

**INFLAMMATION AND NEUTROPHIL RECRUITMENT
IN AGEING SUBJECTS AND PATIENTS WITH
CHRONIC OBSTRUCTIVE PULMONARY DISEASE**

by

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ABSTRACT

The neutrophil is believed to be central to the development of COPD. Whilst critical in the clearance of bacteria, neutrophils also have the capacity to cause extensive tissue damage, as the substances contained within their cytoplasmic granules are able to degrade all components of the extra-cellular matrix. To enter lung tissue, neutrophils must migrate accurately from the circulation to areas of inflammation. There have been few studies examining neutrophil migration in COPD, and due to contradictory results, it is unclear which migratory stimuli are important. It is also unclear whether neutrophils from patients with COPD vary in their migratory behaviour, either to age-matched controls (as the majority of studies have not stringently age-matched) or patients with similar lung disease. It is also unclear whether neutrophil migration is compatible with emerging theories of advanced ageing as a component of the development of COPD.

Sputum and plasma samples were collected on eleven occasions during one month from patients with COPD. There was significant variability in all inflammatory indices but variability could be reduced by using a rolling mean of individual patient data points. Significant correlations were demonstrated both between the inflammatory biomarkers themselves and between inflammatory biomarkers and markers of disease. Some relationships were not apparent when results from a single sample were used and the reliability of inter-relationships improved as more data points were used for each patient. IL-8 correlated the most strongly with other inflammatory mediators, neutrophil counts and clinical indices of disease, suggesting that it is an important migratory signal in COPD.

To assess the affect of ageing on neutrophil migration, neutrophils were isolated from healthy young and older subjects. Although the speed of migration was intact, neutrophils from older subjects migrated with less velocity, less directional persistence and less

accuracy in the presence of IL-8 and GRO α . Differences in migration could not be accounted for by cell surface receptor expression or shedding, but an inhibitor of CXCR2 receptor function gave young neutrophils and old migratory phenotype, suggesting altered downstream signalling.

Neutrophils from patients with COPD did not display changes compatible with increased ageing, but instead migrated with increased speed and reduced accuracy compared with age-matched controls or patients with a similar lung disease, in the presence of IL-8, GRO α and sputum. Structurally, COPD neutrophils formed less pseudopodia when migrating, and had reduced surface expression of CXCR1 and CXCR2. These changes were not seen in healthy controls, healthy smokers, or patients with a similar lung disease (who were matched for age, gender, lung physiology, smoking status and medications), suggesting that smoking, lung disease, inflammation or its treatment were not the cause of the altered behaviour. Studies of the migratory function of CXCR1 and CXCR2 suggested that both were important in neutrophil migration, but that CXCR2 was the predominant receptor when migrating in gradients of biological samples. Inhibition of CXCR1 and CXCR2 could not give healthy neutrophils a COPD phenotype, suggesting that a reduction in receptor expression was not the only factor potentially altering migratory dynamics. Treating COPD cells with a PI3 Kinase inhibitor differentially altered their migratory behaviour, reducing the speed, but increasing the accuracy, so that cells now resembled those from healthy individuals. This suggests strongly that the mechanism of altered migration lies within this migratory pathway and potentially provides a new therapeutic target for patients with COPD.

TABLE OF CONTENTS

| | | |
|----------|---|-----------|
| 1 | INTRODUCTION..... | 1 |
| 1.1 | THE NORMAL BRONCHIAL TREE..... | 2 |
| 1.1.1 | Anatomy..... | 2 |
| 1.1.2 | Blood Supply..... | 3 |
| 1.1.3 | Immune function..... | 4 |
| 1.2 | THE NEUTROPHIL..... | 10 |
| 1.2.1 | Differentiation, maturation and apoptosis..... | 10 |
| 1.2.2 | Neutrophil migration..... | 15 |
| 1.3 | ANTI-PROTEINASES..... | 31 |
| 1.3.1 | α 1-antitrypsin..... | 31 |
| 1.3.2 | Secretory leukoprotease inhibitor..... | 31 |
| 1.3.3 | Other anti-proteinases that inhibit neutrophil proteinases..... | 32 |
| 1.4 | AGEING AND NEUTROPHIL FUNCTION..... | 33 |
| 1.5 | CHRONIC OBSTRUCTIVE PULMONARY DISEASE..... | 37 |
| 1.5.1 | Definition and classification of disease severity..... | 37 |
| 1.5.2 | Prevalence and impact..... | 37 |
| 1.5.3 | Pathogenesis..... | 38 |
| 1.5.4 | Pro-migratory stimuli..... | 42 |
| 1.5.5 | Neutrophil migration in COPD..... | 48 |
| 1.5.6 | Proteinases in COPD..... | 50 |
| 1.5.7 | Other Inflammatory Cells..... | 62 |
| 1.5.8 | Oxidative Stress..... | 62 |
| 1.5.9 | Bacteria and Viruses..... | 64 |
| 1.6 | THE RELATIONSHIP BETWEEN COPD AND AGEING..... | 65 |
| 1.7 | ALPHA 1-ANTITRYPSIN DEFICIENCY..... | 68 |
| 1.8 | SUMMARY..... | 70 |
| 1.9 | STRUCTURE OF THIS THESIS..... | 71 |
| 2 | METHODS..... | 74 |
| 2.1 | ETHICAL APPROVAL..... | 75 |
| 2.2 | PATIENTS..... | 75 |
| 2.3 | PULMONARY FUNCTION TESTING..... | 75 |
| 2.4 | SPUTUM AND BLOOD SAMPLE COLLECTION..... | 75 |
| 2.5 | SPUTUM PROCESSING..... | 75 |
| 2.5.1 | Sputum Colour..... | 76 |
| 2.5.2 | Quantitative bacterial culture..... | 76 |
| 2.5.3 | Sputum sol phase collection..... | 76 |
| 2.5.4 | Cytospin preparation..... | 76 |
| 2.5.5 | Peripheral blood processing..... | 77 |
| 2.5.6 | Plasma sample..... | 77 |
| 2.5.7 | Isolation of Neutrophils..... | 77 |
| 2.6 | BIOCHEMICAL ASSAYS..... | 80 |
| 2.6.1 | Growth Related Oncogene..... | 80 |
| 2.6.2 | Interleukin-1 β | 81 |
| 2.6.3 | High sensitivity Interleukin-1 β | 83 |
| 2.6.4 | Interleukin-8..... | 85 |
| 2.6.5 | Leukotriene-B4..... | 86 |
| 2.6.6 | Tumour Necrosis Factor Alpha..... | 88 |
| 2.6.7 | Myeloperoxidase..... | 90 |
| 2.7 | CHEMOTAXIS ASSAYS..... | 91 |
| 2.7.1 | Agarose Gel Chemotaxis Assay..... | 92 |
| 2.7.2 | Modified Dunn Chamber Chemotaxis Assay..... | 93 |
| 2.8 | FLUORESCENT ANTIBODY CELL SORTING..... | 98 |
| 2.8.1 | Immunofluorescent labelling of CXCR1 and CXCR2 receptors on isolated neutrophils..... | 98 |
| 2.8.2 | Flow cytometry..... | 98 |
| 2.9 | STATISTICAL ANALYSIS..... | 99 |

3 THE QUANTIFICATION AND VARIABILITY OF NEUTROPHILS AND INFLAMMATORY MEDIATORS ASSOCIATED WITH NEUTROPHIL MIGRATION IN COPD

| | |
|-------|---|
| 100 | |
| 3.1 | BRIEF INTRODUCTION.....101 |
| 3.2 | EXPERIMENTS102 |
| 3.2.1 | Study subjects102 |
| 3.2.2 | Study Design.....102 |
| 3.2.3 | Sample collection & processing.....103 |
| 3.2.4 | Measurement of mediators103 |
| 3.2.5 | Statistical Analysis104 |
| 3.3 | RESULTS.....105 |
| 3.3.1 | Subject characteristics.....105 |
| 3.3.2 | Assay Variability and sputum characterisation108 |
| 3.3.3 | Intra-patient variability in sputum cell and mediator concentrations108 |
| | Figure 3.1a: Changes in raw data over one month for Patient 1 for the sputum sol mediators IL-1beta, TNF alpha and IL-8109 |
| 3.3.4 | Intra-patient variability in plasma cells and mediator concentrations113 |
| | Figure 3.2: Changes in raw data over one month for Patient 1 for the plasma mediators IL-1beta, TNF alpha and absolute neutrophil and monocyte counts.....114 |
| 3.3.5 | Reducing intra-patient variability in sputum samples.....117 |
| 3.3.6 | Inter-patient variability and the effects of a rolling mean.121 |
| 3.3.7 | Determining the sample size required to power interventional studies.123 |
| 3.3.8 | The relationship between sol phase inflammatory mediators and sputum cell counts125 |
| 3.3.9 | The relationship between sol phase inflammatory mediators, cell counts and disease129 |
| 3.4 | DISCUSSION.....130 |
| 4 | NEUTROPHIL MIGRATION IN THE HEALTHY ELDERLY139 |
| 4.1 | BRIEF INTRODUCTION.....140 |
| 4.2 | EXPERIMENTS141 |
| 4.2.1 | Study subject.....141 |
| 4.2.2 | Isolation of blood neutrophils142 |
| 4.2.3 | Neutrophil Chemotaxis142 |
| 4.2.4 | Flow Cytometry.....143 |
| 4.2.5 | Statistical analysis144 |
| 4.3 | RESULTS.....145 |
| 4.3.1 | Under agarose assay: Validation145 |
| 4.3.2 | Under agarose assay: Results.....146 |
| 4.3.3 | Modified Dunn Assay : Validation.....149 |
| 4.3.4 | Modified Dunn Chamber: Results155 |
| 4.3.5 | CXCR1 and 2 receptor surface expression: validation.....169 |
| 4.4 | DISCUSSION.....176 |
| 5 | ABERRANT NEUTROPHIL MIGRATION IN COPD.....182 |
| 5.1 | BRIEF INTRODUCTION.....183 |
| 5.2 | EXPERIMENTS185 |
| 5.2.1 | Study subjects185 |
| 5.2.2 | Isolation of blood neutrophils186 |
| 5.2.3 | Neutrophil Chemotaxis186 |
| 5.2.4 | Statistical analysis187 |
| 5.3 | RESULTS.....188 |
| 5.3.1 | Under Agarose and Modified Dunn Assays: validation.....188 |
| 5.3.2 | Under Agarose assay: Results188 |
| 5.3.3 | Modified Dunn Assay: Results192 |
| 5.3.4 | Model of lung damage in COPD and A1ATD.....202 |
| 5.3.5 | Migration towards GRO α204 |
| 5.4 | DISCUSSION.....209 |
| 6 | MECHANISMS OF ABERRANT MIGRATION: PSEUDOPOD FORMATION IN COPD ..214 |
| 6.1 | BRIEF INTRODUCTION.....215 |

| | | |
|-----------|--|------------|
| 6.2 | EXPERIMENTS | 217 |
| 6.2.1 | <i>Study subjects</i> | 217 |
| 6.2.2 | <i>Isolation of blood neutrophils</i> | 217 |
| 6.2.3 | <i>Assessment of pseudopod formation</i> | 218 |
| 6.2.4 | <i>Statistical analysis</i> | 219 |
| 6.3 | RESULTS | 219 |
| 6.3.1 | <i>Validation</i> | 219 |
| 6.3.2 | <i>Smoking and Non smoking health controls</i> | 220 |
| 6.3.3 | <i>Pseudopod formation in neutrophils isolated from patients with COPD, A1ATD and healthy controls</i> | 223 |
| 6.4 | DISCUSSION | 226 |
| 7 | MECHANISM OF ABERRANT MIGRATION. SURFACE EXPRESSION OF CXCR1 AND CXCR2 AND THE AFFECTS OF INHIBITION OF CXCR1 AND CXCR2 IN COPD | 229 |
| 7.1 | BRIEF INTRODUCTION | 230 |
| 7.2 | EXPERIMENTS | 234 |
| 7.2.1 | <i>Study subjects</i> | 234 |
| 7.2.2 | <i>Isolation of blood neutrophils</i> | 235 |
| 7.2.3 | <i>Flow Cytometry</i> | 235 |
| 7.2.4 | <i>Neutrophil Chemotaxis</i> | 236 |
| 7.2.5 | <i>Statistical analysis</i> | 238 |
| 7.3 | RESULTS | 239 |
| 7.3.1 | <i>Baseline characteristics</i> | 239 |
| 7.3.2 | <i>CXCR1 and CXCR2 receptor surface expression</i> | 240 |
| 7.3.3 | <i>The affects of IL-8 and GROα antibodies and a CXCR2 antagonist: validation</i> | 248 |
| 7.3.4 | <i>The affects of IL-8 and GROα monoclonal antibodies and a CXCR2 antagonist: Results</i> | 254 |
| 7.3.5 | <i>The affects of CXCR2 and CXCR1 monoclonal antibodies: validation</i> | 276 |
| 7.3.6 | <i>The affects of CXCR2 and CXCR1 monoclonal antibodies: results</i> | 279 |
| 7.4 | DISCUSSION | 292 |
| 8 | MECHANISM OF ABERRANT MIGRATION. | 297 |
| 8.1 | BRIEF INTRODUCTION | 298 |
| 8.2 | EXPERIMENTS | 302 |
| 8.2.1 | <i>Study subjects</i> | 302 |
| 8.2.2 | <i>Isolation of blood neutrophils</i> | 302 |
| 8.2.3 | <i>Neutrophil Chemotaxis</i> | 302 |
| 8.2.4 | <i>Statistical analysis</i> | 303 |
| 8.3 | RESULTS | 303 |
| 8.3.1 | <i>The affect of a type 1 PI3 Kinase inhibitor on neutrophil migration</i> | 303 |
| 8.4 | DISCUSSION | 313 |
| 9 | GENERAL DISCUSSION | 318 |
| 9.1 | THE QUANTIFICATION AND VARIABILITY OF INFLAMMATORY MEDIATORS AND CELLS IN COPD | 322 |
| 9.2 | NEUTROPHIL MIGRATION IN THE HEALTHY ELDERLY | 326 |
| 9.3 | NEUTROPHIL MIGRATION IN COPD | 329 |
| 9.4 | PSEUDOPOD FORMATION IN COPD | 332 |
| 9.5 | CXCR1 AND CXCR2 FUNCTION IN COPD | 333 |
| 9.6 | PI3 KINASE ACTIVITY IN COPD | 335 |
| 9.7 | FUTURE WORK | 337 |
| 10 | REFERENCES | 340 |
| 11 | APPENDICES | 368 |
| 11.1 | ABBREVIATIONS IN ALPHABETICAL ORDER | 369 |
| 11.2 | PUBLICATIONS ARISING FROM THIS THESIS | 370 |
| 11.2.1 | <i>Published Articles</i> | 370 |
| 11.2.2 | <i>Published Abstracts</i> | 370 |
| 11.2.3 | <i>Submitted articles</i> | 372 |

1 INTRODUCTION

1.1 THE NORMAL BRONCHIAL TREE

1.1.1 Anatomy

The airways of the respiratory tract are classified as either conductive or respiratory, based upon their participation in gaseous exchange. The largest conducting airway is the trachea; this divides into the main bronchi, sub-segmental bronchi and then bronchioles. The final, purely conductive airways are the terminal bronchioles, beyond which gas exchange occurs.

There are approximately three generations of respiratory bronchioles following the terminal bronchioles. The respiratory bronchioles are transitional, as they conduct gas and participate in gaseous exchange. After the respiratory bronchioles are two to nine generations of alveolar ducts, which terminate in the alveolar sacs.

The conducting airway walls contain epithelial, lymphoid, muscular, vascular and nervous elements which are placed in a connective tissue supportive frame which is arranged as follows: firstly there is a lining mucosa of surface epithelium, basement membrane and supporting elastic lamina propria. Secondly, there is the submucosa in which lie the glands, muscle and cartilage; and finally, there is the adventitial coat.

The alveoli are lined with squamous epithelium that is in continuity with the columnar epithelium of the conducting airways. The alveolar epithelium consists of two principle cell types (type I and type II pneumocytes). These cells are separated from the connective tissue and capillaries of the interstitium by a basement membrane. Type I cells provide a continuous but thin covering of the alveolus and prevent fluid loss while facilitating rapid gas exchange. Type II cells are twice as numerous as Type I, but

because of their cuboidal shape and size, they cover only 7% of the alveolar surface (Crapo, Barry et al. 1982). Type II cells have an important role in surfactant production and also retain some plasticity, as they can differentiate to replace damaged type I cells (Evans, Cabral et al. 1975).

1.1.2 Blood Supply

The trachea is supplied with systemic blood through branches of the inferior thyroid and bronchial arteries, which anastomose with each other. The bronchial and bronchiole walls receive systemic blood through the bronchial arteries, which are derived from the descending aorta and the intercostal and internal mammary arteries (Deffebach, Charan et al. 1987). There is a rich vascular network supplying the airway wall (Laitinen, Laitinen et al. 1987) which is able to participate in gas exchange, via broncho-pulmonary anastomoses. The network also helps to control heat exchange and contributes transudate to airway secretions.

Each airway is accompanied by a branch of the pulmonary artery that divides with the airways, forming a capillary meshwork in the alveolar wall, which is in close contact with the alveolar space. The capillaries converge at the periphery of the acinus to form pulmonary veins. Blood leaves the lungs via the hilar and bronchial veins from the bronchi; via the pulmonary veins deep in the lung; and via the thyroid veins from the trachea (Jeffrey 1995).

1.1.3 Immune function

As well as gas exchange, the airways also warm and humidify inhaled air, and have important host defence functions against potentially harmful inhaled particles and micro-organisms. The primary host defence system is outlined below.

1.1.3.1 Airway epithelium

The epithelial cells of the conducting airways provide an important mechanical barrier to inhaled particles and micro-organisms. The epithelium is primarily composed of pseudostratified, ciliated and columnar cells, although many other cell types are present, including goblet cells, serous cells, clara cells, endocrine, basal and brush cells, as well as a variety of transitional forms (Jeffrey and Reid 1975; Breeze and Wheeldon 1977; Jeffrey 1983). Most airway pathogens will not adhere to bronchial epithelium unless it has been damaged (Bredt, Feldner et al. 1982; Tuomanen and Hendley 1983). Where cells are damaged bacteria are able to adhere (Plotkowski, Beck et al. 1989; Feldman, Read et al. 1992) via lectin like substances, exopolysaccharide secretion and the expression of hair like fimbriae (Wilson and Rayner 1997).

1.1.3.2 Airways secretions

Airways secretions form an important component of the primary host defence system. In the trachea, there are approximately 4000 submucosal glands which produce mucus (Tos 1970), antibacterial proteins (including lysozyme (Bowes and Corrin 1977) and lactoferrin (Bowes, Clark et al. 1981)), the secretory component necessary for immunoglobulin (Ig) A transport (Brandtzaeg 1974), and the antiproteinase, secretory leukoprotease inhibitor (SLPI) (Kramps, Franken et al. 1981). Submucosal glands are composed of a central acinus consisting of serous cells, and a tubule lined with mucous cells. Plasma cells (responsible for the production of IgA) are also found in the submucosal glands (Soutar 1976).

The serous and mucous cells of the bronchial glands secrete the majority of the bronchial secretions, although goblet cells, and both the serous and clara cells of the airway epithelium make important contributions. Secretions are further diluted by alveoli surfactant and plasma fluid transudate (Puchelle, Girod de Bentzmann et al. 1995).

Bronchial mucus comprises of a continuous watery sol layer which overlays the bronchial epithelium and in which the cilia beat; and a more viscous gel layer, which lies on the tips of the cilia. The sol layer is 5 - 10 μ m deep, and is derived from the clara cells in the airway epithelium at the bronchiolar level with some contribution from fluid transudation. The major control mechanism of the water content of the sol phase of airway secretions relates to the movement of sodium and chloride ions, and the balance between the water content of the interstitium and the lumen. The sol layer enables the cilia to propel the gel layer over its surface, and is fundamental to mucociliary clearance. The mucus gel layer is derived from several sources including goblet and serous cells in the airway epithelium, clara cells at the bronchiolar level (Widdicombe and Pack 1982) and the submucosal glands (Shimura and Takishima 1994). The sol phase contains soluble bronchial proteins and serum proteins, whilst the gel phase contains the mucinous glycoproteins, other serum proteins and also proteins bound to mucins (Kim 1997). The mucins are high molecular mass mucoglycoproteins (King and Rubin 1994), and confer viscoelastic properties on the gel phase which enable the mucociliary escalator to function even in areas where ciliated epithelium is interrupted. A number of different mucin proteins have been identified, and the quantitative expression of individual mucins appears to alter with disease, especially in the presence of inflammation, altering the nature of the gel phase and potentially its ability to clear

pathogens and particles from the lung. Phospholipids appear to be important in weakening adhesion of the mucus to the epithelium, aiding mucociliary clearance (Rubin, Ramirez et al. 1992).

Bronchial mucus has many functions. It reduces evaporative loss from the respiratory tract, provides a protective barrier over the bronchial epithelium and removes trapped inhaled particles via ciliary action. The mucus also provides a medium for immunoglobulins and other protective proteins.

1.1.3.3 Mucociliary clearance

In healthy individuals, airway secretions are moved up to the mouth by ciliary action in the mucociliary escalator. Ciliated cells are found primarily in the tracheo-bronchial epithelium, although they are also present in the bronchioles (Shimura and Takishima 1994; Widdicombe and Widdicombe 1995). There are approximately 200 – 300 cilia per cell; each is 4 – 6 μm long and 0.1 – 0.2 μm in diameter. The cilia beat 1000 times per minute, and in health the action of the cilia is co-ordinated, both within a single cell and between adjacent cells (Sanderson and Sleight 1981). The ciliary beat cycle has two components. The first is movement towards the larynx; this is the effective stroke, and is followed by a recovery stroke in the opposite direction where the cilia bend and disengage from the mucus (Sleight, Blake et al. 1988). Microvilli project between the cilia and are believed to regulate the depth of the periciliary fluid level.

The clearance of mucus depends on ciliary action (Puchelle, Zahm et al. 1980), cough, mucus volume, and the viscoelasticity and adhesiveness of the mucus to the airway epithelium. Mucus transportation has two phases, a fast phase related to ciliary

clearance and cough, which is completed after a few hours in healthy individuals, and a slower phase which represents alveolar clearance and occurs over weeks or months (Pavia 1984; Hasania and Pavia 1989).

Mucociliary clearance is impaired in a variety of conditions including bronchitis and bronchiectasis. There are many reasons for the impairment, including inhibition of ciliary activity by proteinases such as neutrophil elastase (NE) (Smallman, Hill et al. 1984), the presence of bacterial products (Wilson, Pitt et al. 1987) and epithelial damage. In chronic lung disease the excess and accumulated secretions are expectorated in the form of sputum, which is a mixture of bronchial secretions, cells, cellular debris, cleared organisms and saliva.

1.1.3.4 Secretory proteins

There are many proteins in airway secretions including albumin, lysozyme, lactoferrin, immunoglobulins, antioxidants and antiproteinases such as alpha-1-antitrypsin (α 1AT), alpha-1-chymotrypsin and SLPI (Stockley 1997). Lactoferrin and lysozyme are involved in host defence (Ellison and Giehl 1991), other molecules such as catalase and glutathione have anti-oxidant and anti-inflammatory properties (Cantin, Fells et al. 1990). The roles of anti-proteinases will be discussed in section 1.3.

Immunoglobulin A (IgA) is also an important component of airway secretions, predominantly in the upper respiratory tract (Morgan, Hussein et al. 1980). In contrast, the other immunoglobulins contribute little to bronchial mucus. In the lung, most of IgA is dimeric, with 70% being of the IgA1 subclass and 30% being of the IgA2 subclass (Burnett 1986). This local IgA is thought to be secreted by submucosal plasma cells and is released as a dimer joined by a 15kDa protein linker called the “J”

chain. The dimer binds to the polymeric IgA receptor on the basal surface of epithelial cells, is internalised and transported to the apical surface where the receptor is cleaved leaving a small IgA bound fragment (the secretory component).

The dimeric form of IgA is believed to reduce bacteria and viral adherence to epithelial cells (Jefferis 1997). The secretory component of IgA enhances macrophage phagocytosis (Richards and Gauldie 1985) and (working with IgG) facilitates cell mediated cytotoxicity (Shen and Fanger 1981). IgA also appears to stabilise bronchial mucus by forming cross-links with the mucopolysaccharides.

1.1.3.5 Immune cells and the lung

Monocytes in the systemic circulation are continuously recruited to the lung tissues where they differentiate into pulmonary macrophages and dendritic cells (Poulter 1997). These cells are further classified by their location, and include pleural, interstitial, alveolar and airway macrophages, and dendritic cells (found solely within the bronchial epithelium). Inhaled bacteria are able to reach the alveolar (as is any particulate matter less than 3 μm in diameter), where they are recognised and phagocytosed by macrophages. The process of phagocytosis begins with the bacteria adhering to the surface of the macrophage by receptors that recognise bacterial mannose sugars or the C3b complement receptors (once C3 has been activated by binding to the organism). Following phagocytosis, the phagosome fuses with a lysosome, exposing the bacterium to anaerobic acid hydrolases and products of the respiratory burst, resulting in its death. Macrophages also release free oxygen radicals, proteinases and acid hydrolases, which may contribute to extracellular bacterial killing (Poulter 1997).

It appears that bacterial loads of less than 10^5 organisms can be eradicated by macrophages and other components of the innate host defence. However, greater bacterial loads require neutrophil recruitment and the involvement of the secondary acquired immune response (Onofrio, Toews et al. 1983). Macrophages and dendritic cells facilitate this in a variety of ways. They are able to migrate to the bronchial lymph nodes, particularly to the T cell paracortical areas (Thepen, Claassen et al. 1993) where the antigen they carry is available for primary stimulation of the T cell clones. T cell derived cytokines then amplify the effector function of macrophages by enhancing their phagocytic and anti-microbial capacity (Skerrett and Martin 1994). Interactions between neutrophils and macrophages are described in section 1.5.6.4.

Macrophages interact with different bacteria using similar mechanisms, but their ability to clear and kill varies with each pathogen. In the main, gram positive bacteria are readily killed after phagocytosis, whereas gram negative bacteria are often resistant to macrophage killing. Mycobacteria, Listeria and Legionella are readily ingested but are able to withstand the respiratory burst and pathogen clearance can only be achieved by removal of the affected macrophages (Horwitz and Silvrstein 1981). Macrophages laden with phagocytosed bacteria are removed from the lungs by transport up the bronchial tree until they are swallowed with airway secretions.

Healthy epithelium also contains migratory lymphocytes and mast cells. Lymphocytes remove allergenic particles from the airways and occur singly or in aggregated lymphoid follicles (known collectively as bronchus associated lymphoid tissue) (McDermott, Befus et al. 1982). The T cell surface markers found in bronchial

epithelium include the universal lymphocyte marker CD3, as well as the more specific CD4⁺ (helper) and CD8⁺ (cytotoxic) cells. In the epithelium CD8⁺ cells are predominant, while in the lamina propria this is reversed and there is an preponderance of CD4⁺ cells (Azzawi, Bradley et al. 1990). B lymphocytes make up only a small proportion of cells seen in the lung.

1.2 THE NEUTROPHIL

The neutrophil is the most abundant circulating leukocyte. The average peripheral blood neutrophil count is $2.5 - 7.5 \times 10^6$ /ml and when inactivated, its' circulating half life is only 6 – 8 hours, which means that the bone marrow is required to produce and release more than $5 - 10 \times 10^{10}$ neutrophils daily, with the capacity to increase production further if needed.

1.2.1 Differentiation, maturation and apoptosis

Neutrophils have a characteristic multi-lobed nucleus and abundant storage granules in their cytoplasm. The mature neutrophil has three chemically distinct granule types, which appear at different stages of maturation (see table 1.1).

The neutrophil differentiates and matures within bone marrow, developing from a bipotential progenitor cell, the granulocyte-macrophage colony forming unit. In the first developmental stage, the cell divides and differentiates from myeloblasts to promyelocytes. During this stage the azurophilic or primary granules are produced. These granules contain myeloperoxidase (MPO), antibacterial proteins (such as defensins, lysozyme and azurocidin) and three serine proteinases, neutrophil elastase (NE), cathepsin G (CG) and proteinase 3 (PR3).

Table 1.1: Enzymes and other constituents of human neutrophil granules

| Constituents | Granules | | |
|--------------------------------|---|--|--|
| | Azurophil | Specific | Small storage |
| Antimicrobial | Myeloperoxidase Lysozyme Defensins Bacterial permeability-increasing protein (BPI) | Lysozyme Lactoferrin | |
| Neutral proteinases | Elastase Cathepsin G Proteinase 3 | Collagenase Complement activator Phospholipase A2 | Gelatinases Plasminogen activator |
| Acid Hydrolases | Cathepsin D β -D-Glucuronidase α -Mannosidase Phospholipase A2 | | Cathepsin B and D β -D-Glucuronidase α -Mannosidase |
| Cytoplasmic membrane receptors | | CR3, CR4 fMLP receptors Laminin receptors | |
| Others | Chondroitin-4-sulphate | Cytochrome b558 Monocyte chemotactic factor Histaminase Vitamin B12 binding protein | Cytochrome b558 |

The enzymes and other constituents of human neutrophil granules.
fMLP = N-formylmethionyl-leucyl-phenylalanine

The serine proteinases are produced as pre-proenzymes, with gene expression stopping at the metamyelocyte stage (Fouret, du Bois et al. 1989) and are activated by a lysosomal cysteine proteinase, dipeptidyl peptidase (Adkison, Raptis et al. 2002). The specific or secondary granules are formed as the cell enters the myelocytic stage. These granules contain lysozyme, lactoferrin, collagenase and various membrane receptors. The small storage (or tertiary) granules are formed last, at the metamyelocyte stage, and contain Gelatinases (matrix metalloproteinases) and Cathepsin B and D. At maturation

(which takes approximately 2 weeks) each neutrophil contains a full complement of proteins which provide the mechanisms of cell migration, opsonophagocytosis and a formidable arsenal against pathogens. However, the neutrophil proteinases (especially NE) also have the capacity to be intensely destructive, degrading structural lung proteins (including elastin, collagen and gelatine) and appear to be involved in the post-translational processing of enzymes, cytokines and receptors (Stockley 2002). Fully mature neutrophils leave the bone marrow in a non-activated state and have a half life of 4 – 10 hours before marginating and entering tissue pools (Burnett 1997). Once in tissue, neutrophils are usually removed by apoptosis leading to their recognition and phagocytosis by macrophages in the main and by other neutrophils when the macrophage clearance system is overwhelmed (Rydell-Tormanen, Uller et al. 2006). This mechanism prevents cell necrosis and the release of the remaining cellular content of proteinase and other mediators.

Apoptosis is regulated cell death that allows the elimination of unwanted or damaged cells. Currently, three caspase dependent apoptosis pathways have been described. The first pathway is triggered in response to extracellular signals (the receptor mediated extrinsic pathway), mediated by the binding of tumour necrosis factor related proteins (such as Fas ligand) to death receptors on the cell surface. This results in the formation of the death inducing signalling complex (DISC), that activates a series of Caspases, leading to DNA fragmentation by DNase (Muzio, Chinnaiyan et al. 1996; Hirata, Takahashi et al. 1998; Muzio, Stockwell et al. 1998; Scaffidi, Fulda et al. 1998; Tang and Kidd 1998). A second pathway, the mitochondrial intrinsic pathway, responds to stress signals by the release of cytochrome C from mitochondria. Cytochrome C, apoptotic protease activating factor-1 (Apaf-1) and caspase-9 activate caspase-3, leading to apoptosis (Liu, Kim et al. 1996; Zou, Henzel et al. 1997; Slee, Harte et al. 1999).

Finally, in the endoplasmic reticulum pathway, caspase-12 is activated in response to stress signals such as hypoxia (Rao, Hermel et al. 2001; Szegezdi, Fitzgerald et al. 2003), leading to apoptosis.

Clearance following apoptosis physiologically has no inflammatory sequelae. However, if phagocytosis fails, apoptotic cells undergo secondary necrosis with cell rupture, which is inflammatory in nature and increases both the pro-inflammatory proteins and proteinase burden in the lung (Rydell-Tormanen, Uller et al. 2006). Figure 1.1 describes the appearance of neutrophils during induced lung inflammation, showing both activated and apoptotic cells, apoptotic cells undergoing secondary necrosis and neutrophils containing phagosomes with neutrophilic cell remnants.

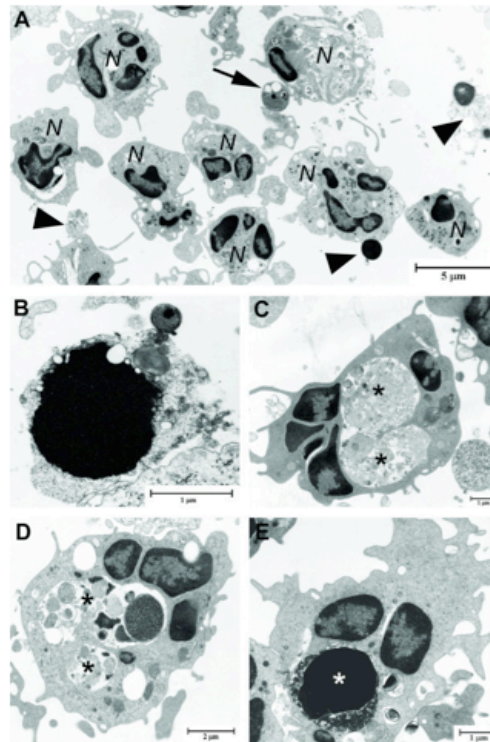


Figure 1.1. Neutrophils during an LPS induced lung inflammation

Legend.

Electron micrographs displaying neutrophils during an LPS-induced lung inflammation. Highly activated neutrophils are shown (N) (characterized by phagosomes and/or cytoplasmic protrusions), lying amongst apoptotic neutrophils (black arrow) and cell debris (black arrowhead) (Figure A). Secondary necrosis (characterized by membrane rupture of cells with an otherwise apoptotic morphology) of neutrophils is shown in figure B. Neutrophils containing large phagosomes (asterisks) enclosing neutrophilic cell remnants such as apoptotic nuclei and neutrophil granulae are shown in images C-E.

Used with kind permission from Rydell-Tormanen et al, Respir research 2006; 7: 143

1.2.2 Neutrophil migration

Neutrophils are present at both the bronchial and alveolar level in COPD, and therefore it is likely that neutrophil migration occurs from both the bronchial and pulmonary circulation. Most of our understanding of neutrophil migration has been derived from *in vitro* studies using systemic vessels in the mesentery and dermis. Studies using samples of lung tissue suggests that while migration in the bronchial circulation occurs in a similar fashion to migration in other tissues, migration in the pulmonary capillaries may depend on distinct mechanisms.

1.2.2.1 Neutrophil migration in the bronchial circulation

Initiation of migration

In the bronchial circulation, neutrophils migrate from vessel to tissue in a step-like process, dictated by the sequential activation of adhesive proteins and their ligands on neutrophils and endothelial cells.

Migration begins with the capture of neutrophils from flowing blood, causing the cell to roll along the endothelial surface. Tethering and rolling of the neutrophil along the vessel wall is a normal feature of circulating neutrophils and is due to reversible binding of transmembrane glycoprotein adhesive molecules called “selectins”, which are found both on neutrophils and endothelial cells (Wagner and Roth 2000).

Leukocyte Selectin (L-Selectin) is constitutively expressed on projecting microvilli on the surface of neutrophils and binds an endothelial ligand that is believed to be a sialomucin oligosaccharide (potentially a fucosylated variant of CD34). L-Selectin induced neutrophil capture can be transient in non-inflamed tissue (“stick and release”),

however, during both transient and prolonged rolling interactions, L-Selectin, once bound, is shed from neutrophils (Walchek, Kahn et al. 1996). Both L-Selectin binding and shedding is enhanced in the presence of inflammatory products such as Tumour Necrosis factor alpha (TNF α), Interleukin 8 (IL-8), the bacterial peptide Formyl-methionyl-leucyl-phenylalanine (fMLP) and Lipopolysaccharide (LPS) (Drost and MacNee 2002). Once bound and cleaved, L-Selectin molecules cannot be replaced and low expression has been associated with neutrophilic apoptosis (Matsuba, Van Eeden et al. 1997).

In the presence of inflammation, at least two further endothelial bound selectins are expressed, Platelet Selectin (P-Selectin) and Endothelial Selectin (E-Selectin). P-Selectin is stored intracellularly in Weibel-Palade bodies in endothelial cells (Malik and Lo 1996) and can be mobilised rapidly in response to various inflammatory mediators including oxygen free radicals, components of the complement cascade and many cytokines (Spertini, Luscinskas et al. 1991). Its neutrophilic counterligand is P-Selectin glycoprotein ligand-1 (PSGL1) which is uniformly expressed on the surface of neutrophils. P-Selectin–PSGL1 interactions occur after L-Selectin-ligand interactions, and have greater longevity. In the presence of other adhesion molecules, P-Selectin/PSGL1 binding slows neutrophil rolling velocities and eventually causes cell tethering to the endothelium but in the absence of other adhesive events, binding is also transient (Alon, Chen et al. 1997).

E Selectin is not stored, and peak expression is seen 4 – 6 hours after endothelial exposure to inflammatory mediators (Klein, Bittenger et al. 1995). It binds to E-Selectin ligand 1

(Steeigmaier, Levinovitz et al. 1995) and it is thought to maintain neutrophil tethering after P-selectin has been down regulated.

Firm Adhesion

The next step in neutrophil migration is the transition from reversible rolling to firm adhesion with the endothelium. This is achieved by the sequential activation of neutrophil receptors called Integrins (Crockett-Torabi and Fantone 1995; Williams and Solomkin 1999).

The Integrins are heterodimeric transmembrane glycoproteins that comprise an alpha and beta subunit, which together form an extra cellular binding site. Integrins are found on many haematopoietic cells, with differing alpha and beta subunits. The two most important integrins on neutrophils share a beta2 subunit (CD18), and are called Macrophage antigen 1 (MAC1; CD11b/CD18) and Lymphocyte-associated function antigen 1 (LFA-1; CD11a/CD18). A third CD18 integrin, p150,95 can also promote neutrophil migration, but MAC1 appears to be the most important integrin in models of neutrophil migration (Diamond, Staunton et al. 1990; Rainger, Buckley et al. 1997).

MAC1 is stored in secretory granules (Borregaad and Cowland 1997) and is rapidly mobilised to the cell surface after exposure to inflammatory stimuli (including fMLP, TNF- α and LPS). Inflammatory stimuli also promote transcription and translation of the MAC1 gene via a G-protein, Rho (Laudanna, Campbell et al. 1996), further increasing its surface expression (Spertini, Luscinskas et al. 1991). Some MAC1 is expressed constitutively on the neutrophil, but these proteins are incapable of binding ligands unless activated by intra or extracellular signals, where conformational changes occur exposing a requisite binding epitope (Michishita, Videm et al. 2003).

MAC1 has a high affinity for Intercellular adhesion molecule 1 (ICAM1), an immunoglobulin-like protein that is expressed in low numbers on the endothelial cell surface, but is rapidly induced in inflammation (Hashimoto, Shingu et al. 1994). Vascular cell adhesion molecule-1 (VCAM-1) is also an immunoglobulin-like molecule expressed by endothelial cells which it binds selectively to $\alpha 4\beta 1$ -integrin, called Very Late Antigen-4 (VLA-4) on neutrophils (Davenpeck, Steeber et al. 1988). MAC1/ICAM1 interactions cause enhanced expression of both ICAM1 and VCAM1 on endothelial cells, suggesting that both may be important in inflammatory driven neutrophil migration (Clayton, Evans et al. 1998).

Migration

The final step of neutrophil recruitment from the bronchial circulation to the lungs is transendothelial migration. This was believed to occur preferentially at tricellular junctions (Burns, Walker et al. 1997), requiring the activation of Platelet endothelial cell adhesion molecule (PECAM1) (Newman 1997) which is distributed evenly around the neutrophil and at intercellular junctions of endothelial cells. PECAM1 is thought to act as a homing beacon that directs migration towards cellular junctions and blocking PECAM1 on either neutrophils or endothelial cells using antibodies does not prevent adhesion but does prevent migration through the basement membrane both *in vitro* and *in vivo* (Muller 1995). A recent study has suggested an alternative route for transmigrating neutrophils, with 70% crossing the endothelial barrier by an active process of engulfment by endothelial cells that is dependent on the small GTPase RhoG (van Buul, Allingham et al. 2007). The role and implications of this second process have yet to be pursued.

Once through the endothelial cell layer, leukocytes bind to matrix components such as collagen and laminin via $\beta 1$ integrins, with VLA-6 and 9 being perhaps the most important in allowing neutrophils to move through venule basement membrane and lung tissue (Shang and Issekutz 1997; Shang, Yednock et al. 1999; Yadav, Larbi et al. 2002). Endothelial/neutrophil PECAM1 interactions lead to increased neutrophil surface expression of VLA-6 ($\alpha 6\beta 1$) and VLA-6 facilitates passage thorough the basement membrane and beyond. To support this, neutrophils from PECAM1 knock out mice do not have the associated rise in VLA-6 which is seen in the wild type (Thompson, Noble et al. 2001). Migration is accompanied by release of neutrophil proteinases especially neutrophil elastase (Wright and Gallin 1979), which may facilitate passage by matrix degradation, exposing laminin for VLA-6 binding. Selectin and Integrin interactions are summarised in figure 1.2.

Direction of migration and neutrophil movement

Neutrophils migrating within the lung encounter multiple chemoattractants signals in complex spatial and temporal patterns as endothelial, epithelial cells and immune cells respond to infection or injury. Individual chemoattractants can vary in their ability to affect neutrophils and *in vitro* models have demonstrated that neutrophils can migrate up and down chemical gradients, responding to one signal, migrating to its concentration peak and then migrating up a novel, more distant chemoattractant gradient, from endothelium to tissue.

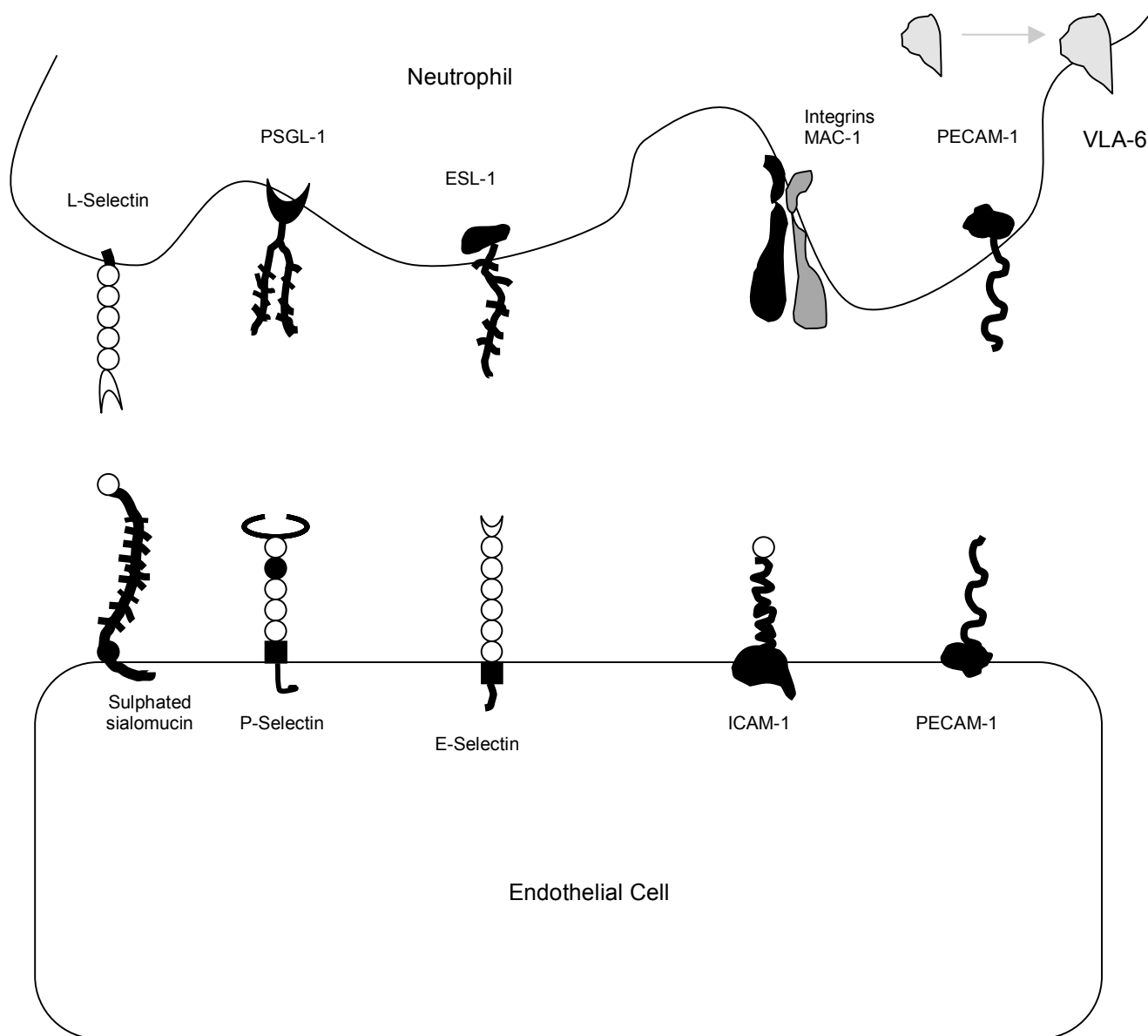
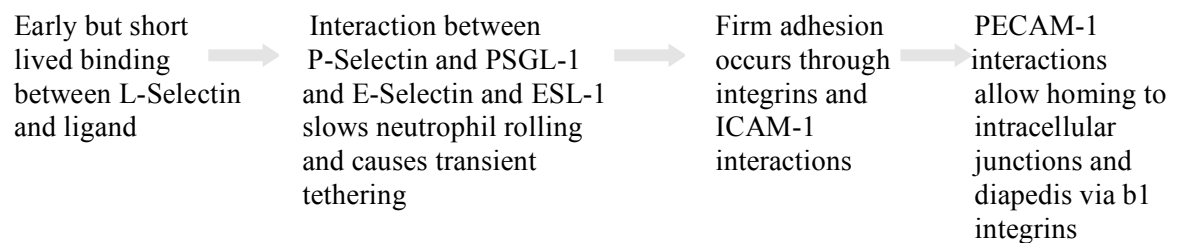


Figure 1.2: Schematic summary of Neutrophil and Endothelial Cell Adhesion

Molecules and their ligands in neutrophil transendothelial migration.



Modified from (Wagner and Roth 2000)

Neutrophils can ignore a high concentration source (due to receptor saturation, desensitization and /or receptor sequestration) and preferentially respond to novel chemoattractants (Foxman, Campbell et al. 1997). Under agarose studies have suggested that neutrophils are able to migrate in the direction of the vector sum of two or more differing signals (Foxman, Kundel et al. 1999). If two sources of the same agonist are used, migration towards the second attractant is poor (Foxman, Campbell et al. 1997), so two distinct agonists are required for precise targeting. These findings may account for the observation that activated cells characteristically secrete multiple induced chemoattractants concurrently. Cells can regain their prior sensitivity, but this process takes time, requiring recycling of receptors. For example neutrophils preincubated with low chemotactic levels of Leukotriene B4 (LTB4) showed a significant reduction in chemotaxis to LTB4 which improved after 10 minutes (Foxman, Campbell et al. 1997).

Neutrophil movement itself is achieved by the formation of locally protruding actin-rich pseudopods while retracting other regions of the cell body (Devreotes and Janetopoulos 1988). In order to achieve this, directed neutrophil chemotaxis begins with cell polarisation. Following stimulation, neutrophils acquire polarity through a self-organising process involving positive “frontness” and negative “backness” feedback loops. “Backness” is enhanced by inhibiting the formation of a leading edge and with the development of a uropod. This is achieved by actin-myosin II meshworks, regulated by Rho-A and PTEN. The leading edge is composed of F-actin rich lamellipodia, and highly polarized distributions of lipids that are up-regulated during cell alignment (including PIP₃, Cdc42, PAK1 and Akt). In keeping with the role of the leading and

trailing edge of the neutrophil, receptors for chemoattractants and integrins are redistributed to pseudopods (Gomez-Mouton, Lacelle et al. 2001) and those for adhesion (such as PSGL-1 and ICAM-3) are redistributed to the uropod (Fais and Malorni 2003).

The cell-signaling pathway involved in neutrophil chemotaxis has been described. Chemoattractants bind to their specific cell surface receptors and activate G_i proteins, which are heterotrimeric G proteins (subunits α , β and γ). Ligand bound receptors promote exchange of GDP with GTP on the α subunits, and GTP-bound $G\alpha$ dissociates from the $\beta\gamma$ subunits. The $G\beta\gamma$ subunit up-regulates PLC, PI3K γ , ion channels, G-protein coupled receptor kinases and PAK1. The $G\alpha$ subunit also couples with chemoattractants, leading to activation of RhoA.

PI3K is required for PIP₃ production, and PIP₃ is required to orient the cell's leading edge in accordance with the chemoattractant cue by localizing the formation of F actin-rich lamellipodia using PAK/PIX α activation of Cdc42. (a member of the Rho family of small GTPases), and this confers cell motility (Wu 2005). The persistent presence of PIP₃ at a particular site on the plasma membrane causes cells to acquire an elongated shape in which one F-actin enriched pseudopod becomes the leading edge of the migrating cell, whereas retraction of pseudopods at the rear and sides of the cell is mediated by cortical myosin II (Charest and Firtel 2007).

Although there may be many proximal signal pathways by which polarisation is regulated, the key event appears to be activation of the RhoGTPases, Rho and Rac via integrins (Huttunlocher 2005). The $\alpha 4\beta 1$ integrin forms a complex with paxillin and GIT1 which inhibits Rac activation at the sides and rear of the cell, but this inhibitory

complex is impeded at the leading edge of the cell by phosphorylation of the $\alpha 4$ integrin cytoplasmic tail, which allows Rac activation (Nishiya, Kiosses et al. 2005). At the front of the cell Rac activation leads to the f-actin polymerisation while at the rear of the cell Rho activation leads to assembly of myosin, with both GTPases working in a co-operative manner to establish and stabilise cell polarity (Ohta, Hartwig et al. 2006).

PTEN is a negative regulator of this pathway, dephosphorylating PIP_3 . PTEN is localized at the posterior of cells (localization is dependent upon $\text{PIX}\alpha$) and may also be involved in cell directionality. While PIP_3 and Cdc42 localise F actin formation, they are not required for F actin formation (which is Rac dependent). Rac activation appears to depend on P-Rex-1 (Wu 2005). Figure 1.3 summarises the pathways believed to be of importance.

The neutrophil appears to “steer” by sustaining pseudopods that are aligned with the chemoattractant gradient. Pseudopods are made in spatially restricted sites by splitting of the leading edge of the cell, and the generation of these protrusions appears to be random in both their direction and timing. In a study where cells were exposed to a pro-migratory stimulus which was then relocated once an accurate trajectory was established, pseudopod generation occurred randomly, but pseudopods that extended towards the stimulus were more likely to be retained, while those which extended in inaccurate directions were retracted (see figure 1.4). Therefore it appears that neutrophils migrate up chemoattractant gradients by choosing the best aligned of competing randomly generated pseudopods (Andrew and Insall 2007).

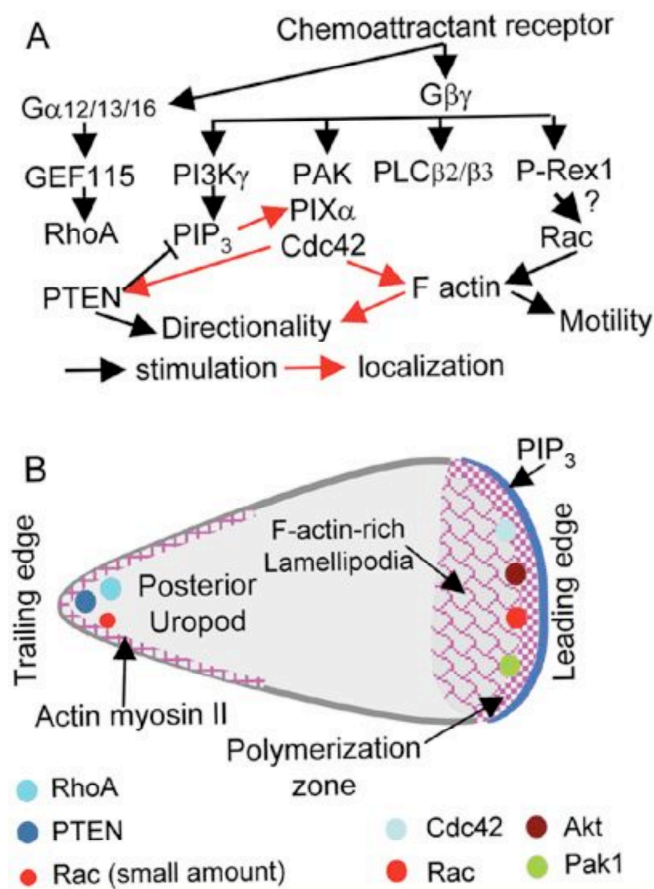


Figure 1.3. Outline of the chemoattractant signalling pathways and neutrophil cell polarisation involved in neutrophil chemotaxis

Legend

A. The signalling pathway involved in neutrophil chemotaxis

B. The proteins involved in the polarisation and mechanics of cell movement.

Figure reproduced from (Wu 2005) with kind permission. See text for full explanation.

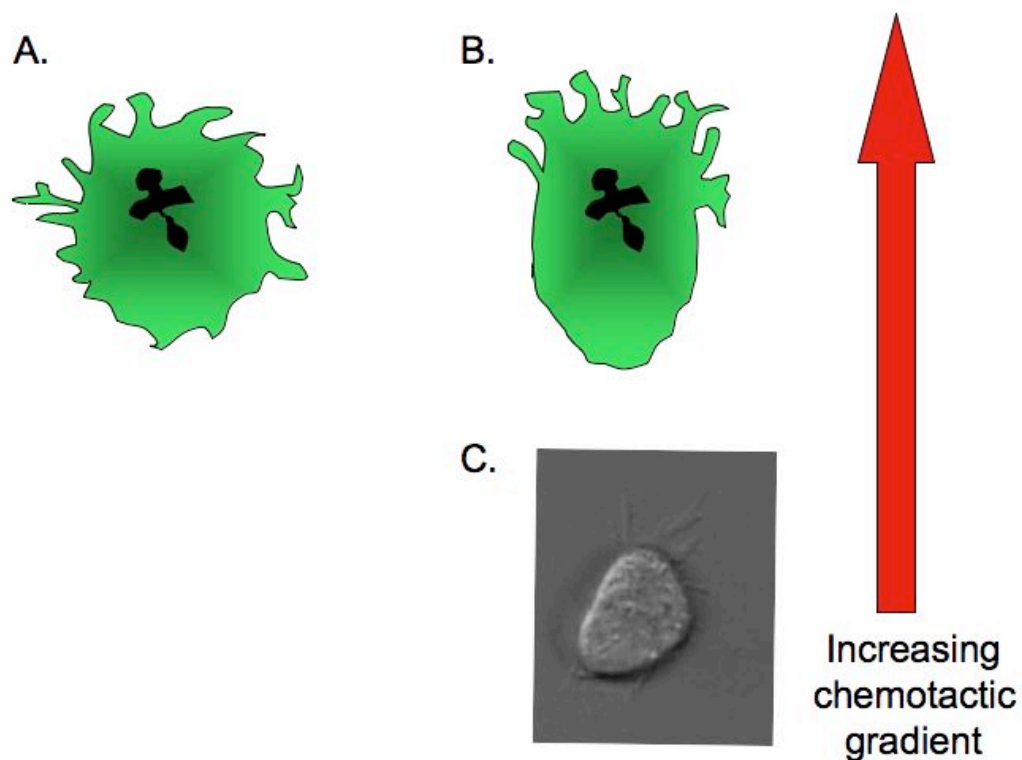


Figure 1.4. Neutrophil migration by pseudopod generation towards a pro-migratory stimulus

Legend.

A representation of pseudopods generated over the time course. **A.** When cells were quiescent, pseudopod generation was noted across the breadth of the leading edge of the cell in a random fashion. **B.** The direction of the chemoattractant gradient is shown by the large red arrow. Pseudopods generated towards the stimulus were more likely to be retained while pseudopods that extended in inaccurate directions were more likely to be retracted (the sustained pseudopods over the time course is shown schematically). **C.** A polarised (elongated) neutrophil migrating towards Interleukin 8 in a Zigmond chamber. It appears that neutrophils migrate up chemoattractant gradients by choosing the best aligned of competing randomly generated pseudopods.

Transmigration through extra cellular matrix

Neutrophil proteinases are released during migration through extra cellular matrix (Wright and Gallin 1979) but it has been difficult to ascertain whether proteinases are necessary for neutrophil migration. Chemotaxis through artificial substrates in response to fMLP can be inhibited by 50% using α 1AT (Stockley, Shaw et al. 1990) and CG antibodies; synthetic inhibitors of CG and α 1ACT also reduce neutrophil migration (Lomas, Stone et al. 1996). Furthermore, fMLP-stimulated migration across an artificial basement membrane is also reduced by inhibitors of both NE and Matrix metalloproteinase 9 (MMP-9) (Declaux, Delacourt et al. 1996). However, *in vitro* studies of endothelial monolayers and basement membrane matrices have shown consistently that proteinase inhibitors are ineffective at stopping neutrophil migration (Allport, Ding et al. 1997; Mackeral, Cottell et al. 1999) although degradation of basement membrane components is reduced (Steadman, St John et al. 1997). In animal studies, neutrophils from mice whose genes for NE and CG had been “knocked out” showed normal migration both *in vitro* and *in vivo* when exposed to LPS although pathogen clearance was impaired (Tkalecivic, Novelli et al. 2000) and mice deficient in Gelatinase B had normal neutrophilic migration into the lungs (Betsuyaku, Shipley et al. 1999). However, animal studies of cigarette smoke inhalation suggest that neutrophil influx into the lung is reduced in the presence of proteinase inhibitors (Delacourt, Herigault et al. 2002; Wright, Farmer et al. 2002).

The mechanisms remain unresolved, but the conflicting results may well reflect the study model used, including the density of the ECM studied and in particular the presence or absence of cross linked collagen (compare, for example (Chun, Sabeh et al. 2004) and (Sabeh, Ota et al. 2004)). The majority of recent studies have used tumour cells, but there

is evidence to suggest that migrating cells can change their behaviour depending on the matrix and stimuli they are exposed to. For example, in a non-cross linked collagen 3D matrix, cells migrate along fibres, but can squeeze between fibres in the presence of anti-proteinases, therefore migration is not inhibited but redirected. On the other hand in a dense cross linked collagen 3D matrix, cells require proteinases to migrate and migration is inhibited in the presence of anti-proteinases. Cell derived matrices are cross linked but have gaps and migrating cells appear to preferentially seek out these gaps to migrate through. In this scenario proteinases may or may not be required, depending on the size of the gap (Even-Ram and Yamada 2005). If neutrophil migration *in vivo* is partially proteinase-dependent, migration may not require degradation of “loose” *in vitro* extra cellular matrix by proteinases to allow cell passage, but instead the generation/activation of inflammatory chemokines and cytokines or modulation of adhesion molecules (enhancing expression, increasing their activation or exposing binding sites (Yadav, Larbi et al. 2002)) by proteinases, and this may be more easily decreased or prevented by inhibitors, explaining the apparent experimental contradictions.

1.2.2.2 Neutrophil migration in the pulmonary circulation

The majority of neutrophils appear to enter the lung from the pulmonary capillary network rather than the post capillary venules (Doerschuk, Allard et al. 1989; Downey, Worthen et al. 1993). Neutrophil emigration into lung from the pulmonary circulation is less well understood than from the bronchial circulation, however there seem to be important differences in mechanism, probably due to the size of capillary networks. Forty to sixty percent of pulmonary capillary segments are narrower than a spherical neutrophil, which on average is 7 to 8 μm in diameter (Hogg 1987; Doerschuk, Beyers

et al. 1993), so neutrophil rolling is unlikely because of size constraints. Neutrophils entering the pulmonary capillary network have to undergo a shape change, from a sphere to an oblong, to allow passage through these narrow vessels. This slows their transit time, and indeed, radio labelled neutrophils from patients with stable COPD have been shown to have a slower transit time in the pulmonary circulation compared with red blood cells (Selby, Drost et al. 1994). When activated, neutrophils become less deformable due to actin polymerization, and this slows their progression through the capillary network further (Erzurum, Downey et al. 1992). In such circumstances there is no need for transient adhesion via the selectins to initiate neutrophil rolling along the endothelium, as the leukocytes are already in close contact with the endothelium, and indeed there is evidence to suggest that some migration can occur without conventional adhesion molecules (Doerschuk 1992; Morland, Morland et al. 2000).

In the bronchial circulation, adhesion and migration appears to be primarily dependent upon ICAM1 interactions, however, in the pulmonary circulation both CD18 dependent and independent adhesion pathways have been described and the path utilised appears to be stimulus specific. For example, in animal models, bacteria such as *streptococcus pneumoniae* and *Staphylococcus aureus* and hydrochloric acid have induced CD18 independent neutrophil migration, while human studies have demonstrated CD18 independent migration towards host-derived chemoattractants such as Leukotriene B4 (LTB4), IL8 and sputum (Mackeral, Russell et al. 2000; Morland, Morland et al. 2000). In contrast, Interleukin 1 (IL-1), phorbol myristate acetate and gram negative bacterial stimuli including LPS elicit migration via pathways predominantly mediated by CD18 (Doerschuk, Winn et al. 1990; Hellewell, Young et al. 1994; Qin, Quinlan et al. 1996; Ramamoorthy, Saski et al. 1997). CD18 dependent migration may not only be stimulus

driven but also inflammatory mediator driven. Rabbits produce both IL8 and TNF- α during a bacterial pneumonia but CXCL8 and TNF- α production during a gram negative pneumonia are 2 and 10 fold greater respectively than seen during a gram positive pneumonia (Shoburg, Quinlan et al. 1994). It may be that the varying concentrations of these pro-inflammatory stimuli elicit a CD18 dependent response, perhaps by inducing nuclear factor- κ B (Doerschuk, Winn et al. 1990) rather than the bacterial insult itself. Animal models have suggested that selectins are required where CD18 dependent migration occurs, but not for CD18 independent migration. For example, L-Selectin knock out mice had significantly less neutrophil recruitment into the lungs in response to LPS (a CD18 dependent stimulus) while recruitment in response to *Streptococcus pneumoniae* (a CD18 independent stimulus) was unaffected (Doyle, Bhagwan et al. 1997).

1.2.2.3 Phagocytosis and bacterial killing

The role of the neutrophil is to kill and remove micro-organisms. The neutrophil achieves this by a process of phagocytosis, the respiratory burst and the release of cytotoxic peptides and proteins.

Phagocytosis

Ingestion of bacteria precedes intracellular killing. Immunoglobulins bind to bacterial surface antigens (a process of opsonisation), enhancing phagocytosis and causing the activation of the complement system. The neutrophil then binds both the immunoglobulin (via the Fc γ R receptor) and complement factor C3 (via the Complement receptor type 3 receptor, CD11b/CD18, Mac-1) (Burnett 1997). Ingested bacteria are encapsulated within the phagolysosome, where they are killed and

degraded. Bacterial killing is achieved via the respiratory burst and the action of cytotoxic proteins while bacterial degradation is achieved via the release of granule proteases.

Respiratory burst

The respiratory burst describes the release of oxidative molecules that originated from the membrane-bound NADPH oxidase system. Superoxide production starts with the reduction of oxygen by NADPH to form the superoxide radical, O_2^- . The radicals can react spontaneously with water to form molecular oxygen and hydrogen peroxide, or the reaction can be catalysed by superoxide dismutase. MPO (stored in azurophil granules) catalyses the reduction of hydrogen peroxide to water or to hyperchlorous acid when chloride is present. These powerful products are released into the phagolysosome, where they destroy ingested bacteria. These products are also capable of damaging adjacent cells when released outside of the neutrophil (Weiss, Young et al. 1981), a process that is limited by the relevant antioxidants in healthy individuals (MacNee 2000). The importance of the respiratory burst as a means to destroy bacteria is amply illustrated in chronic granulomatous disease, where phagocytic cells are unable to generate the products of the respiratory burst, leading to recurrent bacterial infections.

Cytotoxic peptides and proteins

The neutrophil also contains cytotoxic proteins, most of which are stored in the azurophil granules. These proteins include human neutrophil peptides 1 – 4 (collectively known as “the defensins”). They account for 50% of the total protein

content of azurophil granules and are highly toxic to fungi, enveloped viruses and bacteria (Burnett 1997).

The three most important neutrophil serine proteinases are elastase, cathepsin G and proteinase 3. The serine proteinases are located within the primary granules, whereas metalloproteinases are located within the specific granules. The serine proteinases will be discussed in section 1.5.6.

1.3 ANTI-PROTEINASES

1.3.1 α 1-antitrypsin

α 1-antitrypsin (α 1AT) is the major plasma inhibitor of neutrophil elastase and probably the most important antiproteinase in the distal airways and alveoli (Stockley 1997). It is a 54 kDa glycoprotein that is secreted by hepatocytes, with little secretion occurring in the lung (Stockley, Mistry et al. 1979). Inflammation leads to an acute rise in the plasma concentration of α 1AT with a corresponding increase in passive diffusion into the lung. α 1AT inactivates free neutrophil elastase, cathepsin G and proteinase 3 in the airways (Lee and Downey 2001). Its importance becomes obvious in patients with a severe hereditary deficiency associated with a low serum concentration of α 1AT, in whom uncontrolled elastase activity leads to the early onset of emphysema (see section 1.7).

1.3.2 Secretory leukoprotease inhibitor

Secretory leukoprotease inhibitor (SLPI) is the main inhibitor of elastase in the proximal airways. It is of low molecular mass (12 kDa) and is produced locally by lung epithelial cells and is present in serous glands and clara cells (Mooren, Kramps et al. 1983; De Water, Willems et al. 1986; Maruyama, Hay et al. 1994). SLPI can also be

produced by type II pneumocytes (Sallenave J.M., Shulman et al. 1994), is secreted from the basolateral surface of submucosal glands and has been detected in the lung interstitium (Willems, Otto-Verberne et al. 1986; Dupuit, Jacquot et al. 1993) suggesting a more widespread role throughout the lung. As well reversibly inhibiting NE and CG (Sallenave J.M. 2000), SLPI has anti-viral, anti-bacterial and anti-fungal properties (Tomee, Koeter et al. 1998). Its regulation in the lung is mainly unknown, although elastase can reduce its release from epithelial cells (Sallenave J.M., Shulman et al. 1994).

1.3.3 Other anti-proteinases that inhibit neutrophil proteinases.

α 1-antichymotrypsin (A1ACT) is produced by hepatocytes and present in serum and rapidly inactivates CG (Stockley 1997). A1ACT is present in the lung due to passive diffusion, active transport and some local production (Stockley 1983).

Other proteinase inhibitors that have been identified in the lung include elafin, α 2-macroglobulin (A2M) and monocyte/neutrophil elastase inhibitor (MNEI). Table 1.2 provides a summary of the anti-proteinases which inhibit neutrophil proteinases

Table 1.2. The inhibitors of neutrophil proteinases

| Inhibitor | Source | Target proteinase | Inhibition |
|---|--|-------------------------------|--------------|
| Alpha 1 anti trypsin | Produced in the liver Found in serum | NE, CG, PR3 | Irreversible |
| Alpha 1 antichymotrypsin | Liver and macrophages | CG | Reversible |
| Alpha 2 macroglobulin | Liver and macrophages | NE, CG, PR3, MMP 8, MMP 9 | Irreversible |
| Secretory leucoproteinase inhibitor | Mucosa | NE, CG | Reversible |
| Elafin | Mucosa | NE, PR3 | Reversible |
| Monocyte-neutrophil elastase inhibitor | Neutrophils / monocytes | NE, CG, PR3 | Irreversible |
| Tissue Inhibitors of metalloproteinases | Many cell types including epithelial cells, fibroblasts, neutrophils and monocytes | Gelatinases (MMP 8 and MMP-9) | Irreversible |

1.4 Ageing and neutrophil function

Developments in medicine, public health and socioeconomics, has led to an increase in our average lifespan. Statistics from the World Health Organisation website shows that 10% of the world's population is made up of individuals aged 60 years or older, and this figure is expected to rise to 22% in 2050 (Dorshkind, Montecino- Rodriguez et al. 2009).

It is well established that the efficiency of the immune system declines with increasing age. This phenomenon has been termed “immunesenescence” and is most obviously demonstrated by the increased risk of infection-associated morbidity and mortality experienced by the elderly (Fein 1999; Bonomo 2002; Gavazzi and Krause 2002). Bacterial dysentery is three times more common in older adults, and there is a 50% higher mortality from gram negative bacterial sepsis in the elderly (Martin, Mannino et al. 2006). Furthermore, deaths from gastrointestinal infections, pneumonia and influenza

are largely confined to patients above the age of 65 (Liang and Mackowiak 2007). In addition, older adults are at increased risk of post-surgical complications such as lower respiratory tract infections and show a delayed resolution of inflammation, which is associated with an increased risk of tissue damage and the onset of physical frailty (Butcher, Killampalli et al. 2003).

The high incidence of bacterial infections in older adults is highly suggestive of a sub-optimal neutrophil response, as these cells are fundamental in clearing bacteria, and recent studies have described functional differences in neutrophil behaviour in older adults.

With advancing age, neutrophil priming and activation is reduced in response to a number of ligands including fMLP, GM-CSF, G-CSF and LPS (Fortin 2008). These receptors have distinct signalling pathways and their modulation during aging suggests a common underlying mechanism. *Ex vivo* animal studies have suggested that neutrophils from older populations demonstrate increased membrane fluidity that seems to be related to a reduced cholesterol content within the cell (Alvarez 2001). This was associated with a decrease in superoxide generation in response to fMLP but not to the phorbol ester, phorbol myristic acid (PMA). fMLP acts via a specific receptor, while PMA activates downstream signalling events via direct activation of protein kinase C (PKC), suggesting a proximal signalling defect, potentially within the lipid raft.

Lipid rafts are regions of reduced fluidity within the phospholipid bilayer and are enriched for cholesterol and phospholipids with long saturated fatty acid side chains. They are important for the regulation of cell signalling because they provide a means to

segregate receptors and their proximal signalling components within the membrane, with receptor activity modulated by the inclusion or exclusion of signalling elements in the lipid raft-associated signalsome (Simons 2000). Alterations in cell fluidity would interfere with lipid raft formation and thus could impact on a wide range of cellular signalling pathways, including those involved with migration, phagocytosis, and apoptosis. Currently, there are no published studies assessing membrane fluidity in human neutrophils, however, there is indirect evidence of alterations in lipid raft function with increasing age. The negative regulator of GM-CSF receptor signalling (SHP-1) is excluded from lipid rafts within one minute of stimulation of neutrophils from young donors with GM-CSF, but remains associated with lipid rafts in neutrophils from old donors (Fortin, Larbi et al. 2006). Furthermore, agonist receptors such as TREM-1 and TLR4 show reduced recruitment to lipid rafts in neutrophils from old donors, compromising their signalling function (Fulop, Larbi et al. 2004; Fortin, Larbi et al. 2007).

Comparisons of neutrophils from healthy young and older subjects have demonstrated that bactericidal (superoxide generation and degranulation) and phagocytic function are dramatically reduced in circulating cells isolated from older subjects (Lipschitz, Udupa et al. 1984; Fulop, Komaromi et al. 1986; Wenisch, Patruta et al. 2000; Butcher, Chahal et al. 2001). This may have major implications for their role in infection in the elderly.

Superoxide generation in response to the bacterial peptide fMLP and to gram negative bacteria appear to be maintained in older subjects (Corberand, Ngyen et al. 1981; Esparza, Sanchez et al. 1996; Butcher, Chahal et al. 2001). However, superoxide generation in response to gram positive stimuli (such as *Staphylococcus aureus*) appear to

be significantly reduced (Wenisch, Patruta et al. 2000) and it is known that older adults have a reduced ability to resolve infections caused by Gram positive organisms (Whitelaw, Rayner et al. 1992). Furthermore, the mechanisms underpinning these alterations to function have been partially characterised. A reduction in cell surface expression of CD16 may account for reduced phagocytic function (Butcher, Chahal et al. 2001), and alterations in intra-cellular signalling through the Jak-STAT and SHP-1 pathways due to changes in membrane fluidity may contribute to reduced superoxide generation, particularly in response to GM-CSF (Fortin, Larbi et al. 2006; Fortin, Larbi et al. 2007; Tortorella, Simone et al. 2007).

In contrast, the effects of ageing upon neutrophil migration is poorly understood. Some studies have shown no effects on migration in aging adults, with unaltered neutrophil adherence to endothelium (MacGregor and Shalit 1990; Esparza, Sanchez et al. 1996) and comparable expression of adhesion molecules in older subjects (Rao, Currie et al. 1992) (Esparza, Sanchez et al. 1996). In other reports, peripheral neutrophils from aging donors have demonstrated reduced movement towards chemotactic signals (Niwa, Kasama et al. 1989; Wenisch, Patruta et al. 2000; Fulop, Larbi et al. 2004). Interestingly, one study reported intact chemokinesis, but reduced chemotaxis in neutrophils from older subjects (Wenisch, Patruta et al. 2000). However, studies performed to date have not been able to characterise individual neutrophil migration in detail and so it still remains unclear of ageing impacts upon neutrophil chemotaxis.

Ageing in association with chronic obstructive pulmonary disease is discussed in section 1.6.

1.5 Chronic Obstructive Pulmonary Disease

1.5.1 Definition and classification of disease severity

Chronic obstructive pulmonary disease (COPD) is characterised by airflow obstruction with related symptoms including cough, shortness of breath, expectoration and wheeze. The widely accepted Global Initiative for Chronic Obstructive Lung Disease (GOLD) has classified COPD as “a disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases” (Pauwels, Buist et al. 2001). The GOLD definition for airflow limitation is a forced expiratory volume in 1 second (FEV_1 / forced vital capacity (FVC) ratio of $< 70\%$ and disease severity is classified into four stages: stage 1 ($FEV_1 \geq 80\%$ predicted); stage 2 ($FEV_1 \geq 50$ to $< 80\%$ predicted); stage 3 ($FEV_1 \geq 30$ to $< 50\%$ predicted); stage 4 ($FEV_1 < 30$ or $< 50\%$ predicted in the presence of chronic respiratory failure) (Global Initiative for chronic obstructive lung disease. 2007).

COPD also includes chronic bronchitis, small airways disease (bronchiolitis) and emphysema, and these conditions can co-exist in varying proportions between affected individuals (Saetta, Turato et al. 2001). Chronic bronchitis is defined clinically as the presence of chronic productive cough for 3 months in each of 2 successive years (Medical Research Council. 1965). Emphysema is defined pathologically as the destruction of alveolar walls and the permanent enlargement of the airspaces distal to the terminal bronchioles.

1.5.2 Prevalence and impact

Chronic Obstructive Pulmonary Disease (COPD) is one of the foremost causes of chronic morbidity and mortality worldwide. Globally, it affected 44 million people in

1990 (Lomas 2002) and recent estimates have suggested that currently COPD affects approximately 210 million people (World Health Organization 2007) with the prevalence continuing to rise. In 2007, COPD accounted for 5% of all deaths (World Health Organization 2007) but the WHO predicts an increase in COPD related deaths of more than 30% in the next 10 years, emphasising the continued impact this disease will have internationally (Mannino and Holguin 2006).

1.5.3 Pathogenesis

Cigarette smoking remains the most important risk factor for the development of COPD (Doll, Peto et al. 1994) although only approximately 20% of smokers develop clinically significant disease (Tashkin, Clark et al. 1984). This suggests that a combination of genetic and environmental factors interact to cause COPD, and there has been much research aiming to identify candidate genes that may confer genetic susceptibility. To date, however, only deficiency alleles on the α 1AT gene have been robustly identified as predisposing to disease (Sanford and Silverman 2002).

Pathologically, COPD is characterised by widespread inflammation of the peripheral and central airways with destruction of the lung parenchyma. Oedema, fibrosis, smooth muscle hypertrophy and loss of elastic recoil lead to bronchial wall thickening, which cause airflow obstruction (Saetta, Turato et al. 2001).

COPD, while primarily a lung disease, is associated with increased co-morbidity including cardiovascular disease, and systemic pathology such as muscle wasting and dysfunction. It has been hypothesised that persistent low-grade inflammation may drive the co-morbidity and the systemic effects noted with this disease (Sin, Anthonisen et al.

2006). The systemic manifestations of COPD are important, as they are not only associated with increased morbidity, but are also predictive of disease outcome, especially Body Mass Index (BMI) which forms part of the BODE index (Body Mass, airflow obstruction, dyspnoea and exercise capacity) (Celli, Cote et al. 2004).

There is controversy concerning whether increased and sustained pulmonary inflammation causes increased systemic inflammation. However C-reactive protein (CRP) (a generic marker of systemic inflammation) is raised in patients with COPD compared to healthy controls (Pinto-Plata, Mullerova et al. 2004) irrespective of cigarette smoke exposure and ischaemic heart disease (an independent cause of a raised CRP). It is unclear whether these systemic changes are incidental or driven by pulmonary inflammation.

1.5.3.1 Neutrophilic presence in the lung

There is a substantial body of evidence to support the hypothesis that the neutrophil is the primary effector cell in Chronic Obstructive Pulmonary Disease (COPD) and that neutrophil proteinases, especially NE are responsible for the main pathological features seen.

Studies have shown that patients with COPD have increased numbers of neutrophils in proximal airway secretions (Stanescu, Sanna et al. 1996; Rutgers, Timens et al. 2000) and bronchoalveolar lavage fluid (BALF) (Martin, Raghu et al. 1985) compared with asymptomatic smokers, and that the percentage of neutrophils in BALF is higher in patients with the greatest degree of airflow obstruction (Thompson, Daughton et al. 1989; Lacoste, Bousquet et al. 1993). Furthermore, in a recent study of bronchial

biopsies, small airways intra-epithelial neutrophil counts were greater in patients with COPD compared with both smoking and non-smoking controls, and correlated with airway obstruction (Pilette, Colinet et al. 2007). Increased neutrophil counts have been found in the bronchial walls and in BALF samples from patients during exacerbations of COPD (Balbi, Bason et al. 1997; Tsoumakidou, Tzanakis et al. 2005), with increased neutrophil sequestration in the pulmonary microcirculation and such episodes relate to subsequent progression of airflow obstruction (Selby, Drost et al. 1991). Resolution of the neutrophilic inflammation occurs approximately 5 days after appropriate treatment of the exacerbation, which coincides with clinical recovery (Gompertz, O'Brien et al. 2001). In addition, a recent study by Donaldson et al (Donaldson, Seemungal et al. 2005) reported that patients with higher numbers of neutrophils in their sputum had a faster decline in FEV1 compared with those with lower neutrophil counts, losing approximately 1% more than predicted each year. Parr et al (Parr, White et al. 2006) demonstrated that baseline markers of neutrophilic inflammation relate to subsequent decline of lung function and CT quantification of emphysema over the subsequent four years. Gas transfer (the most direct physiological measure of emphysema) has been shown to be inversely proportional to levels of neutrophil associated markers, such as MPO and human neutrophil lipocalin in patients with COPD (Ekberg-Jansson, Andersson et al. 2001). Both clinical and sub-clinical emphysema (noted on HRCT) are associated with an increase in NE and other neutrophil proteins in BALF (Betsuyaku, Nishimura et al. 1999; Yoshioka, Betsuyaku et al. 2005) and in established emphysema, severity is proportional to NE immuno-reactivity in tissue (Ge, Zhu et al. 1990) and enzyme activity in BALF (Fujita, Nelson et al. 1990). Finally, both neutrophil counts and NE concentration appear to decline with smoking cessation (Rennard, Daughton et al. 1990) which is consistent with the benefits of this intervention.

The increased numbers of neutrophils seen in the lungs of patients with COPD may accumulate because of an increased influx from peripheral blood or because of prolonged neutrophil survival. Exposure to cigarette smoke appears to stimulate neutrophil differentiation and maturation, causing a peripheral leucocytosis (Corre, Lellouch et al. 1971; Van Eeden and Hogg 2000) which has been found to correlate with the severity of airflow obstruction (Yeung and Buncio 1984). This suggests that a peripheral neutrophilia facilitates an increased neutrophil transmigration into the lung. Platz and colleagues (Platz, Ioanas et al. 2004) described a reduction of peripheral neutrophil apoptosis (based on Annexin V-PE binding and nuclear morphology) during exacerbations of COPD compared with healthy controls, which returned to control levels upon resolution of symptoms. This increased cell longevity would facilitate increased neutrophil accumulation into the lung during exacerbations, again predisposing to enhanced lung damage. Once in the lungs, it has been suggested that reduced neutrophil apoptosis may account for the high numbers seen in the lung and the increased inflammation described in COPD, although recent studies present conflicting results (Uller, Persson et al. 2006). Ryttilä et al (Ryttilä, Plataki et al. 2006) found no differences in the proportion of apoptotic neutrophils in induced sputum, or in the *in vitro* anti-apoptotic activity detected in the sputum fluid phase in patients with COPD, healthy smokers or healthy controls despite patients with COPD having a significantly higher percentages of neutrophils in the samples. The authors suggested that the increased neutrophilic presence in COPD was more likely due to an increased influx rather than a reduction in apoptosis.

1.5.4 Pro-migratory stimuli

Neutrophils migrate into the lung in response to soluble mediators. Pro-migratory stimuli can be classified as non-chemotactic cytokines, chemotactic cytokines (chemokines) or chemoattractants.

1.5.4.1 Non-chemotactic Cytokines

Tumour Necrosis Factor alpha (TNF- α) is thought to be one of the most important pro-adhesive cytokines in COPD. It is produced by activated monocytes and macrophages (but also epithelium, endothelium and probably smooth muscle cells) and has been implicated in the pathogenesis of COPD (Churg A., Dai et al. 2002; Sakao, Tatsumi et al. 2002). Increased levels of TNF α have been measured in serum, sputum (Hacievliyagil, Gunen et al. 2006) and in bronchoalveolar lavage samples from patients with COPD, demonstrating a dose dependent relationship with cigarette exposure (Kuschner, D'Alessandro et al. 1996), with further increases during exacerbations (Aaron, Angel et al. 2001). TNF α has also been associated with the systemic manifestations of COPD, including a low BMI (Di Francia, Barbier et al. 1994) (perhaps via leptin); (Calikoglu, Sahin et al. 2004) and abnormal resting energy expenditure (Nguyen, Bedu et al. 1999). TNF α over-expression (due to genetic polymorphism) has been linked to early COPD development and rapid progression (Brogger, Steen et al. 2006). In support of this, mouse models with an inducible TNF α gene construct have shown that over-expression of TNF α is associated with the development of emphysema associated with a general increase in lung inflammation (Vuilleminot, Rodriguez et al. 2004) probably by inducing MMP production (Wright, Tai et al. 2007). TNF α receptor knock out mice demonstrate reduced smoking or elastase induced emphysema in comparison with the wild type (Lucey, Keane et al. 2002; Churg A., Wang et al. 2004) suggesting a central role for this cytokine.

IL-1 β is also produced by macrophages (although neutrophils and epithelial cells can also produce the cytokine) and increased levels have been found in sputum of patients with stable COPD, which increase further during exacerbations (Chung 2001). IL-1 β production is enhanced by cells cultured from smokers with COPD following cigarette smoke exposure compared with controls (Rusznak, Mills et al. 2000) and there is some evidence that over-expression of IL-1 β (caused by polymorphisms such as the -511 SNP with a cytosine/thymine transition) may increase susceptibility to COPD (Asada, Yamaya et al. 2005). Furthermore, animal studies using inducible IL-1 β systems have described a neutrophilic infiltrate, distal airspace enlargement, increased thickness of the conducting airways and enhanced mucin production when IL-1 β was over-expressed (Lappalainen, Whitsett et al. 2005).

TNF α and IL-1 β are not directly chemotactic, but they act by increasing expression of integrins on the neutrophil surface and increasing endothelial expression of selectins and I-CAM1, aiding capture and firm adhesion of neutrophils to the vascular endothelium (Burke-Gaffney and Hellewell 1996; Scholz, Devaux et al. 1996). They also increase production of directly chemotactic mediators from endothelial cells to enhance the migration process (Smart and Casale 1994).

1.5.4.2 Chemoattractants

Neutrophils possess at least five different receptors for chemotactic stimuli, including unique receptors for leukotriene B₄ (LTB₄), complement protein C5a, bacterial peptides including Formyl-methionyl-leucyl-phenylalanine (fMLP) and platelet activating factor (PAF).

Leukotriene B4

LTB4 is mainly produced by monocytes, alveolar macrophages and activated neutrophils and its production is up-regulated by a number of inflammatory mediators including C5a, IL-1 β , TNF- α , granulocyte-macrophage colony stimulating factor (GM-CSF), PAF, NE and even LTB4 itself (Crooks and Stockley 1998; Borgeat, Krump et al. 2003). LTB4 enhances neutrophil aggregation and chemotaxis via two neutrophil surface receptors. A low affinity receptor induces degranulation and increases oxidative metabolism whereas a high affinity receptor induces aggregation, chemokinesis and adhesion via the integrin Mac-1 (Tonnesen, Anderson et al. 1989). LTB4 may also activate endothelial cell monolayers *in vitro*, enhancing neutrophil emigration (Nohgawa, Sasada et al. 1997). LTB4 concentrations are elevated in sputum (Hill, Bayley et al. 2000) and exhaled breath condensate (Montuschi, Kharitonov et al. 2003) from patients with COPD. Concentrations correlate with the degree of airway neutrophilia (Bhowmik, Seemungal et al. 2000) and increase further during bacterial exacerbations (Gompertz, O'Brien et al. 2001), returning to baseline once bacteria have been successfully eradicated (White, Gompertz et al. 2003). In addition, LTB4 concentrations also correlate with the subsequent decline in gas transfer implicating neutrophilic infiltration in the pathophysiology of emphysema (Parr, White et al. 2006).

In vitro chemotaxis studies, using functional antibodies against IL-8 and an LTB4 receptor antagonist, have demonstrated that over 60% of the chemotactic activity of sputum can be accounted for by contributions from both IL-8, and LTB4 (Woolhouse, Bayley et al. 2002; Beeh, Kornmann et al. 2003).

LTB₄ is degraded by oxidation in myeloid cells and hepatocytes rendering it biologically inactive (Feinmark, Lindgren et al. 1981). There is also a negative feedback loop where LTB₄ stimulates the production of degradative enzymes, limiting its own duration of activity (Crooks and Stockley 1998).

Complement protein C5a

Cleavage of complement protein C5 yields C5a, a component of the membrane attack complex, and C5b, a powerful chemoattractant. Circulating C5a is produced during the activation of the classical complement cascade in blood and can bind to circulating neutrophils (Kohl and Bitter-Suermann 1983). Tissue macrophages and epithelial cells produce proteins of the alternative complement pathway (Strunk, Eidlen et al. 1988) (including C5), and so C5a is able to promote neutrophil migration by the formation of a concentration gradient. C5a has been shown to enhance adhesion molecule expression (especially ICAM-1) in airway epithelial cells and interestingly, this effect was exaggerated in the presence of cigarette smoke (Floreani, Wyatt et al. 2003). There have been few *in vivo* studies monitoring C5a levels in COPD, and to date, levels have not appeared elevated in COPD patients (McLeod, Mack et al. 1985).

Formyl-methionyl-leucyl-phenylalanine

Formylated bacterial peptides are produced following the cleavage of the NH₂–terminal portions of common bacterial proteins during synthesis; there are no similar formylated peptides in mammalian cells, which makes these proteins highly specific targets for neutrophil activation and degranulation. The fMLP receptor consists of seven transmembrane-spanning loops and they are expressed on neutrophils and monocytes both when quiescent and activated, however, the presence of fMLP (or other agents)

causes the mobilisation of secretory vesicles, leading to a five fold increase in surface receptor expression (Borregaard and Cowland 1997). The presence of fMLP has multiple affects on neutrophils, including degranulation, oxidative burst, cytoskeletal changes, and chemotaxis (Wagner and Roth 2000).

The numbers of fMLP receptors are elevated in both healthy smokers and subjects with COPD who smoked, but not non-smoking patients with COPD (Matheson, Rynell et al. 2003) suggesting that fMLP may play a role in the recruitment of neutrophils leading to the development of disease and its progression whilst smoking and especially during bacterial exacerbations (where bacterial formyl peptides may be most important).

Platelet activating factor

PAF is produced by endothelial cells, platelets, neutrophils and macrophages. It is pro-inflammatory and enhances neutrophil adhesion to the endothelial surface, promoting migration (Mathiak, Szewczyk et al. 1997). Its' role or relevance in the pathogenesis of COPD is unknown.

1.5.4.3 Chemokines

Chemokines are a group of small proteins (approximately 40 in number) with similar cysteinyl-containing structures. They are classified by the sequence of the two most NH₂ –proximal cysteines; in the CXC chemokines, the cysteines are separated by an amino acid whereas in the CC chemokines, the cysteines are adjacent to one another. In general, CC chemokines are more strongly chemotactic for monocytes and CXC chemokines are more strongly chemotactic for neutrophils. CXC chemokines include interleukin 8 (IL-8); Growth related oncogene (GRO) alpha, beta and gamma; epithelial

cell derived neutrophil activating peptide (ENA)-78 and neutrophil activated peptide (NAP)-2. Interleukin 8 is the most well studied and is believed to be one of the most important chemoattractants in COPD.

Interleukin-8

IL-8 is a 16kDa protein that is primarily produced by leukocytes (monocytes, T cells, neutrophils and natural killer cells) and airway epithelial cells. Production is not constitutive but is induced by pro-inflammatory cytokines such as IL-1 β and TNF α (Matsushima, Morishita et al. 1988), bacteria and bacterial products (DiMango, Zar et al. 1995; Khair, Davies et al. 1996), viruses such as adenovirus and rhinovirus (Alcorn, Booth et al. 2001) and oxidants from cigarette smoke (Deforge, Preston et al. 1993). Once secreted, IL-8 binds to CXC Receptors on leukocytes resulting in activation of protein kinase B and GTPases, which lead to enhanced neutrophil adherence to endothelial cells (by increasing expression of β_2 -integrins) and directed cell migration. IL-8 also activates Ras and mitogen activated protein kinases and extracellular signal-related kinases in neutrophils, causing degranulation.

In COPD, sputum IL8 correlates with levels of neutrophil activation markers such as myeloperoxidase (MPO) and neutrophil elastase (NE) (Hill, Bayley et al. 1999) and relates to airflow obstruction (Yamamoto, Yoneda et al. 1997), oxygen saturation, cigarette exposure (Hacievliyagil, Gunen et al. 2006) and progression of emphysema quantified by CT scan (Parr, White et al. 2006). It has been suggested that oxidative stress (caused by cigarette smoke and bacterial and viral infections) induces IL-8 production in both airway epithelial and endothelial cells, leading to neutrophil adhesion, chemotaxis and degranulation.

Growth Related Oncogene Alpha

GRO α is detectable in bronchial secretions (Traves, Culpitt et al. 2002) and both GRO α and ENA-78 have been measured in BAL fluid (Morrison, Strieter et al. 1998). One study has suggested that chemotaxis towards GRO α and NAP2 is increased in COPD compared with healthy controls and smokers (Traves, Smith et al. 2004), but their contribution to the pathogenesis of COPD is unknown.

Neutrophils possess two chemokine receptors, CXCR1 and CXCR2. IL-8 can bind both with high affinity, but the other chemokines exert effects primarily through CXCR2, and have only a low affinity for CXCR1 (Wagner and Roth 2000).

The individual importance of each pro-inflammatory or pro-migratory protein in COPD has yet to be unravelled, certainly many of these proteins appear to have similar actions and a degree of overlap or redundancy is likely to exist in the inflammatory cascade. However, there is increasing interest in identifying the key inflammatory mediators in COPD as potential targets for therapeutic interventions. To date, trials which have aimed to block a specific mediator have not proved helpful (de Boer 2005).

1.5.5 Neutrophil migration in COPD

Given the increased presence of neutrophils in the lungs of patients with COPD, it has been hypothesised that neutrophils in COPD reflect an enhanced chemotactic response. However, there is conflicting evidence for both overall neutrophil migration and for an up-regulation of adhesion molecules in COPD. Burnett and colleagues demonstrated a clear increase in chemotaxis and extracellular proteolysis compared with matched

controls (Burnett, Chamba et al. 1987), whereas Yoshikawa et al found the reverse, and described a reduction in overall chemotaxis in COPD (Yoshikawa, Dent et al. 2007).

Noguera et al (Noguera, Batle et al. 2001) measured MAC-1, LFA-1 and L-Selectin expression on neutrophils from controls and patients with COPD, prior and post stimulation with TNF- α . Neutrophils from patients with COPD had enhanced expression of these adhesion molecules compared with controls, and differences were even more pronounced following stimulation with chemoattractants. Woolhouse et al (Woolhouse, Bayley et al. 2005) demonstrated up-regulation of CD11b (a component of MAC1) on neutrophils from smokers with COPD compared with controls. Furthermore Di Stefano et al demonstrated increased expression of E-selectin and ICAM-1 on basal epithelial cells in the bronchial mucosa of chronic bronchitis with airflow obstruction (Di Stefano, Maestrelli et al. 1994). However, Gonzalez (160) found no differences between levels of adhesion molecules in smokers with and without airflow obstruction and Noguera et al found decreased levels of soluble ICAM-1 in patients with COPD compared with healthy controls (Noguera, Busquets et al. 1998).

Despite the conflicting results, neutrophil function does appear to be different in COPD, with enhanced reactive oxygen species production (Noguera, Batle et al. 2001), enhanced phagocytosis and potentially enhanced migration (Burnett, Chamba et al. 1987) with increased adhesion to endothelial cells under flow conditions (Woolhouse, Bayley et al. 2005). The differences may be accounted for by genetic polymorphisms and be apparent during maturation in bone marrow. Alternatively neutrophils could be “primed” following their release into the circulation, perhaps by inflammatory cytokines, so that they are more responsive, with an increased ability to respond and

degranulate compared with those of healthy individuals. This priming may also occur during transmigration and studies have confirmed differences between neutrophils prior to and following migration even in healthy controls with increased expression of proteinases on the cell surface, increased adhesion molecule expression and enhancement of the respiratory burst (Dangerfield, Larbi et al. 2002; Yadav, Larbi et al. 2002). However, these processes have not been studied in COPD. It remains unclear whether current observations reflect a predisposing factor for COPD or a consequence of the disease.

The contradictory findings in migration and adhesion molecule expression may be explained by differences in patient groups and variations in methodologies, however, it is difficult to formulate a robust theory of the pathogenesis of COPD without these issues being resolved.

1.5.6 Proteinases in COPD

Table 1.3 provides an overview of neutrophil serine proteinases.

Table 1.3: An overview of neutrophil serine proteinases

| | Neutrophil elastase | Cathepsin G | Proteinase 3 |
|-----------------------|-------------------------|-------------------------------|--|
| Size | 218 amino acids | 235 amino acids | 222 amino acids |
| Mass (kDa) | 39 | 28.5 | 29 |
| Substrate specificity | Val- Xaa > Ala - Xaa | Aromatic acids in P1 position | Same as NE, accepts basic amino acids in P1 position |
| Elastolytic activity | | 20% potency of NE | 40% potency of NE |

1.5.6.1 Neutrophil Elastase

A significant physiological role of NE is bacterial killing. This is achieved when opsonised bacteria are ingested in phagosomes that fuse with lysosomes containing proteinases and oxidants. NE is also intensely destructive and this is believed to be the primary cause of lung damage seen in both emphysema and chronic bronchitis. NE was the first serine proteinase to be shown to produce emphysema in animal models. Intra-tracheal instillation of purified NE induced emphysema in dogs and hamsters (Janoff, Sloan et al. 1977; Senior, Tegner et al. 1977) and intra-tracheal instillation of other elastases have given similar results in various animal models - Papain (pineapple elastase) in rats (Gross, Pfizer et al. 1964), neutrophil lysates in dogs (Marco, Mass et al. 1971) and porcine pancreatic elastase in rats and hamsters (Blackwood, Hosannah et al. 1973; Snider, Hayes et al. 1974). In fact the development of emphysema seems specific to elastase activity with emphysema severity relating proportionally to the elastolytic potency of the elastase used (Blackwood, Hosannah et al. 1973; Snider, Hayes et al. 1974) and in these models emphysema can be prevented by specific elastase inhibitors (Rudolphus, Kramps et al. 1994; Lucey, Stone et al. 1998).

NE acts upon a wide range of proteins and can degrade elastin, fibronectin and collagen (Beith 1986; Kafienah, Buttle et al. 1998), and also decrease the function of immunoglobins and activate components of the complement cascade (Niederman, Merrill et al. 1986; Vogt 2000). NE may also effect wound healing, by its actions on transforming growth factor β and the epithelins (Ashcroft, Lei et al. 2000). During activation, azurophil granule proteinases (including NE) are expressed on the neutrophil membrane (Owen, Campbell et al. 1995; Owen, Campbell et al. 1997) and *in vitro*, over 95% remains associated with the cell by a charge dependent mechanism, while less than 5% is released into the liquid milieu or directly onto tissue, causing damage by close

contact between cells and matrix (Owen, Campbell et al. 1997; Campbell, Campbell et al. 2000). Recent elegant studies have shown that NE polarises towards the leading edge of the neutrophil as it migrates. Some is then left behind as the cell moves on where it may cause collateral connective tissue damage (Cepinskas, Sandig et al. 1999).

Free NE activity has been detected in secretions of patients with COPD (Hill, Bayley et al. 1999) and this is felt to be fundamental in the development of the condition *in vivo*. Free NE may accumulate from degranulating neutrophils, or (in contrast with apoptotic cells) may be freely released during cell necrosis (Fadok, Bratton et al. 2001). Also the process of phagocytosis may cause the release of significant quantities of proteinases into the media (“sloppy eating”), especially during “frustrated phagocytosis”, when cells attempt to ingest large particles (Ohlsson, Linder et al. 1996). Free NE can also be released from activated macrophages, which scavenge the proteinase from apoptotic neutrophils via endocytosis and subsequently release it during the first 24 hours of their own inflammatory response (Weitz, Huang et al. 1987). This is important, as although cell-associated proteinases have partial resistance to native inhibitors such as α 1AT (Owen, Campbell et al. 1997), free NE is more readily inactivated by both serum and tissue-based inhibitors (Weitz, Huang et al. 1987) if sufficient quantities are present, which means that in health, free NE should be completely inactivated at a short distance from the activated cell.

Indeed, it has been shown that the concentration of free NE released from neutrophils falls exponentially away from the cell (Liou and Campbell 1996). In healthy subjects, the serum concentration of α 1-AT, which inhibits NE on a one to one molar basis, is 30 μ Mol. This is at least two orders of magnitude lower than NE concentrations in the neutrophil

granule. Therefore following degranulation NE cannot be completely inhibited until it has diffused far enough to reduce its concentration to 30 μ Mol. This phenomenon is called quantum proteolysis (Liou and Campbell 1996) and was clearly demonstrated in a series of experiments using serum from patients with normal or deficient α 1AT where an area of obligate enzyme activity existed even in the presence of serum from healthy subjects, but was far greater using serum from patients with α 1 antitrypsin deficiency (Campbell, Campbell et al. 1999). Membrane bound NE is less susceptible to anti-proteinases (Owen, Campbell et al. 1997) and the combination of increased free and membrane bound NE present in COPD may be sufficient to overcome local inhibitors, causing the tissue destruction which is characteristic of the disease.

