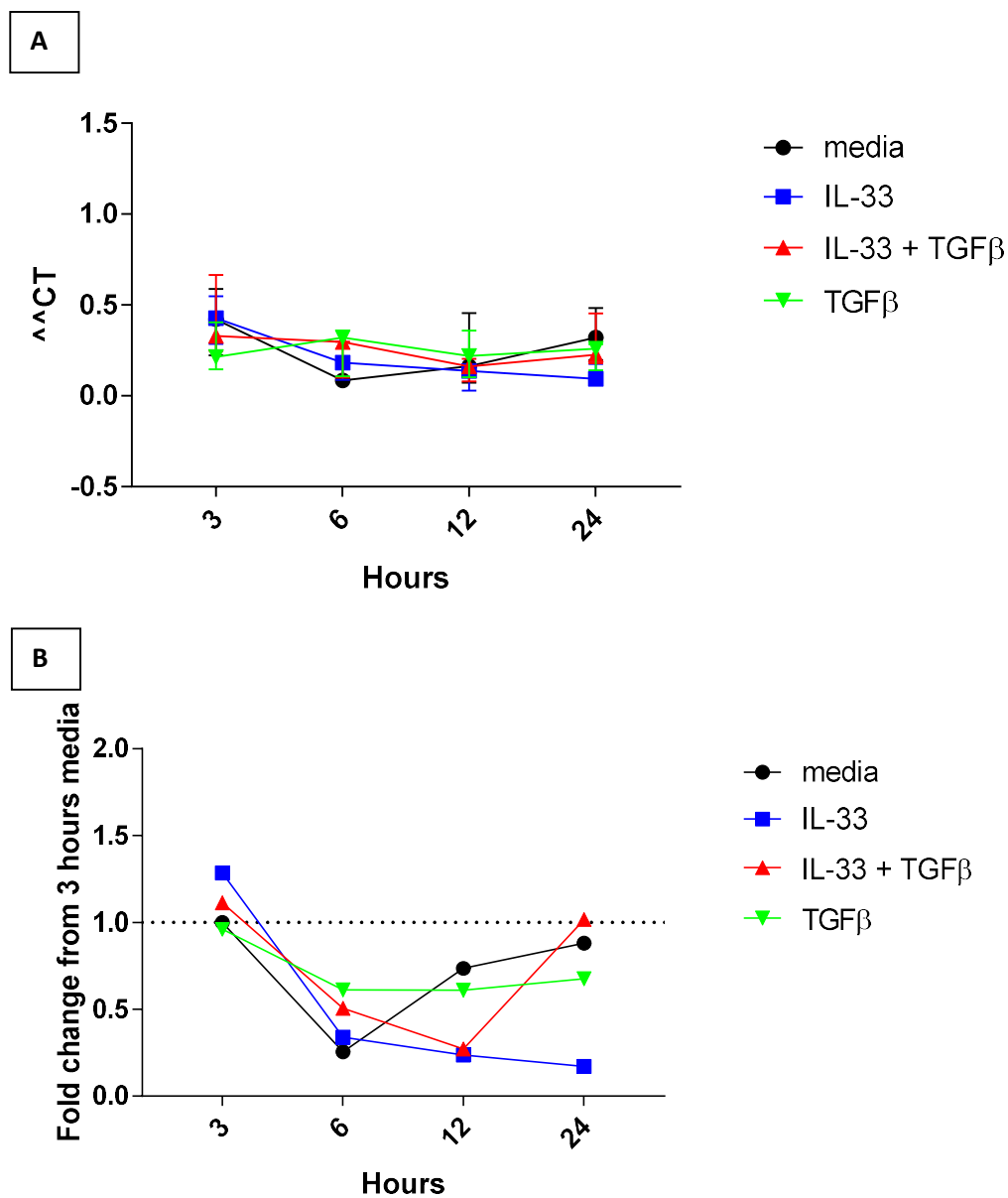


Figure 5.16 COL1A1 expression in stimulated fibroblasts; IPF HLFs (n=3), stimulated in triplicate over 24 hour time-course with media alone, IL-33, IL-33 + TGF- β and TGF- β . COL1 α 1 expression measured by RT-PCR and values represented as $\Delta\Delta$ CT values of TGF- β expression with data expressed as median and interquartile range of triplicate Wells **A)** $\Delta\Delta$ CT values of TGF- β expression normalised to the earliest unstimulated timepoint (media only at 3 hours) **B)**

Figure 5.17

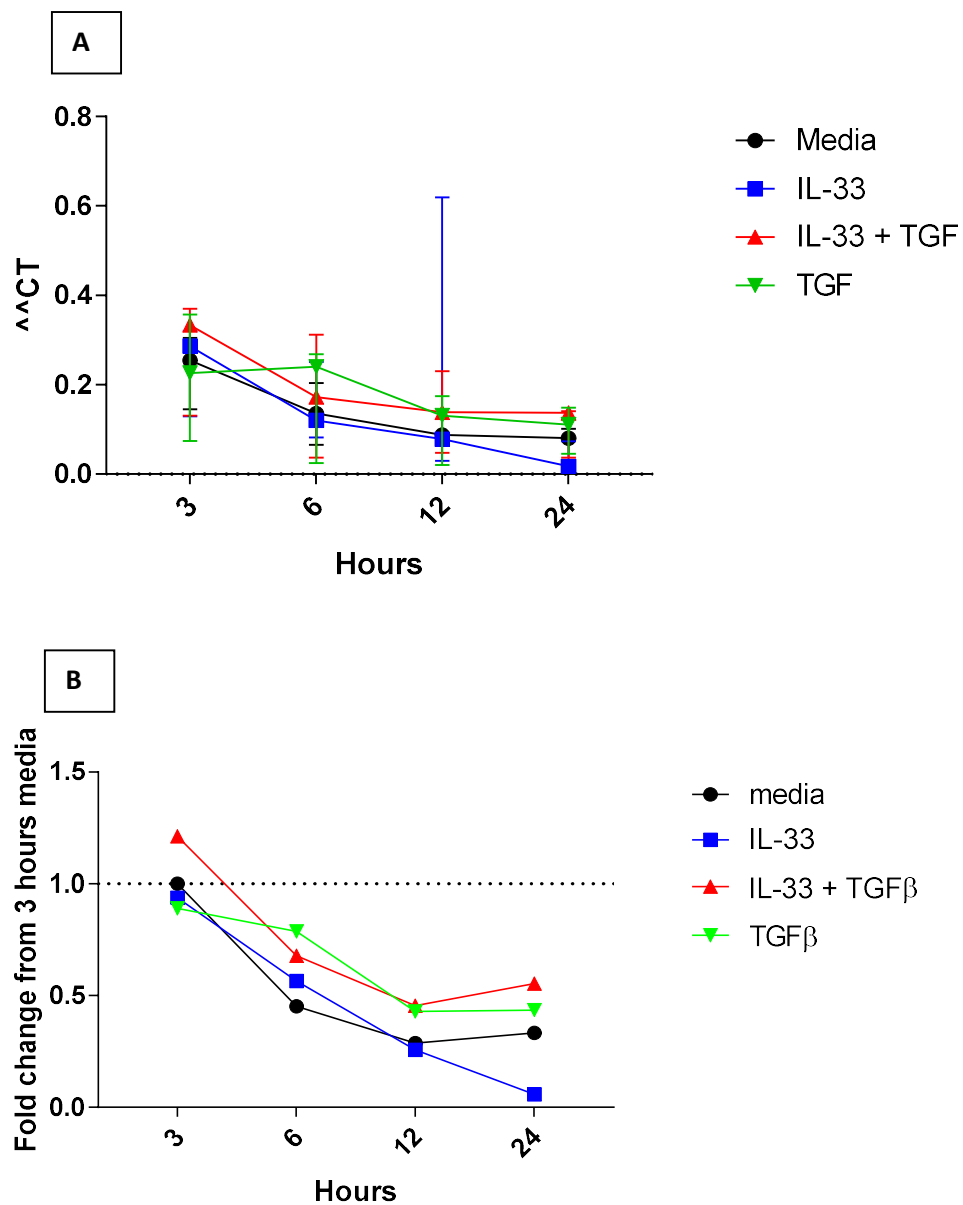


Figure 5.17 α -SMA expression in stimulated IPF;IPF HLFs (n=3), stimulated in triplicate over 24 hour time-course with media alone, IL-33, IL-33 + TGF- β and TGF- β . α -SMA expression measured by RT-PCR and values represented as $\Delta\Delta$ CT values of α -SMA expression with data expressed as median and interquartile range of triplicate Wells **A)** $\Delta\Delta$ CT values of TGF- β expression normalised to the earliest unstimulated timepoint (media only at 3 hours) **B)**.

