PHARMACOLOGICAL TARGETING OF NEUTROPHILIC AIRWAY INFLAMMATION IN COPD

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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Vandana Gupta

School of Medicine
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Abstract
A Thesis submitted by Vandana Gupta for the degree of Doctor of Philosophy at the University of Manchester.
Title: Pharmacological targeting of neutrophilic airway inflammation in COPD.
Month and year of submission: September 2014

Background: COPD is characterised by increased neutrophilic inflammation which further increases during exacerbations. Corticosteroids are currently one of the mainstays of treatment but they have limited effectiveness; there is a great need to develop new anti-inflammatory pharmacotherapies for use in COPD. Inhaled LPS has been used as a model of increased neutrophilic inflammation in healthy patients, smokers and asthmatics. Its use in patients with COPD as a model of exacerbations has not yet been evaluated. PI3 kinase is a vital intracellular enzyme, which upon activation leads to a number of cellular processes; the γ and δ isoforms of the enzyme are of particular importance in leucocyte migration, development and activation. There is increasing evidence for upregulation of this pathway in COPD.

Aims: (1) To test the safety of the use of inhaled LPS in patients with COPD for use as a model of exacerbation and to investigate the systemic and airway inflammatory response in vivo. (2) To investigate the action of PI3 kinase enzyme inhibitors and dexamethasone in vitro on neutrophilic inflammation in COPD patients during the stable state and exacerbations.

Methods: (1) 12 patients with mild to moderate COPD inhaled 5µg LPS; safety measurements and airway and systemic biomarkers were collected up to 24 hours post inhalation. (2) The effect of PI3 kinase enzyme inhibitors and dexamethasone on MMP-9 and ROS release from peripheral and airway neutrophils from stable COPD and exacerbations was examined in vitro. The effect of PI3 kinase enzyme inhibitors and dexamethasone on cytokine release from peripheral neutrophils from stable COPD patients was also investigated.

Results: (1) Inhaled LPS (5µg) caused a significant fall in FEV1 and increase in sputum neutrophil numbers. There was an associated increase in systemic IL-6, CRP and CC-16, all with differing temporal patterns. No patients reported any significant symptoms. (2) PI3 kinase enzyme inhibitors significantly reduced MMP-9 and ROS release from airway and peripheral neutrophils from COPD patients in the stable state and during exacerbations; dexamethasone had minimal effect. Cytokine release from peripheral neutrophils from COPD patients in the stable state was also significantly inhibited by PI3 kinase enzyme inhibitors and dexamethasone.

Conclusions: (1) Inhaled LPS in patients with COPD is a safe model to induce acute on chronic neutrophilic inflammation and therefore could be used as a model to study COPD exacerbations. (2) PI3 kinase enzyme inhibitors reduce COPD neutrophil MMP-9, ROS and cytokine release in vitro and are therefore are a promising new anti-inflammatory pharmacotherapy.
Declaration

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http://www.manchester.ac.uk/library/aboutus/regulations) and in The University’s policy on presentation of Theses.
Preface

To recruit the patients for these studies, a search was performed on the Medicines Evaluation Unit volunteer database to identify COPD patients that could potentially be used in the study. I screened the patients involved in these studies which included performing lung function measurements and the collection and processing of all biological samples. However, I did receive help from my colleagues at the Medicines Evaluation Unit and the Education Research Centre in collecting some of this data. Elastase assays were performed at GlaxoSmithKlein, Stevenage, UK, and CRP was analysed at the Clinical Sciences Building, University Hospital South Manchester, UK. The cell apoptosis experiments were performed in conjunction with a fellow PhD student, Jonathan Lemon, who was also investigating the effect of PI3 kinase enzyme inhibitors on neutrophil functions. I also had help from research scientists in performing western blot assays and flow cytometry experiments. Dr Abid Khan helped me in particular with flow cytometry experiments and in designing and performing some cell culture experiments.

Finally, I was fully responsible for the statistical analysis and the interpretation of all the data from the experimental chapters.
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I wish to thank Dr Jonathan Plumb for all of the advice and guidance he has given me throughout my PhD. My colleagues at the Education and Research Centre and the Medicines Evaluation Unit have been invaluable over the last few years; in particular I wish to thank Matt Rossall, Umme Kolsum, Naimat Khan, Abid Khan and Jonathan Lemon for all their patience, encouragement and assistance when I needed it the most.

Finally, I dedicate this thesis to my wonderful family. Without their unconditional love and support this thesis would not have been possible.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
</tr>
<tr>
<td>AP</td>
<td>Activator protein</td>
</tr>
<tr>
<td>ARDS</td>
<td>Adult Respiratory Distress Syndrome</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell-activating factor</td>
</tr>
<tr>
<td>BAL</td>
<td>Broncho-alveolar Lavage</td>
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<tr>
<td>BIRB796</td>
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</tr>
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<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
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<td>Clara cell protein 16</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine (C-C motif) receptor</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-guanine dinucleotide</td>
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<td>CRP</td>
<td>C Reactive Protein</td>
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<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCC</td>
<td>Differential cell count</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropylfluorophosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECLIPSE</td>
<td>Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophilic cationic protein</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>eNO</td>
<td>Exhaled nitric oxide</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin</td>
</tr>
<tr>
<td>EU</td>
<td>Endotoxin unit</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>FOXO</td>
<td>Foxhead box protein O</td>
</tr>
<tr>
<td>FPR</td>
<td>fMLP receptor</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>GAP</td>
<td>GTPase accelerating protein</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanosine nucleotide exchange factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GOLD</td>
<td>Global Initiative for Obstructive Lung Disease</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid response elements</td>
</tr>
<tr>
<td>GRO</td>
<td>Growth related oncogene</td>
</tr>
<tr>
<td>GSK</td>
<td>GlaxoSmithKlein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacyt lase</td>
</tr>
<tr>
<td>HEX</td>
<td>Healthy ex smoker</td>
</tr>
<tr>
<td>HNL</td>
<td>Human neutrophil lipocalin</td>
</tr>
<tr>
<td>HNS</td>
<td>Healthy non smoker</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Healthy smoker</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>ICC</td>
<td>Intraclass coefficient</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor associated kinases</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LABA</td>
<td>Long acting β agonist</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipopolysaccharide binding protein</td>
</tr>
<tr>
<td>LFA</td>
<td>Lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTB4</td>
<td>Leukotriene B4</td>
</tr>
<tr>
<td>MAC</td>
<td>Macrophage-1 antigen</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose Binding Lectin</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid leukaemia cell differentiation protein</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MEF</td>
<td>Myocyte enhancer factor</td>
</tr>
<tr>
<td>MEKK</td>
<td>Mitogen activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inhibitory protein</td>
</tr>
<tr>
<td>MKK</td>
<td>Mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>mMRC</td>
<td>Modified medical research council</td>
</tr>
<tr>
<td>MPIF</td>
<td>Myeloid progenitor inhibitory factor</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>Myd88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>NRF2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PARC</td>
<td>Pulmonary and activation-regulated chemokine</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline containing 0.05% tween 20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDE4</td>
<td>Phosphodiesterase 4</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PGP</td>
<td>Proline-glycine-proline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Phospho</td>
<td>Phosphorylated</td>
</tr>
<tr>
<td>PI3</td>
<td>Phosphoinositide-3</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol 3, 4, 5-triphosphate [PI(3,4,5)P₃]</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PSB</td>
<td>Protected sheath brush</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted on chromosome 10</td>
</tr>
<tr>
<td>Rac</td>
<td>Ras-related C3 botulinum toxin substrate</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised controlled trial</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SABA</td>
<td>Short acting β agonist</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Sd</td>
<td>Syndecin</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal cell-derived factor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2 domain containing 5 inositol phosphatase</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble-N-ethylmaleimide sensitive accessory-protein receptors</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SPD</td>
<td>Surfactant protein D</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TAK</td>
<td>Transforming growth factor activated kinase</td>
</tr>
<tr>
<td>TCC</td>
<td>Total cell count</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TH₂</td>
<td>Helper T lymphocyte</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteins</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumour necrosis factor receptor associated factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRIF</td>
<td>Toll-IL-1R domain containing adaptor inducing IFN-β</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigen</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 General introduction

Chronic Obstructive Pulmonary Disease (COPD) causes significant morbidity and mortality and is predicted to become the third commonest cause of death in the western world by 2020. COPD is characterised by progressive airflow obstruction which is poorly reversible and by increased airway and systemic neutrophilic inflammation. Exacerbations or acute worsenings are common, and patients who frequently exacerbate have a more rapid decline in lung function and poorer quality of life. Exacerbations are characterised by a further increase in neutrophilic inflammation. At present there are very few pharmacotherapies available to clinicians to treat exacerbations.

Neutrophils have a vital role in antimicrobial responses. They interact with microbial ligands to initiate a number of responses including phagocytosis, degranulation and the release of oxidants, proteinases and other cytotoxic products. In COPD, these functions also significantly contribute to tissue damage and lead to excessive oxidative stress.

Inhaled Lipopolysaccharide (LPS) has been used as a model of increased airway and systemic inflammation in healthy smokers, non-smokers and asthmatics; inhalation causes increased airway and systemic neutrophilic inflammation via Toll-Like-Receptor-4 (TLR4). Therefore, when administered to patients with COPD may lead to acute on chronic neutrophilic inflammation and thus serve as a model of exacerbations with which to investigate new treatments in vivo.
One of the mainstays of current treatment for COPD is corticosteroids, which reduce airway inflammation; however there is a degree of steroid resistance associated with COPD. Another pathway which could be targeted is the Phosphoinositide-3 (PI3) kinase enzyme pathway. The PI3 kinase enzyme is an essential intracellular enzyme which has a role in cell differentiation, growth, immune responses and metabolism. The γ and δ isoforms of the enzyme are of particular importance in leucocytes. There is evidence for up-regulation of the PI3 kinase enzyme pathway in COPD and in neutrophilic inflammation. This pathway could be targeted to reduce excessive inflammation both in stable disease and in the prevention of exacerbations and therefore requires further investigation of pharmacological effects in vitro.

This thesis pharmacologically investigates the neutrophilic activation associated with COPD in vivo and in vitro. The use of inhaled LPS in patients with COPD as model of exacerbation is evaluated. In addition, the effects of PI3 kinase enzyme inhibitors on a number of neutrophil functional responses in vitro are investigated.

1.2 Clinical aspects

COPD is mainly linked to cigarette smoking (1), but in developing countries exposure to biomass fuels is also a major risk factor (2). In addition, there is evidence for a gene-environment interaction; not all smokers will develop clinical disease due to some patients having a genetic predisposition to COPD (3). An important example of genetic predisposition is the autosomal recessive inherited disorder α1 anti-trypsin deficiency (4). α1 anti-trypsin is a protease inhibitor and therefore deficiency leads to parenchymal destruction and emphysema.
Patients with COPD commonly encounter progressive shortness of breath, wheeze and chronic cough (5). Shortness of breath, or dyspnoea, is due to a combination of hyperinflation and impaired gas exchange. This has a major impact on health related quality of life due to its effect on activities of daily living and due to the symptom itself (6). Wheeze and chest tightness may be variable and are non-specific symptoms due to airflow obstruction. Some patients may develop a chronic cough, which may or may not be productive of sputum. The cough may be intermittent initially and then become more persistent. Patients may produce variable quantities and purulence of sputum. Chronic bronchitis is defined as daily production of sputum for 3 or more months for 2 consecutive years (5). Smokers under the age of 50 with chronic bronchitis and normal lung function are at a 2 fold increased risk of developing COPD (7).

COPD is diagnosed by assessment of clinical symptoms combined with the presence of airflow obstruction, as defined by spirometry (5). In 2007, the Global Initiative for Obstructive Lung Disease (GOLD) guidelines defined airflow limitation as the presence of a post-bronchodilator forced expiratory volume in 1 second (FEV1) / forced vital capacity (FVC) ratio of less than 0.7, with COPD severity based on post-bronchodilator FEV1; GOLD stage I (mild, FEV1 ≥80% predicted), stage II (moderate, FEV1 ≥50 and < 80% predicted), stage III (severe, FEV1 ≥30 and <50% predicted) and stage IV (very severe, FEV1<30% predicted) (8). Currently, in the 2012 GOLD guidance, a combined assessment of COPD is recommended on a scale of A-D based on airflow obstruction, symptoms and risk of exacerbations (5); patients at low risk of exacerbations and with few symptoms are graded A whilst patients at high risk of exacerbations with more symptoms are graded D.
There is increasing evidence for patients with COPD also having a number of extra-pulmonary effects of the disease (9). These include significant weight loss and skeletal muscle dysfunction due to a combination of inflammation, hypoxia and inactivity. Other illnesses often found in patients with COPD are depression, osteoporosis, lung cancer and ischaemic heart disease (10-12). These conditions may be true co-morbidities i.e. co-existing disorders not related to COPD, or systemic manifestations of COPD i.e. caused by the underlying pathological processes associated with COPD. These extra-pulmonary effects and co-existing illnesses further lead to reduced physical activity and increased morbidity and mortality associated with the disease (9).

Different phenotypes of COPD exist, which are defined as “single or a combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes” (13). Phenotypes may be identified by clinical, radiological or physiological characteristics. Recently identified clinical phenotypes include frequent exacerbators (14), patients who exacerbate ≥2 times per year, patients with increased systemic inflammation (9) and patients with rapid decline in lung function (15).

Exacerbations or “flares” are a common feature of COPD (14). These may be triggered by bacterial and viral infections or environmental exposures. Patients may experience increased dyspnoea, cough, sputum volume and sputum purulence (16). Frequent exacerbators have reduced health related quality of life (17), faster decline in FEV1 (18) and severe exacerbations are associated with increased mortality (19). Exacerbations will be discussed in more detail in section 1.4.
1.3 Pathophysiology of COPD

1.3.1 Histopathology

The hallmark of COPD is three pathological processes: chronic bronchitis, emphysema and small airways disease. Most patients have all three pathological processes to varying degrees, but there is debate as to which is the most important. In addition, these 3 pathological processes may represent different phenotypes of disease. Chronic bronchitis is defined as daily sputum production for 3 months of the year for 2 consecutive years (5). The term emphysema derives from the Greek word emphyema, meaning inflation (from the verb emphysaein, to inflate, or blow in). It is defined as abnormal, permanent enlargement of air spaces distal to the terminal bronchioles, accompanied by the destruction of their walls and without obvious fibrosis. Small airways are those <2mm in diameter; inflammatory processes cause narrowing of these airways in addition to larger bronchi. These processes affect different anatomical locations of the lung and bronchial tree, as described in Figure 1.1.

Chronic bronchitis affects 14-74% of patients with COPD (20, 21); this varying prevalence may be due to differing definitions and populations studied. Cigarette smoke (22), bacterial and viral infections (23) and inflammatory cell influx (24) lead to mucous hypersecretion by goblet cells. In addition, patients have poor cough, reduced ciliary function and plugging which leads to reduced mucous clearance from the airway (25, 26). This leads to airflow obstruction by causing obstruction of the bronchial lumen, thickening of the epithelial layer and altering surface tension therefore predisposing it to collapse on expiration. Patients with chronic bronchitis have an accelerated decline in lung function (7), worse health related quality of life (27) and increased exacerbations (17). There is also some evidence that chronic
bronchitis increases mortality (28), although this has not been a universal finding (29).

A major site of airway obstruction is the small airways (less than 2mm in diameter) (30), which are spread between the fourth and fourteenth generation of airway branches. As the number of airways increases, cross-sectional area also vastly increases and resistance to airflow falls in smaller airways. There are structural abnormalities in the small airways of smokers with or without COPD caused by the accumulation of mucous (26). COPD progression has also been shown to be related to inflammatory exudates in small airway lumens in surgically resected lung tissue (26, 31). Submucosal inflammation, airway remodelling and peribronchiolar fibrosis also contributes to airway narrowing (32). This reduction in lumen calibre therefore leads to an increase in airways resistance and airflow obstruction.

Emphysema is defined as the permanent destructive enlargement of airspaces distal to terminal bronchioles. Emphysema may be centrilobular, resulting from destruction of the respiratory bronchiole, panacinar, causing dilatation of the entire acinus or paraseptal, on the periphery of the lobule (33, 34). The pathogenesis of emphysema has been largely attributed to an imbalance between proteases and anti-proteases, mainly since the discovery that severe emphysema was linked to α1 antitrypsin deficiency (4). Proteases cause degradation of elastin and collagen in the lung extracellular matrix which then leads to the destruction of lung parenchyma characteristic of emphysema. Airflow limitation and tissue destruction leading to loss of elastic recoil causes air trapping and a phenomenon known as hyperinflation (35). Furthermore, inflammatory cell influx into small airways may destroy their attachment to alveoli, predisposing it to closure (36).
The major proteases involved are neutrophil elastase (NE) released from neutrophils and matrix-metalloproteinases (MMP) released from neutrophils and macrophages. Tracheal installation of NE in animal models induced emphysema and neutrophilic inflammation (37). MMP-8 and 9 have been implicated in the pathogenesis of COPD (38), with MMP-9 levels elevated in lung (39) and alveolar macrophages of patients with COPD (40). An imbalance between MMPs and tissue inhibitors of metalloproteins (TIMPs) has also been shown to be associated with COPD (41).

Other proposed inflammatory mechanisms of emphysema development include the inhibition of histone deacetylase by cigarette smoke (42), oxidative stress (43), malnutrition (44), surfactant instability (45) and alveolar cell apoptosis (46).
Figure 1.1: The pathogenesis of COPD. COPD is characterised by the pathological changes of chronic bronchitis and emphysema causing narrowing of the airways and destruction and enlargement of air spaces.
1.3.2 Inflammatory cells and mediators

COPD is known to be associated with chronic airway and systemic inflammation, and is characterised by increased numbers of neutrophils, activated macrophages and T-lymphocytes particularly in the small airways. The degree of inflammation increases with severity of disease (26).

The role of neutrophils will be discussed in detail in a later section, 1.4.

Macrophages have an important role in COPD pathogenesis through release of reactive oxygen species (ROS), extracellular matrix proteins and lipid mediators. There are increased macrophage numbers in the airways, lung parenchyma, bronchoalveolar lavage (BAL) fluid and sputum in patients with COPD (47); there is also a correlation between numbers in the bronchial mucosa and severity of disease (48). Macrophages are derived from monocytes in the circulation and there may be increased recruitment of monocytes in patients with COPD as there is elevated monocyte chemotactic protein (MCP)–1 (49). Macrophages are found at sites of alveolar wall destruction in patients with emphysema (50) and clusters have been found around small airways associated with peri-bronchiolar fibrosis in smokers and ex-smokers (51).

Cigarette smoke stimulates macrophages to release inflammatory cytokines and chemokines such as interleukin (IL)–8, and proteases such as MMP–9 (52). Hypoxia, which is important in COPD exacerbations and severe disease, also stimulates IL-8 release from macrophages in Adult Respiratory Distress Syndrome (ARDS) (53).

Defective phagocytosis of bacteria such as *Haemophilus influenzae* by COPD alveolar macrophages has also been reported (54), implicating a role in bacterial
colonisation seen in some patients. Exposure of alveolar macrophages to cigarette smoke also impairs their ability to phagocytose apoptotic neutrophils (55), a process known as efferocytosis, again contributing to persistent inflammation.

Other leucocytes are involved in COPD pathogenesis: T-lymphocytes are increased in the airways of COPD patients compared to smoking controls (56), and the number of CD8+ lymphocytes has been found to be directly related to the degree of airflow obstruction (57). Increased numbers of dendritic cells have also been observed in the small airway epithelia and adventitia in COPD compared to smokers and non-smokers and is associated with disease severity (58).

There has been increasing recent interest in the role of eosinophils in COPD. Eosinophilic inflammation has traditionally been associated with T helper 2 cell (TH2) mediated responses and therefore asthma. However, it has been noted that 20-40% of COPD patients also have >3% sputum eosinophils (59) and therefore may represent an eosinophilic phenotype. BAL eosinophil cationic protein (ECP) levels have been found to be lower in COPD than in asthma (60), but higher levels have been demonstrated in sputum of patients with moderate to severe disease (59). Thus, the cells may be more activated in severe disease. The relationship between eosinophils and disease severity remains unclear, although increased numbers may be associated with viral exacerbations, which will be discussed later.

C reactive protein (CRP) is produced by the liver in response to stimulation by IL-6 (61). In stable COPD, serum CRP is increased compared to healthy controls (62-64), and higher levels are associated with severe disease (65), frequent exacerbations (9, 66) and increased mortality (9, 67). In addition, there is evidence that sputum (65), and lung (68) CRP is increased in COPD. A major disadvantage of CRP as a
biomarker is its lack of specificity; it is increased during infections as well as other chronic disease. Tumour necrosis factor (TNF) α is another protein shown to be increased in COPD compared to stable patients (69, 70); in addition polymorphisms of TNFα are associated with disease progression (71).

A number of inflammatory cytokines, chemokines and other mediators are also involved in the pathogenesis of COPD. These are summarised in Table 1.1.
### Inflammatory mediator

<table>
<thead>
<tr>
<th>Inflammatory mediator</th>
<th>Functions</th>
<th>COPD Airway</th>
<th>COPD Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pro-inflammatory cytokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>Released by macrophages, neutrophils, T cells, epithelial cells and airway smooth muscle cells. An important neutrophil chemotactic protein.</td>
<td>Increased in sputum (69, 72).</td>
<td>Increased in serum (70, 73). Associated with weight loss and muscle wasting (74).</td>
</tr>
<tr>
<td>IL-6</td>
<td>Link between innate and adaptive immunity. Secreted by leucocytes, epithelial cells and fibroblasts.</td>
<td>Increased in sputum (75).</td>
<td>Increased in serum (73) and associated with increased mortality (76) and poor outcomes (9).</td>
</tr>
<tr>
<td>IL-8/CXCL-8</td>
<td>Neutrophil recruiting and activating factor exerting effect through CXCR1, CXCR2.</td>
<td>Increased in sputum (69) and BAL (77).</td>
<td>Increased in serum (70).</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Activates macrophages from patients with COPD to release pro-inflammatory mediators.</td>
<td>Bacterial colonisation associated with increase in sputum (78).</td>
<td>Correlates with FEV1 and reduced antagonists in COPD (79).</td>
</tr>
<tr>
<td>IL-17</td>
<td>Upregulates IL-8. Mostly released by TH17 cells, a subset of CD4 cells.</td>
<td>Increased expression in airway tissue, neutrophils and epithelial cells (80, 81).</td>
<td>Increased TH17 cell numbers in the circulation (82).</td>
</tr>
</tbody>
</table>

Table 1.1: A summary of inflammatory mediators involved in COPD in the airway and systemic circulation. (CCL: chemokine C-C ligand; CCR: chemokine C-C receptor; CXCL: chemokine C-X-C ligand; CXCR: chemokine C-X-C receptor; GM-CSF: Granulocyte macrophage colony stimulating factor; GRO: growth related oncogene; IL: Interleukin; IFN: Interferon; MCP: monocyte chemotactic protein; MIP: macrophage inhibitory protein; MMP: matrix metalloproteinase; RANTES: regulated on activation T cell expressed; TGF: Transforming growth factor; TH: Helper T cell; TNF: Tumour necrosis factor).
<table>
<thead>
<tr>
<th>Inflammatory mediator</th>
<th>Functions</th>
<th>COPD Airway</th>
<th>COPD Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18</td>
<td>Produced by activated macrophages. IL-18 has an important role in T cell polarization.</td>
<td>Increased expression in COPD airway (83) and induced sputum (84). Increased receptor gene expression in macrophages (85).</td>
<td>Increased in serum COPD (83, 86) and smokers (83).</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Produced by T lymphocytes, induces chemokine release from alveolar macrophages and epithelial cells.</td>
<td>Increased production from BAL T cells in COPD and smokers (87).</td>
<td>Increased production from peripheral T cells (87).</td>
</tr>
</tbody>
</table>

### 1. Growth factors

| GM-CSF | Has role in differentiation and survival of neutrophils, eosinophils and macrophages. | Increased in sputum (88). | No evidence |
| TGF-β  | Induces proliferation of airway smooth muscle and fibroblasts. | Increased expression in airway epithelial cells (89). | Increased in serum (90). |

### 2. Chemokines

<p>| CCR2 agonists | CCL2/MCP-1 is activating factor of monocytes and T lymphocytes. | Increased in sputum (91) and airway epithelium (92). Increased in COPD and smoker BAL (49). | Increased in serum (93). |</p>
<table>
<thead>
<tr>
<th>Inflammatory mediator</th>
<th>Functions</th>
<th>COPD Airway</th>
<th>COPD Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR3 agonists</td>
<td>CCL11/eotaxin 1, RANTES, MCP-5 attract and activate eosinophils by binding to CCR3.</td>
<td>Increased CCL11 in sputum (94) and BAL (95).</td>
<td>Decreased CCL11 in plasma (96), increased CCL11 in serum (97).</td>
</tr>
<tr>
<td>CCR5 agonists</td>
<td>CCL5/RANTES, CCL3/MIP-1α, CCL4/MIP-1β bind to CCR5, expressed by macrophages and lymphocytes.</td>
<td>Increased CCL5 in BAL (95) and sputum in COPD and smokers (98).</td>
<td>No evidence</td>
</tr>
<tr>
<td>CXCR2 agonists</td>
<td>Activated by IL-8, CCL1/GROα and CXCL5. Expressed on neutrophils and involved in chemotaxis.</td>
<td>Increased GROα in sputum (91).</td>
<td>No difference between smokers and COPD (93).</td>
</tr>
<tr>
<td>CXCR3 agonists</td>
<td>Activated by CXCL9, CXCL10 and CXCL11, all induced by IFNγ.</td>
<td>CXCL9-11 increased in sputum (98) and CXCL10 in airway epithelium (99).</td>
<td>Increased CXCR3 expression on T cells (100).</td>
</tr>
<tr>
<td><strong>1. Anti-inflammatory cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>Inhibits production of TNF-α, GM-CSF, IL-5 and MMP-9.</td>
<td>Decreased sputum levels in COPD and smokers (101, 102).</td>
<td>Decreased serum levels in COPD and smokers (101).</td>
</tr>
</tbody>
</table>
1.3.3 Bacterial colonisation

Even when clinically stable, 20-70% of COPD patients have positive sputum cultures (78, 103-105), using quantitative culture techniques. Bronchoscopic studies using protected sheath brushings (PSB) (106) and BAL (107, 108) have also identified evidence of colonisation in stable COPD using this method. However, culture based techniques may underdiagnose bronchial colonisation as bacteria are not detected below the limit of detection (less than $10^3$ colony forming units/ml). Bacteria have been detected in 10% of culture negative samples (109).

The emergence of the molecular method using polymerase chain reaction (PCR) amplification of the 16S ribosomal ribonucleic acid (rRNA) gene has allowed the identification of bacteria previously undetectable by culture techniques (110). PCR techniques are increasingly used to detect pathogens in sputum (111), and can detect the presence of differences in genotype and therefore new strains of bacteria (112). A PSB study of COPD patients and healthy smokers using PCR identified that even the smokers have changes in the airway microbiome, which changes in COPD (113). Further studies using lung tissue, bronchial aspirates and BAL have also demonstrated a significant diversity in the microbiome of the lower COPD airway (114-117); two of these studies also included analysis of healthy non-smokers which showed that even in this population the lower airway was not sterile (115, 117).

Colonisation has been shown to be related to airflow obstruction and current cigarette smoking (118). Relationships between colonisation and airway inflammation (104), lung function decline (119) and quality of life (120) have been identified. There also appears to be an association between bacterial colonisation and exacerbation frequency and severity (105). The mechanisms behind these associations remain unclear; there is debate as to whether colonisation causes
deterioration in COPD or whether colonisation is a marker of severe disease with impaired mucociliary clearance.

The most common colonising bacteria are *Haemophilus influenzae, Streptococcus pneumoniae, Moraxella catarrhalis* and *Pseudomonas aeruginosa* (78, 105, 121). Colonisation is associated with increased neutrophilic inflammation in the airway, with increased myeloperoxidase (MPO), IL-8, leukotriene (LT)-B4 and neutrophil counts (78, 105, 107, 122) and increased systemic inflammation, with increased CRP (78).

1.4 The Neutrophil

In the 19th century, Paul Elrich discovered several novel leucocyte subtypes and named a group characterised by a polymorphous nucleus the “neutrophil”. Their function, as antimicrobial cells, was discovered by his colleague Elie Metchinoff who also renamed them polymorphonuclear leucocytes (PMNs). Neutrophils are vital in the innate immune response and play a key role in the elimination of pathogens. In humans, neutrophils constitute 40-60% of the white blood cell population and reduced neutrophil numbers leads to marked immunodeficiency (123). There is now increasing evidence that neutrophil activation contributes to the pathogenesis of several inflammatory disorders such as rheumatoid arthritis and COPD. It is also now recognised that neutrophils also have a role in adaptive immunity, acting as antigen presenting cells (124). Neutrophil functions are summarised in Figure 1.2.
Figure 1.2: A summary of key neutrophil functions. At areas of inflammation, neutrophils are initially primed and then undergo full activation. They release pro-inflammatory mediators, phagocytose bacteria, release neutrophil extracellular traps (NETs) and degranulate in response to infectious stimuli, leading to the release of destructive oxidants and proteases.
1.4.1 Neutrophil production and release

The average life span of a neutrophil is only 6-8 hours; therefore the bone marrow must produce the cells at a rapid rate of $5 \times 10^{10}$ per day. Neutrophils and macrophages originate from a common myeloid precursor cell and neutrophils differentiate under the action of granulocyte-colony stimulating factor (G-CSF) (125). The neutrophil goes through several stages in its maturation: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell and finally PMN cell. Mature neutrophils migrate across bone marrow endothelium and release is regulated by interaction between chemokine (C-X-C motif) receptor (CXCR)-4 on neutrophils and syndecin (Sd)-1 on stromal cells (126).

When injected into healthy volunteers, 50% $^{32}$ Diisopropylfluorophosphate (DFP)-labelled autologous granulocytes disappear within seconds (127), which suggests that there is a marginated pool of neutrophils. These are largely in the bone marrow, spleen, liver and lung. The lung does seem to be enriched with neutrophils, but some argue that this is because the pulmonary vasculature receives all of the cardiac output (128). The distribution of neutrophils has also been shown to depend upon their activation and stage of maturation (129).

1.4.2. Neutrophil extravasation and migration

Neutrophils move from the circulation to sites of inflammation, if required. This usually involves the steps of tethering, rolling, adhesion, crawling, and finally transmigration. The vascular endothelium expresses a number of adhesion molecules including E-selectin, P-selectin and P-selectin glycoprotein ligand 1 in response to local inflammatory mediators (130). These molecules tether to L-selectin on the surface of neutrophils and allow subsequent rolling of the neutrophil in the direction of blood flow.
There are then further conformational changes in a number of integrins on the neutrophil surface: very late antigen (VLA)-4 (CD49d/CD29b), lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18) and Macrophage-1 antigen (MAC-1) (CD11b/CD18). These molecules are expressed at high levels during the resting state, but once they have undergone conformational changes they can bind to endothelial cell surface molecules intracellular adhesion molecule (ICAM)-1 and ICAM-2 leading to strong adherence (131). Once adhered, some neutrophils may crawl towards endothelial-endothelial cell junctions prior to transmigration; crawling is dependent on interaction between ICAM-1 and MAC-1 (131).

The neutrophil then transmigrates across the vascular endothelial cells in one of two ways: para-cellularly (between cells) or trans-cellularly (through the cell). The process requires integrins, ICAM-1, ICAM-2 and CD31 and members of the junctional adhesion molecule (JAM) family (132). Once through the endothelium, the neutrophil then migrates towards inflamed tissue across a chemotactic gradient, a process known as chemotaxis. Chemoattractant molecules include the bacterial derived N-formyl-methionyl-leucyl-phenylalanine (fMLP), the complement component 5a (C5a) and the chemotactic stimuli IL-8 and leukotriene B4 (LTB4). Exposure to fMLP or C5a induces polarization of receptors at the leading edge of the cell (133).

1.4.3. Neutrophil activation

The main function of the neutrophil is as an antimicrobial cell; in order to complete these functions the cell needs to become activated. Chemotactic stimuli begin to activate the cell as it moves towards an area of inflammation, in preparation for respiratory burst and degranulation. fMLP binds to the G protein coupled receptor (GPCR) fMLP receptor FPR1 initiating a downstream signal cascade, leading to the
release of adenosine triphosphate (ATP), which is critical for the initiation of immune responses by the neutrophil (134). Infective stimuli also stimulate other receptors known as pattern-recognition receptors; the best known of these are TLRs which will be discussed in a later section.

However, some chemotactic stimuli exert only a “priming” effect: on their own they stimulate the cell only mildly but when the cell is further stimulated they dramatically enhance the response. This priming effect has been demonstrated with the effect of LPS and TNFα on the fMLP stimulation of respiratory burst (135-138). Therefore neutrophils are present in one of three states: quiescent, primed or active. Contact with activated epithelium and foreign surfaces or hypoxia may also prime neutrophils. In addition, priming of neutrophils causes shedding of L-selectin, upregulation of MAC-1, inhibition of apoptosis, shape change, fusion of granule proteins with the cell membrane prior to exocytosis and increased expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (139-145). Differential priming effects are seen, with LPS causing loss of surface CD62-L but no change in CD11b and TNFα causing upregulation of CD11b and loss of surface CD62-L (139). Priming may be reversible leading to de-priming (146, 147), depending on the agent used.

1.4.3.1 Cytokine release

On arrival at sites of infection and inflammation, neutrophils secrete cytokines and chemokines to stimulate the innate immune response. These proteins are mostly synthesised de novo in response to bacterial stimuli (148), and cells undergo an increase in transcription. Some cytokines are stored in intracellular pools and are secreted upon stimulation of the cell, these include TNF-Related Apoptosis-Inducing Ligand (TRAIL), Chemokine (C-C motif) ligand (CCL20), B cell-activating factor
(BAFF) and IL-1 (149). In comparison to other immune cells, neutrophils release small amounts of cytokine although the number of circulating neutrophils means that they contribute significantly to total cytokine production.