NE interacts with matrix proteins and cells, and this affects not only its own activity, but also the efficacy of its inhibitors and other proteinases. For example, although free NE is irreversibly inhibited by α 1AT, elastin bound NE is poorly inhibited by α 1AT, while the inhibitory effect of SLPI is unaffected (Morrison, Kramps et al. 1987; Rice and Weiss 1990). Adhesion to goblet cells appears to alter the neutrophil membrane, enhancing the release of membrane bound NE into the inter-cellular space (Takeyama, Agusti et al. 1998) potentially causing mucus secretion. Furthermore, animal models have demonstrated that NE can induce secretory cell metaplasia which is prevented by NE specific inhibitors (Lanone, Zheng et al. 2002). It has been suggested that mucous gland epidermal growth factor receptor interacts with NE as part of a signalling cascade to mediate this effect (Shim, Dabbagh et al. 2001). In human studies, there is a clear relationship between the amount of mucus production and the concentration of active neutrophil elastase in the lung secretions.

NE can damage the respiratory epithelium *in vitro*, reduce ciliary beating (Smallman, Hill et al. 1984; Amitani, Wilson et al. 1991) and trigger a state of oxidative stress in cells (Aoshiba, Yasuda et al. 2001) all of which are abrogated by NE inhibitors. NE can also induce apoptosis of epithelial cells (Nakajoh, Fukushima et al. 2002) and detachment of bronchial epithelial cells from the extra cellular matrix (Rickard and Rennard 1989) and both PR3 and NE induce detachment and apoptosis of endothelial cells (Ballieux, Hiemstra et al. 1994) which has also been implicated in the pathogenesis of COPD (Tuder, Zhen et al. 2003).

NE stimulates the release of LTB₄ by macrophages (Hubbard, Fells et al. 1991) and may cause release of IL8 from bronchial epithelial cells which enhances neutrophil migration into the lung. This inflammatory response is greater in patients with α 1-AT deficiency, who have higher levels of both LTB₄ and elastase. The increased inflammatory burden amplifies neutrophil recruitment, increasing the potential for tissue destruction compared with patients with normal anti-proteinase function, which explains in part the more severe and rapidly progressive disease of α 1 – AT deficiency patients (Hill, Bayley et al. 2000).

The most important risk factor for COPD is cigarette smoke exposure and the relationships between smoke inhalation and NE have been studied in depth. In animal models NE knock out mice are partially protected (45%) against the development of emphysema (Shapiro 2000), however, these models are limited, as CG and PR3 persist, and CG has also been shown to cause secretory cell metaplasia (Lucey, Stone et al. 1985) whereas PR3 causes both emphysema and secretory cell metaplasia (Kao, Wehner et al. 1988). Furthermore, animal models have suggested that both synthetic (Lucey, Stone et al. 1989) and natural NE inhibitors (Lucey, Stone et al. 1990; Stone, Lucey et al. 1990;

Rudolphus, Kramps et al. 1994) can limit emphysema development when delivered simultaneously with the elastase insult. Most animal models suggest that neutrophil influx is greatest during the early stages of lung damage with macrophage influx, and their metalloproteinases accumulating at a later stage (Ofulue, Ko et al. 1998; Zay, Loo et al. 1999). However, even when given in established disease, NE inhibitors still limit inflammation and connective tissue breakdown (Wright, Farmer et al. 2002) suggesting that this enzyme plays a key role throughout disease development and progression.

COPD is characterised by periods of stability of symptoms punctuated with exacerbations (which are intermittent worsening of symptoms and (most probably) of the inflammatory load (Sapey and Stockley 2006)). NE activity relates to sputum purulence, which is due to MPO and can be graded visually (Gompertz, O'Brien et al. 2001). During bacterial exacerbations of COPD, sputum purulence, neutrophil influx, NE activity and tissue degradation products increase (Dowson, Guest et al. 2001; Gompertz, O'Brien et al. 2001; Donaldson, Seemungal et al. 2002) and therefore it is likely that proteinase induced tissue damage also increases. More frequent exacerbations are associated with a faster decline in lung function as well as increased morbidity and mortality and it may be that the increases in NE load and activity during these periods causes progressive lung damage which is reflected in markers of disease severity (Dowson, Guest et al. 2001). Table 1.4 summarises the actions of NE in COPD.

Table 1.4. The actions of neutrophil elastase in COPD

| | |
|--|---|
| Bacterial Killing | Intra-cellular : engulfed organisms in phagosome Extra cellular: Targeting and cleaving bacterial virulence factors in released granule proteins |
| Degradation | ECM components Cystatin C TIMPs T Lymphocyte surface antigen |
| Activation | MMP-2, MMP-3, MMP-9, Cathepsin B |
| Modification of inflammatory mediators | Enhances epithelial secretion of CXCL8 Enhances macrophage secretion of LTB4 Inhibits cellular response to TNFR1 prolonging half life of TNF Increases SLPI expression but reduces secretion of SLPI Increases elafin expression Increases alpha1-AT expression by monocytes and alveolar macrophages |
| Cell migration | NE/alpha 1 antitrypsin complexes are chemotactic for neutrophils Modification of ICAM1 expression enhancing adhesion |
| Cell apoptosis | Increases epithelial and endothelial cell apoptosis |
| Cell function | Disruption and detachment of epithelial cells Reduces ciliary beating of columnar epithelium Enhances oxidative stress Increases mucin MUC5AC protein content Increases bacterial adherence and colonisation |

Legend

References included in text.

1.5.6.2 Matrix metalloproteinases

Both neutrophils and macrophages produce large amounts of Metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). The MMPs are proteolytic enzymes that are secreted as proenzymes (activated by other MMPs and NE) and remain bound to cell membranes. MMPs not only degrade matrix proteins, but also inactivate anti-proteinases such as α 1-AT and α 1-ACT, activate enzymes involved in the clotting cascade and interact with cytokines and adhesion molecules, so their potential role in the pathogenesis of COPD is complex and wide ranging.

Over 24 mammalian MMPs have been described, and although they are primarily grouped according to the proteins they degrade (collagenases, gelatinases etc.), most can degrade all extra cellular substrates. MMPs are inhibited by α 2-macroglobulin and by the four TIMPs described to date (Baker, Edwards et al. 2002). The main MMPs secreted by neutrophils are MMP-9 (Gelatinase B), which degrades collagen, elastin and gelatine; and MMP-8 (Neutrophil collagenase) which degrades collagen types I-III.

MMP-9 is increased in lung tissue, BALF and plasma taken from patients with COPD (Segura-Valdez, Pardo et al. 2000), levels are negatively correlated with airflow obstruction and relate to the number of sputum neutrophils (Vignola, Bonanno et al. 1998; Beeh, Kornmann et al. 2003). MMP-9 not only acts as a proteinase, but also modifies cellular functions by regulating cytokines and matrix bound growth factors and therefore may have a role in lung remodelling after the inflammatory insult resolves (Li, Cui et al. 2002; Atkinson and Senior 2003). MMP-9 deficient mice exposed to intra-tracheal LPS showed no differences in histological tissue damage, neutrophil migration or infiltration than the wild type mouse, suggesting a limited role for this MMP in the pathogenesis of COPD (Betsuyaku, Shipley et al. 1999). In a further study MMP-9

knock out mice displayed greater neutrophil influx than the wild type, perhaps because MMP-9 can degrade neutrophil chemoattractants (Lanone, Zheng et al. 2002). However smoke exposed guinea pigs displayed a reduction in the severity of emphysema and MMP-9 activity in BALF after introduction of a broad spectrum MMP inhibitor, although this may also have reflected inhibition of other metalloproteinases (Selman, Cisneros-Lira et al. 2003). Genetic polymorphisms of MMP-9 have been identified that cause enhanced protein expression, and in a Japanese study, polymorphism -1562C/T was associated with an increased risk of smoking induced emphysema (Minematsu, Nakamura et al. 2001) although this has yet to be replicated in other populations (Wallace and Sandford 2002).

MMP-8 has been less studied in COPD compared with MMP-9, however one study described both raised concentrations and increased activity of MMP-8 in the induced sputum of patients with COPD compared with smokers without evidence of COPD. Furthermore both the levels and activity of MMP-8 correlated inversely with FEV1 and positively with sputum neutrophil counts (Vernooy, Lindeman et al. 2004). There are currently no relevant animal studies to elucidate the importance of MMP-8 in COPD.

1.5.6.3 Macrophages

Macrophage numbers are elevated in the lungs of patients with COPD, where they accumulate in the alveoli, bronchioli and small airways (Saetta, Turato et al. 2001). There is also a correlation between alveolar wall macrophage numbers and the degree of small airways obstruction in COPD (Jeffery 1998). Macrophage metalloproteinases potentially contribute to the breakdown of connective tissue in COPD, with increased elasteolytic activity seen in smoke exposed cells (Russell, Thorley et al. 2002). “Knock

out” mouse models have demonstrated a role for macrophage metalloproteinases as well as neutrophil elastase in the development of tobacco smoke induced emphysema, although the relationship between these models to human disease remains unknown. Interactions between neutrophils and macrophages are described in section 1.5.6.4 and summarised in figure 1.5.

1.5.6.4 Interactions between Neutrophil Elastase and Matrix metalloproteinases

It is likely that the serine proteinases and MMPs act synergistically in lung disease. NE degrades TIMPs (Itoh and Nagase 1995) facilitating MMP activity, and can activate several MMPs converting pro-enzymes, including MMP-9, to the active form (Ferry, Lonchampt et al. 1997). Conversely MMP-12 inactivates α 1-AT thereby enhancing NE activity (Desrochers, Jeffrey et al. 1991). The majority of animal “knock out” models of emphysema support a multi-faceted pathogenic process in the disease, as inhibition of serine, cysteine or matrix metalloproteinases all show partial protection from the development of emphysema (Lanone, Zheng et al. 2002; Wright, Farmer et al. 2002). The noticeable exception is MMP-12 and there have been several conflicting animal studies comparing MMP-12 activity, macrophage and neutrophil influx and emphysema. MMP-12 knock-out mice (Line 129 mice) exposed to cigarette smoke did not develop emphysema and lung inflammation was reduced, although macrophage recruitment to the lungs was normal (Hautamaki, Kobayashi et al. 1997). In a study of Sprague-Dawley rats, neutrophil numbers increased in the first month following smoke exposure, but breakdown of connective tissue and the development of emphysema occurred later and related to a subsequent increase in macrophages (Ofulue, Ko et al. 1998). However, in a further studies, cigarette exposed mice showed an acute influx of neutrophils with evidence of both elastin and collagen degradation in a dose dependent manner. This was

reduced by neutrophil depletion or the administration of the serine proteinase inhibitor α 1-AT (Zay, Loo et al. 1999). Other animal models have also found an early rise in neutrophil number and activity, and a corresponding rise in matrix breakdown products after exposure to cigarette smoke (Wright, Farmer et al. 2002).

In order to understand the relationship between macrophages and neutrophils in COPD, MMP-12 knock out mice were studied further. Cigarette smoke exposed MMP-12 knock out mice did not display the early neutrophilic infiltrate or the release of desmosine and hydroxyproline (matrix breakdown products) that is characteristic of wild type mice, although low levels of macrophage infiltration did occur. When the MMP-12 knock-out mice underwent intra-tracheal instillation of normal macrophages, a neutrophil influx was seen and the use of an MMP inhibitor also prevented a neutrophilic infiltrate and subsequent matrix breakdown (Churg A., Dai et al. 2002). Following these observations, studies demonstrated that cigarette smoke induced production of TNF- α from alveolar macrophages in wild type mice, but not in MMP-12 knock out mice while levels of TNF- α mRNA were the same in both groups. It was surmised that MMP-12 processes TNF- α after secretion and it is likely, therefore, that MMP-12 is needed to activate TNF- α which in turn initiates neutrophil recruitment, leading to degranulation and tissue damage (Churg, Wang et al. 2003)..

When interpreting results from *in vitro* work and animal models, it is crucial to consider that there may be important variations in the pathogenesis of COPD between cell types and differing species, and indeed, conflicting results are common. However it seems likely that neutrophil infiltration is an early event and that both neutrophil numbers and elastolytic activity relate well to lung damage in the early stages of disease.

Macrophages seem central to neutrophil recruitment, probably via activation of TNF- α by metalloproteinases, and may be needed to sustain the inflammatory process and hence development of emphysema.

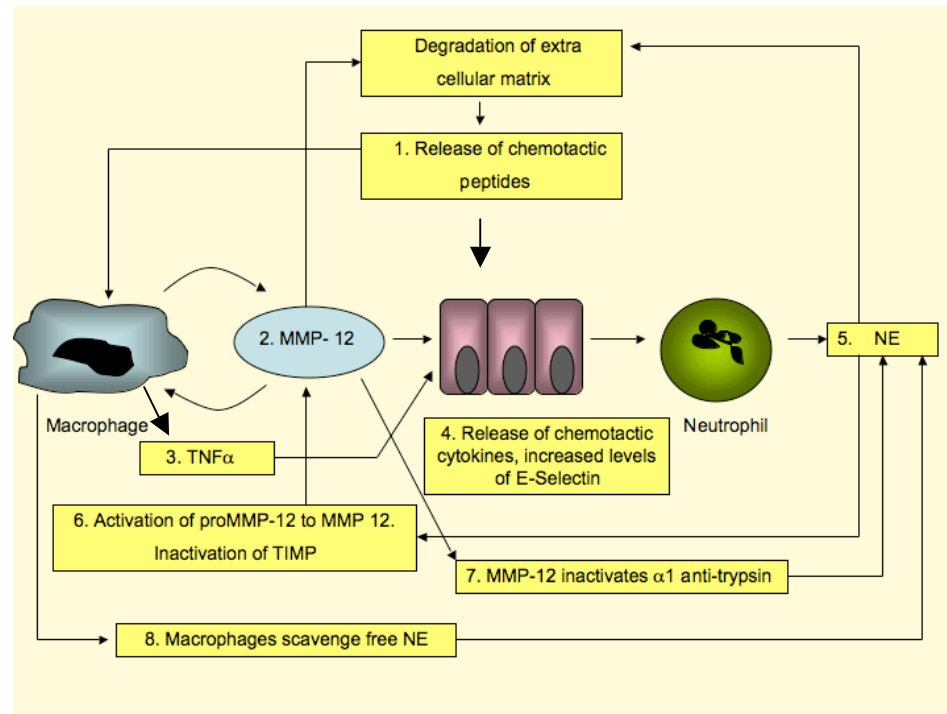


Figure 1.5. The pro-inflammatory properties of MMP-12 and macrophage / neutrophil interactions.

Legend.

1. Cigarette smoke causes the release of chemotactic peptides which recruit macrophages and neutrophils to airways.
2. Activated macrophages secrete MMP-12 which activates epithelial cells and causes degradation of extracellular matrix components.
3. MMP-12 facilitates the release of active TNF α which activates epithelial cells further.
4. Activated epithelial cells release chemotactic cytokines and increase the expression of pro-adhesive proteins such as E-Selectin.
5. Neutrophils migrate to areas of activated epithelium and release free NE during frustrated phagocytosis or sloppy eating. NE promotes epithelial cell apoptosis and increases expression of pro-inflammatory mediators such as LTB₄ and CXCL8.
6. NE proteolytically converts proMMP-12 to active MMP-12 and proteolytically inactivates TIMP, both of which enhance MMP-12 activity.
7. MMP-12 proteolytic inactivation of α 1AT causes the further release of pro-migratory proteins and allows increased NE activity.
8. Macrophages scavenge free NE which is then reused to further degrade extracellular matrix components

1.5.7 Other Inflammatory Cells

1.5.7.1 Lymphocytes

There are increased numbers of T lymphocytes (predominantly CD8+) in the lungs of smokers with COPD (Saetta, Di Stefano et al. 1998) (O'Shaughnessy, Ansari et al. 1997), although the role of the lymphocyte in the pathogenesis of COPD has yet to be established. Supporting published data is limited, nevertheless, one theory suggests that smoking induced lung injury leads to structural alterations to self antigens leading to the activation and proliferation by auto-reactive T cells. T cells could then damage the lungs further by promoting cellular inflammation, as well as cellular necrosis and apoptosis, leading to airway remodelling (Cosio, Majo et al. 2002).

1.5.7.2 Eosinophils

The absolute numbers of eosinophils found in the lung are much lower than other cell types, but levels appear to be raised in bronchial lavage fluid, biopsies and alveolar tissue taken from some patients with COPD (Lacoste, Bousquet et al. 1993; Pesci, Balbi et al. 1998; Retamales, Elliot et al. 2001). Eosinophils are classically associated with asthma, and it appears that eosinophils are a feature of a clinically distinct subset of patients with COPD with a more “asthmatic” phenotype, as corticosteroid related reversibility of airflow obstruction correlates with eosinophilic cationic protein (Chanez, Vignola et al. 1997).

1.5.8 Oxidative Stress

There is evidence of oxidative stress in smoking related lung disease. Cigarette smoke contains approximately 10^{15} oxidant molecules per puff (Church and Pryor 1985), and oxygen free radicals and hydrogen peroxide are released from activated neutrophils and

macrophages (Ludwig and Hoidal 1982; Schaberg, Haller et al. 1992). In health, free reactive species are scavenged by anti-oxidants and anti-oxidant enzymes as part of the oxidant/antioxidant balance (MacNee 2000), however, smokers have increased lipid peroxidation and reduced plasma antioxidant capacity, indicating an increased oxidative burden (Rahman, Morrison et al. 1996).

An increase in oxidative burden appears to be capable of causing emphysema in mouse models. Antioxidant transcription factor nuclear erythroid-related factor 2 (which among other genes regulates the expression of glutathione peroxidase 2 (Singh, Rangasamy et al. 2006)) knock out mice are susceptible to cigarette smoke exposure. These mice developed airspace enlargement as well as more pronounced inflammation and apoptosis of alveolar septal epithelial and endothelial cells (Rangasamy, Cho et al. 2004). Furthermore, oxidant/antioxidant imbalance is able to cause emphysematous change, even in the absence of cigarette smoke. Zhang et al. (Zhang, Shan et al. 2006) followed TLR4-knockout mice (*Tlr4*^{-/-} mice) into middle age and found that the animals spontaneously developed emphysema due to increased *Nox3* (a novel NADPH oxidase) gene expression and subsequent elastin degradation. Interestingly, the elastin breakdown was caused by oxidants, since NADPH oxidoreductase inhibition prevented the elastin breakdown. Also of interest, the *Tlr4*^{-/-} mice did not show infiltration of the lung parenchyma with inflammatory cells, and the authors speculate that the source of oxidants and of proteolytic activity may be the alveolar septal endothelial cells.

The presence of oxidative stress could contribute to the pathogenesis of COPD by a number of other mechanisms. Firstly, by the oxidative inactivation of anti-proteinases (Johnson and Travis 1979); by the increased sequestration of neutrophils in the

pulmonary microvasculature (Drost, Selby et al. 1992); and by increase gene expression of pro-inflammatory mediators (Antonicelli, Parmentier et al. 2002).

1.5.9 Bacteria and Viruses

A proportion of patients with COPD have airways that are colonised with bacteria, even when clinically stable. Colonisation is associated with increased sputum concentrations of inflammatory mediators including IL-8, LTB₄ and NE (Hill, Campbell et al. 2000). Lower airway bacterial colonisation in the stable state appears to increase the frequency and alter the character of COPD exacerbations (Patel, Seemungal et al. 2002). Exacerbation frequency relates to subsequent decline in lung function (Donaldson, Seemungal et al. 2002) and health status (Seemungal, Donaldson et al. 1998); suggesting that colonisation may be important in disease progression.

Viruses have also been implicated in the pathogenesis of COPD. Alveolar epithelial expression of adenovirus E1A protein has been associated with emphysema, and in particular inflammatory cell tissue destruction (Retamales, Elliot et al. 2001), potentially by amplification of lung inflammation (for example, increased transcription of IL-8 (Gilmour, Rahman et al. 2001)). Furthermore, cell culture studies suggest that 20% of COPD exacerbations may be virally related (PCR studies place this figure closer to 40%), which again may be associated with lung damage by many mechanisms including enhanced neutrophil recruitment, increased release of reactive oxygen species, and increased expression of pro-inflammatory mediators (Sapey and Stockley 2006).

1.6 The relationship between COPD and ageing

There has been recent speculation that many chronic disease states, including COPD, may represent a form of advanced ageing. Certainly, there is a higher prevalence of COPD in ageing subjects, even when cigarette smoke exposure is accounted for (Fukuchi, Nishimura et al. 2004; Buist, McBurnie et al. 2007). Greater burden of COPD in the elderly may be attributable to two hypotheses. The first is that age-associated structural and functional changes within the body *per se* may cause an increase in patho-genetic susceptibility to COPD, potentially even causing COPD. The second hypothesis suggests that the repeated insults endured by an organ so intimately associated with the environment over a prolonged life may cumulatively make lung disease more apparent or progress faster, but that ageing itself does not contribute to the development of the disease. The ability of the immune system to respond to pathogens appears diminished in the elderly, and it is known that the elderly experience more pulmonary infections than the young. Perhaps these repeated insults alongside with damage caused by smoking make COPD more apparent in the elderly.

All organs see a decline in function with advancing age, and natural ageing (as opposed to a pathological decline in function) must satisfy four principles. It is intrinsic, universal, progressive and unfortunately detrimental to function (Strehler, Mark et al. 1959). Pulmonary physiology changes with normal ageing, characterised by a significant reduction in the elastic recoil of the lung, a loss of power of the muscles of respiration and increased chest wall rigidity (Turner, Mead et al. 1968; Knudson, Clark et al. 1977). These physiological changes lead to a reduction in the FEV₁/FVC ratio and a greater measured residual volume, changes that are also seen in COPD which could lead to an over-diagnosis of COPD in healthy elderly subjects. However, histologically, the changes seen with advancing age are distinguishable from COPD. With advancing

age, there is alveolar enlargement without the alveolar wall destruction and distal duct ectasia seen in emphysema (Verbeken, Cauberghs et al. 1992). These changes have been termed “Ageing Lung”.

Senescence accelerated mouse models (including senescent prone and senescent resistant mice strains) have been used to study age dependent changes in the lung. Firstly, these studies confirmed that normal ageing was associated with alveolar enlargement, but not emphysema. In these models, it seems clear that ageing *per se* does not cause COPD. Secondly, when both age-prone and age-resistant strains were exposed to cigarette smoke, accelerated ageing was associated with more emphysema for matched smoke exposure compared with age-resistant strains. Advancing age made the mice more sensitive to extrinsic insults (Fukuchi 2009), but these studies could not determine whether this was due to changes in the lung alone, or age related changes in other body systems.

In pulmonary emphysema, there is believed to be an imbalance between increased alveolar cell apoptosis and increased alveolar cell proliferation, which favours alveolar wall destruction (Calabrese, Giacometti et al. 2005; Imai, Mercer et al. 2005). *In vitro* and *in vivo* studies suggest that cigarette smoke induces cellular senescence, increasing the expression of markers of senescence such as β -galactosidase (Tsuji, Aoshiba et al. 2004). Human studies have found increased levels of the senescent markers p16^{INK4a} and p21^{CIP1/WAF1/Sd1} (antagonists of cyclin dependent kinase, which is required for cell cycle turnover) in type II alveolar cells in patients with emphysema compared with age matched controls (Tsuji, Aoshiba et al. 2006). Cellular senescence is associated with a decrease in cellular proliferation (Aoshiba. K. and Nagai 2009). If senescence causes an

imbalance in cellular degradation and proliferation, this could explain some of the changes seen in ageing lung. Furthermore, if the combination of natural ageing and smoke exposure increases cellular senescence, the usual compensatory rise in cellular proliferation that one would expect following alveolar cell damage would be diminished, leading to a greater degree of lung damage than one would normally expect.

Ageing is pro-inflammatory, and is believed to be the result of prolonged exposure to damaging insults with a high antigenic burden (Franceschi, Bonafe et al. 2000). In addition, studies of senescent cells suggest that they produce more inflammatory mediators both when quiescent and stimulated, including $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-8 (Shelton, Chang et al. 1999; Mariotti, Bernardini et al. 2006). Senescent human type II alveolar cells express more phosphorylated $\text{I}\kappa\text{B}$ and $\text{TNF}\alpha$ and there is a positive relationship between the degree of $\text{p16}^{\text{INK4a}}$ positive cell senescence and the severity of inflammation in patients with emphysema (Aoshiba. K. and Nagai 2009).

In view of all these data, we proposed that the pathogenesis of COPD may be an interplay between genetic susceptibility, environmental exposure and age. Potentially, smoking and ageing (in susceptible individuals) leads to alveolar and airway cell senescence, causing arrested tissue repair. Cellular senescence leads to chronic inflammation with increased inflammatory cell recruitment, and associated tissue damage. This may be heightened in COPD due to aberrant inflammatory cell responses, in particular, neutrophilic responses, enhancing inflammatory tissue damage. Furthermore, the deterioration of immune function with age might increase susceptibility to infection (and COPD exacerbations), with further inflammation and tissue damage.

Finally, studies of neutrophil migration in COPD have not always applied rigorous age matching when comparing patients with controls (for example, (Strassburg, Droemann et al. 2004; Yoshikawa, Dent et al. 2007)). Without an appreciation of the affects of age on neutrophil migration, it is difficult to interpret these studies as one cannot differentiate whether reported differences are due to disease, age or both.

1.7 Alpha 1-Antitrypsin Deficiency

Alpha-1 antitrypsin deficiency (A1ATD) is a hereditary condition, where the inheritance of two deficiency alleles on the α 1AT gene (located on chromosomal segment 14q 32.1 (Darlington, Astrin et al. 1982)) result in a severe deficiency of A1AT. There are more than 100 phenotypes of the A1AT protein, many of which are caused by a single amino acid substitution. The variants are described alphabetically and have differing electrophoretic properties that are detectable by isoelectric focusing (Hoffman and van der Brock 1977). The most common phenotype is “M”, of which there are at least four subtypes, although none are thought to lead to ill health. The “Z” variant is the most common of the deficiency phenotypes, with an estimated gene frequency in Europe of 2 - 24 per 1000 (Hutchinson 1998). The homozygous form of the “Z” phenotype has an estimated prevalence of 0.02 – 0.06% in Europe (Hutchinson 1998) and this variant results in very low serum concentrates of α 1AT (typically less than 11 μ M). The mutation affects the tertiary structure of α 1AT, resulting in the formation of chains or polymers. These polymers become entangled in the endoplasmic reticulum of hepatocytes, effectively preventing secretion (Lomas, Evans et al. 1992).

Severe deficiency of A1AT is associated with early onset panacinar emphysema, which can occur in non-smokers (Piitulainen, Tornling et al. 1997), although morbidity and

mortality are increased in patients who smoke (Larsson 1978). A1ATD is also associated with cirrhosis of the liver, hepatocellular carcinoma and the vasculitides (Eriksson, Carlson et al. 1986; Fortin, Fraser et al. 1991).

The pulmonary disease is believed to develop due to an imbalance in the levels of neutrophil elastase present in the lung, compared with the low concentration of A1AT. This imbalance leads to an inability to neutralise elastase during neutrophil migration and degranulation, resulting in a wider area of tissue destruction. The presence of elastase enhances the release of other pro-inflammatory mediators (for example; elastase stimulates the secretion of LTB₄ from macrophages), which further enhances neutrophil migration, with further release of neutrophil elastase that is poorly inactivated due to the deficiency of A1AT, leading to a perpetual amplification of inflammation (Hubbard, Fells et al. 1991). This cycle of inflammation is supported by studies demonstrating increased numbers of neutrophils and increased concentrations of inflammatory mediators in samples taken from patients with A1ATD compared to patients with COPD without the genetic deficiency in A1AT (Morrison, Kramps et al. 1987; Hill, Bayley et al. 2000).

There are few studies of neutrophil migration in A1ATD, but those carried out to date suggest that neutrophil migration towards sputum in A1ATD may be enhanced, but this is likely to be due to increased concentrations of pro-inflammatory mediators (in particular LTB₄ and IL8) (Woolhouse, Bayley et al. 2002) rather than aberrant neutrophil behaviour. However, there have been no detailed studies of the mechanics of neutrophil migration in this disease, and further studies are awaited. Furthermore, patients with A1ATD form a sound control group when studying neutrophil migration

in COPD, as these patients can be matched for the presence of lung disease, pulmonary inflammation, smoking exposure and treatments, all of which may alter immune cell function in disease.

1.8 Summary

COPD is an important and complex, chronic disease. The neutrophil, and its proteolytic enzymes, have been shown to cause all of the pathological features of COPD, and neutrophil numbers (and their associated products) relate to disease severity and disease progression. However, it remains unclear if these cells are primarily and directly responsible for the development of COPD, or whether other cell types and disease processes are also involved. The inefficacy of inflammatory mediator inhibitors (such as anti-TNF α) suggest that the disease is not caused by a single mediator, but little is known of the day to day concentrations of these mediators, making it difficult to predict the outcome of therapeutic interventions. Although a wealth of data supports the neutrophil as being the most likely effector cell in COPD, little is understood of its migratory behaviour in the disease, which may explain the preponderance of cells in the lungs. These may well prove to be important issues in identifying points of intervention in order to treat the disease.

It is known that ageing affects neutrophil function and that advancing age is associated with COPD. While some studies have examined the effects of senescence on lung tissue in relation to COPD, none have focused on the neutrophil. Given the likely importance of this cell in the pathogenesis of COPD, it is of importance to understand

how ageing effects the neutrophil, and how any functional differences compare with those noted in COPD.

Patients with A1ATD have a similar spectrum of disease as usual COPD and have similar patterns of lung inflammation (although this can be heightened). Current treatments for A1ATD-related symptoms are identical to those used in usual COPD, and they have a similar burden of bacterial colonisation. In light of this, it was felt that these patients were an ideal secondary control group for cellular studies of usual COPD, as this would help determine if any differences noted were specific to COPD or common to lung disease, pulmonary and systemic inflammation and their treatments.

1.9 Structure of this thesis

The studies presented in this thesis were performed to examine some of the aspects of neutrophil recruitment and migration in healthy aging and COPD. In particular, we wished to answer three questions.

1. What are the key inflammatory mediators that are involved in neutrophil recruitment in COPD and are likely to be important in neutrophil migration, and how variable is their expression in pulmonary secretions?

In order to study this, Chapter 3 examines the expression pro-inflammatory mediators in pulmonary secretions from patients with COPD, and explores relationships between mediators and neutrophils and markers of disease severity, to ascertain which mediators were of likely importance in studies of neutrophil migration.

2. Does ageing affect neutrophil migration towards the chemoattractants that are believed to be physiologically important in COPD?

In order to study this Chapter 4 examines, *in vitro*, neutrophil migration towards IL-8 and GRO α using both the well established under agarose assay and a newly validated assay using a Modified Dunn Chamber. Surface expression of receptors is semi-quantified and migratory antagonists are used in order to identify potential mechanisms. The work presents evidence of aberrant neutrophil migration in the healthy elderly and proposes that directed neutrophil movement (chemotaxis) is selectively diminished with increasing age, with no alterations in random cellular movement (chemokinesis). This work highlights the importance of age matching in studies of neutrophil function.

3. Are there functional and structural differences in migratory neutrophils isolated from patients with COPD compared with age matched healthy controls and disease matched patients with A1ATD?

In order to study this, Chapters 5 and 6 include work that examines quantitative differences in neutrophil migratory pathways and neutrophil structure during migration towards IL-8 and GRO α . Chapter 7 and 8 examine potential mechanisms for the differences seen, including receptor expression and function, and down stream signalling in the migratory pathway. The work presents evidence of aberrant neutrophil migration in patients with COPD and proposes that this is distinct from that seen in healthy ageing. Namely, there is an increase in neutrophil chemokinesis in usual COPD, coupled with a reduction in chemotaxis. These differences appear unique to this group, and cannot be explained by age, cigarette smoke exposure (past or

present), lung inflammation nor its treatments. Furthermore, these differences can be corrected using antagonists of the migratory pathway.

2 METHODS

2.1 Ethical Approval

All studies reported in this thesis were approved by the South Birmingham Health Authority ethics committee and all subjects provided written informed consent.

2.2 Patients

The characteristics of the individual patient groups are described in the relevant section of the thesis. COPD was defined clinically using accepted definitions (GOLD criteria (Pauwels, Buist et al. 2001), patients with α 1ATD were homozygotes for the Z variant. Patients with chronic bronchitis were defined by daily sputum production for at least 3 months of 2 consecutive years (Medical Research Council. 1965).

2.3 Pulmonary Function Testing

Pulmonary function tests were performed according to national guidelines (British Thoracic Society and the Association of Respiratory Technicians and Physiologists, 1994). All pulmonary function tests were performed by trained Respiratory Physiologists at the Lung Investigation Unit, University Hospital Birmingham NHS Foundation Trust.

2.4 Sputum and Blood Sample Collection

Spontaneously expectorated sputum samples were collected from waking for four hours, into a sterile container. Patients undertook mouth washing procedures to minimise saliva contamination. Blood samples were taken peripherally using the vacutainer® system of blood collection (Becton Dickson Ltd, Oxford, UK)

2.5 Sputum Processing

Sputum samples were processed within 2 hours of collection completion.

2.5.1 Sputum Colour

Each sputum sample was allocated a number referenced to a standard colour chart (Stockley, Bayley et al. 2001) according to its macroscopic appearance. Values of 1 -2 (colourless to white) were classified as mucoid and values of 3-5 (yellow to green) were classified as mucopurulent to purulent.

2.5.2 Quantitative bacterial culture

Where appropriate an aliquot of sputum (with a minimum weight of 1g) was removed from the sputum sample prior to any processing, and the presence and number of viable organisms was determined (as described previously (Pye, Stockley et al. 1995). Results are expressed in terms of the colony forming units per ml of sputum (cfu/ml).

2.5.3 Sputum sol phase collection

The major aliquot of sputum (with a minimum weight of 1g) was ultracentrifuged at 50,000g for 90 minutes at 4°C and the supernatant stored at -70°C until required.

2.5.4 Cytospin preparation

A final aliquot of sputum was taken and used for cytospin preparation using a modified protocol from that previously described (Pavord, Pizzichini et al. 1997). In brief, freshly prepared dithiothreitol (DTT, Sputolysin; Calbiochem corp, CA, USA) was added to the sputum at a ratio of 4:1 (volume/weight). The mixture was briefly vortexed and then rocked for 15 minutes at room temperature. A further 4 volumes of 0.9% sodium chloride (NaCl) was added to the aliquot which was then rocked for 5 minutes. The sample was filtered (using a 48µm nylon gauze) and the filtrate was centrifuged at 400g for 10 minutes at 4°C. The resultant cell pellet was resuspended in 1ml phosphate buffered saline (PBS, Gibco Invitrogen Corp, Paisley, UK). Total cell counts and viability assessments were performed following the addition of 20µl trypan blue (Sigma Chemicals Ltd, Poole, UK) to an equal volume of the suspension. Ten microlitres of the

mixture was pipetted onto a haemocytometer for cell counting using low power light microscopy and cells that excluded trypan blue were classified as viable. Total cell count per ml of sputum was calculated using the following formula:

Total cell count = total number of non-squamous cells x trypan blue dilution factor x 10^4 .

The cell suspension was diluted to give a concentration of 200,000 cells/ml and 100 μ l aliquots were added to pre-prepared cytopsin blocks. The blocks were centrifuged at 450rpm for 3 minutes in a Shandon cytopsin centrifuge (Thermo Shandon Inc, PA, USA) resulting in a thin film of approximately 20,000 cells per slide. Two slides were stained using Diff Quick® (Gamidor Ltd, Oxford, UK) and 300 cells were counted to produce a differential cell count. The cell sample was considered adequate if the slides contained greater than 50% neutrophils and less than 10% squamous cells.

2.5.5 Peripheral blood processing

Blood samples were processed with 30 minutes of collection.

2.5.6 Plasma sample

A plasma sample was obtained by centrifuging the blood at 3000rpm for 10 minutes at 4°C and stored at -70°C until analysed.

2.5.7 Isolation of Neutrophils

Neutrophils were purified from peripheral blood by discontinuous percoll density gradients, using the method of Jepsen and Skottun (1982).

An isotonic stock solution of percoll (Sigma-Aldrich Company Ltd., Poole, UK), was prepared in a ratio of 9:1 (v/v) with 1.5 M sterile saline solution (NaCl). The stock percoll was further diluted with sterile physiological saline (0.15 M NaCl) to 78% (density 1.096 g/ml) and 54% (1.075 g/ml). The discontinuous gradients were prepared by careful underlaying of 2 ml 54% percoll with 3 ml 78% percoll in 10 sterile 15 ml test tubes.

Peripheral venous blood samples were collected into lithium heparin Vacutainer® tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, USA), and then diluted with an equal volume of sterile saline (0.15 M NaCl) and 7 ml was layered onto the gradients. The tubes were then centrifuged at 200 g for 25 minutes in a Heraeus Christ Bactifuge at 20°C. After centrifuging distinct bands were present with mononuclear cells at the interface of the plasma and the 54% percoll. The band at the interface of the 54% and 78% Percoll contained neutrophils and the basal pellet consisted of erythrocytes. The neutrophil layer (extending into the 78% percoll) was removed using a sterile pastette and placed in a sterile universal tube. The cells were then washed twice with sterile 0.15 M NaCl, centrifuging at 1500 g. The cells were counted using a haemocytometer with improved Neubaur markings and then resuspended in Roswell Park Memorial Institute 1640 medium (RPMI, Sigma Chemicals Ltd, Poole, UK) at the correct concentration for the assays.

Lithium heparin blood tubes were utilised for blood collection. Each tube contained 90 Unit Standard Potency (USP) lithium heparin per 6mL blood. This is the equivalent of 15 USP of heparin per ml blood, where 1 USP = 1 U_{mg}⁻¹/ml, with the heparin having been sprayed on to the internal walls of the tube. Tubes were medical grade and sterile (information from manufacturer). All methods of isolating neutrophils are associated

with a risk of activation with the potential for alteration in receptor expression and function.

The majority of studies investigating the affects of heparin have treated endothelial cells with heparin, and then observed neutrophil adhesion. There is clear evidence that unfractionated heparin and fractionated low molecular weight heparins (at concentrations of $20 - 1000 \text{ U ml}^{-1}$ (with $1 \text{ U} = 125 - 180 \text{ U mg}^{-1}/\text{ml}$)) have been shown to reduce neutrophil adhesion to Human Umbilical Vein Endothelial Cells (HUVEC), without significantly altering expression of ICAM-1 or E-Selectin (Lever, Hoult et al, 2000; Xie et al, 1997; Silvestro et al, 1994). Neutrophils are also capable of binding to heparin (as described by Diamond, et al (1995), potentially through integrins receptors. However the majority of these studies have used concentrations of heparin that are much higher than those seen in standard blood tubes.

A recent study attempted to address this by comparing techniques for neutrophil isolation (including the use of heparin, EDTA and citrate) and found that heparin and citrate increased PMA-induced calcium signalling and respiratory burst more than EDTA (Freitas, Portp et al, 2008), however, once again, higher concentrations of heparin were used than are present in standard lithium heparin tubes.

Despite the lack of data regarding the affect of low dose heparin on neutrophil function, it is likely that even 90 USP of heparin may alter neutrophil responses. However, this body of work aimed to identify differential effects of neutrophils isolated from young and old healthy subjects, and patients with COPD, and it was reasoned that as long as neutrophil isolation was standardized, the results would be comparable.

2.6 Biochemical Assays

2.6.1 Growth Related Oncogene

Growth related oncogene alpha (GRO α) was measured by ELISA using a commercially available kit (R&D Systems Ltd, Abingdon, UK). Two hundred microlitres of standard or sample (diluted by diluent RD5K) were added to each well. The wells were covered with an adhesive strip and incubated at room temperature for one and a half hours. Wash buffer was prepared by diluting 20ml of wash buffer concentrate (R&D Systems) with 480ml of distilled water and each well was washed three times. Two hundred microlitres of GRO α conjugate were then added to each well and the plate was incubated for a further one hour at 2 - 8° C after which each well was washed a further three times with wash buffer. Two hundred microlitres of substrate solution (equal volumes of colour reagents A and B) were then added to each well and the plate incubated for 15 minutes at room temperature, protected from the light. The reaction was stopped using 50 μ l of stop solution and the absorbance read at 450nm, with 540 nm correction, using a Dynatech MR 5000 microplate reader (Dynatech Corporation, Burlington, VT, USA). The GRO α concentration was calculated by interpolation from the standard curve.

For this, and subsequent assays, single samples from separate sputum sol phase pools collected from patients with chronic bronchitis were assayed on 4 to 6 occasions to obtain the intra-assay co-efficient of variation. A known quantity of pure mediator was then added to the sample and it was re-assayed. The result of this “spiked” sample was obtained by interpolation from the standard curve and compared to the expected value to obtain the proportion recovered. The lower limit of detection, intra-assay co-efficient of variation and recovery of pure GRO α from samples spiked with a known

concentration are shown in Table 2.1. The standard curve for GRO α is shown in Figure 2.1.

Table 2.1. The lower limit of detection, intra-assay coefficient of variation (CV) and spike recovery for the GRO α assay.

| Lower limit of detection | Intra-assay CV (%) | Spike recovery (%) |
|--------------------------|--------------------|--------------------|
| 10 pg/ml | 3.75 | 88.9 |

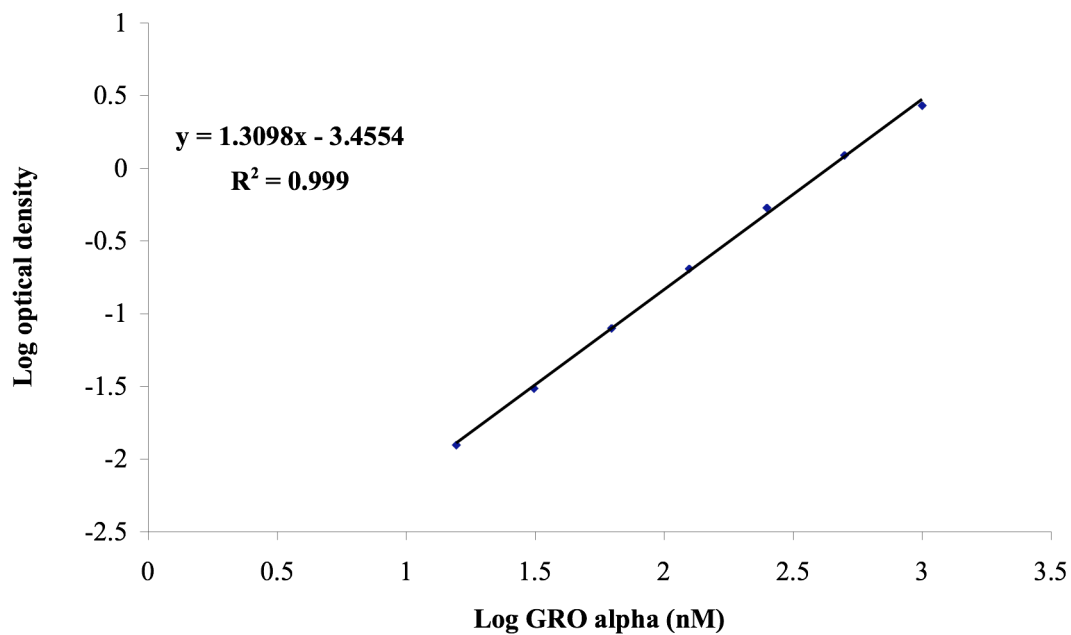


Figure 2.1 Standard curve for GRO α

2.6.2 Interleukin-1 β

Interleukin -1 β (IL-1 β) was measured in sputum sol phase by ELISA using a commercially available kit (R&D Systems Ltd, Abingdon, UK). Two hundred microlitres of standard or sample (dissolved in calibrator diluent RD5) were added to each well. The wells were covered in an adhesive strip and allowed to incubate for 2 hours at room temperature. Five hundred ml of wash buffer were produced by dissolving 20ml of wash buffer concentrate with 480ml distilled water. Each well was

then washed 3 times with wash buffer. Two hundred microlitres of IL-1 β conjugate were then added to each well and the plate was incubated for a further 1 hour at room temperature and then each well was washed 3 times with wash buffer. Two hundred microlitres of substrate solution (equal volumes of colour reagents A and B) were then added to each well and the plate incubated for 20 minutes at room temperature, protected from the light. The reaction was stopped using 50 μ l of stop solution and the absorbance read at 450nm with a 540nm correction, using a Dynatech MR 5000 microplate reader. The IL-1 β concentration in the sample was calculated by interpolation from the standard curve.

The lower limit of detection, intra-assay coefficient of variation and recovery of pure IL-1 β from samples spiked with a known amount are shown in Table 2.2. The standard curve is shown in Figure 2.2.

Table 2.2. The lower limit of detection, intra-assay coefficient of variation (CV) and spike recovery for the IL-1 β assay.

| Lower limit of detection | Intra-assay CV (%) | Spike recovery (%) |
|--------------------------|--------------------|--------------------|
| 0.207 pM | 3.9 | 95.5 |

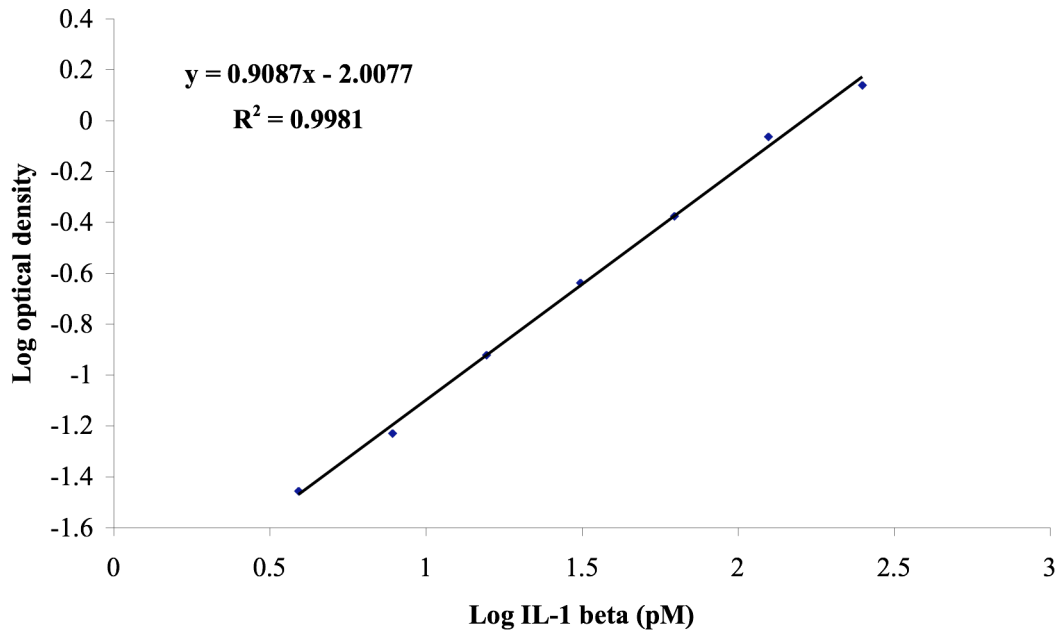


Figure 2.2. Standard curve for IL-1 β in sputum sol

2.6.3 High sensitivity Interleukin-1 β

Interleukin -1 β (IL-1 β) was measured in plasma by ELISA using a commercially available high sensitivity kit (R&D Systems Ltd, Abingdon, UK). One hundred microlitres of diluent RD1-82 were added to each well followed by 150 μ l of standard or sample (diluted by diluent RD6-44). The wells were covered in an adhesive strip and allowed to incubate for 3 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm. One litre of wash buffer was produced by dissolving 100ml of wash buffer concentrate with 900ml distilled water and each well was washed 6 times with wash buffer (200 μ l per well). Two hundred microlitres of IL-1 β HS conjugate were added to each well and the plate was incubated for a further 2 hours at room temperature on the shaker. Each well was then washed a further 6 times with wash buffer. Fifty microlitres of substrate solution were then added to each well and the plate was incubated for one hour at room temperature on the shaker. Fifty μ l of amplifier solution were then added to each well and allowed to incubate for 30 minutes at room

temperature on the shaker. The reaction was stopped using 50µl of stop solution and the absorbance read at 490nm, with a 650nm correction, using a Dynatech MR 5000 microplate reader. The IL-1β concentration in the samples was calculated by interpolation from the standard curve.

The lower limit of detection, intra-assay coefficient of variation and recovery of pure IL-1β from samples spiked with a known amount are shown in Table 3.3. The standard curve is shown in Figure 2.3.

Table 2.3. The lower limit of detection, intra-assay coefficient of variation (CV) and spike recovery for the IL-1β assay.

| Lower limit of detection | Intra-assay CV (%) | Spike recovery (%) |
|--------------------------|--------------------|--------------------|
| 0.057 pg/ml | 7.9 | 87.9 |

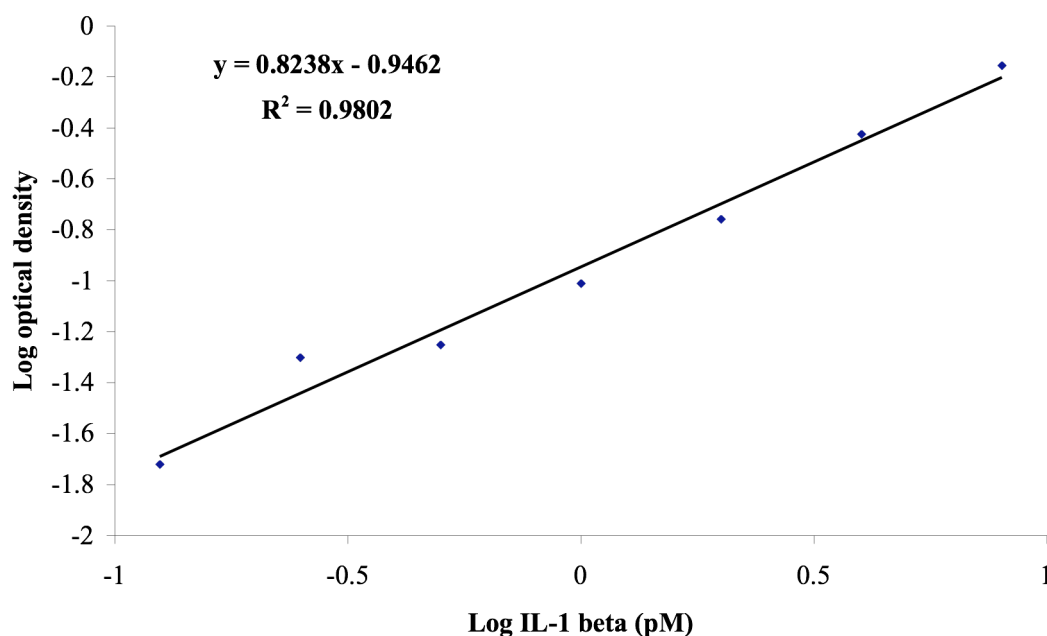


Figure 2.3. Standard curve for High sensitivity IL-1β in plasma

2.6.4 Interleukin-8

Interleukin -8 (IL-8) was measured in sputum sol by ELISA using a commercially available kit (R&D Systems Ltd, Abingdon, UK). One hundred microlitres of diluent RD1-8 were added to each well followed immediately by 50µl of standard or sample (dissolved in calibrator diluent RD5-P) and 100µl of IL-8 conjugate. The wells were covered by an adhesive strip and allowed to incubate for 2.5 hours at room temperature. Wash buffer (500ml) was produced by dissolving 20ml of wash buffer concentrate with 480ml of distilled water and each well was washed 6 times (200µl per well per wash). Two hundred microlitres of substrate solution (equal volumes of colour reagent A and B) were then added to each well and the plate was incubated for 30 minutes at room temperature. The reaction was stopped using 50µl of stop solution and the absorbance read at 450nm, with a 540nm correction, using a Dynatech MR 5000 microplate reader. The IL-8 concentration in the samples was calculated by interpolation from the standard curve.

The lower limit of detection, intra-assay coefficient of variation and recovery of pure IL-8 from samples spiked with a known amount are shown in Table 2.4. The standard curve is shown in Figure 2.4. Plasma IL-8 concentrations were measured by AstraZeneca using a kit this company have developed and validated. Results are included with their permission.

Table 2.4. The lower limit of detection, intra-assay coefficient of variation (CV) and spike recovery for the IL-8 assay.

| Lower limit of detection | Intra-assay CV (%) | Spike recovery (%) |
|--------------------------|--------------------|--------------------|
| 0.0125 nM | 6.18 | 98.9 |

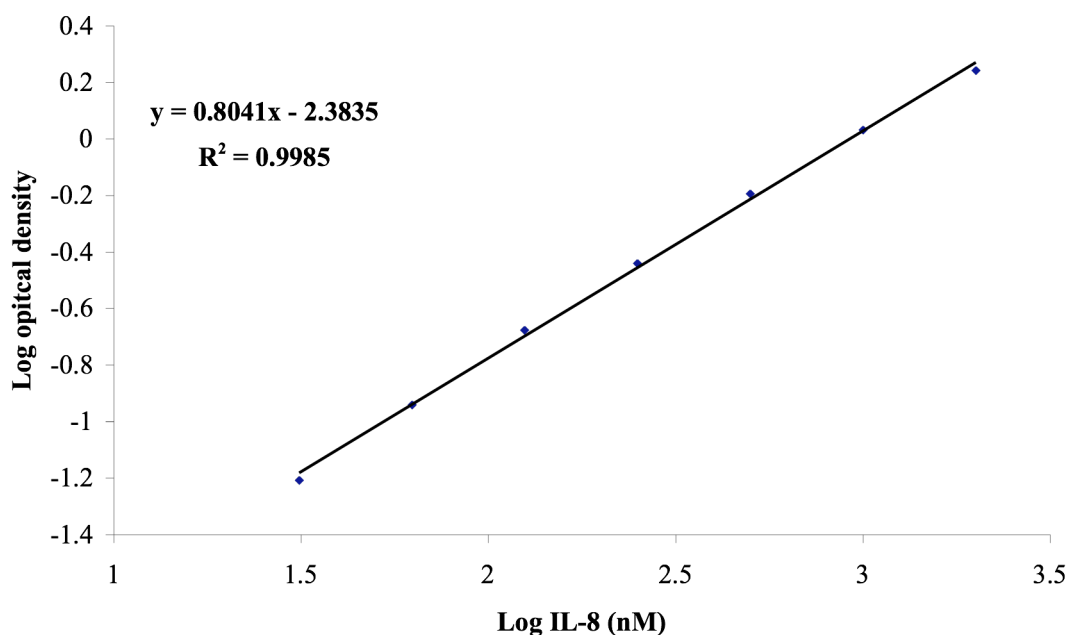


Figure 2.4. The standard curve for IL-8 in sputum sol

2.6.5 Leukotriene-B4

Leukotriene-B4 (LTB4) was measured by ELISA using a commercially available kit (Amersham International plc, Buckinghamshire, UK). One hundred microlitres of assay buffer were added to the non-specific binding wells, followed immediately by 50 µl of standard or sample (dissolved in assay buffer). Fifty microlitres of LTB4 antiserum were then added to all wells except the non-specific binding wells and the plate was covered with an adhesive strip. The plate was incubated for two hours at room temperature whilst shaking at 150 rpm on a rotary shaker (Fisher Scientific, Loughborough, UK). Fifty microlitres of LTB4 peroxidase conjugate were then added to each well and the plate covered with an adhesive strip. The plate was then incubated for a further 1 hour at room temperature whilst shaking at 150 rpm on the rotary shaker. Twenty millilitres of wash buffer concentrate were dissolved in 480 ml of distilled water to produce 500 ml of wash buffer. Each well was then washed 4 times with wash buffer and 150µl of enzyme substrate were then added to each well and the plate was covered

with an adhesive strip before a final incubation period of 30 minutes at room temperature, whilst shaking at 150 rpm on a rotary shaker. The reaction was stopped using 100 µl of 1M sulphuric acid and the absorbance read at 450 nm using a Dynatech MR 5000 microplate reader. The LTB4 concentrate in the samples was calculated by interpolation from the standard curve.

The lower limit of detection, intra-assay coefficient of variation and recovery of pure LTB4 from samples spiked with a known amount are shown in Table 2.5. The standard curve is shown in Figure 2.5.

Table 2.5. The lower limit of detection, intra-assay coefficient of variation (CV) and spike recovery for the LTB4 assay.

| Lower limit of detection | Intra-assay CV (%) | Spike recovery (%) |
|--------------------------|--------------------|--------------------|
| 137 pM | 9.67 | 105.7 |

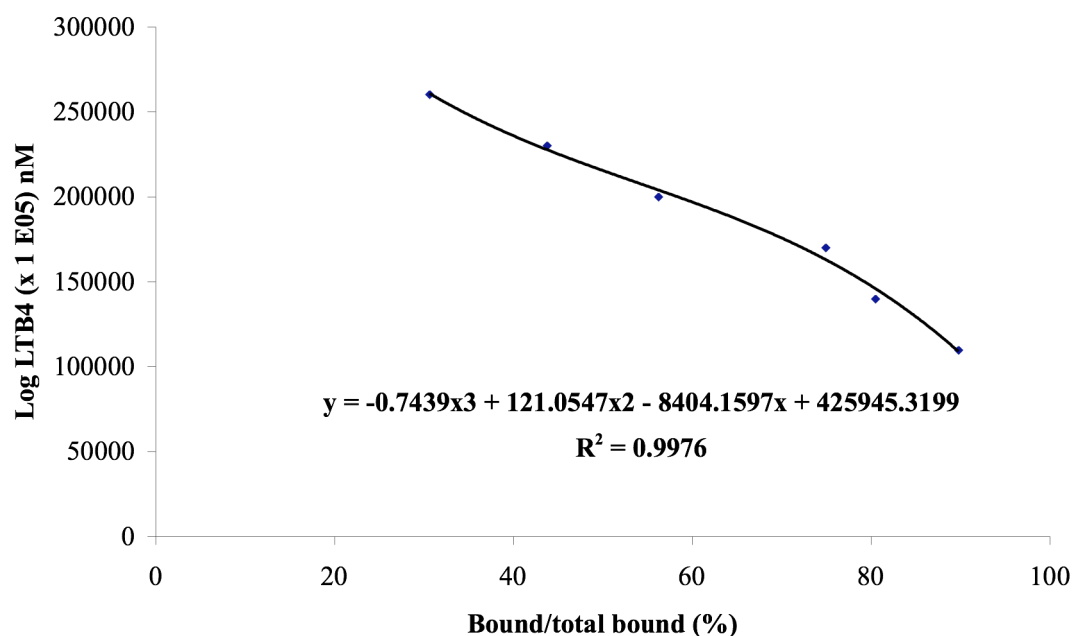


Figure 2.5. The standard curve for LTB4

2.6.6 Tumour Necrosis Factor Alpha

Tumour necrosis factor alpha (TNF α) was measured by ELISA in sputum sol phase and plasma, using a commercially available high sensitivity kit (R&D Systems Ltd, Abingdon, UK). Fifty microlitres of assay diluent RD1F were added to each well followed immediately by 200 μ l of standard or sample (dissolved in calibrator diluent RD6 – 13). The wells were covered in an adhesive strip and the plate was incubated for 3 hours at room temperature. Wash buffer concentrate (100ml) was added to 900 ml of distilled water to make 1 litre of wash buffer and each well was washed 6 times (200 μ l per well per wash). Two hundred millilitres of TNF α HS conjugate were added to each well and the plate incubated for a further 2 hours at room temperature. Each well was then washed a further 6 times with wash buffer and 50 μ l of substrate solution (lyophilised substrate mixed with 6 ml of substrate solution) were added to each well. The plate was then covered with an adhesive strip and incubated for one hour, after which 50 μ l of amplifier solution (lyophilised amplifier dissolved in 6 ml of amplifier substrate) were added to each. The plate was again covered in an adhesive strip and incubated for a further 30 minutes at room temperature. The reaction was then stopped using 50 μ l of stop solution and the absorbance read at 490 nm with a 650 nm correction, using a Dynatech MR 5000 microplate reader. The TNF α concentration in the samples were calculated by interpolation from the standard curve.

The lower limit of detection, intra-assay coefficient of variation and the recovery of pure TNF α from samples spiked with a known amount are shown in Table 2.6 for sputum and 2.7 for plasma. The standard curve for sputum is shown in Figure 2.6 and for plasma is shown in Figure 2.7.

Table 2.6. The lower limit of detection, intra-assay coefficient of variation (CV) and spike recovery for the TNF α assay in sputum sol phase.

| Lower limit of detection | Intra-assay CV (%) | Spike recovery (%) |
|--------------------------|--------------------|--------------------|
| 0.052pM | 6.12 | 111.1 |

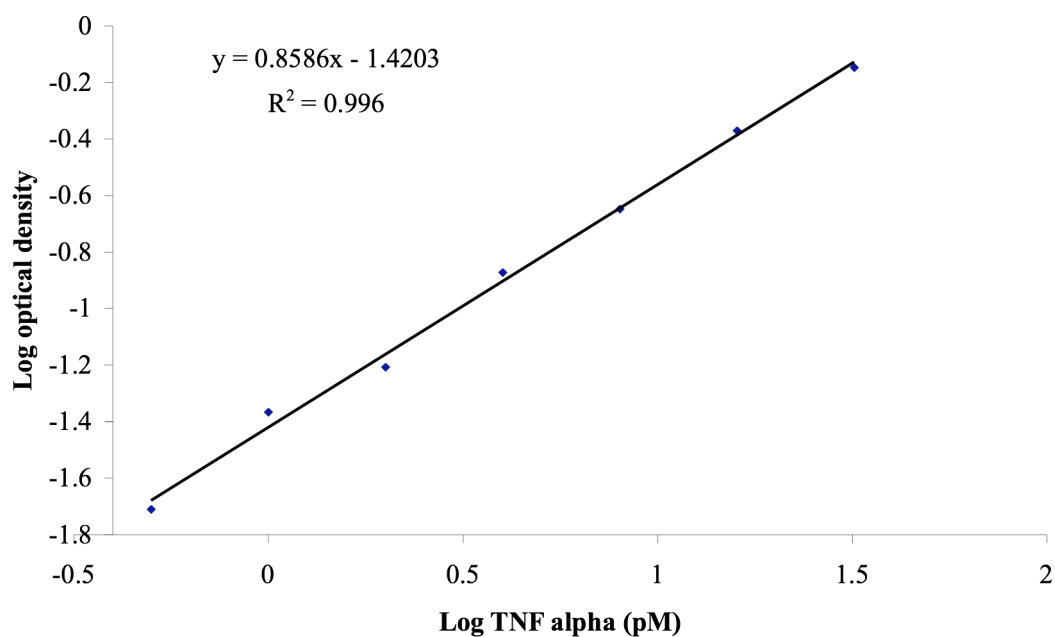


Figure 2.6. Standard curve for TNF α in sputum sol phase

Table 2.7. The lower limit of detection, intra-assay coefficient of variation (CV) and spike recovery for the TNF α assay in plasma.

| Lower limit of detection | Intra-assay CV (%) | Spike recovery (%) |
|--------------------------|--------------------|--------------------|
| 0.052pM | 3.92 | 100.15 |

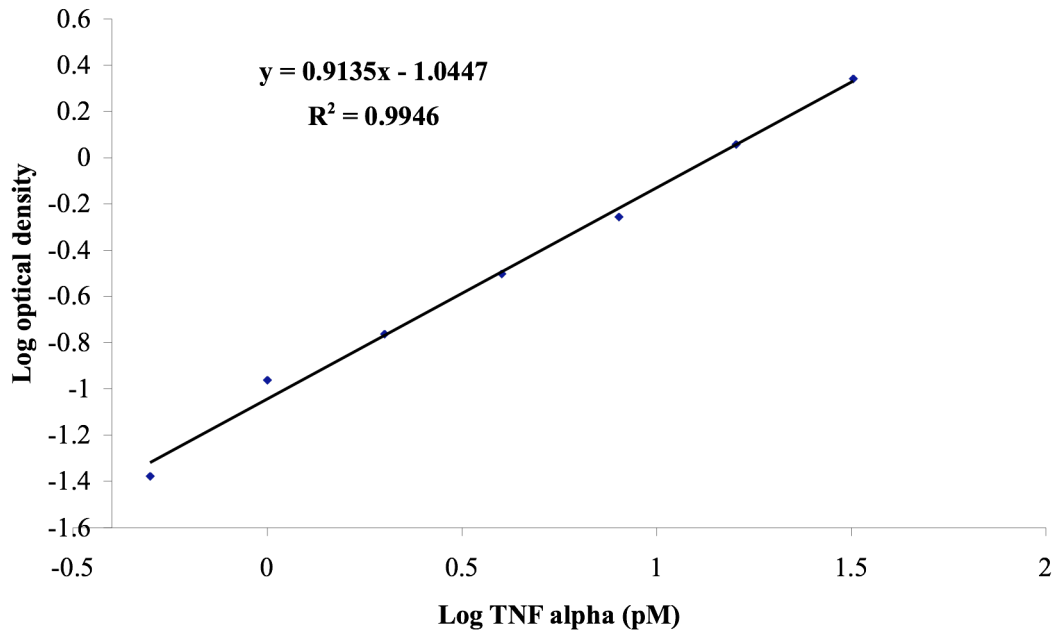


Figure 2.7. The standard curve for TNF α in plasma

2.6.7 Myeloperoxidase

Sputum myeloperoxidase measurements were used as a marker of neutrophil influx and activation. MPO activity was measured by chromogenic substrate assay relative to a preparation of lysed neutrophils of known concentration. Ten microlitres of standard or sample were added to the wells in a microtitre plate, followed by 150 μ l of 1 mg/ml (w/v) O-dianisidine dihydrochloride (Sigma chemicals Ltd., Poole, UK), 0.01% (v/v) 30% H₂O₂ in 50nM K₃HPO₄ and 0.5% w/v hexadecyl trimethyl ammonium bromide (pH 6) (Sigma Chemicals Ltd., Poole, UK). The plate was then incubated for 15 minutes at 25°C and the absorbance measured at 450nm using a Dynatech MR 5000 microplate reader. The MPO concentration in the samples was calculated by interpolation from the standard curve and expressed as mg/l.

The lower limit of detection, intra-assay coefficient of variation and the recovery of pure MPO from samples spiked with a known amount are shown in Table 2.8. The standard curve for sputum is shown in Figure 2.8.

Table 2.8. The lower limit of detection, intra-assay coefficient of variation (CV) and spike recovery for the MPO assay in sputum sol phase.

| Lower limit of detection | Intra-assay CV (%) | Spike recovery (%) |
|--------------------------|--------------------|--------------------|
| 2.93 mg/l | 4.59 | 94.89 |

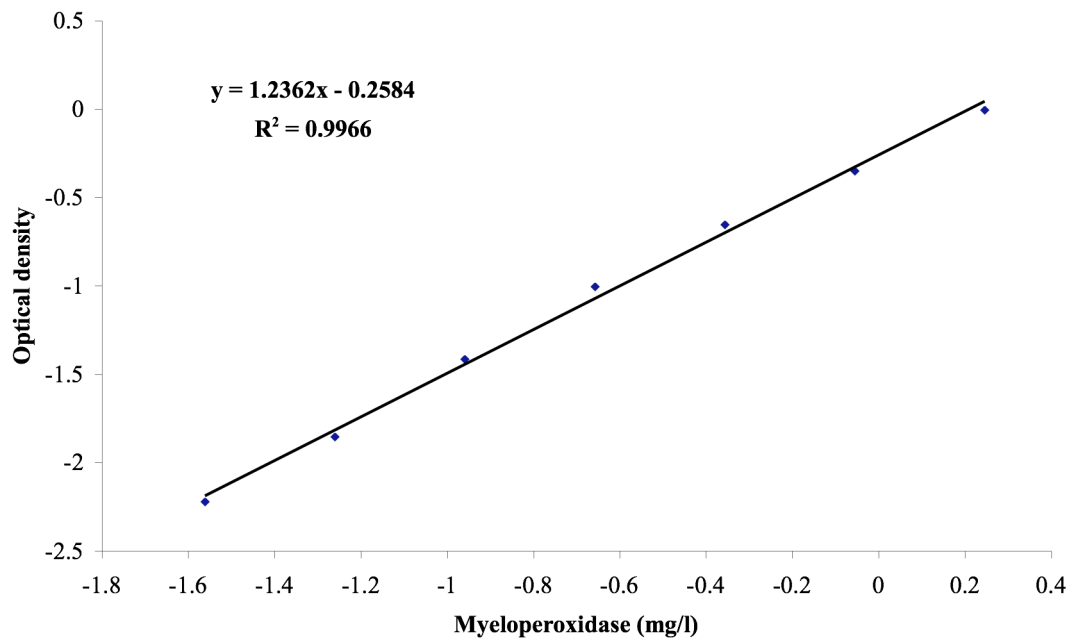


Figure 2.8. The standard curve for MPO

2.7 Chemotaxis Assays

Neutrophil chemotaxis was assessed using two methods. The first employed modifications of the agarose chemotaxis assays of Cutler (1974), Nelson *et al.* (1975) and John and Sieber (1976) and the second employed a modified Dunn Chamber and time lapse photography (Andrew and Insall 2007).

2.7.1 Agarose Gel Chemotaxis Assay

Agarose chemotaxis plates were prepared as follows: agarose (0.5g, Sigma Aldrich, Poole, UK) were boiled at 100°C in 25ml distilled water and allowed to cool to 56°C. RPMI (Sigma Aldrich) (0.82g) and 0.25g gelatin (Sigma Aldrich) were dissolved in 25ml distilled water at 56°C in a water bath and the two solutions were mixed and kept at 56°C until required. The mixture (6ml) was pipetted into a 50 x 15mm tissue culture dish (Griener UK, Gloucester, UK). The plates were left to set at room temperature for 20 minutes and placed at 4°C for 10 minutes. Wells were cut into the agarose gel with a 3.2mm diameter punch using the template illustrated in figure 2.9

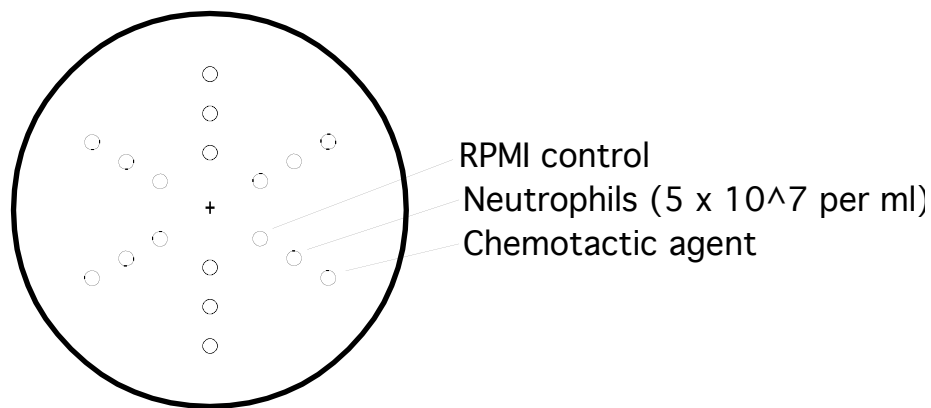


Figure 2.9. Template for under agarose chemotaxis assay

Freshly harvested neutrophils were resuspended at 5×10^7 cells/ml in RPMI. Ten μ l of RPMI were placed into each of the inner wells, 10 μ l of cells were placed into each of the centre wells and 10 μ l of chemoattractant (10^{-7} FMLP in RPMI) into each of the outer wells. Blank wells had RPMI in the inner and outer wells. The plates were incubated at 37°C (5% CO₂) for 2 hours and then flooded with methanol for overnight fixation of the cells. The gel was carefully removed and the plates washed under slow running tap water. The plates were then stained with Diff Quick® and left to dry. For

each well the chemotactic and chemokinetic responses were read using an eye-piece graticule. The overall response to the chemoattractant (chemotactic differential) was calculated by subtracting the chemokinetic from the chemotactic response (see Figure 2.10).

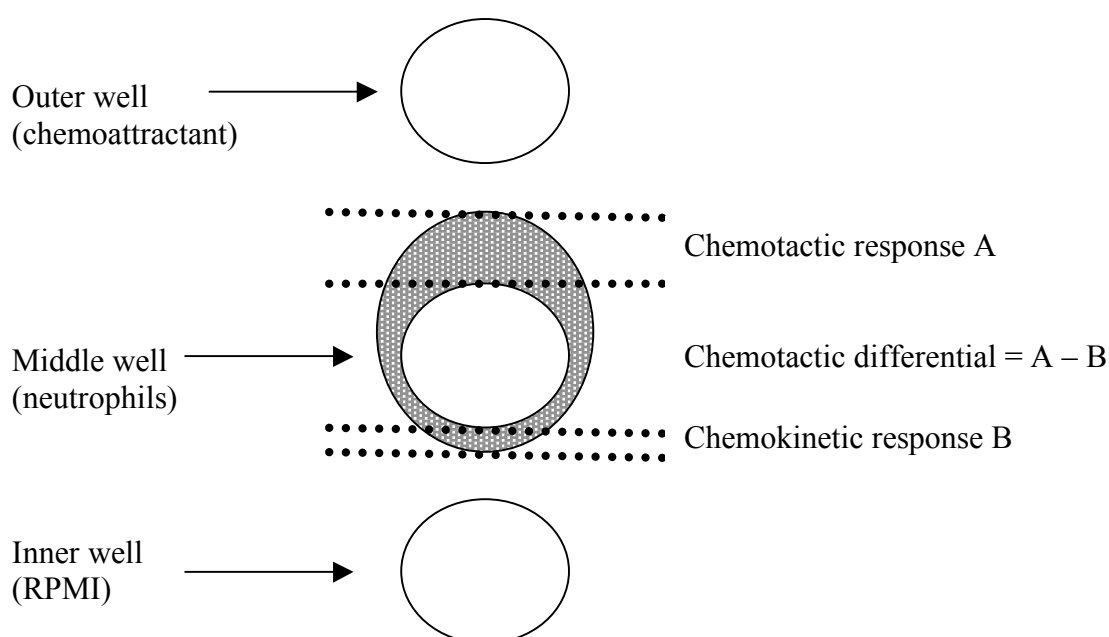


Figure 2.10. Calculation of the chemotactic differential (chemotactic response A – chemokinetic response B)

2.7.2 Modified Dunn Chamber Chemotaxis Assay

2.7.2.1 Video microscopy

All DIC and phase time-lapse recordings were made using wither a Zeiss Axiovert 100 or S100 inverted microscope fitted with a Mono-10bit or a *FAST* Mono-12bit QICAM digital camera respectively. Images were captured using Improvision OpenLab software, at 30 second intervals for 20 minutes.

2.7.2.2 Modified Dunn Chamber Assay

The chemotaxis assay was performed using a modified Dunn Chamber (Weber Scientific International Ltd, Teddington) with an adaptation of methods described

previously (Zicha, Dunn et al. 1991; Andrew and Insall 2007). Dunn chamber coverslips (Weber Scientific International Ltd, Teddington) were prepared by acid etching and baking before being coated with 7.5% culture-tested bovine serum albumin (Sigma-Aldrich, UK). Neutrophils (suspended at 5×10^6 /ml) were allowed to adhere to this surface for 20 minutes at room temperature. The coverslip was then inverted on to the modified Dunn chamber, with the channel pre-filled with buffer (RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK) containing 0.15% bovine serum albumin (Sigma-Aldrich, UK)). The buffer was replaced with either the same control buffer or containing IL-8 (Sigma-Aldrich, UK). The gradient was allowed to develop for 10 minutes at room temperature before filming. Analysis was performed on 10 randomly assigned cells from each experiment. Figure 2.11 is a diagrammatic representation of the modified Dunn chamber. Figure 2.12 is a diagrammatic representation of the assay.

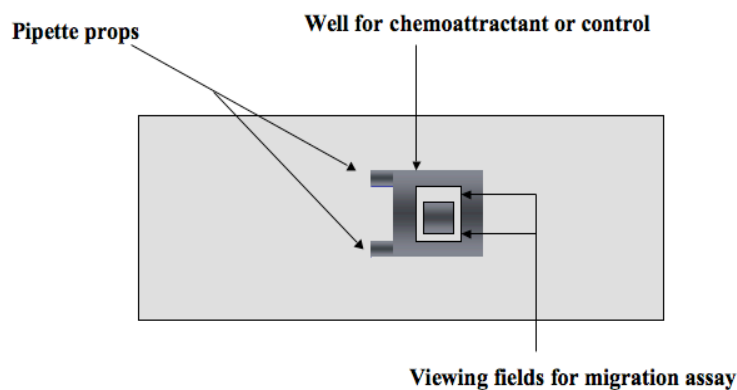


Figure 2.11. Diagrammatic representation of the modified Dunn Chamber

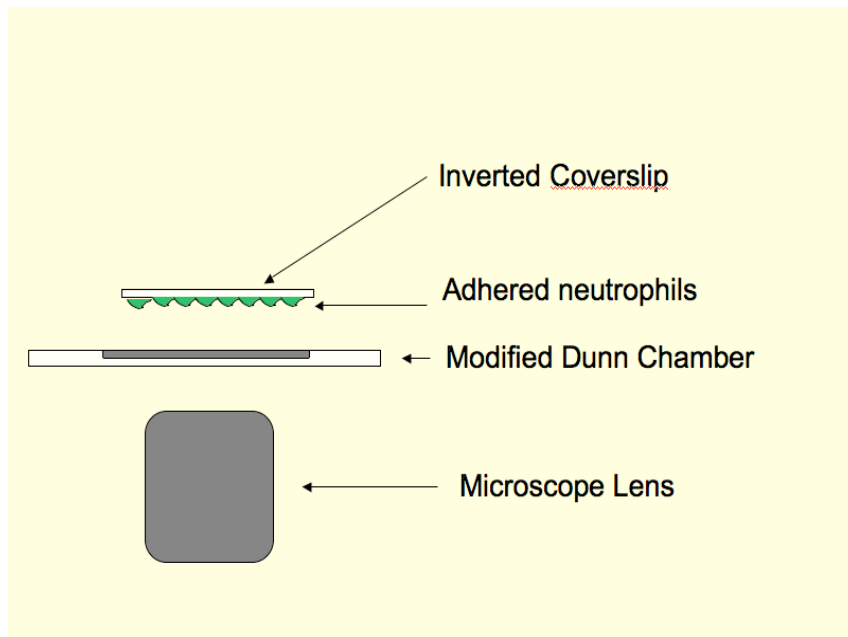


Figure 2.12. Diagrammatic representation of the Modified Dunn Chamber migration assay

2.7.2.3 Image analysis

Analysis of cell tracks

The java software ImageJ (Wayne Rasband, NIH, Bethesda) was used to outline whole cells in time lapse images using plugs-in developed previously (Andrew and Insall 2007). The 10 cells analysed were chosen at random and all analyses were carried out blinded (unaware of which patient group or treatment the cells belonged to). Each chosen cell was digitally tagged and the migratory pathways were used to analyse specific migratory parameters. See figure 2.13a and 2.13b.

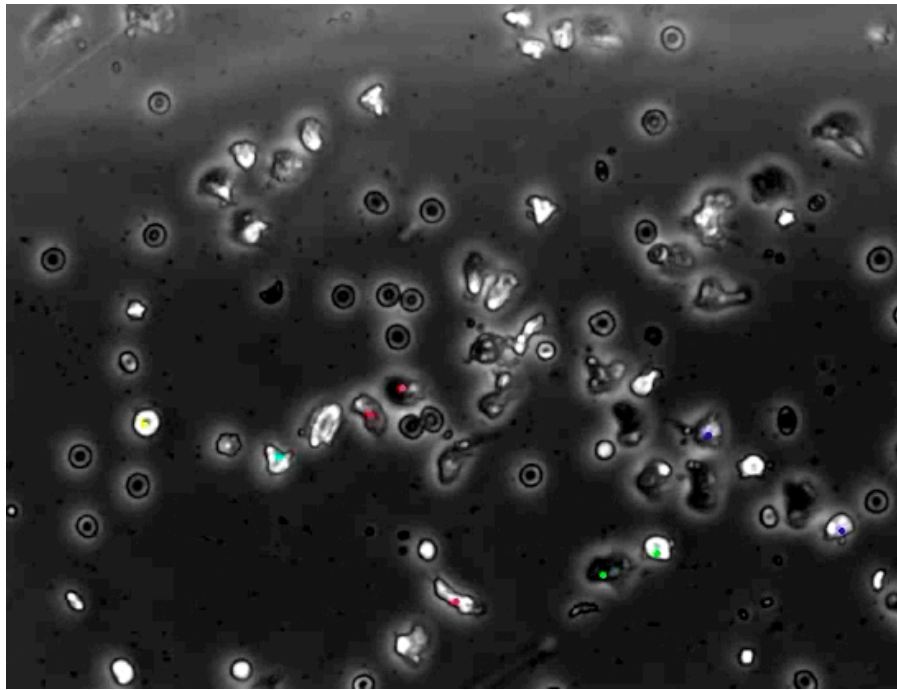


Figure 2.13a. Digital tagging of initial cell position

Legend. Each randomly chosen cell was digitally tagged using Image J software at the start of the assay (as shown by a coloured dot).

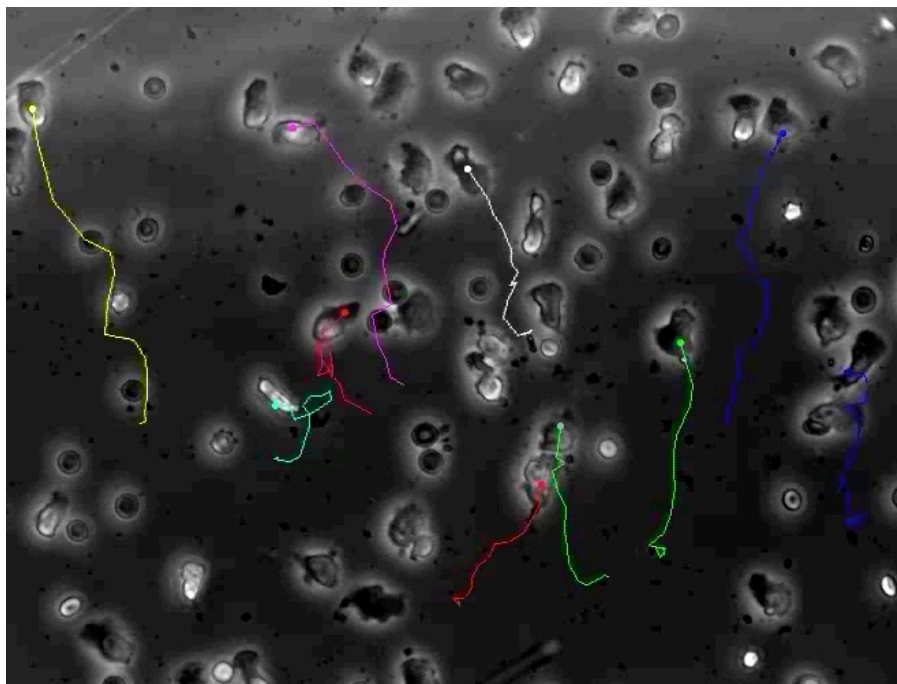


Figure 2.13b. Neutrophil migration pathways identified using digital tagging.

Legend. Each cell was followed during migration using the digital tags. The migratory pathway is shown as a coloured line. Each point on the line has digital co-ordinates to allow quantification of migratory parameters.

Analysis of pseudopods

The java software ImageJ (Wayne Rasband, NIH, Bethesda) was used to outline individual pseudopods of time lapse images using plug-ins developed by Natasha Andrew (Biosciences, University of Birmingham). Pseudopods were defined as being granule free regions of the cell that had some protrusive activity. New pseudopods were defined as “daughter” or “new lateral” pseudopods. Daughter pseudopods were identified after having split from the parent pseudopod (noted when a granular region divided a granule free area into two regions), sharing pixels with their parents in the previous frame. Any new pseudopod that did not have a parent defined by this criterion was defined as a new lateral pseudopod.

Analysis of migrational parameters

Differences in chemotaxis were assessed using the mean data of the 10 cells per experiment. Chemotaxis was assessed using 4 parameters. The first was cell speed of movement (measured from the distance travelled between two frames in $\mu\text{m}/\text{minute}$). The second was cell velocity (speed in a consistent direction towards the chemoattractant source measured in $\mu\text{m}/\text{minute}$). The third was directional persistence (a measure of the continuity of cell orientation over time, calculated by the cosine of the angle between directions in consecutive frames, expressed as 0 to 1). Cells that tend to move in a straight line, or cells that execute slow changes in direction, have a high persistence value; whereas cells that move randomly and rapidly change direction do not. The fourth parameter was chemotactic index, a measure of the accuracy of the cell's directional orientation, calculated by the cosine of the angle between the cell's direction and the orientation of the chemoattractant gradient, expressed as a score between -1 and 1.

2.8 Fluorescent antibody cell sorting

2.8.1 Immunofluorescent labelling of CXCR1 and CXCR2 receptors on isolated neutrophils

Neutrophils, isolated from peripheral blood as described in section 2.5.7, were resuspended at 5×10^6 /ml in EBM with 0.1% Bovine Serum Albumin. The appropriated wells of a 96 well plate were coated with Foetal Calf Serum and 100µl of cell solution were added to each well. The plate containing the cells was spun in a pre-cooled centrifuge for 3 min, at 1200 rpm at 4°C and the plate was then washed once with PBS.

Immunofluorescent staining was performed by adding 100ml Fluorescein Isothiocyanate Isomer (FITC) conjugated monoclonal antibodies (mAb) directed against CXCR1 or CXCR2 receptors, (both IgG2a, R&D Systems). Controls were incubated using isotype matched irrelevant antibodies directed against human IgG2a (supplied by R&D Systems, UK).

The neutrophils were incubated with the CXCR1 or 2 receptor monoclonal antibody for 30 minutes at 4°C, protected from the light. Following this, the cells were spun in the precooled centrifuge for 3 min, at 1200 rpm at 4°C and again washed once with PBS. The neutrophils were then resuspended in 300ml of PBS and 2% bovine serum albumin, ready for analysis.

2.8.2 Flow cytometry

Flow cytometry was performed using a Coulter Epics XL machine (Coulter Corp., FL, USA). Cells (100,000 per sample) were analysed by flowing them through a laser at an

excitation wave length of 488nm and the fluorescence emission from each cell was measured at 530nm. Different cell populations were identified from their patterns of forward and 90° light scattering. These signals were used for gating so that the fluorescence signals from the neutrophils alone were analysed. The intensity of fluorescence for neutrophils labelled with specific antibody was determined relative to the intensity of fluorescence for cells labelled with the non-specific antibody.

2.9 Statistical analysis

Statistical analyses were performed using the SPSS statistical program (version 16.0 Chicago, USA). Data were tested for normality using the Shapiro-Wilk test and the appropriate paired or unpaired statistical test used to compare groups. Relationships between variables were examined using the Pearson rank correlation test and a p value < 0.05 was considered to be statistically significant.

Power calculations for sample size were performed using the formula

$$n = 1 + 2C \left(\frac{S}{D} \right)^2 \text{ for a two group parallel comparison and } n = 1 + C \left(\frac{S}{D} \right)^2$$

for a cross over design. Here, **D** was the smallest difference to be detected (for example, a 50% decrease in mediator concentration or cell count) and **s** represented the standard deviation of the observations. **C** = 7.85 to provide an 80% power of detecting a reduction in mean mediator concentration or cell counts at the 5% level of significance (Snedecor and Cochran 1989).

3 The quantification and variability of neutrophils and inflammatory mediators associated with neutrophil migration in COPD

3.1 Brief introduction

It is generally accepted that COPD is an inflammatory condition, and that the neutrophils is a key effector cell. Neutrophils migrate into the lung following chemoattractant gradients formed by the secretion of pro-inflammatory mediators and these mediators and their corresponding receptors have been highlighted as potential targets for specific anti-inflammatory therapeutic intervention (de Boer 2005).

The mediators which have attracted most attention for study are IL-8, LTB₄, IL-1, TNF α and GRO α , with MPO as a marker of neutrophilic inflammation. However the daily variability is unknown, and their inter-relationships have not been clearly documented.

The studies described in this chapter were performed to assess the relationship between neutrophils and mediators that have been implicated in the pathogenesis of COPD, especially those thought to drive neutrophilic inflammation, in order to determine those that may be of primary importance in COPD assessment and intervention.

Before this could be achieved, it was necessary to clarify the variability of neutrophils and related inflammatory mediators and cells in spontaneous sputum and plasma in stable COPD that is seen with daily sampling. The current studies propose that multiple sampling significantly reduces this variability and that these data can be used for calculations to power clinical trials accurately with a primary end-point of a specified reduction in mediator concentration or inflammatory cell number.

Plasma mediator measurements were limited to those mediators that appeared of interest following analysis of the data gathered from sputum.

3.2 Experiments

3.2.1 Study subjects

Patients aged 50 – 78 years with a diagnosis of GOLD stage III COPD (Pauwels, Buist et al. 2001) were recruited. Patients were daily sputum producers with a history of chronic bronchitis as defined by the MRC criteria (Medical Research Council. 1965) and were current or ex-smokers. All patients were confirmed to have obstructive airways disease at screening by spirometric measurements and had stable symptoms of COPD for at least eight weeks prior to recruitment with no changes in medications during this time. Alternative and concomitant lung disease was excluded clinically and by high resolution computed tomography.

3.2.2 Study Design

Patients were seen on 11 occasions over four weeks (daily for 5 days, then twice weekly for three weeks). On each visit, patients underwent review, symptoms were noted and samples of spontaneous sputum and blood were collected. Spirometry was assessed weekly to confirm disease stability.

Patients were asked to complete daily diary cards, described previously (Woolhouse, Hill et al. 2001), throughout the study. Symptoms (including dyspnoea, sputum volume and colour) were recorded using an ordinal scale that allowed differentiation between normal for the patient and better or worse than usual to ensure that there were no changes suggestive of an exacerbation.

3.2.3 Sample collection & processing

Spontaneous sputum samples were collected as described in section 2.4. Sputum collection and analysis occurred at the same time on each visit. The samples were divided into 2 aliquots: the first was used to prepare a sol phase sample to determine mediator concentrations. The second was treated with dithiothreitol to assess total cell numbers and cytopins prepared for total and differential cell counts of squamous cells, neutrophils, eosinophils, macrophages and lymphocytes (Mikami, Llewellyn-Jones et al. 1998) and cytopins were prepared for total and differential cell counts. Where relevant patients were asked to abstain from smoking from waking until sputum collection was complete. Blood samples were collected in order to obtain a plasma sample to measure mediator concentrations.

3.2.4 Measurement of mediators

Mediators were measured using Enzyme Amplified Sensitivity Immunoassay (R&D Systems, Abingdon, UK) and are expressed in molar concentrations. All assays were validated to determine their working range and the variability of mediator measurements both within and between assay plates using methods described previously (Woolhouse, Bayley D.L. et al. 2002).

In order to reduce intra-patient variability in biomarkers, the effects of employing a rolling mean was assessed. A three-day rolling mean was determined for each mediator and cell count in each patient by averaging the results from visits one, two and three (“rolling mean 3a”), then from visits two, three and four (“rolling mean 3b”) etc., until the final visit was incorporated into a rolling mean (“rolling mean 3i”). A five-day rolling mean was also determined for each biomarker in each patient by averaging the results from visits one to

five (“rolling mean 5a”), then from visits two to six (“rolling mean 5b”), etc., until the final visit (“rolling mean 5g”). In order to overcome bias induced by the previous two days of a subsequent mean, comparisons were also made using random groups of 3 or 5 samples.

3.2.5 Statistical Analysis

Data analysis was performed using SPSS 12.0 for Windows (SPSS, Chicago, IL, USA). Normally distributed data are expressed as mean and standard deviations, categorical data as percentages and non-normally distributed data as median and inter-quartile range (IQR). Non-normally distributed continuous variables (such as mediator concentrations and cell counts) were log transformed to achieve normality and allow calculation of co-efficient of variation (CV). The correlations between logged mediator concentrations and cell counts were evaluated by Pearson’s correlation coefficient (PCC). Sample size calculations were performed as described previously.

3.3 Results

3.3.1 Subject characteristics

14 patients were enrolled and their baseline characteristics are summarized in Table 3.1. The medications each patient was taking during the study are listed in Table 3.2. Exacerbations, defined clinically (Anthonisen, Manfreda et al. 1987) were excluded on each visit by review of daily dairy cards, clinical examination, confirmation of unchanged therapy (Rodriguez-Roisin 2000) and weekly spirometry. None of the patients experienced an exacerbation during the study period and there were no significant changes in diary scores or lung function between visits.

Table 3.1. Intra-patient variability: Baseline characteristics of the study patients

| | |
|--|-------------|
| Age, yr | 65.5 ± 8.5 |
| Male, % | 6 (43) |
| Diagnosis, yr | 6.4 ± 5.4 |
| FEV ₁ , L | 0.89 ± 0.3 |
| FEV ₁ , % of predicted | 40 ± 8.3 |
| FVC, L | 2.28 ± 0.61 |
| FEV ₁ /FVC | 42 ± 11 |
| Increase in FEV ₁ following 400µg inhaled salbutamol, | |
| mL | 132 ± 77 |
| % predicted | 5 ± 3.3 |
| BMI, kg/m ² | 23.8 ± 4.5 |
| Current smokers, (%) | 6 (43) |
| Pack year history | 45 ± 20.7 |
| Receiving inhaled steroids at enrolment, (%) | 11 (79) |

Legend.

Results are presented as average ± standard deviation or percentage (shown in parentheses).
The length of time from a formal diagnosis of COPD is shown.

Table 3.2: The list of medications taken by each patient during the study period.

| Patient | Medications | Patient | Medications |
|---------|--|---------|---|
| 1 | Salbutamol 100mcg inh bd Tiotropium 18mcg inh od Beclometasone dipropionate 200mcg inh bd Aspirin 75mg po od | 8 | Salbutamol 100mcg inh qds Ipratropium 20mcg inh bd Theophylline modified release 175mg po bd Seretide (250mcg/50mcg) inh bd Gliclazide 80mg po od |
| 2 | Salmeterol 50mcg inh bd Salbutamol 100mcg inh prn Budesonide 200mcg inh bd Ipratropium 40mcg inh bd Theophylline modified release 250mg po bd | 9 | Salbutamol 100mcg inh prn |
| 3 | Salmeterol 100mcg inh bd Salbutamol 100mcg inh bd Ipratropium 40mcg inh bd Fluticasone propionate 100mcg inh bd Ranitidine 300mg po od | 10 | Salmeterol 50mcg inh bd Salbutamol 100mcg inh prn Fluticasone propionate 200mcg inh bd |
| 4 | Seretide (250mcg/ 50mcg) inh bd Salbutamol 100mcg inh prn | 11 | Salbutamol 100mcg inh qds Ipratropium 20mcg inh bd Fluticasone propionate 250mcg inh bd |
| 5 | Salmeterol 50mcg inh bd Salbutamol 100mcg inh prn Ipratropium 20mcg inh bd Beclometasone dipropionate 20mcg inh bd Paracetamol 1g po QDS | 12 | Salmeterol 50mcg inh bd Salbutamol 100mcg inh prn Fluticasone propionate 250mcg inh bd |
| 6 | Salmeterol 50mcg inh bd Salbutamol 100mcg inh prn Ipratropium 20mcg inh qds | 13 | Combivent inh (100mcg/20mcg) inh qds Beclometasone dipropionate 200mcg inh bd |
| 7 | Ipratropium 20mcg inh bd Fluticasone propionate 250mcg inh bd Lansoprazole 15mg po od Warfarin 3mg po od (variable dose) Furosemide 20mg po od | 14 | Formoterol fumarate 4.5mcg inh bd Tiotropium 18mcg inh od |

Legend.

Medications taken during the study period. Medications did not change for 8 weeks prior to the study or during the study period.

3.3.2 Assay Variability and sputum characterisation

In all cases, the mediator intra and inter-assay variability was less than 10%. The median percentage of squamous cells in the sputum samples was 0.6% (IQR = 0 – 7.9%) and the median percentage of viable cells (assessed by trypan blue exclusion) was 83% (IQR = 63 – 92%) which is comparable to that seen with induced sputum (Bhowmik, Seemungal et al. 1998).

3.3.3 Intra-patient variability in sputum cell and mediator concentrations

There was considerable variability in the concentrations of neutrophils, mediators and other cells within individual patients over the study period. Figure 3.1 demonstrates the variability seen for one patient and Table 3.3 describes the intra-patient variability for all mediators, and sputum neutrophil and macrophage counts for each patient.

The variability in sputum inflammatory markers seen within patients was wide, irrespective of the inflammatory mediator or cell type measured. The median intra-patient co-efficient of variation for each mediator (calculated from each patient's co-efficient of variation) is shown in Table 3.4. This variability was not related to any changes in individual symptoms as recorded in the patients' diary card scores, clinical features, sputum microbiology or lung function (all of which remained stable).

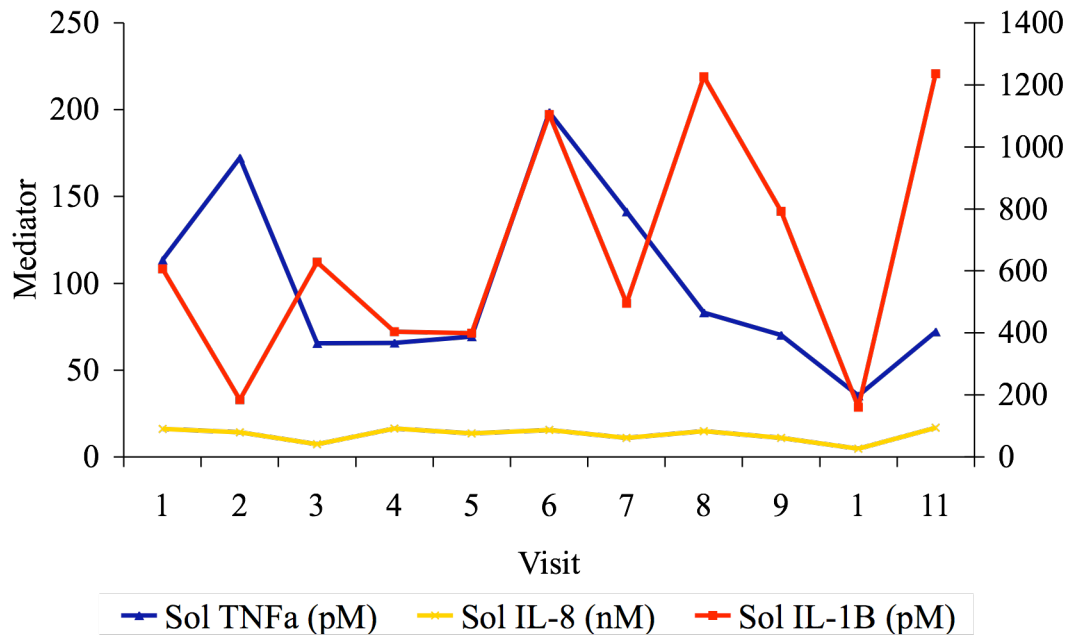


Figure 3.1a: Changes in raw data over one month for Patient 1 for the sputum sol mediators IL-1beta, TNF alpha and IL-8

Each data point represents the measured concentration of one mediator for a single patient on a single visit (in units as per figure legend). IL-1 β concentrations are given on the right hand axis, TNF α and IL-8 concentrations are given on the left hand axis.

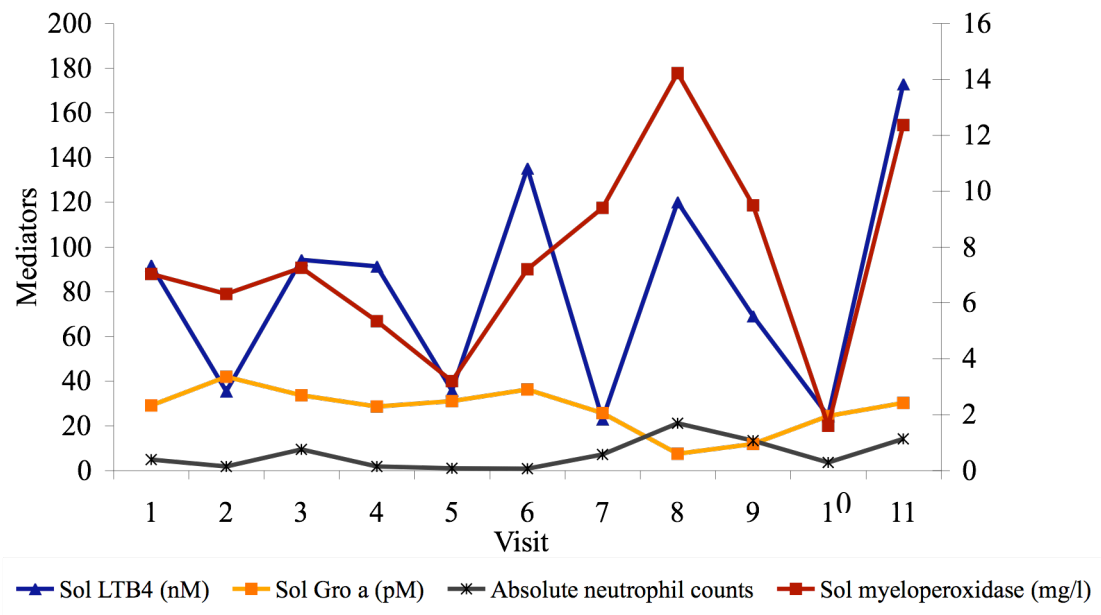


Figure 3.1b: Changes in raw data over one month for Patient 1 for the sputum sol phase mediators MPO, LTB4, GRO alpha and absolute neutrophil counts

Each data point represents the measured concentration of one mediator for a single patient on a single visit (in units as per figure legend). MPO concentrations are given on the right hand axis, LTB4, GRO α concentrations and absolute neutrophil counts ($10^6/\text{ml}$) are given on the left hand axis.

