The Role of p38 Mitogen-Activated Protein Kinase in Corticosteroid-Insensitivity in Severe Asthma and COPD

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Declaration of Originality & of Copyright

I declare that this thesis is entirely my own work. Information derived from the published and unpublished work of others has been acknowledged in the text and full references are provided.

Nadia Mohamed Khorasani

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Abstract

Severe asthma and chronic obstructive pulmonary disease (COPD) are chronic inflammatory diseases of the lung both characterised by airway limitations but with differing pathophysiology. Asthma is normally well controlled with the use of corticosteroids (CS). However in 5 – 10% of all patients with asthma - severe asthmatics - and in most patients with COPD, the response to CS is poor and these patients are relatively CS-insensitive. p38 mitogen-activated protein kinase (MAPK) is a kinase cascade whose heightened activity has been reported in severe asthma and COPD. In this thesis I hypothesised that increased p38 MAPK activity contributes to CS-insensitivity in severe asthma and COPD.

Induced p38 MAPK activity was increased in peripheral blood mononuclear cells (PBMC) from patients with severe asthma compared with non-severe asthma and in patients with COPD compared with smoking subjects. The ability of dexamethasone to suppress induced pro-inflammatory cytokine release was impaired in PBMC from patients with severe asthma compared with those with non-severe asthma, and in patients with COPD compared with smoking subjects, demonstrating CS-insensitivity in these patients. The inhibition of p38 MAPK activity with GW856553 improved the ability of dexamethasone to suppress induced cytokine release in PBMC from patients with severe asthma or COPD. To investigate the molecular mechanisms by which p38 MAPK activity may be involved in CS-insensitivity, the effect of p38 MAPK inhibition on the phosphorylation of glucocorticoid receptor (GR) at serine 211 residue was determined and was revealed to be p38 MAPK dependent in PBMC from patients with COPD.

MAPK phosphatase (MKP)-1 is an anti-inflammatory mediator that is induced by CS and can regulate p38 MAPK activity through dephosphorylation of its serine/threonine residues. Baseline and induced expression of MKP-1 was reduced in PBMC and monocytes from patients with severe asthma compared with non-severe asthma. Impairment of MKP-1 induction by CS may therefore be a mechanism through which CS-insensitivity manifests itself in severe asthma, however, siRNA
knockdown of MKP-1 in monocytes from normal subjects resulted in an increase of induced pro-inflammatory cytokines, but it did not reach significance.

CS and long-acting β₂ agonists (LABA) are used in combination to treat patients with COPD or severe asthma. The effect of fluticasone propionate (FP) or the novel CS, fluticasone furoate (FF), alone or in combination, with a novel ‘ultra-LABA’ vilanterol trifenatate, on suppression of induced cytokine release was examined and compared between severe asthmatics and non-severe asthmatics, and between patients with COPD and healthy smokers. FF, compared with FP, was superior in its ability to suppress induced cytokine release in PBMC from all groups. Moreover, suppressibility of FF in combination with vilanterol, on induced cytokine release, was significantly enhanced, compared with FP in combination with vilanterol, or vilanterol alone, in PBMC from severe asthmatics or COPD.
Acknowledgments

First and foremost I would like to give thanks and praise to God.

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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ACD</td>
<td>Acid citrate dextrose</td>
</tr>
<tr>
<td>AF1</td>
<td>Activation function-1</td>
</tr>
<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
</tr>
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<td>AP-1</td>
<td>Activator protein-1</td>
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<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
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<tr>
<td>ASM</td>
<td>Airway smooth muscle</td>
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<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchial alveolar lavage</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BDP</td>
<td>Beclomethasone dipropionate</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CAAT/enhancer binding protein</td>
</tr>
<tr>
<td>CBP</td>
<td>cAMP response element binding (CREB)-binding protein</td>
</tr>
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<td>CCL</td>
<td>CC-chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC-chemokine receptor</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinases</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
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<tr>
<td>DUSP</td>
<td>Dual-specificity phosphatase</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>ERS</td>
<td>European respiratory society</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume in one second</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>FF</td>
<td>Fluticasone furoate</td>
</tr>
<tr>
<td>FP</td>
<td>Fluticasone Propionate</td>
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<tr>
<td>FVC</td>
<td>Forced Vital Capacity</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GINA</td>
<td>Global Initiative for Asthma</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GOLD</td>
<td>Global initiative for Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>GRE</td>
<td>Glucocorticoid-response elements</td>
</tr>
<tr>
<td>GRO-α</td>
<td>Growth-related oncogene-α</td>
</tr>
<tr>
<td>GSK</td>
<td>Glycogen synthase kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GW-A</td>
<td>p38 MAPK inhibitor provided by GSK (commercial name: losmapimod)</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferases</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered saline solution</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IL-6R</td>
<td>Interleukin-6 receptor</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<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>LBD</td>
<td>Ligand-binding domain</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTB₄</td>
<td>Leukotriene B₄</td>
</tr>
<tr>
<td>LXA₄</td>
<td>Lipoxin A₄</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKAP-K2</td>
<td>MAPK-activated protein kinase 2</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MgCl</td>
<td>Magnesium chloride</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein 1α</td>
</tr>
<tr>
<td>MKK</td>
<td>MAPK Kinase</td>
</tr>
<tr>
<td>MKKK</td>
<td>MAPK Kinase Kinases</td>
</tr>
<tr>
<td>MKP-1</td>
<td>Mitogen-activated protein kinase phosphatase-1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MSK1</td>
<td>Mitogen and stress-activated kinase 1</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nGRE</td>
<td>Negative glucocorticoid response element</td>
</tr>
<tr>
<td>NICE</td>
<td>National institute for health and care excellence</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation sequence</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonyl phenoxypolyethoxylethanol 40</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCAF</td>
<td>p300/CBP-associated factor</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PTM</td>
<td>Post translational modifications</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell Park Memorial Institute media 1640</td>
</tr>
<tr>
<td>RTPCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SABA</td>
<td>Short acting β-agonist</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>SAPK</td>
<td>Stress-activated protein kinases</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>sIL-6R</td>
<td>Soluble IL-6 receptor</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcriptions</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin like modifiers</td>
</tr>
<tr>
<td>TAB1</td>
<td>TAK1-binding protein</td>
</tr>
<tr>
<td>TACE</td>
<td>Tumour-necrosis factor receptor α-converting enzyme</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor-β-activated protein kinase 1</td>
</tr>
<tr>
<td>TBP</td>
<td>Tata binding protein</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T cells</td>
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<tr>
<td>Th</td>
<td>T-helper cells</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour-necrosis factor receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour-necrosis factor α</td>
</tr>
<tr>
<td>TTP</td>
<td>Tristetraprolin</td>
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</table>
Chapter 1
Introduction
Chapter 1 - Introduction

1.1 Asthma

Asthma is a common disease affecting patients of all ages, ethnic backgrounds and both genders. Usually beginning in the pre-teen or teenage years, the onset of asthma can occur at any age. An estimated 300 million people worldwide have asthma and the frequency of this disease has significantly increased since the 1980s (Bousquet et al. 2007), with the potential for an additional 100 million more cases to be diagnosed by 2025 (Masoli et al., 2004). The World Health Organization (WHO) estimates that 15 million disability-adjusted life years are lost annually due to asthma, representing 1% of the total global disease burden, and annual worldwide deaths from asthma are estimated at 250,000 per year. In 1989 the Global Initiative for Asthma (GINA) programme was established, not only to raise awareness amongst public health workers, government officials and the general public, of the increased prevalence of asthma, but also to recommend management programs and provide effective medical care to local healthcare systems, based on the best available scientific evidence (Masoli et al., 2004).

1.1.1 Definition of asthma

The most recently updated formal definition of asthma as published by the GINA guideline states:

“Asthma is a heterogeneous disease, usually characterised by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation.” (GINA, 2017)
1.1.2 Characteristics of Asthma

Asthma is a disorder of the airways characterised by underlying airway inflammation, the predominant feature of which is episodic shortness of breath, particularly at night, often accompanied by cough, chest tightness and wheeze. There is a complex interaction of airflow obstruction, airway hyperresponsiveness and underlying inflammation that varies in duration and intensity within patients and which can lead to the chronicity of the disorder.

1.1.2.1 Bronchoconstriction and reversibility

The dominant physiological event that leads to the clinical symptoms of asthma is bronchoconstriction which is the contraction of the bronchial smooth muscle which narrows the airways. Exacerbations of asthma that lead to bronchoconstriction and acute airflow obstruction can be triggered by many stimuli such as allergens, irritants, exercise or exposure to cold air (NHLBI Guideline, 2007). Although it appears that underlying airway inflammation is responsible for the mechanisms of airway obstruction, it is noted that some non-steroidal anti-inflammatory drugs can trigger airway obstruction through a non-immunoglobulin (IgE) dependent pathway. Furthermore, asthma exacerbations may also be triggered by psychosocial stress (Ritz et al., 2011).

These episodes of variable airflow obstruction in asthma are often at least partially reversible, either with treatment or spontaneously. However this reversibility of airflow limitation may be incomplete in some patients due to permanent structural changes of the airway (remodelling). Spirometry can be used to assess the reversibility of obstruction in patient with asthma and is observed by an increase in lung function after inhalation of a short-acting bronchodilator (NHLBI Guideline, 2007).
1.1.2.2 Airway Oedema

As asthma progresses other factors that limit airflow include oedema, inflammation, mucus hypersecretion and the development of mucus plugs. Structural changes to the airway may also occur in the form of hypertrophy (enlargement) and hyperplasia (increase in the number) of airway smooth muscle (ASM) cells (NHLBI Guidelines, 2007).

1.1.2.3 Airway hyperresponsiveness

Airway or bronchial hyperresponsiveness is the heightened sensitivity of the airways to triggering stimuli which induce deterioration in measures of lung function. Although airway hyperresponsiveness does exist in other conditions such as chronic bronchitis or sarcoidosis, it is a major feature of asthma (Chung and Durham, 1992). The hyperresponsiveness deteriorates lung function due to narrowing of the airways and the severity of asthma can be correlated with the clinical response to methacholine for example (Cockcroft, 2010).

1.1.2.4 Airway Remodelling

As part of the repair process in asthma, airway remodelling sometimes occurs whereby permanent changes in the structure of the airway wall take place. This is characterised by thickening of the subepithelial layer of the airway wall from increased depositions of extracellular matrix proteins such as collagens and goblet cell metaplasia (Manuyakorn et al., 2013) as well as a substantial increase in both the large and small airways (Chung and Adcock, 2001). Remodelling features differ between asthma and chronic obstructive pulmonary disease (COPD), in allergic and non-allergic asthma and with the severity of asthma (Figure 1.2) (Busse and Lemanske, 2001). Moreover, airway remodelling in asthma has been found to be promoted by repeated mechanical bronchoconstriction, independent of inflammation, suggesting that the management of asthma should not necessarily solely focus on the inflammatory component of the disease (Grainge et al.,
The result of these permanent structural changes by airway remodelling can lead to only partial reversibility of airflow limitation in patients with asthma.

1.1.3 Causes of asthma

The question of the initiation of the inflammatory process in asthma has not yet been fully answered and to date there is no definitive cause for the pathogenesis observed in the disease. The interplay however, between host factors (such as innate immunity and a genetic component) and environmental exposures (airborne allergens and respiratory viral infections) that occur in a crucial stage of the development of the immune system, are important and always under intense investigation (NHLBI Guidelines, 2007).

1.1.4 Treatment of Asthma

For the last 20 years, the most effective mainstay treatment of asthma is the combined use of a bronchodilator, a long-acting β-adrenergic agonist (LABA) and anti-inflammatory corticosteroid (CS) (Chung, 2013). GINA outlines a stepwise approach, consisting of five steps, for the management of asthma, with pharmacological options falling into three main categories: Controller medications, used for regular daily maintenance treatment (normally low dose inhaled CS); reliever (rescue) medications, to relieve breakthrough symptoms or to prevent exercise-induced bronchoconstriction, usually in the form of short-acting β-adrenergic agonists (SABA) or anticholinergics, as needed; Add-on therapies (usually high dose inhaled CS and a LABA), for patients considered to have ‘severe’ asthma (GINA Report, Global Strategy for Asthma Management and Prevention, 2017). It is suggested that the controller medication is issued as soon as possible after diagnosis of asthma. A cycle of assessment, adjustment of treatment and review of response should then be implemented to achieve good control of symptoms and to minimise the risks of future exacerbations, airflow limitations and / or side-effects of medications. After an initial 2 -3 month period of controller
treatment, the patient response is then reviewed and the treatment is either ‘stepped up’ or ‘stepped down’, as per the GINA stepwise approach, based on how well symptom control is achieved (Figure 1.1) (GINA Report, Global Strategy for Asthma Management and Prevention, 2017).
**Preferred Controller Choice**

<table>
<thead>
<tr>
<th><strong>STEP 1</strong></th>
<th><strong>STEP 2</strong></th>
<th><strong>STEP 3</strong></th>
<th><strong>STEP 4</strong></th>
<th><strong>STEP 5</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low dose ICS</strong></td>
<td><strong>Low dose ICS/LABA</strong></td>
<td><strong>Med/High ICS/LABA</strong></td>
<td><strong>Refer for Add-on treatment e.g. Tiotropium</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Other Controller Options**

- Consider low dose ICS
- Leukotriene receptor antagonists (LTRA)
- Low dose theophylline

**Reliever**

- As needed SABA
- As needed SABA
- As needed SABA or low dose ICS/formoterol
- As needed SABA or low dose ICS/formoterol
- As needed SABA or low dose ICS/formoterol

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**Figure 1.1:** GINA 2017 Stepwise approach to control asthma symptoms and minimise future risk in adults and children older than 12 years (Adapted from the GINA Report, Global Strategy for Asthma Management and Prevention, 2017). ICS – inhaled corticosteroid; OCS – oral corticosteroid; Med – medium dose; LABA - long-acting β-adrenergic agonist; SABA - short-acting β-adrenergic agonist.
1.1.5 Severe Asthma

Although asthma symptoms, in their mild and moderate forms, are normally well managed with inhaled CS and/or LABA, a small proportion of between 5 - 10% of asthmatics do not respond well if at all, to current treatments. These individuals fall under the umbrella term “severe asthmatics” and account for over half the healthcare costs associated with asthma (Antonicelli et al., 2004). Patients with the most severe asthma consume more healthcare resources and require more medical attention. Additionally, there is the concern of the substantial socio-economic costs associated with the loss of productivity at work and the reduced social function of these patients (Sterling and Chung, 2001). In the United Kingdom it is estimated that 50% of asthma healthcare costs originate from the most severe 20% of the asthmatic population (Masoli et al., 2004).

‘Severe’ asthma is distinct from ‘difficult-to-treat’ asthma. Recent GINA guidelines have described difficult-to-treat asthma as when the disease continues to be symptomatic despite the use of reliever medication plus two or more controllers (GINA, 2012). The definition of severe asthma has yet to be universally agreed upon. When patients cannot control their asthma despite the high levels of treatment, it is referred to as ‘therapy-resistant’, ‘refractory’ or ‘severe’ asthma. In 1999, a Task Force from the European Respiratory Society (ERS) defined severe asthma as: asthma that is poorly controlled in terms of chronic symptoms, episodic exacerbations, persistent and variable airway obstruction and a continued requirement for SABA despite the use of a maximal dose of inhaled CS (Chung et al., 1999). The American Thoracic Society (ATS) is one of the leading organisations working worldwide to improve those affected by respiratory disease, critical illness and sleep disorders (www.thoracic.org). In 2000, the proceedings of an ATS-sponsored workshop developed a working definition of refractory/severe asthma that was dependent on the basis of medication requirements, asthma symptoms, frequency of asthma exacerbations and degree of airflow limitations. There were two major and seven minor criteria put forward, with refractory/severe asthma being defined as one or both major criteria and at least two minor criteria,
so long as other conditions had been excluded, exacerbating factors optimally treated and poor adherence was not a confounding factor (Table 1.1).

**Table 1.1: Refractory / Severe asthma workshop consensus for typical clinical features.** FEV₁ – Forced Expiratory Volume in 1 second. (Proceedings of the ATS Workshop on Refractory Asthma; July 2000)

A more recent Task Force supported by the ERS and ATS has suggested the following reviewed definition of the disease:

“When the diagnosis of asthma is confirmed and the comorbidities addressed, severe asthma is defined as asthma that requires treatment with high dose inhaled corticosteroids plus a second controller and/or systemic corticosteroids to prevent it from becoming ‘uncontrolled’ or that remains ‘uncontrolled’ despite this therapy” (Chung et al., 2014).

The ERS/ATS Task Force has also outlined an updated step-wise guideline for the identification and recommended management of severe asthma, as the process has always been
complicated and difficult, which has undoubtedly lead to the misdiagnosis and therefore inadequate management of disease. The steps involve determining and eliminating the presence of ‘difficult-to-treat’ asthma first before confirming ‘severe asthma’ based on the following: 1) the updated definition of severe asthma; 2) the identification of potential mechanisms/phenotypes of severe asthma; 3) the evaluation of the diagnosis of asthma (based on confounding factors and comorbidities, phenotype data and patient adherence to treatment) and 4) the recommended treatment based on these previous steps (Chung et al., 2014).

1.2 Chronic Obstructive Pulmonary Disease (COPD)

COPD is an increasingly prevalent disease of the lungs and is the fourth leading cause of death worldwide. More than 65 million people are estimated to have moderate to severe COPD (www.who.int). In the UK alone, the National Institute for Health and Care Excellence (NICE) has estimated that 3 million people have COPD, 2 million of who are undiagnosed (www.nice.org.uk). More than 3 million people worldwide died from the disease in 2005 and it is set to become the third leading cause of global death by 2030 (www.who.int). COPD has become a much greater drain on healthcare resources than asthma and exceeds the healthcare spending on asthma by approximately three-fold in industrialised countries (Barnes and Stockley, 2005). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) was set up in 1997 in collaboration with the National Heart, Lung and Blood Institute (NHLBI) and the WHO to recommend effective management and prevention of COPD in all countries, increase awareness of COPD, decrease deaths that occur from COPD and to promote study of COPD in order to understand its increasing prevalence and its relationship with the environment (www.goldcopd.org).
1.2.1 Definition of COPD

The most up-to-date definition of the disease according to GOLD is as follows:

“Chronic Obstructive Pulmonary Disease is a common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases.” (GOLD, 2017).

The ATS/ERS Task Force of 2004 outlined a similar working definition of COPD: “a preventable and treatable disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and is associated with an abnormal inflammatory response of the lungs to noxious particles or gases, primarily caused by cigarette smoking. Although COPD affects the lungs, it also produces significant systemic consequences”, (Celli and MacNee et al., 2004). In the UK, NICE has stated in its updated guidelines that COPD is now the preferred term for the conditions in patients with airflow obstruction who were previously diagnosed as having chronic bronchitis (chronic cough and sputum production) or emphysema (destruction of the lung alveoli) (www.nice.org.uk). Yet this is an ongoing debate, as emphysema describes only one of many structural abnormalities observed in COPD and chronic bronchitis is an independent disease entity in itself, which can exist in patients with normal spirometry (GOLD, 2014).

Since 2001, GOLD have developed a staging system for COPD based on the simple lung function measurements, the FEV₁ ( Forced Expiratory Volume in one second) and the FEV₁ / FVC ratio ( Forced Expiratory Volume in one second / Forced Vital Capacity), after a bronchodilator been administered (Table 1.2). Additionally a formal symptomatic assessment is performed as the correlation between lung function, symptoms and impairment of quality of life is weak, and therefore spirometric data alone is not necessarily enough to make a correct diagnosis of stage of COPD. The symptomatic assessments that are suitable for use in routine practice (as opposed to the more comprehensive and complex Modified British Medical Research Council (mMRC) Questionnaire or St George’s Respiratory Questionnaire (SGRQ)), include the COPD Assessment Test (CAT™) and
The COPD Control Questionnaire (The CCQ©) which provide measures of the symptomatic impact of COPD and not just the level of breathlessness a patient experiences. In addition, the assessment of COPD exacerbation risk, based on frequency of annually treated exacerbations, is also considered when defining the stage of the disease. With these factors in mind, GOLD updated the COPD grading system in 2011 to include the “ABCD” assessment tools which takes into consideration the simple spirometric data, as well as patient-reported outcomes (through mMRC or CAT™) and exacerbation risks, in order to better stage, manage and treat the disease. The number (1-4) is related to the severity of airflow limitation, as measured by spirometry and the letter (A, B, C, or D) provides information on burden of symptoms and risk of exacerbation. By this method of assessment, the stage of COPD can be better defined based on symptoms and not only spirometry, at any given time, and therefore a more precise treatment can be implemented (Table 1.2), (GOLD, 2017).
Table 1.2: COPD stages as defined by spirometry and the refined ABCD assessment tool. GOLD classifies the different stages of COPD based on simple spirometric data post-bronchodilator administration, in addition to symptomatic assessment. mMRC – modified British Medical Research Council Questionnaire; CAT – COPD Assessment Test. (Adapted from GOLD, 2017)

1.2.2 Characteristics of COPD

COPD is characterised by poorly reversible airflow obstruction and an abnormal inflammatory response in the lungs (MacNee, 2006), the clinical symptoms of which include exertional breathlessness, chronic cough, regular sputum production, frequent winter bronchitis and wheeze (GOLD, 2014). The main pathological changes associated with COPD occur in four different compartments of the lungs – namely the central airways, peripheral airways, lung parenchyma and pulmonary vasculature (Celli and MacNee et al., 2004). Chronic airflow limitation characteristic in COPD is caused by a mixture of small airway inflammation and fibrosis (obstructive bronchiolitis),
parenchymal destruction (emphysema), and mucous hypersecretion (chronic bronchitis) (Rabe et al., 2007; MacNee, 2006).

1.2.2.1 COPD exacerbation

A COPD exacerbation defined as “an event in the natural course of the disease characterised by a change in the patients’ baseline dyspnoea, cough and /or sputum that is beyond normal day-to-day variations, is acute in onset and may warrant a change in the regular medication in a patient with underlying COPD”. COPD exacerbation is one of the commonest reasons for hospital admissions, including in the UK, and is associated with a poorer health outcome as well a greater financial burden on any given healthcare system (Rabe et al., 2007). Exacerbations can be caused by bacterial or viral infection, air pollution and changes in ambient temperatures. In mild exacerbations airflow obstruction is unchanged or slightly decreased contrasted by severe exacerbations where there is a worsening of pulmonary gas exchange as a result of airway inflammation, oedema, mucus hypersecretion and bronchoconstriction (MacNee, 2006).

1.2.3 Causes of COPD

Tobacco smoking is the cause of the vast majority of cases of COPD. The WHO estimates that in high-income countries, 73% of COPD mortality is related to smoking with 40% related to smoking in nations of low and middle income. Occupational exposure to dust, chemicals, vapours or fumes can impact on patients with COPD. Excessive exposure to biomass fuels, such as coal, animal dung or wood, used to heat and/or cook in poorly ventilated homes, also contributes towards a higher prevalence of COPD in some countries. Outdoor air pollution can also impact on the development of COPD and/or may worsen disease symptoms (Mannino and Buist, 2007). It is rare for people who have never smoked to develop COPD although a severe hereditary deficiency of the
α1-antitrypsin gene has been documented as a genetic risk factor - it has been shown to cause premature and accelerated development of emphysema in both smokers and non-smokers but particularly in the former (GOLD, 2014).

1.2.4 The “Dutch Hypothesis”

In 1961, Professor N.G.M. Orie and colleagues from Groningen, the Netherlands, proposed that all obstructive airway diseases including asthma, emphysema and chronic bronchitis (now included under the umbrella of COPD) should be considered different manifestations of a single disease which had common genetic origins. This was later coined the “Dutch hypothesis” on account of its source (Postma and Quanjer, 2006). It was strongly disputed by British and American researchers, led by Vermeire and Pride, who countered with the “British hypothesis” that asthma and COPD were in fact distinct diseases with separate causal mechanisms. Asthma tends to develop early on in childhood, is episodic in nature and does not progress, showing good responses to bronchodilators and CS treatment. By contrast, COPD is slow in its progression with most cases diagnosed after 60 years old and patients have a poor response to bronchodilators and CS. Confusingly however, and giving strength to the Dutch hypothesis, some patients with COPD have more reversibility and some patients with asthma are progressive in their course (Barnes, 2006a). The British hypothesis states that the presence of cough and sputum is the key factor in COPD and the Dutch hypothesis points to the presence of increased airway hyperresponsiveness in asthma that leads to the development of COPD (Mannino and Buist, 2007). Both diseases are heterogeneous in their nature which may explain the overlaps. However, despite the similarities of some of the clinical features of asthma and COPD, there are marked differences in the pattern of inflammation within the respiratory tract including differential recruitment of inflammatory cells, mediator production and responses to treatments (Barnes, 2008). Ultimately, it is important to distinguish asthma from
COPD in order to conduct more efficient research and to eventually treat patients with these diseases more effectively.

1.2.5 Treatment of COPD

As smoking is the main causative factor in the development of COPD, the first line of treatment is usually smoking cessation. This can slow down, although not halt, the decline in lung function and may improve the other symptoms observed in patients with COPD. In terms of pharmacologic therapy for COPD these include the use of inhaled bronchodilators in the form of LABA or SABA, anticholinergics, methylxanthines such as theophylline, phosphodiesterase-4 inhibitors or inhaled CS. Despite the use of these therapies giving some management in the symptoms of COPD, none to date have been shown to reduce the progression of COPD.

GOLD guidelines stipulate bronchodilators such as LABA as the preferred treatment of COPD as they have been shown to give some symptomatic relief through the dilation of the distal airways, albeit that their use does not modify the progression of the disease. Improvement in bronchodilator development is ongoing - ultra-LABA such as vilanterol and indacaterol, which last at least 12-24 hours (significantly longer than traditional LABA), are a recent addition to this category (Ngkelo and Adcock, 2013). Inhaled CS are routinely administered for asthma because of their anti-inflammatory effects. When similarities in the patterns of inflammation were observed between asthma and COPD, CS were administered to patients with COPD with the hope that they would gain the same beneficial effects as asthmatics. However, most patients with COPD do not respond well to CS treatment (Suissa et al, 2008).
### Inflammation

<table>
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**Figure 1.2: Comparison of histopathology between normal, asthma and COPD airways.** Airway from a normal individual (shown on the left), small airways from a patient who died of asthma (middle picture) and from a patient with severe COPD (shown on the right) are compared. Inflammatory immune cells are heavily present in both asthma and COPD airways. Airway smooth muscle is more thickened in asthma than normal and COPD. The basement membrane in asthma is thicker than normal and COPD due to subepithelial collagen deposition. Fibrosis (obstructive bronchiolitis) is more pronounced in COPD. There is alveolar wall disruption in COPD, but not in asthma, due to emphysema. Asthma and COPD airway images courtesy of Dr J. Hogg (adapted from Barnes, 2008).

1.3 Inflammation

Inflammation is the protective response that has evolved against the constant warfare that we are engaged in with the microbes that surround us. Eliminating or neutralising foreign organisms or materials, which is the resolution of inflammation, involves endogenous anti-inflammatory mechanisms and the protective influx of white cells, complement, antibody and other plasma proteins into a site of infection or injury. The processes of capillary dilatation (redness), exudation of plasma proteins and also of fluid (oedema) due to hydrostatic and osmotic pressure changes, and accumulation of neutrophils are collectively termed the acute inflammatory response (Rabson et al., 2005). When correctly functioning and balanced, the innate inflammatory response provides the eventual beneficial restoration of tissue function, usually within hours. However, when the inflammatory response ceases to function normally, this inflammation can become chronic and may last much longer, persisting for weeks or even years (Lawrence and Gilroy, 2007). The prolonged presence of the inflammatory response, due to persistent invading matter or stimulus, can create a chronic inflammatory disease state whereby destruction of the cellular matrix can occur, leading to loss of tissue and or, organ function. Examples of such conditions include rheumatoid arthritis, whereby inflammatory cells accumulate at synovial joints causing damage (Lawrence et al., 2002), as well as myocardial infarction, asthma and chronic obstructive pulmonary disease (COPD) (Barnes, 2000; Lawrence et al., 2002).

1.3.1 Inflammatory cells involved in severe asthma and COPD

There are several types of cells that are involved in the inflammatory process that come under the umbrella of white blood cells or leukocytes including lymphocytes, neutrophils, eosinophils, blood monocytes and macrophages. In asthma or COPD, there are specific patterns of inflammation that create their pathophysiology involving particular cells to predominate over others in their number, intensity and duration of activation. There are distinct patterns of inflammation between asthma and COPD.
1.3.1.1 Lymphocytes

The lymphocytes are a subset of the leukocyte population and can be any one of three subsets: natural killer (NK) cells, B cells or T-cells.

1.3.1.1.1 T-Cells

T-cells originate from bone marrow but mature in the thymus and can be differentiated into T-helper cells (T\(_{\text{H}}\) / CD4\(^+\) cells) or cytotoxic T cells (T\(_{\text{C}}\) / CD8\(^+\) cells) depending on which glycoprotein they express on their surfaces. CD4\(^+\) cells are activated when presented with peptide antigens by major histocompatibility complex (MHC) class II molecules that are expressed on the surface of antigen-presenting cells (APCs) (including dendritic, mononuclear phagocytes and B cells). CD8\(^+\) cells bind to antigen associated with MHC class I molecules (all nucleated cells). CD4\(^+\) cells then differentiate into either T-helper 1 (T\(_{\text{H}}\)1) or T-helper 2 (T\(_{\text{H}}\)2) cells, and all are heavily involved in cell-mediated adaptive immunity through the production of cytokines or chemokines. T\(_{\text{H}}\)1 cells produce interferon-\(\gamma\) and tumour-necrosis factor (TNF) cytokines whereas T\(_{\text{H}}\)2 cells produce mainly interleukin (IL)-4, IL-5 and IL-13 that counteract a T\(_{\text{H}}\)1 cell response to control immunity (Rabson et al., 2005).

In asthma, a preference towards the CD4\(^+\) type T\(_{\text{H}}\)2 cell cytokine profile, specifically IL-5 production, results in an eosinophilic inflammatory state (Saetta et al., 1996). In peripheral blood from patients with asthma, eosinophil numbers were increased, correlating to disease severity (Bousquet et al., 1990). In bronchial alveolar lavage (BAL) fluid of asthmatics, increased levels of cells expressing T\(_{\text{H}}\)2 type cytokines (IL-3, IL-4 and IL-5) compared with control subjects has been shown (Robinson et al., 1992). T\(_{\text{H}}\)2 cells, in allergic inflammation, secrete IL-4 and IL-13 to drive B-cell immunoglobulin (IgE) production, and IL-9 which drives mast cell migration (Figure 1.3) (Barnes, 2008). However, in sputum from asthmatic patients experiencing acute exacerbation, there is an increase in neutrophil numbers compared with eosinophils (Fahy et al., 1995) and in severe
asthmatics high numbers and percentages of neutrophils in BAL fluid and biopsies has been shown, compared with moderate asthmatics (Wenzel et al., 1997). In COPD, eosinophils are also not prominent, except during exacerbation, or if patients have concomitant asthma (Fabbri et al., 1998).

$CD8^+$ cells target and kill virus infected pathogens and tumour cells. Viral infections are a frequent occurrence in COPD. In patients with COPD compared with control subjects, studies have shown an increase in number of $CD8^+$ cells in the peripheral airways (Saetta et al., 1998) and an increase in the activity of sputum $CD8^+$ cells (Chrysofakis et al., 2004). Similarly, in asthmatics, numbers of $CD8^+$ cells in bronchial biopsies were associated with disease outcome determined by increased cytokine production of sputum $CD8^+$ cells and was related to asthma severity (Cho et al., 2004).

1.3.1.1.2 NK Cells

NK cells are morphologically classified as lymphocytes (which are generally associated with adaptive immunity) but they do however, form part of the innate immune defence system due to their lack of antigen-specific cell surface receptors. NK cells can directly induce the death of tumour cells and virus-infected cells without specific immunisation and are major producers of several cytokines (particularly interferon-$\gamma$ (IFN-$\gamma$)), chemokines and growth factors. Secretion by NK cells of IFN-$\gamma$ and chemokines such as ‘monocyte chemoattractant protein-1’ (MCP-1) and ‘regulated on activation, normal T cell expressed, and secreted’ (RANTES), shape the responses of other immune cells such as dendritic cells and T-cells, to areas of inflammation (Vivier et al., 2011). Approximately 10% of resident lymphocytes in the lung are NK cells and when activated by infection or inflammation, NK cells are recruited from the blood stream to the lung and secrete cytokines, predominantly IFN-$\gamma$. In asthmatics, NK cells appear to be differentially activated, depending on the source of exacerbation, and different NK cell subset activations may contribute to the balance of Th1 and Th2 cytokine production in asthma and allergy. In COPD, partially due to the effects of smoking,
NK cell function was found to be impaired but this reduction in function was also observed in ex-smokers compared with control, suggesting a disease association (Culley, 2009).

1.3.1.1.3 B Cells

In mammals, B cells develop from lymphoid progenitor cells and mature in the bone marrow and are involved in adaptive immunity with their main functions being antibody secretion, antigen presentation and cytokine secretion. B cell activation is induced when a naïve B cell binds to either a soluble antigen or antigen presented by macrophage or dendritic cell with its B cell receptor. Th cells then assist in the maturation of B cells into plasma and memory B cells expressing CD20. In the lymphoid follicles, mature B cells and T cells are segregated into different areas depending in CD40 (B cell expressed) or CD40 ligand (T–cell expressed) binding. Environmental insults to the airway epithelium and macrophages, such as bacteria, result cytokine production recruiting immature B cells, T-cells and dendritic cells and chronic inflammation due to, for example persistent antigen exposure or tissue damage, leads to the aggregation of lymphoid follicles separated by B or T cells. T cells, macrophages and dendritic cells then activate the B cells through the ‘B cell activating factor of the TNF family’ (BAFF) receptor. B cells activated through antigen binding proliferate and form plasma cells that produce antibodies promoting immunity to increase pathogen clearance (Polverino et al., 2016). It has been reported that in patients with COPD, that infiltrating B cells and percentage of small airways containing B cell-rich lymphoid follicles, were associated with disease severity (Hogg et al., 2004) and that B cell counts were increased (Gosman et al., 2006).
Figure 1.3: Cell and cytokine interactions in asthmatic airways. Dendritic cells present antigens to T-cells, with the subsequent divergence to Th2 cells. Roles of other cells are also crucial e.g. eosinophils, neutrophils, macrophages, airway epithelium and airway smooth muscle cells. (GM-CSF - granulocyte-monocyte colony stimulating factors; IFN - interferon; IgE - immunoglobulin E; IL - interleukin; MCP-1 - monocyte chemoattractant protein-1; PDGF - platelet-derived growth factor; RANTES - regulated on activation, normal T cell expressed, and secreted; TCR - T cell receptor; Th2 - T-cell helper type 2; TGF - transforming growth factor; TNF - tumour necrosis factor). (Chung and Adcock, 2001)

1.3.1.2 Eosinophils

Eosinophils account for approximately 2-4% of the white blood cell population and are a type of granulocyte, a name given due to the presence of granules in their cytoplasm. They are produced and matured in the bone marrow before circulating in the blood to sites of inflammation or infection in response to chemotactic cytokines (chemokines) or leukotrienes. Eosinophilia is generally presented in asthma but not COPD, implicating these cells in the type of inflammation
displayed in asthma. In asthma, T_{H2} mediated eosinophilia is orchestrated by IL-5 release from T_{H2} cells and further synergised by mast cell secretion of chemokines such as CC-chemokine ligand 11 (CCL11 or eotaxin 1) (Pelaia et al., 2015). Increased eosinophil numbers in patients with severe asthma have been associated with sub-basement membrane thickening and raised numbers of inflammatory cells, including lymphocytes, mast cells and macrophages (Wenzel et al., 1999). Interestingly, the level of eosinophilia acts as a general marker of CS-responsiveness – in patients with severe asthma, a neutrophilic, rather than eosinophilic, inflammatory pattern is seen and these patients are more CS-insensitive (Green et al., 2002). In patients with stable COPD displaying sputum eosinophilia, CS-therapy improved lung function (Papi et al., 2000; Brightling et al., 2005) suggesting these patients may have concomitant asthma.

However, in more recent years the recognition of an asthma-COPD overlap syndrome (ACOS) has given strength to the “Dutch Hypothesis” where it was acknowledged that there were several problems when trying to distinguish between asthma and COPD due to overlapping features, which were especially dependent on age (usually older patients), sex and environmental factors (Postma and Rabe, 2015). There is currently a global expert panel discussion taking place to define ACOS based on a phenotyping criteria approach and a recent GOLD-GINA document describes the syndrome as persistent airflow limitation with several features usually associated with asthma and several features usually associated with COPD (Barrecheguren et al., 2015). Taking this into account, sputum eosinophilia, normally associated with asthma, when observed in COPD is now one of the most important diagnostic features of recognising ACOS in COPD (with other criteria including persistent airflow limitations in patients over 40 years old despite the adequate administration of short-acting bronchodilator, at least 10 pack-years of smoking or equivalent in exposure to biomass fuels and a documented history of asthma before 40 years old) (Sin et al., 2016). Interestingly, in one study where patients with COPD who displayed ‘asthma-like’ symptoms such as episodic breathlessness and wheeze, they also displayed higher than average peripheral eosinophils counts and this also correlated with these patients being more likely to respond to ICS compared with COPD.
patients without asthma, giving strength to using eosinophil counts as a possible biomarker for ACOS (Kitaguchi et al., 2012).

### 1.3.1.3 Neutrophils

Neutrophils are granulocytes representing the largest population of white blood cells at approximately 60 – 70% of the total. Produced in the bone marrow, neutrophils stay mainly in blood circulation until an inflammatory or infectious episode initiates their migration to that site. CXC-chemokine ligand-8 (CXCL8) or leukotriene B4 (LTB₄), are examples of the pro-inflammatory mediators that attract neutrophils to the site of inflammation through a chemotactic gradient (or chemotaxis). In severe asthma, induced sputum display neutrophilia, indicative of a non-Tₐ₂ driven mechanism and although inconclusive, neutrophilic asthmatics show a poor response to CS suggesting a link between neutrophilia and CS-insensitivity (Chung, 2013). BAL fluid from patients with severe asthma displayed a higher percentage of neutrophils compared with patients with non-severe asthma (Bhavsar et al., 2008). In smokers with mild asthma, a neutrophilic pattern of lung inflammation was observed compared with non-smokers (Chalmers et al., 2001). In COPD patients, BAL fluid and sputum samples show increased levels of activated neutrophils and in induced sputum, this correlated with COPD disease severity (Keatings et al., 1996). Various neutrophil chemotactic factors have also been shown to be elevated in COPD airways such as growth-related oncogene (GRO)-α (Traves et al., 2002), TNFα and CXCL8 (Daldeegan et al., 2005).

### 1.3.1.4 Monocytes

Monocytes are the largest type of white blood cell in the body, originating from the bone marrow and circulate in the blood stream. They are able to phagocytose (engulf microbes and particles following their destruction) and their role in inflammation is to release cytokines, respond quickly to the site of infection and in those tissues, to differentiate into macrophage or dendritic cells (Ziegler-Heitbrock, 2015). In COPD, irritants to the lung activate epithelial cells to release
several chemotactic factors such as CC-chemokine ligand 2 (CCL2) which acts on CC-chemokine receptor 2 (CCR2) to attract monocytes to the lung and CXCL1 and CXCL8 which attract both neutrophils and monocytes to the lung. Monocytes differentiate into macrophages to continue the inflammatory response (Traves et al., 2004). Lipopolysaccharide (LPS)-induced cytokine production of blood monocytes from patients with asthma was increased compared normal subjects (Hallsworth et al., 1994).

Peripheral blood mononuclear cells (PBMC) are a mixed population of single nucleus cells, derived from and easily isolated from whole blood, and are a rich source of blood monocytes. In the lung, blood monocytes differentiate into pulmonary macrophages, which are the predominate cells involved in the immune response in lung disease and therefore the use of PBMC, as an easy-to-isolate cell model, is the ideal surrogate for exploration of macrophage responses in-vitro.

1.3.1.5 Macrophages

Macrophages are a subset of the white blood cell population whose primary function is to perform phagocytosis during the inflammatory response. They are found in almost all tissues of the body but there are distinct subsets depending on their location. Macrophages also form part of the mononuclear phagocyte system (MPS) which consists of monocytes, monocyte-derived cells, macrophages or dendritic cells (DCs).

1.3.1.5.1 Alveolar Macrophages

In the lungs, pulmonary macrophages are the most numerous immune-cell present and within the alveolar spaces of the lung are the alveolar macrophages (AM), that represent 90% of the total pulmonary lung macrophage population (Vlahos and Bozinovski, 2014). Pulmonary macrophages can modulate the ASM contractility suggesting a direct link between them and airway hyperresponsiveness, a hallmark feature of asthma (Yang et al., 2012a). In patients with asthma compared with normal subjects, LPS-induced AM suppressive activity was comparatively weaker.
(Pujol et al., 1990) but AM cytokine production (Hallsworth et al., 1994) and basal AM activity (Viksman et al., 1997) was increased. In patients with severe asthma compared to those with mild-moderate asthma or normal subjects, a decrease in AM LPS responsiveness manifested in defective apoptotic cell uptake and reduced secretion of inflammatory mediators (Huynh et al., 2005). In severe asthmatics compared with non-severe asthmatics, AM numbers were reduced in the BAL fluid (Bhavsar et al., 2008). In the same report, CS suppression of LPS-treated AM from patients with severe asthma was impaired compared with AM from non-severe asthmatics and this was associated with increased p38 MAPK activity (Bhavsar et al., 2008). The persistent inflammation in severe asthma has been linked to an imbalance in anti-inflammatory lipoxin A4 (LXA4) biosynthesis and the pro-inflammatory LTB4. In AM from severe asthmatics, decreased LXA4 and increased LTB4 generation associated with impaired CS of LPS-induced LTB4 supports a role for AM involvement in the pro-inflammatory state of severe asthma (Bhavsar et al., 2010a). Furthermore it was reported that there was a reduced phagocytic capacity of macrophages for bacteria in AM from patients with severe asthma compared with those with non-severe asthma or control subjects, suggesting that a bacterial clearance defect in AM may be contributing to the airway colonisation and persistent inflammation in severe asthma (Liang et al., 2014).

