

NEUTROPHIL DYNAMICS IN IDIOPATHIC PULMONARY FIBROSIS

By

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ABSTRACT

Introduction: The pathophysiological drivers of epithelial damage and fibrosis in idiopathic pulmonary fibrosis (IPF) remain poorly understood. Novel therapeutic targets need to be identified to improve patient management. Neutrophils are the most common immune cells in the body and are critical responders to tissue injury; housing an array of enzymes critical for repair and remodelling. However, the role of neutrophils in IPF development and progression remains unknown.

Hypotheses: This thesis describes experiments which explored the following three hypotheses: 1) does the pro-fibrotic cytokine, transforming growth factor-beta 1 (TGF β 1), drive neutrophil chemotaxis; 2) do circulating neutrophils from patients with IPF display a primed phenotype and altered migratory and effector functions; 3) are neutrophils and their products resident in fibrotic lung tissue.

Results: Chapter Four demonstrates using *ex vivo* neutrophil migration and chemotaxis assays (validated in Chapter Three) that TGF β 1 increases neutrophil chemokinesis (direction independent movement), but not chemotaxis. This likely represents neutrophils transitioning into a primed state, with TGF β 1 also amplifying movement responses to classical chemoattractants.

Chapter Five outlines the development of the prospective cohort study entitled 'The Hypoxic Burden in Interstitial Lung Diseases (HYBRID)', which allowed the assessment of phenotype and function of circulating neutrophils derived from IPF patients, as well as the measurement of day and nighttime oximetry. The latter was of relevance as hypoxia has been implicated in neutrophil dysfunction in this disease. The 16 control and 26 IPF participants recruited to

this study were well matched. IPF participants were mostly of mild to moderate disease severity and experienced an increased frequency of episodic hypoxaemia.

Chapter Six describes the characterisation of circulating neutrophils derived from IPF and control participants of the HYBRID study. IPF patients had a greater circulating neutrophil count than control participants and a higher percentage of CD10 negative neutrophils, suggesting an expansion of immature neutrophil populations and emergency granulopoiesis. There was no evidence that IPF derived neutrophils were in a primed state. However, TGF β 1 appeared to drive IPF neutrophils to move less directly than control neutrophils. Furthermore, provisional analysis of bronchoalveolar lavage fluid samples was suggestive of increased NETs in IPF samples. Further expansion of this project is needed to improve the statistical power of this work.

Chapter Seven describes the development of a multiplex immunofluorescence panel to allow the localisation of neutrophils within IPF lung tissue. Descriptive analyses of the staining is described here; confirming the presence of neutrophils and their contents (neutrophil elastase and neutrophil extracellular traps) in the alveolar spaces and interstitium of IPF lung tissue.

Conclusions: This thesis highlights for the first time that there appears to be alterations in the phenotype of circulating neutrophils in IPF patients, and confirms the presence of neutrophils within fibrotic lung tissue using state-of-the-art multiplex technology. Future IPF research requires detailed characterisation of neutrophil activity in the pulmonary environment, for example using multiplex technology as described here, to aid discovery of novel therapeutic targets for this condition.

DEDICATION

This thesis is in memory of my grandpa, John Eric Rule.

Grandpa taught me the importance of an inquisitive nature, but also the need to balance
hard work with the fun things in life.

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List of Abbreviations

α SMA	Alpha-smooth muscle actin
AAT	Alpha-1 antitrypsin
AHI	Apnoea-hypopnoea index
AIP	Acute interstitial pneumonia
ALAT	Latin American Thoracic Society
ARDS	Acute respiratory distress syndrome
ATS	American Thoracic Society
AUC	Area under the curve
BALF	Bronchoalveolar lavage fluid
BD	Becton Dickinson
BLT-1	Leukotriene B ₄ receptor 1
BMI	Body mass index
BOOP	Bronchiolitis obliterans organising pneumonia
bpm	Beats per minute
BSA	Bovine serum albumin
cfDNA	Cell free DNA
cH3	Citrullinated histone H3
CO ₂	Carbon dioxide
COP	Cryptogenic organising pneumonia
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CXCR	C-X-C motif chemokine receptor
DAMP	Danger-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DIP	Desquamative interstitial pneumonia
DLCO	Diffusing capacity of the lungs for carbon monoxide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular matrix
ERS	European Respiratory Society
F	Female
FEF 25-75%	Forced mix-expiratory flow
FEV1	Forced expiratory volume in one second
FFPE	Formalin-fixed and paraffin-embedded
fM	Femtomolar
fMLP	N-Formylmethionine-leucyl-phenylalanine
FPR1/2	Formylpeptide receptor 1/2
FSC	Forward scatter
FSC-A	Forward scatter-area

FSH-H	Forward scatter-height
FVC	Forced vital capacity
GAP	Gender, Age and Physiology
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCP	Good Clinical Practice
GCP-2	Granulocyte chemotactic protein 2
G-CSF	Granulocyte– colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte-macrophage progenitor
GORD	Gastroesophageal reflux disease
H&E	Hematoxylin and eosin
HBSS	Hank's Balanced Salt Solution
HCL	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIER	Heat induced epitope retrieval
HIF1 α	Hypoxia inducible factor –1 alpha
HK	Heat killed
HP	Hypersensitivity pneumonitis
HRCT	High-resolution computed tomography
HRP	Horseradish peroxidase
HYBRID	The Hypoxic Burden in Interstitial Lung Diseases
IC50	Half maximum inhibitory concentration
Ig	Immunoglobulin
IL-1 β	Interleukin-1 beta
IL-8	Interleukin-8
ILD	Interstitial lung diseases
IPF	Idiopathic pulmonary fibrosis
IQR	Interquartile range
JNK	c-jun N-terminal kinases
JRS	Japanese Respiratory Society
KBILD	King's Brief Interstitial Lung Disease
KCO	Carbon monoxide transfer coefficient
kDa	Kilodalton
LAM	Lymphangioleiomyomatosis
LAP	Latency associated peptide
LCH	Langerhans cell histiocytosis
LIP	Lymphocytic interstitial pneumonia
LPS	Lipopolysaccharide
LUT	Lookup table
Ly6G	Lymphocyte antigen 6 complex locus G6D
$\mu\text{g}/\text{mL}$	micrograms per millilitre
μL	Microlitre
μM	Micromolar

M	Male
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MDSC	Myeloid-derived suppressor cell
MDT	Multi-disciplinary team
MFI	Median fluorescence intensity
Min	Minutes
mL	Millilitre
mM	Millimolar
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MRC	Medical Research Council
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
ng/ mL	Nanograms per millilitre
NHS	National Health Service
NIHR	National Institute for Health Research
NLR	Neutrophil-lymphocyte- ratio
nM	Nanomolar
NOX2	NADPH oxidase 2
NSIP	Non-specific interstitial pneumonia
O ₂ ⁻	Superoxide
ODI	Oxygen desaturation index
OH	Hydroxyl free radical
OSA	Obstructive sleep apnoea
PAD	Pan-peptidyl arginine deaminase
PAD4	Peptidyl arginine deaminase-4
pAHI	Peripheral arterial tone apnoea-hypopnea index
PAT®	Peripheral arterial tone
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1 receptor
PD-L1	Programmed death-ligand 1
PF-ILD	Progressive fibrotic interstitial lung disease
pg/ mL	Picograms per millilitre
PI	Propidium iodide
Pi3K	Phosphoinositide 3-kinase
pM	Picomolar
PMA	Phorbol 12-myristate 13-acetate
PPF	Progressive pulmonary fibrosis
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis

RA-ILD	Rheumatoid arthritis – interstitial lung disease
RB-ILD	Respiratory bronchiolitis- interstitial lung disease
REC	Research Ethics Committee
REM	Rapid eye movement
REMAP-ILD	Randomised Embedded Multifactorial Adaptive Platform in fibrotic interstitial lung diseases
RESBP	Respiratory effort snore and body position
RFU	Relative fluorescence unit
RGB	Red-green-blue
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
SD	Standard deviation
SGRQ	St George’s Respiratory Questionnaire
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
SpO2	Peripheral arterial blood oxygen saturation
SSC	Side Scatter
SSc	Systemic sclerosis
TAK1	Transforming growth factor beta-activated kinase 1
TBST	Tris-buffered saline supplemented with 3% Tween® 20 detergent
TGFβ	Transforming growth factor-beta
TGFβR1/2	Transforming growth factor-beta type 1 or 2 receptor
TIMPs	Tissue inhibitors of metalloproteinase
TLCO	Transfer capacity of the lung
TNFα	Tumour necrosis factor alpha
UIP	Usual interstitial pneumonia
UK	United Kingdom
uPAT	Unified peripheral arterial tone and pulse oximeter
USA	United States of America
VATS	Video assisted thoracoscopic surgery
WHO	World Health Organization

Chapter 1. INTRODUCTION

Parts of this chapter are published in the review article:

1. **“Neutrophil Dynamics in Pulmonary Fibrosis: Pathophysiological and Therapeutic Perspectives”** European Respiratory Review, 2024 [1].

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1.1. Idiopathic pulmonary fibrosis

Interstitial lung diseases (ILD) are a group of non-malignant lung parenchymal disorders encompassing a range of clinical phenotypes with varying degrees of fibrosis and/or inflammation (see Figure 1.1). Idiopathic pulmonary fibrosis (IPF) is the most common subtype, characterised by progressive collagen deposition and lung architectural destruction, of unknown origin.

1.2. IPF: a growing health burden

IPF is a growing public health concern, with prevalence increasing globally and conservatively estimated at 3- 9 cases per 100 000 per year in North America and Europe [2]. IPF most frequently affects Caucasian men of older age, the reason for this remains unclear. Although the aetiology of IPF is unknown, various environmental factors (smoking, occupational exposures, such as asbestos) and genetic variants (for instance, MUC5B and telomere length) have been associated with disease development [3–6]. The median survival of IPF patients from diagnosis is approximately 3 years, worse than many cancers [7]. Analysis of the World Health Organization (WHO) mortality database summarised that mortality increased in much of Europe between 2001 and 2013 [8]. However, this study was performed prior to the licensing of the anti-fibrotic, nintedanib, in Europe. Hopefully, widespread administration of the anti-fibrotic therapies, nintedanib and pirfenidone, which were found to slow lung function decline in phase III clinical trials (measured as forced vital capacity (FVC)) in IPF, are associated with improved overall patient survival [9,10]. Furthermore, Ryerson *et al* suggested that a perceived increase in mortality may reflect an increased number of patients being diagnosed with IPF rather than an actual increase in IPF prevalence or death [11].

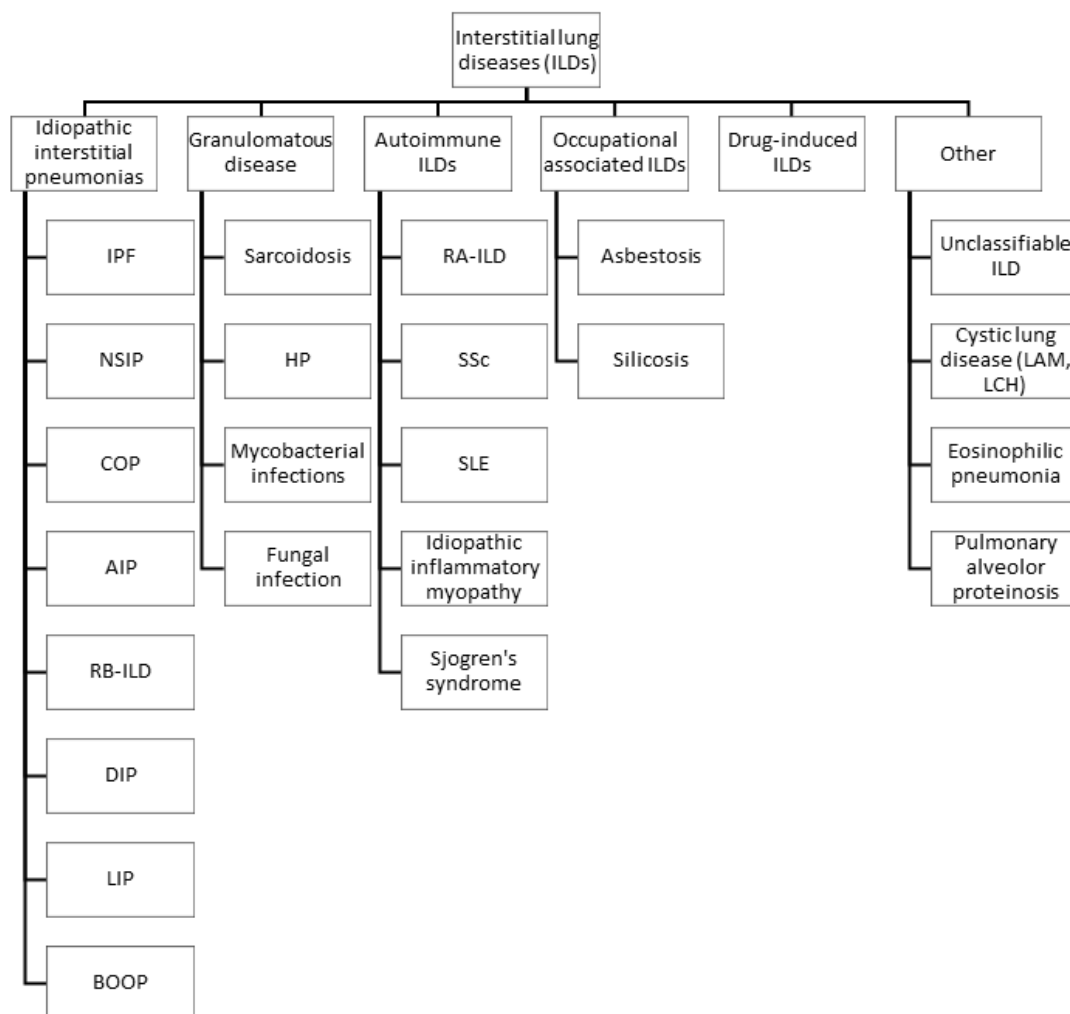


Figure 1.1: Classification of interstitial lung diseases.

Interstitial lung diseases (ILDs) encompass a group of conditions that affect the lung parenchyma. These can be divided into different types including idiopathic interstitial pneumonias, granulomatous disease, autoimmune ILDs, occupational associated ILDs, drug-induced ILDs and other unique entities. Idiopathic pulmonary fibrosis (IPF) is the most common type of idiopathic interstitial pneumonia. AIP - acute interstitial pneumonia, BOOP – bronchiolitis obliterans organising pneumonia, COP - cryptogenic organising pneumonia, DIP - desquamative interstitial pneumonia, HP – hypersensitivity pneumonitis, LAM – lymphangioleiomyomatosis, LCH – langerhans cell histiocytosis, LIP – lymphocytic interstitial pneumonia, NSIP – non-specific interstitial pneumonia, RA-ILD – rheumatoid arthritis – interstitial lung disease, RB-ILD – respiratory bronchiolitis- interstitial lung disease, SLE – systemic lupus erythematosus, SSc – systemic sclerosis.

1.3. Progressive pulmonary fibrosis: shared mechanisms

Over a third of other fibrosing ILDs, when progressive, have a similar prognosis to IPF [12]. The collective term ‘progressive fibrotic interstitial lung diseases (PF-ILDs)’, or newly defined ‘progressive pulmonary fibrosis (PPF)’ by the latest American Thoracic Society (ATS)/European Respiratory Society (ERS)/Japanese Respiratory Society (JRS)/Latin American Thoracic Society (ALAT) Clinical Practice Guideline [13], describes all fibrotic ILDs that demonstrate a progressive pattern based on fibrosis, symptoms, lung function and/or radiological features. The shared disease course and histo-radiological patterns seen in PF-ILDs support the notion that these conditions likely have at least some shared pathobiological mechanisms. Thereby, translational research in IPF, the most studied ILD, should help to identify novel therapeutic targets not only in IPF, but other types of PPF. This has already come to fruition with nintedanib, which is now licensed for the management of non-IPF PPF disorders.

1.4. Diagnosis and management of IPF – a multidisciplinary approach

1.4.1. Diagnosis

Patients with IPF usually present with respiratory symptoms, such as new or worsening shortness of breath, and/or cough. Bilateral reticular shadowing of the lung peripheries, which are lower zone predominant, are usually seen on chest radiographs. High-resolution computed tomography (HRCT) of the thorax is the gold standard diagnostic tool for IPF. Usual interstitial pneumonia (UIP) is the hallmark histo-radiological pattern of IPF. UIP is characterised on HRCT by honeycombing, with or without traction bronchiectasis/bronchiolectasis, and irregularly thickened interlobular septa, which are basal

and subpleural predominant [13]. The distribution is often heterogeneous and mild ground glass opacification (an area of increased attenuation, which can be a sign of inflammation) may be present [13]. Histology is no longer mandatory for diagnosis, reserved usually for indeterminate cases, as UIP pattern on HRCT is thought to provide a high confidence (>90% described in the latest ATS/ERS/JRS/ALAT Clinical Practice Guideline [13]) for UIP histology. Diagnosis is determined in a multi-disciplinary team meeting (including radiologists and respiratory physicians) by both the presence of UIP on HRCT and exclusion of alternative causes of ILD (such as negative immunological tests and absence of rheumatological conditions and occupational exposures).

1.4.2. Management

A holistic, multi-disciplinary team approach is critical to the management of patients with IPF. Lung transplant remains the only cure for this condition but is limited by organ availability and patient suitability. The anti-fibrotic therapies, nintedanib and pirfenidone, are the sole licensed treatments for IPF. Nintedanib is an anti-vascular endothelial growth factor receptor -2, fibroblast growth factor receptor-1 and platelet derived growth factor receptor – alpha, -beta multi-tyrosine kinase inhibitor, having multi-factorial effects *in vitro* including suppressing fibroblast proliferation and activation [14]. Conversely, pirfenidone's precise mechanism of action is currently unclear.

Patients with IPF have a significant symptom burden, which can negatively impact quality of life [15]. Drugs, such as low dose opiates and benzodiazepines, are commonly prescribed for the modulation of symptoms. However, phase III clinical trials demonstrating the efficacy of

these treatments are lacking. There is increasing understanding of the benefits of non-pharmacological therapies, such as pulmonary rehabilitation, which are becoming part of the treatment pathway [16]. Multi-disciplinary, supportive and palliative care services are thereby critical in the management of this fatal condition.

1.5. Oxygen therapy – a contentious issue

As the disease progresses, patients with IPF become increasingly short of breath and develop hypoxaemia, on exertion and at rest. The true burden of hypoxaemia over the disease course is unclear. It is thought to be evident not only in the day, but also at night with one prospective study noting that nearly 20% of IPF patients experienced $\geq 10\%$ of the night with oxygen saturations less than 90% [17]. Studies have observed expression of surrogate markers of hypoxia in IPF lung tissue, including cells such as fibroblasts and neutrophils, which could potentiate the disease for example through hypoxia inducible factor –1 alpha (HIF1 α) mediated mechanisms [18,19]. Ambulatory and long-term oxygen therapy are frequently prescribed to counteract the hypoxaemia. However, there has been no randomised controlled trial evidence to date confirming the efficacy of either of these treatments on mortality or quality of life.

1.6. Pathophysiology of IPF

1.6.1. Fibrosis driven by alveolar epithelial damage and myofibroblasts.

Although the aetiology of IPF is unknown, its pathogenesis is highly characterised. It is considered that repetitive cellular injury drives type II alveolar epithelial cell apoptosis,

prompting a cascade of dysregulated lung tissue repair, remodelling and neo-angiogenesis (see Figure 1.2) [20]. An array of repetitive insults is thought to drive the initial damage including, gastroesophageal reflux, cigarette smoke and infection [21]. Profibrotic growth factors such as transforming growth factor-beta ($TGF\beta$) and alterations to the extracellular environment promote a positive feedback loop of fibroblast proliferation, myofibroblast transdifferentiation and dysregulated collagen turnover [20]. Lung injury and subsequent fibrogenesis in this context were thought to be related to epithelial-mesenchymal interactions with minimum involvement from the innate immune system. However, the importance of macrophages in this process is becoming increasingly evident. This includes fibroblast activation through the release of pro-fibrotic cytokines, such as $TGF\beta$ [22].

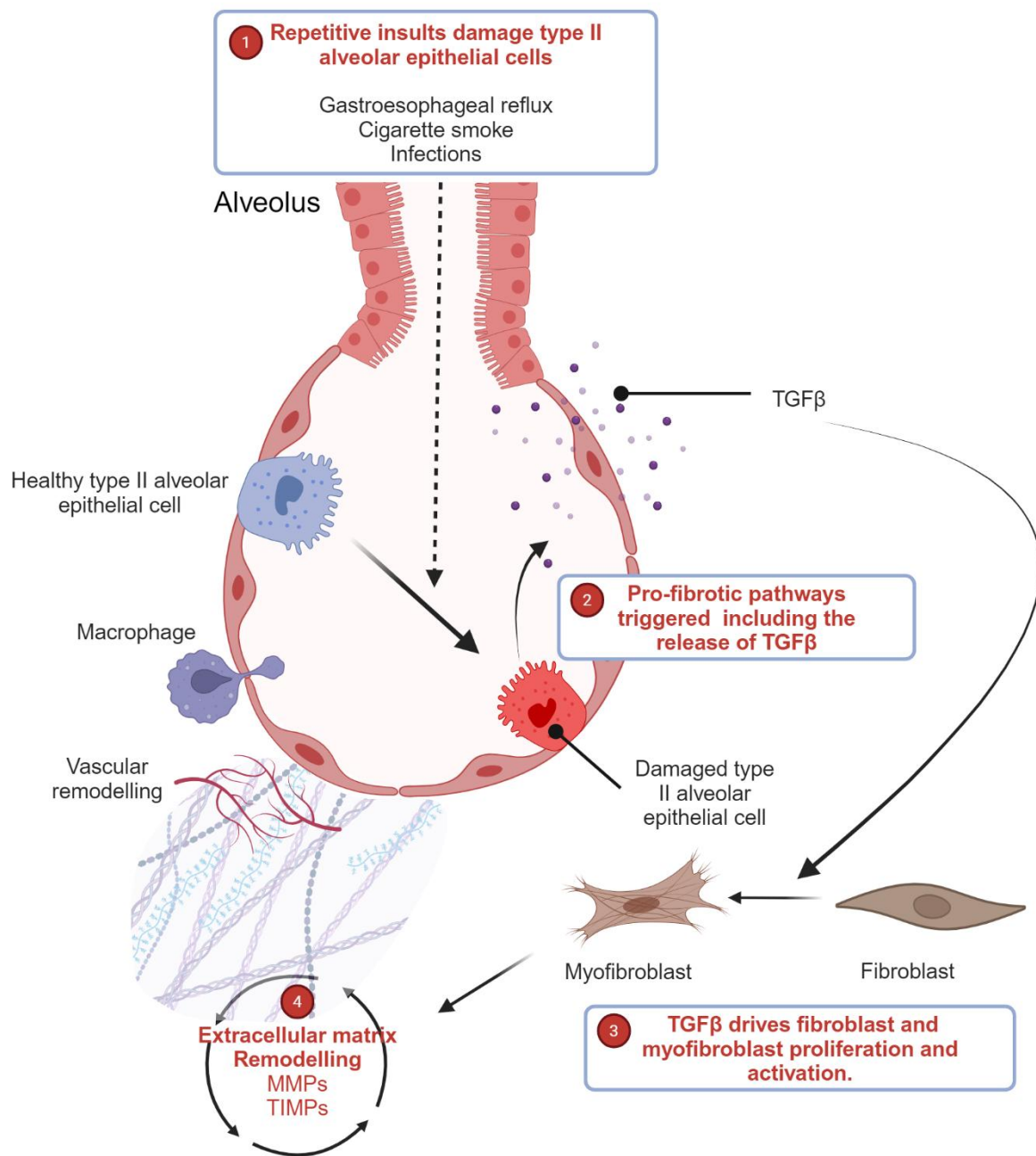


Figure 1.2: Outline of the pathophysiology of IPF.

(1) Repetitive insults damage type II alveolar epithelial cells that undergo dysregulated apoptosis and repair [20], which triggers **(2)** pro-fibrotic pathways including release of the pro-fibrotic growth factor transforming growth factor- β (TGF β) [23]. **(3)** TGF β drives mesenchymal cell, particularly fibroblast and myofibroblast proliferation and activation [24]. **(4)** This results in a self-perpetuating cycle of remodelling, regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) and increasing deposition of extracellular matrix material [25]. The role of macrophages is becoming increasingly implicated in this cycle [22]. *Figure created using Biorender.com, taken from [1].*

1.6.2. The proposed role of TGF β

TGF β is the most studied pro-fibrotic growth factor and has three isoforms (TGF β 1, -2 and -3), with TGF β 1 considered the most relevant in ILD [26]. TGF β 1 is a ubiquitous molecule that is secreted and sequestered in the extracellular matrix as a large latent complex (See Chapter 4 for more detail) [27]. TGF β bioavailability is largely regulated by the activation of TGF β 1 when it is released from the latent complex. An array of environmental changes, such as increased mechanical tension has been reported to activate latent TGF β 1, all of which are related to changes in the extracellular matrix [27]. Immunohistochemistry of lung sections from patients with IPF has confirmed increased TGF β 1 protein expression in both early and advanced lung fibrosis [28] and serum levels of TGF β 1 are raised approximately 3-fold in patients with ‘idiopathic interstitial pneumonia’ compared to healthy controls [29]. This also occurs in other ILD types with serum and sputum TGF β 1 levels significantly increased in patients with systemic sclerosis and rheumatoid arthritis (RA) associated lung fibrosis [30,31].

TGF β signalling (see Chapter 4 for further detail) plays a key role in the lung epithelial cellular response to injury, promoting FasL mediated apoptosis [32] and epithelial-to-mesenchymal transition [33]. Impaired regeneration of alveoli can result in elevated mechanical tension leading to further activation of the TGF β signalling pathways in type II alveolar epithelial cells [23]. TGF β can then drive collagen deposition and extracellular matrix remodelling through the activation of fibroblasts, into alpha-smooth muscle actin (α SMA) expressing myofibroblasts [24], with an imbalance between matrix metalloproteinases (MMPs) and their cognate inhibitors (tissue inhibitors of metalloproteinases (TIMPs)) [25] either increasing or reducing collagen degradation.

1.7. The role of neutrophils in fibrotic lung diseases?

Neutrophils are the most common immune cell in the blood and are key members of the innate immune system, including the body's first responders to infection and tissue damage. Following chemotaxis and extravasation into target tissues, neutrophils can activate an array of inflammatory and antimicrobial responses [34]. These include phagocytosis, containment and destruction of organisms by proteinases and reactive oxygen species (ROS), and release of neutrophil extracellular traps (NETs). All these processes have the ability not only to clear infection but are critical in driving tissue injury and repair [35,36].

Neutrophils have largely been neglected in the pathological paradigm of lung fibrosis. The clinical importance of increased neutrophils in the bronchoalveolar lavage fluid (BALF) of patients with ILD is largely unknown. Although, neutrophils and their contents have been localised within fibrotic lung tissue [37], the temporal relationship of neutrophil mobilisation and disease pathophysiology is unknown. The depletion of neutrophils and their enzymatic contents impacts bleomycin induced pulmonary collagen deposition in some animal models [38–40]. However, results vary and translate poorly to the disease process in humans. The impact of neutrophils in the injured and fibrotic lung in patients with ILD remains unknown. This raises the question of whether neutrophils amplify the fibrotic process, or are part of a physiological attempt to repair the damaged pulmonary architecture?

To explore this conundrum, firstly classical neutrophil functions will be discussed. Then the current literature surrounding the role of the neutrophil as a biomarker and contributor to the pathogenesis of ILD will be summarised, including gaps that warrant further investigation and novel, neutrophil-centric therapeutic options.

1.8. Classical neutrophil response

1.8.1. Neutrophil granulopoiesis and maturation

The lifespan of neutrophils is brief, initially thought to be less than one day [41], although recent data has revealed that they can survive for up to 5 days, with a variety of stimuli/environmental factors prolonging [42–44] their survival. Neutrophils are continuously generated in the bone marrow, with an estimated $1-2 \times 10^{11}$ cells produced daily during steady-state conditions [45]. Neutrophils develop from haematopoietic stem cells and mature through three pools: the stem cell pool, mitotic pool and post-mitotic pool (see Figure 1.3) [46]. During neutrophil development, morphology, antigen expression and granular content changes. For example, on maturation, the nucleus becomes segmented, and CD10 expression becomes evident on the cell surface [47,48]. Neutrophil production and egress into the circulation are tightly regulated. Granulocyte– colony stimulating factor (G-CSF) is the main driver of neutrophil production, maturation and release from the bone marrow [49,50]. Other regulators include the chemokine receptors on the neutrophil surface, C-X-C motif chemokine receptor- 2 (CXCR2) and CXCR4, which promote and prevent egress into the circulation, respectively [51]. In states of severe infection and/or inflammation, to contain the insult, neutrophil production can be rapidly amplified in a process termed emergency granulopoiesis. This often results in blood neutrophilia and increased levels of both circulating mature (CD10 positive) and immature (CD10 negative) neutrophils, as seen in patients with sepsis [52].

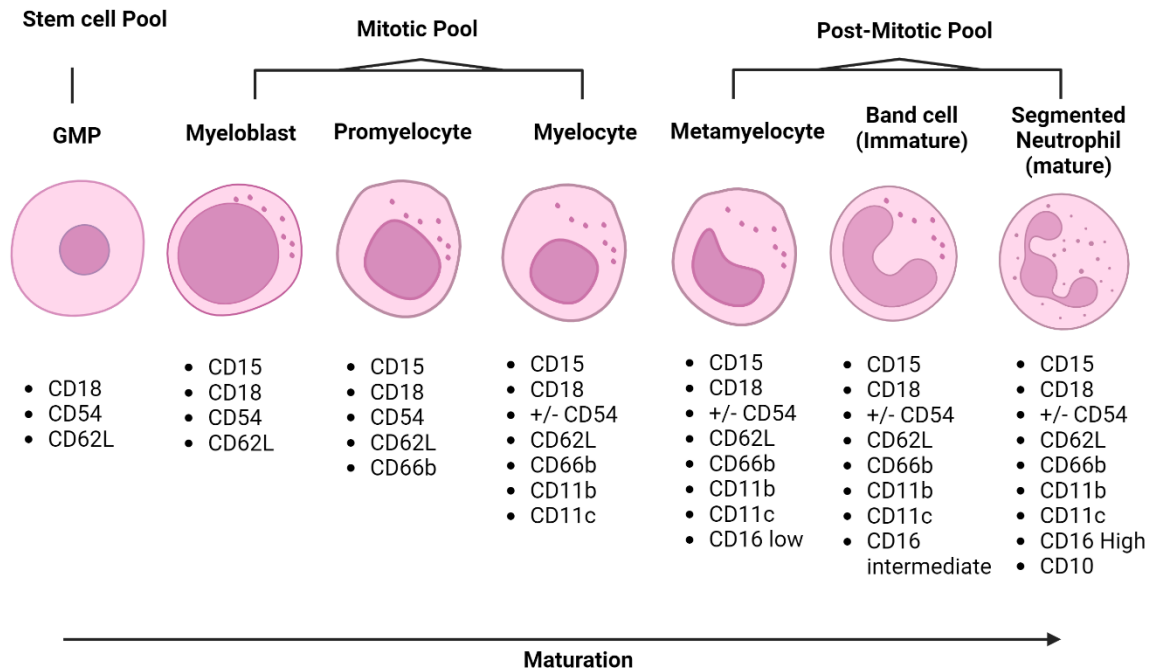


Figure 1.3: Stages of neutrophil development.

Morphology and antigens expressed on the cell surface at each stage of neutrophil development are shown [53,54]. GMP - Granulocyte-macrophage progenitor. *Figure created using Biorender.com.*

1.8.2. Neutrophil priming

On release into the circulation, neutrophils are initially in a quiescent state, displaying minimal propensity to adhere to endothelial cells or respond to stimuli. Neutrophil priming is the next critical step in the neutrophil response to infection. Priming is defined as where a stimulus does not on its own 'activate' the neutrophil but induces changes (intracellular signalling pathways/receptor upregulation), which renders the neutrophil more susceptible to a subsequent stimulus [55]. This 'amber' or high-alert state allows for maximal response to activating agents [56]. A primed phenotype can be demonstrated *in vitro* by augmented effector functions in response to stimuli (such as amplified ROS production or degranulation) and/or changes in cell surface receptors that occur on priming, such as increased CD11b expression or CD62L (also called L-selectin) shedding [56,57].

Many priming agents exist including pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF α) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and bacterial products, such as lipopolysaccharide (LPS) [58], which are released into the circulation from injured or infected tissues. Signalling pathways that occur on priming vary on the cytokine/agent [59]. For example, TNF α and GM-CSF enhance N-Formylmethionine-leucyl-phenylalanine (fMLP) induced ROS production, by phosphorylating p47, one of the key cytoplasmic components of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [60,61]. However, upstream of p47, TNF α acts principally via p38 mitogen activated protein kinase (MAPK), whereas GM-CSF phosphorylates p44/42 MAPK signalling pathways [61].

1.8.3. Neutrophil migration

1.8.3.1. Transendothelial migration

Migration from the circulation into tissue occurs through a series of sequential interactions between neutrophils and endothelial cells (see Figure 1.6 describing migration in the context of ILD) [62]. Selectins on the neutrophil surface (such as CD62L) and endothelial cells (P-selectin and E-selectin) mediate the initial capture and rolling of neutrophils on the vessel wall [63–65]. This selectin engagement prompts signalling pathways within neutrophils that are critical to migration, for example E-selectin drives beta-2 integrin activation in neutrophils via phosphorylation of p38 MAPK [66].

Local factors impact neutrophil migration for instance L-selectin requires a minimum level of shear stress to support adhesion [67] and selectin expression can be amplified on endothelial cells through activation by pro-inflammatory stimuli, such as TNF α and LPS [68].

The interaction of integrins present on the neutrophil surface, such as CD11b- CD18 (also known as mac-1 and $\alpha_m\beta_2$ integrin) and molecules on the endothelial surface, such as CD54 (also called intracellular adhesion molecule- 1), plays an important role in neutrophil rolling, arrest and firm adhesion to the endothelium [69,70]. Integrin activation is crucial to this, with chemoattractants, such as the chemokine interleukin-8 (IL-8) being the strongest physiological activators [62,71]. Transendothelial migration is the last step in neutrophil migration into target tissue. This can occur either transcellularly (the most common method) or paracellularly and is also dependent on integrin ligation and chemoattractants [72].

1.8.3.2. Transepithelial migration into the alveolar space

Following arrival into the pulmonary interstitium, neutrophils can migrate into the alveolar space via transepithelial migration. Transepithelial migration follows three main steps: 1) epithelial adhesion to the basal surface, 2) paracellular migration and 3) postmigration adhesion/detachment to the apical surface where neutrophils can then provide an antimicrobial barrier [73]. Integrins, such as CD11b/CD18, are also important here [74]. As neutrophils undertaking this process have just traversed the endothelium to reach the pulmonary interstitium, neutrophils are most likely in a primed state prior to epithelial adhesion [75].

1.8.4. Neutrophil chemotaxis

Chemoattractants are a group of molecules (described in Table 1.1) that drive neutrophil migration in a concentration dependent manner, referred to as chemotaxis.

Group	Example	Receptor	End or intermediate- target chemoattractant
Anaphylatoxins	Complement 5a	Complement 5a receptor	End-target
Chemokines	Interleukin-8	CXCR1, CXCR2	Intermediate- target
Chemotactic lipids	Leukotriene B ₄	Leukotriene B ₄ receptor 1 (BLT-1)	Intermediate- target
Formyl peptides	fMLP	FPR1	End-target

Table 1.1: Neutrophil chemoattractants.

There are four broad groups of neutrophil chemoattractants [76]. Examples of each and respective cognate receptors are described. BLT-1 - CXCR - C-X-C motif chemokine receptor, fMLP - N-Formylmethionine-leucyl-phenylalanine, FPR1 - formylpeptide receptor 1.

IL-8 was one of the first chemokines discovered [77,78] and acts via two seven-transmembrane G-protein-coupled receptors located on the neutrophil surface called CXCR1 and CXCR2 [79,80]. fMLP is a formylated peptide derived from bacteria that acts on formylpeptide receptor 1 (FPR1), the first G-protein-coupled receptor discovered on human neutrophils [81,82]. It is now thought that a hierarchy of chemoattractants orchestrates neutrophil migration [83,84]. Initially, host generated intermediate- target chemoattractants (such as IL-8 and leukotriene B₄ [85]) drive neutrophil migration towards the general site of injury/infection. End- target chemoattractants (such as fMLP and complement 5a) then take precedence ensuring final localisation more specifically to the target site [86].

Chemotaxis is dependent on neutrophils being able to temporarily project the leading edge of cytoplasm and membrane called pseudopodia in the direction of the highest concentration of chemoattractant [87]. Targeted actin polymerisation at these sites is also critical for the directional migration of neutrophils [88]. Chemoattractants drive actin polymerisation via various signalling pathways (such as p38 MAPK, p42/44 (ERK) MAPK) following receptor binding [89,90]. Chemoattractants not only drive migration but have a role in neutrophil priming and subsequent activation/effector functions.

1.8.5. Neutrophil effector functions

Neutrophils have an array of intracellular and extracellular anti-microbial mechanisms, namely phagocytosis, degranulation, neutrophil extracellular trap (NET) and ROS formation.

1.8.5.1. Phagocytosis

Phagocytosis is the mechanism by which neutrophils engulf pathogens into a phagosome and subsequently leads to intracellular bacterial killing through the release of proteinases and

ROS. The process commences following engagement of endogenous opsonic ligands, for example, complement proteins, immunoglobulins and/or pathogen-associated molecular patterns [91]. Subsequent phagosome formation is dependent on rearrangement of the actin cytoskeleton [91].

1.8.5.2. Degranulation

Neutrophils contain intracellular granules and secretory vesicles, which upon stimulation can be released within the phagosome or extracellularly to facilitate antimicrobial activity and assist in other processes, such as endothelial adhesion, migration and phagocytosis [92]. These intracellular organelles contain an array of bactericidal and proteolytic proteins, as well as membrane proteins, such as integrins, which can be rapidly mobilised to the cell surface on activation (see Table 1.2) [93]. Secretory vesicles are the most readily released from neutrophils followed by tertiary granules, secondary granules, and finally primary granules [92,94].

Primary (azurophilic) granules	Secondary (specific) granules	Tertiary (gelatinase) granules	Secretory Vesicles
Defensins Neutrophil serine proteases: - Cathepsin G - elastase - Proteinase-3 Lysozyme Myeloperoxidase	CD11b, CD66 Collagenase Cytochrome b558 (NADPH-oxidase component) Lactoferrin Lysozyme	CD11b Cytochrome b558 (NADPH-oxidase component) Gelatinase Lysozyme MMP-9	Alkaline phosphatase CD11b Cytochrome b558 (NADPH-oxidase component) fMLP receptor Lysozyme

Table 1.2: Contents of neutrophil granules and secretory vesicles.

The contents of each type (primary (azurophilic), secondary (specific) and tertiary (gelatinase)) of neutrophil granules and secretory vesicles are described [95–97] although this is not an exhaustive list (see [97]). fMLP - N-Formylmethionine-leucyl-phenylalanine, NADPH - nicotinamide adenine dinucleotide phosphate, MMP-9 – matrix metalloproteinase -9.

1.8.5.3. Neutrophil extracellular traps (NETs)

In 2004, Brinkmann *et al* discovered using high-resolution scanning electron microscopy and immunofluorescence that neutrophils have a further extracellular antimicrobial ability [98]. Stimulation by a variety of activators including, phorbol 12-myristate 13-acetate (PMA – a synthetic protein kinase C activator), IL-8, LPS, and bacteria, led to the release of fragile extracellular net- like fibers composed of chromatin and granules called NETs [98]. This is a rapid process that can occur within 10 minutes of neutrophil stimulation and is distinct from phagocytosis; continuing in the presence of the actin polymerisation inhibitor, cytochalasin D [98,99]. NET production can be divided into; 1) ‘classical or suicidal NETosis’ whereby NETs containing nuclear deoxyribonucleic acid (DNA) are produced during a process of active cell death that is distinct from apoptosis and necrosis [100], 2) ‘vital NETosis’ where viable neutrophils extrude mitochondrial DNA and/or nuclear DNA [99,101].

The granular contents of NETs are complex, containing primary granule proteins such as myeloperoxidase, neutrophil elastase (NE) as well as secondary and tertiary granule contents, with the list ever growing [98]. Proteomic analysis of proteins within NETs has demonstrated that content changes between disease states and stimuli [102]. Quantity and timing of NET formation is also variable and dependent on numerous factors including internal machinery and the local microenvironment [100].

NET integrity and function are dependent on the chromatin structure (interwoven DNA and histones) [103]. NE and myeloperoxidase work synergistically in chromatin decondensation [104]. The enzyme peptidyl arginine deaminase-4 (PAD4), which catalyses the generation of citrullinated histone 3, is also considered important for NET formation [105].

1.8.5.4. Reactive oxygen species

NADPH oxidase, a surface multi protein, consisting of the catalytic subunit NADPH oxidase 2 (NOX2) (gp91phox) along with the small guanosine triphosphatase, Rac, and 4 regulatory subunits (p67phox, p22phox, p47phox, p40phox), is critical for neutrophils' simultaneous rapid consumption of oxygen and production of ROS, commonly known as respiratory burst [106]. The free radical of oxygen, superoxide (O_2^-) is generated by NADPH oxidase and is important in the cells' phagocytic bacterial activity [107]. Superoxide dismutase (SOD) catalyses the dismutation of superoxide to hydrogen peroxide, which can then be converted to oxygen and water via catalase as part of deactivation [108] and noted to reduce bacterial killing [109]. Alternatively, myeloperoxidase can transform hydrogen peroxide into more toxic secondary oxidants, such as hypochlorous acid [108,110].

ROS generation is critical to other neutrophil functions, including NET formation. Fuchs *et al* demonstrated that NADPH oxidase and ROS are critical for PMA and staphylococcus aureus induced NETosis in neutrophils isolated from patients with chronic granulomatous disease [100,111]. However, other stimuli, such as calcium ionophore are considered "NOX2 independent" [112]. It is likely not as simple as a single stimulus being dependent or independent of a particular component, as Pilszek *et al* observed that staphylococcus aureus was also able to rapidly induce NET formation in an oxidant-independent manner [99].

1.8.5.5. The importance of oxygen

The importance of the NADPH oxidase system and ROS formation on neutrophil anti-microbial functions is clear. However, despite this the effect of restricting the key component, oxygen, on neutrophil function remains unclear. One study observed that LPS induced NET release *ev vivo* was significantly reduced in a 1% oxygen atmosphere [113]. Surprisingly, despite its

dependence on oxygen for functionality, NADPH oxidase, was noted to be important in both normoxic and hypoxic conditions [113]. Others have noted that 0.8% oxygen has no effect on NET formation, as measured by extracellular DNA release, but that it did increase neutrophil degranulation [114]. In contrast Khawaja *et al* observed increased NET release when neutrophils were exposed to 1% oxygen [19].

Manipulation of HIF1 α , a transcription factor that is upregulated in low oxygen environments, is another means of mimicking the effects of hypoxia *in vitro*. McInturff *et al* noted that knockdown of HIF1 α attenuated NET formation suggesting that this downstream molecule is also important [115]. Further work is needed to understand the effects of both severe hypoxia as well as clinically relevant hypoxia of disease states on neutrophil function.

1.8.6. Neutrophil clearance/homeostasis.

Once the pathogen is contained, neutrophil clearance is required to retain homeostasis. This can occur by apoptosis and subsequent efferocytosis by macrophages and/or reverse transmigration back into the circulation, identified by upregulated expression of the cell surface receptor CD54 [116]. Factors within the microenvironment have been shown to increase neutrophil longevity, which will be discussed in the lung fibrosis context later, alongside neutrophil priming and/or activation [43,56,117]. Tight regulation of these factors is critical as persistent activation can be detrimental as seen in acute respiratory distress syndrome (ARDS) [118].

1.9. Neutrophils and fibrotic lung tissue

Pathological studies have identified both peripheral and local neutrophilic abnormalities in ILDs [37,119,120] raising the possibility that this reflects either a reaction to the fibrotic process or that neutrophils have a direct role in disease progression.

1.9.1. Neutrophil counts in BALF

Elevated neutrophil content in the BALF of ILD patients was first reported in the 1990s [119–121] followed by numerous subsequent studies. The clinical impact of these observations was subsequently described in the 2012 official ATS clinical practice guideline where IPF is amongst the differential diagnoses of a BALF ‘neutrophil cellular pattern’ [122]. Most studies have reported BALF neutrophil percentage rather than count, making it difficult to interpret the real change in neutrophil recruitment. One study noted that both absolute neutrophil count and lymphocyte count were significantly greater in IPF BALF than healthy controls [123] and another that both neutrophil percentage and total cell count were elevated in IPF patients [124]. Nevertheless, some studies have failed to confirm this with IPF BALF neutrophil count similar to controls [125].

It is, however, evident that a significant number of IPF patients have an elevated neutrophil percentage in the BALF. In IPF, 67% and 56% of patients were noted to have a BALF neutrophil percentage >3% [126] and >5% [127], respectively. In systemic sclerosis-associated ILD, 47% of patients were noted to have a BALF neutrophil proportion of >4% [128]. These data suggest that neutrophil numbers are raised in the BALF of ILD patients, but it is important to consider that a reduction in other cell types could reflect this proportional increase. For example, some have suggested that BALF lymphocytosis is protective against fibrosis [127,129]. An increase

in lymphocytes could result in a proportional decrease in neutrophils but not necessarily the count.

It is important therefore, to determine the reasons and clinical significance of elevated neutrophil percentage/count on disease progression. Several studies have reported an association between neutrophil content in BALF and the extent of segmental/lobar fibrosis and mortality [130–133]. This includes the observation that neutrophil count as well as percentage were higher in BALF collected from lobes that were seen to have more extensive disease on imaging in IPF patients [132]. In addition, it has been reported that with each doubling of neutrophil percentage in the BALF of IPF patients, there was a 30% increased risk of mortality [134]. Mortality was not associated with either BALF eosinophil or lymphocyte %, suggesting the importance of the neutrophilia [134], although not confirmed by others [128].

The prognostic importance of neutrophils is likely not limited to IPF, with Watase *et al* finding that increased neutrophil percentage in BALF in PF-ILD was also associated with subsequent mortality [133].

However, neutrophil activity may be more critical than number. Increased NE/alpha-1 antitrypsin (AAT) complexes have been observed in IPF patients, implying increased local neutrophil degranulation [125]. Citrullinated histone 3, a marker of NET formation (a feature of neutrophil activation and also capable of tissue damage), is elevated in the BALF of patients with IPF [135]. However, incomplete demographic matching of IPF and control groups likely influenced interpretation of these findings.

1.9.2. Neutrophil counts in blood

Several studies have reported that circulating neutrophil counts are increased in patients with IPF and RA-ILD [136–139] although average counts are within the “normal reference range”. Careful consideration of the clinical significance of this potentially small but statistically significant increase in ILD patients compared to controls, is needed to avoid over interpretation. The lack of information concerning the spread of data makes the current literature difficult to interpret. For instance, an elevated blood neutrophil count defined by some as $>7.5 \times 10^9/L$ reflects lung function decline/progression [140,141] although the proportion of patients above this threshold is not described and hence patient specific impact is not clear.

As in BALF, it may be that the relative contribution of neutrophils, rather than absolute count, is critical in ILD patients. It is important to determine this as the former could merely reflect a reduction of other immune cells as mentioned above. There have been numerous studies of the association of neutrophil-lymphocyte- ratio (NLR) [133]. A metanalysis summarised that NLR was greater in ILD cohorts, compared to non-ILD participants, and associated with worse prognoses [142]. Many studies that have identified NLR as a potential biomarker, either did not comment on neutrophil count alone or reported no association with outcomes [143–145], suggesting that a reduction in lymphocyte count may have reflected changes seen.

However, it is inappropriate to dismiss circulating neutrophil levels as biomarkers in this disease process. For example, Achaiah *et al* noted that a raised peripheral neutrophil count (defined as $> 7.5 \times 10^9/L$) in IPF patients was associated with mortality, whereas NLR was not [141]. Furthermore, circulating neutrophil count has been noted in other studies to be associated with IPF development, severity and mortality [138–141], and survival in RA-ILD

[146]. A large prospective study may help clarify the role of peripheral neutrophil count alone as a prognostic biomarker in ILD and together with paired BALF samples determine whether blood data reflects both BALF and tissue neutrophil counts and activity. In summary, interpreting blood data alone is complex as this reflects neutrophil granulopoiesis, partitioning, extravasation into target organs, tissue clearance, and does not necessarily reflect the degree of local neutrophil recruitment and activation in situ.

Overall, the evidence suggests that neutrophils are an important part of the immune landscape of ILD and appear to be associated with fibrosis severity. Whether this is reflected solely by an increase in absolute number and/or a reduction in other immune cells is not clear and needs further in depth study. Importantly the role of neutrophils needs greater clarification in the pathophysiology of ILD itself but also any relationship to acute exacerbations of ILD, which are frequent and associated with significant morbidity and mortality [147]. For instance, in Coronavirus Disease 2019 (COVID-19) amplified systemic neutrophil counts, enzymes and NETs are associated with post-COVID-19 interstitial lung changes [148]. Whether the neutrophil response in acute exacerbations of ILD has such an effect is unknown.

1.9.3. Neutrophil localisation within the fibrotic lung

The localisation of neutrophils in the lung parenchyma in ILD remains unclear and there is no quantitative data of neutrophil infiltration in fibrotic lung. One of the characteristics of the histopathological pattern of UIP that defines IPF and is present in many non-IPF PF-ILDs, is scarcity of inflammatory features, including the presence of neutrophils [149,150]. However,

others dispute this [37] and have identified NE and markers of NETs in fibrotic lung tissue [19,125]. The spatial characterisation of neutrophils and its contents within IPF lung tissue is discussed in further detail in Chapter 7.

1.10. Are neutrophil-mediated repair mechanisms disrupted or dysfunctional in lung fibrosis?

There is growing evidence that neutrophils play a ubiquitous role in tissue repair (see Figure 1.4). A murine two-hit (acid aspiration and mechanical ventilation) pulmonary injury model demonstrated that neutrophils rapidly migrate to damaged lung, with tissue remodelling and collagen deposition occurring soon after [151]. The role of neutrophils in lung repair is further supported by murine ventilator induced lung injury models where lungs from neutropenic mice demonstrate delayed repair [152]. This is also seen in thermal hepatic injury where neutrophils are crucial for the reduction of injury size, collagen deposition and revascularisation [36]. Dermal wound closure models investigating the effect of deficiencies in the chemotactic receptors FPR1 and FPR2 have also demonstrated that early neutrophil recruitment is critical for normal wound healing [153].

Neutrophil dysfunction has been noted in ageing [154]. The importance of neutrophil mediated repair mechanisms may therefore become more apparent with increasing age, as evidenced by neutrophil depletion markedly delaying wound healing in aged mice [155]. This is relevant as many ILDs, such as IPF occur more commonly in older adults.

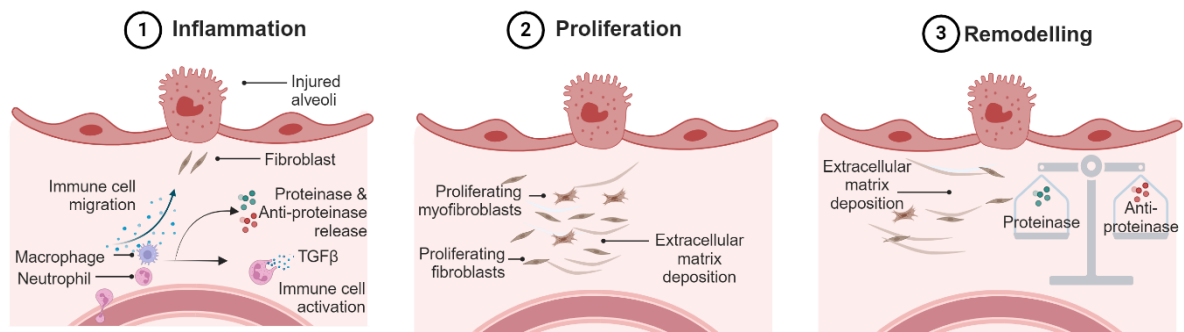


Figure 1.4: Stages of wound repair.

Following tissue injury, immune cells, including homeostasis, neutrophils are recruited **(1)** to remove pathogens/unwanted debris, and release cytokines, such as interleukin-8 and transforming growth factor- β (TGF β), and proteinase/anti-proteinases. **(2)** Fibroblasts/myofibroblasts migrate, proliferate and deposit extracellular matrix material. **(3)** Extracellular matrix material, particularly collagen, is remodelled resulting in increased tensile strength and organised fibrotic tissue. This process is dependent on a delicate balance of proteinases and anti-proteinases[156]. *Figure created using Biorender.com, taken from [1].*

Mechanisms of neutrophil driven repair mechanisms are currently unclear. Neutrophil derived proteinases, such as MMP-9 [152] appear important in lung injury repair in animal models. Alveolar epithelial damage is considered an early step in IPF pathogenesis. Activation of beta-catenin signalling through NE mediated cleavage of E-cadherin is a proposed mechanism by which neutrophils regulate alveolar epithelial repair [157]. Neutrophil depletion on the other hand impairs type II alveolar epithelial cell proliferation [158]. It is unknown whether neutrophil mediated repair mechanisms such as these are functional or dysregulated in patients with ILD.

Pro-resolution effects can also be detrimental. A further murine model demonstrated that neutrophilic inflammation at the saccular stage of lung development can result in aberrant elastic fibre generation likely through inhibitory effects of NE on elastic fibre assembly [159]. Therefore, neutrophil repair processes may, in part, 'plug the damage' but with abnormal extracellular matrix material and structure. This needs to be explored in ILDs as any disruption to the delicate alveolar- capillary interface required for efficient gas exchange could have major clinical effects.

1.11. Potential impact of neutrophils in the fibrotic microenvironment?

Experimental work to date investigating the impact of neutrophils in the pulmonary fibrotic microenvironment have been limited to animal models and simple *in vitro* cell culture systems.

1.11.1. Animal models

The few animal models used to investigate lung fibrosis pathophysiology poorly reflect progressive pulmonary fibrosis in humans. The bleomycin model is the most established as it develops some of the histological hallmarks seen in IPF, is reproducible and readily accessible [160]. However, it is far from ideal as it displays a significant degree of reversibility unlike human disease and is associated with a markedly greater inflammatory response [160].

Table 1.3 summarises animal models that have been developed to investigate the effect of neutrophil manipulation on lung fibrosis. Neutrophils are recruited to the BALF within 24 hours of bleomycin administration in murine models [161]. Neutrophils remain elevated alongside increased NE activity even at day 14 [162]. The effect of this neutrophil recruitment is unclear with neutrophil depletion either having a negligible effect or increasing collagen content within 1-2 weeks [38,39,163,164]. This suggests that neutrophils may be either protective or amplify bleomycin associated damage. However, inhibition/absence of NE consistently reduces lung collagen content in bleomycin models suggesting the enzyme is a potential driver or amplifier of fibrosis [40,165,166].

To understand the role of the neutrophil in disease it is important to characterise the recruitment process, cell maturity and activation status both peripherally and within the lung

microenvironment, as this will influence the presence and degree of effector functions. This information is currently lacking in patients with ILD.

A sustained increase in 'aged' (CXCR4 high and CD62L low) neutrophils in the blood and BALF of bleomycin treated mice has been noted [167]. Neutrophil activation markers such as NE and myeloperoxidase are elevated in the BALF of bleomycin-treated mice, although the neutrophil activation/adhesion marker CD11b is unaffected [167]. The same study suggested that tissue-resident macrophages may be central to conducting neutrophil recruitment to the alveolar spaces in lung fibrosis [167]. With the described limitations of animal models, it is important to determine the state and associated effector functions of neutrophils from patients with ILDs, not only to determine any concordance with animal models, but to identify putative modulation pathways.

Author, year, reference	Fibrosis model	Type of neutrophil manipulation	Impact on lung collagen content	Other effects
Neutrophil Depletion				
Thrall <i>et al</i> , 1981[38]	Bleomycin rat model	Neutrophil depletion: anti-neutrophil serum	Increased at 1 week. No effect at 1 month (neutrophil count restored at week 1).	-
Clark and Kuhn, 1982 [39]	Bleomycin hamster model	Neutrophil depletion: anti-neutrophil serum	Increased at day 8 and day 12.	-
Manoury <i>et al</i> , 2007 [163]	Bleomycin mouse model	Neutrophil depletion: anti-neutrophil antibody	No effect at day 14.	Reduced pro-MMP-9 activity at day 1.
Lv <i>et al</i> , 2017 [164]	Bleomycin mouse model	Neutrophil depletion: anti-mouse Ly6G mAb	No effect at day 21.	-
Neutrophil Elastase				
Chua <i>et al</i> , 2007 [165]	Bleomycin mouse model	NE -null mice	Reduced at day 30	Reduced active TGFβ1 levels in lung tissue at day 7.
Takemasa <i>et al</i> , 2012 [40]	Bleomycin mouse model	NE inhibitor (sivelestat)	Reduced at day 7 and day 14.	Sivelestat reduced bleomycin induced increase in active TGFβ1 levels and phospho-Smad2 in lung homogenates.
Gregory <i>et al</i> , 2015 [166]	Asbestos mouse model	NE -null mice Small molecule inhibitor of elastase.	Reduced at day 14 Reduced at day 14	Reduced fibroblasts and myofibroblasts. -
Cheng <i>et al</i> , 2019 [168]	Bleomycin +/- particulate matter mouse model	NE inhibitor (sivelestat)	Reduced at day 14	Sivelestat reduced bleomycin induced phosphorylation Smad2/Smad3 and αSMA.

Neutrophil extracellular trap manipulation				
Suzuki <i>et al</i> , 2020 [169]	Bleomycin mouse model	PAD inhibitor (Cl-amidine) PAD4 knock out mice	Reduced at day 21 in PAD4 knock out mice.	Cl-amidine reduced bleomycin induced NET formation in the blood, alveolar and interstitial spaces. Haematopoietic cell grafts from PAD4 knock out mice reduced bleomycin-induced lung fibrosis.
Riehl <i>et al</i> , 2023 [135]	Bleomycin mouse model	Neutralising monoclonal anti-histone H2A/H4 antibodies	Reduced at 4 weeks.	TGFβ1 reduced in BALF at weeks 2 and 4.

Table 1.3: Animal models investigating the effects of neutrophils and related contents on lung fibrosis.

αSMA - alpha-smooth muscle actin, BALF - bronchoalveolar lavage fluid, cH3 - citrullinated histone H3, Ly6G - Lymphocyte antigen 6 complex locus G6D, MMP-9 - matrix metalloproteinase-9, mAb - monoclonal antibody, NE - neutrophil elastase, NET - neutrophil extracellular trap, PAD - pan-peptidyl arginine deaminase, TGFβ1 - transforming growth factor-β1.

1.11.2. Proteinases

Neutrophils contain a variety of granules, storing serine proteinases such as NE, and MMPs (described in Table 1.2). These are all released extracellularly during recruitment and activation and especially during cell death leading to NET formation [102]. Whether these functions differ in patients with lung fibrosis is unknown and important to determine as homeostasis of extracellular matrix deposition and degradation is likely determined by the balance with their cognate inhibitors, TIMPs and AAT/secretory leucocyte protease inhibitor, in the lung [170].

NE is the most characterised neutrophil derived proteinase in ILD with increasing evidence that it is important in fibrosis development. NE levels are elevated in IPF BALF [171]. Animal and *in vitro* studies have demonstrated the importance of NE in models of lung fibrosis, highlighting it as a potential therapeutic target. NE null mice are protected from asbestos and bleomycin induced lung fibrosis [165,166]. NE promotes lung fibroblast proliferation and myofibroblast transdifferentiation *in vitro*, the latter appearing independent of TGF β and phosphoinositide 3-kinase (Pi3K), but dependent on Smad signalling [166]. Furthermore, NE inhibition by sivelestat reduces bleomycin induced pulmonary fibrosis and neutrophilic inflammation [40]. Finally, NE has also been noted to induce collagen deposition in a bleomycin model perpetuated by particulate matter exposure, through activation of the Smad2/Smad3/ α SMA pathway [168]. NE can itself impact the MMP-TIMP balance by inactivating TIMP [172] and activating MMPs which is worthy of further study as a mechanism in ILD. Figure 1.5 summarises the proposed mechanisms by which neutrophils, including proteinases may drive fibrosis.

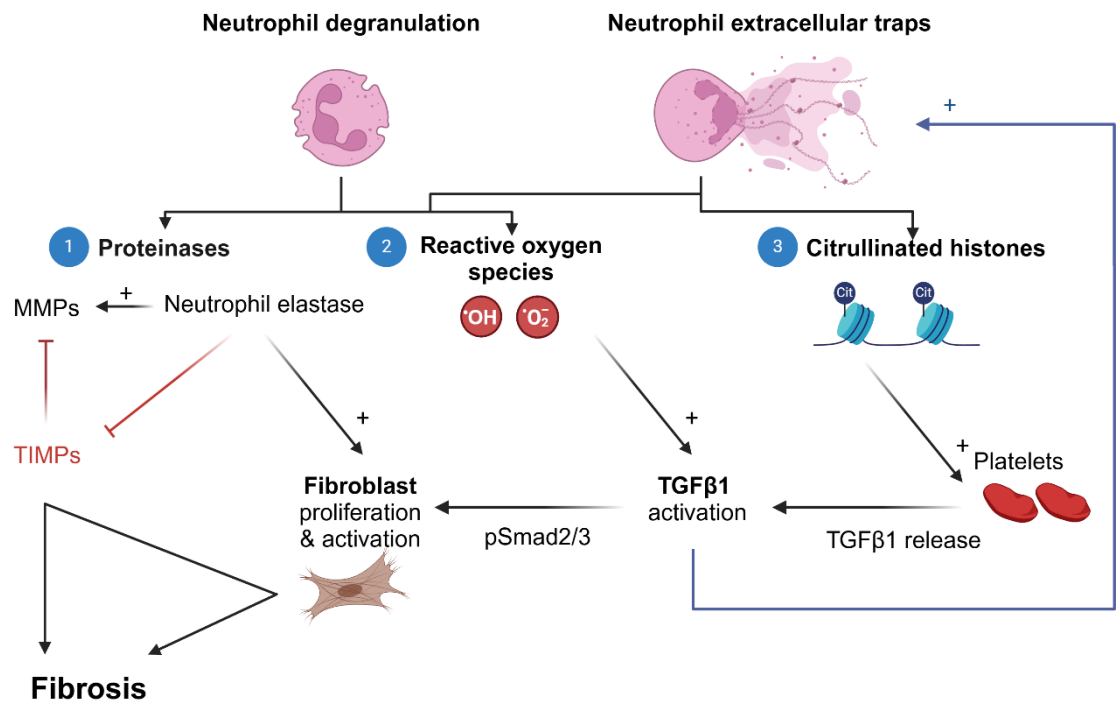


Figure 1.5: Potential mechanisms by which neutrophils drive lung fibrosis.

On neutrophil activation (degranulation and/or neutrophil extracellular trap (NET) formation) there may be release of (1) proteinases, such as neutrophil elastase and matrix metalloproteinases (MMPs), (2) reactive oxygen species and (3) citrullinated histones. All these substances may promote fibrosis through altering the balance of MMPs/tissue inhibitors of metalloproteinases (TIMPs), driving fibroblast proliferation and activation, and increasing the bioavailability of transforming growth factor- β 1 (TGF β 1). Active TGF β 1 may perpetuate this process by driving NET formation. $\text{O}_2^{\cdot-}$ - superoxide anion, $\text{OH}\cdot$ - hydroxyl free radical, pSmad2/3 - phosphorylated smad2/3. *Figure created using Biorender.com, taken from [1].*

Neutrophil release of other proteinases may also contribute to tissue and vascular remodelling in the lung. MMP dysregulation is thought to play a critical role in alveolar permeability and deranged collagen turnover, with MMP-7 silencing significantly attenuating fibrosis in bleomycin rat models [173]. In response to tissue injury, the best *in vivo* evidence shows MMP-7 drives re-epithelialisation and neutrophil influx [174]. This may be helpful in an acute setting, but a prolonged/excessive response, in the context of fibrosis, can also be damaging [174]. Neutrophils have been reported as an important cellular source of MMP-9 in lung inflammation [175]. MMP-8 and -9 are elevated in the BALF of IPF patients, especially in those with rapid lung function decline and MMP-8 relates to neutrophil count and BALF collagenolytic activity [170,176]. MMP8 knock out mice treated with bleomycin develop less lung fibrosis, (despite greater lung inflammation) than wildtype mice [177]. However, MMP-9 knock out had little effect in an experimental lung fibrosis model [178] suggesting the MMP-8 was more important in this process.

Targeting proteinases as a potential therapeutic strategy in IPF, therefore seems logical, but potentially at risk of offsetting their beneficial or regulatory roles. Clearly a better understanding of the potential roles and interaction of these individual enzymes and their relationship to neutrophil inflammation and fibrosis is essential in the development of such specific mediator therapy.

1.11.3. TGF β activation

As indicated above, TGF β is accepted as a central mediator of the fibrotic process and may be modulated by neutrophils for two main reasons.

(A) NE can activate TGF β 1 in the extracellular matrix whilst sivelestat (a specific NE inhibitor) reduces active TGF β 1 levels and its downstream signalling molecule phosphorylated smad2, though not total TGF β 1 [162]. NE mediated downstream signalling molecules have been demonstrated in bleomycin animal models [168,179]. NE is also able to release latent TGF β 1 from epithelial and endothelial cell matrices *in vitro* but is unable to activate latent TGF β 1 in this setting [180]. NE directly activates MMPs, which themselves activate TGF β 1 although this interplay in the fibrotic lung remains to be confirmed [181,182].

(B) Neutrophils may contribute to TGF β bioavailability via alternative pathways. The bleomycin murine model suggested externalised histones released on NET formation may promote TGF β 1 release from platelets [135]. Peripheral blood neutrophils themselves contain and secrete active TGF β 1 in response to PMA stimulation [183]. Asbestos-derived ROS have been shown to activate latent TGF β 1 in A549 cells [184], although it is unknown whether neutrophil generated ROS also increases TGF β 1 activation especially in the lung. Oxidant driven activation of TGF β has been demonstrated in fibroblasts, wherein cellular influx of superoxide through chloride channels caused ERK1/2 and p38 MAPK dependent activation and subsequent upregulation of TGF β 1 [185]. By this rationale activated neutrophils, which produce superoxide as a primary microbe killing mechanism, are capable and perhaps likely to induce the same processes of oxidant driven TGF β 1 activation as seen in fibroblasts.

1.11.4. Neutrophil extracellular traps (NETs)

NETs have been identified in IPF lung tissue and are increased in ILD BALF [19]. The key determinants of NET formation are unknown here, although TGF β may be important [186]. Primary human lung fibroblasts stimulated with NETs undergo increased myofibroblast differentiation, collagen production and promote alveolar epithelial cell damage through histone release [187,188]. However, the mechanism by which NETs influence other changes in pulmonary epithelial and mesenchymal cells remains unclear. Again persistence of neutrophils and NETs in the lungs of IPF patients may enhance and perpetuate the fibrosis element and/or impair wound healing [189].

1.12. What drives neutrophil recruitment to the lung in ILD?

1.12.1. Alveolar epithelial cell damage?

Neutrophil mobilisation has been suggested as a physiological response to the type II alveolar epithelial cell damage that occurs early in the pathogenesis of IPF. Figure 1.6 summarises the potential stages of neutrophil migration to and from damaged alveoli in ILDs. Damaged epithelial cells release danger-associated molecular patterns (DAMPs) that act on pattern recognition receptors (PRRs) in order to drive an immune and potentially reparative response [190]. Expression of these danger signals may account for migration of neutrophils to fibrotic lungs in close proximity to areas of alveolar damage in IPF [191]. This danger signalling may be an essential component of the physiological pro-healing response [153].

However, once in the lung, the neutrophil driven “remodelling” may also be detrimental to the interstitial architecture. For example, the PRR, FPR1, heavily expressed on neutrophils,

and its ligands, appear to be critical in bleomycin-induced neutrophil recruitment and subsequent fibrosis in the lung, but not in the kidney or liver [190]. Neutrophils themselves may drive DAMP release, thereby amplifying and perpetuating neutrophil infiltration, through proteinase mediated extracellular matrix degradation products, such as fibronectin, having the propensity to activate toll-like receptors [192]. Although this has yet to be confirmed in the lung.

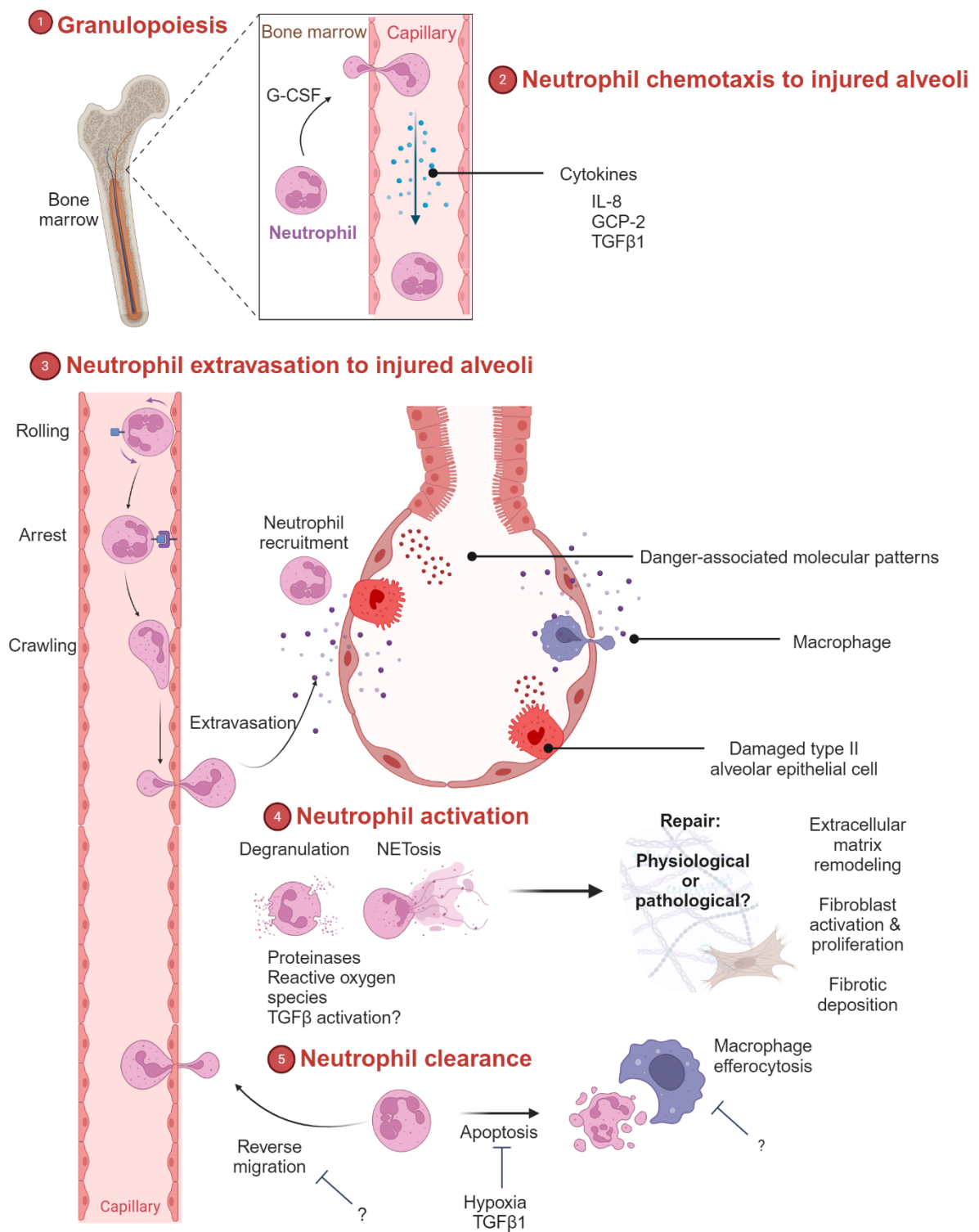


Figure 1.6: Proposed pathway of neutrophil response to injured alveoli in ILDs.

Neutrophils are produced by the bone marrow **(1)** in response to factors, such as G-CSF (granulocyte– colony stimulating factor) [49,193], and migrate to the site of alveoli injury down a chemotactic gradient **(2)**. Neutrophils extravasate from the pulmonary capillaries into the alveoli interstitium [194] **(3)** in response to chemokines and danger signalling molecules released from damaged type II alveolar epithelial cells [21,190,191]. **(4)** On arrival neutrophils are activated, degranulating and/or undergoing neutrophil extracellular trap formation (NETosis) [19], which releases enzymes, such as proteinases, transforming growth factor-beta 1 (TGFβ1) and reactive oxygen species (ROS). These mediators attempt to drive repair processes including extracellular matrix remodelling. This may activate fibroblasts and result in fibrotic deposition [166,188]. Following activation, **(5)** neutrophil clearance through reverse transmigration out of the lungs and/or apoptosis is required to retain homeostasis. Factors, such as hypoxia [43], may reduce the homeostatic clearance of senescent neutrophils. GCP-2 - granulocyte chemotactic protein 2, IL-8 - interleukin-8. *Figure created using Biorender.com, taken from [1].*

1.12.2. Increased granulopoiesis?

Greater levels of the granulopoiesis regulator, G-CSF, have been noted in IPF BALF than in health [195]. BALF G-CSF levels were related to the BALF neutrophil count and negatively associated with disease severity as determined by FVC [195]. Neutrophil chemotaxis was increased towards BALF derived from IPF patients, and was abrogated by G-CSF neutralisation indicating that G-CSF is also likely an important chemoattractant in this condition [195]. G-CSF exacerbates lung injury in bleomycin rat models through a neutrophil dependent mechanism [193]. Such a mechanism suggests a process whereby neutrophil production and recruitment to the damaged lung by G-CSF is increased, potentially exacerbating the tissue damage. However, G-CSF may also act as an anti-fibrotic by promoting bone marrow mesenchymal stem cell mobilisation to injured tissue [196]. Whether this is important in human disease development and how it alters neutrophil phenotype, recruitment and activation are clearly worth further study.

1.12.3. Altered neutrophil migration and chemotaxis?

The chemokine and endothelial environment changes in response to bleomycin in animal models, increasing the release of (A) chemoattractants, IL-8 and monocyte chemoattractant protein-1, and (B) upregulating expression of adhesion molecules such as E-selectin, CD54 and vascular adhesion molecule 1 by pulmonary microvascular endothelial cells [194]. Using an *in vitro* flow system, neutrophil adhesion to human umbilical vein endothelial monolayers was increased by bleomycin treatment, independent of CD54, CD18, and e-selectin [194]. Changes in the fibrotic microenvironment due to alveolar/capillary integrity may also be influenced by

hypoxia which increases neutrophil adhesion, transendothelial migration and expression of $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins *in vitro* [19].

Although these chemokine and endothelial changes have been seen in animal models and *in vitro*, the pertinent mediators in human ILDs are less clear. There is evidence that the chemokine milieu in BALF is dysregulated increasing neutrophil chemotactic response in IPF compared to health [124]. IL-8 is one of the major chemoattractants generated in the lung, acting via the neutrophil cell surface receptors, CXCR1 and CXCR2. One study demonstrated significantly greater IL-8 concentrations in the supernatant from cultured IPF derived endothelial progenitor cells than control cells [197]. Greater neutrophil transwell migration was noted *ex vivo* towards the IPF conditioned media than control and was abolished by an anti-IL-8 antibody [197]. Furthermore, IL-8 staining was noted in the vessels of IPF lung tissue but not control [197]. IL-8 levels in the serum and BALF are significantly higher in patients with ILD and serum IL-8 levels relate to the percentage of neutrophils in the BALF, supporting its role in neutrophil mobilisation [198,199]. In addition, serum IL-8 levels are negatively related to lung function indicators of disease severity, FVC, gas transfer, partial pressure of oxygen and survival [198,200]. These data suggest IL-8 is a marker of the severity and progression of IPF possibly reflecting neutrophil recruitment and activation.

Other chemokines may be important in neutrophil mobilisation in ILDs. The chemokine granulocyte chemotactic protein 2 (GCP-2) is upregulated in the BALF of IPF patients and bleomycin challenged mice [201]. Furthermore, neutralisation of GCP-2 reduces early inflammation, neutrophil influx, TIMP-1 expression and fibrosis in bleomycin murine models suggesting the inflammatory activity of this cytokine in this milieu likely has a role [201].

1.12.4. Amplified/abnormal cell recruitment (TGFβ1)?

The generation of classical neutrophil chemoattractants may be central to the physiological neutrophil response in IPF. However, in the complex inflammatory milieu it remains possible that cytokines not conventionally recognised as chemoattractants may prime neutrophil responses or the destructive processes in the lung matrix may amplify the neutrophil response.

TGFβ1 (a key cytokine in the fibrotic response) may influence overall neutrophil recruitment in IPF but it remains unclear (discussed in further detail in Chapter 4). Further studies are required to explore its contribution in neutrophil influx in ILDs, clarify the mechanism and the relationship to progression and severity.

1.12.5. Impaired neutrophil clearance/resolution?

As mentioned previously, following recruitment as part of a physiological response to injury neutrophils need to undergo clearance as persistence may enhance fibrosis, tissue damage and inflammation in the pulmonary interstitium as in models of lung fibrosis and fibroproliferative ARDS [202–204].

Macrophage efferocytosis is a major mechanism of neutrophil clearance in the lung as part of the pro-resolution response. It has been suggested that the efferocytosis of apoptotic cells by alveolar macrophages is impaired in IPF patients [205]. It is unknown whether there is a similar defect specifically in the efferocytosis of senescent neutrophils. Although TGFβ1 has been noted to increase macrophage mediated clearance of apoptotic neutrophils *in vitro*, its effects on ILD derived cells are unknown [206].

Pro-survival signals in the fibrotic microenvironment may contribute to prolonged residence and activity of neutrophils in lung tissue. These may include TGF β and hypoxia (with subsequent activation of the HIF1 α hydroxylase oxygen-sensing pathway) increasing neutrophil longevity [43,207]. In addition, increased neutrophil retention upon priming [208] could also be potentially damaging to the tissues.

The complex nature of neutrophil mobilisation and clearance has only been covered briefly here with tissue injury likely the primary trigger. The persistent neutrophilia is recognised but the mechanisms by which this carefully controlled process of recruitment and removal apply to or are altered in ILDs are relatively unexplored. In addition, the impact of the fibrotic milieu on neutrophil phenotype and function is unknown; does it drive a more damaging rather than reparative role?

1.13. Therapeutic potential in neutrophil modulation

Anti-inflammatory and immunomodulatory treatments continue to be the mainstay pharmacological therapies for ILDs that demonstrate significant inflammatory radiological changes. This was previously true for IPF until a landmark combination trial, of azathioprine, N-acetylcysteine and prednisolone was prematurely discontinued following interim results revealing increased mortality and hospitalisation in the intervention arm (www.ClinicalTrials.gov identifier NCT00650091) [209].

Currently, the only approved pharmacological therapies for IPF are the two anti-fibrotic agents, nintedanib and pirfenidone. Both drugs show signs of immunomodulatory effects for example dampening bleomycin induced neutrophil infiltration and fibrosis in animals models

[210,211] but a direct anti-neutrophil effect is not clear. With current therapeutic strategies only slowing disease decline, targeting alternative pathways, such as neutrophil effector functions, may potentially provide an important unmet need.

Failed attempts to safely antagonise TGF β 1 is an example of how problematic it can be to generate a therapeutic strategy against a target that has multifunctional homeostatic effects. The same concept is true with an antineutrophilic strategy, where careful consideration is needed of potential adverse reactions that may occur with neutrophil modulation. The most notable risk is the increased susceptibility of serious recurrent infections as seen in patients with neutropenia. Neutrophil manipulation would need to be limited to clear pro-fibrotic pathways without adversely suppressing granulopoiesis, physiological recruitment and effector functions to minimise off-target effects. Table 1.4 describes potential strategies of neutrophil manipulation in ILD. None of these drugs have been tested so far in patients with ILD. All these strategies depend on the concept that at least part of the neutrophil response is causative thus it is essential to dissect the physiological neutrophil responses from any potentially pathological ones to develop a logical strategy.

Targets	Drugs	Animal models/Clinical trials
Neutrophil proteinase activation	Brensocatib (Dipeptidyl peptidase 1 inhibitor)	Phase III clinical trial (STOP-COVID19 ISRCTN30564012 [212]) – brensocatib was not beneficial in the acute management of patients hospitalised with COVID-19. Brensocatib did not increase the rate of infection. Phase II clinical trial (WILLOW: www.ClinicalTrials.gov identifier NCT03218917 [213]) - brensocatib extended time to first exacerbation in bronchiectasis patients. Phase III clinical trial (ASPEN: www.ClinicalTrials.gov identifier NCT04594369) - results awaited.
Neutrophil elastase	Sivelestat (Neutrophil elastase inhibitor)	Bleomycin animal models – sivelestat reduced pulmonary fibrosis development [40,168]. Phase IV clinical trial in acute lung injury associated with systemic inflammatory response syndrome patients [214] - reasonable safety profile.
NET formation, such as peptidyl arginine deaminase (PAD)	PAD4 inhibitor (JBI-589 - not yet trialed in humans)	Bleomycin murine models – PAD4 knock out mice generated less NETs and lung fibrosis in response to bleomycin instillation than wild type mice [169]. JBI-589 blocked NET formation in an animal arthritis model [215].
Granulocyte-macrophage colony stimulating factor	Namilumab (granulocyte-macrophage colony stimulating factor inhibitor)	Phase II clinical trial (CATALYST ISRCTN40580903 [216]) - namilumab reduced c-reactive protein levels in COVID-19 patients. Namilumab increased the rate of infection.

Table 1.4: Potential therapeutic strategies of neutrophil manipulation in interstitial lung diseases.

Described here are targets/drugs that have shown promise in animal models and/or clinical trials at manipulating neutrophil responses. COVID19 - Coronavirus disease 2019, NET – neutrophil extracellular trap, PAD4 - Peptidyl arginine deaminase-4.

In designing a clinical trial careful consideration of ILD type and study participants will be required to attempt to enrol participants who are most likely to benefit from the proposed outcome (i.e. rapid progressors) and thereby minimise the risk of a false negative trial. Investigation of these products may be best suited within an adaptive platform trial design, such as Randomised Embedded Multifactorial Adaptive Platform in fibrotic interstitial lung diseases (REMAP-ILD) [217].

1.14. Barriers to neutrophil research in ILDs

Neutrophils are vulnerable to activation and degranulation *ex vivo* and these features are heavily influenced by processing time and neutrophil isolation methods [218]. This fragility is likely a significant reason for our relatively poor understanding of neutrophils' contribution to lung fibrosis including lack of single cell ribonucleic acid-sequencing/proteomic data, which could provide important insights.

1.15. Summary

This introduction has highlighted numerous plausible mechanisms by which neutrophils may play a role in the pathophysiology of lung fibrosis. The body of evidence appears sufficient to support neutrophils being present not solely as a reaction to the alveolar epithelial injury. However, their impact, whether reparative or pathological on the delicate lung architecture has yet to be clarified. It is critical to understand any role as manipulation of neutrophil

recruitment and function could provide a novel and much needed therapeutic strategy for patients with fibrosing ILDs.

There are many research questions that need answering before considering any neutrophil related therapeutic targets in IPF. BALF and circulating neutrophil counts appear to be associated with disease severity. However, it is unknown; 1) what is driving neutrophil infiltration into the fibrotic lung, 2) where neutrophils and their contents localise in lung tissue and 3) how these change with disease severity and progression. The effect of the pro-fibrotic growth factor, TGF β 1, on neutrophil recruitment and function remains unclear. Considering TGF β 1's critical role in the pathobiology of IPF, it is important that any contribution to neutrophil migration to the fibrotic lung is determined. Other cytokines, known to influence neutrophil migration and priming and/or activation also appear to be upregulated in patients with IPF. It is therefore important to determine if peripheral neutrophils from IPF patients display an altered phenotype and function, which may affect their anti-microbial and repair functions. Of the minimal literature available in this area, hypoxia has been highlighted as of potential importance in neutrophil biology in IPF [19]. Therefore, the burden of disease related hypoxia on these functions also needs to be considered.

1.16. Thesis Hypotheses

This thesis describes experiments aimed at exploring the following three hypotheses:

- 1) The pro-fibrotic cytokine, TGF β 1, drives neutrophil migration into the fibrotic lung.
- 2) Circulating neutrophils from patients with IPF display a primed phenotype and altered migratory and effector functions.
- 3) Neutrophils and their products (NETs and proteinases) are resident in fibrotic lung tissue and are associated with fibrosis severity.

1.17. Thesis Aims

This thesis aims to:

1. Investigate the effect of the pro-fibrotic cytokine, TGF β 1, on neutrophil chemotaxis and priming.
2. Investigate whether circulating neutrophils from patients with IPF display an aberrant phenotype and function, compared to age-matched control participants.
3. Spatially characterise neutrophils and neutrophil products (NETs and proteinases) in IPF lung tissue.

Chapter 2. MATERIALS AND METHODS

2.1. Ethical approval

All studies reported in this thesis were approved by the West Midlands – South Birmingham Research Ethics Committee (REC). All participants provided written informed consent to the relevant study prior to enrolment (see Table 2.1).

Participants	Study Title	REC number
Control participants (18- 60 years of age)	Investigations of the Ageing Immune System.	10/H1208/48
IPF patients and age-matched controls	Investigating the Hypoxic Burden in Interstitial Lung Disease (HYBRID), RRK7934.	22/WM/0274
Control lung tissue blocks	Use of Tissue from Lung Resections to Investigate the impact of Smoking and Vaping on Cellular Function in Human Lung Disease, RRK6192.	17/WM/0272
IPF patients' lung tissue blocks	Use of tissue from lung resections to investigate the molecular and functional mechanisms of human lung disease, RRK6083.	07/MRE08/42

Table 2.1: Outline of the ethically approved studies included in this thesis.

IPF- Idiopathic pulmonary fibrosis. REC – Research Ethics Committee.

2.2. Peripheral blood sample collection

Following informed written consent, venous blood samples were collected peripherally using Becton Dickinson (BD) Vacutainer® Push Button Blood Collection Sets and BD Vacutainer® Blood Collection tubes (*Becton, Dickinson and Company, United States of America (USA)*). The type of BD Vacutainer® Blood Collection tube used was dependent on the desired sample required for experimental analysis (See Table 2.2). All venous blood samples were processed within 30 minutes of collection.

Blood Collection tube	Desired sample
BD Vacutainer® Lithium heparin Tube (367885)	Percoll Density Gradient neutrophil isolation. Plasma sample.
BD Vacutainer® Serum Tube (368975)	Serum sample.
BD Vacutainer® Sodium Citrate Tube (367691)	Full blood count (Sysmex XN-1000 analyser). Double spun plasma sample for cell free DNA assays.

Table 2.2: Description of blood collection tubes.

Outline of BD Vacutainer® Blood Collection tubes (Becton, Dickinson and Company, USA) used to collect peripheral venous blood for experimental samples. BD – Becton Dickinson.

2.3. Peripheral blood sample processing

2.3.1. Full blood count analysis using Sysmex XN-1000 analyser

BD Vacutainer® Sodium Citrate Tubes (*Becton, Dickinson and Company, USA: 367691*) were inverted gently 8 - 10 times. Two hundred µl of whole blood was subsequently transferred using a sterile pipette into a 1.5 millilitres (mL) Eppendorf tube. The Eppendorf tube was placed on the Sysmex XN-1000 analyser (*Sysmex United Kingdom (UK) Ltd, Milton Keynes, UK*) and full blood count analysis subsequently performed. A printed report for each sample was filed in the secure site file.

2.3.2. Plasma processing

BD Vacutainer® Lithium heparin Tubes (*Becton, Dickinson and Company, USA: 367885*) were stood upright at room temperature for 30 minutes. The sample was centrifuged at 560 x g for ten minutes at room temperature. Supernatant was collected and stored at -80°C.

2.3.3. Double spun plasma processing

BD Vacutainer® Sodium Citrate Tubes (*Becton, Dickinson and Company, USA: 367691*) were inverted gently 8 - 10 times and centrifuged at 2500 x g for 15 minutes without a brake at room temperature. The supernatant was then pipetted into a fresh 15ml centrifuge tube (*Corning, Mexico: 430791*) and centrifuged again at 2500 x g for 15 minutes. Using a fresh pipette, the top 80% of the supernatant was collected and stored immediately at -80°C.

2.3.4. Serum processing

BD Vacutainer® Serum Tubes (*Becton, Dickinson and Company, USA: 368975*) were stood upright at room temperature for 30 minutes and centrifuged at 560 x g for ten minutes at room temperature. The supernatant was collected and immediately stored at -80°C.

2.4. Peripheral neutrophil isolation

Neutrophils were isolated using dextran sedimentation and discontinuous Percoll density gradients, as described previously [219].

2.4.1. Dextran sedimentation

Whole blood from BD Vacutainer® Lithium heparin Tubes was decanted into 50 mL centrifuge tubes and dextran (*2% working concentration, Sigma-Aldrich, Germany: 31392*) in 0.514 M sodium chloride (*Scientific Laboratory Supplies, UK: CHE3326*) added at a 6: 1 ratio. The contents of the 50 mL centrifuge tube were gently mixed and then stood upright at room temperature for 30 minutes to sediment the erythrocytes.

2.4.2. Percoll density gradient centrifugation

Towards the end of this 30 minute period, a Percoll® (*Cytiva, Sweden: 17089101*) density gradient was made (see Figure 2.1). Three concentrations (90%, 80%, 56%) of isotonic Percoll solutions were prepared (see Table 2.3). 5.5 mL of 56% Percoll was subsequently pipetted into a sterile 15 mL centrifuge tube. 2.5 mL of 80% Percoll was then layered carefully underneath the 56% Percoll layer using an extended fine tip sterile Pastette® (*Alpha lab, UK: LW4232*).

Following the 30 minutes blood incubation, the top buffy coat (plasma) created from the dextran sedimentation was pipetted using an extended fine tip sterile Pastette® slowly on top of the 56% Percoll (see Figure 2.1A).

Concentration of Percoll Solution	Solution A	Solution B	Dilution Ratio Solution A: Solution B
90%	Percoll® (<i>Cytiva, Sweden: 17089101</i>)	Sterile sodium chloride (1.54 M)	9: 1
80%	90% Percoll	Sterile sodium chloride (0.154 M)	4: 1
56%	90% Percoll	0.9% Sodium Chloride	1.27: 1

Table 2.3: Constituents of Percoll concentrations used.

90% Percoll was made first using a 9: 1 dilution of neat Percoll® (*Sigma-Aldrich, USA: P4937*) and 10x saline. 80% and 56% Percoll were subsequently made using a 4: 1 and 1.27: 1 ratio of the 90% Percoll and 0.9% sodium chloride, respectively.

Once all the plasma was added, the 15 mL centrifuge tube was centrifuged for 20 minutes without acceleration or brake at 470 x g at room temperature. This centrifugation step separated the cells present in the plasma into distinct bands dependent on density (see Figure 2.1B).

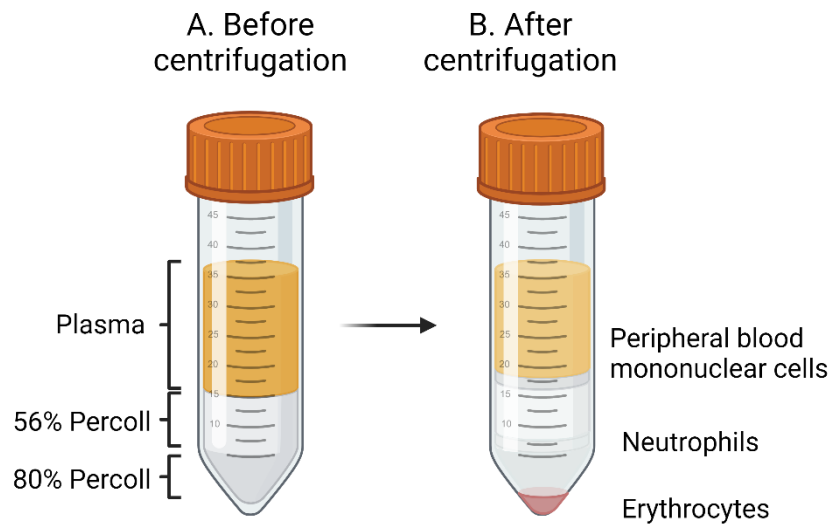


Figure 2.1: Percoll density gradient centrifugation.