Table 3.3. The within patient variability in pulmonary inflammatory mediators and cells seen in stable patients with COPD over one month

| Patient | | IL-1 β (pM) | TNF α (pM) | CXCL8 (nM) | MPO (mg/ml) | LTB4 (nM) | GRO α (nM) | PMN (10 ⁶ /ml) | Macrophage (10 ⁶ /ml) |
|---------|--------|----------------------|----------------------|---------------|----------------|--------------|----------------------|------------------------------|-------------------------------------|
| 1 | Median | 606 | 72.2 | 14.27 | 7.2 | 91.2 | 29.1 | 4.8 | 0.09 |
| | IQR | 399 - 1104 | 66 - 141 | 10.9-16.1 | 5.34-9.5 | 35-120 | 24-33 | 1.8-13.4 | 0.06 – 0.17 |
| | CV | 11 | 11.3 | 15.9 | 32.9 | 16.9 | 16 | 77.3 | 31.8 |
| 2 | Median | 19.1 | 0.4 | 4.8 | 1.8 | 1.4 | 14.7 | 1.3 | 0.18 |
| | IQR | 9.3 - 26.6 | 0.19 - 0.69 | 3.85-6.68 | 1.24-1.8 | 0.85-1.96 | 12-20.4 | 0.4-1.95 | 0.14 – 0.34 |
| | CV | 19.6 | 87 | 25.4 | 162.5 | 194 | 9.6 | 471 | 37.1 |
| 3 | Median | 5.8 | 0.3 | 1.4 | 1.7 | 5.9 | 18.1 | 0.9 | 0.12 |
| | IQR | 3.4 - 6.6 | 0.2-0.6 | 1.2-1.5 | 1.4-1.9 | 5-6.5 | 16.6-19.7 | 0.7-1.4 | 0.07 – 0.13 |
| | CV | 30.3 | 45.3 | 85 | 61.2 | 22.2 | 12.3 | 804 | 44 |
| 4 | Median | 501 | 52.2 | 24.4 | 2.41 | 28 | 16.8 | 5.35 | 0.2 |
| | IQR | 417 - 865 | 48.5 - 68.8 | 19.8-27.5 | 1.6-4.5 | 20.5-42 | 14.7-18.7 | 2.5-7.4 | 0.13 – 0.42 |
| | CV | 8.3 | 6.6 | 20.7 | 84.3 | 13.5 | 9.5 | 44.9 | 51.7 |
| 5 | Median | 697 | 74.6 | 26 | 4.13 | 10.23 | 3.16 | 6.25 | 0.4 |
| | IQR | 375 - 775 | 33.2-105 | 18.4-43 | 2-7.2 | 7.4-23.4 | 1.5-3.8 | 5.2-25.9 | 0.26 – 0.81 |
| | CV | 10 | 31 | 13.9 | 74.3 | 25.1 | 96.8 | 44.8 | 81.7 |
| 6 | Median | 120.2 | 4.32 | 5.37 | 4.27 | 11.17 | 12.3 | 3.4 | 0.27 |
| | IQR | 65.9 - 343 | 0.4-42 | 2.5-6.9 | 3.4-6.4 | 2.2-17.6 | 10.2-16.5 | 2.2-5.9 | 0.16 – 0.3 |
| | CV | 21.2 | 159 | 44.3 | 35.4 | 47.9 | 18.5 | 38.7 | 30.4 |
| 7 | Median | 606 | 253 | 40.1 | 5.79 | 49.04 | 12.3 | 18.1 | 0.46 |
| | IQR | 504-1128 | 212-301 | 27.7-61.4 | 3.4-13.8 | 21.7-75.7 | 10.6-21.2 | 12.7-24.9 | 0.31 – 0.73 |
| | CV | 12 | 5.6 | 12.5 | 59.2 | 21.9 | 16.4 | 17.3 | 98.3 |
| 8 | Median | 5.1 | 0.1 | 0.33 | 1.15 | 0.95 | 4.87 | 0.4 | 0.03 |
| | IQR | 3.1-5.9 | 0.03-0.14 | 0.24-0.41 | 1.04-1.43 | 0.6-1.5 | 3.5-6.4 | 0.3-0.8 | 0.03 – 0.06 |
| | CV | 39.8 | 29.8 | 29 | 224 | 1299 | 189 | 92.5 | 31.3 |
| 9 | Median | 9.26 | 0.25 | 1.04 | 1.93 | 0.49 | 8.12 | 0.4 | 0.33 |
| | IQR | 3.5-43.4 | 0.02-1.1 | 0.5-2.6 | 1.1-2.6 | 0.32-0.7 | 4.7-9.8 | 0.2-1.6 | 0.17 – 0.65 |
| | CV | 55.5 | 113 | 633 | 70.5 | 159.4 | 20.6 | 236.5 | 86.7 |

Table 3.3. (continued). The within patient variability in pulmonary inflammatory mediators and cells seen in stable patients with severe COPD over one month

| Patient | IL-1 β (pM) | TNF α (pM) | CXCL8 (nM) | MPO (mg/ml) | LTB4 (nM) | GRO α (nM) | PMN (10 ⁶ /ml) | Macrophage s (10 ⁶ /ml) |
|---------|----------------------|----------------------|---------------|----------------|--------------|----------------------|------------------------------|--|
| 10 | Median | 156.9 | 5.25 | 2.11 | 2.03 | 10.81 | 5.04 | 2.15 |
| | IQR | 88 - 417 | 3 -10.4 | 1.7-3 | 1.9-2.4 | 7.1-15.1 | 3.9-5.3 | 0.95-12.6 |
| | CV | 19 | 37.4 | 49.2 | 22.7 | 27.9 | 11.6 | 181 |
| 11 | Median | 10.6 | 0.06 | 0.11 | 1.38 | 6.74 | 0.3 | 1.65 |
| | IQR | 7.5 - 11.8 | 0.04-0.2 | 0.06-0.14 | 1.2-2.6 | 3.8-11.7 | 0.1-0.4 | 1.1-2.2 |
| | CV | 11.4 | 29.2 | 24.3 | 85.7 | 32 | 54.1 | 92.4 |
| 12 | Median | 31 | 4.89 | 3.7 | 0.88 | 3.07 | 24 | 5.1 |
| | IQR | 18 - 41.9 | 2.7-10 | 2.8-4.3 | 0.6-1.1 | 2.3-7.6 | 21.4-28.4 | 4.1-6.7 |
| | CV | 16.3 | 87 | 32.7 | 186.7 | 111.2 | 4.4 | 20 |
| 13 | Median | 13.8 | 0.19 | 2.05 | 1.28 | 1.77 | 16.8 | 1.8 |
| | IQR | 6.7-30.8 | 0.1-0.3 | 1.3-3.7 | 1 - 1.4 | 0.9-1.8 | 7.8-21.8 | 0.8-5.3 |
| | CV | 31.2 | 29.3 | 73.8 | 107.9 | 111.8 | 4.4 | 178 |
| 14 | Median | 3.12 | 0.16 | 2.03 | 0.78 | 0.49 | 19.7 | 2.65 |
| | IQR | 2 - 4.9 | 0.1-0.2 | 1.4-2.6 | 0.3-0.9 | 0.2-1.6 | 18.4-22 | 2.2-5.2 |
| | CV | 46.9 | 13.4 | 60.1 | 129.5 | 180 | 5.5 | 50.1 |

Legend

The median biomarker concentration (with corresponding inter-quartile range) is given for each patient. The co-efficient of variation (CV) for each patient was determined from log transformed data and is expressed as a percentage.

Table 3.4: The median intra-patient co-efficient of variation (CV) in sputum

| Mediator | CV (%) | IQR |
|---|--------|------------|
| IL-1 β (pM) | 19.4 | (11 – 33) |
| TNF α (pM) | 30.3 | (13 – 88) |
| LTB4 (nM) | 39.9 | (22 – 165) |
| IL-8 (nM) | 30.8 | (19 – 63) |
| GRO α (nM) | 16.2 | (10 – 29) |
| Absolute neutrophil count (10 ⁶ /ml) | 77.3 | (42 – 180) |
| Absolute macrophage count (10 ⁶ /ml) | 74.2 | (41 - 102) |
| MPO (mg/ml) | 79.3 | (53 – 138) |

Legend

The median intra-patient CV is derived from each individual patient's CV. Each patient's mediator-specific CV was calculated using the average data from eleven sequential visits.

3.3.4 Intra-patient variability in plasma cells and mediator concentrations

Plasma concentrations of IL-8, LTB4, GRO α and MPO were below the lower limit of detection for the assays available when writing this thesis, (12.5pM for IL-8, 130pM for LTB4, 10pM for GRO α and 2.93mg/ml for MPO). Given the inability to accurately quantify these mediators, they were excluded from further analysis. Plasma concentrations of TNF α and IL-1 β could be accurately assessed for all patients, as could cell counts of neutrophil and monocytes, and so these were included in the analysis of variability.

There was less variability in the plasma concentrations of neutrophils, monocytes and mediators within individual patients over the study period. Figure 3.2 demonstrates the

variability seen for the same patient as shown in figure 3.1 and Table 3.5 describes the intra-patient variability for $\text{TNF}\alpha$, $\text{IL-1}\beta$, and peripheral blood neutrophil and macrophage counts for each patient.

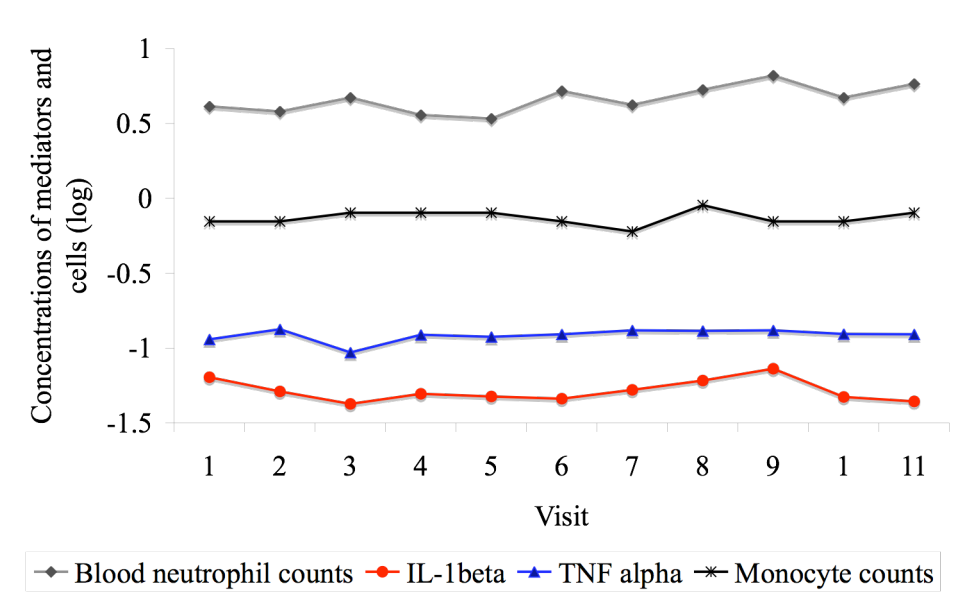


Figure 3.2: Changes in raw data over one month for Patient 1 for the plasma mediators IL-1beta, TNF alpha and absolute neutrophil and monocyte counts.

Each data point represents the measured concentration of one mediator for a single patient on a single visit. $\text{IL-1}\beta$ and $\text{TNF}\alpha$ concentrations are given in pM (log), neutrophil and monocyte counts are $10^6/\text{ml}$.

Table 3.5. The within patient variability in measurable plasma inflammatory mediators and cells seen in stable patients with severe COPD over one month

| Patient | | IL-1 β (pM) | TNF α (pM) | Neutrophils (10 ⁶ /ml) | Monocytes (10 ⁶ /ml) |
|---------|--------|----------------------|----------------------|--------------------------------------|------------------------------------|
| 1 | Median | 0.05 | 0.12 | 4.7 | 0.7 |
| | IQR | 0.04 – 0.07 | 0.12 – 0.13 | 3.9-5.2 | 0.7 – 0.8 |
| | CV | 10 | 9 | 21 | 11 |
| 2 | Median | 0.05 | 0.1 | 4.5 | 0.6 |
| | IQR | 0.04 – 0.05 | 0.1 - 0.12 | 4.3-4.8 | 0.5 – 0.6 |
| | CV | 11 | 10 | 10 | 13 |
| 3 | Median | 0.06 | 0.11 | 6.5 | 0.8 |
| | IQR | 0.05 – 0.06 | 0.1-0.2 | 6.3-6.9 | 0.8 – 0.9 |
| | CV | 17 | 12 | 6 | 10 |
| 4 | Median | 0.05 | 0.08 | 7.1 | 0.92 |
| | IQR | 0.04 – 0.08 | 0.08 – 0.09 | 6.7 – 7.2 | 0.9 – 0.97 |
| | CV | 22 | 10 | 11 | 13 |
| 5 | Median | 0.06 | 0.7 | 4.9 | 0.7 |
| | IQR | 0.05 – 0.07 | 0.7 – 0.8 | 4.4 – 5.3 | 0.7 – 0.8 |
| | CV | 22 | 13 | 18 | 13 |
| 6 | Median | 0.03 | 0.07 | 5.8 | 0.8 |
| | IQR | 0.02 – 0.04 | 0.05 – 0.08 | 4.5 – 7.4 | 0.8 – 0.9 |
| | CV | 20 | 31 | 33 | 12 |
| 7 | Median | 0.02 | 0.07 | 5.2 | 0.7 |
| | IQR | 0.02 – 0.03 | 0.06 – 0.07 | 5.0 – 5.3 | 0.6 – 0.7 |
| | CV | 15 | 11 | 8 | 17 |
| 8 | Median | 0.03 | 0.6 | 4.8 | 0.6 |
| | IQR | 0.02 0.03 | 0.5 – 0.65 | 4.4 – 4.9 | 0.5 – 0.65 |
| | CV | 10 | 14 | 8 | 14 |
| 9 | Median | 0.02 | 0.06 | 4.8 | 0.5 |
| | IQR | 0.01 – 0.02 | 0.04 – 0.07 | 4.8 – 5.1 | 0.5 – 0.6 |
| | CV | 30 | 20 | 6 | 18 |

Table 3.5. (continued). The within patient variability in plasma inflammatory mediators and cells seen in stable patients with severe COPD over one month

| Patient | | IL-1 β (pM) | TNF α (pM) | Neutrophils (10 ⁶ /ml) | Macrophages (10 ⁶ /ml) |
|---------|--------|----------------------|----------------------|--------------------------------------|--------------------------------------|
| 10 | Median | 0.06 | 0.09 | 6.2 | 1 |
| | IQR | 0.05 – 0.07 | 0.08 – 0.09 | 5.2 – 6.6 | 0.8 – 1.1 |
| | CV | 12 | 8 | 13 | 44 |
| 11 | Median | 0.05 | 0.04 | 3.1 | 0.4 |
| | IQR | 0.04 – 0.05 | 0.04 – 0.05 | 2.9 – 3.2 | 0.3 – 0.4 |
| | CV | 6.9 | 9.8 | 10 | 5 |
| 12 | Median | 0.05 | 0.04 | 6.5 | 0.5 |
| | IQR | 0.02 – 0.05 | 0.04 – 0.05 | 6.1 – 7.0 | 0.5 – 0.6 |
| | CV | 32 | 15 | 9 | 17 |
| 13 | Median | 0.06 | 0.05 | 4.8 | 0.65 |
| | IQR | 0.05 – 0.07 | 0.04 – 0.05 | 4.3 – 5.7 | 0.6 – 0.7 |
| | CV | 14 | 30 | 16 | 8.4 |
| 14 | Median | 0.03 | 0.05 | 3 | 0.4 |
| | IQR | 0.03 – 0.04 | 0.04 – 0.05 | 2.7 – 3.4 | 0.3 – 0.4 |
| | CV | 6 | 10 | 19 | 20 |

Legend

The median biomarker concentration (with corresponding inter-quartile range) is given for each patient. The co-efficient of variation (CV) for each patient was determined from log transformed data and is expressed as a percentage.

In comparison with sputum, there was little intra-patient variability in plasma inflammatory markers and cells (the values were similar to the inherent variability seen within each assay, which is approximately 10%). The median intra-patient co-efficient of variation for each mediator (calculated from each patient's co-efficient of variation) is shown in Table 3.6. This variability did not relate to any changes in individual symptoms as recorded in the patients' diary card scores, clinical features, sputum microbiology or lung function (all of which remained stable).

Table 3.6: The median intra-patient co-efficient of variation (CV) in blood.

| Mediator/ Cell | CV | IQR |
|-----------------------------------|-----|---------|
| IL-1 β (pM) | 14% | 10 - 19 |
| TNF α | 10% | 8 - 15 |
| Neutrophils (10 ⁶ /ml) | 10% | 8 - 17 |
| Monocytes (10 ⁶ /ml) | 13% | 11 - 17 |

Given the stability of blood inflammation in this group of patients, further analysis to reduce intra-patient variability was limited to measurements taken from sputum.

3.3.5 Reducing intra-patient variability in sputum samples

It was proposed that multiple sampling would significantly reduce intra-patient variability, and so a rolling mean technique was adopted to test this hypothesis. Table 3.7 compares the effects of a rolling mean on intra-patient variability for all patients and all sputum mediators (expressed as CVs). When comparing a single mediator within patients, a significant reduction in variability of the raw data (as determined by the CV) was obtained using a three-day rolling mean in all biomarkers except for sputum neutrophil counts. A further reduction in variability was seen using a five-day rolling mean in all biomarkers, including

sputum neutrophil counts. Figures 3.3a – 3.3c compare graphically the variability in mediator concentrations and the effect of rolling means for one representative patient.

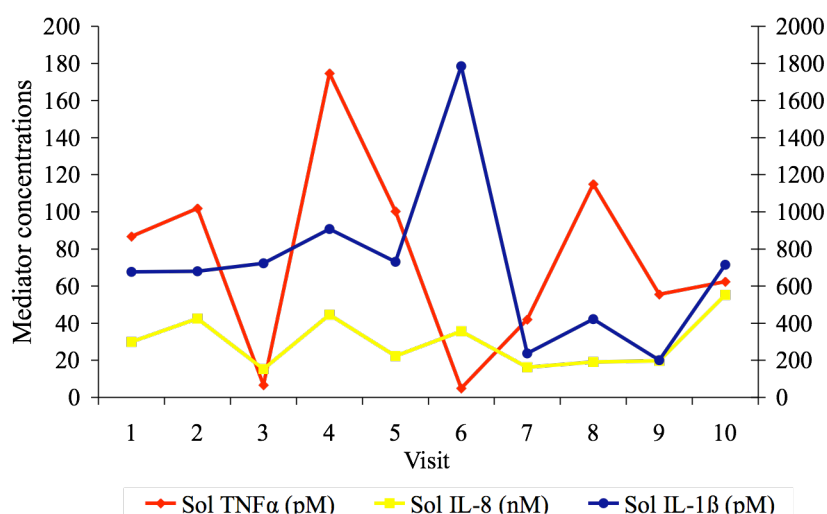


Figure 3.3a: Rolling means. Changes in raw data over one month for a representative patient (here, Patient 5) for the sputum sol phase mediators IL-1beta, TNFα and IL-8. IL-1β concentrations are given on the right hand axis, TNFα and IL-8 concentrations are given on the left hand axis. Data points represent the measured concentration of one mediator for a single patient on a single visit (in units as per figure legend).

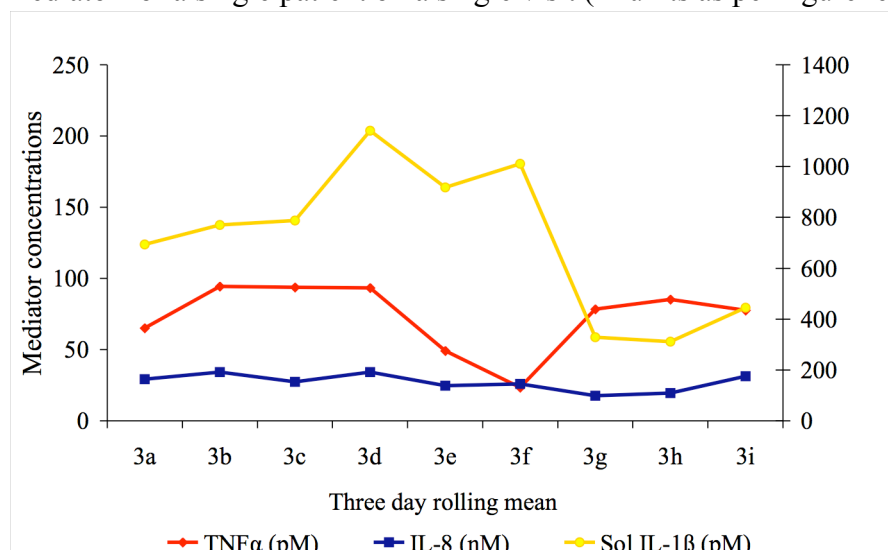


Figure 3.3b: Changes in mediators over one month for Patient 5 using a three day rolling mean

IL-1β concentrations are given on the right hand axis, TNFα and IL-8 concentrations are given on the left hand axis. Data points represent the mean measured concentration of one mediator for three consecutive days for a single patient. A three-day rolling mean was determined for each mediator and cell count in this patient by averaging the results from visits one, two and three (rolling mean “3a” on x axis), then from visits two, three and four (rolling mean “3b”) etc., until the final 3 visits were incorporated into a rolling mean (“3i”).

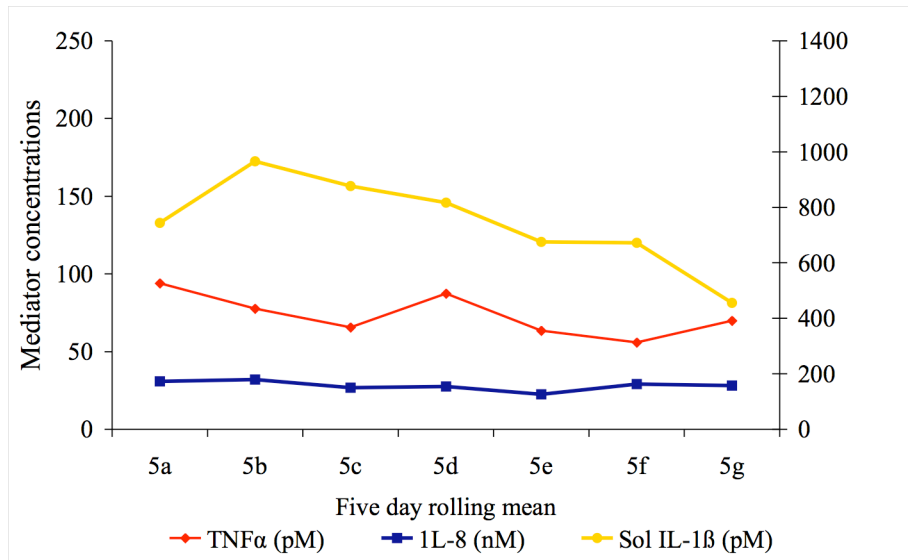


Figure 3.3c: Changes in mediators over one month for a Patient 5 using a five day rolling mean

Data points represent the mean measured concentration of one mediator for five consecutive days for a single patient. A five-day rolling mean was determined for each biomarker in this patient by averaging the results from visits one to five (rolling mean “5a” on x axis), then from visits two to six (rolling mean “5b”), etc., until the final visit (“rolling mean 5g”).

Table 3.7. Comparison of the median intra-patient coefficient of variation for each mediator using daily data, a three-day rolling mean and a five-day rolling mean

| | | IL-1 β (pM) | TNF α (pM) | IL-8 (nM) | MPO (mg/l) | LTB4 (nM) | GRO α (pM) | Sputum neutrophils (10 ⁶ /ml) | Sputum macrophages (10 ⁶ /ml) |
|--------------------------|---------------------------|----------------------|----------------------|---------------------|---------------------|----------------------|------------------------|--|--|
| Raw data | Median CV (%) (IQR) | 19.4 (11 - 33) | 30.3 (13 - 88) | 30.8 (19 - 63) | 79.3 (53 - 138) | 39.9 (22 - 165) | 16.2 (10 - 29) | 77.3 (42 - 180) | 74.2 (41 - 102) |
| 3 day rolling mean | Median CV (%) (IQR) | 11.2 * (6 - 17) | 20.6 * (8 - 50) | 17.3 * (12.6) | 44.5 * (20 - 60) | 22.5 * (10 - 96) | 7.6 * (4 - 11) | 46.7 (13 - 218) | 33.9 * (26 - 59) |
| 5 day rolling mean | Median CV (%) (IQR) | 6.9 * ‡ (4 - 9) | 12.2 * ‡ (2 - 37) | 9.5 * ‡ (4 - 20) | 22 * ‡ (13 - 39) | 13.8 * ‡ (4 - 53) | 4.1 * ‡ (2.6 - 6.8) | 13.4 * ‡ (8 - 124) | 20 * (13 - 32) |

Legend

The median coefficient of variation has been derived from the marker specific CV from each patient. Differences in CVs were assessed using the Friedman's matched samples test and the Wilcoxon's matched pairs signed rank test. * = significant reduction in mean CV from raw data ($p < 0.05$). ‡ = significant reduction in mean CV from 3 day rolling mean ($p < 0.05$).

3.3.6 Inter-patient variability and the effects of a rolling mean.

There was marked variability in inflammatory biomarkers between patients with COPD using data from a single day. Using a three-day or five-day rolling mean for each patient did not alter the inter-patient variability suggesting that it relates to individual differences in the degree of airway inflammation. When all mediators were considered together, the median variability between patients using daily data (expressed as the CV) was 101% (IQR 90 – 123%) and the median variability between patients using the first five day rolling mean was only slightly reduced to 91% (IQR 73 – 115%) (see Table 3.8).

Table 3.8: Comparison of the inter-patient coefficient of variation for each mediator using

| | IL-1 β (pM) | TNF α (pM) | IL-8 (nM) | MPO (mg/ml) | LTB4 (nM) | GRO α (nM) | Sputum macrophag es (10 ⁶ /ml) | Sputum neutrophils (10 ⁶ /ml) | Median CV (IQR) |
|--|----------------------|----------------------|-------------------|------------------|-------------------|----------------------|--|--|-----------------------|
| Median IQR | 43.3 7.5 – 413 | 2.07 0.2 – 55 | 3.6 1.4 - 14.4 | 1.8 1.1 - 3.5 | 6.1 1.5 - 18.6 | 12.3 5 - 19.5 | 0.29 (0.13 – 0.75) | 2.65 1 - 6.4 | |
| CV calculated from raw data | 53.5 | 407.8 | 145.4 | 102 | 99.8 | 60.9 | 100 | 115.4 | 101% (90-123) |
| CV calculated using a 5- day rolling mean | 52.15 | 234.6 | 131 | 92.15 | 78.2 | 58.5 | 110 | 89.4 | 91% (73 – 115) |

Legend

The median biomarker concentration (and IQR) is calculated from all measurements for all patients. In order to calculate the inter-patient CVs, the mean logged concentrations were calculated for each biomarker in each patient (either using daily data or data from the first 5 day rolling mean).

3.3.7 Determining the sample size required to power interventional studies.

Using a cross-over design, the number of patients needed to confirm a 50% reduction in sputum neutrophil counts with 80% power and at the 5% significance level using a single data point per patient would be 72. However, if a three-day average is used, the number needed to confirm the same reduction would be 23 and 20 using a five-day average. For a parallel design, the number needed to confirm a 50% reduction in sputum neutrophil counts using a single data point would be 140 (70 per group), which would be reduced to 106 and 103 using a three or five-day average respectively.

Table 3.9 summarises the average number of patients required (with SEM) to detect similar changes in other mediators when a single sputum sample is collected or when the average of three or five consecutive samples is used for power calculations.

Using the same formula for a cross-over design, the number of patients needed to confirm a 50% reduction in plasma $\text{TNF}\alpha$ with 80% power and at the 5% significance level using a single data point per patient would be 6. To see the same reduction in $\text{IL-1}\beta$ with the same power and at the same significance, one would need to include 4 patients. In comparison with sputum samples, fewer subjects would be required for studies with adequate power, due to the reduction in variability. However, it must be recalled that both $\text{IL-1}\beta$ and $\text{TNF}\alpha$ are at the lower limits of detection for both of the assays that were used in this thesis, and so a further reduction is likely to be unquantifiable if measured using the same techniques..

Table 3.9: A comparison of the numbers needed in a cross-over study design when one day's data, the mean of three days' data or the mean of five days' data are used for calculations

| | | IL-1 β | TNF α | IL-8 | MPO | LTB4 | GRO α | Absolute macrophage counts | Absolute neutrophil counts |
|-------------|-------------------------|--------------|--------------|------|-------|------|--------------|----------------------------|----------------------------|
| 1 days data | Mean number of patients | 10 | 330 | 61 | 62 | 41 | 20 | 52 | 72 |
| | SEM | 0.76 | 74.72 | 7.14 | 15.90 | 4.79 | 4.95 | 11.2 | 11.11 |
| 3 days data | Mean number of patients | 2 | 23 | 7 | 16 | 7 | 5 | 12 | 23 |
| | SEM | 0.73 | 9.08 | 1.93 | 4.01 | 2.13 | 1.94 | 1.7 | 6.69 |
| 5 days data | Mean number of patients | 2 | 15 | 5 | 13 | 7 | 5 | 6 | 20 |
| | SEM | 0.07 | 3.12 | 0.30 | 1.49 | 0.45 | 1.34 | 0.6 | 2.25 |

Legend.

Numbers required to provide an 80% chance of detecting a 50% decrease in mediators or cells at the 5% level of significance. For 1 day's data, power calculations were performed for each mediator on each visit, and the mean of this is presented (with the standard error of the mean (SEM)). For three days' data; power calculations were performed on the first three days (visit 1 to 3), then the next three days (visit 2 to 4) until visit 9 – 11. These were used to calculate the mean number of patients required.

For five days' data; power calculations were performed on the first five days (visit 1 to 5), then the second five days (visit 2 to 6) until visit 7 to 11. These were used to calculate the mean number of patients required.

3.3.8 The relationship between sputum phase inflammatory mediators and sputum cell counts

All biomarkers measured showed inter-relationships with each other of varying significance. Of the chemoattractants studied, IL-8 (followed by LTB₄) was most closely associated with neutrophils and other inflammatory mediators, with the most significant correlations. None of the mediators correlated with sputum macrophage counts. The weakest and often non-significant correlations were obtained using a single days sample. The strength of correlations between mediators and neutrophils (and the significance of these correlations) increased as more data points were used for each patient, see table 3.10. Table 3.11. shows the relationships between cytokines. With the exception of GRO α , good correlations were seen however many days data were used. However, in general, there was an increase in the correlation coefficient using the mean of three days' data as opposed to one day's data (mean difference in Pearson's correlation coefficient between one day's data and the mean of three days data = 0.11, CI = 0.04 – 0.18, p = 0.003), however there was no further increase in the strength of the correlation using the mean of more data points. The exception to this was GRO α , which showed no correlation with neutrophils or any other mediator measured in sputum, apart from CXCL8.

Figure 3.4a – 3.4c demonstrates the correlations seen between the absolute sputum neutrophil count and IL-8, TNF α and IL-1 β respectively, using the average value for all eleven samples for each patient.

Table 3.10: Correlations between cells and mediators

| Mediator or cell line | Data used for correlations PCC (p) | Sputum neutrophils | IL-8 | LTB4 | TNF α | MPO | IL-1 β | GRO α |
|--------------------------|---|-----------------------|--------------|-------------|---------------|--------------|---------------|--------------|
| Sputum neutrophils | 1 day | | 0.6 (0.02) | 0.5 (ns) | 0.46 (0.09) | 0.18 (0.53) | 0.60 (0.02) | 0.13 (0.7) |
| | 3 days | | 0.8 (<0.001) | 0.61 (0.01) | 0.84 (<0.001) | 0.2 (0.5) | 0.77 (0.001) | 0.16 (0.6) |
| | 5 days | | 0.66 (0.005) | 0.65 (0.01) | 0.71 (0.003) | 0.2 (0.5) | 0.77 (0.001) | 0.3 (0.36) |
| | 11 days | | 0.7 (0.004) | 0.7 (0.008) | 0.81 (<0.001) | 0.13 (0.64) | 0.83 (<0.001) | 0.25 (0.4) |
| Sputum macrophages | 1 day | 0.38 (0.17) | 0.1 (0.7) | 0.006 (0.9) | 0.08 (0.8) | -0.35 (0.22) | -0.08 (0.8) | 0.08 (0.8) |
| | 3 days | 0.45 (0.1) | 0.22 (0.44) | 0.05 (0.89) | 0.09 (0.73) | -0.37 (0.2) | 0.09 (0.73) | 0.22 (0.44) |
| | 5 days | 0.29 (0.3) | 0.11 (0.7) | -0.15 (0.6) | 0.05 (0.8) | -0.14 (0.6) | 0.07 (0.8) | 0.16 (0.6) |
| | 11 days | 0.4 (0.12) | 0.19 (0.51) | -0.19 (0.5) | 0.09 (0.8) | -0.39 (0.16) | 0.03 (0.9) | 0 (0.9) |

Legend

Data describes the relationships between mediators and cell counts when one days data is used (here, the first day), or the mean of the first 3, 5 or all 11 days' data. Relationships are expressed as Pearsons Correlation Co-efficient (PCC) and p value.

Table 3.11: Correlations between mediators

| Mediator or cell line | Data used for correlations PCC (p) | CXCL8 | LTB4 | IL-1 β | TNF α | MPO | GRO α |
|-----------------------|------------------------------------|---------------|---------------|---------------|---------------|--------------|--------------|
| CXCL8 | 1 day | | 0.52 (0.06) | 0.85 (<0.001) | 0.84 (<0.001) | 0.41 (0.07) | 0.22 (0.5) |
| | 3 days | | 0.66 (0.01) | 0.84 (<0.001) | 0.83 (<0.001) | 0.5 (0.07) | 0.23 (0.5) |
| | 5 days | | 0.60 (0.02) | 0.85 (<0.001) | 0.8 (<0.001) | 0.51 (0.04) | 0.6 (0.03) |
| | 11 days | | 0.64 (0.02) | 0.82 (<0.001) | 0.83 (<0.001) | 0.53 (0.04) | 0.56 (0.02) |
| LTB4 | 1 day | 0.52 (0.06) | | 0.78 (0.002) | 0.87 (<0.001) | 0.51 (0.04) | 0.12 (0.7) |
| | 3 days | 0.66 (0.01) | | 0.84 (<0.001) | 0.84 (<0.001) | 0.6 (0.012) | 0.16 (0.6) |
| | 5 days | 0.60 (0.02) | | 0.8 (<0.001) | 0.87 (<0.001) | 0.62 (0.011) | 0.01 (0.99) |
| | 11 days | 0.64 (0.02) | | 0.8 (<0.001) | 0.82 (<0.001) | 0.6 (0.02) | 0.03 (0.9) |
| IL-1 β | 1 day | 0.85 (<0.001) | 0.78 (0.002) | | 0.84 (<0.001) | 0.34 (0.2) | 0.1 (0.8) |
| | 3 days | 0.84 (<0.001) | 0.84 (<0.001) | | 0.96 (<0.001) | 0.4 (0.1) | 0.06 (0.9) |
| | 5 days | 0.85 (<0.001) | 0.8 (<0.001) | | 0.87 (<0.001) | 0.4 (0.1) | 0.15 (0.6) |
| | 11 days | 0.82 (<0.001) | 0.8 (<0.001) | | 0.88 (<0.001) | 0.51 (0.06) | 0.13 (0.6) |

Legend

Data describes the relationships between mediators and cell counts when one days data is used (here, the first day), or the mean of the first 3, 5 or all 11 day's data. Relationships are expressed as Pearson's Correlation Co-efficient (PCC) and p value.

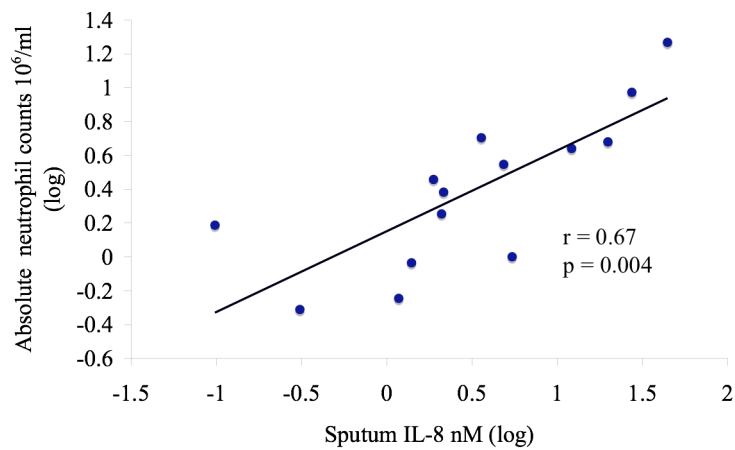


Figure 3.4a

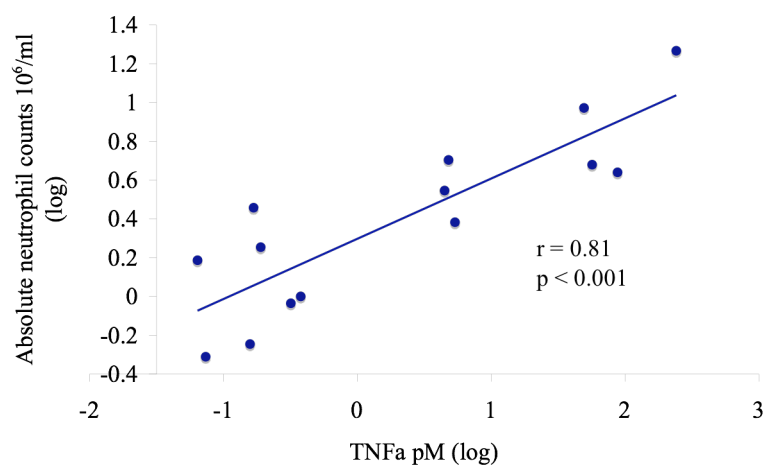


Figure 3.4b

Figure 3.4: The relationship between sputum neutrophil counts and sputum IL-8 (a), and TNF α (b).

In all figures the data has been log transformed. Each point represents the mean of eleven sequential samples for each patient. The correlation is described using Pearson's correlation coefficient (r) together with the significance of the relationships (p).

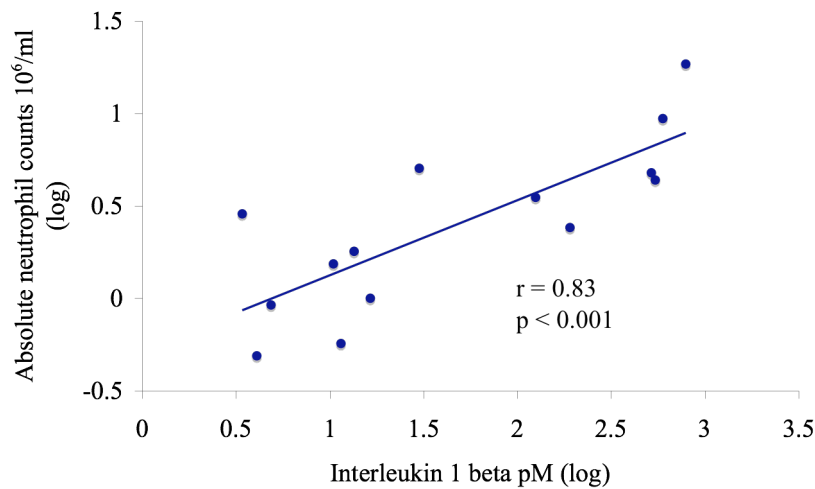


Figure 3.4: The relationship between sputum neutrophil counts and sputum IL-1 β .

In all figures the data has been log transformed. Each point represents the mean of eleven sequential samples for each patient. The correlation is described using Pearson's correlation coefficient (r) together with the significance of the relationships (p).

3.3.9 The relationship between sol phase inflammatory mediators, cell counts and disease

Correlations were seen between neutrophil counts, inflammatory mediators and markers of disease. IL-8 correlated with airflow obstruction (FEV₁/FVC ratio, $r = -0.4$, $p = 0.04$), but no other chemoattractant correlated with markers of airways disease. Neutrophil counts correlated both with smoking history (pack years, $r = 0.61$, $p = 0.008$) and negatively with FEV₁ ($r = -0.6$, $p = 0.01$).

Once again, the strength of the correlation (and the significance value) increased as more data points were used for each patient. Using the first days data for each patient, only IL-1 β correlated with BMI ($r = -0.5$, $p = 0.03$). Using the mean of the first three days for each patient, significant correlations were seen between BMI and IL-1 β ($r = -0.5$, $p = 0.02$) and TNF α ($r = -0.48$, $p = 0.03$). However, using the average of eleven data points per person, significant correlations were present between BMI and IL-1 β ($r = -0.41$, $p =$

0.05), $\text{TNF}\alpha$ ($r = -0.4$, $p = 0.05$) and LTB_4 ($r = -0.51$, $p = 0.03$); smoking history with sputum neutrophils (see above) and sputum macrophages ($r = 0.46$, $p = 0.05$); FEV_1 with sputum neutrophils (see above) and $\text{TNF}\alpha$ ($r = -0.45$, $p = 0.04$) and FEV_1/FVC ratio with $\text{IL-1}\beta$ ($r = -0.5$, $p = 0.03$) and IL8 (see above).

3.4 Discussion

There is marked variability in inflammatory mediators and cells, both between patients and within each patient over the course of a four week period in well characterised patients with GOLD stage III COPD and chronic bronchitis. Given the heterogenous nature of COPD, it is perhaps unsurprising that inflammatory cells and mediators vary between individuals and indeed this finding is in agreement with a wealth of previously published data (Rutgers, Timens et al. 2000; Biernacki, Kharitonov et al. 2003) .

The variability of the raw data within individuals was also marked, even in samples taken on consecutive days; and the fluctuations did not relate to changes in symptom scores, clinical findings or changes in FEV_1 (all of which remained stable). It is likely that the variability reflects changes in sampling methodology, contamination with oropharyngeal secretions or sputum humidification over the study period rather than individual patients showing wide fluctuations in the daily inflammatory load of the lungs without this impacting on daily symptoms. It may also be that only when changes in lung inflammatory load are sustained at a higher magnitude over consecutive days that it relates to changes in clinical status. A similar relationship has been demonstrated in patients with prednisone-dependent asthma where clinical indices deteriorate much later than the inflammatory changes in sputum (Pizzichini, Pizzichini et al. 1999). The studies confirm that serial sampling can be used to dramatically reduce within patient variability, and this reduction

decreases the number of subjects who would need to be recruited to adequately power putative studies where a reduction in neutrophil counts (or other sputum mediator concentrations) was the primary outcome.

The current study demonstrated clear inter-relationships between neutrophils and the majority of measured inflammatory mediators and some important clinical parameters of disease activity. Previous studies have reported indirect evidence that some biomarkers may be involved in the pathogenesis of COPD, including the presence of higher concentrations of mediators in disease compared with matched controls and the increases seen during exacerbations (Hill, Campbell et al. 2000; Sætta, Turato et al. 2001; Traves, Culpitt et al. 2002). This study is the first to describe the relationships seen between several biomarkers and the strength of these correlations in lung secretions.

Many cells and mediators have been found to be raised in COPD, but it is unlikely that all will be central to the pathogenesis of the disease. FEV₁ is the most consistent single predictor of survival and quality of life in COPD (Wise 2006). The relationships described between absolute sputum neutrophil counts and FEV₁, airflow obstruction and smoking history is highly suggestive that neutrophils are of importance in the pathogenesis of airflow obstruction.

Three neutrophil chemoattractants were studied, IL-8, LTB₄ and GRO α . In the current study, sputum IL-8 concentrations correlated with all other mediators and airflow obstruction in stable COPD; while previous work has found associations between IL-8 and various disease parameters (Yamamoto, Yoneda et al. 1997; Hacıevliyagil, Gunen et al. 2006; Parr, White et al. 2006), making it a clear candidate for modification of neutrophil

dependent pathophysiological parameters. IL-8 binds to both CXCR1 and CXCR2 on neutrophils with equal affinity. The inflammatory effects of GRO α are mediated mainly via CXCR2 receptors, and although this chemokine did not relate to other mediators or markers of disease, it maybe of importance in planned studies of CXCR2 antagonists. LTB₄ did not correlate with lung physiology, but did correlate inversely with BMI, perhaps suggesting a more systemic reflection of this mediator.

IL-1 β and TNF α also correlated with neutrophils, the chemoattractants and some clinical parameters of disease (in particular, BMI). It is recognized that weight loss and low body weight are common in COPD, and that low body weight is associated with increased mortality independent of lung function (Wilson, Rogers et al. 1989; Gray-Donaldson, Gibbons et al. 1996). Further studies have suggested that loss of muscle mass is the most important component of weight loss in COPD, and it is this which affects patients functionally (Mador 2002). Previous studies have linked a low BMI to systemic inflammation (Eid, Ionescu et al. 2001), however, in the current study, IL-1 β and TNF α in particular correlated negatively with BMI, supporting the hypothesis that an increase in lung inflammation is associated with a lower body weight.

This study used spontaneous sputum to assess pulmonary inflammation. Sampling techniques vary between studies of inflammation in COPD, however, the intra and inter-patient variability of measured mediators is unknown for most methodologies. Spontaneous sputum collection is non-invasive and has no inflammatory sequelae to limit sequential sampling. Furthermore, spontaneous sputum samples are the only medium where an artificial dilution factor is absent. However, there is still an inherent physiological dilution

present in all lung secretions, which will have an impact upon the ability to obtain accurate serial measurements.

Sputum is a mixture of lower airways and oropharyngeal secretions. Lower airway secretions are affected by exudation of plasma-derived proteins into the airway lumen (van Zuijlen, van de Graaf et al. 2001) which occurs both when the epithelial mucosa is damaged (Persson, Erjefait et al. 1998) and intact both structurally and functionally (Erjefalt and Sundler 1996) and presents a dilutional factor inherent in all sampling methodologies.

Oropharyngeal contamination can be reduced by patient education and supported by measuring the percentage of squamous cells in the sample, which should typically be less than 10%. In our study, squamous cells accounted for less than 1% of cells seen in the samples, which suggests that oropharyngeal contamination was low. Although we took the precaution of the patients rinsing their mouths prior to expectoration the rinse itself or any remaining oropharyngeal secretions could still dilute the sputum samples. Indeed this could explain some of the intra-patient variability seen day to day and similar effects could influence the between patient variability.

If mediator and cell values were overwhelmingly influenced by dilution, it would be predicted that mediator concentrations between patients would correlate well, as dilute samples would have low measured concentrations of mediators and concentrated samples would have high measured concentrations of mediators, even if all patients were in the same inflammatory state and mediator secretion was identical. The data for GRO α however suggests that this is not the case. In our study GRO α failed to correlate with any other mediator except CXCL8. This suggests firstly that the remaining correlations reflect

different inflammatory states per patient, and secondly that GRO α does not reflect inflammatory differences between patients nor the neutrophil content of airway secretions. Thus the data suggests that GRO α may be less important in the development or progression of COPD than other mediators. It is possible that GRO α has a different spectrum of activity than previously suspected (Traves, Culpitt et al. 2002), or that GRO α is maximally expressed in COPD patients with chronic bronchitis irrespective of the subsequent degree of inflammation. However, the fact that GRO α does not correlate with other biomarkers provides sound evidence that the relationships seen between other mediators are not purely dilutional in nature. Furthermore the correlations with systemic markers of disease (BMI, and FEV₁) and the use of repeated sampling (when any variable dilutional factors would be minimised) also makes this explanation unlikely.

By studying spontaneous sputum, we limited our recruitment to patients with chronic bronchitis. Although there remains some controversy as to the importance of sputum production in COPD, a body of evidence is emerging that suggests that chronic bronchitis may be a predictor of mortality and morbidity independent of FEV₁. Previous work has concluded that the inflammatory burden is higher in patients with chronic bronchitis compared with matched patients without (Gompertz, Hill et al. 2006). Furthermore, chronic mucus hyper-secretion is consistently associated with both an excess FEV₁ decline, an increased risk of subsequent hospitalization (Vestbo, Prescott et al. 1996) and death from respiratory infections (Prescott, Lange et al. 1995). Given the importance of this symptom in COPD and its relationship to inflammation, it is important to understand the relationships between inflammatory mediators in this group of patients. Studying spontaneous sputum samples prevents comparison with healthy controls, which is a limitation of the current study. However, it has been established that the mediators and cells measured in the current

study are raised in COPD compared with healthy controls even in studies using induced sputum (Gompertz, Bayley D et al. 2001; Franciosi, Page et al. 2006).

The wide inter-patient variability is determined from a relatively small cohort of patients. It is possible that others may have inflammation vastly outside the range documented here, which could affect the power calculations for a parallel study. However the single day data is consistent with stable state results in previous larger studies of over 100 patients with a wide range of FEV₁ (Gompertz and Stockley 2002) (Hill, Campbell et al. 2000; Gompertz, O'Brien et al. 2001; Gompertz, Hill et al. 2006) suggesting that the current data is representative. Of importance, the inter-patient variability was not significantly affected even by the use of the average of 11 collections per patient, suggesting that it represents true inter-patient variability.

It is difficult to comment on the likely bioavailability of any of the mediator measured in this thesis. The relationships seen with other biomarkers and with disease markers could be indicative of their overall activity, suggesting a central role in the pathogenesis of COPD. Alternatively, the relationships could be a confounding association, and other, as yet unidentified mediators may drive both the disease and the expression of the two cytokines. Certainly, further studies are warranted in order to try and determine the likelihood of activity in the stable state, but would be beyond the scope of this body of work.

Interestingly, the present study has found some similarity in results to the recently published ECLIPSE study (Singh, Edwards, et al, 2010). In this study, annual induced sputum samples and a blood sample were collected in 166 patients with COPD for two years. Here, the percentage of neutrophils in sputum correlated positively with disease severity (as characterised by GOLD) and negatively (albeit weakly) with FEV₁. There was no association with absolute neutrophil count and FEV₁, or association between the percentage neutrophil count and systemic inflammatory mediators. Sputum mediators were not studied. The systemic mediators measured in plasma in the ECLIPSE dataset differed from those measured in the present study. However, given the low concentration of plasma mediators, their bio-availability and short half-life it is perhaps unsurprising that plasma concentrations did not correlate with neutrophil percentages. It is disappointing that sputum mediators were not studied, as this may have reflected the inflammatory burden of disease better.

The ECLIPSE authors suggest that a lack of correlation between sputum numbers and FEV₁ may be due to the wide inter-patient variability in neutrophil numbers in sputum, even in patients with similar disease burdens. The current study is consistent with this hypothesis, as, in spontaneous sputum, the inter-patient CV of neutrophil counts was 115%. However, the ECLIPSE study does not comment on intra-patient variability, which would also affect results. In contrast with inter-patient variability, intra-patient variability can be reduced by serial sampling, increasing likelihood of identifying significant clinical associations. As stated, it is likely that serial sampling reduces variability by reducing the effects of variable sample dilution. Unlike spontaneous sputum, induced sputum has an artificial dilution factor (that of the inhaled hypertonic saline), which may impact upon sputum dilution and the variability of sputum cell counts and mediator concentrations, however, due to

inflammatory nature of the technique of sputum induction, it cannot be repeated on a daily basis. The ECLIPSE study suggests that over 125 patients would be needed to sufficiently power a study to identify a 50% reduction in induced sputum neutrophils. This is significantly greater than the numbers needed to identify the same differences using a single sample of spontaneous sputum, and over 5 fold higher than needed if serial sampling is employed. The ECLIPSE data may have failed to find an association between absolute neutrophil counts and FEV₁ either because no association truly exists, or because it was insufficiently powered to identify this relationship using a single induced sputum sample per patient. Neutrophil percentages in sputum are not as variable, both in terms of inter and intra-patient variability, and this reduction in variability will enhance the identification of associations, and may account for the association between percentage of neutrophils and FEV₁ in the ECLIPSE dataset. Finally, differences in sampling methodologies may account for differences seen between the current work and the ECLIPSE data, but there are currently insufficient studies comparing the two sampling techniques to allow for further comment.

In conclusion, this study documents the variability in neutrophil and mediator concentrations both between and within patients with stable severe COPD. Of importance, intra-patient variability is reduced significantly by averaging sequential biomarker measurement, which minimizes this variation irrespective of alterations in the inflammation signal. In addition, averaging sequential samples enabled relationships between mediators to be identified with more confidence providing at least 3 samples were obtained. This protocol would therefore be ideal for studying inflammatory change in COPD and can be used to power short term proof of concept studies.

The study demonstrates that positive correlations exist between neutrophil counts and inflammatory mediators, and inverse correlations exist between neutrophils, some mediators and clinical markers of disease severity. Out of the three chemoattractants studied, IL-8 appears to be more clinically relevant, correlating strongly with other mediators and lung function. Further studies of neutrophil migration focused upon these chemokines and its' receptors are indicated. The strong correlations seen with $\text{TNF}\alpha$ and $\text{IL-1}\beta$ have been pursued separately.

4 Neutrophil migration in the healthy elderly

4.1 Brief introduction

The increased morbidity and mortality from bacterial infections in older adults is highly suggestive of a sub-optimal neutrophil response and in support of this, there is clear evidence of a reduction in bactericidal (superoxide generation and degranulation) and phagocytic function in circulating cells isolated from older subjects (Lipschitz, Udupa et al. 1984; Fulop, Komaromi et al. 1986; Wenisch, Patruta et al. 2000; Butcher, Chahal et al. 2001).

In contrast, the affects of ageing upon neutrophil migration is poorly understood with studies showing contrasting results, including no differences (MacGregor and Shalit 1990; Esparza, Sanchez et al. 1996), and reduced movement towards chemotactic signals (Niwa, Kasama et al. 1989; Wenisch, Patruta et al. 2000; Fulop, Larbi et al. 2004). Reassessing this issue is of importance because studies of neutrophilic inflammation in COPD involves patients middle aged and above.

Previous studies used under agarose and Boyden chamber assays, and while these techniques can provide information about overall migratory patterns, they are not designed to study the migratory parameters of individual cells, such as speed or accuracy of movement and they cannot characterise any differences in migration.

Previous studies of neutrophil migration in COPD have not included closely age-matched controls; for example (Yoshikawa, Dent et al. 2007). Without a sound understanding of the impact of healthy ageing on neutrophil migration, it is difficult to be sure if differences reported reflect true disease-based processes or differences that are

seen generally with increasing age. Furthermore, to explore if chronic diseases such as COPD are driven by pathological or heightened ageing, it is important to identify and quantify any neutrophil behavioural differences that are age dependent .

The studies described in this chapter were performed to examine, firstly, overall migratory patterns of circulating neutrophils using the Under Agarose assay. This would provide a point of comparison with previous work. Secondly, a newer migrational assay was validated in order to provide quantitative detail of individual cell behaviour. Finally, a CXCR2 inhibitor was used and surface expression of CXCR1 and CXCR2 were measured to explore potential mechanisms or features that influence results.

Given the relevance of IL-8 in COPD (as proposed in Chapter 3) this was used as the main chemoattractant for studies included in this chapter. GRO α was also included to determine if any differences seen were CXCR1 or CXCR2 dependent.

4.2 Experiments

4.2.1 Study subject

Healthy older subjects consisted of volunteers over the age of 65, who had never smoked, had no evidence of chronic disease, normal spirometry and were medication free.

Healthy younger controls (who were under 35), had the same entry criteria and were gender matched to the older subjects. All studies were approved by the local Research and Ethics Committee and all subjects gave their informed written consent.

4.2.2 Isolation of blood neutrophils

Neutrophils were isolated from the whole blood as described previously (methods 2.5.7) (Mikami, Llewellyn-Jones et al. 1998). The neutrophils (>95% pure, > 97% viable, by exclusion of trypan blue) were resuspended in buffer (RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK) containing 0.15% bovine serum albumin (Sigma-Aldrich, UK) at a concentration of 2 million cells/ml.

4.2.3 Neutrophil Chemotaxis

4.2.3.1 Under Agarose Assay

Neutrophil chemotaxis was firstly assessed using the under-agarose assay, as described previously (methods section 2.7.1) (Nelson, Quie et al. 1975). Following dose response experiments, peripheral neutrophils isolated from old (n=6) or young (n=6) subjects migrated towards 100nM IL-8 and both a negative control (Assay buffer: RPMI 1640 (Flow Laboratories, UK) containing 0.15% bovine serum albumin (Sigma-Aldrich)) and positive control (fMLP 100nM, Sigma-Aldrich). Migration is expressed in mm, with differentiation between chemotaxis and chemokinesis. Each experimental condition was repeated three times per subject and averaged to form an intra-subject mean. Each intra-subject mean was also averaged as an overall mean for both subject groups (Healthy older subjects or healthy young controls).

4.2.3.2 Modified Dunn Chamber

Neutrophil chemotaxis was then measured using a modification of the Dunn chamber. Full methodological details are included in the methods (section 2.7.2). Chemotaxis was assessed as described in general methods (section 2.7.2.3).

Following appropriate dose responses, neutrophils migrated in gradients of a negative control (Assay buffer, see above), IL-8 (RnDSystems) (10nM) and GRO α (RnDSystems) 100nM. Neutrophil migration was then assessed towards IL-8 (10nM), following neutrophilic incubation with 5nM of a CXCR2 antagonist (kindly provided by AstraZeneca) (5nM) for 45 minutes at room temperature. The CXCR2 antagonist provided by AstraZeneca was an unknown compound, with no documentation of its structure, specificity or potency.

4.2.4 Flow Cytometry

CXCR1 and 2 receptor expression on isolated neutrophils was measured as described in section 2.8 of the general methods. All measurements were carried out in triplicate, and a median result used for each subject. Anti-CXCR1 (IgG2A stock concentration 50 μ g/ml) diluted to 2ng/ml in 2% BSA PBS, Anti-CXCR2 (IgG2A stock concentration 50 μ g/ml) diluted to 3ng/ml in 2% BSA PBS and an isotype matched irrelevant FITC labelled IgG_{2A} (stock concentration 200 μ g/ml), diluted to 2ng/ml in 2% BSA PBS, was used as a control. Both were incubated on ice for 20 minutes and the resuspended cells were analysed following FACS within twenty minutes of preparation. Samples from young and older subjects were read at the same time point to allow comparison of receptors.

CXCR1 and 2 receptor expression over time was assessed by measuring the receptors at 3 time points (time 0, +1 hour and + 2 hours). Samples were incubated on ice between readings. The effects of cell stimulation on CXCR1 and CXCR2 expression were investigated by measuring CXCR2 receptors following neutrophil incubation with three

concentrations of IL-8 (1, 10 and 100nM) and a negative control (RPMI 1640). Neutrophils were incubated with IL-8 for ten minutes prior to preparation for cell sorting. The dose range for IL-8 was chosen as this represented a physiological spread of concentrations that the neutrophil would encounter on migrating towards the sputum and were chosen following appropriate validity experiments (see section 4.3.5).

4.2.5 Statistical analysis

Migrational differences seen using the Under Agarose assay were compared using the mean chemotactic response, chemokinetic response and chemotactic differential (see figure 2.10) of experiments completed in triplicate for each individual. Migrational differences seen using the Modified Dunn Chamber were compared using the mean data of 10 cells per experiment, per subject. Differences in chemotaxis criteria were compared using an Independent *t* test. Differences in CXCR2 surface expression were compared using a Mann Whitney U test. A *p* value of less than 0.05 was considered to be statistically significant.

Under agarose studies were exploratory, and sample size calculations were not performed. For the Modified Dunn chamber, sample size calculations were based upon a two group parallel comparison, to provide an 80% power of detecting a 25% difference in mean speed at the 5% level of significance. For CXCR2 expression, sample size calculations were based upon a two group parallel comparison, to provide an 80% power of detecting a 25% difference in expression at the 5% level of significance (both as described in section 2.9).

4.3 Results

4.3.1 Under agarose assay: Validation

The neutrophil chemotactic response was maximal towards IL-8 at a concentration of 100nM. See figure 4.1.

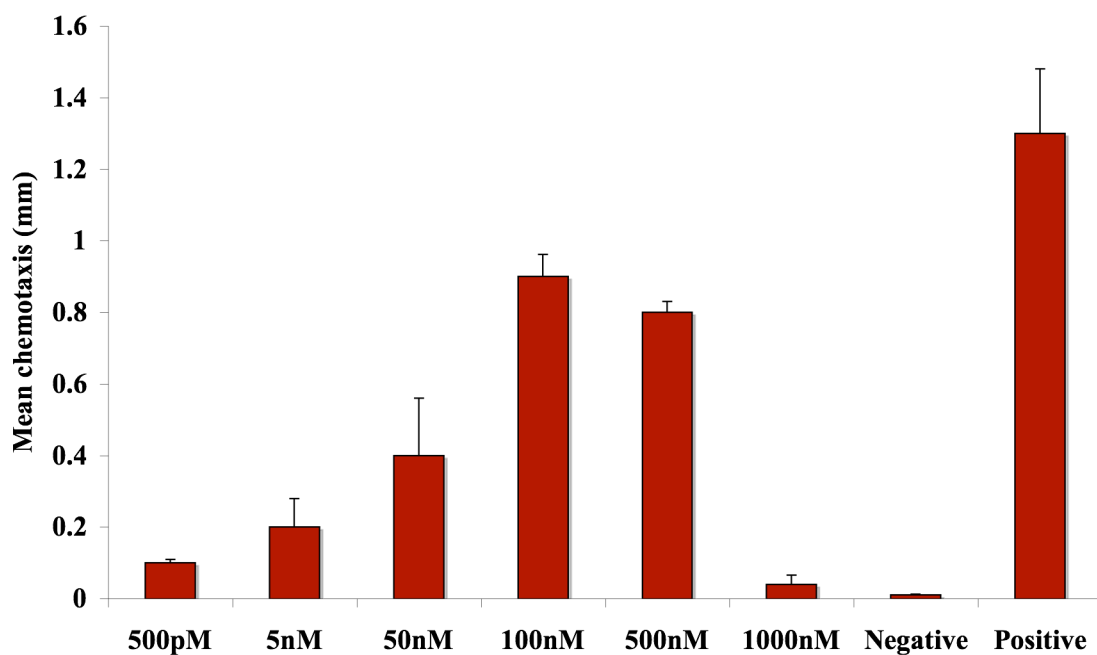


Figure 4.1. The chemotactic response of neutrophils towards increasing concentrations of IL-8

The results shown are the mean (and SEM) values of six experiments. Negative control is buffer (RMPI (Sigma-Aldrich)). Positive control is fMLP (Sigma-Aldrich) 10^{-7} M.

4.3.2 Under agarose assay: Results

4.3.2.1 Baseline characteristics

Demographic data for the old and young healthy controls are shown in Table 4.1.

Table 4.1. Ageing studies Under agarose: Demographic data for healthy old and young controls

| | <u>Healthy older subjects</u> | <u>Young Controls</u> |
|--------------------------------------|-------------------------------|-----------------------|
| N | 6 | 6 |
| Age (years) | 71 (66 - 84) | 27 (21 – 33) * |
| Male | 4 (66%) | 3 (50%) |
| Smoking status | All never | All never |
| Pack years | 0 | 0 |
| FEV ₁ (percent predicted) | 94 (7.9) | 113 (4.6) |
| FEV ₁ /FVC (%) | 82 (5.3) | 85 (3.9) |
| BMI | 23 (2.7) | 21 (3.2) |

Legend

Normally distributed data is presented as mean (with range or SEM). Categorical data is presented with percentages given in parentheses. * = significantly different from healthy older subjects ($p \leq 0.05$).

4.3.2.2 Neutrophil migration towards IL-8

Table 4.2 describes the mean chemotaxis, mean chemokinesis and mean chemotactic differential for each group. The chemotactic differential for circulating neutrophils from older subjects was reduced in gradients of both IL-8 and fMLP ($p = 0.001$ for both). This was due to a reduction in chemotaxis towards both inflammatory mediators ($p = 0.04$ and 0.001 respectively), without any difference in chemokinesis. See figure 4.2.

Table 4.2. The mean chemotaxis, chemokinesis and chemotactic differential for each subject group.

| | <u>Healthy older subjects</u> | <u>Healthy young controls</u> |
|--------------------------------|-----------------------------------|-----------------------------------|
| <u>Negative control</u> | | |
| Chemotaxis | 0.39 (0.02) | 0.41 (0.02) |
| Chemokinesis | 0.41 (0.02) | 0.38 (0.02) |
| Chemotactic differential | -0.02 (0.02) | 0.02 (0.02) |
| <u>IL-8</u> | | |
| Chemotaxis | 0.71 (0.03) | 0.85 (0.03) * |
| Chemokinesis | 0.44 (0.01) | 0.41 (0.01) |
| Chemotactic differential | 0.26 (0.03) | 0.43 (0.03) * |
| <u>Positive Control</u> | | |
| Chemotaxis | 0.79 (0.02) | 1.06 (0.03)* |
| Chemokinesis | 0.44 (0.03) | 0.43 (0.02) |
| Chemotactic differential | 0.39 (0.03) | 0.59 (0.06)* |

Legend.

Results are overall means for each group (migration in mm). * = significantly different from healthy older subjects.

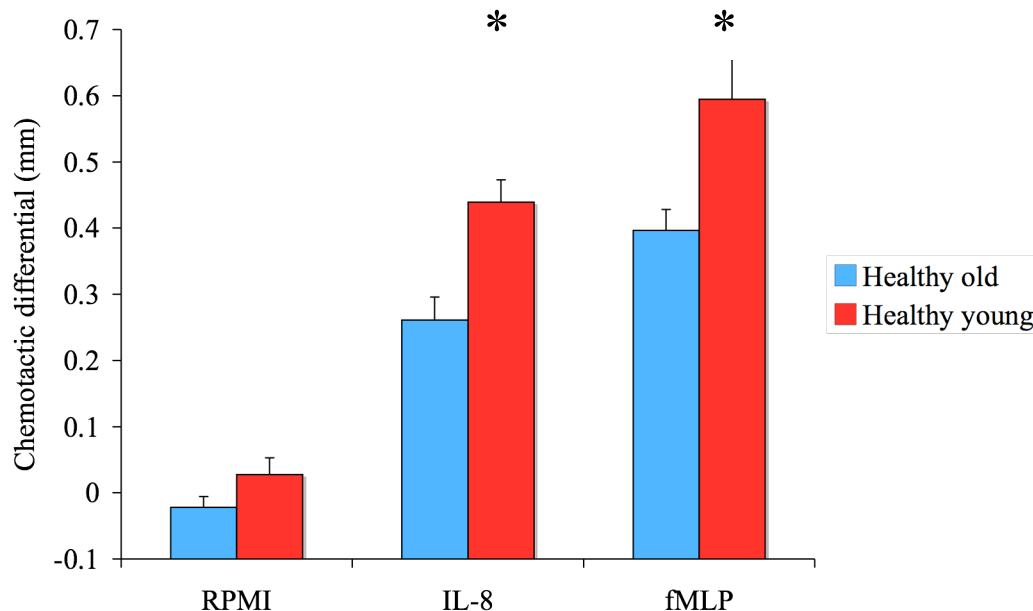


Figure 4.2a. Under agarose chemotaxis in the healthy elderly: The chemotactic differential response of circulating neutrophils from older and younger subjects (legend after figure 4c).

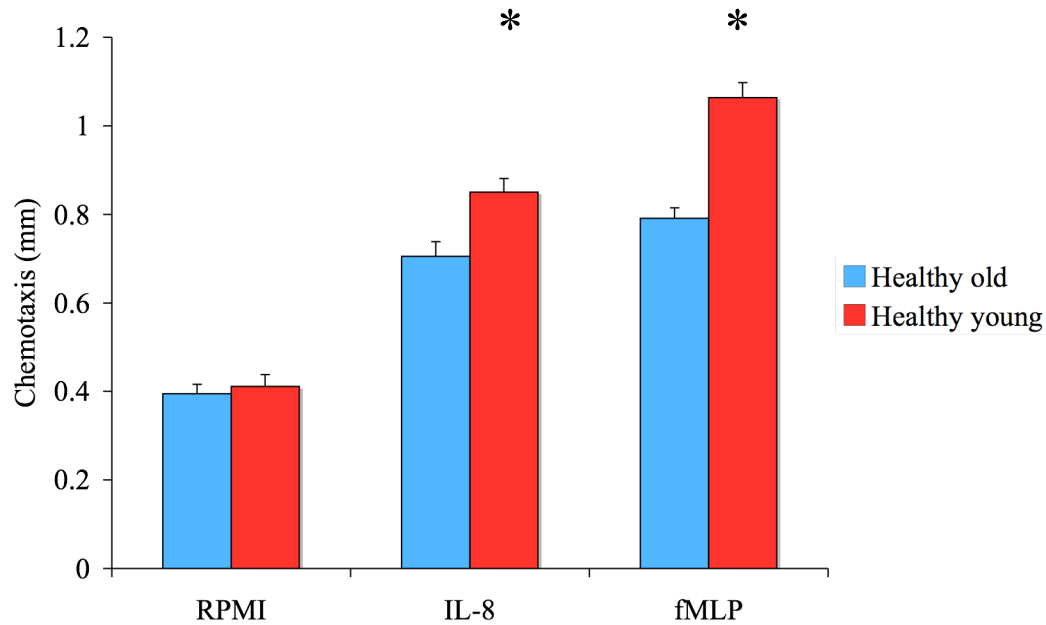


Figure 4.2b. The chemotactic response of circulating neutrophils from older and younger subjects (legend after figure 4c).

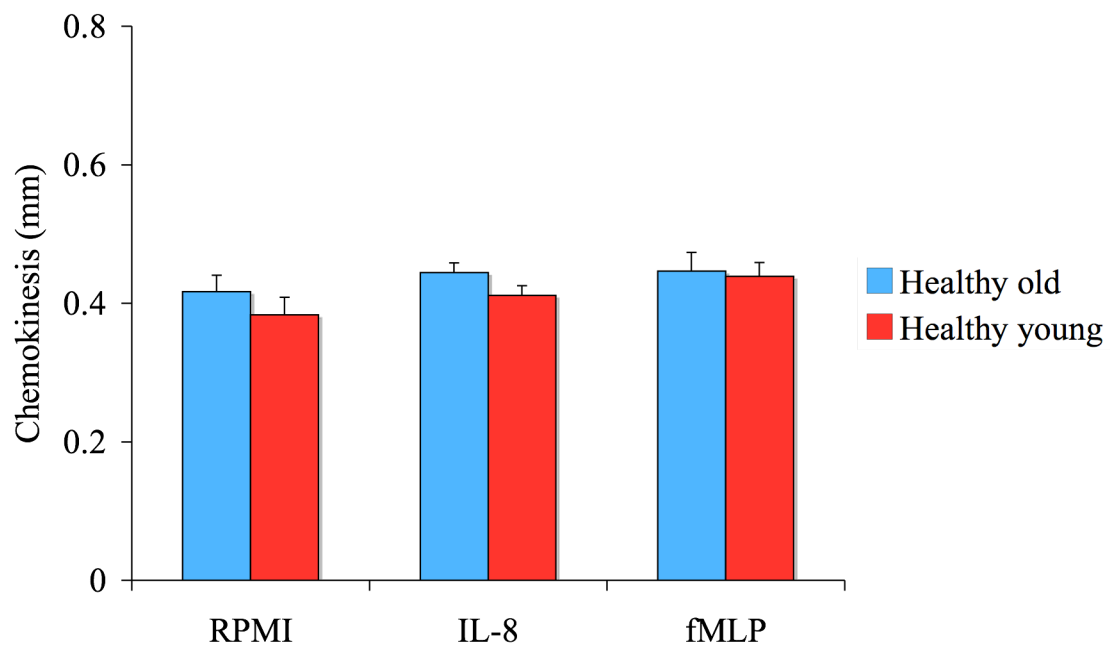


Figure 4.2c. The chemokinetic response of circulating neutrophils from older and younger subjects.

Legend for figures 4.2a, b and c

The results show the overall mean migratory parameter (and SEM) for both groups. RPMI 1640 (Sigma-Aldrich) is the negative control, and the positive control is fMLP 10^{-7} M (Sigma-Aldrich). See Methods 2.7.1 for an explanation of terms. * = significant difference from healthy older subjects ($p < 0.05$).

These data confirm that healthy ageing is associated with differences in neutrophil migration, including a reduction in chemotactic differential. This was caused by a reduction in chemotaxis with preserved chemokinesis.

4.3.3 Modified Dunn Assay : Validation

A Modified Dunn chamber was used to characterise individual cell behaviour and study separate components of migration, including speed, velocity, changes in directional orientation (persistence) and overall migratory accuracy (chemotactic index). This assay needed to be modified prior to use with neutrophils (see below for validation).

4.3.3.1 Coverslip preparation

For optimal migration, neutrophils needed to adhere to the coverslip, but overcome static forces to enable chemotaxis. In order to ascertain the optimal conditions for neutrophil adhesion to the coverslip, migration was tested using uncoated coverslips, or those coated in 0.1% fibronectin from human plasma (Sigma-Aldrich, UK) or albumin (1%, 7.5%, 15%, 30%) (Sigma-Aldrich, UK). Optimal adherence was assessed by determination of the percentage of adhered neutrophils and the percentage of adhered cells that were able to migrate towards IL-8 and was achieved using 7.5% bovine serum albumin for coating of the coverslips (Sigma-Aldrich) (see figure 4.3).

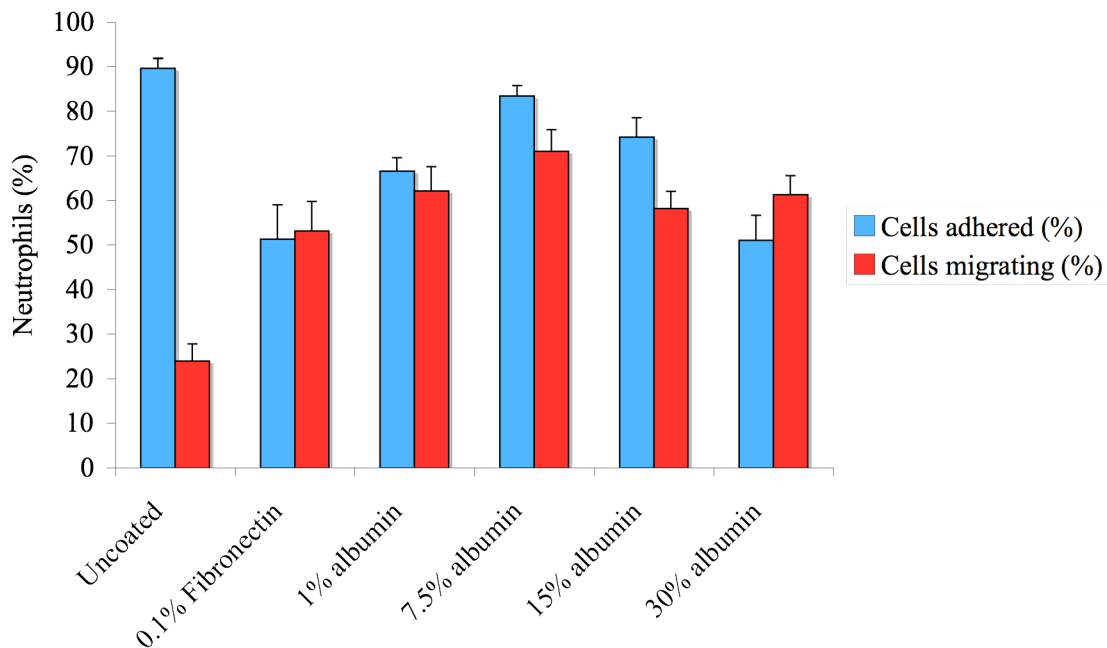


Figure 4.3. Assessing optimal neutrophil adherence to coverslip

Legend.

The figure demonstrates the effect of uncoated or coated coverslips on neutrophil adherence and subsequent migration to IL-8. Adhered neutrophils = adhered neutrophils/total neutrophil count per field x 100. Migrating cells = migrating neutrophils / adhered neutrophils per field x 100. The results shown are the mean (and SEM) of 6 experiments.

4.3.3.2 Assay buffer

The intra-patient percentage of neutrophils migrating towards IL-8 was found to differ over time using HBSS (1.3mM CaCl_2 , 5.0mM KCL, 0.3mM KH_2PO_4 , 138mM NaCl, 0.3mM Na_2HPO_4 , 0.8mM MgSO_4 , 10mM HEPES-Na, pH7.6) (the original buffer for the assay). It was theorised that this may be due to a lack of glucose in the buffer. To determine the optimal buffer for migration, neutrophils were suspended in either HBSS or RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK). RPMI 1640 (Sigma-Aldrich) was used in all experiments following time response experiments, where neutrophils were incubated with either buffer for varying times from purification

before being placed on the coverslips (see figure 4.4 and 4.5). These time response experiments also proved that neutrophils were able to be stored in RPMI for up to 4 hours without it effecting adherence (figure 4.4) migratory function (figure 4.5).

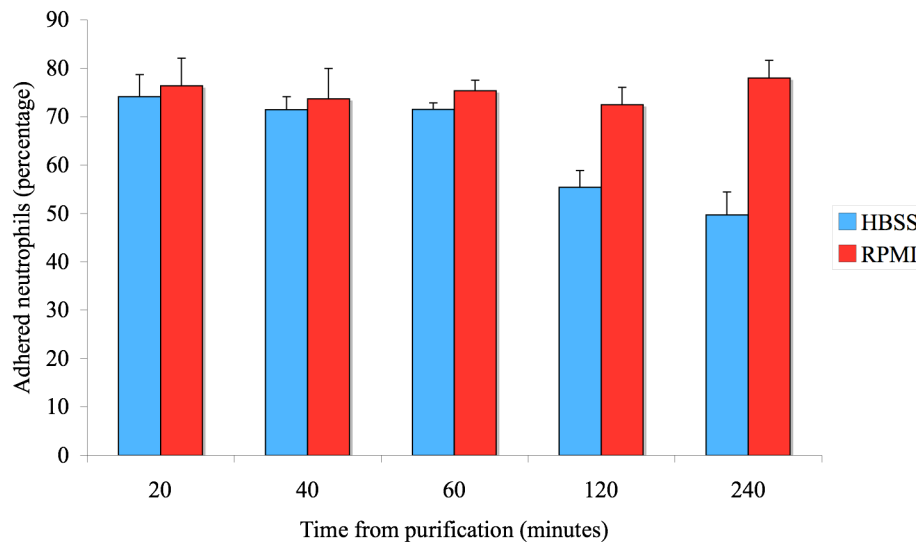


Figure 4.4. The affects of differing buffers on neutrophil adherence over time (legend following figure 4.5).

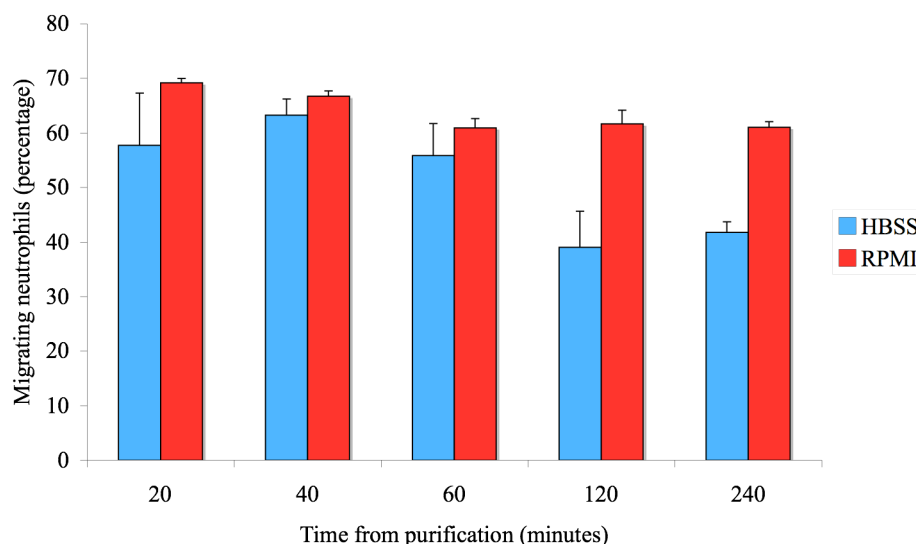


Figure 4.5. The affects of differing buffers on cell migration over time.
Legend for figure 4.4 and 4.5.

The graphs demonstrate the effect of HBSS or RPMI buffer on neutrophil adherence and migration to IL-8 over time. Adherent cells = adherent cells/ total number of cells per field x 100. Migrating cells = migrating neutrophils / adhered neutrophils per field x 100. The results shown are the mean (and SEM) of 3 experiments.

4.3.3.3 Dose response experiments

Dose response experiments for increasing concentrations of IL-8 and GRO α were performed to establish the concentrations where neutrophil chemotaxis was maximal. The neutrophil chemotactic response was maximal towards IL-8 at a concentration of 10nM, therefore this concentrations was used for subsequent experiments (see figure 4.6). The neutrophil chemotactic response was maximal towards GRO α at a concentration 100nM and so this concentration was used for subsequent experiments (see figure 4.7).

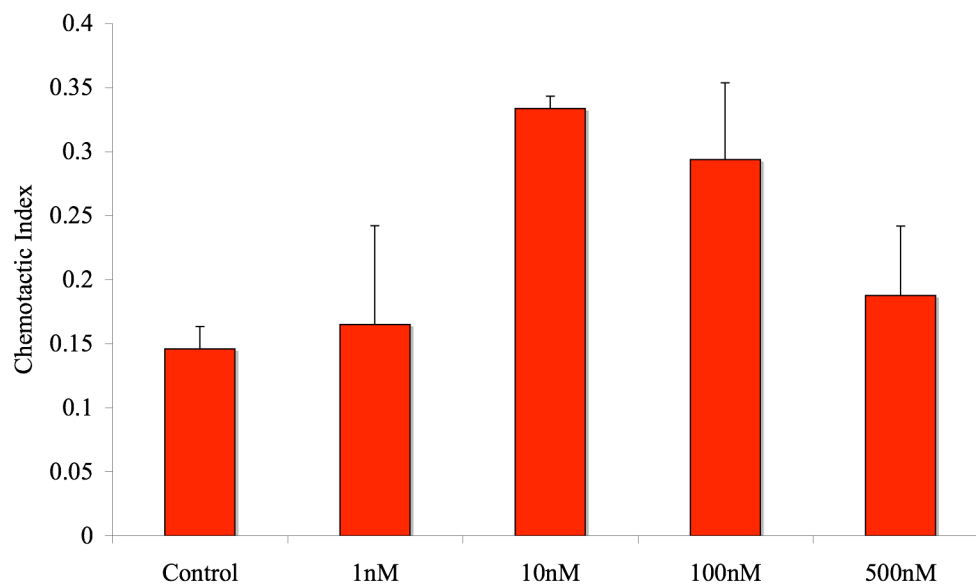


Figure 4.6. The chemotactic index of neutrophils towards increasing concentrations of IL-8 in the modified Dunn chamber (legend after figure 4.7).

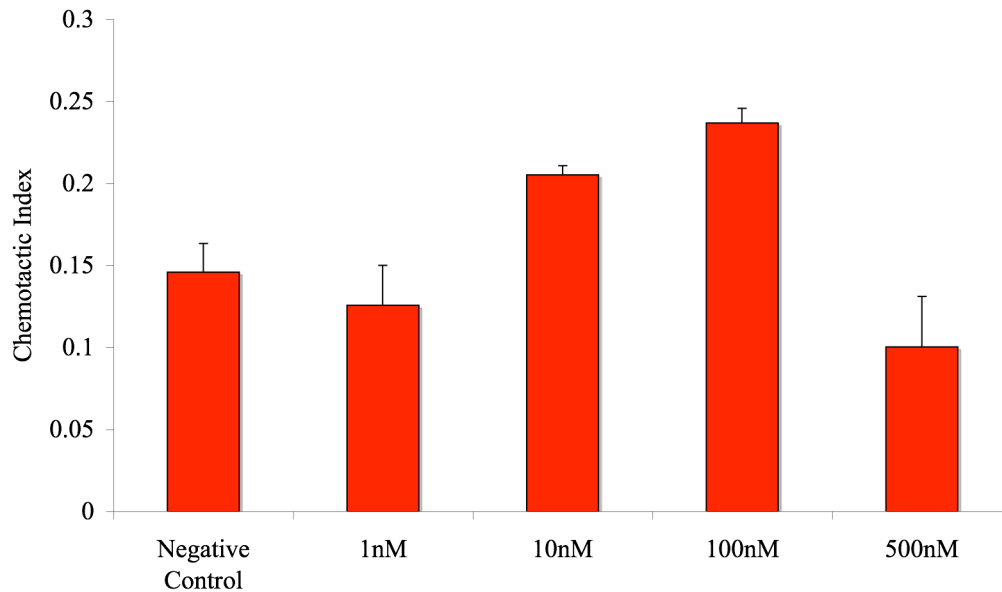


Figure 4.7. The chemotactic index of neutrophils towards increasing concentrations of GRO α in the modified Dunn chamber.

Legend for figure 4.6 and 4.7

The results shown are the mean chemotactic index (and SEM) values of four experiments related to chemoattractant concentration. For an explanation of terms see section 2.7.2.3.

Dose response experiments were also performed on neutrophils pre-incubated with increasing concentrations of the CXCR2 antagonist to establish a dose where chemotaxis inhibition was maximal without impairing adherence or random chemokinesis (see figure 4.8 and 4.9). Concentrations of 5nM were found to be optimum and were used for all subsequent experiments for this partial inhibition of migration.

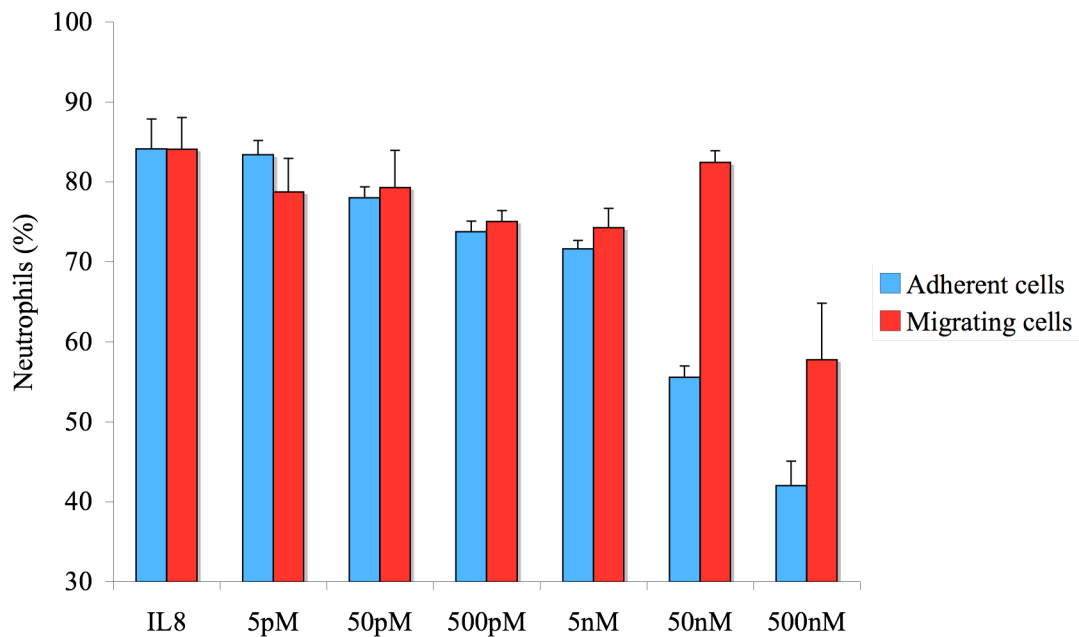


Figure 4.8. The affect of increasing concentrations of CXCR2 antagonist on neutrophil adherence to the coverslip and migration

Legend. The graph demonstrates the effect of increasing concentrations of a CXCR2 antagonist on neutrophil adherence and subsequent migration to IL-8 10nM. Adhered neutrophils = adhered neutrophils/total neutrophil count per field x 100. Migrating cells = moving neutrophils / adhered neutrophils per field x 100. The results shown are the mean (and SEM) of 4 experiments.

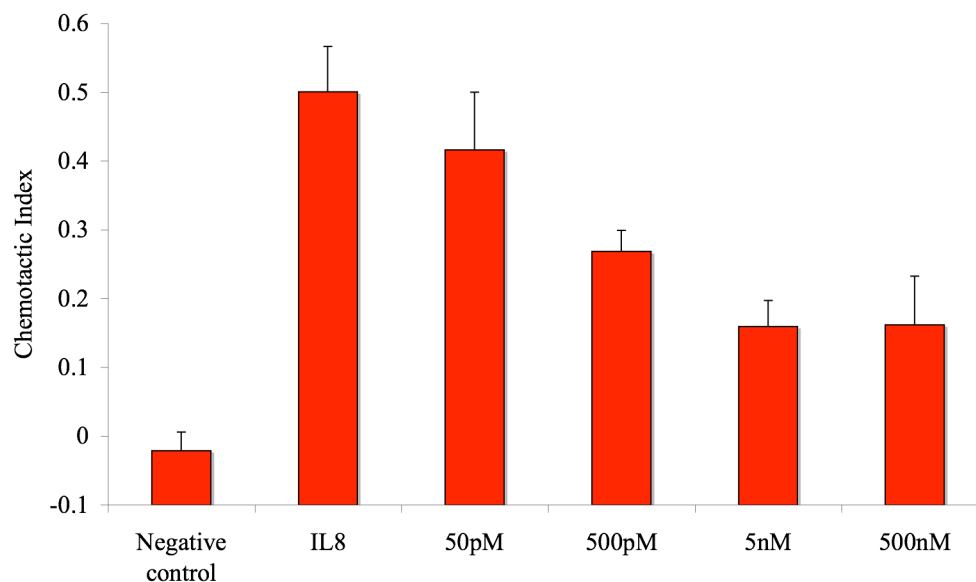


Figure 4.9. The chemotactic response of neutrophils towards 10nM IL-8 following incubation with increasing concentrations of the CXCR2 antagonist.

Legend. The results shown are the mean chemotactic index (and SEM) values of four experiments. For an explanation of terms see section 2.7.2.3.

4.3.3.4 Power calculations

Power calculations were performed to determine the numbers needed to confirm a 50 – 25% reduction in migrating neutrophil speed with 80% power and at the 5% significance level, using the mean of data from 10 cells per subject. Table 4.3 summarises the mean speed of neutrophil migration, the variation in inter-patient migratory speeds and the number of subjects required in each group. Based on these data, 20 participants were recruited to each subject group, to provide a clear opportunity to see any differences in migration of this magnitude.

Table 4.3. The numbers needed in a parallel design study to detect varying difference in neutrophil migratory speed between groups

| | |
|--|------|
| Mean speed ($\mu\text{m}/\text{minute}$) | 3.36 |
| SEM | 0.28 |
| Co-efficient of variation | 20% |
| Number of subjects needed in each group to detect: | |
| 50% difference in speed | 4 |
| 40% difference in speed | 5 |
| 35% difference in speed | 6 |
| 30% difference in speed | 8 |
| 25% difference in speed | 11 |

4.3.4 Modified Dunn Chamber: Results

4.3.4.1 Baseline characteristics

Demographic data for the healthy elderly and young controls is shown in Table 4.4. The results shown are for FEV₁ expressed as a percentage of the value predicted for the subject's age, gender and height (British Thoracic Society 1994) and the ratio of FEV₁ to forced vital capacity (FEV₁/FVC).

4.3.4.2 Neutrophil migration towards IL-8

Figure 4.10 and 4.11 provide examples of the cell tracks that were recorded for a representative subject in each group.

Table 4.4: Demographic data for elderly and young healthy subjects

| | Younger subjects | Older subjects |
|------------------------------|------------------|-----------------|
| N | 20 | 20 |
| Age (range) | 28 (21 – 34) | 76 (66 - 89) |
| Male (%) | 10 (50%) | 10 (50%) |
| Smoking history (pack years) | 0 | 0 |
| FEV1 percent predicted | 113 (93 - 131) | 117 (101 - 144) |
| FVC percent predicted | 121 (108 - 132) | 130 (109 - 145) |

Legend

Age and lung function parameters are presented as the mean (with the range in parentheses). Gender is the number of males in each group, together with the percentage in parentheses.

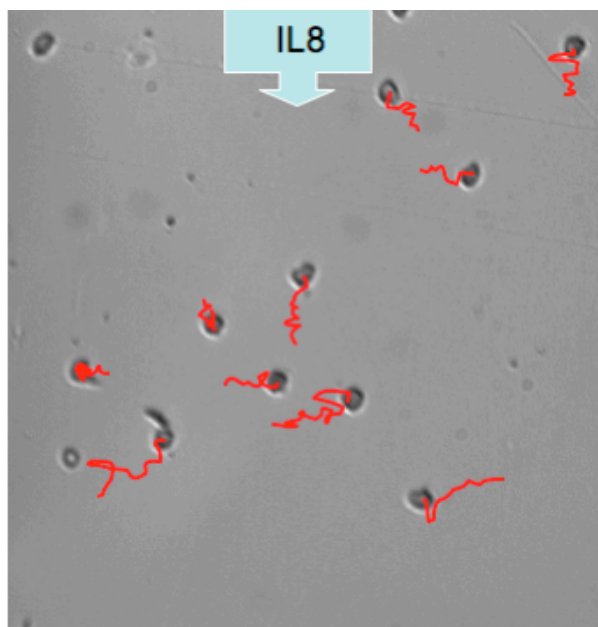


Figure 4.10 Neutrophil migratory tracks from a representative subject aged > 65.

Legend: The images show the final neutrophil positions within the Dunn chemotaxis chamber following 20 minutes of time-lapse recording. The blue arrow at the top of each picture represents the direction of the source of the chemotactic signal (IL-8 at a concentration of 10nM for all figures). The tracks (shown in red) indicate the path of migration for each cell analysed: each track begins where the cell was positioned at the start of recording and ends where the cell was positioned at the end of recording.

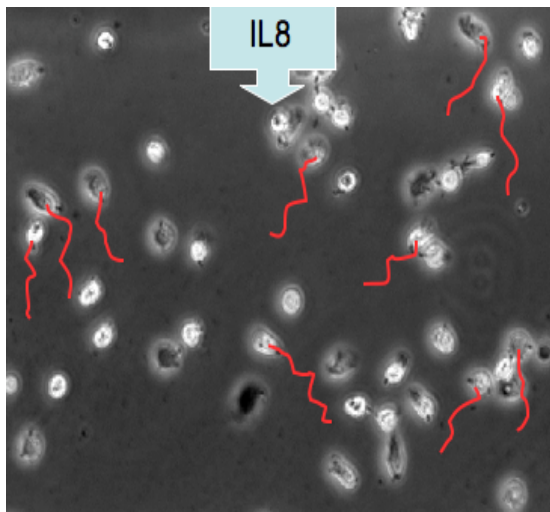


Figure 4.11. Neutrophil migratory tracks from a representative subject aged < 35

Legend: The images show the final neutrophil positions within the Dunn chemotaxis chamber following 20 minutes of time-lapse recording. The blue arrow at the top of each picture represents the direction of the source of the chemotactic signal (IL-8 at a concentration of 10nM for all figures). The tracks (shown in red) indicate the path of migration for each cell analysed: each track begins where the cell was positioned at the start of recording and ends where the cell was positioned at the end of recording.

The migratory tracks from neutrophils from older subjects appeared to migrate along a more convoluted pathways than those isolated from young subjects.

Speed

Peripheral neutrophils from both young and elderly subjects demonstrated increased speed of migration in the presence of IL-8 compared with the negative control (mean difference between speed of migration in RPMI or IL-8: elderly 0.7 $\mu\text{m}/\text{minute}$, 95% confidence intervals 0.48 – 0.92 $p = 0.0001$; young 1.2 $\mu\text{m}/\text{minute}$, 95% confidence intervals 0.9 – 1.4, $p = 0.0001$). However, elderly neutrophils displayed increased random migration (increased speed) in the presence of the negative control (mean difference 0.47 $\mu\text{m}/\text{minute}$, 95% confidence intervals 0.24 – 0.7, $p = 0.0001$) but this

was not the case in the presence of IL-8, where the speed of migration was the same.

See figure 4.12.

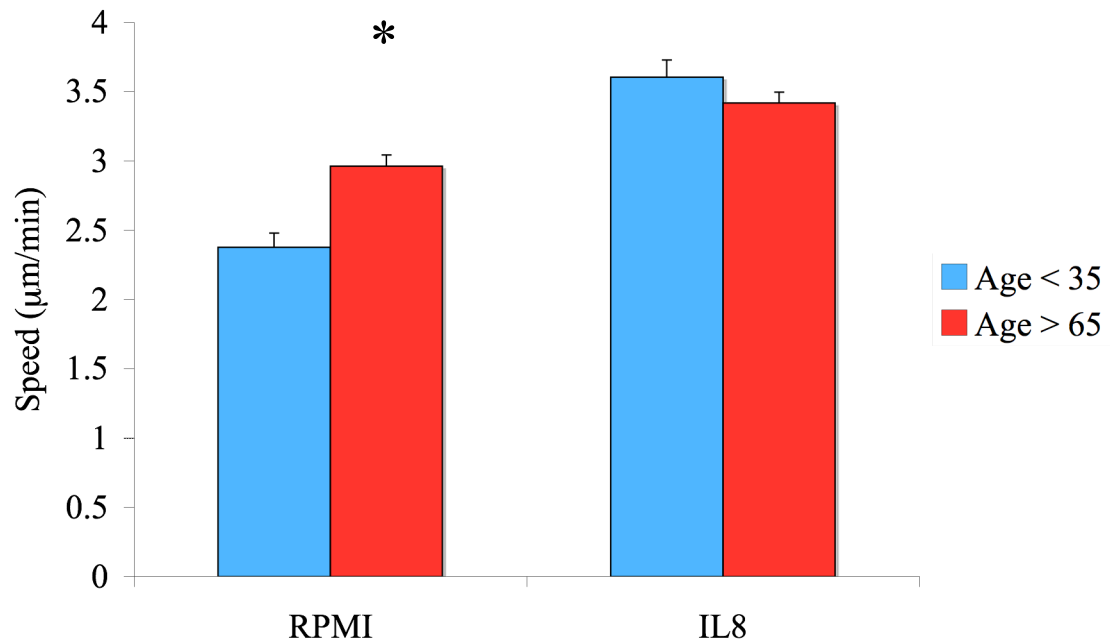


Figure 4.12: The mean speed of migrating neutrophils from elderly and younger subjects in shallow gradients of IL-8.

Legend: Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. * = $p < 0.05$. Neutrophils from older subjects moved with significantly greater speed in the presence of RPMI than the younger controls (mean difference $0.47 \mu\text{m per minute}$, $p = 0.0001$) but not in the presence of IL-8, where there was no difference.

