Glucocorticoid Resistance in COPD: The Role of p38 MAPK

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List of Abbreviations

ABC Avidin Biotin Complex

AF-1-2 Activation Functions 1-2

ANOVA Analysis of Variance

BAL Bronchoalveolar Lavage

BCL2 B cell Lymphoma 2

BSA Bovine Serum Albumin

cAMP Cyclic Adenosine Monophosphate

CBP CREB Binding Protein

CDNA Complementary DNA

COPD Chronic Obstructive Pulmonary Disease

COX-2 Cyclooxygenase 2

CREB CAMP-Response Element-Binding Protein

CRP C Reactive Protein

DAB 3,3 Diaminobenzidine

DAPI 4',6 Diamidino-2-phenylindole

DBD DNA Binging Domain

DMSO Dimethyl Sulfoxide

DNA Deoxyribonucleicacid

DTT Dithiothreitol

EDTA Ethylenediaminetetraacetic Acid

EGR-1 Epidermal Growth Factor-1

ELISA Enzyme Linked Immunosorbent Assay

ERK Extracellular regulated signal kinase

FEV1 Forced expiratory Volume in 1 second

FFPE Formalin Fixed Paraffin Embedded

fMLP Formyl-Methionyl-Leucyl-Phenylalanine

FOXP3 Forkhead Box p3

FVC Forced Vital Capacity

GAPDH Glyceraldehyde 3-Phosphate Dehydrogenase

GC Glucocorticoid

GILZ Glucocorticoid Induced Leucine Zipper

GM-CSF Granulocyte Macrophage Colony Stimulating Factor

GOLD Global Initiative for Chronic Obstructive Pulmonary Disease

GR Glucocorticoid Receptor

GRE Glucocorticoid Response Element

GRO-α Growth Regulated Oncogene Alpha

GSK3 Glycogen Synthase Kinase 3

HAT Histone Acetyl Transferase

HBEs Human Bronchial Epithelial Cells

HDAC Histone Deacetylases

HIER Heat-Induced Epitope Retrieval

HRV Human Rhinovirus

HSP Heat Shock Protein

ICAM-1 Intercellular Adhesion Molecule 1

ICS Inhaled Corticosteroid (Glucocorticoid)

IFNγ Interferon gamma

IKK IκB Kinase

INOS Inducible Nitric Oxide Synthase

IRAK Interleukin-1 Receptor-Associated Kinase

JNK c-Jun-N-terminal kinases

LAAC Long Acting Anticholinergic

LABA Long Acting Beta Agonist

LBD Ligand Binding Domain

LPS Lipopolysaccharide

LSP1 Lymphocyte Specific Protein 1

LTB4 Leukotriene B4

MAC-1 Macrophage 1 Antigen

MAPK Mitogen Activated Protein Kinase

MCP-1 Monocyte chemotactic protein

MEK MAP kinase kinase

MEM Minimum Essential Medium

MIP Macrophage Inhibitory Protein

MMPs Matrix Metalloproteinases

MPK-1-9 MAPK Phosphatase 1-9

mRNA Messenger Ribonucleic Acid

MyD88 Myeloid Differentiation Primary Response Gene (88)

NADPH Nicotinamide Adenine Dinucleotide Phosphate

NE Neutrophil Elastase

NEMO NFκB Essential Modulator

NFκB Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells

NS Non-smoker

PBMCs Peripheral Blood Mononuclear Cells

PBS Phosphate Buffered Saline

PCAF P300/CBP-Associated Factor

PDE4 Phosphodiesterase 4

PHA Phytohaemagglutinin

PI3K Phosphoinositide 3-kinase

PMA Phorbol 12-Myristate 13-Acetate

PMN Polymorphonucleocyte

PP5 Protein Phosphatase 5

QPCR Quantitative Polymerase Chain Reaction

RANTES Regulated Upon Activation, Normal T cell Expressed and Secreted

RIPA Radioimmunoprecipitation Assay

RNA Ribonucleic Acid

S Smoker

SARM Selected Androgen Receptor Modulators

SDS Sodium Dodecyl Sulfate

SLP Synaptotagmin-Like Protein

SRC Proto-OncogeneTyrosine-Protein Kinase

TAB 1-3 TAK1 Binding Protein 1-3

TANK TRAF-Family Member Associated NFκB Activator

TAK1 TGF beta Activated Kinase 1

TBS Tris-Buffered Saline

TBK TANK Binding Kinase

TEMED Tetramethylethylenediamine

TGFβ Transforming Growth Factor Beta

TICAM-1 TIR Domain-Containing Adaptor Molecule 2

TIR Toll/Interleukin-1 Receptor

TLR Toll-Like Receptor

TNFα Tumour Necrosis Factor Alpha

TNFR TNF Receptor

Th1/Th2 T Helper subsets

TRADD TNF Receptor Type 1-Associated Death Domain Protein

TRAF TNF Receptor Associated Factors

TRAM TRIF-Related Adaptor Molecule

TRIF TIR-Domain–Containing Adaptor Inducing Interferon-β

TTP Tristetrapolin

UTR Untranslated Region

VEGF Vascular Endothelial Growth Factor

WHO World Health Organisation

Abstract

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Chronic Obstructive Pulmonary Disease (COPD) is a chronic, inflammatory condition, characterised by airflow limitation. The use of glucocorticoids (GC) as an anti-inflammatory treatment in COPD has limited clinical benefits, and as such, new treatments are needed. Identifying key pathways involved in the inflammatory response in COPD may enable the development of novel treatments. The aims of this thesis were to examine the steroid sensitivity of an *in vitro* mixed sputum culture cell model, comparing COPD cells to smoking and non-smoking controls, examine expression of the intracellular signalling molecule p38 Mitogen Activated Protein Kinase (MAPK) in COPD lungs compared with controls, examine the GC and p38 MAPK inhibitor and dual therapy sensitivity of a bronchial epithelial cell line and finally, to understand the mechanisms by which a p38 MAPK inhibitor in combination with a GC synergistically inhibit pro-inflammatory mediator production in a bronchial epithelial cell line. Dexamethasone inhibits mixed sputum cell pro-inflammatory mediator release, with no differences in sensitivity observed between COPD and control cells. Isolated sputum neutrophils demonstrate modest sensitivity to dexamethasone, which is in contrast to blood neutrophils. There are increased numbers of cells positive for activated p38 MAPK in COPD lungs compared with controls, specifically localised to follicular B and CD8+ T cells, bronchial epithelial cells and alveolar and sputum macrophages. Lung and sputum neutrophils are devoid of activated p38 MAPK, and a pharmacological p38 MAPK inhibitor has no effect on pro-inflammatory mediator production from these cells. This is in contrast to blood neutrophils, whereby p38 MAPK activation can be induced following LPS stimulation and *in vitro* cell culture, and pro-inflammatory mediator release is inhibited by a p38 MAPK inhibitor. Dexamethasone and birb 796 inhibit stimulated pro-inflammatory mediator release from a bronchial epithelial cell line in a dose-dependent manner. Sensitivity to either drug is dependent on stimuli and the pro-inflammatory mediator analysed. There is additive and synergistic inhibition of pro-inflammatory mediator production when combination therapy comprising dexamethasone and birb 796 is used compared with either drug alone. This may be due to Birb 796 enhancing dexamethasone-mediated nuclear translocation of the glucocorticoid receptor, which may enhance the GC-mediated anti-inflammatory effects. Combination therapy may therefore be a useful therapeutic in the treatment of COPD.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

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Chapter 1

Introduction

1.1 COPD

Chronic obstructive pulmonary disease (COPD) is an inflammatory condition of the lungs, characterised by progressive airflow limitation that is not fully reversible. COPD encompasses a number of inflammatory conditions including chronic bronchitis, bronchiolitis and emphysema. Chronic bronchitis is a common condition affecting the upper respiratory tract. Clinically, chronic bronchitis is diagnosed as the presence of a productive cough for more than three months over a two-year period and is often linked to cigarette smoking. Bronchiolitis affects small airways and is progressive and non-reversible. Emphysema is a permanent, abnormal enlargement of the lung-gas-exchanging structures, and results in the destruction of the respiratory alveolar tissue. COPD causes considerable morbidity and mortality, with increasing mortality rates observed in recent years despite the advancements made in disease management over the last 30 years. This is in contrast to other diseases, including heart disease and cancer, where mortality has fallen in the last few decades (Halpin and Miravitlles, 2006).

COPD is diagnosed using spirometry, with patient histories and physical examinations also forming part of the diagnostic process. Spirometry is also used for monitoring disease progression.

Disease severity is classified by the Global Strategy for the Diagnosis, Management and Prevention of COPD (GOLD) guidelines.

In very early stages of the disease often there are no characteristic symptoms of the disease, and in later stages, the symptoms are partially, but not fully reversible.

1.1.1 Epidemiology

Predictions suggest that COPD will be the third leading cause of death worldwide by 2020 (Chapman et al, 2006). It is estimated that around 210 million people worldwide are currently affected by the disease (Chapman et al, 2006). In addition, chronic lower respiratory diseases, which are primarily thought to be COPD, are already the third leading cause of death in the United States, with around 5% of adults reporting a diagnosis of emphysema or chronic bronchitis (Akinbami and Lui, 2011). In the United Kingdom, COPD is currently the fifth leading cause of death, after ischaemic heart disease, stroke, lung cancer and pneumonia (Halpin and Miravitlles, 2006). Interestingly, estimates suggest that between 45–65% of patients with COPD are never formally diagnosed with the disease, due to feelings of breathlessness and limited exercise tolerance seen as part of the usual aging processes and ‘smoker’s cough’ being a normal symptom of smoking (Halpin and Miravitlles, 2006). Smoking is the primary cause of COPD development, and at least 75% of deaths resulting from COPD can be attributed to smoking (Brown, 2011).

The main symptoms of COPD are dyspnoea, chronic cough, sputum production, wheezing and chest tightness. Additional symptoms experienced

by patients with more severe disease include fatigue, weight loss and anorexia.

1.1.2 Burden of COPD

COPD inflicts a high burden upon patients, both in terms of health-related quality of life and overall health status. Poor physical functions and distressing symptoms that require frequent hospitalisations are experienced by patients with COPD on a regular basis. In addition, patients are frequently unable to work, thus becoming socially isolated and depressed in some cases. Around two thirds of patients experience difficulties in performing everyday tasks, including climbing stairs and getting washed and dressed due to breathlessness (Rennard et al, 2002).

1.1.3 Risk factors in COPD development

The risk of development of COPD is related to an interaction between both genetic and environmental exposures.

1.1.3.1 Genetic factors

The most common genetic factor linked to COPD is a deficiency of the serine protease α1 antitrypsin, which occurs in approximately 1–3% of patients with COPD (Stoller and Aboussouan, 2005). This protease inhibitor protects

tissues from neutrophil elastase (NE), and a deficiency results in NE-induced elastin degradation. Having reduced concentrations of this enzyme, especially in combination with exposure to cigarette smoke or other environmental dusts, increases the risk of developing emphysema (Stoller and Aboussouan, 2005). A number of other genes have also been implicated in COPD development including transforming growth factor beta one (TGFβ1) (Celedon et al, 2004), tumour necrosis factor alpha (TNFα) (Keatings et al, 2000), microsomal epoxidehydrolase 1 and glutathione transferase (Cheng et al, 2004). To date there have been a large number of studies investigating genetic predisposition to COPD, although the results are still mostly inconclusive.

1.1.3.2 Cigarette smoke

Cigarette smoke is the most common cause of COPD. In developed countries, estimates suggest that around 73% of COPD mortality is related to smoking, and approximately 40% of COPD-related deaths related to smoking in developing nations (Lopez et al, 2006). There are around 5000 different chemicals present in cigarette smoke, many of which have the potential to damage the airways, including nicotine and acrolein. Chemical interactions between the different chemicals occurring may also contribute to inflammation in the lung through the formation of free radicals and DNA adducts. However, not all smokers develop COPD. Indeed, suggestions that up to half of all smokers develop COPD (Mannino et al, 2006, Cheng et al, 2004, Lunnback et al, 2003) supports the hypothesis that genetic susceptibility is also an important factor in the development of COPD. In addition, research has

shown that smoking during pregnancy can also lead to foetal lung growth abnormalities and the subsequent development of lung disease (Gilliland et al, 2003), indicating that early life exposure to cigarette smoke may potentially contribute to the development of COPD later in life.

1.1.3.3 Occupational dusts

Population-based and occupational cohort studies have demonstrated that occupational exposure to dusts, gas and fumes can cause COPD irrespective of smoking (Hnizdo et al, 2002). Indeed, it is estimated that in around 15% of non-smokers and 31% of smokers, COPD can be attributed to occupational exposures. The influence of this occupational exposure on the clinical and functional characteristics of COPD are not well understood, although exposure is associated with increased respiratory symptoms, including dyspnoea and wheeze, as well as with airflow obstruction (Hnizdo et al, 2002).

1.1.3.4 Indoor air pollutants

Exposure to biomass fuels, including coal, straw, animal dung, crop residues and wood, is also linked to COPD and may in fact be the most important risk factor globally (Salvi and Barnes, 2009). Estimates suggest that in poorer countries, exposure to indoor smoke from biomass fuels was the cause of approximately 35% of people developing COPD (Hnizdo et al, 2002).

1.1.3.5 Outdoor air pollutants

Outdoor air pollutants contribute a much smaller risk to the development of COPD, with around 1–3% of COPD cases attributed (Lopez et al, 2006). Air pollution is linked to lower respiratory infections and thus may contribute to the development of COPD (Manninio and Buist, 2007).

1.1.3.6 Aging

The prevalence of COPD morbidity and mortality increases with age. In addition, lung function declines with age, and studies suggest that elderly people with high levels of lung function are likely to live much longer than those with reduced lung function levels (Mannino and Davis, 2006). The increased prevalence of COPD in recent years is partly due to the changing demographics of the world's population, with people living longer and therefore at greater risk of developing COPD (Jamal et al, 2002).

1.1.3.7 Infections

Infections are believed to play an important role in the development and progression of COPD and are specifically linked to COPD exacerbations (De Serres et al, 2009). The increased prevalence of B cell-containing follicles in COPD lungs (Hogg et al, 2004) also lends support to infections contributing to the development of COPD. Latent adenovirus infections are associated with COPD development (Hogg et al, 2001) and, indeed, in patients with

emphysema, excess inflammation is associated with an increase in alveolar epithelial cell expression of the adenoviral E1A protein compared with control subjects with similar smoking histories (Ratemales et al, 2001). *Chlamydia pneumoniae* is an established cause of acute and chronic upper and lower respiratory tract infections, and *in vivo* and *in vitro* data indicate that infection can lead to the development of both small airways disease and emphysema (Branden et al, 2005). Tuberculosis is also a risk factor for COPD development (Lee et al, 2012). There are also indications that lower respiratory tract infections in children may, in some instances, also predispose to chronic airflow limitation later in life, potentially leading to COPD development (Martinez, 1999).

1.1.3.8 Gender

Historically, COPD was more prevalent in males than females, likely due to differences in exposures to cigarette smoke and occupational dusts and chemicals (Mannino et al, 2002, Silverman et al, 2000). More recently, however, the prevalence of COPD appears to be more equal in men and women, in particular in areas where smoking habits are equal between the sexes. There is evidence to suggest that when exposures are equal, women are actually more susceptible to COPD development (Buist et al, 2007, Watson et al, 2006), although the mechanisms underlying this are not well understood.

1.1.3.9 Socioeconomic factors

The risk of developing COPD appears to be greater in poor populations, and indeed, poor nutrition, over-crowding, exposure to pollutants, high smoking rates, poor access to healthcare and early respiratory tract infections increase the risk of COPD (Anto et al, 2001, Shohaimi et al, 2004, Lawlor et al, 2004).

1.1.4 Pathogenesis

The development of COPD is believed to occur as a result of an abnormal innate and adaptive immune response to inhaled noxious particles, with cigarette smoke and other air pollutants being the main sources of these toxic products (Brusselle et al, 2011). Inhalation of these toxic gases and particles leads to the infiltration of inflammatory cells into the lung, as demonstrated by the increased number of inflammatory cells, including CD8+ T cells (Di Stefano et al, 2001) and macrophages (Saetta et al, 2001) in the lungs of smokers compared with non-smoker patients. When injury occurs and exceeds the ability of lung tissue to repair, an abnormal tissue repair and remodelling process occurs. Alterations in normal tissue structure results in compromised function, which, in the airways, leads to fibrosis development. Contraction of fibrotic tissues then results in narrowing of the luminal space (Brusselle et al, 2011).

Alveolar tissue injury can lead to mild fibrosis, which is often seen in the early stages of COPD (Cosio et al, 2002). More extensive injury can lead to alveolar wall destruction, which is the defining feature of emphysema. Loss of alveolar wall reduces lung surface area and therefore compromises gas

exchange. Loss of lung elastic recoil and alveolar tethering of the small airways can cause the small airways to collapse (Saetta, 1985), which causes the expiratory airflow limitation in emphysema (Han et al, 2010).

Airway oedema and inflammation may also contribute to airflow limitation. Mucin secretions can accumulate within the narrowed lumen and as such are believed to be particularly important in exacerbations and in more severe disease (Hogg et al, 2004).

1.1.5 The role of Cytokines in COPD

Cytokines are extracellular signalling proteins produced by a variety of different cell types. They function in a paracrine way, exerting effects upon cells that are located in close proximity. In addition, cytokines can also act at a distance (endocrine) as well as affect the cell of origin (autocrine). The effects of cytokines are mediated via high affinity cell surface receptors which are usually present in lower numbers and subsequently upregulated upon cell activation.

A number of pro-inflammatory cytokines are believed to play a role in the pathogenesis of COPD (**Table 1.1**). The predominant pro-inflammatory cytokines found in the sputum and bronchoalveolar lavage (BAL) fluid of patients with COPD are TNFα, interleukin- (IL-) 1β, and IL-6, granulocyte macrophage-colony stimulating factor (GM-CSF) and TGFβ, and levels of these mediators are increased compared with subjects without COPD, suggesting a role for these pro-inflammatory mediators in COPD development and progression (Culpitt et al, 2003, Di Stefano et al, 2004). Levels of TNFα in

sputum correlate directly with smoking pack years and inversely with forced expiratory volume in one second (FEV1) (Hacievliyagil et al, 2006) and serum levels of TNFα positively correlate with COPD disease severity (von Haehling et al, 2009). In support of a key role for TNFα, a polymorphism in the TNFα promoter has been shown to result in increased TNFα production and has been linked to COPD (Sakao et al, 2001) although other studies have not confirmed this (Higham et al, 2000). Anti-TNFα therapies in COPD have also shown little clinical benefit (Van der Vaart et al, 2005, Rennard et al, 2007, Dentener et al, 2008), although an observational study investigating the use of infliximab and etanercept in patients with COPD found that etanercept reduced COPD-associated hospitalisations (Suissa et al, 2008). Taken together, these data indicate that although TNFα levels are directly associated with COPD development and progression and anti-TNFα therapies may be effective in a subgroup of patients with COPD who have high levels of TNFα, other pro-inflammatory mediators are also important. Increased TNFα (Aaron et al, 2001) and IL-6 (Browmich et al, 2000) levels are also observed in patients experiencing COPD exacerbations, linking these pro-inflammatory cytokines to the onset of an exacerbation in patients with COPD. IL-6 levels are also higher in induced sputum from patients with COPD and negatively correlate with FEV1, FEV1% and forced vital capacity (FVC) (Grubek-Jaworska et al, 2012). Recent studies have found that increased levels of IL-18 (Rovina et al, 2009), and the novel cytokine IL-32 (Calabrese et al, 2008) are also upregulated in patients with COPD compared with controls, and are associated with airflow limitation, thus suggesting a possible role in the pathogenesis of COPD.

COPD is a T helper (Th) 1-mediated disease, with Th1 cells, in addition to neutrophils and macrophages, being the predominant cell types found in bronchial biopsies. Specifically, interferon gamma (IFNγ)-secreting T cells are increased in the airways of patient's with COPD (Di Stefano et al, 2004), which may have an important role in inducing chemokine release, causing the infiltration of other inflammatory mediators into the airways (Di Stefano et al, 2004). Conversely, increased IL-4 in BAL fluid of patients with COPD compared with controls has also been demonstrated suggesting a potential role for Th2 cells in COPD development and progression (Barczyk et al, 2006).

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases, which degrade extracellular matrix proteins, cleave cell surface receptors, induce the release of apoptotic ligands and activate a number of pro-inflammatory mediators. In COPD, MMP-1, -2, -3, -7 and -9 are upregulated in serum, and specifically, levels of MMP-1, -8 and -9 correlate with disease severity (Navratilova et al, 2012), suggesting a role for these systemic proteins in disease pathogenesis.

1.1.6 The role of Chemokines in COPD

Chemokines are small cytokines (8–10 kDa) involved in inducing chemotaxis in responsive cells. Chemokines also have an important role in COPD as they are involved in directing the recruitment of inflammatory cells to the airways. CCL2 (also known as monocyte chemotactic protein-1 [MCP-1]) levels are increased in the sputum and BAL fluid of patients with COPD, and are

believed to be involved in macrophage accumulation in the lungs (Traves et al, 2002). CXCL1 and CXCL8 levels are upregulated in the sputum of patients with COPD compared with control subjects, and levels correlate with the increased neutrophil numbers present in the sputum (Traves et al, 2002), indicating that these chemokines may be involved in the neutrophillic inflammation present in the lungs of patients with COPD. In addition, leukotriene B4 (LTB4), a potent neutrophil chemoattractant, is also elevated in patients with COPD compared with control subjects, especially in more severe cases (Marian et al, 2006) and levels correlate with neutrophil numbers found in induced sputum in patients with COPD (Profita et al, 2005), suggesting a role for this chemokine in neutrophil-driven COPD pathogenesis. A large number of other chemokines have also been implicated in COPD, including growth-related oncogene alpha (GROα), regulated on activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein 1α, monocyte chemotactic protein (MCP-) 1, -3 and -4, and eotaxin (Chung 2001).

Table 1.1 Summary of key pro-inflammatory mediators and their potential role in COPD

Pro-inflammatory mediator	Association with COPD	Role in COPD
TNFα	Increased sputum concentrations Increased BAL concentrations Increased serum levels; correlate with disease severity Increased levels in exacerbations	Activate NFκB pathway Activate MAPK pathways Induce apoptosis
IL-1β	Increased sputum concentrations; correlates with disease severity Decreased IL-1 receptor antagonist Decreased soluble IL-1 receptor	Activates macrophages to secrete pro-inflammatory mediators
IL-6	Increased sputum concentrations; correlate with disease severity Increased exhaled breathe concentrations Increased serum levels	Stimulates release C reactive protein from liver Involved in systemic features of disease
IL-32	Increased expression in epithelial cells, macrophages, CD8+ T cells; correlates with disease severity	Induces TNFα and IL-1β release
GM-CSF	Increased BAL concentrations; correlate with neutrophil numbers	Differentiation and survival of neutrophils, eosinophils and macrophages
TGFβ	Increased expression on bronchial epithelial cells and macrophages	Fibroblast and airway smooth muscle proliferation Deposition extracellular matrix Epithelial repair
CCL2	Increased sputum concentrations Increased BAL concentrations	Monocyte chemoattractant
CXCL8	Increased sputum concentrations; correlate with neutrophil numbers Increased BAL concentrations	Neutrophil chemoattractant

GM-CSF, granulocyte macrophage-colony stimulating factor; IL-, interleukin; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; TGFβ, transforming growth factor beta; TNFα, tumour necrosis factor alpha

1.1.7 Inflammatory cells in COPD

A number of distinct inflammatory cell types play a role in COPD, including macrophages, neutrophils, T and B lymphocytes, and eosinophils. In addition, bronchial epithelial cells are also implicated in COPD. Each cell type plays a distinct role in the development and progression of COPD.

1.1.7.1 Macrophages

Macrophages form part of the mononuclear phagocyte population and are generated from a committed progenitor cell in the bone marrow. Circulating monocytes differentiate into tissue macrophages when they enter tissues and are sub-divided based on their anatomical location and phenotype. Typically, macrophages have proteolytic and catabolic activities and ingest pathogens by phagocytosis, scavenging dead cells and cellular debris, as well as involvement in tissue remodelling following injury. Airway macrophages are known as alveolar macrophages and as such they are the first line of defence against inhaled pathogens and toxins. In patients with COPD, increased numbers of macrophages are found in the bronchial sub-epithelium (Rutgers et al, 2000, Di Stefano et al, 1998, Saetta et al, 1993, O'Shaugnhessy et al, 1997 and Di Stefan et al, 1996), bronchial glands, (Saetta et al, 1997), small airway epithelium (Turato et al, 2002), sputum (Keatings et al, 1997) and BAL fluid (Linden et al, 1993, Kuschner et al, 1996 and Capelli et al, 1999), indicating a key role for these cells in COPD development and progression. In further support of a key role in COPD, parenchymal alveolar macrophage

density is linked to the severity of lung destruction in emphysematous lung tissue from human subjects (Finkelstein et al, 1995). In addition, a higher rate of monocyte recruitment from the peripheral blood is also seen in patients with COPD (Corrigan and Kay, 1991), possibly as a result of increased cytokine and chemokine production, as discussed previously.

Although increased numbers of macrophages are present in the lungs of patients with COPD, exacerbations associated with bacterial colonisation of the airways are common in patients with COPD. Indeed, COPD macrophages phagocytose fewer *Escherichia coli* (Prieto et al, 2001) and *Haemophilus influenzae* (Berenson et al, 2006), as well as apoptotic epithelial cells through efferocytosis (Hodge et al, 2007), suggesting that certain cell-specific functions of macrophages are impaired in COPD. More recently, monocyte-derived macrophages in COPD have been found to have a reduced ability to phagocytose *Haemophilus influenzae* and *Streptococcus pneumoniae* but no defect in their ability to phagocytose inert particles (Taylor et al, 2010), suggesting that this phagocytic defect is specific to pathogenic bacteria phagocytosis and may explain the increase in bacterial colonisation in COPD.

A number of *in vitro* studies have shown that cigarette smoke activates the release of pro-inflammatory mediators from alveolar macrophages, including CXCL8 (Walters et al, 2005, Culpitt et al, 2003, Culpitt et al, 2003), GM-CSF (Culpitt et al, 2003, Culpitt et al, 2003), and MMP-9 (Russell et al, 2002). In contrast, cigarette smoke has been shown to down-regulate the production of a number of pro-inflammatory cytokines in peripheral blood mononuclear cells (PBMCs), including TNFα and IL-1β (Ouyang et al, 2000). A more recent study investigating the effects of cigarette smoke extract on COPD

macrophage cytokine, chemokine and signal transduction gene expression profiles showed that exposure to cigarette smoke down-regulates IL-1β, IL-6, IL-10 and IL-18 expression, and upregulates CXCL8 expression. (Kent et al, 2008). In addition, the expression of nuclear factor kappa-light-chain-enhancer of activated B cell (NFκB) signalling pathway components were suppressed by cigarette smoke, whilst the expression of a number of AP-1 components were enhanced. The authors concluded that exposure to cigarette smoke appears to upregulate chemotactic mechanisms through the increased CXCL8 expression whilst down-regulating components of the innate immune system. Taken together these studies indicate that cigarette smoke exposure alters the pro-inflammatory response *in vitro,* which may be replicated *in vivo,* thus altering the normal immune response generated against inhalation of cigarette smoke.

Some data suggest there is increased pro-inflammatory cytokine production released from the bacterial cell wall component lipopolysaccharide- (LPS-) stimulated COPD alveolar macrophages compared with control cells (Cosio, 2004); whilst others have shown reduced cytokine release from LPS-stimulated COPD and control alveolar macrophages (Armstrong et al, 2009). This is similar to findings from a number of studies showing that smoking reduces pro-inflammatory mediator production from alveolar macrophages (Kent et al, 2008, Chen et al, 2007, Brown et al, 1989, Yamaguchi et al, 1989, Yamaguchi et al, 1993, Soliman et al, 1992, Ohta et al, 1998).

Sputum induction is a non-invasive method of sampling cells from the upper airways, including macrophages and neutrophils. As such, sputum cell culture provides an alternative model to investigate the functional properties of both

airway macrophages and neutrophils (Quaedvlieg et al, 2005, Profita et al, 2003). Previous mixed sputum cell culture studies have demonstrated the spontaneous release of pro-inflammatory cytokines, including TNFα, CXCL8 and GM-CSF from patients with COPD, (Profita et al, 2003), with increased spontaneous production of TNFα and CXCL8 in mixed sputum cultures from patients with COPD compared with control subjects (Profita et al., 2003). In contrast to the *ex vivo* response of alveolar macrophages (Armstrong et al, 2009; Kane et al, 2009), stimulation of mixed sputum cells with LPS has been shown to have no effect on pro-inflammatory cytokine production from both COPD and control cells (Dentener et al, 2006). Since mixed sputum contains both neutrophils and macrophages, the relative contribution of each cell in relation to pro-inflammatory mediator production cannot be determined, and the functional properties of COPD isolated sputum macrophages has not yet been described.

To summarise, COPD is characterised by increased numbers of macrophages in the lung that have altered functional properties compared with macrophages in non-COPD lungs. The role of macrophages present in other areas of the lung is required to fully understand the role they play in disease onset and progression.

1.1.7.2 Neutrophils

Neutrophils are the most abundant leukocyte and are primarily involved in the release of granules by exocytosis and respiratory burst, which contribute towards an inflammatory response against pathogens. Neutrophils have much

shorter life spans than macrophages and are released into the bloodstream as mature cells that do not proliferate.

The neutrophil is widely believed to be the primary effector cell in COPD. Indeed, experimental models of COPD can be induced by NE (Stockley, 2002), and deficiency of endogenous NE protects against emphysema following exposure to cigarette smoke (Shapiro et al, 2004). In addition, the link between α1 antitrypsin deficiency and COPD further supports a key role for the neutrophil in COPD.

High levels of neutrophil chemoattractants LTB4 (Marian et al, 2006) and CXCL8 (Traves et al, 2002) are elevated in patients with COPD compared with control subjects, and these correlate with neutrophil numbers and COPD severity. Patients with COPD have elevated numbers of neutrophils in their sputum and BAL fluid (Keatings and Barnes, 1997; Lacoste et al, 1993; Vlahos et al, 2012), and bronchial biopsies (Keatings et al, 1996), which also correlate with disease severity (Keatings et al, 1996; Vlahos et al, 2012), suggesting that neutrophils are principally involved in the development and progression of the disease. Increased expression of galectin-3, a neutrophil activator, in patients with COPD is associated with increased neutrophil accumulation, epithelial proliferation and also airway obstruction (Pilette et al, 2007). Cigarette smoke prevents macrophages ingesting neutrophils by phagocytosis (Minematsu et al, 2001), which may account for the increased numbers of neutrophils found in the lungs of patients with COPD. Neutrophils are also implicated in COPD disease progression, as neutrophil numbers in sputum are associated with reduced FEV1 (Singh et al, 2010, Stanescu et al, 1996).