Macrophages play a key role in the pathology of COPD and are activated by cigarette smoke extract (CSE) to release pro-inflammatory mediators including TNFα, CXCL8 and LTB4 which provides a cellular mechanism linking smoking with COPD inflammation(Figure 1.4) (Barnes, 2014). However acute CSE exposure can also have a suppressive effect on macrophage inflammatory gene expression, apart from CXCL8, reflecting an upregulation of cell chemotaxis (through CXCL8) but down-regulation of the innate immune response (Kent et al., 2008). Most inflammatory proteins upregulated in COPD macrophages are regulated by the transcription factor, ‘nuclear factor kappa-light-chain-enhancer of activated B cells’ (NF-κB) which is activated in the AM of patients with COPD, in particular during exacerbation (Caramori et al., 2003). AM from patients with COPD release more CXCL8 at baseline, and when stimulated with cigarette smoke extract, than those from smokers,
suggesting AM are more active in COPD. Additionally, in COPD AM compared with those of smokers and normal subjects, CS are ineffective in inhibiting AM release of CXCL8 or TNFα (Culpitt et al., 2003). AM and monocyte derived macrophages from patients with COPD display reduced phagocytic uptake of bacteria (Taylor et al., 2009) and AM show defective apoptotic cell uptake (Hodge et al., 2003) which may be determining factors in the chronic colonization of the lower airways of COPD which may lead to exacerbation.

1.3.1.5.2 Macrophage phenotypes and functions

AM can be further characterised by the specific gene expression profile of surface markers exhibited by the macrophage, after cytokine or microbial stimulation. Using this approach several macrophage subsets have been described, each with distinct functions (Murray and Wynn 2011). Exposure to pathogens induces the polarization of AM into M1 or M2 cells. M1 cells are ‘classically’ activated macrophages, activated by either IFN-γ or LPS and responsible for inflammation and for protection against invading pathogens, expressing high levels of proinflammatory cytokines such as TNF-α and inducible nitric oxide synthase (iNOS). M2 cells are ‘alternatively’ activated macrophages and a recent development has shown that these AM can be further subdivided into ‘alternative activated’ cells (M2a), ‘type II alternatively activated’ cells (M2b) or ‘acquired deactivated’ cells (M2c) and M2d cells. With regards to recently identified functions, M2a, M2b and M2c macrophages have been shown to, so far, be broadly involved in allergic inflammation, tissue remodelling and fibrosis, and anti-inflammatory actions, respectively. Furthermore their polarization and function are substantially influenced by the microenvironment, including several cytokines and chemokines (Jiang and Zhu, 2016). The MPS cells of the lung tissue have recently been characterised in healthy subjects whereby five pulmonary subsets were identified by specific cell surface markers (including those for dendritic cells and macrophages), different from previously described blood MPS cells, using flow cytometry (Desch et al., 2016). The discovery of these tissue subsets, including the alveolar macrophages, suggests that external stimuli such as cytokines, different locations and varying local
environments, may influence cell function. Therefore in disease such as COPD, the influences on these cells may be altered, for example in the case of macrophages, whereby phagocytosis is one of the functions that becomes defective (Singh and Donnelly, 2016).

Figure 1.4: Interactions between cells and cell mediators in COPD. Irritants such as inhaled cigarette smoke activate epithelial cells and macrophages to release chemotactic factors including CC-chemokine ligand 2 (CCL2), which acts on CC-chemokine receptor 2 (CCR2) to attract monocytes, CXC-chemokine ligand 1 (CXCL1) and CXCL8, acting on CCR2 to attract neutrophils and monocytes (which differentiate into macrophages in the lungs) and CXCL9, CXCL10 and CXCL11, which act on CXCR3 to attract T helper 1 (Th1) cells and type 1 cytotoxic T (Tc1) cells. These inflammatory cells along with macrophages and epithelial cells release proteases including matrix metalloproteinase 9 (MMP9) which cause elastin degradation and emphysema. Neutrophil elastase causes mucus hypersecretion. Epithelial cells and macrophages release transforming growth factor-β (TGFβ), to stimulate fibroblast proliferation resulting in fibrosis in the small airways. (Barnes, 2008)
1.3.2 Cytokines

The recruitment of inflammatory cells (eosinophils, neutrophils, macrophages and lymphocytes) to the airways in asthma and COPD triggers amongst other factors, the specific release of small proteins called cytokines that activate and coordinate cell signalling in the immune response of these diseases. Structural cells such as epithelial and smooth muscle cells also secrete cytokines in the immune response. Cytokines act through cell-surface receptors, targeting their own cell type from which they were released (autocrine), nearby cells (paracrine) or distant cells through the lymph (endocrine). Excessive or insufficient production of particular cytokines may contribute to the pathophysiology of asthma and COPD and they are therefore, useful to study in-vitro.

1.3.2.1 Tumour necrosis factor (TNF)-α

The pleiotropic cytokine TNFα was first isolated as a cytotoxic factor from a human myeloid cell line and thereafter, found to be produced by a wide variety of cell types in response to inflammatory stimuli – its major involvement in growth regulation, differentiation, regulation of apoptosis, inflammation, autoimmune diseases and its response to bacterial, viral, fungal and parasitic infections has been identified (Aggarwal, 2000). TNFα was one of the first of many members of what is coined the TNF superfamily, including inhibitors and homologues and their receptors. It is produced primarily by activated macrophages, NK, T and B-cells, in response mainly to bacterial lipopolysaccharides, and its ligands bind to the membrane receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), being expressed in either all cells or endothelial and immune cells, respectively. All TNF superfamily members activate the transcription factor NF-κB, which is a key component of TNF signal transduction, and implicated in many processes including, suppression of apoptosis, cell survival, proliferation, inflammation and metastasis. Cells exposed to TNF undergo both pro-apoptosis and anti-apoptosis processes simultaneously, and proliferation is also induced in some cells, suggesting different pathways are concurrently activated under TNF signalling (Gaur and
Aggarwal, 2003). This differential regulation of response to TNF depends on binding of the receptors TNFR1 or TNFR2 and occurs in various cell types, in normal and diseased cells. Cell culture and knockout studies in mice have so far revealed that TNF induced pro-inflammatory and programmed cell death pathways, associated with tissue repair, are largely mediated through TNFR1, whereas signalling promoting tissue repair and angiogenesis are mainly TNFR2 mediated, although the latter is less well characterised (Bradley, 2007). TNFα receptor-ligand interaction can cause extensive intracellular signalling leading to induction of the mitogen activated kinase (MAPK) pathways, along a kinase phosphorylation cascade (Liu, 2005) which can eventually activate NF-κB, increasing the transcription of other pro-inflammatory cytokines and immune regulatory proteins such CXCL8, IL-6 and TNFα itself (Brightling et al., 2008).

The consequences of an imbalance of TNFα production, or dysregulation, can contribute to the pathogenesis of inflammatory disease conditions including asthma and COPD. In the airways of patients with asthma, BAL fluid mRNA (Ying et al., 1991) and mast cell protein (Bradding et al., 1994) levels of TNFα were found to be increased. In bronchial epithelial cells and mast cells from bronchial biopsy specimens, TNF immunostaining was detected in asthmatic specimens (Ackerman et al., 1994). In young patients with asthma compared with control subjects, increased sputum TNFα was associated with severity of airway hyperresponsiveness (Obase et al., 2001). Normal subjects who inhaled recombinant TNFα developed airway hyperresponsiveness and airway neutrophilia (Thomas et al., 1995) and in patients with mild asthma, sputum neutrophils and eosinophils increased in number (Thomas and Heywood, 2002) suggesting TNFα may contribute to airway inflammation in asthma. In patients with severe asthma, TNFα was increased in BAL fluid and in bronchial mRNA expression compared with patients with non-severe asthma and asthma symptoms were additionally improved with the use of an anti-TNFα treatment, identifying TNFα as a therapeutic target in this disease (Howarth et al., 2005). In a clinical trial, peripheral blood monocytes from patients with refractory (severe) asthma, compared to control subjects, membrane bound TNFα, TNFR1 and TNFα-converting enzyme (TACE) were increased in expression at baseline. Anti-TNFα
treatment reduced membrane bound TNFα expression, reduced airway hyperresponsiveness and improved asthma symptoms, as compared with placebo (Berry et al., 2006).

TNFα also plays a central role in the pathophysiology of COPD. In smokers compared with non-smokers, airway macrophages produced more TNFα and the anti-inflammatory IL-10 (Lim et al., 2000). Also in smokers versus non-smokers, BAL fluid TNFα was increased although not significantly but other pro-inflammatory cytokines and chemokines were significantly higher in expression (IL-6, CXCL8, IL-1β) (Kuschner et al., 1996). These data indicate that cigarette smoking is likely to directly initiate cytokine/chemokine release in COPD (Chung, 2001). Compared with non-smoking subjects, TNFα and CXCL8 levels and neutrophil numbers were increased in induced sputum from patients with COPD (Keatings et al., 1996). Also in induced sputum, patients with COPD displayed increased concentration of TNFα and CXCL8 correlating with increased sputum neutrophils, compared with healthy controls (Daldeegan et al., 2005).

1.3.2.2 Interleukin 6 (IL-6)

Human IL-6 was originally identified in the culture supernatant of mitogen or antigen stimulated peripheral mononuclear cells, as a T-cell derived cytokine that induced the final maturation B-cells into antibody producing cells. It has since been found to be involved in a wide variety of biological activities in various tissues and cells (Kishimoto, 1989). In response to infection or tissue injury, IL-6 is synthesised and activates an acute immune response. IL-6 induces differentiation of activated B-cells into plasma cells and also acts as a growth factor for hybridoma and myeloma cells. Additionally, IL-6 induces naïve CD4⁺ T-cells into specific effector T-cell subsets. IL-6 stimulates acute phase production of C-reactive protein (CRP) and serum amyloid A (SAA) which are biomarkers of inflammation and mainly regulated by IL-6 (Tanaka and Kishimoto, 2014).
IL-6 production can be triggered by other cytokines including IL-1β and TNFα, in addition to bacterial or viral infections and can be synthesised by (but not limited to) monocytes, macrophages, lymphocytes, endothelial cell and some tumour cells. Its signalling occurs upon IL-6 binding to the IL-6 receptor (IL-6R) which exist in two forms – the membrane bound and soluble forms. Membrane bound IL-6R is only expressed in leukocytes and hepatocytes whereas the soluble form, sIL-6R is expressed by hepatocytes, neutrophils, macrophages and some CD4+ cells. Both forms of receptor signalling require mediation by transmembrane glycoprotein, gp130, and the consequent IL-6/receptor complex of either form will activate identical intracellular pathways. These include the tyrosine kinases (JAK1 and JAK2) which lead to phosphorylation of ‘signal transducers and activators of transcriptions’ 1 and 3 (STAT1 and STAT3), as well as the RAS-MAPK pathway, all of which are involved in tumour functions, in addition to inflammatory and autoimmune diseases (Yao et al., 2014).

As with all pro-inflammatory cytokines, the dysregulation and overproduction of IL-6 can contribute to the pathogenesis of various inflammatory diseases such as rheumatoid arthritis (RA) - increased amounts of IL-6 were reported to be produced in the synovial tissues of the joints of patients with RA (Kishimoto, 2010). IL-6 and CXCL8 expression were increased in bronchial epithelium of patients with asthma compared to normal subjects (Marini et al., 1992). Also in patients with asthma, levels of serum IL-6 (Yokoyama et al., 1995) and serum sIL-6R (Yokoyama et al., 1997) were elevated compared to control subjects. IL-6 levels in BAL fluid from patients with ‘active’ asthma were significantly higher than those from patients with stable asthma, healthy non-smokers or patients without asthma and on mechanical ventilation (Tillie-Leblond et al., 1999). In patients with ‘intrinsic’ (more severe asthma) compared with allergic (non-active) asthma, BAL fluid IL-6 levels were higher (Virchow et al., 1996) and in induced sputum from patients with mild allergic asthma compared with healthy subjects, IL-6 levels were increased, but not IL-1β nor TNFα (Neveu et al., 2010). These studies suggest that the presence of IL-6 in the airways of asthmatic patients may
be independent of inflammation, and be more associated with an “activated” state of pulmonary lung cells (Rincon and Irvin, 2012).

In patients with COPD, exacerbations increased serum IL-6 levels and elevated plasma fibrinogen, which is associated with increased risk of coronary heart disease (Wedzicha et al., 2000). In induced sputum from patients with COPD exacerbation (severe COPD), IL-6 as well as CXCL8 and TNF were significantly increased compared with patients with mild-moderate COPD. IL-6 levels also correlated with lung function and disease duration (Hacievliyagil et al., 2006). In another study, in patients with COPD, levels of sputum IL-6 that increased over time, inversely correlating with a faster decline in lung function therefore suggesting a role for IL-6 in COPD to be marker of rate of inflammation (Donaldson et al., 2005; Grubeck-Jaworska et al., 2012). A cytokine biomarker profile of patients with COPD, or cystic fibrosis, compared with healthy subject showed several pro-inflammatory cytokines to be increased in sputum, including IL-6, CXCL8 and monocyte chemoattractant protein (MCP)-1 and these cytokine levels demonstrated significant negative correlation with FEV₁ and FVC lung function parameters (Eickmeier et al., 2010), suggesting pulmonary inflammation can be assessed by cytometric analysis of induced sputum.

1.3.3 Chemokines

Chemokines are a subset of cytokines that have chemoattractant capabilities - they are able to attract particular cell types along a chemical gradient via chemotaxis. The overwhelming majority of chemokines are split between two subfamilies, distinguished according to the arrangement of the first two cysteines in their molecular structure, which are either separated by one amino acid (CXC chemokines) or adjacent to each other (CC chemokines). CXC chemokines are chemoattractant for mainly neutrophils and monocytes whereas CC chemokines induce the migration of monocytes, NK cells and dendritic cells (Baggiolini et al., 1995). Two further subfamilies of chemokines are C
chemokines (lacking cysteines one and three of the typical chemokine structure) and CX3C chemokines (cysteines are separated by three amino acids) – the only member of which is fractalkine (Zlotnik and Yoshie, 2000).

Chemokines exert their effects through over 20 distinct G protein coupled seven-transmembrane receptors. Most chemokine receptors are able to bind to multiple ligands (e.g. CCR3 can bind CCL5, CCL7, CCL8, CCL11, CCL13, CCL24 and CCL26) whereas some chemokines can bind to multiple receptors (e.g. CXCL8 can bind to CXCR1 and CXCR2) and a few chemokines and chemokine receptors demonstrate exclusive binding. Chemokines bind to the extracellular portion of the chemokine receptor activating the heterotrimeric G protein to dissociate into its subunits. This triggers firm adhesion of the leukocyte to endothelial cells followed by migration of the cells into the direction of the chemotactic gradient (Palmqvist et al., 2007).

1.3.3.1 CXCL8 (Interleukin (IL)-8)

CXCL8 is a potent neutrophil chemoattractant and activator that was first identified in LPS stimulated monocytes (Baggiolini et al., 1995). CXCL8 acts through the CXCR1 and CXCR2 receptors found exclusively in neutrophils and therefore these cells are recruited exclusively in specific inflammatory processes (Baggiolini, 2001). However, CXCL8 can be also be produced by monocytes, T-cells, NK cells and additionally, endothelial cells, fibroblasts and epithelial cells. CXCL8 production is induced by a variety of stimuli including the pro-inflammatory cytokines IL-1 and TNFα, bacterial products such as LPS and viruses (Mukaida, 2003). Production of CXCL8 is controlled at the gene transcription level as its promoter contains binding motifs for the transcription factors, NF-κB, activator protein-1 (AP-1) and CAAT/enhancer binding protein (C/EBP) (Mukaida, 1994).

CXCL8 production is readily associated with inflammatory disease and its role in the pathophysiology of asthma and COPD has been reported numerously. In the airways of patients with mild to moderate asthma, elevated IL-5 production associated with increased eosinophilia is the
classic phenotypical presentation. In severe asthma, it has been shown that neutrophilia is more common, driven by increased CXCL8 production. Fahy and colleagues were of the first to describe this phenomenon – in induced sputum from patients with acute severe asthma, neutrophils predominated more frequently than eosinophils and this was associated with high CXCL8 levels (Fahy et al., 1995). In another study, sputum neutrophils were increased and associated with elevated levels of CXCL8 in patients with severe asthma compared with patients with mild asthma. In the same study, eosinophil numbers were increased in mild and severe asthma, however, eotaxin (IL-5) levels were highest in sputum from patients with mild asthma therefore suggesting a role for neutrophils (and their recruiter, CXCL8) in severe asthma (Jatakanon et al., 1999). Similarly, there were significantly higher neutrophil numbers, positively correlating with increased CXCL8 levels, in tracheal aspirates from patients with acute severe asthma (Ordoñez et al., 2000), induced sputum from patients with persistent asthma (Gibson et al., 2001), and induced sputum from patients with severe asthma (Shannon et al., 2008), compared with normal subjects or patients with moderate asthma. Serum levels of CXCL8 were also elevated in patients with severe asthma compared with those with mild-moderate asthma, associated with increased number of circulating neutrophils suggesting a CXCL8 to be a marker of systemic inflammation in severe asthma (Silvestri et al., 2006). Monocytes isolated from patients with moderate-severe asthma displayed higher baseline and LPS-stimulated levels of CXCL8 compared with normal subjects (Gangemi et al., 2002). CXCL8 and eotaxin expression in ASM cells was increased in patients with severe asthma compared with moderate asthma (Pepe et al., 2005).

Cigarette smoke induced CXCL8 release from human bronchial epithelial cells (Mio et al., 1997) and A549 cells (human alveolar epithelial cell line) (Masubuchi et al., 1998). As smoking is one of the main causes of COPD, in BAL fluid from smokers compared with non-smokers, CXCL8 was elevated (Kuschner et al., 1996; Mio et al., 1997) including neutrophils (Morrison et al., 1998). Furthermore, as COPD is possibly considered a neutrophilic disease, it is as no surprise that several groups have shown increased CXCL8 associated with neutrophilia in patients with COPD compared
to control subjects, in induced sputum (Keatings et al., 1996; Yamamoto et al., 1997), BAL fluid (Nocker et al., 1996; Pesci et al., 1998) and PBMC (Khorasani et al., 2015). In the bronchiolar epithelium (de Boer et al., 2000) and peripheral lung tissue (Tomaki et al., 2007) of patients with COPD, CXCL8 mRNA levels were elevated compared to control subjects.

In exacerbation of COPD, more common in patients with advanced COPD and commonly associated with bacterial or viral infection, further activation of the inflammatory and immune response and immune cells is triggered (Chung and Adcock, 2008). Plasma levels of CXCL8 and IL-6 were increased in patients with COPD exacerbation compared with recovery, correlated with worsening lung function (Pinto-Plata et al., 2007). Bronchial biopsy tissue from patients who had an acute COPD exacerbation showed increased neutrophilia associated with elevated levels of CXCL8, IL-5 and the receptor CXCR2 (but not CXCR1), compared with patients with stable COPD and smoking controls (Qiu et al., 2003), suggesting differential roles for the receptors depending on the condition of COPD.

### 1.3.4 Cell Signalling pathways involved in severe asthma and COPD

To orchestrate the pathogenesis of severe asthma or COPD there are intracellular mechanisms that regulate inflammatory mediator production, inflammatory cell survival and cell apoptosis. Such cell signalling (signal transduction) pathways can operate through kinase cascades and it has been found that specific pathways are more activated within differing inflammatory diseases.

#### 1.3.4.1 Mitogen-activated Protein Kinase (MAPK) Pathways

MAPK pathways are activated by varying extracellular stimuli with specific biological consequences including cell proliferation, gene expression, cell survival and apoptosis. It is a three-
member protein kinase cascade whereby MAPKs are phosphorylated and consequently activated by MAPK kinases (MKKs). MKKs are typically dual-specificity protein kinases activating the MAPKs through threonine and tyrosine residue phosphorylation. MKKs are also subsequently phosphorylated and activated through serine or threonine kinases which are referred to as MKK kinases (MKKKs) (Garrington and Johnson, 1999). Within the MAPK family three distinct subgroups have been identified in mammals to date: extracellular signal-regulated kinases (ERKs); c-jun N-terminal kinases (JNK) and the p38 MAPKs. ERKs can be further subdivided into the classic ERKs (ERK1 and ERK2) or the larger ERKs (e.g. ERK5) dependent on the size of the extended carboxy-terminal sequence. ERK1/2 families typically respond to growth factors and mitogens to induce cell growth and differentiation. JNKs are activated by environmental stresses (ionising radiation, heat, oxidative stress and DNA damage), inflammatory cytokines and growth factors and participate in apoptosis, inflammation, cytokine production and metabolism (Morrison, 2012). p38 MAPK members include p38α, p38β, p38γ, and p38δ and are strongly activated by environmental stresses and inflammatory cytokines and are also known as the Stress-Activated Protein Kinases (SAPKs) (Cuenda and Rousseau, 2007).

1.3.4.2 p38α MAPK

The first member of the p38 MAPK family was identified by four separate research groups as a 38 kDa protein (p38) and of the four p38 MAPK subgroups, p38α (MAPK14) is the best characterised and has been shown to be most physiologically relevant in inflammatory responses. The other p38 MAPK family members (p38β, p38γ, and p38δ) are approximately 60% identical in their amino acid sequences. Human p38α MAPK was originally identified in LPS stimulated human monocytes as the molecular target of the pyridinyl imidazole class of compounds that inhibited inflammatory cytokines such as IL-1 and TNFα (Lee et al., 1994). p38α MAPK is ubiquitously and
substantially expressed in most human cell types whereas the other isoforms are expressed in a more tissue specific manner (Cuenda and Rousseau, 2007).

Figure 1.5: The p38 MAPK signalling pathway Different environmental stresses, growth factors and inflammatory cytokines are amongst the stimuli that can activate the p38 MAP Kinases. Upstream regulators and downstream phosphorylation targets are represented in the figure. (CHOP - C/EBP-homologous protein; DLK1 - dual-leucine-zipper-bearing kinase 1; EEA1 - early-endosome antigen 1; eEF2K - eukaryotic elongation factor 2 kinase; eIF4E - eukaryotic initiation factor 4E; HMG-14 - high-mobility group 14; NHE-1 - Na+/H+ exchanger 1; PLA2 - phospholipase A2; PSD95 - postsynaptic density 95; Sap1 - SRF accessory protein 1; STAT - signal transducer and activator of transcription; TAO - thousand-and-one amino acid; TPL2 - tumour progression loci 2; TTP - tristetraprolin; ZAK1 - leucine zipper and sterile-α motif kinase 1; ZNHIT1 - zinc finger HIT-type 1. (Cuadrado and Nebreda, 2010)
1.3.4.2.1 Activation, downregulation and substrates of p38α MAPK

It has been proposed that upstream regulators of MAPK kinases, MKK3 and MKK6, can activate p38 MAPK, although this activation is differential dependant on the p38 isoform (Parker, 1998). Additionally MKK4, an upstream kinase of JNK, has also been shown to aid activation of p38α and p38δ in specific cell types (Jiang et al., 1997). This suggests p38 isoforms are activated and specifically controlled by varying upstream regulators. A diversity of MKK kinases also activate p38 including TAK1, ASK1/MAPKKK5 and MEKK4 in addition to low molecular weight Rho GTP-binding proteins such as Rac1 and Cdc42 (Zarubin and Han, 2005).

An MKK-independent mechanism of activation of p38α has also been proposed involving TAB1 (transforming growth factor-β-activated protein kinase 1 (TAK1)-binding protein) mediated auto-phosphorylation of p38α (Ge et al., 2002). TAB1 is an adaptor protein that has no known catalytic activity (Johnson and Lapadat, 2002). This unusual mechanism has been further described in mouse and rat ischemic heart models (Tanno et al., 2003; Li et al., 2005; DeNicola et al., 2014) suggesting therapeutic relevance in myocardial injury. Furthermore these reports describe p38 MAPK activation mechanisms which may vary under different physiological or pathological circumstances.

p38 MAPK activity is ‘switched off’ or downregulated by dephosphorylation through protein phosphatases such as protein phosphatase (PP) 2A that target the MAPK activation loop threonine and tyrosine residues individually (serine/threonine residues or tyrosine residues). Dual-specificity phosphatases (DUSPs) also known as MAPK phosphatases (MKPs) can dephosphorylate both threonine and tyrosine residues of the MAPK groups (English et al., 1999). MKP1, 4, 5 and 7 can dephosphorylate p38α and p38β MAPK, and JNKs (Cuadrado and Nebreda, 2010).

Downstream substrates of p38α are numerous and the use of chemical inhibitors and more recently, targeted deletion of the pathway components, has allowed better identification and definition of p38 MAPK substrates. p38α substrates include protein kinases such as MAPKAP-
K2/MK2 (MAPK-activated protein kinase 2), MK3, MSK1 (mitogen and stress-activated kinase 1) and MSK2. MSK1 and MSK2 are important for the rapid induction of immediate-early genes, responding to stress or mitogen stimuli, through chromatin remodelling or transcription machinery recruitment. MK2 and MK3 are involved in mainly post-transcriptional control of gene expression. Other p38α substrates include nuclear proteins and cytosolic proteins, defining many functions for p38α within the cell such as protein degradation, mRNA stability, chromatin remodelling or apoptosis (Figure 1.5) (Cuadrado and Nebreda, 2010).

1.3.4.2.2 Role of p38α MAPK in inflammation

Inhibition of p38 MAPK by pyridinyl imidazole compounds has elucidated its role in inflammation. The first study to report this observation included a pharmacological screen of compounds that modulated TNFα and IL-1 production from an LPS-stimulated human monocytic cell line and p38 MAPK inhibition by was found to inhibit cytokine production (Lee et al., 1994). This suggested p38 MAPK was critical for pro-inflammatory cytokine production. Further studies using improved pyridinyl imidazole compounds, SB203580 and SB202190, confirmed p38α and p38β MAPK (the specific isoforms blocked by these inhibitors) as crucial for the production and activity of pro-inflammatory TNFα, IL-1, IL-6 and CXCL8 in various cell types, including macrophages, monocytes, synovial and endothelial cells (Schindler et al., 2007). Another p38α selective inhibitor SD282 for example, inhibited LPS induced TNFα and granulocyte macrophage colony-stimulating factor (GM-CSF) release from macrophages originating from smokers (Smith et al., 2006).

Knockout mice models have also been used to uncover the in-vivo differences in function between the p38α and p38β MAPK isoforms, as the inhibitors block activities of both simultaneously. Four reports described p38α deficiency to result in embryonic lethality but this also, however, revealed roles for p38α in placental development and erythropoietin expression (Allen et al., 2000; Adams et al., 2000; Mudgett et al., 2000; Tamura et al., 2000). p38β mice were found to be
viable and interestingly, in p38β−/− knockouts, MK2 and MSK1 activation (downstream substrates of p38α MAPK) was normal as was the transcription of immediate-early genes and LPS-induced cytokine production therefore suggesting p38α, but not p38β, as the dominant isoform involved in the immune response (Beardmore et al., 2005). p38γ or p38δ knockouts were viable as well as double knockouts of p38γ and p38δ (Sabio et al., 2005).

In addition to inhibition and knockout studies, animal models of inflammatory diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), atherosclerosis, asthma and COPD, have also been used to further confirm the relationship between inflammation and p38 MAPK (Gupta and Nebreda, 2015). In a mouse model for asthma, p38 inhibitor SB2309063, selective for p38α and p38β, inhibited LPS-induced TNFα production (Underwood et al., 2000). In another asthma mouse model, respirable anti-inflammatory p38α MAPK antisense oligonucleotide reduced ovalbumin (OVA)-induced: eosinophil numbers; IL4, IL-5 and IL-13 levels; lung tissue eosinophilia, airway mucus hypersecretion and airway hyperresponsiveness (Duan et al., 2005). In other mouse models SD282 inhibited bronchial hyperresponsiveness and reduced ASM and goblet cell hyperplasia in OVA-induced, allergen exposed animals (Nath et al., 2006) and ozone-induced airway hyperresponsiveness, lung neutrophilia and cyclooxygenase-2 (COX-2), IL-6 and IL-1β mRNA expression (Williams et al., 2008). In an acute lung inflammation induced mouse model, a new generation p38 MAPK inhibitor, LASSBio-998 inhibited LPS-induced TNFα and IL-1β production as well as decrease neutrophilia of the lung tissues (Lima et al., 2011). In a COPD mouse model, using tobacco smoke to induce airway inflammation, SD282 decreased mRNA expression of pro-inflammatory COX-2 and CXCL8 and inhibited tobacco-smoke induced increase of neutrophils and macrophages (Medicherla et al., 2008). More recently, an LPS and cigarette smoke solution was used to mimic COPD in mice. Transgenic mice expressing a constitutive-active form of MKK6 (activator of p38 MAPK) developed enlargement of alveolar spaces, destruction of lung parenchyma (emphysema) and increased BAL cytokine induction compared with wild-type (WT) strongly suggesting the role of p38 activation in the development of COPD (Amano et al., 2014), (Table 1.3).
1.3.4.2.3 Increased p38 MAPK activity in severe asthma and COPD

In ASM cell from biopsies of patients with severe asthma compared with non-asthmatics, there was increased staining of phospho-MAPKAP-K2 (a substrate of p38 MAPK) (Robins et al., 2011). In alveolar macrophages (AM) from patients with severe asthma, p38 MAPK activation was increased compared with non-severe asthmatics and normal subjects (Bhavsar et al., 2008). In epithelial cells from patients with severe asthma compared with those with mild asthma and healthy controls, increased phospho-p38 MAPK staining was detected, correlating with disease severity (Liu et al., 2008). In patients with COPD, p38 MAPK activity was increased in AM and the alveolar walls, compared with control subjects (Renda et al., 2008). Furthermore, phospho-p38 expression was increased in cell-specific manner – B-cells, CD8+ lymphocytes, small airway bronchial epithelial cells and alveolar and sputum macrophages - within the lungs of patients with COPD compared with smoking or non-smoking controls (Gaffey et al., 2013). Moreover, in sputum from patients with COPD, increased p38 MAPK activity correlated with increased CXCL8 expression and decreased lung function (Huang et al., 2013). In addition, PBMC from patients with COPD also displayed increased p38 MAPK activity, compared with smokers (Khorasani et al., 2015). These observations suggest that activated p38 MAPK is involved in the pathogenesis of severe asthma and COPD.
<table>
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<th>Mouse Lines</th>
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<tr>
<td>WT</td>
<td>LPS induced lung inflammation; p38α and p38β MAPK inhibitor (SB239063)</td>
<td>Reduced lung inflammation</td>
<td>Reduced TNFα</td>
<td>(Underwood et al., 2000)</td>
</tr>
<tr>
<td>WT</td>
<td>OVA induced asthma; p38α antisense oligonucleotide</td>
<td>Reduced disease symptoms</td>
<td>Reduced IL-4, IL-5, IL-13 and eosinophil recruitment</td>
<td>(Duan et al., 2005)</td>
</tr>
<tr>
<td>WT</td>
<td>OVA and allergen induced lung inflammation; p38α MAPK inhibitor (SD282)</td>
<td>Reduced lung inflammation</td>
<td>Reduced bronchial hyperresponsiveness, ASM and goblet cell hyperplasia</td>
<td>(Nath et al., 2006)</td>
</tr>
<tr>
<td>WT</td>
<td>Ozone induced lung inflammation; p38α MAPK inhibitor (SD282)</td>
<td>Reduced lung inflammation</td>
<td>Reduced airway hyperresponsiveness, lung neutrophilia, COX-2, IL-6 and IL-1β</td>
<td>(Williams et al., 2008)</td>
</tr>
<tr>
<td>WT</td>
<td>LPS induced lung inflammation; p38 MAPK inhibitor (LASSBio-998)</td>
<td>Reduced lung inflammation</td>
<td>Reduced TNFα, IL-1β and neutrophil accumulation</td>
<td>(Lima et al., 2011)</td>
</tr>
<tr>
<td>WT</td>
<td>Tobacco smoke induced COPD; p38α MAPK inhibitor (SD282)</td>
<td>Reduced lung inflammation</td>
<td>Reduced COX-2, IL-6, neutrophils and macrophage accumulation</td>
<td>(Medicherla et al., 2008)</td>
</tr>
<tr>
<td>S-C-MKK6 CA (transgenic)</td>
<td>Cigarette smoke+LPS induced COPD</td>
<td>Enhanced disease severity</td>
<td>Increased IL-16, CXCL-1, MMP-12, TCA-3, Leptin</td>
<td>(Amano et al., 2014)</td>
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Table 1.3: p38α MAPK signalling in mouse models of asthma and COPD. WT – wild type; (Adapted from Gupta and Nebreda, 2015).
1.3.4.3 MAP Kinase phosphatase (MKP)-1

The MKP family are dual specificity protein phosphatases that are involved in the control of MAP kinase through dephosphorylation of the tyrosine and serine/threonine residues in the active kinase, rendering it inactive. This leads to regulation of downstream processes such as pro-inflammatory cytokine gene transcription. The mammalian MKP family members share common features including an extended active site motif, two short regions in their amino termini that are homologous to sequences in yeast Cdc25 phosphatase catalytic site and a cluster of basic amino acids that are important for binding to MAP kinase. The MKP family are sub-grouped mainly based on their substrate preference but also on their subcellular location. DUSP1 (MKP-1) is expressed exclusively in the nucleus and belongs to subgroup I, on the basis of its exon number and location of active-site motif (Theodosiou and Ashworth, 2002). It was named MKP-1 as it was the first dual specificity phosphatase that dephosphorylated a MAP kinase to be identified in-vivo (Sun et al., 1993). MKP-1 was initially thought to be specific phosphatase for the ERK pathway. However, in a U937 cell line which was modified to conditionally express MKP-1, it was found that p38 and JNK were far more sensitive to inhibition by MKP-1, than ERK (Franklin and Craft, 1997). MKP-1 was also shown to associate directly with p38 MAPK which enhanced the catalytic activity of MKP-1, in-vitro and in-vivo, in human epithelial and human embryonic kidney cell lines (Hutter et al., 2000). Further studies showing MKP-1 preferential deactivation of p38 MAPK have been reported in mouse embryonic cells where MKP-1 knockout did not affect ERK activation after stimulation (Dorfman et al., 1996). In LPS-stimulated primary macrophages from MKP-1−/− mice, prolonged JNK and p38 MAPK activity ensued with little effect on ERK activity (Chi et al., 2006; Zhao et al., 2006). MKP-1 is strongly induced by mitogenic stimulations and genotoxic stresses such as UV irradiation, that potently activate the JNK and p38 MAPK pathways that it also deactivates, and therefore MKP-1 is regarded as an important feedback control mechanism for p38 MAPK and JNK (Figure 1.6) (Wang and Liu 2007).
Figure 1.6: The function of MKP-1. Extracellular stimuli trigger the activation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK) and p38 MAP kinases. Upon activation the MAPKs translocate to the nucleus whereby they phosphorylate and activate transcription factors to initiate gene transcription. The MAPKs can activate MKP-1. MKP-1 protein has the ability to dephosphorylate the MAPKs therefore inactivating MAPK dependent gene transcription. Equally, phosphorylation of MKP-1 protein by the MAPKs can regulate the stability of MKP-1 thus a negative feedback loop is created (Wang and Liu 2007).

MKP-1 deficiency in mice leads to upregulation of pro-inflammatory mediators - in a murine macrophage cell line, MKP-1 inhibition by triptolide prevented activation of JNK and p38 MAPK and overexpression of MKP-1 resulted in accelerated inactivation of both pathways and decreased TNFα and IL-6 production (Chen et al., 2002a). In activated murine cell lines and primary macrophages, MKP-1 inhibition prolonged p38 MAPK and JNK activation and MKP-1 knockdown increased IL-1β and TNFα (Shepherd et al., 2004). Although MKP-1⁻/⁻ mice used for knockout studies were able to
reproduce and grow normally compared with wild-type animals, they did display numerous physiological alterations at baseline and developed distinct disease phenotypes in experimental models (Wancket et al., 2012). In primary macrophages from MKP-1−/− mice, LPS induced prolonged p38 MAPK phosphorylation and increased rapid TNFα and IL-6 production compared with wild-type mice (Zhao et al., 2005; Zhao et al., 2006; Chi et al., 2006). Systemic pro-inflammatory cytokines levels were also increased in MKP-1−/− mice, as well as Toll-like receptor (TLR)-induced p38 activation (Salojin et al., 2006). The half-lives of IL-6, IL-10 and TNFα mRNAs were significantly reduced in macrophages from MKP-1−/− mice compared with wild-type, suggesting MKP-1 regulation of cytokines at the level of signalling of mRNA stability (Yu et al., 2011). Exposure of MKP-1−/− mice to Gram-positive bacterial products increased cytokine production and inducible nitric-oxide synthase expression, in addition to a higher mortality rate, increased neutrophil infiltration in the lungs and more severe organ damage (Wang et al., 2007).

MKP-1 has been implicated in human inflammatory diseases with altered expression in asthma, rheumatoid arthritis, cancer and sarcoidosis. MKP-1 has also been found to contribute to depression (Duric et al., 2010) and multiple sclerosis (Eljaschewitsch et al., 2006). Fewer studies, however, have been performed in human cells to investigate of the role of MKP-1 in the regulation of pro-inflammatory gene expression. In a human lung epithelial cell line it was found that MKP-1, negatively regulated the cytokine induced expression of IL-6, CXCL8 and COX-2 through p38 MAPK (Turpeinen et al., 2010). In human ASM cells, TNFα induced MKP-1 and p38 MAPK with p38 regulating MKP-1 mRNA stability and therefore confirming a negative feedback loop in these cells and mechanism whereby pro-inflammatory cytokine expression is regulated (Manetsch et al., 2012). CS upregulate MKP-1 expression and this is believed to be one of the modes of action through which CS exert their anti-inflammatory effects (Lasa et al., 2002; Clark, 2003). However, despite the use of high concentrations of oral CS, which are meant to induce anti-inflammatory targets such as MKP-1, patients with severe asthma do not gain the benefits of the therapy. In bronchial biopsies from allergen exposed patients with mild asthma, MKP-1 mRNA is upregulated, but not to significance, by
inhaled CS treatment compared with placebo (Kelly et al., 2012). In LPS-stimulated alveolar macrophages from patients with severe asthma (who were treated with high dose CS), p38 MAPK activity is increased compared with non-severe asthma and this was associated with a downregulation in CS+ LPS stimulated MKP-1 mRNA expression (Bhavsar et al., 2008). In AM and PBMC from patients with asthma, decreased CS-induced MKP-1 mRNA expression correlating with increasing body mass index was shown, compared with subjects without asthma (Sutherland et al., 2008). In contrast, in ASM cells from patients with severe asthma compared with non-asthmatic subjects, baseline MKP-1 mRNA expression was increased. The proposed explanation by the authors is that although MKP-1 is induced in these cells, it may not be post-translationally active, as evidenced by the observation that the same cells displayed increased MAPKAP-K2 activity – a downstream substrate and marker of active p38 MAPK – compared with normal subjects (Robins et al., 2011).

1.3.4.4 Nuclear Factor-kappa B (NF-κB)

The inducible transcription factor NF-κB is crucial in its role in the immune system and broadly influences the gene expression to impact cell survival, differentiation and proliferation, the dysregulation of which leads to severe consequences, in the form of disease, for example. Aberrant activation of NF-κB has been associated with cancer, neurodegenerative diseases and cardiovascular disease amongst others but it is most understood in chronic inflammatory disease (such as: rheumatoid arthritis; inflammatory bowel disease; asthma; COPD) whereby its activation drives pro-inflammatory cytokine production and pro-inflammatory cytokines can activate NF-κB (Hayden and Ghosh 2012).
1.3.4.4.1 Structure and activation of NF-κB

First identified in murine B-lymphocyte cells, NF-κB is present in most cell types and consists of two subunits which belong to the Rel family – p65 (Rel A) and p50 (NF-κB1) are the usual subunits that are ubiquitously expressed and form a heterodimer when NF-κB is in a resting state. Other Rel proteins, Rel-B, p52 and c-Rel may also form part of the heterodimer with differential regulatory effects (Barnes and Adcock, 1997). Inactive resting NF-κB is cytoplasmic and bound to varying isoforms of the inhibitory protein, IκB, (IκBα, IκBβ, IκBγ, IκBδ and IκBε) which masks the nuclear localisation sequence (NLS), preventing NF-κB from entering the nuclei (Baldwin, 1996). It is generally believed that the IκB isoforms associate with specific Rel protein dimers for example IκBα and IκBβ associate with the p65-p50 or p50-c-Rel dimers (Edwards et al., 2009). When a cell is stimulated, specific kinases phosphorylate and ubiquinate IκB protein and it is rapidly degraded by the proteasome. This releases NF-κB from the IκB and allows its translocation into the nucleus where it binds to specific sequences in the promoter region of target genes. In asthma and COPD, some of the stimuli of NF-κB include cytokines (TNFα, IL-1β), oxidants (hydrogen peroxide, ozone) and viruses (rhinovirus, influenza) and the pro-inflammatory target genes of NF-κB in these diseases include cytokines (TNFα, IL-1β, IL-6), chemokines (CXCL8, eotaxin), enzymes (inducible nitric oxide synthase, 5-lipoxygenase) and adhesion molecules (Barnes and Karin, 1997).

