NEUTROPHIL MIGRATION AND INFLAMMATION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

by

JAMES STOCKLEY

A thesis submitted to
The University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

School of Clinical and
Experimental Medicine
The University of Birmingham
June 2015

ACKNOWLEDGEMENTS

Firstly, I would like to thank Prof. Janet Lord and Dr. Elizabeth Sapey for the opportunity to undertake this research and their invaluable support and guidance throughout it. I have learnt a great deal about many aspects of research and I'm very grateful.

I would also like to thank my family, particularly my wife, Gemma, for her understanding and my father for providing further moral support.

Thank you also to the other members of Prof. Lord's research group for their help with experimental assays as well as members of the respiratory research team and lung function staff that have assisted along the way.

ABSTRACT

COPD is a leading cause of morbidity and mortality worldwide and it is believed that the neutrophil is key to the pathology. Evidence to date suggests that neutrophils migrate less accurately in patients with COPD, although the precise mechanisms by which this occurs have not been defined.

We have shown that COPD neutrophils migrate faster (chemokinesis) but less accurately (chemotaxis) in various chemokine gradients. It appears to be an intrinsic cell defect, as incubation of healthy neutrophils in COPD plasma did not alter migratory dynamics. This phenomenon does not occur in other respiratory diseases and is unrelated to age, disease severity, smoking status or the presence of emphysema. Furthermore, there were no differences in markers of neutrophil activation or maturity, although degranulation markers were higher in COPD.

Expression of certain chemokine receptors was lower on quiescent COPD neutrophils, but these differences were abolished after stimulation. Receptors localised to the leading edge effectively in COPD neutrophils and there were no differences in receptor shedding. PI3K phosphorylation was increased in COPD and inhibition of γ and δ isoforms improved chemotaxis. Neither Akt phosphorylation nor intracellular calcium signalling was altered. Simvastatin also improved chemotaxis of COPD neutrophils but CXCR1/2 inhibitors did not.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

ABBREVIATIONS IN ALPHABETICAL ORDER

α_1-AT	Alpha-1 Antitrypsin
α_1-ATD	Alpha-1 Antitrypsin Deficiency
ADAM-17	A Disintegrin and Metalloprotease-17
Akt	Protein Kinase B
ARF	ADP Ribosylation Factors
BALF	Bronchoalveolar Lavage Fluid
BSA	Bovine Serum Albumin
C5a	Complement Protein 5a
C5aR	Complement Protein 5a Receptor
Ca^{2+}	Calcium Ion
Cdc42	Cell Division Control Protein 42
CD	Cluster of Differentiation
CG	Cathepsin G
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-Reactive Protein
DAG	Diacylglycerol
DC	Dendritic Cell
ECL	Enhanced Chemiluminescence
EGTA	Ethylene Glycol tetra-Acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
Erk	Extracellular Signal-Related Kinase
E-Selectin	Endothelial Cell Selectin
ESI	Elastase-Specific Inhibitor
FACS	Fluorescence-Activated Cell Sorting
F-Actin	Filamentous Actin
Fc Receptor	Fragment Crystallizable Receptor
FEV_1	Forced Expiratory Volume in 1 Second
FITC	Fluorescein Isothiocyanate Isomer
FVC	Forced Expiratory Volume
fMLP	N- Formyl-Methionyl-Leucyl-Phenylalanine
FPR1	Formyl Peptide Receptor 1
GAP	GTPase-Activating Proteins
G-CSF	Granulocyte Colony-Stimulating Factor
GDI	Guanine Nucleotide Dissociation Inhibitors
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factors
GM-CSF	Granulocyte-Monocyte Colony-Stimulating Factor
GPCR	G Protein-Coupled Receptor
GRO*α*	Growth-Related Oncogene Alpha
GTP	Guanosine-5'-Triphosphate
HBSS	Hank's Buffered Saline Solution
HC	Healthy Control

HRCT	High Resolution Computerised Tomography
IC_{50}	50% Maximal Inhibitory Concentration
ICAM-1	Intercellular Adhesion Molecule-1
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol 1,4,5-Trisphosphate
IQR	Interquartile Range
JNK	c-Jun N-terminal kinases
LFA-1	Lymphocyte-Associated Function Antigen-1
LPS	Lipopolysaccharide
L-Selectin	Leukocyte Selectin
LTB_4	Leukotriene B_4
LTB_4R1	Leukotriene B_4 Receptor 1
Mac-1	Macrophage Antigen-1
MAPK	Mitogen-Activated Protein Kinase
MFI	Median Fluorescence Intensity
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
mTORC	Mammalian Target of Rapamycin Complex 2
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NE	Neutrophil Elastase
NEP	Neutral Endopeptidase
NETs	Neutrophil Extracellular Traps
NK Cell	Natural Killer Cell
NKT Cell	Natural Killer T-Cell
PAF	Platelet Activating Factor
PAK-1	P21-Activated Kinase-1
PBS	Phosphate Buffered Saline
PDK1	phosphoinositide dependent kinase 1
PDK2	Pyruvate Dehydrogenase Kinase
PE	Phycoerythrin
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PFA	Paraformaldehyde
PH	Pleckstrin Homology
PIP_2	Phosphatidylinositol 4,5 Bisphosphate
PIP_3	Phosphatidylinositol 3,4,5 Trisphosphate
PI3K	Phosphatidylinositol 3-Kinase
PLC	Phospholipase C
PKC	Protein Kinase C
PR3	Proteinase 3
P-Selectin	Platelet Selectin
PSGL-1	P-Selectin Glycoprotein Ligand-1
PTEN	Phosphatase and Tensin Homologue
RBD	Ras-Binding Domain
ROCK	Rho Kinase

ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SH	Src Homology
SHIP1	SH2 Domain-Containing Inositol Phosphatase 1
SLPI	Secretory Leukoprotease Inhibitor
SOD	Superoxide Dismutase
Sol	Sputum Sol Phase
TLR	Toll-Like Receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNFα	Tumour Necrosis Factor Alpha
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-6	Very Late Activation Antigen-6
WASp	Wiskott–Aldrich Syndrome Protein

CHAPTER 1

INTRODUCTION

NORMAL RESPIRATORY PHYSIOLOGY

1.1.1 Anatomy & Physiology

The primary function of the respiratory system is the exchange of respiratory gases between the external atmosphere and the body. This involves the uptake of oxygen, which is required by the tissues for aerobic respiration, and the removal of carbon dioxide, which is the waste product of cellular respiration. The mammalian respiratory system is highly evolved, and consists of two subsystems. The external respiratory system is comprised of the lungs (the organs of respiration) and the pulmonary circulation, and the internal respiratory system consists of the tissue cells. The lungs are specifically adapted to gaseous exchange by virtue of their large internal surface area, relative permeability, and extensive blood supply.

The lungs are contained within the thoracic cavity, which is a closed compartment consisting of the thoracic muscles, ribcage, sternum, thoracic vertebrae, and connective tissue. The anatomy of the thorax is illustrated in Figure 1.1.

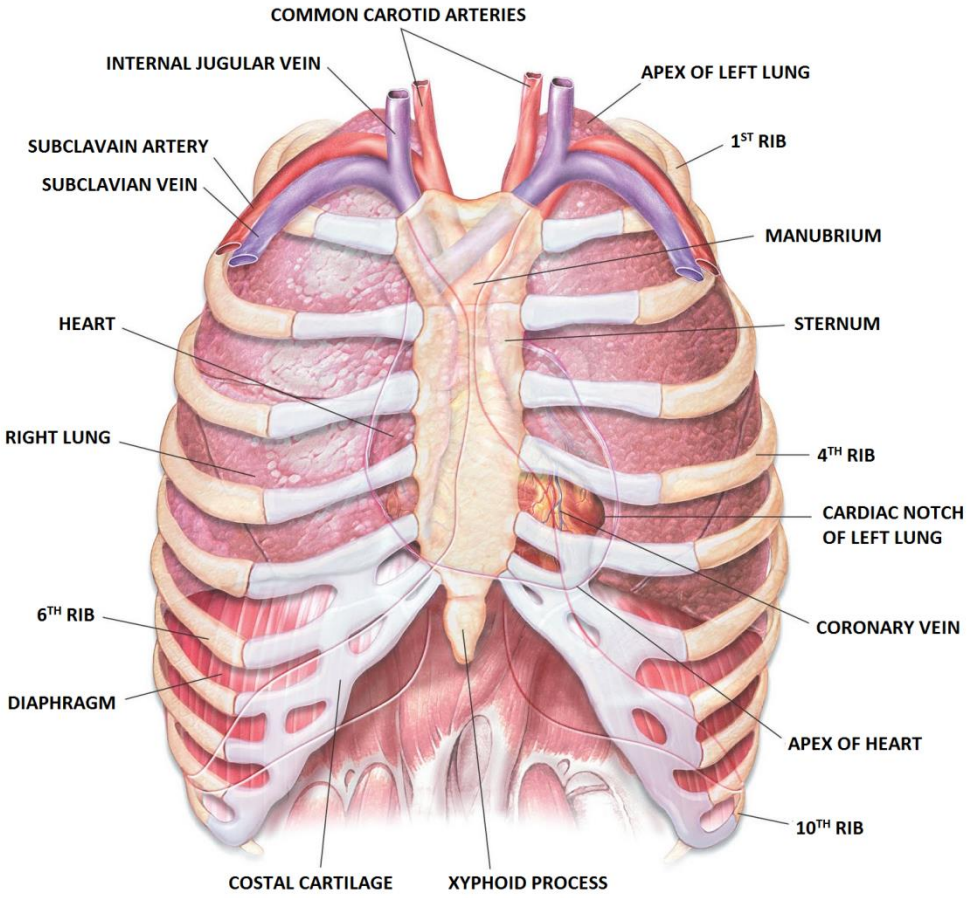

Figure 1.1: A diagram to show the viscera of the thorax.

Legend: An anterior view of including the lungs, diaphragm and cardiovascular system as well as the skeletal structures encasing the thoracic cavity (Adapted from http://infogrphic.blogspot.com/2011/04/viscera-of-thorax.html).

The process of respiration begins by air entering the respiratory passage via the nose or mouth, and passing through the pharynx and larynx into the trachea, which subsequently leads into the thorax. The trachea then branches at the major carina into the left and right bronchi, which connect to the respective left or right lung. Following a series of successive branching leading to a fine network of bronchioles, the airways finally terminate at the

alveoli, which are the respiratory acini. Each progressive division of the bronchial tree is referred to as a "generation". There are twenty-four generations in total, although gaseous exchange only occurs within the last eight, which include the terminal respiratory bronchioles, alveolar ducts, and the alveoli.

Structural support in the larger airways is provided by cartilage, which surrounds the epithelial, muscular, nervous, lymphoid and vascular components of the airway wall. As the generations increase and the airways become smaller, the cartilaginous support is progressively lost and is replaced by smooth muscle and elastic connective tissue. The alveoli are lined with two types of squamous epithelial cells; type I and type II pneumocytes. Type I cells form a thin, permeable layer throughout the alveoli that is primarily involved in gaseous exchange but also prevents fluid loss. Type II cells are granular and cuboidal and, although more numerous than type I cells, constitute only a small percentage of the alveolar surface area (Crapo et al. 1982). Their main function is to produce surfactant, a group of phospholipids which lower the surface tension within the alveoli. In addition, they can replicate in the alveoli and replace type I cells, which are susceptible to toxic insults and are unable to replicate (Evans et al. 1975).

The structure and physical properties of the lungs are elastic in nature, comprising both elastin and collagen fibers, hence they have a tendency to collapse inward. In contrast, the chest wall has a tendency to spring outwards (up to approximately 65% of full inspiration). These two opposing forces balance each other at rest, thus maintaining the overall shape of the thoracic cage. Ventilation is achieved by the expansion and relaxation of the lungs within the thoracic cavity. During tidal ventilation, or "quiet" breathing, inspiration is an active

process involving muscular contraction, whereas expiration is a normally passive, occurring due to the relaxation of inspiratory muscles and the elastic properties of the lung.

1.1.2 Pulmonary Blood Supply

Cells continually use oxygen for the metabolic processes that release energy from nutrient molecules. Therefore, oxygen must be continually absorbed from the atmosphere into the circulation and carbon dioxide (a waste product of metabolism) must be continually excreted. This is achieved during normal ventilation, which is increased during periods of activity/exercise to match the increased respiratory demands.

The pulmonary circulation is a separate component of the cardiovascular system that carries oxygen-depleted blood from the heart to the lungs and returns oxygenated blood back to the heart. Each airway within in the pulmonary tree is supplied by a branch of the pulmonary artery that divides at each progressive bifurcation, ultimately forming an intricate capillary network in the alveolar wall. The extent of this blood supply and the proximity of the capillary network to the alveolar airspaces both serve to maximise gaseous exchange.

A separate system, called the bronchial circulation, supplies blood to the conducting airways in the lungs down to the terminal bronchioles. The bronchial arteries originate from either the aorta or the intercostal arteries and supply arterial blood to the tissues and structures throughout the lungs. This circulation is also important in the “air-conditioning” of inspired air and controlling heat exchange in the lungs.

1.1.3 Immunity

To accomplish gaseous exchange, the large surface area of the lungs must be in continual contact with ambient air. As a result, the thin, permeable layer of cells that comprise the lung epithelium is continually exposed to airborne pathogens that could potentially lead to infection. Therefore, the respiratory tract requires effective mechanisms to prevent microbial proliferation. The lung has therefore evolved with effective physical barriers, mechanical defences and inflammatory cell responses to prevent microbial colonisation and infection.

Pathogens that enter the lower respiratory tract are initially trapped in the epithelial lining fluid, which comprises complex secretions, including a mucus blanket and surfactant. These secretions are then moved upwards on the "mucociliary escalator" (Green et al. 1977) towards the oropharynx where the cough and swallow mechanism clears the pathogens physically from the respiratory tract. Pathogens that evade this clearance mechanism can adhere to the lung epithelium, begin to proliferate and initiate a more specific immune/inflammatory response designed to combat the infection at a cellular level.

The immune system includes many specific and non-specific mechanisms and diverse cell types, each with individual functions that provide defence against microorganisms and other foreign antigens. A rapid innate response is the first line of defence and is relatively non-specific. This primary response includes resident airway phagocytes, local non-specific (IgA) immunity and a variety of antimicrobial peptides. This is followed (when the primary innate immune system is overwhelmed) by a secondary inflammatory cell response including recruitment of circulating neutrophils and monocytes, as well as leakage of plasma proteins such as complement and more specific immunoglobulins. If the infection persists, a highly specific adaptive response, which offers a cognate/specific recognition of individual antigens,

develops over days to weeks although, on subsequent exposure to the same antigen, this response occurs earlier as part of the "memory" function.

Innate and adaptive immunity are interlinked in part via toll-like receptors (TLRs), which are pattern recognition receptors found on the cell surface and in endosomal compartments of the various host inflammatory cells (Akira et al. 2006). Once TLRs recognise microbial-derived molecules common to foreign pathogens (such as bacterial endotoxin), they initiate many subsequent stages of the immune response, including secretion of pro-inflammatory cytokines (Galanos et al. 1993). Innate immunity involves several cell types including monocytes/macrophages, neutrophils, eosinophils, basophils, natural killer (NK), natural killer T (NKT) cells and dendritic cells (DCs), some of which are resident, although most are recruited from the circulation in response to infection. The process is mediated by a vast number of pro-inflammatory cytokines that modulate the features of inflammation and, hence, bacterial clearance. The final stage of adaptive immunity is antigen-specific and predominantly based on T and B lymphocyte-mediated mechanisms.

The mechanical defences of the lungs are thus continuously supported by the immune system and lung immunity is dynamically mediated by both the innate and adaptive arms of the immune system (Meyer 2001). This complex, interrelated sequence of events is summarised in Figure 1.2. More detailed accounts of these processes are described in (Martin et al. 2005, Zaas et al. 2005, Sheehan et al. 2006).

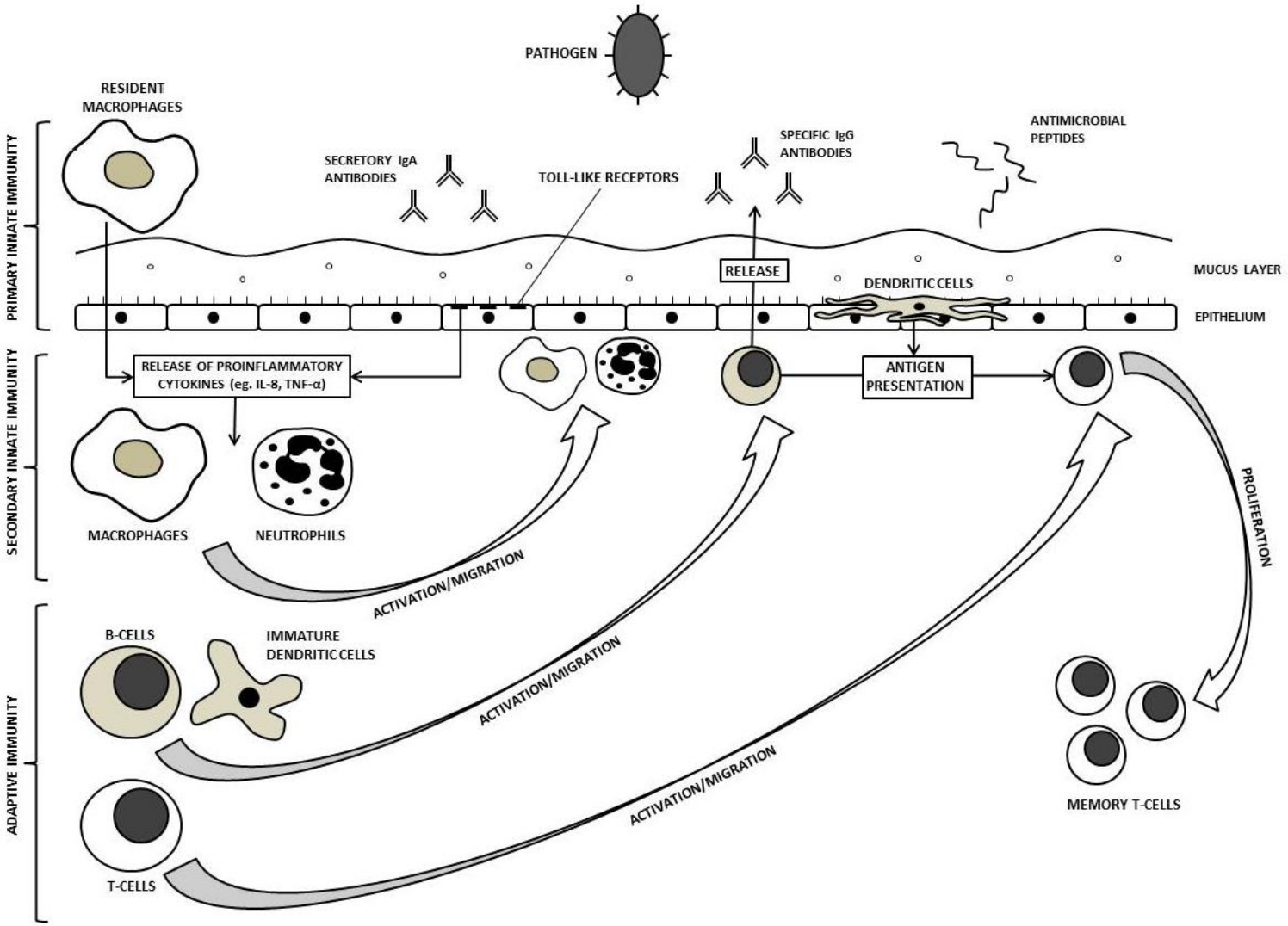

Figure 1.2: A simplified model of the interrelated sequence of events involved in pulmonary immunity.

Legend: The inflammatory processes include the release of regulatory cytokines (such as TNFα and IL-8), recruitment and proliferation of numerous inflammatory cells as well as specific defence mechanisms of the epithelium.

1.2 THE NEUTROPHIL

1.2.1 Development & Structure

Neutrophil polymorphonuclear leukocytes or "neutrophils" are the most abundant white blood cell in the circulation, accounting for 40-65% of all leukocytes. Normal concentrations range between 3 and 5 x 10^6 cells/ml of blood, though this number can increase dramatically during periods of infection.

Traditionally, it was believed that their circulating half-life is relatively short at 8-20 hours (Dancey et al. 1976), which may extend to several days if they leave the circulation and enter the tissue. A recent *in vivo* study suggests a much longer neutrophil lifespan of 5.4 days (Pillay et al 2010) although, due to the method by which the neutrophils were labelled with ^2H_2O, neutrophils present in the bone marrow were almost certainly labelled as well as peripheral blood neutrophils, hence the longevity of blood neutrophils was likely to be overestimated. Furthermore, the majority of recent data also suggest the lifespan of circulating neutrophils is relatively short, though the evolutionary advantage of this rapid turnover remains unclear (Summers et al. 2010).

The development and maturation of neutrophils has been well described (Edwards 2005) but key points will be summarised here. Neutrophils differentiate and mature within the bone marrow. Due to the large number of neutrophils and their short half-life, large numbers of neutrophils must be produced and released daily by the bone marrow, a process which is regulated by cytokines such as Granulocyte Colony-Stimulating Factor (G-CSF) and may be amplified as much as tenfold during periods of infection. The development of mature neutrophils in the bone marrow occurs via the differentiation of generic stem cells into

precursor, or "progenitor" cells that follow the developmental lineage specific to neutrophils. The first identifiable cells in this lineage are myeloblasts, which are relatively undifferentiated and contain a large nucleus. These then proliferate and differentiate into promyelocytes, at which stage the cells develop a large number of "primary" or "azurophile" granules. These contain a number of different enzymes that become important for the specific immune functions of mature neutrophils. They include myeloperoxidase (MPO), antibacterial peptides (azurocidin, defensins and lysozyme) and serine proteinases such as Cathepsin G (CG), neutrophil elastase (NE) and Proteinase-3 (PR3). As the cells mature into myelocytes, they synthesise a large number of peroxidise-negative granules termed "secondary" or "specific" granules. These granules also contain enzymes as well as various membrane receptors. From here, the cells mature further to form metamyelocytes and subsequently band cells, where they lose their ability to divide. At this stage, the last of the granules are formed, namely the "tertiary" or "small storage" granules, which contain cathepsins and gelatinases. In fully mature neutrophils, there are over 200 granules with vast array of antibacterial enzymes, collectively forming a robust defence against pathogens. The developmental stages of mature neutrophils are summarised in Figure 1.3.

When mature neutrophils leave the bone marrow, they are in a quiescent, non-activated state. At the end of their lifespan (if not activated), they return to marginated pools within the vasculature of various organs such as the spleen and bone marrow (Burnett 1997). Here, they undergo apoptosis, or "programmed cell death", and are phagocytosed by macrophages. This process is important as a number of neutrophil granular contents are damaging to structural tissue proteins (for instance, elastin degradation by NE). Apoptosis and subsequent

phagocytosis prevents the contents of the neutrophil being released into the local environment as the cell enters secondary necrosis.

Sites of margination include the liver, spleen and bone marrow (Peters et al. 1985, Ussov et al. 1995). It was originally believed that the lungs were the major site of neutrophil margination (Hogg et al. 1995), though subsequent work by a different group challenged this and it is now accepted that the pulmonary involvement is only modest (Peters et al. 1998).

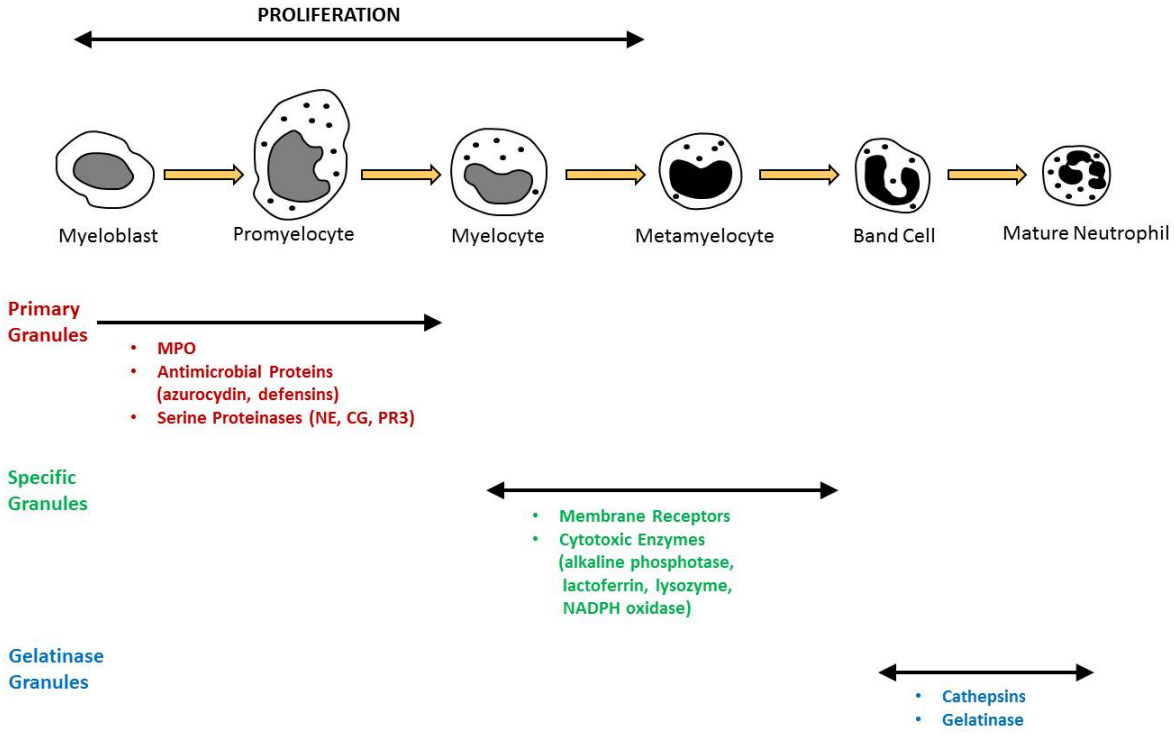

Figure 1.3: A summary of the stages involved in neutrophil differentiation and maturation.

Legend: As neutrophils mature (left to right), they develop various types of granules. A mature neutrophil contains over 200 granules that are essential for immune functions such as migration and bacterial killing (Adapted from Edwards 2005).

1.2.2 Neutrophil Migration

The migration of leukocytes from the circulation into the tissues occurs through a series of distinct steps. The cell must adhere to the blood vessel wall (margination), pass through the endothelial layer (transmigration/diapedesis) and subsequently migrate through the tissue to the site of inflammation. The process is initiated by the interaction activation of adhesion molecules and their respective ligands on the surface of both leukocytes and endothelial cells (Lawrence et al. 1991, von Andrian et al. 1991, Konstantopoulos et al. 1996). Neutrophils, monocytes and lymphocytes all migrate in this manner but differences occur in their response to chemoattractants and other inflammatory signals.

Adhesion

Neutrophil migration begins with the "capture" of neutrophils from the blood flow as they roll along the surface of the endothelium. This initial tethering is a result of the reversible binding of transmembrane glycoprotein adhesion molecules called selectins, which are present on both neutrophils and endothelial cells (Bevilacqua et al. 1993, Albelda et al. 1994, Luscinskas et al. 1994, Crockett-Torabi et al. 1995; Tedder et al. 1995a, Wagner et al. 2000). Selectins contain three different domains: A C-type lectin domain, an epidermal growth-factor-like domain and several regulatory domains. The dominant selectin expressed on the surface of a neutrophil is Leukocyte Selectin (L-Selectin). Under normal, non-pathological conditions, quiescent neutrophils can bind briefly to endothelial cells as they make contact in a "stick and release" manner. This leads to a progressive loss of L-Selectin molecules as they are shed and cannot be replaced (Walchek et al. 1996) and low expression of L-Selectin is associated with neutrophil apoptosis and may incite their removal from the

circulation (Matsuba et al. 1997). Cleavage of L-Selectin from the plasma membrane is predominantly regulated *in vivo* by the sheddase; A Disintegrin and Metalloprotease-17 (ADAM-17) (Li et al. 2006). This process is amplified in the presence of various pro-inflammatory cytokines including Tumour Necrosis Factor Alpha (TNFα), Lipopolysaccharide (LPS), Interleukin-8 (IL-8) and Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) (Drost et al. 2002).

The two other selectins that are known to facilitate neutrophil-endothelial cell adhesion are Platelet Selectin (P-Selectin) and Endothelial Cell Selectin (E-Selectin). These are both bound to the endothelium and are only expressed when certain inflammatory stimuli are present. P-Selectin (CD62P) is stored intracellularly in Weibel-Palade bodies of endothelial cells as well as the α-granules of platelets (Malik et al. 1996). In response to inflammatory stimuli, it is rapidly mobilised to the cell surface where, following the initial interactions with L-Selectin (CD62L), it binds to P-Selectin Glycoprotein Ligand-1 (PSGL-1; CD162) on the surface of neutrophils, forging more durable neutrophil-endothelium associations and eventual tethering (Alon et al. 1997, Davenpeck et al. 1997). As with L-Selectin however, this interaction is not permanent and is reversed in the absence of subsequent adhesive mechanisms (Lawrence et al. 1991, Finger et al. 1996, Davenpeck et al. 1997). E-Selectin (CD62E) is not stored intracellularly and requires gene transcription for expression. Peak expression *in vitro* is achieved between 4 and 6 hours after exposure to inflammatory cytokines (Klein et al. 1995, Scholz et al. 1996). E-Selectin also promotes the rolling and tethering of neutrophils to the endothelium by binding to E-Selectin ligand 1 (Steegmaier et al. 1995), which may help to maintain these functions after P-Selectin has been down-regulated (Malik et al. 1996, Yang et al. 1999).

The next stage is the "firm adhesion" of neutrophils to the endothelium. This is achieved by the activation of additional neutrophil receptors called integrins, which mediate cell-cell and cell-extracellular matrix adhesions (Hynes 1992, Luscinskas et al. 1994, Crockett-Tobari et al. 1995, Williams et al. 1999). Integrins are a ubiquitous group of heterodimeric transmembrane glycoproteins found on all hematopoietic cells. They comprise an α- and β-subunit, which form the extracellular ligand binding site and a cytoplasmic tail, which provides phosphorylation sites and linkages to cytoskeletal proteins involved in signal transduction (Alpin et al. 1998). There are a number of different α- and β-subunits that associate in different combinations to form at least 23 known integrins that are found in a variety of cells including leukocytes, lymphocytes and platelets. In neutrophils, the two most important integrins are Macrophage Antigen-1 (Mac-1; CD11b/CD18) and Lymphocyte-Associated Function Antigen-1 (LFA-1; CD11a/CD18), which both have the same β-subunit (CD18). Of these, Mac-1 appears to be the dominant integrin involved in neutrophil migration (Diamond et al. 1990, Rainger et al. 1997).

In neutrophils, Mac-1 is stored intracellularly in the secretory, specific and gelatinase granules (Sengelov 1996, Borregaad et al. 1997). Following exposure to degranulation stimuli (such as fMLP) or weaker stimuli (including TNFα and LPS that only act on secretory vesicles), it is quickly mobilised to the cell surface where it binds with high affinity to Intercellular Adhesion Molecule-1 (ICAM-1) on the endothelium. However, only a small percentage (approximately 10%) of Mac-1 incorporated into the cell membrane may be capable of ligand binding. This was suggested by *in vitro* work using a monoclonal antibody (CBRM1/5), which recognised only a fraction of Mac-1 on activated neutrophils, but still blocked Mac-1-dependent adhesion to fibrinogen and ICAM-1 (Diamond et al. 1993). Mac-

1/ICAM-1 interactions in turn causes an increase in endothelial surface expression of both ICAM-1 and a similar molecule, Vascular Cell Adhesion Molecule-1 (VCAM-1), suggesting that both molecules may be integral to neutrophil migration (Clayton et al. 1998). In addition, inflammatory stimuli can promote transcription/translation of the Mac-1 genes, thereby extending integrin involvement during inflammation.

Transmigration/Diapedesis

Once firmly attached, neutrophils must then pass through the endothelial cell barrier before progressing through the surrounding tissue to the site of inflammation. Initial evidence suggests that transendothelial migration occurs paracellularly, where three or more adjacent endothelial form a "tricellular corner" (Burns et al. 1997, Su et al. 2002). At these junctions, there is a localisation of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1; CD31), which is thought to attract neutrophils (that also express PECAM-1) to these specific areas. Selective blocking of PECAM-1 with antibodies on neutrophils or endothelial cells prevents transmigration (Muller et al. 1995). Furthermore, Mac-1 deficient neutrophils are unable to crawl along the endothelium and subsequently develop pseudopods that probe the endothelial cell membrane (Phillipson et al. 2006, Phillipson et al. 2008). Under these experimental conditions, neutrophils are seen to migrate transcellularly through the body of endothelial cells, a process that may occur to a greater extent when the paracellular route is unavailable. The proportion of neutrophils that normally undergo transcellular migration remains unclear. Some studies suggest that the majority utilise this route (Feng et al. 1998, Williams et al. 2010), but this is at variance with other research (Ionescu et al. 2003, Yang et al. 2005, Carman et al. 2007, Phillipson et al. 2008), particularly Woodfin et al. who have quantified

by real-time confocal imaging *in vivo* that 90% of neutrophils migrate via the paracellular route (Woodfin et al. 2011).

Endothelial/neutrophil PECAM-1 interactions result in an increased expression of Very Late Activation Antigen-6 (VLA-6; CD49f) on the surface of neutrophils. This integrin binds to components of the extracellular matrix (such as collagen and laminin), which facilitates neutrophil migration beyond the vasculature and through the tissue. Migration through the matrix is further aided by the release of neutrophil proteinases, particularly NE (Wright et al. 1979). However, it is currently unclear as to whether proteinase release is actually necessary for migration through the extracellular matrix. Some studies have shown that chemotaxis through artificial substrates in the presence of fMLP can be partially inhibited by inhibitors of NE and Matrix Metalloproteinase-9 (MMP-9) (Stockley et al. 1990, Declaux et al. 1996). In contrast, studies of neutrophil migration across endothelial monolayers and basement membrane matrices show that chemotaxis is unaffected by proteinase inhibitors (Allport et al. 1997, Mackeral et al. 1999). However, the role of CG and NE in neutrophil migration is unclear as the evidence is conflicting. In one study, CG and NE knockout animal models exhibit normal neutrophil migration both *in vitro* and *in vivo* (Tkalcevic et al. 2000), whereas a more recent animal model demonstrated an attenuation of neutrophil influx into areas of inflammation following the administration of a similar peptidase inhibitor (Oliveira C et al. 2010).

The variability of these results may be reflective of the type of substrate used but also different migratory processes adopted by neutrophils. In non-cross linked collagen 3D matrix, neutrophils will preferentially migrate along the fibres but can squeeze through the gaps between fibres when proteinases are inhibited. In contrast, in a more densely

concentrated cross-linked collagen 3D matrix, neutrophils seem to require proteinases to migrate through as the gaps are not large enough to facilitate proteinase-independent migration. Therefore, it seems that neutrophils may prefer to use proteinases to ease their migratory passage through artificial or biological matrices but can still migrate effectively through them when gaps between fibres are sufficiently large. The processes involved in neutrophil adhesion and migration are summarised in Figure 1.4.

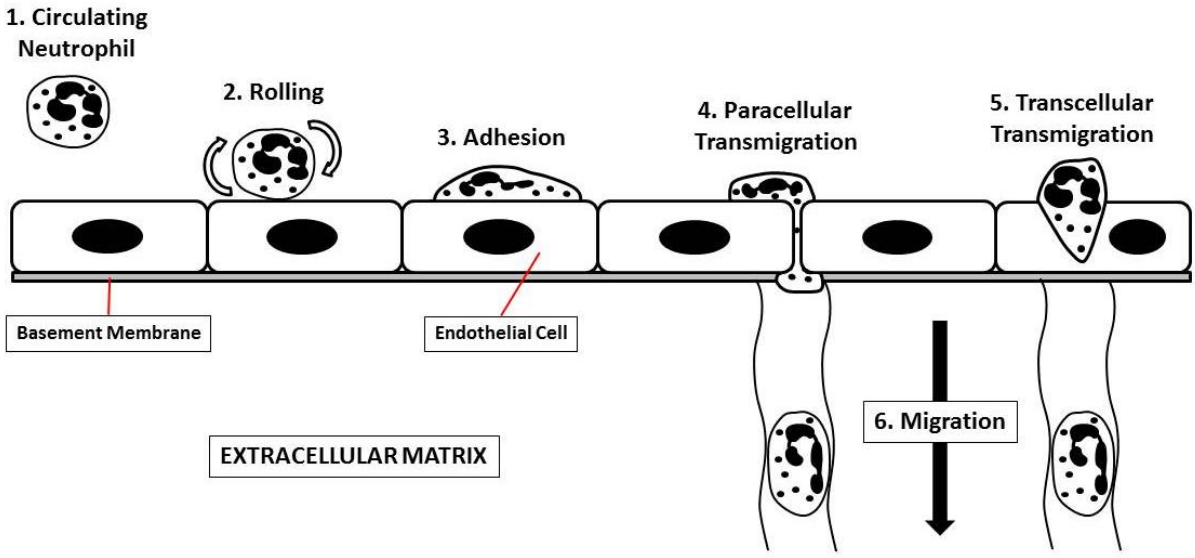

Figure 1.4: A summary of the sequential processes involved in neutrophil adhesion and migration.

Legend: Peripheral blood neutrophils (1) begin their adhesion to the endolthelium by rolling along the suface in a "stick and release" manner (2) before they become firmly adhered (3). They then crawl along the endothelium until a suitable point for transmigration is found. This may either occur between endothelial cells (4. paracellular transmigration) or through an endothelial cell (5. transcellular migration). Following passage through the endothelium and basement membrane, neutrophils migrate through the extracellular matrix to the site of inflammation (6).

Chemotaxis

Chemotaxis is fundamental to neutrophil inflammatory function. Without accurate chemotaxis, the ability of neutrophils to reach the site of inflammation and subsequently locate invading pathogens would be significantly impaired.

Neutrophils migration is mediated by a large number of different chemical stimuli. Pro-migratory stimuli can be classed as either non-chemotactic cytokines or chemoattractants. For instance, TNFα and Interleukin-1 (IL-1) may induce expression of adhesion molecules on neutrophils and endothelial cells, but they do not possess chemoattractive qualities. Neutrophils migrating towards a site of inflammation are continually exposed to a large number of chemoattractants from a variety of different sources (including endothelial, epithelial and inflammatory cells as well as pathogenic organisms), with each chemical eliciting a different response. At the start of migration, neutrophils follow a chemoattractive gradient toward an initial signal up to its peak. At this point, the concentration of chemoattractant is so high that the receptors become saturated and the signal is ineffective (Foxman et al. 1997). Neutrophils then start along a new gradient from a second signal and so on until they reach the target inflammatory site. The resulting effect is a "chemoattractant hierarchy", where neutrophils initially follow relatively weak host chemoattractants, then proceed to followed more potent ones and, finally, terminal chemoattractants that originate from invading pathogens.

Neutrophil movement is achieved by the formation of actin-rich protrusions called "pseudopods" (Devreotes et al. 1988). Following stimulation by chemoattractants, the cell polarises into a leading edge, which is made up of numerous small cytoskeletal projections, rich in filamentous Actin (F-Actin), called "lamellipodia" and a trailing edge or "uropod". To

facilitate effective migration, there is a reorganisation of cell surface receptors, where the chemoattractant and integrin receptors are localised to the pseudopods (Gomez-Mouton et al. 2001) and the adhesion molecules to the uropod (Fais et al. 2003). Pseudopods are continually and randomly generated at the leading edge of the cell. For a neutrophil to direct itself down a concentration gradient, the pseudopods projecting in the direction of the gradient are sustained, thereby allowing the cell to alter its trajectory and follow the correct course. (Andrew et al. 2007).

1.2.3 Signalling to Migration

Once chemoattractants bind to their specific receptors on the neutrophil cell surface, they initiate a signal pathway that results in cytoskeletal rearrangement and motility. The signalling pathways involved are similar for all chemoattractant receptors. The first step in the cascade is the activation of heterotrimeric Gi proteins, which comprise an α-,β- and γ-subunit. Activation leads to the hydrolysis of Guanosine-5'-Triphosphate (GTP) to Guanosine Diphosphate (GDP) on the α-subunit, resulting in its dissociation from the βγ-subunit (Hofmann et al. 2009). Following this, the βγ-subunit activates a number of cellular mediators including Phospholipase-C (PLC) and calcium ions (Boyer et al. 1992), Phosphatidylinositol 3-Kinase-γ (PI3Kγ) (Stephens et al. 1994), G Protein-Coupled Receptor (GPCR) Kinases, P21-Activated Kinase-1 (PAK-1) (Li et al. 1993).

Protein phosphorylation is a widely used post-translational biological signalling mechanism. Families of protein phosphatases also catalyse opposite reactions, making phosphorylation–dephosphorylation a reversible process. Protein kinases play a key role in

the regulation of almost every aspect of cell biology. The human protein kinase family is one of the largest gene families, consisting of 518 genes (Manning et al. 2002).

PI3K/Akt Pathway

Receptor-ligand binding activates a number of cellular mediators including phosphatidylinositol-3 kinase (PI3K). PI3Ks are a family of enzymes that catalyse the phosphorylation of one or more inositol lipids in the 3 position of the inositol ring early in the signalling cascade (Rickert et al. 2000). They are intimately involved in a number of cellular functions such as growth, proliferation, differentiation, and survival, and are key regulators of neutrophil migration (Stephens et al. 2002, Hannigan et al. 2002). There are eight mammalian PI3K isoforms that are classified into three families defined by their structure, substrate specificity and function. These include four Class I isoforms (PI3Kα, β, γ and δ), three Class II isoforms (PI3KCα, β, γ) and one Class III (Vanhaesebroeck 2010, Okkenhaug 2013). Each Class I isoform catalyses the same reaction but are distinct in their upstream activation by receptor transduction pathways (Vanhaesebroeck 2010, Fritsch et al 2013). PI3K catalyses the phosphorylation of phosphatidylinositol 4,5 bisphosphate (PIP_2) to phosphatidylinositol 3,4,5 trisphosphate (PIP_3) at the plasma membrane.

Class I PI3Ks are soluble enzymes that are activated by a number of factors, resulting in their recruitment to the membrane and conformational changes that increase their catalytic efficiency. The α and β isoforms are broadly expressed in a variety of tissues and have a fundamental role in cellular function (such as proliferation), whereas the δ and γ isoforms have a more restricted cellular distribution and have a more focussed mediatory role in an inflammatory response. All class I isoforms are heterodimers that comprise both a catalytic

subunit and one of four different regulatory subunits. Depending on the coupling of these subunits, Class I PI3Ks are further classified into Class 1a and Class 1b. Class Ia isoforms (PI3Kα, β, and δ) comprise a p110 catalytic subunit and a p85 regulatory subunit (Carpenter et al. 1990). There are five variants of the p85 regulatory subunit variants (p85α, p55α, p50α, p85β, and p55γ) and three variants of the p110 catalytic subunit (p110α, β, or δ), resulting in fifteen potential heterodimers. The first three p85 regulatory subunits (p85α, p55α, p50α) are all splice variants of the same gene (PIK3R1), whereas p85β and p55γ are encoded by the PIK3R2 and PIK3R3 genes, respectively. The p85 regulatory subunits contains several domains, including two Src Homology (SH)2 domains and a SH3 domain (Krasilnikov et al. 2000). Together, these regulate the binding of Class Ia PI3Ks to tyrosine-phosphorylated receptor tyrosine kinases. This interaction has two consequences that must both occur in order for PI3K to become fully activated (Songyang et al. 1993, Yoakim et al. 1994). Firstly, PI3K translocates from the cytosol to the inner leaflet of the plasma membrane, which allows the p110 catalytic subunit access to PIP_2 (Gillham et al. 1999). Secondly, conformational changes of the p85 regulatory subunit occur that negate its inhibitory effect of catalytic activity of p110 (Yu et al. 1998).

At present, only one Class Ib isoform (PI3Kγ) has been identified. PI3Kγ comprises a p110γ catalytic subunit (Stoyanov et al. 1995) and either a p84 (Suire et al. 2005, Voight et al. 2005, Voight et al. 2006) or p101 regulatory subunit (Stephens et al. 1997). PI3Kγ is activated by chemokines through GPCRs and direct interaction with the G-βγ subunit (Ferguson et al. 2007, Stephens et al. 2008). Therefore, the primary chemokine-mediated responses occur through PI3Kγ, although PI3Kδ may also play a later role by sustaining this response in a "biphasic" manner (Condliffe et al. 2005). Interaction between regulatory p101

and catalytic p110 subunits is very tight, although the precise activation mechanism remains unclear. The p101 regulatory subunit binds readily to G-βγ and it has been proposed that this acts as an essential anchor linking G-βγ with the catalytic p110 subunit (Stephens et al. 1997). In contrast, a different study demonstrated that p101 is not required for G-βγ activation of PI3Kγ (Stoyanov et al. 1995). Later studies support this hypothesis by showing that binding of the G-βγ subunit to the different subunits of PI3Kγ actually induces different mechanisms of its activation. They demonstrated that direct stimulation of the catalytic subunit p110 was sufficient to induce PIP_3 formation but that binding to the p101 regulatory subunit mediates PI3Kγ recruitment to the membrane (Brock et al. 2003) and substrate selectivity (Maier et al. 1999). Far less is understood about p84/p110 interactions, which are less well described, although studies have shown that, despite strong association, the p84/p110γ complex is less sensitive to G-Protein βγ activation than p101/p110γ (Suire et al. 2005, Kurig et al. 2009). p110γ also contains a Ras-Binding Domain (RBD), which binds selectively to Ras-GTP. Ras-GTP synergises the co-localise PI3K and PIP_2 before its phosphorylation occurs (Suire et al. 2006) and *in vitro* studies have shown that it can activate p110γ directly as well as the p84/p110γ and p101/p110γ complexes (Pacold et al. 2000). A more recent study suggests that Ras-GTP exclusively activates p84/p110γ, whereas p101/p110γ activation only occurs via the G-Protein βγ-subunit binding (Kurig et al. 2009). Although this evidence is conflicting (at least partially), collectively it suggests that p84/p110γ activation may be controlled by ligands capable of activating Ras (such as tyrosine kinases) rather than GPCRs.

Formation of PIP_3 results in the recruitment of Akt to the cell membrane during neutrophil polarisation (Burelout et al. 2007). Akt comprises an amino-terminal pleckstrin

homology (PH) domain, a serine-threonine kinase domain and a COOH-terminal regulatory tail. Phosphorylation of Akt at both S473 by the pyruvate dehydrogenase kinase 2 (PDK2) class of kinases (including mammalian Target of Rapamycin Complex 2 (mTORC2)) and, subsequently, T308 by phosphoinositide dependent kinase 1 (PDK1) is necessary for full kinase activity (Scheid et al. 2003, Hanada et al. 2004, Ananthanarayanan et al. 2005). Furthermore, Akt stabilises the organisation of the actin cytoskeleton during cell migration, where its activity is regulated by PI3K as well as mTORC2 (Lee et al. 2005).

PIP_3 and Akt localisation at the leading edge results in the formation of F actin-rich pseudopodia (Yoo et al. 2010). These may either induce forward movement (in migration) or pathogenic engulfment (in phagocytosis). A number of studies have implicated Akt as a key regulator of neutrophil chemotaxis (Hannigan et al. 2002, Heit et al. 2002, Sasaki et al 2006, Lane et al. 2006, Kolsch et al. 2008). The phosphorylation of Akt is also considered to be a strong indicator of the activation of PI3K and its pathway (Weiner 2002, Inoue et al. 2008).

Rho GTPases

PI3K signalling is also mediated by small (~21kDa) G-proteins belonging to the Rho family of GTPases that act as molecular "switches" for many other biological processes, including survival and organelle development (Boureux et al. 2007, Bustelo et al. 2007). They are regulated by GTPase-activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and guanine nucleotide dissociation inhibitors (GDIs). GAPs mediate the hydrolysis of GTP to GDP by GTPase, thereby regulating the rate of transition from the active to inactive status. GEFs activate Rho proteins by catalysing the exchange of GDP for GTP. GDIs combine with Rho-GTPases to form a large complex and prevent their diffusion

into the cytosol, thereby allowing for tightly regulated spatial control of rho activation (Ellenbroek et al 2007). Spatially regulating Rho GTPases in this manner at the leading edge of the cell is important in polarity but may also act as a directional compass during chemotaxis (Rickert et al. 2000).

In mammals, the Rho family contains 22 members (Ridley et al. 2006) and a number of these are essential for neutrophil migration. These include Rac1 and Rac2, which mediate actin polymerisation at the leading edge of the cell (Srinivasan et al. 2003), Cdc42, which limits PTEN activity to the rear of the cell and is involved in the formation of the PIP_3 gradient and cell polarity (Srinivasan et al. 2003), Rho and Rho Kinase (ROCK), which drive cell propulsion by orchestrating myosin light chains and Myosin II contraction at the rear of the cell (Wu et al. 2005, Charest et al. 2007).

Neutrophil Polarity

There is substantial evidence suggesting that PI3K signalling has a pivotal role in neutrophil polarisation. Servent et al. showed that Akt is selectively mobilised to the leading edge of neutrophils following exposure to a chemoattractive stimulant (Servent et al. 2000). In a follow-up study, Weiner at al. investigated the spatial distribution of PIP_3 using neutrophil-like differentiated HL-60 cells. They demonstrated that, using a cationic lipid shuttling mechanism, exogenous delivery of PIP_3 elicits accumulation of endogenous PIP3 at the cell's leading edge. They describe the paradox that PIP_3 and Rho GTPases (Rac and Cdc42) function both upstream and downstream of one another appears to be a positive feedback loop mechanism that results in a self-organised intracellular PIP_3 gradient that leads to cell polarity (Weiner et al. 2002).

In a similar study using exogenous introduction of PIP_3 to the inner leaflet of the neutrophil plasma membrane, Tian et al. showed a number of separate effects related to neutrophil polarity (Tian et al. 2003). Firstly, exogenous PIP_3 was incorporated into the membrane in distinct regions and this increased the random formation of small pseudopodia and, later, the formation of the single, large pseudopod that is associated with cell polarisation. During this process, PIP_3 was subsequently excluded from the site of pseudopod extension to the uropod, where it was inactivated, only to return the original site following pseudopod relaxation. Interestingly, they found that pseudopodia did not form in the PIP_3-containing regions when neutrophils were stimulated by fMLP. They suggest this may be because the exogenous PIP_3 induced the formation of an artificial 'uropod-like' structure. Lastly, the introduction of exogenous PIP_3 resulted in repetitive global calcium (Ca^{2+}) signalling event, the onset of which precedes morphological polarisation. Importantly, the Ca^{2+} signalling was inhibited by a PI3K inhibitor (LY294002), suggesting that it was mediated (at least in part) by increased PI3K.

PI3K activity is regulated by both SH2 domain-containing inositol phosphatase 1 (SHIP1) and phosphatase and tensin homologue (PTEN), which both hydrolyse PIP_3 (to phosphatidylinositol 3,4 bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$), respectively) and prevent pseudopod extension (Sly et al. 2003, Heit et al. 2008). Evidence suggests that SHIP1 is essential for chemoattractant-mediated neutrophil migration. It is believed that PTEN and SHIP1 are both important in establishing a PIP_3 gradient, with PTEN co-ordinating a front-to-back gradient and SHIP1 co-ordinating a top-to-bottom gradient, together ensuring PIP_3 is exclusively expressed at the leading edge (Mondal et al. 2012). It has also been suggested that SHIP1 is the predominant phospholipid phosphatase in

this process as disruption of SHIP1 (not PTEN) results in uniform F-actin polymerisation across the cell membrane rather than localisation to the leading edge. As a result, these neutrophils become flattened, do not correctly polarise and migrate at a dramatically slower rate (Nishio et al. 2007, Mondal et al. 2012).

A recent *in vivo* study using zebrafish embryos has also provided novel insight into how neutrophil polarity is regulated by PI3K (Yoo et al. 2010). The authors firstly demonstrated that neutrophil recruitment to a laser-induced wound was inhibited by both LY294002 and a PI3Kγ-specific inhibitor. Secondly, high-resolution ratiometric imaging revealed that both PIP_3 and $PI(3,4)P_2$ (following hydrolysis by SHIP1) were located at the leading edge pseudopods during neutrophil migration. Lastly, activation of Rac at specific areas rapidly induced pseudopod extension and directional migration, supporting the hypothesis that there is a positive feedback loop between Rac and PIP_3 at the leading edge. Interestingly, activation of Rac at the leading edge of PI3K-inhibited neutrophils was sufficient to induce pseudopod extension but not migration or uropod contraction (due to an inability to rescue normal polarity of F-actin dynamics).

Bound integrins traffic to the rear of the cell, where they detach for recycling (Pierini et al. 2000). Integrins also facilitate cell polarity, activating the Rho family of GTPases (Huttunlocher et al. 2005, Caswell et al. 2009). Phagocytosis is also PI3K and β2 integrin-dependent (Schnitzler et al. 1999, Dewitt et al. 2003), leading to PI3K-driven PIP_3 accumulation at the leading edge of the cell where engulfment occurs (Botelho et al. 2000, Hannigan et al. 2003, Kennedy et al. 2008).

The orientation of the predominant signalling mediators and the movement machinery within a polarised neutrophil are represented graphically in Figure 1.5.

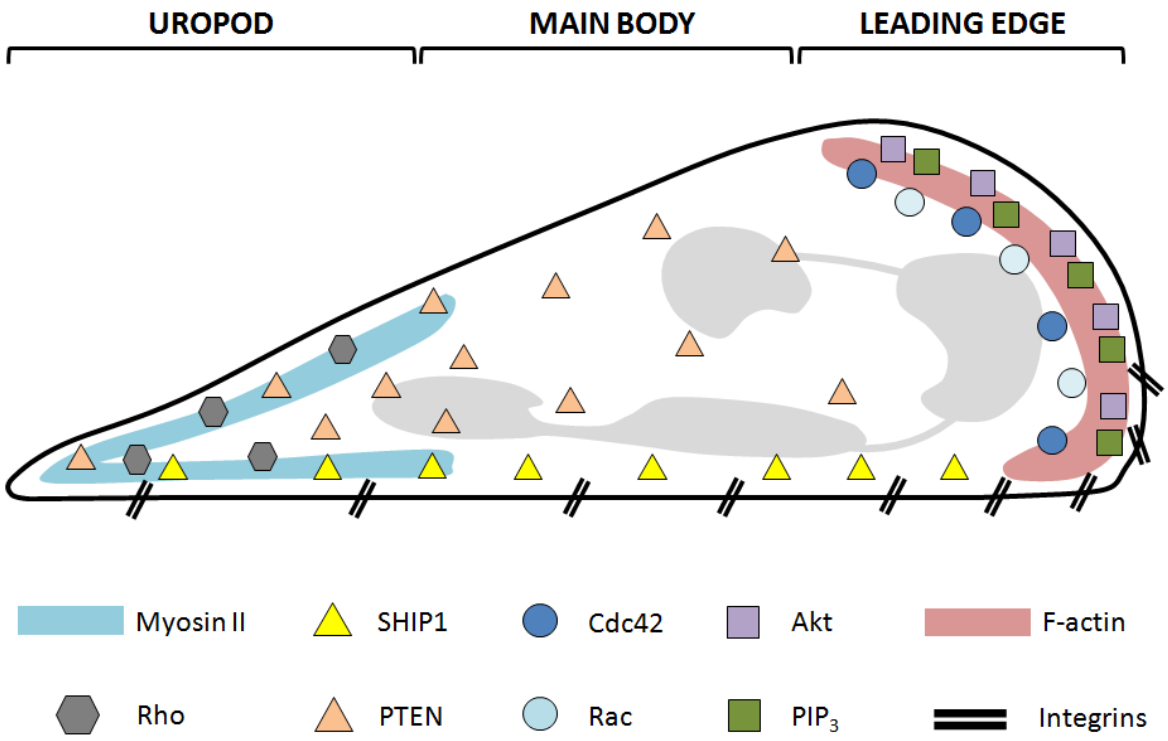

Figure 1.5: A polarised neutrophil showing the localisation of signalling mediators and movement machinery.

The signalling molecules associated with F-actin polymerisation, including Akt, PIP3, Rac and Cdc42 are located at the leading edge to facilitate pseudopod extension. In contrast, myosin II and Rho localise at the rear of the cell to detach the uropod and enable continual migration. PTEN and SHIP1 orientate towards the back and at the bottom of the cell, respectively to limit PIP3 to the leading edge.

Mitogen-Activated Protein Kinases

Until relatively recently, it was believed that chemotaxis was exclusively regulated by PI3K. However, a plethora of other signalling molecules have subsequently been implicated in regulating neutrophil chemotaxis, thereby creating a complex web of inter-related signalling pathways. The Mitogen-Activated Protein Kinases (MAPKs) are family of serine/threonine protein kinases involved regulating a variety of cellular activities ranging from gene expression to mitosis, proliferation, apoptosis, phagocytosis and chemotaxis (Heit et al. 2007). They include c-Jun N-terminal kinases (JNK) which phosphorylates paxillin (inhibiting Rac at the cell sides and back), Extracellular Signal-Related Kinase (Erk) which phosphorylates myosin light chain kinase and p38 which phosphorylates MAPK-activated protein kinase 2/3 that is involved in directionality (Huang et al. 2004). It has also been suggested that p38 and Erk act as the migratory "stop" and "go" signal, respectively (Li et al. 2012).

The PI3K pathway is together with the involvement of these other associated signalling pathways is summarised diagrammatically in Figure 1.6.

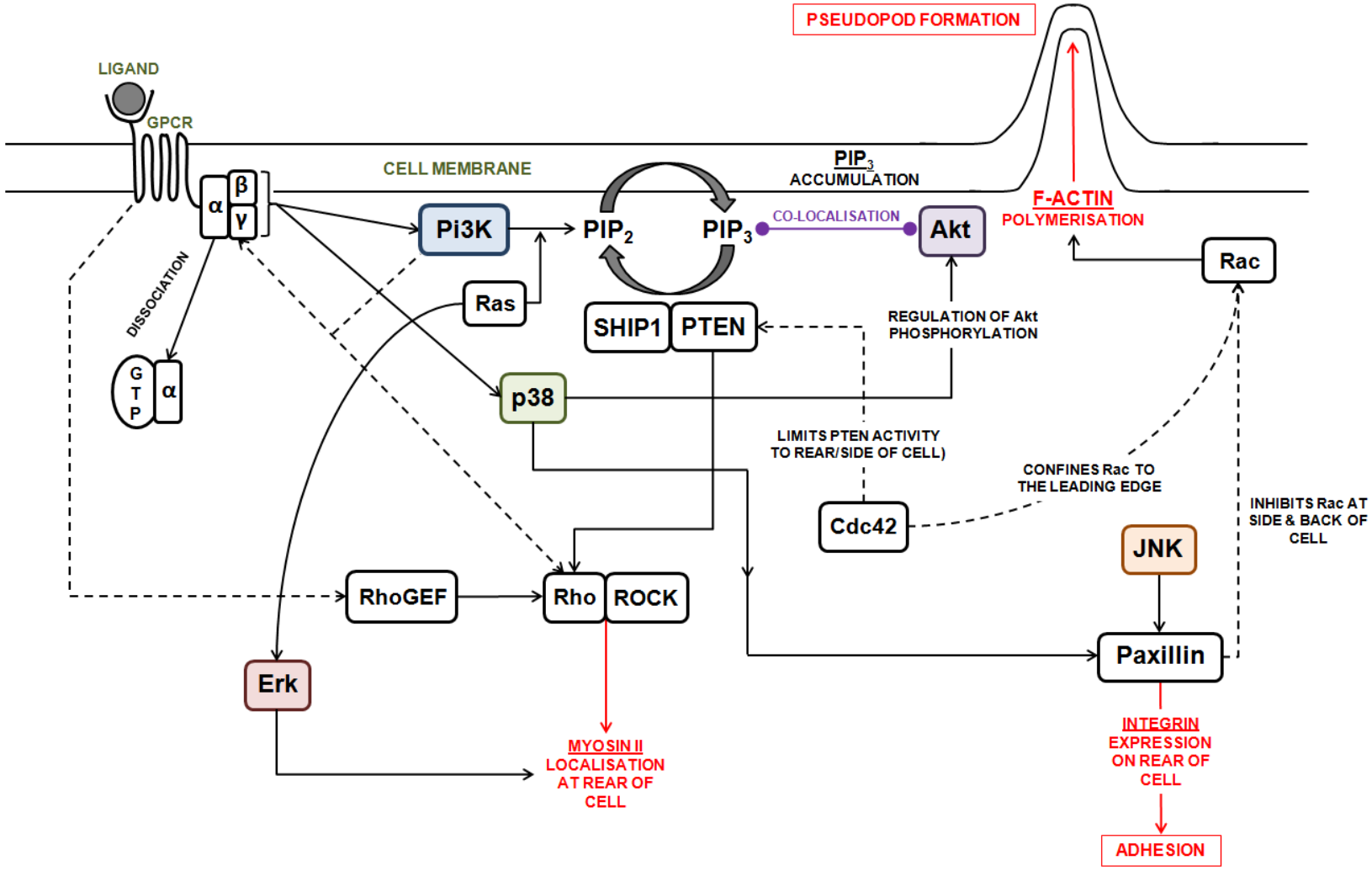

Figure 1.6: A simplified diagram of the PI3K signalling pathway.

Legend: G-Protein coupled receptor (GPCR) binding with ligand up-regulates PI3K phosphorylation of PIP_2 to PIP_3. PIP_3 accummulation at the leading edge of the cell leads to F-actin localisation and pseudopod protrusion, which is coordinated by Rac and Cdc42. PI3K and PTEN activate Rho at the rear of the cell, which inhibits F-actin polymerisation while inducing Myosin localisation. PI3K up-regulates integrin expression and recycling at the rear of the cell and increases GPCR recycling to the leading edge. The Mitogen-Activated Protein Kinases (MAPKinases) JNK, Erk and p38 are also directly involved in cell migration by regulating the phosphorylation of downstream mediators.

Calcium Signalling

Calcium (Ca^{2+}) is a simple and incredibly diverse second messenger and is one of the most extensively studied signalling molecules in biology. Ca^{2+} signalling has been implicated in a wide variety of important physiological roles (Allbritton et al. 1992). Most notably, these include muscle contraction, neuronal transmission, fertilisation, cell growth and proliferation and cell motility. The level of intracellular (cytoplasmic) calcium has been specifically linked to neutrophil polarisation and migration and is believed to play a key role in chemotaxis (Meshulam et al. 1986, Marks et al. 1990). Neutrophil polarisation is an essential precursor to accurate cell migration, which contributes to an adequate innate immune response (O'Donnell et al. 1992, Onsum et al. 2007). Other recent studies (Evans et al. 2007, Wei et al. 2011) further support this model, where local calcium signals were shown to occur at the leading edge of polarised and migrating cells. Collectively, these studies suggest that calcium is a critical component of cell polarisation and/or directed migration.

The resting concentration of cytoplasmic Ca^{2+} is generally maintained at low levels between 10–100 nM. To maintain this, Ca^{2+} is actively pumped from the cytosol to the extracellular space and into the endoplasmic reticulum (ER), and sometimes in the mitochondria (Clapham 2007). Specific signals can induce rapid and dramatic increases in the cytoplasmic Ca^{2+} level (up to 1000 nM) by opening channels in the ER or the plasma membrane. Indeed, calcium spikes at the plasma membrane have been shown to synchronise neutrophil arrest and migration during rolling and adhesion by integrating chemotactic and adhesive signals (Schaff et al. 2008). In addition, Ca^{2+} accumulation in the uropod has been demonstrated, which may be important for cell polarisation (Clark et al. 2008) and Ca^{2+}-

dependent myosin II activation may be required for uropod retraction during neutrophil migration (Eddy et al. 2000).

The most common signalling pathway by which this occurs is the PLC pathway. Numerous surface receptors, including G protein-coupled receptors activate PLC, which in turn hydrolyses PIP_2 to form the downstream messengers, inositol 1,4,5-trisphosphate **(**IP_3) and diacylglycerol (DAG). IP_3 then diffuses to the ER, where it binds to its receptor and induces the release of Ca^{2+}. DAG activates protein kinase C (PKC), which is important in several other signal transduction cascades as well as PI3K. The GPCR/Ca^{2+} signalling cascade is illustrated simply in Figure 1.7.

Many of calcium-mediated physiological functions occur when released Ca^{2+} binds to and activates the regulatory protein calmodulin (calcium-modulated protein**)**. In migration, this includes the regulation of calcium/calmodulin-dependent myosin light chain kinase and suppression of β_1 and β_3 integrin release at the rear of the cell (Niggli 2003). A number of the proteins that calmodulin binds to are unable to bind calcium themselves, and use calmodulin as a calcium sensor and signal transducer (Chin et al. 2000).

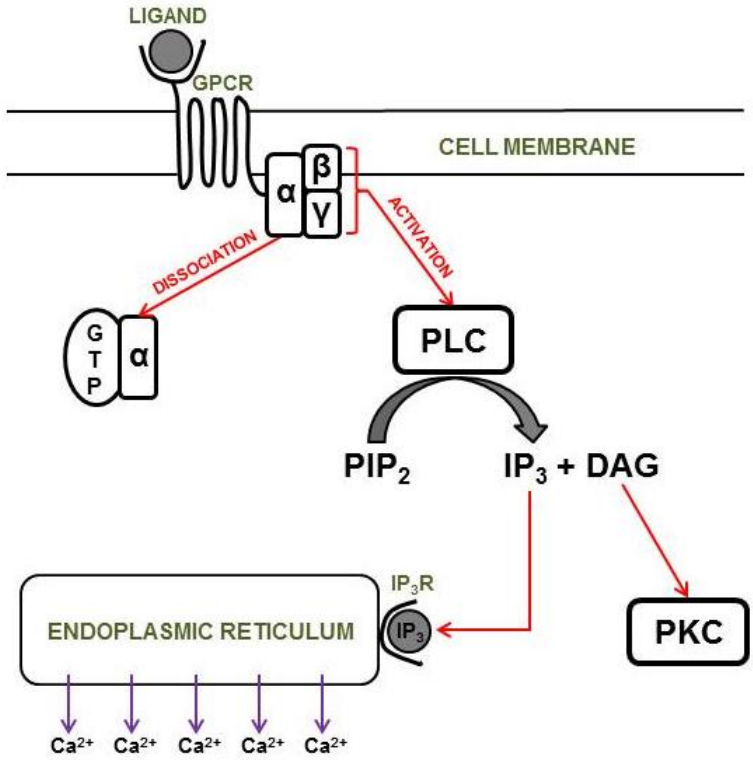

Figure 1.7: A simplified diagram of the Ca^{2+} signalling pathway.

Legend. G-Protein coupled receptor (GPCR) binding with ligand activates PLC. This catalyses the hydrolysis of PIP_2 to IP_3 and DAG. IP_3 diffuses to the ER, where it binds to its receptor and induces Ca^{2+} release, while DAG activates PKC (a messenger of other signalling cascades).

The importance of Ca^{2+} in cell migration has been highlighted in a number of previous studies. Calpain is an intermediate signalling molecule downstream of chemoattractant-induced membrane lipid reorganisation and has previously been implicated as an important Ca^{2+}-dependent regulator of actin and the cytoskeletal rearrangement associated with cell migration (Huttenlocher et al. 1997, Kulkarni et al. 1999, Dourdin et al 2001). A later study showed that the intracellular Ca^{2+}-dependent protease calpain is active in quiescent neutrophils and its inhibition enhances random cell movement (Lokuta et al. 2003). In a

follow-up study, the same group demonstrated that calpain 2 (but not calpain 1) was associated with the "frontness" signal during neutrophil polarisation (Nuzzi et al. 2007). A more recent study using differentiated HL-60 neutrophil-like cells showed that filling of Ca^{2+} stores in the endoplasmic reticulum (termed store-operated Ca^{2+} entry (SOCE) activates Akt, Rac and Cdc42 and this could be the primary Ca^{2+} mechanism involved in cell polarisation (Zou et al. 2012).

1.2.4 Chemoattractants

Gradient sensing is achieved by receptor occupancy, distribution, and desensitisation (Wu et al. 2007). Neutrophils have at least five different types of receptor for chemoattractive stimuli, which include unique receptors for Complement Protein 5a (C5a), Leukotriene B_4 (LTB_4), Platelet Activating Factor (PAF), and fMLP. A number of other receptors also exist that share similarities in structure and bind to a group of chemoattractants called "chemokines". Chemokines contain similar cysteinyl-containing structures at the NH_2 terminus that form disulphide bridges. They are classified into CC chemokines (where the cysteines are adjacent) and CXC chemokines (where the cysteines are separated by an amino acid). CXC chemokines include, amongst others, IL-8 and Growth-Related Oncogene Alpha (GROα).

Interleukin-8

IL-8 (CXCL8) is the most extensively studied and one of the most important chemokines in neutrophil migration. It is a small 8 kDa protein primarily produced by leukocytes (monocytes, NK cells, neutrophils and T-Cells), though it is also produced by epithelial and

endothelial cells. Its production is induced by a variety of factors including pro-inflammatory cytokines such as IL-1β and TNFα (Matsushima et al. 1988), bacteria and their products such as fMLP (Dimango et al. 1995, Khair et al. 1996), certain viruses and oxidants in tobacco smoke (Deforge et al. 1993). On neutrophils, IL-8 binds with high affinity to both neutrophil chemokine receptors, CXCR1 and CXCR2. Once bound, it may induce a number of effects, although the dominant function appears to be the recruitment of neutrophils to sites of inflammation (Roberts et al. 1993). In support of this hypothesis, it has been demonstrated that IL-8 increases adhesion through up-regulation of integrin expression (Von Andrian et al. 1992). Furthermore, IL-8 induces migration via induction of the PI3K pathway (Knall et al. 1997), which also contributes to cell directionality during chemotaxis (Stephens et al. 2002, Hannigan et al. 2002, Wang et al. 2002, Wu 2005, Sapey et al. 2011).

Growth-Related Oncogene Alpha

GROα (CXCL1), together with a number of other chemokines, acts primarily through CXCR2 as they have only a low affinity for CXCR1 (Wagner et al. 2000, Tsai et al. 2002). It is secreted by human melanoma cells, has mitogenic properties and has been implicated in melanoma pathogenesis (Richmond et al. 1988, Anisowicz et al. 1987). It is expressed by macrophages, neutrophils and epithelial cells (Iida et al. 1990, Becker et al. 1994) and has neutrophil chemoattractant properties (Moser et al. 1990, Schumacher et al. 1992).

Leukotriene B_4

Leukotrienes are generated via the activities of lipoxygenases on arachadonic acid. LTB_4 is a powerful chemoattractant for neutrophils that is primarily produced by monocytes,

macrophages and activated neutrophils (Werz 2002). In addition, it can also induce neutrophil adherence to the endothelium, aggregation and, at higher concentrations, degranulation. The LTB_4 receptor (LTB_4R1) may exist in a high affinity state (where binding leads to chemotaxis and adhesion through Mac-1 (Tonnesen et al. 1989) and a low affinity state (where binding leads to secretion and oxidase activity).

Complement Fragment C5a

The binding of an antibody to its antigen often triggers this complement system, which helps or "complements" the ability of antibodies and phagocytes to clear pathogens. When stimulated by one of several triggers, proteinases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. C5-derived products are chemoattractants for various leukocytes including basophils, eosinophils, monocytes and neutrophils. C5a is the most potent chemoattractant of these products and can bind to neutrophils in the circulation (Kohl et al. 1983). In addition, epithelial cells and tissue macrophages produce C5a via the alternative complement system (Strunk et al. 1988).

Formyl-Methionyl-Leucyl-Phenylalanine

Peptides of bacterial origin, such as fMLP, are potent activators of neutrophils and fMLP is commonly used for this role in *in vitro* studies. The fMLP receptor (Formyl Peptide Receptor 1; FPR1) is expressed on the surface of quiescent neutrophils, although activation induces a dramatic and rapid increase in expression via the mobilisation of internal secretory vesicles (Borregard et al. 1997). It binds rapidly to the fMLP receptor, causing polarisation even in the absence of a concentration gradient. Depending on the concentration of fMLP, a

variety of effects on neutrophils may be activated. These include aggregation, Reactive Oxygen Species (ROS) production, degranulation, cytoskeletal changes and chemotaxis (Wagner et al. 2000).

1.2.5 Receptor Recycling

Following ligand binding, GPCRs become reversibly desensitised and are internalised for recycling or degradation or are shed. Internalisation, therefore, results in a reduction in surface receptor expression (Painter et al. 1995, Fan et al. 2003). This process occurs via GPCR Kinases (GRKs), which phosphorylate the respective receptors (Prossnitz et al. 1995) and allow accessory proteins (such as arrestins) to bind, inducing cleavage of the receptor from its G protein. If internalisation of one receptor is high enough, migration may be attenuated, at which point the neutrophil may follow a new gradient formed by a more potent chemoattractant. The importance of receptor recycling in the maintenance of chemotaxis has been demonstrated, whereby interruption of recycling results in reduced cell migration (Samanta et al. 1990).

FPR1 is endocytosed into receptosomes while still bound to fMLP. Receptosomes contain surface membrane neutral endopeptidase (NEP) that degrades fMLP, thereby preventing it from rebinding to unoccupied receptors. The receptors are then recycled to the cell surface (Painter et al. 1995). CXCR1 and CXCR2 are recycled by the same processes, though evidence suggests that CXCR1 and CXCR2 may recycle through different endosomes; CXCR1 is re-expressed far more quickly than CXCR2 following stimulation with and subsequent removal of IL-8. Only partial re-expression of CXCR2 was observed 60

minutes after IL-8 removal (Chuntharapai et al. 1995). The mechanisms of receptor internalisation and re-expression are illustrated in Figure 1.8.