Altered neutrophil functions are also associated with COPD. Airway neutrophils have been reported to release increased levels of MMP-9 and NE, with levels correlating to disease severity (Vlahos et al, 2012). Systemic blood neutrophils from patients with COPD have been demonstrated to have enhanced respiratory burst compared with control cells (Noguera et al, 2001). In addition, reduced chemotactic activity has also been reported in blood neutrophils (Yoshikawa et al, 2007), although this has recently been disputed as neutrophils from smokers with and without COPD have been found to have increased neutrophil chemotaxis towards CXCL8 (Blidberg et al, 2012). Some studies also report decreased apoptosis in COPD blood neutrophils (Pletz et al, 2004; Brown et al, 2009), whereas others demonstrate increased apoptosis (Makris et al, 2009) or no differences in the apoptotic activity of COPD neutrophils (Noguera et al, 2004; Rytila et al, 2006). These differences may be due to differences in glucocorticoid (GC) treatments between patient groups (Zhang et al, 2001). Cigarette smoke extract also impairs the phagocytic activity of systemic blood neutrophils, (Stringer, 2007) as well induces the release of CXCL8 (Mortaz et al, 2009).

Taken together these data suggest that neutrophils play a central role in the development and progression of COPD, and as such, targeting these cells pharmacologically may be useful in the treatment of COPD. There is very limited published information regarding the functional role that resident lung or airway neutrophils play in COPD, and as such, further research regarding these cells is necessary to fully understand their contribution to the inflammation present in COPD.

1.1.7.3 Epithelial cells

The airway epithelium is a primary interface with the outside world and is targeted by toxic particles and gases from cigarette smoke and other environmental agents. Cigarette smoke activates epithelial cells to produce a variety of inflammatory mediators and proteases, including TNFα, TGFβ, IL-1β, IL-6 and CXCL8 (Hellerman et al, 2002; Mio et al, 1997; Takizawa et al, 2001; Beisswenger et al, 2004), indicating a primary role for epithelial cells in the development of COPD through the recruitment of inflammatory cells into the lungs.

In vitro studies demonstrate that cigarette smoke activates the NFκB pathway in bronchial epithelial cells, which in turn induces the expression of CXCL8, which then acts as a chemoattractant for neutrophils into the lung, (Hellermann et al, 2002) along with the expression and release of IL-6 (Beisswenger et al, 2004), which itself acts on a number of pro-inflammatory cell types to induce the release of pro-inflammatory mediators. Indeed, patients with COPD have enhanced NFκB expression (Tagi et al, 2006), which may account for the increased release of pro-inflammatory mediators from COPD bronchial epithelial cells.

Reynolds et al, (2006), showed that the cigarette smoke-induced increases in pro-inflammatory mediator release from epithelial cells were induced by early growth response gene-1 (EGR-1), which is significantly up-regulated in the lungs of smokers with COPD (Ning et al, 2004). A study by Pierrou et al showed that epithelial expression of genes involved in oxidant and antioxidant responses is altered in COPD and smoker subjects compared with non-

smokers (Pierrou et al, 2007). Cigarette smoke induces EGR-1 expression (Reynolds et al, 2006), as well as down-regulating the expression of vascular endothelial growth factor (VEGF) (Suzuki et al, 2008), which is thought to be important for maintaining structural homeostasis in the adult lung (Voelkel et al, 2006).

Cigarette smoke induces a number of other effects on bronchial epithelial cells including mitochondrial dysfunction (van der Toorn et al, 2007), repair process abnormalities (Wang et al, 2001) and mucin production (Shao et al, 2003; Shao et al 2004; Baginski et al, 2006). A recent study also showed that cigarette smoke reduces epithelial integrity, which is likely to be mediated through the epithelial growth factor receptor (EGFR) and the endogenous protease calpain (Heijink et al, 2012).

COPD bronchial epithelial cells have also been demonstrated to have increased TNFα-induced CXCL8 and GROα production (Schulz et al, 2004). This suggests that the initial exposure to cigarette smoke alters the phenotypic properties of bronchial epithelial cells, inducing an enhanced pro-inflammatory phenotype in response to different stimuli. A large number of studies have shown that stimulation of bronchial epithelial cells with TNFα, IL-1β and rhinovirus induces the release of a variety of pro-inflammatory mediators including MMP-9, CXCL8, IL-6, RANTES, GM-CSF and IL-10 (see **Table 1.2** for details). In addition, TNFα stimulation has also been found to inhibit production of the anti-inflammatory cytokine TGFβ from bronchial epithelial cells, suggesting that pro-inflammatory stimulation of bronchial epithelial cells may contribute to altered repair processes observed in COPD (Hodge et al, 2001).

These studies highlight the key role that bronchial epithelial cells play in COPD development. There have been no studies to date that have compared the effects of different stimuli on the release of a variety of pro-inflammatory mediators from the same bronchial epithelial cell line. This would be useful in understanding the relative contribution of pro-inflammatory cytokine-, bacterial- and/or virus-induced effects on these cells. Targeting these cells with anti-inflammatory therapeutics may halt the progression of COPD by dampening down the inflammatory response seen in patients with COPD.

1.1.7.4 Lymphocytes

T lymphocytes

T lymphocytes play a central role in cell-mediated immunity and form part of the adaptive immune response. CD4+ T cells produce cytokines including, but not limited to, IFNγ, IL-2 and IL-10, which are involved in the initiation and regulation of immune responses. CD8+ T cells, also known as cytotoxic T cells are involved in the destruction of virally infected cells. There are increased numbers of $CD8^+$ T cells in the lungs and airways of patients with COPD (Hogg, 2001; O'Shaughnessy et al, 1997; Saetta et al, 1999), which are linked to alveolar destruction and airflow limitation (Finkelstein et al, 1995, Saetta et al, 1999). There are also increased numbers of CD8+ T cells with an altered phenotype in the airway epithelium of patients with COPD (Mikko et al, 2012). CD4+ T cells have also been recently been hypothesised to play a role in COPD, both as effector cells, particularly in severe disease (Sullivan et al,

2005), and also as regulatory cells (Lane, 2010). A recent study by Zhu and colleagues examining peripheral T cell functions in patients with COPD showed that fewer $CD4^+$ T cells produced the anti-inflammatory cytokine IL-10, while $CD8^+$ T cells produced increased IFNγ and IL-4 (Zhu et al, 2009), indicating that the balance between pro- and anti-inflammatory mediators is altered in patients with COPD. This is in contrast to lung tissue T lymphocytes, as these cells have been shown to release significantly lower levels of IFNγ compared with control cells (Kaur et al, 2012). This suggests that T lymphocytes entering the lung undergo phenotypic changes, altering their functional properties compared with systemic T cells in the circulation. In addition, COPD severity is significantly and inversely associated with the proportion of circulating $CD4^+$ T lymphocytes, and directly correlates with CD4+ IL-2 production (Zhu et al, 2009). COPD disease severity is also directly associated with the frequency of $CD8^+$ T cell activation and IFNγ production (Zhu et al, 2009).

Increased CD8+ T lymphocyte expression of the chemokine receptors CCR3 and CCR5 has been described in patients with COPD compared with controls (Smyth et al, 2008). In addition, CD8+ T cell expression of CCR5 was found to correlate with smoking pack years. CD3+ T lymphocytes also have increased CCR3 expression in patients with COPD, suggesting that cigarette smoke upregulates chemokine expression on T lymphocytes. Increased CXCR3 expression on T cells infiltrating the peripheral airways of smokers with COPD has also been shown by Saetta and colleagues (Saetta et al, 2002). These studies demonstrate that COPD development and progression is associated with increased chemokine receptor expression, which may then be involved in

the recruitment of T lymphocytes into the lungs, further exacerbating the inflammatory environment.

COPD may have an autoimmune component, as supported by increased numbers of clonogenic T cells found in patients with COPD (Lambers et al, 2009). Patients with COPD have reduced numbers of forkhead box 3- (FoxP3-) expressing T cells in their small airways compared with smokers and non-smokers, which is negatively correlated with airflow obstruction (Isajevs et al, 2009). Reduced CD4+CD25+ T regulatory cells in COPD lungs compared with smokers subjects has also been demonstrated (Barcelo et al, 2008). Smokers without COPD have increased CD4+CD25+ T regulatory cells compared with non-smokers, indicating that whilst exposure to cigarette smoke upregulates T regulatory cell numbers, the development of COPD coincides with a reduction in T regulatory cell numbers. In addition, significantly reduced numbers of T regulatory cells have been observed in the lungs of emphysema patients compared with control subjects, as well as reduced FoxP3 mRNA in emphysematous lungs compared with controls (Lee et al, 2007). CD8/CD28 (null) cells are also increased in patients with COPD and express increased co-stimulatory molecules compared with CD4/CD28(+) cells, indicating a role in autoimmune responses in patients with COPD (Hodge et al, 2011). These studies suggest that a breakdown in T cell-mediated immune regulation in COPD, which may play a role in disease progression. Interestingly, Lee et al also showed the presence of auto-reactive T cells in the periphery of patients with COPD, indicating a breakdown in self-tolerance that is associated with tissue destruction. Elastin breakdown products may be the potential antigen responsible (Lee et al,

2007). Conversely, increased numbers of follicular FoxP3 T cells (Plumb et al, 2009) and increased numbers of CD4+ T regulatory cells (Smyth et al, 2007) have been found in COPD lungs compared with controls. These differences in T regulatory cell distribution may be due to changes in microenvironments in different areas of the lung, although the functional properties of regulatory T cells remain to be elucidated in COPD.

B lymphocytes

B lymphocytes are principally involved in the humoral immune response, producing antibodies against pathogens. Increased numbers of B cells have been found in the airways of patients with COPD compared with controls (Hogg et al, 2004, van der Strate et al, 2006), suggesting a role in COPD pathogenesis. Interestingly, oxidative stress, which is induced by cigarette smoke, has been shown to induce leukotriene synthesis in B cells (Werz et al, 2001), which may implicate B cells in the COPD inflammatory response. Further research into the role of B lymphocytes in COPD is required to fully understand their role in disease pathogenesis.

1.1.7.5 Eosinophils

Eosinophils differentiate from myeloid precursor cells in response to IL-13, IL-5 and GM-CSF and produce and store secondary granule proteins including major basic protein, eosinophil cationic protein, eosinophil peroxidise and eosinophil-derived neutrotoxin. Interestingly, increased sputum eosinophil

counts have been described in smokers compared with non-smokers, and numbers correlate to smoking pack years and FEV1 (Dippolito et al, 2001). There is conflicting evidence in regards to the importance of eosinophils in COPD. Some studies report increased numbers in Patients with COPD compared with controls (Lacoste et al, 1993; Papi et al 2000), while others dispute this and show reduced numbers of eosinophils in COPD (Keatings and Barnes, 1997; Maestrelli et al, 1995), which indicates that discreet phenotypes of COPD may exist. Elevated levels of eosinophillic cationic proteins (Fiorini et al, 2000) and eotaxin (Balzano et al, 1999) in the BAL fluid and sputum from Patients with COPD compared with controls has been observed. In addition, increased numbers of eosinophils are associated with COPD exacerbations (Saha and Brightling, 2006) suggesting a role for eosinophils in COPD, although further research into their precise role is required.

1.1.8 COPD disease exacerbations

An acute exacerbation is a sustained increase in cough, production of sputum and/or dyspnoea (Macintyre and Huang, 2008) Exacerbations are usually inflammatory events, with a number of airay and systemic inflammatory markers increasing. Exacerbations are caused by complex interactions between the host, respiratory viruses, airway bacterial pathogens, and environmental pollution, resulting in an increase in inflammatory burden. Common bacterial pathogens include *Haemophilus influenzae*, *Moraxella*

catarrhalis, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* (Anzueto et al, 2007). Frequently isolated viral pathogens from patients with COPD exacerbations include rhinovirus, coronavirus, influenza, parainfluenza, adenovirus, and respiratory syncytial virus. Exacerbations that are triggered by respiratory viral infections are often more severe and associated with longer recovery times than those triggered by other factors. The precise role of bacteria at COPD exacerbations has been difficult to assess, since airway bacterial colonisation in the stable state is associated with the same organisms as those isolated at exacerbations. Indeed, in many patients with exacerbations, both respiratory viruses and bacteria can be isolated (Wedzicha and Seemungal, 2007).

In about 30% of acute exacerbation cases, no specific aetiology can be identified (Macintyre and Huang, 2008). Studies suggest the frequencies of exacerbations are related to disease severity, with patients diagnosed with moderate to severe COPD experiencing more exacerbations per year than patients with mild COPD (Anzueto et al, 2007). Exacerbations are typically associated with an increase in inflammation, with increased cellular infiltration and pro-inflammatory cytokine production, which can therefore result in more tissue damage (Sapey and Stockley, 2006). As mentioned previously, higher numbers of neutrophils are found in the BAL fluid and bronchial walls of patients experiencing acute exacerbations, indicating a key role for neutrophils in exacerbations. Studies have also shown that there is increased sequestration of neutrophils in the pulmonary microcirculation prior to migration to the airways (Sapey and Stockley, 2006). A number of inflammatory markers have also been found to be increased during

exacerbations compared with stable disease, including IL-6, CXCL8, endothelin 1, LTB4 and NE (Sapey and Stockley, 2006).

1.1.9 Systemic effects

Several complications of COPD have been associated with a systemic inflammatory response including ischaemic heart disease, heart failure, osteoporosis, normocytic anaemia, lung cancer, depression, diabetes, metabolic syndrome, skeletal muscle wasting and weakness.

A systemic inflammatory response is characterised by mobilisation and activation of inflammatory cells into the circulation, as well as the production of acute phase proteins and circulating pro-inflammatory mediators (Sinden and Stockley, 2010). C-reactive protein (CRP) is a marker of the acute phase inflammatory response and levels are elevated in Patients with COPD and associated with airflow obstruction (Sin and Man, 2003). Patients with COPD also have increased circulating levels of fibrinogen, another acute phase protein (Jousilahti et al, 1999; Wedzicha et al, 2000), as well as increased circulating levels of pro-inflammatory mediators including TNFα and IL-6 (Di Francia et al, 1994, Chung, 2001, Eid et al, 2001).

Increased systemic inflammation in COPD may explain the increased frequency of comorbid conditions in patients with COPD and may result from ‘overspill’ of disruptive inflammatory events occurring in the lung, or alternatively because COPD develops as part of a multi-organ disease rather than a disease characterised solely by lung inflammation. The high frequency

of concurrent disease in patients with COPD may also be partly explained by the fact that the majority of patients with COPD are of an older generation and thus are more likely to suffer from poor health in general as well as their exposure to cigarette smoke, which is a major risk for COPD and many other chronic diseases (Corsonello et al, 2011).

1.1.10 Current treatments

Current therapies for COPD focus on alleviating symptoms, reducing exacerbations, and reducing lung function decline, with the overall goal to improve quality of life for sufferers. Approaches include the use of bronchodilators and GC, which are delivered via inhalation so they can act directly within the lung. According to the GOLD strategy, the main medications employed in the treatment of COPD include short-acting β2 adrenoreceptor agonists, long-acting β2 adrenoreceptor agonists, short- and long-acting anticholinergics, phosphodiesterase 4 inhibitors and inhaled and systemic GC.

1.1.10.1 Bronchodilators

Bronchodilators are effective in COPD as the airflow obstruction is partially reversible. A number of different bronchodilator treatments are employed as a therapeutic strategy in COPD to help alleviate symptoms and improve overall quality of life.

β_2 adrenoreceptor agonists

Adrenergic receptors are part of the sympathetic nervous system and are characterised by their interaction with adrenaline and noradrenaline. Stimulation of the β receptor is associated with vasodilation, and inhibition of the bronchial smooth muscle (Burgess et al, 1997). β_2 adrenoreceptors are present on inflammatory and resident pulmonary cells including macrophages, neutrophils, lymphocytes, eosinophils, epithelial and endothelial cells (Zhang et al, 2011), and have been shown to exert a variety of effects on these cells, including inhibition of pro-inflammatory mediator release from bronchial epithelial cells (Miyabayashi et al, 2006, Loven et al, 2007, Skevaki et al, 2009, Chiu et al, 2007), alveolar macrophages (Donnelly et al, 2010) and inhibition of superoxide anion O_2^- release from neutrophils (Tachibana et al, 2001).

Short-acting β_2 adrenoreceptor agonists

Short-acting β2 adrenoreceptor agonists include salbutamol, fenoterol and terbutaline and are used in both chronic and stable management of COPD. They are also used in exacerbations, and have been shown to improve FEV1 compared with placebo in addition to reducing breathlessness (Sestini et al, 2009).

Long-acting β2 adrenoreceptor agonists

The most prescribed long-acting β2 adrenoreceptor agonist (LABA) in COPD is salmeterol, which, when used in combination with fluticasone, can reduce inflammation (Calverley et al, 2007). LABAs also increase skeletal muscle mass and strength, improving muscle weakness in Patients with COPD (Barnes, 2008). LABAs are also associated with increased FEV1 and larger changes in lung volumes, which reduces breathlessness (Celli et al, 2004). LABAs have side effects, primarily associated with the cardiovascular system and include increased heart rate and reductions in potassium concentrations (Rossi et al, 2008), and as such the use of LABAs in COPD are associated with increased mortality (Wood-Baker et al, 2010).

1.1.10.2 Anticholinergics

Stimulation of the muscarinic receptors results in generalised vasodilation and bronchoconstriction, increased secretion from all exocrine glands, and increased tracheobronchial secretions, amongst others (Roffell et al, 1990). Anticholinergics also have direct effects on a number of cells within the lung including on alveolar macrophages (Sato et al, 1998, Buhling et al, 2007), bronchial epithelial cells (Koyama et al, 1998, Koyama et al, 1992, Profita et al, 2008) and on COPD sputum cells (Profita et al, 2005).

Long-acting anticholinergics

Tiotropium, the first long-acting anticholinergic (LAAC) developed, reduces COPD exacerbations and associated hospitalisations, improving quality-of-life and symptoms as well as reducing the decline in FEV1 (Barr et al, 2006).

Short-acting anticholinergics

Although short-term use of short-acting anticholinergics has been shown to increase lung function (Gross and Skorodin 1984), long-term use has no effect on lung function over time (Anthonisen et al, 1994). In addition, long-term use is linked to an increase in mortality, particularly from cardiovascular disease (Singh et al, 2008)

1.1.10.3 PDE4 inhibitors

Phosphodiesterase- (PDE)-4 is expressed on inflammatory cells involved in COPD. PDE4 degrades cyclic adenosine monophosphate (cAMP), a secondary messenger involved in pro-inflammatory mediator production. cAMP activates protein kinase A, which phosphorylates proteins, thus inhibiting many inflammatory cells. Increased cAMP levels exert an anti-inflammatory effect due to decreased PDE4-induced cAMP degradation. PDE4 inhibitors increase cAMP levels and the subsequent down-regulation of a variety of inflammatory cell activities (Vignola, 2004).

Theophylline

Theophylline is a weak non-selective inhibitor of PDEs and has been used in the treatment of airway disease for over 70 years. Its mechanism of action and effectiveness in COPD is still not fully understood, although data suggest that theophylline increases histone deacetylase (HDAC) activity (Barnes, 2010).

1.1.10.4 Inhaled Glucocorticoids

GC are used in combination with β-agonists to inhibit airway inflammation and potentiate the bronchodilatory effects of LABAs and have been shown to reduce exacerbations and improve quality-of-life compared with using either treatment alone, (Calverley et al, 2007, Barnes, 2008).

1.2 Glucocorticoids

1.2.1 Natural glucocorticoids

Endogenous GC are released by the adrenal cortex and have a wide range of effects on many natural processes within the body, exerting effects on almost every cell. Estimates suggest GC modulate the expression of approximately 10% of all genes (Buckingham, 2006).

1.2.2 Glucocorticoid receptor

GC diffuse across the cell membrane into the cell cytoplasm and bind to a single 777 amino acid cytosolic glucocorticoid receptor (GR). The GR, a member of the nuclear receptor superfamily, is localised to the cytoplasm of nearly every single cell in the human body (Rhen and Cidlowski, 2005). GR is a modular protein, and each domain has distinct functions associated with it. The N-terminal region contains the constitutive transcriptional activation function (AF-1) (**Figure 1.1**). The central region of 65 amino acids make up a highly conserved zinc finger DNA-binding protein (DBD), which plays an essential role in receptor homo-dimerisation and cofactor interactions. Finally, the C-terminus encodes the ligand binding domain (LBD) and also contains the motif for ligand-dependent transcriptional activation function (AF-2). The DBD and LBD also contain nuclear localisation signals.

1.2.2.1 Alternative splicing

The gene encoding GR is located on chromosome 5q31-32 and made up of 9 exons that are highly conserved among species (**Figure 1.1**) (Stolte et al, 2006). Alternative splicing gives rise to a number of GR isoforms, including the most common, GRα, as well as GRβ, GRδ and GRγ. GRβ is believed to act as a dominant negative inhibitor of GC action by interfering with GR binding to DNA (Lewis-Tuffin and Cidlowski, 2006) and may, therefore, affect the GC responses in a number of inflammatory diseases (Chikanza, 2002; Hamid, 1999, Honda et al, 2000).

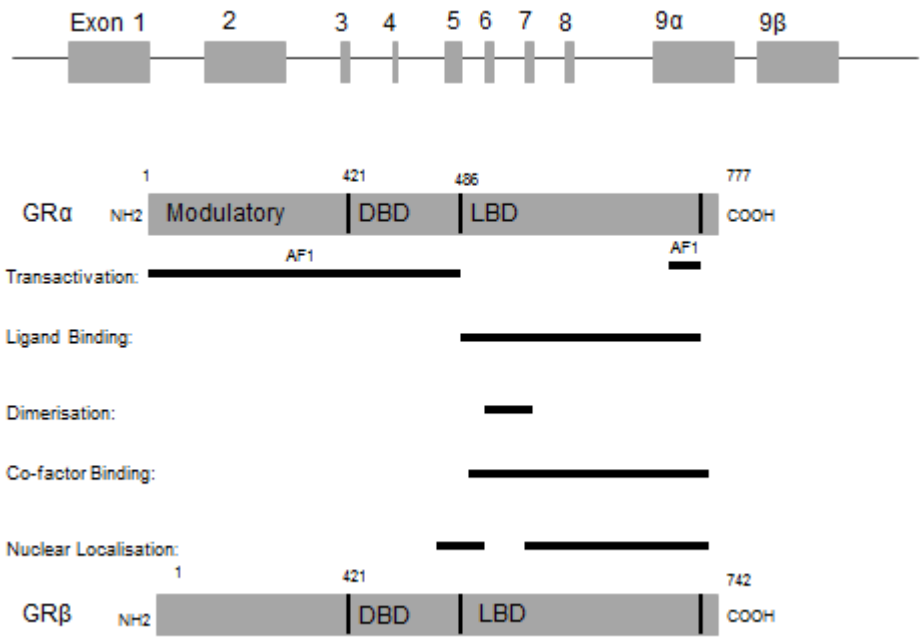


Figure 1.1 Structure of glucocorticoid receptor and alternative splicing
GR mRNA is made up of nine exons that are able to translate from alternative splicing into GRα (1-9α) or GRβ (1-9β). GRα is a 777 amino acid containing a transcriptional activation function (AF1) with 421 amino acid modulatory region. The DNA-binding domain (DBD) is made up of 65 amino acids. The C terminus domain is involved in ligand binding, dimerization, co-factor binding and nuclear localisation. GRβ is a 742 amino acid which does not bind GC due to a truncated C-terminus within the ligand binding domain (LBD).

1.2.2.2 Cytoplasmic glucocorticoid receptor

In the absence of ligand, GR exists as a multiprotein heterocomplex containing heat shock protein- (HSP-) 90, HSP-70, an acidic 23 KDa protein, the small p23 phosphoprotein, the p59 immunophillin molecule and protein phosphatise 5 (PP5) (Kanelakis et al, 2002). HSP-90 prevents nuclear localisation of unbound GR. Upon ligand binding, HSP-90 disassociates leading to the rapid translocation of the GR/GC complex into the nucleus (**Figure 1.2**).

1.2.3 Mechanism of action

1.2.3.1 Transactivation

The GC/GR complex binds to specific GC response elements in DNA chromatin, known as GC response elements (GREs) (**Figure 1.2**). This results in gene transcription, the production of mRNA molecules, and the synthesis of specific proteins.

The GC/GR complex modulates gene transcription by three molecular mechanisms;

1. Direct binding of the GC/GR complex to simple GREs in the promoter regions of target genes;

2. Binding to composite GREs in gene promoters in combination with other transcription factors;

3. Binding to tethering GREs on DNA sites for other transcription factors (such as NFκB, AP-1, and STAT3) (Chinenov and Rogatsky, 2007).

For transactivation mechanisms to occur GR recruits CBP/p300 through AF-1 or indirectly through co-factors with AF-2 (Kurihara et al, 2002). The SRC family are responsible for ligand-dependent interactions with GR in AF-2 with SRC-1, which then recruits PCAF and results in specific histone H3 modifications (Ito et al, 2000).

Genes activated by GC have anti-inflammatory properties and include genes encoding mitogen-activated protein kinase phosphatase-1 (MKP-1) (Lasa et al, 2002), annexin A1 (also known as lipocortin-1) (Perretti and D'Acquisto 2009), secretory leukocyte peptidase inhibitor (SLPI) (Hayashi et al, 2004), IL-10 (Dandona et al, 1998), β2 adrenergic receptor (Mak et al, 1995), GC-induced leucine zipper (GILZ) (Beaulieu et al, 2010), and G-protein signalling 2 (RGS2) (Holden et al, 2011).

1.2.3.2 Transrepression

The GC/GR complex can also regulate gene transcription via negative GREs (nGREs) (Dostert and Heinzel, 2004). To date, only a small number of genes, including, but not limited to, interleukin-1β (IL-1β), osteocalcin, and corticotropin-releasing hormone, have been shown to contain nGREs.

The GC/GR complex can also bind to other transcription factors, such as AP-1, NFκB, and interferon regulatory factor 3 (IRF3) (Heck et al, 1994, Ogawa et al, 2005, Reily et al, 2006). This enables the GC/GR complex to inhibit gene

transcription induced by these proteins. Importantly, all of these factors, which act as tethering GREs (Chinenov and Rogatsky, 2007), can modulate the transcription of pro-inflammatory genes, and this underpins many of the immune regulatory effects of GC.

The GC/GR complex physically interacts with NFκB in the cytoplasm to block its nuclear translocation (Widén et al, 2003) or in the transcription complex to prevent gene transcription (McKay and Cidlowski, 1998, Ray and Prefontaine, 1994). The GC/GR complex also represses transcriptional activation mediated by AP-1, through a direct interaction between GR and c-Jun/c-Fos, the two subunits comprising AP-1 (Schule et al, 1990, Touray et al, 1991). These processes act to decrease histone acetylation, chromatin remodelling, as well as the action of RNA polymerase II (Ito et al, 2000). Protein-protein interactions between the GC/GR complex and NFκB and AP-1 result in repression of the production of a variety of pro-inflammatory mediators including, but not limited to, IL-1β, IL-2, IL-3, IL-6, CXCL8, TNFα, and GM-CSF (Almawi and Melemedjian, 2002). GR is also able to recruit histone deacetylase (HDAC)-2 to the activated inflammatory gene complex in order to reduce histone acetylation, resulting in inhibition of activated pro-inflammatory genes in the nucleus. In addition, GC inhibit both nuclear translocation and p38 mitogen-activated protein kinase (MAPK)-mediated phosphorylation of the transcription factor GATA3, which regulates Th2 genes. (Maneechotesuwan et al, 2007).

1.2.3.3 Post-translational effects

GC are also involved in the destabilisation of mRNA, resulting in rapid degradation of target mRNA and as a result, reduced pro-inflammatory protein production (Bergmann et al, 2004). This may be mediated through increased expression of proteins involved in the destabilisation of pro-inflammatory proteins, such as the zinc finger protein tristetrapolin (TTP), which binds to the 3' AU-rich untranslated region of mRNAs, resulting in their degradation. Dexamethasone induces TTP mRNA in a pulmonary epithelial cell line (Smoak and Cidlowski, 2006), which may be another GC-mediated transactivation mechanism. GC may also induce expression of other proteins involved in mRNA stability including HuR and T cell intracellular antigen-1 (TIA-1) (Anderson et al, 2004). Indeed, GC are known to be involved in the destabilisation of IL-6 (Amano et al, 1993; Quante et al, 2008), cyclooxygenase 2 (COX-2 (Lasa et al, 2001)), TNFα (Smoak and Cidlowski, 2006) and CXCL8 (Chang et al, 2001).

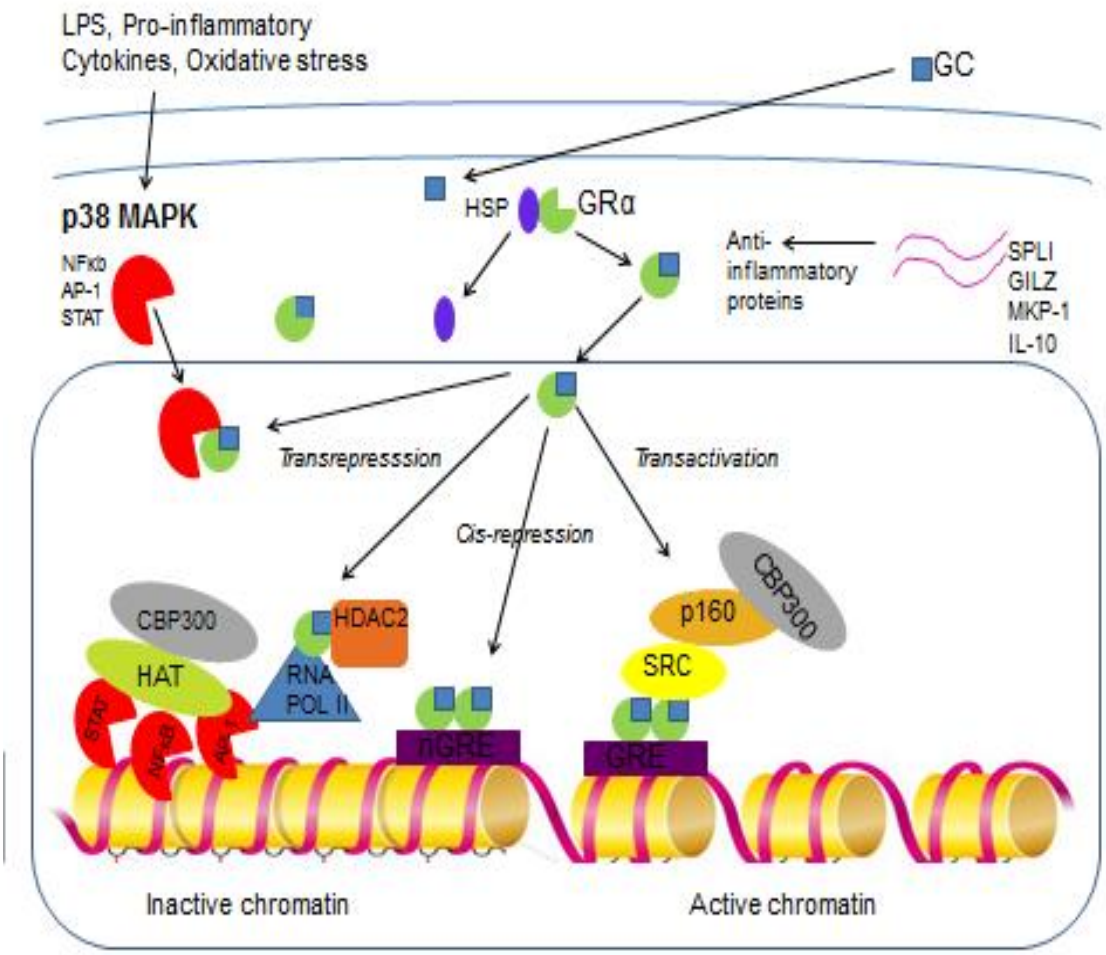


Figure 1.2 The mechanism of action of glucocorticoids
Inactive GR is bound to chaperone proteins including HSPs in the cytoplasm. Upon ligand binding, GR disassociates from HSPs and translocates into the nucleus. Bound GR can dimerise and act as a transcription factor binding to GREs found in promoter regions of target genes. To do this co-factors including SRC are required, which then enables recruitment of p160 and CBP/p300 with HAT activity. This enables DNA unwinding from histones, binding to RNA Polymerase II, and subsequent transcriptional activation. Anti-inflammatory genes activated by GR include SLPI, IL-10, MKP-1 and GILZ. GR dimers are also able to bind negative GRE, resulting in the repression of gene transcription via cis-repression. A number of pro-inflammatory stimuli can induce inflammatory mediators, including p38 MAPK. AP-1, NFκB and STAT recruit co-factors with HAT activity resulting in enhanced transcriptional activation of pro-inflammatory genes via RNA Polymerase II recruitment. GR is also to act as a monomer, binding AP-1 and repress its function through sequestration. Transrepression is also mediated by recruitment of HDAC2, resulting in the inactivation of chromatin.