Graphical outline of layers of Percoll and plasma (following dextran sedimentation) before **(A)** and after centrifugation **(B)**. Following centrifugation, peripheral blood mononuclear cells were first removed. Neutrophils were located at the junction between the 56% and 80% Percoll layers and were carefully removed without disrupting the erythrocyte pellet at the base of the tube. *Figure created with BioRender.com.*

The peripheral blood mononuclear cells (PBMCs) were typically located in the top layer and were the first to be removed using a 3 mL sterile graduated transfer pipet (*Fisherbrand™, UK: 1346-9108*). The classical neutrophil layer was situated on the boundary of the 80% and 56% Percoll and was carefully removed using an extended fine tip sterile Pastette®. Neutrophils were then washed in sterile Dulbecco's Phosphate Buffered Saline (DPBS) (not containing magnesium or phosphate) (*Gibco, UK: 14190-04*) and centrifuged at 250 x g for 10 minutes at room temperature and the neutrophil pellet was resuspended in RPMI (Roswell Park Memorial Institute Medium) 1640 containing 2 mM L-glutamine (*Gibco, UK: 21875-034*), supplemented with 1% penicillin-streptomycin (*Sigma-Aldrich, USA: P4333*), which is referred to as RPMI in this thesis.

2.4.3. Cell counting

Neutrophils and other cells were counted using a FastRead 102™ Counting Slide (*Immune systems, UK: BVS100*) and light microscope (*Wilovert, Germany*). Nine microlitres (µl) of cell suspension was pipetted into a single chamber and the number of cells in 16 small squares was counted. The number of cells per mL was subsequently determined using the equation:

$$\text{Count of cells per mL} = \text{Number of cells counted in 16 small squares multiplied by 10,000.}$$

2.4.4. Assessment of neutrophil purity and morphology

Neutrophil purity and morphology was assessed using a cytopsin. One hundred μL of neutrophil suspension (at 1 million cells per mL) was transferred to a cytopsin chamber attached to a white filter card (*Epredia, USA: 5991022*) and a Superfrost™ microscope slide (*Epredia, China: AG00008232E01MNZ10*). This cytopsin chamber was subsequently centrifuged at 300 revolutions per minute in a Cytospin 4 (*ThermoFisher Scientific, UK*) for 5 minutes. The microscope slide was then removed, fixed and stained using a REASTAIN QUICK-DIFF KIT (*Reagen, Finland: 102164*) whereby the slide was dipped in REASTAIN Quick-Diff fix containing methanol for 10 seconds, REASTAIN Quick-Diff red for 10 seconds and REASTAIN Quick-Diff blue for 5 seconds. The slide was subsequently rinsed in distilled water and allowed to dry. The slides were imaged using a light microscope (*Wilovert, Germany*) at 20x magnification. The number of cells and percentage of neutrophils were calculated. Neutrophil purity was typically >95%. Figure 2.2 displays a representative image of a stained neutrophil cytopsin slide.

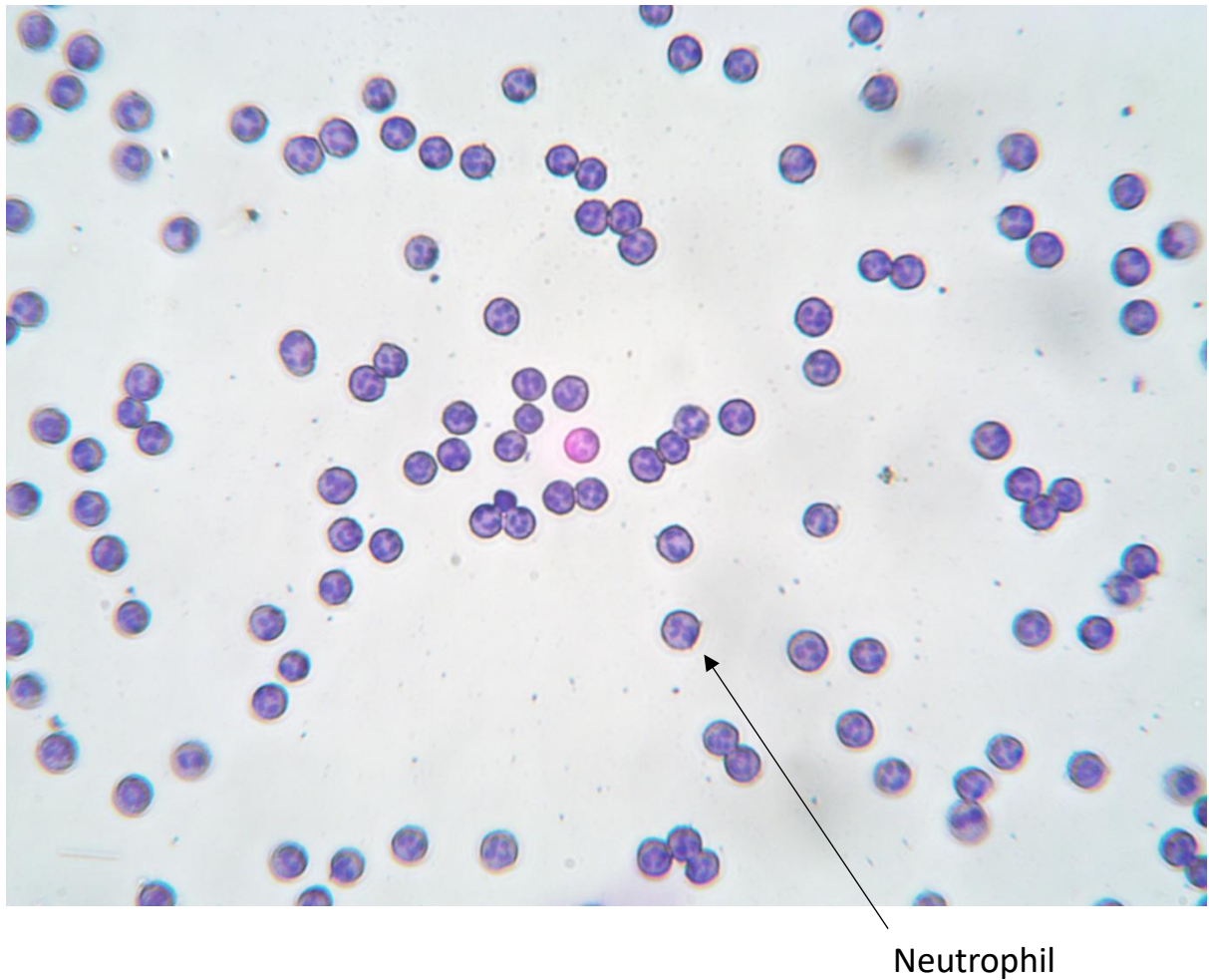


Figure 2.2: Representative image of a stained neutrophil cytospin slide.

Peripheral neutrophils were isolated from a venous blood sample collected from a healthy participant. Neutrophils were transferred to a microscope slide via cytospin and stained using REASTAIN QUICK-DIFF KIT (*Reagen, Finland: 102164*). Neutrophils are identified by the characteristic multi-lobe nucleus. Image taken using a light microscope (Wilovert, German) at 20x magnification.

2.5. Generation of a 1% oxygen *in vitro* culture environment

A 1% oxygen environment was modelled *in vitro* by placing neutrophil suspensions in the relevant culture plates in a Whitley H37 Hypoxystation (*Don Whitley Scientific, UK*) with an atmosphere containing: 1% oxygen, 5% carbon dioxide (CO₂) and 75- 85% humidity. Regular calibration of the oxygen sensor was performed as per the manufacturer instructions. After the desired culture time, neutrophils were fixed in paraformaldehyde (2% *working concentration*, *ThermoScientific, Belgium: J19943-K2*) for 10 minutes to minimise reoxygenation of the cells prior to removal from the Whitley H37 Hypoxystation.

2.6. Viability assays

2.6.1. Annexin V/Propidium Iodide viability staining

Neutrophils were resuspended in ice cold Annexin V binding buffer. Annexin V binding buffer was made by diluting 1: 10 using distilled water a 10 times concentrated solution of filter sterilised: 25 mM calcium chloride (*Sigma Life Science, Japan: C7902*), 1.4 M sodium chloride and 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4). Annexin V-FITC stain (*BD Bioscience, UK: 556420*) or Annexin V-Vioblue antibody (*BioLegend®, USA: 640924*) were added at a 1: 100 dilution and incubated for 20 minutes in the dark on ice. Neutrophils were then washed twice by; adding 500 µl ice cold Annexin V binding buffer, centrifugation at 250 x g for 5 minutes at 4 °C and disposal of the supernatant. Neutrophils were finally resuspended in 200 µl ice cold Annexin V binding buffer and stored on ice in the dark ready for data acquisition. Propidium iodide (PI) (*Sigma-Aldrich, USA: P4864-10ML*) was added at a 1: 100 dilution immediately prior to data acquisition.

Heat exposed neutrophils labelled as 'heat killed (HK)' were used as positive controls for both Annexin V/PI and Zombie NIRTM fixable viability assays. HK controls were created by incubating an aliquot of neutrophil suspension in a 70°C heat block for 2 minutes and 15 seconds and were stained with the relevant dyes as described in the relevant sections. '100% HK' controls included solely heat exposed neutrophils and '50% HK' contained a 1: 1 ratio of heat exposed and untreated neutrophils.

Samples were analysed on the MACSQuant[®] Analyzer 10 (*Miltenyi Biotec, UK*). Five to 10,000 neutrophil events were recorded. Fluidics were set to medium or high and to minimise variation, the fluidics setting was consistent in comparable experiments. FlowJo_v10.9.0 Software was used to analyse the data. First a scatter plot was created of forward scatter (FSC) versus side scatter (SSC) to allow for gating of neutrophils (Figure 2.3A). Single cells were then identified from this population from scatter FSC–area (FSC-A) versus FSC-height (FSC-H) parameters (Figure 2.3B).

Due to two fluorophores being used in a single sample, compensation analysis was performed on FlowJo_v10.9.0 Software to correct for any spillover of fluorescence emissions. Single stained (Annexin V or PI) HK control samples were used to determine clear positive and negative populations for compensation analyses. Figure 2.4 describes representative scatter and histogram plots of how a compensation matrix was performed and resultant spillover values.

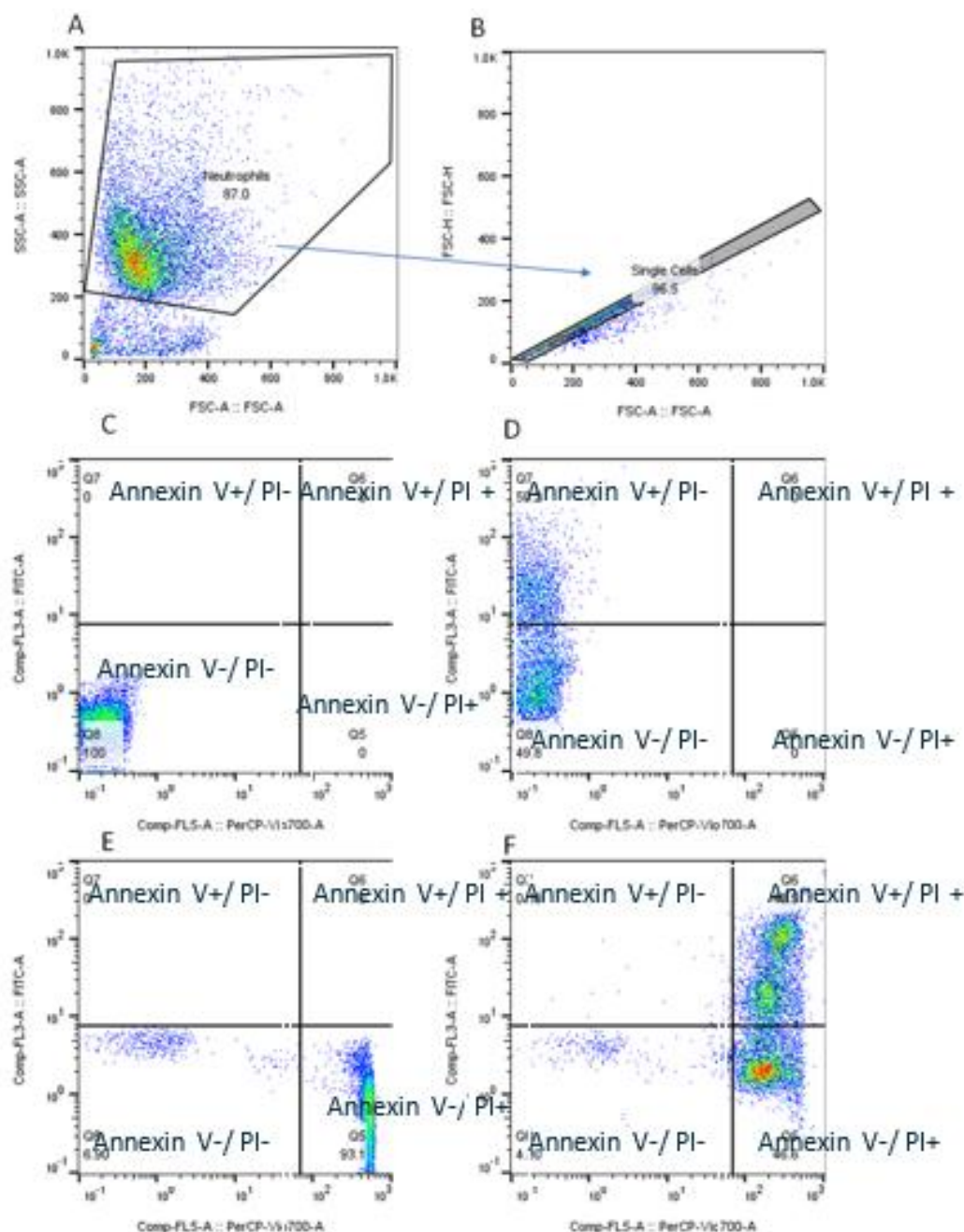


Figure 2.3: Representative scatter plots displaying gating of Annexin V/PI viability data.

(A) Neutrophils were gated using scatter SSC-A versus FSC-A parameters. (B) Single cells were identified in the neutrophil gate using scatter FSC-A versus FSC-H parameters. The percentage of viable (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+, PI+) and necrotic (Annexin V-, PI+) cells were determined by creating four quadrants from single cells using scatter compensated PerCP-Vio700 – area versus compensated FITC-A or VioBlue. Representative scatter plots of compensated PerCP-Vio700 – area versus compensated FITC-A of (C) unstained neutrophils (D) Annexin V stained HK neutrophils (E) PI stained HK neutrophils (F) Annexin V and PI stained HK neutrophils.

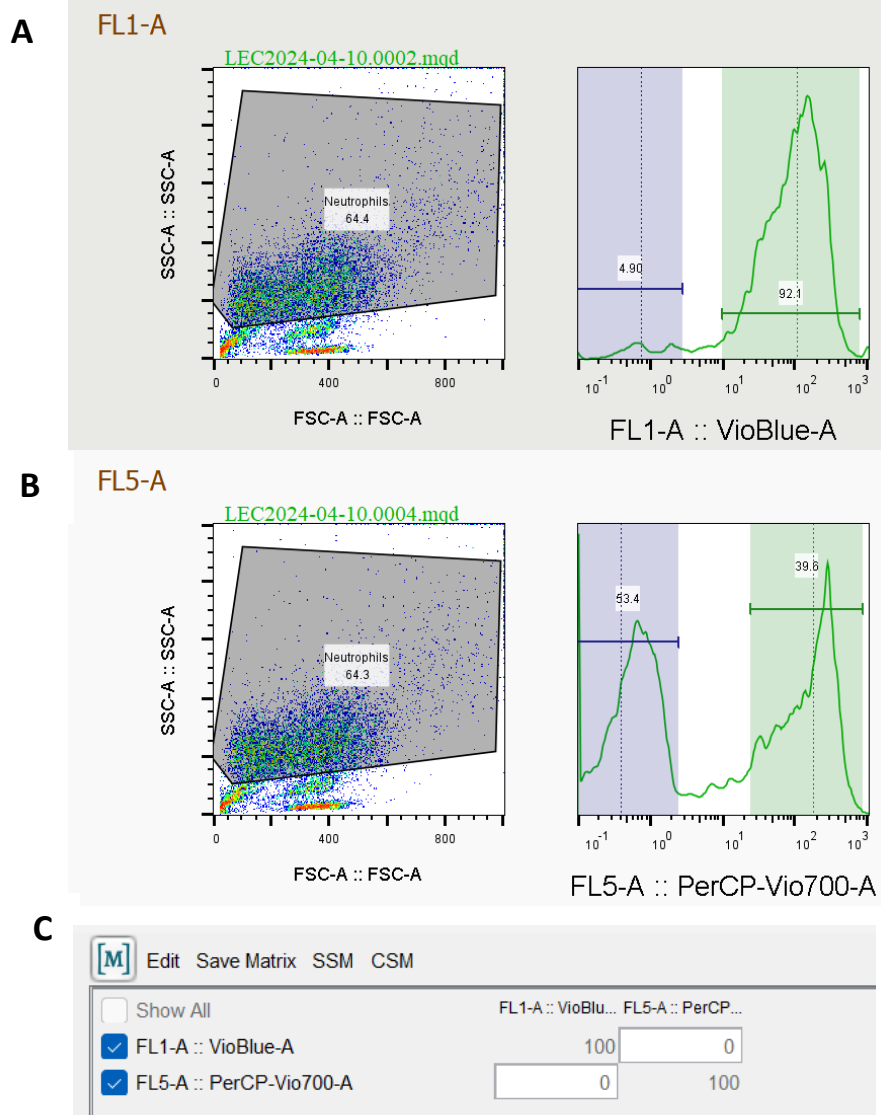


Figure 2.4: Representative scatter plots and histograms describing compensation matrix creation.

(A) Annexin V single stained 50% HK neutrophil sample: FSC-A versus SSC-A scatter plot (left) and corresponding VioBlue-A histogram (right) displayed. Annexin V negative population gated (highlighted in blue) and annexin V positive population gated (highlighted in green).

(B) PI single stained 50% HK neutrophil control sample: FSC-A versus SSC-A scatter plot (left) and corresponding PerCP-Vio700-A histogram (right) displayed. PI negative population gated (highlighted in blue) and PI positive population gated (highlighted in green).

(C) Compensation matrix created from figures A and B describing compensation spillover values (zero) between VioBlue-A and PerCP-Vio700-A.

2.6.2. Zombie NIR™ fixable viability stain

Neutrophils were resuspended in RPMI at a concentration of 1 million per mL and incubated with Zombie NIR™ fixable viability stain (*BioLegend, San Diego, USA: 423105*) at a 1: 100 dilution for 30 minutes at 37°C. Neutrophils were subsequently fixed in paraformaldehyde (2% *working concentration*) for 10 minutes at 37°C. Neutrophils were washed twice by; adding 500 µL ice cold sterile DPBS, centrifuged at 250 x g for 5 minutes at 4 °C and the supernatant discarded. Neutrophils were finally resuspended in 200 µl ice cold DPBS and stored on ice in the dark. Samples were analysed on the MACSQuant® Analyzer 10 (*Miltenyi Biotec, UK*) and acquired as described in the Annexin V/PI viability assay section.

Data analysis was performed using FlowJo_v10.9.0 Software and single cells identified from the neutrophil gate. Histograms were created to display the frequency distribution of APC-Vio770A intensity of neutrophil singlets. HK neutrophils were used as positive controls to gate cells that stained positively for Zombie NIR™ dye and hence deemed not viable (see Figure 3.1).

2.7. Migration and chemotaxis assays

2.7.1. Transwell migration assay

Six hundred μ l RPMI was pipetted into wells of a 24 well plate and pre-warmed in a 37°C CO₂ incubator. The media was supplemented with the chemoattractant agent under investigation (see Table 2.5) while ensuring that the final volume was 600 μ l. Three μ m pore size Nunc™ (*ThermoFisher Scientific, UK: 140620*) or Corning® Transwell® (*Corning, USA: CLS3415*) polycarbonate cell culture inserts were added (see Figure 2.5 and Table 3.1) to the 24 well plate. A comparison of neutrophil transwell migration through these two types of inserts is shown in Figure 3.3. One hundred μ l of neutrophil suspension (resuspended in RPMI at a concentration of 10 million cells per mL) was immediately pipetted onto the centre of each transwell insert. The 24 well plate was then placed in a 37°C 5% CO₂ incubator for 90 minutes. Subsequently, the transwell inserts were discarded and the contents of each well were transferred to individual Falcon® round-bottom polystyrene tubes (*Falcon™, UK: 10100151*). Following this, where stated, cells were fixed by adding paraformaldehyde (2% *working concentration*) for 10 minutes at 37°C. Samples were supplemented with 500 μ l 1% bovine serum albumin (BSA) (*Sigma-Aldrich, USA: A7906*) – DPBS, centrifuged at 250 x g for 5 minutes, resuspended in 200 μ l 1% BSA- DPBS and placed on ice. Data acquisition was performed using the MACSQuant® Analyzer 10 (*Miltenyi Biotec, UK*). A 100 μ l sample volume was processed with the fluidics set to high for all samples and FlowJo_v10.9.0 Software was used to analyse the data. Neutrophil singlets were counted in each sample by using gating strategies (SSC-A versus FSC-A, and FSC-A versus FSC-H) described previously, and these represented cells that had migrated through the transwell membrane. The number of cells counted was multiplied by 2 to determine total number of cells within the initial 200 μ L sample.

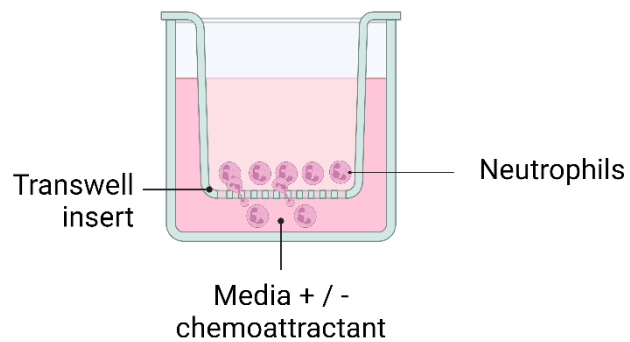


Figure 2.5: Transwell migration assay.

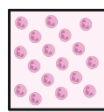
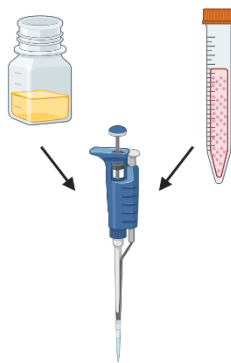
Graphical illustration of the transwell migration assay. Six hundred μl RPMI with or without chemoattractant was present in the bottom well of a 24 well plate. A 3 μm pore sized transwell insert was then placed into the well and 100 μl neutrophil suspension pipetted into the centre of the transwell membrane. The number of neutrophils that migrated through the transwell membrane to the bottom of the well was then counted using a flow cytometer. *Figure created with BioRender.com.*

2.7.2. Insall chamber assay

The Insall chamber assay was performed using a modified Dunn chamber [220]. See Figure 2.6 for a graphical illustration. Neutrophils were resuspended at a concentration of 2 million per mL in RPMI supplemented with 0.01% BSA (7.5% BSA (*Gibco, USA: 15260-037*) added at a 1:666.7 dilution). Four hundred μ L neutrophil suspension was added to a 7.5% BSA coated 22 mm-by-22 mm glass coverslip (*Hawksley, UK: FG-22204*) and incubated for 20 minutes at room temperature. Meanwhile the Insall chamber was cleaned with 5% ChemGene (*Medi-Mark, UK: XTM301*), 70% ethanol (*VWR Chemicals, UK: 20821.310*) and RPMI. RPMI was pipetted into the central well and 'chemoattractant well'. After 20 minutes of incubation at room temperature, the coverslip was inverted onto the Insall chamber. The 'chemoattractant well' was gently emptied using filter paper and refilled with RPMI supplemented with or without a chemoattractant or relative vehicle. The Insall chamber slide was subsequently imaged every 20 seconds for a total of 12 minutes using the Leica DMI6000 B video capture microscope (*Leica Microsystems*) at 20x magnification.

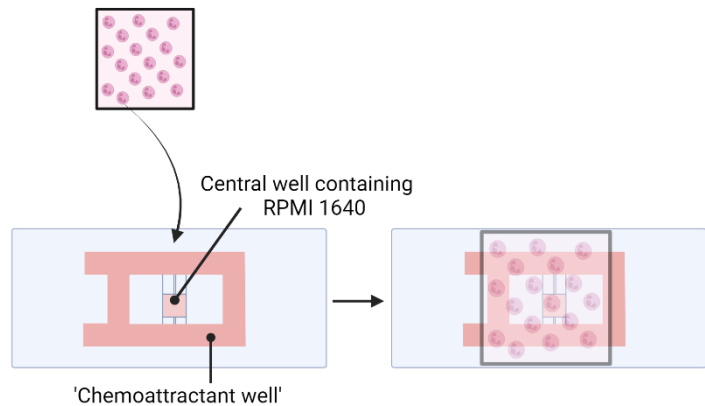
1. Pipette onto glass coverslip:

- a) 7.5% BSA b) Neutrophil suspension



2. Incubate 20 min at room temperature

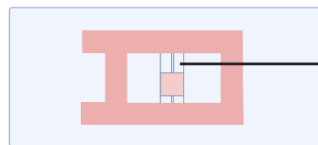
3. Invert glass coverslip onto Insall chamber



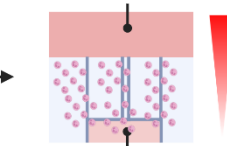
4. Time-lapse microscopy

Image specific area between lines where gradient can be generated.

Image every 20 seconds over 12 minutes.



'Chemoattractant well'



Central well containing RPMI 1640

Chemoattractant gradient

5. Analyse neutrophil movement using FIJI IMAGE J software.

- a) Remove every other image using Slice Remover

- b) Manually track 10 random neutrophils over the 12 minutes and calculate chemotaxis and chemokinesis parameters using Excel template.

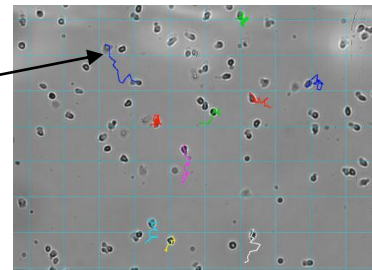


Figure 2.6: Insall Chamber Assay.

Graphical illustration of the Insall chamber assay. **(1)** Neutrophil suspension was added to a 7.5% BSA coated glass coverslip and **(2)** incubated for 20 minutes at room temperature. **(3)** The coverslip was inverted onto the Insall chamber. The 'chemoattractant well' was gently emptied using filter paper and refilled with RPMI supplemented with or without a chemoattractant to create a chemoattractant gradient. **(4)** The Insall chamber slide was subsequently imaged every 20 seconds for 12 minutes using the Leica DMI6000 B video capture microscope at 20x magnification. **(5)** Using FIJI Image J software, every other image was removed using the 'Slice Remover' plug-in and 10 randomly selected neutrophils were tracked on each slide. Chemotaxis and chemokinesis parameters were calculated using an Excel template. Min – minutes. *Figure created with BioRender.com.*

Images were analysed using FIJI Image J (*Version 1.8.0_322 National Institutes of Health, USA*). Every other image was removed using the 'Slice Remover' plug-in and then 10 randomly selected neutrophils were tracked on each slide using the 'Manual Tracking' plug-in. An eleven by nine grid was overlayed onto the images and a random number generator determined the location of neutrophil selection. An example grid is displayed in Figure 2.6. Table 2.4 defines the parameters used to assess neutrophil chemokinesis and chemotaxis as defined by Jasper et al 2022 [221] and Hughes et al 2021 [222]. Only one Insall chamber slide could be imaged at a single time on the microscope. To allow for sequential conditions to be assessed from a single experimental sample efficiently, coverslip coating and subsequent loading and imaging of the Insall Chamber were performed in staggered succession using a prewritten timesheet. Where an experiment was designed to investigate the chemoattractant effects of a molecule, the order of testing was alternated between vehicle control and investigative molecule to minimise any potential confounding effect of time since isolation.

Parameter	Definition	Direction
Speed	The average cumulative distance travelled by 10 neutrophils divided by time. Reported as $\mu\text{m}/\text{minute}$.	Independent
Distance	The average cumulative distance travelled by 10 neutrophils. Reported as μm .	Independent
Displacement	The average distance between the start point and endpoint of 10 neutrophil tracks. Reported as μm .	Independent
Directness	Calculated by the equation: Displacement / cumulative distance is a measure of the average straightness of 10 neutrophil tracks.	Independent
Velocity	The average speed travelled by 10 neutrophils relative to the chemotactic gradient (y-direction) only. Reported as $\mu\text{m}/\text{minute}$.	Dependent
Chemotactic index	A measure of the accuracy of neutrophil movement towards the chemoattractant. Average of 10 neutrophils calculated. Reported on a scale of -1 to 1 where -1 and 1 equate to all movements directly opposite or towards the chemoattractant in all images, respectively.	Dependent

Table 2.4: Definitions of neutrophil chemotaxis and chemokinesis parameters.

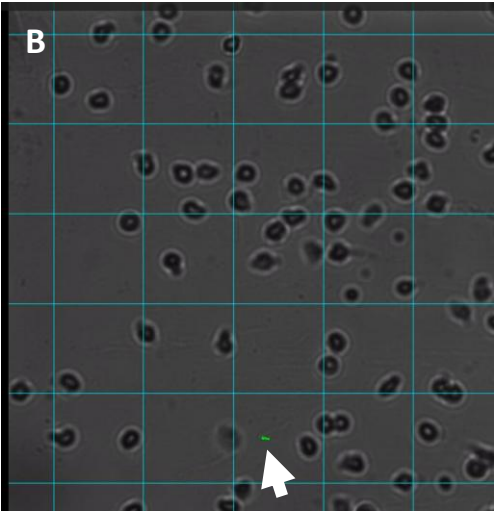
Definitions of neutrophil chemotaxis and chemokinesis parameters reported on analysis of Insall chamber neutrophil tracking data, as defined by Jasper *et al* 2022 [221] and Hughes *et al* 2021 [222]. Time-lapse microscopy of neutrophil movement within the Insall Chamber was performed, where an image was taken every 20 seconds over 12 minutes. Using FIJI Image J and 'manual tracking' plug in software, 10 random neutrophils were tracked and the average speed, distance, displacement, directness, velocity and chemotactic index calculated using Excel (*Microsoft 365*) as described in this table.

2.7.2.1. Adjustment for slide movement

A subtle but sudden background movement in the neutrophil coated slide was noted on analysis of some of the time-lapse microscopy images performed during the Insall chamber assay. Vibration artefact in particular and changes in atmospheric conditions during imaging (such as temperature) were considered as possible causes. Attempts to minimise vibration artefact were included, such as bench damping technology *in situ* and the room door closure mechanism adjusted to prevent slamming. Despite these efforts, it was not possible to stop all events. Therefore, a method to quantify and adjust for background slide movement was determined.

The background movement was calculated using the same methods of manual tracking described previously, but instead of neutrophils, a fixed point identified as a bubble or defect on the slide was tracked (see Figure 2.7A and 2.7B). The change in X- axis and Y- axis movement was then calculated for this non-cell target and subtracted from the corresponding coordinates of each neutrophil tracked on the same image (see Figure 2.7C). The degree of background movement in either the X- or Y- axis was independent of the chemoattractant present in the Insall chamber (p- value > 0.05, One-way Anova (X- axis) or Friedman test (Y- axis) see Figure 2.7D and 2.7E).

A	Coordinates of slide		Slide movement	
	X	Y	X	Y
Image number				
1	386	669		
2	386	669	0	0
3	385	669	-1	0
4	383	669	-2	0
5	383	669	0	0
6	382	668	-1	-1



C	Neutrophil coordinates		Slide movement		Adjusted neutrophil coordinates			
	X	Y	X	Y	Track number	Image number	X	Y
Image number								
1	878	633	0	0	1	1	878	633
2	869	641	0	0	1	2	869	641
3	866	649	-1	0	1	3	867	649
4	869	640	-2	0	1	4	872	640
5	866	644	0	0	1	5	869	644
6	866	647	-1	-1	1	6	870	648

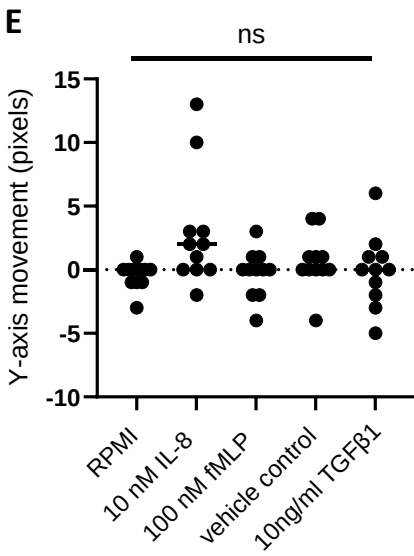
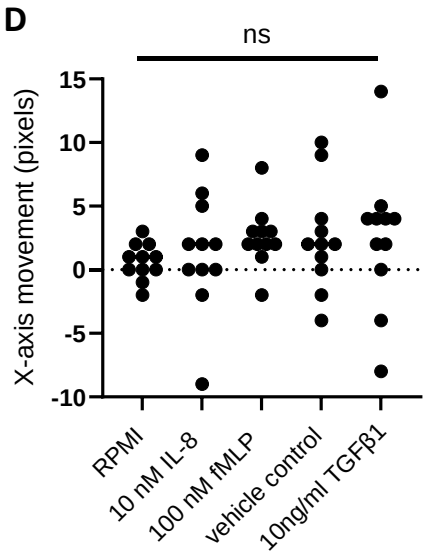


Figure 2.7: Measurement and adjustment of background slide movement in the Insall chamber assay.

Table A and C, and image B are data from the same Insall chamber experiment performed on neutrophils isolated from a control participant from the HYBRID study and exposed to 10 nanograms per millilitre (ng/ mL) transforming growth factor- beta (TGFβ1).

Table A displays the coordinates of a fixed point on an Insall chamber slide that was tracked over successive images. Note that for demonstration purposes, only data collected from the first 6 images are displayed here. The columns in blue display the calculated change in X- axis and Y- axis slide position between each image performed.

Image B displays the manual tracking (green coloured track highlighted by a white arrow) of a fixed point on a slide. This track represents the change in slide position over the 12 minutes that time-lapse microscopy was performed. In this example the slide moved 8 pixels left and 1 pixel up.

Table C displays the coordinates of a single neutrophil tracked on the same slide over the first 6 images and the corresponding updated coordinates following adjustment for slide movement.

Figure D and E display change in (D) X-axis and (E) Y-axis slide position in pixels (1 pixel = 0.46 μm) between the first and final image slice for each chemoattractant (or control) under investigation. Neutrophils were isolated from control participants (n= 11) recruited to the HYBRID study. No statistical difference was noted by chemoattractant condition in X- axis or Y- axis movement (p-values > 0.05). Dot per participant. X- axis data was normally distributed. Y- axis data was skewed. Repeated measures one-way ANOVA and Friedman test were performed to assess statistical significance for X-axis and Y-axis data, respectively.

fMLP - N-formylmethionine-leucyl-phenylalanine, HYBRID – Investigating the Hypoxic Burden in Interstitial Lung Disease, IL-8 – interleukin-8, TGFβ1 - transforming growth factor- β1.

The effect of this background adjustment on neutrophil chemotaxis and chemokinesis results was then assessed. Figure 2.8 displays the Insall chamber assay findings of control neutrophils (from the HYBRID study) exposed to vehicle or 10 nanograms per millilitre (ng/ mL) TGF β 1, pre or post adjustment for background movement. Adjustment for slide movement had minimal effect on the average Insall chamber assay results, except for directness. For both data sets 10 ng/ mL TGF β 1 significantly increased the speed and distance travelled of neutrophils compared to vehicle control (p- value < 0.05, paired T-tests), and neutrophil velocity and chemotactic index were unaffected (p- value > 0.05, paired T-tests). Slide adjustment reduced the variability of cellular data, as seen by the standard deviations of the direction dependent parameters shown in Figure 2.8 Table A and the distribution of data plots and standard deviations in Figure 2.8B. Using this approach enabled small changes to be identified with confidence for instance, the minor effect of TGF β 1 on displacement was only statistically confirmed with slide adjustment as shown in Figure 2.8 Table A (p = 0.048). Statistical comparisons in the measure of directness between vehicle and TGF β 1 exposure did change upon slide adjustment (p-value no longer significant). On review of the data values in Figure 2.8 Table A, this likely reflects the reduction in displacement values (artefact removed) following slide adjustment, thereby giving a more genuine reflection of events.

The following conclusions were drawn from this validation analysis, the background slide movement was a random event and had little general impact on the Insall chamber assay results. However, accounting for this potential effect by correcting for non-cell related artefact improved data precision and hence adjustment for slide movement was undertaken for ongoing studies unless otherwise stated.

A	Not adjusted for slide movement			Adjusted for slide movement		
	Vehicle control (mean, SD)	10 ng/ mL TGFβ1 (mean, SD)	p-value	Vehicle control (mean, SD)	10 ng/ mL TGFβ1 (mean, SD)	p-value
Speed (μm/ min)	2.659 (1.358)	3.888 (1.876)	0.020	2.707 (1.342)	3.932 (1.850)	0.020
Distance travelled (μm)	30.140 (15.390)	44.070 (21.260)	0.020	30.680 (15.210)	44.560 (20.960)	0.020
Displacement (μm)	9.547 (4.725)	13.350 (7.732)	0.085	8.503 (5.442)	12.950 (7.770)	0.048
Velocity (μm/ min)	0.133 (0.393)	0.141 (0.343)	0.957	0.169 (0.343)	0.119 (0.301)	0.749
Chemotactic index (Units)	0.022 (0.221)	0.032 (0.104)	0.895	0.060 (0.118)	-0.001 (0.083)	0.257
Directness	0.383 (0.095)	0.310 (0.070)	0.030	0.276 (0.066)	0.270 (0.047)	0.806

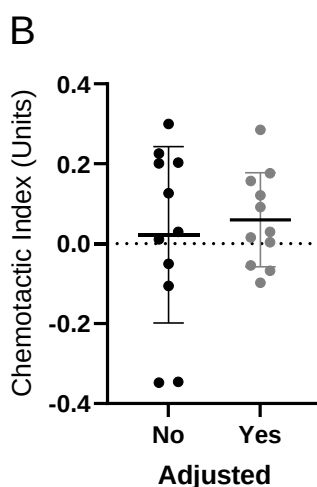


Figure 2.8: Investigating the effect of slide movement on Insall chamber assay results.

Movement towards 10 ng/ mL TGFβ1 or vehicle control was assessed over 12 minutes using time-lapse microscopy within the Insall chamber of neutrophils isolated from control participants (n = 11) of the HYBRID research study.

Table A displays the movement parameters measured with and without adjustment for background slide movement. Data was normally distributed, paired T-tests were performed. Figure B is a dot plot (dot per participant) displaying the unadjusted and adjusted chemotactic index data of neutrophils in response to vehicle control, respectively. Bars are mean and standard deviation. This figure shows that the standard deviation is smaller following adjustment for slide movement.

2.7.3. Chemoattractants (established or under investigation)

Recombinant human IL-8 (*R&D Systems, UK: 208-IL/CF*), fMLP (*Sigma, UK: F3506*) or recombinant human TGFβ1 (*R&D Systems, UK: 240-B/CF*) were added directly to RPMI at the relevant concentration. Recombinant human TGFβ1 was reconstituted in filter sterilised 0.1% BSA containing DPBS supplemented with 4mM hydrochloric acid (Fisher Chemical™, Canada: A508-P500). This solvent is described as the vehicle control for experiments that include TGFβ1 treatment. Table 2.5 summarises the chemoattractants investigated in this thesis.

Chemoattractant under investigation	Vehicle solution	Stock concentration	Working Concentration
fMLP (<i>Sigma, UK: F3506</i>)	RPMI 1640	7617 µM	10 nM
Recombinant human IL-8 (<i>R&D Systems, UK: 208-IL/CF</i>)	Sterile PBS – 0.1% BSA	6.25 µM	78.1 pM – 100 nM
Recombinant human TGFβ1 (<i>R&D Systems, UK: 240-B/CF</i>)	0.1% BSA–DPBS supplemented with 4 mM HCl	10 or 20 µg/ml [§]	1 pg/mL – 10 ng/mL [§]

Table 2.5: Summary of chemoattractants (established or under investigation).

Two established chemoattractants, N-formylmethionine-leucyl-phenylalanine (fMLP) and interleukin-8 (IL-8) were used to assess chemotactic responses by human neutrophils. The chemotactic effect of transforming growth factor- β1 (TGFβ1) on neutrophils was also investigated. The vehicle solution used to reconstitute fMLP, IL-8 and TGFβ1, and stock and working concentrations are described. [§]TGFβ1 concentrations are described using grams per mL (rather than molarity) as this is most recognised/reported in the literature. HCL – hydrochloric acid, µM – micromolar, nM – nanomolar, pM – picomolar, pg/ mL – picograms per millilitre, µg/ ml – micrograms per millilitre.

2.8. Measuring neutrophil extracellular trap release *in vitro*.

2.8.1. Measuring cell free DNA release using a fluorescence plate reader.

Two hundred µl of neutrophil suspension (at a concentration of 1 million per mL in RPMI) was pipetted into individual wells of a flat bottom 96 well cell culture plate (*Corning Inc., USA: 3596*), ensuring three technical replicates per condition. Neutrophils were treated with proteins/cytokines (see Table 2.6) and incubated for the desired time at 37°C and 5% CO₂. Following this, where stated, cells were fixed by adding paraformaldehyde (*2% working concentration*) for 10 minutes at 37° C. The neutrophil suspension was subsequently transferred into individual 500 µl Eppendorfs and centrifuged at 2200 x g for 10 minutes at 4° C. The top 100 µl supernatant was pipetted into a flat black bottom 96 well assay plate (*Corning Inc., USA: 3917*) and 10 µL SYTOX™ Green (*1µM working concentration, Invitrogen, UK: S7020*) added. Cells were then incubated for 10 minutes in the dark. Fluorescence was measured using the BioTek Synergy 2 Fluorometric plate reader; excitation 485nm/emission 528nm. The mean background fluorescence (namely the relative fluorescence unit (RFU) of wells not containing cells) was subtracted from each RFU of experimental wells containing neutrophils. Results are presented as mean RFU of technical replicates or mean RFU of treated wells minus mean RFU of control (RPMI with or without vehicle) wells.

Treatment	Vehicle	Stock concentration	Working concentration
IL-1 β (<i>R&D Systems, UK: 201-LB/CF</i>)	Sterile DPBS	5.9 μ M	588.2 pM – 5.9 nM
Recombinant human IL-8 (<i>R&D Systems, UK: 208-IL/CF</i>)	0.2 μ m-filtered DPBS – 0.1% BSA	6.25 μ M	0.5 - 50 nM
PMA (<i>Cayman Chemical, USA: 10008014</i>)	Dimethyl sulfoxide (DMSO) (<i>Sigma-Aldrich, UK: D2650</i>)	1.62 mM	25 nM
Recombinant human TGF β 1 (<i>R&D Systems, UK: 240-B/CF</i>)	0.2 μ m-filtered 0.1% BSA-DPBS supplemented with 4mM HCl	10 - 20 μ g/ ml ^{\$}	1 pg/ mL – 10 ng/mL ^{\$}
Tumour necrosis factor- α (TNF α) (<i>Sigma-Aldrich, UK: T6674</i>)	0.2 μ m- filtered distilled water	5.7 μ M	574.7 pM

Table 2.6: Protein and cytokine treatments used to assess neutrophil extracellular trap and reactive oxygen species release.

The protein and cytokine treatments that were used to investigate neutrophil extracellular trap and reactive oxygen species release are described, including vehicle solutions used for reconstitution, stock and working concentrations. ^{\$}TGF β 1 concentrations are described using grams per mL (rather than molarity) as this is most recognised/reported in the literature. HCL – hydrochloric acid, IL-1 β – interleukin-1 beta, PMA – phorbol 12-myristate 13-acetate.

A DNA standard curve was performed to demonstrate that this fluorometric method can successfully measure DNA by adding a serial dilution (1: 2) of lambda DNA (*ThermoScientific, UK: SD0011*) using DPBS and SYTOXTM Green (*1 μ M working concentration*) to a flat black bottom 96 well assay plate (see Figure 2.9). Fluorescence was measured using the BioTek Synergy 2 Fluorometric plate reader; excitation 485nm/emission 528nm. A line of best fit was calculated using GraphPad Prism (*Version 9.5.0, San Diego, USA*) and interpolated DNA concentrations calculated following inputting raw sample RFU values.

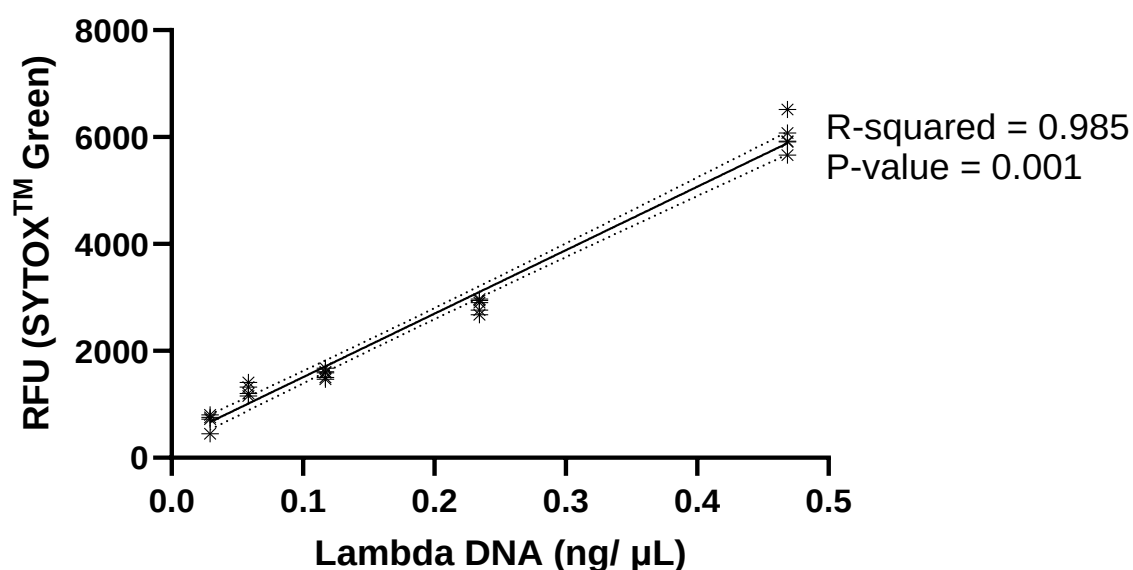


Figure 2.9: Example of a deoxyribonucleic acid standard curve.

A serial dilution (1: 2 from 0.469 ng/ μ l to 0.029 ng/ μ l using DPBS) of lambda DNA (*ThermoScientific, UK: SD0011*) was added to a 96 well plate and treated with SYTOX™ Green. Individual relative fluorescence units (RFU) from 5 experiments are presented. A line of best fit (solid line, R squared 0.985 and $p = 0.001$) with 95% confidence interval (dotted line) is displayed. Using this standard curve, 2571 RFU (neutrophils isolated from a patient with IPF exposed to 25 nM PMA for four hours) results in an interpolated DNA concentration of 0.189 ng/ μ L.

2.8.2. Visualising nucleic acid release using fluorescence microscopy.

2.8.2.1. Immunofluorescence staining protocol.

Two hundred μl of neutrophil suspension (at a concentration of 1 million per mL in RPMI) and 200 μl RPMI were pipetted onto a 22 mm-by-22 mm glass coverslip (*Hawksley, UK: FG-22204*) located within a 6 well plate. Cells were incubated at 37°C, 5% CO₂ for 20 minutes to allow cell adherence. A further 1600 μl RPMI with or without stimulant was added (see Table 2.6). Neutrophils were incubated for the desired time at 37°C, 5% CO₂ and subsequently fixed by adding paraformaldehyde (4% *working concentration*) for 30 minutes at 37°C. The neutrophil coated cover slips were washed three times with DPBS, permeabilised with Triton X-100 (0.1% *working concentration, Sigma Life Science, USA: T8787*) for 1 minute and washed again with DPBS. Neutrophils were stained for 10 minutes with 1 μM SYTOX™ Green and washed with DPBS. The neutrophil coated cover slips were mounted using Fluomount-G™ mounting medium (*Invitrogen, USA: 15586276*) onto Superfrost™ microscope slides (*Epredia, USA: AG00008232E01MNZ10*). Cells were visualised using the Leica DMI6000 B microscope at 20x magnification. Images were taken at random using bright field microscopy and fluorescence microscopy. Images were opened using the 'Bio-Formats Importer' plug-in on FIJI Image J (*Version 1.8.0_322 National Institutes of Health, USA*). FIJI Image J displays single fluorophore images as default in grey scale, whereby the brighter the colour, the brighter the fluorescence. A lookup table (LUT) is where a colour map can be applied to represent the degree of fluorescence. The default grey images are here described as having a greys LUT. 3-3-2 red-green-blue (RGB) is an alternative LUT where the colours, red, green and blue, represent the level of fluorescence in descending order. This 3-3-2 RGB LUT enables the lower fluorescent signals (blue) to be seen more clearly to the human eye. An example of this is displayed in Figure 2.10.

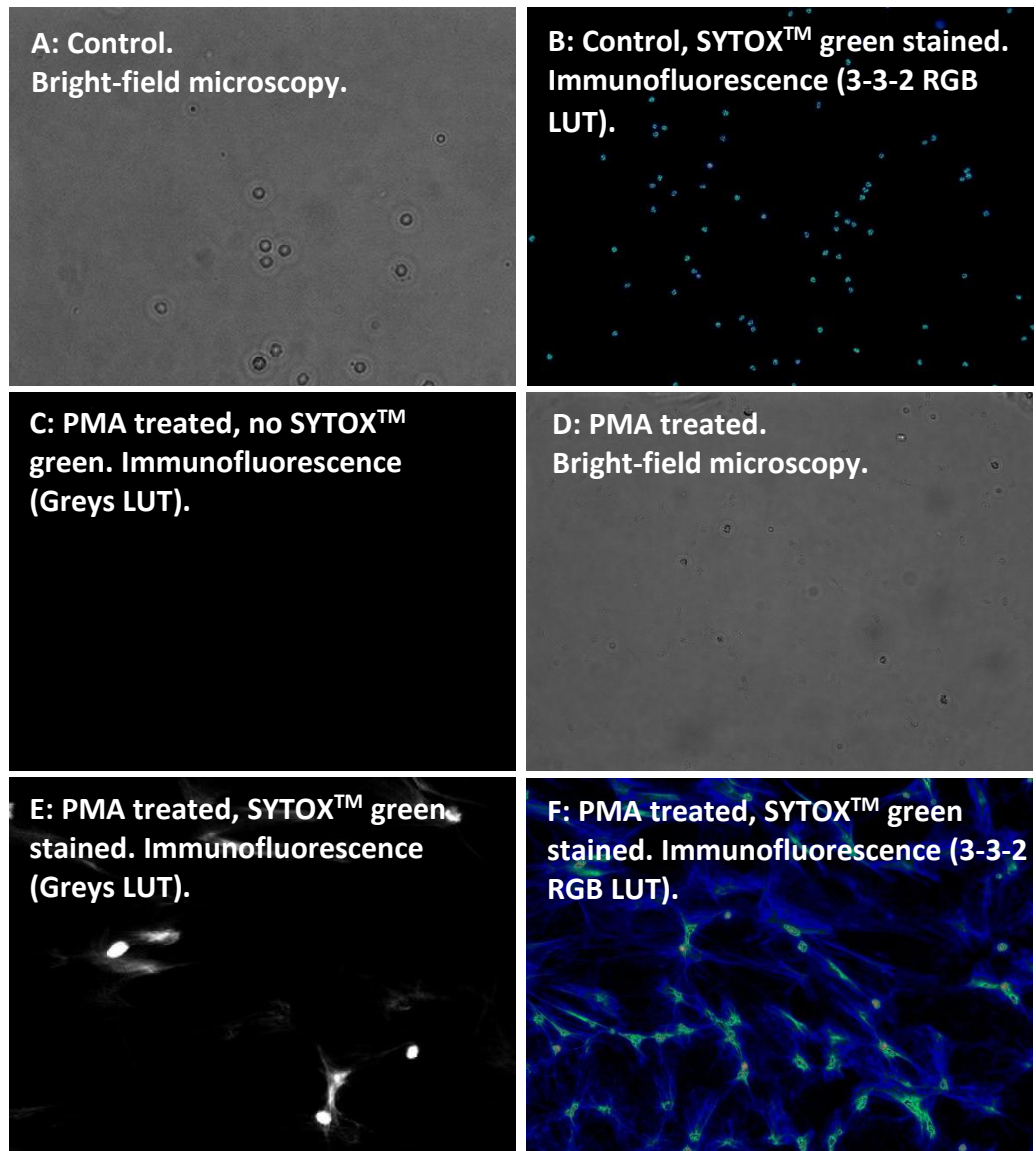


Figure 2.10: Representative brightfield and immunofluorescence images displaying nucleic acid release by neutrophils.

Peripheral neutrophils were isolated from one healthy control. Neutrophils were treated with or without 25 nM PMA for four hours, fixed in 4% paraformaldehyde, permeabilised and stained with SYTOX™ green. Images were captured using a Leica DMI6000 B microscope, 20x magnification. (A) Bright-field image of untreated neutrophils (control). (B) Immunofluorescence image (3-3-2 RGB LUT applied) of untreated neutrophils (control) stained with SYTOX™ green. Fluorescence is noted in the nuclei of cells. (C) Immunofluorescence image of PMA-treated neutrophils not stained with SYTOX™ green. Note that no fluorescence is visible. (D) Bright-field image of PMA treated neutrophils. (E) and (F) Immunofluorescence images of PMA-treated neutrophils stained with SYTOX™ green. (E) and (F) are the same image displayed using greys LUT and 3-3-2 RGB LUT, respectively.

2.8.2.2. Semi-quantitative analysis of SYTOX™ Green staining

Semi-quantitative analysis of NET formation was performed on the immunofluorescence images using Image J (*Version 1.8.0_345 National Institutes of Health, USA*). Images were opened using the 'Bio-Formats Importer' plug-in. Two codes were created and applied using the 'Macro' plug-in to first count the number of nuclei on each image to determine cell number (Figure 2.11A), and second to determine the total area of nucleic acid staining on each image as a surrogate marker of NET area (Figure 2.11B). For both analyses, contrast was automatically enhanced and a Gaussian Filter (sigma value = 3.5) applied. An 'Otsu threshold' (8, 255) was then set to identify the focal areas of nucleic acid staining in keeping with nuclei and a Watershed Transformation to separate touching spots. The number of nuclei was determined by using the 'Analyze particles' function, which counted any spot with an area of ≥ 3 micron². To determine the total area of NET formation, a 'Percentile threshold' (1, 255) was applied instead, the image was converted to RGB colour and the total area of nucleic acid staining was calculated using the measure function. See Figure 2.12 for representative images of these thresholds applied.

The following calculation was then performed to estimate the area of NET formation per cell on each image:

Total area of SYTOX™ Green stained nucleic acid per slide

divided by number of cells per slide.

The average area of NETs per cell of all images taken of each slide was then calculated.

(A) Code for counting nuclei

```
File Edit Font Examples Macros Debug
Run Install Macro
/// counting nuclei
run("Duplicate...", "");
//run("Brightness/Contrast...");
run("Enhance Contrast", "saturated=0.35");
run("Close");
run("Gaussian Blur...", "sigma=3.5");
setAutoThreshold("Default dark no-reset");
//run("Threshold...");
setAutoThreshold("Otsu dark no-reset");
//setThreshold(88, 255);
setOption("BlackBackground", true);
run("Convert to Mask");
run("Watershed");

run("Analyze Particles...", "size=3-Infinity summarize");
```

(B) Code for measuring NET area.

```
File Edit Font Examples Macros Debug
Run Install Macro
/// Measuring NET area
run("Bio-Formats Importer", "open=C:/Users/spencekl/Desktop/LC_practisenets.
//run("Brightness/Contrast...");
run("Enhance Contrast", "saturated=0.35");

run("Gaussian Blur...", "sigma=3.5");
setAutoThreshold("Default dark no-reset");
//run("Threshold...");
setAutoThreshold("Percentile dark no-reset");
//setThreshold(1, 255);
setOption("BlackBackground", true);
run("Convert to Mask");
run("RGB Color");
run("Color Threshold...");
//then manually press select
// then manually press measure and save image and data
```

Figure 2.11: Codes utilised to semi-quantify NET formation on immunofluorescence images.

Snapshots of 'Macro' codes used to (A) count the number of nuclei and (B) measure the area of SYTOXTM green stained nucleic acid in each image. These codes were created using the 'Macro' plug-in on Image J (*Version 1.8.0_345 National Institutes of Health, USA*).

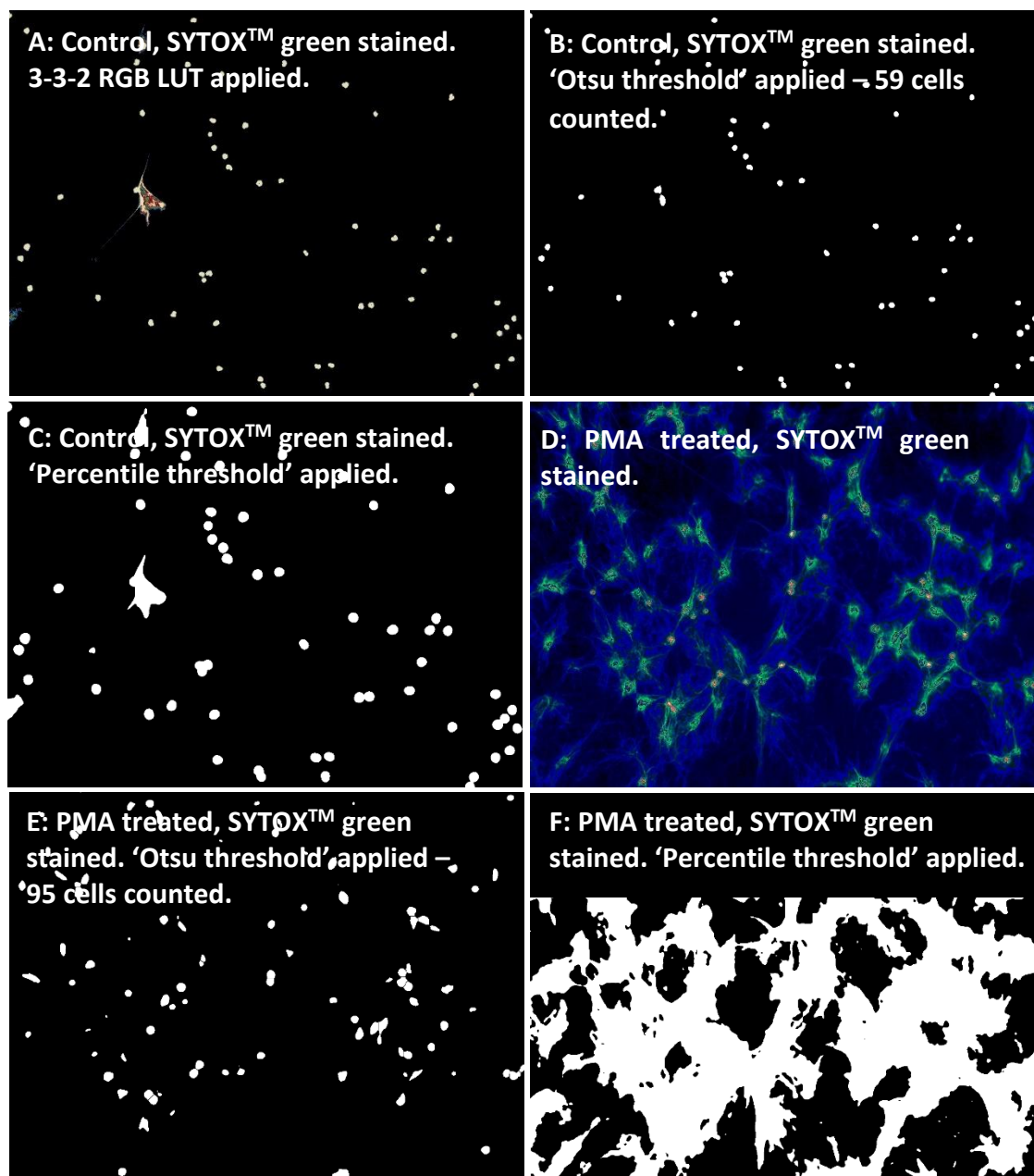


Figure 2.12: Representative immunofluorescence images with analysis thresholds applied.

Peripheral neutrophils were isolated from one healthy control. Neutrophils were treated with or without 25 nM PMA for four hours, fixed in 4% paraformaldehyde, permeabilised and stained with SYTOX™ green. Images were captured using a Leica DMI6000 B microscope, 20x magnification. Immunofluorescence images of untreated neutrophils (control) stained with SYTOX™ green with (A) 3-3-2 RGB LUT applied (no threshold) or (B) an 'Otsu threshold' applied to determine cell count or (C) a 'Percentile threshold' applied to measure area of nucleic release. Immunofluorescence images of PMA- treated neutrophils stained with SYTOX™ green with (D) 3-3-2 RGB LUT applied (no threshold) or (E) an 'Otsu threshold' applied to determine cell count or (F) a 'Percentile threshold' applied to measure area of nucleic acid release.

2.9. Reactive oxygen species

2.9.1. Reactive oxygen species detection using CellROX™

CellROX™ Reagents are fluorogenic probes that fluoresce upon oxidation by ROS and this fluorescence is maintained upon cell fixation. CellROX™ Deep Red Reagent (*ThermoFisher Scientific, Oregon, USA: C10422, excitation/emission: 644/655 nm*) is permeable to the cell membrane and thereby reflects cytoplasmic ROS. Neutrophils were resuspended in RPMI at a concentration of 1 million per mL. Neutrophils were incubated with 5 μ M CellROX™ Deep Red Reagent for 30 minutes at 37°C. Neutrophils were subsequently fixed in paraformaldehyde (2% *working concentration*) for 10 minutes at 37°C. Neutrophils were washed twice by adding 500 μ L ice cold sterile DPBS and centrifugation at 250 x g for 5 minutes at 4 °C. Neutrophils were finally resuspended in 200 μ L ice cold DPBS and stored on ice in the dark. Samples were analysed on the MACSQuant® Analyzer 10 (*Miltenyi Biotec, UK*) with fluidics set to medium. Voltages on the flow cytometer were maintained for comparable experiments. APC median fluorescence intensity (MFI) of CellROX™ Deep Red stained neutrophils were determined of singlet cells within the neutrophil gated population using FlowJo_v10.9.0 Software. Any samples where the number of neutrophils analysed on the flow cytometer was less than 2500 were excluded from analyses. See Figure 2.13 for example histograms.

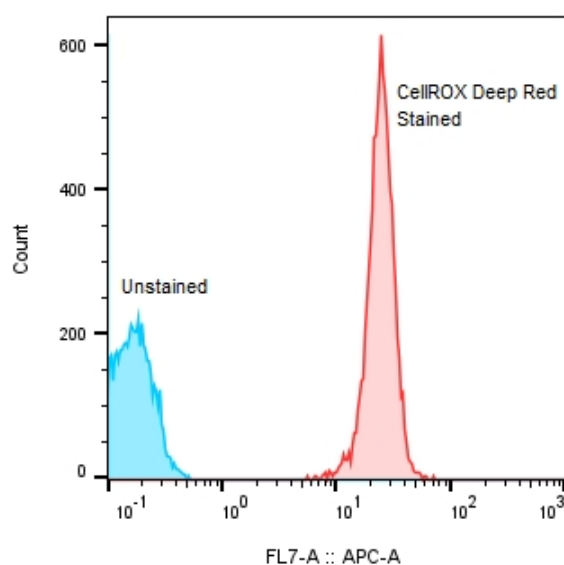


Figure 2.13: Example histograms displaying CellROX™ deep red staining.

Peripheral neutrophils isolated from one control participant were stained with CellROX™ Deep Red for 30 minutes and fixed with paraformaldehyde. Neutrophils were analysed on the MACSQuant® Analyzer 10 (*Miltenyi Biotec*). APC median fluorescence intensity (MFI) of CellROX™ Deep Red stained neutrophils were recorded of neutrophil singlets. Histograms are displayed of CellROX™ Deep Red stained (red) and unstained neutrophil singlets (blue), APC-A on X-axis and cell count on y-axis.

2.9.2. Lucigenin-amplified chemiluminescence

Lucigenin-amplified chemiluminescence is a method of measuring contemporaneous extracellular superoxide anion release by neutrophils [223]. Neutrophils were resuspended in Hank's Balanced Salt Solution (HBSS) (*GibcoTM*, UK: 15266355) containing magnesium chloride and calcium chloride at a concentration of 1 million per mL. One hundred μ l neutrophil suspension, 50 μ l HBSS and 25 μ l lucigenin (*N,N'*-Dimethyl-9,9'-biacridinium dinitrate, 200 μ M working concentration, *Sigma-Aldrich: M8010*, USA) were pipetted into wells of a pre-coated 2% BSA-DPBS white bottomed 96 well plate (*Corning[®]*, USA: 3917). A further 25 μ l HBSS was added to control wells. Treatments (25 μ l volume, described in Table 2.6) were added to relevant wells via automated injection. ROS generation was then assessed at 1-minute intervals for 200 minutes using a Berthold Centro LB 960 luminometer (*Berthold Technologies, UK*). Area under the curve (AUC) and peak chemiluminescence intensity were calculated using area under the curve analysis on GraphPad Prism (*Version 9.5.0*, San Diego, USA) (see Figure 2.14).

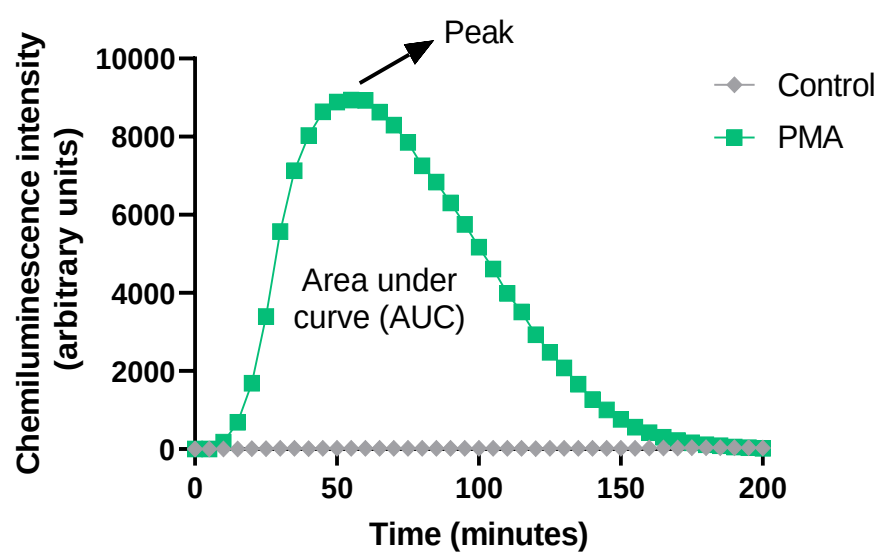


Figure 2.14: Example histogram of lucigenin-amplified chemiluminescence assay.

The lucigenin-amplified chemiluminescence intensity of peripheral neutrophils isolated from one healthy control that were unstimulated (control – grey diamonds) or stimulated with 25 nM PMA (green squares) was measured over 200 minutes. This figure highlights the location of the area under the curve (displayed underneath the green line on the figure) and peak chemiluminescence intensity that were calculated for each experiment.

2.10. Immunophenotyping panel

2.10.1 Antibody panels and staining

Three separate antibody mixtures were devised to assess neutrophil cell surface protein expression. Panel 1 included antibodies against markers that commonly change on activation or priming namely, CD66b, CD11b and CD62L. Panel 2 contained solely an anti-CXCR2 antibody. Panel 3 included antibodies against: CD16, CD10, CD54, CXCR4, programmed death-ligand 1 (PD-L1) and CD11c. Further detail regarding the selection of these targets are described in Chapter 3. For each experiment to assist analysis of non-specific binding, neutrophils were stained with an isotype control mixture for each panel containing antibodies of the same immunoglobulin class/subclass and conjugated to the identical fluorophore.

Neutrophils were resuspended in 1% BSA-DPBS at a concentration of 1 million per mL and incubated with the relevant antibodies manufactured by BioLegend® (UK) (see Table 2.7) for 30 minutes on ice in the dark. Neutrophils were then washed twice by adding 500 µL ice cold 1% BSA-DPBS and centrifugation at 250 x g for 5 minutes at 4°C. Neutrophils were finally resuspended in 200 µl ice cold 1% BSA-DPBS and stored on ice in the dark ready for data acquisition.

Target	Conjugated fluorophore	Cat. no	Volume (μL)	Isotype	Isotype control Cat. no
Panel 1					
CD66b	APC	305118	1	Mouse IgM, κ	401616
CD11b	BV786	301346	2.5	Mouse IgG1, κ	400170
CD62L	BV605	304834	1	Mouse IgG1, κ	400102
Panel 2					
CXCR2	PE	320706	2.5	Mouse IgG1, κ	400112
Panel 3					
CD16	PerCP	302030	1	Mouse IgG1, κ	400148
CD10	BV510	312220	5	Mouse IgG1, κ	400172
PD-L1	BV605	329724	1	Mouse IgG2b, κ	400350
CXCR4	PE Cy7	306514	1	Mouse IgG2a, κ	400232
CD11c	FITC	371516	2.5	Mouse IgG2b, κ	400310
CD54	AF700	353126	1	Mouse IgG1, κ	400144

Table 2.7: Immunophenotyping panels.

Antibodies and matching isotype controls used within the three immunophenotyping panels are described. The volume of each antibody added to 100 μL neutrophil suspension are outlined. All antibodies were supplied by BioLegend® (UK). Cat. – catalogue, Ig – immunoglobulin, no – number.

2.10.2 Data analysis

Prior to data acquisition, compensation analysis was performed using panel 1 and panel 3 antibody mixtures at regular intervals using BD™ anti-Mouse Ig, κ and negative control beads (*BD Biosciences, UK: 552843*) on the BD LSR Fortessa™ X-20 cell analyser. This was performed as per the BD LSRFortessa™ X-20 Cell Analyzer User's Guide [224]. Neutrophils were analysed on the BD LSR Fortessa™ X-20 cell analyser with the fluidics set to medium or high.

FlowJo_v10.9.0 Software was used to analyse the data. Neutrophil singlets were identified as described previously by gating relevant cells within FSC-A versus SSC-A, and FSC-A versus FSC-H scatter plots. Scatter plots displaying spread of fluorescence intensity of each target against SSC-A were created. Data is presented as percentage of cells that are positive for each surface marker and MFI. A positive gate for each cell surface marker was determined using the isotype control sample, whereby a gate was placed to the right of the main neutrophil population (typically only 2-3% of the isotype control-stained neutrophil population would be within this 'positive' gate) (see Figure 2.15). Note an additional step was performed to analysis CD10 expression in the HYBRID study samples, which is described in Chapter 6.

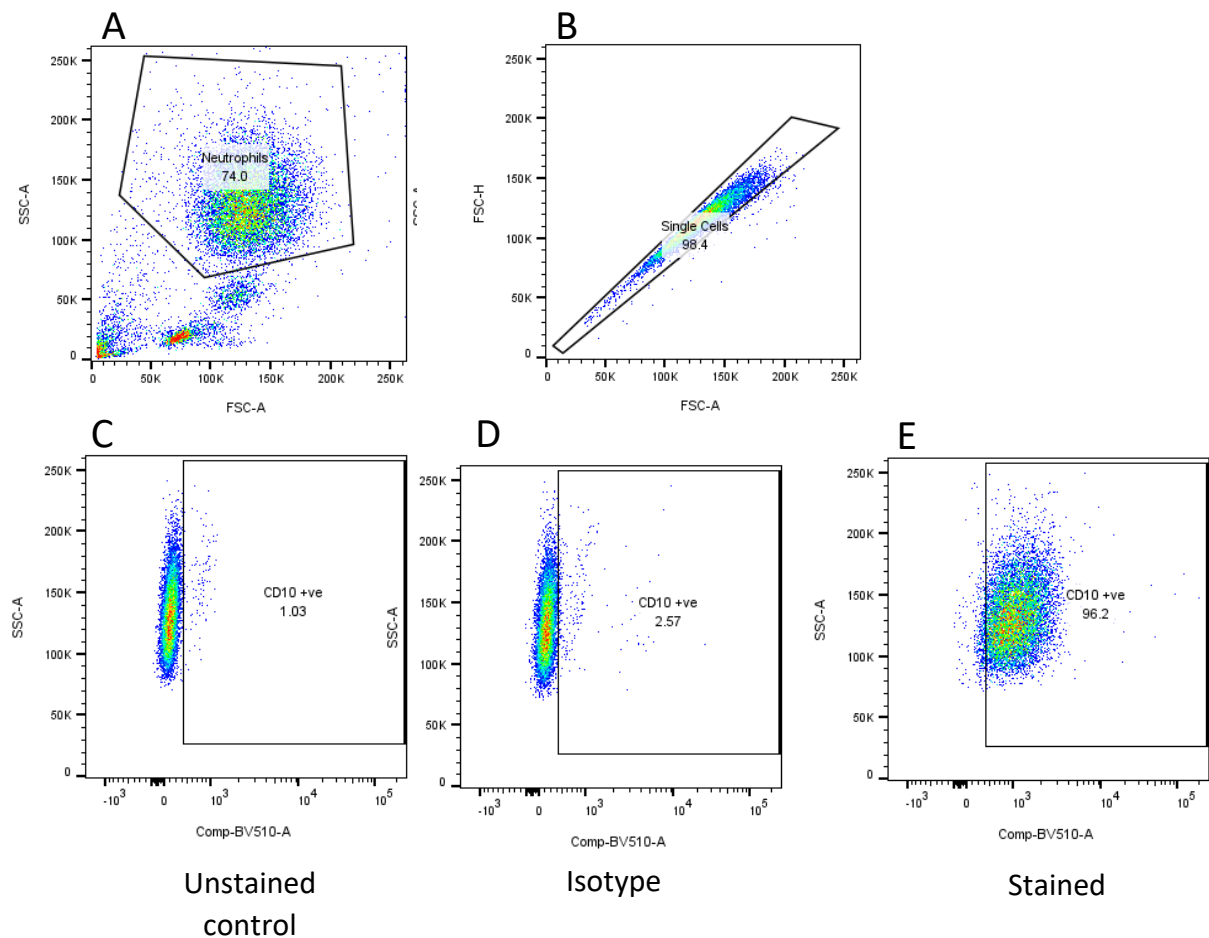


Figure 2.15: Gating strategy used in immunophenotyping data analysis.

This figure displays example immunophenotyping scatter plots and gates created using FlowJo_v10.9.0 Software. Neutrophils used from this experiment were isolated from a control participant and either not stained, stained with the isotype control mixture or panel 3 antibody mixture.

(A) The neutrophil population was first selected by creating a SSC-A versus FSC-A scatter plot. (B) Single cells were then identified by plotting FSC-H versus FSC-A. Fluorescence intensity of compensated BV510 are plotted against SSC-A in (C) to (E). A gate outlining cells that are positive for CD10 (described as CD10 +ve) was determined using the isotype control sample (D). This location of this gate is also seen on the unstained control (C) and antibody-stained sample (E: 96.2% positive).

2.11. Western blot

2.11.1. Protein lysate collection

Isolated neutrophils were centrifuged at 1000 x g for 10 minutes at 4 °C and resuspended in 200 µL RIPA buffer (*Sigma-Aldrich: R0278*) supplemented with the phosphatase and proteinase inhibitors: PhosSTOP (*working concentration 0.63X, Roche, Germany: 4906845001*), cOmplete™ Protease Inhibitor Cocktail (*working concentration 1.56X, Roche, Germany: 04693116001*), phenylmethylsulfonyl fluoride, sodium fluoride, β-glycerophosphate and sodium orthovanadate. This neutrophil lysate solution was then vortexed, placed on ice for 10 minutes and centrifuged at 10,000 x g for 2 minutes at 4 °C. Following centrifugation, the supernatant (protein lysate) was collected and stored at -80°C until analysed.

2.11.2. Gel electrophoresis and protein transfer.

Separating and stacking gels were prepared (see Table 2.8) and placed in the electrophoresis tank (*AE-6500 Dual Mini Slab kit; Atto Corporation, Japan*) containing running buffer (1: 10 dilution of 500mL distilled water supplemented with Trizma base 15 g (*Sigma Life Science, USA: T1503*), 72 g glycine (*Sigma Life Science, USA: G8898*) and 5 g SDS.

	10% Separating Gel	Stacking Gel
Acrylamide (<i>Sigma- Aldrich, USA: A3699</i>)	5 mL	1 mL
Distilled water	5.84 mL	4.5 mL
Separating buffer (1.5M Trizma base, pH 8.8)	3.76 mL	-
Stacking buffer (0.5M Trizma base, pH 6.8)	-	1.9 mL
10% sodium dodecyl sulfate (<i>Sigma Life Science: 05030</i>)	200 μ L	100 μ L
10% Ammonium persulfate (<i>Thermo Scientific, USA: 17874</i>)	200 μ L	200 μ L
N, N, N', N' - Tetramethylethylenediamine (TEMED) (<i>Sigma- Aldrich, USA: T9281</i>)	20 μ L	20 μ L

Table 2.8: Constituents of separating and stacking gels.

Constituents required to make two 10% separating gels and two stacking gels are described. The 10% separating gel was added first to two glass plates held together with a gasket and clips. The stacking gel was then layered on top of the set separating gel and a comb inserted. Once the stacking gel was set, loading wells were created by carefully removing the comb.

Each protein lysate sample was supplemented with SDS sample treatment buffer (containing 0.05 M Trizma base, glycerol (*Sigma Life Science, USA: 49781*), 2% SDS (*Sigma Life Science, USA: 05030-500ML-F*) and 10% beta- mercaptoethanol (*Sigma, Germany: M3148*)) and then boiled for 5 minutes on a 100°C heat block. A colour prestained protein standard, broad range (10-250 kilodalton (kDa)) (10 μ L, *New England Biolabs, UK: P7719S*) and protein lysate samples were loaded to the stacking gel. Equal concentration of neutrophil protein lysate was loaded for each condition for comparable experiments. For example, 30 μ L of protein lysate created from 5 million neutrophils lysed in 200 μ L RIPA buffer. An electric current was subsequently applied across the gels to drive protein separation.

Protein transfer from gel to nitrocellulose membrane (*Cytiva, Germany: 15219804*) was performed using a semi-dry transfer blotting system (either WSE-4115; *Atto Corporation, Japan* or SD10; *Cleaver Scientific Limited, UK*). A sandwich was assembled of gels, nitrocellulose

membranes and filter paper in the transfer tank (see Figure 2.16). Stacking buffer (containing 800 mL distilled water and 200 mL methanol (*VWR Chemicals, France: 20847.307*) supplemented with 3 g Trizma, 14.4 g glycine and 1 mL 10% SDS was generously added and the lid of the transfer tank closed. An electric current was applied to the tank at 25 volts for 55 min.

2.11.3. Membrane washing, blocking and antibody staining

The membrane was removed from the transfer tank and stained with Ponceau S Solution (*Sigma Life Science, USA: P7170-1L*) to visualise the protein bands (see Figure 2.17). The membrane was then washed in tris-buffered saline (*ITW Reagents, Germany: A1087*) supplemented with 3% Tween® 20 (*MP Biomedicals, UK: TWEEN201*) detergent (TBST) and incubated for 1 hour at room temperature in 5% BSA-TBST to block non-specific binding. The membrane was then probed with the primary antibody made up in 5% BSA-TBST for the required time (specified in Table 2.9). The membrane was subsequently washed three times with TBST at room temperature and incubated for 1 hour at room temperature with the secondary antibody (specified in Table 2.9) made up in TBST. The membrane was then washed three times with TBST at room temperature ready for visualisation using enhanced chemiluminescence.

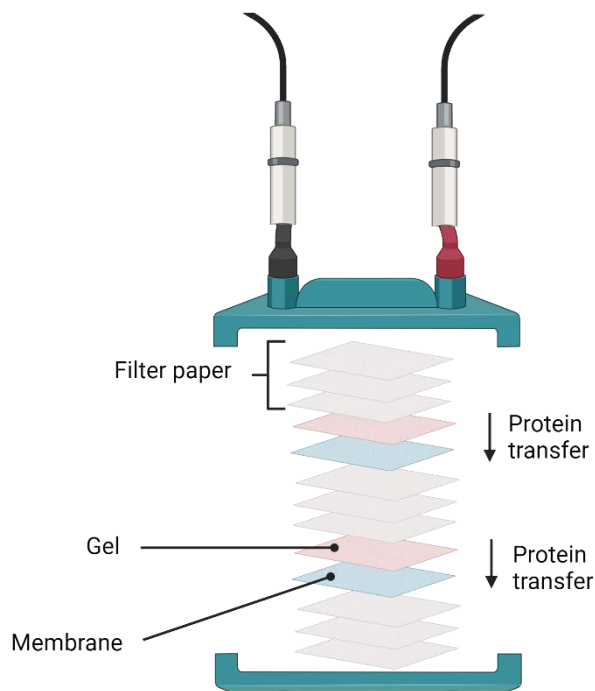


Figure 2.16: Assembly of a 'sandwich' used in protein transfer.

This is a graphical illustration of filter paper, gel and membrane layers in a sandwich-like formation that were set up within the semi-dry transfer tank. These layers were soaked in transfer buffer and an electrical current applied to ensure effective protein transfer from gel to membrane. *Figure created with BioRender.com.*

Target	Molecular weight (kDa)	Primary Antibody		Secondary Antibody	
		Antibody, host species	Concentration, time, temperature.	Antibody	Concentration
p-p38 MAPK	43	9211S (<i>Cell Signaling Technology</i>), Rabbit	1: 1000, overnight, 4 °C	Anti-Rabbit HRP conjugated (<i>Dako: P0448</i>)	1: 2000
p38 MAPK	40	8690S (<i>Cell Signaling Technology</i>), Rabbit	1: 1000, overnight, 4 °C		
p-p42/44 MAPK	42, 44	4376S (<i>Cell Signaling Technology</i>), Rabbit	1: 1000, overnight, 4 °C		
p42/44 MAPK	42, 44	9102S (<i>Cell Signaling Technology</i>), Rabbit	1: 1000, overnight, 4 °C		
GAPDH	36	ab181602 (<i>Abcam</i>), Rabbit	1: 10,000, 1 hour, room temperature		
TGFβR1	53	mab5871 (<i>R&D Systems</i>), Rat	1 µg/mL (1: 500 stock), overnight, 4 °C	Anti-rat IgG HRP conjugated (<i>R&D Systems: HAF005</i>)	1: 1000

Table 2.9: Primary and secondary antibodies used in western blotting.

The primary and secondary antibodies used to probe membranes in western blots are summarised, including expected molecular weight, antibody supplier, catalogue number and concentration, incubation time and temperature. GAPDH – glyceraldehyde-3-phosphate dehydrogenase, HRP – horseradish peroxidase, Ig – immunoglobulin, kDa – kilodalton, MAPK – mitogen-activated protein kinase, ‘p’ – phosphorylated, TGFβR1 - TGFβ type 1 receptor.

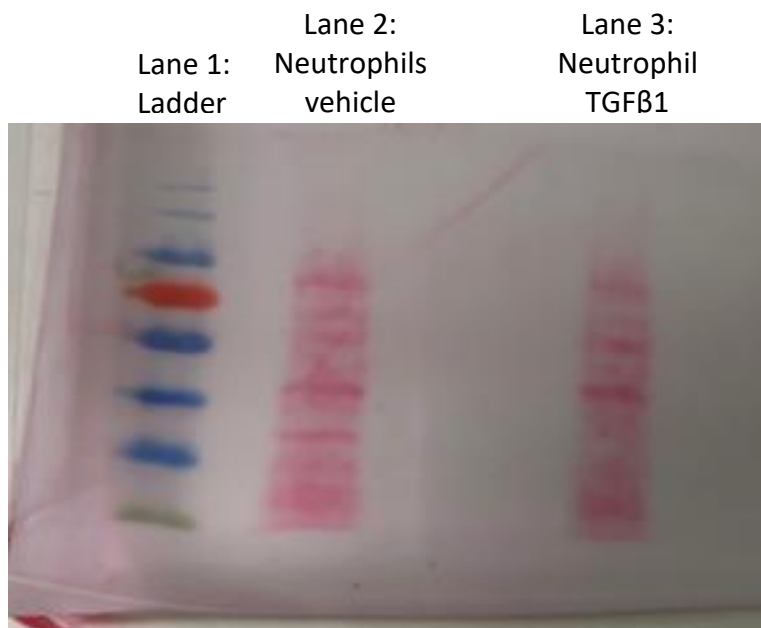


Figure 2.17: Example of a membrane stained with ponceau S solution.

This is an example image of protein bands visible on a membrane stained with ponceau S solution. The molecular weight ladder is seen in lane one. Lane two and three displays protein bands from peripheral neutrophils isolated from a healthy control where neutrophils were exposed *ex vivo* to vehicle control and 10 ng/mL transforming growth factor- β 1 (TGFB1) for 15 minutes, respectively.

2.11.3. Protein visualisation using enhanced chemiluminescence.

Proteins were visualised using enhanced chemiluminescence (*Amersham ECL-Prime, GE healthcare, UK*), Chemidoc™ MP (*BioRad, UK*) and Image Lab 5.2.1 software (*BioRad, UK*).

2.11.4. Semi-quantification of protein expression

Semi-quantitative analysis was performed of protein bands developed by enhanced chemiluminescence using FIJI Image J (*Version 1.8.0_322 National Institutes of Health, USA*).

Firstly, a region of interest was selected by drawing a yellow rectangle around the bands at the relevant molecular weight (see Figure 2.18). Lane profile plots were generated from this rectangle (region of interest) and the line drawing tool used to ensure each peak had a closed area from which the AUC was calculated. Relative expression of each protein was calculated by dividing the AUC by the AUC of the loading control, for example phosphorylated-p38 mitogen-activated protein kinase (MAPK) divided by GADPH or phosphorylated-p38 MAPK divided by p38 MAPK to determine expression of total p38 MAPK that was phosphorylated.

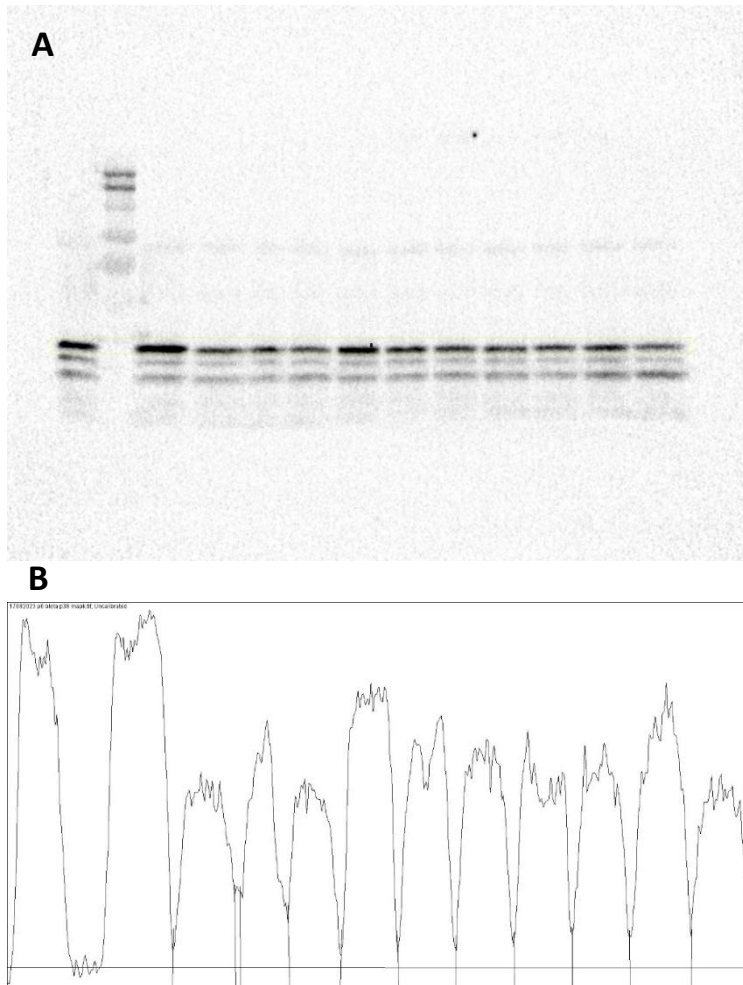


Figure 2.18: Example of semi-quantitative western blot analyses of neutrophil protein lysates.

Protein lysates were generated from human neutrophils that were isolated from a healthy control and exposed *ex vivo* to media (control), 1 μ M fMLP, 10 ng/mL TGF β 1 or vehicle for 1 minute, 5 minutes or 15 minutes. The membrane was probed with an anti-p38 MAPK antibody and secondary anti-rabbit conjugated horseradish peroxidase antibody.

(A) An image of protein bands on a membrane generated using enhanced chemiluminescence. Image was analysed using FIJI Image J and a region of interest selected by drawing a yellow rectangle around the bands at relevant molecular weight (43 kDa).

(B) Lane profile plots generated from rectangle (region of interest) on FIJI Image J. The line drawing tool was used to ensure each peak had a closed area from which the AUC was calculated. Relative expression of each protein was calculated by dividing the AUC by the AUC of the housekeeping protein and/or non-phosphorylated protein on the same blot, for example phosphorylated-p38 MAPK divided by p38 MAPK to determine expression of total p38 MAPK that is phosphorylated.

2.12. Phosphoflow cytometry assay measuring pSmad2/3 expression.

Neutrophils were resuspended at a concentration of 1 million per mL in RPMI and treated with 10 ng/mL TGF β 1 or vehicle for 5 to 60 minutes at 37°C. Neutrophils were subsequently washed with 300 μ L ice cold DPBS and centrifuged at 250 x g for 5 minutes at 4°C. Cells were resuspended in 100 μ L ice cold DPBS and fixed with paraformaldehyde (*2% working concentration*) for 20 minutes at room temperature. Following fixation, neutrophils were washed with 300 μ L ice cold DPBS and centrifuged at 250 x g for 5 minutes at 4°C. Neutrophils were subsequently permeabilised by treating with 500 μ L ice cold methanol for 30 minutes in the dark at room temperature and then washed twice with 500 μ L ice cold DPBS. Neutrophils were consequently treated with 100 μ L ice cold 1% BSA-DPBS supplemented with BD Phosflow™ PE-mouse anti-Smad2 (pS465/pS467)/Smad3 (pS423/pS425) antibody (*BD Biosciences, UK: 562586*) at a 1: 20 dilution or PE-mouse IgG1, κ isotype control (*BioLegend, UK: 400112*) at a 1: 2500 dilution for 30 minutes in the dark at 4°C. Finally, cells were washed with 500 μ L ice cold DPBS, centrifuged at 250 x g for 5 minutes at 4°C and resuspended in 200 μ L ice cold DPBS ready for flow cytometric analysis.

Data analysis was performed using FlowJo_v10.9.0 Software and single cells identified from the neutrophil gate as described previously. Histograms were created to display the frequency distribution of PE-A intensity of neutrophil singlets (see Figure 2.19).

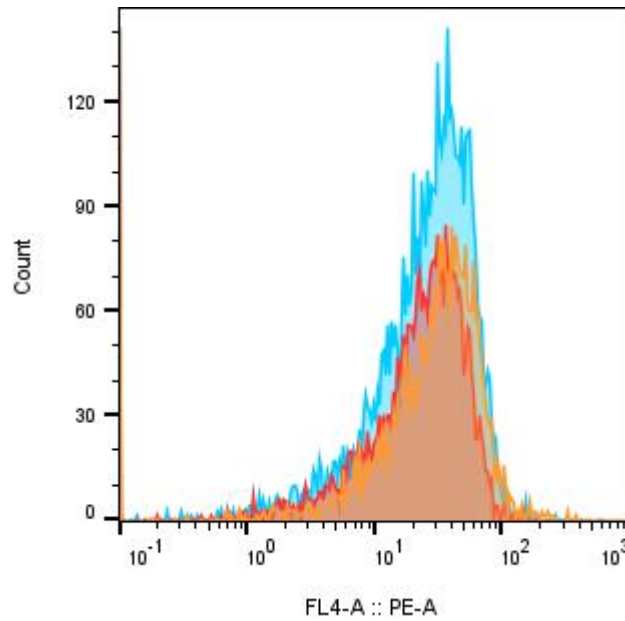


Figure 2.19: Representative histograms of phosphoflow cytometry assay.