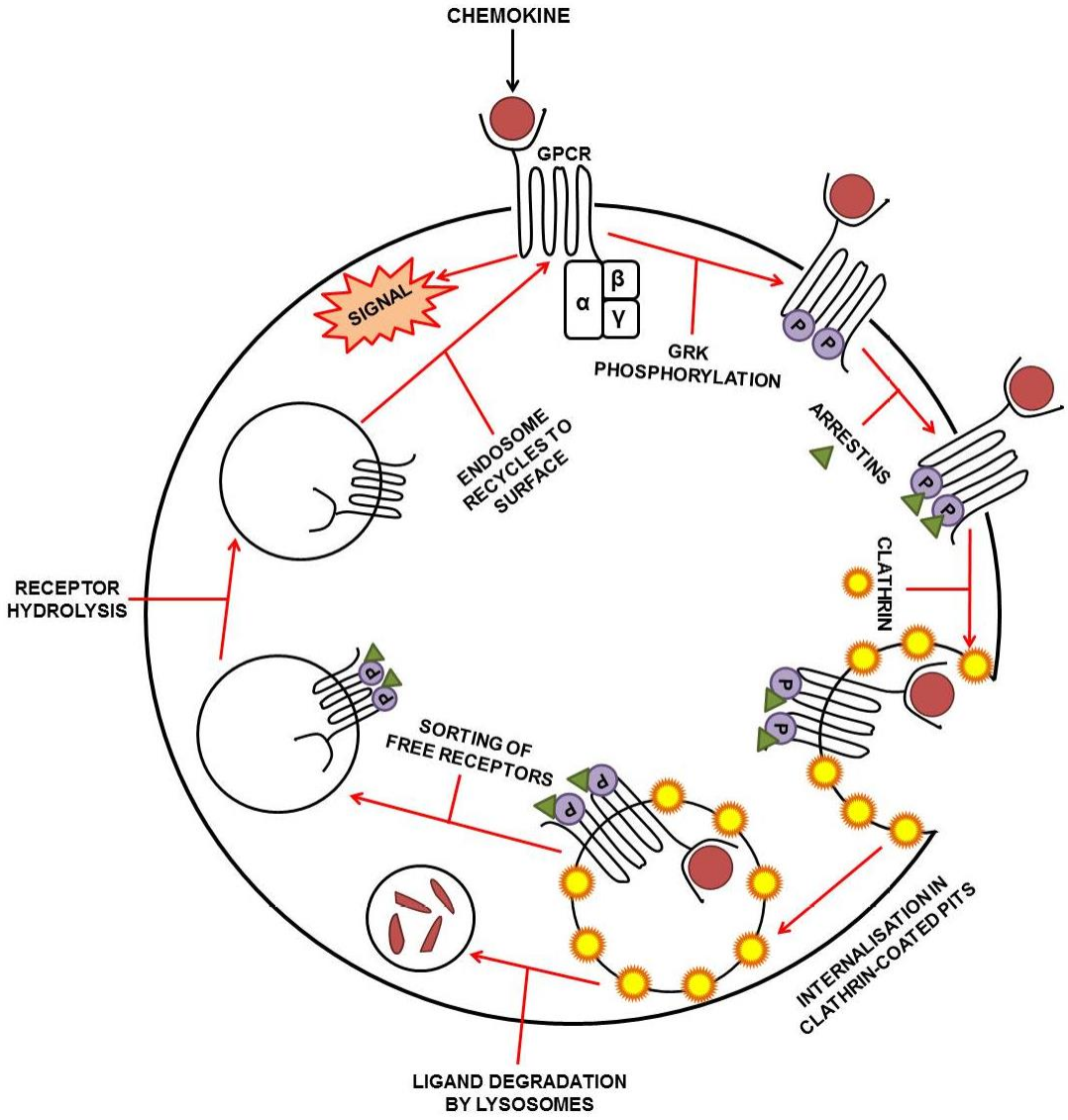

Figure 1.8: The mechanisms of receptor internalisation and recycling.

Legend: Once ligands bind to their respective GPCRs, they are internalised in clathrin-coated pits that subsequently form vesicles. Once internalised, the ligand is degraded and the receptor can be recycled back to the cell surface in endosomes. This process is actin-dependant.

1.2.6 Neutrophil Defence Mechanisms

Clearance of inhaled bacteria is essential in maintaining a sterile environment within the lungs. In addition to mechanical clearance via the mucocilliary escalator, inflammatory cells also assist in the clearance and killing of invading pathogens. Neutrophils have a number of defence mechanisms which are outlined below.

Phagocytosis

Phagocytosis is an important defence mechanism. The dominant phagocytes in the lungs are macrophages (Martin et al. 2005), though during certain periods (such as infection) when the bacterial load becomes overwhelming, neutrophils are recruited to assist with this clearance process (Zhang et al. 2000).

Before an invading particle can be engulfed by a phagocyte it must be recognised as foreign. Most commonly, this occurs through opsonisation, where invading particles are coated with soluble proteins in the serum such as complement fragments or immunoglobulins. These are then recognised by their specific complement or immunoglobulin receptors on the phagocyte cell surface, such as Mac-1 and CD16 respectively. Activation of these receptors induces cytoskeletal rearrangement, formation of a phagosome and particle engulfment. This process varies slightly depending on which receptors are activated. Complement-mediated phagocytosis results in a pit formation on the cell surface into which the foreign particle sinks (Allen et al. 1996, Aderem et al. 1999) whereas, during immunoglobulin-mediated phagocytosis, pseudopods form and reach out to capture opsonised particles (Allen et al. 1996). Both processes lead to the formation of a phagosome, which fuses with lysosomes and results in bacterial killing via bombardment

from an array of cytotoxic molecules, including proteinases and ROS. However, within the airways of healthy lungs, serum is not present in great quantities and phagocytosis predominantly occurs through non-opsonic mechanisms. The precise mechanisms by which this occurs have yet to be elucidated, though Mac-1 may still be involved (Heale et al. 2001).

Studies have shown that PI3Ks are required at certain stages of phagocytosis. It has been demonstrated that PIP_3 occurs transiently on regions of the phagosomal cup close to the ingested particle (Marshall et al. 2001, Vieira et al. 2001). This PI3K activity is related to both the extension of pseudopodia around target particles and the formation of phagosome by their closure (Araki et al. 1996, Marshall et al. 2001). In support of this, myosin X (a downstream PIP_3-binding protein) is required for optimal pseudopodal extension (Cox et al. 2002). Moreover, the addition of new membranes to an extending pseudopod is governed by PI3K-dependant control of ADP Ribosylation Factors (ARF) GTPases where, in the formation of a phagosome, PI3K activates ARF1 and inhibits ARF6 (Beemiller et al. 2006). The precise mechanisms of PI3K involvement have not been determined and PI3K is not required for all phagocytosis (Anderson et al. 2008). However, it does seem that PI3K signalling is necessary for optimal phagocytosis to occur in most instances.

Anti-Microbial Peptides (Proteinases)

There are over 500 different proteinases in humans, most of which are serine, cysteine and metalloproteinases (López-Otín et al. 2006). They act by degrading large proteins into smaller protein fragments and amino acids. Neutrophils store three major serine proteinases (NE, PR3 and CG), predominantly within the azurophile granules, which they use for (amongst other things) bacterial killing. Both NE and CG are stored within the granules, PR3

is also constitutively expressed on the cell surface (Csernok et al. 1990, Halbwachs-Mecarelli et al. 1995). Upon neutrophil activation, these granular proteinases are mobilised to both intracellular phagosomes (where they assist in the digestion microorganisms) and to the plasma membrane (where they are secreted extracellularly to kill free-roaming or trapped pathogens) (Owen et al. 1999; Owen 2008). Although proteinases play a significant role in innate immune defence, they are also destructive. Extracellular proteinase activity is tightly regulated by anti-proteinases (see Section 1.3), though chronic dysfunction of this system can result in disease (discussed in greater detail in Sections 1.5.4 and 1.6).

Oxidative Burst

In addition to microbicidal peptides and proteinases, neutrophils also generate ROS upon activation in a process called oxidative or respiratory burst. Activation leads to the production of superoxide anions ($O2^{\bullet -}$), which dismutate either spontaneously or via the catalyst superoxide dismutase (SOD). This process generates other ROS including hydrogen peroxide (H_2O_2), which is subsequently converted into hypochlorous acid (HOCl) by MPO and hydroxyl radicals ($OH^{\bullet}$). All of these contribute to bacterial killing (Hampton et al. 1998, Baboir 2000, Klebanoff 2005), although HOCl in particular has a strong effect as it oxidises a wide variety of bacterial components (Weiss 1999).

The initial production of superoxide is catalysed by the multicomponent nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Chanock et al. 1994, Baboir 1999, Quinn et al. 2004, El-Benna et al. 2005, Groemping et al. 2005), which oxidises NADPH and transfers two electrons onto an O_2 molecule. NADPH oxidase comprises 6 subunits; a Rho GTPase (Rac2 in human neutrophils) and five "phox" units including $p91^{phox}$ (also known as

NOX2), $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$. After activation, the cytosolic heterotrimeric $p47^{phox}$-$p67^{phox}$-$p40^{phox}$ complex is phosphorylated by protein kinases such as p38 MAPK and PI3K (Yamamori et al. 2000). Following this, it translocates to the plasma membrane, where it interacts with the membrane and other oxidases (Nauseef 1999). Activation also leads to the translocation of phox components (together with Rac2) to specific granules, where they can produce superoxide for short periods (Ambruso et al. 2004). Within these granules, NADPH oxidase activity is regulated by PKCδ and PI3K (Sergeant et al. 1997, Brown et al. 2003). The microbicidal effects of ROS once granules have fused with phagosomes contribute to bacterial killing following phagocytosis.

In addition to their microbicidal function, ROS at non-toxic levels are also implicated in the regulation of signalling pathways involved in various cellular functions including differentiation, proliferation, homeostasis and immune function (Touyz 2005). Furthermore, alteration of the redox state by ROS can influence many stages of a number of intracellular signalling pathways such as receptor function, enzyme activity, transcription factors and gene expression (Suzuki et al. 2005, Tonks 2005, Fialkow et al. 2007).

Neutrophil Extracellular Traps

Neutrophil Extracellular Traps (NETs) are complex, extracellular structures comprising a chromatin "backbone" together with anti-microbial peptides originating from intracellular neutrophil granules (Brinkman et al. 2004). They are made up of a series of threads that have globuli at intervals along their length. Single threads are often wound together to form thicker cables that, in turn, form large web-like structures that are capable of trapping microbes. NET

formation (NETosis) occurs in response to various stimuli that collectively stimulate external neutrophil receptors including TLRs, cytokine receptors and Fragment Crystallizable (Fc) receptors (Brinkman et al. 2004, Clark et al. 2007). Receptor stimulation then activates PKC which, in turn, leads to the activation of the NADPH oxidase complex. ROS, such as H_2O_2, have been implemented in NET formation, and can induce NET release *in vitro* at physiological concentrations (Fuchs et al. 2007). However, the processes that connect ROS production and the changes in morphology preceding cell rupture and NET release are not understood. Not only can NETosis immobilise pathogens, thereby preventing further spread and facilitating phagocytosis, but it is thought to kill pathogens directly though the action of antimicrobial histones and peptides (Papayannopoulos et al. 2009). Despite the immunological advantages of NETs, their accumulation due to chronic, increased activity and/or ineffective clearance could have pathological effects. For example, the accumulation of neutrophil DNA in the mucus of cystic fibrosis patients was traditionally though to originate exclusively from necrotic cells (Lethem et al. 1990). More recently, however, studies have suggested that the source of this DNA is actually from NETs (Marcus et al. 2010, Marcus et al. 2011, Manzenreiter et al. 2012).

The various processes by which neutrophils can eliminate pathogens are illustrated in Figure 1.9.

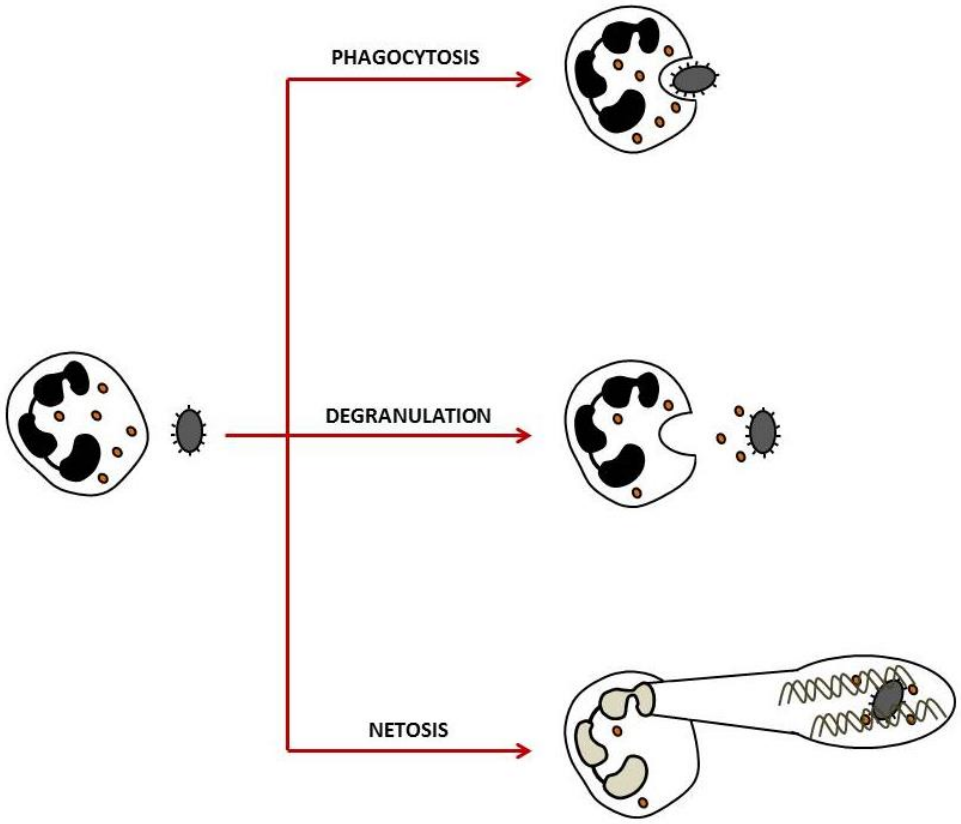

Figure 1.9: Neutrophil defence mechanisms.

Legend: Neutrophils can eliminate pathogens through various mechanisms, both intra- and extracellularly. When a pathogen is encountered, they phagocytose them and kill them by release of toxic agents (such as ROS & microbicidal peptides) from granules into the phagosome. These ROS and antibacterial peptides may also be releases into the extracellular milieu (degranulation), although this is often harmful to the host. NETs can immobilise pathogens preventing their proliferation and aiding subsequent phagocytosis as well as killing pathogens directly. However, NETosis also results in the death of the neutrophil.

1.3 ANTI-PROTEINASES

Anti-proteinases are important inhibitors of enzymatic damage mechanisms that minimise (by neutralisation) the proteinase destruction to tissues as neutrophils migrate

through the extracellular matrix. The three major serine anti-proteinases in the lungs are discussed below in order of prominence.

α_1-Antitrypsin

α_1-Antitrypsin (α_1-AT) is a proteinase inhibitor of the serpin superfamily. It is a 52 kDa, 418 amino acid glycoprotein primarily synthesised in the liver by hepatocytes (Rogers et al. 1983). It inhibits a wide variety of proteinases (Gettins 2002) and is the dominant inhibitor of free NE as well as CG and PR3 (Lee et al. 2001). It is considered to be the predominant elastase inhibitor in tissues and has a fundamental role in the distal (small) airways and the alveoli (Stockley 1997). During inflammation, the plasma concentration of α_1-AT increases causing increased diffusion into the lungs. In its absence, the breakdown of elastin by NE is uninhibited and contributes to a far greater degree of tissue destruction and accelerated emphysematous change, which may in turn lead to respiratory complications (see Section 1.6; "Alpha 1-Antitrypsin Deficiency").

Secretory Leukoprotease Inhibitor

Secretory Leukoprotease Inhibitor (SLPI) is an 11.7 kDa, highly cationic protein that is present in fluids lining mucosal surfaces (McElvany et al. 1997). It inhibits a number of proteinases including Leukocyte Elastase, CG, Trypsin and NE and is considered to be the major elastase inhibitor in secretions. In the lungs, it is secreted by club cells (previously referred to as "Clara" cells) and goblet cells epithelium and serous cells in the submucosal glands (Hiemstra 2002) and inhibits elastase in the proximal (larger) airways. In addition to

this protective role, SLPI is also recognised as an anti-microbial factor, possessing anti-bacterial, anti-fungal and anti-viral qualities (Tomee et al. 1998).

Elafin

The third most prominent proteinase in the lungs is Elafin, or Elastase-Specific Inhibitor (ESI). It is a small (6 kDa), cationic non-glycosylated member of the chelonianin family that also includes SLPI (Zani et al. 2004). It is a secreted protein, primarily expressed by epithelial surfaces such as skin (Alkemade et al. 1994, Nonomura et al. 1994, Pfundt et al. 1996) or lung epithelium (Sallenave et al. 1994, van Wetering et al. 2000), though it can also be expressed by various inflammatory cells including alveolar macrophages (Mihaila et al. 2001) and neutrophils (Sallenave et al. 1997). It is also found in plasma (Alkemade et al. 1995) and bronchial secretions (Sallenave et al. 1992, Nara et al. 1994), where it constitutes up to 20% of all anti-elastase molecules in Bronchoalveolar Lavage Fluid (BALF) from healthy individuals.

1.4 AGEING AND IMMUNE FUNCTION

1.4.1 The Ageing Lung

Ageing is associated with a gradual systemic decrease in organ and cell function, which includes both a loss of immune function as well as physiological lung impairment and throughout life there is a gradual loss of lung structure and function (Knudson 1991, Crapo 1993, Janssens et al. 1999). For example, it has been demonstrated that the ability of the lungs to exchange gases decreases significantly with age (Guenard et al. 1996), although the

patient cohort studied contained ex-smokers, some of whom may have had smoke-related damage, thereby exaggerating the true effects of age on gas transfer. However, a subsequent study, including only never-smokers demonstrated a similar decline in gas exchange (Neas et al. 1996), although the average age of this group was younger making it difficult to predict the true change in more elderly subjects. The reduction in gas transfer appears to be due to a loss of alveolar surface volume (Butler et al. 1970) and a gradual increase in ventilation-perfusion mismatching (Sorbini et al. 1968). In general, the decline in pulmonary diffusing capacity is unlikely to lead to significant symptoms due to the adoption of a more sedentary lifestyle in old age and a large remaining pulmonary reserve.

Another factor that may contribute to the loss of lung function with age is lifetime neutrophil migration through the lung parenchyma, where neutrophils release proteolytic enzymes (such as NE) during their passage, inflicting non-specific damage to healthy tissue as they migrate (Cepinskas et al. 1997). It is widely accepted that this process is central to the development of Chronic Obstructive Pulmonary Disease (COPD), especially the emphysematous process (Blackwood et al. 1973, Snider et al. 1974, Damiano et al. 1986), and similar structural changes are observed in the aged lung. However, there still remains uncertainty because of a potential cause and effect relationship between ageing and COPD (Fukuchi 2009, Sharma et al. 2009). This is discussed in greater detail in Section 1.5.8.

In addition to structural changes, there is a decrease in both mucociliary clearance of the lungs and bronchial secretions in older subjects (Gyetko et al. 1993, Puchelle et al. 1979), which may not only increase the risk of pathogen adherence and, hence, lung epithelial damage, but also prolong the retention time. This is most likely a key predisposing factor for the higher incidence of respiratory infections in elderly people. Studies have also suggested

that the total number of immune cells (such as neutrophils and T-cells) in the pulmonary airspace may be significantly increased with age (Thompson et al. 1992, Meyer et al. 1996).

1.4.2 The Ageing Immune System

During normal human ageing, many of the innate and adaptive immune functions and their interactions become less efficient. Collectively, the degradation of the immune system with age is termed "immunosenescence" and is largely responsible for the reduced ability of elderly subjects to mount an effective secondary innate or adaptive immune response to antigenic insult. Although our current knowledge on the effects of immunosenescence is incomplete, many specific functional changes in various cell types have been observed and documented. There is a wealth of knowledge on the various aspects of immunosenescence available (Panda et al. 2009, Agarwal et al. 2010). However, certain key issues will be discussed briefly to summarise the potential problems of altered immunity with ageing.

It has been demonstrated that the absolute monocyte numbers are increased in elderly subjects (Della Bella et al. 2007) and there have been several studies into the effects of ageing on macrophage function, though the outcomes are somewhat conflicting. Earlier studies showed impairment in both chemotaxis and phagocytosis in humans and mice (De La Fuenta et al. 1985, Fietta et al. 1993) whereas, more recently, studies using rats showed the opposite effect (Corsini et al. 2005, Hilmer et al. 2007) and one study found no change in aged macrophage function (Miller et al. 2007). However, increased levels of circulatory inflammatory cytokines, such as TNFα and IL-6 are observed in aged humans (so-called "inflammaging" (Franceschi et al. 2000)), but output of these cytokines by monocytes is decreased. This is associated with a decreased expression of associated TLRs on the surface

of monocytes (van Duin et al. 2007) meaning that, although the basal activity of macrophages may be increased in ageing, a reduction their TLR expression could attenuate their activation and lead to overall reduction in macrophage function.

Granulocytes, including neutrophils, eosinophils and basophils are also recruited to the tissues as an early part of the secondary innate response. The suggestion that granulocyte function may be altered with age is strongly supported by the presence of increased morbidity and mortality as a result of bacterial infections in the elderly (Snider et al. 1974, Laupland et al. 2003) and that fact that age is an independent risk factor for the development of a number chronic inflammatory diseases, where these cells play a central role. To date, there have been few studies of any age-associated changes in eosinophils (Leng et al. 2005, Mathur et al. 2008) and basophils (Schwarzenbach et al. 1982, Marone et al. 1986). In contrast, there is far more information available on the loss of neutrophil function with age (Lord et al. 2001, Schroder et al. 2003, Tortorella et al. 2007, Fortin et al. 2008, Lord et al. 2009). Evidence to date suggests that most aspects of neutrophil function decrease with age, although findings are variable. The majority of previous work indicates that neutrophil phagocytosis is impaired in the elderly (Antonaci et al. 1984, Fulop et al. 1985, Esparza et al. 1996, Butcher et al. 2000, Tortorella et al. 2000, Wenisch et al. 2000, Alonzo-Fernandes et al. 2008), with only one study reporting no difference (Placket et al. 2004). In contrast, the effects of ageing on neutrophil oxidative burst are less clear. Some *in vitro* studies describe a constant ROS production by neutrophils from elderly donors following stimulation by fMLP or gram-negative bacteria (Ito et al. 1998, Butcher et al. 2000, Lord et al. 2001), whereas a number of other studies found that the processes that lead to intracellular oxidative killing could not be fully activated by multiple stimuli (McLaughlin et al. 1986, Whitelaw et al.

1992, Seres et al. 1993, Fulop et al. 1994, Polignano et al. 1994, Braga et al. 1997, Tortorella et al. 1999, Tortorella et al. 2000, Tortorella et al. 2007, Panda et al. 2009). The effect of ageing on neutrophil migration is also poorly understood as findings are variable. Some studies have shown that neutrophil migration is unaltered in aged individuals (MacGregor et al. 1990, Esparza et al. 1996), while others report a reduced chemotaxis with age (Niwa et al. 1989, Wenisch et al. 2000, Fulop et al. 2004). Recent, data from our research group also clearly demonstrates reduced chemotaxis in neutrophils from peripheral blood of aged individuals compared to young control subjects (Sapey et al. 2014).

It has also been demonstrated that the ability of neutrophils to respond to survival signals, particularly granulocyte-monocyte colony stimulating factor (GM-CSF), may also be reduced in the elderly (Fortin et al. 2007) leading to premature apoptosis. Since these cells provide the major phagocytic function in the airways via non-specific receptors or specific opsonophagocytic receptors involving antibodies provided by adaptive immunity, defects in their function is likely to have a marked effect on host defence.

In addition to innate immunosenescence, there are also changes in adaptive immunity with age. For instance, thymic involution results in a diminished production of new T-cells with age, thereby reducing or limiting overall T-cell receptor diversity (Naylor et al. 2005). Therefore, there is a slow shift in T-cell population towards fewer naïve T cells and more memory T-cells (Akbar et al. 2005), which reduces the ability of the host to efficiently recognise new or mutated pathogen epitopes. Memory responses to pathogens encountered previously are also reduced, as repeated stimulation leads to the shortening of telomeres and cellular senescence (Moro-García et al. 2012). Other effects of adaptive immunosenescence include reduced T-cell survival (Aggarwal et al. 1998) and T-cell signalling due to increased

differentiation of T-cells and loss of co-stimulating molecules such as CD28 (Haynes et al. 1999, Eaton et al. 2004, Larbi et al. 2004, Fulop et al. 2005, Sadighi et al. 2005, Kovaiou et al. 2006, Weng et al. 2009). The effects of ageing on these and the other cells of the immune system are summarised in Table 1.1.

Immune Cell Type	Changes With Age
Monocytes	↑ numbers, ↓ surface expression of TLRs
Macrophages	↑ basal activity, ↓ activation
Neutrophils	↓ chemotaxis, ↓ phagocytosis, ↓ ROS production, ↓ response to survival signals
Basophils	Generally maintained degranulation
Eosinophils	↓ ROS production, ↓ neurotoxin production
Natural Killer Cells	↑ numbers, ↓ cytotoxic function, ↓ cytokine/chemokine production
Natural Killer T-Cells	↓ cytotoxic function (certain types), ↑ cytotoxic function (TCRγδ+ cells)
T-Cells	↓ naïve T-cell numbers, ↑ memory T-cell numbers, ↓ survival, ↓ signalling
B-Cells	↓ numbers, ↓ antibody diversity
Dendritic Cells	Maintained antigen presentation, ↓ TLR-induced cytokine production in pDCs

Table 1.1: A summary of the effects of ageing on the cells of the immune system.

Legend: This phenomenon, collectively referred to as immunosenescence, is largely responsible for the impaired ability of elderly subjects to mount an effective immune response.

1.5 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic Obstructive Pulmonary Disease (COPD) is a disorder that encompasses a number of different disease pathologies and clinical syndromes, including emphysema (tissue destruction), chronic bronchitis (mucous gland hyperplasia) and bronchiolitis (small airways disease) that may exist concurrently (Saetta et al. 2001). It has been defined by the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) as "a disease state characterised by airflow limitation [on exhalation] that is not fully reversible [by bronchodilation]. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases" (Pauwels et al. 2001). In 2007, GOLD defined COPD by the degree of airflow limitation measured by spirometry. Spirometric measures of ventilation include the Forced Expired Volume in 1 Second (FEV_1) and Forced Vital Capacity (FVC), where an FEV_1/FVC percentage ratio post-maximal bronchodilation below 70% is indicative of airflow limitation. GOLD further characterised COPD severity into Stage 1 ($FEV_1 \geq 80\%$ predicted), Stage 2 (FEV_1 = 50-79% predicted), Stage 3 (FEV_1 = 30-49% predicted) and Stage 4 ($FEV_1 < 30\%$ predicted) (GOLD 2007). Although widely accepted, these definitions were limited as they did not account for the age-related predicted normal range of spirometric parameters (where an FEV_1/FVC ratio slightly below 70% may be normal for elderly subjects), individual variability of associated symptom severity (including cough, expectoration, wheeze, shortness of breath and exercise limitation) or other more direct measures of lung function such as Gas Transfer and High Resolution Computerised Tomography (HRCT) scan. More recently, GOLD have revised their guidelines to include the degree of patient dyspnoea and their exacerbation history (GOLD 2011).

1.5.1 Epidemiology

COPD is a leading cause of mortality and morbidity worldwide. Recent estimates in 2007 by the World Health Organisation (WHO) suggested that, globally, it affected 210 million people and accounted for 5% of all deaths (WHO 2007). Furthermore, the WHO predicts these values are likely to increase significantly in the next 10 years, particularly the number of related deaths which is predicted to rise by more than 30% (WHO 2007).

1.5.2 Pathogenesis

COPD is a chronic inflammatory disease of the lungs with an insidious progression. Over the last 50 years, there have been an abundance of comprehensive publications regarding the pathology of COPD (Reid 1967, Heard 1969, Thurlbeck 1976). Since the first experimental model of emphysema was inadvertently induced in guinea pigs (Gross et al. 1964), understanding of COPD pathology has grown significantly and there is now substantial evidence implicating the neutrophil as the dominant effector cell, with particular reference to the destructive effect of NE. Chronic tobacco smoke inhalation is the single most important risk factor for COPD (Doll et al. 1994), though only 20% of smokers are susceptible to the disease (Tashkin et al. 1984) and the reasons for this still remain unclear. It is likely that there may be an element of genetic predisposition to COPD susceptibility, although specific genes have yet to be identified and current evidence is conflicting (Hallberg et al. 2008, Smolonska et al. 2009, van Durme et al. 2009).

1.5.3 Neutrophils in the COPD Lung

A wealth of cumulative evidence implicates the neutrophil as the key effector cell in the disease pathology. Studies have shown that neutrophils are present in greater numbers throughout the lungs in patients with COPD compared to healthy controls in large airway secretions (Stanescu et al. 1996, Rutgers et al. 2000), BALF (Martin et al. 1985) and biopsies of the lung epithelium (Pilette et al. 2007). Furthermore, increased neutrophil numbers in these areas of the COPD lung positively correlate with the degree of airflow limitation, the severity of emphysema on high resolution computer tomography scanning (Ekberg-Jansson et al. 2003, Parr et al. 2006) and the presence of chronic bronchitis (Hill et al. 2000). NE is of particular importance as it causes all of the pathological features of COPD *in vitro* and in animal models; emphysema (Senior et al. 1977), secretory cell metaplasia (Lucey et al. 1985), endothelial activation, damage (Amitani et al. 1991) and apoptosis (Ballieux et al 1994) and a reduction in ciliary beat frequency on the respiratory epithelium (Smallman et al. 1984). NE triggers a state of oxidative stress in cells (Aoshiba et al. 2001), degrades all of the components of the extracellular matrix (Kafienah et al. 1998), activates the clotting cascade (Vogt 2000), damages the opsonophagocytic receptor, C3bi (Berger et al. 1989), and activates matrix metalloproteinases (Ferry et al. 1997) while inhibiting tissue inhibitors of metalloproteinases (Itoh et al. 1995) thereby enhancing the destructive capacity of macrophage proteinases. Both neutrophil numbers and NE concentration in the COPD lung decrease following smoking cessation (Rennard et al. 1990), supporting not only the association between smoking and increased neutrophil activity in the lungs but also highlighting the importance of smoking cessation as the major therapeutic option COPD. Moreover, exposure to cigarette smoke results induces an inflammatory cascade including

the release of numerous inflammatory cytokines and growth factors that lead to the recruitment of inflammatory cells such as neutrophils (Saetta et al. 1997). However, as only 20% of smokers develop COPD (Tashkin et al. 1984), it is clear that this inflammation is not a universal response to smoking.

The increased neutrophil presence in the lungs of COPD patients appears to be due primarily to an increased transmigration from the peripheral blood. Tobacco smoke induces an increase in the production and release of neutrophils from the bone marrow into the circulation (Corre et al. 1971, Van Eeden et al. 2000) which, in turn, results in a greater degree of influx to the airways in smokers. The increased neutrophil migration through the lung parenchyma in smokers susceptible to COPD appears to result in an accelerated rate of alveolar and airway destruction by NE, although the precise mechanisms by which this occurs have yet to be elucidated. It has also been suggested that neutrophils that have migrated to the lungs survive longer, which would further increase lung damage, although the current evidence is conflicting (Pletz et al. 2004, Uller et al. 2006, Rytila et al. 2006). A HRCT scan showing the destruction of lung tissue and enlargement of airspace in the COPD lung compared to the preserved airspaces in a healthy lung is shown in Figure 1.10 (Taken from; http://www.ctsnet.org/portals/thoracic/newtechnology/article-4.html).

There is both direct and indirect evidence of abnormal neutrophil function in patients with COPD. Altered functions include an increased ROS production (Noguera et al. 2001), increased phagocytosis with potentially enhanced migration (Burnett et al. 1987) and improved adhesion to endothelial cells under flow (Woolhouse et al. 2005). The reasons for these differences remain unclear. They may either arise during neutrophil maturation in the bone marrow or at some point after their release into the circulation, for example by

cytokines in the circulation or during transmigration. It has been demonstrated that neutrophils change phenotype between initial activation and the end of migration in healthy subjects (Dangerfield et al. 2002, Yadav et al. 2002) but this has not yet been studied in COPD.

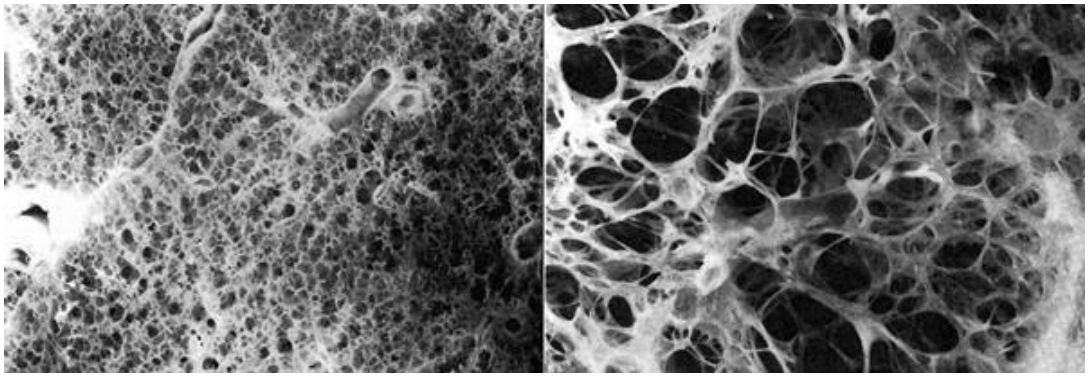

Figure 1.10: HRCT scans comparing healthy and COPD lungs.

Legend: Image showing the preserved airspaces in a healthy lung (A) and the enlarged airspaces in a COPD lung caused by persistent tissue destruction (B).

1.5.4 Proteinases and COPD

Proteinases are believed to play a key role in the pathogenesis of COPD. The proteinase/anti-proteinase theory suggests that lung damage occurs when the level of anti-proteinase is insufficient to neutralise the presence of proteinases (Tetley 1993). This imbalance may occur due to a loss of anti-proteinases (by genetic origin (α_1-Antitrypsin Deficiency) or proteolytic/oxidative damage) or excessive recruitment or activation of proteinases.

Proteinases are classified into three groups; the serine proteinases (including NE, CG and PR3), cysteine proteinases (Cathepsins) and Matrix Metalloproteinases (MMPs). The serine proteinases are stored with MPO in the azurophile granule (Ohlsson et al. 1977). Of these enzymes, NE was the first to be shown to produce emphysema in animal models (Janoff et al. 1977, Senior et al. 1977). The main physiological role of NE is bacterial killing, although it has the potential to be extremely destructive and its role in the pathogenesis of COPD (particularly emphysema) is well documented in animal models and *in vitro* (Snider et al. 1974, Stockley 2002). In further support, studies have also shown that NE damage in the lung can be prevented by elastase inhibitors (Lucey et al. 1989, Rudolphus et al. 1994).

The mechanisms of neutrophil migration are not fully understood. It may be that serine proteinases are not required for migration through tissue or that their function is not specific to matrix degradation and tissue destruction is merely a side-effect. The imbalance of proteinase and anti-proteinase may allow NE to diffuse further from the neutrophil before it is neutralised, thereby causing the breakdown of elastin (Campbell et al. 1999). NE has also been implicated in mucous cell metaplasia and mucus hypersecretion that is associated with the chronic bronchitis element of COPD (Lucey et al. 1985), which may be inhibited by NE inhibitors (Takeyama et al. 1998).

Far less is understood about the role of cysteine proteinases in COPD, though they possess similar properties to serine proteinases. Of particular note is Cathepsin B, which is produced by macrophages, is present in the BALF of COPD patients and increasing during periods of exacerbation (Burnett et al. 1983). It is known that NE induces Cathepsin B activity in sputum via activation of pro-enzymes (Buttle et al. 1991) and this may form a

positive feedback loop as Cathepsin B also inactivates SLPI (the local NE inhibitor) (Taggart et al. 2001).

In contrast, more is understood about the potential role of MMPs in COPD. Both macrophages and neutrophils secrete large amounts of MMPs and their inhibitors; "Tissue Inhibitors of Metalloproteinases" (TIMPs). To date, there are over 24 mammalian MMPs described, although only a few have been implicated in COPD. They have a variety of functions including degradation of extracellular matrix proteins as well as the breakdown of anti-proteinases (such as α_1-AT) and other proteins (such as adhesion factors), activation of clotting cascade enzymes and modification of cytokines. Examination of BALF from patients with emphysema has demonstrated a presence of MMPs compared to a control group of healthy smokers (Burnett et al. 1988). Tissue destruction by MMPs may be associated with macrophages more so than neutrophils as alveolar macrophages that reside within the lungs are able to continually secrete MMPs. However, it is likely that there is a high degree of interaction between neutrophils, macrophages, MMPs and their other respective secretory enzymes. For example, NE can not only activate various MMPs (Okada et al. 1989, Rice et al. 1995, Ferry et al. 1997) but also degrade TIMPs (Itoh et al. 1995). In addition, a number of macrophage enzymes have been shown to degrade NE inhibitors such as α_1-AT (Banda et al. 1980, Sires et al. 1994, Lui et al. 2000).

1.5.5 Oxidative Damage

ROS are products of normal metabolism and are produced primarily by mitochondria (Halliwell et al. 1999) and are found throughout the lungs. The predominant defence against ROS and other free radicals are anti-oxidants, which rapidly neutralise ROS. The toxicity of

oxygen in the lung has recognised for many years (Halliwell et al. 1999, van der Vliet et al. 1999) and there is mounting evidence for potential oxidative damage in COPD (Rahman et al 1996, Rahman et al. 1999, Anderson et al. 2004). In the lungs and other organs there is a balance between ROS and anti-oxidants and an increase in ROS and/or depletion of anti-oxidants induces disequilibrium and results in "oxidative stress".

Cigarette smoke comprises a mixture of at least 5000 different chemical compounds including free radicals and other oxidants, both present in high concentrations (10^{15} free radical molecules per inhalation) (Church et al. 1985, Pryor et al. 1993). Direct exposure of the lung epithelium to inhaled smoke makes it particularly vulnerable to associated oxidative damage. Oxidants in cigarette smoke can directly damage elastin and collagen in the lung matrix (Cantin et al. 1985) as well as impairing the ability to regenerate elastin (Laurent et al. 1983), which may enhance emphysematous damage. However, smokers who are not susceptible to COPD do not endure lung damage through smoking alone, so tobacco smoke-induced ROS alone is not sufficient to cause COPD. The oxidative burden in COPD is amplified by release of ROS from macrophages and neutrophils, which migrate in greater numbers into the lungs of smokers, particularly those susceptible to COPD (Hunninghake et al. 1983). Furthermore, there is also evidence supporting increased systemic oxidative stress as circulating neutrophils in COPD patients appear to have an increased capacity to release ROS (Noguera et al. 1998). The effects of cigarette smoke-induced oxidative stress within the lung environment are summarised in Figure 1.11 (Adapted from; http://healthy-lifestyle.most-effective-solution.com/2007/10/01/oxidative-stress-and-free-radicals/).

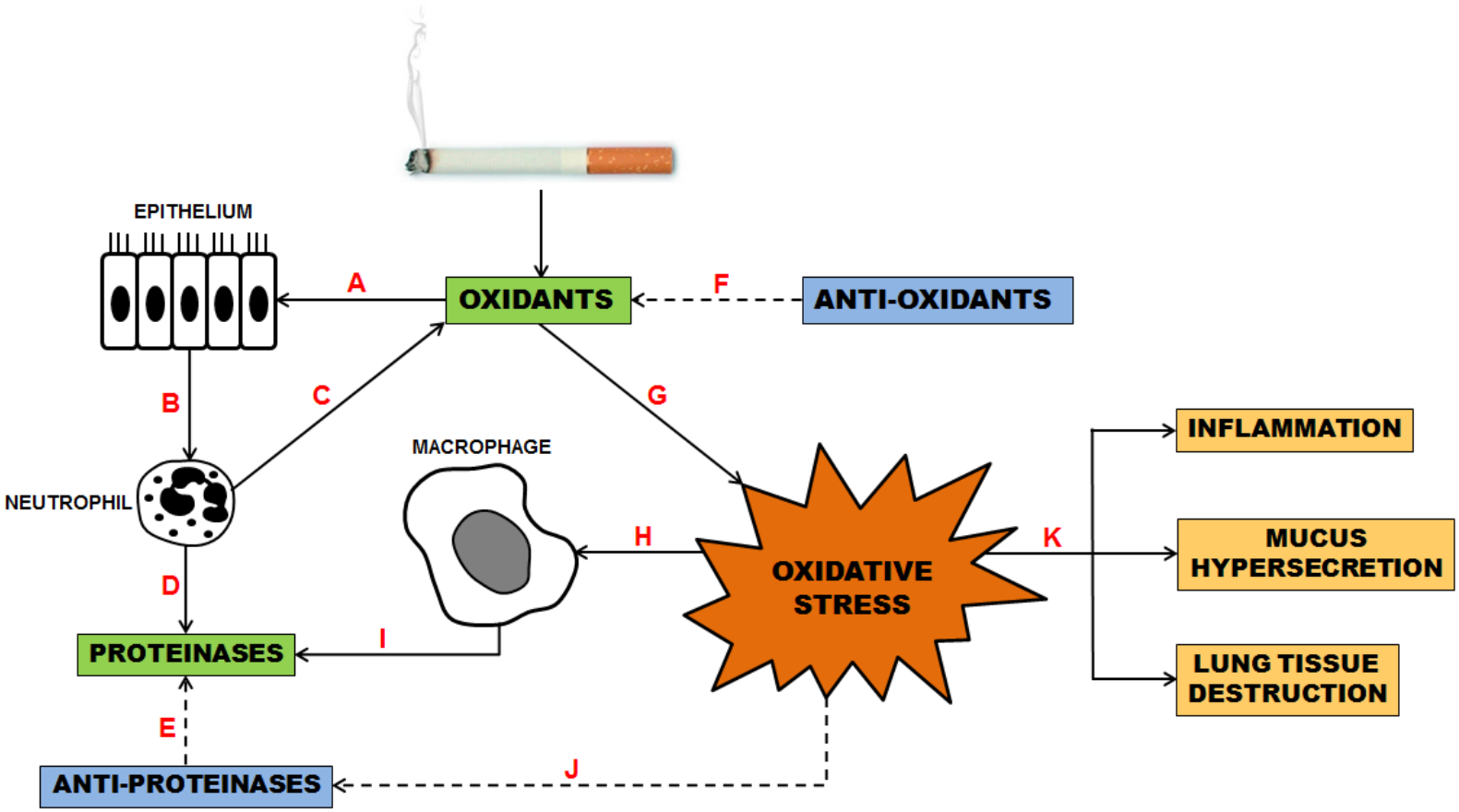

Figure 1.11: Oxidative damage in COPD.

Legend: Oxidants in cigarette smoke aggravate epithelial cells in the lung (A), causing the release of inflammatory cytokines (B). This prompts the recruitment of neutrophils that release cell-derived oxidants (C) and proteinases (D). Anti-proteinases inhibit proteinases (E) and anti-oxidants inhibit damage caused by oxidants (F). Due to an oxidant/anti-oxidant imbalance, oxidative stress occurs (G), resulting in activation of macrophages (H) that release more proteinases (I) and inhibition of anti-proteinases (J). This ultimately leads to inflammation, mucus hypersecretion and lung tissue destruction (K).

1.5.6 Bacteria and Viruses in COPD

Bacterial colonisation can occur in the airways of patients with COPD (particularly those with severe disease) even when clinically stable (Monso et al. 1995). As the lungs are continuously in contact with the external environment, they are not completely sterile in healthy subjects. However, the differential speciation and increased bacterial load in the lungs of COPD patients (Hassett et al. 2014) suggests that they may potentially have a causative role in the disease pathogenesis. The contribution of infection to the pathogenesis and progression of COPD may be multifactorial (Murphy et al. 1992). However, the specific role of bacteria has been a topic of debate for some years, with hypotheses ranging from a fundamental and causative role to more secondary and coincidental one (Leeder 1975, Tager et al. 1975, Hirschmann 2000, Murphy et al. 2000).

Latent adenoviral infection has also been implicated in the pathogenesis of COPD. Evidence of persistent adenoviral infection has been demonstrated in the lungs of asymptomatic adults (Matsuse et al. 1992) and expression of the adenovirus E1A protein on lung epithelial cells persists even after the virus has ceased replication and symptoms have abated (Elliot et al. 1995). In addition, the adenovirus E1A protein has been associated with an amplification of inflammation in emphysema (Retamales et al. 2001).

Studies also suggest that 20-40% of exacerbation in COPD have a viral genesis, which would also amplify lung damage through different mechanisms such as increased neutrophil influx, increased ROS release and increased expression of pro-inflammatory cytokines (Sapey et al. 2006).

1.5.7 Systemic Effects of COPD

Although COPD is primarily a disorder of the lungs, it is also associated with a variety of systemic effects and comorbidities (Decramer et al. 2008, Fabri et al. 2008). The most common comorbidities include ischaemic heart disease, osteoporosis, cachexia, chronic kidney disease and lung cancer as well as cognitive and affective manifestations such as depression (Decramer et al. 2008, Casanova et al. 2010). Diabetes was also originally thought to be a primary comorbidity of COPD but is now more frequently associated with restrictive lung disease (van den Borst et al. 2010). These comorbidities clearly affect health outcomes and may also provide plausible explanation, at least in part, as to the heterogeneic nature of COPD and why its clinical features do not correlate strongly with FEV_1 (Celli et al. 2004).

The causal link between COPD and its comorbidities is difficult to establish. In some instances, comorbidities could simply be a reflection of poor lifestyle as smokers may lead a more sedentary lifestyle and eat less healthily (Booth et al. 2000, Health 2008). However, it has been suggested that there is a direct link between COPD and its comorbidities (Fabbri et al. 2007), either due to a systemic "spillover" of pro-inflammatory mediators from the lungs, or because of a general systemic effect impacting on multiple organs (Barnes et al. 2009). All of the comorbidities can develop in patients without COPD but, as they are commonly associated with the disorder (particularly severe cases), common risk factors seem likely. Chronic tobacco smoke inhalation is a major risk factor for COPD but it is also a risk factor for cardiovascular disease, osteoporosis and lung cancer (Edwards 2004). Lastly, ageing is a major risk factor for the development of chronic disease and nearly half of the world's population over the age of 65 have three or more chronic medical disorders (Boyd et al. 2005).

The common factor between all comorbidities is the presence of systemic inflammation. Increased plasma levels of pro-inflammatory cytokines (TNFα, IL-6 and IL-8), adipokines (leptin, ghrelin) and acute-phase proteins (C-Reactive Protein (CRP), fibrinogen) are seen in the majority of these diseases and the independent risk factors have all been linked to the presence of systemic inflammation (Fabbri et al. 2007, Mora et al. 2007, Yanbaeva et al. 2007). However, whether these systemic inflammatory markers are in fact overspill from the pulmonary system or reflect a more general inflammatory state remains unclear (Fabbri et al. 2004, Barnes et al. 2009, Sinden et al. 2010).

1.5.8 Ageing and COPD

It has been proposed that the pathology of many chronic diseases, including COPD, may in fact be an accelerated form of the processes that occur with natural ageing. The incidence of COPD is clearly greater in older individuals (Fukuchi et al. 2004, Buist et al. 2007), although this could simply be because COPD usually progresses very slowly and, by the time symptoms have manifested and patients are diagnosed, they are often of middle to old age. Hence, the cause and effect relationship between ageing and COPD is unclear. It could be that the natural changes in lung structure associated with ageing, coupled with the progressive loss of immune function with age (immunosenescence) may increase susceptibility to or even cause COPD. In contrast, it could be that the repetitive toxic insults associated with COPD (most commonly tobacco smoke inhalation) directly influence the pulmonary environment and amplify natural inflammatory processes (such as leukocyte migration) that, over time, exaggerate pulmonary damage and manifest as disease.

The changes that occur in the ageing lung in the absence of disease lead to a detrimental shift in pulmonary physiology. A reduction in the elastic recoil of the lungs is coupled with an increase in cell wall rigidity and there is also a loss of respiratory muscle strength (Turner et al. 1968, Knudson et al. 1977). These can lead to a modest impairment in ventilation that is indicated by a reduced FEV_1/FVC ratio and an increased residual volume (RV) but the fact that elderly individuals usually adopt a more sedentary lifestyle means that these changes are generally asymptomatic.

Importantly, the histological changes observed in lung with advanced age are not analogous to those in COPD. Even though there is alveolar enlargement at autopsy in human aged lungs, the concurrent alveolar wall destruction and distension of alveolar ducts seen in COPD is absent (Verbeken et al. 1992). Mouse models of accelerated senescence also demonstrate that normal ageing is associated with alveolar enlargement without emphysema, indicating that ageing *per se* did not cause COPD (Fukuchi et al. 2009). However, this study also showed that mice with accelerated ageing developed more emphysema than age-resistant mice when exposed to equal amounts of tobacco smoke, which suggests that ageing does increase susceptibility to COPD.

1.5.9 Current and Emerging Therapies

Despite the huge global impact of COPD on mortality and the burden on healthcare, there are currently no drug therapies that have been shown to significantly prevent the progression of the disease, reduce exacerbation frequency or reduce mortality. For many years, bronchodilators have been the cornerstone of COPD treatment and the only significant

advancements in therapy have been the development of long-acting β_2-agonists (LABAs) and long-acting muscarinic agents (LAMAs) (Cazzola et al. 2008).

The once-daily muscarinic agent, tiotropium bromide, has been used as a first line treatment for moderate-severe COPD since 2002 in over 100 countries (Boehringer Ingelheim GmbH 2011). It has been shown to improve a variety of outcome measures including expiratory airflow, lung hyperinflation, exercise capacity and quality of life as well as a reduction in exacerbation frequency and mortality (Tashkin et al. 2008, GOLD 2011). In addition, *in vitro* studies have shown that tiotropium bromide may possess anti-inflammatory properties, such as suppression of IL-8 release from bronchial epithelial cell lines (Suzaki et al. 2011) as well as reduction of ROS production by alveolar macrophages and inhibition of alveolar macrophage-mediated neutrophil migration (Vacca et al. 2011). Until recently, tiotropium bromide was the only once-daily LAMA available but, due to its commercial success and efficacy in the treatment of COPD symptoms, two other products have now been licensed (glycopyrronium bromide and aclidinium bromide).

Salmeterol and formoterol are both LABAs with an extended duration of action that may last up to 12 hours following administration (Ball et al. 1991). These twice-daily bronchodilators have been in use for many of years but there are now a number of new once-daily LABAs in production. Indacaterol is currently the only bronchodilator of this type on the market, though others are in development such as vilanterol trifenatate (Kempsford et al. 2013). Indacaterol is an ultra-long acting LABA that has a rapid onset of action as well as a 2-fold greater efficacy than salmeterol at the target β_2 adrenergic receptors in airway smooth muscle cells (Batram et al. 2006).

As useful as bronchodilators may be in relieving symptoms, they offer no long-term solutions to disease progression. Smoking cessation is certainly the most important and effective intervention for halting or slowing disease progression (Godtfredsen et al. 2008), though current strategies are relatively ineffective. Furthermore, in moderate-severe COPD, smoking cessation may not be sufficient to resolve established neutrophil inflammation and the disease can often progress in a self-perpetuating manner (Willemse et al. 2005).

Development of new pharmaceutical agents for the treatment of COPD has proven difficult. This is predominantly due to limited understanding of the underlying molecular mechanisms behind the diseases pathology (in contrast to asthma, for example). Furthermore, it is unclear how to test drugs for COPD. Animal models to date have focused exclusively on emphysema and have not investigated other components of COPD, such as small airways disease (which may relate more closely to certain physiological measures and symptoms (Boswell-Smith et al. 2007)). Moreover, animal models of drug testing are generally unreliable as positive therapeutic effects are rarely mirrored in subsequent human trials (Seok et al. 2013). For human subjects, the large phenotypic variation in COPD make it difficult to differentiate which patient groups may benefit from certain treatments, as clinical trials have included numerous clinical phenotypes (Barnes 2012). In addition, a large proportion of COPD patients have co-morbidities (such as cardiovascular disease and diabetes) that may exclude them from participating in clinical trials of new treatments (Majo et al. 2001). Lastly, as COPD progression is slow and insidious, clinical trials can require long study periods and prove extremely costly (Banner et al. 1995).

Due to the large number of inflammatory mediators implicated in the pathophysiology of COPD (Cortijo et al. 1993, Hatzelmann et al. 2001), blockade of these mediators seemed an

attractive target for novel therapies. Unfortunately, clinical trials to date have yielded no truly effective anti-inflammatory drugs, where results have been either negative or shown only modest improvement in outcome measures but often with undesirable side-effects. Of particular note, the phoshodiesterase 4 (PDE4) inhibitor, roflumilast, has been the subject of a number of clinical trials with moderate-severe COPD patients. The studies reported a number of promising outcomes including decreased exacerbation frequency, improved health-related quality of life and improved post-bronchodilator FEV_1 (Calverley et al. 2007, Calverley et al 2009, Fabri et al. 2009, Martinez et al. 2010, AURA Study, HERMES Study, OPUS Study, Ratio Study) and roflumilast has now reached the market for treatment of COPD. However, the clinical benefits were frequently coupled with significant side effects including diarrhoea, nausea and weight loss, resulting in intolerance for many COPD patients. As a result, roflumilast is not currently recommended for the general treatment of COPD and is not available in the UK.

Blockage of both LTB_4R1 (with a major receptor antagonist) and TNFα (with infliximab) had no clinical benefit in COPD (Wollin et al. 2006, European Medicines Agency 2010). Blocking antibody to IL-8 had a small effect in reducing dyspnoea (Kumar et al. 2003) and there has been great interest utilizing CXCR2 blocking strategies to modify neutrophil recruitment in COPD (Barnes 2005). CXCR2 blockade is associated with a reduction in neutrophil migration (Del Rio et al. 2001) with a resulting decrease in inflammation in animal models of inflammatory disease (Farooq et al. 2009). Two recent clinical trials in humans demonstrated that a CXCR2 antagonist (AZD8309) attenuated neutrophil airway recruitment after LPS challenge in healthy humans (Virtala et al. 2011,

Leaker et al. 2013). If a similar effect were to be observed in COPD patients, it could potentially reduce lung damage caused by neutrophil transmigration and possibly slow the emphysematous process.

Due to the increased bacterial colonisation commonly seen in COPD and the association between chronic inflammation and acute exacerbations (Hassett et al. 2014), the use of macrolide therapy (such as erythromycin, clarithromycin and azithromycin) as a treatment has been investigated. Their antibacterial effects include the inhibition of bacterial protein synthesis, impaired bacterial biofilm synthesis and the attenuation of bacterial virulence factors and their effectiveness in treating infectious respiratory diseases is well established (Woodhead et al. 2011). In addition, it has also been demonstrated that macrolides have a number of immunoregulatory effects, including control of mucus hypersecretion, resolution of inflammation, and modulation of host defence mechanisms (Lopez-Boado et al. 2008, Altenburg et al. 2011a, Altenburg et al. 2011b). Yao et al. have recently published a meta-analysis of previous research into the effectiveness of macrolide maintenance therapy in reducing exacerbation rate in COPD (Yao et al. 2013). They concluded that treatment with macrolides significantly lowered the frequency of acute exacerbations in COPD, although treatment for at least 6 months was required to elicit a positive therapeutic effect.

As mentioned previously (see Section 1.2.3), PI3K activity is intimately involved in neutrophil inflammatory functions (Stephens et al. 2002) and has been proposed as a putative target for therapeutic interventions in COPD. *In vitro* and transgenic animal models with increased PI3K activity appear to mimic the COPD neutrophil migratory phenotype: PTEN knockout (KO) mouse neutrophils demonstrate increased speed of migration (Sarraj et al. 2009); SHIP1 KO mice have greater neutrophil accumulation following inflammatory insult

(Helgason et al. 1998); and PTEN knockout *Dictyostelium* demonstrate reduced chemotaxis, corrected by reducing PI3K output (Tang et al. 2011). In keeping with this, previous *in vitro* studies by our group demonstrated that incubation of COPD neutrophils with a non-selective PI3K inhibitor (LY29004) restored normal migratory behaviour by reducing their speed and increasing their accuracy, although LY29004 is not specific to PI3K so its effect could not be exclusively attributed to PI3K activity (Sapey et al. 2011).

However, these are likely to be associated with various side effects as PI3K is associated with many cellular functions. However, the γ- and δ-isoforms are the dominant isoforms in leukocytes (Ferguson et al. 2007, Martin et al. 2010), so their anti-inflammatory effects may target these cells more exclusively. Data on specific isoform inhibition and neutrophil migration is limited. One study investigating blockade of PI3Kγ in mouse models of rheumatoid arthritis demonstrated that neutrophil recruitment and joint inflammation is suppressed in both PI3Kγ knockout mice and those treated with an oral PI3Kγ inhibitor (Camps et al. 2005). In contrast, a later murine study demonstrated that GROα-mediated recruitment of neutrophils to the lung was only inhibited in PI3Kγ knockout mice when treated with a PI3Kδ inhibitor and not by PI3Kγ or PI3Kδ inhibitor in wild type mice (Pinho et al. 2007). Another group observed that inhibition of PI3K by LY294002 and PI3Kγ by a specific isoform inhibitor, blocked neutrophil recruitment driven by the chemokine regulated on activation normal T-cell expressed and secreted (RANTES) in a murine model of peritoneal chemotaxis (Ferrandi et al. 2007). Collectively, these data suggest that specific PI3K isoform inhibition can modulate neutrophil chemotaxis, although the effects of PI3K isoform inhibition in human COPD patients are currently unknown. In addition, PI3Kδ may also reverse the corticosteroid resistance in COPD (Hamblin et al. 2008) and its inhibition

attenuates neutrophil migration into inflamed tissue by 20-40% in a murine model of acute lung injury (Puri et al. 2004). This partial inhibition would be of particular importance if the same effect was observed in human subjects with COPD as the desired outcome would be to provide a degree of relief to the lung parenchyma from neutrophil influx (thereby reducing lung damage and slowing disease progression), while not significantly compromising the innate immune defence within the pulmonary airspace.

There have also been a number of studies investigating the effects of statins in COPD. Statins are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors that are traditionally used to treat hypercholesterolaemia by blocking the mevalonate pathway. In addition to blocking cholesterol synthesis, all products downstream of HMG-CoA are reduced, including the isoprenoids farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), which regulate Ras and Rho activity respectively. A number of studies suggest Ras and Rho inactivation by statins have a number of pleiotropic anti-inflammatory and immunomodulatory effects (Fromigue et al. 2006, Dai et al. 2007). These effects are shown in Table 1.2 (summarised from Blanco-Colio et al. 2003).

Anti-Inflammatory Effects	Immunomodulatory Effects
↓ Inflammatory Cell activation/recruitment	↓ Proliferation of lymphoid cells
↓ Inflammatory Cell proliferation/differentiation	↓ Natural killer cell activity
↓ Pro-inflammatory cytokine release	↓ Major histocompatibility class II antigens
↓ Adhesion molecule expression	↓ Organ rejection
↓ Transcription factors	
↓ ROS production	

Table 1.2: A summary of the potential pleiotropic effects of statins.

Legend: Statins are thought to have many anti-inflammatory and immunomodulatory effects in addition to their effect on cholesterol. Many of these arise through the inhibition of GTPase activity.

There have been a number of studies into the effects of statins on neutrophil inflammation and migration, although the majority of these simply investigate overall cell recruitment rather than migratory dynamics. It has previously been shown that statins inhibit neutrophil influx into the lungs in both human transplant recipients (Maher et al. 2009) and murine models of emphysema (Lee et al. 2005). One study in atherosclerosis found that simvastatin inhibits neutrophils adhesion to the endothelium under flow (Eccles et al. 2008). In addition, a longitudinal study by Guasti et al. showed that a four-week treatment course of 20 mg simvastatin in high-risk atherosclerosis patients attenuated the pro-inflammatory properties of neutrophils by reducing chemotaxis (using a Boyden Chamber) as well as IL-8 production (Guasti et al. 2006). A similar study in healthy individuals also showed a reduction in *ex vivo* neutrophil chemotaxis following a two-week course of 40 mg simvastatin (Kinsella et al. 2011). Animal studies have also shown that statins reduce neutrophil recruitment to the lungs of rats following one-lung ventilation and re-expansion

(Leite et al. 2013) and mice following inflammatory insult with LPS (Grommes et al. 2012). Clinical trials of statin in respiratory disease are limited. One recent trial in COPD found no positive effect of 40 mg simvastatin on exacerbation frequency over two years, although no assessment of neutrophil function was included (Criner et al. 2014). A similar trial on ventilator-acquired pneumonia (VAP) showed no difference in 8-day mortality with 60 mg simvastatin (Papazian et al. 2013). At present, the effects of statins on the migratory dynamics of COPD neutrophils remains unexplored, although statins could potentially alter chemotaxis neutrophils, at least in part, by modulating PI3K activity through GTPase prenylation.

1.6 ALPHA 1-ANTITRYPSIN DEFICIENCY

Alpha 1-Antitrypsin Deficiency (α_1-ATD) was first described by Laurell and Eriksson in 1963 when they found missing α_1 bands in electrophoretic patterns of serum samples from some patients with emphysema (Laurell et al. 1963). It is a hereditary condition resulting from the inheritance of two recessive alleles on the α_1-AT gene (Darlington et al. 1982). There are over 100 different phenotypes of the α_1-AT protein, with "PiZ" being the most common deficiency. It causes a mutation the tertiary structure of α_1-AT protein, where a single substitution of lysine for glutamic acid widens the β-sheet. This results in polymerisation of α_1-AT that prevents its secretion by hepatocytes (Lomas et al. 1992) and leads to severely limited serum concentration.

In PiZ individuals, there is a proteinase/anti-proteinase imbalance between the concentrations of NE (normal) and α_1-AT (reduced). Uncontrolled NE activity leads to a

greater degree of lung tissue destruction during neutrophil migration and degranulation (Campbell et al. 1999). This process is illustrated in Figure 1.12.

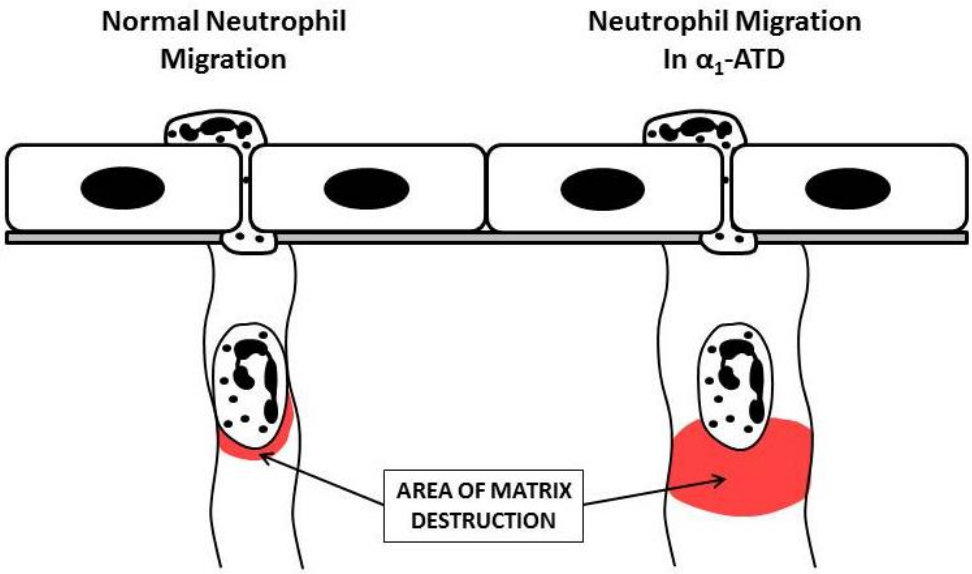

Figure 1.12: A comparison of collateral damage to the extracellular matrix during neutrophil migration in a health and α_1-ATD.

Legend: Neutrophil migration through the lung matrix in health (small area of destruction due to effective neutralisation of NE by α_1-AT) and in α_1-ATD (large area of destruction due to poor control of NE by low serum levels of α_1-AT).

The presence of NE further increases the release of pro-inflammatory mediators (such as LTB_4 by macrophages), thereby increasing the neutrophil influx and indirectly amplifying the destructive processes. Over time, these processes can manifest physiologically as panaciner emphysema with basal distribution, which may not only occur at a relatively early age, but also in non-smokers (Gishen et al. 1982, Brantly et al. 1988). Studies of neutrophil migration in α_1-ATD are limited but one study suggests migration towards sputum in α_1-ATD

could be enhanced (Woolhouse et al. 2002). However, this may be explained by the increased concentration of chemoattractants in the α_1-ATD sputum (such as IL-8 and LTB_4) rather than altered neutrophil function.

Patients with α_1-ATD provide a useful control group when studying neutrophil inflammation and migration in COPD as they can be matched for all elements of lung disease and inflammation as well as smoking history and therapeutics.

1.7 SUMMARY

COPD is chronic inflammatory condition of the lungs. The pathophysiology is complex, involving a number of inflammatory cells and a multitude of mediators. It is believed that the neutrophil is the key effector cell in the disease pathology and numerous experimental models have demonstrated that they can cause all of the pathological features of COPD. Furthermore, neutrophil numbers within the lung correlate with disease severity and progression. However, the exact role of neutrophils in COPD and the precise mechanisms of their dysfunction (particularly migratory behaviour) remain unclear. Further definition of the cellular pathways involved in neutrophil chemotaxis and migratory morphology may yield a greater understanding of their role in COPD and ultimately lead to the development of much-needed novel therapeutic interventions.

1.8 AIMS OF THE THESIS

The experiments in this thesis were designed to investigate different aspects of inflammation and migration of COPD neutrophils compared to those from age-matched healthy control subjects and patients with α_1-ATD. There are many aspects of neutrophil

function and their inflammatory environment that could potentially explain their dysfunction in COPD, though the experiments of this thesis were designed to investigate five main questions;

1. *Is the aberrant migration of COPD neutrophils to IL-8, GROα and sputum previously described (Sapey et al. 2011) a generic phenomenon and is it inherent to the cell or influenced by the local inflammatory environment? (Chapter 3).*

To study this, two-dimensional neutrophil migratory dynamics were determined *in vitro* using a specific chamber for direct visualisation of chemotaxis (Muinonen-Martin et al. 2010) in response to five chemoattractants; IL-8, GROα, fMLP, LTB_4 and C5a. These were chosen to represent different arms of host immunity (some of which have been shown to be increased in COPD BALF) as well as those commonly released by invading bacteria. Initial concentration responses for these chemoattractants were performed to determine the most effective concentration for the final chemotaxis experiments.

In separate, follow-on assays, COPD neutrophils were pre-incubated with plasma from a healthy donor and vice versa to determine if the inflammatory environment may be influencing chemotaxis. Furthermore, expression of surface markers of cell activation (CD11b, CD62L), maturity (CD16) and degranulation (CD63) was quantified on neutrophils in whole blood by flow cytometry to determine of COPD neutrophils were of a different phenotype to those from healthy subjects.

2. *Does the migratory accuracy of neutrophils worsen with disease severity or relate to other factors that comprise the clinical phenotype of COPD patients (Chapter 3).*

To study this, migratory data from the current study was collated with data from collaborative researchers to generate a large cohort of both healthy individuals and COPD patients with GOLD Stages I-IV (GOLD 2007). Chemotaxis in each GOLD Stage was compared in a group-wide analysis and linear regression was conducted comparing chemotaxis to FEV_1 % Predicted and FEV_1/FVC. Comparisons of chemotaxis to age, smoking status and the presence of emphysema were also made.

3. *Can any differences in migratory dynamics be explained by an altered cell surface expression of chemokine receptors or differences in their localisation and/or rates of recycling and shedding? (Chapter 4).*

To study this, surface expression of receptors for the five chemoattractants used in the original chemotaxis assay were semi-quantified using immunohistochemistry and flow cytometry. This was performed on both quiescent neutrophils (for all five chemoattractants) and then for selected receptors over a two-hour time-course following stimulation. Initial concentration responses for the fluorescent antibodies and their respective isotype control antibodies were performed to determine the most effective concentration for the final analysis.

Receptor shedding at specific time-points throughout the time-course was later quantified by Enzyme-linked Immunosorbent Assay (ELISA). Receptor localisation on

quiescent and activated, polarised neutrophils from COPD and healthy donors was determined by fluorescence microscopy.

4. *Can any differences in migratory dynamics be explained by altered intracellular signalling? (Chapter 4).*

To study this, both PI3K and Akt phosphorylation was determined by western blot. Lysates from COPD and healthy neutrophils were compared from both unstimulated neutrophils and those stimulated with various chemokines. The length of stimulation time was predetermined through time-course assays for each antibody.

In addition, intracellular calcium signalling was quantified by real-time fluorometric measurement of both peak and total calcium ion release following stimulation with IL-8.

5. *Can the dysfunctional neutrophil migration in COPD be improved with novel therapeutic interventions? (Chapter 5).*

To study this, the chemotaxis assays were repeated in selected chemokine gradients after pre-incubation in a variety of different pharmaceutical agents. These included CXCR1 and CXCR2 agonists, specific PI3K isoform inhibitors (α, β, γ & δ), Erk and p38 inhibitors and simvastatin. The concentrations used were either based on those currently used in medical prescription (simvastatin), half maximal inhibitory concentration (IC_{50}) (PI3K isoform inhibitors) or maximal effect (CXCR1 and CXCR2 agonists) as determined by concentration response experiments.

CHAPTER 2

METHODS

2.1 HUMAN PARTICIPANTS

All experiments in the current study were approved by the South Birmingham Health Authority ethics committee (Ethics Number: LREC3359A). All subjects included provided informed and written consent for the donation of blood samples.

COPD was defined clinically in accordance with the GOLD guidelines (Pauwels et al. 2001). All patients with α_1-ATD were of the PiZ phenotype. Due to the potential effects of ageing on neutrophil function, all subjects were aged-matched within five years.

2.2 PULMONARY FUNCTION TESTING

All subjects undertook pulmonary function testing in the Lung Investigation Unit, University Hospital Birmingham NHS Foundation Trust. Tests were performed by trained Respiratory Physiologists in accordance with the national guidelines recommended by the British Thoracic Society (BTS) (BTS 1997). As a minimum, all subjects undertook Spirometry (including FEV_1 and FVC), though COPD and α_1-ATD patients often undertook Static Lung Volumes and Gas Transfer tests as part of their routine lung function assessments.

2.3 PERIPHERAL BLOOD PROCESSING

Venous blood samples were taken using lithium heparin Vacutainer® tubes (Becton Dickinson Vacutainer System, Franklin Lakes, USA) and processed within 30 minutes of collection.

2.3.1 Plasma Samples

Plasma samples were obtained by centrifugation of blood at 3000rpm for 10 minutes at 20°C (Jouan C3i Centrifuge, DJB Labcare, Buckinghamshire, UK). If they were not used on the same day, the samples were stored at -80°C until required.

2.3.2 Neutrophil Isolation

Neutrophils were isolated in accordance with a previously established protocol (Afford et al. 1992). Whole blood was first treated with 2% Dextran T500 (500 kDa molecular weight) (Pharmacosmos, Holbaek, Denmark) in Phosphate Buffered Saline (PBS) (Sigma-Aldrich, Poole, UK) at a ratio of 1 ml 2% Dextran to 6 ml whole blood. Once the erythrocytes had sedimented, the remaining plasma containing the leukocytes was extracted and layered onto a discontinuous Percoll density gradient. 100% Percoll (Sigma-Aldrich, Poole, UK) was diluted in a ratio of 9:1 with sterile 10x Saline solution (1.5M NaCl) to give 90% Percoll, or "100% working Percoll" (wPercoll). 100% wPercoll was further diluted into 80% wPercoll (by dilution in a ratio of 4:1 with 1x Saline solution (0.15M NaCl)) and 56% wPercoll (by dilution in a ratio of 14:11 with 1x Saline solution). The discontinuous gradient was prepared by carefully underlaying 5 ml 56% wPercoll with 2.5 ml 80% wPercoll in a 15 ml Falcon™ Tube (BD Biosciences, Oxford, UK). Up to 6.5 ml the plasma containing leukocytes was carefully layered onto the gradient and the tube was then centrifuged at 1100rpm for 20 minutes at 20°C (Jouan C3i Centrifuge, DJB Labcare).

Following centrifugation, the plasma and peripheral blood mononuclear cell layer was removed using a pasteur pipette and the isolated neutrophil layer was then extracted with a fresh pipette and then washed with sterile PBS by centrifugation at 1600rpm for 10 minutes

at 20°C (Jouan C3i Centrifuge, DJB Labcare). The cells were then re-suspended in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich) for all experiments except calcium signalling, where they were re-suspended in calcium-containing Hanks Buffered Saline Solution (HBSS) (Sigma-Aldrich). Neutrophils were counted in a haemocytometer and, if necessary, diluted further to achieve the desired concentration (between 1 and $4x10^6$ neutrophils/ml, depending on the assay). Purity was verified by differential Giemsa staining and only viable samples (neither activated nor apoptotic) with a neutrophil purity of >95% were used. Neutrophil gradients before and after centrifugation are illustrated in Figure 2.1.

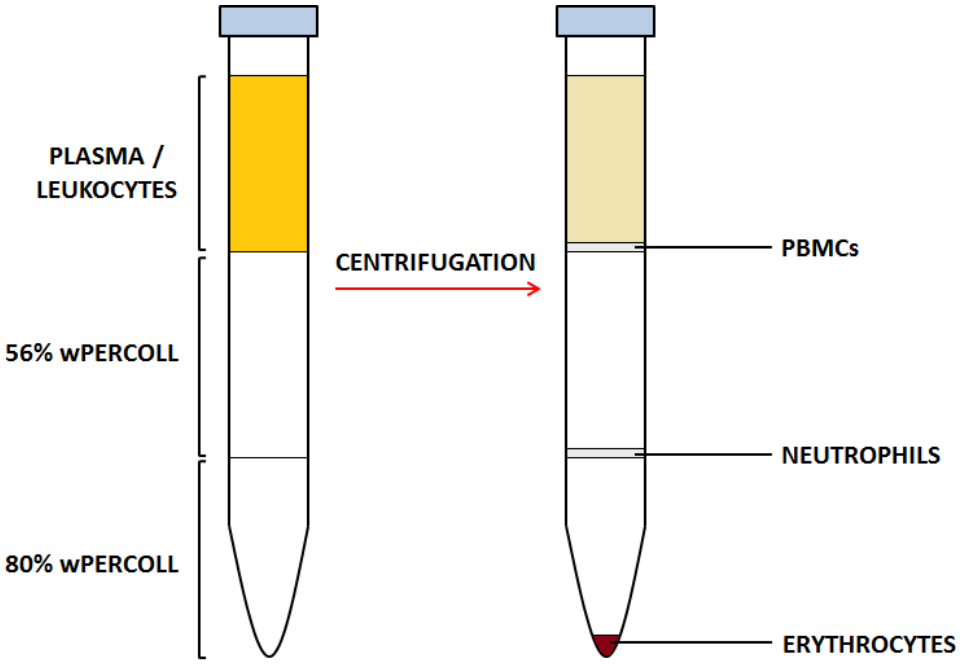

Figure 2.1: A diagram illustrating the discontinuous Percoll gradient.