1.2.4 Post translational modifications of glucocorticoid receptor

1.2.4.1 Phosphorylation

GR is a phospho-protein and contains a number of phosphorylation sites at serines 113, 114, 203, 211, 226 and 404 (Zhou and Cidlowski, 2005). The receptor is constitutively phosphorylated under physiological conditions, but undergoes agonist-induced, as well as cell cycle-dependent, hyperphosphorylation (Zhou and Cidlowski, 2006).

Phosphorylation at serine 211 is critical for GR activation (Wang et al, 2002). GR phosphorylation may also be important in affecting HSP-90 interactions (Hu et al, 1994), subcellular localisation (Somers and DeFranco, 1992) and transactivation potential (Wang et al, 2007). GR serines can be phosphorylated by MAPKs, such as p38 (Irusen et al, 2002, Szatmary, 2004, Miller et al, 2005, Nader et al, 2010), c-jun N-terminal kinase (JNK) (Rogatsky et al 1998, Itoh et al, 2002) and extracellular signalling kinases (ERK) (Rogatsky et al, 1998, Takabe et al, 2008), as well as cyclin-dependent kinases (Krstic et al, 1997), and glycogen synthase kinase-3 (GSK-3) (Rogatsky et al, 1998).

1.2.4.2 Other post translational modifications

GR can also be acetylated, which may attenuate its repressive effect on NFκB (Ito et al, 2006), nitrated, which may enhance its activity (Paul-Clark et al, 2003), ubiquitinated, which regulates its transcriptional activity (Wallace and

Cidlowski, 2001), and sumoylated, which may regulate GR stability (Drean et al, 2002).

1.2.5 Glucocorticoid insensitivity in COPD

GC are the drug of choice in the treatment of many inflammatory conditions but patients with COPD show limited clinical benefit in response to such treatments. A subgroup of patients with COPD with a particularly high sputum eosinophil count have been shown to respond favourably to GC, with short-term prednisolone (Brightling et al, 2000) and mometasone (Brightling et al, 2005) demonstrating favourable effects. However, other studies have shown that high doses of GC fail to reduce inflammatory markers in sputum or bronchial biopsies of patients with COPD (Keatings et al, 1997; Culpitt et al, 1999; Loppow et al, 2001; Hattotuwa et al, 2002; Bourbeau et al, 2007). In addition, GC have no effect on systemic inflammatory markers including IL-6 and C-reactive protein (Sin et al, 2008), as well as having few effects on neutrophillic inflammation (Barnes et al, 2008).

A number of large interventional studies assessed the effectiveness of GC in patients with COPD compared with placebo, with no effect on disease progression observed (Pauwels et al, 1999, Vestbo et al, 1999, Burge et al, 2000, Lung Health Research Group, 2000), although a number of short-term studies investigating the use of inhaled GC in Patients with COPD have demonstrated some clinical benefits (Weir et al, 1990; Kerstjens et al, 1993; Weiner et al, 1999).

A number of studies have shown that use of GC can reduce the frequency of exacerbations (Alsaeedi et al, 2002, (Jenkins et al, 2009, Calverley et al, 2007). GC have also been shown to reduce the risk of rehospitalisation for exacerbations amongst patients previously hospitalised with an exacerbation (Sin et al, 2001), other studies dispute this (Boureau et al, 2003).

GC have been demonstrated to improve airflow in Patients with COPD with acute exacerbations (Maltais et al, 2002), and are also associated with a reduction in all-cause mortality (Sin and Man, 2003; Sin and Tu, 2001). Regular use of fluticasone propionate alone and in combination with salmeterol is associated with increased survival (Soriano et al, 2002).

Taken together these studies indicate that GC may offer some clinical benefit in a subset of patients, particularly in regards to reducing the frequency of exacerbations. However, in many patients with COPD, there is little clinical benefit in regards to reducing lung inflammation observed with the use of GC, indicating that COPD is a partially GC insensitive disease. There are a number of mechanisms believed to be involved in GC insensitivity in patients with COPD.

1.2.6 Mechanisms of glucocorticoid insensitivity

1.2.6.1 Abnormal histone acetylation

Gene expression is regulated by acetylation of core histones, which enables chromatic remodelling to occur, transcription factors and RNA polymerase binding and subsequent gene transcription to occur. Coactivator molecules

regulate gene expression, which have intrinsic histone acetyltransferase (HAT) activity. Interestingly, in patients with COPD, increased histone acetylation at the promoter region of certain inflammatory genes, including CXCL8 has been described (Ito et al, 2005), and disease severity correlates with increased acetylation. Histone acetylation is reversed by HDACs, which therefore play a crucial role in suppressing gene expression (Barnes 2008). HDAC activity is reduced in smokers, and is associated with increased expression of pro-inflammatory genes (Ito et al, 2001). In addition, patients with COPD have lower levels of HDAC-2 activity, which also correlates with disease severity (Ito et al, 2005). Overexpression of HDAC-2 in COPD bronchoalveolar macrophages restored GC sensitivity (Ito et al, 2006), suggesting decreased HDAC-2 plays a role in GC insensitivity in COPD. HDAC-2 plays a key role in the regulation of the GR. In patients with COPD, the reduction in HDAC-2 activity means that there is increased acetylation of the GR, which may account for the GC resistance observed (Ito et al, 2006).

This reduction in HDAC in COPD may be due to oxidative and nitrative stress inactivating the enzyme (Barnes et al, 2004; Rahman et al, 2004). Oxidative and nitrative stress, generated by cigarette smoke and also some inflammatory cells, leads to the formation of peroxynitrate, which induces the nitration of particular tyrosine residues on proteins, including HDAC (Barnes, 2008). Interestingly, oxidative and nitrative stress is higher in patients with COPD, and is associated with the severity of disease (Bowler et al, 2004), and also HDAC-2 has increased tyrosine nitration in these patients, which again correlates with the increased expression of CXCL8 (Ito et al, 2004). Oxidative stress activates the phosphoinositide-3-kinase (PI3K) pathway,

which also inactivates HDAC-2 (Failla et al, 2007). Nitrative and oxidative stress reduce HDAC-2 expression via the nitration of distinct tyrosine residues *in vitro* (Ito et al, 2004; Osoata et al, 2009) and *in vivo* (Marwick et al, 2004), possibly due to increased proteasomal degradation following ubiquitination (Osoata et al, 2009). This hypothesis is supported by work showing degradation of HDAC-2 by an HDAC inhibitor through ubiquitination (Kramer et al, 2003). Cigarette smoke causes the dephosphorylation of HDAC-2 leading to decreased activity and increased degradation in macrophages, human bronchial and airway epithelial cells (Adenuga et al, 2009). As a result, patients with COPD have reduced HDAC activity, resulting in enhanced inflammatory gene expression which may be involved in GC insensitivity (Barnes, 2007). Although theophylline has been shown to increase HDAC2 and restore GC insensitivity in COPD cells (Cosio et al, 2004), there is little evidence to suggest that this drug has a clinical benefit in patients with COPD (Rabe and Hiemstra, 2010), suggesting other mechanisms are involved.

1.2.6.2 Defective glucocorticoid receptor binding and nuclear translocation

Increased levels of IL-2 and IL-4 are associated with GC-insensitive asthma (Leung et al, 1995) and *in vitro* combinations of IL-2 and IL-4 attenuate GR nuclear translocation and binding affinity within the nucleus of T lymphocytes (Sher et al, 1994; Spahn et al, 1996; Irusen et al, 2002; Matthews et al, 2004). This effect on GR function may be a result of p38 MAPK-induced GR phosphorylation, which can be blocked by pharmacological p38 MAPK inhibition (Irusen et al, 2002). This p38 MAPK-induced GR phosphorylation

may be at serine residue 211 or 226, or it may be an indirect effect of p38 MAPK (Irusen et al, 2004; Szatmary et al, 2004; Miller et al, 2005). Other kinases may also phosphorylate GR, thus altering GR binding, stability, nuclear translocation, DNA and other protein interactions, for example, with transcription factors or other chaperone proteins (Weigel and Moore, 2007). Other MAPK-induced GR phosphorylation has also been reported. JNK is activated by a number of pro-inflammatory cytokines implicated in COPD, such as TNFα, and has been shown to directly phosphorylate GR at serine residue 226, which inhibits GRE binding (Rogatsky et al, 1998). In addition, ERK-mediated GR phosphorylation at serine 203 may inhibit GR function, as inhibition of ERK, causing reduced phosphorylation at serine 203, leads to increased nuclear translocation and subsequent binding to GRE (Takabe et al, 2008). The role of GR phosphorylation in GC insensitivity in COPD requires further investigation.

1.2.6.3 Delayed neutrophil apoptosis

GC induce anti-inflammatory actions in a number of ways, one of which is by inducing apoptosis in a number of inflammatory cell types, such as lymphocytes and eosinophils. These cells are then removed by the process of phagocytosis. However, in contrast to eosinophils and lymphocytes, dexamethasone has been shown to actively delay apoptosis in blood neutrophils (Cox et al, 1995; Liles et al; 1995 and Meagher et al, 2001). This may be due to the ability of GC to alter the degradation of MCL-1L, an anti-apoptotic protein which is part of the BCL-2 family (Siverson et al, 2007).

Therefore, the treatment of patients with COPD with GC may have little effect on the neutrophillic inflammation seen in the airways of these patients (Barnes, 2008), therefore resulting in little overall clinical improvement.

1.2.6.4 Cell- and cytokine-specific insensitivity

Cytokine production from COPD alveolar macrophages *ex vivo* is reported to be GC resistant (Culpitt et al, 2003; Cosio et al, 2004). Culpitt and colleagues investigated the effects of dexamethasone on pro-inflammatory mediator release from macrophages isolated from BAL fluid. They showed that dexamethasone had little effect on basal and cigarette smoke- and IL-1β-stimulated CXCL8 release from COPD macrophages, whereas dexamethasone inhibited CXCL8 release from macrophages isolated from smoker patients. In addition, dexamethasone inhibited basal and IL-1β-stimulated GM-CSF release from both COPD and smoker patients, but had little effect on cigarette smoke-induced GM-CSF. They also showed that IL-1β-stimulated GM-CSF release from COPD macrophages was less sensitive to dexamethasone, compared with smokers (Culpitt et al, 2003). In agreement with this study, Cosio et al showed that CXCL8 and TNFα release from COPD alveolar macrophages stimulated with LPS were also less sensitive to dexamethasone compared with smokers and non-smoker cells (Cosio et al, 2004). These studies suggest that COPD alveolar macrophages are less sensitive to the effects of dexamethasone compared with smoking and non-smoking control cells, however only a limited number of pro-inflammatory mediators were investigated in these studies.

More recent data, however, suggests GC insensitivity is a cytokine- and cell-specific phenomenon present in alveolar macrophages regardless of disease or smoking status (Hew et al, 2006; Bhavsar et al, 2008; Armstrong et al, 2009; Kent et al, 2009). Armstrong and colleagues analysed the release of a number of pro-inflammatory mediators from LPS-stimulated alveolar macrophages (Armstrong et al, 2009). They showed that a number of pro-inflammatory mediators, including IL-6 and TNFα, were sensitive to dexamethasone, whereas other mediators, including CXCL8 and GM-CSF, showed limited sensitivity. They also showed that the sensitivity of COPD macrophages was similar to control cells. This is agreement with a previous study using gene arrays in COPD monocyte-derived macrophages (Kent et al, 2009). Other studies have used multiplex protein profiling to show that pro-inflammatory mediators each have different sensitivities towards GC in both LPS-stimulated macrophages (Bhavsar et al, 2008) and PBMCs in asthma (Hew et al 2006). Taken together these studies indicate that GC sensitivity is a cytokine- and cell-specific phenomenon irrespective of disease status. There are increased numbers of macrophages in COPD lungs and therefore increased levels of a number of inflammatory mediators present, some of which appear to be less responsive to inhibition by GC, highlighting a potential mechanism of GC insensitivity in patients with COPD.

Mixed sputum cells (mostly sputum macrophages, and neutrophils) are also insensitive to GC, with a recent study demonstrating only modest inhibition (40%) of IL-6 from healthy and asthmatic patients (Manise et al, 2010), indicating that cells in the upper airways are also GC insensitive, regardless of

disease status. The GC sensitivity of COPD mixed sputum cells has not been investigated.

A recent study has demonstrated that both COPD and control airway neutrophils do not express GR and incubation with dexamethasone has modest effects on pro-inflammatory mediator release (Plumb et al, 2011). Previous studies that have shown that airway neutrophils from cystic fibrosis (Corvol et al, 2003) and bronchial sepsis patients (Pang et al, 1997) are also resistant to GC. Taken together these studies suggest that neutrophils leaving the bloodstream and entering the lung undergo phenotypic changes, including reduced GR expression. Patients with COPD have increased numbers of neutrophils in the lungs and airways. Although there have been limited studies investigating the functional properties of airway and/or lung tissue neutrophils, they likely contribute to the inflammation within the lung environment in patients with COPD, and as such, this is likely to be resistant to the effects of GC.

Phytohaemagglutinin- (PHA-) and phorbol 12-myristate 13-acetate- (PMA-) stimulated release of IFNγ from T lymphocytes isolated from the lungs of patients with COPD has been shown to be GC-insensitive compared with control cells, which was not due to differences in GRα or GRβ expression (Kaur, et al, 2012). In addition, the authors also showed differences in pro-inflammatory mediator GC sensitivity, with IFNγ being more resistant to GC than IL-2. This indicates that T lymphocytes in COPD lungs have an altered phenotype compared with control cells, which is in contrast to both neutrophils and macrophages, whereby GC insensitivity appears to be an inherent functional property of the cells rather than a disease-specific functionality. In

addition, as T lymphocytes numbers are increased in COPD lungs, the lack of effect of GC on these cells is likely to contribute to the overall reduced effectiveness of GC in patients with COPD.

TNFα-stimulated GM-CSF secretion from human bronchial epithelial cells is also reported to be GC insensitive, with 40% inhibition achieved with dexamethasone (Korn et al, 2001), although other studies have shown GC inhibit TNFα-stimulated GM-CSF release by as much as 80% (Rossios et al, 2011). Other studies have demonstrated varying effects of GC on stimuli-induced pro-inflammatory mediator production in both primary cells and cell lines (see **Table 1.2** for details). Studies comparing the effects of GC on bronchial epithelial pro-inflammatory mediator release induced by different stimulants are required to determine whether GC sensitivity in epithelial cells is dependent on stimulus and/or pro-inflammatory mediator release.

Further work assessing the GC sensitivity of lung tissue and airway cells in COPD is required to fully understand the mechanisms of GC insensitivity in COPD. However, taken together these studies strongly support the hypothesis that in patients with COPD GC do not suppress the production of certain GC insensitive cytokines from the increased numbers of macrophages, neutrophils and lymphocytes in the airways.

Table 1.2. Summary of *in vitro* effects of glucocorticoids on bronchial epithelial cells

Author	Cell type	Stimulus	GC-mediated effect
Tacon et al, 2012	BEAS2B	HRV-16	Inhibition of MMP-9 release via MEK/ERK pathway
	Primary HBEs		
Rebeyrol et al, 2012	16HBES	TNFα	Inhibition of CXCL8 secretion, AP-1 and NFκB activity
Nasreen et al, 2012	Bronchial airway epithelial cells	Cigarette smoke	Inhibition of IL-6
Rossios et al, 2011	BEAS2B	TNFα and/or IL-1β	Inhibition of GM-CSF (max 80% inhibition) Increased MKP-1 expression Inhibition phospho-p38 MAPK expression
	Primary bronchial epithelial cells		
Galleli et al, 2010	Primary bronchial epithelial cells	TNFα	Inhibition CXCL8 release Inhibition phospho-p38 MAPK expression
Boero et al, 2012	BEAS2B	TNFα	Inhibition ICAM-1 expression (maximum 30% inhibition) Inhibition GM-CSF (60% maximum inhibition) Inhibition IL-5 (70% maximum inhibition)
Newton et al, 2010	Human bronchial epithelial cells	IL-1β	Inhibition GM-CSF release Increased MKP-1 expression
Skevaki et al, 2009	BEAS2B	Rhinovirus	Inhibition of RANTES Inhibition of CXCL8 Inhibition of IL-10
	Primary normal human bronchial epithelial cells		
Edwards et al, 2005	BEAS2B	IL-1β	Inhibition CXCL8 production
Korn et al, 2001	Human bronchial epithelial cells	TNFα	Inhibition GM-CSF (40% maximum inhibition) Inhibition CXCL8 (40% maximum inhibition)
Chang et al, 2001	HBE1 cells	Unstimulated	Inhibition CXCL8 protein and MRNA (60% maximum inhibition)
Wang et al, 1997	Human bronchial epithelial cells	TNFα	Inhibition RANTES
Wang et al, 1996	Human bronchial epithelial cells	TNFα	Inhibition RANTES
Stellato et al, 1995	BEAS2B	TNFα	Inhibition RANTES
Levine et al, 1993	BEAS2B	TNFα	Inhibition IL-6 protein and MRNA Inhibition CXCL8 protein and MRNA

The implications of this present a major problem for clinicians as GC are the mostly commonly prescribed and one of the most effective anti-inflammatory therapies available, yet they provide little benefit to patients with COPD. Currently there are no other anti-inflammatory treatments available. Understanding the molecular mechanisms of GC insensitivity will potentially provide new therapeutics to combat this resistance and treat patients with COPD more effectively.

1.3 Mitogen Activated Protein Kinases (MAPKs)

MAPKs are a family of intracellular signalling molecules involved in the regulation of many cellular activities including gene expression, mitosis, differentiation and apoptosis, and all eukaryotic cells possess multiple MAPK pathways to coordinate these cellular activities (Schindler et al, 2007). They include ERK 1 and 2, JNK 1–3, the p38 MAPK (p38 α, β, γ, and δ) and ERK 5. Activation of the MAPKs occurs through a variety of stimuli, but in general, ERK 1 and 2 are activated in response to growth factors and phorbol esters, while JNK and the p38 kinases are more responsive to stress stimuli, including osmotic shock, ionising radiation and also cytokine stimulation (Schindler et al, 2007). The MAPKs share approximately 70% homology to each other, but differ in their activation loop sequences and sizes (Roux and Blenis, 2004). Each family of MAPK is composed of three evolutionary conserved, sequentially acting kinases; a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (**Figure 1.3**). Activation of MAPKKK usually

occurs through phosphorylation and/or the binding of a small GTP-binding protein of the Ras/Rho family in response to an extracellular stimulus. MAPKKK activation leads to the subsequent activation and phosphorylation of MAPKK, which in turn leads to stimulation of MAPK. MAPK are activated by dual phosphorylation of their activation loop, which enables active site residues to be repositioned and substrate binding and catalysis to occur (Roux and Blenis, 2004). The MAPK can then phosphorylate their target substrates. Downstream substrates include phospholipases, cytoskeleton proteins and transcription factors, and phosphorylation of these regulates the expression of specific genes (Schindler et al, 2007). Therefore, MAPK activation is involved in inflammation, apoptosis, differentiation and proliferation. MAPK also catalyse the phosphorylation and activation of a number of protein kinases (MKs), which represents an additional enzymatic and amplification step. MKs are a family of ribosomal kinases that mediate a wide range of biological functions in response to mitogens and stress stimuli (Roux and Blenis, 2004).

1.3.1 p38 MAPK

p38 MAPK exists in 4 isoforms, p38α, p38β, p38γ and p38δ, each encoded by a separate gene, each with different tissue expression; α and β are ubiquitously expressed, γ is expressed mostly in skeletal muscle, whereas δ is expressed in the kidney, pancreas and testes. The p38 MAPK pathway is involved in the synthesis and regulation of a number of pro-inflammatory mediators including TNFα, IL-1, IL-6 and CXCL8 in macrophages, monocytes,

synovial cells and also endothelial cells, as well as COX2 and inducible nitric oxide synthase (iNOS) (Underwood et al, 2000; Dean et al, 1999). In addition, the expression of MMPs is also regulated by the p38 MAPK pathway (Underwood et al, 2000). Activation of the p38 MAPK pathway occurs via ligand binding to a range of different receptors, including G-protein coupled receptors, cytokine receptors, Toll-like receptors (TLRs), growth factor receptors and also other receptors that are associated with different environmental and genotoxic stresses (**Figure 1.3**). These include oxidative stress, ulta-violet radiation, hypoxia, ischemia, and various cytokines (Schindler et al, 2007). Once activated p38 MAPK activates a variety of substrates, mainly kinases and transcription factors (Chopra et al, 2008). The kinase substrates include MK2, MK3, MK5, MSK1, MNK1, MNK2, and CK2. Studies using MK2 knockout mice suggest that MK2 is the most important p38 MAPK substrate in mediating its proinflammatory effect (Chopra et al, 2008). p38 MAPK is present in both the cytoplasm and nucleus of quiescent cells, and following activation, it translocates to the nucleus, although even in activated cells some still remains in the cytoplasm (Blenis and Roux, 2004). Evidence suggest that it is critical for normal immune function as well as being critical in inflammatory responses, including the production of a number of different cytokines and chemokines. It is activated in macrophages, neutrophils, and T cells (Ono and Han, 2000). It also functions as part of neutrophil and macrophage responses, including the respiratory burst, chemotaxis, granular exocytosis, adherence, and apoptosis, and also T cell differentiation and apoptosis via IFNγ production. In addition, p38 MAPK stabilises specific cellular mRNAs also involved in the immune response (Ono

and Han, 2000) including CXCL8, IL6, TNFα, and COX-2, possibly through phosphorylation of its substrate MAPKAPK2 (Clark et al, 2003).

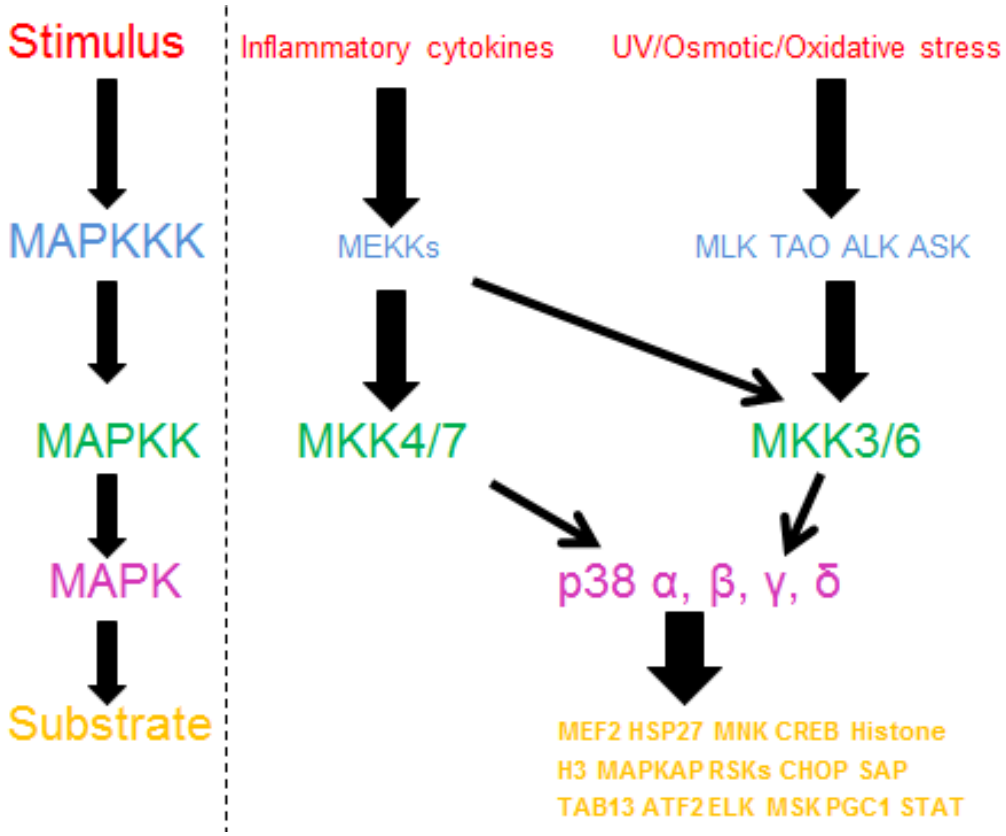


Figure 1.3 The p38 MAPK signalling pathway
The generic pathway is shown on the left. ALK, anaplastic lymphoma kinase; ASK, apoptosis signal-regulatory kinase 1; ATF-2, activating transcription factor 2; C/EBP, CCAAT enhancer binding protein; CHOP, c/EBP-homologous protein; CREB, cAMP response element binding; ELK, extracellular signal regulated-like kinase; HSP, heat shock protein; MAPK, mitogen-activated kinase; MAPKAPK, MAPK activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MEF, myocyte-specific enhancer factor; MEK, mitogen-activated protein kinase kinase; MKK, mitogen-activated protein kinase kinase; MKKK, mitogen-activated protein kinase kinase kinase; MLK, mixed lineage kinase; MNK, MAP kinase interacting kinase; MSK, mitogen- and stress-activated protein kinase 1; PGC-1, proliferator-activated receptor gamma family of transcriptional coactivators; RSK, ribosomal S6 kinase; SAP, stress-activated protein; STAT1, signal transducers and activators of transcription 1; TAB, transforming growth factor-β-activated protein 1-binding protein; TAO, serine/threonine-protein kinase; UV, ultraviolet.

1.3.2 MAPK phosphatases

A family of 10 dual-specificity MKPs act to dephosphorylate and inactivate the MAPKs, thereby playing an important role in the regulation of MAPK signalling (Reviewed by Owens and Keyse, 2007). Inducible expression of MKP-1 showed that MKP-1 regulates all three classes of MAPK, although it preferentially inactivates p38 kinase, then JNK, followed by ERK 1 and 2 (Franklin et al, 1997; Franklin et al, 1998). A number of studies have shown that MKP-1 plays a critical role in inactivating p38 MAPK. Animals lacking MKP-1 show over-production of pro- and anti-inflammatory cytokines (Chi et al, 2006; Hammer et al, 2006; Salojin et al, 2006; Zhao et al, 2006). In addition, macrophages from these animals show increased p38 and JNK MAPK activities in response to LPS, as well as increased TNFα production (Zhao et al, 2006). The livers of MKP-1-deficient mice also have hyperactivation of ERK, JNK and p38 MAPK (Chang and Karin, 2001). MKP-1-deficient fibroblasts stimulated from serum also exhibit hyperactivation of p38 MAPK and JNK (Wu and Bennett, 2005). Dexamethasone, via its transactivation mechanisms, is able to induce MKP-1 expression (Lasa et al, 2002), which acts to de-activate phospho-p38 MAPK, thus inhibiting its pro-inflammatory actions (Lasa et al, 2002, King et al, 2009).

1.3.3 p38 MAPK expression and inflammation

Increased expression of phospho-p38 MAPK is associated with a number of inflammatory conditions including glomerulonephritis (Polzer et al, 2008), inflammatory skin disorders (Wang et al, 2009) severe asthma (Liu et al, 2008) and COPD (Renda et al, 2008). In severe asthma, increased activated p38 MAPK has been observed in severe asthma epithelial cells compared with patients with mild asthma and healthy controls (Liu et al, 2008). In COPD lungs the numbers of alveolar macrophages and the number of cells in the alveolar wall expressing phosphorylated p38 MAPK was significantly higher compared with smoking and non-smoking healthy controls (Renda et al, 2008). Western blotting analyses of cell protein extracts of alveolar macrophages obtained at bronchoscopy were also found to have increased phosphorylation of p38 MAPK compared with healthy controls, suggesting that patients with COPD have increased levels of activated p38 MAPK (Renda et al, 2008). The expression of phospho-p38 MAPK by other cell types in the lung, including neutrophils and lymphocytes has not yet been investigated.

1.3.4 Cellular effects of p38 MAPK inhibition

1.3.4.1 Macrophages

As mentioned previously, macrophages are key cell types involved in the development and progression of COPD, and as such represent a viable target to target novel anti-inflammatory drugs in the treatment of COPD. LPS induces activation of p38 MAPK in macrophages (Meja, 2000; Armstrong et al, 2011). A number of studies have shown that pharmacological p38 MAPK

inhibitors can inhibit LPS-induced release of a large number of pro-inflammatory mediators including TNFα (Birrell et al, 2006, Smith et al, 2006, Gruenbaum et al, 2009, Kent et al, 2009), CXCL8 (Birrell et al, 2006, Gruenbaum et al, 2006, Kent et al, 2009), and GM-CSF (Meja et al, 2000, Smith et al, 2006, Gruenbaum et al, 2009, Kent et al, 2009). Interestingly, p38 MAPK inhibitors appear to have less effect on gene expression of pro-inflammatory mediators, suggesting that the predominant role of p38 MAPK is at the translational rather than transcriptional level (Birrell et al, 2006). Indeed, a number of genes in monocyte-derived macrophages, including IL-1β, IL-15, IL-18, were insensitive to p38 MAPK inhibition (Kent et al, 2009). Recent studies have also shown that a p38 MAPK inhibitor used in combination with dexamethasone inhibits pro-inflammatory mediator release from macrophages more effectively compared with using either drug alone (Bhavsar et al, 2010, Armstrong et al, 2011). In the study by Armstrong and colleagues LPS-stimulated alveolar macrophages were incubated with dexamethasone (0–1000nM), birb 796 (0–1000nM) or a combination of these. Whilst dexamethasone and birb 796 alone inhibited pro-inflammatory mediator release in a dose-dependent manner, increasing the concentrations of birb 796 in combination with dexamethasone induced significantly greater inhibition of pro-inflammatory mediator production compared with using either drug alone. Statistical analyses showed that there were significant efficacy-enhancing benefits and synergistic dose-sparing effects. The mechanism underlying this synergistic effect is unknown, and thus further research is required to elucidate this.

Data also suggests that the p38 MAPK pathway is also important in mediating the effects of respiratory virus infections on alveolar macrophages. For example, the human rhinovirus HRV-16 induces p38 MAPK activation in alveolar macrophages and the subsequent release of MCP-1 (Hall et al, 2005).

The effect of pharmacological p38 MAPK on macrophages is therefore well characterised, and demonstrates that, therapeutically, p38 MAPK may be a useful target in COPD macrophages.