Representative flow cytometry histograms are displayed of neutrophils isolated from a healthy participant, which were either: 1) unstained (red histogram), 2) stained with PE-mouse IgG1, κ isotype control (blue histogram) or, 3) treated with 10ng/ mL TGF β 1 for 20 minutes and stained with BD PhosflowTM PE-mouse anti-Smad2 (pS465/pS467)/Smad3(pS423/pS425) antibody (orange histogram).

2.13. Statistical Analyses

Statistical analyses were performed using GraphPad Prism (*Version 9.5.0, San Diego, USA*).

Shapiro-Wilk tests were performed to assess the normality of data distribution.

2.13.1. Comparing differences between two groups (continuous data)

Paired T-test or Wilcoxon rank tests were used to assess differences between samples from the same donor for normally distributed or skewed data, respectively. Unpaired T-test or Mann-Whitney tests were used to assess differences between independent samples for normally distributed or skewed data, respectively. Two-tailed tests were used in all cases, except for positive controls where stated. Statistical significance was determined by p-value < 0.05.

2.13.2. Comparing differences between three or more groups (continuous data)

Repeated measures one-way ANOVA or Friedman tests were performed to compare differences between three or more matched groups that contained normally distributed or skewed data, respectively. Ordinary one-way ANOVA or Kruskal-Wallis tests were performed to compare differences between three or more unmatched groups that contained normally distributed or skewed data, respectively.

If the previously described statistical tests identified a difference across the three or more groups, a further statistical test was performed to assess differences between individual groups. This included those described in Chapter 2.13.1 or multiple comparisons tests. Dunnett's or Dunn's multiple comparisons tests were performed for parametric and skewed data, respectively.

2.13.3. Categorical data

Fisher's exact test was used to analyse two or more groups containing categorical data.

2.13.4. Regression

Pearson and spearman rank correlations were performed for parametric and non-parametric data, respectively. A line of best-fit was calculated and r or r squared values and p -values displayed.

2.13.5. Post-hoc power and sample size calculations.

Post-hoc power and sample size calculations were performed using calculators provided on the ClinCalc.com website [225,226]. Mean and standard deviations were inputted. Power was set at 80% and alpha value = 0.05.

Chapter 3. VALIDATION OF EXPERIMENTS MODELLING NEUTROPHIL PHENOTYPE AND FUNCTION *EX VIVO*.

3.1. Introduction

3.1.1. The importance of validation

Many of the techniques performed in this thesis have been used previously to assess neutrophil viability, movement and NET formation [227,228]. However, neutrophils are fragile cells and prone to activation *ex vivo*, which can influence experimental work and introduce increased inter-researcher variability [229]. Therefore, it was important to confirm that these experiments could appropriately examine functions/parameters in neutrophils isolated from healthy donors, prior to testing hypotheses to be studied in the current thesis. Positive controls defined as an exposure with an expected/known outcome, were used to validate the techniques and the results of these background experiments are described in the current chapter.

In addition, alternative stimuli to PMA (a specific protein kinase C activator) deemed more physiological in IPF were sought to assess NET formation by IPF derived neutrophils. These included the pro-inflammatory cytokines, IL-8 and IL-1 β , which are elevated in the circulation and/or BALF of patients with lung fibrosis [230–233], and known to induce NET formation in healthy donor neutrophils and other disease states [234–236].

3.1.2. Immunophenotyping panels – markers explained

The assessment of neutrophil cell surface markers was considered an important method of determining the effects of TGF β 1 on human neutrophils and understanding neutrophil populations in patients with IPF. Therefore, three immunophenotyping panels were developed to examine this by flow cytometry (described in Chapter 2.10 and Table 2.7 and outlined below).

3.1.2.1. Panel 1 – neutrophil activation and/or priming markers

The cell surface expression of adhesion molecules and glycoproteins change on neutrophil priming and activation. Therefore, immunophenotyping ‘panel 1’ targeting the adhesion/activation molecules, CD11b, CD66b and CD62L, was devised to aid assessment of whether 1) TGFβ1 primes and/or activates human neutrophils, and 2) IPF donor neutrophils display an altered state of priming and/or activation.

The cell surface expression of CD11b (an integrin alpha M subunit) and CD66b (glycoprotein) increases on priming/activation, whereas CD62L (L-selectin) is shed [237–239]. The activating agents, IL-8 and fMLP, and priming agent, TNFα, all drive such changes on the neutrophil surface [237–243]. These stimuli were therefore used as positive controls to test if the expression of markers in ‘panel 1’ changes on priming and/or activation.

3.1.2.2. Panel 2 – CXCR2 expression

Immunophenotyping panel 2 included a single antibody against the chemokine receptor CXCR2. CXC chemokines, such as IL-8, exert their chemotactic effects by binding to the seven transmembrane G-protein coupled receptors, CXCR1 and CXCR2. These receptors are internalised following chemokine binding, resulting in reduced extracellular expression [244]. CXCR2 has been implicated in the pathogenesis of IPF with lung fibrosis attenuated in a bleomycin murine model following CXCR2 antagonism [245]. However, CXCR2 antagonism reduced neutrophil transmigration into the BALF, but not into the lung parenchyma [245]. Assessment of CXCR2 expression on neutrophils was therefore of importance in this thesis as it could improve our understanding of its role in neutrophils in IPF and aid mechanistic investigation of how TGFβ1 does or does not affect neutrophil movement.

3.1.2.3. Panel 3 – maturation, activation, ageing and naïve neutrophils.

An additional antibody cocktail labelled immunophenotyping ‘panel 3’ (see Table 2.7) was created to assess the expression of other cell surface markers to provide insight into neutrophil maturity and activation, as well as ‘aged’ and tissue naïve neutrophils. These markers (described in detail below) included: CD16, CD10, CD11c, PD-L1, CXCR4 and CD54. The expression of these neutrophil markers is unknown in patients with IPF.

3.1.2.3.1. CD16

CD16 (also called Fc gamma-receptor III) is one of the most abundant transmembrane receptors present on the neutrophil surface and through binding of immunoglobulin-G containing immune complexes, provides an important link between the innate and adaptive immune systems [246–248]. CD16 binding can contribute to neutrophil activation processes, such as cell adhesion and degranulation [249–251]. Cell surface expression of CD16 increases as neutrophils mature (see Figure 1.3) and varies between different neutrophil subtypes (low density neutrophils noted to have higher CD16 expression [252]) and states (shed on neutrophil apoptosis [253] and stimulation [254]). Therefore, CD16 expression is a useful identifier of neutrophils and can provide insight into neutrophil phenotype/status.

3.1.2.3.2. CD11c

CD11c is an integrin alpha X chain protein that is present intracellularly in human neutrophils, and to a lesser extent expressed on the cell surface where it contributes to neutrophil adhesion [255,256]. Upregulated cell surface expression of CD11c on human circulating neutrophils has been associated with conditions, such as sepsis [257]. Cell surface expression of CD11c increases when neutrophils are stimulated, for example with fMLP, but also when isolated using Percoll density gradient centrifugation [258]. CD11c was used as marker of neutrophil activation in this panel.

3.1.2.3.3. PD-L1

PD-L1 is a co-inhibitory molecule, which acts via the programmed cell death protein 1 receptor (PD-1). The role of PD-L1 in neutrophil responses is still being clarified with it first noted to have an immunosuppressive effect on T cell responses [259]. PD-L1 is upregulated on neutrophils in various disease states, such as sepsis and SLE, and by pro-inflammatory stimuli (interferon-gamma and LPS) [260–262]. PD-L1 may itself drive neutrophil mediated inflammation with one study noting that it delays neutrophil apoptosis and that PD-L1 knock down reduces lung injury in murine sepsis models [260]. Elsewhere, neutrophil specific knockout of PD-L1 attenuated lung injury in an LPS induced ARDS animal model, alongside reduced NET release [262]. As with all the neutrophil cell surface markers in these panels, the expression and importance of PD-L1 in neutrophils is unknown in the context of IPF.

3.1.2.3.4. CD10

The transmembrane glycoprotein CD10 (also known as common acute lymphoblastic leukaemia antigen) is a useful marker of maturity, only expressed on the surface of mature, segmented neutrophils (see Figure 1.3 for antigen expression at the different states of neutrophil development) [263,264].

3.1.2.3.5. CXCR4

The chemokine receptor, CXCR4, has been used in this panel to highlight ‘aged’ neutrophils. During maturation, CXCR4 prevents neutrophil egress from the bone marrow and CXCR4 expression subsequently drops to allow neutrophil release into the circulation [265,266]. However, when neutrophils become senescent or ‘aged’, CXCR4 is upregulated on the cell surface, which promotes return and subsequent clearance in the bone marrow [265]. As described in Chapter 1, increased expression of ‘aged’ (CXCR4 high and CD62L low) neutrophils

have been noted in bleomycin murine models [267]. However, the expression of CXCR4 on circulating neutrophils derived from IPF patients is unknown and requires further study.

3.1.2.3.6. CD54

The final marker in this panel, the transmembrane glycoprotein, CD54 is sparsely expressed on the surface of circulating neutrophils derived from healthy individuals, as it is thought to only be upregulated following transmigration through the vascular endothelium into tissues, and subsequent reverse migration back into the circulation [116]. Factors that trigger CD54 expression remain unclear, although *in vitro* GM-CSF has been noted to increase expression [268]. CD54 appears to contribute to superoxide production and neutrophil adherence and aggregation [268,269]. Buckley *et al* defined tissue 'naïve' cells as circulating CD54 low and CXCR1 (chemokine receptor) low neutrophils [116]. Therefore, in this thesis CD54 was quantified to help distinguish between cells that are tissue naïve (CD54 low) or have reverse migrated back into the circulation (CD54 high).

3.2. Chapter Aims

1. To demonstrate that the Zombie NIR™ fixable stain provides comparable neutrophil viability results to the Annexin V/PI stain.
2. To demonstrate that the transwell assay can model neutrophil migration *ex vivo*.
3. To demonstrate that the Insall chamber assay can interrogate neutrophil chemotaxis and chemokinesis properties *ex vivo*.
4. To demonstrate that the immunophenotyping panels devised in this thesis can detect changes in neutrophil activation state.
5. To demonstrate that NET formation can be assessed by fluorometric measurement and microscopic imaging of SYTOX™ green nucleic acid staining.
6. To demonstrate that IL-8 and IL-1 β drive NET formation in healthy donor neutrophils.

3.3. Materials and methods linked to Chapter 3

3.3.1. Ethical approval and study participants

All venous blood samples were collected from volunteers recruited to the 'Investigations of the Ageing Immune System' study (10/H1208/48). Participants were self-reported non-smokers, healthy and aged between 18- 60 years of age.

3.3.2. Neutrophil isolation

Neutrophil isolation was performed as described in Chapter 2.4.

3.3.3. Neutrophil assays

Neutrophil viability was assessed by Annexin V/PI and Zombie NIR™ fixable viability staining as described in Chapter 2.6. The transwell migration assay and Insall chamber chemotaxis assay was performed as described in Chapter 2.7. Cell free DNA (cfDNA) release was assessed using a fluorescence plate reader and fluorescence microscopy as described in Chapter 2.8. Immunophenotyping was performed using flow cytometry as described in Chapter 2.10.

3.4. Results

3.4.1. Validating Zombie NIRTM fixable viability stain

Neutrophil viability was assessed by two methods in this thesis: annexin V/PI viability staining and Zombie NIRTM fixable viability staining. Annexin V is a phospholipid binding protein that has a high affinity for phosphatidylserine, a phospholipid that is translocated to the external surface of the cell during apoptosis [270]. PI is a viability stain that only penetrates cells that have lost cell membrane integrity. Annexin V/PI staining has the benefit of discriminating between viable (annexin negative, PI negative) early apoptotic (annexin positive, PI negative), late apoptotic (annexin positive, PI positive) and necrotic cells (PI positive, annexin negative) [270]. However, limitations of this method are that samples cannot be fixed and need to be processed immediately on the flow cytometer for optimum results. This provides technical difficulties where multiple assays are being performed on the same donor neutrophils and it is helpful to have the increased flexibility associated with running fixed samples.

The Zombie NIRTM fixable stain is a fluorescent dye, which only binds to cells that have permeable membranes. Cells can be fixed following staining with this dye thereby allowing greater flexibility regarding timing of data acquisition. Cell fixation can also be beneficial in minimising the effects of reoxygenation when culturing in 1% oxygen. Although the Zombie NIRTM fixable stain has been used previously to assess viability of human neutrophils *ex vivo*, it is less well reported than Annexin V/PI staining [218,271].

Subsequently, neutrophil viability determined by Zombie NIRTM and Annexin V/PI staining were compared. Representative scatter plots of an Annexin V/PI assay (Figure 3.1 A-C) and histogram plots of Zombie NIRTM staining (Figure 3.1 D-F) performed on neutrophils isolated from the same single healthy donor are described.

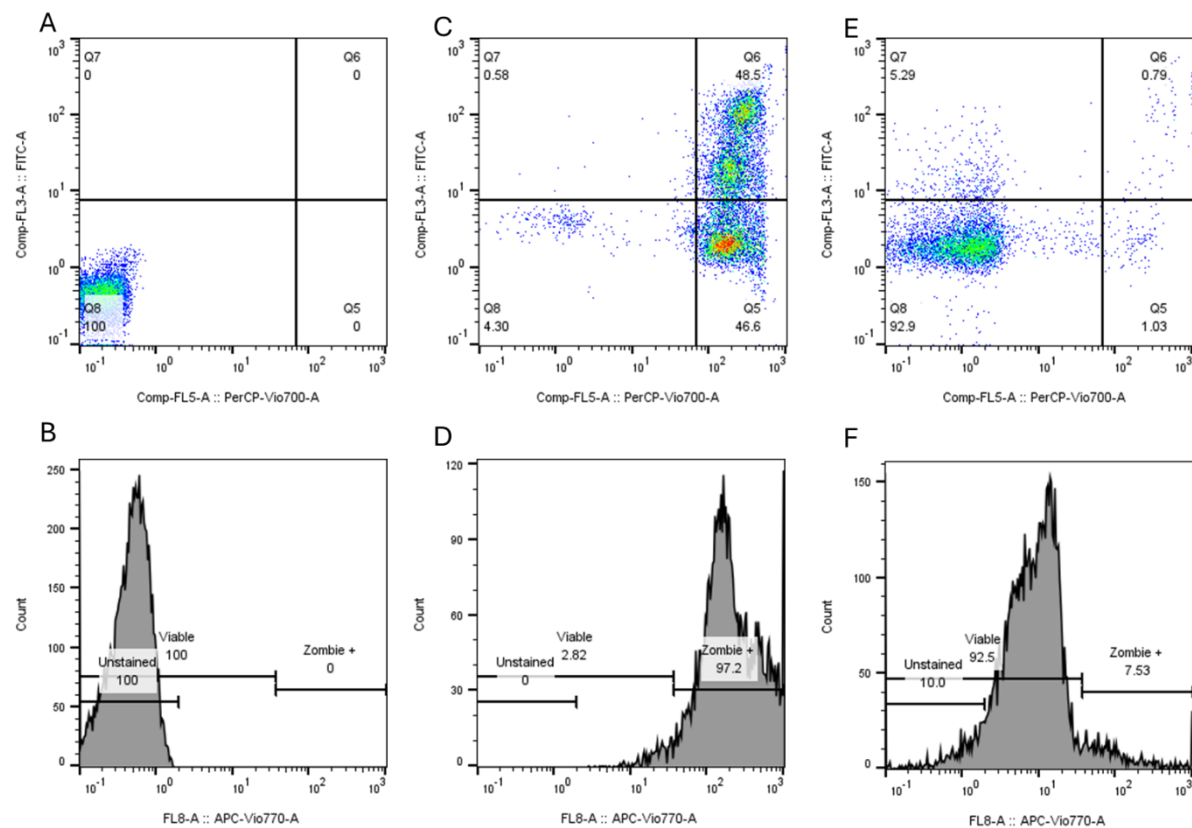


Figure 3.1: Representative scatter plots of Annexin V/PI staining (A - C) and histogram plots of Zombie NIRT™ staining (D - F).

Isolated neutrophils or heat killed neutrophils used as a positive control (cells exposed to 70° C for 2 minutes and 15 seconds) from a single healthy donor, were stained with Annexin V/PI or Zombie NIRT™ stain. The gating strategy outlined in Figure 2.3 was applied and the percentage of cells falling into each quadrant was recorded to determine; live cells, total apoptotic cells and necrotic cells as appropriate. Unstimulated neutrophils as shown in (A) dot plot, (B) histogram. Positive control heat killed neutrophils stained with Annexin V/PI (C) or Zombie NIRT™ stain (D). Untreated neutrophils stained with Annexin V/PI (E) or Zombie NIRT™ stain (F) revealed 92.9% and 92.5% of cells were viable, respectively.

Combined results of three experimental replicates show no difference in neutrophil viability determined by Zombie NIR™ or Annexin V/PI stains in untreated (mean 95.9% (SD 3.2%) versus 96.1% (SD 2.7%), respectively, $p = 0.809$, $n = 3$) or heat killed neutrophils (mean 8.3% (SD 7.9%) versus 1.6% (SD 2.4%), respectively, $p = 0.692$, $n = 3$) (see Figure 3.2).

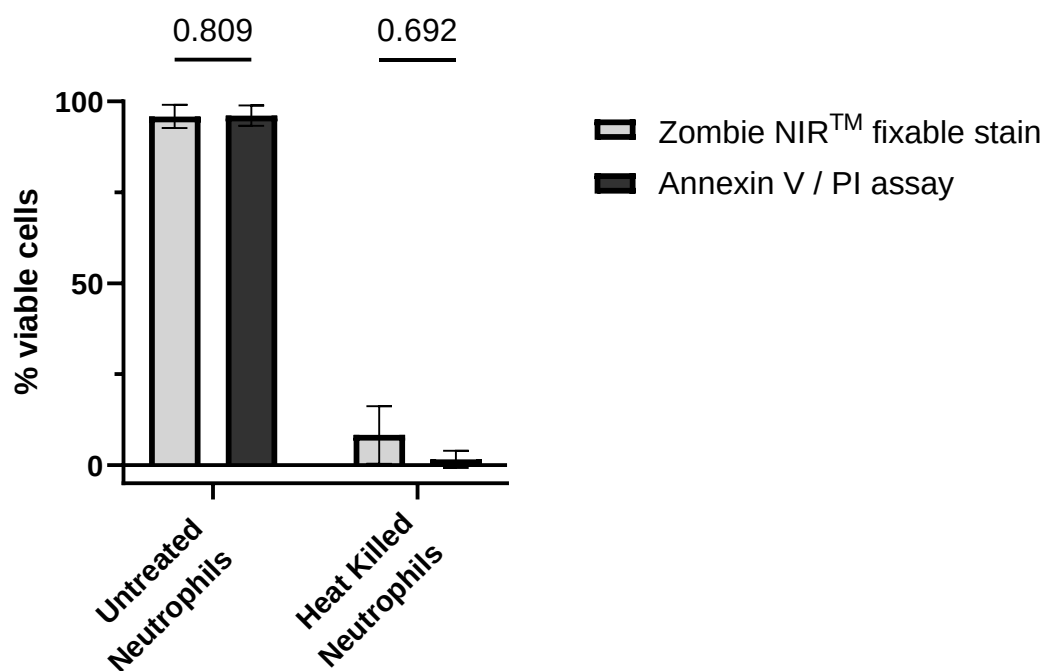


Figure 3.2: Comparison of neutrophil viability assays.

Neutrophils isolated from healthy donors (n = 3) were assessed for viability using the Zombie NIR™ fixable stain and Annexin V/PI stain. Neutrophils that stained negative for Zombie NIR™ stain and Annexin V negative/PI negative were deemed viable. No statistical difference was noted between neutrophil viability determined by Zombie NIR™ or Annexin V/PI stains in untreated or heat killed neutrophils. Data bars are mean and standard deviation. Paired T-tests were performed.

3.4.2. The transwell system can model *ex vivo* neutrophil migratory responses towards a classical chemoattractant.

Neutrophil transmigration can be modelled *ex vivo* by counting the number of cells that pass through a transwell membrane over time (modified Boyden chamber method) [271]. The two types of polycarbonate cell culture inserts used in this thesis are described in Table 3.1.

	Nunc™ polycarbonate cell culture inserts (ThermoFisher Scientific, UK: 140620)	Corning® Transwell® polycarbonate cell culture inserts (Corning, USA: CLS3415)
Pore size	3 µm	3 µm
Surface area	0.47 cm ²	0.33 cm ²

Table 3.1: Polycarbonate cell culture inserts used in transwell migration assays.

Two types of polycarbonate cell culture inserts were used in the transwell migration assays: Initially, Nunc™ polycarbonate cell culture inserts were used, and later, Corning® Transwell® polycarbonate cell culture inserts due to supply issues. Both inserts had 3 µm sized pores.

As described in Chapter 1, IL-8 is a potent chemokine and others have demonstrated that 100 nanomolar (nM) IL-8 drives neutrophil migration in this transwell system [271,272]. The ability of this assay to model neutrophil migratory responses *ex vivo* was assessed using IL-8, which increased the number of neutrophils that transmigrated through the transwell inserts over 90 minutes in a dose-dependent manner (Figure 3.3A, n = 3). One hundred nM IL-8 increased the number of cells that migrated through Nunc™ polycarbonate cell culture inserts (mean response = 226958 (SD 101940) versus control = 36921 (SD 22543); p = 0.028, n = 4) and Corning® Transwell® polycarbonate inserts (mean response = 66454 (SD 46337) versus control = 1502 (SD 896); p = 0.035, n = 5) (Figure 3.3B). This demonstrates that the transwell system (using either type of transwell insert) can appropriately detect amplified neutrophil migration in response to a known chemoattractant.

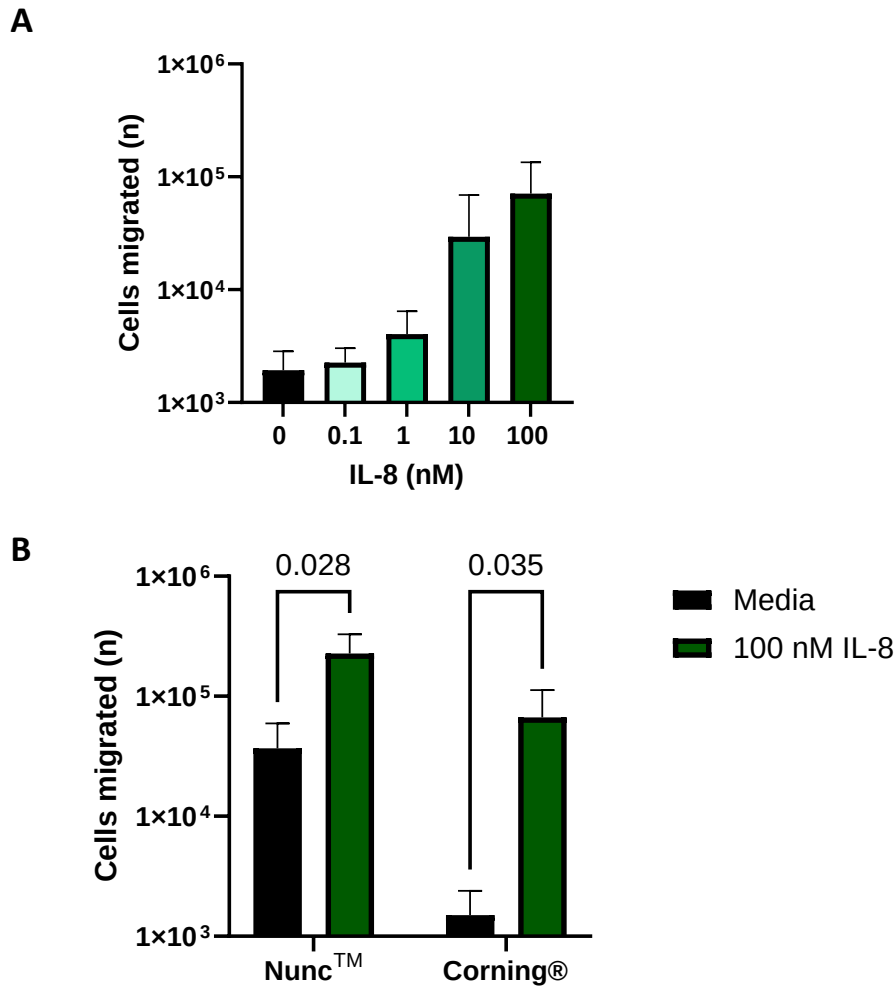


Figure 3.3: Effect of IL-8 on neutrophil migration across transwell inserts.

(A) Neutrophils isolated from healthy volunteers ($n = 3$) were added to a Corning® Transwell® polycarbonate cell culture insert. The number of neutrophils that had migrated over 90 minutes through the transwell insert into the bottom well containing media only or 0.1- 100 nM IL-8 was counted by flow cytometry. Histograms represent mean with standard deviation bars.

(B) Neutrophils isolated from healthy volunteers were added to Nunc™ polycarbonate cell culture inserts ($n = 4$) or Corning® Transwell® ($n = 5$) polycarbonate cell culture inserts for direct comparison to determine differences related to transwell construction. Histograms represent mean with standard deviation bars. Paired T-tests were used to determine the statistical significance of the effect.

3.4.3. The Insall chamber assay can model *ex vivo* neutrophil chemokinetic and chemotactic responses to a classical chemoattractant.

The Insall chamber assay is an alternative method of assessing neutrophil movement *ex vivo* and provides additional information regarding both direction independent (chemokinesis: speed, distance, displacement) and direction dependent (chemotactic: velocity and chemotactic index) movement (see Table 2.4 for definitions) [220]. Increased neutrophil chemokinesis and chemotaxis in response to IL-8 has previously been demonstrated in the modified Dunn chamber [273,274].

The ability of this assay to model neutrophil migratory responses *ex vivo* was assessed using IL-8. The chemoattractant well was filled with either media (control), 78.1 pM IL-8, 781 pM IL-8 or 100 nM IL-8. The upper concentration, 100 nM IL-8, was used as a positive control [275]. These two lower concentrations were chosen as these reflect the physiological levels of IL-8 measured in the BALF of patients with IPF [198,276]. In addition, these concentrations are equivalent molar concentrations of TGF β 1 (78.1 pM: 1ng/mL, 781 pM: 10ng/mL) that are investigated in this thesis, allowing for the effects of TGF β 1 on neutrophil movement to be compared with the equivalent molar concentrations of IL-8.

IL-8 increased neutrophil chemokinesis (Figure 3.4A (speed), B (distance travelled), C (displacement) and chemotaxis (Figure 3.4D (velocity) and E (chemotactic index)). Speed and distance travelled were significantly increased by 78.1 pM (mean speed 4.1 μ m/ min (SD 1.3) versus control = 1.8 μ m/ min (SD 1.0); $p < 0.001$, $n = 9$) (mean distance 46.8 μ m (SD 15.2) versus control = 20.5 μ m (SD 11.2); $p < 0.001$, $n = 9$).

Similar changes were seen in response to 781 pM (speed 4.0 μ m/ min (SD 1.1) versus control = 1.8 μ m/ min (SD 1.0); $p = 0.001$, $n = 8 - 9$), (mean distance 45.0 μ m (SD 12.9) versus control

= 20.5 μm (SD 11.2); $p = 0.001$, $n = 8 - 9$). However, 78.1 pM and 781 pM did not increase neutrophil chemotaxis parameters ($p > 0.05$, $n = 8/9$).

In comparison, 100 nM IL-8 increased all neutrophil responses further; speed (mean speed 6.3 $\mu\text{m}/\text{min}$ (SD 1.0) versus control = 1.8 $\mu\text{m}/\text{min}$ (SD 1.0); $p < 0.001$, $n = 9$), displacement (median 29.1 μm (interquartile range (IQR) 20.3 – 37.4) versus control = 8.6 μm (IQR 5.1 – 11.0); $p < 0.001$, $n = 9$), velocity (mean 1.4 $\mu\text{m}/\text{min}$ (SD 0.9) versus control = -0.1 $\mu\text{m}/\text{min}$ (SD 0.4); $p < 0.001$, $n = 9$) and chemotactic index (mean 0.2 units (SD 0.8) versus control = 0.0 units (SD 0.1); $p < 0.001$, $n = 9$) as compared to media alone. Dunnett's and Dunn's multiple comparisons tests were used to test significance for normally distributed and skewed data, respectively.

These data demonstrate that the Insall chamber assay can detect increased neutrophil chemokinesis and chemotaxis ex vivo in response to 100 nM IL-8. The lower concentrations of IL-8 tested (78.1 pM and 781 pM) increased neutrophil chemokinesis (direction and speed), but not chemotaxis in this in vitro system.

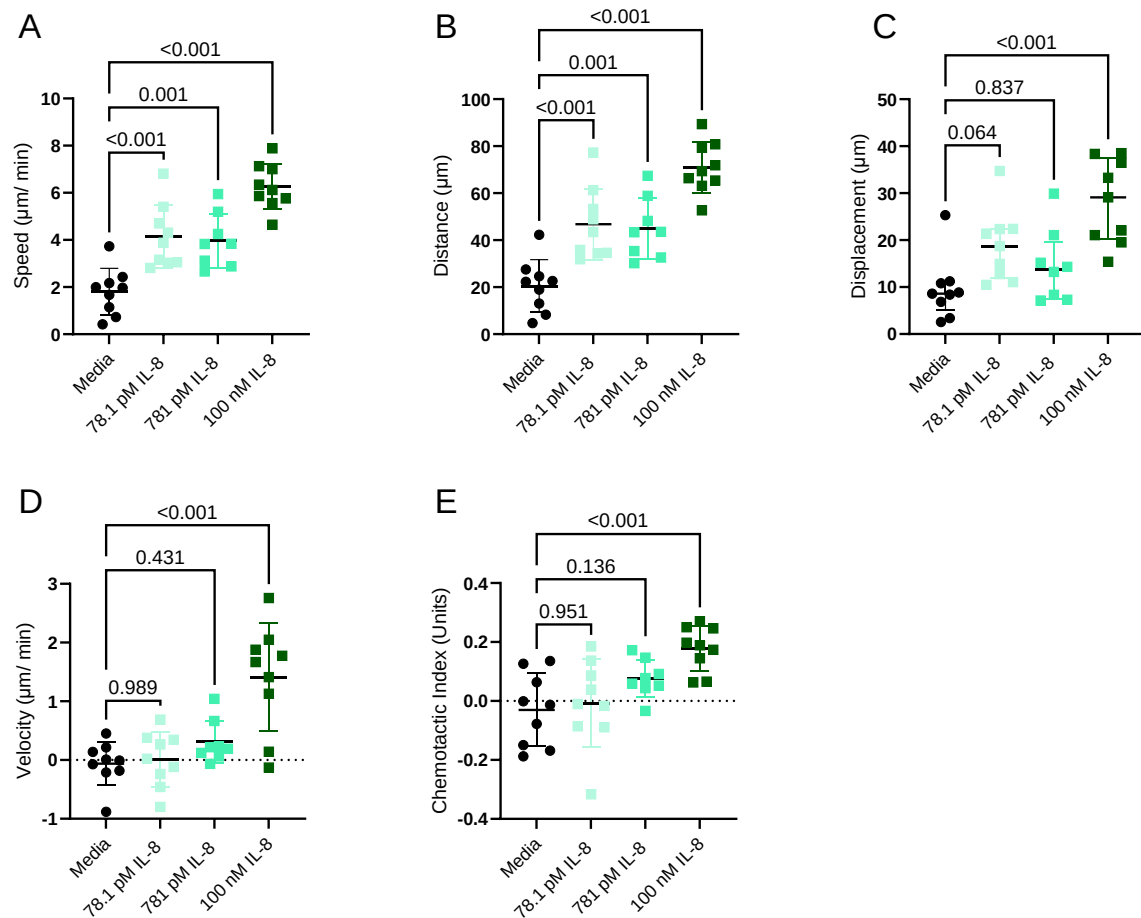


Figure 3.4: Effect of IL-8 on neutrophil chemokinesis and chemotaxis within an Insall chamber.

Chemokinetic ((A) speed, (B) distance, (C) displacement) and chemotactic ((D) velocity and (E) chemotactic index) responses are shown to 78.1 pM, 781 pM and 100 nM IL-8. These were assessed using the Insall chamber assay. Neutrophils were isolated from healthy donors ($n = 8/9$) and movement assessed over 12 minutes using time-lapse microscopy. Dot per participant. Results in (A), (B), (D) and (E) were normally distributed; data bars are mean and standard deviation. Results in (C) were skewed; data bars are median and interquartile range. Dunnett's and Dunn's multiple comparisons tests were performed to assess the statistical difference between each IL-8 concentration and media for normally distributed and skewed data, respectively.

3.4.4. Neutrophil extracellular trap formation assessed by nucleic acid staining.

3.4.4.1. *NET formation can be assessed by fluorometric measurement and visualisation of SYTOX™ green stained nucleic acid that is released by neutrophils.*

NET formation can be assessed by fluorometric measurement of cfDNA release (DNA is a key component of these net-like structures – see Chapter 1 for further detail) in neutrophil supernatant or visualisation of DNA released by neutrophils using microscopy. Both of these techniques use the nucleic acid stain, SYTOX™ green. Stimulation of neutrophils with 25 nM PMA has previously been shown to induce NET formation [227].

Here it was also noted that 25 nM PMA significantly increased cfDNA release from human neutrophils cultured *ex vivo* for 4 hours as compared to control (media) (Figure 3.5A: mean RFU 5211 (SD 1646) versus control = 1949 (SD 1327); $p = 0.044$, $n = 5$, paired T-test). Figure 3.5C and Figure 3.5D are representative microscopy images of neutrophils stained with SYTOX™ green following culture in media or media supplemented with 25 nM PMA for 4 hours *ex vivo*, respectively. Neutrophils cultured in media alone (control) displayed limited NET formation, with staining restricted to the retained multi-nuclear structures within the neutrophils (Figure 3.5C). However, there was loss of nuclear integrity and widespread NET formation observed in response to 25 nM PMA (Figure 3.5D). Mean NET area on the immunofluorescence images was semi-quantified relative to cell number (See Chapter 2.8.2.2 for details) and summarised in Figure 3.5B. In keeping with the fluorometric data, 25 nM PMA significantly increased mean NET area relative to cell number as compared to control (mean 1501 (SD 465) versus mean control = 199 (SD 70); $p < 0.001$).

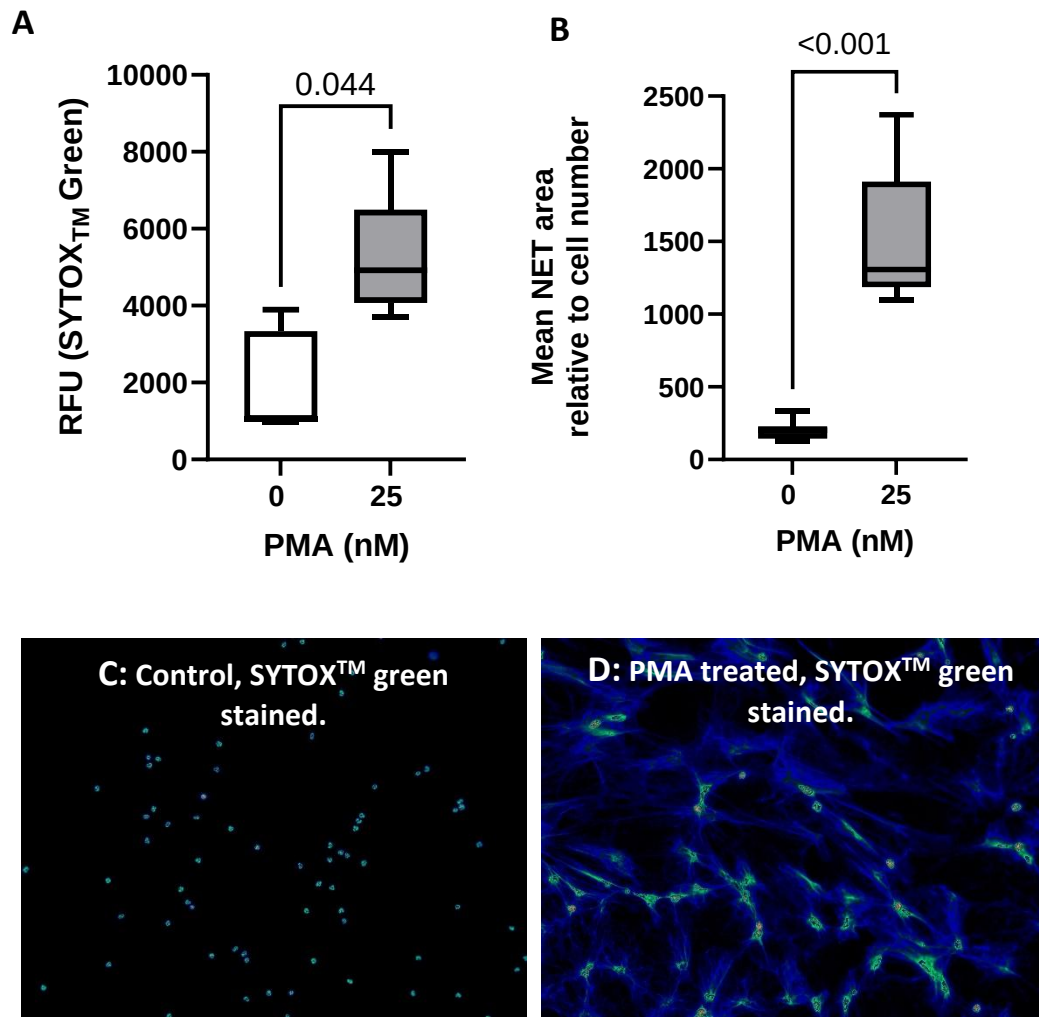


Figure 3.5: Effect of PMA on neutrophil extracellular trap formation.

(A) Extracellular DNA release from neutrophils isolated from healthy donors ($n = 5$) that were cultured for 4 hours *ex vivo* in RPMI with or without 25 nM PMA was measured using a fluorometric plate reader. The fluorescence of DNA stained with SYTOX™ green is presented as RFU. Twenty five nM PMA significantly increased cfDNA release compared to control ($p = 0.044$).

(B) NET release was visualised by fluorescence microscopy from neutrophils isolated from healthy donors ($n = 7$) that were cultured for 4.5 hours *ex vivo* in RPMI (control) with or without 25 nM PMA and stained with SYTOX™ green.

Paired T-tests were performed. P-values are shown above the Tukey box and whiskers plots.

(C) and (D) are representative microscopy images (20x magnification) of SYTOX™ green stained neutrophils following 4.5 hours in RPMI or 25 nM PMA, respectively. 3-3-2 RGB look up table is applied to the images.

3.4.4.2. IL-8 and IL-1 β are physiological cytokines upregulated in IPF patients that can drive NET formation.

Alternative stimuli to PMA were then sought that were considered more physiological in IPF.

First, neutrophils isolated from healthy donors were exposed to 0.5, 5 and 50 nM IL-8 for 4 hours *ex vivo*, cfDNA measured and DNA release visualised using fluorometry and microscopy, respectively. Five nM IL-8 significantly increased cfDNA release as compared to media alone (Figure 3.6A: mean RFU = 2343 (SD 1218) versus control = 2037 (SD 1129); $p = 0.024$, $n = 6$, paired T-test). Fifty nM IL-8 also significantly increased cfDNA release ($p = 0.014$, $n = 6$, paired T-test), although 0.5 nM IL-8 did not ($p = 0.054$, $n = 6$, paired T-test). NET release was observed using microscopy in neutrophils exposed to IL-8 (Figures 3.6B – 3.6E).

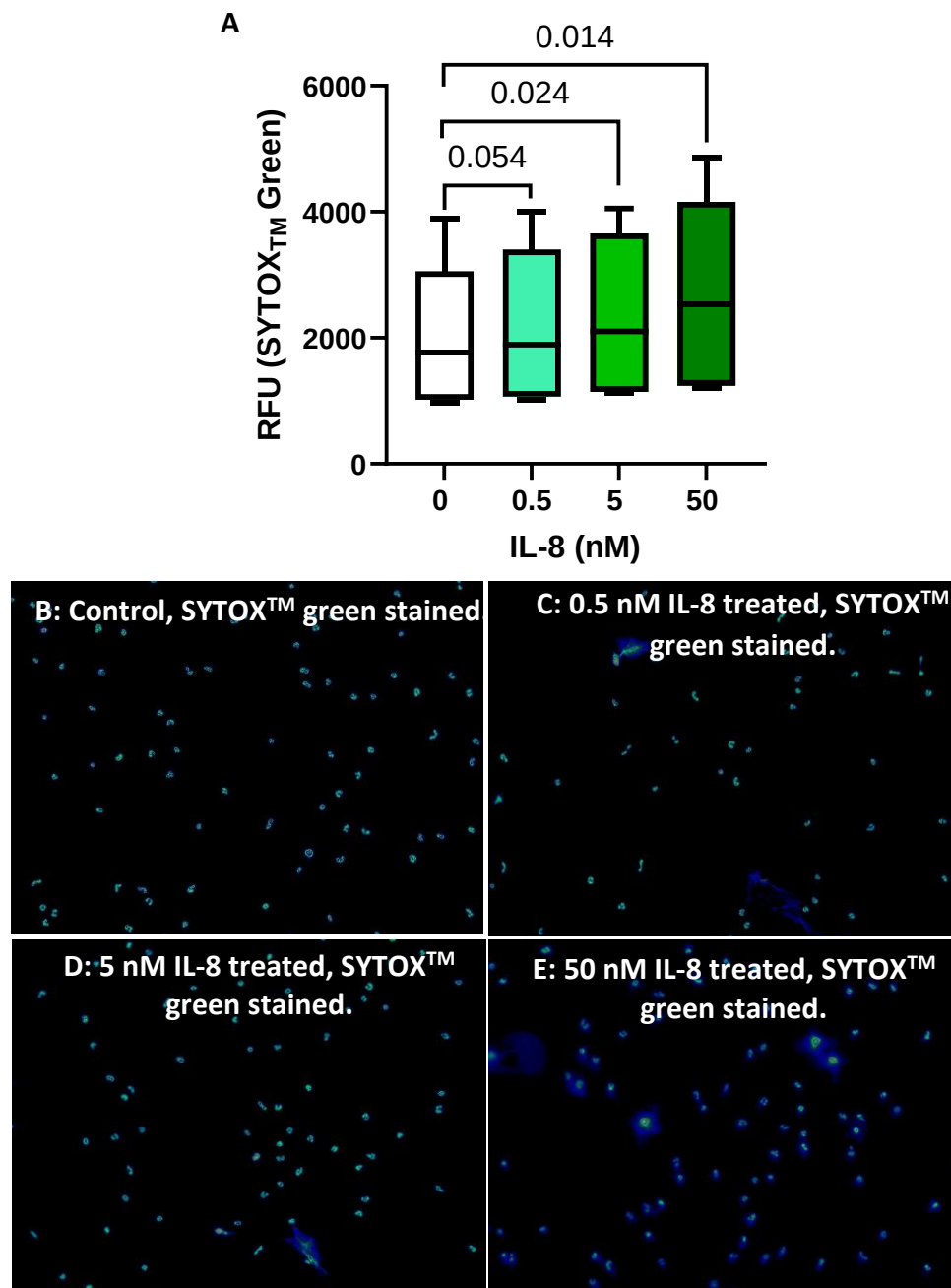


Figure 3.6: Effect of IL-8 on neutrophil extracellular trap formation.

DNA release of healthy donor neutrophils ($n = 6$) cultured for 4 hours ex vivo was measured (A) using a fluorometric plate reader as the fluorescence of DNA stained with SYTOX™ green, presented as RFU. Statistical difference from control is indicated for each dose. One-way ANOVA was performed, followed by paired T-tests. P-values are shown above the Tukey box and whiskers plots. DNA release was also visualised using fluorescence microscopy (B - E show representative images). Neutrophils were cultured in media only (B) or supplemented with (C) 0.5 nM (D) 5 nM or (E) 50 nM IL-8. Images were taken using a Leica DMI6000 B microscope and presented with 3-3-2 RGB look up table applied.

Second, the effect of IL-1 β on NET formation was assessed. Exposure for 4 hours with all three concentrations (588.2 pM, 2.9 nM and 5.8 nM) of IL-1 β significantly increased cfDNA release from neutrophils *ex vivo* compared to media only ($p < 0.05$, all comparisons, $n = 4$, paired T-tests). The data for each dose is summarised in Figure 3.7A. The mean RFU of neutrophils increased to 1497 (SD 459.5) from RFU 1394 (SD 473.9) ($p = 0.011$) in the presence of 2.9 nM IL-1 β . This was confirmed using microscopy in neutrophils exposed to IL-1 β (Figures 3.7B – 3.7D).

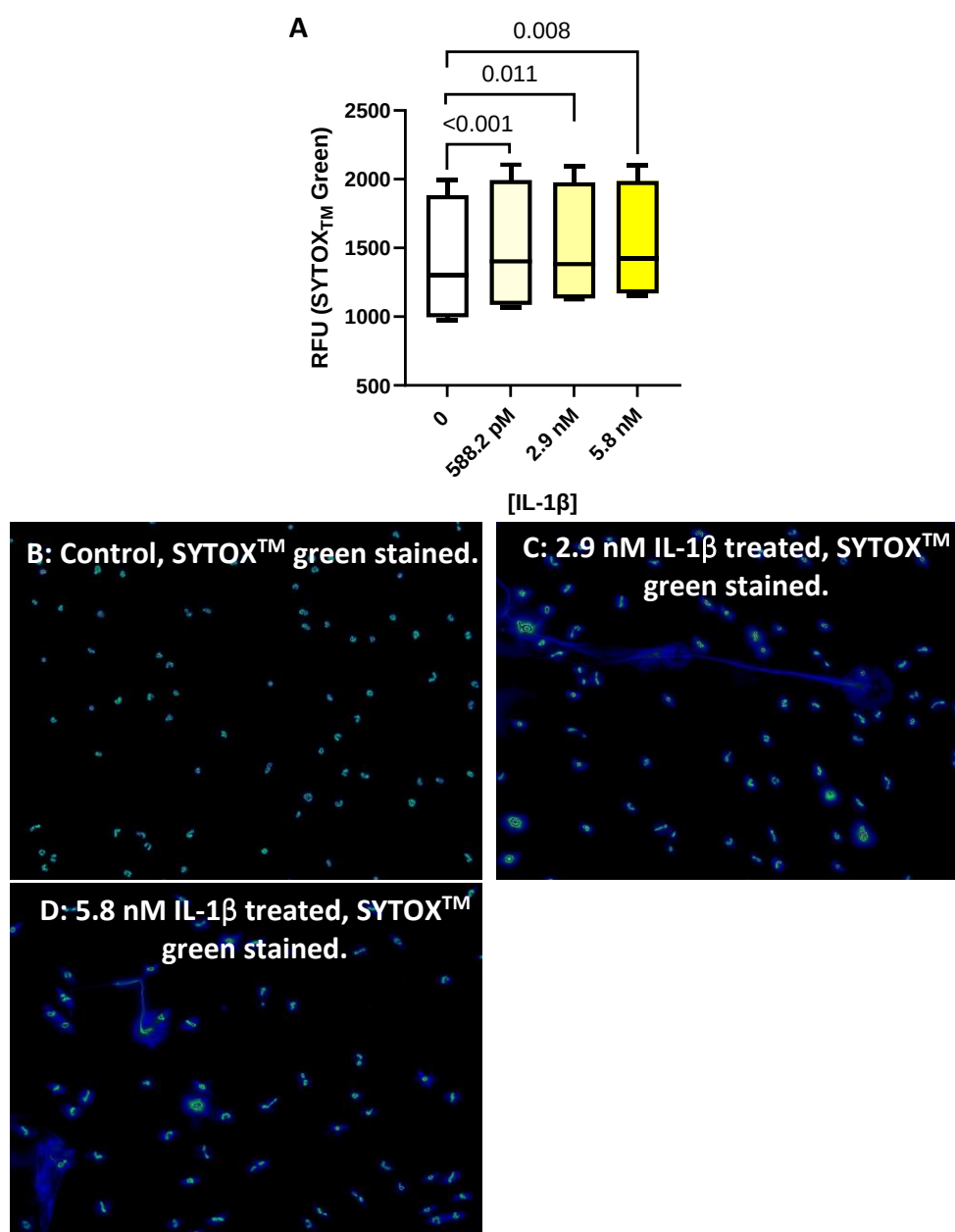


Figure 3.7: Effect of IL-1 β on neutrophil extracellular trap formation.

DNA release of healthy donor neutrophils ($n = 4$) cultured for 4 hours ex vivo was measured (A) using a fluorometric plate reader as the fluorescence of DNA stained with SYTOX™ green, presented as RFU. All three concentrations (588.2 pM, 2.9 nM, 5.8 nM) of IL-1 β significantly increased cfDNA release from neutrophils ex vivo compared to media only as shown on the figure. One-way ANOVA was performed, followed by paired T-tests. P-values are shown above the Tukey box and whiskers plots. DNA release was also visualised using fluorescence microscopy (Leica DMI6000 B microscope, 20X magnification) and presented with 3-3-2 RGB look up table applied. Representative images are shown of neutrophils cultured in media only (B) or supplemented with (C) 2.9 nM or (D) 5.8 nM IL-1 β .

3.4.5. Changes in cell surface protein expression that occur on neutrophil priming and/or activation can be detected using flow cytometry.

Neutrophils isolated from healthy donors were cultured *ex vivo* for up to 60 minutes in media supplemented with either 100 nM IL-8, or equivalent vehicle control (RPMI 1640 supplemented with 0.002% BSA-PBS).

Treatment with 100 nM IL-8 for up to 30 minutes significantly increased CD11b (Figure 3.8A: mean 59.6% (SD 26.5) versus control = 25.2% (SD 10.1); $p = 0.019$, $n = 5/6$, paired T-test) and CD66b expression (Figure 3.8C: mean 97.7% (SD 2.4) versus control = 87.6% (SD 6.4); $p = 0.023$, $n = 5/6$, paired T-test).

CD62L expression was not affected by IL-8 treatment (Figure 3.8E and 3.8F: $p > 0.05$ for all timepoints, $n = 4/5$, Wilcoxon matched-pairs signed rank tests). The data for each timepoint is summarised in Figure 3.8.

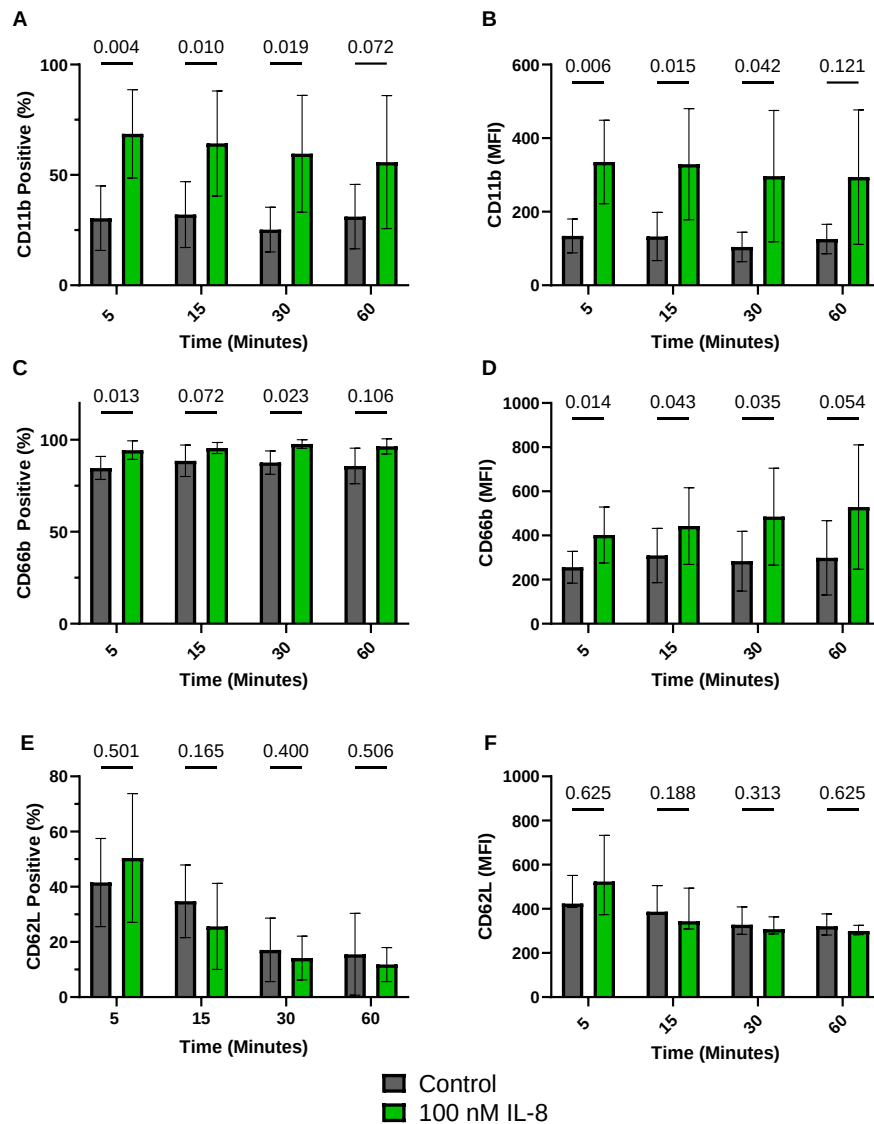


Figure 3.8: Effect of IL-8 on the expression of neutrophil activation markers.

Neutrophils isolated from healthy donors ($n = 4/5$) were cultured *ex vivo* with media containing 100 nM IL-8 or vehicle control for 5 – 60 minutes. Cell surface expression of CD11b (A and B), CD66b (C and D) and CD62L (E and F) were assessed by flow cytometry following staining with the immunophenotyping panel 1 antibody cocktail and fixation with 2% paraformaldehyde. Percentage of positive cells and median fluorescence intensity (MFI) are presented for each marker. Five to 30 minutes exposure to 100 nM IL-8 significantly increased CD11b and CD66b expression (% positive cells and MFI) on the neutrophil surface as compared to vehicle control. CD62L expression was unaffected by IL-8 at all timepoints. Histograms represent mean and standard deviation, except CD62L (MFI) which was median and interquartile range (due to skewed data). Analysis was performed using paired T-test, except CD62L (MFI) where Wilcoxon matched-pairs signed rank test was used. P-values are shown above the histograms.

In contrast, 30 minutes exposure to the alternative stimulus, 1 μ M fMLP, significantly reduced the percentage of neutrophils expressing CD62L on the cell surface (Figure 3.9A: from 75.6 (SD 2.6) to 9.2 (SD 8.1), $p = 0.036$, $n = 2$, paired T-test). CD62L MFI also appeared to reduce over the 30 minutes of exposure to 1 μ M fMLP, but this was not statistically significant (Figure 3.9B: $p = 0.206$, $n = 2$, paired T-test).

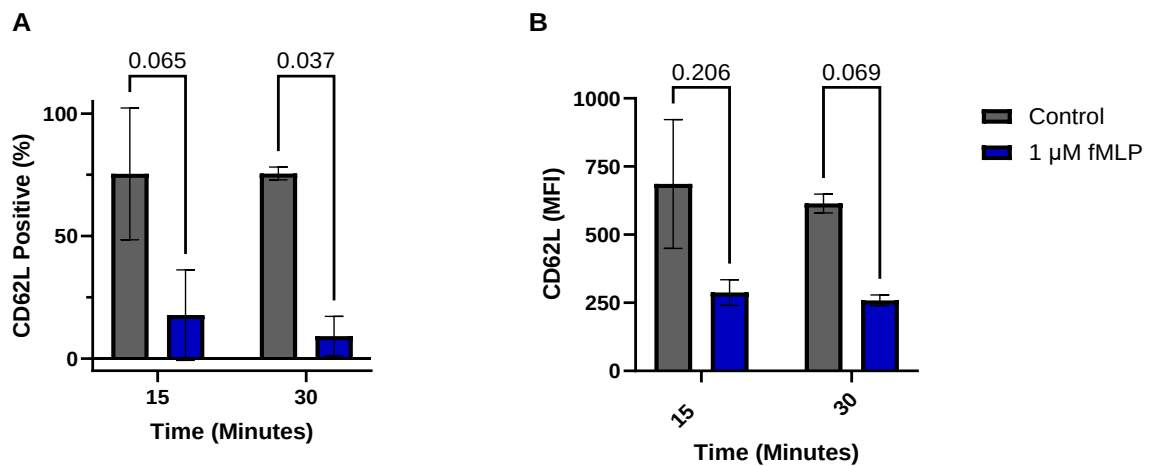


Figure 3.9: Effect of fMLP on CD62L expression on the neutrophil surface.

Neutrophils isolated from healthy donors ($n = 2$) were cultured *ex vivo* with RPMI alone (control) or RPMI containing 1 μ M fMLP for 15 or 30 minutes. Cell surface expression of CD62L was assessed by flow cytometry. (A) shows the percentage of cells expressing CD62L on the cell surface. (B) represents corresponding median fluorescence intensities (MFI). Bars represent mean and standard deviation. Analyses were performed using paired T-tests. P-values are shown above the histograms.

The effect of the priming agent, TNF α on these markers was then assessed. TNF α (100 ng/ mL) appeared to increase CD11b and CD66b expression at 30 minutes, but was not statistically significant (n = 2, paired T-test) (Figure 3.10).

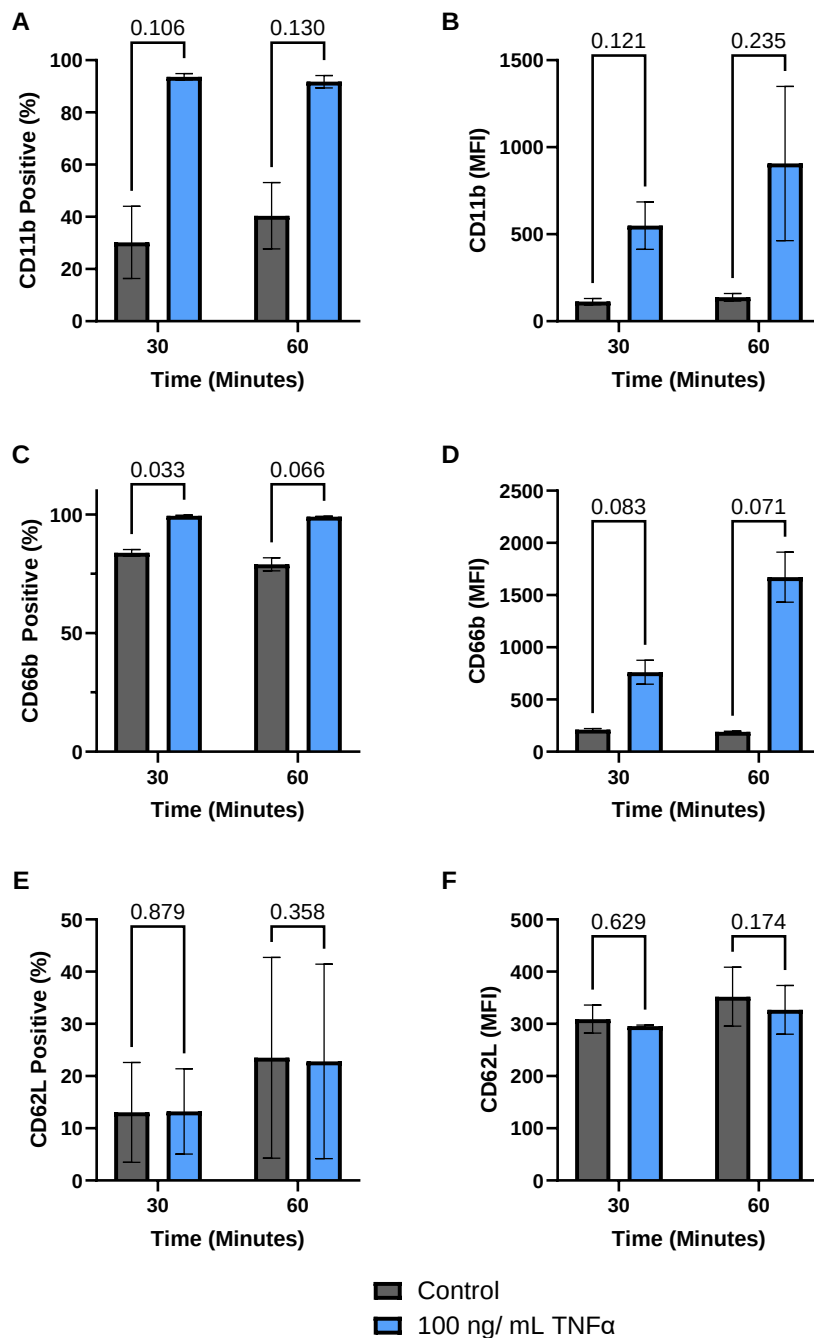


Figure 3.10: Effect of TNFα on the expression of cell surface markers of neutrophil priming.

Neutrophils isolated from healthy donors (n = 2) were cultured *ex vivo* with media alone (control) or media containing 100 ng/mL TNFα for 30- 60 minutes. Cell surface expression of CD11b (A and B), CD66b (C and D) and CD62L (E and F) were assessed by flow cytometry. Percentage of positive cells and median fluorescence intensity (MFI) are presented for each marker. Bars represent mean and standard deviation. Analyses were performed using paired T-tests. P-values are shown above the histograms.

3.4.6. CXCR2 blocking/internalisation on chemokine (IL-8) binding is detected by flow cytometry.

As shown above (Chapter 3.4.3), 100 nM IL-8 (which binds to CXCR2) was shown to drive neutrophil chemotaxis and chemokinesis. This cytokine was therefore used as a positive control for demonstrating CXCR2 blocking/internalisation. Vehicle control described in the remaining flow cytometry experiments contained RPMI 1640 supplemented with 0.002% BSA-PBS. The percentage of neutrophils that expressed CXCR2 on the cell surface was significantly reduced after 5 minutes of exposure (and persisted to 60 minutes) to 100 nM IL-8 compared vehicle control (Figure 3.11: mean 4% (SD 4.4) versus vehicle control = 62.6% (SD 17.8); $p = 0.004$, $n = 4/5$, paired T-tests).

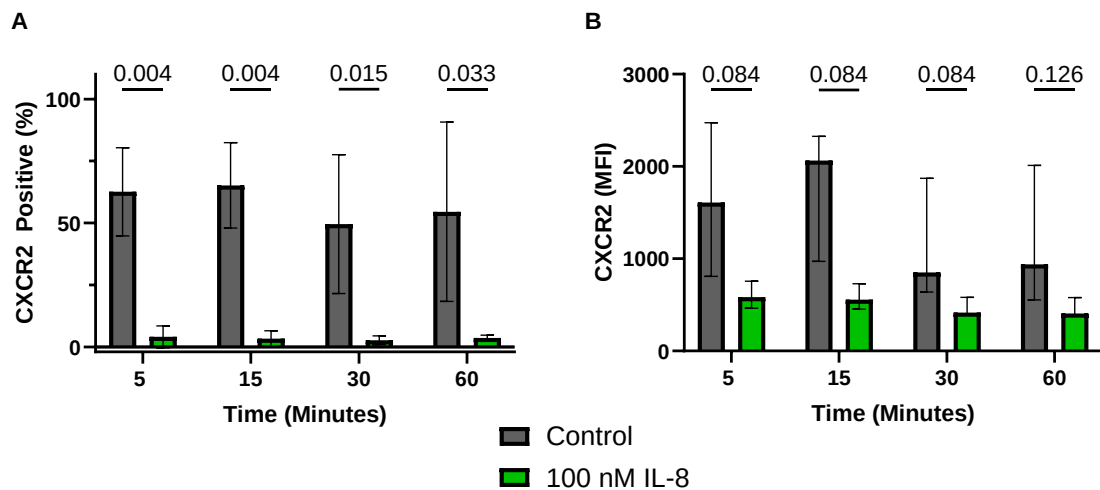


Figure 3.11: Effect of IL-8 on neutrophil surface expression of CXCR2.

Neutrophils isolated from healthy donors (n = 4/ 5) were cultured *ex vivo* with vehicle control or 100 nM IL-8 for 5 – 60 minutes. Cell surface expression of CXCR2 was assessed by flow cytometry following staining with PE conjugated anti-CXCR2 antibody and fixation with 2% paraformaldehyde.

Percentage of cells expressing CXCR2 (A) was significantly reduced after 5 minutes of exposure to 100 nM IL-8 compared to vehicle control. Bars represent mean and standard deviation. Statistical analyses were performed using paired T-tests.

(B) displays CXCR2 median fluorescence intensity (MFI). Bars represent median and interquartile range. Wilcoxon matched-pairs signed rank test was used. P-values are shown above the histograms.

3.4.7. Cell surface markers of neutrophil maturity, activation, ageing and tissue naivety; effects of stimulation with IL-8.

One hundred nM IL-8 was chosen to test changes in cell surface markers *in vitro* that are in the immunophenotyping panel 3 (CD16, CD11c, PD-L1, CD10, CXCR4 and CD54). The rationale is that neutrophil responses to 100 nM IL-8 (amplified chemotaxis, NET formation and CD11b and CD66b expression) have been confirmed. Therefore, it was postulated that this stimulus would also increase other markers that translocate to the cell surface on activation, such as CD11c, PD-L1 and CD54.

One hundred nM IL-8 had no effect on CD16 expression over the 5 to 60 minutes exposure time (Figure 3.12A and Figure 3.12B: $p > 0.05$, $n = 4/6$, Wilcoxon matched-paired signed rank tests). In parallel with the activation markers (CD11b and CD66b) that were examined earlier, 100 nM IL-8 significantly increased the percentage of neutrophils expressing CD11c compared to vehicle control over 30 minutes (Figure 3.12C: mean 98.9% (SD 0.65) versus vehicle control = 90.7% (SD 6.3), $p = 0.019$, paired T-test, $n = 6$) and increased CD11c MFI at all time-points (Figure 3.12D: $n = 4 - 6$, paired T-tests). In addition, PD-L1 expression was significantly increased by 100 nM IL-8 (both percentage positive cells and MFI) from 5 to 60 minutes (Figure 3.12E and Figure 3.12F: $n = 4 - 6$, paired T-tests).

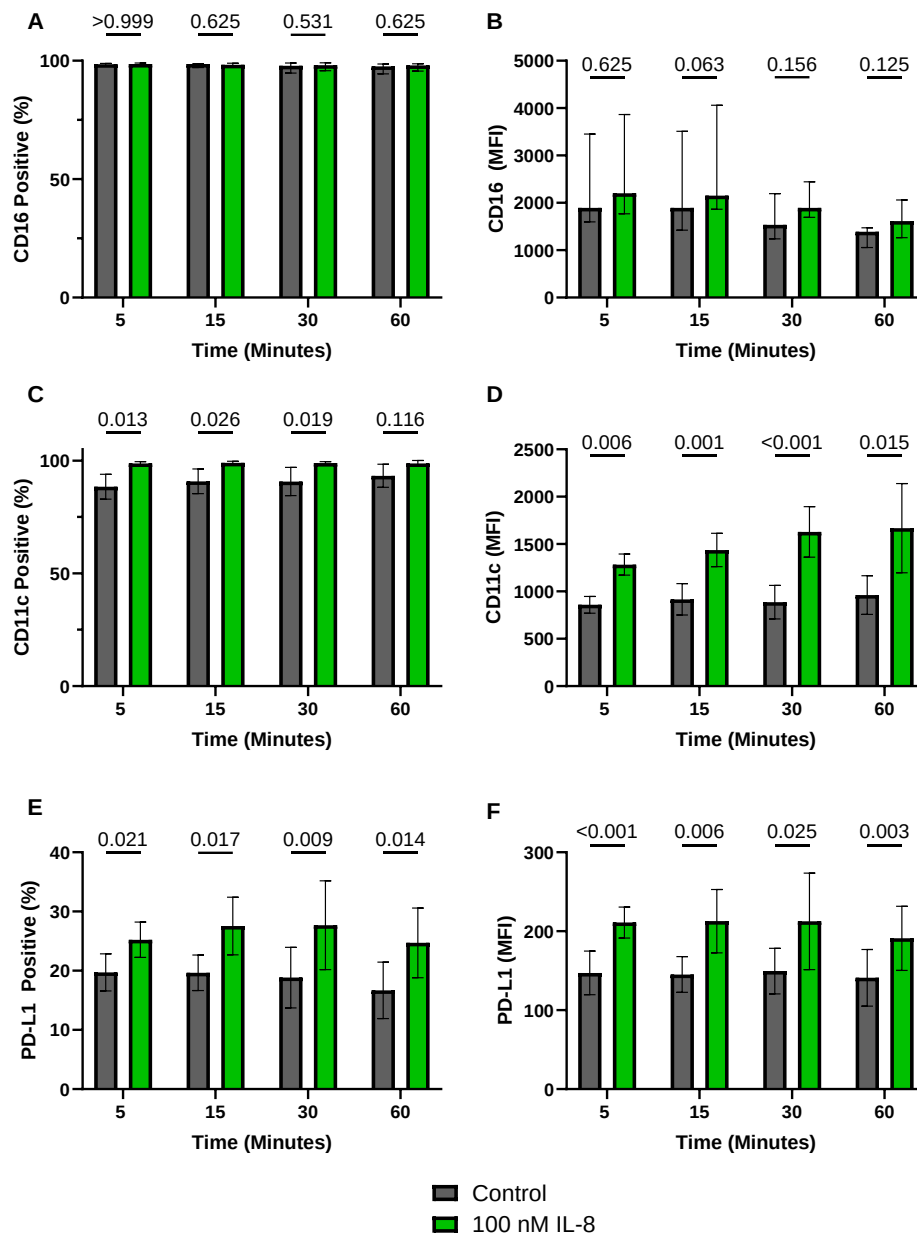


Figure 3.12: Effect of IL-8 on neutrophil surface expression of CD16, CD11c and PD-L1.

Neutrophils isolated healthy donors (n = 4/ 6) were cultured *ex vivo* with media containing vehicle control or 100 nM IL-8 for 5 – 60 minutes. Cell surface expression of CD16 (A and B), CD11c (C and D) and PD-L1 (E and F) were assessed by flow cytometry following staining with the immunophenotyping panel 3 antibody cocktail and fixation with 2% paraformaldehyde. Percentage of positive cells and median fluorescence intensity (MFI) are presented for each marker. Bars represent mean and standard deviation, except for CD16 (skewed data - presented as median and interquartile range). Analyses were performed using paired T-tests, except for CD16 where Wilcoxon matched-pairs signed rank test was used. P-values are shown above the histograms.

The MFI of CD10 was significantly increased on exposure to 100 nM IL-8 compared to vehicle control over all timepoints (Figure 3.13B: 60 minutes: 2036 (SD 329) versus control = 1053 MFI (SD 196); $p = 0.002$, paired T-test, $n = 4$).

CXCR4 expression (percentage positive cells and MFI) was significantly increased on exposure to 100nM IL-8 at all timepoints (Figure 3.13C and Figure 3.13D: $n = 4 - 6$, paired T-tests), as well as the percentage of neutrophils and MFI expressing CD54 (Figure 3.13E: p -values < 0.05 , $n = 4 - 6$, paired T-tests).

The data for each timepoint is summarised in Figure 3.13.

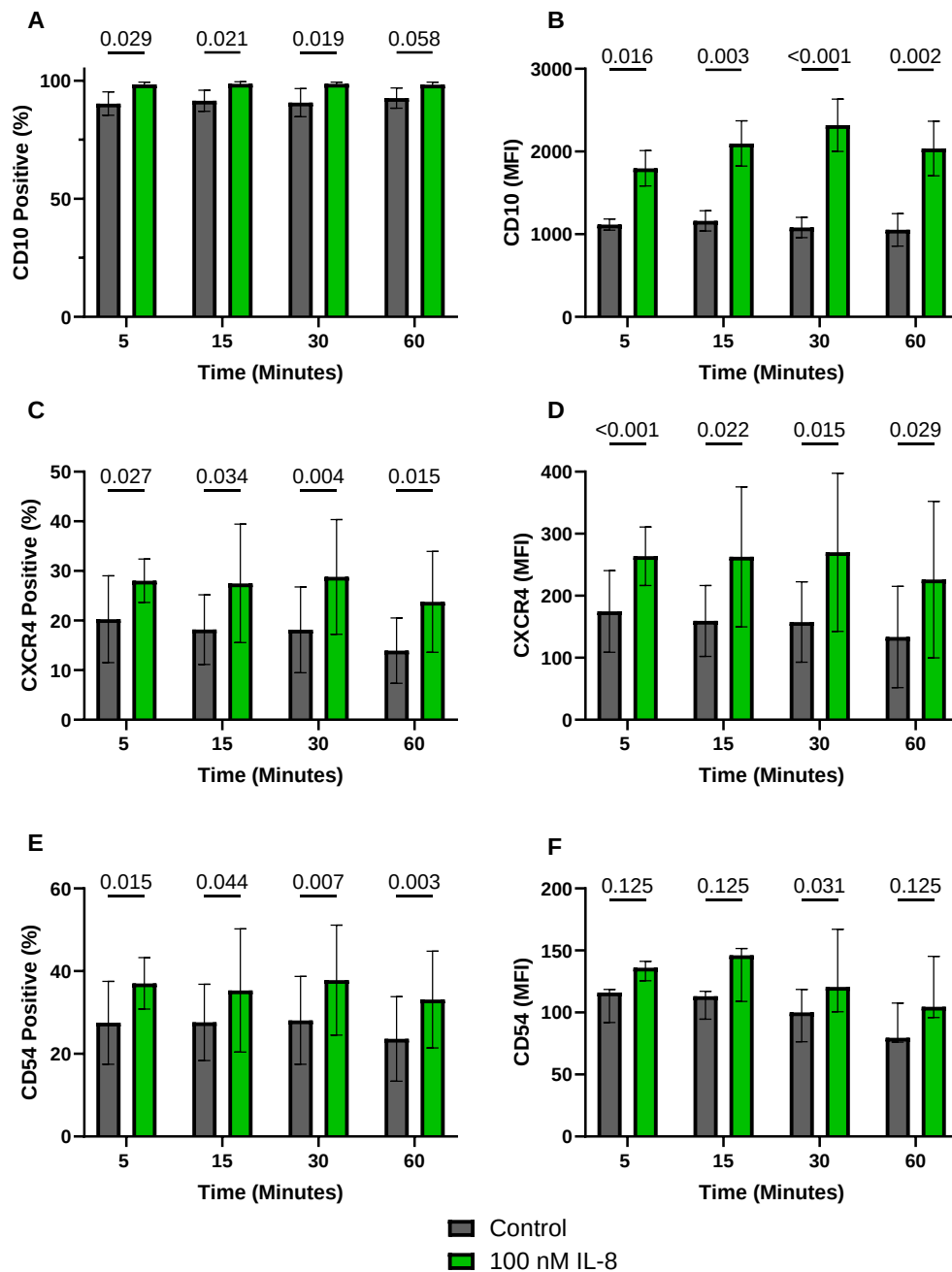


Figure 3.13: Effect of IL-8 on neutrophil surface expression of CD10, CXCR4 and CD54.

Neutrophils isolated from healthy donors (n = 4/ 6) were cultured *ex vivo* with media containing vehicle or 100 nM IL-8 for 5 – 60 minutes. Percentage of positive cells and median fluorescence intensity (MFI) are presented for each marker. Histograms represent mean and standard deviation, except for CD54 MFI (skewed data - presented as median and interquartile range). Analyses were performed using paired T-tests, except for CD54 MFI where Wilcoxon matched-pairs signed rank test was used. P-values are shown above the histograms.

3.5. Discussion

This chapter has demonstrated that the experiments can model neutrophil phenotype and function *ex vivo*. This includes firstly showing that both viability assays, annexin V/PI and Zombie NIR™ fixable stain, are equally able to identify live and dead cells. This gives confidence in using the latter technique in experiments where cells need fixation.

Secondly, the data demonstrated that both the transwell migration system and Insall chamber assay are able to model neutrophil movement *ex vivo*, with IL-8 demonstrating chemotactic capabilities as shown previously [236].

Thirdly, NET release could be induced by PMA, IL-8 and IL-1 β demonstrated by increased cfDNA release also visualised using fluorescence microscopy. The concentrations, 5 nM IL-8 and 2.9 nM IL-1 β , significantly increased cfDNA release and will be used as more physiological stimuli, alongside 25 nM PMA, to assess NET formation in IPF and age-matched neutrophils in subsequent chapters.

Assessment of surface marker expression on neutrophils is an important aspect of investigating the effects of TGF β 1 and understanding potential neutrophil phenotypes in IPF. Here, the three immunophenotyping panels devised to assess several relevant phenotypes have demonstrated responsiveness to stimuli. IL-8 led to the rapid reduction in CXCR2 cell surface expression, suggestive of receptor binding and/or internalisation. CD16 was expressed on nearly 100% of neutrophils, which was unaffected by 100 nM IL-8 confirming its valid use as a neutrophil identifier. CD11b, CD66b and CD11c increased on stimulation with IL-8 (as expected) and CD62L reduced with fMLP [71,277,278]. The following markers also increased with IL-8 treatment; PD-L1, CD10, CXCR4 and CD54, which has not been previously reported

and their role and response in the presence of inflammatory states is worthy of further study. The cell surface expression of PD-L1 and CD54 increases on stimulation with other molecules, such as LPS and GM-CSF, respectively [260,268]. Further investigation of this, is outside the scope of the current thesis.

Chapter 4. INVESTIGATING THE EFFECT OF TGF β 1 ON THE CHEMOKINETIC AND CHEMOTACTIC FUNCTIONS OF CIRCULATING NEUTROPHILS.

4.1. Abstract

Neutrophil contents, such as neutrophil elastase and markers of neutrophil extracellular traps, are elevated in fibrotic lung tissue [19,279]. This suggests that an altered chemokine environment is present in the circulation and tissue that promotes neutrophil migration to the fibrotic lung. However, the key effectors of neutrophil movement in this disease system are unknown.

The pro-fibrotic growth factor, TGF β 1, is significantly elevated in the circulation and lungs of IPF patients [28,29,280]. It is unclear whether TGF β 1 is a neutrophil chemoattractant [281–283]. The effects of TGF β 1 on the movement of healthy donor neutrophils is investigated *ex vivo* in this chapter.

This data demonstrates that TGF β 1 increases neutrophil chemokinesis. This effect is likely representative of neutrophils transitioning into a primed state, with TGF β 1 also amplifying chemokinetic and chemotactic responses to the chemoattractants, IL-8 and fMLP, respectively. TGF β 1 likely acts via the TGF β type 1 receptor and phosphorylates the canonical signalling molecules, Smad2/3.

4.2. Background

The pro-fibrotic growth factor, TGF β 1, is elevated in the circulation and lung tissue of patients with IPF and plays a critical role in disease pathophysiology [28,29,280]. In Chapter 1, the concept of TGF β 1 activation, including its importance in TGF β 1 bioavailability was introduced. Now, TGF β 1 signalling will be discussed together with our current understanding of how it impacts neutrophil effector functions and movement.

4.2.1. TGF β 1 activation and signalling

TGF β 1 is produced as a pro-peptide, which is subsequently cleaved into a small latent complex incorporating mature TGF β non-covalently associated to a latency associated peptide (LAP) (See Figure 4.1A) [284,285]. This small latent complex then binds to latent TGF β binding proteins to form a large latent complex [286]. This large latent complex is secreted extracellularly and rapidly stored in the extracellular matrix [287]. Prior to TGF β binding to cell surface receptors, it needs to be activated, which occurs on release from the LAP [288,289]. There are numerous mechanisms liberating TGF β from its latent form, such as proteolytic release by MMPs [181] and NE [162] (as discussed in Chapter 1.11.3), integrin activation [290,291], and alterations in the microenvironment, such as extracellular acidification (a common means of TGF β activation *in vitro*) [292].

Active TGF β homodimers initiate TGF β signalling by binding to the cell membrane serine and threonine kinase receptors, TGF β type 1 and 2 receptors (TGF β R1, also known as alk5, and TGF β R2), forming a heterocomplex [293] (Figure 4.1A). TGF β R2 binding phosphorylates and activates TGF β R1, which then mediates intracellular signalling. TGF β receptor internalisation by endocytosis has been observed in various cell types, which is amplified on TGF β binding, but can also occur in the absence of a ligand [294–297]. Receptor internalisation is not mandatory for downstream signalling but can both increase downstream signalling or suppress TGF β activity as receptor binding is reduced [296,298].

There are two broad signalling pathways driven by TGF β , namely canonical (Smad dependent) and non-canonical (Smad independent) (see Figure 4.1B). In canonical signalling, TGF β R1 binds and phosphorylates Smad proteins (Smad2/3), which interact with Smad4 and shuttles to the nucleus to regulate transcription [293,299–301].

Alternatively, TGF β drives non-canonical (Smad-independent) signalling pathways [302]. These include activation of MAPK cascades, such as phosphorylated p44/42 MAPK via Ras/Raf/MEK1/2 or phosphorylated p38 MAPK via transforming growth factor beta- activated kinase 1 (TAK1) [303–306]. TGF β can also rapidly activate Pi3K resulting in AKT activation and regulation of protein synthesis via mammalian target of rapamycin (mTOR) [307,308]. Crosstalk between these canonical and non-canonical pathways that was initially considered mutually exclusive is now also thought to take place [309,310]. These pathways are well characterised in other cell types within the fibrotic microenvironment, such as fibroblasts and epithelial cells (see Chapter 1) but remain unclear in neutrophils. Our current understanding of the effect of TGF β on neutrophil signalling and activity is described below.

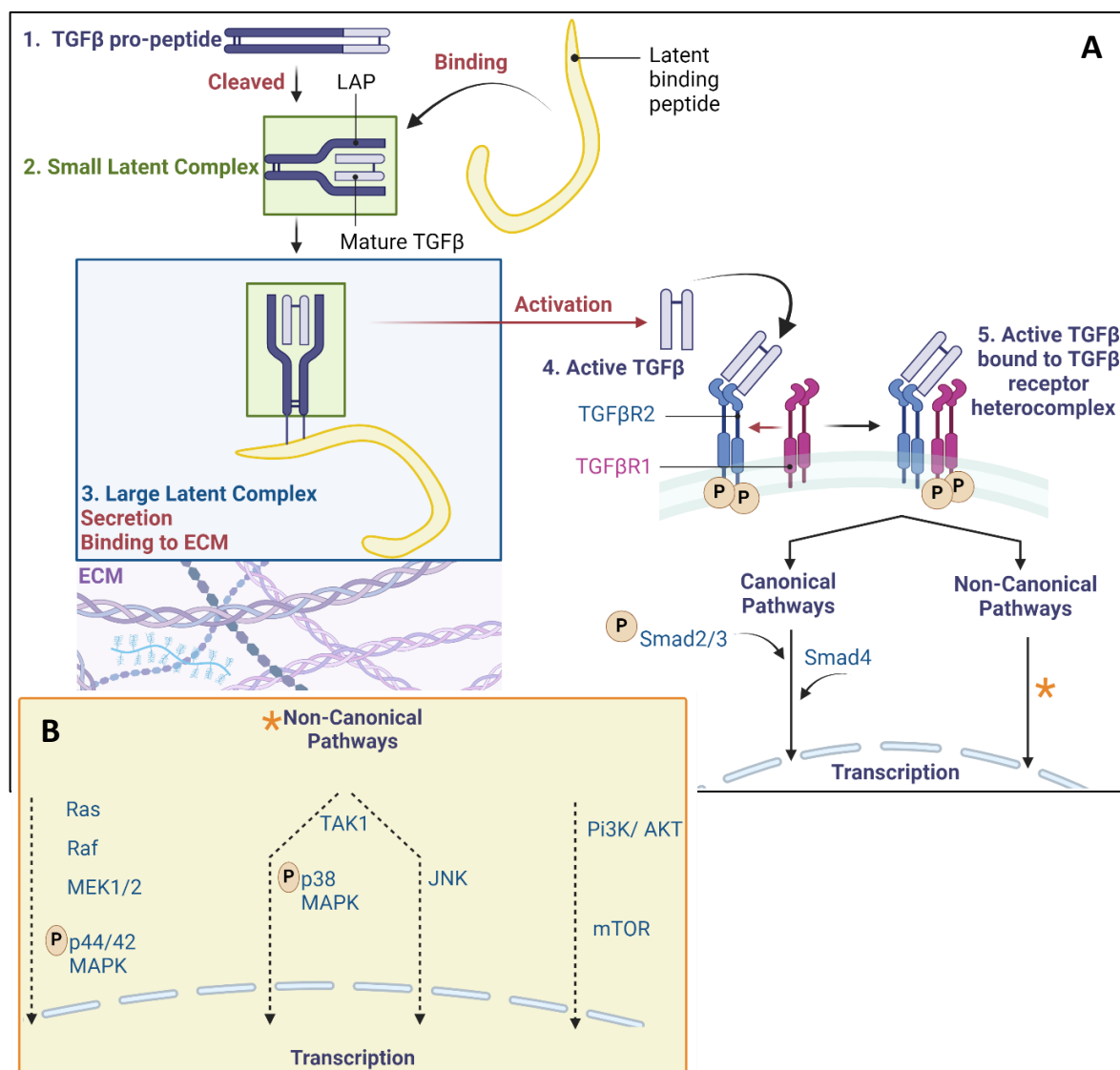


Figure 4.1: TGFβ activation and signalling.

Figure 4.1A are graphical representations of TGFβ activation and signalling pathways. (1) The TGFβ1 pro-peptide is (2) cleaved into a small latent complex which is made up of mature TGFβ non-covalently associated to a latency associated peptide (LAP) [284,285]. This small latent complex binds to latent TGFβ binding proteins to form a (3) large latent complex [286]. This large latent complex is secreted extracellularly and rapidly stored in the extracellular matrix (ECM). (4) TGFβ is activated following release from the LAP and can then bind to TGFβR2 [288,289]. (5) TGFβR1 and TGFβR2 form a heterocomplex [293], which results in phosphorylation and subsequent activation of TGFβR1. Intracellular signalling takes place via canonical (Smad dependent) or non-canonical (Smad independent) pathways.

Figure 4.1B describes examples of common TGFβ driven non-canonical signalling pathways, such as activation of MAPK cascades and regulation of protein synthesis via Pi3K/AKT/mTOR [302]. JNK – c-jun N-terminal kinases, LAP – latency associated peptide, mTOR – mammalian target of rapamycin, Pi3K – phosphoinositide 3- kinase, TAK1 - transforming growth factor beta-activated kinase 1. *Figure created using BioRender.com.*

4.2.2. TGFβ1 signalling in neutrophils.

The effects of inflammatory cytokines/chemokines, such as TNFα and IL-8, on neutrophil priming and/or activation, including their critical role in anti-microbial responses are well documented. However, this is not the case for TGFβ. Evidence of TGFβ receptor expression in human neutrophils is weak, which may be compounded by the fragility of neutrophils *ex vivo* and the selection of antibodies that target these epitopes poorly. TGFβR1 and TGFβR2 ribonuclease transcripts have been detected by real-time quantitative polymerase chain reaction [311]. Brandes *et al* found using affinity binding studies that primarily TGFβR1 was present on human neutrophils [312].

The signalling pathways downstream of these receptors are also poorly described in neutrophils. A recent study observed that 10- 75 minutes exposure to 2 ng/ mL TGFβ1 increased expression of pSmad3c in human neutrophils compared to baseline [313]. There was also greater phosphorylation of the non-canonical signalling molecule, p44/42 MAPK, in neutrophils exposed for 20 minutes to TGFβ1 as compared to baseline [313]. However, the authors did not report whether TGFβ1 increased phosphorylation relative to vehicle control at either of these timepoints, despite doing so in other cell lines, questioning the validity of these conclusions.

A separate study reported that p38-MAPK signalling is important in TGFβ1 mediated neutrophil chemotaxis [314]. Although as discussed later, the chemotactic effects of TGFβ1 are still unclear.

4.2.3. Does TGFβ1 affect neutrophil effector functions?

Cancer research has helped support the notion that neutrophils are responsive to TGFβ, with TGFβ thought to direct neutrophils into a “N2” pro-tumourigenic phenotype [315]. Mouse model data suggested that TGFβ1 does this by promoting transformation into low-density neutrophils that have reduced cytotoxic, migratory and oxidative burst capabilities [315,316]. Elsewhere, Shen *et al* similarly noted that TGFβ1 reduces LPS induced lactoferrin (a glycoprotein present in neutrophil granules) release, which was reversed by TGFβR1 inhibition [317]. However, this was only evident at lower concentrations of TGFβ1 (1- 100 picograms per millilitre (pg/ mL)) [317].

The suppressive effects of TGFβ1 on neutrophil function have been infrequently replicated. Many studies have demonstrated no effect on neutrophil degranulation or ROS production [312,318–320]. In contrast, Lagraoui *et al* reported that TGFβ1 increased neutrophil phagocytosis and respiratory burst [321]. Another study noted that pg/ mL concentrations of TGFβ1 promoted lactoferrin and hydrogen peroxide (a type of ROS) release when neutrophils were adherent to fibrinogen [322]. Differences in the experimental environment, such as neutrophil adherence via integrin binding, could account for the differential responsiveness to TGFβ1 *in vitro* and hence requires further validation. TGFβ1 may have other effects, such as working synergistically with GM-CSF to suppress neutrophil apoptosis and promote granulopoiesis [207,323]. This may be of importance in the fibrotic microenvironment, which in conjunction with hypoxia mediated HIF1α upregulation, could increase neutrophil survival [43].

NET formation has been observed in fibrotic lung tissue, but the factors responsible remain unknown including the potential role of TGFβ1 [19]. One study (using fluorescence

microscopy) observed that more cells released NETs in response to TGF β , but other measures of NET release (myeloperoxidase and citrullinated histone 3 release into the supernatant) were unaffected [186]. This suggests that the role of TGF β 1 in NET formation in IPF is worthy of further study.

4.2.4. Does TGF β 1 affect neutrophil movement?

In 1991 Fava *et al* reported that intra-articular injection of TGF β 1 promoted local neutrophil recruitment in rats, and *in vitro* TGF β 1 appeared to be more potent than fMLP in driving neutrophil movement within a Boyden chamber [324]. Several papers since have observed that TGF β 1 drives human neutrophil chemotaxis using the under agarose method; with maximal effects at approximately 40 femtomolar (fM) [320,325]. However, Chong *et al* demonstrated that TGF β 1 only promoted neutrophil movement through a polyester transwell *in vitro* at a concentration of 78.1 pM (equivalent of 1 ng/ mL) TGF β 1, with no effect between 7.8 fM – 7.8 pM (equivalent of 0.1 – 100 pg/ mL) [326]. Parekh *et al* noted that all three isoforms of TGF β (-1, -2 and -3) stimulated neutrophil movement in the under agarose method at fM concentrations [325].

TGF β acting as an additional chemoattractant in the fibrotic microenvironment is thus plausible. However, TGF β 1 inhibition in one study did not attenuate the chemotactic effects of supernatant derived from human lung fibroblasts that had been treated with bleomycin [327]. Whereas, anti-IL-8 and anti-G-CSF antibodies did reduce this response [327] suggesting minimal TGF β 1 contribution to the overall effect. In addition, others noted that TGF β 1 had no

chemotactic activity *in vitro* providing further conflicting evidence of an effect on this process [282,328].

If TGF β 1 does affect neutrophil movement, the mechanism remains unclear.

Like many established chemoattractants, TGF β 1 does appear to drive redistribution and polymerisation of actin filaments, which may be via the p38 MAPK pathway [314,320]. However, unlike fMLP, TGF β 1 does not appear to drive neutrophil movement by G protein activation or calcium mobilisation [320].

Furthermore, as described earlier, unlike potent classical chemoattractants, including IL-8 and fMLP, TGF β 1 does not appear to activate neutrophils [320].

Therefore, the current evidence remains unclear regarding any pro-migratory effects of TGF β 1 on human neutrophils, with any underlying mechanism and clinical significance in the context of pulmonary fibrosis unknown. Differences in the methodology of modelling neutrophil movement may account for the described variations in the literature. Neutrophil movement can be random (chemokinesis) or direction dependent (chemotaxis). The *in vitro* modelling systems used by previous studies (such as Boyden chambers/transwells) are unable to distinguish direction dependent movement. However, these can be discriminated using the Insall chamber assay described in Chapter 2.7.2, which is able to manually track neutrophils over the desired time course using time-lapse microscopy.