Figure 5.18

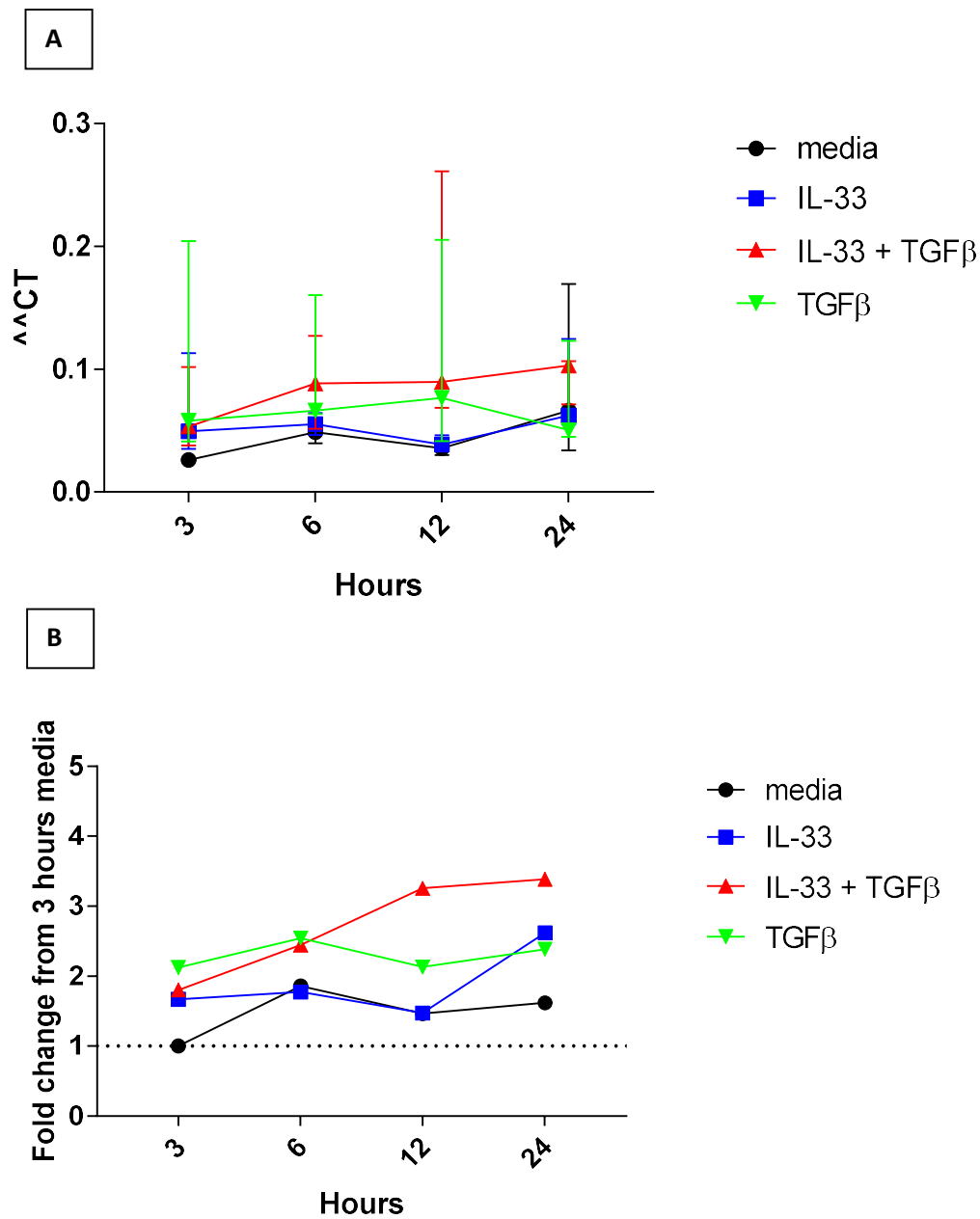


Figure 5.18 TGF-β expression in stimulated fibroblasts; IPF HLFs (n=3), stimulated in triplicate over 24 hour time-course with media alone, IL-33, IL-33 + TGF-β and TGF-β. with TGF-β expression measured by RT-PCR and values represented as ΔCT values of TGF-β expression with data expressed as median and interquartile range of triplicate Wells **A)** ΔCT values of TGF-β expression normalised to the earliest unstimulated timepoint (media only at 3 hours) **B).**

Figure 5.19

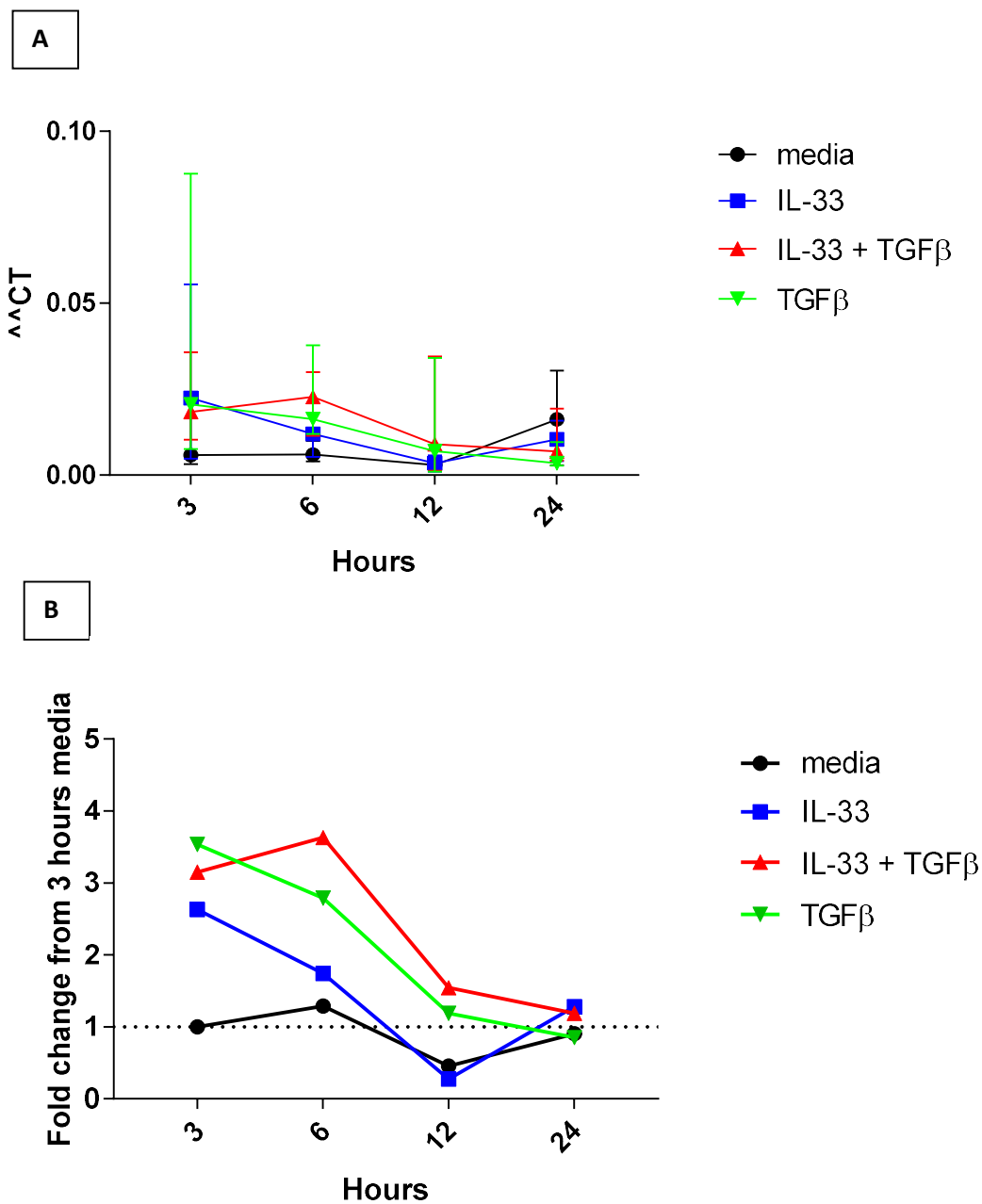


Figure 5.19 TGF- β expression in stimulated fibroblasts; IPF HLFs (n=3), stimulated in triplicate over 24 hour time-course with media alone, IL-33, IL-33 + TGF- β and TGF- β . TGF- β r1 expression measured by RT-PCR and values represented as ΔCT values of TGF- β r1 expression with data expressed as median and interquartile range of triplicate Wells **A)** ΔCT values of TGF- β r1 expression normalised to the earliest unstimulated timepoint (media only at 3 hours) **B).**

Figure 5.20

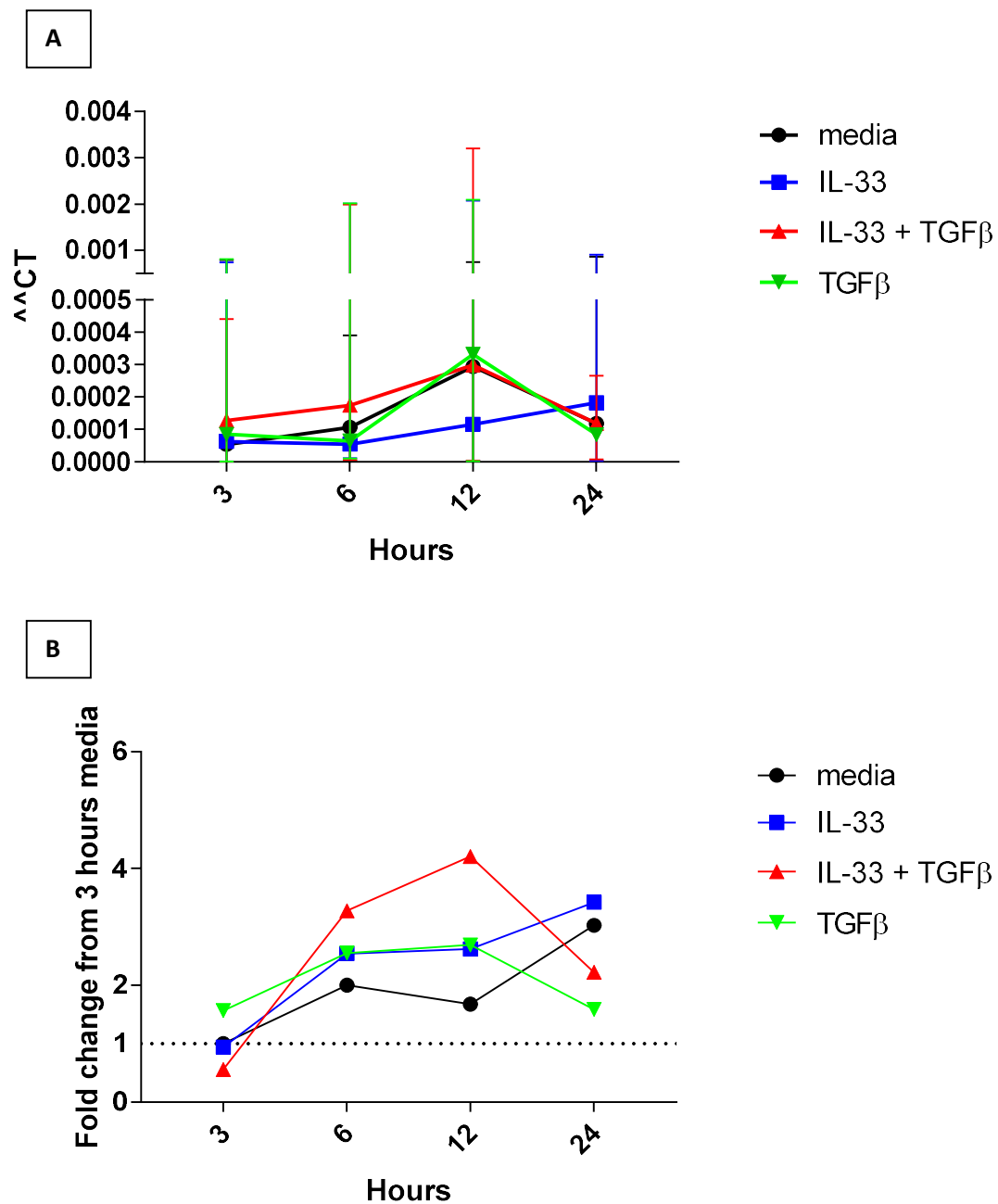


Figure 5.20 ST2 expression in stimulated fibroblasts; IPF HLFs (n=3) were stimulated in triplicate over a 24 hour time course with ST2 gene expression measured at 3, 6, 12 and 24 hours. $\Delta\Delta$ CT values of ST2 expression with data expressed as median and interquartile range of triplicate Wells **A)** $\Delta\Delta$ CT values of ST2 expression normalised to the earliest unstimulated timepoint (media only at 3 hours) **B).**