1.3.4.2 Lymphocytes

Inhibitors of the p38 MAPK pathway have stimulus- and cytokine-dependent effects on lymphocytes. For example, inhibition of p38 MAPK in lymphocytes attenuates lFNγ (Rincon et al, 1998; Koprak et al, 1999), IL-4 (Schafer et al, 1999), IL-5 (Mori et al, 1999) and IL-10 release (Veiopaulo et al, 2004, Koprak et al, 1999). Conversely, inhibition has been shown to have little or no effect on IL-4 (Rincon et al, 1998, Korpak et al, 1999, Mori et al, 1999), or lFNγ (Mori et al, 1999, Schafer et al, 1999). This may be due to different stimuli being used in different studies or due to differences between CD4+ and CD8+ T cells that aren't always identified in studies.

In regards to B lymphocytes, although p38 MAPK inhibition has been shown to prevent oxidative stress-induced leukotriene release (Werz et al, 2001), there are few other studies that have investigated the effects of p38 MAPK inhibition of B cell functions.

1.3.4.3 Neutrophils

Stimulation of neutrophils with TNFα, GM-CSF, formyl-fethionyl-leucyl-phenylalanine (fMLP), PMA or ionomycin induces the phosphorylation and subsequent activation of p38 MAPK (Zu et al, 1998). As such there has been a considerable amount of research done into the effects of p38 MAPK inhibitors on blood neutrophils.

- Respiratory burst

Pharmacological p38 MAPK inhibition prevents the IL-1β- (Suzuki et al, 2001), TNFα, GM-CSF- (Zu et al, 1998; Suzuki et al, 1999), and fMLP- (Zu et al, 1998; Lal et al, 1999; Sakamoto et al, 2006) -induced release of O_2^-, possibly by down-regulating the TNFα-induced expression of CR3, a receptor involved in phagocytosis (Forsberg et al, 2001) or by attenuating activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Lal et al, 1999).

- Chemotaxis

Research has also shown that p38 MAPK inhibitors significantly reduce the neutrophil recruitment in a number of different model systems, including endotoxemia (Badger et al, 1996; Nick et al, 2000), collagen-induced arthritis (Badger et al, 1996), pulmonary inflammation (Nick et al, 2000, Nick et al, 2002) and gastritis (Takahashi et al, 2001). *In vitro*, p38 MAPK inhibitors

reduce the emigration and chemotaxis of neutrophils (Cara et al, 2001; Nick et al, 1997; Nick et al, 2002; Fujita et al, 2005, Spisani et al, 2005, Montecucco et al, 2006), thereby reducing the recruitment of neutrophils into areas of inflammation (Nick et al, 2002). This may be due to inhibition of L-selectin shedding (Rizoli et al, 1999; Smolen et al, 2000, Kaba et al, 2008), or inhibition of CD18 and MAC-1 expression (Tandon et al, 2000), thus causing a reduction in neutrophil rolling on E-selectin and intercellular adhesion molecule 1 (ICAM-1) (Chesnutt et al, 2006). Paradoxically, p38 MAPK inhibition of TNFα-stimulated blood neutrophils had reduced adhesion and increased migration (Lokuta and Hutteblocher, 2005). In addition, a p38 MAPK inhibitor has also been shown to have no effect on LPS-induced neutrophil migration, unless used in combination with an MEK inhibitor (U0126), suggesting p38 MAPK has more of a regulatory role in neutrophil migration (Aomatsu et al, 2007).

Leukocyte-specific-protein-1 (LSP-1) is expressed by neutrophils and has a variety of functions, including mediating chemotaxis (Wu et al, 2007). Inhibition of p38 MAPK reduces the phosphorylation of LSP-1 in neutrophils, which may cause altered F-actin polarization/stabilisation, thus impairing the chemotaxis of these cells (Wu et al, 2007). This suggests that the ability of p38 MAPK inhibitors to inhibit neutrophil chemotaxis may be, in part, due to its inhibition of LSP-1 phosphorylation and subsequent F-actin stabilisation and polarization.

- Pro-inflammatory mediator modulation

LPS-induced release of CXCL8 and macrophage-inhibitory protein (MIP) -1β from neutrophils is also inhibited by p38 inhibitors *in vivo* (Nick et al, 2002). *In vitro*, p38 MAPK inhibition attenuates CXCL8 production from LPS- (Zu et al, 1998; Coxon et al, 2003; Ribeiro et al, 2003, Cloutier et al, 2007) and TNFα- (Zu et al, 1998, Cloutier et al, 2007, Gruenbaum et al, 2009) stimulated blood neutrophils as well as inhibiting CXCL8 mRNA expression (Ribeiro et al, 2003). This suggests that the reduced neutrophil migration seen with p38 MAPK inhibitors may also be due to the reduction in CXCL8 production and secretion by neutrophils, as CXCL8 is a powerful chemoattractant for these cells. In addition, MIP-1α (Cloutier et al, 2007, Ekman et al, 2009), TNFα, IL-1β, and CCL4 release are also attenuated in LPS- and TNFα-stimulated blood neutrophils by p38 MAPK inhibitors (Cloutier et al, 2007).

- Apoptosis

Neutrophils undergo spontaneous apoptosis, which prevents unwanted tissue damage being caused by excessive numbers of activated neutrophils (Haslett, 1992). The p38 MAPK pathway has been shown to be implicated in the mechanism of spontaneous apoptosis, as inhibition results in delayed apoptosis *in vitro* (Aoshiba et al, 1999; Saffar et al, 2008). Inhibition of p38 MAPK increases LPS-induced inhibition of apoptosis in neutrophils isolated from blood, possibly by activating the ERK pathway (Sheth et al, 2001; Nolan et al, 1999). This may be due to impairment of HSP-27 which is involved in neutrophil apoptosis (Niwa et al, 2003). Association of p38 MAPK with caspase-8 and caspase-3 occurs during neutrophil apoptosis, and levels of

p38 MAPK directly correlate with caspase 8 and caspase 3 phosphorylation (Alvadaro-Kristensson et al, 2004). Inhibition of p38 MAPK increases caspase-3 and 8 activities in neutrophils, inducing their apoptosis, highlighting an alternative pathway for p38 MAPK regulation of apoptosis.

These studies highlight the potential for pharmacological p38 MAPK inhibition in down-regulating neutrophil functions in COPD (**Table 1.3**). To date, most research has examined the effects of p38 MAPK inhibitors on blood neutrophils, and as such these demonstrate the systemic effects of p38 MAPK inhibition in these cells. There are no studies investigating the effects of pharmacological p38 MAPK inhibition on lung neutrophils, which would be beneficial in regards to understanding the potential benefits of a therapeutic p38 MAPK inhibitor in patients with COPD.

Table 1.3 Summary of *in vitro* data showing effects of p38 MAPK inhibition on blood neutrophils

Author	Stimulus	Effect of p38 MAPK inhibitor
Suzuki et al, 2001	IL-1β	Inhibition of superoxide release
Zu et al, 1998 Suzuki et al, 2001	TNFα GM-CSF	
Zu et al, 1998 Lal et al, 1999 Sakamoto et al, 2006	fMLP	
Cara et al, 2001 Nick et al, 1997 Nick et al, 2002 Fujita et al, 2005 Spisani et al, 2005 Montecucco et al, 2006		Inhibition of chemotaxis
Zu et al, 1998 Coxon et al, 2003 Ribeiro et al, 2003, Cloutier et al, 2007	LPS	Inhibition of CXCL8 release
Zu et al, 1998 Cloutier et al, 2007 Gruenbaum et al, 2009	TNFα	
Cloutier et al, 2007	LPS TNFα	Inhibition of TNFα release Inhibition of IL-1β release Inhibition of CCL4 release
Sheth et al, 2001 Nolan et al, 1999	LPS	Enhanced apoptosis

fMLP, formyl-methionyl-leucyl-phenylalanine; IL, interleukin; GM-CSF, granulocyte-macrophage colony stimulating factor; LPS, lipopolysaccharide; TNFα, tumour necrosis factor alpha.

1.3.4.4 Epithelial cells

Due to their anatomical location, targeting bronchial epithelial cells with novel anti-inflammatory therapeutics may prove useful in attenuating lung inflammation in patients with COPD. Indeed, previous studies have shown that cigarette smoke (Nasreen et al, 2012) and pro-inflammatory cytokines (Matsumoto et al, 1998, Beisswenger et al, 2004, Berube et al, 2009) activate the p38 MAPK pathway in bronchial epithelial cells, highlighting the p38 MAPK pathway as a novel target in these cells.

- Pro-inflammatory mediator modulation

Pharmacological inhibition of p38 MAPK in bronchial epithelial cells attenuates the release of a number of pro-inflammatory mediators implicated in COPD including CXCL8 (Matsumoto et al, 1998, Laan et al, 2001, Cui et al, 2002, Li et al, 2002, Prause et al, 2003, Lee et al, 2008, Berube et al, 2009), GM-CSF (Hashimoto et al, 2000), RANTES (Hashimoto et al, 2000, Cui et al, 2002, Lui et al, 2008, Berube et al, 2009), and IL-6 (Beisswenger et al, 2004, Wang et al, 2005, Lee et al, 2008) (summarised in **Table 1.4**). Although these studies used different stimuli and different cell lines, they demonstrate the importance of the p38 MAPK pathway in pro-inflammatory mediator release from bronchial epithelial cells under different conditions.

A recent study has shown that a p38 MAPK inhibitor in combination with dexamethasone has a greater suppressive effect on TNFα-induced CXCL8 release from a bronchial epithelial cell line compared with using either drug alone (Rebeyrol

et al, 2011), indicating that the synergistic interaction between the GR and p38 MAPK pathway described earlier in alveolar macrophages is also present in other cell types. Rebeyrol and colleagues used only a single concentration of dexamethasone and p38 MAPK inhibitor, thus potential dose-sparing and synergistic mechanism could not be fully analysed.

- Mucin production

MUC5AC is a major airway mucin, implicated in the pathogenesis of mucin hypersecretion seen in many inflammatory diseases of the airways, including COPD. IL-1β (Kim et al, 2002; Song et al, 2003) and TNFα (Song et al, 2003) induce MUC5AC expression and secretion in epithelial cells, which is inhibited with the use of a p38 MAPK inhibitor (Kim et al, 2002; Song et al, 2003). Pharmacological p38 MAPK inhibition also reduces goblet cell density in human bronchial epithelial cell cultures, which is indicative of a mucin hypersecretory phenotype (Atherton et al, 2003).

TGFβ is produced by bronchial epithelial cells and is critically involved in airway remodelling and inflammation (Pelaia et al, 2003). TGFβ induces p38 MAPK activation. Inhibition of p38 MAPK blocks TGFβ-induced apoptosis, suggesting that p38 MAPK plays a critical role in transducing the apoptotic signal in epithelial cells (Pelaia et al, 2003).

Table 1.4 Summary of *in vitro* data showing effects of p38 MAPK inhibition on bronchial epithelial cell lines

Author	Stimulant	Effect of p38 MAPK inhibitor
Matsumoto et al, 1998 Laan et al, 2001 Cui et al, 2002 Li et al, 2002 Prause et al, 2003 Lee et al, 2008 Lui et al, 2008 Berube et al, 2009	Various	Inhibition CXCL8 release
Hashimoto et al, 2000	Various	Inhibition GM-CSF release
Hashimoto et al, 2000 Cui et al, 2002 Lui et al, 2008 Berube et al, 2009	Various	Inhibition RANTES release
Beisswenger et al, 2004, Wang et al, 2005 Lee et al, 2008	Various	Inhibition IL-6 release
Song et al, 2003	TNFα	Inhibition mucin secretion
Kim et al, 2002 Song et al, 2003	IL-1β	
Pelaia et al, 2003	TGFβ	Inhibition of apoptosis

GM-CSF, granulocyte macrophage colony stimulating factor; IL-, interleukin; RANTES, regulated upon activation, normal T cell expressed and secreted

Activation of p38 MAPK is, therefore, of critical importance to the function of epithelial cells, in particular to their inflammatory mediator release and subsequent recruitment of other cells into inflammatory areas. As highlighted above, the use of p38 MAPK inhibitors may therefore be useful in inhibiting many of their actions and could therefore be a useful therapeutic in the treatment of COPD.

1.3.5 Clinical trial development

A number of p38 MAPK inhibitors have been used in clinical trials with significant success. Birb 796 has been shown to inhibit TNFα and IL-6 (Regan et al, 2002), whilst RWJ67657 has been shown to inhibit neutrophil and endothelial activation

against disease biomarkers (Fijen et al, 2002). More recently, SB681323 was shown to inhibit TNFα production from patients with COPD (Singh et al, 2010). In addition, oral losmapimod has been shown to significantly reduce circulating fibrinogen in Phase 2 clinical trial, although no statistically significant effects on other systemic biomarkers of inflammation such as IL-6, CXCL8 or C-reactive protein were observed (Lomas et al, 2012).

Despite these positive results, a major setback in the development of a pharmacological p38 MAPK inhibitor has been the increase in liver toxicity. As a result, new second generation p38 MAPK inhibitors have focused on reducing the effects of cytochrome p450 (Barnes, 2006).

To summarise, much research has been carried out assessing the potential use of pharmacological p38 MAPK inhibitors as a pharmaceutical agent for use in the treatment of GC-insensitive inflammatory conditions. In the most part the use of p38 MAPK inhibitors appears to inhibit the production of pro-inflammatory cytokines from macrophages, neutrophils, lymphocytes and epithelial cells, as well as inhibiting certain cell-specific functions.

1.4 Hypothesis

Patients with COPD have increased numbers of lung resident cells positive for phospho-p38 MAPK, resulting in an overall reduced effectiveness of GC in these patients compared with patients without COPD. Combination therapy comprising GC and a p38 MAPK inhibitor may be a more effective therapeutic than using either drug alone. The mechanisms underlying this interaction may be due to enhanced repression of phospho-p38 MAPK, reduced p38-MAPK-induced GR phosphorylation, enhanced destabilisation of pro-inflammatory mRNA transcripts and/or enhanced GR nuclear translocation.

1.5 Aims and objectives

The overall aim of this thesis is to understand the role of the p38 MAPK pathway in GC-insensitivity in patients with COPD.

1. The first aim is to use a mixed sputum cell culture model to examine GC sensitivity in patients with COPD compared with controls.

2. The next aim is to examine and quantify the cell-specific presence of activated p38 MAPK in lung tissue; in CD4+ and CD8+ T lymphocytes, CD20+ B lymphocytes, neutrophils, macrophages and bronchial epithelial cells.

3. The effect of pharmacological p38 MAPK inhibition on pro-inflammatory mediator release from neutrophils isolated from COPD sputum compared with blood neutrophils will be investigated.

4. The effect of dexamethasone, the pharmacological p38 MAPK inhibitor birb 796, and a combination of these on pro-inflammatory mediator release from a bronchial epithelial cell line will be examined.

5. The mechanistic interactions between a p38 MAPK inhibitor and GC will be examined, specifically looking at the effects of combination treatment on stimuli-induced CXC8 mRNA stability, GR phosphorylation at serine 203, 211 and 226 and on GR nuclear translocation in a bronchial epithelial cell line.

Chapter 2

Materials and Methods

2.1 Study subjects

For mixed sputum culture experiments a total of 10 COPD, 10 smokers (S) with normal lung function and 12 lifelong non-smokers (NS) were recruited. Eight of these Patients with COPD were recruited for isolated neutrophil experiments. For immunohistochemical analysis, 20 patients with COPD, 12 S, and 12 NS subjects undergoing surgical resection for suspected or confirmed lung cancer were recruited. The diagnosis of COPD was by the GOLD definition, while S were defined as >10 pack year smoking history and normal pulmonary function. Demographics are shown in individual results chapters where appropriate. All patients gave written informed consent. All studies were approved by the local ethics committee.

2.2 Sputum induction and processing

2.2.1 Sputum Induction

Patients/subjects underwent spirometry followed by administration of 200mcgs of salbutamol through a spacer. Patients/subjects were then left for 20 minutes before reversibility was checked and spirometry was performed again. The induction commenced with patients/subjects inhaling increasing concentrations (3, 4, and 5%) of nebulised saline using an ultrasonic nebuliser for three 5 minute periods. To ensure no bronchospasm occurred during the procedure spirometry was performed after each inhalation. If no change in FEV_1 was noted (defined as a drop in FEV_1 of less than 10% from post nebuliser values, an increase in FEV_1, or no change), the patient/subject inhaled the next concentration of saline. If a drop of greater than 20% from the post nebuliser value was observed induction was terminated and the

patient/subject received nebulised salbutamol (200mcgs). If a drop in FEV_1 of less than 20% but greater than 10% was recorded the patient/subject underwent repeated nebulisation at the same saline concentration. After each inhalation patients/subjects were asked to rinse their mouth and blow their nose prior to expectorating into a sterile container. Samples were kept on ice prior to processing.

2.2.2 Sputum processing

Sputum plugs were isolated from the sample using forceps and placed into a pre-weighed sterile container and the weight of the sample noted. Samples less than 0.1g were discarded. 0.1% dithiothreitol (DTT; Sigma Aldrich, Dorset, UK) in phosphate buffered saline (PBS) was added to the sample at a volume of four times the sample weight. The sample was then vortexed and placed on a rolling mixer for 15 minutes at room temperature. PBS at a volume of four times the sample weight was then added, and the sample was filtered through 48µm sterile nylon gauze into a fresh sterile container. The sample was then centrifuged at 790g for 10 minutes at 4°C. The supernatant was frozen at -80°C for future cytokine analysis. The remaining cell pellet was resuspended in supplemented RPMI 1460 (Sigma Aldrich) containing 10% fetal calf serum (Fisher, Leicestershire, UK), 1mM/L L-glutamine (Fisher) and 1% penicillin/streptomycin (Sigma Aldrich). A cell count was performed by placing 10µl cell suspension and 10µl trypan blue (Sigma Aldrich) onto a neubauer haemocytometer. Total numbers of cells in the upper left, middle and bottom right square were counted and a mean calculated. This value was then multiplied by the dilution factor with trypan blue, multiplied by the total volume of sample and multiplied by 100 to obtain a cell count x 10^6. The cells were then resuspended at a concentration of 1 x 10^6/ml.

2.2.3 Sputum differential cell counts

50µl of sputum cells at a concentration of 0.5 x 10^6/ml were used to produce cytospins. These were air dried, fixed in methanol for 30 minutes then stained with Rapi-Diff (Triangle, Skelmersdale, UK); briefly, cells were incubated in red solution for 1 minute, rinsed in distilled water, followed by incubation in blue solution for 1 minute. Cells were then rinsed in tap water and mounted in DPX mounting solution (Sigma Aldrich). The differential cell count was performed by counting a total of 400 cells per cytospin. Total numbers of neutrophils, macrophages, eosinophils, lymphocytes and squamous cells were recorded and calculated as a percentage of total cells.

2.3 Mixed sputum cell culture

Sputum cells at a concentration of 1 x 10^6/ml were added to a 96 well flat bottomed plate at a volume of 100µl per well (100,000 cells per well). Dexamethasone (final concentration of 0.1–1000nM; Sigma Aldrich) or dimethyl sulfoxide (DMSO) control were each added at a volume of 10µl per well where appropriate and the total volume per well made up to 200µl using supplemented RPMI 1460 as described earlier. For some experiments, cells were incubated with LPS (0.1–10,000ng/ml; Sigma Aldrich). Cells were cultured for between 2 and 24 hours at 37°C, 5% CO_2. After this time, plates were centrifuged at 2000rpm for 10 minutes and supernatants removed and stored at -80°C for future cytokine analysis.

2.3.1 Isolated sputum neutrophil cell culture

To isolate neutrophils from the mixed sputum cell population the method described by Scheicher et al, (2007) was followed. Sputum cells at a concentration of 1 x 10^6 cells/ml were added to a 24 well plate at a volume of 400µl (400,000 cells per well) and cultured for 2 hours at 37°C, 5% CO_2 to allow macrophage adherence. After this time, the non-adherent cells were removed by taking up a volume of 380µl from each well. These cells were centrifuged at 400g for 10 minutes at 4°C. The supernatant was discarded and the cell pellet resuspended in supplemented RPMI 1460 as described earlier. Cell counts were performed as before and cells resuspended at a concentration of 1 x 10^6 cells/ml. Cells were plated out at a volume of 400µl per well of a 24 well plate. Dexamethasone or the p38 MAPK inhibitor SB731445 (GSK, Herefordshire, UK; final concentrations 1–1000nM) or DMSO control were added to the wells at a volume of 40µl per well where appropriate. For some experiments cells were also stimulated with LPS (final concentrations 0.1–10,00ng/ml) then the total volume per well made up to 800µl using supplemented RPMI 1460, as described earlier. Cells were cultured for 24 hours at 37°C, 5% CO_2. After this time, plates were centrifuged at 2000rpm for 10 minutes and supernatants removed and stored at -80°C for future cytokine analysis.

2.3.2 Isolated sputum neutrophil differential cell counts

50µl of isolated sputum neutrophil cells at a concentration of 0.5 x 10^6/ml were used to produce cytospins. These were air dried, fixed in methanol for 30 minutes then stained with Rapi-Diff (Triangle). The differential cell count was performed by counting a total of 400 cells per cytospin. Total numbers of neutrophils, macrophages, eosinophils, lymphocytes and squamous cells were recorded and calculated as a percentage of total cells.

2.3.3 Sputum neutrophil cytospins

For some experiments, sputum neutrophils were harvested after culture, centrifuged at 400g for 10 minutes at 4°C, resuspended in supplemented RPMI 1460 and cell counts established as before. Sputum neutrophils at a concentration of 0.5 x 10^6 cells/ml were then used to prepare cytospins for apoptosis analysis and for immunohistochemical analysis described further on in the Methods section.

2.3.4 Isolated sputum macrophage culture

Sputum cells at a concentration of 1 x 10^6 cells/ml were added to the wells of a 24 well plate at a volume of 400µl (400,000 cells per well) and cultured for 2 hours at 37°C, 5% CO_2 to enable macrophage adherence. After this time, the non-adherent cells were removed by taking up a volume of 380µl from each well. The remaining adherent cells were then used to perform experiments. Cells were incubated in dexamethasone (final concentrations 0.1–1000nM; Sigma Aldrich), or DMSO control at a volume of 40µl per well where appropriate and the total volume per well made up to 800µl using supplemented RPMI 1460, as described earlier. Cells were cultured for 24 hours at 37°C, 5% CO_2. After this time, plates were centrifuged at 2000rpm for 10 minutes and supernatants removed and stored at -80°C for future cytokine analysis.

2.4 Isolation of blood neutrophils

5ml heparinised venous blood was layered over 3ml mono-poly resolving medium (MP Biomedicals, Cambridgeshire, UK). After centrifuging at 800g for 45 minutes at 18°C the polymorphonucleocyte (PMN) layer was removed and placed into

supplemented RPMI 1460 and centrifuged at 400g for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in supplemented RPMI 1460. A cell count was performed as described earlier. The cells were then resuspended at a concentration of 1 x 10^6 cells/ml.

2.4.1 Blood neutrophil differential cell counts

50µl of blood neutrophils at a concentration of 0.5 x 10^6 per millilitre were used to produce cytospins. These were air dried, fixed in methanol for 30 minutes then stained with Rapi-Diff. The differential cell count was performed by counting a total of 400 cells per cytospin. Total numbers of neutrophils, eosinophils and lymphocytes were recorded and calculated as a percentage of total cells.

2.4.2 Blood neutrophil cell culture

Cells at a concentration of 1 x 10^6 cells/ml were plated out at a volume of 400µl per well of a 24 well plate. Cells were stimulated with or without LPS (final concentrations 0–10,000nM) for 24 hours. For some experiments cells were also pre-incubated for 2 hours with dexamethasone (final concentrations 0.1–1000nM) or the p38 MAPK inhibitor SB731445 (final concentrations 1–1000nM) or DMSO controls. Stimulants, drugs and/or DMSO were added to the wells at a volume of 40µl per well where appropriate and the total volume per well made up to 800µl using supplemented RPMI 1460 as described earlier. Cells were cultured for 24 hours at 37°C, 5% CO_2. After this time, plates were centrifuged at 2000rpm for 10 minutes and supernatants removed and stored at -80°C for future cytokine analysis.

2.4.3 Blood neutrophil cytospins

For some experiments, blood neutrophils, were harvested after culture, centrifuged at 400g for 10 minutes at 4°C, resuspended in supplemented RPMI 1460 and cell counts established as before. Blood neutrophils at a concentration of 0.5 x 10^6 cells/ml were then used to prepare cytospins for apoptosis analysis and for immunohistochemical analysis described further on in the Methods section.

2.5 Apoptosis Analysis

Isolated blood and sputum neutrophils, both basal and following culture with and without drugs and/or stimulants, were used to prepare cytospins as described earlier. These were air dried prior to fixation.

2.5.1 Morphological Analysis

Isolated blood and sputum neutrophils were fixed in methanol for 30 minutes at room temperature then stained in Rapi-Diff (Triangle). Apoptosis was assessed by examining the disappearance of chromatin bridges between nuclear lobes (early apoptosis), and shrinkage of or fragmentation of the nucleus (late apoptosis) in a total of 400 cells per cytoslide.

2.5.2 Tunel Assay

Isolated blood and sputum neutrophil cytoslides were fixed in 4% paraformaldehyde (Sigma Aldrich) in PBS at room temperature for 20 minutes. The Tunel assay kit (Roche Diagnostics, Hertfordshire, UK) was used as per the manufacturer's instructions. Briefly, cells were washed 3 x 3 minutes in PBS. The cells were then incubated in permeabilisation buffer (0.1% Triton X in 0.1% sodium citrate) for 2 minutes on ice. After washing in PBS the cells were then incubated in Tunel reaction

mixture and incubated at 37°C for 1 hour. The cells were then rinsed in PBS. To enable identification of neutrophils in the mixed sputum cytospins dual labelling with NE, (clone NP57, Dako, Cambridge, UK) was carried out. Cytospins were incubated in 1.5% normal goat serum (Vector Labs, Peterborough, UK) in PBS for 30 minutes at room temperature, followed by primary antibody diluted in 1.5% normal goat serum (Vector Labs) overnight at 4°C. Detection was by Alexa 568 conjugated goat anti-mouse IgG antibody (Invitrogen, Renfrewshire, UK). Cells were counter-stained in 4', 6-diamidino-2-phenylindole (DAPI) (Invitrogen). A total of 400 cells per slide were examined and identified as positive or negative for apoptosis. For dual-label images, fluorescent images from the same field were captured and digitally merged. Digital micrographs were obtained through the use of a Nikon Eclipse 80i microscope (Nikon UK Ltd, Surrey, UK) equipped with a QImagining digital camera (Media cybernetics, Marlow, UK) and ImagePro Plus 5.1 software (Media Cybernetics).

2.6 Immunohistochemistry

2.6.1 Tissue processing

Lung tissue was obtained from subjects undergoing surgery for suspected or confirmed lung cancer. Tissue was taken as far distally from the tumour as possible. Tissue was then fixed for 48 hours in 10% neutral buffered formalin before being embedded in paraffin.

Lung tissue was cut into 4µm sections using a Leica monotome. The cut sections were floated on water at 37°C and lifted on to a polysine coated glass slide (Leica

Microsystems, Buckinghamshire, UK). Slides were then dried overnight at 45°C and subsequently stored at room temperature prior to immunohistochemical staining.

2.6.2 Haematoxylin and eosin staining

Tissue sections were de-waxed in xylene for 2 x 5 minutes, then hydrated in decreasing alcohol concentrations; 100% for 3 minutes, 90% for 3 minutes, 75% for 2 minutes and 50% for 1 minute followed by 5 minutes in distilled water. Sections were placed in Mayer's Haematoxylin (Sigma Aldrich) for 15 minutes followed by washing in tap water. Tissue was then counterstained in eosin (Sigma Aldrich) for 2 minutes. Sections were then placed back in running water for 5 minutes prior to de-hydration through alcohol; 50% for 1 minute, 75% for 2 minutes, 90% for 3 minutes and 100% for 3 minutes. After a final 2 x 5 minutes in xylene sections were mounted in DPX mountant and stored at room temperature prior to analysis.

Tissue sections were selected from a large database of formalin fixed, paraffin embedded (FFPE) lung tissue held at the Translational Research Facility at the University Hospital of South Manchester. Tissue sections stained for haematoxylin and eosin for each demography were selected if they contained at least one inflammatory follicle, in order to enable the detection of phospho-p38 MAPK in lymphocyte follicles.

2.6.3 Phosphorylated p38 MAPK immunohistochemical analysis

Tissue sections were de-waxed in xylene for 2 x 5 minutes, then hydrated in decreasing alcohol concentrations; 100% for 3 minutes, 90% for 3 minutes, 75% for 2 minutes and 50% for 1 minute followed by 5 minutes in distilled water. Heat induced epitope retrieval (HIER) was then performed by microwaving slides in 0.01M

citrate buffer pH 6 for 20 minutes at 800W. Sections were then left to cool before being placed in running water for 5 minutes. After incubation in 1.5% normal goat serum (Vector Labs) in tris-buffered saline (TBS) for 30 minutes at room temperature, sections were then incubated with phospho-p38 MAPK antibody (New England Biolabs, Hertfordshire, UK) diluted at 1 in 100 in 1.5% goat serum in TBS overnight at 4°C. Sections were washed in TBS containing 0.1% tween (TBST) for 3 x 3 minutes. Endogenous peroxidises were quenched using 3% H_20_2 in methanol for 30 minutes at room temperature. After being placed in running water for 5 minutes, secondary antibody was applied at a concentration of 1 in 200 in TBS using goat anti-rabbit immunoglobulin (IgG) secondary antibody (Vector Labs) for 30 minutes at room temperature. After washing in TBST for 3 x 3 minutes sections were incubated in Avidin Biotinylated enzyme complex (ABC; Vector Labs) for 30 minutes at room temperature followed by washing in TBST for 3 x 3 minutes. Finally, sections were incubated in 3'3 Diaminobenzadine (DAB; Invitrogen) substrate. The amount of chromagen development was determined microscopically and the reaction stopped by placing tissue into water. Sections were then placed in running water for 5 minutes followed by counterstaining in haematoxylin (Sigma Aldrich) for 1 minute. Sections were then placed back in running water for 5 minutes prior to de-hydration through alcohol; 50% for 1 minute, 75% for 2 minutes, 90% for 3 minutes and 100% for 3 minutes. After a final 2 x 5 minutes in xylene sections were mounted in DPX mountant and stored at room temperature prior to analysis.

2.6.4 Dual label phosphorylated p38 MAPK immunofluorescent analysis

Tissue sections were dewaxed, rehydrated, HIER-treated and incubated in phospho-p38 MAPK antibody as described above. Detection of phospho-p38 MAPK was by

Alexa 488 conjugated goat anti rabbit (Invitrogen) for 90 minutes at 37°C at a concentration of 1 in 200 in TBS. Sections were then incubated with a number of cell specific markers including mouse monoclonal anti-human CD20 (Vector Labs) for detection of B lymphocytes, CD8 (clone C8/144B; Dako, Cambridgeshire, UK) for the detection of CD8+ T lymphocytes and CD4 (clone 4B12, Dako) for the detection of CD4+ T lymphocytes. All primary antibodies were diluted 1:50 in TBS. Incubation was at 4°C overnight. After washing 3 x 3 minutes in TBST, detection of the cell specific markers was by Alexa 568 conjugated goat anti-mouse (Invitrogen) diluted 1 in 200 in TBS for 90 minutes at 37°C. Finally, sections were incubated in DAPI, (1 in 50,000 in TBS), for 5 minutes in the dark. Sections were mounted using immunofluorescent mounting medium (Invitrogen) and stored in the dark at 4°C until analysis.