IL-8 is the most abundantly produced neutrophil cytokine (150), which leads to the recruitment of other neutrophils and is produced after stimulation with a number of different biological agents, including TNFα, granulocyte macrophage-colony stimulating factor (GM-CSF), C5a, platelet activating factor (PAF) and LTB4. Some newly synthesised IL-8 remains cell associated and may act as a signal during efferocytosis (151). Neutrophils also produce IL-1β, TNFα, interferon (IFN)-γ, macrophage inflammatory protein (MIP)-1α, MIP-1β, growth related oncogene (GRO)α and IL-10 (152). MIP-1α and β, like IL-8, are released by neutrophils in response to a number of stimulants (153, 154) and their release further amplifies the immune response. Importantly, the magnitude and pattern of cytokine production varies depending on the stimulus used; LPS and TNFα induce the release of IL-8, GROα and MIP-1α, while GM-CSF and fMLP only cause minimal production of IL-8 (155). However, a further study demonstrated that fMLP and IL-8 stimulation did not cause increased neutrophil gene expression of cytokines (156).

1.4.3.2 Degranulation

Neutrophils possess a number of granules containing cytotoxic molecules; the 4 major granules are secretory vesicles, azurophilic (primary) granules, specific (secondary) granules and gelatinase (tertiary) granules. Granules can also be divided into myeloperoxidase positive (i.e. azurophilic) or myeloperoxidase negative (i.e. specific or gelatinase). There is a hierarchical pattern of degranulation due to different sensitivities of the granules to intracellular calcium (157). Granules formed at later stages of development in the bone marrow such as secretory vesicles and
gelatinase granules undergo exocytosis more readily than azurophilic granules. Neutrophil granules and their constituents are summarised in Table 1.2.

On priming and then activation of the neutrophil, granules are mobilised and fuse with the cell membrane or phagosome, releasing their contents and altering the molecular composition of the membrane. The fusion of secretory vesicles with the cell membrane increases adhesion and activation capacity of the cell (139, 158). Fusion of specific and azurophilic granules lead to increased membrane CD66b and CD63 expression respectively (159). Fusion of specific granules is particularly important for activation of the respiratory burst as flavocytochrome b558, which forms part of the NADPH oxidase unit, is on the granule membrane (160).

<table>
<thead>
<tr>
<th></th>
<th>AZUROPHILIC</th>
<th>SPECIFIC</th>
<th>GELATINASE</th>
<th>SECRETORY</th>
</tr>
</thead>
<tbody>
<tr>
<td>SURFACE MOLECULE</td>
<td>CD63 CD68</td>
<td>CD66b CD11b/CD18</td>
<td>CD11b/CD18</td>
<td>CD11b/CD18 Complement receptor 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CD16</td>
</tr>
<tr>
<td>PROTEASES</td>
<td>Elastase</td>
<td>Collagenase</td>
<td>MMP-9 Arginase-1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Proteinase-3</td>
<td>Heparinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cathepsin G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTHER ANTIMICROBIAL</td>
<td>Defensin</td>
<td>Lysozyme</td>
<td>β2.microglobulin</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>PROTEINS</td>
<td>MPO</td>
<td>Lactoferrin</td>
<td>CRISP3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
<td>β2.microglobulin</td>
<td>HNL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B-glucuronidase</td>
<td>CRISP3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2: The constituents of the 4 major neutrophil granules. (CRISP3: Cysteine-rich secretory protein 3; HNL: human neutrophil lipocalin; MMP: Matrix metalloproteinase; MPO: myeloperoxidase)
Table 1.2 summarises some of the main granulocyte peptides; over 800 have been described in total. These peptides may be charged, facilitating contact with the surface of microbes and they may interfere with microbial deoxyribonucleic acid (DNA) replication, transcription or energy production. NE, contained in azurophilic granules, degrades elastin, fibronectin, collagen and tissue inhibitors of matrix metalloproteinase and as discussed previously contributes significantly to the pathogenesis of COPD. There is also evidence that NE can specifically cleave enterobacterial virulence factors (161). MMP-9/gelatinase is stored as an inactive proform in gelatinase granules and undergoes proteolytic activation following exocytosis; it degrades major structural components of the extracellular matrix including collagens, fibronectin and leptin. There is also evidence that MMP-9 is of particular importance in the degradation of basement membranes and interstitium during neutrophil extravasation and migration (162).

**1.4.3.3 Respiratory burst**

When activated, neutrophils produce ROS in a process known as respiratory burst. Patients with chronic granulomatous disease are prone to bacterial and fungal infections and have reduced microbial killing associated with reduced oxygen consumption (163). NADPH oxidase is assembled on the cell membrane and consists of one membrane bound and 4 cytosolic components. On activation of the cell the cytosolic components ras-related C3 botulinum toxin substrate (Rac)-2, p40phox, p47phox and p67phox migrate to the membrane bound component cytochrome b558 (164). Phosphorylation of the component subunits results in enzyme activation and the formation of a multi-component electron transfer system.

Reduced NADPH oxidase catalyses the production of superoxide (O$_2^-$) from oxygen and NADPH (165). Other oxidising agents generated by NADPH oxidase include
hydrogen peroxide (H$_2$O$_2$), ozone and the hydroxyl radical (OH$^-$). MPO, a constituent of azurophilic granules, also reacts with hydrogen peroxide to produce a number of reactive species, including hypochlorous acid which is produced from chloride ions (Cl$^-$) and H$_2$O$_2$. ROS have direct antibacterial action and are released either extracellularly or directly into the phagosome. However, in addition to beneficial antimicrobial effects, ROS increase oxidative stress and increase inflammation. ROS may also have a role regulating other immune cells (166), regulating apoptotic processes (167) and in activating granule proteases (168). There is also evidence that oxygen free radicals act as intracellular signalling molecules for a number of cytokines including LTB4, TNF$\alpha$ and IL-1 (169).

1.4.3.4 Phagocytosis and Neutrophil extracellular traps

When neutrophils encounter microorganisms they may be internalised by the cell membrane forming vacuole inside the cell called the phagosome. The pathogens are then killed by the release of ROS or antibacterial proteins. Internalisation depends on interaction between the pathogen and the neutrophil which may be direct, via pattern-recognition receptors, or indirect, through opsonisation (170).

Highly activated neutrophils may also eliminate microbes by releasing neutrophil extracellular traps (NETs). NETosis is an active form of cell death which leads to the release of decondensed chromatin into the extracellular space (171). NETs also contain antimicrobial proteins and proteins released from neutrophil granules. NETs immobilise microbes therefore preventing spread; they kill pathogens by direct action through proteins and proteases and also facilitate phagocytosis (172).
1.4.3.5 Apoptosis

The resolution of neutrophilic inflammation occurs by apoptosis, or programmed cell death. Apoptosis minimises and prevents any further damage of neutrophilic inflammation. The process is actively regulated by the pro-apoptotic proteins bcl-2-like protein 4 (Bax) and bcl-2-associated death promoter (Bad), the anti-apoptotic induced myeloid leukaemia cell differentiation protein (Mcl-1), and the activation of caspases (173). There is increasing evidence that lipoxin, a lipid mediator, has an important role in apoptosis causing reduced neutrophil recruitment, inhibiting L-selectin shedding and by inhibiting ROS production and IL-8 expression (174). Apoptosis results in nuclear and cytoplasmic condensation, DNA fragmentation, dilation of the endoplasmic reticulum and leakage of cytochrome c from the mitochondria. If apoptosis does not occur correctly there is prolonged inflammation. Phagocytosis of apoptotic neutrophils by macrophages leads to the release of the anti-inflammatory cytokines, such as IL-10 and IL-12 (175).

1.4.4 The Neutrophil in COPD

COPD is characterised by increased neutrophilic inflammation. There are increased numbers of neutrophils in the airways of patients with COPD; this has been demonstrated in BAL (77), bronchial epithelium (176), smooth muscle (177) and small airways (26). Hogg et al demonstrated that the numbers of neutrophils in the small airways increases with COPD severity (26). Furthermore, there are increased numbers of neutrophils in COPD sputum compared with smokers and healthy non-smokers and studies comparing these groups are summarised in Figure 1.3.
Figure 1.3: A summary of studies comparing percentage neutrophils in sputum from COPD patients versus healthy smoking and non-smoking controls. Data from Rytila et al (178), O’Donnell et al (179) expressed as mean, Keatings et al (69) expressed as median. (*p<0.05, **p<0.001 between groups as indicated).
Healthy non-smokers have <50% sputum neutrophils (69, 178, 180, 181), but this proportion increases with chronic cigarette smoking (182). Patients with COPD usually have >70% sputum neutrophils (69, 178, 180, 181, 183), although their relationship with disease severity and lung function decline is yet to be defined. Several studies, including analysis of the Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) cohort, have demonstrated a weak relationship between FEV1 % predicted and sputum neutrophils (180, 183, 184). Sputum neutrophil percentage was related to lung function decline over 15 years in 38 smokers (185). However, this study studied sputum only at the end of the 15 year period so it is difficult to draw robust conclusions. There was no relationship between sputum neutrophil % and FEV1 decline in the ECLIPSE cohort, although the follow up period for this study was short (183).

Increased airway neutrophils in COPD may be explained by increased production by the bone marrow and increased pulmonary sequestration. Systemic inflammation with increased LTB4, IL-8 and TNFα has been shown to cause neutrophilia in experimental animals (186), which may be due to increased stromal cell-derived factor (SDF)-1 expression (187). Inflammatory mediators and hypoxia may also prime neutrophils thus altering their shape and deformability and therefore increase retention in the pulmonary vasculature (147, 188). Unprimed neutrophils pass through the pulmonary vasculature quickly (147); there is evidence that the de-priming mechanism of the lungs may be defective in ARDS (147) therefore this may also be the case in COPD. Priming also induces increased expression of the adhesion molecule Mac-1 (139, 142) facilitating neutrophil extravasation. Several studies have demonstrated increased expression of Mac-1 on the surface of neutrophils in the
circulation (189-192) and in the bronchial tissue (193), indicating that COPD neutrophils are already primed and therefore more readily activated.

There have been conflicting reports on COPD neutrophil chemotaxis. It has been reported that peripheral and airway neutrophils display reduced chemotactic activity towards IL-8 and fMLP compared to healthy controls (194). In contrast, another study reported increased chemotaxis towards IL-8 by COPD and healthy smoker peripheral blood neutrophils compared with healthy non-smokers, indicating that this may be a smoking effect rather than a disease effect (195). It has recently been reported that COPD peripheral neutrophils move with greater speed but less accuracy than cells from smoking and non-smoking controls (196). Increased chemotaxis combined with an increase neutrophil chemoattractants (IL-8, LTB4 and GROα) in the airway (69, 91, 197) leads to increased neutrophil migration.

There is also evidence for increased neutrophil derived inflammatory mediators: IL-8, MMP-9 and NE have been found to be elevated in the sputum of patients with COPD compared to smokers and their levels correlated with disease severity and lung function decline at 2 years (198). MPO activity has been shown to be increased in COPD sputum (199) and MPO levels are increased in COPD serum compared with smokers and non-smokers (200); serum levels were also found to be correlated with disease severity.

A number of studies have demonstrated increased levels of the proteases MMP-9 and NE in the airways of patients with COPD (38, 201-206) and subclinical disease (38). MMP-9 levels also correlate with the degree of neutrophilia (204, 205, 207) and lung function (202, 205), although the relationship to disease severity has not been a universal finding (206). NE levels in BAL has also been shown to be correlated to
disease severity (205). MMP-9 is also elevated in the blood of COPD patients compared to controls (208, 209). Other neutrophil associated proteins elevated in COPD airway and circulation are human neutrophil lipocalin (HNL) (210, 211) and the collagen fragment proline-glycine-proline (PGP) (212). There is also evidence that peripheral blood neutrophils from patients with COPD exhibit enhanced extracellular proteolytic activity (213, 214) and respiratory burst (190, 215) compared to controls. Therefore, in addition to increased numbers there is increased protease release and oxidative stress, contributing to the pathogenesis of COPD.

The ability of neutrophils to ingest and kill bacteria may be compromised in COPD. Cigarette smoke extract impairs phagocytosis of neutrophils in vitro (216), and 2 studies have demonstrated that isolated neutrophils from COPD patients have reduced phagocytic ability (217, 218). A further study has shown that hypoxia impairs respiratory burst activity and causes a defect in Staphylococcus aureus killing (219). Sputum neutrophils from patients with COPD also express less TLR2, which may impair anti-microbial function (220). Hence, a defect in microbial killing may cause colonisation (104) and frequent exacerbations (14). NET formation in COPD neutrophils has not been studied to date; however there is evidence that neutrophils from older patients display an age related decline in NET formation (221).

Apoptosis in COPD neutrophils has been investigated, with conflicting results; there was no difference in apoptosis between the airway neutrophils of patients with mild COPD compared to healthy controls (178), but a further study investigating more severe patients revealed significantly increased apoptosis in the COPD group compared to controls (222). However, another study demonstrated reduced apoptosis in COPD and healthy smoker sputum neutrophils compared to non-smokers (223).
No differences in apoptosis in circulating neutrophils between COPD patients and healthy controls have been shown in 2 studies (189, 224). Therefore the increased neutrophil numbers seen in COPD airways may be due to increased chemotaxis or by the reduced clearance of these cells, leading to prolonged inflammation.

1.5 COPD Exacerbations

Exacerbations or acute worsenings of COPD cause a significant economic burden (225). Frequent exacerbators have a more rapid decline in lung function (18) and poorer health related quality of life (17), and severe exacerbations are associated with increased mortality (19). Exacerbations may be caused by bacterial or viral infections or by environmental factors. They are characterised by increased airway and systemic neutrophilic inflammation.

No airway or systemic biomarker has been identified as yet for an exacerbation. A biomarker would be useful not only to diagnose and identify aetiology but also to grade severity in clinical and research settings. Many previous studies have been cross-sectional only studying patients during exacerbations and not in the stable state. In addition, invasive sampling during an exacerbation using bronchoscopy is restricted due to obvious ethical and safety reasons. However, induced sputum has been shown to be safe in severe airflow obstruction (226) and other techniques like exhaled breath condensate are emerging (227).

There are currently few therapies available to clinicians during exacerbations and the mechanisms responsible for the clinical manifestations such as prolonged duration and secondary bacterial infection are poorly understood. A particular difficulty is that many drugs used in exacerbations are usually studied during the stable state and
therefore may be ineffective during acute increases in inflammation. Bacterial versus viral exacerbations can be difficult to differentiate and there is often a delay in obtaining sputum cultures, leading to unnecessary use of antibiotics. The development of specific and sensitive biomarkers to diagnose bacterial and viral exacerbations would also help guide treatment and prevent use of ineffective drugs with potentially significant side effects.

1.5.1 Definition

There is currently no standardised definition of an exacerbation and the subject is controversial. Differing approaches include those based on respiratory symptoms alone, or those based upon symptoms and an event such as the prescription of a medication or hospital admission. Previous studies have demonstrated that 50-70% of exacerbations are unreported (17, 228).

The most widely used definition of an exacerbation is based on the criteria described by Anthonisen et al (16); the cardinal symptoms were increased dyspnoea, increased sputum volume and increased sputum purulence. Anthonisen et al proposed 3 grades of exacerbation depending on the number of symptoms: type 1 (all 3), type 2 (2 symptoms) and type 3 (1 cardinal and 1 minor symptom). Minor symptoms included an upper respiratory tract infection in the previous 5 days, fever, increased wheeze, cough and increase in respiratory rate or heart rate by 20% above baseline.

1.5.2 Aetiology of exacerbations

The majority of exacerbations are caused by bacteria, viruses and environmental factors. 78% of hospitalised patients have evidence of bacterial or viral infections, with up to 25% of having evidence of both (229). 10% of exacerbations may be
caused by environmental pollution (230) and in up to 30% the cause is unknown (231). Causes of exacerbations are represented in Figure 1.4.

The most common bacteria found in the airway during COPD exacerbations are *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and occasionally Gram negative bacteria. During an exacerbation 7-70% of patients have bacteria isolated from sputum (111, 229, 232-238) and 20-45% from bronchoscopic samples (236, 239). These differences in detection rates may be due to the differing methodology used to confirm the presence of bacteria (109) and some studies being done in hospitalised patients, with more severe exacerbations (229). The acquisition of a new bacterial strain increases the risk of having an exacerbation (112) and new strain exacerbations are associated with increased airway and systemic inflammation (235). Exacerbation strains of *Haemophilus influenzae* induce more airway inflammation than colonisation strains in mouse models of airway infection (240).

Higher bacterial load has also been associated with greater decrease in FEV1, in particular in exacerbations associated with a change in bacteria (119). The presence of purulent sputum has been shown to be associated with bacterial infection (241), which may guide clinicians when deciding whether to prescribe antibiotic therapy. Anthonisen et al studied 300 patients with exacerbations, and showed a greater success in treatment in patients who were given antibiotics, especially if they presented with the three cardinal symptoms of increased sputum, dyspnoea and sputum purulence (16).

Exacerbations are commonly triggered by respiratory viral infections (242), which are more common during the winter. Viral infections are also associated with a
longer duration of symptoms, increased exacerbation frequency and more severe exacerbations (243, 244). There is also a higher chance of hospital admission in patients with respiratory viruses than those without (245). The development of molecular techniques has enabled viral infections to be detected; around 40-50% of exacerbations are thought to be caused by viruses (229, 233, 244) .

The most common viruses detected during exacerbations are rhinovirus, respiratory syncytial virus and influenza. 23% of patients have evidence of rhinovirus infection during an exacerbation, but only <1% stable patients (246). Experimental models administering rhinovirus to patients with COPD have been shown to produce symptoms of an exacerbation (247). Viral infections like bacterial infections are associated with increased inflammation, with increased sputum neutrophils and eosinophils (229), and increased serum IL-10 (232).

Many patients with exacerbations have evidence of viral and bacterial infections (229, 233). In addition, a recent study demonstrated that when COPD patients were infected with rhinovirus 60% went on to develop secondary bacterial infection (248). Patients with concomitant *Haemophilus influenzae* and rhinovirus infection have been shown to have a greater fall in lung function, rise in serum IL-6 and sputum IL-8 (237).

Exacerbations may also be caused by increased levels of atmospheric sulphur dioxide, ozone and nitrogen dioxide (249). A fall in temperature is also associated with increased frequency of exacerbations and decline in lung function (250), although this may be due to an increased risk of upper airway viral infection.
Figure 1.4: A diagramatic representation of causes of COPD exacerbations. Exacerbations may be caused by bacteria, viruses, atypical organisms or pollutants.
1.5.3 Exacerbation phenotypes

Current treatments available for COPD exacerbations include antibiotics and systemic corticosteroids. However, their beneficial effect is marginal (251, 252) and they may be of more benefit in specific types of exacerbation. This has led to increased interest in the identification of specific disease phenotypes.

The frequent exacerbator has recently been described through analysis of 2138 patients enrolled in the ECLIPSE study (14); the best predictor for exacerbations is a history of exacerbations. This phenotype was also associated with disease severity, poorer quality of life and elevated white cell count. Polymorphisms in the Mannose binding lectin 2 (MBL2) gene are associated with frequent infective exacerbations (253). Frequent exacerbators also have a smaller reduction in systemic inflammation at recovery than infrequent exacerbators (254).

A further study of 182 exacerbations identified 4 biological exacerbation phenotypes: bacterial (associated with sputum IL-1β), viral (associated with serum IL-10), eosinophilic sputum (associated with peripheral eosinophils) and pauciinflammatory (232). In addition, Bafadhel et al investigated the use of blood eosinophils to direct corticosteroid therapy in a randomised controlled trial. There was no difference in health status improvement between patients given corticosteroids regardless of their eosinophil count and those given corticosteroids based on their eosinophil count (255). There was also significantly more treatment failures in patients who were biomarker negative but received corticosteroids compared to those biomarker negative receiving placebo. A further randomised controlled trial (RCT) investigating the use of the anti-TNFα treatment etanercept also demonstrated that patients with raised blood eosinophils benefited from treatment with oral corticosteroids (256). These studies provide evidence that raised
eosinophils may be potential biomarker for targeted therapy, but further work is required in more severe exacerbations before drawing robust conclusions.

1.5.4 Inflammation in COPD exacerbations

COPD exacerbations are associated with increased systemic and airway inflammation. A summary of important studies is shown in Table 1.3.

Studies investigating changes in inflammation have largely been small and diverse in their methodology. Furthermore, definitions of exacerbations vary. There is little information on changes in lower airway inflammation due to the obvious difficulty in invasive bronchoscopic sampling during exacerbations. Studies investigating lower airway inflammation during exacerbations have demonstrated increased neutrophilic inflammation with increased neutrophils, NE, IL-8 and CXCR1/2 expression in BAL, lower airway aspirates or bronchial biopsies (257, 258). These studies both used patients with very severe exacerbations who were intubated so it must be said that these patients may have also had other secondary infections.

During exacerbations, there may be increased sequestration of neutrophils in the pulmonary circulation prior to release into the airways (259). Increased sputum neutrophils have been demonstrated in the sputum of exacerbators (94, 229, 234, 260), and the severity of exacerbation has been shown to be related to sputum neutrophils (229). There is increased expression of the neutrophil adhesion molecule ICAM-1 in the plasma (261) and serum (262) of exacerbating patients, although this has not been a universal finding (189). There are also increased levels of the neutrophil chemoattractants IL-8 and LTB4 in sputum from patients with COPD exacerbations (234, 235, 238, 263). Thus, the increased number of airway neutrophils during exacerbation may be due to increased sequestration in the
pulmonary circulation, increased expression of adhesion molecules and increased chemoattractants within the airway.

In addition to increased numbers, there is also evidence for increased neutrophil activation in the airways during exacerbations, with increased sputum NE, MMP-9 and MPO (94, 207, 229, 235, 260, 263, 264). Patients with frequent exacerbations have higher baseline sputum IL-6 and IL-8 than those who exacerbate infrequently (265). Other inflammatory mediators are also elevated during exacerbations; studies have reported increased TNFα (235, 238), endothelin (ET)-1 (266), MMP-8 (260), eosinophilic cationic protein (ECP) and regulated on activation normal T cell expressed and secreted (RANTES) (94). Studies measuring exhaled breath condensate during exacerbations have also reported increased IL-6, IL-8 and TNF-α (267) and increased markers of oxidative stress (268).

There is evidence of increased systemic inflammation during exacerbations which may be a result of overspill from the airway, and in contrast to the stable state there appears to be a correlation between the degree of airway inflammation and the size of the systemic response (264). A number of studies have demonstrated elevated serum or plasma IL-6, CRP, fibrinogen, IL-8 and LTB4 (234, 235, 261, 263, 264, 269-271) in exacerbations. A study investigating the use of plasma biomarkers to confirm exacerbation or severity found increased CRP, IL-6, myeloid progenitor inhibitory factor (MPIF)-1, pulmonary and activation-regulated chemokine (PARC) and ICAM-1, but only CRP had value in confirming an exacerbation (261). Elevated CRP 14 days after an initial exacerbation is a predictor for a further exacerbation within 50 days (254). One study has reported increased systemic TNFα (272), although this has not been replicated in subsequent studies (261, 269).
There is also evidence for increased systemic neutrophilic inflammation with increased numbers (234) and activation: one study has shown increased numbers of segmented neutrophils (269) and one has demonstrated increased ROS release for patients with bacterial exacerbations compared to the stable state (273). In addition peripheral neutrophils undergo reduced spontaneous apoptosis during exacerbations (224, 274), leading to prolonged inflammation.

There are conflicting reports on patterns of inflammation and correlation with bacterial and viral infections. Exacerbations associated with infective aetiologies have been associated with increased blood neutrophil counts (229) and raised plasma IL-6 and fibrinogen (270). Bacterial infections have been associated with sputum IL-1β, IL-8, LTB4, MPO, NE and serum IL-6 and CRP (232, 234, 263, 264); CRP has been particularly associated with new strain exacerbations (235). However Aaron et al did not find a relationship between infection and inflammation (238), although the numbers of patients enrolled in this study was small (n=14 exacerbations) and only 3 patients had evidence of viral or bacterial infection.

Few studies have examined inflammation following viral exacerbations, finding: elevated serum IL-10 (232, 275), sputum eosinophilia (229) and sputum IL-6 (246, 271). However, another study has demonstrated that the presence of rhinovirus did not cause any changes in inflammation (264). Experimental rhinovirus infection in patients with COPD leads to increased sputum neutrophils, NE and IL-8, BAL IL-6, blood neutrophils and serum CRP (247). Therefore it seems that viral infection does cause an inflammatory response, but the exact nature of this is yet to be determined.
<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Sample</th>
<th>Raised marker (Exacerbated vs stable COPD)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bathoorn et al (2009)</td>
<td>45 matched exacerbation and stable</td>
<td>Sputum, Blood, Serum</td>
<td>Neutrophils, Eosinophils, Lymphocytes, LTB4, MCP-1, ECP, Neutrophils, IL-6</td>
<td>(234)</td>
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<tr>
<td>Sethi et al (2008)</td>
<td>177 exacerbation, 148 matched pre and 133 post exacerbation</td>
<td>Sputum, Serum</td>
<td>IL-8, TNF-α, NE, CRP, Further increase in new strain exacerbations</td>
<td>(235)</td>
</tr>
<tr>
<td>Ilumets et al (2008)</td>
<td>12 Hex, 28 HS, 15 stable COPD, 15 exacerbations and 8 matched stable</td>
<td>Sputum</td>
<td>Neutrophils, MMP-9, MMP-8, NE</td>
<td>(260)</td>
</tr>
<tr>
<td>Rohde et al (2008)</td>
<td>36 exacerbations, 20 stable</td>
<td>Serum, Blood, Sputum</td>
<td>CRP, Leucocytes, IL-6 (viral only)</td>
<td>(271)</td>
</tr>
<tr>
<td>Pinto-Plata et al (2007)</td>
<td>20 matched exacerbation and stable</td>
<td>Plasma, Blood</td>
<td>IL-6, IL-8, LTB4, Segmented neutrophils</td>
<td>(269)</td>
</tr>
<tr>
<td>Authors</td>
<td>Study Design</td>
<td>Sample Type</td>
<td>Biomarkers</td>
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<td>Papi et al (2006)</td>
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<td>Neutrophils, Eosinophils (viral only), ECP, NE</td>
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<td>Hurst et al (2006a)</td>
<td>90 matched exacerbation and stable</td>
<td>Plasma</td>
<td>CRP, IL-6, MPIF-1, PARC, s-ICAM-1</td>
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<tr>
<td>Hurst et al (2006b)</td>
<td>41 exacerbations, 21 matched stable, 26 stable</td>
<td>Sputum, Nasal wash, Serum</td>
<td>Leucocytes, MPO, Leucocytes, IL-6, MPO, IL-6, CRP</td>
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<tr>
<td>Fujimoto et al (2005)</td>
<td>30 matched exacerbations and stable, 32 stable, 11 HNS</td>
<td>Sputum</td>
<td>Neutrophils, Eosinophils, Lymphocytes, IL-8, ECP, NE, RANTES</td>
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</tr>
<tr>
<td>Study</td>
<td>Exacerbations/Control</td>
<td>Sample Type</td>
<td>Findings</td>
<td>Reference</td>
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<tr>
<td>Drost et al (2005)</td>
<td>14 exacerbations (7 intubated), 7 stable COPD, 10 HNS, 12 HS</td>
<td>BAL Large airway secretions</td>
<td>Percentage neutrophils Glutathione (reduced levels) IL-8</td>
<td>(258)</td>
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<tr>
<td>Calikoglu et al (2004)</td>
<td>16 exacerbations, 26 stable, 15 HNS</td>
<td>Serum</td>
<td>TNFα</td>
<td>(272)</td>
</tr>
<tr>
<td>Qiu et al (2003)</td>
<td>15 intubated exacerbations, 7 stable COPD, 15 HNS</td>
<td>Bronchial epithelium Bronchial subepithelium</td>
<td>NE NE IL-8 IL-5 CXCR1 CXCR2</td>
<td>(257)</td>
</tr>
<tr>
<td>Aaron et al (2001)</td>
<td>14 matched exacerbation and stable, 36 stable</td>
<td>Sputum</td>
<td>IL-8 TNFα</td>
<td>(238)</td>
</tr>
<tr>
<td>Gompertz et al (2001)</td>
<td>84 matched exacerbations and stable</td>
<td>Sputum (purulent group only) Serum</td>
<td>MPO NE LTB4 CRP</td>
<td>(263)</td>
</tr>
<tr>
<td>Roland et al (2000)</td>
<td>22 exacerbations, 14 paired stable, 7 stable</td>
<td>Sputum</td>
<td>Endothelin-1</td>
<td>(266)</td>
</tr>
<tr>
<td>Wedzicha et al (2000)</td>
<td>120 exacerbations in 67 patients, 67 matched stable</td>
<td>Plasma</td>
<td>Il-6 Fibrinogen</td>
<td>(270)</td>
</tr>
<tr>
<td>Bhowmik et al (2000)</td>
<td>37 matched exacerbations and stable, 7 stable</td>
<td>Sputum</td>
<td>IL-6 (Associated with cold symptoms)</td>
<td>(265)</td>
</tr>
</tbody>
</table>

Table 1.3: A summary of studies demonstrating elevated inflammatory cells and mediators in COPD exacerbations compared to the stable state. (BAL: bronchoalveolar lavage; CRP: C reactive protein; CXCR: chemokine (C-X-C motif) receptor; ECP: eosinophilic cationic protein; HEX: healthy ex smoker; HNS: healthy non smoker; HS: healthy smoker; ICAM: intracellular adhesion molecule; IL: interleukin; LTB4: leukotriene B4; MCP: monocyte chemotactic protein; MMP: matrix metalloproteinase; MPIF: myeloid progenitor inhibitory factor; MPO: myeloperoxidase; NE: neutrophil elastase; PARC: pulmonary and activation-regulated chemokine; TIMP: tissue inhibitors of metalloproteins; RANTES: regulated on activation T cell expressed; TNF: tumour necrosis factor)
1.6 Inhaled Lipopolysaccharide and Toll-Like Receptors

1.6.1 Toll-Like Receptors

TLRs are pattern recognition receptors (PRRs) which sense microbial stimuli such as LPS and other pathogen associated molecular patterns (PAMPs). TLRs are expressed on multiple airway cells including epithelial cells, fibroblasts, airway smooth muscle, endothelial cells, macrophages and neutrophils (276). TLRs are key members of the innate immune system and play an important role in detecting invading pathogens and inducing host antibacterial defences; therefore activation may also cause excessive inflammation.

10 TLRs have been identified in humans and 12 in mice, each interacting with distinct agonists. TLRs may be differentiated based on those responding to bacteria (TLR1, 2, 4, 5, 6 and 9) or viruses (TLR3, 7 and 8). However, some viruses may activate TLR4 and TLR9 and therefore this differentiation is not exclusive. TLRs may also be distinguished by the location of their interaction with their associated ligand; TLR1, 2, 4, 5 and 6 interact on the cell surface while the others bind ligands in intracellular endosomes (277).

TLR2 interacts with a number of ligands including gram positive bacteria, mycobacteria and fungi (278). TLR1 and 6 are structurally similar to TLR2, and stimulation of TLR2 leads to cross linking between these receptors which may explain the large variation in microbial ligands detected. TLR4 is essential in the recognition of LPS (279); this interaction requires recruitment of the accessory molecules CD14 and MD-2 (280). CD14 polymorphisms have been associated with increased prevalence of gram negative infections in critically ill patients (281) as well as atopy and immunoglobulin (Ig)E levels in children (282). Other implicated
PAMPs associated with TLR4 include *Streptococcus pneumoniae* pneumolysin (283), heat shock proteins (hsp) (284) and respiratory syncytial virus (RSV) (285). TLR5 recognizes flagellin, a constituent of bacterial flagella. Flagellin stimulates bronchial epithelial cells to produce IL-8 and polymorphisms in TLR5 are associated with susceptibility to the flagellated bacterium *Legionella pneumophilia* (286).

TLR3, 7 and 8 have been implicated in antiviral activity and upon stimulation induce strong type 1 interferon responses. TLR3 recognizes double stranded (287) and TLR7 and 8 recognize single stranded viral RNA (288). TLR9 can distinguish between human and non-human DNA through its ability to recognise cytosine-guanine dinucleotide (CpG) motifs (289). It is principally antibacterial but also recognizes viral RNA. The function of TLR10 is still unknown.

Signalling via TLRs occurs via a complex process. TLR4 signalling is represented in Figure 1.5. The receptor has 2 domains: an extracellular domain and a cytoplasmic domain. The extracellular domain directly recognizes PAMPs and upon activation lead to the recruitment of downstream signalling molecules (290); this process is either dependent on or independent of the protein myeloid differentiation factor 88 (MyD88). TLR3 can only signal through the MyD88 independent pathway, TLR4 acts through both and all other TLRs act through the MyD88 dependent pathway.

MyD88 facilitates interaction between the IL-1 receptor associated kinases (IRAK)-1 and 4, which leads to phosphorylation of IRAK 1 (291). This induces activation of TNF receptor associated factor (TRAF)-6 which in turn activates transforming growth factor activated kinase (TAK)-1. Activated TAK-1 ultimately leads to stimulation of the mitogen activated protein (MAP) kinase (292) cascade and the nuclear factor kappa beta (NF-ĸB) pathway (293), leading to inflammatory cytokine
production. The MyD88 independent pathway signals through Toll-IL-1R domain containing adaptor inducing IFN-β (TRIF) which phosphorylates IFN regulatory factor (IRF) 3. This then induces expression of IFN-β and IFN inducible genes (294). This pathway also leads to activation of the NF-κB pathway, although this is slightly delayed. There is increasing evidence for a direct link between TLR stimulation and activation of the PI3 kinase enzyme pathway; this may be via direct interaction between phosphorylated tyrosine kinase on TLRs and the p85 subunit (295) or via TLR adaptor proteins.

Neutrophils express all TLRs except for the intracellular TLRs 3 and 7 (296). Stimulation of TLR2 and 4 leads to upregulation of CD11b on the cell surface (297) and shedding of L selectin (298), thus TLRs in neutrophils may have a role in neutrophil recruitment. In addition there is evidence to support TLR stimulation causing neutrophil activation, in terms of production of ROS (298), cytokine release (299, 300) and phagocytosis (298). Finally, stimulation of TLR4 delays neutrophil apoptosis therefore causing persistent neutrophilic inflammation (297).