IκB phosphorylation and the activation of the NF-κB Rel proteins can be classical (canonical) or alternative (noncanonical). In the classical pathway, the IκB kinase (IKK) complex phosphorylates IκB protein. IKK is formed of two catalytic subunits (IKK-α/IKK-1 and IKK-β/IKK-2) and a regulatory subunit IKK-γ/NF-κB essential modulator (NEMO). IKK-β possesses 20-fold more activity than IKK-α in phosphorylating IκB and is therefore more important in NF-κB activation, in the classical pathway. The classical pathway incorporates signalling from Toll-like receptors / IL-1R family members, TNFR and T-cell receptor ligation (Edwards et al., 2009). The alternative NF-κB pathway is induced by specific TNF cytokine family member including CD40 ligand and lymphotoxin-β. In contrast to the classical pathway, the alternative pathway, which is believed to be involved in adaptive immunity, is
dependent on IKK-α and is independent of NEMO. IKK-α activation phosphorylates p100 (precursor to Rel protein, p52) and allows nuclear translocation of the p52-RelB heterodimer (Figure 1.7) (Hayden and Ghosh 2012).

Compared with control subjects, NF-κB activity was found to be increased in patients with asthma (Hart et al., 1998) and in patients with COPD and smokers (Di Stefano et al., 2002) based on positive staining for p65 protein in cells from bronchial biopsies. Additionally in PBMC from patients with severe uncontrolled asthma, there was increased expression of p65 and IKK-β proteins correlating with augmented p65-DNA binding affinity, compared with normal subjects (Gagliardo et al., 2003). Furthermore, immunostaining revealed increased NF-κB expression in BAL fluid from patients with severe asthma compared to those with mild asthma (Abdulamir et al., 2009). More recently, in human ASM, induced p65 expression was greater in patients with severe asthma compared with control subjects (Chang et al., 2015).

1.3.4.4.2 Post-translational modifications of NF-κB

A further control mechanism of the NF-κB pathway is through post-translational modifications such as phosphorylation, (small ubiquitin like modifiers) SUMOylation, ubiquitinylation and acetylation which affect the localisation, stability and ability of NF-κB proteins to interact with DNA and other transcriptional cofactors (Hayden and Ghosh, 2012). The NF-κB Rel proteins can often be phosphorylated to influence its activation – p65 phosphorylation may enhance NF-κB transcriptional activation whereas p105 (precursor to p50) can reduce its dissipation into p50 (Naumann and Schidereit, 1994). Acetylation of p65 on two specific lysine sites resulted in differential regulation of distinct biological activities of NF-κB (Chen et al., 2002b).
Figure 1.7: NF-κB signalling and activation The canonical pathways signal through: i) TLR/IL-1 receptors, leading to IRAK activation and IKK-β phosphorylation; ii) intracellular viral receptors including RNA helicases and PKR, which activate IKK-β and iii) the TCR pathway, leading to IKK-α/β activation; TNFR pathway, which signals via TRADD to activate IKK-β. The alternative pathway is induced by CD40-CD40L activation, lymphotoxin-β or RANKL, which leads to activation of NIK and IKK-α. IRAK: IL-1 receptor-associated kinase, PKR: dsRNA-dependent protein kinase R, TNFR: TNF receptor, TRADD: TNFR-1 associated death domain, RANKL: receptor activator of NF-κB ligand, NIK: NF-κB-inducible kinase (Edwards et al., 2009)
1.4 Corticosteroids (CS)

Corticosteroids (CS) are extremely effective at controlling the symptoms of many inflammatory conditions and immune diseases. Synthetic CS, referred to as glucocorticoids or glucocorticosteroids, were developed on the basis of the natural endogenous CS, namely, steroid hormones, which are small lipophilic compounds derived from a common cholesterol precursor. The four major groups of steroids are progestins, androgens, estrogens and corticoids, differing in the carbon atom number they contain, receptors they bind to and biological activities they are involved in. Corticoids are either mineralocorticoids (regulating ion transport) or corticosteroids (regulating metabolism, immunosuppressive and anti-inflammatory effects) (Liberman et al., 2007). The predominant effect of CS as a treatment is to switch off multiple genes, which can encode cytokines, chemokines, adhesion molecules, enzymes, receptors and proteins, which may all be activated in the chronic inflammatory process (Barnes, 2006b).

1.4.1 Side-effects of CS

Although effective, CS can also have additional unwanted side-effects on the body as the administered pharmacological doses mimic a chronic stress response in which the adrenal glands produce high serum cortisol concentrations resulting in the signs and symptoms associated with Cushing's disease (Table 1.4) (Miner et al., 2005). The higher concentrations of oral CS for the treatment of COPD, for example, can also cause skin thinning and easy bruising as well as increase the risk of pneumonia, tuberculosis, bone fractures in these patients (Price et al., 2013). Local side-effects, specifically associated with the use of inhaled CS include bronchospasm, oral thrush, reflex cough and pharyngitis (Dahl, 2006). The most serious systemic side-effect that can occur is suppressed hypothalamic-pituitary-adrenal (HPA) axis function which can arise as CS can downregulate corticotrophin production by the same negative feedback loop that controls endogenous glucocorticoid production, therefore leading to adrenal suppression (Dahl, 2006).
Furthermore, CS are not always effective, as some patients may become CS-insensitive, are inherently CS-insensitive or are even CS-resistant, although the latter scenario is very rare.

<table>
<thead>
<tr>
<th>Side Effect</th>
<th>Clinical Manifestation</th>
<th>Clinical Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor wound healing</td>
<td>Delayed recovery/infection</td>
<td>N/A</td>
</tr>
<tr>
<td>Psychosis</td>
<td>Behaviour</td>
<td>Survey</td>
</tr>
<tr>
<td>Glucose intolerance</td>
<td>Hyperglycaemia and diabetes</td>
<td>Fasting serum glucose, serum insulin, Glucose tolerance test and impaired glucose tolerance test</td>
</tr>
<tr>
<td>Increased fat deposition and redistribution</td>
<td>Weight gain altered</td>
<td>Lipid profiles, weight and body–mass index</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td>Blood pressure</td>
</tr>
<tr>
<td>Muscle wasting</td>
<td>Weakness/falls</td>
<td>Nitrogen wasting and strength</td>
</tr>
<tr>
<td>Adrenal suppression</td>
<td>Impaired stress response and asthenia</td>
<td>Serum cortisol and Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>Fractures</td>
<td>Osteocalcin, bone mineral density and urinary collagen peptides</td>
</tr>
<tr>
<td>Suppressed growth hormone secretion</td>
<td>Stunted growth</td>
<td>Short stature</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>Susceptibility to infections</td>
<td>Leukocytopenia</td>
</tr>
</tbody>
</table>

Table 1.4: Corticosteroid use and some associated side effects. The table indicates the clinical markers and manifestations associated with the side effects that often appear with chronic CS use (Adapted from Miner et al., 2005).
1.4.2 Mechanisms of action of CS

Increased expression of pro-inflammatory genes is a recognised hallmark of chronic inflammatory diseases such as rheumatoid arthritis, asthma and COPD, with the pattern of inflammation, and involvement of inflammatory mediators, differing between each disease. Increased mediator expression is primarily controlled at the level of gene transcription through the transcription factors such as NF-κB and AP-1 which when activated, play a critical role in the amplification and longevity of the inflammatory process.

1.4.2.1 Chromatin remodelling

Chromatin consists of highly organised DNA, histone proteins and nucleosomes, which form the backbone of the chromosome. The histones are imperative regulators of the expression of genes that can determine their activity or suppression. The nucleosomes are particles of DNA associated with an octamer of two molecules each of the core histone proteins, (H2A, H2B, H3 and H4) around which 146 base pairs of DNA are wound. Whilst the cell is in a resting state, the DNA around the basic core histones is tightly wound which prevents binding of the enzyme RNA polymerase II and thus prevents activation of mRNA and gene transcription. This ‘closed’ chromatin structure is associated with suppression of gene expression. Gene transcription only occurs when the chromatin structure ‘opens’ and the DNA unwinds allowing the association with RNA polymerase II and basal transcription machinery (Barnes et al., 2005). Remodelling of the chromatin structure by enzymatic post-translational modification of the core histone protein (usually by acetylation of the lysine residues located on histone N-terminal tails) can affect the suppression or expression of genes (Figure 1.8)(Barnes, 2006b).
1.4.2.2 Histone acetyltransferases (HATs), histone deacetylases (HDACs) and coactivators

When the NF-κB or AP-1 become activated they bind to specific recognition sites in DNA which attracts interaction with coactivator molecules such as cAMP response element binding (CREB)-binding protein (CBP), p300 and p300/CBP-associated factor (PCAF). These coactivator molecules all possess intrinsic histone acetyltransferase (HAT) activity and as such can control gene transcription (Ogryzko et al., 1996). Acetylation of the core histones by HATs allows the chromatin structure to open, for DNA to unwind and be accessible for the basal transcription machinery and RNA pol II, to initiate gene transcription. This process is reversible through the deacetylation of acetylated histones, by histone deacetylases (HDACs), which is generally associated with gene silencing (Gao et al., 2002). The balance between HAT and HDAC activity can therefore control gene transcription.

Figure 1.8: The regulation of gene transcription by histone acetylation. Coactivator molecules such as cAMP response element binding (CREB)-binding protein (CBP), interact with transcription factors such as CREB, AP-1 and NF-κB, which induces their intrinsic HAT activity. This leads to acetylation (Ac) of the core histones, which opens up the chromatin structure and allows binding on RNA polymerase II, initiating gene transcription (Barnes, 2006b).
1.4.2.3 Glucocorticoid Receptor (GR)

CS exert their biological effects through binding to the ubiquitously expressed glucocorticoid receptor (GR) which is a ligand activated transcription factor, able to suppress or repress the expression of target genes (Liberman et al., 2007). GR is a member of the nuclear receptor (NR) superfamily and a modular protein of 777 amino acids, with three major motifs, each related to a distinct function (Giguère et al., 1986). The N-terminus with 421 amino acids, encodes primarily constitutive transcriptional activation function-1 (AF1). The central domain is 65 amino acid residues composed of a highly conserved zinc finger DNA-binding domain (DBD) which is crucial for GR homodimerisation and cofactor interaction. The C-terminus region encodes the ligand-binding domain (LBD) containing the motif for ligand-dependent transcriptional activation function-2 (AF2). The C-terminal is also involved in protein-protein interactions within the cytoplasm. Within those motifs are embedded NLS, which are covered and protected by molecular chaperones when GR is inactive (Zhou and Cidlowski, 2005). Cytoplasmic GR is inactive and associated with molecular chaperones – heat shock protein (hsp) 90 and hsp70 and several immunophilins. CS binding activates the ligand leading to a conformational change in the receptor which triggers the dissociation of the molecular chaperones and exposure of its NLS. A group of nuclear translocation proteins recognise the NLS and shuttle GR into the nucleus and once inside, GR forms homodimers then interacts with specific DNA sequences at target gene promoters known as the glucocorticoid-response elements (GREs) leading to gene transcription (Figure 1.9) (Zhou and Cidlowski, 2005).
Figure 1.9: The anti-inflammatory effects of corticosteroids in the cell. The glucocorticoid ligand binds to the glucocorticoid receptor (GR) in the cytoplasm which results in the translocation of this complex into the nucleus. 

(A) A direct inhibitory action by GR on transcription factor, activator protein-1 (AP-1) leads to pro-inflammatory gene repression. 

(B) GR binding to the glucocorticoid responsive element (GRE) of the promoter region of an anti-inflammatory gene, such as MKP-1 or IL-10, causes anti-inflammatory gene induction. 

(C) An inhibitory interaction of GR with CBP and recruitment of HDAC to promoter regions of inflammatory genes, which results in reduced histone acetylation and subsequent inflammatory gene repression. 

(CBP – CREB binding protein; HDAC – histone deacetylase; hsp90 - heat shock protein 90; IL-10 - interleukin-10; MKP-1 - MAP kinase phosphatase-1). (Hew and Chung, 2010)
1.4.2.3.1 GR transactivation and transrepression

‘Transactivation’ refers to the transcriptional (genomic) regulation in which homodimers of ligand-bound GR bind to the GRE DNA sequences. The GRE consensus sequence is GGAACAnnnTGTTCT, which is an imperfect palindrome containing two half-sites with a critical three base pair spacer. One of each GR homodimer binds to each half-site of the GRE. GR binding to the GRE results in recruitment of RNA pol II and basal transcription machinery and typically transactivation, where CS induce target gene expression (Cain and Cidlowski, 2015). Coactivators and co-regulators in the form of HATs (such as PCAF) and steroid receptor coactivator (SCR)-1 are involved in this process (Jenkins et al., 2001). Direct binding of GR to GREs can also induce ‘transrepression’ which is the repression of target genes (Uhlenhaut et al., 2013). GR can repress genes by ‘tethering’ itself, in an activated monomer form, to other transcription factors such as NF-κB or AP-1 (without itself binding directly to the DNA) and thus reducing their capacity to induce the transcription of pro-inflammatory genes (De Bosscher et al., 2000). There also exist negative GRE (nGRE) sites which mediate gene suppression or cis-repression on target genes and have recently been shown to directly recruit GR, promoting the assembly of a corepressor complex and HDACs to orchestrate CS-dependent repression of specific genes (Surjit et al., 2011). nGREs are generally associated with CS side effects, including genes that regulate the hypothalamic-pituitary axis, bone metabolism and skin structure (Barnes, 2006c). Finally, GR can regulate gene expression of target genes in a ‘composite’ manner whereby direct GR-GRE binding occurs followed by interaction with neighbouring DNA-bound NF-κB or AP-1, (Figure 1.10) (Oakley and Cidlowski, 2013). GR can also signal its effects outside of the nucleus in a rapid non-genomic (transcription-independent) manner by directly modulating signal transduction pathways whereby membrane-bound GR or cytosolic GR can interact with members of the MAPK family, although a detailed mechanism for this process is yet to be fully elucidated (Cruz-Topete and Cidlowski, 2015).
Figure 1.10: Glucocorticoid signalling through GR. When GR is activated in the cytoplasm by ligand binding, three mechanisms of genomic effects can occur following nuclear translocation: (A) direct binding of GR to DNA via GREs and nGREs to ‘transactivate’ or ‘transrepress’ gene transcription; (B) tethering to DNA-bound transcription factors to modulate transcription indirectly; or (C) composite activity of DNA binding with interaction with adjacent DNA-bound transcription factors to affect gene transcription. Furthermore, non-genomic effects of GR can occur following ligand-induced dissociation of the GR multiprotein complex in the cytosol. BTM, basal transcription machinery. (Cain and Cidlowski, 2015).

1.4.2.3.2 Post-translational effects and modifications of GR

CS are able to reverse the effect of mRNA stabilisation of certain pro-inflammatory genes such as TNFα. CS can affect rapid degradation of the mRNA and reduce inflammatory protein secretion. This process may be mediated through increased gene expression of destabilising mRNA proteins such as tristetraprolin (TTP) which can bind to the 3’AU-rich untranslated region of mRNAs (Barnes, 2011; Smoak and Cidlowski, 2006). Post translational modifications (PTMs) represent another layer of the regulatory mechanism of GR activity. PTMs of GR include its ubiquitination, SUMOylation, acetylation, methylation or phosphorylation (Liberman et al., 2007).
1.4.2.3.2.1 Phosphorylation of GR

GR is a phosphoprotein and phosphorylation modulates its activity, occurring in a hormone-dependent manner, at serine/threonine residues located within its DBD (Liberman et al., 2007). There are several serine residues (S113, S134, S141, S143, S203, S211, S226 and S404) within the human GR that have been reported to be phosphorylated by a variety of protein kinases including cyclin-dependent kinases (CDKs), glycogen synthase kinase–3β and the MAPK family (Cruz-Topete and Cidlowski, 2015) (Figure 1.11). In rat neuronal cells, GR phosphorylation on ser224 and ser232 by cyclin-dependent kinase 5 (CDK5) resulted in GR suppression, and these residues correspond to ser203 and ser211, respectively, in humans (Kino et al., 2007). To demonstrate the involvement of p38 MAPK in GR inhibition, Wang and colleagues showed in mouse fibroblast cells that IL-1α induced p38 MAPK activity inhibited CS-GR binding therefore decreasing its function (Wang et al., 2004). Furthermore, in a human epithelial cell line, transfected with constitutively active MKK6 (an upstream activator of p38), GR activation was inhibited and this effect was completely reversed by use of a p38 MAPK inhibitor. This suggested therefore, that sustained specific activation of p38 MAPK resulted in a strong inhibition of GR mediated transcriptional activation (Szatmary et al., 2004).

Ser203, ser211 and ser226 are located in the AF1 region of the N-terminal domain of GR. It has been proposed that phosphorylation at GR ser211 is associated with nuclear translocation and transcriptional activation of GR after hormone treatment and there is some evidence to suggest that phosphorylation at GR ser203 is a determinant of ligand-dependent downregulation of GR (Wang et al., 2002). GR ser211 was found to be a substrate for p38 MAPK in a human lymphoid cell line (Miller et al., 2005) and mutation of GR ser211 to alanine greatly diminished the p38 mediated, GR-induced gene transcription capabilities and apoptosis of lymphoid cells (Miller et al., 2005). In contrast however, ser226 phosphorylation by JNK, inhibited GR transactivation (Rogatsky et al., 1998) and ser226 phosphorylation was found to reduce GR signalling transduction (Chen et al., 2008) (Table 1.5). JNK-mediated phosphorylation of GR ser226 was also reported, which enhanced GR nuclear
export in a human epithelial cell line with a possible role in downregulation of GR-mediated transcription (Itoh et al., 2002). GR ser203 is phosphorylated in both the absence and presence of agonists, whereas phosphorylation of ser211 is only observed upon agonist treatment (Wang et al., 2002). Dexamethasone treatment phosphorylates ser211 (Wang et al., 2002) and ser226 (Lee et al., 2005) before translocation to the nucleus, suggesting that they contribute to transcriptional regulation, whereas the majority of the ser203 was confined to the perinuclear region of the cell (Wang et al., 2002). GR ser404 was found to alter co-factor recruitment, modulating the transcriptional response of GR to attenuate CS signalling (Galliher-Beckley et al., 2008) and GR ser134 was identified as a p38 MAPK-dependent, but ligand-independent site (Galliher-Beckley et al., 2011). Finally, GR phosphorylation at ser203 and ser211 was found to be differentially regulated by p38 MAPK in ASM, with blockade of p38 leading to downregulation of GR dependent gene transcription through decreased phosphorylation of ser203 and not ser211 (Bouazza et al., 2012).

Figure 1.11: Possible post-translational modification sites and domains of the Glucocorticoid Receptor (GR). A – alanine; G – glycine; K – lysine; L – leucine; N – asparagine; S – serine; T – threonine; Y – tyrosine; GCR – glucocorticoid receptor; zf – zinc finger binding domain. (Figure extracted from PhosphoSitePlus®; www.phosphosite.org).
<table>
<thead>
<tr>
<th>Phosphorylation Site</th>
<th>Kinase(s)</th>
<th>Ligand-dependent activity</th>
<th>Ligand-independent activity*</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser134</td>
<td>p38 MAPK</td>
<td></td>
<td></td>
<td>Modulates promoter selectivity; Gene-specific effect on ligand-dependent transcription</td>
<td>Galliher-Beckley et al., 2011</td>
</tr>
<tr>
<td>Ser203</td>
<td>ND (basal and hormone mediated)</td>
<td></td>
<td></td>
<td>Cytoplasmic localization; DNA binding</td>
<td>Chen et al., 2008</td>
</tr>
<tr>
<td>Ser211</td>
<td>ND (hormone mediated)</td>
<td>B; C</td>
<td></td>
<td>Receptor conformational change; Gene-specific effect on transcription</td>
<td>Chen et al., 2008</td>
</tr>
<tr>
<td>Ser211</td>
<td>p38 MAPK</td>
<td>A</td>
<td></td>
<td>Regulation of apoptosis</td>
<td>Miller et al., 2005</td>
</tr>
<tr>
<td>Ser226</td>
<td>ND (hormone-mediated)</td>
<td>B; C</td>
<td></td>
<td></td>
<td>Chen et al., 2008</td>
</tr>
<tr>
<td>Ser226</td>
<td>JNK</td>
<td>B</td>
<td></td>
<td>Enhanced nuclear export (ligand-independent)</td>
<td>Itoh et al., 2002</td>
</tr>
<tr>
<td>Ser404</td>
<td>GSK3β</td>
<td></td>
<td></td>
<td>Enhanced nuclear export; Decreased protein stability; Decreased coregulator interaction; Gene-specific effect on transcription</td>
<td>Galliher-Beckley et al., 2008</td>
</tr>
</tbody>
</table>

Table 1.5: Summary of candidate kinases and functions for some serine phosphorylation sites on GR. ND – not determined; * - defined as activation by kinase signalling cascade and does not refer to basal activity; A – increased expression of a reporter gene; B – decreased expression of a reporter gene; C – decreased endogenous gene expression; (Adapted from Trevino and Weigel, 2013).
1.4.3 Crosstalk between CS, p38 MAPK and MKP-1

The ability of CS to exert their anti-inflammatory effects through GR can occur in a genomic or non-genomic manner. The inhibition of pro-inflammatory MAPK activity by CS is mainly due to the induction of factors that negatively regulate the MAPK signalling (Ayroldi et al., 2012) and this is a form of crosstalk that exemplifies a genomic transrepression mechanism of CS-GR action (Figure 1.11). Dexamethasone is a CS that readily induces MKP-1 to exert its anti-inflammatory effects and this induction has been reported in several cell-types including: i) primary and cell line macrophages (Bhavsar et al., 2008; Chen et al., 2002a; Zhao et al., 2005); ii) primary human ASM cells (Issa et al., 2007; Quante et al., 2008); iii) primary human neutrophils (Mortaz et al., 2008) and iv) primary human epithelial cells (Kaur et al., 2008; Wilson et al., 2009). In mechanistic detail, induction of MKP-1 requires GR activation and CS dimerization (Vandevyver et al., 2012) in addition to p300 recruitment and rapid modulation of the chromatin structure (via acetylation of histone H3 and H4) surrounding the GRE (Shipp et al., 2010). In mice macrophages expressing a conditional GR deletion, dexamethasone was unable to induce MKP-1, however p38 MAPK inhibition overturned this (Bhattacharyya et al., 2007). Additionally, dexamethasone induction of MKP-1 expression and inhibition of its proteasomal degradation were reported to be essential for CS-mediated MAPK inactivation (Kassel et al., 2001).

Upregulation of MKP-1 by dexamethasone leads to the dephosphorylation and inhibition of p38 MAPK activity as reported for example, in Hela cells (Lasa et al., 2002) and human ASM cells (Quante et al., 2008). Furthermore, in MKP-1−/− mouse macrophages, dexamethasone mediated inhibition of p38 MAPK, and several pro-inflammatory genes, was abrogated (Abraham et al., 2006) confirming, in-vivo, in these cells, that MKP-1 is required for CS action. In human bronchial epithelial and alveolar epithelial cell lines, dexamethasone induced MKP-1 correlated with impaired TNFα-stimulated: p38 activity; NF-κB transcription and CXCL8 expression (King et al., 2009). This study suggests that MKP-1 may be involved, at least partially, in the regulation of CS mediated NF-κB...
function. Conversely, in bone-marrow derived mast cells from both MKP-1−/− and wild-type mice, there was no difference in the level of CS-mediated inhibition of ERK-1/2 activity and pro-inflammatory cytokines, and only a slight impairment of p38 activity, suggesting in these cells, GC-mediated anti-inflammatory action is only partially MKP-1 dependent (Maier et al., 2007).

Taken together, these reports propose that deficient MKP-1 expression may be associated with impairment of CS function. In murine models of asthma, ozone-induced airway inflammation and recruitment of pro-inflammatory mediators was associated with increased p38 MAPK activity, decreased CS effectiveness and down-regulation of MKP-1 expression and CS-induced MKP-1 expression (Liang et al., 2013; Bao et al., 2014). In clinical samples from patients with insensitivity to CS-therapy, persistent JNK and p38 activation was displayed compared with those from CS-sensitive control subjects (Bantel et al., 2002). Moreover, in BAL macrophages from patients with severe asthma, increased p38 activity and susceptibility to LPS-induced pro-inflammatory cytokine release, correlated with decreased induced MKP-1 expression, compared to the more CS-sensitive patients with non-severe asthma (Bhavsar et al., 2008).
Figure 1.12: The relationship between CS, MKP-1 expression and p38 MAPK. The anti-inflammatory agents, corticosteroids (CS), bind to the glucocorticoid receptor (GR) to induce MKP-1 expression leading to inhibition of the p38 MAPK cascades and inactivity of the inflammatory response. p38 MAPK can also induce MKP-1 expression, therefore perpetuating the anti-inflammatory effects of the CS-GR binding. (Adapted from Wang and Liu, 2007).
1.4.4 CS-insensitivity in severe asthma

As previously mentioned, treatment of severe asthma is difficult as it is increasingly considered to be a heterogeneous disease, with several causes and environmental factors accounting for its manifestation. The responses of asthmatics to the effects of CS can be categorised into either CS-sensitive or CS-insensitive patients. In severe asthma cohorts, one third of patients are on a daily oral dose of prednisolone and they are termed ‘corticosteroid-sensitive’ as a reduction in steroid maintenance leads to a worsening of asthma control. Conversely, increasing the CS dose improves the control of asthma, therefore the term ‘relative corticosteroid-insensitivity’ is applied as the patients are not entirely resistant to the effects of steroids (Hew and Chung, 2010). Relative CS-insensitivity has been demonstrated in vitro, in AM (Bhavsar et al., 2008; Lea et al., 2015), PBMC (Hew et al., 2006; Rossios et al., 2012) human ASM cells (Chang et al., 2012) from patients with severe asthma compared with those with non-severe asthma, in term of CS suppression of induced pro-inflammatory cytokine release. Additionally, asthmatic smokers also displayed a level of CS-insensitivity compared with non-smokers (Spears et al., 2013). To improve the sensitivity to CS in severe asthma, two approaches have been taken. Developing a ‘dissociated’ CS that gives the beneficial outcome of a steroid without the harmful side-effects has been one approach. The side-effects of excessive steroid use are numerous (Table 1.4) and those taken orally are more potent than those that are inhaled. This is unfortunate for patients with severe asthma who mainly use oral CS such as prednisolone. Another approach taken to improve the effect of CS in severe asthma is to reverse the insensitivity to CS in these patients and ultimately restore CS-sensitivity.

1.4.5 CS-insensitivity in COPD

High doses of systemic CS (oral/intravenous) provide modest benefit in reducing the hospital stay, the time until next exacerbation and hospital re-admission rates of patients with COPD (Niewoehner, 2001; Aaron et al., 2003). However this administration is short-term due to the
harmful side-effects of prolonged systemic CS use (Barnes and Stockley, 2005). Although several large scale studies have shown inhaled CS use to reduce the frequency of exacerbations in patients with stable COPD, the effects on lung function tests were variable and overall there were no consistent statistical differences between treatment and placebo groups (Yang et al., 2012b). CS-insensitivity in cell models of patients with COPD has been previously observed in PBMC (Kobayashi et al., 2013; Rossios et al., 2012), airway lymphocytes (Kaur et al., 2012) and AM (Culpitt et al., 2003; Cosio et al., 2004). Insensitivity to the anti-inflammatory effects of CS is a major barrier to the effective treatment of COPD, therefore similarly with severe asthma, a better understanding of the molecular mechanisms involved in CS-insensitivity will allow for an improvement in the therapeutic strategies for these diseases.

1.4.6 Reversal of CS-insensitivity by p38 MAPK inhibition

The observation of heightened p38 MAPK activity and its possible contribution to CS-insensitivity in severe asthma and COPD marks it as an obvious drug target for inhibition, not only to reduce inflammation but to also improve the anti-inflammatory effects of CS in these diseases.

It has been reported in human epithelial cell line that activated p38 MAPK indirectly targets the GR ligand binding domain to reduce its anti-inflammatory function (Szatmary et al., 2004). Similarly in mouse fibroblast cells, IL-1α mediated inhibition of GR function through activated p38 MAPK was reversed when p38 was inhibited (Wang et al., 2004). These reports suggest that p38 MAPK inhibition can restore GR function. p38 MAPK activation may also increase phosphorylation and phospho-acetylation of the core histones (such as H3) in the promoter regions of NF-κB-dependant genes such as CXCL8 and MCP-1 (Saccani et al., 2002). As previously mentioned (section 1.4.3) MKP-1 downregulation may contribute to CS-insensitivity through increased deregulation of p38 MAPK activity. However as p38 MAPK and MKP-1 are target substrates for each other, inhibition of p38 may rebalance MKP-1 activity to reverse CS-insensitivity. In PBMC from healthy subjects, the
combination of IL-2 and IL-4 transiently induced CS-insensitivity which was completely reversed by p38 MAPK inhibition (Irusen et al., 2002).

### 1.4.6.1 p38 MAPK inhibitors

An initial series of pyridinyl imidazole agents that were screened for their anti-inflammatory potential were the first tools that revealed the role of p38 MAPK in inflammation (Lantos et al., 1984). Subsequently, the refinement of the imidazole template of the p38 MAPK inhibitors, related to the first generation of inhibitors, has improved their selectivity. For example replacement of the pyridine with 2-pyrimidines significantly reduced the inhibitory effects of the imidazoles on cytochrome P450s (related to hepatotoxicity). In addition, kinase selectivity was improved, the effects on the enzymes of the arachidonic acid pathway were reduced and their in-vivo and in-vitro potencies improved (Kumar et al., 2003). The pyridinyl imidazole inhibitors bind competitively at the ATP-binding site and target p38α and β isoforms but not the δ or γ isoforms, with SB203580 being the prototype inhibitor that is selective for both p38α and β MAPK isoforms. Other inhibitors in this category, but that possess more selectivity for the p38α isoform, include: SD282; an indole-5-carboxamide; SCIO469 and SB202190 (Chung, 2011).

### 1.4.6.2 p38 MAPK inhibitors in clinical trials

p38 MAPK inhibitors have been most commonly trialled in rheumatoid arthritis (RA), in phase I and phase II clinical studies. Yet to date, they have only been modestly beneficial in RA with adverse effects reported. For example VX-754 (a first generation p38 inhibitor selective for p38α, β and γ) was dosed orally in patients with active RA and it was well tolerated, producing significant clinical effect compared with placebo. However, adverse effects that were reported with its use included liver toxicity and its crossing into central nervous system (Kumar et al., 2003). Nevertheless, as RA
and chronic lung disease share several similar inflammatory features, these studies can be taken as examples from which lessons can be learned for future p38 inhibition trials.

More recently, clinical trials using p38 MAPK inhibitors in patients with COPD, have been reported. A single oral dose of a second generation p38 inhibitor, mainly selective for p38α (Dilmapimod / SB681323), inhibited the activity of HSP27 (a p38 MAPK substrate) and the production of TNFα in whole blood (Singh et al., 2010). Further studies in these patients revealed a reduction in IL-1β gene expression in both whole blood and sputum samples, post-dilmapimod administration (Betts et al., 2015). In another clinical trial using a different, diarylpyridinone p38α selective inhibitor, PH-797804, lung function was improved and CRP levels suppressed in a sustained manner, in patients with moderate to severe COPD, compared with placebo. This occurred without the usual adverse effects on liver function, associated with other p38 MAPK inhibitors (MacNee et al., 2013). Moreover, p38 MAPK inhibitor Losmapimod reduced plasma fibrinogen levels in patients with COPD and when administered with an inhaled salmeterol/FP combination, significantly improved lung function versus placebo (Lomas et al., 2012).

1.5 Long-Acting β₂ Agonists (LABA)

1.5.1 The β₂-adrenoceptor

Found in almost all peripheral tissues and several neuronal populations within the central nervous system, the adrenoceptors (or adrenergic receptors) mediate the central and peripheral actions of noradrenaline (primary sympathetic neurotransmitter) and adrenaline (primary adrenal medullary hormone and central neurotransmitter), (Bylund et al., 1994). Noradrenaline and adrenaline partake in several functions including control of blood pressure, airway reactivity and myocardial contraction and as such have been of major interest as targets for drug actions. Synthetic
drugs have differing affinities for adrenoceptors and based on this the receptors have been subdivided into distinct groups, primarily α and β. Drug interaction with these subtyped receptors, have proven useful in diseases such as hypertension, congestive heart failure, depression, glaucoma and asthma (Bylund et al., 1994). The β-adrenoceptors have been further subdivided into at least three distinct groups, β₁, β₂ and β₃, identified mainly in cardiac, ASM and adipose tissue, respectively. β₂-adrenoceptors are widely distributed, not only in ASM but also in lung epithelial and endothelial cells and mast cells (Johnson, 1998).

1.5.2 Activation and signalling of the β₂-adrenoceptor

The β₂-adrenoceptor is a member of the G-protein coupled receptor family, which all have 7 transmembrane spanning α–helices. Three extracellular loops, one of which is the amino terminus are interlinked with three intracellular loops with a carboxy-terminus (Figure 1.12). It is proposed that β₂-adrenoceptors oscillate between activated and inactivated forms and when resting, the two forms are in equilibrium, with the inactive state predominating (Johnson, 2006). The receptor becomes activated when it associates with the α-subunit of the Gs protein and with a molecule of guanosine triphosphate (GTP). Guanosine diphosphate (GDP) replaces the GTP which radically reduces the binding affinity of the α-subunit for the receptor, which leads to dissociation and induction of the receptor to return to an inactive state (Onaran et al., 1993).

Binding of the β₂-adrenoceptor β₂-agonist leads to its activation, which mediates the increase in intracellular cAMP levels. cAMP increases due to stimulation of adenylate cyclase which catalyses its conversion from adenosine triphosphate. A trimeric Gₛ protein can regulate the coupling of β₂ receptor to adenylate cyclase. cAMP is then degraded to 5′AMP (Johnson, 2006). Intracellular cAMP is thought to activate protein kinase A (PKA) the result of which is the direct phosphorylation of several intracellular regulatory proteins involved in, for example, the control of muscle tone in
ASM cells (Johnson, 1998). β₂-adrenoceptor activation may also occur through coupling to Gᵢ, rather than Gₛ, proteins which results in activation of ERK and p38 MAPK pathways. In this mechanism, the β₂-adrenoceptor is phosphorylated by PKA and mediated by βγ-subunits of the G protein which act as scaffolding for several upstream regulators of the MAPK family such as Raf and RAS – this culminates in MAPK activation (Daaka et al., 1997).

Figure 1.13: Structure of the human β₂-adrenergceptor (Johnson, 1998).

1.5.3 Mechanism of action of β₂-agonists

Site directed mutagenesis has identified that the active site of the β₂-adrenoceptor where β₂-agonists bind and exert their biological effect is approximately one third down into the receptor core. The molecular structure of a β₂-agonist determines its interaction with the β₂-adrenoceptor. For example SABA, that are hydrophilic, are able to interact with the active site of the receptor by
direct entry through its extracellular aqueous compartment. By contrast, LABA are lipophilic and form a depot before entry into the cell membrane where it then progressively filters out towards the active site of the receptor (Johnson, 2006). The onset times of SABA are very rapid in comparison to LABA, however they have a briefer agonist-receptor interaction as well as shorter duration of action (4-6 hours) compared with LABA, which can last up to 12 hours and now much longer with the new generation ultra-LABA (Ngkelo and Adcock, 2013).

1.5.4 LABA interactions with CS

The β₂-adrenoceptor gene is responsive to transcriptional upregulation when exposed to CS and although LABA primarily function on the β₂-adrenoceptors to exert bronchodilatory action, they can also complement the effects of CS through interaction with their signal transduction (Tamm et al., 2014). LABA were reported to enhance dexamethasone, budesonide or fluticasone propionate (FP) induced GR-GRE transactivation of anti-inflammatory genes (Kaur et al., 2008). In ASM, formoterol and budesonide in combination directly enhanced GR activation more than either treatment alone. Furthermore, the combination of LABA and CS also activated GR through the transcription factor CCAT enhancer binding protein (C/EBPα), which stimulated the promoter p21Waf1/Cip1 (cell cycle inhibitor protein) to suppress proliferation of smooth muscle (Roth et al., 2002). LABA may also activate MAPK cascades which can phosphorylate GR to enhance CS-dependent transactivation (Adcock et al., 2002). Moreover, nuclear translocation of GR from the cytosol was enhanced in-vivo with the combination of salmeterol and FP compared with FP alone (Usmani et al., 2005). These reports, a few of many, demonstrate a steroid sparing effect of LABA, when used in combination with inhaled CS which is one of the main treatment goals for CS-insensitive diseases such as severe asthma and COPD.
1.6 Hypothesis

There is a poor response to the beneficial therapeutic effects of CS in patients with severe asthma and COPD, suggesting a degree of CS-insensitivity in these chronic respiratory diseases. Increased p38 MAPK activity has been reported in severe asthma and COPD, correlating with decreased CS effectiveness. The role of p38 MAPK in the relative CS-insensitivity of severe asthma and COPD is unknown.