4.3. Hypothesis

TGF β 1 is a neutrophil chemoattractant.

4.4. Chapter Aims

1. To determine whether TGF β 1 affects the viability of neutrophils *in vitro*.
2. To determine whether TGF β 1 affects neutrophil chemotaxis and/or chemokinesis *in vitro*.
3. To determine whether TGF β 1 primes neutrophils, resulting in an amplified chemotactic response to classical chemoattractants (IL-8 and fMLP).
4. To investigate the mechanism by which TGF β 1 affects neutrophil movement.

4.5. Materials and methods linked to Chapter 4

4.5.1. Ethical approval and study participants

All venous blood samples were collected from volunteers recruited to the 'Investigations of the Ageing Immune System' study (10/H1208/48). Participants were self-reported non-smokers, healthy and aged between 18- 60 years of age.

4.5.2. Neutrophil assays

Briefly neutrophil isolation was performed as described in Chapter 2.4. Neutrophil viability was assessed by Annexin V/PI and Zombie NIR™ fixable viability staining as described in Chapter 2.6. Immunophenotyping and phosphoflow assays were performed as described in Chapter 2.10 and 2.12, respectively. The transwell and Insall chamber assays were performed as described in Chapter 2.7, and adjustment for slide movement was performed for all Insall chamber assay results presented here. NET release was assessed using a fluorescence plate reader and fluorescence microscopy as described in Chapter 2.8. ROS release was assessed by lucigenin-amplified chemiluminescence as described in Chapter 2.9.2.

4.5.3. Western blotting

Wester blotting was performed as described in Chapter 2.11.

4.5.4. TGFβ receptor 1 inhibitor

The ATP-competitive cell permeable inhibitor of TGFβR1, LY364947 (*Selleck, US: S2805*), was reconstituted in DMSO as described by others [329].

4.6. Results

4.6.1. The vehicle used to reconstitute TGFβ1 does not affect the pH of RPMI 1640 or HBSS.

Recombinant human TGFβ1 was reconstituted in filter sterilised 0.1% BSA-DPBS supplemented with 4 mM hydrochloric acid to ensure full recovery and activation of TGFβ1 [330]. The pH of RPMI 1640 and HBSS were unaltered by the addition of vehicle at both concentrations, 1: 1000 and 1: 2000.

4.6.2. TGFβ1 and vehicle do not affect neutrophil viability *ex vivo*.

The effects of TGFβ1 and vehicle on neutrophil viability over the maximum exposure time during the transwell and Insall chamber assays were assessed using Annexin V/PI and Zombie NIR™ fixable viability staining. Neutrophil viability (double negative for Annexin V and PI) over 60 minutes was unaffected by vehicle (both 1: 1000 and 1: 2000 dilutions) and 0.1 – 10 ng/mL TGFβ1 compared to RPMI alone (Figure 4.2A - D) ($p > 0.05$, Kruskal-Wallis test, $n = 4$). Furthermore, an average of >93% of neutrophils were alive (defined by the absence of Zombie NIR™ staining) following 90 minutes exposure to vehicle (described as 0 on the x- axis) or 0.001 – 10 ng/ mL TGFβ1 (Figure 4.2E) ($p > 0.05$, one-way ANOVA, $n = 3/ 4$).

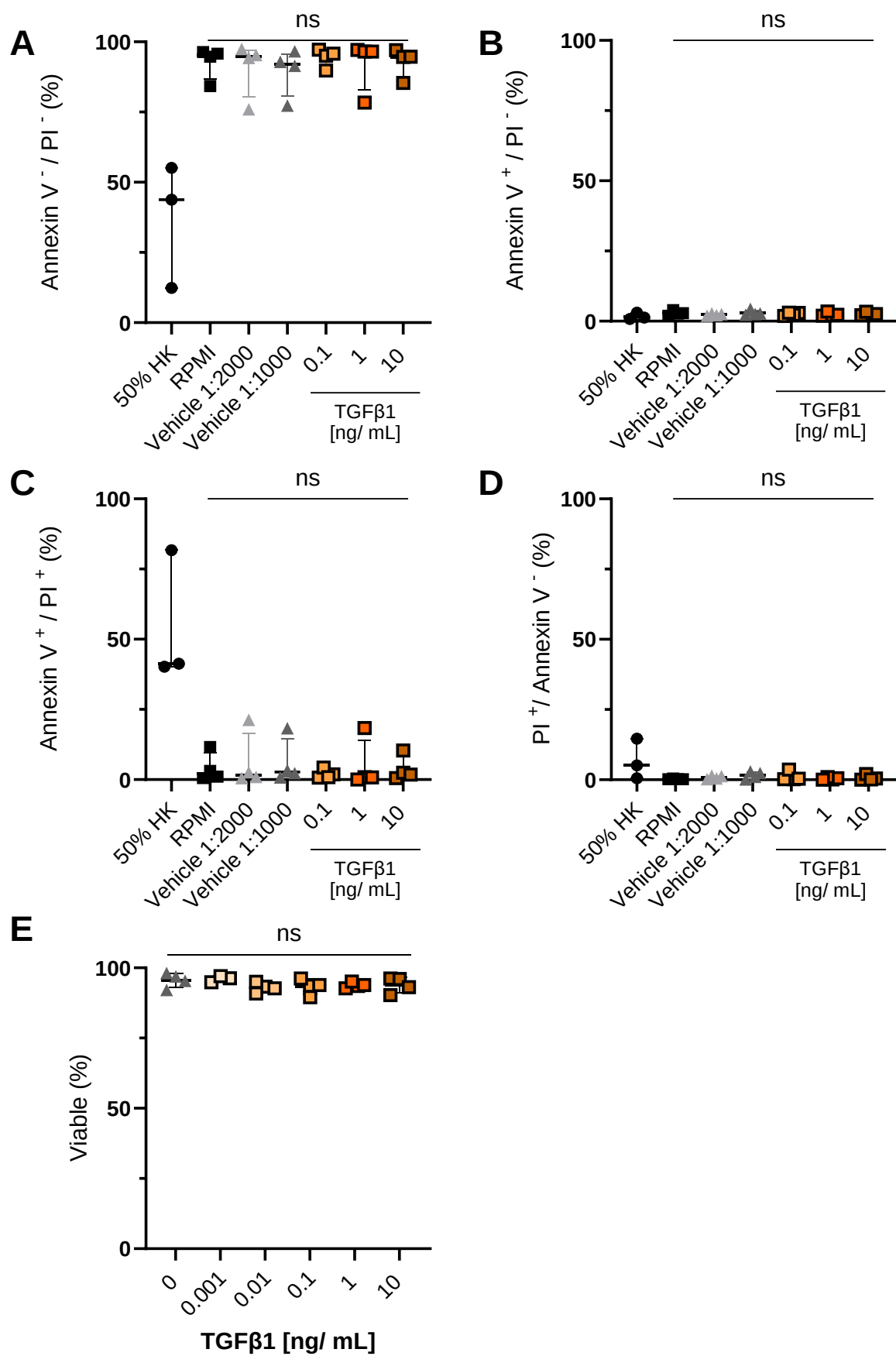


Figure 4.2: TGFβ1 does not affect neutrophil viability ex vivo over 60 to 90 minutes.

Using Annexin V/PI staining (A – D), isolated neutrophils from healthy participants (n = 3/ 4) were assessed for the percentage of cells that were viable (A), early apoptotic (B), late apoptotic (C) and necrotic (D) after 60 minutes exposure to 0.1 – 10 ng/ mL TGFβ1 or vehicle control. A positive control was also performed, labelled 50% heat killed (HK), which contained 50% neutrophils exposed to 70° C for 2 minutes and 15 seconds.

Using Zombie NIR™ fixable viability stain (E), isolated neutrophils from healthy participants (n = 3/ 4) were assessed for the percentage of viable cells (stain negative) after treatment with vehicle or 0.001 – 10 ng/ mL TGFβ1 for 90 minutes.

Data point per donor. Data bars are median and interquartile range for skewed data (A, C, D) or mean and standard deviation for normally distributed data (B and E). One-way ANOVA and Kruskal-Wallis tests were performed for normally distributed and skewed data, respectively.

4.6.3. Investigating the effect of TGF β 1 on neutrophil chemokinesis and chemotaxis *ex vivo*.

4.6.3.1. TGF β 1 increases neutrophil migration across transwell membranes.

The effect of TGF β 1 on neutrophil movement was first investigated using the transwell migration assay. Media containing vehicle or 0.001 to 10 ng/ mL TGF β 1 was added to the bottom well of a 24 well plate. The number of neutrophils that passed through the transwell membrane over 90 minutes was counted by flow cytometry. A greater number of neutrophils migrated through the membrane ($p = 0.014$, Dunnett's multiple comparisons, $n = 6$) in response to 1 ng/ mL TGF β 1 as compared to vehicle (mean TGF β 1 = 45638 cells (SD 20600) vs control = 33826 cells (SD 16128) (see Figure 4.3). However, the other concentrations of TGF β 1 did not significantly affect neutrophil migration ($p > 0.05$, Dunnett's multiple comparisons, $n = 6$).

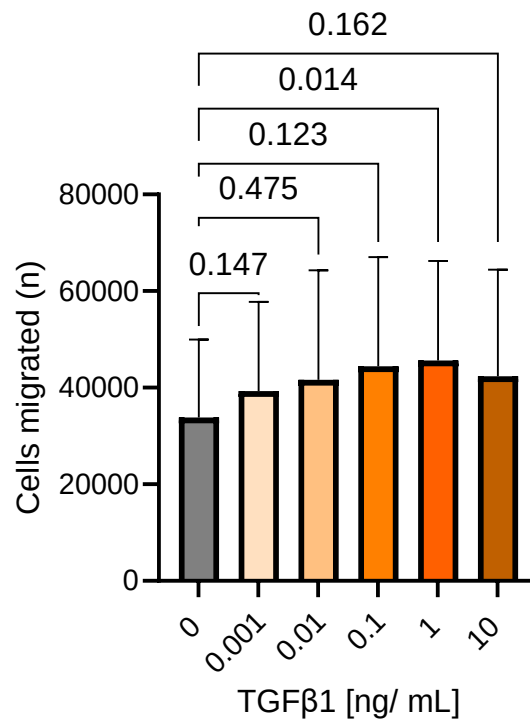


Figure 4.3: Effect of TGFβ1 on neutrophil migration across transwell membranes *ex vivo*.

Neutrophil transmigration was assessed in response to TGFβ1 using the transwell migration assay. One ng/ mL TGFβ1 increased the number of cells that had migrated across the membrane as compared to vehicle ($p = 0.014$, $n = 6$). Although other concentrations of TGFβ1 did not. The significance of changes for each dose is shown on the figure compared to control. Data histograms are mean with standard deviation bars. Kruskal-Wallis test and Dunnett's multiple comparisons tests were performed.

4.6.3.2. TGF β 1 increases neutrophil chemokinesis, but not chemotaxis.

The effect of TGF β 1 on neutrophil movement was then assessed using the Insall chamber assay, to provide further information on any impact of TGF β 1 on direction dependent (chemotaxis) and/or independent (chemokinesis) manner. The chemoattractant well was filled with increasing doses of TGF β 1 or equal vehicle (diluted 1: 1000) to create a chemoattractant gradient and neutrophils were tracked over 12 minutes as described in Chapter 2.7.2. The direction independent parameters (speed, distance travelled and displacement) were significantly increased in response to 10 ng/ mL TGF β 1 as compared to vehicle ($p < 0.001$, Dunnett's multiple comparisons tests, $n = \geq 12$) (Figure 4.4A – C) but not the other concentrations. Direction dependent parameters (velocity and chemotactic index) and directness were all unaffected by TGF β 1 (Dunnett's multiple comparisons tests, $n = \geq 8$) (Figure 4.4D – F).

Representative images of neutrophils tracked using time- lapse microscopy within the Insall chamber in response to 100 nM IL-8 (positive control), 10 ng/ mL TGF β 1 and respective controls are displayed in Figure 4.5.

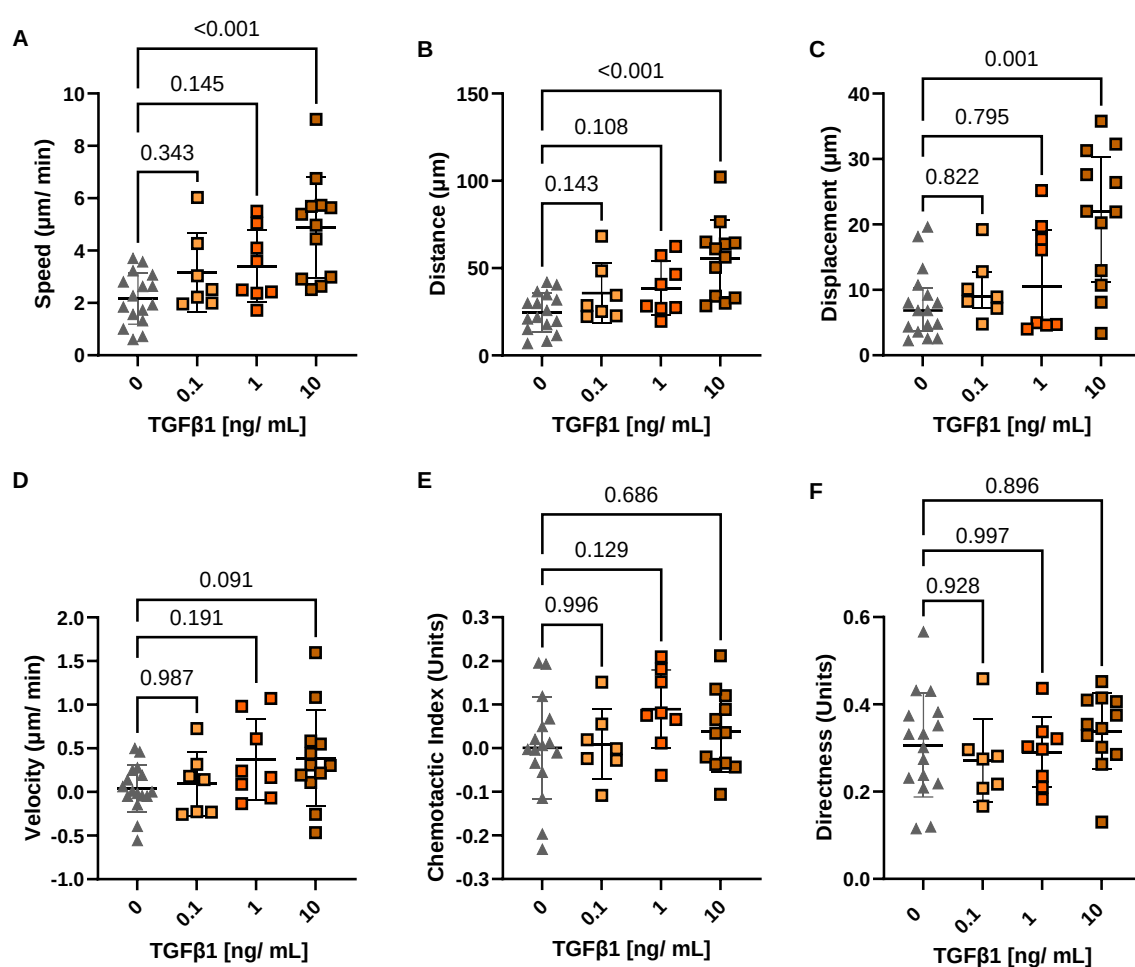


Figure 4.4: Effect of TGFβ1 on neutrophil chemokinesis and chemotaxis *ex vivo*.

Neutrophil chemotactic and chemokinetic responses to TGFβ1 using the Insall chamber assay. Neutrophils were isolated from healthy participants (n = 8 – 16). Data point per donor. Data bars are mean and standard deviation, except displacement, which is shown as median and interquartile range (due to non-normal distribution). One-way ANOVA and Dunnett's multiple comparisons tests were performed, except displacement where Kruskal-Wallis and Dunn's multiple comparisons tests were performed. Cell tracking was adjusted for background slide movement as described in Chapter 2.7.2.1.

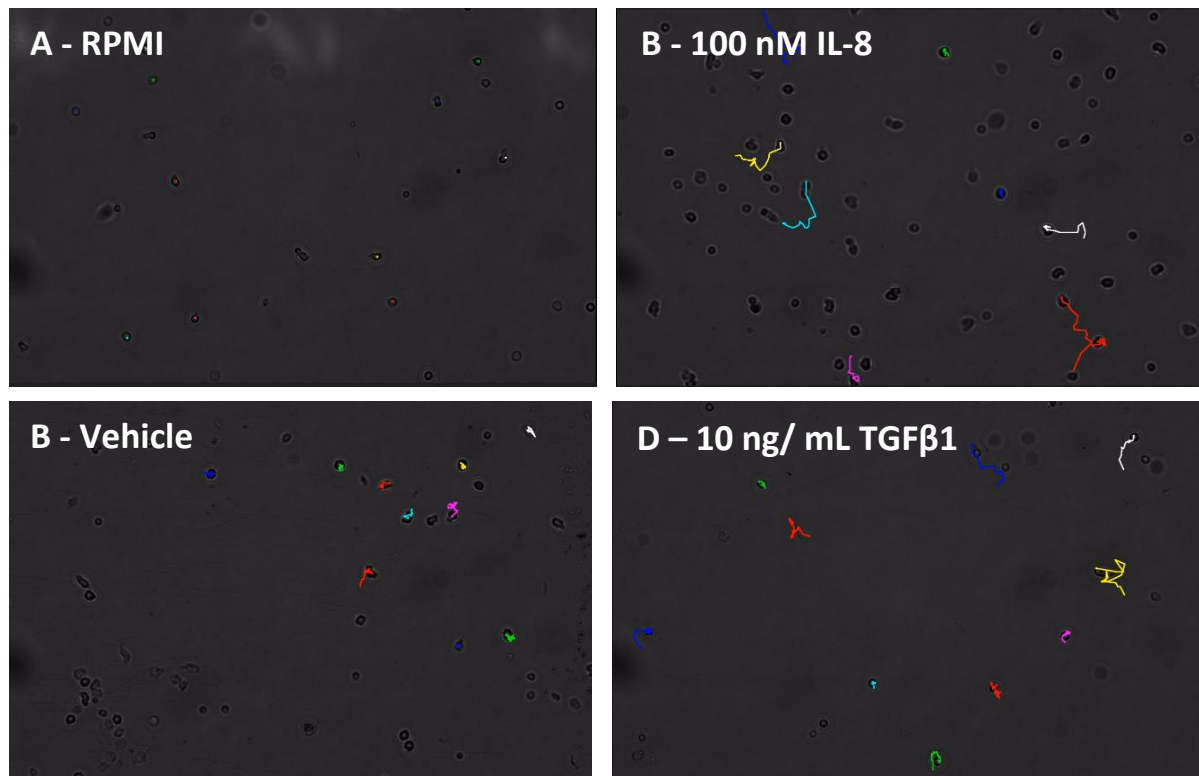


Figure 4.5: Representative images of neutrophils tracked within the Insall chamber using time-lapse microscopy.

Neutrophils isolated from a healthy participant were loaded to an Insall chamber slide containing either (A) RPMI, (B) 100 nM IL-8, (C) vehicle control for TGF β 1 or (D) 10 ng/ mL TGF β 1 within the chemoattractant well. This created a concentration gradient whereby a higher concentration of the chemoattractant under investigation was present at the upper area of each image. Neutrophil movement was tracked using the manual tracking plug-in on FIJI Image J using images taken by time- lapse microscopy (at 20x magnification). Each image here shows the tracks of 10 randomly selected neutrophils over 12 minutes of imaging.

The effect of 30 ng/ mL TGF β 1 on neutrophil movement was also investigated as this is reflective of the upper limit of the physiological range of TGF β 1 that has been seen in the BALF of IPF patients [331]. To create this upper TGF β 1 working concentration, a higher vehicle concentration was required (1: 333). This distinct vehicle concentration was tested at 30 ng/ mL TGF β 1 and thus the results of this are presented separately in Figure 4.6.

Speed and distance travelled were significantly increased by 30 ng/ mL TGF β 1 compared to vehicle ($p = 0.046$, paired T-tests, $n = 7$). However, 30 ng/ mL TGF β 1 did not significantly impact the other movement parameters ($p > 0.05$, paired T-tests, $n = 7$). This suggests that TGF β 1 preferentially affects neutrophil chemokinesis, rather than chemotaxis.

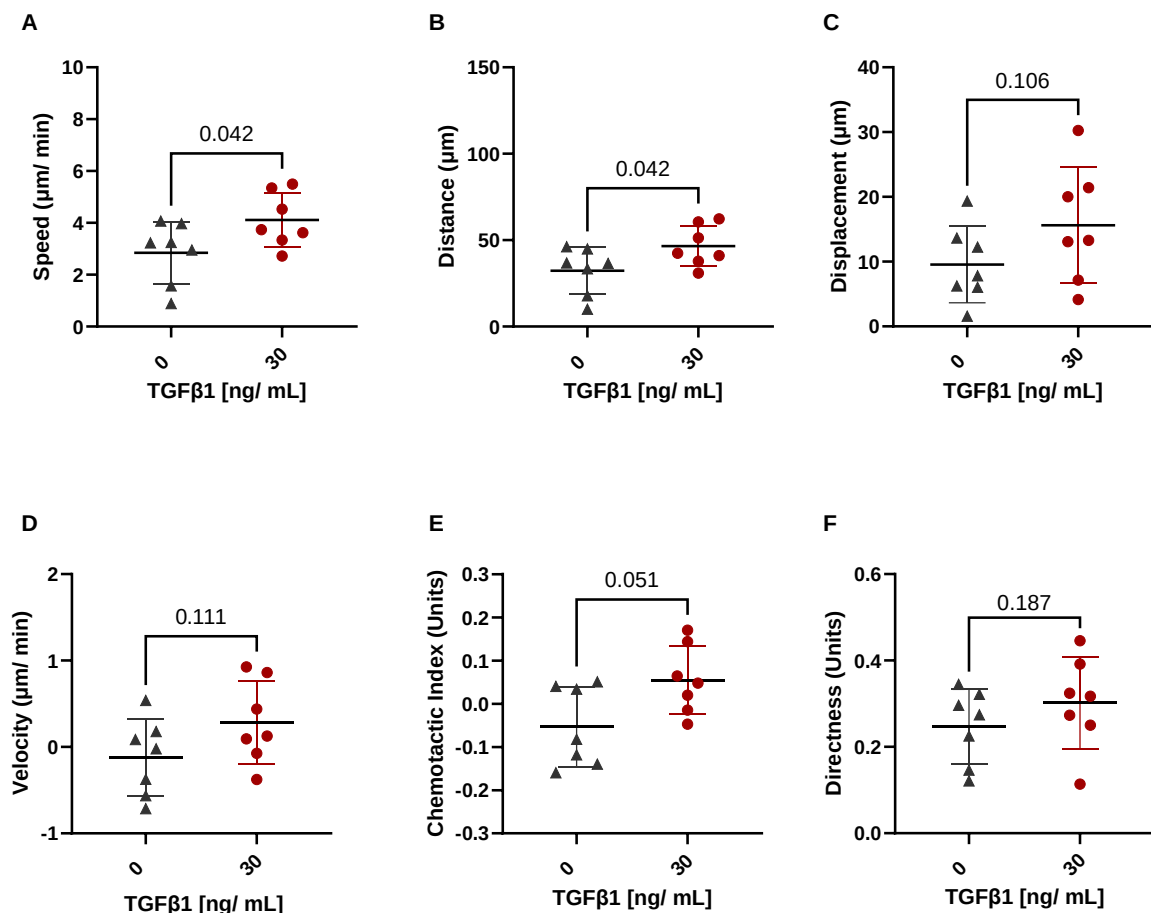


Figure 4.6: Effect of 30 ng/ mL TGFβ1 on neutrophil chemokinesis and chemotaxis *ex vivo*.

Neutrophil chemotaxis and chemokinesis in response to 30 ng/ mL TGFβ1 using the Insall chamber assay. Neutrophils were isolated from 7 healthy participants. Data point per donor. Data bars are mean and standard deviation. Paired T-tests were performed. Cell tracking was adjusted for background slide movement as described in Chapter 2.7.2.1. Thirty ng/ mL TGFβ1 significantly increased neutrophil speed (A) and distance travelled (B) compared to vehicle ($p = 0.042$). Displacement (C), velocity (D), chemotactic index (E) and directness (F) were not affected by 30 ng/ mL TGFβ1 ($p > 0.05$).

4.6.4. Does TGF β 1 prime neutrophils?

It was hypothesised that the chemokinetic effects of TGF β 1 reflected neutrophils transitioning into a primed state. This was investigated first by assessing if TGF β 1 treatment changed the expression of adhesion molecules and degranulation markers on the neutrophil surface that are affected by priming agents.

4.6.4.1. TGF β 1 does not alter the expression of the adhesion molecules: CD11b or CD62L.

Ten ng/ mL TGF β 1 had no effect compared to vehicle on the percentage expression or MFI of the cell surface adhesion markers, CD11b or CD62L (Figure 4.7: $p > 0.05$ paired T-tests or Wilcoxon matched-pairs signed rank tests, $n = 5$).

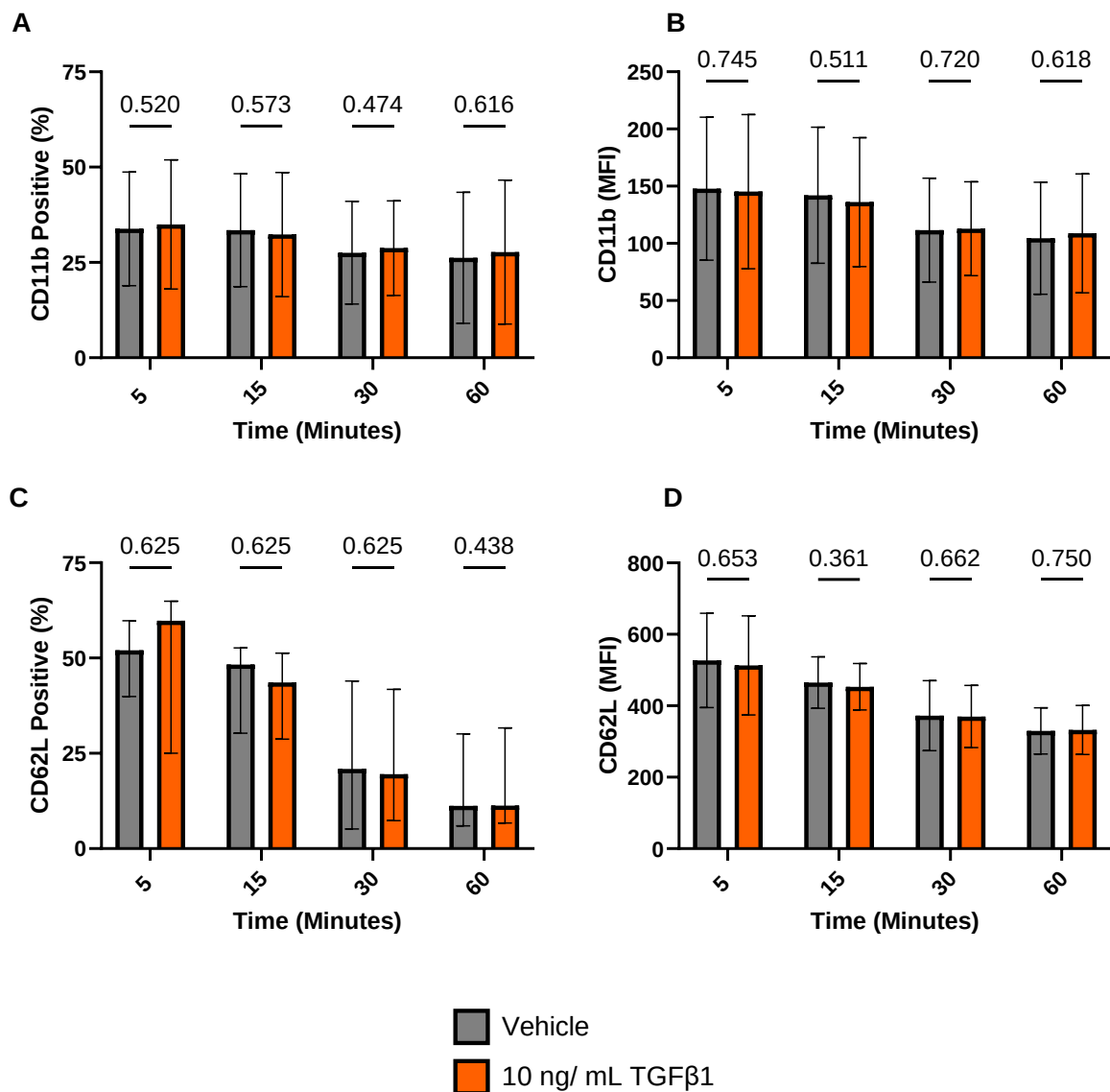


Figure 4.7: Effect of TGFβ1 on the neutrophil surface adhesion markers, CD11b and CD62L.

Neutrophils isolated from healthy donors (n = 5) were cultured *ex vivo* with media containing 10 ng/ mL TGFβ1 or vehicle for 5 – 60 minutes. Cell surface expression of CD11b (A and B) and CD62L (C and D) were assessed by flow cytometry following staining with the immunophenotyping panel 1 antibody cocktail and fixation with 2% paraformaldehyde. Percentage of positive cells and median fluorescence intensity (MFI) are presented for each marker. Histograms represent mean and standard deviation, except (C) which was median and interquartile range (due to skewed data). Analysis was performed using paired T-tests, except (C) where Wilcoxon matched-pairs signed rank test was used. P-values compared to control are shown above the matched histograms. There was no statistical difference in percentage expression or MFI of any of the cell surface markers between TGFβ1 and vehicle exposed neutrophils ($p > 0.05$).

4.6.4.2. TGF β 1 does not upregulate cell surface expression of CD66b.

Trafficking of CD66b from intracellular granules to the cell surface is a sign of degranulation.

Some priming agents, such as TNF α , can upregulate CD66b expression on their own, whereas others, including GM-CSF require a second stimulus [332].

CD66b expression on the cell surface, measured as percentage of positive cells (Figure 4.8A) and MFI (Figure 4.8B) were unaffected after 5 to 60 minutes exposure to 10 ng/ mL TGF β 1 ($p > 0.05$; paired T-tests or Wilcoxon matched-pairs signed rank tests, $n = 5$).

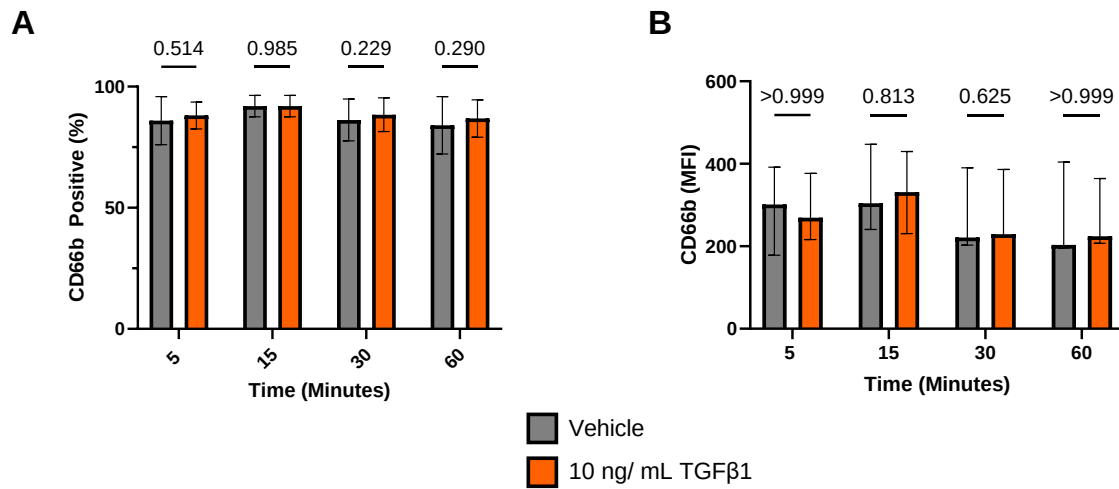


Figure 4.8: Effect of TGFβ1 on the degranulation marker, CD66b.

Neutrophils isolated from healthy donors (n = 5) were cultured *ex vivo* with media containing 10 ng/ mL TGFβ1 or vehicle for 5 – 60 minutes. Cell surface expression of CD66b was assessed by flow cytometry following staining with the immunophenotyping panel 1 antibody cocktail and fixation with 2% paraformaldehyde. (A) describes the percentage of cells expressing CD66b on the cell surface and (B) represents median fluorescence intensity (MFI). Histograms represent mean and standard deviation in (A). Paired T-test was performed in (A). Histograms represent median and interquartile range in (B). Wilcoxon matched pairs signed rank tests were performed in (B). P-values are shown above the matched histograms. There was no statistical difference in percentage expression or MFI of CD66b between TGFβ1 and vehicle exposed neutrophils.

4.6.4.3. *TGFβ1 amplifies chemotactic/chemokinetic responses.*

The effect of pre-treatment with TGFβ1 on neutrophil chemotactic responses to established chemoattractants was subsequently investigated using the Insall chamber assay. Neutrophils were initially treated with 10 ng/ mL TGFβ1 or vehicle for 15 minutes, centrifuged and resuspended in RPMI. Neutrophil movement within the Insall chamber was then assessed in response to 100 nM IL-8 and 10nM fMLP (as intermediate and end- target chemoattractants, respectively as described in Chapter 1.8.4).

Ten ng/ mL TGFβ1 pretreatment had differential effects on neutrophil responses to IL-8 and fMLP *in vitro*. TGFβ1 pretreatment significantly increased the speed (Figure 4.9A: mean = 7.1 μm/ min (SD 1.6) vs vehicle = 5.7 μm/ min (SD 1.3); $p = 0.02$, paired T-test, $n = 9$) and distance moved in response to 100 nM IL-8 (Figure 4.9B: mean = 80.1 μm (SD 17.8) vs vehicle = 64.6 μm (SD 15.0); $p = 0.02$, paired T-test, $n = 9$). The velocity of neutrophils towards 10 nM fMLP was significantly increased following exposure to 10 ng/ mL TGFβ1 compared to vehicle (Figure 4.10D: mean = 2.0 μm/ min (SD 1.4) vs vehicle = 1.0 μm/ min (SD 1.2); $p = 0.026$, paired T-test, $n = 9$). However, neutrophil directness in response to IL-8 or fMLP was not affected by 10 ng/ mL TGFβ1 ($n = 9$). These data indicates that TGFβ1 can prime neutrophils; amplifying chemokinetic and chemotactic responses to IL-8 and fMLP, respectively.

However, neutrophil movement was similar between those exposed to vehicle or 10 ng/ mL TGFβ1 for 15 minutes, then washed and added to the Insall chamber without any chemoattractant or TGFβ1 present within the system ($p > 0.05$, paired T-tests, $n = 9$). This is in contrast with the earlier findings in Chapter 4.6.3. whereby concurrent exposure to TGFβ1 increased neutrophil chemokinesis. This suggests that the effects of TGFβ1 on neutrophils requires continuous TGFβ1 bioavailability and is short lived.

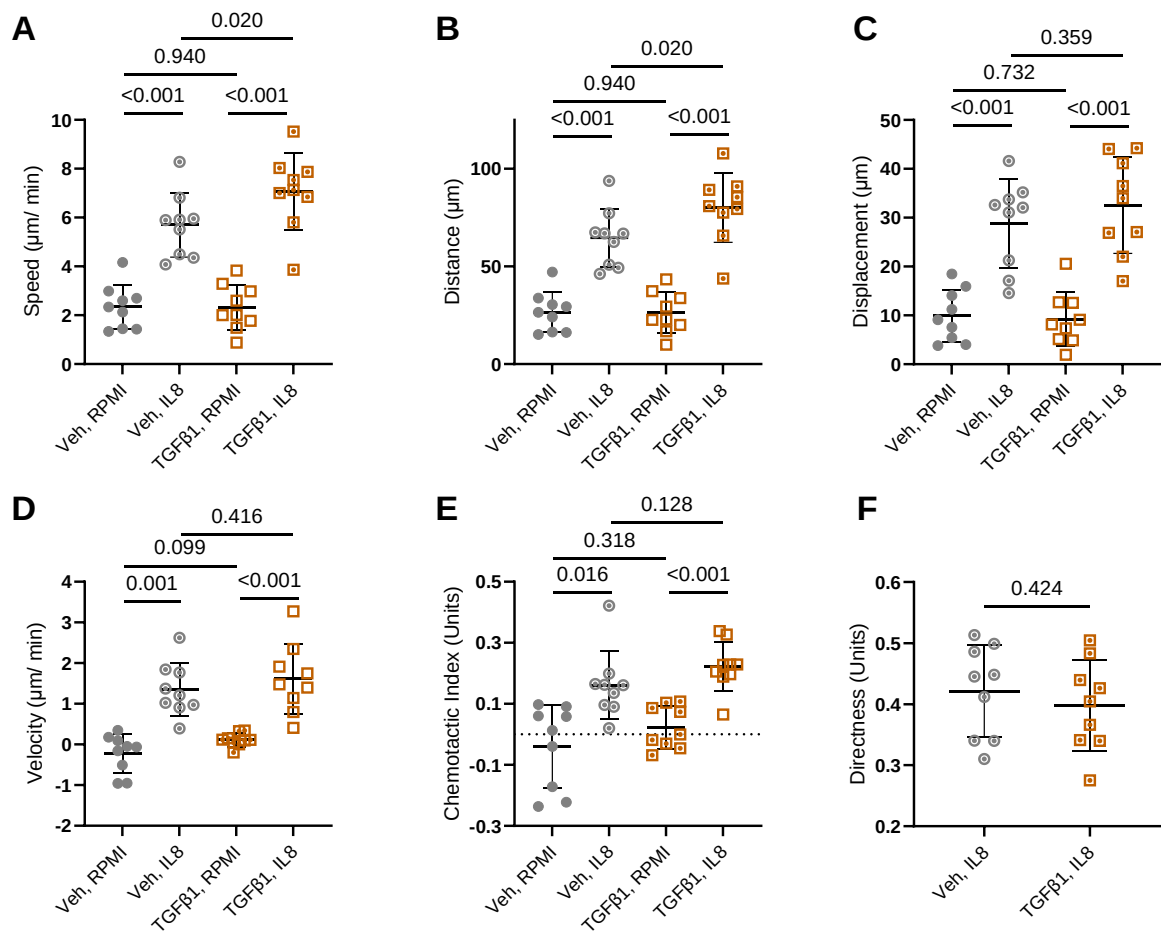


Figure 4.9: Effect of TGF β 1 pre-treatment on neutrophil chemotactic responses to 100 nM IL-8.

Neutrophils isolated from healthy participants ($n = 9$) were treated for 15 minutes with 10 ng/mL TGF β 1 or vehicle, centrifuged and resuspended in RPMI. Neutrophil chemotactic and chemokinetic responses to 100 nM IL-8 were then assessed in the Insall chamber. Pretreatment with TGF β 1 increased speed and distance measures (A – B), but not chemotactic responses to IL-8. P values indicate differences between experimental conditions compared. Data point per donor. Data bars are mean and standard deviation. One-way ANOVA and paired T-tests were performed. Cell tracking was adjusted for background slide movement as described in Chapter 2.7.2.1.

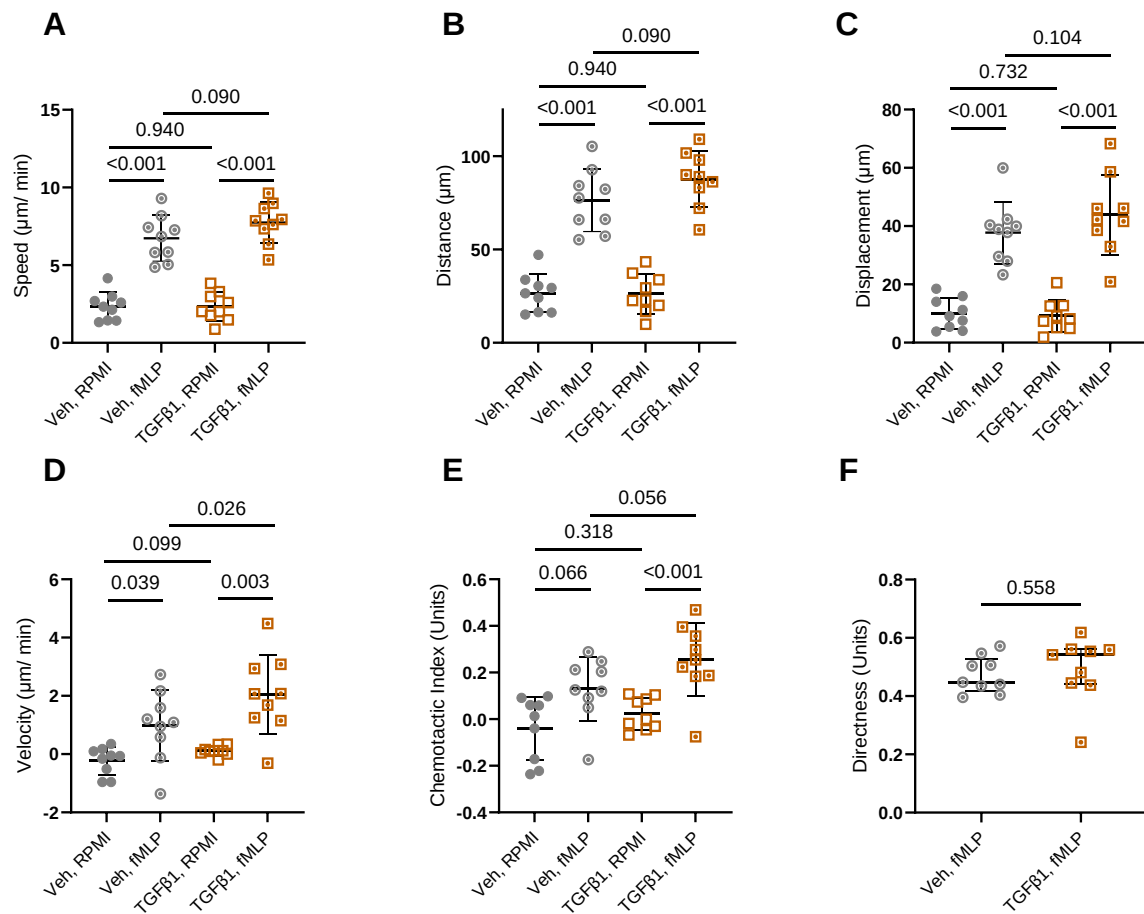


Figure 4.10: Effect of TGF β 1 pre-treatment on neutrophil chemotactic responses to 10nM fLMP.

Neutrophils isolated from healthy participants ($n = 9$) were treated for 15 minutes with 10 ng/mL TGF β 1 or vehicle control, centrifuged and resuspended in RPMI. Neutrophil chemotactic and chemokinetic responses to 10 nM fMLP were then assessed in the Insall chamber. TGF β 1 pre-treatment had no effect on chemokinetic features (A - C) although the effect on chemotactic parameters (D - velocity) reached borderline levels of significance. P values indicate differences between experimental conditions compared. Data point per donor. Data bars are mean and standard deviation. One-way ANOVA and paired T-tests were performed. Cell tracking was adjusted for background slide movement as described in Chapter 2.7.2.1.

4.6.4.4. TGF β 1 does not amplify PMA- mediated NET formation.

To examine a further aspect of priming, the effects of TGF β 1 pre-treatment on PMA induced NET formation was assessed. Neutrophils isolated from healthy participants were cultured *ex vivo* in RPMI supplemented with vehicle or 1 ng/ mL TGF β 1 for 30 minutes. Following this, 25 nM PMA was added, and neutrophils were cultured for a further 4 hours in 37° C and 5% CO₂.

NET formation was assessed by two techniques: measuring the cfDNA that had been released into the supernatant using a fluorometric plate reader (Figure 4.11A) or immunofluorescence microscopy (Figure 4.11B). Twenty five nM PMA stimulated NET formation in all samples. Pre-treatment with 1 ng/ mL TGF β 1 had no effect on PMA driven cfDNA release ($p = 0.299$, paired T-test, $n = 4$) or area of NET formation ($p = 0.416$, paired T-test, $n = 6$). This suggests that TGF β 1 does not prime for PMA driven NET formation.

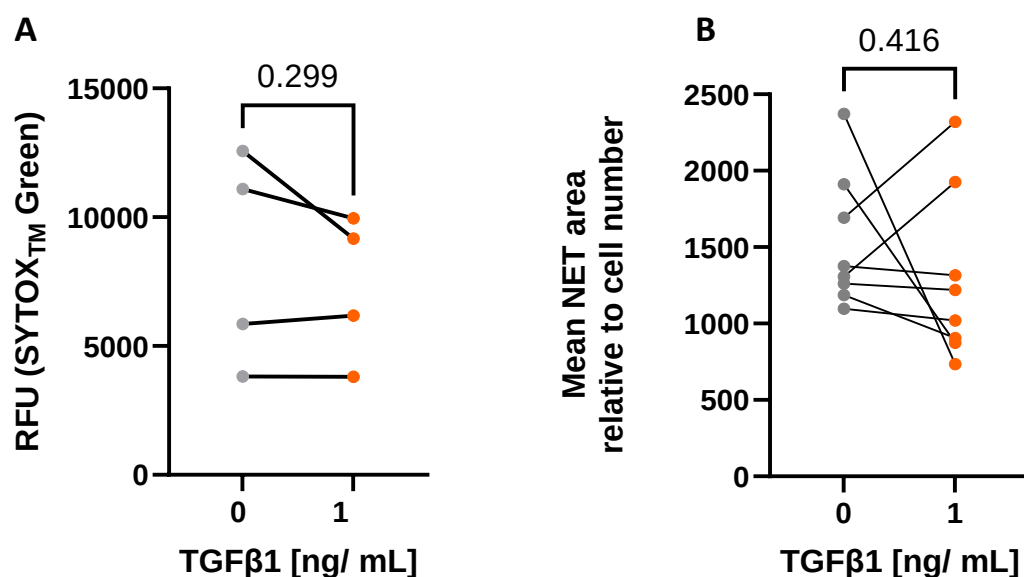


Figure 4.11: The effect of TGFβ1 on PMA- induced NET formation.

Neutrophils isolated from healthy participants (n= 4/ 6) with and without 1 ng/ mL TGFβ1 exposure for 30 minutes followed by 4 hours exposure to 25 nM PMA were assessed for NET formation by measuring cell free DNA present in the supernatant using fluorescence of SYTOX™ green staining and expressed as RFU (A) or immunofluorescence microscopy (B) as described in Chapter 2.8.

One ng/ mL TGFβ1 treatment prior to 25 nM PMA stimulation had no effect on NET formation when assessed by fluorometry (p = 0.299, n = 4) or immunofluorescence (p = 0.416, n = 6). Dot per participant sample. Lines join paired samples. Paired T-tests were performed.

4.6.5. Can TGFβ1 drive NET formation, without the presence of a second stimulus?

TGFβ1 was tested alone to determine any effect on NET formation. Neutrophils isolated from healthy participants were cultured *ex vivo* in RPMI containing 1 ng/ mL TGFβ1 or vehicle for 4.5 hours. DNA release was then measured following SYTOX™ green nucleic acid staining using fluorometry or microscopic visualisation, respectively. Fluorometric analysis (Figure 4.12) detected greater cfDNA within the supernatant collected from neutrophils that had been exposed to 1 ng/ mL TGFβ1 compared to vehicle (mean = 5130 RFU (SD 1740) vs vehicle = 4393 RFU (SD 1546); $p = 0.004$, paired T-test, $n = 6$).

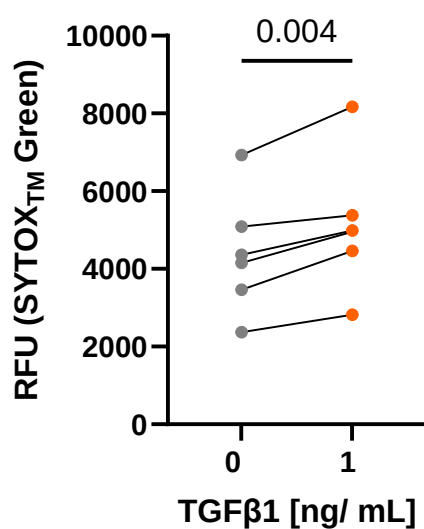


Figure 4.12: Effect of TGFβ1 on release of cell free DNA from neutrophils *ex vivo*.

Extracellular DNA release from neutrophils isolated from 6 healthy donors following *ex vivo* culture in RPMI containing vehicle or 1 ng/ mL TGFβ1 for 4.5 hours was measured using a fluorometric plate reader. Lines join paired control and treated samples from each donor. One ng/ mL TGFβ1 significantly increased extracellular DNA release compared to vehicle ($p = 0.004$ by paired T-test).

However, analysis of NET formation by immunofluorescence imaging (mean NET area relative to cell number semi-quantified as described in Chapter 2.8.2.2) revealed no significant difference in NET area between TGF β 1 and vehicle treated neutrophils (Figure 4.13A). This is despite visually noting increased NET formation with TGF β 1 exposure in 4 out of the 6 samples as compared to vehicle (representative images displayed in Figures 4.13B and 4.13C).

Differential causes for extracellular DNA release from neutrophils include apoptosis and necrosis. It was previously shown that up to 90 minutes of exposure with 1 pg/ mL – 10 ng/ mL TGF β 1 did not induce cell death (see Figure 4.2). The effect of TGF β 1 on neutrophil viability over the 4.5 hour NET assay exposure time was subsequently assessed. There was no difference between the percentage of neutrophils that were viable, early apoptotic, late apoptotic or necrotic following exposure to vehicle or 0.1 – 10 ng/ mL TGF β 1 ($p > 0.05$, one-way ANOVA, $n = 3$). The mean percentage of viable (annexin V negative, PI negative) cells was 91.9% (SD 1.6%) and 90% (SD 3.7%) in neutrophils exposed to 10 ng/ mL TGF β 1 or vehicle for 4.5 hours, respectively.

This suggests that a) TGF β 1 does not induce neutrophil apoptosis or necrosis and, b) if the elevated cfDNA release on TGF β 1 exposure is attributed to NET formation, this is likely a type of non-suicidal NETosis.

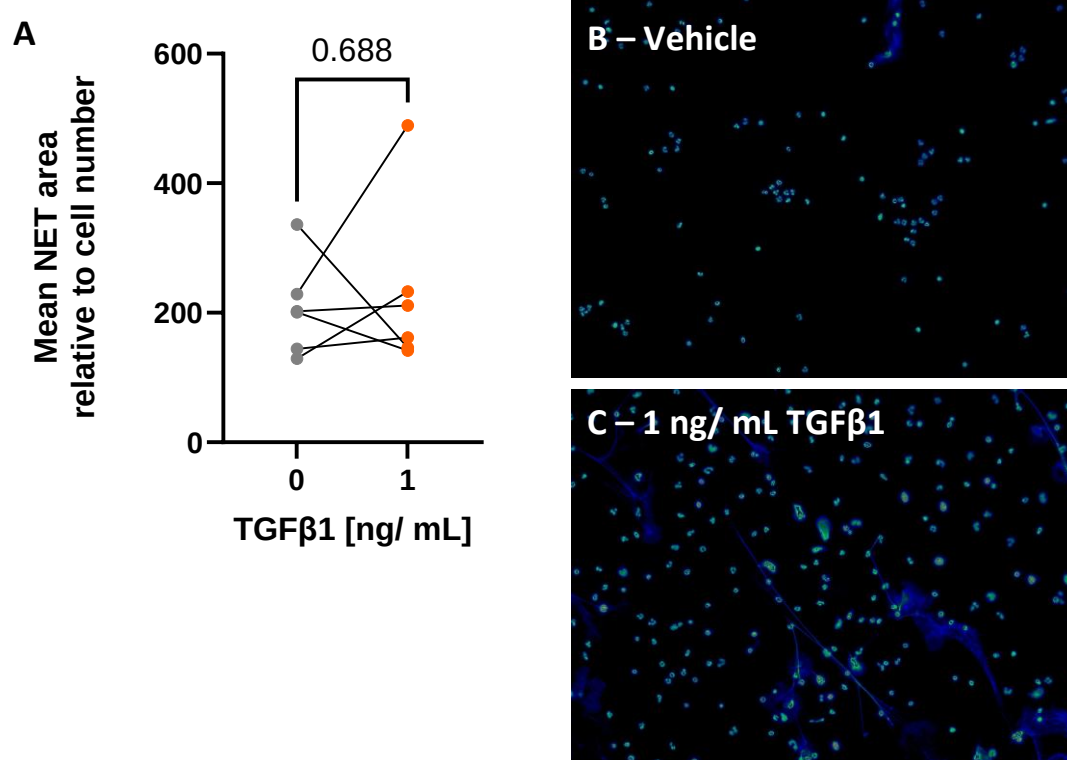


Figure 4.13: The effect of TGFβ1 on NET formation.

Neutrophils isolated from healthy donors ($n = 6$) were cultured *ex vivo* on a cover slip in RPMI containing vehicle or 1 ng/ mL TGFβ1 for 4.5 hours and then stained with SYTOX™ green. Cells were imaged and analysed as described in Chapter 2.8.2.

(A) displays the mean NET area relative to cell number for each condition. Dot per donor. Lines join paired samples. There was no significant difference in NET area between vehicle and TGFβ1 exposed neutrophils ($p = 0.688$ by paired T-test, $n = 6$).

(B) and (C) are representative microscopy images of neutrophils exposed to RPMI containing vehicle or 1 ng/ mL TGFβ1, respectively. These images are from a donor where mean NET area relative to cell number increased from 129 to 233. Images are presented with 3-3-2 RGB look up table applied. Red, green and blue colours represent SYTOX™ green stained nucleic acid.

4.6.6. Does TGF β 1 act on the NADPH oxidase system?

The effect of TGF β 1 on respiratory burst was assessed to explore this as a mechanism for priming neutrophils. It was investigated by measuring extracellular superoxide anion production every 1 minute for 200 minutes using the lucigenin-amplified chemiluminescence assay. NADPH oxidase mediated superoxide production is essential for PMA induced NET formation by driving chromatin decondensation [333]. The chemoattractant, fMLP, has also been shown to increase ROS release [334]. Therefore, PMA and fMLP were used as positive controls in this chemiluminescence assay. Twenty five nM PMA (Figure 4.14) increased the total AUC compared to unstimulated neutrophils (median PMA = 722212 (IQR 538210 – 726771) vs control = 4169 (IQR 2236 – 19954; $p = 0.031$, single-tailed Wilcoxon test, $n = 5$).

Although 1 μ M fMLP (Figure 4.15) increased total superoxide anion production in all samples relative to unstimulated neutrophils, this failed to reach statistical significance ($p > 0.05$, single-tailed Wilcoxon test, $n = 5$).

Histogram plots displaying mean superoxide anion production by neutrophils over 200 minutes following exposure to 25 nM PMA or 1 μ M fMLP are displayed in Figure 4.14B and Figure 4.15B, respectively.

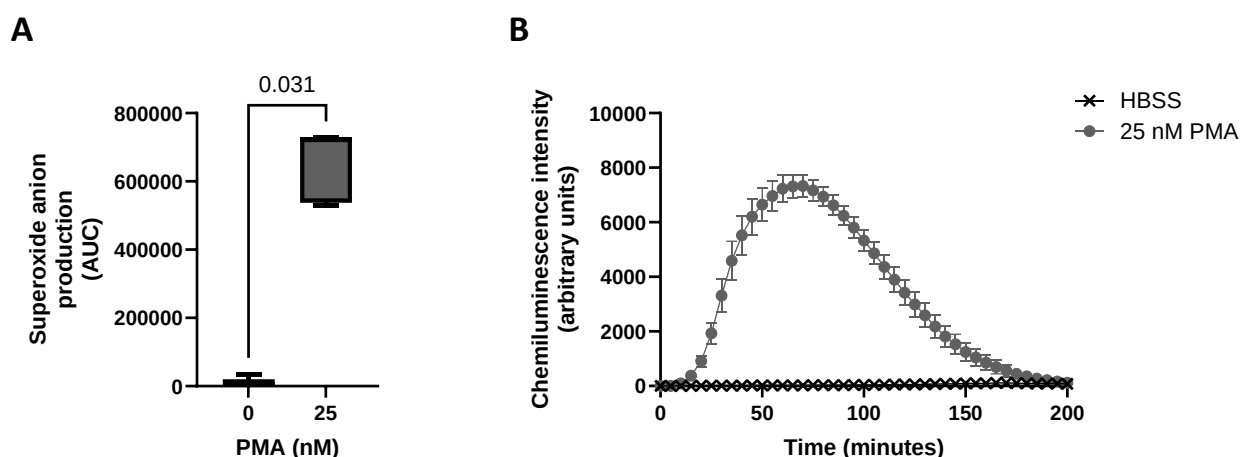


Figure 4.14: Effect of PMA on extracellular superoxide anion production by neutrophils.

Neutrophils from healthy donors ($n = 5$) were resuspended in HBSS containing lucigenin and treated with or without 25 nM PMA. Superoxide anion generation was then measured over 200 minutes using a luminometer. The total superoxide anion production was determined by measuring the area under the curve (AUC).

- (A) Box and whisker plots (Tukey) are shown for control and PMA treated cells. The AUC increased with 25 nM PMA exposure compared to unstimulated neutrophils ($p = 0.031$, single-tailed Wilcoxon test).
- (B) Mean and standard error time course of the combined chemiluminescence intensity values of the neutrophil samples ($n = 5$) after stimulation with HBSS alone (black crosses) or 25 nM PMA (grey circles).

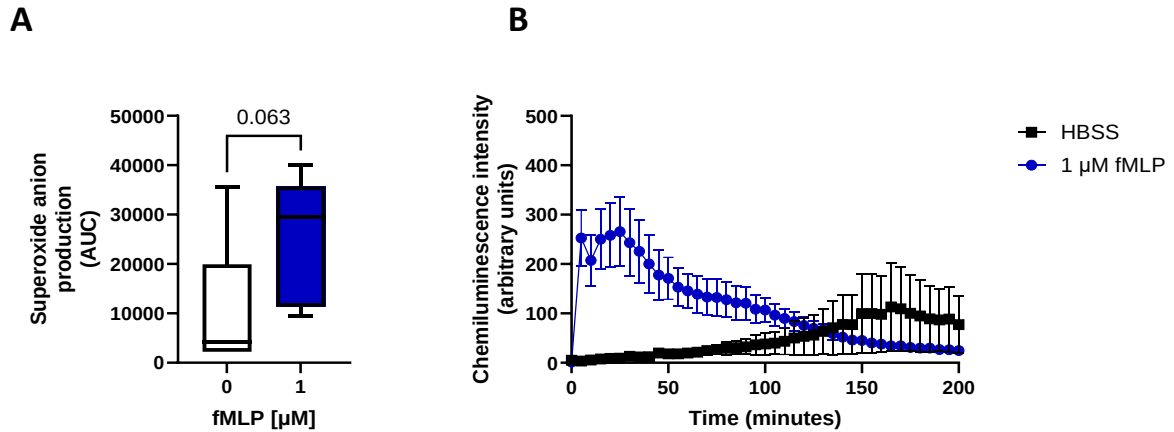


Figure 4.15: Effect of fMLP on extracellular superoxide anion production by neutrophils.

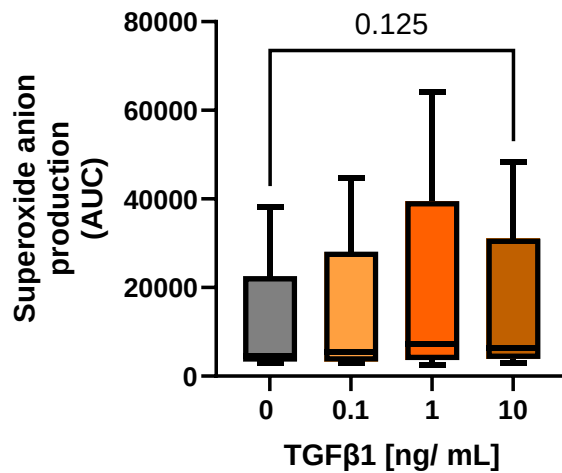
Neutrophils from healthy donors ($n = 5$) were resuspended in HBSS containing lucigenin and treated with or without 1 μ M fMLP. Superoxide anion generation was then measured over 200 minutes using a luminometer. The total superoxide anion production was determined by measuring the AUC.

(A) Box and whisker plots (Tukey) are shown. The AUC increased with 1 μ M fMLP exposure compared to unstimulated neutrophils in all biological samples (median fMLP = 29452 (IQR 11335 – 35694); median control = 4169 (IQR 2236- 19954)). Data was not statistically significant by single-tailed Wilcoxon test; $p = 0.063$.

(B) Mean and standard error time course of the combined chemiluminescence intensity values of the neutrophil samples ($n = 5$) in HBSS alone (black squares) or with 1 μ M fMLP (blue circles).

Neutrophils from healthy donors were exposed to 0.1, 1 or 10 ng/ mL TGF β 1 for 15 minutes, centrifuged (1500 x g for 2 mins) then chemiluminescence readings performed once the cells were resuspended in HBSS. Total superoxide anion production was not significantly increased at any of the concentrations of TGF β 1 compared to vehicle (Figure 4.16A: $p > 0.05$, Wilcoxon tests, $n = 5$). Flow plots displaying the mean superoxide anion production by neutrophils in response to vehicle or 0.1 – 10 ng/ mL are displayed in Figure 4.16B.

A



B

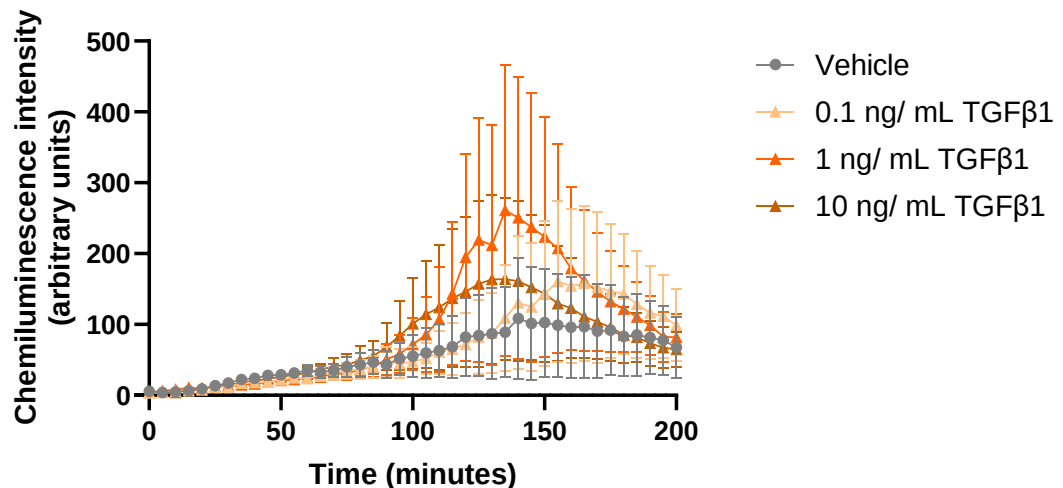


Figure 4.16: Effect of TGFβ1 exposure on extracellular superoxide anion production by neutrophils.

Neutrophils isolated from healthy donors ($n = 5$) were resuspended in HBSS containing lucigenin and treated with vehicle or 0.1 – 10 ng/ mL TGFβ1 for 15 minutes. Neutrophils were then centrifuged at 1500 x g for 2 minutes and resuspended in HBSS. Superoxide anion generation was measured for 200 minutes using a luminometer. The total superoxide anion production was determined by measuring the AUC.

(A) Box and whisker plots (Tukey) are presented. The AUC was not increased with any of the concentrations of TGFβ1 compared to vehicle (p -value > 0.05 by Wilcoxon tests).

(B) Mean and standard error time course of the combined chemiluminescence intensity values of the neutrophil samples ($n = 5$) after exposure with vehicle or 0.1 – 10 ng/ mL TGFβ1.

4.6.7. The effect of TGF β 1 pre-treatment on PMA induced ROS.

It was considered that a time point of 4 hours was too late to assess whether TGF β 1 amplifies PMA driven NET formation as this was likely to be after the maximum level of NETosis. ROS production is critical to PMA mediated NET formation and the chemiluminescence assay allows for real time measurements of ROS production immediately following stimulant exposure. Therefore, this assay was performed to determine whether TGF β 1 could accelerate/enhance PMA mediated functions. Neutrophils from healthy donors (n = 5) were exposed to vehicle or 0.1 – 10 ng/ mL TGF β 1 for 15 minutes, centrifuged then resuspended in HBSS containing lucigenin. Neutrophils were then treated with 25 nM PMA and superoxide anion production measured over 200 minutes using a luminometer.

Pre-treatment with 10 ng/ mL TGF β 1 significantly reduced PMA mediated total ROS generation (Figure 4.17A, p = 0.022, paired T-test, n = 5) and peak chemiluminescent intensity (Figure 4.17B, p = 0.009, paired T-test, n = 5) compared to control. Figure 4.17C displays representative chemiluminescence intensity histograms of neutrophils exposed to 25 nM PMA over time. The data representing neutrophils pre-treated with TGF β 1 (orange triangles) have visibly smaller AUCs and lower peak chemiluminescent intensities compared to the control data (grey circles).

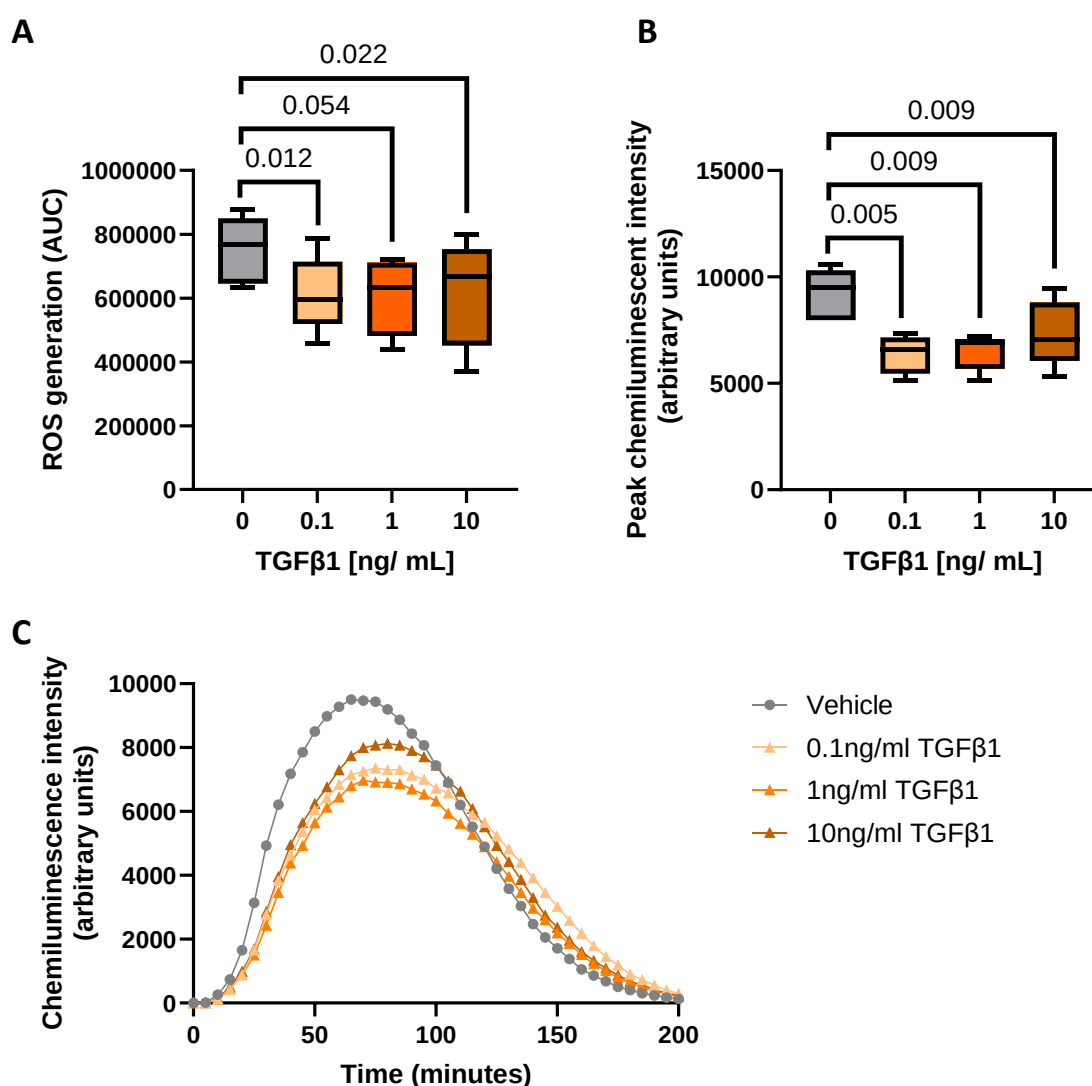


Figure 4.17: TGFβ1 pre-treatment reduces PMA mediated extracellular superoxide anion production by neutrophils.

Neutrophils from healthy donors ($n = 5$) were exposed to vehicle or 0.1 – 10 ng/ mL TGFβ1 for 15 minutes, centrifuged then resuspended in HBSS containing lucigenin and subsequently treated with 25 nM PMA.

(A) The total superoxide anion production and (B) peak chemiluminescence intensity determined using AUC analyses. Box and whisker plots (Tukey) are shown. One-way ANOVA and paired T-tests were performed. TGFβ1 reduced total ROS production compared to vehicle ($p < 0.05$) and reduced the peak chemiluminescent intensity compared to vehicle alone ($p < 0.05$).

(C) Representative chemiluminescence intensity values from a single donor pre-treated with vehicle or 0.1 – 10 ng/ mL TGFβ1, followed by 25 nM PMA.

4.6.8. TGF β 1 signalling in human neutrophils.

4.6.8.1. *TGF β 1 is expressed by human neutrophils.*

Western blotting was performed to assess TGF β 1 expression by human neutrophils (Figure 4.18). Neutrophils from healthy participants were lysed in RIPA buffer cocktail. Western blotting was performed as described in Chapter 2.11. A band at the expected molecular weight for TGF β 1 (53 kDa) was noted in all three neutrophil lanes. This suggests that TGF β 1 is expressed by human neutrophils.

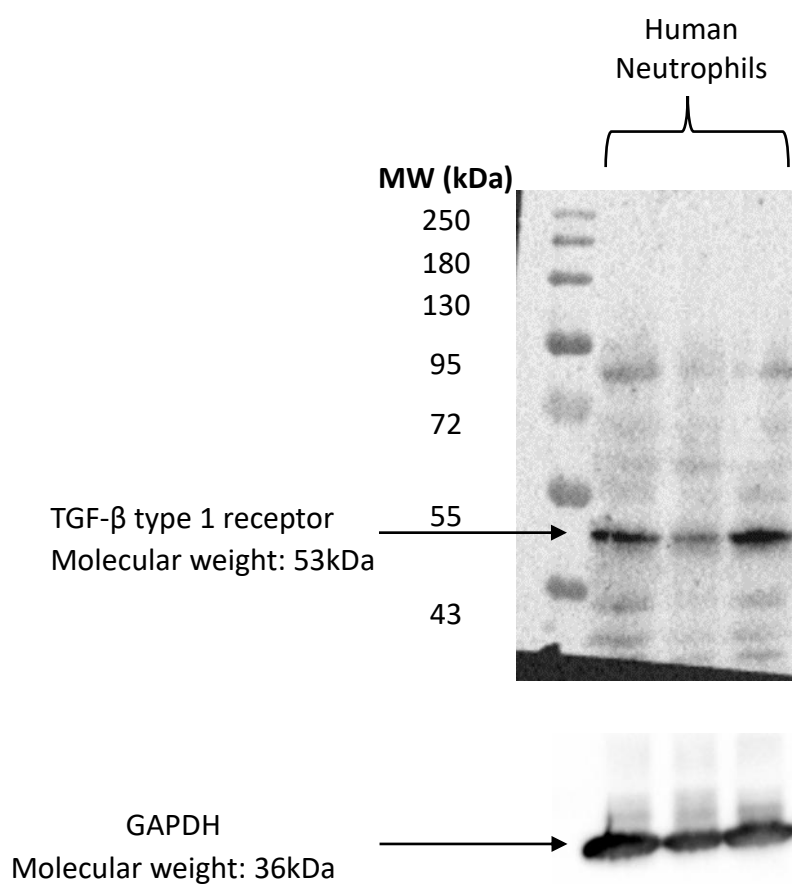


Figure 4.18: TGFβ type 1 receptor expression by human neutrophils.

Neutrophils isolated from three healthy donors (lane per donor) were lysed in RIPA buffer cocktail. Equal volume of lysate was loaded to each lane. Membranes were sequentially probed for TGFβ type 1 receptor (expected molecular weight (MW) 53kDa – mab5871 (*R&D Systems*)) and GAPDH (expected MW 36kDa – ab181602 (*Abcam*)). GAPDH – glyceraldehyde-3-phosphate dehydrogenase.

4.6.8.2. Effect of TGF β R1 antagonism on cfDNA release by human neutrophils.

An ATP-competitive cell permeable inhibitor of the TGF β R1, LY364947, was used to determine if TGF β 1 exerts its priming effects via the TGF β R1 [329]. First, Annexin V/PI staining was performed to assess if LY364947 affects cell viability (See Figure 4.19). The half maximum inhibitory concentration (IC₅₀) of LY364947 reported by the suppliers is 59 nM [335]. Twenty nine point five nM to 1 μ M LY364947 had no significant effect on the percentage of cells that were viable (annexin V negative, PI negative) as compared to vehicle (DMSO, described as 0 nM) ($p > 0.05$, One-way Anova, $n = 3$). Mean neutrophil viability was 88- 90% for vehicle and LY364947 treated neutrophils after 4 hours 30 minutes exposure time.

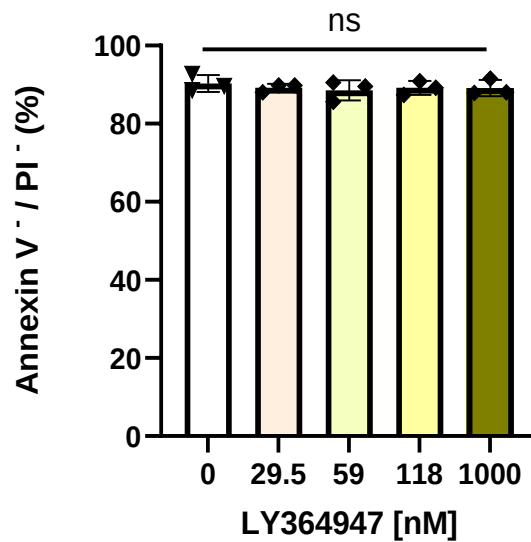


Figure 4.19: Effect of the TGF β type 1 receptor inhibitor, LY364947, on neutrophil viability *ex vivo*.

Using Annexin V/PI staining, isolated neutrophils from healthy donors ($n = 3$) were assessed for cell viability (Annexin V negative and PI negative) following treatment with the TGF β type 1 receptor inhibitor, LY364947 (Selleck, US: S2805, IC₅₀: 59nM), for 4 hours 30 minutes. Histograms are mean with standard deviation bars. Each data point represents a donor. There was no difference in viability of neutrophils exposed to vehicle (DMSO), indicated as 0 nM) or any concentration of LY364947.

The effect of LY364947 pre-treatment on TGF β 1 mediated cfDNA release was then assessed using fluorometry. Neutrophils isolated from healthy donors were exposed for 45 minutes to RPMI or RPMI supplemented with vehicle (DMSO) or 59 nM LY364947. Subsequently, neutrophils were treated with vehicle or 10 ng/ mL TGF β 1 for 4 hours in 37° C and 5% CO₂. The cfDNA present in the supernatant was then stained with SYTOX_{TM} green and measured using a fluorometric plate reader as described in Chapter 2.8.1. The difference in SYTOX_{TM} green RFU between 10 ng/ mL TGF β 1 and vehicle treated neutrophils was calculated. DMSO pre-treatment had no effect on cfDNA release ($p > 0.05$, paired T- test, $n = 6$) (see Figure 4.20). There was a significant reduction in cfDNA release from neutrophils pre-treated with 59 nM LY364947, compared to DMSO control (LY364947 mean = -168.9 (SD 184.6) vs DMSO mean = 2.5 (SD 253); $p = 0.045$, paired T-test, $n = 6$).

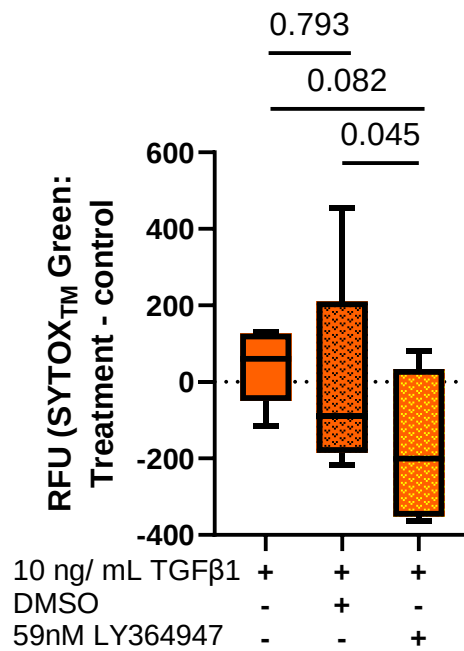


Figure 4.20: Effect of TGFβ type 1 receptor inhibition on TGFβ1 mediated cell free DNA release from neutrophils.

Neutrophils isolated from healthy donors (n = 6) were exposed to media only, TGFβ type 1 receptor inhibitor, 59 nM LY364947 (*Selleck, US: S2805, IC50: 59nM*) or vehicle (DMSO) for 45 minutes, followed by 10 ng/ mL TGFβ1 or vehicle for 4 hours. DNA release *ex vivo* was measured using a fluorometric plate reader as described. Box and whisker plots (Tukey) and p-values are shown. Paired T-tests were performed.

4.6.8.3. Does TGF β 1 act via the CXCR2 receptor?

CXCR2 is an important chemokine receptor on human neutrophils [336]. In Chapter 3.4.5, it was demonstrated that treatment for 5 to 60 minutes with 100 nM IL-8 significantly reduced CXCR2 expression on the neutrophil cell surface, which is suggestive of CXCR2 internalisation or blockage following receptor binding. The effect of TGF β 1 on CXCR2 expression on the neutrophil surface was assessed by flow cytometry to determine if a) TGF β 1 binds to CXCR2 and, b) TGF β 1 amplifies the chemokinetic effects of IL-8 by impacting extracellular expression of CXCR2.

Neutrophils isolated from healthy donors were exposed to 10 ng/ mL TGF β 1 or vehicle for 5 to 60 minutes and stained with a PE conjugated anti-CXCR2 antibody. Following fixation with 2% paraformaldehyde, cell surface expression of CXCR2 was measured using flow cytometry and the percentage of cells expressing CXCR2 (Figure 4.21A) and MFI (Figure 4.21B) was determined. Ten ng/ mL TGF β 1 had no effect on CXCR2 expression over any of the treatment times as compared to vehicle ($p > 0.05$, paired T-tests, $n = 5/6$). This is in contrast to results in Chapter 3 that demonstrated rapid reduction of cell surface expression of CXCR2 or blocking of this receptor with IL-8 exposure. This suggests that a) TGF β 1 does not act via the CXCR2 receptor as receptor blocking/internalisation was not noted and b) TGF β 1 does not increase IL-8 mediated chemokinesis by increasing CXCR2 expression on the cell surface.

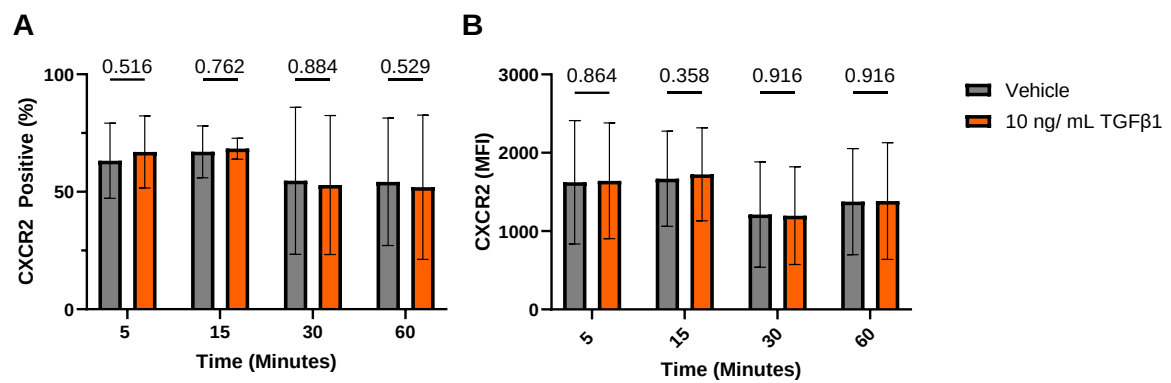


Figure 4.21: Effect of TGFβ1 on CXCR2 expression on the surface of neutrophils.

Neutrophils isolated from healthy donors (n = 5/ 6) were cultured *ex vivo* with media containing 10 ng/ mL TGFβ1 or vehicle for 5 – 60 minutes. (A) shows the percentage of cells expressing CXCR2 on the cell surface and (B) represents the MFI of CXCR2. Histograms represent mean with standard deviation. There was no statistical difference in percentage expression or MFI of CXCR2 at any of the time-points between TGFβ1 and vehicle exposed neutrophils (paired t-test). Individual p values for each comparison are shown.

4.6.8.4. Downstream signalling pathways

TGF β 1 signalling is broadly divided into canonical (Smad- dependent) and non-canonical (Smad- independent) pathways [337,338]. These pathways are well documented in other cell types in the human body, but poorly understood in neutrophils.

4.6.8.4.1. Canonical pathways

Western blotting was first attempted to assess the effect of TGF β 1 on the phosphorylation of the canonical signalling molecules, Smad2/3. However, despite repeated attempts and adjustments to the western blotting protocol to try to reduce non-specific binding, specific blotting for p-Smad2/3 on neutrophil lysates failed.

Therefore, a phosphoflow cytometry assay was performed to measure pSmad2/3 expression as described in Chapter 2.12. Neutrophils from healthy donors (n= 3/ 4) were exposed to 10 ng/ mL TGF β 1 for up to 60 minutes and were stained with a PE conjugated anti-Smad2 antibody or isotype control antibody following fixation and permeabilisation. pSmad2/3 expression was then assessed using flow cytometry and % of positive cells (Figure 4.22A) and MFI (Figure 4.22B) was determined. Sixty minutes exposure to 10 ng/ mL TGF β 1 significantly increased pSmad2/3 MFI compared to vehicle treated neutrophils (p = 0.038, unpaired T-test, n = 3/ 4). This suggests that TGF β 1 acts via canonical pathways in human neutrophils.

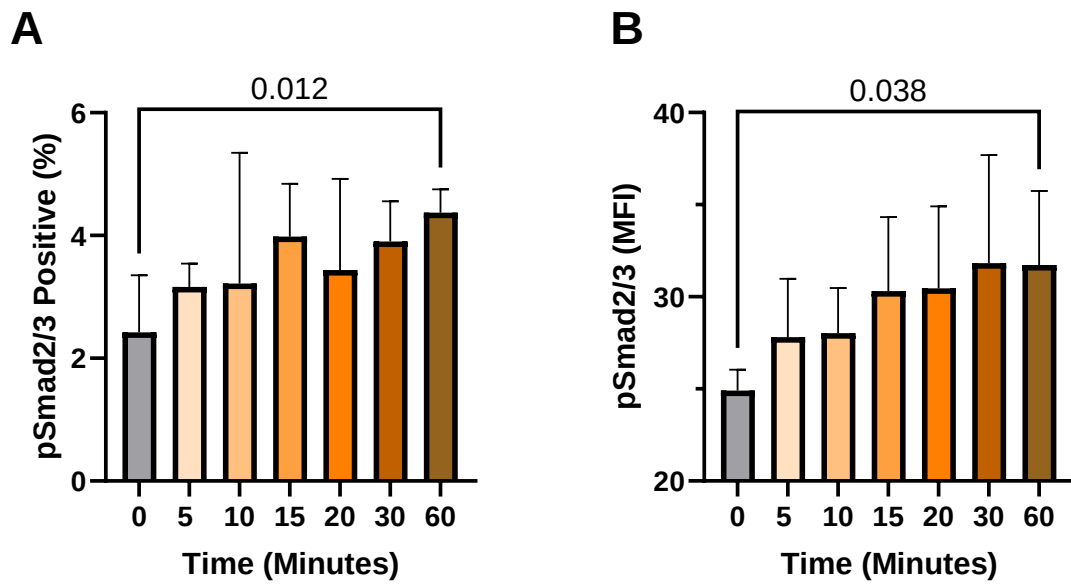


Figure 4.22: Effect of TGFβ1 on pSmad2/3 phosphorylation in human neutrophils.

Neutrophils isolated from healthy donors ($n = 3/4$) were cultured *ex vivo* with media containing vehicle or 10 ng/mL TGFβ1 for 5 – 60 minutes. pSmad2/3 expression was assessed by flow cytometry. (A) shows the percentage of cells expressing pSmad2/3 and (B) represents the median fluorescence intensity (MFI) of pSmad2/3. Histograms represent mean with standard deviation. Analyses were performed using unpaired T-tests. The significance of changes from baseline are shown. Timepoint 0 represents vehicle exposed neutrophils cultured for 60 minutes *ex vivo*.

4.6.8.4.2. Non-canonical pathways

The effect of TGF β 1 on the phosphorylation of the non-canonical signalling molecules, p38 MAPK and p44/42 MAPK, was assessed using western blotting. Studies have previously reported that 1 μ M fMLP increases phosphorylation of both p38 MAPK and p44/42 MAPK in human neutrophils [90] and was used as a positive control for these experiments. Neutrophils isolated from healthy donors were exposed to 1 μ M fMLP, 10 ng/ mL TGF β 1 or vehicle for 1 to 15 minutes followed by immediate centrifugation at 4° C and lysis in RIPA buffer cocktail. Western blotting was performed and protein expression semi-quantified using FIJI Image J as described in Chapter 2.11.

One μ M fMLP significantly increased the expression of phosphorylated p38 MAPK (relative to p38 MAPK) after 1 minute (mean fMLP = 1.06 (SD 0.10) vs RPMI = 0.32 (SD 0.11); $p = 0.002$, paired T-test, $n = 2$, see Figure 4.23).

One μ M fMLP also increased the expression of phosphorylated p44/42 MAPK (relative to p44/42 MAPK) over 5 minutes (mean fMLP = 1.46 (SD 0.38) vs RPMI = 0.07 (SD 0.07); $p = 0.020$, paired T-test, $n = 3$, see Figure 4.24).

However, 10 ng/ mL TGF β 1 had no effect on the phosphorylation of p38 MAPK or p44/42 MAPK over 1 to 15 minutes compared to vehicle control ($p > 0.05$, paired T-tests, $n = 2 - 5$). Findings were equivalent when the expression of phosphorylated p38 MAPK and p44/42 MAPK was measured relative to GADPH.

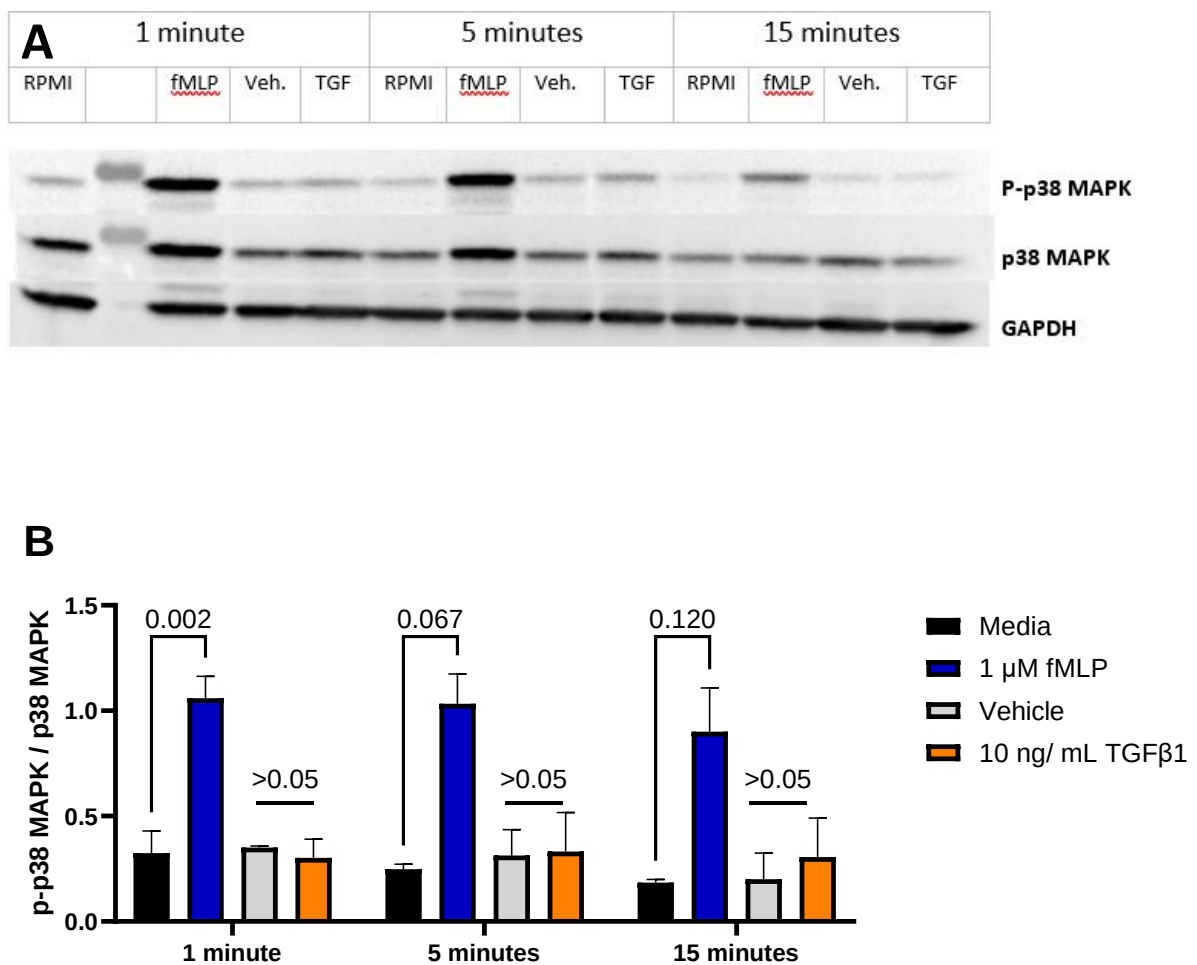


Figure 4.23: Effect of TGF β 1 on the phosphorylation of p38 MAPK in neutrophils.

Neutrophils isolated from healthy donors were treated with 1 μ M fMLP (positive control), 10 ng/ mL TGF β 1 or vehicle for 1 - 15 minutes and subsequently lysed in equal volume of RIPA buffer cocktail. Equal volume of lysate was loaded to each lane. Membranes were probed sequentially for phosphorylated-p38 MAPK (P-p38 MAPK), p38 MAPK and GAPDH.

Figure A is a representative image of a blot sequentially probed for phosphorylated-p38 MAPK (row 1), p38 MAPK (row 2) and GAPDH (row 3).

Figure B displays the combined results of phosphorylated-p38 MAPK expression relative to p38 MAPK of human neutrophils ($n = 2 - 4$). Histograms represent mean and standard deviation bar lines. Statistical analyses were performed using paired T-tests. One μ M fMLP increased the expression of phosphorylated-p38 MAPK (relative to p38 MAPK) over 1 minute compared to media control ($p = 0.002$, $n = 2$). Ten ng/ mL TGF β 1 had no effect on phosphorylated p38 MAPK levels compared to control over any of the treatment times ($p > 0.05$, $n = 2 - 4$).