Legend: Before (left) and after (right) centrifugation at 1100rpm for 20 minutes at 20°C. The PBMC layer is disposed of before the neutrophil layer is removed, washed in PBS and re-suspended at the desired concentration in the appropriate medium.

2.4 SPUTUM COLLECTION AND SOL PHASE PREPARATION

Spontaneously expectorated sputum samples were collected from COPD patients in sterile containers within four hours of waking. Patients undertook mouth washing procedures prior to expectoration to minimise saliva contamination. A large aliquot (1 g minimum weight) was ultracentrifuged at 50,000 g for 90 minutes at 4ºC. The supernatant was removed and stored at -80ºC until required.

2.5 NEUTROPHIL MIGRATION ASSAYS

2.5.1 Neutrophil Migration to Different Chemoattractants

Neutrophils were isolated and re-suspended in RPMI-1640 at a concentration of $2x10^6$/ml. 7.5% Bovine Serum Albumin (BSA) (Sigma-Aldrich) was then added to the neutrophil suspension at a volume quantity of 0.15%. Coverslips were prepared by submersion in 0.4M sulphuric acid and then twice in fresh distilled water before being coated on one side with 7.5% BSA. After 20 minutes, the BSA was tipped off and 300 ml of neutrophil suspension at $2x10^6$/ml (equivalent to $6x10^5$ cells) was then added to the coverslip and a 20 minute incubation period at room temperature was allocated to allow sufficient time for the cells to adhere.

The assay was performed using an Insall Chamber (Muinonen-Martin et al. 2010) at room temperature. The channels were cleaned twice with RPMI-1640 and then re-filled before each use. Excess neutrophils were tipped off the coverslip after 20 minutes incubation and the coverslip was inverted over the Insall Chamber channels. The RPMI-1640 in the loading channels was extracted and replaced by pipette with either the same medium (as a

control) or one of the five chemoattractants; IL-8 (R&D Systems, Abingdon, UK), GROα (R&D Systems), fMLP (Sigma-Aldrich), LTB_4 (Sigma-Aldrich) or C5a (Sigma-Aldrich). Phase time-lapse recordings were made using a Zeiss Axiovert 200 Microscope (Zeiss, Welwyn Garden City, UK) fitted with a Hammamatsu C4742-95 digital camera (Hammamatsu, Welwyn Garden City, UK). Images were captured every 20 seconds for 12 minutes (this was chosen as previous unpublished data from our group showed that migrational behaviour beyond this time did not alter). Figure 2.2 is a diagrammatic representation of the Insall Chamber, showing a magnified concentration gradient and microscopic image of the viewing field containing migrating neutrophils.

Images were then analysed to determine cell movement using the Java software ImageJ (Wayne Rasband, National Institutes of Health, Bethesda). A total of 10 cells were chosen at random and digitally tracked in every other image captured (a total of 18 images spanning 12 minutes). The resulting neutrophil migratory parameters included four separate indices. Firstly, the speed of migration, or "chemokinesis" (the distance travelled between two frames in μm/min), which equates to non-specific movement in any direction. Secondly, the chemotaxis (migrational speed in a consistent direction towards the chemoattractant source measured in μm/minute), which is a measure of the speed of migration towards the chemotactic source. The third was the directional persistence (the continuity of cell orientation over time, calculated by the cosine of the angle between directions in consecutive frames, on a scale of 0 to 1), which is a determinate of how frequently and rapidly the cells change direction during migration (the closer to a straight line and the lower the number of directional changes, the higher the persistence). Lastly, was the chemotactic index (cosine of the angle between the cell's direction and the orientation of the chemoattractant gradient, on

a scale of -1 and 1), which is an overall measure of how accurately and rapidly the cell migrates towards the chemotactic source (a higher score indicates more accurate migration).

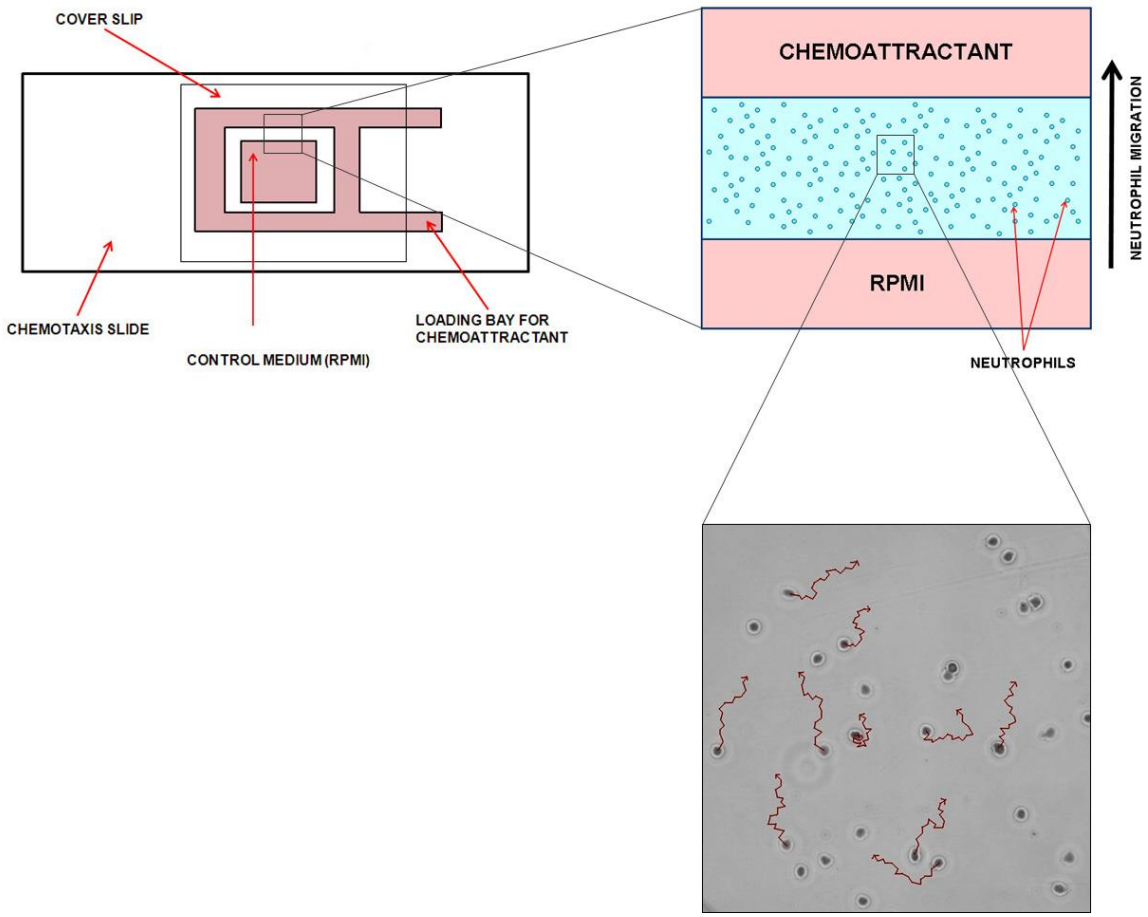

Figure 2.2: A diagrammatic representation of the Insall Chamber.

Legend: An overhead view of the chamber (top left) with magnified regions showing the chemotactic gradient (top right) with an arrow indicating the expected trajectory of neutrophil migration towards the chemoattractant. Further magnification shows a microscopic image of migrating neutrophils (bottom right). Examples of the migratory tracks are shown in red on the microscopic image with the start point at the cell and the endpoint at the arrowhead.

2.5.2 Effect of Donor Plasma on Neutrophil Migration

For this "cross-over" assay, neutrophil migration was assessed in RPMI-1640 and chemoattractants that were limited to 100 nM IL-8 (an endogenous cytokine) and 100 nM fMLP (a bacterial cytokine). For COPD neutrophils, migration was assessed with cells bathed in RPMI-1640 medium, 100% plasma from the same patient and 100% plasma from healthy control subject that was closely age-matched. For healthy control neutrophils, migration was assessed with cells bathed in RPMI-1640 medium, plasma from the same donor and plasma from an age-matched COPD patient. Incubation in the various media was done at room temperature for 40 minutes (including 20 minutes in solution and 20 minutes on the albumin-coated coverslip). Images were analysed and results reported as described previously (Section 2.5.1).

2.5.3 Neutrophil Migration with CXCR1 and CXCR2 Agonists

COPD neutrophil migration was assessed in RPMI-1640, 100 nM IL-8 and 10 nM GROα. Migration in IL-8 and GROα was assessed with and without pre-incubation for 40 minutes in either 2 μg/ml of CXCR1 inhibitor (Monoclonal Mouse IgG2A Clone # 42705) or 50 μg/ml of CXCR2 inhibitor (Monoclonal Rat IgG2A Clone # 242216) (both R&D Systems). In addition, migration in IL-8 was also assessed following incubation for 40 minutes with a dual CXCR1/2 inhibitor (SCH527123) (AbMole Bioscience, Kowloon, Hong Kong). SCH527123 was diluted initially in DMSO then RPMI-1640 to two different IC_{50} concentrations; 3nM (specific to CXCR2) and 42nM (required for concurrent CXCR1 inhibition). These concentrations were recommended by the manufacturer. Neutrophils that

were not incubated with the CXCR1/2 inhibitors were incubated with the equivalent concentration of DMSO for the same duration of time as a carrier control.

2.5.4 Neutrophil Migration with PI3K Isoform Inhibitors

COPD neutrophil migration was assessed in RPMI-1640, 100nM IL-8 alone and 100 nM IL-8 following pre-incubation for 40 minutes with PI3Kα, -β, -γ and -δ isoform inhibitors (Stratech Scientific Ltd, Newmarket, UK) or a the equivalent concentration of DMSO as a carrier control. PI3K inhibitors were used at the IC_{50} final concentrations (prepared initially in DMSO then diluted with RPMI-1640) recommended by the manufacturer, which are shown in Table 2.1.

Isoform Inhibitor	Product Name	IC_{50} (nM)
α	PIK-75	7.8
β	TGX-221	8.5
γ	AS-25	33
δ	CAL-101	65

Table 2.1: PI3K isoform inhibitors.

Legend: The four PI3K isoform inhibitors used in the chemotaxis assay together with their IC_{50} as recommended by the manufacturer.

2.5.5 Neutrophil Migration with p38 and ERK Inhibitors

COPD neutrophil migration was assessed in RPMI-1640, 100nM IL-8 alone and 100 nM IL-8 following pre-incubation for 40 minutes with either an ERK inhibitor (UO126) or one of two different p38 inhibitors (SCIO469 or VX745) (Tocris Bioscience, Abingdon, UK) or the equivalent concentration of DMSO as a carrier control. The ERK and p38 inhibitors were diluted initially in DMSO then RPMI-1640 to the IC_{50} final concentrations recommended by the manufacturer, which are shown in Table 2.2.

Inhibitor	Product Name	IC_{50} (μM)
Erk	UO126	0.07
p38	SCIO469	0.09
p38	VX745	0.01

Table 2.2: ERK and p38 inhibitors.

Legend: The ERK and p38 inhibitors used in the chemotaxis assay together with their IC_{50} as recommended by the manufacturer.

2.5.6 Neutrophil Migration with Simvastatin

Neutrophils from COPD patients were incubated for 40 minutes with simvastatin (Sigma-Aldrich) before their migratory dynamics in gradients of IL-8 and fMLP were assessed. The concentrations of simvastatin used were based on doses used in general therapy, with 1 nM equivalent to the minimum therapeutic dose of 20 mg/day and 1 μM

equivalent to the maximum therapeutic dose of 80 mg/day. Patients were excluded from this experiment if they were currently on statin therapy.

2.6 NEUTROPHIL PHENOTYPE

To determine if neutrophils from the peripheral blood of COPD patients were of a different phenotype to those from healthy controls, the expression of different surface markers was quantified by flow cytometry. These included markers of cell activation (CD11b, CD62L), neutrophils maturity (CD16) and a marker of degranulation (CD63). A*ctive* CD11b was used as, only when it dimerises with CD18 to form Mac-1, does it become a marker of neutrophil activation. To minimise the risk of activation during the assay preparation, neutrophils were not isolated. Instead, whole blood was used and the neutrophil population was gated for analysis.

50 µl of whole blood was added to each FACS tube. 500 µl of 1% PBS-BSA was then added to each tube and the cells were then pelleted by centrifugation at 4˚C for 5 minutes at 250g (Jouan C3i Centrifuge, DJB Labcare). The supernatant was then carefully removed with a pasteur pipette and Fluorescein Isothiocyanate Isomer (FITC) conjugated monoclonal antibody for 20 µl Active CD11b (Clone CBRM1/5, 400 µg/L), 5 µl CD16 (Clone 3G8, 400 µg/L) and 20 µl CD63 (Clone H5C6, 200 µg/L) were added into the appropriate FACS tubes (all from BioLegend, London, UK). 20 µl Phycoerythrin (PE) conjugated CD62L (BioLegend, Clone DREG-56, 25 µg/L) was added into the tube containing the FITC CD16. IgG1 isotype-matched irrelevant antibodies were also used at the same concentrations to control for non-specific binding. FITC IgG1 (BioLegend, Clone MOPC-21, 500 µg/L) alone was added to one FACS tube of whole blood, and both FITC and PE IgG1 (Clone MOPC-21,

200 µg/L) was added to the other. The FACS tubes were then left on ice for 30 minutes in the dark to allow for antibody/receptor binding. The cells were then washed twice with 500 µl 1% PBS-BSA by centrifugation at 4˚C for 5 minutes at 250g (Jouan C3i Centrifuge, DJB Labcare), with the supernatant removed and replaced with fresh 1% PBS-BSA between washes. After the second spin, the supernatant was removed and replaced with 2 ml of BD FACS lysing solution (BD Biosciences, Oxford, UK) (diluted 1:10 with distilled water) to remove the erythrocytes. After 10 minutes, the tubes were centrifuged at 4˚C for 5 minutes (Jouan C3i Centrifuge, DJB Labcare) and then washed twice more with 500 µl 1% PBS-BSA (between washes, the excess fluid was simply tipped off). After the final wash, the cells were re-suspended in 300 µl 1% PBS-BSA, placed on ice and analysed immediately with a BD Accuri C6 Flow Cytometer (BD Biosciences).

10,000 cells were analysed in each sample and the neutrophil population was identified by assessing forward and sideways light scattering, with forward scatter equating to cell size and side scatter to cell granularity. Gating was used to ensure only the signals from the neutrophil population were included. Neutrophil fluorescence from those labelled with specific receptor antibodies were determined relative to those labelled with the analogous non-specific antibody by subtraction of the isotype Median Fluorescence Intensity (MFI) value from that of the receptor antibody. A representative image of fluorescent cells (showing forward and side scatter) and neutrophil gating is shown in Figure 2.3.

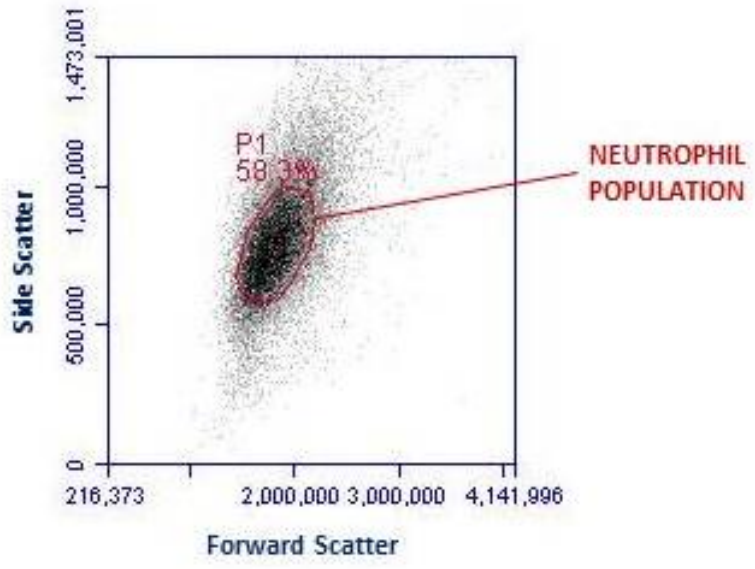

Figure 2.3: A graphical display of a typical FACS plot.

Legend: The neutrophil population was determined by cell size (forward scatter) and granularity (side scatter). The neutrophils were gated around in order to exclude debris and other cell types in the analysis. Each pixel is one cell and a total of 10,000 neutrophils were analysed for each sample.

2.7 CHEMOKINE RECEPTORS

2.7.1 Receptor Expression on Quiescent Neutrophils

$2x10^5$ isolated neutrophils in 100 µl RPMI-1640 (Sigma-Aldrich) were added to appropriate wells of a 96 well plate. An additional 100 µl of cold 1% PBS-BSA was added to each well. The plate was then spun in a centrifuge pre-cooled to 4˚C for 5 minutes at 250g (Jouan C3i Centrifuge, DJB Labcare). Pelleted cells were then resuspended in 50 µl of cold 1% PBS-BSA, followed by the addition of appropriate Fluorescein Isothiocyanate Isomer (FITC) conjugated monoclonal antibody for CXCR1 2 µg/ml (Clone 42705), CXCR2

3 μg/ml (Clone 48311), fMLP receptor (FPR1) 3 μg/ml (Clone 350418), LTB_4 receptor (LTB_4R1) 3 μg/ml (Clone 203/14F11) and C5a receptor (C5aR) 3 μg/ml (Clone 347214) (all from R&D Systems). An IgG2A isotype-matched irrelevant antibody (Dako, Ely, UK, Clone DAK-GO5) was used as a control for CXCR1, CXCR2, FPR1 and C5aR antibodies and an IgG1 isotype-matched irrelevant antibody (Dako, Clone DAK-GO1) was used as a control for LTB_4R1 antibody. The suspension was mixed and incubated in the dark at 4˚C for 15 minutes.

After incubation, an additional 150 μl of cold 1% PBS-BSA was added to each well and the plate was spun again in a centrifuge pre-cooled to 4˚C for 5 minutes at 250g (Jouan C3i Centrifuge, DJB Labcare). Following this spin, the pelleted cells were resuspended in 200 μl of cold 1% PBS-BSA and transferred to individual tubes (already containing 200 μl of cold 1% PBS-BSA) for analysis by flow cytometry using a Coulter Epics XL machine (Coulter Corp., Florida, USA).

2.7.2 Receptor Expression Following Stimulation

The change in neutrophil surface receptor expression of CXCR1, CXCR2 and FPR1 was measured over a two hour time-course following stimulation with either IL-8 or fMLP at various time-points. These chemoattractants were selected based on their potency and the fact that they originate from both an endogenous and bacterial source. The assay was performed using neutrophils from COPD patients, healthy subjects and patients with α_1-ATD.

The wells of a 96 well U-bottomed FACS plate were pre-coated with heat-inactivated Fetal Calf Serum (FCS) (Sigma-Aldrich) to minimise cell adhesion. 50 μl of neutrophil

suspension ($4x10^6$ neutrophils/ml suspended in RPMI-1640 +10% FCS) was then added to each well of the FACS plate required for the time-course. Stimulants used were IL-8 and fMLP at final concentrations of 100 nM, which were added to specific wells at the appropriate time-points. The FACS plate was incubated at 37°C for the duration of the time-course to allow receptor internalisation, shedding and re-expression to occur. The cells were kept in suspension by gently tapping the FACS plate every 30 minutes.

At the end of the time-course, the FACS plate was removed from the incubator and immediately placed on ice while specific anti-human antibodies were added to the appropriate wells. These included CXCR1 PE conjugated (at 2 µg/ml), CXCR2 FITC conjugated (at 3 µg/ml) and FPR1 FITC conjugated at (3 µg/ml). The isotype-matched irrelevant antibody, IgG2A (R&D Systems, Clone MOPC-21) was used at the same concentration to each of the respective anti-receptor antibodies.

The FACS plate was then left on ice in the dark for 15 minutes to allow surface binding of the antibodies to occur. Each well was then topped up with 150 µl of cold 2% PBS with Bovine Serum Albumin (PBS-BSA) (Sigma-Aldrich) and centrifuged at 250g for 5 minutes in a pre-cooled centrifuge at 4°C (Jouan C3i Centrifuge, DJB Labcare). Following centrifugation, the supernatant was carefully collected (ensuring the pellet was not disturbed) from each time-point and placed in labelled eppendorf tubes. These were stored at -80°C for future quantification of receptor shedding by ELISA (see Section 2.6.4). Neutrophils were then re-suspended in 200 µl of 2% cold PBS-BSA and placed into labelled FACS tubes already containing 200 µl of cold 2% PBS-BSA and kept on ice for immediate analysis with a BD Accuri C6 Flow Cytometer (BD Biosciences).

2.7.3 Chemokine Receptor Recycling

It has previously been demonstrated that chemokine receptor re-expression occurs on the cell surface once the stimulus is removed (Chuntharapai et al. 1995). To assess this mechanism, 100 µl of neutrophil suspension (2 x 10^6/ml) in FACS tubes was treated with either 100 nM IL-8 or 100 nM fMLP at 37°C for 30 minutes. Cells were then washed with RPMI-1640 containing 10% FCS and the cells re-suspended in 100 µl RPMI-1640+ 10% FCS. 50 µl of the neutrophil suspension was then placed in corresponding wells on the FACS plate for 90 minutes at 37°C in an incubator. The cells were then stained for CXCR1, CXCR2 and FPR1 described in Section 2.7.1.

2.7.4 Chemokine Receptor Shedding

CXCR1

A commercial CXCR1 ELISA kit (Cusabio Biotech Co. Ltd, East Sussex, UK) was used to quantify CXCR1 shedding during the two hour time-course. All reagents were removed from 4°C storage and allowed to warm to room temperature for 30 minutes prior to use. All vials were vortexed before use to ensure the contents were mixed well. The working wash buffer was prepared by diluting 20 ml of the wash buffer concentrate into 480 ml of deionised water. The Biotin-antibody working solution was made by diluting the Biotin-antibody 100-fold using the Biotin-antibody diluent. Working HRP-Avidin was made by diluting HRP-Avidin 100-fold using the HRP-Avidin diluent.

Supernatants collected from COPD patients and healthy control subjects at the 30 and 120 minute time-points during the time-course (as described in Section 2.7.2) were removed

from -80°C storage and allowed to warm to room temperature. 100 µl of the standard, blank or patient sample were added in duplicate to corresponding wells on a 96 well ELISA plate. 50 µl of conjugate was then added to each well and mixed thoroughly. The plate was then sealed with an adhesive strip and left to incubate for 2 hours at 37°C. Following this, the liquid from each well was carefully removed with a pipette and the remaining drops were removed by patting the plate on a paper towel. 100 µl of the Biotin-antibody working solution was then added to each well and left to incubate for 1 hour at 37°C. The wells were then washed with an automated microplate washer (Asys Atlantis 4, Biochrom, Cambridge, UK) with 200 µl of working wash buffer and left to stand for 150 seconds. This was repeated 3 times, after which the remaining liquid was removed by patting the plate on a paper towel. 100 µl of HRP-Avidin working solution was then added to each well and the microtitre plate was then left to incubate for 1 hour at 37°C. The same aspirating and wash cycle was then repeated 5 times. Following this, 90 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added to each well and left to incubate in the dark 37°C. Once the wells containing the highly concentrated standards (S7-S6) turned blue, 50 µl of the stop solution was added to each well. The optical density was then determined immediately using a microplate reader (BioTek EL808, Potton, UK) set at 450nm.

CXCR2

A commercial CXCR2 ELISA kit (Novateinbio, Massachusetts, USA) was used to quantify CXCR2 shedding during the two hour time-course. The assay procedure was very similar to the CXCR1 kit, although there were slight differences. As before, all reagents were removed from 4°C storage and allowed to warm to room temperature for 30 minutes prior to

use and all vials were vortexed before use to ensure the contents were well mixed. All standards and solutions were provided pre-prepared in the kit apart from the 10 ml of wash solution, which had to be diluted with 990 ml distilled water to form 1 litre. The concentrations of the standards were as follows; S7 = 50 ng/ml, S6 = 25 ng/ml, S5 = 12.5 ng/ml, S4 = 6.25 ng/ml, S3 = 3.125 ng/ml, S2 = 1.563 ng/ml and S1 = 0.781 ng/ml.

100 µl of the standard, blank or patient sample were added in duplicate to corresponding wells on a 96 well ELISA plate. 50 µl of conjugate was then added to each well and mixed thoroughly. The plate was then sealed with an adhesive strip and left to incubate for 1 hour at 37°C. The plate was then washed five times with an automated microplate washer (Biochrom) with 400 µl of working wash buffer with a soaking time of 10 seconds and a shaking time of 5 seconds in between washes. 50 µl Chromogenic Substrate A and then 50 µl Chromogenic Substrate B was added to each well. The plate was then covered and incubated for 10 minutes at room temperature away from sunlight. 50 µl of the stop solution was then added to each well and mixed thoroughly. The optical density was then determined immediately using a microplate reader (BioTek) set at 450nm.

2.7.5 Chemokine Receptor Localisation

Chemokine receptor localisation was assessed by fluorescence microscopy following binding of conjugated monoclonal antibodies for CXCR1 and CXCR2. This was done with quiescent and polarised neutrophils from both healthy and COPD donors.

Coverslips were prepared by submersion in 0.4M sulphuric acid and then twice in fresh distilled water before being coated on one side with 80 µg/ml of fibronectin (Sigma-Aldrich), after which they were left for 45 minutes at room temperature. The fibronectin was then

removed and the coverslip immersed in PBS to rinse. 300 µl of neutrophil suspension ($2x10^6$/ml in RPMI-1640) was immediately layered onto the fibronectin and 15 minutes were allowed for the cells to adhere. The excess RPMI (including any neutrophils that had not adhered) was then removed and replaced with either 100 nM fMLP (to induce polarisation without affecting the receptors of interest) or RPMI-1640. After 5 minutes, the fMLP or RPMI-1640 was replaced with 4% Paraformaldehyde (PFA) and the coverslips were left for 30 minutes to fix the neutrophils. The PFA was then replaced with either 2 µg/ml CXCR1 PE conjugated or 3 µg/ml CXCR2 FITC conjugated. The coverslips were then left in the dark for 15 minutes to allow sufficient time for antibody/receptor binding to occur.

The FITC-stained neutrophils showed weak fluorescence. Therefore, these cells were subsequently bathed in 300 µl of Anti-FITC Alexa Fluor 488 (Life Technologies Ltd., Paisley, UK) (diluted from stock 1:100 with PBS) in the dark for 20 minutes. Excess solution was then removed and the coverslips were inverted and fixed to labelled microscope slides with one drop of Fluoromount (Sigma-Aldrich) and left to dry for 10 minutes before observation under a fluorescence microscope (Leica Microsystems, Milton Keynes, UK) using a x40 objective lens. Cells were selected at random starting at the bottom left corner and gradually moving upwards (by traversing the viewing field left to right then right to left) until the first clearly visible population of neutrophils was found.

2.8 PI3K & AKT PHOSPHORYLATION BY WESTERN BLOT

2.8.1 Neutrophil Stimulation and Lysis

Once isolated, neutrophils were suspended at $4x10^6$ cells/ml in RPMI-1640. 1 ml ($4x10^6$ cells) was added to each of four eppendorf tubes, which were then centrifuged at 4000 rpm for 5 minutes in a Micro Centaur (Sanyo, UK). Excess RMPI-1640 was discarded and the cells were resuspended in 90 µl of RPMI-1640. 10 µl of stimulant (to give final concentrations of 100 nM IL-8, 100 nM fMLP and 10 nM GROα) or RPMI-1640 (control) were added to appropriate eppendorf tubes. For both $pPI3K_{T458}$ and $pAkt_{S473}$, neutrophils were stimulated at room temperature for 3 minutes whereas, for $pAkt_{T308}$, they were stimulated at room temperature for 20 seconds (see Section 4.4.1 for stimulation time-courses). After this time, 900 µl of cold PBS was added to each eppendorf and the samples were quickly transferred (in an ice tray) to a centrifuge (Jouan C3i Centrifuge, DJB Labcare, Buckinghamshire, UK) and spun at 4000 rpm for 3 minutes at 4°C. Following this spin, excess PBS was removed and the neutrophils were resuspended in 64 µl of MOPS-based lysis buffer (8 µl per 0.5 x 10^6 cells). The components of the MOPS-based lysis buffer are shown in Table 2.3.

Chemical	Function
20mM 3-[N-Morpholino] Propanesulfonic Acid (MOPS) (Sigma-Aldrich)	Buffer
50mM Sodium Fluoride (Sigma-Aldrich)	Phosphatase Inhibitor
50mM β-glycerophosphate (Sigma-Aldrich)	Phosphatase Inhibitor
10mM Sodium Orthovanadate (Sigma-Aldrich)	Phosphatase Inhibitor
1% Triton X-100 (Sigma-Aldrich)	Detergent
7.3ml dH_2O	
1mM 4-(2-Aminoethyl) Benzenesulfonyl Fluoride Hydrochloride (AEBSF) (Sigma-Aldrich)	Protease inhibitor
1mM Dithiothreitol (DTT) (Sigma)	Reducing agent
1:100 Calbiochem Protease inhibitor Set III (Millipore, Watford, UK)	Protease inhibitor
1:100 Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich)	Phosphatase Inhibitor
1:100 Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich)	Phosphatase Inhibitor

Table 2.3: Components of the MOPS-Based Lysis Buffer

Legend: A solution containing the first 6 components was made up in advance and stored at 4°C. The other components were added just before use.

The eppendorf tubes were then vortexed and left on ice for 5 minutes. This step was repeated twice more. 64 µl of hot 2X SDS lysis buffer was then added to each eppendorf tube, which were then transferred into a heating block and boiled for 10 minutes at 100°C. The samples were then stored at -80°C until required for western blotting. The components of the SDS-lysis buffer are shown in Table 2.4.

2X Sodium Dodecyl Sulfate (SDS) Lysis Buffer
4% SDS (Sigma-Aldrich)
0.1M DTT (Sigma-Aldrich)
20% Glycerol (Sigma-Aldrich)
0.0625M Tris-HCL (pH 6.8)
0.004% Bromophenol Blue (Sigma-Aldrich)

Table 2.4: Components of the 2x SDS Lysis Buffer

Legend: The buffer solution was made up in advance, stored at -20°C and heated before use.

2.8.2 Running Samples by Western Blot

Subject samples (COPD and healthy control) were removed from -80°C storage, allowed to warm to room temperature and subsequently boiled at 100°C for 5 minutes. Samples were then centrifuged at 13,000 rpm for 5 minutes to pellet any debris and 20 µl of each sample were added to corresponding lane in a Criterion TGX Precast Gel (Bio-Rad, Hemel Hempstead, UK). The first lane was allocated for 20 µl of protein marker (Bio-Rad) and samples from a COPD patient and a healthy control were run in parallel.

Gels were run for 3½ hours at 100 volts in a running buffer comprising 1X Tris/Glycine/SDS (Sigma-Aldrich) and 9X distilled H_2O (dH_2O). Once the gel had run, proteins were transferred to a nitrocellulose membrane (Geneflow, Lichfield, UK) in a blotting buffer (1X Tris/Glycine (Sigma-Aldrich), 1X Methanol and 8X dH_2O) using the Bio-Rad transfer kit, standing in ice to minimise the formation of large bubbles between the gel and blotting membrane. The membrane was then blocked with 1X Tris-Buffered Saline (TBS)-1%Tween-20 containing 5% BSA under agitation for one hour at room temperature. Anti-phospho-Akt_{S473} or anti-phospho-Akt_{T308} (both New England Biolabs, Hitchin, UK) was

then added at the recommended concentration of 1:1000 and the membrane was left under agitation overnight at 4°C. The following morning, the membrane was washed for 15 minutes in 1X TBS-1%Tween-20 three times before the addition of Anti-Rabbit IgG Horseradish Peroxidase-Linked secondary antibody (GE Healthcare, Chalfont St Giles, UK) at a concentration of 1:2000. After one hour incubation under agitation at room temperature, the membrane was once again washed three times with 1X TBS-1%Tween-20. It was then covered in Enhanced Chemiluminescence (ECL) solution (Geneflow) for 5 minutes before analysis in a Bio-Rad Chemidoc system.

To determine the cellular loading of each lane, the membrane was subsequently stripped with a mild stripping buffer (15 g glycine, 1 g SDS, 10 ml Tween20, adjusted to pH to 2.2 and brought to 1 L with ultrapure water (all from Sigma-Aldrich)), re-blocked with 1X TBS-1%Tween-20 containing 5% BSA under agitation for one hour at room temperature. Total (pan)-Akt (New England Biolabs, Hitchin, UK) was then added at the recommended concentration of 1:1000 and the membrane was left under agitation overnight at 4°C. The following morning, the same protocol was followed for washing, probing with Anti-Rabbit IgG Horseradish Peroxidase-Linked secondary antibody, ECL exposure and analysis.

For phospho-PI3K$_{T458}$ analysis, the same methods were used with an anti-phospho PI3K$_{T458}$ antibody (New England Biolabs) with equal loading checked using an anti-β-Actin antibody (Sigma-Aldrich) at a 1:5000 dilution with an Anti-Mouse IgG Horseradish Peroxidase-Linked secondary antibody (GE Healthcare).

Optical density of phospho-Akt, phospho-PI3K and their respective loading controls were analysed using the Image J freeware Version 1.43u (National Institute of Health, USA).

2.9 INTRACELLULAR CALCIUM SIGNALLING

Neutrophils were isolated as described previously but re-suspended at $1x10^6$ cells/ml in RPMI-1640 + 0.5% BSA. One vial of the a UV light-excitable, ratiometric Ca^{2+} indicator, Indo-1 AM (Life Technologies Ltd, Paisley, UK) was reconstituted with 110 µl of anhydrous Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich). This was then added to the neutrophil suspension in the ratio of 7 µl per 1 ml of suspension, mixed well and incubated in the dark (in a foil-wrapped 50 ml Falcon Tube) at room temperature. Following this, the cell suspension was increased to 50 ml with additional RPMI-1640 + 0.5% BSA and then centrifuged at 1600 rpm for 10 minutes (Jouan C3i). The neutrophils were then re-suspended in fresh RPMI-1640 + 0.5% BSA and left in the dark for a further 30 minutes. They were then centrifuged again at 1600 rpm for 10 minutes and then re-suspended in calcium-containing HBSS (Sigma-Aldrich) at 1×10^6/ml.

For real-time analysis of Ca^{2+} release following stimulation, 2 ml of neutrophils suspension was transferred into a cuvette and placed in a pre-warmed water bath at 37°C for 5 minutes. The cuvette was then transferred to the fluorimeter (Perkin Elmer LS50B Luminscence Spectrometer, Beakonsfield, UK) and a magnetic stirrer was added to ensure the neutrophils remained in suspension. Once a stable baseline had established (approximately 1 minute), the neutrophils were stimulated with 100 nM IL-8 to induce intracellular Ca^{2+} release. Once the signal had returned to baseline (indicating that Ca^{2+} release had ceased), the neutrophils were stimulated with 2.7 mM ionomycin (a positive control stimulant) was added. Once Ca^{2+} release had peaked and plateaued, ethylene glycol tetra-acetic acid (EGTA) (1mM final concentration) was added to return the signal to baseline. This process is illustrated in Figure 2.4.

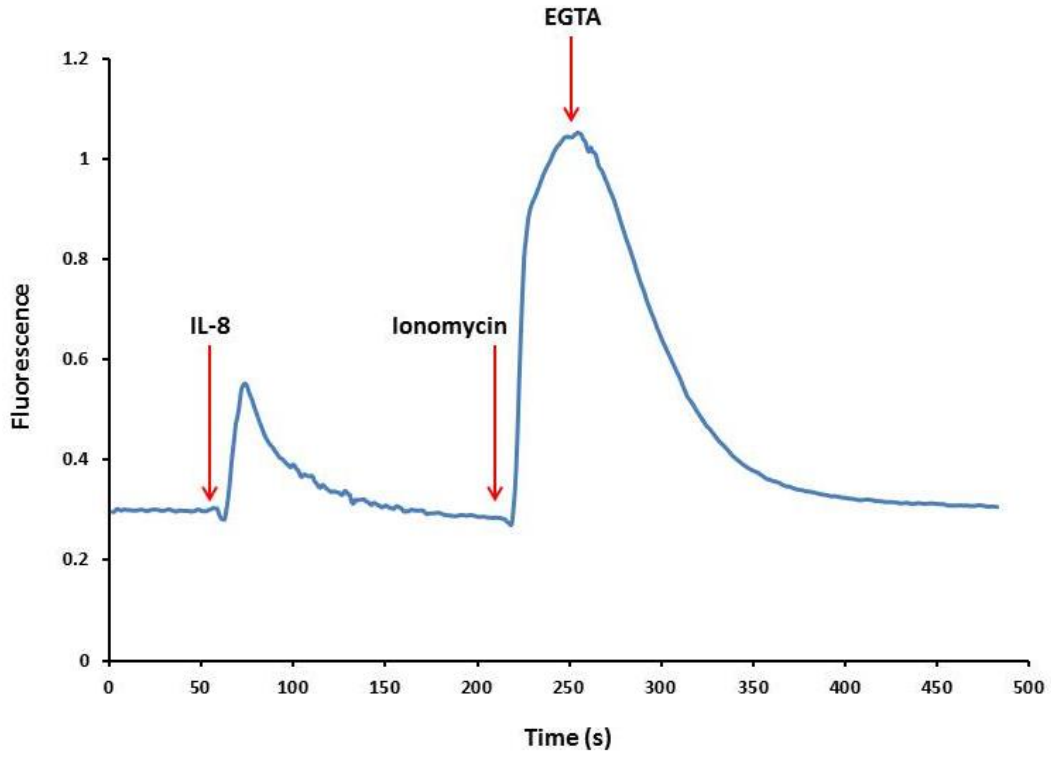

Figure 2.4: The process of Ca^{2+} signal measurement.

Legend: A graphical representation of real-time Ca^{2+} analysis. 2 x 10^6 neutrophils were stimulated with IL-8 to induce Ca^{2+} release from intracellular stores. Once Ca^{2+} release had ceased, ionomycin was added as a positive control and, at its peak, Ca^{2+} release was halted with the addition of EGTA.

Fluorescence measurements were then converted into Ca^{2+} concentration by comparison of the maximum and minimum fluorescence at two emission wavelengths in relation to the positive control (ionomycin). From these data, both peak (increase from baseline) and total (area under the curve) calcium release were determined and compared between neutrophils from healthy, COPD and α_1-ATD subjects.

2.10 STATISTICAL ANALYSIS

Statistical analyses were performed using either the SPSS statistical program (version 16.0, Chicago, USA) or GraphPad Prism (version 5.03, La Jolla, USA). Initially, a Kolmogorov-Smirnov test was used to determine if data sets were normally distributed. The distribution of data then dictated whether parametric tests (for normally distributed data) or non-parametric tests (for data not normally distributed) were used. For group comparisons, the specific test was then dependent on; (i) the number of data groups that were to be compared and (ii) whether or not the data groups were from different patients (unpaired) or separate data from the same patients (paired; e.g. pre- and post-therapeutic intervention). Lastly, the application of a one-tailed or two-tailed test depended on the expected outcome. For example, if results from one group were expected to be higher or expected to be lower (e.g. an increase from baseline following the application of a validated intervention such as a functional inhibitor) the test was one-tailed. In instances where the outcome could not be predicted, a two-tailed test was used, where the "p" value was twice that of a one-tailed test (meaning the probability of the outcome occurring by chance was essentially doubled).

Therefore, for data that were normally distributed, either a t-test (unpaired for comparisons of two separate cohorts or paired for comparisons of two data sets from the same patients) or a one-way Analysis of Variance (ANOVA) (for comparisons between 3 cohorts) with subsequent Tukey's test (to determine which comparisons were significantly different from each other) were performed to compare the results. When the data were not normally distributed, either a Mann-Whitney U test (unpaired for comparisons of two separate cohorts or paired for comparisons of two data sets from the same patients) or a Kruskal-Wallis test (for comparisons between 3 cohorts) with subsequent Dunn's test (to

determine which comparisons were significantly different from each other) were conducted. For linear regression, a Pearson's correlation was used for data that were normally distributed and a Spearman's Rank correlation was used for data that were not normally distributed. In this thesis, a p value of 0.05 or less ($p \leq 0.05$) was classified as statistically significant.

To ensure the experiments were adequately powered, power calculations for sample size were performed using the following formula;

$$n = 1+2\mathbf{C} \times (\mathbf{s} / \mathbf{D})$$

D is the smallest difference detected, **s** represents the standard deviation of the observations and C = 7.85 to provide an 80% power of detecting a difference at the 5% level of significance (Snedecor and Cochran 1989).

CHAPTER 3

ABERRANT NEUTROPHIL MIGRATION IN COPD

3.1 BRIEF INTRODUCTION

COPD is a chronic inflammatory disease and there is a wealth of evidence implicating the neutrophil as a key effector cell in its pathogenesis. Neutrophils migrate towards the lung along a series of chemotactic gradients that are established through the secretion of pro-inflammatory cytokines by the endothelium, host inflammatory cells and invading pathogens.

Previous work investigating neutrophil migration in COPD has yielded conflicting results. An early study (Burnett et al. 1987) suggested that neutrophils from COPD patients migrate more efficiently than neutrophils from healthy donors, whereas a later study described a reduced chemotaxis of COPD neutrophils (Yoshikawa et al. 2007). More recently, a study looking specifically at how the neutrophils migrate by two-dimensional tracking has demonstrated that neutrophils from the peripheral blood of COPD patients migrate with increased speed but impaired accuracy in comparison to those from healthy subjects (Sapey et al. 2011). Although this study provided far more insight into how the cells were migrating rather than how many moved from one location to another, only IL-8 and GROα were used to generate chemotactic gradients. Therefore, it is unclear whether the aberrant neutrophil migration in COPD is a general phenomenon or simply related to IL-8, GROα and their respective receptors (CXCR1 and CXCR2).

The studies described in this chapter were conducted to initially investigate whether the aberrant migration of COPD neutrophils occurs in gradients of other endogenous chemoattractants as well as those of bacterial origin. Further studies were then conducted to determine if any differences in migration between neutrophils from COPD and healthy donors were due to intrinsic cell defects or a result of systemic inflammation.

3.2 SUBJECT DEMOGRAPHICS

Demographic data for the COPD patients, healthy age-matched control subjects and patients with α_1-ATD included in this chapter are shown in Table 3.1.

	COPD	Healthy Control	α_1-ATD
Number	71	70	10
Age (years)	66 (41 - 85)	58 (35 - 87)	61 (47 - 71)
Sex (% Male)	65	64	60
Smoking status	31 CS, 50 XS	6 NS, 14 CS, 21 XS	5 NS, 5 XS
Pack years	60 (4.8)	5 (1.1)	13 (4.6)
FEV1 % predicted	49.5 (2.1)	101.2 (2.0)	40.6
FEV1 /FVC %	42.9 (1.5)	73.6 (0.9)	30.0 (2.3)

Table 3.1: Subject Demographics.

Legend: A table showing the demographic data for all subjects included in this chapter. Data are normally distributed and presented as the mean and SEM, except the age which is mean (range). NS = Never-smokers, CS = Current smokers, XS = Ex-smokers.

3.3 NEUTROPHIL MIGRATION

3.3.1 Validation: Selection of Chemoattractant Concentrations

To determine the most appropriate concentration of each chemoattractant (IL-8, GROα, fMLP, LTB_4 and C5a), neutrophil chemotaxis was assessed in response to a range of concentrations using neutrophils from healthy donors (n=5). The chemotactic index (migratory accuracy) data for the concentration responses of each chemoattractant are shown in Figure 3.1.

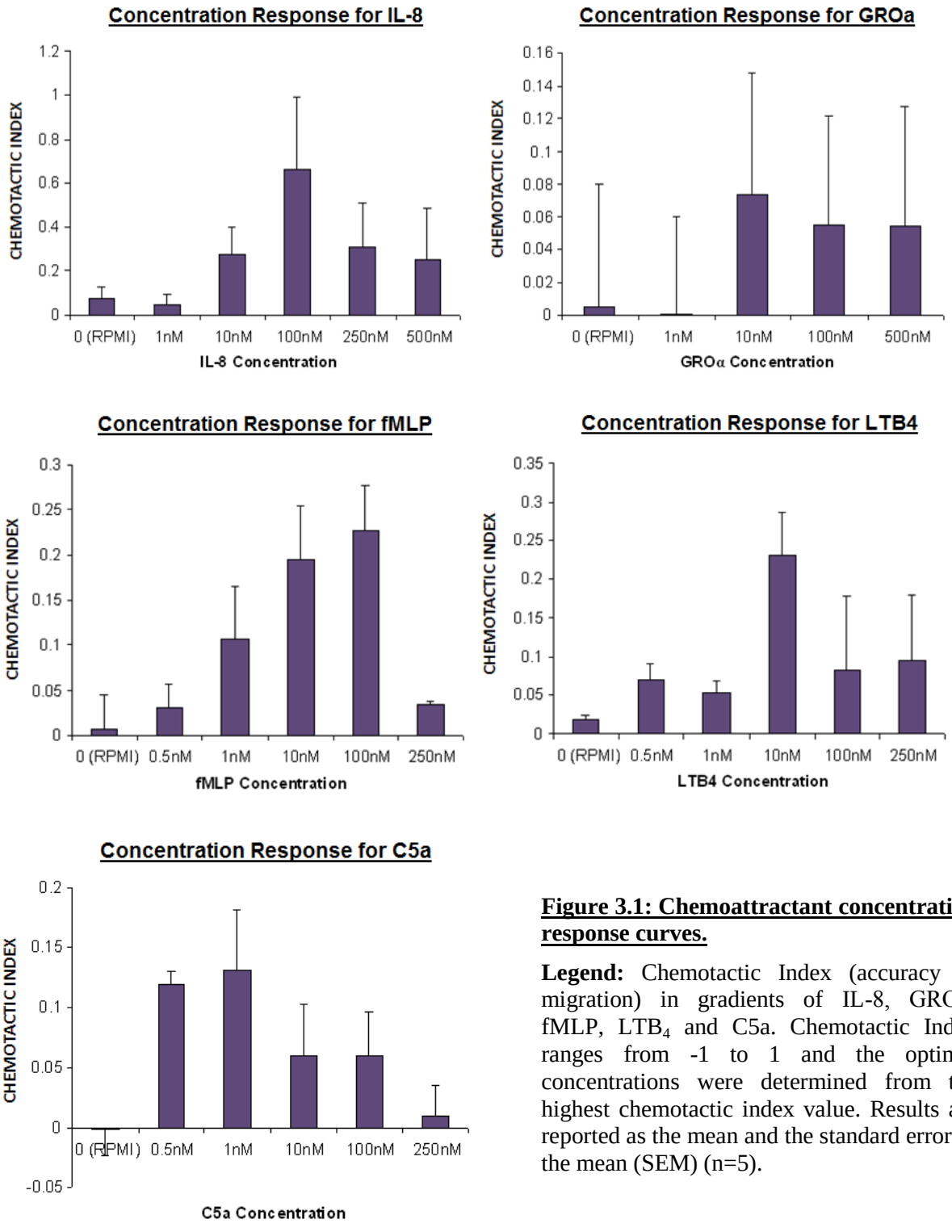

Figure 3.1: Chemoattractant concentration response curves.

Legend: Chemotactic Index (accuracy of migration) in gradients of IL-8, GROα, fMLP, LTB_4 and C5a. Chemotactic Index ranges from -1 to 1 and the optimal concentrations were determined from the highest chemotactic index value. Results are reported as the mean and the standard error of the mean (SEM) (n=5).

From these assays, the optimal concentration of each chemoattractant was determined from the highest chemotactic index value, which equates to the most accurate neutrophil migration. IL-8, fMLP and C5a displayed a typical "bell-shaped" concentration response curve, though this was less obvious for LTB_4 (though 10 nM seemed most effective) and absent for GROα, which also displayed high variability. The optimal concentrations of the five chemoattractants were as follows; IL-8: 100nM, GROα: 10nM, fMLP: 100nM, LTB_4: 10nM, C5a: 1nM. Henceforth, subsequent assays were performed using the chemoattractants at these concentrations.

3.3.2 Neutrophil Migration in COPD, Health and α_1-ATD

Neutrophil migration was assessed using the Insall Chamber in the presence of medium alone (RPMI), IL-8, GROα, fMLP, LTB_4 and C5a (as described in Section 2.5.1) in a total of 10 subjects in each group.

Chemokinesis

The random speed of migration (chemokinesis) of COPD neutrophils was significantly higher in comparison to healthy control subjects for each of the five chemoattractants; IL-8 ($p = 0.01$), GROα ($p = 0.009$), fMLP ($p = 0.004$), LTB_4 ($p = 0.008$) and C5a ($p = 0.02$). The same phenomenon was also observed when comparing the COPD to the α_1-ATD group, where COPD neutrophils moved at greater speed than those from α_1-ATD patients; IL-8 ($p = 0.001$), GROα ($p = 0.006$), fMLP ($p = 0.01$), LTB_4 ($p = 0.01$) and C5a ($p = 0.009$). No differences were observed in neutrophil chemokinesis between healthy subjects and α_1-ATD patients. These data are summarised in Figure 3.2.

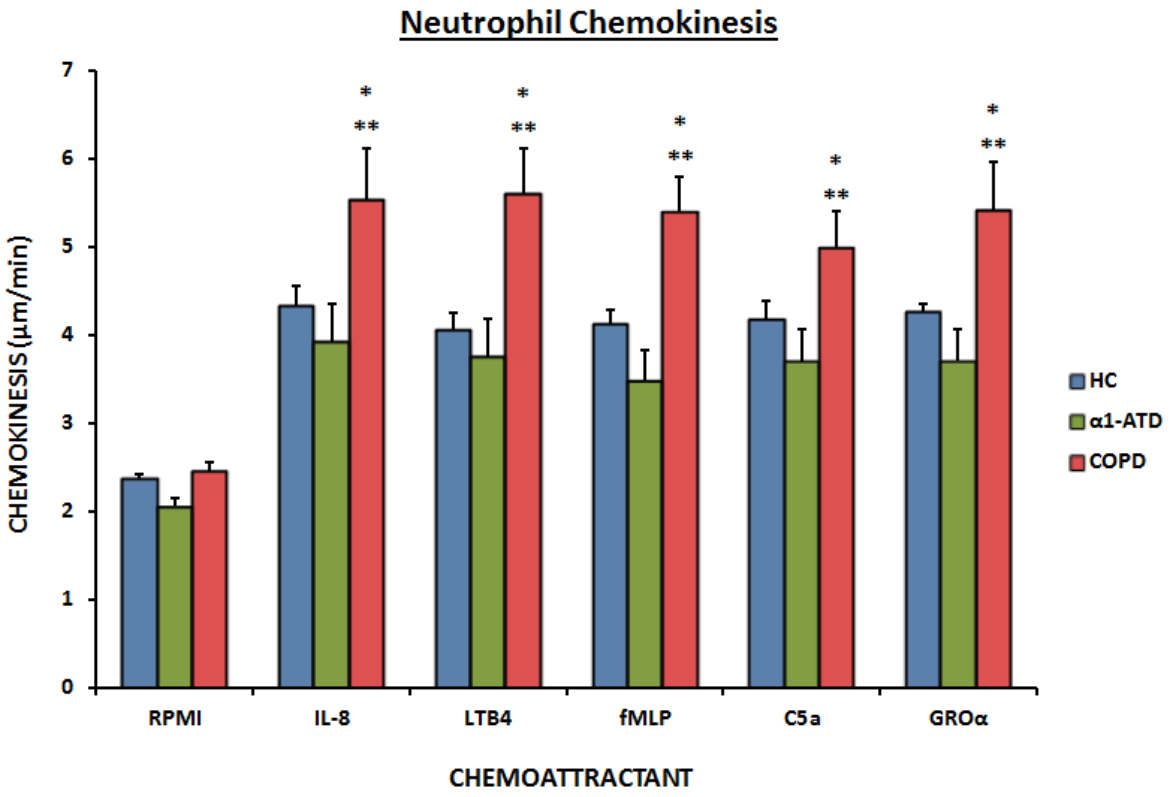

Figure 3.2: Neutrophil chemokinesis in health, COPD and α_1-ATD.

Legend: Chemokinesis (speed of movement) in the presence of medium alone (RPMI), IL-8, GROα, fMLP, LTB_4 and C5a. Results are shown as the mean and SEM (n=10). * indicates $p \leq 0.05$ for COPD compared to Healthy Control (HC) and ** indicates $p \leq 0.05$ for COPD compared to α_1-ATD patients. (Analysed by one-way ANOVA).

Chemotaxis

Significant differences were also observed in neutrophil chemotaxis (speed of migration towards the chemoattractant source), for all chemoattractants. COPD neutrophils moved with markedly reduced chemotaxis compared to neutrophils from healthy subjects; IL-8 ($p = 0.001$), GROα ($p = 0.04$), fMLP ($p = 0.007$), LTB_4 ($p = 0.02$) and C5a ($p = 0.03$). The same reductions in chemotaxis were observed when comparing COPD neutrophils to those from α_1-ATD patients; IL-8 ($p = 0.006$), GROα ($p = 0.04$), fMLP ($p = 0.01$), LTB_4 (p

= 0.01) and C5a (p = 0.02). Interestingly, neutrophils from α_1-ATD patients showed a tendency to move with greater chemotaxis than those from healthy control subjects and, in the presence of IL-8 and fMLP, these differences were significant; IL-8 (p = 0.04), fMLP (p = 0.05). The neutrophil migratory chemotaxis data are summarised in Figure 3.3.

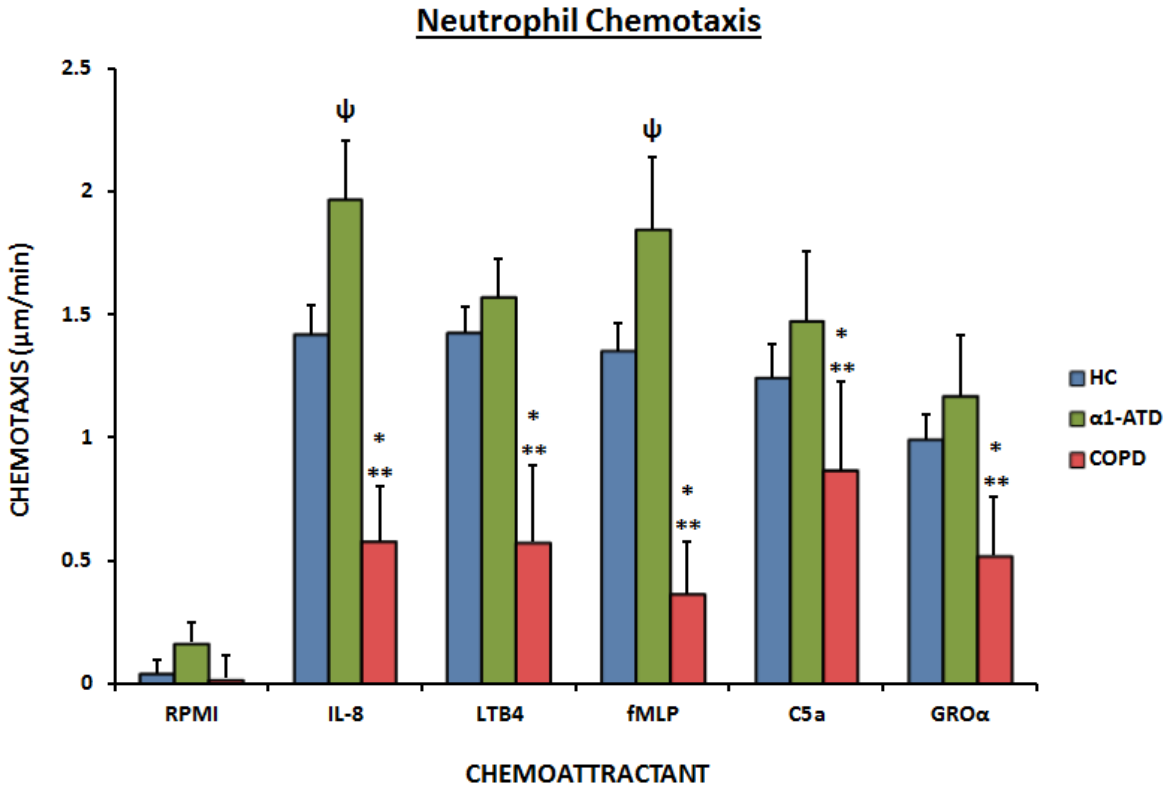

Figure 3.3: Neutrophil chemotaxis in health, COPD and α_1-ATD.

Legend: Chemotaxis (directed cell movement) towards medium alone (RPMI), IL-8, GROα, fMLP, LTB_4 and C5a. Results are shown as the mean and SEM (n=10). * indicates $p \leq 0.05$ for COPD compared to HC and ** indicates $p \leq 0.05$ for COPD compared to α_1-ATD patients. ψ indicates ≤ 0.05 for α_1-ATD patients compared to HC. (Analysed by one-way ANOVA).

Persistence

No significant differences were observed in neutrophil persistence for any of the five chemoattractants in comparisons between any group. These results are summarised in Figure 3.4.

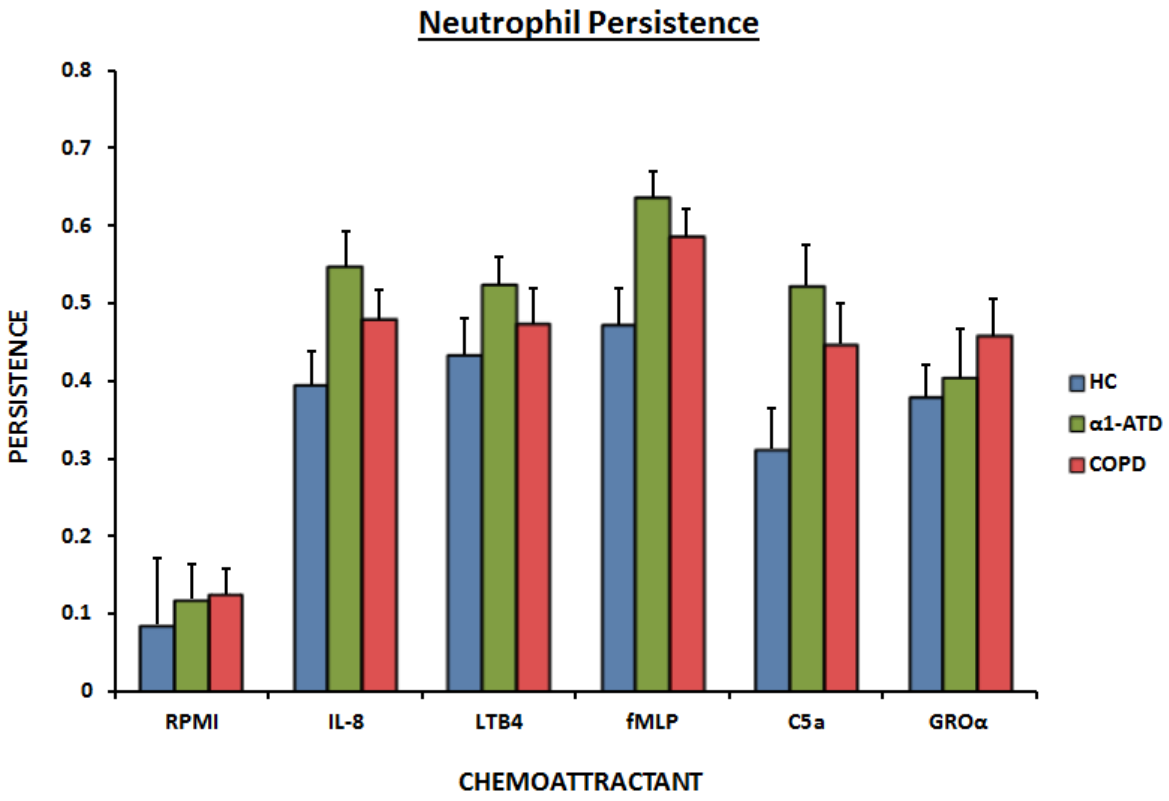

Figure 3.4: Neutrophil persistence in health, COPD and α_1-ATD.

Legend: Persistence in the presence of medium alone (RPMI), IL-8, GROα, fMLP, LTB_4 and C5a. Results are shown as the mean and SEM (n=10). No significant differences were observed in comparisons between groups for any of the chemoattractants. (Analysed by one-way ANOVA).

Chemotactic Index

Significant differences in the chemotactic index (accuracy of migration) were observed for all chemoattractants. COPD neutrophils moved less accurately compared to those from healthy subjects; IL-8 ($p = 0.004$), GROα ($p = 0.003$), fMLP ($p = 0.002$), LTB_4 ($p = 0.03$) and C5a ($p = 0.01$). Even greater differences in migratory accuracy were observed when comparing the α_1-ATD group to the COPD; IL-8 ($p = 0.004$), GROα ($p = 0.002$), fMLP ($p < 0.001$), LTB_4 ($p = 0.01$) and C5a ($p = 0.005$). Although neutrophils from α_1-ATD donors appeared to be moving with greater accuracy in some instances than those from healthy donors, the comparisons did not achieve statistical significance. The migratory accuracy data are summarised in Figure 3.5.

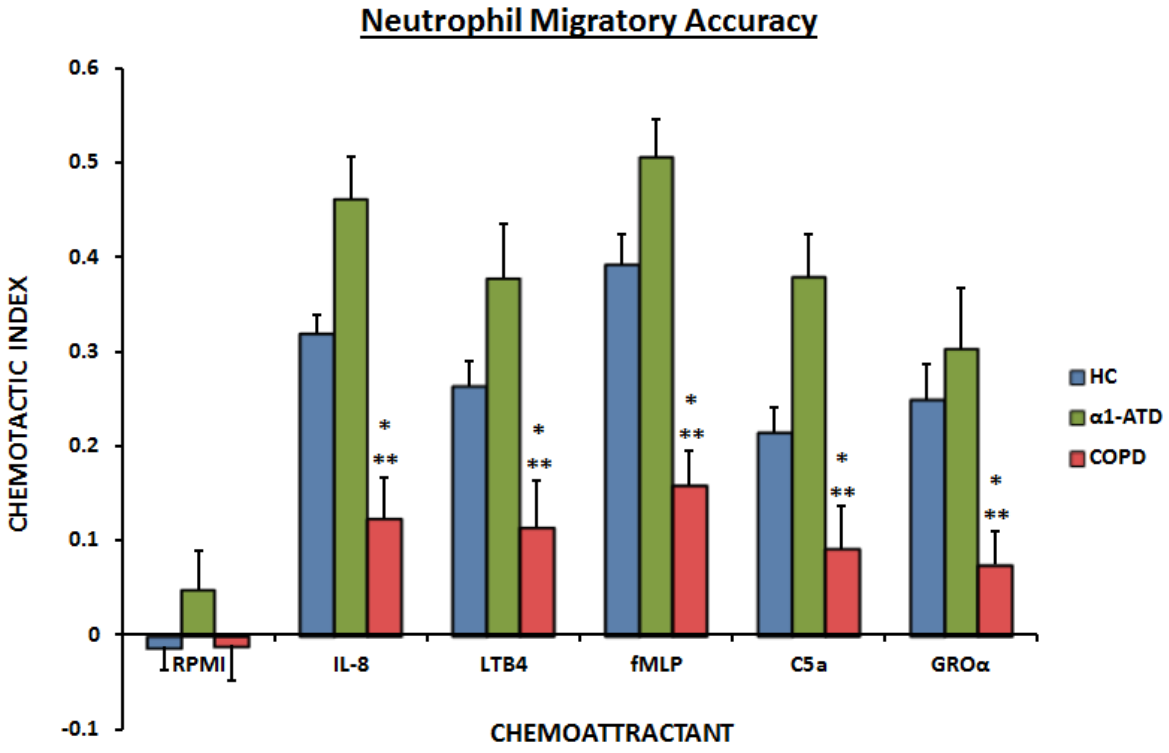

Figure 3.5: Chemotactic Index in health, COPD and α_1-ATD.

Legend: Chemotactic index (overall migratory accuracy) in the presence of RPMI, IL-8, GROα, fMLP, LTB_4 and C5a. Results are shown as the mean and SEM (n=10). * indicates $p \leq 0.05$ for COPD compared to HC and ** indicates $p \leq 0.05$ for COPD compared to α_1-ATD patients. (Analysed by one-way ANOVA).

3.3.3 Effect of Donor Plasma on Neutrophil Migration

To determine if the local inflammatory environment could be influencing neutrophil migration through a systemic effect, migratory dynamics of COPD neutrophils were re-assessed following pre-incubation in healthy plasma and vice versa in shallow gradients of IL-8 and fMLP. Cells were also incubated in autologous plasma or RPMI-1640 for control comparisons. Since migration was aberrant to all chemoattractants studied, IL-8 and fMLP were chosen in order to replicate chronic inflammation and bacterial infections,

respectively. Furthermore, previous work on neutrophil chemotaxis in COPD had also predominantly focussed on these two chemokines.

When the migratory dynamics of neutrophils from COPD donors was assessed following incubation in RPMI-1640, autologous plasma or plasma from an age-matched healthy control subject, no significant differences were observed in their chemokinesis in the presence of either IL-8 or fMLP (Figure 3.6). Pre-incubation in the different media also had no effect on the chemotaxis of COPD neutrophils (Figure 3.7).

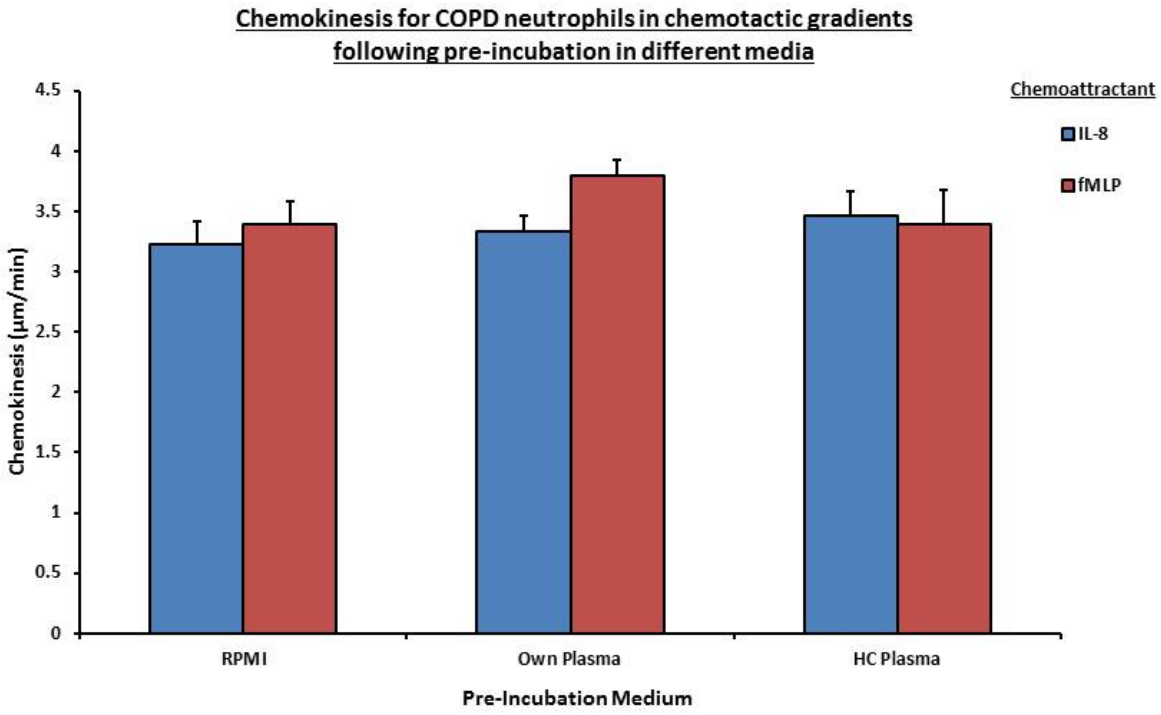

Figure 3.6: The effect of healthy plasma on the chemokinesis of COPD neutrophils.

Legend: The chemokinesis of COPD neutrophils in the presence of IL-8 and fMLP following pre-incubation in RPMI, plasma from the same donor and plasma from an age-matched healthy donor. Results are displayed as the mean and SEM (n=5). (Analysed by one-way ANOVA).

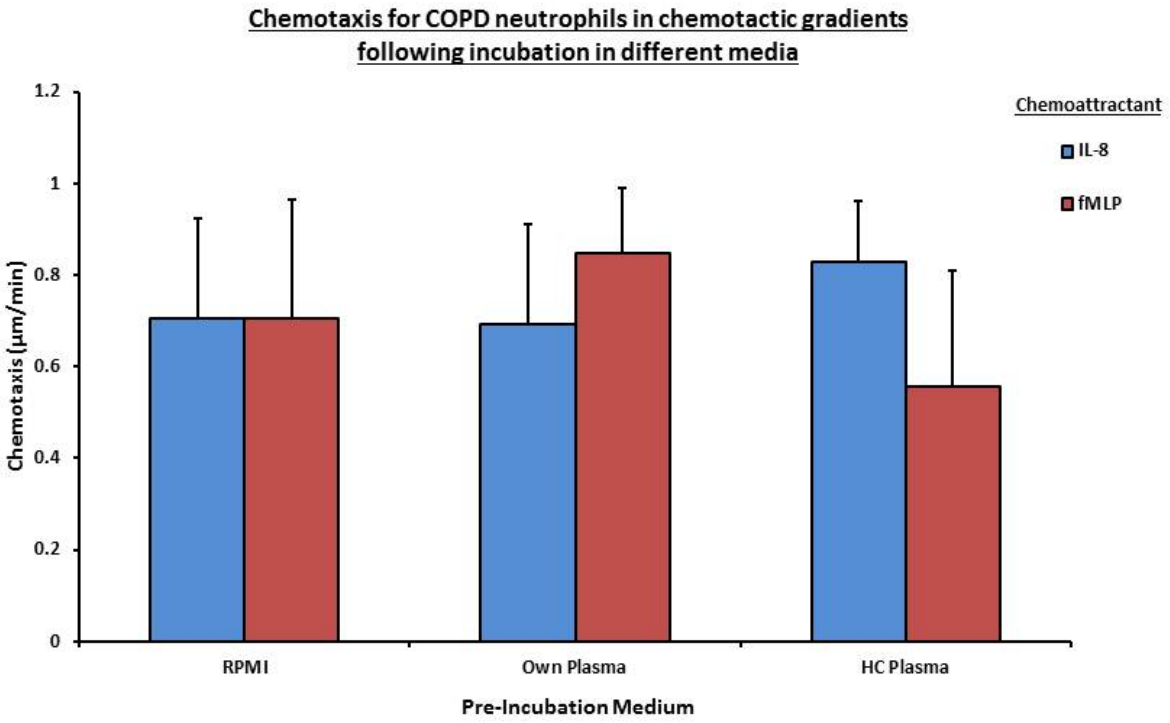

Figure 3.7: The effect of healthy plasma on the chemotaxis of COPD neutrophils.

Legend: The chemotaxis of COPD neutrophils in the presence of IL-8 and fMLP following pre-incubation in RPMI, plasma from the same donor and plasma from an age-matched healthy donor. Results are displayed as the mean and SEM (n=5). (Analysed by one-way ANOVA).

When the migratory dynamics of neutrophils from healthy control subjects was assessed in the presence of either IL-8 or fMLP, neither chemokinesis (Figure 3.8) nor chemotaxis (Figure 3.9) was altered by pre-incubation in plasma from an age-matched COPD patient compared to autologous plasma or RPMI-1640.

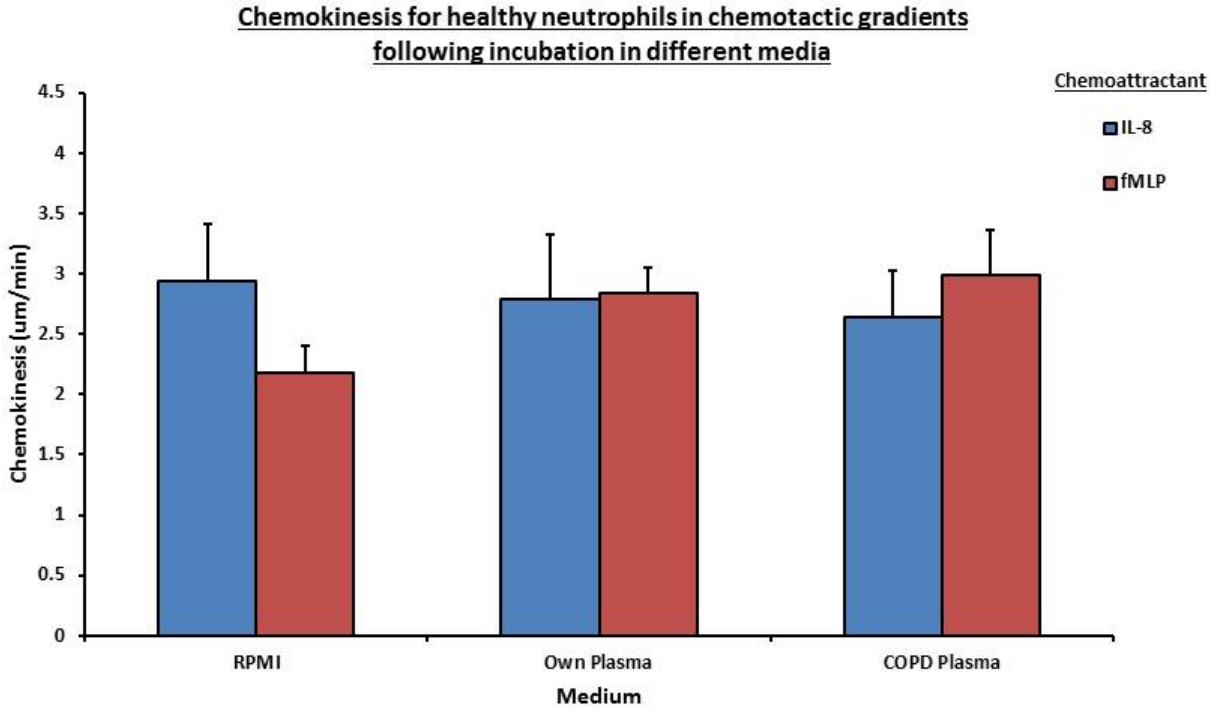

Figure 3.8: The effect of COPD plasma on the chemokinesis of healthy neutrophils.

Legend: The chemokinesis of healthy neutrophils in the presence of IL-8 and fMLP following pre-incubation in RPMI, plasma from the same donor and plasma from an age-matched COPD patient. Results are displayed as the mean and SEM (n=5). (Analysed by one-way ANOVA).