*I hypothesise that heightened activation of p38 MAPK influences CS sensitivity in severe asthma and COPD.*

1.7 Aims of thesis

To test this hypothesis, I will investigate and compare:

i. The activity of p38 MAPK between severe asthmatics with non-severe asthmatics and COPD patients with healthy smokers

ii. The effect of p38 MAPK inhibition on CS function (measured by suppression of induced cytokine release) in severe asthmatics and patients with COPD

iii. The impact of p38 MAPK inhibition on the phosphorylation of GR serine 211 in severe asthmatics and patients with COPD

iv. The expression and function of MKP-1 in severe asthmatics compared with non-severe asthmatics

v. The effect of the suppressive actions and potencies of 444 alone or in combination with FF or FP (measured by suppression of induced cytokine release) between severe asthmatics with non-severe asthmatics and COPD patients with healthy smokers
Chapter 2

Materials and Methods
Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 Cell Culture Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Citrate Dextrose (ACD)</td>
<td>Reagents from Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>(See Table 2.3 for recipe)</td>
<td></td>
</tr>
<tr>
<td>Ficoll Paque™ Plus</td>
<td>GE Healthcare, Buckinghamshire, UK</td>
</tr>
<tr>
<td>Hanks’ Buffered Saline Solution (HBSS)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Heat-inactivated, fetal bovine serum (FBS)</td>
<td>PAA Laboratories Ltd, UK</td>
</tr>
<tr>
<td>HemoHes 6% (w/v)</td>
<td>B Braun Medical Ltd AG, Switzerland</td>
</tr>
<tr>
<td>Kimura Stain</td>
<td>Reagents from Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>(See Table 2.3 for recipe)</td>
<td></td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Penicillin-Streptomycin solution</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Percoll™</td>
<td>GE Healthcare, Buckinghamshire, UK</td>
</tr>
<tr>
<td>Phosphate-buffered saline (PBS)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Roswell Park Memorial Institute (RPMI) 1640 medium</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Sodium chloride 0.9% (w/v) IV</td>
<td>Baxter Healthcare, Berkshire, UK</td>
</tr>
<tr>
<td>Sterile tissue culture grade water</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
</tbody>
</table>

Table 2.1: Cell culture materials list and suppliers
### 2.1.2 Molecular Biology Reagents

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x siRNA buffer</td>
<td>Dharmacron, Colorado, USA</td>
</tr>
<tr>
<td>AMV reverse transcriptase enzyme</td>
<td>Promega, Southampton, UK</td>
</tr>
<tr>
<td>AMV reverse transcriptase reaction buffer</td>
<td>Promega, Southampton, UK</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>cOmplete Protease Inhibitor Cocktail Tablets</td>
<td>Roche Diagnostics Ltd, Burgess Hill, UK</td>
</tr>
<tr>
<td>Deoxynucleotide triphosphates (dNTPs)</td>
<td>Bioline, London, UK</td>
</tr>
<tr>
<td>Dimethylsulphoxide (DMSO)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate (Na₂HPO₄)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>Active Motif, Rixensart, Belgium</td>
</tr>
<tr>
<td>Full-Range Rainbow Molecular Weight Marker</td>
<td>GE Healthcare, Buckinghamshire, UK</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Hanks' Buffered Saline Solution (HBSS)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>HEPES buffer</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Herring Sperm DNA</td>
<td>Active Motif, Rixensart, Belgium</td>
</tr>
<tr>
<td>Luminata Forte Western HRP substrate</td>
<td>Millipore, MA, USA</td>
</tr>
<tr>
<td>Magnesium chloride (MgCl₂)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Marvel Milk Powder</td>
<td>Waitrose</td>
</tr>
<tr>
<td>Methylthiazolylphenyl-tetrazolium bromide (MTT)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Monopotassium phosphate (KH₂PO₄)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>NP-40</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>NuPAGE® LDS Sample Buffer (4x)</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>NuPAGE® MOPS SDS Running Buffer (20x)</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>NuPAGE® Novex® Bis-Tris 4-12% mini gels</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Phosphatase inhibitor cocktail</td>
<td>Active Motif, Rixensart, Belgium</td>
</tr>
<tr>
<td>Phosphate-buffered saline (PBS) solution</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Phosphate-buffered saline (PBS) tablets</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Potassium chloride (KCL)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Radioimmunoprecipitation assay (RIPA) buffer</td>
<td>Millipore, MA, USA</td>
</tr>
<tr>
<td>Random primers</td>
<td>Promega, Southampton, UK</td>
</tr>
<tr>
<td>ReBlot™ Plus antibody stripping solution</td>
<td>Millipore, MA, USA</td>
</tr>
<tr>
<td>Recombinant RNasin ribonuclease inhibitor</td>
<td>Promega, Southampton, UK</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>Bioline, London, UK</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Sodium orthovanadate (Na₃VO₄)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Tween® 20</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
</tbody>
</table>

**Table 2.2: Molecular biology reagents list and suppliers**
2.1.3 Buffer, Solution or Stain Recipe

<table>
<thead>
<tr>
<th>Buffer, Solution or Stain</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>5M NaCl</td>
<td>292 g NaCl; up to 1 L with deionised H$_2$O.</td>
</tr>
<tr>
<td>Acid citrate dextrose solution (ACD; anti-</td>
<td>4.2 g di-sodium hydrogen citrate; 5 g glucose; up to 100 ml with deionised H$_2$O; filter-sterilise before use.</td>
</tr>
<tr>
<td>coagulant)</td>
<td></td>
</tr>
<tr>
<td>Kimura Stain</td>
<td>11 ml Toluidine Blue; 0.8 ml Light Green; 0.5 ml Saponin saturated in 50% (v/v) ethanol; 5 ml phosphate-buffered saline solution; filter-sterilise before use.</td>
</tr>
<tr>
<td>Ponceau S stain</td>
<td>1 g Ponceau S; 50 ml acetic acid; up to 1 L with deionised H$_2$O.</td>
</tr>
<tr>
<td>Stop Solution (for ELISA)</td>
<td>11 ml Sulphuric Acid; up to 100 ml with deionised H$_2$O.</td>
</tr>
<tr>
<td>Tris Buffered Saline + Tween (TBS-Tween; for western blotting)</td>
<td>10 ml Tris-HCl; 30 ml 5M NaCl; up to 1 L with deionised H$_2$O; 500 µl Tween$^\circ$ 20.</td>
</tr>
<tr>
<td>Tris-HCl (pH 7.4)</td>
<td>121 g Tris base; adjust to pH 7.4 with HCl; up to 1 L with deionised H$_2$O.</td>
</tr>
<tr>
<td>Wash Buffer (for ELISA)</td>
<td>5 phosphate buffer tablets diluted in 1 L deionised H$_2$O; 500 µl Tween$^\circ$ 20.</td>
</tr>
</tbody>
</table>

Table 2.3: Buffers, solutions or stain recipes

2.1.4 Kits and Assays

<table>
<thead>
<tr>
<th>Kit</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicinchoninic acid (BCA) protein assay</td>
<td>Pierce Biotechnology, Rockford, IL, USA</td>
</tr>
<tr>
<td>Bio-Rad Protein Assay</td>
<td>Bio-Rad, Hemel Hempstead, UK</td>
</tr>
<tr>
<td>Duoset ELISA Kits (for detection of IL-6, CXCL8 or TNFα)</td>
<td>R&amp;D Systems, Abingdon, UK</td>
</tr>
<tr>
<td>Human Monocyte Nucleofector® Kit</td>
<td>Lonza, Cologne, Germany</td>
</tr>
<tr>
<td>MACS Monocyte Isolation Kit II</td>
<td>Miltenyi Biotec Ltd., Surrey, UK</td>
</tr>
<tr>
<td>Nuclear Extraction Kit</td>
<td>Active Motif, Rixensart, Belgium</td>
</tr>
<tr>
<td>Phospho-p65 ELISA Kit (Cell Signaling)</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>QIAshredder Spin Columns</td>
<td>Qiagen, Crawley, UK</td>
</tr>
<tr>
<td>QuantiTect SYBR Green PCR Kits</td>
<td>Qiagen, Crawley, UK</td>
</tr>
<tr>
<td>QuantiTect Sybr-Green PCR Kit</td>
<td>Qiagen, Crawley, UK</td>
</tr>
<tr>
<td>RNase-Free DNase Set</td>
<td>Qiagen, Crawley, UK</td>
</tr>
<tr>
<td>RNeasy Mini Kit</td>
<td>Qiagen, Crawley, UK</td>
</tr>
<tr>
<td>TransAM GR-GRE DNA Binding Assay</td>
<td>Active Motif, Rixensart, Belgium</td>
</tr>
</tbody>
</table>

Table 2.4: Kits and assays list and suppliers
2.1.5 Oligonucleotides

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p38 MAPKα forward</td>
<td>AACCTGTCTCCAGTGGGCTCT</td>
</tr>
<tr>
<td>p38 MAPKα reverse</td>
<td>CGTAACCCCGTTTTTTGTGCA</td>
</tr>
<tr>
<td>18s forward</td>
<td>CTTAGAGGGACAAGTGGCG</td>
</tr>
<tr>
<td>18s reverse</td>
<td>AGCCTGAGCCAGTCAGTGT</td>
</tr>
<tr>
<td>MKP-1 forward</td>
<td>GTACATCAAGTCCATCTGAC</td>
</tr>
<tr>
<td>MKP-1 reverse</td>
<td>GGTTCCTCTAGGAGTAGACA</td>
</tr>
<tr>
<td>CXCL8 forward</td>
<td>GCCAACACAGAAATTATTGTAAAGCT</td>
</tr>
<tr>
<td>CXCL8 reverse</td>
<td>CCTCTGCACCCAGTTTTCTT</td>
</tr>
<tr>
<td>MAPK14 siRNA – four Target sequences</td>
<td>GGAAUUCAAUGAUGUGUAU</td>
</tr>
<tr>
<td></td>
<td>UCUCCGAGGUCUAAAGUAU</td>
</tr>
<tr>
<td></td>
<td>GUAAUCUAGCUGUGAAUGA</td>
</tr>
<tr>
<td></td>
<td>GUCCAUCAUCAUGCGAAA</td>
</tr>
<tr>
<td>DUSP1 siRNA - four Target sequences</td>
<td>CCAAUUGUCCCAACCAUUU</td>
</tr>
<tr>
<td></td>
<td>GCAUACUGCCUUGAUCAA</td>
</tr>
<tr>
<td></td>
<td>GCGCAAGUCUUUCUUCCUCA</td>
</tr>
<tr>
<td></td>
<td>GAAGGGUGUUUGUCCACUG</td>
</tr>
</tbody>
</table>

Table 2.5: Oligonucleotides list and their primer sequences
### 2.1.6 Western Blotting Antibodies

<table>
<thead>
<tr>
<th>1° Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>phospho p65 (serine 276)</td>
<td>1/1000</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>phospho p65 (serine 536)</td>
<td>1/1000</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Total p65</td>
<td>1/1000</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>phospho p38 (Thr180/Tyr182)</td>
<td>1/1000</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Total p38</td>
<td>1/2000</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>phospho GR (serine 211)</td>
<td>1/1000</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Total GR (E-20)</td>
<td>1/1000</td>
<td>Santa Cruz, Heidelberg, Germany</td>
</tr>
<tr>
<td>Total MKP-1</td>
<td>1/500</td>
<td>Santa Cruz, Heidelberg, Germany</td>
</tr>
<tr>
<td>phospho MSK-1</td>
<td>1/1000</td>
<td>AbCam, Cambridge, UK</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>1/1000</td>
<td>AbCam, Cambridge, UK</td>
</tr>
<tr>
<td>β-actin</td>
<td>1/10000</td>
<td>AbCam, Cambridge, UK</td>
</tr>
<tr>
<td>Tata-Binding Protein</td>
<td>1/2000</td>
<td>AbCam, Cambridge, UK</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2° Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal Goat Anti-Rabbit</td>
<td>1/2000</td>
<td>DAKO, Cambridgeshire, UK</td>
</tr>
<tr>
<td>Polyclonal Goat Anti-Mouse</td>
<td>1/2000</td>
<td>DAKO, Cambridgeshire, UK</td>
</tr>
</tbody>
</table>

Table 2.6: Western blotting antibodies list with dilutions used and suppliers
2.1.7 Corticosteroids, inhibitors and stimulants used in this project

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stock Solution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW642444 <em>Ultra Long-Acting β₂ Agonist</em></td>
<td>10⁻² M in DMSO</td>
<td>GSK, Stevenage, UK</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>10⁻³ M in sterile H₂O</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td><em>Fluticasone Furoate (FF)</em> <em>Ultra long-acting corticosteroid</em></td>
<td>10⁻² M in DMSO</td>
<td>GSK, Stevenage, UK</td>
</tr>
<tr>
<td>Fluticasone Propionate (FP)</td>
<td>10⁻² M in DMSO</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>GW856553 <em>p38 MAP Kinase inhibitor</em></td>
<td>10⁻² M in DMSO</td>
<td>GSK, Stevenage, UK</td>
</tr>
<tr>
<td>Lipopolysaccharide (LPS; from <em>Escherichia coli</em>)</td>
<td>1 mg / mL in PBS</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
</tbody>
</table>

Table 2.7: Corticosteroids inhibitors and stimulant list with suppliers

2.1.8 Equipment and Devices

<table>
<thead>
<tr>
<th>Equipment or Device</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well MicroWell™ NUNC plates</td>
<td>Fisher Scientific UK Ltd., Loughborough, UK</td>
</tr>
<tr>
<td>FUJIFILM medical X-ray film</td>
<td>Genetic Research Instrumentation Ltd., Rayne, UK</td>
</tr>
<tr>
<td>Gel Doc-It™ imaging system and LabWorks software</td>
<td>Ultra-Violet Products Ltd., Cambridge, UK</td>
</tr>
<tr>
<td>Inverted light microscope</td>
<td>Leica Microsystems, Buckinghamshire, UK</td>
</tr>
<tr>
<td>Luminex100 laser spectrophotometer</td>
<td>Luminex corporation, Austin, TX, USA</td>
</tr>
<tr>
<td>NanoDrop</td>
<td>Labtech International, East Sussex, UK</td>
</tr>
<tr>
<td>Nucleofector™ II Device</td>
<td>Lonza, Cologne, Germany</td>
</tr>
<tr>
<td>PowerEase® 500 Power Supply for Electrophoresis</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Reacti-Bind™ Streptavidin High Binding Capacity Coated 96-Well Plates</td>
<td>Pierce Biotechnology, Rockford, IL, USA</td>
</tr>
<tr>
<td>RotorGene RG3000 (for Realtime PCR)</td>
<td>Qiagen, (Corbett Research), Crawley, UK</td>
</tr>
<tr>
<td>SpectraMax microplate reader</td>
<td>Molecular Devices, Sunnyvale, CA, USA</td>
</tr>
<tr>
<td>Thermocycler Block (for RT-PCR)</td>
<td>G-Storm, Somerset, UK</td>
</tr>
<tr>
<td>XCell SureLock™ Mini-Cell Electrophoresis System</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
</tbody>
</table>

Table 2.8: Equipment and devices list with suppliers
2.2 Methods

2.2.1 Subject Selection

Normal healthy subjects, healthy smokers, non-severe asthmatics, severe asthmatics and patients with COPD, were recruited. All the studies were approved by the Ethics committee of the Royal Brompton & Harefield hospitals NHS trust and all subjects gave written, informed consent.

2.2.2 Cell isolation methods

2.2.2.1 Peripheral Blood Mononuclear Cell (PBMC) isolation using Ficoll®

80 ml of venous blood was collected into syringes containing acid citrate dextrose (ACD; an anti-coagulant) to give a final concentration of 1 mM. The blood was divided into 4 x 20 ml aliquots each of which were then diluted 1:1 with Hanks’ Buffered Saline Solution (HBSS). 20 ml of this mixture was then layered onto 15 ml of Ficoll-Hypaque® in a 50 ml Falcon tube. After centrifugation, 400 x g for 30 min at 22 °C, PBMC were collected from the interface between the Ficoll® and plasma, washed with HBSS and centrifuged again at 400 x g for 10 min at 22 °C. After this spin, the PBMC were resuspended in RPMI culture medium (0.5% v/v Foetal-Bovine Serum, 2 mM L-glutamine, 100 U Penicillin-Streptomycin) and a cell count was conducted using Kimura staining and a haemocytometer.

2.2.2.2 Peripheral Blood Mononuclear Cell (PBMC) isolation using Percoll™

In order to isolate PBMC for transfection, 80 ml of venous blood was collected into syringes containing ACD to give a final concentration of 1 mM. The blood was divided into 4 x 20 ml aliquots each of which were then mixed with 6 ml of 0.9% sodium chloride solution and 20 ml of 6% HemoHes solution. The tubes were inverted to mix and then left for 60 min to allow sedimentation
of the red blood cells. After this time, the top leukocyte-rich layer was then transferred to clean 50 ml falcon tubes and the volume adjusted to 50 ml with, Dulbecco's phosphate buffer solution (PBS) followed by centrifugation at 400 x g for 10 min at 22 °C. The pellets were resuspended in PBS to wash and centrifugation was repeated at 400 x g for 10 min at 22 °C.

The cell pellet contains a mixture of leukocytes therefore granulocytes were separated from the PBMC fraction using Percoll™ gradients. A 100% (v/v) working solution was prepared by combining 43.2 ml of Percoll™ and 4.8 ml of 10x PBS. From this solution, Percoll™ fractions of 81%, 68% and 55% (v/v) were prepared in PBS. Gradients were prepared by transferring 4 ml of the 81% fraction into a 15 ml falcon tube which was then overlaid with 4 ml of the 68% fraction. The cell pellet from above was resuspended in 3 ml of the 55% fraction which was then overlaid onto the pre-prepared Percoll™ gradient. Cells were separated according to density by centrifugation, 800 x g for 30 min at 22°C. PBMC harvested from the 55%-68% interface were washed and a cell count was performed using a 1:10 dilution of Kimura stain and a haemocytometer.

2.2.2.3 Monocyte isolation from PBMC population using MACS® Cell Separation Technology

Following Percoll™ isolation of PBMC, MACS® Cell Separation Technology was used to isolate monocytes from the mixed population of cells. Using the Monocyte Isolation Kit II, human monocytes were isolated by depletion of non-monocytes by a process referred to as ‘negative selection’. The principle works on the basis of indirect magnetic labelling of non-monocytes to a cocktail of biotin-conjugated monoclonal antibodies, as a primary labelling reagent and anti-biotin monoclonal antibodies conjugated to microbeads, as secondary labelling reagent. The magnetically labelled non-monocytes are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabelled monocytes pass through the column. Cells were resuspended in 30 µl of separation buffer per 1 x 10⁷ cells. 10 µl of FCR blocking reagent and biotin—
antibody cocktail were also added per $1 \times 10^7$ cells and the mixture was incubated on ice for 10 min. After this time, 30 µl of separation buffer and 20 µl of anti-biotin microbeads were added per $10^7$ cells in total. Cells were then incubated for 15 min on ice followed by washing with the separation buffer and then resuspended in 500 µl of buffer. An LS-MACS column was attached to a magnet and rinsed with 3 ml of buffer. The cell suspension was then added to the column and the run-through from the column (the negative fraction, representing the enriched monocytes) was collected. A further 9 ml of buffer was run through the column and collected. The resulting effluent was centrifuged and resuspended in complete medium. A cell count was performed using a 1:10 dilution of Kimura stain and a haemocytometer.

### 2.2.3 PBMC plating and stimulation for steroid/inhibitor experiments

PBMC were diluted in media, to a stock solution of $1 \times 10^6$ cells/ml then plated at a density of $0.75 \times 10^6$ cells per well of 12-well cell culture plates. Cells were pre-incubated with +/- dexamethasone, Fluticasone Propionate (FP), or Fluticasone Furoate (FF) +/- p38 MAPK inhibitor SB856553 (GW856553) or 444 (vilanterol), all between concentrations of $10^{-14} - 10^{-5}$ M, specified for each experiment, for 30 min. The ranges of steroid or inhibitor doses selected are anecdotal values as advised by GlaxoSmithKline based on pKa values, with $10^{-6}$ M - $10^{-5}$ M chosen as maximal doses and $10^{-10}$ M - $10^{-9}$ M (for steroids) equating to therapeutically achievable nanomolar quantities. The PBMC were then stimulated with LPS (10 µg/ml or 100 ng/ml) and incubated overnight (37°C; 5% CO$_2$; 90% humidity). After 24 h cell supernatants were harvested and stored at -20 °C until ready for cytokine analysis. The range of doses used for each compound is specified in the methods section of each chapter.
2.2.4 Cytokine analysis using ELISA

IL-6, CXCL8 and TNFα cytokine release levels were measured using an enzyme-linked immunosorbent assay (ELISA) based method, using DuoSet ELISA kits, according to manufacturer’s instructions. ELISA is a sensitive immunodetection method which uses biotinylated detection antibodies to identify specific antigens in a complex of proteins, to accurately measure the concentration of e.g. a specific cytokine, in a given sample. It has been termed a sandwich assay because an antibody-antigen-antibody complex is formed. Wells of a microtitre plate are coated with a ‘capture antibody’ directed against the protein of interest. After immobilisation of the antigen (protein of interest) in the unknown sample to the capture antibody in the well, biotin-labelled ‘detection antibody’ is added which is also directed against the protein of interest and which binds to an exposed site on the protein. The biotin tag allows conjugation to streptavidin horseradish peroxidase (HRP) enzyme, which when exposed to a substrate, such as tetramethylbenzidine, produces a visible colour change. This enzymatic reaction is halted upon addition of an acid solution. Furthermore, the colour intensity is proportional to the concentration of protein of interest and this can then be measured on a microplate reader.

A 96-well microtitre plate was coated with 100 μl of capture antibody solution at the working concentration in PBS and incubating for 24 h at room temperature. After this time, the capture antibody solution was removed and each well was washed three times with 300 μl of wash buffer. The wells were thoroughly dried by blotting the plate several times against a pad of paper towels. The wells were then blocked with 300 μl of Blocking Buffer (1% BSA and 0.05% NaN3 in PBS) and the plate was incubated for 1 h at room temperature. Another wash step was performed as described above. A seven-point protein standard was then prepared with 2-fold serial dilutions of the stock protein standard in Reagent Diluent buffer (0.1% BSA and 0.05% Tween 20 in PBS). 100 μl of the standards and diluted supernatants were then aliquotted into the wells of the plate which was then incubated for 2 h at room temperature. The wash step was repeated and 100 μl of detection
antibody, diluted to the working concentration in Reagent Diluent buffer, was added to each well and the plate further incubated for 2 h at room temperature. After another wash step, 100 μl of streptavidin-HRP solution, diluted 1/200 in Reagent Diluent buffer, was dispensed into each well and the plate was incubated for 20 min at room temperature, protected from light. After the final wash step, 50 μl of the substrate solution, prepared by mixing equal volumes of Colour Reagents A (H₂O₂) and B (tetramethylbenzidine), was added to each well and incubated until a blue product developed. The substrate reaction was then stopped by adding 50 μl of stop solution (2N H₂SO₄) to each well. The absorbance of each well was then measured at 450 nm with a 540 nm correction using a microplate reader and a four-parameter logistic standard curve was automatically generated using the microplate reader software. The cytokine concentration of each sample was determined from the standard curve and corrected for the dilution by multiplying by the dilution factor.

2.2.5 Luminex Beadlyte™ Assay

PBMC were plated at 4 x 10⁶ cells per well of a 6-well cell culture plate. Post-stimulation and harvesting, whole cell extracts were prepared and were analysed for total-p38 MAPK and phosphorylated p38 MAPK (active form) using a commercially available kit and according to manufacturer’s instructions (Beadlyte® Phospho-p38 (Thr180/Tyr182) Beadmates™). A 96 well filter plate was pre-wet before addition of 25 μl of whole cell extract diluted with 25 μl of 1x p38 bead solution, to each well. After vortexing, the plate was left in the dark, on a plate-shaker overnight at 4°C. The following day each well was washed with 100 μl of Beadlyte® Cell Signalling Assay Buffer followed by addition of 25 μl of 1x Phospho-p38 reporter solution. The plate was vortexed and left to shake in the dark for 1 h at room temperature. The reporter solution was removed and 25 μl of diluted Beadlyte® Streptavidin-Phycoerythrin was added to each well. The plate was vortexed and left to shake in the dark for 30 min at room temperature. The Streptavidin-Phycoerythrin was removed and the wells resuspended in 100 μl of Beadlyte® Cell Signalling Assay Buffer 1. The plate
was vortexed and placed on a plate shaker for 1 min and read in a Luminex® 100 laser spectrophotometer, as detailed below in section 2.2.6.

2.2.6 Fluorescence Spectrophotometry by Luminex® 100

A Luminex® 100 laser spectrophotometer (Luminex Corporation™) was used. This machine allows the simultaneous detection of multiple cytokines in each microplate well because microsphere beads for each cytokine emit a unique ratio of two other fluorophores. Beads are processed individually and undergo two sequential readings. The first reading measures the ratio of these two identifying fluorophores allowing identification of the cytokine to which the bead is active. The second reading measures the emission of fluorescence by streptavidin-phycoerythrin thereby quantifying the concentration of cytokine to which the bead was exposed. Cytokine concentrations were expressed as pg/ml converted from mean fluorescence intensity (MFI) derived from standard curves using the provided standards of known concentration for each cytokine which were provided in the BeadMaster kit.

2.2.7 Determination of mRNA expression by real-time PCR

For the determination of mRNA expression, total RNA was extracted from the cells and complimentary DNA (cDNA) was prepared by reverse transcription polymerase chain reaction (RT-PCR). mRNA levels were quantified by real-time quantitative PCR amplification of the cDNA using SYBR Green dye.

2.2.7.1 Total RNA extraction

Cells plated at 4 x 10^6 per well, were scraped on ice and transferred into fresh 2 ml eppendorfs. To pellet, the cells were centrifuged for at 300 x g for 5 min at 4°C. The supernatant was
removed and the pellet then resuspended in 350 μl of RLT buffer containing 1% β-mercaptoethanol. RNA extraction was then carried out immediately or cell lysates were stored at -80°C. Total RNA was isolated from PBMC or monocytes by using the RNeasy Mini Kit according to the manufacturer’s instructions and all steps were performed at room temperature. In brief, cell lysates were homogenised by centrifugation through QIAshredder spin columns at 13,000 rpm for 2 min. An equal volume of 350 μl of 70% ethanol was then mixed with the lysate and this was then run through the RNeasy Mini spin column by centrifugation at 10,000 rpm for 15 sec. This step allowed any total RNA to be retained on the silica-based membrane of the column. The membrane bound RNA was washed using suitable buffers to remove contaminants and incubated with a DNAse enzyme for 15 min at room temperature to remove any DNA contamination. RNA was eluted from the column with 30 μl of nuclease-free H₂O and centrifugation at 10,000 rpm for 1 min. The absorbance at 260 nm and 280 nm of each RNA eluate was measured by placing 1 μl onto the NanoDrop device. The purity of the RNA was determined by calculating the ratio of absorbance at 260 nm over that at 280 nm (A260/A280).

2.2.7.2 Reverse-transcriptase PCR (RT-PCR)

0.3 μg of total RNA in a volume of 10 μl was setup for each RT-PCR reaction. Using a thermal cycler, the primary denaturation step was carried out for 5 min at 70°C. After denaturation, the reverse-transcription reaction was set-up in a total volume of 20 μl, which contained 1 mM dNTPs, 1x AMV reverse transcriptase reaction buffer, 1 μg random primers, 40 U recombinant RNasin ribonuclease inhibitor, 10 U AMV reverse transcriptase enzyme and denatured RNA. The reaction mixture was incubated at 42°C for 60 min followed by incubation at 90°C for 4 min to inactivate the reverse transcriptase enzyme. The final cDNA product was then diluted 3-fold in nuclease-free H₂O.
2.2.7.3 Quantitative real time PCR (qPCR)

mRNA expression was quantified by means of a two-step qPCR experiment. It is a technique based on the 5'-3' exonuclease activity of the Taq DNA polymerase and allows a researcher to accurately quantify the amount of gene of interest (GOI) in a given nucleic acid sample. SYBR green I is a DNA-binding dye often used in conjunction with real time PCR, which is undetectable in its free form, however once preferentially bound to double-stranded DNA, starts to emit fluorescence. In qPCR, fluorescence is measured during each PCR cycle and an accumulation of a fluorescent signal defines a positive reaction. The cycle threshold (Ct) value is the number of cycles required for the fluorescent signal to exceed background level fluorescence. Ct values are inversely proportional to the amount of GOI in the sample therefore a lower Ct value denotes a greater amount of GOI in a sample. The qPCR experiment is normalised to a constitutively expressed housekeeping gene (e.g. 18S rRNA) in order to correct for experimental variation in each individual RT or qPCR reaction.

Briefly, 2.5 μl of cDNA were added to capillary tubes containing 7.5 μl of PCR reaction mixture, which consisted of SYBR Green PCR Master Mix Reagent (containing HotStarTaq DNA polymerase, dNTPs and SYBR Green I dye) and gene specific primers. The qPCR reaction was carried out in a real-time cycler. The reaction involved incubation at 95°C for 15 min to activate the heat-activated DNA polymerase followed by 30-50 cycles consisting of a denaturation step (94°C for 20 sec), an annealing step (55 - 60°C for 20 sec) and an extension step (72°C for 20 sec). Fluorescence emitted from DNA-bound SYBR Green was detected at 510 nm after excitation at 470 nm during the extension step. Data from the reaction were analysed using the computer software Rotor-Gene 6 v.6. Once the run was completed, the Ct value threshold was set manually. The Ct of the GOI was normalised to the housekeeping gene and the relative expression calculated in one of two ways, depending on the experiment. The first was to convert the Ct into copies of the gene per reaction, by using a standard curve created by serial dilution of a suitable sample. The second way was to use the ΔΔCt (delta-delta Ct) algorithm.
Specific primers for CXCL8, p38 MAPK, MKP-1 and 18S rRNA (Table 2.5) were designed according to their published sequences using the GenScript online primer design software and synthesised by Sigma-Genosys.

### 2.2.8 Whole cell protein extraction

PBMC plated at 4 x 10^6 cells per well, were kept on ice, scraped in media and transferred into pre-chilled 2 ml eppendorfs. The cells were pelleted by centrifugation at 5000 rpm for 5 min at 4°C and the supernatant was removed. After washing the cells once with ice cold PBS, containing 1x phosphatase inhibitor cocktail (to retain the phosphorylation status of the proteins of interest), the supernatant was removed and the cell pellet was resuspended in 100 μl of RIPA buffer containing 1 mM PMSF and 1x Complete protease inhibitor cocktail. After leaving on ice for 30 min, the lysates were centrifuged at 13,000 rpm for 15 min at 4°C and the resulting supernatant (the whole cell extract) was transferred into new pre-chilled tubes and stored at -80°C.

### 2.2.9 Cytoplasmic and nuclear protein extraction

PBMC pellets, plated at 4 x 10^6 cells per well, were lysed with 100 μl of ice-cold lysis buffer (10 mM TrisHCl pH 7.4, 150 mM NaCl, 0.75% (v/v) NP40, 1 mM PMSF, 1 mM DTT and 1x phosphatase inhibitor cocktail) for 10 min on ice and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant (the cytoplasmic fraction) was collected into a fresh Eppendorf. The pellet was re-suspended in 20 μl of nuclei extraction buffer (20 mM HEPES, 1.5 mM MgCl_2, 0.42 M NaCl, 0.2 mM EDTA, 25% Glycerol, 1 mM PMSF, 1 mM DTT and 1x phosphatase inhibitor cocktail) and the tube was scraped against a tube rack to help break the nuclear membrane. The lysate was left on ice for 30 min then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant (the nuclear fraction) was collected into a fresh Eppendorf and mixed with 40 μl of neutralising buffer (20 mM HEPES, 50 mM KCl, 0.2 mM
EDTA, 1 mM PMSF, 1 mM DTT and 1x phosphatase inhibitor cocktail). The cytoplasmic and nuclear fractions were stored at -80°C until required.

2.2.10 Bicinchoninic acid (BCA) protein assay

Protein concentration of whole cell extracts, cytoplasmic extracts or nuclear extracts was determined by using the BCA protein assay kit, in a 96-well plate according to the manufacturer’s instructions. Briefly, protein extracts were diluted 2-fold in a total volume of 10 μl cell-culture grade water in the plate. BSA standards (0.016, 0.125, 0.25, 0.5, 1 and 2 mg/ml) were also prepared in cell culture grade water to a total volume of 10 μl in each well. The BCA protein assay reaction mixture was then prepared by mixing reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and B (4% cupric sulphate). 200 μl of the mixture was added to the protein extracts and standards and the reaction was incubated for 30 min at 37°C. The absorbance of the product was measured at 562 nm using a microplate reader and the protein concentrations were determined by comparison to the standard curve.

2.2.11 Determination of protein expression by SDS PAGE and western blotting

10-50 μg of protein extracts were transferred into tubes before NuPAGE® LDS Sample Buffer (4x) containing 3.8% β-mercaptoethanol was added. The samples were boiled for 5 min at 100°C to denature the proteins. The proteins were then loaded onto a 4 - 12% Bis-Tris Novex® pre-cast mini-gel. The gels were placed in an electrophoresis cell connected to a power supply and were electrophoresed at 200 V for 50 min in 1x NuPAGE® MOPS SDS running buffer. The gel cassette was opened using a gel knife and the gel was placed into the iBlot™ device. The iBlot™ Dry Blotting system uses a semi-dry method to blot proteins from polyacrylamide gels onto a nitrocellulose membrane in 7 minutes. The gel was placed on top of a ‘Bottom’ stack with a copper anode (negative) layer and a nitrocellulose membrane. A mini-roller was used to remove any bubbles
between the gel and the membrane. A piece of filter paper soaked in deionised H\textsubscript{2}O was placed onto
the gel and again, bubbles rolled out. A ‘Top’ stack consisting of a copper cathode (positive) layer
was placed on top of the sandwich in addition to a layer of sponge and the iBlot™ device was tightly
closed and the transfer commenced. After the transfer had completed, the sandwich was
disassembled and the nitrocellulose membrane, onto which the proteins had now transferred, was
cut to size using a scalpel. To check that the transfer of protein was successful, the membrane was
incubated in Ponceau-S stain for 5 min before rinsing with deionised H\textsubscript{2}O.

To prevent any non-specific binding the membrane was rinsed in TBS-Tween (0.1% Tween)
and blocked by incubating in 5% milk for 1 hr at 4°C on a shaker. The membrane was then rinsed in
TBS-Tween and incubated with a suitable dilution of the primary antibody for 16 h at 4°C on a
shaker. After the primary antibody incubation, the membrane was washed three times in TBS-Tween
for 5 min each time then incubated with the suitable secondary antibody for 45 min at room
temperature. At the end of the incubation, the membrane was washed 3 times for 10 min each time
and then covered with 2 ml of Luminata™ western HRP solution for 5 min at room temperature.
Excess Luminata™ solution was then removed and the membrane was wrapped in cling-film and
exposed to a medical X-ray film in a dark room. Exposed films were then developed and the relevant
band intensities were quantified using the Gel Doc-It™ imaging system by scanning densitometric
analysis using LabWorks™ software. The membranes were stripped by incubating in mild antibody
stripping solution for 15 - 20 min then blocked in 5% milk and TBS-tween solution and then re-
probed with a β-actin, α-tubulin or Tata-binding protein (TBP) monoclonal antibodies, to control for
equal loading of protein, in each lane, for whole cell extracts, cytoplasmic extracts or nuclear
extracts, respectively. Densitometric data were normalised to that of the appropriate loading
control.
2.2.12 siRNA transfection of PBMC and monocytes

RNA interference (RNAi) is the process of mRNA degradation induced by double-stranded RNA in a sequence-specific manner. In mammalian cultured cells, RNAi is typically induced by short-interfering RNA (siRNA) that induce targeted knock-down of gene expression, resulting in partial to full loss of function. Monocytes were transiently transfected with ON-TARGETplus SMARTpool™ siRNA specific to either the MAPK14 (p38 MAPK α) gene or MKP-1 gene. As a negative control for the siRNA, ON-TARGETplus™ Non-targeting ‘Scramble’ siRNA was used. The Human Monocyte Nucleofector® kit and Nucleofector™ device were used to transfect the cells through electroporation.

After isolation, pelleted monocytes were resuspended in the appropriate volume of Human Monocyte Nucleofector® solution. 100 µl of sample for transfection, containing between 2 x 10^6 - 4 x 10^6 cells, was transferred into 0.5 ml eppendorfs. The siRNA was made up to 0.3 µg, 0.6 µg, or 1.0 µg in 1X siRNA buffer. These concentrations were used to optimise transfection conditions and a single concentration was chosen thereafter. After addition of the siRNA, the cells were transferred to a cuvette that was then placed into the Nucleofector™ device and the ‘Y001’ program, specific for monocytes, was used to electroporate the cells. A negative control for the transfection process, a ‘mock’ sample was also electroporated (cells without siRNA). Each electroporated sample was then transferred into 2ml of pre-warmed complete culture medium (10% v/v FBS, 2mM L-glutamine) in 6-well cell culture plates. Transfected cells were left to incubate (37°C; 5% CO₂; 90% humidity), for 48 h, 72 h or 96 h depending on the experiment. Cells were treated with dexamethasone (10⁻⁶ M) and stimulated with LPS (100 ng/ml) for the times indicated for specific experiments.

To examine the functional effect of the knockdown, transfected cells were harvested after 48 h, for quantitative real-time PCR analysis. To check efficiency of the knockdown, transfected cells were harvested after 72 h or 96 h for western blot analysis. At both 48 h and 72 h, cell supernatants were harvested for later analysis of IL-6 or CXCL8 release using ELISA.
2.2.13 GR-GRE binding assay

GR-GRE binding in protein extracts was measured using a method which was developed in-house. PBMC pellets, plated at 4 x 10^6 cells per well were stimulated according to the experiment and after harvesting, nuclear extracts were prepared as previously described. Nuclear extracts were added to the wells of 96-well streptavidin coated plates conjugated with biotinylated oligonucleotide duplexes which contain two glucocorticoid response-elements (GRE). Activated GR from the nuclear extracts binding to the GRE-containing oligonucleotides was then detected colourmetrically by an enzyme-immunosorbent assay method using an anti-GR primary antibody followed by a secondary horse radish peroxidase (HRP) conjugated antibody. After addition of the developing solution any colour change was quantified using a spectrophotometer.

2.3 Statistical Analysis

The statistical software package GraphPad Prism (Version 5.03) was used for data analysis throughout. Data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. To compare differences between non-parametric data the Mann-Whitney U-test was used. For intra-group analysis of e.g.: i) the effect of dexamethasone and inhibitor compared with dexamethsone, or ii) repeated concentration-dependent response measurements, one-way analysis of variance (ANOVA) (Kruskal-Wallis test) followed by Dunnet’s multiple comparison test was used. For intra-group analysis of parametric data, paired t-test was used. For correlations between parameters, Spearman’s rank correlation was used. \( P < 0.05 \) was considered as statistically significant.

Interaction ratios were calculated to estimate synergistic reactions between compounds using the Abbott formula whereby the expected efficacy \( (I_E) \) of a combination of compounds can be predicted based on the observed efficacy \( (I_o) \) and taking this ratio. The Abbott formula is: \[ I_E = A + B - (AB/100) \],
whereby ‘A’ is the efficacy of compound A and ‘B’ is the efficacy of compound B. An interaction ratio of 0.5 – 1.5 is interpreted as an additive effect (Grundy et al., 2016; Gisi 1996).

2.4 Recruitment of healthy subjects and patients with non-severe or severe asthma or patients with COPD

The specific number of selected patients and patient demographics for each set of experiments are outlined in the methods section of each chapter of this thesis. All healthy subjects were normal volunteers and members of the department aged between 20 – 65 years and who were spirometrically normal, with a negative PC20 to methacholine challenge and no history of any significant medical problems. Patients with severe or non-severe asthma or COPD were selected, without bias, from a pool of subjects undergoing bronchoscopy procedures, whilst attending the Respiratory Unit at Royal Brompton Hospital. Healthy smokers were recruited by local advertisements.

Patients with severe asthma were defined according to guidelines developed by ATS criteria (ATS, 2000; Table 1.1) with the presence of at least one of two major criteria for CS usage (>1000 µg/day or equivalent of inhaled beclomethasone dipropionate) and at least two minor criteria. Patients with non-severe asthma were defined as those who did not meet the criteria for severe asthma and used <1000 µg/day or equivalent of inhaled beclomethasone dipropionate. All participants gave informed consent to protocols approved by the Ethics Committee of Royal Brompton and Harefield NHS Trust/National Heart and Lung Institute (Ethics Committee approval number: 08/H0708/29).

Patients with COPD were defined on the basis of spirometry (ratio of FEV1 / FVC < 70%), a more than 10 pack-years smoking history and characterised according to GOLD criteria (www.goldcopd.org) based on predicted FEV1. Healthy smokers were selected based on a smoking
history of > 10 pack-years but with an $\text{FEV}_1 / \text{FVC} > 70\%$ and $\text{FEV}_1 > 80\%$ predicted. All participants gave informed consent to protocols approved by the Ethics Committee of Royal Brompton and Harefield NHS Trust/National Heart and Lung Institute (Ethics Committee approval number: 09/H0708/19).
CHAPTER 3

Reversal of CS-insensitivity by p38 MAPK inhibition in severe asthma
Chapter 3 – Reversal of CS-insensitivity by p38 MAPK inhibition in severe asthma

3.1 Introduction

For over 50 years corticosteroids (CS) have been the mainstay treatment for various inflammatory conditions. However, in severe asthma, characterised by chronic airflow obstruction, airway and lung remodelling, and chronic inflammation, CS do not exert their effects efficiently and hence severe asthma is often referred to as a refractory CS-insensitive disease. Accounting for approximately 10% of 5.4 million asthmatics in the UK (asthma.org.uk, 2012) severe asthmatics suffer with increased mortality and morbidity from the disease (Moore et al., 2007). CS therapy is not effective, neither at high dose administrations nor when combined with long-acting β-agonists (LABA). Furthermore, the associated healthcare costs are excessive, with the increased consumption and use of medicines, hospital facilities and medical manpower. Additionally, there are the indirect socio-economic costs linked with loss of productivity and impairment of social function (Sterling and Chung, 2001). To that end, there is an urgent need to improve current therapies for severe asthma and an understanding of the molecular mechanisms underlying CS-insensitivity is therefore vital.

During the innate immune response, the p38 mitogen activated protein kinase (MAPK) pathway, is a key signalling cascade and regulator of pro-inflammatory mediator expression that has been implicated in chronic inflammation (Cuenda and Rousseau, 2007). We have previously shown that lipopolysaccharide (LPS)-induced levels of the active form of p38 MAPK, phosphorylated p38 MAPK (phospho-p38 MAPK) were higher in alveolar macrophages of severe asthmatics compared with non-severe asthmatics and normal subjects (Bhavsar et al., 2008). In airway tissue, phospho-p38 MAPK staining was found to be significantly increased in severe asthmatics compared with mild asthmatics (Liu et al., 2008). The evidence indicates that increased p38 MAPK activity is involved in the severity of asthma. In peripheral blood mononuclear cells (PBMC) of normal subjects, where transient CS-
insensitivity had been introduced, CS binding ability to nuclear glucocorticoid receptor (GR) was reduced. However, p38 MAPK inhibition restored GR-CS binding and effectively reversed CS-insensitivity. Furthermore, phosphorylated GR, detected in transiently induced CS-insensitive PBMC, was inhibited in the presence of a p38 MAPK inhibitor (Irusen et al., 2002). Taken together, these data suggested that p38 MAPK may be linked with CS-insensitivity and that this link may be mediated through phosphorylation of GR.

The aims of this chapter are to:

1) Compare in PBMC, p38 MAPK activity between non-severe and severe asthmatics

2) Determine the effect of p38 MAPK inhibition on the suppressive action of CS in severe asthmatics

3) Elucidate the molecular mechanisms by which p38 MAPK inhibition may be improving CS function in severe asthmatics.
3.2 Methods

3.2.1 Cell isolation protocol for experiments performed with dexamethasone and GW856553

PBMC were isolated from the whole blood of non-severe asthmatics and severe asthmatics (for whom the clinical characteristics are outlined in Tables 3.1 and 3.2) and normal volunteers. PBMC were isolated from whole blood using a Ficoll™ gradient (as described in section 2.2.2.1). A cell count was conducted using Kimura staining and a haemocytometer.

3.2.2 Comparison of p38 MAPK activity in PBMC

PBMC were used to compare the phosphorylated p38 MAPK protein levels between non-severe and severe asthmatics. After isolation using a Ficoll™ gradient (as described in section 2.2.2.1) the stock concentration of the PBMC was adjusted to $2 \times 10^6$ cells per ml. The cells were then plated at 2 ml per well of 6-well culture plates, thus giving a total of $4 \times 10^6$ PBMC per well, per treatment. The cells were treated with LPS (100 ng/ml) for 1 h to induce p38 MAPK activity. Whole cell extracts were prepared (as described in section 2.2.8) and protein levels examined by western blotting (as described in section 2.2.11) with anti-phospho p38 MAPK, anti-total p38 MAPK and anti-β-actin antibodies.

3.2.3 Treatment conditions for p38 MAPK inhibition experiments in PBMC

PBMC were used to observe the effect of p38 MAPK inhibition on dexamethasone mediated suppression of LPS-induced CXCL8 or IL-6 release. The stock concentration of the PBMC after isolation was adjusted to $1 \times 10^6$ cells per ml. The cells were plated with 0.75 ml per well of 12-well culture plates to give $0.75 \times 10^6$ PBMC per well, per treatment. Cells were pre-treated with dexamethasone ($10^{-10} \text{ M} - 10^{-6} \text{ M}$) and / or GW856553 (p38 MAPK inhibitor) ($10^{-10} \text{ M} - 10^{-6} \text{ M}$) for 30 min. Lipopolysaccharide (LPS; 10 µg/ml) was used to stimulate and induce CXCL8 or IL-6 cytokine
release. All experiments were harvested for cell supernatants 24 h post-treatment. ELISA was used to quantify CXCL8 or IL-6 release from PBMC (as described in section 2.2.4).

3.2.4 p38 MAPK activity as measured by Beadlyte™ protocol

The phosphorylation of p38 MAPK in the presence of GW856553 was investigated in PBMC. After isolation, PBMC were plated at 4 x 10^6 PBMC per well of a 6-well plate. Cells were pre-treated with dexamethasone (10^{-6} M) and / or GW856553 (10^{-6} M) for 30 min. LPS (10 µg/ml) was used to induce p38 MAPK activation. At harvest, the contents of each well were stored in Beadlyte™ cell lysis buffer, followed by assay for phosphorylated and total p38 MAPK using microsphere beads coated with antibodies to phosho-p38 and total p38 MAPK using the Beadlyte™ protocol (as described in section 2.2.5).

3.2.5 Preparation of cells preceding p38 MAPK siRNA knockdown

p38 MAPK siRNA knockdown experiments described at the end of this chapter (Sections 3.3.3), were performed in monocytes. To obtain monocytes, a Percoll™ gradient was prepared to first separate the PBMC from whole blood (as described in section 2.2.2.2) followed by use of MACS® Cell Separation Technology to specifically isolate the monocytes from the PBMC fraction (as described in section 2.2.2.3). A cell count was performed using a 1:10 dilution of Kimura stain and a haemocytometer.