There is also evidence for the involvement of TLRs in the pathogenesis of COPD. Mouse models of COPD implicate TLR4 in cigarette smoke induced inflammation (301). In addition there is increased expression of TLR1, 2, 4 and 6 in CD8 lymphocytes in lung tissue from patients with COPD compared with controls (302). However, there have been conflicting reports regarding TLR2 and 4 expression in COPD; one study reported reduced TLR2 expression on alveolar macrophages (303) and sputum in COPD (220), while others have reported increased expression in peripheral blood monocytes (304). Furthermore, neutrophil numbers and TLR4 but not TLR2 expression was increased in the BAL fluid from intubated COPD patients (305), implicating TLR4 in airway neutrophilia. There is also increased CD14
expression in the sputum of smokers with and without COPD (220). Polymorphisms in TLR2 and 4 are associated with increased decline in FEV1 and increased sputum inflammatory cells in patients with COPD (306). Therefore, TLRs appear to be associated with the inflammation seen in COPD and are involved in its pathogenesis; the exact mechanisms of this process, however, are yet to be elucidated.
Upon activation with the LPS/MD-2 complex, Toll-like receptor 4 (TLR4) signals via the MyD88 dependent or independent pathway. The MyD88 dependent pathway involves phosphorylation of IL-1 receptor associated kinases (IRAK1) and activation of nuclear factor-κB (NF-κB) and mitogen activated protein (MAP) kinase downstream. The MyD88 independent pathway involves phosphorylation or IFN regulatory factor (IRF) 3 and induces the production of interferon (IFN)-β and IFN-inducible gene.
1.6.2 Inhaled Lipopolysaccharide

Endotoxin and its purified constituent LPS are bacterial constituents of gram negative cell walls and act upon TLR4, which stimulates a number of pro-inflammatory signalling pathways. Endotoxin has been implicated as an aetiological factor in occupational lung diseases such as byssinosis and swine worker’s disease (307). There is also a relationship between levels of endotoxin in house dust and the severity of allergic asthma (308). Endotoxin is present in the domestic and occupational environment, in addition to cigarette smoke.

LPS causes a systemic inflammatory response when administered intravenously, causing increased circulating cytokines and flu like symptoms; thus this has been used as an experimental model of sepsis (309). Intra-segmental application of LPS via a bronchoscope has also been used (310, 311), although this method is invasive and widespread use is limited. LPS, when administered systemically or via the inhaled route causes increased neutrophilic inflammation (309, 312). Thus inhaled LPS may be a model in which to test the use of new pharmacotherapies targeting neutrophils.

LPS has been administered via inhalation to asthmatics, healthy smokers and non-smokers in doses of 0.5-300µg (312-316) to induce airway and systemic neutrophilic inflammation. Initial LPS provocation studies involved exposing healthy volunteers to swine dust or making subjects inhale solutions of cotton dust, which unfortunately involved a high incidence of side effects and the exact dose of LPS delivered could not be quantified (317). Lyophilized LPS was then developed, which was LPS bound to the walls of *Escherichia coli*; these preparations also caused significant side effects as the LPS would also be bound to other proteins. Rylander et al administered
300µg LPS from *Enterobacter agglomerans*, leading to 88% of subjects experiencing physical side effects (313).

More recently, LPS has been purified to avoid bacterial contaminants. The most common source used is *Escherichia coli* serotype 026:B6. A number of studies have used purified LPS as a model of airway and systemic inflammation (summarised in Table 1.4) and have demonstrated that inhaled LPS is a safe and reproducible method of studying airway inflammation (315, 316).

LPS inhalation causes airway neutrophilia in healthy volunteers, smokers and asthmatics (314, 316, 318-324), which has been associated with an increase in IL-8 (319, 321, 323, 324) and markers of neutrophil activation such as MPO, MMP-9 and NE (314, 321, 322, 324, 325). There is also an increase in the number of circulating neutrophils (312, 314, 315, 322, 324-327), along with an increase in systemic IL-6 and CRP (316, 328). Some studies have also reported increased airway TNFα, IL-6, ECP, MCP-1, MIP-1α, MIP-1β and IL-6 (314, 321-323, 325) and systemic TNFα (325, 326) post LPS challenge. However, there are some inconsistencies between studies which may be due to different serotypes and doses of LPS used. Other newer biomarkers studied by Aul et al (316) will be discussed later.

These changes in inflammation are sometimes associated with flu-like symptoms, but these are short lived and well tolerated (314, 315). Falls in FEV1 (312, 316, 323, 325, 329) and rises in temperature (312, 314-316, 319, 323, 324) have also been demonstrated with varying doses of LPS, although this has not been a universal finding (315, 322, 330).

Some studies report a dose dependent increase in inflammation and symptoms (314, 316, 325). However, some report distinct phenotypes of patients with some being
sensitive, depending on fall of FEV1 in response to LPS, and others hypo-responsive or non-sensitive (312, 329). Therefore, even in healthy volunteers, differing responses to LPS may be expected perhaps due to genetic factors or sex differences. In addition to TLR4, responses to LPS are mediated by interaction with MD2, CD14 and lipopolysaccharide binding protein (LBP). Polymorphisms in these mediators may also lead to the differential responses to LPS seen (280).

Inhaled LPS has been used as a model of inflammation to investigate pharmacotherapies in healthy volunteers (321, 322, 324, 330). There is currently sparse literature on the use of inhaled LPS in patients with COPD. LPS inhalation in COPD could be a model of acute on a background of chronic neutrophilic inflammation, as seen in COPD exacerbations (229, 234). Thus, new pharmacotherapies for use during exacerbations could be investigated in vivo using this model.
<table>
<thead>
<tr>
<th>Study</th>
<th>Patients</th>
<th>LPS dose (µg)</th>
<th>Airway changes</th>
<th>Systemic changes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandstrom et al (1992)</td>
<td>8 HNS</td>
<td>25</td>
<td>↑BAL neutrophils, lymphocytes and fibronectin ↓BAL macrophage phagocytosis</td>
<td>Not measured</td>
<td>(318)</td>
</tr>
<tr>
<td>Michel et al (1992)</td>
<td>8 Asthma</td>
<td>20</td>
<td>↑Bronchial hyper-responsiveness</td>
<td>↑Neutrophils ↑Leucocytes ↑TNFα ↑CRP</td>
<td>(326)</td>
</tr>
<tr>
<td>Michel et al (1995)</td>
<td>8 HNS</td>
<td>20</td>
<td>↔FEV1</td>
<td>↑Leucocytes ↑Neutrophils ↑Neutrophil chemiluminescence ↑ACTH ↑CRP ↔TNFα, C3, haptoglobin</td>
<td>(327)</td>
</tr>
<tr>
<td>Michel et al (1997)</td>
<td>9 HNS</td>
<td>0.5, 5, 50</td>
<td>↔FEV1</td>
<td>↑Temp (50µg) ↑Neutrophils ↑CRP</td>
<td>(314)</td>
</tr>
<tr>
<td>Study</td>
<td>Patients</td>
<td>Challenge Duration</td>
<td>Outcomes</td>
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<tr>
<td>Nightingale et al (1998)</td>
<td>11 HNS</td>
<td>60</td>
<td>↔FEV1</td>
<td></td>
<td></td>
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<td></td>
<td>7 Atopic</td>
<td></td>
<td>↑sputum neutrophils (HNS and asthma)</td>
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<td>↑IL-8 from sensitive AMs compared with hyporesponsive</td>
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<td>↓IL-6 and IL-8 from peripheral blood monocytes from sensitive compared with hyporesponsive</td>
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<td>Alexis et al (2003)</td>
<td>10 Asthma</td>
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</tr>
<tr>
<td>Michel et al (2005)</td>
<td>15 HNS</td>
<td>50</td>
<td></td>
<td>↔FEV1</td>
<td>↔Temp</td>
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<tr>
<td>Roos-Engstrand et al (2005)</td>
<td>15 HNS</td>
<td>50</td>
<td>↑p38 expression bronchial epithelial cells ↑BAL neutrophils, TNFα, IL-6</td>
<td>Not measured</td>
<td></td>
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<tr>
<td>Kitz et al (2006)</td>
<td>30 HNS</td>
<td>100 (cumulative)</td>
<td>↓FEV1 in 12 “sensitive” (↓FEV1 &gt;12.5%) HNS and 6 “sensitive” asthmatics ↑eNO asthmatics</td>
<td>↑CRP ↑Leucocytes ↑Neutrophils ↑LBP ↑Temp</td>
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<tr>
<td>Michel et al (2007)</td>
<td>18 HNS</td>
<td>50</td>
<td></td>
<td>↔FEV1 ↑sputum neutrophils ↑MMP9 ↑TNFα ↔MPO, TIMP1</td>
<td>↔Temp ↑CRP ↑LBP ↑Neutrophils ↑E-selectin</td>
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<tr>
<td>Kitz et al (2008)</td>
<td>20 HNS</td>
<td>100 (cumulative)</td>
<td>↔eNO, FEV1</td>
<td></td>
<td>↑Temp ↑Leucocytes ↑Neutrophils ↑CRP ↑LBP ↔IL-8</td>
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<tr>
<td>Fouassier et al (2009)</td>
<td>12 HNS</td>
<td>50</td>
<td></td>
<td>Not measured</td>
<td>↑IL-6 ↑CRP</td>
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<tr>
<td>Study</td>
<td>Group Description</td>
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<td>Changes</td>
<td>Inflammation Markers</td>
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<tr>
<td>Aul et al (2012a)</td>
<td>14 HNS</td>
<td>30</td>
<td>↓FEV1</td>
<td>↑Sputum neutrophils ↑IL-8 ↑IL-6 ↑MCP-1 ↔GROα</td>
<td>(323)</td>
</tr>
<tr>
<td>Aul et al (2012b)</td>
<td>12 Healthy smokers</td>
<td>5, 30</td>
<td>↓FEV1</td>
<td>↑Sputum neutrophils ↓sputum macrophages ↑nuclear p65</td>
<td>(316)</td>
</tr>
<tr>
<td>Korsgren et al (2012)</td>
<td>19 HNS</td>
<td>5, 50</td>
<td>↔α1-antitrypsin, NE ↑TNFα ↑NE activity</td>
<td>↑Neutrophils ↑Leucocytes ↑CRP, ↑TNFα ↑α1-antitrypsin</td>
<td>(325)</td>
</tr>
<tr>
<td>Janssen et al (2013)</td>
<td>12 HNS</td>
<td>2</td>
<td>↑sputum neutrophils ↑sputum monocytes ↑FEV1 ↑IL-8 ↑MPO</td>
<td>↑Temp ↑Neutrophils ↑Leucocytes</td>
<td>(324)</td>
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**Table 1.4: A summary of studies using inhaled Lipopolysaccharide and the effects on airway and systemic inflammation.** (ACTH: adrenocorticotropic hormone; AM: alveolar macrophage; BAL: bronchoalveolar lavage; CC-16: clara cell protein 16; CCL: Chemokine (C-C motif) ligand; CRP: C reactive protein; ECP: eosinophilic cationic protein; eNO: exhaled nitric oxide; FEV1: Forced expiratory volume in 1 second; GM-CSF: granulocyte macrophage- colony stimulating factor; GRO: growth related oncogene; HNS: healthy non smoker; IL: Interleukin; LBP: lipopolysaccharide binding protein; MCP: monocyte chemotactic protein; MIP: macrophage inhibitory protein; MMP: matrix metalloproteinase; MPO: myeloperoxidase; NE: neutrophil elastase; SPD: Surfactant protein D; TIMP: tissue inhibitors of metalloproteins; TNF: tumour necrosis factor).
1.6.3 Novel systemic biomarkers of COPD

No blood based biomarker of COPD has been proven to be a reliable endpoint for clinical trials to date. A number of novel proteins have recently been suggested as potential biomarkers, although further validation and research is necessary before any robust conclusions can be drawn. Some of these proteins are secreted by the lung and therefore are more specific to pulmonary disease, unlike others secreted by the liver and bone marrow which may be elevated in other chronic inflammatory disease.

Serum Clara cell secretory protein-16 (CC-16) is secreted by Clara cells in the respiratory bronchioles; although some is produced in the urogenital tract serum levels largely reflect production in the respiratory tract. Analysis from the ECLIPSE study demonstrated lower levels of serum CC-16 in COPD patients compared with healthy smokers (332). Lower levels are also seen in healthy smokers compared to non smokers (333), indicating that cigarette smoke may be in part responsible for the effect seen. LPS inhalation also causes an acute increase in serum CC-16 in healthy non smokers (330) and smokers (316). Recently low serum CC-16 has been shown to be associated with rapid decline in FEV1 (334), therefore may be a marker of poor prognosis.

PARC/CCL-18 is expressed by macrophages and dendritic cells in the lungs (335). Serum PARC is elevated in pulmonary fibrosis (336) and acute coronary syndromes (337). In COPD, serum levels are raised and associated with an increased risk of cardiovascular hospitalisation and increased mortality (338). Increased serum PARC has been demonstrated post LPS inhalation in smokers (316) and has also been associated with acute exacerbations of COPD (261).
Surfactant protein D (SPD) is produced by type II pneumocytes and clara cells in the lung (339). Levels are elevated in patients with COPD and in smokers compared to non-smokers (340). High SPD levels are also associated with increased risk of COPD exacerbations (340). β-defensin 2 is produced by lung epithelial cells and is a neutrophil chemoattractant (341); levels are increased in the BAL of patients with COPD compared to controls (305). LPS inhalation in smokers causes an increase in serum levels of both these biomarkers (316).

Plasma fibrinogen is also elevated in patients with COPD compared to healthy controls (342). It is an acute phase soluble glycoprotein which is produced by the liver. During coagulation it is converted into fibrin under the action of thrombin. High levels are associated with increased disease severity (343), increased exacerbations (344) and higher risk of death (342).

1.7 Glucocorticoids

1.7.1 Mechanisms of action

Glucocorticoid action is summarised in Figure 1.6. Glucocorticoids (GC) act by diffusing across the cell membrane and activating a cytoplasmic glucocorticoid receptor (GR). The GR is held in a resting state in the cytoplasm by associating with heat shock protein 90 (hsp90). There are 2 form of GR: GRα and GRβ; GRβ may act as a dominant negative inhibitor of GC action by interfering with binding of DNA to GRα (345). When the GC binds to the GR, the hsp90 complex dissociates leading to GR phosphorylation. The activated GR then translocates to the nucleus where it directly associates with DNA at GC response elements (GREs) leading to the expression of anti-inflammatory genes, a process known as transactivation.
The major action of GC is to inhibit the production of inflammatory genes in the nucleus by acting at promoters (346) by a process known as transrepression. Transcription factors such as activator protein (AP)-1 and NF-κB regulate the production of inflammatory genes. Activated GR binds to these transcription factors and recruits corepressor proteins such as histone deacetylase (HDAC) 2, and therefore prevents these transcription factors from switching on inflammatory genes (347).

1.7.2 Glucocorticoid resistance in COPD

There has been no proven benefit of the long-term use of inhaled corticosteroids (ICS) alone in unselected COPD patients (348, 349). ICS alone also appear to have little effect on sputum inflammatory cells (350, 351). However, in combination with long acting beta agonists (LABA) there is evidence of a reduction in exacerbations, improved quality of life and lung function (352, 353). Current guidelines therefore recommend that ICS are not prescribed to all patients with COPD, and are only given to specific phenotypes: those patients with severe disease and frequent exacerbations (5). Therefore there is a degree of steroid insensitivity associated with COPD.

In exacerbations, oral GC therapy is currently recommended as there is evidence of improved clinical outcome, reduced length of hospital stay and rapid improvement in FEV1 (251, 354, 355). A recent study has suggested that systemic GC only benefit patients with eosinophilic exacerbations (255). In addition, GC have significant side effects including weight gain, osteoporosis, diabetes mellitus and hypertension. Thus there is a great need to develop new therapies for use in stable and exacerbated COPD.
A number of studies have investigated the effects of corticosteroids in vitro. One study demonstrated that dexamethasone had a reduced effect on the release of IL-8 from COPD alveolar macrophages compared to healthy smokers (356). However, a further study by Armstrong et al failed to replicate this (357). Furthermore this study suggested a number GC resistant cytokines: in healthy smoker, non-smoker and COPD alveolar macrophages, dexamethasone did not inhibit the release of G-CSF, GM-CSF and IL-8 (357). These findings support the theory that GC do not target the whole inflammatory genome; even in healthy cells some genes are regulated by transcriptional mechanisms not affected by the action of GC (358).

GC resistance may be due to a number of other factors. Neutrophilic inflammation predominates in COPD which is relatively steroid resistant (359); some studies have suggested that these cells have relatively high expression of the GRβ isoform (360). Higher levels of GRβ relative to GRα may impair GRα function. Recently it has been demonstrated that COPD airway neutrophils have low levels of GR expression (361) which may also explain GC insensitivity.

Genetic mutations may also contribute to steroid insensitivity, and culprit genes have been identified in asthma (362). However, the link in COPD has not been established as yet. Genetic polymorphisms in GRβ may also be responsible for reduced transrepression (363), but these have not been definitely linked with GC resistance in inflammatory disease.

Oxidative stress is thought to play a key role in the development of inflammatory responses and GC insensitivity. Increased oxidative stress in COPD is due to a combination of cigarette smoke and endogenous factors such as reactive oxygen species produced by activated neutrophils and macrophages. In animal models,
exposure to cigarette smoke leads to GC resistance (364), and there is also relative GC insensitivity in asthmatics who smoke (365). Oxidative stress acutely reduces HDAC2 activity in *in vivo* and *vitro* models (359), which in turn leads to an imbalance in histone acetylation. As a result there is reduced inhibition of inflammatory genes and therefore GC resistance. Low levels of HDAC2 are expressed in alveolar macrophages, airways and peripheral lung of patients with COPD (366). Increasing HDAC2 levels using low doses of theophylline leads to restoration of GC sensitivity (367).

Other potential therapies which may increase HDAC2 include nuclear factor erythroid 2-related factor 2 (NRF2) activators (368) and PI3 kinase enzyme inhibitors (364). The PI3 kinase enzyme pathway is activated by oxidative stress; inhibitors of the PI3Kδ isoform restore GC sensitivity both in animal models of COPD (364) and in COPD blood monocytes (369). Other kinases may play a role in GC resistance: the p38 MAP kinase pathway is upregulated in COPD (370), which may cause phosphorylation of GRα and therefore interfere with its ability to bind to ligands (371).
Figure 1.6: The mechanism of action of glucocorticoids by transrepression and transactivation. Glucocorticoids diffuse across the cell membrane (A) and activate a cytoplasmic Glucocorticoid Receptor (GR) (B), held in a resting state by chaperone proteins e.g. Heat Shock Protein (hsp) 90. The activated GR translocates to the nucleus (C) and binds to transcription factors such as nuclear factor-κB (NF-κB) (D), preventing them from switching on inflammatory genes (X), a process called transrepression. Activate GR also binds directly with glucocorticoid response elements (GRE) (E) and stimulate the production of anti-inflammatory genes, a process known as transactivation.
1.8 The p38 MAP kinase enzyme pathway

1.8.1 Pathway overview

To date five different MAP kinase enzyme pathways have been identified, each activated by distinct stimuli and causing distinct downstream cascades. The classical MAP kinase enzyme pathway involves the enzyme extracellular signal-regulated kinases (ERK)-1 or ERK-2 and is typically activated by mitogenic stimuli. This pathway has a key role in proliferation and growth and therefore has been most extensively studied in tumorgenesis (372). The enzymes c-Jun N-terminal kinases (JNK) or stress-activated protein kinase (SAPK) pathways are stimulated by ultraviolet (UV) light and stress and have a key role in apoptosis and inflammation (373). The p38 MAP kinase enzyme pathway and the effect of small molecule inhibitors of this pathway have been studied in COPD.

4 enzyme isoforms make up the p38 MAP kinase enzyme (MAPK) family: p38α, p38β, p38γ and p38δ. α, β, and δ are widely expressed whereas γ is limited to muscle. Osmotic shock, UV light, GPCR ligands and hormones cause activation through dual tyrosine phosphorylation. This phosphorylation occurs through the upstream enzyme MAPK kinases (M KKs) 3 and 6, which are activated by the enzyme MAPK kinase kinases (MEKKs) such as MEKK3, apoptosis signal regulatory kinase 1 and TAK-1. The p38 pathway promotes translation and stabilises inflammatory protein messenger RNA (mRNA) (374); this results in the expression of inflammatory cytokines such as IL-8, TNF-α and MMPs from immune cells. This occurs through activation of a number of transcription factors including actifacting transcription factor (ATF)-2, Signal Transducers and Activators of Transcription (STAT)-1 and myocyte enhancer factor (MEF)-2. The wide variety of MEKKs involved in activation of the pathway allows interaction of the p38 MAP kinase
enzyme pathway with other signalling pathways. The p38 MAP kinase enzyme pathway is summarised in Figure 1.7.

1.8.2 The p38 MAP kinase enzyme pathway in COPD

Cigarette smoke, oxidative stress and cytokines activate p38 MAP kinase enzymes (375-377), implicating the pathway in the pathogenesis of COPD. Furthermore, there is increased p38 MAP kinase expression in the airways of patient with COPD compared with healthy controls (370, 378). Inhibitors of the α and β isoforms of p38 MAP kinase enzyme have been shown to reduce cigarette smoke induced IL-6 and cyclooxygenase-2 in murine airways (379); inhibition of LPS induced TNFα and GM-CSF release from alveolar macrophages from smokers has also been demonstrated (380). In addition, a number of studies have shown that p38 MAP kinase enzyme inhibitors reduce cytokine production from COPD airway cells in vitro (370, 381).

LPS, fMLP and TNFα stimulation of peripheral human neutrophils leads to activation of the p38 MAP kinase enzyme pathway (382, 383) in vitro. P38 MAP kinase enzyme inhibitors reduce the release of and gene expression of IL-8, CCL3 and CCL4 in response to LPS or TNFα stimulation, which is associated with reduced p38 MAP kinase enzyme activation (384). Furthermore, p38 MAP kinase enzyme inhibition has been shown to reduce fMLP mediated neutrophil chemotaxis and superoxide generation (383). Exocytosis of primary and secondary granules has also been shown to be diminished by p38 MAP kinase enzyme inhibitors (385). Therefore, the p38 MAP kinase enzyme appears to be involved in neutrophilic inflammation, again indicating a role in COPD pathogenesis. However, its role in the neutrophilic airway inflammation is yet to be clarified as lung and sputum
neutrophils have been shown to be devoid of the phosphorylated (phospho-) p38 enzyme (370).

*In vivo* studies have also demonstrated oral administration of p38 MAP kinase enzyme inhibitors reduces systemic inflammation in patients with COPD (386-388). There has been no proven reduction in sputum neutrophils and airway inflammation (386) and only one study has demonstrated an improvement in FEV1 after 6 weeks treatment (388). However, further longer term trials are necessary and there have been unacceptable side effects such as liver toxicity in rheumatoid arthritis trials; inhaled therapy may provide a safer delivery option.

There is also evidence that viral infection with rhinovirus causes p38 MAP kinase enzyme activation and release of inflammatory chemokines (389). Bacterial infection, notably *Moraxella catarrhalis* and *Haemophilus influenzae*, have also been shown to exert their effect on airway cells by potentiating the p38 MAP kinase enzyme pathway (390, 391). *Haemophilus influenzae* associated with exacerbations is associated with increased pathway stimulation compared with colonising bacteria (240). Thus, there appears to be a role for the p38 MAP kinase enzyme in COPD exacerbations and inhibition could be a future pharmacological approach.

1.9 The PI3 kinase enzyme pathway

1.9.1 Pathway overview

PI3 kinase is an important intracellular enzyme, which upon activation leads to a number of inflammatory processes (392). The enzyme was initially identified as a potential cancer target as it was purified with an oncogene in 1987 (393). In more
recent years the enzyme has been implicated in many disease processes due to its vital role in cell differentiation, growth, immune responses and metabolism.

PI3 kinase enzymes are divided into 3 subclasses depending on their structure: class I, II and III. PI3 kinase enzymes phosphorylate the D-3 position of the inositol ring of target lipids. Class I are the most extensively investigated and are activated by multiple cell receptors. They convert phosphatidylinositol 4, 5-biphosphate to phosphatidylinositol 3, 4, 5-triphosphate [PI(3,4,5)P₃], a ubiquitous lipid second messenger on the cell membrane. PI(3,4,5)P₃ acts as a docking site to recruit and activate proteins which contain phospholipid binding domains. These downstream proteins include protein kinases that promote cell growth, survival and proliferation (such as the serine/threonine kinase Akt, phosphoinositide-dependent kinase 1 (PDK1), and the Tec family kinases). In turn, Akt phosphorylates many downstream substrates, including forkhead box protein O (FOXO), glycogen synthase kinase-3 (GSK-3), Bad and mammalian target of rapamycin (mTOR). Activated mTOR stimulates protein synthesis by phosphorylating the ribosomal kinase protein S6K1 and S6K2 (394). mTOR also activates Akt (395), therefore indicating a crucial role for the enzyme in PI3 kinase enzyme signalling both upstream and downstream of Akt. In addition proteins that regulate guanosine triphosphate (GTPases) controlling cell motility and trafficking e.g. GTPase accelerating proteins (GAPs) and guanosine nucleotide exchange factors (GEFs) (396) as well as key scaffolding proteins regulating cell signalling e.g. Gab2 (397) are activated by PI(3,4,5)P₃.

Therefore the PI3 kinase enzyme is a central enzyme through which many secondary signals are transmitted through the cell. The challenge for targeting this process with enzyme inhibitors is to try to control pathological activation whilst minimising off-
target effects; this may be achieved by specifically inhibiting PI3 kinase enzyme isoforms.

The class I enzymes consist of a regulatory (p50, p55, p85 or p101) and a catalytic subunit (p110α, p110β, p110γ and p110δ) (392). Class Ia consists of α, β, and δ isoforms and is activated by receptor tyrosine kinases (RTK). Class Ib consists of the γ isoform and is activated by GPCR. The regulatory subunits mediate membrane localization, receptor binding, and activation, whereas the catalytic subunit phosphorylates phosphatidylinositol 4,5 bisphosphate to yield PI(3,4,5)P3. The PI3 kinase enzyme is activated by bacterial lipoproteins such fMLP, cytokines such as IL-8 (398) and TNFα (137) and also ROS (399).

The PI3K enzyme pathway is negatively regulated by the phosphatases phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and SH2 domain containing 5 inositol phosphatase (SHIP). Hydrolysis of PI(3,4,5)P3 by PTEN generates phosphatidylinositol 4, 5-biphosphate thereby terminating downstream PI3K signaling. In contrast, SHIP modulates but does not terminate PI3K signalling. The class I PI3K enzyme signalling pathway is represented in Figure 1.7.

The use of drugs targeting α and β isotypes are therapeutically limited as α and β knockouts in mice are lethal (400), suggesting a vital role for these isoforms in cell proliferation during development; however δ and γ knockouts are physiologically normal. In addition, the α and β isoforms are expressed by all cells and therefore potential toxicity is high, but the γ and δ isoforms are of particular importance in leucocytes (137). There is increasing evidence that the γ and δ isoforms are the main isoforms involved in PI3 kinase enzyme mediated innate and adaptive immune responses and cell migration. Specifically, the δ enzyme isoform has been implicated
in B cell development (401), cytokine receptor signalling (402), B-cell migration (403), T-cell development (404), cell proliferation (405), B- and T- cell antigen receptor signalling (401), smooth muscle tone and hypertension (406), and neutrophil migration and degranulation (137). The γ isoform has been reported to have a role in thymocyte development (407), T cell development (404), T cell migration (403), neutrophil migration (407), macrophage and dendritic cell migration (408), mast cell degranulation (409), neutrophil degranulation (137), insulin secretion (410) and myocardial contractility (411). The role of the PI3 kinase enzyme in neutrophils will be discussed in more detail in section 1.9.2.

Class II PI3K enzymes are widely expressed and consist of 3 isoforms although their exact function remains unknown. They are activated by a number of stimuli including CCL-2, TNFα, insulin and epidermal growth factor (EGF) (412). Class III PI3K enzymes consist of a single family member and are important in endocytosis and the trafficking of vesicles (413); in addition a role in TLR signalling has recently been identified (414).
Figure 1.7: A representation of class 1 PI3K and p38 MAPK enzyme signalling. Class I Phosphoinositide-3 kinases convert phosphatidylinositol 4, 5-biphosphate to PI(3,4,5)P$_3$. PI(3,4,5)P$_3$ acts as a docking site and activates a number of proteins including Akt. This then leads to the activation of multiple inflammatory downstream processes involved in cell growth, motility, proliferation, differentiation, chemotaxis and survival. The PI3K pathway is negatively regulated by PTEN and SHIP. TAK-1 or MEKK3 activate MKK3 or 6 which in turn activates the downstream p38 MAP kinase enzyme. This then leads to activation of a number of transcription factors including STAT-1, MEF-2 and ATF-2 leading to a number of inflammatory processes.
1.9.2 The PI3 kinase enzyme pathway in COPD

To date there are no published clinical trials of PI3 kinase enzyme inhibitors in COPD although there is increasing evidence for its role in disease pathogenesis and associated neutrophilic inflammation. Alveolar macrophages from COPD patients express increased PI3Kδ isoforms and phosphorylated Akt compared with healthy controls (364). Furthermore selective PI3Kδ but not γ enzyme inhibition reversed GC insensitivity in peripheral blood monocytes from COPD patients by restoration of HDAC2 (364). In addition, PIP3 levels and PI3Kδ isoform gene expression are increased in COPD blood neutrophils compared to healthy controls (214). Oxidative stress has also been shown to activate the PI3K enzyme pathway; mechanisms behind this may be through direct activation of receptor tyrosine kinases (415) or by inactivation of PTEN (399). MMP-9 also contributes significantly to COPD pathogenesis and its release may be upregulated by the PI3 kinase enzyme pathway (416).

PI3K enzyme activation appears to have a role in neutrophil chemotaxis. There is accumulation of PI(3,4,5)P3 at the leading edge of the migrating neutrophil and the faster, less accurate movement seen in COPD neutrophils towards a variety of chemoattractants can be reversed by the addition of a pan PI3K enzyme inhibitor (196). However, the exact role of the different isoforms is yet to be clarified. Some studies have demonstrated a reduction in fMLP induced directional movement in healthy neutrophils by the addition of a PI3Kδ isoform inhibitor (417); in addition PI3Kδ isoform inhibition reduced neutrophil influx in vivo in a murine model of acute lung injury (418). Other studies involving murine neutrophils suggest that PI3Kγ is the more important isotype in neutrophil migration (419, 420). PI3K enzyme activation in neutrophils may be chemoattractant specific as one study has
shown that the PI3K enzyme potentiated initial responses to fMLP but had no effect on actual migration towards it (421).

Class I PI3K enzymes are thought to play a major role in the generation of reactive oxygen species from neutrophils (398, 422, 423). fMLP mediated ROS release from human neutrophils is thought to involve both PI3Kδ and γ isoforms in a biphasic manner; the initial phase is PI3Kγ dependent and the later δ dependent phase also dependent upon initial γ activation (137). This study also demonstrated significant augmentation of the later δ mediated fMLP stimulated ROS release by initial TNFα priming (137). This model of neutrophil priming and stimulation has also been shown to cause increased PIP₃ accumulation which parallels ROS production (424).

The mechanisms behind PI3K enzyme activation causing ROS release from neutrophils may be three fold: phosphorylation of p47\textsuperscript{phox} via phosphoinositide dependent kinases (425), direct interaction of cytosolic subunits with phosphoinositides (426) or by activation of Rac (427). NADPH oxidase is composed of the membrane protein cytochrome b\textsubscript{558} and the 4 cytosolic proteins p47\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox} and Rac2 which assemble on cell membranes upon activation.

p47\textsuperscript{phox} in the resting cell is in the cytoplasm and held in an auto-inhibited state (428). Upon activation, p47\textsuperscript{phox} is phosphorylated on a number of serines (Ser303, Ser304, Ser315, Ser320, Ser328, Ser345, Ser348, Ser359, Ser370 and Ser379) (429). In vitro, phospho-akt phosphorylates p47\textsuperscript{phox} in human neutrophils stimulated by fMLP or by bacterial phagocytosis; this occurs at Ser304 and Ser328 (425, 430). Inhibition of Akt decreases ROS release in response to fMLP (425). 2 different isoforms of Akt have been demonstrated in neutrophils, and it is Akt2 which appears to be associated with p47\textsuperscript{phox} phosphorylation and NADPH assembly (431).
TNFα and GM-CSF priming prior to fMLP stimulation of neutrophils has also been shown to result in more intense phosphorylation of $p47^{phox}$ (432), mirroring the increase seen in PIP$_3$ (424) and ROS (137). TNFα priming causes phosphorylation at Ser345, an effect abrogated by p38 MAP kinase inhibition (433). There is also increased expression of $p47^{phox}$ in synovial fluid neutrophils from patients with rheumatoid arthritis (433), indicating a potential role in the associated inflammatory process. $p47^{phox}$ has a crucial role in host defence as knockout mice develop lethal infections and develop granulomatous inflammation (434).

TNFα and LPS stimulated release of IL-8, MIP-1α and MIP-1β from healthy neutrophils has been shown to be PI3 kinase enzyme dependent, particularly involving PI3Kδ (435). Moreover, this study demonstrated that phospho-Akt was downstream of the enzymes p38 MAP kinase and TAK-1, indicating a possible therapeutic axis of inhibition (435). Pan PI3K enzyme inhibition also inhibits human neutrophil degranulation in an in vitro model of allergic asthma (436). Peptidoglycan induces MMP-9 release from human neutrophils which is dependent on the p38 MAP kinase and PI3K enzyme pathways (372). Furthermore, NE and MMP-9 release from healthy neutrophils is reduced by pan PI3K enzyme inhibition (205, 437).

The role for the PI3K enzyme in neutrophil apoptosis is yet to be established: earlier studies suggested that GM-CSF and TNFα mediated delayed apoptosis was PI3K enzyme dependent (438) but a more recent study has suggested no role for class I isoforms in this process (274). In addition, this study showed that the delayed neutrophil apoptosis in COPD exacerbations was PI3K enzyme independent (274). Importantly PI3Kδ isoform inhibition had no effect on bactericidal activity in vitro (422). Thus, the PI3K enzyme appears to have an important role in neutrophil
recruitment, activation and inflammation and is an attractive future therapeutic target.