Figure 5.21

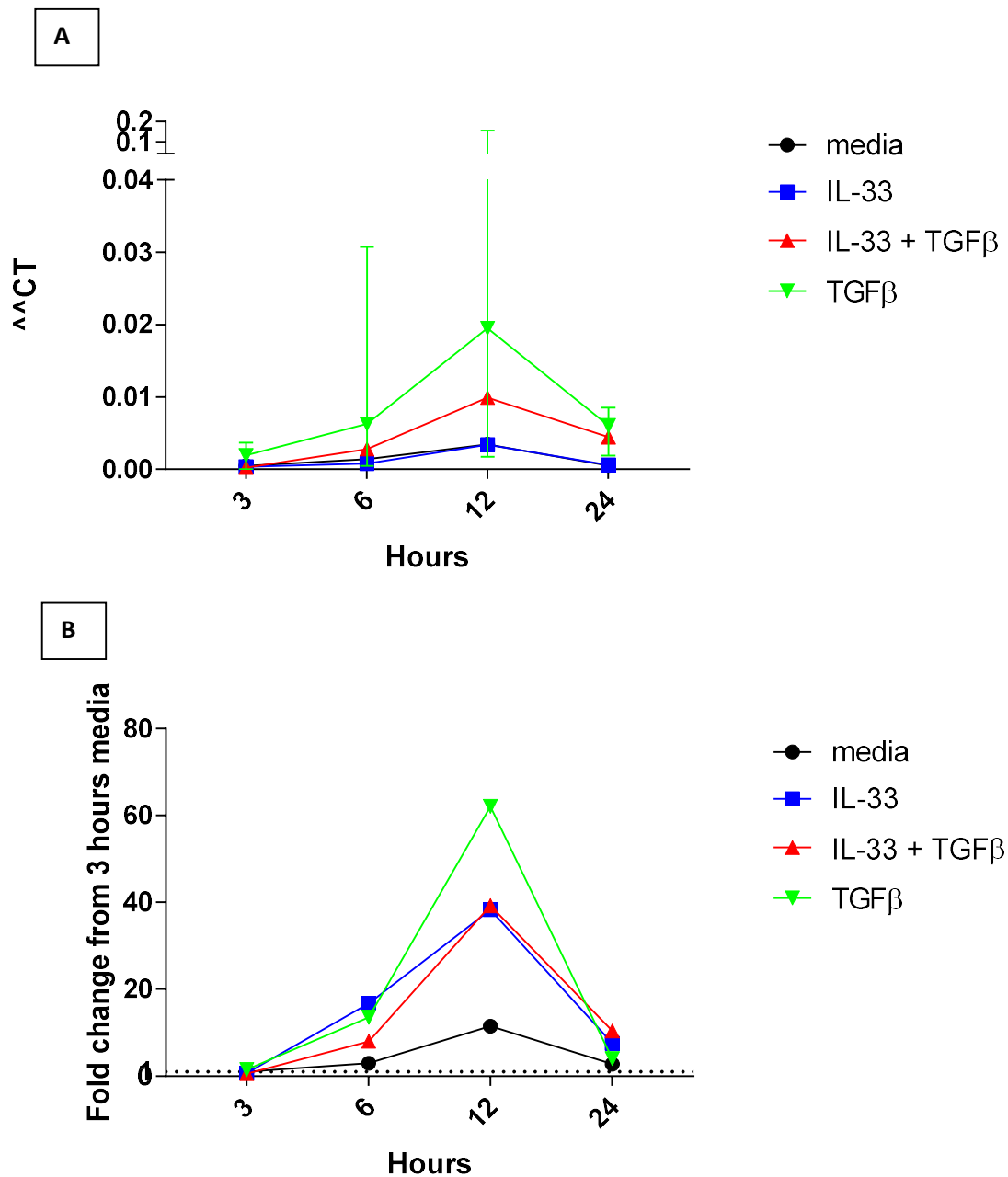


Figure 5.21 IL-33 expression in stimulated fibroblasts; IPF HLFs (n=3) stimulated in triplicate over a 24 hour time course with IL-33 gene expression measured at 3, 6, 12 and 24 hours^{ΔΔCT} values of IL-33 expression with data expressed as median and interquartile range of triplicate Wells **A)** ^{ΔΔCT} values of IL-33 expression normalised to the earliest unstimulated timepoint (media only at 3 hours **B).**

5.2.5.3 Soluble mediators secreted by fibroblasts on stimulation

IL-33 did not exert any effect on the genes regulating extracellular matrix deposition that I interrogated in this chapter. I wished to further characterise the impact upon collagen deposition by measuring secreted soluble collagen and also analysed MMP 9 from our treated fibroblasts. I sought to investigate the possibility that IL-33 has a direct effect upon extracellular matrix turnover by its effect on fibroblast production of MMP9.

Fibroblast supernatant was taken at 48 and 72 hours were taken for measurement of secreted collagen. Sircol assay was performed on IPF (n=3) and healthy control (n=2) fibroblasts supernatant with results suggesting that, at these time points, IL-33 did not effect any change in collagen deposition over our untreated control (figure 5.22), however, statistical analysis was not performed on these experiments due to the low number of healthy controls.. Later time points were taken as our 24 hour gene expression analysis did not suggest the presence of an early upregulatory effect, however there does remain the possibility that an earlier effect on collagen deposition could be present which this study may not have detected.

Released bioactive MMP9 was measured in cell supernatant of IPF (n =3)and healthy control (n =2) fibroblasts stimulated with IL-33 and TGF- β by gelatine zymogram, with the gelatinolytic activity quantified by measuring the integrated density of the bands, visualised by coomassie blue staining. No differences were shown between the treatment groups in IPF or control (figure 5.23).

Further analysis of the secretory effect of IL-33 and TGF- β stimulation in IPF was directed towards investigating its effect upon soluble ST2 and monocyte chemoattractant protein-1 (MCP-1) production by HLFs and HBECs in IPF and control.

MCP-1 is a pro-inflammatory chemokine which also promotes collagen deposition and TGF β production in fibroblasts. It is elevated in lung tissue in individuals with IPF and has been shown to be produced from epithelial cells and fibroblasts (Liu et al, 2015). Using cell supernatant from

stimulated IPF and normal fibroblasts and bronchial epithelial cells at 24 hours, I examined the levels of MCP-1 in response to IL-33 and TGF- β stimulation.

This work demonstrated both IPF and normal HBECs stimulated with IL-33 showed a statistically significant reduction of measured secreted soluble ST2 as compared with control. This effect was also seen with combined IL-33 and TGF- β stimulation in IPF samples but not in unstimulated control samples. The trend towards a decrease in soluble ST2 in IL-33 treated cell supernatant was also seen in the supernatant of stimulated fibroblasts but did not reach statistical significance and this may be related to the smaller n number in this experiment.

This effect could represent a negative feedback mechanism of IL-33 stimulation which acts to enhance its effect, or could be due to reduced binding of free soluble ST2 as a result of it having sequestered IL-33.

MCP-1 was secreted by both control and IPF and healthy control HBECs and HLFs. Upon stimulation with IL-33 and TGF β , there were no significant differences between the levels of MCP-1 secreted by IPF and control HBECs or HLFs.

IL-33 stimulation did not produce any demonstrable effect on human lung fibroblasts in the parameters examined. It showed no effect on fibroblast wound healing, ECM critical gene regulation or collagen deposition. Furthermore I did not find any evidence of its previously reported potential upregulation of MMP9 in IPF or normal human lung fibroblasts. These data, although based on work performed with small n numbers, did not provide evidence to support the hypothesis of IL-33 being a direct signal to which fibroblasts respond by upregulation of factors critical in ECM turnover.

Figure 5.23

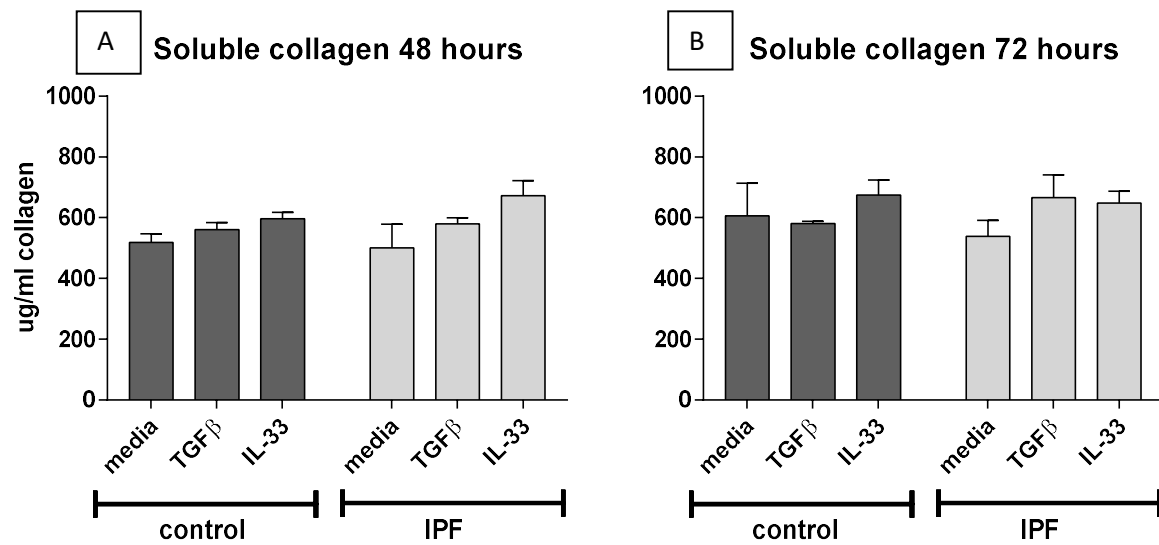


Figure 5.22 showing soluble collagen deposition in IL-33 and TGF-β treated healthy control and IPF fibroblasts. HLFs were seeded onto culture plates and grown to 90% confluence then stimulated with IL-33 and TGF-β for 48 and 72 hours and supernatant then removed.. Soluble collagen was measured in supernatant of stimulated IPF (n=3) and healthy control lung fibroblasts (n=2) at 48 hours and **A**) 72 hours using Sircol assay **B**).