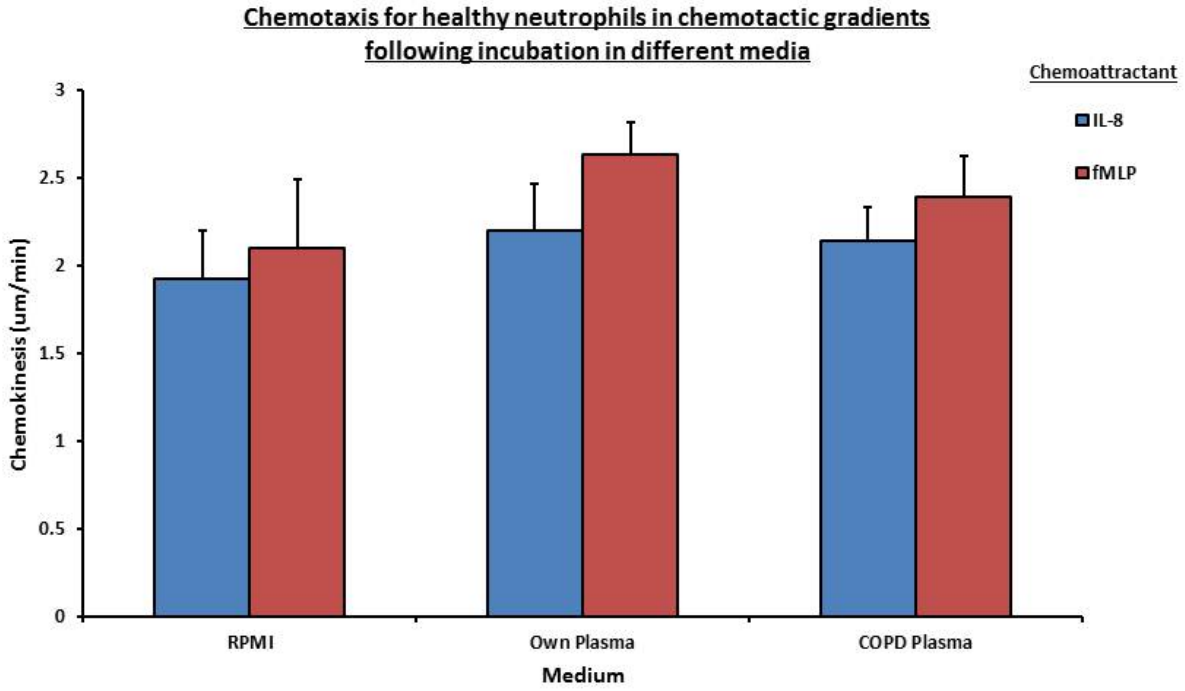

Figure 3.9: The effect of COPD plasma on the chemotaxis of healthy neutrophils.

Legend: The chemotaxis of healthy neutrophils in the presence of IL-8 and fMLP following pre-incubation in RPMI, plasma from the same donor and plasma from an age-matched COPD patient. Results are displayed as the mean and SEM (n=5). (Analysed by one-way ANOVA).

3.4 NEUTROPHIL PHENOTYPING

Due to the inflammatory nature of COPD, it is possible that neutrophils from the peripheral blood of COPD patients may adopt a different phenotype compared to those from healthy subjects and this may, in turn, affect cellar functions such as migration. This could potentially occur either through a systemic inflammatory effect or be due to early release of immature neutrophils from the bone marrow to compensate for the increased neutrophil influx from the circulatory system into the lungs. To determine if neutrophils

from COPD patients were of a different phenotype, expression of surface markers of cell activation (active CD11b, CD62L), degranulation (CD63) and maturity (CD16) were measured in whole blood samples.

3.4.1 Validation: Selection of Antibody Titre

To determine the most appropriate concentration of each receptor antibody, immunostaining was quantified by flow cytometry using a range of concentrations with whole blood from 2 healthy donors. The concentrations recommended by the manufacturer were 20 µl for CD11b and CD62L and 5 µl for CD16 and CD63 into cells isolated from 50 µl of whole blood (following centrifugation and removal of the supernatant). Therefore, concentration responses were performed for all antibodies using volumes of 5 µl, 10 µl and 20 µl. Neutrophils were gated relative to the isotype control so that only cells positive for each antibody were included in the analysis. This gating strategy is shown in Figure 3.10.

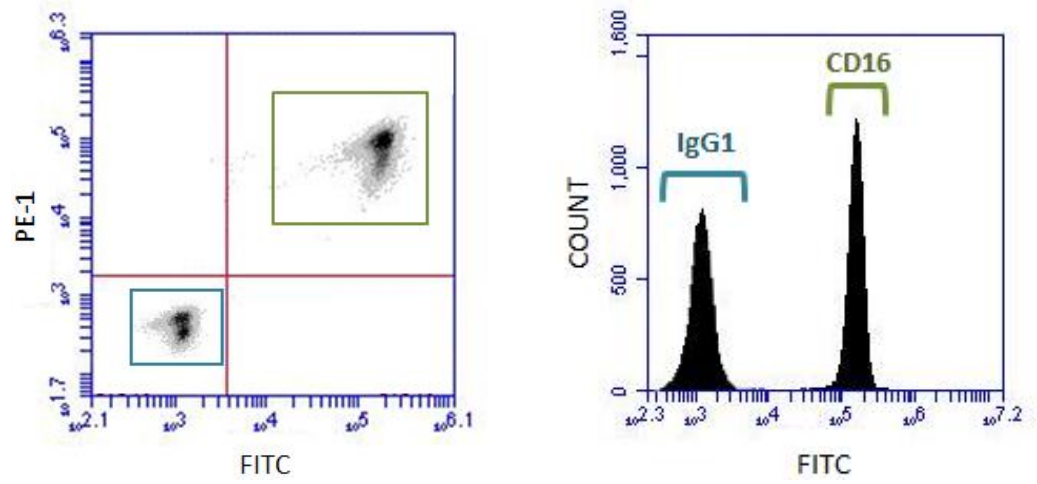

Figure 3.10: A representative FACS plot of isotype control and FITC/PE positive neutrophils.

Legend: The gating strategy (left) was designed to account for non-specific FITC or PE binding. Gates were positioned around neutrophils stained with the isotype control antibody IgG1 (example population indicated by the blue box), so that only neutrophils positive for FITC- and/or PE-stained receptor expression (outside the lower left quadrant) were included in the final analysis (example population indicated by the green box). An example of the cell count display is shown on the right for IgG1 vs. CD16 ($FITC^{+}$).

The MFI data for each antibody are shown in Figure 3.11. From these concentration response assays, the optimal concentration of each antibody was determined from the highest MFI value. The optimal volumes of the four antibodies per 50 µl of whole blood were as follows; CD11b (active) (400 µg/L): 20 µl, CD16 (400 µg/L): 5 µl, CD62L (25 µg/L): 20 µl and CD63 (200 µg/L) 20 µl. Henceforth, the subsequent assay was performed using these volumes of the antibodies.

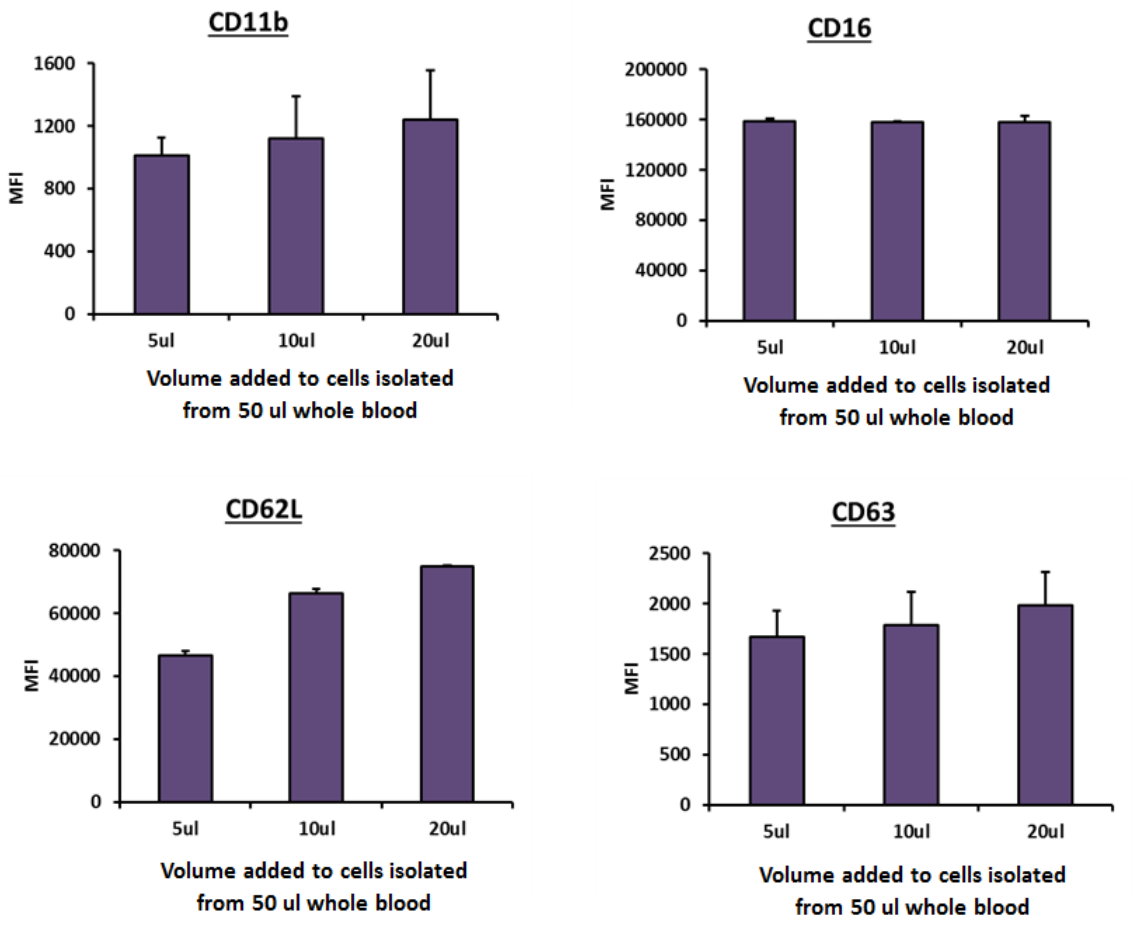

Figure 3.11: Antibody concentration response curves for neutrophil markers of activation and maturity.

Legend: Neutrophil surface expression of CD11b (active) (400 µg/L), CD16 (400 µg/L), CD62L (25 µg/L) and CD63 (200 µg/L) in whole blood from healthy donors. The optimal concentrations were determined from the highest MFI value. Results are reported as the mean and SEM (n=2).

3.4.2 Markers of Neutrophil Activation and Maturity

The surface expression of active CD11b, CD16, CD62L and CD63 on neutrophils in whole blood was compared between 15 COPD patients and 15 healthy control subjects. No differences were observed between comparisons of COPD and healthy neutrophils for the

MFI of active CD11b, CD16 or CD62L. However, a modest but significantly higher expression of CD63 was observed in COPD neutrophils. These data are summarised in Figure 3.12.

In addition, the percentage of neutrophils with a positive expression of each of the four markers was determined. As expected, virtually all neutrophils from every subject were highly positive for CD16 (Healthy control = 99.7%, COPD 99.4%) and CD62L (Healthy control = 99.8%, COPD 99.8%) expression but there were no differences between COPD and health. Unexpectedly, a few subjects had a small percentage of neutrophils that were positive for active CD11b. This was observed more frequently in COPD than health (Healthy control = 3.5%, COPD 5.1%), although there was no statistically significant difference. This phenomenon was more pronounced for CD63 expression (Healthy control = 5.2%, COPD 11.4%) but, again, this did not reach statistical significance. These data are summarised in Figure 3.13.

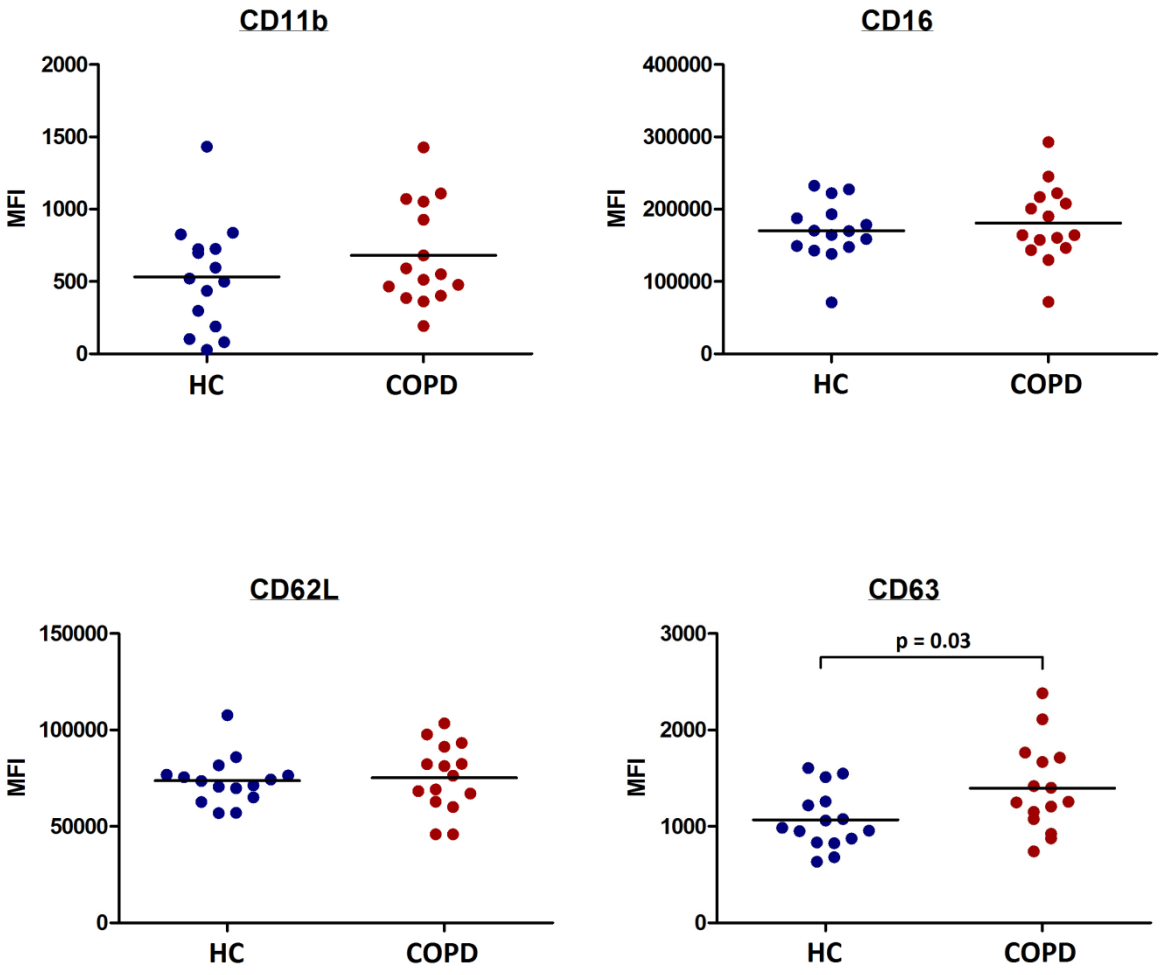

Figure 3.12: Surface expression of active CD11b, CD16, CD62L and CD63.

Legend: Surface expression was semi-quantified in populations of neutrophils in whole blood from 15 HC subjects and 15 COPD patients by flow cytometry. Mean MFI values for each patient group are indicated by the horizontal line. (Analysed by unpaired Mann-Whitney U test).

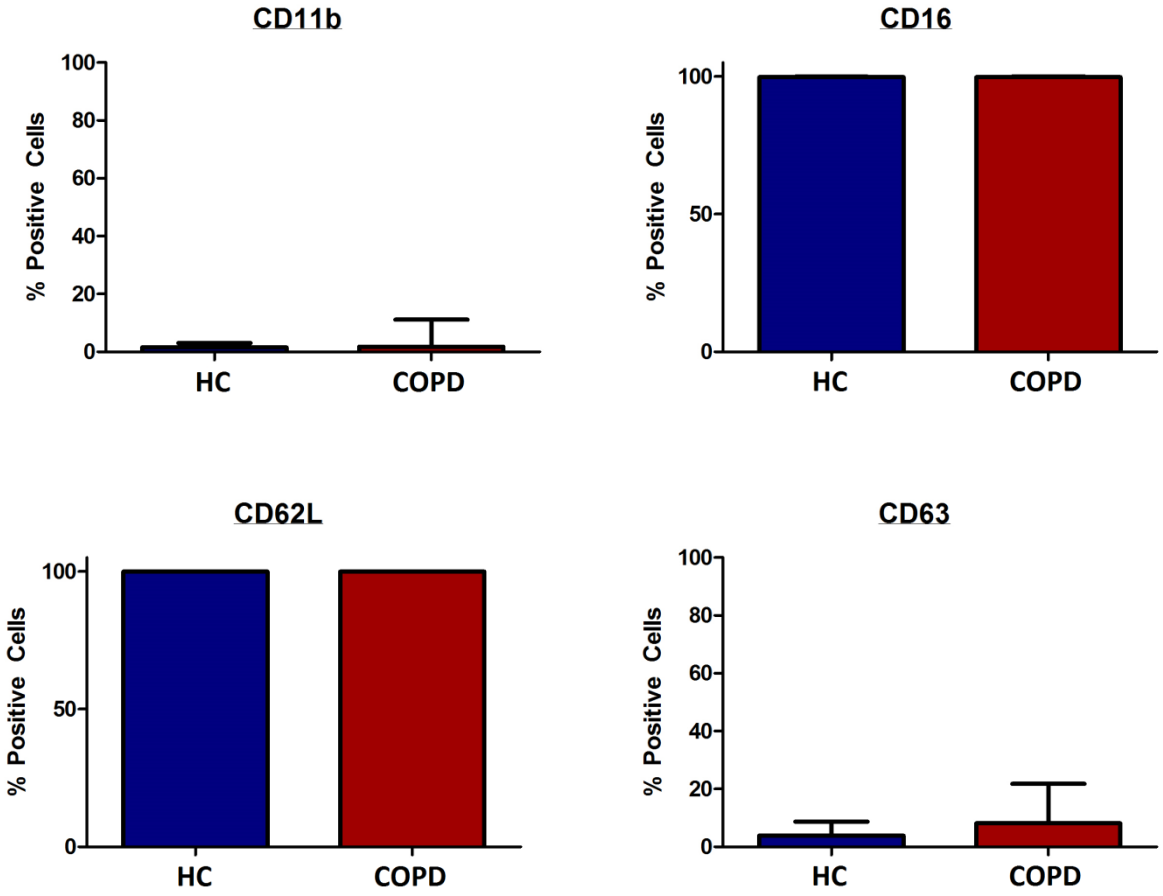

Figure 3.13: The percentage of $CD11b^+$, $CD16^+$, $CD62L^+$ and $CD63^+$ neutrophils.

Legend: The percentage of neutrophils positive for these markers was determined by a gating strategy whereby only neutrophils that fell outside the gate of the isotype-matched irrelevant antibodies (IgG1). Results are displayed as the median with standard deviation (data within 95% confidence intervals). (Analysed by unpaired Mann-Whitney U test).

3.5 NEUTROPHIL MIGRATION AND CLINICAL PHENOTYPE

Currently, it is unknown whether or not the aberrant neutrophil migration in COPD is a genetically predetermined phenomenon or caused by other factors that relate to the disease, such as smoking status, the presence of emphysema and age. It is also unknown if

migratory accuracy of neutrophils changes with the severity of COPD. This is important as it may help clarify whether the aberrant neutrophil migration in COPD is a secondary effect of chronic inflammation or, in fact, a hereditary condition, where neutrophils always migrate with poor accuracy in susceptible individuals due to altered gene expression, but COPD only manifests as a result of increased transmigration in response to chronic toxin inhalation.

Although the initial chemotaxis data was sufficiently powered to discern differences in the migratory dynamics of neutrophils in COPD as a whole, it was not sufficiently powered to allow for the analysis of individual stages of disease severity or other factors that contribute to the clinical phenotype of COPD, such as smoking status, the presence of emphysema and age. Therefore, a much larger cohort of both COPD patients with varying disease severity (n = 52) and healthy individuals (n = 55) was generated through the addition of chemotaxis data (in 100nM IL-8) from collaborative researchers within our group.

3.5.1 Neutrophil Chemotaxis and COPD Severity

Group comparison of migratory data in COPD Stages I-IV revealed no significant differences between neutrophil chemotaxis in IL-8 in any group. When a large healthy cohort was included in a subsequent comparison, chemotaxis was only significantly different in COPD Stages II-IV compared to health. However, the fact that chemotaxis was not different in COPD Stage I compared to health was most likely due to a large discrepancy in patient numbers (n = 55 for healthy controls compared to n = 8 in COPD Stage I). These data are summarised in Figure 3.14.

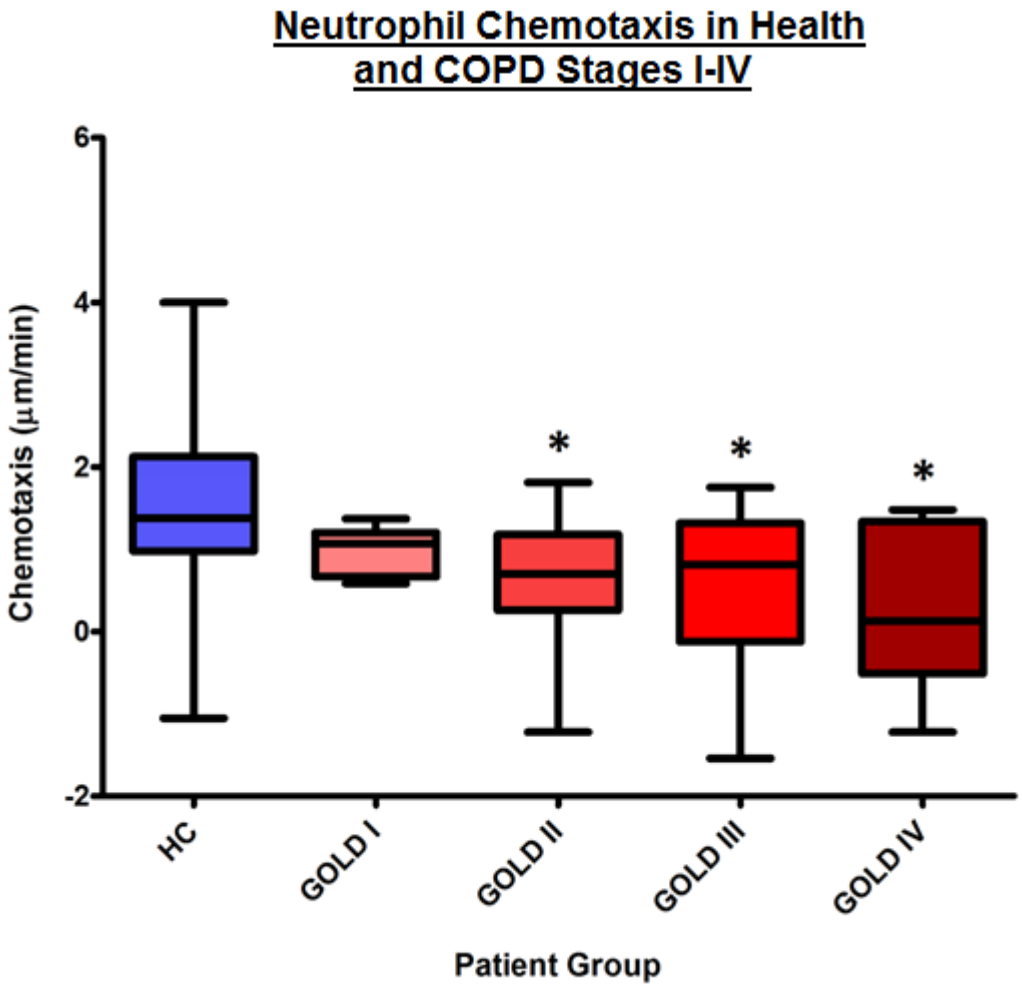

Figure 3.14: Neutrophil chemotaxis in health and COPD Stages I-IV.

Legend: Chemotaxis data to 100nM IL-8 was collated healthy controls (n=55) and for COPD GOLD Stage I (n=8), Stage II (n=19), Stage III (n=14) and Stage IV (n=11). Data are presented as a Box and Whisker Plot with the median, quartiles and range. * $p \leq 0.05$ compared to health. (Analysed by Kruskal-Wallis test).

COPD Gold Stages I-IV are defined by the presence of airflow obstruction (FEV_1/FVC less than 70%) and further classified by FEV_1 % Predicted. A linear regression plot of chemotaxis and FEV_1 % Predicted for all COPD patients (n = 52) was used to determine if

there was a relationship between chemotaxis and the severity of COPD. There was no significant correlation between chemotaxis and FEV_1 % Predicted (r^2 = -0.042, p = 0.144). These data are summarised graphically in Figure 3.15.

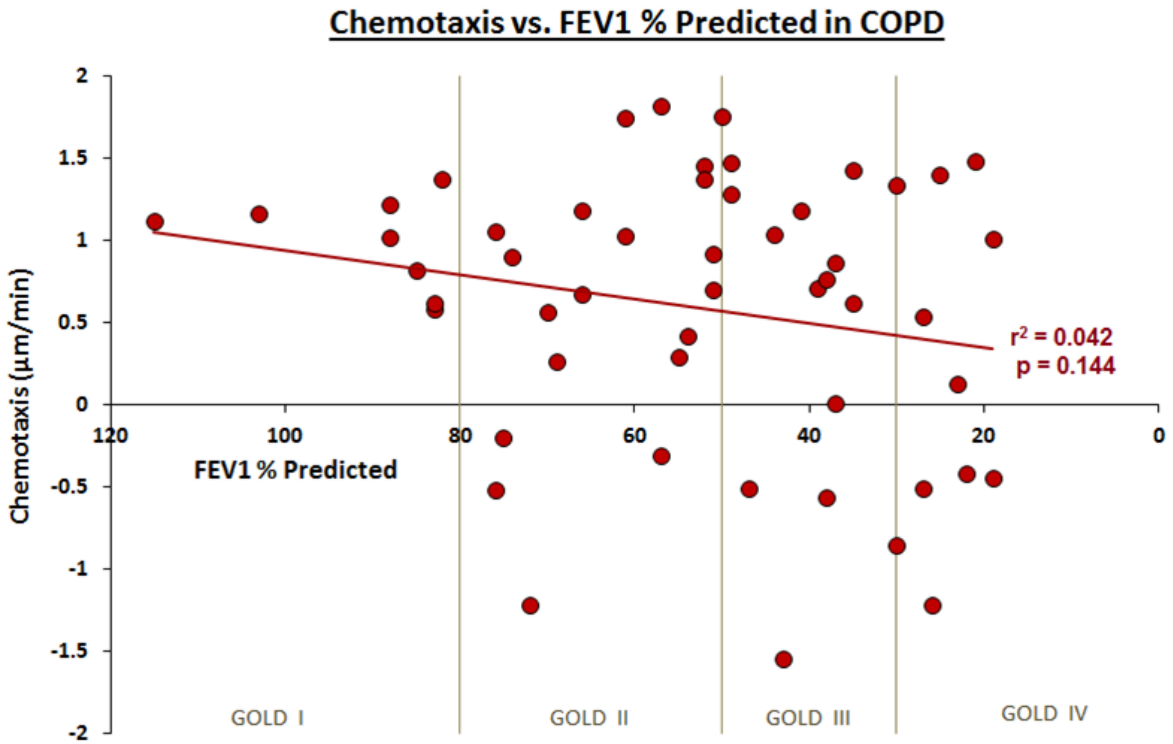

Figure 3.15: Linear regression of neutrophil chemotaxis and FEV_1 % Predicted in COPD.

Legend: Chemotaxis data (to 100nM IL-8) from a large cohort of COPD patients with varying degrees in severity was compared to FEV_1 % Predicted by linear regression. Statistically, there was no significant relationship (r^2 = -0.042, p = 0.144). (Analysed by Spearman's Rank).

Although the severity of airflow obstruction in COPD is defined by the FEV_1 % Predicted, the FEV_1/FVC ratio is perhaps a better measure of airflow obstruction. Indeed, it

is possible for a COPD patient to have "mild" COPD (FEV_1 > 80% predicted) but with a markedly reduced FEV_1/FVC. Therefore, a linear regression plot of chemotaxis and FEV_1/FVC for all COPD patients was used to determine if there was a relationship between chemotaxis and the degree of airflow obstruction. There was no significant correlation between chemotaxis and FEV_1/FVC (r^2 = 0.016, p = 0.430). These data are summarised in Figure 3.16.

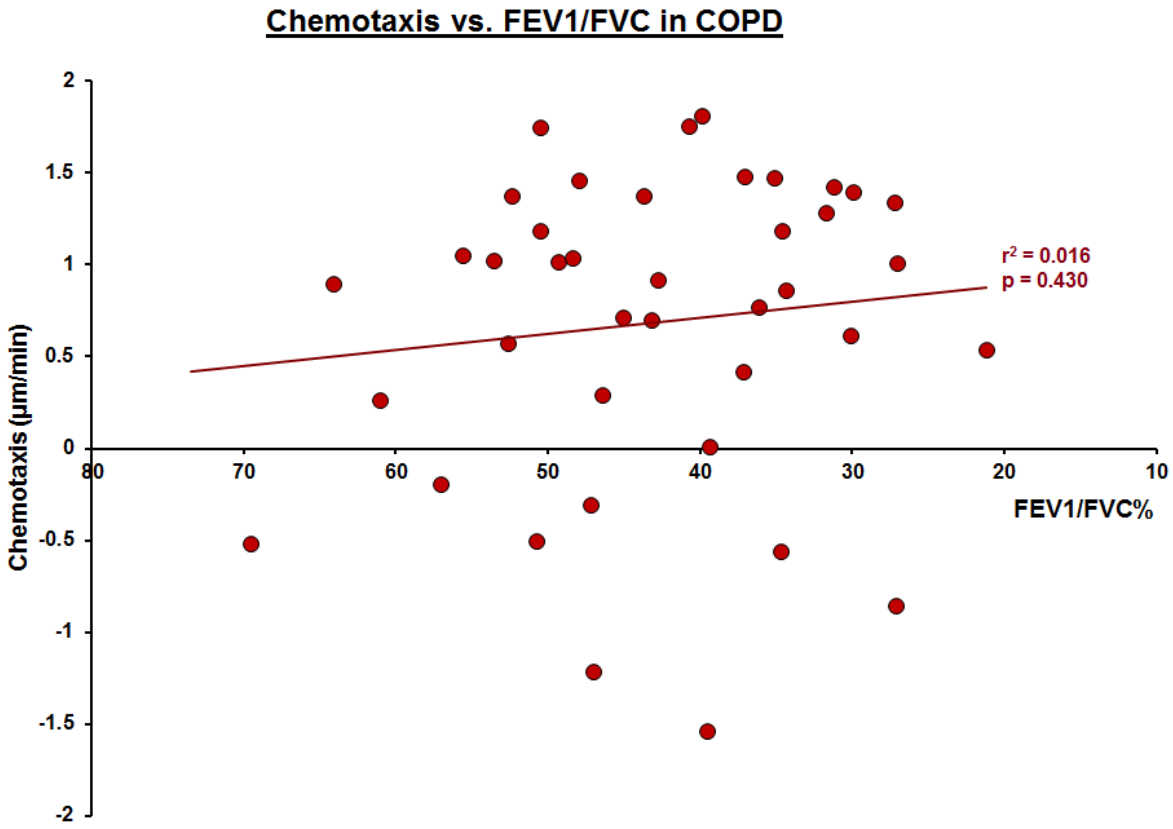

Figure 3.16: Linear regression of neutrophil chemotaxis and FEV_1/FVC in COPD.

Legend: Chemotaxis data (to 100nM IL-8) from a large cohort of COPD patients with varying degrees in severity was compared to FEV_1/FVC by linear regression. Statistically, there was no significant relationship (r^2 = 0.016, p = 0.430). (Analysed by Spearman's Rank).

3.5.2 Neutrophil Chemotaxis and Smoking Status

To determine if smoking status influenced neutrophil migration, the healthy and COPD cohorts were divided into groups related to their smoking status. These included never smokers (NS; healthy cohort only), current smokers (CS) and ex-smokers (XS). In agreement with the previous data (Section 3.3.2), neutrophils from COPD patients moved with significantly increased chemokinesis and decreased chemotaxis when compared to those from healthy control subjects. However, no differences in either chemokinesis (Figure 3.17) or chemotaxis (Figure 3.18) were observed between never smokers, current or ex-smokers in either health or COPD.

3.5.3 Neutrophil Chemotaxis and the Presence of Emphysema

As COPD is a disorder of different disease pathologies and clinical syndromes (emphysema, chronic bronchitis and bronchiolitis) (Saetta et al. 2001), the next analysis sought to determine if the presence of emphysema (which seems to be related to neutrophil transmigration (Woolhouse et al. 2005)) had any relationship to aberrant neutrophil migration in COPD. Patients within the COPD cohort were divided into those with or without emphysema in accordance with a previously validated HRCT Scan protocol, where a Voxel Index below -910 Hounsfield Units (HU) defines the presence of emphysema (Parr et al. 2008). Comparisons of chemokinesis (Figure 3.19A) and chemotaxis (Figure 3.19B) showed no difference in these COPD sub-groups.

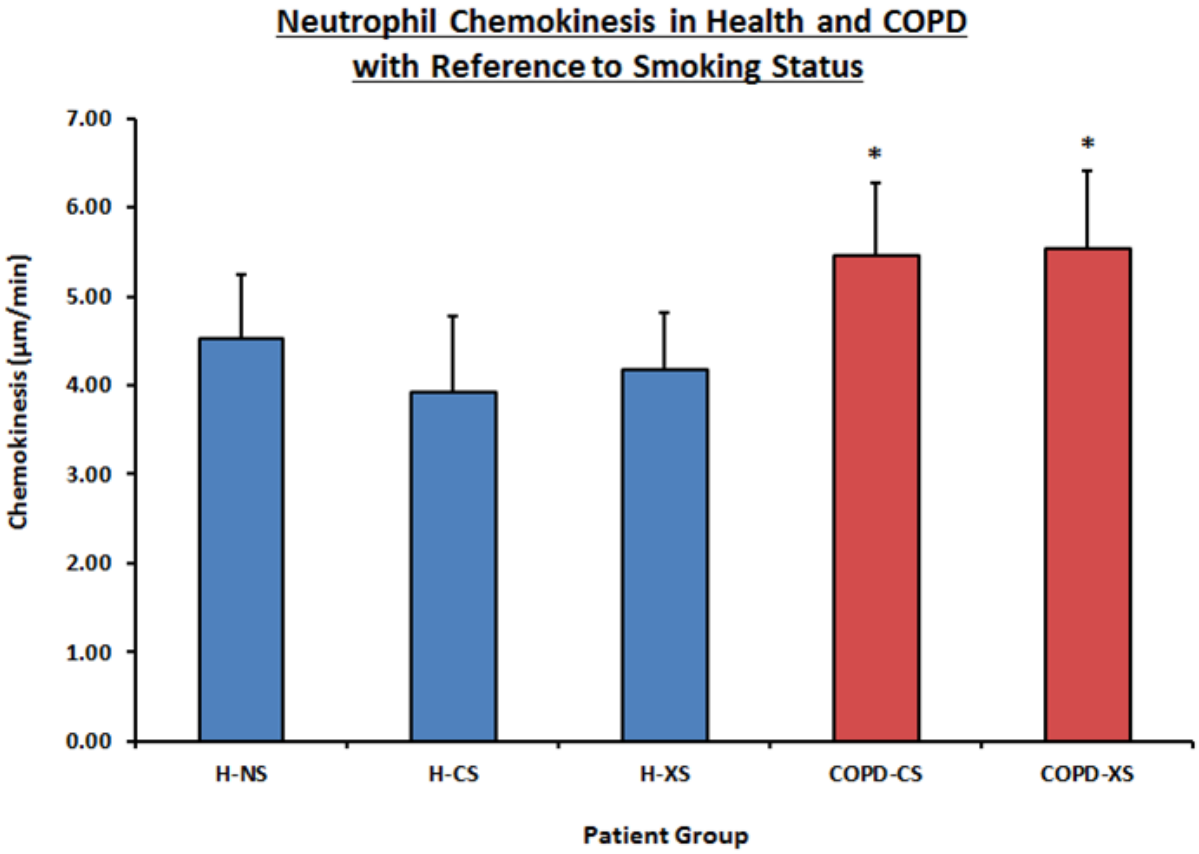

Figure 3.17: The effect of smoking status on neutrophil chemokinesis in health and COPD.

Legend: Chemokinesis data (to 100nM IL-8) from the large cohorts of healthy subjects and COPD patients split by their smoking status. Neutrophils from both COPD current smokers (CS) and ex-smokers (XS) migrated with increased chemokinesis when compared to those from healthy never smokers (NS), CS and XS ($p<0.05$). No differences were observed between COPD CS and XS or between healthy NS, CS and XS. Results are displayed as the median with standard deviation (data within 95% confidence intervals). (Analysed by Kruskal-Wallis test).

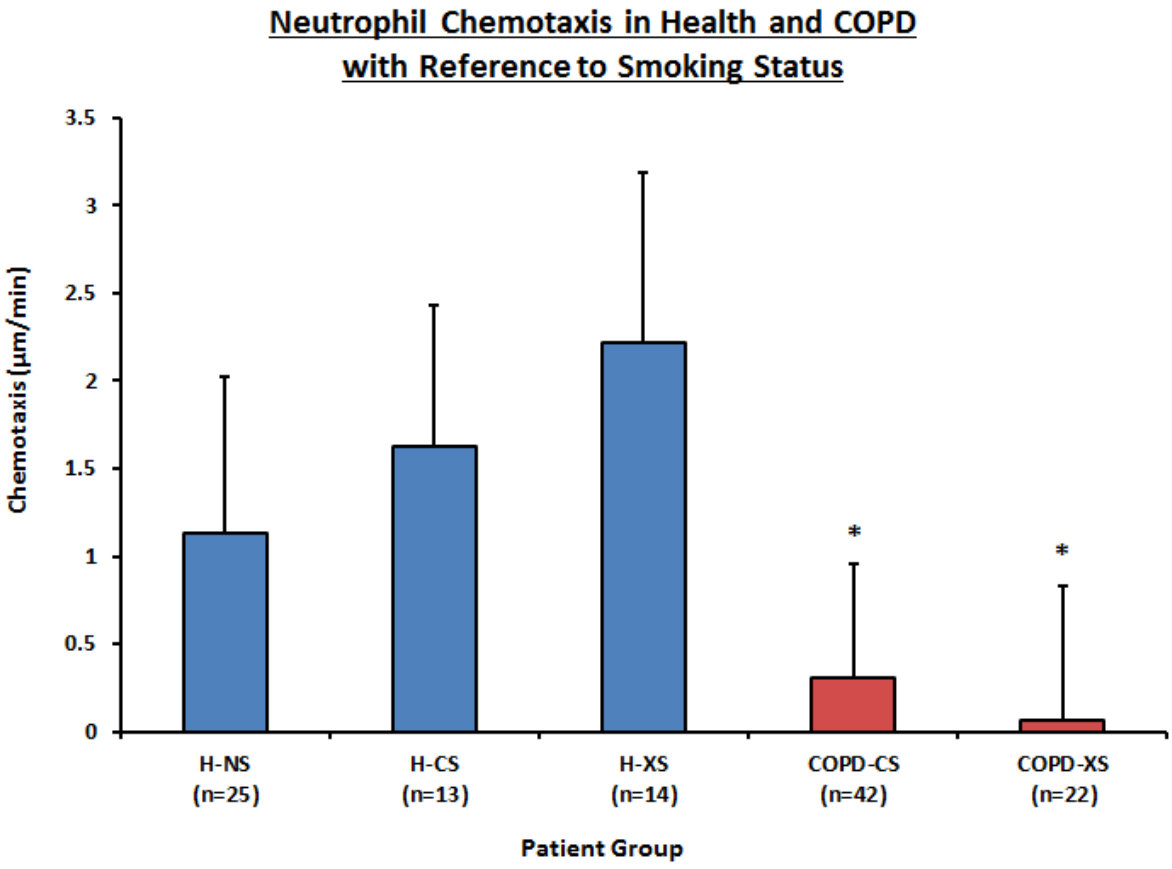

Figure 3.18: The effect of smoking status on neutrophil chemotaxis in health and COPD.

Legend: Chemotaxis data (to 100nM IL-8) from the large cohorts of healthy subjects and COPD patients split by their smoking status. Neutrophils from both COPD current smokers (CS) and ex-smokers (XS) migrated with decreased chemotaxis when compared to those from healthy never smokers (NS), CS and XS ($p<0.05$). No differences were observed between COPD CS and XS or between healthy NS, CS and XS. Results are displayed as the median with standard deviation (data within 95% confidence intervals). (Analysed by Kruskal-Wallis test).

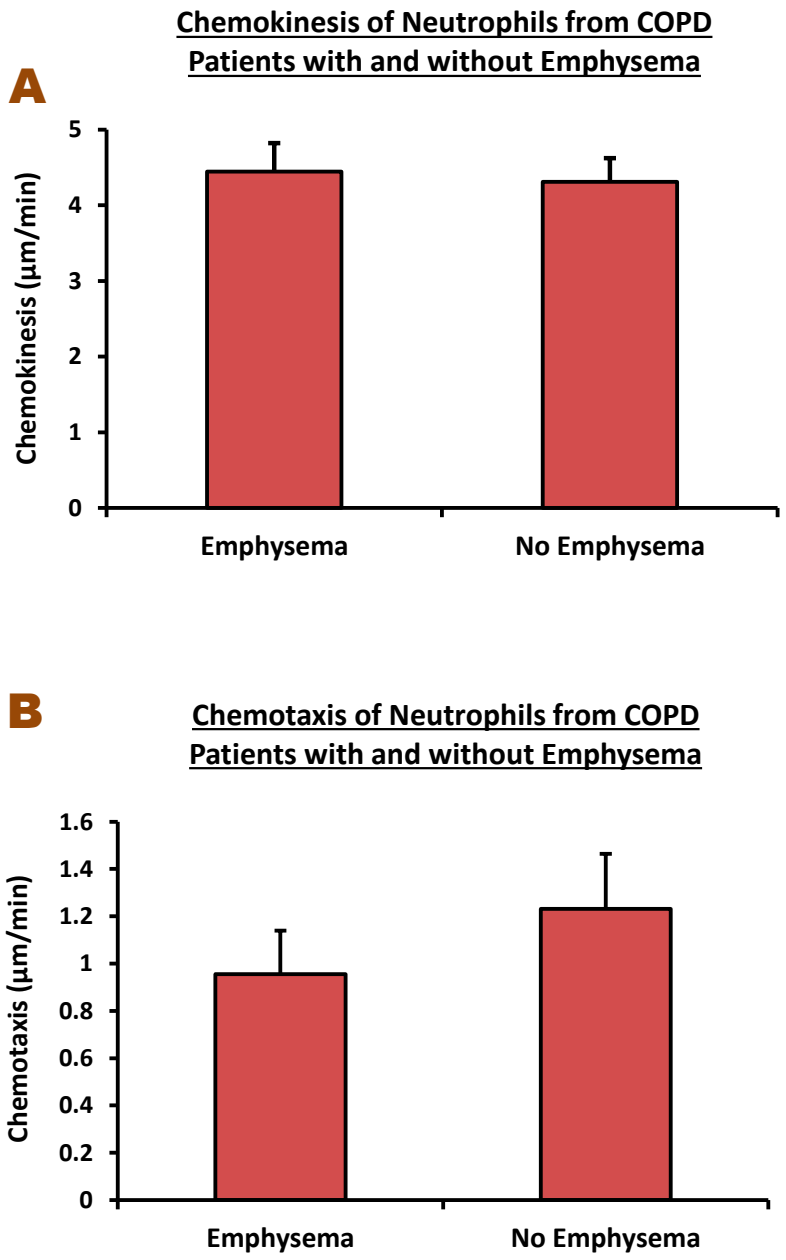

Figure 3.19: The relationship between emphysema and neutrophil migration in COPD.

Legend: Chemotaxis data (to 100nM IL-8) from COPD patients was divided into those patients with or without emphysema (confirmed by HRCT Scan). There was no significant difference in either chemokinesis (A) or chemotaxis (B) between groups. Data are displayed as the mean and SEM (n=18). (Analysed by 2-tailed unpaired t-test).

3.5.4 Neutrophil Chemotaxis and Age

Linear regressions were performed for both neutrophil chemokinesis and chemotaxis versus age in healthy control subjects and patients with COPD. There was no correlation between chemokinesis and age in either group (Figure 3.20). There was a significant negative correlation between chemotaxis and age in the healthy control cohort (r^2 = -0.4, p=0.003), although a similar relationship was not observed in COPD (Figure 3.21).

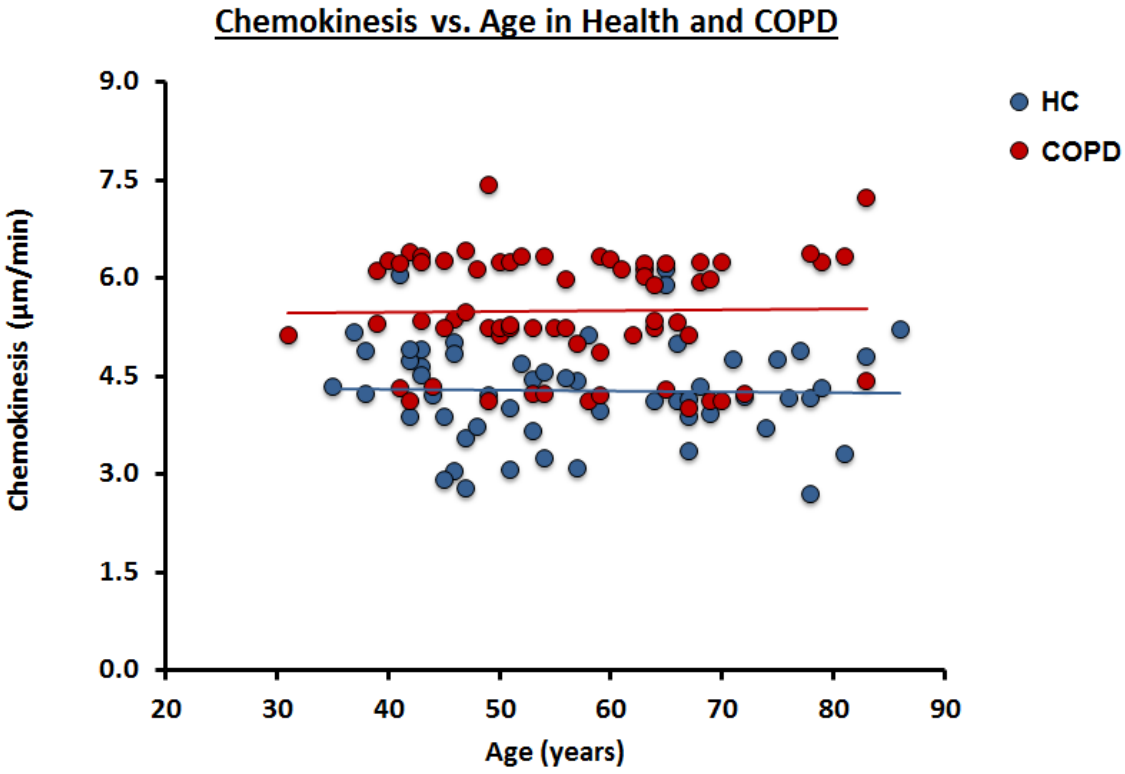

Figure 3.20: Linear regression of neutrophil chemokinesis and age in health and COPD.

Legend: Chemokinesis data (to 100nM IL-8) from large cohorts of Healthy Control (HC) subjects and COPD patients was compared to subject age by linear regression. There was no statistical correlation. (Analysed by Spearman's Rank).

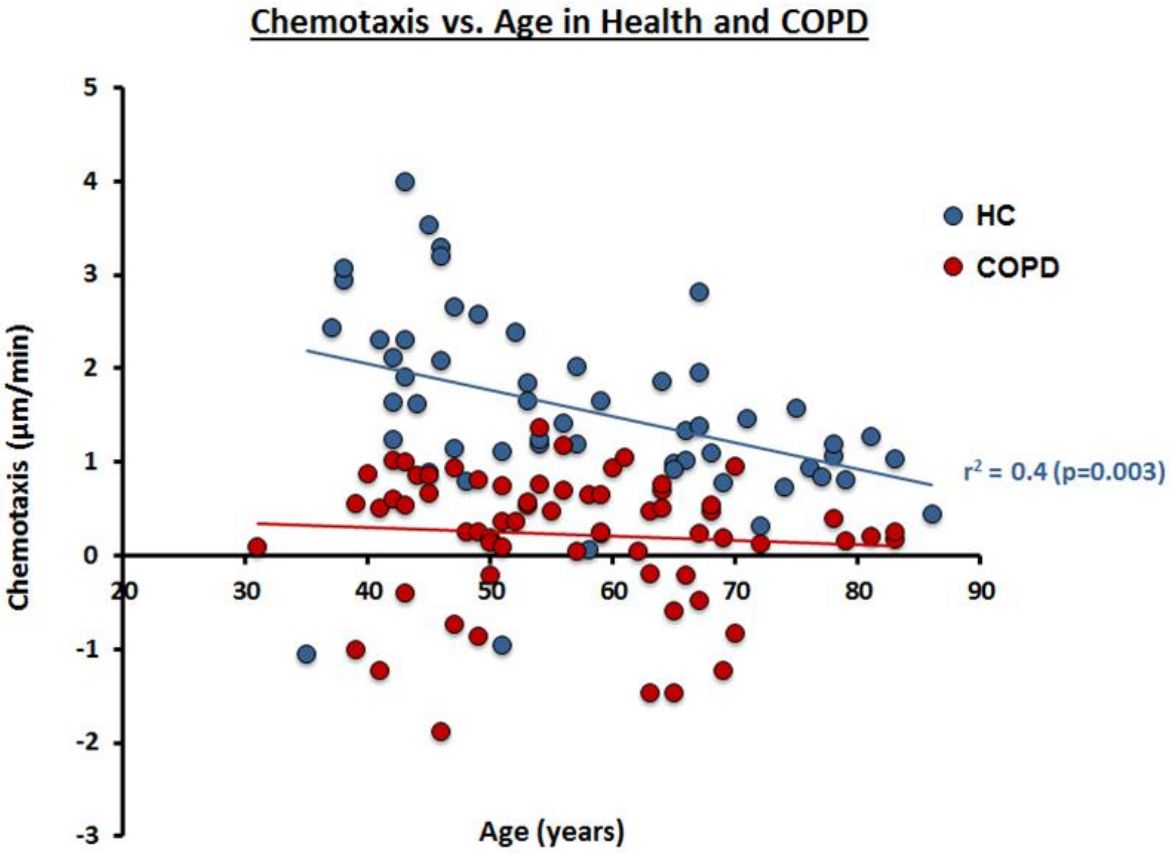

Figure 3.21: Linear regression of neutrophil chemotaxis and age in health and COPD.

Legend: Chemotaxis data (to 100nM IL-8) from large cohorts of Healthy Control (HC) subjects and COPD patients was compared to subject age by linear regression. There was a significant negative correlation between neutrophil chemotaxis and age in health (r^2 = -0.4, p=0.003), but this relationship was not observed in COPD. (Analysed by Spearman's Rank).

3.6 DISCUSSION

3.6.1 Aberrant Neutrophil Chemotaxis in COPD

The ability of neutrophils to migrate accurately from the circulation, through the extracellular matrix and onwards to an inflammatory source is paramount for a rapid innate immune response. Moreover, once at the site of migration, their ability to locate and destroy invading pathogens is also essential for the resolution of bacterial infection. Neutrophils migrate in response to a series of chemokine gradients produced by both host cells and pathogens themselves.

Previous work by our group demonstrated that neutrophils from COPD patients migrated with increased speed but reduced accuracy *in vitro* compared to those from age-matched healthy donors and patients with α_1-ATD in shallow gradients of IL-8 and GROα (Sapey et al. 2011). However, whether this effect was a general phenomenon or exclusively related to the IL-8 and GROα receptors, CXCR1 and CXCR2 could not be determined. Therefore, the first aim of the current study was to assess the migratory dynamics of neutrophils from patients with COPD, age-matched healthy control subjects and patients with α_1-ATD not only in the in the presence of IL-8 and GROα, but also three other chemoattractants, C5a, fMLP and LTB_4. Although there are a vast number of chemoattractive stimuli produced by both host inflammatory cells and invading pathogens, C5a, fMLP and LTB_4 each have different, specific receptors on the cell surface of neutrophils and we believe that these, together with IL-8 and GROα represent a good variety of host and bacterial chemokines that signal through different receptors.

In the current study, COPD neutrophils consistently demonstrated clear differences in migratory behaviour to all five chemoattractants when compared to those from age-matched healthy donors and patients with α_1-ATD, migrating with increased speed (chemokinesis) but impaired accuracy (chemotaxis and chemotactic index). This suggests that the aberrant migration of COPD neutrophils *in vitro* is a generic phenomenon and not specific to particular chemokines and their respective receptors.

Previous studies of neutrophil migration in COPD have yielded conflicting results. One group (Burnett et al. 1987) used a Boyden Chamber to assess neutrophil migration in the presence of an fMLP gradient. In this assay, cells migrate from an upper well through two membranes to a lower well that contains a chemoattractive source. This study demonstrated an increased migration of neutrophils from COPD patients compared to those from healthy controls and patients with bronchiectasis (another lung disease involving neutrophil inflammation). More recently, a similar study described a reduced chemotaxis to IL-8 and fMLP in neutrophils from patients with Stage II-IV COPD (though not Stage I COPD) compared to healthy neutrophils (Yoshikawa et al. 2007).

The differences in results from these studies and those reported here may be reflective of the assay used. The basic mechanism behind the Boyden chamber is to calculate the proportion of leukocytes migrating into a chemoattractant filled chamber through a porous membrane (Chen 2005). The migration of cells through the membrane of the Boyden chamber relies on the formation of a chemical gradient between the chambers which is determined by pore size and pore spacing. However, Burnett et al. used a small pore double membrane, whereas Yoshikawa et al. used a large pore single membrane. These variations in pore size can change the decay rates of chemical gradients, which could result in an

altered neutrophil migratory response. Also, in the absence of a non-standardised Boyden assay membrane, it is suggested that sample sizes should be large (n=50) to reduce the effects of artefact. However, both Yoshikawa et al. and Burnett et al. used study groups of less than 20 subjects. Therefore, it is difficult to determine whether differing results between studies using non-homogenous membranes are real. Furthermore, these assays provide limited information on neutrophil migration, as they only assess the overall number of neutrophils that have migrated from the upper to lower well. The Insall chamber used here provides more insight into how the neutrophils are moving during two-dimensional migration in a chemotactic gradient. Although computer tracking is capable of providing information on four migratory parameters (chemokinesis, chemotaxis, persistence and chemotactic index), there was no difference in persistence between COPD, health and α_1-ATD. As a result, the chemotaxis and chemotactic index were almost exact mirrors of one another. Therefore, the subsequent assays of neutrophil migration focussed solely on chemokinesis and chemotaxis for simpler interpretation.

The Insall chamber is not without limitations. Firstly, it is possible that the neutrophils that adhere to the albumin-coated coverslip are of a different phenotype to those that do not adhere (for instance their quiescent expression of adhesion molecules, such as integrins, could be higher). Secondly, it is an *in vitro* assay that only measures two-dimensional migration. Migration of cells on albumin-coated glass predominantly relies on β2 integrins (Sixt et al. 2001), which also are important in mediating adhesion to many extracellular matrix molecules (Hansell et al. 1994, Kubes et al. 1995, Frieser et al. 1996). In contrast, migration through the basement membrane appears to rely on β1 and β3 integrins (Frieser et al. 1996). This means that, although the *in vitro* data from the current study may reflect

neutrophil migration along the endothelium and within pulmonary airspaces, it may not necessarily reflect migration through the extracellular matrix (which could potentially involve different processes).

Another limitation is that the chemotaxis assays were performed at room temperature and not 37ºC, which would be more representative of an *in vivo* environment. Lower temperatures could potentially influence neutrophil function, particularly integrin-dependent adhesion (Nash et al. 2001). Ambient temperature is also subject to variation (particularly winter to summer months), although the migration assays using healthy and COPD neutrophils were performed at the same time of year and would, therefore, have been subject the same variations in temperature. Importantly, neutrophils from both healthy and COPD neutrophils were migrating and clear differences in their migratory dynamics were observed. However, preliminary chemotaxis assays at 37ºC from our group suggest the differences in chemotaxis between healthy and COPD neutrophils are maintained (unpublished data from G. Walton).

Inaccurate neutrophil migration may have implications in the pathogenesis of COPD as, if neutrophils are migrating less directly through the lung parenchyma to the airways, the area of collateral damage resulting from the release of destructive enzymes (such as NE) would be increased. Interestingly, neutrophils from patients with α_1-ATD seem to migrate more accurately in the presence of certain chemoattractants (IL-8 and fMLP), which cannot be accounted for by an age effect (as there is no age difference between groups). One previous study has suggests that α_1-AT actually inhibits neutrophil chemotaxis (Lomas et al. 1995). Therefore, it is possible that low serum α_1-AT has had a residual effect on the neutrophils of α_1-ATD patients and improves their chemotaxis.

Incubation of AATD neutrophils or (even those from healthy or COPD neutrophils) in α_1-AT prior to the chemotaxis assay may help confirm or refute this effect. As no impairment was demonstrated in the migration of α_1-ATD neutrophils, it can be inferred that the analogous emphysematous pathology in COPD and α_1-ATD occur via different mechanisms. As COPD neutrophils migrate, the area of residual proteolytic damage along their migratory track is comparable to healthy neutrophils (as it is sufficiently controlled by anti-proteinases). However, the tracks may be much longer as the cells seem to move more randomly, which would result in a net increase in the area of damage. In α_1-ATD, the cells migrate normally along a more direct path but the area of tissue destruction is amplified due to a proteinase/anti-proteinase imbalance. These theoretical effects are illustrated in Figure 3.22.

Assessment of neutrophil migration through artificial matrices (such as collagen) may clarify whether or not COPD neutrophils migrate with the same impaired accuracy within a three-dimensional environment.

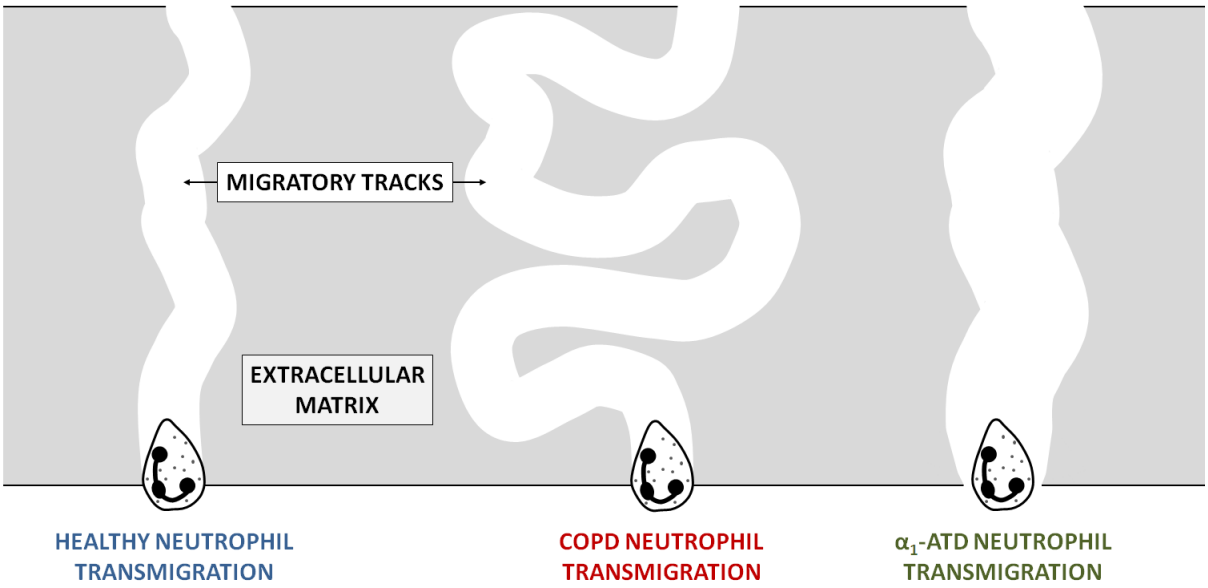

Figure 3.22: A theoretical model of neutrophil transmigration in health, COPD and α_1-ATD.

Legend: Healthy neutrophil transmigration is accurate with sufficient antiproteinase levels to minimise collateral damage to the surrounding tissue. COPD neutrophils migrate less accurately, leading to longer migratory tracks and increased tissue damage. α_1-ATD neutrophils migrate accurately but, due to reduced levels of α_1-AT, their migratory tracks are wider and the resulting tissue damage is also increased.

In addition, the inability of COPD neutrophils to migrate accurately along two-dimensional chemoattractive gradients (particularly fMLP) could result in an impaired "hunting" ability when tracking invading pathogens. Ultimately, this could lead to an increased bacterial presence within the lungs, although unpublished data by our group suggests that neutrophil migration does not relate to exacerbation frequency. The fact that there is an increased neutrophil presence within the pulmonary airways of COPD patients (Martin et al. 1985, Stanescu et al. 1996, Rutgers et al. 2000, Pilette et al. 2007) could mean that, although they may be less able to locate bacteria, their increased numbers

(Stanescu et al. 1996, Rutgers et al. 2000) and phagocytic capacity (Burnett et al. 1987) may balance this effect.

3.6.2 Effect of Donor Plasma on Neutrophil Chemotaxis

As mentioned previously (Section 1.5.7), inflammation in COPD not only occurs within the lungs, but also systemically. It has previously been shown that the pro-inflammatory cytokine, TNFα, is increased in the plasma (Takabatake et al. 2000, Broekhuizen 2005) and serum (Karadag et al. 2008) of COPD patients. Furthermore, serum levels of TNFα correlate with disease severity (von Haehling et al. 2009). Therefore, it was hypothesised that soluble factors in the circulation (such as increased levels of inflammatory mediators) could potentially alter the neutrophil phenotype in COPD and possibly lead to impaired migration.

Comparisons of migratory dynamics were performed following incubation of COPD neutrophils in their own plasma and plasma from an age-matched healthy control. In the presence of an IL-8 chemotactic gradient, no significant differences in migration were observed and in fMLP, COPD neutrophils appeared to move more accurately in their own plasma than in healthy plasma or medium alone. This demonstrates that a COPD phenotype cannot be induced in healthy neutrophils by incubation in COPD plasma and, also, that dysfunctional COPD neutrophil migration cannot be normalised or even improved by transferring the cells into healthy plasma. These data suggest that the immediate systemic inflammatory environment may not influence neutrophil migration in COPD and the aberrant migration observed in COPD neutrophils could be due to an intrinsic cell defect. The null effect of donor plasma incubation is further supported by the fact that incubation

in 100% plasma did not affect the ability of healthy or COPD neutrophil to adhere to the albumin-coated coverslips or their ability to initiate migration. However, the effect of chronic exposure in the bone marrow during neutrophil development cannot be extrapolated from these data, which only reflects acute exposure in the plasma.

On reflection, it may also have been useful to measure plasma concentration of pro-inflammatory cytokines (such as TNFα, IL-1β, IL-6 and IL-8) as well as other soluble inflammatory markers (such as CRP) in the plasma of each subject included in this experiment to confirm the presence or absence of systemic inflammation.

3.6.3 Neutrophil Phenotype

Despite of the null effect of incubating healthy neutrophils with COPD plasma and vice versa on migratory dynamics, previous work has provided evidence that at least a proportion of circulating neutrophils in patients with stable COPD may be activated. In earlier studies, one group consistently showed an increase in surface expression of Mac-1 (an integrin dimer comprising active CD11b and CD18) on neutrophils isolated from COPD patients compared to those from healthy controls. In their first study, this was true under both basal conditions and following stimulation with TNFα and, importantly, expression of healthy non-smokers and healthy smokers was no different (Noguera et al. 1998). In a subsequent study, basal expression of Mac-1 was higher in stable COPD compared to health but, surprisingly, decreased to levels comparative to health during exacerbations of COPD (Noguera et al. 2001). In both of these studies, the neutrophil surface expression of other adhesion molecules, namely L-Selectin (CD62L) and

Lymphocyte Function-Associated Antigen (CD11a/CD18) was the same in health and COPD. A later study by a different group also found increased expression of CD11b on isolated COPD neutrophils compared to health, although CD18 was not differentially expressed (Yamagata et al. 2007). The most recent study analysed various activation markers on isolated neutrophils from patients with COPD as well as healthy smokers at baseline and post-rhinovirus infection (Mallia et al. 2013). These markers included CD62L and CD163 (P-Selectin) (involved in rolling and capture), CD11a and CD11b (required for firm adhesion), CD31 and CD54 (ICAM-1) (involved in transmigration) and CD63 and CD66b (activation markers). In contrast to the previous studies, this group reported no difference in any of these neutrophil activation markers at baseline between COPD and healthy smokers. Although CD11a was reduced in both cohorts and CD11b, CD62L and CD162 expression significantly reduced in COPD following rhinoviral infection, no differences were observed between healthy smokers and COPD.

In the current study, no difference in either CD11b or CD62L surface expression on quiescent neutrophils from patients with COPD compared to those from healthy subjects. While none of the previous studies have found a difference in CD62L expression, the data on CD11b is at variance with the majority of previous studies that found an increased expression on COPD neutrophils (Noguera et al. 1998, Noguera et al. 2001, Yamagata et al. 2007) and only in agreement with one study (Mallia et al. 2013). This could potentially be due to differences in methodology as, in all previous studies, neutrophils have been isolated from whole blood. In order to minimise the risk and the number of neutrophils that could become activated, the analysis was conducted using neutrophil populations in whole blood. It is possible that COPD neutrophils are more susceptible to activation in COPD

(particularly if they are primed with increased plasma levels of TNFα), and the proportion of neutrophils that express CD11b following isolation may, therefore, be increased in COPD compared to health.

In contrast, the data reported here indicate that the surface expression of CD63 is higher on circulating neutrophils from COPD patients compared to those from healthy individuals, although the difference was only modest. CD63 is a membrane marker of azurophile granules and its translocation to the plasma membrane is associated with PMN stimulation and azurophilic granule exocytosis (Mahmudi-Azer et al. 2003). For this reason, upregulation of CD63 on the surface of neutrophils is considered a cogent marker of azurophilic degranulation and, by inference, neutrophil activation (Kuijpers et al. 1991).

One study has investigated CD63 expression in isolated neutrophils from COPD patients compared to healthy controls (Zhang et al. 2007). In addition to surface expression, they also measured total CD63 content of permeabilised neutrophils and found an increased expression on the surface of COPD neutrophils compared to health but a lower overall content. The data in this chapter are in agreement with Zhang et al., although they are at variance with a later study by Malia et al. that did not show difference (Mallia et al. 2013). A trend towards a higher percentage of cells with a CD63 positive phenotype was also found but this did not reach statistical significance. Whether or not the increase in CD63 expression is a true reflection of increased neutrophil activation in COPD is difficult to determine, as other markers of activation were not different when compared to health. If the higher CD63 surface expression is a genuine phenomenon, it is unlikely to be related to overall neutrophil migration, as only a modest difference was observed. However, it could influence the pathology of COPD because, as CD63 is a marker of azurophilic

degranulation, there is potential for an increased collateral damage during migration through the parenchyma as well as during the respiratory burst if granular exocytosis is also increased. However, as Zhang et al. actually found a lower overall CD63 content in COPD neutrophils compared to those from healthy subjects (Zhang et al. 2007), this may not be the case. CD63 interacts with a number of molecules, including MHC Class II molecules (found on antigen presenting cells and monocytes) (Hammond et al. 1998), integrins (Berditchevski et al. 2001) and Phosphatidylinositol 4-kinase (Berditchevski et al. 1997, Yauch et al. 2000) (which is involved in intracellular membrane trafficking). Therefore, increased expression on the cell surface (particularly in fully activated neutrophils) could affect cell adhesion as well as the interaction with other inflammatory cells (such as dendritic cells), which may augment chronic inflammation in COPD.

Measurement of the surface expression of CD63 on activated neutrophils in COPD and health may provide further insight, though the precise mechanisms by which CD63 is involved in granule mobilisation and release have yet to be established. Furthermore, it would important to assess CD63 expression on adhered and fixed cells by confocal microscopy to determine if this phenotype is robustly present during migration. It was surprising to see an increased expression of CD63 on COPD neutrophils when more sensitive markers of neutrophil activation, CD62L and CD11b, were not different. To confirm this phenomenon, it would be logical to repeat this experiment and include other markers of the azurophile granule (such as NE, MPO and lysozyme).

Two recent studies by a group in the Netherlands have identified sub-populations of neutrophils with differential expression of CD16 and CD62L. Firstly, Pillay et al. identified a population of $CD16^{bright}/CD62L^{dim}$ neutrophils in humans *in vivo* during acute systemic

inflammation induced by either LPS challenge or severe injury. These were considered a unique population of myeloid cells with a functional difference in that they were able to suppress human T cell proliferation (Pillay et al. 2012). In the follow-on study, Kamp et al. further classified sub-populations of circulating neutrophils into $CD16^{bright}/CD62L^{dim}$, $CD16^{dim}/CD62L^{bright}$ (immature, banded phenotype), and $CD16^{bright}/CD62L^{bright}$ (phenotypically mature) (Kamp et al. 2012). When various aspects of neutrophil migration (including endothelial capture, integrin binding and chemotaxis) were assessed, functional differences were recorded. Of note, the immature, $CD16^{dim}/CD62L^{bright}$ population had impaired Mac-1 binding in fMLP and IL-8 but increased chemotaxis in fMLP (though not in IL-8 or C5a) compared to the more mature ($CD16^{bright}$) sub-populations. However, chemotaxis was assessed using a Boyden Chamber, and information on migratory dynamics was not determined.

As COPD is a chronic inflammatory disorder and is associated with persistent neutrophil migration from the circulation into the lungs, it was hypothesised that, under this burden, immature neutrophils may be released from the bone marrow to balance the number of neutrophils that have left the circulation. If the neutrophils in the circulation of COPD patients are less mature than those of healthy subjects, they may not have developed the capacity to direct themselves accurately along a chemotactic gradient. However, no difference was found in either CD16 or CD62L expression on the surface of neutrophils from COPD patients and healthy subjects and the percentage of $CD16^{+}$ and $CD62L^{+}$ was virtually 100% in every subject (with negative cells most likely artefact). Furthermore, there did not seem to be any "double populations" of $CD16^{dim}$ and $CD16^{bright}$ neutrophils that were observed by the aforementioned studies (Pillay et al. 2012, Kamp et al. 2012).

Therefore, it can be concluded that, regardless of the potential differences in migratory accuracy between immature and mature neutrophils, there seems to be no difference in the neutrophil CD16/CD62L phenotype between COPD and health.

3.6.4 Clinical Phenotype

COPD is a complex, heterogeneous disorder, which presents clinical and functionally with large variability in patients with comparable airflow obstruction (Wedzicha 2000, Agusti 2005, GOLD 2009). This study sought to determine if factors relating to the clinical phenotype of COPD were related to the degree of chemotactic impairment.

It seems logical to hypothesise that, as COPD is a progressive disease, neutrophil function may become worse as the disease becomes more severe. However, no statistically significant differences were observed in chemotaxis between COPD GOLD Stages I-IV. Moreover, there was no correlation between chemotaxis and FEV_1 % Predicted (the severity of COPD) or FEV_1/FVC (degree of airflow obstruction). This suggests that neutrophil chemotaxis may be equally impaired at any stage of the disease and could even be a congenital disorder, where COPD only manifests due to a chronic inflammatory insult (such as tobacco smoke inhalation). Despite no observed statistical differences between neutrophil chemotaxis and either GOLD Stage or FEV_1 % Predicted in COPD, a visual trend for decreasing chemotaxis with disease severity was observed (both through COPD Stages I-IV and with decreasing FEV_1 % Predicted). Although this potential relationship is biologically plausible, it is purely speculative (as the groups were clearly underpowered) and a further investigation with a much larger study is needed to confirm or refute this hypothesis.

This theory is partly supported by the fact that no differences in neutrophil migratory dynamics were observed between current and ex-smokers with COPD. In ex-smokers with more severe COPD, the degree of lung damage may be extensive enough for the inflammation to become self-perpetuating, which may continually influence neutrophil function. From the data in the current study, it is not possible to accurately describe the effects of smoking in mild COPD as, although this group included both current and ex-smokers, it was not highly powered. In addition, the negative correlation between age and chemotaxis observed in healthy ageing was not observed in COPD. This suggests that the same degree of chemotactic impairment is present in all COPD patients regardless of their age (as young as 31). The data are rather widespread and markedly reduced neutrophil chemotaxis was even observed in a small proportion of younger healthy adults. The data from these healthy subjects are most likely outliers. However, if the hypothesis that COPD susceptibility is hereditary is correct, they could be individuals that would develop COPD if they had a longer smoking history.

In patients with COPD (even in severe disease), airflow obstruction can result from inflammation and a gradual thickening of the small airways (< 2mm diameter) (Hogg et al, 1668). Furthermore, in a proportion of these patients, the small airways disease can occur in the absence of emphysema. It is plausible that different pathophysiological phenotypes of COPD may have different degrees of inflammatory cell functional impairment. However, no differences in neutrophil migration in COPD patients were seen between patients with emphysema compared to those without. This suggests that neutrophil chemotaxis impaired to the same degree in different “forms” of COPD and that it does not

matter where in the pulmonary environment the inflammatory signal originates from, neutrophils may always migrate to it with poor accuracy.

Although the data are collated from a number of experiments conducted by different individuals, the current author either performed or supervised the majority. Moreover, each individual performed the assays according to standardised protocol although, admittedly, there may be slight methodological differences between researchers that could account for some of the variability in the data. However, the fact that every separate set of experiments observed the same reduced chemotaxis of COPD neutrophils compared to health strongly supports our initial observations. Importantly, the large variability in chemotaxis data in the healthy cohort as a whole (as seen in Figure 3.14) can be accounted for by the large age range and the fact that there is a progressive loss of neutrophil migratory accuracy with age. Therefore, the inclusion of younger healthy individuals in the comparison to separate COPD GOLD Stages actually introduces a degree of bias. A more statistically robust assessment would age match separate healthy cohorts to each COPD Gold Stage, which would be a consideration for similar future investigations.