Velocity

Peripheral neutrophils from both young and elderly subjects demonstrated increased velocity of migration in the presence of IL-8 compared with the negative control (mean difference between velocity of migration in RPMI or IL-8: elderly $0.52 \mu\text{m/minute}$, 95% confidence intervals $0.29 - 0.71$, $p = 0.001$; young $1.28 \mu\text{m/minute}$, 95% confidence intervals $0.6 - 1.5$, $p < 0.0001$). However, elderly neutrophils migrated with less velocity towards IL-8 (mean difference $0.73 \mu\text{m/minute}$, 95% confidence intervals $0.48 - 0.98$, $p < 0.0001$). See figure 4.13.

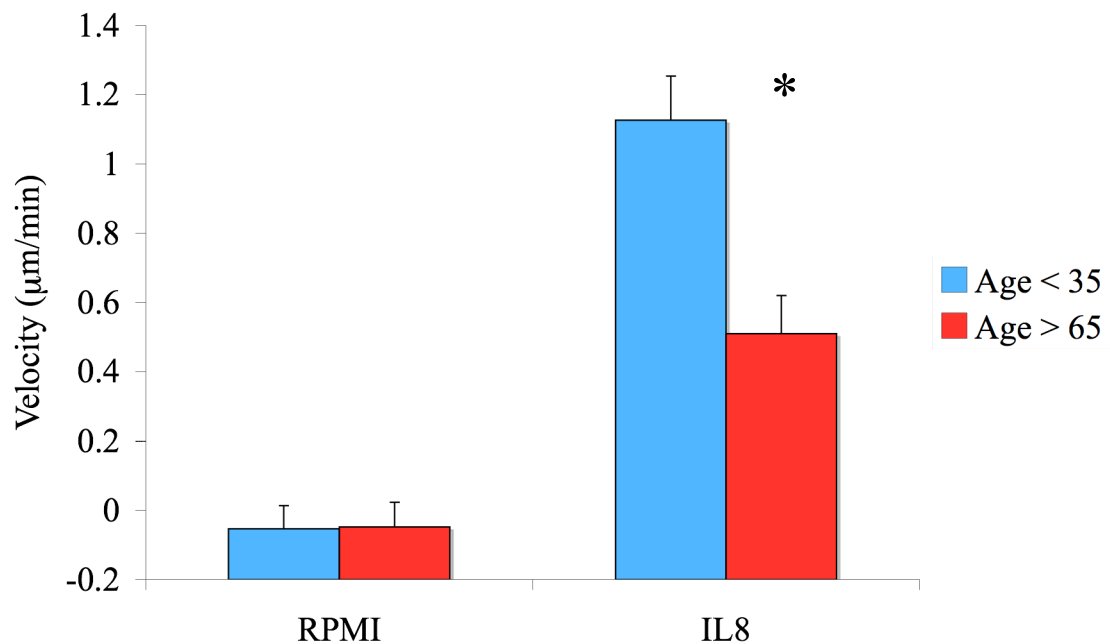


Figure 4.13. The mean velocity of migrating neutrophils from elderly and younger subjects in the presence of IL-8

Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. * = $p < 0.05$. Neutrophils from older subjects moved with significantly less velocity in the presence of IL-8 than those from the younger controls (mean difference $0.73 \mu\text{m}$ per minute, $p < 0.0001$).

Directional Persistence

Peripheral neutrophils from both young and elderly subjects demonstrated less changes of cellular direction when migrating towards IL-8 compared with the negative control (mean difference between persistence of migration in RPMI or IL-8: elderly mean difference 0.09, 95% confidence intervals 0.04 – 0.15, $p = 0.001$; young, mean difference 0.31, 95% confidence intervals 0.24 – 0.38, $p < 0.001$). However, elderly neutrophils migrated with less directional persistence towards IL-8 (mean difference 0.14, 95% confidence intervals 0.12 – 0.25, $p=0.0001$). See figure 4.14.

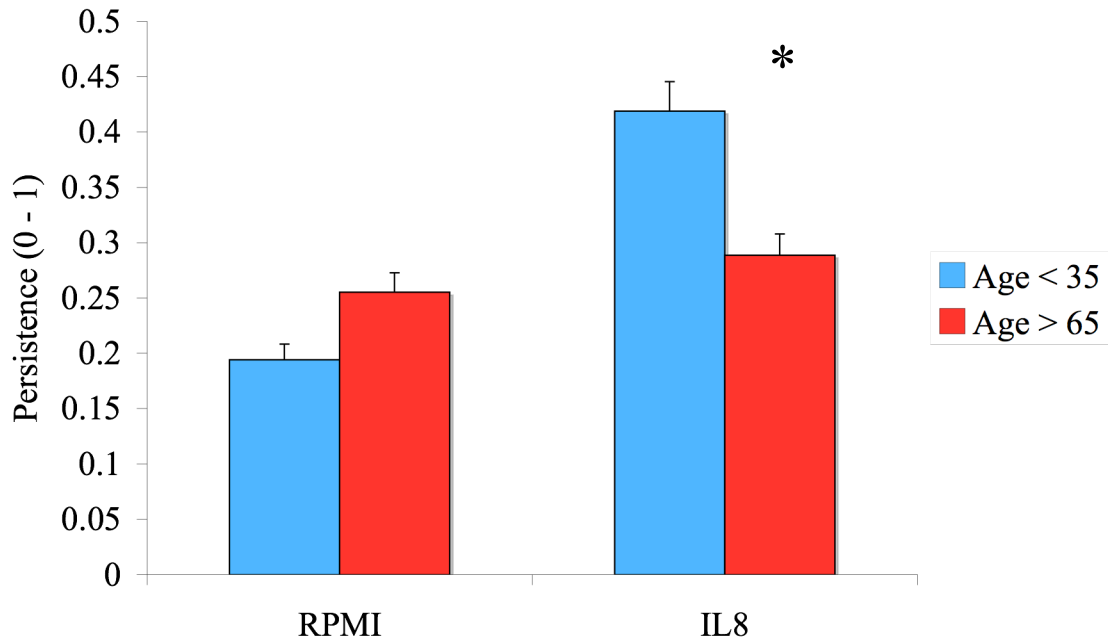


Figure 4.14. The mean persistence of migrating neutrophils from elderly and younger subjects in gradients of IL-8.

Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. * = $p < 0.05$. Neutrophils from older subjects moved with significantly less directional persistence in the presence of IL-8 than the younger controls (mean difference 0.14, $p = 0.0001$).

Chemotactic accuracy

Peripheral neutrophils from both young and elderly subjects demonstrated more chemotactic accuracy when migrating towards IL-8 compared with the negative control (mean difference between accuracy of migration towards the source of RPMI or IL-8: elderly 0.17, 95% confidence intervals 0.1 – 0.2, $p = 0.001$; young 0.4, 95% confidence intervals 0.34 – 0.47, $p < 0.0001$). However, elderly neutrophils migrated with less accuracy towards IL-8 (mean difference 0.23, 95% confidence intervals 0.16 – 0.29, $p < 0.0001$). See figure 4.15.

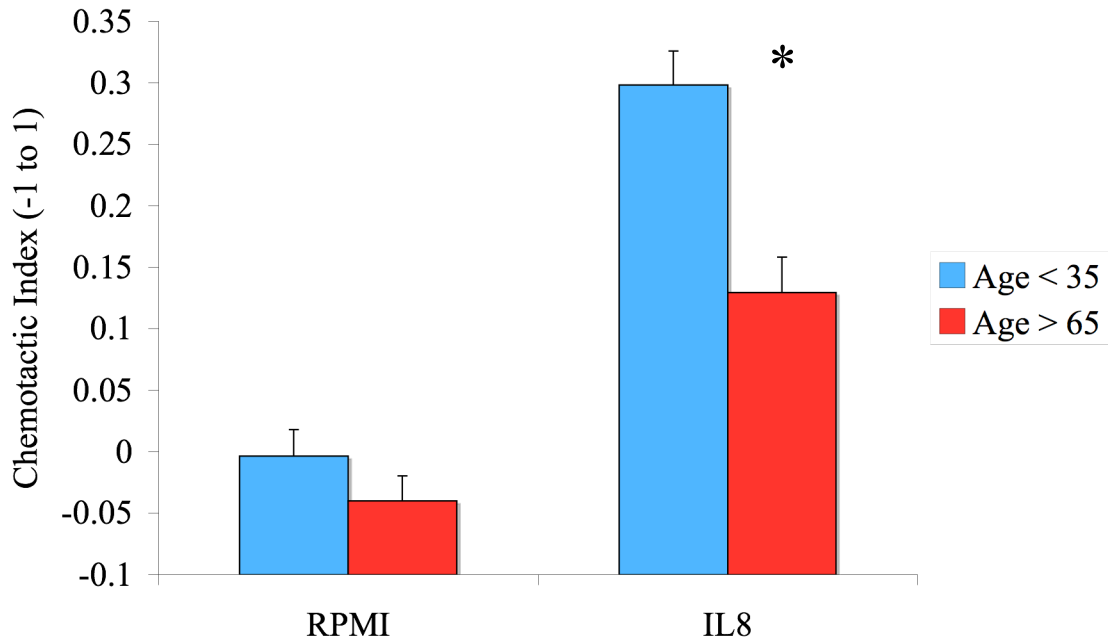


Figure 4.15: The mean chemotactic accuracy of migrating neutrophils from elderly and younger subjects in gradients of IL-8

Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. * = $p < 0.05$. Neutrophils from older subjects moved with significantly less accuracy in the presence of IL-8 than the younger controls (mean difference 0.23, $p < 0.0001$).

In summary, neutrophils from the elderly moved randomly with the same speed (chemokinesis) as those taken from younger subjects in the presence of IL-8, but with significantly less velocity (chemotaxis). Also, the neutrophils from older subjects changed direction more than cells from younger subjects, and hence displayed less chemotactic accuracy.

4.3.4.3 Neutrophil migration towards $\text{GRO}\alpha$

IL-8 acts upon CXCR1 and CXCR2 with equal efficacy. Further migratory studies were performed in the presence of $\text{GRO}\alpha$ (which acts preferentially via CXCR2) to explore if mechanisms were CXCR2 or CXCR1 dependent. Here, neutrophils from older subjects

migrated with similar speed (see figure 4.16) but with decreased velocity (mean difference 0.25 $\mu\text{m}/\text{min}$, confidence intervals 0.001 – 0.41, $p = 0.04$, see figure 4.17), decreased persistence (mean difference 0.17, confidence intervals 0.003 – 0.34, $p = 0.04$, see figure 4.18), and decreased chemotactic accuracy (mean difference 0.16, confidence intervals 0.02 – 0.3, $p = 0.03$, see figure 4.19) than neutrophils isolated from younger subjects.

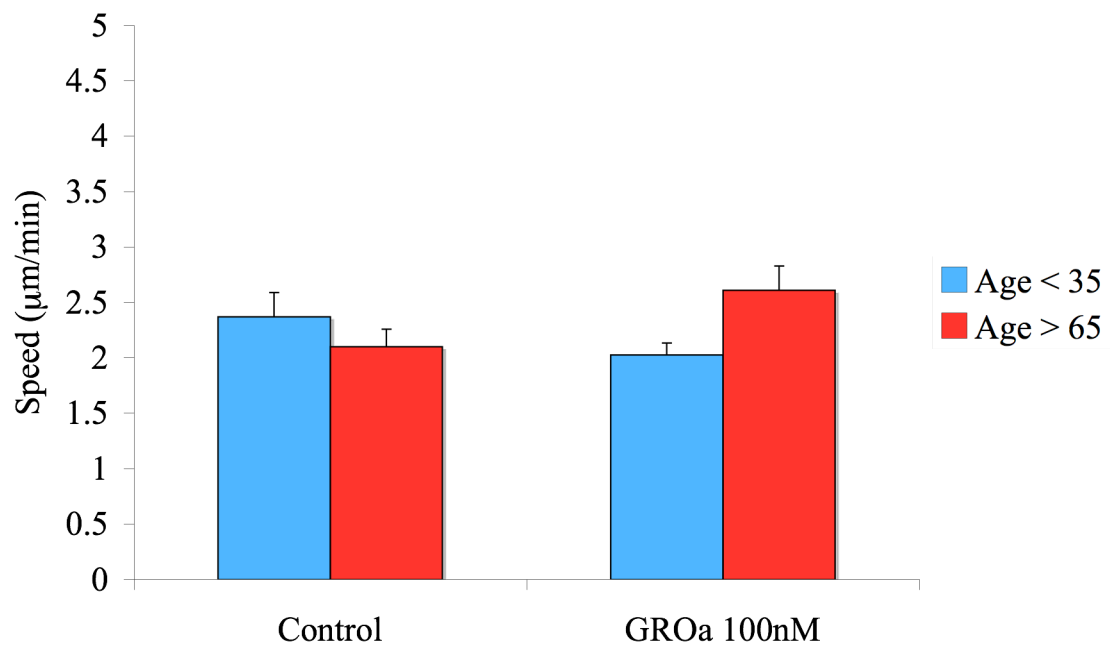


Figure 4.16: The mean speed of migrating neutrophils from elderly and younger subjects in shallow gradients of $\text{GRO}\alpha$.

Legend: Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. Standard error bars are displayed.

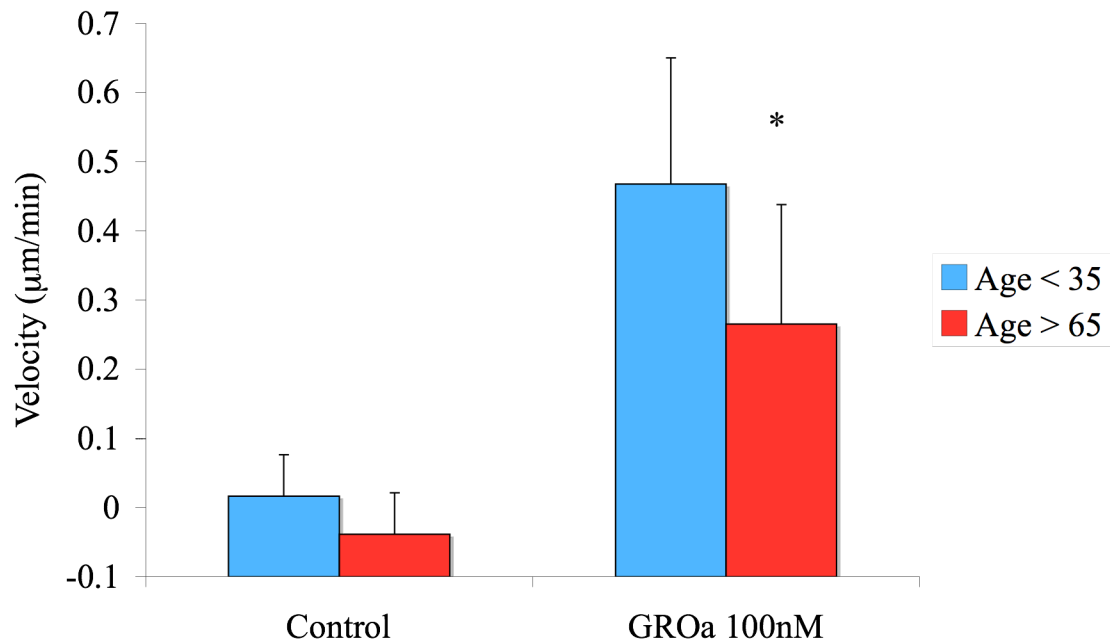


Figure 4.17: The mean velocity of migrating neutrophils from elderly and younger subjects in shallow gradients of GRO α .

Legend: Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. Standard error bars are displayed. * = $p < 0.05$.

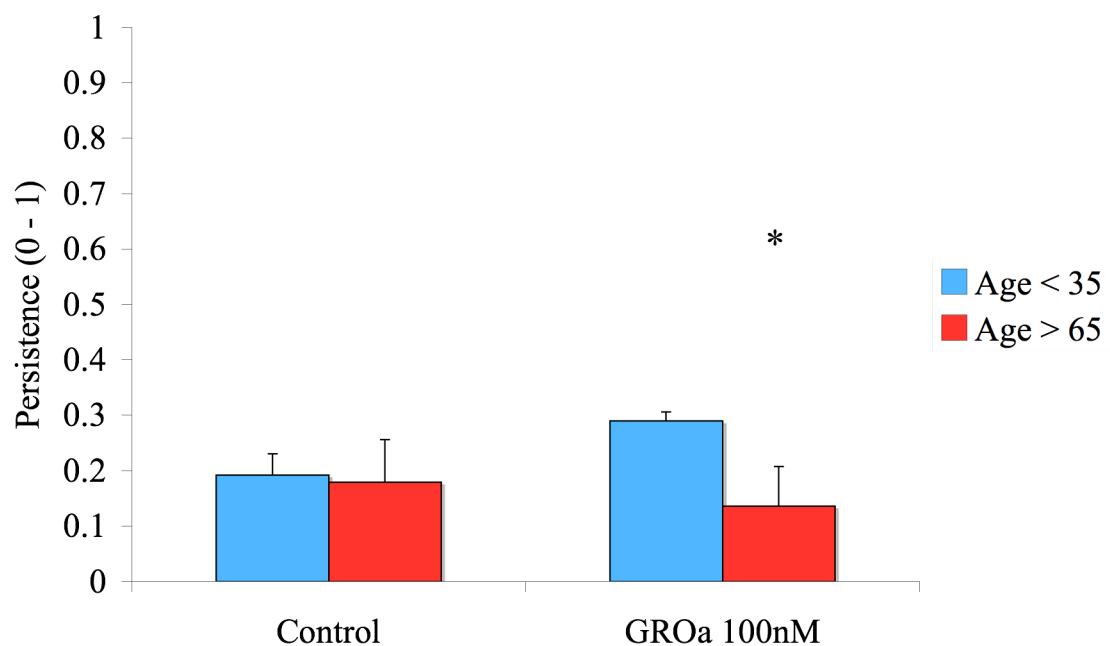


Figure 4.18: The mean persistence of migrating neutrophils from elderly and younger subjects in shallow gradients of GRO α .

Legend: Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. Standard error bars are displayed. * = $p < 0.05$.

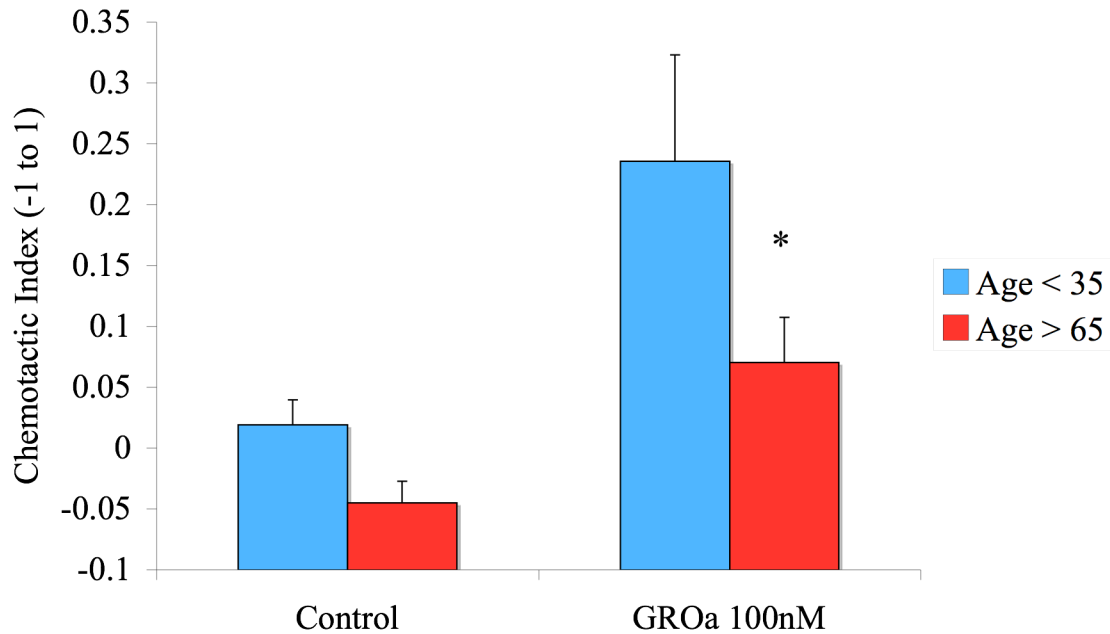


Figure 4.19: The mean chemotactic index of migrating neutrophils from elderly and younger subjects in shallow gradients of GRO α .

Legend: Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. Standard error bars are displayed. * = $p < 0.05$.

Given the similar migratory patterns seen in neutrophils from elderly individuals to both IL8 and GRO α , the CXCR2 receptor was initially focused upon for the initial exploration of the underlying mechanism.

4.3.4.4 Neutrophil migration towards IL-8 following incubation with a CXCR2 antagonist

A CXCR2 receptor antagonist (a small molecule antagonist kindly provided by AstraZeneca, UK) was used to test the hypothesis that the aberrant migration seen in elderly subjects may reflect alterations in CXCR2 receptor expression or function. 5nM of the CXCR2 antagonist was incubated with isolated neutrophils, and migration studies were repeated towards IL-8 (10nM).

Some differential effects were seen following pre-incubation with a CXCR2 antagonist in elderly and young subjects. There was a trend towards the CXCR2 antagonist reducing the speed of migration in both groups (young cells: from $3.6 \pm 0.1 \mu\text{m}/\text{min}$ to $2.8 \pm 0.1 \mu\text{m}/\text{min}$, $p=0.05$. Older cells: from $3.4 \pm 0.1 \mu\text{m}/\text{min}$ to $2.7 \pm 0.1 \mu\text{m}/\text{min}$, $p=0.06$) (see figure 4.20).

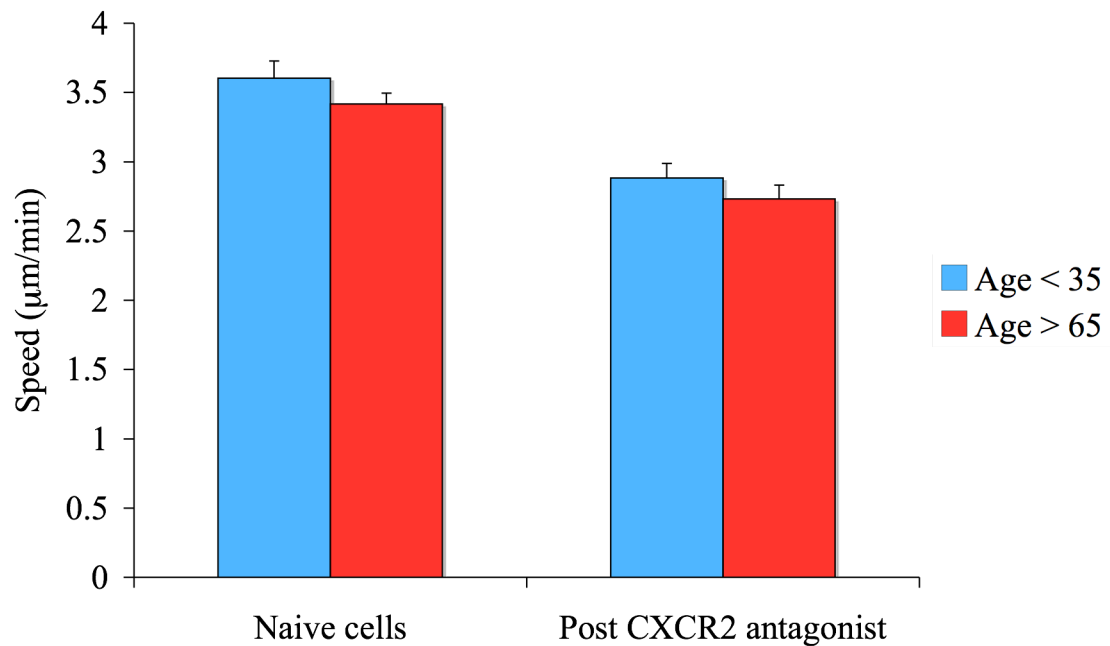


Figure 4.20: The mean speed of migrating neutrophils from elderly and younger subjects in shallow gradients of IL-8 following incubation with CXCR2 antagonist.
Legend: Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. Standard error bars are displayed.

However, incubation with the CXCR2 antagonist did decrease the velocity of migration towards IL-8 in both groups (Young subject: from $1.1 \pm 0.1 \mu\text{m}/\text{min}$ to $0.4 \pm 0.1 \mu\text{m}/\text{min}$, $p < 0.0001$. Elderly subjects: from 0.5 ± 0.1 to $0.1 \pm 0.1 \mu\text{m}/\text{min}$, $p = 0.001$). See figure 4.21.

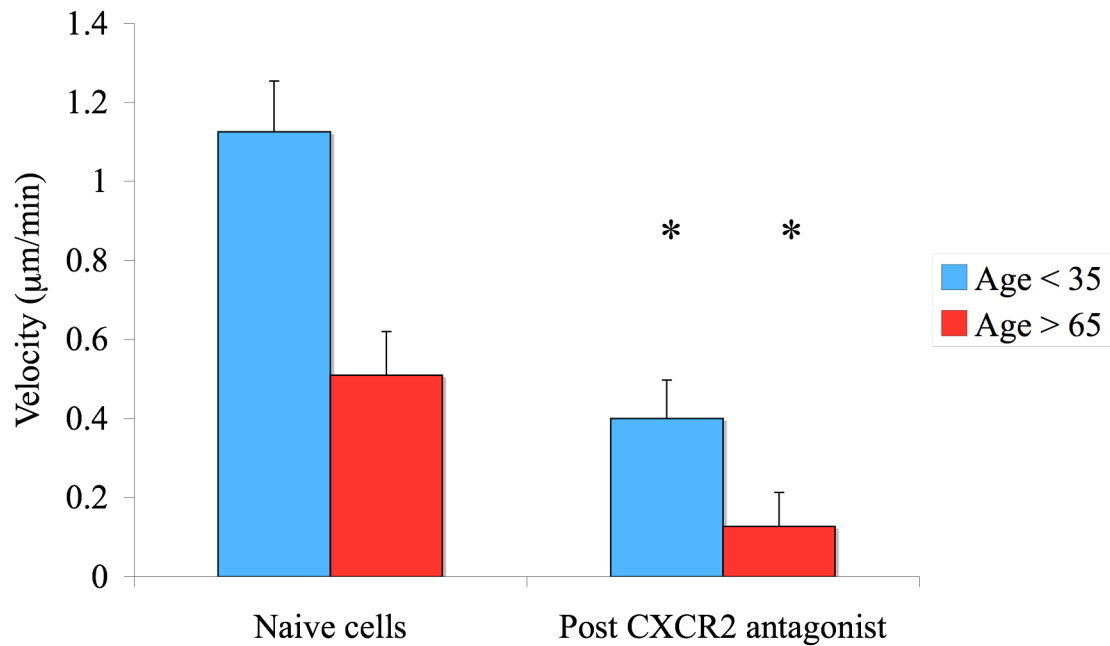
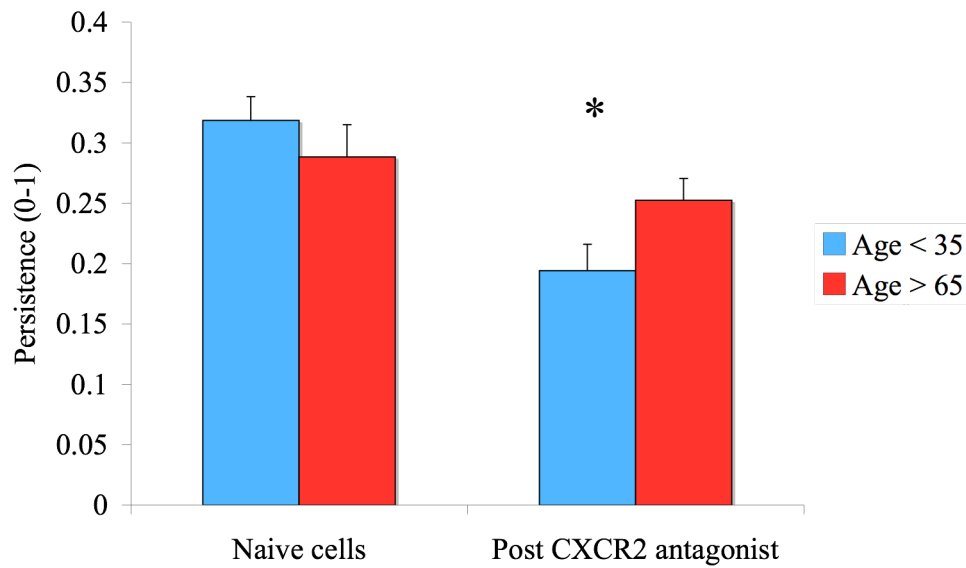


Figure 4.21: The mean velocity of migrating neutrophils from elderly and younger subjects in shallow gradients of IL-8 following incubation with 5nM CXCR2 antagonist.

Legend: Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. Standard error bars are displayed. * = $p = 0.001$ or less.

The CXCR2 antagonist reduced persistence in young (from 0.32 ± 0.01 to 0.19 ± 0.02 , $p = 0.001$) but not elderly subjects (from 0.28 ± 0.02 to 0.25 ± 0.02), see figure 4.22. Furthermore, the antagonist reduced chemotactic accuracy in both groups (young: from 0.3 ± 0.03 to 0.06 ± 0.02 , $p < 0.0001$. Elderly: from 0.1 ± 0.03 to 0.002 ± 0.02 , $p = 0.0001$); see figure 4.23.



Figure

4.22: The mean persistence of migrating neutrophils from elderly and younger subjects in shallow gradients of IL-8 following incubation with 5nM CXCR2 antagonist.

Legend: Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. Standard error bars are displayed. * = $p < 0.001$.

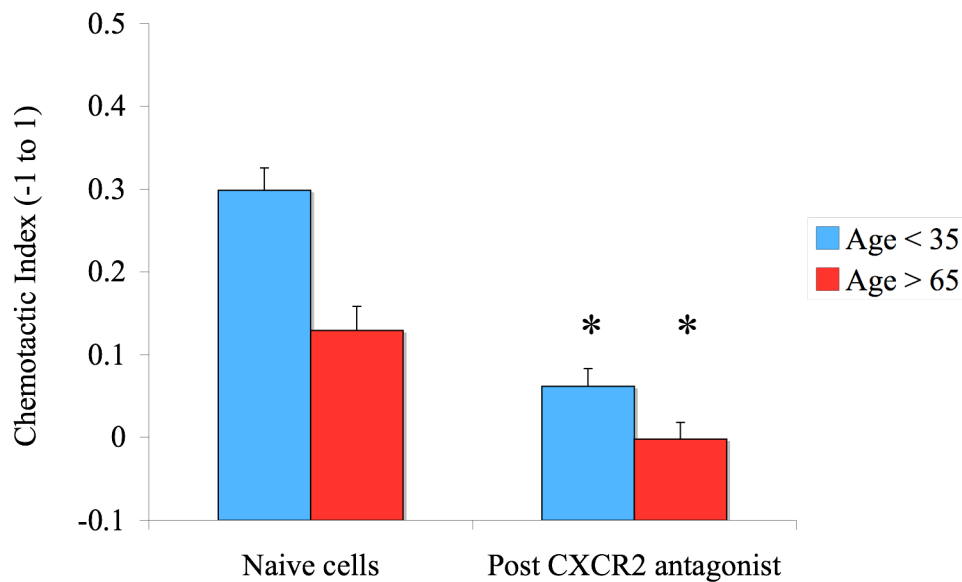


Figure 4.23: The mean chemotactic index of migrating neutrophils from elderly and younger subjects in shallow gradients of IL-8 following incubation with 5nM CXCR2 antagonist.

Legend: Measurements were taken from 10 cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. Standard error bars are displayed. * = $p = 0.0001$ or less.

Expressed as a percentage (where naïve neutrophil migration towards IL-8 is 100%), pre-incubation with a CXCR2 antagonist reduced speed (chemokinesis) by a small amount in both old and young neutrophils ($20 \pm 8\%$ for both groups), however, migrational velocity (chemotaxis) was reduced by $74 \pm 7\%$ in neutrophils from elderly subjects, $p=0.001$ and by $65 \pm 9\%$ in neutrophils from younger subjects, $p < 0.0001$. Incubation with the CXCR2 inhibitor reduced directional persistence by $12 \pm 4\%$ in the elderly (which was not significant), but by $40 \pm 9\%$ in the young, $p=0.001$. Furthermore, the CXCR2 antagonist reduced chemotactic accuracy by $98 \pm 2\%$ in the elderly, $p = 0.0001$ and $75 \pm 4\%$ in the young, $p=0.0001$. See figure 4.24

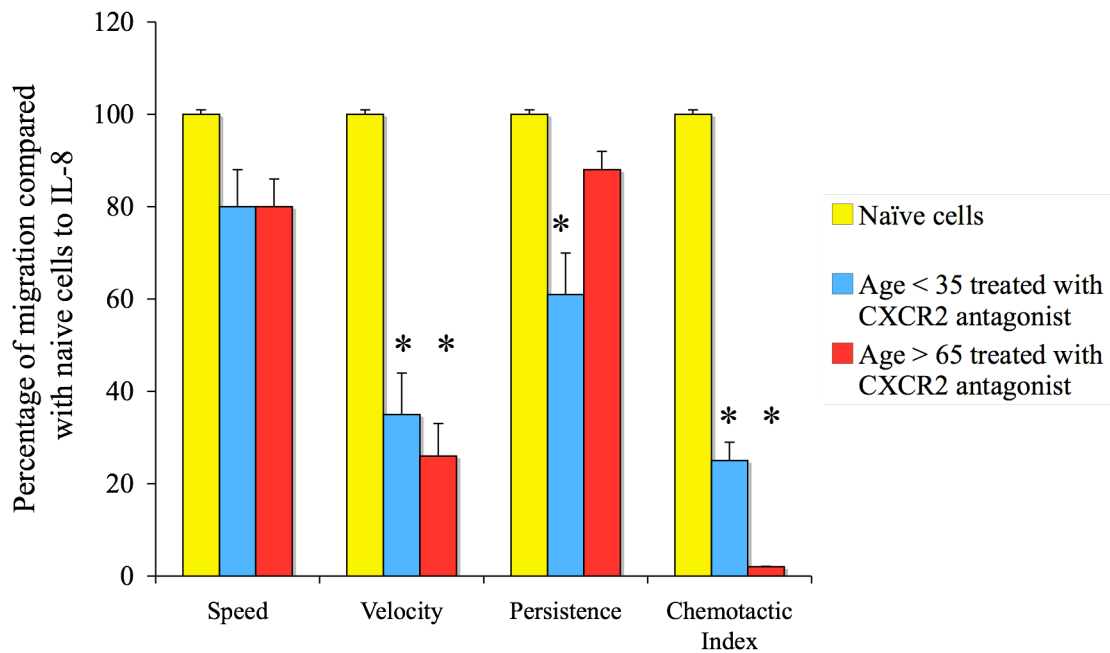


Figure 4.24. The affect on migration following pre-incubation with a CXCR2 antagonist.

Legend. Data is presented as the percentage of the migratory parameter following incubation with the CXCR2 antagonist, compared to migration of naïve cells (naïve cells are expressed as 100% of migratory parameter, migration following incubation with the CXCR2 antagonist is expressed as the percentage of remaining migration). * = $p=0.001$ or less (see preceding text)).

Pre-treating neutrophils from young subjects with the CXCR2 antagonist appeared to adjust migration so that it resembled migratory pathways from older subjects, giving credence to the hypothesis that migratory flaws may be in part due to CXCR2 expression or function. To explore this further, CXCR1 and CXCR2 surface expression was measured on circulating neutrophils.

4.3.5 CXCR1 and 2 receptor surface expression: validation

Dose response experiments determined that antiCXCR1 2ng/ml and antiCXCR2 3ng/ml were the most appropriate to measure surface expression of CXCR1 and CXCR2 (see figure 4.25 and 4.26).

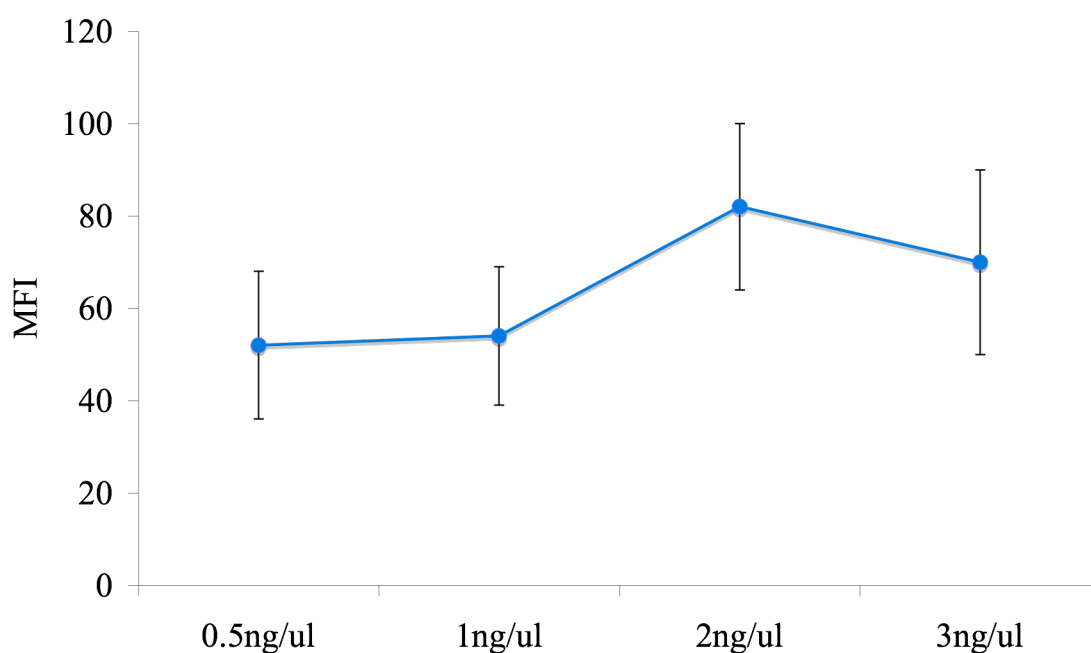


Figure 4.25. CXCR1 surface expression using differing concentrations of antiCXCR1.

Legend: Data is the median fluorescence intensity for six young subjects in anti-human CXCR1 (IL-8 RB)-Fluorescein supplied as 50 µg antibody in 1 mL PBS containing 0.1% sodium azide. The anti-CXCR1 was serially diluted using PBS 2% BSA. Error bars represent interquartile range.

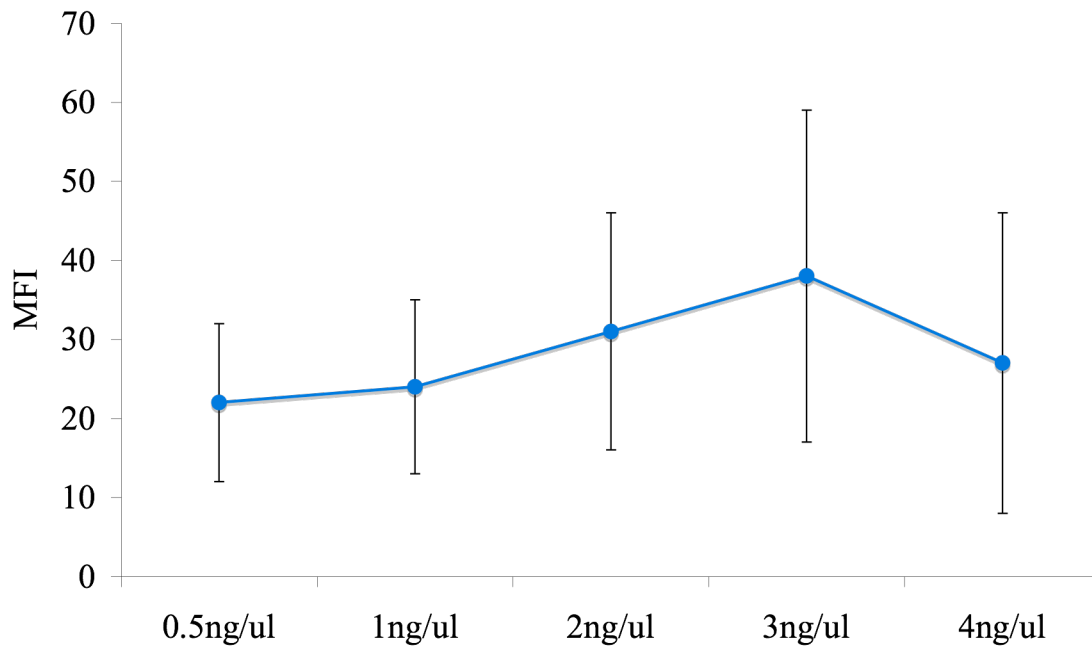


Figure 4.26. CXCR2 surface expression using differing concentrations of antiCXCR2.

Legend: Data is the median fluorescence intensity for six young subjects in anti-human CXCR2 (IL-8 RB)-Fluorescein supplied as 50 µg antibody in 1 mL PBS containing 0.1% sodium azide. The anti-CXCR2 was serially diluted using PBS 2% BSA. Error bars represent interquartile range.

Time course experiments determined that neutrophil CXCR1 and CXCR2 expression in both young and old subjects (n = 6 for both) was greatest immediately following neutrophil isolation (0 Hour). For these experiments, neutrophils were prepared as documented in the methods, but were kept at room temperature (21°C) for the duration of the time course, with the appropriate antibody being added 30 minutes before the measured time point. Neutrophil surface expression of both receptors was seen to decrease in both subject groups in a time dependent manner. In light of these results, expression was measured at the same time in young and old subjects, as soon as the neutrophils were isolated, and not longer than 20 minutes from preparation. See figure 4.27 and 4.28.

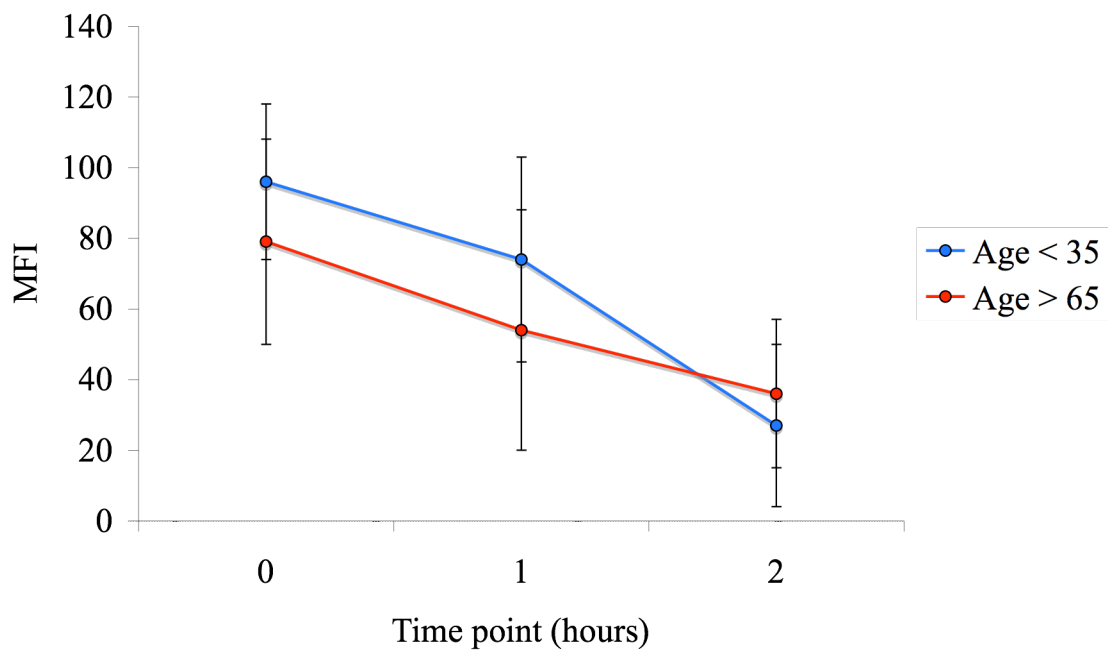


Figure 4.27. Time course for CXCR1 surface expression.

Legend: Data is the median fluorescence intensity for six young subjects in anti-human CXCR1 (IL-8 RB)-Fluorescein 2ng/μl over 3 time points. Error bars represent interquartile range.

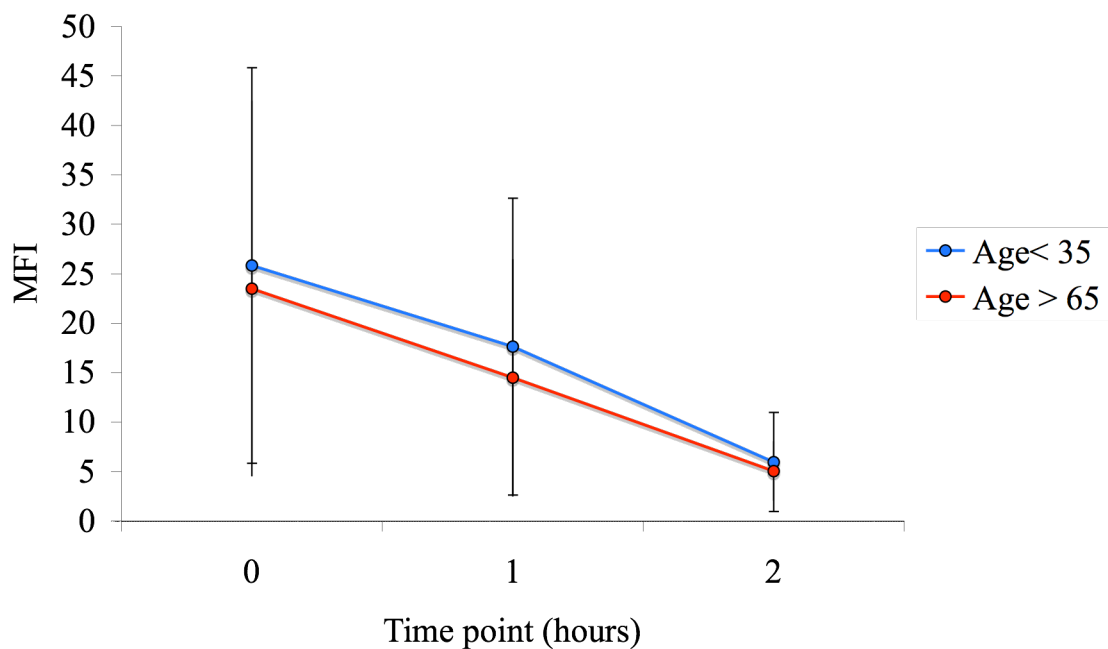


Figure 4.28. Time course for CXCR2 surface expression.

Legend: Data is the median fluorescence intensity for six young subjects in anti-human CXCR2 (IL-8 RB)-Fluorescein 3ng/μl over 3 time points. Error bars represent interquartile range.

4.3.5.1 Power calculations

Power calculations were performed to determine the numbers needed to confirm a 50 – 10% difference in CXCR1 and CXCR2 surface expression with 80% power and at the 5% significance level. Table 4.5 summarises the median fluorescence intensity, the variation in inter-patient expression and the number of subjects required in each group. 20 participants were recruited to each subject group, to provide an 80% chance of detecting a 20% difference in CXCR1 and a 25% difference in CXCR2.

Table 4.5. The numbers needed in a parallel design study to detect varying difference in CXCR1 and CXCR2 surface expression

| | CXCR1 | CXCR2 |
|---------------------------|----------|---------|
| Median expression | 82 | 46 |
| Inter-quartile Range | 64 - 100 | 35 – 53 |
| Co-efficient of variation | 22% | 28% |

Numbers needed in each group to detect

| | | |
|----------------|----|-----|
| 50% difference | 4 | 6 |
| 30% difference | 10 | 14 |
| 25% difference | 14 | 21 |
| 20% difference | 21 | 32 |
| 10% difference | 80 | 126 |

4.3.5.2 Quiescent CXCR1 and CXCR2 expression

There were no differences in surface receptor expression of CXCR1 (young; median fluorescence intensity 82, inter-quartile range 61 – 104: old; median fluorescence intensity 76, inter-quartile range 60 – 98) or CXCR2 (young; median fluorescence intensity 28, inter-quartile range 17- 48: old; median fluorescence intensity 39, inter-quartile range 29 – 53), on quiescent neutrophils isolated from either groups. See figure 4.29 and 4.30.

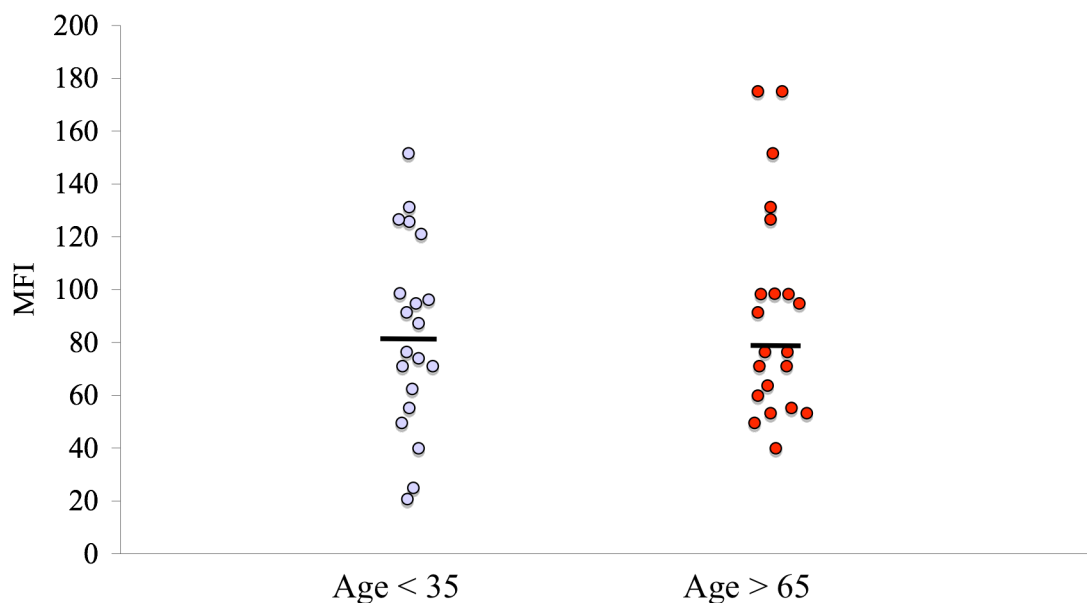


Figure 4.29. Median CXCR1 surface expression.

Legend: Data is the median fluorescence intensity for each of 20 young subjects and 20 older subjects in anti-human CXCR1 (IL-8 RB)-Fluorescein 2ng/μl. The horizontal line is the group median value.

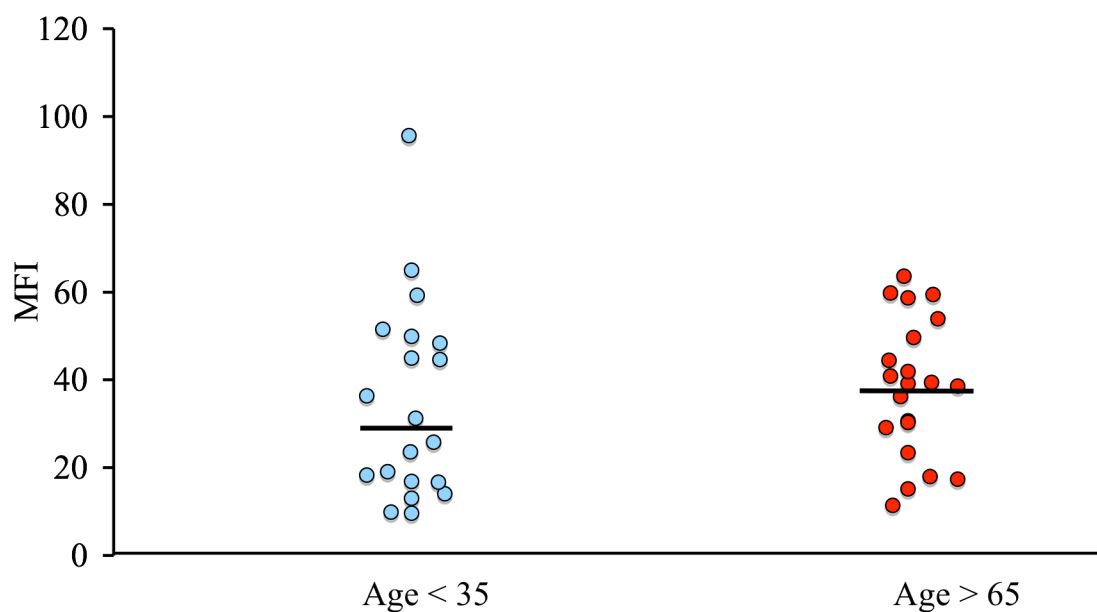


Figure 4.30. Median CXCR2 surface expression.

Legend: Data is the median fluorescence intensity for each of 20 young subjects and 20 older subjects in anti-human CXCR2 (IL-8 RB)-Fluorescein 3ng/μl. The horizontal line is the group median value.

4.3.5.3 Surface expression of CXCR1 and CXCR2 over time

Graphs of surface receptor expression over three time points for CXCR1 and CXCR2 are shown previously (graph 4.27 and 4.28). There was a reduction in surface expression of CXCR1 in both young and older groups over time (Young Vs Older, median results (IQR): time point zero, 96 (74 – 118) Vs. 79 (50 – 108); 1 hour, 74 (45 – 103) Vs 54 (20 – 88); 2 hours, 27 (4 – 50) Vs 36 (15 – 57). However, there was no differential decline in surface expression of CXCR1 between young and older subjects over the time course.

There was a reduction in surface expression of CXCR2 in both young and older groups over time (Young Vs Older, median results (IQR): time point zero, 26 (5 - 45) Vs. 23 (4 - 42); 1 hour, 18 (2 - 32) Vs 14 (3 - 26); 2 hours, 6 (1 - 11) Vs 5 (2 – 8). However, there was no differential decline in surface expression of CXCR2 between young and older subjects over the time course.

This suggested that in the quiescent state, neutrophils from young and older subjects reduced surface expression to a similar degree.

4.3.5.4 Surface expression of CXCR1 and CXCR2 following stimulation with IL-8

Surface expression of CXCR1 following neutrophil stimulation with three concentrations of IL-8 are shown in figure 4.31. Surface expression of CXCR2 following neutrophil stimulation with three concentrations of IL-8 are shown in figure 4.32.

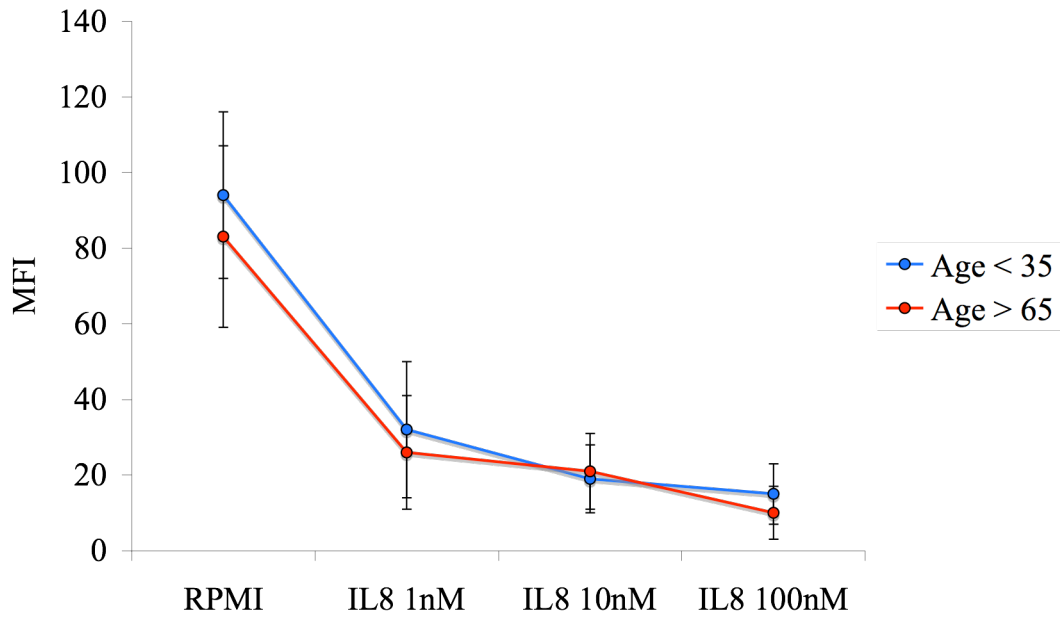


Figure 4.31. Median CXCR1 surface expression following stimulation with IL-8.

Legend: Data is the median fluorescence intensity for 20 young subjects and 20 older subjects in anti-human CXCR1 Fluorescein 2ng/μl stimulated with the stated doses of IL-8. Error bars represent the inter quartile range. There were no differences in expression between groups.

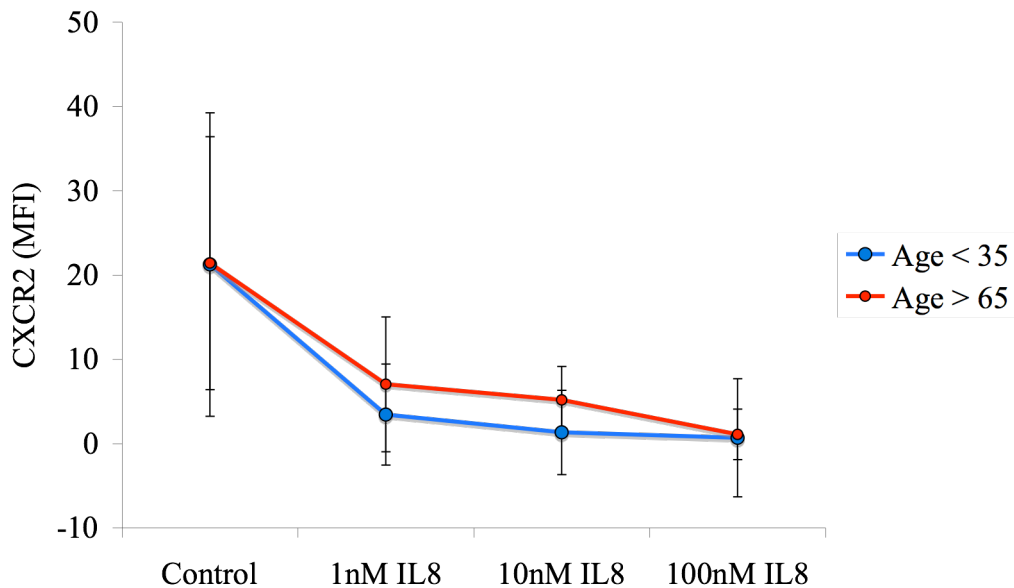


Figure 4.32. Median CXCR2 surface expression following stimulation with IL-8.

Legend: Data is the median fluorescence intensity for 20 young subjects and 20 older subjects in anti-human CXCR2 Fluorescein 3ng/μl stimulated with the stated doses of IL-8. Error bars represent the inter quartile range. There was no difference in expression between groups.

Although stimulation with IL-8 led to a decline in CXCR1 surface expression in a dose dependent manner, there was no difference in CXCR1 expression between neutrophils isolated from young and older subjects (Young Vs older median MFI (IQR); Negative control, 94 (72 – 116) Vs 83 (59 – 107); 1nM IL-8, 32 (14 – 50) Vs 26 (11 – 41); 10nM IL-8, 19 (10 – 28) Vs 21 (11 – 31); 100nM, 15 (7 – 23) Vs 10 (3 – 17). Furthermore, there was no difference in CXCR2 expression between neutrophils isolated from young and older subjects (Young Vs older median MFI (IQR); Negative control, 21 (5 - 36) Vs 22 (3 - 39); 1nM IL-8, 4 (1 - 16) Vs 7 (1 - 9); 10nM IL-8, 1 (0 .1- 4) Vs 5 (1 - 7); 100nM, 0.7 (0.1 - 4) Vs 1 (0.1 – 5). These data suggest that there is no differential expression of CXCR1 or CXCR2 following stimulation with IL-8, when semi-quantified using MFI.

4.4 Discussion

These data describe significant differences in the migratory behaviour of neutrophils from healthy older subjects compared with gender matched healthy younger controls. This was firstly demonstrated using the simple under agarose technique and subsequently studied in more detail using the Dunn chamber. Neutrophils from the older subjects moved randomly with a similar speed (chemokinesis), but were less chemotactic, exhibiting a lower migratory velocity and a lower chemotactic index than younger controls. Neutrophils did not vector towards IL-8 or GRO α as well as neutrophils from young controls, making them less accurate in their migratory pathways. Neutrophils from older subjects also changed their directional orientation more, resulting in lower levels of persistence. The cells made fine alterations in directional orientation while moving towards the source of IL-8 and GRO α , whereas neutrophils from younger subjects migrated with more directional purpose.

In summary, clear differences were observed in the migratory dynamics of neutrophils from older subjects. Although speed of movement (chemokinesis) was preserved, the cells demonstrated a decrease in chemotactic accuracy (denoted by the decrease in velocity, decrease in persistence and decrease in chemotactic index). The cells appeared unable to respond optimally to chemotactic signals which should have changed their direction. This observation has been suggested previously in a study where chemokinesis and chemotaxis were assessed separately using the under agarose assay (Wenisch, Patruta et al. 2000) and confirmed in studies reported here.

This is a crucial distinction as longitudinal and cross-sectional studies (Niwa, Kasama et al. 1989; Egger, Aigner et al. 2004) have suggested that reduced neutrophil chemotaxis, rather than chemokinesis, is associated with higher mortality in elderly subjects. As previously described, bacterial infections are associated with increased mortality and an increased burden of morbidity in elderly people (Liang and Mackowiak 2007). It is clear that neutrophil phagocytic function and in some situations, superoxide production is reduced with increasing age (Wenisch, Patruta et al. 2000; Butcher, Chahal et al. 2001). If chemotaxis is significantly impaired, (as was clearly demonstrated in the current study) the combination of aberrant neutrophil function is likely to impact on bacterial clearance in disease.

Ageing is also associated with a decline in organ function. For example, there is a clear reduction in lung function with increasing age, which has been associated with a higher circulating inflammatory load in a dose dependent manner (Fogarty, Jones et al. 2007; Jiang, Burke et al. 2008). It could be hypothesised that the decline in organ function may be due, in part, to low-grade neutrophilic inflammation. During activation, a high

proportion of neutrophil proteinases are expressed on the neutrophil membrane (Owen, Campbell et al. 1995; Owen, Campbell et al. 1997; Campbell, Campbell et al. 2000), polarising towards the leading edge of the neutrophil as it migrates (Cepinskas, Sandig et al. 1999). A proportion of the proteinase is left behind as the cell moves on (Clayton, Evans et al. 1998; Cepinskas, Sandig et al. 1999) and it has been clearly demonstrated that an area of obligate enzyme activity always exists following the secretion of free NE until concentrations have decreased by diffusion to match the concentration of surrounding proteinase inhibitors (Liou and Campbell 1996; Campbell, Campbell et al. 1999).

Inaccurate chemotaxis would lead to neutrophils from elderly subjects spreading further during migration compared to those from younger controls. If migratory routes taken by elderly neutrophils are more circuitous than normal (as shown here), the potential for collateral damage would be increased, as more membrane bound NE would be released during the prolonged journey to the inflammatory source. The net result would be an increase in the distribution of obligate enzyme activity, with an associated increase in collateral tissue damage. Furthermore, the presence of this extra NE, and any resultant tissue and cell damage, could increase the secretion of other pro-inflammatory mediators (such as LTB₄ and IL-8 (Hubbard, Fells et al. 1991)), amplifying the migration of inflammatory cells into the surrounding tissue (including more neutrophils and macrophages) with their resultant pro-inflammatory interactions (Itoh and Nagase 1995; Ferry, Lonchamp et al. 1997). This would establish a low grade, self perpetuating cycle of damaging inflammation which may (in part) account for the age dependent development of structural lung damage (Verbeken, Cauberghs et al. 1992).

We hypothesised that intact chemokinesis with aberrant chemotaxis was suggestive of a fault with the process of determining cellular orientation within a chemical gradient because of an inability to compare pro-inflammatory receptor occupancy distributed spatially around the cell body (Jin and Hereld 2006; Levine and Rappel 2006). Theoretically, this would reduce the accuracy of pseudopod selection and therefore directed motility. Such a fault could conceivably be caused by hypersensitivity or insensitivity to spatial signals, driven by an alteration in receptor numbers or activity. Since similar migratory patterns were seen in the presence of IL-8 and GRO α , it was hypothesised that the aberrant migration may be dependent upon CXCR2 receptor expression or function.

CXCR2 is a member of a superfamily of G protein-coupled receptors (GPCRs) that transduce signals to the interior of the cell through heterotrimeric guanine nucleotide-binding proteins (G proteins). These receptors share a common putative structural topology composed of seven-transmembrane domains separated by three extracellular and three intracellular loops. Upon agonist binding, CXCR2 activates G protein-mediated phosphoinositide hydrolysis to generate diacylglycerol and inositol 1,4,5-trisphosphate, thereby activating protein kinase C and mobilizing Ca²⁺ to initiate a variety of cellular response. Receptor activation is followed by receptor phosphorylation on multiple serine residues and subsequent desensitization of the receptor to further stimulations. The receptors then either undergo dephosphorylation and recycle back to the cell surface, becoming resensitized or are degraded and down-regulated (Yang, Wang et al. 1999).

To assess CXCR2 function, neutrophils were incubated with a selective CXCR2 antagonist, which is a small molecule antagonist that binds to the intracellular receptor domain, preventing downstream signalling. The overall effects of the antagonist were a significant decrease in velocity of migration (chemotaxis) but not speed (chemokinesis). This is highly suggestive of a role for CXCR2 receptors in sensory awareness of pro-inflammatory mediators during migration rather than movement itself. Migratory persistence and accuracy were also significantly decreased following incubation with a CXCR2 antagonist, which again suggests that CXCR2 receptors are involved in the perception of pro-inflammatory gradients rather than the mechanics of cellular movement *per se*. Overall, the addition of a CXCR2 antagonist to neutrophils from younger subjects resulted in them adopting the migratory dynamics of cells from older subjects, with reduced velocity, persistence and accuracy with a maintained speed, reinforcing the hypothesis that CXCR2 expression or function may be altered on older cells.

CXCR1 and CXCR2 receptor surface expression was semi-quantified on neutrophils isolated from young and older subjects using FACS analysis, and found to be comparable. At this time, there are no other published data regarding CXCR1 or CXCR2 receptor expression on neutrophils in healthy ageing subjects from which to make comparisons. The current study had sufficient power to detect a 25% difference in expression between groups, which was felt to be the smallest difference likely to be clinically relevant, so it is unlikely that the differences seen in migration are due to receptor expression alone. Furthermore, there were no differences in surface expression of either receptor over time, or when stimulated with IL-8, again suggesting no obvious fault with cell surface receptor shedding or internalisation. However, given the complex

nature of CXCR1 and CXCR2 endocytosis and recycling, it is overly simplistic to assume that there are no differences in receptor function. It is also possible that deficits in migration are caused by differences in the migratory pathway downstream to the CXCR receptors and this could be assessed by measuring other components of the migratory pathway in these two groups. Identifying potential mechanisms that underpin aberrant migration is being pursued separately. Nevertheless, the inaccurate migration of neutrophils from elderly subjects may be of importance in the defence against infection, with poor bacterial capture leading to the increased incidence of clinically severe infections in the elderly. Further studies are warranted to investigate this further.

In conclusion, clear differences were seen in the migratory dynamics of neutrophils from older healthy subjects, with reduced chemotaxis but preserved chemokinesis moving towards IL-8 and GRO α . Neutrophils from younger subjects displayed similar migratory patterns following incubation with a CXCR2 antagonist, leading to the hypothesis that CXCR2 receptor expression or function is aberrant in cells from elderly subjects, and indeed, a CXCR2 antagonist heightened the migratory flaws, consistent with this hypothesis. There was no difference in CXCR1 or CXCR2 surface expression, suggesting that the problem may relate to intracellular processing of the receptor, or its signalling pathway. The aberrant migration could theoretically delay bacterial clearance and increase by-stander tissue damage during migration.

These data confirm that migrational studies of neutrophils in COPD need to ensure that control subjects are age matched. Furthermore, using these data, it will be possible to assess whether the patterns of neutrophil migration in COPD are in keeping with theories that the pathophysiology is related to advanced ageing.

5 Aberrant neutrophil migration in COPD

5.1 Brief introduction

It is widely accepted that airway inflammation is central to the development and progression of COPD, leading to destruction of lung parenchyma, goblet cell hypertrophy and tissue remodelling. There is a wealth of data supporting the hypothesis that the neutrophil is of fundamental importance in the pathophysiology of the disease, including an increased cellular presence in the lung (Martin, Raghu et al. 1985; Selby, Drost et al. 1991; Stanescu, Sanna et al. 1996; Rutgers, Timens et al. 2000) and correlations with clinical markers of disease severity and progression (Thompson, Daughton et al. 1989; Ge, Zhu et al. 1990; Lacoste, Bousquet et al. 1993; Betsuyaku, Nishimura et al. 1999; Yoshioka, Betsuyaku et al. 2005; Parr, White et al. 2006; Pilette, Colinet et al. 2007). Furthermore, Neutrophil elastase (NE) has been shown to cause all of the pathological features of COPD (Blackwood, Hosannah et al. 1973; Snider, Hayes et al. 1974; Smallman, Hill et al. 1984; Rickard and Rennard 1989; Amitani, Wilson et al. 1991; Ballieux, Hiemstra et al. 1994; Nakajoh, Fukushima et al. 2002). However, it remains unclear whether the increased number of neutrophils present in the lung is an effect of the disease (due to increased inflammatory signal) or central to the pathophysiology as a result of increased or aberrant migration.

Observations of neutrophil migration in COPD to date have employed modifications of the under agarose assay, the Boyden Chamber assay (in which cells migrate through a filter in a transwell dish) or migration under flow, and results have been contradictory. For example, Burnett et al (Burnett, Chamba et al. 1987) described increased neutrophil migration in patients with COPD compared to patients with bronchiectasis and healthy controls. In a more recent study by Yoshikawa et al (Yoshikawa, Dent et al. 2007), the number of neutrophils that had successfully migrated towards IL-8 was reduced in patients

with stage II - IV COPD compared with healthy controls and healthy smokers, although interestingly, neutrophils from patients with early (stage 1) COPD demonstrated more migration than all other groups.

These contradictory results could be explained by the assays used or the diverse group of patients included. The characteristics of the migration platform in the Boyden chamber assay can cause variations in migration (for example pore size and associated chemotactic gradient formation), differences in cell activation has a profound affect, and the assay only assesses overall migratory patterns and not individual cell behaviour. Studies have also not always included rigorously age matched controls, which, as demonstrated in the previous chapter, can have a profound effect on neutrophil migration. These studies were, therefore, unable to address the fundamental question of whether and how cells from COPD patients differ from healthy controls.

The studies described in this chapter were performed to firstly examine overall migratory patterns in COPD under agarose, to provide a point of comparison with previous work. Secondly, (using the modified Dunn Chamber) to provide quantitative detail of individual neutrophil migration, assessing the effects of smoking, and the role of lung inflammation and its treatment on chemotaxis and chemokinesis, testing the hypothesis that neutrophil migration in COPD would be compatible with a state of advanced ageing.

It is known that COPD is associated with heightened pulmonary and systemic inflammation and thus any differences seen in neutrophil migration between healthy controls and COPD patients could be a non specific response to inflammation, lung disease itself or both. For these reasons we carefully selected the control groups and

specifically included patients with A1ATD as these patients can be selected with the same degree of functional impairment, cigarette exposure and similar pulmonary inflammation and therapy to usual COPD (all of which may influence results). Thus, if neutrophil migration in A1ATD resembles health more than COPD, it could be surmised that any differences in migratory patterns was not due to the presence of pulmonary and systemic inflammation or lung disease alone.

IL-8 was chosen as the main chemoattractant in these studies as this chemokine has been shown to have intimate relationships with both neutrophil numbers in lung secretions and COPD disease burden (Sapey, Bayley et al. 2008), as proposed previously. GRO α was also used to help to differentiate whether affects were driven by signalling via CXCR1 or CXCR2.

5.2 Experiments

5.2.1 Study subjects

COPD patients had moderate to severe disease defined by GOLD criteria (Pauwels, Buist et al. 2001). They were ex-smokers, aged between 50 and 70. All patients were clinically stable for at least eight weeks prior to recruitment with no changes in medication. Alternative and concomitant disease was excluded clinically, physiologically and radiologically.

Controls consisted of age and gender matched healthy volunteers and patients with A1ATD. Patients with A1ATD (PiZ) were chosen as they have a similar phenotype of lung disease and a similar inflammatory signal in the lung but with a known genetic cause

of disease. The A1ATD patients were ex-smokers and matched for the severity of airflow obstruction, age, gender and medications. Healthy volunteers were divided into two groups. The first had never smoked, had no evidence of chronic disease (including lung disease) and were medication free. The second group of healthy controls were current smokers who had no evidence of lung disease (or other co-morbidities) and were also medication free, the so called “healthy smokers”. These two control groups were chosen as comparisons with healthy controls would allow an understanding of differences from normal, including the effects of smoking and comparisons with patients with A1ATD would differentiate the effects of pulmonary inflammation and lung disease *per se*.

5.2.2 Isolation of blood neutrophils

Neutrophils were isolated from the whole blood as described previously (methods 2.5.7). The neutrophils (>95% pure, > 97% viable, by exclusion of trypan blue) were resuspended in buffer (RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK) containing 0.15% bovine serum albumin (Sigma-Aldrich, UK).

5.2.3 Neutrophil Chemotaxis

5.2.3.1 Under agarose assay

Neutrophil chemotaxis was firstly assessed using the under-agarose assay, (methods section 2.7.1). Following dose response experiments, peripheral neutrophils isolated from patients with COPD, healthy non smoking controls and patients with A1ATD (n=6 for all groups) migrated towards 100nM IL-8 and both a negative control (Assay buffer: RPMI 1640 (Flow Laboratories, UK) containing 0.15% bovine serum albumin (Sigma-Aldrich)) and a positive control (fMLP 100nM (Sigma Aldrich)). Migration is expressed in mm, with differentiation between chemotaxis and chemokinesis. Each experimental condition was repeated three times per subject to provide the mean result for the

individual. Each subject mean result was used to form an overall mean for the subject group (Healthy control, PiM COPD or PiZ COPD).

5.2.3.2 Modified Dunn Chamber

Neutrophil chemotaxis was then measured using a modification of the Dunn chamber. Full methodological details are included in the methods (section 2.7.2). Each subject's neutrophils were studied with a negative control (assay buffer, see above) and with IL-8 10nM and GRO α 100nM (concentrations were chosen following appropriate dose response experiments, see 4.3.3.3). Migratory dynamics were firstly compared between healthy non-smokers and "healthy smokers" (n=6 for each group). Finally, neutrophil migration was assessed for patients with COPD, healthy controls and patients with A1ATD (n=12 for each group).

5.2.4 Statistical analysis

Migrational differences seen using the Under Agrose assay were compared using the mean chemotactic response, chemokinetic response and chemotactic differential (see figure 2.10) of experiments completed in triplicate for each individual. Migrational differences seen using the Modified Dunn Chamber were compared using the mean data for 10 random cells per experiment, per subject. Differences in chemotaxis criteria were compared using an Independent *t* test. A p value of less than 0.05 was considered to be statistically significant.

Under agarose studies were exploratory, and formal sample size calculations were not performed. For the Modified Dunn chamber, sample size calculations were based

upon a two group parallel comparison, to provide an 80% power of detecting a 35% change in mean speed at the 5% level of significance between healthy smokers and non smokers and a 25% change in mean speed at the 5% level of significance between patients with COPD, healthy controls and patients with A1ATD (both as described in section 2.9).

5.3 Results

5.3.1 Under Agarose and Modified Dunn Assays: validation

For both assays, dose response experiments were performed with increasing concentrations of IL-8 (R&D Systems) to establish the concentration at which neutrophil chemotaxis was maximal (see 4.3.1 and 4.3.3).

5.3.2 Under Agarose assay: Results

5.3.2.1 Baseline characteristics

Demographic data for the COPD patients, healthy controls and patients with A1ATD are shown in Table 5.1.

Table 5.1. COPD neutrophil migration. Demographic data for COPD patients and control groups

| | <u>COPD</u> | <u>Healthy Controls</u> | <u>A1ATD</u> |
|--------------------------------------|--------------|-------------------------|--------------|
| N | 6 | 6 | 6 |
| Age (years) | 65 (57 - 75) | 59 (55 - 66) | 53 (45 - 67) |
| Male | 4 (66%) | 3 (50%) | 3 (50%) |
| Smoking status | All ex | All never | All ex |
| Pack years | 33 (5.8) | 0 * | 13.2 (3) |
| FEV ₁ (percent predicted) | 45 (2.9) | 107 (3.4) * | 47 (13) |
| FEV ₁ /FVC (%) | 56 (6.1) | 80 (1.49) * | 63 (11) |
| Inhaled steroids | 6 (100%) | 0 (0%) | 6 (100%) |
| Theophyllines | 1 (16.5%) | 0 | 0 |
| Sputum colonization | 1 (16.5%) | 0 | 0 |
| BMI | 22 (1.6) | 25 (1.22) | 24 (1.7) |

Legend

Normally distributed data is presented as mean (with range or SEM). Categorical data is presented with percentages given in parentheses. * = significantly different from PiM COPD patients ($p \leq 0.05$). The 1 COPD patient where bacteria were isolated was colonised with *Moraxella. Catarrhalis* 6×10^6 . Including or excluding this patient had no effect on overall results and hence data from this subject was included in the analysis.

5.3.2.2 Neutrophil migration towards IL-8

Table 5.2 summarises the mean chemotaxis, mean chemokinesis and mean chemotactic differential for each group. The chemotactic differential for circulating neutrophils from patients with COPD was similar to both control groups migrating towards IL-8 and 100nM fMLP (used as a positive control). There was an increase in chemokinesis in gradients of both inflammatory mediators in COPD patients compared to healthy controls ($p < 0.0001$ for both IL-8 and fMLP) and compared to patients with A1ATD (IL-8, $p = 0.005$; fMLP $p < 0.0001$). There was also a corresponding increase in chemotaxis towards both IL-8 and fMLP compared to healthy controls (IL-8, $p = 0.002$; fMLP, $p = 0.001$) and A1ATD patients ($p = 0.01$ and $p < 0.001$ respectively). See figure 5.1 – 5.3.

Table 5.2. The mean chemotaxis, chemokinesis and chemotactic differential for each subject group.

| | <u>COPD</u> | <u>Healthy controls</u> | <u>A1ATD</u> |
|---|-----------------------------|-------------------------|--------------|
| <u>Negative control (Assay buffer)</u> | | | |
| Chemotaxis | 0.5 (0.02) | 0.44 (0.03) | 0.44 (0.02) |
| Chemokinesis | 0.47 (0.03) | 0.42 (0.02) | 0.42 (0.02) |
| Chemotactic differential | 0.03 (0.03) | 0.02 (0.02) | 0.02 (0.02) |
| <u>Positive control (fMLP)</u> | | | |
| Chemotaxis | 1.24 (0.05) * ^{\$} | 1.1 (0.1) | 1.00 (0.09) |
| Chemokinesis | 0.73 (0.02) * ^{\$} | 0.52 (0.03) | 0.47 (0.02) |
| Chemotactic differential | 0.51 (0.06) | 0.58 (0.08) | 0.53 (0.09) |
| <u>IL-8</u> | | | |
| Chemotaxis | 1.14 (0.05) * ^{\$} | 0.84 (0.04) | 0.89 (0.05) |
| Chemokinesis | 0.67 (0.02) * ^{\$} | 0.41(0.03) | 0.48 (0.03) |
| Chemotactic differential | 0.47 (0.04) | 0.43 (0.04) | 0.41 (0.03) |

Legend.

Results are overall means for each group (migration in mm). Standard errors are given in parentheses. * = significantly different from Healthy control. ^{\$} = significantly difference from patients with A1ATD.

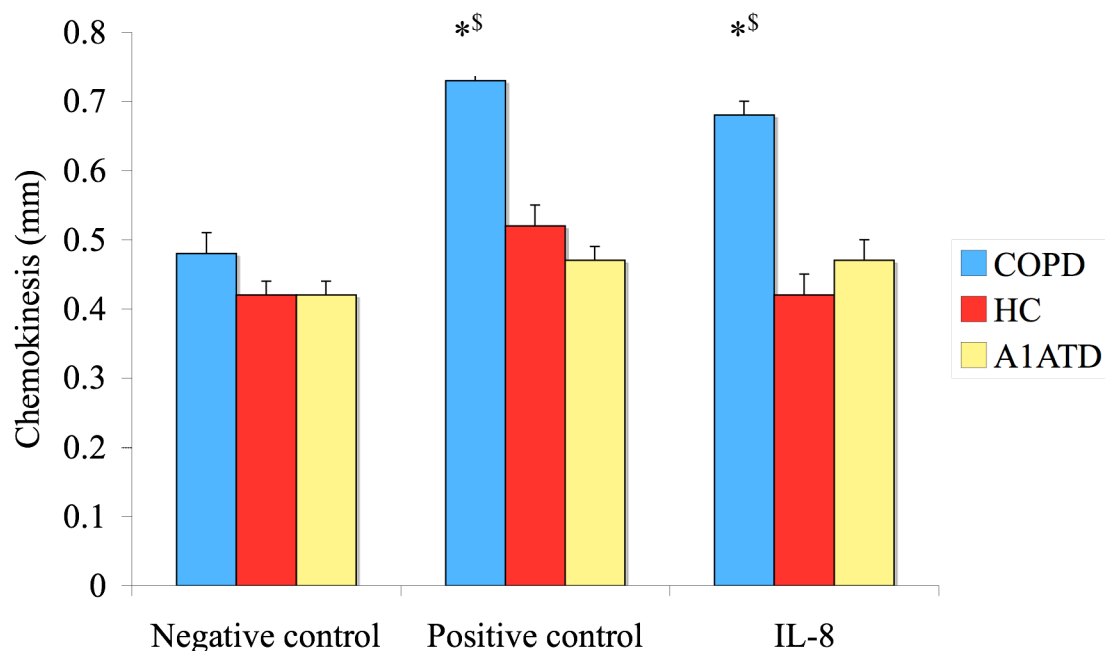


Figure 5.1. The chemokinetic response of COPD neutrophils

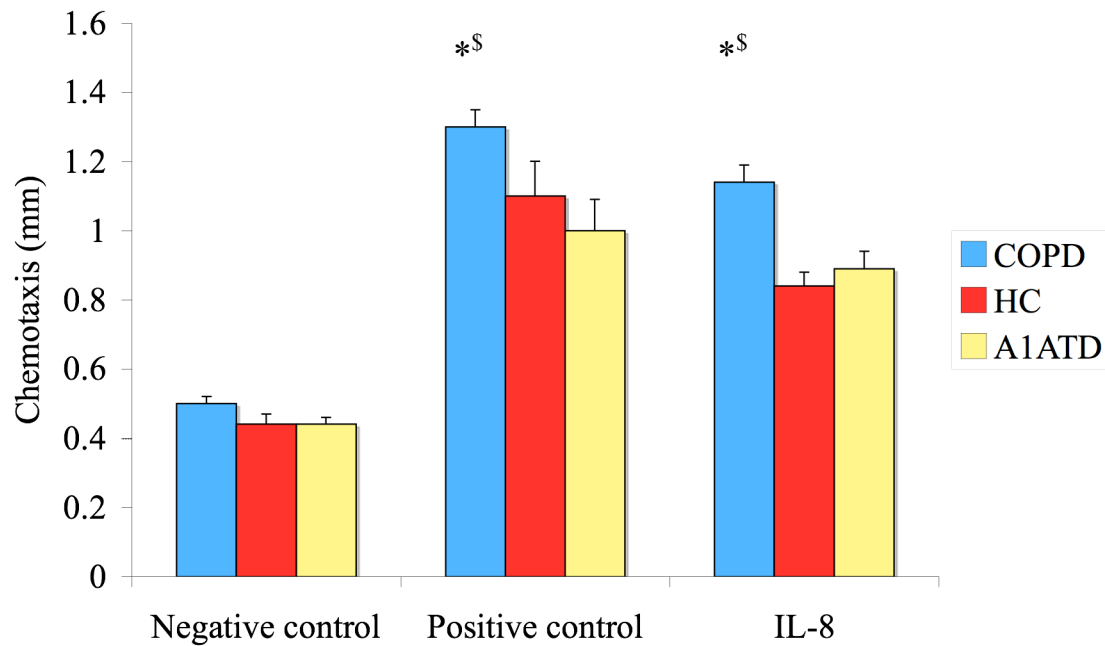


Figure 5.2. The chemotactic response of neutrophils

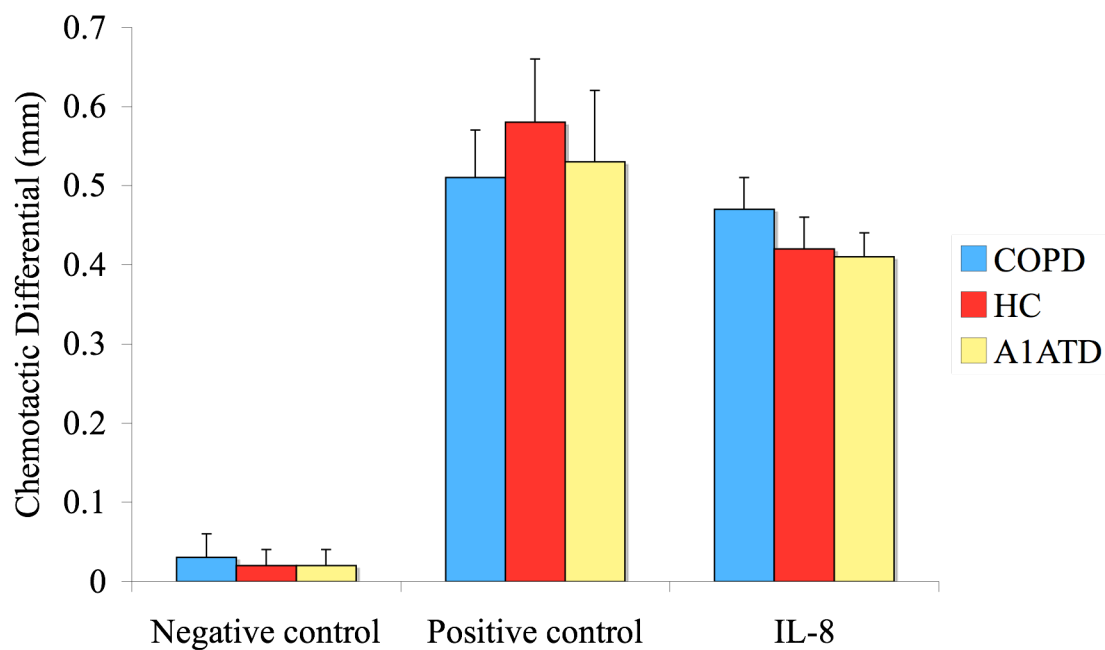


Figure 5.3. The chemotactic differential of circulating neutrophils.

Legend for figures 5.1, 5.2 and 5.3.

The results show the overall mean (and SEM) for each group. Negative control is RPMI 1640 (Sigma-Aldrich), positive control is 10^{-7} M fMLP (Sigma-Aldrich).

* = significant difference compared with healthy controls (HC), \$ = significant difference from patients with A1ATD.

The overall migratory pattern seen with the Under Agarose assay appeared to suggest that neutrophils isolated from patients with COPD spread out more during migration (with more movement both towards and away from the chemotactic stimulus) than neutrophils isolated from healthy controls and patients with A1ATD. There were no clear differences in migratory patterns between patients with A1ATD and healthy controls. The Modified Dunn chamber was used to explore these differences further.

5.3.3 Modified Dunn Assay: Results

5.3.3.1 Migratory differences between healthy smokers and non smokers

The Under Agarose assay experiments included non-smoking healthy controls and ex-smoking patients with COPD and A1ATD. There were clear migratory differences between patients with COPD and the other groups, however, the COPD group also had greater smoking exposure (although this was not significantly different between COPD and A1ATD patients). In order to explore the impact of smoking on neutrophil migration, healthy never smokers and matched healthy smokers (n=6 in each group) were recruited, and neutrophil migratory patterns were explored.

Baseline characteristics

Demographic data for the “healthy smokers” and healthy non smokers are shown in table 5.3. The results shown are for post-bronchodilator forced expiratory volume in one second (FEV₁) expressed as a percentage of the value predicted for the patient’s age, gender and height (British Thoracic Society 1994) and the ratio of FEV₁ to forced vital capacity (FEV₁/FVC).

Table 5.3. Demographic data for healthy never smokers and smokers

| | Healthy never smokers | Healthy smokers |
|------------------------------|-----------------------|------------------|
| N | 6 | 6 |
| Age (years) | 58 (53 - 68) | 62 (56 - 65) |
| Male | 3 | 2 |
| Smoking status | All never | All current |
| Pack years | 0 | 26 (15 - 47) |
| FEV ¹ | 2.46 (2.1- 2.8) | 2.63 (2.4 - 3.1) |
| FEV ¹ % predicted | 98 (94 – 108) | 106 (100 - 111) |
| FEV ¹ /FVC | 82 (75 - 85) | 80 (78 - 83) |
| BMI | 26 (21 - 33) | 23 (21 - 29) |

Legend. Age is presented as the mean (with the range in parenthesis). Gender is the number of males in each group. The remaining data is presented as the median (with the interquartile range in parentheses). Healthy non-smokers differed from healthy smokers only in terms of smoking exposure. There were no other differences between groups.

Migratory patterns of neutrophils from healthy never smokers and healthy smokers

Figure 5.4a shows pathways of neutrophil migration for a healthy non-smoker and figure 5.4b for a healthy smoker.

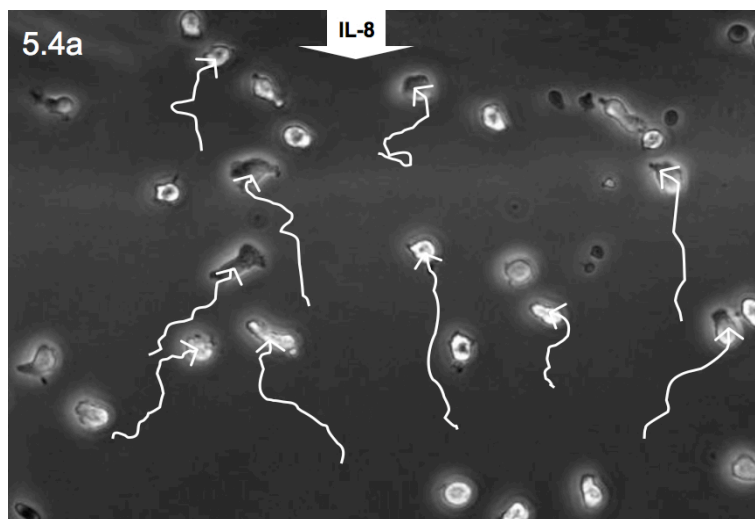


Figure 5.4a. Migratory pathways of neutrophils isolated from a non-smoking healthy control.

See below for legend

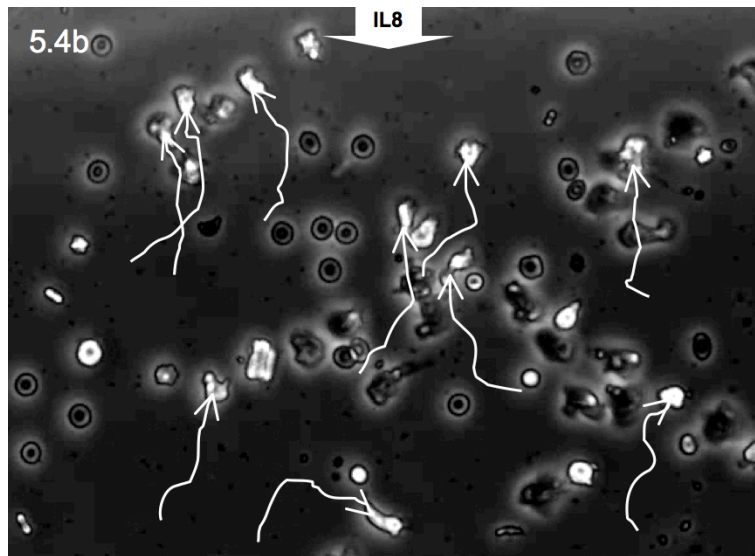


Figure 5.4b Migratory pathways of neutrophils isolated from a smoking healthy control.

Legend. Each image is a photograph taken at the end of migration, with migratory pathways for each neutrophil added using digital tagging. The large arrow at the top of each image is the source of the chemoattractant (IL-8 10nM). The small arrows show the neutrophils migratory pathway. The neutrophil starting position is the beginning of the small arrows, the arrow tip represents the cells orientation at the end of migration.

There were no significant differences in the migratory pathways of cells from either group.

When individual migratory parameters were studied, there were no migratory differences in circulating neutrophils from age and gender matched healthy non smokers and the “healthy smokers”, suggesting that smoking per se does not effect neutrophil migration in this assay (see Figure 5.5). The mean chemokinesis (speed) was $4.28 \pm 0.4 \mu\text{m}/\text{min}$ (non-smoker) Vs. $4.30 \pm 0.4 \mu\text{m}/\text{min}$ (smoker), mean chemotaxis (velocity) was $1.09 \pm 0.1 \mu\text{m}/\text{min}$ (non-smoker) Vs $1.2 \pm 0.3 \mu\text{m}/\text{min}$ (smoker), persistence was 0.28 ± 0.04 (non-smoker) Vs 0.38 ± 0.06 (smoker) and chemotactic accuracy was 0.21 ± 0.05 (non smoker) Vs. 0.22 ± 0.06 (smoker), respectively.

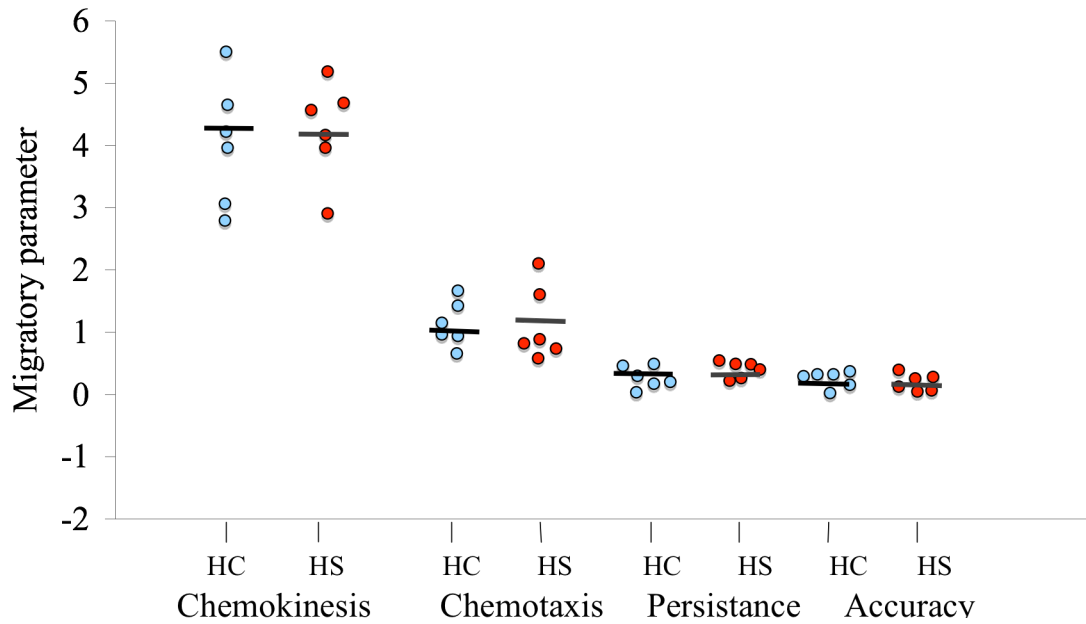


Figure 5.5: Differences in migratory dynamics of peripheral neutrophils from healthy non smokers and healthy smokers

Legend: Each data point represents the mean migratory parameter for one individual, taken from the analysis of 10 neutrophils with each experiment repeated three times. Chemokinesis is the random speed of movement in any direction (measured in $\mu\text{m}/\text{minute}$), chemotaxis is the velocity of movement towards the chemoattractant (measured in $\mu\text{m}/\text{minute}$). Persistence is a measure of the number of changes of direction made by a cell during migration (scored between 0 and 1, the higher the score, the less directional changes). Accuracy is a vector analysis of the accuracy of cell migration, with 1 equalling movement straight up the chemoattractant gradient, and -1 equalling movement in the opposite direction to the chemoattractant gradient. The bar represents the overall group mean. There were no differences in migratory parameters in neutrophils isolated from healthy non smokers (HC) and healthy smokers (HS).

5.3.3.2 Migratory differences between patients with COPD, healthy controls and patients with A1ATD

Baseline characteristics

Demographic data for the COPD patients with and without A1ATD and healthy controls are shown in Table 5.4. The results shown are for postbronchodilator forced expiratory volume in one second (FEV_1) expressed as a percentage of the value predicted for the patient's age, gender and height (British Thoracic Society 1994) and the ratio of FEV_1 to vital capacity (FEV_1/FVC).

Table 5.4. Demographic data for COPD patients, healthy controls and patients with A1ATD.

| | COPD | Healthy subjects | A1ATD |
|---|------------------|--------------------|-----------------|
| N | 12 | 12 | 12 |
| Age (years) | 60 (55 - 68) | 59 (53 - 68) | 53 (45 – 67) |
| Gender (Male) | 7 | 5 | 7 |
| Smoking status | All ex-smokers | Mixed | All ex-smokers |
| Pack years | 36 (24 - 43) | 13 (0 - 47) * | 29 (15 – 38) |
| FEV ₁ (l) | 0.96 (0.8 - 1.1) | 2.54 (2.5 - 2.9) * | 1.2 (0.9 – 1.6) |
| FEV ₁ % predicted | 44 (39 - 49) | 101 (94 – 111) * | 41 (34 – 49) |
| FEV ₁ /FVC | 38 (31 - 47) | 79 (75 - 83) * | 42 (35 – 43) |
| BMI (kg/m ²) | 26 (24 - 28) | 24 (21 - 33) | 25 (23 – 27) |
| Receiving inhaled steroids | 83% | 0% * | 83% |
| Receiving theophyllines | 0% | 0% | 0% |
| Receiving long acting β 2 agonist | 75% | 0% * | 83% |
| Bacterial colonization | 0% | N/A | 0% |

Legend

Age is presented as the mean (with the range in parenthesis). Gender is the number of males in each group. Patients receiving listed medications are denoted as a percentage of the group. The remaining data is presented as the median (with the interquartile range in parentheses). Healthy subjects differed significantly from both patient groups in all lung function, smoking and medication parameters.

* = significant difference from COPD and A1ATD groups ($p < 0.05$)

Chemotaxis towards IL-8

Figure 5.6, 5.7 and 5.8 provide examples of the cell migratory tracks that were recorded for a representative subjects in each group.