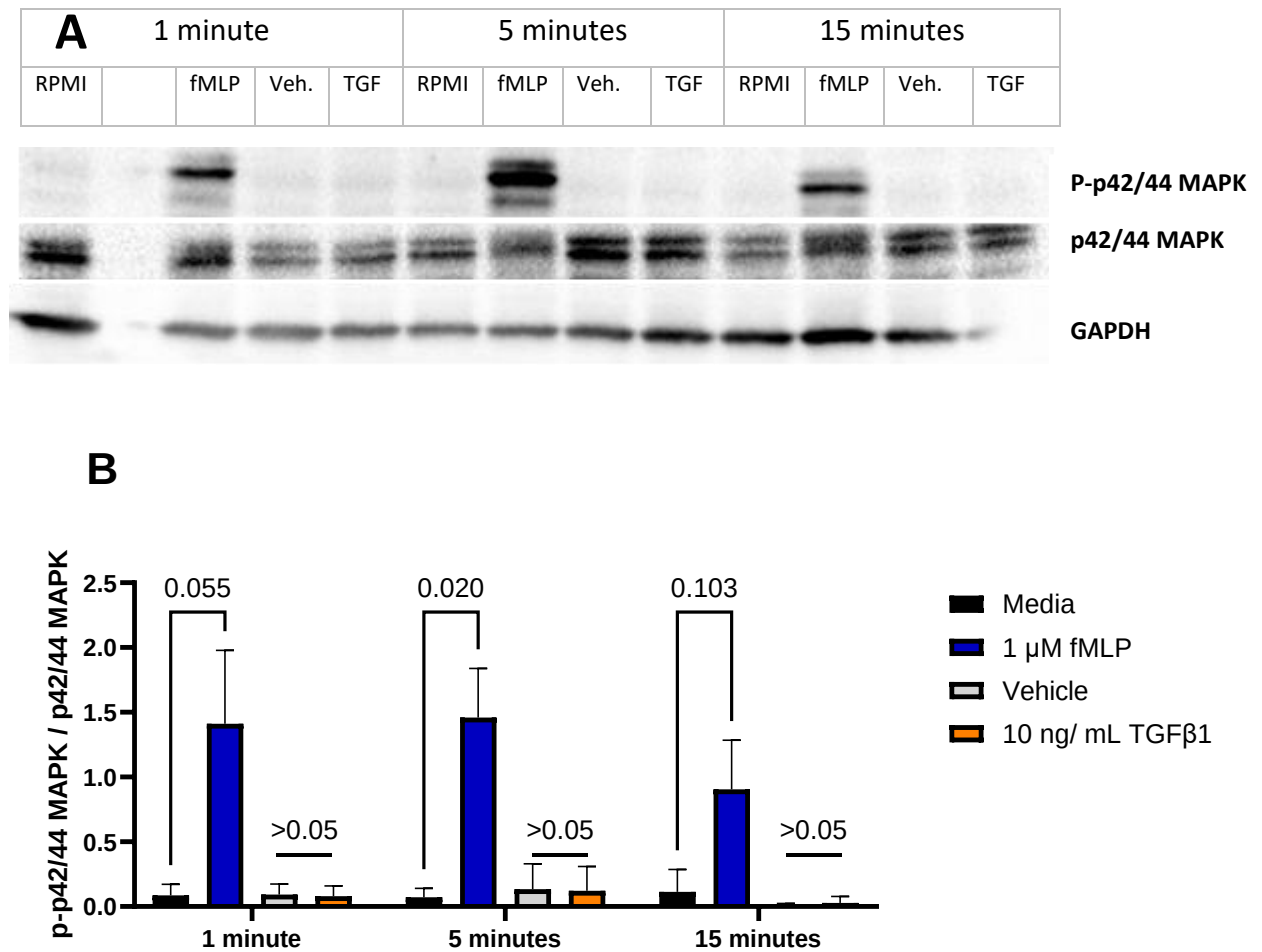


Figure 4.24: Effect of TGF β 1 on the phosphorylation of p42/44 MAPK in neutrophils.

Neutrophils from healthy donors were treated with 1 μ M fMLP (positive control), 10 ng/ mL TGF β 1 or vehicle for 1 - 15 minutes and subsequently lysed in equal volume of RIPA buffer cocktail. Equal volume of lysate was loaded to each lane. Membranes were probed sequentially for phosphorylated p42/44 MAPK (p-p42/44 MAPK), p42/44 MAPK and GAPDH.

(A) are representative images of a blot sequentially probed for phosphorylated-p42/44 MAPK (row 1), p42/44 MAPK (row 2) and GAPDH (row 3).

(B) displays the combined results of phosphorylated p42/44 MAPK expression relative to p42/44 MAPK of human neutrophils (n = 3 - 5). Histograms represent mean and standard deviation bar lines. Analyses were performed using paired T-tests. One μ M fMLP increased the expression of phosphorylated p44/42 MAPK (relative to p44/42 MAPK) compared to media control over 5 minutes (p = 0.020, n = 3). Ten ng/ mL TGF β 1 had no effect on phosphorylated p44/42 MAPK levels as compared to control over any of the treatment times (p > 0.05, n = 3 - 5).

4.7. Discussion

4.7.1. Neutrophil movement

This chapter aimed to assess whether TGF β 1 affects neutrophil movement. Briefly TGF β 1 induced neutrophil transmigration across a transwell membrane. Results using the Insall chamber assay demonstrated that TGF β 1 increases direction independent movement parameters namely, speed, distance moved and displacement, but not direction dependent parameters. This suggests that TGF β 1 affects neutrophil chemokinesis, but not chemotaxis.

Neutrophil movement across the transwell membrane was increased by 1 ng/ mL TGF β 1. Although this same concentration did not significantly increase movement within the Insall chamber, whereas greater concentrations (10 ng/ mL and 30 ng/ mL) did. These differences in concentration effect most likely reflect sensitivity of the assay and sample size, with post-hoc analyses of the Insall chamber assay data indicating that the sample size of 12 for 10 ng/ mL TGF β 1 and 8 for 1 ng/ mL TGF β 1 provided statistical power of 99.9%, and 63.2%, respectively. This work is consistent with previous studies that demonstrated 1 ng/ mL TGF β 1, but not lower concentrations, affected neutrophil movement through a polyester transwell membrane [326]. Others have reported that TGF β 1 affects neutrophil chemotaxis using the Boyden chamber assay or under agarose method [320,324,325]. However, use of the word 'chemotaxis' is non-specific particularly with the Boyden chamber method. This method does not distinguish between chemotaxis or chemokinesis with movement calculated by counting the number of cells that have travelled through or become adherent to the polycarbonate filter [324]. In contrast, the under agarose method presents some insight into a specific chemotaxis response; (a 'chemotactic index') by comparing the distance travelled by neutrophils towards the well containing the chemoattractant under investigation, and the

distance travelled by neutrophils towards a well containing buffer control [339]. The work described in this thesis, by using real time neutrophil tracking data, as described previously [275], has allowed clear separation of a chemokinetic response, from a specific chemotactic response, towards TGF β 1.

Several papers have reported that TGF β 1 is equally as potent at affecting neutrophil movement as an established chemoattractant, such as fMLP [324,326]. The results here do not confirm this conclusion. In Chapter 3, 100 nM IL-8, a widely recognised chemoattractant [275], was used as a positive control to demonstrate that both the transwell and Insall chamber assays can measure neutrophil movement. One hundred nM IL-8 increased the number of cells that traversed the transwell membrane 36.8 fold (Figure 3.3A) and increased neutrophil speed within the Insall chamber 3.5 fold (Figure 3.4) compared to RPMI control. However, 1 ng/ mL TGF β 1 increased the total number of neutrophils that passed through the transwell membrane only 1.3 fold, and 10 ng/ mL TGF β 1 increased neutrophil speed by approximately 2.3 fold compared to control. This suggests that TGF β 1 is less potent than IL-8 at increasing neutrophil movement. It is possible that the maximum effective concentration of TGF β 1 was not tested in experiments reported in this thesis. However, this is unlikely for 2 reasons; a) 1 pg/ mL TGF β 1 did not affect neutrophil migration across the transwell membrane and b) 30 ng/ mL TGF β 1, which is approximately the upper limit of the physiological range in IPF, only increased neutrophil speed in the Insall chamber 1.5 fold. Furthermore, two previous papers have reported no effect of TGF β 1 on neutrophil movement [282,317]. Although both were likely underpowered with only three or four experimental replicates [282,317]. Variability in the biological activity of TGF β 1 used in studies could also account for the

discrepancies in the literature, with other papers not describing the supplier or method of activating the recombinant human TGF β 1 [282,317,326].

4.7.2. Neutrophil priming

Exposure to a stimulus can result in neutrophils transitioning from a resting phenotype to a primed or activated state. This primed state is critical for ensuring optimal effector functions on arrival at the site of tissue injury. Amplified neutrophil priming by dysregulated circulating cytokines has been noted in other chronic lung diseases [340]. Although priming agents are recognised for their ability to augment responses to activating stimuli, they can themselves induce functional changes in neutrophils. For example, the inflammatory cytokine TNF α , has been shown on its own to drive neutrophil chemokinesis, but also amplify chemotactic responses to fMLP *in vitro* [341]. Therefore, it was considered that the chemokinetic effect of TGF β 1 was reflective of neutrophil priming alone. This was investigated by assessing whether TGF β 1 treatment a) changes the expression of cell surface molecules commonly seen on priming and/or, b) amplifies subsequent responses to known stimuli.

TGF β 1 had no effect on the adhesion molecules CD11b or CD62L, which can increase and decrease, respectively, in response to priming and activating agents [71,277]. The glycoprotein CD66b, which is shuttled to the cell surface during exocytosis of granules, was also unaffected by TGF β 1 [278]. This is in keeping with previous studies that had noted no effect of TGF β 1 on granule enzyme release [314,342]. Although TGF β 1 does not affect the expression of these markers, this does not exclude priming capabilities. For example, the priming agent TNF α does not consistently increase CD11b expression [341], and GM-CSF has been shown to have no effect on cell surface CD66b expression [340] despite their effect as priming agents.

However, in the experiments described in this thesis pre-treatment with 10 ng/ mL TGFβ1 amplified direction independent and dependent responses to IL-8 and fMLP, respectively. This supports the concept that TGFβ1 can prime neutrophils. The differential effects of TGFβ1 on chemokinetic and chemotactic measures in response to these recognised chemoattractants could reflect the experiments being underpowered. Post-hoc analysis calculated a statistical power (alpha value 0.05) of only 51% from the 9 experimental replicates performed that compared the effect of TGFβ1 compared to control pre-treatment on neutrophil speed towards IL-8. The calculated sample size of 26 would be required for 80% statistical power (alpha value 0.05) to assess the effect of 10 ng/ mL TGFβ1 pre-treatment on neutrophil speed in response to 10 nM fMLP.

If TGFβ1 does have differential priming effects on IL-8 and fMLP mediated cell movement, this could reflect variability in signalling pathways driving neutrophil movement towards these intermediate and end target chemoattractants [343]. For example, IL-8 driven chemotaxis is mediated by a Pi3K dominant pathway [343], whereas although Pi3K signalling accelerates fMLP mediated chemotaxis, it has a lesser role than p38 MAPK [344].

Pre-treatment experiments also gave some insight into the dynamics of TGFβ1's chemokinetic effects. Movement was similar between neutrophils exposed to vehicle or 10 ng/ mL TGFβ1 for 15 minutes prior to loading to an Insall chamber in the absence of a classical chemoattractant. In these experiments neutrophils were then centrifuged at 1500 x g for 2 minutes and resuspended in RPMI without any TGFβ1, prior to loading into the Insall chamber. This would remove any unbound TGFβ1 from the local microenvironment although it is unknown whether this centrifugation step might lead to dissociation of TGFβ1 from its

receptor. Nevertheless, this suggests that the chemokinetic effects that TGFβ1 exerts on its own are short-lived and/or rely on a continuous environmental supply of the ligand.

However, TGFβ1's ability to amplify movement responses to IL-8 and fMLP remained even after the centrifugation/wash step. Further work is needed to explore this result in greater detail. For example does continuous TGFβ1 exposure (i.e. no centrifugation/wash step) further amplify chemokinetic responses to IL-8, and does TGFβ1 drive chemokinesis alone/amplify responses to other chemoattractants by distinct mechanisms?

In contrast to neutrophil chemokinesis, TGFβ1 pre-treatment did not amplify PMA- mediated NET formation. Twenty five nM PMA is a potent inducer of NET formation (demonstrated in Figure 3.5) with a systematic review reporting that PMA has a '100% success rate for inducing NETosis' [345]. Remijnsen *et al* observed that plasma membrane permeabilisation and release of decondensed chromatin is evident within only 40 minutes exposure to 100 nM PMA [333]. Therefore, it is possible that NET formation had peaked following 4 hours of 25 nM PMA exposure with no ability to detect amplification by priming agents. Future investigation would include assessing the effect of TGFβ1 exposure prior to an alternative, physiological and less potent NET forming stimuli, such as IL-8 or IL-1β (described in Chapter 3), or assess DNA release at an earlier timepoint to determine whether TGFβ1 accelerates PMA- induced NET formation.

4.7.3. Neutrophil extracellular trap formation

Many agents that drive neutrophil chemokinesis, such as IL-8, also promote NET formation without the requirement of a second stimulus [236]. In this chapter, it was noted that 1 ng/mL TGFβ1 increased cfDNA release from human neutrophils. However, semi-quantification of

NET area relative to cell number of the microscopy images did not show a statistically significant increase with TGF β 1 treatment. This is despite visually noting increased NET formation in 4 out of the 6 samples as compared to vehicle. Low sample size and poor assay sensitivity could account for the lack of effect noted with this second method. Post-hoc analysis revealed that these six experimental replicates provided only 6% statistical power (alpha value 0.05). This lack of consistency with methodology is similar to a study performed in lichen planus, where there appeared to be an increased number of neutrophils undergoing NETosis on immunofluorescence imaging, but no increase in other markers of NET release within the supernatant, namely myeloperoxidase and citrullinated histone 3 [186]. It is important that further experimental replicates are performed to appropriately power both methods of NET measurements in this thesis, alongside a third measure, such as granular enzyme release, before any conclusions can be made of the effects of TGF β 1 on NET formation.

It was demonstrated in this chapter that TGF β 1 does not increase neutrophil apoptosis or necrosis. Therefore, if TGF β 1 does drive NET formation, it is likely a non-suicidal form, whereby neutrophils maintain the integrity of their plasma membranes following NET release. This is in contrast to PMA, which induces suicidal NETosis [103,333]. If TGF β 1 drives NET formation by an alternative (non-suicidal) pathway to PMA, this could be an alternative explanation to peak effect suggested above for TGF β 1 not amplifying PMA driven NETosis. The chemiluminescence results showed that TGF β 1 pre-treatment significantly reduces PMA mediated superoxide anion release providing some support for this concept.

Two types of non-suicidal NET formation have been described; one type involves rapid exocytosis of vesicles containing DNA (within minutes after exposure to *Staphylococcus*

Aureus), which appears NADPH oxidase independent [346] whereas a second type is dependent on ROS production which expels mostly mitochondrial DNA [347]. SYTOXTM green was used to stain nucleic acid in the experiments reported in this thesis and cannot distinguish between mitochondrial and nuclear DNA. Future studies using polymerase chain reaction-based methods and mitochondrial DNA staining, such as MitoSOXTM, could help distinguish between these two processes providing firmer evidence.

4.7.4. NADPH oxidase system

Respiratory burst describes the conversion of oxygen into superoxide anions by the NADPH oxidase system present in neutrophils. Amplified respiratory burst activity is an essential step for many priming agents and subsequent effector functions. In this chapter it was shown that TGF β 1 did not significantly increase extracellular superoxide anion release from neutrophils. These chemiluminescence experiments were significantly underpowered (post-hoc power calculation 5% for 5 samples of 10 ng/ mL TGF β 1 vs control) likely due to recognised inter-donor variability (displayed graphically as error bars representing standard error of the mean in Figure 4.16B). Unfortunately, no further experimental replicates could be performed for the remainder of this PhD time due to the luminometer breakdown.

4.7.5. TGF β 1 signalling in human neutrophils

TGF β R1 expression was identified in human neutrophils using western blot analysis. This is in keeping with two previous studies that demonstrated TGF β R1 expression by real-time quantitative polymerase chain reaction and affinity binding studies [283,311].

Inhibition of the TGF β R1 by the small molecule inhibitor, LY364947, appeared to reduce cfDNA release from neutrophils exposed to TGF β 1, suggesting that TGF β 1 plays a role in NET formation via this receptor. However, CXCR2 does not appear to be involved in TGF β 1 mediated chemokinesis, with no signs of internalisation or blocking following TGF β 1 treatment.

The effect of TGF β 1 on canonical and non-canonical signalling pathways was first explored using western blot. TGF β 1 did not affect the phosphorylation of the non-canonical signalling molecules; p38 MAPK or p44/42 MAPK. This is at variance with a previous study that noted increased phosphorylated-p44/42 MAPK expression in human neutrophils following 20 minutes exposure to 2 ng/ mL TGF β 1 [313]. However, this is not likely to represent experimental failure in the current thesis as the expression of these two signalling molecules was clearly increased by the positive control, 1 μ M fMLP, which suggests that the experiment design should appropriately detect these molecules [90]. However, it remains possible that western blot was insufficiently sensitive enough to detect these proteins if TGF β 1 induced only low quantities of phosphorylation. This would be likely if signalling components changed by the same relative amount as the functional changes precipitated by TGF β 1 (up to 15-20%). Non-specific antibody binding prevented reliable detection of phosphorylated Smad1 or Smad2/3 in human neutrophils via western blot. Therefore, a phosphoflow cytometry assay was developed, which showed that TGF β 1 increased the expression of phosphorylated Smad2/3 in human neutrophils consistent with previous work that showed TGF β 1 phosphorylated Smad3c [313].

The findings described in this chapter are summarised graphically in Figure 4.25.

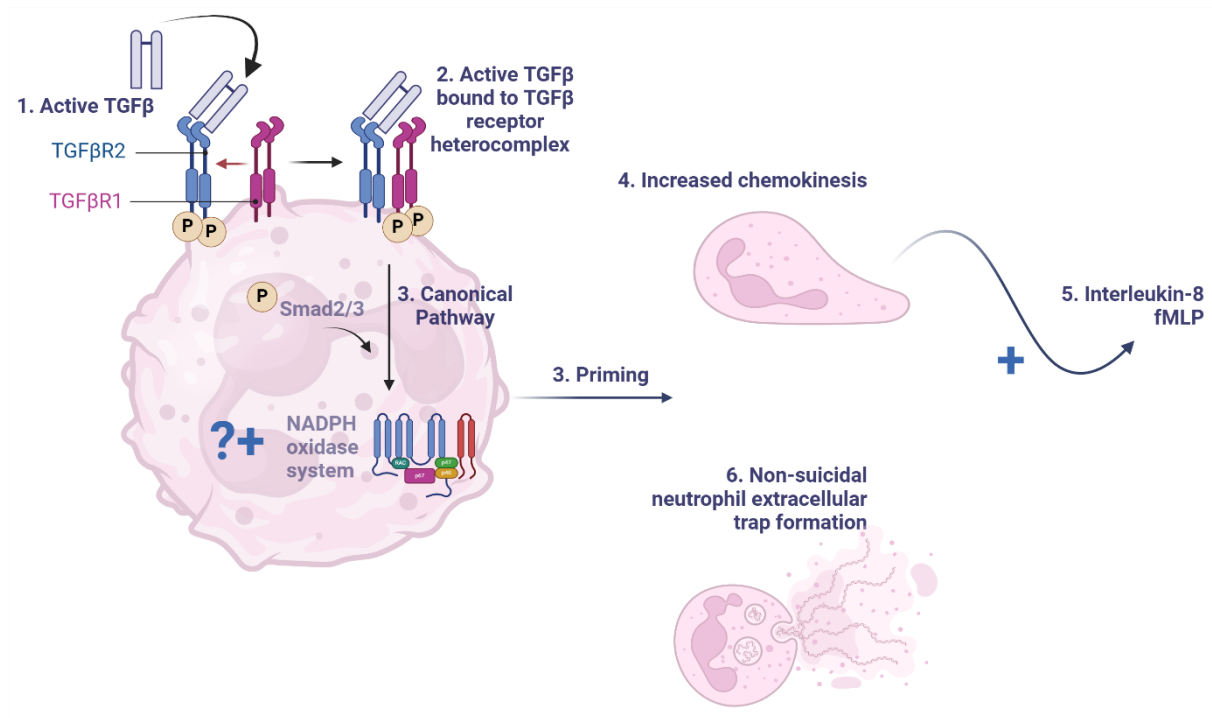


Figure 4.25: Proposed effects of TGFβ1 on human neutrophils.

This figure summarises the findings in this chapter. Here it is proposed that **(1)** active TGFβ1 binds to TGFβR2 on the surface of human neutrophils, which forms an activated heterocomplex with TGFβR1 **(2)**. This drives signalling via the canonical pathway **(3)**, namely phosphorylated Smad2/3. Canonical signalling and downstream molecules/mechanisms (yet to be determined) results in **(3)** neutrophil priming, whereby neutrophils demonstrate **(4)** increased chemokinesis, and **(5)** amplifies chemokinetic and chemotactic responses towards the chemoattractants, interleukin-8 and fMLP, respectively. TGFβ1 may also drive **(6)** non-suicidal neutrophil extracellular trap formation. Further work is required to confirm whether TGFβ1 drives NET formation and explore whether the NADPH oxidase system is important in TGFβ1 mediated priming. *Figure created using BioRender.com.*

4.7.6. Limitations

There are some natural issues that provide limitations for the interpretation of data presented within the current thesis.

4.7.6.1. Donor heterogeneity

It is widely recognised that neutrophil heterogeneity provides a challenge to small replicate studies even using cells from healthy individuals.

Neutrophil donors in this chapter were male or female and were self-reported healthy, non-smokers. Nevertheless a heterogeneous donor population may have contributed to the variability in neutrophil responses to TGF β 1 and resultant low statistical power reported in some experiments (such as chemiluminescence assays). For example, donor age has been shown to impact neutrophil responses, which was variable here (20 to 60 years of age), although most participants were less than 40 years old [348]. Polymorphic sequence changes in TGF β R1 may be an additional reason for inter-donor variability in TGF β 1 responses, as identified in primary squamous cell carcinomas of the head and neck [349]. Unfortunately, there was no preceding data on such variability prior to studies performed here that could have enabled pre study power calculations for all the experiments although post-hoc analysis enables some caution in the interpretation of borderline statistical data.

4.7.6.2. Neutrophil isolation and handling

Human neutrophils were isolated from venous blood using dextran sedimentation followed by Percoll density gradient centrifugation as described previously [350]. Neutrophils are difficult to examine *ex vivo* due to their high susceptibility during isolation to activation that can be influenced by numerous factors, such as anticoagulation type, isolation methods,

processing time and incubation temperature [218]. Care was taken to minimise neutrophil activation, which included ensuring gentle physical handling of neutrophils, minimising processing delays, and ensuring that all materials were sterile. Connelly *et al* noted that neutrophil apoptosis becomes evident after 6 hours incubation time *ex vivo* [351]. So here all attempts were made to minimise processing time, and all experimental assays were complete within 6 hours of isolation. Connelly *et al* also showed that neutrophil activation appeared to relate to the number of manipulation steps undertaken during isolation and processing [351]. For example, gradient centrifugation using Ficoll (an alternative to Percoll) recovered fewer neutrophils and appeared to increase neutrophil degranulation and activation as compared to whole blood methods [351]. Connelly *et al* therefore recommended assessing cell surface markers on whole blood, which included a red blood cell lysis step [351]. However, there remains no clear consensus on optimum neutrophil isolation methods, with others recommending against the use of red blood cell lysis, finding that it reduced neutrophil responses to milder stimuli and activates neutrophils [352]. This same paper showed that histopaque (an alternative to dextran)/Percoll density gradient centrifugation resulted in neutrophil recovery and purity equivalent to negative selection immunomagnetic bead-based kits [352]. Furthermore, cell surface markers of activation (CD11b, CD66b and CD63) were similar between unprocessed whole blood neutrophils and neutrophils isolated via histopaque/Percoll density gradient centrifugation [352]. Therefore, the inconsistency in neutrophil isolation and handling methods between studies are likely to explain some of the variability here and in the published literature in neutrophil responsiveness to TGF β 1.

4.7.6.3. *Simplistic models – far from physiological*

Neutrophils interact with a complex milieu of cell types (immune cells, red blood cells and endothelial cells) and an exhaustive array of molecules within the circulation. Blood flow within vessels also results in shear stress, which can impact neutrophil adhesion [67]. The transwell and Insall chamber assays used in this chapter are very simplistic models of neutrophil movement that lack the physiological complexity, in particular; 1) presence of other circulating cell types, 2) endothelial cells and their activation and 3) shear stress itself.

In this thesis, a neutrophil purity of >95% was sought due to the importance of high purity for experimental assays *in vitro* [353]. However, excluding other cell types that are usually present within the physiological system, could prevent identification of both direct and indirect effects of TGF β 1 on neutrophils via interaction with other cell types.

Endothelial cell - neutrophil interactions through selectins and integrins are critical for neutrophil migration into tissues as described in Chapter 1.8.3 [63–65,69,70]. The two models of neutrophil movement used in this thesis lack endothelial cells. Next steps to give greater physiological insight could include performing a flow-based adhesion assay (to also provide shear stress) as described by Butler *et al* [354] over an endothelial cell layer. The pro-fibrotic cytokine, IL-1 β , could be used to prime the endothelial cells in this model (an important step in this system) in light of its physiological relevance in IPF [233]. TGF β 1 might also drive neutrophil transmigration by acting directly on endothelial cells and upregulating expression of adhesion molecules, such as ICAM-1 and VCAM-1, as has been noted in murine models [355]. Establishing such a more complicated system would provide better insight to the physiological mechanisms of cell activation and migration.

An extension to the model that resembles migration through the complex parenchymal microenvironment would provide information on whether/how TGFβ1 drives neutrophil movement through fibrotic lung tissue following extravasation. Neutrophil interaction and movement within precision cut lung slice models of lung fibrosis would be an important developmental method [356].

4.7.6.4. TGFβ1 bioactivity

The bio-activity of TGFβ1 was not assessed for any of the industrial batches used in this thesis. As discussed previously, human recombinant TGFβ1 needs to be activated. This was performed in the current thesis by acidification (4 mM hydrochloric acid) as per manufacturer's instructions. TGFβ1 was not used beyond the maximum storage time (3 months) recommended by the manufacturer. However, it is unknown whether TGFβ1 activity remained stable even over no more than three months of storage. TGFβ reporter cell lines could be used to confirm continuing TGFβ1 bioactivity for all future studies.

4.7.7. Future work

It is critical that the current work is consolidated and expanded as this will expand our knowledge of how TGFβ1 affects neutrophils, including the underlying mechanisms/pathways. This will allow the examination of any aberrancies that may be present in the context of IPF and crucially may identify novel treatment targets.

4.7.7.1. Consolidate current experiments

It is of primary importance that further experimental replicates are performed where already indicated (for example chemiluminescence and NET immunofluorescence microscopy) to improve statistical power of these experiments. Where there is incongruency between assays, a third method needs to be sought to tease out true effects from those seen solely due to chance.

4.7.7.2. Neutrophil movement

Next steps to investigate the effects of TGF β 1 on neutrophil movement would include utilising more complex and physiological models of migration and chemotaxis as described in Chapter 4.7.6.3. Subsequently, a step-wise approach would be required to determine the mechanism by which TGF β 1 promotes neutrophil chemokinesis. This would include firstly assessing the effect of TGF β R1 blockade and downstream signalling molecules on TGF β 1 mediated neutrophil movement. The effect of TGF β 1 on actin polymerisation (a crucial process of neutrophil movement) using phalloidin staining should be measured.

4.7.7.3. Neutrophil priming

Various pathways should be explored to clarify how TGF β 1 amplifies IL-8 and fMLP mediated chemokinetic or chemotactic responses. This would include whether TGF β 1 increases fMLP mediated induction of CD11b and shedding of CD62L, as seen with TNF α priming [341]. In addition, examination of the process of receptor transactivation between the TGF β receptors and the G-protein coupled receptors for IL-8 and fMLP, namely CXCR1/2 and FPR1, respectively as activation of G-protein coupled receptors induces rapid cell surface translocation of cytosolic TGF β receptors in other cell types [357]. It is currently unknown if this occurs in neutrophils.

4.7.7.4. NET formation

Understanding of non-suicidal NET formation remains limited. Various methods should be adopted to identify the features of this pathway and confirm whether/how TGF β affects NET formation via this pathway. Electron microscopy imaging would visualise morphological changes in the nuclear membrane, vesicle formation and exocytosis of NETs as used by Brinkmann *et al* and Fuchs *et al* [103,358]. High resolution time-lapse microscopy would also characterise the biomechanics of NET extrusion [359]. In addition, localisation of mitochondrial DNA and other components of NETs, such as citrullinated histone, using fluorogenic dyes/antibodies would provide a more comprehensive understanding of the process. Finally, it is important to assess the contribution of the NADPH oxidase system pathway in TGF β 1 mediated mechanisms to complete understanding of the process.

4.7.8. Conclusions

This chapter identified that TGF β 1 primes neutrophils and increases neutrophil chemokinesis. TGF β 1 exposure amplifies chemokinetic and chemotactic responses to the chemoattractants, IL-8 and fMLP, respectively and likely acts via the cell membrane serine and threonine kinase receptor, TGF β R1 and canonical pathways. Future work is required to assess if TGF β 1 drives neutrophil movement in more physiological models, clarify the mechanisms, and provide more rigorous evidence that TGF β 1 induces non-suicidal NETosis. This chapter has led to the support of the following hypothesis that will be investigated in Chapter 6 of this thesis: circulating neutrophils in IPF patients display a primed phenotype that is precipitated, in part, by TGF β 1. Chapter 5 will now describe the protocol and participant characteristics of the prospective cohort study, “HYBRID”, that was devised to investigate this.

Chapter 5. DESIGN AND CHARACTERISTICS OF THE PROSPECTIVE COHORT STUDY, “HYBRID”.

5.1. Abstract

This chapter outlines the development of the prospective cohort study entitled ‘The Hypoxic Burden in Interstitial Lung Diseases (HYBRID)’. This study was devised to investigate a) the hypothesis that circulating neutrophils in IPF patients display a primed phenotype and b) to assess day and nighttime patterns of peripheral arterial blood oxygen saturation (SpO₂) of IPF patients. This study also included an optional research bronchoscopy for the collection of bronchoalveolar lavage fluid. The study protocol and methodology are described below. The detailed characterisation of control and IPF participants is outlined and indicates that these cohorts were well matched for demographics and other parameters. IPF patients were mostly of mild to moderate disease severity and experienced increased frequency of exertional and intermittent hypoxaemia compared to control participants. The results of the neutrophil related experimental assays performed from biological samples obtained from participants in this study are described in Chapter 6.

5.2. Background

5.2.1. Neutrophils in IPF – a prospective cohort study is needed.

Neutrophil content in the blood and BALF of IPF patients may be associated with fibrosis severity and mortality (see Chapter 1 for further detail) [360–362]. However, most of the research to date is retrospective in nature and of the few prospective studies performed, IPF and control cohorts are often poorly matched, introducing potential confounding factors [361]. This has resulted in little confidence that neutrophils are a disease biomarker and a lack of translational research investigating the role of neutrophils in IPF pathophysiology. A prospective cohort study is needed where IPF and control cohorts are well matched to assess whether neutrophils, their function and contents, differ in this disease state.

5.2.2. Hypoxia may alter neutrophil function in ILDs.

Khawaja *et al* performed one of the few studies that has attempted to characterise neutrophil activity in IPF patients [19]. This study observed more NETs in IPF tissue and in BALF derived from patients with ILD (IPF, chronic hypersensitivity pneumonitis, fibrotic non-specific interstitial pneumonia, unclassifiable ILD) compared to control participants [19]. However, the control participants in this study were poorly matched to ILD participants regarding demographics and smoking status [19]. Khawaja *et al* proposed that hypoxic mechanisms may drive neutrophil recruitment and NET formation in the fibrotic lung [19]. This was supported by: a) HIF-1 α (an hypoxia-dependent transcription factor) expression in neutrophils present within IPF tissue and b) hypoxia increased NET formation and neutrophil adhesion and migration *in vitro* [19]. Extensive research outside the ILD setting has demonstrated that hypoxia is indeed an important regulator of neutrophil function, increasing neutrophil degranulation [363] and longevity [43].

Resting and exertional hypoxaemia are more frequent in patients with IPF [364] reflecting alterations in the pulmonary gas exchange unit. This takes place early in the disease process, with impairment of the diffusing capacity of the lungs for carbon monoxide (DLCO), present in nearly 100% of IPF patients at diagnosis [365]. Furthermore, exertional and nocturnal hypoxaemia appear to be predictive of mortality [366,367]. However, the extent of hypoxaemia during day to day activities remains unknown in IPF and how this alters cellular functions, such as neutrophils, and potentially contributes to disease pathophysiology.

5.2.3. The development of the HYBRID study.

In the previous chapter, I demonstrated that the key pro-fibrotic growth factor, TGF β 1, primes neutrophils; amplifying neutrophil chemokinetic and chemotactic responses. Studies elsewhere have noted that TGF β 1 is elevated in the circulation of patients with IPF [29,280]. Therefore, the following hypothesis was considered:

Circulating neutrophils in IPF patients display a primed phenotype.

The prospective observational study, HYBRID, was created to investigate this hypothesis by comparing the phenotype and function of circulating neutrophils derived from IPF patients and carefully matched control participants. As hypoxia may be an important regulator of neutrophil function, characterisation of study participants included the measurement of daytime and overnight peripheral SpO₂.

This chapter, describes the methodology and participant characteristics of the HYBRID study.

5.3. Chapter Aims

1. To develop a prospective observational study that includes control and IPF cohorts, closely matched for demographics, exposure history and comorbidities.
2. To characterise the severity of IPF in the disease cohort, which included physiological measures and symptom and health related quality of life questionnaires.
3. To compare exertional, daytime and overnight SpO₂ between IPF and control participants.

5.4. Methods linked to Chapter 5

5.4.1. Ethical Approval

The HYBRID study received ethical approval by the Research Ethics Committee West Midlands, reference 22/WM/0274 (RRK7934). The study was performed at the Queen Elizabeth Hospital, University Hospitals Birmingham National Health Service (NHS) Foundation Trust, and sponsored by the University of Birmingham, England. All research was conducted in accordance with the Good Clinical Practice (GCP) guidelines.

5.4.2. Participant identification and recruitment

5.4.2.1. Inclusion and exclusion criteria

Inclusion and exclusion criteria are outlined in Table 5.1. IPF and control participants were enrolled concurrently with careful attention to ensure demographic features, smoking history and comorbidities (except IPF diagnosis) were comparable between the two groups.

Inclusion Criteria	Exclusion Criteria
Age $\geq$ 18 years, and able to provide informed consent, and MDT diagnosis of IPF (IPF cohort)*, Or no diagnosis of ILD (Control cohort).	• Resting SpO₂ <92% • Lung cancer diagnosis • Imminent treatment withdrawal for palliation • Intercurrent oral corticosteroid use* • Intercurrent infection*

Table 5.1: Participant inclusion and exclusion criteria.

All participants enrolled in this study met the described inclusion and exclusion criteria. These criteria are outlined as per the HYBRID study Protocol V1.6. Additional criteria that are marked with an asterisk were implemented for the work in this thesis. MDT – multi-disciplinary team.

5.4.2.2. IPF patient recruitment

Patients with a multi-disciplinary team diagnosis of IPF due to be reviewed or under follow-up in the respiratory clinic were screened for eligibility. Patients who fulfilled the inclusion and

exclusion criteria (see Table 5.1) were introduced to the HYBRID study by a member of the respiratory team. Patients who were interested in the study were provided with a participant information sheet and later invited to attend study visit 1.

5.4.2.3. Control participant recruitment

The HYBRID study was introduced to potential control participants who were currently/previously enrolled in 'The Birmingham 1000 Elders group' via email or in person. Those expressing interest, were provided with a participant information sheet and invited to attend study visit 1.

5.4.3. Study schedule

The study schedule is outlined in Figure 5.1. Written informed consent was completed upon participant arrival for study visit one. Subsequently, clinical data was collected (see Chapter 5.4.4), healthy related quality of life questionnaires completed (see Chapter 5.4.5) and a six-minute walk test was performed (see Chapter 5.4.6). Participants were provided with two wristwatch devices to perform daytime and overnight oximetry (see Chapter 5.4.7). These devices were subsequently returned by hand to the Queen Elizabeth Hospital, Birmingham. A 20 mL urine sample and 40 mL venous blood sample were collected and processed immediately (see Chapter 5.4.9). Completion of health-related quality of life questionnaires, six-minute walk test, day and overnight oximetry and urine sample collection were optional aspects of the study. Therefore, these activities were not performed for all study participants either due to patient choice, logistics on the day or if deemed not clinically appropriate to be performed. Study visit two was an optional visit for those who provided written informed consent to undertake a research bronchoalveolar lavage procedure.

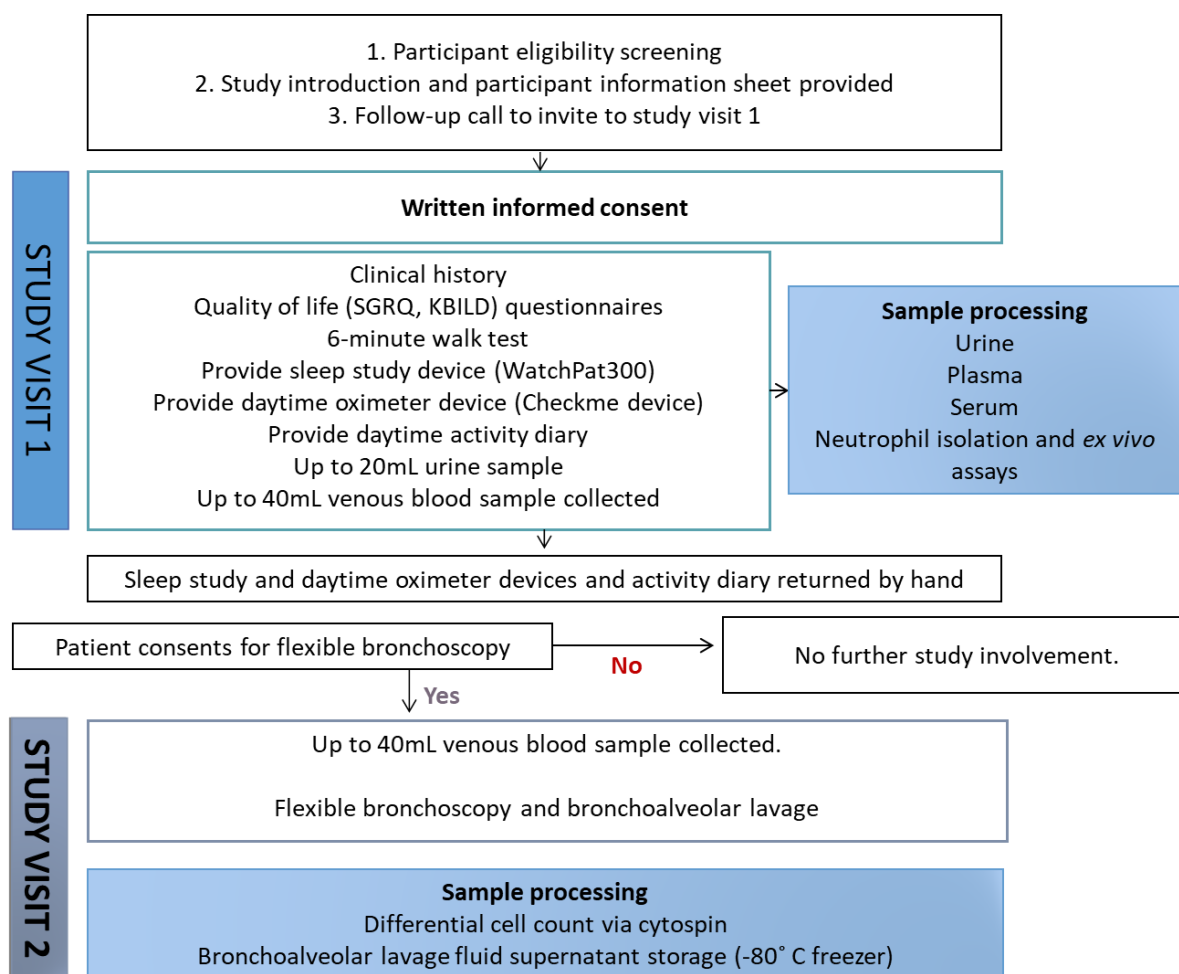


Figure 5.1: HYBRID Study Schedule.

HYBRID study schedule is outlined. Informed consent was provided at study visit one. Study visit two was an optional visit for those who provided informed consent to undertake a research flexible bronchoscopy procedure. KBILD – King’s Brief Interstitial Lung Disease, SGRQ – St George’s Respiratory Questionnaire.

5.4.4. Clinical and pulmonary function data collection

Relevant clinical data was collected by a GCP-trained member of the research team by obtaining a verbal medical history from the participant and review of the electronic patient records. Data was collected using paper case report forms and following pseudo-anonymisation then transcribed onto a secure, electronic database. All study records were archived in accordance with the applicable regulatory requirements.

The closest pulmonary function data performed within 9 months of study visit 1 was retrieved from the electronic patient records. The mean time between lung function tests and study visit one was ± 3.8 months (SD 2.5). Dependent on the site that the pulmonary function tests were performed, gas transfer was reported as either DLCO or transfer capacity of the lung (TLCO) (both were corrected for haemoglobin unless otherwise stated). Carbon monoxide transfer coefficient (KCO) was also reported, alongside forced expiratory volume in one second (FEV1) and forced mix-expiratory flow (FEF 25-75%) (measure of small airway dysfunction [368]).

The Gender, Age and Physiology (GAP) score [369], which predicts mortality in IPF patients was determined for IPF participants using the lung physiology measures of FVC and gas transfer. A score from 0 to 8 points is calculated and then divided into three stages as described by others [369] – see Table 5.2.

Stage	Points	1- year Mortality
I	0 - 3	6%
II	4 - 5	16%
III	6 - 8	39%

Table 5.2: Gender, age and physiology (GAP) score.

Gender, age and physiology (GAP) score was developed by Ley *et al* as a multidimensional prognostic staging system using readily measured physiological and clinical variables for patients with idiopathic pulmonary fibrosis [369]. The three stages calculated by points and relative predicted 1- year mortality as determined by the authors of the GAP score are outlined [369].

5.4.5. Questionnaires

5.4.5.1. Medical Research Council dyspnoea scale

Control and IPF participants self-administered the Medical Research Council (MRC) dyspnoea scale adapted from Gough, *et al* [370,371], as described in Table 5.3.

Medical Research Council Scale	Description
1	Not troubled by breathlessness except during strenuous exercise.
2	Short of breath when hurrying or walking up a slight hill.
3	Walks slower than contemporaries on the level because of breathlessness, or has to stop for breath when walking at own pace.
4	Stops for breath after walking about 100 metres or after a few minutes on the level.
5	Too breathless to leave the house, or breathless when dressing or undressing.

Table 5.3: Medical research council (MRC) dyspnoea scale.

The medical research council (MRC) dyspnoea scale adapted from Gough, *et al* [370,371]. Participants self-administered this questionnaire, which scores the patient's perspective of the effect of breathlessness on their physical activity.

5.4.5.2. Health related quality of life questionnaires

IPF participants, following informed consent, self-administered two health related quality of life questionnaires at study visit one: the St George's Respiratory Questionnaire (SGRQ) and the King's Brief Interstitial Lung Diseases (KBILD) questionnaire.

Permission was given by St George's, University of London (St George's Hospital Medical School) to use the SGRQ in this study. The SGRQ was one of the first health related quality of life questionnaires in respiratory medicine and was developed for patients with chronic airways disease [372]. SGRQ has since been shown to be predictive of mortality in patients with IPF [373] The SGRQ is divided into three domains: 1) symptoms, 2) activity and 3) impacts. A score of zero represents best health status and a score of 100 points represents worst possible health status.

The more recent KBILD questionnaire was specifically designed for patients with ILDs and divides questions into three domains: 1) psychological, 2) breathlessness and activities, 3) chest symptoms [374,375]. A score of zero represents worst health status, whereas a score of 100 points represents best possible health status [376]. A non-commercial licensing agreement was completed prior to use of the KBILD questionnaire in this observational study. The results of each questionnaire were pseudoanonymised, transcribed to a secure Microsoft Excel sheet and scored as per the questionnaire provider recommendations.

5.4.6. Six-minute walk test

The six-minute walk test is a reliable measure of exercise tolerance in patients with IPF [377]. During this test, the maximum distance walked over six minutes is measured. It has been

shown previously that total walk distance weakly relates to FVC and gas transfer, and subsequent decline in walk distance is highly predictive of mortality [377]. Over the six minutes, pulse oximetry, heart rate, level of dyspnoea using the Borg scale [378] and leg fatigue are also measured at 1-minute intervals. Therefore, this test also provides data on SpO₂ during exertion. Six-minute walk test is commonly used by lung physiology departments to guide commencement of ambulatory oxygen therapy, and desaturation during six-minute walk test is predictive of mortality in patients with IPF [379].

The six-minute walk test was performed in this study by control and IPF participants except those who declined, those unable due to physical limitations, or those receiving ambulatory or long-term oxygen therapy. Verbal instructions were provided to the participant prior and during the test as described in the 'American Thoracic Society Statement: Guidelines for the Six-Minute Walk Test' [378]. The number of shuttles walked between a 10-metre course were recorded using a mechanical counter and multiplied by 10 to determine total distance walked. The number and duration of rests were also recorded. Pulse oximetry and heart rate were measured using the Checkme O₂ wristwatch oximeter device (*Viatom Technology, China*) as described in the following section.

5.4.7. Oximetry devices

5.4.7.1. Checkme O₂ pulse oximeter device

SpO₂ was measured during the six-minute walk test and daytime oximetry study using the Checkme O₂ device. The Checkme O₂ device is a wristwatch (see Figure 5.2), which measures heart rate and SpO₂ non-invasively using spectrophotometry. Briefly, oxyhaemoglobin and deoxyhaemoglobin absorb near-infrared and red light differentially (oxyhaemoglobin absorbs

near-infrared better than red, and vice-versa for deoxyhaemoglobin). Pulse oximeter devices have light-emitting diodes on one side of the finger and detectors on the opposite side that measure the relative amount of near infra-red and red light absorbed [380]. Light absorbance is also impacted by rising and falling arterial volume that occurs with systole and diastole, [380]. These factors are all sensed and processed by the oximeter to provide SpO₂ and heart rate measurements.

The Checkme O₂ device was used in 'monitor mode' during the 6-minute walk test as this ensured that SpO₂ and heart rate were visible on the screen at each one-minute time point.

5.4.7.2. Daytime oximetry studies

The daytime oximetry studies were performed by both control and IPF participants, excluding any patients who declined or were receiving ambulatory or those on long term oxygen therapy. Participants were given a Checkme O₂ device to take home. Participants were asked to use the device for a single 'typical' day for approximately ten hours. For the daytime studies, the device was placed in 'sleep mode', as this mode ensured the device recorded for the entire duration that the finger probe was worn. Oximetry and heart rate readings were not displayed during this 'sleep mode' which had the benefit of blinding participants to the readings and preserving device battery. A paper 'activity diary' was provided to each participant, with instructions to write the start time and end time of using the device, timing of physical activities, for example walking, cleaning, gardening, and any periods of rest. Following completion of the daytime recording, participants returned the CheckMe O₂ device and activity diary to the research team. Data from the CheckMe O₂ devices were downloaded and summarised into a report using Visi-Download (*Stowood, Oxford, UK*). Results were summarised in graphical form and statistical tests performed using GraphPad Prism (*Version 9.5.0, San Diego, USA*).

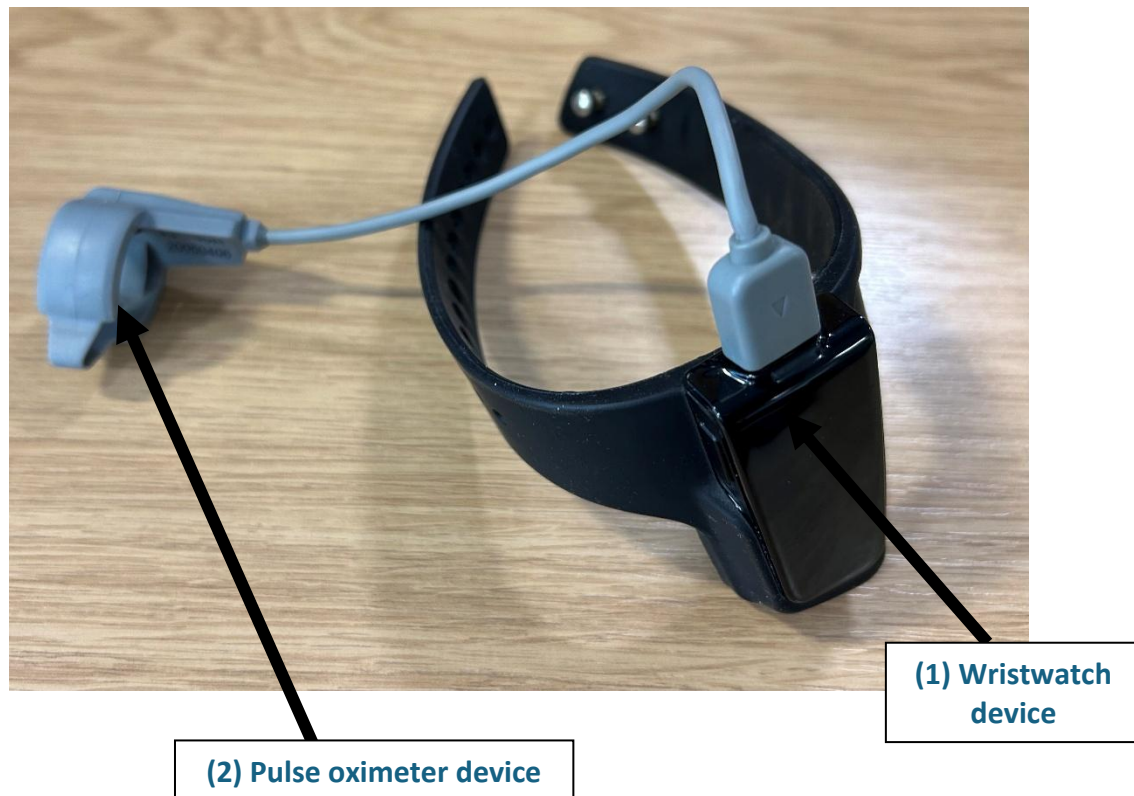


Figure 5.2: Checkme O2 pulse oximeter device.

Photograph of a Checkme O2 pulse oximeter device used for oximetry and pulse recording during the six-minute walk test and daytime oximetry studies. This is a wristwatch device **(1)** placed on the wrist using the watch strap, and the pulse oximeter device **(2)** was placed on either a thumb or finger for comfort and security. The screen displayed heart rate and peripheral arterial blood oxygenation readings. The button was used to turn on the device and cycle between modes.

5.4.7.3. Sleep study device – WatchPAT™300

Sleep studies were performed using the WatchPAT™300 (*Itama Medical Ltd., Israel*) device, which is a non-invasive home sleep apnoea testing device. Detection of oxygen desaturation and apnoea-hypopnoea index (AHI) from WatchPAT devices highly correlate with the gold-standard overnight polysomnograms [381]. WatchPAT devices are now commonly used in clinical practice due to their ease of use, and lower cost compared to traditional polysomnography techniques. They have also previously been used in an observational study, which investigated whether nocturnal hypoxaemia and obstructive sleep apnoea (OSA) related to clinical outcomes in patients with fibrotic ILD [382].

The WatchPAT™300 device is worn on the wrist (See Figure 5.3), and the finger-mounted probe (called a unified peripheral arterial tone (PAT®) and pulse oximeter (uPAT) probe) measures both PAT® and SpO2 [383]. The PAT® signal reflects relative arterial vasomotor activity, whereby a reduction in PAT® signal amplitude reflects peripheral arterial vasoconstriction secondary to sympathetic activation. The combination of sympathetic activation, rise in pulse rate and desaturation that occurs on termination of a respiratory event are used to detect apnoea/hypopnoea events [384]. A combination of PAT® and actigraphy signals aids characterisation of sleep, such as light/deep/rapid eye movement (REM) sleep (data not shown). Chest movement, body position and snoring are also detected by a WatchPAT™ integrated respiratory effort snore and body position (RESBP) sensor that is placed on the sternum (data not shown). An actigraph that detects activity/rest is built into the watch device.

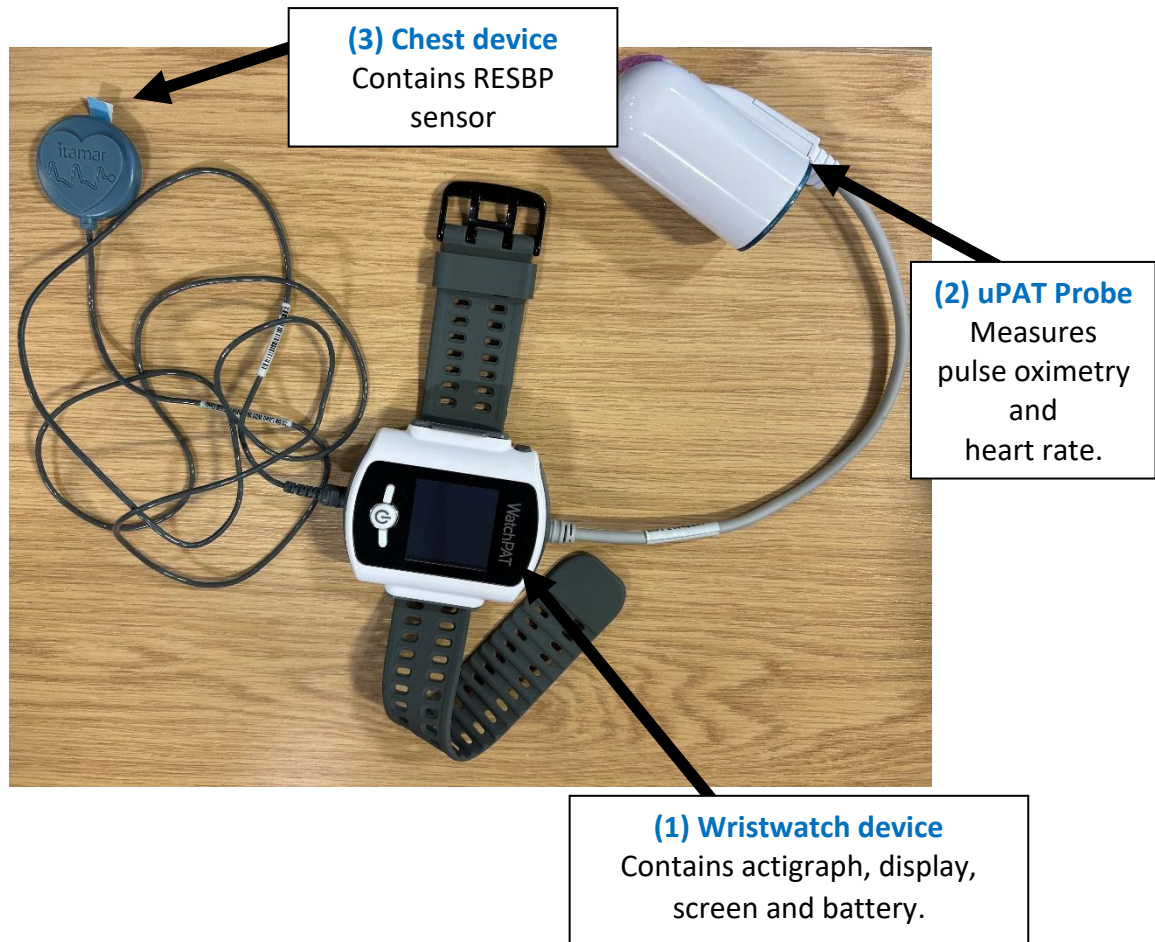


Figure 5.3: WatchPAT™300 device.

Photograph of a WatchPAT™300 device used to perform overnight oximetry studies. This is a wristwatch device (1) secured in place using the strap. The unified peripheral arterial tone (PAT®) and pulse oximeter (uPAT) probe (2) is placed on the finger with the sticky tag removed prior to use. WatchPAT™ integrated respiratory effort snore and body position (RESBP) sensor is present within the chest device (3), which is placed on the sternum and measures chest movement, position and can detect snoring. Built-in actigraph, buttons (to turn the device on and perform device checks), display screen and battery (one AAA battery) are present in the wristwatch device (1).

Prior to use, the device was registered to the study participant (using zzzPAT® Software (*Itama Medical Ltd., Israel*)), a new uPAT probe (single use only) and AAA battery applied and an electronic device check performed. Participants were advised to start the device when about to go to sleep and to remove it in the morning. Following completion of the study, the participant returned the device to the research department. Data from the WatchPAT™300 device was downloaded and summarised into a report using zzzPAT® Software. Results were summarised in graphical form and statistical tests performed using GraphPad Prism (*Version 9.5.0, San Diego, USA*).

5.4.8. Bronchoalveolar lavage fluid collection

5.4.8.1. Bronchoscopy procedure

Flexible bronchoscopy was performed by myself under supervision by Dr Dhruv Parekh (Chief Investigator of the HYBRID study), and supported by trained endoscopy nurses. All procedures were performed in a dedicated endoscopy suite at the National Institute for Health Research (NIHR)/Wellcome Trust Clinical Research Facility, University Hospitals Birmingham. Only participants who provided informed written consent for the research bronchoscopy, including the completion of a local consent form, and were deemed suitable for the procedure by the Chief and sub-investigators of the study were included. Participants with a gas transfer less than 50% predicted or receiving ambulatory or long-term oxygen therapy were not invited for a research bronchoscopy. The research bronchoscopy was performed under conscious sedation (midazolam +/- fentanyl) and local anaesthetic instilled into the upper respiratory tract and bronchial tree using an EVIS X1 video bronchoscope (*Olympus, UK: BF-H1200*). Bronchoalveolar lavage fluid samples were collected by instilling 3 x 50 mL sterile saline

(*Baxter, UK: FKE1323*) into a segmental bronchus within the right middle or lower lobe (until the bronchoscope was gently wedged). Previous imaging was reviewed prior to the procedure being performed on patients with IPF to check that the region of lavage showed features of UIP. Following instillation, the lavage fluid was aspirated into 20 mL specimen traps (*Pennine, UK: MST-3000*). These BALF samples were then immediately placed on ice and transferred for processing in the laboratory.

5.4.8.2. BALF processing

BALF was strained through non-woven sterile swabs (*Unisurge International, UK: F821034*) and then centrifuged at 560 x g for 10 minutes at 4 ° C. The supernatant was removed and stored immediately at -80 ° C until analysed. The cell pellet was resuspended in RPMI and BALF differential cell count was determined using a cytospin and REASTAIN QUICK-DIFF KIT (*Reagent, Finland: 102164*) as described in Chapter 2.4.4. The percentage of neutrophils, mononuclear cells and alveolar macrophages was determined by imaging the stained slide by light microscope (*Wilovet, Germany*) at 20x magnification. Total cell number was also calculated as described in Chapter 2.4.3. See Table 6.3 describing BALF yield and differential count.

5.4.9. Processing of other biological samples

5.4.9.1. *Urine*

Urine was centrifuged at 560 x g for 10 minutes and the supernatant collected and stored immediately at -80°C until analysed.

5.4.9.2. *Venous blood*

Briefly, venous blood samples were collected peripherally into blood collection tubes as described in Chapter 2.2. A variety of collection tubes (outlined in Table 2.2) were used to allow for the isolation of neutrophils (experimental assays outlined in Chapter 6) and collection of plasma (single and double spun) and serum supernatant (centrifugation methods described in Chapter 2.3).

5.5. Results

5.5.1. Patient characteristics

Eighteen control and 26 IPF participants were recruited to the HYBRID study between September 2023 and September 2024. Two control participants were excluded from analyses due to the incidental identification of abnormal lung physiology (see Figure 5.4). One patient desaturated significantly on six-minute walk test and was subsequently noted to have restrictive spirometry in keeping with suspected undiagnosed ILD (investigations ongoing). A second patient was noted to have a high neutrophil percentage in the BALF (72%), which on further investigation was diagnosed as untreated small airways inflammation (bronchial thickening on thoracic imaging and moderately raised fractional exhaled nitric oxide). A further BALF sample was excluded due to features of acute viral infection on differential immune cell count (low alveolar macrophage (21%) and high lymphocyte (57%) percentages with a high number of ciliated columnar epithelial cells).

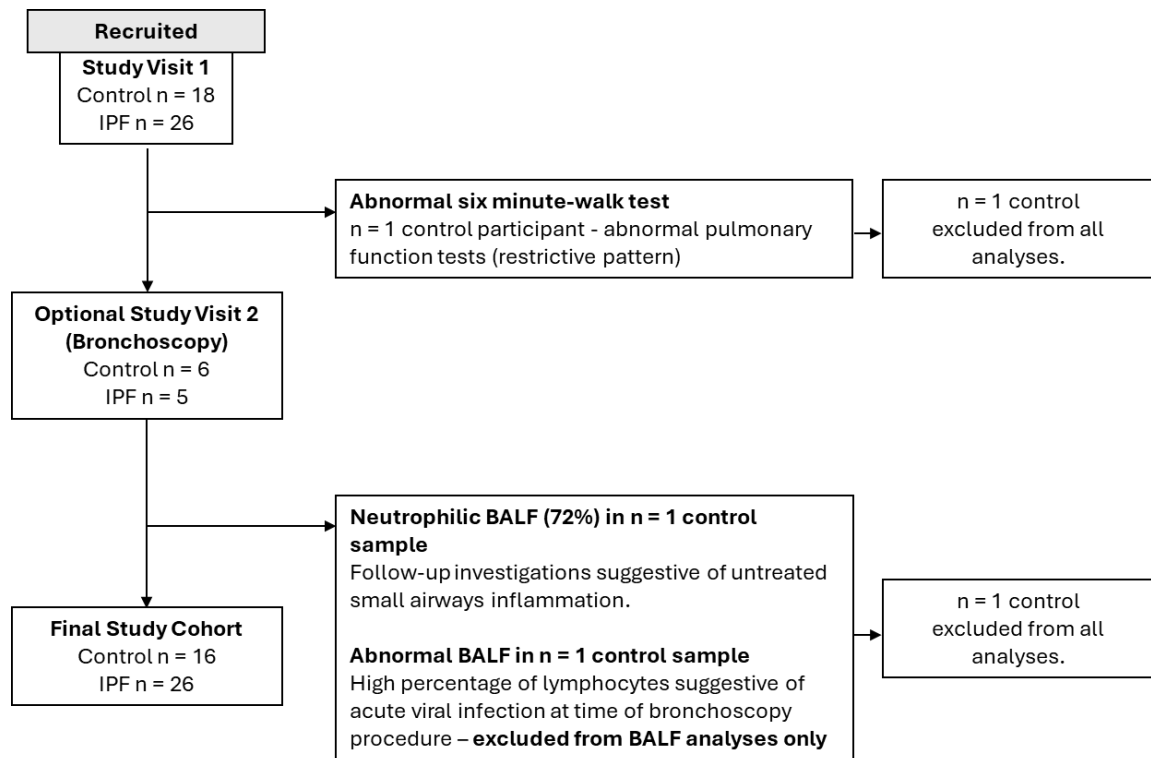


Figure 5.4: Study inclusion flow chart.

Eighteen control and 26 IPF participants were recruited to the HYBRID study at visit 1. Two control participants desaturated by a considerable amount during the six-minute walk test. One had normal lung function tests so was included in the study. The other participant was excluded from all study analyses as pulmonary function tests revealed a restrictive pattern. Six control and 5 IPF participants undertook a research bronchoscopy at study visit 2. One control sample displayed an abnormally high neutrophil percentage (72%). Subsequent investigations suggested underlying untreated small airways inflammation. This participant was thus excluded from all study analyses. One control BALF sample contained a high percentage of lymphocytes (57%) suggestive of acute viral infection at time of bronchoscopy. This sample was excluded from BALF analyses only. This led to a final study cohort of 16 control and 26 IPF participants.

Therefore, a total of 16 control participants and 26 IPF patients were included in the study analyses. Participants were well matched for demographics, smoking history, comorbidities and concomitant medications, except for body mass index (BMI) which was higher in patients with IPF versus control participants (28.9 (SD 4.1) versus 26.1 (SD 4.1), $p = 0.04$, unpaired t-test). The mean age was approximately 75 years for both groups and over 70% of participants were men in both cohorts. Approximately two thirds of participants were ex-smokers with a similar pack year history and the average time since stopping smoking was 43.1 years (SD 14.8) and 29.7 years (SD 20.8) for control and IPF participants, respectively. None of the participants were current smokers. These data are summarised in Table 5.4.

Charlson comorbidity index was not significantly different between cohorts ($p > 0.05$, Fisher's exact test), although the IPF cohort unlike controls, did include 4 patients with a Charlson comorbidity index of 5 or more. Hypertension and gastroesophageal reflux disease (GORD) were the most common comorbidities in both cohorts. None of the participants had an alternative respiratory condition that was deemed clinically significant, defined as being symptomatic/uncontrolled/requiring polypharmacy. Three patients with IPF reported a diagnosis of asthma (either well-controlled or misdiagnosed), requiring only a single preventer inhaler with no recent asthma exacerbations. Three patients incidentally were noted to have minimal emphysema on previous HRCT imaging (upper lobe predominant). However, no patients had underlying chronic bronchitis or chronic obstructive pulmonary disease (COPD). Cohort participants had a similar medication burden (except anti-fibrotic therapy) with the most common medication types outlined in Table 5.4. Anti-hypertensive therapies, proton pump inhibitors and statins were the most common medications being administered in both cohorts.

	Control (n = 16)	IPF (n = 26)	p-value
Demographics			
Age (years), mean (SD)	74.8 (5.8)	75.4 (5.9)	ns
Sex ratio, M : F	11 : 5	20 : 6	ns
Caucasian, n (%)	15 (93.8%)	26 (100%)	ns
Black, n (%)	1 (6.3%)	0 (0%)	ns
BMI, Kg/ m ² , mean (SD)	26.1 (4.1)	28.9 (4.1)	0.04
Smoking history			
Never-smoker, n (%)	5 (31.3%)	9 (34.6%)	ns
Current smoker, n (%)	0 (0%)	0 (0%)	ns
Ex-smoker, n (%)	11 (68.8%)	14 (65.4%)	ns
Years since stopped smoking, mean (SD)	43.1 (14.8)	29.7 (20.8)	ns
Pack history, median (IQR)	0.9 (0- 9.7)	6.2 (0- 30.0)	ns
Never vaper, n (%)	16 (100%)	26 (100%)	ns
Comorbidities			
Asthma, n (%)	0 (0%)	3 (11.5%)	ns
Cancer, n (%)	0 (0%)	1 (3.8%)^	ns
Cardiovascular disease, n (%)	0 (0%)	2 (7.7%)	ns
Chronic Kidney Disease Stage 3- 5, n (%)	1 (6.3%)	3 (11.5%)	ns
COPD, n (%)	0 (0%)	0 (0%)	ns
Diabetes, n (%)	1 (6.3%)	5 (19.2%)	ns
Emphysema [§] , n (%)	0 (0%)	3 (11.5%)	ns
GORD, n (%)	9 (56.3%)	14 (53.8%)	ns
Hypertension, n (%)	7 (43.8%)	17 (65.4%)	ns
Charlson Comorbidity Index			
≤ 2, n (%)	1 (6.3%)	4 (15.4%)	ns
3, n (%)	10 (62.5%)	7 (26.9%)	
4, n (%)	5 (31.3%)	11 (42.3%)	
≥ 5, n (%)	0 (0%)	4 (15.4%)	
Medications			
Anti-coagulation, n (%)	1 (6.3%)	4 (15.4%)	ns
Anti-hypertensives, n (%)	8 (50%)	18 (69.2%)	ns
Anti-platelet therapies, n (%)	2 (12.5%)	7 (26.9%)	ns
Oral steroids/immunosuppression, n (%)	0 (0%)	0 (0%)	ns
Proton pump inhibitors, n (%)	7 (43.8%)	10 (38.4%)	ns
Statins, n (%)	5 (31.3%)	17 (65.4%)	ns
Anti-fibrotic therapies			
Nintedanib, n (%)	0 (0%)	10 (38.4%)	0.007
Pirfenidone, n (%)	0 (0%)	6 (23.1%)	ns
Ambulatory oxygen therapy, n (%)	0 (0%)	5 (19.2%)	ns
Long-term oxygen therapy, n (%)	0 (0%)	0 (0%)	ns

Table 5.4: Participant characteristics.

Characteristics of control and IPF participants are described. Participants were well matched for demographics, smoking status, co-morbidities and medication history. IPF patients had a significantly higher BMI than control participants ($p=0.04$, unpaired T-test). [§]Three patients with IPF had minimal emphysema (upper lobe predominant) incidentally noted on HRCT imaging, in the absence of chronic bronchitis and COPD. [^]One patient with IPF had a history of localised liposarcoma under outpatient monitoring. Normally distributed and skewed data are presented as mean (SD) or median (IQR), respectively. Unpaired T-tests and Mann-Whitney tests were performed for normally distributed and skewed data, respectively. Fisher's exact test was used to analyse categorical data. BMI – body mass index, COPD – chronic obstructive pulmonary disease, F - female, GORD – gastroesophageal reflux disease, M – male.

5.5.2. IPF characterisation

All IPF patients recruited to the HYBRID study had a definite or probable UIP pattern on thoracic HRCT imaging and a diagnosis confirmed at the ILD multi-disciplinary team meeting.

Pulmonary function results revealed that IPF patients had a mean FVC of 77.8% predicted for age, sex and height (SD 12.8). FEV1 was 84.0% predicted (SD 13.0) and FEV1/FVC ratio was > 70% for all IPF patients. Mean gas transfer was 56.2% predicted (SD 19.4) and mean KCO 81.1% predicted (SD 25.2) (see Figure 5.5A). Note that three patients' gas transfer results were not corrected for haemoglobin (as not provided in report). FVC was $\geq 50\%$ predicted for all IPF patients and only 3 patients had a gas transfer $\leq 30\%$ predicted. Three patients were missing recent gas transfer results and two patients were missing recent spirometry data. Median FEF 25-75% for the IPF patients was 111.0 % predicted (IQR 97.5 - 144.5); 5 patients were missing recent data (see Figure 5.5B).

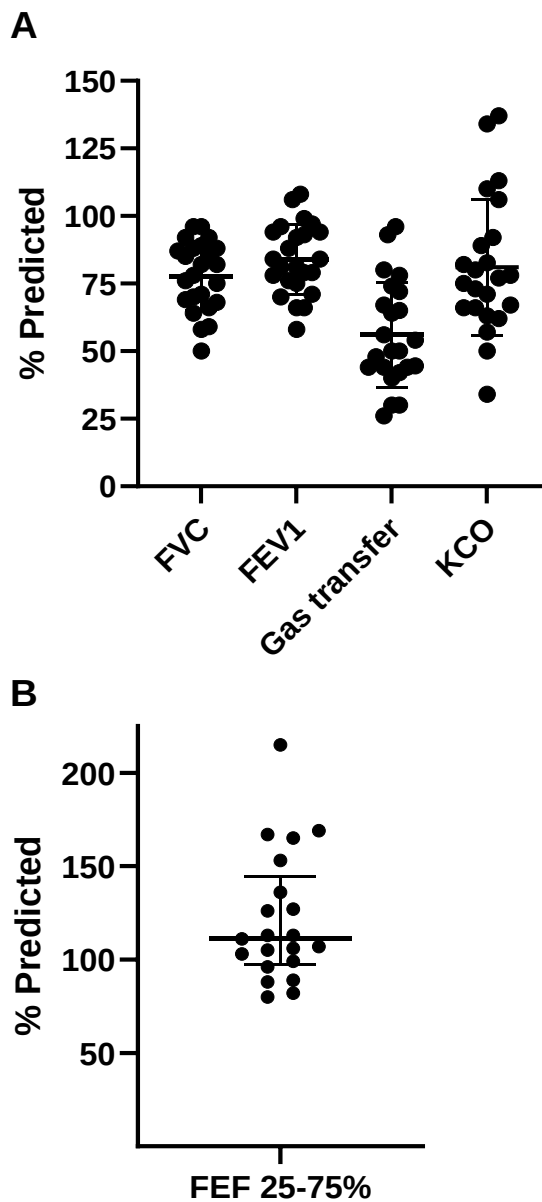


Figure 5.5: Pulmonary function test results.

Pulmonary function test results are displayed of the 26 patients with IPF recruited to the HYBRID study.

(A) displays percentage predicted values of FVC, FEV1, gas transfer and KCO.

(B) displays FEF 25-75% predicted.

Each dot represents a single patient. Data bars are mean and standard deviation. FEV1 – forced expiratory volume in one second, FEF 25-75% - Forced mix-expiratory flow, FVC – forced vital capacity, KCO – carbon monoxide transfer coefficient.

Ten (38.4%) and 6 (23.1%) IPF patients were currently receiving the anti-fibrotic therapies, nintedanib or pirfenidone, respectively. Four patients had previously discontinued nintedanib therapy; three of whom are now receiving pirfenidone therapy. Five patients (19.2%) were prescribed ambulatory oxygen therapy but none were receiving long term oxygen therapy.

The prognostic staging system, the GAP score, was determined for each IPF patient (excluding three participants missing recent FVC or gas transfer results). Twelve (46.2%) had a stage I GAP score. Nine (34.6%) and two (7.7%) participants had a GAP score of stage II and stage III, respectively.

5.5.3. Health related quality of life questionnaires

IPF patients had a significantly higher MRC dyspnoea score than control participants ($p = 0.002$, Fisher's exact test). Control participants reported an MRC dyspnoea score of either one or two. Over 50% of IPF participants reported a MRC dyspnoea score of two and 42.3% of patients self-reported a score of 3 or more (see Figure 5.6).

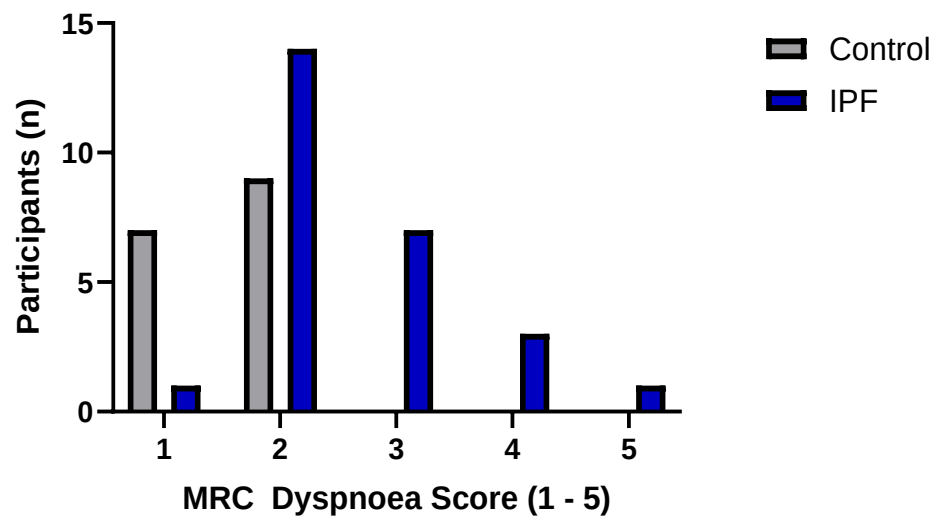


Figure 5.6: Medical research council (MRC) dyspnoea scale.

Medical research council (MRC) dyspnoea scores of control (grey, n = 16) and IPF participants (blue, n = 26). The MRC score was significantly lower in the control cohort than the IPF cohort (p = 0.002, Fisher's exact test).

IPF patients' total scores and scores by domain for the health related quality of life questionnaires, KBILD and SGRQ, are described in Table 5.5.

King's Brief Interstitial Lung Disease (KBILD) Questionnaire	Score (0 – 100)
Psychological	62.1 (17.1)
Breathlessness and Activities	43.7 (20.7)
Chest symptoms	73.4 (63.7- 100)
Total	59.3 (12.2)
St George's Respiratory Questionnaire (SGRQ)	Score (0 – 100)
Symptoms	48.6 (18.3)
Activity	58.8 (23.0)
Impacts	24.4 (14.7 – 39.4)
Total	42.1 (19.9)

Table 5.5: Health related quality of life questionnaires.

This table summarises domain scores and total scores of the King's Brief Interstitial Lung Disease (KBILD) and St George's Respiratory Questionnaires (SGRQ) for the 26 patients with idiopathic pulmonary fibrosis (IPF) recruited to the HYBRID study.

The KBILD is divided into three domains: psychological, breathlessness and activities, chest symptoms. Domain and total score ranges are 0- 100. Zero represents worst health status, whereas 100 points represents best possible health status [376].

The SGRQ is divided into three domains: symptoms, activity and impacts. Zero represents best health status and 100 points represents worst possible health status. A domain score and total score were missing for two participants.

Data is presented as mean (standard deviation) and median (interquartile range) for normally distributed and skewed data, respectively.

There was a strong negative linear association between total SGRQ and total KBILD scores ($r^2 = 0.696$, $p < 0.0001$, $n = 24$, Figure 5.7A). There was a weak but positive linear association between KBILD total score and FVC % predicted ($r^2 = 0.234$, $p = 0.020$, $n = 23$, Figure 5.7B) and gas transfer % predicted ($r^2 = 0.292$, $p = 0.008$, $n = 23$, Figure 5.7C). On review of the SGRQ, there was a negative association between total score and gas transfer % predicted ($r^2 = 0.378$, $p = 0.003$, $n = 21$, data not shown), but no association with FVC % predicted ($r^2 = 0.104$, $p = 0.144$, $n = 22$, data not shown).

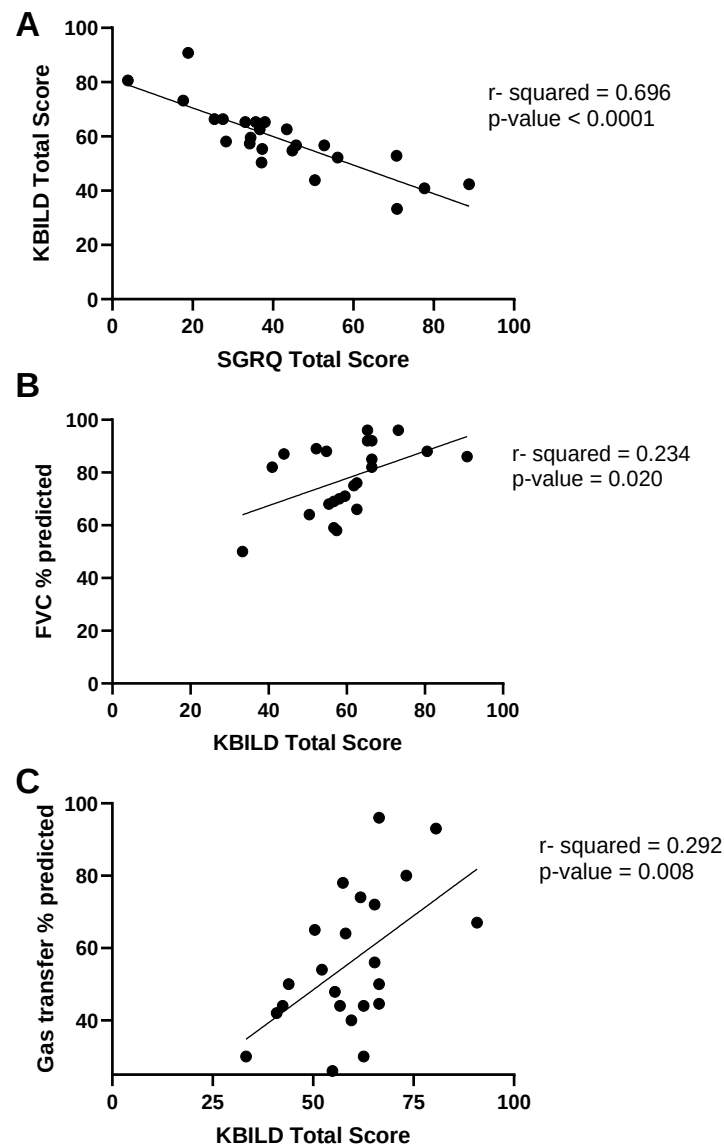


Figure 5.7: Health related quality of life questionnaires.

(A) Scatter plot displaying a strong negative association between IPF participants' total SGRQ and total KBILD scores ($r\text{-squared} = 0.696$, $p < 0.0001$, $n = 24$). For the KBILD score, zero points represents worst health status, whereas 100 points represents best possible health status [376]. For the SGRQ, zero points represents best health status and 100 points represents worst possible health status. Scatter plots present dot per participant. Line of best fit calculated using simple linear regression.

(B) Scatter plot displaying positive association between KBILD total score and FVC % predicted ($r\text{-squared} = 0.234$, $p = 0.020$, $n = 23$).

(C) Scatter plot displaying positive association between KBILD total score and gas transfer % predicted ($r\text{-squared} = 0.292$, $p = 0.008$, $n = 23$).

5.5.4. Peripheral arterial oximetry (SpO₂)

5.5.4.1. Six-minute walk test

Sixteen of the control and 18 of the IPF patients who completed six-minute walk tests were included in the analyses summarised in Table 5.6. Resting SpO₂ was not different between IPF and control subjects ($p > 0.05$, Mann-Whitney test).

IPF patients' SpO₂ dropped to a lower percentage during the six-minute walk test than the control participants (median 87% (IQR 83- 90%) versus median 93% (IQR 91- 96%) respectively, ($p < 0.001$, Mann-Whitney test). IPF patients also walked a shorter distance than control subjects (362.1 metres (SD 63.3) versus 442.5 metres (SD 67.1), respectively, ($p = 0.001$, unpaired T-test).

These results are summarised in Table 5.6

Six-minute walk test parameters	Control (n = 16)	IPF (n = 18)	p-value
Resting SpO ₂ , median (IQR)	97 (96- 97)	96 (94- 97)	ns
Lowest SpO ₂ , median (IQR)	93 (91- 96)	87 (83- 90)	<0.001
Walk distance (metres), mean (SD)	442.5 (67.1)	362.1 (63.3)	0.001
Peak Borg Score (1- 10), median (IQR)	0.8 (0- 1.8)	0.8 (0- 3.3)	ns
Peak Heart rate (bpm), mean (SD)	111.6 (13.7)	106.4 (7.9)	ns

Table 5.6: Six-minute walk test results.

The table summarises the six-minute walk test results for patients and control subjects. None of the participants stopped to rest during the six-minute walk test. Normally distributed and skewed data are presented as mean and SD, and median and IQR, respectively. Unpaired T-tests and Mann-Whitney tests were performed for normally distributed and skewed data, respectively. Bpm – beats per minute. The significance of differences between groups is shown (p-value or ns for no difference).

Figure 5.8A displays mean SpO₂ measurements during the six-minute walk tests at one minute intervals in IPF patients and control subjects. Resting and lowest SpO₂ recordings are displayed graphically in Figure 5.8B.

The pulmonary function measures, FVC, gas transfer and KCO, all positively (but not strongly) associated with lowest SpO₂ recorded by IPF patients during the six-minute walk test (FVC: r-squared = 0.368, p = 0.013, n = 16; gas transfer: r-squared = 0.454, p = 0.003, n = 17; KCO: r-squared = 0.267, p = 0.034, n = 17), see Figure 5.9).

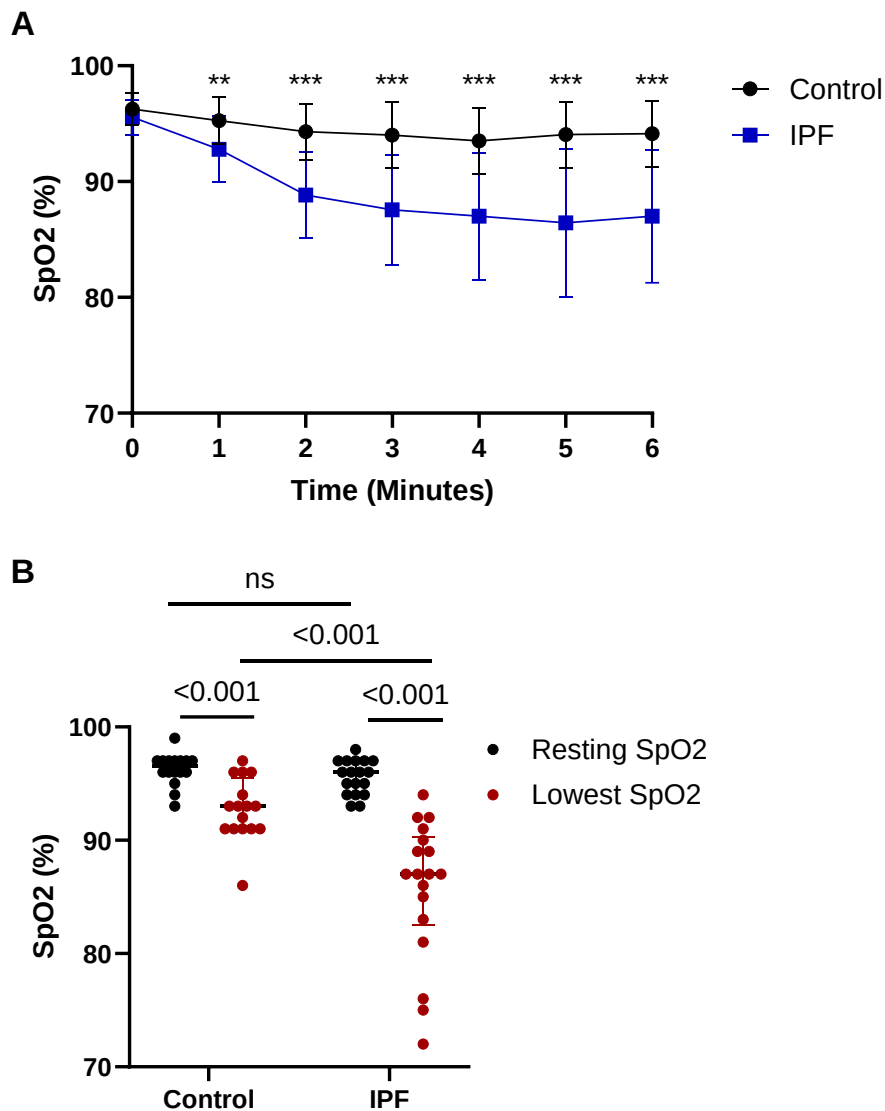


Figure 5.8: Peripheral arterial blood oxygenation saturations during the 6-minute walk test.

(A) SpO2 measurements collected at 1 minute intervals during the 6-minute walk test are displayed for control (black circles, $n = 16$) and IPF (blue squares, $n = 18$) cohorts. Mean and standard deviation are presented for each cohort. SpO2 was significantly lower between control and IPF cohorts after walking commenced (timepoints: 1 – 6 minutes) as denoted by the asterisks (** $p < 0.01$, *** $p < 0.001$ by Mann-Whitney tests).

(B) Resting SpO2 and lowest SpO2 measured in control subjects ($n = 16$) and IPF patients ($n = 18$) during the six-minute walk tests are displayed (each point represents a single subject). There was a significant difference between the resting and lowest SpO2 measured during the 6-minute walk test in both cohorts ($p < 0.001$, Wilcoxon tests). IPF patients' SpO2 dropped to lower values during the six-minute walk test than control participants ($p < 0.001$, Mann-Whitney test). Data bars represent median and IQR.

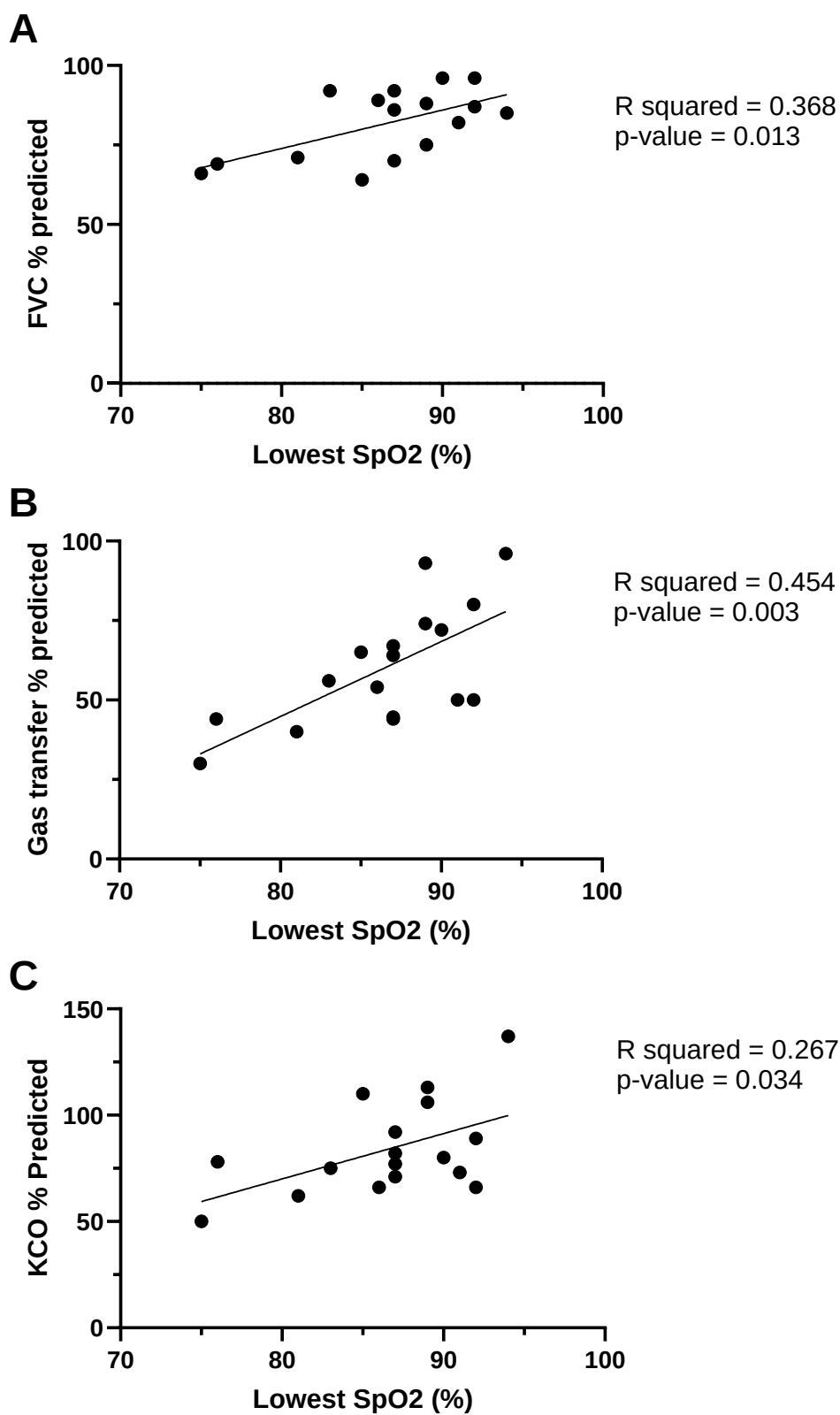


Figure 5.9: Relationship between lowest SpO2 during six-minute walk test and pulmonary function measures.

Scatter plots display the linear relationships between lowest SpO₂ recorded during the six-minute walk test and pulmonary function results in IPF patients.

(A) There was a positive association with forced vital capacity (FVC) percentage (%) predicted (r-squared = 0.368; p = 0.013, n = 16).

There was also a positive association with (B) gas transfer % predicted (r-squared = 0.454; p = 0.003, n = 17) and (C) KCO % predicted (r-squared = 0.267; p = 0.034, n = 17).

Each dot represents a single patient where data was available. Two IPF patients had missing FVC and/or gas transfer/KCO results.

5.5.4.2. Daytime oximetry

SpO₂ was measured during the daytime on a single day in 14 control and 16 IPF participants. Total analysis time for each study was a median 10 hours for both control and IPF cohorts. Mean FVC, gas transfer and KCO for the IPF patients who undertook daytime oximetry, were 79.8% predicted (SD 12.3), 58.7% predicted (SD 17.9) and 82.2% predicted (SD 22.4), respectively. Results are summarised in Table 5.7. Mean SpO₂ during the day was 95.9% (SD 0.8%) in control participants and slightly lower (94.0%; SD 1.9%) in IPF participants ($p = 0.002$, unpaired T-test). IPF patients spent a significantly greater percentage of the day with SpO₂ less than; 94%, 92% and 90%, than control participants ($p < 0.05$, Mann-Whitney tests). However, there was no difference in the percentage of time spent with SpO₂ $< 88\%$ ($p > 0.05$, Mann-Whitney test). IPF patients also experienced a greater number of dips in SpO₂ of $> 2-4\%$ per hour from baseline than control participants ($p < 0.01$, Mann-Whitney tests).