1.10 Hypotheses and Aims

1.10.1 Hypotheses

1. LPS inhalation in patients with COPD could be used as a model of COPD exacerbation in order to investigate new pharmacotherapies.

2. Increased neutrophilic inflammation associated with COPD in the stable state and during exacerbations is glucocorticoid insensitive but may be sensitive to inhibition of the PI3 kinase enzyme pathway.

1.10.2 Aims

Based upon these hypotheses I plan to use in vivo and in vitro models to pharmacologically assess neutrophil function in COPD. The following are the aims of this thesis:

1. To perform a pilot study using LPS inhalation in patients with COPD to test the safety of this model and to investigate the systemic and airway inflammatory response in vivo.

2. To investigate the action of PI3 kinase enzyme inhibitors and dexamethasone in vitro on neutrophilic inflammation in COPD patients during the stable state and exacerbations.
1.10.3 Experimental objectives

In order to meet these aims experiments were designed to meet the following objectives:

1. To investigate the safety of inhaled LPS in patients with mild to moderate COPD by recording symptoms and by measuring changes in FEV1, temperature, blood pressure, pulse and oxygen saturations post inhalation. In addition to investigate changes in sputum and systemic biomarkers of inflammation post LPS inhalation.

2. To investigate the effect of selective PI3 kinase enzyme isoform inhibitors and dexamethasone on MMP-9 and ROS release from airway and blood COPD neutrophils in the stable state.

3. To investigate the effect of selective PI3 kinase enzyme isoform inhibitors and dexamethasone on MMP-9 and ROS release from blood neutrophils in COPD exacerbations.

4. To investigate the effect of selective PI3 kinase enzyme isoform inhibitors and dexamethasone on MMP-9 release from LPS primed COPD neutrophils in the stable state.

5. To investigate the effect of selective PI3 kinase enzyme isoform inhibitors and dexamethasone on cytokine release from blood COPD neutrophils in the stable state.
2. Materials and Methods

2.1 Patient recruitment

Patients were recruited from a large clinical database of volunteers held at the Medicines Evaluation Unit and from respiratory wards at the North West Lung Centre, University Hospital South Manchester. Written informed consent was obtained from all patients and ethical approval was gained from all studies by the local ethics committee. Specific ethical approvals are outlined in the relevant chapters. All procedures were carried out in accordance with recommendations found in the Declaration of Helsinki (439). Height was measured to the nearest cm and weight to the nearest 100 grams. Body mass index (BMI) was calculated as weight (kg) / height$^2$ (m).

2.1.1 Inclusion and exclusion criteria

All patients with COPD fulfilled the following criteria:

- Physician diagnosis of COPD.
- Smoking history greater than 10 pack years.
- Over 40 years of age.
- FEV1/FVC ratio less than 70%.
- No respiratory infections in the preceding 6 weeks (stable patients only).

The following were exclusion criteria:

- History of other significant respiratory illness such as asthma.
- History of malignancy in the last 5 years.
- Any inflammatory disorders such as rheumatoid arthritis or inflammatory bowel disease.
• History of α1 antitrypsin deficiency

Other inclusion criteria specific to each study are described in the corresponding chapter.

2.2 Health status questionnaires

The modified Medical Research Council (mMRC) dyspnoea score was used to evaluate disability associated with symptoms of breathlessness. Patients were attributed to 1 of 5 statements encompassing the entire range of respiratory disability, from none (Grade 0) to incapacity (Grade 4). mMRC complements FEV1 in describing disability in COPD and can also predict survival (440).

2.3 Lung function

2.3.1 Spirometry

Before all lung function tests patients were asked to withhold use of short acting beta agonists (SABA) for 6 hours and/or LABA for 12 hours. Spirometry was performed using the vitalograph spirometer (Vitalograph, Buckinghamshire, UK). Equipment was calibrated daily as per manufacturer’s instructions. Patients were instructed to form a tight seal around a plastic mouth piece containing a filter and wear a nose clip and forcibly exhale for a minimum of 6 seconds to gain FEV1 and FVC measurements. Measurements were performed in triplicate and the highest value was recorded according to American Thoracic Society (ATS) guidelines (441).
2.3.2 Reversibility

FEV1 and FVC was measured before and 20 minutes after administration of 200µg salbutamol (Ventolin inhaler, Baker Norton, London, UK) via a spacer. Reversibility in millilitres was calculated by subtracting pre-bronchodilator FEV1 from post-bronchodilator FEV1. The reversibility percentage was then calculated by dividing the reversibility volume by the pre-bronchodilator FEV1 and multiplying by 100.

2.4 Sputum collection and processing

2.4.1 Sputum induction

Sputum induction was performed according to standard methods (442), a method also safe in patients with COPD (443). Increasing concentrations of saline (3%, 4% and 5%) were administered using an ultrasonic nebuliser (Ultraneb 2000, Medix, Harlow, UK). Patients were asked to rinse their mouth with water and clear nasal secretions to reduce squamous cell contamination. Patients were then asked to expectorate sputum into a pot. Serial FEV1 measurements were performed for safety and the procedure abandoned if FEV1 fell by greater than 20% of baseline.

2.4.2 Sputum processing

Induced sputum was placed on ice and processed within 2 hours of expectoration according to methods previously described (444). Sputum plugs were selected to separate sputum from saliva. The plugs were then weighed and 4 volumes of 0.1% dithiothreitol (DTT, Sigma Aldrich, Poole, UK) was added. The sample was then vortexed for 15 seconds and then agitated on a rocker for 15 minutes. 4 volumes of phosphate buffered saline (PBS, Sigma Aldrich) were then added, the solution vortexed for 15 seconds and filtered through a 48µm nylon mesh (Sefar, Bury, UK).
The sample was centrifuged at 790g for 10 minutes at 4°C. Supernatants were removed and stored at -80°C for further analysis.

The cell pellet was resuspended in 0.3-1ml of PBS, depending on the cell pellet size. Cells were counted and viability assessed using trypan blue exclusion. Squamous cells were also counted. Samples were excluded if there were greater than 50% non-viable cells or greater than 30% squamous cells. Total cell count (TCC) and TCC per gram were calculated. The cell suspension was then adjusted to a final concentration of 0.5 x 10^6 cells/ml using PBS. For experiments investigating the release of reactive oxygen species (ROS) from sputum cells, cell suspension was made up to 2 x 10^6 cells/ml, these experiments are described separately. Cytospins were prepared from 50-100µl 0.5 x 10^5 cell suspension for differential cell counts (DCC).

### 2.4.3 Differential cell counts

Cytospins were air dried for 30 minutes and then fixed with methanol and stained with Rapi-diff (Triangle, Skelmersdale, UK). 400 non squamous cells were counted and differential counts obtained. Differential counts were expressed as a percentage of the totals cell counted.

### 2.5 Blood collection and processing

#### 2.5.1 Blood collection

Blood was collected in vacutainers (BD, Oxford, UK) containing clot activator and gel for serum separation. The tubes were inverted a few times after collection to allow mixing. Blood used for neutrophil isolation was collected in 50ml Falcon tubes containing 50µl sodium heparin (Wockhardt, Wrexham, UK).
2.5.2 Serum processing

Vacutainer tubes were left for 30 minutes at room temperature to allow a clot to form and were then centrifuged at 1500g for 10 minutes at 21°C. If a clot had not formed by 30 minutes, samples were left for a further 30 minutes. The resulting serum was then aliquoted into labelled cryo-tubes and immediately stored at -80°C.

2.5.3 Neutrophil isolation

Whole blood (5ml) was carefully layered over 3ml Mono-Poly Resolving medium (MP Biomedicals, Cambridge, UK) in a 15ml Falcon tube, within 60 minutes of blood collection. The samples were then centrifuged at 450g for 45 minutes at 19°C. Two distinct cell phases were then formed, the lower of which consisted of neutrophils; this is represented in Figure 2.1. The lower phase was carefully removed using a fine Pasteur pipette, and the removed cells suspended in RPMI (Sigma Aldrich) supplemented with L-glutamine (2mM), penicillin (100U/ml) and streptomycin (100µg/ml) (Invitrogen, Paisley, UK). RPMI without phenol red was used for experiments measuring ROS. The suspension was then centrifuged at 1000g for 10 minutes at 4°C.

The resulting cell pellet was resuspended and adjusted to a concentration of 1.0 (cell culture experiments) or 2.0 (ROS experiments) x 10^6 cells/ml in RPMI. Cytospins were also prepared as described for sputum cells with the cell suspension and differential cell counts obtained.
Whole blood (5ml) was carefully layered over 3ml Mono-Poly Resolving medium in a 15ml Falcon tube. The samples were then centrifuged at 450g for 45 minutes at 19°C. Two distinct cell phases were then formed, the lower of which consisted of neutrophils and the upper of which contained mononuclear cells. The yellow layers between the cell layers represent serum.
2.5.4 Neutrophil cell culture

General neutrophil cell culture is described here and will be described in more detail in relevant chapters.

4 x 10^5 cells were seeded in each well of a 24 well plate for a specified time (30 minutes to 24 hours) at 37°C in humidified 5% carbon dioxide (CO₂). Cells were cultured in the presence/absence of inhibitors and/or stimulants. After incubation the plates were centrifuged at 2000g for 10 minutes at 4°C. Cell free supernatants were then harvested and stored immediately at -80°C.

2.6 Supernatant analysis

2.6.1 Enzyme Linked Immunosorbent Assay (ELISA)

The following biomarkers were measured in serum, sputum or neutrophil cell culture supernatants using standard commercially available ELISA kits, as per manufacturer’s instructions. Briefly, a sandwich ELISA was used with the substrate sandwiched between capture and detection antibodies. Capture antibody was used to coat flat bottomed 96 well ELISA plates (Immulon 2 HB, Thermo-Fisher scientific, Loughborough, Leicestershire, UK) and incubated overnight at room temperature. The plates were then washed in PBS containing 0.05% tween 20 (PBST). The plate was then incubated with 1% bovine serum albumin (BSA) (Sigma-Aldrich) for 1 hour to prevent non specific binding. After further washing with PBST, samples and standards in various concentrations were then added to the plate; all were diluted using recommended sample diluents. The plates were incubated again for 2 hours and then washed again to remove any excess cytokine not bound to capture antibody. Appropriate detection antibody was added followed by a further 2 hour incubation.
After a further wash with PBST streptaviridin conjugated to horse radish peroxidase (HRP) was added and incubated for 20 minutes. A further wash was performed followed by the addition of substrate solution, consisting of a 1:1 mixture of colour reagent A (H₂O₂) and colour reagent B (Tetramethylbenzidine). Plates were left in the dark for 20 minutes until colour changed to blue. Stop solution (2N H₂SO₄, Sigma-Aldrich) was used to terminate the reaction.

The plates were read using a microplate reader at 450nM and 570nM using Fluostar Optimax plate reader (FLUOstar Optima; BMG Labtech LTD). A table of optical densities at 450nM and 570nM was generated, with each value adjusted by subtracting the optical density of blank wells to remove background noise. FLUOstar Optima software produced a standard curve of optical densities (y axis) against cytokine using a 4 parameter log curve. Any sample with values outside the linear curve were reanalysed following adjustment of the sample dilution.

**2.6.1.1 Cytokines**

IL-8 and MIP-1α were measured using Duoset ELISA kits from R&D systems (Abingdon, UK). The limits of detection were 31.2-2000 pg/ml for IL-8 and 7.81-500 pg/ml for MIP-1α. IL-6 was measured using a high sensitivity Quantikine ELISA kit with limits of detection 0.156-10 pg/ml.

**2.6.1.2 Proteases**

MMP-9 was measured using a Duoset ELISA kit from R&D systems (Abingdon, UK), with limits of detection 31.2-2000 pg/ml.

**2.6.1.3 Other biomarkers**

CC-16 was measured using a commercially available ELISA kit from Biovendor, (Modrice, Czech Republic) as per manufacturer’s instructions. The limits of
detection were 2-40 ng/ml. Serum CRP was measured on the Abbott’s Architect Analyser (Abbott, Maidenhead, UK), using an immunoturbidimetric technique, with the lower limit of detection 0.2mg/ml. CRP was analysed at the Clinical Sciences Building, University Hospital South Manchester, Manchester, UK.

2.6.2 Luminex
Sputum supernatants were analysed for the chemokines MIP-1β and MCP-1 on luminex plates (Millipore, Watford, UK), as per manufacturer’s instructions. Plates were read on the Millipore Magpix system. Full details of the methodology used are described in Appendix 1.

2.6.3 Elastase assay
Sputum supernatants were added to the molecular probe Rhodamine 110, bis-(CBZ-L-Alanyl-L-Alanyl-L-Alanyl-L-Alanine Amide) (10µM; Invitrogen). Upon cleavage of the substrate by elastase, an increase in fluorescence were measured on a microplate reader and expressed as nM. Elastase activity was measured by subtracting the baseline fluorescence from fluorescence at 30 minutes and read on a Safire spectrofluorometer (TECAM, Mannedorf, Switzerland). XFluor software was used for analysis. Elastase was analysed at GlaxoSmithKlein, Stevenage, UK.

2.7 Detection of Intracellular ROS
Blood neutrophils, sputum cells or alveolar macrophages (2 x 10^6 /ml) were incubated with the ROS sensitive dye CM-H2DCFDA (5µM) (Invitrogen) for 20 minutes at 37°C. All cells were processed in phenol red free unsupplemented media or PBS. Cells were then centrifuged at 400g for 10 minutes at 4°C and re-suspended in PBS (2 x10^6/ml). Cells were seeded at 2 x 10^5 cells per well on a 96 well flat
bottomed plate. Inhibitors were added 5 minutes prior to the addition of fMLP (Sigma Aldrich) (1µM). fMLP was added via pumps inside the POLARstar Omega plate reader. Intracellular ROS was measured using MARS data analysis software version 2.10R3 by calculating changes in intracellular fluorescence over 60 minutes.

2.8 Cell apoptosis

DNA fragmentation and presence of condensed nuclei were assessed by the TUNEL method in conjunction with the microscopic analysis of the nuclear morphology. Cytospins of blood neutrophils were prepared as described in the relevant chapters. Apoptosis was determined by examining the disappearance of chromatin bridges between nuclear lobes (early apoptosis) and shrinkage or fragmentation of the nucleus (late apoptosis). Neutrophils displaying signs of early and late apoptosis are demonstrated in Figure 2.2. The percentage of viable neutrophils as well as early and late apoptotic neutrophils was assessed by counting a total of 400 neutrophils.

The TUNEL assay was used as per the manufacturer’s instructions (Roche Diagnostics, Hertfordshire, UK). Cells were labelled with TUNEL and counterstained in 4’,6-diamidino-2-phenylindole (DAPI) (Invitrogen). A total of 300 neutrophils per cytospin were analysed and classified as positive or negative for tunel staining. Digital micrographs were obtained through the use of a Nikon Eclipse 80i microscope (Nikon UK Ltd, Surrey, UK) equipped with a QImaging digital camera (Media Cybernetics, Marlow, UK) and ImagePro Plus 5.1 software (Media Cybernetics).
Figure 2.2: Apoptotic blood neutrophils identified under light microscopy. As indicated, normal cells, cells in early apoptosis demonstrating loss of chromatin bridges and in late apoptosis demonstrating nuclear shrinkage are represented.

2.9 Statistical analysis

Detailed statistical analysis is outlined in each chapter. Analysis was carried out using GraphPad InStat software version 3.06 (GraphPad Software, Inc., San Diego, CA, USA).
3. Characterisation of the inflammatory response to inhaled LPS in mild to moderate COPD

3.1 Introduction

Patients with COPD suffer with progressive airflow obstruction (5). A hallmark feature of this condition is increased airway inflammation, involving neutrophils, macrophages and lymphocytes (180). A subgroup of COPD patients also has significant systemic inflammation (9). Exacerbations are defined as acute worsenings of COPD beyond the normal day to day variation; COPD patients who frequently exacerbate have increased mortality (19), accelerated decline in lung function (18) and worse quality of life (17). Exacerbations may be caused by viral or bacterial infections, and are characterised by increased airway and systemic inflammation (264).

LPS, also known as endotoxin, is a constituent of the outer cell membrane of gram negative bacteria, and triggers an innate immune response after binding to TLR4 (445). Inhaled LPS has been used experimentally in healthy subjects and patients with asthma to cause acute neutrophilic airway inflammation (314, 319, 323), which is accompanied by a short lived systemic immune response involving an increase in CRP and IL-6 levels (312, 314, 328). This model has been used safely in healthy subjects in order to investigate the effects of anti-inflammatory drugs on neutrophilic airway inflammation (321, 322, 446). Novel drugs in early clinical development for the treatment of COPD can be tested using this healthy volunteer model before proceeding to larger studies involving COPD patients.
A potential criticism of the LPS model in healthy non-smokers is that acute inflammation in healthy lungs does not resemble COPD lung inflammation. The numbers of airway neutrophils are increased in healthy smokers compared with healthy non-smokers (182), and are further increased in COPD patients (69). It has recently been demonstrated that inhaled LPS causes an acute increase in airway neutrophil numbers in healthy smokers (316). This might more closely resemble the acute increase in airway neutrophil numbers that occurs on a background of chronic neutrophilic airway disease during COPD exacerbations (229). This study further developed this model by characterising the immune response to inhaled LPS in COPD patients. This could serve as a model of bacterial exacerbations in COPD patients, in order to test the efficacy of novel pharmacotherapies.

Recently, there has been much interest in COPD biomarkers that are secreted by the lung and measurable systemically. An example is CC-16, which is produced by lung Clara cells and has anti-inflammatory actions (332). CC-16 levels are reduced in serum and sputum of COPD patients compared to controls (332). There is also an increase in CC-16 levels in healthy non-smokers after inhalation of LPS and ozone (330, 447), and healthy smokers after LPS inhalation (316). These findings suggest that CC-16 levels are increased by acute lung inflammation but decreased during the chronic inflammation that occurs in COPD.

In healthy non-smokers and smokers, 5µg LPS has been shown to be the minimum threshold dose of LPS to induce an increase in sputum absolute neutrophils and serum CRP without any significant side effects (314, 320). This dose has also been used in healthy smokers to induce increased sputum neutrophil percentage and serum IL-6 (316). Doses of ≥ 50µg have been shown to be associated with more clinical side effects (314). 30µg has been used to induce increased airway and systemic
inflammation in healthy smokers (316, 448). There is little data on the use of inhaled LPS in patients with COPD. Therefore in this study, it was planned to use 5µg as an initial dose to test safety in mild COPD and then go on to use a 30µg dose.

The aims of this study were to examine the safety of LPS inhalation in COPD patients, and to characterise changes in airway and systemic inflammation. Changes in sputum cellular counts, sputum supernatant IL-8, MCP-1 and MIP-1β levels, and peripheral blood IL-6, CRP and CC-16 levels were measured.

3.2 Methods

3.2.1 Patient recruitment

Twelve COPD patients with a FEV1 ≥70% and <100% predicted and an FEV1 /FVC ratio <70% were recruited from a database held at the Medicines Evaluation Unit, University Hospital South Manchester, UK; demographic details are shown in Table 3.1. Eight patients were taking ICS. Patients were excluded if they had any other significant respiratory disease including asthma, any other inflammatory disease, oxygen saturations <92%, a <10 pack year history of smoking or an exacerbation within 6 weeks of screening. All patients were previously diagnosed with COPD by their primary care physician, and the clinical history of COPD was checked by myself at the research unit. In order to be eligible for the study patients had to produce an adequate sputum sample at the screening visit; all 12 patients produced adequate samples at subsequent visits. Written informed consent was obtained from all patients and the study was approved by the Greater Manchester South ethics committee (reference 11/H1003/1).
3.2.2 Study design

The protocol specified that the safety and effects of 5µg and 30µg LPS inhalation on systemic and airway inflammation in COPD patients would be assessed, with all patients completing the lower dose before proceeding to the higher dose; the study design involved five visits (see Figure 3.1).

Visit 1 (Screening):

Informed consent was obtained and eligibility was ascertained by the following assessments: spirometry, physical examination, oxygen saturations and pregnancy test if female and of child bearing potential. The patients underwent baseline sputum induction. Sputum was collected and processed as described in section 2.4.

Visit 2 (5µg LPS challenge):

After an interval of at least 48 hours, an inhaled LPS challenge (Escherichia coli serotype 026:B6, Sigma-Aldrich) was performed. LPS was obtained as a lyophilized powder and reconstituted in 9ml 0.9% saline to a concentration of 0.11mg/ml. The Mefar dosimeter (Markos Mefar, Bresica, Italy) was used to deliver 9µl of aerosol at each inhalation; each patient was asked to take 5 inhalations and therefore a total of 5µg. Sputum induction was repeated at 6 hours post LPS inhalation. Pulse, blood pressure, oxygen saturations, temperature, symptoms and spirometry (as described in section 2.3.1) were performed pre-challenge, 5 and 30 minutes post challenge and then every hour for 8 hours post challenge, with the exception of spirometry, which stopped after sputum induction at 6 hours when patients were administered salbutamol. Blood samples for biomarker analysis were collected at 0, 4 and 8 hours. Patients were asked to refrain from smoking for 2 hours prior to LPS inhalation.
Visit 3 (24 hours post LPS inhalation):

Spirometry and vital signs were also measured prior to sputum induction at 24 hours. Patients were asked to report any new symptoms up to 24 hours after LPS inhalation. Blood samples for biomarker analysis were collected 24 hours post LPS challenge.

Visit 4 and 5 (30µg LPS inhalation):

The protocol planned for patients to go on to inhale 30µg LPS if 5µg was well tolerated, as per visit 2 and 3. This would be a minimum of 2 weeks post 5µg inhalation.
VISIT 1
Informed consent, eligibility, sputum induction

VISIT 2
5µg LPS inhalation, sputum induction 6 hours, blood biomarkers 0, 4 and 8 hours

VISIT 3
Sputum induction, blood biomarkers 24 hours post 5µg

VISIT 4
30µg LPS inhalation, as per visit 2

VISIT 5
24 hours post 30µg LPS inhalation, as per visit 3

**Figure 3.1: LPS challenge in patients with COPD study design.** 5 patient visits were planned, with an initial screening visit and then 2 LPS challenges.
3.2.3 Sputum supernatant biomarkers
Sputum was induced and processed with DTT using established methods as described in section 2.3.2. Sputum supernatants were analysed for IL-8 by ELISA, along with MCP-1 and MIP-1β by Luminex assay (see sections 2.5.1.1 and 2.5.2). The effect of DTT on MCP-1 and MIP-1β recovery by immunoassay were assessed by reconstituting standards with and without 0.05% DTT. It is already known that DTT does not affect IL-8 recovery (449). NE activity was quantified using Rhodamine (see section 2.6.3).

3.2.4 Serum biomarkers
Serum was collected and processed as described in section 2.4.1 and 2.4.2. Serum levels of IL-6 (see section 2.5.1.1) and CC-16 were measured by ELISA (see section 2.5.1.3). CRP was measured as described in section 2.6.1.3.

3.2.5 Statistical analysis
No formal sample size calculation was performed, as there is no previous LPS challenge data in COPD patients to perform such a calculation. A sample size of 12 was chosen based on previous studies with similar sample sizes that were able to (316, 323) detect changes in FEV1 and sputum inflammatory markers.

Safety data was analysed using one way ANOVA with Bonferroni correction. Neutrophil and macrophage differential counts, and serum CC-16 levels were normally distributed using the Kolmogorov-Smirnov test, with LPS effects analysed by students paired t-tests. Other sputum cell counts, sputum supernatant measurements, and serum IL-6 and CRP were non-parametric, so were log transformed and then compared using students paired t-tests. P<0.05 was considered significant. Two sputum slides obtained after LPS challenge from 10 patients were
counted, in order to assess within sample repeatability; Bland-Altman analysis and intraclass correlation coefficient (ICC) were performed.
<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.2 ± 4.6</td>
</tr>
<tr>
<td>M/F</td>
<td>10/2</td>
</tr>
<tr>
<td>FEV1 (litres)</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>85.3 ± 9.3</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>60.4 ± 6.3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.0 ± 3.2</td>
</tr>
<tr>
<td>PACK YRS</td>
<td>38.8 (11.4-122.2)*</td>
</tr>
<tr>
<td>ICS (Y/N)</td>
<td>8/4</td>
</tr>
<tr>
<td>LAMA (Y/N)</td>
<td>4/8</td>
</tr>
<tr>
<td>LABA (Y/N)</td>
<td>8/4</td>
</tr>
<tr>
<td>Current/Ex smoker</td>
<td>5/7</td>
</tr>
<tr>
<td>Chronic bronchitis (Y/N)</td>
<td>8/4</td>
</tr>
<tr>
<td>No of exacerbations prev 12 months</td>
<td>0 (0-2)*</td>
</tr>
<tr>
<td>mMRC (n=7)</td>
<td>1 ± 0.2</td>
</tr>
</tbody>
</table>

Table 3.1: A summary of baseline demographics of the 12 patients. All values are expressed as mean ± SD, apart from *, expressed as median (range). BMI: Body mass index, FEV1: Forced expiratory volume in 1 second post 200µg inhaled salbutamol, FVC: Forced Vital Capacity, ICS: Inhaled corticosteroids, LAMA: Long acting muscarinic antagonist, LABA: Long acting beta agonist, mMRC: modified Medical Research Council score (available on n=7 patients).
3.3 Results

3.3.1 Safety data
Following inhalation of 5µg LPS, 5 patients experienced minor symptoms of headache (n=4) and mild cough (n=1), which resolved at 24 hours. Figure 3.2A shows there was a significant fall in FEV1 from 0.5 to 6 hours after LPS inhalation, which returned to normal at 24 hours. The greatest mean change in FEV1 was 11.7% at 1 hour (95% CI 5.1-18.2%). The maximal fall in FEV1 varied between patients (2.5% to 37.7%; Figure 3.2B), and occurred between 5 minutes and 5 hours post LPS inhalation (mean 155.4 ± 30.7 minutes). Given this large fall in FEV1 observed in some patients after inhalation of 5µg LPS, it was unsafe to proceed to inhalation of 30µg. There were no significant relationships between smoking status or inhaled corticosteroid use and maximal fall in FEV1.

There were no significant changes in the mean temperature and pulse after LPS inhalation (Figure 3.2C and 3.2D), nor oxygen saturations and blood pressure (data not shown). There was, however, a large range in observed changes in temperature and pulse post LPS; maximal increase in temperature and pulse varied between 0 and 1.1°C and 1 and 28 beats per minute respectively.

3.3.2 Airway inflammation

3.3.2.1 Sputum cell counts
LPS inhalation induced significant increases in total cell count / gram of sputum after 6 and 24 hours (2.5 and 2.7 fold respectively), and also total neutrophil count / gram of sputum after 6 and 24 hours (2.5 and 2.3 fold respectively; see Table 3.2). There were no significant changes in sputum neutrophil percentage or the percentage or absolute number / gram of any other cell type after LPS inhalation. There were
few squamous cells present in the cytospins (mean 0.6 %). There were no significant relationships between maximal fall in FEV1 and total neutrophil count/gram sputum. There were also no significant relationships between smoking status or inhaled corticosteroid use and total neutrophil count/gram sputum. The mean difference between neutrophil percentage between slides from the same patient sample was -1.8%, with limits of agreement -8.1 to 4.5%. ICC was 0.95, indicating excellent repeatability.

3.3.2.2 Sputum supernatant

There were no significant changes in elastase activity, IL-8, MCP-1 or MIP-1β in sputum supernatants after 6 and 24 hours post LPS inhalation (see Figure 3.3). The recovery rates of the standards after addition of 0.05% DTT (mean ± standard deviation (SD)) were 83.6 ± 13.2% for MCP-1 and 69.7 ± 4.3% for MIP-1β.

3.3.3 Systemic inflammation

Figure 3.4 shows a significant increase in CC-16 at 4 hours after LPS inhalation, which then returned to below baseline levels at 24 hours. IL-6 levels were increased at 4 and 8 hours after inhalation, with CRP increased at 24 hours after challenge.
<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>6 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neutrophil %</strong></td>
<td>72.0 (±14.6)</td>
<td>78.5 (±11.6)</td>
<td>76.6 (±10.8)</td>
</tr>
<tr>
<td><strong>Macrophage %</strong></td>
<td>22.3 (±11.0)</td>
<td>18.6 (±10.9)</td>
<td>18.6 (±10.4)</td>
</tr>
<tr>
<td><strong>Eosinophil %</strong></td>
<td>0.8 (0.3-13.5)</td>
<td>0.8 (0-3.3)</td>
<td>0.8 (0-11)</td>
</tr>
<tr>
<td><strong>Lymphocyte %</strong></td>
<td>0.0 (0-0.8)</td>
<td>0.0 (0.0-1.0)</td>
<td>0.0 (0-1.0)</td>
</tr>
<tr>
<td><strong>Epithelial cell %</strong></td>
<td>1.8 (0-9.8)</td>
<td>1.4 (0-4.8)</td>
<td>2.4 (0-5.7)</td>
</tr>
<tr>
<td><strong>Total Cell Count (x10^6) per gram</strong></td>
<td>2.1 (0.3-13.9)</td>
<td>5.2 (2.1-25.2)*</td>
<td>5.6 (1.9-17.3)*</td>
</tr>
<tr>
<td><strong>Neutrophil Cell Count (x10^6) per gram</strong></td>
<td>1.6 (0.3-11.3)</td>
<td>4.1 (1.1-22.4)*</td>
<td>3.7 (1.4-15.5)*</td>
</tr>
<tr>
<td><strong>Macrophage Cell Count (x10^6) per gram</strong></td>
<td>0.4 (0.1-4.4)</td>
<td>0.9 (0.2-2.7)</td>
<td>0.1 (0.0-1.8)</td>
</tr>
<tr>
<td><strong>Eosinophil Cell Count (x10^6) per gram</strong></td>
<td>0.04 (0.00-0.16)</td>
<td>0.04 (0.00-0.15)</td>
<td>0.04 (0-0.75)</td>
</tr>
<tr>
<td><strong>Lymphocyte Cell Count (x10^6) per gram</strong></td>
<td>0.00 (0.00-0.01)</td>
<td>0.00 (0.00-0.02)</td>
<td>0.00 (0.00-0.05)</td>
</tr>
<tr>
<td><strong>Epithelial Cell Count (x10^6) per gram</strong></td>
<td>0.06 (0.00-0.46)</td>
<td>0.07 (0.00-0.13)</td>
<td>0.11 (0.00-0.69)</td>
</tr>
</tbody>
</table>

Table 3.2: Sputum cell counts at baseline, 6 hours and 24 hours post LPS inhalation. Neutrophil and macrophage percentage are presented as mean (± SD) and results compared with baseline using the students paired t test. Data are presented as median (range). These results have been natural log transformed and then compared using the students paired t test. * indicates p≤0.05.
Figure 3.2: Safety assessments following LPS inhalation. Percentage change in FEV1 (A), maximal fall in FEV1 for each individual patient (B), change in temperature (C) and change in pulse rate (D). All data points in (A), (C) and (D) represent mean and error bars standard error of the mean (SEM). * indicates p≤0.05 and ** indicates p≤0.001 compared to baseline.
Figure 3.3: Changes in sputum supernatant biomarkers post LPS inhalation; IL-8 (A; n=12), elastase activity (B; n=10), MCP-1 (C; n=8) and MIP-1β (D; n=8). Individual data points are represented, and red lines represent median.
Figure 3.4: Changes in systemic biomarkers over time following LPS inhalation; CC-16 levels post challenge (A), IL-6 levels post challenge (B) and CRP levels post challenge (C). (A) is presented as mean, and error bars represent SEM. (B) and (C) are presented as median, and error bars represent range. * indicates $p \leq 0.05$, ** indicates $p \leq 0.001$. 

3.4 Discussion

LPS administered to COPD patients increased the total number of sputum neutrophils, with associated changes in serum levels of CC16, IL-6 and CRP. LPS was safely administered to COPD patients, and suggests that this is a model of COPD bacterial exacerbations that can be used to investigate disease mechanisms or the effects of new pharmacotherapies.

The 5µg inhaled LPS dose was symptomatically well tolerated, although there were large decreases in FEV1 in some patients; therefore it was felt to be unsafe to proceed to the higher LPS dose stated in the protocol. Previous studies involving healthy volunteers have administered inhaled LPS doses up to 300µg (312-314, 319), which have been associated with an increased incidence of side effects, such as decreases in lung function and flu-like symptoms. I used the same LPS serotype that was studied in healthy smokers, and observed that 5µg caused greater decreases in FEV1 in some of the COPD patients compared to healthy smokers (316) where there was a mean maximal decrease in FEV1 of 8.2% (range 1.2 -19.1%) . This suggests that the presence of airflow obstruction in COPD predisposes to an increased tendency to bronchoconstriction after inhaled LPS. For this reason, it may be recommended that inhaled LPS challenges are only performed in patients with mild to moderate COPD; for safety reasons COPD patients with FEV1 ≥70% predicted were included. As with FEV1, there was a wide range in change in temperature (0-1.1°C) and pulse (1-28bpm) again indicating significant variation in systemic LPS responses.

The large variation in the FEV1 response to inhaled LPS may be due to LPS tolerance (450), as the airways of some COPD patients are colonised with bacteria that stimulate TLR4 (107). Current smoking status may also be important, as
cigarette smoke contains LPS (451) and therefore could lead to tolerance; however, there was no relationship between current smoking status and response to LPS, although the numbers involved in this sub-analysis were small. Alternatively, genetic polymorphisms in the TLR4 receptor complex may be responsible for this diversity (452). Furthermore, although there was a standardised method of LPS inhalation, the delivered dose of LPS may have varied between patients.

Inhaled LPS increased the total sputum cell count and absolute number of neutrophils at 6 and 24 hours post-inhalation. However, there was no change in sputum neutrophil percentage. This contrasts with studies in healthy non-smokers where inhaled LPS increased both the percentage and absolute number of sputum neutrophils (314, 319, 322, 323). Healthy non-smokers have a neutrophil percentage that is generally <50%, affording an adequate “window” for LPS to increase the neutrophil percentage (69). It is well known that healthy smokers have an increased neutrophil percentage caused by cigarette smoking, often in the range 50 – 70% (316). It has previously been shown that inhaled LPS causes a further small increase in the neutrophil percentage in healthy smokers, and also increases the total neutrophil cell count (316). The high pre-LPS challenge sputum neutrophil percentage in COPD patients (71%) prevented a statistically significant increase in this measurement being observed after LPS challenge. However, a > 2 fold increases in the total neutrophil count / gram of sputum was observed. In addition, previous studies investigating sputum neutrophils in COPD exacerbations compared to stable patients have also found increased sputum neutrophil numbers but not percentages (94, 234, 453); thus the sputum changes seen in this model resembles those seen during exacerbations. Within sample repeatability of sputum neutrophil percentage was excellent in this study, with an ICC of 0.95. Furthermore, there are multiple
reports demonstrating both short term (454, 455) and long term (456) reproducibility of sputum cell counts; these results support the use of sputum neutrophils as a biomarker in COPD.