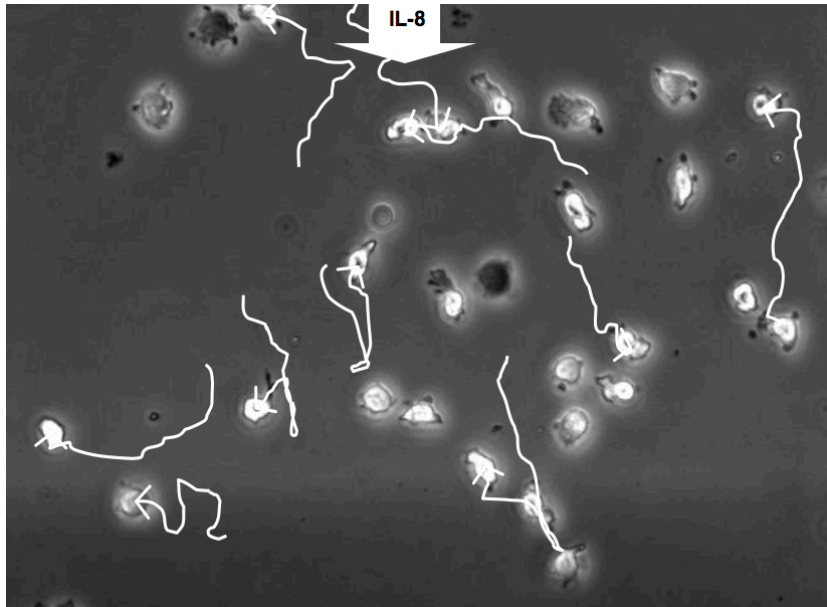


Figure 5.6. Tracks of neutrophil migration from a patient with COPD

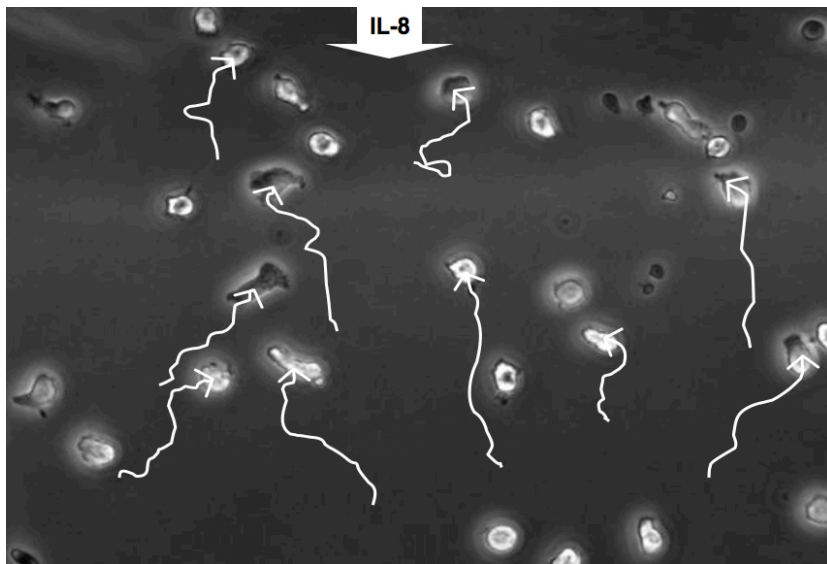


Figure 5.7. Tracks of neutrophil migration from a healthy control

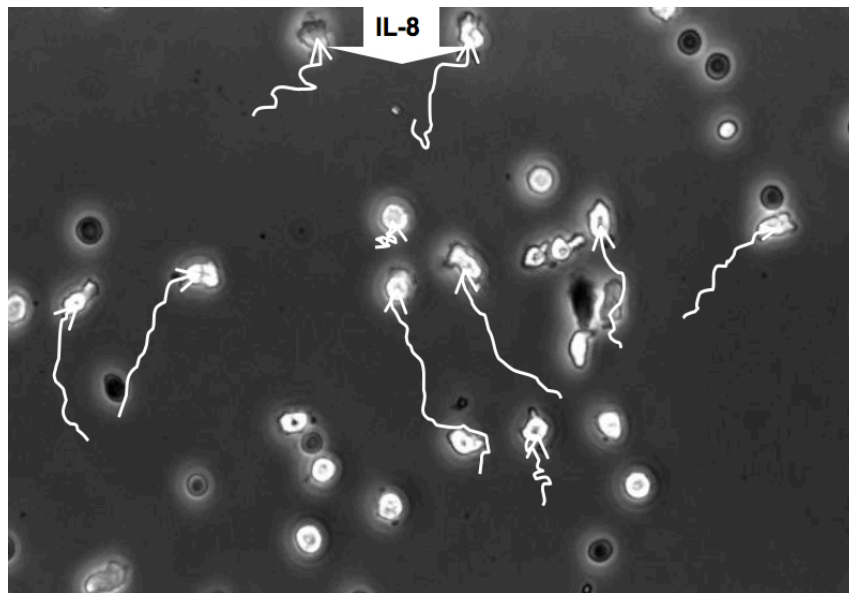


Figure 5.8. Tracks of neutrophil migration from a subject with A1ATD

Figure 5.6 – 5.8: Tracks of neutrophil migration from a subject with COPD, a healthy control and a subject with COPD due to A1ATD.

The images show the final neutrophil positions within the Dunn chemotaxis chamber following 20 minutes of phase time lapse recording. The large white arrow at the top of each picture represents the direction of the source of the chemotactic signal (here IL-8 at a concentration of 100nM for all figures). The tracks (shown in white) indicate the path of migration for each cell analysed: each track begins where the cell was positioned at the start of recording and ends where the cell was positioned at the end of recording. The arrow at the end of each track (again shown in white) indicates the last direction of movement.

Figure 5.6: Tracks of neutrophil migration from a subject with COPD

This picture is representative of all recordings taken of neutrophils from patients with COPD across all concentrations. In this instance, mean cell speed was $5.12\mu\text{m}/\text{min}$, mean velocity was $-1.09\mu\text{m}/\text{min}$, mean persistence was 0.43 and mean chemotactic index was -0.19.

Figure 5.7: Tracks of neutrophil migration from an age and gender matched healthy control This picture is representative of all recordings taken of neutrophils from healthy controls across all concentrations. In this instance, mean cell speed was $3.77\mu\text{m}/\text{min}$, mean velocity was $2.14\mu\text{m}/\text{min}$, mean persistence was 0.34 and mean chemotactic index was 0.49.

Figure 5.8: Tracks of neutrophil migration from a patient with A1ATD matched for FEV₁

This picture is representative of all recordings taken of neutrophils from patients with A1ATD across all concentrations. In this instance, mean cell speed was $3.04\mu\text{m}/\text{min}$, mean velocity was $1.6\mu\text{m}/\text{min}$, mean persistence was 0.38 and mean chemotactic index was 0.47

Speed of migrating neutrophils

Circulating neutrophils from patients with COPD moved with significantly greater speed (chemokinesis) than both healthy controls ($5.91\mu\text{m}/\text{min} \pm 0.17$ Vs. $4.37\mu\text{m}/\text{min} \pm 0.14$, $p < 0.0001$) and the disease-matched patients with A1ATD ($3.26\mu\text{m}/\text{min} \pm 0.16$, $p < 0.0001$); summarised in figure 5.9.

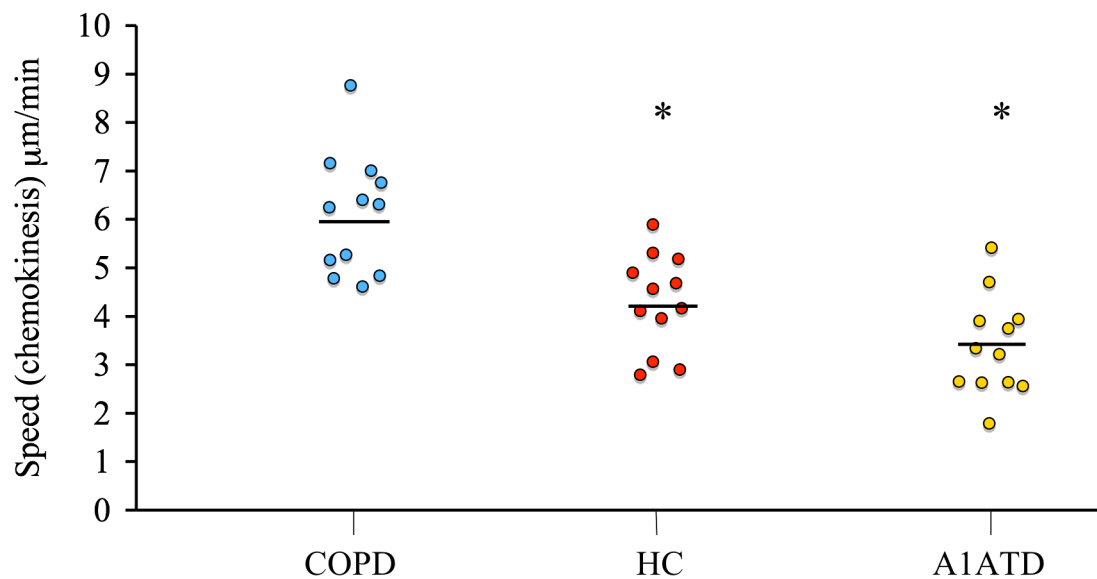


Figure 5.9. Comparisons of average speed of migrating neutrophils from patients with COPD, age matched controls and patients with A1ATD: IL8

Legend: Measurements were taken from 10 cells from each individual (migrating in the presence of a chemotactic gradient formed from 10nM of IL-8). Each experiment was repeated three times and the average results for each subject were calculated, and an overall average was used for comparisons between groups. Each data point represents the average migratory parameter for one individual. * = significant difference from COPD neutrophils (see text).

Velocity of migrating neutrophils

COPD neutrophils demonstrated a decreased velocity (directed speed movement or chemotaxis) compared with healthy controls ($0.87\mu\text{m}/\text{min} \pm 0.16$ Vs. $1.43\mu\text{m}/\text{min} \pm 0.12$,

$p = 0.04$) but not compared to the A1ATD patients ($1.1\mu\text{m}/\text{min} \pm 0.26$, $p = 0.8$); figure 5.10.

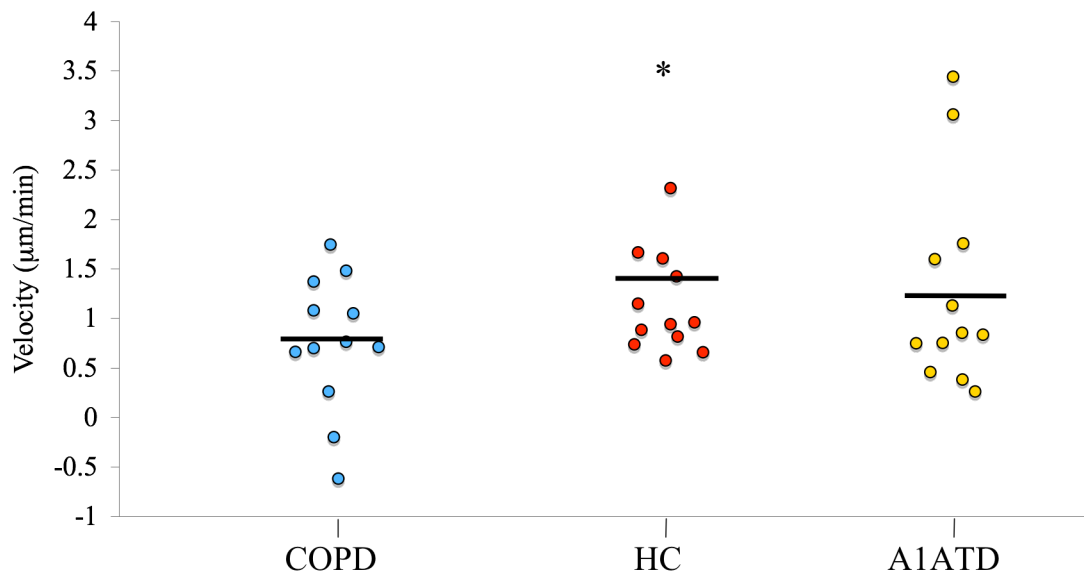


Figure 5.10. Comparisons of average velocity of migrating neutrophils from patients with COPD, age matched controls and patients with A1ATD: IL8

Legend: Measurements were taken from 10 random cells from each individual (migrating in the presence of a chemotactic gradient formed from 10nM of IL-8). Each experiment was repeated three times and the average results for each subject were calculated, and an overall average was used for comparisons between groups. Each data point represents the average migratory parameter for one individual. * = significant difference from COPD neutrophils (see text).

The persistence of migrating neutrophils

There were no differences in the number of changes of direction made by circulating COPD neutrophils during migration compared to both control groups (they displayed the same degree of directional persistence); COPD mean persistence 0.28 ± 0.05 ; Healthy control mean persistence 0.32 ± 0.04 ; A1ATD mean persistence 0.28 ± 0.05 .

Chemotactic Accuracy

Neutrophils from patients with COPD were significantly less accurate during migration (displaying a lower chemotactic index) compared with the healthy controls (0.15 ± 0.02 Vs. 0.27 ± 0.02 , $p = 0.006$) and patients with A1ATD (0.26 ± 0.03 , $p = 0.04$), summarised in figure 5.11.

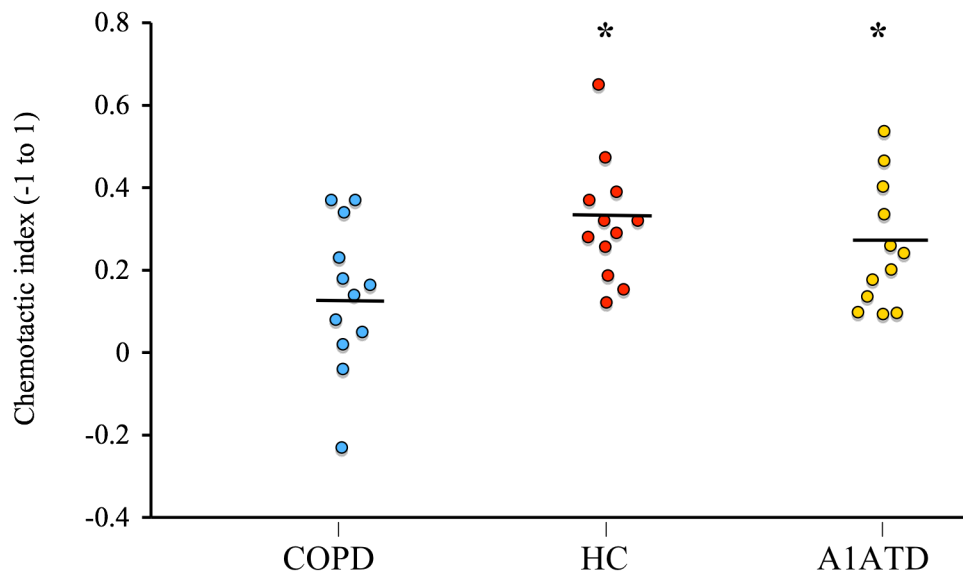


Figure 5.11. Comparisons of average chemotactic accuracy of migrating neutrophils from patients with COPD, age matched controls and patients with A1ATD: IL8

Legend: Measurements were taken from 10 random cells from each individual (migrating in the presence of a chemotactic gradient formed from 10nM of IL-8). Each experiment was repeated three times and the average results for each subject were calculated, and an overall average was used for comparisons between groups. Each data point represents the average migratory parameter for one individual. * = significant difference from COPD neutrophils (see text).

These data show that neutrophils isolated from patients with COPD clearly have movement differences involving excessive, continuous migration, as demonstrated by their increased speed of migration, coupled with a reduction in velocity and chemotactic accuracy. Knowing the speed and distance of the neutrophil migratory tracks enabled a

theoretical comparison of the potential lung damage neutrophils could produce if migratory patterns *in vivo* resembled those seen *in vitro*.

5.3.4 Model of lung damage in COPD and A1ATD

The observations included in the current study are consistent with a substantial body of work implicating the neutrophil in the pathogenesis of COPD and provide a potential model incorporating the physiological effects of neutrophil proteinases and the widespread damage seen in lung tissue. During activation, it is known that a high proportion of neutrophil proteinases are expressed on the neutrophil membrane (Owen, Campbell et al. 1995; Owen, Campbell et al. 1997; Campbell, Campbell et al. 2000), polarising towards the leading edge of the neutrophil as it migrates (Cepinskas, Sandig et al. 1999). A proportion of the proteinase is left behind as the cell moves on (Clayton, Evans et al. 1998; Cepinskas, Sandig et al. 1999) and it has been clearly demonstrated that an area of obligate enzyme activity (or “collateral damage”) always exists following the secretion of free NE from activated neutrophils until concentrations have decreased by diffusion to match the concentration of surrounding proteinase inhibitors (Liou and Campbell 1996; Campbell, Campbell et al. 1999). This explains the excess lung damage that occurs during neutrophil migration in A1ATD where the low level of antitrypsin results in a wider area of obligate enzyme activity around a normal migrating neutrophil.

The more circuitous migratory routes taken by COPD neutrophils (as shown here) would increase the potential for collateral damage by a different mechanism, as more NE would be released during the lengthier journey to the source of inflammation, thereby increasing the area of obligate enzyme activity.

Furthermore, the presence of this extra NE, and any resultant tissue and cell damage, would also increase the secretion of other pro-inflammatory mediators such as LTB₄ and IL-8 (Hubbard, Fells et al. 1991), amplifying the migration of inflammatory cells (including more neutrophils and macrophages) into the surrounding tissue with their resultant pro-inflammatory interactions (Itoh and Nagase 1995; Ferry, Lonchamp et al. 1997),

Diffusion analysis predicts that the obligate catalytic activity (excess of local NE over inhibitor concentration) extends to 1.33 μm from the site of granule extrusion in subjects with normal plasma levels of α_1 antitrypsin, and 3.29 μm from the site of granule extrusion in subjects with A1ATD and reduced levels of A1AT (Liou and Campbell 1995). Measurements from our data suggest that the average diameter of a polarised migrating neutrophil is 3 μm . The average migratory distances of neutrophils were significantly greater in COPD than in health ($0.98 \mu\text{m} \pm 0.03$ Vs. $0.72 \mu\text{m} \pm 0.02$ in ten seconds) or A1ATD ($0.54\mu\text{m}$ in ten seconds ± 0.03).

Using a simplistic mathematical model, and applying the formula distance $\times (\pi r^2)$, where r equals the average radius of a cyclindrical neutrophil including an allowance for obligate catalytic activity based on the calculations by Liou et al (Liou and Campbell 1995), the theoretical volume of lung damage in ten seconds of a migrating neutrophil in COPD is $76\mu\text{m}^3$ compared to $56\mu\text{m}^3$ in health. If one accepts that neutrophils are releasing proteinases throughout their migratory tracks in order to penetrate through tissue, these data suggest that the COPD neutrophil would cause approximately 30% more damage compared to control cells and this may explain the increased susceptibility of such

individuals to develop COPD features. In A1ATD there is also an increased potential for tissue damage, with the theoretical volume of lung damage in ten seconds of a migrating neutrophil in A1ATD being $110\mu\text{m}^3$ compared to $56\mu\text{m}^3$ in health (an increase of 90%), because of the low prevailing concentration of A1AT rather than abnormal migratory function.

5.3.5 Migration towards GRO α

To determine if migratory patterns seen were selectively dependent on CXCR1 or CXCR2, similar migratory studies were performed on a smaller sample of patients with COPD compared to healthy controls and patients with A1ATD (n=6 per group), using GRO α as the chemoattractant.

5.3.5.1 Baseline characteristics

Demographic data for the COPD patients, healthy controls and patients with A1ATD are shown in Table 5.5. The results shown are for postbronchodilator forced expiratory volume in one second (FEV₁) expressed as a percentage of the value predicted for the patient's age, gender and height (British Thoracic Society 1994) and the ratio of FEV₁ to vital capacity (FEV₁/FVC).

Table 5.5. Demographic data for COPD patients, healthy controls and patients with A1ATD

| | COPD | HC | A1ATD |
|---|------------------|--------------------|------------------|
| N | 6 | 6 | 6 |
| Age (years) | 62 (57 - 66) | 60 (55 – 64) | 59 (54 – 65) |
| Gender (Male) | 4 | 4 | 3 |
| Smoking status | All ex-smokers | Mixed | All ex-smokers |
| Pack years | 37 (31 - 53) | 16 (0 – 29) * | 28 (20 – 36) |
| FEV ₁ (l) | 0.84 (0.7 – 1.0) | 2.15 (2.0 – 2.7) * | 0.96 (0.8 – 1.2) |
| FEV ₁ % predicted | 46 (41 - 50) | 91 (85 – 106) * | 50 (46 – 52) |
| FEV ₁ /FVC | 52 (36 - 59) | 81 (76 – 85) * | 60 (40 – 66) |
| BMI (kg/m ²) | 22 (19 - 27) | 24 (21 – 32) | 21 (20 – 23) |
| Receiving inhaled steroids | 100% | 0% * | 100% |
| Receiving theophyllines | 0% | 0% * | 0% |
| Receiving long acting β 2 agonist | 66% | 0% * | 50% |
| Receiving anti-cholinergics | 50% | 0% * | 50% |
| Bacterial colonization | 0% | N/A | N/A |

Legend

Age is presented as the mean (with the range in parenthesis). Gender is the number of males in each group. Patients receiving listed medications are denoted as a percentage of the group. The remaining data is presented as the median (with the interquartile range in parentheses). Healthy subjects differed significantly from COPD and A1ATD patients in all lung function, smoking and medication parameters.

* = significant difference from COPD ($p < 0.05$)

Speed of migrating neutrophils

Circulating neutrophils from patients with COPD moved with significantly greater speed (chemokinesis) in gradients of GRO α compared to healthy controls ($3.97 \mu\text{m}/\text{min} \pm 0.13$ Vs. $2.34 \mu\text{m}/\text{min} \pm 0.2$, $p = 0.0001$) and patients with A1ATD ($3.01 \mu\text{m}/\text{min} \pm 0.2$, $p=0.008$); summarised in figure 5.12.

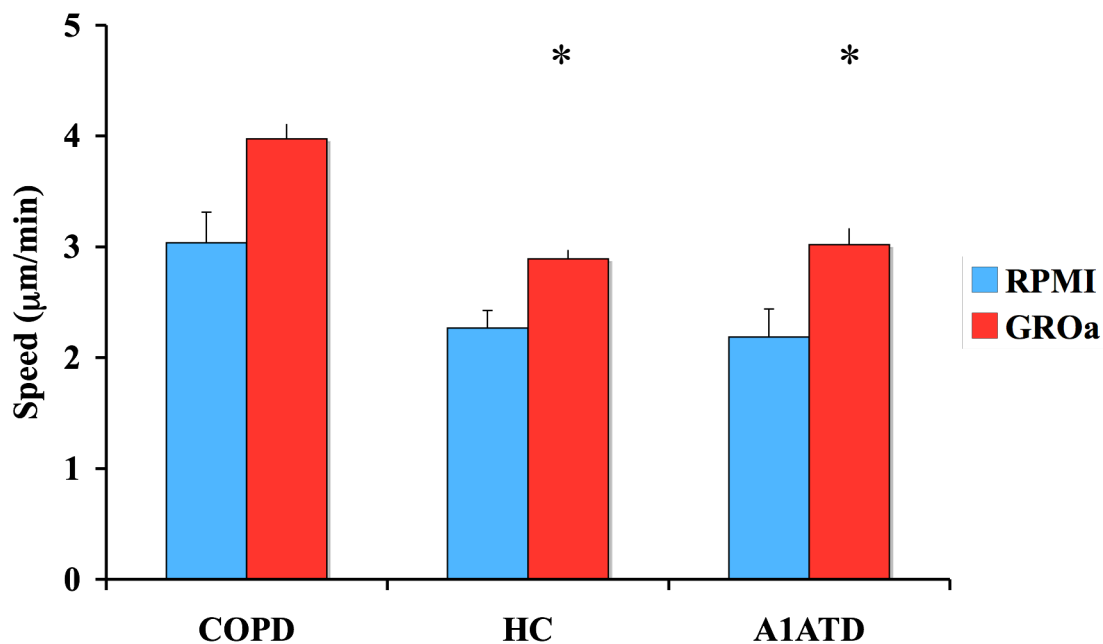


Figure 5.12. Comparisons of average speed of migrating neutrophils from patients with COPD, age matched controls and patients with A1ATD: GRO α

Legend: Measurements were taken from 10 cells from each individual (migrating in the presence of a chemotactic gradient formed from 100nM of GRO α). Each experiment was repeated three times and the average results for each subject were calculated, and an overall average was used for comparisons between groups. Each column represents the average migratory speed and the error bar is the standard error. * = significant difference from COPD neutrophils (see text).

Velocity of migrating neutrophils

COPD neutrophils demonstrated a decreased velocity (directed speed movement or chemotaxis) compared with healthy controls ($0.21\mu\text{m}/\text{min} \pm 0.2$ Vs. $0.32\mu\text{m}/\text{min} \pm 0.1$, $p = 0.02$) and patients with A1ATD (0.35 ± 0.1 , $p = 0.006$); see figure 5.13.

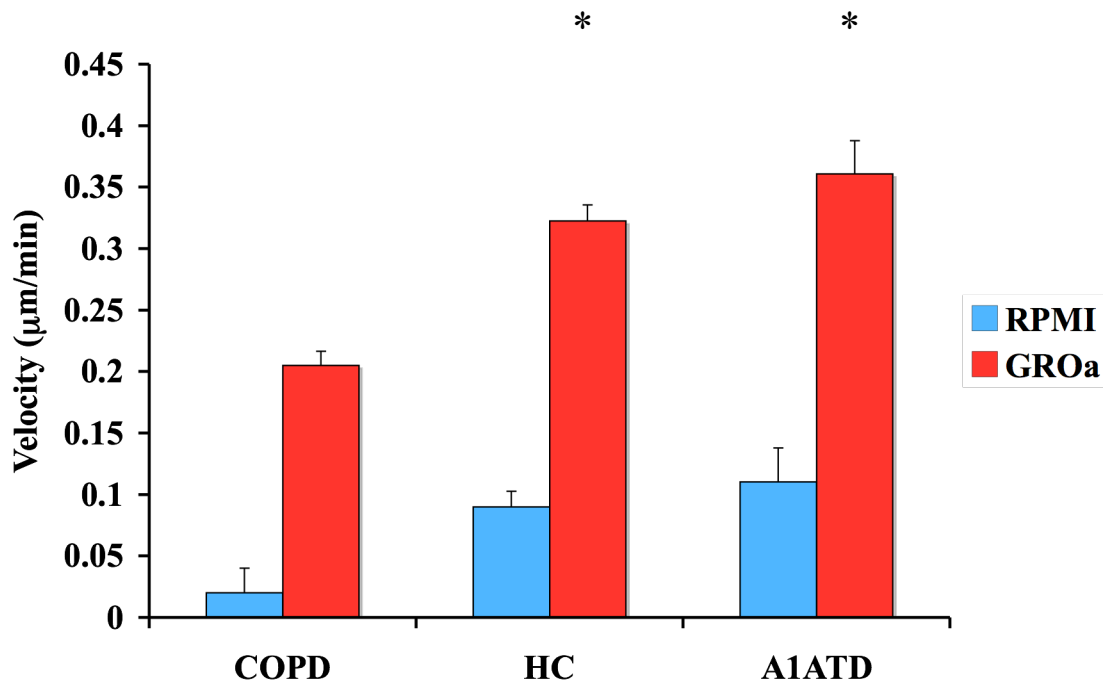


Figure 5.13. Comparisons of average velocity of migrating neutrophils from patients with COPD, age matched controls and patients with A1ATD: GROα

Legend: Measurements were taken from 10 random cells from each individual (migrating in the presence of a chemotactic gradient formed from 100nM of GROα). Each experiment was repeated three times and the average results for each subject were calculated, and an overall average was used for comparisons between groups. Each column represents the mean velocity and the error bars are the standard error. * = significant difference from COPD neutrophils (see text).

The persistence of migrating neutrophils

There were no differences in the number of changes of direction made by circulating COPD neutrophils during migration compared to the control groups (they displayed the same degree of directional persistence); COPD mean persistence 0.17 ± 0.03 ; healthy control mean persistence 0.19 ± 0.1 ; patients with A1ATD 0.19 ± 0.06 .

Chemotactic Accuracy

Neutrophils from patients with COPD were significantly less accurate during migration (displaying a lower chemotactic index) compared with the healthy controls (0.03 ± 0.03

Vs. 0.21 ± 0.05 , $p = 0.004$) and patients with A1ATD (0.25 ± 0.08 , $p < 0.0001$), summarised in figure 5.14.

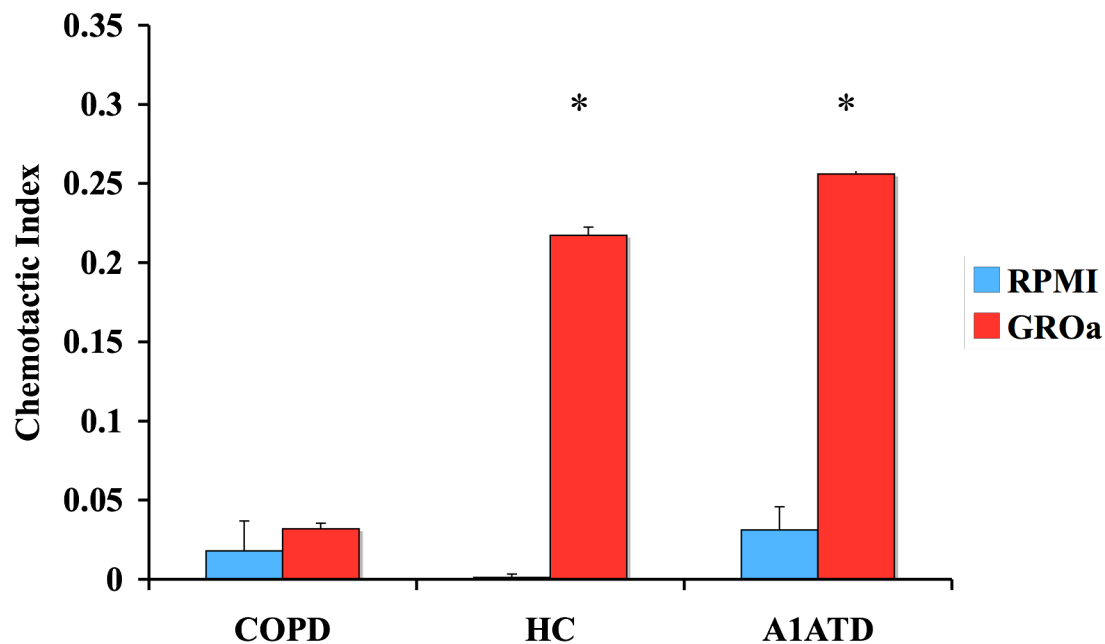


Figure 5.14. Comparisons of average chemotactic accuracy of migrating neutrophils from patients with COPD, age matched controls and patients with A1ATD: GROα

Legend: Measurements were taken from 10 random cells from each individual (migrating in the presence of a chemotactic gradient formed from 100nM of GROα). Each experiment was repeated three times and the average results for each subject were calculated, and an overall average was used for comparisons between groups. Each column is the mean chemotactic index and the error bars are the standard error. * = significant difference from COPD neutrophils (see text).

These data also show that neutrophils isolated from patients with COPD are clearly different comprising excessive but inaccurate migration, as demonstrated by their increased speed of migration, coupled with a reduction in velocity and chemotactic accuracy towards GROα. There was no difference in migration between healthy controls and patients with A1ATD. These data gave the same pattern of results as was seen with IL-8.

5.4 Discussion

This body of work proposes that lung damage in COPD could be driven by aberrant migration in COPD, and that the migrational differences seen in this disease cannot be explained by cigarette smoke, lung inflammation or its treatment per se, but instead are likely to represent an intrinsic flaw with the neutrophil in this patient population. This work also suggests that the migrational differences seen in COPD are not purely an expression of advanced ageing, since they differ from the patterns of ageing neutrophils seen in the previous chapter.

The current study describes clear differences in the migratory behaviour of neutrophils from patients with COPD compared with two control groups; age and gender matched healthy subjects, and patients with A1ATD with COPD due to a genetic deficiency of alpha 1-antitrypsin, but with a similar severity of lung disease.

The under agarose studies demonstrated that neutrophils from patients with COPD (without A1ATD) displayed greater chemotaxis and chemokinesis than either control group, when migrating in the presence of IL-8 and fMLP. It appeared that the neutrophils were spreading out further during their migration, both towards and away from the chemotactic source. The net chemotactic movement (chemotaxis – chemokinesis) however was not different.

Previous studies have reported that neutrophils from patients with A1ATD display a greater chemotactic differential (chemotaxis – chemokinesis) than patients with usual COPD, when migrating towards their own sputum (Woolhouse, Bayley et al. 2002). However, the A1ATD sputum samples contained greater concentrations of both IL-8

and LTB₄ than those from the PiM COPD patients, suggesting that migratory differences could have been driven by increased mediator content. There have been no other studies directly commenting on the migratory differences between COPD and A1ATD using this methodology. To further explore the migratory differences seen, individual cells were studied using the modified Dunn Chamber.

Firstly, using this assay in smoking and non smoking healthy controls who were age matched to the COPD group, established that active smoking does not influence neutrophil migration. Secondly, it was shown that neutrophils from the COPD patients displayed clear differences in their migratory dynamics compared to other controls groups. The cells were faster in their migratory speed but less accurate (exhibiting a lower velocity and chemotactic index) than both the healthy controls and patients with A1ATD. Overall the data suggests cells from COPD patients move more randomly in the presence of a chemotactic gradient, as demonstrated by the results of both the agarose and Dunn chamber method.

When migratory pathways were mapped out, a rudimentary volume of tissue damage (allowing for theories of quantum proteolysis) per neutrophil was calculated. COPD neutrophils had the capacity to cause 30% more tissue damage than healthy controls (due to convoluted migratory tracks but a normal area of obligate damage), while A1ATD neutrophils had the capacity to cause 90% more damage (due to less regulated proteolysis in the presence of normal migration). Interestingly, when one compares studies of FEV₁ decline, the approximate annual decline in absolute FEV₁ in healthy subjects (age matched to the COPD patients included in this study) is 45ml/year (Fletcher and Peto 1977; Kerstjens, Rijcken et al. 1997). Studies of COPD have given

variable results, but in patients with similar characteristics to those included in this body of work, the average annual decline in absolute FEV₁ ranges from 59 – 74 ml/year (Burge, Calverley et al. 2000; Simmons, Connett et al. 2005; Makris, Moschandreas et al. 2007) and in patients with A1ATD and moderate severity airflow obstruction, the decline is approximately 90ml/year (Dawkins, Dawkins et al. 2009). This equates to a 45% increase in FEV₁ decline in COPD and a 100% increase in FEV₁ decline in A1ATD, compared to that seen in health. These figures are similar to the estimated increased potential for tissue damage given above using migratory tracks, suggesting a common paradigm of neutrophil mediated damage in both groups.

The current study included patients with COPD related to a genetic anti-proteinase deficiency (A1ATD), in order to determine any impact of lung disease alone or its treatment on neutrophil migration. In the current study, cells from these A1ATD patients had similar migratory parameters to healthy controls. Previously, neutrophils from patients with A1ATD also displayed overall adhesion and migratory patterns under flow conditions that were the same as healthy controls (Woolhouse, Bayley et al. 2005). In the same study, neutrophils from matched COPD patients showed enhanced adhesion and migration consistent with the findings of the current study. On the other hand, neutrophils from patients with bronchiectasis (another more marked inflammatory lung disease) also had normal chemotactic responses (Burnett, Chamba et al. 1987). Together, these data suggest that lung inflammation or severity *per se* does not alter the mechanics of circulating neutrophil migration. In the current study, the patients with A1ATD were also matched for smoking status, medication, the absence of bacterial colonisation and lung function, suggesting that the differences seen in migration cannot

be accounted for any of these factors, and hence are likely to be central to the pathophysiology of COPD.

Of note, the migratory parameters measured in neutrophils from the healthy controls throughout this series of experiments (and in particular when using the modified Dunn chamber) did not closely resemble those seen in the healthy elderly subjects in the preceding chapter (with both velocity and chemotactic index being higher when migrating in gradients of IL-8). Nor did they closely resemble those from younger subjects (as they demonstrated reduced persistence), and instead were a mixture of both. Subjects in this body of work were age matched to the COPD patient group, and were much younger than the elderly subjects included in the experiments described in chapter 4. It is likely that any age-related changes in neutrophil behaviour develop progressively rather than suddenly, although there is no published data to confirm this. If patterns of migration documented in this younger group are representative, it appears as though persistence is the first parameter to change with age (with cells making more directional changes during migration), with velocity altering at a later stage. Interestingly, neutrophil migratory behaviour in COPD does not appear to be advanced ageing, as there were notable differences in the dynamics of migration (namely, the increased speed).

In conclusion, the current study presents clear differences in migratory behaviour of circulating neutrophils in COPD compared with healthy age matched controls and disease-matched patients with A1ATD. Cells from COPD patients migrated with greater speed, but with less accuracy towards both IL-8 and GRO, and changes seen could not be explained by smoking, inflammation, bacterial colonisation or treatment

regimes. The findings are in keeping with theories of bystander cell and tissue damage in COPD and provide an important insight into potential mechanisms of the increased lung damage noted in this disease. In order to study these differences further, analysis of the structural properties of migratory neutrophils was undertaken (see chapter 6).

6 Mechanisms of aberrant migration: Pseudopod formation in COPD

6.1 Brief Introduction

Work described in chapter 5 found clear differences in the migratory dynamics of circulating neutrophils from patients with COPD, namely increased speed of movement but decreased accuracy, when migrating in the presence of chemotactic gradients.

As described previously, neutrophil movement occurs via the formation of locally protruding actin-rich pseudopods while retracting other regions of the cell body (Devreotes and Janetopoulos 1988). In order to achieve this, directed neutrophil chemotaxis begins with cell polarisation, where, following stimulation, neutrophils acquire polarity through a self-organising process involving positive “frontness” and negative “backness” feedback loops. The leading edge is composed of F-actin rich lamellipodia, and highly polarized distributions of lipids that are up-regulated during cell alignment (including PIP_3 , Cdc42, PAK1 and Akt). “Backness” is enhanced by inhibiting the formation of a leading edge and with the development of a uropod. This is achieved by actin-myosin II meshworks, regulated by Rho-A and PTEN. In keeping with the role of the leading and trailing edge of the neutrophil, receptors for chemoattractants are redistributed to pseudopods (Gomez-Mouton, Lacelle et al. 2001) and those for adhesion (such as PSGL-1 and ICAM-3) are redistributed to the uropod (Fais and Malorni 2003).

No studies to date have examined the structural nature of individual migrating cells in COPD to determine if differences in migratory behaviour equate to differences in migratory structure. Neutrophils (as with most eukaryote cells) randomly protrude actin-rich pseudopods at the leading edge of the cell and sustain those pseudopods that are best

placed in chemotactic gradients (Andrew and Insall 2007). The direction of chemical gradients are believed to be perceived by receptor occupancy, and those pseudopodia orientated towards the gradient are maintained, determining the direction of migration (Andrew and Insall 2007).

Most studies of cellular migration have focused upon *Dictyostelium* cells, and caution must be applied when making comparisons, however, studies of mutated *Dictyostelium* strains demonstrate that abnormally increased formation of pseudopodia is associated with less migration (Weber 2006). Differences in pseudopodia generation are likely, therefore, to be a determinant of migratory dynamics.

The aims of the present study were to examine, in quantitative detail, pseudopod formation in subjects with COPD, compared to non smoking healthy subjects, smoking subjects with normal lung function and no respiratory symptoms (so called “healthy smokers”) and patients with A1ATD matched for lung disease, severity and treatment.

Once again, the control groups were carefully selected and specifically included patients with A1ATD as these patients can be selected with the same degree of functional impairment, cigarette exposure and similar pulmonary inflammation and therapy to usual COPD (all of which may influence results). Thus, if pseudopod formation in A1ATD resembles health more than COPD, it could be surmised that any differences were not due to the presence of pulmonary and systemic inflammation or lung disease alone.

6.2 Experiments

6.2.1 Study subjects

COPD patients had moderate to severe disease defined by GOLD criteria (Pauwels, Buist et al. 2001). They were ex-smokers, aged between 50 and 70. All patients were clinically stable for at least eight weeks prior to recruitment with no changes in medication. Alternative and concomitant disease was excluded clinically, physiologically and radiologically.

Controls consisted of age and gender matched healthy volunteers and patients with A1ATD. The A1ATD patients were ex-smokers and matched for the severity of airflow obstruction, age, gender, bacterial colonisation and medications. Healthy volunteers were divided into two groups. The first had never smoked, had no evidence of chronic disease (including lung disease) and were medication free. The second group of healthy controls were current smokers who had no evidence of lung disease (or other co-morbidities) and were also medication free, the so called “healthy smokers”. These two control groups were chosen as comparisons with healthy controls would allow an understanding of differences from normal, including the effects of smoking and comparisons with patients with A1ATD would differentiate the effects of pulmonary inflammation and lung disease *per se*.

6.2.2 Isolation of blood neutrophils

Neutrophils were isolated from the whole blood as described previously (methods 2.5.7). The neutrophils (>95% pure, > 97% viable, by exclusion of trypan blue) were resuspended in buffer (RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK) containing 0.15% bovine serum albumin (Sigma-Aldrich, UK).

6.2.3 Assessment of pseudopod formation

6.2.3.1 Modified Dunn Chamber

Pseudopod formation was assessed using a modification of the Dunn chamber and DIC time lapse recordings. Full methodological details are included in the methods (section 2.7.2). Each subject's neutrophils were studied with a negative control (assay buffer, see above) and with IL-8 10^{-7} M (concentrations were chosen following appropriate dose response experiments, see 4.3.3.3).

Analysis of pseudopods

The java software ImageJ (Wayne Rasband, NIH, Bethesda) was used to outline individual pseudopods of time lapse images using plug-ins developed by Natasha Andrew (Biosciences, University of Birmingham), as described in section 2.7.2.3. In brief, pseudopods were defined as being granule free regions of the cell that had some protrusive activity. New pseudopods were defined as “daughter” or “new lateral” pseudopods. Daughter pseudopods were identified after having split from the parent pseudopod (noted when a granular region divided a granule free area into two regions), sharing pixels with their parents in the previous frame. Any new pseudopod that did not have a parent defined by this criterion was defined as a new lateral pseudopod.

Pseudopod formation was firstly compared between healthy non-smokers and “healthy smokers” (n=6 for each group). With this as a background, it was then assessed in patients with COPD, healthy controls and patients with A1ATD (n=12 for each group).

6.2.4 Statistical analysis

Pseudopod generation between groups was compared using the mean data of 5 random cells per experiment, with each experiment being repeated three times for each subject. Differences in pseudopod generation were compared using an Independent *t* test. A *p* value of less than 0.05 was considered to be statistically significant.

Sample size calculations were carried out following a pilot study to assess inter and intra patient variability in pseudopod generation. Based upon a two group parallel comparison, the inclusion of 6 subjects to each arm provided an 80% power of detecting a 30% change in pseudopod formation at the 5% level of significance between healthy smokers and non smokers and the inclusion of 12 subjects to each arm provided an 80% power of detecting 20% change in pseudopod formation at the 5% level of significance between patients with COPD, healthy controls and patients with A1ATD (both as described in section 2.9).

6.3 Results

6.3.1 Validation

A pilot study of healthy volunteers determined that there was little intra or inter-subject variability in pseudopod formation on neutrophils migrating towards IL-8. Power calculations were performed to determine the numbers needed to confirm a 50 – 25% change in pseudopod formation with 80% power and at the 5% significance level, using the mean of data from 5 cells per subject, with each experiment repeated in triplicate. Table 6.1 summarises the mean pseudopod formation in healthy subjects, the variation in inter-patient pseudopod formation and the number of subjects required in each group.

six participants were recruited to study differences in healthy controls and 12 subjects were recruited to study differences in COPD and A1ATD, to provide the maximum opportunity to see differences in migration.

Table 6.1. The numbers needed in a parallel design study to detect varying difference in neutrophil pseudopod formation between groups

| | |
|--|------|
| Mean new pseudopod generation (per minute) | 6.9 |
| SEM | 0.24 |
| Co-efficient of variation | 8.5% |
| Number of subjects needed to detect: | |
| 50% difference in pseudopod formation | 2 |
| 40% difference in pseudopod formation | 3 |
| 30% difference in pseudopod formation | 4 |
| 20% difference in pseudopod formation | 6 |
| 10% difference in pseudopod formation | 12 |

6.3.2 Smoking and Non smoking health controls

Pseudopod formation was studied in non healthy and “healthy smokers” to assess the effect of active smoking on pseudopod generation, both when cells were quiescent and activated in chemotactic gradients of IL-8 (10nM).

6.3.2.1 Baseline characteristics

Demographic data for the “healthy smokers” and healthy non smokers (who had never smoked) are shown in table 6.2. The results shown are for postbronchodilator forced expiratory volume in one second (FEV₁) expressed as a percentage of the value predicted for the patient’s age, gender and height (British Thoracic Society 1994) and the ratio of FEV₁ to forced vital capacity (FEV₁/FVC). Subjects were recruited if they had no chronic disease, were on no medication, were symptom free and had no evidence of pathology on physical examination or spirometry.

Table 6.2. Pseudopod extension. Demographic data for healthy never smokers and smokers

| | Healthy never smokers | Healthy smokers |
|------------------------------|-----------------------|------------------|
| N | 6 | 6 |
| Age (years) | 61 (56 - 69) | 63 (58 – 71) |
| Male | 3 | 3 |
| Smoking status | All never | All current |
| Pack years | 0 | 36 (20 – 46) |
| FEV ¹ | 2.51 (2.3- 2.9) | 2.49 (2.3 – 3.2) |
| FEV ¹ % predicted | 93 (90 - 106) | 96 (92 – 110) |
| FEV ¹ /FVC | 81 (73 - 86) | 83 (76 – 86) |
| BMI | 26 (23 – 30) | 25 (22 – 29) |

Legend. Age is presented as the mean (with the range in parenthesis). Gender is the number of males in each group. The remaining data is presented as the median (with the interquartile range in parentheses). Healthy non-smokers differed from healthy smokers only in terms of smoking exposure. There were no other differences between groups.

6.3.2.2 Pseudopod generation

Figure 6.1 depicts typical pseudopodia on a neutrophil from a healthy control, migrating towards IL-8. Pseudopods shown in figure 6.1 are typical for those counted throughout this body of work. There are some differences in appearances of these pseudopods and those shown in other works (for example, Andrew et al, 2007). This is due to differences in filming techniques (those shown in the Andrew paper were filmed under agar), differences in focusing (with films included in this thesis being focused higher than the substratum, which emphasises the cell body rather than the pseudopod), and differences in cell types (with current observations suggesting that neutrophil pseudopodia being less substantial than those seen in migrating *Dictyostelium* cells).

There were no differences in pseudopod generation between healthy non-smokers and healthy smokers, either in the presence of the negative control (quiescent cells) or in the presence of IL-8 10nM, indicating that smoking per se does not effect neutrophil pseudopod projection (see figure 6.2).

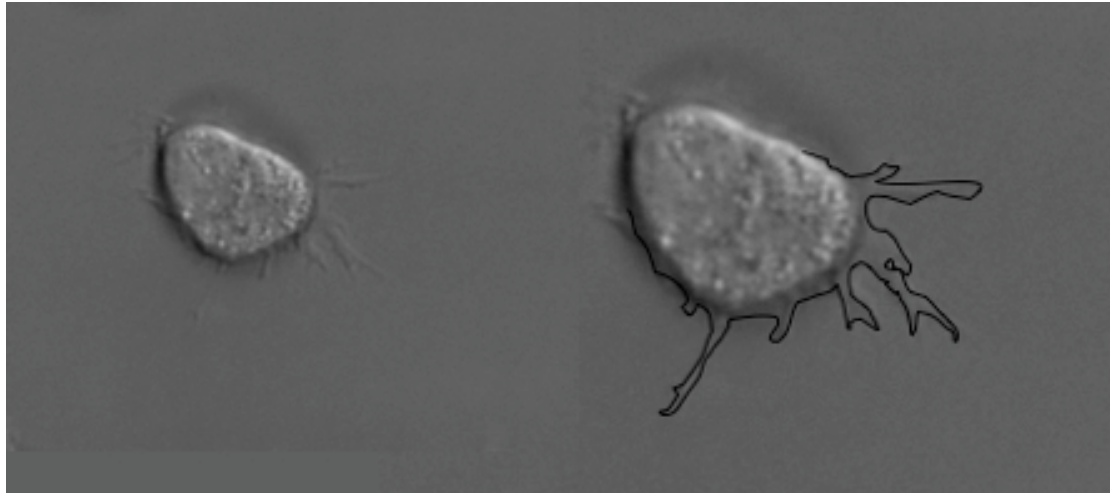


Figure 6.1: Pseudopod structure

Legend: This is an example of a DIC image of a migrating peripheral neutrophil from a healthy control. The image has then been enlarged (on the right) and the pseudopods on the leading edge of the cell have been outlined. Pseudopods (pseudopodia) are temporary projections of eukaryotic cells. Pseudopodia extend and contract by the reversible assembly of actin subunits into microfilaments, while filaments near the cell's end interact with myosin which causes contraction, the combination propels the cell forward. Pseudopodia are thought to be retracted or maintained depending on their orientation in a chemical gradient.

When inactive, mean cellular pseudopod extension was 5.4 ± 0.4 per minute for healthy non-smokers and 5.0 ± 0.3 per minute for healthy smokers. In the presence of a chemotactic gradient of IL-8, mean cellular pseudopod extension increased to 7.0 ± 0.5 per minute for healthy non-smokers ($p = 0.002$) and 7.2 ± 0.3 per minute for healthy smokers ($p=0.001$).

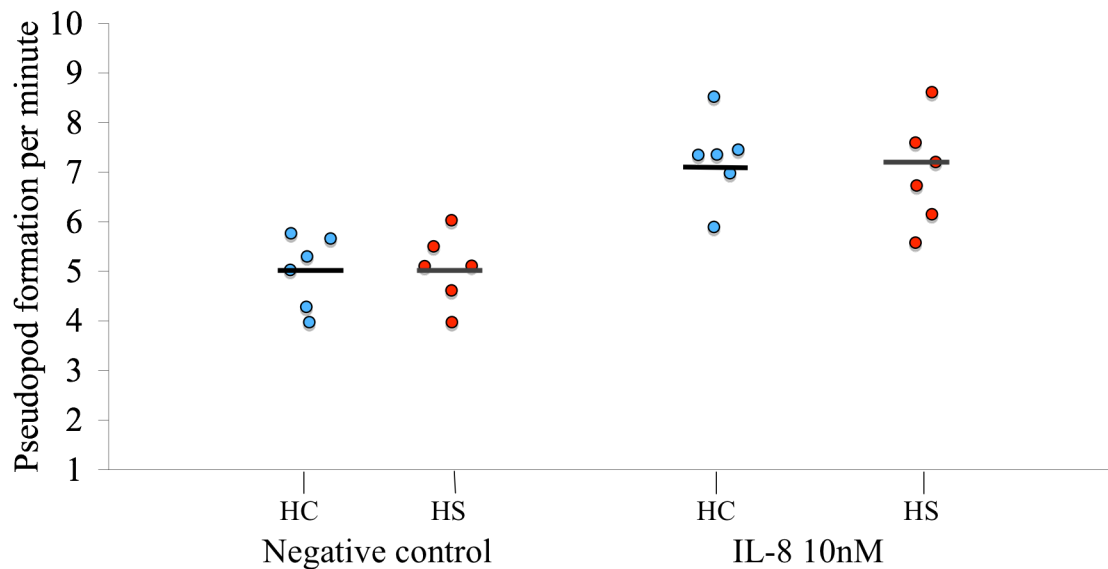


Figure 6.2. Pseudopodia formation on neutrophils from healthy non-smokers and healthy current smokers.

Legend: Pseudopodia formation per minute by in neutrophils isolated from healthy non-smokers (HC) and healthy smokers (HS) when cells were quiescent (negative control) or activated (IL-8). Each data point is the mean pseudopod count per individual over 20 minutes of filming. IL-8 increases pseudopod formation (see text) but there were no differences between groups.

As pseudopod formation was the same in healthy smokers and non-smokers, it was surmised that smoking *per se* does not alter pseudopod formation during neutrophil migration towards IL-8.

6.3.3 Pseudopod formation in neutrophils isolated from patients with COPD, A1ATD and healthy controls

Pseudopod formation was then studied in patients with COPD, A1ATD and compared to the healthy controls to see if there were differences both when cells were quiescent and activated.

6.3.3.1 Baseline characteristics

Demographic data for the COPD patients with and without A1ATD and healthy controls are shown in Table 6.3. The results shown are for postbronchodilator forced expiratory volume in one second (FEV₁) expressed as a percentage of the value predicted for the patient's age, gender and height (British Thoracic Society 1994) and the ratio of FEV₁ to vital capacity (FEV₁/FVC).

Table 6.3. Demographic data for COPD patients, healthy controls and patients with A1ATD.

| | COPD | Healthy subjects | A1ATD |
|---|------------------|--------------------|-----------------|
| N | 12 | 12 | 12 |
| Age (years) | 60 (55 - 68) | 59 (53 - 68) | 53 (45 - 67) |
| Gender (Male) | 7 | 5 | 7 |
| Smoking status | All ex-smokers | Never and current | All ex-smokers |
| Pack years | 36 (24 - 43) | 13 (0 - 47) * | 29 (15 - 38) |
| FEV ₁ (l) | 0.96 (0.8 - 1.1) | 2.54 (2.5 - 2.9) * | 1.2 (0.9 - 1.6) |
| FEV ₁ % predicted | 44 (39 - 49) | 101 (94 - 111) * | 41 (34 - 49) |
| FEV ₁ /FVC | 38 (31 - 47) | 79 (75 - 83) * | 42 (35 - 43) |
| BMI (kg/m ²) | 26 (24 - 28) | 24 (21 - 33) | 25 (23 - 27) |
| Receiving inhaled steroids | 83% | 0% * | 83% |
| Receiving theophyllines | 0% | 0% | 0% |
| Receiving long acting β 2 agonist | 75% | 0% * | 83% |
| Bacterial colonisation | 0% | N/A | 0% |

Legend

Age is presented as the mean (with the range in parenthesis). Gender is the number of males in each group. Patients receiving listed medications are denoted as a percentage of the group. The remaining data is presented as the median (with the interquartile range in parentheses). Healthy subjects differed significantly from both patient groups in all lung function, smoking and medication parameters.

* = significant difference from COPD and A1ATD groups ($p < 0.05$, see text for values)

6.3.3.2 Pseudopod formation

Pseudopod generation was studied when cells were quiescent and activated. In total, peripheral neutrophils from healthy controls produced 5.1 ± 0.2 pseudopods per minute when the cells were quiescent (negative control), which increased to 7.1 ± 0.3 pseudopods per minute in the presence of 10nM IL-8 ($p = 0.001$).

When quiescent, neutrophils from patients with COPD produced less pseudopods per minute than those from healthy control (3.9 ± 0.1 pseudopods per minute, $p < 0.0001$). There was no significant difference compared with patients with A1ATD (4.1 ± 0.1 pseudopods per minute, $p = 0.06$).

However, when activated and migrating towards IL-8, neutrophils from patients with COPD did not increase the rate of pseudopod formation compared with quiescent cells. COPD neutrophils produced significantly less pseudopods while migrating than cells from healthy controls (3.9 ± 0.1 Vs 7.1 ± 0.3 pseudopods per minute, $p < 0.0001$) and cells from patients with A1ATD (4.9 ± 0.1 pseudopods per minute, $p = 0.0001$). See Figure 6. 3.

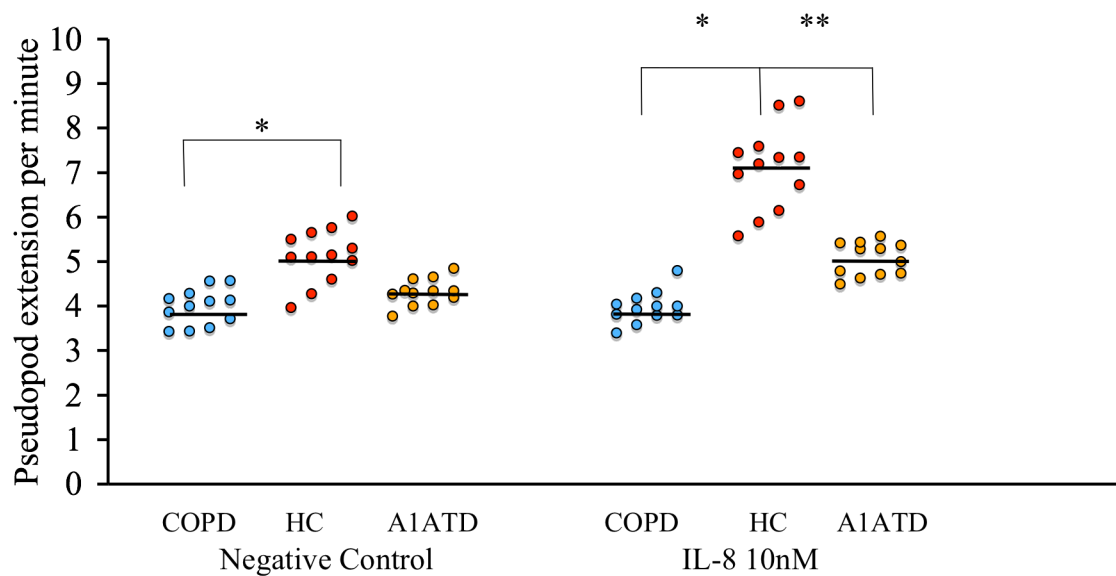


Figure 6.3. Pseudopodia formation on neutrophils from patients with COPD, healthy controls and patients with A1ATD.

Legend. Pseudopodia formation per minute are shown for each individual for each subject group when cells were quiescent (negative control) or activated (IL-8). Each data point is the mean pseudopod count per individual over 20 minutes of filming. * = significant difference from HC cells. ** = significant difference from A1ATD cells. COPD neutrophils produced less pseudopodia than cells from healthy controls when quiescent ($p < 0.0001$), and less than cells from healthy controls and patients with A1ATD when activated ($p < 0.0001$ and $p=0.0001$, respectively).

6.4 Discussion

Circulating neutrophils isolated from subjects with COPD display clear differences in migratory behaviour compared with control groups. This series of experiments also suggest that they display different structural qualities during migration. Pseudopod counts were lower on COPD neutrophils compared with healthy controls both when quiescent and when activated. As stated, it is known that some mutated *Dictyostelium* strains demonstrate abnormally increased pseudopodia formation and that this is associated with less migratory speed (Weber 2006). It is therefore possible that the reverse is also true, and that fewer pseudopods on neutrophils might be consistent with

faster migratory speed. Pseudopod generation is limited by enzymatic reactions in the signalling pathway (Zhelev, Alteraifi et al. 2004), suggesting a defect within this pathway in COPD patients might explain the increased speed of migration.

It is generally accepted that pseudopods are also a means by which motile cells experience their surroundings and orientate themselves via receptor occupancy (Andrew and Insall 2007). It is known that pseudopod extension frequency increases with chemoattractant mediators in a dose dependent manner (Zhelev, Alteraifi et al. 2004). Fewer pseudopods would reduce chemotactic sensitivity, due to a smaller surface area for receptor binding. This would result in less points of comparison to ascertain the origin of a chemotactic signal accurately (both spatially or temporally) by pro-inflammatory receptor occupancy around the cell (Jin and Hereld 2006; Levine and Rappel 2006). This therefore might represent the mechanism of reduced migratory accuracy.

It is likely that the differences in pseudopod formation are unique to COPD. Studies of healthy smokers and non-smokers demonstrate that pseudopod formation is not altered by smoking. Furthermore, comparison of activated neutrophils between patients with COPD and A1ATD suggest that differences seen are not driven by lung disease alone, the treatment of lung disease or the presence of inflammation, as these were comparable between groups.

It is known that patients with A1ATD often have higher concentrations of inflammatory mediators in their sputum (such as IL-8 and LTB₄) compared to matched patients with COPD (Woolhouse, Bayley et al. 2002). The presence of inflammation may effect

pseudopod development when cells are quiescent, as there was no difference in pseudopod formation between patients with A1ATD and COPD in the presence of a negative control, and both groups displayed significantly less pseudopod formation than age and gender matched healthy controls.

One hypothesis of immunosenescence is that changes in immune function with advanced ageing are driven by raised systemic inflammation (termed “inflammaging” (Giunta 2006)). It is known that patients with COPD and A1ATD have increased pulmonary inflammation (Sapey, Bayley et al. 2008) and there is some evidence that this is associated with increased systemic inflammation (for example, a raised high sensitivity CRP (Sapey, Bayley et al. 2008) although evidence for a direct “over spill” from lung to plasma remains mixed (Sapey, Ahmad et al. 2009). It is possible that the presence of continued low-grade inflammation in COPD and A1ATD may reduce pseudopod formation by adaptive mechanisms (preventing neutrophils from migrating and degranulating where there is no specific inflammatory trigger, a process which would limit unnecessary bystander tissue damage). However, if anything, the airway inflammation in A1ATD is greater than in COPD in the stable state (Woolhouse, Bayley et al. 2002), suggesting that this alone does not explain the results for COPD neutrophils. Furthermore, in the presence of an over-riding inflammatory source (here, IL-8), A1ATD neutrophils (unlike COPD cells) are able to increase pseudopod formation (although to a lesser degree than cells from healthy controls), perhaps enabling more accurate migration. In the presence of IL-8, the COPD neutrophils did not increase pseudopod formation, suggesting that there is an underlying problem with migratory processes. In order to study these differences further, analysis of some of the components of the migratory pathway was undertaken.

7 Mechanism of aberrant migration. Surface expression of CXCR1 and CXCR2 and the affects of inhibition of CXCR1 and CXCR2 in COPD

7.1 Brief Introduction

Previous studies in the current thesis have described clear differences in migratory parameters in neutrophils isolated from patients with COPD, as well as the presence of structural differences (with reduced pseudopod formation). Migration is controlled by a pathway, which begins with chemoattractant receptor occupancy at the leading edge of the cell, and through a series of complex interactions, ends with myosin and actin mobilisation (outlined in greater depth in the main introduction). During polarisation, receptors for chemoattractants are mobilised to pseudopods at the leading edge of the cell (Gomez-Mouton, Lacelle et al. 2001).

IL-8 is a member of the ELR+ CXC subfamily of chemokines. These have the characteristic ability to specifically recruit neutrophils into inflamed tissue. Recruitment from the bronchial circulation (although perhaps not from the pulmonary circulation) requires rolling, adhesion and transmigration, which is initiated by the expression of immobilized ELR+ chemokines on the surface of the endothelium (Rainger, Fisher et al. 1997).

CXCR1 and CXCR2 are specific receptors on the cellular surface for the ELR+ chemokines (Holmes, Lee et al. 1991; Murphy and Tiffany 1991). Both receptors bind IL-8 with high and similar affinity, whereas CXCR2 binds the remaining ELR+ with high affinity, with minimal binding from CXCR1 (Lee, Hpruk et al. 1992). In both cases, down stream signalling is initiated once the receptor-ligand complex is internalised via clathrin-mediated endocytosis (Signoret, Hewlett et al. 2005). The binding of ligand results in phosphorylation of Ser and Thr residues in the intracellular loops and the C-terminus of the chemokine receptor by G-protein-coupled receptor

kinases (GRKs) which results in the uncoupling of the G protein subunits from the receptor and receptor desensitization in some cases (Ferguson 2001).

Phosphorylation of these residues is important for the recruitment of adaptor molecules that link the receptor to a lattice of clathrin that facilitates receptor internalization. The association of receptors with these adaptor molecules results in recruitment of clathrin and formation of clathrin-coated pits which pinch-off from the membrane through the action of dynamin and become clathrin-coated vesicles (Barlic, Khandaker et al. 1999; Colvin, Campanella et al. 2004). The clathrin-coated vesicle is then uncoated and the receptor-ligand complex enters the early endosome. The receptor can either enter the recycling compartment and traffic back to the plasma membrane to bind ligand, or enter the late endosome where it will be sorted to the lysosome for degradation (Neel, Lapierre et al. 2008).

Prior studies show that the length of stimulation with ligand plays a role in the recycling/degradation sorting decision (Signoret, Pelchen-Matthews et al. 2000; Fan, Lapierre et al. 2003; Neel, Schutyser et al. 2005). Initially, following CXCL8 stimulation of CXCR1 or CXCR2, the receptor enters the recycling compartment; however, following extended periods of stimulation, the receptor enters the late endosome and lysosome (Fan, Lapierre et al. 2003). The ability of internalised CXCR2 to recycle is crucial for continued gradient sensing and chemotactic response to ligand. When CXCR2 recycling is inhibited, chemotaxis and signaling are impaired (Fan, Lapierre et al. 2003; Fan, Lapierre et al. 2004).

Although both receptors have been characterised, their roles in mediating biological effects has not been well defined, including their role in IL-8 mediated cell chemotaxis. Indeed, studies have produced contradictory results as to the composite role of each receptor in migration. Loetscher et al, monitored the migration of Jurkat cell lines that expressed CXCR1 or CXCR1 and CXCR2. There was an unquantified degree of migration towards IL8 in the absence of CXCR2, that was not seen when GRO α or NAP-2 were used as migratory stimulants, suggesting that CXCR2 is not necessary for migration towards IL-8 in this cell line (Loetscher, Seitz et al. 1994). Chuntharapai et al semi-quantified the surface expression of CXCR2 on neutrophils following migration towards IL-8, and concluded that reduced expression was evidence of a role of CXCR2 in migration (Chuntharapai and Kim 1995). Quan et al utilised a CXCR1 monoclonal antibody and found reduced migration under agarose towards IL-8, but did not assess CXCR2 (Quan, Martin et al. 1996). Ben-Barach described increased phosphorylation of neutrophil CXCR2 following stimulation with IL-8, and suggested that this was suggestive of its' importance in migration, but did not assess CXCR1 (Ben-Barach, Grimm et al. 1997). Finally, Godaly et al treated epithelial layers with antibodies to CXCR1 or CXCR2 and found that only epithelial cells treated with CXCR1 decreased neutrophil migration when the epithelial cells were stimulated with IL-8. The authors suggested that this demonstrated a role for CXCR1 but not CXCR2 in neutrophil migration (Godaly, Hang et al. 2000). However, this oft cited conclusion is flawed. The antibodies were not incubated with the migrating cells, but with the static epithelial cells, and so what was being tested was the affect of antiCXCR1 and antiCXCR2 on the epithelial cell response to IL-8 (perhaps altering autocrine expression of chemokines and adhesion molecules), not neutrophil migration. The authors then showed that neutrophils from an IL-8 receptor homolog knock out mice (mice do not possess

CXCR1 and CXCR2) did not migrate towards IL-8, but here there could be no differentiation between receptor types (Godaly, Hang et al. 2000). To date, therefore, there have been no erudite studies of the role of CXCR1 or CXCR2 on neutrophil migration.

IL8 and CXCR2 expression appears to vary with chronic disease, for example, expression appears to be increased in inflammatory bowel disease (Banks, Bateman et al. 2003) and cystic fibrosis (Hay and Sarau 2001). In pulmonary secretions from patients with COPD, IL8 has been shown to account for 30% of the chemotactic activity of sputum (Woolhouse, Bayley et al. 2002) and IL8 concentrations correlate with neutrophil numbers in sputum (see section 4.7.7), airflow obstruction (see section 4.7.8), and progression of emphysema (Parr, White et al. 2006).

There have been some studies measuring CXCR1 and 2 receptors on neutrophils in COPD, but results have been contradictory. A study by Pignatti et al (Pignatti, Moscato et al. 2005) found lower expression of CXCR2 on the surface of neutrophils from 13 patients with COPD compared with those with neutrophilic asthma and healthy controls. A study by Yamagata et al (Yamagata, Sugiura et al. 2007), found higher median fluorescent intensity for CXCR1, but not CXCR2 in 16 patients with COPD compared with healthy controls. Differences may be explained by the heterogeneity of COPD as a disease, but certainly the numbers included would only be sufficient to determine a 30% change in CXCR1 expression and a 50% change in CXCR2, based upon the power calculations included in this thesis (see section 4.3.5.1).

Potentially differences in pseudopod formation may alter CXCR1 and CXCR2 surface expression or the ability to recycle receptors following stimulation with their ligand. We

hypothesise that a reduction in pseudopod formation may lead to reduced surface receptor expression in COPD (including surface expression of the receptors CXCR1 and CXCR2) as a direct consequence of the reduced pseudopod surface area. If this were the case, it would be predicted that a CXCR2 or CXCR1 antagonist would have differential effects in COPD compared with age matched controls towards CXC chemokines or sputum if perception of receptor occupancy is altered by the migratory or structural differences seen.

The current studies aimed to explore the functional role of CXCR receptors in neutrophil chemotaxis in COPD and age and gender matched healthy subjects (including smoking and non smoking healthy controls). CXCR1 and 2 receptors were measured on the surface of circulating neutrophils taken from these groups, to determine if there was any disparity in surface expression when quiescent and activated. Migrational studies were performed utilising the ELR+ chemokines IL-8 and GRO α , their respective monoclonal antibodies, sputum and following specific inhibition of CXCR1 and CXCR2.

7.2 Experiments

7.2.1 Study subjects

Healthy controls consisted of healthy volunteers, age and gender matched to the COPD group, who had never smoked, had no evidence of chronic disease and were medication free. Healthy smokers were subjects matched to healthy controls, but had a significant smoking history

COPD patients had moderate to severe disease defined by GOLD criteria (Pauwels, Buist et al. 2001). They were ex-smokers, aged between 50 and 70. All patients were clinically

stable for at least eight weeks prior to recruitment with no changes in medication. Alternative and concomitant disease was excluded clinically, physiologically and radiologically. Patients had normal circulating levels of A1AT and were PiMM genotype.

7.2.2 Isolation of blood neutrophils

Neutrophils were isolated from the whole blood as described previously (general methods 2.5.7, (Mikami, Llewellyn-Jones et al. 1998)). The neutrophils (>95% pure, > 97% viable, by exclusion of trypan blue) were resuspended in sterile buffer (RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK) containing 0.15% bovine serum albumin (Sigma-Aldrich, UK) for migrational studies or PBS for semi-quantification of surface receptors by flow cytometry (see section 2.8).

7.2.3 Flow Cytometry

CXCR1 and CXCR2 receptor expression on isolated neutrophils was measured as described in section 2.8 of the general methods. All measurements for an individual were carried out in triplicate, and a median result used for each subject.

Anti-CXCR1 (IgG2A stock concentration 50 µg/ml) diluted to 2ng/ml in 2% BSA PBS, Anti-CXCR2 (IgG2A stock concentration 50 µg/ml) diluted to 3ng/ml in 2% BSA PBS and an isotype matched irrelevant FITC labelled IgG_{2A} (stock concentration 200 µg/ml), diluted to 2ng/ml in 2% BSA PBS, was used as a control. All were incubated on ice for 20 minutes and the resuspended cells were read on FACS within twenty minutes of preparation. Samples from COPD patients and healthy subjects were read at the same time point to allow comparison of receptors.

CXCR1 and 2 receptor expression over time was assessed by measuring the receptors at 3 time points (Time zero, 1 hour, and 2 hours). The effects of cell stimulation on CXCR1 and 2 expression were investigated by measuring receptor surface expression following neutrophil incubation with three concentrations of IL-8 (1, 10 and 100nM) and a negative control (RPMI 1640). The dose range and exposure time were chosen following appropriate validity experiments (as detailed in section 4.3.5).

7.2.4 Neutrophil Chemotaxis

7.2.4.1 Modified Dunn Chamber

Neutrophil chemotaxis was measured using a modification of the Dunn chamber. Full methodological details are included in the general methods (section 2.7.2). Chemotaxis was assessed as described in general methods (section 2.7.2).

7.2.4.2 Chemo-attractants and antagonists

Neutrophils migrated in gradients of a negative control (RPMI (Sigma-Aldrich)), IL-8 (RnDSYSTEMS) (10nM) and IL-8 (at the stated dose) that had been preincubated for 45 minutes at room temperature with IL-8 monoclonal antibody (RnDSYSTEMS) (1.6µM). Studies were also performed with GROα (RnDSYSTEMS) (100nM), GROα that had been pre-incubated for 45 minutes at room temperature with GROα monoclonal antibody (RnDSYSTEMS) (1.6µM) and sputum from patients with COPD. Neutrophils then migrated towards IL-8 (10nM), GROα (100nM) and sputum sol phase both before and following neutrophil incubation with CXCR2 antagonist (kindly provided by AstraZeneca) at two concentrations, 5nM and 500nM for 45 minutes at room

temperature. All doses were chosen following appropriate initial dose response experiments.

Neutrophils then migrated towards IL-8, GRO α (concentrations as above) and sputum sol phase before and following 45 minute incubation with CXCR1 and CXCR2 antibodies (both at 1.6 μ M).

7.2.4.3 Sputum collection and processing

Sputum samples were collected from 15 patients with moderate to severe COPD (according to GOLD criteria). Patients were daily sputum producers with a history of chronic bronchitis as defined by the MRC criteria (Medical Research Council, 1965) and were current or ex-smokers. All patients were confirmed to have obstructive airways disease at screening by spirometric measurements and had stable symptoms of COPD for at least eight weeks prior to recruitment with no changes in medications during this time. Alternative and concomitant lung disease were excluded clinically and by high resolution computed tomography.

Sputum was collected and processed as described in the general methods (section 2.4 and 2.5). All sol samples were pooled, and the same pool of sputum sol was used throughout all studies. Sputum was diluted in RPMI (Sigma-Aldrich) following appropriate initial dose response experiments.

7.2.4.4 Mediator measurement

Concentrations of IL-8 and GRO α were measured in triplicate in the pooled sputum sol samples using commercially available ELISA kits that had been validated previously (see section 2.6).

7.2.5 Statistical analysis

Differences in CXCR1 and 2 surface expression were compared using a Mann Whitney U test. A p value of less than 0.05 was considered to be statistically significant. Migrational differences seen using the Modified Dunn Chamber were compared using the mean data of 10 cells per experiment, per subject. Differences in chemotaxis criteria were compared using an Independent *t* test.

For CXCR1 and 2 surface expression, sample size calculations were based upon a two group parallel comparison, to provide an 80% power of detecting a 20% change of expression in CXCR1 and a 25% change of expression in CXCR2 at the 5% level of significance (both as described in section 2.9). For the Modified Dunn chamber, sample size calculations were based upon a two group parallel comparison, to provide an 80% power of detecting a 25% change in mean speed at the 5% level of significance.

7.3 Results

7.3.1 Baseline characteristics

Demographic data for healthy smoking and non-smoking subjects is shown in table 7.1.

Demographic data for patients with COPD and age and gender matched healthy controls is shown in Table 7.2.

Table 7.1. Demographic data for healthy controls

| | Healthy non smoking subjects | Healthy smoking subjects |
|------------------------------|---------------------------------|-----------------------------|
| N | 12 | 12 |
| Age (years) | 58 (53 - 71) | 64 (55 – 76) |
| Gender (Male) | 5 | 6 |
| Smoking status | All never smokers | All ex or current |
| Pack years | 0 | 19 (12 – 26) * |
| FEV ₁ (l) | 2.34 (2.2 – 2.7) | 2.48 (2.2 – 2.9) |
| FEV ₁ % predicted | 101 (89 – 106) | 94 (90 – 103) |
| FEV ₁ /FVC | 82 (76 – 93) | 81 (70 – 85) |
| BMI (kg/m ²) | 23 (19 – 27) | 24 (21 – 30) |

Legend

Age is presented as the mean (with the range in parenthesis). Gender is the number of males in each group. The remaining data is presented as the median (with the interquartile range in parentheses). Healthy smoking subjects differed significantly from non smoking subjects in smoking history alone. * = significant difference from non smoking subjects ($p < 0.05$)

Table 7.2. Demographic data for COPD patients and healthy controls

| | COPD | Healthy subjects |
|---|------------------|--------------------|
| N | 20 | 20 |
| Age (years) | 66 (55 - 74) | 69 (57 – 78) |
| Gender (Male) | 10 | 10 |
| Smoking status | All ex-smokers | Mixed |
| Pack years | 38 (22 - 47) | 18 (0 – 35) * |
| FEV ₁ (l) | 1.04 (0.8 - 1.3) | 2.48 (2.6 – 2.9) * |
| FEV ₁ % predicted | 47 (38 - 51) | 96 (92 – 107) * |
| FEV ₁ /FVC | 42 (31 - 52) | 78 (74 – 82) * |
| BMI (kg/m ²) | 28 (23 - 32) | 27 (21 – 34) |
| Receiving inhaled steroids | 95% | 0% * |
| Receiving theophyllines | 0% | 0% |
| Receiving long acting β 2 agonist | 80% | 0% * |
| Receiving anti-cholinergics | 75% | 0% * |
| Bacterial colonization | 0% | N/A |

Legend

Age is presented as the mean (with the range in parenthesis). Gender is the number of males in each group. Patients receiving listed medications are denoted as a percentage of the group. The remaining data is presented as the median (with the interquartile range in parentheses). Healthy subjects differed significantly from COPD patients in all lung function, smoking and medication parameters.

* = significant difference from COPD ($p < 0.05$)

7.3.2 CXCR1 and CXCR2 receptor surface expression**7.3.2.1 Validation**

Dose response experiments determined that antiCXCR1 2ng/ml and antiCXCR2 3ng/ml were the most appropriate to measure surface expression of CXCR1 and CXCR2 (see section 4.3.5). Time course experiments determined that neutrophil CXCR1 and CXCR2 expression was greatest immediately following neutrophil isolation (0 Hour). Neutrophil surface expression of both receptors then decreased in a time dependent manner. In light of these results, expression was measured at the same time in neutrophils isolated from COPD patients and healthy subjects, as soon as the neutrophils were isolated, and not longer than 20 minutes from preparation.

7.3.2.2 Quiescent CXCR1 and CXCR2 expression

CXCR1 and CXCR2 surface receptors were semi-quantified on neutrophils isolated from smoking and non smoking healthy controls to assess the impact of smoking behaviour on expression. There was no significant difference in surface expression between these two groups, suggesting that smoking alone has no impact upon surface expression (healthy non smoker, CXCR1 MFI 50, IQR 34 – 58, healthy smoker CXCR1 MFI 52, IQR 36 – 66; healthy non smoker CXCR2 MFI 38, IQR 26 – 46, healthy smoker CXCR2 MFI 41, IQR 35 – 51). See Figure 7.1 and 7.2.

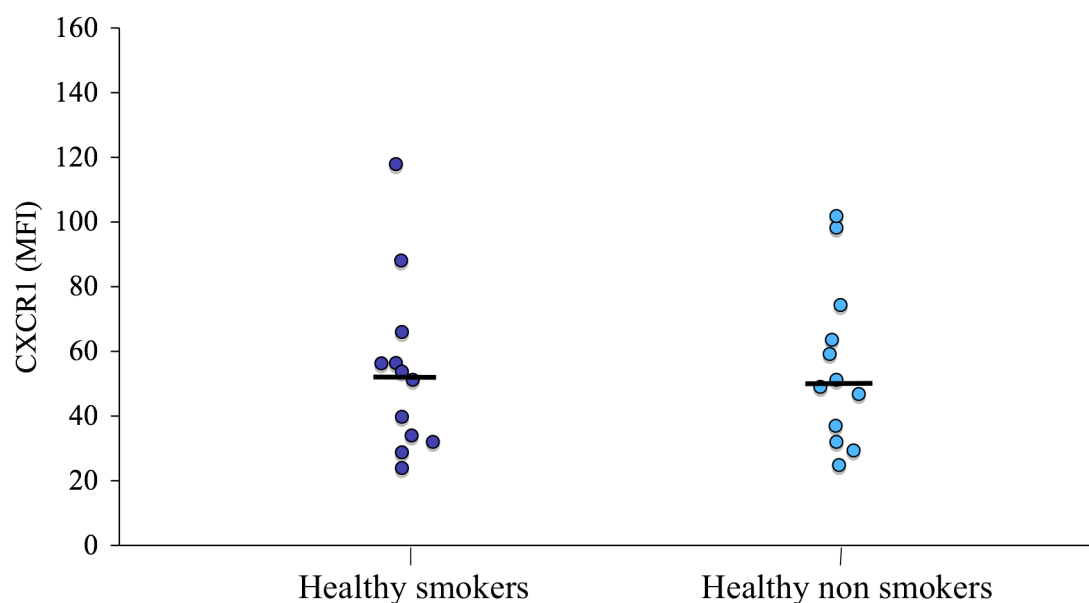


Figure 7.1 Median CXCR1 surface expression

Legend. Data is the median fluorescence intensity for each of 12 healthy non smoking and 12 healthy smoking subjects in anti-human CXCR1. The group medians are shown as the horizontal line.

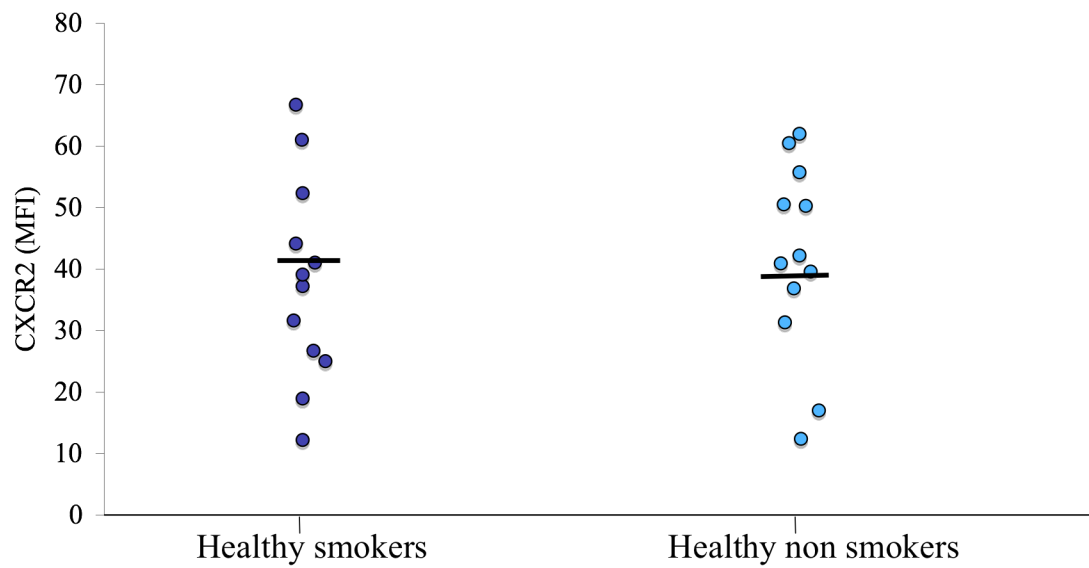


Figure 7.2 Median CXCR2 surface expression

Legend. Data is the median fluorescence intensity for each of 12 healthy non smoking and 12 healthy smoking subjects in anti-human CXCR2. The group medians are shown as the horizontal lines.

There was significantly less CXCR1 expression in COPD compared with age and gender matched healthy controls (median MFI (inter-quartile range), Healthy subjects 49 (32 – 68), COPD patients 33 (22 – 45), $p=0.02$). See figure 7.3.

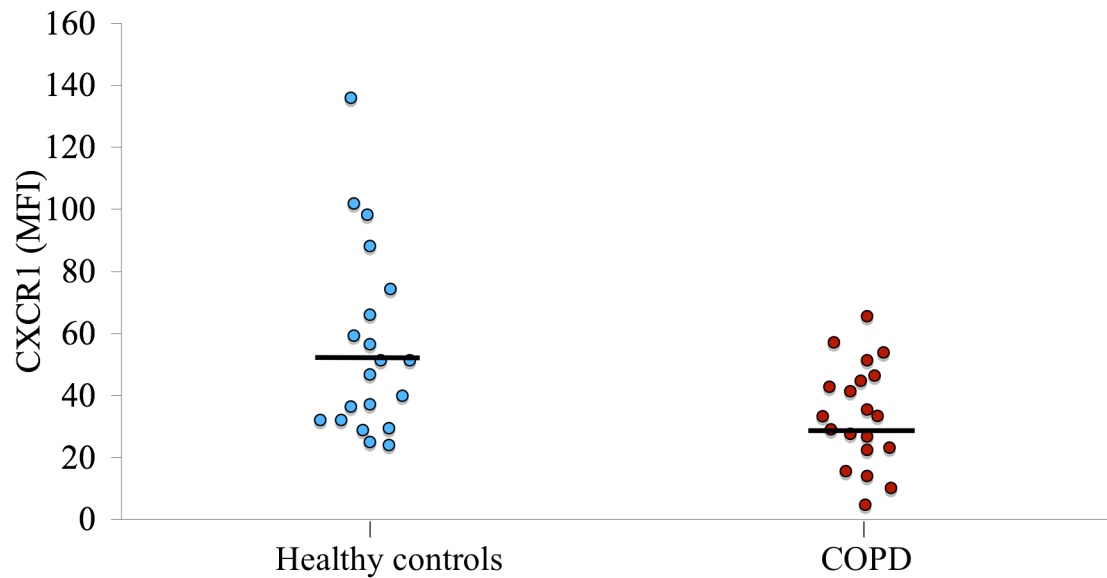


Figure 7.3 Median CXCR1 surface expression in COPD

Legend. Data is the median fluorescence intensity for each of 20 healthy controls and 20 COPD patients in anti-human CXCR1. The group median value is shown as the horizontal line. Data from one healthy control is not included due to a technical error.

There was also significantly less CXCR2 expression in COPD compared with age and gender matched healthy controls (median MFI (inter-quartile range), Healthy subjects 40 (30 – 51), COPD patients 27 (21 – 34), $p=0.01$). See figure 7.4.

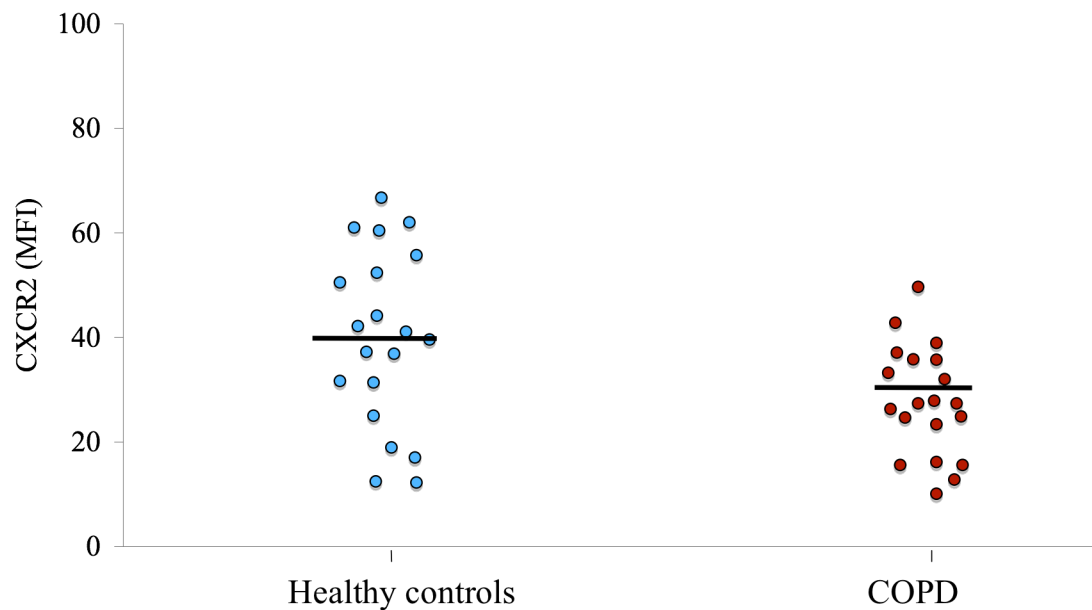


Figure 7.4 Median CXCR2 surface expression in COPD

Legend. Data is the median fluorescence intensity for each of 20 healthy controls and 20 COPD patients in anti-human CXCR2. The group median value is shown as the horizontal line.

7.3.2.3 CXCR1 and CXCR2 receptor expression over time.

In order to assess the time course of CXCR1 and CXCR2 surface expression on quiescent cells, neutrophils were incubated in a negative control (RPMI) and surface expression of receptors was semi-quantified over three time points (time zero, one hour and two hours). There was a decline in surface expression of both receptors in a time dependent manner, however there was no evidence of differential changes in receptor expression between patients with COPD and healthy controls. See table 7.3 and figures 7.5 and 7.6.

Table 7.3. Changes in surface expression in CXCR1 and CXCR2 in quiescent cells over time in COPD.

| | | CXCR1 | | |
|------------------|--|--------------|--------------|--------------|
| | | Time 0 | 1 hour | 2 hours |
| Healthy controls | | 43 (39 - 46) | 25 (20 - 26) | 14 (13 - 17) |
| COPD | | 28 (25 - 29) | 14 (12 - 15) | 6 (5 - 8) |

| | | CXCR2 | | |
|------------------|--|--------------|--------------|--------------|
| | | Time 0 | 1 hour | 2 hours |
| Healthy controls | | 48 (42 - 55) | 39 (34 - 40) | 24 (20 - 27) |
| COPD | | 29 (26 - 31) | 16 (15 - 18) | 10 (9 - 11) |

Legend. Data is the median fluorescence intensity for 20 healthy controls and 20 COPD patients in anti-human CXCR1 and CXCR2 following incubation with a negative control over three time points. The inter-quartile range is shown in parentheses.

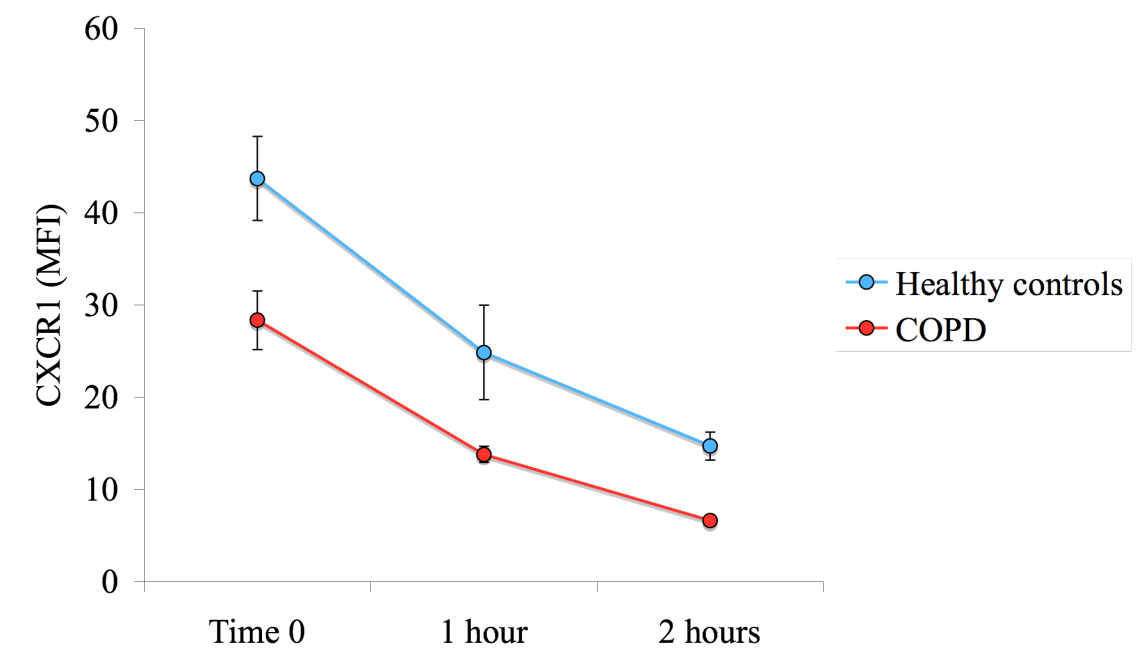


Figure 7.5 Median CXCR1 surface expression over time in COPD

Legend. Data is the median fluorescence intensity for 20 healthy controls and 20 COPD patients in anti-human CXCR1 over three time points (time 0, 1 hour and 2 hours). The interquartile range is shown as the vertical bars.

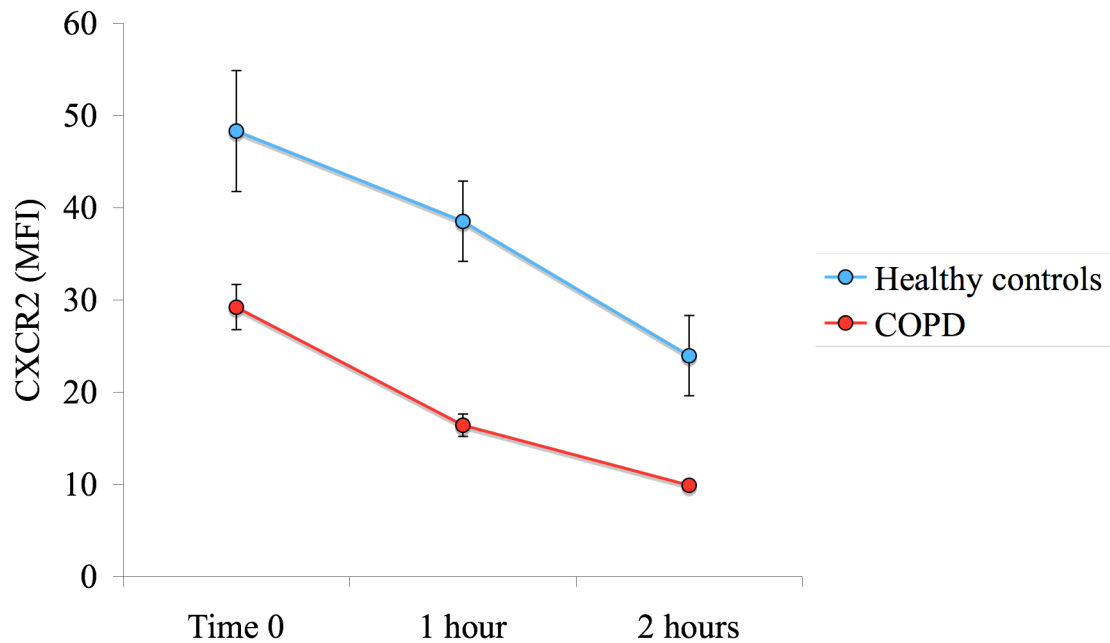


Figure 7.6 Median CXCR2 surface expression over time in COPD

Legend. Data is the median fluorescence intensity for 20 healthy controls and 20 COPD patients in anti-human CXCR2 over three time points (time 0, 1 hour and 2 hours). The interquartile range is shown as the vertical bars.

7.3.2.4 CXCR1 and CXCR2 receptor expression when activated by IL-8

In order to assess the impact of cell activation on CXCR1 and CXCR2 surface expression, cells were incubated in three concentrations of IL-8 or a negative control and surface expression of receptors was semi-quantified. There was a decline in surface expression of both receptors in a dose dependent manner with increasing concentrations of IL-8, however there was no evidence of differential changes in receptor expression between patients with COPD and healthy controls. See table 7.4 and figures 7.7 and 7.8.

Table 7.4. Changes in surface expression in CXCR1 and CXCR2 in quiescent and activated cells in COPD.

| | | CXCR1 | | | |
|------------------|--|------------------|--------------|--------------|-------------|
| | | Negative control | IL8 1nM | IL8 10nM | IL8 100nM |
| Healthy controls | | 43 (39 - 46) | 31 (28 - 32) | 23 (21 - 25) | 10 (8 - 11) |
| COPD | | 28 (25 - 29) | 14 (13 - 16) | 10 (9 - 11) | 6 (5 - 7) |

| | | CXCR2 | | | |
|------------------|--|------------------|--------------|--------------|--------------|
| | | Negative control | IL8 1nM | IL8 10nM | IL8 100nM |
| Healthy controls | | 48 (42 - 55) | 36 (32 - 38) | 18 (16 - 17) | 11 (10 - 12) |
| COPD | | 29 (27 - 31) | 14 (13 - 15) | 10 (9 - 11) | 5 (5 - 6) |

Legend. Data is the median fluorescence intensity for 20 healthy controls and 20 COPD patients in anti-human CXCR1 and CXCR2 following incubation with three concentrations of IL-8 and a negative control. The interquartile range is shown in parentheses.

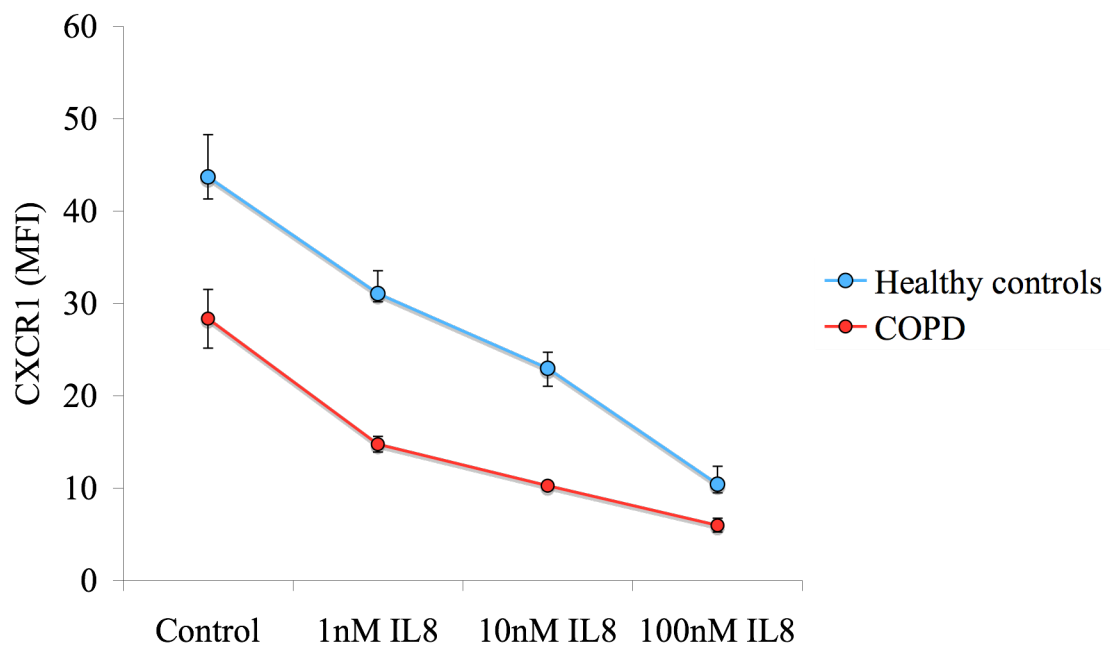


Figure 7.7 Median CXCR1 surface expression in COPD following cell activation

Legend. Data is the median fluorescence intensity for of 20 healthy controls and 20 COPD patients in anti-human CXCR1 when incubated in negative control (RPMI) or three concentrations of IL-8 (1nM, 10nM, 100nM). The inter-quartile range is shown as the vertical bars.

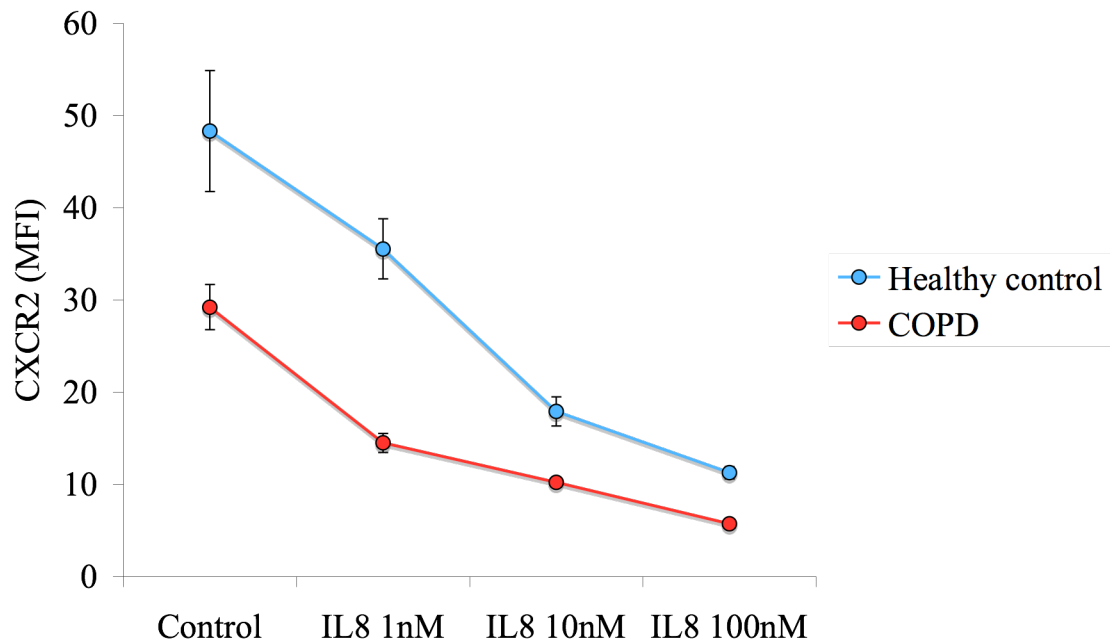


Figure 7.8 Median CXCR2 surface expression in COPD following cell activation

Legend. Data is the median fluorescence intensity for of 20 healthy controls and 20 COPD patients in anti-human CXCR2 when incubated in negative control (RPMI) or three concentrations of IL-8 (1nM, 10nM, 100nM). The inter-quartile range is shown as the vertical bars.