	Control (n = 14)	IPF (n = 16)	p-value
Analysis time (hours), median (IQR)	10.0 (9.9- 10.0)	10.0 (9.7 – 10)	ns
Mean SpO2 (%), mean (SD)	95.9 (0.8)	94.0 (1.9)	0.002
% Time spent with SpO2, median (IQR)			
<94%	9.5 (5.7- 16.7)	28.6 (12.9- 49.7)	0.002
<92%	4.1 (1.2- 8.1)	11.2 (4.0- 22.7)	0.008
<90%	1.8 (0.1- 4.3)	6.2 (1.1- 13.7)	0.042
<88%	0.5 (0.0- 2.5)	2.2 (0.2- 6.8)	ns
<86%	0.2 (0.0- 0.8)	0.5 (0.0- 4.0)	ns
Dips in SpO2 per hour, median (IQR)			
>2%	6.1 (4.6- 7.8)	10.6 (8.2- 13.8)	0.003
>3%	2.8 (2.3- 3.7)	6.1 (4.7- 7.3)	0.002
>4%	1.8 (1.2- 2.2)	3.5 (2.6- 4.3)	0.002

Table 5.7: Daytime oximetry results.

Summary results of SpO2 measures collected using the Checkme O2 device during the daytime of a single day for 14 control and 16 IPF participants. Normally distributed and skewed data are presented as mean and standard deviation, and median and interquartile range, respectively. Unpaired T-tests and Mann-Whitney tests were performed for normally distributed and skewed data, respectively.

Simple linear regression was performed to assess if daytime oximetry results were associated with pulmonary function measures. The average number of dips per hour in SpO2 from baseline by > 4% was significantly associated with gas transfer (r-squared = 0.563, p = 0.002, n = 14, Figure 5.10A) and KCO (r-squared = 0.655, p < 0.001, n = 14, Figure 5.10B) but not FVC (r-squared = 0.102, p = 0.265, n = 14, Figure 5.10C).

Percentage of the daytime that SpO2 was less than 88% was not associated with FVC (r = -0.464, p = 0.096 by non-parametric spearman rank correlation, n = 14, data not shown) or gas transfer (r = -0.469, p = 0.092 by non-parametric spearman rank correlation, n = 14, data not shown).

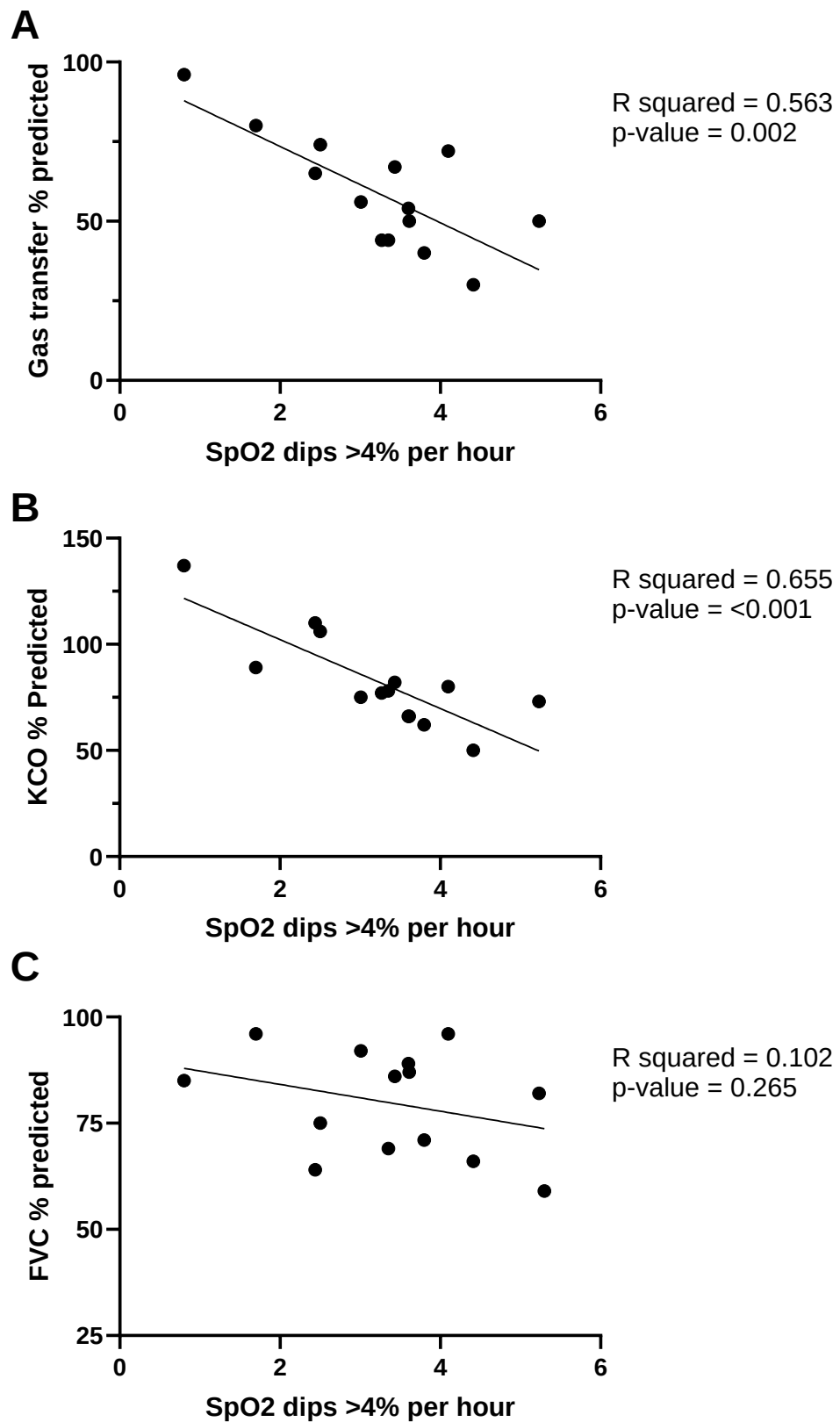


Figure 5.10: Relationship between dips in daytime SpO2 and pulmonary function.

Scatter plots displaying the relationship between frequency per hour of dips in SpO₂ by >4% from baseline during the daytime and pulmonary function results in IPF patients.

(A) There was a negative association between dips per hour by >4% SpO₂ and gas transfer % predicted (r-squared = 0.563, p = 0.002, n = 14).

(B) There was a negative association between dips per hour by >4% SpO₂ and KCO % predicted (r-squared = 0.655, p < 0.001, n = 14).

(C) There was no association between dips per hour by >4% SpO₂ and FVC percentage predicted (r-squared = 0.102, p = 0.265, n = 14).

Each dot represents data from a single patient. Three IPF patients had missing FVC and/or gas transfer/KCO data.

KCO – carbon monoxide transfer coefficient, FVC – forced vital capacity, SpO₂ - peripheral arterial blood oxygen saturation.

5.5.4.3. Overnight oximetry

Fifteen control and 21 IPF patients completed overnight oximetry (see Table 5.8). The mean technically valid sleep time was comparable between both groups (approximately 6.6- 6.8 hours). Average overnight SpO₂ was statistically lower in IPF patients (median 93%) compared to control participants (median 94%, $p = 0.002$, Mann-Whitney test). Two IPF patients met overnight hypoxaemia (defined as $\geq 10\%$ of sleep with SpO₂ < 90% as described by Myall *et al* [382]). None of the control participants had overnight hypoxaemia. The median percentage of sleep with SpO₂ < 90% in IPF patients was 1% and was statistically greater than control participants ($p = 0.009$, Mann-Whitney test). There was no difference in the percentage of sleep with SpO₂ $\leq 88\%$ ($p > 0.05$, Mann-Whitney test) between the groups.

Oxygen desaturation index (ODI) 4% is defined as the number of times per hour of sleep that $\geq 4\%$ oxygen desaturation from baseline [385]. Peripheral arterial tone apnoea-hypopnoea index (pAHI) 4% is defined as the number of apnoeas and hypopnoeas per hour of sleep identified using PAT technology [385]. ODI 4% and pAHI % were significantly higher in IPF patients than control participants ($p < 0.05$, Mann-Whitney tests). There was no association noted between ODI 4% or the percentage of sleep with SpO₂ <90% and the pulmonary function measures, FVC or gas transfer ($p < 0.05$, data not shown).

	Control (n = 15)	IPF (n = 21)	p-value
Technically valid sleep time (hours), mean (SD)	6.6 (0.8)	6.8 (1.4)	ns
Mean SpO2 (%), median (IQR)	94 (94- 95)	93 (92- 93.5)	0.002
Overnight hypoxaemia, n (%)	0 (0%)	2 (9.5%)	ns
% Sleep with SpO2 < 90%, median (IQR)	0 (0- 0.4)	1 (0.1- 1.45)	0.009
% Sleep with SpO2 ≤ 88%, median (IQR)	0 (0- 0.3)	0.1 (0- 0.6)	ns
ODI 4%, median (IQR)	2.5 (0.6- 7.9)	6.6 (4.2- 14.3)	0.015
pAHI 4%, median (IQR)	3.5 (0.6- 8.7)	7.5 (4.2- 15.8)	0.012
pAHI ≥ 15, n (%)	0 (0%)	5 (23.8%)	ns

Table 5.8: Overnight oximetry results.

SpO2 measurements during sleep are summarised in 15 control and 21 IPF patients. Normally distributed and skewed data are presented as mean and SD, and median and IQR, respectively. Unpaired T-tests and Mann-Whitney tests were used to analyse normally distributed and skewed data, respectively. Fisher's exact test was used to analyse categorical data. Significance of differences between the 2 groups are shown (p-value). ODI - oxygen desaturation index, pAHI – peripheral arterial tone apnoea-hypopnoea index.

5.6. Discussion

5.6.1. Study cohorts are well matched.

Participants in this study were mostly Caucasian males with an average age of ~75 years consistent with the patient demographics reported in the UK IPF Registry [386] including smoking status with most being ex-smokers.

The most common comorbidities (hypertension and GORD) and medications (anti-hypertensives, statins, proton pump inhibitors) were also equivalent between control and IPF patients. Therefore, the IPF patients were representative of the IPF population in the UK together with being well matched to the control subjects. This careful matching aimed to minimise effects of any confounding factors on differences in neutrophil phenotype and function noted for the two groups.

The only significant difference in characteristics, other than IPF, were 1) BMI which was significantly higher in IPF (although both groups were in the overweight category between 25 and 29.9) and 2) anti-fibrotic therapy administration for IPF.

Increased adiposity has been associated with increased neutrophil blood count and a more activated neutrophil phenotype [387–390]. Therefore, this needs to be considered when interpreting the experimental data presented in Chapter 6, including whether both adiposity and the presence of lung fibrosis in this patient cohort could be driving a dysregulated neutrophil state. A third cohort of IPF patients with the same BMI as controls is required to determine this.

Both anti-fibrotic therapies, pirfenidone and nintedanib, may have immunomodulatory effects, for example both reduce bleomycin induced neutrophil infiltration as well as fibrosis

in animals models [391,392]. Assessing this possible confounding factor would require treatment naïve IPF patients as a third cohort by recruiting participants at or soon after IPF diagnosis. This should be considered as part of future studies.

5.6.2. The IPF cohort were mostly mild-moderate disease severity.

The mean FVC and gas transfer of the IPF cohort was 78% predicted and 56% predicted, respectively. Furthermore, all patients had an FVC $\geq$ 50% predicted and the majority had a gas transfer >30% predicted indicating patients were mostly of mild to moderate disease severity if referring to pulmonary function reference ranges utilised in clinical trials [393,394]. In addition, nearly half of patients had a stage I GAP score. In order to identify changes in neutrophil phenotype/function over IPF progression, expansion of the cohort to include the whole range of disease severity is required, as well as longitudinal follow-up to assess rate of changes.

Symptom and health related quality of life questionnaires provide further information of the clinical severity and/or prognosis of IPF patients. As expected, IPF patients had a higher MRC dyspnoea score than control subjects and over half of the control subjects had a score of 2 points. This is likely related to the age associated deterioration/reduction in activity associated with this closely age matched control cohort (mean age 75 years).

Even in this small IPF cohort, a weak positive association was noted between KBILD and both the pulmonary function measures, FVC and gas transfer, as reported previously in larger cohorts [375]. This demonstrates that even though the study presented in this chapter has only a small sample size, it is consistent with larger cohorts.

5.6.3. IPF patients experience intermittent hypoxaemia.

Overall, the IPF patients experienced only mild hypoxaemia during the day and night with averages being statistically lower than control subjects, but remaining relatively normal (93-94%). When SpO₂ was less than 94% during the day, it was mostly between 92 and 94%. Patients on average experienced significant hypoxia (SpO₂ < 88%) for only 2% of the daytime.

The most notable finding was that the IPF patients experienced around twice the number of dips per hour during both the day and night in SpO₂ (by > 4% from baseline) than control subjects. The average number of dips in SpO₂ more than 4% per hour during the day was statistically associated with gas transfer/KCO, but not FVC. This data suggests that episodic or intermittent hypoxaemia appears to be evident early in disease, which may become more prominent as the diffusing capacity of the lung deteriorates.

Results of the six-minute walk test highlighted that IPF patients have a reduced exercise tolerance (walked a shorter distance) and experience more exertional hypoxaemia than control participants. Therefore, it is likely that these episodic desaturations during the daytime are related to exertion. Detailed examination and coding of the activity diaries in the future will aid determination of this, alongside use of daytime actigraph technology.

Pulmonary hypertension often develops in patients with IPF associated with impaired gas transfer [395]. None of the patients in this study had an established diagnosis of pulmonary hypertension. Non-invasive markers of pulmonary hypertension, such as echocardiography data or B-Type Natriuretic Peptide levels, were not collected during this study. Therefore, it is unknown if concomitant presence of pulmonary hypertension may be contributing to these dips in SpO₂ but is clearly an issue that needs to be addressed in future studies.

OSA, defined as an AHI of ≥ 15 , is a feature of some patients with fibrotic ILD, reported to be present in more than a third of patients in one study. [382] The same study found that nearly 20% of the 102 fibrotic ILD patients assessed had nocturnal hypoxaemia (spending over 10% of the night with an SpO₂ <90%), and this was associated with increased 1-year mortality [382]. In my study 23.8% of IPF patients had an pAHI of ≥ 15 but only two experienced overnight hypoxaemia. Average gas transfer was higher in the participants studied by me compared to the study reported by others (26), suggesting more advanced disease influences this feature. Expansion of this study to include the full spectrum of disease would give clearer insight into this.

5.6.4. Limitations

The study reported here includes a relatively small cohort of patients with mild to moderate disease and a selection bias in control and IPF patients. Firstly, only patients with a SpO₂ of 92% or more were included. This inclusion criteria was implemented to a) set an initial safety threshold for those recruited for the optional bronchoscopy aspect of the study and b) to allow measurement of daytime, exercise and overnight SpO₂ patterns in those without resting hypoxaemia. Secondly, participation in the study involved an additional visit to the hospital solely for research. Therefore, patients who consented to the study were those who felt well enough to participate and was likely to mainly include those with greater physical capacity.

Selection bias may also have influenced control subject recruitment. The controls were all contacted via the 'The Birmingham 1000 Elders group' mailing list. Therefore, all were already involved in other clinical research of health in the elderly and were likely more conscious of

‘healthy living’ than the general population. Furthermore, pulmonary function tests and thoracic imaging were not routinely performed on control subjects and thus mild but undiagnosed chronic lung disease cannot be completely excluded. However, if any of the study procedures during visit one or two were suggestive of an underlying heart or pulmonary condition, appropriate clinical investigations were performed and affected subjects were excluded from study analyses. Future studies should include more comprehensive clinical screening and simple lung function (spirometry) to at least rule out significant respiratory impairment.

5.6.5. Conclusions

This chapter demonstrated that control and IPF subjects enrolled into the prospective cohort study, HYBRID, were well matched for basic demographics, smoking history and comorbidities. The IPF patients mostly had mild to moderate disease severity and showed evidence of intermittent hypoxaemia during the day and night, with the former reflecting, gas transfer. Further work needs to be performed to assess whether intermittent hypoxaemia contributes to disease pathophysiology and its role together with health status and lung physiology as biomarkers of IPF clinical impact, progression and mortality. This would require a greater sample size and more comprehensive clinical/radiological and physiological characterisation to ensure sufficient cross sectional statistical power and longitudinal follow-up of patients.

Having outlined the study protocol and participants characteristics, the following chapter (Chapter 6) outlines the results of the *ex vivo* experiments performed on venous and BALF

samples from these individuals to explore the primary aim of the HYBRID study, 'circulating neutrophils in IPF patients display a primed phenotype'.

**Chapter 6. INVESTIGATING THE PHENOTYPE,
CHEMOTACTIC AND EFFECTOR FUNCTIONS OF
CIRCULATING NEUTROPHILS IN IDIOPATHIC
PULMONARY FIBROSIS.**

6.1. Abstract

It is unknown if neutrophils in the circulation of IPF patients have a dysregulated phenotype. Previous work in this thesis observed that TGF β 1, a key pro-fibrotic growth factor, which is elevated in the circulation of patients with IPF [29,280] likely primes neutrophils. This study subsequently investigated the hypothesis that circulating neutrophils in IPF patients display a primed phenotype. This was explored by assessing the phenotype (cell surface markers), movement and NET responses of circulating neutrophils derived from IPF and control participants recruited to the HYBRID study.

This prospective study demonstrated that IPF patients have a greater circulating neutrophil count than control participants and a higher percentage of CD10 negative neutrophils, suggesting an expansion of immature neutrophil populations and emergency granulopoiesis. Expression of the chemokine receptor, CXCR4, and co-inhibitory molecule, PD-L1, were higher in IPF derived neutrophils. Movement towards classical chemoattractants, IL-8 and fMLP, were maintained in IPF derived neutrophils. However, TGF β 1 appeared to drive IPF neutrophils to move less directly than control neutrophils. Cell free DNA release in response to PMA, but not other stimuli, was reduced in IPF derived neutrophils.

However, provisional analysis of BALF samples identified greater cfDNA in IPF BALF than control participants, which is suggestive of greater NETs within the alveolar space.

This study highlights for the first time that there appears to be alterations in the phenotype of circulating neutrophil populations in patients with IPF. Recruitment is ongoing to this study to improve the statistical power of results presented here and to gain understanding of the underlying drivers of the increased granulopoiesis and change seen.

6.2. Background

The role of neutrophils in IPF pathophysiology remains unknown. The importance of investigation into this field and the current limited knowledge base was described in Chapter 1. The observational study, HYBRID, was devised to allow the prospective comparison of neutrophil phenotype and function derived from IPF patients with that of control participants. The results in Chapter 5 demonstrated that both control and IPF cohorts were well matched in regard to demographics, smoking status and comorbidities. Therefore, comparison of neutrophil populations between the two cohorts in the HYBRID study, will give insight into whether the disease itself is associated with aberrant neutrophil function in early disease (reducing the effects of confounding).

Neutrophils are resident within three broad sites in IPF patients: 1) the bone marrow, 2) the circulation and 3) the lung. BALF and tissue samples are two sources in which neutrophil populations within the lung can be examined. However, isolating neutrophils from these pulmonary sources is challenging for several reasons. Firstly, BALF and tissue samples are not readily available, as these are now infrequently obtained in the clinical setting. Secondly, isolation and identification of neutrophils is difficult due to low absolute neutrophil counts and sharing of cell surface markers with other immune cells (discussed in Chapter 7). BALF samples have been obtained on a subset of HYBRID study participants. The sample size is small at present due to the invasiveness of the procedure, but provisional measurement of markers of NET formation will be described in this chapter.

Venous blood is the most readily available source for sampling neutrophils. Neutrophil populations in the circulation can be highly variable between disease states and give insight into the systemic inflammatory response, for example amplification of immature circulating

neutrophils suggests a state of emergency granulopoiesis [396]. Furthermore, alterations in the cytokine milieu in the circulation can prime neutrophils, which can then impact neutrophil movement and effector responses in target tissue [397]. It was observed in Chapter 4 that the pro-fibrotic cytokine TGF β 1 appears to prime neutrophils. Therefore, it was proposed that IPF neutrophils may be primed in the circulation of patients with IPF, which results in altered movement responses and subsequent effector functions on arrival within the fibrotic lung.

6.3. Hypothesis

Circulating neutrophils derived from patients with IPF display a primed phenotype.

6.4. Aims

1. To determine whether peripheral neutrophil count is elevated in IPF patients as compared to control participants.
2. To determine whether circulating neutrophils from IPF patients express different cell surface marker patterns (phenotype) as compared to neutrophils derived from control participants.
3. To determine whether neutrophils from IPF patients are in a more primed or activated state as compared to neutrophils derived from control participants.
4. To determine whether circulating neutrophils from IPF patients have differing chemokinetic and chemotactic responses than neutrophils derived from control participants.

5. To determine whether NET release is different from IPF derived neutrophils as compared to neutrophils from control participants.
6. To determine whether there are increased markers of NET formation in the circulation and BALF of IPF patients as compared to control participants.

6.5. Methods linked to Chapter 6

6.5.1. Ethical approval

All venous blood samples were collected from participants enrolled to the HYBRID study as described in Chapter 5. Except for the data shown in Figure 6.26, which was obtained from volunteers recruited to the ‘Investigations of the Ageing Immune System’ study (10/H1208/48).

6.5.2. Blood sample collection, processing and neutrophil isolation.

Briefly, venous blood samples were collected and processed as described in Chapter 2 and Chapter 5. Full blood count analysis using the Sysmex XN-1000 analyser was performed as described in Chapter 2.3.1. Neutrophil isolation was performed, and neutrophil purity assessed (typically >95%) as described in Chapter 2.4. Figure 6.1 displays representative images of neutrophils isolated from control and IPF participants.

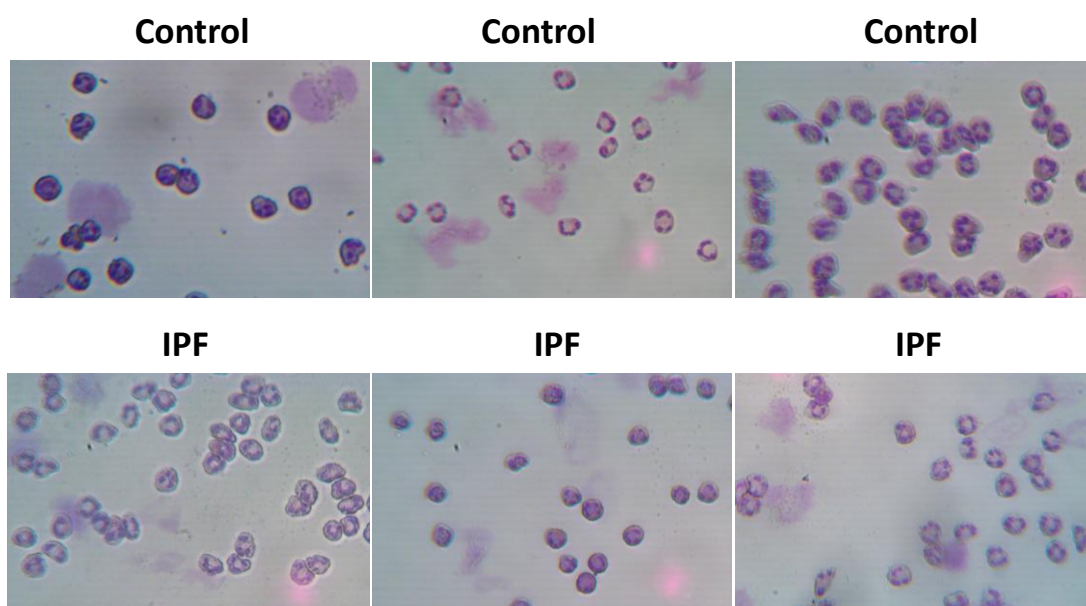


Figure 6.1: Representative images of isolated neutrophils.

Peripheral neutrophils isolated from control and IPF participants of the HYBRID study. Neutrophils were transferred to a microscope slide via cytopsin and stained using REASTAIN QUICK-DIFF KIT. Images taken using a light microscope at 20x magnification.

6.5.3. Neutrophil experimental assays.

The neutrophil assays performed in the HYBRID study are outlined graphically in Figure 6.2. Immunophenotyping was performed as described in Chapter 2.10. Neutrophil chemotaxis was assessed using the Insall chamber assay, with all experiments adjusted for slide movement, as described in Chapter 2.7.2. Any samples where there was an issue loading the neutrophils to the chamber or a visibly large shift in the slide during the 12 minutes of live imaging, defined as a ≥ 15 pixel difference in X or Y position between the first and last image, were excluded from analysis (a single TGF β 1 condition and 5 vehicle experimental conditions were excluded in total).

Neutrophil viability was assessed using Zombie NIRTM or Annexin/PI stain stains as described in Chapter 2.6. CfDNA release was assessed using a fluorescence plate reader as described in Chapter 2.8.1. Intracellular ROS detection using CellROXTM Deep Red staining was performed as described in Chapter 2.9.1. Any samples where the total number of neutrophil events analysed on the flow cytometer were less than 2500 were excluded from analyses.

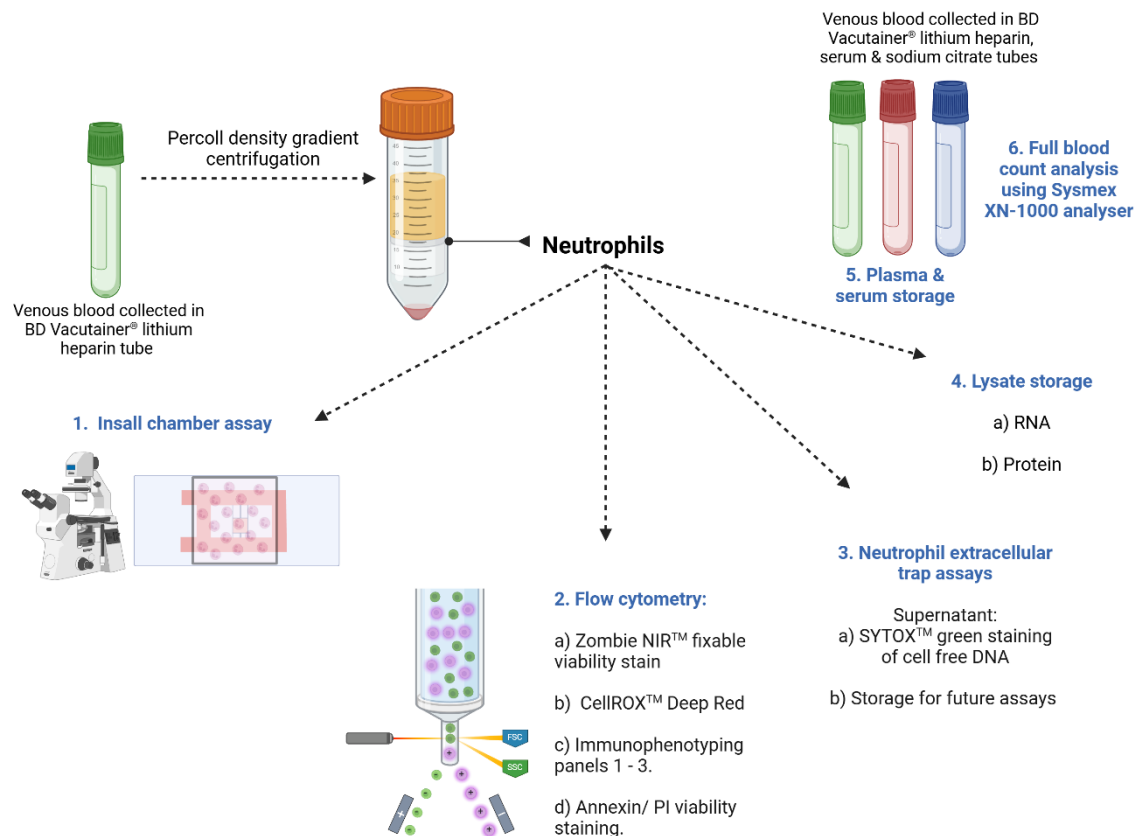


Figure 6.2: Summary of experimental assays performed from venous blood samples collected in the HYBRID study.

Neutrophils were isolated using Percoll density gradient centrifugation.

Neutrophil chemotaxis was assessed using the Insall chamber assay **(1)** and flow cytometry was performed to assess viability, reactive oxygen species content and cell surface marker expression using the **(2a)** Zombie NIR™ fixable viability stain, **(2b)** cellROX™ Deep Red and **(2c)** immunophenotyping panels 1 - 3, respectively.

(2d) Annexin/PI staining was later added to the experimental protocol to assess viability after 4 and 24 hours of culture *ex vivo*.

Neutrophil extracellular trap formation was assessed by measuring **(3a)** cell free DNA in the supernatant using SYTOX™ green staining. The remaining neutrophil supernatant **(3b)** was stored in -80°C freezers, alongside **(4)** neutrophil ribonucleic acid (RNA) and protein lysates for future analyses.

(5) Plasma and serum were collected by centrifugation of venous blood collected in BD Vacutainer® lithium heparin, serum and sodium citrate tubes. Double spun plasma samples were then used to measure cell free DNA concentrations.

(6) Full blood count analysis was performed on venous blood collected in BD Vacutainer® sodium citrate tubes using a Sysmex XN-1000 analyser. *Figure created using Biorender.com.*

6.5.4. Power Calculations

No studies have been published to date that have explored neutrophil phenotype or function in patients with IPF. This makes determination of sample size difficult. Therefore, power calculations were estimated here using the Insall Chamber Assay results in Chapter 4. For example, if aiming for a statistical power 80%, $\alpha = 0.05$, a sample size of between 8 (using data comparing effect of 10 ng/ mL TGF β 1 vs vehicle on neutrophil speed) and 26 (assessing effect of TGF β 1 pre-treatment on neutrophil chemokinetic responses to fMLP) in each group is required. Therefore, a minimum of 8 participants in each group were required for each experiment. However, the final recruitment target for this study is 26 in each group. The experimental assays performed on each donor sample were limited by a variety of factors: neutrophil yield, experimental time and machinery. As not all of the neutrophil assays could be performed on all donor samples, a summary table comparing the participant characteristics of both cohorts is provided in the relevant results of each assay in this chapter.

6.5.5. Analysis of CD10 expression.

An additional analysis step was performed to determine the percentage of cells that were positive for CD10 in the HYBRID study samples. A discrete population was noted within the isotype control antibody stained samples, which had a higher BV510 fluorescence intensity, suggesting non-specific binding. An example is displayed in Figure 6.3A where 10.5% of isotype control antibody stained neutrophils were noted to have this higher fluorescence intensity. This population was not found with any of the other fluorescence markers. Cells within this population did appear to be neutrophils following review of SSC and FSC parameters (Figures 6.3B and 6.3C).

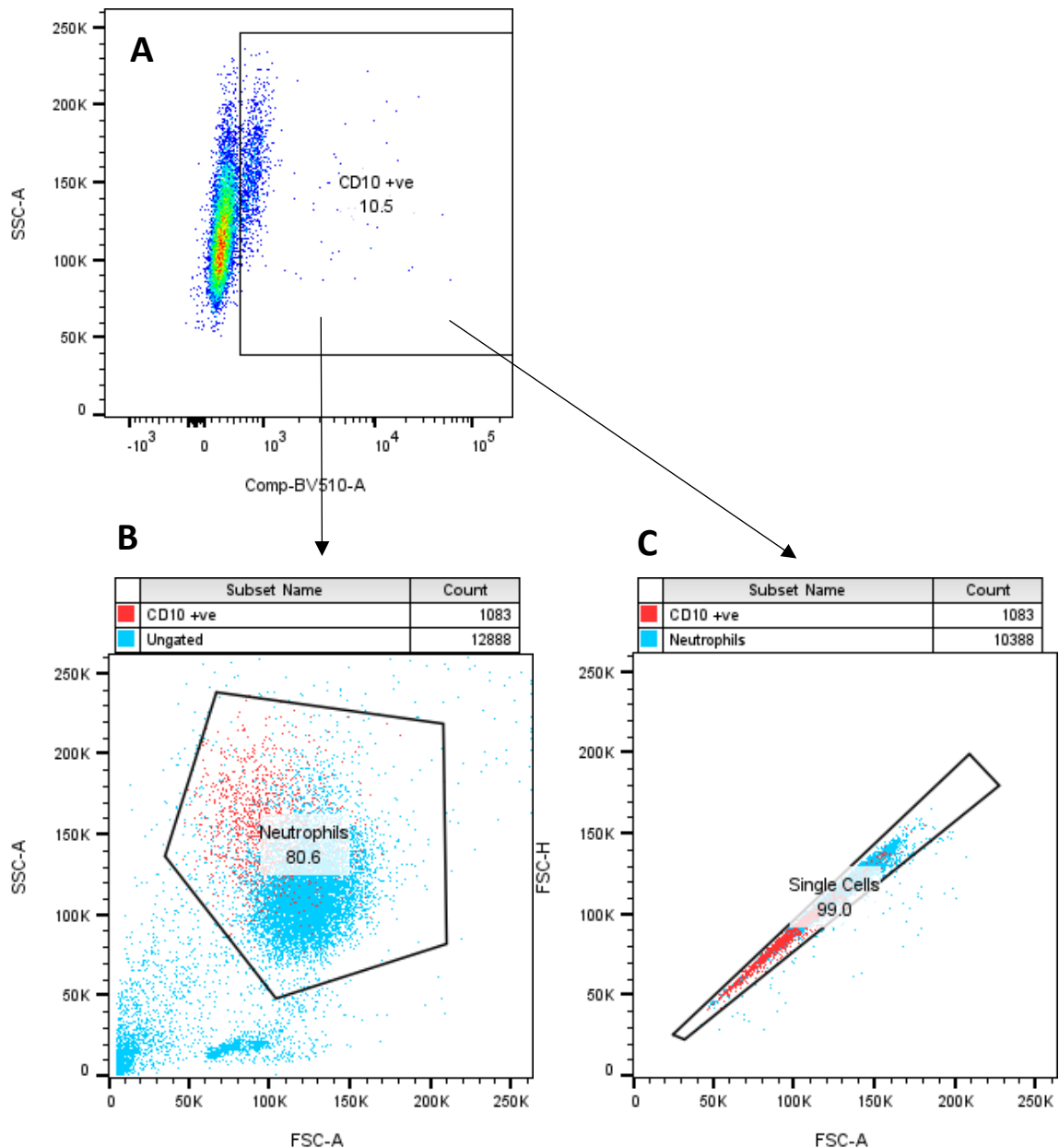


Figure 6.3: A high BV510 fluorescence population was noted within isotype control stained neutrophils.

(A) Representative scatter plot displaying the BV510 fluorescence intensity of isotype stained neutrophils from an IPF participant. The CD10 positive (CD10 +ve) gate outlines the 10.5% of neutrophils with a higher BV510 fluorescence.

(B) and (C) display all the isotype control stained neutrophils from an IPF participant (blue) and respective CD10+ve neutrophils (red – gated from Figure (A) in (B) SSC-A vs FSC-A and (C) FSC-H vs FSC-A scatter plots. These figures demonstrate that this high BV510 fluorescence population does appear to be neutrophil singlets.

If a positive gate was applied to the right of the main isotype population (as performed for all other cell surface markers), this led to a variable percentage of isotype control stained neutrophils present within this positive gate, most of which were >3% and the highest being 15.4%. The percentage of isotype control stained neutrophils with this higher BV510 fluorescence intensity was equivalent between control and IPF donor samples (median control = 3.9% (IQR 2.9 – 4.9) vs IPF = 4.8% (IQR 2.7 – 8.3); $p = 0.238$, by Mann-Whitney test). However, an appropriate gating strategy was sought to take into account this group of cells to which there may be non-specific antibody binding.

Two gating strategies were compared, which are displayed graphically in Figure 6.4. In Figure 6.4A the CD10 positive gate was placed just to the right of the main neutrophil population, whereas in Figure 6.4B the gating strategy was such to ensure that less than 3% of the isotype control neutrophils were within the CD10 positive gate. Following transfer of these gating strategies to the panel 3 stained neutrophils (bottom scatter plots in Figure 6.4A and 6.4B), these two methods resulted in different CD10 positive values (81.8% vs 28.5%). This large reduction in the percentage of CD10 positive cells noted on using the second gating strategy did not appear a true representation of CD10 expression when CD10 is such a ubiquitous marker of neutrophil maturity [263].

Therefore, the following strategy was utilised in the HYBRID study samples: a gate was applied to the right of the main neutrophil population (as per Figure 6.4A). However, an additional step was performed to attempt to account for potential non-specific binding, whereby the percentage of CD10 positive cells within the isotype control stained samples was subtracted off the value. Using Figure 6.4A as an example: $81.8 - 4.8 = 77\%$.

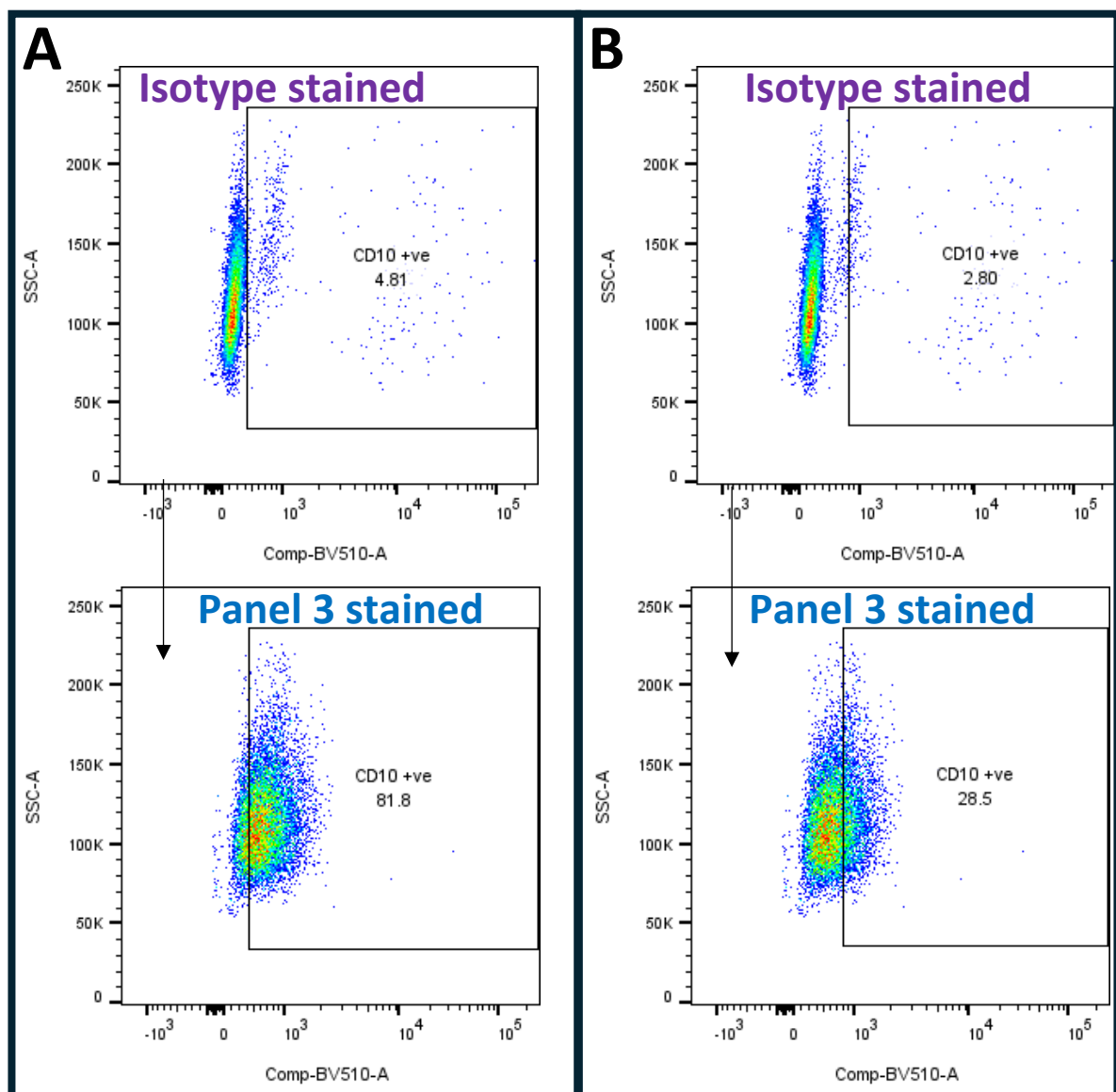


Figure 6.4: Comparison of two gating strategies to calculate the CD10 positive population.

Representative scatter plots display two potential gating strategies to calculate the CD10 positive neutrophil population.

(A) CD10 positive gate was placed just to the right of the main neutrophil population in the isotype control stained sample (top scatter plot). Application of this gate on the panel 3 stained sample resulted in a CD10 positive (CD10 +ve) population of 81.8% (bottom scatter plot).

(B) CD10 positive gate was placed in a location that ensured that less than 3% of the isotype control neutrophils were within the CD10 positive gate (top scatter plot). Application of this gate on the panel 3 stained sample resulted in a CD10 positive (CD10 +ve) population of 28.5% (bottom scatter plot).

6.5.6. BALF collection and processing.

BALF was collected by flexible bronchoscopy, and subsequently processed, including determination of differential cell count, as described in Chapter 5.4.8.

6.5.7. Protein quantification in BALF.

The protein concentration in the BALF samples was quantified using a Pierce™ BCA Protein Assay Kit (*ThermoScientific, USA: 23225*). Twenty five µL of BALF or protein standard (provided with BCA assay kit and diluted in PBS to create a standard curve) were added to wells of a clear flat bottomed 96 well plate, ensuring two technical replicates per condition. Reagents A and B were combined and then 200 µL added to each well. The plate was placed on a shaker for 1 minute and then incubated at 37°C for 30 minutes. Absorbance was measured using the BioTek Synergy 2 Fluorometric plate reader; 562 nm. A standard curve was plotted using Excel (*Microsoft 365*) and the protein concentration of each BALF sample calculated using a line of best fit equation. BALF samples were normalised to protein content where stated, using sterile saline.

6.5.8. CfDNA quantification in BALF and plasma.

Ten µL of BALF, double spun plasma (processed as described in Chapter 2.3.3) or lambda DNA (*ThermoScientific, UK: SD0011*) were pipetted into individual wells of a flat bottom 96 well cell culture plate (*Corning Inc., USA: 3596*), ensuring two technical replicates per condition. One hundred and forty µL of SYTOX™ Green (*1µM working concentration, Invitrogen, UK: S7020*) was added to each well. Cells were then incubated for 10 minutes in the dark. Fluorescence was measured using the BioTek Synergy 2 Fluorometric plate reader; excitation 485 nm/

emission 528 nm. The mean background fluorescence (namely the RFU of a well containing saline or PBS only) was subtracted from the RFU of each BALF and lambda DNA well. A DNA standard curve was then calculated (see example in Figure 2.9) from the serial lambda DNA RFUs and the DNA concentration of each BALF sample interpolated using GraphPad Prism (*Version 9.5.0, San Diego, USA*).

6.6. Results

6.6.1. Peripheral neutrophil counts are higher in patients with IPF.

Circulating immune cell counts were first measured in participants of the HYBRID study using the Sysmex XN-1000 analyser. Peripheral neutrophil count was greater in the IPF cohort (mean = 4.6×10^9 /L (SD 1.2)) as compared to control participants (mean = 3.3×10^9 /L (SD 0.8)); $p = 0.001$, unpaired T-tests, $n = 14$, Figure 6.5). Lymphocyte and monocyte counts were also greater in the IPF group ($p < 0.05$, unpaired T-tests, $n = 14$). However, there was no difference in NLR between the two cohorts ($p > 0.05$, Mann-Whitney test, $n = 14$, Figure 6.5C). Participants characteristics of IPF and control participants (age, gender, smoking history and Charlson Comorbidity Index) included in these analyses were comparable (Table A in Figure 6.5).

A	Control (n = 14)	IPF (n = 14)	p-value
Age (years), mean (SD)	74.6 (6.1)	76.7 (5.2)	ns
Sex ratio, M : F	9 : 5	13 : 1	ns
Current: Ex: Never smoker	0: 9: 5	0: 9: 5	ns
Pack history, median (IQR)	0.6 (0- 5.9)	8.1 (0- 30.0)	ns
Charlson Comorbidity Index			ns
≤ 2, n (%)	1 (7.1%)	1 (7.1%)	
3, n (%)	8 (57.1%)	4 (28.6%)	
4, n (%)	5 (35.7%)	7 (50.0%)	
≥ 5, n (%)	0 (0%)	2 (14.3%)	

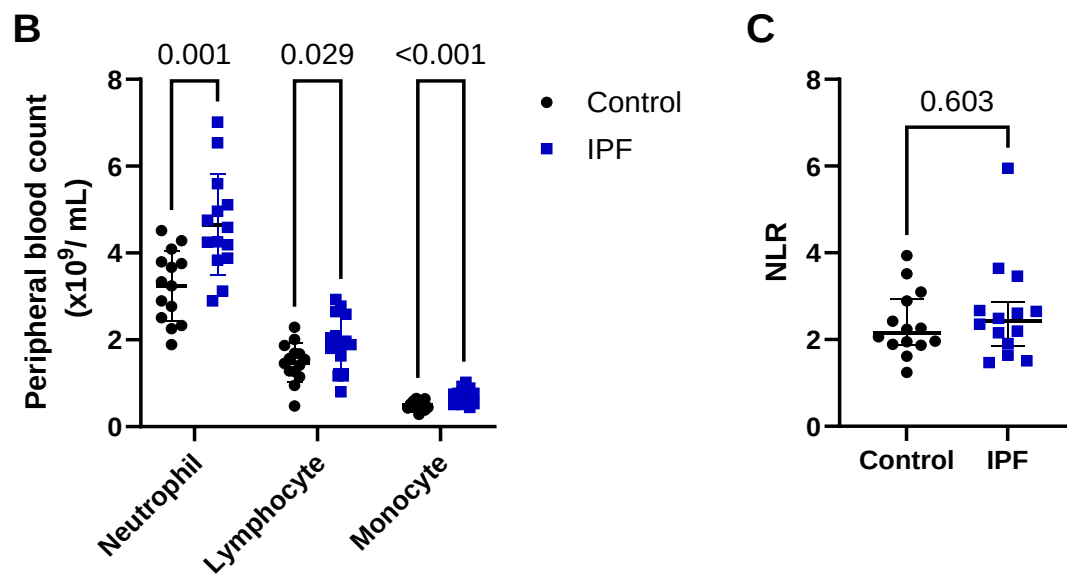


Figure 6.5: Peripheral immune cell counts in IPF and control participants.

Table A describes the characteristics of 14 control and 14 IPF participants from which peripheral immune cell count was calculated using the Sysmex XN-1000 analyser.

(B) summarises neutrophil, lymphocyte and monocyte counts in the circulation of control (black dots) and IPF (blue squares) participants. All three cell counts were higher in the IPF group – p-values comparing to control are displayed above the data points.

(C) displays the neutrophil-lymphocyte- ratio (NLR) (= neutrophil count divided by lymphocyte count) in control (black dot) and IPF (blue squares) participants.

Dot/square per participant. Data bars are mean and SD or median and IQR, for (B) and (C), respectively. Unpaired t-tests (B) and Mann-Whitney (C) tests were performed. Fisher's exact test was used to analyse categorical data. F – female, M – male.

6.6.2. Neutrophil viability was equivalent on isolation between the two cohorts.

Neutrophil characterisation first involved assessment of viability immediately following isolation using Zombie NIR™ staining. The percentage of viable cells was similar between the two cohorts (mean control = 97.3% (SD 1.8) vs IPF = 96.3% (SD 3.1); $p > 0.05$, unpaired T test, Figure 6.6). Participant characteristics were comparable between the two donor cohorts (outlined in Table A in Figure 6.6).

A	Control (n = 9)	IPF (n = 11)	p-value
Age (years), mean (SD)	72.8 (6.5)	75.4 (5.3)	ns
Sex ratio, M : F	6 : 3	11 : 0	ns
Current: Ex: Never smoker	0: 5: 4	0: 6: 5	ns
Pack history, median (IQR)	0.5 (0- 9.3)	1.3 (0- 37.5)	ns
Charlson Comorbidity Index			
≤ 2, n (%)	1 (11.1%)	1 (9.1%)	ns
3, n (%)	5 (55.6%)	4 (36.4%)	
4, n (%)	3 (33.3%)	5 (45.5%)	
≥ 5, n (%)	0 (0%)	1 (9.1%)	

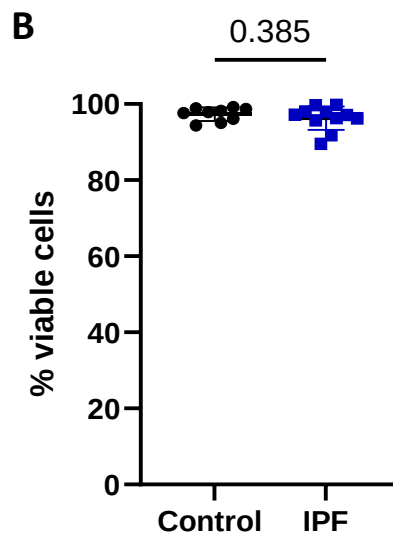


Figure 6.6: Viability of neutrophils isolated from control and IPF participants.

Table A describes the characteristics of 9 control and 11 IPF participants from which neutrophils were isolated and viability assessed using Zombie NIR™ staining. Unpaired T-Test and Mann-Whitney tests were performed for normally and skewed continuous data, respectively. Fisher's exact test was used to analyse categorical data.

(B) displays the percentage of neutrophils that were viable, defined as Zombie NIR™ negative. P-value presented above data points (unpaired T-test). Dot/square per participant. Data bars are mean and SD.

6.6.3. Characterisation of neutrophil cell surface markers from the circulation of IPF patients.

Flow cytometry was performed using the three immunophenotyping panels that were described in Table 2.7 and validated in Chapter 3, to compare the expression of markers on the surface of peripheral neutrophils derived from IPF and control participants. Participant characteristics of control and IPF donors included in this assay were comparable (see Table 6.1).

	Control (n = 14)	IPF (n = 20)	p-value
Age (years), mean (SD)	74.0 (5.5)	75.1 (6.0)	ns
Sex ratio, M : F	9 : 5	16 : 4	ns
Current: Ex: Never smoker	0: 9: 5	0: 11: 9	ns
Pack history, median (IQR)	1.5 (0- 10.1)	3.7 (0- 30.0)	ns
Charlson Comorbidity Index			ns
≤ 2, n (%)	1 (7.1%)	3 (15.0%)	
3, n (%)	9 (64.3%)	7 (35.0%)	
4, n (%)	4 (28.6%)	6 (30.0%)	
≥ 5, n (%)	0 (0%)	4 (20.0%)	

Table 6.1: Immunophenotyping assay: HYBRID study participant characteristics.

Characteristics of the 14 control and 20 IPF participants from which neutrophils were isolated and cell surface markers assessed. Unpaired t-test and Mann-Whitney tests were performed for normally and skewed continuous data, respectively. Fisher's exact test was used to analyse categorical data.

6.6.3.1. Neutrophils from IPF patients do not appear to be in a primed or activated state.

Cell surface expression of adhesion; CD11b, CD62L, and degranulation markers; CD66b, which change on neutrophil priming/activation were assessed. Expression of all three proteins was equivalent between control and IPF participants ($p > 0.05$, unpaired T-test/Mann-Whitney tests, Figure 6.7).

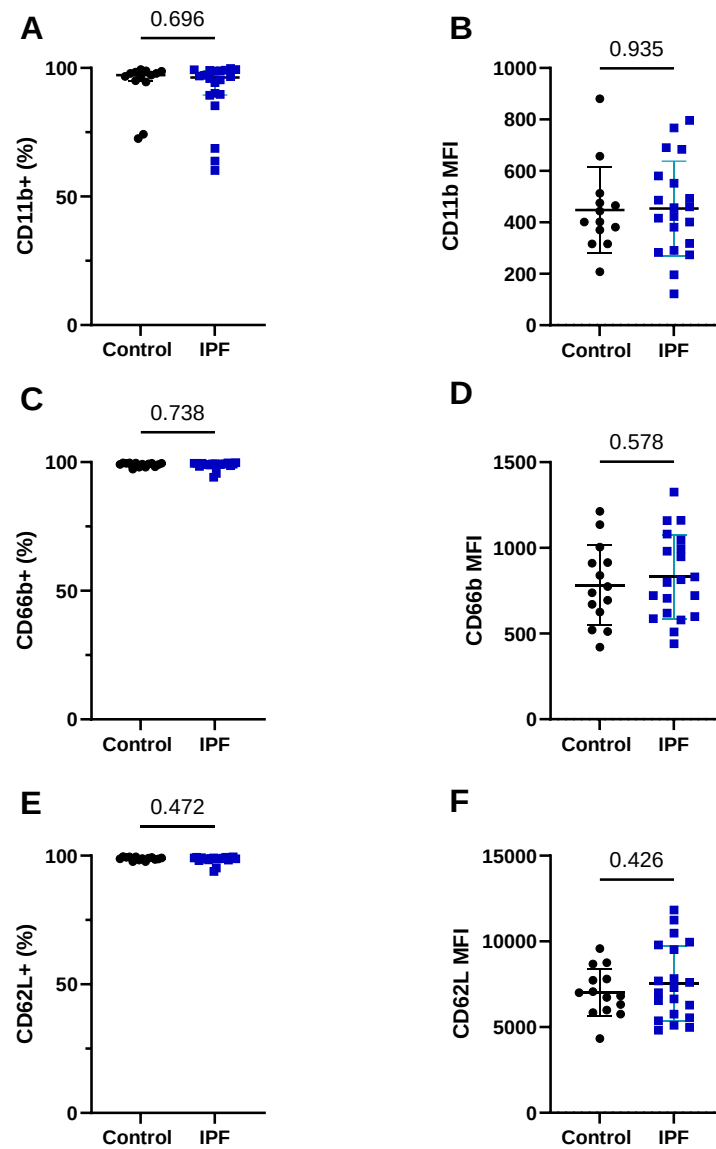


Figure 6.7: Cell surface expression of CD11b, CD66b and CD62L on control and IPF donor neutrophils.

Peripheral neutrophils isolated from 14 control and 20 IPF participants were stained with the panel 1 immunophenotyping antibody cocktail (detailed in Table 2.7). No difference was noted in percentage positive cells or median fluorescence intensity (MFI) of the markers: CD11b (A and B), CD66b (C and D) or CD62L (E and F). P-values comparing IPF to control are displayed above the data points. Dot/square per participant. Data bars are mean and standard deviation for (B), (D) and (F). Data bars are median and interquartile range for (A), (C) and (E). Unpaired T-test (B, D and F) and Mann-Whitney tests (A, C and E) were performed.

6.6.3.2. Expression of the chemokine receptor, CXCR2, was comparable between IPF and control cohorts.

Cell surface expression of the chemokine receptor, CXCR2, the receptor predominantly responsible for detection of neutrophil chemoattractants was assessed. Again, MFI and percentage of cells expressing CXCR2 was equivalent between IPF and control cohorts ($p > 0.05$, unpaired T-test/Mann-Whitney test - Figure 6.8). A median 96.9% and 95.5% of IPF and control neutrophils expressed CXCR2, respectively.

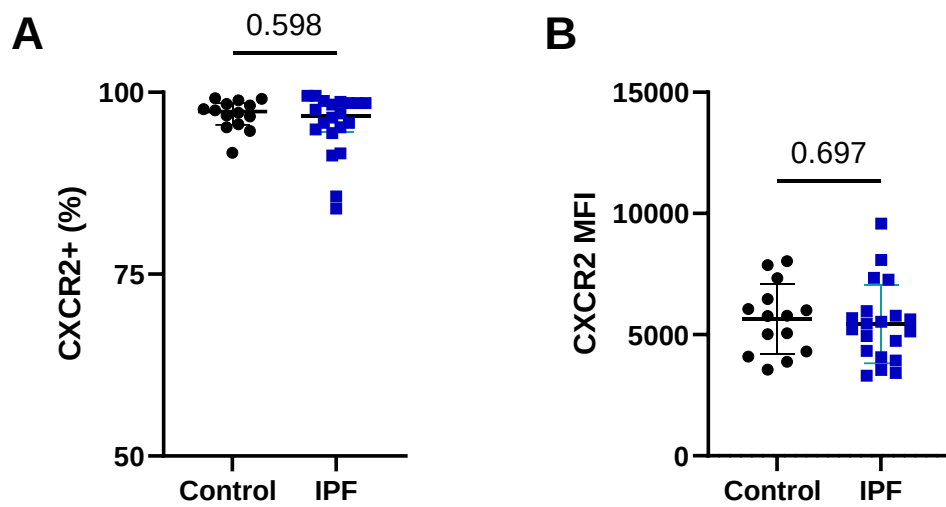


Figure 6.8: Cell surface expression of CXCR2 on control and IPF donor neutrophils.

Peripheral neutrophils isolated from 14 control and 20 IPF participants were stained with a PE conjugated anti-CXCR2 antibody. No difference was noted in percentage of positive cells (A) or MFI (B) of CXCR2. P-values comparing IPF to control are displayed above the data points. Dot/square per participant. Data bars are median and interquartile range and analysed using Mann-Whitney test in (A). Data bars are mean and standard deviation and analysed using unpaired T-test in (B).

6.6.3.3. The markers: CD16, CD54 and CD11c were equivalent between control and IPF donor neutrophils.

Nearly all neutrophils expressed the FC gamma receptor and common neutrophil identifier, CD16 (Figure 6.9A and 6.9B). MFI values of CD16 were the largest of all the cell surface markers examined in this study (mean of approximately 12 thousand), reflecting the high level of expression.

CD11c, an integrin alpha X chain protein, which is trafficked to the cell surface on activation (see Chapter 3.1.2.3), was expressed in approximately 96% of control and IPF donor neutrophils (Figure 6.9C).

No difference was noted in the MFI or percentage of cells expressing CD16, or CD11c between IPF and control participants ($p > 0.05$, unpaired T-test/ Mann-Whitney test- Figure 6.9A-D).

The transmembrane glycoprotein, CD54, was expressed by a mean 80% (SD 10.3) of control neutrophils and mean 73.4% (SD 12.8) of IPF donor neutrophils, with again no statistical difference noted between cohorts ($p > 0.05$, unpaired T-tests (Figure 6.9E and F).

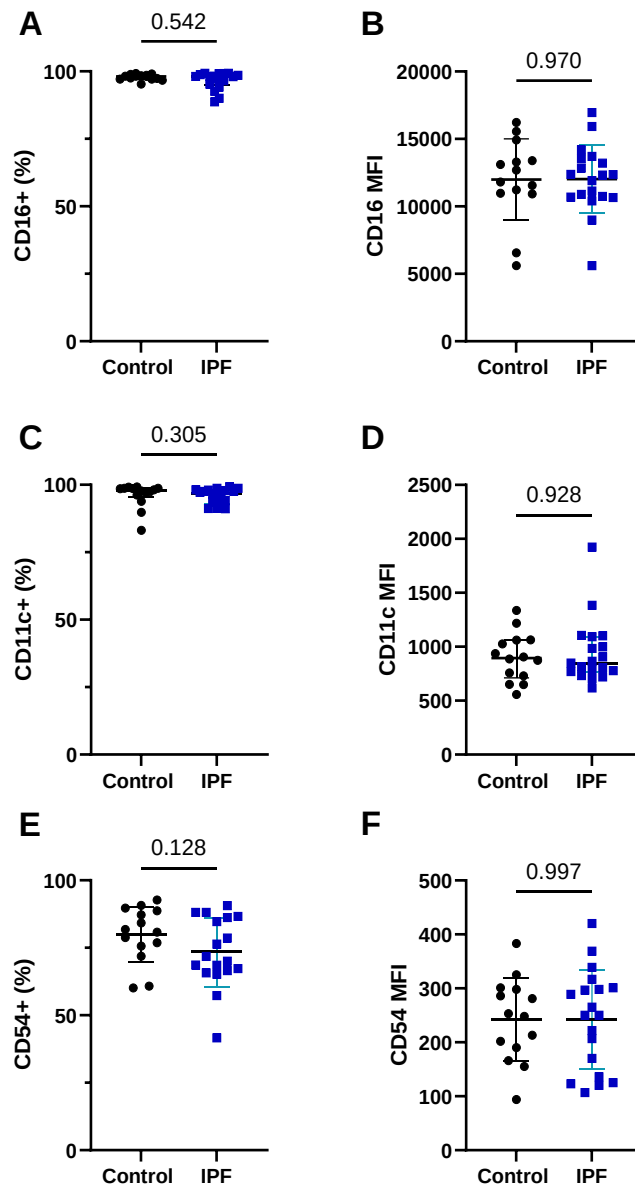


Figure 6.9: Cell surface expression of CD16, CD11c and CD54 on control and IPF donor neutrophils.

Neutrophils isolated from 14 control and 19 IPF participants were stained with the panel 3 immunophenotyping antibody cocktail (detailed in Table 2.7). Percentage positive cells and MFI of the markers: CD16 (A and B), CD11c (C and D) or CD54 (E and F) are presented. P-values comparing IPF to control are displayed above the data points. Dot/square per participant. Data bars are mean and standard deviation for (B), (E) and (F). Data bars are median and interquartile range for (A), (C) and (D). Unpaired T-test (B, E and F) and Mann-Whitney tests (A, C and D) were performed.

6.6.3.4. CXCR4 and PD-L1 expression appears higher in IPF donor neutrophils.

Cell surface expression of the neutrophil ageing/senescence marker, CXCR4, and co-inhibitory molecule, PD-L1 was then assessed. The median percentage of cells that were positive for CXCR4 and PD-L1 was 5% and 6% in both cohorts, respectively. Despite no difference in the proportion of positive cells, CXCR4 MFI and PD-L1 MFI were higher in the IPF cohorts as compared to control cohorts (Figure 6.10). CXCR4 MFI: median control = 61.7 (IQR 44.9 – 124.8) vs IPF = 112.0 (IQR 80.9 – 112.0); $p = 0.028$ by Mann-Whitney Test, $n = 14/19$. PD-L1 MFI: median control = 90.6 (IQR 87.4 – 109.5) vs IPF = 127 (IQR 114.0 – 149.0); $p = 0.02$, $n = 14/19$.

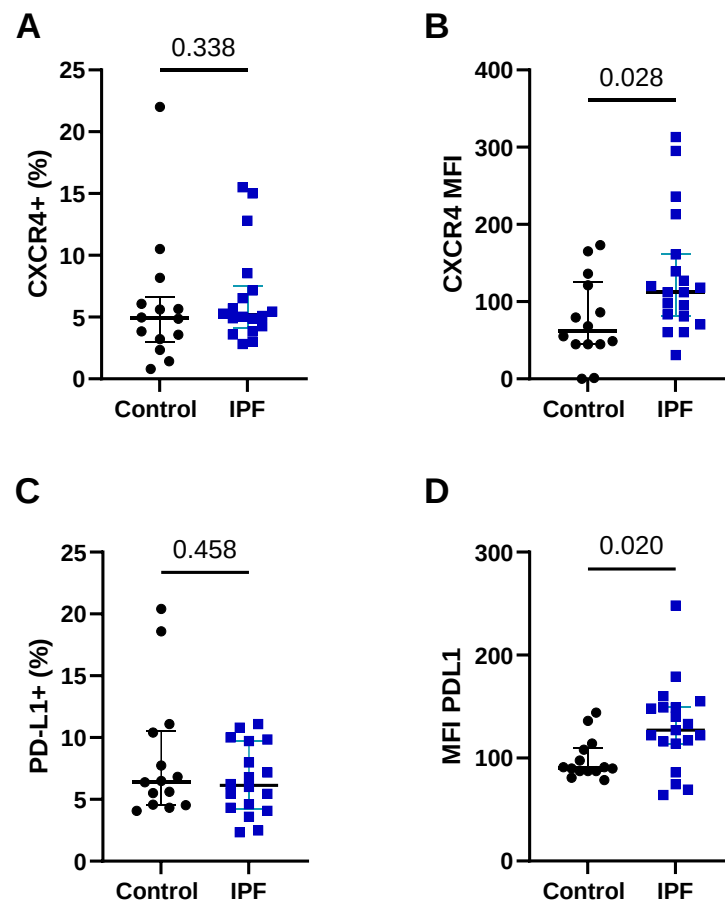


Figure 6.10: Cell surface expression of CXCR4 and PD-L1 on control and IPF donor neutrophils.

Neutrophils isolated from 14 control and 19 IPF participants were stained with the panel 3 immunophenotyping antibody cocktail (detailed in Table 2.7). No difference was noted in the percentage of cells expressing CXCR4 (A) or PD-L1 (C). MFI of CXCR4 (B) and PDL-1 (D) were higher in IPF donor neutrophils than control neutrophils. P-values comparing IPF to control are displayed above the data points. Dot/square per participant. Data bars are median and interquartile. Mann-Whitney tests were performed.

6.6.3.5. The percentage of CD10 positive neutrophils appears lower in the circulation of IPF patients.

CD10 expression was assessed to give insight into neutrophil maturity [47,48]. The percentage of CD10 positive neutrophils was lower in those derived from IPF patients as compared to control participants (median IPF = 85.8% (IQR 77.5- 90.0) vs control = 91.8%; $p = 0.018$ by Mann-Whitney test, $n = 14/ 18$, Figure 6.11A). However, CD10 MFI was comparable between the two cohorts ($p > 0.05$, unpaired T-test, $n = 14/ 19$, Figure 6.11B).

CD16 expression is also increased during neutrophil maturation (from intermediate to high) [398]. It was noted here that the CD10 positive cells had a higher CD16 MFI than CD10 negative cells in both control and IPF cohorts ($p < 0.001$, paired T-tests, $n = 14/ 17$, Figure 6.11C). This supports that CD10 expression in this panel can delineate between mature and immature neutrophil subsets.

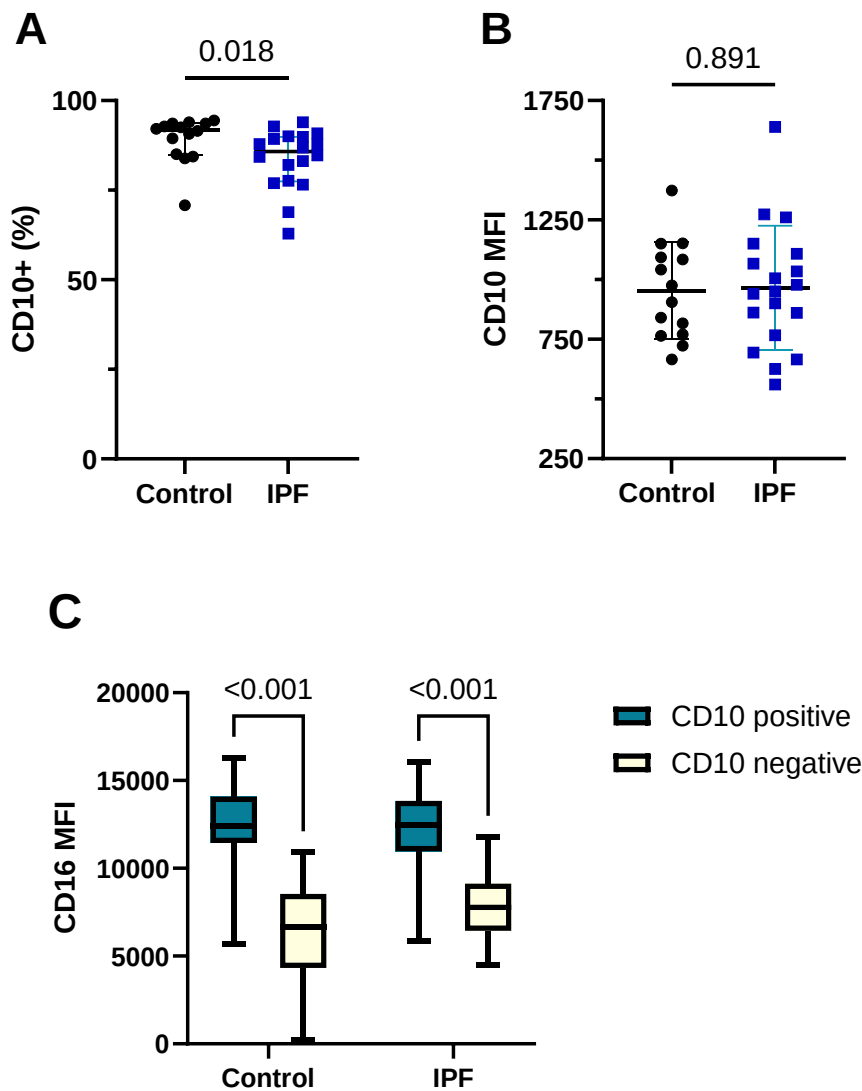


Figure 6.11: Cell surface expression of CD10 on IPF and control derived neutrophils.

Neutrophils isolated from 14 control and 19 IPF participants were stained with the panel 3 immunophenotyping antibody cocktail.

(A) Percentage of CD10 positive neutrophils were lower in the IPF cohort as compared to control cohort. One data point is missing in the IPF cohort due to an issue with the isotype control stain. (B) No difference was noted in MFI between the two groups.

P-values comparing IPF to control are displayed above the data points. Dot/square per participant. Data bars are median and interquartile range for (A), analysed using Mann-Whitney test. Data bars are mean and standard deviation for (B), analysed using unpaired T-test.

(C) Box and whisker plots (Tukey) displaying CD16 MFI of CD10 positive and CD10 negative neutrophils in control (n = 14) and IPF (n = 17) cohorts. P-values are displayed above the Box and whisker plots (Tukey). Paired T-tests were performed.

6.6.4. Chemotactic and chemokinetic responses of neutrophils derived from the circulation of IPF and control participants.

The chemotactic and chemokinetic responses of peripheral neutrophils isolated from 11 control and 19 IPF participants enrolled in the HYBRID study were assessed using the Insall chamber assay. The participant characteristics of the neutrophil donors were well matched and are described in Table 6.2.

	Control (n = 11)	IPF (n = 19)	p-value
Age (years), mean (SD)	73.4 (6.0)	74.3 (6.3)	ns
Sex ratio, M : F	6 : 5	16 : 3	ns
Current: Ex: Never smoker	0: 5: 4	0: 6: 5	ns
Pack history, median (IQR)	0.5 (0- 8.6)	3 (0- 37.5)	ns
Charlson Comorbidity Index			
≤ 2, n (%)	1 (9.1%)	4 (21.1%)	ns
3, n (%)	6 (54.5%)	5 (26.3%)	
4, n (%)	3 (27.3%)	7 (36.8%)	
≥ 5, n (%)	0 (0%)	3 (15.8%)	

Table 6.2: Insall chamber assay: HYBRID study participant characteristics.

Characteristics of the 11 control and 19 IPF participants from which neutrophils were isolated and movement assessed within the Insall chamber. Unpaired T-test and Mann-Whitney tests were performed for normally and skewed continuous data, respectively. Fisher's exact test was used to analyse categorical data.

6.6.4.1. Neutrophil movement is equivalent in the absence of a stimulus/chemoattractant between IPF and control donors.

RPMI containing no stimulus/chemoattractant was the first condition tested in every Insall chamber assay performed in the HYBRID study. This aimed to assess, alongside cell surface marker expression described in Chapter 6.6.3, whether neutrophils from IPF patients are in a primed/activated state as compared to neutrophils from control participants. Neutrophil movement of control (n = 11) and IPF (n = 19) donor neutrophils was equivalent for all parameters ($p > 0.05$, unpaired T-test/ Mann-Whitney tests, Figure 6.12).

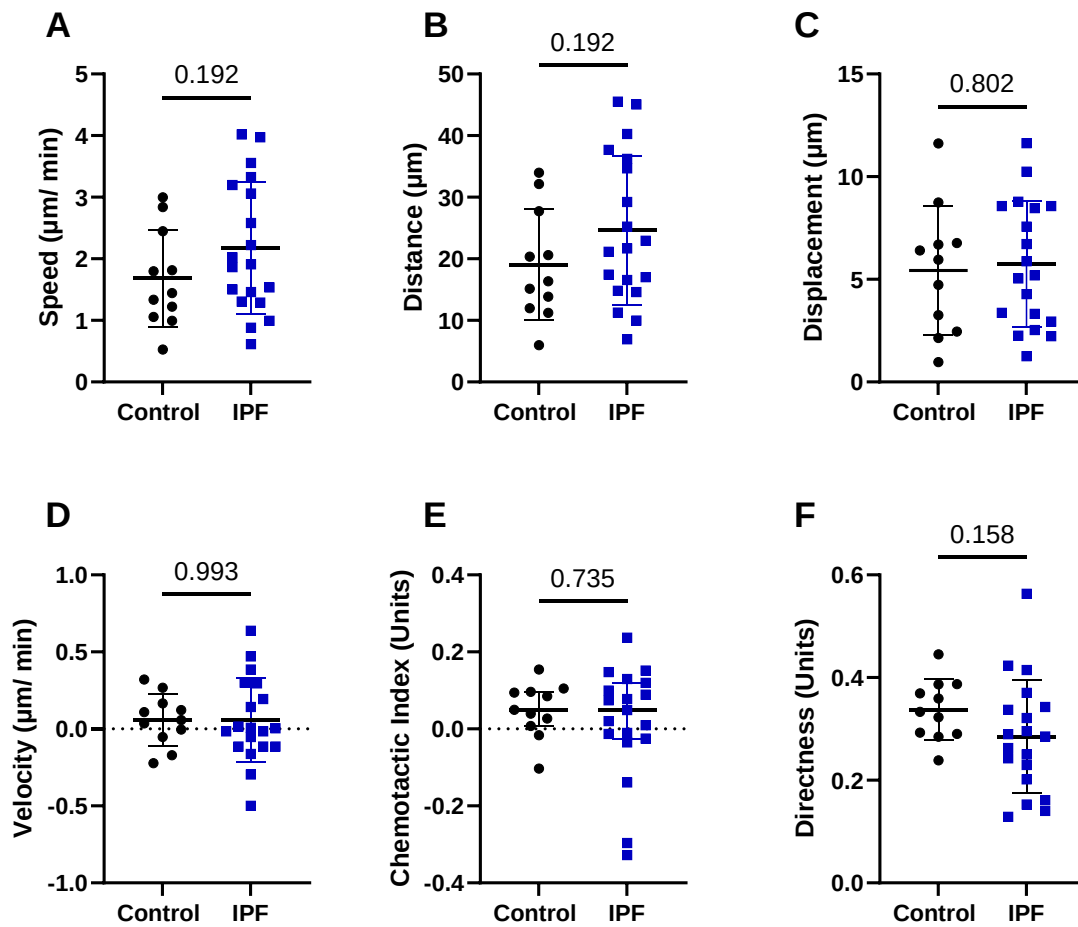


Figure 6.12: Movement of control and IPF donor neutrophils within the Insall chamber in the absence of a stimulus/chemoattractant.

Movement patterns within the Insall Chamber in the absence of a stimulus was compared between neutrophils isolated from 11 control and 19 IPF participants. P-values compared to control are shown above the data points. Dot/square per participant. Data bars are mean and standard deviation for all, except (E) which are median and interquartile range. Unpaired T-tests were performed for all, except (E) in which Mann-Whitney test was carried out.

6.6.4.2. IPF donor neutrophils maintain chemotactic responses to classical chemoattractants.

Movement of control and IPF donor neutrophils towards the classical chemoattractants, 100 nM IL-8 and 10 nM fMLP, was then assessed within the Insall chamber. Exposure to 100 nM IL-8 and 10 nM fMLP within the Insall chamber assay increased both chemokinetic and chemotactic responses of control (Figure 6.13) and IPF derived neutrophils (Figure 6.14) as compared to RPMI ($p < 0.05$, paired T-tests, $n = 10/16$).

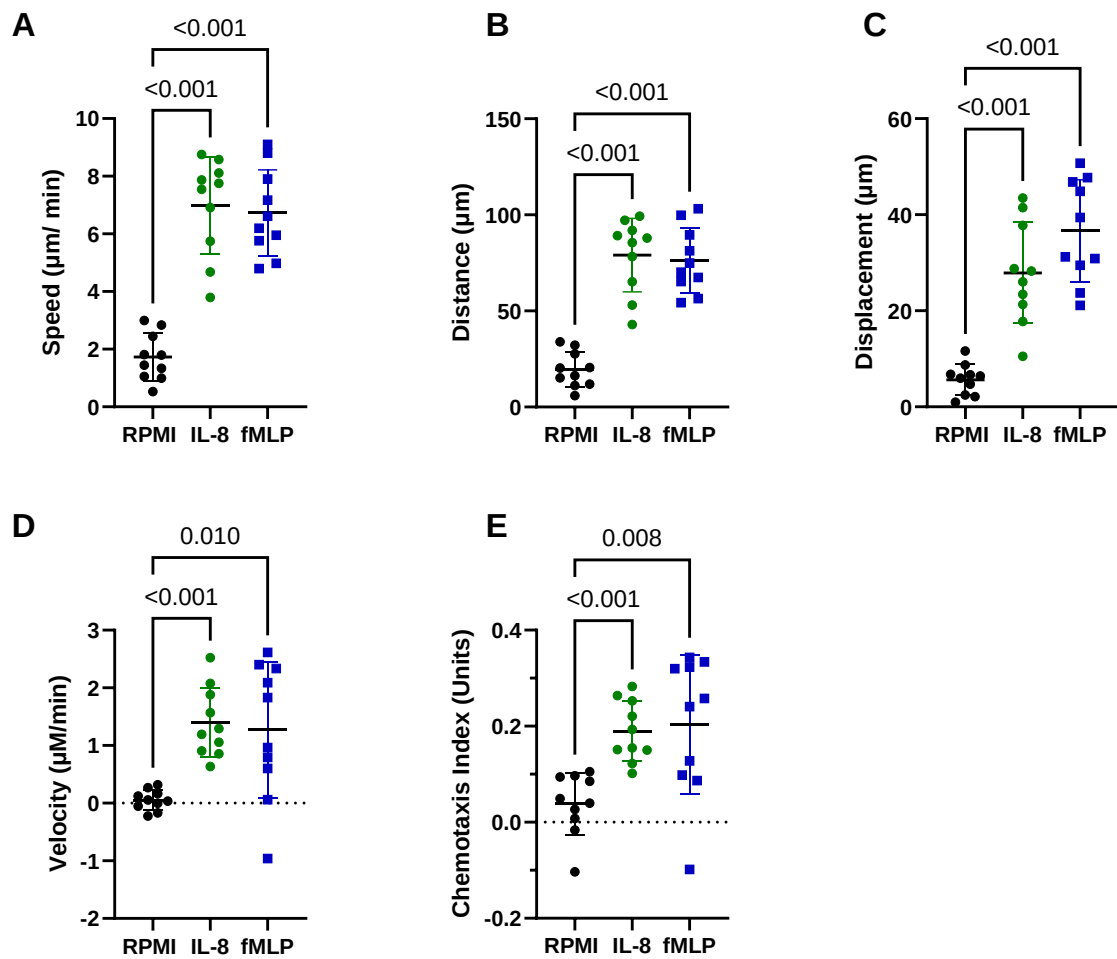


Figure 6.13: Chemotaxis of control donor neutrophils towards IL-8 and fMLP.

Comparison of chemotactic responses within the Insall chamber of neutrophils derived from 10 control participants towards the classical chemoattractants, 100 nM IL-8 and 10nM fMLP. IL-8 (green dots) and fMLP (blue squares) increased all direction dependent and independent movement parameters as compared to RPMI (black dots; $p < 0.05$ by paired T-tests). P-values compared to RPMI are shown above the data points. Dot/square per participant. Data bars are mean and standard deviation.

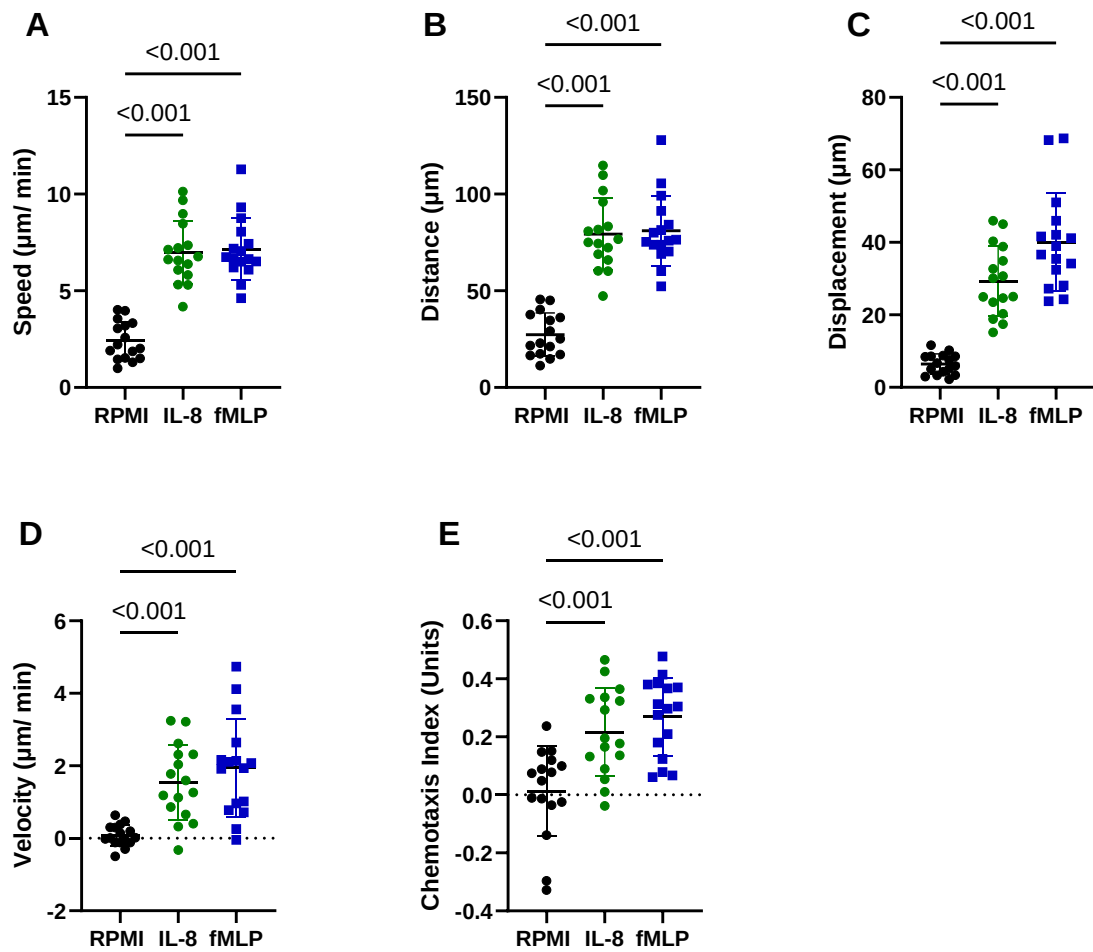


Figure 6.14: Chemotaxis of IPF derived neutrophils towards IL-8 and fMLP.

Comparison of chemotactic responses within the Insall chamber of neutrophils derived from 16 IPF participants towards the classical chemoattractants, 100 nM IL-8 and 10nM fMLP. IL-8 (green) and fMLP (blue) increased all direction dependent and independent movement parameters as compared to RPMI (black dots; $p < 0.001$ by paired T-tests). P-values compared to RPMI are shown above the data points. Dot/square per participant. Data bars are mean and standard deviation.

Furthermore, direct comparison of control and IPF donor neutrophils, showed no difference in chemotactic or chemokinetic responses to 100 nM IL-8 (Figure 6.15) or 10 nM fMLP (Figure 6.16). This suggests that neutrophil movements towards these classical chemoattractants are not impaired in IPF patients relative to control participants of similar age and comorbidities.

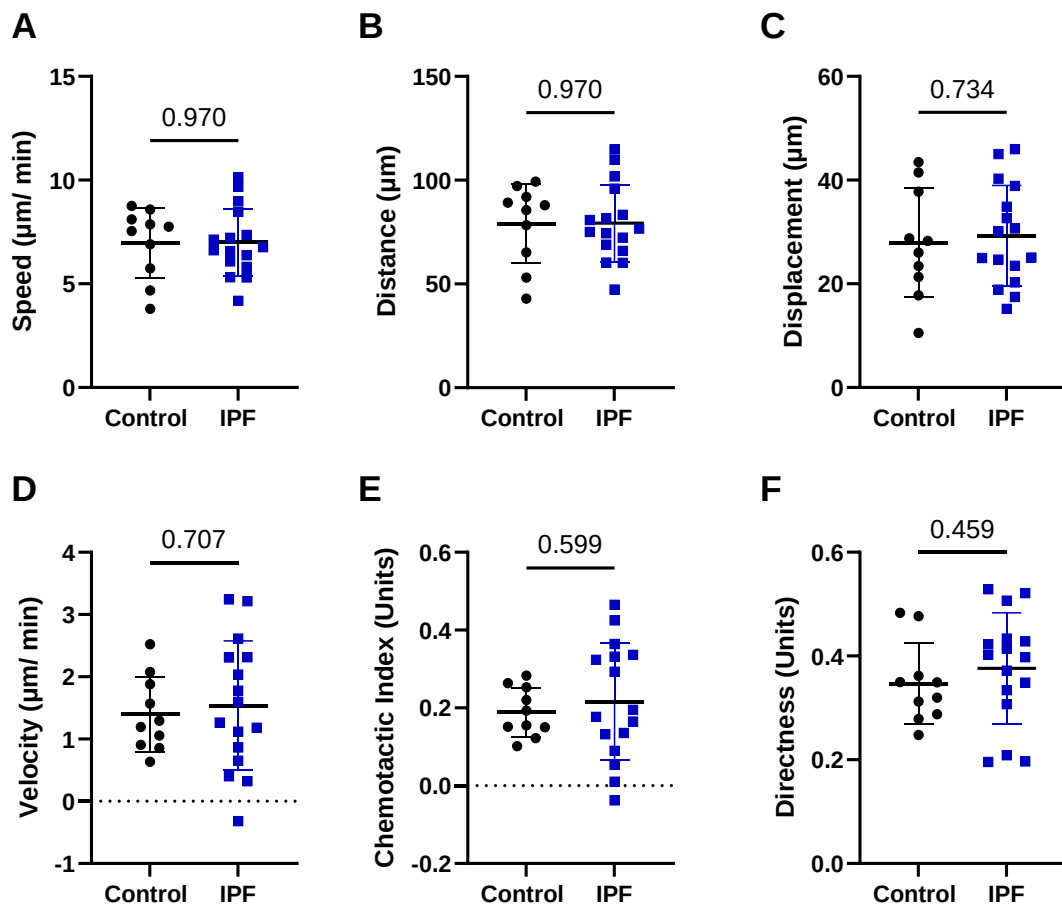


Figure 6.15: Comparison of chemotactic and chemokinetic responses to IL-8 between control and IPF derived neutrophils.

Chemotactic and chemokinetic responses of control ($n = 10$) and IPF ($n = 16$) derived neutrophils towards 100 nM IL-8 within the Insall chamber are described. P-values compared to control are shown above the data points. Dot/square per participant. Data bars are mean and standard deviation. Unpaired T-tests were performed.

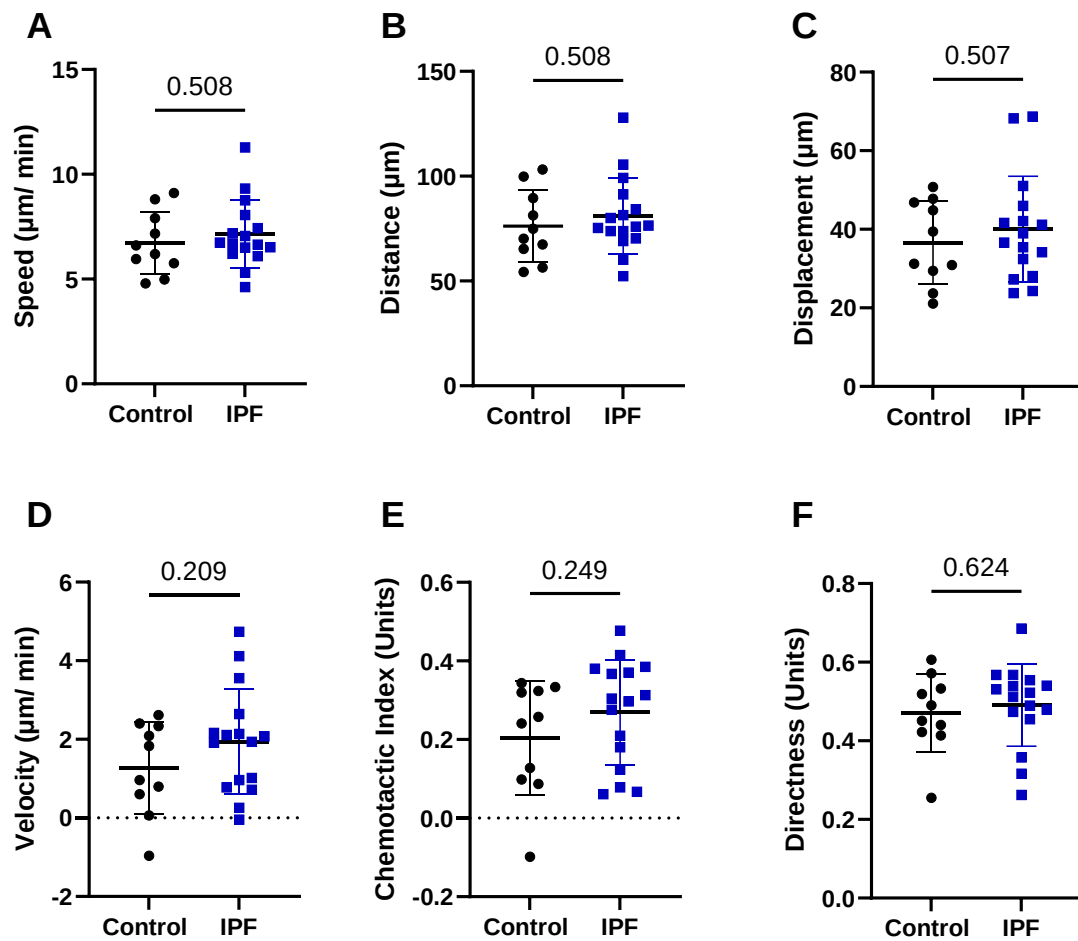


Figure 6.16: Comparison of chemotactic and chemokinetic responses to fMLP between control and IPF derived neutrophils.

Chemotactic and chemokinetic responses of control ($n = 10$) and IPF ($n = 16$) derived neutrophils towards 10 nM fMLP within the Insall chamber are described. P-values compared to control are shown above the data points. Dot/square per participant. Data bars are mean and standard deviation. Unpaired T-tests were performed.

6.6.4.3. TGF β 1 increased neutrophil chemokinesis of control donor neutrophils.

It was demonstrated in Chapter 4 that 10 ng/ mL TGF β 1 increased the chemokinesis, but not chemotaxis, of healthy donor neutrophils. The movement of IPF and control participant neutrophils in response to this pro-fibrotic growth factor was subsequently assessed in the Insall chamber. Ten ng/ mL TGF β 1 significantly increased speed, cumulative distance and displacement of neutrophils derived from control participants as compared to vehicle ($p < 0.05$, paired T-tests, $n = 11$, Figure 6.17A-C). Furthermore, as seen in healthy donor neutrophils, direction dependent parameters and directness were unaffected by TGF β 1 in this age-matched control group ($p > 0.05$, paired T-tests, $n = 11$, Figure 6.17D-F).