The majority of patients in this study (8 out of 12) were taking regular inhaled corticosteroids. A subgroup analysis showed no influence of inhaled corticosteroid use on the LPS response, although it is clear that the sample size for this analysis is small. It is unlikely that inhaled corticosteroids influenced the LPS response in this study, as oral corticosteroids do not affect LPS induced airway inflammation in healthy subjects (322).

Studies in healthy volunteers and patients with asthma have shown that inhaled LPS increases the levels of IL-8 and MCP-1 in induced sputum (319, 323), while in BAL there is an increase in IL-8 and MIP-1 concentrations after LPS inhalation (321, 457). There were no observed changes in the levels of these proteins in induced sputum after LPS challenge. Recovery rates of approximately 70% or above for MCP-1 and MIP-1β were observed in DTT standards, which meet the standards of acceptability set out in guidelines for sputum supernatant analysis (458). COPD patients have higher levels of IL-8 and MCP-1 (69, 91) in induced sputum compared to healthy controls, and perhaps the reason for a lack of induction of IL-8 in COPD patients was that the levels were already elevated before LPS inhalation. Another possible explanation for this finding is that the study was terminated after a low dose of LPS, while a higher LPS dose may have increased the levels of these proteins. However, the increased sputum neutrophil cell count caused by the low dose of LPS in the current study suggests that LPS increased neutrophilic chemokine levels in the airways of COPD patients. Perhaps this study was underpowered to detect such changes in the sputum supernatant.
$5 \mu g$ LPS was administered to patients in this study; the LPS serotype *Escherichia coli* 026:B26 used contained 500,000 endotoxin units (EU)/mg according to the product datasheet which would equate to a total of 2,500 endotoxin units delivered to each patient at inhalation. Environmental endotoxin exposure has been measured and described using different methods in the literature, with some studies describing EU/m$^2$ (459) and some describing EU/g (460). Studies examining the relationship between endotoxin exposure and the development of atopy in children have reported 22.8 EU/g and 37.8 EU/g house dust in non-farming and farming households respectively (460). Therefore although $5 \mu g$ LPS is a relatively low dose, the amount of endotoxin inhaled would have been higher than the normal environmental exposure.

Sputum NE activity has been shown to be elevated in patients with COPD compared to controls (204) and is further increased during exacerbations, in particular bacterial exacerbations (235). There was no significant increase in NE activity post LPS inhalation. Again, this may be due to a higher baseline in COPD patients to begin with, and the low dose of LPS used.

LPS inhalation causes an acute increase in serum CC-16 levels in healthy smokers followed by a decrease to below pre-challenge levels by 24 hours (316). The same pattern was observed in COPD patients in this study. In the stable state, it is known that smokers and COPD patients have lower concentrations of CC-16 in serum and sputum compared to healthy non-smokers (332). This suggests that acute inflammation caused by TLR4 signalling in COPD causes an acute upregulation of CC-16 secretion, followed by chronic downregulation.
Serum IL-6 and CRP levels are increased during exacerbations of COPD (264). Inhaled LPS increased serum IL-6 and CRP in COPD patients; this has also been observed in healthy volunteers (312, 314, 328). IL-6 regulates CRP production by the liver (61), explaining the temporal difference in the increase in these biomarkers. Although an increase in CRP levels was detected at 24 hours only, this increase may have occurred at any time between the blood sampling times of 8 and 24 hours. These changes in systemic inflammatory biomarkers indicate that the airway inflammatory changes caused by the low dose of LPS were associated with significant systemic inflammation. The airway and systemic inflammatory changes caused by LPS inhalation are similar to those observed during COPD exacerbations (235, 264), and suggest that this model could be used to test the effects of novel drugs designed to prevent the inflammatory response during COPD exacerbations.

A significant limitation of this study is that it was carried out in mild COPD patients (FEV>70%) and therefore the results may not be generalizable to patients with more severe disease. However, the FEV1 reductions observed in some patients raises safety issues if this model were used in patients with more severe COPD. This study design involved unblinded challenges, without a placebo challenge; an alternative design would have involved randomised, placebo controlled challenges. However, it should be noted that inhaled challenges in clinical trials, including LPS, are performed in an unblinded manner (321, 322, 446, 461-463). I did not investigate reproducibility of challenges performed on different days, although a previous similar study demonstrated good reproducibility in healthy smokers (316).

This study has demonstrated increased systemic and airway inflammation which resembles COPD exacerbations, but did not show increased airway IL-8 or sputum neutrophil percentage which are both known to be associated with exacerbations.
This study demonstrates initial safety data for using LPS challenges in COPD, and the changes in IL-8 and neutrophil percentage could be addressed in further larger studies.

In conclusion, inhaled LPS challenges were safely conducted using a low dose (5µg), which increased neutrophilic airway inflammation in COPD patients. This was associated with changes in systemic inflammation, and overall the inflammatory changes observed resembled those seen in COPD exacerbations. Inhaled LPS challenge in COPD patients can therefore be used as a model to evaluate novel anti-inflammatory drugs designed to prevent COPD exacerbations.
4. The effect of PI3 kinase δ isoform inhibition on MMP-9 and ROS release from COPD neutrophils

4.1 Introduction

There is evidence of increased activity of COPD neutrophils, with enhanced secretion of pro-inflammatory mediators involved in the pathophysiology of COPD, as such as proteases (213, 214) and ROS (190, 215). Corticosteroids are the most widely used anti-inflammatory therapy for COPD patients, but these drugs have limited clinical benefits (464). Lung neutrophils show reduced expression of the glucocorticoid receptor (361) thus limiting the effects of corticosteroids on neutrophilic lung inflammation. There is a need for novel therapies to treat neutrophilic inflammation in COPD.

PI3 kinase enzymes are a potential therapeutic target for the treatment of COPD. These intracellular enzymes are involved in cell metabolism, growth and repair (392). Class I PI3 kinase enzymes catalyse the formation of PIP3; this lipid second messenger controls cell metabolism and activation pathways through phosphorylation of Akt and other proteins. Class I PI3 kinase enzymes are composed of a regulatory subunit and a catalytic subunit; either p110α, p110β, p110γ or p110δ (392). The γ and δ isoforms are leucocyte specific (392), and so there is currently much interest in developing drugs that selectively inhibit these isoforms for the treatment of inflammatory diseases.

Studies using peripheral blood neutrophils from healthy subjects have shown that both δ and γ isoforms are involved in ROS release (137), and that pan PI3K enzyme inhibition suppresses cytokine (435) and MMP-9 (205) secretion. In addition, PIP3
levels and PI3Kδ isoform gene expression are increased in COPD blood neutrophils compared to healthy controls (214). This suggests an important role for the PI3Kδ isoform in the regulation of neutrophil activity in COPD patients. However, the effect of PI3 kinase δ isoform selective inhibition on the secretion of MMP-9 and ROS from COPD neutrophils has not been investigated.

The δ and γ isoforms of the enzyme do have significant roles in leucocyte maturation, migration and activation; these functions are important in normal immunity and cell homeostasis. However, further understanding of the crystal structures and development of specific isoform inhibitors could lead to dampening of the pathological inflammatory response associated with disease whilst maintaining important cellular functions.

ROS release from neutrophils results from the assembly of NADPH on the cell membrane: on activation of the cell the cytosolic components Rac2, p40phox, p47phox and p67phox migrate to the membrane bound component cytochrome b558 and become phosphorylated. In vitro, phospho-akt phosphorylates p47phox in human neutrophils stimulated by fMLP or by bacterial phagocytosis; this occurs at Ser304 and Ser328 (425, 430). Therefore PI3 kinase enzyme activation may lead to ROS release by phosphorylation of p47phox.

The aim of this study was to understand the anti-inflammatory effects of selective PI3Kδ isoform inhibition on COPD neutrophils. Importantly, neutrophils from the blood and sputum were studied, with investigations performed in the stable clinical state and during exacerbations. The study focused on ROS and MMP-9 release from neutrophils, as these are relevant to the pathophysiology of COPD (465).
4.2 Methods

4.2.1 Patient recruitment

33 patients were recruited from a database of patients held at the Medicines Evaluation Unit, Manchester, UK. All patients had a previous diagnosis of COPD, with an FEV1/FVC ratio <70%, age >40 and at least a 10 pack year history of smoking. Patients were excluded if there was a history of active malignancy, asthma or any inflammatory disease. Stable patients were recruited only if they had been free from respiratory infections in the preceding 6 weeks. Stable patient demographics are summarised in Table 4.1. All patients gave written informed consent. Approval was obtained from the local ethics committee GM East (05/Q1402/41) and North West- Preston (10/H1016/25).

4.2.2 Exacerbating patients

COPD patients presenting with exacerbations were recruited from outpatient clinics and respiratory wards at the University Hospital South Manchester, UK. Exacerbations were defined as increased respiratory symptoms for 2 days with at least 1 major symptom (dyspnoea, sputum purulence and sputum volume), and another major or a minor symptom (wheeze, cold, sore throat and cough) (16). Exacerbating patients seen in the clinic had been asked to keep diary cards of their symptoms and were only included if they had not received antibiotics or corticosteroids for 6 weeks prior to their clinic visit; an example of a diary card is demonstrated in Figure 4.1. Patients recruited from wards were sampled within 24 hours of admission and receiving treatment. Demographics of exacerbating patients are shown in Table 4.1. Patients were sampled a mean (± SD) of 2.9 ± 0.5 days after symptoms started. All patients gave written informed consent. Approval was obtained from local ethics committee GM South (10/H1003/108).
4.2.3 Spirometry, reversibility and health questionnaires
This was carried out as described in section 2.2 and 2.3.

4.2.4 Blood collection and neutrophil isolation
This was carried out as described in section 2.5. Cytospins were prepared for DCC as described.

4.2.5 MMP-9 release
4 x 10^5 blood neutrophils were seeded in 24 well plates; each condition was performed in triplicate.

4.2.5.1 MMP-9 release from fMLP stimulated neutrophils; dose response experiment
Blood neutrophils (n=3) were incubated with fMLP (Sigma-Aldrich) (0.01-1000nM) or appropriate control (0.01% dimethyl sulphoxide, DMSO) at 37°C in humidified 5% CO₂ for 30 minutes. After incubation the plates were centrifuged at 2000g for 10 minutes at 4°C. Cell free supernatants were then harvested and stored immediately at -80°C for later MMP-9 analysis.

4.2.5.2 MMP-9 release from fMLP stimulated neutrophils; a time course experiment
fMLP (1000nM) or appropriate control was added to isolated neutrophils (n=3). Plates were incubated at 37°C in humidified 5% CO₂ for 30 minutes, 4 and 24 hours and then centrifuged and supernatants removed as described above.
4.2.5.3 MMP-9 release from fMLP stimulated neutrophils; the dose response effect of PI3 kinase enzyme inhibitors

Isolated neutrophils (n=11) were pre-treated (1 hour) with a pan PI3K enzyme inhibitor; ZSTK474 (Selleck chemicals, Suffolk, UK) (1-1000nM), a selective PI3K δ isoform inhibitor; GSK045 (GlaxoSmithKline, Stevenage, UK) (1-1000nM), a selective p38 α and β isoform inhibitor; BIRB796 (1-(5-tert-Butyl-2-p-tolyl-2H-pyrazol-3-yl)-3-[4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl]urea) (Selleck chemicals) (1–1000nM), and dexamethasone (Sigma-Aldrich) (1-1000nM). Thereafter, cells were stimulated (30 minutes) with fMLP (10nM) at 37ºC in humidified 5% CO₂. Plates were then centrifuged and supernatants harvested as described above.

pIC50s for the PI3 kinase enzyme inhibitors are shown in Appendix 2. The pIC50 value is the –log of the concentration (in moles, M) needed to inhibit the PI3 kinase enzyme by 50% in a biochemical or cellular assay. Therefore, if a compound has a pIC50 1 log unit higher than another it is 10 times more potent for that PI3 kinase isoform. pIC50 of ZSTK474 for the δ and γ isoforms were 8.5M and 7.4M respectively and of GSK045 were 9.1M and 6.8M respectively. IC50 values may vary depending on experimental conditions. pKi values are also shown in Appendix 2; the pKi is the –log of the binding affinity of the receptor (in M). Ki refers to the concentration of the drug (can be either an agonist or antagonist) which would occupy 50% of the receptors if there was no other ligand present. These values were provided by GlaxoSmithKlein.

4.2.5.4 Supernatant analysis

MMP-9 was measured in supernatants as described in section 2.6.
4.2.6 Intracellular ROS assays

4.2.6.1 ROS release from fMLP stimulated neutrophils; dose response experiment

Blood neutrophils from patients with stable COPD (n=3) were incubated at 37ºC in humidified 5% CO₂ with the ROS sensitive dye, as described in section 2.7.

2 x 10⁵ cells per well were seeded on a 96 well flat bottomed plate. Thereafter fMLP (0.1-1000nM) or appropriate control (0.01% DMSO) was added. The plate was then immediately placed on the POLARstar Omega plate reader and changes in fluorescence over an hour were calculated.

4.2.6.2 ROS release from fMLP stimulated neutrophils; the dose response effects of PI3 kinase enzyme inhibitors

Experiments were carried out as described in section 2.7. Stable COPD blood neutrophils (2 x 10⁵/well; n=6) were pre-treated (5 minutes) with ZSTK474 (1-1000nM), GSK045 (1-1000nM), BIRB796 (1-1000nM) and dexamethasone (1-1000nM). fMLP (1000nM) was then added via pumps on the POLARstar Omega plate reader and changes in fluorescence measured as described. 5 minutes was chosen as the time period for incubation with inhibitors as this has previously been used in investigation of the PI3 kinase enzyme pathway in neutrophil ROS release (137).

4.2.6.3 ROS release from fMLP stimulated sputum cells; the effect of PI3 kinase enzyme inhibitors

Sputum from stable COPD patients (n=11) was obtained and processed using established methods (DTT) as described in section 2.4.2; cytospins for DCCs were prepared. ROS assays were carried out as described in section 2.7. Sputum cells (2 x
10^5 (well) were pre-treated (5 minutes) with ZSTK474 (1000nM, n=7), GSK045 (1000 nM, n=8), BIRB796 (1000nM, n=8) and dexamethasone (1000nM, n=8). fMLP (1000nM) was then added via pumps on the POLARstar Omega plate reader and changes in fluorescence measured as described. Only 1 concentration of each inhibitor was used in this experiment due to a limited number of sputum cells available.

4.2.6.4 ROS release from alveolar macrophages stimulated with fMLP or PMA

To demonstrate that the neutrophil population was responsible for the observed changes in sputum ROS, alveolar macrophages from 3 donors undergoing lung resection were incubated with the ROS sensitive dye CM-H2DCFDA (5µM) for 20 minutes and then stimulated with fMLP (1000nM), phorbol myristate acetate (PMA) (25ng/ml) or appropriate control (0.01% DMSO). ROS release was measured as described in section 2.7.

Alveolar macrophages from donors undergoing lung resections were obtained as follows: resected lung tissue was washed with 0.1M sodium chloride (NaCl). The fluid was centrifuged (400g for 10 minutes at room temperature) and the resulting cell pellet resuspended in supplemented media. The cell suspension was then layered over a Ficol gradient, centrifuged (400g for 35 minutes at room temperature) and counted by trypan blue exclusion. Cells were then resuspended at a concentration of 1 x 10^6/ml. 4 x 10^5 cells were seeded into each well in a 24 well plate and incubated for 24 hours in 5% CO_2 at 37°C. Adherent macrophages were removed, washed and resuspended in unsupplemented media without phenol red. Following further centrifugation (400g for 10 minutes at room temp) the cells were recounted and resuspended in supplemented media without phenol red at a concentration of 2 x 10^6/ml. Cytospins were prepared as described earlier.
4.2.7 MMP-9 and ROS release from blood neutrophils from exacerbating COPD patients

All experiments in this section were performed using blood neutrophils from patients with exacerbations of COPD. Only 1 concentration of inhibitor was used in these experiments as it was felt to be unsafe to venesect large volumes of blood from patients who were clinically unwell.

4.2.7.1 MMP-9 release from blood neutrophils from exacerbating COPD patients; the effect of PI3 kinase enzyme inhibitors

Isolated neutrophils were pretreated (1 hour) with ZSTK474, GSK045, BIRB796, and dexamethasone (all 1000nM). 1000nM was chosen as this concentration caused significant inhibition of MMP-9 from neutrophils from stable patients. Thereafter, cells were incubated in the presence or absence of fMLP (10nM; 30 minutes at 37°C in humidified CO₂) to measure unstimulated (n=10) and stimulated (n=11) MMP-9 release. Plates were then centrifuged and supernatants harvested as described above, for MMP-9.

4.2.7.2 ROS release from blood neutrophils from exacerbating COPD patients; the effect of PI3 kinase enzyme inhibitors

Experiments were carried out as described in section 2.7. 2 x 10⁵ blood neutrophils were pre-treated (5 minutes) with ZSTK474, GSK045, BIRB796 and dexamethasone (all 1000nM). Cells were stimulated with fMLP (1000nM; n=7), which was then added via pumps on the POLARstar Omega plate reader and changes in fluorescence measured as described. The effect of the inhibitors on ROS release from unstimulated cells (n=6) was also examined.
4.2.8 Cell apoptosis

Morphological analysis and TUNEL staining was carried out as described in section 2.8.

4.2.8.1 Neutrophil death curve

4 x 10⁵ blood neutrophils from 3 healthy donors (n=3) were seeded in each well in a 24 well plate and incubated in 5% CO₂ at 37°C for a number of timepoints (0, 1, 2, 4, and 6 hours). At each time point, plates were centrifuged (2000g for 10 minutes at 4°C), supernatants removed and the remaining cells used to prepare cytospins. 2 slides were used for DCCs and 2 slides immediately frozen at -80°C for later TUNEL analysis.

4.2.8.2 The effect of the compounds on neutrophil apoptosis

4 x 10⁵ blood neutrophils from 3 healthy donors were seeded in 24 well plates. Cells were pre-treated (1 hour) with ZSTK474 (1000nM), GSK045 (1000nM), BIRB796 (1000nM), dexamethasone (1000nM) or appropriate control (DMSO 0.02%), followed by stimulation with fMLP (1000nM) and incubated in 5% CO₂ at 37°C. After 6 hours, plates were centrifuged and cytospins prepared as described in section 4.2.8.1.

4.2.8.3 The effect of the ROS reagent CM-H2DCFDA on cell apoptosis

2 x 10⁵ blood neutrophils from 3 healthy donors were seeded in 96 well plates. Cells were treated with CM-H2DCFDA (5µM), DMSO (5%) or PBS for 20 minutes in 5% CO₂ at 37°C. 5% DMSO was the concentration of DMSO the ROS reagent was required to be dissolved in. Plates were then centrifuged and cytospins prepared as described in section 4.2.8.1.
4.2.9 Detecting intracellular phospho-Akt in blood neutrophils by flow cytometry

To investigate the effects on intracellular phospho-Akt in neutrophils following fMLP stimulation a time course assay detecting intracellular phospho-Akt by flow cytometry was performed.

4.2.9.1 Time course assay to detect changes in intracellular phospho-Akt following fMLP stimulation

To investigate whether fMLP stimulation causes neutrophil activation via activation of the PI3 kinase enzyme, intracellular levels of the PI3K enzyme downstream marker; phosphorylated Akt (AKT-p) was analysed. Isolated neutrophils from patients with COPD (n=3) were stimulated (0, 5, 10, 20, 40 and 60, 80, 100 and 120 minutes) with fMLP (1000nM) or with DMSO (0.01%). At the appropriate time point cells were washed (PBS), fixed (4% paraformaldehyde) and incubated (30 minutes) with Anti-CD16-PE (BD Biosciences, Oxford, UK) or appropriate isotype control. Thereafter, cells were washed, fixed again followed by permeabilisation (0.5% Tween-20) (30 minutes). After a further wash, cells were incubated with AKT-p-XP-A647 (Cell Signalling, Hitchen, UK) or the appropriate isotype control. Cells were analysed using BD Canto II flow cytometer with FACsDiva software (v6). Neutrophils were gated using High side scatter (SSc) and CD16 positive profile in the PE channel (FL-2). These settings were applied for acquisition of 10,000 gated CD16 positive events. AKT-p levels in CD16 positive events were expressed as percentage positive events for AKT-p. Cytospins were prepared from cell suspensions for DCCs.
4.2.10 The effect of fMLP stimulation on neutrophil intracellular phospho-p47phox by Western blotting; a time course experiment

To investigate whether fMLP stimulation caused neutrophil ROS release via phosphorylation of p47phox western blot experiments were performed. This method was chosen as a commercially available antibody was found specific for phospho-p47phox at serine 304; previous literature has demonstrated that phospho-Akt leads to phosphorylation of p47phox at serine 304 and 328 (425, 430). This antibody had been optimised for use in western blot and had also been used in published literature (466). Isolated blood neutrophils (1 x 10⁶) from stable patients with COPD (n=1) were stimulated (0, 5, 10, 20, 40 and 60 minutes) with fMLP (1000nM) or with DMSO (0.01%). Cells were then lysed with RIPA buffer (10mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% Nonidet P-40) containing phosphatase (Sigma-Aldrich) and protease (Calbiochem, Nottingham, UK) inhibitors. Samples were then denatured by adding loading buffer (62.5mM Tris-HCl pH 6.95, 10% glycerol, 1% sodium dodecyl sulphate (SDS), 1% β-mercaptoethanol and 0.01% bromphenol blue) and boiling to 90°C. Samples were then stored at -20°C prior to western blot analysis.

Samples were then loaded and electrophoresed on SDS polyacrylamide gels (10%) at 150V in 1 x tris-glycine SDS (TGS) running buffer. The Precision Plus Protein Kaleidoscope Standard (Bio-Rad Laboratories Ltd, Hampshire, UK) was used as the molecular weight marker. Separated proteins were transferred to Hy-bond ECL membranes (Whatman, Amersham, UK) at 400mA in 1x TGS, 20% methanol transfer for 1 hour. Membranes were incubated with blocking buffer (1% dried milk, 150mM NaCL containing 0.1% Tween 20) for 1 hour at room temperature and then
incubated with primary antibody, rabbit anti-phospho-NCF1/p47\textsuperscript{phox} (Ser304) (Abcam, Cambridge, UK), diluted in blocking buffer at 4°C overnight.

Membranes were washed in Tris-buffered saline containing 0.1% Tween 20 (TBS/Tween 20). The membranes were then incubated for 60 minutes with horseradish peroxidase-conjugated goat anti-rabbit (Cell Signalling) diluted in wash buffer, washed again, and the antibody labelled proteins were visualised by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

4.2.11 Statistical analysis

In stable blood neutrophils, the dose response effects of each drug on MMP-9 and ROS release were analysed by repeated measures ANOVA followed by Bonferroni correction. The effects of the drugs on sputum cell ROS release were compared using paired students t tests. For blood neutrophils obtained during exacerbations, drug effects on MMP-9 and ROS release were evaluated using paired students t tests, with the exception of unstimulated MMP-9 secretion which was non-parametrically distributed and so analysed using the wilcoxon signed rank test. Unstimulated blood neutrophil MMP-9 release from stable and exacerbation patients was compared using the Mann Whitney U test and stimulated MMP-9 release compared using the unpaired students t test. The correlation between unstimulated MMP-9 release and percentage inhibition of MMP-9 release by the compounds in exacerbation blood neutrophils was analysed using the Spearman test. The effect of fMLP on intracellular phospho-Akt measured by flow cytometry was compared using the students paired t test.
Stimulated ROS release was calculated relative to unstimulated values. The percentage inhibition caused by compounds was calculated using the following formula:

\[
\frac{(\text{Stimulated value} - \text{Compound effect value})}{(\text{Stimulated value} - \text{Unstimulated value})} \times 100
\]

The percentage inhibition of the compounds on unstimulated ROS release was calculated using the following formula:

\[
\frac{(\text{Unstimulated value} - \text{Compound effect value})}{\text{Unstimulated value}} \times 100
\]
<table>
<thead>
<tr>
<th></th>
<th>Stable COPD</th>
<th>Exacerbations</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood MMP-9</td>
<td>Blood ROS</td>
<td>Blood MMP-9</td>
</tr>
<tr>
<td>(n=11)</td>
<td>(n=6)</td>
<td>(n=11)</td>
<td>(n=15)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>67 (54-74)*</td>
<td>65 (56-69)*</td>
<td>66 (60-84)*</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>7/4</td>
<td>4/2</td>
<td>11/4</td>
</tr>
<tr>
<td>FEV1 (litres)</td>
<td>1.77 ± 0.63</td>
<td>1.90 ± 1.45</td>
<td>1.13 ± 0.38</td>
</tr>
<tr>
<td>FEV1 % pred</td>
<td>66.12 ± 13.68</td>
<td>58.86 ± 34.15</td>
<td>44.67 ± 11.44</td>
</tr>
<tr>
<td>FEV1/FVC ratio</td>
<td>51.37 ± 10</td>
<td>45.47 ± 16.28</td>
<td>36.27 ± 5.87</td>
</tr>
<tr>
<td>Pack years</td>
<td>40.13 (20-67)*</td>
<td>44 (31.8-101)*</td>
<td>41.8 (20-82.8)*</td>
</tr>
<tr>
<td>Smoking (Ex/C)</td>
<td>6/5</td>
<td>3/3</td>
<td>51.8 (40.1-60)*</td>
</tr>
<tr>
<td>ICS (Y)</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>LAMA (Y)</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>LABA (Y)</td>
<td>7</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>mMRC score</td>
<td>1.55 ± 0.34</td>
<td>3 ± 1.0</td>
<td>2.2 ± 0.6</td>
</tr>
</tbody>
</table>

Table 4.1: A summary of patient demographics. All values are expressed as mean ± SD, apart from *, expressed as median (range). BDP: beclomethasone dipropionate equivalent, FEV1: Forced expiratory volume in 1 second post 200µg inhaled salbutamol, FVC: Forced Vital Capacity, ICS: Inhaled corticosteroids, LAMA: Long acting muscarinic antagonist, LABA: Long acting beta agonist, mMRC: Modified Medical Research Council Questionnaire.
Figure 4.1: An example of a diary card COPD clinic patients used to record their symptoms. Patients were asked to telephone the research team if they experienced any worsenings in their symptoms before taking antibiotics or corticosteroids. An appointment was made the same day to see a clinician and appropriate treatment was prescribed after blood and sputum sampling.
4.3 Results

4.3.1 Neutrophil purity

DCCs of cytospins prepared from blood neutrophil cell suspensions are shown in Table 4.2. Neutrophil isolation using Mono-Poly differentiating gradient achieved 96.6 % neutrophils.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Mean percentage ± SD (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>96.6 ± 0.5</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1.2 ± 2.4</td>
</tr>
</tbody>
</table>

Table 4.2: Differential cell counts from blood neutrophil cell suspension after separation using Mono-Poly differentiating media (n=5). Data are represented as mean ± SD.

4.3.2 fMLP stimulated MMP-9 and ROS; dose response and time course experiments

4.3.2.1 MMP-9 release

fMLP (0.01-1000nM) caused a dose dependent increase in MMP-9 release from blood neutrophils (see Figure 4.2A; n=3). 10-1000nM fMLP all increased MMP-9 release by around 10-12 fold from baseline. 10nM was therefore chosen for further experiments as there was minimal difference between these concentrations.

MMP-9 release after stimulation with fMLP was unchanged at 30 minutes, 4 and 24 hours; there was an increase in 3 fold from baseline at each timepoint. Therefore a
stimulation time of 30 minutes was chosen as an optimal time point to measure MMP-9 release.

4.3.2.2 ROS release

fMLP (0.1-1000nM) caused a dose dependent increase in ROS release from blood neutrophils (Figure 4.3; n=3). 1000nM fMLP caused a 1.5 fold increase in ROS release and therefore was chosen as a stimulatory concentration for further experiments.
Figure 4.2: MMP-9 release from COPD blood neutrophils (n=3) in response to increasing doses of fMLP (A) and time course experiment (B). Mean ± SEM are represented; V: vehicle control (DMSO 0.01%), S: stimulated value (fMLP 1000nM).
Figure 4.3: ROS release from COPD blood neutrophils (n=3) in response to increasing doses of fMLP. Data is presented relative to the control value. Mean ± SEM are represented; V: vehicle control (DMSO 0.01%).
4.3.3 Peripheral blood neutrophils from stable COPD patients

4.3.3.1 MMP-9 release

fMLP significantly increased MMP-9 secretion from COPD peripheral blood neutrophils (Figure 4.4; n=11). Concentration dependent inhibition of fMLP induced MMP-9 secretion was caused by the PI3Kδ isoform inhibitor GSK045 and the P38 MAPK enzyme inhibitor BIRB796, with similar effects observed at 1000nM (53.4% and 59.3% inhibition respectively; both p<0.001). The pan-PI3K enzyme inhibitor ZSTK474 and dexamethasone did not inhibit MMP-9 secretion.

Based on these experiments 1000nM of the inhibitory compounds was chosen as an optimal concentration for further experiments using single inhibitor concentrations.

4.3.3.2 Intracellular ROS release

fMLP significantly increased ROS release (1.7 fold increase) from COPD peripheral blood neutrophils (Figure 4.5; n=6). ZSTK474 (1000nM) completely inhibited stimulated ROS production, while the same concentration of GSK045 inhibited stimulated ROS release by 74.0% (p<0.001). In contrast to effects on MMP9 release, BIRB796 had no significant effect on ROS release, while dexamethasone caused minimal inhibition.
Figure 4.4: Release of MMP-9 and percentage inhibition of MMP-9 from COPD peripheral neutrophils. Isolated neutrophils were treated with ZSTK474 (1-1000nM, n=11), GSK045 (1-1000nM, n=11), BIRB796 (1-1000nM, n=11) and dexamethasone (1-1000nM, n=11) for 1 hour prior to stimulation with fMLP (10nM) for 30 minutes. Supernatants were harvested and analysed for MMP-9 release. Data are expressed as mean ± SEM with percentage inhibition above; V: vehicle control (DMSO 0.02%), S: stimulated value (fMLP 10nM). * p<0.05, **p<0.001.
Figure 4.5: Intracellular ROS release from COPD peripheral neutrophils. After incubation with the ROS sensitive dye CM-H2DCFDA (5 µM, all n=6), isolated neutrophils were treated with ZSTK474 (1-1000nM), GSK045 (1-1000nM), BIRB796 (1-1000nM), and dexamethasone (1-1000nM) for 5 minutes prior to stimulation with fMLP (1000nM) for 1 hour. Changes in fluorescence were detected and are expressed relative to baseline unstimulated values as mean ± SEM; S: stimulated value (fMLP 1000nM). *p<0.05, **p<0.001.
4.3.4 Peripheral blood neutrophils from exacerbating COPD patients

4.3.4.1 MMP-9 release

Neutrophils were isolated from COPD patients during exacerbations (n=15); Unstimulated MMP-9 release was higher compared to stable COPD patients (n=15) (Figure 4.6A); medians 16.5 ng/ml versus 12.7 ng/ml respectively (p=0.02). This difference between groups appeared to be driven by a subset of COPD exacerbations with increased MMP-9 levels. fMLP stimulated MMP-9 release was similar in COPD patients during the stable state and in exacerbations (Figure 4.6B).

ZSTK474 significantly inhibited MMP-9 release from unstimulated neutrophils obtained during exacerbations by 38.0% (p<0.05; Figure 4.7A). There were trends for BIRB796 and GSK045 to cause inhibition, but these did not reach statistical significance (p=0.08 and 0.08 respectively). There was a significant correlation between unstimulated MMP-9 levels from neutrophils and percentage inhibition of MMP-9 caused by ZSTK474 and GSK045 in samples obtained during exacerbations (Figure 4.8; r=0.95 and 0.93 respectively, both p<0.001).

ZSTK474 and GSK045 significantly inhibited fMLP stimulated MMP-9 release by 53% (p<0.001) and 56.5% (p<0.05) of the stimulated value respectively (see Figure 4.7B). BIRB796 and dexamethasone had no significant effect.

4.3.4.2 Intracellular ROS release

Neutrophils from patients with COPD exacerbations were isolated (n=12); unstimulated and stimulated ROS release was measured (Figure 4.7C and D). ZSTK474 and GSK045 caused complete inhibition of fMLP induced ROS release (p<0.05; Figure 3D). BIRB796 and dexamethasone had no effect. ZSTK474, GSK045 and dexamethasone has small but statistically significant inhibitory effects
on unstimulated ROS release; 19.3%, 12.6% and 11.8% reductions respectively (Figure 4.7C; p<0.05). BIRB796 had no effect.

### 4.3.5 Sputum neutrophils

Table 4.3 shows the sputum differential cell counts; the cell suspensions used for sputum ROS experiments were composed of 76.7% neutrophils. fMLP significantly increased ROS release from sputum cells (n=11) by 1.6 fold (p<0.05). 1000nM ZSTK474 and GSK045 caused significant inhibition (70.8% and 48.1% respectively) of stimulated ROS release (p<0.05), while BIRB796 and dexamethasone had no effect (Figure 4.9 A and B).

The cell suspension used to evaluate the response of alveolar macrophages to fMLP or PMA was composed of 86% macrophages. Changes in fluorescence in alveolar macrophages stimulated with fMLP and PMA and are shown Figure 4.9C: there was no significant change in fluorescence after stimulation with fMLP indicating that changes in ROS seen in sputum cells are attributable to neutrophils.