Values presented as median and range.

Figure 5.23

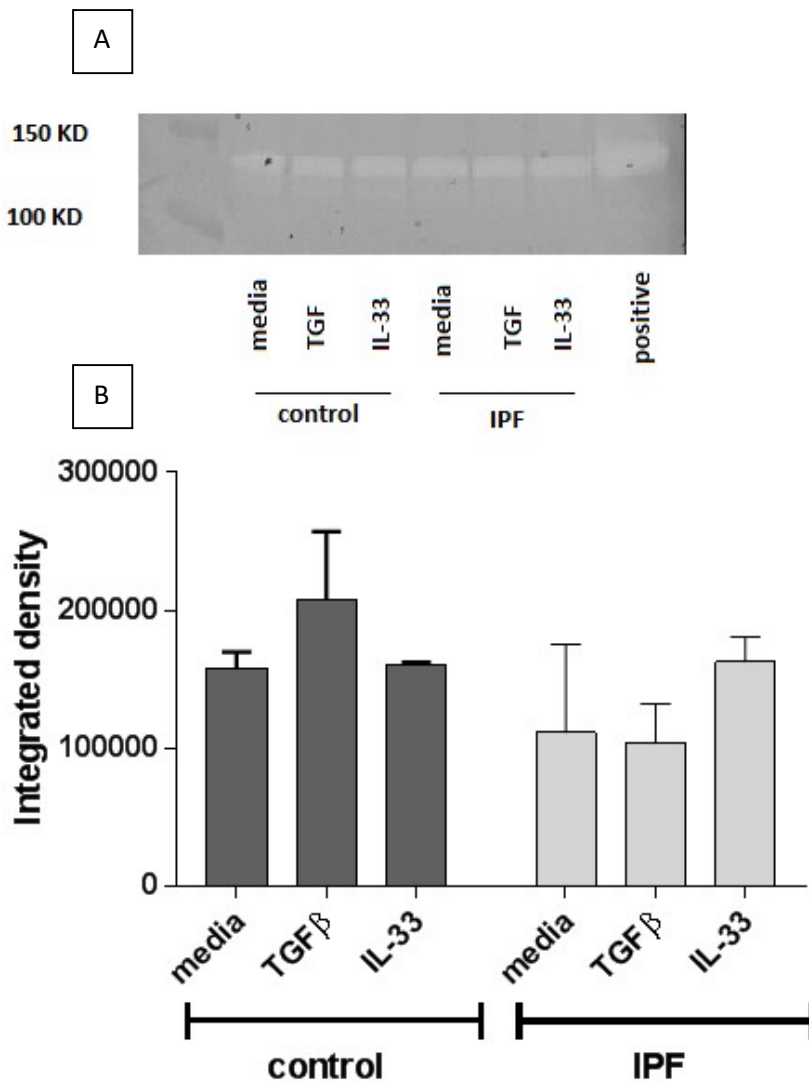


Figure 5.23 Bioactive MMP9 in supernatant of stimulated fibroblasts; Bioactive MMP9 was measured by gelatine zymography in the supernatant of control and IPF HLFs, were stimulated with TGF- β , IL-33 or with fibroblast media alone. Supernatant was collected at 24 hours post stimulation. Representative micrograph of gelatine zymogram of MMP9 release **A)** MMP9 levels secreted by stimulated IPF (n=3) and control lung fibroblasts (n=2) **B).**

Values are shown as median and range.

Figure 5.24

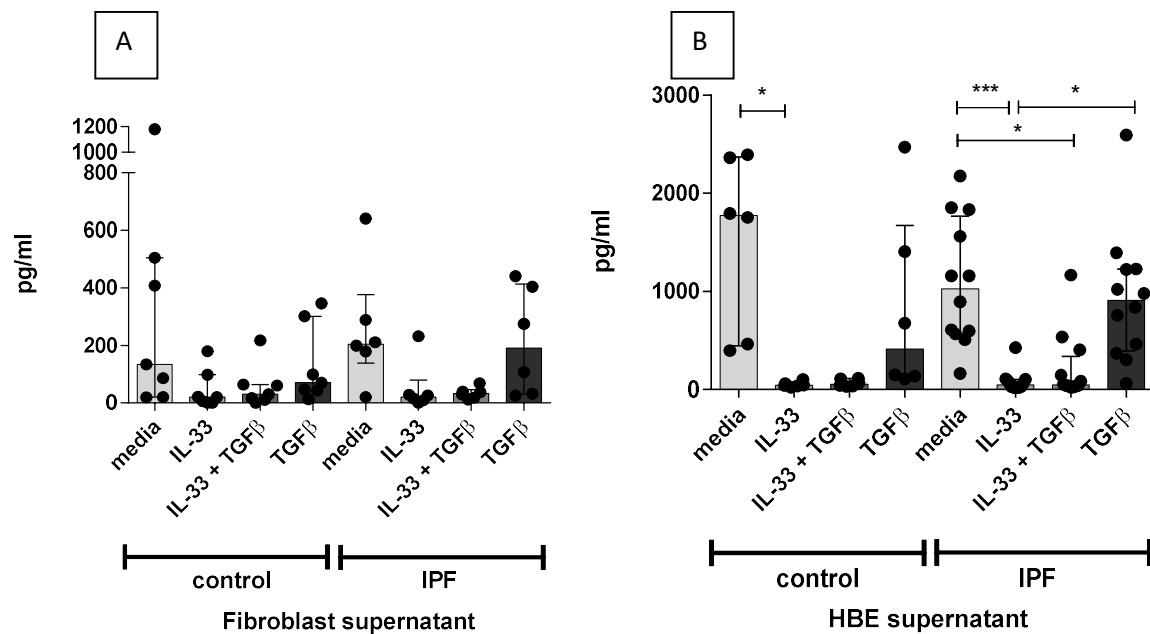


Figure 5.24 Soluble ST2 in supernatant of stimulated IPF and healthy control HBECS and lung fibroblasts; soluble ST2 in supernatant of Fibroblasts from IPF and (n=6) and healthy control (n=7) and **A)** HBECS from IPF (n=13) and control (n=6) stimulated with IL-33 and TGF-β **B)** .

Data shown as median and range, comparisons made using one way ANOVA and Dunn's multiple comparisons test. *=p<0.05, **=P<0.01, *** =p<0.001

Figure 5.25

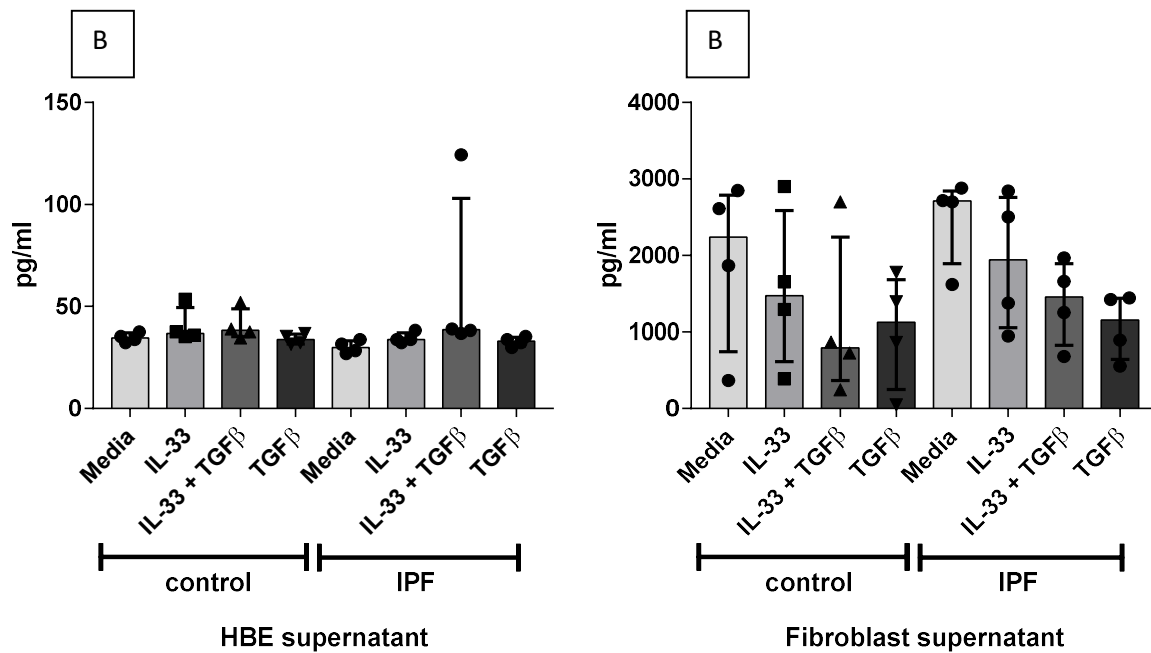


Figure 5.25 MCP-1 secretion from IPF and healthy control fibroblasts and bronchial epithelial cells stimulated with IL-33 or TGF-β; Fibroblasts and HBEs were seeded into culture Wells and grown to confluence then stimulated with IL-33 and TGF-β as per previous experimental protocol (figure 5.15) and MCP-1 was measured in supernatant removed at 24 hours from IPF (n=4) and healthy control (n=4) IPF HBEs and **A**) IPF (n=4) and healthy control (n=4) IPF HLFs **B**).

Values shown as median and interquartile range, comparisons made using one way ANOVA and Dunn's multiple comparisons test

5.3 Discussion

In this chapter I described the distribution of IL-33 and its receptor ST2 in lung tissue, and secreted into the alveolar space, comparing IPF and healthy control. I examined the effect IL-33 has upon fibroblast function and gene expression as well as protein expression. The results demonstrated some evidence of minor dysregulation of the IL-33/ST2 signalling axis in IPF, but did not show any direct link between IL-33 and a conventionally pro-fibrotic response, as measured by collagen gene regulation, collagen secretion, proliferation rates or fibrosis associated mediator release.

I demonstrated immunoreactivity of IL-33 predominantly in the basal cells of the bronchial epithelium, with IL-33 staining also detected in endothelium, tissue macrophages and fibroblasts. ST2 was predominantly detected on ciliary cells lining the airways and was also found on macrophages and lymphocytes distributed throughout the parenchymal tissue. Heavy staining of both was found in lymphoid aggregates and low level staining was detected in fibroblastic foci. I did not find any difference between IPF and control. Further immunostaining showed IL-33 to be found both in the nucleus and cytoplasm of HBECs. This data is in keeping with previously published work which has shown IL-33 to be widely distributed in humans with protein expression found in bronchial epithelium and endothelium, airway smooth muscle (Kaur et al, 2015) (Moussion et al, 2008), lymphoid tissues, macrophages (Schmitz et al, 2005), fibroblasts (Velickovic et al, 2016) and mast cells (Masamune et al, 2010). Further staining of cultured HBECs demonstrated IL-33 to localise to nucleus and cytoplasm of IPF and healthy control which is in accordance with data from Gordon et al (Gordon et al, 2016) showing that, in asthma, IL-33 shows alternative splicing, with one of the discovered alternatively spliced isoforms localising to the cytoplasm allowing for extracellular secretion.