NE (clone NP57; Dako) was also dual labelled with phospho-p38 MAPK to allow the identification of neutrophils expressing phospho-p38 MAPK. As this antibody did not require HIER, sections were treated as above but first incubated with NE primary antibody overnight, followed by detection with Alexa 468 as above. Sections then underwent HIER, followed by incubation with phospho-p38 MAPK antibody and detection with Alexa 488 and DAPI as described above.

2.6.5 Phosphorylated p38 MAPK immunocytochemical analysis

Cytospins to be stained for phospho-p38 MAPK were fixed in 4% paraformaldehyde at room temperature for 10 minutes. After washing 3 x 3 minutes in PBS, cells were incubated in 1.5% normal goat serum (Vector Labs) in PBS for 30 minutes at room temperature followed by incubation in phospho-p38 MAPK primary antibody (Cell Signalling) diluted in PBS overnight at 4°C. Cells were then washed 3 x 3 minutes in

PBS. Endogenous peroxidises were quenched using 3% H_20_2 in methanol for 30 minutes at room temperature followed by running water for 5 minutes. Phospho-p38 MAPK was detected using goat anti-rabbit immunoglobulin (IgG) secondary antibody (Vector Labs) at a concentration of 1 in 200 in PBS for 30 minutes at room temperature. After washing in PBS for 3 x 3 minutes sections were incubated in ABC (Vector Labs) for 30 minutes at room temperature followed by washing in PBS for 3 x 3 minutes. Bound antibody was detected by incubating sections in 3'3 DAB substrate. The amount of chromagen development was determined microscopically. Sections were then placed in running water for 5 minutes followed by counterstaining in haematoxylin (Sigma Aldrich) for 1 minute. Sections were placed back in running water for 5 minutes prior to dehydration through alcohol; 50% for 1 minute, 75% for 2 minutes, 90% for 3 minutes and 100% for 3 minutes. After a final 2 x 5 minutes in xylene sections were mounted in DPX mountant and cover slips and stored at room temperature prior to analysis.

2.6.6 Analysis of phosphorylated p38 MAPK expression

2.6.6.1 Lung tissue

For each patient 300 macrophages, identified by their morphology, in consecutive fields of vision, were analysed for phospho-p38 MAPK expression. Small airway epithelial expression of phospho-p38 MAPK was also quantified by counting total numbers of small airway epithelial cells per small airway per patient.

For dual label phospho-p38 MAPK expression total numbers of CD20+, CD8+ and CD4+ cells within all inflammatory follicles were counted and the percentage expressing phospho-p38 MAPK was calculated. CD20+, CD8+ and CD4+

expression of phospho-p38 MAPK was also quantified in the sub-epithelial layer of small airways by counting total numbers of lymphocytes in all small airway sub-epithelia. Due to the low numbers of neutrophils present in the lung tissue, total numbers of neutrophils were counted per patient and percentage expression of phospho-p38 MAPK calculated.

2.6.6.2 Cytospins

A total of 400 sputum cells per cytoslide were counted and analysed for phospho-p38 MAPK expression.

2.6.6.3 Image analysis

Digital micrographs were obtained using a Nikon Eclipse 80i microscope equipped with a QImaging digital camera and ImagePro Plus 5.1 software. Quantification of individual cell counts, follicle size, and length of airway luminal perimeter was carried out using the ImagePro Plus 5.1 software. Cell counts were calculated and standardised to the number of positive cells per mm2of the area of interest (follicle, epithelia, sub-epithelia).

2.7 16HBE cell culture

16HBE, an immortalised normal bronchial epithelial cell line, were maintained in minimum essential medium (MEM; Sigma Aldrich) supplemented with 10% fetal calf serum (Fisher), 1mM/L L-glutamine (Fisher) and 1% penicillin/streptomycin (Sigma Aldrich). Cells were grown to confluence in a 75cm^2 flask and were maintained in 5% CO_2 at 37°C. When the cells were confluent the media was removed and the cells were washed twice in PBS before being incubated with trypsin-

ethylenediaminetetracetic acid (EDTA; Sigma Aldrich) to break the hydrogen bonds adhering the cells to the flask. After 5 minutes the cells were placed in supplemented media and centrifuged at 400g for 10 minutes at 4°C. The cells were then resuspended in fresh supplemented MEM, a cell count was obtained as described earlier, and the cells were sub-cultured at a ratio of 1:10. Cells for experiments were resuspended in supplemented media at a concentration of 1 x 10^6/ml.

2.7.1 Cytokine release assays

25µl of cell suspension (25,000 cells per well) was added to a 96 well flat-bottomed plate and cells left to adhere on day 1. On day 4, cells were washed twice with supplemented MEM, before being treated with dexamethasone (final concentrations 0.1–1000nM; Sigma Aldrich) and/or the p38 MAPK inhibitor birb 796 (final concentrations 0.1–1000nM; Stratech Scientific, Surrey, UK). After 2 hours the cells were then stimulated with LPS (final concentration 1µg/ml; Sigma Aldrich), TNFα (final concentration 10ng/ml; Peprotech, London, UK) or the toll like receptor 3 ligand (TLR3) Poly IC (final concentration 100µg/ml; Source Bioscience, Nottingham, UK) and incubated for 24 hours, 5% CO_2 at 37°C. Plates were centrifuged at 2000rpm for 10 minutes at 4°C and supernatants removed and stored at -80°C for cytokine analysis. For each experiment, all conditions were run in triplicate and each experiment was repeated a minimum of 4 times with different cell passages.

2.7.2 Protein expression analysis

On day 1 400µl of cell suspension (400,000 cells per well) was added to a 6 well plate and left to adhere. On day 3 cells were washed twice in PBS and serum starved overnight prior to experiments on day 4. Cells were placed in fresh serum

free media before stimulation with either LPS (final concentration 1µg/ml; Sigma Aldrich), TNFα (final concentration 10ng/ml; Peprotech, London, UK) or Poly IC (final concentration 100µg/ml; Source Bioscience). For some experiments cells were pre-incubated with dexamethasone (final concentrations 0.1–1000nM; Sigma Aldrich) and/or the p38 MAPK inhibitor birb 796 (final concentrations 0.1–1000nM; Stratech) prior to stimulation. The cells were then lysed in RIPA buffer (10mM Tris-HCL, PH 7.4, 150mM NaCl, 1mM EDTA, 1%Nonidet P-40, 0.25%) containing phosphatase (Sigma Aldrich) and protease (Calbiochem, Nottinghamshire, UK) inhibitors. Cell lysates were stored at -80°C until analysis. Each experiment was repeated a minimum of 3 times with different cell passages.

2.7.3 mRNA stability assay

On day 1, 100µl of cell suspension (100,000 cells per well) was added to a 24 well plate and left to adhere. On day 3 cells were washed twice in PBS and serum starved overnight prior to experiments on day 4. Cells were placed in fresh serum free media before stimulation with either LPS (final concentration 1µg/ml; Sigma Aldrich), TNFα (final concentration 10ng/ml; Peprotech) or Poly IC (final concentration 100µg/ml; Source BioScience) for 4 hours. Dexamethasone (final concentrations 1000nM; Sigma Aldrich) and/or the p38 MAPK inhibitor birb 796 (final concentrations 1000nM; Stratech) along with the transcriptional inhibitor actinomycin D (final concentration 5µg/ml; Sigma Aldrich) were then added to appropriate wells. At set time points (0.25, 0.5, 1, 2, 3 and 4 hours), supernatants were harvested and stored at -80°C for cytokine release analysis and cells were harvested in Trizol and stored at -80°C for future PCR analysis. Each experiment was repeated a minimum of 4 times with different cell passages.

2.7.4 Glucocorticoid receptor translocation assay

On day 1 25µl of cell suspension (25,000 cells per well) were added to a chamber slide (Fisher). On day 3 cells were washed twice in PBS and serum starved overnight prior to experiments on day 4. Cells were placed in fresh serum free media before stimulation with either LPS (final concentration 1µg/ml; Sigma Aldrich), TNFα (final concentration 10ng/ml; Peprotech, London, UK) or Poly IC (final concentration 100µg/ml; Source Bioscience). For some experiments cells were pre-incubated with dexamethasone (final concentrations 0.1–1000nM; Sigma Aldrich) and/or the p38 MAPK inhibitor birb 796 (final concentrations 0.1–1000nM; Stratech) prior to stimulation. At the end of the experiment, cells were washed in PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Immunofluorescence was then used to detect GR translocation. Cells were washed 3 x 3 minutes in PBS followed by incubation in 1.5% normal goat serum (Vector Labs) in PBS. Incubation in primary antibody; purified mouse anti-GR (clone 41; BD Transduction Laboratories) diluted 1:200 in PBS, followed and incubation overnight at 4°C occurred. After washing 3 x 3 minutes in PBS cells were then incubated in Alexa 568 conjugated goat anti-mouse antibody (Invitrogen) 1 in 200 in PBS for 90 minutes at 37°C. Finally cells were incubated in DAPI, 1 in 50,000 in TBS, for 5 minutes in the dark. Sections were mounted using immunofluorescent mounting medium (Invitrogen) and stored in the dark at 4°C until analysis. Each experiment was repeated a minimum of 4 times with different cell passages.

2.7.5 Glucocorticoid receptor translocation analysis

Bronchial epithelial cells were analysed by assessing the location of the GR. Location was classed as either being all cytoplasmic, cytoplasmic and nuclear; where some cellular GR was found to be present in the nucleus but there was also some present in the cytoplasm or all nuclear, whereby there was no longer any GR present in the cytoplasm of a particular cell. 400 cells were counted per condition.

Digital micrographs were obtained using a Nikon Eclipse 80i microscope equipped with a QImagining digital camera and ImagePro Plus 5.1 software.

2.8 Western Blotting

2.8.1 Bradford Assay

Protein concentrations from whole cell extracts were determined using Bradford Reagent (Sigma Aldrich). Serial dilutions of bovine serum albumin (concentrations 5, 2.5, 1.25, 0.625, 0.3125µg/ml and blank) were used as standards and added at 5µl per well in duplicate to a 96 well flat bottomed plate. Protein samples were added to the plates in triplicate at 5µl per well. Bradford reagent was added neat at 300µl per well to samples and standards and then left to incubate for 20 minutes at room temperature. The plate was read at 600nm wavelength using a microplate reader. Using the optical densities of the standards, a graph of optical density against protein concentration was plotted and extrapolated to calculate the protein concentration of each sample.

2.8.2 Polyacrylamide gel electrophoresis

Cell lysates were diluted 1 in 3 in sample buffer (62.5mM Tris, 10% glycerol, 1% SDS, 1% beta-mercaptoethanol, and 0.1% bromphenol blue, pH 6.8). Samples were

boiled for 10 minutes prior to electrophoresis on SDS-polyacrylamide gels (10%; detailed in **Tables 2.1** and **2.2**). Proteins were then transferred to Hy-Bond ECL membranes (Whatman, Kent, UK).

Table 2.1 Reagent details to make up 10% SDS polyacrylamide separating and stacking gels

10% SDS polyacrylamide gel reagents	
Separating gel	18ml distilled H20 9.375ml buffer B (details below) 9.375ml acrylamide bis solution (Sigma Aldrich) 375µl 10% APS (Sigma Aldrich) in H20 37.5µl TEMED (Sigma Aldrich)
Stacking gel	10ml distilled H20 3.75ml buffer A (details below) 1.13ml acrylamide bis solution 150µl 10% APS 15µl TEMED

Table 2.2 Reagent details to make up buffer A and buffer B in 10% SDS polyacrylamide separating and stacking gels

Reagents for buffers in 10% polyacrylamide gel			
Buffer A	0.53M Trizma base (Sigma Aldrich), 0.01M SDS, (Sigma Aldrich) pH 6.8	Buffer B	1.56M Trizma base, 0.01M SDS, pH 8.8

Non-specific binding sites were blocked by incubating membranes in blocking buffer as described in **Table 2.3**. Membranes were then incubated in primary antibody diluted in blocking buffer on a continuous roller overnight at 4°C. Primary antibodies used include phospho-p38 MAPK (Cell Signalling), total p38 MAPK (Cell Signalling), phosphorylated glucocorticoid receptor at 203 (Abcam, Cambridgeshire, UK), phosphorylated glucocorticoid receptor at serine 211 (Cell Signalling), phosphorylated glucocorticoid receptor at serine 226 (Abcam), and beta actin (Abcam). After washing, the membranes were incubated with a peroxidise-conjugated secondary antibody for 60 minutes at room temperature. After washing the membranes were then incubated in lumigen TMA-6 enhanced chemiluminescent (Bioquote, Yorkshire, UK) to allow detection of horseradish peroxides conjugated secondary antibody. Densitometric analysis was performed by normalising band density to that for total antibody using Quantity One v4.6.1 software (Bio-Rad, Hertfordshire, UK).

Table 2.3 Individual antibody dilutions and buffers for western blot protocol

Western blotting protocol for each antibody used			
	Block	**Primary antibody**	**Secondary antibody**
Phospho-p38 MAPK (cell signalling)	5% milk in TBSt 1h at RT	1:1000 in block buffer overnight at 4°C	Anti-Rabbit HRP 1:1000 in TBSt
p38 MAPK (cell signalling)	5% milk in TBSt 1h at RT	1:1000 in block buffer overnight at 4°	Anti-Rabbit HRP IgG 1:1000 in TBSt for 1h at RT
Phospho-GR serine 203 (Ab cam)	0.5% milk, 0.08M NaCl, 0.01% tween, for 4h at RT	1:1000 in block buffer overnight at 4°	Rabbit true-blot: anti rabbit IgG HRP 1:1000 for 1 h at RT
Phospho-GR serine 211 (cell signalling)	0.5% milk, 0.08M NaCl, 0.01% tween, for 4h at RT	1:1000 in block buffer overnight at 4°	Rabbit true-blot: anti rabbit IgG HRP 1:1000 for 1 h at RT
Phospho-GR serine 226 (Abcam)	0.5% milk, 0.08M NaCl, 0.01% tween, for 4h at RT	1:1000 in block buffer overnight at 4°	Rabbit true-blot: anti rabbit IgG HRP 1:1000 for 1 h at RT
Bet actin (Abcam)	0.5% milk, 0.08M NaCl, 0.01% tween, for 4h at RT	1:1000 in block buffer overnight at 4°	Rabbit true-blot: anti rabbit IgG HRP 1:1000 for 1 h at RT

HRP, horse raddish peroxidase; RT, room temperature; TBSt, tris-buffered saline with tween

2.8.3 Membrane stripping and re-probing

For some experiments, membranes were stripped and re-probed with alternative primary antibodies. Membranes were incubated in stripping buffer (62.5mM Tris-HCL (pH 6.8), 2% SDS, 10mM 2-beta-mercaptoethanol) for 40 minutes at 60°C. Membranes were then washed in distilled water and the protocol continued as described earlier from the blocking step. To check that the membranes were stripped of both primary and secondary antibodies, after blocking some membranes were incubated with lumigen TMA-6 enhanced chemiluminescent to ensure the secondary antibody had been stripped from the membrane, or by incubating in the corresponding secondary antibody followed by detection all as described earlier to check the primary antibody had also been stripped from the membrane. This was done on a number of membranes in order to optimise the stripping buffer incubation time.

2.9 Polymerase Chain Reaction

2.9.1 RNA extraction

16HBEs were harvested for total RNA isolation. Commercially available kits were used to extract total RNA (RNeasy Mini Kit, Qiagen, Sussex, UK). Briefly, cells harvested in trizol were homogenised by passing the lysate through a 21G needle and 1ml syringe and mixed 5:1 with chloroform. Samples were then left to stand at room temperature for 5 minutes before being centrifuged at 12000g for 15 minutes at 18°C. The upper phase was carefully removed and placed into fresh sterile eppendorfs, followed by the addition of 70% ethanol at a dilution of 1:1. The homogenised sample was then placed in to an RNeasy spin column in a 1.5ml

collection tube and centrifuged at 12000g for 15 seconds. The flow through was discarded and the sample washed in buffer RW1 before being centrifuged again and flow through discarded. 80µl DNase 1 (1:7 in RDD buffer) was then added to the sample and left to incubate for 15 minutes at room temperature. The sample was then washed in RW1 buffer, centrifuged and the flow through discarded before being washed twice in RPE buffer and again centrifuged and flow through discarded. Finally the spin column was transferred to a fresh tube and RNase free water was added to the spin column, followed by centrifugation for 2 minutes, eluting the RNA sample. The concentration of RNA was measured with a spectrophotometer at 260nm wavelength, with samples being diluted 1:50 in RNase free water. The RNA sample was then stored at -80°C until further analysis.

2.9.2 Reverse Transcription

To synthesise cDNA from whole RNA reverse transcription was performed on 50ng of RNA from 16HBEs using TaqMan reverse transcription PCR with the Verso-2-step QRT-PCR kit (Thermo-Scientific, Surrey, UK). Briefly, extracted RNA (5µl) was incubated with 5x cDNA synthesis buffer (4µl), dNTP mix (2µl), random hexamer primers (1µl), RT Enhancer (1µl) Verso enzyme mix (1µl) and RNAse free water (6µl) at 37°C for 1 hour. The reaction was terminated at 93°C for 5 minutes; cDNA was synthesised and subsequently used for quantitative polymerase chain reaction (qPCR).

2.9.3 Real time quantitative polymerase chain reaction (qPCR)

Gene transcript level of CXCL8 and the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time PCR using

ABsolute blue qPCR mix (Thermo Scientific) on a Stratagene MX3005P (Agilent Technologies, West Lothian, UK). Premade ABI Taqman gene expression assays for CXCL8 and GAPDH were purchased from Thermo Scientific as per the manufacturer's instructions. Briefly, previously prepared CDNA (1µl) was mixed with primer probe (0.5µl) and qPCR mix (12.5µl) and RNase free water (11µl) for GAPDH analysis and cDNA (1µl) was mixed with primer probe (0.3µl) and qPCR mix (12.5µl) and RNase free water (11.2µl) for CXCL8 analysis. The PCR cycle was as follows; 50°C for 2 minutes, 95°C for 10 minutes then 40 to 50 cycles of denaturing at 95°C for 15 seconds and 60°C for 60 seconds for annealing and extension. Each transcript was analysed by delta (Δ) CT method and variations in cDNA concentrations between different samples were corrected using GAPDH. Controls without RT-enzyme showed there was no genomic DNA amplification.

Primers used:

CXCL8 reference code: Hs00174103_m1

GAPDH reference code: Hs02758991_g1

2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

IL-6, CXCL8, TNFα and RANTES levels in cell supernatants were measured using a kit-based sandwich ELISA assay (R and D Systems, Abingdon, UK) using a standard curve to obtain concentrations from OD values. 50µl of purified capture antibody was used to coat an Immunolon IV flat bottomed 96 well ELISA plate and incubated overnight at room temperature. Unbound capture antibody was removed

by washing the plates in PBS containing 0.1% tween 20 (PBST). 150µl of 1% bovine serum albumin (BSA; Sigma Aldrich) in PBS was then added to each well to block any non-specific binding sites and incubated at room temperature for at least 1 hour. Plates were then washed in PBST. Control blanks of media, standards of known recombinant cytokine concentrations (see **Table 2.4** for details, serially diluted in complete medium through 8 dilutions) and samples at various dilutions were then added in duplicates/triplicates at 50µl per well. These were then incubated at room temperature for 2 hours. The plates were then washed 3 times in PBST to remove any excess cytokine not bound to capture antibody. 50µl of secondary biotinylated detection antibody was then added to each well and incubated for 2 hours at room temperature. Streptavidin-conjugated horseradish peroxidise enzyme was then added at 50µl per well for 20 minutes at room temperature. Any unbound enzyme-antibody was then removed by 3 washes in PBST. Substrate solution, consisting of a 1:1 mixture of colour reagent A (H_2O_2) and colour reagent B (tetramethylbenzidine), was then added at 50µl per well and plates were left to develop in the dark. After development, 25µl of Stop solution (2N H_2SO_4, Sigma Aldrich) was used to terminate the reaction. The plates were then read using a microplate reader at 450nm.

Table 2.4 ELISA protocol details

	IL-6	CXCL8	TNFα	RANTES
Capture antibody	1:180 in PBS	1:180 in PBS	1:180 in PBS	1:360 in PBS
Standard concentration range (pg/ml)	600-9.375	2000-31.25	1000-15.65	1000-15.65
Detection antibody	1:180 in 1%BSA in PBS	1:180 in 0.1%BSA in TBS	1:180 in 1%BSA in PBS	1:360 in 1%BSA in PBS
Streptavidin HRP	1:200 in 1% BSA in PBS	1:200 in 0.1% BSA in TBS	1:200 in 1% BSA in PBS	1:200 in 1% BSA in PBS
Lower limits of detection (pg/ml)	9.375	31.25	15.65	15.65

BSA, bovine serum albumin; HRP, horse raddish peroxidase; IL, interleukin; PBS, phosphate buffered saline; RANTES, regulated upon activation, normal t cell expressed and secreted; TBS, tris-buffered saline; TNFα, tumour necrosis factor alpha

2.11 Statistical analysis

Details of statistical analyses used are described in each chapter. All analyses were carried out using GraphPad InStat software version 3.06 (GraphPad Software, Inc., San Diego, CA, USA.).

Chapter 3

The glucocorticoid sensitivity of airway cells

3.1 Introduction

COPD is a progressive disease characterised by persistent airway inflammation (Barnes, 2000). The lungs of patients with COPD have increased numbers of macrophages (Hogg et al, 2004), and neutrophils (Lacoste et al, 1993; Keatings et al, 1996; Keatings and Barnes, 1997) compared with control lungs. These cells form part of the innate immune system, providing the first line of defence against invading pathogens. Neutrophils and macrophages produce a range of pro-inflammatory mediators and tissue destructive proteases, which are implicated in the pathogenesis of COPD. Neutrophils are particularly important as sputum neutrophil counts and levels of the neutrophil chemoattractant CXCL8 in sputum supernatants correlate with disease progression (Quint and Wedzicha, 2007; Profita et al, 2005; Traves et al, 2002). In patients with COPD, the innate immune response appears to be amplified and persistently activated, thus contributing to increased airway inflammation.

GC are the most commonly used anti-inflammatory therapy in COPD but they offer limited clinical benefits (Soriano et al, 2007). GC bind to GRα, forming a complex that inhibits activity by two mechanisms of action; 1. transactivation whereby anti-inflammatory gene expression is induced, and 2. transrepression, whereby pro-inflammatory transcription factors are repressed causing reduced expression of pro-inflammatory genes.

Cytokine production from COPD alveolar macrophages *ex vivo* is reported to be GC insensitive (Culpitt et al, 2003; Cosio et al, 2004). More recent data, however, suggests GC insensitivity is a cytokine- and cell-specific phenomenon present in

alveolar macrophages regardless of disease or smoking status (Hew et al, 2006; Bhavsar et al, 2008; Armstrong et al, 2009; Kent et al, 2009). Airway neutrophils isolated from induced sputum have been shown to be resistant to GC in patients with cystic fibrosis (Corvol et al, 2003) and bronchial sepsis (Pang et al, 1997). The GC sensitivity of sputum cells from patients with COPD in comparison with control cells is relatively unexplored. In addition, neutrophils are thought to be the primary effector cell in COPD, and as such represent an important target with which to direct anti-inflammatory therapies. There are limited studies investigating the phenotype of isolated sputum neutrophils from patients with COPD.

Increased levels of pro-inflammatory mediators such as TNFα, CXCL8 and GM-CSF have been reported within the sputum supernatants from patients with COPD (Keatings et al, 1996; Profita et al, 2003), however little is understood about the cellular inflammatory properties of the mixed sputum cells. Sputum induction is a commonly used non-invasive method of sampling cells from the upper airways, including macrophages and neutrophils, and sputum cell culture provides a suitable model to investigate *ex vivo* cytokine production from these cells (Quaedvlieg et al, 2005; Profita et al, 2003). Previously, mixed sputum cell culture studies have shown the spontaneous release of pro-inflammatory cytokines, for example, IL-4, IL-6, IL-10, TNFα and IFNγ, in samples from asthmatic cells (Quaedvlieg et al, 2005), TNFα, CXCL8 and GM-CSF from COPD cells (Profita et al, 2003), and IL-4, IL-6, IL-10, TNFα, IFNγ, CXCL8 and GM-CSF from smoking and non-smoking control subjects (Profita et al, 2003; Quaedvlieg et al, 2005). Increased spontaneous production of TNFα and CXCL8 has been reported in mixed sputum cultures from patients with COPD compared with control subjects (Profita et al., 2003). Unlike the *ex vivo*

response of alveolar macrophages (Armstrong et al, 2009; Kane et al, 2009) stimulation of mixed sputum cells with LPS (1ng/ml and 10ng/ml) has been shown to have no effect on pro-inflammatory cytokine production (Dentener et al, 2006), suggesting a differential inflammatory signalling mechanism between cells from the upper and lower airways.

The GC flunisolide has been shown to decrease production of the MMP-9 and the special class of tissue inhibitors of MMPs (TIMP-) 1 in asthmatic mixed sputum cultures over 24 hours (Profita et al, 2004). The only study investigating the effect of GC on COPD mixed sputum cultures demonstrated a modest inhibition (40%) of IL-6 from either healthy or asthmatic mixed sputum cells at the highest concentration of prednisolone (100ng/ml), suggesting a degree of GC insensitivity within this mixed cell culture model (Manise et al, 2010). However, the nature of GC sensitivity has not yet been fully described.

Sputum culture provides a physiologically relevant *in vitro* model using the combination of cell types that are present in the upper airways. Therefore, the aim of this study was to fully characterise mixed sputum cell culture *in vitro* by analysing the spontaneous and LPS stimulated cytokine response from airway cells over time. The effect of the GC dexamethasone was evaluated using mixed sputum cells from patients with COPD, as well as smoking and non-smoking control subjects. The effects of GC were also investigated on isolated sputum and blood neutrophils and on sputum macrophages in order to fully understand the inflammatory profile of individual airway cells involved in COPD.

3.2 Methods

3.2.1 Subjects

11 patients with COPD, 10 S and five NS control subjects with normal lung function were recruited. For isolated neutrophil and macrophage cultures, seven of the patients with COPD were used. See **Table 3.1** for details of demography. Patients with COPD were classified as defined by GOLD guidelines, and S had a greater than 10 pack year smoking history and normal pulmonary function. Patients gave written informed consent. The study was approved by the local research ethics committee.

Table 3.1 Patient demographics

	COPD (N=11)	S (N=10)	NS (N=5)
Sex	9M 2F	3M 7F	4M 1F
Age	70.73 (7.3)	53.60 (5.60)	48.6 (17.53)
Smoking history (Pack Years)	40.82 (9.99)	29.70 (6.57)	0
FEV 1	1.17 (0.32)	3.04 (0.64)	4.00 (1.20)
FEV 1% predicted	44.83 (13.88)	99.02 (7.95)	110.93 (13.07)
FEV/FVC	46.04 (10.93)	76.85 (5.37)	76.88 (5.28)
Number on ICS	7	0	0

Demographics of patients used in the mixed sputum cell culture assays: spirometry, smoking history and GC use of COPD, smokers (S) and non-smokers (NS). All data are normally distributed and presented as mean $\pm$ standard deviation. FEV 1: forced expiratory volume in 1 second; FVC: forced vital capacity. ICS: inhaled corticosteroid

3.2.2 Sputum induction

Sputum was induced and processed to obtain a cell pellet. Supplemented RMPI media was added to the pellet and the cells resuspended. Cell counts and cell

viability were established using the trypan blue exclusion method in a Neubauer haemocytometer. Cytospins were prepared, air dried, fixed in methanol and stained with Rapi-diff for differential cell counts. Cell counts are reported as cells x 10^6.

3.2.3 Sputum Cell culture

Sputum cells were adjusted to a concentration of 1 x 10^6/ml using supplemented RPMI. Cells were cultured at a concentration of 1 x 10^5 cells per well in a 96 well plate for up to 24 hours at 37°C in humidified 5% CO_2 in the presence or absence of LPS (final concentrations 0.1–10,000ng/ml) and/or dexamethasone (final concentrations; 0.1–1000nM). After incubation, plates were centrifuged at 400g for 10 minutes at 4°C, and cell-free supernatants were removed and stored at -80°C for future cytokine analysis.

3.2.4 Isolation of neutrophils

3.2.4.1 Sputum neutrophils

Mixed sputum cells were added to 24 well plates at a volume of 4x10^5 cells per well and incubated for 2 hours to enable the macrophages to adhere to the wells. The non-adherent cells were then removed, centrifuged, resuspended in supplemented media and cell counts obtained. Cells (89.4% neutrophil purity as determined by cell counts using RapiDiff) were then added to a fresh 24 well plate at 4x10^5 cells per well and cultured for 24 at 37°C in humidified 5% CO_2 in the presence or absence of LPS (final concentrations 0.1–10,000ng/ml) and/ or dexamethasone (0.1–1000nM).

After incubation, plates were centrifuged at 400g for 10 minutes at 4°C, and cell-free supernatants were removed and stored at -80°C for future cytokine analysis.

3.2.4.2 Blood neutrophils

PMNs were isolated from whole blood using mono-poly resolving medium. $4x10^5$ cells per well were cultured for 24h at 37°C in humidified 5% CO_2 in the presence or absence of LPS (final concentrations 0.1–10,000ng/ml) and/ or dexamethasone (final concentrations; 0.1–1000nM). After incubation, plates were centrifuged at 400g for 10 minutes at 4°C, and cell-free supernatants were removed and stored at -80°C for future cytokine analysis.

3.2.5 Isolated sputum macrophages

The adherent cells left after the removal of neutrophils were also cultured for 24 hours at 37°C in humidified 5% CO_2 in the presence or absence of LPS (final concentrations 0.1–10000ng/ml) and/ or dexamethasone (final concentrations; 0.1–10,00nM). After incubation, plates were centrifuged at 400g for 10 minutes at 4°C, and cell-free supernatants were removed and stored at -80°C for future cytokine analysis.

3.2.6 Cytokine release analysis

ELISA was used to determine the supernatant level of TNFα and CXCL8, according to the manufacturer's instructions. Lower limits of detection were 32.5pg/ml for CXCL8 and 15.63pg/ml for TNFα.

3.2.7 Apoptosis analysis

3.2.7.1 Morphological analysis

At 0 and 24 hours, cell viability was assessed using trypan blue exclusion. Cytospins were also prepared as described earlier. Apoptosis was assessed in Rapi-diff stained cytospins as described previously (Pletz et al, 2004) by examining the disappearance of chromatin bridges between nuclear lobes (early apoptosis) and shrinkage or fragmentation of the nucleus (late apoptosis). The percentage of apoptotic neutrophils was assessed by counting a total of 400 neutrophils per cytospin.

3.2.7.2 Tunel assay

The tunel assay was also used to assess levels of apoptosis in mixed sputum cultures. Cytospins were prepared at 0 and 24 hours, air dried then fixed in 4% paraformaldehyde. The tunel assay was used as per the manufacturer's instructions. To enable identification of neutrophils in the mixed sputum cytospins dual labelling

with tunel and a mouse anti-human NE was carried out. Cytospins were incubated in primary antibody diluted in 1.5% normal serum overnight at 4°C. NE was detected by Alexa 568 conjugated goat anti-mouse IgG antibody. Cells were counter-stained in DAPI. For dual-label images, fluorescent images from the same field were captured and digitally merged. Digital micrographs were obtained through the use of a Nikon Eclipse 80i microscope equipped with a QImagining digital camera and ImagePro Plus 5.1 software.