3.2.6 siRNA knockdown of p38 MAPK in monocytes

siRNA transfection by the Human Monocyte Nucleofector® Kit from Lonza was used to induce targeted knockdown of p38 MAPK gene expression in monocytes (as described in section 2.2.12). Following isolation, at least 2 x 10^6 monocytes were transfected with 0.3, 0.5, 0.8, 1 or 2 µg of p38 MAPK or scramble siRNA (as specified for each experiment). Cells were electroporated using the Nucleofector™ device as well as a mock sample (cells without siRNA). To confirm knockdown of p38 MAPK in monocytes, cells were harvested at 24 h or 48 h or 72 h post transfection and either,
western blot (as described in section 2.2.11) analysis was used to determine the level of total p38 MAPK protein expression or quantitative real-time PCR (as described in section 2.2.7) was used to determine the level of p38 MAPK mRNA transcript. To examine the functional effect of the knockdown, cells were: i) pre-treated with dexamethasone (10^{-6} M or 10^{-8} M) for 1 h and stimulated with LPS (100 ng/ml) for 24 h before cell supernatants were harvested for CXCL8 or IL-6 determination by ELISA (as described in section 2.2.4) and ii) harvested for western blot analysis (as described in section 2.2.11) to quantify protein levels of phospho-p38 MAPK, phospho-MSK-1 or β-actin.
3.3 Results

3.3.1 Results Part I: Comparison of p38 MAPK activity between severe and non-severe asthmatics

3.3.1.1 Non-severe and severe asthmatic patient demographics

For this comparative study, 6 non-severe asthmatics and 5 severe asthmatics were recruited (Table 3.1). Previous literature has shown this sample size to be appropriate for this type of study, whereby a similar number of asthmatic subjects with CS controlled symptoms (n=9) were used in experiments in which isolated PBMC were utilised as an ex-vivo model for asthma (Irusen et al., 2001).

<table>
<thead>
<tr>
<th></th>
<th>Non-severe Asthmatics</th>
<th>Severe Asthmatics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>51 ± 3</td>
<td>43 ± 13</td>
</tr>
<tr>
<td><strong>Gender (F:M)</strong></td>
<td>4:2</td>
<td>4:1</td>
</tr>
<tr>
<td><strong>FEV₁ (% Predicted)</strong></td>
<td>81 ± 9</td>
<td>80 ± 13</td>
</tr>
<tr>
<td><strong>FEV₁/FVC (ratio)</strong></td>
<td>73 ± 7</td>
<td>75 ± 4</td>
</tr>
<tr>
<td><strong>Bronchodilator response (%)</strong></td>
<td>13 ± 10</td>
<td>10 ± 5</td>
</tr>
<tr>
<td><strong>Log PC₂₀ (mg/ml)</strong></td>
<td>2 ± 3</td>
<td>1 ± 0.7</td>
</tr>
<tr>
<td><strong>Prednisolone Dose (mg/ml)</strong></td>
<td>N/A</td>
<td>All severe asthmatics were on prednisolone</td>
</tr>
<tr>
<td><strong>BDP equivalent (μg/day)</strong></td>
<td>700 ± 794</td>
<td>1640 ± 409</td>
</tr>
</tbody>
</table>

Table 3.1: Clinical characteristics of severe and non-severe asthmatics involved in comparisons of p38 MAPK activity. All severe asthma patients were on prednisolone however dosing information is unavailable. Non-severe asthmatics were not on prednisolone. Data are presented as mean ± Standard Deviation (SD); F = female; M = male; FEV₁ = Forced Expiratory Volume in 1 sec; FVC = Forced Vital Capacity in 1 sec; bronchodilator response = measured as % increase over baseline FEV₁ after 400 mg albuterol aerosol; PC₂₀ = provocative concentration of methacholine causing a 20% fall in FEV₁; BDP = beclomethasone dipropionate.
3.3.1.2 Comparison of p38 MAPK activity between severe and non-severe asthmatics

Comparison of p38 MAPK activation in PBMC between severe and non-severe asthmatics was investigated. There were no significant differences in baseline p38 MAPK activation levels between severe and non-severe asthmatics (Figure 3.1). Cells were also stimulated with lipopolysaccharide (LPS) for 30 min to induce p38 MAPK activation. LPS-induced p38 MAPK activation was significantly increased in severe asthmatics compared with non-severe asthmatics (P = 0.03), (Figure 3.2).

![Figure 3.1: Comparison of baseline levels of phosphorylated p38 MAPK protein expression between and non-severe severe asthmatics. PBMC were isolated from the whole blood of severe asthmatics (n=5) and non-severe asthmatics (n=6). Whole cell extracts were prepared and protein levels examined by western blotting with anti-phospho-p38 MAPK, anti-total p38 MAPK and anti-β-actin antibodies, as represented by the blots (A). Band density was quantified using densitometric software analysis (B). For baseline levels phospho-p38 MAPK expression was normalised to total p38 MAPK expression. Bars represent the median value; ns = no statistical difference between non-severe and severe asthmatics.](image)
Figure 3.2: Comparison of LPS-induced levels of p38 MAPK activity between severe and non-severe asthmatics. PBMC were isolated from the whole blood of non-severe asthmatics (n=6) and severe asthmatics (n=5). Cells were stimulated with LPS (100 ng/ml) for 30 min. Whole cell extracts were prepared and protein levels examined by western blotting with anti-phospho-p38 MAPK, anti-total p38 MAPK and anti-β-actin antibodies, as represented by the blots (A). Band density was quantified using densitometric software analysis (B). Phospho-p38 MAPK expression was normalised to total p38 MAPK expression. Bars represent the median value. * P < 0.05 compared to non-severe asthmatics (Mann-Whitney U-test). LPS = lipopolysaccharide.
3.3.2 Results Part II: The effect of p38 MAPK inhibition on corticosteroid suppression of LPS-induced cytokine release from PBMC

3.3.2.1 Severe asthmatic patient demographics

For the experiments performed in this section, 8 severe asthmatics were recruited (Table 3.2).

<table>
<thead>
<tr>
<th>Severe Asthmatics</th>
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</thead>
<tbody>
<tr>
<td>N</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Gender (F:M)</td>
</tr>
<tr>
<td>FEV₁ (% Predicted)</td>
</tr>
<tr>
<td>FEV₁/FVC (ratio)</td>
</tr>
<tr>
<td>Bronchodilator response (%)</td>
</tr>
<tr>
<td>Log PC&lt;sub&gt;20&lt;/sub&gt; (mg/ml)</td>
</tr>
<tr>
<td>Prednisolone Dose (mg/day)</td>
</tr>
<tr>
<td>BDP equivalent (μg/day)</td>
</tr>
</tbody>
</table>

Table 3.2: Clinical characteristics of severe asthmatics involved in p38 inhibition studies

Data are presented as mean ± SD; F = female; M = male; FEV₁ = Forced Expiratory Volume in 1 sec; FVC = Forced Vital Capacity in 1 sec; bronchodilator response = measured as % increase over baseline FEV₁ after 400 mg albuterol aerosol; PC<sub>20</sub> = provocative concentration of methacholine causing a 20 % fall in FEV₁; BDP = beclomethasone dipropionate.
3.3.2.2 The effect of p38 MAPK inhibition on dexamethasone mediated suppression of LPS-induced cytokine release, in severe asthmatics

The effect of p38 MAPK inhibition on LPS-induced cytokine release from PBMC of severe asthmatics was investigated. p38 MAPK inhibition was achieved by treating the cells with an inhibitor highly specific for the p38α isoform of the p38 MAPK pathway (GW856553). Dexamethasone suppressed LPS-induced CXCL8 in a concentration dependent manner, with maximal suppression at 52 ± 4%. The median inhibitory concentration (IC$_{50}$) was $3.9 \times 10^{-7}$ M. Similarly, maximal suppression of CXCL8 by GW856553 alone was 52 ± 8% with an IC$_{50}$ value of $3.7 \times 10^{-7}$ M. To determine whether there was an additive or synergistic suppressive effect between dexamethasone and GW856553, the cells were co-treated with both and the percentage suppression of cytokine release was calculated. The effect of dexamethasone at $10^{-6}$ M in suppressing CXCL8 release was increased in the presence of GW856553 at $10^{-10}$ - $10^{-6}$ M ($P < 0.0001$, one-way ANOVA) with maximal percentage suppression with the combination of dexamethasone and GW856553, both at $10^{-6}$ M, significantly increased compared with dexamethasone alone at $10^{-6}$ M (90 ± 3% vs. 52 ± 4% ($P < 0.01$)). Maximal suppression of CXCL8 release achieved with dexamethasone at $10^{-6}$ M and GW856553 at $10^{-7}$ M was also significantly increased compared with dexamethasone alone (68 ± 6% vs. 52 ± 4% ($P < 0.05$)). A lower concentration of dexamethasone at $10^{-8}$ M in combination with GW856553 at $10^{-6}$ M, also displayed a significant increase in the maximal suppressive ability of the corticosteroid compared with its action alone (68 ± 6% vs. 31 ± 4% ($P < 0.05$)). Furthermore, with the lowest concentration of dexamethasone assayed, at $10^{-9}$ M, the combination with GW856553 also significantly increased the action of the corticosteroid (59 ± 5% vs. 14 ± 4% ($P < 0.01$)) (All Figure 3.3A).

The suppression of LPS-induced IL-6 was also investigated. GW856553 suppressed IL-6 in a concentration dependent manner with a maximal suppression of 76 ± 7%. The maximal suppression by dexamethasone alone at $10^{-6}$ M, $10^{-8}$ M and $10^{-9}$ M was 75 ± 7%, 37 ± 3% and 18 ± 7%, respectively, and when combined at these concentrations with GW856553 at $10^{-6}$ M, there was
significant increase in maximal suppression for every dexamethasone concentration (97 ± 2 vs. 75 ± 7% ($P < 0.05$); 93 ± 3% vs. 37 ± 3% ($P < 0.01$); 81 ± 7% vs. 18 ± 7% ($P < 0.05$), respectively; Figure 3.3B).
Figure 3.3: The effect of p38 MAPK inhibition on dexamethasone mediated suppression of LPS-induced CXCL8 or IL-6 release. PBMC isolated from the whole blood of severe asthmatics (n=8) were pre-treated with dexamethasone (dex) alone (single coloured symbols) or GW856553 alone (black curve) or in combination (coloured curves) at the concentrations indicated, for 30 min. After stimulation with LPS (10 µg/ml) for 24 h, cell supernatants were harvested and CXCL8 release (A) or IL-6 release (B) was quantified by ELISA. Suppression of CXCL8 or IL-6 by dexamethasone or GW856553 is represented as a percentage of LPS induced release. Bars represent mean ± SEM. * P < 0.05; ** P < 0.01 (ANOVA); compared with dexamethasone alone. LPS = lipopolysaccharide; Dex = dexamethasone; GW-A = GW856553.
3.3.2.3 The effect of p38 MAPK inhibition on dexamethasone mediated suppression of LPS-induced cytokine release compared with the inhibitory effect of GW856553 alone

In the previous section (3.3.2.2), the cytokine release data was plotted to show the effect of p38 MAPK inhibition GW856553 combined with dexamethasone, on the suppression of LPS-cytokine release compared three single concentrations of dexamethasone alone. For this section, the same data has been re-plotted to show the effect of the combination on suppression, compared with that of two concentrations of GW856553 alone which each had an inhibitory effect of less than 5%. With GW856553 $10^{-9}$ M and at $10^{-10}$ M, which alone had an inhibitory effect of $3.9 \pm 2.1\%$ and $5.1 \pm 2.3\%$, respectively, CXCL8 suppression by dexamethasone at $10^{-6}$ M was significantly increased to $77 \pm 4\%$ and $66 \pm 4\%$, respectively ($P < 0.001$). At a 100-fold lower concentration ($10^{-8}$ M), dexamethasone suppression of CXCL8 in the combination was still significantly increased compared with GW856553 alone at both $10^{-9}$ M and at $10^{-10}$ M ($54 \pm 5\%$ vs. $3.9 \pm 2.1\%$ ($P < 0.001$); $42 \pm 6\%$ vs. $5.1 \pm 2.3\%$ ($P < 0.01$), respectively; Figure 3.4A). Similar results were obtained with LPS-induced IL-6 release. The combination of dexamethasone at $10^{-6}$ M with GW856553 at both $10^{-9}$ M and $10^{-10}$ M significantly increased the maximal suppression of IL-6 by dexamethasone ($67 \pm 10\%$ vs. $26 \pm 10\%$ and $82 \pm 7\%$ vs. $14 \pm 7\%$, respectively ($P < 0.05$)) (Figure 3.4B). To determine whether the suppression of CXCL8 or IL-6 by the combination was additive or synergistic, interaction ratios for the combination of dexamethasone and GW856553 (at both $10^{-9}$ M and $10^{-10}$ M) were calculated. The ratios ranged from 0.6 to 0.9, consistent with an additive and not synergistic effect. (Table 3.3).
Figure 3.4: The effect of p38 MAPK inhibition on dexamethasone mediated suppression of LPS-induced CXCL8 or IL-6 release. PBMC isolated from the whole blood of severe asthmatics (n=8) were pre-treated with dexamethasone or GW856553 alone, or both in combination, at the concentrations indicated, for 30 min. After stimulation with LPS (10 µg/ml) for 24 h, cell supernatants were harvested and CXCL8 release (A) or IL-6 release (B) were quantified by ELISA. Suppression of CXCL8 or IL-6 by dexamethasone or GW856553 is represented as a percentage of LPS induction. Bars represent mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001 (ANOVA); compared with GW856553 alone. LPS = lipopolysaccharide; Dex = dexamethasone; GW-A = GW856553.
Table 3.3: Interaction Ratios for the combination of dexamethasone and GW856553 on PBMC from patients with severe asthma

Data are presented as % inhibition as compared with LPS-stimulated cells. Unshaded section refers to combination with GW856553 at $10^{-9}$ M. Light grey shaded section refers to combination with GW856553 at $10^{-8}$ M. $I_O$ – Observed efficacy; $I_E$ – expected efficacy; IR – interaction ratio; an interaction ratio value of $0.5 – 1.5$ is considered an additive effect.

<table>
<thead>
<tr>
<th>Dex (Log M)</th>
<th>CXCL8</th>
<th></th>
<th></th>
<th>IL-6</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$I_O$</td>
<td>$I_E$</td>
<td>IR</td>
<td>$I_O$</td>
<td>$I_E$</td>
<td>IR</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>23.7</td>
<td>34.6</td>
<td>0.7</td>
<td>27.9</td>
<td>38.0</td>
<td>0.7</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>53.6</td>
<td>68.1</td>
<td>0.8</td>
<td>58.3</td>
<td>71.6</td>
<td>0.8</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>76.9</td>
<td>88.9</td>
<td>0.9</td>
<td>82.3</td>
<td>94.2</td>
<td>0.9</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>18.1</td>
<td>29.8</td>
<td>0.6</td>
<td>20.7</td>
<td>31.7</td>
<td>0.7</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>42.2</td>
<td>60.3</td>
<td>0.7</td>
<td>48.2</td>
<td>64.7</td>
<td>0.7</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>66.3</td>
<td>83.8</td>
<td>0.8</td>
<td>76.0</td>
<td>92.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>
3.3.2.4 The effect of p38 MAPK inhibition on the attenuation of p38 MAPK activity, in severe asthmatics

To determine whether GW856553 (10⁻⁹ M) demonstrated inhibitory effects on p38 MAPK activity, the ratio of phosphorylated p38 MAPK to total p38 MAPK was measured in PBMC from severe asthmatics. GW856553 (10⁻⁹ M) alone or in combination with dexamethasone (10⁻⁸ M) attenuated p38 MAPK activity induced by LPS stimulation (Figure 3.5).

![Figure 3.5: The effect of p38 MAPK inhibition on attenuation of p38 MAPK activity in severe asthmatics.](image)

PBMC isolated from the whole blood of severe asthmatics (n=3) were pre-treated for 30 min with dexamethasone (10⁻⁸ M) or GW856553 (10⁻⁹ M) alone, or both in combination. After stimulation with LPS (10 µg/ml) for 24 h, cells were lysed and then assayed for levels of phosphorylated and total p38 MAPK using microsphere beads coated with antibodies to phospho-p38 MAPK and total p38 MAPK using the Beadlyte protocol. p38 MAPK activation is represented as a ratio of phospho p38 to total p38 and then as a fold change over unstimulated cells. Bars represent the mean ± SEM. * P < 0.05; ** P < 0.01 (t-tests). Unstim = unstimulated cells; LPS = lipopolysaccharide; Dex = dexamethasone; GW·A = GW856553.
3.3.3 Results Part III: p38α MAPK siRNA knockdown in monocytes using Nucleofection™ Technology

3.3.3.1 The effect of p38α MAPK siRNA knockdown on total p38 MAPK protein expression in monocytes

After several attempts to optimise a protocol for the knockdown of p38α MAPK in PBMC, the protocol was amended to use monocytes instead. The benefit of using monocytes over PBMC for these experiments was that as a single cell population, monocytes were better suited for use with the Human Monocyte Nucleofector™ Kit (Lonza), as it was designed specifically for use with blood monocytes and this should therefore improve the efficiency of the knockdown. A Percoll™ gradient was used to isolate the PBMC from whole blood (prior to monocyte separation). Monocytes were isolated from the PBMC population using the MACS® Monocyte Isolation Kit II. Cells were harvested 72 h post-transfection as optimisation by the kit manufacturers showed this to be the most appropriate time point.

Monocytes were isolated from whole blood of normal healthy volunteers (n=6) and siRNA knockdown was performed using the Human Monocyte Nucleofector™ Kit. PBMC were mock transfected or transfected with scramble or p38α MAPK siRNA (1 µg). After 72 h, whole cell extracts were prepared and total p38 MAPK protein was semi-quantified by western blot with anti-total p38 MAPK and anti-β-actin antibodies (Figure 3.6A). Densitometric analysis revealed that p38α MAPK siRNA transfected cells expressed significantly reduced total p38 MAPK protein compared with scramble transfected cells (P = 0.031). However there was no difference in p38 MAPK protein between p38α MAPK transfected cells compared with mock control cells (Figure 3.6B).
Figure 3.6: The effect of p38α MAPK siRNA knockdown on total p38 MAPK protein expression in monocytes. Monocytes were isolated from the whole blood of normal volunteers (n=6). Cells were nucleofected in the absence of siRNA (mock) or transfected with scramble or p38α MAPK siRNA (1 µg). Cells were harvested after 72 h then whole cell extracts were prepared and protein levels examined by western blotting with anti-total p38 MAPK and anti-β-actin antibodies as represented by the blots (A). Band density was quantified using densitometric software analysis (B). Total p38 MAPK protein expression was normalised to β-actin loading control then expressed as a fold change with respect to mock-transfected control. Bars represent the mean ± SEM. * P < 0.05 (Wilcoxon T test); compared with scramble siRNA transfected monocytes.
3.3.3.2 The effect of p38α MAPK siRNA knockdown on dexamethasone mediated suppression of LPS-induced cytokine release from monocytes

The effect of p38α MAPK siRNA knockdown on the function of dexamethasone mediated suppression of cytokine release was subsequently investigated. Monocytes from normal healthy volunteers (n=6) were transfected using the Primary Cell Nucleofector™ Kit. After 72 h, there were no significant differences between p38α MAPK and scramble siRNA transfected cells in terms of their LPS-induced or their dexamethasone suppressed, cytokine release. This was observed for both CXCL8 (Figure 3.7A) and IL-6 (Figure 3.7B) release.
Figure 3.7: The effect of p38α MAPK siRNA knockdown on pro-inflammatory cytokine release. Monocytes were isolated from the whole blood of normal volunteers (n=6). Cells were nucleofected in the absence of siRNA (mock) or transfected with scramble or p38α MAPK siRNA (1 µg). Cells were treated with LPS (100 ng/ml) or LPS + dexamethasone (10⁻⁶ M) + LPS at 48 h. Cells were then harvested at 72 h for CXCL8 release (A) or IL-6 release (B). Cytokine release was expressed as a fold change with respect to unstimulated scramble transfected controls. Bars represent the mean ± SEM. There were no significant statistical differences between p38 MAPK transfected and scramble transfected monocytes. Unstim = unstimulated cells; LPS = lipopolysaccharide; Dex = dexamethasone.
3.3.3.3 The effect of p38α MAPK siRNA knockdown on the activity of p38 MAPK in monocytes

The effect of p38α MAPK siRNA knockdown on the activity of p38 MAPK protein was determined in monocytes. Monocytes of normal healthy volunteers (n=6) were transfected using the Primary Cell Nucleofector™ Kit and after harvesting at 72 h, whole cell extraction was performed. The activity of p38 MAPK was represented by the measurement of phosphorylated p38 MAPK by western blot (Figure 3.8A). Densitometric analysis revealed no differences in p38 MAPK activity between p38α MAPK and scramble siRNA transfected monocytes (Figure 3.8B). This result was observed both at baseline and for cells treated with LPS or with LPS and dexamethasone (Figure 3.6).
Figure 3.8: The effect of p38α MAPK siRNA knockdown on p38 MAPK activity in monocytes. Monocytes were isolated from the whole blood of normal volunteers (n=6). Cells were nucleofected in the absence of siRNA (mock) or transfected with scramble or p38α MAPK siRNA (1 µg). Cells were treated with LPS (100 ng/ml) or LPS + dexamethasone (10^{-6} M) at 48 h. Cells were then harvested at 72 h, whole cell extracts were prepared and protein levels examined by western blotting with anti-phospho p38 MAPK, anti-total p38 MAPK and anti-β-actin antibodies, as represented by the blots (A). Band density was quantified using densitometric software analysis (B). Phospho p38 MAPK protein expression was normalised to total p38 MAPK then expressed as a fold change with respect to mock-transfected control. Bars represent the mean ± SEM. There were no significant statistical differences between p38 MAPK transfected and scramble transfected monocytes. Unstim = unstimulated cells; LPS = lipopolysaccharide; Dex = dexamethasone.
3.3.3.4 Effect of p38α MAPK siRNA knockdown on MSK-1 activity in monocytes

To further investigate the effect of p38α MAPK siRNA knockdown on the activity of p38 MAPK, phosphorylated mitogen and stress-activated protein kinase (MSK-1; a direct target of p38 MAPK) protein levels were determined. Of the western blots that were prepared from normal volunteers whose monocytes were transfected and harvested for measurement of phosphorylated p38 MAPK (section 3.3.3.3), one blot was chosen (n=1) and was reprobed for phosphorylated MSK-1 (Figure 3.9A). Densitometric analysis revealed that there were no differences in phosphorylated MSK-1 protein expression levels between p38α MAPK and scramble siRNA transfected monocytes (Figure 3.9B). This was observed both at baseline and for cells treated with LPS or with LPS and dexamethasone.
Figure 3.9: The effect of p38α siRNA knockdown on MSK-1 activity in monocytes. Monocytes were isolated from the whole blood of normal volunteers (n=1). Cells were nucleofected in the absence of siRNA (mock) or transfected with scramble or p38α MAPK siRNA (1 µg). Cells were treated with dexamethasone (10^{-6} M) + LPS (100 ng/ml) at 48 h. Cells were then harvested at 72 h, whole cell extracts were prepared and protein levels examined by western blotting with anti-phospho MSK-1 and anti-β-actin antibodies, as represented by the blots (A). Band density was quantified using densitometric software analysis (B). Phosphorylated MSK-1 protein expression was normalised to β-actin then expressed as a fold change with respect to mock-transfected control. Unstim = unstimulated cells; LPS = lipopolysaccharide; Dex = dexamethasone.
3.4 Discussion

3.4.1 Summary of Findings

In this chapter, it has been shown that LPS-induced p38 MAPK activity is heightened in PBMC of severe asthmatics compared with non-severe asthmatics. Previous findings from our group have shown that blood and lung cells of severe asthmatics were CS-insensitive compared with non-severe asthmatics. To reveal the link between heightened p38 MAPK activity and CS-insensitivity in severe asthmatics, a p38 MAPK inhibitor, highly selective for the p38α isoform was utilised. p38 MAPK inhibition significantly increased dexamethasone’s ability to suppress LPS-induced CXCL8 and IL-6 release in PBMC from severe asthmatics. PBMC are a mixed population of single nucleus cells, derived from and easily isolated from whole blood, and are a rich source of blood monocytes. In the lung, blood monocytes differentiate into pulmonary macrophages, which are the predominate cells involved in the immune response in lung disease and therefore the use of PBMC, as an easy-to-isolate, primary cell model, is an ideal surrogate for exploration of macrophage responses in-vitro.

3.4.2 Increased p38 MAPK activity in severe asthmatics

The p38 MAPKs have four isoforms, α, β, γ and δ of which the α-isoform is expressed most abundantly in cells, has been best characterised and has been specifically linked with inflammation. As key regulators of the immune response and inflammatory cytokine expression, p38 MAPKs appear to be involved in chronic inflammation and as such are involved in the manifestation of many inflammatory conditions (Johnson and Lapadat, 2002; Cuenda and Rousseau, 2007). For example, increased p38 MAPK activity has been shown to correlate with disease severity in T-cells of rheumatoid arthritis (López-Santalla et al., 2011). With regard to airway diseases, our group have previously shown that LPS-induced levels of the active phosphorylated form of p38 MAPK was heightened in AM of severe asthmatics compared with non-severe asthmatics (Bhavsar et al., 2008). In addition to this, phospho-p38 staining in the epithelial walls of severe asthmatics was increased...
compared with non-severe asthmatics (Liu et al., 2008) and increased phospho-MAPKAP-K2 (a direct target of activated p38 MAPK) was recently observed in human airway smooth muscle (ASM) cells of severe asthmatics compared with non-asthmatics (Robins et al., 2011). Also in human ASM cells, TNFα-induced p38 MAPK activity was significantly increased in severe asthmatics compared with non-severe asthmatics and non-asthmatic subjects (Chang et al., 2012).

Concurring with previous studies, this study is the first to show in PBMC from severe asthmatics that LPS-induced p38 MAPK activation is significantly elevated compared with non-severe asthmatics (Figure 3.2). The evidence therefore suggests that increased p38 MAPK activity may be involved in the severity of asthma.

### 3.4.3 CS-insensitivity in severe asthmatics

Our group have previously shown CS-insensitivity is present in PBMC and alveolar macrophages (AM) of severe asthmatics compared with non-severe asthmatics. This was measured by the ability of dexamethasone to suppress the release of LPS-induced pro-inflammatory mediators MIP-1α, IL-1β, IL-6 and CXCL8 in PBMC (Hew et al., 2006) and LPS-induced MCP-1, MIP-1α, IL-1β, IL-6 and CXCL8 in AM (Bhavsar et al., 2008). In asthmatics, impaired CS suppression of serum-induced human ASM cells was observed compared with non-asthmatics (Roth et al., 2004). More recently, dexamethasone inhibition of TNFα-induced and of TNFα/anti-CD3/CD28-induced CXCL8 release, was significantly reduced in PBMC from severe asthmatics compared with non-severe asthmatics or healthy volunteers (Mercado et al., 2011) again demonstrating CS-insensitivity in these cells. Moreover it was found that the reduction in CS responsiveness correlated with a reduction in lung function suggesting that patients with decreased CS sensitivity in PBMC displayed a more severe phenotype (Mercado et al., 2011). Furthermore, in human ASM cells, dexamethasone suppression of TNFα-induced eotaxin and CXCL8 release was impaired in severe asthmatics compared with non-severe asthmatics and non-asthmatic subjects, demonstrating the CS-insensitive nature of this disease (Chang et al., 2012).
3.4.4 Increased CS function with p38 MAPK inhibition in severe asthmatics

As CS efficiency was shown to be reduced in AM and PBMC of severe asthmatics and this appeared to be linked with increased p38 MAPK activity, it was examined whether p38 inhibition could modulate CS-suppressed LPS-induced cytokine release. In AM from severe and non-severe asthmatics, LPS-induced IL-1β, IL-6 and MIP-1α release is better inhibited by the combination of SD282 and dexamethasone than with either compound alone (Bhavsar et al., 2010b). In PBMC from severe asthmatics, dexamethasone suppression of LPS-induced CXCL8 release was significantly improved in the presence of a highly selective p38 MAPK inhibitor of the α-isoform, GW856553 (Losmapimod) which is currently in clinical development (Figures 3.3 and 3.4; Bhavsar et al., 2010b). The maximal suppression of CXCL8 achieved by dexamethasone alone was 50% and maximal suppression with GW856553 alone was minimal compared with that of the combination which was nearing 90%, suggesting a synergistic effect with the combination (Figure 3.3A and 3.4A; Bhavsar et al., 2010b). However, after calculating the interaction ratios for the combination of these drugs, the findings were consistent with an additive and not synergistic effect (Table 3.3). The results for IL-6 release were similar and equally significant as those for CXCL8 release (Figure 3.3B, Figure 3.4B; Bhavsar et al., 2010b) and again, additivity was determined from interaction ratio calculations (Table 3.3). Similar results with GW856553 were shown in human ASM cells from severe asthmatics, whereby dexamethasone suppression of TNFα-induced eotaxin and CXCL8 release was significantly improved in its presence compared with either compound alone (Chang et al., 2012).

The LPS-induced activity of p38 MAPK was significantly attenuated by GW856553 in PBMC from severe asthmatics (Figure 3.5). This measurement confirmed previous studies that have demonstrated that p38 inhibition can prevent in-vitro phosphorylation of p38 MAPK (Galán et al., 2000; Matsuguchi et al., 2000). Additionally, p38α MAPK has been shown to auto-phosphorylate (Ge et al., 2002) and trans-phosphorylate (Koch et al., 2004) suggesting a mechanism by which p38 MAPK inhibitors may be working in-vitro. Finally, SB203508, a similar p38 MAPK inhibitor to
GW856553, has been shown to inhibit the enzymatic activity of activated and inactivated forms of p38α (Frantz et al., 1998).

The mechanisms behind why p38 MAPK inhibition may be improving the ability of CS to perform more efficiently may be associated with one of the targets of p38 MAPK, the glucocorticoid receptor (GR). GR is phosphorylated at its serine 211 residue, a marker of the transcriptional activity of GR. The impact of p38 MAPK inhibition on GR ser211 phosphorylation was examined in severe asthmatics, the results of which are further debated in the discussion section of Chapter 4 in this thesis.

3.4.5 Determining synergistic interactions between drug compounds

Several methodologies have been established to determine if combinations of drug compounds display synergism (as opposed to additivity or antagonism) for reasons such as increasing the therapeutic effect of a given drug, or to reduce the dosage whilst improving or maintaining efficacy to avoid drug toxicity. One method, as utilised in this chapter, is the calculation of an interaction ratio based on the Abbott formula, which can be used without mathematical transformation, to estimate the expected efficacy of a drug combination (Giss, 1996). This method has been used to determine synergistic interaction of the combination of dexamethasone with a phosphodiesterase-4 inhibitor on cytokine release from pulmonary CD8 cells, of patients with COPD and smokers (Grundy et al., 2016). Isobolograms are another empirical means by which to determine synergy whereby the concentrations of both drug A and drug B required to give a particular effect (such as the IC_{50}) are used to indicate two points on the x and y –axes of a two coordinate plot. A line that connects these two points becomes the line of additivity. The concentrations of drug A and drug B in combination, that give the same IC_{50} values as when alone, are then placed on the same plot. The placement of this line below, on, or above the line of additivity is consistent with synergy, additivity or antagonism, respectively (Zhao et al., 2010). This methodology follows the Loewe definition of additivity which in drug terms can be translated as
when the dose required to achieve the effect in combination is the same as required in monotherapy. If the dose required is less, then it is defined as synergy and if it is more, then it is antagonism (Harbron, 2010). This approach has been demonstrated well in a study of AM from asthma patients, where LPS-induced cytokine release was inhibited at significantly greater levels by the combination of dexamethasone and a p38 MAPK inhibitor (BIRB-796), than with either drug alone. This combination also had a significant dose-sparing synergy on TNFα and IL-6 inhibition, specifically (Lea et al., 2014). Taken together it would be useful to perform such analysis on future drug combination studies as the determination of true synergy adds valuable robustness to the data.

3.4.6 siRNA knockdown of p38α MAPK in blood monocytes

In order to further investigate the role of p38α MAPK inhibition on CS-function, RNAi technology was used to knockdown p38α gene expression in blood cells. p38α siRNA was transfected into PBMC using electroporation. PBMC were chosen for the transfection experiments as the initial inhibition experiments had been performed in them, however as they are non-adherent primary cells, they are typically ‘difficult-to-transfect’ and as such, the level of efficacy of the knockdown was low. To overcome this issue, monocytes were isolated from the PBMC population and were transfected as a single cell population. The level of efficacy of the transfection was greatly improved in monocytes compared with that in the PBMC population, likely due to the Human Monocyte Nucleofector™ Kit, designed specifically for use with blood monocytes. The p38α MAPK protein expression was significantly reduced in monocytes treated with p38α MAPK siRNA compared with cells transfected with the control, scramble siRNA (Figure 3.6A) confirming that the knockdown of p38α in these cells was successful.

To determine the effect of corticosteroids on a cell model where p38α MAPK expression was reduced, transfected monocytes were pre-treated with dexamethasone then stimulated with LPS. CXCL8 and IL-6 cytokine release was not significantly different between transfected and control transfected cells (Figure 3.7). These data suggest that although p38α MAPK protein expression is
reduced, this does not, as had been expected, improve the ability of dexamethasone to reduce LPS-induced inflammatory release from these cells. This could be because the efficiency of the transfection of p38α MAPK siRNA in monocytes was not great enough to have an impact on the functionality of the cell. Alternatively, perhaps all isoforms of the p38 MAPK pathway need to be targeted to ensure the gene is fully silenced. To further investigate this, activated p38α MAPK protein expression levels were compared between siRNA transfected and scramble transfected monocytes and the results showed that there were no significant differences between the two (Figure 3.8). This suggests that although total p38α MAPK protein expression was reduced, the activated form of the pathway was still ‘switched on’ after transfection and this could be the reason why the functionality of the cell was unaffected.

A downstream target of p38 MAPK is mitogen and stress activated protein kinase 1 (MSK-1). The activity of MSK-1 was measured after transfection as a marker of the activity of p38α MAPK pathway after transfection. Figure 3.9 confirms that after transfection in monocytes, there was no significant difference in phosphorylated MSK-1 protein expression levels between p38α transfected cells and scramble transfected cells i.e. p38α MAPK was still functioning after transfection suggesting that the transfection did not completely silence this pathway in these cells. Therefore in this cell model, the knockdown of p38α MAPK by transfection to examine its role in CS-insensitivity, may not be the most appropriate method and an alternative would be to use a dominant negative mutant expression vector to reduce the expression of p38α MAPK gene. This again however, would not completely silence p38 MAPK protein expression. A further option to study the biological function of p38α MAPK is to generate p38α knockout mice however in previously performed studies, this has resulted in embryonic death due to defective placental angiogenesis or impaired erythropoietin expression (Allen et al., 2000; Adams et al., 2000; Mudgett et al., 2000; Tamura et al., 2000).
Chapter 4
Reversal of CS-insensitivity by p38 MAPK inhibition in COPD
Chapter 4 - Reversal of CS-insensitivity by p38 MAPK inhibition in COPD

4.1 Introduction

Predicted to become the third leading cause of death worldwide by 2020 (Rabe et al., 2007), COPD is progressive in its nature and is caused mainly by cigarette smoke inhalation. It is characterised by chronic airflow obstruction of the small airways and is poorly-responsive to inhaled CS and LABA which are the mainstay treatments for the relief from symptoms of COPD (Chung et al., 2009). To date, these approaches have not been able to abrogate the progressiveness of the disease nor its global impact on morbidity and mortality hence the unmet need for novel anti-inflammatory therapies.

As a key signalling cascade and regulator of the innate immune response, the p38 MAPK pathway has been implicated in chronic inflammation (Cuenda and Rousseau, 2007). Increased phosphorylated p38 MAPK staining was observed in AM and the alveolar walls of COPD subjects, compared with individuals with normal lung function (Renda et al., 2008). More recently elevated levels of phosphorylated p38 MAPK were observed in bronchial epithelial cells, macrophages and CD20\(^+\) and CD8\(^+\) lymphocytes in COPD lungs compared with control samples (Gaffey and Reynolds et al., 2013). This evidence suggests that p38 MAPK is up-regulated in COPD and therefore may be involved in its pathophysiology.

Patients with COPD remain symptomatic despite the use of high-dose of inhaled corticosteroids (Bourbeau et al., 2007). In PBMC where CS-insensitivity had been induced, p38 MAPK inhibition restored the ability of CS to bind to GR - effectively reversing CS-insensitivity in these cells - and also inhibited levels of detected phosphorylated GR in these cells (Irusen et al., 2002). Furthermore, p38 MAPK inhibitors have been shown to be effective in suppressing inflammatory mediators in AM from patients with COPD (Kent et al., 2009; Armstrong et al., 2011). Taken together
this data suggests a link between increased p38 MAPK and CS-insensitivity in COPD that may be mediated through phosphorylation of GR.

The aims of this chapter are to:

1) Compare in PBMC, p38 MAPK activity between healthy smokers and COPD subjects

2) Determine the effect of p38 MAPK inhibition on the suppressive action of CS in PBMC of COPD subjects

3) Elucidate the molecular mechanisms by which p38 MAPK inhibition may improve CS function in COPD subjects.
4.2 Methods

4.2.1 Cell isolation and plating conditions

PBMC were isolated from the whole blood of healthy smokers and patients with COPD, for whom the clinical characteristics are outlined in Table 4.1, and normal volunteers. A Ficoll™ gradient was used to isolate PBMC from whole blood (as described in section 2.2.2.1). A cell count was conducted using Kimura staining and a haemocytometer.

4.2.2 Comparison of p38 MAPK activity in PBMC

Phosphorylated p38 MAPK protein levels were used as a representation of p38 MAPK activity to compare levels between healthy smokers and COPD subjects. After isolation using a Ficoll™ gradient (as described in section 2.2.2.1) the cells were plated to give 4 x 10^6 PBMC per treatment. The cells were treated with LPS (100 ng/ml) for 1 h to induce p38 MAPK activity. Whole cell extracts were prepared (as described in section 2.2.8) and protein levels examined by western blotting (as described in section 2.2.11) with anti-phospho p38 MAPK, anti-total p38 MAPK and anti-β-actin antibodies.

4.2.3 Treatment conditions for p38 MAPK inhibition experiments in PBMC

PBMC were used to observe the effect of p38 MAPK inhibition on dexamethasone mediated suppression of LPS-induced CXCL8 or IL-6 release. The stock concentration of the PBMC after isolation was adjusted to 1 x 10^6 cells per ml. The cells were plated at 0.75 ml per well of 12-well culture plates to give 0.75 x 10^6 PBMC per well, per treatment. Cells were pre-treated with dexamethasone (10^-10 M to 10^-6 M) and / or GW856553 (p38 MAPK inhibitor) (10^-10 M to 10^-6 M) for 0.5 h. Lipopolysaccharide (LPS) (10 µg/ml) was used to stimulate and induce CXCL8 or IL-6 cytokine release. All experiments were harvested for cell supernatants 24 h post-treatment. ELISA was used to quantify CXCL8 or IL-6 release from PBMC (as described in section 2.2.4).
4.2.4 GR ser211 phosphorylation status in the presence of p38 MAPK inhibition

PBMC were used to compare the effect of p38 MAPK inhibition on phosphorylated GR ser211 protein levels in patients with COPD or severe asthma. After isolation using a Ficoll™ gradient (as described in section 2.2.2.1) the cells were plated to give $4 \times 10^6$ PBMC per treatment. The cells were pre-treated with dexamethasone ($10^{-6}$ M) and / or GW856553 ($10^{-6}$ M) for 1 h. Treatment with LPS (100 ng/ml) for 30 min was used to induce p38 MAPK activity. Whole cell extracts were prepared (as described in section 2.2.8) and protein levels examined by western blotting (as described in section 2.2.11) with anti-phospho GR ser211, anti-total GR and anti-β-actin antibodies.
4.3 Results

4.3.1 Healthy smoker and COPD subject clinical characteristics

For the experiments in this chapter, 10 healthy smokers and 11 COPD subjects were recruited (Table 4.1).

<table>
<thead>
<tr>
<th></th>
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<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
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<td>11</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55 ± 3</td>
<td>66 ± 2 **</td>
</tr>
<tr>
<td>Gender (F:M)</td>
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<td>3:8</td>
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<td>FEV&lt;sub&gt;1&lt;/sub&gt;/FVC (ratio)</td>
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<td>63 ± 4 **</td>
</tr>
<tr>
<td>Smoking History (Pack years)</td>
<td>36 ± 8</td>
<td>66 ± 17 **</td>
</tr>
</tbody>
</table>

Table 4.1: Clinical characteristics of healthy smokers and COPD subjects involved in p38 studies

Data are presented as mean ± SEM; F = female; M = male; FEV<sub>1</sub> = Forced Expiratory Volume in 1 sec; FVC = Forced Vital Capacity in 1 sec; ** P < 0.01 (Mann-Whitney U-test) compared with healthy smokers.
4.3.2 Comparison of p38 MAPK activity between healthy smokers and COPD subjects

A comparison of p38 MAPK activity was investigated between healthy smokers and COPD subjects. COPD subjects expressed significantly increased levels of p38 MAPK activity compared with healthy smokers, at both baseline (4.0 vs. 0.9; \( P = 0.016 \)) (Figure 4.1) and when induced with LPS (3.0 vs. 1.0; \( P = 0.032 \)) (Figure 4.2).