There are clear differences in initial CXCR1 and CXCR2 surface expression in patients with COPD compared with healthy controls, which may exacerbate flaws in migration, potentially due to a reduced ability to utilise receptor occupancy in the detection of a chemoattractant gradient. In order to study this further, monoclonal antibodies for IL8, GRO α and a CXCR2 antagonist were used to look for differential effects during migration to these chemokines or sputum.

7.3.3 The affects of IL-8 and GRO α antibodies and a CXCR2 antagonist: validation

Dose response experiments for increasing concentrations of IL-8, GRO α (see section 4.3.3.3.) and increasing dilutions of sputum sol phase were performed to establish the

concentrations where neutrophil chemotaxis was maximal. Dose response experiments were performed for chemokine antibodies (anti-IL-8 with IL-8, and anti-GRO α with GRO α) to establish the concentration where chemotaxis to the pure chemoattractant was maximally inhibited. Dose response experiments were also performed on neutrophils pre-incubated with increasing concentrations of the CXCR2 antagonist to establish doses where chemotaxis inhibition was initiated and where it could be fully inhibited (see section 4.3.3.3.).

The dose response to varying dilutions of pooled sputum sol phase from COPD patients is shown in Figure 7.9. For this pooled sputum, maximal chemotaxis was achieved when sputum was diluted 1 in 2 in RPMI. This dilution was used for all subsequent experiments.

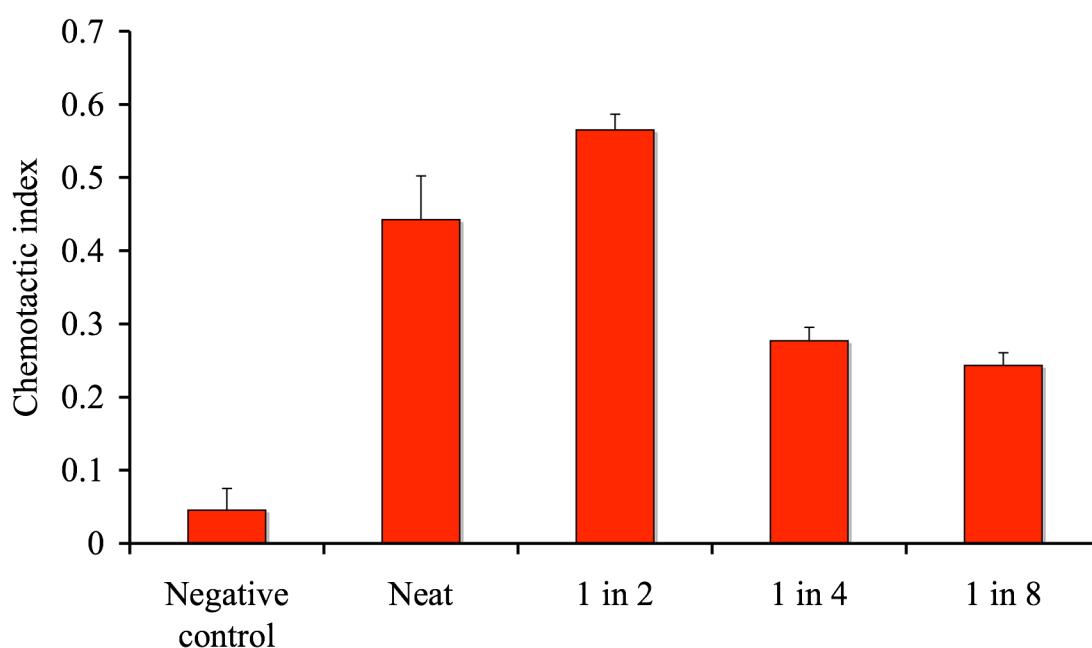


Figure 7.9. The chemotactic response of increasing dilutions of pooled sputum from COPD patients

Legend. The results shown are the mean (and SEM) values of six experiments. Negative control is RPMI (Sigma-Aldrich). Migration is assessed in the presence of increasing dilutions of pooled sputum sol phase (diluted in RPMI).

The neutrophil chemotactic response was inhibited maximally towards IL-8 when an IL-8 antibody concentration of 1.6 μ M was used. See figure 7.10.

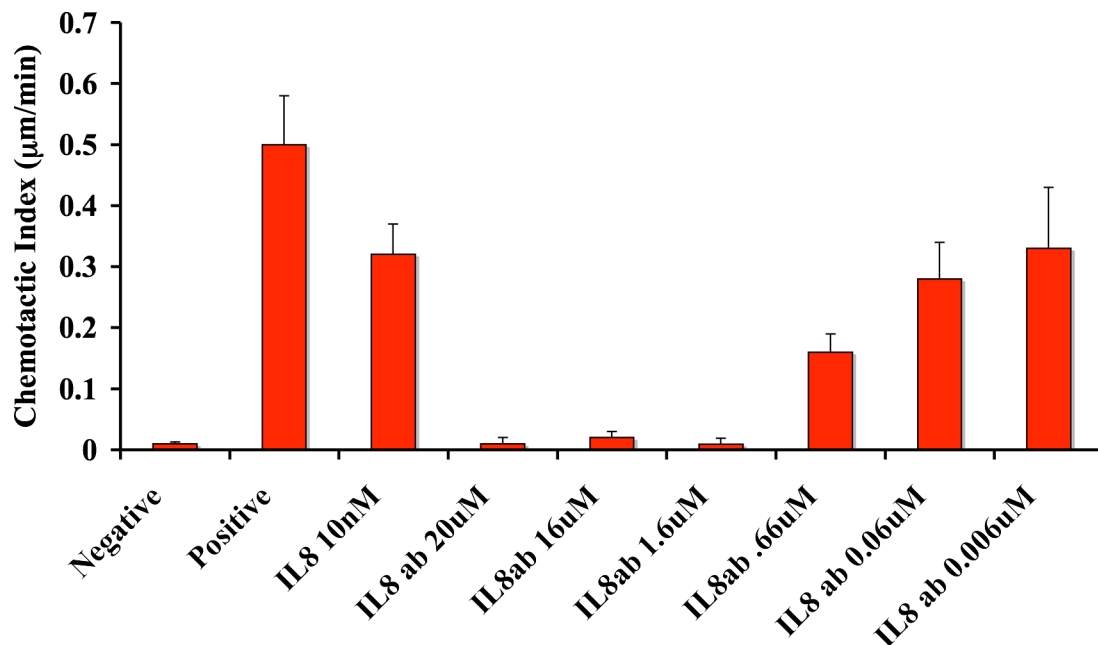


Figure 7.10. The chemotactic response of neutrophils towards IL-8 mixed with increasing concentrations of antihuman IL-8 antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. Negative control is buffer (RMPI (Sigma-Aldrich)). Positive control is fMLP (Sigma-Aldrich) 10^{-7} M. IL-8 10nM is migration in the presence of IL-8 alone, while concentrations of 20 – 0.006 μ M represents migration in the presence of IL-8 (10nM) and the given concentration of IL-8 antibody.

The neutrophil chemotactic response was inhibited maximally towards GRO α when a GRO α antibody concentration of 1.6 μ M was used. See figure 7.11.

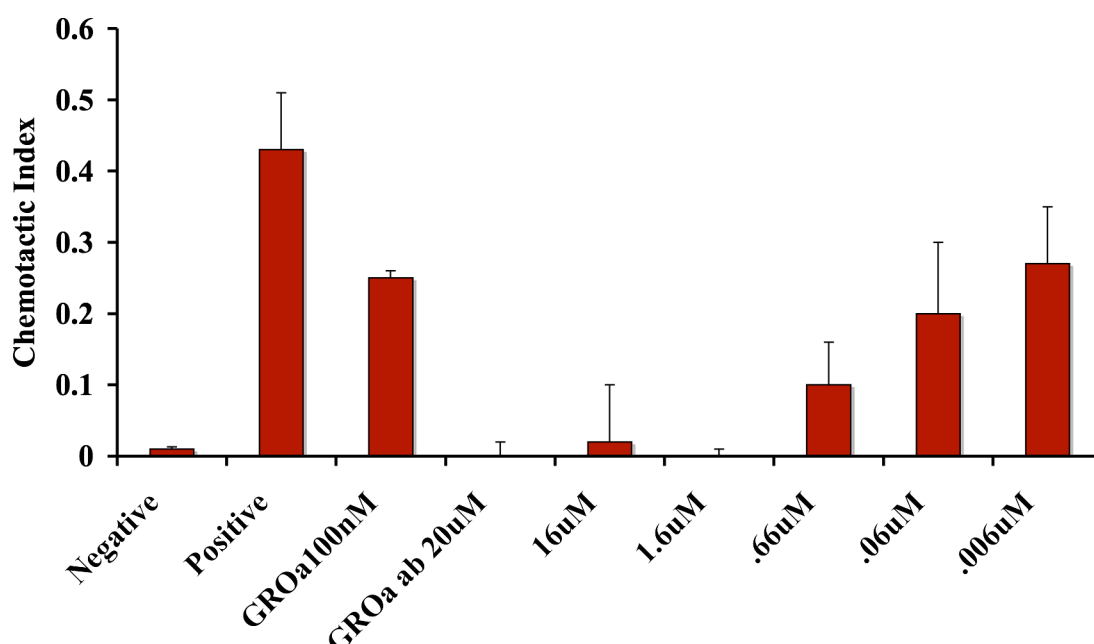


Figure 7.11. The chemotactic response of neutrophils towards GRO α mixed with increasing concentrations of anti-human GRO α antibody

Legend. The results shown are the mean (and SEM) values of six experiments. Negative control is buffer (RMPI (Sigma-Aldrich)). Positive control is fMLP (Sigma-Aldrich) 10^{-7} M. GRO α 100nM is migration in the presence of GRO α alone, while concentrations of 20 – 0.006 μ M represents migration in the presence of GRO α (100nM) and the given concentration of GRO α antibody.

Validatory experiments using both the specific and isotype matched but irrelevant antibodies (Anti-human goat IgG for both IL-8 and GRO α , RnD Systems) were performed to ensure that the presence of antibody alone did not effect migration to either chemokines or sputum. There was no evidence of non-specific antibody effects on neutrophil migration (see figure 7.12). Cross-reactivity experiments were performed with the IL-8 and GRO α antibody to assess the specificity of each antibody to its' ligand compared with the other CXC chemokines. There was no cross-reactivity between IL-8

antibody and GRO α , and only minimal cross-reactivity between GRO α antibody and IL-8 (< 10%). See figure 7.13.

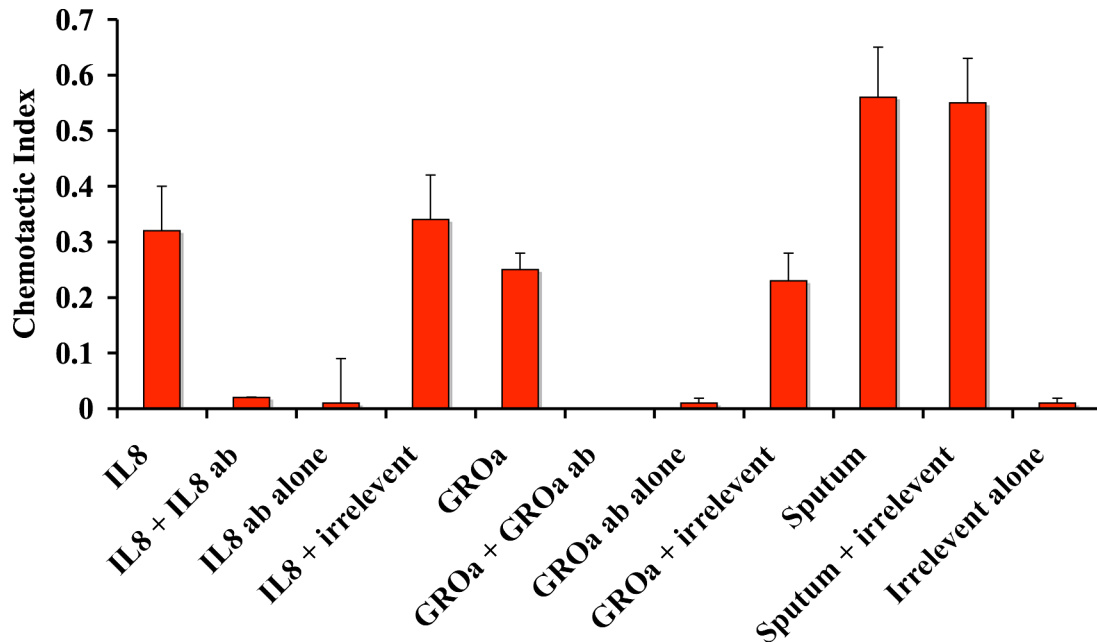


Figure 7.12. The chemotactic response of neutrophils towards IL-8, GRO α or sputum mixed with specific or irrelevant anti-human antibodies.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 is migration in the presence of IL-8 alone (10nM), IL-8 + IL-8 antibody is migration towards IL-8 (10nM) mixed with IL-8 antibody (1.6 μ M). Ab alone is migration in the presence of the antibody alone (either the IL-8 and GRO α antibodies or the irrelevant). IL-8 + irrelevant is IL-8 (10nM) mixed with the irrelevant antibody (1.6 μ M). Similar nomenclature is used for GRO α and sputum studies.

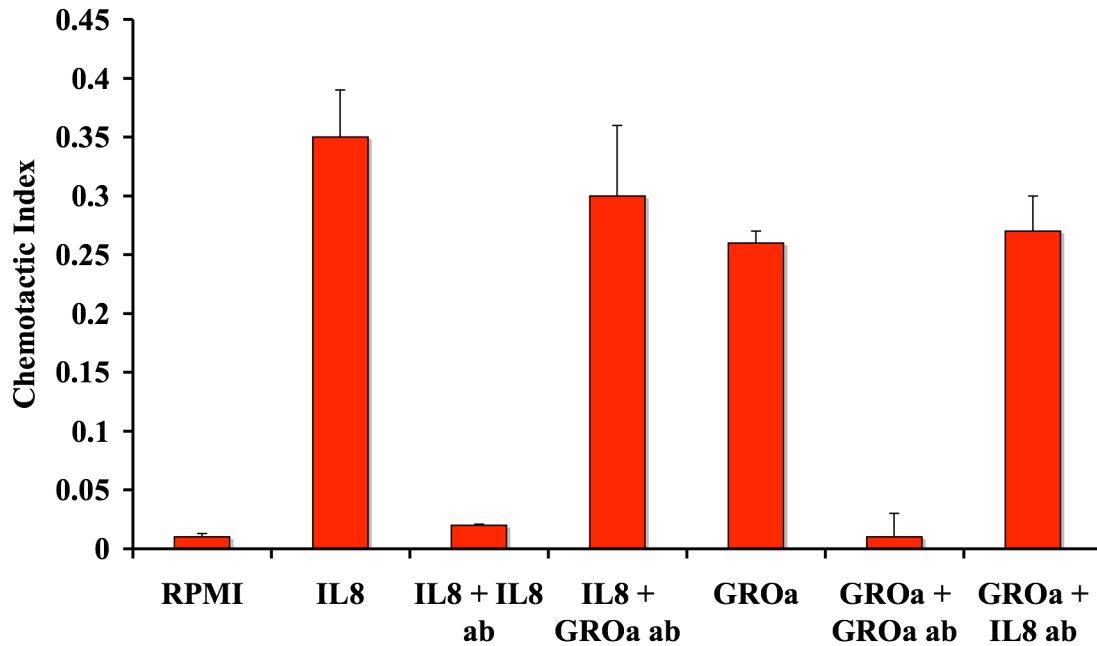


Figure 7.13. The chemotactic response of neutrophils towards IL-8 and GRO α mixed with IL-8 and GRO α anti-human antibodies.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 is migration in the presence of IL-8 alone (100nM), IL-8 + IL-8 antibody is migration towards IL-8 (10nM) mixed with IL-8 antibody (1.6 μ M). IL-8 + GRO α antibody is migration towards IL8 (10nM) mixed with GRO α antibody (1.6 μ M). Similar nomenclature is used for GRO α studies, except that 100nM of GRO α was used.

The neutrophil chemotactic response was partially inhibited following incubation with 5nM CXCR2 antagonist and maximally inhibited following incubation with 500nM CXCR2 antagonist, towards IL-8 and GRO α , and so both concentrations were used for migratory studies. Two concentrations were chosen to look for differential effects of the antagonist in the two groups, as there were clear differences in CXCR2 receptor surface expression. See section 4.3.3.3.

7.3.4 The affects of IL-8 and GRO α monoclonal antibodies and a CXCR2 antagonist: Results

7.3.4.1 Baseline characteristics

Healthy controls and COPD

Demographic data for the COPD patients and healthy controls are shown in Table 7.2.

Pooled sputum was collected from patients with COPD and a known chronic bronchitis phenotype. These samples were used to obtain a sol phase, which was then pooled. The demographic data of these patients is described in Table 7.5.

Table 7.5. Demographic data of COPD patients whose sputum samples formed the pooled sputum sol.

| | |
|--|--------------|
| N | 15 |
| Age | 65 (56 - 78) |
| Male | 6 (40%) |
| Stable state FEV1 (% predicted) | 40 \pm 8.3 |
| Stable state FEV1/VC (%) | 42 \pm 11 |
| Current smokers | 6 (40%) |
| Inhaled corticosteroids | 11 (73 %) |
| Inhaled long acting B2 agonists | 6 (40%) |
| Inhaled anti-cholinergics | 5 (33%) |
| Theophyllines | 0 |
| Sputum bacterial load > 10 ⁷ (cfu/ml) | 0 |

Legend. Age is presented as the mean age (with the range in parenthesis). Gender is the number of males in each group. Patients receiving inhaled steroids, theophyllines, anticholinergics or long acting beta 2 agonists are denoted as a number (with percentage of the group in parentheses). The remaining data is presented as the mean with the standard deviation.

7.3.4.2 Concentrations of mediators in pooled sputum

Concentrations of IL-8 and GRO α were measured (in triplicate) in the pooled sputum samples to compare the concentrations present in these biological samples to those used in the chemotaxis assay. Furthermore, it was necessary to ensure that concentration of each monoclonal antibody was sufficient to inhibit the IL-8 and GRO α present in the

pooled sputum sol. The median of the three samples is given with the range. There was 6.8nM of IL-8 (range 6.4 – 7.3nM) and 29nM of GRO α (range 25 – 32nM) in the pooled sputum. 10nM of IL-8 and 100nM of GRO α was found to be the optimal concentrations of the chemokines for the chemotaxis assays, therefore the concentrations of the antibodies and the CXCR2 antagonist would be sufficient to inhibit the IL-8 and GRO α present in the pooled sputum.

7.3.4.3 The affect of a CXCR2 antagonist in neutrophil migration towards IL-8 in COPD

Speed of migration (Chemokinesis)

In the control group, there was a significant increase in speed of migration in IL-8 compared with negative control (RPMI 2.3 $\mu\text{m}/\text{min} \pm 0.2$ Vs IL-8 3.2 ± 0.3 , $p < 0.0001$) but incubating IL-8 with the IL-8 antibody significantly reduced overall migratory speed to that seen with the negative control. There was no difference in the speed of neutrophils migrating towards IL-8 after treatment with the CXCR2 receptor antagonist at the lower concentration (untreated cells 3.2 ± 0.3 Vs treated cells 3.0 ± 0.1) and a trend towards a decrease in speed when neutrophils were incubated with the higher concentration (2.5 ± 0.2 , $p=0.06$), a reduction of 20%.

In the COPD group, there was a significant difference in speed of migration in IL-8 compared with negative control (RPMI 3.0 $\mu\text{m}/\text{min} \pm 0.3$ Vs. IL-8 6.2 ± 0.6 , $p < 0.0001$) but incubating IL-8 with the IL-8 antibody significantly reduced overall migratory speed to that seen with the negative control. Following treatment with the CXCR2 receptor antagonist, there was a significant decrease in the speed of neutrophils from COPD patients migrating in IL-8 at the lower concentration (untreated cells 6.2 $\mu\text{m}/\text{min} \pm 0.6$ Vs. treated cells 4.6 $\mu\text{m}/\text{min} \pm 0.6$, $p=0.01$), by approximately 25%.

There was no further reduction using the higher concentration of the CXCR2 antagonist (3.8 ± 0.3).

As seen in previous studies, COPD neutrophils migrated with greater speed than those from healthy controls (MD 3.02, CI 1.2 – 3.8, $p=0.0002$). The monoclonal antibody reduced migratory speed to baseline levels in both groups. The CXCR2 antagonist was not as efficacious and reduced migratory speed by approximately 25% in both groups, however, a lower dose was required to achieve this in COPD. See figure 7.14.

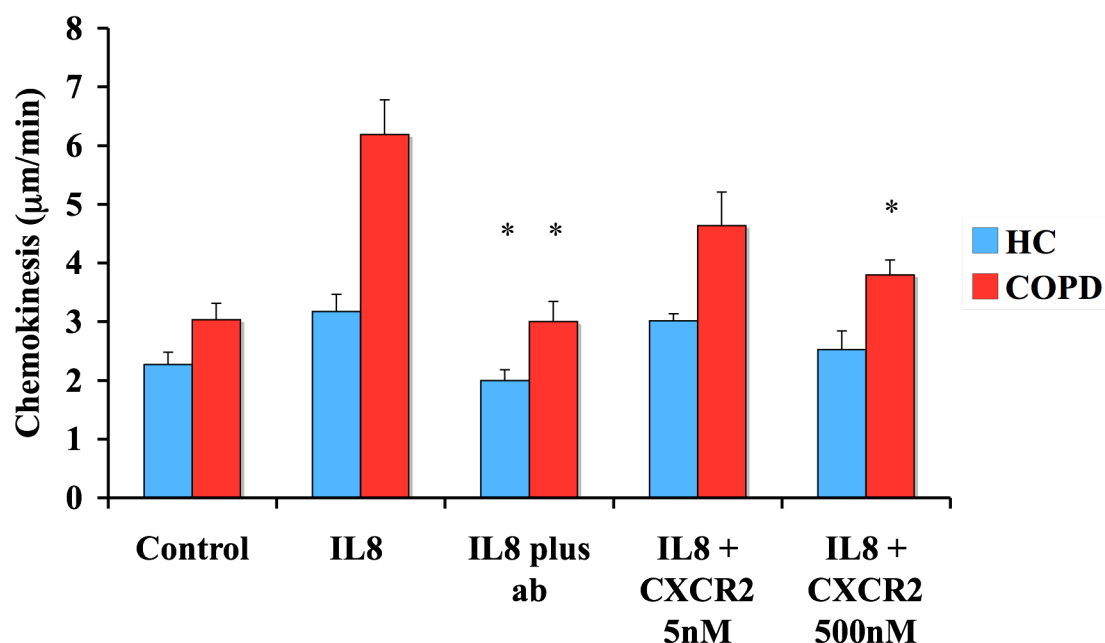


Figure 7.14. The affect of an IL-8 antibody and CXCR2 antagonist on the speed of migration of neutrophils in the presence of IL-8

Legend. The figure describes the mean speed for each condition (with SEM). On the horizontal axis, control is RPMI (Sigma-Aldrich), IL8 is migration in shallow gradients of this chemokine (10nM). IL-8 plus ab is migration in shallow gradients of IL-8 mixed with IL-8 monoclonal antibody. IL-8 plus CXCR2 antagonist is neutrophil migration towards IL-8 following neutrophil incubation with the CXCR2 receptor antagonist at the two given concentrations. * = significant difference from IL-8 alone.

Velocity of migration (Chemotaxis).

In the control group there was a significant increase in migratory velocity towards IL-8 compared with the control (RPMI $0.01 \mu\text{m}/\text{min} \pm 0.06$ Vs IL-8 $1.56 \mu\text{m}/\text{min} \pm 0.39$, $p < 0.0001$). The velocity of neutrophils migrating towards IL-8 was reduced to control levels when mixed with its' antibody. Pre-incubating neutrophils with the 5nM CXCR2 antagonist also reduced the velocity of migration towards IL-8 (to $0.82 \mu\text{m}/\text{min} \pm 0.26$, $p = 0.009$) by approximately 46%. There was a further reduction in chemotaxis (62%) using 500nM of the CXCR2 antagonist (reduced to 0.58 ± 0.4 , $p = 0.03$).

In the COPD group there was a significant increase in velocity towards IL-8 compared with the control (RPMI $0.2 \mu\text{m}/\text{min} \pm 0.3$ Vs IL8 $1.1 \mu\text{m}/\text{min} \pm 0.3$, $p = 0.001$). The velocity of neutrophils migrating towards IL-8 was reduced to control levels when mixed with its' antibody. Pre-incubating neutrophils with the 5nM CXCR2 antagonist significantly reduced chemotaxis (65%) (to $0.37 \mu\text{m}/\text{min} \pm 0.3$, $p = 0.002$). There was no further reduction in chemotaxis using the higher concentration of the CXCR2 antagonist ($0.3 \mu\text{m}/\text{min} \pm 0.2$).

As seen in previous studies, COPD neutrophils migrated with less velocity than those from healthy controls (HC $1.6 \mu\text{m}/\text{min} \pm 0.4$ Vs. COPD $1.1 \mu\text{m}/\text{min} \pm 0.3$, $p = 0.01$). The IL-8 monoclonal antibody reduced velocity to baseline levels in both groups. The CXCR2 antagonist also reduced velocity in both groups, but to a lesser degree. Again, COPD neutrophils differentially responded to a lower dose of the CXCR2 antagonist.

See figure 7.15.

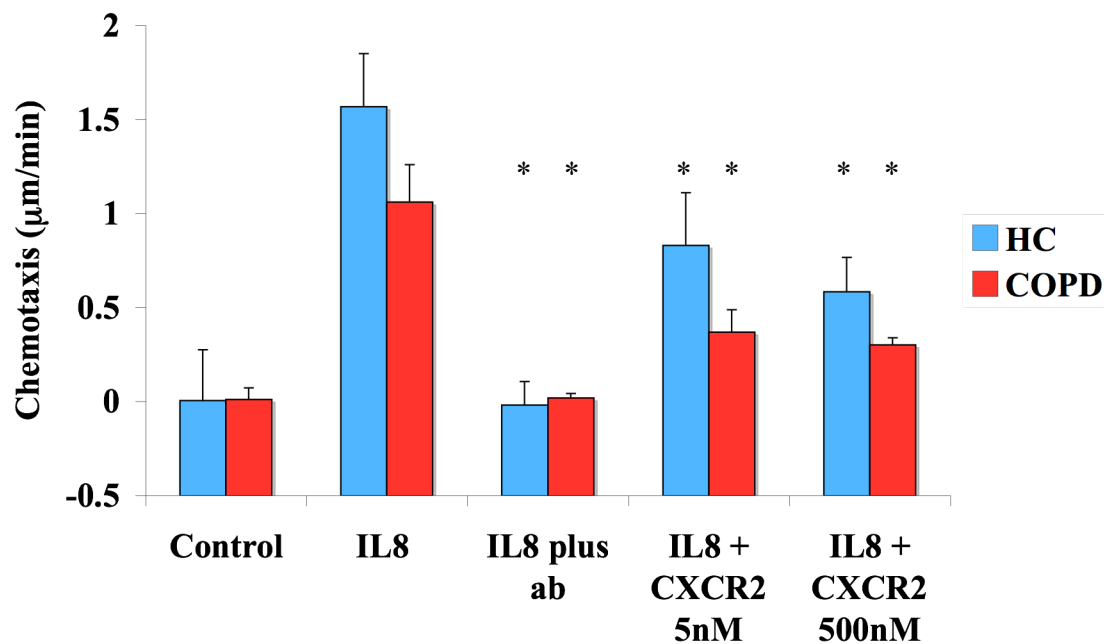


Figure 7.15. The affect of an IL-8 antibody and CXCR2 antagonist on the velocity of neutrophils towards IL-8

Legend. The figure describes the mean velocity for each condition (with SEM). On the horizontal axis, control is RPMI (Sigma-Aldrich), IL8 is migration in shallow gradients of this chemokine (10nM). IL-8 plus ab is migration in shallow gradients of IL-8 mixed with IL-8 monoclonal antibody. IL-8 plus CXCR2 antagonist is neutrophil migration towards IL-8 following neutrophil incubation with the CXCR2 receptor antagonist at the given concentration. * = significant difference from IL-8 alone.

Directional persistence

In the healthy controls, directional persistence of neutrophils migrating towards IL-8 was reduced to levels comparable to baseline in the presence of the IL-8 antibody (Control 0.05 ± 0.01 , IL-8 0.36 ± 0.09 , IL-8 + IL-8 antibody 0.05 ± 0.04 , $p < 0.0001$). Pre-incubation of neutrophils with the CXCR2 antagonist reduced directional persistence when migrating towards IL-8 both at 5nM (to 0.18 ± 0.04 , $p = 0.04$, by 50%), and at 500nM (to 0.13 ± 0.05 , $p = 0.02$, by 63%).

In the COPD group, directional persistence of neutrophils migrating towards IL-8 was reduced to levels comparable with baseline in the presence of the IL-8 antibody (Control 0.07 ± 0.1 , IL-8 0.29 ± 0.11 , IL-8 + IL-8 antibody 0.006 ± 0.09 , $p=0.005$). Pre-incubation of neutrophils with the 5nM CXCR2 antagonist also reduced directional persistence (to 0.004 ± 0.07 , $p < 0.0001$). The 500nM CXCR2 antagonist had no further affect on persistence.

As seen in previous studies, COPD neutrophils migrated with similar persistence to those from healthy controls (HC 0.36 ± 0.09 Vs. COPD 0.29 ± 0.11). The monoclonal antibody reduced persistence to baseline levels in both groups. The CXCR2 antagonist reduced persistence to baseline levels in COPD neutrophils, but not in neutrophils from healthy controls (5nM: difference in percent, healthy controls $50\% \pm 7$, COPD $98\% \pm 12$, $p < 0.001$). See figure 7.16.

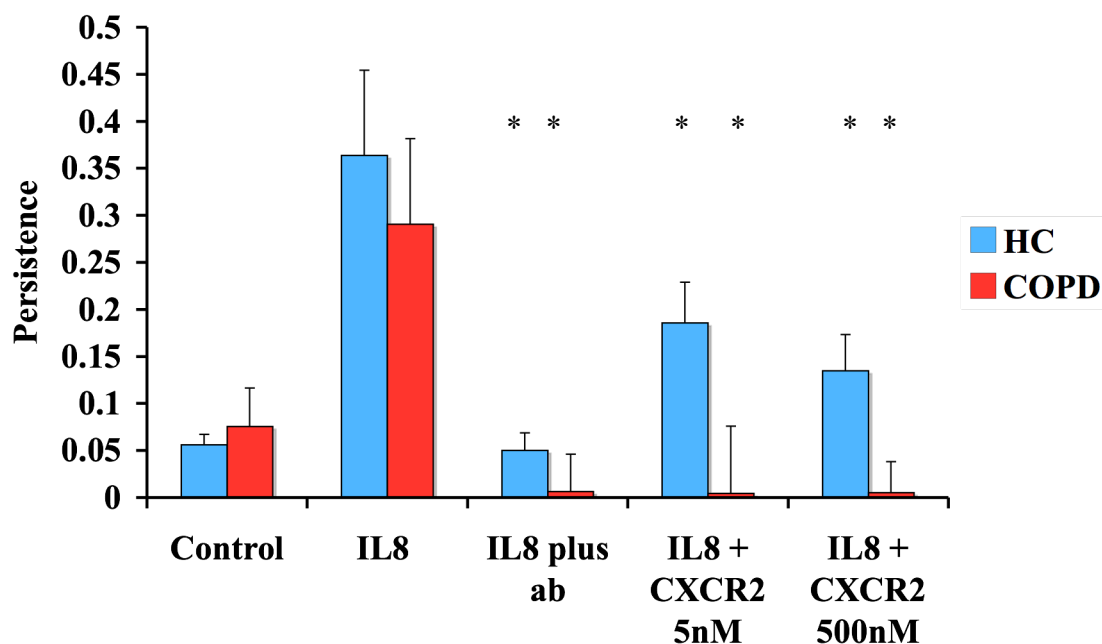


Figure 7.16. The affect of an IL-8 antibody and CXCR2 antagonist on the directional persistence of neutrophils towards IL-8

Legend. The figure describes the mean persistence for each condition (with SEM). On the horizontal axis, control is RPMI (Sigma-Aldrich), IL8 is migration in shallow gradients of this chemokine (10nM). IL-8 plus ab is migration in shallow gradients of IL-8 mixed with IL-8 monoclonal antibody. IL-8 plus CXCR2 antagonist is neutrophil migration towards IL-8 following neutrophil incubation with the CXCR2 receptor antagonist at the given concentration. * = significant difference from IL-8 alone.

Chemotactic Index

In the healthy controls, the addition of IL-8 monoclonal antibody completely reversed the chemotactic index of neutrophils migrating towards IL-8 to control levels (Control 0.02 ± 0.02 , IL-8 0.4 ± 0.07 , IL-8 + IL-8 antibody 0.01 ± 0.02 , $p < 0.0001$). Pre-incubation of neutrophils with the 5nM CXCR2 antagonist also reduced the chemotactic index (52%) towards IL-8 (to 0.2 ± 0.04 , $p = 0.002$), with a further reduction (69%) using the 500nM CXCR2 antagonist (0.12 ± 0.02 , $p = 0.03$).

In the COPD group, the addition of IL-8 antibody completely reversed the chemotactic index of neutrophils migrating towards IL-8 to control levels (Control 0.01 ± 0.06 , IL-8

0.22 ± 0.05 , IL-8 + IL-8 antibody 0.01 ± 0.02 , $p=0.005$). Pre-incubation of neutrophils with the 5nM CXCR2 antagonist reduced the chemotactic index (70%) towards IL-8 (to 0.07 ± 0.03 , $p=0.007$) with no further decrease using the 500nM CXCR2 antagonist (0.06 ± 0.02).

As seen in previous studies, COPD neutrophils migrated with less accuracy than those from healthy controls (HC 0.4 ± 0.07 Vs. COPD 0.22 ± 0.05 , $p=0.002$). The IL-8 antibody reduced the accuracy of migration to baseline levels, but this was not achieved by the CXCR2 antagonist. Treating cells with 5nM CXCR2 antagonist reduced the accuracy of neutrophils in COPD patients more than was seen with healthy controls (difference in percent, healthy controls $52\% \pm 6$, COPD $69\% \pm 11$, $p < 0.01$), but not to comparable levels. See Figure 7.17.

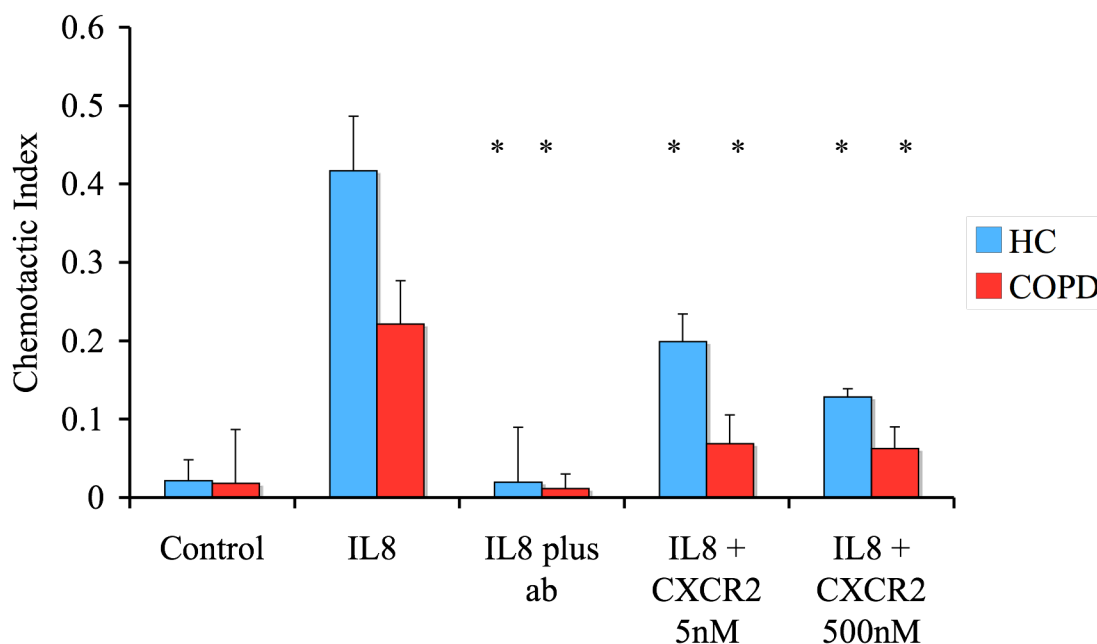


Figure 7.17. The affect of an IL-8 antibody and CXCR2 antagonist on the overall accuracy of neutrophils towards IL-8

Legend. The figure describes the mean chemotactic index for each condition (with SEM). On the horizontal axis, control is RPMI (Sigma-Aldrich), IL8 is migration in shallow gradients of this chemokine (10nM). IL-8 plus ab is migration in shallow gradients of IL-8 mixed with IL-8 monoclonal antibody. IL-8 plus CXCR2 antagonist is neutrophil migration towards IL-8 following neutrophil incubation with the CXCR2 receptor antagonist at the given concentrations. * = significant difference from IL-8 alone.

In summary, neutrophils from patients with COPD moved with greater speed, but less accuracy than those from healthy controls, mirroring the results of the previous chapters. In both groups, mixing IL-8 with its monoclonal antibody reduced the speed, velocity, directional persistence and accuracy of migration to baseline levels, effectively abrogating the affect of the chemokine. The CXCR2 antagonist was not as effective, having a smaller affect on chemokinesis (random speed), chemotaxis (directed speed or velocity) and overall accuracy of movement. The residual migration could be due to the binding of IL-8 to CXCR1 (which the antagonist would not inhibit) or by a partial efficacy of the CXCR2 antagonist (although doses chosen were based upon dose

response experiments). There were differential effects between COPD and controls, in that COPD neutrophils responded to a lower concentration of CXCR2 antagonist compared with those from healthy controls. This is in keeping with a lower surface expression of CXCR2. To further assess the role of CXCR2, migrational studies were repeated using GRO α , which preferentially binds to CXCR2.

7.3.4.4 The affect of a CXCR2 antagonist in neutrophil migration towards GRO α in COPD

Speed of migration (Chemokinesis)

In the control group, there was a small but significant increase in speed of migration in shallow gradients of GRO α compared with the negative control (RPMI $2.3 \mu\text{m}/\text{min} \pm 0.2$ Vs GRO α 2.9 ± 0.2 , $p = 0.04$). Incubating GRO α with the GRO α antibody and incubating neutrophils with the higher concentration of the CXCR2 antagonist reduced this to control levels (GRO α antibody, $2.3 \mu\text{m}/\text{min} \pm 0.2$, $p = 0.03$; CXCR2 5nM, $2.5 \mu\text{m}/\text{min} \pm 0.2$; CXCR2 500nM, $2.2 \mu\text{m}/\text{min} \pm 0.3$, $p = 0.01$).

In the COPD group, there was an increase in speed of migration with GRO α compared with negative control (RPMI $3.0 \mu\text{m}/\text{min} \pm 0.3$ Vs. GRO α 4.4 ± 0.2 , $p = 0.003$). Incubating GRO α with the GRO α antibody reduced overall migratory speed to control levels (GRO α Ab $3.0 \mu\text{m}/\text{min} \pm 0.1$, $p = 0.004$). There was a decrease in speed following treatment with 5nM CXCR2 receptor antagonist (untreated cells $4.4 \mu\text{m}/\text{min} \pm 0.2$ Vs. treated cells $2.8 \mu\text{m}/\text{min} \pm 0.4$, $p = 0.001$). There was no additional affect with 500nM CXCR2 antagonist ($2.7 \mu\text{m}/\text{min} \pm 0.3$).

As seen in previous studies, COPD neutrophils migrated with greater speed (were more chemokinetic) than those from healthy controls (average difference $1.9\mu\text{m}/\text{minute}$, CI $0.7 - 2.4$, $p=0.001$). The $\text{GRO}\alpha$ antibody reduced speed to baseline levels in both groups, as did the CXCR2 antagonist, however a differential effect was seen, with COPD cells responding maximally to a lower concentration. See Figure 7.18.

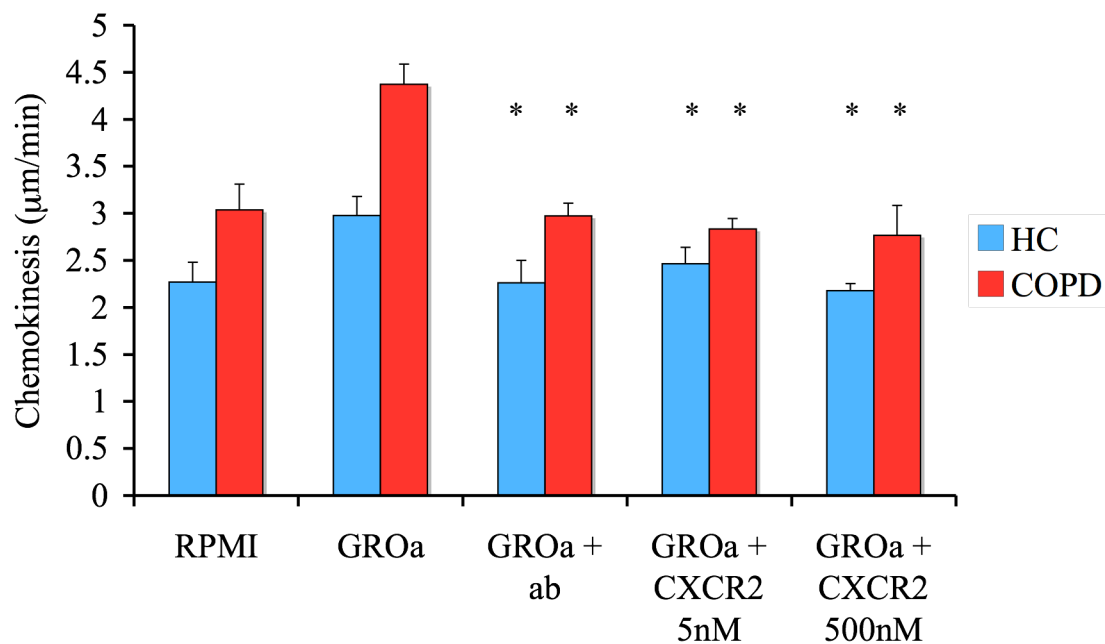


Figure 7.18. The affect of a $\text{GRO}\alpha$ antibody and CXCR2 antagonist on the speed of migration of neutrophils in the presence of $\text{GRO}\alpha$

Legend. The figure describes the mean speed for each condition (with SEM). On the horizontal axis, RPMI is the negative control, $\text{GRO}\alpha$ is migration in shallow gradients of this chemokine (100nM). $\text{GRO}\alpha$ plus ab is migration in shallow gradients of $\text{GRO}\alpha$ mixed with $\text{GRO}\alpha$ monoclonal antibody. $\text{GRO}\alpha$ plus CXCR2 antagonist is neutrophil migration in the presence of $\text{GRO}\alpha$ following neutrophil incubation with the CXCR2 receptor antagonist at the given concentration. * = significant difference from $\text{GRO}\alpha$ alone.

Velocity of migration (Chemotaxis)

In the control group there was a significant increase in velocity (chemotaxis) towards $\text{GRO}\alpha$ compared with the control ($\text{RPMI } 0.03 \mu\text{m}/\text{min} \pm 0.01$ Vs $\text{GRO}\alpha \ 0.38\mu\text{m}/\text{min} \pm 0.07$, $p=0.002$). This was reduced to control levels when $\text{GRO}\alpha$ was mixed with its'

antibody. Pre-incubating neutrophils with 5nM CXCR2 antagonist reduced the velocity of migration towards GRO α (50%) (to 0.2 $\mu\text{m}/\text{min} \pm 0.01$, $p=0.01$). The 500nM CXCR2 antagonist reduced this further to baseline (0.03 $\mu\text{m}/\text{min} \pm 0.08$ ($p = 0.02$))

In the COPD group there was a significant increase in velocity towards GRO α compared with the control (RPMI 0.02 $\mu\text{m}/\text{min} \pm 0.02$ Vs GRO α 0.14 $\mu\text{m}/\text{min} \pm 0.01$, $p=0.03$). This was reduced to control levels when mixed with its' antibody. Pre-incubating neutrophils with 5nM CXCR2 antagonist significantly reduced the velocity of migration towards GRO α to baseline (to 0.04 $\mu\text{m}/\text{min} \pm 0.02$, $p=0.01$). The 500nM CXCR2 antagonist did not reduce this further (0.01 $\mu\text{m}/\text{min} \pm 0.03$).

As seen in previous studies, COPD neutrophils migrated with less velocity than those from healthy controls (HC 0.38 $\mu\text{m}/\text{min} \pm 0.01$ Vs. COPD 0.14 $\mu\text{m}/\text{min} \pm 0.01$, $p=0.008$). Mixing GRO α with its monoclonal antibody could reduce the velocity to baseline levels in both groups. The CXCR2 antagonist also reduced chemokinesis to baseline in both groups, although neutrophils from healthy controls required a higher dose of the antagonist. See figure 7.19.

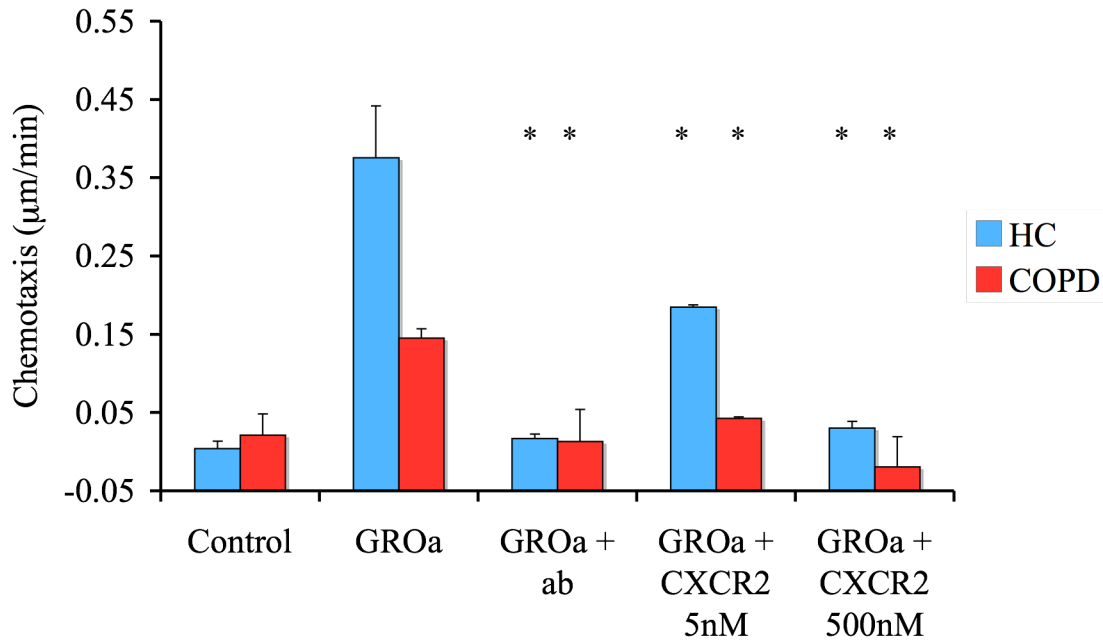


Figure 7.19. The affect of a GRO α antibody and CXCR2 antagonist on the chemotaxis of neutrophils towards GRO α

Legend. The figure describes the mean velocity for each condition (with SEM). On the horizontal axis, control is RPMI (Sigma-Aldrich), GRO α is migration in shallow gradients of this chemokine (100nM). GRO α plus ab is migration in shallow gradients of GRO α mixed with GRO α monoclonal antibody. GRO α plus CXCR2 antagonist is neutrophil migration towards GRO α following neutrophil incubation with the CXCR2 receptor antagonist at the given concentration. * = significant difference from GRO α alone.

Directional persistence

In the healthy controls, the directional persistence of neutrophils migrating towards GRO α was reduced to baseline levels when GRO α was incubated with the GRO α monoclonal antibody (Control 0.07 ± 0.01 , GRO α 0.14 ± 0.009 , GRO α with GRO α antibody 0.05 ± 0.01 , $p=0.009$). Pre-incubation of neutrophils with 5nM CXCR2 antagonist reduced the directional persistence when migrating towards GRO α (34%) (to 0.09 ± 0.01), with a further reduction using the higher concentration of CXCR2 antagonist (0.06 ± 0.02 , $p=0.02$) to baseline.

In the COPD group, directional persistence of neutrophils migrating towards $\text{GRO}\alpha$ was reduced to baseline when $\text{GRO}\alpha$ was incubated with the $\text{GRO}\alpha$ monoclonal antibody (Control 0.07 ± 0.01 , $\text{GRO}\alpha$ 0.16 ± 0.03 , $\text{GRO}\alpha$ + $\text{GRO}\alpha$ antibody 0.06 ± 0.01 , $p=0.04$). Pre-incubation of neutrophils with 5nM CXCR2 antagonist reduced directional persistence (to 0.08 ± 0.002 , $p = 0.03$) to baseline levels, but there was no further reduction with the higher concentration of the antagonist (0.06 ± 0.02).

As seen in previous studies, COPD neutrophils migrated with similar persistence to those from healthy controls (HC 0.14 ± 0.09 Vs. COPD 0.16 ± 0.03). The monoclonal antibody and CXCR2 antagonist reduced persistence to baseline in both groups, however, cells from healthy controls required a higher dose of antagonist to achieve this. See figure 7.20.

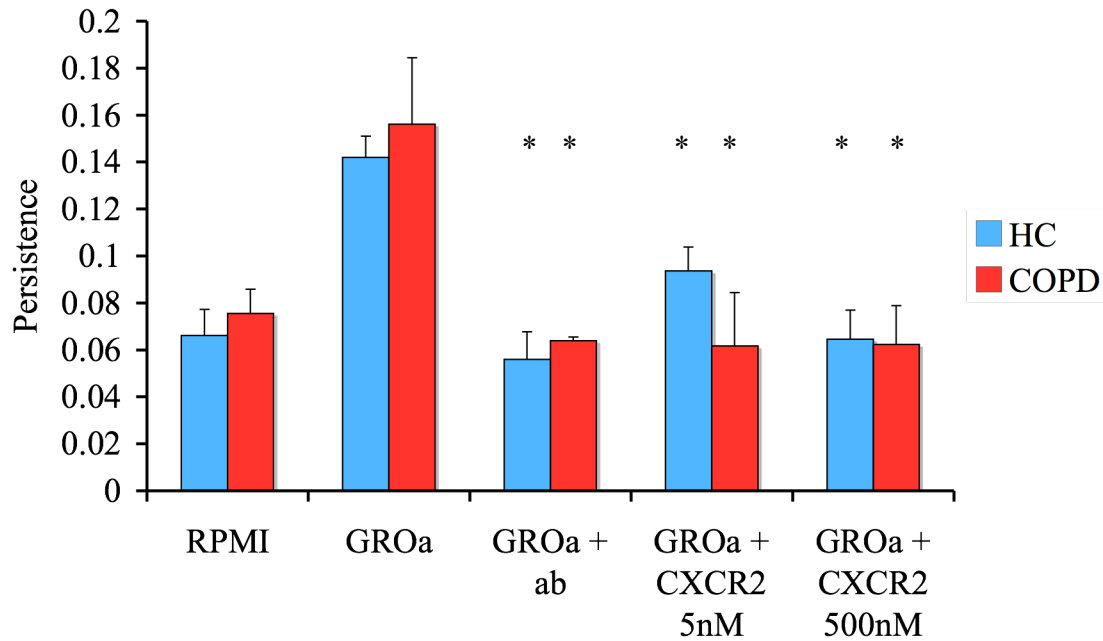


Figure 7.20. The affect of a GRO α antibody and CXCR2 antagonist on the directional persistence of neutrophils towards GRO α

Legend. The figure describes the mean persistence for each condition (with SEM). On the horizontal axis, RPMI (Sigma-Aldrich) is the negative control, GRO α is migration in shallow gradients of this chemokine (100nM). GRO α plus ab is migration in shallow gradients of GRO α mixed with GRO α monoclonal antibody. GRO α plus CXCR2 antagonist is neutrophil migration towards GRO α following neutrophil incubation with the CXCR2 receptor antagonist. * = significant difference from GRO α alone.

Chemotactic Index

In the healthy controls, the addition of GRO α monoclonal antibody reduced the chemotactic index of neutrophils migrating towards GRO α to control levels (Control 0.02 ± 0.002 , GRO α 0.13 ± 0.02 , GRO α + GRO α antibody 0.02 ± 0.003 , $p=0.012$). Pre-incubation of neutrophils with 5nM CXCR2 antagonist also reduced the migrational accuracy towards GRO α (to 0.07 ± 0.01 , $p=0.02$), but 500nM CXCR2 were required to reduce this to baseline (0.03 ± 0.01).

In the COPD group, GRO α did not significantly increase migrational accuracy compared with the negative control (Control 0.02 ± 0.009 , GRO α 0.03 ± 0.003 , GRO α + GRO α antibody 0.009 ± 0.001).

As seen in previous studies, COPD neutrophils migrated with less accuracy than those from healthy controls (HC 0.13 ± 0.002 Vs. COPD 0.03 ± 0.003 , $p=0.008$). In healthy controls, both the monoclonal antibody and CXCR2 antagonist (at the higher concentration) could reduce accuracy to baseline levels. The effect of a CXCR2 antagonist on migratory accuracy in COPD could not be evaluated as GRO α did not significantly increase accuracy from baseline in the COPD group. See figure 7.21.

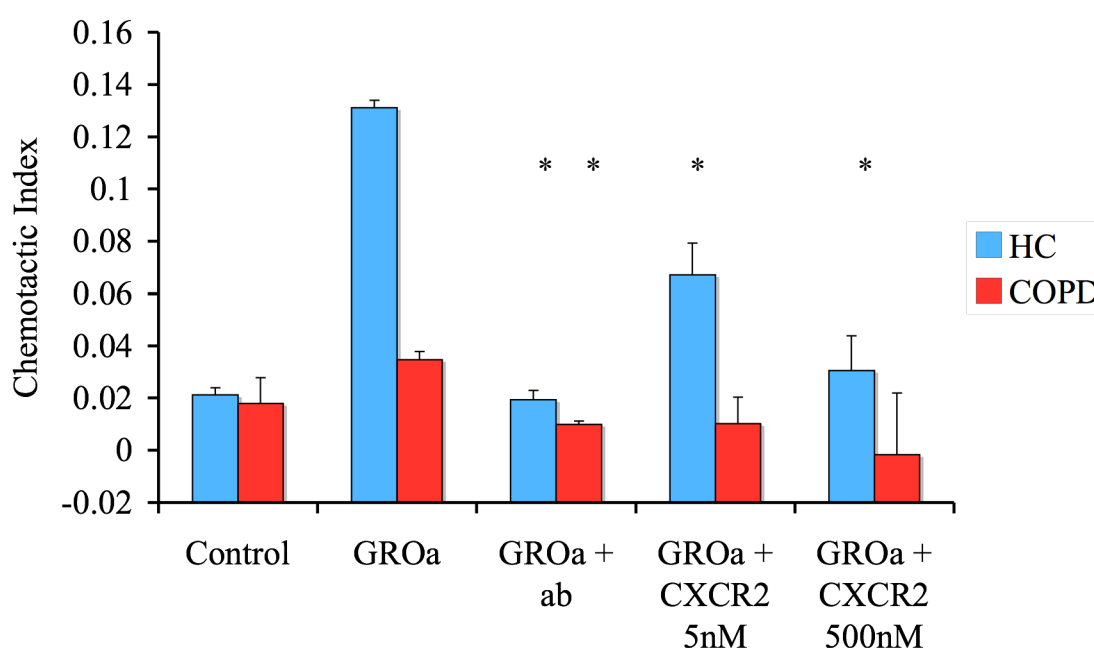


Figure 7.21. The affect of a GRO α antibody and CXCR2 antagonist on the overall accuracy of neutrophils towards GRO α

Legend. The figure describes the mean chemotactic index for each condition (with SEM). On the horizontal axis, control is RPMI (Sigma-Aldrich), GRO α is migration in shallow gradients of this chemokine (100nM). GRO α plus ab is migration in shallow gradients of GRO α mixed with GRO α monoclonal antibody. GRO α plus CXCR2 antagonist is neutrophil migration towards GRO α following neutrophil incubation with the CXCR2 receptor antagonist at the given concentration. * = significant difference from GRO α alone.

In summary, COPD neutrophils also displayed greater speed, but reduced accuracy when migrating towards $\text{GRO}\alpha$ compared with control cells. Here, the CXCR2 antagonist was as effective in abrogating migration as the monoclonal antibody, suggesting that migration towards $\text{GRO}\alpha$ is CXCR2 dependent, and that the antagonist is effective in abrogating this effect. This data is supportive of the likelihood that the residual migration seen towards IL-8 in the previous experiments was CXCR1 dependent. When studying this residual IL-8 response, we would predict that 40% of the migratory response to IL-8 in health and 25% of the migratory response to IL-8 in neutrophils from patients with COPD (measured by velocity, directional persistence or accuracy) is CXCR1 dependent, if the receptors exert separate affects. However, specific inhibition of CXCR1 would be required to confirm this hypothesis. Again, in the current series of experiments, COPD neutrophils responded to a lower dose of the CXCR2 antagonist than controls, which would be consistent with lower surface expression of CXCR2, shown earlier in this chapter.

7.3.4.5 The affect of a CXCR2 antagonist in neutrophil migration towards sputum in COPD

Speed of migration (Chemokinesis)

There was a significant increase in the speed of healthy control neutrophil movement in the presence sputum compared with the negative control (RPMI $2.3 \mu\text{m}/\text{min} \pm 0.2$; Sputum $3.14 \mu\text{m}/\text{min} \pm 0.1$, $p=0.04$). However, the addition of IL-8 and $\text{GRO}\alpha$ antibody and incubation with either concentration of the CXCR2 antagonists did not significantly reduce this speed of movement (IL-8 ab; 3.1 ± 0.3 ; $\text{GRO}\alpha$ ab 2.9 ± 0.07 , CXCR2 5nM 2.9 ± 0.2 ; CXCR2 500nM 2.7 ± 0.3).

When COPD neutrophils migrated in shallow gradients of sputum, there was an increase of speed in comparison with cells from healthy controls ($3.14 \mu\text{m}/\text{min} \pm 0.1$ Vs. $5.31 \mu\text{m}/\text{min} \pm 0.6$, $p=0.0001$). Again the addition of IL8 antibody ($5.2\mu\text{m}/\text{min} \pm 0.7$) and $\text{GRO}\alpha$ antibody ($5.2\mu\text{m}/\text{min} \pm 0.3$) did not significantly affect the speed of movement, nor did 5nM ($4.8 \mu\text{m}/\text{min} \pm 0.1$) or 500nM ($4.7\mu\text{m}/\text{min} \pm 0.3$) of the CXCR2 antagonist.

See figure 7.22.

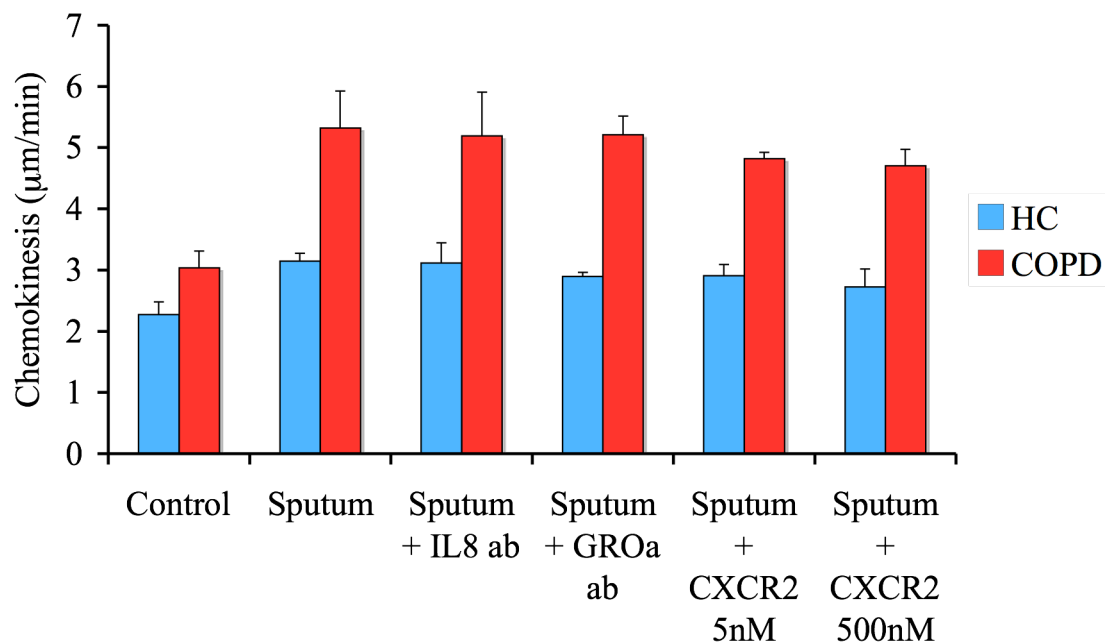


Figure 7.22. The affect of an IL-8 and $\text{GRO}\alpha$ antibody and CXCR2 antagonist on the overall speed of neutrophils migrating in gradients of sputum

Legend. The figure describes the mean speed for each condition (with SEM). On the horizontal axis, control is RPMI (Sigma-Aldrich), sputum is migration in sputum sol phase, Sputum plus ab is migration in shallow gradients of sputum mixed with the named monoclonal antibody. Sputum plus CXCR2 antagonist is neutrophil migration towards sputum sol phase following neutrophil incubation with the CXCR2 receptor antagonist at the given concentration.

Velocity (Chemotaxis)

There was a significant increase in the velocity of healthy control neutrophil movement in the presence sputum compared with the negative control (RPMI $0.01 \mu\text{m}/\text{min} \pm 0.2$; Sputum $1.44 \mu\text{m}/\text{min} \pm 0.1$, $p=0.002$). The addition of IL-8 antibody reduced velocity by 56% (to $0.63 \mu\text{m}/\text{min} \pm 0.05$, $p=0.004$) and GRO α antibody by 30 % (to $1 \mu\text{m}/\text{min} \pm 0.08$, $p=0.04$). Incubation with 5nM CXCR2 antagonist did not alter the velocity, but there was a significant reduction of 42% using 500nM (CXCR2 5nM $1.29 \mu\text{m}/\text{min} \pm 0.02$; CXCR2 500nM $0.8 \mu\text{m}/\text{min} \pm 0.08$, $p = 0.003$).

When COPD neutrophils migrated in sputum, velocity of movement was reduced in comparison with cells from healthy controls (Controls $1.44 \mu\text{m}/\text{min} \pm 0.06$ Vs. COPD $1.1 \mu\text{m}/\text{min} \pm 0.06$, $p=0.001$). The addition of IL8 antibody reduced velocity by 58% (to $0.4 \mu\text{m}/\text{min} \pm 0.02$, $p=0.002$) as did GRO α antibody (by 28%, $0.8 \mu\text{m}/\text{min} \pm 0.02$, $p=0.02$). There was a significant decrease of 41% following incubation with 5nM CXCR2 antagonist, with no further changes seen using 500nM (5nM $0.64 \mu\text{m}/\text{min} \pm 0.03$, $p = 0.006$, 500nM $0.55 \mu\text{m}/\text{min} \pm 0.1$). See figure 7.23.

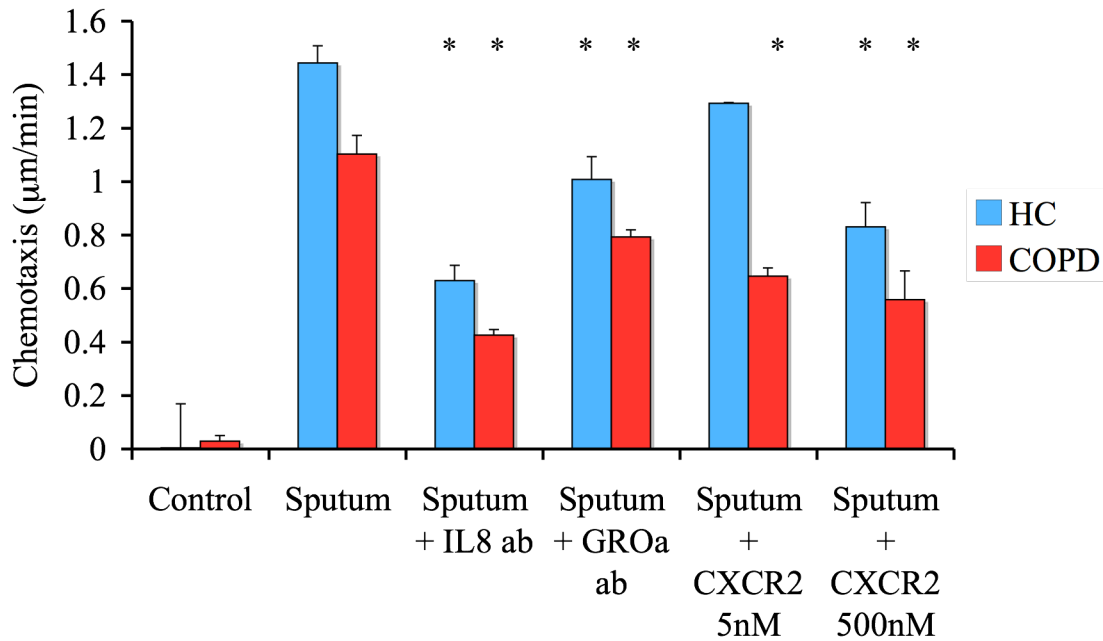


Figure 7.23. The affect of an IL-8 and GRO α antibody and CXCR2 antagonist on the overall velocity of neutrophils migrating towards sputum

Legend. The figure describes the mean velocity for each condition (with SEM). On the horizontal axis, control is RPMI (Sigma-Aldrich), sputum is migration in sputum sol phase, Sputum plus ab is migration towards sputum mixed with the named monoclonal antibody. Sputum plus CXCR2 antagonist is neutrophil migration towards sputum sol phase following neutrophil incubation with the CXCR2 receptor antagonist at the given concentration. * = significant difference from sputum alone.

Persistence

There were no differences in the directional persistence of neutrophils from healthy controls or patients with COPD migrating in sputum (controls: 0.42 ± 0.01 Vs COPD 0.37 ± 0.08). The addition of IL-8 antibody reduced persistence by 18% in healthy controls (to 0.34 ± 0.01 , $p=0.04$) and by 17% in COPD (to 0.31 ± 0.07 , $p=0.04$). The addition of GRO α antibody did not significantly reduce persistence in either group. Incubation with 5nM CXCR2 antagonist decreased persistence by 12% in COPD neutrophils (to 0.32 ± 0.02 , $p=0.02$), but there was no further decrease using 500nM (0.34 ± 0.03). A similar decrease in persistence was not seen until 500nM of the CXCR2 antagonist was used in healthy controls (13% reduction, to 0.36 ± 0.04 , $p=0.04$) See figure 7.24.

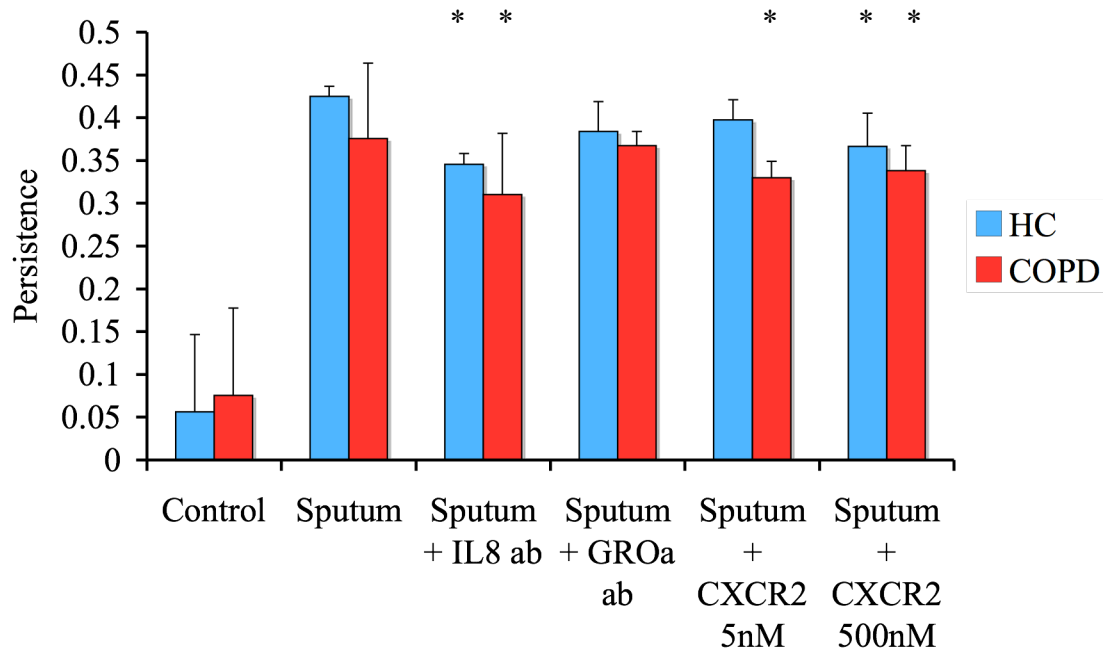


Figure 7.24. The affect of an IL-8 and GRO α antibody and CXCR2 antagonist on the persistence of neutrophils migrating in gradients of sputum

Legend. The figure describes the mean migratory parameter for each condition (with SEM). On the horizontal axis, control is RPMI (Sigma-Aldrich), sputum is migration in sputum sol phase, Sputum plus ab is migration towards sputum mixed with the named monoclonal antibody. Sputum plus CXCR2 antagonist is neutrophil migration towards sputum sol phase following neutrophil incubation with the CXCR2 receptor antagonist at the given concentration. * = significant difference from sputum alone.

Chemotactic Index (Migratory accuracy)

Neutrophils from patients with COPD migrated with less accuracy towards sputum compared with those from healthy controls (Controls: 0.46 ± 0.01 Vs COPD: 0.16 ± 0.08 , $p=0.002$). The addition of IL-8 antibody reduced migratory accuracy by 52% in healthy controls (to 0.23 ± 0.02 , $p=0.003$) and by 55% in COPD neutrophils (to 0.07 ± 0.02 , $p=0.004$). The addition of GRO α antibody reduced accuracy by 28% in healthy control neutrophils (to 0.33 ± 0.06 , $p=0.009$) and by 25% in COPD (to 0.12 ± 0.04 , $p=0.01$). Incubation with 5nM CXCR2 antagonist decreased accuracy by 39% in COPD neutrophils (to 0.1 ± 0.01 , $p=0.008$), but there was no further decrease using 500nM (0.09 ± 0.06). A significant decrease in accuracy was not seen until 500nM of the

CXCR2 antagonist was used in healthy controls (54% reduction, to 0.21 ± 0.01 , $p=0.004$). See figure 7.25.

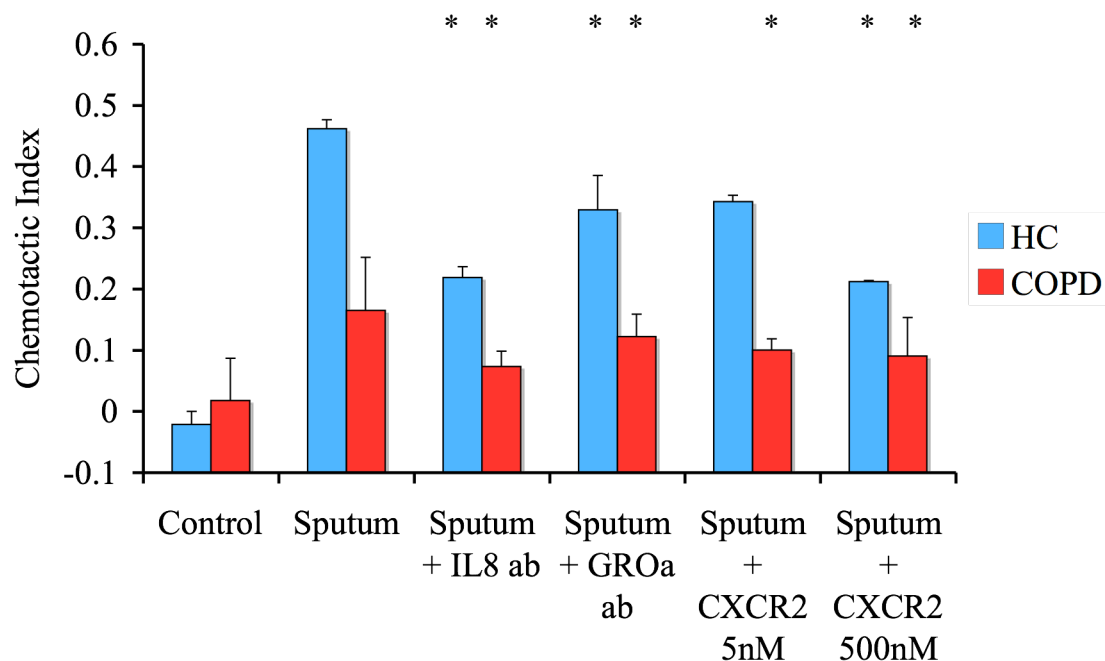


Figure 7.25. The affect of an IL-8 and GRO α antibody and CXCR2 antagonist on the chemotactic index of neutrophils migrating in gradients of sputum

Legend. The figures describes the mean chemotactic index for each condition (with SEM). On the horizontal axis, control is RPMI (Sigma-Aldrich), sputum is migration in sputum sol phase, Sputum plus ab is migration towards sputum mixed with the named monoclonal antibody. Sputum plus CXCR2 antagonist is neutrophil migration towards sputum sol phase following neutrophil incubation with the CXCR2 receptor antagonist at the given concentration. * = significant difference from sputum alone

In summary, blocking the affects of IL-8 and GRO α in the migratory response to sputum (using either monoclonal antibodies or the antagonist) did not alter chemokinesis. However, the IL-8 antibody reduced the velocity and accuracy of migration by approximately 50% in both groups, again emphasizing the importance of this chemokine in the migratory drive of neutrophils. Abrogating GRO α reduced velocity and accuracy by a smaller amount (28%). Abrogating the CXCR2 receptor (which should specifically target migration caused by GRO α , NAP-2, ENA 78 and a proportion of migration caused by IL-8) reduced migration by approximately 50% in

healthy controls and 40% in patients with COPD. Again, a lower dose of the CXCR2 antagonist was required in COPD, and this, coupled with the observation that the CXCR2 antagonist had less of an effect in migration towards sputum in COPD would be consistent with lower surface expression of the receptors in this group.

It was interesting to note that the increased speed of migration, reduced velocity and reduced accuracy of COPD neutrophils towards sputum was still present following the addition of IL-8 antibody, GRO α antibody or the CXCR2 antagonist. Sputum contains other mediators that act independently of CXCR1 or CXCR2 receptors (including, for example, LTB₄). That these migratory differences are still present when CXCR1 and 2 receptors (or their ligands) are blocked, strongly suggests that this phenomenon is not specific to CXCR1 or 2, and is a more generalised defective response, perhaps localised at a common downstream pathway of these specific receptors.

To identify the differential affects of CXCR1 and CXCR2 inhibition on neutrophil migration in COPD, studies were repeated using CXCR1 and CXCR2 monoclonal antibodies. Monoclonal antibodies were used, as there was no small molecule antagonist of CXCR1 available at the time of study, and it was felt important to compare like with like.

7.3.5 The affects of CXCR2 and CXCR1 monoclonal antibodies: validation

Dose response experiments were performed for anti-CXCR2 (RnD Systems, UK) and anti-CXCR1 (RnD Systems, UK) with IL-8 to establish the concentration where chemotaxis was maximally inhibited. 1.6 μ m was found to be the most effective antibody concentration for both antibodies (see figures 7.26 and 7.27). Validation

experiments carried out by RnDSystems, UK have previously confirmed that these antibodies do not cross-react. Both antibodies are IgG2a and an irrelevant IgG2a antibody was used to assess any non-specific migrational affects caused by the presence of antibody alone. There were no changes in migration following incubation with the irrelevant IgG2a antibody, see figure 7.28.

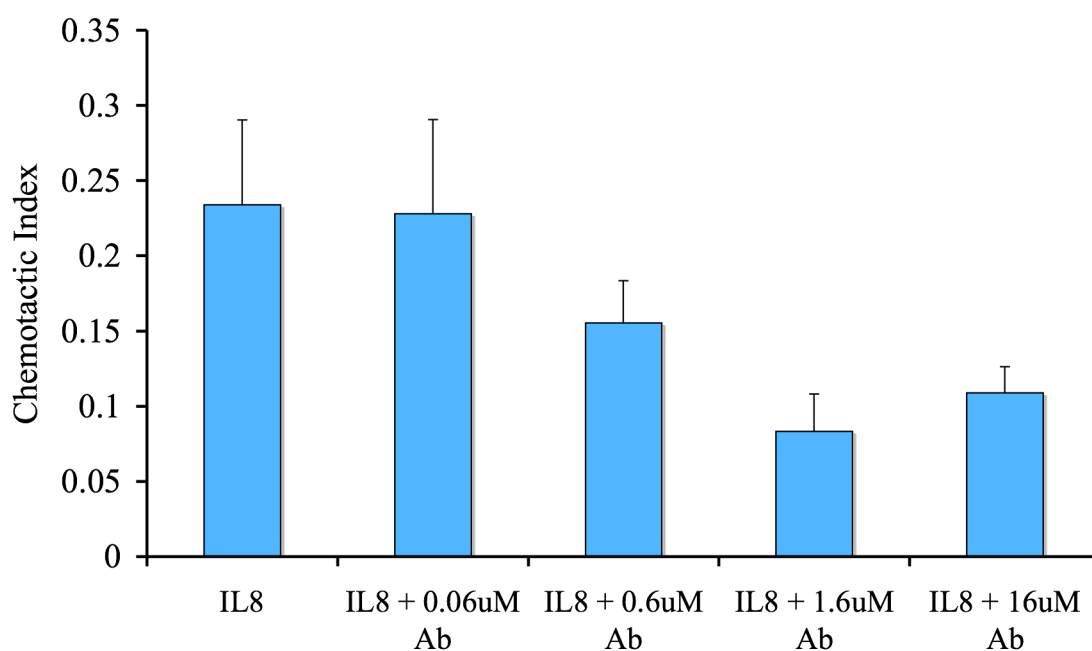


Figure 7.26. The chemotactic response of neutrophils towards IL-8 after incubation with increasing concentrations of anti human CXCR2 antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 10nM is migration in the presence of IL-8 alone, while concentrations of 16 – 0.06 μ M represents migration in the presence of IL8 (10nM) following neutrophil incubation with the given concentration of CXCR2 antibody.

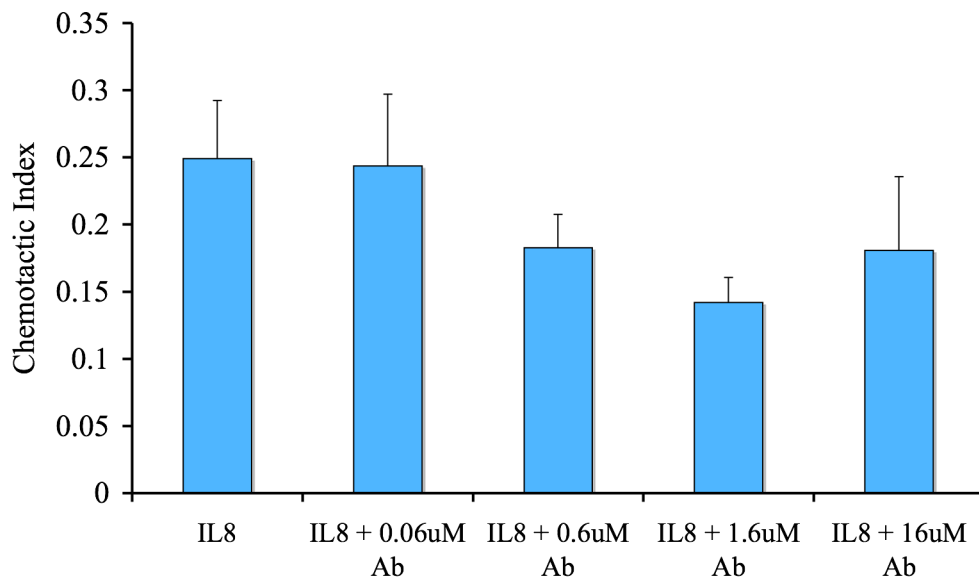


Figure 7.27. The chemotactic response of neutrophils towards IL-8 after incubation with increasing concentrations of anti human CXCR1 antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 10nM is migration in the presence of IL-8 alone, while concentrations of 16 – 0.06μM represents migration in the presence of IL8 (10nM) following neutrophil incubation with the given concentration of CXCR1 antibody

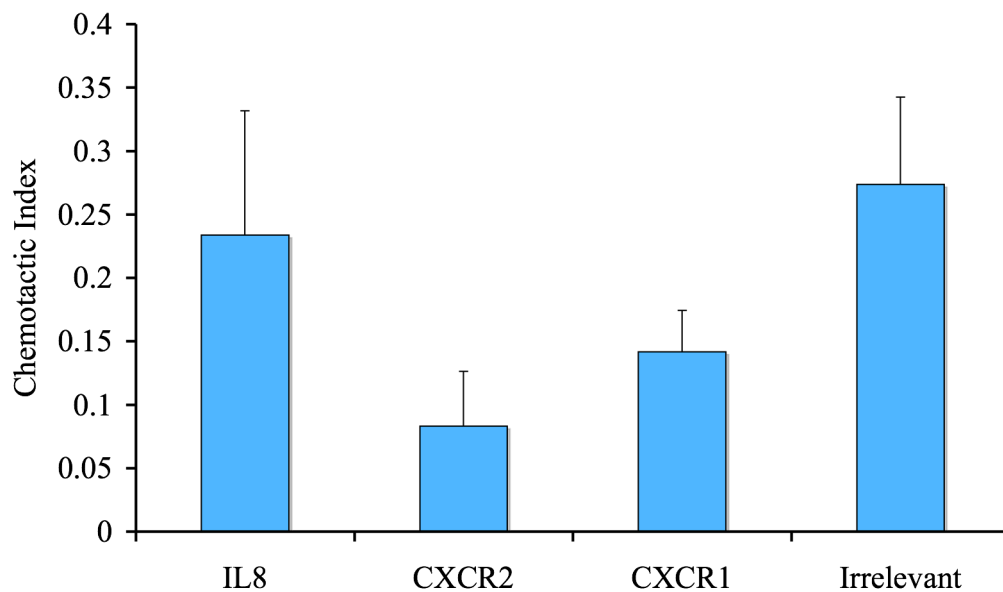


Figure 7.28. The chemotactic response of neutrophils towards IL-8 after incubation with anti human CXCR1 or CXCR2 antibody or a matched irrelevant antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 10nM is migration in the presence of IL-8 alone, CXCR2, CXCR1 and Irrelevant is migration in the presence of IL8 (10nM) following neutrophil incubation with the named (CXCR1, CXCR2, Irrelevant).

7.3.6 The affects of CXCR2 and CXCR1 monoclonal antibodies: results

7.3.6.1 CXCR2

Speed of migration (Chemokinesis)

As seen with the small molecule CXCR2 antagonist, when neutrophils from healthy controls were incubated with the CXCR2 antibody, there was a significant reduction in the speed of movement in IL-8 (IL-8 $3.6 \mu\text{m}/\text{min} \pm 0.2$; IL-8 + CXCR2 Ab $2.5 \mu\text{m}/\text{min} \pm 0.2$, $p = 0.01$) and GRO α (GRO α $3.0 \mu\text{m}/\text{min} \pm 0.2$; GRO α + CXCR2 Ab $2.2 \mu\text{m}/\text{min} \pm 0.1$, $p=0.04$). However, the CXCR2 antibody did not reduce the speed of migration towards sputum (Sputum $3.1 \mu\text{m}/\text{min} \pm 0.2$; Sputum + CXCR2 Ab $2.7 \mu\text{m}/\text{min} \pm 0.2$).

With neutrophils from COPD patients, a similar pattern was seen, with a reduction in speed towards the chemokines (IL-8, $6.2 \mu\text{m}/\text{min} \pm 0.4$; IL8 + CXCR2 Ab, $4.2 \mu\text{m}/\text{min} \pm 0.2$, $p=0.008$; GRO α , $4.4 \mu\text{m}/\text{min} \pm 0.2$; GRO α + CXCR2 Ab, $3.0 \mu\text{m}/\text{min} \pm 0.1$, $p = 0.009$). Incubation with the CXCR2 antibody did not significantly affect the speed of movement in gradients of sputum (Sputum, $5.3 \mu\text{m}/\text{min} \pm 0.1$; Sputum + CXCR2 Ab, $4.6 \mu\text{m}/\text{min} \pm 0.1$). COPD neutrophils were faster compared with those from healthy controls, see figure 7.29, but there were no differential affects using the small molecule antagonist or the CXCR2 antibody.

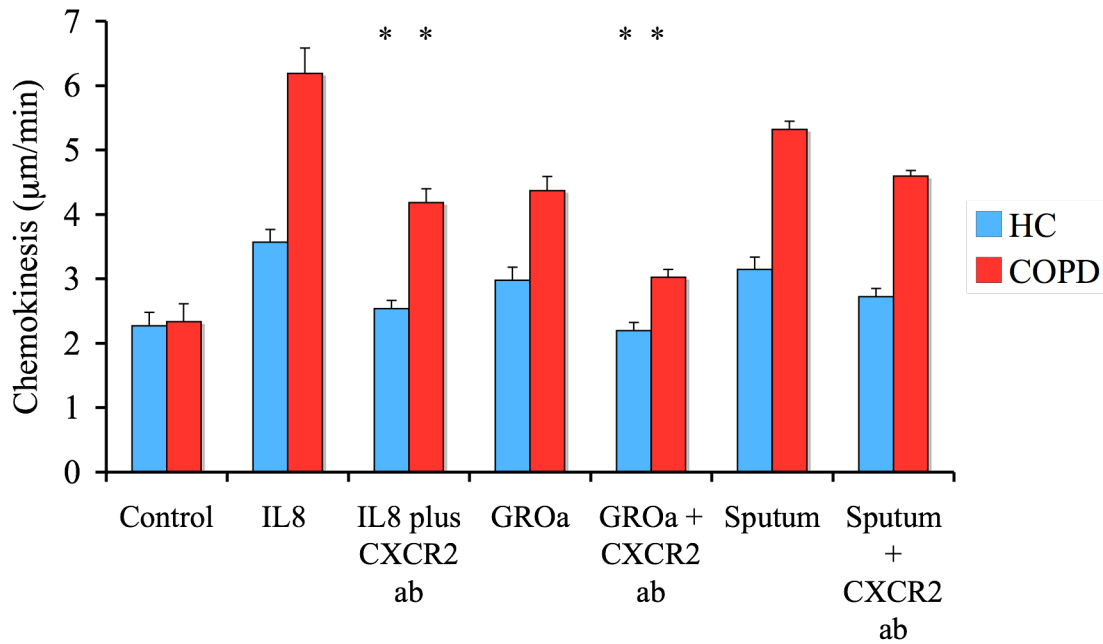


Figure 7.29. The chemokinetic response of neutrophils towards IL-8, GRO α and sputum after incubation with anti human CXCR2 antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 10nM is migration in the presence of IL-8 alone, IL8 + CXCR2 Ab is in the presence of IL8 (10nM) following incubation with the antibody. The nomenclature is the same for GRO α (100nM) and Sputum (2:1 dilution). * = significant difference from chemoattractant alone.

Velocity (Chemotaxis)

As seen with the small molecule CXCR2 antagonist, when neutrophils from healthy controls were incubated with the CXCR2 antibody, there was a significant reduction in the velocity of movement towards IL-8 of approximately 40% (IL-8 $1.6 \mu\text{m}/\text{min} \pm 0.2$; IL-8 + CXCR2 Ab $0.9 \mu\text{m}/\text{min} \pm 0.1$, $p = 0.009$) and GRO α by approximately 90% (GRO α $0.4 \mu\text{m}/\text{min} \pm 0.2$; GRO α + CXCR2 Ab $0.02 \mu\text{m}/\text{min} \pm 0.01$, $p=0.02$). The CXCR2 antibody also reduced chemotaxis towards sputum by 50% (Sputum $1.4 \mu\text{m}/\text{min} \pm 0.1$; Sputum + CXCR2 Ab $0.7 \mu\text{m}/\text{min} \pm 0.1$, $p=0.008$).

With neutrophils from COPD patients, a similar pattern was seen, with a reduction in velocity towards the chemokines (IL-8, $1.1 \mu\text{m}/\text{min} \pm 0.1$; IL8 + CXCR2 Ab, 0.3

$\mu\text{m}/\text{min} \pm 0.1$, $p=0.006$: $\text{GRO}\alpha$, $0.14\mu\text{m}/\text{min} \pm 0.2$; $\text{GRO}\alpha + \text{CXCR2 Ab}$, $0.003\mu\text{m}/\text{min} \pm 0.1$, $p = 0.03$). There was also a significant reduction in velocity towards sputum (Sputum, $1.1\mu\text{m}/\text{min} \pm 0.1$; Sputum + CXCR2 Ab, $0.5\mu\text{m}/\text{min} \pm 0.1$, $p=0.008$). Once again, COPD neutrophils had less velocity compared to those from healthy controls, (see figure 7.30).

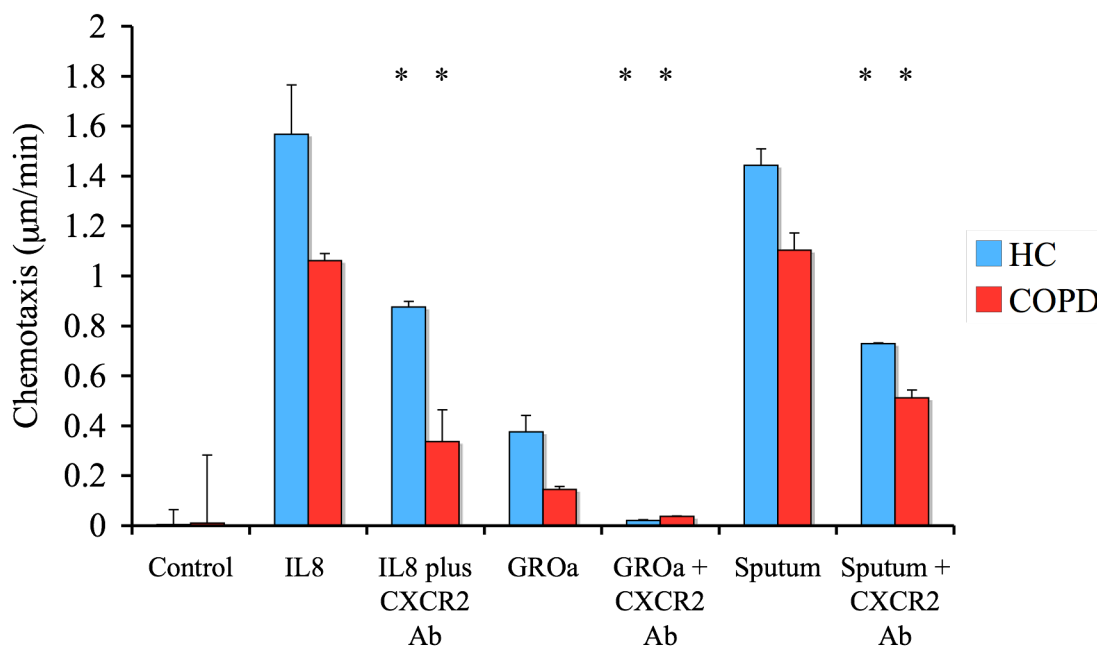


Figure 7.30. The velocity of neutrophils towards IL-8, $\text{GRO}\alpha$ and sputum after incubation with anti human CXCR2 antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 10nM is migration towards IL-8 alone, IL8 + CXCR2 Ab is towards IL8 (10nM) following incubation with the antibody (1.6 μM). The nomenclature is the same for $\text{GRO}\alpha$ (100nM) and Sputum (2:1 dilution). * = significant difference from chemoattractant alone.

Persistence

As seen with the small molecule CXCR2 antagonist, when neutrophils from healthy controls were incubated with the CXCR2 antibody, there was a significant reduction in the persistence of movement towards IL-8 of approximately 55% (IL-8 0.36 ± 0.1 ; IL-8 + CXCR2 Ab 0.16 ± 0.1 , $p = 0.01$) and $\text{GRO}\alpha$ to baseline levels ($\text{GRO}\alpha$ 0.14 ± 0.1 ; $\text{GRO}\alpha + \text{CXCR2 Ab}$ 0.06 ± 0.01 , $p=0.03$). The CXCR2 antibody did not reduce

directional persistence towards sputum (Sputum 0.4 ± 0.1 ; Sputum + CXCR2 Ab 0.3 ± 0.1).

With neutrophils from COPD patients, a similar pattern was seen, with a reduction in persistence towards the chemokines to baseline levels (IL-8, 0.3 ± 0.1 ; IL8 + CXCR2 Ab, 0.03 ± 0.1 , $p=0.004$; GRO α , 0.16 ± 0.1 ; GRO α + CXCR2 Ab, 0.06 ± 0.1 , $p = 0.04$). However, the CXCR2 antibody did not alter persistence towards sputum (Sputum, 0.37 ± 0.1 ; Sputum + CXCR2 Ab, 0.3 ± 0.1). Once again, there were no differences in the persistence of neutrophils from COPD patients or controls, (see figure 7.31). There were also no differential affects using the CXCR2 antibody.

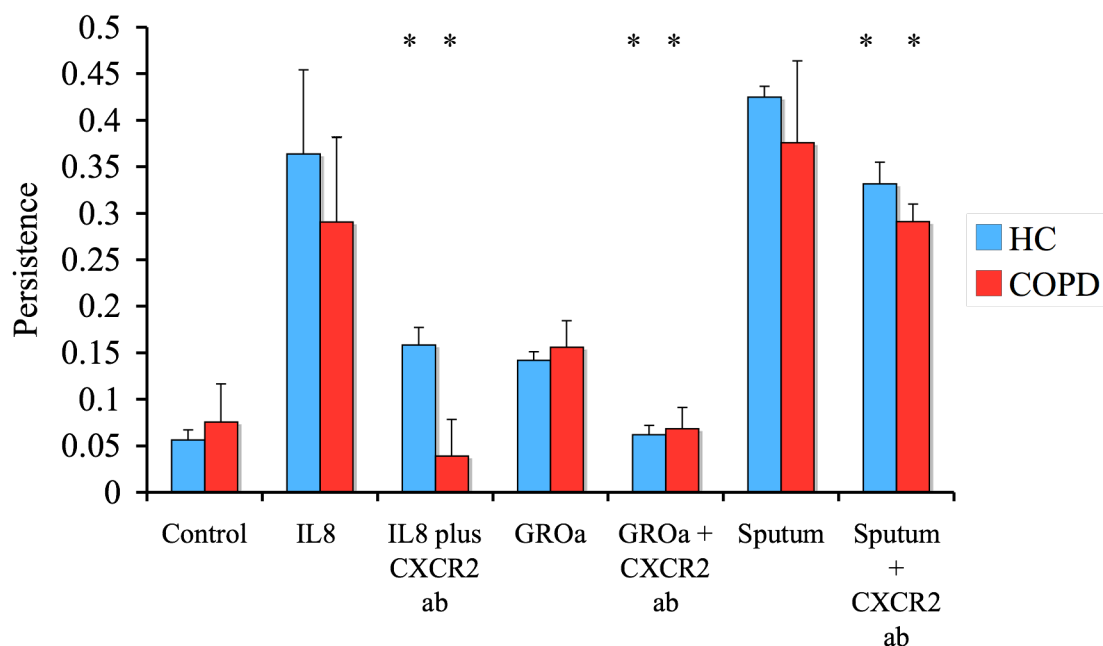


Figure 7.31. The persistence of neutrophils towards IL-8, GRO α and sputum after incubation with anti human CXCR2 antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 10nM is migration towards IL-8 alone, IL8 + CXCR2 Ab is towards IL8 (10nM) following incubation with the antibody (1.6 μ M). The nomenclature is the same for GRO α (100nM) and Sputum (2:1 dilution). * = significant difference from chemoattractant.

Chemotactic Index (Accuracy)

As seen with the small molecule CXCR2 antagonist, when neutrophils from healthy controls were incubated with the CXCR2 antibody, there was a significant reduction in the chemotactic index of neutrophils migrating towards IL-8 (64%) (IL-8 0.41 ± 0.1 ; IL-8 + CXCR2 Ab 0.15 ± 0.1 , $p = 0.009$) and GRO α by approximately 71% (GRO α 0.13 ± 0.1 ; GRO α + CXCR2 Ab 0.03 ± 0.01 , $p=0.01$). The CXCR2 antibody also reduced the accuracy of migration towards sputum by 56% (Sputum 0.46 ± 0.1 ; Sputum + CXCR2 Ab 0.20 ± 0.1).

With neutrophils from COPD patients, there was a reduction in accuracy towards IL-8, but as there was no increase in accuracy in the presence of GRO α compared to baseline, the affect of the antibody could not be assessed (Control 0.02 ± 0.1 ; IL-8, 0.22 ± 0.1 ; IL8 + CXCR2 Ab, 0.05 ± 0.1 , $p=0.004$; GRO α , 0.02 ± 0.1 ; GRO α + CXCR2 Ab, 0.004 ± 0.1). There was a significant reduction in accuracy of movement towards sputum (Sputum, 0.17 ± 0.1 ; Sputum + CXCR2 Ab, 0.09 ± 0.1 , $p=0.02$). Once again, COPD neutrophils were less accurate compared to those from healthy controls, (see figure 7.32), but there were no differential affects using the small molecule antagonist or the CXCR2 antibody.

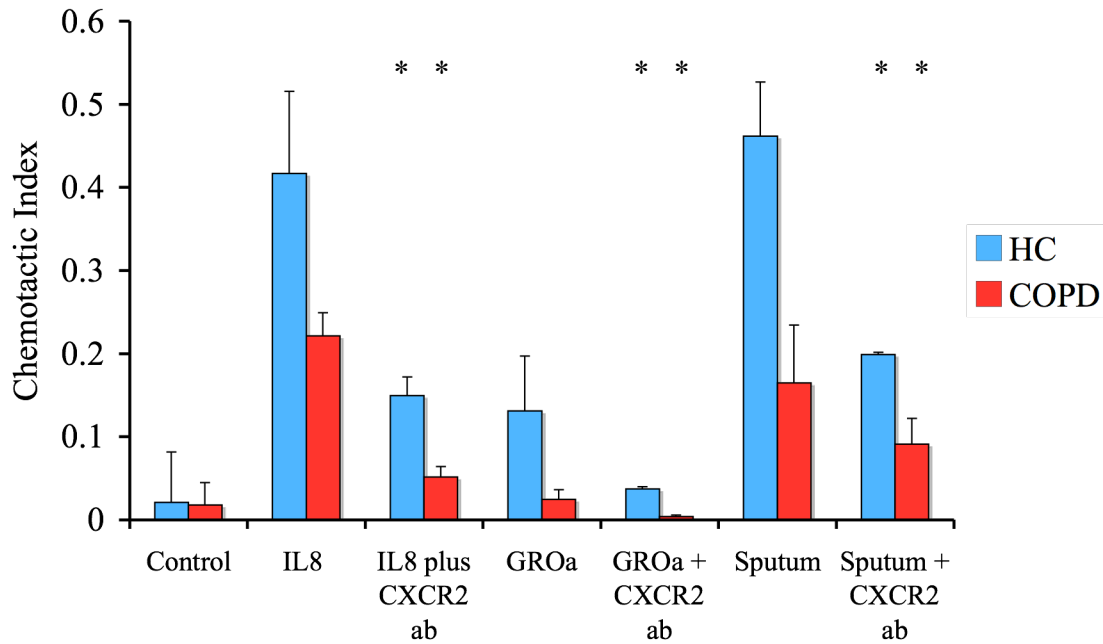


Figure 7.32. The chemotactic index of neutrophils towards IL-8, GRO α and sputum after incubation with anti human CXCR2 antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 10nM is migration towards IL-8 alone, IL8 + CXCR2 Ab is towards IL8 (10nM) following incubation with the antibody (1.6 μ M). The nomenclature is the same for GRO α (100nM) and Sputum (2:1 dilution). * = significant difference from chemoattractant.