The observation of IL-33 staining within fibroblastic foci is a novel observation, which has not been reported before. Fibroblastic foci are small aggregates of actively proliferating myofibroblasts and fibroblasts which are considered the hallmark lesion of IPF; they represent organisation of prior foci

of acute lung injury (Fukuda et al, 1995) and have been shown to be the sites of active collagen synthesis (Kuhn et al, 1989). Fibroblastic foci have been established as a manifestation of ongoing lung injury in patients with pulmonary fibrosis and fibroblastic foci are thought to be the key lesions in IPF pathogenesis. The data presented here shows that staining of IL-33 and ST2 is found in fibroblastic foci, but at low intensity. The most intense regions of staining for both cytokine and receptor in IPF tissue was in areas of lymphoid aggregation, as well as in airway epithelium. Taken together these insights do not provide clear evidence of IL-33 having a direct non-immune cell mediated effect in the IPF lung, but point towards it exerting its signalling effect through immune cell mediated mechanisms and a more clear association with the inflammatory response than with areas of clear tissue fibrosis..

I examined BAL fluid for secreted IL-33 by Western blot of BAL fluid, with early work suggesting detectable levels of 2 isoforms of IL-33, of differing molecular weights, secreted into the alveolar space. Relative density of both detected bands was quantified by ImageJ technology and I found a statistically significant increase in levels of the higher molecular weight band at ~50kDa with no difference detected between the lower bands of ~30kDa. Our findings did not clearly correlate to the published body of work, with IL-33 isoforms having been found to be found at ~18kDa for the spliced version of IL-33 and the full length form of IL-33 found at 30-32 kDa (Hong et al, 2011) (Talbot-Ayer et al, 2009) (Gautier et al, 2016), findings also replicated by the localisation of our IL-33 positive control as well. There exist many isoforms of IL-33 which can vary in terms of localisation, biological activity and molecular weight, however a higher molecular weight variant ~50kDa isoform of IL-33 has not been previously reported. I proceeded to further investigate this finding by mass spectrometry of proteins, electrophoretically separated to molecular weight. Numerous proteins were detected at ~30kDa and ~50kDa, but IL-33 was not detected in either band. This finding suggests that IL-33 is not present in high enough abundance to be detectable in our samples at the molecular weight corresponding to the bands evident with staining with IL-33 (R&D), or/and there is a problem with the specificity of the antibody binding in these samples. The results could also indicate

a low level of secretion of IL-33 into the alveolar space in our samples, below the limit of detection of the method of mass spectrometry.

The detectable bands I found in our BAL samples suggest non-specific binding with the R&D IL-33 antibody. These findings were then confirmed with repeat Western blot of the same samples using a different IL-33 antibody (Enzo, Life Sciences), which did not reveal any binding at either molecular weight. This antibody has been widely used in IL-33 research but has not, to our knowledge, been published with respect to findings in human BAL samples.

I further investigated expression of the IL-33/ST2 signalling axis in BAL by examining the levels of IL-33 and soluble ST2 (sST2) by ELISA. sST2 has been reported to be elevated in the IPF lung during acute exacerbations (Tajima, 2003) but no data has hitherto compared the levels compared with control. Our data showed that, both IL-33 and sST2 (expressed as μg of protein loaded) were detectable in BAL samples, but did not demonstrate any significant difference compared with healthy control. Taken together with the data from mass spectrometry of BAL fluid –which showed IL-33 not to be present in the ~ 30 kD band- the finding of detectable BAL IL-33 by ELISA means this ELISA must be detecting the lower molecular weight proteolytically cleaved variant of IL-33 rather than the full length variant.

As detailed in the previous chapter, gene expression of IL-33, ST2, IRAP and SIGIRR by qPCR of HBECS revealed a statistically significant up-regulation of SIGIRR and a trend towards increase in IL-33 and ST2. When taken together with our characterisation of IL-33 and ST2 in lung tissue and BAL samples, these data suggest a possible up-regulation of the signalling axis at a transcript level in HBECS, but no clear detected increase in detectable protein levels. Given the higher sensitivity of qPCR quantification compared with methods of immunohistochemical quantification, this could suggest subtle changes which have not been detectable at a protein level. The immunoreactivity of IL-33 and ST2 in tissue showed similar distribution in IPF and control lung tissue suggesting any up-regulation present is not detectable using this method. The difference in SIGIRR gene expression,

could be a feedback regulator in response to HBECs being exposed to higher levels of IL-33, but could also be a function of the slightly different demographic profiles of the IPF samples compared to our control samples. No data is available on how ageing affects the regulation of this axis and our disease and control groups were not age matched.

Having not demonstrated a clear or unequivocal change in expression of IL-33 or the receptor ST2, I proceeded to investigate the functional effect exerted by IL-33 upon the principal fibrosis-effecting cell, the lung fibroblast. Wound healing was measured by real time cell imaging of scratch assays with IPF compared to control. Our data showed that at baseline, IPF HLFs exhibited an enhanced wound-healing rate when compared with healthy control, and this effect was abrogated by the addition of TGF- β . With the addition of IL-33 to TGF- β stimulation, this effect was partly overridden, returning the IPF HLF wound healing towards its normal rate. Normal HLFs showed no response to the addition of TGF- β or IL-33.

This effect however did not reach statistical significance in this dataset, and were it true it would seem surprising given the widely reported prominence of TGF- β as a prominent pro-fibrotic cytokine. However, published data has shown the role of TGF- β to be potentially bi-modal, with conflicting data showing it can both inhibit and promote fibroblast wound healing (Goldberg et al, 2002). The effects of TGF- β have been previously demonstrated to be dose dependent (Chen et al, 2012) (Lal et al, 2003) with maximal effects seen between 1 and 10ng/ml and the dose of TGF- β used for stimulation compares with other published literature, which have widely used a range between 1 and 10ng/ml (Kissin et al, 2002) (Ghatak et al, 2017) (Krupsky et al, 1996).

TGF- β has been shown to promote fibroblast survival (Kulasekaran et al, 2009), and has also been reported to both increase fibroblast proliferation via platelet derived growth factor (Fernandez et al, 2012), and down-regulate the expression of the PDGF receptor on lung fibroblasts (Bonner et al, 1995). TGF- β is a pleiotropic cytokine with its many functions seemingly dependent on both concentration, and tissue environment. This study looked at monoculture and, therefore, our

results reflect only the direct effect upon HLFs, without allowing examination of the wider and secondary effects TGF- β may have upon wound healing when taking its roles in cell to cell interaction into account. This work focused on characterising the functional effects of IL-33 upon fibroblasts, with TGF- β added to allow detailing of the effects of IL-33 when compared to and alongside it. A fuller investigation into the bi-modal effects TGF- β alone can exert was outwith the scope of this work.

The interaction between IL-33 and TGF- β has been shown to be complex and tissue specific. IL-33 has been found to enhance TGF- β release from murine lung epithelium, and in turn, TGF- β enhanced IL-33 mediated inflammation via ILC2 recruitment (Denney et al, 2015). TGF- β can suppress IL-33 induced mast cell function in the lung (Ndaw et al, 2017) and suppresses IL-33 function in the inflamed gut (Rani et al, 2011).

The counteractive effect of IL-33 upon TGF- β suppression of HLF wound healing demonstrated by our data has not previously been reported and does not easily fit into the current picture of IL-33/ TGF- β interaction. It could be inferred that this suggests a pro-fibrotic role, opposing -as it does- the restoration of IPF wound healing, to an over exuberant phenotype. However IL-33 itself does not have a pro-wound healing effect, and the significance of this action is difficult to interpret fully at present.

Further characterisation of the functional effect of IL-33 upon HLFs was detailed by direct stimulation with recombinant IL-33. Subsequent qPCR analysis did not demonstrate any effect upon the key fibrotic genes, COL1 α 1, COL3a1 or α -SMA, suggesting that stimulation with IL-33 does not exert any direct effect upon collagen production or fibroblast-myofibroblast transformation.

I also examined the effect upon ST2 and IL-33 gene expression. Over the 24-hour time course, the peak of IL-33 gene up-regulation was seen in all stimulated groups at 12 hours, with a considerable upregulation seen in response to TGF- β , IL-33, and TGF- β plus IL-33 combined when compared to the

unstimulated control. TGF- β stimulation provoked the greatest upregulation, with the addition of IL-33 effecting an upregulation from control, but at a lower level than with TGF- β alone and when IL-33 and TGF- β were combined in stimulation, the effect seen was similar to that of IL-33 alone. ST2 expression increased in a time dependent manner with a small peak seen at 12 hours with IL-33 and TGF- β treatment combined. I explain the absence of a clear effect of TGF- β on COL1A1 and α -SMA (as would be expected) by the fact that all cell stimulations were performed in media with fetal bovine serum. Therefore, our untreated control was not denuded of TGF- β , but our TGF- β treatment groups contained higher concentrations of serum.

To further investigate our findings with respect to collagen regulation, I measured soluble collagen in cell supernatant and did not find IL-33 to exert any pro-collagen effect. In contrast to previously published work, I did not find any directly pro-fibrotic effect of TGF- β with our samples. I feel this is likely due to the presence of fetal bovine serum (FBS) in the cell media of all stimulation conditions. The concentration of TGF- β in FBS means that the TGF- β effect would be detectable in all conditions, rather than just those containing supplemented levels of TGF- β .