3.2.8 Statistics

Sputum differential cell counts were compared between groups using (ANOVA) for normally distributed data and Kruskall Wallis for non-parametric data. Absolute levels of cytokine and per cent inhibition at each dexamethasone concentration were normally distributed and compared using ANOVA followed by bonferroni's post-test for mixed sputum culture, isolated macrophage and isolated neutrophil pro-inflammatory mediator release. Per cent inhibitions of TNFα and CXCL8 at each dexamethasone concentration were compared between sputum neutrophil and PMN (stimulated and unstimulated) using ANOVA followed by bonferroni's post-test. Numbers of normal, early and late apoptotic neutrophils were also normally distributed and compared at 0 and 24 hours and between control and dexamethasone treated cells using ANOVA followed by Tukey Kramer's post-test. All statistical analysis was performed using Graphpad InStat version 3.0 for Windows 95.

3.3 Results

3.31 Mixed sputum culture

3.3.1.1 Differential cell counts

Differential cell counts for the mixed sputum cell culture are shown in **Table 3.2**. Total sputum cell count and sputum neutrophil percentages were significantly higher in COPD compared with S and NS ($p<0.001$). The percentage of sputum macrophages was significantly lower in COPD samples compared with S and NS ($p<0.001$). There were no statistical differences in eosinophil, lymphocyte or squamous cell numbers between the three patient groups.

Table 3.2 Sputum differential cell counts

	COPD	S	NS
Total cell count	17.96 (9.55)**	3.23 (1.21)	2.06 (0.63)
Total neutrophil count	12.67 (6.43)**	1.10 (0.45)	0.46 (0.47)
Neutrophil %	71.20 (5.18)	34.21 (8.28)	19.47 (12.71)
Total macrophage	3.73 (2.10)**	2.07 (0.86)	1.44 (0.22)
Macrophage %	21.22 (5.35)	64.03 (8.24)	72.82 (11.71)
Eosinophil %	2.24 (1.50)	0.53 (0.78)	0.42 (0.37)
lymphocyte %	0.76 (0.7–2.1)	0.1 (0–1.1)	0.1 (0.1–1.5)
squamous %	4.17 (2.35)	1.1 (1.08)	5.98 (1.44)

Normal data are presented as mean ± SD. Non parametric data are presented as median (range). Data was compared between all groups ANOVA and Kruskall Wallis. ** $p<0.01$ COPD vs S and COPD vs NS

3.3.1.2 Pro-inflammatory mediator production from mixed sputum cells

The time course and dose-dependent effect of LPS on pro-inflammatory mediator release was analysed in mixed sputum cells from eight patients with COPD. Cells were stimulated with or without LPS (final concentrations 1 and 10ng/ml) for 2, 6 and 24 hours. Unstimulated COPD mixed sputum cells spontaneously released TNFα and CXCL8 at all time points examined (**Figure 3.1.1** and **3.1.2**). One way ANOVA was used to compare levels of TNFα and CXCL8 at 2, 6, and 24 hours for each LPS

concentration (unstimulated, 10ng/ml, 100ng/ml). There were no significant differences in levels of TNFα or CXCL8 between unstimulated cells, cells stimulated with 10ng/ml or 100ng/ml at any time points ($p>0.05$ for all comparisons). Unstimulated levels of TNFα and CXCL8 were compared between time points, with significantly higher levels of unstimulated TNF release at 6 hours compared with 2 hours (one way ANOVA $P=0.046$; bonferroni post-test $p<0.01$), and levels of CXCL8 significantly higher at 6 and 24 hours compared with 2 hours (one way ANOVA $p=0.0025$; bonferroni post-test $p<0.05$ 2 hours vs 6 hours; $p<0.01$ 6 hours vs 24 hours), therefore all future experiments were conducted after 24 hours of culture. The effect of increasing concentrations of LPS (0–10,000ng/ml) at 24 hours was also assessed in mixed sputum cells from six patients with COPD. Increasing concentrations of LPS had no effect on TNFα or CXCL8 production (one way ANOVA $p>0.05$ for all comparisons) (**Figure 3.1.3** and **3.1.4**); therefore all future experiments with sputum cells were performed without LPS stimulation.

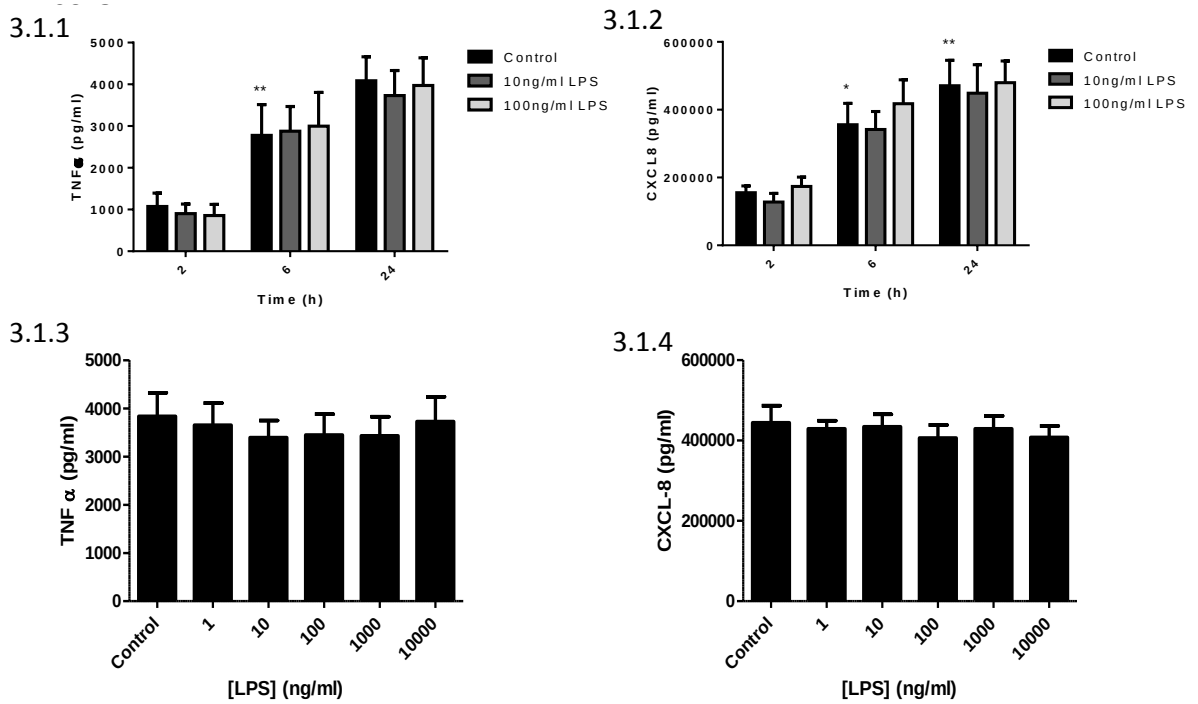


Figure 3.1 The release of pro-inflammatory mediators from mixed sputum cells
COPD mixed sputum cells (n=8; 3.1.1 and 3.1.2) were cultured for 2, 6, and 24 hours in the presence of LPS (0, 10, and 100ng/ml). Supernatant levels of 3.1.1) TNFα and 3.1.2) CXCL8 were measured by ELISA. COPD mixed sputum cells (n=6; 3.1.3 and 3.1.4) were cultured for 24 hours in the presence of LPS (0–10,000ng/ml). Supernatant levels of 3.1.3) TNFα and 3.1.4) CXCL8 were measured by ELISA. Error bars represent SEM. One-way ANOVA tests followed by bonferroni post tests were performed comparing absolute levels of cytokine at 2, 6 and 24 hours for each LPS concentration. Levels of unstimulated TNFα and CXCL8 were also compared between time points. *p<0.05 **p<0.01 denotes differences in levels of unstimulated cytokine vs 2 hours. One way ANOVA was used to compare levels of TNFα and CXCL8 in unstimulated sputum cells at 24 hours.

3.3.1.3 Pro-inflammatory mediator production from COPD and control mixed sputum cells

Unstimulated sputum cells from 11 COPD, 10 S and five NS were cultured for 24 hours. Absolute levels of TNFα production from COPD mixed sputum cells were numerically lower than S and NS mixed sputum cells (**Figure 3.2.1**) , and absolute levels of CXCL8 from COPD mixed sputum cells were numerically higher than S and NS (**Figure 3.2.2**). Statistical analysis showed that there were no significant

differences in unstimulated pro-inflammatory mediator release between patient groups ($p>0.05$ for both TNFα and CXCL8).

3.3.1.4 Effects of dexamethasone on pro-inflammatory mediator release

The sputum cells from these 11 COPD, 10 S and five NS were cultured in the presence of dexamethasone (final concentrations 0–1000nM) for 24 hours. Dexamethasone caused a dose-dependent inhibition of both TNFα and CXCL8 release in all three groups (**Figure 3.2.3** and **3.2.4**). The mean maximum per cent inhibition of TNFα in COPD samples (54.7%) was similar to S (55.96%) and NS (60.95%). Similarly, there was no difference between per cent inhibition in COPD samples compared with S and NS for CXCL8; 63.6% in COPD compared with 57.1% and 48.9% in S, and NS subjects, respectively. There were no significant differences in mean per cent inhibition between any of the groups at any of the dexamethasone concentrations examined ($p>0.05$ for all comparisons).

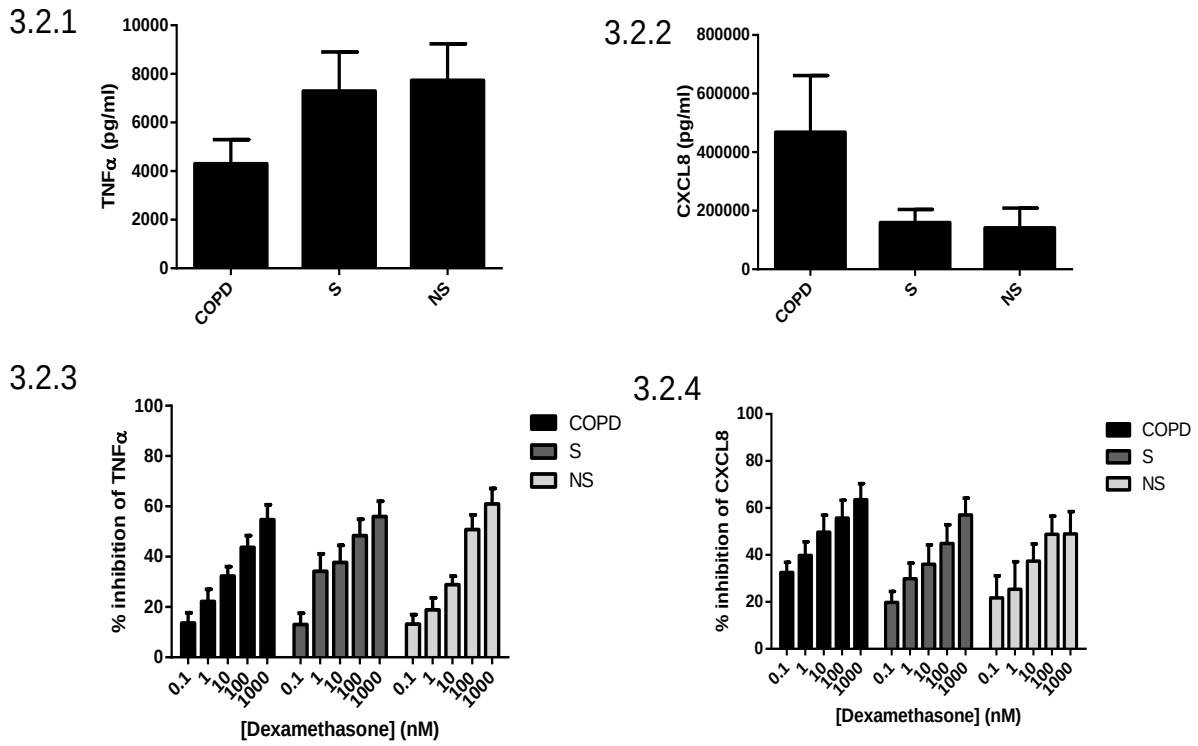


Figure 3.2 The release of pro-inflammatory mediators from COPD and control mixed sputum cells and the effect of dexamethasone
Mixed sputum cells from COPD (n=10), S (n=10) and NS (n=5) were cultured in the presence of dexamethasone (0–1000nM) for 24 hours. Supernatant levels of TNFα (3.2.1 and 3.2.3) and CXCL8 (3.2.2 and 3.2.4) were measured by ELISA. Basal absolute levels of cytokine (3.2.1 and 3.2.2) and per cent inhibition (3.2.3 and 3.2.4) data are shown. One-way ANOVA was performed to look for differences between patient groups. Error bars represent SEM.

3.3.1.5 Cell viability and apoptosis analysis

Neutrophils are prone to apoptosis during cell culture (Savill et al, 1989; Whyte et al, 1993) and, specifically, GC have been shown to induce apoptosis in neutrophils (Meagher et al, 1996). Therefore, cell viability and neutrophil apoptosis was examined in sputum cells with and without dexamethasone.

Trypan blue exclusion was used to examine cell death at 0 and 24 hours, with and without dexamethasone (1000nM). One-way ANOVA demonstrated there were no significant differences in numbers of dead cells in basal mixed sputum cells

compared with cells cultured for 24 hours in the presence or absence of dexamethasone (ANOVA p=0.0783).

There was a significant difference between numbers of early and late apoptotic neutrophils over time by morphological analysis (ANOVA p<0.0001 for both comparisons). Tukey Kramer's multiple comparisons post test showed there were significantly higher numbers of early apoptotic neutrophils at 24 hours in both control and dexamethasone-incubated cells compared with numbers of early apoptotic neutrophils at 0 hours (p<0.001 for both comparisons; **Figure 3.3.1** and **Figure 3.4**). In addition, there was significantly higher numbers of late apoptotic cells at 24 hours in cells cultured with and without dexamethasone compared with cells at 0 hours (p<0.001 for all comparisons). There were no significant differences between the numbers of early and late apoptotic neutrophils cultured in the presence or absence of dexamethasone (Tukey-Kramer multiple comparisons post-test p>0.05 for all comparisons).

To further interrogate cell viability a tunel assay was carried out. One way ANOVA demonstrated that there was no significant differences in the number of apoptotic neutrophils over time (ANOVA p=0.0783; **Figure 3.3.2**).

Taken together these data demonstrate that although cell viability decreased over time and numbers of apoptotic cells increased over time, there were no differences in the numbers of apoptotic cells following culture with dexamethasone compared with cells cultured in control media when examined by morphology or tunel assay.

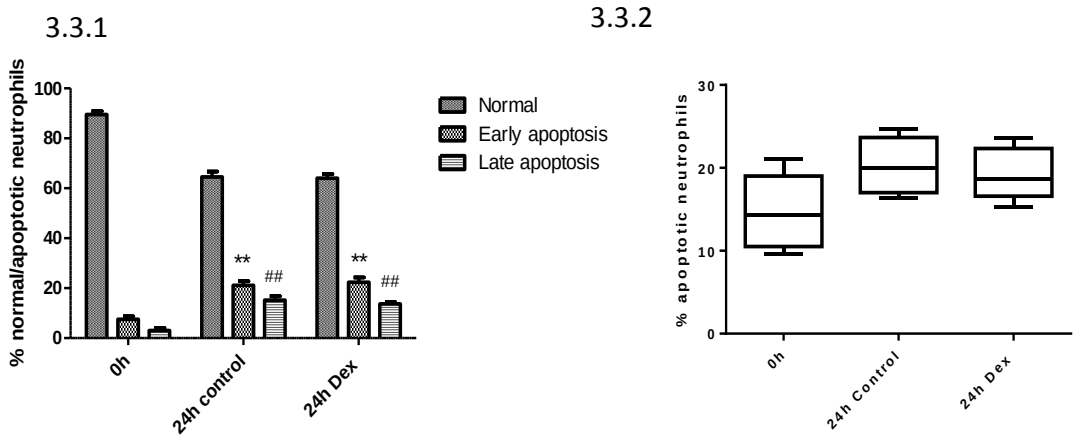


Figure 3.3 The effect of dexamethasone on cultured mixed sputum cell apoptosis

COPD mixed sputum cells (n=5) were cultured in the presence of dexamethasone (0 or 1000nM) for 24 hours. Cytospins were prepared and cells analysed for signs of apoptosis by 3.3.1) morphological analysis and 3.3.2) tunel assay. Error bars represent SEM. One-way ANOVA followed by Tukey Kramer post-tests were used to compare percentages of apoptotic neutrophils between time points, and to compare the percentage of early and late apoptotic neutrophils in the presence or absence of dexamethasone at 24 hours compared with 0 hours. ** $p<0.01$ compared with 0 hours. ## $p<0.01$ compared with 0 hours.

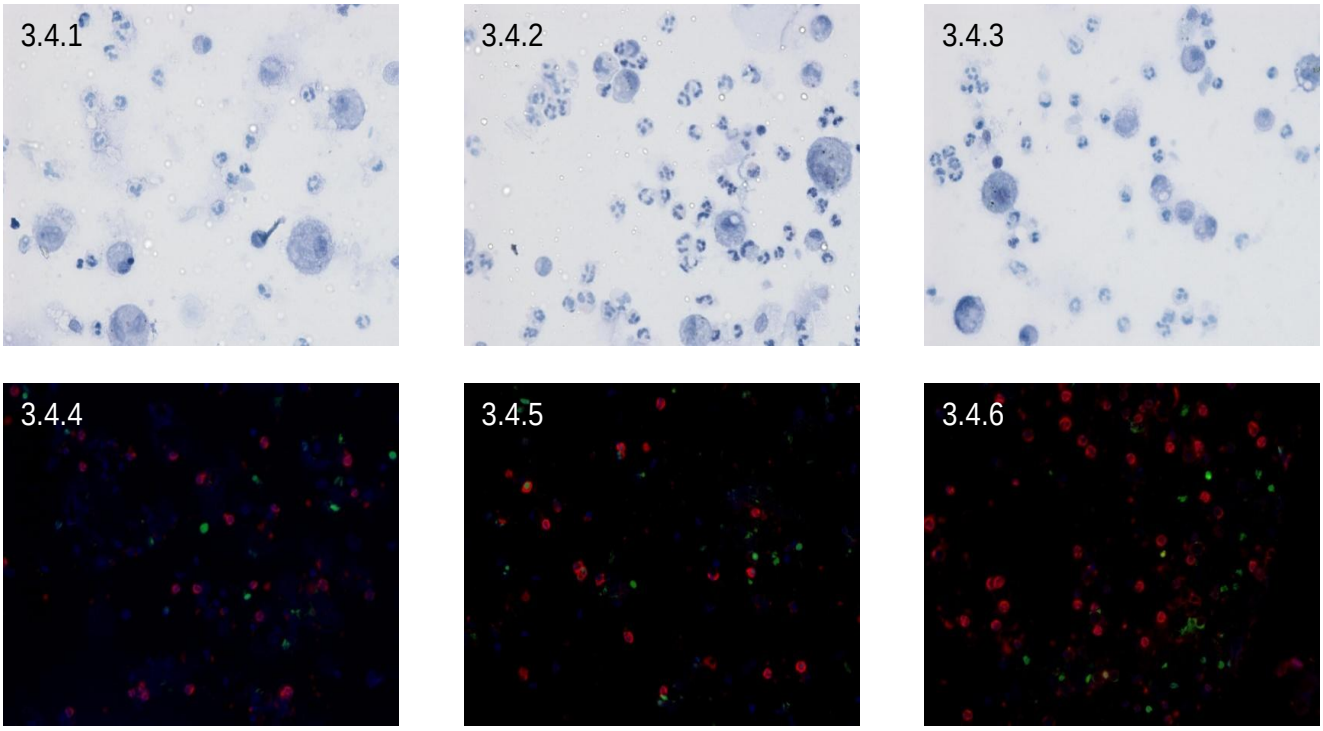


Figure 3.4 Photomicrographs depicting the effect of dexamethasone on apoptosis in cultured mixed sputum cells
COPD mixed sputum cells (n=5) were cultured in the presence of dexamethasone (0 or 1000nM) for 24 hours. Basal cells are also shown. Cytospins were prepared and cells analysed for signs of apoptosis by 3.4.1–3.4.3) rapi-diff morphological analysis and 3.4.4–3.4.6) tunel assay. For morphological analysis neutrophils were categorised as normal (chromatin bridges intact), early apoptosis (disappearance of chromatin bridges between nuclear lobes) or late apoptosis (shrinkage or fragmentation of the nucleus). For tunel assay analysis, cells were categorised as apoptotic by the presence of positive tunel staining (green). To enable the identification of neutrophils, cytoslides were dual-labelled with neutrophil elastase (red) and counterstained with DAPI (blue).

3.3.2 Isolated sputum neutrophil culture

3.3.2.1 Pro-inflammatory mediator production from isolated sputum neutrophils

Sputum neutrophils isolated from mixed sputum cells (n=7 COPD) were cultured in the presence or absence of LPS (final concentration 100ng/ml) for 24 hours. Mean unstimulated levels of TNFα and CXCL8 were 756.65 (± 46.34) pg/ml and 4388.50

(± 654.75) pg/ml, respectively. LPS had no effect on TNFα or CXCL8 release. These data suggest that sputum neutrophils do not require LPS stimulation in order to produce pro-inflammatory mediator release.

3.3.2.2 Effects of dexamethasone on pro-inflammatory mediator release

These isolated sputum neutrophils were cultured in the presence or absence of dexamethasone (final concentrations 0–1000nM) for 24 hours. Dexamethasone had a modest inhibitory effect on TNFα and CXCL8 release, with no significant differences observed (ANOVA p>0.05 for TNFα and CXCL8; **Figure 3.5.1** and **3.5.2**). The mean maximum per cent inhibition of TNFα and CXCL8 were 41.53% and 39.65%, respectively.

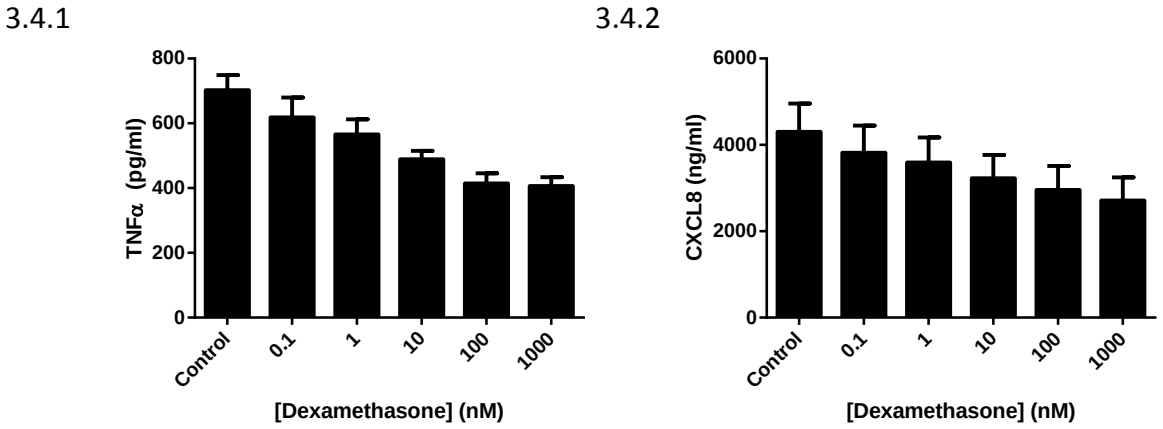


Figure 3.5 The effect of dexamethasone on pro-inflammatory mediator release from sputum neutrophils
Isolated sputum neutrophils (n=7 COPD) were cultured in the presence of dexamethasone (0–1000nM) for 24 hours. Supernatant levels of 3.5.1) TNFα and 3.5.2) CXCL8 were measured by ELISA. Statistical analysis using one way-ANOVA was carried out to examine differences between cytokine levels at each dexamethasone concentration. Error bars represent SEM.

3.3.3 Isolated blood neutrophil culture

3.3.3.1 Pro-inflammatory mediator production from isolated blood neutrophils

Neutrophils isolated from the whole blood of eight patients with COPD were cultured in the presence of LPS (final concentrations 0–10,000ng/ml) for 24 hours. PMNs spontaneously released TNFα and CXCL8, with levels of 577.24 (± 162.45) pg/ml and 3474.18(± 577.84) pg/ml released after 24 hours, respectively (**Figure 3.6.1** and **3.6.2**). LPS significantly increased both TNFα (ANOVA p=0.0086) and CXCL8 release (ANOVA p=0.0296). A sub-optimal LPS concentration of 100ng/ml was chosen for all subsequent experiments with PMNs.

3.3.3.2 Effects of dexamethasone on pro-inflammatory mediator release

Unstimulated and LPS stimulated (final concentration 100ng/ml) PMNs were incubated with dexamethasone (final concentrations 0–1000nM) for 24 hours. Dexamethasone caused a dose-dependent inhibition of both TNFα and CXCL8 in both unstimulated (TNFα ANOVA p<0.0001; CXCL8 ANOVA p<0.0001, **Figure 3.6.3** and **3.6.4**) and LPS stimulated (TNFα ANOVA p<0.05; CXCL8 ANOVA p<0.001) PMNs. In unstimulated PMNs the mean maximum per cent of inhibition of TNFα and CXCL8 was 80.07% and 78.09%, respectively. In LPS stimulated PMNs, the mean maximum per cent inhibition of TNFα and CXCL8 was 88.72% and 91.41%, respectively.

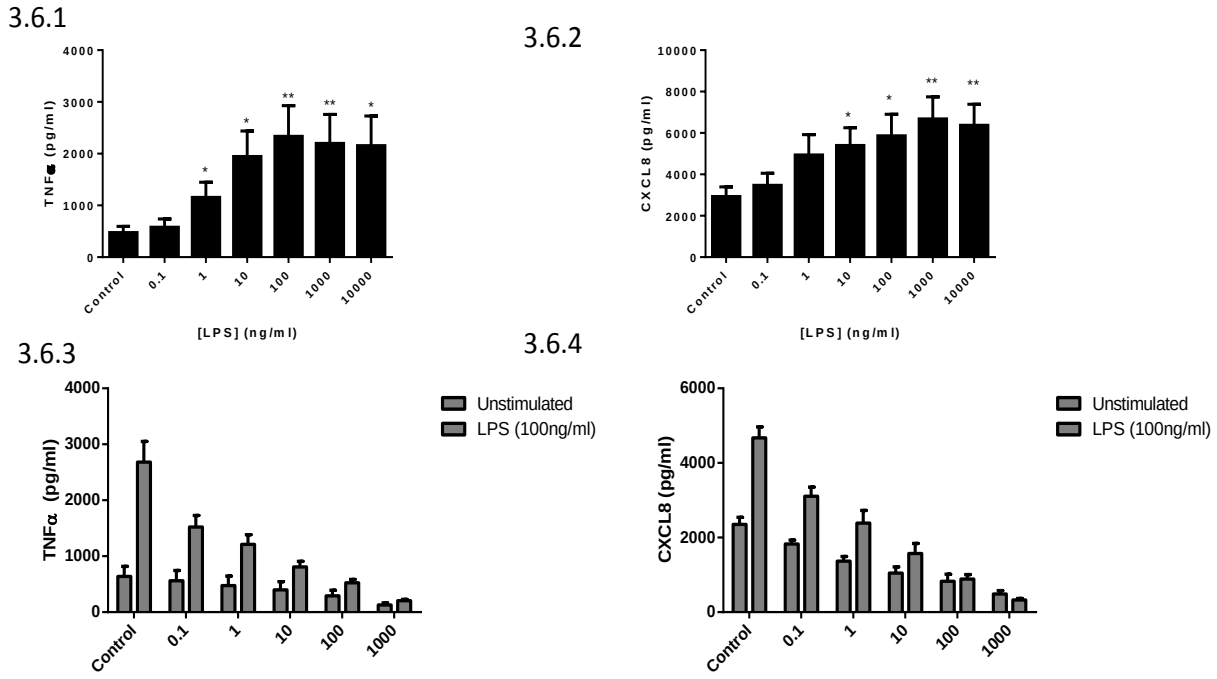


Figure 3.6 The release of pro-inflammatory mediators from unstimulated and LPS stimulated PMNs and the effect of dexamethasone

Blood neutrophils (n=7 COPD) were cultured with LPS for 24 hours (0–10µg/ml) and levels of 3.6.1) TNF and 3.6.2) CXCL8 were measured by ELISA. LPS-stimulated (100ng/ml) and unstimulated neutrophils were cultured with dexamethasone for 24 hours (0–1000nM) and levels of 3.6.3) TNFα and 3.6.4) CXCL8 levels were measured by ELISA. Error bars represent SEM.*p<0.05. *p<0.05; **p<0.01 compared with control levels.

3.3.4 Effects of dexamethasone on pro-inflammatory mediator release in sputum and blood neutrophils

Statistical analyses showed that dexamethasone had a significantly greater effect on LPS stimulated TNFα release in PMNs compared with sputum neutrophils at all dexamethasone concentrations (Bonferroni multiple comparisons test p<0.001 for all concentrations, **Figure 3.7.1** and **3.7.2**) and at 1000nM in unstimulated PMNs (p<0.001). Inhibition of CXCL8 release was significantly higher in LPS-stimulated PMNs compared with sputum neutrophils at all dexamethasone concentrations (p<0.01 at 0.1nM and 1nM; p<0.001 at 10nM, 100nM, and 1000nM) and in unstimulated PMNs compared with sputum neutrophils at 1000nM (p<0.001).

3.8.1 3.8.2

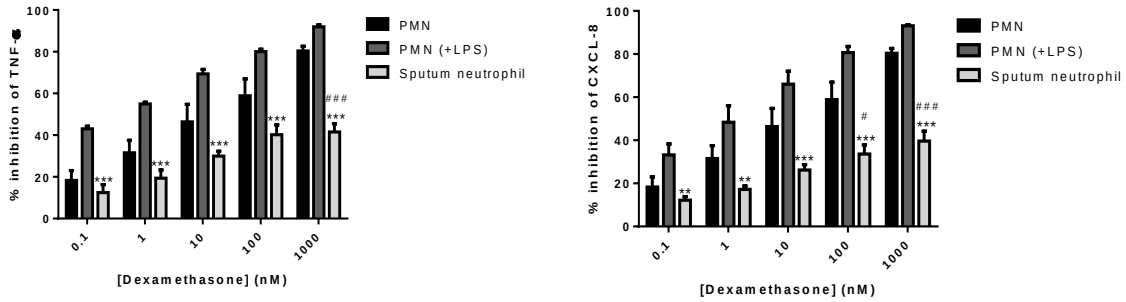


Figure 3.7 The effect of dexamethasone on pro-inflammatory mediator release from sputum and blood neutrophils
Isolated blood neutrophils (unstimulated and stimulated with LPS [100ng/ml]) and sputum neutrophils (unstimulated) were cultured for 24 hours in the presence of dexamethasone (0–1000nM) and 3.7.1) TNFα and 3.7.2) CXCL8 levels were measured by ELISA. Data are presented as mean per cent inhibition. Error bars represent SEM. One-way ANOVA followed by Bonferroni's post-test were performed comparing sputum and PMN. ** p < 0.001 and *** p < 0.0001 for sputum neutrophils vs LPS stimulated PMNs. # p < 0.05, and ### p < 0.0001 for sputum neutrophils vs unstimulated PMNs.