7.3.6.2 CXCR1

Speed of migration (Chemokinesis)

When neutrophils from healthy controls were incubated with the CXCR1 antibody, there was a significant reduction in the speed of movement of approximately 25% in IL-8 (IL-8 4.8 μ m/min \pm 0.2; IL-8 + CXCR1 Ab 3.6 μ m/min \pm 0.2, p = 0.04). There was no significant reduction towards GRO α (GRO α 4.2 μ m/min \pm 0.2; GRO α + CXCR1 Ab 4.1 μ m/min \pm 0.1) nor towards sputum (Sputum 4.3 μ m/min \pm 0.2; Sputum + CXCR1 Ab 4.3 μ m/min \pm 0.2).

With neutrophils from COPD patients, a similar pattern was seen, with a 22% reduction in speed towards IL-8 (IL-8, 5.3 μ m/min \pm 0.4; IL8 + CXCR1 Ab, 4.1 μ m/min \pm 0.2,

$p=0.03$), but no significant reduction towards $\text{GRO}\alpha$, ($4.8\mu\text{m}/\text{min} \pm 0.2$; $\text{GRO}\alpha + \text{CXCR1 Ab}$, $4.7\mu\text{m}/\text{min} \pm 0.1$) or sputum (Sputum, $4.9\mu\text{m}/\text{min} \pm 0.1$; Sputum + CXCR1 Ab, $4.6\mu\text{m}/\text{min} \pm 0.1$). Once again, COPD neutrophils were faster compared with those from healthy controls, see figure 7.33, but there were no differential affects using the CXCR1 antibody.

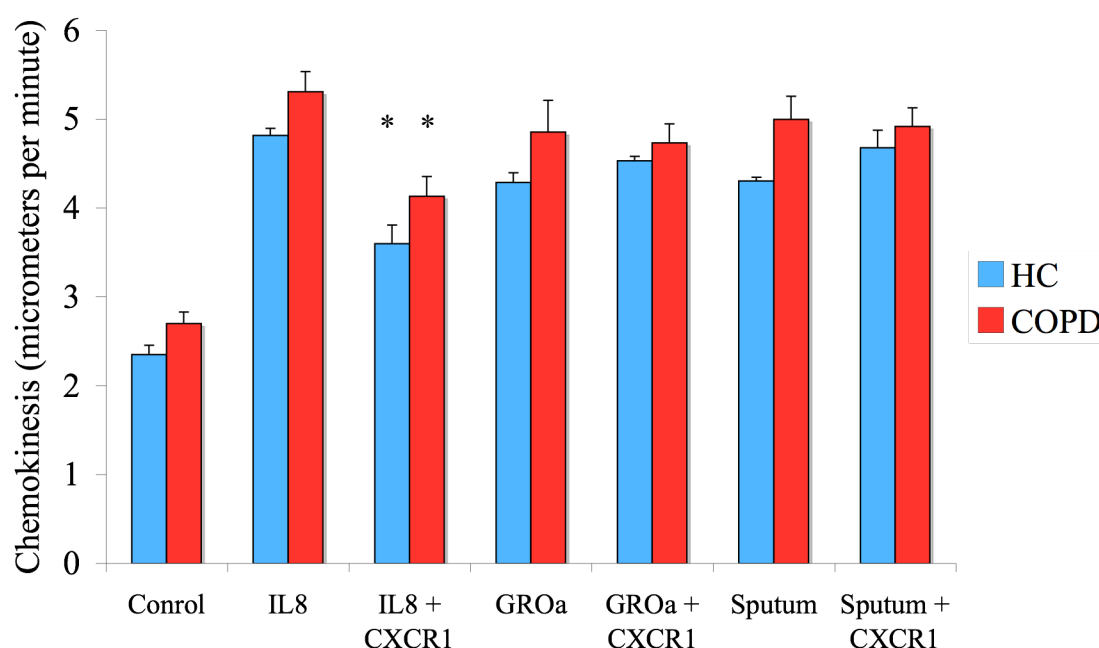


Figure 7.33. The chemokinetic response of neutrophils towards IL-8, $\text{GRO}\alpha$ and sputum after incubation with anti human CXCR1 antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 is migration in the presence of IL-8 alone, IL8 + CXCR1 Ab is in the presence of IL8 (10nM) following neutrophil incubation with the antibody. The nomenclature is the same for $\text{GRO}\alpha$ (100nM) and Sputum (2:1 dilution). * = significant difference from chemoattractant alone.

Velocity (Chemotaxis)

When neutrophils from healthy controls were incubated with the CXCR1 antibody, there was a significant reduction in the velocity of movement towards IL-8 of approximately 40% (IL-8 $1.9\mu\text{m}/\text{min} \pm 0.2$; IL-8 + CXCR1 Ab $1.1\mu\text{m}/\text{min} \pm 0.1$, $p = 0.001$). The CXCR1 antibody did not alter velocity towards $\text{GRO}\alpha$ ($\text{GRO}\alpha$ $1.2\mu\text{m}/\text{min} \pm 0.2$; $\text{GRO}\alpha$

+ CXCR1 Ab $1.3\mu\text{m}/\text{min} \pm 0.01$), however, the CXCR1 antibody reduced velocity towards sputum by 20% (Sputum $1.2\mu\text{m}/\text{min} \pm 0.2$; Sputum + CXCR1 Ab $0.9\mu\text{m}/\text{min} \pm 0.1$, $p=0.04$).

With neutrophils from COPD patients, a similar pattern was seen, with a 30% reduction in velocity towards IL-8 (IL-8, $0.5\mu\text{m}/\text{min} \pm 0.1$; IL8 + CXCR1 Ab, $0.3\mu\text{m}/\text{min} \pm 0.1$, $p=0.03$) but not GRO α (GRO α , $0.6\mu\text{m}/\text{min} \pm 0.2$; GRO α + CXCR1 Ab, $0.5\mu\text{m}/\text{min} \pm 0.1$). There was a significant reduction in velocity towards sputum (Sputum, $0.6\mu\text{m}/\text{min} \pm 0.1$; Sputum + CXCR1 Ab, $0.4\mu\text{m}/\text{min} \pm 0.1$, $p=0.04$). Once again, COPD neutrophils had less velocity towards all chemoattractants than those from healthy controls, (see figure 7.34), but there were no differential affects using the CXCR1 antibody.

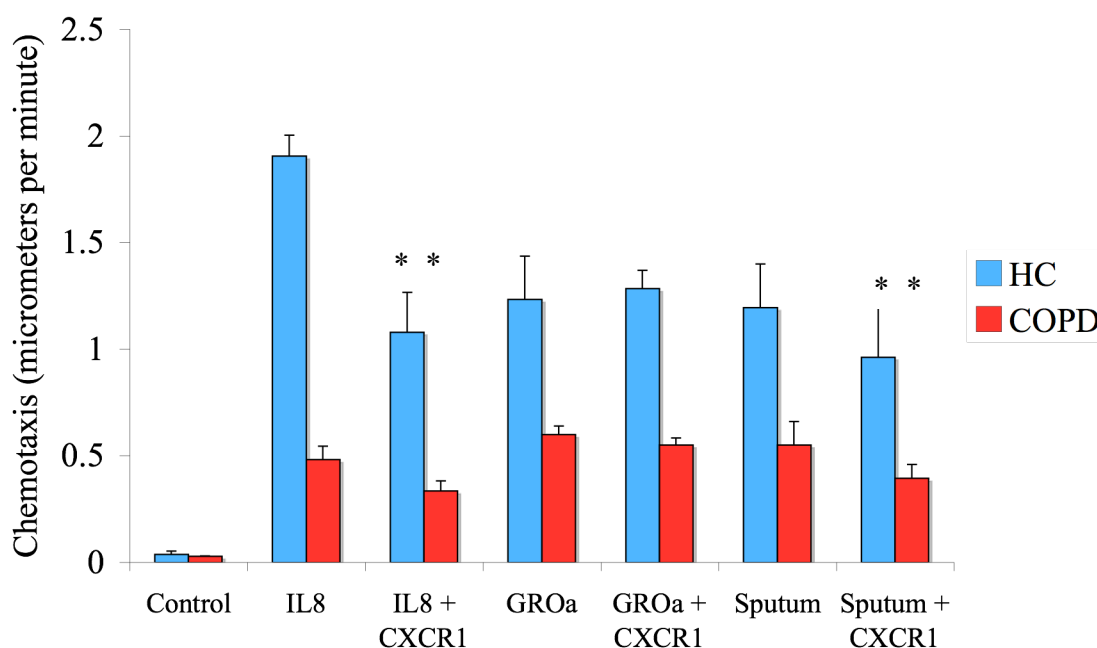


Figure 7.34. The velocity of neutrophils towards IL-8, GRO α and sputum after incubation with CXCR1 antihuman antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 is migration towards IL-8 alone, IL8 + CXCR1 Ab is towards IL8 (10nM) following incubation with the antibody (1.6 μM). The nomenclature is the same for GRO α (100nM) and Sputum (2:1 dilution). * = significant difference from chemoattractant alone.

Persistence

When neutrophils from healthy controls were incubated with the CXCR1 antibody, there was a small but significant reduction in the persistence of movement towards IL-8 of approximately 20% (IL-8 0.35 ± 0.1 ; IL-8 + CXCR1 Ab 0.27 ± 0.1 , $p = 0.04$). CXCR1 antibody did not change persistence towards GRO α (GRO α 0.34 ± 0.1 ; GRO α + CXCR1 Ab 0.32 ± 0.1) or sputum (Sputum 0.4 ± 0.1 ; Sputum + CXCR1 Ab 0.4 ± 0.1).

With neutrophils from COPD patients, a similar pattern was seen, with a small reduction in persistence towards IL-8 (IL-8, 0.3 ± 0.1 ; IL8 + CXCR2 Ab, 0.2 ± 0.1 , $p=0.04$) but the CXCR1 antibody did not change persistence towards GRO α (GRO α , 0.3 ± 0.1 ; GRO α + CXCR1 Ab, 0.3 ± 0.1) or towards sputum (Sputum, 0.2 ± 0.1 ; Sputum + CXCR2 Ab, 0.2 ± 0.1). Once again, there were no differences in the persistence of neutrophils from COPD patients or controls, (see figure 7.35).

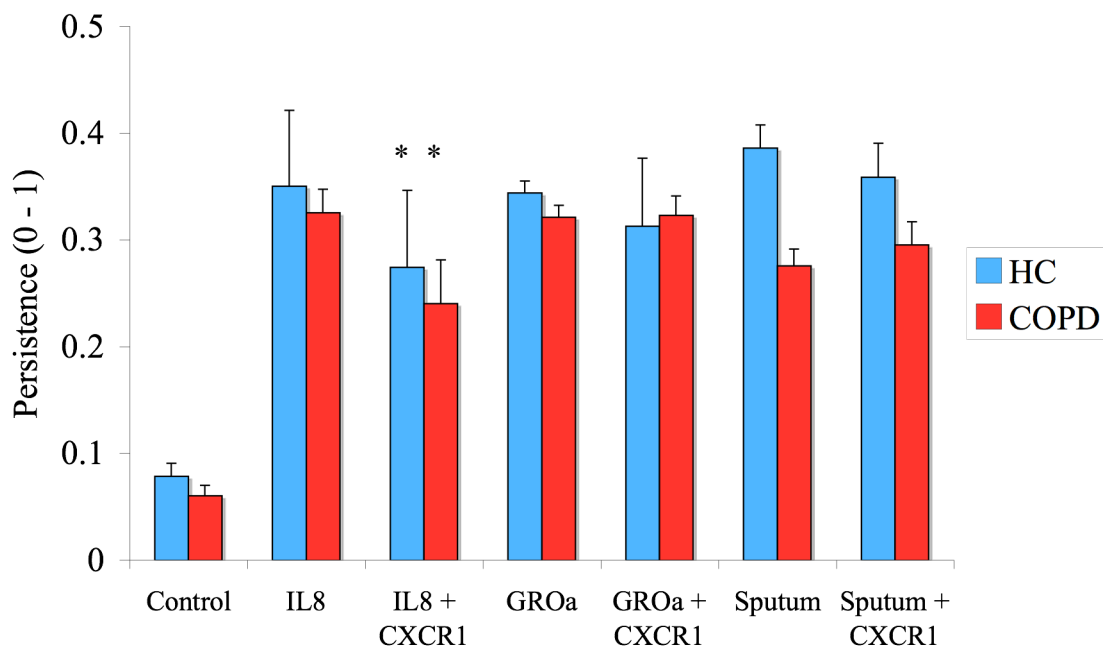


Figure 7.35. The persistence of neutrophils towards IL-8, GRO α and sputum after incubation with anti human CXCR1 antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 is migration towards IL-8 alone, IL8 + CXCR1 Ab is towards IL8 (10nM) following incubation with the antibody (1.6 μ M). The nomenclature is the same for GRO α (100nM) and Sputum (2:1 dilution). * = significant difference from chemoattractant alone.

Chemotactic index (Accuracy)

When neutrophils from healthy controls were incubated with the CXCR1 antibody, there was a significant reduction in the accuracy of movement towards IL-8 of approximately 30% (IL-8 0.25 ± 0.1 ; IL-8 + CXCR1 Ab 0.18 ± 0.1 , $p = 0.03$). The CXCR1 antibody did not affect the accuracy of migration towards GRO α (GRO α 0.21 ± 0.1 ; GRO α + CXCR1 Ab 0.23 ± 0.01) but it did reduce the accuracy of migration towards sputum by 20% (Sputum 0.27 ± 0.1 ; Sputum + CXCR1 Ab 0.19 ± 0.1 , $p=0.02$).

With neutrophils from COPD patients, similar patterns were seen, with a reduction in accuracy towards IL-8 of 20% (IL-8, 0.14 ± 0.1 ; IL8 + CXCR1 Ab, 0.09 ± 0.1 , $p=0.04$). The CXCR1 antibody did not reduce accuracy towards GRO α (GRO α , 0.12 ± 0.1 ; GRO α + CXCR1 Ab, 0.11 ± 0.1). There was a significant reduction in accuracy of movement towards sputum of 50% (Sputum, 0.16 ± 0.1 ; Sputum + CXCR1 Ab, 0.07 ± 0.1 , $p=0.002$). Once again, COPD neutrophils were less accurate compared to those from healthy controls, (see figure 7.36).

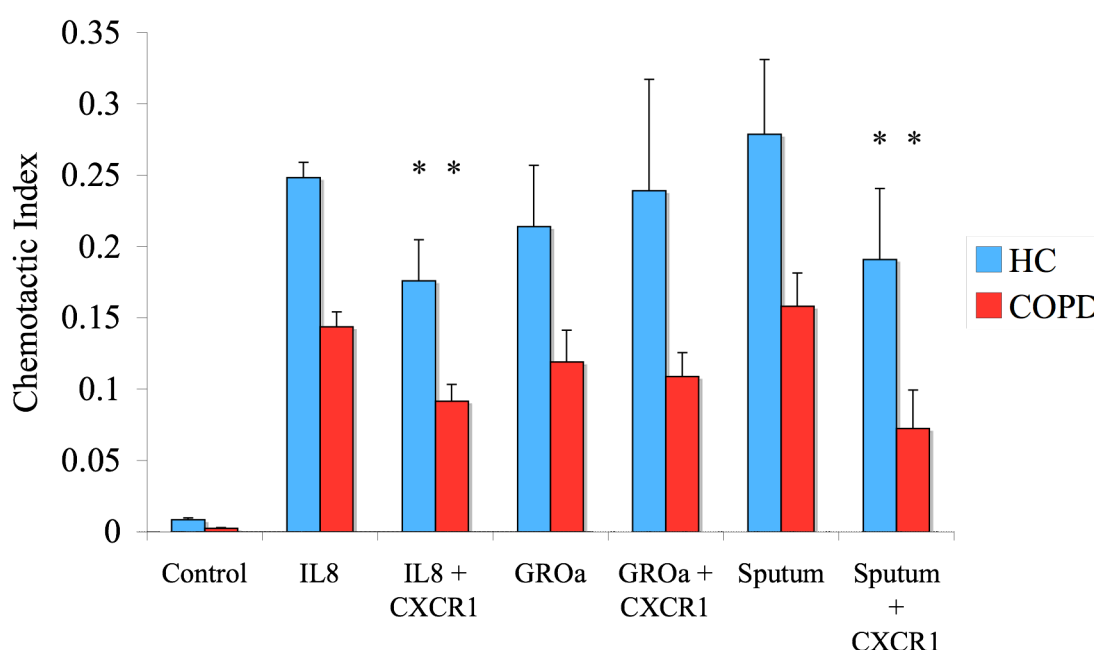


Figure 7.36. The chemotactic index of neutrophils towards IL-8, GRO α and sputum after incubation with anti human CXCR1 antibody.

Legend. The results shown are the mean (and SEM) values of six experiments. IL-8 is migration towards IL-8 alone, IL8 + CXCR1 Ab is towards IL8 (10nM) following neutrophil incubation with the antibody (1.6 μ M). The nomenclature is the same for GRO α (100nM) and Sputum (2:1 dilution). * = significant difference from chemoattractant alone.

7.3.6.3 Comparing the affects of CXCR1 and CXCR2 antibodies in neutrophil migration from healthy controls

IL-8

Data from these studies suggest that CXCR1 accounted for $25\% \pm 3$ of neutrophil chemokinesis with IL-8, while CXCR2 accounted for $30\% \pm 5$. Neutrophils are not static, even in the absence of a chemoattractant, and a combination of CXCR1 and CXCR2 inhibition exerting these effects would reduce movement to baseline levels. Inhibition of both receptors decreased chemotaxis towards IL-8 by 40% (CXCR1, $\pm 9\%$, CXCR2 $\pm 8\%$), suggesting an equally important role for CXCR1 and CXCR2, and again, a combination of the two should theoretically reduce chemotaxis to baseline. CXCR2 appeared differentially important in directional persistence (with a $56\% \pm 7$ increase in directional change Vs. a $20\% \pm 4$ increase for CXCR1, $p=0.04$) and overall accuracy (with a $64\% \pm 11$ reduction in chemotactic index Vs. $30\% \pm 6$ reduction with CXCR1, $p=0.02$) but again, the receptors in combination should reduce both parameters to baseline.

GRO α

As expected, given the preferential binding of GRO α to CXCR2, inhibition of CXCR1 had no affects on migration towards GRO α . CXCR2 inhibition reduced all parameters to baseline levels, confirming that GRO α mediated migration is via CXCR2.

Sputum

Speed or chemokinesis in the presence of sputum was not altered by CXCR1 or CXCR2 inhibition, suggesting that neither of the receptors are of predominant importance for non-directional movement. There was a significant decrease in velocity, but CXCR2

inhibition had a greater affect compared to CXCR1 ($49\% \pm 4$ Vs $19\% \pm 5$, $p=0.03$). Neither antibody altered directional persistence towards sputum. However, although anti-CXCR1 reduced overall accuracy of migration towards sputum, it did so less than CXCR2 ($30\% \pm 6$ Vs. $56\% \pm 8$, $p=0.01$).

7.3.6.4 Comparing the affects of CXCR1 and CXCR2 antibodies in neutrophil migration from patients with COPD

In patients with COPD, similar patterns of the migratory affects of inhibiting CXCR1 or CXCR2 were seen. There was a significant and similar reduction in IL-8 mediated chemokinesis following CXCR1 and CXCR2 inhibition, reducing speed to baseline. This was not seen with GRO α or sputum driven chemokinesis. In COPD, as with controls, inhibition of CXCR2 had a greater affect on neutrophil velocity ($68\% \pm 10\%$ Vs $30\% \pm 4$, $p=0.009$), persistence ($86\% \pm 16$ Vs $19\% \pm 3$, $p=0.007$) and accuracy $74\% \pm 9$ Vs. $35\% \pm 12$, $p=0.04$) driven by IL-8, compared to CXCR1 inhibition. This confirmed that CXCR2 is more important in accurate and efficient migration towards IL-8. A similar pattern was seen in migration towards sputum, with no significant reduction of chemokinesis or persistence with either antibody, but a greater reduction in velocity with CXCR2 inhibition compared with CXCR1 inhibition ($53\% \pm 9$ Vs. $28\% \pm 10$, $p=0.03$), although not chemotactic index (CXCR2 inhibition $44\% \pm 5$ Vs CXCR1 inhibition $54\% \pm 7$). As seen with neutrophils from healthy controls, CXCR2 inhibition reduced all migration mediated by GRO α to baseline levels, while CXCR1 inhibition did not.

In summary, theoretically, in health and in COPD, a combination of CXCR1 and CXCR2 would reduce all migratory parameters towards IL-8 to baseline, and reduce chemotaxis and accuracy of migration towards sputum by 65 – 70%. Here, both

receptors seem able to moderate migration, as neither inhibitor alone was able to stop it completely. However, CXCR1 inhibition altered migration less than CXCR2., suggesting that CXCR2 is the predominant migratory receptor involved in migration to airway secretions.

There were no significant differential affects between the responses of healthy controls and patients with COPD, and neither antibody altered migratory dynamics of neutrophils from healthy controls to resemble those from patients with COPD.

7.4 Discussion

These data describe further differences in circulating neutrophils from patients with COPD compared with those isolated from healthy controls. Firstly, COPD neutrophils expressed significantly less CXCR1 and CXCR2 receptors on their surface compared with healthy controls, and these differences remained both over time and when cells were activated. There were no differences in surface expression of either receptor on neutrophils from healthy smokers and non-smokers, suggesting that expression is independent of smoking status. These differences are not necessarily specific to COPD, as the healthy controls also differ in terms of their medications, inflammatory burden and lung function, all of which may alter the expression of receptors involved in migration. However, it is interesting that in some inflammatory conditions (including inflammatory bowel disease (Banks, Bateman et al. 2003) and Behçets eye disease (Qiao, Sonoda et al. 2005)) CXCR2 expression is increased, and so down-regulation of CXCR2 is not a universal response to inflammation.

It is also interesting that receptor numbers are reduced in COPD when in a previous chapter it was established that pseudopod counts were reduced in neutrophils from this

patient population. It is known that the receptors for chemoattractants are localised to the leading edge of a cell, and expressed on pseudopods (Weber 2006). Although no causal link can be established, it could be hypothesised that reduced receptor expression may be linked with reduced pseudopod formation, and might, in combination, explain the decreased accuracy of migration due to a reduced ability to compare sites of receptor occupancy.

There have been three previous studies of CXCR1 and CXCR2 expression in COPD, one reporting reduced expression of CXCR2 (Pignatti, Moscato et al. 2005), one describing increased expression of CXCR1 but not CXCR2 (Yamagata, Sugiura et al. 2007) and one describing no difference between groups, although there was a trend towards a decrease in CXCR2 (Traves, Smith et al. 2004). In all studies, a wide range of COPD patients were included, rather than the more homogenous patient group included in the current thesis, and it may be that different phenotypes of patients or differing disease severities may affect receptor expression. Further studies of a wider group of highly characterised patients, in larger numbers would be required to examine this.

It is also overly simplistic to assume that receptor expression on quiescent neutrophils equates to receptor function, given the complex nature of receptor recycling and degradation. Studies of surface expression when cells were activated, and studies of expression over time, were undertaken to start to explore functional differences in CXCR1 and 2, and no differential affects were seen. To explore this further, studies of a CXCR2 small molecule antagonist and CXCR1 and CXCR2 monoclonal antibodies were undertaken.

To summarise the migratory components of these studies, neutrophils from patients with COPD moved with greater speed, but less accuracy towards IL-8, GRO α and sputum. This remained even in the presence of CXCR1 or CXCR2 inhibition. Incubating the IL-8 monoclonal antibody with IL-8 or the GRO α antibody with GRO α reduced all facets of migration towards the chemokines to baseline levels in both groups. Inhibition of CXCR2 by the CXCR2 antagonist or antibody also reduced all migratory parameters to baseline when used with GRO α (in keeping with its preferential binding to CXCR2), but not when used with IL-8 where inhibition of CXCR2 achieved only a 25% reduction in chemokinesis, but approximately a 40 - 60% reduction in chemotaxis, persistence and accuracy. Inhibition of CXCR1 also reduced chemokinesis towards IL-8 by 25%, but reduced persistence and accuracy of migration to a lesser degree (20 – 30%)

None of the chemokine specific antibodies, the receptor antibodies nor the antagonist reduced the speed of movement (chemokinesis) in sputum, suggesting that CXCR1 and CXCR2 are not important components of non specific cell activation and random movement in this biological fluid. However, it is likely that these receptors have a role in directed migration, as chemotaxis and overall accuracy could be reduced by 50% following inhibition of IL-8 and 30% following inhibition of GRO α . CXCR2 inhibition reduced the chemotaxis and overall accuracy by approximately 50%, while CXCR1 inhibition reduced chemotaxis and accuracy by 20 – 30% .

These data allow some comment about the specific role of CXCR1 and CXCR2 in neutrophil migration. It is apparent that both receptors are required for accurate migration, but not chemokinesis. However, that CXCR2 signalling accounts for a

greater proportion of directed cell movement (approximately 60% CXCR2 compared with 30% CXCR1) suggests that it is the dominant receptor in neutrophil migration.

It is known that CXCR2 is of fundamental important in neutrophil recruitment to areas of inflammation and infection, as demonstrated *in vitro* and *in vivo* in animal studies (Del Rio, Bennouna et al. 2001; Farooq, Stillie et al. 2009). The role of CXCR1 is less clear, but CXCR1 has been associated with bacterial killing, the respiratory burst and the release of α defensins by neutrophils from subjects with cystic fibrosis and COPD although CXCR2 was not associated with these functions (Hartl, Latzin et al. 2007). It has also been hypothesised that these closely related G protein receptors are involved in cross-talk, as the down-regulation seen in surface expression on neutrophils following activation, is reduced when both receptors are present (Attal, Cohen-Hillel et al. 2007). In addition the CXCR2 carboxyl terminus phosphorylation sites are required for inducing inhibition of CXCR1 down-regulation, and *vice versa* (Attal, Cohen-Hillel et al. 2007). Certainly, the data included in the current chapter provides some evidence of synergistic actions in migration, as neutrophil migration should theoretically only be maximal towards IL-8 when both receptors are available.

It is interesting to note that inhibiting CXCR1 and CXCR2 on neutrophils from healthy controls did not produce a COPD phenotype, because although this reduced the accuracy of migration, it did not increase migratory speed. This suggests that the defect in COPD migration is not only caused by a lack of or impaired function of CXCR1 and CXCR2 receptors. Indeed, the fact that neutrophils isolated from COPD patients continued to display excessive speed but reduced accuracy of migration towards sputum, even when IL-8 and GRO α were abrogated, and following inhibition of CXCR1 and CXCR2

demonstrates very clearly that the aberrant migration is not purely dependent upon CXCR1 and CXCR2 signalling. This suggests very strongly that the defect involves a wider range of chemoattractants (sputum will contain a variety of other chemoattractants including LTB₄) and hence is likely to be due to an alteration in downstream signalling, in a shared component of the migratory pathway. In order to study this further, the last series of studies have focused upon the common migratory pathway, in particular on phosphoinositide-3-kinase (PI3K).

8 Mechanism of aberrant migration.

Phosphoinositide 3-Kinase inhibition

8.1 Brief Introduction

Data described in previous chapters have consistently demonstrated that neutrophils from patients with COPD migrate with increased speed but reduced accuracy towards IL-8, GRO α and sputum and with reduced pseudopod formation. This was associated with reduced surface expression of CXCR1 and CXCR2, but did not seem to be attributable to the decrease in expression of these receptors alone. Firstly, because the aberrant migration towards sputum was seen when IL-8 and GRO α were blocked by monoclonal antibodies, suggesting that other pathways may be involved, and secondly because inhibiting CXCR1 and CXCR2 in healthy controls did not produce a “COPD” migration phenotype.

Although further studies of CXCR1 and CXCR2 expression, placement and recycling are warranted, pro-migratory ligands act via phosphoinositide 3 kinase (PI3K). PI3Ks are a family of related enzymes that play a pivotal role in cellular regulatory mechanisms, by phosphorylating the 3'-OH position of phosphoinositide lipids, generating lipid second messengers.

The PI3K family is divided into three classes, termed I, II and III. All class I members are heterodimers consisting of a catalytic subunit and a non-catalytic subunit. They phosphorylate phosphatidylinositol (PI), phosphatidylinositol-4-mono-phosphate (PIP) and phosphatidylinositol-4,5- biphosphate (PIP₂) in vitro, but have a strong preference for PIP₂ in vivo. Class one members are further subdivided into class1A and 1B PI3Ks. Class 1A consist of three isoforms (p100 α , p110 γ and p100 δ) whereas the only class1B member is p110 γ . Class1A are commonly activated by tyrosine-kinases, which generate docking sites for the p85/p55 adapter subunits by phosphorylating tyrosines

within the p85/p55 consensus binding motifs on a large number of proteins (Cantrell 2001). In contrast, the class 1B member (p110 γ) is activated by G-protein-coupled receptors (GPCRs) (Foster, Traer et al. 2003), such as CXCR1 and CXCR2, but also including the receptors for LTB₄, fMLP, and C5a.

All class 1 PI3Ks are further activated by the small GTPase, Ras via a direct interaction between Ras-GTP and the catalytic p110 gamma subunits (Suire et al, 2006). Effectors of class 1 PI3Ks are pleckstrin-homology domain containing proteins such as Akt/PKB, BTK, TEC, ITK and small GTPases (e.g. Cdc42, Rac or Ras). The action of PI3Ks is antagonised by the phosphatidyl-inositol-3,4,5-trisphosphate phosphatases SHIP and PTEN (Foster, Traer et al. 2003).

PI3K gamma is predominantly expressed in haematopoietic cells, and is important in cell migration (Faerrandi, Ji et al. 2003; Del Prete, Vermi et al. 2004). In *Dictyostelium*, studies of *pi3k1*- and *pi3k2*- double knock-out cells have suggested that PI3K is important in regulating cell speed and locomotion (Kolsch, Charest et al, 2008) and is involved in directional sensing in shallow gradients of chemoattractants (although in steep gradients, other pathways may be sufficient to allow migration) (Hoeller and Kay, 2007). Importantly, Class 1 PI3K knock out strains of *Dictyostelium* are still able to migrate, but show reduced speed (Hoeller and Kay, 2007).

PI3K γ is believed to orientate the cell by initiating and maintaining the leading edge (cell polarity) (Wang, Herzmark et al. 2002), allowing directed motility by the formation of pseudopodia (Wu 2005). Cell migration involves local production and degradation of phosphatidylinositol (3,4,5) trisphosphate (PtdIns(3,4,5)P₃) at the plasma membrane,

resulting in a net accumulation of PtdIns(3,4,5)P₃ at the leading edge of the cell, leading ultimately to actin polymerization, with the formation of pseudopodia. PtdIns(3,4,5)P₃ accumulation at the leading edge is PI3Kγ dependent (Ferguson et al, 2007; Nishio et al, 2007), as is stabilisation of the leading edge (Sasaki et al, 2004).

PI3Kγ probably controls pseudopod generation by the localisation of PtdIns(3,4,5)P₃ signalling, however PI3K is not responsible for pseudopod placement. Instead it is likely that PTEN suppresses lateral pseudopods (by degrading PtdIns(3,4,5)P₃) maintaining migratory direction (Wessels et al, 2007), and helping prevent the migrating cell from “going off track”. The balance of activity between PI3K and PTEN may establish gradients of PtdIns(3,4,5)P₃ in chemotaxing cells, that can facilitate (PI3K) or suppress (PTEN) pseudopod generation in accordance with chemotactic stimuli.

A study of *pten*- mice by Subramanian et al (2007) suggested that neutrophils without PTEN activity had enhanced PtdIns(3,4,5)P₃ levels, Akt phosphorylation and actin polymerization, with increased speed of migration, but the cells also had directional migratory defects (Subramanian et al, 2007), potentially due to a lack of lateral pseudopod suppression. SHIP1 (Src homology 2 domain-containing inositol-5-phosphatase 1) is also believed to be important in PtdIns(3,4,5)P₃ degradation (perhaps especially so in human neutrophils), but in recent studies of neutrophils lacking SHIP1, migration was slower (in contrast to neutrophils depleted in PTEN), with reduced cellular polarity (Nishio et al, 2007).

Therefore, PtdIns(3,4,5)P₃ signalling via PI3K and inhibition of signalling via PTEN/SHIP1 does not affect the sensing of a chemotactic stimuli or gradient, but will affect the

speed of migration and potentially the overall efficiency of migration due to effective sustainment of aligned pseudopodia and the suppression of unaligned lateral pseudopodia. It is unclear whether over-activity or under-activity of either enzyme would change rate of pseudopod formation.

Neutrophils from patients with COPD have displayed increased speed but reduced accuracy of migration towards chemoattractants in shallow gradients (as shown in previous chapters). This could be consistent with an alteration in $\text{PtdIns}(3,4,5)\text{P}_3$ signalling.

The PI3 kinase Class 1 inhibitor, LY294002, has been shown to reduce chemokinesis and chemotaxis towards IL-8 in the Boyden chamber assay using neutrophils from healthy young controls (Knall, Worthen et al. 1997). Furthermore, studies by Andrew et al suggest that LY294002 reduces the efficiency of migration without removing the ability to chemotax up chemotactic gradients, potentially by altering pseudopod placement on the leading edge (Andrew and Insall 2007).

Differences in PI3K/PTEN activity could be a potential mechanism for the migratory differences seen in COPD, however, the affects of a PI3K Class 1 inhibitor has not been assessed in COPD, compared with healthy controls and patients with A1ATD.

The studies included in this chapter aimed to determine whether a PI3K inhibitor (LY294002) had differential affects in neutrophil migration in COPD.

8.2 Experiments

8.2.1 Study subjects

Healthy controls consisted of healthy volunteers, age and gender matched to the COPD group, who had never smoked, had no evidence of chronic disease and were medication free. COPD patients had moderate to severe disease defined by GOLD criteria (Pauwels, Buist et al. 2001). They were ex-smokers, aged between 50 and 70. All patients were clinically stable for at least eight weeks prior to recruitment with no changes in medication. Alternative and concomitant disease was excluded clinically, physiologically and radiologically. Patients had normal circulating levels of A1ATD and were PiMM genotype. Patients with A1ATD (PiZ) were matched for age, gender, lung physiology and treatments to patients with usual COPD.

8.2.2 Isolation of blood neutrophils

Neutrophils were isolated from the whole blood as described previously (general methods 2.5.7) (Mikami, Llewellyn-Jones et al. 1998). The neutrophils (>95% pure, > 97% viable, by exclusion of trypan blue) were resuspended in sterile buffer (RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK) containing 0.15% bovine serum albumin (Sigma-Aldrich, UK) for migrational studies.

8.2.3 Neutrophil Chemotaxis

8.2.3.1 Modified Dunn Chamber

Neutrophil chemotaxis was measured using a modification of the Dunn chamber. Full methodological details are included in the general methods (section 2.7.2). Chemotaxis was assessed as described in general methods (section 2.7.2).

8.2.3.2 Chemo-attractants and antagonists

In both assays, neutrophils migrated in gradients of a negative control (RPMI (Sigma-Aldrich)) and IL-8 (RnDSystems) (10nM). Subsequently, neutrophils were pre-incubated the type 1 PI3 kinase inhibitor, LY294002 (SABiosciences, tebu-bio, Peterborough, UK) (1nM) made up with buffer (RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK), at room temperature for 45 minutes. The pre-treated neutrophils were studied in gradients of IL-8 (10nM) or control buffer as before. Concentrations of LY294002 were chosen following appropriate dose response experiments to slow but not prevent migration. Chemotaxis was assessed as described in general methods (section 2.7.2).

8.2.4 Statistical analysis

Migrational differences seen using the Modified Dunn Chamber were compared using the mean data of 10 cells per experiment, per subject. Differences in chemotaxis criteria were compared using an Independent *t* test. Sample size calculations were based upon a two group parallel comparison, to provide an 80% power of detecting a 25% change in mean speed at the 5% level of significance.

8.3 Results

8.3.1 The affect of a type 1 PI3 Kinase inhibitor on neutrophil migration

8.3.1.1 Validation.

Initially, neutrophilic adherence and migration was assessed in the presence of increasing concentrations of LY294002 by determination of the percentage of cells that had adhered to the coverslip (compared with cells that had not) and the percentage of

adhered cells that were able to migrate towards IL-8. Using this data, dose response experiments were performed with increasing concentrations of LY294002 to establish concentrations where migration was decreased, but not inhibited.

LY294002 appeared to profoundly effect neutrophil adherence to coverslips and migration at concentrations over 1 μ M (see figure 8.1). Dose responses to assess the effects of LY294002 on migratory speed were limited to a dosing range of 1nM to 1 μ M in view of the above results. Neutrophil migratory speed was slowed, but not fully inhibited when neutrophils were pre-incubated with LY294002 concentrations of 1nM, 0.01 μ M and 0.1 μ M (see figure 8.2a). Further dose responses were carried out using neutrophils isolated from patients with COPD. These data showed that COPD neutrophils migrated with reduced speed at concentrations of LY294002 of 1nM, and that this effect was more pronounced than seen with neutrophils isolated from healthy controls. Given that this seemed to be a differential affect, a concentration of 1nM was utilised for further experiments.

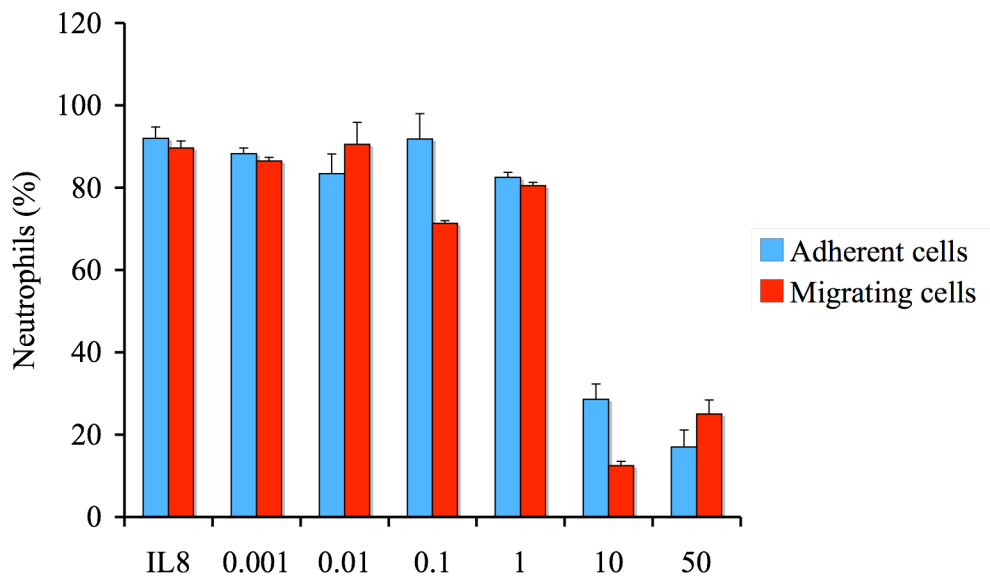


Figure 8.1. The affects of incubation with increasing doses of LY294002 on neutrophil adherence and migration

Legend: The graph demonstrates the effect of increasing concentrations of LY294002 on neutrophil adherence and migration to IL-8. Adherent cells = adherent cells/ total number of cells per field x 100. Migrating cells = number of migrating neutrophils / adherent neutrophils per field x 100. On the x axis, IL-8 represents naïve neutrophils, while 0.001 – 50 represents neutrophils pre-treated with the corresponding concentration of LY294002 (μ M). The results shown are the mean (and SEM) of 6 experiments.

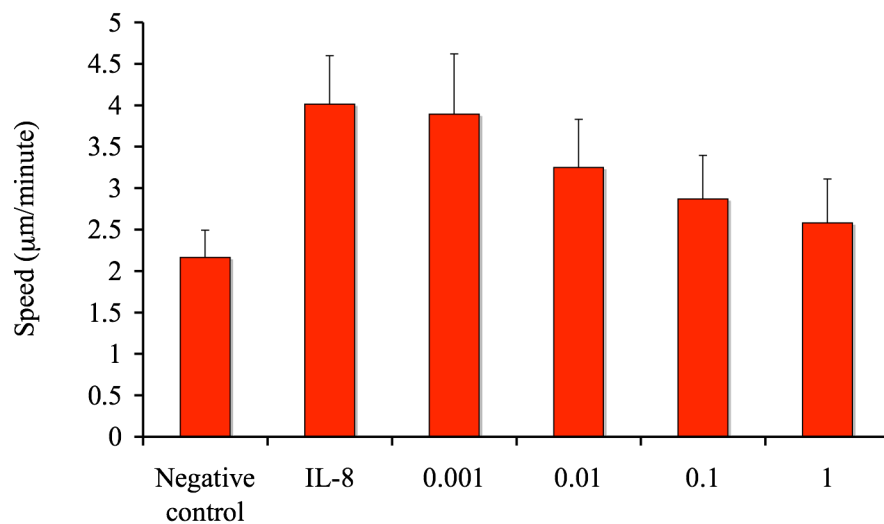


Figure 8.2a. The speed of migrating neutrophils from healthy controls pre-incubated in increasing concentrations of LY294002

Legend: Neutrophils migrated in shallow gradients of 10nM IL-8. On the x axis, negative control is RPMI buffer, IL-8 is migration of naïve neutrophils towards IL-8, 0.001 – 1 represent increasing concentrations of LY294002 (in μ M). The results shown are the mean (and SEM) values of six experiments.

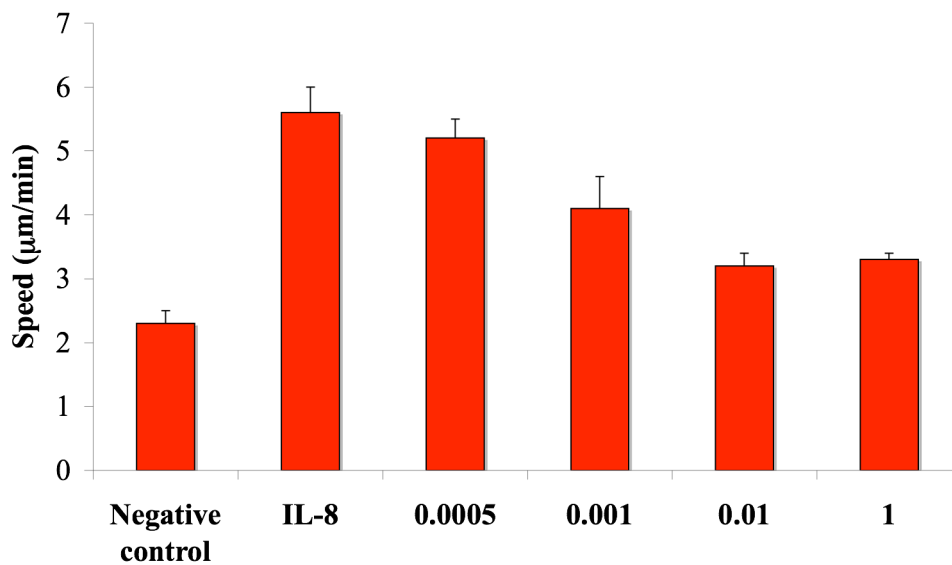


Figure 8.2b. The speed of migrating neutrophils from patients with COPD pre-incubated in increasing concentrations of LY294002

Legend: Neutrophils migrated in shallow gradients of 10nM IL-8. On the x axis, negative control is RPMI buffer, IL-8 is migration of naive neutrophils towards IL-8, 0.001 – 1 represent increasing concentrations of LY294002 (in µM). The results shown are the mean (and SEM) values of six experiments

The affects of LY294002 on neutrophil migration in COPD

Migrational studies and comparisons of PI3K expression were conducted on neutrophils isolated from 6 patients with COPD and 6 age and gender matched healthy controls.

Demographic data is presented in Table 8.1.

Table 8.1: Demographic data for COPD patients, healthy controls, healthy smokers and patients with A1ATD for studies of LY294002

| | COPD | HC | HS | A1ATD |
|------------------------------|------------------|--------------------|--------------------|------------------|
| N | 6 | 6 | 6 | 6 |
| Age (years) | 62 (57 - 66) | 60 (55 – 64) | 58 (55 - 61) | 59 (54 – 65) |
| Gender (Male) | 4 | 3 | 3 | 3 |
| Smoking status | All ex-smokers | Never * | Current | All ex-smokers |
| Pack years | 29 (24 - 35) | 0 * | 25 (18 - 29) | 24 (19 – 30) |
| FEV ₁ (l) | 0.97 (0.8 – 1.1) | 2.25 (2.0 – 2.7) * | 1.98 (1.8 - 2.5) * | 1.02 (0.7 – 1.3) |
| FEV ₁ % predicted | 49 (43 - 54) | 91 (85 – 106) * | 90 (84 - 101) * | 51 (46 – 53) |
| FEV ₁ /FVC | 56 (44 - 58) | 81 (76 – 85) * | 78 (76 - 83)* | 58 (51 – 63) |
| BMI (kg/m ²) | 21 (20 - 22) | 21 (20 – 22) | 22 (20 - 23) | 22 (20 – 23) |
| Inhaled steroids | 83% | 0% * | 0% * | 66% |
| Theophyllines | 0% | 0% * | 0% * | 0% |
| Long acting b2 agonist | 50% | 0% * | 0% * | 50% |
| Anti-cholinergics | 50% | 0% * | 0% * | 50% |
| Bacterial colonisation | 0% | N/A | N/A | 0% |

Legend. Age is presented as the mean age (with the range in parentheses). Gender is the number of males in each group. The remaining data is presented as the median (with the interquartile range in parentheses). Healthy non-smoking controls (HC) and healthy smokers (HS) differed from COPD patients in terms of lung function and treatments. There were no other differences between groups.

* = significant difference from COPD patients ($p < 0.05$)

Speed (Chemokinesis)

Pre-incubation with LY294002 significantly reduced the speed of migrating COPD neutrophils from $4.8 \pm 0.2 \mu\text{m}/\text{min}$ to $2.4 \pm 0.5 \mu\text{m}/\text{min}$ ($p = 0.001$) without slowing cells from healthy controls (unaltered at $3.7 \pm 0.4 \mu\text{m}/\text{min}$), healthy smokers (from 3.6 ± 0.2 to $3.4 \pm 0.3 \mu\text{m}/\text{min}$) or patients with A1ATD (from 3.5 ± 0.2 to $3.4 \pm 0.2 \mu\text{m}/\text{min}$), see figure 8.3.

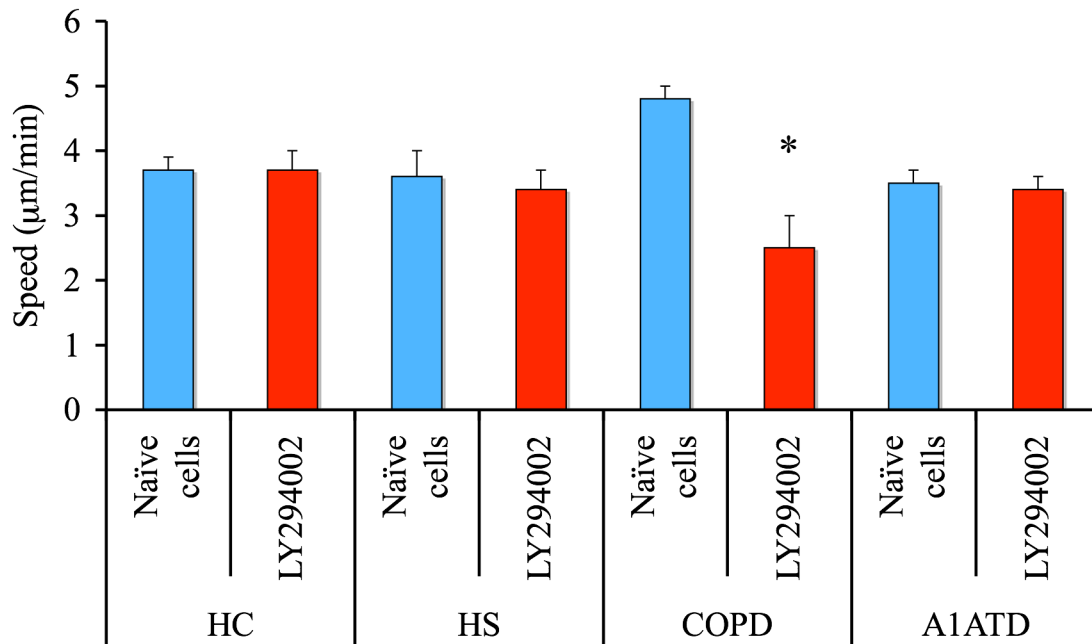


Figure 8.3. Speed of neutrophils pre-incubated with the PI3K inhibitor LY294002.

Legend. For graphs 8.3 – 8.5 measurements were taken from 10 cells from each individual (migrating in the presence of a chemotactic gradient formed from 10nM of IL-8). Neutrophils are migrating in IL-8 alone (naïve cells), or in IL-8 following incubation with LY294002. Each experiment was repeated three times and the average results for each subject were calculated, and an overall average was used for comparisons between groups. HC = healthy non-smoking, HS = healthy-smoking. For graph 8.3: a significant reduction in speed following treatment with LY294002 is annotated * where $p < 0.05$.

Velocity (Chemotaxis)

LY294002 increased the velocity of neutrophils from COPD patients (from $0.5 \pm 0.1 \mu\text{m/min}$ to $0.9 \pm 0.1 \mu\text{m/min}$ $p = 0.03$) while decreasing the velocity of healthy controls (from $1.2 \pm 0.1 \mu\text{m/min}$ to $0.8 \pm 0.1 \mu\text{m/min}$, $p=0.03$) and healthy smokers ($1.3 \pm 0.4 \mu\text{m/min}$ to $0.7 \pm 0.3 \mu\text{m/min}$, $p=0.02$), with a trend towards a reduction in patients with A1ATD (from $1.1 \pm 0.1 \mu\text{m/min}$ to $0.8 \pm 0.1 \mu\text{m/min}$, $p=0.06$). See figure 8.4.

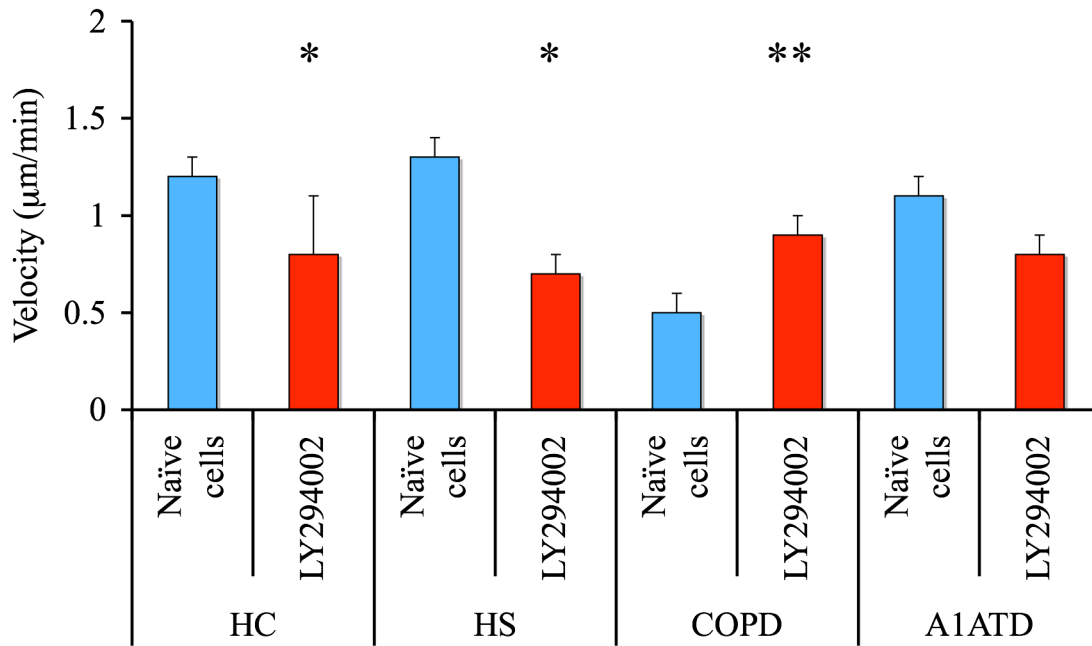


Figure 8.4. Velocity of neutrophils pre-incubated with the PI3K inhibitor LY294002.
Legend. See legend for graph 8.3. Neutrophils are migrating towards IL-8 alone (naïve cells), or towards IL-8 following incubation with LY294002. * = significant decrease from naïve cells. ** = significant increase from naïve cells.

Persistence

The PI3kinase inhibitor did not alter the directional persistence of neutrophils from patients with COPD (naïve cells 0.43 ± 0.08 , treated cells 0.48 ± 0.06), healthy controls (naïve cells 0.44 ± 0.01 , treated cells 0.41 ± 0.05), healthy smokers (naïve cells 0.36 ± 0.03 , treated cells 0.35 ± 0.04), or patients with A1ATD (naïve cells 0.39 ± 0.04 , treated cells 0.37 ± 0.06).

Chemotactic Accuracy

LY294002 significantly increased the chemotactic index of COPD neutrophils compared with untreated cells (from 0.2 ± 0.06 to 0.3 ± 0.01 , $p=0.03$), while there was a trend towards a decrease in chemotactic index following incubation with the PI3 kinase inhibitor in healthy controls (from 0.29 ± 0.06 to 0.22 ± 0.05 , $p=0.06$), healthy smokers (from 0.31

± 0.02 to 0.27 ± 0.04 , $p=0.09$) and patients with A1ATD (from 0.28 ± 0.07 to 0.22 ± 0.06 , $p=0.07$) as summarised in figure 8.5.

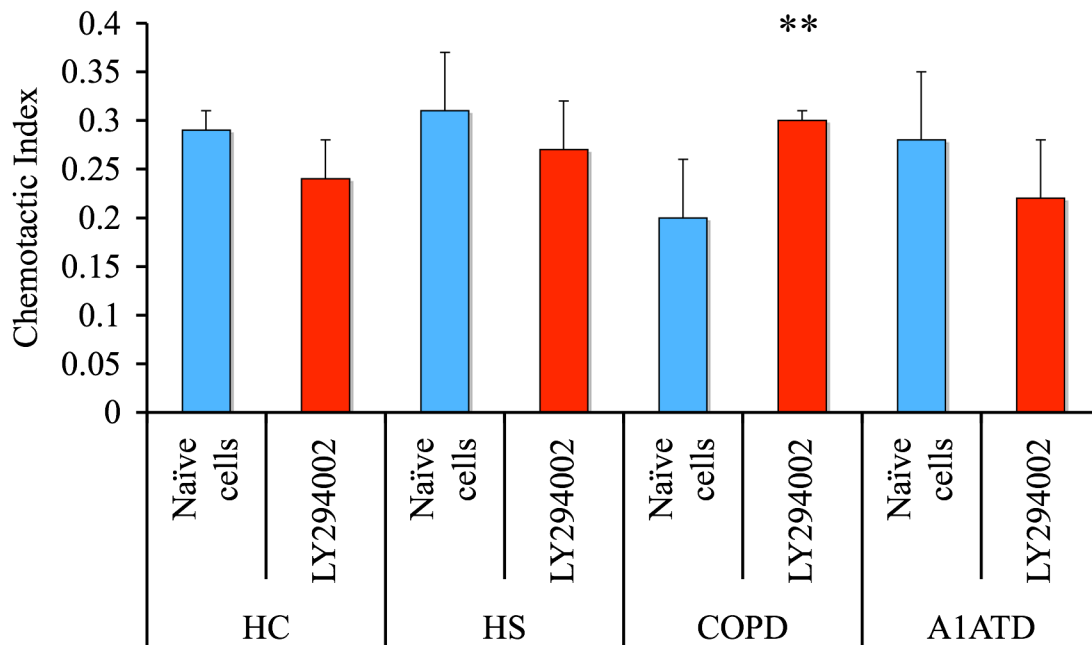


Figure 8.5. Chemotactic Accuracy of neutrophils pre-incubated with the PI3K inhibitor LY294002.

Legend. See legend for graph 8.3. Neutrophils are migrating towards IL-8 alone (naïve cells), or towards IL-8 following incubation with LY294002. * = significant decrease in chemotactic index from naïve cells. ** = significant increase in chemotactic index from naïve cells.

Figure 8.6 and 8.7 provide representative examples of the circulating neutrophils migratory tracks that were recorded for an individual COPD patient both with and without pre-incubation with LY294002 in response to IL-8.

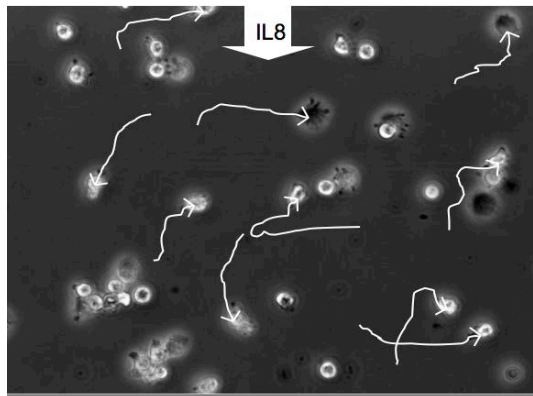


Figure 8.6

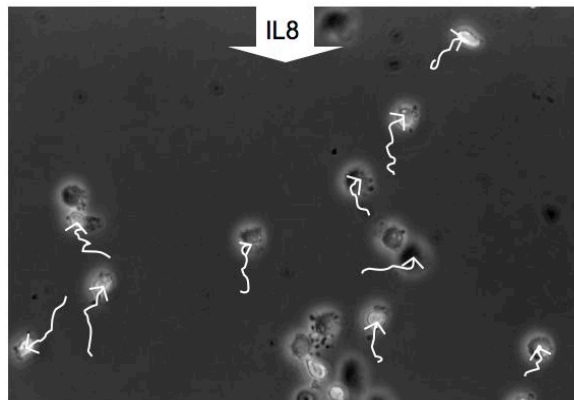


Figure 8.7

Figures 8.6 and 8.7 Tracks of neutrophil migration from a subject with COPD, with and without neutrophil incubation with the PI3 Kinase Inhibitor, LY294002.

Legend. The images show the final neutrophil positions within the Dunn chemotaxis chamber following 20 minutes of phase time lapse recording. The large white arrow at the top of each picture represents the direction of the source of the chemotactic signal (here IL-8 at a concentration of 10nM for all figures). In Figure 8.6, mean cell speed was $5\mu\text{m}/\text{min}$, mean velocity was $-1\mu\text{m}/\text{min}$, mean persistence was 0.43 and mean chemotactic index was -0.19. In Figure 8.7, (treated with LY294002) mean cell speed was $1.97\mu\text{m}/\text{min}$, mean velocity was $0.63\mu\text{m}/\text{min}$, mean persistence was 0.48 and mean chemotactic index was 0.373.

In summary, there were no differences in the migratory patterns seen between patients with A1ATD and healthy controls. The addition of LY294002 to COPD neutrophils reduced migratory speed to levels below those seen in the healthy controls ($p = 0.0001$ for

both). The inhibitor increased migratory velocity to that of healthy controls. Furthermore, although treating cells with the inhibitor did not alter migratory persistence, it enhanced overall chemotactic accuracy to levels comparable to healthy controls. See Table 8.2.

Table 8.2: A comparison of migratory parameters in 6 patients with COPD (with and without incubation with LY294002) and 6 healthy subjects

| | Healthy Controls | COPD without Inhibitors | LY294002 |
|-------------------|---------------------|----------------------------|--------------|
| Speed | 3.66 (0.2) | 4.88 (0.1) * | 2.44 (0.5) ± |
| Velocity | 1.18 (0.1) | 0.46 (0.1) * | 1.0 (0.1) ± |
| Persistence | 0.44 (0.1) | 0.43 (0.01) | 0.48 (0.1) |
| Chemotactic Index | 0.29 (0.1) | 0.17 (0.1) * | 0.30 (0.1) ± |

Legend. The table compares the untreated and treated circulating neutrophils from patients with COPD with age and gender matched healthy controls. The mean data for each group is displayed (with the SEM in parentheses). Neutrophils migrated in the presence of a chemotactic gradient formed using 10nM IL-8. Speed and velocity are measured in $\mu\text{m}/\text{minute}$. Persistence is scaled from 0 to 1, where 1 equates to no change of cell orientation during migration (no change of direction) and 0 equates to many changes in cell orientation (see text). Chemotactic Index is measured from -1 to 1, where 1 equates to direct movement towards the chemoattractant source and -1 equates to direct movement away from the chemoattractant source. * = significant difference ($p < 0.05$) in comparison with neutrophils from healthy controls. ± = significant difference ($p < 0.05$) in comparison with untreated neutrophils from patients with COPD.

This series of experiments suggested that the PI3 kinase inhibitor was able to change the migratory dynamics of COPD neutrophils, so that they now resembled those from healthy controls.

8.4 Discussion

The studies included in this chapter once again demonstrate that neutrophils isolated from patients with COPD display altered migratory dynamics compared with those from healthy controls: namely an increase in speed of migration and a decrease in chemotactic accuracy. Previous work has also described that pseudopodia counts were lower on COPD neutrophils compared with healthy controls both when quiescent and when activated, a finding that could be consistent with the enhanced speed but reduced accuracy of migration (Weber 2006). Pseudopod generation is altered by enzymatic reactions in the signalling pathway (Zhelev, Alteraifi et al. 2004), suggesting that a defect within this pathway in COPD patients might explain the increased speed of migration.

Incubation of COPD neutrophils with a Type 1 PI3 Kinase inhibitor altered migratory dynamics, so that they resembled those from healthy controls. Migratory speed was reduced, but accuracy was increased, both in terms of migratory velocity and chemotactic index (a vector analysis of migration).

LY294002 was chosen as it has its' main actions on the pathway that drives migration, non selectively inhibiting all Class 1 sub types (El-Kholy, MacDonald et al. 2003). PI 3-kinase is central to neutrophil chemotaxis (Knall, Worthen et al. 1997; Sasaki, Irie-Sasaki et al. 2000), in particular for directed migration through cell polarisation (Sadhu, Masinovsky et al. 2003). It is activated by multiple extracellular stimuli and acts via the intracellular localisation of proteins such as AKT and thus F-actin to the leading edge of the cell (Hannigan, Zhan et al. 2002). PI3K is also believed to be an integral regulator

of pseudopod generation (Andrew and Insall 2007) and has been found to accelerate neutrophil movement towards chemoattractants (Heit, Liu et al. 2008).

Class I PI3Ks are heterodimeric enzymes consisting of a 110kDa catalytic subunit in complex with a regulatory subunit (Vanhaesebroeck, Leivers et al. 2001). Tissue distribution and the regulation of PI3K expression has recently been reviewed (Kok, Geering et al. 2009). Whereas p110 α and p110 β appear to have a broad tissue distribution, p110 γ is highly expressed in leukocytes but also found at lower levels in other cell types. Class 1 PI3 kinases have genes for 8 catalytic and 6 regulatory subunits.

IL-8 has its' inflammatory actions via G protein receptors which activate p110 γ . The gene for this subunit is *PIK3CG* found on chromosome 7q22.3. Its' regulatory subunits are p101 and p84, the genes for which are *PIK3R5* and *PIK3R6*, both placed upon chromosome 17p13.1. A promoter region for murine p110 γ has been identified (Zhao, Cheng et al. 2006). Multiple transcriptional start sites exist for p110 γ , resulting in transcripts with varying 5' untranslated regions (5'UTRs), up to 874 bp in length. Analysis of the genomic p110 γ DNA up to 1.2 kb upstream from the transcription start site revealed that the putative promoter region contains consensus sites for housekeeping transcription factors such as AP1 and SP1, as well as several putative binding sites for leukocyte-specific transcription factors. There have been no studies of gene regulation of p101 or p84.

The data included in this chapter identify a potential mechanistic action for aberrant migration: one of altered PtdIns(3,4,5)P₃ signaling by potentially increased PI3 Kinase

expression (or potentially reduced PTEN/ SHIP1 activity, preventing PtdIns(3,4,5)P₃ degradation, leading to an effectual increase in PI3K activity).

It is known that inflammatory stimuli, including TNF α and fMLP, cause an increase in p110 γ expression and activity (Cadwallader, Condliffe et al. 2002). In p110 γ -deficient mice, LPS (lipopolysaccharide)-induced lung oedema, neutrophil recruitment and pro-inflammatory cytokine production are all reduced *in vivo* when compared with wild-type mice (Yum, Arcaroli et al. 2001). p110 γ has also been associated with some inflammatory conditions such as atheroma formation (Chang, Sukhova et al. 2007) and ulcerative colitis, both of which involve neutrophil recruitment (Gonzalez-Garcia, Sanchez-Ruiz et al. 2010).

It is unclear if inflammation alone drives PI3 kinase expression, or whether PI3 kinase over-activity is a recognized phenomenon on its own. However, it is interesting that the inhibitor did not improve migratory dynamics in neutrophils from patients with A1ATD, suggesting that inflammation alone cannot account for migratory differences seen. There are no studies that report polymorphisms of p110 γ , or p101 and p84, however, potentially, genetic variations could lead to alterations in expression.

If excessive PI3 kinase activity causes aberrant neutrophil migration, potentially, the PI3 kinase inhibitor would correct it. It is known that the inhibitor can slow migration and alter pseudopod extension, (as seen with *Dictyostelium* strains (Andrew and Insall 2007)). However, PI3 kinase is thought to have more widespread effects in migratory cells. Of note, it can diminish cell polarisation (Eddy, Plerinl et al. 2000; Fais and Malorni 2003), and may alter pseudopod placement (Andrew and Insall 2007; Wessels,

Lusche et al. 2007) which would enhance the efficiency of directional locomotion, potentially focusing signalling capacity at the leading edge of the cell (Wessels, Lusche et al. 2007).

It is also very interesting that COPD neutrophils responded differentially to very low concentrations of LY294002 (nM). Previous studies of this inhibitor have used much higher concentrations (μ M) (for example Andew and Insall, 2007) however, these studies sought to determine the maximal affect of the inhibitor, whereas the studies included in this chapter aimed for modulation of migration (a partial inhibition). Furthermore, the reduction of cell adhesion to the albumn-coated coverslip prevented higher concentrations being used in this assay. This is in-keeping with previous studies suggesting that PI3K is important in regulating integrin-based adhesion (Ferguson et al, 2007). It could be that COPD neutrophils are sensitive to lower concentrations of the antagonist, however, this should be confirmed in separate studies including a range of COPD severity to determine if this is a progressive phenomenon. Furthermore, it is known that chemotaxis involves pathways that are parallel to $\text{PtdIns}(3,4,5)\text{P}_3$ signalling, and it would be important to determine (using other antagonists, such as those acting on MAP kinases) whether PI3K inhibition was both necessary and sufficient to alter cellular migration.

Certainly, the findings reported here warrant further investigation, including studies of PI3 kinase, PTEN and SHIP1 activity and expression in neutrophils from COPD patients and other related neutrophilic inflammatory conditions. It would also be of interest to repeat these studies using neutrophils from healthy controls that had been activated using inflammatory mediators (such as $\text{TNF}\alpha$) or plasma from COPD patients, to

explore if behavioural differences were due to an intrinsic flaw in COPD neutrophils or whether they could be induced by an inflammatory stimulus or environment. If these studies suggest that PI3 Kinase activity is excessive in COPD it would provide a novel target for therapeutic intervention.

9 General Discussion

Current estimations suggest that 10% of the world's population aged over 40 have COPD (Zhong, Ran et al. 2004) and by the year 2020, it is predicted that COPD will be the fifth leading cause of morbidity and mortality worldwide (World Health Organization 2007). Despite this immense burden of current and future disease, there is still no pharmacological therapy available that alters disease progression. It is clear that COPD is associated with an inflammatory process in both the small and large airways and in the lung parenchyma (Saetta, Turato et al. 2001), which is known to continue even following smoking cessation (Retamales, Elliot et al. 2001).

Although many cells and mediators have been implicated in the pathogenesis of COPD, it is clear that the neutrophil is central to its development and progression (Sapey and Stockley 2008). There is an increased presence of neutrophils in the lungs of patients with COPD (Stanescu, Sanna et al. 1996), and the degree of neutrophilia correlates with airflow obstruction (Sparrow, Glynn et al. 1984) and emphysema (Parr, White et al. 2006). Furthermore, neutrophils have been shown to cause all of the pathological features of COPD, including emphysema (Janoff, Sloan et al. 1977), mucous gland hyperplasia (Lucey, Stone et al. 1985) and mucus hypersecretion (Shimura and Takishima 1994). These features suggest that neutrophil products and neutrophil chemoattractants are potential targets for novel therapeutic strategies. However, many mediators are raised in COPD, and there is likely to be an element of redundancy (Barnes, Chowdhury et al. 2006). It is important to assess which mediators are more closely associated with disease (which could qualify as biomarkers), and to understand the variability in day to day expression, in order to target the most promising candidates and to power studies adequately.

Neutrophils migration is of fundamental importance in the inflammatory response, and the leukocyte's ability to sense and move towards a chemoattractant gradient is finely controlled. The detection of a chemotactic stimulus leads to the activation of signalling pathways, cytoskeletal rearrangement and changes in cell surface molecules that facilitate neutrophil migration. In the bronchial circulation, it is believed that neutrophils are recruited by the "rolling-tethering" paradigm (explained extensively in the introduction and in (Wagner and Roth 2000)). In the pulmonary circulation, where the majority of neutrophil migration occurs at the capillary level, cell rheology and not surface receptors may be of greater importance (Yoshida, Kondo et al. 2006). In a recent study, 70% of neutrophil transmigration was achieved by direct engulfment of neutrophils by endothelial cells, by a process dependent on RhoG (van Buul, Allingham et al. 2007). Migration through the basement membrane is less well understood, but may require proteinase release (Cepinskas, Sandig et al. 1999) with cells targeting areas of reduced matrix protein density (Wang, Voisin et al. 2006).

Directional neutrophil recruitment appears to be reliant on the temporal and spatial occupancy of chemoattractant mediator receptors expressed preferentially in pseudopods at the front of a polarised cell (Weber 2006). G-protein receptors act via PI3 Kinase (p110 γ), which is known to modulate both cell polarity and pseudopod extension, facilitating neutrophil migration (Andrew and Insall 2007).

Studies of neutrophil migration in COPD have shown variable results. An initial study by Burnett et al demonstrated increased neutrophil migration and enhanced extracellular proteolysis compared to healthy controls (Burnett, Chamba et al. 1987). In keeping with this, increased expression of adhesion proteins including CD11b and L-selectin have

been found in some studies of patients with COPD (Noguera, Batle et al. 2001; Woolhouse, Bayley et al. 2005). However, Yoshikawa et al (Yoshikawa, Dent et al. 2007) found higher numbers of neutrophils in induced sputum from patients with COPD compared with healthy controls but described a reduced neutrophilic chemotactic response to interleukin 8 and fMLP in the COPD patients which worsened with increasing disease severity. Furthermore, Gonzalez (Gonzalez, Hards et al. 1996) found no differences between levels of adhesion molecules in smokers with and without airflow obstruction.

Increased or aberrant neutrophil migration could be of fundamental importance in the pathogenesis of COPD. Neutrophils express membrane bound proteinases during migration, a proportion of which is released as free elastase (Cepinskas, Sandig et al. 1999). This causes an area of obligate tissue damage due to quantum proteolysis (Liou and Campbell 1996). If COPD is associated with increased numbers of migrating neutrophils due to enhanced inflammatory signal (Barnes, Chowdhury et al. 2006), or aberrant migration due to faulty cell signalling, (or both), the potential for tissue damage maybe amplified.

However, studies published to date have been unable to dissect differences in neutrophil migration in COPD because of many important factors. Firstly, COPD is a heterogenous disease, and a general lack of in-depth characterization of the small number of the patients included in each study will increase the variability of the data. Secondly, the methodologies utilised (such as under agarose studies and the Boyden chamber) have been unable to describe individual cell behaviour, and instead comment on an overall pattern of cell behaviour, quoting the numbers of cells able to migrate

(Boyden Chamber) or the distance of overall migration (Under Agarose). Newer modalities exist (such as the zebra fish model (Renshaw, Loynes et al. 2006)) provide exciting opportunities to monitor *in vivo* neutrophil migration. However, these cannot be applicable to COPD until any molecular mechanism of aberrant migration is known and transferable to the appropriate model. The same is true of murine models of COPD. These can provide insight into the physiological importance of over or under expression of a mediator or a receptor, however their use remains limited until a defining mechanism of COPD is identified. Therefore, in the initial phases of understanding neutrophil migration in COPD, a simple assay, where individual cell migratory dynamics can be filmed and analysed, is essential. Finally, although there is some evidence that ageing affects neutrophil migration (Fulop, Larbi et al. 2004), studies of migration in COPD to date have not always applied rigorous age matching with control groups. The impact of healthy ageing on neutrophil function is poorly understood, but any differences in migration between age groups needs to be established to determine the validity of previous studies in COPD. The studies included in this thesis were specifically designed to address some of these issues.

9.1 The quantification and variability of inflammatory mediators and cells in COPD

In order to characterise inflammation in COPD, there must be an understanding of the mediators that are raised in the condition, the variability of their expression and the relationships that exist between the mediators and parameters of disease. While there is interest in the systemic components of COPD, it is primarily understood to be a disease of the lung, and so sampling of the respiratory system remains critical. In the experiments described in this thesis, the variability of mediator and cell concentrations were studied by collecting daily spontaneous sputum samples for five sequential days,

and then twice weekly for three weeks in patients with stable COPD and a chronic bronchitis phenotype.

There are many techniques that have been employed in sampling lung secretions, including bronchial biopsies, bronchoalveolar lavage, spontaneous and induced sputum and exhaled gas analysis (Barnes, Chowdhury et al. 2006). Bronchial biopsies have been used to document the structural changes, cellular patterns and mediator profiles seen in the tissues in COPD (O'Shaughnessy, Ansari et al. 1997) (Saetta, Turato et al. 2001). This technique has the advantage of directly sampling airway tissue providing spatially intact components of the airway wall. However, the procedure is invasive, can only sample proximal airways and is not well tolerated by those with severe disease or those with significant cardiac co-morbidity (Hattotuwa, Gizycki et al. 2002) and biopsy only documents those cells retained in the tissues and not migratory cells. Variability in sampling is high. A recent study demonstrated that 132 patients would be required in order to have an 80% chance of detecting a 50% reduction in cells which were mRNA positive for IL-8, using a parallel study design. (Gamble, Qiu et al. 2006). Therefore obtaining meaningful results requires large numbers and repeated sampling.

Bronchoalveolar lavage (BAL) has the advantage of sampling the distal airways and the resultant cell counts have been used in interventional studies of corticosteroids (Verhoeven, Hegmans et al. 2002) (Balbi, Majori et al. 2000). However, BAL is also an invasive procedure, and causes transient fever in some patients (Hattotuwa, Gamble et al. 2002). Furthermore, quantification of biomarkers is complicated by dilution from the saline lavage which is operator dependent and variable (Barnes, Chowdhury et al. 2006). In addition, the variability of biomarkers using this sampling technique is currently unknown, as this

requires repeated sampling, and the lavage itself induces an inflammatory response, which may impact on biomarker concentrations (Hattotuwa, Gamble et al. 2002).

Sputum induction using hypertonic saline provides samples which are essentially derived from the more proximal airways (Holz, Richter et al. 1998). There have been many studies reporting on the cellular and inflammatory mediator concentrations in COPD using induced sputum, including a number of interventional studies assessing the affect of inhaled corticosteroids (for example (Keatings, Jatakanon et al. 1997)). To date, only one study has provided data regarding reproducibility (Beeh, Kornmann et al. 2003). However, sputum induction is poorly tolerated by patients (especially those with an FEV1 < 30% predicted), causes transient airflow obstruction in some patients (Ryttila, Lindqvist et al. 2000) (Taube, Holz et al. 2001) and induces a neutrophilic inflammation for at least twenty four hours, making repeat sampling within this time impractical (Holz, Richter et al. 1998) (Nightingale, Rogers et al. 1998). This technique will not necessarily provide information of the processes underway in the broncho-alveolar region.

Exhaled breath condensate (EBC) is a non-invasive technique (and hence can be repeated). The main problem with this technique remains that the concentrations of mediators in samples are low, (often at levels close to the lower level of detection and usually below the lower limit of quantification for assays that are currently available). This makes accurate measurements of mediators extremely difficult and makes comparisons between groups almost impossible (Bayley, Abusriwil et al. 2008; Sapey, Bayley D et al. 2008). There is also a relatively high variability in repeated measurements of EBC inflammatory indices (Borrill, Starkey et al. 2005), which may be dependent (at least in part) upon the difficulty in quantification of biomarkers or the

dilution caused by exhaled water vapour, and these problems need to be overcome before this technique can be routinely used in research and clinical trials.

Spontaneous sputum samples have been extensively used in the study of COPD (Hill, Bayley et al. 2000; Hill, Campbell et al. 2000). Unlike other techniques, this is non-invasive, is acceptable to most patients who expectorate, and has no inflammatory sequelae to limit sequential sampling. It is diluted by oropharyngeal secretions, but mouth washing procedures reduce this and squamous cell quantification provides some measure of this. The largest limitation of spontaneous sputum is that it only allows study of patients with chronic bronchitis, and is more frequent in moderate to severe disease. However, this group of patients have increased levels of inflammation in induced samples whereas inflammation is not raised in those who do not (Gompertz, Hill et al. 2006) and expectoration is a predictor of disease progression and a tendency to recurrent exacerbation. This suggests that chronic expectoration should be documented to clarify the interpretation of all studies (Gompertz, Hill et al. 2006). Finally, studies have shown that the inflammatory profile in sputum is similar in bronchial and bronchoalveolar lavage in the same patient indicating that it reflects processes throughout the bronchial tree (Wiggins, Hill et al. 1983)

The studies presented in this thesis demonstrate the wide variability in mediators and cells in spontaneous sputum both between patients and within each patient, in the stable clinical state. This finding could explain the wide range of cell counts and mediator concentrations described in individual studies of COPD with similar patient cohorts (Rutgers, Timens et al. 2000; Gompertz and Stockley 2002; Woolhouse, Bayley et al. 2002), by suggesting that the “normal range” of inflammatory load in the lungs of patients, even with very similar disease burdens, may be vast. The variability of raw data did not relate to any fluctuations in symptoms scores, clinical examination findings or lung function, and, therefore, did not

impact upon any clinical parameter that would be studied in clinical trials. Changes most likely relate to contamination with oropharyngeal secretions or sputum humidification, although it remains possible this reflects real fluctuations in inflammatory load. However, patients were followed for a month, with no clinical deterioration suggesting that the later possibility is less likely. It may be that inflammatory changes need to be more sustained or of a greater magnitude before there is a change in clinical status (Pizzichini, Pizzichini et al. 1999). The studies confirm that sequential sampling and averaging three days data significantly reduces the impact of the fluctuations of mediators and cells, providing more individual data stability and thereby reducing the numbers needed to power putative studies where a primary endpoint is a reduction in an inflammatory protein or cell (in the case of IL-8, 61 patients would be required in a cross-over study aiming to detect a 50% change in IL-8 expression in single sputum samples, whereas only 7 would be required if the mean of 3 days data were used). Sequential sampling also improved the strength of correlations between inflammatory mediators, cells and clinical parameters.

Three neutrophil chemoattractants were studied, IL-8, LTB₄ and GRO α . Consistently, IL-8 correlated with other mediators, inflammatory cells and some markers of clinical severity (for example, airflow obstruction). These findings complement those of other researchers (Woolhouse, Bayley et al. 2002; Hacievliyagil, Gunen et al. 2006; Parr, White et al. 2006) making it a clear candidate for future studies of neutrophil recruitment and neutrophil mediated pathology.

9.2 Neutrophil migration in the healthy elderly

The vast majority of studies in COPD include patients with a mean age of greater than 60 (e.g., (Hill, Bayley et al. 1999; Sapey, Bayley et al. 2008; Borrill, Roy et al. 2009). In the majority of cases, great pains are taken to adequately match control subjects for

many parameters, including age which is an important consideration in any study of COPD. Age is associated with changes in the structure and the function of the lung, which give rise to spirometric changes consistent with airflow obstruction (Turner, Mead et al. 1968; Knudson, Clark et al. 1977). Age appears to make the lung more susceptible to inflammatory insults, with a reduction in cell proliferation following injury (Calabrese, Giacometti et al. 2005; Aoshiba. K. and Nagai 2009) causing alveolar enlargement which shares some, but not all of the features of emphysema (Verbeken, Cauberghs et al. 1992; Tsuji, Aoshiba et al. 2006). Age affects nearly all of the immune system functions that have been studied to date (Panda, Arjona et al. 2009). With increasing age, there is an increase in cytokine expression in plasma (Mariotti, Bernardini et al. 2006), and alterations in both the initiation and sustainment of neutrophilic inflammation (Fulop, Larbi et al. 2004). Finally, it is well recognised that ageing alters drug metabolism, therapeutic responses to drugs and the susceptibility to adverse effects, including drugs used in the treatment of COPD (Hall, Reed et al. 1974; Ray, Griffin et al. 1990; Feinberg 1993; Garbe, Suissa et al. 1998).

Despite these important considerations, some studies of neutrophil migration in COPD have not provided closely age matched controls (for example (Woolhouse, Bayley et al. 2002; Strassburg, Droemann et al. 2004; Yoshikawa, Dent et al. 2007)). Often the differences in the mean age of the cohorts studied have been no more than 10 or 15 years, but it is unclear how much impact this might have on neutrophil migration. Furthermore, there is an emerging theory of COPD as a form of advanced pulmonary ageing (Buist, McBurnie et al. 2007; Fukuchi 2009), despite there being differences in the pathological features of the so called “senile lung” and COPD (see above). Given these factors, it was felt important to evaluate the affect of ageing on neutrophil

migration using both a traditional assay from which comparisons could be made to previous work, and an assay that allowed direct visualisation of single cells and the measurement of individual migratory parameters. These studies were conducted firstly to determine the affect of ageing on neutrophil migration towards physiologically relevant chemoattractants in healthy subjects. Secondly, to determine if there were similarities between the patterns of ageing neutrophil migration and COPD neutrophil migration, in order to assess whether neutrophil behaviour in COPD could represent an advanced ageing phenotype.

The under agarose assays determined that there were significant differences in the migratory patterns of neutrophils from older subjects, in that there was a reduction in chemotaxis (directed migration towards the chemoattractant) with preserved chemokinesis (random movement away from the chemoattractant). The Modified Dunn Chamber was able to characterise this further, demonstrating that the speed of cellular movement was preserved with ageing, but there was a reduction in velocity (chemotaxis). This coupled with an increase in the number of directional changes each cell made during migration (a reduced persistence), resulted in an overall reduction in chemotactic accuracy. This was consistently found using both IL-8 and GRO α as chemoattractants. The net result of the more circuitous migratory pathways of neutrophils from ageing subjects is of potential importance. Firstly, there might be an important delay in reaching the site of infection, as previous studies have suggested that reduced chemotaxis may be associated with worsening outcomes in bacterial infections (Niwa, Kasama et al. 1989; Egger, Aigner et al. 2004). Secondly, these more circuitous migratory pathways may lead to an increase in by-stander tissue damage because of the expression and release of proteinases on the surface of neutrophils during migration

(Liou and Campbell 1996; Cepinskas, Sandig et al. 1999). It is therefore also possible likely that neutrophil activation and migration in the elderly begets further inflammation by the release of further chemoattractants such as IL-8 and LTB₄ (Hubbard, Fells et al. 1991). This could establish a low grade, self perpetuating cycle of damaging inflammation which may (in part) account for the age dependent development of mild emphysematous-like changes referred to as “senile lung”.

IL-8 signals via CXCR1 and CXCR2, while GRO α signals preferentially via CXCR2. Interestingly, a CXCR2 small molecule antagonist changed migratory dynamics of neutrophils from young controls, giving them an ageing phenotype. This suggested that reduced CXCR2 expression or function could account for the changes seen in migration of cells from the elderly. However, there were no differences in surface expression of CXCR1 or CXCR2 on quiescent cells indicating that the defect was more likely to be downstream from the CXCR2 receptor. However, given the complex nature of receptor endocytosis and recycling, it is overly simplistic to state that there are not changes in CXCR1 or 2 function with advancing age. The studies do not, at this stage, enable the identification of a potential mechanism for the migratory changes seen. However, the migratory phenotype of neutrophils from ageing subjects has now been described and can be used in studies assessing migrating neutrophils from subjects with COPD. The experiments contained within this thesis suggest that previous studies of neutrophil function in COPD where controls are not age matched should therefore be interpreted with a degree of caution.

9.3 Neutrophil migration in COPD

There is no doubt that there are increased numbers of neutrophils in the lungs of patients with COPD (Hill, Bayley et al. 1999; Sapey and Stockley 2008). Their presence appears

to correlate with an increased pro-inflammatory signal from the lungs (Crooks and Stockley 1998; Hill, Bayley et al. 1999), without clear evidence of delayed apoptosis (Ryttila, Plataki et al. 2006; Uller, Persson et al. 2006). There has also been some suggestion that neutrophil function in COPD, including migration, may be altered (Burnett, Chamba et al. 1987; Strassburg, Droemann et al. 2004; Yoshikawa, Dent et al. 2007). It is known that an inflammatory signal remains in the lungs of patients with COPD, even after the cessation of smoking (Retamales, Elliot et al. 2001) and this may represent the presence of genetic predispositions towards disease or continuing environmental triggers. Aberrant neutrophil migration could potentially lead to a continuation of inflammation even after the initial stimulus for disease is gone, by perpetuating an inflammatory response to neutrophil proteinases released during inaccurate migration (as outlined in the previous section).

Neutrophil migration could be affected by a number of parameters, including smoking status, the presence of lung disease, lung inflammation and the therapies used to treat inflammation and airflow obstruction. In order to account for these factors, a second control group was included in this section of work, namely patients with A1ATD. These patients were selected as they have a similar pattern of lung disease, but a known genetic predisposition with a recognised pathophysiological process, and hence can be chosen to match with age, gender, lung function, and treatments.

Neutrophil migration was assessed firstly by the under agarose assay. Here neutrophils from patients with COPD demonstrated a preserved chemotactic differential, with both enhanced chemotaxis (movement towards the chemoattractant) and enhanced chemokinesis (movement away from the chemoattractant) resulting in the neutrophils

spreading further. This already demonstrated that the migratory dynamics of neutrophils from patients with COPD was different from the patterns noted in healthy ageing. The same spreading pattern was not seen in patients with A1ATD, where migrational patterns resembled those from healthy controls. Furthermore, detailed studies confirmed that there were no differences in the migratory pathways of neutrophils from healthy smokers and never smokers, suggesting that active smoking status does not affect this facet of neutrophil function. These comparative studies confirmed that the neutrophil dynamics were a feature of COPD and not recognised potential confounding factors.

Throughout the studies included in this thesis, COPD neutrophils migrated with increased speed (chemokinesis), but with reduced velocity (chemotaxis) and a reduced chemotactic index (overall accuracy) towards IL-8 and GRO α (and in later chapters, towards sputum). This was not seen when the studies were replicated in A1ATD, where migratory patterns again resembled those from healthy controls. Based on the work of Liou and Campbell (Liou and Campbell 1996; Campbell, Campbell et al. 1999), measurements of the dimensions of migrating neutrophils and maps of their migratory pathways, it is possible to estimate the potential volume of tissue damage that could occur during migration. Using a simple mathematical model, it was estimated that neutrophils from patients with COPD have the potential to cause 30% extra tissue damage, purely because of the theoretical release of proteinase during inaccurate migration. Neutrophils from patients with A1ATD also had an increased potential for tissue damage (90% more than healthy controls), but this is due to low prevailing concentrations of circulating A1AT rather than aberrant migration. Interestingly, these differences are comparable to the relative increase in decline of FEV₁ seen in COPD and A1ATD (Burge, Calverley et al. 2000; Simmons, Connett et al. 2005; Dawkins,

Dawkins et al. 2009), suggesting a common paradigm of neutrophil mediated damage in both groups.

9.4 Pseudopod formation in COPD

Differences in migratory behaviour in neutrophils isolated from patients with COPD were accompanied by structural differences in migration, with a reduction in pseudopod formation following neutrophil activation with IL-8. This was not seen in matched patients with A1ATD, and there were no differences in pseudopod formation between healthy smokers and never smokers. This is the first evaluation of pseudopod formation in a disease state, but as formation was not altered by smoking or the presence of pulmonary inflammation (as seen in the healthy smokers and A1ATD groups) or therapy (as A1ATD patients were matched for this), it appears to be a feature of usual COPD. Further studies of other chronic inflammatory disease states would be of great interest to determine whether this observation is unique to COPD.

Studies contained within the current body of work cannot prove that a decrease in pseudopod formation is causally associated with an increase in migratory speed or a reduced migratory accuracy. However, this association fits well with current understanding of the function of pseudopodia (Wang, Herzmark et al. 2002; Weber 2006; Wessels, Lusche et al. 2007). In particular that the rate of pseudopod formation is central to both the speed and accuracy of migration (Zhelev, Alteraifi et al. 2004; Weber 2006) and pseudopod generation is altered in the presence of chemoattractants and by components of the migratory pathway (Zhelev, Alteraifi et al. 2004; Andrew and Insall 2007).

It may be that pseudopod formation is altered by differential activation of signalling pathways in COPD or that altered pseudopod formation is the primary event, due to changes in microtubule formation or actin and myosin function and that this leads to changes in signalling. Certainly, it is less likely that alterations in pseudopod formation are a product of exposure to inflammation, as patients with A1ATD are known to have in general a higher burden of inflammation (Woolhouse, Bayley et al. 2002), and there were no differences in pseudopod formation between the A1ATD patients and healthy controls.

9.5 CXCR1 and CXCR2 function in COPD

Studying cell signalling pathways suggested further differences in circulating neutrophils isolated from patients with COPD. There was a significant reduction in surface expression of CXCR1 and CXCR2 when receptor levels were semi quantified in COPD neutrophils. This is not a universal phenomenon in inflammatory disease, as most reports in chronic inflammation suggest increased expression, especially of CXCR2 (Banks, Bateman et al. 2003; Qiao, Sonoda et al. 2005). Studies of CXCR1 and CXCR2 expression in COPD have been inconsistent, although most have found either a decrease or a trend towards a decrease in CXCR2 expression (Traves, Smith et al. 2004; Pignatti, Moscato et al. 2005).

Interestingly, the reduction in pseudopod formation seen in activated neutrophils (with IL-8 10nM) in COPD compared with healthy controls was approximately 45%. The reduction in surface expression of CXCR1 or CXCR2 in activated cells (again, using 10nM IL-8) in COPD compared with healthy controls was approximately 56% and 45% respectively. These reductions are comparable and it is interesting to speculate that a reduction in pseudopod formation may limit chemoattractant receptor surface expression

due to constraints of space. Work in this series of experiments does not allow for a comment on the whole cellular content of CXCR1 or CXCR2, nor potential differences in receptor recycling or endocytosis, but these mechanisms warrant further study.

Studies using antagonists and antibodies to CXCR1 and CXCR2 demonstrated that both have a role in neutrophil migration. Inhibiting both CXCR1 and CXCR2 reduced chemokinesis to levels comparable to controls, whereas CXCR2 inhibition had the most profound reduction on chemotaxis, directional persistence and overall accuracy. There have been few comparative studies of the roles of CXCR1 and CXCR2 receptors in neutrophils (Hartl, Latzin et al. 2007), and there remain concerns about the design of some of the studies which limit interpretation (for example, (Godaly, Hang et al. 2000)). However, it seems clear that CXCR2 is important in neutrophil recruitment (Del Rio, Bennouna et al. 2001; Farooq, Stillie et al. 2009).

CXCR1 or CXCR2 blocking strategies did not have large differential effects on neutrophil migration between COPD and healthy controls, and neutrophils from healthy subjects did not display a COPD phenotype when CXCR1 or CXCR2 function was down-regulated. Furthermore, COPD neutrophils retained their phenotypic migratory characteristics towards sputum, even when IL-8, GRO α or CXCR1 and CXCR2 were inhibited. Together, these data suggest that expression or function of these receptors is not the underlying cause of migratory differences in COPD and instead suggest that these migrational patterns may be part of a generalised phenomenon to many chemoattractants. To clarify this possibility, similar migrational studies would need to be carried out using other chemoattractants, acting via other surface receptors. One such study reported that fMLP receptor expression (measured indirectly in subjects who were

not age matched) was dependent upon smoking status rather than COPD (Matheson, Rynell et al. 2003), while another found an increase in LTB₄ receptors in tissue from patients with COPD, but did not measure receptors on neutrophils (Marian, Baraldo et al. 2006).

Presently, it is unclear whether neutrophil migration in COPD is unusually fast but inaccurate in the presence of a wider range of stimuli. However, given that receptor expression and function (as studied in this body of work) did not account for changes in migratory pathways, a more generic component of the migratory pathway was studied.

9.6 PI3 Kinase activity in COPD

PI3 kinase is central to many cellular functions, including neutrophil migration (Knall, Worthen et al. 1997; Sasaki, Irie-Sasaki et al. 2000), and especially directed migration through cell polarisation (Sadhu, Masinovsky et al. 2003). Incubation of COPD neutrophils with a Type 1 PI3 Kinase inhibitor altered migratory dynamics, so that they resembled those from healthy controls. Migratory speed was reduced, but accuracy was increased, both in terms of migratory velocity and chemotactic index (a vector analysis of migration).

There are no comparable studies in the literature to facilitate interpretation of these findings. However, studies of PI3 kinase function suggest that it is an integral regulator of the rate of pseudopod generation (Andrew and Insall 2007) and accelerates neutrophil movement towards chemoattractants (Heit, Liu et al. 2008). IL-8 and GRO α are ligands G protein receptors that activate p100 γ . It is known that inflammatory stimuli cause an

increase in p110 γ expression and activity (Cadwallader, Condcliffe et al. 2002) and that some chronic inflammatory conditions have been associated with increased expression of this subunit (Chang, Sukhova et al. 2007; Gonzalez-Garcia, Sanchez-Ruiz et al. 2010).

The studies contained within the current thesis suggest that inflammation alone does not drive the differences in responses to a PI3 kinase inhibitor, as cells from patients with A1ATD resembled those from healthy controls, rather than patients with COPD. This suggests that there is something unique or unusual about the behaviour of COPD neutrophils. There are no studies that report polymorphisms of p110 γ , or p101 and p84, however, potentially, genetic variations could lead to alterations in expression or activity, as with other genetic polymorphisms in COPD (Sapey, Wood et al. 2010).

If excessive PI3 kinase activity causes aberrant neutrophil migration, it is expected that the PI3 kinase inhibitor would correct it. The inhibitor is known to slow migration and alter pseudopod extension (Andrew and Insall 2007), diminish cell polarisation (Eddy, Plerinl et al. 2000; Fais and Malorni 2003), and may alter pseudopod placement (Andrew and Insall 2007; Wessels, Lusche et al. 2007). All of these factors would theoretically improve migratory dynamics and explain the results of the current experiments.

Although the studies contained in this thesis have not identified an exact mechanism for aberrant neutrophil migration in COPD, a pathway has been identified, with a means to alter migration so that it resembles that seen in health. This is potentially an important starting point in understanding neutrophil function and the role of the neutrophil in the

development and progression of COPD, and may, in time, offer a new therapeutic paradigm in this important disease.

9.7 Future work

This thesis has identified many features of the inflammation and neutrophil function in COPD. It provides a series of important pathophysiologically relevant results that provide potential mechanisms to explain disease susceptibility. However many further studies are needed to clarify the exact mechanisms involved and their specificity both in the field of ageing and immunosenescence and in COPD.

The work on migrational studies in neutrophils from ageing subjects was instigated to help interpret studies of migration in COPD. However, it is also important to characterise the affects and mechanisms of neutrophil migration in the elderly further.

1. Initially, migrational studies should be repeated using physiologically important chemoattractants such as LTB₄, fMLP and C5a, to determine if the less accurate migration is a general feature to all stimuli, acting via different receptors. The interpretation should also include an assessment of pseudopod formation and placement to determine whether there are structural differences in cell migration with advancing age.
2. Receptor function and expression should be characterised more fully, including studies of receptor placement in the lipid raft, surface Vs cellular content of receptors and studies of receptor recycling. This data would identify if receptor placement, endocytosis and /or recycling was involved in migrational defects.

3. All of the studied receptors act via calcium signalling, and this can be quantified and compared between groups as a study of the initial components of cell signalling.
4. The migratory pathway has many generic components, and it is possible to study each one in a step-wise, progressive manner, including PI3 kinase expression and activity, Phospho-ERK expression and activity and the role of PTEN. This would determine if there was a single step responsible for the observations found.
5. Further studies could also include the activation of small GTPases, such as Rho and Rac, and interactions between actin and myosin, as this is the final step in migration.
6. *In vitro* and *in vivo* studies are needed to assess the clinical importance of aberrant migration in the elderly, including studies of the migration in gel matrices and the inflammatory sequelae of an inflammatory challenge to the lungs (such as inhaled LPS).
7. In addition real time observations of bacterial phagocytosis by neutrophils from the elderly will determine whether these differences impact upon the final step of bacterial clearance and hence relate to the increased susceptibility to infection.
8. It would also be of importance to repeat key studies following incubation of neutrophils from young or older subjects with serum or plasma from young or old donors. This would help to determine if differences were caused by the neutrophils alone or by the pro-inflammatory environment of the cells in older subjects.

The same series of experiments should be pursued in COPD, but here an additional control group is needed to account for the presence of inflammation. A1ATD patients have proven to be suitable for this, as they have a similar lung insult as usual COPD, a similar pattern of inflammation (although inflammatory markers are often higher in pulmonary secretions from A1ATD (Woolhouse, Bayley et al. 2002)) and can be

matched for smoking status, disease phenotype (such as chronic bronchitis) and therapies.

In conclusion, the studies contained within this body of work have demonstrated unique differences in migrating neutrophils isolated from healthy ageing subjects and patients with COPD. In healthy ageing, cells become less accurate during migration, but maintain their overall speed. In COPD, inaccuracy is coupled with enhanced speed of migration. Theoretically, both migratory flaws would lead to worsening clinical outcomes. Firstly, inaccurate migration may delay the cell's response to infection (by potentially delaying bacterial clearance). Secondly, inaccurate migration may lead to an increase in by-stander tissue damage mediated by the release of free proteinases, as neutrophils migrated along more meandering pathways. Both may explain the poorer response to infections and the decline in end organ function seen in these groups. The studies provide some insight into potential mechanism, but no definitive mechanism is identified. There is a clear need for further study, both in the healthy ageing and in COPD.