Altogether, these data strongly suggest that none of the clinical features of COPD significantly influence the migratory dynamics of neutrophils. It is possible that migratory accuracy may worsen with disease severity, but the data cannot confirm this theory. It is also possible that aberrant neutrophil migration is a congenital disorder that only causes COPD in individuals who chronically inhale tobacco smoke or other airborne particles.

3.6.5 Summary

In conclusion, this chapter has demonstrated clear differences in the migratory accuracy of neutrophils from COPD (which migrated with increased speed but reduced accuracy) compared to those from healthy subjects and patients with α_1-ATD. This appears to be an intrinsic cell defect as incubation of healthy neutrophils in COPD plasma did not induce a COPD phenotype. Furthermore, it appears to be unrelated to the clinical features of COPD or the activation status and maturity of COPD neutrophils. Therefore, it seems logical to assume that the migratory dysfunction of COPD neutrophils is related to chemokine receptor function and/or downstream intracellular signalling.

CHAPTER 4

MECHANISMS UNDERLYING ABERRANT NEUTROPHIL MIGRATION IN COPD

4.1 BRIEF INTRODUCTION

The studies described in Chapter 3 revealed clear differences in the migratory dynamics of neutrophils from COPD patients compared to those from both healthy donors and patients with α_1-ATD. These included an increased migratory speed but a decreased migratory accuracy. This was a generic phenomenon that occurred in the presence of a number of different chemoattractants. Furthermore, it appears to be to an intrinsic cell defect and is not the result of a higher level of neutrophil activation in COPD.

As described previously, the initiation of intracellular signalling that ultimately leads to migration occurs when chemotactic ligands bind to their respective receptors on the cell surface. Many endogenous and exogenous molecules can act as neutrophil chemoattractants and neutrophils have at least five different types of receptor for these stimuli, which include unique receptors for C5a, LTB_4, PAF and fMLP, as well as a number of CC and CXC receptors that bind other chemokines such as IL-8 and GROα.

Previous work on neutrophil chemokine receptor expression has only included studies on CXCR1 and CXCR2 with sole focus on IL-8 as a chemoattractant. The evidence for differing expression of these receptors in COPD is conflicting, where one group reported no difference between expression of CXCR1 in COPD compared to health but a decrease in CXCR2 expression (Pignatti et al. 2005) and, in contrast, another group reported no difference in CXCR2 expression but an overexpression of CXCR1 in COPD (Yamagata et al. 2007). Therefore, the roles of CXCR1 and CXCR2 in neutrophil migration, particularly in COPD, remain unclear and no previous work has been conducted to investigate the expression of other chemokine receptors. Furthermore, previous studies have only looked at chemokine expression on quiescent cells. This yields limited information, as migrating neutrophils are

not quiescent and surface receptor expression is likely to change due to internalisation, shedding and mobilisation to the plasma membrane from internal stores. This is important as it seems logical that the number of receptors available on the cell surface and their location relative to the leading edge at any one time would govern neutrophil migration, not simply their expression in a quiescent state. Rates of receptor recycling (internalisation and re-expression) and, potentially, receptor shedding both dictate the degree of surface expression following stimulation and during migration. In turn, this could influence how effectively the cell responds to a chemotactic stimulus and how accurately it migrates along the gradient.

The aberrant neutrophil migration observed in this study could also be a result of altered downstream signalling. Following chemokine/GPCR binding, the βγ-subunit activates a variety of cellular mediators and signalling pathways. Of particular interest is the PI3K signalling pathway, which has been heavily implicated in neutrophil chemotaxis and directionality (Stephens et al. 2002, Hannigan et al. 2002, Wang et al. 2002, Wu 2005, Sapey et al. 2011). Importantly, evidence suggests that, although components of the PI3K pathway (particularly PIP_3) influence cell migration, they are not essential for chemotaxis. *Dictyostelium* lacking PI3K are still able to migrate accurately in steep chemotactic gradients, though their speed of movement is reduced (Loovers et al. 2006, Hoeller et al. 2007). In shallow chemotactic gradients, inhibition of PI3K in *Dictyostelium* affects the rate at which pseudopods are generated which, in turn influences migration (Andrew et al. 2007). Although not essential for cell migration, the PI3K signalling pathway may be preferential and differences in the activity of PI3K itself and/or other key components could be a potential mechanism for the aberrant neutrophil migration in COPD.

Calcium is a diverse second messenger that also has an important role in many physiological functions (Meshulam et al. 1986, Marks et al. 1990), though its role in neutrophil chemotaxis is less well defined. Neutrophil polarisation is an essential precursor to accurate cell migration (O'Donnell et al. 1992, Onsum et al. 2007) and local calcium signals have been shown to occur at the leading edge migrating neutrophils (Evans et al. 2007, Wei et al. 2011). Therefore, dysfunctional calcium signalling could also provide a reason for the impaired migratory accuracy of COPD neutrophils.

The studies described in this chapter were conducted firstly to investigate expression of CXCR1, CXCR2, FPR1, LTB_4R1 and the C5aR receptor on quiescent neutrophils. Following this, CXCR1, CXCR2 and FPR1 expression was measured on neutrophils that had been stimulated with either IL-8 or fMLP over a two hour time-course. Receptor shedding throughout the time-course was quantified by ELISA and receptor localisation was determined by fluorescent labelling of stimulated neutrophils and microscopy. Secondly, intracellular signalling was investigated; PI3K and Akt phosphorylation was determined by Western Blot and real-time intracellular calcium ion release following stimulation was quantified by fluorimetry.

4.2 SUBJECT DEMOGRAPHICS

Demographic data for the COPD patients, healthy age-matched control subjects and patients with α_1-ATD included in this chapter are shown in Table 4.1.

	COPD	Healthy Control	α_1-ATD
Number	51	49	23
Age (years)	68 (49 - 85)	60 (41 - 87)	61 (47 - 71)
Sex (% Male)	53	47	48
Smoking status	18 CS, 33 XS	38 NS, 3 CS, 8 XS	16 NS, 7 XS
Pack years	49 (4.4)	2 (1.1)	9 (3.0)
FEV1 % predicted	44.7 (3.1)	101.0 (1.3)	57.5 (6.2)
FEV1 /FVC %	38.8 (2.1)	73.2 (0.5)	37.5 (3.7)

Table 4.1: Subject Demographics.

Legend: A table showing the demographic data for all subjects included in this chapter. Data are normally distributed and presented as the mean and SEM, except the age which is mean (range). NS = Never-smokers, CS = Current smokers, XS = Ex-smokers.

4.3 CHEMOKINE RECEPTOR EXPRESSION

4.3.1 Validation: Receptor Antibody Titrations

To determine the most appropriate concentration of each chemokine receptor antibody (CXCR1, CXCR2, FPR1, LTB_4R1 and C5aR), receptor expression was assessed over a range of concentrations using neutrophils from 3 healthy donors. Data for the median fluorescence

intensity (MFI = level of receptor expression) for each antibody at each concentration are shown in Figure 4.1. Neutrophils were gated relative to the isotype control so that only cells positive for each antibody were included in the analysis.

From these data, the optimal concentration of each receptor antibody was determined from the highest MFI value. With some of the antibodies (such as FPR1 and C5aR), a maximal binding was not achieved by 3μg/ml. The optimal concentrations of the five receptor antibodies were as follows; CXCR1: 2μg/ml, CXCR2: 3μg/ml, FPR1: 3μg/ml, LTB_4R1: 3μg/ml, C5aR: 3μg/ml. Henceforth, subsequent assays were performed using the antibodies at these concentrations.

Isotype-matched irrelevant antibodies (IgG1 and IgG2A) did not demonstrate a concentration response and were therefore used at the same concentration to each of the respective anti-receptor antibodies.

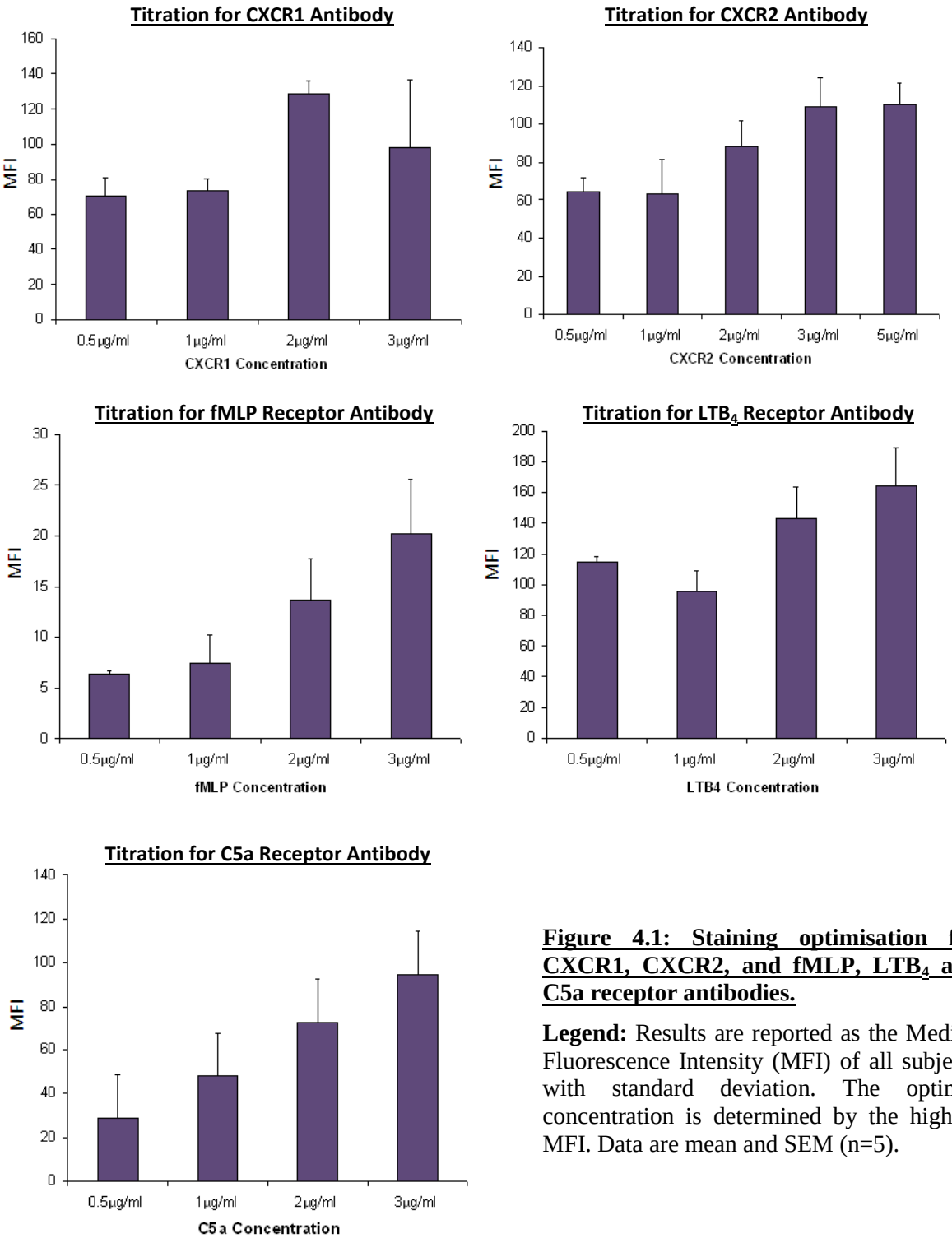

Figure 4.1: Staining optimisation for CXCR1, CXCR2, and fMLP, LTB_4 and C5a receptor antibodies.

Legend: Results are reported as the Median Fluorescence Intensity (MFI) of all subjects with standard deviation. The optimal concentration is determined by the highest MFI. Data are mean and SEM (n=5).

4.3.2 Quiescent Chemokine Receptor Expression in COPD, Health and α_1-ATD

Quiescent neutrophil receptor expression was measured for CXCR1, CXCR2, and FPR1, LTB_4R1 and C5aR in 20 age-matched subjects from each cohort. Surface expression of CXCR1 was significantly lower in COPD neutrophils compared to those from healthy control subjects ($p = 0.02$) and a trend towards a similar reduction in CXCR2 expression was also observed in COPD, although this did not achieve statistical significance ($p = 0.06$). No significant differences were observed between healthy and α_1-ATD neutrophils for expression of either CXCR1 or CXCR2. When comparing neutrophils from α_1-ATD to COPD patients, no differences were observed in CXCR1 expression, although there was a lower expression of CXCR2 in COPD ($p = 0.04$). These data shown in Figure 4.2.

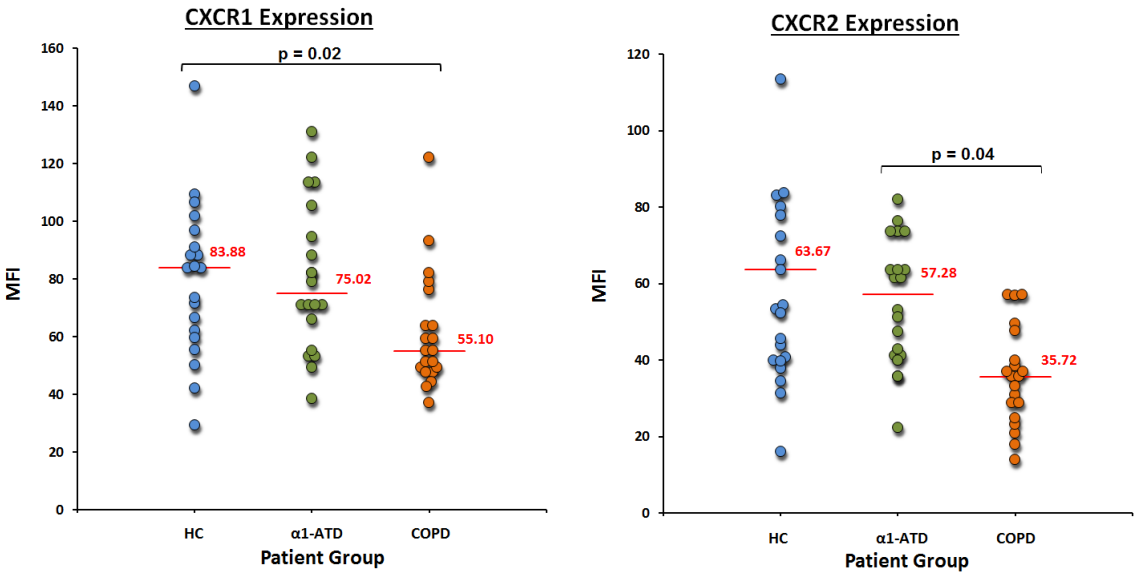

Figure 4.2: Quiescent neutrophil surface expression of CXCR1 and CXCR2.

Legend: CXCR1 and CXCR2 expression was semi-quantified in populations of isolated, quiescent neutrophils from 20 Healthy Control (HC) subjects, α_1-ATD patients and COPD patients by immunostaining and flow cytometry. Median MFI values for each patient group are indicated by the red horizontal line. (Analysed by Kruskal-Wallis test).

No significant differences were observed between any group comparison for surface expression of FPR1 or LTB_4R1. Expression of C5aR was significantly lower in COPD neutrophils compared to healthy controls ($p = 0.04$), although no significant differences were observed for C5aR expression in α_1-ATD compared to healthy controls or the COPD patients (Figure 4.3).

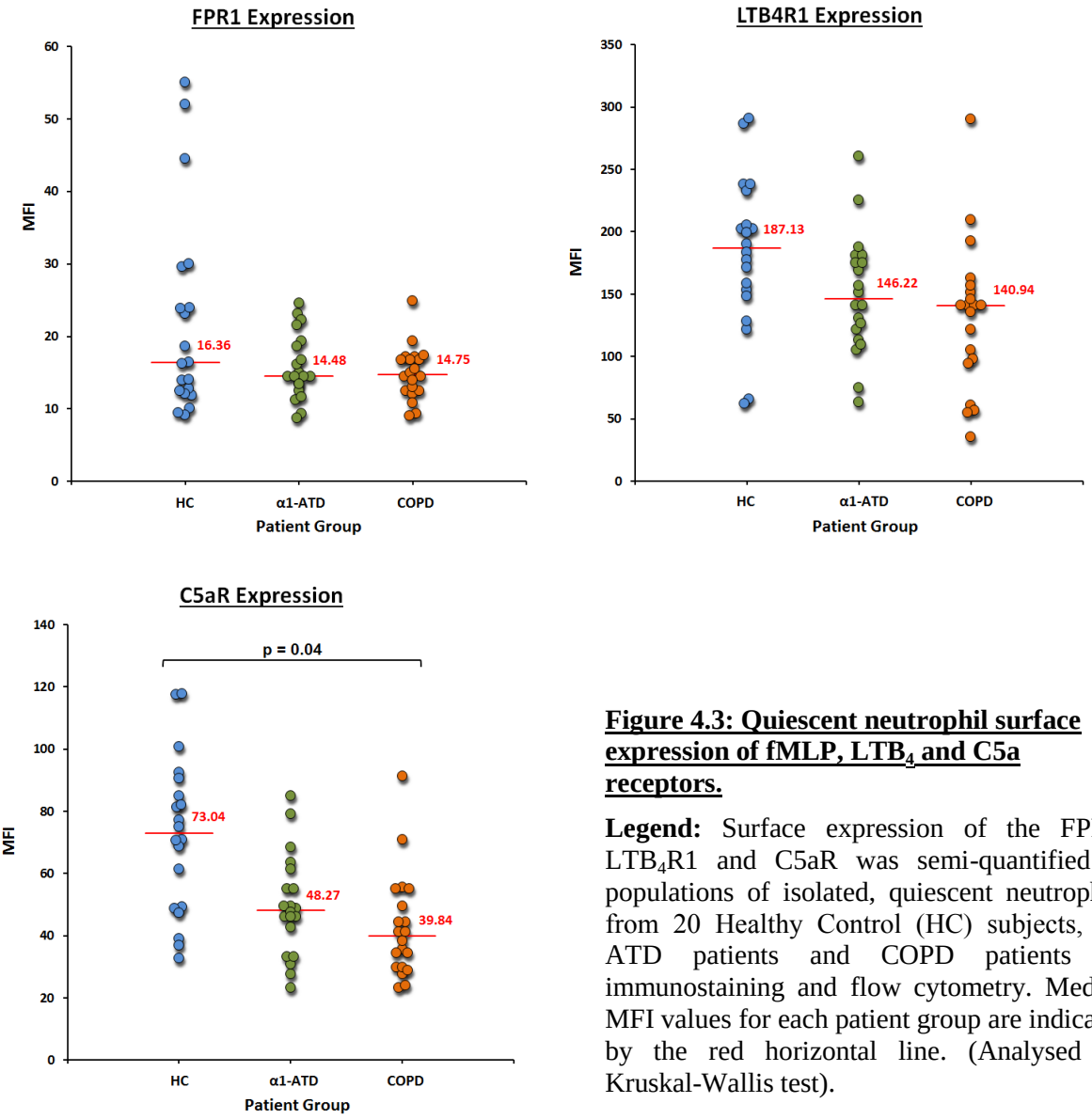

Figure 4.3: Quiescent neutrophil surface expression of fMLP, LTB_4 and C5a receptors.

Legend: Surface expression of the FPR1, LTB_4R1 and C5aR was semi-quantified in populations of isolated, quiescent neutrophils from 20 Healthy Control (HC) subjects, α_1-ATD patients and COPD patients by immunostaining and flow cytometry. Median MFI values for each patient group are indicated by the red horizontal line. (Analysed by Kruskal-Wallis test).

4.3.3 Chemokine Receptor Expression Following Neutrophil Stimulation

Although differences in chemokine receptor expression on quiescent neutrophils may be indicative of a mechanism of aberrant migration, when neutrophils migrate down chemotactic gradients they are not in a quiescent state. Following stimulation by chemokines, surface expression their respective receptors may potentially increase due to mobilisation from intracellular stores or decrease due to internalisation and/or shedding. Furthermore, it is unclear whether or not surface expression fluctuates with time.

To determine if the differences observed in chemokine receptor expression between COPD and healthy neutrophils persist following stimulation, CXCR1, CXCR2 and FPR1 expression was measured at various time-points throughout a two hour stimulation with IL-8 or fMLP (n=13). CXCR1 and CXCR2 shedding was also measured in neutrophils from COPD and healthy donors at various time-points by ELISA (n=11).

Quiescent Expression

In agreement with the previous results (Section 4.3.2), CXCR1 expression was significantly lower in quiescent COPD neutrophils when compared to those from healthy controls. Interestingly, CXCR1 expression was also significantly lower in neutrophils from COPD patients when compared to those from patients with α_1-ATD. This difference was not observed in the previous experiment. No differences were observed between CXCR2, which is also at variance with the previous data, where a significantly lower expression of CXCR2 on COPD neutrophils compared to those from α_1-ATD patients was reported. There were no differences in FPR1 expression, which is in agreement with the previous results.

Kinetics of Receptor Loss Upon Stimulation

Surface expression of both CXCR1 decreased continually in all cohorts following stimulation with IL-8 throughout the time-course. Although there was still a trend towards lower expression of CXCR1 in COPD neutrophils compared to healthy controls, the significant difference observed in quiescent neutrophils was rapidly abolished (within 5 minutes). CXCR1 expression remained significantly lower in COPD neutrophils compared to those from patients with α_1-ATD at the 10 minute time-point but was no longer present 15 minutes after stimulation. These data are summarised in Figure 4.4.

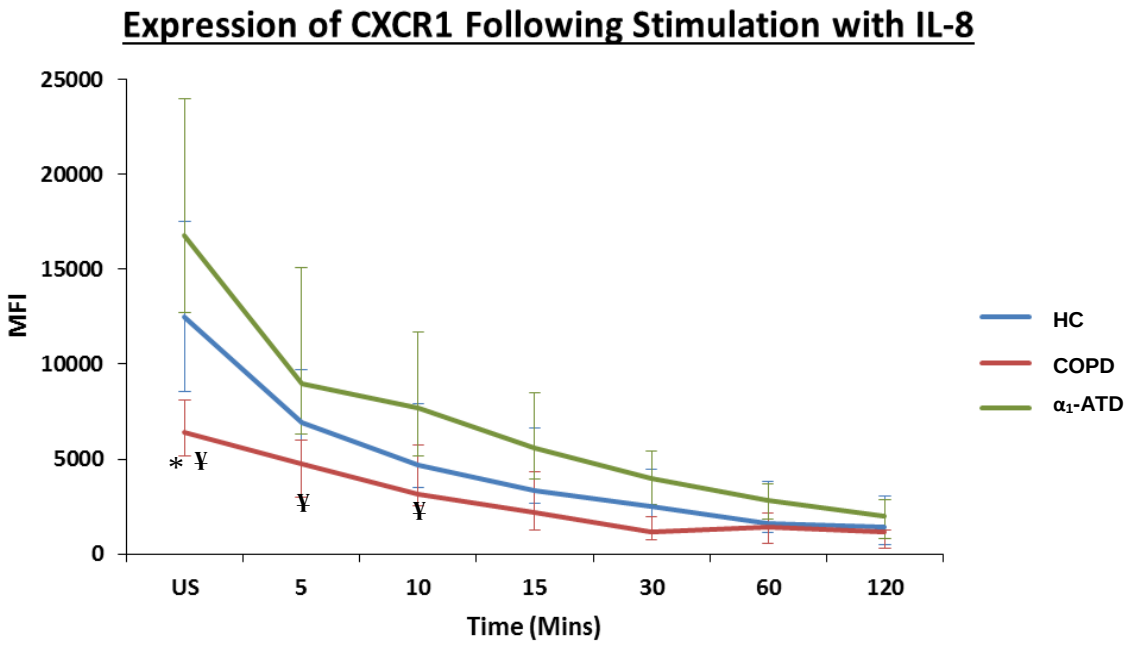

Figure 4.4: CXCR1 expression following stimulation with IL-8.

Legend: CXCR1 surface expression was measured following stimulation with 100nM IL-8. The difference in expression in unstimulated (US) neutrophils between Healthy Controls (HC) and COPD (*) ($p \leq 0.05$) was abolished within 5 minutes. The difference between α_1-ATD and COPD (¥) ($p \leq 0.05$) remained for longer but was not present after 15 minutes. Results are median and IQR (n=13). (Analysed by Kruskal-Wallis test).

CXCR2 expression decreased more rapidly following stimulation in all cohorts (compared to CXCR1) and remained the same throughout the time-course for all patient groups. These data are summarised in Figure 4.5.

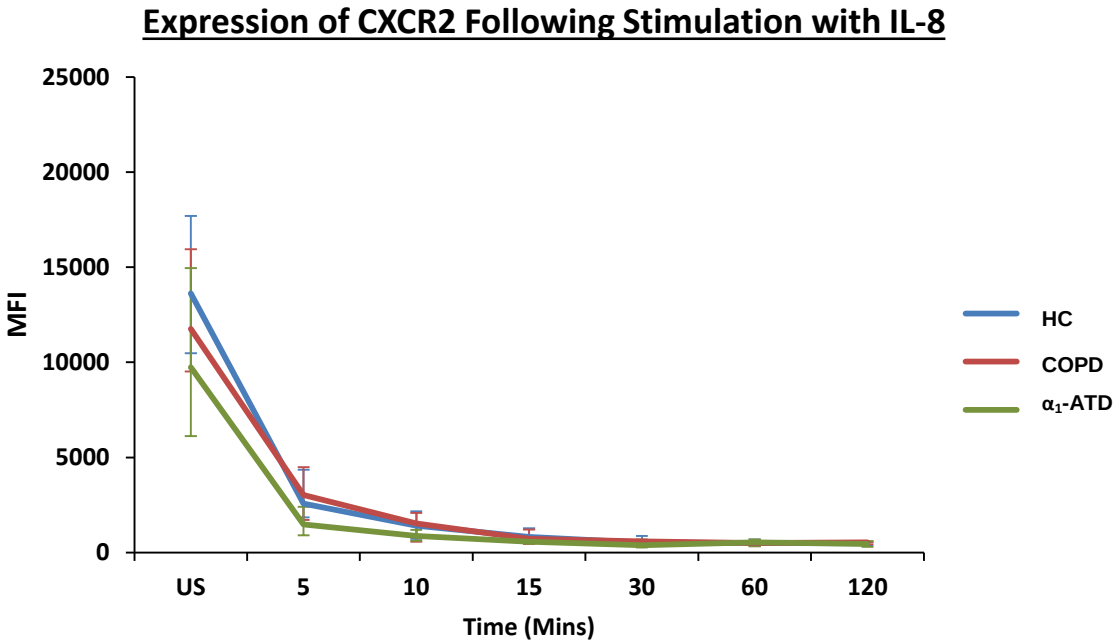

Figure 4.5: CXCR2 expression following stimulation with IL-8.

Legend: CXCR2 surface expression was measured upon stimulation with 100nM IL-8. Expression declined rapidly in all cohorts following stimulation. No differences were observed in surface expression at any time-point. Results are median and IQR (n=13). (Analysed by Kruskal-Wallis test).

FPR1 surface expression was initially relatively low in all cohorts and remained so throughout the time-course. No differences in FPR1 expression were observed between any patient group at any time-point. These are summarised in Figure 4.6.

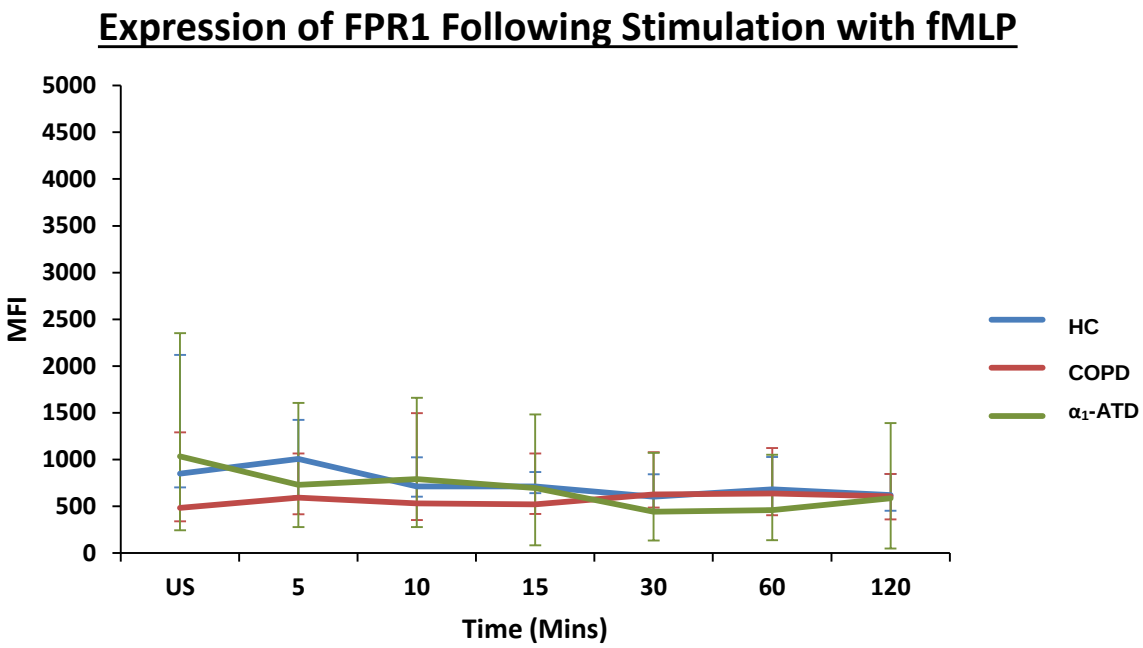

Figure 4.6: FPR1 expression following stimulation with fMLP.

Legend: FPR1 expression was measured after stimulation with 100nM fMLP. Expression was initially low in unstimulated neutrophils in all cohorts (n=13). Expression did not increase or decrease following stimulation with fMLP and no differences in expression were observed between cohorts at any time-point. Results are median and IQR (n=13). (Analysed by Kruskal-Wallis test).

4.3.4 Chemokine Receptor Recycling

In order to verify that receptor recycling was occurring, CXCR1 and CXCR2 expression on neutrophils from selected healthy and COPD donors was measured 90 minutes after the IL-8 stimulus was removed. Surface expression of both CXCR1 and CXCR2 had increased from expression at 30 minutes post-stimulation in both healthy and COPD subjects. Although the surface expression in each case had not returned to baseline levels observed in quiescent neutrophils, a clear trend for surface re-expression was observed in each instance (Figure 4.7).

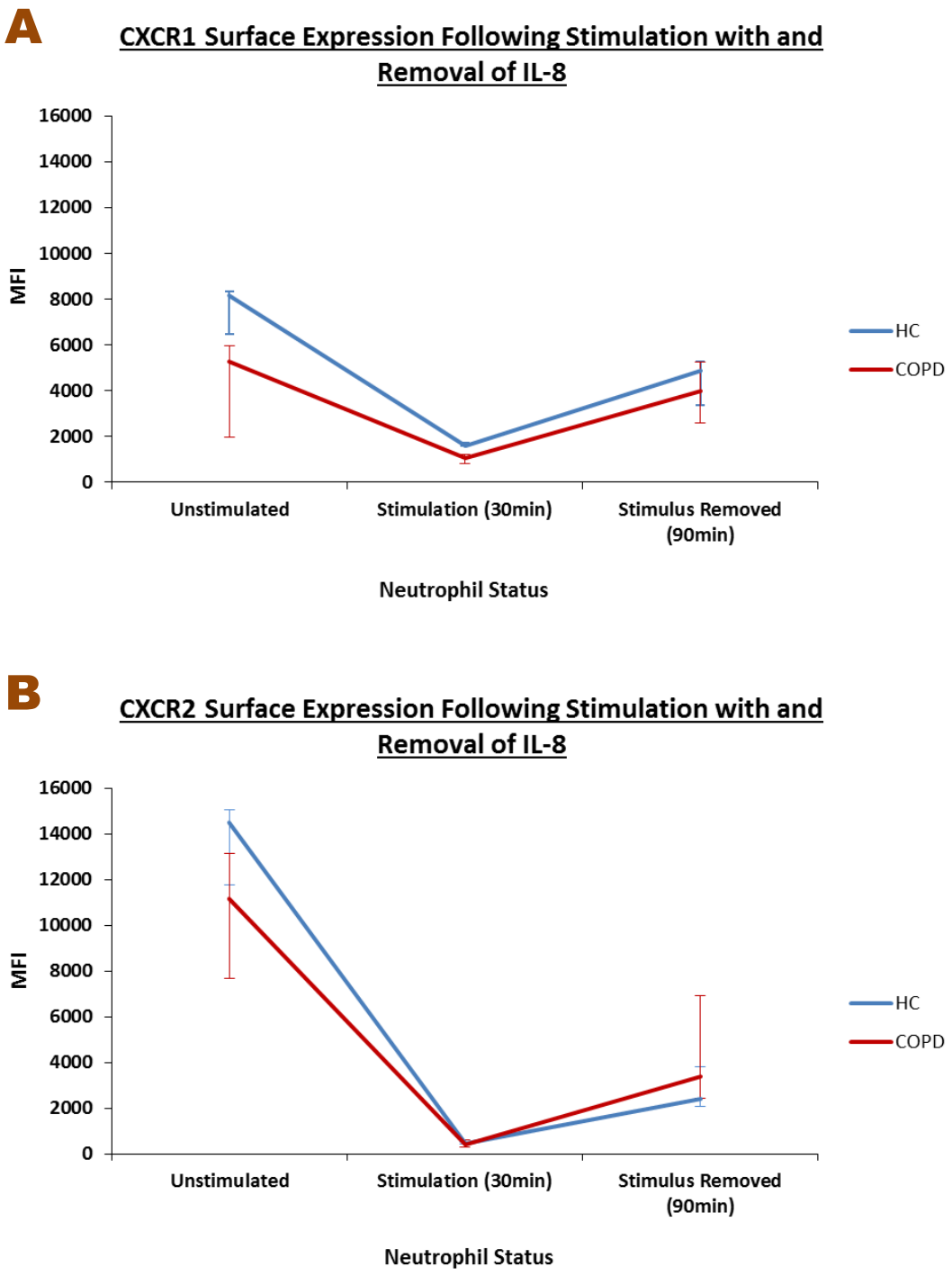

Figure 4.7: CXCR1 recycling following removal of IL-8 stimulus.

Legend: Surface expression of CXCR1 (A) and CXCR2 (B) was quantified on unstimulated cells, 30 minutes after stimulation with 100 nM IL-8 and, finally, 90 minutes after the IL-8 stimulus was removed. This was done with neutrophils from COPD patients (n=8) and healthy subjects (n=5). Data are median and IQR. (Analysed by unpaired Mann-Whitney U test).

4.3.5 Chemokine Receptor Shedding

The degree of CXCR1 and CXCR2 shedding was assessed by ELISA using supernatants collected at the 30 and 120 minute time-points. These time-points were chosen as receptor expression had decreased dramatically by 30 minutes (and had plateaued in COPD patients) and by 120 minutes receptor expression had plateaued in all groups. Therefore, if any shedding had occurred, it would most likely be detected at these times. The standard curve, which indicates the optical density of known CXCR1 concentration-dependent manor, is shown in Figure 4.8.

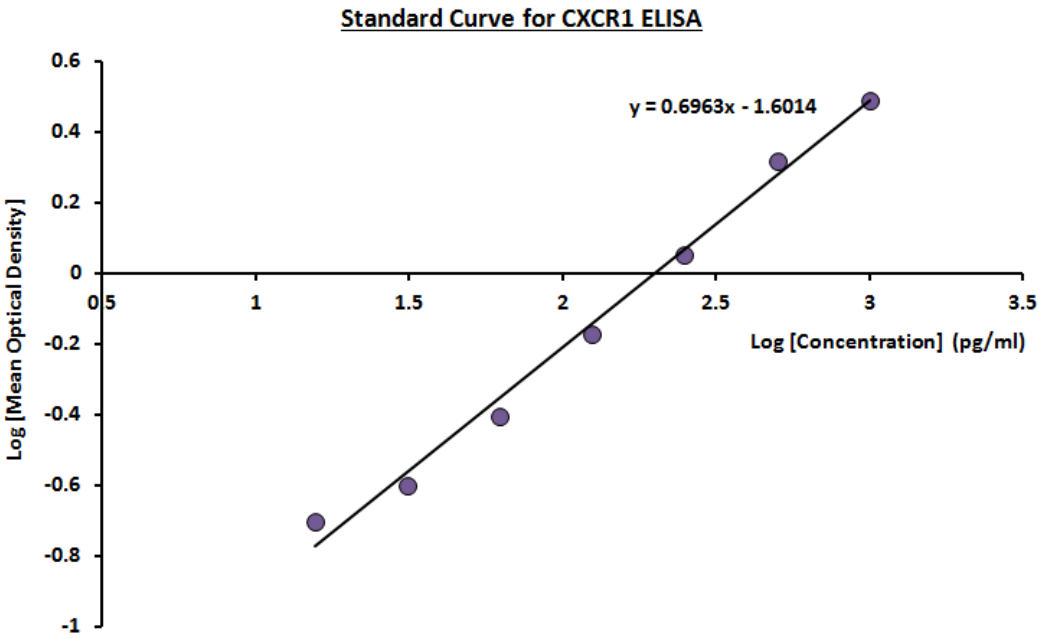

Figure 4.8: CXCR1 ELISA Standard Curve.

Legend: The Log concentration of the standard samples (S1 to S7) were plotted against the mean Log optical density of duplicate samples to produce a standard curve. As expected, the relationship was linear. This graph was then used to determine the concentrations of CXCR1 in the neutrophil supernatants from healthy and COPD donors following stimulation.

For CXCR1, the concentration in the supernatant of healthy controls was 8.6 pg/ml after 30 minute stimulation and 9.5 pg/ml after 120 minutes. For COPD patients it was 7.6 pg/ml at 30 minute stimulation and 8.3 pg/ml at 120 minutes. These values were actually less than the blank reference value (15.6 pg/ml), essentially indicating that no CXCR1 was detectable in supernatants from either COPD neutrophils suspension or those from healthy controls.

In contrast, CXCR2 was detected in the majority of supernatants for both COPD and healthy controls, though the results were still relatively low and rather variable. The concentration in the supernatant of healthy controls was 2.1 ng/ml after 30 minute stimulation and 1.6 ng/ml after 120 minutes. For COPD patients it was 1.1 ng/ml at 30 minute stimulation and 1.4 ng/ml at 120 minutes. No differences were observed between COPD and health at either time-point. These data are summarised in Figure 4.9.

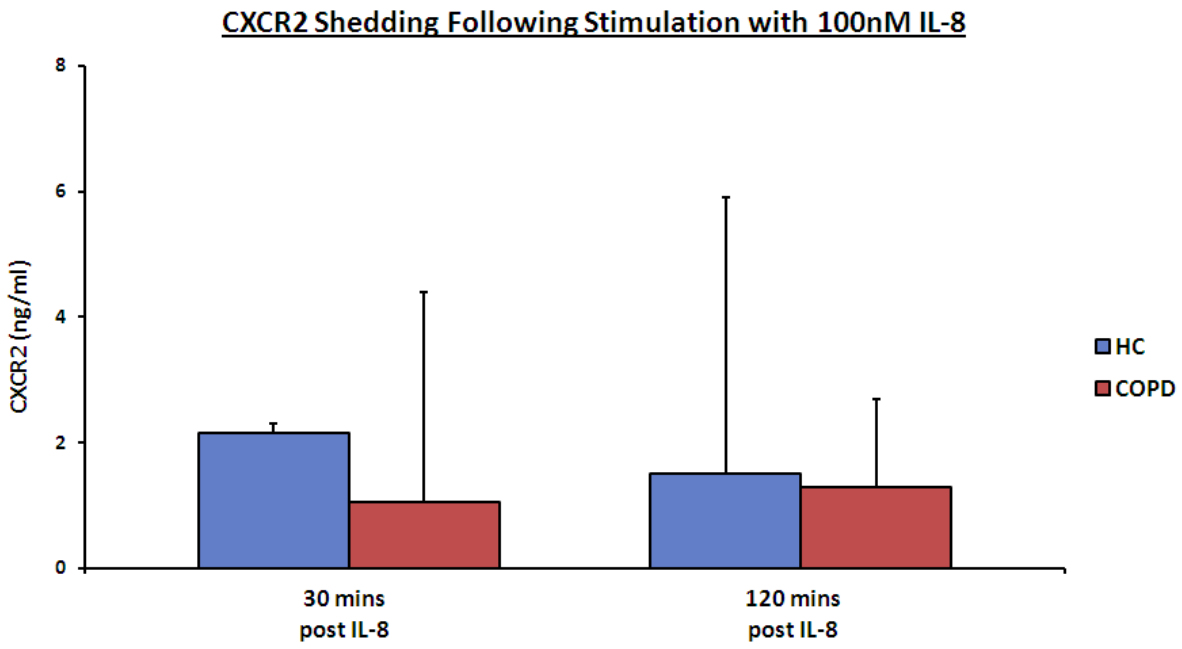

Figure 4.9: CXCR2 shedding following stimulation with IL-8.

Legend: Supernatants were collected from stimulated neutrophils over a two-hour time-course. CXCR1 shedding was quantified at 30 and 120 minutes by ELISA. Results are reported as the median and IQR (n=11). (Analysed by unpaired Mann-Whitney U test).

4.3.6 Chemokine Receptor Localisation

As expected, CXCR1 and CXCR2 were uniformly distributed across the cell surface in quiescent neutrophils in health and COPD. After polarisation, both CXCR1 and CXCR2 were highly expressed at the leading edge on neutrophils from healthy donors. Neutrophils from COPD donors also appeared to localise CXCR1 and CXCR2 to the leading edge to the same degree as those from healthy subjects. Microscopic images showing CXCR1 and CXCR2 localisation on quiescent and polarised neutrophils are shown in Figure 4.10 and Figure 4.11, respectively and results are representative of separate experiments for four individual COPD patients and healthy subjects.

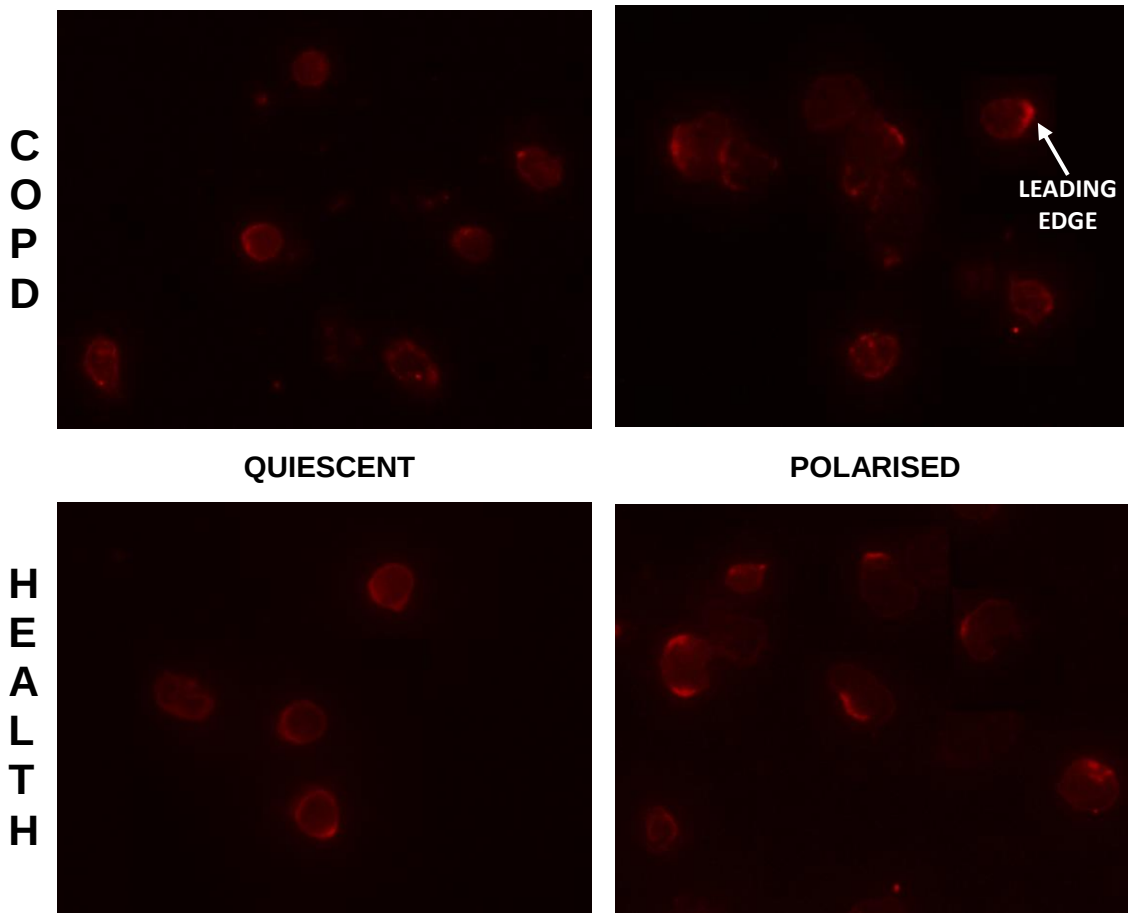

Figure 4.10: CXCR1 localisation on quiescent and polarised neutrophils.

Legend: Microscopic images showing the distribution of PE-labelled CXCR1 across the surface of neutrophils in quiescent (left) and polarised (right) neutrophils from COPD patients (top) and healthy control subjects (bottom). Images were taken using a Leica Microsystems fluorescence microscope using a x40 objective lens and are representative of experiments from 4 separate individuals for each cohort.

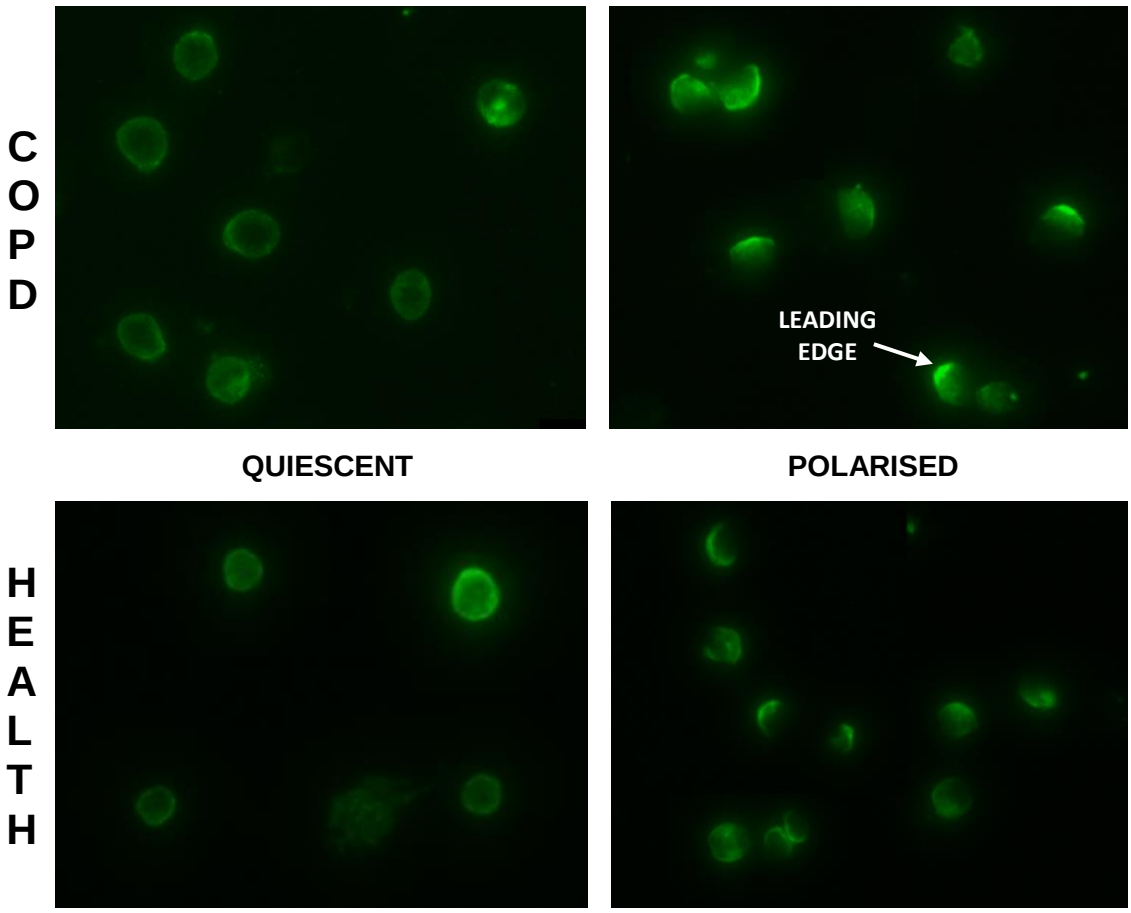

Figure 4.11: CXCR2 localisation on quiescent and polarised neutrophils.

Legend: Microscopic images showing the distribution of FITC-labelled CXCR2 across the surface of neutrophils in quiescent (left) and polarised (right) neutrophils from COPD patients (top) and healthy control subjects (bottom). Images were taken using a Leica Microsystems fluorescence microscope using a x40 objective lens and are representative of experiments from 4 separate individuals for each cohort.

4.4 PI3K & AKT PHOSPHORYLATION

4.4.1 Stimulation time-courses

Expression of the phosphorylated, active form of both PI3K at tyrosine 458 (T458) and Akt (at both phosphorylation sites; serine 473 (S473) and threonine 308 (T308)) was determined by western blotting. Initial time-course experiments were conducted to establish the optimal length of chemokine stimulation for PI3K and Akt phosphorylation. For $PI3K_{T458}$ and Akt_{S473}, time-points included 1, 2, 3, 5, 10, 15 and 30 minutes. These experiments indicated a good degree of phosphorylation in stimulated cells by 1 minute, with optimal expression occurring at 3 minutes for both phospho(p)-$PI3K_{T458}$ and $pAkt_{S473}$. Therefore, neutrophils were stimulated for 3 minutes on every subsequent assay with these antibodies. An example blot from a time-course for $pAkt_{S473}$ is shown in Figure 4.12.

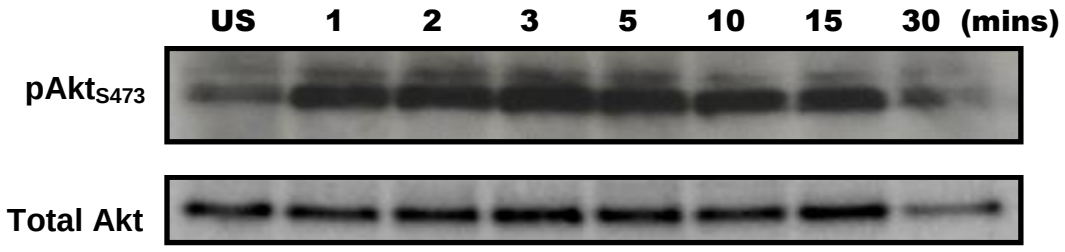

Figure 4.12: Akt phosphorylation at S473 over a time-course of IL-8 stimulation.

Legend: A western blot of $pAkt_{S473}$ and total Akt from neutrophil lysates from unstimulated neutrophils and those stimulated with 100 nM IL-8 over a 30 minute time-course (stimulation time in minutes is indicated at the top. US = Unstimulated). The image is representative of 3 separate experiments.

From a personal communication from Dr. Philip Hawkins in The Babraham Institute, Cambridge, it was indicated that phosphorylation of Akt at T308 occurs far more rapidly and transiently than at S473. Therefore, the time-course for $pAkt_{T308}$ was adjusted accordingly to include 0, 10, 20, 30, 40, 50 and 60 second stimulations (with "0 seconds" being where IL-8 was added immediately before washing by centrifugation). The time-courses were of lower quality than pPI3K or $pAkt_{T308}$ but optimal expression occurring at 20 seconds. Therefore, neutrophils were stimulated for 20 seconds on subsequent assays with $pAkt_{T308}$. An example blot from a time-course for $pAkt_{T308}$ is shown in Figure 4.13.

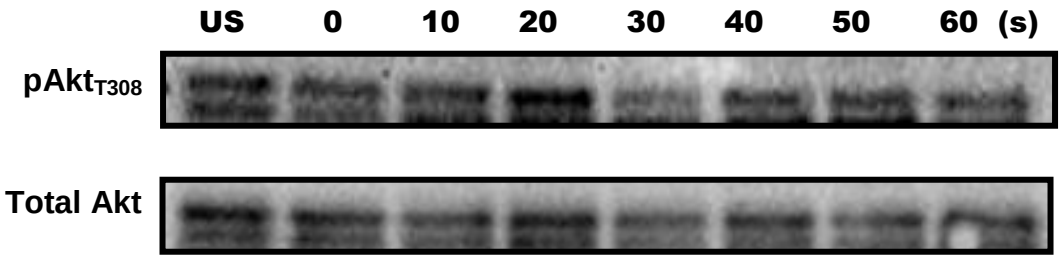

Figure 4.13: Akt phosphorylation at T308 over a time-course of IL-8 stimulation.

Legend: A western blot of $pAkt_{T308}$ and total Akt from neutrophil lysates from unstimulated neutrophils and those stimulated with 100 nM IL-8 over a 60 second time-course (stimulation time in seconds is indicated at the top. US = Unstimulated). The image is representative of 3 separate experiments.

Initial western blots, including the time-courses were unreliable and often of very poor quality. Therefore, a number of modifications were made to the lysis protocol, most importantly the rapid cooling of neutrophil suspensions post-stimulation with ice-cold PBS (to halt cellular processes) and the addition of MOPS-based lysis buffer (with additional

phosphatase inhibitors that further prevent the breakdown of the enzymes of interest) before boiling with the SDS buffer. Following these modifications, results were far more reliable and reproducible.

4.4.2 PI3K$_{T458}$ Phosphorylation

Baseline PI3K$_{T458}$ phosphorylation was relatively high in unstimulated (RPMI control) neutrophils for both healthy control subjects and COPD patients. There was a trend towards higher phosphorylation in COPD patients under all stimulatory conditions, though it was only significantly higher in COPD following stimulation with GROα. A typical pPI3K$_{T458}$ western blot is shown in Figure 4.14 and the collated data for 6 COPD patients and 6 age-matched healthy control subjects are summarised graphically in Figure 4.15.

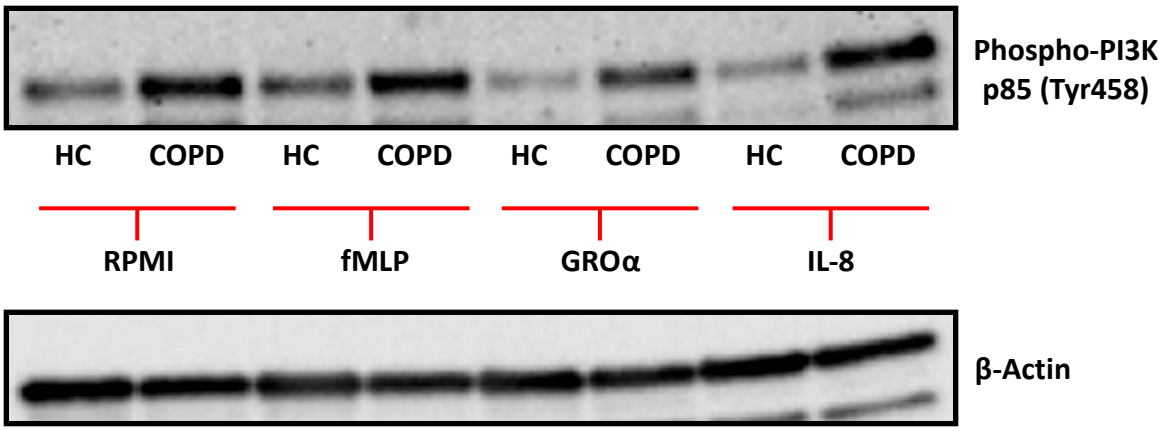

Figure 4.14: A representative western blot for pPI3K$_{T458}$.

Legend: A western blot from phospho-PI3K in lysates of unstimulated neutrophils (RPMI control) and those stimulated with fMLP, GROα and IL-8 from a Healthy Control (HC) subject and a patient with COPD. The blot is representative of 6 separate experiments.

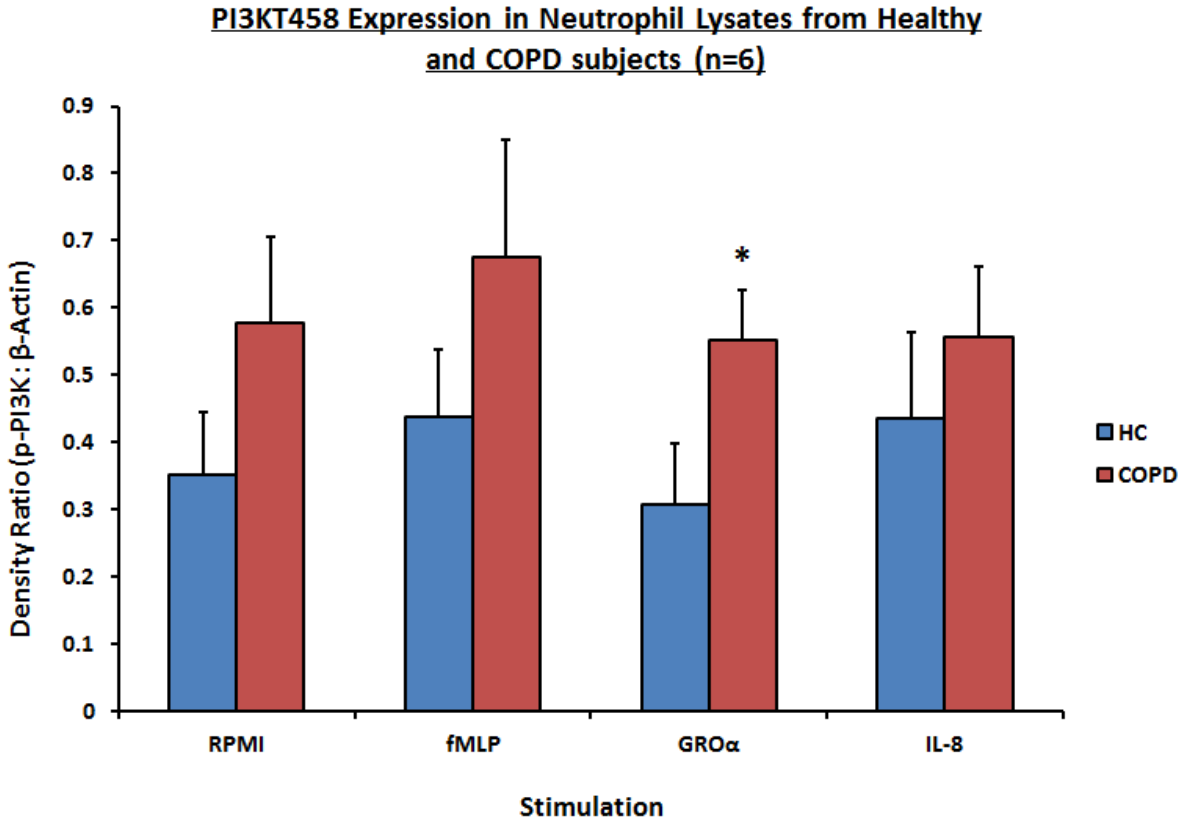

Figure 4.15: $PI3K_{T458}$ phosphorylation in neutrophils from healthy and COPD subjects.

Legend: COPD and healthy neutrophils were either unstimulated (RPMI control) or stimulated for 3 minutes with fMLP (100 nM), GROα (10 nM) and IL-8 (100 nM). Density ratio was calculated by dividing the optical density of $pPI3K_{T458}$ by that of the loading control, β-Actin. Data are mean and SEM (***** indicates phosphorylation is significantly higher in COPD compared to Healthy Control (HC) with the same stimulus ($p \leq 0.05$)). (Analysed by unpaired Mann-Whitney U test).

4.4.3 Akt_{S473} Phosphorylation

Baseline Akt_{S473} phosphorylation was relatively low in unstimulated (RPMI control) neutrophils for both healthy control subjects and COPD patients. Akt_{S473} phosphorylation increased significantly with fMLP, GROα and IL-8 stimulation in healthy control neutrophils and with fMLP and IL-8 stimulation in COPD neutrophils. However, there was no difference in Akt_{S473} phosphorylation between COPD and healthy control neutrophils under any experimental condition. A typical $pAkt_{S473}$ western blot is shown in Figure 4.16 and the collated data for 6 healthy and 6 COPD subjects are summarised graphically in Figure 4.17.

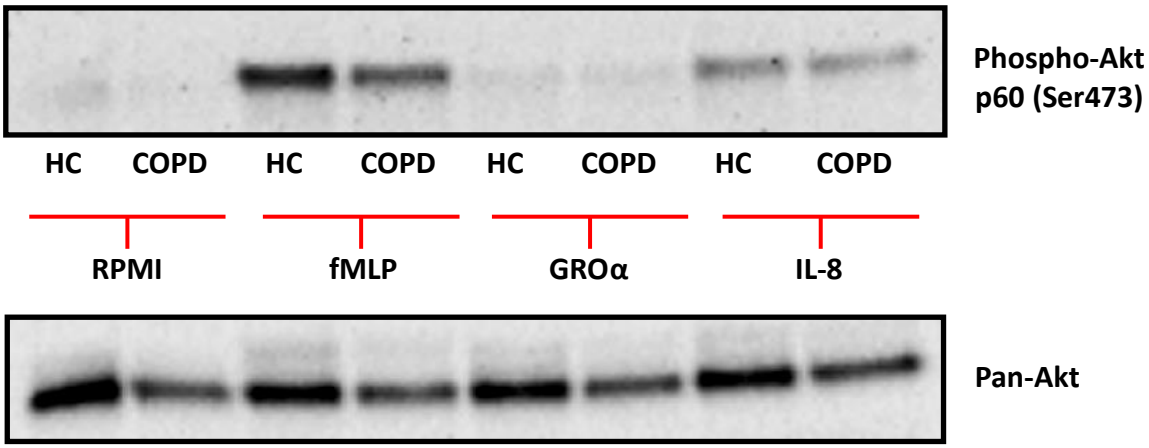

Figure 4.16: A representative western blot for $pAkt_{S473}$.

Legend: A western blot of $pAkt_{S473}$ from lysates of unstimulated neutrophils (RPMI control) and those stimulated with fMLP, GROα and IL-8 from a Healthy Control (HC) subject and a patient with COPD. The blot is representative of 6 separate experiments.

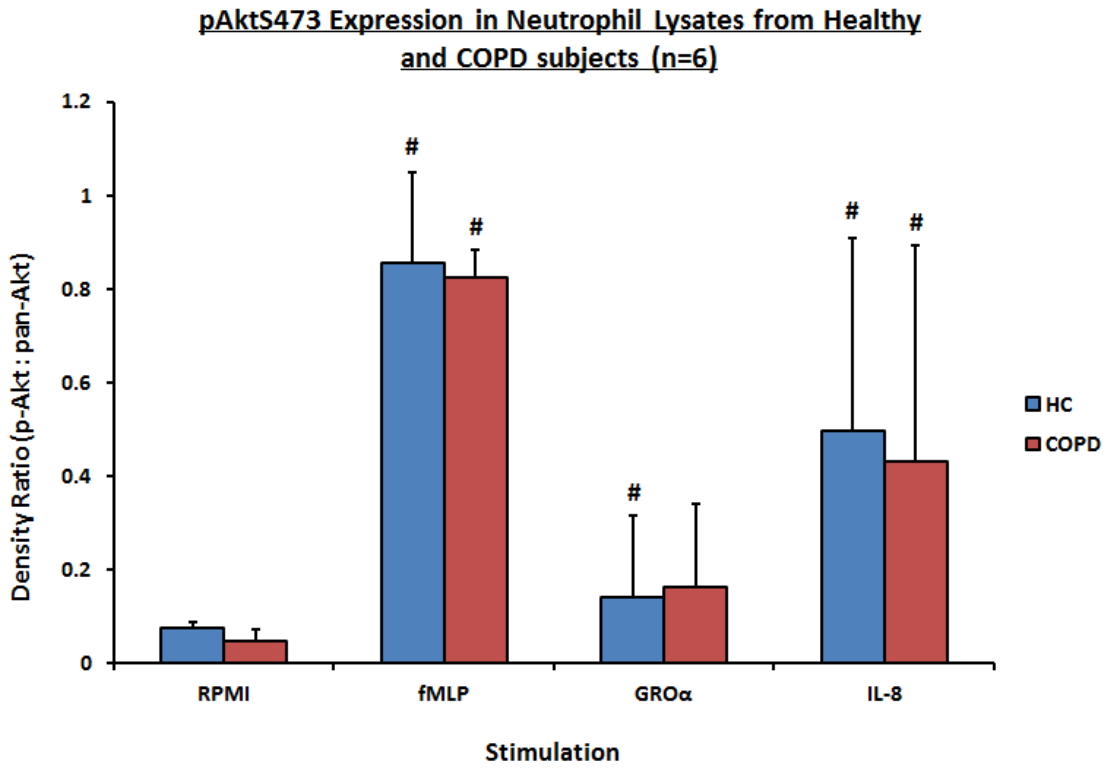

Figure 4.17: Akt phosphorylation at S473 in neutrophils from healthy and COPD subjects.

Legend: COPD and healthy control (HC) neutrophils were either unstimulated (RPMI) or stimulated for 3 minutes with fMLP (100 nM), GROα (10 nM) and IL-8 (100 nM). Density ratio was calculated by dividing the optical density of phospho-Akt by that of the loading control, Pan-Akt. Data are displayed as median and IQR (# indicates phosphorylation is significantly higher in stimulated cells compared to unstimulated cells of the same cohort ($p \leq 0.05$)). (Analysed by unpaired Mann-Whitney U test).

4.4.5 Akt_{T308} Phosphorylation

Baseline Akt_{T308} phosphorylation was comparatively high in unstimulated (RPMI control) neutrophils for both healthy control subjects and COPD patients. This did not increase significantly when neutrophils were stimulated with fMLP, GROα or IL-8 in health or COPD. Furthermore, no significant differences in Akt_{T308} phosphorylation were observed when comparing health to COPD. A typical $pAkt_{T308}$ western blot is shown in Figure 4.18 and the collated data for 3 healthy and 3 COPD subjects are summarised graphically in Figure 4.19.

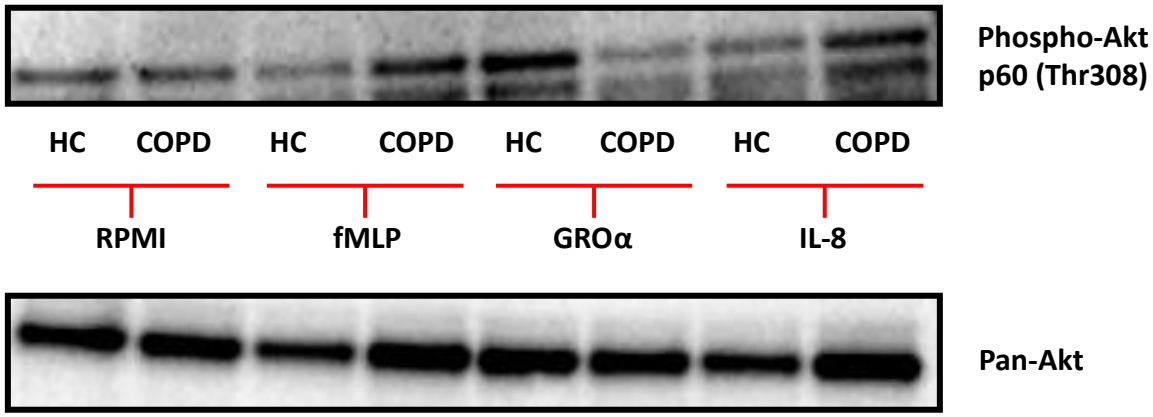

Figure 4.18: A representative western blot for $pAkt_{T308}$.

Legend: A western blot of $pAkt_{T308}$ from lysates of unstimulated neutrophils (RPMI) and those stimulated with fMLP, GROα and IL-8 from a healthy control (HC) subject and a patient with COPD. The blot is representative of 3 separate experiments.

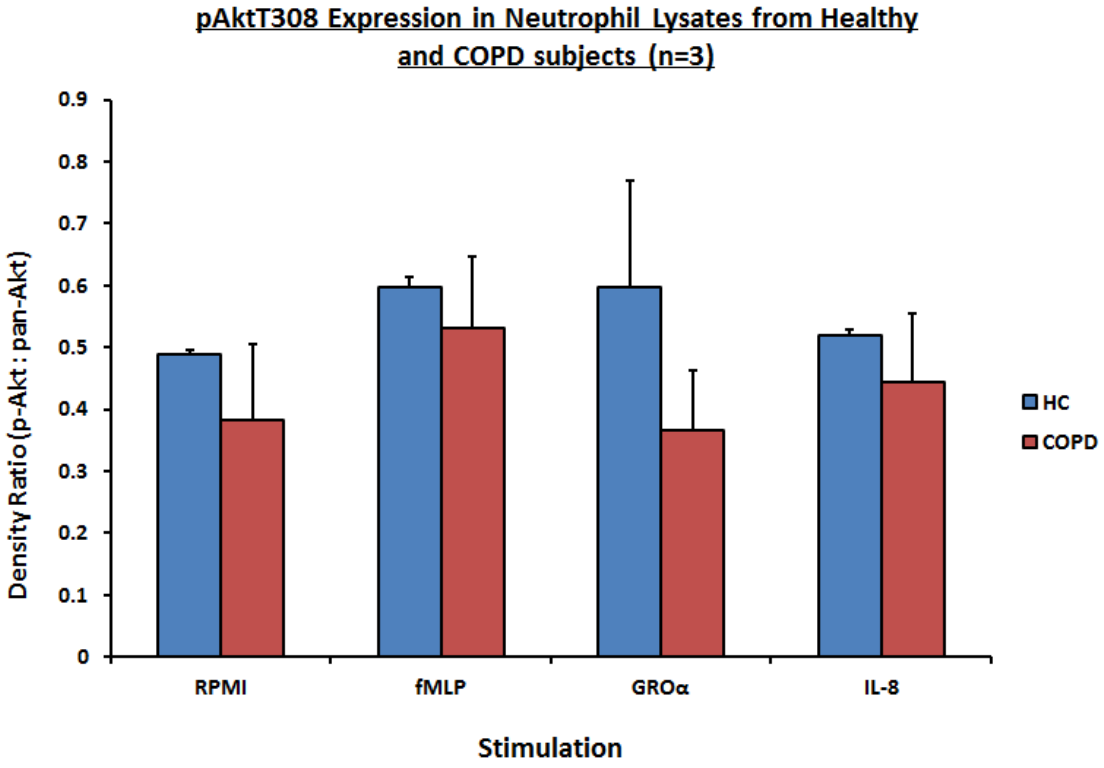

Figure 4.19: Akt phosphorylation at T308 in neutrophils from healthy and COPD subjects.

Legend: COPD and healthy neutrophils were either unstimulated (RPMI) or stimulated for 3 minutes with fMLP (100 nM), GROα (10 nM) and IL-8 (100 nM). Density ratio was calculated by dividing the optical density of phospho-Akt by that of the loading control, Pan-Akt. Data are displayed as median and IQR. No significant differences were observed between comparisons of Akt_{T308} phosphorylation between COPD and health. (Analysed by unpaired Mann-Whitney U test).

4.5 INTRACELLULAR CALCIUM SIGNALLING

Intracellular calcium release following stimulation with IL-8 was measured by fluorimetry in neutrophils from age-matched healthy subjects, patients with COPD and patients with α_1-ATD (n=10). Both peak calcium release (increase from baseline) and total

calcium release (area under the curve) were calculated for each patient. As expected, the addition of IL-8 caused a rapid increase in neutrophil intracellular calcium in every subject and there was only modest variability in the initial surge of calcium release and the length of time it took for the intracellular calcium to return to basal levels. There was no significant difference in the peak calcium release between the three cohorts (Figure 4.20). Furthermore, the total calcium release was also comparable between groups (Figure 4.21).

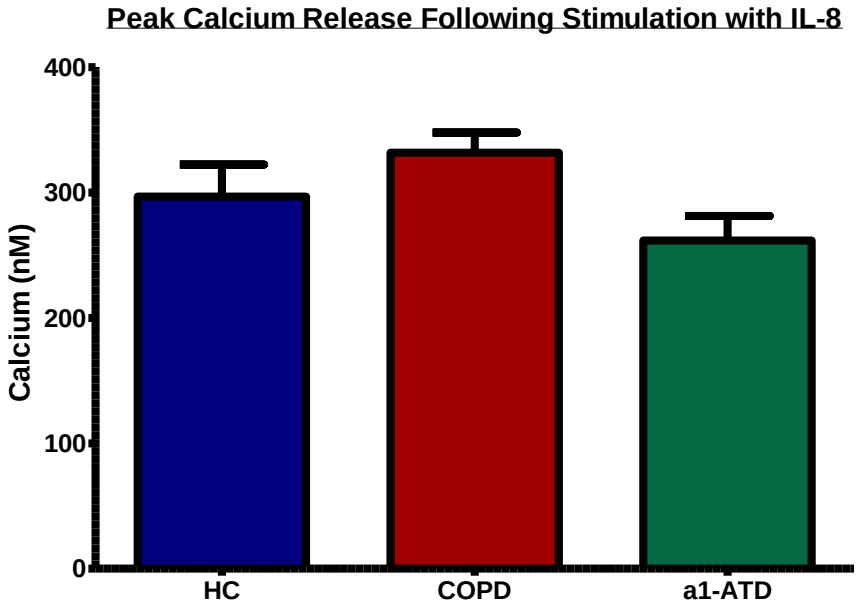

Figure 4.20: Peak Intracellular calcium release following stimulation with IL-8.

Legend: Neutrophils from healthy, COPD and α_1-ATD subjects were stimulated with 100 nM IL-8 to induce calcium release from intracellular stores. Peak calcium release was taken as the highest value achieved. Data are mean and SEM (n=10). (Analysed by one-way ANOVA).

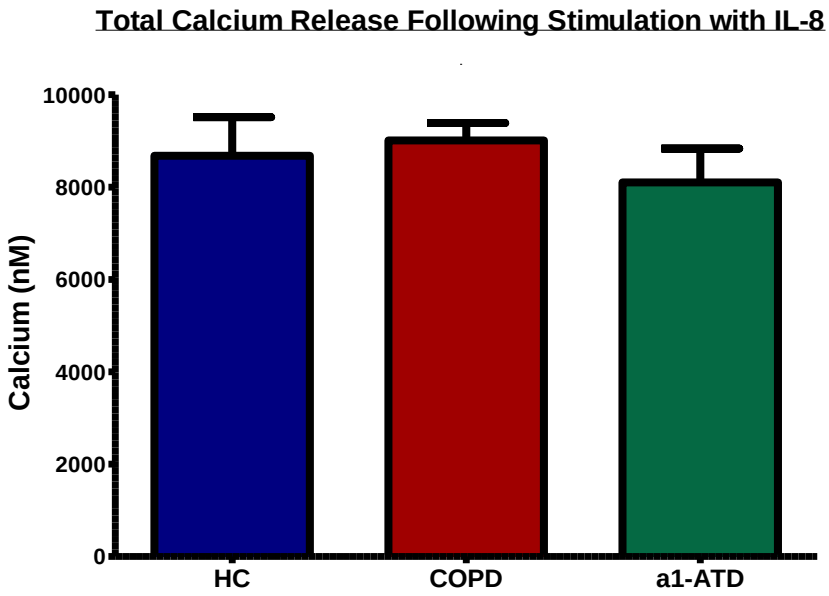

Figure 4.21: Total Intracellular calcium release following stimulation with IL-8.