3.3.5 Isolated sputum macrophage culture

3.3.5.1 Pro-inflammatory mediator production from isolated sputum macrophages

The adherent cells present after the isolation of sputum neutrophils were cultured in the absence or presence of LPS (100ng/ml) for 24 hours. Mean unstimulated levels of TNFα and CXCL8 were 3426.86 (± 1215.3) pg/ml and 16128.40 (± 6081.2) pg/ml, respectively (**Figure 3.8.1** and **3.8.2**). LPS stimulation had no effect on TNFα or CXCL8 release from isolated sputum macrophages (ANOVA $p>0.05$); suggesting as with neutrophils from mixed sputum that these cells do not require stimulation in order to release CXCL8 or TNFα.

3.3.5.2 Effects of dexamethasone on pro-inflammatory mediator release

The effect of dexamethasone on pro-inflammatory mediator release from sputum macrophages was then assessed. Cells were cultured for 24 hours in the presence of increasing dexamethasone concentrations (0–1000nM). Dexamethasone caused a concentration-dependent inhibition of both TNFα (ANOVA $p=0.0036$) and CXCL8 (ANOVA $p=0.0068$, **Figure 3.8**), with mean maximum per cent inhibitions of 56.46% and 47.81%, respectively. Bonferroni multiple comparisons analysis showed that dexamethasone inhibited TNFα release at concentrations of 10nM ($p=0.0428$), 100nM ($p=0.0143$) and 1000nM ($p=0.0054$) and significantly inhibited CXCL8 release at concentrations of 100nM ($p=0.0426$) and 1000nM ($p=0.0149$).

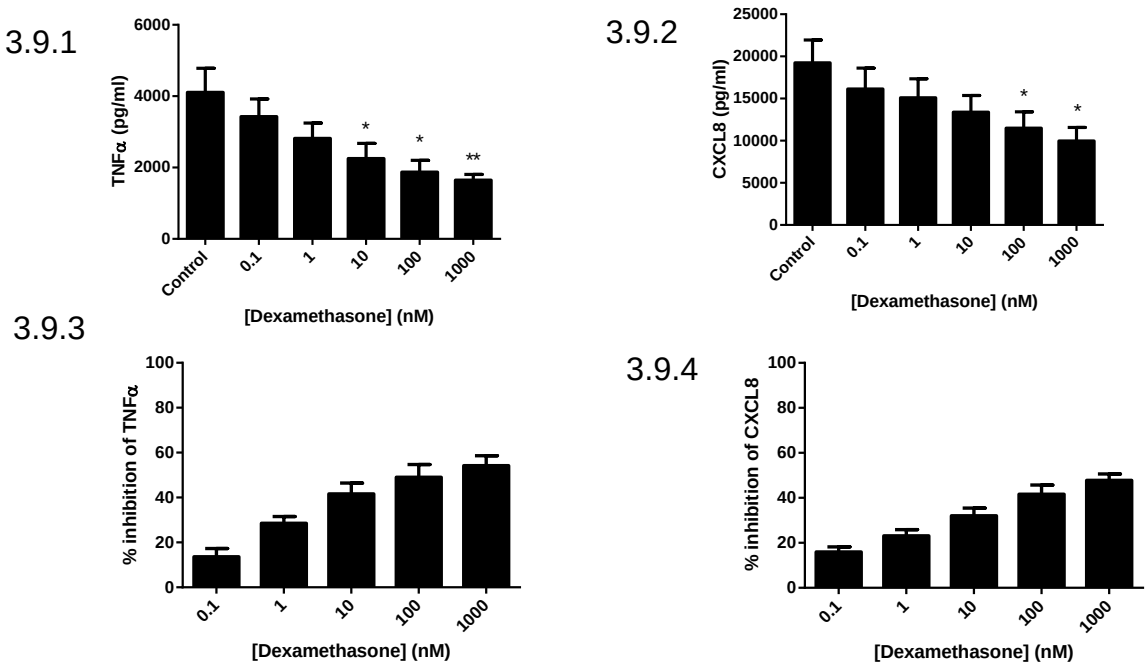


Figure 3.8 Pro-inflammatory mediator release from isolated sputum macrophages and the effect of dexamethasone

Isolated sputum macrophages were cultured for 24 hours with dexamethasone (0–1000nM). Supernatant levels of 3.8.1 and 3.8.3) TNFα and 3.8.2 and 3.8.3) CXCL8 were measured by ELISA. Absolute levels (3.8.1 and 3.8.2) and per cent inhibition (3.8.3 and 3.8.4) are shown. Error bars represent SEM. Bonferroni multiple comparisons test were performed comparing absolute levels of cytokine at each dexamethasone concentration to control levels. *p<0.05, **p<0.01.

3.4 Discussion

The aim of this chapter was to investigate the effects of GC on the innate immune response in patients with COPD compared with control samples, using cultured mixed sputum cells, which are predominantly neutrophils and macrophages. Dexamethasone induced a dose-dependent inhibition of pro-inflammatory mediator release from mixed sputum cells, which was not dependent on disease status, as no differences in inhibition levels were seen in COPD compared with control groups. The GC sensitivity of isolated sputum neutrophils and macrophages was also investigated. Dexamethasone induced, at best, modest effects on sputum neutrophil pro-inflammatory mediator production, with maximum inhibition levels for TNFα and CXCL8 of 41.53 and 39.65%, respectively, whereas sputum macrophages demonstrated sensitivity similar to that of the mixed sputum cell fraction. In contrast to sputum neutrophils, unstimulated- and stimulated-pro-inflammatory mediator production from blood neutrophils was inhibited by GC. This study also shows that mixed sputum cells provide a viable method of sampling cells from the upper airways from COPD and healthy smoking and non-smoking control subjects and could therefore be used to assess the effects of novel anti-inflammatory drugs on upper airway cells in COPD lungs.

The data presented here show that mixed sputum cells are unresponsive to LPS stimulation which is in agreement with previous cultured sputum cell studies (Pang et al, 1997; Bettiol et al, 2002; Dentener et al, 2006; Scheicher et al, 2007). The T cell mitogen PHA has been shown to induce IFNγ and IL-5 production from sputum cells (Liu et al, 2000) whilst the chemoattractant fMLP has been shown to induce oxidative burst in mixed sputum cells (Beeh et al, 2004). This suggests that the lack of

stimulus-induced response seen in mixed sputum cells is specific to LPS. Sputum neutrophils have been shown to lack expression of TLR4 in severe asthma subjects, (Baines et al, 2010), although whether airway neutrophils from other subject groups express TLR4 is unknown. In addition, macrophages exposed to continual low concentrations of LPS can be rendered tolerant to subsequent LPS stimulation (Fujihara et al, 2003). A lack of TLR4 expression on airway neutrophils together with macrophages exhibiting tolerance to LPS as a result of bacterial colonisation in the airways may account for the lack of overall unresponsiveness to LPS seen in sputum cells, although further research is needed to fully understand the lack of sputum cell responsiveness to LPS.

Mixed sputum cells secrete high levels of pro-inflammatory cytokines without any subsequent stimulation, which suggests that the cells are already in an activated state, possibly as a result of pro-inflammatory mediators in the airways stimulating these cells. This may be due to *in vivo* activation as a result of bacterial colonisation in the airways, particularly in the case of patients with COPD. However, absolute levels of pro-inflammatory cytokines were similar from COPD, smoker and non-smoker sputum cells, suggesting that bacterial colonisation cannot be the only reason for the spontaneous secretion. The use of DTT as a mucolytic agent to homogenise the sputum during processing may also induce activation of the cells. Processing the sputum without DTT to assess differences in pro-inflammatory cytokine release would determine whether DTT activates the cells, although much smaller cell yields would make it difficult to do any meaningful analysis. In contrast to airway cells systemic neutrophils isolated from whole blood were responsive to LPS, which has been shown previously (Dubravec et al, 1990; Blidberg et al, 2011). Blood

neutrophils also spontaneously released pro-inflammatory mediators, in agreement with a previous study (Blidberg et al, 2011) indicating that pro-inflammatory mediator release from blood neutrophils may be a result of pre-formed granules and *de novo* synthesis.

There were lower levels of TNFα release and higher levels of CXCL8 release from COPD cells compared with control cells, which is in contrast to previous research showing increased TNFα levels in peripheral blood, bronchial biopsies, induced sputum and BAL fluid of patients with COPD compared with healthy control subjects (De Francia et al, 1994; Keatings et al, 2004; Mueller et al, 1996; Keatings et al, 1996; Daldegan et al, 2005). Previous sputum cell culture studies have also found lower TNFα release from COPD cells compared with controls (Dentener et al, 2006) although increased TNFα from COPD mixed sputum cells has also been described (Profita et al, 2003). CXCL8 levels are typically higher in BAL fluid and sputum compared with healthy controls (Riise et al, 1996) and previous sputum cell culture studies have also found increased CXCL8 from COPD cells compared with healthy controls (Profita et al, 2003). The differences may due to patients with COPD undergoing ICS treatment, which may be lowering TNFα levels, but having less of an effect on CXCL8, which has been shown to be more GC insensitive in macrophages (Armstrong et al, 2009).

Cytokine production from COPD alveolar macrophages *ex vivo* is reported to be GC resistant (Culpitt et al, 2003; Cosio et al, 2004). More recent data, however, suggests GC insensitivity is a cytokine- and cell-specific phenomenon present in alveolar macrophages regardless of disease or smoking status (Hew et al, 2006; Bhavsar et al, 2008; Armstrong et al, 2009; Kent et al, 2009). Airway neutrophils from patients

with cystic fibrosis (Corvol et al, 2003) and bronchial sepsis (Pang et al, 1997) have also been shown to be insensitive to GC. In LPS stimulated alveolar macrophages dexamethasone at 1000nM inhibited CXCL8 production by approximately 50%, with no differences in inhibition in COPD, S and NS (Armstrong et al, 2010). In another study, airway neutrophils exhibited a similar inhibition of CXCL8 release with approximately 60% inhibition achieved with 100nM dexamethasone (Pang et al, 1997). In the current study, dexamethasone inhibited CXCL8 release by 63.55% in COPD mixed sputum cells, although no differences in GC sensitivity between patient groups were observed. TNFα release was inhibited to 60.95% in NS subjects, similarly with no differences in the GC sensitivity between groups. This suggests that mixed sputum cells are partially GC insensitive, which agrees with other recent studies showing that GC sensitivity is a cell specific phenomenon present in patients with COPD and control subjects.

In isolated sputum neutrophils the effects of GC were modest, with mean maximum per cent inhibition of 41.53% seen with the maximum (1000nM) dexamethasone concentration. Recently, sputum neutrophils have been shown to have low expression of GR compared with other cells such as alveolar and sputum macrophages (Plumb et al, 2011), which would account for the limited response to dexamethasone observed. In contrast, blood neutrophils express the GR (Pujols et al, 2002), and pro-inflammatory mediator production was suppressed by GC by around 91.99% with 1000nM dexamethasone in the current study. A previous study showed that fMLP-stimulated MMP-9 and NE release from COPD blood neutrophils is insensitive to inhibition by dexamethasone, indicating that GC sensitivity in blood neutrophils may also be cytokine- and stimuli-dependent (Vlahos et al, 2012). The differences in the responses of blood and sputum neutrophils to LPS and GC

highlight a potential pitfall of using blood neutrophils as a model of lung neutrophils. Research suggests that lung neutrophils have an altered phenotype compared with systemic neutrophils in the blood (Baines et al, 2009), which may be indicative of cells undergoing phenotypic changes as they cross the endothelial barrier and enter the lung.

Unstimulated lung macrophages secrete low levels of pro-inflammatory mediators, which can be induced following LPS stimulation (Armstrong et al, 2009; Kent et al, 2009). This is in contrast to sputum macrophages, which, in the current study, were found to be unresponsive to LPS and to secrete pro-inflammatory mediators without prior stimulation. These data suggest that macrophages present in the upper airways display a different phenotype to resident lung macrophages. Previous data has shown that macrophages isolated from sputum are much smaller (Frankenberger et al, 2004) and have a different receptor expression profile compared with macrophages present in the lung (Lensmar et al, 1998), again suggesting that cells in distinct areas of the lung have altered phenotypic properties compared with their systemic cell counterparts.

Previous studies have shown that GC can induce (Meagher et al, 1996) and inhibit (Cox et al, 1995 and Liles et al; 1995) apoptosis in neutrophils. In this study morphological analysis and a tunel assay were used to examine apoptosis specifically in sputum neutrophils. Although increased numbers of apoptotic neutrophils were found following 24 hours of culture compared with non-cultured cells, no differences in the numbers of apoptotic cells exposed to dexamethasone compared with control cells were observed. This suggests that the inhibition of pro-inflammatory cytokine release observed was due to the anti-inflammatory actions of

GC rather than the induction of apoptosis. Previous studies investigating the effect of GC on neutrophils used circulating PMNs rather than airway neutrophils isolated from sputum, which may explain the differences in GC-mediated neutrophil apoptosis observed. As discussed earlier, airway neutrophils have low expression of GR (Plumb et al, 2012), which may account for the lack of GC-mediated apoptosis in the current study.

Induced sputum is a non-invasive technique that can be used to sample cells from the upper airways, which can then be cultured and the effects of anti-inflammatory therapies assessed. Anti-inflammatory drugs used in the treatment of COPD are likely to target the various cell types found in the airways, including macrophages and neutrophils, rather than just a single cell population. Mixed sputum cell culture therefore provides a clinically relevant way of assessing the use of anti-inflammatory therapies in the airways of patients with COPD using a mixed cell population. The main disadvantages of this technique are that whilst patients with COPD typically produce high amounts of sputum with high cell yields, it is more difficult to obtain samples from smoking and non-smoking control subjects, particularly with regards to cell yields. However, since new anti-inflammatory therapies are required specifically for the treatment of COPD, it is important to fully understand the effects of new therapeutics on COPD cells.

In summary, cultured mixed sputum cells are a partially GC insensitive model that can be used to investigate novel anti-inflammatory drugs on pro-inflammatory cytokine release from cells of the innate immune system. There is no difference in the GC sensitivity of COPD disease cells compared with healthy smoking and non-

smoking control cells, suggesting that GC sensitivity is a cell-specific phenomenon regardless of smoking or disease status, in agreement with previous studies (Hew et al, 2006; Bhavsar et al, 2008; Armstrong et al, 2009; Kent et al, 2009). Sputum neutrophils are partially GC insensitive, which may contribute to the lack of GC responsiveness observed in patients with COPD. Since blood neutrophils have been shown to be responsive to GC, it appears that neutrophils leaving the blood and entering the lung undergo phenotypic changes, altering their activation state and/or expression profile. More research is required to understand the functional properties of lung neutrophils, in particularly in COPD lungs, which will enable the identification of alternative inflammatory pathways to direct novel therapeutics against.

Chapter 4

Increased phosphorylated p38 MAPK in COPD lungs

4.1 Introduction

COPD is a progressive inflammatory disease of the airways (Hogg et al, 2004). GC are the most commonly used anti-inflammatory therapy for COPD, but they have limited effects on airway inflammation and disease progression. Novel therapeutic approaches to target the inflammation in COPD are therefore required.

Activation of the p38 MAPK intracellular signalling pathway occurs via a variety of extracellular stimuli which includes pro-inflammatory cytokines such as TNFα as well as TLR agonists such as LPS (Saklatvala, 2004). Activated p38 MAPK can enhance the function of transcription factors such as NFκB and ATF-2, which further amplify gene expression of pro-inflammatory mediators including IL-6, CXCL8 and TNFα. In addition, phospho-p38 MAPK can also act post-transcriptionally through the stabilisation of cellular mRNAs which are involved in the immune response, including IL-6 and CXCL8 (Winzen et al; 1999). Pharmacological p38 MAPK inhibitors attenuate pro-inflammatory mediator production from alveolar macrophages (Birrell et al; 2006, Smith et al; 2006, Kent et al; 2009) and are in clinical development for the treatment of COPD (Singh et al; 2010).

Increased numbers of inflammatory cells are present in the lungs of patients with COPD, including lymphocytes (Hogg 2001), macrophages (Hogg et al, 2004) and neutrophils (Lacoste et al, 1993; Keatings et al, 1996; Keatings and Barnes, 1997). There are also increased numbers of organised inflammatory lymphoid structures called follicles within the lung parenchyma and associated with the bronchial tree (Hogg et al, 2004). These follicles may function as sites of antigen presentation,

supporting previous suggestions that COPD has an auto-immune component (Van der Strate et al, 2009; Lambers et al, 2009). There is limited published information regarding the presence of activated p38 MAPK within inflammatory cells and follicles within the lungs of patients with COPD compared with control subjects. Renda et al (2008) showed that the expression of activated p38 MAPK was increased in the alveolar macrophages of patients with COPD compared with control subjects as well as increased numbers of phospho-p38-positive cells within alveolar walls. The presence of activated p38 MAPK on other relevant inflammatory cells types such as lymphocytes and neutrophils has not yet been described.

The aim of the current study was to characterise the cell-specific presence of activated p38 MAPK in COPD lung tissue compared with smoking and non-smoking controls. Dual labelled immunofluorescence was used to quantify phospho-p38+ CD20+, CD8+ and CD4+ lymphocytes and phospho-p38 positive neutrophils. In addition, phospho-p38 positive alveolar macrophages and airway epithelial cells were also quantified. Phospho-p38 MAPK was absent in lung neutrophils. Previous studies have shown that blood neutrophils express phospho-p38 (Zu et al, 1998), and that cytokine production from blood neutrophils can be suppressed by inhibition of the p38 MAPK pathway (Zu et al, 1998; Coxon et al, 2003; Ribeiro et al, 2003). Therefore, functional studies comparing the effect of p38 MAPK inhibition on pro-inflammatory mediator production from isolated lung and blood neutrophils were also performed.

4.2 Methods

4.2.1 Study subjects

For immunohistochemical analysis 20 patients with COPD, 12 S with normal lung function and 12 NS subjects undergoing surgical resection for suspected or confirmed lung cancer were recruited. For isolated neutrophil culture studies seven patients with COPD were recruited. The diagnosis of COPD was by the GOLD definition, while S were defined as greater than 10 pack year smoking history and normal pulmonary function. Demographics are shown in **Table 4.1**. All patients gave written informed consent. The study was approved by the local ethics committee.

Table 4.1 Patient demographics

	Immunohistochemistry						**Cell culture**
	Lung tissue			Sputum cells			Neutrophils
	COPD	S	NS	COPD	S	NS	COPD
Male/Female	14/6	6/6	6/6	5/3	0/6	3/1	4/3
Age (years)	68.3 (6.4)	62 (9.8)	55.8 (15.8)	72.5 (5.5)	52 (6.8)	54 (14.7)	74.1 (3.1)
Smoking History (Pack years)[#]	43.0 (13-110)	47.4 (25-116)	0	50.0 (38-159)	29.8 (8.6)	0	47.57 (6.9)
FEV1 (L)	1.9 (0.5)	2.3 (1.55-3.73)	2.2 (0.5)	1.1 (0.6-1.3)	2.8 (0.6)	3.6 (0.9)	1.1 (0.3)
FEV1% predicted	68 (13.3)	83.2 (13.7)	90.2 (12.4)	43.6 (17.4)	101.0 (9.1)	106.4 (9.6)	47.0 (15.7)
FEV1/FVC ratio	57.3 (5.9)	72.0 (6.8)	75.6 (5.5)	42.0 (11.6)	76.2 (4.2)	75.0 (3.6)	46.7 (8.7)
Number on ICS	12	0	0	6	0	0	7

Data are presented as mean (SEM) unless otherwise stated. COPD, chronic obstructive pulmonary disease; S,: smokers; NS, non-smokers; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; ICS, inhaled corticosteroids.

4.2.2 Induced sputum

Sputum was induced and processed using DTT. The resulting cell pellet was resuspended in supplemented RPMI 1460. Cell counts and viability were established using trypan blue method in a Neubauer haemocytometer. Cytospins were prepared and stained with Rapi-diff for differential cell counts. Cell counts are reported as cells per gram of sputum (**Table 4.2**).

4.2.3 Sputum neutrophil isolation

To isolate neutrophils from the mixed sputum cell fraction, mixed sputum cells were cultured for 1 hour in 24 well plates ($4x10^5$/well) at 37°C, 5% CO_2 to allow the macrophages to adhere to the wells. Non-adherent cells enriched for neutrophils (89.6% mean purity [**Table 4.2**]) were then removed and resuspended at $1x10^6$/ml in supplemented RPMI.

Table 4.2 Sputum differential cell counts

	Before enrichment	After enrichment
Total cell count/g sputum *	14.2 (13.5)	8.84 (8.9)
Total neutrophil count/g sputum *	10.3 (9.7)	8.0 (8.2)
Neutrophil %	73.5 (4.6)	89.5 (3.1)
Total macrophage count/g sputum *	3.3 (3.5)	0.7 (0.6)
Macrophage %	22.5 (4.4)	9.0 (3.6)
Eosinophil % #	1.0 (0.5-1.8)	0 (0-1.75)
Lymphocyte % #	0.3 (0-1.8)	0 (0-0.5)
Squamous cell% #	1 (0-1.75)	0.25 (0-3.75)

Differential cell counts for sputum samples used for cell culture pre- and post- neutrophil isolation step (n=7). Data are presented as mean (SD). Cell numbers are normalised per gram of sputum, * denotes number of cells x10^6 and # denotes data presented as median (range).

4.2.4 Blood neutrophil isolation

Venous blood (5ml) was layered over 3ml of mono-poly resolving medium and centrifuged at 800g for 45mins at 18°C. PMNs were removed, washed and resuspended in RPMI 1460. Cell counts and viability were determined and cytospins were prepared as described earlier. Cells were resuspended in supplemented RPMI at a concentration of 1 x 10^6 per millilitre.

4.2.5 Cell culture

Neutrophils were cultured in 24 well plates for 24 hours at 37°C, 5% CO_2 in the presence or absence of LPS (final concentration 100ng/ml). The p38 MAPK inhibitor SB731445 (IC_{50} in PBMCs: 30nM [GSK data on file], therefore final concentrations 10–1000nM to be used) was added for 2 hours before the addition of LPS. Cell-free supernatants were removed and stored at -80°C for cytokine analysis.

In addition, after 24 hours culture cells were removed and centrifuged at 400g for 10 mins at 4°C. Cytospins were prepared and air dried. Cytospins were fixed in methanol and stained using RapiDiff for differential cells counts. Some cytospins were fixed in 4% paraformaldehyde prior to immunocytochemical analysis.

4.2.6 Cytokine release analysis

Supernatant levels of TNFα and CXCL8 were determined using ELISA according to the manufacturer's instructions (R and D systems). The lower limits of detection for TNFα and CXCL8 were 15.6pg/ml and 32.5 pg/ml, respectively.

4.2.7 Immunohistochemistry

Tissue blocks were obtained from an area of the lung as far distal to the tumour as possible, and processed as described in the methods section. Blocks were labelled

using anti-phospho-p38 MAPK primary antibody. PMN and sputum neutrophil cytoslides were also labelled using anti-phospho-p38 MAPK antibody. Dual label immunofluorescence with phospho-p38 was performed with one of the following primary antibodies: neutrophil elastase, CD20, CD8 or CD4.

4.2.7.1 Image analysis

The percentage of CD20+, CD8+ and CD4+ cells positive for phospho-p38 MAPK was calculated within inflammatory follicles and within the sub-epithelium. Total numbers of neutrophils, small airway epithelial cells and 300 macrophages (identified by morphology) positive for phospho-p38 MAPK were also quantified. For dual-label images, fluorescent images from the same field were captured and digitally merged to determine the phospho-p38 positive cells. Digital micrographs were obtained through the use of a Nikon Eclipse 80i microscope equipped with a QImaging digital camera and ImagePro Plus 5.1 software. Cell counts, follicle area and epithelial and sub-epithelial length were quantified using the ImagePro Plus 5.1 software. Cell counts were standardised to the number of positive cells.mm^{-2} of the area of interest.

4.2.8 Statistical Analysis

Immunohistochemistry data were normally distributed. One-way ANOVA tests were performed to determine differences between groups. Significant differences were further analysed using the Bonferroni multiple comparisons test. Paired t tests were

used to compare expression levels between follicular and sub-epithelial regions. Cell culture data were normally distributed. One way ANOVA tests were performed to determine the effects of SB731445 on cytokine production. Significant differences were further analysed using the Bonferroni multiple comparisons test. One way ANOVA followed by bonferroni's post-test was also used to compare per cent inhibitions of TNFα and CXCL8 between sputum neutrophils and unstimulated and LPS stimulated PMNs at each SB731445 concentration. Analysis was carried out using GraphPad InStat software version 3.0.

4.3 Results

4.3.1 Phosphorylated p38 MAPK in lymphocytes

4.3.1.1 Follicles

Dual label immunofluorescence analysis showed that phospho-p38 MAPK was present within all inflammatory follicles analysed.

Phospho-p38-positive CD20+ B cells as a percentage of total CD20+ B cells within inflammatory follicles was significantly different between COPD, S and NS lung tissue samples (ANOVA $p<0.001$, **Figure 4.1.1** and **4.2**); Bonferroni multiple comparisons test showed that the percentage of phospho p38-positive CD20+ B cells was significantly higher in COPD lungs compared with S and NS ($p<0.001$ for both comparisons, means 86.6%, 44.1% and 30.9% respectively). There was also a significantly greater percentage of phospho p38-positive CD20+ B cells in S compared with NS ($p<0.05$).

Phospho-p38-positive CD8+ T cells as a percentage of total CD8+ T cells within inflammatory follicles was also significantly different between COPD, S and NS lung tissue (ANOVA $p<0.001$, **Figure 4.1.2** and **4.3**); Bonferroni multiple comparisons test showed that phospho-p38-positive CD8+ T cells was significantly higher in COPD lung tissue compared with NS ($p<0.001$) (means 56.2%, and 31.5% respectively). There was also a numerical trend towards an increased phospho-p38-positive CD8+ T cells in COPD vs. S (means 56.2% vs. 43.6%) and in S vs. NS (means 43.6% vs. 31.5%) but these were not statistically significant ($p>0.05$).

The percentages of phospho-p38-positive CD4+ T cells was much lower in all patient groups; generally less than 20% of total CD4+ T cells were phospho-p38-positive (**Figure 4.1.3**). There were no differences in percentages of phospho-p38-positive CD4+ T lymphocytes between any of the patient groups.

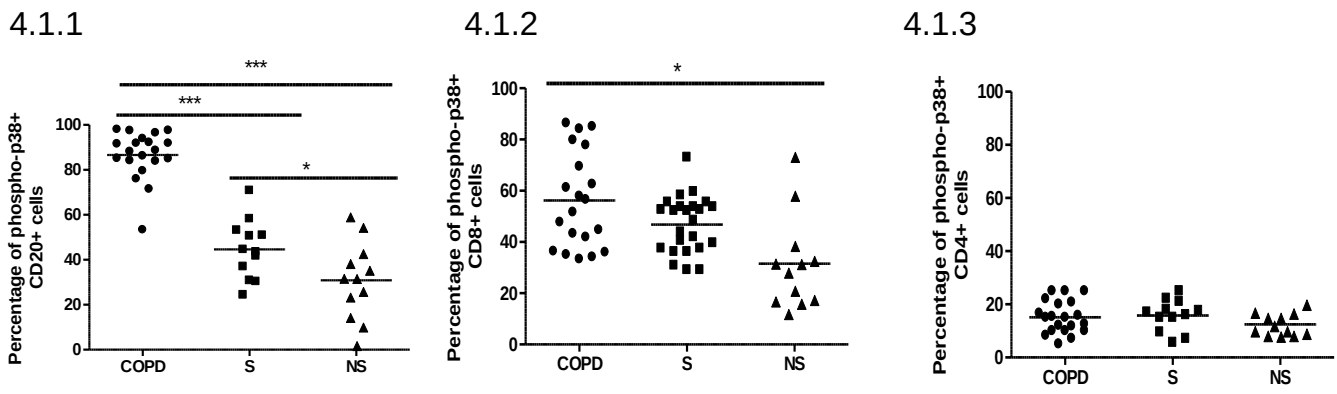


Figure 4.1 The mean percentage of phosphorylated p38 MAPK-positive follicular lymphocytes

The mean percentage of phosphorylated p38 MAPK-positive (4.1.1) follicular CD20+ B cells, (4.1.2) follicular CD8+ T cells, and (4.1.3) follicular CD4+ T cells. ANOVA followed by bonferroni multiple comparisons tests were used to show differences between patient groups for each cell type: *p <0.05, **p<0.01, ***p<0.001

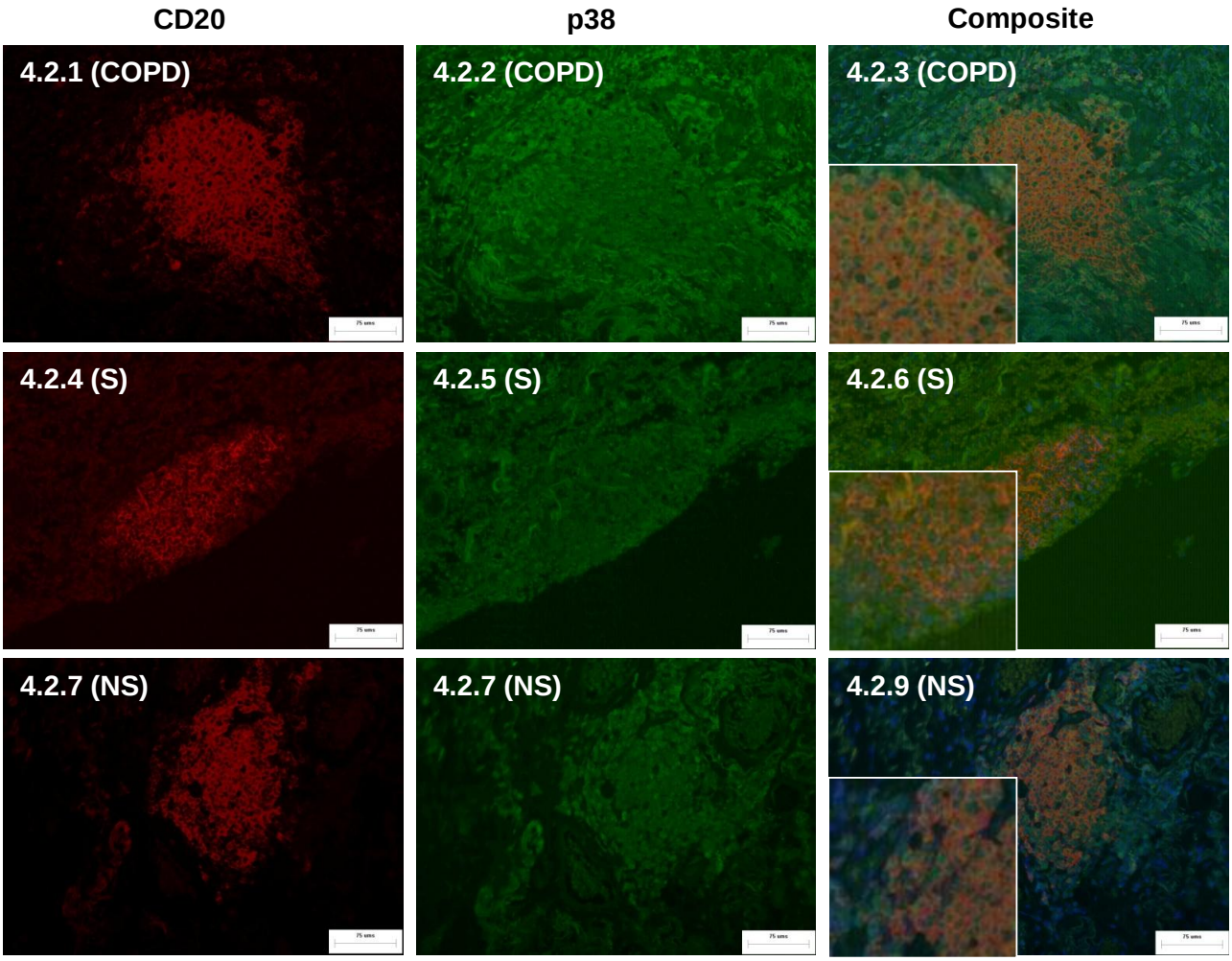


Figure 4.2 Phosphorylated p38 MAPK in CB20+ B lymphocytes
Representative images for the dual label immunofluorescent detection of phosphorylated (phospho-) p38 MAPK in CD20+ B cells in inflammatory follicles within 4µm thick sections of human lung tissue. Representative images from (4.2.1–4.2.3) 20 patients with COPD, (4.2.4–4.2.6) 12 smokers and (4.2.7–4.2.9) 12 non-smokers are shown. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (blue). CD20+ cells were identified using an Alexa-488 conjugated goat anti-mouse secondary antibody (Red; 4.1.1, 4.2.4, 4.2.7) and phospho-p38 MAPK was detected using an Alexa 468 conjugated goat anti-rabbit secondary antibody (Green; 4.2.2, 4.2.5, 4.2.7). Composite images are shown (4.2.3, 4.2.6, 4.2.9). Green/yellow fluorescence is caused by intrinsically fluorescent tissue components such as elastic fibres and red blood cells. Autofluorescence can be distinguished from positive fluorescence by forming a composite image of the red, green and blue channels. Autofluorescence is visible in all 3 channels and so appears an amalgamation of the three colours. Positive fluorescence is visible in only one channel and thus appears as the pure colour. Magnification x200. COPD, chronic obstructive pulmonary disease; S, smoker; NS, non-smoker.