10 References

- Aaron, S. D., J. B. Angel, et al. (2001). "Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease." Am J Respir Crit Care Med **163**: 349 - 355.
- Adkison, A. M., S. Z. Raptis, et al. (2002). "Dipeptidyl peptidase I activates neutrophil-derived serine proteinases and regulates the development of acute experimental arthritis." J Clin Invest **109**: 363 - 371.
- Alcorn, M. J., J. L. Booth, et al. (2001). "Adenovirus type 7 induces interleukin-8 production via activation of extracellular regulated kinase." J Virology **75**: 6450 - 6459.
- Allport, J. R., H. T. Ding, et al. (1997). "L Selectin shedding does not regulate human neutrophil attachment, rolling or transmigration across human vascular endothelium in vitro." J Immunol **158**: 4365 - 4372.
- Alon, R., S. Chen, et al. (1997). "The kinetics of L-selectin tethers and the mechanisms of selectin mediated rolling." J Cell Biol **138**: 1169 - 1180.
- Alvarez, E. (2001). "Age related changes in membrane lipid composition, fluidity and respiratory burst in rat peritoneal neutrophils." Clin Exp Immunol **124**: 95 - 102.
- Amitani, R., R. Wilson, et al. (1991). "Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium." Am J Respir Cell Mol Biol **4**(1): 26 - 32.
- Andrew, N. and R. Insall (2007). "Chemotaxis in shallow gradients is mediated independently of PtdIns-3-kinase by biased choices between random protrusions." Nat Cell Biol **9**(2): 193 - 200.
- Andrew, N. and R. H. Insall (2007). "Chemotaxis in shallow gradients is mediated independently of PtdIns 3-kinase by biased choices between random protrusions." Nature Cell Biol **9**: 193 - 200.
- Anthonisen, N. R., J. Manfreda, et al. (1987). "Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease." Ann Intern Med **106**: 196 - 204.
- Antonicelli, F., M. Parmentier, et al. (2002). "Nacystelyn inhibits oxidant-mediated interleukin-8 expression and NF-kappaB nuclear binding in alveolar epithelial cells." Free Radic Biol Med **32**: 492 - 502.
- Aoshiba, K., K. Yasuda, et al. (2001). "Serine proteinases increase oxidative stress in lung cells." Am J Physiol Lung Cell Mol Physiol **281**: L556 - L564.
- Aoshiba, K. and A. Nagai (2009). "Senescence Hypothesis for the Pathogenetic Mechanism of Chronic Obstructive Pulmonary Disease." Proc Am Thorac Soc **6**: 596 - 601.
- Asada, M., M. Yamaya, et al. (2005). "Interleukin-1beta gene polymorphisms associated with COPD." Chest **128**: 1072 - 1073.
- Ashcroft, G. S., K. Lei, et al. (2000). "Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing." Nat Med **6**: 1147 - 1153.
- Atkinson, J. J. and R. M. Senior (2003). "Matrix metalloproteinase-9 in lung remodeling." Am J Respir Cell Mol Biol **28**(1): 12-24.
- Attal, H., E. Cohen-Hillel, et al. (2007). "Intracellular cross-talk between the GPCR CXCR1 and CXCR2: Role of carboxyl terminus phosphorylation sites." Experimental Cell Research **314**: 352 - 365.
- Azzawi, M., B. Bradley, et al. (1990). "Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. ." Am Rev Respir Dis **142**: 1407 - 1413.

- Baker, A. H., D. R. Edwards, et al. (2002). "Metalloproteinase inhibitors; biological actions and therapeutic opportunities." J Cell Sci **115**: 3719 - 3727.
- Balbi, B., C. Bason, et al. (1997). "Increased bronchoalveolar granulocytes and granulocyte/macrophage colony-stimulating factor during exacerbations of chronic bronchitis." Eur Respir J **10**: 846 - 850.
- Balbi, B., M. Majori, et al. (2000). "Inhaled corticosteroids in stable COPD patients: do they have effects on cells and molecular mediators of airway inflammation?" Chest **117**(6): 1633-7.
- Ballieux, B. E., P. S. Hiemstra, et al. (1994). "Detachment and cytolysis of human endothelial cells by proteinase 3." Eur J Immunol **24**: 3211 - 3215.
- Banks, C., A. Bateman, et al. (2003). "Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in ulcerative colitis and Crohns disease." J Pathol **199**: 28 - 35.
- Barlic, J., M. H. Khandaker, et al. (1999). "beta-arrestins regulate interleukin 8 induced CXCR1 internalization." J Biol Chem **274**: 16287 - 16294.
- Barnes, P. J., B. Chowdhury, et al. (2006). "Pulmonary biomarkers in chronic obstructive pulmonary disease." Am J Respir Crit Care Med **174**(1): 6-14.
- Bayley, D., H. Abusriwil, et al. (2008). "Validation of assays for inflammatory mediators in exhaled breath condensate." Eur Respir J **31**(5): 943 - 948.
- Beeh, K. M., O. Kornmann, et al. (2003). "Neutrophil chemotactic activity of sputum from patients with COPD: role of interleukin 8 and leukotriene B4. ." Chest **123**: 1240 - 1247.
- Beith, J. G. (1986). "Elastases: catalytic and biological properties. In Mecham, R.P., editor." Biology of extracellular matrix; regulation of matrix accumulation **Orlando FL, Academic**: 217 - 320.
- Ben-Barach, A., M. Grimm, et al. (1997). "The different ability of IL-8 and neutrophil-activating peptide-2 to induce attenuation of chemotaxis is mediated by their divergent capabilities to phosphorylate CXCR2 (IL-8 receptor B)." J Immunol **158**: 5927 - 5933.
- Betsuyaku, T., M. Nishimura, et al. (1999). "Neutrophil granule proteins in the bronchoalveolar lavage fluid from subjects with sub clinical emphysema." Am J Respir Crit Care Med **159**(6): 1985 - 1991.
- Betsuyaku, T., J. M. Shipley, et al. (1999). "Neutrophil emigration in the lungs, peritoneum and skin does not require gelatinase B." Am J Respir Cell Mol Biol **20**: 1303 - 1309.
- Bhowmik, A., T. A. R. Seemungal, et al. (2000). "Relation of sputum inflammatory markers to symptoms and lung function changes in COPD." Thorax **55**: 114 - 120.
- Bhowmik, A., T. A. R. Seemungal, et al. (1998). "Comparison of spontaneous and induced sputum for investigation of airway inflammation in chronic obstructive pulmonary disease." Thorax **53**: 953 - 956.
- Biernacki, W. A., S. Kharitonov, et al. (2003). "Increased Leukotriene B4 and 8-isoprotane in exhaled breathe condensate of pateints with exacerbations of COPD." Thorax **58**: 294 - 298.
- Blackwood, C. E., Y. Hosannah, et al. (1973). "Elastolytic titre of inducing enzyme as determinant of the response." Proc Soc Exp Biol Med **144**: 450 - 454.
- Bonomo, R. A. (2002). "Resistant pathogens in respiratory tract infections in older people." J Am Geriatr Soc **50**: S236 - S241.

- Borgeat, P. E., E. Krump, et al. (2003). "The synthesis of leukotrienes by the human neutrophil. In Holgate, S., editor. SRS-A to leukotrienes; the dawning of a new treatment " Blackwell Oxford 69 - 84.
- Borregaard, N. and J. B. Cowland (1997). "Granules of the human neutrophilic polymorphonuclear leukocyte." Blood **89**: 3503 - 3521.
- Borregaard, N. and J. B. Cowland (1997). "Granules of the human neutrophilic polymorphonuclear leukocyte." Blood **89**: 3503 - 3521.
- Borrill, Z., K. Roy, et al. (2009). "Seretide withdrawal increases airway inflammation in moderate COPD patients." European Journal of Clinical Pharmacology **65**: 1165 - 1166.
- Borrill, Z., C. Starkey, et al. (2005). "Reproducibility of exhaled breath condensate pH in chronic obstructive pulmonary disease. ." Eur Respir J **25**: 269 - 274.
- Bowes, D., A. E. Clark, et al. (1981). "Ultrastructural localisation of lactoferrin and glycoprotein in human bronchial glands." Thorax **36**: 108 - 115.
- Bowes, D. and B. Corrin (1977). "Ultrastructural immunocytochemical localisation of lysozyme in human bronchial glands." Thorax **32**: 163 - 170.
- Brandtzaeg, P. (1974). "Mucosal and glandular distribution of immunoglobulin components. Immunohistochemistry with a cold ethanol-fixation technique." Immunology **26**: 1101 - 1114.
- Bredt, W., J. Feldner, et al. (1982). "Adherence of mycoplasma: phenomena and possible role in the pathogenesis of disease. ." Infection **10**: 199 - 202.
- Breeze, R. G. and E. B. Wheeldon (1977). "The cells of the pulmonary airways." Am Rev Respir Dis **116**: 705 - 777.
- British Thoracic Society (1994). "Guidelines for the measurement of respiratory function. Recommendations of the British Thoracic Society and the Association of Respiratory Technicians and Physiologists." Respir Med **88**: 165 - 194.
- Brogger, J., V. M. Steen, et al. (2006). "Genetic association between COPD polymorphisms in TNF, ADRB2 and EPHX1." Eur Respir J **27**: 682 - 688.
- Buist, A. S., M. A. McBurnie, et al. (2007). "International variation in the prevalence of COPD (the BOLD study): a population-based prevalence study." Lancet **370**: 741 - 750.
- Burge, P. S., P. M. Calverley, et al. (2000). "Randomised, double-blind, placebo controlled study of fluticasone propionate in patients with moderate to severe chronic obstructive pulmonary disease." Br Med J **320**: 1297 - 1303.
- Burke-Gaffney, A. and P. G. Hellewell (1996). "Tumour necrosis factor alpha induced ICAM-1 expression in human vascular endothelial and lung epithelial cells: modulation by tyrosine kinase inhibitors." Br J Pharmacol **119**: 1149 - 1158.
- Burnett, D. (1986). "Immunoglobulins in the lung." Thorax **41**: 337 - 344.
- Burnett, D. (1997). "Neutrophils and the pathogenesis of COPD in Stockley, R.A., editor." Pulmonary defences WileyEurope. Chichester.: 113 - 126.
- Burnett, D., A. Chamba, et al. (1987). "Neutrophils from subjects with chronic obstructive lung disease show enhanced chemotaxis and extracellular proteolysis." Lancet **2**(8567): 1043 - 1046.
- Burns, M. J., D. C. Walker, et al. (1997). "Neutrophil transendothelial migration is independent of tight junctions and occurs preferentially at tricellular corners." J Immunol **159**: 2893 - 2903.
- Butcher, S. K., H. Chahal, et al. (2001). "The effect of age on neutrophil function; loss of CD16 associated with reduced phagocytic capacity." Mech Age Dev **122**: 20.
- Butcher, S. K., V. Killampalli, et al. (2003). "Effect of age on susceptibility to post-traumatic infection in the elderly." Biochem Soc Trans **31**: 449 - 451.

- Cadwallader, K. A., A. M. Condliffe, et al. (2002). "Regulation of Phosphatidylinositol 3-kinase activity and phosphatidylinositol 3,4,5-trisphosphate accumulation by neutrophil priming agents." J Immunol **169**: 3336 - 3344.
- Calabrese, F., C. Giacometti, et al. (2005). "Marked alveolar apoptosis/proliferation imbalance in end stage emphysema." Respir Res **6**: 14.
- Calikoglu, M., G. Sahin, et al. (2004). "Leptin and TNF alpha levels in patients with chronic obstructive pulmonary disease and their relationship to nutritional parameters." Respiration **71**: 45 - 50.
- Campbell, E. J., M. A. Campbell, et al. (1999). "Quantum proteolysis by neutrophils; implications for pulmonary emphysema in alpha 1 anti trypsin deficiency." J Clin Invest **104**: 337 - 344.
- Campbell, E. J., M. A. Campbell, et al. (1999). "Quantum proteolysis by neutrophils; implications for pulmonary emphysema in alpha 1 anti-trypsin deficiency." J Clin Invest **104**: 337 - 344.
- Campbell, E. J., M. A. Campbell, et al. (2000). "Bioactive proteinase three on the cell surface of human neutrophils; quantification, catalytic activity and susceptibility to inhibition." J Immunol **165**: 3366 - 3374.
- Campbell, E. J., M. A. Campbell, et al. (2000). "Bioactive proteinase three on the cell surface of human neutrophils: quantification, catalytic activity and susceptibility to inhibition." J Immunol **165**: 3366 - 3374.
- Cantin, A. M., G. A. Fells, et al. (1990). "Antioxidant macromolecules in the epithelial lining fluid of the normal human lower respiratory tract. ." J Clin Invest **86**: 962 - 971.
- Cantrell, D. A. (2001). "Phosphatidylinositol-3-kinase signalling pathway." J Cell Sci **114**: 1439 - 1444.
- Celli, B. R., C. G. Cote, et al. (2004). "The body mass index, airflow obstruction, dyspnoea and exercise capacity index in chronic obstructive pulmonary disease." N Engl J Med **350**: 1005 - 1012.
- Cepinskas, G., M. Sandig, et al. (1999). "PAF induced elastase dependent neutrophil transendothelial migration is associated with mobilisation of elastase to the neutrophil surface and localised to the migrating front." J Cell Sci **112**: 1937 - 1945.
- Cepinskas, G., M. Sandig, et al. (1999). "PAF induced elastase dependent neutrophil transendothelial migration is associated with the mobilisation of elastase to the neutrophil surface and localised to the migrating front." J Cell Sci **112**: 1937 - 1945.
- Chanez, P., A. M. Vignola, et al. (1997). "Corticosteroid reversibility in COPD is related to features of asthma." Am J Respir Crit Care Med **155**(5): 1529-34.
- Chang, J. D., G. K. Sukhova, et al. (2007). "Deletion of the phosphoinositide 3-kinase p110 gamma gene attenuates murine atherosclerosis." Proc Natl Acad Sci USA **104**: 8077 - 8082.
- Charest, P. G. and R. A. Firtel (2007). "Big role for small GTPases in the control of directed cell movement." Biochem J **401**: 377 - 390.
- Chun, T. H., F. Sabeh, et al. (2004). "MTI-MMP dependent neovessel formation with the controls of a 3 dimension extra cellular matrix." J Cell Biol **167**: 757 - 767.
- Chung, K. F. (2001). "Cytokines in chronic obstructive disease." Eur Respir J **34**: 50s - 59s.
- Chuntharapai, A. and K. J. Kim (1995). "Regulation of the expression of IL-8 receptor A/B by IL-8; possible functions for each receptor." J Immunol **155**: 2587 - 2594.

- Church, D. F. and W. A. Pryor (1985). "Free-radical chemistry of cigarette smoke and its toxicological implications." Environ Health Perspect **64**: 111 - 126.
- Churg, A., R. D. Wang, et al. (2003). "Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor- α release." Am J Respir Crit Care Med **167**(8): 1083-9.
- Churg A., J. Dai, et al. (2002). "Tumour necrosis factor α is central to acute cigarette smoke induced inflammation and connective tissue breakdown." Am J Respir Crit Care Med **166**: 849 - 854.
- Churg A., R. D. Wang, et al. (2004). "Tumour necrosis factor α drives 70% of cigarette smoke-induced emphysema in the mouse." Am J Respir Crit Care Med **170**: 492 - 498.
- Clayton, A., R. A. Evans, et al. (1998). "Cellular activation through the ligation of intercellular adhesion molecule 1." J Cell Sci **111**: 443 - 453.
- Clayton, A., R. A. Evans, et al. (1998). "Cellular activation through the ligation of intracellular adhesion molecule 1." J Cell Sci **111**: 443 - 453.
- Colvin, R. A., G. S. Campanella, et al. (2004). "Intracellular domains of CXCR3 that mediate CXCL9, CXCL10 and CXCL11 function." J Biol Chem **279**: 30219 - 30227.
- Corberand, J., F. Ngyen, et al. (1981). "Polymorphonuclear functions and aging in humans." J Am Geriatr Soc **29**: 391 - 397.
- Corre, F., J. Lellouch, et al. (1971). "Smoking and leucocyte counts: results of an epidemiological study." Lancet **2**: 632 - 634.
- Cosio, M. G., J. Majó, et al. (2002). "Inflammation of the airways and lung parenchyma in COPD: role of T cells." Chest **121**(5 Suppl): 160S-165S.
- Crapo, J. D., B. E. Barry, et al. (1982). "Cell number and cell characteristics of the normal human lung." Am Rev Respir Dis **126**: 332 - 337.
- Crockett-Torabi, A. and J. C. Fantone (1995). "The Selectins. Insights into selectin induced intra-cellular signaling in leukocytes." Immunol Res **14**: 237 - 251.
- Crooks, S. W. and R. A. Stockley (1998). "Leukotriene B₄." Int J Biochem Cell Biol **30**: 173 - 178.
- Dangerfield, J., K. Y. Larbi, et al. (2002). "PECAM-1 (CD31) homophillic interaction up-regulates α -6 β -1 on transmigrated neutrophils in vivo and plays a functional role in the ability of α -6 integrins to mediate leukocyte migration through the perivascular basement membrane." J Exp Med **196**: 1201 - 1211.
- Darlington, G. J., K. H. Astrin, et al. (1982). "Assignment of human α 1-antitrypsin to chromosome 14 by somatic cell hybrid analysis." Proc Natl Acad Sci USA **79**: 870 - 873.
- Davenpeck, K. L., D. A. Steeber, et al. (1988). "Rat neutrophils express α 4 and β 1 integrins and bind to vascular adhesion molecule 1 and mucosal adressin cell adhesion molecule." Blood **91**: 2341 - 2346.
- Dawkins, P. A., C. L. Dawkins, et al. (2009). "Rate of progression of lung function impairment in Alpha-1-Antitrypsin deficiency." Eur Respir J Online doi **10.1183/09031936.00061208**.
- de Boer, W. (2005). "Perspectives for cytokine antagonist therapy in COPD." DDT **10**: 93 - 106.
- De Water, R., L. N. Willems, et al. (1986). "Ultrasound localisation of bronchial antileukoprotease in central and peripheral human airways by a gold-labeling technique using monoclonal antibodies." Am Rev Respir Dis **133**: 882 - 890.

- Declaux, C., C. Delacourt, et al. (1996). "Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane." Am J Respir Cell Mol Biol **14**: 288 - 295.
- Deffebach, M. E., N. B. Charan, et al. (1987). "The bronchial circulation. Small, but a vital attribute to the lung." Am Rev Respir Dis **135**: 463 - 481.
- Deforge, L. E., A. M. Preston, et al. (1993). "Regulation of interleukin 8 gene expression by oxidant stress." J Biol Chem **268**: 25568 - 25576.
- Del Prete, A., W. Vermi, et al. (2004). "Defective dendritic cell migration and activation of adaptive immunity in PI3Kinase Gamma deficient mice." EMBO J **23**: 3505 - 3515.
- Del Rio, L., S. Bennouna, et al. (2001). "CXCR2 deficiency confers impaired neutrophil recruitment and increased susceptibility during *Toxoplasma gondii* infection." J Immunol **167**: 6503 - 6509.
- Delacourt, C., S. Herigault, et al. (2002). "Protection against acute lung injury by intravenous or intratracheal pretreatment with EPI-HNE-4; a new potent neutrophil elastase inhibitor." Am J Respir Cell Mol Biol **26**: 290 - 297.
- Desrochers, P. E., J. J. Jeffrey, et al. (1991). "Interstitial collagenase (matrix metalloproteinase-1) expresses serpinase activity." J Clin Invest **87**(6): 2258-65.
- Devreotes, p. and C. Janetopoulos (1988). "Eukaryote chemotaxis; distinctions between directional sensing and polarisation." J Biol Chem **263**: 20445 - 20448.
- Di Francia, M., D. Barbier, et al. (1994). "Tumour necrosis factor alpha levels and weight loss in chronic obstructive pulmonary disease." Am J Respir Crit Care Med **150**: 1453 - 1455.
- Di Stefano, A., P. Maestrelli, et al. (1994). "Up-regulation of adhesion molecules in the bronchial mucosa of subjects with chronic obstructive bronchitis." Am J Respir Crit Care Med **149**: 803 - 810.
- Diamond, M. S., D. E. Staunton, et al. (1990). "ICAM-1 (CD-54): a counter receptor for MAC-1 (CD11b/CD18)." J Cell Biol **111**: 3129 - 3139.
- DiMango, E., H. J. Zar, et al. (1995). "Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produced interleukin 8." J Clin Invest **96**: 1485 - 1490.
- Doerschuk, C. M. (1992). "The role of CD18 mediated adhesion in neutrophil sequestration induced by infusion of activated plasma in rabbits." Am J Respir Cell Mol Biol **7**: 140 - 148.
- Doerschuk, C. M., M. F. Allard, et al. (1989). "Neutrophil kinetics in rabbits during infusion of zymosan-activated plasma." J Appl Physiol **67**: 88 - 95.
- Doerschuk, C. M., N. Beyers, et al. (1993). "Comparison of neutrophil and capillary diameters and their relationship to neutrophil sequestration in the lung." J Appl Physiol **74**: 3040 - 3045.
- Doerschuk, C. M., R. K. Winn, et al. (1990). "CD18 dependent and independent mechanisms of neutrophil emigration in pulmonary and systemic microcirculation of rabbits." J Immunol **144**: 2327 - 2333.
- Doll, R., R. Peto, et al. (1994). "Mortality in relation to smoking: 40 years' observations on male British doctors." BMJ **309**: 901 - 911.
- Donaldson, G. C., T. A. Seemungal, et al. (2002). "Relationship between exacerbation frequency and lung function decline in chronic obstructiv pulmonary disease." Thorax **57**: 847 - 852.
- Donaldson, G. C., T. A. Seemungal, et al. (2005). "Airway and systemic inflammation and decline in lung function in patients with COPD." Chest **128**(4): 1995 - 2004.

- Dorshkind, K., E. Montecino- Rodriguez, et al. (2009). "The aging immune system; is it ever too old to become young again?" Nat Rev Immunol **9**(1): 57 - 62.
- Downey, G. P., G. S. Worthen, et al. (1993). "Neutrophil sequestration and migration in localised pulmonary inflammation." Am Rev Respir Dis **147**: 168 - 176.
- Dowson, L. J., P. J. Guest, et al. (2001). "Longitudinal changes in physiological, radiological, and health status measurements in alpha(1)-antitrypsin deficiency and factors associated with decline." Am J Respir Crit Care Med **164**(10 Pt 1): 1805-9.
- Doyle, N. A., S. D. Bhagwan, et al. (1997). "Neutrophil margination sequestration and emigration in the lungs of L-selectin deficient mice." J Clin Invest **99**: 526 - 533.
- Drost, E. M. and W. MacNee (2002). "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 **32**: 393 - 403.
- Drost, E. M., C. Selby, et al. (1992). "Changes in neutrophil deformability following in vitro smoke exposure; mechanism and protection." Am J Respir Cell Mol Biol **6**: 287 - 295.
- Dupuit, F., J. Jacquot, et al. (1993). "Vectorial delivery of newly synthesized secretory proteins by human tracheal glands in culture. ." Epithelial Cell Biol **2**: 91 - 99.
- Eddy, R. J., L. M. Plerinl, et al. (2000). "Ca²⁺ dependent myosin II activation is required for uropod retraction during neutrophil migration." J Cell Sci **113**: 1287 - 1296.
- Egger, G., R. Aigner, et al. (2004). "Blood polymorphonuclear leukocyte migration as a predictive marker for infections in severe trauma; comparison with various inflammatory parameters." Intensive Care Med **30**: 331 - 334.
- Eid, A. A., A. A. Ionescu, et al. (2001). "Inflammatory response and body composition in chronic obstructive pulmonary disease." Am J Respir Crit Care Med **164**: 1414 - 1418.
- Ekberg-Jansson, A., B. Andersson, et al. (2001). "Neutrophil-associated activation markers in healthy smokers relates to a fall in DL(CO) and to emphysematous changes on high resolution CT." Respir Med **95**: 363 - 373.
- El-Kholy, W., P. E. MacDonald, et al. (2003). "The phosphatidylinositol 3-kinase inhibitor LY294002 potently blocks K(V) currents via a direct mechanism " FASEB J **17**: 720 - 722.
- Ellison, R. T. and T. J. Giehl (1991). "Killing of gram negative bacteria by lactoferrin and lysozyme." J Clin Invest **88**: 1080 - 1091.
- Eriksson, S., J. Carlson, et al. (1986). "Risk of cirrhosis and primary liver cancer in alpha 1-antitrypsin deficiency." N Engl J Med **314**: 736 - 739.
- Erjefalt, J. S. and F. Sundler (1996). "Eosinophils, neutrophils and venular gaps in the airway mucosa at epithelial removal-restitution." Am J Respir Crit Care Med **153**: 1666 - 1674.
- Erzurum, S. C., G. P. Downey, et al. (1992). "Mechanisms of lipopolysaccharide retention." J Immunol **149**: 1526 - 1533.
- Esparza, B., H. Sanchez, et al. (1996). "Neutrophil function in elderly persons assessed by flow cytometry." Immunol Invest **25**: 185 - 190.
- Evans, M. J., L. J. Cabral, et al. (1975). "Transformation of alveolar type 2 cells to type 1 cells following exposure to NO₂." Exp Mol Pathol **22**: 142 - 150.
- Even-Ram, S. and K. Yamada (2005). "Cell migration in a 3D matrix." Curr Opin Cell Biol **17**: 524 - 532.

- Fadok, V. A., D. L. Bratton, et al. (2001). "Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases." J Immunol **166**(11): 6847-54.
- Faerrandi, C., H. Ji, et al. (2003). "The PI3Kinase inhibitor LY294002 decreases RANTES induced peritoneal recruitment of neutrophils in mice." Cell Mol Biol Lett **8**: 579 - 581.
- Fais, S. and W. Malorni (2003). "Leukocyte uropod formation and membrane/cytoskeleton linkage in immune interactions." J Leuk Biol **73**: 556 - 563.
- Fan, G. H., L. A. Lapierre, et al. (2003). "Differential regulation of CXCR2 trafficking by Rab GTPases." Blood **101**: 2115 - 2124.
- Fan, G. H., L. A. Lapierre, et al. (2004). "Rab11 family interacting protein 2 and myosin Vb are required for CXCR2 recycling and receptor mediated chemotaxis." Mol Biol Cell **15**: 2456 - 2469.
- Farooq, S. M., R. Stillie, et al. (2009). "Therapeutic effect of blocking CXCR2 on neutrophil recruitment and dextran sodium sulfate induced colitis." JPET **329**: 123 - 129.
- Fein, A. M. (1999). "Pneumonia in the elderly: Overview of diagnostic and therapeutic approaches." Clin Infect Dis **28**: 726 - 729.
- Feinberg, M. (1993). "The problems of anti-cholinergic adverse effects in older patients." Drugs Ageing **3**: 335 - 348.
- Feinmark, S. J., J. A. Lindgren, et al. (1981). "Stimulation of human leukocyte degranulation by leukotriene B4 and its omega-oxidized metabolites." FEBS Lett **136**: 141 - 144.
- Feldman, C., R. Read, et al. (1992). "The interactions of Streptococcus pneumoniae with intact human respiratory mucosa in vitro." Eur Respir J **5**: 576 - 583.
- Ferguson, S. S. (2001). "Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling." Pharmacol Review **53**: 1 - 24.
- Ferry, G., M. Lonchamp, et al. (1997). "Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury." FEBS **402**: 111 - 115.
- Ferry, G., M. Lonchamp, et al. (1997). "Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury." FEBS Lett **402**(2-3): 111-5.
- Fletcher, C. and R. Peto (1977). "The natural history of chronic airflow obstruction." British Medical Journal **1**: 1645 - 1648.
- Floreani, A. A., T. A. Wyatt, et al. (2003). "Smoke and C5a induce airway epithelial intercellular adhesion molecule-1 and cell adhesion." Am J Respir Cell Mol Biol **29**: 472 - 482.
- Fogarty, A. W., S. Jones, et al. (2007). "Systemic inflammation and decline in lung function in a general population; a prospective study." Thorax **62**: 515 - 520.
- Fortin, C. F. (2008). "Aging and neutrophils: there is still much to do." Rejuvenation Res **11**: 873 - 882.
- Fortin, C. F., A. Larbi, et al. (2006). "Impairment of SHP-1 down regulation in the lipid rafts of human neutrophils under GM-CSF stimulation contributes to their age-related altered functions." J Leuk Biol **79**: 1061 - 1072.
- Fortin, C. F., A. Larbi, et al. (2007). "GM-CSF activates the Jak/STAT pathway to rescue polymorphonuclear neutrophils from spontaneous apoptosis in young but not elderly individuals." Biogerontology **8**: 173 - 187.
- Fortin, P. R., R. S. Fraser, et al. (1991). "Alpha 1-antitrypsin deficiency and systemic necrotising vasculitis." J Rheumatol **18**: 1613 - 1616.

- Foster, F. M., C. J. Traer, et al. (2003). "The phosphoinositide (PI) 3-kinase family." J Cell Sci **116**: 3037 - 3040.
- Fouret, P., R. M. du Bois, et al. (1989). "Expression of the neutrophil elastase gene during human bone marrow cell differentiation." J Exp Med **169**: 833 - 845.
- Foxman, E. F., J. J. Campbell, et al. (1997). "Multi-step navigation and the combinatorial control of leukocyte chemotaxis." J Cell Biol **139**: 1349 - 1359.
- Foxman, E. F., E. J. Kundel, et al. (1999). "Integrating conflicting chemotactic signals; the role of memory in leukocyte navigation." J Cell Biol **147**: 577 - 587.
- Franceschi, C., M. Bonafe, et al. (2000). "Inflamm-aging: an evolutionary perspective on immunosenescence." Ann N Y Acad Sci **908**: 244 - 254.
- Franciosi, L. G., C. P. Page, et al. (2006). "Markers of disease severity in chronic obstructive pulmonary disease." Pulm Pharmacol Ther **19**: 189 - 199.
- Fujita, J., N. L. Nelson, et al. (1990). "Evaluation of elastase and antielastase balance in patients with chronic bronchitis." Am Rev Respir Dis **142**: 57 - 62.
- Fukuchi, Y. (2009). "The aging lung and Chronic Obstructive Pulmonary Diseases." Proc Am Thorac Soc **6**: 570 - 572.
- Fukuchi, Y., M. Nishimura, et al. (2004). "COPD in Japan: the Nippon COPD epidemiology study." Respirology **9**: 458 - 465.
- Fulop, T., I. Komaromi, et al. (1986). "Age dependent variations of intralysosomal enzyme release from human PMN leukocytes under various stimuli." Immunobiol **171**: 302 - 310.
- Fulop, T., A. Larbi, et al. (2004). "Signal transduction and functional changes in neutrophils with aging." Aging Cell **3**: 217 - 226.
- Gamble, E., Y. Qiu, et al. (2006). "Variability of bronchial inflammation in chronic obstructive pulmonary disease: implications for study design." Eur Respir J **27**(2): 293-9.
- Garbe, E., S. Suissa, et al. (1998). "Association of inhaled corticosteroid use with cataract extraction in elderly patients." JAMA **280**: 539 - 543.
- Gavazzi, G. and K. H. Krause (2002). "Aging and infection." Lancet Infectious Diseases **2**: 659 - 666.
- Ge, Y. M., Y. J. Zhu, et al. (1990). "Damaging role of neutrophil elastase in the elastic fibre and basement membrane in human emphysematous lung." Chin Med **103**(7): 588 - 594.
- Gilmour, P. S., I. Rahman, et al. (2001). "Adenoviral E1A primes alveolar epithelial cells to PM(10)-induced transcription of interleukin 8." Am J Physiol Lung Cell Mol Physiol **281**: L598 - L606.
- Giunta, S. (2006). "Is inflammaging an auto (innate) immunity subclinical syndrome?" Immunity and Ageing **3**: 12 - 13.
- Global Initiative for chronic obstructive lung disease. (2007). "Guidelines and resources." www.goldcopd.com/index.
- Godaly, G., L. Hang, et al. (2000). "Trans epithelial neutrophil migration is CXCR1 dependent in vitro and is defective in IL8 receptor knock out mice." J Immunol **165**: 5287 - 5294.
- Gomez-Mouton, C., R. A. Lacelle, et al. (2001). "Segregation of leading edge and uropod components into specific lipid rafts during T cell polarization." Proc Natl Acad Sci USA **98**: 9642 - 9647.
- Gompertz, S., Bayley D, et al. (2001). "Relationship between airway inflammation and the frequency of exacerbations in patients with smoking related COPD. ." Thorax **56**: 36 - 41.

- Gompertz, S., A. T. Hill, et al. (2006). "Effect of expectoration on inflammation in induced sputum in alpha-1-antitrypsin deficiency." Respir Med **100**(6): 1094 - 1099.
- Gompertz, S., C. O'Brien, et al. (2001). "Changes in bronchial inflammation during acute exacerbations of chronic bronchitis." Eur Respir J **17**: 1112 - 1119.
- Gompertz, S., C. O'Brien, et al. (2001). "Changes in bronchial inflammation during acute exacerbations of chronic bronchitis." Eur Respir J **17**(6): 1112 - 1119.
- Gompertz, S. and R. A. Stockley (2002). "A randomised controlled trial of a Leukotriene synthesis inhibitor in patients with COPD." Chest **112**: 289 - 294.
- Gonzalez, S., J. Hards, et al. (1996). "The expression of adhesion molecules in cigarette smoke induced airways obstruction." Eur Respir J **9**: 1995 - 2001.
- Gonzalez-Garcia, A., J. Sanchez-Ruiz, et al. (2010). "Phosphatidylinositol 3-Kinase gamma inhibition ameliorates inflammation and tumour growth in a model of colitis associated cancer." Gastroenterology **138**: 1374 - 1383.
- Gray-Donaldson, K., L. Gibbons, et al. (1996). "Nutritional status and mortality in chronic obstructive pulmonary disease." Am J Respir Crit Care Med **153**: 961 - 966.
- Gross, P., E. H. Pfizer, et al. (1964). "Experimental emphysema; its production with papain in normal and silicotic rats." Arch Environ Health **11**: 50 - 58.
- Hacievliyagil, S. S., H. Gunen, et al. (2006). "Association between cytokines in induced sputum and severity of chronic obstructive pulmonary disease." Respir Med **100**: 846 - 854.
- Hall, D. A., F. B. Reed, et al. (1974). "The relative effects of age and corticosteroid therapy on the collagen profiles of dermis from subjects with rheumatoid arthritis." Age and Ageing **3**: 15 - 22.
- Hannigan, M., L. Zhan, et al. (2002). "Neutrophils lacking phosphoinositide 3-kinase gamma show loss of directionality during N-formyl-Met-Leu-Phe induced chemotaxis." Proc Natl Acad Sci USA **99**: 3603 - 3608.
- Hartl, D., P. Latzin, et al. (2007). "Cleavage of CXCR1 on neutrophils disables bacterial killing in cystic fibrosis lung disease." Nature Medicine **13**: 1423 - 1429.
- Hasania, A. and D. Pavia (1989). "Cough as a clearance mechanism. In Braga, P.C., editor." Cough **Raven Press. New York**.
- Hashimoto, M., M. Shingu, et al. (1994). "Production of soluble ICAM-1 from human endothelial cells induced by IL-1 beta and TNF alpha. ." Inflammation **18**: 163 - 173.
- Hattotuwa, K., E. A. Gamble, et al. (2002). "Safety of bronchoscopy, biopsy, and BAL in research patients with COPD." Chest **122**(6): 1909-12.
- Hattotuwa, K. L., M. J. Gizycki, et al. (2002). "The effects of inhaled fluticasone on airway inflammation in chronic obstructive pulmonary disease: a double-blind, placebo-controlled biopsy study." Am J Respir Crit Care Med **165**(12): 1592-6.
- Hautamaki, R. D., D. K. Kobayashi, et al. (1997). "Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice." Science **277**(5334): 2002-4.
- Hay, W. P. D. and H. M. Sarau (2001). "Interleukin-8 receptor antagonists in pulmonary disease." Curr Opin Pharmacol **1**: 242 - 247.
- Heit, B., L. Liu, et al. (2008). "PI3K accelerates but is not required for neutrophil chemotaxis to fMLP." J Cell Sci **121**: 205 - 209.
- Hellewell, P. G., S. K. Young, et al. (1994). "Disparate role of the integrin CD18 in the local accumulation of neutrophils in pulmonary and cutaneous inflammation in rabbit." Am J Respir Cell Mol Biol **10**: 391 - 398.

- Hill, A. T., D. Bayley, et al. (2000). "Airways inflammation in chronic bronchitis; the effects of smoking and alpha-1 anti-trypsin deficiency." Eur Respir J **15**: 886 - 890.
- Hill, A. T., D. Bayley, et al. (1999). "The interrelationship of sputum inflammatory markers in patients with chronic bronchitis." Am J Respir Crit Care Med **160**: 893 - 898.
- Hill, A. T., E. J. Campbell, et al. (2000). "Association between airway bacterial load and markers of airways inflammation in patients with stable chronic bronchitis." Am J Med **109**: 288 - 295.
- Hirata, H., H. Takahashi, et al. (1998). "Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis." J Exp Med **187**: 587 - 600.
- Hoffman, J. J. and W. G. van der Brock (1977). "Application of isoelectric focusing in alpha 1-antitrypsin phenotyping." Clin Chim Acta **75**: 233 - 242.
- Hogg, J. C. (1987). "Neutrophil kinetics and lung injury." Physiol Rev **67**: 1295 - 1298.
- Holmes, W. E., J. Lee, et al. (1991). "Structure and functional expression of a human interleukin-8 receptor." Science **253**: 1278 - 1280.
- Holz, O., K. Richter, et al. (1998). "Changes in sputum composition between two inductions performed on consecutive days." Thorax **53**(2): 83-6.
- Horwitz, M. A. and S. C. Silverstein (1981). "Activated human monocytes inhibit the intracellular multiplication of Legionnaires' disease bacteria." J Exp Med **154**: 1618 - 1635.
- Hubbard, R. C., G. Fells, et al. (1991). "Neutrophil accumulation in the lung in alpha 1 anti-trypsin deficiency; spontaneous release of leukotriene B4 by alveolar macrophages." J Clin Invest **88**: 891 - 897.
- Hubbard, R. C., G. Fells, et al. (1991). "Neutrophil accumulation in the lung in alpha 1-antitrypsin deficiency. Spontaneous release of leukotriene B4 by alveolar macrophages." J Clin Invest **88**(3): 891-7.
- Hutchinson, D. C. (1998). "Alpha 1-antitrypsin deficiency in Europe; geographical distribution of Pi types S and Z." Respir Med **92**.
- Huttunlocher, A. (2005). "Cell polarisation mechanisms during directed cell migration." Nature Cell Biol. **7**: 336 - 377.
- Imai, K., B. A. Mercer, et al. (2005). "Correlation of lung surface area to apoptosis and proliferation in human emphysema." Eur Respir J **25**: 250- 258.
- Itoh, Y. and H. Nagase (1995). "Preferential inactivation of tissue inhibitor of metalloproteinase-1 that is bound to the precursor of metalloproteinase-9 by human neutrophil elastase." J Biol Chem **270**: 16518 - 16521.
- Itoh, Y. and H. Nagase (1995). "Preferential inactivation of tissue inhibitor of metalloproteinases-1 that is bound to the precursor of matrix metalloproteinase 9 (progelatinase B) by human neutrophil elastase." J Biol Chem **270**(28): 16518-21.
- Janoff, A., B. Sloan, et al. (1977). "Experimental emphysema induced with purified human neutrophil elastase; tissue localisation of the instilled protease. ." Am Rev Respir Dis **115**: 461 - 478.
- Jefferis, R. (1997). "Immunoglobulins. In Stockley, R.A., editor." Pulmonary defences WileyEurope. Chichester.: 39 - 58.
- Jeffery, P. K. (1998). "Structural and inflammatory changes in COPD: a comparison with asthma." Thorax **53**(2): 129-36.
- Jeffrey, P. K. (1983). "Morphologic features of airway surface epithelial cells and glands." Am Rev Respir Dis **128**: S14 - S20.

- Jeffrey, P. K. (1995). "Microscopic structure of the normal lung. In Brewis, R. A. L., editor." Respiratory Medicine **W.B. Saunders Company LTD. London.**: 54 - 72.
- Jeffrey, P. K. and L. Reid (1975). "New observations in rat airway epithelium: a quantitative and electron microscopic study." J Anat. **120**: 295 - 320.
- Jiang, R., G. L. Burke, et al. (2008). "Inflammatory markers and longitudinal lung function decline in the elderly." Am J Epidemiol **168**: 602 - 610.
- Jin, T. and D. Hereld (2006). "Moving towards understanding eukaryotic chemotaxis." Eur J Cell Biol **85**: 905 - 913.
- Johnson, D. and J. Travis (1979). "The oxidative inactivation of human alpha-1-proteinase inhibitor." J Biol Chem **254**: 4022 - 4026.
- Kafienah, W., D. J. Buttle, et al. (1998). "Cleavage of native type 1 collagen by human neutrophil elastase." Biochem J. **330**: 897 - 902.
- Kao, R. C., N. G. Wehner, et al. (1988). "Proteinase 3. A distinct human polymorphonuclear leukocyte proteinase that produces emphysema in hamsters." J Clin Invest **82**(6): 1963-73.
- Keatings, V. M., A. Jatakanon, et al. (1997). "Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD." Am J Respir Crit Care Med **155**(2): 542-8.
- Kerstjens, H. A. M., B. Rijcken, et al. (1997). "Decline of FEV1 by age and smoking status: facts, figures and fallacies." Thorax **52**: 820 - 827.
- Khair, O. A., R. J. Davies, et al. (1996). "Bacterial-induced release of inflammatory mediators by bronchial epithelial cells." Eur Respir J **9**: 1913 - 1922.
- Kim, W. D. (1997). "Lung mucus: a clinician's view." Eur Respir J **10**: 1914 - 1917.
- King, M. and B. K. Rubin (1994). "Epithelial goblet cell secretion. In Shimura, S., editor." Airway secretion, physiological basis for the control of mucous hypersecretion. **Marcel Decker. New York**: 42 - 57.
- Klein, C. L., F. Bittenger, et al. (1995). "Comparative studies on vascular endothelium in vitro. Effects of cytokines on the expression of E-selectin." Pathobiology **65**: 83 - 92.
- Knall, C., G. S. Worthen, et al. (1997). "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 **94**: 3052 - 3057.
- Knall, C., G. S. Worthen, et al. (1997). "PI3 Kinase regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases." Proc Am Thorac Soc **94**: 3052 - 3057.
- Knudson, R. J., D. F. Clark, et al. (1977). "Effect of aging alone on mechanical properties of the normal adult human lung." J Appl Physiol **43**: 1054 - 1062.
- Kohl, J. and D. Bitter-Suermann (1983). "Anaphylotoxins. In Whaley, K., editor." Complement in health and disease. **Kluwer Academic. Boston**: 299 - 324.
- Kok, K., B. Geering, et al. (2009). "Regulation of phosphoinositide 3 kinase expression in health and disease." Trends Biochem Sci **34**: 115 - 127.
- Kramps, J. A., C. Franken, et al. (1981). "Localisation of low molecular weight protease inhibitor in serous secretory cells of the respiratory tract." J Histochem Cytochem **29**: 712 - 719.
- Kuschner, W. G., A. D'Alessandro, et al. (1996). "Dose dependent cigarette smoking-related inflammatory changes in healthy adults." Eur Respir J **9**: 1989 - 1994.

- Lacoste, J. Y., J. Bousquet, et al. (1993). "Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis and chronic obstructive pulmonary disease." J Allergy Clin Immunol **92**(4): 537 - 548.
- Laitinen, L. A., A. Laitinen, et al. (1987). "Effects of inflammatory mediators on airway vascular beds." Am Rev Respir Dis **135**: S67 - S70.
- Lanone, S., T. Zheng, et al. (2002). "Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling." J Clin Invest **110**(4): 463-74.
- Lappalainen, U., J. A. Whitsett, et al. (2005). "Interleukin-1beta causes pulmonary inflammation, emphysema and airway remodeling in the adult murine lung." Am J Respir Cell Mol Biol **32**: 311 - 318.
- Larsson, C. (1978). "Natural history and life expectancy in severe alpha 1-antitrypsin deficiency, Pi Z." Acta Med Scand **204**: 345 - 351.
- Laudanna, C., J. J. Campbell, et al. (1996). "Role of Rho in chemoattractant activated leukocyte adhesion through integrins." Science **271**: 981 - 983.
- Lee, J., R. Hpruk, et al. (1992). "Characterization of two high affinity interleukin-8 receptors." J Biol Chem **267**(23): 16283 - 16287.
- Lee, W. L. and G. P. Downey (2001). "Leukocyte elastase: physical functions and its role in acute lung injury." Am J Respir Crit Care Med **164**: 896 - 904.
- Levine, H., \Kessler, D.D., and W. J. Rappel (2006). "Directional sensing in eukaryotic chemotaxis: a balanced inactivation model." Proc Natl Acad Sci USA **103**: 9761 - 9766.
- Li, H., D. Cui, et al. (2002). "[The role of matrix metalloproteinases in extracellular matrix remodelling in chronic obstructive pulmonary disease rat models]." Zhonghua Nei Ke Za Zhi **41**(6): 393-8.
- Liang, S. Y. and P. A. Mackowiak (2007). "Infections in the elderly." Clinics in Geriatric Medicine **23**: 441 - 446.
- Liou, T. G. and E. J. Campbell (1995). "Nonisotropic Enzyme-Inhibitor Interactions: A novel nonoxidative mechanism for quantum proteolysis by human neutrophils." Biochem **34**: 16171 - 16177.
- Liou, T. G. and E. J. Campbell (1996). "Quantum proteolysis resulting from release of single granules by human neutrophils; a novel, non oxidative mechanism of extra cellular proteolytic activity." J Immunol **157**: 2624 - 2631.
- Liou, T. G. and E. J. Campbell (1996). "Quantum proteolysis resulting from release of single granules by human neutrophils: a novel, nonoxidative mechanism of extracellular proteolytic activity." J Immunol **157**(6): 2624-31.
- Lipschitz, D. A., K. B. Udupa, et al. (1984). "Effect of age on hematopoiesis in man." Blood **63**: 502 - 509.
- Liu, X., C. N. Kim, et al. (1996). "Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome C." Cell **86**: 147 - 157.
- Loetscher, P., M. Seitz, et al. (1994). "Both interleukin-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 **341**: 187 - 192.
- Lomas, D. A. (2002). "Chronic obstructive pulmonary disease. Introduction." Thorax **57**: 735
- Lomas, D. A., D. L. Evans, et al. (1992). "The mechanism of Z alpha 1-antitrypsin accumulation in the liver." Nature **357**: 605 - 607.
- Lomas, D. A., S. R. Stone, et al. (1996). "The control of neutrophil chemotaxis by inhibitors of cathepsin G and chymotrypsin." J Biol Chem **270**: 23437 - 23443.

- Lucey, E. C., J. Keane, et al. (2002). "Severity of elastase induced emphysema decreased in TNF alpha and IL-1 beta receptor deficient mice." Lab Invest **82**: 79 - 85.
- Lucey, E. C., P. J. Stone, et al. (1985). "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 **132**(2): 362-6.
- Lucey, E. C., P. J. Stone, et al. (1990). "Recombinant human secretory leukocyte-protease inhibitor: in vitro properties, and amelioration of human neutrophil elastase-induced emphysema and secretory cell metaplasia in the hamster." J Lab Clin Med **115**(2): 224-32.
- Lucey, E. C., P. J. Stone, et al. (1989). "Amelioration of human neutrophil elastase-induced emphysema in hamsters by pretreatment with an oligopeptide chloromethyl ketone." Eur Respir J **2**(5): 421-7.
- Lucey, E. C., P. J. Stone, et al. (1998). "Amelioration of human neutrophil elastase-induced emphysema in hamsters by pretreatment with an oligopeptide chlormethyl ketone." Eur Respir J **2**: 421 - 427.
- Ludwig, P. W. and J. R. Hoidal (1982). "Alterations in leukocyte oxidative metabolism in cigarette smokers." Am Rev Respir Dis **126**: 977 - 980.
- MacGregor, R. R. and M. Shalit (1990). "Neutrophil function in healthy elderly subjects." J Gerontol **45**: M55 - M60.
- Mackeral, A. J., D. C. Cottell, et al. (1999). "Migration of human neutrophils across human pulmonary endothelial cells is not blocked by matrix metalloproteinase or serine proteinase inhibitors." Am J Respir Cell Mol Biol **20**: 1209 - 1219.
- Mackeral, A. J., K. J. Russell, et al. (2000). "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 **23**: 154 - 161.
- MacNee, W. (2000). "Oxidants / antioxidants and COPD." Chest **117**: 303S - 317S.
- Mador, M. J. (2002). "Muscle mass and not body weight predicts outcome in patients with chronic obstructive pulmonary disease " Am J Respir Crit Care Med **166**: 787 - 789.
- Makris, D., J. Moschandreass, et al. (2007). "Exacerbations and lung function in decline in COPD: New insights in current and ex-smokers." Respir Med **101**: 1305 - 1312.
- Malik, A. R. and S. K. Lo (1996). "Vascular endothelial adhesion molecules and tissue inflammation." Pharmacol Rev **48**: 213 - 229.
- Mannino, D. M. and F. Holguin (2006). "Epidemiology and global impact of obstructive pulmonary disease." Respir Med **1**(4): 114 - 120.
- Marco, V., B. Mass, et al. (1971). "Induction of experimental emphysema in dos using leukocyte homogenates." Am Rev Respir Dis **104**: 595 - 598.
- Marian, E., S. Baraldo, et al. (2006). "Up-regulation membrane and nuclear leukotriene B4 receptors in COPD." Chest **129**: 1523 - 1530.
- Mariotti, M., Castiglioni, S., D. Bernardini, et al. (2006). "Interleukin-1 alpha is a marker of endothelial celular senescent." Immun Ageing **3**: 4.
- Martin, G. S., D. M. Mannino, et al. (2006). "The effect of age on the development and outcome of adult sepsis." Crit Care Med **34**: 15 - 21.
- Martin, T. R., G. Raghu, et al. (1985). "The effects of chronic bronchitis and chronic airflow obstruction on lung cell populations recovered by bronchoalveolar lavage." Am Rev Respir Dis **132**: 260.

- Maruyama, M., J. G. Hay, et al. (1994). "Modulation of secretory leukoprotease inhibitor gene expression in human bronchial epithelial cells by phorbol ester." J Clin Invest **94**: 368 - 375.
- Matheson, M., A. C. Rynell, et al. (2003). "Cigarette smoke increases neutrophil formyl methionyl leucyl phenylalanine receptor numbers." Chest **123**: 1642 - 1646.
- Mathiak, G., D. Szewczyk, et al. (1997). "Platelet activating factor (PAF) in experimental and clinical sepsis." Shock **7**: 391 - 404.
- Matsuba, K. T., S. F. Van Eeden, et al. (1997). "Apoptosis in circulating PMNs; increasing susceptibility in L-selectin deficient PMN." Am J Physiol **272**: H2852 - H2858.
- Matsushima, K., K. Morishita, et al. (1988). "Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumour necrosis factor." J Exp Med **167**: 1883 - 1893.
- McDermott, M. R., A. D. Befus, et al. (1982). "The structural basis for immunity in the respiratory tract." Int Rev Exp Pathol **23**: 47 - 112.
- McLeod, R., D. G. Mack, et al. (1985). "Alveolar macrophage function and inflammatory stimuli in smokers with and without obstructive lung disease." Am Rev Respir Dis **131**: 377 - 384.
- Medical Research Council. (1965). "Definition and classification of chronic bronchitis for clinical and epidemiological purposes. A report to the Medical Research Council by their Committee on the Aetiology of Chronic Bronchitis." Lancet **1**: 775 - 779.
- Michishita, M., V. Vider, et al. (2003). "A novel divalent cation-binding site in the A domain of the beta 2 integrin CR3 (CD11b/CD18) is essential for ligand binding." Cell **72**: 857 - 867.
- Mikami, M., C. G. Llewellyn-Jones, et al. (1998). "The chemotactic activity of sputum from patients with bronchiectasis." Am J Respir Crit Care Med **157**: 723 - 728.
- Minematsu, N., H. Nakamura, et al. (2001). "Genetic polymorphism in matrix metalloproteinase-9 and pulmonary emphysema." Biochem Biophys Res Commun **289**(1): 116-9.
- Montuschi, P., S. Kharitonov, et al. (2003). "Exhaled leukotrienes and prostaglandins in COPD. ." Thorax **58**: 585 - 588.
- Mooren, H. W., J. A. Kramps, et al. (1983). "Localisation of a low molecular weight bronchial protease inhibitor in the peripheral human lung." Thorax **38**: 180 - 183.
- Morgan, K. L., A. M. Hussein, et al. (1980). "Quantification and origin of the immunoglobulins in porcine respiratory tract secretions." Immunology **41**: 729 - 736.
- Morland, C. M., B. J. Morland, et al. (2000). "Migration of CD18 deficient neutrophils in vitro: evidence for a CD18 independent pathway induced by IL8." Biochem Biophys Acta **1500**: 70 - 76.
- Morrison, D., R. M. Strieter, et al. (1998). "Neutrophil chemokines in bronchoalveolar lavage fluid and leukocyte-conditioned medium from smokers and non-smokers." Eur Respir J **12**: 1067 - 1072.
- Morrison, H. M., J. A. Kramps, et al. (1987). "Lung lavage fluid from patients with alpha 1 proteinase inhibitor deficiency or chronic obstructive bronchitis; anti-elastase function and cell profile." Clin Sci (Colch) **72**: 373 - 381.
- Morrison, H. M., J. A. Kramps, et al. (1987). "Lung lavage fluid from patients with alpha 1 proteinase inhibitor deficiency or chronic obstructive bronchitis: anti-elastase function and cell profile." Clin Sci **72**: 373 - 381.

- Muller, W. A. (1995). "The role of PECAM-1 in leukocyte emigration. Studies in vitro and in vivo." J Leuk Biol **66**: 698 - 704.
- Murphy, P. M. and H. L. Tiffany (1991). "Cloning of complementary DNA encoding a functional human interleukin-8 receptor." Science **253**: 1280 - 1283.
- Muzio, M., A. M. Chinnaiyan, et al. (1996). "FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signalling complex." Cell **85**: 817 - 827.
- Muzio, M., B. R. Stockwell, et al. (1998). "An induced proximity model for capase-8 activation." J Biol Chem **273**: 2926 - 2930.
- Nakajoh, M., T. Fukushima, et al. (2002). "Retinoic acid inhibits elastase-induced injury in human lung epithelial cells." Am J Respir Cell Mol Biol **28**(3): 296 - 304.
- Neel, N. F., L. A. Lapierre, et al. (2008). "RhoB plays an essential role in CXCR2 sorting decisions." J Cell Sci **120**: 1559 - 1571.
- Neel, N. F., E. Schutyser, et al. (2005). "Chemokine receptor internalization and intracellular trafficking." Cytokine Growth Factor Review **16**: 637 - 658.
- Nelson, R. D., P. G. Quie, et al. (1975). "Chemotaxis under agarose; a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes." J Immunol **115**: 1650 - 1656.
- Newman, P. J. (1997). "The biology of PECAM-1." J Clin Invest **99**: 3 - 7.
- Nguyen, L. T., M. Bedu, et al. (1999). "Increased resting energy expenditure is related to plasma TNF alpha concentration in stable COPD patients." Clin Nutr **18**: 269 - 274.
- Niederman, M. S., W. W. Merrill, et al. (1986). "Influence of IgA and elastase on trachea cell bacterial adherence." Am Rev Respir Dis **133**: 255 - 260.
- Nightingale, J. A., D. F. Rogers, et al. (1998). "Effect of repeated sputum induction on cell counts in normal volunteers." Thorax **53**(2): 87-90.
- Nishiya, N., W. B. Kiosses, et al. (2005). "An alpha-4 integrin-paxillin-Arf-GAP complex restricts Rac activation to the leading edge of migrating cells." Nature Cell Biol. **7**: 343 - 345.
- Niwa, Y., T. Kasama, et al. (1989). "Neutrophil chemotaxis, phagocytosis and parameters of reactive oxygen species in human aging: cross sectional and longitudinal studies." Life Sci **44**: 1655 - 1664.
- Noguera, A., S. Batle, et al. (2001). "Enhanced neutrophil response in chronic obstructive pulmonary disease." Thorax **56**: 432 - 437.
- Noguera, A., X. Busquets, et al. (1998). "Expression of adhesion molecules and G proteins in circulating neutrophils in chronic obstructive pulmonary disease." Am J Respir Crit Care Med **158**: 1664 - 1668.
- Nohgawa, M., M. Sasada, et al. (1997). "Leukotriene B4 activated human endothelial cells promote transendothelial neutrophil migration." J Leuk Biol **62**: 203 - 209.
- O'Shaughnessy, T. C., T. W. Ansari, et al. (1997). "Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1." Am J Respir Crit Care Med **155**(3): 852-7.
- Ofulue, A. F., M. Ko, et al. (1998). "Time course of neutrophil and macrophage elastolytic activities in cigarette smoke-induced emphysema." Am J Physiol **275**(6 Pt 1): L1134-44.
- Ohlsson, K., C. Linder, et al. (1996). "Release of cytokines and proteases from human peripheral blood mononuclear and polymorphonuclear cells following phagocytosis and LPS stimulation." Scand J Clin Lab Invest **56**(5): 461-70.
- Ohta, Y., J. H. Hartwig, et al. (2006). "A Rho and ROCK regulated GAP for Rac binds filamin A to control actin remodeling." Nature Cell Biol. **8**: 803 - 814.

- Onofrio, J. M., G. B. Toews, et al. (1983). "Granulocyte-alveolar-macrophage interaction in the pulmonary clearance of *Staphylococcus aureus*. ." Am Rev Respir Dis **127**: 335 - 341.
- Owen, C. A., M. A. Campbell, et al. (1997). "Cytokines regulate membrane-bound leucocytes elastase on neutrophils, a novel mechanism for effector activity." Am J Physiol Lung Cell Mol Physiol **272**: L385 - L393.
- Owen, C. A., M. A. Campbell, et al. (1997). "Cytokines regulate membrane-bound leukocyte elastase on neutrophils; a novel mechanism for effector activity." Am J Physiol Lung Cell Mol Physiol **272**: L385 - L393.
- Owen, C. A., M. A. Campbell, et al. (1995). "Cell surface bound elastase and cathepsin G on human neutrophils; a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases." J Cell Biol **131**: 775 - 789.
- Owen, C. A., M. A. Campbell, et al. (1995). "Cell surface bound elastase and cathepsin G on human neutrophils; a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases." J Cell Biol **131**: 775 - 789.
- Panda, A., A. Arjona, et al. (2009). "Human innate immunosenescence: causes and consequences for immunity in old age." Trends Immunol **30**: 325 - 333.
- Parr, D. G., A. J. White, et al. (2006). "Inflammation in sputum relates to progression of disease in COPD: a prospective study." Respir Res **7**: 136
- Patel, I. S., T. A. R. Seemungal, et al. (2002). "Relationship between bacterial colonisation and the frequency, character and severity of COPD exacerbations." Thorax **57**: 759 - 764.
- Pauwels, R. A., A. S. Buist, et al. (2001). "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 **163**: 1256 - 1276.
- Pavia, D. (1984). "Lung mucociliary clearance. In Clarke, S.W., editor." Aerosols and the lung. **Butterworths. Boston**.
- Pavord, I. D., M. M. Pizzichini, et al. (1997). "The use of induced sputum to investigate airway inflammation." Thorax **52**: 498 - 501.
- Persson, C. G. A., J. S. Erjefait, et al. (1998). "Plasma derived proteins in airway defence, disease and repair of epithelial injury." Eur Respir J **11**: 958 - 970.
- Pesci, A., B. Balbi, et al. (1998). "Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease." Eur Respir J **12**: 380 - 386.
- Pignatti, P., G. Moscato, et al. (2005). "Down regulation of CXCL8 receptors on neutrophils after recruitment." J Allergy Clin Immunol **115**: 88 - 94.
- Piitulainen, E., G. Tornling, et al. (1997). "Effect of age and occupational exposure to airway irritants on lung function in non-smoking individuals with alpha 1-antitrypsin deficiency (PiZZ)." Thorax **52**: 244 - 248.
- Pilette, C., B. Colinet, et al. (2007). "Increased galectin-3 expression and intra-epithelial neutrophils in small airways in severe chronic obstructive pulmonary disease." Eur Respir J **29**(5): 914 - 922.
- Pinto-Plata, V., H. Mullerova, et al. (2004). "C-Reactive protein in patients with COPD, control smokers and non-smokers." Thorax **350**: 1005 - 1012.
- Pizzichini, M. M., E. Pizzichini, et al. (1999). "Prednisolone-dependent asthma: inflammatory indices in induced sputum." Eur Respir J **13**: 15 - 21.

- Pletz, M. W., M. Ioanas, et al. (2004). "Reduced spontaneous apoptosis in peripheral blood neutrophils during exacerbations of COPD." Eur Respir J **23**: 532 - 537.
- Plotkowski, M. C., G. Beck, et al. (1989). "Adherence of pseudomonas aeruginosa to respiratory epithelium and the effect of leucocyte elastase." J Med Microbiol **30**: 285 - 293.
- Poulter, L. W. (1997). "Pulmonary macrophages. In Stockley, R.A., editor." Pulmonary defences **WileyEurope. Chichester.**: 77 - 92.
- Prescott, E., P. Lange, et al. (1995). "Chronic mucus hypersecretion in COPD and death from pulmonary infection." Eur Respir J **8**: 1333 - 1338.
- Puchelle, E., S. Girod de Bentzmann, et al. (1995). "Airway secretions and lung liquids. In Brewis, R.A.L., editor." Respiratory Medicine **W.B.Saunders. London**: 97 - 111.
- Puchelle, E., J. M. Zahm, et al. (1980). "Mucociliary transport in vivo and in vitro. Relations to sputum properties in chronic bronchitis." Eur J Respir Dis **61**: 254 - 264.
- Pye, A., R. A. Stockley, et al. (1995). "Simple methods for quantifying viable bacterial numbers in sputum." J Clin Pathol **48**: 719 - 724.
- Qiao, H., K. H. Sonoda, et al. (2005). "CXCR2 expression on neutrophils is upregulated during the relapsing phase of ocular Behcet Disease." Current Eye Research **30**(3): 195 - 203.
- Qin, L., W. M. Quinlan, et al. (1996). "The role of CD11/CD18 and ICAM1 in acute pseudomonas aeruginosa induced pneumonia." J Immunol **157**: 5016 - 5021.
- Quan, J. M., T. R. Martin, et al. (1996). "Antibodies against the N-terminus of IL-8 receptor A inhibit neutrophil chemotaxis." Biochem Biophys Res Commun **219**: 405 - 411.
- Rahman, I., D. Morrison, et al. (1996). "Systemic oxidative stress in asthma, COPD and smokers." Am J Respir Crit Care Med **154**: 1055 - 1060.
- Rainger, G. E., C. Buckley, et al. (1997). "Cross-talk between cell adhesion molecules regulates the migration velocity of neutrophils." Curr Biol **7**: 316 - 325.
- Rainger, G. E., A. C. Fisher, et al. (1997). "Endothelial-borne platelet activating factor and interleukin-8 rapidly immobilise rolling neutrophils." Am J Physiol **272**: H114 - H122.
- Ramamoorthy, C., S. S. Saski, et al. (1997). "CD18 adhesion blockade decreases bacterial clearance and neutrophil recruitment after intrapulmonary E Coli but not after Staph Aureus." J Leuk Biol **61**: 167 - 172.
- Rangasamy, T., C. Y. Cho, et al. (2004). "Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice." J Clin Invest **114**(9): 1248-59.
- Rao, K. M. K., M. S. Currie, et al. (1992). "Age-related alterations in actin cytoskeleton and receptor expression in human neutrophils." J Gerontol **47**: B37 - B44.
- Rao, R. V., E. Hermel, et al. (2001). "Coupling endoplasmic reticulum stress to the cell death program. Mechanisms of caspase activation." J Biol Chem **276**: 33869 - 33874.
- Ray, W. A., M. R. Griffin, et al. (1990). "Adverse drug reactions and the elderly." Health Affairs **3**: 114 - 122.
- Rennard, S. I., D. M. Daughton, et al. (1990). "Short-term smoking reduction is associated with reduction in measures of lower respiratory tract inflammation in heavy smokers." Eur Respir J **3**: 752 - 759.
- Renshaw, S. A., C. A. Loynes, et al. (2006). "A transgenic zebrafish model of neutrophilic inflammation." Blood **108**: 3976 - 3978.

- Retamales, I., M. W. Elliot, et al. (2001). "Amplification of inflammation in emphysema and its association with latent adenoviral infection." **Am J Respir Crit Care Med**: 469 - 473.
- Rice, W. G. and S. J. Weiss (1990). "Regulation of proteolysis at the neutrophil-substrate interface by secretory leukoprotease inhibitor." Science **249**(4965): 178-81.
- Richards, C. D. and J. Gauldie (1985). "IgA-mediated phagocytosis by mouse alveolar macrophages." Am Rev Respir Dis **132**: 82 - 85.
- Rickard, K. and S. Rennard (1989). "Neutrophil elastase causes detachment of bronchial epithelial cells from extracellular matrix." Am Rev Respir Dis **139**: 406.
- Rodriguez-Roisin, R. (2000). "Towards a consensus definition of COPD exacerbation." Chest **117**(5): 398s - 401s.
- Rubin, B. K., O. Ramirez, et al. (1992). "Mucus rheology and transport in neonatal respiratory distress syndrome and the effect of surfactant therapy." Chest **101**: 1080 - 1085.
- Rudolphus, A., J. A. Kramps, et al. (1994). "Intratracheally instilled anti-leukoprotease and alpha 1 proteinase inhibitor; effects on human neutrophil elastase-induced experimental emphysema and pulmonary localisation." Histochemistry **26**: 817 - 827.
- Russell, R. E., A. Thorley, et al. (2002). "Alveolar macrophage-mediated elastolysis: roles of matrix metalloproteinases, cysteine, and serine proteases." Am J Physiol Lung Cell Mol Physiol **283**(4): L867-73.
- Rusznak, C., P. R. Mills, et al. (2000). "Effect of cigarette smoking on the permeability and IL-1 beta and sICAM-1 release from cultured human bronchial epithelial cells of never smokers, smokers and patients with chronic obstructive pulmonary disease." Am J Respir Cell Mol Biol **23**: 530 - 536.
- Rutgers, S. R., W. Timens, et al. (2000). "Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD." Eur Respir J **15**: 109 - 115.
- Rydell-Tormanen, K., L. Uller, et al. (2006). "Direct evidence of secondary necrosis of neutrophils during intense lung inflammation." Eur Respir J **28**: 268 - 274.
- Rydell-Tormanen, K., L. Uller, et al. (2006). "Neutrophil cannibalism - a back up when the macrophage clearance system is insufficient. ." Respir Res **14**: 143 - 149.
- Ryttilä, P., M. Platani, et al. (2006). "Airway neutrophilia in COPD is not associated with increased neutrophil survival." Eur Respir J **28**: 1163 - 1169.
- Ryttilä, P. H., A. E. Lindqvist, et al. (2000). "Safety of sputum induction in chronic obstructive pulmonary disease." Eur Respir J **15**(6): 1116-9.
- Sabeh, F., I. Ota, et al. (2004). "Tumor cell traffic through the extra cellular matrix is controlled by the membrane anchored collagenases." J Cell Biol **167**: 769 - 781.
- Sadhu, C., B. Masinovsky, et al. (2003). "Essential role of Phosphoinositide 3-Kinase delta in neutrophil directional movement." J Immunol **170**: 2647 - 2654.
- Saetta, M., A. Di Stefano, et al. (1998). "CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease." Am J Respir Crit Care Med **157**(3 Pt 1): 822-6.
- Saetta, M., G. Turato, et al. (2001). "Cellular and structural basis of chronic obstructive pulmonary disease." Am J Respir Crit Care Med **163**: 1304 - 1309.
- Sakao, S., K. Tatsumi, et al. (2002). "Association of tumour necrosis factor alpha gene promoter polymorphism with low attenuation areas on high resolution CT in patients with COPD." Chest **122**: 416 - 420.

- Sallenave J.M. (2000). "The role of secretory leukocyte protease inhibitor and elafin as alarm anti proteinases in inflammatory lung disease." Respir Res **1**: 87 - 92.
- Sallenave J.M., J. Shulman, et al. (1994). "Regulation of secretory leukoprotease inhibitor and elastase specific inhibitor in human airway epithelial cells by cytokines and neutrophilic enzymes." Am J Respir Cell Mol Biol **11**: 733 - 741.
- Sanderson, M. J. and M. A. Sleight (1981). "Ciliary activity of cultured rabbit tracheal epithelium: beat pattern and metachrony." J Cell Sci **47**: 331 - 347.
- Sanford, A. J. and E. K. Silverman (2002). "Chronic Obstructive Pulmonary Disease.1: Susceptibility factors for COPD the genotype-environment interaction." Thorax **57**: 736 - 741.
- Sapey, E., A. Ahmad, et al. (2009). "Imbalances between interleukin-1 and tumor necrosis factor agonists and antagonists in stable COPD " J Clin Immunol **29**(4): 508 - 516.
- Sapey, E., Bayley D, et al. (2008). "The validation of assays used to measure biomarkers in exhaled breath condensate." Eur Respir J **32**(5): 1408 - 1409.
- Sapey, E., D. Bayley, et al. (2008). "Inter-relationships between inflammatory markers in stable COPD patients with bronchitis; the intra and inter patient variability." Thorax **63**: 493 - 499.
- Sapey, E. and R. A. Stockley (2006). "COPD exacerbations: Aetiology." Thorax **61**: 250 - 258.
- Sapey, E. and R. A. Stockley (2008). "The neutrophil and its special role in chronic obstructive pulmonary disease. " In Asthma and COPD: Basic Mechanisms and clinical management **Editor: P J Barnes**(Elsevier Publishing).
- Sapey, E., A. M. Wood, et al. (2010). "TNF alpha rs361525 polymorphism is associated with increased local production and downstream inflammation in COPD." Am J Respir Crit Care Med **200912-18460Cv1** **epub ahead of print**.
- Sasaki, T., J. Irie-Sasaki, et al. (2000). "Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration." Science **287**: 1040 - 1046.
- Scaffidi, C., S. Fulda, et al. (1998). "Two CD95 (APO-1/Fas) signaling pathways." Embo J **17**: 1675 - 1687.
- Schaberg, T., H. Haller, et al. (1992). "Superoxide anion release induced by platelet activating factor is increased in human alveolar macrophages from smokers." Eur Respir J **5**: 387 - 393.
- Scholz, D., B. Devaux, et al. (1996). "Expression of adhesion molecules is specific and time-dependent in cytokine stimulated endothelial cells in culture." Cell Tissue Res **284**: 415 - 423.
- Seemungal, T. A. R., G. C. Donaldson, et al. (1998). "Effect of exacerbation on quality of life in patients with chronic obstructive pulmonary disease." Am J Respir Crit Care Med **157**: 1418 - 1422.
- Segura-Valdez, L., A. Pardo, et al. (2000). "Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD." Chest **117**(3): 684-94.
- Selby, C., E. Drost, et al. (1991). "Neutrophil retention in the lungs of pateints with chronic obstructive pulmonary disease." Am Rev Respir Dis **143**(6): 1359 - 1364.
- Selby, C., E. M. Drost, et al. (1994). "Neutrophil sequestration in the lungs removed at surgery; the effect of microscopic emphysema." Am J Respir Crit Care Med **149**: 1526 - 1533.

- Selman, M., J. Cisneros-Lira, et al. (2003). "Matrix metalloproteinases inhibition attenuates tobacco smoke-induced emphysema in Guinea pigs." Chest **123**(5): 1633-41.
- Senior, R. M., H. Tegner, et al. (1977). "The induction of pulmonary emphysema with human leukocyte elastase." Am Rev Respir Dis **166**: 469 - 475.
- Shang, X. Z. and A. C. Issekutz (1997). "Beta-2 (CD18) and beta-1 (CD29) integrin mechanisms in migration of human polymorphonuclear leucocytes and monocytes through lung fibroblast barriers. Shared and distinct mechanisms." Immunol **92**: 527 - 535.
- Shang, X. Z., T. Yednock, et al. (1999). "alpha-9 beta-1 integrin is expressed on human neutrophils and contributes to neutrophil migration through human lung and synovial fibroblast barriers." J Leuk Biol **66**: 809 - 816.
- Shapiro, S. D. (2000). "Animal models for COPD." Chest **117**: 223S - 227S.
- Shelton, D. N., E. Chang, et al. (1999). "Microarray analysis of replicative senescence." Curr Biol **9**: 939 - 945.
- Shen, L. and M. W. Fanger (1981). "Secretory igA antibodies synergise with IgG in promoting ADCC by human polymorphonuclear cells, monocytes and lymphocytes." Cell Immunol **59**(75 - 81).
- Shim, J. J., K. Dabbagh, et al. (2001). "IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils." Am J Physiol Lung Cell Mol Physiol **280**(1): L134-40.
- Shimura, S. and T. Takishima (1994). "Airway submucosal gland secretion. In Shimura, S., editor." Airway secretion, physiological basis for the control of mucous hypersecretion. Marcel Decker. New York: 23 - 35.
- Shoburg, D. S., W. M. Quinlan, et al. (1994). "Cytokine production in response to stimuli that elicit either CD18 dependent or CD18 independent neutrophil emigration." Am J Respir Crit Care Med **147**: A1088.
- Signoret, N., L. Hewlett, et al. (2005). "Agonist-induced endocytosis of CC chemokine receptor 5 is clathrin dependent." Mol Biol Cell **16**: 902 - 917.
- Signoret, N., A. Pelchen-Matthews, et al. (2000). "Endocytosis and recycling of the HIV coreceptor CCR5." J Cell Biol **151**: 1281 - 1294.
- Simmons, M. S., J. E. Connett, et al. (2005). "Smoking reduction and the rate of decline in FEV1: results from the lung health study." Eur Respir J **25**: 1011 - 1017.
- Simons, K. (2000). "Lipid rafts and signal transduction." Nat Rev Mol Cell Biol **1**: 31 - 39.
- Sin, D. D., N. R. Anthonisen, et al. (2006). "Mortality on COPD. Role of co-morbidities." Eur Respir J **28**: 1245 - 1257.
- Singh, A., T. Rangasamy, et al. (2006). "Glutathione peroxidase 2, the major cigarette smoke-inducible isoform of GPX in lungs, is regulated by Nrf2." Am J Respir Cell Mol Biol **35**(6): 639-50.
- Skerrett, S. J. and T. R. Martin (1994). "Intratracheal interferon gamma augments pulmonary defenses in experimental legionellosis." Am J Respir Crit Care Med **149**: 50 - 58.
- Slee, E. A., M. T. Harte, et al. (1999). "Ordering the cytochrome C-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8 and -10 in a caspase-9-dependent manner." J Cell Biol **276**: 281 - 292.
- Sleigh, M. A., J. R. Blake, et al. (1988). "The propulsion of mucus by cilia." Am Rev Respir Dis **137**: 726 - 741.

- Smallman, L. A., S. L. Hill, et al. (1984). "Reduction of ciliary beat frequency in vitro by sputum from patients with bronchiectasis: a serine proteinase effect." Thorax **39**: 663 - 667.
- Smart, S. J. and T. B. Casale (1994). "TNF alpha induced transendothelial neutrophil migration is IL8 dependent." Am J Physiol **266**: L238 - L245.
- Snedecor, G. W. and W. G. Cochran (1989). "Statistical Methods. 8th Edition." Ames Iowa State Press.
- Snider, G. L., J. A. Hayes, et al. (1974). "Relationship between elasteolytic activity and experimental emphysema inducing properties of papain preparations." Am Rev Respir Dis **110**: 254 - 257.
- Soutar, C. A. (1976). "Distribution of plasma cells and other cells containing immunoglobulin in the respiratory tract of normal man and class of immunoglobulins contained therein." Thorax **31**: 158 - 166.
- Sparrow, D., R. J. Glynn, et al. (1984). "The relationship of the peripheral leukocyte count and cigarette smoking to pulmonary function among adult men." Chest **86**: 383 - 386.
- Spertini, O., F. W. Luscinskas, et al. (1991). "Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion." J Immunol **147**: 2565 - 2578.
- Stanescu, D., A. Sanna, et al. (1996). "Airways obstruction, chronic expectoration and rapid decline in FEV1 in smokers are associated with increased levels of sputum neutropils." Thorax **51**: 267 - 271.
- Steadman, R., P. L. St John, et al. (1997). "Human neutrophils do not degrade major basement membrane components during chemotactic migration." Int J Biochem Cell Biol **29**: 993 - 1004.
- Stegmaier, M., A. Levinovitz, et al. (1995). "The E-selectin ligand ESL-1 is a variant of a receptor for fibroblast growth factor." Nature **373**: 615 - 620.
- Stockley, R. A. (1983). "Proteolytic enzymes, their inhibitors and lung diseases." Clin. Sci **64**: 119 - 126.
- Stockley, R. A. (1997). "Soluble proteins in lung defence. In Stockly, R.A., editor." Pulmonary defences WileyEurope. Chicester.: 17 - 38.
- Stockley, R. A. (2002). "Neutrophils and the pathogenesis of COPD." Chest **121**: 151S - 155S.
- Stockley, R. A., D. Bayley, et al. (2001). "Assessment of airway neutrophils by sputum colour: correlation with airways inflammation." Thorax **56**: 366 - 372.
- Stockley, R. A., M. Mistry, et al. (1979). "A study of plasma proteins in the sol phase of sputum from patients with chronic bronchitis. ." Thorax **34**: 777 - 782.
- Stockley, R. A., J. Shaw, et al. (1990). "Effect of alpha 1 proteinase inhibitor on neutrophil chemotaxis." Am J Respir Cell Mol Biol **2**: 163 - 170.
- Stone, P. J., E. C. Lucey, et al. (1990). "Alpha 1-protease inhibitor moderates human neutrophil elastase-induced emphysema and secretory cell metaplasia in hamsters." Eur Respir J **3**(6): 673-8.
- Strassburg, A., D. Droemann, et al. (2004). "Enhanced PMN response in chronic bronchitis and community acquired pneumonia." Eur Respir J **24**: 772 - 778.
- Strehler, B. L., B. L. Mark, et al. (1959). "Rate and magnitude of age pigmented accumulation in human myocardium." J Gerontol **14**: 430 - 439.
- Strunk, R. C., D. M. Eidlen, et al. (1988). "Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. ." J Clin Invest **81**: 1419 - 1426.

- Szegezdi, E., U. Fitzgerald, et al. (2003). "Caspase-12 and ER-stress-mediated apoptosis: the story so far." Ann N Y Acad Sci **1010**: 186 - 194.
- Takeyama, K., C. Agusti, et al. (1998). "Neutrophil-dependent goblet cell degranulation: role of membrane-bound elastase and adhesion molecules." Am J Physiol **275**(2 Pt 1): L294-302.
- Tang, D. and V. J. Kidd (1998). "Cleavage of DFF-45/ICAD by multiple caspases is essential for its function during apoptosis." J Biol Chem **273**: 28549 - 28552.
- Tashkin, D. P., V. A. Clark, et al. (1984). "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 **130**: 707 - 715.
- Taube, C., O. Holz, et al. (2001). "Airway response to inhaled hypertonic saline in patients with moderate to severe chronic obstructive pulmonary disease." Am J Respir Crit Care Med **164**(10 Pt 1): 1810-5.
- Thepen, T., E. Claassen, et al. (1993). "Migration of alveolar macrophages from alveolar space to paracortical T area of draining lymph node." Adv Exp Med Biol **329**: 305 - 310.
- Thompson, A. B., D. Daughton, et al. (1989). "Intraluminal airway inflammation in chronic bronchitis. Characterization and correlation with clinical parameters." Am Rev Respir Dis **140**(6): 1527 - 1537.
- Thompson, R. D., K. E. Noble, et al. (2001). "Platelet-endothelial cell adhesion molecule-1 (PECAM-1) deficient mice demonstrate a transient and cytokine specific role for PECAM-1 in leucocytes migration through perivascular basement membrane." Blood **97**: 1854 - 1860.
- Tkalcevic, J., M. Novelli, et al. (2000). "Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G." Immunity **12**: 210 - 213.
- Tomee, J. F., G. H. Koeter, et al. (1998). "Secretory leukoprotease inhibitor: a native anti-microbial protein presenting a new therapeutic option?" Thorax **53**: 114 - 116.
- Tonnesen, M. G., D. C. Anderson, et al. (1989). "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 **83**: 637 - 642.
- Tortorella, C., O. Simone, et al. (2007). "Age-related impairment of GM-CSF induced signalling in neutrophils; role of SHP-1 and SOCS protein." Aging Res Rev **6**: 81 - 93.
- Tos, M. (1970). "Mucus glands of the trachea in children. Quantitative studies." Anat Anz. **126**: 146 - 160.
- Traves, S. L., S. V. Culpitt, et al. (2002). "Increased levels of the chemokine GRO alpha and MCP-1 in sputum samples from patients with COPD." Thorax **57**: 590 - 595.
- Traves, S. L., S. J. Smith, et al. (2004). "Specific CXC but not CC chemokines cause elevated monocyte migration in COPD. A role for CXCR2." J Leuk Biol **76**: 441 - 450.
- Tsoumakidou, M., N. Tzanakis, et al. (2005). "Changes in T-lymphocyte subpopulations at the onset of severe exacerbations of chronic obstructive pulmonary disease." Respir Med **99**: 572 - 579.
- Tsuji, T., K. Aoshiba, et al. (2004). "Cigarette smoke induces senescence in alveolar epithelial cells." Am J Respir Cell Mol Biol **31**: 643 - 649.

- Tsuji, T., K. Aoshiba, et al. (2006). "Alveolar cell senescence in patients with pulmonary emphysema." Am J Resp Crit Care Med **174**: 886 - 893.
- Tuder, R. M., L. Zhen, et al. (2003). "Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade." Am J Respir Cell Mol Biol **29**(1): 88-97.
- Tuomanen, E. I. and J. O. Hendley (1983). "Adherence of Bordetella pertussis to human respiratory epithelial cells." J Infect Dis **148**: 125 - 130.
- Turner, J. M., J. Mead, et al. (1968). "Elasticity of human lungs in relation to age." J Appl Physiol **25**: 664 - 671.
- Uller, L., C. G. Persson, et al. (2006). "Resolution of airway disease: removal of inflammatory cells through apoptosis, egression or both." Trends Pharmacol Sci **27**(9): 461 - 466.
- van Buul, J. D., M. J. Allingham, et al. (2007). "RhoG regulates endothelial apical cup assembly downstream from ICAM1 engagement and is involved in leukocyte trans-endothelial migration." J Cell Biol **178**: 1279 - 1293.
- Van Eeden, S. F. and J. C. Hogg (2000). "The response of human bone marrow to chronic cigarette smoking." Eur Respir J **15**: 915 - 921.
- van Zuijlen, D. A., E. A. van de Graaf, et al. (2001). "Measuring plasma exudation in nasal lavage fluid and in induced sputum as a tool for studying respiratory tract inflammation." J Immunol Methods **256**: 1 - 10.
- Vanhaesebroeck, B., S. J. Leever, et al. (2001). "Synthesis and function of 3-phosphorylated inositol lipids." Annu Rev Biochem **70**: 535 - 602.
- Verbeken, E. K., M. Cauberghs, et al. (1992). "The senile lung: comparison with normal and emphysematous lungs." Chest **101**: 793 - 799.
- Verhoeven, G. T., J. P. Hegmans, et al. (2002). "Effects of fluticasone propionate in COPD patients with bronchial hyperresponsiveness." Thorax **57**(8): 694-700.
- Vernooy, J. H., J. H. Lindeman, et al. (2004). "Increased activity of matrix metalloproteinase-8 and matrix metalloproteinase-9 in induced sputum from patients with COPD." Chest **126**(6): 1802-10.
- Vestbo, J., E. Prescott, et al. (1996). "Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group. ." Am J Respir Crit Care Med **153**: 1530 - 1535.
- Vignola, A. M., A. Bonanno, et al. (1998). "Increased levels of elastase and alpha1-antitrypsin in sputum of asthmatic patients." Am J Respir Crit Care Med **157**(2): 505-11.
- Vogt, W. (2000). "Cleavage of the fifth component of complement and generation of a functionally active C5b6-like complex by human leukocyte elastase." Immunobiology **201**: 470 - 477.
- Vuilleminot, B. R., J. F. Rodriguez, et al. (2004). "Lymphoid tissue and emphysema in the lungs of transgenic mice inducibly expressing TNF alpha." Am J Respir Crit Care Med **30**: 438 - 448.
- Wagner, J. G. and R. A. Roth (2000). "Neutrophil migration mechanisms with an emphasis on pulmonary vasculature." Pharmacological Review **52**: 349 - 374.
- Walchek, B., J. Kahn, et al. (1996). "Neutrophil rolling altered by inhibition of L-Selectin shedding in vitro." Nature **380**: 720 - 723.
- Wallace, A. M. and A. J. Sandford (2002). "Genetic polymorphisms of matrix metalloproteinases: functional importance in the development of chronic obstructive pulmonary disease?" Am J Pharmacogenomics **2**(3): 167-75.

- Wang, F., P. Herzmark, et al. (2002). "Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils." Nat Cell Biol **4**: 513 - 518.
- Wang, S., M. B. Voisin, et al. (2006). "Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils." J Exp Med **203**: 1519 - 1532.
- Weber, I. (2006). "Is there a pilot in a pseudopod?" Eur J Cell Biol **85**: 915 - 924.
- Weiss, S. J., J. Young, et al. (1981). "Role of hydrogen peroxide in neutrophil mediated destruction of cultured endothelial cells." J Clin Invest **68**: 714 - 721.
- Weitz, J. I., A. J. Huang, et al. (1987). "Elastase-mediated fibrinogenolysis by chemoattractant-stimulated neutrophils occurs in the presence of physiologic concentrations of antiproteases." J Exp Med **166**(6): 1836-50.
- Wenisch, C., S. Patruta, et al. (2000). "Effect of age on human neutrophil function." J Leuk Biol **67**: 40 - 45.
- Wessels, D., D. F. Lusche, et al. (2007). "PTEN plays a role in the suppression of lateral pseudopod formation during Dictyostelium motility and chemotaxis." J Cell Sci **120**: 2517 - 2531.
- White, A. J., S. Gompertz, et al. (2003). "Resolution of bronchial inflammation is related to bacterial eradication following treatment of exacerbations with chronic bronchitis." Thorax **58**: 680 - 685.
- Whitelaw, D. A., B. L. Rayner, et al. (1992). "Community acquired bacteremia in the elderly - a prospective study of 121 cases." J Am Geriatr Soc **40**: 996 - 1004.
- Widdicombe, J. g. and R. J. Pack (1982). "The Clara cell." Eur J Respir Dis **63**: 202 - 220.
- Widdicombe, J. H. and J. G. Widdicombe (1995). "Regulation of human airway surface liquid." Respir Physiol **99**: 3 - 12.
- Wiggins, J., S. L. Hill, et al. (1983). "Lung secretion sol-phase proteins: comparison of sputum with secretions obtained by direct sampling." Thorax **38**: 102 - 107.
- Willems, L. N., C. J. Otto-Verberne, et al. (1986). "Detection of antileukoprotease in connective tissue of the lung." Histochemistry **86**: 165 - 168.
- Williams, M. A. and J. S. Solomkin (1999). "Integrin mediated signaling in human neutrophil functioning." J Leuk Biol **65**: 725 - 736.
- Wilson, D. O., R. M. Rogers, et al. (1989). "Body weight in chronic obstructive pulmonary disease; the National Institutes of Health intermittent positive pressure breathing trial. ." Am Rev Respir Dis **139**: 1435 - 1438.
- Wilson, R., T. Pitt, et al. (1987). "Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia in vitro." J Clin Invest **79**: 221 - 229.
- Wilson, R. and C. F. Rayner (1997). "Pathogenic mechanisms of bacteria causing bronchial infections. In Stockley, R.A., editor." Pulmonary defences WileyEurope. Chichester.: 267 - 287.
- Wise, R. A. (2006). "The value of forced expiratory volume in one second decline in the assessment of chronic obstructive pulmonary disease progression." Am J Med **119**: 4 - 11.
- Woolhouse, I. S., D. Bayley, et al. (2005). "Endothelial interactions of neutrophils under flow in chronic obstructive pulmonary disease." Eur Respir J **25**: 612 - 617.
- Woolhouse, I. S., D. Bayley, et al. (2002). "Sputum chemotactic activity in chronic obstructive pulmonary disease; effect of alpha 1 anti-trypsin deficiency and the role of leukotriene B4 and interleukin 8." Thorax **57**: 709 - 714.

- Woolhouse, I. S., Bayley D.L., et al. (2002). "Effect of sputum processing with dithiothreitol on the detection of inflammatory mediators in chronic bronchitis and bronchiectasis." Thorax **57**(8): 667 - 671.
- Woolhouse, I. S., D. L. Bayley, et al. (2005). "Endothelial interactions of neutrophils under flow in chronic obstructive pulmonary disease." Eur Respir J **25**(4): 612 - 617.
- Woolhouse, I. S., S. L. Hill, et al. (2001). "Symptom resolution assessed using a patient directed diary card during treatment of acute exacerbations of chronic bronchitis." Thorax **56**(12): 947 - 953.
- World Health Organization (2007). "Chronic Obstructive Pulmonary Disease (COPD). Fact sheet 315." www.who.int/mediacentre/factsheet/fs315.
- Wright, D. C. and J. L. Gallin (1979).
- Wright, D. C. and J. L. Gallin (1979). "Secretory responses of human neutrophils: Exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo." J Immunol **123**: 258 - 294.
- Wright, J. L., S. G. Farmer, et al. (2002). "Synthetic serine elastase inhibitor reduces cigarette smoke induced emphysema in guinea pigs." Am J Respir Crit Care Med **166**: 954 - 960.
- Wright, J. L., H. Tai, et al. (2007). "Cigarette smoke up-regulates pulmonary vascular matrix metalloproteinases via TNF alpha signaling." Am J Physiol Lung Cell Mol Physiol **292**: L125 - 133.
- Wu, D. (2005). "Signaling mechanisms for regulation of chemotaxis." Nature Cell Res **15**: 52 - 56.
- Yadav, R., K. Y. Larbi, et al. (2002). "Migration of leukocytes through the vessel wall and beyond." Thromb Haemost **90**: 598 - 606.
- Yamagata, T., H. Sugiura, et al. (2007). "Over expression of CD-11b and CXCR1 on circulating neutrophils; its possible role in COPD." Chest **132**: 890 - 899.
- Yamagata, T., H. Sugiura, et al. (2007). "Overexpression of CD-11b and CXCR1 on circulating neutrophils: Its possible role in COPD." Chest **132**: 890 - 899.
- Yamamoto, C., T. Yoneda, et al. (1997). "Airway inflammation in COPD assessed by sputum levels of interleukin-8." Chest **112**: 505 - 510.
- Yang, W., D. Wang, et al. (1999). "Role of Clathrin-mediated Endocytosis in CXCR2 Sequestration, Resensitization and Signal Transduction." J Biol Chem **274**: 11328 - 11333.
- Yeung, M. C. and A. D. Buncio (1984). "Leukocyte count, smoking and lung function." Am J Med **76**: 383 - 386.
- Yoshida, K., R. Kondo, et al. (2006). "Neutrophil cytoskeletal rearrangements during capillary sequestration in bacterial pneumonia in rats." Am J Respir Crit Care Med **174**: 3040 - 3045.
- Yoshikawa, T., G. Dent, et al. (2007). "Impaired neutrophil chemotaxis in chronic obstructive pulmonary disease." Am J Respir Crit Care Med **175**: 473 - 479.
- Yoshioka, A., T. Betsuyaku, et al. (2005). "Excessive neutrophil elastase in bronchoalveolar lavage fluid from patients with sub-clinical emphysema." Am J Respir Crit Care Med **152**: 2127 - 2132.
- Yum, H. K., J. Arcaroli, et al. (2001). "Involvement of phosphoinositide 3-kinases in neutrophil activation and the development of acute lung injury." J Immunol **167**: 6601 - 6608.
- Zay, K., S. Loo, et al. (1999). "Role of neutrophils and alpha1-antitrypsin in coal- and silica-induced connective tissue breakdown." Am J Physiol **276**(2 Pt 1): L269-79.

- Zhang, X., P. Shan, et al. (2006). "Toll-like receptor 4 deficiency causes pulmonary emphysema." J Clin Invest **116**(11): 3050-9.
- Zhao, J. J., H. Cheng, et al. (2006). "The p110 gammaalpha isoform of PI3K is essential for proper growth factor signalling and oncogene transformation." Proc Natl Acad Sci USA **103**: 16296 - 16300.
- Zhelev, D. V., A. M. Alteraifi, et al. (2004). "Controlled pseudopod extension of human neutrophils stimulated with different chemoattractants." Biophys J **87**: 688 - 695.
- Zhong, N. S., P. X. Ran, et al. (2004). "Burden of chronic obstructive lung disease (BOLD) Project in South China. ." Am J Resp Crit Care Med **169**: A603.
- Zicha, D., G. A. Dunn, et al. (1991). "A new direct-viewing chemotaxis chamber." J Cell Sci **99 (Pt 4)**: 769 - 775.
- Zou, H., W. J. Henzel, et al. (1997). "Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3." Cell **90**: 405 - 413.

11 Appendices

11.1 Abbreviations in alphabetical order

| | |
|--------------|--|
| A1AT | Alpha one anti-trypsin |
| A1ATD | Alpha one anti-trypsin deficiency |
| Akt | Protein kinases B |
| Cdc42 | Cell division control protein 42 |
| CI | 95% confidence intervals |
| COPD | Chronic Obstructive Pulmonary Disease |
| DDT | Dithiothreitol |
| FEV1 | Forced expiratory volume in one second |
| FVC | Forced vital capacity |
| fMLP | N-formyl-methionyl leucylphenylalanine |
| GEF | Guanine nucleotide exchange factor |
| GRO α | Growth related oncogene alpha |
| GTP | Guanosine triphosphate |
| HC | Healthy control |
| ICAM-1 | Intracellular adhesion molecule 1 |
| Ig | Immunoglobulin |
| I κ B | I kappa B |
| IL-1 β | Interleukin-1 beta |
| IL-8 | Interleukin 8 |
| LTB4 | Leukotriene B4 |
| Mac-1 | Macrophage antigen 1 |
| MD | Mean Difference |
| MPO | Myeloperoxidase |
| NE | Neutrophil elastase |
| Pak 1 | P21-activated kinase (Serine/threonine-protein kinase PAK1) |
| PI | Phosphatidylinositol |
| PIP | Phosphatidylinositol-4-phosphate |
| PIP2 | Phosphatidylinositol – 4,5 bisphosphate |
| PIP3 | Phosphatidylinositol – 3,4,5 trisphosphate |
| PI3K | Phosphoinositide 3-kinase |
| PIX α | PAK-associated guanine nucleotide exchange factor |
| PLC | Phospholipase C |
| PMN | Polymorphonuclear cell (neutrophil) |
| P-Rex – 1 | Phosphatidylinositol – 3,4,5 trisphosphate dependent Rac exchanger 1 protein |
| PTEN | Protein Tyrosine Phosphatase |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SLPI | Secretory leukoprotease inhibitor |
| TNF α | Tumour necrosis factor alpha |
| VCAM-1 | Vascular cell adhesion molecule-1 |

11.2 Publications arising from this thesis

11.2.1 Published Articles

Sapey, E., Bayley, D., Ahmad, A., Newbold, P., Snell, N., Stockley, R.A. 2008. Inter-relationships between inflammatory markers in stable COPD patients with bronchitis; the intra and inter patient variability. *Thorax*: 63; 493 – 499.

Sapey E, Stockley RA. The neutrophil and its special role in chronic obstructive pulmonary disease. In *Asthma and COPD; Basic mechanisms and clinical management*. Editor Barnes PJ. Elsevier Publishing. 2008.

Sapey, E., Ahmad, A., Bayley, D., Newbold, P., Snell, N., Stockley R.A. 2009. Imbalances between Interleukin-1 and Tumour Necrosis Factor agonists and antagonists in stable COPD. *Journal of Clinical Immunology*. 29; 508 – 516

Panda A, Arjona A, Sapey E, Bai F, Fikrig E, Montgomery RR, Lord JM, Shaw AC. Human innate immunosenescence: causes and consequences for immunity in old age. *Trends Immunol*. 2009. 30: 325-33

11.2.2 Published Abstracts

Sapey E, Bayley DL, Ahmad A, Griffiths D, Newbold P, Stockley RA. Growth related oncogene alpha does not relate to inflammatory change in COPD. *European Respiratory Society Annual Congress*: 2005, P3170

Sapey E, Bayley DL, Ahmad A, Griffiths D, Newbold P, Stockley RA. Studies of Interleukin-1beta and its receptor IL-1 Receptor 2 and antagonist IL-1 Receptor Antagonist in stable COPD. *British Thoracic Society Winter Meeting*: 2005, P108

Newbold P., Bayley D., Sapey E., Ahmad A., Parker D., Bengtsson T., Snell NJC, Stockley RA. Evidence to support a role for IL-1 β in the COPD disease process. *American Thoracic Society Conference* 2005 J30

Ahmad A, Bayley D, Sapey E, Carribino N, Stockley RA. Myeloid related protein(MRP) 8/14 is associated with increased inflammatory markers in bronchial secretions from patients with COPD. . *European Respiratory Society Annual Congress*: 2005, P3177

Sapey E, Bayley D, Griffiths D, Newbold P, Stockley RA. Evidence of an inflammatory overspill from lung to plasma in chronic obstructive pulmonary disease. *American Thoracic Society Annual Meeting*. 2006, P1547

Sapey E, Bayley D, Griffiths D, Newbold P, Stockley RA. High sensitivity C Reactive Protein levels mark pulmonary and systemic inflammation in stable COPD. *European Respiratory Society Annual Congress*: 2006, E2019

Sapey E, Bayley DL, Gompertz S, Stockley RA. High Sensitivity C Reactive Protein Levels correlate with pulmonary inflammation and FEV1 in stable chronic obstructive

disease. *British Thoracic Society Winter Meeting*. 2006, 61; Supplement II, P166A; ii111.

Sapey E, Bayley DL, Newbold P, Stockley RA. TNF α and its antagonists in plasma and sputum in stable COPD. . *American Thoracic Society Annual Meeting*. 2007, P950355

Sapey E, Bayley DL, Newbold P, Stockley RA. The variability of inflammatory mediators within and between patients with stable COPD. *American Thoracic Society Annual Meeting*. 2007, P950355

Sapey E, Ahmad A, Bayley DL, Newbold P, Snell N, Stockley RA.. The contribution of Growth Related Oncogene alpha to chemotaxis in chronic obstructive pulmonary disease and alpha 1 anti-trypsin deficiency. *British Thoracic Society Winter Meeting*. 2007

Sapey E, Ahmad A, Bayley DL, Insall RH, Stockley RA. Aberrant chemotaxis in neutrophils in COPD. *American Thoracic Society Annual Meeting*. 2008. A605

Sapey E, Ahmad A, Bayley D, Insall RH, Stockley RA. Chemotactic dynamics of COPD neutrophils. *European Respiratory Society Annual Congress*. 2008. E4228

Sapey E, Ahmad A, Newbold, P, Gaw, A., Stockley, R.A. The effects of a CXCR2 antagonist on neutrophil migration in vitro. . *American Thoracic Society Annual Meeting*. 2009 A1331

Sapey E, Ahmad, A., Bayley D., Newbold P, Snell N., Rugman P, Stockley RA. Imbalances between Interleukin-1 and Tumour Necrosis Factor agonists and antagonists in stable COPD. . *American Thoracic Society Annual Meeting*. 2009 A2884.

Sapey E, Ahmad A, Bayley D, Insall RH, Stockley RA. Aberrant chemotaxis of blood neutrophils in COPD: correcting abnormal migration. . *American Thoracic Society Annual Meeting* 2009. A5191

Sapey E, N Aaronson, A Ahmad, H Chahal, A Tatlow, R.H. Insall, R A Stockley, J M Lord. Aberrant peripheral neutrophil migration in the healthy elderly. *British Society for Research on Ageing annual congress*. 2009

Sapey E, Ahmad A, Wood A, Griffiths D, Edgar R, Stockley RA. Imbalances in TNF α in patients with a TNF α polymorphism and a COPD phenotype. *European Respiratory Society Annual Congress*. 2009

Sapey E, H Green, H Chahal, A Love, N Aaronson, R.H. Insall, R A Stockley, J M Lord. Aberrant neutrophil migration in the healthy elderly is a non-specific phenomenon. *American Aging Association Annual Congress* 2010

11.2.3 Submitted articles

Sapey, E., Ahmad, A., Stockley, J., Greenwood, H., Bayley, D., Insall, R.H., Stockley, R.A. 2010. Behavioural and structural differences in migrating peripheral neutrophils from patients with COPD. *Am J Respir Crit Care Med*.

Sapey E., Greenwood H., Love A., Chalal H., Insall, R.A., Stockley, R.A., Lord, J.M. Aberrant neutrophil migration in the healthy elderly. *Aging Cell*.