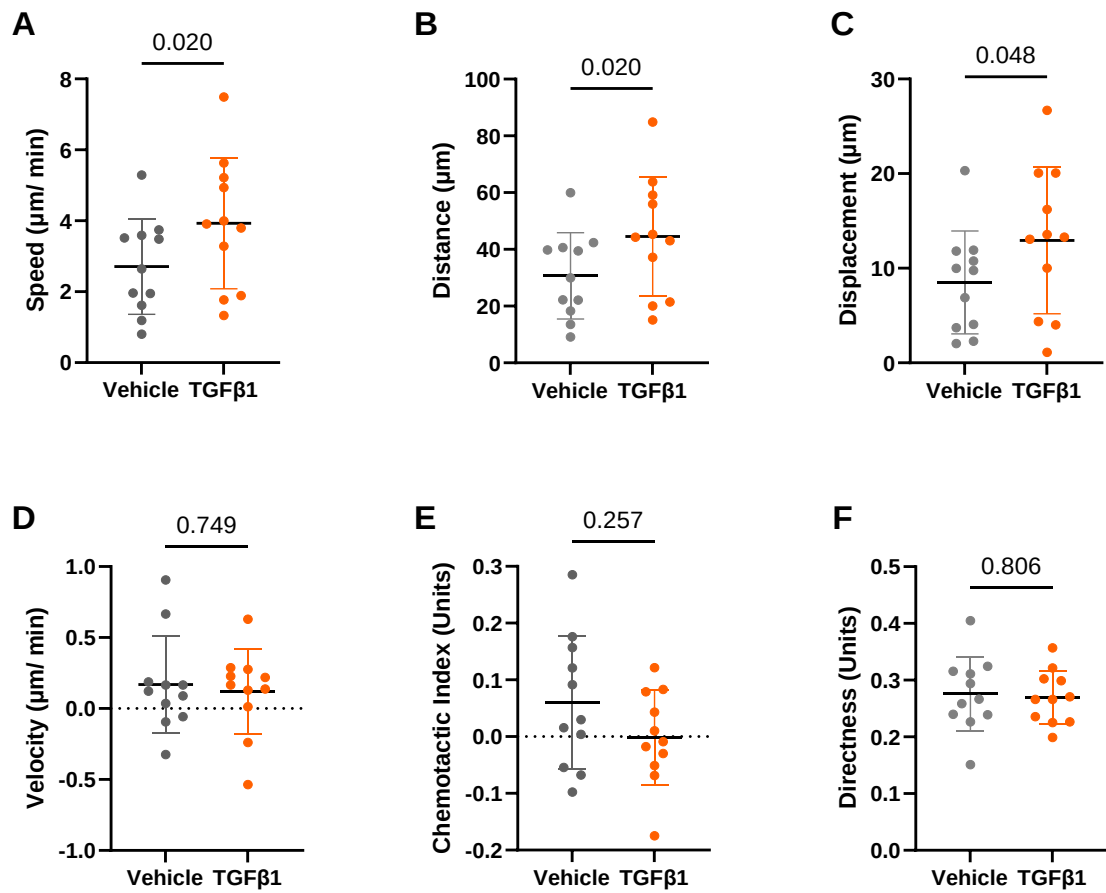


Figure 6.17: Chemokinetic and chemotactic responses of control neutrophils to TGFβ1.

Movement of control donor neutrophils ($n = 11$) was assessed in the Insall chamber in response to 10 ng/ mL TGFβ1. P-values compared to vehicle are shown above the data points. Dot per participant. Data bars are mean and standard deviation. Paired T-tests were performed.

6.6.4.4. TGF β 1 reduces the directness of movement of IPF derived neutrophils.

In contrast to control neutrophils, 10 ng/ mL TGF β 1 did not significantly increase any of the chemokinetic or chemotactic parameters of IPF donor neutrophils as compared to vehicle ($p > 0.05$, paired T-tests/Wilcoxon tests, $n = 13$, Figure 6.18). For example, speed of movement: mean TGF β 1 4.3 $\mu\text{m}/\text{min}$ (SD 2.1) vs vehicle = 3.0 $\mu\text{m}/\text{min}$ (SD 2.0); $p = 0.068$, Wilcoxon test, $n = 11$.

However, directness was significantly lower in IPF donor neutrophils in response to TGF β 1 as compared to vehicle (mean control = 0.28 (SD 0.09) vs IPF = 0.20 (SD 0.05); $p = 0.005$, paired T- test, $n = 13$).

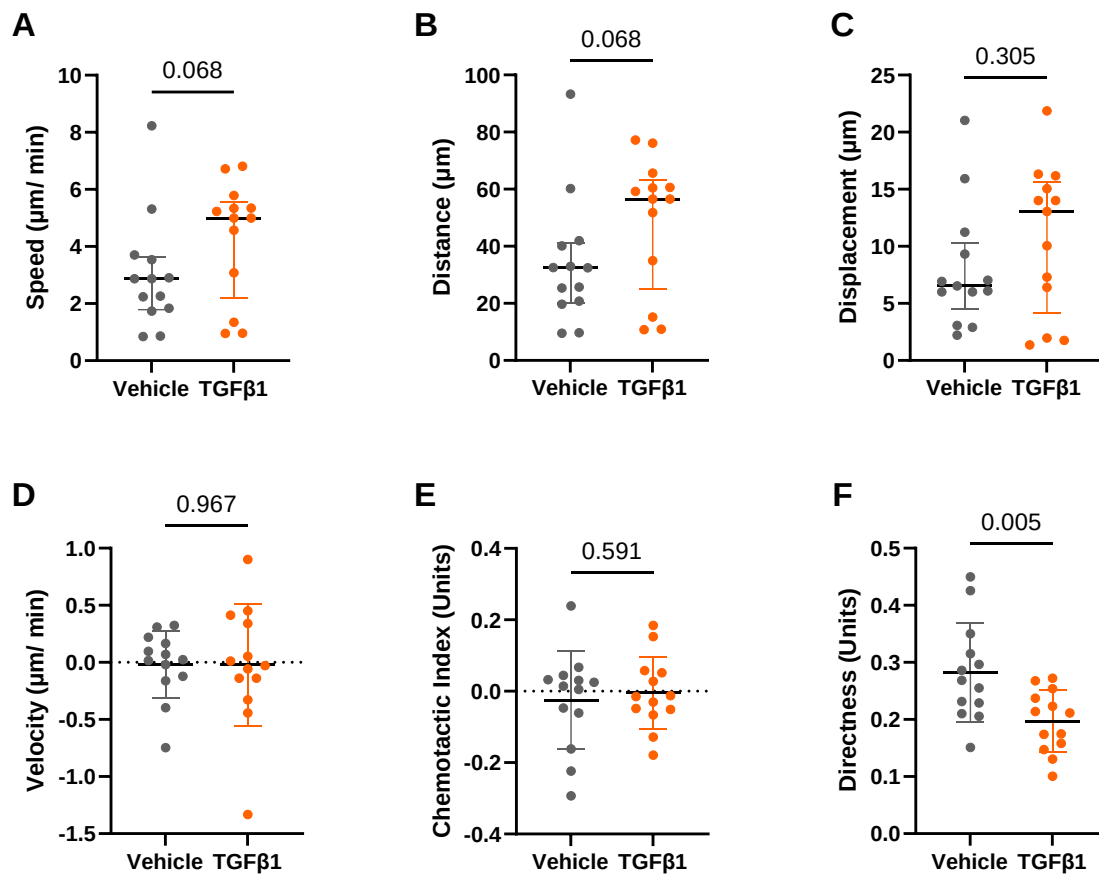


Figure 6.18: Chemotactic and chemokinetic responses of IPF derived neutrophils to TGFβ1.

Movement of IPF (n = 13) donor neutrophils was assessed in the Insall chamber in response to 10 ng/ mL TGFβ1. P-values compared to vehicle are shown above the data points. Dot per participant. Data bars are median and interquartile range (A – C) or mean and standard deviation (D – F). Wilcoxon tests (A – C) or paired T-tests (D – F) were performed.

On direct comparison of IPF and control donor neutrophil responses to 10 ng/ mL TGF β 1, only directness of movement was different between the two cohorts (Figure 6.19). Directness of IPF donor neutrophils was significantly lower than control neutrophils on exposure to TGF β 1 (mean control = 0.27 (SD 0.05) vs IPF = 0.19 (SD 0.05); $p < 0.001$, unpaired T- test, $n = 11/ 18$).

However, neutrophil directness in the presence of vehicle only was equivalent between control and IPF donor neutrophils (mean control = 0.28 (SD 0.07) vs IPF = 0.29 (0.08); $p > 0.05$, unpaired T-tests, $n = 11/ 14$). This suggests it is the TGF β 1 itself that may be driving the altered directness of IPF neutrophils, rather than its vehicle. Representative neutrophil microscopy images displaying the reduced directness of IPF donor neutrophils in response to TGF β 1 as compared to control neutrophils are displayed in Figure 6.20.

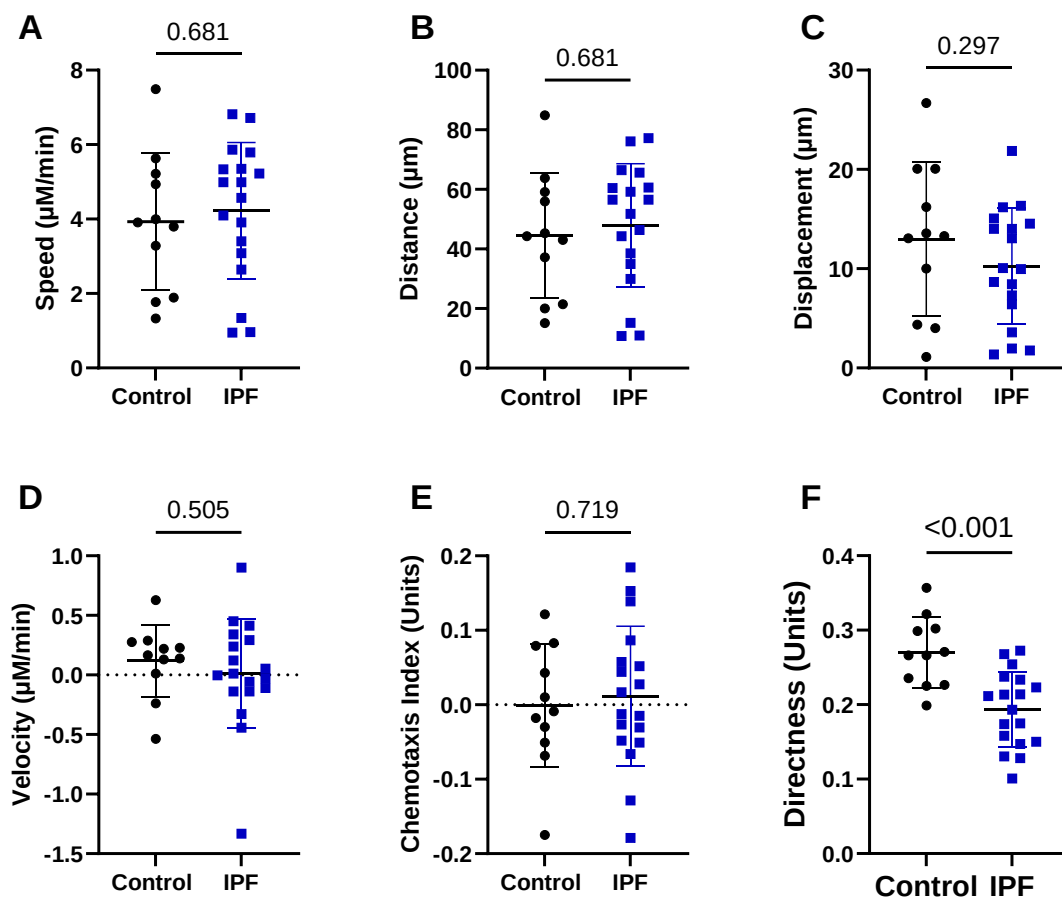


Figure 6.19: Movement of IPF and control derived neutrophils in response to TGF β 1.

Comparison of chemokinetic and chemotactic responses to 10 ng/ mL TGF β 1 between control (n = 11) and IPF (n = 18) derived neutrophils in the Insall chamber. P-values compared to control are shown above the data points. Dot/square per participant. Data bars are mean and standard deviation. Unpaired T-tests were performed.

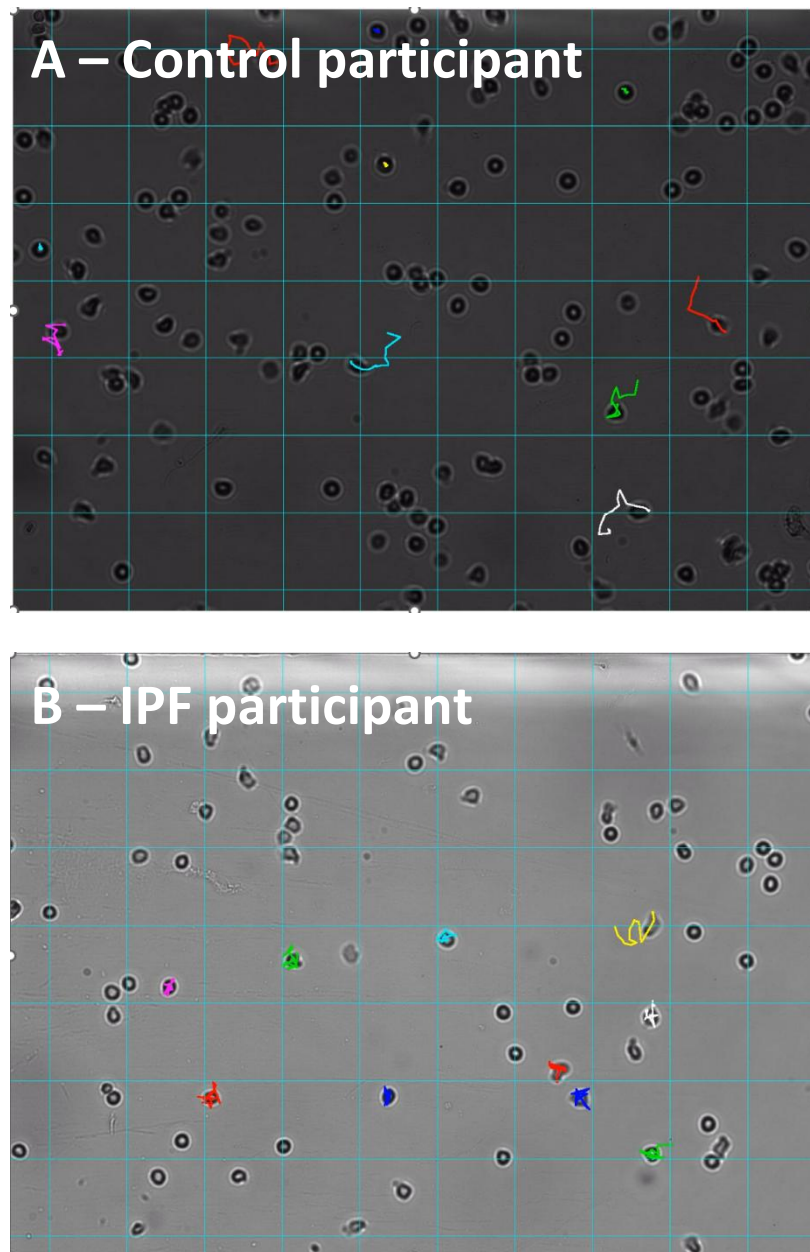


Figure 6.20: Representative images displaying neutrophil movement in response to TGFβ1.

Representative time-lapse microscopy images displaying tracks (coloured lines) of neutrophil movement over 12 minutes in response to 10 ng/ mL TGFβ1. A – control derived neutrophils, B – IPF donor neutrophils. Images taken with a Leica DMI6000 B microscope at 20x magnification.

6.6.5. Neutrophil extracellular traps

6.6.5.1. IPF derived neutrophils release significantly less cfDNA in response to PMA.

NET responses of IPF and control derived circulating neutrophils were assessed by measuring cfDNA release *ex vivo* (see Chapter 2.8.1).

cfDNA release in the absence of a stimulus or in response to 25 nM PMA or 10 ng/ mL TGF β 1 was measured in neutrophils isolated from 11 control and 19 IPF participants. The patient characteristics of these two cohorts are outlined in Table A in Figure 6.21, and were comparable in regards to age, gender, smoking history and Charlson Comorbidity Index.

cfDNA release from unstimulated neutrophils after 4 hours of *ex vivo* culture was equivalent between the two cohorts ($p > 0.05$, Mann-Whitney test, Figure 6.21B). However, IPF neutrophils released significantly less cfDNA in response to 25 nM PMA (mean RFU IPF = 1292 (SD 395) vs control = 1857 (SD 723); $p = 0.011$, unpaired T-test, Figure 6.21C).

A	Control (n = 11)	IPF (n = 19)	p-value
Age (years), mean (SD)	73.4 (6.0)	74.3 (6.3)	ns
Sex ratio, M : F	6 : 5	16 : 3	ns
Current: Ex: Never smoker	0: 6: 5	0: 11: 8	ns
Pack history, median (IQR)	0.5 (0- 8.6)	3.0 (0- 37.5)	ns
Charlson Comorbidity Index			
≤ 2, n (%)	1 (9.1%)	4 (21.1%)	ns
3, n (%)	7 (63.6%)	5 (26.3%)	
4, n (%)	3 (27.3%)	7 (36.8%)	
≥ 5, n (%)	0 (0%)	3 (15.8%)	

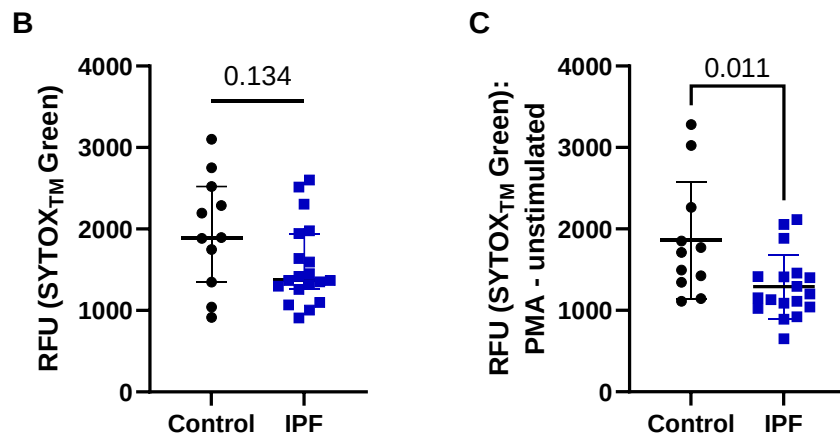


Figure 6.21: Assessment of NET release in response to PMA by control and IPF derived neutrophils.

Table A describes the characteristics of 11 control and 19 IPF participants from which neutrophils were isolated and cell free DNA (cfDNA) release quantified *ex vivo*.

(B) displays cfDNA release (reported as relative fluorescence units (RFU)) from neutrophils that were cultured *ex vivo* for four hours in the absence of a stimulus. (C) displays cfDNA release in response to 4 hours exposure to 25 nM PMA. This was calculated by: SYTOX™ green RFU of PMA stimulated neutrophils minus unstimulated neutrophils. P-values compared to control are shown above the data points. Dot/square per participant. Data bars are median and IQR in (B) and mean and SD in (C). A single IPF data point was excluded from (C) as the incorrect concentration of PMA was added. The participant characteristics remained comparable between the two groups as described in Table A following exclusion of this IPF participant.

Unpaired t-test and Mann-Whitney tests were performed for normally and skewed continuous data, respectively. Fisher's exact test was used to analyse categorical data.

6.6.5.2. cfDNA release was equivalent in response to TGFβ1 between control and IPF derived neutrophils.

In Chapter 4 it was observed that 10 ng/ mL TGFβ1 increased cfDNA release from healthy donor neutrophils.

The absolute change in cfDNA release from 10 ng/ mL TGFβ1 exposure (calculated by RFU of TGFβ1 exposed neutrophils minus RFU of vehicle control treated neutrophils) was equivalent between the two cohorts ($p > 0.05$, unpaired T-test, Figure 6.22). Any value greater than zero (displayed as a dotted line) in Figure 6.22 represents an increase in cfDNA release in the TGFβ1 treated neutrophils relative to vehicle control. Most of the samples were above the line of no effect suggesting that in the majority of samples, as shown in Chapter 4, TGFβ1 increased cfDNA release.

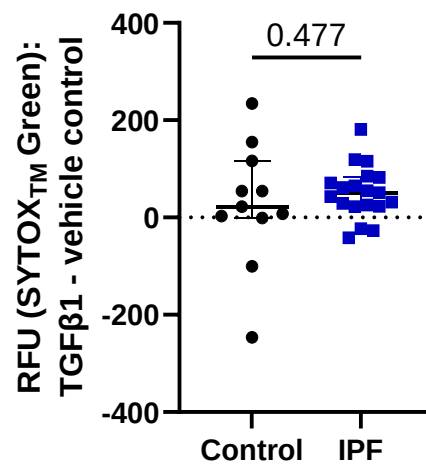


Figure 6.22: NET release in response to 10 ng/ mL TGFβ1.

Cell free DNA (cfDNA) release from 11 control and 19 IPF donor neutrophils was measured following 4 hours exposure *ex vivo* to 10 ng/ mL TGFβ1 or vehicle control.

The absolute change in cfDNA release in response to 10 ng/ mL TGFβ1 between the two cohorts is displayed. This was calculated by: SYTOX™ green RFU of 10 ng/ mL TGFβ1 stimulated neutrophils minus vehicle control exposed neutrophils. P-value compared to control is shown above the data points. Unpaired t-test was performed. Dot/square per participant. Data bars are mean and SD.

6.6.5.2. NET release in response to the pro-inflammatory cytokines, IL-8 and IL-1 β , is equivalent between control and IPF derived neutrophils.

The pro-inflammatory cytokines IL-8 and IL-1 β were chosen as more physiological and relevant stimuli in the context of lung fibrosis to assess NET formation (see Chapter 3). In this study, neutrophils isolated from 9 control and 15 IPF participants were exposed to 5 nM IL-8 or 2.9 nM IL-1 β for four hours *ex vivo* and cfDNA release measured. Participant characteristics were equivalent between the two groups, except for there were fewer female participants in the IPF group (Table A in Figure 6.23).

There was no difference in cfDNA release in response to either 5 nM IL-8 ($p > 0.05$, unpaired T-test, $n = 9/14$, Figure 6.23B) or 2.9 nM IL-1 β ($p > 0.05$, Mann-Whitney test, $n = 9/15$, Figure 6.23C) between IPF and control donor neutrophils.

A	Control (n = 9)	IPF (n = 15)	p-value
Age (years), mean (SD)	74.4 (6.1)	75.0 (6.6)	ns
Sex ratio, M : F	5 : 4	14 : 1	0.047
Current: Ex: Never smoker	0: 4: 5	0: 8: 7	ns
Pack history, median (IQR)	0.0 (0- 7.5)	1.3 (0- 30.0)	ns
Charlson Comorbidity Index			
≤ 2, n (%)	0 (10.0%)	3 (20.0%)	ns
3, n (%)	6 (66.7%)	3 (20.0%)	
4, n (%)	3 (33.3%)	6 (40.0%)	
≥ 5, n (%)	0 (0%)	3 (20.0%)	

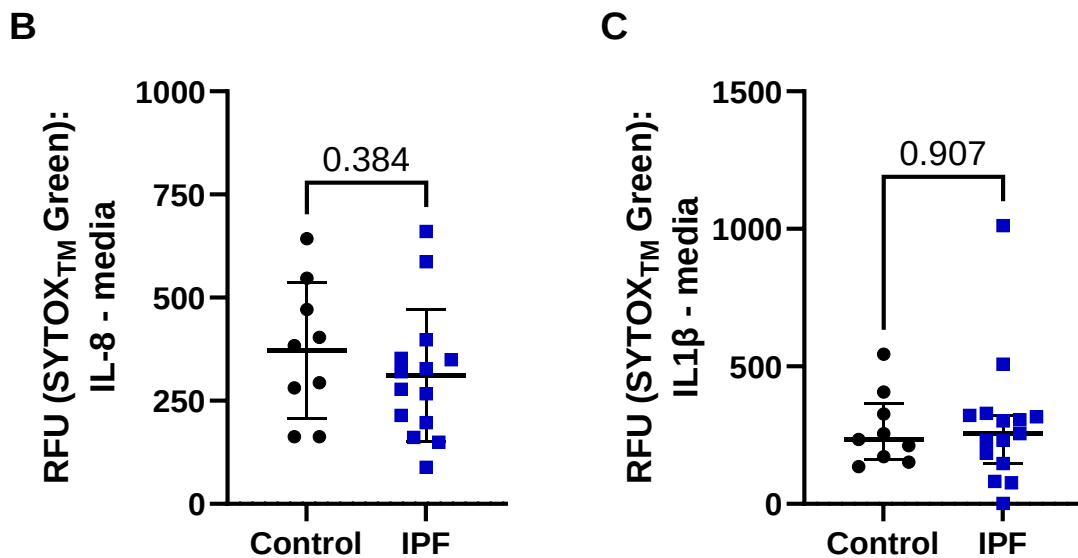


Figure 6.23: NET release from IPF and control neutrophils in response to IL-8 and IL-1 β .

Table A describes the characteristics of 9 control and 15 IPF participants from which neutrophils were isolated and cell free DNA (cfDNA) release quantified *ex vivo*. Unpaired t-test and Mann-Whitney tests were performed for normally and skewed continuous data, respectively. Fisher's exact test was used to analyse categorical data.

(B) and (C) display cfDNA release from IPF and control donor neutrophils in response to 4 hours exposure to 5 nM IL-8 or 2.9 nM IL-1 β , respectively. This was calculated by: SYTOX™ green relative fluorescence units (RFU) of stimulated neutrophils minus unstimulated neutrophils. P-values compared to control are shown above the data points. Dot/square per participant. Data bars are mean and SD in (B) and median and IQR in (C). Unpaired t-test performed in (B) and Mann-Whitney test performed in (C).

6.6.6. Exploring mechanistic drivers of reduced cfDNA release from IPF neutrophils.

6.6.6.1. Neutrophil viability after prolonged culture *ex vivo*.

As described in Chapter 4, DNA can be released from cells during apoptosis/necrosis [399] as well as NET formation. Alterations in the local environment, such as via hypoxia mediated HIF1 α , can lead to prolonged neutrophil survival [43]. Reduced cfDNA release from IPF donor neutrophils was noted midway during HYBRID study recruitment and it was hypothesised that this could be secondary to IPF donor neutrophils being less susceptible to cell death. To investigate this, Annexin V/PI viability staining was performed after 4 hours (NET time-point) and 24 hours of culture in a 37°C 5% CO₂ incubator. The reason for this latter time-point was that neutrophils were expected to show signs of apoptosis by 24 hours. For example, Walmsley *et al* showed that over 50% of human blood peripheral neutrophils displayed signs of apoptosis after 20 hours of culture, which was reduced when in a hypoxic (1% oxygen) atmosphere [43].

The viability of neutrophils from control and IPF donor participants after 4 and 24 hours of culture are presented in Figure 6.24. Unfortunately, due to experimental limitations, only 2 of these experiments were performed on control donor neutrophils (mean percentage viability after 4 and 24 hours were 86.6% and 32.3%, respectively, see Figure 6.24A). Therefore, no comparisons of viability at these timepoints were made between control and IPF donor neutrophils.

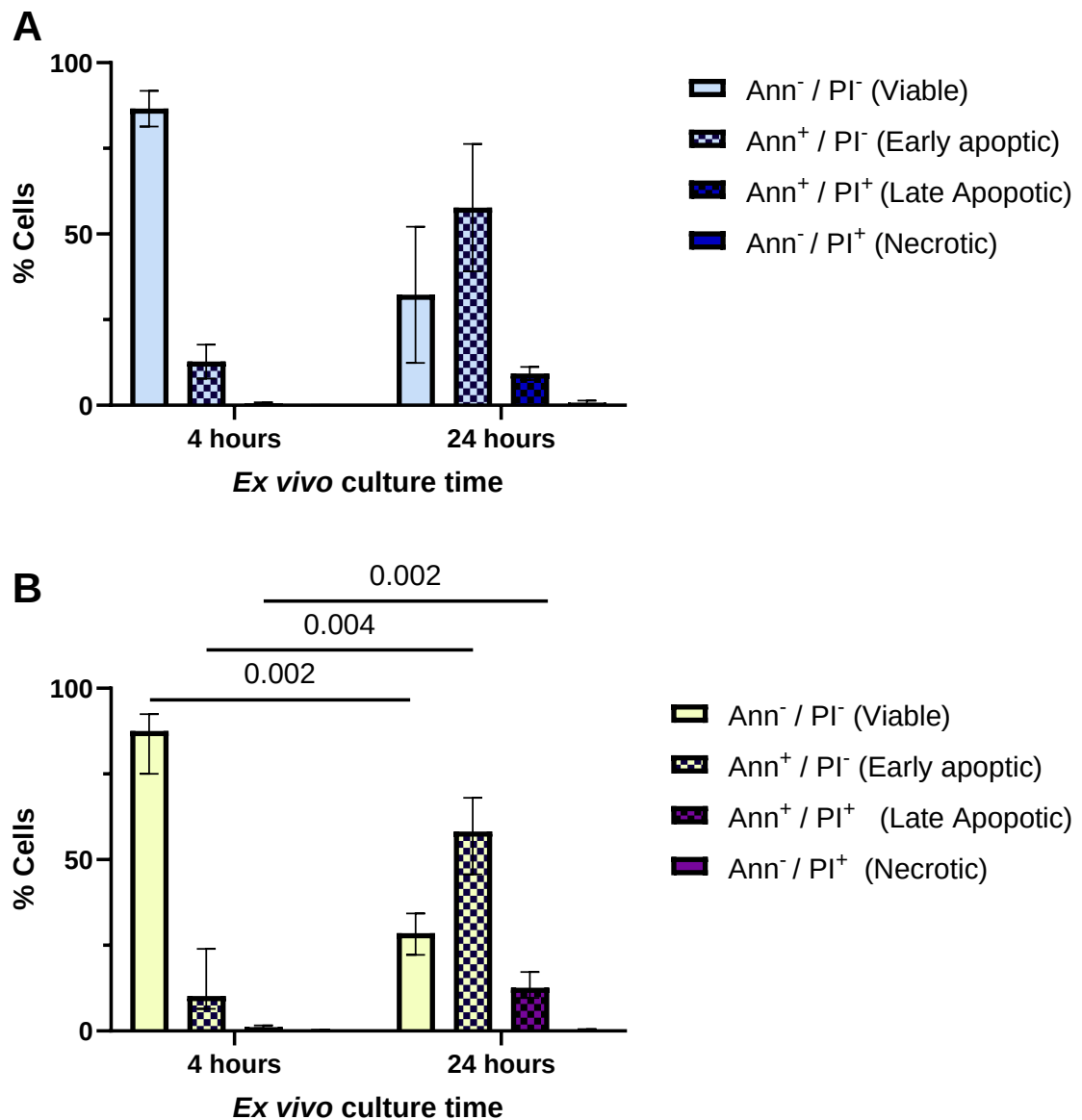


Figure 6.24: Viability of IPF donor neutrophils over prolonged culture *ex vivo*.

Using Annexin V/PI staining, neutrophils from 2 control (A) and 6 IPF patients (B) were assessed for the percentage of cells that were viable, early apoptotic, late apoptotic or necrotic after 4 and 24 hours culture *ex vivo*. Data histograms are median and IQR. Mann-Whitney tests were performed. P-values are displayed above the histograms.

On review of the IPF samples, as expected, the percentage of viable cells (defined as Annexin negative, PI negative) was lower after 24 hours of culture as compared to 4 hours (median 24 hours = 28.6% (IQR 22.2 vs 34.3%) vs median 4 hours = 87.5% (IQR 75.0 – 92.4); $p = 0.002$, Mann-Whitney test, $n = 6$, Figure 6.24B). Furthermore, the percentage of early apoptotic neutrophils (Annexin positive, PI negative) increased from 4 to 24 hours of culture (median 4 hours = 10.2% (IQR 6.4 vs 24.0%) vs median 24 hours = 58.2% (IQR 45.7 – 68.1); $p = 0.004$, Mann-Whitney test, $n = 6$). No difference was noted in the percentage of necrotic cells (Annexin negative, PI positive) between 4 and 24 hours.

6.6.6.2. Baseline ROS levels in IPF derived neutrophils.

Production of ROS by NADPH oxidase is important for PMA mediated NET formation [112,358].

Therefore, isolated neutrophils were stained with CellROX™ Deep Red to assess if there was any disturbance in this process that may be contributing to the difference seen in response to PMA. Participant characteristics were equivalent between the two donor groups, except for sex ratio (fewer female participants in the IPF group, see Table A in Figure 6.25). CellROX™ Deep Red MFI were equivalent between the two groups: median control = 30.1 (IQR 2.42 – 52.2) vs IPF = 47.4 (IQR 40.7 – 58.3); $p = 0.071$ by Mann-Whitney test, $n = 10/19$ (Figure 6.25B).

A	Control (n = 10)	IPF (n = 19)	p-value
Age (years), mean (SD)	73.1 (6.2)	74.3 (6.2)	ns
Sex ratio, M : F	6 : 4	18 : 1	0.036
Current: Ex: Never smoker	0: 6: 4	0: 12: 7	ns
Pack history, median (IQR)	0.8 (0- 9.0)	10.0 (0- 30.0)	ns
Charlson Comorbidity Index			
≤ 2, n (%)	1 (10.0%)	4 (21.1%)	ns
3, n (%)	6 (60.0%)	5 (26.3%)	
4, n (%)	3 (30.0%)	7 (36.8%)	
≥ 5, n (%)	0 (0%)	3 (15.8%)	

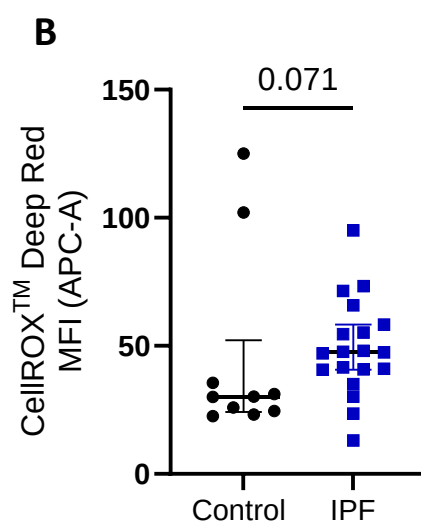


Figure 6.25: Levels of cytoplasmic oxidative stress in control and IPF donor neutrophils.

Table A describes the characteristics of 10 control and 19 IPF participants from which neutrophils were isolated and cytoplasmic reactive oxygen species (ROS) were measured using CellROX™ deep red staining. Unpaired t-test and Mann-Whitney tests were performed for normally and skewed continuous data, respectively. Fisher's exact test was used to analyse categorical data.

(B) displays the CellROX™ deep red median fluorescence intensity (MFI) of neutrophils isolated from control and IPF participants. P-values compared to control are shown above the data points (Mann-Whitney test). Dot/square per participant. Data bars are median and IQR.

6.6.6.3. The effect of low oxygen on NET formation.

An alternative hypothesis for why there appeared to be reduced PMA mediated cfDNA release in IPF neutrophils was the presence of hypoxaemia in IPF patients (as shown in Chapter 5). Perhaps the reduced bioavailability of oxygen, which is the substrate for the NADPH oxidase system, impairs maximal NET formation. This was examined by comparing NET formation in response to PMA while cultured in a Whitley H37 Hypoxystation set at 1% oxygen (described in Chapter 2.5) vs in a 21% oxygen incubator. NET formation was assessed by 1) measuring cfDNA release in the supernatant by fluorometry and 2) visualising SYTOX™ green stained NETs by microscopy. Neutrophils were isolated from young healthy controls for these experiments. Fluorometric and microscopic assays both demonstrated that 25 nM PMA increased NET formation in 1% and 21% oxygen tensions. The former method measured equivalent cfDNA levels in the supernatant of neutrophils stimulated with PMA in either 21% or 1% oxygen ($p > 0.05$, by paired T-test, $n = 4$, data not shown). However, this method was significantly underpowered (post-hoc power = 6%, $n = 128$ required to reach 80% statistical power, $\alpha = 0.05$).

In contrast, semi-quantification of the microscopy images showed that total NET area relative to cell number was significantly less following 4 hours PMA stimulation in 1% oxygen (median NET area in 21% oxygen = 582.9 (IQR 226.2 – 1061) vs 21% oxygen = 1341 (IQR 1205 – 1856); $p = 0.039$, $n = 8$, post-hoc power = 73.6%, Figure 6.26A). Representative microscopy images following application of a 'percentile threshold' using Image J demonstrating NET area (white colour) are displayed in Figures 6.26B and 6.26C. One % oxygen atmosphere did not increase NET area in the absence of a stimulus (RPMI only).

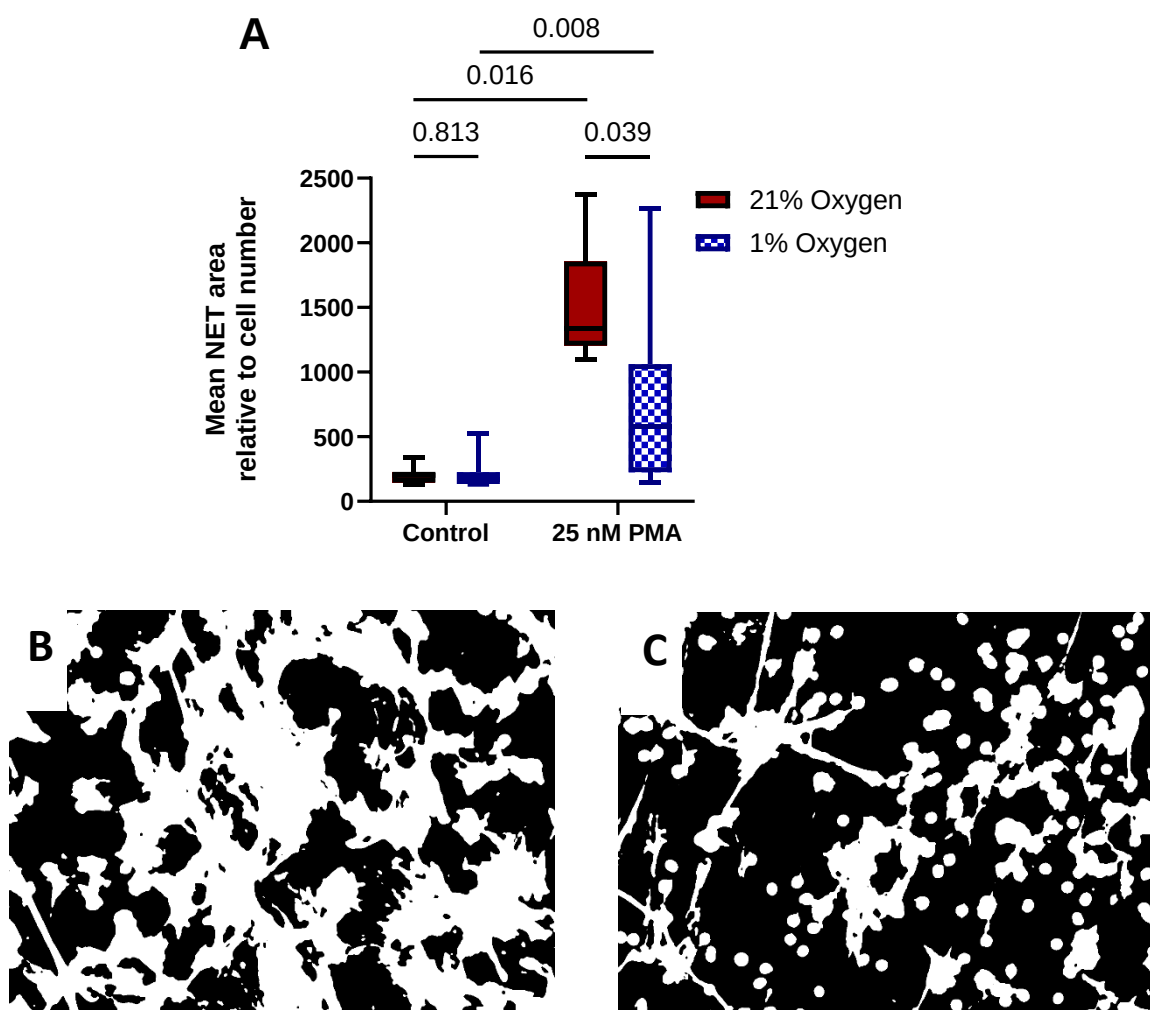


Figure 6.26: Effect of low oxygen environment on NET formation.

Neutrophils isolated from healthy donors ($n = 8$) were cultured *ex vivo* on a glass cover slip in RPMI (control) or 25 nM PMA for 4 hours and then stained with SYTOX™ Green. Cells were imaged as described in Chapter 2.8.2.2.

(A) displays the mean NET area relative to cell number for each condition. Box and whisker plots (Tukey) are displayed. Individual p-values for each comparison are shown. Wilcoxon tests were performed.

(B) and (C) are representative microscopy images of neutrophils exposed to 25 nM PMA in 21% and 1% oxygen, respectively. Neutrophils were isolated from a single healthy donor. Images are displayed with a percentile threshold applied (white regions represent areas of NET formation). Mean NET area relative to cell number for these examples are (B) = 971.5 and (C) = 377.4.

6.6.7. Measurement of NET formation in the circulation and BALF of IPF participants.

6.6.7.1. Differential cell count in the BALF.

Differential immune cell counts in BALF samples derived from 4 control and 5 IPF participants are described in Table 6.3. Mean percentage of neutrophils, alveolar macrophages and lymphocytes in control BALF were 3.7% (SD 2.8), 81.4% (SD 18.5) and 15% (SD 18.0), respectively. Mean percentage of neutrophils, alveolar macrophages and lymphocytes in IPF BALF were 15.2% (SD 17.0), 75.1% (SD 15.2) and 9.7% (SD 3.9), respectively. There was no statistical difference between neutrophil percentage in control and IPF BALF ($p = 0.224$, unpaired T-test, $n = 4/5$). Representative microscopy images of BALF samples are displayed in Figure 6.27.

Cohort, age, sex	FVC % Pred	Gas Trans- fer % Pred	Yield (mL)	Percentage of Immune Cells			Total Cell Count (Millions)
				Neutrophils	Alveolar Macroph -ages	Lympho- cytes	
Control, 70M	-	-	70	0.9	96.6	2.5	6.0
Control, 67F	-	-	50	3.0	92.7	4.2	3.0
Control, 76F	-	-	17.5	3.2	55.6	41.3	1.0
Control, 61M	-	-	42	7.5	80.5	12.0	7.2
IPF, 71M	87	50	75	0.0	89.9	10.1	3.5
IPF, 73M	96	72	37.5	15.5	75.0	9.5	1.7
IPF, 81M	89	54	54	43.1	52.3	4.6	1.2
IPF, 73M	85	96	57	3.4	87.8	8.8	1.5
IPF, 76M	86	67	35	14.1	70.4	15.5	22.0

Table 6.3: Differential immune cell count in BALF.

Characteristics (age (years), sex and lung function) of control and IPF participants who provided BALF samples are described. Total BALF volume retrieved (yield in mL), differential immune cell count and total cell count are also outlined.

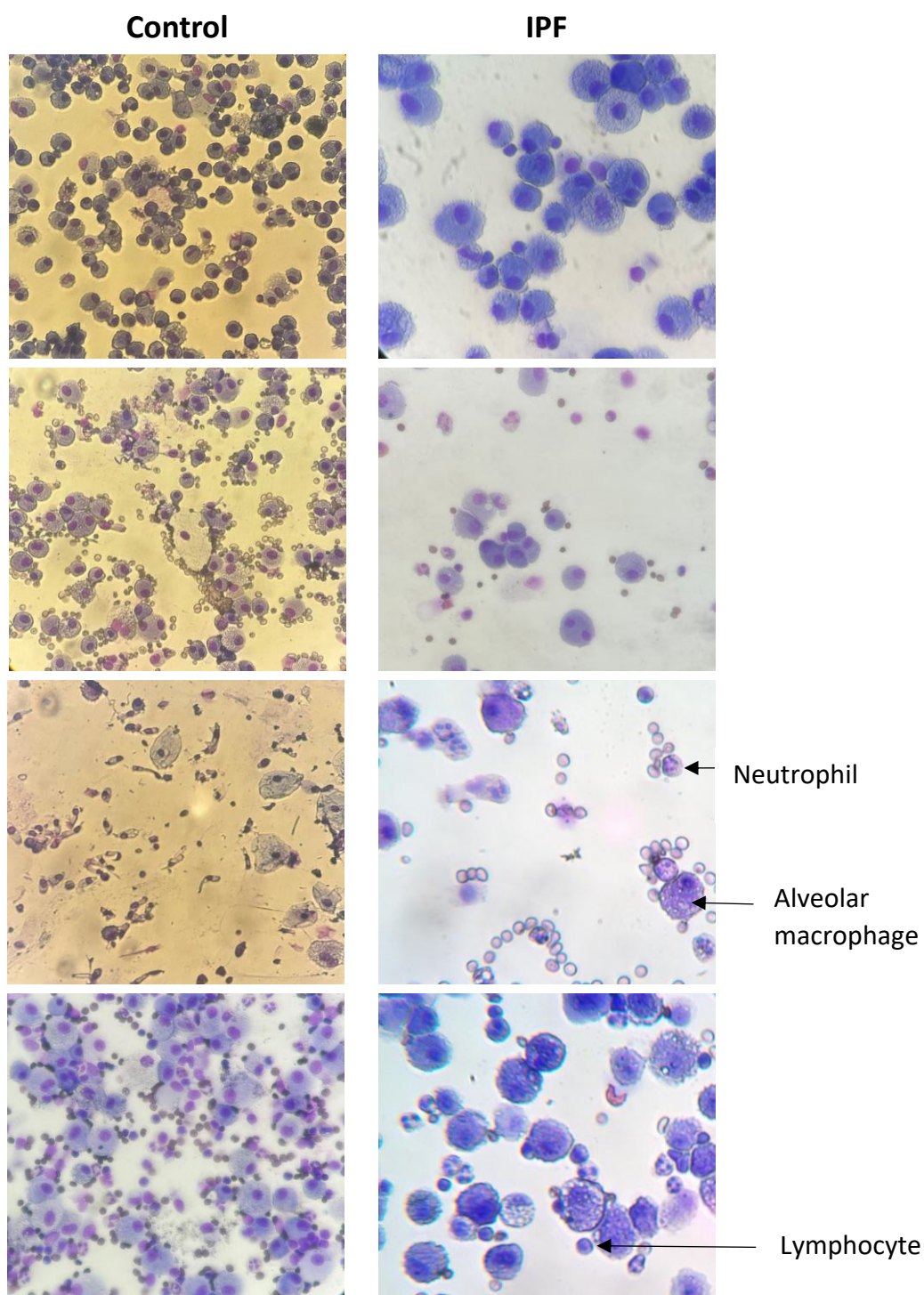


Figure 6.27: Representative microscopy images of BALF samples.

BALF collected from control and IPF participants of the HYBRID study. BALF was transferred to a microscope slide via cytospin and stained using REASTAIN QUICK-DIFF KIT. Images taken using a light microscope at 20x magnification.

6.6.7.2. Protein concentration in the BALF.

The protein concentration in each BALF sample was determined prior to measuring any markers of NET formation (Figure 6.28). Protein concentration was equivalent between IPF and control samples (mean control = 200.3 (SD 101.5) vs IPF = 141.2 (SD 70.6); $p > 0.05$, unpaired T-test, $n = 4/5$). The BALF samples were then normalised by protein concentration using sterile saline prior to measuring cfDNA. This is one method by which to account for the variable dilution effect that takes place on instillation of the sterile saline into the bronchoalveolar spaces when performing the lavage procedure.

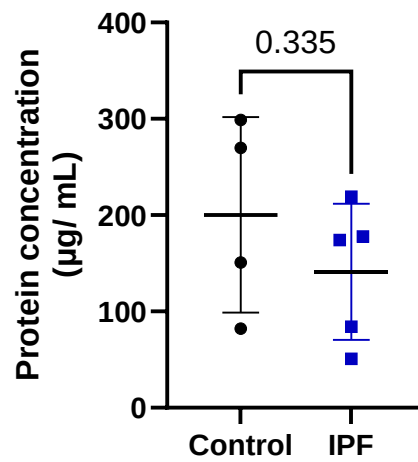


Figure 6.28: Protein content of control and IPF BALF samples.

The protein content of 4 control and 5 IPF BALF samples was determined using the Pierce™ BCA Protein Assay Kit. Dot/square per participant. Data bars are mean and standard deviation. Unpaired t-test performed. P-value compared to control is shown above the data points.

6.6.7.3. CfDNA is higher in IPF BALF than control BALF.

The cfDNA concentration in the BALF samples, with and without normalisation of protein content, was determined by fluorometric analysis of SYTOX™ green stained cell free DNA. The cfDNA content was not significantly different between the control and IPF BALF prior to normalisation of the protein content ($p > 0.05$, unpaired T-test, $n=4/5$, Figure 6.29A). However, upon normalisation of the protein content to the lowest concentration of protein amongst all the samples ($50.6 \mu\text{g/ mL}$), this led to no cfDNA being measured within the control BALF, as compared to a mean 0.56 ng/ mL (SD 0.27) in the IPF BALF ($p = 0.005$, unpaired T-test, $n=4/5$, Figure 6.29B). Therefore, greater cfDNA was noted in the IPF BALF samples.

There was no association observed between cfDNA concentration and neutrophil percentage in the BALF ($r = 0.558$, $p = 0.157$ by non-parametric spearman rank correlation, $n = 9$, Figure 6.29C).

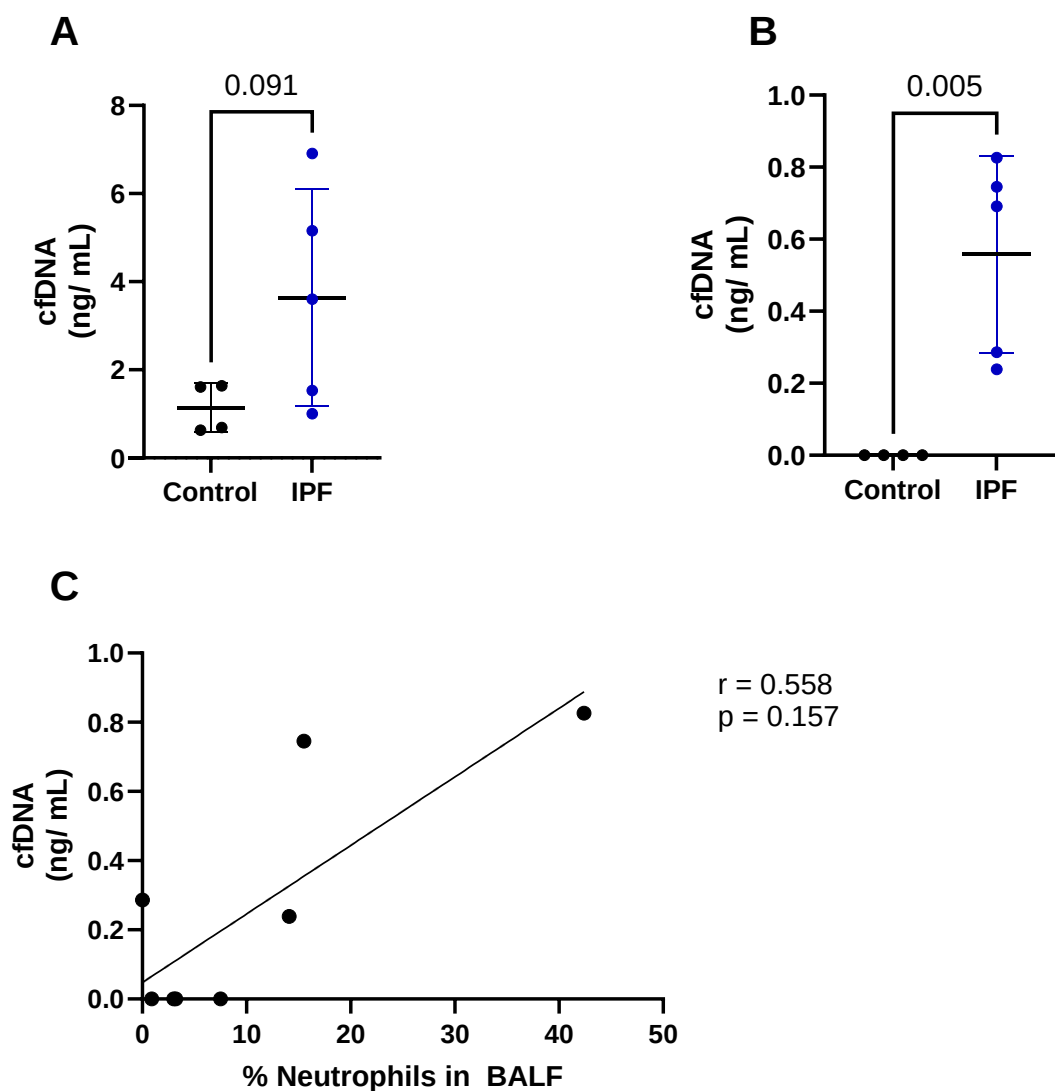


Figure 6.29: Cell free DNA concentrations in BALF derived from control and IPF participants.

Cell free DNA (cfDNA) concentration was measured in the 4 control and 5 IPF BALF samples.

(A) displays cfDNA concentration of BALF that has not been normalised to protein content.

(B) displays cfDNA concentration of BALF that has equal protein concentration.

Dot/square per participant. Data bars are mean and standard deviation. Unpaired t-tests performed. P-values compared to control are shown above the data points.

(C) displays a scatter plot comparing the percentage (%) of neutrophils and cfDNA (adjusted by protein concentration) in the BALF derived from control and IPF participants ($r = 0.558$, $p = 0.157$ by non-parametric spearman rank correlation, $n = 9$).

6.6.7.4. CfDNA concentration in the plasma of IPF and control participants.

The cfDNA concentration in the double spun plasma samples in the HYBRID study was determined by fluorometric analysis of SYTOX™ green stained cell free DNA. The cfDNA concentration was not different between the control and IPF plasma samples (mean control = 7.3 ng/ mL (SD 0.9) vs IPF = 7.6 ng/ mL (SD 1.7); $p > 0.05$, unpaired T-test, $n=16/ 25$, Figure 6.30).

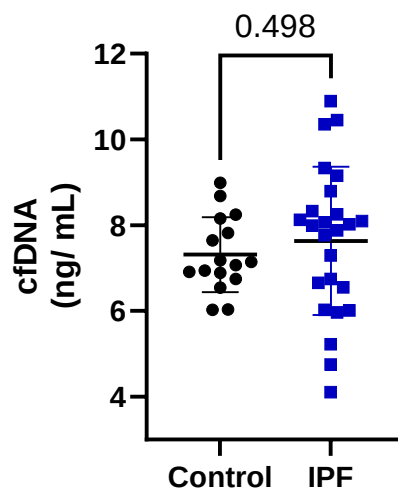


Figure 6.30: Cell free DNA concentrations in the plasma of control and IPF participants.

Cell free DNA (cfDNA) concentration was measured in 16 control and 25 IPF plasma samples.

Dot/square per participant. Data bars are mean and standard deviation. Unpaired t-test performed. P-values compared to control are shown above the data points.

6.7. Discussion

This chapter aimed to assess if circulating neutrophils derived from IPF patients display a primed phenotype.

6.7.1. IPF neutrophils do not appear primed or activated.

This study did not identify any features of priming or activation of IPF donor neutrophils taken from the periphery, following examination of cell surface markers, neutrophil movement or NET formation. Cell surface adhesion and activation markers (CD11b, CD11c, CD62L and CD66b) were equivalent between cohorts. Chemotaxis and chemokinesis responses to the classical chemoattractants, IL-8 and fMLP, were maintained in IPF derived neutrophils. Furthermore, cfDNA release, a marker of NET formation, was comparable between IPF and control donor neutrophils in response to the cytokines, IL-8, IL-1 β and TGF β 1. Conversely, IPF derived neutrophils released less cfDNA than control neutrophils in response to the potent activator, PMA (discussed in Chapter 6.7.6).

6.7.2. IPF – a state of emergency granulopoiesis?

Neutrophil counts were greater in the circulation of IPF patients than control participants, which is in keeping with previous studies [139,360,400]. However, all neutrophil counts measured were below the upper limit of the normal range; $7.5 \times 10^9/L$. Counts above this limit have been associated with lung function decline/fibrosis progression [140,141]. The sample size here was too small to assess the role of neutrophil count as a biomarker in IPF, although this early finding of a statistical difference between these matched cohorts is promising and supports further prospective investigation.

NLR has been explored as an alternative biomarker to neutrophil count in ILDs as it takes into account the relative contribution of neutrophil versus lymphocyte populations. In this study, NLR was comparable between the two cohorts, which opposes the findings of a recent meta-analysis that noted NLR was higher in patients with ILD (included a diverse range of ILDs) as compared to controls [401]. However, only a few of these studies included IPF patients, and only one was prospective in nature [139]. Although this single prospective study by Zinellu *et al* did match age, sex and smoking status of both cohorts, healthy participants did not have any comorbidities, unlike those in the IPF group, thereby introducing confounding [139]. Lymphocyte and monocyte counts were also higher in IPF patients as compared to control participants in the HYBRID study. This accounts for the absence in difference in NLR between the cohorts. The elevated monocyte count in the IPF group was unsurprising, as increased monocyte count has been associated with IPF disease progression and mortality [402,403].

Cell surface marker analysis identified that amongst the expanded neutrophil population was a reduction in mature CD10 positive neutrophils. This suggests emergency granulopoiesis amongst patients with mild to moderate IPF. However, what is driving this is unknown.

6.7.3. Increased CXCR4 expression in IPF neutrophils

There was differential expression of two other cell surface markers between IPF and control donor neutrophils: CXCR4 and PD-L1. The MFI of each of these markers was overall higher in IPF neutrophils, but the percentage of cells was equivalent between groups. Figure 6.31 summarises the potential neutrophil populations identified in this study.

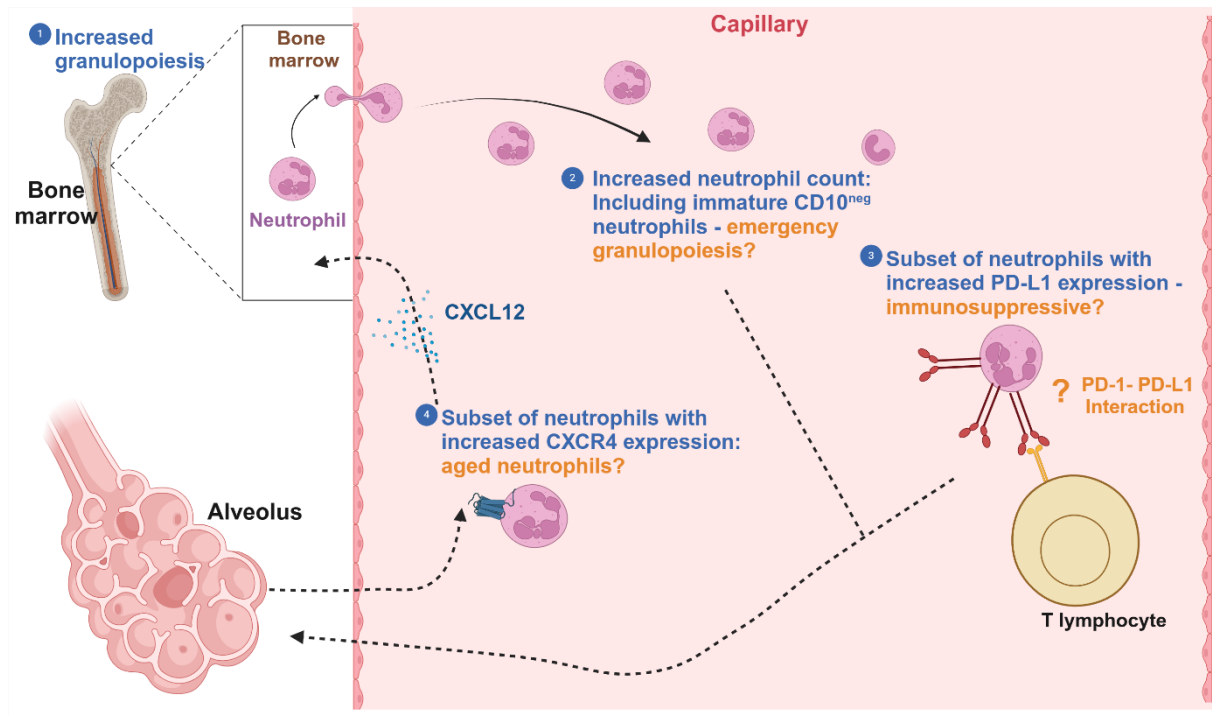


Figure 6.31: Proposed neutrophil populations identified in the circulation of IPF patients.

(1) Increased neutrophil count was noted in IPF participants suggesting increased granulopoiesis. (2) There appeared to be a lower percentage of mature CD10 positive neutrophils in the IPF cohort, which alongside the increased neutrophil count is suggestive of a state of emergency granulopoiesis. (3) PD-L1 expression appeared higher in a subset of IPF derived neutrophils. The role of this co-inhibitory molecule on T lymphocyte activity via PD-1–PD-L1 interactions needs exploration in this context. (4) CXCR4 expression appeared higher in a subset of IPF derived neutrophils. CXCR4 is a marker of neutrophil ageing/senescence so this may represent neutrophils that are trafficking out of the fibrotic lung and homing back to the bone marrow. However, expansion of these study cohorts is required to increase the statistical power of these provisional findings. *Figure created using Biorender.com.*

CXCR4 expression on the neutrophil surface is thought to be a marker of neutrophil ageing/senescence, and on binding of its ligand, CXCL12, homes neutrophils back to the bone marrow [265]. Aged CXCR4 high neutrophils commonly also express low levels of CD62L [404]. A limitation of this study's immunophenotyping assay was that CD62L was in a separate antibody panel to CXCR4, prohibiting the combined assessment of these two markers (I.E. identification of CXCR4 high, CD62L low neutrophils). However, CD62L expression, unlike CXCR4, did not appear different between the two cohorts. The finding of increased CXCR4 expression on the surface of IPF neutrophils is in keeping with a previous animal model of lung fibrosis which showed increased aged (CXCR4 high and CD62L low) neutrophils in the circulation of bleomycin exposed mice [267]. Furthermore, IPF patients appear to have increased circulating levels of its ligand, CXCL12 [405]. Therefore, increased CXCR4 expression appears plausible.

Combined assessment of CXCR4 and CD62L expression in both control and IPF neutrophils would help to confirm if there is an increase in aged neutrophils in the circulation of IPF patients. Flow cytometry and immunohistochemical analysis of CXCR4 expression on neutrophils within BALF and fibrotic lung tissue, respectively, would further characterise these aged/senescent neutrophils within this disease.

6.7.4. PD-L1 expression on IPF derived neutrophils.

Expression of the co-inhibitory molecule, PD-L1, was noted in 6% of control and IPF derived neutrophils in this study. However, MFI of PD-L1 was significantly higher in IPF derived neutrophils. A recent abstract that was presented at the 2024 Winter British Thoracic Society Conference, reported that circulating neutrophils derived from younger IPF patients had a transcriptional signature in keeping with a myeloid-derived suppressor cell (MDSC) phenotype [406]. In the cancer field, PD-L1 expression is increased on MDSCs and on binding to the receptor PD-1 on T lymphocytes, induces T lymphocyte anergy [407,408]. Interestingly, hypoxia has been shown to drive this PD-L1 upregulation in MDSCs via HIF-1 α dependent pathways [408]. Furthermore, the effect that TGF β 1 may have on the MDSC phenotype is worth consideration as TGF β 1 has been shown to transform neutrophils into a potentially immunosuppressive, protumourigenic neutrophil phenotype [409]. The role of T lymphocytes in IPF is still emerging and complex. However, the increased expression of PD-L1 on IPF derived neutrophils seen here may be an important finding as PD-1 expression is increased on lymphocytes present in IPF lung tissue and PD-L1 blockade attenuates bleomycin induced fibrosis in animal models [410].

6.7.5. IPF neutrophils move less accurately in response to TGF β 1.

Neutrophil movement in response to the classical chemoattractants, IL-8 and fMLP, was equivalent between IPF and control donor neutrophils. As seen in Chapter 4, TGF β 1 drove neutrophil chemokinesis, but not chemotaxis, in control donor neutrophils. However, this was not the case for IPF neutrophils. Although the mean speed and distance travelled were not statistically higher on TGF β 1 exposure in IPF donor neutrophils, mean values were higher following TGF β 1 exposure and current data is underpowered (post-hoc power calculation = 55%, n = 26 needed for 80% power, alpha 0.05). Furthermore, chemokinetic responses to TGF β 1 were not statistically different between control and IPF donor neutrophils. This suggests that TGF β 1 may increase neutrophil chemokinesis in IPF donor neutrophils, but further experimental replicates are required to confirm this.

Although neutrophil chemokinesis and chemotaxis responses (namely velocity and chemotactic index) were equivalent between control and IPF donor neutrophils. IPF derived neutrophils moved less directly in response to TGF β 1 as compared to control neutrophils. Directness was calculated by the equation: displacement divided by cumulative distance. I.E. directness is a measure of the straightness of the neutrophil path where one is a straight line. Reduced accuracy of neutrophil movement has been seen in the context of other chronic lung diseases, such as COPD [411]. It is unclear the importance of reduced movement directness in response to TGF β 1, particularly as TGF β 1 appears to drive direction independent movement (chemokinesis) rather than chemotaxis. Perhaps IPF derived neutrophils on arrival into the fibrotic lung are primed by TGF β 1, which results in increased neutrophil chemokinesis that is less direct/more erratic in nature. A more wandering journey through the lung could cause unintended tissue damage, for example due to the actions of proteinases *en route*, as

described in COPD [411–413]. Live cell tracking of TGFβ1 exposed IPF neutrophils in precision cut lung slices could give insight into this. The reason for the altered responsiveness to TGFβ1 in IPF donor neutrophils also needs to be explored. Stored neutrophil RNA and protein lysates from the HYBRID study could be used to assess for the presence of polymorphisms in the TGFβ receptor (introduced in Chapter 4) and expression of key signalling molecules (such as PI3K [275]).

6.7.6. IPF neutrophils show a reduced NETosis response to PMA

PMA was a chosen stimulus in this study as it provided a method to assess maximal NET production. Two potential reasons for the differential response in solely PMA induced NET formation could include: a) a disruption in neutrophil signalling/machinery that is more important in PMA mediated activation, than the other stimuli examined or b) a reduced maximal NET response in IPF neutrophils. The key difference between PMA and the other NET stimuli examined in this thesis is that PMA induces suicidal NETosis [358], whereas the other stimuli most likely (although still poorly understood) drive non-lytic NETosis (as shown in Chapter 4 with TGFβ1). Furthermore, there have been suggestions that NADPH oxidase is more important in suicidal NETosis than other forms, although this may not be the case for IL-8 [236,346].

A first step to give insight into why IPF donor neutrophils released less cfDNA in response to PMA was to assess if these cells were more resistant to cell death after prolonged culture *ex vivo*. This was considered as alterations in the local environment, such as reduced oxygen availability, can prolong neutrophil survival [43]. Approximately 70% of IPF neutrophils showed

signs of either apoptosis or necrosis after 24 hours of culture *ex vivo*, which was similar to Walmsley *et al*'s findings that around 60% of neutrophils isolated from healthy human donors were apoptotic after 20 hours in culture [43]. Unfortunately, too few experimental replicates were performed on control donor neutrophils to compare between groups statistically.

As described in Chapter 1.8.5.4, the surface multi-protein NADPH oxidase converts oxygen into ROS by a process called respiratory burst [414]. NADPH oxidase is critical for PMA mediated NETosis [112,358]. This was supported by the finding here that the area of PMA induced NET formation was reduced when healthy donor neutrophils were in 1% oxygen. In the HYBRID study samples, resting cytoplasmic levels of ROS appeared equivalent between IPF and control donor neutrophils. However, this does not give insight into the capacity of oxidative burst upon stimulation. This could be explored in the future by measuring oxygen consumption rate in response to PMA using the Agilent Seahorse XF Analyser as described by Grudzinska and Jasper *et al* [353] or performing chemiluminescence assays as performed in Chapter 4 of this thesis.

6.7.7. Cell free DNA levels are elevated in IPF BALF

Elevated cfDNA was noted in IPF BALF compared to control BALF. This is in keeping with a study by Khawaja *et al* that noted greater cfDNA and myeloperoxidase-citrullinated histone H3 complexes in BALF derived from ILD patients [19]. Furthermore, a recent study noted that nuclear DNA content in BALF was positively associated with both BALF neutrophil content (%) and lung function impairment in IPF patients [415]. This together suggests that there is increased NET formation in the alveolar space of patients with IPF and questions the clinical relevance of any deficiency in PMA mediated NETosis described previously. It is important that

the causes of this increased NET formation in the fibrotic lung and how this impacts disease development/progression are determined.

Potential reasons for increased NETs in the BALF of IPF patients may include: (a) IPF derived neutrophils have a greater propensity for NET formation, (b) additional cytokines/environmental factors that drive NET formation and/or (c) increased neutrophil infiltration within the alveolar space of IPF patients.

Work presented here points away from (a) as peripheral neutrophils from IPF donors did not release more cfDNA in response to IL-8, IL-1 β or TGF β 1 as compared to control derived neutrophils. However, this is not an exhaustive list of NET stimuli. Furthermore, the numerous steps (for example integrin/selectin binding, intracellular signalling cascades, changes in morphology) that occur during neutrophil migration into the fibrotic lung and subsequent alveolar space likely alters neutrophil responses. Functional assessment of neutrophils derived from IPF BALF is warranted to determine this.

Numerous cytokines are increased within the BALF of IPF patients, which are known NET stimuli, such as IL-8 and IL-1 β [232,233]. Elevated expression of TGF β 1 in IPF tissue [416] may play an important role in neutrophil priming (described in Chapter 4) and NET formation, although further experimental work is required to confirm the latter. Other factors within the fibrotic microenvironment may drive NET formation. Khawaja *et al* proposed that hypoxia (1% oxygen) amplifies NET formation, in the presence or absence of PMA [19]. However, work in this thesis observed that 1% oxygen may reduce PMA induced NET formation, which is unsurprising considering the importance of the NADPH oxidase system in this process [100]. However, this finding was not consistent between the two methods of NETs measurements

(fluorometry and microscopy), which corresponds with the current literature where there is no consensus on the effects of oxygen on this critical effector function [19,113,114]. Further work is needed to tease this out, especially when considering the results described in Chapter 5, which identified intermittent hypoxia was prominent in IPF patients with mild to moderate disease severity.

Neutrophil percentage was not significantly higher in the BALF of IPF patients here as compared to control participants, and was not associated with cfDNA concentration in the BALF. However, this work is underpowered; a common feature of BALF research due to the invasiveness of the bronchoscopy procedure. As discussed in detail in Chapter 1, the previous literature suggests that there is an increased neutrophil count in the BALF of IPF patients [417].

In summary, it is most likely that a combination of factors contributes to the elevated NET content seen in IPF patient BALF, which includes a pro-NETotic milieu alongside an increase in neutrophil number within the alveolar space.

6.7.8. Limitations

The heterogeneity between biological samples and difficulties associated with neutrophil experimental work that were discussed in Chapter 4.7.6 are also relevant here. Appropriately powering a study of this nature is difficult in light of these issues, as well as this being the first time that circulating neutrophils have been examined in IPF. Post-hoc power calculations of PMA-cfDNA data identified a 66.4% power ($\alpha = 0.05$) based on 11 control and 18 IPF samples. Therefore, some of the results here are underpowered (if aiming for a power of $\geq 80\%$). It is therefore important that this is taken into account when reviewing these results to

avoid over-interpretation. Study recruitment is ongoing to increase study power, with a desired final sample size of 26 participants within each cohort.

Another potential issue related to using the Percoll density gradient centrifugation method in this study, is that isolation of neutrophils solely from the neutrophil layer (described in Figure 2.1) may miss low density neutrophil populations that sit in the classical peripheral blood mononuclear cell layer. These lower density neutrophils may represent activated or immature banded neutrophils [418] and therefore may be under identified. Alternative methods would need to be considered to overcome this, such as whole blood staining/cell sorting technology.

The Insall Chamber Assay is a useful technique due to being able to track neutrophil movement in real-time by microscopy. However, this requirement of a microscope is a limitation, as only one chemoattractant condition can be assessed at a single time. Chemoattractant conditions in this assay were performed in the same order for all study participants to minimise variation: First: RPMI (control), second: 100 nm IL-8, third: 10 nM fMLP. The final two conditions (fourth and fifth) alternated between 10 ng/ mL TGF β 1 and its vehicle. The reason for rotating the order of TGF β 1 and its vehicle was to allow the paired comparison between these two conditions to continue to assess how control and IPF neutrophils respond to this growth factor. These last two treatment conditions were performed around 45- 60 minutes after the neutrophils were isolated. This time delay may have impacted neutrophil responses to TGF β 1.

Finally, cfDNA is the only marker of NET formation examined here in IPF BALF. However, cfDNA is also released during cell death. It has been suggested that alveolar macrophages derived from IPF patients have impaired efferocytosis capabilities [205]. It is therefore conceivable that this increased cfDNA is related to an imbalance of cell death and clearance. Other measures

of NET formation need to be explored, such as citrullinated histone or myeloperoxidase, to confirm that NETs are increased in IPF BALF.

6.7.9. Future work

6.7.9.1. *Ongoing participant recruitment.*

The priority of future work is to ensure that the work performed here is sufficiently powered. Contemporaneous recruitment of control and IPF participants is ongoing. More detailed characterisation of the potentially expanded CD10 negative immature neutrophil population could take place by examining nuclear morphology. Furthermore, the phenotyping panel could be adjusted to allow contemporaneous assessment of CXCR4 and CD62L expression for more detailed assessment of aged/senescent neutrophils.

6.7.9.2. *To determine the pathophysiological consequences and drivers of neutrophil alterations in IPF.*

The driving factors and pathophysiological consequences of any changes in neutrophil phenotype and function seen in IPF patients need to be determined as this will aid identification of immunomodulatory treatment targets in this disease.

The latter could be explored by assessing if IPF derived neutrophils differentially impact alveolar epithelial cells and/or fibroblasts as compared to control derived neutrophils in co-culture or precision cut lung slice models. Treatment of lung fibroblasts with NE or NETs has been shown previously to promote lung myofibroblast transdifferentiation *in vitro* [166,419]. Saffarzadeh *et al* also showed that NETs can drive alveolar epithelial cell damage [187]. However, it is unknown how neutrophils from IPF patients interact with pulmonary epithelial and mesenchymal cells.

Alveolar injury is a critical early step in the pathophysiology of IPF [420] and is proposed in Chapter 1 to be the driver of neutrophil mobilisation in this disease context. Investigation of the direct and indirect effects of alveolar epithelial injury on the secretion of established drivers of granulopoiesis, such as IL-6 and G-CSF, could be of interest. Both cytokines have been linked with lung fibrosis in animal models and are elevated in IPF tissue or BALF [421–424]. However, what is driving their release and the impact it has on granulopoiesis in this context is unknown. Bronchial epithelial cells have been shown to secrete IL-6 in response to injury, but is unknown if this is true for alveolar epithelial cells [425].

6.7.9.3. To explore phagocytic capacity of IPF neutrophils.

Phagocytosis has not been examined in this study, but is a critical antimicrobial function performed by neutrophils. It is important that the phagocytic capacity of IPF derived neutrophils is examined in the future as infection is a common cause of acute exacerbation in patients, and carries a high risk of morbidity and mortality [147]. Furthermore, phagocytosis is dependent on intracellular machinery that is shared with PMA mediated NET formation, such as the NADPH oxidase system. Therefore, it is possible that phagocytic capacity may also be altered in IPF neutrophils.

6.7.9.4. To characterise neutrophil populations and contents in the IPF lung

As mentioned previously, the phenotype/function of circulating neutrophils does not necessarily equate to neutrophil populations within the fibrotic lung. Work is ongoing to isolate neutrophils from the BALF using negative selection beads so that phenotype and function of these cells can be assessed. More in-depth analysis of neutrophil sub-sets in each compartment (BALF and circulation) using cell sorting technology would allow downstream functional and subset specific ‘omic’ analysis.

Although BALF provides an insight into the bronchoalveolar space in the lungs of IPF patients, it does not tell us the extent or localisation of neutrophil infiltration within the lung interstitium. Chapter 7 outlines the provisional analysis of multiplex immunofluorescence staining of IPF and control lung tissue, which aims to confirm the presence of neutrophils and their contents within the fibrotic lung.

6.7.10. Summary

This is the first study to explore phenotype and function of circulating neutrophils in patients with IPF. Although further participant recruitment is required to improve the statistical power of this study, the early observation of changes in neutrophil number, cell surface markers and functional responses to stimuli, supports the importance to examine the role of this cell type further in IPF. Most importantly, the presence and contribution of neutrophils within the fibrotic lung needs to be characterised.

Chapter 7. SPATIAL CHARACTERISATION OF NEUTROPHILS IN IPF LUNG TISSUE.

7.1. Abstract

The location of neutrophils and their contents in IPF lung tissue remains poorly understood. Previous staining methods are not specific for neutrophils due to the sharing of surface/intracellular markers with other immune cells, as well as neutrophil fragility *ex vivo*. In this chapter, a multiplex immunofluorescence panel is described that can aid characterisation of neutrophils and their contents within IPF and control lung tissue. Provisional descriptive analysis of this tissue staining is outlined. The data demonstrates that neutrophils are resident within the alveolar spaces and interstitium of fibrotic lung tissue, including within fibroblastic foci. Neutrophil elastase is noted close to/within the hyperplastic alveolar epithelium, including near areas of MMP-7 expression. Markers of NETs (citrullinated histones and myeloperoxidase) are present within the alveolar spaces of IPF tissue, which differs from healthy tissue. However, semi-quantitative analysis of these images using automated imaging software is required to more clearly localise neutrophils and their contents in IPF, and assess any relationship to disease severity and progression.

7.2. Introduction.

7.2.1. Usual interstitial pneumonia (UIP)

The histopathological pattern of IPF, UIP, is characterised by spatially and temporally heterogeneous fibrotic changes including reticulation, honeycombing and fibroblastic foci, with reported scarcity of ground glass change and inflammatory infiltrates including neutrophils [149,150]. Therefore, this traditional definition has pointed towards little or no localised role of neutrophils in these diseases. However, neutrophilic features have been reported by others as 'characteristic' of IPF, with neutrophils present in the lung interstitium, especially dense fibrotic areas, and adjacent to areas of honeycombing, alveolar septa and endothelial cells in the pulmonary microvasculature [37,125]. Degranulation marked by the presence of cell free NE, has been noted in IPF lungs but not control tissue [125]. This enzyme together with myeloperoxidase (another neutrophil granule protein), has also been identified within the pulmonary vasculature of IPF lung tissue [19]. In addition there is colocalisation of NET markers, including citrullinated histones, myeloperoxidase and extracellular DNA in fibrotic lung, but not control tissue [19]. These studies challenge the didactic histopathological definition of UIP and suggest neutrophils have the potential to increase inflammation and tissue destruction in these diseases. However, the spatial relationship of neutrophils and their contents with other cell types within the fibrotic microenvironment remains unexplored.

7.2.2. Multiplex immunofluorescence technology

The temporal nature of neutrophil infiltration is unknown and may change with time, which could explain disparities with previous histopathological reports in IPF. Detailed histological examination of lung tissue at variable disease stages is required to clarify the relationship to

disease trajectory. However, limitations in previous methodology for identifying neutrophils *ex vivo* is likely a significant contributing factor to our lack of clarity.

Neutrophils are prone to degranulation as discussed in Chapter 4, which may hinder the microscopic identification of the polymorphonuclear structure of neutrophils with traditional hematoxylin and eosin (H&E) stains. Furthermore, neutrophils share expression of many of its intracellular and cell surface markers with other resident immune cells, especially monocytes (See Table 7.1). Therefore, single stain immunohistochemical techniques provide a lack of confidence/clarity in the identification of neutrophils within tissue. Specificity of markers for neutrophils versus monocytes is summarised in Table 7.1.

Marker	Neutrophils 	Monocytes 
CD16a [426]	Low/absent	Absent
CD16b [426,427]	High [426]	Absent
CD15 [428]	High	Low
CD11b [427,429]	Intermediate	High
CD66b [427]	Intermediate	Absent, except subset of CD66b+ neutrophils identified in cancer [430]
Myeloperoxidase [431]	High	Absent/low Literature unclear – subset identified that has peroxidase activity [432]
Neutrophil Elastase	Positive	Rare

Table 7.1: Neutrophil and monocyte markers.

Neutrophil and monocyte figures created using Biorender.com.

Novel multiplex immunofluorescence platforms have been devised to enable the expression of a variety of markers to be assessed in a single tissue sample and improve spatial characterisation of cell populations in disease states [433]. Lunaphore COMET™ multiplex immunofluorescence technology was adopted in the current study to overcome the previously nonspecific neutrophil localisation. A sixteen marker antibody panel was devised (see Table 7.2) that targeted: 1) a variety of epitopes expressed by neutrophils to improve confidence of their identification and to give insight into different neutrophil populations residing in the fibrotic lung; 2) markers of degranulation and NETs; 3) markers of hypoxia to assess the relationship between potential hypoxia and neutrophils; 4) other cell types (mesenchymal, epithelial, vascular and macrophage markers) to assist in the outline of lung tissue architecture.

Here I will describe the results of the descriptive analysis of neutrophil related markers of the multiplex immunofluorescence panel within IPF and control lung tissue.

Target	Description
Neutrophil related markers	
CD15	Highly expressed by neutrophils and eosinophils [434,435].
CD11b	Integrin alpha M subunit expressed by neutrophils, eosinophils, monocytes and alveolar macrophages [427,429,435].
CD66b	Glycoprotein expressed by neutrophils and eosinophils [427,436].
Myeloperoxidase	Present within primary granules of neutrophils, released on degranulation and NET formation [431].
Neutrophil Elastase	Present within primary granules of neutrophils and released on degranulation and NET formation [103].
Citrullinated Histone H3 (cH3)	Generated by post-translational conversion of peptidylarginine to citrulline on histone H3 N terminus by PAD4. Critical to chromatic decondensation – a marker of NET formation [437].
CXCR4	Marker of aged/senescent neutrophils [438]. Also expressed by epithelial cells in IPF lung tissue particularly in the airways of hyperplastic epithelium and honeycomb cysts [405].
Markers of other key cell types	
Cytokeratin	Epithelial marker [439]
Vimentin	Cytoskeletal protein – expressed by fibroblasts, myofibroblasts, macrophages [440]. Aid identification of pulmonary interstitium.
α SMA	Within vascular walls and myofibroblasts [441]. Aids identification of vasculature and fibroblastic foci.
CD163	Macrophage marker, expression elevated in IPF tissue and associated with mortality [442].
CD206	Macrophage marker – upregulated in lung fibrosis [443].
Hypoxia related markers	
Carbonic anhydrase 9	Transmembrane enzyme that metabolises carbon dioxide to carbonic acid [444]. Upregulated in the presence of hypoxia [445]. Expression identified in cancers e.g. non-small cell lung cancer [446]. Expression in IPF unknown.
HIF1 α	Expression stabilised in hypoxia. Expression observed in fibrotic areas (less so in fibroblastic foci), neutrophils and endothelial cells within IPF lung tissue [19,447].
Proteinases	
MMP-7	Expression noted in bronchiolar epithelial cells, alveolar epithelial cells, macrophages (strong), fibroblasts and neutrophils (mild) in IPF tissue [448].
MMP-9	Expression noted in bronchiolar epithelial cells, alveolar epithelial cells, macrophages (strong) and neutrophils (mild) in IPF tissue [448].

Table 7.2: Summary of markers examined within the multiplex immunofluorescence panel.

7.3. Hypothesis

Neutrophils and their contents are resident within the alveolar spaces and fibrotic interstitium of IPF lung tissue.

7.4. Aims

1. To devise an antibody panel to use for multiplex immunofluorescence staining that enables spatial characterisation of neutrophils within IPF lung tissue.
2. To localise neutrophils within early and advanced IPF lung tissue.
3. To localise neutrophil derived proteinases, enzymes and NET markers within IPF lung tissue.

7.5. Materials and methods linked to Chapter 7

7.5.1. Ethical approval

All tissue blocks in this chapter were used for research purposes only after each participant had provided written informed consent to enroll in one of the following approved studies:

- 1) Use of tissue from lung resections to investigate the molecular and functional mechanisms of human lung disease (REC: 07/MRE08/42) or,
- 2) Use of Tissue from Lung Resections to Investigate the impact of Smoking and Vaping on Cellular Function in Human Lung Disease (REC: 17/WM/0272).

All tissue samples provided had been surgically removed as part of routine clinical care. No extra tissue was removed for research purposes. The participant characteristics were retrieved from retrospective review of the electronic patient records.

7.5.2. Acknowledgements

Thanks to the Human Biomaterials Resource Centre (Birmingham), University of Hospitals Birmingham NHS Foundation Trust histopathology teams, and Celine Chen (Research Technician in the Department of Inflammation and Ageing, University of Birmingham) for undertaking the tissue block retrieval, processing and slicing. In addition, thanks to the team at Birmingham Tissue Analytics for performing the Lunaphore COMET™ multiplex immunofluorescence and H&E tissue staining.

7.5.3. IPF tissue block retrieval and slicing

Formalin-fixed and paraffin-embedded (FFPE) IPF tissue blocks were derived from lung tissue removed for diagnostic purposes by video assisted thoracoscopic surgery (VATS) or during lung transplantation (explants). Tissue blocks were sliced (4 μ m thickness) by the Human Biomaterial Resource Centre or Heartlands Hospital Histopathology team and stored at 4°C until processed.

7.5.4. Control tissue retrieval and slicing

Control tissue was retrieved from patients undergoing lobectomy for the surgical removal of lung cancer and macroscopically clear of the tumour margin. The retrieved tissue was immediately washed in PBS, and fixed with 10% formalin solution (*Merck, USA: HT501640*) for 48 hours at room temperature. An automatic benchtop tissue processor (*Leica TP1020, USA*) subsequently processed the tissue into FFPE blocks by serial immersion in: formalin, increasing concentrations of ethanol (70%, 90% and 100%), Histo-clear II (*National Diagnostics, USA: NAT1334D2*), Surgipath Paraplast (*Leica Biosystems, USA: 39601006*). The tissue was then placed into a paraffin-filled cassette (*HistoCore Arcadia Embedding Center, Leica Biosystems, USA*) and left to harden.

FFPE blocks were serially sectioned into 4 μ m thick slices using low-profile disposable blades (*Leica biosystems, Germany: DB80 LX*) with a manual rotary microtome (*HistoCore BIOCUT, Leica Biosystems, UK*). The slices were floated onto a water bath at 40°C, lifted onto a X-TRA adhesive microscope slide (*Leica biosystems, Germany: 3800200AE*) and stored at 4°C until analysed.

7.5.5. Lunaphore COMETTM multiplex immunofluorescence.

7.5.5.1. Slide preparation

Tissue slices were baked in an oven (*HerathermTM incubator, Thermo ScientificTM*) at 60°C for 1 hour and treated twice for 10 minutes with xylene (*VWR Chemicals USA: 28975.325*). Tissue slices were then rehydrated with decreasing concentrations of ethanol (100%, 70%, 50%) followed by distilled water (2 minutes each). Slides were then added to a pretreatment module (*EprediaTM PT module*) for 1 hour at 102°C containing Dewax and heat induced epitope retrieval (HIER) buffer H (*EprediaTM, USA: TA-999-DHBH*) and bathed in multistaining buffer (*Lunaphore Technologies, Switzerland: BU06*) prior to loading into the COMETTM (*Lunaphore Technologies, Switzerland: version 1.1.0.0, Software version 1.2.2.0*). The imaging area for each slice was 12.5 mm x 12.5 mm.

7.5.5.2. Staining cycles and antibodies.

All primary antibodies were diluted in multistaining buffer supplemented with 10% BSA-CTM (*working concentration: 1%, Aurion, The Netherlands: 900.022*) and applied to the tissue slices for 8 minutes to allow localisation. All secondary antibodies were added to Intercept[®] (TBS) blocking buffer (*LI-COR Biosciences, USA: 927-60001*) and applied to the tissue slices for 4 minutes. The temperature in the COMETTM was set to 37 °C throughout the procedure. Table 7.3 describes all antibodies used in order of application.

Order	Target	Primary Antibody	Primary Antibody Dilution	Secondary Antibody
1	Carbonic anhydrase 9	NB100-417SS (<i>Novus-Biologicals, USA</i>)	1: 25	Goat Anti-Rabbit IgG ^{\$}
2	Neutrophil Elastase	ab219585 (<i>Abcam, UK</i>)	1: 250	Goat Anti-Rabbit IgG ^{\$}
3	CXCR4	ab181020 (<i>Abcam, UK</i>)	1: 50	Goat Anti-Rabbit IgG ^{\$}
4	CD66b	NB100-77808SS (<i>Novus-Biologicals, USA</i>)	1: 100	Goat anti-mouse IgG (H+L) highly cross-adsorbed Alexa Fluor Plus 647 (<i>Invitrogen, USA: A32728</i>)
5	HIF1 α	48085 (<i>Cell Signalling Technology, USA</i>)	1: 200	Goat Anti-Rabbit IgG ^{\$}
6	Myeloperoxidase	ab9535 (<i>Abcam, UK</i>)	1: 100	Goat Anti-Rabbit IgG ^{\$}
7	MMP-7	ab207299 (<i>Abcam, UK</i>)	1: 2000	Goat Anti-Rabbit IgG ^{\$}
8	MMP-9	ab283575 (<i>Abcam, UK</i>)	1: 5000	Goat Anti-Rabbit IgG ^{\$}
9	CD163	93498T (<i>Cell Signalling Technology, USA</i>)	1: 200	Goat Anti-Rabbit IgG ^{\$}
10	CD11b	ab133357 (<i>Abcam, UK</i>)	1: 400	Goat Anti-Rabbit IgG ^{\$}
11	CD206	91992T (<i>Cell Signalling Technology, USA</i>)	1: 400	Goat Anti-Rabbit IgG ^{\$}
12	α SMA	19245 (<i>Cell Signalling Technology, USA</i>)	1: 200	Goat Anti-Rabbit IgG ^{\$}
13	Vimentin	sc-32322 (<i>Santa Cruz Biotechnology, Inc., USA</i>)	1: 5000	Goat anti-mouse IgG (H+L) highly cross-adsorbed Alexa Fluor Plus 647 (<i>Invitrogen, USA: A32728</i>)
14	Cytokeratin	M351501-2 (<i>Agilent, USA</i>)	1: 200	Goat anti-mouse IgG (H+L) highly cross-adsorbed Alexa Fluor Plus 647 (<i>Invitrogen, USA: A32728</i>)
15	CD15 IgM	4744S (<i>Cell Signalling Technology, USA</i>)	1: 5000	Goat anti-mouse IgM, Alexa Fluor™ 647 (<i>Invitrogen, USA: A21238</i>)
16	Citrullinated Histone H3 (cH3)	97272 (<i>Cell Signalling Technology, USA</i>)	1: 250	Goat Anti-Rabbit IgG ^{\$}

Table 7.3: Primary and secondary antibodies used in the multiplex immunofluorescence panel.

All secondary antibodies were applied at a 1: 400 dilution. ^{\$}Goat Anti-Rabbit IgG^{\$} (H+L) highly cross-adsorbed Alex Fluor Plus 647 (*Invitrogen, USA: A32733*).

The following processes took place after each staining cycle: 1) counterstained with 4',6-diamidino-2-phenylindole (DAPI) (*working dilution 1: 500, Thermo Scientific, USA: 62248*) for 2 minutes and 2) imaged in imaging buffer (*Lunaphore Technologies, Switzerland: BU09*), washed in 3) multi-staining buffer, then 4) elution buffer (*2 minutes, Lunaphore Technologies, Switzerland: BU07*) and finally 5) quenching buffer (30 seconds to quench autofluorescence, *Lunaphore Technologies, Switzerland: BU08*).

7.5.5.3. Tissue imaging

Images were taken by the COMET™ machine at 20x magnification objective after the DAPI staining during each cycle. The COMET™ machine used the DAPI stain to focus on each cycle. Images were then uploaded to HORIZON™ Viewer (*Lunaphore, Switzerland: Version 2.2.0.1*). Autofluorescence was subtracted by the automatic function on HORIZON™ viewer software prior to analysis of the images. Fluorescence intensity was adjusted for brightness and artefact reduced using the histogram function provided for each marker on the HORIZON™ viewer software.

7.5.5.4. H&E staining

Following immunofluorescence staining, slides were unloaded from the COMET™ machine, placed into multistaining buffer and then stained using a H&E staining kit (*Abcam, UK: ab245880*). Stained slices were imaged using a slide scanner (*Aperio AT2, Leica Biosystems*) and visualised using Aperio eSlide manager (*Leica Biosystems: Version 12.4.3.5008*).

7.5.5.5. Tissue staining analysis

The tissue staining was analysed descriptively for the purpose of this thesis. More objective semi-quantifiable analyses should be a feature of future studies.

7.6. Results

7.6.1. Participant characteristics

The characteristics of the 6 IPF patients and 3 control participants included in this study are described in Table 7.4. Three of the IPF tissue blocks were biopsy samples and three were explants.