The cell supernatant was also examined for a selection of secreted mediators to further explore possible avenues of IL-33/ST2 regulation and the mechanisms by which it might provoke a fibrotic reaction. Soluble ST2 production was attenuated from cells that had been treated by IL-33 compared with control and with TGF- β treated cells. IL-33 and the soluble form of the receptor ST2 act in opposing ways as ST2 acts to limit the effect of IL-33 by binding and sequestering it. This data may suggest that IL-33 can have a similarly antagonistic effect upon soluble ST2 by diminishing its expression, however this effect could potentially be due to altered binding of soluble ST2 to detecting antibodies when bound to IL-33. I also examined MCP-1 expression as there is some published work suggesting it may be pathogenic in IPF and has been shown to be induced by IL-33 (Moulin, 2007), however these data showed that although fibroblasts do secrete MCP-1, its regulation is not altered by the effect of IL-33 in lung fibroblasts or HBECs in either the IPF disease

state or in healthy control. Our data did suggest a trend towards an attenuation of MCP-1 secretion in IPF and healthy control fibroblasts post stimulation with TGF β , and, although this did not reach statistical significance, the n numbers in this experiment were small. The effect of TGF β on MCP-1 production has been documented in contexts other than the lung, showing no effect on its own but a downregulation in combination with TNF α in endothelial cells (Wels et al, 1999) and glomerular cells (Wolf et al, 2002) and an upregulation in vascular smooth muscle cells (Lee et al, 2017). However to our knowledge this is the first data examining the effect of TGF β on primary lung cell production of MCP-1, and is suggestive of a potential role in reducing MCP-1 expression in the lung. Given the increased expression of both TGF β and MCP-1 in IPF, it is not clear what significance this finding may have in relation to IPF pathogenesis.

These observations, when taken as a whole, do not provide evidence to support the hypothesis that the IL-33/ST2 axis contributes to the development of pulmonary fibrosis through non immune cell mediated mechanisms. These data have not shown a direct pro-fibrotic effect of IL-33 upon conventional measures of fibroblast dysfunction, with examination of wound healing and collagen gene expression showing no effect in response to stimulation with IL-33. This study found evidence of only minor changes in expression of the components of IL-33 and its receptor complex in the IPF lung, although the wider biological significance of these apparently minor differences has not yet been discerned. The absence of functional effect exerted by IL-33 despite its clear and reproducible role in animal models, may be a consequence of the limitations of translating animal findings in animal models of fibrosis to the setting of IPF in the human lung. The most widely used bleomycin model is one which has a prolonged inflammatory phase before resolution occurs and fibrosis develop. This is a poor replicate of IPF which is believed to be a disease of chronic repetitive micro insult and does, I believe lead, to a profusion of inflammatory pathways being mistakenly implicated in the fibrotic process, when they are, in fact, more likely to be attributable to the overwhelming early inflammation in response to the acute insult of bleomycin administration.

5.4 Future work

The aims of this study were relevant to the hypothesis, and these *in vitro* findings specifically addressed the question of direct fibrogenic effects of IL-33 and looked at numerous functions of lung fibroblasts but with small n numbers. This did not demonstrate any evidence of a pro-fibrotic effect, but further confirmatory work could focus on increasing the numbers included in these experiments to strengthen the power of the observation.

The ability to establish convincing evidence of the levels of IL-33 secreted in the IPF lung is inhibited at present by the limitations of the methods of analysis currently available. Examination with the more sensitive luminex assay could be performed and In light of recently published evidence showing that the biological activity of IL-33 depends on the splice variant, further work could focus on establishing the levels of the different splice variants present, which may shed further light on IL-33 regulation in the IPF lung.

IL-33 predominantly exerts its effects through its induction of Th2 responses via innate lymphoid cells and this study does not provide any data on the indirect immune mediated effects that it may exert. In order to establish if IL-33 may exert an effect via immune cell mediated mechanisms, further work looking at co-culture with ILCs would and fibroblasts would be of interest.

Chapter 6.

Discussion

6.1 Summary of findings and impact of project

The hypothesis tested in the work of this centred around the contribution of airway cells to the development of idiopathic pulmonary fibrosis. I hypothesised that;

- 1) Airway macrophages from individuals with fibrosing lung disease exhibit distinct activation states compared to healthy controls and skew towards an M2 phenotype.
- 2) The phenotype of the bronchial epithelium is altered from the healthy state in idiopathic pulmonary fibrosis, and disruptions are seen in bronchial epithelial proliferation and barrier function, leading directly to extra cellular matrix deposition in the fibrotic lung.
- 3) Direct tissue specific effects of the IL33/ST2 axis contribute to the development of lung fibrosis through non-immune cell mediated mechanisms.

The M1/M2 paradigm was originally observed from murine macrophages which were noted to differentially activate depending on whether they were exposed to IFN γ /and or LPS or IL-4 (Stein et al, 1992). This Well established mouse model has provided supporting evidence for the role of macrophages in lung fibrosis, with M2 specifically having been implicated - airway macrophages are the predominant cell recruited after AEC injury, are involved in collagen uptake, ECM turnover (Dancer et al, 2011) (Atabai et al, 2009) and exacerbate bleomycin induced fibrosis (Gibbons et al, 2011). Data in mice has shown that M2 - believed to be 'wound healing' macrophages - are involved in Th2 immune responses, believed to be pro-fibrotic, and can be induced by a variety of mediators, including IL-4, IL-13, TGF- β and IL-10 (Murray, 2014). Type II alveolar injury in mice produces a macrophage laden infiltrate (Osterholzer, 2013), with the depletion of M2 ameliorating fibrosis, and adoptive transfer on M2 precursors exacerbating fibrosis. There has also been early work suggesting an increase in CD163 positive macrophages in the human fibrotic lung (Gibbons et al, 2011). However, Xue et al have presented extensive transcriptional profiling which has shown macrophages to have a much broader response to signalling than previously thought, and this has led to a more

complex understanding of the diverse and heterogeneous phenotypes that these very plastic cells can exhibit (Xue et al, 2014).

To address the first hypothesis, BAL leucocytes were obtained from individuals with a variety of different ILDs, at bronchoscopy. These cells were then characterised by flow cytometric analysis, with particular focus on airway macrophage phenotype, and correlated to the clinically relevant parameter of serial lung function measurements. The comparison groups were stratified into fibrotic vs non-fibrotic as well as IPF vs non-IPF ILD and compared to healthy control. The results of this analysis demonstrated that, in accordance with previously published data there was an increase in BAL cellular yield of IPF when compared to healthy control individuals (Ohshimo et al, 2009).

These data did not demonstrate any difference in proportions of BAL leucocytes, lymphocytes or airway macrophages between IPF and control, or between IPF and non-IPF ILD, or fibrotic vs non-fibrotic ILDs. There was no difference in proportions of M1/M2 macrophages in any of the analysis groups, but these findings did show a significant difference between the proportions of putative M1 markers CD86 and HLA-DR, and M2 markers CD163 and CD206 on BAL macrophages. This suggests that even these widely published M1/M2 markers are not expressed at the same level on airway macrophages and may call into question their utility as a descriptor of airway macrophage populations. This confirms recent developments in macrophage biology, which have seen a shift away from the M1/M2 polarisation model towards a more nuanced view of macrophage activation.

These data did not show any evidence of a correlation between either extent of fibrosis or subsequent clinical course (as determined by lung function parameters) and BAL cell type proportions or either M1 or M2 macrophage markers in IPF. There was, however, a statistically significant correlation between CD 206 and TLCO decline, and CD86 and TLCO decline in non-IPF ILD. It is not immediately clear why this was found in this diverse, less well defined patient population, but not in IPF, but the n numbers were higher in the non-IPF group and this may have contributed towards reaching statistical significance. If these markers are accepted as truly representing M1 and

M2 then this finding does not show a skew towards an M1 or M2 rich environment in the fibrotic lung but does suggest that in diseases with admixed fibrosis and inflammation there may be an overall increase in macrophage activation as disease advances. This could point towards a role of airway macrophage activation in influencing disease progression in fibro-inflammatory lung disease, but does not offer point towards this activation being polarised along M1/M2 lines.

The work presented here is in keeping with the developing understanding of the activation states of macrophages and the complexity therein, with a complex spectrum of activation states present in tissue macrophages. These data lead to a rejection of the hypothesis that the IPF lung is skewed towards an M2 phenotype do not point towards a clinical utility in phenotyping airway macrophages as being M1 or M2 as reflected by the lack of correlation with lung function parameters over time.

In order to answer the second hypothesis, subsequent work was directed towards a characterisation of bronchial epithelial function. The alveolar epithelium is the primary site of injury in IPF and the key cell in propagating dysfunctional wound healing responses, but a body of work has emerged showing that disorders of gene regulation and mucin production are independent risk factors for IPF and may in fact produce a separate IPF clinical phenotype, with a less aggressive disease course (Peljto et al, 2013) (Seibold et al, 2011) (Noth et al, 2013). The reasons for this are not clear but it has been suggested that overproduction of mucin may dampen down mucociliary escalator function and lead to plugging and reduced clearance of inhaled particles, or alternatively that excess mucin interferes with alveolar repair by disruption of surfactant surface tension. This is the first clear evidence of an upstream mechanism whereby airway (rather than alveolar) dysfunction is associated with the development of fibrosis in IPF and poses questions as to the regulation of other functions of bronchiolar epithelium. These data were obtained from immunostaining of sectioned lung tissue and *in vitro* work with primary HBECs from brushing of individuals with IPF and healthy controls. Phenotypic characterisation of lung tissue demonstrated a statistically significant increase in mucin producing cells and club cells in the bronchioles of the IPF

lung but no discernible differences in the basal cell or ciliated cell populations. The increase in mucin found in the epithelium corresponds to the aforementioned body of work, which first demonstrated MUC5B promoter polymorphism to be an independent risk factor for IPF development and to result in a dysregulation mucin production when compared to control. The finding of an increase in SCGB1A1 expressing club cells is, to our knowledge, a novel observation, although CC16, a protein secreted by club cells, has been shown to be elevated in individuals with IPF compared to other ILDs (Buendia-Roldan et al, 2016). They have been shown - in the murine model - to be able to act as epithelial progenitor populations which can give rise to lineages of alveolar epithelial cells in response to bleomycin injury (Fukumoto et al, 2016), but not in normal pulmonary homeostasis or in response to hyperoxic injury. Akram et al have proposed a model whereby club cells contribute to fibrosis pathogenesis by promoting alveolar epithelial cell apoptosis and subsequent alveolar bronchiolisation in areas of alveolar injury (Akram et al, 2013).