**Figure 4.1:** Baseline levels of p38 MAPK activity in healthy smokers compared with COPD subjects. PBMC were isolated from the whole blood of healthy smokers (n=4) and patients with COPD (n=5). Whole cell extracts were prepared and protein levels examined by western blotting with anti-phospho-p38, anti-total p38 and anti-β-actin antibodies, as represented by the blots (A). Band density was quantified using densitometric software analysis (B). Baseline levels of phospho-p38 MAPK expression were normalised to total p38 MAPK expression. Bars represent the median value; * \( P < 0.05 \) (Mann-Whitney U-test) compared with healthy smokers.
Figure 4.2: LPS-induced levels of p38 MAPK activity in healthy smokers compared with COPD subjects. PBMC were isolated from the whole blood of healthy smokers (n=4) and patients with COPD (n=5). Cells were stimulated with LPS (100 ng/mL) for 30 min. Whole cell extracts were prepared and protein levels examined by western blotting with anti-phospho-p38 MAPK, anti-total p38 MAPK and anti-β-actin antibodies, as represented by the blots (A). Band density was quantified using densitometric software analysis (B). Phospho-p38 MAPK expression was normalised to total p38 MAPK expression. Bars represent the median value. * P < 0.05 (Mann-Whitney U-test) compared to healthy smokers. LPS = lipopolysaccharide.
4.3.3 Comparison of baseline or LPS-induced CXCL8 release and lung function between healthy smokers and patients with COPD

PBMC from healthy smokers and COPD subjects were treated with LPS 10 ng/ml for 24 hours. Baseline ($P \leq 0.01$) and induced CXCL8 ($P \leq 0.05$) release was significantly higher in patients with COPD compared to healthy smokers (Figure 4.3A). Performing spearman rank correlations between baseline or LPS-induced CXCL8 release and % predicted FEV$_1$, both baseline ($r = -0.55; P = 0.04$; Figure 4.3B) and induced ($r = -0.71; P = 0.008$; Figure 4.3C) CXCL8 release correlated inversely with FEV$_1$, such that the greater the CXCL8 release the poorer the lung function.
Figure 4.3: Baseline and LPS-induced CXCL8 release in healthy smokers compared with COPD subjects. PBMC were isolated from the whole blood of healthy smokers (n=10) and patients with COPD (n=11). Cells were stimulated with LPS (10 ng/mL) for 24 h. CXCL8 release was quantified from the supernatants by ELISA (A). Spearman rank correlations were performed between baseline (B) or LPS-induced (C) CXCL8 release and % predicted FEV₁. Horizontal bars represent the median value. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; compared with healthy smokers (Mann-Whitney U-test). Unstim = unstimulated cells; FEV₁ = Forced Expiratory Volume in 1 sec; LPS = lipopolysaccharide.
4.3.4 Comparison of dexamethasone mediated suppression of LPS-induced cytokine release between healthy smokers and patients with COPD

The ability of dexamethasone to suppress LPS-induced CXCL8 or IL-6 release was investigated in PBMC from healthy smokers and patients with COPD. The maximal suppression of LPS-induced CXCL8 release by dexamethasone was significantly lower in COPD subjects compared with that of healthy smokers (37.7 ± 3.1% vs. 59.5 ± 3.7%, respectively; \( P < 0.01 \)) (Figure 4.4A). This result indicates that, in PBMC, COPD subjects are less corticosteroid sensitive compared with smokers. There were no significant differences in dexamethasone suppression levels of LPS-induced IL-6 release between COPD subjects and healthy smokers (Figure 4.4B).
Figure 4.4: Dexamethasone mediated suppression of CXCL8 or IL-6 release in healthy smokers and patients with COPD. PBMC isolated from the whole blood of healthy smokers (n=10) and patients with COPD (n=11), were pre-treated with dexamethasone (at the concentrations indicated) for 30 min then stimulated with LPS (10 µg/mL) for 24 h. Cell supernatants were harvested and CXCL8 release (A) or IL-6 release (B) as quantified by ELISA. Suppression of CXCL8 or IL-6 release by dexamethasone is represented as a percentage of LPS induction. Bars represent the mean ± SEM. ** P < 0.01 (Mann-Whitney U-test) compared with healthy smokers. LPS = lipopolysaccharide; Dex = dexamethasone.
4.3.5 The effect of p38 MAPK inhibition on dexamethasone suppression of LPS-induced cytokine release in patients with COPD

The effect of p38 MAPK inhibition on LPS-induced cytokine release from PBMC of COPD subjects was investigated. GW856553 suppressed LPS-induced CXCL8 release in a concentration dependent manner with maximal suppression at 48 ± 7%. The co-treatment of cells with dexamethasone at $10^{-6}$ M and GW856553 ($10^{-10}$ M – $10^{-6}$ M) significantly increased the effect of the corticosteroid on suppression of LPS-induced CXCL8 ($P < 0.0001$, one-way ANOVA) with maximal suppression at 73 ± 6% compared with 47 ± 6% achieved with dexamethasone alone ($P < 0.05$). The greatest relative improvement on the suppressive effect of dexamethasone was shown with the combination of dexamethasone at $10^{-8}$ M and GW856553, compared with dexamethasone alone (63 ± 6% vs. 25 ± 4%; $P < 0.001$). Additionally, the combination of lower concentrations of GW856553 for example at $10^{-8}$ M combined with low dexamethasone concentration at $10^{-8}$ M, also significantly increased the suppressive ability of the corticosteroid (57 ± 5% vs. 25 ± 4%; $P < 0.01$) (Figure 4.5A).

Investigation of IL-6 release showed that GW856553 percentage suppression of LPS-induced IL-6 was also concentration dependent with maximal suppression observed at 59 ± 7%. The maximal suppression by dexamethasone alone at $10^{-6}$ M, $10^{-8}$ M and $10^{-9}$ M was 49 ± 7%, 32 ± 9% and 13 ± 3%, respectively and when combined, at these concentrations, with GW856553 at $10^{-6}$ M, there was significant increase in maximal suppression for every dexamethasone concentration (87 ± 4 vs. 49 ± 7% ($P < 0.01$); 79 ± 6% vs. 32 ± 9% ($P < 0.01$); 69 ± 7% vs. 13 ± 3% ($P < 0.001$); respectively) (Figure 4.5B).
Figure 4.5: The effect of p38 MAPK inhibition on dexamethasone mediated suppression of LPS-induced CXCL8 or IL-6 release compared with dexamethasone alone. PBMC isolated from the whole blood of patients with COPD (n=11) were pre-treated with dexamethasone alone (single coloured symbols) or GW856553 alone (black curve) or in combination (coloured curves) at the concentrations indicated, for 30 min. After stimulation with LPS (10 µg/mL) for 24 h, cell supernatants were harvested and CXCL8 release (A) or IL-6 release (B), was quantified by ELISA. Suppression of CXCL8 or IL-6 release by dexamethasone or GW856553 is represented as a percentage of LPS induction. Bars represent the mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001 (ANOVA); compared with dex alone. LPS = lipopolysaccharide; Dex = dexamethasone; GW-A = GW856553.
4.3.6 The effect of p38 MAPK inhibition alone compared with its combination with dexamethasone

The suppression of cytokine release from section 4.3.5 was re-plotted to show the effect of the combination of dexamethasone and GW856553 compared with two low concentrations of GW856553 alone which had a suppressive effect of less than 4%. The maximal suppression of CXCL8 by dexamethasone at $10^{-6}$ M was significantly improved, in the presence of GW856553 at $10^{-9}$ M (67 ± 4% vs. 3.7 ± 1.8%; $P < 0.001$) and at $10^{-10}$ M (60 ± 4% vs. 5.1 ± 2.0%; $P < 0.01$). At a 100-fold lower concentration of dexamethasone at $10^{-8}$ M, the suppression of CXCL8 was still significantly improved when combined with GW856553 at both $10^{-9}$ M (47 ± 5% vs. 3.7 ± 1.8%; $P < 0.001$) and at $10^{-10}$ M (36 ± 5% vs. 5.1 ± 2.0%; $P < 0.05$) (Figure 4.6A). Similar results were obtained with LPS-induced IL-6 release. The combination of dexamethasone at $10^{-6}$ M with GW856553 at both $10^{-9}$ M and $10^{-10}$ M significantly improved the maximal suppression of IL-6 by dexamethasone (62 ± 7% vs. 9.1 ± 2.0% and 51 ± 7% vs. 10.3 ± 2.8%, respectively; $P < 0.001$) (Figure 4.6B). To determine an additive or synergistic effect of the combination of dexamethasone and GW856553 (both at $10^{-9}$ M and $10^{-10}$ M) on suppression of cytokine release, the interaction ratios were calculated. The ratios ranged from 0.5 to 0.8 consistent with an additive effect (Table 4.2).
Figure 4.6: The effect of p38 MAPK inhibition on dexamethasone mediated suppression of LPS-induced CXCL8 or IL-6 release. PBMC isolated from the whole blood of patients with COPD (n=11) were pre-treated with dexamethasone or GW856553 alone or with dexamethasone and GW856553 combined, at the concentrations indicated, for 30 min. After stimulation with LPS (10 µg/mL) for 24 h, cell supernatants were harvested and CXCL8 release (A) or IL-6 release (B) was quantified by ELISA. Suppression of CXCL8 or IL-6 release by dexamethasone or GW856553 is represented as a percentage of LPS induction. Bars represent the mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001 (ANOVA); compared with GW856553 alone. LPS = lipopolysaccharide; Dex = dexamethasone; GW-A = GW856553.
Table 4.2: Interaction Ratios for the combination of dexamethasone and GW856553 on PBMC from patients with COPD

Data are presented as % inhibition as compared with LPS-stimulated cells. Unshaded section refers to combination with GW856553 at $10^{-9}$ M. Light grey shaded section refers to combination with GW856553 at $10^{-10}$ M. $I_O$ – Observed efficacy; $I_E$ – expected efficacy; IR – interaction ratio; an interaction ratio value of 0.5 – 1.5 is considered an additive effect.

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<td></td>
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</tbody>
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4.3.7 Comparison of the effect of p38 MAPK inhibition on dexamethasone suppression of LPS-induced CXCL8 release between healthy smokers and patients with COPD

The effect of p38 MAPK inhibition on the improvement of dexamethasone suppression of LPS-induced CXCL8 release was compared in PBMC, between healthy smokers and patients with COPD. Maximal percentage suppression of CXCL8 by dexamethasone alone was significantly improved with p38 MAPK inhibition, in both smokers and COPD (88 ± 3% vs. 56 ± 5% ($P < 0.01$); 73 ± 6% vs. 47 ± 6% ($P < 0.05$); respectively). Significantly, the ability of dexamethasone, in the presence of GW856553, to maximally suppress LPS-induced CXCL8 in COPD subjects was improved to a comparable level to that of healthy smokers (COPD: 88 ± 3% vs. smokers: 73 ± 6%; no significant difference) (Figure 4.7).
Figure 4.7: The effect of p38 MAPK inhibition on dexamethasone mediated suppression of LPS-induced CXCL8 in healthy smokers and COPD subjects. PBMC isolated from the whole blood of healthy smokers (n=6) and COPD subjects (n=11), were pre-treated with dexamethasone or GW856553 alone or with both in combination, at the concentrations indicated, for 30 min. After stimulation with LPS (10 µg/mL) for 24 h, cell supernatants were harvested and CXCL8 release was quantified by ELISA. Suppression of CXCL8 release by dexamethasone and/or GW856553 is represented as a percentage of LPS induction. Bars represent the mean ± SEM. * P < 0.05; ** P < 0.01 (ANOVA); compared with dexamethasone alone. LPS = lipopolysaccharide; Dex = dexamethasone; GW-A = GW856553.
4.3.8 The effect of p38 MAPK inhibition on phosphorylation of glucocorticoid receptor (GR) serine 211

Transcriptional activation of the GR is attributed, in part, to the phosphorylation of its serine 211 residue. The improvement in the ability of dexamethasone to suppress cytokine release by p38 MAPK inhibition, was therefore studied from the perspective of the phosphorylation status of GR ser211 in patients with COPD and severe asthmatics. PBMC were stimulated with LPS and/or dexamethasone in the presence or absence of p38 MAPK inhibitor, GW856553. Western blot analysis revealed in COPD subjects, LPS+Dex treated cells induced phosphorylated GR ser211 by 6-fold compared with baseline levels ($P < 0.05$) and this induction was significantly reduced in the presence of GW856553 to levels comparable to those achieved by dexamethasone alone ($P < 0.05$ vs LPS+Dex induced level) (Figure 4.8A). In severe asthmatics, LPS+Dex treated cells induced phospho GR ser211 by nearly 15-fold compared with baseline ($P < 0.05$). This induction was significantly reduced in the presence of GW856553, to 11-fold compared with baseline ($P < 0.05$ vs LPS+Dex induced level) (Figure 4.8B). These results suggest that LPS+Dex induced phosphorylation of GR ser211 may be p38 MAPK-dependent in patients with COPD or severe asthmatics.
Figure 4.8: The effect of p38 MAPK inhibition on phosphorylation of GR ser211. PBMC were isolated from the whole blood of COPD subjects (n=5) (A), or severe asthmatics (n=4) (B). Cells were treated with dexamethasone and/or GW856553 (10^{-6} M) in the presence or absence of LPS (100 ng/ml). Whole cell extracts were prepared and protein levels examined by western blotting with anti-phospho GR ser211, anti-total GR or anti-β-actin antibodies as represented by the blots. Changes in band density were quantified using densitometric software analysis. phospho GR ser211 protein expression was normalised to β-actin then expressed as a fold change with respect to unstimulated control cells. Bars represent the mean ± SEM. # P < 0.05 (paired t-test) compared with baseline; * P < 0.05 (paired t-test) compared with LPS+dex treated cells. LPS = lipopolysaccharide; Dex = dexamethasone; GW-A = GW856553.
4.4 Discussion

4.4.1 Summary of Findings

In this chapter, it has been shown that p38 MAPK activity, at baseline or when induced with LPS, is heightened in COPD subjects compared with healthy smokers. Moreover, baseline levels of pro-inflammatory chemokine CXCL8 release, were significantly higher in patients with COPD compared with healthy smokers and this directly correlated with impaired lung function as assessed by reduced % predicted FEV$_1$. In addition, it was demonstrated that PBMC from COPD subjects compared with healthy smokers were CS-insensitive in response to LPS. Linking heightened p38 MAPK activity with CS-insensitivity, further investigations were performed using a p38 MAPK inhibitor, highly selective for the p38α isoform. p38α MAPK inhibition improved dexamethasone suppression of LPS-induced IL-6 and CXCL8 release in COPD subjects. To investigate the molecular mechanisms by which p38 MAPK activity may be involved in CS-insensitivity, the effect of p38α MAPK inhibition on the phosphorylation status of GR at the serine 211 residue was determined. Phosphorylation of GR ser211 was shown to be p38 dependent and this may therefore be a mechanism by which CS-insensitivity manifests itself.

4.4.2 Heightened p38 MAPK activity and increased CXCL8 release from patients with COPD

The p38 MAPK pathway represents a crucial signalling cascade in the innate immune response, activated mainly by environmental stress and pro-inflammatory cytokines and subsequently regulating the production of pro-inflammatory cytokines such as TNF, IL-6 and CXCL8, by activating the transcription factors that bind to the promoter regions of these cytokines. Therefore it is unsurprising that increased p38 MAPK activity leads to an increase in pro-inflammatory gene expression, the pathological implications of which appear in the form of
inflammatory conditions such as inflammatory bowel disease, rheumatoid arthritis and asthma (Cuenda and Rousseau, 2007). Baseline and LPS-induced levels of phospho-p38 MAPK were significantly increased in PBMC from patients with COPD compared with those from healthy smokers (Figure 4.1 and 4.2). These findings corroborate what other groups have also observed – increased activity of p38 MAPK, specifically in the lung cells of patients with COPD compared with control groups (Renda et al., 2008; Gaffey and Reynolds et al., 2013). As p38α is the best characterised isoform and is widely expressed in most cell types (Cuenda and Rousseau, 2007) and has been identified as one of the main isoforms to dominate sites of chronic inflammation (Korb et al., 2006), it has been the primary target for the majority of p38 MAPK inhibitor development, including the one used in this study, GW856553. p38α has not only been shown to be the most highly expressed isoform, but also most increased in its expression in lung tissue from patients with COPD and smokers compared with non-smokers (Gaffey and Reynolds et al., 2012) validating it as the correct target for GW856553 inhibition in COPD. However it has also been shown that both p38α and p38δ expression levels were similar in lung macrophages from current smokers (Smith et al., 2006).

Increased release of CXCL8, a neutrophil chemoattractant and activator, has been observed in induced sputum samples from patients with COPD compared with smoking and non-smoking control groups (Keatings et al., 1996). Additionally, basal CXCL8 levels were significantly elevated in AM (Culpitt et al., 2003) and in primary epithelial cells (Schneider et al., 2010) from patients with COPD compared with control subjects. Furthermore, in patients with COPD experiencing exacerbation, baseline sputum CXCL8 levels were found to be higher compared with when clinically stable, suggesting the potential for CXCL8 as a marker of COPD exacerbation (Bhowmik et al., 2000; Aaron et al., 2001). To further extend these findings, baseline and LPS-induced levels of CXCL8 release were significantly higher in PBMC from patients with COPD compared with those from healthy smokers (Figure 4.3) which could indicate systemic inflammation. This correlated inversely with % predicted FEV₁. These significant correlations are driven by the inclusion of healthy smoker controls and it would therefore be useful to compare CXCL8 levels between patients with varying
severities of COPD. However, the progressive airway inflammation contributing to a decline in lung function, that are characterised in COPD, may still impact on circulating PBMC by exposing them to inflammatory stress and elevating CXCL8 levels, as reflected in these patients, at baseline. In lung epithelial cells p38 MAPK activation contributed to a mechanism by which CXCL8 mRNA stability was enhanced and CXCL8 release increased (Bhattacharyya et al., 2011). Heightened p38 MAPK signalling may therefore be the underlying cause for increased CXCL8 expression observed in PBMC from patients with COPD.

4.4.3 Inhibition of p38α MAPK reverses CS-insensitivity in COPD

Although the most commonly used therapy for COPD, the ability of inhaled CS to improve the lung function of patients with COPD has long been debated. Consequently, a meta-analysis of the many placebo-controlled clinical trials that have been conducted with inhaled CS in over 13,000 patients with COPD, found no significant effect on the rate of either FEV1 decline nor mortality (although there was evidence that exacerbations were reduced by approximately 25%), (Barnes, 2010). Correlating with this observation, the ability of dexamethasone to decrease LPS-induced CXCL8 release was significantly reduced in patients with COPD compared with healthy smokers, representing relative CS-insensitivity in PBMC from patients with COPD (Figure 4.4A). COPD CS-insensitivity has been previously observed in PBMC (Rossios et al., 2012) and in AM (Culpitt et al., 2003; Cosio et al., 2004). Interestingly, CXCL8 was identified as one of the pro-inflammatory mediators that is particularly CS-insensitive to dexamethasone in LPS stimulated AM from COPD patients (Armstrong et al., 2009). Contrasting with the CXCL8 result, dexamethasone suppression of LPS-induced IL-6 release was not different between patients with COPD and healthy smokers (Figure 4.4B). The extent of LPS induction of steroid-sensitive intracellular pathways such as the MAPK family or NF-κB, may differ, and this may be one reason why there appears to be selective CXCL8 CS-insensitivity compared with IL-6. Other reasons include cell specificity – although LPS is known to induce monocytes, in the mixed lymphocyte population of PBMC, interactions between cell types...
may impact on overall LPS-mediated steroid-sensitivity. Furthermore, differences in dexamethasone suppression of induced CXCL8 or IL-6 may be due to patient variability and/or their CS current treatment.

To investigate the link between p38 MAPK activation and CS, the selective p38α MAPK inhibitor GW856553 was used and was shown to significantly increase dexamethasone suppression of LPS-induced CXCL8 and IL-6 release in PBMC from patients with COPD (Figure 4.5). For example, maximal suppression of CXCL8 release obtained with the highest concentration of dexamethasone (10−6 M) was also achieved with a 100-fold reduction in dexamethasone concentration (10−8 M) with the addition of GW856553 (10−9 M), 48% vs 67%, respectively. Alone, dexamethasone (10−8 M) and GW856553 (10−9 M) suppressed CXCL8 release at 20% and 4% respectively therefore their combined suppression, which increased to 48% (Figure 4.6A) required further analysis to determine if this was a synergistic effect. This was performed by calculating the interaction ratios between the compounds, which resulted in figures consistent with an additive and not synergistic effect (Table 4.2). There were very similar results observed for LPS-induced IL-6 suppression by dexamethasone and GW856553 (Figure 4.6B) and also consistent with an additive effect (Table 4.2). However it should be noted that increasing concentrations of dexamethasone in the combination are consistent with increased interaction ratio and therefore greater additivily. Furthermore, at specific concentrations of GW856553 (10−9 M) with dexamethasone (10−8 M), the combined suppression of cytokine release was greater than each alone, suggesting a ‘more-than-additive’ effect. Improvement in the suppressive effects of dexamethasone was shown to be identical for both COPD and healthy smokers upon p38 MAPK inhibition, suggesting the reversal of CS-insensitivity in PBMC from COPD patients (Figure 4.7). These results support the use of combination therapy - CS and p38α MAPK inhibitors - as a viable treatment for COPD. In addition, the ‘steroid-sparing’ outcome may be of huge significance for sufferers of a disease where abundant use of CS bears many undesirable side-effects.
Similar combination studies have been recently published also using an ex-vivo approach in patients with COPD, supporting the results in this chapter. In monocyte-derived macrophages (MDM), dexamethasone suppression of several LPS-induced pro-inflammatory mediators was enhanced in the presence of p38 MAPK inhibitor, SB706504 and in AM the same combination achieved near-maximal suppression of LPS-induced TNF release (Kent et al., 2009). Furthermore, dexamethasone suppression of LPS-induced pro-inflammatory mediator release including CXCL8 and IL-6 was significantly improved in the presence of p38 inhibitor, BIRB-796 (Armstrong et al., 2011).

From a clinical view point, oral p38 MAPK inhibition by GW856553 (Losmapimod) significantly reduced levels of plasma fibrinogen in patients with COPD by 11% compared with placebo and decreased levels of CXCL8, IL-6 and C-reactive protein – all systemic biomarkers of inflammation of COPD (Lomas et al., 2012). Most recently, a randomised clinical trial administering an oral p38 inhibitor, PH-797804, to patients with moderate to severe COPD, demonstrated significant improvements in lung function parameters and dyspnoea compared with placebo (MacNee et al., 2013).

4.4.4 Phosphorylation of Ser211 on GR is a p38 MAPK dependent process

GR phosphorylation is enhanced upon CS ligand-binding, suggesting a link between GR hormone-dependent phosphorylation and transcriptional activity (Weigel and Moore, 2007). The human GR phosphorylation sites, Ser203, Ser211 and Ser226 lie within the N-terminal AF1 region of the receptor. GR Ser203 and Ser226 are phosphorylated in both the absence and presence of agonists whereas phosphorylation of Ser211 is only observed in a ligand-dependent manner. Recent studies have suggested that Ser211 is not a primary target for p38 MAPK activity (Chen et al., 2008) and that p38 MAPK primarily phosphorylates Ser134 in a ligand-independent manner (Galliher-Beckley et al., 2011). However, in PBMC from patients with COPD or severe asthma, it has now been shown that the p38 MAPK pathway phosphorylates Ser211 on GR in primary, disease-relevant cells (Figure 4.8).
Hyper-phosphorylation of GR as a result of uncontrolled kinase activity has been shown to inhibit GR transcriptional activation, promote GR export from the nucleus upon hormone withdrawal (Itoh et al., 2002) and can alter co-factor recruitment, modulating the transcriptional response of GR to attenuate CS signalling (Galliher-Beckley et al., 2008). Potential mechanisms of CS-insensitivity involving hyper-phosphorylation of GR include a reduction in GR-GRE interaction, GR ligand binding affinity and GR recycling following nuclear export (Galliher-Beckley et al., 2009). Phosphorylation of proteins can be followed by consecutive SUMOylation and ubiquitination resulting in degradation by the proteasome (Schimmel et al., 2008). This therefore suggests that heightened p38 MAPK leading to hyper-phosphorylation of GR may result in CS-insensitivity through an increase in the degradation of GR. This is a possible mechanism that will require further investigation.
CHAPTER 5

The role of MKP-1 in CS-insensitivity in severe asthma
Chapter 5 – The role of MKP-1 in CS-insensitivity in severe asthma

5.1 Introduction

In innate immunity, dual specificity protein (DUSP) phosphatases primarily deactivate mitogen activated protein kinases (MAPK) through dephosphorylation of the tyrosine and threonine residues that are essential for MAPK activation. As such, these protein kinase phosphatases serve as negative regulators of the innate immune system by controlling the transcription of a myriad of pro-inflammatory cytokines and the inflammatory phenotype, through inactivation of the MAP kinases. MAP kinase phosphatase (MKP)-1 is one such phosphatase that has been shown to preferentially deactivate c-JUN N-terminal kinase (JNK) and p38 MAPK but may also dephosphorylate extracellular signal-regulated kinase (ERK) when highly expressed (Liu et al., 2009). Subsequently, phosphorylation by ERK, JNK and / or p38 MAPK (depending on the stimulation and cellular content) has, in part, been shown to regulate MKP-1 activation, thus forming a feedback control loop. This merits MKP-1 as an essential feedback control regulator that limits MAPK-mediated cytokine production and therefore restores immunologic homeostasis (Wancket et al., 2012). Direct association of p38 MAPK to MKP-1 has been shown, in-vitro and in-vivo, to enhance the catalytic activity of the phosphatase (Hutter et al., 2000; Slack et al., 2001; Li et al., 2009). One of the mechanisms by which CS reduce inflammation involves increasing the expression of anti-inflammatory genes, such as MKP-1, which inactivate MAPK function. For instance, dexamethasone-mediated induction of MKP-1 inhibited p38 activity in HeLa cells (Lasa et al., 2002) and murine macrophages (Abraham et al., 2006) and TNFα induced p38 phosphorylation in human airway smooth muscle cells (Quante et al., 2008). Accordingly, inhibition of MKP-1 results in the excessive production of pro-inflammatory cytokines including TNFα, IL-6 and IL-1β (Chen et al., 2002; Shepherd et al., 2004). Our group previously showed p38 MAPK activity to be increased in the
alveolar macrophages (AM) of severe asthmatics compared with non-severe asthmatics and this correlated with reduced MKP-1 mRNA expression in severe asthmatics (Bhavsar et al., 2008). Despite the use of high dose CS in severe asthma, there still appears to be CS-insensitivity associated with excessive p38 MAPK activity and this may be due to impaired MKP-1 expression in these patients. The negative feedback control loop, crucial for p38 MAPK regulation, may be disrupted by reduced MKP-1 expression, warranting further examination into the role that MKP-1 may be playing in CS-insensitivity in severe asthmatics.

The aims of this chapter are to:

1) Compare in PBMC and monocytes, baseline or induced levels of MKP-1 mRNA expression, between non-severe and severe asthmatics

2) Use siRNA transfection to examine the molecular mechanisms by which MKP-1 may be involved in CS suppression of pro-inflammatory mediators in asthmatics.
5.2 Methods

5.2.1 Cell isolation protocols of PBMC or monocytes

PBMC were isolated from the whole blood of non-severe asthmatics or severe asthmatics, the clinical characteristics of whom are outlined in Tables 5.1 and 5.2. PBMC were isolated from whole blood using a Ficoll™ gradient (as described in section 2.2.2.1). A cell count was conducted using Kimura staining and a haemocytometer. The stock concentration was adjusted to $2 \times 10^6$ cells per ml and PBMC were plated at 2 ml per well of 6-well culture plates to give $4 \times 10^6$ PBMC per well, per treatment.

The latter experiments of this chapter were conducted in blood monocytes. To isolate the monocytes, the ‘adherence’ method was used. PBMC were separated from whole blood with Ficoll™ and plated at $4 \times 10^6$ cells per well, as described above. The PBMC were left to incubate for 2 h, so as to allow the monocyte population of the PBMC fraction to adhere to the bottom of the well. After 2 h, the wells were washed twice with warmed culture media and the remaining monocytes were left in 2 ml of culture media and incubated until time for treatment or harvest. The adherence method assumes that approximately a quarter of the number of PBMC initially plated, will remain as monocytes. Therefore when $4 \times 10^6$ PBMC were plated $1 \times 10^6$ monocytes should remain, per well.

5.2.2 Treatment conditions for MKP-1 induction experiments

PBMC or monocytes that were used in comparison studies between non-severe and severe asthmatics were stimulated with dexamethasone ($10^{-6}$ M) and / or lipopolysaccharide (LPS) (100 ng/ml) to induce MKP-1 mRNA expression. For concentration responses of dexamethasone-induced MKP-1 mRNA, a range of $10^{-12}$ M to $10^{-6}$ M was used. For all experiments harvested at 24 h, cells were pre-treated with dexamethasone for 0.5 h followed by LPS for 24 h. For time-course experiments, the cells were pre-treated with dexamethasone for 0.5 h before treatment with LPS for 0.5 h, 1 h or 24 h.
5.2.3 Total RNA extraction, RT-PCR and quantitative real-time PCR of MKP-1 mRNA expression

For experiments in this chapter, MKP-1 mRNA expression levels were determined in PBMC or monocytes by quantitative real-time PCR. Preceding this step, total RNA was isolated from PBMC or monocytes by using the RNeasy Mini Kit according to the manufacturer’s instructions (as described in section 2.2.7.1). cDNA was then generated from total RNA using reverse-transcriptase PCR (RT-PCR), as described in section 2.2.7.2. Quantitative real-time PCR amplification of the cDNA using SYBR Green dye was used to measure MKP-1 and 18 S rRNA mRNA transcript levels in PBMC or monocyte, as described in section 2.2.7.3. Comparison of MKP-1 expression between non-severe and severe asthmatics was calculated from the ratio of MKP-1 to 18 S mRNA expression levels.

5.2.4 Preparation of monocytes preceding siRNA knockdown

For siRNA knockdown experiments, to obtain as pure a population of monocytes as possible from whole blood, a Percoll™ gradient was prepared to separate the PBMC (as described in section 2.2.2.2) followed by use of MACS® Cell Separation Technology to specifically isolate the monocytes from the PBMC fraction (as described in section 2.2.2.3). Kimura stain was used to perform the final monocyte cell count before the transfection procedure.

5.2.5 siRNA knockdown of MKP-1 in monocytes

siRNA transfection by the Human Monocyte Nucleofector® Kit from Lonza was used to induce targeted knockdown of MKP-1 gene expression in monocytes (as described in section 2.2.12). Following isolation, at least 2 x 10^6 monocytes were transfected with 1 µg of MKP-1 or scramble siRNA (as specified for each experiment). Cells were electroporated using the Nucleofector™ device as well as a mock sample (cells without siRNA). To confirm knockdown of MKP-1, monocytes were harvested 48 h post transfection and quantitative real-time PCR (as described in section 2.2.7.3) was used to determine the level of MKP-1 mRNA transcript levels. To examine the functional effect of the
knockdown, cells were pre-treated with dexamethasone (10^{-6} M) for 1 h and stimulated with LPS (100 ng/ml) for 3 h before real-time PCR was used to determine CXCL8 transcript levels. Also for functional protein analysis of the knockdown, cells were pre-treated with dexamethasone (10^{-6} M) for 1 h and stimulated with LPS (100 ng/ml) for 24 h and cells or cell supernatants were harvested. Transfected cells were harvested for whole cell extraction (as described in section 2.2.8) and phosphorylated p38 MAPK or β-actin protein levels were measured in these lysates using western blot (as described in section 2.2.11). Transfected cell supernatants were harvested for analysis of IL-6 or CXCL8 release using ELISA (as described in section 2.2.4).
5.3 Results

5.3.1 Non-severe and severe asthmatic subjects

11 Non-severe and 18 severe asthmatics were recruited for studies performed in PBMC for this section (Table 5.1). For studies performed in monocytes, 6 non-severe and 9 severe asthmatics were recruited (Table 5.2).

<table>
<thead>
<tr>
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<th>Non-severe Asthmatics</th>
<th>Severe Asthmatics</th>
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<tbody>
<tr>
<td>N</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48 ± 3</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>Gender (F:M)</td>
<td>5:6</td>
<td>11:7</td>
</tr>
<tr>
<td>FEV\textsubscript{1} (% Predicted)</td>
<td>74 ± 6</td>
<td>75 ± 5</td>
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<tr>
<td>FEV\textsubscript{1}/FVC (ratio)</td>
<td>67 ± 4</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>Bronchodilator response (%)</td>
<td>18 ± 4</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Log PC\textsubscript{20} (mg/ml)</td>
<td>0.77 ± 0.3</td>
<td>1.33 ± 0.7</td>
</tr>
<tr>
<td>Prednisolone Dose (mg/ml)</td>
<td>N/A</td>
<td>16 ± 8</td>
</tr>
<tr>
<td>BDP equivalent (μg/day)</td>
<td>963 ± 215</td>
<td>1456 ± 72</td>
</tr>
</tbody>
</table>

Table 5.1: Clinical characteristics of non-severe and severe asthmatics involved in comparisons of MKP-1 expression in PBMC

Data are presented as mean ± SEM; F = female; M = male; FEV\textsubscript{1} = Forced Expiratory Volume in 1 sec; FVC = Forced Vital Capacity in 1 sec; bronchodilator response = measured as % increase over baseline FEV\textsubscript{1} after 400 mg albuterol aerosol; PC\textsubscript{20} = provocative concentration of methacholine causing a 20 % fall in FEV\textsubscript{1}; BDP = beclomethasone dipropionate; N/A = not applicable.
<table>
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<tr>
<th></th>
<th>Non-severe Asthmatics</th>
<th>Severe Asthmatics</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43 ± 6</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>Gender (F:M)</td>
<td>4:2</td>
<td>6:3</td>
</tr>
<tr>
<td>FEV₁ (% Predicted)</td>
<td>80 ± 5</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>FEV₁/FVC (ratio)</td>
<td>76 ± 3</td>
<td>64 ± 2</td>
</tr>
<tr>
<td>Bronchodilator response (%)</td>
<td>13 ± 3</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>Log PC₂₀ (mg/ml)</td>
<td>1.7 ± 1.0</td>
<td>1.6 ± 0.03</td>
</tr>
<tr>
<td>Prednisolone Dose (mg/ml)</td>
<td>N/A</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>BDP equivalent (μg/day)</td>
<td>433 ± 285</td>
<td>1444 ± 137*</td>
</tr>
</tbody>
</table>

Table 5.2: Clinical characteristics of non-severe and severe asthmatics involved in comparisons of MKP-1 expression in monocytes

Data are presented as mean ± SEM; F = female; M = male; FEV₁ = Forced Expiratory Volume in 1 sec; FVC = Forced Vital Capacity in 1 sec; bronchodilator response = measured as % increase over baseline FEV₁ after 400 mg albuterol aerosol; PC₂₀ = provocative concentration of methacholine causing a 20 % fall in FEV₁; BDP = beclomethasone dipropionate; N/A = not applicable; * P < 0.05 (Mann-Whitney U) compared with non-severe asthmatics.
5.3.2 Comparison of baseline MKP-1 mRNA expression between non-severe and severe asthmatics

The mRNA expression of MKP-1 at baseline was compared in PBMC between non-severe and severe asthmatics. The results show that at baseline, there is no significant difference in the level of MKP-1 mRNA expression in PBMC between non-severe and severe asthmatics (Figure 5.1).

Figure 5.1: Comparison of baseline levels of MKP-1 mRNA expression between non-severe and severe asthmatics. PBMC were isolated from the whole blood of non-severe asthmatics (○ n=10) and severe asthmatics (● n=16). RNA extracts were prepared from which cDNA was generated by RT-PCR. MKP-1 mRNA expression levels were examined by real-time PCR with oligonucleotides specific for MKP-1 gene and 18 S rRNA. For baseline levels, the ratio of MKP-1 expression to 18 S expression was calculated. Bars represent the median value; ns = no statistical difference between non-severe and severe asthmatics (Mann-Whitney U-test).
5.3.3  Comparison of LPS induced MKP-1 mRNA expression between non-severe and severe asthmatics

The effect of LPS on MKP-1 mRNA expression was investigated in PBMC from non-severe and severe asthmatics. Real-time PCR indicated that LPS significantly induced MKP-1 mRNA in both non-severe and severe asthmatics compared with baseline levels ($P = 0.0066$ and $P = 0.0008$, respectively; Figure 5.2). There was no difference in induction of MKP-1 between non severe and severe asthmatics.

**Figure 5.2: Comparison of LPS-induced MKP-1 mRNA levels between non-severe and severe asthmatics.** PBMC were isolated from the whole blood of non-severe asthmatics (n=11) and severe asthmatics (n=18). Cells were treated with LPS (100 ng/ml) for 24 h. RNA extracts were prepared from which cDNA was generated by RT-PCR. MKP-1 mRNA expression levels were examined by real-time PCR with oligonucleotides specific for MKP-1 gene and 18 S rRNA. The ratio of MKP-1 gene expression to 18 S expression was plotted as a fold change over that of unstimulated cells. Bars represent the mean ± SEM value; ** $P < 0.01$; *** $P < 0.001$ (paired t-test) compared with unstimulated cells. LPS = lipopolysaccharide.
5.3.4 Comparison of dexamethasone induced MKP-1 mRNA expression between non-severe and severe asthmatics

The effect of dexamethasone on MKP-1 expression was investigated in PBMC from non-severe and severe asthmatics. Dexamethasone induced MKP-1 mRNA in both non-severe and severe asthmatics in a concentration dependent manner (Figure 5.3A). Induction of MKP-1 mRNA, compared with baseline, was achieved with the highest concentration of dexamethasone at $10^{-6}$ M in both non-severe and severe asthmatics ($P = 0.004$ and $P = 0.0016$, respectively; Figure 5.3B). There were no significant differences in dexamethasone-induced levels of MKP-1 mRNA between non severe and severe asthmatics.
Figure 5.3: Comparison of dexamethasone-induced MKP-1 mRNA levels between non-severe and severe asthmatics. PBMC were isolated from the whole blood of non-severe asthmatics (n≤10) and severe asthmatics (n≤14). Cells were treated with dexamethasone at 10^{-12} M to 10^{-6} M (A), or at just 10^{-6} M (B), for 24 h. RNA extracts were prepared from which cDNA was generated by RT-PCR. MKP-1 mRNA expression levels were examined by real-time PCR with oligonucleotides specific for MKP-1 gene and 18 S rRNA. The ratio of MKP-1 gene expression to 18 S expression was plotted as a fold change over that of unstimulated cells. Bars represent the mean ± SEM value; ** P < 0.01 (paired t-test) compared with unstimulated cells. Dex = dexamethasone.
5.3.5 The effect of dexamethasone on LPS-induced MKP-1 mRNA expression in non-severe and severe asthmatics

The effect of dexamethasone on LPS-induced levels of MKP-1 mRNA expression was investigated in PBMC from non-severe and severe asthmatics. Dexamethasone potentiated LPS-induction of MKP-1 mRNA in both non-severe and severe asthmatics in a concentration-dependent manner. Dexamethasone/LPS induction of MKP-1 was significantly increased in both non-severe and severe asthmatics at the higher concentrations of dexamethasone compared with their respective baseline levels. Compared with LPS alone, dexamethasone/LPS induction reached significance when dexamethasone was at $10^{-6}$ M, for both non-severe and severe asthmatics ($P = 0.0054$ and $P = 0.0005$, respectively). Significantly, there was less dexamethasone/LPS induction of MKP-1 mRNA expression in severe asthmatics compared with non-severe asthmatics at concentrations of dexamethasone at $10^{-8}$ M and $10^{-6}$ M ($P = 0.0357$ and $P = 0.0476$, respectively; Figure 5.4).
Figure 5.4: Comparison of dexamethasone/LPS induced MKP-1 mRNA levels expressed between non-severe and severe asthmatics. PBMC were isolated from the whole blood of non-severe asthmatics (n=5) and severe asthmatics (n=6). Cells were treated with dexamethasone at 10^{-12} M to 10^{-6} M then stimulated with LPS (100 ng/ml) for 24 h. RNA extracts were prepared from which cDNA was generated by RT-PCR. MKP-1 mRNA expression levels were examined by real-time PCR with oligonucleotides specific for MKP-1 gene and 18 S rRNA. The ratio of MKP-1 gene expression to 18 S expression was plotted as a fold change over that of unstimulated cells. Bars represent the mean ± SEM value. * P < 0.05; ** P < 0.01 (paired t-test) compared with unstimulated cells. ∞∞ P < 0.01, ∞∞∞∞ P < 0.001 (paired t-test) compared with LPS-induced MKP-1 expression. # P < 0.05 (Mann-Whitney U-test) compared with non-severe asthmatics. LPS = lipopolysaccharide; Dex = dexamethasone.
5.3.6 Comparison of dexamethasone/LPS induction of MKP-1 mRNA expression between non-severe and severe asthmatics

A comparison of dexamethasone/LPS induced MKP-1 mRNA expression was investigated in PBMC from non-severe and severe asthmatics. As described in section 5.3.5, in PBMC, dexamethasone/LPS induction of MKP-1 was significantly less in severe asthmatics compared with non-severe asthmatics. This experiment was repeated, only using dexamethasone at $10^{-6}$ M. The previous section was a pilot experiment to observe the effect of a dexamethasone concentration response on LPS-induced MKP-1 expression (Figure 5.4). As such, the patients used for the experiment in the previous section (non-severe asthmatics, n=5; severe asthmatics, n=6) were included in the total number of patients used for the experiment in the current section. Dexamethasone ($10^{-6}$ M)/LPS induction of MKP-1 was again, significantly less in severe asthmatics compared with non-severe asthmatics ($P = 0.0127$; Figure 5.5A). Additionally, the ratio of dexamethasone/LPS induction of MKP-1 to LPS-induced MKP-1 was calculated as a representation of the effect of dexamethasone alone, on LPS-induced MKP-1 expression. This ratio was also shown to be significantly less in severe asthmatics compared with non-severe asthmatics ($P = 0.0287$; Figure 5.5B).
Figure 5.5: MKP-1 mRNA expression induction by dexamethasone/LPS is significantly less in severe asthmatics. PBMC were isolated from the whole blood of non-severe asthmatics (○ n=10) and severe asthmatics (● n=16). Cells were treated with dexamethasone (10^{-6} M) then stimulated with LPS (100 ng/ml) for 24 h. RNA extracts were prepared from which cDNA was generated by RT-PCR. MKP-1 mRNA expression levels were examined by real-time PCR with oligonucleotides specific for MKP-1 gene and 18 S rRNA. The ratio of MKP-1 gene expression to 18 S expression was plotted as a fold change over that of unstimulated cells (A). The ratio of MKP-1 induction by dexamethasone/LPS to that of LPS alone was plotted (B). Bars in (A) represent the mean ± SEM value. Bars in (B) represent the median value. * P < 0.05, ** P < 0.01, *** P < 0.001 (t-tests); # P < 0.05 (Mann-Whitney U-test) compared with non-severe asthmatics. LPS = lipopolysaccharide; Dex = dexamethasone.
5.3.7 Comparison of MKP-1 mRNA expression in blood monocytes of non-severe and severe asthmatics

MKP-1 mRNA expression in blood monocytes from non-severe and severe asthmatics was investigated. Monocytes compared with PBMC are simpler to investigate due to their homogeneity. The baseline level of MKP-1 mRNA expression was compared in monocytes, between non-severe and severe asthmatics. Real-time PCR results showed that at baseline, severe asthmatics expressed significantly less MKP-1 mRNA than non-severe asthmatics ($P = 0.0420$; Figure 5.6).