### 4.3.6 Summary of PI3 kinase enzyme inhibitor effects

A summary of the effects of ZSTK474, GSK045, BIRB796 and dexamethasone (1000nM) on MMP-9 and ROS release from neutrophils in the stable clinical state and during exacerbations is represented in Table 4.4.
Figure 4.6: Unstimulated (A) and stimulated (B) MMP-9 release from COPD peripheral neutrophils in the stable state and during exacerbations. Isolated neutrophils from stable and exacerbating patients were cultured for 30 minutes in the absence (n=15; A) or presence (n=11; B) of fMLP (10nM). Supernatants were harvested for MMP-9 analysis. Data are expressed as median ± range in (A) and mean ± SEM in (B). *p<0.05.
Figure 4.7: MMP-9 and ROS release from neutrophils in COPD exacerbations. Isolated neutrophils from exacerbating patients were treated (1 hour) with, ZSTK474, GSK045, BIRB796 and dexamethasone (all 1000nM). The effect of these inhibitors on fMLP (10nM, 30 minutes) stimulated (n=11) (B) and unstimulated (n=10) (A) MMP-9 release is demonstrated.

After incubation with the ROS sensitive dye CM-H2DCFDA (5 µM), isolated neutrophils from exacerbating patients were treated (5 minutes) with GSK740, ZSTK474, BIRB796 and dexamethasone (all 1000nM). The effect of these inhibitors on fMLP stimulated (1000nM, 60 minutes) (n=7, D) and unstimulated (n=6, C) ROS release are shown. Changes in fluorescence are expressed relative to baseline unstimulated values.

Data are expressed as median ± range (A) or as mean ± SEM (B, C, D); V: vehicle control (DMSO 0.01-0.02%), S: stimulated value (fMLP 10nM MMP-9, 1000nM ROS). * p<0.05.
Figure 4.8: Correlation between unstimulated MMP-9 release from exacerbating blood neutrophils and the percentage inhibition of MMP-9 release caused by PI3 kinase enzyme inhibitors. Isolated neutrophils from exacerbating patients were treated (1 hour) with 1000nM ZSTK474 (blue triangle) and GSK045 (red circle). The effect of these inhibitors on unstimulated (n=10) MMP-9 release was measured and percentage inhibition was calculated. **p<0.001.
Table 4.3: A summary of sputum differential cell counts. Differential cell counts (n=11) for sputum samples used for ROS experiments. All values expressed as mean ± SD apart from those indicated †, presented as median (range).

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Neutrophil %</td>
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</tr>
<tr>
<td>Macrophage %</td>
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</tr>
<tr>
<td>Eosinophil % †</td>
<td>2.7 (0-27)</td>
</tr>
<tr>
<td>Lymphocyte %</td>
<td>1.5 (±0.4)</td>
</tr>
<tr>
<td>Epithelial cell % †</td>
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</tr>
<tr>
<td>Total Cell Count (x10⁶) per gram</td>
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</tr>
<tr>
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<td>5.0 (±1.3)</td>
</tr>
<tr>
<td>Macrophage Cell Count (x10⁶) per gram</td>
<td>0.9 (±0.4)</td>
</tr>
<tr>
<td>Eosinophil Cell Count (x10⁶) per gram †</td>
<td>0.2 (±0.1)</td>
</tr>
<tr>
<td>Lymphocyte Cell Count (x10⁶) per gram</td>
<td>0.1 (±0.02)</td>
</tr>
<tr>
<td>Epithelial Cell Count (x10⁶) per gram †</td>
<td>0.01 (0-0.2)</td>
</tr>
</tbody>
</table>
Figure 4.9: Intracellular ROS from COPD sputum cells (A and B). After incubation with the ROS sensitive dye CM-H2DCFDA (5 µM), sputum cells were treated with 1000nM, ZSTK474 (n=7), GSK045 (n=8), BIRB796 (n=8) and dexamethasone (n=8) for 5 minutes prior to stimulation with fMLP (1000nM) for 1 hour. A represents absolute values, B represents percentage inhibition. Intracellular ROS from alveolar macrophages (C). After incubation with the ROS sensitive dye CM-H2DCFDA (5 µM), alveolar macrophages (n=3) were stimulated with fMLP (1000nM) or PMA (25ng/ml) for 1 hour. Changes in fluorescence were detected and are expressed relative to baseline unstimulated values as mean ± SEM; V: vehicle control (DMSO 0.01-0.02%) S: stimulated value (fMLP 1000nM). *p<0.05, **p<0.001.
Table 4.4: A summary of percentage inhibition for each compound (1000nM) on each neutrophil function (1000nM fMLP stimulated or unstimulated as indicated). Data are presented as mean ± SD apart from *, represented as median (range).
4.3.7 Cell apoptosis

4.3.7.1 Neutrophil death curve
Apoptosis of cultured peripheral neutrophils from healthy donors (n=3) was assessed at 0, 1, 2, 4, and 6 (Figure 4.10). Between 0 and 6 hours there was little change in percentage cells in early or late apoptosis by cell morphology and staining positive for TUNEL (<10%). Therefore 6 hours was taken as a time point to assess the effect of compounds on cell apoptosis as from these results there was no difference between 0 and 6 hours.

4.3.7.2 The effect of fMLP and inhibitory compounds on neutrophil apoptosis
There was no significant difference in the percentage cells positive for TUNEL staining or percentage cells in early and late positive apoptosis between neutrophils in control conditions (0.02% DMSO) and those treated with fMLP and inhibitory compounds (Table 4.5A; n=3).

4.3.7.3 The effect of the ROS reagent CM-H2DCFDA on cell apoptosis
There was no significant difference in the percentage cells positive for TUNEL staining or the percentage cells in early and late positive apoptosis between neutrophils in control conditions (PBS) and those treated with 5% DMSO or CH-H2DCFDA (Table 4.5B; n=3).
Figure 4.10: The percentage peripheral neutrophils in early and late apoptosis (A) and staining positive for TUNEL (B) at 0, 1, 2, 4 and 6 hours in supplemented media. Neutrophils from 3 healthy donors (n=3) were isolated and cultured for a number of timepoints. At each time point cytospins were prepared and analysis of apoptosis performed by morphological analysis and by the TUNEL assay. Data are displayed as mean ± SEM.
A

<table>
<thead>
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<th>Compound</th>
<th>TUNEL</th>
<th>NEUTROPHIL MORPHOLOGY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% neutrophils positive for TUNEL staining</td>
<td>% early apoptosis</td>
</tr>
<tr>
<td>DMSO (0.02%)</td>
<td>27.0 ± 6.2</td>
<td>6.6 ± 1.4</td>
</tr>
<tr>
<td>fMLP (1000nM)</td>
<td>11.1 ± 6.3</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>ZSTK474 (1000nM)</td>
<td>29.0 ± 9.9</td>
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</tr>
<tr>
<td>GSK045 (1000nM) + fMLP (1000nM)</td>
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<td>5.7 ± 0.9</td>
</tr>
<tr>
<td>BIRB796 (1000nM)</td>
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<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>Dex (1000nM)</td>
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<td>6.0 ± 0.8</td>
</tr>
</tbody>
</table>

B

<table>
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<th>NEUTROPHIL MORPHOLOGY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% neutrophils positive for TUNEL staining</td>
<td>% early apoptosis</td>
</tr>
<tr>
<td>DMSO (5%)</td>
<td>8.1 ± 1.9</td>
<td>7.1 ± 0.7</td>
</tr>
<tr>
<td>PBS</td>
<td>9.2 ± 6.5</td>
<td>7.8 ± 1.6</td>
</tr>
<tr>
<td>CM-H2DCFDA (5µM)</td>
<td>12.1 ± 6.3</td>
<td>7.8 ± 0.6</td>
</tr>
</tbody>
</table>

Table 4.5: Percentage cells in early and late apoptosis or TUNEL positive after inhibitor treatment (A) or CM-H2DCFDA (B). Neutrophils from healthy donors (n=3) were pre-treated (1 hour) with ZSTK474, GSK045, BIRB796 and dexamethasone (1000 nM) followed by fMLP (1000 nM) for 6 hours (A) or with DMSO (5%), PBS or CM-H2DCFDA (5µM) for 20 minutes (B). Cytospins were prepared. The TUNEL method in conjunction with the microscopic analysis of the nuclear morphology was used to assess apoptosis. Data are represented as mean ± SD.
4.3.8 Detecting neutrophil intracellular phospho-Akt by flow cytometry

4.3.8.1 fMLP induced changes in intracellular phospho-Akt; a time course experiment

Peripheral neutrophils from stable COPD patients (n=3) were stimulated with 1000nM fMLP or 0.01% DMSO in a time course experiment (0-120 minutes) and changes in intracellular Akt were detected by flow cytometry. Gating strategy and change in phospho-Akt positive cells post fMLP stimulation is shown in Figure 4.11. At 5 minutes, there was a significant increase in the percentage of phospho-Akt positive cells after fMLP stimulation (Figure 4.12) from 29% to 54% (p=0.008). There was also a trend for percent phospho-Akt cells to be increased at 10 and 20 minutes after fMLP stimulation (p=0.06 and 0.09 respectively). DCCs of neutrophil suspensions used for these experiments demonstrated 96.6% neutrophils.
Figure 4.11: Gating strategy used to identify phospho-Akt positive neutrophils. Neutrophils were gated using High SSc and CD16 positive profile in the PE channel (FL-2). These settings were applied for acquisition of 10 000 gated CD16 positive events. At 5 minutes post fMLP stimulation phospho-Akt positive cells increased from 27% to 49% in this n=1 COPD patient.
Figure 4.12: Intracellular phospho-Akt in stable COPD neutrophils. Blood neutrophils from COPD patients (n=3) were stimulated with 1000nM fMLP or 0.01% DMSO in a time course experiment (0-120 minutes) Changes in the percentage cells expressing phospho-Akt was detected by flow cytometry. Results are expressed as mean ± SEM (S: stimulated value (fMLP 1000nM), V: vehicle control (DMSO 0.01%)). *p<0.05.
4.3.9 The effect of fMLP on neutrophil intracellular phospho-p47\textsubscript{phox}; a time course

To demonstrate whether the fMLP induced increase in ROS release from neutrophils was due to downstream phosphorylation of p47\textsubscript{phox} a time course experiment was performed for western blot protein analysis. The experiment was only done with a single COPD patient and therefore limited by sample size. However, figure 4.13 shows that at 5, 10, 20 and 40 minutes post fMLP (1000nM) stimulation there may be an increase in detected phospho-p47\textsubscript{phox} compared to control conditions (0.01% DMSO). These results are difficult to interpret due to the inconsistency of β-actin results.

Figure 4.13: A time course experiment demonstrating the effect of fMLP stimulation on neutrophil intracellular phospho p47\textsubscript{phox} detected by western blotting. Neutrophils from a COPD patient (n=1) were stimulated with 1000nM fMLP (+) or 0.01% DMSO (-) in a time course experiment (0-60 minutes) and protein extracted for western blot analysis. pp47: phospho-p47\textsubscript{phox}
4.4 Discussion

This study has demonstrated that PI3 kinase enzyme inhibition using a both a pan and δ isoform selective inhibitor suppressed fMLP stimulated MMP-9 and ROS release from COPD neutrophils during both the stable state and exacerbations. In contrast, corticosteroids had little or no effect in these experiments. PI3K enzyme inhibitors have the potential to target corticosteroid insensitive secretion of inflammatory mediators from COPD neutrophils.

MMP-9 and neutrophil elastase are the proteases predominantly responsible for the parenchymal destruction that occurs in emphysema (465). MMP-9 levels are increased in the lungs and blood of COPD patients compared to controls (202, 208, 209) and MMP-9 activity is closely associated with the number of neutrophils in broncho-alveolar lavage from COPD patients (205). It has previously been shown that pan PI3 kinase enzyme inhibition reduces MMP-9 release from healthy blood neutrophils (205, 437). In contrast a further study demonstrated that pan inhibition had no effect on MMP-9 release from COPD neutrophils (214). This study demonstrated that selective PI3 kinase δ isoform inhibition reduced fMLP stimulated MMP-9 release from COPD blood neutrophils obtained during the stable state and during exacerbations; the differences between our results and the previous work (214) may be due to the compound that we used having greater potency for the δ isoform. A further reason for this discrepancy may be that LPS was used as a stimulant by Milara et al (214); the role of the PI3 kinase enzyme in LPS stimulated degranulation will be discussed in a later chapter.

Unstimulated MMP-9 secretion from neutrophils was increased in exacerbations compared to stable samples, with this difference driven by a subset of patients (see Figure 4.6). Indeed, the effects of the PI3 kinase enzyme inhibitors on unstimulated
MMP-9 secretion was strongly related to the absolute level of MMP-9 secretion, indicating that these compounds had greater efficacy in the subset of patients with elevated basal MMP-9 secretion. COPD exacerbations are heterogeneous events, with different aetiologies such as viral or bacterial infections (229); it is possible that MMP-9 up-regulation in a subset of clinical exacerbations is related to a particular aetiology.

The inhibition of MMP-9 observed with the PI3 kinase enzyme inhibitors in unstimulated exacerbation samples was lower than that observed in fMLP stimulated samples. This may be due to the low levels in many exacerbation samples meaning that the “window” to evaluate the effects of these compounds was reduced. fMLP stimulated MMP-9 release from both stable and exacerbation COPD blood neutrophils was inhibited by approximately 50% by GSK045. The effect of ZSTK474 was similar to GSK045 in exacerbation samples, but lower in stable samples. This suggests that the potent PI3 kinase δ isoform selective inhibitor used here more consistently reduced MMP-9 production from COPD neutrophils compared to a pan PI3 kinase enzyme inhibitor with lower δ specific activity.

COPD neutrophils exhibit enhanced ROS release compared to healthy blood neutrophils (190, 215). ROS have essential roles in fighting infection, but also cause increased oxidative stress and therefore contribute to the excessive inflammation observed in COPD (465). It has previously been shown that PI3 kinase δ and γ isoform selective inhibition diminishes fMLP stimulated ROS release from healthy blood neutrophils (137, 423). This study demonstrated that PI3 kinase pan enzyme and δ isoform inhibitors reduce fMLP stimulated ROS release from COPD blood and sputum neutrophils. There appeared to be greater inhibitory effects with ZSTK474 compared to GSK045 in some experiments, such as stable blood and sputum.
samples. This suggests a significant role for other PI3K enzyme subunits in ROS production in stable samples, such as the PI3 kinase γ isoform which has been linked to neutrophil function (423). Interestingly, the effects of these compounds were more similar in unstimulated exacerbation samples suggesting a lesser role for PI3 kinase γ isoform signalling in these samples.

There is other evidence that the PI3K enzyme is involved in the pathophysiology of COPD; pan PI3K enzyme inhibition reverses the reduced migratory accuracy seen in COPD blood neutrophils (196). Furthermore, PI3 kinase δ isoform expression is upregulated in COPD alveolar macrophages, and selective PI3K δ isoform inhibition reverses GC insensitivity in COPD monocytes (369). In COPD animal models, PI3 kinase δ isoform inhibition restores glucocorticoid insensitivity (364) and pan inhibition reduces airway neutrophilia (467). PI3 kinase δ isoform and PIP3 expression is increased in COPD blood neutrophils compared to controls (214). This study has provided further evidence for the role of the PI3K enzyme in COPD, and has shown that selective PI3K δ isoform inhibition can suppress blood and lung neutrophil activity during the stable state and exacerbations.

The treatment options for patients suffering with exacerbations of COPD are limited. The experiments reported here showed little effect of corticosteroids on neutrophil production of ROS and MMP-9 during the stable state or exacerbations. PI3K enzyme inhibition could be used as a therapeutic option against excessive neutrophilic inflammation that occurs in the stable state (69) and during COPD exacerbations (229), and could be used in addition to corticosteroids to target the corticosteroid insensitive neutrophil activities reported here. Furthermore, the potential synergistic interactions between PI3K δ isoform inhibitors and corticosteroids (369) provides a further rationale to combine these drugs.
P38 MAP kinase enzyme inhibitors reduced fMLP stimulated MMP-9 release from stable COPD blood neutrophils, which is in keeping with previous studies in healthy blood neutrophils (205, 385, 437). However, we did not observe any effect on ROS release which is in contrast to a previous report (383). The lack of effect seen in sputum neutrophils is in keeping with the fact that inflammation in airway neutrophils appears to be p38 MAPK enzyme independent as these cells are devoid of phospho-p38 (370). Overall, the effect of p38 MAPK enzyme inhibition in this study was less than PI3K enzyme inhibition, and indicates that PI3K enzyme inhibitors have greater effects on the secretion of mediators from COPD neutrophils.

fMLP has been used extensively to stimulate neutrophil degranulation and ROS release (137, 205, 215). The fMLP receptor is a G protein coupled receptor that signals through various intracellular pathways including PI3 kinase and p38 (468). Studies using neutrophils from healthy subjects have used TNFα to “prime” the cells before stimulation with fMLP (137). However, COPD blood neutrophils display increased surface CD11b (190), indicating increased activation, probably due to inflammatory cytokines present in the blood. fMLP alone was therefore used to stimulate neutrophils; priming is investigated further in a later chapter.

This study demonstrated that fMLP increased intracellular phospho-Akt expression in COPD neutrophils; it is already known that fMLP stimulates ROS and MMP-9 production from neutrophils via the PI3 kinase enzyme (137, 205). To demonstrate that the compounds were inhibiting Akt phosphorylation, further experiments are required showing reduction in phospho-Akt following pre-treatment with ZSTK474 and GSK045. The mechanisms behind PI3K enzyme activation causing ROS release from neutrophils may be three fold: phosphorylation of p47\textsuperscript{phox} via phosphoinositide dependent kinases (425), direct interaction of cytosolic subunits with
phosphoinositides (426) or by activation of Rac (427). In fMLP or bacterial phagocyte stimulated human neutrophils phospho-Akt phosphorylates p47\textsuperscript{phox}; this occurs at Ser304 and Ser328 (425, 430). Therefore this study investigated whether fMLP lead to ROS release by phosphorylation of p47\textsuperscript{phox}. Only 1 patient was used in this experiment and therefore results were difficult to interpret. There was a suggestion that fMLP stimulation did lead to increased p47\textsuperscript{phox} expression, however there were several limitations. Firstly, as the β-actin results were inconsistent, there may have been insufficient protein extract in all samples; a Bradford assay would allow protein quantification. Secondly, no positive control was used in the experiment therefore the results may have included some false positives. Finally, the antibody concentration may have needed optimisation as previous studies have not investigated its use in neutrophils (466).

A further limitation of this study was that sputum ROS analysis was not performed on sputum samples during exacerbations. This was because the ROS experimental protocol required a large number of cells, and during exacerbations it was considered unsafe to induce sputum. In addition we have not compared ROS release from neutrophils from stable versus exacerbating COPD patients; this is because each ROS assay had its own internal control and therefore it was not possible to perform comparisons between experiments.

In conclusion, MMP-9 and ROS release from stable and exacerbation COPD neutrophils is suppressed by PI3 kinase enzyme inhibitors. This study shows for the first time the effects of a PI3K δ isoform specific inhibitor for targeting glucocorticoid insensitive MMP-9 and ROS secretion from COPD neutrophils. These experiments conducted using COPD blood and lung cells support the case for
PI3 kinase enzyme inhibition in addition to current standard therapy to target neutrophilic inflammation in COPD.
5. The effect of PI3 kinase γ isoform inhibition on MMP-9 and ROS release from COPD neutrophils

5.1 Introduction

Inhibition of the PI3 kinase γ isoform has been shown to reduce ROS release from healthy blood neutrophils (137, 423). Selective γ (and δ) inhibition has also been shown to reverse the reduced migratory accuracy seen in healthy neutrophils from older patients compared to younger patients (469); furthermore the γ isoform has a vital role in determining the number of cells which actually move cross a chemotactic gradient to fMLP (419). In animal models, inhibition of the PI3 kinase γ isoform also improves phagocytosis (470) and reduces parasite entry in neutrophils (471). As previously described pan PI3 kinase enzyme inhibitors reduce MMP-9 (314) and cytokine (435) release from neutrophils; however the effect of γ selective inhibition on the release of inflammatory mediators from COPD neutrophils has not been investigated.

In this chapter the effects of PI3 kinase γ isoform inhibition on MMP-9 and ROS release from COPD neutrophils were investigated. Neutrophils in the stable clinical state and during exacerbations have been investigated. This has been presented separately from the pan and δ inhibitor data in chapter 4 because I was given the γ compound later in the course of my PhD and so fewer patient numbers were used in these experiments. This means that the estimate of efficacy for the γ compound is less robust, and the data are not directly comparable in terms of absolute levels as the patients are not matched. However, this chapter does compare the percentage inhibition achieved by γ, δ and pan PI3 kinase enzyme inhibitors.
5.2 Methods

5.2.1 Patient selection
Stable and exacerbating patients were recruited as described in section 4.2.1 and 4.2.2. Patient demographics are presented in Table 5.1.

5.2.3 Spirometry, reversibility and health questionnaires
This was carried out as described in section 2.2 and 2.3.

5.2.4 Blood collection and neutrophil isolation
This was carried out as described in section 2.5.

5.2.5 MMP-9 release
Blood neutrophil culture was carried out as described in section 4.2.5.

5.2.5.1 The effect of PI3 kinase γ isoform inhibition on MMP-9 release from blood neutrophils from stable COPD patients
This was carried out as described in section 4.2.5.3. Isolated neutrophils (n=6) were pre-treated (1 hour) with a γ selective PI3 kinase enzyme inhibitor, GSK723 (GlaxoSmithKlein) (10-1000nM). Supernatants were harvested as described. The pIC50 values for GSK723 were 5.9M and 8.6M for the PI3Kδ and γ isoforms respectively (see Appendix 2).

5.2.5.2 The effect of PI3 kinase γ isoform inhibition on MMP-9 release from blood neutrophils from exacerbating COPD patients
This was carried out as described in section 4.2.7.1; cells were pretreated with GSK723 (1000nM) and unstimulated (n=10) and stimulated (n=11) supernatants were collected for MMP-9 analysis.
5.2.5.3 Supernatant analysis
MMP-9 was measured in supernatants as described in section 2.6.

5.2.6 ROS assays
This was carried out as described in section 2.7.

5.2.6.1 The effect of PI3 kinase γ isoform inhibition on ROS release from blood neutrophils from stable COPD patients
This was carried out as described in section 4.2.6.2. Peripheral blood neutrophils (n=6) were pretreated with GSK723 (10-1000nM).

5.2.6.2 The effect of PI3 kinase γ isoform inhibition on ROS release from sputum cells from stable COPD patients
Sputum from stable COPD patients (n=6) was obtained and processed using methods (DTT) as described in section 2.3.2. The experiment was carried out as described in section 4.2.6.3; sputum cells were pretreated with GSK723 (1000nM).

5.2.6.3 The effect of PI3 kinase γ isoform inhibition on ROS release from blood neutrophils from exacerbating COPD patients
Experiments were carried out as described in section 2.7 and 4.2.7.2; cells were pretreated with GSK723 (1000nM) and unstimulated (n=6) and stimulated (n=7) ROS release was measured.

5.2.7 The effect of the PI3 kinase γ isoform inhibitor on cell apoptosis
The effects of GSK723 (1000nM) on cell apoptosis was assessed as described in section 2.8 and 4.2.8.

5.2.8 Statistical analysis
This was carried out as described in section 4.2.11.
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<tr>
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<td>Blood ROS (n=6)</td>
<td>Sputum ROS (n=6)</td>
</tr>
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<td>65 (56-69)*</td>
<td>66 (58-75)*</td>
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</tr>
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<td>ICS (Y)</td>
<td>4</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>LAMA (Y)</td>
<td>3</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>LABA (Y)</td>
<td>4</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>mMRC score</td>
<td>1.5 ± 1.3</td>
<td>3 ± 1.0</td>
<td>1.2 ± 1.2</td>
</tr>
</tbody>
</table>

**Table 5.1: A summary of patient demographics.** All values are expressed as mean ± SD, apart from *, expressed as median (range). BDP: beclomethasone dipropionate equivalent, FEV1: Forced expiratory volume in 1 second post 200µg inhaled salbutamol, FVC: Forced Vital Capacity, ICS: Inhaled corticosteroids, LAMA: Long acting muscarinic antagonist, LABA: Long acting beta agonist, mMRC: modified Medical Research Council Questionnaire.
5.3 Results

5.3.1 Peripheral blood neutrophils from stable COPD patients

5.3.1.1 MMP-9 release
There was no significant effect on fMLP stimulated MMP-9 release observed with any concentration of GSK723 (Figure 5.1A; n=6).

5.3.1.2 Intracellular ROS release
GSK723 (1000nM) significantly inhibited stimulated ROS release by 66.5% (Figure 5.1B; p<0.001; n=6).

5.3.2 Peripheral blood neutrophils from exacerbating COPD patients

5.3.2.1 MMP-9 release
There was a trend for GSK723 (1000nM) to cause inhibition of unstimulated MMP-9 release, but this did not reach statistical significance (Figure 5.2A; p=0.06). There was also a significant correlation between unstimulated MMP-9 levels from neutrophils and percentage inhibition of MMP-9 caused by GSK723 (Figure 5.3; r=0.70, p=0.03).

GSK723 had no significant effect on stimulated MMP-9 release (Figure 5.2B).

5.3.2.2 Intracellular ROS release
GSK723 (1000nM) caused complete inhibition of fMLP induced ROS release by 100% (Figure 5.2D; p=0.03; n=6). In addition, there was a trend for GSK723 to also reduce unstimulated ROS (Figure 5.2C; p=0.08).
5.3.3 Sputum neutrophils

GSK723 (1000nM) had no significant effect on ROS release from fMLP stimulated COPD sputum neutrophils (Figure 5.4).

5.3.4 Cell apoptosis

GSK723 (1000nM) had no significant effect on cell apoptosis (Table 5.2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>TUNEL</th>
<th>NEUTROPHIL MORPHOLOGY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% neutrophils positive for TUNEL staining</td>
<td>% early apoptosis</td>
</tr>
<tr>
<td>DMSO (0.02%)</td>
<td>27.0 ± 6.2</td>
<td>6.6 ± 1.4</td>
</tr>
<tr>
<td>fMLP (1000nM)</td>
<td>11.1 ± 6.3</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>GSK723 (1000nM)+ fMLP (1000nM)</td>
<td>16.3 ± 8.3</td>
<td>5.8 ± 0.8</td>
</tr>
</tbody>
</table>

Table 5.2: Percentage cells in early and late apoptosis or TUNEL positive after inhibitor treatment. Neutrophils from healthy donors (n=3) were pre-treated (1 hour) with GSK723 (1000nM) followed by fMLP (1000nM) for 6 hours. Cytospins were prepared. The TUNEL method in conjunction with the microscopic analysis of the nuclear morphology was used to assess apoptosis. Data are represented as mean ± SD.

5.3.5 Summary of PI3 kinase γ isoform inhibitor effects

A summary of the effects of GSK723 (1000nM) on MMP-9 and ROS release from neutrophils in the stable clinical state and during exacerbations is represented in Table 5.3.
Figure 5.1: The effect of GSK723 on release of MMP-9 from COPD peripheral neutrophils (A). Isolated neutrophils were treated with GSK723 (10-1000nM, n=6) for 1 hour prior to stimulation with fMLP (10nM) for 30 minutes. Supernatants were harvested and analysed for MMP-9 release. Data are expressed as mean ± SEM. V: vehicle control (DMSO 0.02%), S: stimulated value (fMLP 10nM). The effect of GSK723 on intracellular ROS release from COPD peripheral neutrophils (B). After incubation with the ROS sensitive dye CM-H2DCFDA (5 µM, all n=6), isolated neutrophils were treated with GSK723 (10-1000nM) for 5 minutes prior to stimulation with fMLP (1000nM) for 1 hour. Changes in fluorescence were detected and are expressed relative to baseline unstimulated values as mean ± SEM; S: stimulated value (fMLP 1000nM). *p<0.05, **p<0.001.
Figure 5.2: The effect of GSK723 on MMP-9 and ROS release from neutrophils in COPD exacerbations. Isolated neutrophils from exacerbating patients were treated (1 hour) with GSK723 (1000nM). The effect on fMLP (10nM, 30 minutes) stimulated (n=11) (B) and unstimulated (n=10) (A) MMP-9 release is demonstrated. After incubation with the ROS sensitive dye CM-H2DCFDA (5 µM), isolated neutrophils from exacerbating patients were treated (5 minutes) with GSK723 (1000nM). The effect on fMLP stimulated (1000nM, 60 minutes) (n=7, D) and unstimulated (n=6, C) ROS release are shown. Changes in fluorescence are expressed relative to baseline unstimulated values. Data are expressed as median ± range (A) or as mean ± SEM (B, C, D); V: vehicle control (DMSO 0.01-0.02%), S: stimulated value (fMLP 10nM MMP-9, 1000nM ROS). * p<0.05.
Figure 5.3: Correlation between unstimulated MMP-9 release from exacerbating blood neutrophils and the percentage inhibition of MMP-9 release caused by GSK723. Isolated neutrophils from exacerbating patients were treated (1 hour) with 1000nM GSK723 (grey square). The effect of these inhibitors on unstimulated (n=10) MMP-9 release was measured and percentage inhibition was calculated. Statistically significant correlation is indicated by *p<0.05.

Figure 5.4: The effect of GSK723 on intracellular ROS from COPD sputum cells. After incubation with the ROS sensitive dye CM-H2DCFDA (5 µM), sputum cells were treated with 1000nM GSK723 (n=6) for 5 minutes prior to stimulation with fMLP (1000nM) for 1 hour. Changes in fluorescence were detected and results are expressed relative to unstimulated value. Data are expressed as mean ± SEM; S: stimulated value (fMLP 1000nM).
<table>
<thead>
<tr>
<th></th>
<th>Percentage inhibition (1000nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZSTK474 (PI3 pan)</td>
</tr>
<tr>
<td><strong>STABLE</strong></td>
<td></td>
</tr>
<tr>
<td>BLOOD MMP-9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(n=11)</td>
</tr>
<tr>
<td>BLOOD ROS</td>
<td>104.6 ± 17.0 (p&lt;0.001) (n=6)</td>
</tr>
<tr>
<td>SPUTUM ROS</td>
<td>70.8 ± 15.3 (p=0.002) (n=7)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EXACERBATION</strong></td>
<td></td>
</tr>
<tr>
<td>UNSTIMULATED MMP-9</td>
<td>38.0 (0-91.4)* (p=0.03) (n=10)</td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
</tr>
<tr>
<td>STIMULATED MMP-9</td>
<td>53.0 ± 7.5 (p&lt;0.001) (n=11)</td>
</tr>
<tr>
<td></td>
<td>(n=11)</td>
</tr>
<tr>
<td>UNSTIMULATED ROS</td>
<td>19.3 ± 5.6 (p=0.02) (n=6)</td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
</tr>
<tr>
<td>STIMULATED ROS</td>
<td>191.7 ± 36.1 (p=0.02) (n=7)</td>
</tr>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3: A summary of percentage inhibition for each compound (1000nM) on each neutrophil function (1000nM fMLP stimulated or unstimulated as indicated). Data are presented as mean ± SD apart from *, represented as median (range).
5.4 Discussion

GSK723 only significantly inhibited stimulated ROS release from neutrophils in the stable state and during exacerbations; there was no significant effect on sputum ROS or blood MMP-9 although these experiments were limited by sample size. This suggests that the PI3 kinase γ selective isoform inhibition may have an important role in the release of ROS in COPD blood neutrophils but not in degranulation.

The effect on ROS release is in keeping with previous literature demonstrating that the PI3γ isoform has a critical role in the release of ROS from fMLP stimulated healthy blood neutrophils (137, 423). Studies using knockout mice have also demonstrated ROS production is dependent on the γ isoform (398, 407). Although classically fMLP stimulates G protein coupled receptors which leads to stimulation of the γ isoform and downstream NADPH assembly, there is evidence that fMLP stimulated phospho-Akt andPIP3 formation is dependent on tyrosine kinase activity, therefore implicating the δ isoform (137, 472, 473).

The inhibitory effect of the pan PI3K enzyme inhibitor appeared to be greater that the δ inhibitor in these experiments suggesting that fMLP stimulated ROS release could be reduced by both δ and γ selective inhibitors, as has been shown in an earlier study (137); furthermore, γ selective inhibition did have a significant effect on ROS release. Therefore, fMLP stimulated ROS release from neutrophils may have 2 distinct stimulatory pathways via the PI3 kinase enzyme: a direct stimulatory effect on G coupled receptors via the γ isoform and further stimulatory effect on tyrosine kinases with recruitment of the PI3 kinase δ isoform.

There was a trend for γ selective isoform inhibition to decrease unstimulated ROS release in neutrophils from exacerbating patients (p=0.06) although this did not meet
statistical significance. More patient numbers may have resulted in significance. Alternatively, the neutrophil in a COPD exacerbation *in vivo* is stimulated by a wide range of infectious and inflammatory mediators; these stimulants may have resulted in greater δ isoform dependence on ROS production. The effects of the pan and δ compounds were also similar indicating a possible lesser role for γ. The γ isoform has been shown to be important in neutrophil phagocytosis and reducing parasite entry into cells (470, 471) in knockout mice, therefore although this study did not show a significant reduction in unstimulated ROS release during exacerbations the γ isoform may have a vital role in fighting infections.

The lack of effect seen in sputum neutrophils with the PI3 kinase γ isoform may have been because of the smaller sample size used for this experiment (n=6 compared with n=8 for δ inhibitor). The COPD sputum neutrophil is phenotypically different to the blood neutrophils; it is possible that PI3 kinase enzyme signalling may also be different in the sputum cell, with a larger effect of the δ selective isoform inhibitor on ROS production.

MMP-9 release from COPD neutrophils was not reduced by PI3 kinase γ selective isoform inhibition. Previous studies have demonstrated that pan PI3 kinase enzyme inhibitors diminish MMP-9 release from healthy neutrophils (205, 437), although this has not been a universal finding in COPD neutrophils (214). The mechanisms behind neutrophil degranulation are complex and poorly understood; this study suggests that only δ inhibition is effective in reducing tertiary granule exocytosis and there is a lesser role for γ.
In conclusion ROS but not MMP-9 release from stable and exacerbation COPD neutrophils is suppressed by γ selective PI3 kinase enzyme inhibitors. γ and δ selective inhibition may have specific effects on neutrophilic activation in COPD and may therefore be an attractive future pharmacotherapy for COPD.
6. The effect of LPS priming on fMLP stimulated MMP-9 release from COPD neutrophils

6.1 Introduction

fMLP has been used extensively in the literature to stimulate neutrophil degranulation and ROS release (137, 205, 215). fMLP stimulation activates the PI3 kinase pathway (137, 205). However some publications have primed neutrophils with proteins such as LPS, TNFα and GM-CSF resulting in an augmented response (137, 474, 475). When used alone, these priming agents cause only mild cell stimulation but when the cell is further stimulated they dramatically enhance the response. Possible mechanisms underlying this augmented response include an increase in intracellular calcium (476), increased receptor affinity (477) and activation of tyrosine kinase (478); the exact mechanistic pathway responsible remains unclear. In addition, ROS release from TNFα and LPS primed fMLP stimulated neutrophils has been shown to be P38 MAP kinase enzyme (138, 479) and PI3 kinase enzyme (137, 424) dependent. Some studies suggest that neutrophils in the peripheral circulation of COPD patients are already primed by inflammatory stimuli (156, 190). In human healthy neutrophils, LPS stimulated cytokine production is PI3 kinase enzyme dependent (435) and LPS priming augments fMLP stimulated superoxide and NE production (136, 297, 474).