Legend: Neutrophils from healthy, COPD and α_1-ATD subjects were stimulated with 100 nM IL-8 to induce calcium release from intracellular stores. Total calcium release was derived from the area under the curve. Data are mean and SEM (n=10). (Analysed by one-way ANOVA).

4.6 DISCUSSION

4.6.1 Quiescent Chemokine Receptor Expression

To date, there have been a number of studies into the function of CXCR1- and CXCR2-mediated neutrophil migration, although the specific role of these receptors in chemotaxis is still not clearly defined. In addition, there have been a few studies into the role of CXCR1 and CXCR2 in neutrophil migration toward IL-8 but results are also conflicting and definitive conclusions cannot be drawn. Some studies suggest CXCR2 is not necessary for

IL-8 mediated migration (Loetscher et al. 1994, Godaly et al 2000) while others conclude that CXCR2 is important (Chuntharapai et al. 1995, Ben-Barach et al. 1997). One recent review (Stillie et al. 2009) proposed that both receptors may be capable of stimulating chemotaxis but, as CXCR2 responds to lower concentrations of IL-8 (as well as other chemoattractants) and is internalised/recycled more quickly than CXCR1, it may play a more significant role in migration at further distances from the chemoattractive source. In contrast, CXCR1 is not as sensitive to IL-8 and internalised more slowly but ultimately produces a broader range of physiological responses (such as ROS production) and may be more important nearer the site of inflammation for antimicrobial defence. Moreover, desensitisation of CXCR2 by CXCR1 at the site of inflammation may slow neutrophil migration (Stillie et al. 2009), which could theoretically facilitate more efficient bacterial killing. Currently, there have been no studies investigating neutrophil expression of LTB_4, fMLP or C5a receptors expression in COPD.

Initially, this chapter compared surface expression of CXCR1, CXCR2, FPR1, LTB_4R and C5aR on quiescent neutrophils from healthy subjects, patients with COPD and patients with α_1-ATD. A significant reduction in the surface expression of CXCR1 and C5aR on the neutrophils of COPD patients was seen compared to those from healthy controls. There was also a trend towards reduced expression of CXCR2 in COPD. No differences were observed between chemokine receptor expression in health and α_1-ATD.

During the stimulatory time-course experiments, surface expression of CXCR1, CXCR2 and FPR1 on quiescent neutrophils was also compared. In agreement with the initial data, CXCR1 expression was significantly lower on COPD neutrophils compared to those from

healthy controls. CXCR1 was also significantly lower on COPD neutrophils compared to those from patients with α_1-ATD, which was not observed in the previous experiments. No differences were observed in CXCR2 expression between subject groups, which is again at variance with the previous experiments, where expression was significantly lower on COPD neutrophils compared to α_1-ATD. No significant differences in FPR1 expression were observed, which is in accordance with the previous work.

The discrepancies between these data and the first set of experiments conducted highlight the fact that there is a large variability in published results. There have been a number of studies comparing expression of CXCR1 and CXCR2 on quiescent neutrophils from COPD and healthy subjects, although this is the first study to include patients with α_1-ATD. One recent study reported no difference in either CXCR1 or CXCR2 quiescent surface expression in COPD (Sapey et al. 2011). Pignatti et al. reported no difference between surface expression of CXCR1 in COPD neutrophils compared to those from healthy controls but a decrease in CXCR2 expression (Pignatti et al. 2005). In contrast, Yamagata et al. reported no difference in CXCR2 expression but an increased expression of CXCR1 in COPD neutrophils (Yamagata et al. 2007). It is also important to note that the processes involved in neutrophil isolation may also activate a proportion of neutrophils within the studied population (although cytospin images suggest this was minimal). In retrospect, it may have been better to perform this assay using whole blood and gating around the neutrophil population.

The conflicting results of these studies make it difficult to draw definitive conclusions, though it is reassuring that a lower expression in CXCR1 on COPD neutrophils compared to

those from healthy subjects but similar expression of CXCR2 and FPR1 was observed in two independent experiments. Unfortunately, it was not possible to pool these data as the two experiments were conducted on different flow cytometers following laboratory relocation.

4.6.2 Chemokine Receptor Expression Following Stimulation

Studies into chemokine receptor expression to date have only investigated expression on quiescent neutrophils. While lower quiescent receptor expression may indicate a possible mechanism for aberrant neutrophil migration in COPD, it is impossible to draw conclusions based on this, as migrating neutrophils are not quiescent. Surface receptor expression in activated neutrophils is governed by rates of internalisation and recycling as well as any receptor shedding that may occur. Therefore, it is important to know whether or not these differences in quiescent CXCR1 expression endure following cell activation and whether new differences (for instance in CXCR2 and FPR1) may arise.

Neutrophils were stimulated neutrophils over a 2 hour time-course with fMLP and IL-8 and surface expression of CXCR1, CXCR2 and FPR1 was measured during cell activation. These two chemokines and their respective receptors were chosen over the other chemokines initially investigated (C5a, GROα and LTB_4) as assessment of receptor recycling with all five chemokines was not feasible. Also, IL-8 is a potent endogenous chemoattractant and the majority of previous work in this area has included CXCR1 and CXCR2. fMLP is a bacterial peptide and also a potent activator of neutrophils. Like many chemokines, its levels are elevated during COPD exacerbations (Saetta et al. 2001).

Both CXCR1 and CXCR2 surface expression decreased over time in all patient groups, indicating that either receptor internalisation or shedding was most likely occurring. CXCR1 expression decreased in a more linear fashion than CXCR2, which decreased very rapidly in all patient groups and plateaued after 15 minutes. The only other mechanism by which surface receptor expression can decrease is by shedding. Therefore, CXCR1 and CXCR2 shedding was quantified by ELISA. CXCR1 shedding was negligible at 30 and 120 minutes post-stimulation in both health and COPD, suggesting that the dominant process of CXCR1 removal from the cell surface following IL-8 stimulation was internalisation. A modest amount of CXCR2 shedding was detected but was comparable in both cohorts. It has previously been demonstrated that stimulation of neutrophils with other inflammatory cytokines (such as TNFα) can induce CXCR2 shedding (Asagoe et al. 1998). The data in this chapter suggest that CXCR2 is shed more readily than CXCR1 following stimulation with IL-8. Receptor recycling was confirmed by removal of the IL-8 stimulus and quantification CXCR1 and CXCR2 expression 90 minutes later. Surface expression of both receptors increased on COPD and healthy neutrophils, albeit not back to baseline levels observed on quiescent cells, suggesting recycling and re-expression was occurring at a relatively slow rate.

The initial difference observed in CXCR1 expression on quiescent neutrophils between COPD and healthy controls was rapidly abolished (within 5 minutes) following stimulation with IL-8. CXCR1 expression remained significantly higher in α_1-ATD than COPD for slightly longer (15 minutes), which could potentially explain why more accurate chemotaxis was observed with α_1-ATD neutrophils in the presence of IL-8. CXCR2 expression decreased

at the same rate in all patient groups and no significant differences in surface expression were observed at any time-point.

FPR1 surface expression was relatively low on quiescent neutrophils from COPD patients, healthy controls and patients with α_1-ATD. Activation supposedly induces a dramatic and rapid increase in FPR1 expression via the mobilisation of internal secretory vesicles (Sengelov et al. 1994, Kitchen et al. 1996, Borregard et al. 1997). Therefore, it was surprising to see that FPR1 surface expression did not increase at any stage of the time-course in any patient group. It could be that a large number of internal FPR1 receptors are mobilised to the cell surface before 5 minutes, bind rapidly to fMLP before becoming internalised once more, which may be demonstrated by including additional time points before 5 minutes. Alternatively, a more steady supply of FPR1 receptors may be expressed from internal stores and, again, internalise very rapidly after binding to fMLP. Both mechanisms would account for a relatively constant but low expression of FPR1 on the neutrophils surface during stimulation with fMLP. The latter is less likely based on previously published data showing upregulation of fMLP receptors following stimulation, although the authors used PAF as a stimulant rather than fMLP (Sengelov et al. 1994, Kitchen et al. 1996). The fact that expression (albeit low) was detected in quiescent neutrophils (in two independent assays using two different supplies of FPR1 antibody) suggests that the antibody was most likely reliable. However, it is possible that the fMLP was unreliable or could have degraded in storage, meaning the ability to sufficiently stimulate neutrophils could have been compromised. Therefore, it would be logical to repeat this experiment with fresh fMLP, fresh FPR1 antibody additional and time points before 5 minutes to confirm or refute our observation.

To our knowledge, no previous studies have compared the difference in surface chemokine receptor expression on neutrophils from COPD patients and healthy controls in the presence of a stimulus. One study (Pignatti et al. 2005) compared CXCR1 and CXCR2 expression on neutrophils in the peripheral blood and sputum of COPD patients. They identified a significantly lower expression in sputum neutrophils compared to those in the peripheral blood. In effect, this is somewhat of a similar comparison between quiescent neutrophils (in the blood) and activated neutrophils (that have migrated to the airway) that seems to support findings in this study. It is plausible that down-regulation of surface receptors for endogenous chemokines during migration to an inflammatory site (such as the lungs in COPD) may be important for the sensing of and ability to follow gradients of bacterial chemokines such as fMLP at the site of inflammation. It would be interesting to determine whether or not surface expression of FPR1 is up-regulated on neutrophils following stimulation with IL-8 alone.

4.6.3 Receptor Localisation

The final question this chapter sought to answer regarding chemokine receptors was whether or not these receptors were localising to the leading edge of COPD neutrophils as effectively as would be expected for healthy neutrophils. Therefore, CXCR1 and CXCR2 localisation was investigated on adhered neutrophils following stimulation with fMLP in an assay adapted from a recent study by Mondal et al. (Mondal et al. 2012). Initially, the FITC-stained neutrophils did not fluoresce to a large degree but the addition of Anti-FITC Alexa Fluor 488 resulted in markedly higher fluorescence and clearer images. Although the

PE-labelled neutrophils were relatively clear, it may also have been useful to use an Anti-PE Alexa Fluor to increase the PE fluorescence further.

Unfortunately, densitometric quantification of the receptor expression by pixilation brightness did not seem applicable due to the high variability of CXCR1 and CXCR2 expression observed in the initial flow cytometry assays (between individuals within the same cohort as well as differential expression between COPD and health). Therefore, this method is perhaps a relatively crude means of assessing receptor localisation. However, clear differences in receptor localisation were observed between quiescent neutrophils (where receptors were more uniformly distributed) and stimulated, polarised neutrophils (where receptors localised to one side of the cell) for both COPD and healthy neutrophils. Moreover, the results demonstrate that CXCR1 and CXCR2 appeared to localise to the leading edge of stimulated neutrophils as effectively in COPD as they do in health.

4.6.4 PI3K and Akt Phosphorylation

The PI3K pathway has been implicated in a number of neutrophil inflammatory functions, including cell migration (Knall et al. 1997, Sasaki et al. 2000, Sadhu et al. 2003). Therefore, it was hypothesised that increased activation of key effector proteins such as PI3K and Akt, detected by protein phosphorylation, may account for the aberrant migration of COPD neutrophils.

Ideally, investigation into PI3K phosphorylation and activation in relation to GPCR-mediated neutrophil migration would have included the assessment of the p84/p101 regulatory subunits and, more importantly, the p110 catalytic subunit of PI3Kγ. However, at the time of experimentation, there were no validated phosphospecific antibodies for these

subunits and the only robustly validated antibody was for the Class 1a regulatory subunit, p85 at tyrosine 458 ($PI3K_{T458}$). The biological significance of Tyr458 phosphorylation on PI3K function is currently unknown, although phosphorylation of p85 at serine 83 (by Protein Kinase A) has been shown to influence the interaction of the SH3 domain with binding partners (Cosentino et al. 2007) and it is possible that similar functional modifications could occur through Tyr458 phosphorylation. Therefore, it is important to note that the assessment of $pPI3K_{T458}$ was a pilot study that may be indicative of potential differences in the function of this family of kinases in COPD neutrophils.

A relatively high $PI3K_{T458}$ phosphorylation was detected in unstimulated neutrophils from both healthy controls and COPD patients. Phosphorylation did not increase in either subject group following stimulation by fMLP, GROα or IL-8. While this may seem surprising, published data from our group support these results. Young healthy subjects had low $PI3K_{T458}$ phosphorylation in unstimulated neutrophils, which increased significantly upon stimulation with IL-8, whereas $PI3K_{T458}$ phosphorylation was elevated in unstimulated neutrophils from old healthy subjects and did not increase upon stimulation (Sapey et al. 2014). This suggests that hypothetical neutrophil PI3K over-activity in the elderly may be an independent phenomenon which, once again, highlights the importance of age-matching healthy control groups to COPD patients. In addition, there was a trend towards even higher $PI3K_{T458}$ phosphorylation in both unstimulated and stimulated COPD neutrophils, although the majority of these comparisons failed to reach statistical significance. With greater power, it is possible that all of these comparisons between COPD and health may achieve statistical significance. As the regulatory subunits can influence the stability, localisation and function

of the catalytic subunits, it is possible that dysregulation of the phosphorylation state of different p85 subunits may influence the overall function of Class 1a PI3Ks. Indeed, there have been two studies that have described splice mutations of the PIKR31 gene (that encodes p85α) in lymphocytes that lead to over-activity of PI3K, alterations in lymphocyte structure/function and immunodeficiency (Deau et al. 2014, Lucas et al. 2014). It is possible that this phenomenon could be mirrored in neutrophils, although it is highly speculative to infer that a similar effect may be occurring in the Class 1b PI3Kγ (which signals through the GPCRs involved in chemokine-mediated neutrophil migration). However, the $pPI3K_{TYR458}$ data presented in this thesis suggest that there could be some differences in the phosphorylation and activity of the PI3K family of kinases in COPD and certainly warrants further investigation. Future studies should include assessment of the phosphorylation of the p84/p101 regulatory and p110 catalytic subunits of PI3Kγ. If increased phosphorylation of one or more of these subunits is observed, it could provide a possible reason for the dysfunctional neutrophil migration observed in COPD. Further to this, potential mutations in the genes that encode these subunits could be investigated with whole-exome sequencing, which may identify a genetic cause behind any PI3K over-activity in COPD neutrophils.

The improvement in migratory accuracy with the non-selective PI3K inhibitor LY29004 previously observed (Sapey et al. 2011) infers that PI3K activity could potentially be increased in COPD. Our pilot $PI3K_{T485}$ data demonstrating increased phosphorylation at p85 support this hypothesis, although the information is limited. Increased phosphorylation of PI3K regulatory subunits in quiescent neutrophils could result in a more pronounced association with the plasma membrane and, potentially, augment PIP_3 formation once the catalytic subunits are also phosphorylated upon stimulation. However, this is speculative and

the fact that phosphorylation of the catalytic subunits of Class 1a PI3Ks and, most importantly, the catalytic/regulatory subunits of Class 1b PI3Kγ were not investigated means that the potential alteration of PI3Kγ phosphorylation during GPCR-mediated neutrophil migration could not be truly evaluated. Although our data suggest the phosphorylation of the p85 regulatory subunit is maintained in COPD, it may have been useful to determine if there was a differential expression of the PI3Kδ and, particularly, the PI3Kγ isoforms between health and COPD, as lower PI3Kγ (with either p84 or p101 regulatory subunits) expression may explain why COPD neutrophils were migrating with poor accuracy in chemokines that signal through GPCRs.

In contrast, Akt phosphorylation at the serine 473 site was low in unstimulated neutrophils from both healthy subjects and COPD patients and increased significantly following stimulation with fMLP and IL-8 in both subject groups. A small, but significant increase was seen in healthy neutrophils following stimulation with GROα but this was not seen in COPD. These data help also validate the results for pPI3K, as identical methods were used for stimulation, freezing and western blotting, indicating that the absence of increased PI3K phosphorylation post-stimulation does not seem to be due to methodological limitations. The western blots for Akt phosphorylation at threonine 308 were of poorer quality, which made analysis more difficult and interpretation less clear. This was most likely due to an unreliable primary antibody, which resulted in a high degree of irrelevant binding and made densitometric analysis of the $pAkt_{T308}$ protein bands difficult. It may be expected that a hypothetical loss of p85-mediated catalytic subunit activity of PI3K may increase the downstream signalling of Akt. However, our data suggest that differences in $PI3K_{T458}$

phosphorylation do not appear to relate to Akt phosphorylation (particularly basal levels) and, although Akt is important in cell migration, its activity may not explain the aberrant neutrophil migration observed in COPD. However, with low power (and poor quality western blots for Akt_{T308}), it is difficult to draw definitive conclusions and western blots with larger cohorts and more reliable pAkt antibodies seem warranted. Unfortunately, this experiment was conducted at the end of the thesis and we were unable to repeat it with a different $pAkt_{T308}$ antibody within the timeframe.

Achieving good quality western blots with neutrophils has proved somewhat difficult. Initially, no reliable results were obtained although, with methodological adjustments, clearer blots were ultimately achieved for the majority of the antibodies. Following these amendments, protein bands were clear (with no evidence of proteolysis) but they were still often faint, which may have resulted from a number of factors. Firstly, neutrophils contain high levels of proteinases, which may not be adequately controlled during the lysis protocol (despite the use of protease inhibitors), giving a weaker signal. Secondly, protein phosphorylation could be more transient than the time-courses suggest (particularly in the case of Akt) and the peak phosphorylation time may also be variable between subjects. However, for $pPI3K_{T458}$ and $pAkt_{S473}$, phosphorylation was detected at the majority of time-points throughout the time-course. Lastly, the primary antibodies may not be particularly reliable. Although irrelevant binding was minimal for $pPI3K_{T458}$, this was not the case for pAkt (particularly for T308), which made densitometric quantification of Akt phosphorylation difficult. Stimulation at room temperature could have been a contributing factor to some of the poor responses observed and 37ºC may have been more suitable and enhanced phosphorylation. Other studies have observed clear phosphorylation response to

fMLP with both Akt_{S437} and Akt_{T308} at 37ºC (Yagi et al. 2007). However, a clear phosphorylation of Akt_{S437} as observed at room temperature and increased phosphorylation of $PI3K_{T458}$ following stimulation has been observed in young neutrophils at room temperature (Sapey et al. 2014). Nevertheless, repeating these assays at 37ºC may be important for future experiments.

It is also important to note that these assays were all performed on neutrophils in suspension, which is unlikely to fully replicate the conditions of migration. In hind sight, it may have been more suitable to perform these assays on adhered neutrophils that had been stimulated with a gradient of chemoattractant, rather than non-adhered neutrophils that had chemoattractant simply added to the medium.

In summary, the data shown here suggest that PI3K (but not Akt) phosphorylation may be increased in COPD neutrophils compared to those from healthy controls. Although pPI3K activity cannot be deduced from western blots, this could potentially be a mechanism of aberrant neutrophil migration in COPD.

4.6.5 Intracellular Calcium Signalling

As previously mentioned (Section 1.2.3), current evidence suggests that intracellular calcium signalling may be critical for cell polarisation and/or chemotaxis. However, the precise mechanisms by which calcium is involved in migration have yet to be elucidated. Studies also suggest that calcium transients are only required for neutrophil migration *in vitro* on certain substrates. On fibronectin and vitronectin, which is β1- and β3 integrin-dependant, increases in calcium seem to be required for the detachment of these integrins from the uropod so they may be recycled back to the leading edge (Marks et al. 1990). In contrast,

neutrophil migration on albumin coated glass seems to rely predominantly on β2 integrins (Sixt et al. 2001) and may not require calcium (Marks et al. 1991, Mandeville et al. 1996). However, if the aberrant neutrophil migration observed in COPD is a general phenomenon that occurs during all types of migration *in vivo*, calcium signalling may still be involved.

Here real-time Ca^{2+} release from intracellular stores following stimulation with IL-8 was analysed in COPD patients, healthy control subjects and patients with α_1-ATD. Initially, this assay was very unreliable, primarily due to unstable fluorescence signals (even at baseline). However, by resuspending the neutrophils in calcium-containing HBSS (rather than HBSS alone), signals were far more stable and consistent results were obtained. The disadvantage of suspending neutrophils in calcium-containing HBSS is that they could absorb small amounts of calcium from the medium. This resulted in a very slight increase in the fluorescence at the end of the experiment (following ionomycin and subsequent EGTA administration) compared to baseline. To compensate, the value at the end of the test was taken as the "baseline" value and applied this to the calculation of peak and total calcium release. Although not ideal, the difference in values was minimal and total calcium release (area under the curve) could not be calculated without this modification.

There were no differences in either peak (initial "burst") or total calcium release, indicating that IL-8-induced calcium signalling is unaltered in COPD *in vitro*. To our knowledge, there have been no comparisons between calcium signalling in neutrophils from COPD patients and healthy control subjects. However, regardless of its potential involvement in cell migration, the data here suggest that altered calcium signalling cannot be implicated in the aberrant neutrophil migration in COPD.

4.6.6 Summary

The data in this chapter indicate that the aberrant neutrophil migration in COPD seems unrelated to chemokine receptor expression and localisation on activated neutrophils, regardless of differential expression on quiescent cells. This suggests that downstream signalling is the most likely cause of the chemotactic dysfunction of COPD neutrophils. Intracellular calcium signalling was unaltered, but a clear trend towards increased PI3K (though not Akt) phosphorylation was observed. This suggests that the PI3K pathway may be a suitable target for pharmacological correction of the aberrant neutrophil migration in COPD.

CHAPTER 5

PHARMACOLOGICAL CORRECTION OF ABERRANT NEUTROPHIL MIGRATION

5.1 BRIEF INTRODUCTION

The studies described previously have demonstrated that COPD neutrophils migrate with increased speed but reduced accuracy. This is likely to have implications in the pathophysiology of COPD as, if neutrophils migrate inaccurately through the extracellular matrix, their migratory tracks will be much longer, leading to an amplification of parenchymal destruction by the proteinases released during neutrophil transmigration (Sapey et al. 2014). It is also possible that the ability of neutrophils within the airspaces to locate and kill invading pathogens may also be impaired, which could lead to increased bacterial colonisation and, ultimately, an increase in exacerbation frequency. Current therapies for the treatment of COPD are limited and are predominantly aimed at the short-term relief of symptoms through the improvement of pulmonary ventilation and a reduction of the work of breathing by bronchodilation (Gold 2007). The search for novel anti-inflammatory therapies is ongoing but has, to date, been largely unsuccessful. Theoretically, dysfunctional neutrophil migration may account for part of the pathogenesis of COPD. Therefore, correction of this phenomenon could potentially slow the disease and reduce mortality.

With any medical research, the ultimate goal should be to further the understanding of pathological processes so that novel treatments may be developed. The data presented here indicate that the aberrant neutrophil migration in COPD appears to be an intrinsic cell defect. Therefore, its pharmacological correction should ideally be aimed specifically at neutrophil function rather than a more systemic anti-inflammatory approach. The migratory dysfunction in COPD does not seem to be related to chemokine receptor availability or localisation, in spite of lower surface expression of some receptors on quiescent neutrophils (although this does not necessarily mean that manipulation of chemokine receptor function could not

improve chemotaxis). Therefore, it seems logical to infer that it must be due to a dysfunction in downstream signalling.

Intracellular calcium signalling was unaltered in COPD. However, a trend towards overexpression of pPI3K (but not pAkt) was observed in neutrophils from COPD patients. These data, together with previous work that demonstrated an improvement in migratory accuracy of COPD neutrophils with non-specific PI3K inhibitors (Sapey et al. 2011), highlight the PI3K pathway as an attractive target for therapeutic intervention. Furthermore, the accumulation of PIP_3 at the leading edge of migrating neutrophils provides additional evidence for the involvement of PI3K in neutrophil chemotaxis. However, PI3K is associated with numerous cellular functions, including growth, proliferation, differentiation, motility and survival, meaning non-specific inhibition *in vivo* may result in undesirable side-effects. The γ- and δ-isoforms are more specific to leukocytes, and PI3Kγ in particular is known to be intimately associated with cell migration following stimulation by a number of chemokines and evidence suggests it is necessary for directionality during migration (Hannigan et al. 2002). PI3Kδ involvement is less well described, though it may aid in the sustainment of a chemokine-mediated response (Condliffe et al. 2005). Therefore, it seemed logical that PI3Kγ and/or δ isoform inhibition could prove more therapeutically advantageous than broad range PI3K inhibitors in COPD, where neutrophil directionality is poor.

There has been a growing interest in the potential of PI3Kδ- and/or PI3Kγ-specific inhibitors, which have shown promise in general neutrophil inflammatory responses (Sadhu et al. 2003, Williams et al. 2010) as well as various disease models (Barber et al. 2005, Doukas et al. 2006). Furthermore, the use of these inhibitors in the treatment of respiratory disease such as asthma and COPD has also shown therapeutic potential (Finan et al. 2004,

Adcock et al. 2006, Lee et al. 2006, Ito et al. 2007, Krymskaya 2007), with one group showing that an aerosolised PI3Kγ/δ inhibitor had notable anti-inflammatory effects in a murine model of COPD (Doukas et al. 2010). Importantly, increased PI3Kδ activity as a result of gene mutation has been associated with many adverse physiological effects, including recurrent respiratory infections and progressive airway damage (Angulo et al. 2013). Therefore, if COPD is associated with a general increase in PI3K activity, selective isoform inhibition could potentially have a broader anti-inflammatory effect and even reduce exacerbation frequency. However, further work is required to understand how PI3Kγ and -δ inhibition affects neutrophil function in human COPD.

As mentioned previously (Section 1.2.3), PI3K is not the only kinase involved in neutrophil migration. The MAPKs, including Erk and p38, are involved in migration (Heit et al. 2007), the latter of which is indirectly involved in directionality by phosphorylation of MAPK-activated protein kinase 2/3 (Huang et al. 2004). However, it is currently unknown whether inhibition of these other kinases would also alter the migratory dynamics of neutrophils.

Although chemokine receptor expression may not be altered in migrating COPD neutrophils, it does not necessarily mean that their blockade could not influence neutrophil migration. Indeed, recent clinical trials in healthy subjects have shown that a CXCR2 antagonist (AZD8309) attenuated neutrophil airway recruitment following LPS challenge (Virtala et al. 2011, Leaker et al. 2013). Similarly, a dual CXCR1/2 antagonist has also been shown to inhibit neutrophil recruitment and also reduce mucus production and goblet cell hyperplasia in animal models of pulmonary inflammation (Chapman et al. 2007). However,

the precise effects of chemokine receptor inhibitors such as AZD8309 on the migratory dynamics of COPD neutrophils are currently unknown.

Another therapy that could potentially improve the migratory accuracy of COPD neutrophils is statin, which regulate small GTPase (Ras and Rho) activity further downstream of the PI3K pathway (Fromigue et al. 2006, Dai et al. 2007). As discussed previously (Section 1.5.9), studies into the effects of statins on neutrophil migration are contrasting, although reduced neutrophil influx into the lungs has been demonstrated in both mice and humans following statin treatment (Lee et al. 2005, Maher et al. 2009).

The studies in this chapter were designed to determine if pre-incubation with various pharmacological agents, including CXCR1/2 agonists, specific PI3K isoform inhibitors (α, β, δ, and γ), Erk and p38 inhibitors, or simvastatin could improve the migratory accuracy of neutrophils isolated from the peripheral blood of COPD patients. As the inhibition of CXCR1/2 does not block a generic pathway that can be activated by other chemoattractants, neutrophils could still transmigrate effectively *in vivo* towards other chemoattractants (such as LTB_4) in the sputum. Therefore, the inclusion of Sputum Sol in the CXCR1/ inhibitor assay was deemed essential. Importantly, we have previously shown that COPD neutrophils migrate with comparably poor accuracy in sputum gradients as they do in isolated chemoattractants (Sapey et al. 2011). Neutrophil migration was assessed using the Insall chamber as described previously in Section 2.5.1.

5.2 SUBJECT DEMOGRAPHICS

Demographic data for the COPD patients included in this chapter are shown in Table 5.1.

5.3 CXCR1 AND CXCR2 INHIBITION

Migratory dynamics were measured in neutrophils from 10 COPD patients in medium (RPMI-1640) and shallow gradients of 100 nM IL-8, 10 nM GROα and sputum sol phase (Sol). Migration was re-assessed in IL-8, GROα and Sol following incubation with a CXCR1 inhibitor (2 μg/ml), a CXCR2 inhibitor (50 μg/ml) or a dual CXCR1/2 inhibitor (SCH527123). SCH527123 was used at two concentrations; 3 nM (specific to CXCR2) and 42 nM (required for additional inhibition of CXCR1).

	COPD
N	35
Age (years)	63 (48 - 80)
Gender (Male:Female)	54
Smoking status	12 CS, 23 XS
Pack years	51 (3.5)
FEV1 % predicted	48.3 (4.5)
FEV1 /FVC %	48.3 (3.7)

Table 5.1: Subject Demographics.

Legend: A table showing the demographic data for all COPD patients included in this chapter. Data are normally distributed and presented as the mean and SEM, except the age which is mean (range). CS = Current smokers, XS = Ex-smokers.

5.3.1 CXCR1 Inhibition

Chemokinesis was relatively low in RPMI-1640 medium and increased significantly in gradients of IL-8, GROα and Sol. Pre-incubation with the CXCR1 inhibitor caused a significant reduction in chemokinesis in a gradient of IL-8 ($p<0.0001$) and a modest but significant reduction in chemokinesis in GROα ($p=0.029$). Chemokinesis was also significantly reduced when the COPD neutrophils were migrating towards Sol ($p<0.001$). These data are summarised in Figure 5.1.

Chemotaxis was negligible in medium alone and increased significantly in IL-8, GROα and Sol. However, the CXCR1 inhibitor had no effect on chemotaxis in any of these chemotactic gradients (Figure 5.2).

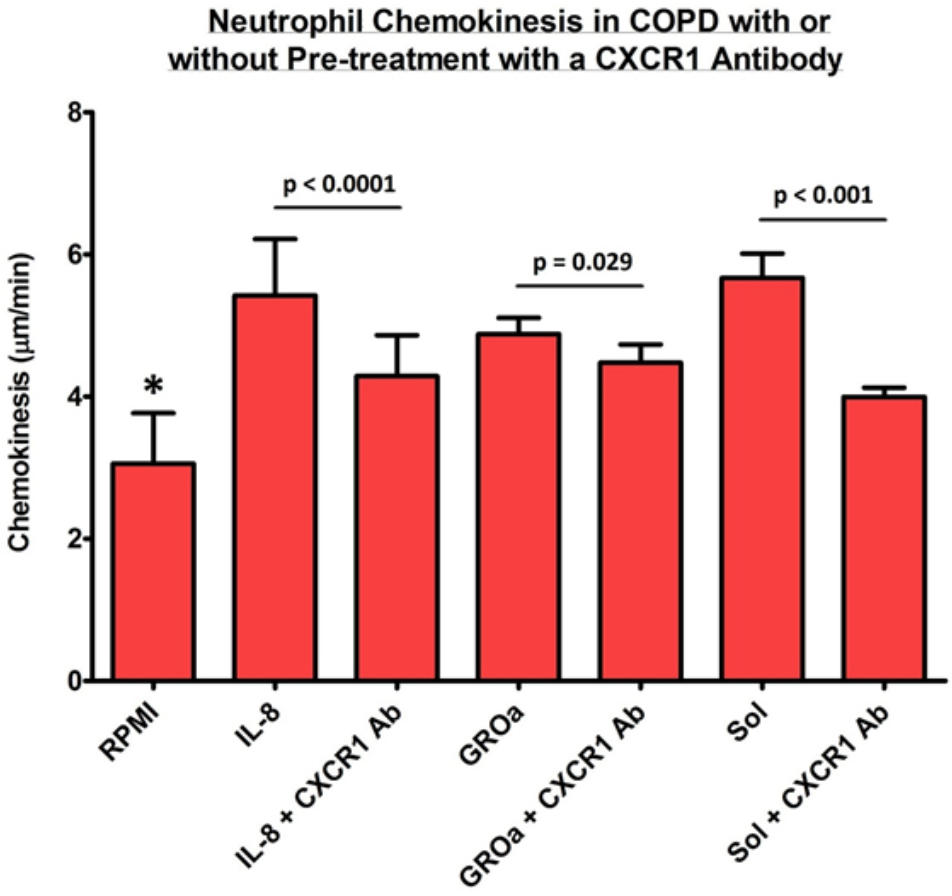

Figure 5.1: Chemokinesis of COPD neutrophils in the presence of a CXCR1 inhibitor.

Legend: The chemokinesis of neutrophils from COPD patients was significantly higher in IL-8, GROα and Sol compared to RPMI (*$p < 0.0001$). Chemokinesis was attenuated with the CXCR1 inhibitor in cells migrating in IL-8, GROα and Sol. Data are median and IQR (n=10). (Analysed by Kruskal-Wallis test).

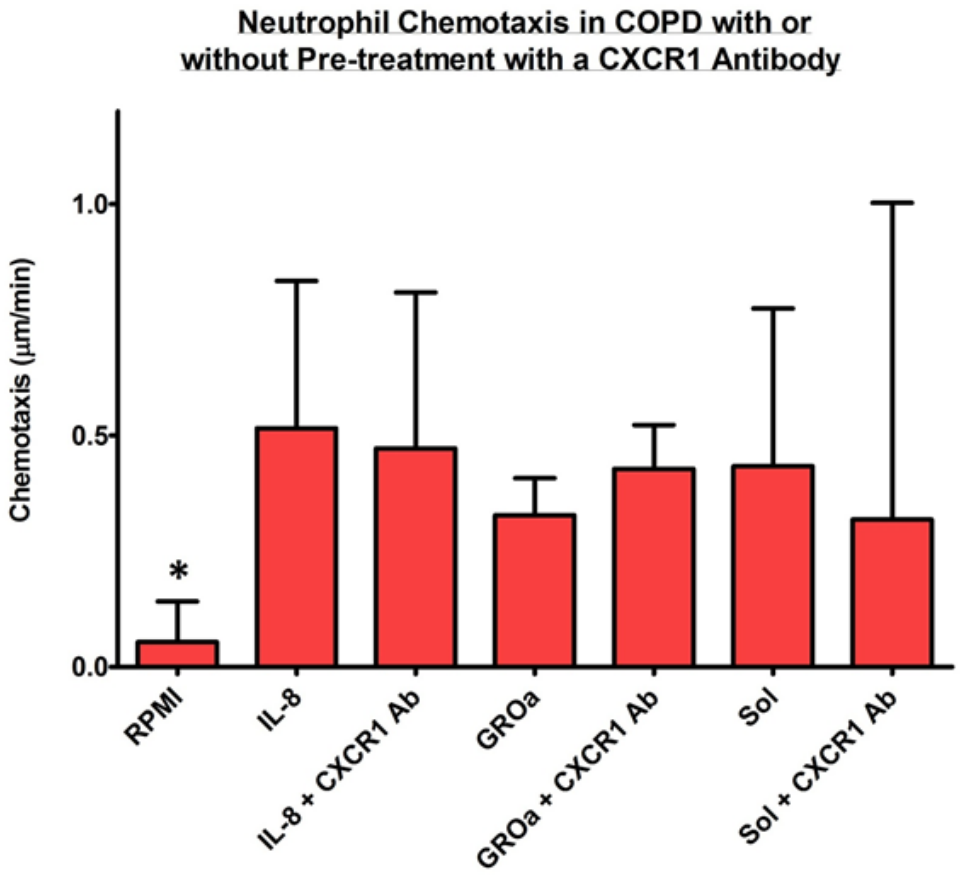

Figure 5.2: Chemotaxis of COPD neutrophils in the presence of a CXCR1 inhibitor.

Legend: The chemotaxis of neutrophils from COPD patients was significantly higher in IL-8, GROα and Sol compared to RPMI (*p < 0.0001). However, chemotaxis was unaltered with the CXCR1 inhibitor in any chemotactic gradient. Data are median and IQR (n=10). (Analysed by Kruskal-Wallis test).

5.3.2 CXCR2 Inhibition

Chemokinesis increased significantly in gradients of IL-8, GROα and Sol compared to RPMI-1640. Pre-incubation with the CXCR2 inhibitor caused a significant reduction in chemokinesis in IL-8, GROα and Sol ($p<0.0001$ for all) (Figure 5.3).

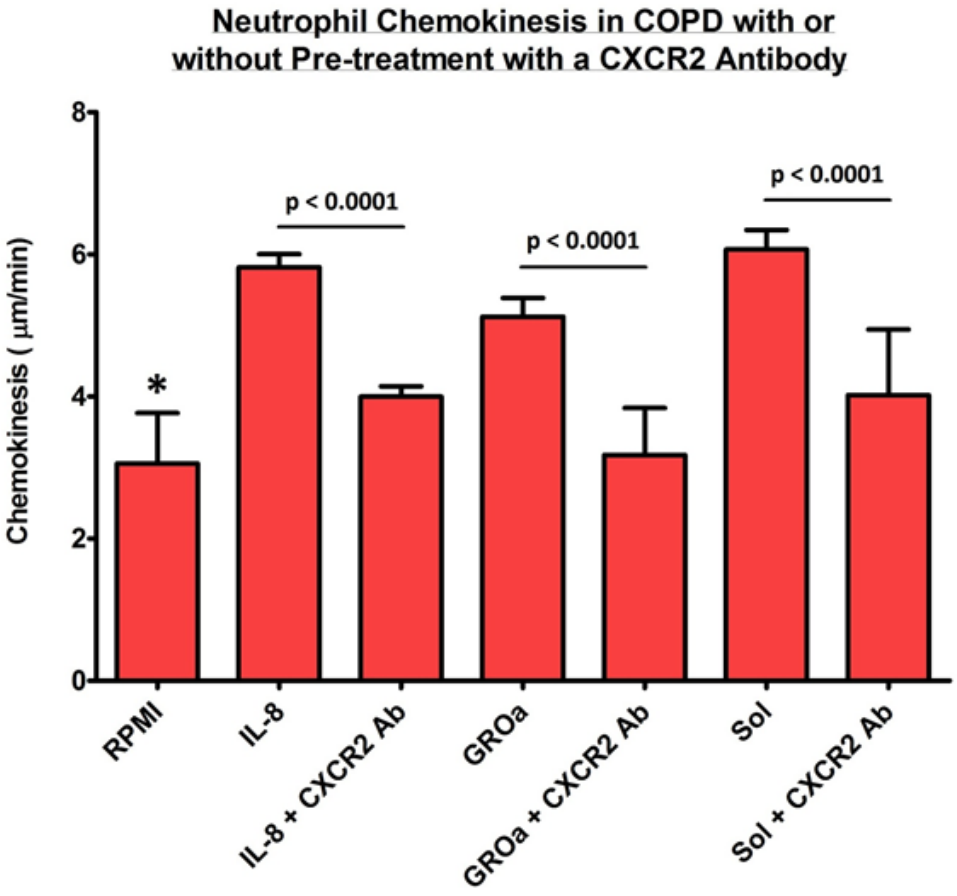

Figure 5.3: Chemokinesis of COPD neutrophils in the presence of a CXCR2 inhibitor.

Legend: The chemokinesis of neutrophils from COPD patients was significantly higher in IL-8, GROα and Sol compared to RPMI (*p < 0.0001). Chemokinesis was attenuated with the CXCR2 inhibitor in all chemotactic gradients. Data are median and IQR (n=10). (Analysed by Kruskal-Wallis test).

Chemotaxis was negligible in RPMI-1640 and increased significantly in gradients of IL-8 and Sol but not GROα. The CXCR2 inhibitor had no effect on chemotaxis in any chemotactic gradient (Figure 5.4).

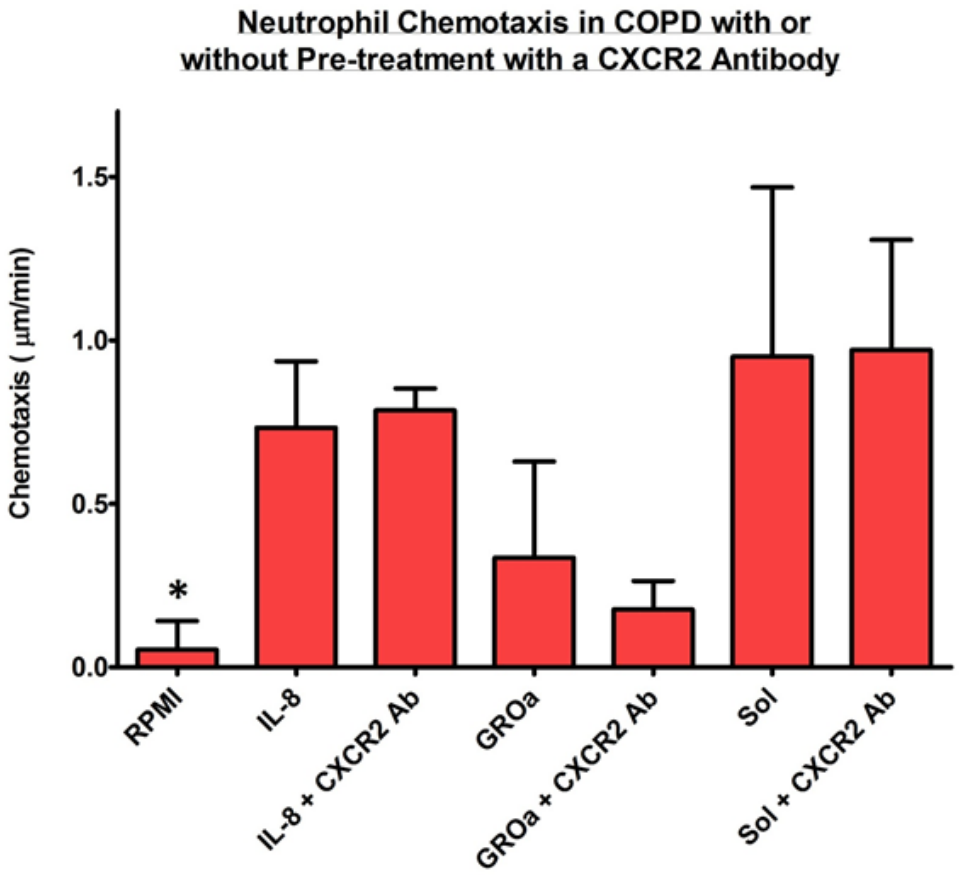

Figure 5.4: Chemotaxis of COPD neutrophils in the presence of a CXCR2 inhibitor.

Legend: The chemotaxis of neutrophils from COPD patients was significantly higher in IL-8 and Sol (not GROα) compared to RPMI (*p < 0.0001). The CXCR2 antibody had no effect on chemotaxis in any chemotactic gradient. Data are median and IQR (n=10). (Analysed by Kruskal-Wallis test).

5.3.3 Dual CXCR1/2 Inhibition

Pre-incubation with the dual CXCR1/2 inhibitor (SCH527123) at both concentrations (3 nM and 42 nM) caused a significant reduction in chemokinesis in gradients of both IL-8 and GROα, but only 3 nM caused a significant reduction of chemokinesis in Sol (Figure 5.5).

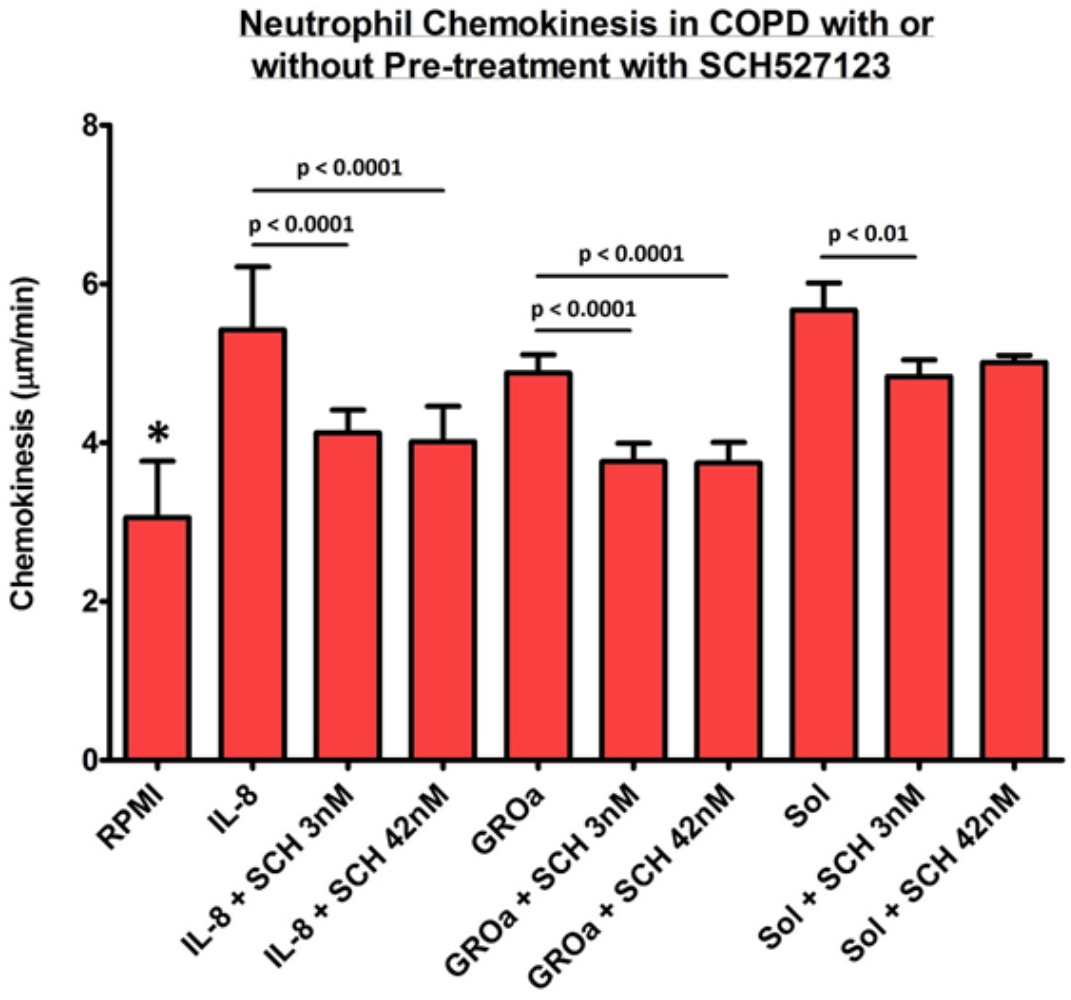

Figure 5.5: Chemokinesis of COPD neutrophils the presence of a CXCR1/2 inhibitor.

Legend: The chemokinesis of neutrophils from COPD patients was significantly higher in IL-8, GROα and Sol compared to RPMI (*p < 0.0001). Chemokinesis was attenuated with both 3 nM and 42 nM of the CXCR1/2 inhibitor (SHC527123) in gradients of IL-8 and GROα but only at 3nM in Sol. Data are median and IQR (n=10). (Analysed by Kruskal-Wallis test).

SCH527123 had no effect at 3 nM on chemotaxis in IL-8, although there was a trend for reduced chemotaxis in 42 nM (p = 0.052). In GROα, 3 nM SCH527123 caused a modest but significant reduction in chemotaxis, but 42 nM had no effect. Neither concentration of SCH527123 affected neutrophil chemotaxis in Sol (Figure 5.6).

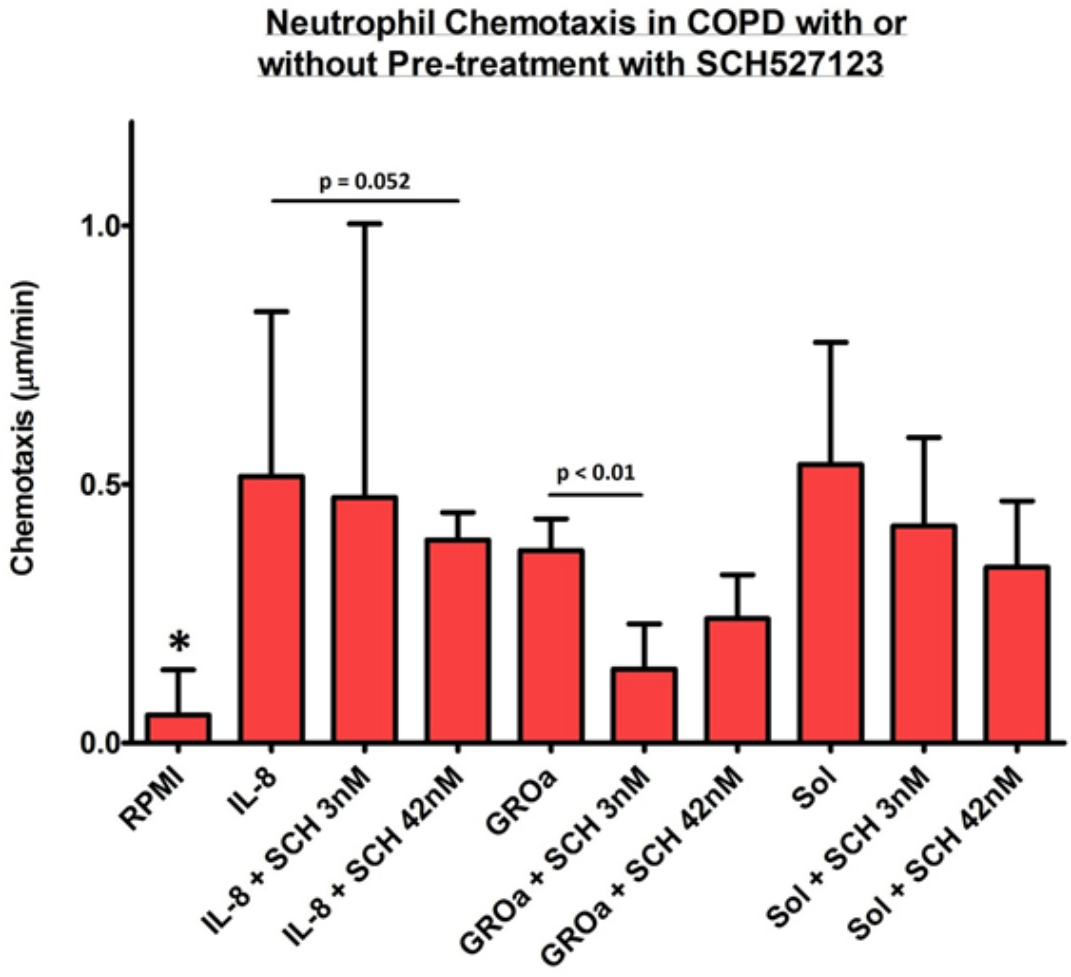

Figure 5.6: Chemotaxis of COPD neutrophils in the presence of a CXCR1/2 inhibitor.

Legend: The chemotaxis of neutrophils from COPD patients was significantly higher in IL-8, GROα and Sol compared to RPMI (*p < 0.0001). The CXCR1/2 inhibitor (SHC527123) did not have any effect on chemotaxis in IL-8 or Sol. In contrast, 3 nM SHC527123 significantly reduced chemotaxis in GROα. Data are median and IQR (n=10). (Analysed by Kruskal-Wallis test).

5.4 PI3K ISOFORM INHIBITION

Migratory dynamics were measured in neutrophils from 5 COPD patients in either medium alone, a shallow gradient of 100nM IL-8 alone or IL-8 following incubation in either α, β, δ, or γ PI3K isoform inhibitors. As expected, chemokinesis was relatively low in RPMI-

1640 and this increased significantly in gradients of IL-8 in all patients. However, chemokinesis was unaltered by incubation in any of the PI3K isoform inhibitors compared to IL-8 alone (Figure 5.7).

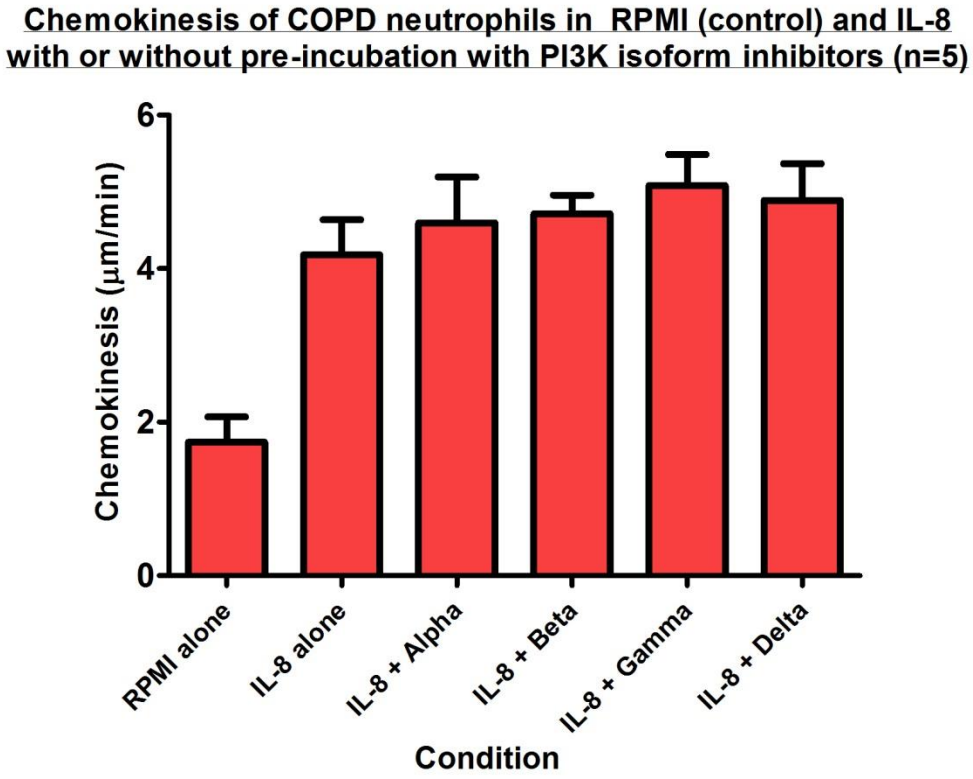

Figure 5.7: Chemokinesis of COPD neutrophils in the presence of PI3K isoform inhibitors.

Legend: The chemokinesis of neutrophils from COPD patients was unaltered following pre-incubation with any of the four PI3K isoform inhibitors. Data are mean and SEM (n=5). (Analysed by one-way ANOVA).

Chemotaxis was negligible in RPMI-1640 and also increased significantly when an IL-8 gradient was applied. This did not improve any further when the neutrophils were pre-incubated with PI3Kα or -β isoform. However, in contrast to chemokinesis, the PI3Kδ and -γ inhibitors significantly improved the chemotaxis of COPD neutrophils (Figure 5.8).

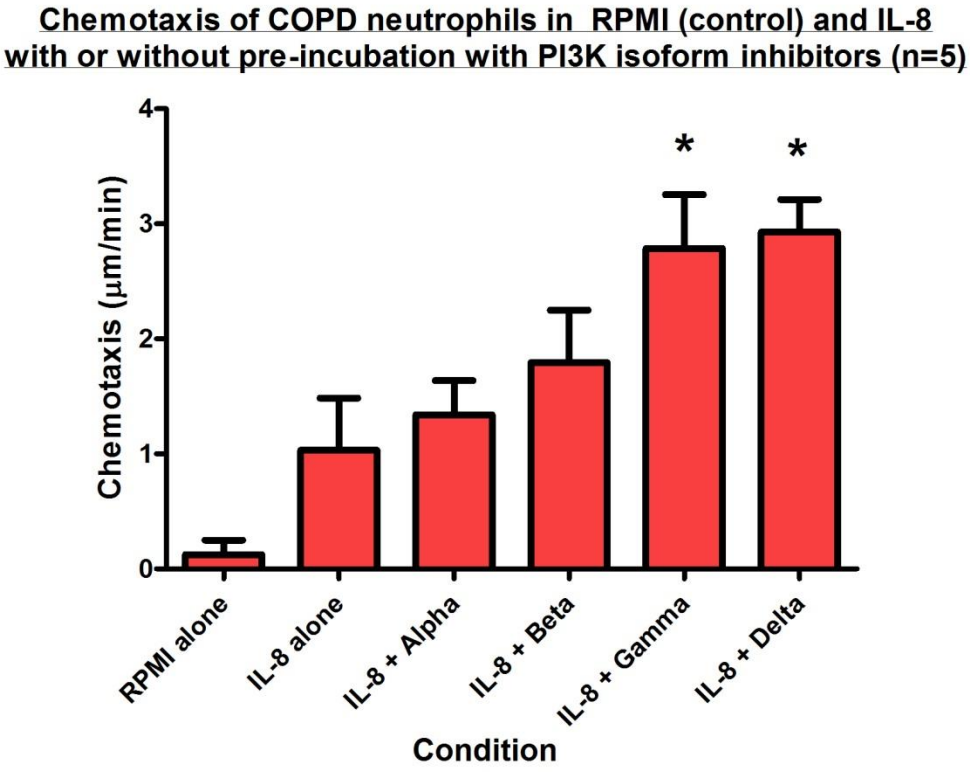

Figure 5.8: Chemotaxis of COPD neutrophils the presence of PI3K isoform inhibitors.

Legend: The chemotaxis of neutrophils from COPD patients was unaltered following pre-incubation with PI3Kα or -β isoform inhibitors. In contrast, both the PI3Kγ and -δ inhibitors significantly improved chemotaxis (* $p<0.05$). Data are mean and SEM (n=5). (Analysed by one-way ANOVA).

5.5 Erk INHIBITION

Migratory dynamics were measured in neutrophils from COPD patients in either medium alone or a shallow gradient of 100nM IL-8 with or without pre-incubation in an Erk inhibitor (UO126). Chemokinesis was unaltered by incubation in the Erk inhibitor (Figure 5.9).

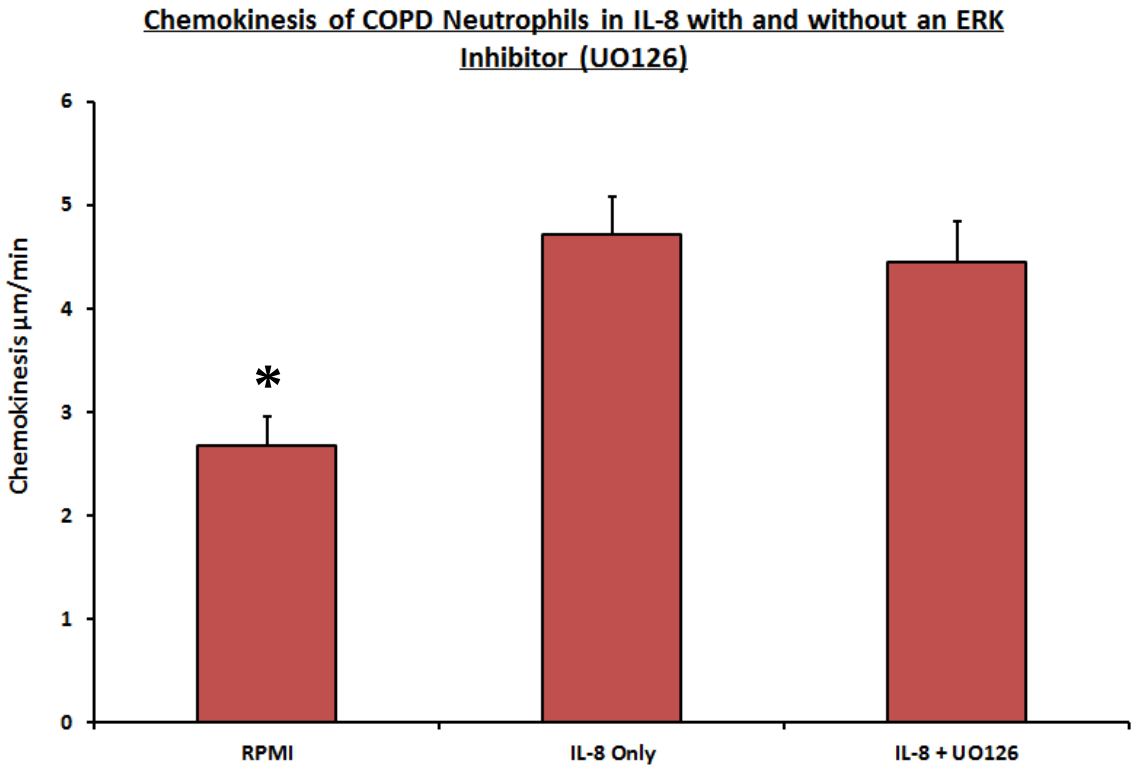

Figure 5.9: Chemokinesis of COPD neutrophils in the presence of an Erk inhibitor.

Legend: The chemokinesis of neutrophils from COPD patients was unaltered following pre-incubation with the Erk inhibitor. Data are mean and SEM (n=6). (*$p < 0.05$ compared to IL-8 ± UO126). (Analysed by one-way ANOVA).

Chemotaxis was negligible in median alone and increased significantly when an IL-8 gradient was applied. This did not alter significantly when the neutrophils were pre-incubated with the Erk inhibitor (Figure 5.10).

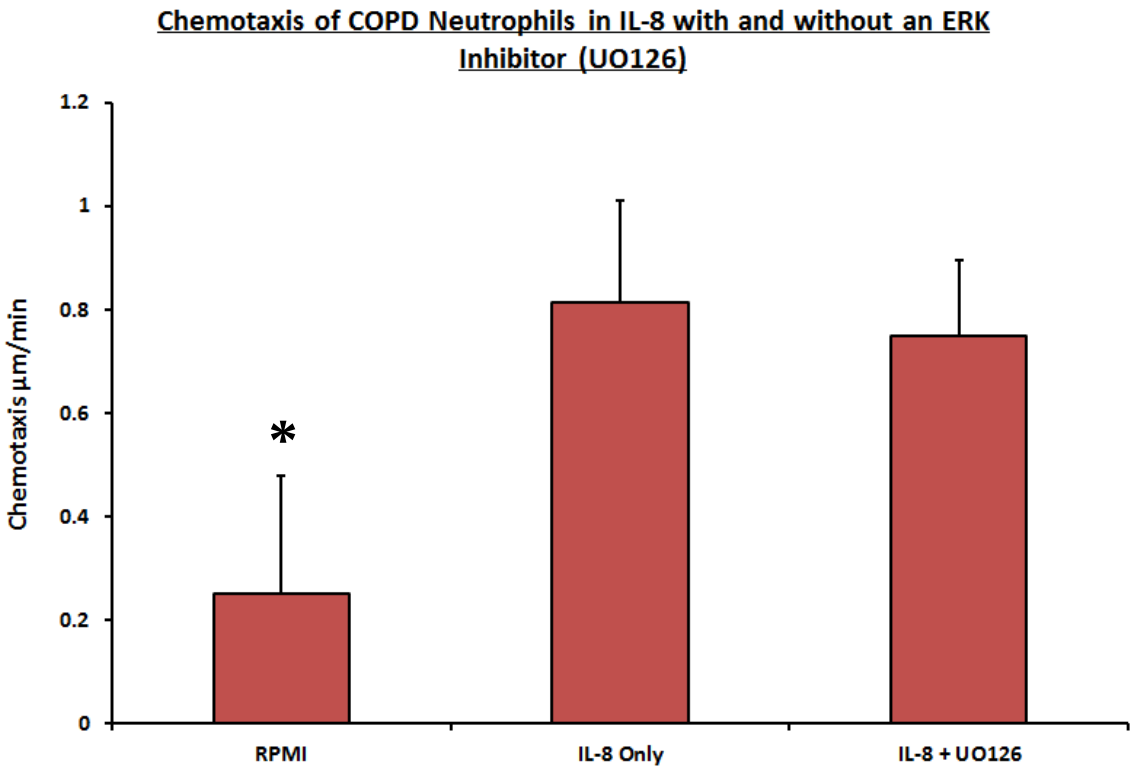

Figure 5.10: Chemotaxis of COPD neutrophils in the presence of an Erk inhibitor.

Legend: The chemotaxis of neutrophils from COPD patients was unaltered following pre-incubation with the Erk inhibitor. Data are mean and SEM (n=6). (*$p < 0.05$ compared to IL-8 ± UO126). (Analysed by one-way ANOVA).

5.6 p38 INHIBITION

Migratory dynamics were measured in neutrophils from COPD patients in either medium (RPMI-1640) or a shallow gradient of 100nM IL-8 with or without pre-incubation in one of two p38 inhibitors (VX745 or SCIO469). As expected, chemokinesis was relatively low in RPMI-1640 and this increased significantly in gradients of IL-8 in all patients. However, chemokinesis was unaltered by incubation in either p38 inhibitor (Figure 5.11).

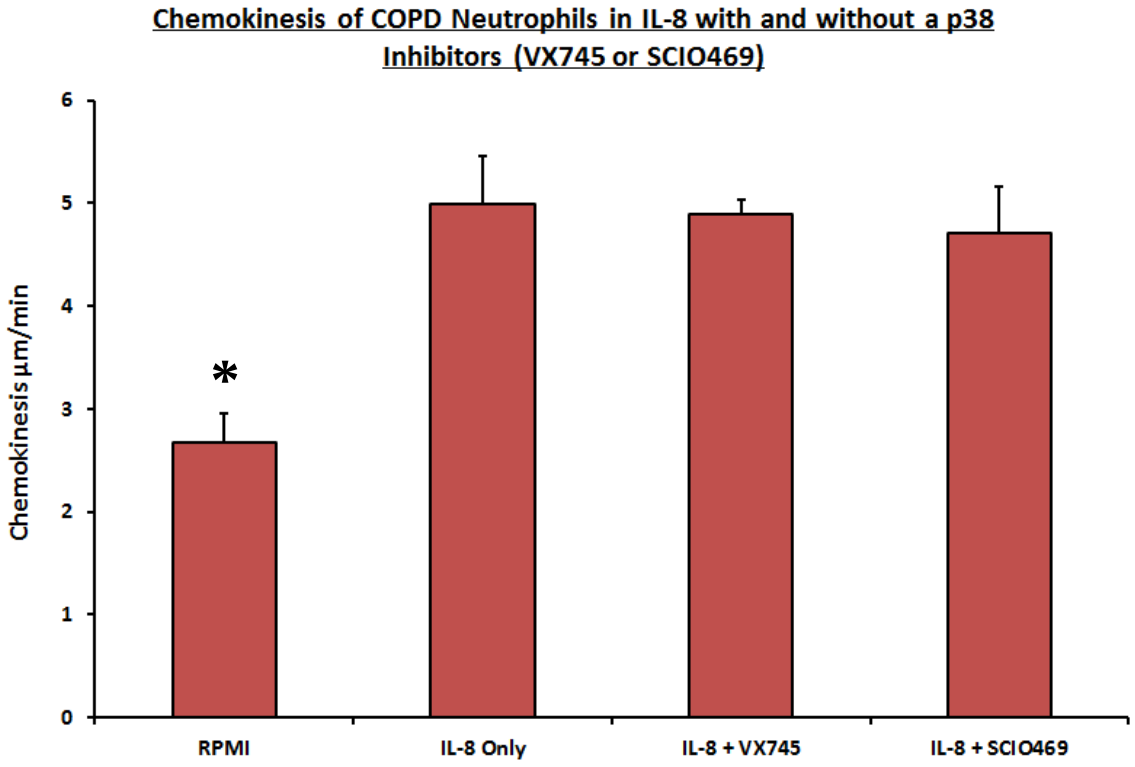

Figure 5.11: Chemokinesis of COPD neutrophils in the presence of p38 inhibitors.

Legend: The chemokinesis of neutrophils from COPD patients was unaltered following pre-incubation with either p38 inhibitor. Data are mean and SEM (n=6). (*$p < 0.05$ compared to IL-8 ± Erk p38 inhibitors). (Analysed by one-way ANOVA).

Chemotaxis was negligible in RPMI-1640 and also increased significantly when an IL-8 gradient was applied. However, it did not alter significantly when the neutrophils were pre-incubated with either p38 inhibitor (Figure 5.12).

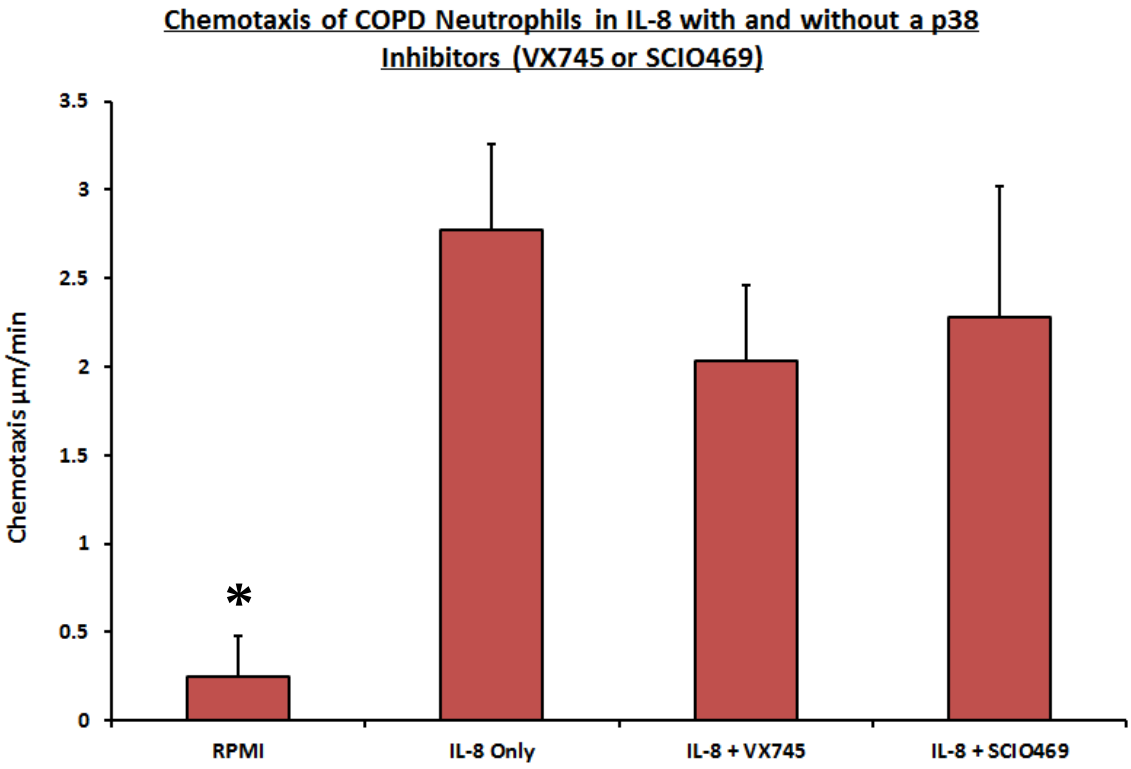

Figure 5.12: Chemotaxis of COPD neutrophils in medium and IL-8 +/- p38 inhibitors.

Legend: The chemotaxis of neutrophils from COPD patients was unaltered following pre-incubation with either p38 inhibitor. Data are mean and SEM (n=6). (*$p < 0.05$ compared to IL-8 ± Erk p38 inhibitors). (Analysed by one-way ANOVA).

5.7 SIMVASTATIN

5.7.1 Validation: Simvastatin Concentration Titration

Chemotaxis was initially assessed using a range of simvastatin concentrations to determine which would yield the highest values for the proposed investigation. Concentrations of simvastatin spanning the normal therapeutic dose range were tested to assess for a concentration response relationship (1nM, 10nM, 100nM, 1μM) using neutrophils from 3 healthy donors. Migration was also measured in RPMI-1640 medium and IL-8 alone as negative controls. Chemotaxis with simvastatin did not show a true concentration response, though migratory accuracy did improve with 1 nM and 10 nM. This returned to values comparable to baseline with 100 nM simvastatin and improved a little (but not significantly) beyond this with 1 μM. These data are summarised graphically in Figure 5.13.

With the increasing concentrations of simvastatin, we obeserved a traditional concentration response, with the peak chemotactic response occuring with 10 nM simvastatin. However, as there as no difference between 1 nM and 10 nM simvastatin, 1 nM (equivalent to 20 mg/day) was used in the final investigation to reflect the minimum therapeutic dose. In addition, 1 μM (equivalent to 80 mg/day) was also used to reflect the maximum therapeutic dose.

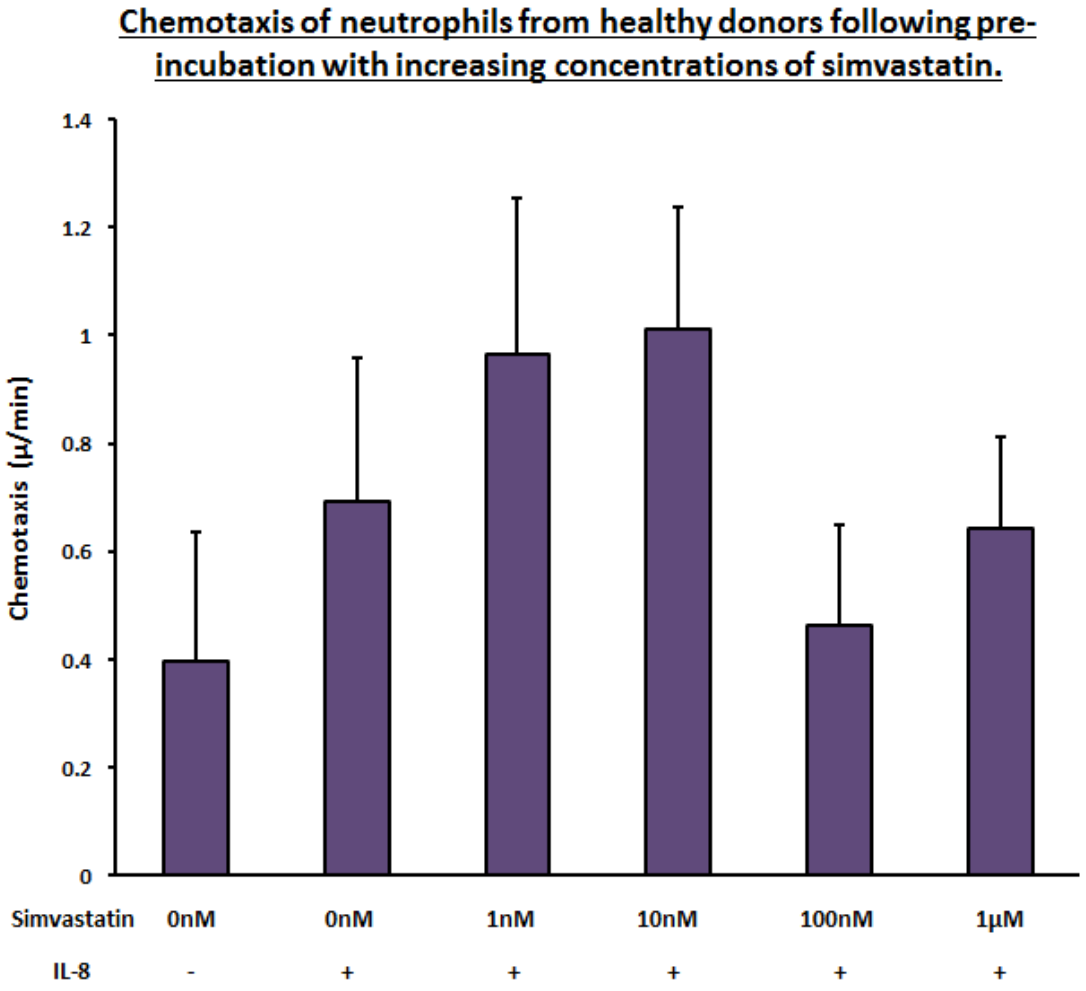

Figure 5.13: Chemotactic concentration response to simvastatin of healthy neutrophils in IL-8.
Legend: Chemotaxis of helathy neutrophils migrating in a shallow gradient of 100 nM IL-8 following pre-incubation with increasing concentrations of simvastatin. Data are mean and SEM (n=3).