Donor ID	Histopathological pattern	Type	Sex, age (years)	Smoking history	FEV1 % Pred.	FVC % Pred.	Gas transfer % Pred.
01	UIP	Biopsy	M, 66	Current	101	118	48
02	UIP	Biopsy	M, 71	Never	89	85	58
03	UIP	Biopsy	M, 72	Current	115	115	Unknown
04	UIP	Transplant	M, 57	Ex	74	70	30
05	UIP	Transplant	M, 59	Ex	45	46	26
06	UIP	Transplant	F, 61	Never	Unknown	Unknown	Unknown
07	Control	Lobectomy	F, 79	Never	103	107	83
08	Control	Lobectomy	F, 66	Never	112	103	133
09	Control	Lobectomy	F, 74	Ex	95	117	81

Table 7.4: Participant characteristics of tissue donors.

FEV1 – forced expiratory volume in one second, FVC – forced vital capacity, pred. – predicted, UIP – usual interstitial pneumonia,

Four out of 6 of the IPF blocks were from lower lobes of the lung (documentation of the lobe site of two biopsy blocks was not found). Smoking history was variable amongst study participants. None of these participants had a diagnosis of COPD. One IPF patient (donor '03') had emphysema.

The three control tissue samples were from non-IPF patients who had undergone VATS lobectomy to remove lung cancer. None of the control donors had a confirmed diagnosis of

ILD/COPD. However, some interstitial/emphysematous changes were incidentally reported on two of the control participants' HRCT imaging and/or lung histology. These included: 1) donor '07' who was reported to have mild lung scarring on HRCT imaging pre surgery, although subsequent histological analysis of the lung tissue identified no fibrosis, but instead the presence of mild emphysema, and 2) donor '09' who had no reports of radiological emphysema, but a histology report of a lung tissue sample used by the clinical laboratory to determine the cancer diagnosis reported emphysematous changes.

7.6.2. Tissue staining.

7.6.2.1. *Classical histopathological features of usual interstitial pneumonia.*

Control and IPF tissue slices were H&E stained to identify classical histopathological features of UIP. H&E staining demonstrated areas of fibrosis/architectural distortion, as well as areas of preserved normal lung architecture in the IPF biopsy samples (Figure 7.1A). More extensive fibrosis was present within IPF explant samples (Figure 7.1B), which is in keeping with these patients' worse lung function parameters (see Table 7.4). The heterogeneous nature of UIP and other features including honeycombing and traction bronchiolectasis are demonstrated in a stained IPF explant sample shown in Figure 7.2. Control samples had preserved alveolar architecture (Figure 7.3).

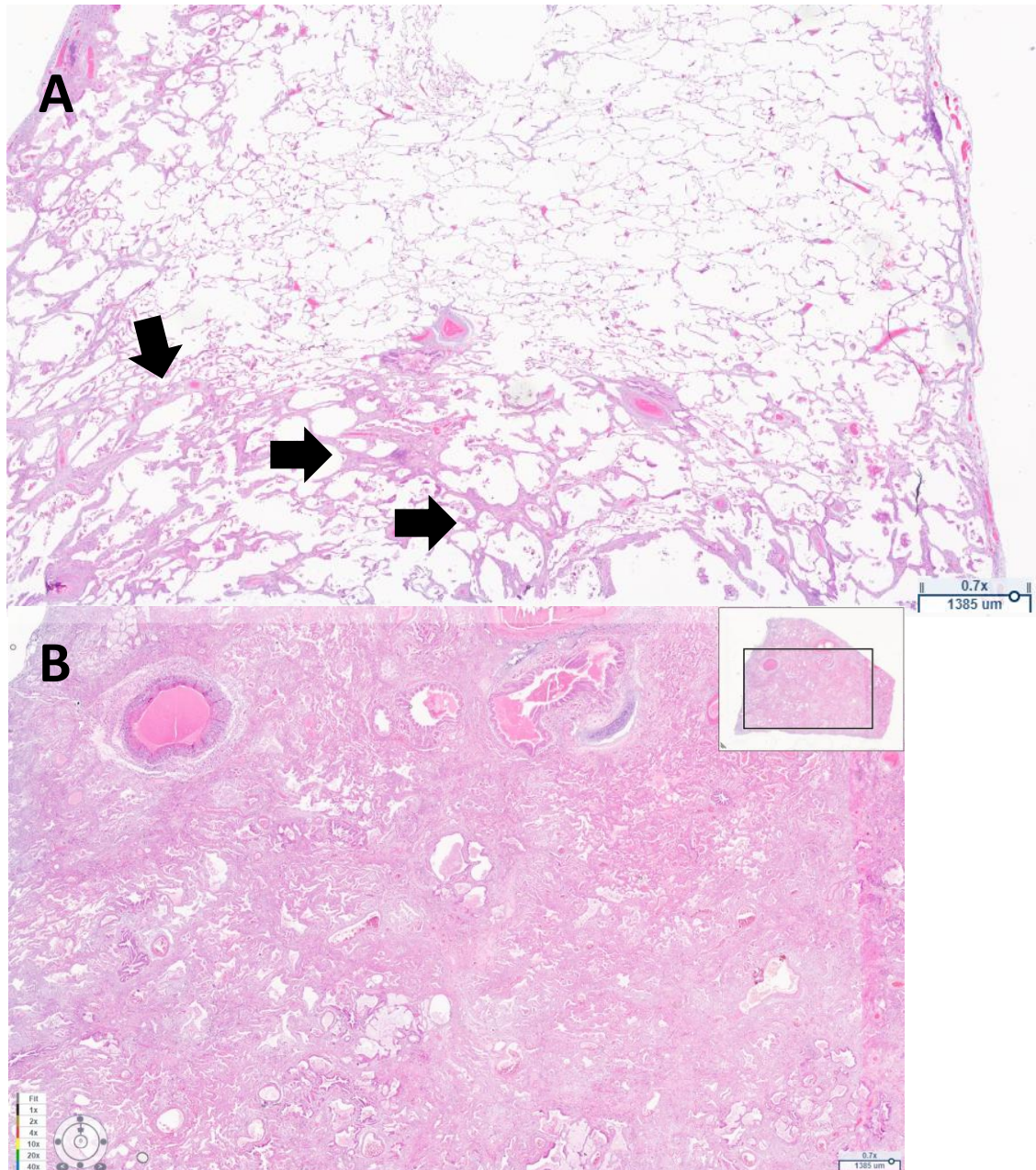


Figure 7.1: H&E stained IPF lung tissue displaying variability in disease severity between biopsy and explant samples.

H&E stained IPF tissue viewed on Aperio eSlide Manager at 0.7x magnification.

(A) Lung biopsy tissue from donor '01' (FVC 118% predicted, gas transfer 48% predicted). A heterogenous pattern of areas of fibrosis (arrows) interspersed with normal alveolar architecture.

(B) Lung explant tissue from donor '04' (FVC 70% predicted and gas transfer 30% predicted) demonstrating advanced fibrosis and architectural distortion.

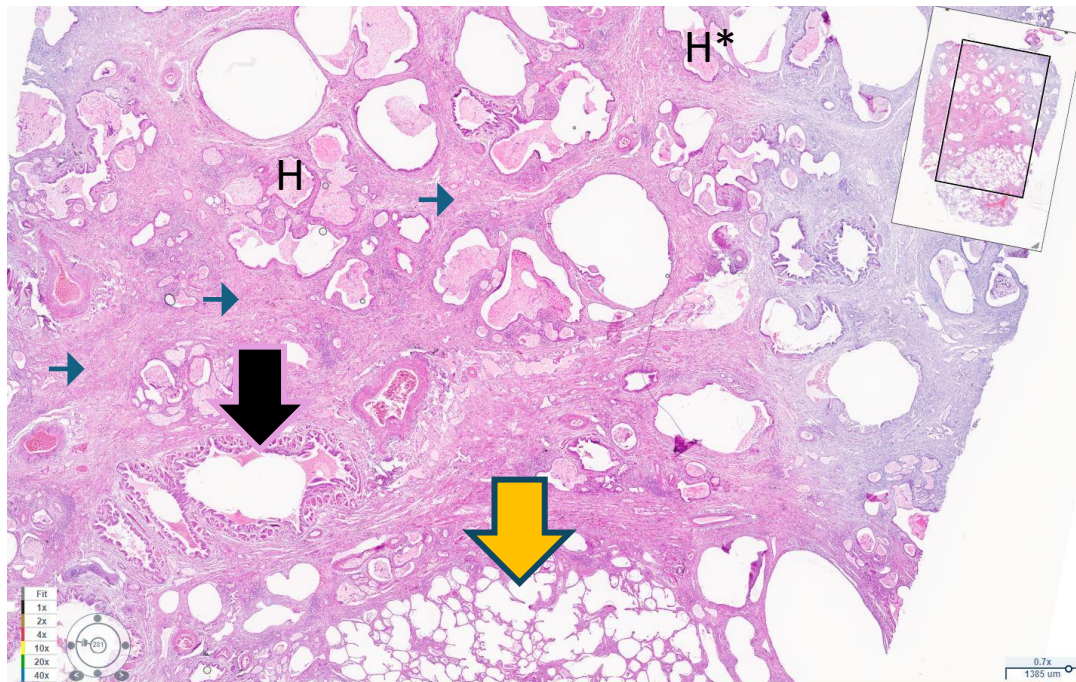


Figure 7.2: Pathognomonic architectural heterogeneity of usual interstitial pneumonia.

H&E stained lung explant tissue (donor '06') showing architectural heterogeneity that is pathognomonic of UIP. Fibrosis and architectural destruction (blue arrows) are critical to diagnosis. More physiological shaped alveolar structures are seen elsewhere (orange arrow). Traction bronchiolectasis is also seen (black arrow). Mucin filled cysts surrounding by hyperplastic/bronchiolar type epithelium called honeycomb cysts [449] are noted, marked as 'H'.

Area of honeycombing outlined by H* is visualised later in Figure 7.15 following staining with the multiplex immunofluorescence panel.

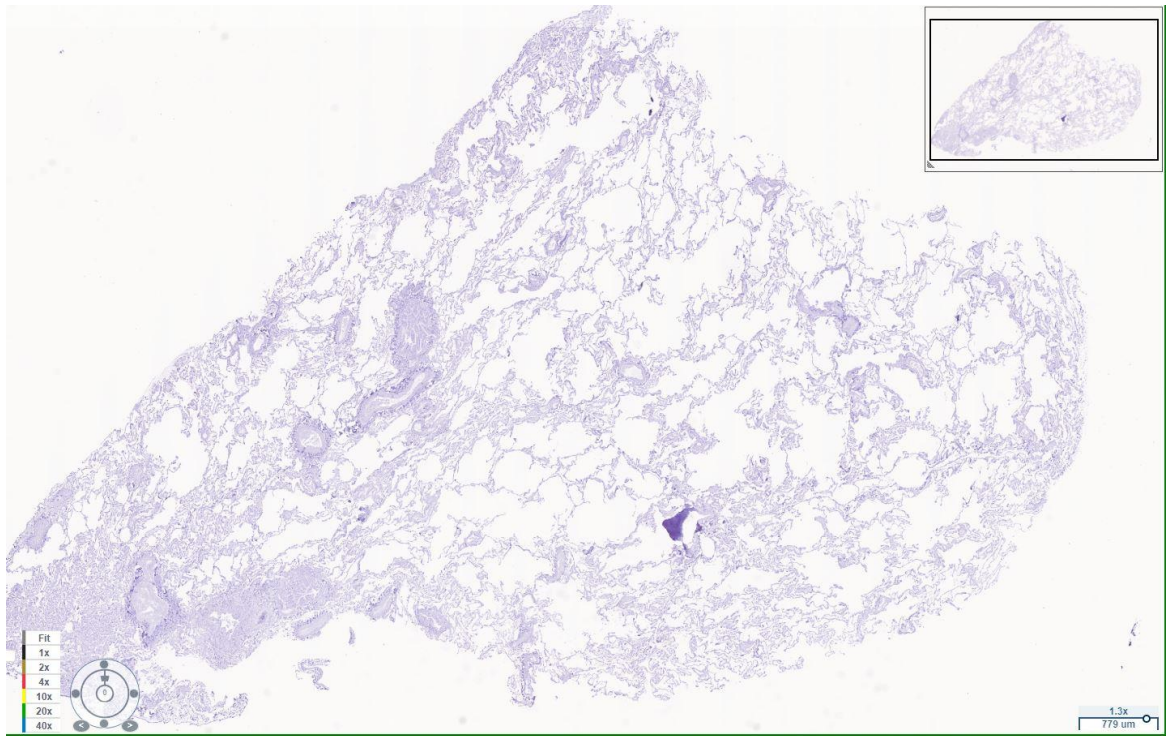


Figure 7.3: H&E stained control tissue.

H&E stained control tissue from donor '08' displayed using Aperio eSlide manager at 1.3x magnification. Preserved alveolar architecture is noted.

Figure 7.4 displays further characteristic features of UIP (fibroblastic foci and fibrosis) on an explant sample that is stained with H&E (Figure 7.4A) and multiplex immunofluorescence (Figure 7.4B).

In the multiplex immunofluorescence stained sample (Figure 7.4B), the vasculature is identified by circumferential α SMA expression (orange in images displayed in this chapter) and the interstitium and alveolar spaces are separated by cytokeratin positive alveolar epithelium (cytokeratin is bright green in all images displayed here). α SMA expression is seen within fibroblastic foci, likely representative of α SMA+ myofibroblasts. α SMA expression is further demonstrated within fibrotic areas in Figure 7.5. In the multiplex immunofluorescence stained slides, alveolar spaces are noted by areas that lack staining (black) that are surrounded by cytokeratin positive alveolar epithelium. Within the alveolar spaces there are cells, which are most commonly vimentin+ alveolar macrophages, neutrophils and other mononuclear cells, such as lymphocytes. Nuclear content is stained with DAPI and assigned a dark blue colour in the images shown here.

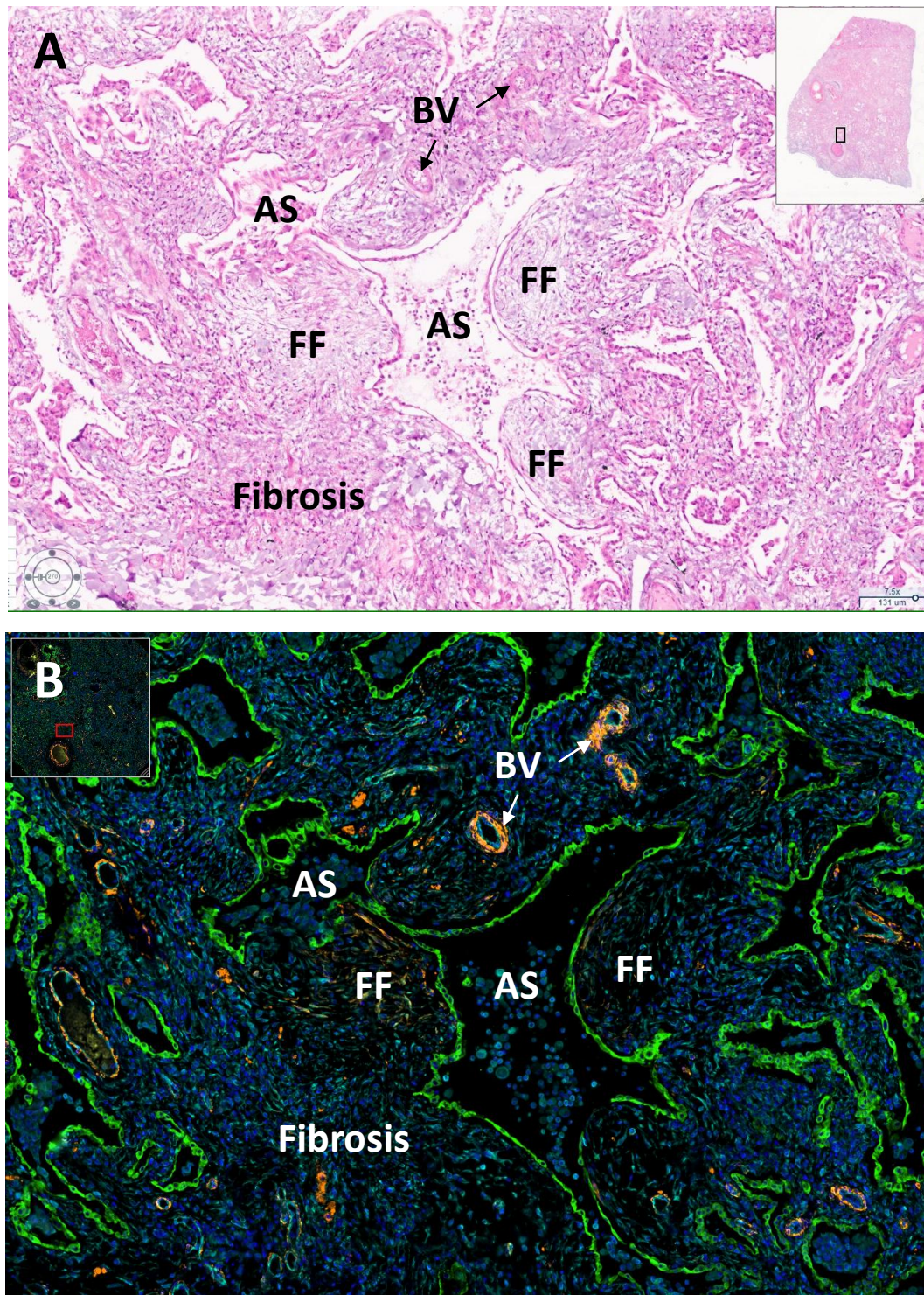


Figure 7.4: Characteristic histological features of usual interstitial pneumonia.

IPF explant lung tissue (donor '04') stained with H&E (A) and the multiplex immunofluorescence panel (B). Dark blue – DAPI, orange – α SMA, green – cytokeratin, turquoise – vimentin. AS – alveolar space, BV – blood vessel, FF – fibroblastic focus.

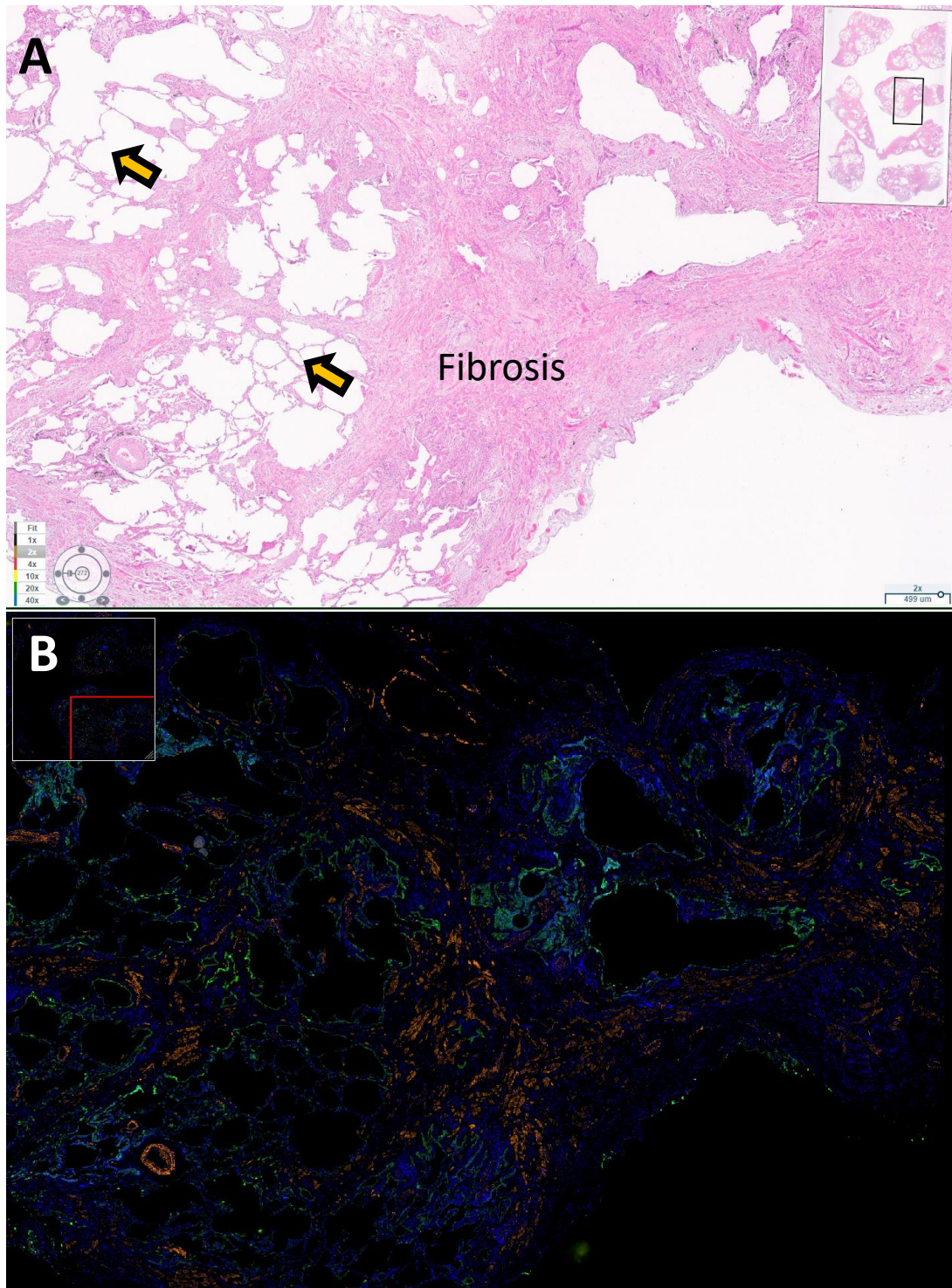


Figure 7.5: IPF lung biopsy tissue displaying extensive α SMA expression within fibrotic areas.

IPF lung biopsy tissue (donor '02') stained with (A) H&E and (B) the multiplex immunofluorescence panel. Orange arrows show areas of milder disease (more characteristic alveolar structures). Fibrosis is noted elsewhere. α SMA (orange) expression is noted within fibrotic areas in (B). Dark blue – DAPI, green – cytokeratin.

7.6.2.2. Localisation of neutrophils (and their contents) in control tissue.

Colocalisation of neutrophil markers, such as, CD15, CD11b, MMP-9, and myeloperoxidase, identifies neutrophils within lung tissue. Examples of this are demonstrated in Figure 7.6 and Figure 7.7, where neutrophils can be seen within the vasculature and stroma of two control tissue samples. Limited expression of neutrophil markers was noted within the alveolar spaces of control tissue samples although analysis using automated imaging software is required for confirmation.

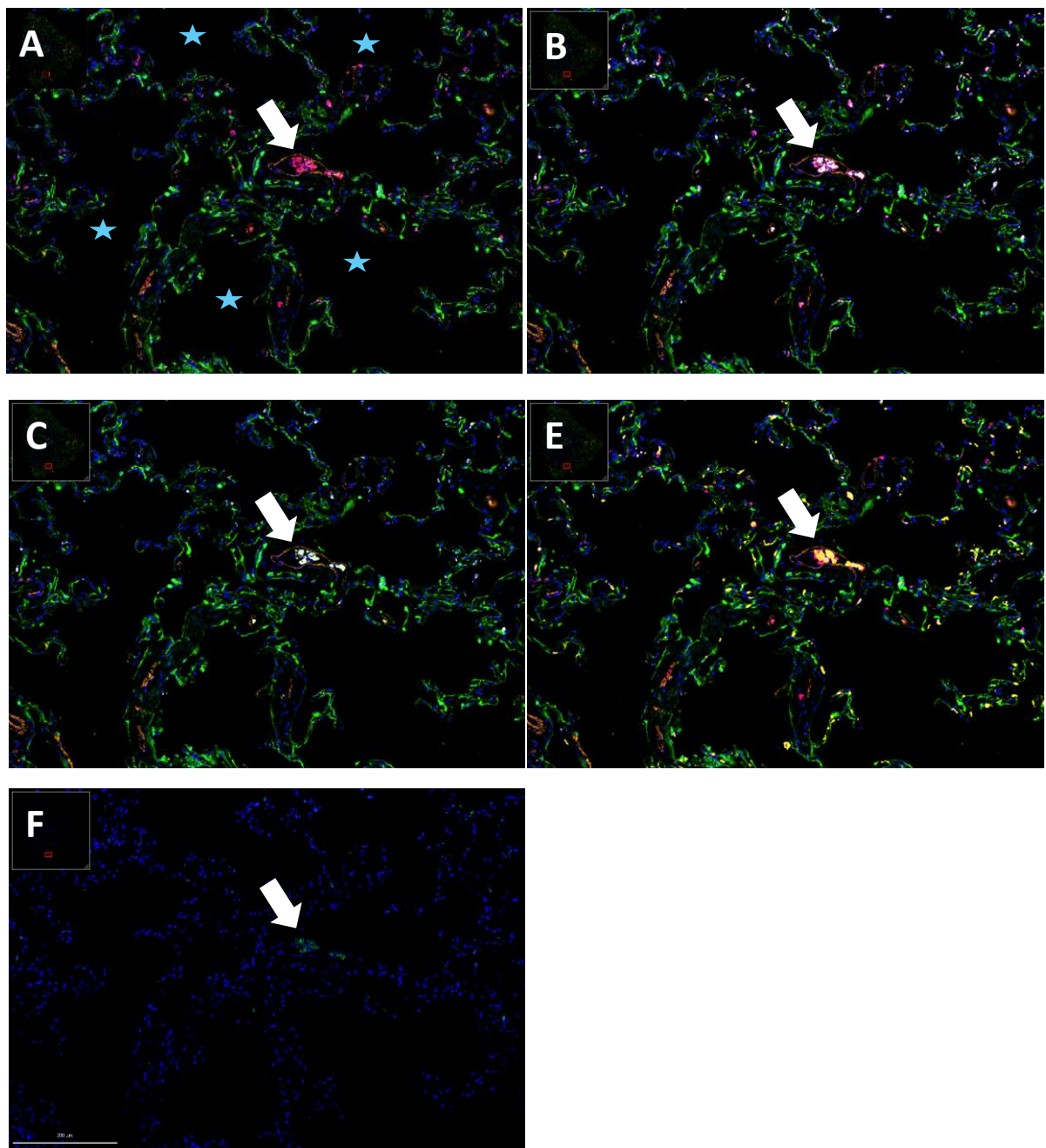


Figure 7.6: Neutrophil localisation within control tissue.

Control tissue (donor '07') stained with the multiplex immunofluorescence panel demonstrating colocalisation of neutrophil markers within the vasculature (white arrow). Few neutrophil markers are noted though within alveolar spaces (blue stars).

(A) CD15+ (pink) cells, dark blue – DAPI, green – cytokeratin, orange – α SMA.

(B) CD11b (white) colocalises with CD15+ cells.

(C) MMP-9 (white) colocalises with CD15+ CD11b+ cells described in (B).

(D) Citrullinated histone H3 (cH3) (yellow) colocalises with CD15+CD11b+ cells.

(E) Myeloperoxidase (green) colocalises with cH3 and CD15+ CD11b+ cells in the vasculature.

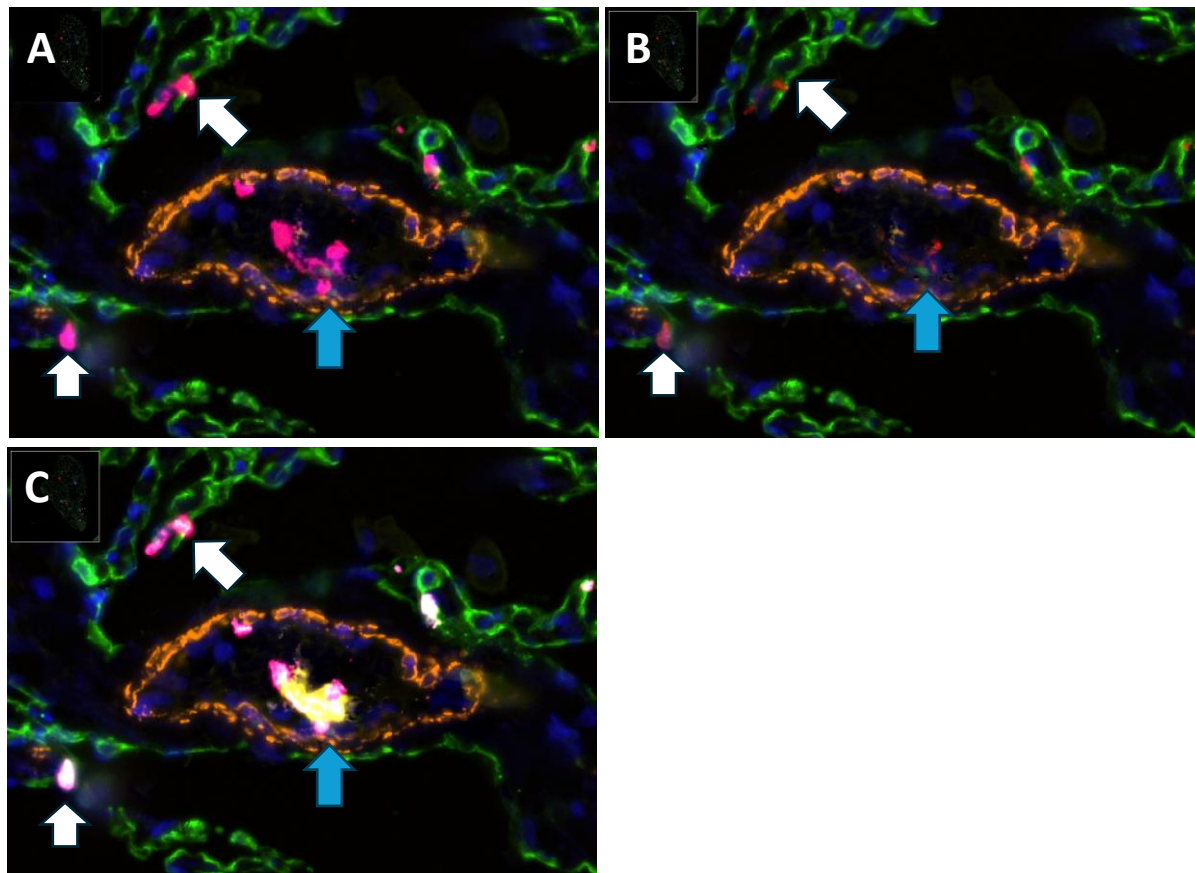


Figure 7.7: Neutrophil localisation within the vasculature and stroma of control tissue.

Multiplex immunofluorescence staining of control tissue (donor '08').

(A) CD15+ (pink) cells are identified within the vasculature (blue arrow) and overlapping the cytokeratin+ (green) alveolar epithelium (white arrows). Dark blue – DAPI, orange – α SMA.

(B) Myeloperoxidase (red) expression in close proximity/colocalising with CD15+ cells seen in (A) within vasculature (blue arrow) and overlapping alveolar epithelium (white arrows).

(C) CD15 (pink), MMP-9 (white) and citrullinated histone H3 (cH3) (yellow) expression in close proximity/colocalising within vasculature (blue arrow). MMP-9 and CD15 colocalise within the alveolar epithelium (white arrows).

Expression of some of the markers, such as citrullinated histone H3 (cH3), was variable within and between control tissue samples as displayed in Figure 7.8. Again, outlining the importance of using objective analytical methods to determine the degree of neutrophil infiltration/activation in lung tissue.

Modest NE expression was noted within control tissue (although again this was variable within/between control tissue samples) (Figure 7.9) and minimal expression of the proteinase, MMP-7 (Figure 7.10) was identified within control samples.

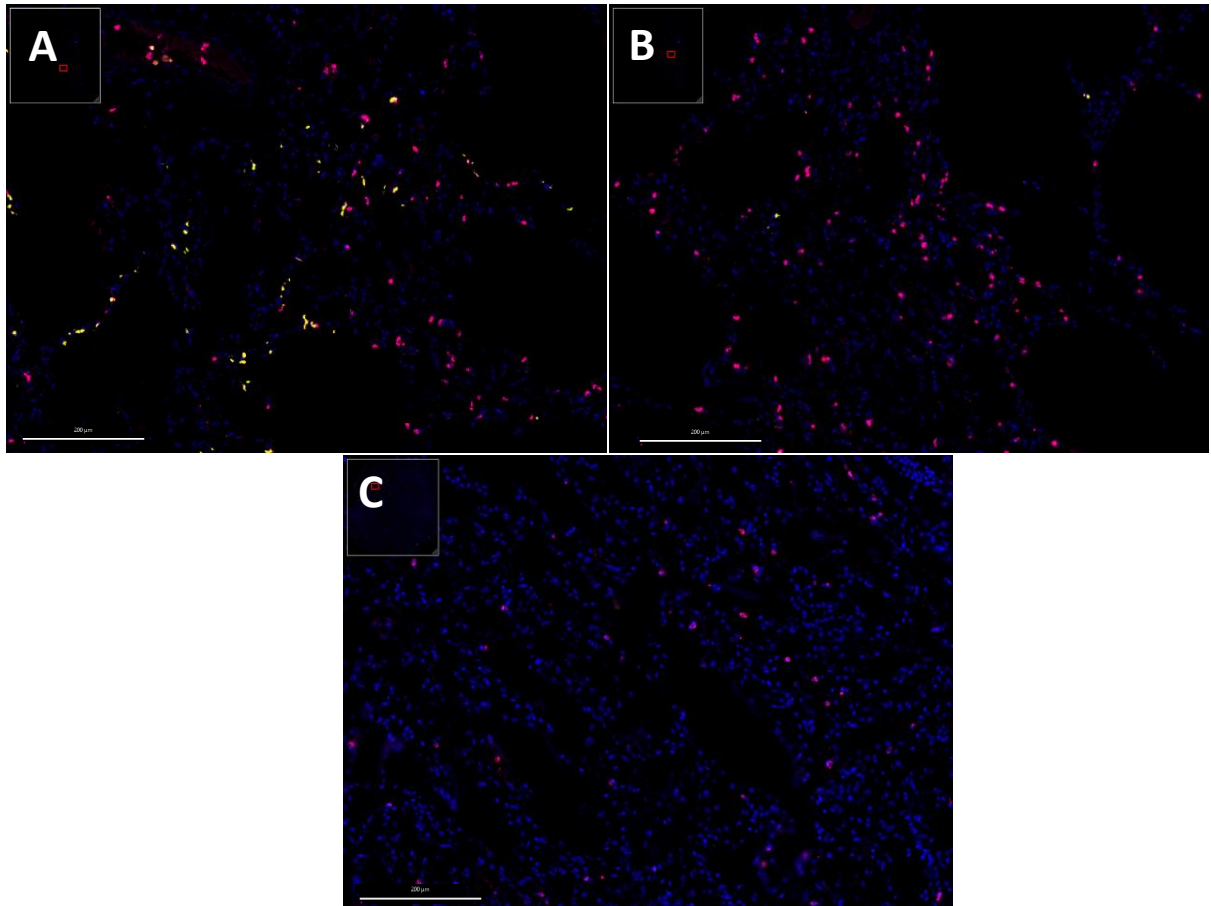


Figure 7.8: Expression of citrullinated histone H3 in control tissue.

Multiplex immunofluorescence staining demonstrates heterogeneous citrullinated histone H3 (cH3 - yellow) expression in control lung tissue. Colocalisation of CD15 (pink) and cH3 (yellow) is variable within the tissue. (A) and (B) are images taken from different areas within the same lung tissue slice (donor '08') demonstrating heterogeneity within the same tissue in cH3 expression. (C) from donor '09'. Dark blue – DAPI.

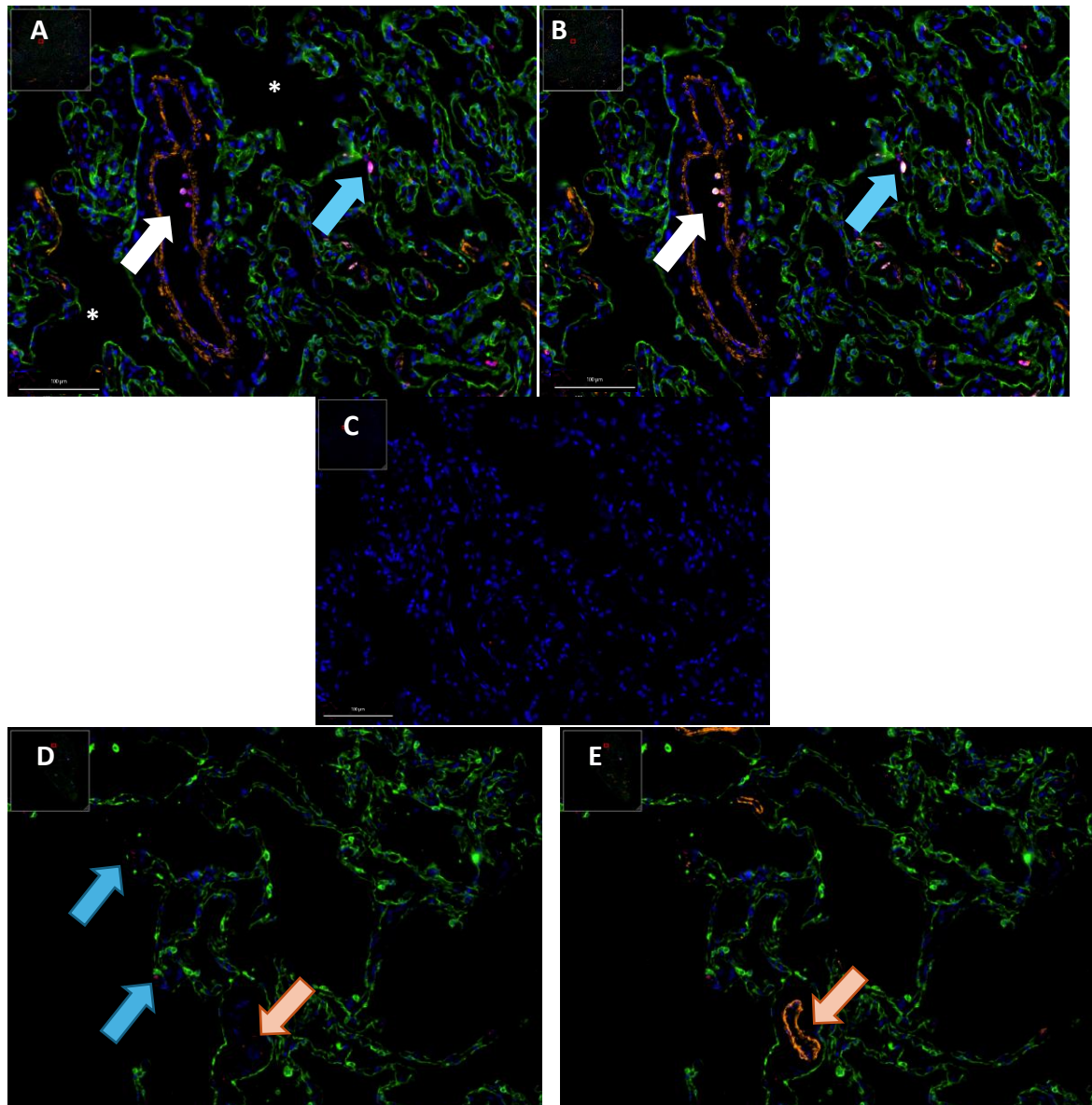


Figure 7.9: Assessment of neutrophil elastase expression in control tissue.

Multiplex immunofluorescence staining demonstrates that neutrophil elastase (NE) expression was variable between control tissue samples (A – C: donor '09', D – E: donor '08'). (A) CD15+ cells within vasculature (white arrow) and interstitium (blue arrow). Dark blue – DAPI, green – cytokeratin, orange – αSMA. White stars highlight some of the alveolar spaces. (B) CD66b (white) colocalises with CD15+ (pink) cells within vasculature (white arrow) and interstitium (blue arrow). (C) DAPI is shown in dark blue and NE in red. There is minimal NE identified in this control sample. (D) NE (red) is noted in donor '08' control lung tissue (blue and orange arrows), green – cytokeratin, dark blue – DAPI. (E) is the same image as (D) but with aSMA (orange) expression added to outline vasculature. NE is noted within/close to vasculature (orange arrow).

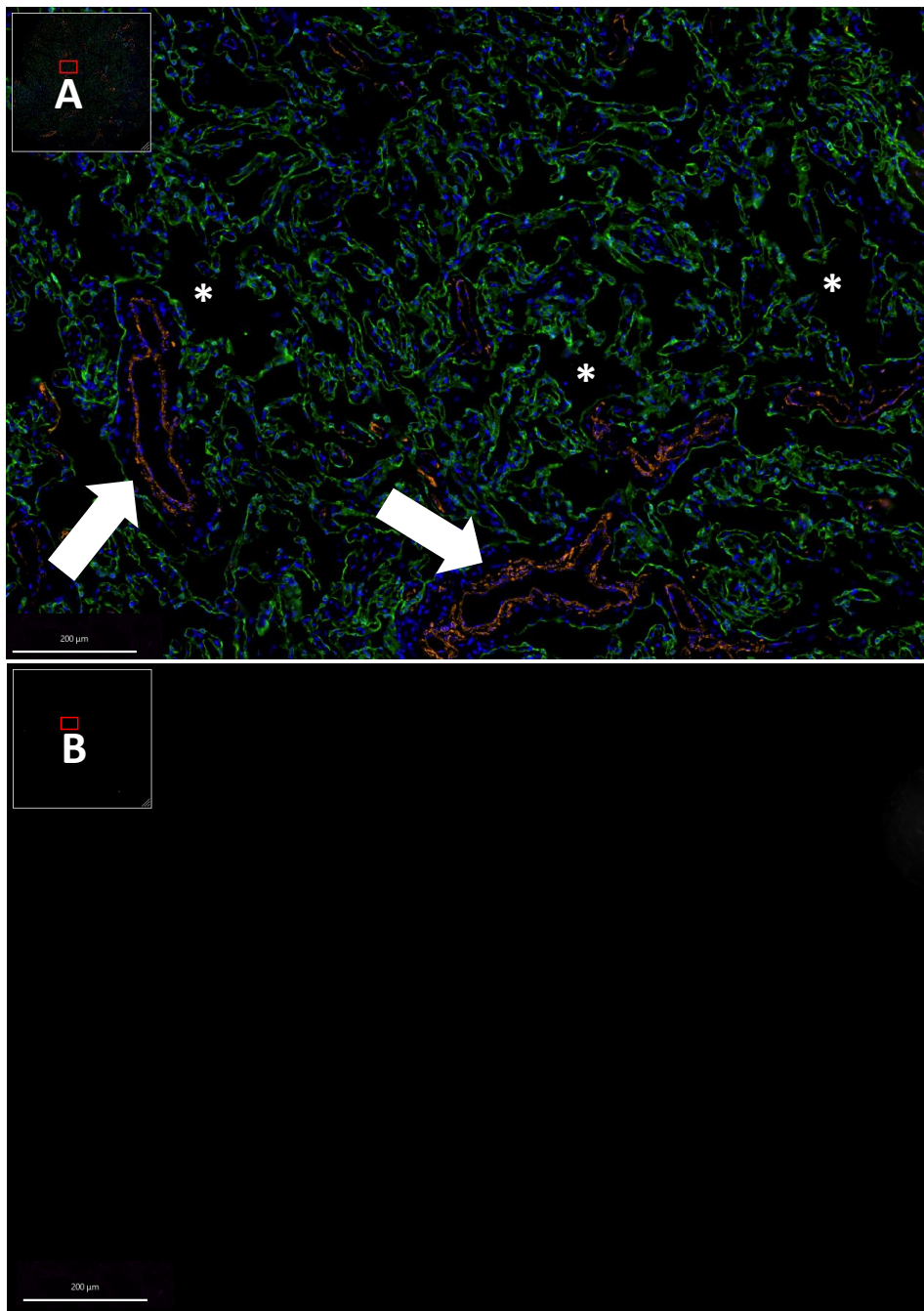


Figure 7.10: Assessment of MMP-7 expression in control tissue.

Multiplex immunofluorescence staining demonstrates minimal MMP-7 expression in control tissue (donor '09').

(A) Outlines structure of control tissue. Dark blue – DAPI, green – cytokeratin, orange – α SMA. White arrows – vasculature, white stars highlight some of the alveolar spaces.

(B) No expression of MMP-7 (assigned white colour) noted.

7.6.2.3. Localisation of neutrophils (and their contents) in IPF lung tissue.

Neutrophils are present within/close proximity to fibroblastic foci and adjacent alveolar spaces as shown in Figure 7.11.

In addition, NE staining is noted within or adjacent to the alveolar epithelium in both IPF biopsy and explant tissue (see Figure 7.12).

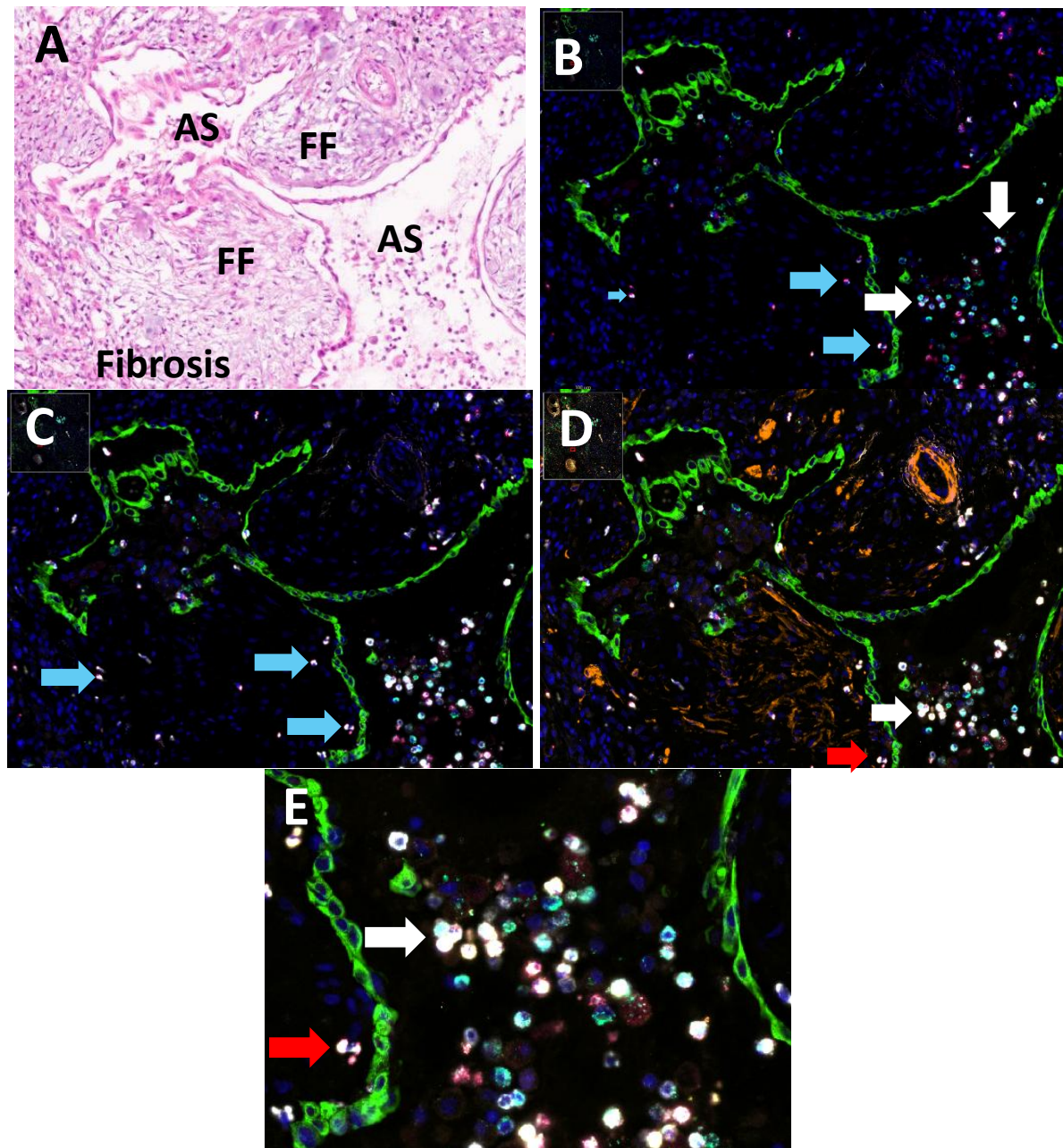


Figure 7.11: Neutrophil localisation within fibroblastic foci and alveolar spaces in IPF tissue.

H&E (A) and multiplex immunofluorescence stained (B – D) IPF explant lung tissue from donor '04' is displayed. (A) Fibroblastic foci (FF), fibrosis and alveolar spaces (AS) are noted.

(B) Blue arrows (within fibroblastic focus/fibrosis) and white arrows (within alveolar space) identify CD15+ (pink), myeloperoxidase (MPO)+ (light green) cells. DAPI – dark blue, cytokeratin – green. White areas represent intense overlap of CD15 and MPO. (C) Blue arrows identify CD15+ (pink), MPO+ (light green), CD11b+ (white) cells. (D and E) Red (within fibroblastic focus) and white (within alveolar space) arrows show CD15+ (pink), MPO+ (light green), CD11b+ (white), MMP-9+ (yellow) cells. α SMA expression (orange) within fibroblastic foci is seen. (E) is a zoomed in version of image D (excluding α SMA).

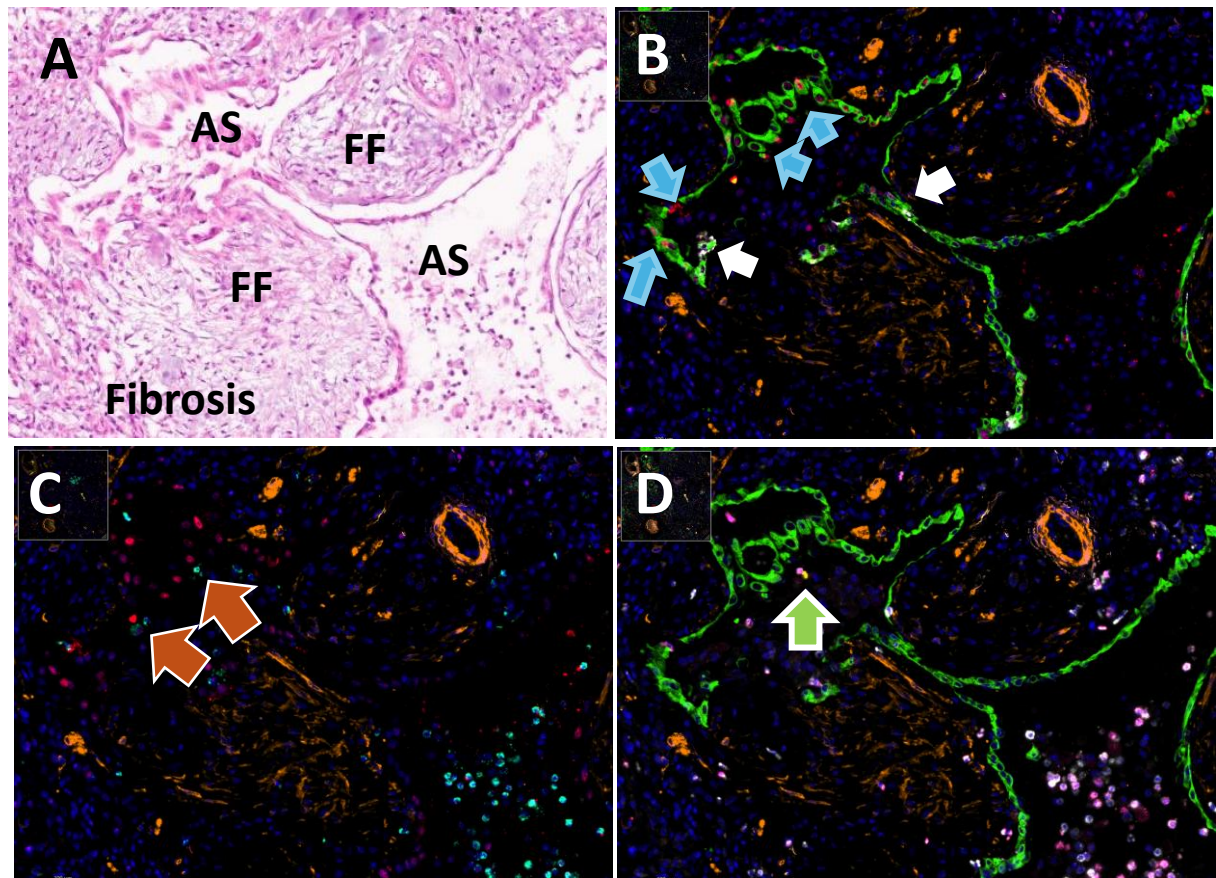


Figure 7.12: Identification of neutrophil elastase and other neutrophil markers around the alveolar epithelium in IPF lung tissue.

H&E (A) and multiplex immunofluorescence stained (B – D) IPF explant lung tissue (donor '04') is displayed. Dark blue – DAPI, bright green – cytokeratin, orange – α SMA.

(A) Fibroblastic foci (FF), fibrosis and alveolar spaces (AS) are noted.

(B) Neutrophil elastase (NE) (red) expression is noted within/adjacent to alveolar epithelium (cytokeratin – bright green), including near areas of MMP-7 (white) expression. Blue arrows highlight the NE expression and white arrows highlight the MMP-7 expression.

(C) Orange arrows identify myeloperoxidase (light green) expression within alveolar spaces near alveolar epithelium. NE – red.

(D) CD15+ (pink) CD11b (white) positive cells with adjacent citrullinated histone H3 (cH3) expression (yellow) is noted in the alveolar space (green arrow).

The most distinct difference between control and IPF tissue samples was the expression of MMP-7 especially in areas (most commonly lining honeycombing) of hyperplastic alveolar epithelium in IPF tissue. This was a feature of both biopsy (see Figure 7.13) and explant tissue (see Figure 7.14). NE expression was frequently noted close to MMP-7+ areas of hyperplastic alveolar epithelium (Figure 7.13D and Figure 7.14B). Other neutrophil activation markers, such as myeloperoxidase and cH3 are noted within the adjacent alveolar space, interstitium and epithelium (Figure 7.14 and Figure 7.15). Colocalisation of DNA, myeloperoxidase and cH3 is shown in Figure 7.15 within the alveolar space suggestive of NET formation in this IPF tissue.

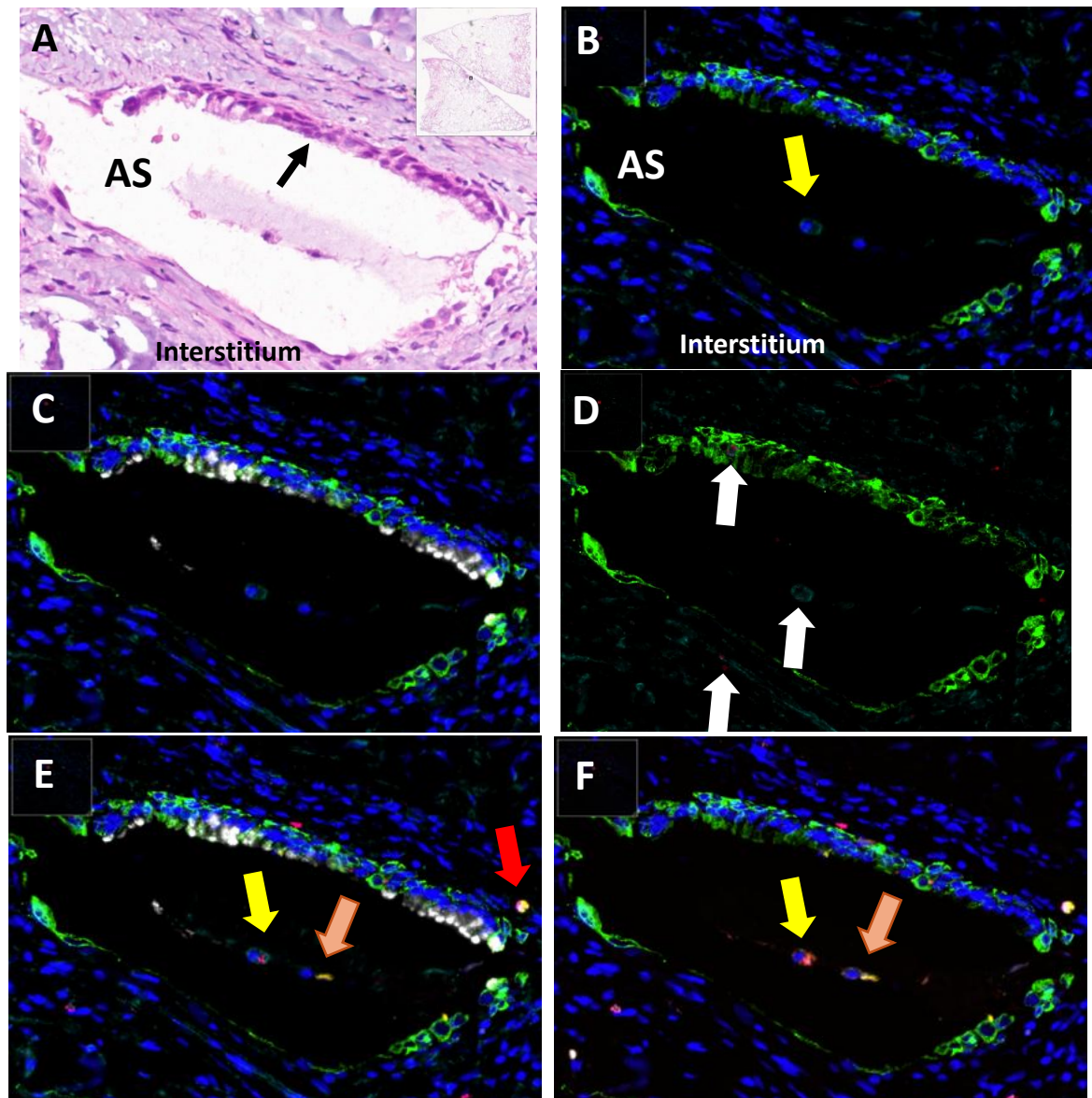


Figure 7.13: Area of hyperplastic alveolar epithelium in IPF lung biopsy tissue.

H&E (A) and multiplex immunofluorescence stained (B – F) IPF biopsy tissue (donor '01').

(A) AS – alveolar space. Black arrow in H&E section A – hyperplastic alveolar epithelium. (B) Cytokeratin (green) outlining alveolar epithelium. Yellow arrow shows CD206+ vimentin+ (turquoise) alveolar macrophage within alveolar space. Dark blue – DAPI.

(C) MMP-7 (white) colocalises with hyperplastic alveolar epithelium.

(D) Neutrophil elastase (red – identified by white arrows) within/interacting with hyperplastic alveolar epithelium, alveolar macrophage and interstitium.

(E) CD15 (pink) appears to be within cytoplasm of alveolar macrophage (yellow arrow). Citrullinated histone H3 (H3) (yellow) within alveolar space (orange arrow). CD15+ cH3+ cell in interstitium (red arrow).

(F) CD15, myeloperoxidase (orange), and cd11b (white) colocalise within cytoplasm of alveolar macrophage (yellow arrow). This is suggestive of alveolar macrophage efferocytosis of a neutrophil. cH3 appears to be in contact with CD11b+ cell (orange arrow).

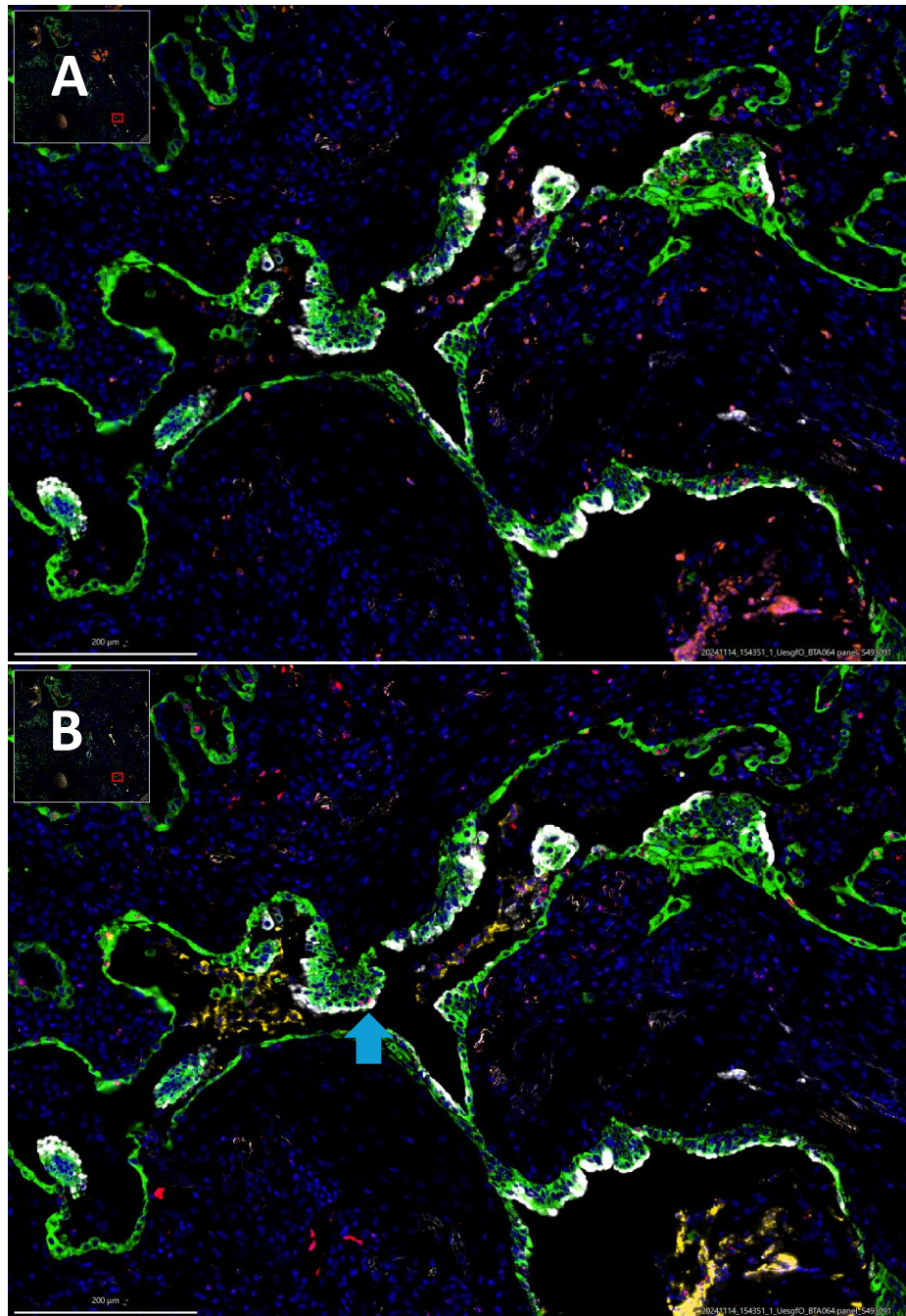


Figure 7.14: Hyperplastic alveolar epithelium in advanced IPF lung tissue: MMP-7 expression and neutrophil activation markers.

Multiplex immunofluorescence stained IPF explant lung tissue from donor '04' is displayed. Dark blue – DAPI, green – cytokeratin. MMP-7 (white) is noted around the hyperplastic alveolar epithelium in (A) and (B).

(A) MPO (orange) expression is noted within the alveolar space, epithelium and interstitial areas. (B) ch3 (yellow) expression, together with neutrophil elastase (NE) (red) is seen within the alveolar spaces, epithelium and interstitial areas. Blue arrow points towards NE within an area of MMP-7 + hyperplastic alveolar epithelium.

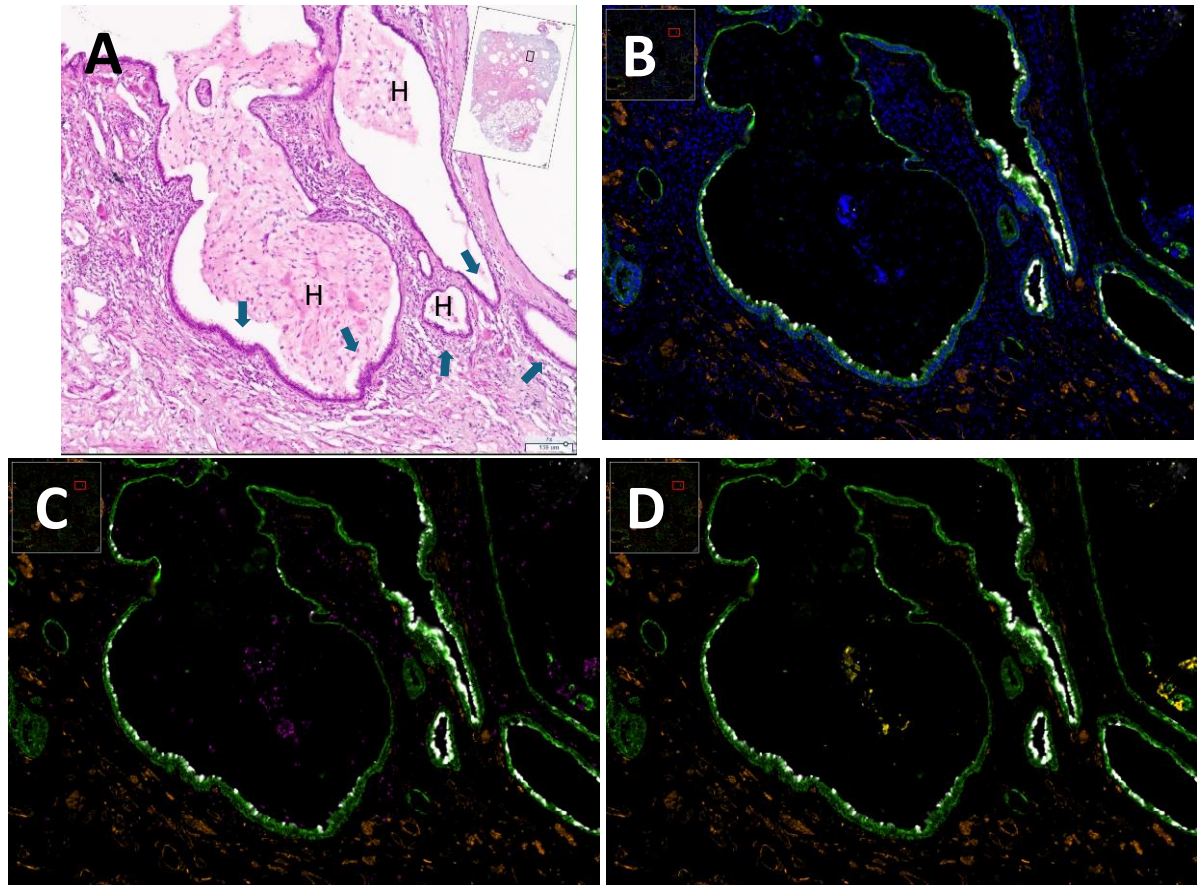


Figure 7.15: MMP-7 expression and markers of NET formation within honeycomb cysts.

Areas of honeycombing (H) present within IPF explant lung tissue (donor '06') stained with H&E (A) and the multiplex immunofluorescence panel (B – D) are displayed. Hyperplastic epithelium that typically lines honeycomb cysts are noted (blue arrows). MMP-7 (white) is noted around the hyperplastic alveolar epithelium in (B – D). Green – cytokeratin, orange – α SMA.

(B) DNA (stained with DAPI –dark blue) is seen with the alveolar space.

(C) MPO (pink) expression is seen within the alveolar space.

(D) cH3 (yellow) expression is seen within the alveolar space – appears to colocalise with the DNA and MPO expression that were seen in (B) and (C), respectively, suggestive of NET formation.

7.7. Discussion

7.7.1. Neutrophils and their contents are resident in IPF lung tissue.

Historical reliance on single stained immunohistochemical techniques has prevented clear localisation of neutrophils and their contents within IPF lung tissue. In this chapter, I have demonstrated that multiplex immunofluorescence technology can provide greater insight and confidence into the identification of this immune cell.

Assessment of paired H&E and multiplex immunofluorescence stained tissue has demonstrated that this novel technique can highlight characteristics features of UIP, such as prominent expression of α SMA in fibroblastic foci, and identification of hyperplastic alveolar epithelial areas (by cytokeratin staining). The ability to identify numerous markers at the same time allows the assessment of the spatial relationship of neutrophils (and contents) with cell types/proteins in these pathological areas.

UIP is characterised by spatial and temporal heterogeneity of fibrosis. Variability in the expression of neutrophil related markers, such as cH3 and NE, was also observed between regions in a single control tissue slice. Therefore, no conclusions can be drawn from this descriptive analysis regarding extent of neutrophil infiltration in IPF versus control tissue and how it changes with disease severity. This requires more objective analysis using automated, pre-trained imaging software, which will be undertaken in the future. The descriptive analysis here provides solely proof of concept of the benefit of creating such an antibody panel, and that neutrophils and neutrophil activation markers are present within IPF tissue. In addition, any clear discriminating features present in UIP tissue that were noted using this staining technology are described.

Here it was noted that neutrophils, by colocalisation of intracellular/cell surface markers, are resident within the alveolar spaces and interstitium of fibrotic lung tissue, including within fibroblastic foci. Expression of NET markers (cH3 and myeloperoxidase) was noted within the alveolar spaces of IPF tissue. This supports Khawaja *et al*'s study that reported increased NETs in IPF tissue but had not provided insight into location within the fibrotic microenvironment [19].

In my study, markers of NETs were seen but rarely noted within the alveolar spaces in control tissue. This is in keeping with my findings in Chapter 6, namely the observation of greater cfDNA in IPF BALF than control samples, and also with that of a previous study which noted increased NET markers in IPF BALF [19]. This suggests that there is increased transepithelial migration and/or activation of neutrophils within the alveolar spaces in this disease. However, this increase in neutrophil content in alveolar spaces needs to be confirmed and quantified by automated analysis.

7.7.2. MMP-7 and neutrophil elastase expression around areas of hyperplastic alveolar epithelium.

A distinct observation on review of the multiplex immunofluorescence staining in this chapter was the localisation of MMP-7 in areas of hyperplastic alveolar epithelium together with prominent NE expression. The latter is in keeping with previous work that has noted increased NE identification in IPF BALF and tissue [171,279].

Abundant MMP-7 expression by bronchiolar and alveolar epithelial cells has been noted previously in IPF lung tissue [448,450]. In addition, gene expression analysis identified that

MMP-7 most distinguished IPF and control lungs [450]. Furthermore, circulating MMP-7 appears to be an important biomarker in this disease; predicting disease progression, mortality and it has a negative relationship to the lung physiological parameters, FVC and gas transfer [451–453]. A role for MMP-7 in disease pathophysiology remains unclear, although supported by the observation that knockout MMP-7 mice are protected from bleomycin induced lung fibrosis [450]. Conversely it is suggested that MMP-7 itself is protective, for example an *in vitro* wound healing assay of A549 cells showed that MMP-7 is important for driving epithelial repair [454] although more physiological models, such as precision cut lung slices are needed to confirm or refute this concept. A phase 1/2 clinical trial is currently recruiting to investigate the effects of ARO-MMP7, a small interfering RNA that moderates MMP7 expression, in healthy volunteers and IPF patients (www.ClinicalTrials.gov identifier NCT05537025) [455,456].

Expression of apoptotic markers (caspase-3, p53 and p21) are increased in hyperplastic alveolar epithelial cells [457]. The localisation of NE around these sites suggests that it may contribute to epithelial damage. The pathological role of NE is supported by animal models (described in Chapter 1.11.1) where lung fibrosis is reduced following its inhibition [40]. However, NE blockade was shown not to affect NET-induced cytotoxicity of A549 cells *in vitro*, suggesting NE may not be a cause of alveolar epithelial cell damage [458].

The observed close localisation of MMP-7 and NE in areas of honeycombing demonstrated here may reflect a study which observed that MMP-7 positively regulated transepithelial neutrophil influx in an acute colon injury model [174]. Therefore, it is possible that MMP-7 also drives neutrophil migration towards the hyperplastic alveolar epithelium in IPF tissue.

Oligonucleotide arrays have identified colocalisation of the matricellular protein, osteopontin, with MMP-7 in the alveolar epithelium and MMP-7 appears to positively regulate the bioactivity of osteopontin [459,460]. Osteopontin has been shown elsewhere (animal model of transfusion-related acute lung injury and *in vitro* migration/*in vivo* mouse model migration assays) to drive neutrophil recruitment/chemotaxis to tissue [461,462]. Therefore, an MMP-7-osteopontin-neutrophil axis may be worth exploration for interpretation of the relevance of these observations in the context of IPF.

7.7.3. Future work

The next step for this work would be to use an automated image analysis pipeline to semi-quantify the multiplex immunofluorescence tissue staining results. This should determine how neutrophil count and neutrophil activation markers compare in IPF versus control tissue, and assess how this changes with fibrosis severity and progression. The two sources of IPF tissue described here (biopsy and explant samples) provide a spectrum of early versus late disease severity which is a surrogate of disease progression. Masson's trichrome staining following stripping of the H&E stain from each slice would allow the semi-quantification of scarring. Further tissue samples should be stained to improve statistical power of this work. In addition, assessment of the expression of neutrophil related markers in the different lung tissue compartments should be performed by training the analysis software to distinguish between tissue regions (alveolar spaces versus interstitium). Colocalisation of common neutrophil markers, such as CD15, CD11b, CD66b and myeloperoxidase, appear to vary on examination of the tissue staining here, this therefore needs semi-quantification to provide clearer and

novel insights into local neutrophil populations, for example which CD15+ cells are CD11b+ versus CD11b-.

Clearance of apoptotic neutrophils is critical for maintaining tissue homeostasis (as described in Chapter 1.12.5). Staining within the alveolar spaces revealed colocalisation of neutrophil markers possibly with the cytoplasm of alveolar macrophages, suggestive of alveolar macrophage efferocytosis or NE ingestion. One study suggested that efferocytosis of apoptotic cells is impaired by IPF derived alveolar macrophages [205]. However, it is unknown whether this defect is true specifically for the clearance of apoptotic neutrophils. Focused analysis of the multiplex tissue staining to assess potential efferocytosis within IPF tissue could provide further insight into the process. Addition of an apoptotic marker to the antibody panel, such as caspase, could more specifically identify neutrophils undergoing apoptosis and hence clearance.

Expression of the hypoxia related markers, HIF1 α and carbonic anhydrase 9, has not been discussed here but could also be assessed in the future to determine whether neutrophils in IPF show HIF1 α expression as has been reported previously [19].

Finally, the relationship of NE, and MMP-7 within areas of hyperplastic alveolar epithelium needs further confirmation as this may indicate a novel axis of the pathological IPF process with potential therapeutic targeting. This would require development of *in vitro* co-culture models followed by more complex precision cut lung slice experiments.

7.7.4. Limitations

The descriptive method of tissue staining analysis described here is the main limitation of this work. This form of analysis is subjective and as described previously the heterogeneous nature of staining in both control and IPF tissue further prohibits any conclusions to be drawn concerning the number and function of neutrophils in IPF without semiquantitative imaging.

In addition, the potential impact of co-existent undiagnosed/subclinical lung disease, such as emphysema, on the localisation/activation of neutrophils in the control and IPF samples used here needs to be considered. As described in Chapter 7.6.1, one of the IPF patients had a background of emphysema (although no diagnosis of COPD). In addition, two of the control participants had minor interstitial/emphysematous changes described on imaging/histology. However, the clinical significance of this is unclear as: 1) there appeared to be inconsistencies between these two diagnostic modalities; 2) the findings were incidental and; 3) neither patient had a confirmed diagnosis of an underlying respiratory disease (except lung cancer). Staining of further control tissue samples, which do not have any interstitial/emphysematous changes on radiology/histology is required. Furthermore, all control participants had lung cancer, which could impact neutrophil migration/activation [463], although samples were macroscopically distinct from the tumour margin to attempt to minimise any confounding effects of this.

A further limitation is that many markers used to identify neutrophils are expressed by other cell types such as monocytes and eosinophils leading to false identification of neutrophils.

Finally, limitations related to the processing of the tissue blocks need to be considered. IPF and control tissue samples were fixed and sliced by different personnel using different

materials/machinery. This is reflected by the control samples appearing less well 'inflated' than the IPF samples. Thus, standard operating procedures should be defined and used for taking this work forward to limit these technical variations.

7.7.5. Summary

Descriptive analysis of the multiplex immunofluorescence staining panel devised here has demonstrated that neutrophils and their contents are present within the fibrotic interstitium (including fibroblastic foci) and alveolar spaces of IPF lung tissue. Of note, NE was present around areas of hyperplastic alveolar epithelium, in close association with MMP-7. More in-depth semi-quantitative analysis using automated imaging software is required to provide further detail of the extent and location of neutrophil infiltration and activation in IPF tissue.

Chapter 8. Thesis Summary

8.1. Summary

Novel therapeutic targets need to be identified to improve the morbidity and mortality of IPF patients. In Chapter 1 it was proposed that neutrophils may play a role in IPF pathophysiology as these cells are important responders to tissue injury; housing machinery that impacts tissue damage, repair and remodelling. Furthermore, there appears to be an association between neutrophil content in BALF and fibrosis severity [361,362], as well as neutrophil activation markers within IPF lung tissue [19,279]. *In vitro* studies have suggested that NETs and NE may drive alveolar epithelial cell damage and/or fibroproliferation [419,458,464]. Therefore, neutrophils pose a novel axis for potential therapeutic manipulation. However, first the role of neutrophils in this disease needs to be ascertained; are they friend or foe?

8.1.1. Examination of circulating neutrophils in IPF.

This thesis aimed to investigate neutrophil dynamics in IPF, specifically: a) whether the key pro-fibrotic growth factor TGF β 1 drives neutrophil chemotaxis; b) assess phenotype and function of circulating neutrophils in IPF patients and; c) localise neutrophils and their contents in fibrotic lung tissue.

8.1.1.1. TGF β 1 primes neutrophils.

In the first instance, it was important to validate the experimental techniques used in this thesis to assess neutrophil movement, phenotype, and NET responses *ex vivo* (detailed in Chapter 3). Subsequently, TGF β 1 was found to drive neutrophil chemokinesis, but not specific chemotaxis. This was thought to be a priming response, with TGF β 1 amplifying chemokinetic and chemotactic responses to the classical chemoattractants, IL-8 and fMLP, respectively. However, TGF β 1 pre-treatment did not amplify PMA-mediated NET formation, although most

likely limited by the experimental conditions (maximal response with solely PMA). Conversely, TGFβ1 alone increased the extracellular release of DNA from neutrophils, a central process for NET formation. However, the alternative method of analysis, immunofluorescence, did not demonstrate that TGFβ1 increased NET formation, which could be explained by the lack of statistical power. It remains important that this is investigated further to determine any TGFβ1 contribution to the increased NETs observed within the fibrotic microenvironment; evidenced by the increased cfDNA measured in IPF BALF (described in Chapter 6) and observation of NET markers in the alveolar space of IPF lung tissue (described in Chapter 7).

TGFβ1 signalling pathways were also investigated. Western blotting and phosphoflow cytometry assays showed that human neutrophils express TGFβR1 and that TGFβ1 exposure leads to phosphorylation of the downstream signalling molecules Smad2/3. Western blotting cannot differentiate cell surface from intracellular expression of proteins. However, antagonism of TGFβR1 with a small molecular inhibitor did reduce TGFβ1 mediated cfDNA release, suggesting that TGFβ1 acts via this receptor.

The mechanism of neutrophil priming needs further clarification, as other priming agents, such as TNFα, work by amplifying the NADPH oxidase system [465,466]. It was considered that this was also likely for TGFβ1. Chemiluminescence assays that measure ROS production in real-time were performed to assess this possibility but remained underpowered due to equipment failure. Further experimental replicates are therefore required to confirm whether TGFβ1 acts via this mechanism.

8.1.1.2. There are differences in IPF donor neutrophil populations compared to controls.

TGFβ1 is a prominent pro-fibrotic cytokine in ILD pathophysiology and is elevated in the serum/plasma of IPF patients [29,280]. Therefore, it was hypothesised that neutrophils in the

circulation of IPF patients would display a primed phenotype. The prospective cohort study, HYBRID, was devised to investigate this possibility. Phenotype and function of neutrophils from IPF patients were compared to control participants who were matched for demographics, exposure history and comorbidities.

IPF neutrophils did not appear primed, with equivalent cell surface expression of priming/activation markers and no signs of increased movement or NETosis responses. This was unexpected as earlier findings showed that TGF β 1 primes neutrophils. The bioavailability of active TGF β 1 in the circulation of IPF patients is relevant. The studies cited here that reported increased concentrations of TGF β 1 in the serum/plasma of IPF patients did not specify whether active or total TGF β 1 was measured [29,280]. If it was the latter this may not reflect an increase in active TGF β 1. For example, a luciferase reporter assay demonstrated that murine serum contained sixty-five times less active than latent TGF β 1 [467]. Therefore, it remains unclear if active TGF β 1 is increased in the circulation of IPF patients, which could explain why priming markers/activity were not increased.

The priming effects of TGF β 1 may be more relevant when neutrophils enter the fibrotic lung where established TGF β 1 driven pro-fibrotic pathways suggest there is increased bioavailability of its active form. Although circulating IPF derived neutrophils did not appear primed, they did respond differently to TGF β 1, moving less directly following exposure, than control derived neutrophils. The mechanisms underlying this difference, indicates assessment of receptor polymorphisms, downstream signalling pathways, and its impact on fibrosis pathogenesis warrants further investigation.

Finally, the observations of a higher neutrophil count and a reduction in the percentage of CD10 positive neutrophils in IPF patients suggests a state of emergency granulopoiesis. Two further differences in cell surface markers were noted in IPF patients: an increase in expression of CXCR4 (marker of aged/senescent neutrophils) and PD-L1 (co-inhibitory molecule). These provisional findings in IPF patients with mild-moderate disease severity highlight the need to continue to expand recruitment to the HYBRID study to ensure sufficient statistical power, and to determine how these changes influence disease pathophysiology to potentially facilitate drug target discovery.

8.1.2. First insights into neutrophil localisation in the lungs of IPF patients.

Characterisation of neutrophil contents within the alveolar spaces and interstitium is critical to understand neutrophils dynamics in IPF. This was explored in two biological samples: BALF and lung tissue. The latter was examined using a novel multiplex immunofluorescence panel. Whilst adding complexity, examining multiple markers at the same time using this technology, avoids the limitations associated with single stain immunohistochemical methods (such as sharing of neutrophil identification markers with other cell types) and gives deeper insight regarding immune cell interactions. Descriptive analysis of this staining demonstrated that neutrophils (identified by co-localisation of several markers) are present within alveolar spaces and the fibrotic interstitium, including fibroblastic foci, within IPF tissue. Markers of neutrophil degranulation/NET formation were also present in these regions. In addition, a distinguishing observation in the IPF tissue (not present in control tissue) was the localisation of NE near areas of MMP-7+ hyperplastic alveolar epithelium. The role of MMP-7 in neutrophil dynamics in IPF and the effect of NE in alveolar epithelial cell damage/repair are worth further

exploration for novel therapeutic development. We await the completion of the phase I/II clinical trial investigating the safety of an inhaled therapy targeting MMP-7 expression [455].

8.1.3. Relevance of hypoxaemia.

Day and nighttime oximetry studies were also performed in control and IPF participants of the HYBRID study. Measurement of hypoxaemia was relevant as one of the few studies that has attempted to characterise neutrophil activity in IPF patients, identified that hypoxia may be an important regulator of neutrophil function [19]. IPF patients in the HYBRID study had only mild hypoxaemia over 24 hours, with average saturations of around 93 – 94%. However, the most notable finding was that IPF patients experienced double the frequency of episodic hypoxaemia, defined here as dips in SpO₂ by > 4% from baseline, which were negatively associated with gas transfer. The relevance of this on disease pathophysiology and as a potential enhancer or biomarker of disease severity/progression needs further examination. If unrecognised episodic hypoxaemia is found to be a driver of disease progression, then this would support a much needed clinical trial to determine the effects of oxygen therapy on disease progression/survival of IPF patients.

8.2. Strengths of this work

Ex vivo experiments were all validated using positive controls as described in Chapter 3. The HYBRID study is prospective in nature, carefully designed to ensure close matching and detailed characterisation of control and IPF cohorts, including measurement of daytime/overnight oximetry which may be relevant in neutrophil biology. It was demonstrated

in Chapter 5 that despite the small study size, findings such as an association between the health-related quality of life questionnaire, KBILD, and lung function parameters, were consistent with those of larger reported studies. Furthermore, inclusion of a second study visit to undertake bronchoscopy allowed the subsequent sampling of both the circulating and pulmonary compartments. Finally, use of state-of-the-art multiple immunofluorescence technology improved the confidence of neutrophil identification in IPF tissue and gave early insight into spatial interactions. Semi-quantitative analysis of this tissue staining in the future should provide specific and deeper insight into neutrophil populations and link to both disease severity and progression.

8.3. General limitations and future work

The biggest limitation for interpretation of these data was the statistical power of some experiments as indicated above and the simplistic nature of the *ex vivo* neutrophil studies.

8.3.1. Statistical power.

There is no published literature examining neutrophil phenotype and function in IPF patients. This alongside natural variability between biological samples, including the unknowns of TGF β 1 responsiveness/receptor polymorphisms, made determining sample size difficult and precluded pre study power calculations. Chemotaxis data from Chapter 4 assisted the determination of some preliminary power calculations for the HYBRID study (a minimum of 8 in each group). However, subsequent post-hoc analysis supports the expansion of this work to 26 participants for each cohort; to improve confidence in accepting/rejecting of the hypotheses. Continued study recruitment to reach the required target is a future priority.

Furthermore, ongoing study expansion to ensure inclusion of the whole spectrum of disease severity will aid determination of potential disease endotypes and time course of IPF specific changes.

8.3.2. All *ex vivo* neutrophil models may create artefact.

Firstly, neutrophil isolation from venous blood using Percoll density gradient centrifugation has potential limitations (for example, neutrophil activation – discussed in Chapter 4, and may not include low density neutrophils - discussed in Chapter 6). Secondly, neutrophil migration and NET assays performed here lack the physiological complexity of the vasculature and relevant pulmonary microenvironment. Thirdly, peripheral neutrophils that have not undergone extravasation into target tissue are not representative of those resident within the fibrotic stroma or alveolar spaces. Whole blood staining experiments could overcome point 1. In Chapter 4, alternative endothelial flow assays were discussed as a more physiological model. Considering the identification here of neutrophils and their contents in alveolar spaces in IPF tissue, modelling of neutrophil migration through the fibrotic interstitium and alveolar epithelium in this disease context is warranted. This could include generation of an air-liquid interface *in vitro* model to assess how neutrophil trafficking affects epithelial and mesenchymal cellular functions. Potential avenues for future work are displayed graphically in Figure 8.1.

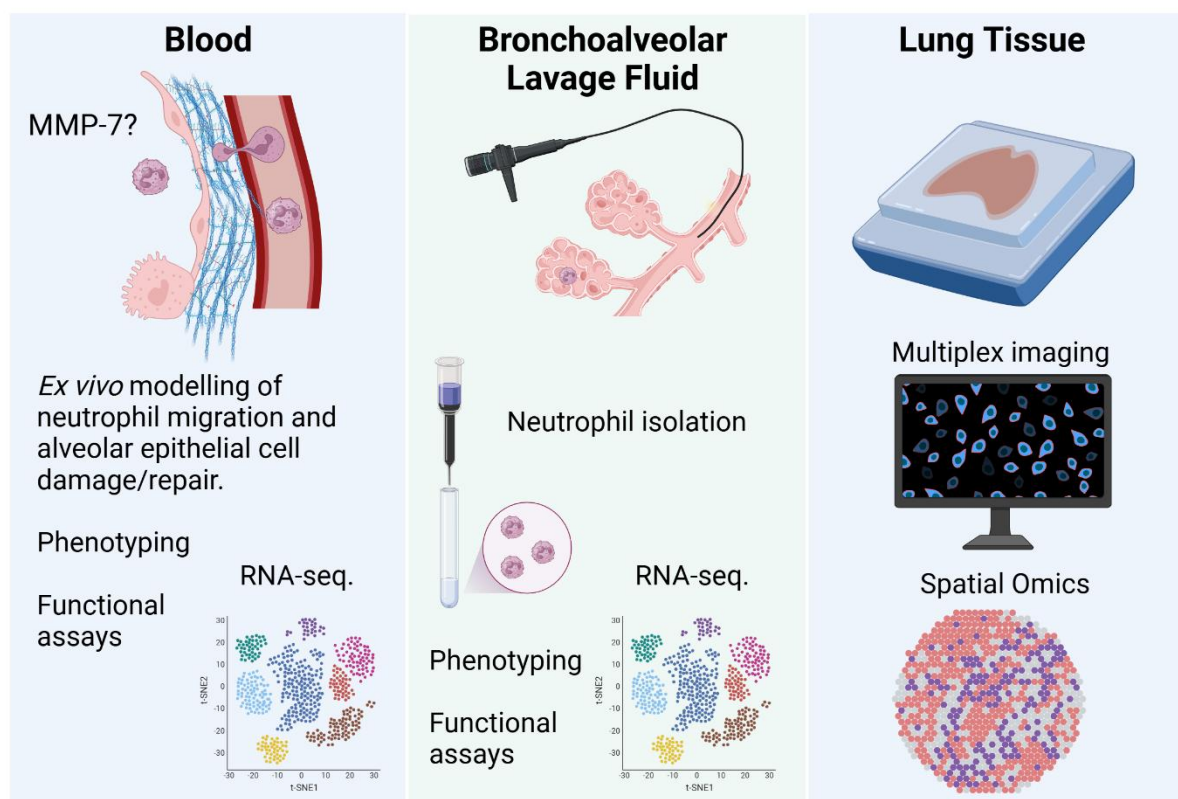


Figure 8.1: Avenues for future investigation into the role of neutrophils in IPF.

Neutrophils should be examined within the blood, bronchoalveolar lavage fluid (BALF) and lung tissue to provide full insight into their role and function in IPF pathophysiology. Phenotyping and functional analysis of neutrophils within these compartments needs continued examination. This includes the development of more complex and physiological *ex vivo* modelling of neutrophil migration through the numerous barriers present within the lung (endothelium, interstitium and alveolar epithelium). In addition, modelling the effects of neutrophils (and neutrophil proteinases) on alveolar epithelial cell damage/repair, and the impact of MMP-7 on neutrophil dynamics. Ribonucleic acid-sequencing (RNA-seq.) of paired blood and BALF samples should give insight into how neutrophils vary in these two biological compartments in health and disease. Neutrophil isolation methods from BALF would include negative bead isolation. Finally, multiplex imaging and spatial 'omics' can provide comprehensive and deep characterisation of neutrophil contents within fibrotic lung tissue at different stages of disease. *Figure created using Biorender.com.*

Techniques to optimally isolate neutrophils from BALF (such as using negative selection immunomagnetic beads and cell sorting technology) would allow a functional assessment of neutrophils that have migrated into the alveolar space. Furthermore other 'omics' methodologies such as, RNA sequencing, proteomics and metabolomics, may provide further insight once the basic pathophysiological process has been established. Plasma and RNA lysates have been stored from peripheral control and IPF donor neutrophils as part of the HYBRID study with such future research in mind.

8.4. Conclusions

This thesis aimed to provide insight into neutrophil dynamics in IPF. This work showed that the pro-fibrotic growth factor, TGF β 1, primes neutrophils and increases neutrophil chemokinesis. The prospective cohort study entitled HYBRID allowed the characterisation of phenotype and function of circulating neutrophils derived from IPF patients. IPF and control cohorts were carefully matched and patients were mostly of mild-moderate disease severity. IPF patients had a higher neutrophil count than controls with reduced percentage expression of CD10 suggesting a state of increased granulopoiesis and neutrophil immaturity. Other alterations in cell surface marker expression were observed (increased CXCR4 and PD-L1). Recruitment is ongoing to understand this further. Although IPF derived neutrophils did not appear primed, they did show a differential response to TGF β 1; moving less directly. Given the importance of this growth factor in disease pathogenesis, the exact mechanisms of this and any effect on disease pathogenesis needs further investigation. Episodic hypoxaemia was more frequent in IPF patients and negatively associated with gas transfer. The potential of this relatively simple, non-invasive measure as a biomarker of disease severity/progression warrants further

validation in an expanded cohort. Finally, multiplex immunofluorescence staining confirmed that neutrophils and their contents (proteinases and NETs) are present within the interstitium and alveolar spaces of IPF lung tissue.

In summary, this thesis has methodically assessed and established the potential role of neutrophils in IPF. This body of work clearly identifies the need for a better understanding of neutrophils either as mechanistic drivers or biomarkers of disease progression outlining avenues for further exploration. Ongoing studies will clarify the potential of neutrophil related pathways as avenues of therapeutic modulation.

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