In previous chapters I have used fMLP alone to stimulate neutrophils and have demonstrated that MMP-9 release is PI3 kinase enzyme dependent. LPS priming prior to fMLP stimulation may activate different cell signalling pathways in the neutrophil to fMLP alone. In a previous study, pan PI3 kinase enzyme inhibitors decreased LPS stimulated primary granule but not secretory vesicle or specific
granule exocytosis (480). In addition pan PI3 kinase enzyme inhibition had no effect on LPS stimulated MMP-9 release (214), suggesting that the mechanisms involved in neutrophil degranulation may be dependent on the stimulus used. The effect of PI3 kinase enzyme inhibitors on LPS primed fMLP stimulated MMP-9 release from neutrophils has not been investigated; this study examined LPS priming in fMLP stimulated COPD blood neutrophils and the effects of PI3 kinase enzyme inhibition on this augmented response.

6.2 Methods

All experiments were carried out with blood neutrophils from patients with stable COPD.

6.2.1 Patient selection

16 patients were recruited from a database of patients held at the Medicines Evaluation Unit, Manchester, UK. Patients were recruited according to the criteria described in section 2.1.1. Patient demographics are summarised in Table 6.1. All patients gave written informed consent. Approval was obtained from the local ethics committee GM East (05/Q1402/41) and North West- Preston (10/H1016/25).

6.2.2 Spirometry, reversibility and health questionnaires

This was carried out as described in section 2.2 and 2.3.

6.2.3 Blood collection and neutrophil isolation

This was carried out as described in section 2.5.

6.2.4 MMP-9 release

Blood neutrophil culture was carried out as described in section 2.5.4.
6.2.4.1 MMP-9 release from neutrophils primed with LPS and stimulated with fMLP; dose response experiment

To examine the dose response effects of LPS neutrophils were incubated with LPS (*Escherichia coli* serotype 026:B6, Sigma-Aldrich), (2.5-250 ng/ml, n=3) or appropriate control (0.01% DMSO) at 37ºC in humidified 5% CO\(_2\) for 30 minutes. After incubation the plates were centrifuged at 2000g for 10 minutes at 4ºC. Cell free supernatants were then harvested and stored immediately at -80ºC for later MMP-9 analysis. The effect of LPS priming followed by fMLP stimulation was also investigated (n=3): neutrophils were incubated with LPS (2.5-250 ng/ml) for 30 minutes. Thereafter fMLP (0.01-1000nM) was added for a further 30 minutes. Plates were centrifuged and supernatants harvested as described above.

6.2.4.2 MMP-9 release from LPS primed followed by fMLP stimulated neutrophils; a time course experiment

A time course experiment was performed to investigate the length of incubation required for dual stimulation (n=3). LPS (25ng/ml) or appropriate control was added to cells; following incubation at 37ºC in humidified 5% CO\(_2\) for 30 minutes fMLP (1000nM) or appropriate control was added. Plates were incubated for 30 minutes, 4 and 24 hours and then centrifuged and supernatants removed as described above.

6.2.4.3 The effect of PI3 kinase enzyme inhibition on MMP-9 release from LPS primed fMLP stimulated blood neutrophils

Isolated neutrophils (n=3) were pre-treated (1 hour) with ZSTK474 (1000nM), GSK045 (1000nM), BIRB796 (1000nM), and dexamethasone (1000nM). Thereafter, cells were stimulated (30 minutes at 37ºC in humidified 5% CO\(_2\)) with LPS (25ng/ml), followed by further stimulation (30 minutes) with fMLP (1000nM).
Plates were centrifuged and supernatants harvested for MMP-9 release as described earlier.

6.2.4.4 The effect of PI3 kinase enzyme inhibition on MMP-9 release from LPS stimulated blood neutrophils

To further understand the effects of LPS, isolated neutrophils (n=10) were pre-treated (1 hour) with ZSTK474 (1000nM), GSK045 (1000nM), GSK723 (1000nM), BIRB796 (1000nM), and dexamethasone (1000nM). Thereafter, cells were stimulated (30 minutes at 37ºC in humidified 5% CO₂) with LPS (100ng/ml). 30 minutes was chosen as this was used for fMLP stimulation and LPS priming followed by fMLP stimulation experiments. Plates were centrifuged and supernatants harvested for MMP-9 analysis as described earlier.

6.2.4.5 Supernatant analysis

MMP-9 was measured in supernatants as described in section 2.6.

6.2.5 Statistical analysis

The effect of the compounds on LPS stimulated MMP-9 release was analysed using the paired t test. All other experiments involved 3 patients and therefore not enough to analyse normality or perform statistics.

6.3 Results

6.3.1 MMP-9 from LPS stimulated neutrophils; dose response curve

LPS (2.5-250 ng/ml) caused a dose dependent increase in MMP-9 release from blood neutrophils (Figure 6.1; n=3). 100 and 250ng/ml LPS increased by 2 and 2.5 fold
respectively, therefore 100ng/ml was chosen for further experiments using LPS alone to stimulate neutrophils.

6.3.2 MMP-9 from LPS primed fMLP stimulated neutrophils

Initial priming with LPS (2.5-250 ng/ml) followed by stimulation with fMLP (0.01-1000nM) caused a dose dependent increase in MMP-9 release from blood neutrophils (see Figure 6.2; n=3). At concentrations of 0-1 nM fMLP, there was little difference in MMP-9 regardless of the LPS concentration used for initial stimulation. fMLP alone (see Figure 4.2) increased MMP-9 production at 10-1000nM, with a dose dependent increase observed. 25ng/ml LPS followed by 1000nM fMLP were therefore chosen for future experiments; these concentrations provided a significant pharmacological window to investigate drug effects.

6.3.3 Time course assessing LPS priming and fMLP stimulation

Unstimulated MMP-9 release was unchanged at 30 minutes, 4 hours and 24 hours (Figure 6.3; n=3). LPS priming followed by fMLP stimulation increased MMP-9 release by approximately 3, 2.5 and 3.5 fold from baseline at 30 minutes, 4 hours and 24 hours respectively. Therefore a stimulation time of 30 minutes was chosen as an optimal time point to measure MMP-9 release from LPS primed fMLP stimulated blood neutrophils.

6.3.4 The effect of PI3 kinase enzyme inhibition on LPS stimulated blood neutrophils

LPS (100ng/ml) increased MMP-9 secretion from COPD peripheral blood neutrophils by approximately 8 fold (Figure 6.4; n=10) (p<0.001). Only BIRB796 caused inhibition of MMP-9 secretion of the order of 19% (p=0.02). ZSTK474, GSK045 and dexamethasone had no significant effect.
6.3.5 The effect of PI3 kinase enzyme inhibition on LPS primed fMLP stimulated blood neutrophils

LPS (25ng/ml) priming followed by fMLP stimulation (1000nM) increased MMP-9 secretion from COPD peripheral blood neutrophils by 4 fold (Figure 6.5; n=3). The compounds had no significant effect on LPS and fMLP stimulated MMP-9 release. Furthermore ZSTK474, GSK045 and dexamethasone appeared to have a stimulatory effect on MMP-9 release although this was not statistically significant.
<table>
<thead>
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<th>Age (years)</th>
<th>64 (56-75)*</th>
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</thead>
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<tr>
<td>Sex (M/F)</td>
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</tr>
<tr>
<td>FEV1 (litres)</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>FEV1 % pred</td>
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<tr>
<td>FEV1/FVC ratio</td>
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<tr>
<td>Pack years</td>
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</tr>
<tr>
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</tr>
<tr>
<td>ICS (Y)</td>
<td>11</td>
</tr>
<tr>
<td>ICS dose (BDP)</td>
<td>1000 (400-2000)*</td>
</tr>
<tr>
<td>LAMA (Y)</td>
<td>7</td>
</tr>
<tr>
<td>LABA (Y)</td>
<td>10</td>
</tr>
<tr>
<td>mMRC score</td>
<td>1.5 ± 1.3</td>
</tr>
</tbody>
</table>

Table 6.1: A summary of patient demographics. All values are expressed as mean ± SD, apart from *, expressed as median (range). BDP: beclomethasone dipropionate equivalent, FEV1: Forced expiratory volume in 1 second post 200μg inhaled salbutamol, FVC: Forced Vital Capacity, ICS: Inhaled corticosteroids, LAMA: Long acting muscarinic antagonist, LABA: Long acting beta agonist, mMRC: Modified Medical Research Council Questionnaire.
Figure 6.1: MMP-9 release from COPD blood neutrophils (n=3) in response to increasing doses of LPS. Mean ± SEM are represented; V: vehicle control (DMSO 0.01%).

Figure 6.2: MMP-9 release from COPD blood neutrophils (n=3) in response to LPS priming (0-250 ng/ml) followed by fMLP stimulation (0-1000nM). Data are expressed as mean; V: vehicle control (DMSO 0.01%).
Figure 6.3: MMP-9 release from COPD blood neutrophils (n=3) after LPS priming (25ng/ml) followed by fMLP stimulation (1000nM) at 30 minutes, 4 hours and 24 hours. Data represent mean ± SEM; V: vehicle control (DMSO 0.01%).
Figure 6.4: The effect of ZSTK474, GSK045, BIRB796 and dexamethasone on LPS stimulated MMP-9 from COPD peripheral neutrophils. Isolated blood neutrophils (n=10) were treated with ZSTK474, GSK045, BIRB796 and dexamethasone (all 1000nM) for 1 hour prior to stimulation with LPS (100ng/ml) for 30 minutes. Supernatants were harvested and analysed for MMP-9 release. Data are expressed as mean ± SEM; V: vehicle control (DMSO 0.02%), S: stimulated value (LPS 100ng/ml). * p<0.05.
Figure 6.5: The effect of ZSTK474, GSK045, BIRB796 and dexamethasone on LPS primed fMLP stimulated MMP-9 from COPD peripheral neutrophils. Isolated blood neutrophils (n=3) were treated with ZSTK474, GSK045, BIRB796 and dexamethasone (all 1000nM) for 1 hour prior to stimulation with LPS (25ng/ml) for 30 minutes followed by fMLP (1000nM) for 30 minutes. Supernatants were harvested and analysed for MMP-9 release. Data are expressed as mean ± SEM; V: vehicle control (DMSO 0.02%).
6.4 Discussion

LPS priming of blood neutrophils augmented fMLP stimulated MMP-9 release. However, in contrast to fMLP stimulation, MMP-9 release in response to LPS stimulation and priming was resistant to the action of PI3 kinase enzyme inhibitors.

A previous study has also demonstrated that LPS stimulated MMP-9 release from COPD neutrophils was not affected by pan PI3 kinase enzyme inhibitors (214). In keeping with these results, in this study pan or δ selective PI3 kinase enzyme inhibition had no significant effect on LPS stimulated MMP-9 release from COPD blood neutrophils. This suggests that signalling via TLR4 leading to neutrophil degranulation is not PI3 kinase enzyme dependent. Pan PI3 kinase enzyme inhibition has been shown to inhibit LPS induced vesicle fusion and phagocytosis in neutrophils (481). However, a further study demonstrated an essential role for the PI3 kinase enzyme in LPS stimulated azurophilic granule release but not in secretory vesicle or specific granule release (480). This suggests a complex hierarchy of degranulation, with separate pathways and signalling mechanisms dependent on the stimulant used. This chapter investigated the effect on MMP-9 release, a component of gelatinase granules; it is therefore conceivable that the PI3 kinase enzyme inhibitors may have significantly reduced LPS stimulated neutrophil elastase, a major constituent of azurophilic granules but had no effect on gelatinase granule exocytosis. This requires further evaluation in experiments examining different endpoints of neutrophil degranulation.

The fMLP receptor is a GPCR that signals through various intracellular pathways including the PI3 kinase and p38 enzymes (468). Studies using neutrophils from healthy subjects have used TNFα and LPS to “prime” the cells before stimulation with fMLP (136-138, 474); the augmented increase in ROS release has been shown
to be PI3 (137, 424) and p38 MAP kinase (138, 479) enzyme dependent. In this study I investigated the effect of LPS priming in fMLP stimulated MMP-9 release. LPS priming increased MMP-9 release and caused the neutrophil to be hyper-responsive to fMLP but this appeared to be resistant to inhibition of the PI3 kinase enzyme. This experiment was however limited by n numbers (n=3). The mechanisms behind priming remain largely unknown, but may be due to mobilisation of intracellular granules allowing more rapid exocytosis; increased fMLP receptor affinity and number (477), increased intracellular calcium (476) and phosphorylation of tyrosine kinases (478) have also been suggested as possible mechanisms. LPS priming may not mobilise gelatinase granules via the PI3 kinase enzyme (480) and therefore the inhibitors had no effect. However, there is evidence that COPD blood neutrophils display increased surface CD11b (190) and other markers of increased activation (156), probably due to inflammatory cytokines present in the blood. Therefore the COPD neutrophil is already primed in the stable state, which may further increase during exacerbations (482); therefore in previous chapters I have used fMLP alone to stimulate neutrophils.

A further reason that the PI3 kinase enzyme inhibitors had no effect on LPS stimulated MMP-9 may be that LPS delays neutrophil apoptosis (140), and this effect is augmented when fMLP is also added (483). LPS and GM-CSF induced delay in apoptosis has been shown to be resistant to Class I PI3 kinase enzyme inhibition (274, 483). However, LPS induced apoptotic delay was reported to be reversed by PI3 kinase enzyme inhibitors (484) in a further study. Therefore the role of the PI3 kinase enzyme in LPS induced prolonged survival appears complex and may be dependent on class II and III enzymes.
p38 MAP kinase enzyme inhibition reduced LPS stimulated MMP-9 release, also in keeping with previous reports suggesting that p38 MAP kinase enzyme activation is necessary for all granule exocytosis (138, 480). The lack of effect seen in LPS primed fMLP stimulated MMP-9 release may have been due to the fact that again it was a limited experiment with only 3 patients.

In conclusion, although LPS priming augmented MMP-9 release from COPD neutrophils in response to fMLP stimulation, LPS primed and stimulated MMP-9 release was resistant to the effect of PI3 kinase enzyme inhibitors. Further investigation is required to examine the mechanisms behind this effect and whether it is a feature of LPS stimulated release of other neutrophil granule proteins.
7. The effect of PI3 kinase enzyme inhibitors on cytokine release from COPD neutrophils

7.1 Introduction
COPD is associated with increased systemic and airway inflammation. There is increased IL-8 in the airway (69, 77) and serum (70) of patients with COPD compared to controls; MIP-1α is also increased in COPD sputum (485). COPD neutrophils release IL-8 and MIP-1α without stimulation (basal release) and in response to inflammatory stimuli (486). Furthermore, peripheral neutrophils from patients with COPD release more IL-8 than those from controls (214).

LPS and TNFα have been widely used in the literature to stimulate cytokine release or inflammatory gene expression from neutrophils (156, 486); in addition these stimulants have also been used to investigate the role of the PI3 kinase enzyme pathway in neutrophil cytokine release (214, 435). TNFα also primes the neutrophil, and leads to upregulation of a number of adhesion molecules and integrins (143, 144). TNFα primed neutrophils also release increased cytokine levels upon full stimulation with opsonized zymosan (487). fMLP, however, has been shown to induce minimal or no cytokine release or expression in neutrophils (155, 156).

In healthy neutrophils, inhibitors of NF-κB and the p38 MAP kinase enzyme pathway have been shown to diminish the release and gene expression of IL-8, TNFα, MIP-1α and MIP-1β (384). However, AP-1 and the JNK pathway appear not to be involved in cytokine release (488). A recent study has demonstrated that pan PI3 kinase enzyme inhibition decreased IL-8, MIP-1α and MIP-1β secretion from healthy neutrophils, with the δ isoform being more important (435).
Neutrophils are regarded as being less glucocorticoid sensitive than other cell types as there is evidence that degranulation and chemotaxis is steroid resistant (205, 489). There are contrasting reports on the effect of corticosteroids on cytokine release from neutrophils. Glucocorticoids have been shown to reduce cytokine release from healthy neutrophils (490) and from COPD peripheral neutrophils (361). In a further study using COPD blood neutrophils, however, IL-8 release was relatively insensitive to dexamethasone and pan PI3 kinase enzyme inhibitors (214). IL-8 release from sputum neutrophils has also been shown to be steroid resistant, due to the airway neutrophil lacking GR (361).

Therefore, there is conflicting evidence regarding the effects of glucocorticoids and PI3 kinase enzyme inhibition on cytokine release from COPD neutrophils. This study investigated the effects of δ isoform selective inhibition on IL-8 and MIP-1α release from COPD blood neutrophils. The effects of the inhibitors on LPS and TNFα stimulated cytokine release and on LPS primed fMLP stimulated cytokine release was examined.

### 7.2 Methods

#### 7.2.1 Patient recruitment

All experiments were performed using blood neutrophils from patients with COPD in the stable state. Patient demographics are summarised in Table 5.1. 15 patients were recruited from a database of patients held at the Medicines Evaluation Unit, Manchester, UK. All patients had a previous diagnosis of COPD, with an FEV1/FVC ratio <70%, age >40 and at least a 10 pack year history of smoking. Patients were excluded if there was a history of active malignancy, asthma or any
inflammatory disease. Patients were recruited only if they had been free from respiratory infections in the preceding 6 weeks. All patients gave written informed consent. Approval was obtained from the local ethics committee GM East (05/Q1402/41) and North West-Preston (10/H1016/25).

7.2.2 Spirometry, reversibility and health questionnaires

This was carried out as described in section 2.2 and 2.3.

7.2.3 Blood collection and neutrophil isolation

This was carried out as described in section 2.5.

7.2.4 Neutrophil cell culture

4 x 10^5 blood neutrophils were seeded in 24 well plates; each condition was performed in triplicate.

7.2.4.1 IL-8 release from neutrophils primed with LPS then stimulated with fMLP; a time course experiment

Blood neutrophils (n=3) were incubated with LPS (25ng/ml) or appropriate control at 37°C in humidified 5% CO_2 for 30 minutes. Thereafter, fMLP (1000nM) or appropriate control (0.01% DMSO) was added. Plates were incubated for 30 minutes, 4 and 24 hours and then centrifuged 2000g for 10 minutes at 4°C. Cell free supernatants were then harvested and stored immediately at -80°C for later IL-8 analysis, as described in section 2.6.1.1.

7.2.4.2 The effect of PI3 kinase enzyme inhibitors on IL-8 and MIP-1α release from LPS primed fMLP stimulated neutrophils

Neutrophils were pretreated (1 hour) with ZSTK474, GSK045 and dexamethasone (all 1000nM) prior to stimulation with LPS (25ng/ml), followed by fMLP (1000nM)
as described in section 5.2.4.1. Plates were incubated for 24 hours and supernatants removed for later IL-8 (n=10) and MIP-1α (n=9) analysis as described in section 2.6.1.1.

7.2.4.3 The effect of PI3 kinase enzyme inhibitors on IL-8 and MIP-1α release from LPS stimulated neutrophils

Neutrophils were pretreated (1 hour) with ZSTK474 (100-1000nM), GSK045 (100-1000nM) and dexamethasone (1000nM) prior to stimulation with LPS (100ng/ml). Plates were incubated for 24 hours and supernatants removed for later IL-8 (n=11) and MIP-1α (n=9) analysis as described in section 2.6.1.1.

7.2.4.4 The effect of PI3 kinase enzyme inhibitors on IL-8 and MIP-1α release from TNFα stimulated neutrophils

Neutrophils were pretreated (1 hour) with ZSTK474 (100-1000nM), GSK045 (100-1000nM) and dexamethasone (1000nM) prior to stimulation with TNFα (10ng/ml; Peprotech, London, UK). Plates were incubated for 24 hours and supernatants removed for later IL-8 (n=10) and MIP-1α (n=9) analysis as described in section 2.6.1.1.

7.2.5 Statistical analysis

The effect of the inhibitors on IL-8 and MIP-1α release from LPS primed fMLP stimulated neutrophils was analysed using the paired students t test. The effects of the inhibitors on IL-8 release and MIP-1α release from LPS only and TNFα only stimulated neutrophils were analysed using the wilcoxon signed rank test as the data was not normally distributed.
7.3 Results

7.3.1 A time course experiment to investigate IL-8 release from neutrophils primed with LPS and stimulated with fMLP

To investigate the most appropriate neutrophil incubation time to analyse IL-8 a time course experiment was performed after initial LPS priming (25ng/ml) followed by fMLP stimulation (1000nM). These concentrations were chosen from previous experiments detailed in chapter 4. Figure 7.1 demonstrates that at 30 minutes there was no detectable IL-8 in unstimulated or stimulated supernatants. Unstimulated IL-8 levels doubled from 4 to 24 hours. LPS (25ng/ml) increased IL-8 secretion by 10 fold at 4 hours and then 70 fold at 24 hours. fMLP alone did not increase IL-8 secretion at any timepoint. Priming with LPS followed by fMLP stimulation increased IL-8 secretion by 30 fold at 4 hours and by 150 fold at 24 hours. Therefore, measuring IL-8 at 24 hours appeared to provide the greatest stimulation window and this was chosen as a timepoint for further experiments.

7.3.2 The effect of PI3 kinase enzyme inhibitors and dexamethasone on IL-8 and MIP-1α from LPS primed fMLP stimulated neutrophils

LPS (25ng/ml) alone or followed by stimulation with fMLP (1000nM) caused a significant increase in IL-8 (n=10) and MIP-1α (n=9) release from COPD blood neutrophils (Figure 7.2; p<0.05). LPS priming followed by fMLP stimulation also significantly increased cytokine release compared to LPS stimulation alone (p=0.002). ZSTK474, GSK045 and dexamethasone significantly decreased LPS primed fMLP stimulated IL-8 release by 66%, 36% and 83% respectively (p<0.001). ZSTK474 and dexamethasone also decreased LPS primed fMLP stimulated MIP-1α release by 57% and 95% respectively (both p=0.004); GSK045 had no significant effect.
<table>
<thead>
<tr>
<th></th>
<th>LPS/fMLP stimulation (n=10)</th>
<th>LPS stimulation (n=11)</th>
<th>TNFα stimulation (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>62 (54-75)*</td>
<td>61 (54-75)*</td>
<td>61 (54-75)*</td>
</tr>
<tr>
<td><strong>Sex (M/F)</strong></td>
<td>9/1</td>
<td>9/2</td>
<td>8/2</td>
</tr>
<tr>
<td><strong>FEV1 (litres)</strong></td>
<td>2.2 ± 0.3</td>
<td>2.2 ± 0.2</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td><strong>FEV1 % pred</strong></td>
<td>66.3 ± 6.7</td>
<td>73.9 ± 5.6</td>
<td>73.4 ± 6.2</td>
</tr>
<tr>
<td><strong>FEV1/FVC ratio</strong></td>
<td>50.7 ± 2.9</td>
<td>55.2 ± 3.0</td>
<td>54.5 ± 3.2</td>
</tr>
<tr>
<td><strong>Pack years</strong></td>
<td>49 (12-84)*</td>
<td>45 (12-100)*</td>
<td>46.3 (12-100)*</td>
</tr>
<tr>
<td><strong>Smoking (Ex/C)</strong></td>
<td>5/5</td>
<td>4/7</td>
<td>3/7</td>
</tr>
<tr>
<td><strong>ICS (Y)</strong></td>
<td>8</td>
<td>8/3</td>
<td>8/2</td>
</tr>
<tr>
<td><strong>ICS dose (BDP)</strong></td>
<td>1000 (400-2000)*</td>
<td>1000 (800-2000)*</td>
<td>1000 (800-2000)*</td>
</tr>
<tr>
<td><strong>LAMA (Y)</strong></td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>LABA (Y)</strong></td>
<td>7</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td><strong>mMRC score</strong></td>
<td>1.3 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.1 ± 0.4</td>
</tr>
</tbody>
</table>

**Table 7.1: A summary of patient demographics.** All values are expressed as mean ± SEM, apart from *, expressed as median (range). BDP: beclomethasone dipropionate equivalent, FEV1: Forced expiratory volume in 1 second post 200µg inhaled salbutamol, FVC: Forced Vital Capacity, ICS: Inhaled corticosteroids, LAMA: Long acting muscarinic antagonist, LABA: Long acting beta agonist, mMRC: Modified Medical Research Council Questionnaire.
Figure 7.1: IL-8 release from COPD blood neutrophils (n=3) after stimulation with LPS (25ng/ml), fMLP (1000nM) or LPS primed followed by fMLP stimulation at 30 minutes, 4 hours and 24 hours. Data represent mean ± SEM; V: vehicle control (DMSO 0.01%).
Figure 7.2: The effect of ZSTK474, GSK045, and dexamethasone on LPS primed followed by fMLP stimulated IL-8 and MIP-1α from COPD peripheral neutrophils. Isolated blood neutrophils were treated with ZSTK474, GSK045, BIRB796 and dexamethasone (all 1000nM) for 1 hour prior to stimulation with LPS (25ng/ml) for 30 minutes followed by fMLP (1000nM) for 30 minutes. Supernatants were harvested and analysed for IL-8 (A; n=10) and MIP-1α (B; n=9) release. Data are expressed as mean ± SEM; V: vehicle control (DMSO 0.02%). * p<0.05, ** p<0.001, *** p<0.0001.
7.3.3 The effect of PI3 kinase enzyme inhibitors and dexamethasone on IL-8 and MIP-1α release from LPS stimulated neutrophils

LPS (100ng/ml) caused a significant increase in IL-8 (n=11; p=0.001) and MIP-1α (n=9; p=0.004) release from neutrophils by 100 and 180 fold respectively (Figure 7.3). 100ng/ml was chosen as a concentration for stimulation based on previous literature (370, 491). 1000nM ZSTK474, GSK045 and dexamethasone all caused significant reduction in LPS stimulated IL-8 release, by 79% (p=0.004), 35% (p=0.004) and 90% (p=0.001) respectively. In addition, 1000nM ZSTK474, GSK045 and dexamethasone all caused significant reduction in MIP-1α secretion, by 62% (0.004), 48% (p=0.008) and 99% (p=0.004) respectively.

7.3.4 The effect of PI3 kinase enzyme inhibitors and dexamethasone on IL-8 and MIP-1α release from TNFα stimulated neutrophils

TNFα (10ng/ml) caused a significant increase in IL-8 (n=10; p=0.002) and MIP-1α (n=9; p=0.004) release from neutrophils by 30 and 70 fold respectively (Figure 7.4). 10ng/ml was chosen as a stimulatory concentration based on previous literature (435). 1000nM ZSTK474, GSK045 and dexamethasone all caused significant inhibition of TNFα stimulated IL-8 release, by 70% (p=0.02), 67% (p=0.02) and 87% (p=0.002) respectively. 1000nM ZSTK474, GSK045 and dexamethasone also significantly reduced MIP-1α release by 63% (p=0.004), 44% (p=0.008) and 72% (p=0.004) respectively.

7.3.5 Summary of compound effects

A summary of the effects of ZSTK474, GSK045 and dexamethasone (1000nM) on IL-8 and MIP-1α release from COPD blood neutrophils is represented in Table 7.2.
Figure 7.3: The effect of ZSTK474, GSK045, and dexamethasone on LPS stimulated IL-8 and MIP-1α from COPD peripheral neutrophils. Isolated blood neutrophils were treated with ZSTK474, GSK045, BIRB796 and dexamethasone (all 1000nM) for 1 hour prior to stimulation with LPS (100ng/ml). Supernatants were harvested and analysed for IL-8 (A; n=11) and MIP-1α (B; n=9) release. Data are expressed as median ± range; V: vehicle control (DMSO 0.01%), S: Stimulated value (LPS 100ng/ml). * p<0.05.
Figure 7.4: The effect of ZSTK474, GSK045, and dexamethasone on TNFα stimulated IL-8 and MIP-1α from COPD peripheral neutrophils. Isolated blood neutrophils were treated with ZSTK474, GSK045, BIRB796 and dexamethasone (all 1000nM) for 1 hour prior to stimulation with TNFα (10ng/ml). Supernatants were harvested and analysed for IL-8 (A; n=10) and MIP-1α (B; n=9) release. Data are expressed as median ± range; V: vehicle control (DMSO 0.01%), S: Stimulated value (TNFα 10ng/ml). * p<0.05.
### Table 7.2: Percentage inhibition (1000nM)

<table>
<thead>
<tr>
<th></th>
<th>ZSTK474 (PI3 pan)</th>
<th>GSK045 (PI3 δ)</th>
<th>Dex (Glucocorticoid)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-8</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS (n=10)</td>
<td>65.9 ± 14.9 (p=0.0009)</td>
<td>36.1 ± 13.6 (p=0.0007)</td>
<td>82.6 ± 14.6 (p&lt;0.0001)</td>
</tr>
<tr>
<td>MIP-1α (n=9)</td>
<td>59.7 ± 3.1 (p=0.004)</td>
<td>-</td>
<td>95.0 ± 34.2 (p=0.004)</td>
</tr>
<tr>
<td><strong>IL-8</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS (n=11)</td>
<td>78.5 (50.9-97.7) (p=0.004)</td>
<td>35.2 (11.2-90.4) (p=0.004)</td>
<td>90.1 (80.1-99.2) (p=0.001)</td>
</tr>
<tr>
<td>MIP-1α (n=9)</td>
<td>62.0 (26.0-80.2) (p=0.004)</td>
<td>47.8 (0.78.7) (p=0.008)</td>
<td>98.6 (95.0-100) (p=0.004)</td>
</tr>
<tr>
<td><strong>IL-8</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS (n=11)</td>
<td>69.8 (4.6-91.8) (p=0.02)</td>
<td>67.1 (5.5-81.7) (p=0.02)</td>
<td>87.2 (55.6-100) (p=0.002)</td>
</tr>
<tr>
<td>MIP-1α (n=9)</td>
<td>62.6 (14.0-100) (p=0.004)</td>
<td>43.6 (0-70.7) (p=0.008)</td>
<td>71.7 (57.9-100) (p=0.004)</td>
</tr>
</tbody>
</table>

**Legend:** LPS primed/fMLP stimulated, LPS stimulated or TNF α stimulated as indicated. Data are presented as mean ± SD apart from *, represented as median (range).
7.4 Discussion

This study has demonstrated that cytokine release from COPD neutrophils is sensitive to the action of dexamethasone and PI3 kinase enzyme inhibitors; the magnitude of this effect was dependent on the stimulus used.

In contrast to MMP-9 and ROS release, the overall inhibitory effect was greater with dexamethasone than the PI3 kinase enzyme inhibitors. Previous studies have demonstrated conflicting results with glucocorticoid effects on IL-8 release from COPD blood neutrophils; one study reported significant inhibition (361), however another reported relative resistance (214). This discrepancy is difficult to explain as both studies used the same stimulus, LPS, both used the same concentrations of dexamethasone (1000nM) and both were investigating COPD peripheral neutrophils. Both studies also used similar severities of COPD (GOLD stage II). The study by Milara et al involved only 3 patients (214) and the study by Plumb et al involved 7 (361) and therefore used only modest sample sizes. In this chapter significant glucocorticoid induced suppression of IL-8 and MIP-1α has been demonstrated in 15 patients and therefore is the largest study to date. IL-8 release from COPD sputum neutrophils (361) and alveolar macrophages (356) has been shown to be steroid insensitive; thus steroid insensitivity may be cell and gene specific (357).

PI3 kinase enzyme inhibitors significantly reduced IL-8 and MIP-1α release from COPD neutrophils and overall the effect of the pan inhibitor was greater than the effect of the δ selective inhibitor. Previous studies investigating the effects of pan PI3 kinase enzyme inhibition on blood neutrophil cytokine release have again revealed conflicting results; one study showed that pan inhibition almost completely inhibited IL-8, MIP-1α and MIP-1β release from healthy neutrophils (435) but a further study showed no significant effect on IL-8 release from COPD neutrophils
The difference between these studies may have been because of the use of COPD patients in the negative study, or again due to the numbers of patients used; both studies used only 3 patients (214, 435).

Potential mechanisms by which PI3 kinase enzyme inhibition diminished cytokine release include inhibition of S6 kinase phosphorylation (435), a downstream target of which is S6 ribosomal protein. Fortin et al also demonstrated that pan PI3 kinase enzyme inhibitors significantly reduced LPS or TNFα stimulated MIP-1α and MIP-1β transcripts but had no effect on IL-8 mRNA (435). Accumulation of inducible mRNA in neutrophils has been shown to be due to increased gene transcription and not increased mRNA stability (492). Therefore there may be a potential role of the PI3 kinase enzyme in protein transcription, specifically in the case of MIP-1α and MIP-1β. The mechanism behind the PI3 kinase enzyme controlling IL-8 protein production remains unclear and further studies are required.

Selective δ inhibition had significant effect on cytokine release whilst γ inhibition had no effect in the study by Fortin et al (493). The δ isoform has also been shown to be important in cytokine release from B and T lymphocytes (494). In this chapter the effect of pan inhibitors was greater than the effect of the δ selective inhibitor indicating a possible role for other isoforms. In cell line experiments, a potential role for the α isoform was also identified (435); the pIC50 value of the pan inhibitor for the α isoform was 7.4M, identical to that for the γ isoform. Therefore part of the difference between pan and δ inhibitors may have been mediated by the α subunit. The addition of other isoform selective inhibitors would help clarify this issue.