5.7.2 Neutrophil Migration Following Treatment with Simvastatin

The migratory dynamics of neutrophils from both COPD patients were assessed in RPMI-1640 medium (control) as well as gradients of 100 nM IL-8 and fMLP with and without a 40 minute pre-incubation with simvastatin (1 nM and 1 µM).

Chemokinesis

As expected, chemokinesis was higher in the presence of IL-8 and fMLP compared to RPMI-1640 medium alone. Pre-incubation with simvastatin (at either 1 nM or 1 μM) did not alter chemokinesis in IL-8. However, there was a significant but modest increase in chemokinesis in fMLP following pre-incubation with 1 nM simvastatin ($p = 0.02$). These data are summarised in Figure 5.14.

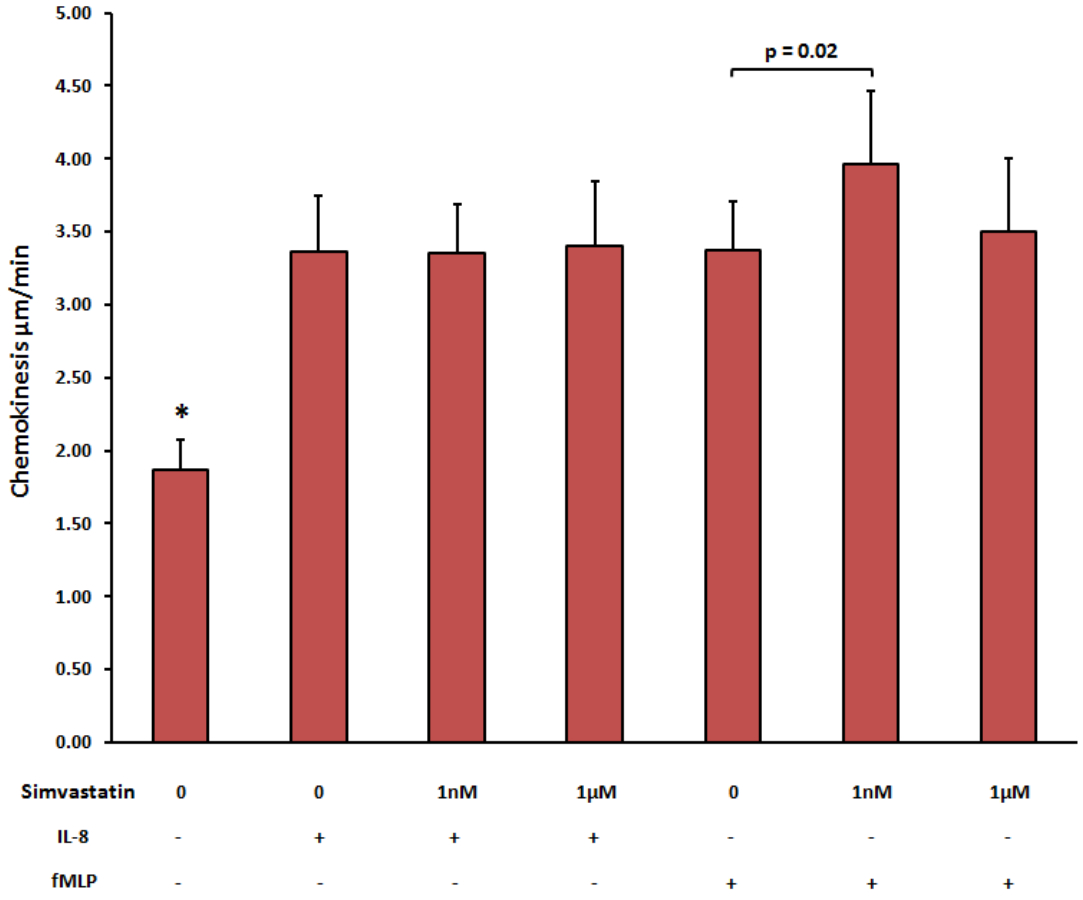

Figure 5.14: The effects of simvastatin on chemokinesis of COPD neutrophils.

Legend: Chemokinesis of COPD and neutrophils migrating in a shallow gradients of 100 nM IL-8 and fMLP following pre-incubation with increasing concentrations of simvastatin. Data are mean and SEM (n=8) (*$p < 0.05$ compared to IL-8 and fMLP). (Analysed by one-way ANOVA).

Chemotaxis

Chemotaxis was significantly higher (although still low) in IL-8 and fMLP alone compared to RPMI-1640 medium. Significant improvements in IL-8 were observed followng incubation with both 1 nM (p = 0.03) and 1 µM (p = 0.04) simvastatin. In addition, a significant improvement in chemotaxis was observed in fMLP with 1 nM simvastatin (p = 0.03), although a statistically significant improvement was not observed with 1 uM simvastatin (p = 0.09). These results are summarised in Figure 5.15.

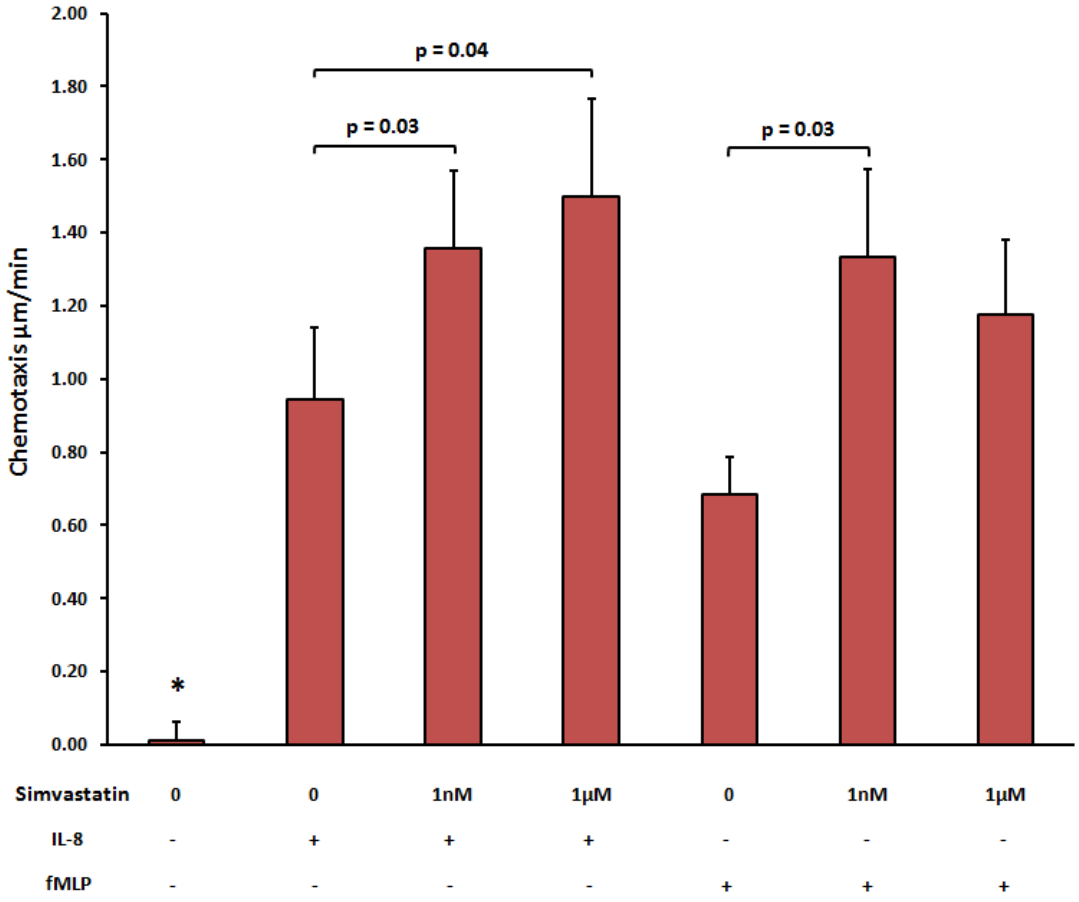

Figure 5.15: The effects of simvastatin on chemotaxis of COPD neutrophils.

Legend: Chemotaxis of COPD migrating in a shallow gradients of 100 nM IL-8 and fMLP following pre-incubation with increasing concentrations of simvastatin. Data are mean and SEM (n=8) (*p < 0.05 compared to IL-8 and fMLP). (Analysed by one-way ANOVA).

5.8 DISCUSSION

5.8.1 CXCR1/2 Inhibition

There has been growing interest into the potential use of CXCR1 and, particularly, CXCR2 antagonists in the treatment of chronic inflammatory diseases such as COPD. The data here suggest that the dominant effect on isolated CXCR1 or CXCR2 inhibition on COPD neutrophils is a reduction in the overall speed of migration with no significant effect on their accuracy. Importantly, the effects seen in IL-8 and GROα were generally mirrored in sputum, a complex physiological secretion more reflective of the *in vivo* environment.

With the dual inhibitor, SHC527123, it would be expected that 3 nM (which favours CXCR2 inhibition) should inhibit migration to GROα and, perhaps to a lesser degree, IL-8 (which can also signal through CXCR1). In contrast, 42 nM (which inhibits both CXCR1 and CXCR) would likely provide comparable inhibition in both GROα and IL-8. On average, chemokinesis in both GROα and IL-8 in both was reduced by approximately half by 3 nM and 42 nM, which seems logical as these were both IC_{50} concentrations. The fact that the effect of SHC527123 on chemokinesis in Sol (although significant) was approximately half when compared to chemokinesis in isolated IL-8 and GROα and accuracy was unaltered in Sol may be due to a migratory response to other chemokines when both CXCR1 and CXCR2 are inhibited. Although chemotaxis appeared to be reduced by SHC527123 in GROα and IL-8, only 3 nM in GROα as significantly lower than it's respective control.

The majority chemotaxis data in the CXCR1/2 inhibitor assays appear highly variable. The reasons for this may be two-fold; firstly the chemotaxis values (in μm/min) are low and, secondly, the data are not normally distributed and, therefore, presented as median with 3rd

quartile error bars (not mean and SEM). The general lack of change in chemotaxis with all CXCR1/2 inhibitors is most likely because these inhibitors seem to slow neutrophil migration and, as the baseline chemotaxis is so poor, it remains unaffected by slower migration. Importantly, the CXCR1/2 inhibitors are certainly not improving chemotaxis, which is perhaps more clinically pertinent.

In summary, it appears that COPD neutrophils were simply migrating more slowly following treatment with CXCR1/2 inhibitors. This may explain why previous studies observed a decrease in airway infiltration following LPS insult (Virtala et al. 2011, Leaker et al. 2013), where the neutrophils may have simply been transmigrating more slowly. In COPD however, this could potentially be detrimental as slower transmigration would logically increase the amount of time that the lung tissue is exposed to the digestive proteinases from each neutrophil (if the rate of degranulation is the same). In theory, this could actually increase collateral tissue damage and accelerate emphysematous change. Furthermore, one recent study that assessed the effects of a CXCR2 antagonist (SB-656933) in patients with cystic fibrosis reported an increase in plasma levels of CRP and IL-8 (Moss et al. 2013). This potentially detrimental physiological response could also be seen in COPD with CXCR2 inhibition, although the implications for general and pulmonary inflammation remain to be determined.

5.8.2 Kinase Inhibition

A previous study by our group demonstrated a significant improvement in the migratory accuracy of neutrophils from COPD patients *in vitro* following treatment with the non-specific PI3K inhibition with LY29004 (Sapey et al. 2011). However, due to the broad

association of PI3K with various functions of numerous cell types, the treatment of COPD with non-specific PI3K inhibitors could result in undesirable side-effects. By this rationale, the experiments in this chapter sought to determine if the advantageous effect of PI3K inhibition on neutrophil migration in COPD could be attributed to specific PI3K isoforms that could potentially be a more specific and suitable therapeutic target.

The data demonstrate that inhibition of both the δ- and γ-isoforms at the recommended IC_{50} significantly improved the chemotaxis of neutrophils from COPD patients. In contrast, inhibition of the α- and β-isoforms had no effect on the migratory accuracy. It was surprising to observe that, in contrast to the initial chemotaxis experiments (Section 3.2.2), the chemokinesis of COPD neutrophils was not significantly greater in IL-8 or fMLP alone than those from healthy control subjects. This could potentially be due to a smaller number of subjects included in this experiment (n = 5) compared to the original experiment (n = 10). On reflection, it may also have been useful to determine whether or not the combination of both the δ- and γ-isoform inhibitors improved the chemotaxis of COPD neutrophils beyond the level achieved when used in isolation.

Importantly, the inhibition of other kinases involved in neutrophil migration (Erk and p38) had no effect on chemokinesis or chemotaxis. This, together with the effects of PI3K inhibition, supports the hypothesis that exclusive dysfunction of the PI3K signalling pathway may be responsible for the aberrant migration of COPD neutrophils.

5.8.3 Simvastatin

Statins have been shown to have beneficial immunomodulatory effects in a variety of diseases. One study demonstrated a reduction the hospitalisation rates of severe COPD

patients with statin treatment (Dobler et al. 2009) and similar effects have been observed in patients with sepsis (Falgas et al. 2008). In addition, statins have been shown to reduce chemokine production (Inoue et al. 2000) and immune cell endothelial capture, which are both important in the excessive inflammatory response that characterises COPD. As it has previously been shown that statins exhibit their immunomodulatory effects through modulation of GTPase activity (which controls many aspects of cell behaviour including migration (Li et al. 2005, Van Keymeulen et al. 2006)), it was hypothesised that the treatment of COPD neutrophils with simvastatin could potentially improve their migratory accuracy.

The data indicated that, while neither 1 nM or 1 μM simvastatin had any major influence on migratory speed, both concentrations improved the migratory accuracy of COPD neutrophils in IL-8 and fMLP, with the greatest benefit achieved with 1 μM (equivalent to 80 mg/day) in IL-8. Moreover, the chemotaxis of COPD neutrophils with simvastatin was comparable to that of the healthy subjects in the initial chemotaxis experiments (Section 3.2.2). This suggests that treatment of COPD neutrophils with simvastatin *in vitro* can improve their migratory accuracy to that observed in healthy ageing. Unpublished data from our group (H. Greenwood personal communication) show that chemotaxis improves in the healthy elderly subjects following treatment with simvastatin, suggesting that neutrophil chemotaxis may not be optimal in healthy ageing. This concept is supported by other data from our group that indicate chemotaxis of neutrophils from healthy elderly subjects is reduced when compared to those from healthy young subjects (Sapey et al. 2014). Once again, this highlights the importance of age-matching healthy control subjects and COPD patients.

The concentration response that we initially observed indicates that the beneficial effects of simvastatin (particularly on neutrophil chemotaxis) may not be achieved if the dose is too high or too low. Therefore, the null effect of simvastatin in previous studies on inflammation and neutrophil function may be because the dose was too low and, perhaps, the assay used and the type of patients studied. For instance, Guasti et al reported a reduction in chemotaxis with 20 mg simvastatin but they used a Boyden Chamber, which does not provide specific information on migratory dynamics and they did not study COPD patients (Guasti et al. 2006). In addition, the large clinical trials of simvastatin also used doses lower than 80 mg/day and, importantly, did not investigate effects on neutrophil function (Papazian et al. 2013, Criner et al. 2014). Recent data from our group has also demonstrated that simvastatin has no effect on neutrophil function during pneumonia with severe sepsis (unpublished data from J. Patel). This suggests that if the host response to infection or injury is too severe, improvements in neutrophil function and, potentially, other anti-inflammatory effects of simvastatin may be lost. Collectively, these data suggest that there may be a tight window of opportunity for statin therapy in respiratory disease, where a high dose is required when patients are not severely ill and clinically stable. This may explain why two other recent clinical trials in Adult Respiratory Distress Syndrome (where the dose was 80 mg/day but patients had severe sepsis) (McAuley et al. 2014) and Community Acquired Pneumonia (where the 20 mg/day dose may have been too low) (Vlasus et al. 2014) were also negative.

As simvastatin did improve the migratory accuracy of COPD neutrophils further implicates the PI3K signalling pathway as a mechanism (albeit indirectly as GTPases such as Ras and Rho are not directly influenced by PI3K but synergise many processes along its signalling pathway). Inhibition of Ras and Rho by simvastatin appears to have a downstream

effect of attenuating the over-active PI3K signalling that we propose as a key mechanism in the aberrant migration of COPD neutrophils.

CHAPTER 6

GENERAL DISCUSSION

COPD is a major disease and a leading cause of mortality and morbidity worldwide (WHO 2007). Its prevalence incurs a huge burden on healthcare and, in spite of its longstanding recognition, relatively little is understood about the pathogenesis of COPD and there are currently no therapies available that can fully reverse its pathological effects. COPD is a disease of chronic inflammation that occurs throughout the airways and the lung parenchyma (Saetta et al. 2001). Numerous inflammatory cells and mediators have been implicated in its pathogenesis, though the neutrophil is believed to be the predominant effector cell (Sapey et al. 2008).

Neutrophil migration is fundamental to their inflammatory function. It is logical to infer that, without accurate migration, the ability of neutrophils to reach the site of inflammation and locate invading pathogens would be impaired. Migration is initially induced via the detection of a chemotactic stimulus by cell surface receptors, which results in the activation of various intracellular signalling pathways that lead to cytoskeletal rearrangement, polarisation and, ultimately, motility (see Section 1.2.3).

The first studies of neutrophil migration in COPD used a Boyden Chamber and yielded variable results, with one study demonstrating an increased migration of neutrophils from COPD patients (Burnett et al. 1987), while a later study described a reduced chemotaxis of neutrophils from patients with more severe COPD (Yoshikawa et al. 2007). The Boyden Chamber assay is limited as it does not allow for the assessment of specific migratory dynamics on a cell-by-cell basis and can only assess net movement. A recent study by our group assessed the two-dimensional migratory dynamics of COPD neutrophils using an Insall Chamber (Sapey et al. 2011). The results indicated that COPD neutrophils consistently migrated with increased speed (chemokinesis) but reduced accuracy (chemotaxis) in the

presence of IL-8, GROα and sputum when compared to neutrophils from healthy donors. Although the data provided far more insight into how the cells were migrating, it was unclear whether the aberrant migration was a generic phenomenon or due specifically to a dysregulation of the IL-8 and GROα receptors, CXCR1 and CXCR2. Moreover, it was unclear whether or not it was an intrinsic cell defect or the result of the systemic inflammatory environment of COPD.

Although migration is only one element of neutrophil-driven inflammation, its dysfunction in COPD could be of fundamental importance to the pathophysiology of the disease. Firstly, the area of collateral, proteolytic tissue damage may be increased if neutrophil migration through the parenchyma is inaccurate due to longer migratory tracks. As elastolytic destruction is central to the emphysematous process, it could be intensified by aberrant, inefficient neutrophil migration through the extracellular matrix. Secondly, an impaired ability of neutrophils to locate pathogens within the airspaces could lead to a further increase in bacterial propagation and, potentially, a more frequent exacerbation rate. Their role in COPD infers that neutrophils, together with their functional bi-products and inflammatory mediators could potentially be targets for novel therapies in COPD. However, the current understanding of the specific processes involved in neutrophil-driven inflammation and migration in COPD is limited. Therefore, this thesis sought to further define these processes with the aim of providing greater insight that may aid in the development of novel therapeutic strategies.

6.1 ABERRANT NEUTROPHIL MIGRATION IN COPD

Following the previous study by our group (Sapey et al. 2011), this study initially compared two-dimensional migration of neutrophils from COPD donors to those from age-

matched healthy donors and patients with α_1-ATD. α_1-ATD mirrors many pathophysiological aspects of COPD but has a known genetic predisposition and a well described pathogenesis. The inclusion of patients with α_1-ATD was important to account for other factors that may influence neutrophil migration, such as smoking status, lung disease/inflammation and pharmacological therapies. In addition to IL-8 and GROα used in the previous study (Sapey et al. 2011), this thesis also assessed migration to other endogenous and bacterial chemoattractants, including LTB_4, C5a and fMLP. These were selected on the basis that they represent inflammatory chemokines from different sources and each have specific receptors on the neutrophil surface distinct from those for IL-8 and GROα.

The data indicate that neutrophils consistently migrate with increased chemokinesis but reduced chemotaxis in the presence of all five chemoattractants. This is important as it suggests that the aberrant neutrophil migration in COPD is a generic phenomenon and not related to specific chemokines and their receptors, which could not be confirmed in previous studies (Yoshikawa et al. 2007, Sapey et al. 2011). There are many mammalian chemokines that induce neutrophil migration. Importantly, the migratory accuracy of COPD neutrophils was as poor in response to sputum Sol as it was to isolated chemokines such as IL-8. If this *in vitro* finding is mirrored *in vivo*, it could mean that neutrophils in COPD patients migrating towards sputum in the airspaces would also migrate inaccurately. As mentioned, this could be a major mechanism by which emphysematous damage and bacterial colonisation is amplified in COPD.

Whether or not this dysfunction develops during neutrophil maturation in the bone marrow or after their release into the circulation has yet to be determined. The chemotaxis data shown here suggest that, although certain inflammatory mediators may be increased in

the plasma of COPD patients, the systemic inflammatory environment is unlikely to be the cause of altered neutrophil migration. It is possible that the time that the healthy neutrophils were exposed to COPD plasma (40 minutes) was not sufficient to have an effect and it may be preferable to expose cells for longer (several hours). However, neutrophils only remain viable for a relatively short time once isolated from peripheral blood (> 60% cell death after 24hrs (Xu et al. 2013)), so they could not be incubated extensively in donor plasma. A more suitable experiment may be the adoptive transfer of radiolabelled neutrophils from healthy animal donor strain into a separate animal strain in which emphysema can be induced before re-extraction and assessment of migration but, again, this is not ideal and the relatively short half-life of neutrophils may prove limiting. Furthermore, the data on neutrophil activation markers suggest that systemic inflammation in COPD is unlikely to have a major effect on neutrophil activation status and migratory dynamics, as the surface expression of the majority of the markers studied were the same in COPD and health.

Lastly, it cannot be excluded that aberrant neutrophil migration is in fact congenital in patients who are predisposed to developing COPD and it is only the continual, inaccurate migration through the lungs in response chronic inhalation of toxic particles that causes COPD to manifest. This could be investigated by studying a large cohort of young never-smokers who, on the whole, appear to have accurate neutrophil migration (Sapey et al. 2014), to determine if there is a proportion that exhibit aberrant migration. Individuals that begin to smoke could then be followed longitudinally with HRCT and lung function tests to see which (if any) develop COPD. Unfortunately, gaining funding for such studies may be difficult.

6.2 MECHANISMS UNDERLYING ABERRANT NEUTROPHIL MIGRATION IN COPD

The initial experiments suggested that the aberrant neutrophil migration in COPD is not influenced by external factors. Therefore, it is logical to assume that the dysfunction is related to the intrinsic factors that mediate chemotaxis, including chemokine receptor function and/or the intracellular signalling pathways used by these receptors.

A slightly reduced expression of CXCR1, CXCR2 and C5aR was observed on quiescent COPD neutrophils compared to those from healthy controls. The mechanisms responsible for the reduced expression of these receptors in COPD are unclear. It has been shown that TNFα, which is present in higher concentrations in the plasma of COPD patients (Matsushima et al. 1988), induces CXCR2 shedding as well as a suppression of chemotaxis *in vitro* (Asagoe et al. 1998). Whether this effect is paralleled *in vivo* and whether similar effects on CXCR1 and C5aR exist remain to be determined. The fact that there was no difference in the surface expression of any chemokine receptor between health and α_1-ATD suggests that inflammation alone is not sufficient to cause this reduction. In support of this, evidence suggests that the concentration of inflammatory cytokines (such as IL-8 and LTB_4) is actually higher in the sputum of α_1-ATD compared to usual COPD (Stone et al. 2012).

As migratory dysfunction of COPD neutrophils was observed in all five chemokine gradients, it seems logical that chemokine receptor expression should be universally decreased if that was the cause of altered migration. However, this was not observed in the experiments investigating chemokine receptor expression. Moreover, quiescent expression may not accurately reflect what occurs during migration, where neutrophils are activated and surface expression of chemokine receptors may alter due to internalisation, re-expression and

shedding. The rapid abolition of differences in quiescent CXCR1 and CXCR2 expression between COPD and healthy neutrophils following their stimulation supports the theory that chemokine expression does not impair migratory accuracy of neutrophils in COPD. Total surface expression of receptors only provides limited information as, for accurate cell migration, these receptors must be localised to the leading edge to allow for optimal gradient sensing and directionality. The data shown here suggest that both CXCR1 and CXCR2 localise to the leading edge as efficiently in COPD compared to health. Although a relatively crude assessment, these experiments further support the proposal that chemokine receptor expression cannot be implicated in the aberrant neutrophil migration of COPD. However, it has previously been show that COPD neutrophils form fewer pseudopods than those from healthy controls (Sapey et al. 2011). In addition, studies on *Dictyostelium* have shown that abnormal or reduced pseudopod formation is associated with altered migration (Weber 2006). It is possible that, although chemokine receptors may be localising to the leading edge on activated, migrating neutrophils as effectively and in similar numbers in COPD compared to health, impaired pseudopod extension could indirectly influence receptor-ligand binding. A more detailed investigation into chemokine receptor localisation on extending pseudopods using differential interference contrast (DIC) microscopy may provide further insight.

PI3K is associated with numerous cellular functions, including directional migration (Knall et al. 1997, Sasaki et al. 2000, Sadhu et al. 2003). A universal trend for increased $PI3K_{T458}$ phosphorylation was observed in both unstimulated and stimulated (with IL-8, GROα and fMLP) COPD neutrophils. Although the biological significance of these pilot data is unclear, they suggest that there could be differences in PI3K signalling in COPD neutrophils. Therefore, the hypothesis that PI3K over-activity may be causing aberrant

neutrophil migration in COPD is speculative (based on the limited data presented in this thesis) but is supported by a previous study by our group that demonstrated that a non-selective PI3K inhibitor (LY29004) corrected the migratory defect (Sapey et al. 2011). Whether potentially increased PI3K activity could be a result of a genetic polymorphism resulting in increased susceptibility to COPD or a result of the disease itself remains to be determined. Regardless, its association with the altered neutrophil migration COPD is becoming more evident and it seems to be the only altered signalling pathway identified to date.

Precisely how a hypothetical increase in PI3K activity could result in increased speed and poor directionality during neutrophil chemotaxis in COPD also remains unclear. As LY29004 is known to reduce migratory speed and affect pseudopod formation (Andrew et al. 2007), it seems logical that there is a downstream effect of PI3K-dependant mediators. Further progress in the area may require mechanistic work in animal models. However, animal models of COPD are currently very limited and only include those with emphysema induced by the acute introduction of pro-inflammatory cytokines into the airways or inhalation of tobacco smoke. These do not truly reflect the chronic and insidious development of COPD in humans, which is also associated with both airflow obstruction and chronic bronchitis. The more recent zebrafish model (Renshaw et al. 2006) could eventually prove useful as it allows for real-time monitoring of neutrophil migration *in vivo*. It may be possible to induce PI3K over-expression in zebrafish by increasing SHIP-1 expression using micro RNA-155 (miR-155) (O'Connell et al. 2009). If aberrant neutrophil migration results, it would support our theory of PI3K involvement. Similarly, if PI3K knock-down animal

models develop less emphysema as a result of cytokine/smoke exposure than those with normal PI3K expression, it would add further support to this theory.

No differences were observed in Akt phosphorylation (at either S473 or T308). Although PI3K binding is important for Akt activity, it is not essential and Akt can be activated in a PI3K-independent manner (Mahajan et al. 2010). In addition, once PI3K binding has occurred, further phosphorylation events are necessary for Akt to become fully activated. Therefore, despite potentially increased PI3K activity in COPD that could lead to a more rapid accumulation of Akt at the plasma membrane, Akt activity may be limited by its phosphorylation by mTORC2 (at S473) and/or PDK1 (at T308). It is important to note that this thesis studied neutrophil migration using adhered cells, whereas PI3K and Akt phosphorylation was determined from non-adherent cells. Personal communications from Dr. Philip Hawkins (The Babraham Institute, Cambridge) have suggested that Akt activity is only up-regulated once neutrophils become adhered. Although an upregulation of $pAkt_{S473}$ from non-adherent neutrophils was observed, it may explain why there was no difference in $pAkt_{T308}$ following stimulation.

The migratory signalling pathway is extremely complex and comprises numerous mediators that each in turn influence and are influenced by other signals. There are many components in the PI3K signalling pathway that have yet to be investigated in COPD. Of particular interest are the regulators of PI3K activity and PIP_3 generation (PTEN and SHIP1), which could potentially be dysregulated and a possible cause of increased PI3K activity in COPD neutrophils. Furthermore, new techniques have recently been developed by Dr. Phillip Hawkins that have been used to directly assess PIP_3 production (unpublished data). As PIP_3

production is catalysed by PI3K, this technique could provide a more direct quantification of PI3K activity in future studies of neutrophil migration.

6.3 PHARMACOLOGICAL CORRECTION OF ABERRANT NEUTROPHIL MIGRATION IN COPD

Current therapies for COPD are limited to symptom relief (primarily through bronchodilation) and the search for new strategies is ongoing. This thesis has demonstrated significant improvements in the accuracy of migrating neutrophils from COPD patients *in vitro* with two independent pharmacological interventions.

CXCR1 and CXCR2 inhibition did not seem to improve chemotaxis. In contrast, there was, generally, a marked reduction in chemokinesis, which in some instances actually resulted in concurrent reductions in chemotaxis. The inclusion of Sol as a chemotactic gradient in these assays was deemed essential. Although the vast majority of chemokines signal through CXCR2, there are a number of other chemoattractants present in lung secretions (such as LTB_4) that signal through other, specific receptors. Therefore, effects of CXCR1/2 inhibitors observed *in vitro* in response to IL-8 or CXCR2 ligands (such as GROα) may not fully represent their potential effects *in vivo*. The fact that effects of CXCR1/2 inhibition on chemokinesis and chemotaxis were generally less pronounced in Sol supports this conclusion. It is difficult to postulate whether or not simply reducing migratory speed would be beneficial or detrimental in COPD. If inhibition of CXCR1/2 reduces the number of neutrophils that initially enter the extracellular matrix and subsequently transmigrate, it would most likely reduce tissue damage and, therefore, be of therapeutic benefit. However, if the same number of neutrophils transmigrate at a slower speed with no improvement in

migratory accuracy, the amount of time each cell remains within the extracellular matrix should be increased. If the rate of proteinase release is unaltered, it could theoretically lead to increase in tissue destruction. It may be possible in future to compare the total number of neutrophils that migrate through 3D matrices with and without CXCR1/2 inhibition. In addition, the resulting density of the matrix could be assessed by micro-CT, a technique that has previously been used to detect early pathophysiological changes in the small airways (Hogg et al. 2013). For example, a lower matrix density following transmigration of neutrophils pre-treated with CXCR1/2 inhibitors would support the hypothesis that reduced transmigratory speed results in increased matrix destruction. However, this may be difficult given the chronic nature of COPD and the time it takes for detectable pathophysiological change to occur.

Specific γ- and δ-isoform inhibitors of PI3K improved chemotaxis, whereas the α- and β-isoform inhibitors did not. This seems logical as PI3K has been shown to regulate cell migration (Hannigan et al. 2002, Sapey et al. 2014) and the -γ and -δ isoforms are dominant in leukocytes (Ferguson et al. 2007, Martin et al. 2010). To our knowledge, this is the first study to investigate the effects of specific PI3K isoform inhibition on the migratory dynamics of COPD neutrophils. The results are promising as, if the improvement in the chemotaxis of COPD neutrophils observed here *in vitro* with PI3Kδ- and γ-isoform inhibitors is mirrored *in vivo*, this could prove an effective therapeutic option for COPD. Clinical trials may be useful to determine firstly, if the short-term treatment (for example 2-4 weeks) of COPD patients with these inhibitors can improve the migratory accuracy of isolated neutrophils *in vitro* and, secondly, if long-term treatment could slow the progression of COPD and improve other factors such as exacerbation frequency. An aerosolised PI3Kγ/δ inhibitor could certainly be

of benefit in COPD (Doukas et al. 2010). Delivery by this method could theoretically improve the migratory dynamics of neutrophils already within the pulmonary airspace and lead to more effective pathogenic location. However, it may not necessarily allow the inhibitor to sufficiently penetrate the alveolar-capillary membrane and improve the chemotaxis of neutrophils migrating through the extracellular matrix from the pulmonary circulation. Future clinical trials of PI3Kγ/δ inhibitors are certainly warranted in COPD, although they may require a more systemic approach such as an oral or intravenous mode of delivery to achieve the optimal therapeutic effect.

Simvastatin had a similar effect on neutrophil chemotaxis in COPD, where the inhibition of small GTPases appears to attenuate PI3K activity. Whether there is a concurrent over-activity of GTPases in the neutrophils of COPD patients has yet to be determined. Further studies could investigate the activity of small GTPase, such as the Ras and Rho. In addition, the Rho GTPase, Cdc42 is a key regulator of cell polarity and lamellipodia formation (Etienne-Manneville 2004). It has recently been shown that Cdc42 regulates neutrophil migration through an intricate mechanism of crosstalk between the Wiskott–Aldrich Syndrome Protein (WASp), CD11b, and microtubules (Kumar et al. 2012). Investigations of these mechanisms may provide further insight into the downstream effects of increased PI3K activity in COPD neutrophils and the possible effects on other cellular functions. For instance, a number the Rho GTPase superfamily members are also involved other neutrophil functions such as phagocytosis and superoxide production (Bokoch 2005). If GTPase activity is increased in COPD, it may explain the increased neutrophil phagocytosis (Burnett et al. 1987) and ROS production (Noguera et al. 2001) previously reported in COPD. Therefore, although simvastatin could potentially improve chemotaxis in COPD, it could possibly have

a detrimental effect on phagocytosis and ROS production. Hence, it would be important to assess the effect of simvastatin (and other pharmacological therapies that may influence GTPase activity) firstly on other neutrophil functions *in vitro* and, secondly, on factors such as exacerbation frequency and symptom scores in future clinical trials.

Simvastatin is already widely used for the treatment of hypercholesterolemia. Because of its long-standing therapeutic use, the side-effects and toxicity range are well documented. Simvastatin is considered a safe and well-tolerated drug and the majority of side-effects are relatively mild (abdominal pain, diarrhoea, indigestion). However, there are a number of drugs that are not recommended to be taken in conjunction with it due to potential adverse interactions. These include a number of commonly used antibiotics (erythromycin, clarithromycin) and antiarrhythmic drugs (amiodarone, verapamil) (FDA 2012). Our data suggest that future clinical trials investigating the effects of simvastatin on neutrophil migration should be with a high-dose (80 mg/day) in clinically stable COPD.

If, as is hypothesised, aberrant neutrophil migration in COPD results in exaggerated parenchymal destruction (due to increased migratory tracks) and impaired pathogenic location within the pulmonary airspace, the improvement of their migratory dynamics with therapies such as PI3Kγ/δ inhibitors and simvastatin could have hugely beneficial effects. These could include slowing the progression of the disease by reducing the tissue destruction (due to improved migrational accuracy through the extracellular matrix) as well as reducing exacerbation frequency (due to improved bacterial clearance). For clinical trials, monitoring outcomes (such as lung function, symptoms and exacerbation frequency) would be relatively simple but current literature suggests that in order to accurately detect an improvement in the

rate of lung function decline (with FEV_1 % predicted), 4 data points over 3 years are recommended (Vestbo et al. 2011).

6.4 THEORETICAL MODEL OF COPD PATHOGENESIS

A theoretical model of neutrophil migration in COPD and its influence on disease pathophysiology is shown in Figure 6.1. This model is predominantly based on findings from the current research project (including hypothetical PI3K over-activity) but also includes hypotheses based on published from our group and other groups.

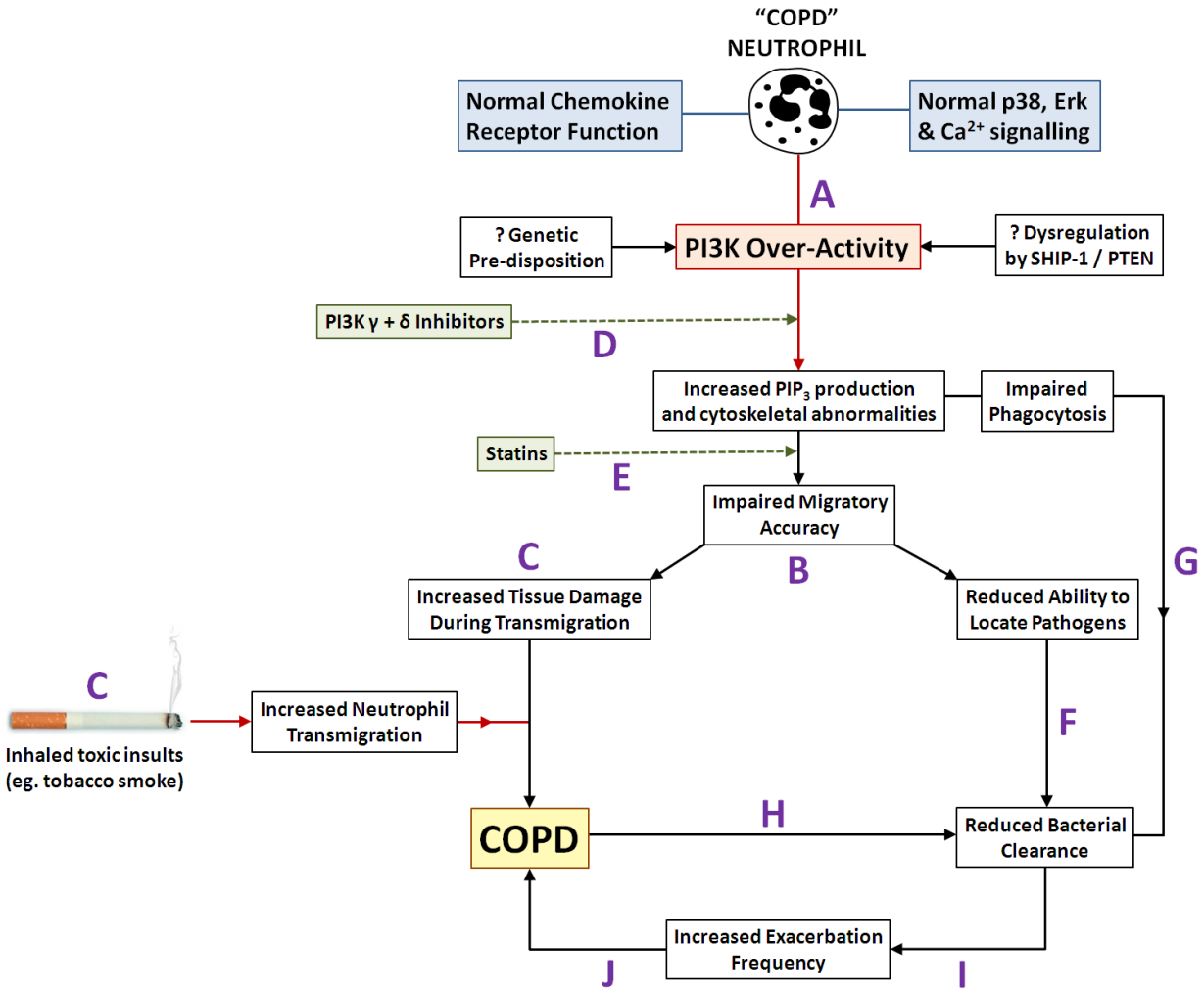

Figure 6.1: A simplified theoretical model of altered neutrophil migration in COPD.

Legend: Although COPD neutrophils appear to have normal chemokine receptor function, p38, Erk and Ca^{2+} signalling, they exhibit PI3K over-activity (A). This could be due to dysregulation (by SHIP1/PTEN) and individuals may be genetically pre-disposed. Increased PI3K activity appears to result in impaired migratory accuracy (B), possibly due to increased PIP_3 production and the loss of a PIP_3 gradient (Platek et al. 2007). If this effect is mirrored *in vivo*, it could increase collateral tissue damage during inaccurate transmigration. This may only induce significant pathological change if coupled with chronic inhalation of toxic particles (C). This thesis has demonstrated that both PI3K inhibitors (D) and Simvastatin (E) can improve chemotaxis and may, therefore, be therapeutically beneficial in slowing disease progress. Poor chemotaxis may also reduce the ability to locate (F) and phagocytose (G) pathogens, which could both impair bacterial clearance. Due to the morphological changes that occur with COPD, the mechanical ability of the pulmonary system to clear pathogens is also affected (H). Reduced bacterial clearance may then lead to increased exacerbation frequency (I), which can perpetuate COPD (J).

6.5 FUTURE WORK

This thesis has provided insight into a number of features of neutrophil-driven inflammation and migration in COPD. The focus has predominantly been on mechanisms of the aberrant neutrophil migration (specifically chemokinesis and chemotaxis). Although the majority of data have yielded negative results, the exclusion of these mechanisms is still important. However, the data have provided strong evidence that implicates increased PI3K-γ and -δ activity in impaired neutrophil chemotaxis in COPD and the potential benefits of PI3Kγ/δ inhibitors and simvastatin in its treatment. However, further studies are required to clarify the exact mechanisms involved downstream of PI3K activation to further define the involvement of this pathway in COPD. Further studies could include;

1. Assessment of three-dimensional neutrophil migration to determine if the aberrant migration observed in two-dimensional planes may still occur during migration through an extracellular matrix. In addition, the effects of therapies such as PI3K inhibitors, statins and CXCR1/2 antagonists could also be assessed in this way. This could be done either *in vitro* using gel matrices or *in vivo* if a suitable animal model (including a PI3K knock-up or knock-down model) is developed.

2. Measurement of the plasma concentration of pro-inflammatory cytokines (such as TNFα, IL-1β, IL-6 and IL-8) as well as other soluble inflammatory markers (such as CRP) in COPD patients to confirm the presence or absence of systemic inflammation and relate this to neutrophil migration.

3. A more detailed investigation to determine if the chemokine receptors that seem to mobilise effectively to the leading edge of polarised COPD neutrophils are actually located on the extending pseudopods. This could be done by fluorescence labelling of chemokine receptors and subsequent observation under a DIC microscope. DIC microscopy would allow for the observation of receptor location not only on fixed neutrophils but also on migrating neutrophils.

4. Assessment of the phosphorylation of additional PI3K subunits, with particular focus on the PI3Kγ p84/p101 regulatory and p110 catalytic subunits.

5. Assessment of gene mutations that encode the regulatory and catalytic subunits of PI3K.

6. The regulators of PI3K activity, SHIP1 and PTEN should also be investigated. These investigations should include western blotting of pSHIP1 and pPTEN, quantification of SHIP1/PTEN activity using commercial PIP_3-phosphatase activity assays and, finally, assessment of migration following treatment with SHIP1 and PTEN inhibitors. Work on SHIP1 phosphorylation and inhibition has already begun within our group and is ongoing.

7. Now that a method of directly assessing PIP_3 activity has been developed, it would be important to quantify this in COPD neutrophils as PIP_3 generation is directly mediated by PI3K and an important mediator of chemotaxis.

8. The data on the improvement in chemotaxis with simvastatin implicate small GTPase involvement in the aberrant neutrophil migration in COPD. Therefore, it would be important to confirm this with Small G-protein Activation Assays ("G-LISA"). It would also be important to determine if this is an effect of PI3K dysregulation or a separate phenomenon.

9. Clinical Trials of both PI3Kγ/δ inhibitors and simvastatin in COPD would be important to determine if the improvement in migratory accuracy of COPD neutrophils can be achieved following a short period of treatment with these therapies (compared to placebo). This principle of this trial is relatively simple, involving *in vitro* assessment of neutrophil migration before and after a short course of treatment. In the long-term, assessment of clinical outcomes (including measures of symptoms and physiology) following extended periods of treatment would be important to determine if these therapies truly have a clinical effect.

6.6 CONCLUSIONS

The studies within this thesis have provided novel evidence that peripheral neutrophils isolated from COPD patients have altered migratory dynamics. They consistently migrate with increased speed and reduced accuracy in a diverse array of chemotactic gradients. This appears to be an intrinsic cell defect that is not influenced by the systemic COPD environment, although it is unclear if this defect is congenital or influenced by chronic inflammatory insult. The aberrant neutrophil migration observed could theoretically affect the pathogenesis of COPD by delaying the inflammatory response, increasing collateral tissue destruction (due to longer migratory tracks through the extracellular matrix) and impairing bacterial clearance in the lungs. The data have also provided evidence for potential intracellular signalling mechanisms (while excluding others) through which pharmacological manipulation can improve the migratory accuracy of COPD neutrophils. This evidence will hopefully lead to future clinical trials of novel therapies that may be of benefit in COPD.

CHAPTER 7

REFERENCES

Adcock IM, Chung KF, Caramori G, and Ito K. Kinase inhibitors and airway inflammation. *Eur J Pharmacol* 2006; 533: 118–132.

Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* 1999; 17: 593–623.

Afford SC, Pongracz J, Stockley RA, et al. The induction by human interleukin-6 of apoptosis in the promonocytic cell line U937 and human neutrophils. *J Biol Chem* 1992; 267: 21612–21616.

Agarwal S, Busse PJ. Innate and adaptive immunosenescence. *Ann Allergy Asthma Immunol* 2010; 104: 183-190.

Aggarwal S, Gupta S. Increased apoptosis of T cell subsets in aging humans: altered expression of Fas (CD95), Fas ligand, Bcl-2, and Bax. *J Immunol* 1998; 160: 1627–1637.

Agusti AG. COPD, a multicomponent disease: implications for management. *Respir Med* 2005; 99: 670-682.

Akbar AN, Fletcher JM. Memory T cell homeostasis and senescence during aging. *Curr Opin Immunol* 2005; 17(5): 480-5.

Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; 124: 783–801.

Albelda SM, Smith CW and Ward PA. Adhesion molecules and inflammatory injury. *FASEB J* 1994; 8: 504–512.

Alkemade JA, Molhuizen HO, Ponec M, et al. SKALP/elafin is an inducible proteinase inhibitor in human epidermal keratinocytes. *J Cell Sci* 1994; 107: 2335-2342.

Alkemade HA, de Jongh GJ, Arnold WP, et al. Levels of skin-derived antileukoproteinase (SKALP)/elafin in serum correlate with disease activity during treatment of severe psoriasis with cyclosporin A. *J Invest Dermatol* 1995; 104: 189-193.

Allbritton N, Meyer T, Stryer L. Range of messenger action of calcium ions and inositol trisphosphate. *Science* 1992; 258: 1812–1814.

Allen LA, Aderem A. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J Exp Med* 1996; 184: 627–637.

Allport JR, Ding HT, et al. L Selectin shedding does not regulate human neutrophil attachment, rolling or transmigration across human vascular endothelium in vitro. *J Immunol* 1997; 158: 4365-4372.

Alon R, Chen S, Puri KD, et al. The kinetics of L-selectin tethers and the mechanics of selectin-mediated rolling. *J Cell Biol* 1997; 138: 1169–1180.

Alonso-Fernandez P, Puerto M, Maté I, et al. Neutrophils of centenarians show function levels similar to those of young adults. *J Am Geriatr Soc* 2008; 56: 2244–2251.

Alpin AE, Howe S, Alahari SK, Juliano RL. Signal transduction and signal modulation by cell adhesion receptors: The role of integrins, cadherins, immunoglobulin- cell adhesion molecules, and selectins. *Pharmacol Rev* 1998; 50: 197–263.

Altenburg J, de Graaff CS, van der Werf TS, Boersma WG: Immunomodulatory effects of macrolide antibiotics. 1. Biological mechanisms. *Respiration* 2011; 81: 67-74.

Altenburg J, de Graaff CS, van der Werf TS, Boersma WG: Immunomodulatory effects of macrolide antibiotics. 2. Advantages and disadvantages of long-term, low-dose macrolide therapy. *Respiration* 2011; 81: 75-87.

Ambruso DR, Cusack N, Thurman G. NADPH oxidase activity of neutrophil specific granules: requirements for cytosolic components and evidence of assembly during cell activation. *Mol Genet Metab* 2004; 81: 313-321.

Amitani R, Wilson R, Rutman A, Read R, Ward C, Burnett D, et al. Effects of human neutrophil elastase and Pseudomonas aeruginosa proteinases on human respiratory epithelium. *Am J Respir Cell Mol Biol* 1991; 4(1): 26-32.

Ananthanarayanan B, Ni Q, Zhang J. Signal propagation from membrane messengers to nuclear effectors revealed by reporters of phosphoinositide dynamics and Akt activity. *Proc Nat Acad Sci USA* 2005; 102: 15081–6.

Anderson D, MacNee W. Targeted treatment in COPD: A multi-system approach for a multi-system disease. *Int J Chron Obstruct Pulmon Dis* 2009; 4: 321-335.

Anderson KE, Boyle KB, Davidson K, et al. CD18-dependent activation of the neutrophil NADPH oxidase during phagocytosis of Escherichia coli or Staphylococcus aureus is regulated by class III but not class I or II PI3Ks. *Blood* 2008; 112: 5202–5211.

Andrew N, Insall R. Chemotaxis in shallow gradients is mediated independently of PtdIns-3-kinase by biased choices between random protrusions. *Nat Cell Biol* 2007; 9: 193-200.

Angulo I, Vadas O, Garçon F, et al. Phosphoinositide 3-kinase δ gene mutation predisposes to respiratory infection and airway damage. *Science* 2013; 342: 866-871.

Anisowicz A, Bardwell L, Sager R. Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. *Proc Natl Acad.Sci USA* 1987; 84: 7188–92.

Antonaci S, Jirillo E, Ventura MT, et al. Non-specific immunity in aging: deficiency of monocyte and polymorphonuclear cell-mediated functions. *Mech. Ageing Dev* 1984; 24: 367–375.

Aoshiba K, Yasuda K, Yasui S, Tamaoki J, Nagai A**.** Serine proteinases increase oxidative stress in lung cells. *Am J Physiol Lung Cell Mol Physiol* 2001; 281: L556-L64.

Araki N, Johnson MT, Swanson JA. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* 1996; 135: 1249–1260.

ARTP. Practical Handbook of Respiratory Function Testing. Eds: Hill SL, Newall C. *Pontaprint* 1999.

Asagoe K, Yamamoto K, Takahashi A, et al. Down-regulation of CXCR2 expression on human polymorphonuclear leukocytes by TNF-alpha. *J Immunol* 1998; 160: 4518-4525.

Babior BM. NADPH oxidase: an update. *Blood* 1999; 93: 1464–1476.

Babior BM. Phagocytes and oxidative stress. *Am J Med* 2000; 109: 33–44.

Ball DI, Brittain RT, Coleman RA, et al. Salmeterol, a novel, long-acting beta 2-adrenoceptor agonist: characterization of pharmacological activity in vitro and in vivo. *Br J Pharmacol* 1991; 104: 665–671.

Ballieux BE, Hiemstra PS, Klar-Mohamad N, Hagen EC, Van Der Woude FJ, Daha MR**.** Detachment and cytolysis of human endothelial cells by proteinase 3. *Eur J Immunol* 1994; 24: 3211-5.

Banda MJ, Clark EJ, Werb Z. Limited proteolysisby macrophage elastase inactivates human α_1-proteinase inhibitor. *J Exp Med* 1980; 152: 1563-1570.

Banner KH, Page CP. Theophylline and selective phosphodiesterase inhibitors as anti-inflammatory drugs in the treatment of bronchial asthma. *Eur Respir J* 1995; 8: 996–1000.

Barber DF, Bartolomé A, Hernandez C, et al. PI3Kgamma inhibition blocks glomerulonephritis and extends lifespan in a mouse model of systemic lupus. *Nat Med* 2005; 11: 933–935.

Barnes PJ. Theophylline in chronic obstructive pulmonary disease: new horizons. *Proc Am Thorac Soc* 2005; 2: 334–339; discussion 340–331.

Barnes PJ**.** New approaches to COPD. *Eur Respir Rev* 2005; 14: 2-11.

Barnes PJ, Celli BR. Systemic manifestations and comorbidities of COPD. *Eur Respir J* 2009; 33: 1165–1185.

Battram C, Charlton SJ, Cuenoud B, et al. In vitro and in vivo pharmacological characterization of 5-[(R)-2-(5,6-diethyl-indan-2-ylamino)-1-hydroxy-ethyl]-8-hydroxy-1H-quinolin-2-o ne (indacaterol), a novel inhaled beta(2) adrenoceptor agonist with a 24-h duration of action. *J Pharmacol Exp Ther* 2006; 317: 762–70.

Becker S, Quay J, Koren HS, Haskill JS. Constitutive and stimulated MCP-1, GRO alpha, beta, and gamma expression in human airway epithelium and bronchoalveolar macrophages. *Am J Physiol* 1994; 266(3 Pt 1): L278–86.

Beemiller, P, Hoppe AD, Swanson JA. A phosphatidylinositol-3-kinase-dependent signal transition regulates ARF1 and ARF6 during Fcgamma receptor-mediated phagocytosis. *PLoS Biol* 2006; 4: e162.

Ben-Barach A, Grimm M, et al. The different ability of IL-8 and neutrophilactivating peptide-2 to induce attentuation of chemotaxis is mediated by their divergent capabilities to phosphorylate CXCR2 (IL-8 receptor B). *J Immunol* 1997; 158: 5927-5933.

Berger M, Sorensen RU, Tosi MF, et al. Complement receptor expression on neutrophils at an inflammatory site, the Pseuodmonas-infected lung in cystic fibrosis. *J Clin Invest* 1989; 84: 1302-13.

Berditchevski F, Tolias KF, Wong K, et al. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J Biol Chem* 1997; 272: 2595-2598.

Berditchevski F. Complexes of tetraspanins with integrins: more than meets the eye. *J Cell Sci* 2001; 114(Pt 23): 4143-4151.

Bevilacqua MP and Nelson RM. Selectins. *J Clin Invest* 1993; 91: 379–387.

Blackwood CE, Hosannah Y, Perman E, *et al*. Elasteolytic titre of inducing enzyme as determinant of the response. *Proc Soc Exp Biol Med* 1973; 144: 450-454.

Blanco-Colio LM, Tunon J, et al. Anti-inflammatory and immunomodulatory effects of statins. *Kidney Int* 2003; 63: 12-23.

Boehringer Ingelheim GmbH. *Annual Report 2011*. Ingelheim: Boehringer Ingelheim GmbH; 2011. http://www.boehringer-ingelheim.com/content/dam/internet/opu/com_EN/document/01_news/08_APC/APC_2012/BoehringerIngelheim_Annual_Report_2011_complete.pdf.

Bokoch GM. Regulation of innate immunity by Rho GTPases. *Trends Cell Biol* 2005; 15: 163-171.

Booth FW, Gordon SE, Carlson CJ, et al. Waging war on modern chronic diseases: primary prevention through exercise biology. *J Appl Physiol* 2000; 88: 774–787.

Borregaad N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* 1997; 89: 3503-3521.

Botelho RJ, Teruel M, Dierckman R, Anderson R, Wells A, York JD, et al. Localised biphasic changes in phosphatidylinositol-4,5-biphosphate at sites of phagocytosis. *JCB* 2000; 151: 1353-68.

Boureux A, Vignal E, Faure S, Fort P. Evolution of the Rho family of ras-like GTPases in eukaryotes *Mol Biol Evol* 2007; 24: 203–16.

Boyd CM, Darer J, Boult C, et al. Clinical practice guidelines and quality of care for older patients with multiple comorbid diseases: implications for pay for performance. *JAMA* 2005; 294: 716–24.

Boyer JL, Waldo GL, Harden TK. βγ-subunit activation of G-protein-regulated phospholipase C. *J Biol Chem* 1992; 267: 25451–25456.

Braga PC, Sala MT, Dal Sasso M, et al. Influence of age on oxidative bursts (chemiluminescence) of polymorphonuclear neutrophil leukocytes. *Gerontology* 1997; 44: 192.

Brantly ML, Paul LD, Miller BH, et al. Clinical features and history of the destructive lung disease associated with α_1-antitrypsin deficiency of adults with pulmonary symptoms. *Am Rev Respir Dis* 1988; 138: 327-336.

Brock C, Schaefer M, Reusch HP, et al. Roles of G beta gamma in membrane recruitment and activation of p110 gamma/p101 phosphoinositide 3-kinase gamma. *J Cell Biol* 2003; 160: 89-99.

Brown GE, Stewart MQ, Liu H, Ha VL, Yaffe MB. A novel assay system implicates PtdIns(3,4)P(2), PtdIns(3)P, and PKC delta in intracellular production of reactive oxygen species by the NADPH oxidase. *Mol Cell* 2003; 11: 35-47.

Broekhuizen R, Grimble RF, Howell WM, et al. Pulmonary cachexia, systemic inflammatory profile, and the interleukin 1b-511 single nucleotide polymorphism. *Am J Clin Nutr* 2005; 82: 1059e64.

BTS guidelines for the management of chronic obstructive pulmonary disease. The COPD Guidelines Group of the Standards of Care Committee of the BTS. *Thorax* 1997; 52: S1–28.

Buist AS, McBurnie MA, et al. International variation in the prevalence of COPD (the BOLD study): a population-based prevalene study. *Lancet* 2007; 370: 741-750.

Burelout C, Naccache PH, Bourgoin SG. Dissociation between the translocation and the activation of Akt in fMLP-stimulated human neutrophils–effect of prostaglandin E2. *J Leukoc Biol* 2007; 81: 1523–34.

Burnett D, Crocker J, Stockley RA. Cathepsin B-like cysteine proteinase activity in sputum and immunohistologic identification of cathepsin B in alveolar macrophages. *Am Rev Respir Dis* 1983; 128: 915-919.

Burnett D, Chamba A, et al. Neutrophils from subjects with chronic obstructive lung disease show enhanced chemotaxis and extracellular proteolysis. *Lancet* 1987; 2(8567): 1043-1046.

Burnett D, Afford SC, Campbell EJ, et al. Evidence for lipid-associated serine proteases and metalloproteases in human bronchoalveolar lavage fluid. *Clin Sci (Lond)* 1988; 75: 601-607.

Burnett D. Neutrophils and the pathogenesis of COPD in Stockley, R.A., editor. Pulmonary defences. *WileyEurope, Chichester* 1997; 113-126.

Burns MJ, Walker DC, et al. Neutrophil transendothelial migration is independent of tight junctions and occurs preferentially at tricellular corners. *J Immunol* 1997; 159: 2893-2903.

Bustelo XR, Sauzeau V, Berenjeno IM. GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *BioEssays* 2007; 29: 356–370.

Butcher S, Chahel H, Lord JM. Aging and the neutrophil: no appetite for killing? *Immunology* 2000; 100: 411–416.

Butler C, Kleinerman J. Capillary density: alveolar diameter, a morphometric approach to ventilation and perfusion. *Am Rev Respir Dis* 1970; 102: 886-894.

Buttle DJ, Abrahamson M, Burnett D, et al. Human sputum cathepsin B degrades proteoglycan, is inhibited by α_2-macroglobulin and is modulated by neutrophil elastase cleavage of cathepsin B precursor and cystatin C. *Biochem J* 1991; 276: 325-331.

Calverley PM, Sanchez-Toril F, McIvor A, et al. Effect of 1-year treatment with roflumilast in severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2007; 176(2): 154–161.

Calverley PM, Rabe KF, Goehring UM, et al. Roflumilast in symptomatic chronic obstructive pulmonary disease: two randomised clinical trials. *Lancet*. 2009 ;374(9691): 685–694.

Campbell EJ, Campbell MA, Boukedes SS, et al. Quantum proteolysis by neutrophils: implications for pulmonary emphysema in α_1-antitrypsin deficiency. *J Clin Invest* 1999; 104: 337-344.

Camps M, Rückle T, Ji H, et al. Blockade of PI3Kgamma suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat Med* 2005; 11: 936-943.

Cantin A, Crystal RG. Oxidants, antioxidants and the pathogenesis of emphysema. *Eur J Respir Dis Suppl* 1985; 139: 7-17.

Carman CV, Sage PT, Sciuto TE, et al. Transcellular diapedesis is initiated by invasive podosomes. *Immunity* 2007; 26: 784–97.

Carman CV. Mechanisms for transcellular diapedesis: probing and pathfinding by invadosome-like protrusions. *J Cell Sci* 2009; 122: 3025–35.

Carpenter CL, Duckworth BC, Auger KR, et al. Purification and characterization of phosphoinositide 3-kinase from rat liver. *J Biol Chem* 1990; 265: 19704–19711.

Caswell PT, Vadrevu S, Norman JC**.** Integrins: masters and slaves of endocytic transport. Nature Reviews: *Molecular Cell Biology* 2009; 10: 843-53.

Cazzola M, Matera MG. Novel long-acting bronchodilators for COPD and asthma. *Br J Pharmacol* 2008; 155: 291–299.

Celli BR, Cote CG, Marin JM, et al. The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. *N Engl J Med* 2004; 350: 1005–12.

Cepinskas G, Noseworthy R, Kvietys PR. Transendothelial neutrophil migration. *Circulation Research* 1997; 81: 618-626.

Chanock SJ, El-Benna J, Smith RM, Babior BM. The respiratory burst oxidase. *J Biol Chem* 1994; 269: 24519–24522.

Chapman RW, Minnicozzi M, Celly CS, et al. A novel, orally active CXCR1/2 receptor antagonist, Sch527123, inhibits neutrophil recruitment, mucus production, and goblet cell hyperplasia in animal models of pulmonary inflammation. *J Pharmacol Exp Ther* 2007; 322: 486-493.

Charest PG, Firtel RA**.** Big role for small GTPases in the control of directed cell movement. *Biochem J* 2007; 401: 377-90.

Chen HC. Boyden chamber assay. *Methods Mol Biol* 2005; 294: 15-22.

Chin D, Means AR. Calmodulin: a prototypical calcium sensor. *Trends Cell Biol* 2000; 10: 322–328.

Chuntharapai A, Kim KJ. Regulation of the expression of IL-8 receptor A/B by IL-8; possible functions for each receptor. *J Immunol* 1995; 155: 2587-2594.

Church DF, Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect* 1985; 64: 111-126.

Clapham DE. Calcium signalling. *Cell* 2007: 131; 1047-1058.

Clark AJ, Petty HR. Observation of calcium microdomains at the uropod of living morphologically polarized human neutrophils using flash lamp-based fluorescence microscopy. *Cytometry A* 2008; 73: 673-678.

Clayton, A, Evans RA, et al. Cellular activation through the ligation of intercellular adhesion molecule 1. *J Cell Sci* 1998; 111: 443-453.

Condliffe AM, Davidson K, Anderson KE, et al. Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils. *Blood* 2005; 106: 1432–1440.

Corre F, Lellouch J, et al. Smoking and leucocyte counts: results of an epidemiological study. *Lancet* 1971; 2: 632-634.

Corsini E, Di Paolo R, Viviani B, et al. Increased carrageenan-induced acute lung inflammation in old rats. *Immunology* 2005; 115: 253-261.

Cortijo J, Bou J, Beleta J, et al. Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with human bronchus. *Br J Pharmacol* 1993; 108: 562–568.

Cosentino C, Di Domenico M, Porcellini A, et al. p85 regulatory subunit of PI3K mediates cAMP-PKA and estrogens biological effects on growth and survival. *Oncogene* 2007; 26: 2095-2103.

Cox D, Berg JS, Cammer M, et al. Myosin X is a downstream effector of PI(3)K during phagocytosis. *Nat Cell Biol* 2002; 4: 469–477.

Crapo, J. D., B. E. Barry, et al. Cell number and cell characteristics of the normal human lung. *Am Rev Respir Dis* 1982; 126: 332-337.

Crapo RO. The aging lung. In: Mahler DA ed. *Pulmonary disease in the elderly patient*. New York: Marcel Dekker Incl.; 1993: 1-25.

Criner GJ, Connett JE, Aaron SD, et al. Simvastatin for the prevention of exacerbations in moderate-to-severe COPD. *N Engl J Med* 2014; 370: 2201-2210.

Crockett-Torabi A, Fantone JC. The selectins: Insights into selectin-induced intracellular signaling in leukocytes. *Immunol Res* 1995; 14: 237–251.

Csernok E, Lüdemann J, Gross WL, and Bainton DF. Ultrastructural localization of proteinase 3, the target antigen of anti-cytoplasmic antibodies circulating in Wegener's granulomatosis. *Am J Pathol* 1990; 137:1113–1120.

Dai Y, Khanna P, et al. Statins synergistically potentiate 7-hydroxystaurosporine (UCN-01) lethality in human leukemia and myeloma cells by disrupting Ras farnesylation and activation. *Blood* 2007; 109: 4415-4423.

Damiano VV, Tsang A, Kucich U, et al. Immunolocalization of elastase in human emphysematous lungs. *J Clin Invest* 1986; 78: 482-493.

Dancey JT, Deubelbeiss KA, Harker LA, et al.. Neutrophil kinetics in man. *J Clin Invest.* 1976;58 (3):705-715.

Dangerfield J, Larbi KY, et al. PECAM-1 (CD31) homophillic interaction up-regulates alpha-6 beta-1 on transmigrated neutrophils in vivo and plays a functional role in the ability of alpha-6 integrins to mediate leukocyte migration through the perivascular basement membrane. *J Exp Med* 2002; 196: 1201-1211.

Darlington GJ, Astrin KH, et al. Assignment of human alpha 1-antitrypsin to chromosome 14 by somatic cell hybrid analysis. *Proc Natl Acad Sci USA* 1982; 79: 870-873.

Davenpeck KL, Steeber DA, Tedder TF, Bochner BS. P- and L-selectin mediate distinct but overlapping functions in endotoxin-induced leukocyteendothelial interactions in the rat mesenteric microcirulation. *J Immunol* 1997; 159: 1977–1986.

Declaux C, Delacourt C, et al. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol* 1996; 14: 288-295.

Decramer M, Rennard S, Troosters T, et al. COPD as a lung disease with systemic consequences-clinical impact, mechanisms, and potential for early intervention. *COPD* 2008; 5: 235–56.

Deforge LE. Preston AM, et al. Regulation of interleukin 8 gene expression by oxidant stress. *J Biol Chem* 1993; 268: 25568-25576.

De La Fuenta M. Changes in macrophage function with aging. *Comp Biochem Physiol A* 1985; 8: 935-938.

Della Bella S, Bierti L, Presicce P, et al. Peripheral blood dendritic cells and monocytes are differently regulated in the elderly. *Clin Immunol* 2007; 122:220-228.

Del Rio L, Bennouna S, Salinas J, Denkers EY**.** CXCR2 deficiency confers impaired neutrophil recruitment and increased susceptibility during Toxoplasma gondii infection. *J Immunol* 2001; 167: 6503-6509

Devreotes P, Janetopoulos C. Eukaryote chemotaxis; distinctions between directional sensing and polarisation. *J Biol Chem* 1988; 128: 20445 - 20448.

De Water R, Willems LN, et al. Ultrasound localisation of bronchial antileukoproteinase in central and peripheral human airways by a gold-labeling technique using monoclonal antibodies. *Am Rev Respir Dis* 1986; 133: 882-890.

Dewitt S, Laffafian I, Hallett MB**.** Phagosomal oxidative activity during B2 integrin mediated phagocytosis by neutrophils is triggered by a non-restricted Ca2+ signal: Ca2+ controls time not space. *JCS* 2003; 116: 2857-65.

Diamond MS, Staunton DE, et al. (1990). ICAM-1 (CD-54): a counter receptor for MAC-1 (CD11b/CD18). *J Cell Biol* 1990; 111: 3129-3139.

Diamond MS, Springer TA. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. *J Cell Biol* 1993; 120: 545–556.

DiMango E, Zar HJ, et al. Diverse Pseudomonas aeruginosa gene products stimulate respiratory epithelial cells to produced interleukin 8. *J Clin Invest* 1995; 96: 1485-1490.

Dobler C, Wong K, et al. Associations between statins and COPD: a systematic review. *BMC Pulmonary Medicine* 2009; 9: 32.

Doll R, Peto R, et al. Mortality in relation to smoking: 40 years' observations on male British doctors. *BMJ* 1994; 309: 901-911.

Doukas J, Wrasidlo W, Noronha G, et al. Phosphoinositide 3-kinase gamma/delta inhibition limits infarct size after myocardial ischemia/reperfusion injury. *Proc Natl Acad Sci U S A* 2006; 103: 19866–19871.

Doukas J, Eide L, Stebbins K, et al. Aerosolized phosphoinositide 3-kinase gamma/delta inhibitor TG100-115 [3-[2,4-diamino-6-(3-hydroxyphenyl)pteridin-7-yl]phenol] as a therapeutic candidate for asthma and chronic obstructive pulmonary disease. *J Pharmacol Exp Ther* 2010; 328: 758-765.

Dourdin N, Bhatt AK, Dutt P, Greer et al. Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. *J Biol Chem* 2001; 276: 48382–48388.

Drost EM, MacNee W. Potential role of IL-8, platelet activating factor and TNF alpha in the sequestration of neutrophils in the lung; effects on neutrophil deformability, adhesion receptor expression and chemotaxis. *Eur J Immunol* 2002; 32: 393-403.

Eaton SM, Burns EM, Kusser K, et al. Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. *J Exp Med.* 2004; 200: 1613–1622.

Eccles KA, Sowden H, Porter KE, et al. Simvastatin alters human endothelial cell adhesion molecule expression and inhibits leukocyte adhesion under flow. *Atherosclerosis* 2008; 200: 69-79.

Eddy RJ, Pierini LM, Matsumura F, et al. Ca2+-dependent myosin II activation is required for uropod retraction during neutrophil migration. *J Cell Sci* 2000; 113: 1287–1298.

Edwards R. The problem of tobacco smoking. *BMJ* 2004; 328: 217–219.

Edwards SW. Biochemistry and physiology of the neutrophil. *Cambridge University Press* 2005; 2: 33-76.

El-Benna J, Dang PM, Gougerot-Pocidalo MA, Elbim C. Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses. *Arch Immunol Ther Exp (Warsz)* 2005; 53: 199–206.

Effect of Roflumilast on Exacerbation Rate in Patients With Chronic Obstructive Pulmonary Disease (COPD): The AURA Study (BY217/M2-124).

Effect of Roflumilast on Exacerbation Rate in Patients With Chronic Obstructive Pulmonary Disease (COPD): The HERMES Study (BY217/M2-125).

Effect of Roflumilast on Exacerbation Rate in Patients With Chronic Obstructive Pulmonary Disease. Ratio-Study. (BY217/M2-112).

Ekberg-Jansson A, Andersson B, Bake B, Boijsen M, Enanden I, Rosengren A, et al. Neutrophil-associated activation markers in healthy smokers relates to a fall in DL(CO) and to emphysematous changes on high resolution CT. *Respir Med* 2001; 95: 363-73.

Ellenbroek S, Collard J. RhoGTPases: functions and association with cancer. *Clin Exp Metastasis* 2007; 24: 657–672.

Elliott W, Hayashi S, Hogg JC. Immunodetection of adenoviral E1A proteins in human lung tissue. *Am J Respir Cell Mol Biol* 1995; 12: 642-648.

Esparza B, Sanchez H, et al. Neutrophil function in elderly persons assessed by flow cytometry. *Immunol Invest* 1996; 25: 185-190.

Etienne-Manneville S. Cdc42–the centre of polarity. *J Cell Sci.* 2004; 117: 1291-1300.
European Medicines Agency. Daxas: EPAR – Product Information 2010.

Evans MJ, Cabral LJ, et al. Transformation of alveolar type 2 cells to type 1 cells following exposure to NO2. *Exp Mol Pathol* 1975; 22: 142-150.

Evans JH, Falke JJ. Ca2+ influx is an essential component of the positive-feedback loop that maintains leading-edge structure and activity in macrophages. *Proc Natl Acad Sci USA* 2007; 104: 16176-16181.

Fabbri LM, Rabe KF. From COPD to chronic systemic inflammatory syndrome? *Lancet* 2007; 370: 797–799.

Fabbri LM, Luppi F, Beghe B, et al. Complex chronic comorbidities of COPD. *Eur Respir J* 2008; 31: 204–12.

Fabbri LM, Calverley PM, Izquierdo-Alonso JL, et al. Roflumilast in moderate-to-severe chronic obstructive pulmonary disease treated with longacting bronchodilators: two randomised clinical trials. *Lancet*. 2009; 374(9691): 695–703.

Fais S, Malorni W. Leukocyte uropod formation and membrane/cytoskeleton linkage in immune interactions. *J Leuk Biol* 2003; 73: 556-563.

Falagas ME, Makris GC, et al. Statins for infection and sepsis: a systematic review of the clinical evidence. *J Antimicrob Chemother* 2008; 61: 774-785.

Fan GH, Lapierre LA, et al. Differential regulation of CXCR2 trafficking by Rab GTPases. *Blood* 2003; 101: 2115-2124.

Farooq SM, Stillie R, Svensson M, et al**.** Therapeutic effect of blocking CXCR2 on neutrophil recruitment and dextran sodium sulfate induced colitis. *JPET* 2009; 329: 123–129.

FDA. Zocor (simvastatin) tablets 2012. From;
http://www.fda.gov/Safety/MedWatch/SafetyInformation/ucm208610.htm.

Feng D, Nagy JA, Pyne K, et al. Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. *J Exp Med* 1998; 187: 903–15.