Further work looking at bronchial epithelial cells in IPF focused on functional behaviour *in vitro* and cell signalling capacity. HBECs cultured from bronchial brushings of individuals with IPF were predominantly basal cells, and, *in vitro* did not demonstrate any difference to healthy control when looking at either proliferative capacity or barrier function. Both IPF and healthy control primary basal HBECs formed confluent monolayers in culture with tight junctions expressing the vital tight junction protein ZO-1. They did not exhibit any differences in barrier permeability through a confluent submerged monolayer, or transepithelial electrical resistance, a surrogate measure of tight junction integrity. An examination of a number of 80 genes(encompassing signalling pathways known to be involved in fibrosis, including growth factors, ECM components remodelling enzymes and inflammatory cytokines and chemokines) revealed few differences between IPF and control HBECs. 4 genes were upregulated in IPF as compared to control, with no genes significantly downregulated. The upregulated genes were; integrin $\alpha 1$ (ITGA1), chemokine receptor type 4 (CXCR4), endoglin (ENG) and thrombospondin1 (THBS1), with only ITGA1 showing a greater than 2 fold up-regulation and with CXCR4 found to be expressed at a very low level. The integrins are a large family of

heterogeneous transmembrane heterodimeric proteins that constitute the main receptors for extracellular matrix components, which are expressed in numerous combinations and permutations in the lung. ITGA1 is known to form a cell surface receptor for collagen and laminin, and also to activate the Ras/MEK/ERK pathway, which has been implicated in TGF- β induced fibrosis. α V β 6 integrin knockout mice are protected from bleomycin induced pulmonary fibrosis and this effect has been shown to be independent of the inflammatory response of bleomycin in this model (Munger et al, 1999), suggesting this integrin may play a prominent role in pulmonary fibrosis. Ablating this pathway has been shown to ameliorate TGF- β induced fibrosis, and increasingly integrins - particularly α V β 6 - have been explored, for their potential to be a lung specific target to inhibit the activation and propagation of the TGF- β pathway. Given the number of pro-fibrotic pathways examined, the lack of difference between healthy control and IPF was striking and argued against a conventionally pro-fibrotic role for IPF HBEs in. This work did not demonstrate a wide dysregulation of the known pro-fibrotic pathways examined, leading to work investigating the IL-33/ST2 pathway, which has not hitherto been believed to be crucially involved in the fibrotic process, but which has been implicated by some recently published data pointing towards a possible pro-fibrotic role. I hypothesised that direct tissue specific effects of the IL33/ST2 axis contribute to the development of lung fibrosis through non-immune cell mediated mechanisms. IL-33 is located in the nucleus of bronchial epithelial cells and released in response to injury or damage. Data published from mice modelling has shown that IL-33 levels are elevated in response to bleomycin, and this leads to M2 macrophage polarisation (Li et al, 2014). Bleomycin fibrosis is ameliorated by inhibition of this pathway and studies have found IL-33 to be elevated over control in BAL of individuals with IPF (Luzina et al, 2013). that Analysis of gene expression of the IPF and control HBEs in this study demonstrated minor evidence of dysregulation of the IL-33 axis with a statistically significant increase in the expression of SIGIRR, the membrane bound negative regulator of this pathway, and with also a trend towards increased expression of the receptor ST2 (without reaching statistical significance). This finding could both represent an inhibition of this signalling

pathway, and therefore a blunting of the signalling response to IL-33, or a feedback mechanism where receptor expression is upregulated in response to higher circulating IL-33 levels. From the data presented here, it is not possible to ascertain the biological significance of this finding. In keeping with previously published work on the localisation of IL-33 and ST2 in lung, I demonstrated immunoreactivity of IL-33 predominantly in the basal cells of the bronchial epithelium, with IL-33 staining also detected in endothelium, tissue macrophages and fibroblasts. ST2 was predominantly detected on ciliary cells lining the airways and was also found on macrophages and lymphocytes distributed throughout the parenchymal tissue. Heavy staining of both was found in lymphoid aggregates and low level staining was detected in fibroblastic foci, with no differences found between IPF and control. IL-33 was shown to localise to nucleus and cytoplasm of HBECs in IPF and control which is in keeping with important work in this field by Gordon et al, who demonstrated the potential for multiple splice variants of IL-33 which govern its cellular localisation, extracellular secretion and biological activity. The finding of IL-33 immunostaining in fibroblastic foci is, to our knowledge, novel. Although this staining was detected at low level when compared to lymphoid aggregates, or basal epithelium, its localisation in these pathologically integral areas in the IPF lung may point towards involvement in this process. Having examined IL-33 and ST2 in lung tissue, further work looked at secreted levels in BAL fluid. Western blot of IL-33 (R&D) in ILD BAL revealed binding in 2 bands corresponding to a molecular weight of ~30kda and ~50kda. Hitherto, IL-33 has been detected at ~18kda in its cleaved form and ~30kda in its full length form. To interrogate this discrepancy mass spectrometry of these areas of antibody binding was performed revealing undetectable levels of IL-33 at both molecular weights. This finding reveals a problem with the specificity of the binding of this antibody using Western blot, demonstrating that the binding revealed in IPF BAL was non-specific and did not represent true IL-33. Furthermore, it may suggest that full length IL-33 (at ~30kda) is not present in high enough quantity to be detected by mass spectrometry in these samples. IL-33 and ST2 in the alveolar space was further characterised by ELISA of BAL fluid and demonstrated no difference in soluble ST2 (a soluble negative regulator that

binds and sequesters IL-33) or IL-33 levels in IPF vs control. IL-33 was expressed at the low end of the detection limit of the ELISA and did show a trend towards increase in IPF but this did not reach statistical significance. This does not correspond with a published dataset with more individuals in the IPF and in the healthy control cohort, which showed an increase in IL-33 in BAL in IPF over control. This may be a result of the larger sample size of this study revealing a significance not revealed by this smaller dataset, but also may be a result of the potential false positives of ELISA in IL-33 (Lee et al, 2017), which has previously been reported (Nygaard et al, 2016) (Ketelaar et al, 2016) (Riviere et al, 2016), with the luminex assay having now shown to be a higher sensitivity assay. The reported ELISA data in IL-33 also needs to be viewed in the context of the discovery of the multiple splice variants of IL-33 (which have been detected in asthma) by Gordon et al. The alternative splicing of IL-33 governs cellular localisation and secretion and it is not clear which variants the commercially available IL-33 ELISAs binds.

Thus far, this work suggested only minor differences in the expression of IL-33 and its receptor axis and did not show direct unequivocal evidence of whether alterations of IL-33 signalling may be evident in the IPF lung. To further characterise the relationship of this signalling pathway to the fibrotic process, the effect of IL-33, in concert with and comparison to the effect of TGF- β , upon the fibrosis effector cell - the lung fibroblast - was investigated. This showed at baseline, IPF HLFs exhibited an enhanced wound-healing rate when compared with healthy control with this effect abrogated by the addition of TGF- β . This abrogation was partly lost when IL-33 was added to TGF- β stimulation. Normal HLFs showed no response to the addition of TGF- β or IL-33. Data on the interaction of IL-33 and TGF- β has hitherto been scarce but the evidence available has shown it to be complex and tissue specific. Furthermore, given that the effects of TGF- β have been shown to be dose dependent, the study is limited by the use of only one (widely published) stimulation dose, and the interaction of IL-33 and TGF- β may potentially result in different effects at different doses. The partially antagonistic effect of IL-33 on the TGF- β mediated slowing of wound healing has not previously been reported and does not easily fit into the existing literature.

The direct non-immune mediated effects of IL-33 upon HLFs were explored in more detail and revealed no effect upon key fibrotic genes COL1a1, COL3a1 and α -SMA. This study did reveal that IL-33 gene expression in fibroblasts evinced an upregulation in response to IL-33 and TGF- β , which peaked at 12 hours, with the greatest effect seen on stimulation with TGF- β . The response of HLFs to stimulation was also scrutinised by measuring the levels of soluble collagen, soluble ST2, MMP9 and MCP-1 secreted into cell supernatant. This revealed the presence of collagen, MMP9 and MCP-1 with no difference between the IL-33 treated groups and control. Soluble ST2 was detected at lower levels in HLFs treated with IL-33 and this could be due to a negative feedback mechanism where IL-33 reduces the secretion of its soluble negative regulator, or may be due to the effect of soluble ST2 sequestering IL-33 in the sample and no longer binding to the antibody.

In summary, this work did not find a clear delineation between putative M1 and M2 in the lung of individuals of ILD or control, and did not show the IPF lung to be an M2 rich environment. The proportions of the lymphocytes, macrophages and M1/M2 did not correlate with lung function parameters over time.

Bronchial epithelium in IPF exhibits higher number of mucin producing cells -as previously reported- and also an increase in club cells. Basal cells do not differ from control in the functions of proliferation, barrier permeability or tight junction integrity and supernatant from IPF epithelial cells does not provoke a fibrotic response in HLFs.

The membrane bound negative regulator of IL-33 (SIGIRR) is upregulated in IPF HBECs but an immunohistochemical examination did not reveal differences in levels of tissue expression between IL-33 and its receptor ST2 in IPF and control, and no difference was detected in levels of measured secreted IL-33 from BAL fluid. Stimulation of IPF HLFs with TGF- β and IL-33 stimulation showed an upregulation in IL-33 with the greatest effect seen with TGF- β . IL-33 did not induce changes to fibroblast wound healing, collagen gene regulation or soluble collagen deposition.

As a whole, this body of work does not support the hypotheses posited, with the presence and contribution of M2 widely seen in mice models, not replicated in this study. Bronchial epithelium, implicated in IPF by the compelling light of evidence of MUC5B dysregulation, did not exhibit differences from control in the functional parameters examined here. Aside from an increase of SIGIRR gene expression in IPF HBECS, the IL-33 axis did not evince clear evidence of difference from control and - in contrast to work published in mice modelling - did not demonstrate any direct pro-fibrotic effects, as measured by the impact of its effect on the collagen regulation of human lung fibroblasts. Given the established animal model evidence underlying the investigation into the M1/M2 phenotype and the IL-33/ST2 axis in fibrosis, this work points to the potential pitfalls of translating animal modelling work to human disease.

6.2 Strengths and Weaknesses of this study

The strengths of the phenotyping of BAL cells lies in the fact that we were able to compare our findings to a healthy control and disease group control cohort. This allowed an examination across a range of diseases, and our dataset contained a wide range of severity at presentation. In addition, the availability of data on clinical parameters allowed the phenotyping markers to be correlated against clinically relevant longitudinal parameters. I believe the use of 2 markers for M1 and M2 ensured the robustness of this analysis. The limitations of our BAL cell investigation lie in the comparatively low numbers (and lower age) of individuals in the control group and the relatively low numbers of individuals with ILD for whom I was able to obtain longitudinal lung function data, and therefore draw our progression based clinical correlations.

Much of the subsequent work I present is subject to the limitations of *in vitro* modelling. The numbers were small in cell culture experiments, and although they did not exhibit any large effects,

these experiments would require repeat with larger numbers to ascertain statistical significance of many of the findings presented herein.