Importantly, the δ selective inhibitor had no significant effect on LPS primed fMLP stimulated cytokine release. As demonstrated in a previous chapter investigating
MMP-9 release, this suggests that priming the neutrophil with LPS and then stimulating with fMLP may result in differential cell signalling pathways leading to cytokine release. This study suggests that this process is dependent on the PI3 kinase enzyme but not the δ isoform; therefore other isoforms may be involved and further investigation is required using other isoform selective inhibitors.

In keeping with previous literature, LPS and TNFα induced a significant increase in IL-8 and MIP-1α from neutrophils (435, 486); there is also evidence that LPS and TNFα induce inflammatory gene expression in human neutrophils (156, 435). TNFα stimulation has been shown to activate the PI3 kinase enzyme pathway (435, 495). TNFα acts upon the TNF receptors TNF1 and TNF2 (496), leading to eventual activation of the JNK and NF-κB pathways and increased inflammatory cytokine production (384). The PI3 kinase enzyme is also involved in this process (497), as selective δ PI3 kinase isoform inhibition was shown to inhibit NF-κB activation in TNFα stimulated neutrophils. LPS acts via TLR4 to increase inflammatory gene transcription in neutrophils (300); TLR signalling in neutrophils has also been shown to be dependent on the PI3 kinase enzyme (484, 498).

In contrast to MMP-9 and ROS release from neutrophils, fMLP did not stimulate increased cytokine release. Other studies have also demonstrated this effect, with fMLP stimulation having modest or no effect on cytokine release (155) or gene expression (156). This suggests that differential neutrophil activation is stimulus specific and that perhaps fMLP increases chemotactic (196) and bactericidal activity (137) rather than cytokine production. Interestingly, initial priming of the neutrophil with LPS lead to significantly increased cytokine release upon stimulation with fMLP. It has been demonstrated that TNFα primed neutrophils release more cytokines upon stimulation with opsonized bacteria than unprimed cells (487), but
this is the first study to show this effect in LPS primed fMLP stimulated cells. This enhanced response was also diminished by PI3 kinase enzyme inhibitors. Possible mechanisms behind this may be that LPS and fMLP synergistically stimulate the neutrophil leading to enhanced cytokine release (499) and perhaps there is cross-talk between TLR4 and the fMLP receptor, as there is evidence of a link between TLR receptors and GPCR in macrophages (500). These mechanisms require further evaluation in neutrophils.

Limitations of this study include the fact that the effects of PI3 kinase enzyme inhibitors on cytokine release from COPD airway cells were not investigated. Airway neutrophils lack GR (361) and they are devoid of phospho-p38 (370). Also, p38 inhibitors and glucocorticoids have minimal effect on cytokine release from sputum neutrophils (361, 370). In a previous chapter, PI3 kinase enzyme inhibitors significantly reduced ROS release from COPD sputum neutrophils; therefore PI3 kinase enzyme inhibition may have also diminished cytokine release. In addition, I did not investigate the effects of the inhibitors on cytokine release from neutrophils during COPD exacerbations. This is due to the fact I was limited in the number of cells I could obtain from patients who were clinically unwell. Therefore I prioritised investigating ROS and MMP-9 release during exacerbations as they have a significant role in COPD pathogenesis (465).

In conclusion, cytokine release from COPD peripheral neutrophils is inhibited by glucocorticoids and by PI3 kinase enzyme inhibitors; more research is required to investigate the specific isoforms and mechanisms involved. Thus this provides further evidence for the development of PI3 kinase enzyme inhibitors in COPD to target specific markers of neutrophilic inflammation.
8. Discussion

COPD is characterised by increased neutrophilic inflammation, which further increases during exacerbations. Currently corticosteroids are the mainstay of treatment but there is a degree of steroid resistance associated with COPD. Novel pharmacotherapies are urgently required for clinical use.

This thesis investigated the effect of inhibition of the PI3 kinase enzyme pathway on the neutrophilic inflammation associated with COPD in vitro. Furthermore, the utility of inhaled LPS in patients with COPD as a model of neutrophilic inflammation has been evaluated in vivo. The key findings of the studies described are now discussed together with possible future areas of investigation.

8.1 LPS inhalation in patients with COPD; a model of COPD exacerbation?

Inhaled LPS, when administered to patients with mild to moderate COPD, caused an increase in airway inflammation with a significant fall in FEV1 and a rise in sputum neutrophil numbers. There was also associated increased systemic inflammation with elevated CRP, IL-6 and CC-16 levels, each with differing temporal patterns. Importantly, none of the patients reported any major side effects. Thus inhaled LPS in patients with mild to moderate COPD may be a safe model of neutrophilic inflammation.

The effects seen in this study mirrors changes seen in healthy patients (322), asthmatics (319) and healthy smokers (316) after LPS inhalation. In addition, the increased neutrophilic inflammation resembles that observed in COPD exacerbations.
(264) demonstrating the potential use in modelling acute on chronic neutrophilic inflammation.

Inhaled LPS has been used as a model to evaluate anti-inflammatory therapies in healthy patients (321, 322). Other models of neutrophilic inflammation exist including inhaled ozone and experimental rhinovirus infection; inhaled ozone has been used to investigate CXCR2 (462, 463) and selectin (461) antagonists in healthy individuals. Inhaled LPS in patients with COPD could be used to test new therapies in vivo that may be effective treatments for exacerbations.

Recently experimental rhinovirus infection has been used to model viral exacerbations in patients with COPD (247). Antibiotics are currently prescribed for most exacerbations of COPD (16), although the underlying precipitant of the exacerbation may be viral or related to pollution and bacteria may only be responsible for up to 50% of acute exacerbations (235). A biomarker which could differentiate between viral and bacterial exacerbations would allow rapid identification of patients most likely to benefit from antibiotics. LPS inhalation could therefore be used to investigate potential biomarkers of bacterial infection to use in this way.

One of the limitations of using inhaled LPS is the wide range of responses seen in healthy volunteers (312, 329) and in asthmatics (312). This effect was also seen in this study of COPD patients; possible explanations include the effect of cigarette smoking (451), differences in the quantity of LPS inhaled, LPS tolerance (450) and polymorphisms in CD14 (452). To completely validate this model as a tool to investigate pharmacotherapies, larger studies are required and perhaps using patients not on inhaled corticosteroids and who are ex-smokers thereby counteracting any
confounding effects. Furthermore, larger studies investigating the reproducibility of LPS challenges in COPD patients are required. The model has been shown to be reproducible in healthy individuals (329) and in healthy smokers (316), but this needs to be confirmed in patients with COPD.

Although increased neutrophilic inflammation was observed, there was no increase in some neutrophil associated markers such as sputum IL-8 and NE which have been associated with COPD exacerbations (94, 235). Perhaps higher doses of LPS would have led to a significant increase in these markers. However, the ethical approval for the study stated that 5µg doses would initially be used followed by 30µg doses and given the large fall in FEV1 observed in some patients it was felt unsafe to proceed to the higher dose. Further studies are required with higher doses of LPS: 15µg could be used in patients whose FEV1 fell by <12% after 5µg to ensure safety.

To further validate this model of exacerbation peripheral neutrophils could be isolated at baseline and after LPS inhalation and MMP-9 and ROS release investigated. As was seen in exacerbating patients, there may be increased MMP-9 and other granule proteins released from the neutrophil post LPS challenge. In addition the effects of PI3 kinase enzyme inhibitors on neutrophil activation markers post LPS challenge could be examined in vitro. There was no observed effect of PI3 kinase enzyme inhibition on LPS stimulated MMP-9 release in keeping with previous literature (214), therefore the effects of PI3 kinase enzyme inhibition on other granule contents could be examined, for example NE. An inhaled PI3 kinase pan enzyme inhibitor has been shown to reduce the neutrophilic inflammation associated with intra-nasally administered LPS in a mouse model of COPD (467). Thus, in the future the utility of PI3 kinase enzyme inhibitors in exacerbations could also be investigated in COPD patients in vivo using the inhaled LPS model.
Inhaled LPS in patients with COPD could also be used to investigate the effects of corticosteroids on the acute on chronic neutrophilic inflammation seen in COPD exacerbations. Roflumilast and cilomilast are new commercially available phosphodiesterase 4 (PDE4) inhibitors, and are currently used to reduce the risk of exacerbations in patients with severe COPD with chronic bronchitis and a history of exacerbations (501). In addition, there is evidence that treatment with roflumilast results in patients switching from the frequent exacerbator phenotype to the infrequent exacerbator state (502). Thus, it would be interesting to examine the role of PDE4 inhibition during exacerbations; the LPS inhalation model could be used in this way.

8.2 Do PI3 kinase enzyme inhibitors reduce neutrophilic inflammation in COPD?

There is evidence that the PI3 kinase enzyme pathway is upregulated in COPD, with alveolar macrophages (369) and peripheral neutrophils (214) from patients with COPD expressing increased levels of the δ isoform. In addition, peripheral neutrophils express higher levels of PIP₃ (214). Pan PI3 kinase enzyme inhibition reverses the reduced migratory accuracy (196) in COPD neutrophils and inhibits MMP-9 and NE release (205) in healthy neutrophils. Cytokine release and ROS release from healthy neutrophils are also diminished by PI3 kinase enzyme inhibitors (137, 435).

In this thesis I observed that MMP-9 and ROS release from COPD neutrophils in the stable state and during exacerbations were significantly reduced by PI3 kinase enzyme inhibition; overall, the δ selective inhibitor had the greatest inhibitory effect
on both MMP-9 and ROS. As described, increased expression of the δ isoform in COPD neutrophils and alveolar macrophages has been demonstrated (214, 369); in addition, δ isoform selective inhibition reverses the glucocorticoid insensitivity in COPD blood monocytes (364). There is also evidence for a role for the δ isoform in monocyte adhesion and recruitment into the lungs (503). Thus, this thesis adds further support to the targeting of the δ isoform in COPD as an anti-inflammatory treatment.

I observed significant inhibition on ROS release with the PI3 kinase γ isoform inhibitor; this is in keeping with previous literature demonstrating γ dependent ROS release (137, 423). The lack of effect of the PI3 kinase γ isoform seen in MMP-9 release may have been due to the fewer patient numbers used in these experiments compared with the δ and pan inhibitor. Therefore in future work it would be important to increase the sample size to fully determine the role of the γ inhibitor.

PI3 kinase enzyme inhibition also significantly diminished cytokine release, the effects of the δ inhibitor appeared less than in MMP-9 and ROS release. The effects of the γ inhibitor were not investigated in this model. Previous studies in healthy neutrophils have demonstrated a predominant role of the δ and α isoforms in cytokine release (435). This is the first study to demonstrate a role for the PI3 kinase enzyme in cytokine release from COPD blood neutrophils. Future work should examine the effects of other PI3 kinase enzyme isoform inhibitors on cytokine release from COPD blood neutrophils. Although neutrophils are important sources of cytokines, the relative role of this cell release in the pathogenesis of COPD is not fully clear as many other immune cells also release numerous cytokines, and neutrophils release relatively small amounts of cytokine in vitro compared to other cells. Therefore targeting MMP-9 and ROS release from neutrophils may be more
important than targeting neutrophil cytokine release in the development of novel anti-inflammatory therapies.

The neutrophil has a vital role in antimicrobial responses. These cells interact with microbial ligands to initiate a number of responses including phagocytosis, degranulation and the release of oxidants, proteinases and other cytotoxic products. In COPD, these functions also contribute to tissue damage (465). To non-specifically inhibit all neutrophil functions may however leave the host vulnerable to infection. Therefore targeting specific functions and granule contents may be a more realistic way to develop anti-neutrophil therapies whilst not interfering with susceptibility to infection; PI3 kinase isoform specific inhibitors may be one way to approach this. There is evidence that pathways driving tertiary granule release differ from those behind primary and secondary granules (205, 504). Further examination of the effect of PI3 kinase enzyme inhibition on other neutrophil functions is therefore required, for example the effects on NE, lactoferrin and secretory vesicle release and the effects on phagocytosis or NET release. Each neutrophil function may have a specific PI3 kinase enzyme isoform underlying it and therefore selective inhibition may be possible, minimising side effects.

In addition to investigating the effects of PI3 kinase enzyme inhibitors on other neutrophil functions in blood neutrophils, the effects in sputum cells should also be examined. The effect of the inhibitory compounds on MMP-9 release and other granule proteins from sputum cells could be evaluated. Furthermore, the effects of PI3 kinase enzyme inhibitors on MMP-9 and ROS release from sputum neutrophils from exacerbating COPD patients should be investigated.
PI3 kinase isoforms have also been implicated in other inflammatory disease. In rheumatoid arthritis there is increased γ expression in the synovial fluid (505), and pan enzyme inhibition has been shown to diminish osteoclast generation in mice (506). Both δ and γ isoforms have been shown to contribute to the pathogenesis of experimental models of multiple sclerosis (507). In addition, an aerosolized pan inhibitor has been shown to reduce airway inflammation in models of asthma (467). Thus, as further understanding is gained regarding the crystal structure of specific isoforms, selective isoform inhibitors could be developed to target these isoforms for use not only in COPD but in other inflammatory disorders.

Although I observed significant inhibitory effects with the PI3 kinase inhibitory compounds, in order to fully attribute neutrophil functions to specific isoforms I would need to initially detect the specific isoforms in neutrophils and then examine the effect of the selective inhibitors on these isoforms and other downstream proteins. There are a number of isoform specific antibodies currently available to investigate specific isoforms using flow cytometry and immunohistochemistry.

A potential flaw in studying neutrophils in this way is that the process of isolation has been shown to prime them and therefore stimulate increased activation (508). Other techniques using whole blood to investigate surface markers such as CD63 and CD66b, markers of primary and secondary granule release respectively, have been developed (508, 509). However, in this thesis all neutrophils were isolated using the same method and therefore comparisons have been made between the effects of the compound on the cells, all of which will have been activated in the same way.
8.3 What mechanisms may underlie the effects of the PI3 kinase enzyme inhibitors in neutrophils?

The mechanisms behind neutrophil degranulation are complex and unclear. In this thesis I demonstrated that PI3 kinase enzyme inhibitors decreased fMLP stimulated MMP-9 release from COPD neutrophils both in the stable state and during exacerbations. Granule exocytosis is dependent on a number of steps: initially actin cytoskeleton remodelling and microtubule assembly recruits granules from the cytoplasm to the cell membrane (510). The vesicle then tethers and docks resulting in the outer surface of the lipid bilayer of the cell membrane coming into contact with the inner surface of the cell membrane. The granules then fuse with the cell membrane by developing a fusion pore between the granule and the target membrane.

Intracellular calcium is increased during granule exocytosis (157), along with ATP and guanosine triphosphate (GTP) hydrolysis; these processes are dependent on effector molecules such as calcium and GTP binding proteins, although the specific nature of these protein has not yet been determined. The fMLP receptor is a G protein coupled receptor which is known to increase intracellular calcium. G protein coupled receptors are known to stimulate the PI3 kinase γ isoform, therefore implicating the PI3 kinase enzyme pathway in degranulation (392). In addition fMLP stimulated tyrosine kinases, in particular the src family, have been shown to be implicated in neutrophil exocytosis (511). Tyrosine kinases are known to activate the δ isoform (321), again implicating the PI3 kinase enzyme pathway. Rac is a member of the GTPase family and consists of 3 different isoforms: Rac1, Rac2 and Rac3. In addition to its role in activating NADPH and respiratory burst (164), there is also evidence that Rac2 has a crucial role in neutrophil degranulation (512). fMLP
stimulated Rac2 activation is diminished by PI3 kinase enzyme inhibitors in human neutrophils (513), suggesting another possible mechanism by which the PI3 kinase enzyme is responsible for degranulation.

A final possible mechanism by which the PI3 kinase enzyme may play a role in neutrophil degranulation may be via soluble-N-ethylmaleimide sensitive accessory-protein receptors (SNARES). SNARE molecules are thought to facilitate binding of neutrophil granules to the cell membrane (514). PI3 kinase enzyme inhibition has been shown to reduce fMLP stimulated SNARE protein phosphorylation and degranulation in rat mast cells; these effects in neutrophils have not yet been investigated but it is plausible that the PI3 kinase enzyme pathway is also involved in SNARE protein phosphorylation in human neutrophils.

The hierarchical manner in which neutrophils degranulate appears to be under differential mechanistic controls; selective recruitment of src kinases has been shown to be responsible for primary and secondary granule release (511, 515). In addition LPS stimulated degranulation has been shown to be dependent on the PI3 kinase enzyme for primary granule release only, whereas the P38 MAP kinase enzyme pathway was required for the mobilisation of all granules (480). Thus, as previously suggested in future work it would be important to determine the effects of the PI3 kinase enzyme inhibitors on other granule proteins.

The mechanisms behind PI3K enzyme activation causing ROS release from neutrophils may be three fold: phosphorylation of p47phox via phosphoinositide dependent kinases (425), direct interaction of cytosolic subunits with phosphoinositides (426) or by activation of Rac (427). NADPH oxidase is composed
of the membrane protein cytochrome b$_{558}$ and the 4 cytosolic proteins p47$^{\text{phox}}$, p67$^{\text{phox}}$, p40$^{\text{phox}}$ and Rac2 which assemble on cell membranes upon activation.

p47$^{\text{phox}}$ in the resting cell is in the cytoplasm and held in an auto-inhibited state (428). In vitro, phospho-Akt phosphorylates p47$^{\text{phox}}$ in human neutrophils stimulated by fMLP or by bacterial phagocytosis; this occurs at Ser304 and Ser328 (425, 430). Inhibition of Akt decreases ROS release in response to fMLP (425). 2 different isoforms of Akt have been demonstrated in neutrophils, and it is Akt2 which appears to be associated with p47$^{\text{phox}}$ phosphorylation and NADPH assembly (431). Thus, the stimulation of the PI3 kinase enzyme pathway may be critical in p47$^{\text{phox}}$ phosphorylation. In this thesis in an experiment using western blotting in a single COPD patient, there was a suggestion that fMLP stimulation increased p47$^{\text{phox}}$ phosphorylation in blood neutrophils. This experiment requires further optimisation, but if fMLP stimulated p47$^{\text{phox}}$ phosphorylation could be reliably identified in blood neutrophils, the next step would be to investigate whether the addition of PI3 kinase enzyme inhibitors decreases this phosphorylation.

p47$^{\text{phox}}$ has a crucial role in host defence as knockout mice develop lethal infections and develop granulomatous inflammation (434). There is also increased expression of phosphorylated p47$^{\text{phox}}$ in synovial fluid neutrophils from patients with rheumatoid arthritis (433), indicating a potential role in the associated inflammatory process. Thus phosphorylated p47$^{\text{phox}}$ expression may be increased in the neutrophils of COPD patients compared to controls; this may further be increased during exacerbations. This would be an interesting area for future investigation. In addition, MMP-9 has been shown to be correlated with neutrophil numbers and disease severity (205) so perhaps levels of phosphorylated p47$^{\text{phox}}$ may also correlate with FEV1.
There is limited information available on the mechanisms driving COPD exacerbations. NF-κB has been implicated, with increased nuclear p65 detected in sputum macrophages but not neutrophils during exacerbations (453, 516). In addition, PI3 kinase enzyme inhibition does not reverse the reduced neutrophil apoptosis seen in exacerbations (274). In this thesis I have shown that PI3 kinase enzyme inhibition suppresses both basal and stimulated MMP-9 and ROS release from blood neutrophils during exacerbations and this is the first study to demonstrate a potential role for PI3 kinase enzyme inhibitors for COPD exacerbation treatment.

The mechanisms by which PI3 kinase enzyme inhibitors decrease cytokine release from neutrophils may be by inhibition of the phosphorylation of S6 kinase and therefore the S6 ribosomal protein (435). The S6 ribosomal protein is involved in regulating protein translation and is responsible for many cell processes including cell proliferation and growth. The exact translational targets of the PI3 kinase enzyme are yet to be elucidated. Furthermore in this thesis I investigated the effects of PI3 kinase enzyme inhibitors on LPS and TNFα stimulated cytokine release; the PI3 kinase enzyme has been implicated in LPS and TNFα mediated cell signalling in neutrophils, via TLR4 and the TNFR respectively (484, 497).

P38 MAP kinase enzyme inhibition significantly reduced fMLP and LPS stimulated MMP-9 release from COPD neutrophils, in keeping with previous literature (205, 385, 437, 480). There is evidence that the PI3 kinase and P38 MAP kinase enzyme pathways interact in neutrophils; phosphorylation of Akt has been shown to be dependent on P38 MAP kinase (517). In addition the PI3 kinase enzyme may act downstream of the P38 MAP kinase enzyme in LPS or TNFα stimulated cytokine release (435). Although I did not investigate the effects of P38 MAP kinase enzyme inhibition on cytokine release, it has been demonstrated that selective MAP kinase α
and β inhibition significantly reduces cytokine release from COPD blood neutrophils (370). Therefore perhaps the effects of P38 MAP kinase enzyme inhibition are mediated partly by downstream inhibition of the PI3 kinase enzyme pathway.

8.4 What is the importance of neutrophil priming?

Some publications have used proteins such as TNFα, LPS, IL-8 and GM-CSF to prime neutrophils prior to stimulation with fMLP (137, 474, 475) resulting in an augmented response. In keeping with this previous literature I observed that LPS priming significantly augmented fMLP stimulated MMP-9 and cytokine release from COPD neutrophils. However, in contrast to fMLP only stimulated MMP-9 release, LPS priming and stimulation was resistant to PI3 kinase enzyme inhibition as previously reported (214). In a previous study, PI3 kinase enzyme inhibitors decreased LPS stimulated primary granule but not other granule exocytosis. IL-8 mediated MMP-9 release is also dependent on different pathways for primary and tertiary granule release (504). Thus neutrophil degranulation is complex and is dependent on a number of stimulant specific, granule specific signalling pathways and LPS priming may stimulate a cell signalling cascade independent of the PI3 kinase enzyme.

Although pan and δ selective PI3 kinase enzyme inhibitors diminished LPS and TNFα stimulated cytokine release, only pan inhibitors significantly reduced cytokine release from LPS primed fMLP stimulated cells. Again this may be due to differential cell signalling pathways; LPS primed fMLP stimulated neutrophil cytokine release may be dependent on other PI3 kinase enzyme isoforms such as γ or α.
COPD neutrophils may already be primed in the circulation (156, 189); these priming markers are further increased during exacerbations (482). Therefore, it can be argued that in vitro priming of COPD neutrophils is not necessary. These differential effects of LPS and fMLP on cell signalling have been observed in vitro, however their significance in vivo is not established: the circulating neutrophil in COPD is likely to be exposed to a number of inflammatory mediators at the same time each activating multiple intracellular pathways. Nevertheless, I have observed that PI3 kinase enzyme inhibitors significantly reduce MMP-9, ROS and cytokine release from COPD neutrophils and are therefore a promising future therapy to target neutrophilic inflammation.

8.5 What are the effects of corticosteroids on neutrophil activation in COPD?

In this thesis I have demonstrated that MMP-9 and ROS release from COPD neutrophils appears to be relatively resistant to the action of glucocorticoids, however cytokine release is glucocorticoid sensitive. Therefore the effects of corticosteroids appear to be specific for each neutrophil function.

Glucocorticoids bind to the GR in the cytoplasm, this complex then translocates to the nucleus and interferes with the action of pro-inflammatory transcription factors such as NF-κB therefore preventing pro-inflammatory gene transcription; this process in known as transrepression. Clinical trials have shown that patients with severe COPD and frequent exacerbations are most likely to benefit from inhaled corticosteroids (352, 353). However, there is a degree of steroid resistance associated with COPD, as inhaled corticosteroids have been shown not to affect the airway inflammation associated with COPD (350). Furthermore, IL-8 release from COPD
alveolar macrophages is resistant to the action of dexamethasone (356, 357). Various mechanisms of steroid resistance have been suggested including a reduction of HDAC2 caused by oxidative stress (366) and overexpression of GRβ (360). Furthermore, COPD airway neutrophils do not express GR and therefore may be phenotypically different from peripheral neutrophils (361).

In keeping with previous literature COPD neutrophil MMP-9 release was resistant to the action of dexamethasone (205). In addition a further study demonstrated that fluticasone propionate did not affect MMP-9 or ROS release from healthy neutrophils exposed to cigarette smoke (518). These results suggest that the mechanisms underlying neutrophil degranulation and respiratory burst are steroid resistant. A possible explanation is that unlike cytokines, the release of granule products and ROS is not dependent on gene transcription and is therefore unaffected by transrepression. Granule proteins are already synthesised and present in vesicles stored in the neutrophil; upon cell activation it is the mechanisms driving granule exocytosis that control granule protein release.

Cytokine release from neutrophils was sensitive to the action of dexamethasone, with dexamethasone causing around 90% inhibition of stimulated cytokine release. IL-8 release from alveolar macrophages has been reported to be relatively steroid resistant (356, 357); furthermore release of GM-CSF, G-CSF and IL-8 even from healthy macrophages was insensitive to glucocorticoids (357). In contrast, TNFα and IL-6 release in all patient groups was dexamethasone sensitive indicating that there may be some glucocorticoid insensitive and sensitive cytokines in macrophages. There may be some genes that are regulated by glucocorticoid independent transcription mechanisms. This differential response to corticosteroids may also be cell specific, explaining the results seen in COPD neutrophils. IL-8 release from peripheral blood
neutrophils has previously been demonstrated to be sensitive to dexamethasone (361). Thus, the IL-8 gene transcription in neutrophils may be regulated by steroid sensitive mechanisms involving transrepression. To clarify the role of glucocorticoids in cytokine release perhaps a further array of cytokines needs to be investigated. In addition it would be prudent to examine the effects of corticosteroids on gene transcription by detecting changes in mRNA expression.

Glucocorticoids have significant systemic side effects such as cataracts, diabetes and osteoporosis (519, 520) and therefore their use should be limited to those patients whom obtain most benefit. In addition although systemic corticosteroids in exacerbations have been shown to improve outcomes (251, 355), recent studies suggest that it is patients with eosinophilic exacerbations who gain the most clinical benefit (255, 256). Thus alternative therapies are required for patients with non-eosinophilic exacerbations; perhaps PI3 kinase enzyme inhibition could be used to target neutrophilic inflammation. Further larger studies are required investigating potential biomarkers and phenotypes of exacerbation in order to develop new targeted pharmacotherapies.

Previous studies have investigated the combined effects of glucocorticoids and other anti-inflammatory compounds. I did not do this in this thesis but it would be an important area for further investigation. Selective inhibition of PI3 kinase δ isoform has been shown to reverse the steroid insensitivity associated with IL-8 release from COPD blood monocytes (369), possibly by a restoration in HDAC2 (364). Also, the combination of a P38 MAP kinase enzyme inhibitor and dexamethasone synergistically reduced the release of inflammatory cytokines from COPD alveolar macrophages (381). Therefore, the addition of a PI3 kinase enzyme inhibitor with
glucocorticoid may reverse the steroid resistance seen in COPD and allow lower doses of steroid to be used, minimising side effects.

8.6 What are the potential pitfalls of PI3 kinase enzyme inhibition?

Studies using knockout mice have demonstrated that animals devoid of the PI3 kinase γ isoform have a reduced host response to *Streptococcus pneumoniae* infection (521). Furthermore, mice deficient in the δ isoform have reduced B cell development and activation (402, 522). In addition, T regulatory cells are retained in the thymus of δ deficient mice; T regulatory cells have an important role in controlling immune responses (523). Therefore in COPD these effects may leave the host more susceptible to bacterial and viral infection and further exacerbations.

Thus, further understanding of the exact crystal structure of PI3 kinase enzyme isoforms is required and the development of highly selective inhibitors to target neutrophilic inflammation whilst minimising unwanted effects. In COPD, local administration into the lungs i.e. via the inhaled route may also be a solution to overcome systemic side effects.

8.6 Final conclusions

I have conducted a number of experiments pharmacologically investigating neutrophilic inflammation in COPD, both *in vivo* and *in vitro*. From the work presented in this thesis, the following are the main conclusions which can be drawn:

- Inhaled LPS at low dose (5μg) administered to patients with COPD increases neutrophilic airway inflammation and associated systemic inflammation and
therefore resembles COPD exacerbations. Therefore this may be used as a model of exacerbation in which to test novel pharmacotherapies in vivo.

- fMLP stimulated MMP-9 and ROS release from stable and exacerbation COPD neutrophils is suppressed by PI3 kinase enzyme inhibitors. In addition, unstimulated MMP-9 and ROS release from exacerbation COPD neutrophils is also suppressed. In particular, PI3Kδ specific inhibition is a promising therapy to target glucocorticoid insensitive MMP-9 and ROS secretion from COPD neutrophils.

- ROS but not MMP-9 release from stable and exacerbation COPD neutrophils is suppressed by γ isoform selective PI3 kinase inhibitors. These experiments were limited by small patient numbers.

- LPS priming augments MMP-9 release from COPD neutrophils in response to fMLP stimulation. LPS primed and stimulated MMP-9 release is resistant to the effect of PI3 kinase enzyme inhibitors, suggesting that LPS priming results in differential cell signalling pathways independent of the PI3 kinase enzyme.

- Cytokine release from COPD peripheral neutrophils is inhibited by glucocorticoids and by PI3 kinase enzyme inhibitors; δ selective inhibitors overall had a lesser effect than in MMP-9 and ROS release. Other PI3 kinase enzyme isoforms may be primarily involved in cytokine release.

There are a number of experiments which could be performed in future work, as discussed. PI3 kinase enzyme inhibition appears to be a promising strategy to target the neutrophilic inflammation associated with COPD.
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Appendix 1: Method for performing Luminex assay

The Luminex Kit HCYTOMAG-60k-02 (Merck-Millipore, UK) was used. The assay was read using the Luminex Magpix analyser (Luminex, USA) using Luminex Xponent (Luminex, USA) software.

All reagents were allowed to warm to room temperature (20-25°C) before use in the assay. 200μl assay buffer was added into each well of the plate for a initial plate wash. The plate was sealed and placed on a plate shaker at 650 rpm for 10 minutes at room temperature (20-25°C). Assay buffer was decanted and the residual amount was removed from all wells by inverting the plate and tapping it onto absorbent towels several times. 25μl of each standard or control was added into the appropriate wells. Assay buffer was used for 0 pg/ml standard. 25μl of assay buffer was added to the sample wells. 25μl of sputum supernatant matrix solution (PBS) was added to the background, standards, and control wells. 25μl of neat sputum supernatant sample was transferred from the sample plate into the appropriate wells of the Luminex plate. The bead mixing bottle was vortexed for 60 seconds and 25μl of the mixed beads were added to every well of the assay plate. The plate was sealed with a plate sealer, wrapped with foil and incubated with agitation on a plate shaker for 16 hours at 4°C at 650 rpm.

After 16 hours the plate was washed using a luminex magnetic plate mount. 25μl of detection antibodies were added into each well, the plate was covered with foil and incubated with agitation on a plate shaker at 650 rpm for 1 hour at room temperature (20-25°C). Without aspiration, 25μl of Streptavidin-Phycoerythrin was added to each well containing the 25μl of detection antibodies. The assay plate was sealed, covered with foil and incubated with agitation on a plate shaker at 650 rpm for 30 minutes at room temperature (20-25°C). Well contents were removed and the assay
plate was washed 2x using the magnetic plate mount as previously. 100µl of sheath fluid was added to all wells and the beads were resuspended on a plate shaker for 5 minutes at 650 rpm prior to reading.

The Luminex Magpix system was calibrated and aspirated a total sample volume of 100µl from every well for injection and analysis.

**Preparation of Reagents:**

All reagents were removed from the kit and left on the laboratory bench at room temperature (20-25°C) for at least 30 minutes before performing the assay.

**Preparation of Antibody-Immobilized beads**

- Each antibody-bead vial was sonicated for 30 seconds and then vortex for 1 minute
- 150 µl of NE antibody beads were added to the mixing bottle and then 2850 µl of bead diluent was added. Thereafter, the beads were vortexed.

**Preparation of Quality Controls**

- Before use, Quality Control 1 and Quality Control 2 were reconstituted with 250 µl deionized water and then vortexed.
- After 10 minutes then transfer the controls were transferred to appropriately labelled tubes.

**Preparation of wash buffer**

- The wash buffer was brought to room temperature (20-25°C) and mixed.
- 60 ml of 10X wash buffer was diluted with 540 ml deionized water.
Preparation of Human Cytokine Standard

- Prior to use, the human sepsis panel 3 standard was reconstituted with 250μl deionized water. After 10 minutes the standard was transferred to an appropriately labelled tube (Standard 6).

- The working standards were prepared by labelling five polypropylene tubes Standard 5, Standard 4, Standard 3, Standard 2 and Standard 1.

- 150μl of assay buffer was added to each of the five tubes. 1:4 serial dilutions were performed by adding 50μl of reconstituted standard 6 to the standard 5 tube, mixing and transferring 50 μl of the Standard 5 to the Standard 4 tube, and so on. The 0 pg/ml standard (background) was assay buffer.
Appendix 2: pIC50 and pKi values of the PI3 kinase enzyme inhibitors

<table>
<thead>
<tr>
<th></th>
<th>ZSTK740 (pan-inhibitor)</th>
<th>GSK045 (δ inhibitor)</th>
<th>GSK723 (γ inhibitor)</th>
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<tbody>
<tr>
<td>pIC50</td>
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<tr>
<td>PI3Kδ</td>
<td>8.5M</td>
<td>9.1M</td>
<td>5.9M</td>
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<tr>
<td>PI3Kγ</td>
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<tr>
<td>PI3Kγ</td>
<td>7.7M</td>
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</table>

A summary of pIC50 and pKi values for each PI3 kinase enzyme inhibitor on specific isoforms (in M): the pan PI3 kinase inhibitor ZSTK474, the PI3Kδ inhibitor GSK045 and the PI3Kγ inhibitor GSK723.
Appendix 3: Publications

The work presented in this thesis has resulted in the following publications.

Poster presentations:

1. Inhaled Lipopolysaccharide (LPS) in patients with COPD
   V Gupta, M Kaur, C Jagger, A Banyard, WY Lee, J Sutula, P Hitchen, D Singh

2. Matrixmetalloproteinase-9 and reactive oxygen species release from COPD airway and peripheral neutrophils; the effect of PI3 kinase and p38 MAP kinase inhibitors.
   V Gupta, A Khan, S Worsley, A Amour, J Lemon, T Southworth, D Singh

Papers:

1. Characterisation of the inflammatory response to inhaled LPS in mild to moderate COPD
   V Gupta, A Banyard, A Mullan, S Sriskantharajah, T Southworth, D Singh