Ferguson GJ, Milne L, Kulkarni S, et al. PI(3)Kgamma has an important context-dependent role in neutrophil chemokinesis. *Nat Cell Biol* 2007; 9: 86–91.

Ferrandi C, Ardissone V, Ferro P, et al. Phosphoinositide 3-kinase gamma inhibition plays a crucial role in early steps of inflammation by blocking neutrophil recruitment. *J Pharmacol Exp Ther* 2007; 322: 923-930.

Ferry G, Lonchampt M, Pennel L, et al. Activation of MMP-9 by neutrophil elastase in an *in vivo* model of acute lung injury. *FEBS Lett* 1997; 402: 111-115.

Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signalling molecules regulating neutrophil function. *Free Radic Biol Med* 2007; 42: 153-164.

Fietta A, Merlini C, De Bernardi PM, et al. Non specific immunity in aged healthy subjects and in patients with chronic bronchitis. *Aging (Milano)* 1993; 5: 357-361.

Finan PM and Thomas MJ. PI 3-kinase inhibition: a therapeutic target for respiratory disease. *Biochem Soc Trans* 2004; 32: 378–382.

Finger EB, Bruehl RE, Bainton DF, Springer TA. A differential role for cell shape in neutrophil tethering and rolling on endothelial selectins under flow. *J Immunol* 1996; 157: 5085–5096.

Fortin CF, Larbi A, Lesur O, et al. GM-CSF activates the Jak/STAT pathway to rescue polymorphonuclear neutrophils from spontaneous apoptosis in young but not elderly individuals. *Biogerontology* 2007; 8: 173-187.

Fortin CF, McDonald PP, Lesur O, et al. Aging and neutrophils: there is still much to do. *Rejuvenation Res* 2008; 11: 873-882.

Foxman EF, Campbell JJ, et al. Multi-step navigation and the combinatorial control of leukocyte chemotaxis. *J Cell Biol* 1997; 139: 1349-1359.

Franceschi C, Bonafe M, Valensin S, et al. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci* 2000; 908: 244-254.

Frieser M, Hallmann R, Johansson S, et al. Mouse polymorphonuclear granulocyte binding to extracellular matrix molecules involves beta 1 integrins. *Eur J Immunol* 1996; 26: 3127–3136.

Fritsch R, Downward J. Snapshot: Class I PI3K isoform signalling. *Cell* 2013; 154: 940-940.

Fromigue O, Hay E, et al. RhoA GTPase inactivation by statins induces osteosarcoma cell apoptosis by inhibiting p42/p44-MAPKs-Bcl-2 signaling independently of BMP-2 and cell differentiation. *Cell Death Differ* 2006; 13: 1845-1856.

Fukuchi Y, Nishimura M, et al. COPD in Japan: the Nippon COPD epidemiology study. *Respirology* 2004; 9: 458-465.

Fukuchi Y. The aging lung and chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2009; 6: 570-572.

Fulop T, Larbi A, et al. Signal transduction and functional changes in neutrophils with aging. *Aging Cell* 2004; 3: 217-226.

Fulop T, Larbi A, Wikby A, et al. Dysregulation of T-cell function in the elderly: scientific basis and clinical implications. *Drugs Aging* 2005; 22: 589–603.

Fulop T Jr, Fóris G, Wórum I, Leövey A. Age-dependent alterations of Fcg receptor-mediated effector functions of human polymorphonuclear leucocytes. *Clin Exp Immunol* 1985; 61: 425–432.

Fulop T Jr, Seres I. Signal transduction changes in granulocytes and lymphocytes with aging. *Immunol Lett* 1994; 40: 259.

Galanos C, Freudenberg MA. Mechanisms of endotoxin shock and endotoxinhypersensitivity. *Immunobiology* 1993; 187: 346–356.

Gettins PG. Serpin structure, mechanism, and function. *Chem Rev* 2002; 102(12): 4751–804.

Gillham H, Golding MC, Pepperkok R. Intracellular movement of green fluorescent protein-tagged phosphatidylinositol 3-kinase in response to growth factor receptor signaling. *J Cell Biol* 1999; 146: 869–880.

Gishen P, Saunders AJ, Tobin MJ, et al. α_1-Antitrypsin deficiency: the radiological features of pulmonary emphysema in in subjects of Pi type Z and Pi type SZ: a survey by the British Thoracic Association. *Clin Radiol* 1982; 33: 371-377.

Global Initiative for Chronic Obstructive Lung Disease. Guidelines and resources 2007; www.goldcopd.com/index.

Global Strategy for Diagnosis, Management, and Prevention of COPD. [Updated 2009]. http://www.goldcopd.com/Guidelineitem.asp?l1=2&l2=1&intId=2003.

Global Initiative for Chronic Obstructive Lung Disease (GOLD). Global strategy for the diagnosis, management, and prevention of COPD [webpage on the Internet]. Vancouver, WA: Global Initiative for Chronic Obstructive Lung Disease; 2011 [updated Dec 2011]. http://www.goldcopd.org/uploads/users/files/GOLD_Report_2013.pdf.

Global Initiative for Chronic Obstructive Lung Disease (GOLD). Global strategy for the diagnosis, management and prevention of chronic pulmonary disease, 2011. http://www.goldcopd.org/Guidelines/guidelines-resources.html.

Godal, G, Hang L, et al. Transepithelial neutrophil migration is CXCr1 dependent in vitro and is defective in IL8 receptor knock out mice. *J Immunol* 2000; 165: 5287-5294.

Godtfredsen NS, Lam TH, Hansel TT, et al. COPD related morbidity and mortality after smoking cessation: status of the evidence. *Eur Respir J* 2008; 32: 844-853.

Gold PM. The 2007 GOLD Guidelines: a comprehensive care framework. *Respir Care* 2009; 54: 1040-1049.

GOLD Workshop Report (Updated 2008): Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease. www.goldcopd.com.

Gomez-Mouton C, Lacelle RA, et al. Segregation of leading edge and uropod components into specific lipid rafts during T cell polarization. *Proc Natl Acad Sci USA* 2001; 98: 9642-9647.

Green GM, Jakab GJ, Low RB, et al. Defense mechanisms of the respiratory membrane. *Am Rev Respir Dis* 1977; 115: 479-514.

Groemping Y, Rittinger K. Activation and assembly of the NADPH oxidase: a structural perspective. *Biochem J* 2005; 386: 401–416.

Grommes J, Vijayan S, Drechsler M, et al. Simvastatin reduces endotoxin-induced acute lung injury by decreasing neutrophil recruitment and radicalformation. *PLoS One* 2012; 7: e38917.

Gross P, Babyak MA, Tolker E et al. Enzymatically produced pulmonary emphysema. A preliminary report. *J Occup Med* 1964; 6: 481-484.

Guasti L, Marino F, Cosentino M, et al. Simvastatin treatment modifies polymorphonuclear leukocyte function in high-risk individuals: a longitudinal study. *J Hypertens* 2006; 24: 2423-2430.

Guenard H, Marthan R. Pulmonary gas exchange in elderly subjects. *Eur Respir J* 1996; 9: 2573-2577.

Gyetko MR, Toews GB. Immunology of the aging lung. *Clin Chest Med* 1993; 14: 379-391.

Halbwachs-Mecarelli L, Bessou G, Lesavre P, et al. Bimodal distribution of proteinase 3 (PR3) surface expression reflects a constitutive heterogeneity in the polymorphonuclear neutrophil pool. *FEBS Lett* 1995; 374: 29–33.

Hallberg J, Dominicus A, Erkisson UK et al. Interaction between smoking and genetic factors in the development of chronic bronchitis. *Am J Respir Crit Care Med* 2008; 177: 486-490.

Halliwell B, Gutteridge JM. Free Radicals in Biology and Medicine, 3rd Edn. *Oxford: Oxford University Press* 1999.

Hamblin JN, Angell TD, Ballantine SP, et al. Pyrazolopyridines as a novel structural class of potent and selective PDE4 inhibitors. *Bioorg Med Chem Lett* 2008; 18: 4237–4241.

Hammond C, Denzin LK, Pan M, et al. The tetraspan protein CD82 is a resident of MHC class II compartments where it associates with HLA-DR, -DM, and -DO molecules. *J Immunol* 1998; 161: 3282-3291.

Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* 1998; 92: 3007–3017.

Hanada M, Feng J, Hemmings BA. Structure, regulation and function of PKB/AKT–a major therapeutic target. *Biochim Biophys Acta* 2004; 1697: 3–16.

Hannigan M, Zhan L, Li Z, et al. Neutrophils lacking phosphoinositide 3-kinase γ show loss of directionality during *N*-formyl-Met-Leu-Phe-induced chemotaxis. *Proc Natl Acad Sci USA* 2002; 99: 3603-3608.

Hannigan MO, Huang CK, Wu DQ**.** Roles of PI3K in neutrophil function. *Curr Top Microbiol Immunol* 2003; 282: 165-75.

Hansell P, Berger E, Chambers JD, et al. Influence of acridine tracer dyes on neutrophil function. *J Leukocyte Biol* 1994; 56: 464–468.

Hassett DJ, Borchers MT, Panos RJ. Chronic obstructive pulmonary disease (COPD): evaluation from clinical, immunological and bacterial pathogenesis perspectives. *J Microbiol* 2014; 52: 211-226.

Hatzelmann A, Schudt C. Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. *J Pharmacol Exp Ther* 2001; 297: 267–279.

Haynes L, Linton PJ, Eaton SM, et al. Interleukin 2, but not other common gamma chain-binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice. *J Exp Med* 1999; 190: 1013–1024.

Heale JP, Pollard AJ, et al. Two distinct receptors mediate nonopsonic phagocytosis of different strains of Pseudomonas aeruginosa. *J Infect Dis* 2001; 183: 1214–20.

Heard BE. Pathology of chronic bronchitis and emphysema. Baltimore: *Williams and Wilkins* 1969.

Heath GW. Physical activity transitions and chronic disease. *Am J Lifestyle Med* 2009; 3: 27S–31S.

Helgason CD, Damen JE, et al. Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. *Genes Dev* 1998; 12: 1610-20.

Heit B, Tavener S, Raharjo E, Kubes P. An intracellular signalling hierarchy determines direction of migration in opposing chemotactic gradients. *J Cell Biol* 2002; 159: 91–102.

Heit B, Liu L, Colarusso P, Puri KD, Kubes P**.** PI3K accelerates, but is not required for neutrophil chemotaxis to fMLP. *JCS* 2007; 121: 205-14.

Heit B, Robbins SM, Downey CM, Guan Z, Colarusso P, Miller JD, et al. PTEN functions to prioritize chemotactic cues and prevent distraction in migrating neutrophils. *Nat immunol* 2008; 9: 743-52.

Hiemstra PS. Novel roles of proteinase inhibitors in infection and inflammation. *Biochem Soc Trans* 2002; 30: 116–120.

Hill AT, Bayley D, Campbell EJ, Hill SL, Stockley RA**.** Airways inflammation in chronic bronchitis; the effects of smoking and alpha-1 anti-trypsin deficiency. *Eur Respir J* 2000; 15: 886-90.

Hilmer SN, Cogger VC, Le Couteur DG. Basal activity of Kupffer cells increases with old age. *J Gerentol A Biol Sci Med Sci* 2007; 62: 973-978.

Hirschmann JV. Do bacteria cause exacerbation of COPD? *Chest* 2000; 118: 193-203.

Hoeller O, Kay RR. Chemotaxis in the absence of PIP3 gradients. *Curr. Biol* 2007; 17: 813–817.

Hofmann KP, Scheerer P, Hildebrand PW, et al. A G protein-coupled receptor at work: the rhodopsin model. *Trends Biochem Sci* 2009; 34: 540-552.

http://healthy-lifestyle.most-effective-solution.com/2007/10/01/oxidative-stress-and-free-radicals/

http://infogrphic.blogspot.com/2011/04/viscera-of-thorax.html

http://www.ctsnet.org/portals/thoracic/newtechnology/article-4.html

Hogg JC, Macklem PT, Thurlbeck WM. Site and nature of airway obstruction in chronic obstructive lung disease. *N Engl J Med* 1968; 278: 1355-1360.

Hogg JC, Doerschuk CM. Leukocyte traffic in the lung. *Annu Rev Physiol* 1995; 57: 97–114.

Hogg JC, McDonough JE, Suzuki M. Small airway obstruction in COPD: new insights based on micro-CT imaging and MRI imaging. *Chest* 2013; 143: 1436-1443.

Huang C, Jacobson K, Schaller MD**.** MAP kinases and cell migration. *J Cell Sci* 2004; 117: 4619-28.

Hunninghake GW, Crystal RG. Cigarette smoke and lung destruction: accumulation of neutrophils in the lungs of cigarette smokers. *Am Rev Respir Dis* 1983; 128: 833-888.

Huttenlocher A, Palecek SP, Lu Q, et al. Regulation of cell migration by the calcium-dependent protease calpain. *J Biol Chem* 1997; 272: 32719–32722.

Huttunlocher A**.** Cell polarisation mechanisms during directed cell migration. *Nature Cell Biol* 2005; 7: 336-377.

Hynes RO. Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* 1992; 69: 11–25.

Iida N, Grotendorst GR. Cloning and sequencing of a new gro transcript from activated human monocytes: expression in leukocytes and wound tissue. *Mol Cell Biol* 1990; 10(10): 5596–9.

Incalzi RA, Corsonello A, Pedone C, et al. Chronic renal failure: a neglected comorbidity of COPD. *Chest* 2010; 137: 831–837.

Inoue I, Goto S, Mizotani K, et al. Lipophilic HMG-CoA reductase inhibitor has an anti-inflammatory effect: reduction of MRNA levels for interleukin-1beta, interleukin-6, cyclooxygenase-2, and p22phox by regulation of peroxisome proliferator-activated receptor alpha (PPARalpha) in primary endothelial cells. *Life Sci* 2000; 67: 863-876.

Inoue T, Meyer T. Synthetic activation of endogenous pi3k and rac identifies an and-gate switch for cell polarization and migration. *PLos One* 2008; 3: e3068.

Ionescu CV, Cepinskas G, Savickiene J, et al. Neutrophils induce sequential focal changes in endothelial adherens junction components: role of elastase. *Microcirculation* 2003; 10: 205–20.

Ito K, Caramori G, and Adcock IM. Therapeutic potential of phosphatidylinositol 3-kinase inhibitors in inflammatory respiratory disease. *J Pharmacol Exp Ther* 2007; 321: 1–8.

Ito Y, Kajkenova O, Feuers RJ, et al. Impaired glutathione peroxidase activity accounts for the age-related accumulation of hydrogen peroxide in activated human neutrophils. *J Gerontol A Biol Sci Med Sci* 1998; 53: M169–M175.

Itoh Y, Nagase H. Preferential inactivation of tissue inhibitor of metalloproteinase-1 that is bound to the precursor of matrix metalloproteinase 9 (progelatinase B) by human neutrophil elastase. *J Biol Chem* 1995; 270: 16 518-521.

Janoff A, Sloan B Weinbaum G, et al. Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled proteinase. *Am Rev Respir Dis* 1977; 115: 461-478.

Janssens JP, Pache JC, Nicod LP. Physiological changes in respiratory function associated with ageing. *Eur Respir J* 1999; 13: 197-205.

Jin SL, Goya S, Nakae S, et al. Phosphodiesterase 4B is essential for T(H)2-cell function and development of airway hyperresponsiveness in allergic asthma. *J Allergy Clin Immunol* 2010; 126:1252–1259 e1212.

Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. *Nature reviews. Cancer* 2004; 4: 253–65.

Kafienah W, Buttle DJ, Burnett D, Hollander AP**.** Cleavage of native type 1 collagen by human neutrophil elastase. *Biochem J* 1998; 330: 897-902.

Kamp VM, Pillay J, Lammers JW, et al. Human suppressive neutrophils CD16bright/CD62Ldim exhibit decreased adhesion. *J Leukoc Biol* 2012; 92: 1011-1020.

Karadag F, Karul AB, Cildag O, et al. Biomarkers of systemic inflammation in stable and exacerbation phases of COPD. *Lung* 2008; 186: 403e9.

Kempsford R, Norris V, Siederer S. Vilanterol trifenatate, a novel inhaled long-acting beta2 adrenoceptor agonist, is well tolerated in healthy subjects and demonstrates prolonged bronchodilation in subjects with asthma and COPD. *Pulm Pharmacol Ther* 2013; 26: 256–264.

Kennedy AD, DeLeo FR**.** PI3K and NADPH oxidase: a class act. *Blood* 2008; 112: 4788-4789.

Khair OA, Davies RJ, et al. Bacterial-induced release of inflammatory mediators by bronchial epithelial cells. *Eur Respir J* 1996; 9: 1913-1922.

Kinsella A, Raza A, Kennedy S, et al. The impact of high-dose statin therapy on transendothelial neutrophil migration and serum cholesterol levels in healthy male volunteers. *Eur J Clin Pharmacol* 2011; 67: 1103-1108.

Kitchen E, Rossi AG, Condliffe AM, et al. Demonstration of reversible priming in human neutrophils using platelet-activating factor. *Blood* 1996; 88: 4330-4337.

Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol* 2005; 77: 598–625.

Klein CL, Bittinger F, Kohler H, Wagner M, Otto M, Hermanns I, Kirkpatrick CJ. Comparative studies on vascular endothelium in vitro. 3. Effects of cytokines on the expression of E-selectin, ICAM-1 and VCAM-1 by cultured human endothelial cells obtained from different passages. *Pathobiology* 1995; 65: 83–92.

Knall C, Worthen GS, Johnson GL. Interleukin 8-stimulated phosphatidylinositol-3-kinase regulates the migration of human neutrophils independent of extracellular signal-related kinase and p38 mitogen-activated protein kinases. *Proc Natl Acad Sci USA* 1997; 94: 3052-3057.

Knudson RJ, Clark DF, et al. Effect of aging alone on mechanical properties of the normal adult human lung. *J Appl Physiol* 1977; 43: 1054-1062.

Knudson RJ. Physiology of the aging lung. In: Crystal RG, West JB eds. *The Lung*. New York: Raven Press; 1991: 1749-1759.

Kohl, J. and D. Bitter-Suermann. Anaphylotoxins. In Whaley K, editor. Complement in health and disease. *Kluwer Academic. Boston* 1983: 299-324.

Kolsch V, Charest PG, Firtel RA. The regulation of cell motility and chemotaxis by phospholipid signaling. *J Cell Sci* 2008; 121: 551–9.

Konstantopoulos K, McIntire LV. Effects of fluid dynamics on vascular cell adhesion. *J Clin Invest* 1996; 98: 2661–2665.

Kovaiou RD, Grubeck-Loebenstein B. Age-associated changes within CD4+ T cells. *Immunol Lett* 2006; 107: 8–14.

Krasilnikov MA. Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation. *Biochemistry* 2000; 65: 59–67.

Kubes P, Niu XF, Smith CW, et al. A novel beta 1-dependent adhesion pathway on neutrophils: a mechanism invoked by dihydrocytochalasin B or endothelial transmigration *FASEB J* 1995; 9: 1103–1111.

Kulkarni S, Saido TC, Suzuki K, et al. Calpain mediates integrin-induced signaling at a point upstream of Rho family members. *J Biol Chem* 1999; 274: 21265–21275.

Kumar S, Xu J, Perkins C, et al. Cdc42 regulates neutrophil migration via crosstalk between WASp, CD11b, and microtubules. *Blood* 2012; 120: 3563-3575.

Kurig B, Shymanets A, Bohnacker T, et al. Ras is an indispensable coregulator of the class IB phosphoinositide 3-kinase p87/ p110gamma. *Proc Natl Acad Sci USA* 2009; 106: 20312–20317.

Krymskaya VP. Targeting the phosphatidylinositol 3-kinase pathway in airway smooth muscle: rationale and promise. *BioDrugs* 2007; 21: 85–95.

Kuijpers TW, Tool AT, van der Schoot CE, et al. Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood* 1991; 78: 1105-1111.

Kumar RK, Herbert C, Thomas PS, et al. Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma. *J Pharmacol Exp Ther* 2003; 307: 349–355.

Lane HC, Anand AR, Ganju RK. Cbl and Akt regulate CXCL8-induced and CXCR1- and CXCR2-mediated chemotaxis. *Int Immunol* 2006; 18: 1315-25.

Larbi A, Douziech N, Dupuis G, et al. Age-associated alterations in the recruitment of signal-transduction proteins to lipid rafts in human T lymphocytes. *J Leukoc Biol* 2004; 75: 373–381.

Laudanna C, Melotti P, Bonizzato C, et al. Ligation of members of the β_1 or the β_2 subfamilies of integrins by antibodies triggers eosinophil respiratory burst and spreading. *Immunology* 1993; 80: 273– 280.

Laupland KB, Church DL, Mucenski M, et al. Population-based study of the epidemiology of and the risk factors for invasive staphylococcus aureus infections. *J Infect Dis* 2003; 187: 1452-1459.

Laurent P, Janoff A, Kagan HM. Cigarette smoke blocks the cross-linking of elastin *in vitro*. *Am Rev Respir Dis* 1983; 127: 189-192.

Laurell C-B, Eriksson A. The electrophoretic α_1-globulin pattern of serum in α_1-antitrypsin deficiency. *Scand J Clin Lab Invest* 1963; 15: 132-140.

Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: Distinction and prerequisite for adhesion through integrins. *Cell* 1991; 65: 859–873.

Lawrence MB and Springer TA. Neutrophils roll on E-selectin. *J Immunol*; 1994; 151: 6338–6346.

Leaker BR, Barnes PJ, O'Connor B. Inhibition of LPS-induced airway inflammation in healthy volunteers with an oral CXCR2 antagonist. *Respir Res* 2013; 14: 137.

Lee JH, Lee DS, et al. Simvastatin Inhibits Cigarette Smoking–induced Emphysema and Pulmonary Hypertension in Rat Lungs. *Am J Respir Crit Care Med* 2005; 172: 987-993.

Lee KS, Lee HK, Hayflick JS, et al. Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *FASEB J* 2006; 20: 455–465.

Lee S, Comer FI, Sasaki A, McLeod IX, et al. TOR complex 2 integrates cell movement during chemotaxis and signal relay in Dictyostelium. *Mol Biol Cell* 2005; 16: 4572–83.

Lee WL, Downey GP. Leukocyte elastase: physical functions and its role in acute lung injury. *Am J Respir Crit Care Med* 2001; 164: 896-904.

Leeder SR. Role of infection in the cause and course of chronic bronchitis and emphysema. *J Infect Dis* 1975; 131: 731-742.

Leite CF, Marangoni FA, Camargo EA, et al. Simvastatin attenuated neutrophil recruitment in one-lung ventilation model in rats. *Acta Cir Bras* 2013; 8: 245-250.

Leng S, Xue QL, Huang Y, et al. Total and differential white blood cell counts and their associations with circulating interleukin-6 levels in community dwelling older women. *J Gerentol A Biol Sci Med Sci* 2005; 60: 195-199.

Lethem MI, James SL, et al. The origin of DNA associated with mucus glycoproteins in cystic fibrosis sputum. *Eur Respir J* 1990; 3: 19–23.

Li Y, Brazzell J, Herrera A, Walcheck B. ADAM17 deficiency by mature neutrophils has differential effects on L-selectin shedding. *Blood* 2006; 108: 2275-2279.

Li Z, Hannigan M, Mo Z, et al. Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. *Cell* 2003; 114: 215-227.

Li, Z., X. Dong, Wang Z, et al. Regulation of PTEN by Rho small GTPases. *Nat Cell Biol* 2000; 7: 399-404.

Loetscher P, Seitz M, et al. Both interlukin-8 receptors independently mediate chemotaxis. Jurkat cells transfected with IL-8R1 or IL-8R2 migrate in response to IL-8, GRO alpha and NAP-2/. *FEBS Lett* 1994; 341: 187-192.

Lokuta MA, Nuzzi PA, Huttenlocher A. Calpain regulates neutrophil chemotaxis. *Proc Natl Acad Sci USA* 2003; 100: 4006-4011.

Lomas DA, Evans DL, Finch JT, et al. The mechanism of Z alpha 1-antitrypsin accumulation in the liver. *Nature* 1992; 357: 605-607.

Lomas DA, Stone SR, Llewellyn-Jones C, et al. The control of neutrophil chemotaxis by inhibitors of cathepsin G and chymotrypsin. *J Biol Chem* 1995; 270: 23437-23443.

Loovers HM, Postma M, Keizer-Gunnink I, et al. Distinct roles of PI(3,4,5)P3 during chemoattractant signaling in Dictyostelium: a quantitative in vivo analysis by inhibition of PI3-kinase. *Mol Biol Cell* 2006; 17: 1503–1513.

Lopez-Boado YS, Rubin BK. Macrolides as immunomodulatory medications for the therapy of chronic lung diseases. *Curr Opin Pharmacol* 2008; 8: 286-291.

López-Otín C, Bond JS. Proteases: multifunctional enzymes in life and disease. *J Biol Chem* 2008; 283: 30433–30437.

Lord JM, Butcher S, Killampali V, et al. Neutrophil ageing and immunesenescence. *Mech Ageing Dev* 2001; 122: 1521-1535.

Lord JM, Phillips AC, Arlt W. Synergistic effects of ageing and stress on neutrophil function. In: Fulop T et al. eds. *Handbook of Immunosenescence*: Springer, 2009: 475-498.

Lucey EC, Stone PJ, Breuer R, et al. Effect of combined human neutrophil cathepsin G and elastase on induction of secretory cell metaplasia and emphysema in hamsters, with *in vitro* observations on elastolysis by these enzymes. *Am Rev Respir Dis* 1985; 132: 362-366.

Lucey EC, Stone PJ, Powers JC, et al. Amelioration of human neutrophil elastase-induced emphysema in hamsters by pretreatment with an oligopeptide chloromethylketone. *Eur Respir J* 1989; 2: 421-427.

Lui Z, Zhou X, Shapiro SD, et al. The serpine α_1-proteinase inhibitor is a critical substrate for gelatinase B/MMP-9 *in vivo*. *Cell* 2000; 102: 647-655.

Luscinskas FW and Lawler J. Integrins as dynamic regulators of vascular function. *FASEB J* 1994; 8: 929–938.

McElvaney NG, Crystal RG. *The Lung*. Lippincott-Raven 1997: New York.

MacGregor RR, Shalit M. Neutrophil function in healthy elderly subjects. *J Gerontol* 1990: 45: M55-M60.

McLaughlin B, O´Malley K, Cotter TG. Age-related differences in granulocyte chemotaxis and degranulation. *Clin Sci* 1986; 70: 59.

Mackeral AJ, Russell KJ, et al. Interleukin 8 and leukotriene B4 but not formylmethionyl leucylphenylalanine stimulate CD18 independent migration of neutrophils across human pulmonary endothelial cells in vitro. *Am J Respir Cell Mol Biol* 2000; 23: 154-161.

Mahajan K, Coppola D, Challa S, et al. Ack1 mediated AKT/PKB tyrosine 176 phosphorylation regulates its activation. *PLoS One* 2010; 5: e9646. doi: 10.1371/journal.pone.0009646.

Maher BM, Dhonnchu TN, et al. Statins alter neutrophil migration by modulating cellular Rho activity - a potential mechanism for statins-mediated pleotropic effects? *J Leukoc Biol* 2009; 85: 186-193.

Mahmudi-Azer S, van Eeden SF. Neutrophil 'connectivity': key to neutrophil-mediated tissue injury? *Crit Care* 2003; 7: 285-287.

Maier U, Babich A, Nürnberg B. Roles of non-catalytic subunits in gbetagamma-induced activation of class I phosphoinositide 3-kinase isoforms beta and gamma. *J Biol Chem* 1999; 274: 29311-29317.

Majo J, Ghezzo H, Cosio MG. Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. *Eur Respir J* 2001; 17: 946–953.

Mallia P, Message SD, Contoli M, et al. Neutrophil adhesion molecules in experimental rhinovirus infection in COPD. *Respir Res* 2013; 14: 72. doi: 10.1186/1465-9921-14-72.

Malik AR, Lo SK. Vascular endothelial adhesion molecules and tissue inflammation. *Pharmacol Rev* 1996; 48: 213–229.

Mandeville JT, Maxfield FR. Calcium and signal transduction in granulocytes. *Cur Opin Hematol* 1996; 3: 63–70.

Manning G, Whyte DB, Martinez R, et al. The protein kinase complement of the human genome. *Science* 2002; 298: 1912–34.

Manzenreiter R, Kienberger F, et al. Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electronmicroscopy. *J Cyst Fibros* 2012; 11: 84–92.

Marcos V, Zhou Z, et al. CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation. *Nat Med* 2010; 16: 1018–1023.

Marcos V, Zhou Z, et al. Retraction: CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation. *Nat Med* 2011; 17: 899.

Marks PW, Maxfield FR. Transient increases in cytosolic free calcium appear to be required for the migration of adherent human neutrophils. *J Cell Biol* 1990; 110: 43-52.

Marks PW, Hendey B, Maxfield FR. Attachment to fibronectin or vitronectin makes human neutrophil migration sensitive to alterations in cytosolic free calcium concentration. *J Cell Biol* 1991; 112: 149–158.

Marone G, Poto S, di Martino L, et al. Human basophil releasability. I. Age-related changes in basophil releasability. *J Allerg Clin Immunol* 1986; 77: 377-383.

Marshall JG, Booth JW, Stambolic V, et al. Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fc gamma receptor-mediated phagocytosis. *J Cell Biol* 2001; 153: 1369–1380.

Martin TR, Raghu G, et al. The effects of chronic bronchitis and chronic airflow obstruction on lung cell populations recovered by bronchoalveolar lavage. *Am Rev Respir Dis* 1985; 132: 260.

Martin TR, Frevert CW. Innate immunity in the lungs. *Proc Am Thorac Soc* 2005; 2: 403–411.

Martin EL, Souza DG, Fagundes CT, et al. Phosphoinositide-3 kinase gamma activity contributes to sepsis and organ damage by altering neutrophil recruitment. *Am J Respir Crit Care Med* 2010; 182(6): 762–773.

Martinez F, Calverley PMA, Goehring UM, Rennard SI. Defining patient populations in COPD: experience with roflumilast. 2010.

Maruyama M, Hay JG, et al. Modulation of secretory leukoproteinase inhibitor gene expression in human bronchial epithelial cells by phorbal ester." *J Clin Invest* 1994; 94: 368-375.

Mathur SK, Schwantes EA, Jarjour NN, et al. Age-related changes in eosinophil function in human subjects. *Chest* 2008; 133: 412-419.

Matsuba, KT, Van Eeden SF, et al. Apoptosis in circulating PMNs; increasing susceptibility in L-selectin deficient PMN. *Am J Physiol* 1997; 272: H2852-H2858.

Matsuse T, Hayashi S, Kuwano K, et al. Latent adenoviral infection in the pathogenesis of chronic airways obstruction. *Am Rev Respir Dis* 1992; 146: 177-184.

Matsushima K, Morishita K, et al. Molecular cloning of a human monocytederived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumour necrosis factor. *J Exp Med* 1988; 167: 1883-1893.

McAuley DF, Laffey JG, O'Kane CM, et al. Simvastatin in the acute respiratory distress syndrome. *N Engl J Med* 2014; 371: 1695-1703.

Meshulam T, Proto P, Diamond RD, et al. Calcium modulation and chemotactic response: Divergent stimulation of neutrophil chemotaxis and cytosolic calcium response by the chemotactic peptide receptor. *J Immunol* 1986; 137: 1954-1960.

Meyer KC, Ershler W, Roenthal NS, et al. Immune dysregulation in the aging human lung. *Am J Respir Crit Care Med* 1996; 153: 1072-1079.

Meyer KC. The role of immunity in susceptibility to respiratory infection in the aging lung. *Respir Physiol* 2001; 128: 23-31.

Mihaila A, Tremblay GM. Human alveolar macrophages express elafin and secretory leukocyte proteinase inhibitor. *Z Naturforsch C* 2001; 56: 291–297.

Miller AP, Xing D, Feng W, et al. Aged rats lose vasoprotective and anti-inflammatory actions of estrogen in injured arteries. *Menopause* 2007; 14: 251-260.

Mondal S, Subramanian KK, Sakai J, et al. Phosphoinositide lipid phosphatase SHIP1 and PTEN coordinate to regulate cell migration and adhesion. *Mol Biol Cell* 2012; 23: 1219-30.

Monso E, Ruiz J, Rosell A, et al. Bacterial infection in chronic obstructive pulmonary disease: a study of stable and exacerbated outpatients using the protected specimen brush. *Am J Respir Crit Care Med* 1995; 152: 1316-1320.

Mooren HW, Kramps JA, et al. Localisation of a low molecular weight bronchial proteinase inhibitor in the peripheral human lung. *Thorax* 1983; 38: 180-183.

Mora S, Cook N, Buring JE, et al. Physical activity and reduced risk of cardiovascular events: potential mediating mechanisms. *Circulation* 2007; 116: 2110–18.

Moro-García MA, Alonso-Arias R, López-Larrea C. Molecular mechanisms involved in the aging of the T-cell immune response. *Curr Genomics* 2012; 13: 589-602.

Moser B, Clark-Lewis I, Zwahlen R, Baggiolini M. Neutrophil-activating properties of the melanoma growth-stimulatory activity. *J Exp Med* 1990; 171(5): 1797–802.

Moss RB, Mistry SJ, Konstan MW, et al. Safety and early treatment effects of the CXCR2 antagonist SB-656933 in patients with cystic fibrosis. *J Cyst Fibros* 2013; 12: 241-248.

Muinonen-Martin AJ, Veltman DM, Kalna G, Insall RH. An improved chamber for direct visualisation of chemotaxis. *PLoS One* 2010; 14;5(12) :e15309.

Murphy TF, Sethi S. Bacterial infection in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1992; 146: 1067-1083.

Murphy TF, Sethi S, Niederman MS. The role of bacteria in exacerbations of COPD: a constructive view. *Chest* 2000; 118: 204-209.

Nara K, Ito S, Ito T, Suzuki Y, Ghoneim MA, Tachibana S et al. Elastase inhibitor elafin is a new type of proteinase inhibitor which has a transglutaminase-mediated anchoring sequence termed 'cementoin.' *J Biochem* 1994; 115: 441–448.

Nash GB, Abbitt KB, Tate K, et al. Changes in the mechanical and adhesive behaviour of human neutrophils on cooling in vitro. *Pflugers Arch* 2001; 442: 762-770.

Nauseef WM. The NADPH-dependent oxidase of phagocytes. *Proc Assoc Am Physicians* 1999; 111: 373-382.

Naylor K, Li G, Vallejo AN, et al. The influence of age on T cell generation and TCR diversity. *J Immunol* 2005; 174: 7446 –7452.

Neas LM, Schwartz J. The determinants of pulmonary diffusing capacity in a national sample of US adults. *Am J Respir Crit Care Med* 1996; 15: 656-664.

Niggli V. Signaling to migration in neutrophils: importance of localized pathways. *Biochem Cell Biol* 2003; 35: 1619-1638.

Nishio M, Watanabe K, Sasaki J, et al. Control of cell polarity and motility by the PtdIns(3,4,5)P3 phosphatase SHIP1. *Nat Cell Biol* 2007; 9: 36–44.

Niwa Y, Kasama T, et al. Neutrophil chemotaxis, phagocytosis and parameters of reactive oxygen species in human aging: cross sectional and longitudinal studies. *Life Sci* 1989; 44: 1655-1664.

Noguera A, Busquets X Sauleda J, et al. Expression of adhesion molecules and G proteins in circulating neutrophils in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998; 158: 1664-1668.

Noguera A, Batle S, et al. Enhanced neutrophil response in chronic obstructive pulmonary disease. *Thorax* 2001; 56: 432-437.

Nonomura K, Yamanishi K, Yasuno H, Nara K, Hirose S. Up-regulation of elafin/SKALP gene expression in psoriatic epidermis. *J Invest Dermatol* 1994; 103: 88-91.

Nuzzi PA, Senetar MA, Huttenlocher A. Asymmetric localization of calpain 2 during neutrophil chemotaxis. *Mol Biol Cell* 2007; 18: 795-805.

O'Connell RM, Chaudhuri AA, Rao DS, et al. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc Natl Acad Sci USA* 2009; 106: 7113-7118.

O'Donnell NG, McSharry CP, Wilkinson PC, Asbury AJ. Comparison of the inhibitory effect of propofol, thiopentone and midazolam on neutrophil polarisation in vitro in the presence of human serum albumin. *Br J Anaesth* 1992; 69: 70-74.

Ohlsson K, Olsson I, Spitznagel K. Localization of chymotrypsin-like cationic protein, collagenase and elastase in azurophile granules of human neutrophilic ploymorphonuclear leukocytes. *Hoppe-Seyler's Z Phsiol Chem* 1977; 358: 361-366.

Okada Y, Nakanishi I. Activation of matrix metalloproteinase 3 (stromelysin) and matrix metalloproteinase 2 ('gelatinase') by human neutrophil elastase and cathepsin G. *FEBS Lett* 1989; 249: 353-356.

Okkenhaug K. Signaling by the phosphoinositide 3-kinase family in immune cells. *Annu Rev Immunol* 2013; 31: 675-704.

Oliveira C, Navarro-Xavier RA, Anjos-Vallota EA, et al. Effect of plant neutrophil elastase inhibitor on leucocyte migration, adhesion and cytokine release in inflammatory conditions. *Br J Pharmacol* 2010; 161: 899-910.

Onsum M, Rao CV. A mathematical model for neutrophil gradient sensing and polarization. *PLoS Comput Biol* 2007; 3: e36

OPUS Study: Effect of Roflumilast on Exacerbation Rate in Patients With Chronic Obstructive Pulmonary Disease (BY217/M2-111).

Owen CA, Campbell EJ. The cell biology of leukocyte-mediated proteolysis. *J Leukoc Biol* 1999; 65: 137–150.

Owen CA. Leukocyte cell surface proteinases: regulation of expression, functions, and mechanisms of surface localization. *Int J Biochem Cell Biol* 2008; 40: 1246–1272.

Pacold ME, Suire S, Perisic O, et al. Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. *Cell* 2000; 103: 931–943.

Painter RG, Aiken ML. Regulation of N-formyl-methionyl-leucyl-phenylalanine receptor recycling by surface membrane neutral endopeptidase-mediated degradation of ligand. *J Leukoc Biol* 1995; 58: 468-476.

Panda A, Arjona A, Sapey E, et al. Human innate immunosenescence: causes and consequences for immunity in old age. *Trends Immunol* 2009; 30: 325-333.

Papayannopoulos V, Zychlinsky A. NETs: a new strategy for using old weapons. *Trends Immunol* 2009; 30: 513–521.

Papazian L, Roch A, Charles PE, et al. Effect of statin therapy on mortality in patients with ventilator-associated pneumonia: a randomized clinical trial. *JAMA* 2013; 310: 1692-1700.

Parr DG, White AJ, Bayley DL, Guest PJ, Stockley RA**.** Inflammation in sputum relates to progression of disease in COPD: a prospective study. *Respir Res* 2006; 7: 136.

Parr DG, Sevenoaks M, Deng C, et al. Detection of emphysema progression in alpha 1-antitrypsin deficiency using CT densitometry; methodological advances. *Respir Res* 2008, 9: 21.

Pauwels RA, Buist AS, et al. Global Strategy for the diagnosis, management and prevention of Chronic Obstructive Pulmonary Disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001; 163: 1256-1276.

Peters AM, Saverymuttu SH, Keshavarzian A, et al. Splenic pooling of granulocytes. *Clin Sci (Lond)* 1985; 68: 283–289.

Peters AM. Just how big is the pulmonary granulocyte pool? *Clin Sci (Lond)* 1998: 94: 7–19.

Pettit EJ, Hallett MB. Temporal and spatial resolution of Ca^{2+} release and influx in human neutrophils using a novel confocal laser scanning mode. *Biochem Biophys Res Commun* 1996; 229: 109–113.

Pfundt R, van Ruissen F, van Vlijmen-Willems IM, Alkemade HA, Zeeuwen PL, Jap PH et al. Constitutive and inducible expression of SKALP/elafin provides anti-elastase defense in human epithelia. *J Clin Invest* 1996; 98: 1389–1399.

Phillipson M, Heit B, Colarusso P, et al. Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J Exp Med* 2006; 203: 2569–75.

Phillipson M, Kaur J, Colarusso P, et al. Endothelial domes encapsulate adherent neutrophils and minimize increases in vascular permeability in paracellular and transcellular emigration. *PLoS One* 2008; 3: e1649.

Pierini LM, Lawson MA, Eddy RJ, Hendey B, Maxfield FR**.** Oriented endocytic recycling of alpha 5 beta 1 in motile neutrophils. *Blood* 2000; 95: 2471-80.

Pignatti P, Moscato G, Casarini S, et al. Downmodulation of CXCL8/IL-8 receptors on neutrophils after recruitment in the airways. *J Allergy Clin Immunol* 2005; 115(1): 88-94.

Pilette C, Colinet B, et al. Increased galectin-3 expression and intra-epithelial neutrophils in small airways in severe chronic obstructive pulmonary disease *Eur Respir J* 2007; 29(5): 914-922.

Pillay J, den Braber I, Vrisekoop N, et al. *In vivo* labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood* 2010; 116(4): 625-627.

Pillay J, Kamp VM, van Hoffen E, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest* 2012; 122: 327–336.

Pinho V, Russo RC, Amaral FA, et al. Tissue- and stimulus-dependent role of phosphatidylinositol 3-kinase isoforms for neutrophil recruitment induced by chemoattractants in vivo. *J Immunol* 2007; 179: 7891-7898.

Placket TP, Boehmer ED, Faunce DE, Kovacs EJ. Aging and innate immune cells. *J Leukoc Biol* 2004; 76: 291–299.

Platek A, Vassilev VS, de Diesbach P, et al. Constitutive diffusive activation of phophoinositide 3-kinase at the plasma membrane by v-Src suppresses the chemotactic response to PDGF by abrogating the polarity of PDGF receptor signalling. *Exp Cell Res* 2007; 313: 1090-1105.

Pletz MW, Ioanas M, et al. Reduced spontaneous apoptosis in peripheral blood neutrophils during exacerbations of COPD. *Eur Respir J* 2004; 23: 532-537.

Polignano A, Tortorella C, Venezia A, et al. Age-associated changes of neutrophil responsiveness in a human healthy elderly population. *Cytobios* 1994; 80: 145–153.

Prossnitz ER, Kim CM, et al. Phosphorylation of the N-formyl peptide receptor carboxyl terminus by the G protein-coupled receptor kinase, GRK2. *J Biol Chem* 1995; 270: 1130-1137.

Pryor WA, Stone K. Oxidants in cigarette smoke: radicals, hydrogen peroxide, peroxynitrate and peroxynitrite. *Ann N Y Acad Sci* 1993; 686: 12-27.

Puchelle E, Zahm JM, Bertrand A. Influence of age on bronchial mucociliary transport. *Scand J respire Dis* 1979; 60: 307-313.

Puri KD, Doggett TA, Douangpanya J, et al. Mechanisms and implications of phosphoinositide 3-kinase delta in promoting neutrophil trafficking into inflamed tissue. *Blood* 2004; 103: 3448–3456.

Quinn MT, Gauss KA. Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases. *J Leukoc Biol* 2004; 76: 760–781.

Rahman I, MacNee W. Role of oxidants/antioxidants in smoking-induced lung diseases. *Free Radic Biol Med* 1996; 21: 669-681.

Rahman I, MacNee W. Lung glutathione and oxidative stress: Implications in cigarette smoke-induced airway disease. *Am J Physiol* 1999; 277: L1067-1088.

Rainger, GE, Buckley C, et al. Cross-talk between cell adhesion molecules regulates the migration velocity of neutrophils. *Curr Biol* 1997; 7: 316-325.

Reid L. The pathology of emphysema. Philadelphia: *JB Lippincott* 1967.

Rennard SI, Daughton DM, et al "Short-term smoking reduction is associated with reduction in measures of lower respiratory tract inflammation in heavy smokers. *Eur Respir J* 1990; 3: 752-759.

Renshaw SA, Loynes CA, et al. A transgenic zebrafish model of neutrophilic inflammation. *Blood* 2006; 108: 3976-3978.

Retamales IM, Elliott WM, Meshi B, et al. Amplification of inflammation in emphysema and its association with latent adenoviral infection. *Am J Respir Crit Care Med* 2001; 164: 469-473.

Rice A, Banda MJ. Neutrophil elastase processing of gelatinase A is mediated by extracellular matrix. *Biochemistry* 1995; 34: 9249-9256.

Richmond A, Thomas HG. Melanoma growth stimulatory activity: isolation from human melanoma tumors and characterization of tissue distribution. *J Cell Biochem* 1988; 36 (2): 185–98.

Rickert P, Weiner OD, Wang F, et al. Leukocytes navigate by compass: roles of PI3K gamma and its lipid products. *Trends Cell Bio*. 2000; 10: 466-473.

Ridley AJ. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol* 2006; 16: 522-529.

Roberts PJ, Pizzey AR, Khwaja A, et al. The effects of interleukin-8 on neutrophil fMetLeuPhe receptors, CD11b expression and metabolic activity, in comparison and combination with other cytokines. *Br J Haematol* 1993; 84: 586–594.

Rogers J, Kalsheker N, Wallis S, Speer A, Coutelle CH, Woods D et al. The isolation of a clone for human alpha 1-antitrypsin and the detection of alpha 1-antitrypsin in mRNA from liver and leukocytes. *Biochem Biophys Res Commun* 1983; 116: 375–382.

Rudoplhus A, Kramps JA, Mauve I, et al. Intratracheally-instilled antileukoproteinase and α_1-proteinase inhibitor effect on human neutrophil elastase-induced experimental emphysema and pulmonary localization. *Histochem J* 1994; 26: 817-824.

Rutgers SR, Timens W, et al. Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. *Eur Respir J* 2000; 15: 109-115.

Rytila P, Plataki M, et al. Airway neutrophilia in COPD is not associated with increased neutrphil survival. *Eur Respir J* 2006; 28: 1163-1169.

Sadhu C, Dick K, Tino WT, and Staunton DE. Selective role of PI3K delta in neutrophil inflammatory responses. *Biochem Biophys Res Commun* 2003; 308: 764–769.

Sadhu C, Masinovsky B, Dick K, et al. Essential role of Phosphoinositide 3-Kinase delta in neutrophil directional movement. *J Immunol* 2003; 170: 2647-2654.

Sadighi Akha AA, Miller RA. Signal transduction in the aging immune system. *Curr Opin Immunol* 2005; 17: 486–491.

Saetta M, Turato G, Facchini FM, et al. Inflammatory cells in the bronchial glands of smokers with chronic bronchitis. *Am J Respir Crit Care Med* 1997; 156: 1633–1639.

Saetta M, Turato G, et al. Cellular and structural basis of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001; 163: 1304-1309.

Sallenave JM, Marsden MD, Ryle AP. Isolation of elafin and elastase-specific inhibitor (ESI) from bronchial secretions. Evidence of sequence homology and immunological cross-reactivity. *Biol Chem Hoppe Seyler* 1992; 373: 27–33.

Sallenave JM, Shulmann J, Crossley J, Jordana M, Gauldie J. Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes. *Am J Respir Cell Mol Biol* 1994; 11: 733–741.

Sallenave JM, Si Tahar M, Cox G, Chignard M, Gauldie J. Secretory leukocyte proteinase inhibitor is a major leukocyte elastase inhibitor in human neutrophils. *J Leukoc Biol* 1997; 61: 695–702.

Samanta AK, Oppenheim JJ, et al. Interleukin 8 (monocyte-derived neutrophil chemotactic factor) dynamically regulates its own receptor expression on human neutrophils. *J Biol Chem* 1990; 265: 183-189.

Sapey E. Stockley RA. COPD exacerbations: Aetiology. *Thorax* 2006; 61: 250-258.

Sapey E, Bayley D, et al. Inter-relationships between inflammatory markers in stable COPD patients with bronchitis; the intra and inter patient variability. *Thorax* 2008; 63: 493-499.

Sapey E, Stockley JA, Greenwood H, et al. Behavioural and structural differences in migrating peripheral neutrophils from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2011; 183(9): 1176-1186.

Sapey E, Greenwood H, Walton G, et al. Phosphoinositide 3-kinase inhibition restores neutrophil accuracy in the elderly: toward targeted treatments for immunosenescence. *Blood* 2014; 123: 239-248.

Sarraj B, Massberg S, et al. Myeloid-specific deletion of tumor suprressor PTEN augments neutrophil transendothelial migration during inflammation. *J Immunol* 2009; 182: 7190-200.

Sasaki AT, Firtel RA. Regulation of chemotaxis by the orchestrated activation of Ras, PI3K, and TOR. *Eur J Cell Biol* 2006; 85: 873–95.

Sasaki T, Irie-Sasaki J, Jones RG, et al. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* 2000; 287: 1040-1046.

Schaff UY, Yamayoshi I, Tse T, et al. Calcium flux in neutrophils synchronizes beta2 integrin adhesive and signaling events that guide inflammatory recruitment. *Ann Biomed Eng* 2008; 36: 632-646.

Scheid MP, Woodgett JR. Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS Lett* 2003; 546: 108–12.

Schnitzler N, Haase G, Podbielski A, Lutticken R, Schweizer KG**.** A co-stimulatory signal through ICAM b2 integrin-binding potentiates neutrophil phagocytosis. *Nat Med* 1999; 5: 231-235.

Scholz D, Devaux B, Hirche A, Potzsch B, Kropp B, Schaper W, Schaper J. Expression of adhesion molecules is specific and time-dependent in cytokinestimulated endothelial cells in culture. *Cell Tissue Res* 1996; 284: 415–423.

Schroder AK, Rink L. Neutrophil immunity of the elderly. *Mech Ageing Dev* 2003; 124: 419-425.

Schumacher C, Clark-Lewis I, Baggiolini M, Moser B. High- and low-affinity binding of GRO alpha and neutrophil-activating peptide 2 to interleukin 8 receptors on human neutrophils. *Proc Natl Acad Sci USA* 1992; 89(21): 10542–6.

Schwarzenbach HR, Nakagawa T, Conroy MC, et al. Skin reactivity, basophil degranulation and IgE levels in ageing. *Clin Allergy* 1982; 12: 465-473.

Sergeant S, McPhail LC. Opsonized zymosan stimulates the redistribution of protein kinase C isoforms in human neutrophils. *J Immunol* 1997; 159: 2877-2885.

Sengelov H, Boulay F, Kjeldson L, et al. Dubcellualr localization and translocation ofthe receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. *Biochem J* 1994; 299: 473-479.

Sengelov H. Secretory vesicles of human neutrophils. *Eur J Haemotol* 1996; 57 (Suppl 58): 6–24.

Senior RM, Tegner H, Kuhn C, et al. The induction of pulmonary emphysema with human leukocyte elastase. *Am Rev Respir Dis* 1977; 116: 469-475.

Seres I, Csongor I, Mohacsi A. Age-dependent alterations of human recombinant GM-CSF effects on human granulocytes. *Mech Ageing Dev* 1993; 71: 143.

Servant G, Weiner OD, Herzmark P, et al. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* 2000; 287: 1037–1040.

Seok J, Warren HS, Cuenca AG et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA* 2013; 110: 3507-3512.

Sharma G, Hanania NA, Shim YM. The aging immune system and its relationship to the development of chronic obstructive pulmonary disease. *Pro Am Thorac Soc* 2009; 6: 573-580.

Sheehan JK, Kesimer M, Pickles R. Innate immunity and mucus structure and function. *Novartis Found Symp* 2006; 279: 155-166.

Sinden NJ, Stockley RA. Systemic infl ammation and comorbidity in COPD: a result of 'overspill' of infl ammatory mediators from the lungs? Review of the evidence. *Thorax* 2010; 65: 930–936.

Sires UI, Murphu G, Baragi VM, et al. Matrilysin is much more efficient than other matrix metalloproteinases in the proteolytic inactivation of α_1-antitrypsin. *Biochem Biophys Res Commun* 1994; 204: 613-620.

Sixt M, Hallmann R, Wendler O, et al. Cell adhesion and migration properties of beta 2-integrin negative polymorphonuclear granulocytes on defined extracellular matrix molecules. Relevance for leukocyte extravasation. *J Biol Chem* 2001; 276: 18878-18887.

Sjaastad MD, Nelson WJ. Integrin-mediated signalling and regulation of cell adhesion by intracellular calcium. *Bioessays* 1997; 19: 47–55.

Sly LM, Rauh MJ, Kalesnikoff J, Buchse T, Krystal G**.** SHIP, SHIP2 and PTEN activities are regulated in vivo by modulation of their protein levels: SHIP is up-regulated in macrophages and mast cells by lipopolysaccharide. *Exp Hematol* 2003; 31: 1170-81.

Smallman LA, Hill SL, Stockley RA**.** Reduction of ciliary beat frequency in vitro by sputum from patients with bronchiectasis: a serine proteinase effect. *Thorax* 1984; 39: 663-7.

Smolonska J, Wijmenga C, Postma DS, Boezen HM. Meta-analyses on suspected chronic obstructive pulmonary disease genes: a summary of 20 years' research. *Am J Respir Crit Care Med* 2009; 180: 618–631.

Snedecor GW, Cochran G. Statistical Methods. 8th Edition. 1989 *Ames Iowa State Press*.

Snider GL, Hayes JA, Franzblau C, et al. Relationship between elastolytic activity and experimental emphysema inducing properties of papain preparation. *Am Rev Respir Dis* 1974; 110: 254-262.

Songyang Z, Shoelson SE, Chaudhuri M et al. SH2 domains recognize specific phosphopeptide sequences". *Cell* 1993; 72: 767–778.

Sorbini CA, Grassi V, Solinas E, et al. Arterial oxygen tension in relation to age in healthy subjects. *Respiration* 1968; 25: 3-13.

Southwick FS, Dabiri GA, Paschetto M, et al. Polymorphonuclear leukocyte adherence induces actin polymerisation by a transduction pathway which differs from that used by chemoattractants. *J Cell Biol* 1989; 109: 1561–1569.

Srinivasan S, Wang F, Glavas S, et al. Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. J Cell Biol 2003;160: 375-85.

Stansecu D, Sanna A, et al. Airways obstruction, chronic expectoration and rapid decline in FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax* 1996; 51: 267-271.

Steegmaier, M, Levinovitz A, et al. The E-selectin ligand ESL-1 is a variant of a receptor for fibroblast growth factor. *Nature* 1995; 373: 615-620.

Stephens LR, Eguinoa A, Erdjument-Bromage H, et al. The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell* 1997; 89: 105–114.

Stephens L, Smrcka A, Cooke FT, et al. A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein βγ subunits. *Cell* 1994; 77: 83–93.

Stephens LR, Ellson C, Hawkins PT**.** Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. *Curr Opin Cell Biol* 2002; 14: 203-213.

Stephens L, Milne L, Hawkins P. Moving towards a better understanding of chemotaxis. *Curr Biol* 2008; 18: R485–R494.

Stille R, Farooq SM, Gordon J, et al. The functional significance behind expressing two IL-8 receptor types on PMN. *J Leukoc Biol* 2009; 86(3): 529-43.

Stockley RA, Shaw J, et al. Effect of alpha 1 proteinase inhibitor on neutrophil chemotaxis. *Am J Respir Cell Mol Biol* 1990; 2: 163-170.

Stockley RA. Soluble proteins in lung defence. In: Stockley RA, editor. Pulmonary Defences. *Wiley Europe Chicester* 1997: 17-38.

Stockley RA. Neutrophils and the pathogenesis of COPD. *Chest* 2002; 121: 151S-155S.

Stone H, McNab G, Wood AM, et al. Variability of sputum inflammatory mediators in COPD and α1-antitrypsin deficiency. *Eur Respir J* 2012; 40: 561-569.

Stoyanov B, Volinia S, Hanck T, Rubio et al. Cloning and characterization of a G protein-activated human phosphoinositide- 3 kinase. *Science* 1995; 269: 690–693.

Strunk, R. C., D. M. Eidlen, et al. Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. *J Clin Invest* 1988; 81: 1419-1426.

Su WH, Chen HI, Jen CJ. Differential movements of VE-cadherin and PECAM-1 during transmigration of polymorphonuclear leukocytes through human umbilical vein endothelium. *Blood* 2002; 100: 3597–603.

Suire S, Coadwell J, Ferguson GJ, et al. p84, a new Gbetagamma-activated regulatory subunit of the type IB phosphoinositide 3-kinase p110gamma. *Curr Biol* 2005; 15: 566–570.

Suire S, Condliffe AM, Ferguson GJ et al. Gbetagammas and the Ras binding domain of p110gamma are both important regulators of PI(3)Kgamma signalling in neutrophils. *Nat Cell Biol* 2006; 8: 1303-1309.

Summers C, Rankin SM, Condliffe AM, et al. Neutrophil kinetics in health and disease. *Trends Immunol* 2010; 31: 318-324.

Suzaki I, Asano K, Shikama Y, et al. Suppression of IL-8 production from airway cells by tiotropium bromide in vitro. *Int J Chron Obstruct Pulmon Dis* 2011; 6: 439-448.

Suzuki YJ, Nagase H, Nie K, Park AM. Redox control of growth factor signaling: recent advances in cardiovascular medicine. *Antioxid Redox Signal* 2005; 7: 829-834.

Tager I, Speizer FE. Role of infection in chronic bronchitis. *N Engl J Med* 1975; 292: 563-571.

Taggart CC, Lowe GJ, Greene CM et al. Cathepsin B, L, and S cleave and inactivate secretory leucoprotease inhibitor. *J Biol Chem* 2001; 276: 33345-33352.

Takabatake N, Nakamura H, Abe S, et al. The relationship between chronic hypoxemia and activation of the tumour necrosis factor alpha system in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000; 161: 1179e84.

Takeyama K, Agustí C, Ueki IF, et al. Neutrophil-dependant goblet cell degranulation: role of membrane-bound elastase and adhesion molecules. *Am J Physiol* 1998; 19: L294-L302.

Tang M, Lijima M, et al**.** Disruption of PKB signaling restores plraity to cells lacking tumor suppressor PTEN. *Mol Biol Cell* 2011; 22: 437-47.

Tashkin DP, Clark VA, et al. The UCLA population studies of chronic obstructive respiratory disease. VIII. Effects of smoking cessation on lung function; a prospective study of a free-living population. *Am Rev Respir Dis* 1984; 130: 707-715.

Tashkin DP, Celli B, Senn S, et al; for UPLIFT Study Investigators. A 4-year trial of tiotropium in chronic obstructive pulmonary disease. *N Engl J Med* 2008; 359: 1543–1554.

Tedder TF, Steeber DA, Chen A and Engel P. The selectins: Vascular adhesion molecules. *FASEB J* 1995a; 9: 866–873.

Tetley TD. New perspectives on basic mechanisms in lung disease. 6. Proteinase imbalance: its role in lung disease. *Thorax* 1993; 48: 560-565.

Thompson AB, Scholer SG, Daughton DM, et al. Altered epithelial lining fluid parameters in old normal individuals. *J Gerontol* 1992; 47: M171-176.

Thurlbeck WM. Chronic airflow obstruction in lung disease. Philadelphia: *WB Saunders* 1976.

Tian W, Laffafian I, Dewitt S, et al. Exclusion of the exogenous phosphatidylinositol-3,4,5-trisphosphate from the neutrophil-polarizing pseudopodia: stabilization of the uropod and cell polarity. *EMBO Rep* 2003; 4: 982-988.

Tkalcevic J, Novelli M, et al. Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G. Immunity 2000; 12: 210-213.

Tomee, JF, Koeter GH, et al. Secretory leukoproteinase inhibitor: a native anti-microbial protein presenting a new therapeutic option? *Thorax* 1998; 53: 114-116.

Tonks NK. Redox redux: revisiting PTPs and the control of cell signaling. *Cell* 2005; 121: 667-670.

Tonnesen, M. G., D. C. Anderson, et al. Adherence of neutrophils to cultured human microvascular endothelial cells. Stimulation by chemotactic peptides and lipid mediators and dependence upon the Mac-1, LFA-1, p150,95 glycoprotein family. *J Clin Invest* 1989; 83: 637-642.

Tortorella C, Piazzolla G, Spaccavento F, et al. Age-related effects of oxidative metabolism and cyclic AMP signaling on neutrophil apoptosis. *Mech Ageing Dev* 1999; 110: 195–205.

Tortorella C, Piazzolla G, Spaccavento F, et al. Regulatory role of extracellular matrix proteins in neutrophil respiratory burst during aging. *Mech Ageing Dev* 2000; 119: 69–82.

Tortorella C, Simone O, Piazzolla G, et al. Age-related impairment of GM-CSF-induced signalling in neutrophils: role of SHP-1 and SOCS proteins. *Ageing Res Rev* 2007; 6: 81-93.

Touyz RM. Reactive oxygen species as mediators of calcium signaling by angiotensin II: implications in vascular physiology and pathophysiology. *Antioxid Redox Signal* 2005; 7: 1302-1314.

Tsai HH, Frost E, To V, et al. The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. *Cell* 2002; 110: 373–83.

Turner JM, Mead J, et al. Elasticity of human lungs in relation to age. *J Appl Physiol* 1968; 25: 664-671.

Uller L, Persson CG, et al. Resolution of airway disease: removal of inflammatory cells through apoptosis, egression or both. *Trends Pharmscol Sci* 2006; 27(9): 461-466.

Ussov WY, Aktolun C, Myers MJ, et al. Granulocyte margination in bone marrow: comparison with margination in the spleen and liver. Scand J Clin Lab Invest 1995; 55: 87–96.

Vacca G, Randerath WJ, Gillissen A. Inhibition of granulocyte migration my tiotropium bromide. *Respir Res* 2011 27; 12: 24.

Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, et al. The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* 2010; 11: 329-341.

van den Borst B, Gosker HR, Zeegers MP, Schols AM. Pulmonary function in diabetes: a metaanalysis. *Chest* 2010; 138: 393–406.

Van der Vliet, O'Neill CA, Cross CE, et al. Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *Am J Physiol* 1999; 276: L289-L296.

van Duin D, Mohanty S, Thomas V, et al. Age-associated defect in humanTLR-1/2 function. *J Immunol* 2007; 178: 970-975.

van Durme YM, Verhamme KM, Aarnoudse AJ, et al. C-reactive protein levels, haplotypes, and the risk of incident chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2009; 179: 375–382.

Van Eeden SF, Hogg JC. The response of human bone marrow to chronic cigarette smoking. *Eur Respir J* 2000; 15: 915-921.

Van Keymeulen A, Wong K, Knight ZA, et al. To stabilize neutrophil polarity, PIP3 and Cdc42 augment RhoA activity at the back as well as signals at the front. *J Cell Biol* 2006; 174: 437-445.

van Wetering S, van der Linden AC, van Sterkenburg MA, et al. Regulation of SLPI and elafin release from bronchial epithelial cells by neutrophil defensins. *Am J Physiol Lung Cell Mol Physiol* 2000; 278: L51–L58.

Verbeken EK, Cauberghs M, et al. The senile lung: comparison with normal and emphysematous lungs. 1. Structural apects. *Chest* 1992; 101: 793-799.

Vestbo J, Edwards LD, Scanlon PD, et al. Changes in forced expiratory volume in 1 second over time in COPD. *N Engl J Med* 2011; 365: 1184-1192.

Viasus D, Garcia-Vidal C, Simonetti AF, et al. The effect of simvastatin on inflammatory cytokines in community-acquired pneumonia: a randomised, double-blind, placebo-controlled trial. *BMJ Open* 2015; 5: e006251.

Vieira OV, Botelho RJ, Rameh L, et al. Distinct roles of class I and class III phosphatidylinositol 3-kinases in phagosome formation and maturation. *J Cell Biol* 2001; 155: 19–25.

Virtala R, Ekman AK, Jansson L, et al. Airway inflammation evaluated in a human nasal lipopolysaccharide challenge model by investigating the effect of a CXCR2 inhibitor. *Clin Exp Allergy* 2012; 42: 590-596.

Vogt W**.** Cleavage of the fifth component of complement and generation of a functionally active C5b6-like complex by human leukocyte elastase. *Immunobiology* 2000; 201: 470-477.

Voigt P, Brock C, Nurnberg B, et al. Assigning functional domains within the p101 regulatory subunit of phosphoinositide 3-kinase gamma. *J Biol Chem* 2005; 280: 5121–5127.

Voigt P, Dorner MB, Schaefer M. Characterization of P87Pikap, a novel regulatory subunit of phosphoinositide 3-kinase gamma that is highly expressed in heart and interacts with PDE3B. *J Biol Chem* 2006; 281: 9977–9986.

von Andrian UH, Chambers JD, McEvoy LM, Bargatze RF, Arfors KE and Butcher EC. Two-step model of leukocyte-endothelial cell interaction in inflammation: Distinct roles for LECAM-1 and the leukocyte b2-integrins in vivo. *Proc Natl Acad Sci USA* 1991; 88: 7538–7542.

Von Andrian UH, Hansell P, Chambers JD, et al. L-selectin function is required for β2 integrin-mediated neutrophil adhesion at physiological shear rates in vivo. *Am J Physiol* 1992; 263: H1034.

von Haehling S, Hopkinson NS, Polkey MI, et al. Elevated TNF alpha production in whole blood in patients with severe COPD: the potential link to disease severity. *Wien Klin Wochenschr* 2009; 121: 303e8.

Wagner JG, Roth RA. Neutrophil migration mechanisms with an emphasis on pulmonary vasculature. *Pharmacological Review* 2000; 52: 349-374.

Walchek B, Kahn J, et al. Neutrophil rolling altered by inhibition of L-Selectin shedding in vitro. *Nature* 1996; 380: 720-723.

Wang, F., P. Herzmark, et al. Lipid products of PI(3)Ks mainatin persistent cell polarity and directed motility in neutrophils. *Nat Cell Biol* 2002; 4: 513-518.

Weber I. Is there a pilot in a pseudopod? *Eur J Cell Biol* 2006; 85: 915–924.

Wedzicha JA. The heterogeneity of chronic obstructive pulmonary disease. *Thorax* 2000; 55: 631-632.

Wei C, Wang X, Chen M, et al. Calcium gradients underlying cell migration. *Curr Opin Cell Biol* 2011. 24: 254-261.

Weiner OD. Regulation of cell polarity during eukaryotic chemotaxis: The chemotactic compass. *Curr Opin Cell Biol* 2002; 14: 196-202.

Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med* 1989; 320: 365-376.

Weng NP, Akbar AN, Goronzy J. CD28(-) T cells: their role in age-associated decline of immune function. *Trends Immunol* 2009; 30(7): 306-12.

Wenisch C, Patruta S, et al. Effect of age on human neutrophil function. *J Leuk Biol* 2000; 67: 40-45.

Werz O. 5-lipoxygenase: cellular biology and molecular pharmacology. *Curr Drug Targets Inflamm Allergy* 2002; 1: 23–44.

Whitelaw DA, Rayner BL, Willcox PA. Community-acquired bacteremia in the elderly-a prospective study of 121 cases. *J Am Geriatr Soc* 1992; 40: 996.

Willemse BW, ten Hacken NH, Rutgers B, et al**.** Effect of 1 year smoking cessation on airway inflammation in COPD and asymtomatic smokers. *Eur Respir J* 2005; 26: 835-845.

Williams MA, Solomkin JS. Integrin mediated signaling in human neutrophil functioning. *J Leuk Biol* 1999; 65: 725-736.

Williams O, Houseman BT, Kunkel EJ, et al. Discovery of dual inhibitors of the immune cell PI3Ks p110delta and p110gamma: a prototype for new anti-inflammatory drugs. *Chem Biol* 2010; 17: 123-134.

Williams SL, Milne IR, Bagley CJ, et al. A proinflammatory role for proteolytically cleaved annexin A1 in neutrophil transendothelial migration. *J Immunol* 2010; 185: 3057–63.

Wollin L, Bundschuh DS, Wohlsen A, et al. Inhibition of airway hyperresponsiveness and pulmonary inflammation by roflumilast and other PDE4 inhibitors. *Pulm Pharmacol Ther* 2006; 19: 343–352.

Woodfin A, Voisin MB, Beyrau M, et al. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. *Nat Immunol* 2011; 12: 761-769.

Woodhead M, Blasi F, Ewig S, et al. Guidelines for the management of adult lower respiratory tract infections - full version. *Clin Microbiol Infect* 2011; 17: E1-E59.

Woolhouse IS, Bayley DL, Stockley RA. Sputum chemotactic activity in chronic obstructive pulmonary disease: effect of alpha(1)-antitrypsin deficiency and the role of leukotriene B(4) and interleukin 8. *Thorax* 2002; 57(8):709-714.

Woolhouse IS, Bayley DL, et al. Endothelial interactions of neutrophils under flow in chronic obstructive pulmonary disease. *Eur Respir J* 2005; 25(4): 612-617.

World Health Organization. Chronic Obstructive Pulmonary Disease (COPD). Fact sheet 315. 2007; www.who.int/mediacentre/factsheet/fs315.

Wright DC, Gallin JL. Secretory responses of human neutrophils: Exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. *J Immunol* 1979; 123: 258-294.

Wu D**.** Signaling mechanisms for regulation of chemotaxis. *Nature Cell Res* 2005; 15: 52-6.

Wu D, Lin F**.** Modeling Cell Gradient Sensing and Migration in Competing Chemoattractant Fields. *PLoS ONE* 2011; 6: e18805.doi:10.1371/journal.pone.0018805.

Yadav R, Larbi KY, et al. Migration of leukocytes through the vessel wall and beyond. *Thromb Haemost* 2002; 90: 598-606.

Xu Y, Li H, Bajrami B, et al. Cigarette smoke (CS) and nicotine delay neutrophil spontaneous death via suppressing production of diphosphoinositol pentakisphosphate. *Proc Natl Acad Sci U S A* 2013; 110(19): 7726-7731.

Yagi M, Kantarci A, Iwata T, et al. PDK1 Regulates Chemotaxis in Human Neutrophils. *J Dent Res* 2009; 88: 1119–1124.

Yamagata T, Sugiura H, Yokoyama T, et al. Overexpression of CD-11b and CXCR1 on circulating neutrophils: its possible role in COPD. *Chest* 2007; 132(3); 890-899.

Yamamori T, Inanami O, Nagahata H, Cui Y, Kuwabara M. Roles of p38 MAPK, PKC and PI3-K in the signalling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes. *FEBS Lett* 2000; 467: 253-258.

Yanbaeva DG, Dentener MA, Creutzberg EC, et al. Systemic effects of smoking. *Chest* 2007; 131: 1557–1566.

Yang J, Hirata T, Croce K, et al. Targeted gene disruption demonstrates that P-selectin glycoprotein ligand (PSGL-1) is required for P-selectin-mediated but not E-selectin-mediated neutrophil rolling and migration. *J Exp Med* 1999; 190: 1769–1782.

Yang L, Froio RM, Sciuto TE, et al. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood* 2005; 106: 584–92.

Yao GY, Ma YL, Zhang MQ, et al. Macrolide therapy decreases chronic obstructive pulmonary disease exacerbation: a meta-analysis. *Respiration* 2013; 86: 254-260.

Yauch RL, Hemler ME. Specific interactions among transmembrane 4 superfamily (TM4SF) proteins and phosphoinositide 4-kinase. *Biochem J* 2000; 351 Pt 3: 629-637.

Yoakim M, Hou W, Songyang Z, et al. Genetic analysis of a phosphatidylinositol 3-kinase SH2 domain reveals determinants of specificity. *Mol Cell Biol* 1994; 14: 5929–5938.

Yoo SK, Deng Q, Cavnar PJ, et al**.** Differential regulation of protrusion and polarity by PI3K during neutrophil motility in live zebrafish. *Dev Cell* 2010; 18: 226-36.

Yoshikawa T, Dent G, Ward J, et al. Impaired neutrophil chemotaxis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007; 175: 473-479.

Yu J, Zhang Y, McIlroy J, et al. Regulation of the p85/p110 phosphatidylinositol 3-kinase: stabilization and inhibition of the p110 catalytic subunit by the p85 regulatory subunit. *Mol Cell Biol* 1998; 18: 1379–1387.

Zaas AK, Schwartz D. Innate immunity and the lung: Defense at the interface between host and environment. *Trends Cardiovasc Med* 2005; 15(6): 195-202.

Zani ML, Nobar SM, Lacour SA, et al. Kinetics of the inhibition of neutrophil proteinases by recombinant elafin and pre-elafin (trappin-2) expressed in *Pichia pastoris*. *Eur J Biochem* 2004; 271: 2370–2378.

Zhang P, Summer WR, Bagby GJ, Nelson S. Innate immunity and pulmonary host defense. *Immunol Rev* 2000; 173: 39–51.

Zhang XZ, Paré PD, Sandford AJ. PMN degranulation in relation to CD63 expression and genetic polymorphisms in healthy individuals and COPD patients. *Int J Mol Med* 2007; 19: 817-822.

Zou W, Meng X, Cai C, et al. Store-operated Ca2+ entry (SOCE) plays a role in the polarization of neutrophil-like HL-60 cells by regulating the activation of Akt, Src, and Rho family GTPases. *Cell Physiol Biochem* 2012; 30: 221-237.

CHAPTER 8

APPENDICES

8.1 PUBLICATIONS ARISING FROM THIS THESIS

Stockley JA, Walton GM, Lord JM, et al. Aberrant neutrophil functions in stable chronic obstructive pulmonary disease: the neutrophil as an immunotherapeutic target. *Int Immunopharmacol* 2013; 17(4): 1211-1217.

Sapey E, Stockley JA, Greenwood H, et al. Behavioral and structural differences in migrating peripheral neutrophils from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2011; 183(9): 1176-1186.

8.2 PUBLISHED ABSTRACTS

Stockley JA, Peat L, Walton GM, Lord JM, Sapey E. The role of chemokine receptors in the aberrant migration of COPD neutrophils. *European Respiratory Society International Congress* 2014 (P3859).

Sapey E, Stockley JA, Walton GM, Usher A, Bettaney B, Stockley RA. Abnormal neutrophil migration is a feature of early COPD, present across disease phenotypes and causally related to increased Phosphoinositide-3-Kinase signalling. *American Thoracic Society Annual Meeting* 2013 (A3492)

Sapey E, Stockley JA, Greenwood H, Insall R, Lord JM, Stockley RA. *In vitro* aberrant neutrophil migration in COPD is a generic phenomenon and is not related to surface-expression of chemoattractant receptors or systemic inflammation. *American Thoracic Society Annual Meeting* 2011 (A4347)

8.3 SUBMITTED ARTICLES

Stockley JA, Peat L, Walton GM, Lord JM, Sapey E. The Role of chemokine receptors in the aberrant migration of COPD neutrophils. *Thorax*.

Stockley JA, Walton GM, Greenwood H, Usher A, Sadhra C, Ungurs M, Clondiffe AM, Lord JM, Stockley RA, Sapey E. Correcting aberrant neutrophil migration in COPD: PI3K delta and gamma inhibitors as focused immunomodulatory therapies. *Am J Respir Crit Care Med.*