The population of cells cultured from bronchial epithelium was found to be a basal cell population and , thus, without taking the cultures to air liquid interface, I was not able to investigate the mucociliary or secretory function of club or ciliated cells. The conclusions drawn from these experiments can only be applied to the function of basal cells and therefore do not pertain to the bronchial epithelium as a whole. Furthermore, cell stimulation experiments were performed with fixed concentrations of recombinant protein and, given that TGF- β has been shown to have dose dependent, differing effects, this could have influenced the results observed.

6.3 Future work

The healthy control group in the macrophage phenotyping work presented herein is small compared to the IPF group and increasing the size (and age matching the healthy control volunteers as I was unfortunately able to do) would provide stronger evidence for the conclusions drawn here. Recent interesting new insights into our understanding of the ontogeny of tissue macrophages have raised the possibility that, in the lung, these cells self replenish from a locally resident pool and are not , as previously thought, recruited from circulating haematogenous precursors. In the context of IPF as a disease of ageing, this novel insight could point towards a pool of senescent airway macrophages self perpetuating in-situ and driving a pro-fibrotic local lung micro-environment in the absence of a systemic factors. Future work in this area could be aimed at investigating the ontogeny and maturity of IPF airway macrophages. Furthermore, a 3D co-culture model with AECs and fibroblasts could offer fresh insights into how airway macrophages fit into the self-propagating cycle of epithelial cell injury, fibroblast activation and collagen deposition which occurs in IPF. This may bring new insights

into how AMs can influence this process and how they are able to contribute to the regulation of ECM deposition.

The origin of the SCGB1A1 bronchial epithelial cells found to be upregulated in this study cells is not yet known. Mice modelling has shown them capable of acting as a pool of progenitor cells which can phenoconvert and migrate to the alveolus where they function as alveolar epithelial cells and following work should be directed at identifying the signals which induce the proliferation of SCGB1A1 positive cells and their differentiation into cells of alveolar lineage as well as their relationship to established fibrotic signalling pathways, such as TGF- β .

Given the emergence of recent data showing that IL-33 has many splice variants which determine its biological activity and localisation within the cell, a comprehensive characterisation of the variants present in the airways of individuals with IPF would help elucidate the biological significance of the molecule in IPF. Having not detected any direct non-immune cell mediated mechanisms by which IL-33 influences the fibrotic process, further work could focus on the effect this molecule has on indirect mechanisms via its key effector cells, innate lymphoid cells (ILCs). Furthermore, the *in vitro* observations presented in this study are based on small n-value experiments would require further investigation with larger sample sizes, according to sample size calculations.

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Supplementary data

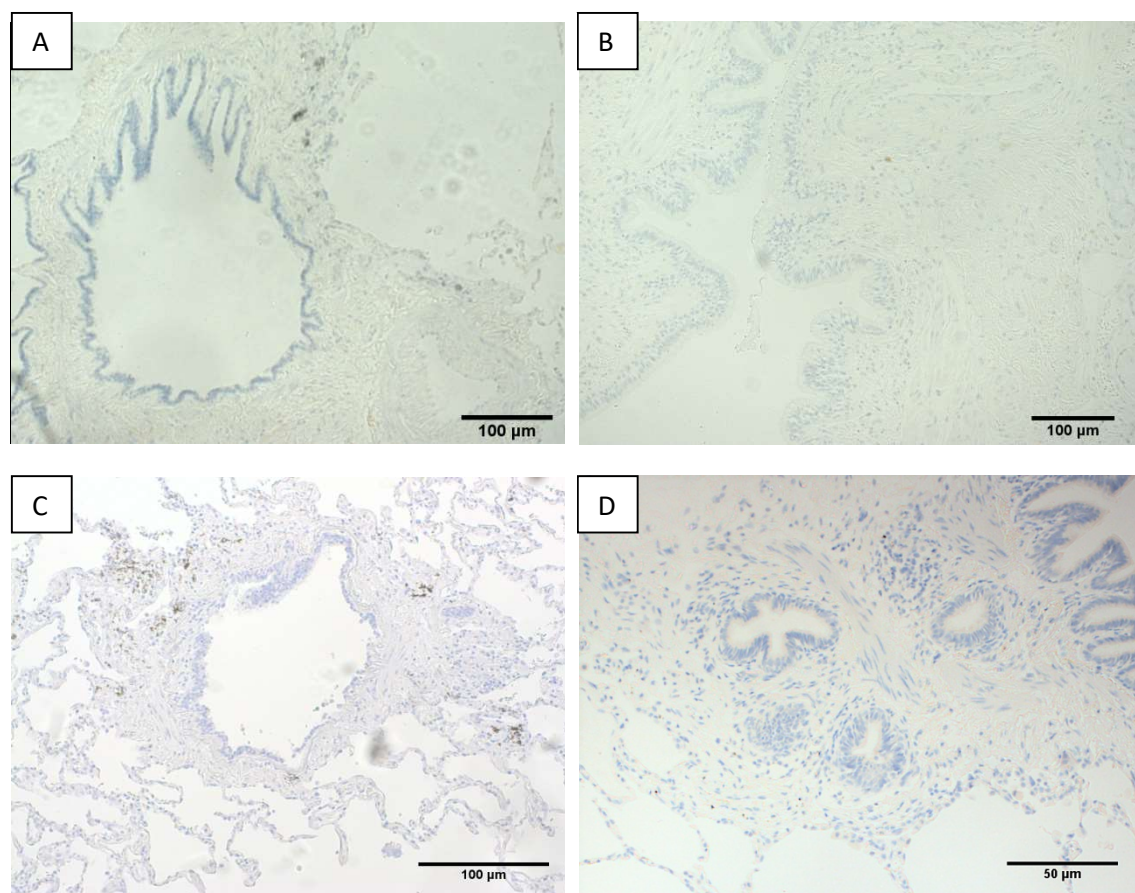
Supplementary Data

Supplementary table S.1 genes examined in fibrosis array

Pro-Fibrotic: ACTA2 (α -SMA), AGT, CCL11 (Eotaxin), CCL2 (MCP-1), CCL3 (MIP-1a), CTGF, GREM1, IL13, IL13RA2, IL4, IL5, SNAIL (Snail)
Anti-Fibrotic: BMP7, HGF, IFNG, IL10, IL13RA2
Extracellular Matrix & Cell Adhesion: <u>ECM Components:</u> COL1A2, COL3A1 <u>Remodeling Enzymes:</u> LOX, MMP1 (Collagenase 1), MMP13, MMP14, MMP2 (Gelatinase A), MMP3, MMP8, MMP9 (Gelatinase B) PLAT (tPA), PLAU (uPA), PLG, SERPINA1 (α 1-antitrypsin), SERPINE1 (PAI-1), SERPINH1, TIMP1, TIMP2, TIMP3, TIMP4 <u>Cellular Adhesion:</u> ITGA1, ITGA2, ITGA3, ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, ITGB8
Inflammatory Cytokines and Chemokines: CCL11 (Eotaxin), CCL2 (MCP-1), CCL3 (MIP-1a), CCR2, CXCR4, IFNG, IL10, IL13, IL13RA2, IL1A, IL1B, IL4, IL5, ILK, TNF
Growth Factors: AGT, CTGF, EDN1, EGF, HGF, PDGFA, PDGFB, VEGFA
Signal Transduction: <u>TGFβ Superfamily:</u> BMP7, CAV1, DCN, ENG (EVI-1), GREM1, INHBE, LTBP1, SMAD2, SMAD3, SMAD4, SMAD6
Transcription Factors: CEBPB, JUN, MYC, NFKB1, SP1, STAT1, STAT6
Epithelial-to-Mesenchymal Transition AKT1, BMP7, COL1A2, COL3A1, ILK, ITGAV, ITGB1, MMP2 (Gelatinase A), MMP3, MMP9, SERPINE1 (PAI-1), SMAD2, SNAIL (Snail) TGFB1, TGFB2, TGFB3, TIMP1
Others:

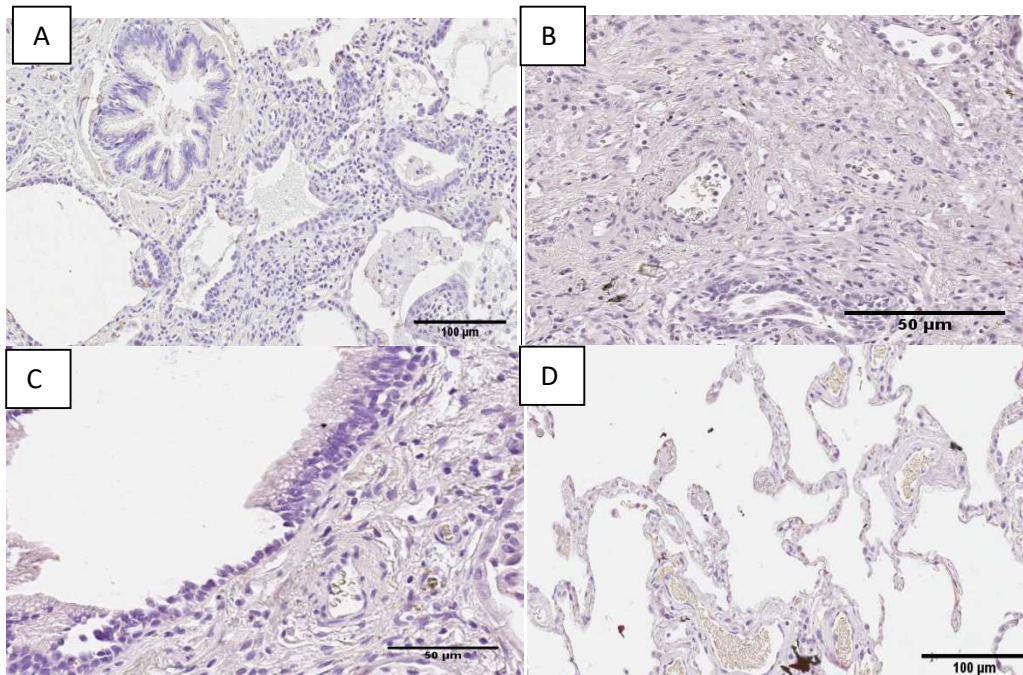
Supplementary table S.1 showing the full list of genes examined in Profiler PCR array of IPF and control HBECs

Supplementary Figure S.1 Isotype control staining of lung tissue for HBEC phenotyping



Supplementary figure S.1 showing isotype control staining of human lung tissue for A) ACT B) MUC5AC C) CK5 D) SCGB1A1

Supplementary figure S.2 Isotype control staining of lung tissue for IL-33/ST2 expression



Supplementary figure S.2 showing isotype control staining for A) ST2 in airway B) ST2 in tissue and C) IL-33 in airway D) and IL-33 in tissue in human lung tissue.