![Comparison of MKP-1 mRNA expression in blood monocytes of non-severe and severe asthmatics](image)

Figure 5.6: Comparison of baseline levels of MKP-1 mRNA expression between monocytes of non-severe and severe asthmatics. Monocytes were isolated from the whole blood of non-severe asthmatics (○ n=5) and severe asthmatics (● n=8). RNA extracts were prepared from which cDNA was generated by RT-PCR. MKP-1 mRNA expression levels were examined by real-time PCR with oligonucleotides specific for MKP-1 gene and 18S rRNA. For baseline levels, the ratio of MKP-1 expression to 18S expression was calculated. Bars represent the median value; # $P < 0.05$ (Mann-Whitney U-test) compared with non-severe asthmatics.
5.3.8 Comparison of a time course of MKP-1 mRNA induction between non-severe and severe asthmatics

A time-course of MKP-1 mRNA induction was performed in blood monocytes from non-severe and severe asthmatics. Real-time PCR analysis revealed that MKP-1 mRNA expression was induced by LPS alone and dexamethasone alone and that dexamethasone/LPS potentiated this induction, at all time-points in asthmatics. Maximal dexamethasone/LPS induction of MKP-1 mRNA was significant compared with baseline, at 1 h in non-severe asthmatics ($P < 0.01$). In severe asthmatics, maximal dexamethasone/LPS induction of MKP-1 mRNA was significant compared with baseline at 1 h ($P < 0.001$) and similarly so at 24 h ($P < 0.001$; Figure 5.7A). At 1 h, dexamethasone/LPS induction of MKP-1 mRNA was significantly less in severe compared with non-severe asthmatics ($P = 0.0091$; Figure 5.7B).
Figure 5.7: Comparison of induced levels of MKP-1 mRNA expression between monocytes of non-severe and severe asthmatics. Monocytes were isolated from the whole blood of non-severe asthmatics (n=6) and severe asthmatics (n=9). Cells were treated with dexamethasone (10^{-6} M) and / or LPS (100 ng/ml) for 0.5, 1 or 24 h as shown in (A) or only 1 h as shown in (B). RNA extracts were prepared from which cDNA was generated by RT-PCR. MKP-1 mRNA expression levels were examined by real-time PCR with oligonucleotides specific for MKP-1 gene and 18 S rRNA. The ratio of MKP-1 to 18 S expression was calculated as a fold change over unstimulated cells. Bars represent the mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001 compared with unstimulated cells (paired t-test). ## P < 0.01 (Mann-Whitney U-test) compared with non-severe asthmatics. LPS = lipopolysaccharide; Dex = dexamethasone.
5.3.9 siRNA knockdown of MKP-1 in blood monocytes

To more closely examine its role on the effect of corticosteroids, siRNA transfection was used to knockdown MKP-1 expression in monocytes from normal volunteers. The most appropriate way to confirm knockdown of a gene in transfected cells, is to measure its protein expression by western blot analysis. However, the only available antibody against total MKP-1 was unable to reliably detect any bands from any of the western blot membranes that were processed. Therefore, to validate confirmation of MKP-1 knockdown, real-time PCR was used to measure MKP-1 mRNA expression in the samples. The results showed that MKP-1 mRNA expression was significantly reduced in MKP-1 siRNA treated samples compared with the mock control ($P = 0.0087$) and the scramble siRNA control ($P = 0.0087$), indicating that the knockdown in monocytes was successful (Figure 5.8).

![Figure 5.8](image)

**Figure 5.8: The effect of MKP-1 siRNA knockdown on MKP-1 mRNA expression in monocytes.** Monocytes were isolated from the whole blood of normal volunteers (n=6). Cells were nucleofected in the absence of siRNA (mock) or transfected with scramble or MKP-1 siRNA (1 µg). Cells were harvested after 48 h then RNA extracts were prepared from which cDNA was generated by RT-PCR. MKP-1 mRNA expression levels were examined by real-time PCR with oligonucleotides specific for MKP-1 gene and 18 S rRNA. The ratio of MKP-1 to 18 S mRNA was calculated as a fold change over mock control. Bars represent the mean ± SEM. ** $P < 0.01$ (t-tests).
5.3.10 Effect of MKP-1 siRNA knockdown on phosphorylated p38 MAPK protein in monocytes

The functional effect of MKP-1 siRNA knockdown on the inflammatory response in was investigated in monocytes. p38 MAPK is a known substrate of MKP-1. MKP-1 transfected cells displayed increased LPS-induced p38 MAPK activation compared with scramble transfected cells, however this observation did not reach significance \( (P = 0.4) \). Similarly, dexamethasone/LPS induction p38 MAPK activation was also increased in the knockdown cells compared with the scramble transfected cells, but this outcome did not reach significance (Figure 5.9).

**Figure 5.9: The effect of MKP-1 siRNA knockdown on p38 MAPK activity in monocytes.** Monocytes were isolated from the whole blood of normal volunteers \((n=3)\). Cells were nucleofected in the absence of siRNA (mock) or transfected with scramble or MKP-1 siRNA \((1 \mu g)\). Cells were treated with LPS \((100 \text{ ng/ml})\) or LPS/dexamethasone \((10^{-6} \text{ M})\) at 48 h. Cells were then harvested at 72 h, whole cell extracts were prepared and protein levels examined by western blotting with anti-phospho p38 MAPK and anti-β-actin antibodies, as represented by the blots (A). Changes in band density were quantified using densitometric software analysis (B). Phospho-p38 MAPK protein expression was normalised to β-actin then expressed as a fold change with respect to unstimulated scramble transfected cell. Bars represent the mean ± SEM. There were no significant differences between MKP-1 transfected and scramble transfected monocytes. LPS = lipopolysaccharide; Dex = dexamethasone.
5.3.11 Effect of MKP-1 siRNA knockdown on CXCL8 mRNA expression in blood monocytes

The functional effect of MKP-1 siRNA knockdown on CXCL8 mRNA expression was investigated in monocytes from normal volunteers. Real-time PCR revealed that LPS induced CXCL8 mRNA expression was increased in MKP-1 compared with scramble transfected monocytes. Similarly, dexamethasone mediated suppression of LPS-induced CXCL8 mRNA was slightly increased in MKP-1 knockdown cells compared with that of scramble transfected cells. However, the increases between knockdown and scramble transfected cells did not reach significance (Figure 5.10).

![Figure 5.10: The effect of MKP-1 siRNA knockdown on CXCL8 mRNA in monocytes](image)

Monocytes were isolated from the whole blood of normal volunteers (n=6). Cells were nucleofected in the absence of siRNA (mock) or transfected with scramble or MKP-1 siRNA (1 µg). Cells were treated with LPS (100 ng/ml) or LPS/dexamethasone (10^{-6} M) at 48 h, then harvested. RNA extracts were prepared from which cDNA was generated by RT-PCR. MKP-1 mRNA expression levels were examined by real-time PCR with oligonucleotides specific for MKP-1 gene and 18 S rRNA. The ratio of MKP-1 to 18 S mRNA was calculated as a fold change over scramble transfected control. Bars represent the mean ± SEM. LPS = lipopolysaccharide; Dex = dexamethasone.
5.3.12 Effect of MKP-1 siRNA knockdown on CXCL8 and IL-6 release in monocytes

The effect of MKP-1 siRNA knockdown on dexamethasone mediated suppression of LPS-induced CXCL8 or IL-6 release in monocytes was investigated in normal volunteers. ELISA was used to measure LPS or dexamethasone/LPS induced CXCL8 or IL-6 release from MKP-1 siRNA transfected monocytes. Compared with scramble transfected cells, LPS induced CXCL8 or IL-6 release was increased in MKP-1 transfected cells but this did not reach significance for either cytokine ($P = 0.4$ for CXCL8 and IL-6). Also, in MKP-1 siRNA transfected cells, dexamethasone suppressed LPS-induced cytokine release to the same level as that in scramble transfected cells for both CXCL8 and IL-6 ($P = 0.68$ and $P = 0.90$, respectively) (Figure 5.11A and Figure 5.11B).

![Figure 5.11: The effect of MKP-1 siRNA knockdown on CXCL8 and IL-6 released mRNA in monocytes.](image)

Monocytes were isolated from the whole blood of normal volunteers ($n=6$). Cells were nucleofected in the absence of siRNA (mock) or transfected with scramble or MKP-1 siRNA (1 µg). Cells were treated with LPS (100 ng/ml) or LPS/dexamethasone ($10^{-6}$ M) at 48 h, then harvested at 72 h. CXCL8 (A) or IL-6 (B) release levels were examined by ELISA. The CXCL8 or IL-6 release was calculated as a fold change over that of the scramble transfected control. Bars represent the mean ± SEM. LPS = lipopolysaccharide; Dex = dexamethasone.
5.4 Discussion

5.4.1 Summary of findings

In this chapter it was shown that dexamethasone and LPS, individually, significantly induced MKP-1 mRNA expression, compared with baseline levels, in PBMC from severe and non-severe asthmatics. In combination, dexamethasone potentiated LPS induction of MKP-1 mRNA expression which was significantly less in PBMC from severe asthmatics compared with non-severe asthmatics. Additionally the ratio of dexamethasone/LPS induced to LPS-induced MKP-1 mRNA expression was also significantly less in PBMC from severe asthmatics compared with non-severe asthmatics. In monocytes, baseline levels of MKP-1 mRNA expression were significantly less in severe asthmatics compared with non-severe asthmatics. A time course of MKP-1 mRNA induction performed in monocytes revealed that dexamethasone potentiated LPS-induced MKP-1 mRNA, maximally at 1 hour, and this induction was significantly less in severe asthmatics compared with non-severe asthmatics. Knockdown of MKP-1 gene expression by siRNA was successfully performed in monocytes. To examine the functional effect of MKP-1 gene knockdown, p38 MAPK activity was induced by LPS. LPS increased p38 MAPK activity in MKP-1 siRNA transfected monocytes and dexamethasone slightly decreased this, however these results did not reach significance compared with scramble siRNA transfected monocytes. LPS-induced CXCL8 mRNA expression was increased in MKP-1 siRNA transfected monocytes and this was reduced by dexamethasone but it did not reach significance compared with control. LPS-induced CXCL8 and IL-6 release from MKP-1 siRNA transfected cells was increased compared with control however this also was not statistically significant. Finally, dexamethasone reduced LPS-induced CXCL8 or IL-6 release in MKP-1 siRNA transfected cells but there were no differences compared with control.
5.4.2 MKP-1 mRNA expression is impaired in severe asthma

Previous findings from our group reported a reduced induction of MKP-1 expression by dexamethasone in AM from severe asthmatics compared with non-severe asthmatics (Bhavsar et al., 2008). Now, supporting this observation, dexamethasone/LPS induced MKP-1 mRNA expression was significantly lower in PBMC (Figure 5.4 and 5.5) and in monocytes (Figure 5.7) from severe asthmatics compared with non-severe asthmatics. There were also significantly lower MKP-1 expression levels in monocytes, at baseline, in severe asthmatics suggesting MKP-1 as a potential biomarker for asthma severity in these patients (Figure 5.6). Similarly, dexamethasone induction of MKP-1 was also lower in obese asthmatics compared with non-asthmatics (Sutherland et al., 2008).

Conversely, MKP-1 mRNA expression was significantly higher in bronchoscopic biopsy tissues from severe asthmatics compared with non-severe asthmatics, despite the fact that MAP kinase activated protein kinase-2 (MAPKAP-K2) on residue threonine 334 (a target of active p38 MAPK) and ERK1/2 phosphorylation, were also significantly increased in the same tissues (Robins et al., 2012). This would suggest that although induced, not all MKP-1 protein may be active in these tissues (Robins et al., 2012). Of note, dexamethasone potentiated LPS induction of MKP-1 mRNA expression to great levels in both severe and non-severe asthmatics (Figure 5.4). This may be explained by the enhancement by GR of the recruitment of transcription factors activated by LPS, required for CS-independent mediated MKP-1 gene activation.

5.4.3 CS control of p38 through induction of MKP-1

As previously described (Section 1.4.3), CS such as dexamethasone, are one of the key inducers of MKP-1 and as such are able to exert their effects through MKP-1 gene transcription (Kassel et al., 2001; Lasa et al., 2002; Abraham et al., 2006). Once induced, MKP-1 regulates MAPK pathways, including p38 MAPK, forming a negative feedback loop with the kinase to tightly control its intensity and duration (Franklin and Craft, 1997; Hutter et al., 2000; Slack et al., 2001; Salojin et al., 2006). Altering MKP-1 induction therefore affects MAPK regulation. For example, murine AM
treated with the MKP-1 inhibitor triptolide showed prolonged p38 and JNK activation (Chen et al., 2002a). Several MKP-1 \textsuperscript{−/−} mouse cell model studies that have described an increase in induced pro-inflammatory mediators, including increased p38 activity, compared with wild-type (Chen et al., 2002a; Zhao et al., 2005; Chi et al., 2006; Zhao et al., 2006) as well as systemic increases (Salojärvi et al., 2006). In a human lung epithelial cell line, MKP-1 siRNA knockdown increased the activation of p38 MAPK and JNK, as well as expression of IL-6, CXCL8 and COX-2, when induced with cytokines (Turpeinen et al., 2010). At the other extreme, MKP-1 overexpression significantly attenuated TNFα levels in murine AM (Zhao et al., 2005) and the overexpression of MKP-1 in LPS-stimulated rat macrophages, significantly reduced the half-lives of IL-6, IL-10 and TNFα mRNA compared to controls (Yu et al., 2011).

In agreement with these reports, baseline and induced levels of MKP-1 expression were significantly reduced in severe asthmatics compared with non-severe asthmatics (Figure 5.4 – Figure 5.7) correlating with heightened p38 MAPK activation in severe asthmatics (3.3.1.2). p38 MAPK inhibition improved the suppressive abilities of dexamethasone in severe asthmatics (3.3.2.2) suggesting that heightened p38 MAPK activation, due to lowered MKP-1 expression may be involved in the CS-insensitivity seen in severe asthma. To strengthen this hypothesis, siRNA knockdown of MKP-1 was performed in monocytes from normal volunteers (Figure 5.8) and baseline and induced levels of p38 MAPK activation were found to be increased - although not significantly - in the knockdown cells compared with scramble-siRNA treated cells (Figure 5.9). Similarly, LPS-induced CXCL8 mRNA expression (Figure 5.10) and LPS-induced CXCL8 and IL-6 release (Figure 5.11) were measured in the knockdown cells and, although they were found to be increased compared with scramble siRNA-transfected cells, this did not reach significance. Lastly, MKP-1 knockdown did not significantly affect dexamethasone suppression of LPS-induced CXCL8 or IL-6. Although siRNA knockdown of MKP-1 was validated by its decreased mRNA expression compared with control, the lack of an appropriate antibody at the time, to measure its protein level has meant that it cannot be confirmed whether MKP-1 protein expression was truly reduced in the monocytes, post-
transfection. This may explain the observation, that though MKP-1 knockdown in monocytes did lead to an increased pro-inflammatory state, as expected, perhaps the level of knockdown was insufficient to render these measurements statistically significant. Another factor is the relatively low n-numbers used for these experiments. However, it must also be considered that other phosphatase pathways (such as other members of the MKP family or protein phosphatase family) may have also been involved in the final result, in a compensatory manner, therefore masking the effect of the knockdown of MKP-1.

5.4.4 Molecular mechanisms of MKP-1 involvement in CS action

Most of the anti-inflammatory effects of CS are facilitated through GR-GRE interaction resulting in either transactivation or transrepression of CS sensitive genes (Barnes, 2006b). The mechanism underlying the correlation between CS anti-inflammatory action and diminished MAPK activation and pro-inflammatory mediator expression, may be through the induction of MKP-1 gene expression. For example, in MKP-1−/− mouse macrophages, dexamethasone mediated inhibition of p38 MAPK and expression of several pro-inflammatory genes, was abrogated (Abraham et al., 2006). In human ASM cells, CS destabilisation of IL-6 mRNA transcript and subsequent inhibition of its expression, was mediated through MKP-1 down-regulation of p38 MAPK (Quante et al., 2008). Furthermore, in GR−/− mice, dexamethasone did not induce MKP-1 in peritoneal macrophages demonstrating the importance of GR in CS mediated MKP-1 induction (Bhattacharyya et al., 2007). In human and mouse MKP-1 genes, several putative consensus GRE binding sites have been identified in their promoter regions (Tchen et al., 2010). In AS49 cells, a functional GRE, on the MKP-1 gene promoter, was identified and the HAT, p300, a transcriptional cofactor with intrinsic histone acetylase (HAT) properties, was required for CS-mediated MKP-1 transcription (Shipp et al., 2010).

In contrary to the above, several reports do also describe CS-mediated action that is partially or wholly MKP-1-independent. It has also been reported that MKP-1 levels may be differentially expressed depending on the cell type (Tephly and Carter, 2007). For instance, some pro-
inflammatory genes, such as inducible nitric oxide synthase (iNOS), were inhibited by dexamethasone in MKP-1/− mouse macrophages in a seemingly MKP-1 independent manner (Abraham et al., 2006). In MKP-1/− mouse bone-marrow derived mast cells, CS-mediated inhibition of p38 activity was not significantly different compared with wild-type (Maier et al., 2007). Furthermore in MKP-1 siRNA transfected human ASM cells, CS mediated inhibition of induced JNK (but not p38 MAPK), and pro-inflammatory cytokine release was reversed, suggesting only partial dependence on MKP-1 (Issa et al., 2007).

It still therefore remains unclear whether reduced MKP-1 expression, which is observed in cells of severe asthmatics, is a systemic feature of the disease or if it is a result of dephosphorylation by heightened p38 MAPK in these patients. Indeed, AM from non-severe asthmatics also displayed low levels of induced MKP-1 expression albeit more than in the severe asthmatics (Bhavsar et al., 2008) suggesting that this may be a biomarker of the asthmatic state itself. Finally to partially answer the question of the in-vivo effects of CS induced gene transcription, Kelly and colleagues examined bronchial biopsies from allergen exposed patients with mild asthma. After inhaled CS treatment, anti-inflammatory GILZ, FKBP51 and MKP-1 mRNA gene expression was upregulated compared with placebo, however only MKP-1 expression was not to significance (Kelly et al., 2012).
CHAPTER 6

Alternative combination therapies for the improvement of CS sensitivity
Chapter 6 – Alternative combination therapies for the improvement of CS sensitivity

6.1 Introduction

Inhaled corticosteroids have been the most effective anti-inflammatory treatment for the treatment of asthma. For COPD, inhaled CS have been shown to have a small effect on improving symptoms but not to the extent observed in asthma due to the differing manifestations of the two diseases. Since the discovery that the combination of inhaled CS with LABA improves the action of the inhaled CS, and therefore the effectiveness of the treatment in asthma and COPD, this has now become the mainstay therapy for these diseases (Chung et al., 2009). In severe asthmatics however, the use of inhaled CS is not wholly effective, despite their use at high doses and even with the use of a LABA (Sterling and Chung, 2001). It has been previously demonstrated, in AM and PBMC, severe asthmatics to be relatively CS-insensitive compared with non-severe asthmatics (Hew et al., 2006; Bhavsar et al., 2008). In patients with COPD, inhaled CS do not show much effect at controlling the symptoms and are unable to reverse the progression of the disease (Chung, 2009). Moreover, as shown in Chapter 4 of this thesis, PBMC from patients with COPD display relative CS-insensitivity compared with those from healthy smokers. Taken together, despite the current CS/LABA combination therapies that already exist, there is still an unmet need for more effective therapies for severe asthmatics and for patients with COPD.

When administered, CS penetrate the cell membrane binding to GR whereby translocation to the nucleus occurs and GRE binding ensues resulting in the transactivation of CS-responsive genes. Alternatively, direct CS-GR interactions with transcription factors such as AP-1 or NF-kB and the subsequent recruitment of HDACs and promotion of chromatin remodelling, leads to the down-regulation of pro-inflammatory genes through transrepression (Barnes and Adcock, 2003). All LABA exert their airway smooth muscle (ASM) relaxant effects through the cell surface β2-
adrenoreceptors. Upon binding to the active site of the receptor, the α-component of the associated G protein dissociates, activating adenylate cyclase and producing intracellular cAMP which subsequently activates PKA the result of which is the phosphorylation of several intracellular regulatory proteins involved in for example in ASM cells, the control of muscle tone (Johnson, 1998).

In terms of gene transcription, β2-agonists, through cAMP and subsequent increased translocation of PKA to the nucleus may lead to phosphorylation of CREB-binding protein, enhancing its DNA-binding and transactivation effects. Alternatively, binding of the LABA to the β2-adrenoreceptors may lead to coupling to a G protein. The β2-adrenoceptor is phosphorylated by PKA and mediated by βγ-subunits of the G protein which act as scaffolding for several upstream regulators of the MAPK family such as Raf and RAS – this culminates in MAPK regulation, for example, p38 MAPK (Johnson, 2006; Daaka et al., 1997). LABAs, through β2-adrenoceptors, can also regulate nuclear GR translocation (and therefore GR activity) (Eickleberg et al., 1999; Roth et al., 2002; Usmani et al., 2005; Mercado et al., 2011). As CS effects are also exerted through GR (promoting, for example, the transactivation of anti-inflammatory genes such as MKP-1) LABA/GR nuclear translocation mechanism may explain the enhancement of CS when combined with LABA. CS-insensitivity in asthma and COPD may be associated with hyper-phosphorylation of GR through increased activation of p38 MAPK (Figure 4.8; Miller et al., 2005; Mercado et al., 2011), which may in turn, be linked with reduced MKP-1 expression (Figure 5.5; figure 5.6; Abraham et al., 2006; Bhavsar et al., 2008; Quante et al., 2008). However, LABA in combination with CS may alleviate CS-insensitivity due to their ability to regulate GR nuclear translocation which may restore GR function.

Fluticasone furoate (FF, GW685698X) is a novel, enhanced-affinity CS, with a unique combination of pharmacodynamics and physiochemical properties (Salter et al., 2007). Sharing similar properties with fluticasone propionate (FP) apart from a substitution of a 17α hydroxyl group, FF was originally developed as a topically active glucocorticoid (Valotis and Högger, 2007) for the treatment of allergic rhinitis (Salter 2007). Despite the name and nomenclature, FF and FP are in fact structurally distinct drug substances with FF possessing superior properties (Biggadike 2011).
Vilanterol trifenate (GW642444M) is a novel, ‘ultra-LABA’ that has recently been developed to be used in combination with FF as a once-daily treatment for asthma and COPD and is currently undergoing clinical trials (Procopiou et al., 2010; Tashkin and Fabbri, 2010).

The aims of this chapter are to:

1) Compare, in PBMC, the suppressive action and potencies of FF and FP on the inflammatory response between non-severe asthmatics and severe asthmatics, and between patients with COPD and healthy smokers

2) Determine the anti-inflammatory effect of 444 alone or in combination with FF or FP during the inflammatory response, in non-severe asthmatics compared with severe asthmatics, and in patients with COPD compared with healthy smokers

3) Examine the effect of FF or FP on the duration of induction of MKP-1, in asthmatics.
6.2 Methods

6.2.1 Cell isolation and plating conditions for experiments performed with FF, FP and Vilanterol

PBMC were isolated from the whole blood of non-severe asthmatics, severe asthmatics, healthy smokers, and patients with COPD, the characteristics of whom are outlined in Table 6.1 and 6.2 below. PBMC were isolated from whole blood using a Ficoll™ gradient, as fully described in section 2.2.2.1. PBMC were diluted in media, to a stock solution of $1 \times 10^6$ cells per mL then plated at a density of $0.75 \times 10^6$ cells per well of 12-well cell culture plates. Plated cells were allowed to settle for 2 h ($37^\circ C; 5\% CO_2; 90\%$ humidity) before treatment.

6.2.2 Treatment conditions for experiments with performed with FF, FP and Vilanterol

Cells were pre-incubated with Fluticasone Propionate (FP), or Fluticasone Furoate (FF) +/- vilanterol, all between $10^{-14}$ M – $10^{-7}$ M, as specified for each experiment, for 30 min. Stimulation with LPS at 100 ng/mL, to induce CXCL8 or TNFα release, was followed by incubation overnight ($37^\circ C; 5\% CO_2; 90\%$ humidity). After 24 h cell supernatants were harvested and stored at -20°C until ready for cytokine analysis.

6.2.3 Cytokine analysis of CXCL8 or TNFα from PBMC supernatants

CXCL8 or TNFα release was quantified using DuoSet ELISA kits, as fully described in section 2.2.4.

6.2.4 Treatment conditions for FF or FP induced MKP-1 experiments

The stock concentration of the PBMC was adjusted to $2 \times 10^6$ cells per mL then in 6-well culture plates, $4 \times 10^5$ PBMC per well was plated for each treatment. Following incubation for 2 h PBMC were treated with FF or FP ($10^{-6}$ M) and / or lipopolysaccharide (LPS) (100 ng/mL) to induce MKP-1 mRNA expression. Cells were harvested at either 4 h or 24 h.
6.2.5 Quantification of MKP-1 mRNA expression

MKP-1 mRNA expression levels were determined in PBMC or monocytes by quantitative real-time PCR. Preceding this step, total RNA was isolated from cells using the RNeasy Mini Kit according to the manufacturer’s instructions and as fully described in section 2.2.7.1. cDNA was generated from total RNA using reverse-transcriptase PCR as fully described in section 2.2.7.2. Quantitative real-time PCR amplification of the cDNA using SYBR Green dye was used to measure MKP-1 mRNA and 18S rRNA mRNA transcript levels in PBMC, as described in section 2.2.7.3. Comparison of MKP-1 mRNA expression between induced and baseline levels were calculated from the ratio of MKP-1 to 18S mRNA expression levels.
6.3 Results

6.3.1 Results Part I: The effect of the combination of CS with LABA on steroid efficacy in PBMC from severe asthmatics or patients with COPD.

6.3.1.1 Non-severe and severe asthmatic subjects

For this comparison study, 14 non-severe asthmatics and 20 severe asthmatics were recruited (Table 6.1) and patients were chosen from this pool.

<table>
<thead>
<tr>
<th></th>
<th>Non-severe Asthmatics</th>
<th>Severe Asthmatics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>49 ± 4</td>
<td>43 ± 3</td>
</tr>
<tr>
<td><strong>Gender (F:M)</strong></td>
<td>7:7</td>
<td>15:5</td>
</tr>
<tr>
<td><strong>FEV₁ (% Predicted)</strong></td>
<td>85 ± 4</td>
<td>76 ± 4</td>
</tr>
<tr>
<td><strong>FEV₁/FVC (ratio)</strong></td>
<td>73 ± 3</td>
<td>67 ± 2</td>
</tr>
<tr>
<td><strong>Bronchodilator response (%)</strong></td>
<td>10 ± 1</td>
<td>20 ± 4*</td>
</tr>
<tr>
<td><strong>Log PC₂₀ (mg/ml)</strong></td>
<td>2.1 ± 0.7</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td><strong>Prednisolone Dose (mg/ml)</strong></td>
<td>N/A</td>
<td>19 ± 3</td>
</tr>
<tr>
<td><strong>BDP equivalent (μg/day)</strong></td>
<td>983 ± 129</td>
<td>1545 ± 82**</td>
</tr>
</tbody>
</table>

Table 6.1: Clinical characteristics of non-severe and severe asthmatics involved in FF/FP studies

Data are presented as mean ± SEM; F = female; M = male; FEV₁ = Forced Expiratory Volume in 1 sec; FVC = Forced Vital Capacity in 1 sec; bronchodilator response = measured as % increase over baseline FEV₁ after 400 mg albuterol aerosol; PC₂₀ = provocative concentration of methacholine causing a 20 % fall in FEV₁; BDP = beclomethasone dipropionate; N/A = not applicable. * P < 0.05, ** P < 0.01 (Mann-Whitney U-test) compared with non-severe asthmatics.
6.3.1.2 Healthy smokers and patients with COPD

For this comparison study, 8 healthy smokers and 13 patients with COPD were recruited (Table 6.2).

<table>
<thead>
<tr>
<th></th>
<th>Healthy Smokers</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Age (yrs.)</td>
<td>50 ± 4</td>
<td>64 ± 2</td>
</tr>
<tr>
<td>Gender (F:M)</td>
<td>3:5</td>
<td>11:2</td>
</tr>
<tr>
<td>FEV₁ (% Predicted)</td>
<td>88 ± 4</td>
<td>60 ± 6**</td>
</tr>
<tr>
<td>FEV₁/FVC (ratio)</td>
<td>78 ± 1</td>
<td>56 ± 3**</td>
</tr>
<tr>
<td>Smoking History (Pack years)</td>
<td>36 ± 8</td>
<td>34 ± 2</td>
</tr>
</tbody>
</table>

Table 6.2: Clinical characteristics of healthy smokers and patients with COPD involved in FF/FP studies

Data are presented as mean ± SEM; F = female; M = male; FEV₁ = Forced Expiratory Volume in 1 sec; FVC = Forced Vital Capacity in 1 sec; ** P < 0.01 compared with healthy smokers (Mann-Whitney U-test).
6.3.1.3 Comparison of FF with FP mediated suppression of LPS-induced CXCL8 release in non-severe and severe asthmatics

The effect of FF or FP mediated suppression of LPS-induced CXCL8 release was compared in PBMC from non-severe asthmatics and severe asthmatics. The inhibitory effect (% suppression) of FF or FP on LPS-induced CXCL8 release was calculated as the % of cytokine suppressed as compared with that maximally induced by LPS alone. The results indicated that both FF and FP suppressed LPS-induced CXCL8 in a concentration dependent manner. However, FF displayed greater potency compared with FP in both non-severe (IC\textsubscript{50}: 0.72 vs 12.0 nM) and severe asthmatics (IC\textsubscript{50}: 0.46 vs 180 nM) at all concentrations of CS tested (10\textsuperscript{-14} M to 10\textsuperscript{-7} M) (Table 6.3; figure 6.1). Although there were no differences in maximal efficacy between FF and FP (61.65 vs 54.2% in non-severe asthmatics (Figure 6.1A); 63.1% vs 46.0% in severe asthmatics (Figure 6.1B)), suppression by FF was significantly increased compared with that of FP at 10\textsuperscript{-10} M in non-severe asthmatics (50 ± 8% vs. 29 ± 8%; \( P < 0.05 \); Figure 6.1A) and at 10\textsuperscript{-9} M in severe asthmatics (60 ± 4% vs. 46 ± 5%; \( P < 0.05 \); Figure 6.1B).

The IC\textsubscript{50} value and maximal % inhibition values for FF or FP suppression of CXCL8 or TNF\( \alpha \), related to asthmatics, are outlined in Table 6.3

<table>
<thead>
<tr>
<th>Patient asthma status</th>
<th>FF ( \text{IC}_{50} ) (nM)</th>
<th>Maximal inhibition (%)</th>
<th>FP ( \text{IC}_{50} ) (nM)</th>
<th>Maximal inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe (CXCL8)</td>
<td>0.46</td>
<td>63.1</td>
<td>180</td>
<td>46.0</td>
</tr>
<tr>
<td>Non-severe (CXCL8)</td>
<td>0.72</td>
<td>61.6</td>
<td>12</td>
<td>54.2</td>
</tr>
<tr>
<td>Severe (TNF( \alpha ))</td>
<td>0.02</td>
<td>83.9</td>
<td>0.078</td>
<td>82.4</td>
</tr>
<tr>
<td>Non-severe (TNF( \alpha ))</td>
<td>0.1</td>
<td>75.5</td>
<td>0.3</td>
<td>73.8</td>
</tr>
</tbody>
</table>

Table 6.3: IC\textsubscript{50} values and maximal inhibitions by FF or FP on LPS-induced CXCL8 or TNF\( \alpha \) release in patients with asthma

Unshaded section refers to CXCL8 release. Light grey shaded section refers to TNF\( \alpha \) release. Maximal inhibition – inhibition of CXCL8/TNF\( \alpha \) by FF/FP presented as percentage of LPS induced release; IC – inhibitory concentration; FF - Fluticasone Furoate; FP - Fluticasone Propionate.
Figure 6.1: The effect of FF or FP on the suppression of LPS-induced CXCL8 release. PBMC isolated from the whole blood of non-severe asthmatics (n=8) (A) and severe asthmatics (n=11) (B), were pre-treated with FF or FP (at the concentrations indicated) for 30 min then stimulated with LPS (100 ng/mL) for 24 h. Cell supernatants were harvested and CXCL8 levels quantified by ELISA. Suppression of CXCL8 by FF or FP is presented as a percentage of LPS induced release. Bars represent the mean ± SEM. * P < 0.05 (Mann-Whitney U-test); compared with FP. LPS = lipopolysaccharide; FF = Fluticasone Furoate; FP = Fluticasone Propionate.
6.3.1.4 **FF and FP suppress LPS-induced TNFα release in a concentration dependent manner in both non-severe and severe asthmatics**

The effect of FF and FP on suppression of LPS-induced TNFα release was investigated in PBMC from non-severe asthmatics (Figure 6.2A) and severe asthmatics (Figure 6.2B). The results showed both CS suppressed TNFα in a concentration dependent manner in both non-severe and severe asthmatics and there were no differences in efficacy between FF and FP in either group.

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**Figure 6.2**: The effect of FF or FP on suppression of LPS-induced TNFα release. PBMC isolated from the whole blood of non-severe asthmatics (n=5) (A) and severe asthmatics (n=12) (B), were pre-treated with FF or FP (at the concentrations indicated) for 30 min then stimulated with LPS (100 ng/mL) for 24 h. Cell supernatants were harvested and TNFα levels quantified by ELISA. Suppression of TNFα by FF or FP is presented as a percentage of LPS induced release. Bars represent the mean ± SEM. LPS = lipopolysaccharide; FF = Fluticasone Furoate; FP = Fluticasone Propionate.
6.3.1.5 Comparison of the suppression of LPS-induced CXCL8 or TNFα release by FF or FP between non-severe and severe asthmatics

The suppressive effects of FF on LPS-induced CXCL8 (Figure 6.3A) or TNFα (Figure 6.3B) release and effects of FP on LPS-induced CXCL8 (Figure 6.3C) or TNFα (Figure 6.3D) release, were compared in PBMC between non-severe and severe asthmatics. This was to determine whether CS-insensitivity could be demonstrated between the disease groups with the use of FF or FP. The results showed that there were no significant differences between non-severe and severe asthmatics in relation to the ability of either FF or FP to suppress induced cytokine release in these cells. The IC_{50} values and maximal % inhibition for FF or FP suppression of CXCL8 or TNFα, related to asthmatics, are outlined in Table 6.3.
Figure 6.3: The effect of FF or FP on the suppression of LPS-induced CXCL8 or TNFα release in severe and non-severe asthmatics. PBMC isolated from the whole blood of non-severe asthmatics (n=10) and severe asthmatics (n=12), were pre-treated with FF (A/B) or FP (C/D) (at the concentrations indicated) for 30 min then stimulated with LPS (100 ng/mL) for 24 h. Cell supernatants were harvested and CXCL8 (A/C) or TNFα (B/D) levels quantified by ELISA. Suppression of LPS-induced CXCL8 or TNFα by FF or FP is presented as a percentage of LPS induction. Bars represent the mean ± SEM. LPS = lipopolysaccharide; FF = Fluticasone Furoate; FP = Fluticasone Propionate.
6.3.1.6 The effect of Vilanterol Trifenatate on suppression of LPS-induced TNFα release, by FF or FP, in severe asthmatics

The combinatorial suppressive effects of the LABA, vilanterol trifenatate (‘vilanterol’ or ‘GW642444M’) with FF or FP were investigated in PBMC from severe asthmatics. Specifically, the suppressive effect of a FF+vilanterol combination or FP+vilanterol combination on LPS-induced TNFα release, were compared with vilanterol alone and with FP or FF alone, respectively. The FF/FP concentrations used were $10^{-12} \text{M}$ to $10^{-9} \text{M}$ combined with vilanterol concentration range of $10^{-12} \text{M}$ to $10^{-7} \text{M}$. The percentage suppression of the FF+vilanterol or FP+vilanterol combination on LPS-induced TNFα release was calculated as the percentage of TNFα release as compared with that induced by LPS alone. The percentage suppression was then compared between the combination and each component alone to determine whether vilanterol enhanced the suppressive abilities of either FF or FP. Vilanterol alone inhibited LPS-induced TNFα release in a concentration-dependent manner with maximal efficacy of $37 \pm 4\%$ at $10^{-8} \text{M}$, (Figures 6.4 A-D). The inhibitory effect of the combination of either FF or FP with vilanterol was increased compared with the LABA alone. For all the concentrations of CS used ($10^{-12} \text{M} – 10^{-9} \text{M}$) in combination with vilanterol, there was greater potency with FF+vilanterol compared with FP+vilanterol on the suppression of induced TNFα release. There were no differences in TNFα suppression between the combination FP+vilanterol and vilanterol alone at any concentration of the steroid. The inhibitory effect of the FF+vilanterol combination however, was greater than that of FP+vilanterol, with maximal efficacy at FF $10^{-9} \text{M} +$ vilanterol $10^{-12} \text{M}$ compared with vilanterol alone ($86 \pm 3\%$ vs. $30 \pm 5\%; P < 0.05$; Figure 6.4A). Furthermore, at the lower concentrations of CS ($10^{-12} \text{M}$), FF but not FP, in combination with vilanterol, was significantly more efficient in TNFα suppression compared with FF alone suggesting a steroid sparing effect with the FF+vilanterol combination (maximal efficacy: $53 \pm 5\%$ vs. $21 \pm 3\%; P < 0.001$; Figure 6.4D).
Figure 6.4: Comparison of the effect of vilanterol inhibition on FF or FP mediated suppression of LPS-induced TNFα release compared with vilanterol alone. PBMC isolated from the whole blood of severe asthmatics (n ≤ 14) were pre-treated for 30 min, with FF or FP alone (single coloured circles) or vilanterol alone (black curve) or with FF+vilanterol or FP+vilanterol combined (coloured curves) at the concentrations indicated, (FF/FP at 10⁻⁹ M (A); FF/FP at 10⁻¹⁰ M (B); FF/FP at 10⁻¹¹ M (C); FF/FP at 10⁻¹² M (D)). After stimulation with LPS (100 ng/mL) for 24 h, cell supernatants were harvested and TNFα release quantified by ELISA. Suppression of TNFα by vilanterol and/or FF/FP is represented as a percentage of LPS induction. Bars represent the mean ± SEM. * P < 0.05, ** P < 0.01 (ANOVA); compared with vilanterol alone. # P < 0.05, ## P < 0.01 (ANOVA); compared with FF alone. LPS = lipopolysaccharide; FF = Fluticasone Furoate; FP = Fluticasone Propionate; 444 = vilanterol trifenate.
6.3.1.7 Comparison of the effect of FF or FP on LPS-induced CXCL8 release in PBMC from patients with COPD and healthy smokers

The effect FF or FP mediated suppression of LPS-induced CXCL8 release was compared in PBMC from healthy smokers and patients with COPD. There was a concentration dependent suppression of induced CXCL8 release by both FF and FP in all subjects. The IC$_{50}$ value for FP was greater than FF for both healthy smokers (IC$_{50}$: 1.8 vs 0.12 nM) and patients with COPD (IC$_{50}$: 7.3 vs 1.7 nM) representing greater potency of FF over FP (Table 6.4). Although there were no differences between the maximal efficacies of FF and FP in healthy smokers (68.4% vs 64.9% (Table 6.4)), there was at a sub-maximal concentration of $10^{-10}$ M (55 ± 9% vs. 24 ± 7%; $P < 0.05$; Figure 6.5A). Similarly, in patients with COPD, although the difference in maximal efficacy of FF or FP on CXCL8 inhibition was negligible (57.6% vs 54.9%) suppression by FF was significantly greater than that of FP at a submaximal concentration of $10^{-10}$ M (43 ± 5% vs. 22 ± 4%; $P < 0.05$; Figure 6.5B).

<table>
<thead>
<tr>
<th>Patient asthma status</th>
<th>FF IC$_{50}$ (nM)</th>
<th>Maximal inhibition (%)</th>
<th>FP IC$_{50}$ (nM)</th>
<th>Maximal inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COPD (CXCL8)</strong></td>
<td>1.7</td>
<td>57.6</td>
<td>7.3</td>
<td>54.9</td>
</tr>
<tr>
<td><strong>Smokers (CXCL8)</strong></td>
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<td>1.8</td>
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</tr>
<tr>
<td><strong>COPD (TNFα)</strong></td>
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<td>81.8</td>
</tr>
<tr>
<td><strong>Smokers (TNFα)</strong></td>
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<td>93.6</td>
<td>0.13</td>
<td>91.4</td>
</tr>
</tbody>
</table>

Table 6.4: IC$_{50}$ values and maximal inhibitions by FF or FP on LPS-induced CXCL8 or TNFα release in patients with COPD or healthy smokers

Unshaded section refers to CXCL8 release. Light grey shaded section refers to TNFα release. Maximal inhibition – inhibition of CXCL8/TNFα by FF/FP presented as percentage of LPS induced release; IC – inhibitory concentration; FF - Fluticasone Furoate; FP - Fluticasone Propionate.
Figure 6.5: The effect of FF or FP on the suppression of LPS-induced CXCL8 release. PBMC isolated from the whole blood of healthy smokers (n=8) (A) and patients with COPD (n=13) (B), were pre-treated with FF or FP (at the concentrations indicated) for 30 min then stimulated with LPS (100 ng/mL) for 24 h. Cell supernatants were harvested and CXCL8 levels quantified by ELISA. Suppression of CXCL8 by FF or FP is presented as a percentage of LPS induced release. Bars represent the mean ± SEM. * P < 0.05 (Mann-Whitney U-test) compared with FP. LPS = lipopolysaccharide; FF = Fluticasone Furoate; FP = Fluticasone Propionate.
6.3.1.8 Comparison of the effect of FF and FP mediated suppression of LPS-induced TNFα release in PBMC of patients with COPD and healthy smokers

The effect of FF or FP mediated suppression of LPS-induced TNFα release in PBMC was examined in healthy smokers (Figure 6.6A) and patients with COPD (Figure 6.6B). The results showed that both FF and FP suppressed induced TNFα release in a concentration dependent manner and the potency of FF was greater than that of FP for smokers (IC$_{50}$: 0.13 vs 0.046 nM) but not for patients with COPD (IC$_{50}$: 0.041 vs 0.047 nM) (Table 6.4). In smokers, the effect of FF was significantly greater than FP at 10$^{-10}$ M (68 ± 6% vs. 42 ± 7%; $P < 0.05$; Figure 6.6A) but there were no differences in maximal inhibition of TNFα by FF or FP, for either group (Table 6.4).
Figure 6.6: The effect of FF or FP on the suppression of LPS-induced TNFα release. PBMC isolated from the whole blood of healthy smokers (n=8) (A) and patients with COPD (n=13) (B), were pre-treated with FF or FP (at the concentrations indicated) for 30 min then stimulated with LPS (100 ng/mL) for 24 h. Cell supernatants were harvested and TNFα levels quantified by ELISA. Suppression of TNFα by FF or FP is presented as a percentage of LPS induced release. Bars represent the mean ± SEM. * P < 0.05 (Mann-Whitney U-test); compared with FP. LPS = lipopolysaccharide; FF = Fluticasone Furoate; FP = Fluticasone Propionate.
6.3.1.9 Comparison between healthy smokers and patients with COPD, of the effect of FF or FP mediated suppression of LPS-induced CXCL8 or TNFα release

In order to attempt to demonstrate CS-insensitivity in patients with COPD compared with healthy smokers, the ability of FF to suppress LPS-induced CXCL8 (Figure 6.7A) or TNFα (Figure 6.7B) release and that of FP to suppress induced CXCL8 (Figure 6.7C) of TNFα (Figure 6.7D) release, were determined. The IC\textsubscript{50} of FF inhibition of CXCL8 was greater in patients with COPD compared with healthy smokers (IC\textsubscript{50}: 1.7 nM vs 0.12 nM; Table 6.4). Furthermore, FF at $10^{-7}$ M inhibited CXCL8 significantly less in patients with COPD compared with healthy smokers (68 ± 5% vs. 52 ± 3%; $P < 0.05$; Figure 6.7A). FP inhibition of CXCL8 in patients with COPD was also less potent compared with smokers (IC\textsubscript{50}: 7.3 nM vs 1.8 nM; Table 6.4) with significantly less suppression observed with FP at $10^{-8}$ M (65 ± 5% vs. 51 ± 4%; $P < 0.05$; Figure 6.7C). FF or FP inhibition of TNFα did not differ significantly between patients with COPD and healthy smokers, particularly with FP.
Figure 6.7: The effect of FF or FP mediated suppression of LPS-induced CXCL8 or TNFα release in healthy smokers and patients with COPD. PBMC isolated from the whole blood of healthy smokers (n=8) and patients with COPD (n=13), were pre-treated with FF (A/B) or FP (C/D) (at the concentrations indicated) for 30 min then stimulated with LPS (100 ng/mL) for 24 h. Cell supernatants were harvested and CXCL8 (A/C) or TNFα (B/D) levels quantified by ELISA. Suppression of LPS-induced CXCL8 or TNFα by FF or FP is presented as a percentage of LPS induction. Bars represent the mean ± SEM. * P < 0.05 (Mann-Whitney U-test); compared with healthy smokers. LPS = lipopolysaccharide; FF = Fluticasone Furoate; FP = Fluticasone Propionate.
6.3.1.10 Comparison of the suppression of LPS-induced TNFα release by Vilanterol in combination with FF or FP in PBMC from patients with COPD

To examine whether CS action, in terms of suppression of induced TNFα release, could be improved in patients with COPD, the levels of suppression by the combination of FF+vilanterol or FP+vilanterol were determined in PBMC. Vilanterol alone suppressed LPS-induced TNFα release in patients with COPD in a concentration-dependent manner and maximal suppression was 37 ± 3% (Figure 6.8 A - D). Combining either FF or FP with vilanterol increased the inhibition of induced TNFα release. Compared with vilanterol alone, there were no differences in inhibition of induced TNFα with most concentrations of the FF+vilanterol or FP+vilanterol combinations. The exceptions were the combinations of FF (10⁻⁹ M)+vilanterol (10⁻¹¹ M) and FF (10⁻¹⁰ M)+vilanterol (10⁻¹¹ M) where there was a significant increase in suppression of TNF compared with the LABA alone (75 ± 4% vs. 19 ± 2%; \( P < 0.01 \); Figure 6.8A and 62 ± 5% vs. 19 ± 2%; \( P < 0.001 \); Figure 6.8B, respectively).
Figure 6.8: Comparison of the effect of vilanterol on FF or FP mediated suppression of LPS-induced TNFα release compared with vilanterol alone. PBMC isolated from the whole blood of patients with COPD (n ≤ 10) were pre-treated for 30 min, with FF or FP alone (single coloured circles) or vilanterol alone (black curve) or with FF+vilanterol or FP+vilanterol combined (coloured curves) at the concentrations indicated, (FF/FP at 10^{-9} M (A); FF/FP at 10^{-10} M (B); FF/FP at 10^{-11} M (C); FF/FP at 10^{-12} M (D)). After stimulation with LPS (100 ng/mL) for 24 h, cell supernatants were harvested and suppression of TNFα levels were quantified by ELISA. Suppression of TNFα by vilanterol and/or FF/FP is represented as a percentage of LPS induced release. Bars represent the mean ± SEM. * P < 0.05, ** P < 0.01 (ANOVA) compared with vilanterol alone. # P < 0.05 (ANOVA) compared with FF alone. LPS = lipopolysaccharide; FF = Fluticasone Furoate; FP = Fluticasone Propionate; 444 = vilanterol trifenatate.
6.3.2 Results Part II: Comparison of the effect of FF and FP on induction of MKP-1 mRNA in non-severe and severe asthmatics

CS can induce anti-inflammatory genes such as MKP-1. To investigate this in PBMC, cells from non-severe and severe asthmatics were treated with LPS and/or FF or FP and the resulting induced MKP-1 mRNA levels, as a measure of CS anti-inflammatory action, were measured at 4 h and 24 h. Induced MKP-1 mRNA levels by either CS, in the presence of LPS, were minimal at 4 hours with less than 15-fold induction compared with unstimulated cells. Additionally, there were no differences between FF and FP in inducing MKP-1 mRNA at 4 hours in both severe and non-severe asthmatics. At 24 hours, there was a trend to suggest that LPS+FF induced MKP-1 mRNA levels were greater than those of LPS+FP. Although not significant, this trend was observed in both non-severe (Figure 6.9A) and severe asthmatics (Figure 6.9B).
Figure 6.9: Comparison of the induction of MKP-1 mRNA by FF or FP and/or LPS in non-severe and severe asthmatics. PBMC were isolated from the whole blood of non-severe asthmatics (n=3) (A) and severe asthmatics (n=5) (B). Cells were treated with FF or FP at $10^{-7}$ M and / or then stimulated with LPS (100 ng/ml) for 4h or 24 h. RNA extracts were prepared from which cDNA was generated by RT-PCR. MKP-1 mRNA expression levels were examined by real-time PCR with oligonucleotides specific for MKP-1 gene and 18S rRNA as the housekeeping gene. The ratio of MKP-1 gene expression to 18S expression was plotted as a fold change over that of unstimulated cells. Bars represent the mean ± SEM value. * $P < 0.05$ (paired t-test) compared with unstimulated cells; # $P < 0.05$ (paired t-test) compared with FP alone at 24 h. LPS = lipopolysaccharide; FF = Fluticasone Furoate; FP = Fluticasone Propionate.
6.4 Discussion

6.4.1 Summary of findings

In this chapter, the effect of the novel corticosteroid FF, on an anti-inflammatory response and its potency, was compared to FP in PBMC from asthmatics and patients with COPD. FF was shown to have a significantly greater ability than FP, to suppress LPS-induced CXCL8 in PBMC from severe and non-severe asthmatics, healthy smokers and patients with COPD. Additionally, FF suppression of LPS-induced TNFα release was significantly greater than with FP, in healthy smokers, but not patients with COPD. In an attempt to present CS-insensitivity in PBMC, between non-severe and severe asthmatics, and between healthy smokers and patients with COPD, the effects of FF and FP were investigated separately on LPS-induced CXCL8 or TNFα release. There were no significant differences in the suppression of LPS-induced CXCL8 or TNFα between non-severe and severe asthmatics when treated with either FF or FP. However, CS-insensitivity was demonstrated in PBMC from patients with COPD as FF and FP suppression of LPS-induced CXCL8 release was significantly less in these cells than in those from healthy smokers. Additionally in this chapter, the effect of the novel ultra-LABA, vilanterol trifenatate, on FF or FP mediated suppression of an induced release of inflammatory markers in PBMC from severe asthmatics and from patients with COPD, was also examined. The moderate effect of vilanterol alone on suppression of LPS-induced TNFα release was significantly increased in the presence of FF, but not FP, in severe asthmatics and patients with COPD. Finally, the potency of FF and FP was examined in terms of LPS-induction of MKP-1 mRNA and was quantified in PBMC from severe and non-severe asthmatics. Although not significant, at 24 h, FF in the presence of LPS, induced MKP-1 mRNA expression was greater than compared with FP suggesting a higher potency of the novel CS.
6.4.2 Current treatments for severe asthma or COPD

CS show anti-inflammatory action in mild to moderate asthmatics by reversing specific chronic airway inflammation, reducing airway hyperresponsiveness and targeting mainly the eosinophilic characteristic of the disease (Chung, 2009). The use of β2-agonists as a monotherapy in asthmatic patients has been a source of controversy as it has been associated with increased asthma deaths and worsening asthma control (Chung, 1993). However, the use of inhaled CS and LABA, in combination, in asthmatics, has proven to be beneficial in reducing exacerbations and improving lung function parameters, more so than either component alone (Chung, 2009). In severe asthmatics the beneficial effects of inhaled CS are conversely, not observed, despite the additional administration of LABA. Asthmatic symptoms may persist possibly due to eosinophilic and neutrophilic inflammation (Jatakanon et al., 1999). Moreover, an abnormal cellular response to CS is observed in severe asthmatics and a relative CS-insensitivity, as demonstrated in-vitro in AM and PBMC (Hew et al., 2006; Bhavsar et al., 2008).

In patients with COPD, the beneficial effects of inhaled CS alone on the improvement of symptoms such as airflow obstruction, is minimal (Chung 2009). In this thesis it has been shown that PBMC from patients with COPD are relatively CS-insensitive compared with healthy smokers (Section 4.3.4). Furthermore, reduced HDAC activity in patients with COPD, may cause the low response to CS, as a result of diminished repression of NF-κB mediated gene expression (Ito et al., 2005). The use of β2-agonists in patients with stable COPD has been shown to improve lung function, exercise tolerance and dyspnoea and to reduce exacerbations (Cazzola, 2011). Despite this, no single treatment to date can effectively decrease the rate of decline of lung function in COPD. For more severe cases of the disease, combination therapy between LABA and inhaled CS has been recommended for its treatment (Celli and MacNee, 2004). In patients with COPD, salmeterol and fluticasone, in combination, significantly improved FEV1 and the health status of patients, compared with either drug alone, with no additional adverse effects (Calverley et al., 2003). The TORCH
(Towards a Revolution in COPD Health) trial also negated fears regarding the adverse effects from the use of CS and LABA and showed that the combination of salmeterol and fluticasone did not increase the risk of mortality in patients with COPD, over a three year period (Calverley et al., 2007).

6.4.3  **FF, compared with FP, improves CS action to a greater extent in severe asthmatics and patients with COPD**

With the need still unmet for a better inhaled CS, for treatment of these CS-insensitive conditions, the first part of this chapter focused on the novel enhanced-affinity and long-acting corticosteroid, Fluticasone Furoate (FF), and (compared to Fluticasone Propionate (FP)), in its ability to suppress LPS-induced cytokine release in PBMC of severe asthmatics and patients with COPD. In both, severe asthmatics and non-severe asthmatics (Figure 6.1) and in patients with COPD and healthy smokers (Figure 6.5), FF, compared with FP, suppressed LPS-induced CXCL8 release to a significantly greater extent. There were no differences in FF or FP suppression of TNFα, with the exception of one clinically relevant concentration, $10^{-10}$ M, in healthy smokers whereby the effect of FF was significantly greater than FP at $10^{-10}$ M and the potency of FF was greater (Figure 6.6). The differences in the effects of CS on CXCL8 and TNFα may be due to differential signalling pathways involved when induced by LPS, as the pathways themselves may be sensitive to CS to varying degrees. The results in this chapter provide further evidence that PBMC of patients with COPD are CS-insensitive. The ability of either FF or FP to suppress LPS-induced CXCL8 release was significantly decreased in patients with COPD compared with healthy smokers (Figures 6.7A and 6.7C, respectively), with IC$_{50}$ values agreeing accordingly (Table 6.4). Although it did not reach significance, FF suppression of LPS-induced TNFα release was also less in patients with COPD (Figure 6.7B) however FF displayed the same level of potency for both COPD and smokers (Table 6.4). The results from this thesis, now shows that three different CS (FF, FP and dexamethasone), are impaired in their ability to suppress induced cytokine release in cells from patients with COPD compared with
control cells, further strengthening the argument that CS alone are an ineffective treatment for this disease.

The result of improved CS action by FF compared with FP is in accordance with many of the published findings about this novel CS when examined against currently used CS. Valotis and Högger compared the binding affinity to the human lung GR, GR binding kinetics and human lung tissue retention of FF against currently used corticosteroids FP, mometasone furoate (MF), ciclesonide and budesonide. They found FF compared to other CS: a) showed the highest ever described relative receptor affinity for GR; b) showed very rapid association and slow dissociation from GR and c) displayed pronounced retention to human lung tissue in-vivo (Valotis and Högger, 2007). Additionally, Salter and colleagues further characterised FF’s superiority over other clinically used corticosteroids, namely FP, MF and budesonide, and in comparison found that FF had: a) the highest potency to inhibit NF-κB mediated gene transcription and LPS-induced TNFα production in human lung epithelial cell lines; b) a very potent ability to activate GR-GRE binding and thus activate anti-inflammatory mediators; c) a highly effective cellular protection and a potent epithelial permeability reduction against elastase-induced and mechanically-induced cell damage; d) the highest selectivity of affinity for GR; e) a high degree of binding to human lung epithelial cells; f) the longest association time and slowest rate of flux out of human lung epithelial cells therefore greater cellular retention and finally g) at a low dose almost completely inhibited lung eosinophilia in rat model of ovalbumin-induced respiratory allergic eosinophilia (Salter et al, 2007). In terms of studies in primary cells, Rossios and colleagues showed in PBMC of mild asthmatics and from patients with COPD, that FF was more potent than FP at inhibiting TNFα-induced CXCL8 release (Rossios et al., 2011). This observation is in agreement with the results in this chapter: FF inhibits LPS-induced CXCL8 release more potently, in PBMC from non-severe asthmatics, severe asthmatics and patients with COPD. Additionally, it has been shown that FF compared with FP has a longer duration of CS action in BEAS2B and A549 (epithelial cell lines), primary bronchial epithelial cells and U937 (monocytic cell
lines) (Rossios et al., 2011). Furthermore, under conditions of oxidative stress, efficacy of FF was greater than that of FP in primary bronchial epithelial cells (Rossios et al., 2011).

In recent clinical trials, many studies have observed that FF boasts a longer duration of action when compared with other CS such as FP, or with placebo. In asthmatics suffering from adenosine 5′-monophosphate-induced (AMP) airway hyperresponsiveness, FF but not FP, provided up to 26 h of prolonged protection (van den Berge et al., 2010). In patients with moderate persistent asthma, once daily 200-800 µg dose of inhaled FF produced effective asthma control through lung function improvement in patients where previously, medium doses of inhaled CS were ineffective (Busse et al., 2012). Further Phase IIb clinical trials observed that in patients with mild to moderate asthma, FF, administered as a single 400µg daily evening dose, showed similar efficacy to FF when administered at 200 µg twice daily (Woodcock et al., 2011a) and similarly, in patients with moderate asthma, FF, administered at 200 µg once daily in the evening, was not inferior to those administered 100 µg twice daily (Woodcock et al., 2011b). In all studies, FF was well tolerated in patients and there were no serious adverse effects when compared with placebo. Furthermore they provided support for FF as suitable for once-daily dosing (Bateman et al., 2012). The reasons for the enhanced and longer duration of action of FF compared with any other CS can be explained by its structure. As FF is not a ‘pro-drug’ and does not need to undergo any changes to be active (such as with ciclesonide which requires de-esterification before becoming active) the whole of its structure is associated with its activity (Valotis and Hogger, 2007; Salter et al., 2007). Although FF is structurally related to FP – its development was based on modification of the 17α propionate ester of FP – they are distinct drug substances with distinct properties (Biggadike, 2011). X-ray crystallography revealed FF and GR interactions showing the furoate 17α-ester occupying a discrete pocket on the receptor much more completely than the smaller propionate 17α-ester of FP (Biggadike et al., 2008), hence explaining the superior receptor affinity compared with other CS.
6.4.5 The action of vilanterol is better improved when combined with FF compared with FP

The suppressive effect of the novel ultra-LABA vilanterol trifenatate (‘vilanterol’ of ‘GW642444M’) alone on LPS-induced TNFα release was modest in PBMC from severe asthmatics or patients with COPD, with maximal suppression of approximately 37 % for both groups. Combining FF or FP with vilanterol showed that suppression of induced TNFα release was improved compared with vilanterol alone, with both CS at the higher concentrations (10^{-9} M and 10^{-10} M), significantly, the FF+vilanterol combination was better compared to vilanterol alone, in both severe asthmatics (Figure 6.4A and 6.4B) and patients with COPD (Figure 6.8A and 6.8B). Interestingly, it was at the lower concentrations of FF, but not FP, when combined with vilanterol, which showed significant improvement in TNFα suppression, when compared with FF alone, in both severe asthmatics (Figure 6.4C and 6.4D) and patients with COPD (Figure 6.8C) suggesting a potential steroid-sparing effect of vilanterol when combined with FF. The examination of the combination of FF and vilanterol is based on the development of the ultra LABA, to be used in combination with FF as a once-daily treatment for asthma and COPD that is currently undergoing clinical trials (Procopiou et al., 2010; Tashkin and Fabbri, 2011). Vilanterol is an antedrug analogue of salmeterol and in-vitro assays have found vilanterol to have a high selectivity for the β_{2}-adrenergic receptor, with greater intrinsic efficacy than salmeterol in addition to increased potency, compared with salbutamol and indacaterol (the first ultra LABA to be approved for the treatment of COPD in Europe) (Procopiou et al., 2010; Tashkin and Fabbri, 2011).

The effects of the combination of CS and vilanterol in PBMC follow on from several previous studies where ex-vivo macrophage cell models of COPD have been used with preceding generations of LABAs. In PBMC, CS-dependent inhibition of allergen-induced cytokine was enhanced with salmeterol (Oddera et al., 1998). In monocyte-derived macrophages (MDMs) cigarette smoke-induced CXCL8 release was significantly inhibited by salmeterol, the effect of which was potentiated in the presence of FP (Sarir et al., 2007). Salmeterol and formoterol inhibited LPS-induced TNFα and
GM-CSF (but not CXCL8) in MDMs. Formoterol and not salmeterol induced anti-inflammatory cAMP levels and in combination with budesonide, formoterol decreased formoterol-induced cAMP, suggesting different activation mechanisms of β₂-agonists in macrophages (Donnelly et al., 2010). Interestingly, in differentiated THP-1 cells, neither salmeterol nor formoterol had any effect on LPS-induced macrophage-derived chemokine or CXCL10 (both upregulated in allergen-induced inflammation), again suggesting differential activation mechanisms of LABA based on cell type. In sputum macrophages from mild asthmatics, inhaled formoterol combined with low dose budesonide has the same effect on GR activation compared with high dose of inhaled budesonide alone (Essilfie-Quaye et al., 2011). A similar study in sputum macrophages from COPD patients showed FP and salmeterol combination enhanced GR nuclear translocation to levels equivalent to those by a five-fold higher concentration of FP alone (Haque et al., 2013). Although LABA are generally associated with decreased levels of pro-inflammatory mediator, it is of note that they can also increase pro-inflammation. For example, salmeterol induced IL-6 in ASM through the β₂-receptor, the effects of which were supressed by FP (Edwards et al., 2007). The undesirable effects of LABA may therefore be regulated by CS when in combination (Newton and Giembycz, 2016).

The effectiveness of a treatment lies in the patients’ compliance levels as much as it does in the treatment itself. One of the obstacles to successfully managing asthma or COPD is adherence to the use of the various CS and/or LABA or any of the other inhalants administered, especially if treatment procedures are complicated or too frequent (Cazzola, 2011). One way therefore, of overcoming this obstacle, and this is the current goal in asthma and COPD therapy, would be to develop combinatory inhaled treatments that could be used just once daily. The results herein show significant improvement in suppression of LPS-induced TNFα in-vitro, by FF+vilanterol compared with vilanterol alone in both severe asthmatics and in patients with COPD, concurring with recent data from several clinical trials. The first publication to present clinical data on the effects of inhaled FF and vilanterol in patients with COPD, showed that over a 4-week duration, in subjects with moderate-to-severe COPD, the combination when administered once daily, improved lung function.
(FEV\textsubscript{1}) compared with placebo, without any additional adverse effects - as commonly reported with inhaled CS and LABA combination therapy (Lötvall et al., 2011). In a recent phase III trial in patients with COPD, FF in combination with vilanterol, inhaled once daily in the morning for 28 days produced significant improvements in lung function and prolonged (over 24 h) duration of action in the patients (Boscia et al., 2012). Once-daily administered vilanterol alone was shown to statistically, and clinically improve, lung function compared with placebo in patients with moderate to severe COPD without any serious adverse effects (Hanania et al., 2012). In patients with persistent asthma, vilanterol alone was well tolerated and the data suggested that there was no advantage of a twice-daily administration of vilanterol compared with once-daily administration in the improvement of observed lung function (Sterling et al., 2012). In patients with mild asthma, FF administered alone or in combination with vilanterol provided significant protection to the lungs from the early asthmatic response and over a 23 h period (Oliver et al., 2012) and in asthmatics who remained symptomatic despite the use of inhaled CS, once-daily administered vilanterol resulted in prolonged bronchodilation of at least 24 h (Lötvall et al., 2012).

6.4.6 The mechanism of superior CS action of FF over FP may be partially dependent on increased MKP-1 gene expression

MAP kinase phosphatase (MKP)-1 mRNA was induced by LPS and / or FF or FP in PBMC of non-severe and severe asthmatics. There was minimal induction of MKP-1 mRNA at 4 h post stimulation with either FF or FP, however at 24 h, there was a trend to suggest that LPS+FF induced MKP-1 mRNA levels were higher than compared with LPS+FP induced levels in both non-severe and severe asthmatics (Figure 6.9A and 6.9B). MKP-1 is a dual specificity protein phosphatase that possesses anti-inflammatory action by dephosphorylating and therefore deactivating the MAP Kinases, specifically c-JUN and p38 MAPK (Li et al., 2009). Induction of anti-inflammatory mediators by CS can occur when the CS activates GR-GRE binding and subsequent transactivation of CS-responsive genes such as MKP-1. The results suggest that long-acting CS FF, may be able to prolong
its anti-inflammatory actions \textit{in-vitro}, through GR associated activation of MKP-1 gene transcription. These results in PBMC are in agreement with those found in A549 cells, whereby potent GR-GRE binding induced by FF was associated with increased MKP-1 gene induction compared with FP or budesonide. Additionally, MKP-1 induced gene induction by FF was significantly more potent than that by FP and was observed for 16 h while that induced by FP peaked at 4 h, returning to baseline by 16 h, even after a double addition of FP. Moreover, FF treatment in BEAS2B cells activated nuclear translocation of GR which remained in the nuclei after 30 h, at which point in FP treated cells, GR was predominantly cytoplasmic (Rossios \textit{et al.}, 2011).

\textbf{6.4.7 Understanding the mechanisms underlying CS and LABA combination interactions}

The molecular mechanisms involved in the improved suppression of inflammation by the combination of inhaled CS and LABA has not yet been clearly defined. There is a suggestion that inhaled CS can improve the efficacy of the LABA but also that the LABA can enhance the action of the inhaled CS. The presence of several GRE sequences in the promoter region of the human $\beta_2$-receptor gene may be transcriptionally more active when CS are present as CS would up-regulate $\beta_2$-receptor expression through GRE. Indeed in patients treated intra-nasally with CS, the density of $\beta_2$-adrenoreceptor in the nasal mucosa was significantly increased. Additionally, the down regulation of $\beta_2$-receptor density by regular LABA use may be reversed in the presence of CS, which restores responsiveness to bronchodilators (Sin and Man, 2006). There is evidence to suggest that LABA alone can promote GR nuclear translocation. In sputum epithelial and macrophage cells of healthy controls and mild asthmatics and in U-937 and BEAS-2B cell lines, the rate of GR nuclear translocation by salmeterol combined with FP, was significantly higher than for either drug alone. Furthermore the FP induction of CS-inducible genes, MKP-1 and secretory leuko-proteinase inhibitor (SLPI) in U-937 and BEAS-2B cells was significantly enhanced in the presence of salmeterol, when compared with FP induction alone (Usmani \textit{et al.}, 2005). In human bronchial smooth muscle cells, the rate of GR nuclear translocation and GR-GRE binding was much greater with a formoterol and
budesonide combination than with either drug alone. Additionally, synergy was observed between formoterol and budesonide in inhibiting proliferation of bronchial smooth muscle cell and this was reasoned to occur through the p21, cyclin dependent kinase inhibitor, pathway (Roth et al., 2002). Moreover, asthmatics were observed to be deficient in the CCAAT/enhancer binding protein alpha (C/EBPalpha), which is active through the p21 pathway, may be CS-insensitive (Roth et al., 2004). Furthermore, p21 expression was abnormally high in severe asthmatic bronchial epithelium (Puddicombe et al., 2003). Therefore in the case of severe asthmatics, LABA may enhance the effect of CS to inhibit smooth muscle proliferation, by overcoming C/EBPalpha deficiency through modulation of p21 activation. It would be interesting to examine CS-insensitivity in severe asthmatics through this angle, using the newer generations of CS and LABA. Furthermore, in bronchial epithelial cells, TNFα induced GM-CSF inhibition by formoterol and salmeterol was demonstrated in cells with siRNA-mediated GR knockdown, inferring that there may be an alternative method by which LABA action can occur, which is independent of GR (Loven et al., 2007).

Better understanding of the molecular mechanisms underlying CS-insensitivity as well as the enhanced, and sometimes synergistic, improvement of action when CS and LABA are in combination would enable more effective drug development and therefore better treatment for the patients who suffer with the symptoms of diseases such as severe asthma or COPD.
Chapter 7

General Discussion
Chapter 7 – General Discussion

7.1 Summary of findings

Severe asthma and COPD remain serious global chronic inflammatory health conditions. The impact of these diseases on the lives of the patients and their families is immense, as are the huge socio-economic implications and financial burdens on healthcare resources. The relative CS-insensitivity observed in these patients presents a major challenge and is part of the reason for these difficulties, as there is an unmet need for effective treatment of these diseases.

In this thesis, I hypothesised that the heightened activity of p38 MAPK influenced CS sensitivity in patients with severe asthma and in those with COPD. p38 MAPK activity was indeed increased in PBMC from patients with severe asthma compared with those with non-severe asthma (3.3.1.2) Heightened p38 MAPK activity was also confirmed in patients with COPD compared with healthy smokers (4.3.2) at baseline and when induced suggesting an inherent pro-inflammatory state in these patients. Baseline and induced levels of CXCL8 release were increased in patients with COPD compared with controls and this directly correlated with a decrease in lung function in these patients, as measured by reduced % predicted FEV1 (4.3.3), signifying a role for CXCL8 in the pathogenesis of this disease.

As the presence of CS-insensitivity in severe asthmatics had already been demonstrated in PBMC, (Hew et al., 2006), it followed that CS-insensitivity should also be demonstrated in the same cell model from patients with COPD. CS-insensitivity was therefore represented by impaired dexamethasone suppression of LPS induced CXCL8 release in COPD compared with healthy smokers (4.3.4). This observation of CS-insensitivity was reproduced using two additional corticosteroids, FP and FF, in PBMC from patients with COPD (6.3.1.9). COPD CS-insensitivity has now been demonstrated several times in-vitro, in PBMC (Kobayashi et al., 2013; Rossios et al., 2012), airway
lymphocytes (Kaur et al., 2012) and alveolar macrophages (Culpitt et al., 2003; Cosio et al., 2004), further strengthening the argument that CS alone are an ineffective treatment for this disease.

As heightened p38 MAPK activity and impaired CS action was now confirmed in these cell models, it suggested that there was a link between the two, therefore the next question asked, was what impact p38 MAPK inhibition would have on CS function. The p38α MAPK selective inhibitor, GW856553 (GW-A or Losmapimod) significantly improved dexamethasone suppression of LPS-induced CXCL8 and IL-6 release in PBMC from severe asthmatics (3.3.2.2 - 3.3.2.3) and patients with COPD (4.3.5 – 4.3.6), in a synergistic manner, compared with the effect of the inhibitor alone. This observation confirmed my hypothesis that heightened p38 MAPK activity did indeed influence CS sensitivity in severe asthma and COPD, as p38 MAPK inhibition appeared to restore the anti-inflammatory effect of dexamethasone in these cells. Similar observations using other p38α/β selective MAPK inhibitors have been reported in monocyte-derived macrophages and AM from patients with COPD (Kent et al., 2009, Armstrong et al., 2011) further proving a role for p38 MAPK in CS-insensitivity.

There was an obligation to further confirm the effect of p38 MAPK inhibition in the appeared reversal of CS-insensitivity in this cell model, at a molecular level. RNAi technology and siRNA was utilised to knockdown p38α MAPK in monocytes from healthy volunteers to determine if, in CS-sensitive cells, the absence of p38 MAPK would elicit a pro-inflammatory response (3.3.3). Although total p38 MAPK was successfully knocked down in monocytes compared with the control siRNA, as determined by protein measurement (3.3.3.1) p38 MAPK was, surprisingly, still functional in these knockdown cells, in its response to LPS and dexamethasone (3.3.3.2). Furthermore the activity of p38 MAPK had not been completely silenced (3.3.3.3) and this was additionally confirmed by the presence of active MSK-1 (a downstream target of p38 MAPK) in these cells at the same levels as those from control-transfected cells (3.3.3.4). This suggests that this method of transfection did not completely silence p38α MAPK pathway in these cells and alternative methods would need to be
employed to suppress its activity (3.4.5). Moreover the results suggest that there may be other pathways acting in compensation for p38 MAPK, such as ERK or JNK, and this would require further investigation.

The next parts of my thesis were dedicated to investigating the molecular mechanisms through which p38 MAPK may be partaking, to have such sway on CS sensitivity. It has been reported that post-translational modification of GR may impact on its ability to function effectively (Liberman et al., 2007). In particular, phosphorylation of GR on serine 211 has been linked with nuclear translocation and transcriptional activation of GR after hormone treatment (Wang et al., 2002). Additionally GR ser211 was found to be a substrate target for p38 MAPK in a human lymphoid cell line (Miller et al., 2005). This suggests that increased p38 MAPK activity may influence GR and even reduce GR activity. On this basis, the phosphorylation status of GR was measured in PBMC protein extracts from patients with severe asthma or COPD (4.3.8). Induced phosphorylation of GR ser211 was significantly reduced in the presence of GW-A in both severe asthma and COPD samples. This suggests that phosphorylation of GR is a p38 MAPK-dependent process and that there may be a degree of hyper-phosphorylation of GR by p38 MAPK, in CS-insensitive settings, which impairs GR function and leads toward a pro-inflammatory state. Whether the effect of p38 MAPK on GR ser211 is direct or indirect, remains to be determined.

To extend the investigations into the molecular mechanisms of CS-insensitivity, the possible role of MAPK phosphatase (MKP)-1 was examined. Corticosteroids can induce the transcriptional activation of protein kinase phosphatases such as MKP-1 to serve as negative regulators of the innate immune system through dephosphorylation and inactivation of MAP kinases. MKP-1 has been shown to preferentially deactivate JNK and p38 MAPK (Li et al., 2009). Direct association of p38 MAPK to MKP-1 has been shown to enhance the catalytic activity of the phosphatase thus forming a feedback control loop (Wancket et al., 2012). It has been previously described that increased p38 MAPK activity correlated reduced MKP-1 mRNA expression in AM from severe asthmatics compared
with non-severe asthmatics (Bhavsar et al., 2008) This observation warranted further investigation in my cell model. Induced expression of MKP-1 was reduced in PBMC from severe asthmatics compared with non-severe asthmatics (5.3.5 - 5.3.6). Induced and baseline MKP-1 mRNA expression levels were reduced in monocytes from severe asthmatics (5.3.7 – 5.3.8) suggesting a systemic deficiency of MKP-1 in these patients. siRNA transfection was used to knockdown MKP-1 activity in monocytes from normal volunteers (5.3.9 – 5.3.11). Although induced levels of p38 MAPK activity and cytokine mRNA expression and release, were slightly increased in the knockdown cells, this was not statistically significant. It did however suggest that a lack of MKP-1 may lead to, and perpetuate, a pro-inflammatory state, such as increased activation of p38 MAPK, and that this may overwhelm the immune response, despite the presence of CS.

The final chapter of this thesis focused on alternative CS combinatory treatments (alternative to p38 MAPK inhibition) that could potentially be used inflammatory airway disease. Since the discovery that the combination of inhaled CS with LABA improves the action of the CS, and therefore the effectiveness of the treatment in asthma and COPD, this has now become the mainstay therapy for these diseases (Chung et al., 2009). However, even with the use of a LABA, high dose inhaled CS are still not wholly effective (Sterling and Chung, 2001). The effect of FP or the novel long-acting CS, FF, alone or in combination, with a novel ‘ultra-LABA’ vilanterol trifenatate, on suppression of induced cytokine release was examined and compared between severe asthmatics and non-severe asthmatics, and between patients with COPD and healthy smokers. FF, compared with FP, was significantly superior in its ability to suppress induced CXCL8 release in PBMC from all groups (6.3.1.3 and 6.3.1.7). Moreover, FF when in combination with vilanterol, significantly enhanced the suppression of induced cytokine release, compared with FP in combination with vilanterol, or vilanterol alone, in PBMC from severe asthmatics or COPD (6.3.1.6 and 6.3.1.10). Curiously, there were no differences in suppressive abilities between FF and FP on induced TNFα release in any of the groups apart from healthy smokers (6.3.1.8) suggesting possible selective targeting of pro-inflammatory pathways of these CS or highlighting differential LPS-induced
pathways of CXCL8 and TNFα. Induction of MKP-1 mRNA expression by LPS and FF was greater than that of LPS and FP, at 24hrs, in PBMC from both severe and non-severe asthmatics, although not significantly so (6.3.1.11). This result suggests that FF may be exerting its enhanced actions through prolonging GR-mediated MKP-1 activity.

In conclusion, my hypothesis was proven as heightened p38 MAPK activity was observed in PBMC from patients with severe asthma and those with COPD. Augmented p38 MAPK activity was associated with CS-insensitivity and reduced MKP-1 expression. Inhibition of p38 MAPK restored CS-function in PBMC from patients with severe asthma or COPD. The p38 MAPK pathway may therefore be a potential drug target for the reversal of CS-insensitivity in patients with chronic inflammatory lung diseases.

7.2 Limitations of the thesis

There are some limitations throughout this thesis that warrant explanation. The first is regarding the chosen cell model for the majority of experiments performed in this thesis. PBMC are a plentiful cell population and their extraction from whole blood is a relatively simple and non-invasive method. As PBMC include monocytes (macrophage precursor) amongst their cell population they can be used as a disease-relevant ex-vivo cell model for asthma or COPD. However, PBMC are a heterogeneous population of cells, and as well as monocytes (5%), include B cells (15%), T cells (70%) and NK cells (10%) (Corkum et al., 2015). It is therefore obvious that a major limitation is the high proportion of other cell types present in a given sample which will be releasing their own mediators alongside the target monocytes. Although it is assumed that by targeting the monocytes in the PBMC population, by stimulating with LPS, that the monocytes (possessing LPS receptors) will produce the dominant result, it cannot be ruled out that the final observations of an experiment may be influenced by the presence of the other cell types. Additionally, the proportions of cell populations differ between individuals, so two samples of the same number of PBMC may have differing numbers of monocytes and this is another limitation.
Another limitation of this study are the experiments where monocytes were utilised for siRNA transfection (Chapter 3 and Chapter 4). When investigating the role of p38α MAPK inhibition on CS-function, RNAi technology was used to knockdown the p38α gene. As PBMC were the cell model used to confirm differences in p38 activity between severe and non-severe asthmatics, they were the first choice of cell to transfect. However after several attempts with no success, monocytes were isolated from the PBMC population and used for transfection instead. This resulted in improved levels of efficacy of transfection and although confirmation of knockdown of p38α in monocytes was achieved (reduced protein expression), subsequent functional assays measuring showed no differences between p38α transfected monocytes and controls. A similar scenario occurred when determining the function of MKP-1 in dexamethasone treated monocytes. Although knockdown of MKP-1 had been confirmed in transfected monocytes compared with control (as determined by MKP-1 mRNA), subsequent functional assays did not show any difference. Limitations in these cases include (specifically for p38 transfection): only p38α was targeted for knockdown and therefore all isoforms of the p38 MAPK pathway may need to be targeted to ensure the gene is fully silenced as the other pathways may be compensating and rendering the pathway still ‘switched on’. In the case of MKP-1 transfection a specific limitation was the lack of an appropriate total MKP-1 antibody, at the time, to determine knockdown in monocytes at an MKP-1 protein expression level. The general limitations may the choice of cell model used for transfection – the efficiency of the transfection of either p38α or MKP-1 in monocytes may not have been great enough to have an impact on the functionality of the cell. Without these confirmations it is therefore difficult to draw strong conclusions on molecular functions of either pathway in CS-insensitivity.

The phosphorylation status of GR at Ser211 was investigated (Chapter 4) in patients with COPD and severe asthmatics and the results suggested that LPS+Dex induced phosphorylation of GR ser211 may be p38 MAPK-dependent in these patients. However the limitations in this regard are that no functional experiments were performed to confirm the activity of GR. Although GR phosphorylation is enhanced by ligand binding with CS and in this case p38, its transcriptional
activity would still need to be determined. Therefore it would have been important to measure in these cells, GR dependent gene expression of glucocorticoid-induced leucine zipper protein (GILZ) for example to determine GR functional activity.

7.3 Directions for future studies

There are obvious advantages to using primary cell models over cell line models, such as a closer representation of the \textit{in-vivo} state and heterogeneous nature of the human population. However, major limitations include the number of cells one can gain from a given blood sample, for example, and the low $n$-numbers. It would be useful therefore to increase the $n$-numbers for the studies performed in this thesis, particularly the examination of phosphorylation status of GR in PBMC from patients groups, and the siRNA knockdown of MKP-1 or p38 MAPK in normal subjects. Other areas of this work that may warrant further investigation are listed below.

1. **The phosphorylation of GR in CS-insensitive patients with severe asthma or COPD.** The phosphorylation status of other residues of GR, which may potentially be affected in a CS-insensitive setting, should be investigated in patients compared with controls. Namely, serine 203 and serine 226 and serine 404 should be examined as they have been implicated in the transcriptional activity of GR; also, what the effect of p38 MAPK inhibition is on their activities.

2. **The nuclear retention of p65 (NF-κB) in CS-insensitive patients with severe asthma or COPD.** Investigate the differences, between patients and control groups in terms of the activity of cytosolic and nuclear p65. Lengthy nuclear retention of p65 or increased shuttling of p65 to and from the nucleus (leading to over–activity of NF-κB-dependent gene transcription) has been suggested to be involved in CS-insensitivity. Also, investigate what impact inhibition of p38 MAPK may have on recruitment of p65 to pro-inflammatory gene promoters, in presence of CS, in disease groups compared with controls.
3. **Mechanisms through which augmented p38 activity perpetuates CS-insensitivity in severe asthma and COPD.** Examine the effect of CS, in the presence or absence of p38 MAPK inhibition, on the mRNA half-lives of induced CXCL8, TNFα, and IL-6, in addition to MKP-1 mRNA.

4. **The role of MKP-1 in CS-insensitivity in COPD.** As the mRNA expression of MKP-1 has now been shown to be reduced in lung and blood cells from severe asthmatics compared with controls, it follows that this should also be investigated in patients with COPD to determine a role for MKP-1 in the manifestation of CS-insensitivity.
Publications


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