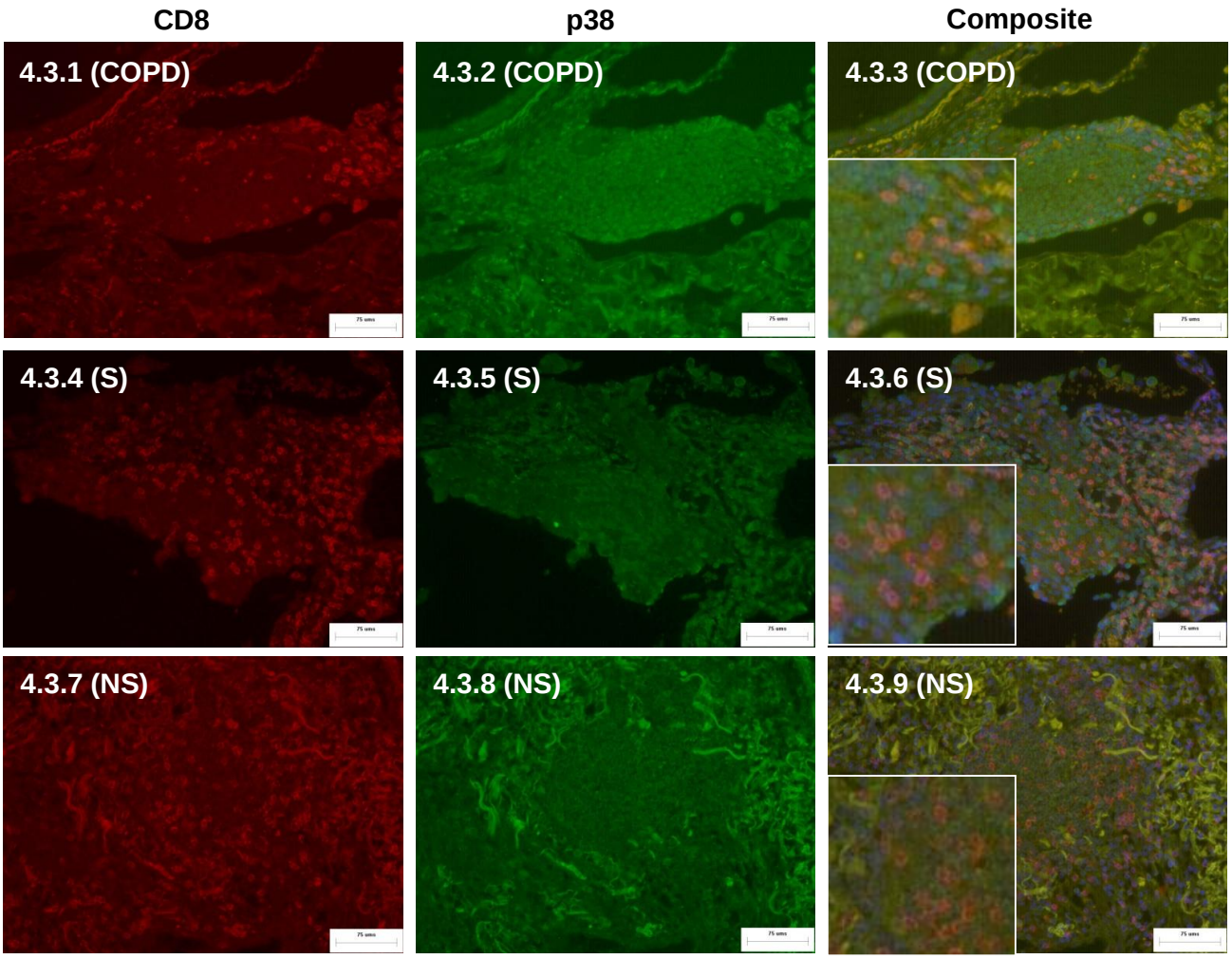


Figure 4.3 Phosphorylated p38 MAPK in CD8+ T cells

Representative images for the dual immunofluorescent detection of phosphorylated (phospho-) p38 MAPK in CD8+ T cells within inflammatory follicles in lung tissue. Representative images from (4.3.1–4.3.3) 20 patients with COPD, (4.3.4–4.3.6) 12 smokers and (4.3.7–4.3.9) 12 non-smokers are shown. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (Blue). CD8+ cells were identified using an Alexa 488 conjugated goat anti-mouse secondary antibody (Red; 4.3.1, 4.3.4, 4.3.7) and phospho-p38 MAPK was detected using an Alexa 468 conjugated goat anti rabbit secondary antibody (Green; 4.3.2, 4.3.5, 4.3.8). Composite images are also shown (4.3.3, 4.3.6, 4.3.9). Magnification x200. COPD, chronic obstructive pulmonary disease; S, smoker; NS, non-smoker.

4.3.1.2 Sub-epithelium

Numerically higher numbers of CD20+, CD8+ and CD4+ lymphocytes were found in the sub-epithelium of COPD lung tissue compared with control tissue samples, although these differences were not statistically significant (see **Table 4.3**). Phospho-p38-positive sub-epithelial lymphocyte percentages were similar across COPD, S and NS. Phospho-p38 MAPK was not found in any sub-epithelial CD4+ T lymphocytes in any patient group. Phospho-p38-positive sub-epithelial CD20+ and CD8+ lymphocytes as a percentage of total sub-epithelial CD20+ and CD8+ lymphocytes were significantly lower compared with follicular phospho-p38-positive CD20+ and CD8+ cells (ANOVA $p<0.0001$ for comparisons of both cell types).

Table 4.3 Phosphorylated p38 MAPK in subepithelial lymphocytes

	Number of lymphocytes per mm^2 sub epithelia				Percentage phospho-p38 MAPK expression			
	COPD n=20	S n=12	NS n=12	ANOVA p value	COPD n=20	S n=12	NS n=12	ANOVA p value
CD20+	181.64	113.47	108.81	0.55	4.35	4.84	3.24	0.90
CD8+	438.82	400.31	380.93	0.62	15.43	8.91	3.93	0.86
CD4+	84.44	60.36	57.4	0.98	0	0	0	0

Mean number of CD20+, CD8+ and CD4+ lymphocytes/mm^2 sub-epithelia and percentage expression of phosphorylated p38 MAPK. Summary of the mean number of CD20+/CD8+CD4+ lymphocytes identified per mm^2 of sub-epithelial tissue. The percentage of phospho-p38+ sub-epithelial lymphocytes are also shown. ANOVA p values shown for comparisons between patient groups for each tell types. COPD, chronic obstructive pulmonary disease; S, smokers; NS, non-smokers.

4.3.2 Phosphorylated p38 MAPK in macrophages

4.3.2.1 Alveolar macrophages

The percentage of phospho-p38-positive alveolar macrophages was significantly different between COPD, S and NS (ANOVA p<0.0001, **Figure 4.4.1** and **4.5.1–4.5.3**); Bonferroni multiple comparisons test showed that the percentage of phospho-p38-positive alveolar macrophages was significantly greater in COPD lungs compared with both S and NS (means 70.0%, 56.4% and 28.5% respectively, COPD vs. S p<0.01, COPD vs. NS p<0.001), and in S compared with NS (p<0.001).

4.3.2.1 Sputum macrophages

There was also a significant difference in the percentage of phospho-p38-positive sputum macrophages in COPD, S and NS (ANOVA p<0.0001, **Figure 4.4.2** and **4.5.4–4.5.6**); Bonferroni multiple comparisons test analysis showed that the percentage of phospho-p38-positive sputum macrophages was significantly greater in COPD samples (n=8) compared with both S (n=6) and NS samples (n=4) (means 92.8%, 59.3%, 31.8%, respectively; COPD vs. S and COPD vs. NS p<0.001 for both comparisons), and in S compared with NS (p<0.001).

4.3.3 Phosphorylated p38 MAPK in epithelial cells

A high percentage of total bronchial epithelial cells were positive for activated p38 MAPK in all three patient groups (**Figure 4.4.3** and **4.5.7–4.5.9**). There was a significant difference in the percentage of phospho-p38-positive bronchial epithelial cells in COPD, S and NS (ANOVA p=0.0112); Bonferroni multiple comparisons test

showed that there was a significantly higher percentage of phospho-p38-positive epithelial cells in COPD lungs compared with NS ($p<0.01$; means 96% and 81%, respectively).

4.3.4 Phosphorylated p38 MAPK in neutrophils

Phospho-p38 MAPK was not present in any lung (**Figure 4.5.10–4.5.12**) or sputum neutrophil analysed (**4.5.1–4.5.3**). The presence of phospho-p38 MAPK in PMNs isolated from the whole blood of patients with COPD was also analysed (**Figure 4.6.1**). Phospho-p38 MAPK was not present in PMNs examined immediately after isolation from blood, but phospho-p38 MAPK was induced following culture with and without LPS (100ng/ml) (**Figure 4.6.2**). Culture of sputum neutrophils with and without LPS stimulation (100ng/ml) did not induce phospho-p38 MAPK, indicating that the p38 MAPK pathway is not active in lung neutrophils (**Figure 4.6.3 and 4.6.4**).

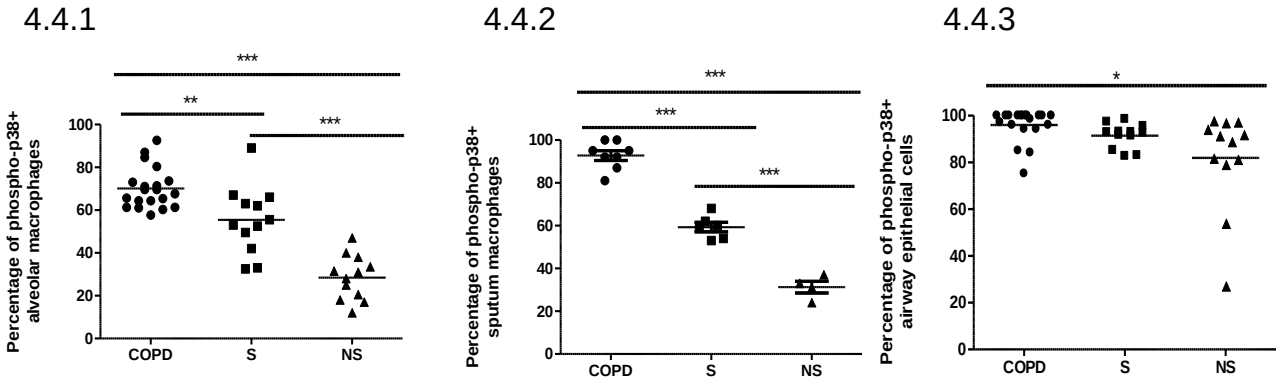


Figure 4.4 The mean percentage of phosphorylated p38 MAPK-positive alveolar macrophages, sputum macrophages and bronchial epithelial cells
The mean percentage of phosphorylated (phospho-) p38 MAPK+ (4.4.1) alveolar macrophages, (4.4.2) sputum macrophages, and (4.4.3) bronchial epithelial cells. ANOVA followed by bonferroni multiple comparisons tests were used to show differences between patient groups for each cell type: *p <0.05, **p<0.01, ***p<0.001.

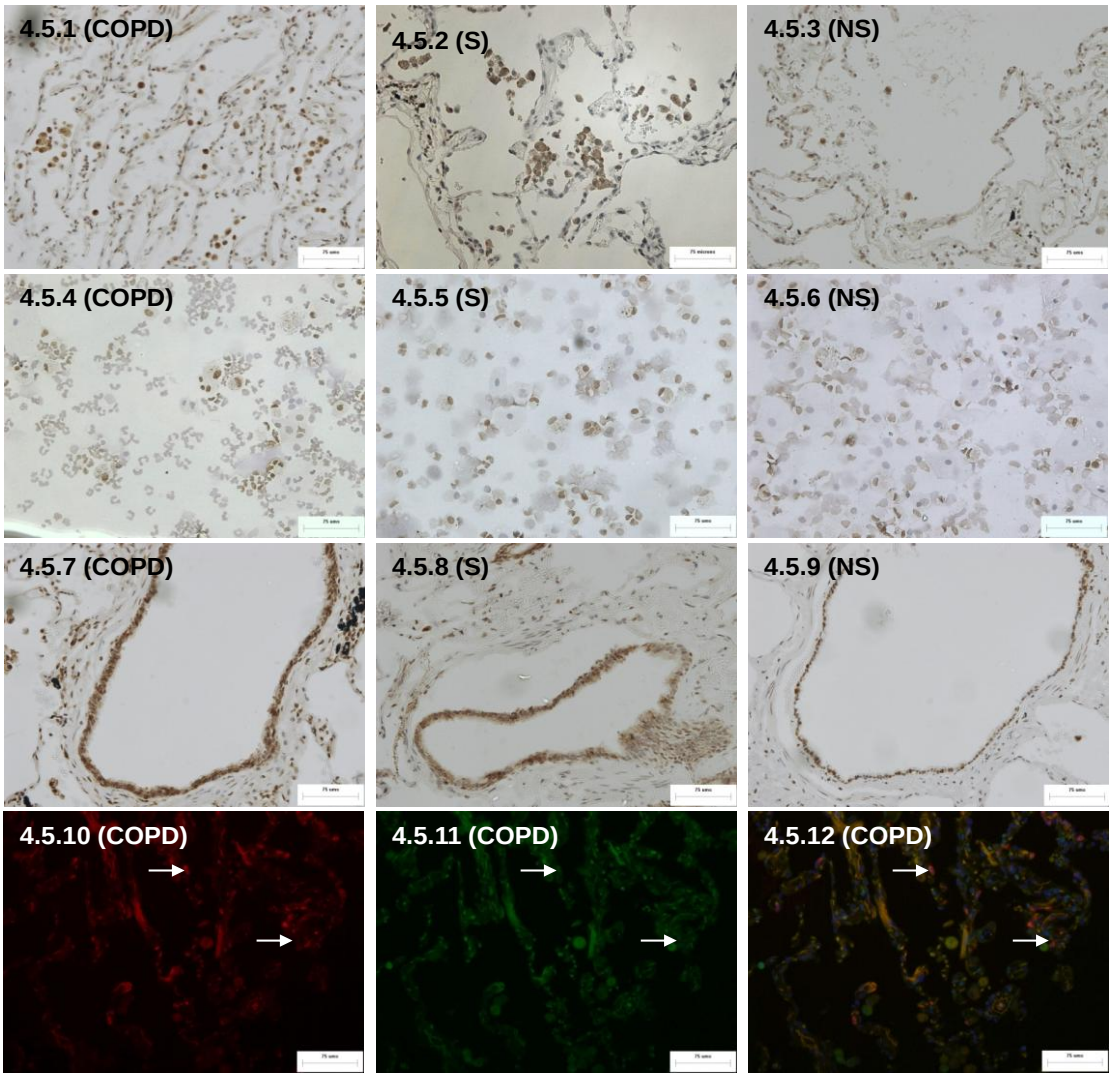


Figure 4.5 Phosphorylated p38 MAPK in alveolar and sputum macrophages, bronchial epithelial cells and lung tissue neutrophils

Representative images for the immunohistochemical and dual label immunofluorescent detection of phosphorylated (phospho-) p38 MAPK in human lung tissue and sputum cytospins. Representative images shown for (4.5.1–4.5.9) immunocytochemical and (4.5.10–4.5.12) Immunofluorescence tissue analysis from (4.5.1, 4.5.4, 4.5.7; n=20, D; n=8) patients with COPD, (4.5.2, 4.5.5, 4.5.8; n=12, E; n=6) smokers and (4.5.3, 4.5.6, 4.5.9; n=12 F; n=4) non-smokers are shown. Cell nuclei were counterstained with either Mayer's haematoxylin (Blue; 4.5.1–4.5.9) or 4', 6-diamidino-2- phenylindole (Blue; 4.5.10–4.5.12). For immunohistochemical analysis phospho-p38 MAPK expression was detected using 3, 3'-diaminobenzadine (Brown; 4.5.1–4.5.9). For dual label immunofluorescence (4.5.10–4.5.12) lung tissue neutrophils were identified using an Alexa-488 conjugated goat anti mouse secondary antibody (Red; 4.5.10) and phospho-p38 MAPK was detected using an Alexa 468 conjugated goat anti rabbit secondary antibody (Green; 4.5.11). Composite images for dual label immunofluorescence are also shown (4.5.12). Phospho-p38 MAPK expression in alveolar macrophages (Brown; top panel), sputum macrophages (Brown; 2nd from top panel) and small airway epithelial cells (Brown; 3rd from top panel). Lung tissue neutrophils (Red; shown by arrows) expressing phospho-p38 MAPK (Green; bottom panel). Magnification x200. COPD, chronic obstructive pulmonary disease; S, smoker; NS, non-smoker.

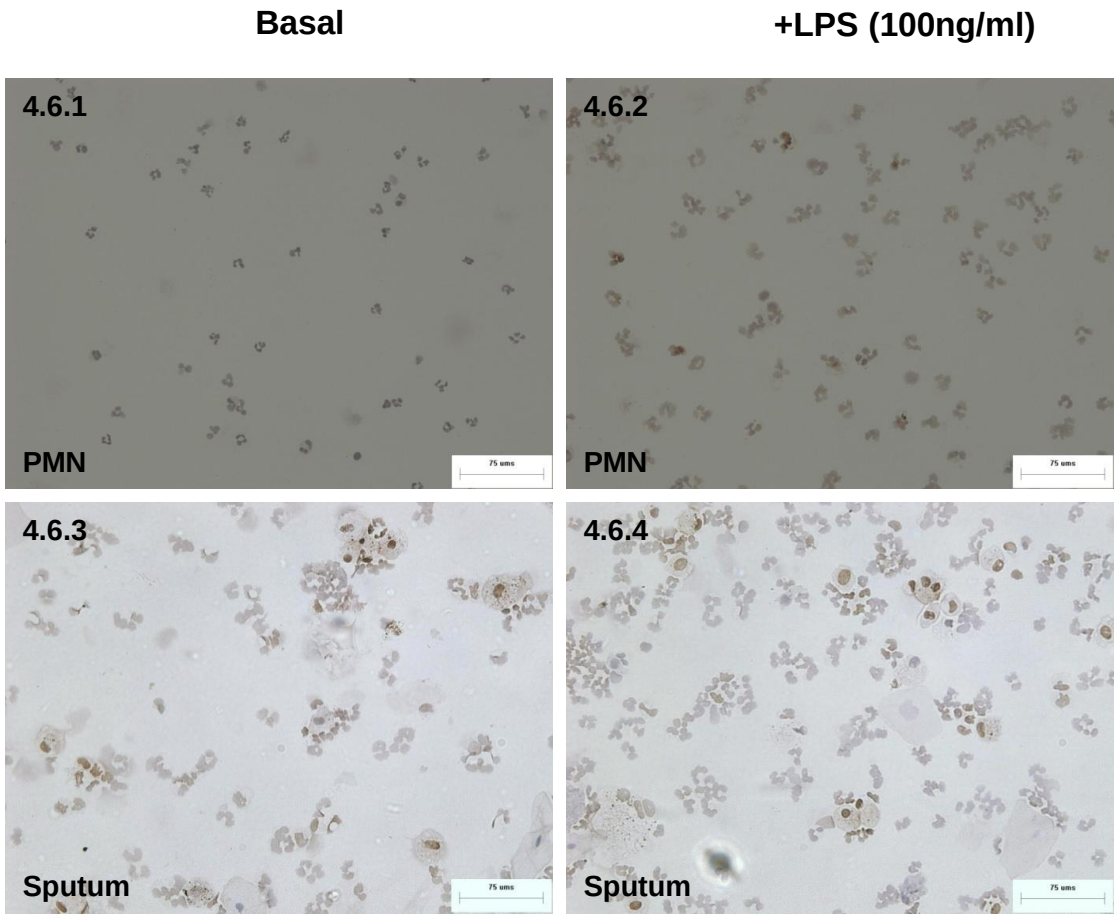


Figure 4.6 Phosphorylated p38 MAPK in isolated COPD blood and sputum neutrophils

Representative images for the immunohistochemical detection of phosphorylated (phospho-) p38 MAPK in isolated COPD blood and sputum neutrophils. Cell nuclei were counterstained with Mayer's haematoxylin (Blue). Phospho-p38 MAPK expression was detected using 3, 3'-diaminobenzadine following direct immunohistochemistry (Brown). (4.6.1) Phospho-p38 MAPK is absent in basal blood neutrophils. (4.6.2) Phospho-p38 MAPK (Brown) is induced in blood neutrophils following stimulation with 1µg/ml LPS. Phospho-p38 MAPK is absent in (4.6.3) basal and (4.6.4) LPS stimulated sputum neutrophils. Magnification x200. PMN, polymorphonucleocyte.

4.3.5 Effect of p38 MAPK inhibition on neutrophils

Neutrophils isolated from the blood of eight patients with COPD were cultured for 24 hours with and without LPS. The mean levels of basal TNFα and CXCL8 release were 577.24 (± 162.45) pg/ml and 3474.18(± 577.84) pg/ml, respectively, increasing to 1673 (± 591.74) pg/ml and 5866.44 (± 1035.3) pg/ml, respectively, after LPS stimulation (**Figure 4.7.1–4.7.4**). The p38 MAPK inhibitor caused a concentration dependant inhibition of TNFα (ANOVA $p<0.0001$) and CXCL8 (ANOVA $p<0.001$) in stimulated PMNs and CXCL8 in unstimulated PMNs (CXCL8 ANOVA $p<0.0001$). There were no differences between per cent inhibition of TNFα and CXCL8 in stimulated or unstimulated PMNs.

Previous studies have shown that LPS has minimal effects on pro-inflammatory mediator production from isolated sputum neutrophils (Pang et al, 1997; Baines et al, 2009) which I also observed (see Chapter 3). The mean levels of unstimulated TNFα and CXCL8 release were 702.41 (± 46.35) pg/ml and 4303.50 (± 654.75) pg/ml in sputum neutrophils. In unstimulated neutrophils isolated from sputum, the pharmacological p38 MAPK inhibitor had no effect on TNFα and CXCL8 production (TNFα ANOVA $p=0.1425$, CXCL8 ANOVA $p=0.8840$) (**Figure 4.7.5** and **4.7.6**).

One way ANOVA followed by post bonferroni's post-tests demonstrated that SB731445 has a significantly greater effect on the inhibition of both TNFα and CXCL8 released by unstimulated and LPS stimulated PMNs compared with sputum neutrophils (**Figure 4.8.1** and **4.8.2**).

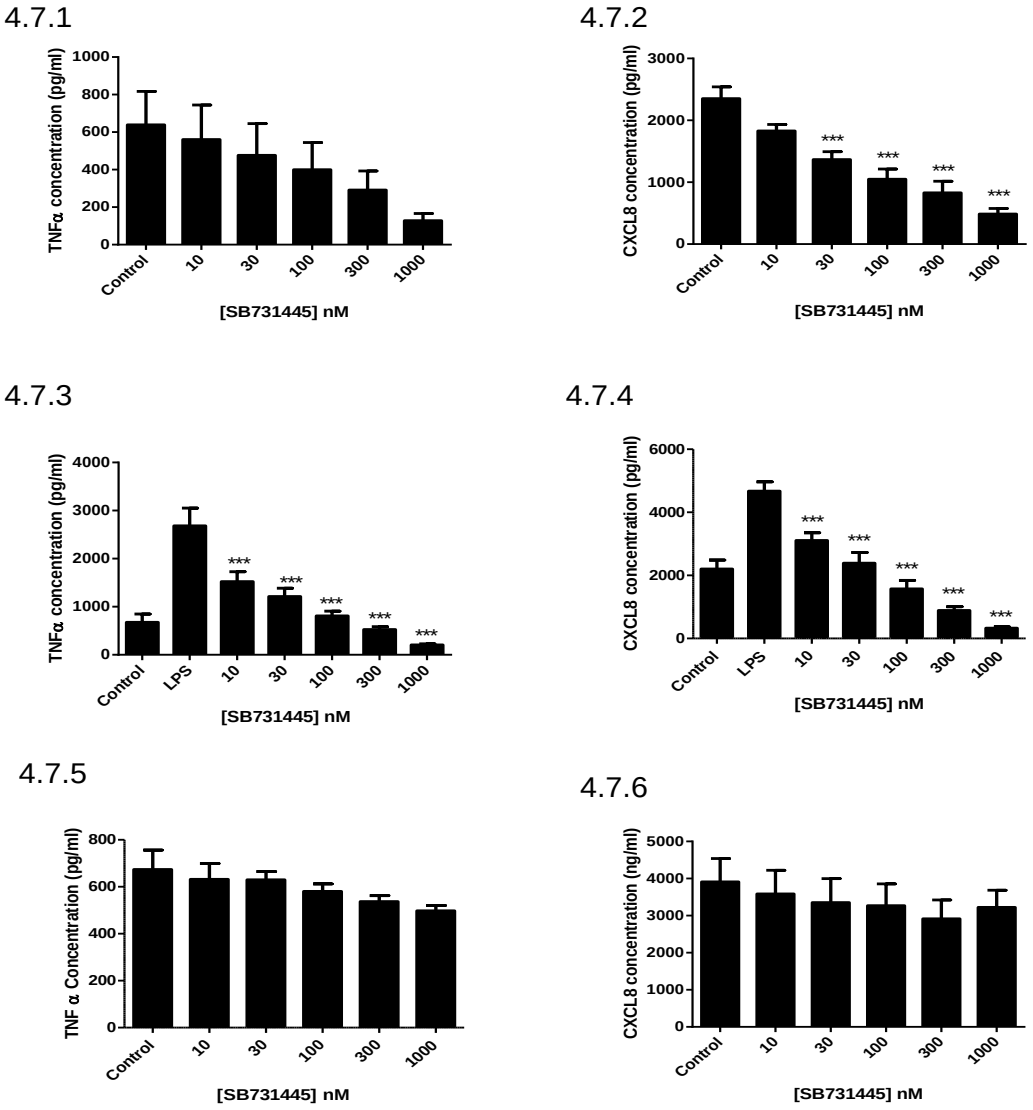
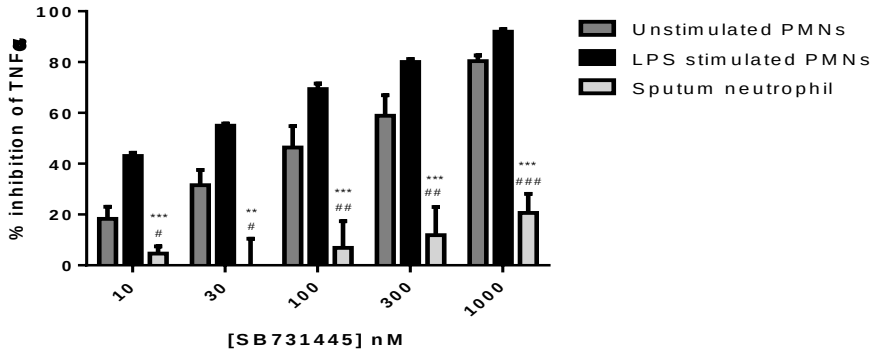


Figure 4.7 The release of pro-inflammatory mediators from isolated COPD blood and sputum neutrophils and the effect of SB731445

Absolute levels of (4.7.1, 4.7.3, 4.7.5) TNFα and (4.7.2, 4.7.4, 4.7.6) CXCL8 in isolated COPD blood and sputum neutrophils (n=7) following incubation with SB731445 (10–1000nM) for 24h. Supernatant levels of TNFα and CXCL8 were measured by ELISA. Data are presented as mean ± SEM. (4.7.1 and 4.7.2) unstimulated PMNs, (4.7.3 and 4.7.4) LPS stimulated PMNs, (4.7.5 and 4.7.6) unstimulated sputum neutrophils. ANOVA followed by Bonferroni multiple comparison post-test comparing absolute levels of pro-inflammatory mediator at each SB731445 concentration compared with control. ***p<0.001.

4.8.1



4.8.2

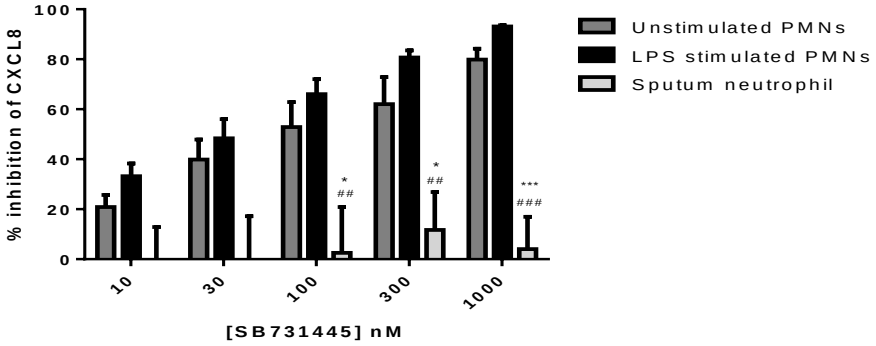


Figure 4.8 The effect of SB731445 on inhibition of TNFα and CXCL8 release from COPD isolated blood and sputum neutrophils

Inhibition of (4.8.1) TNFα and (4.8.2) CXCL8 in isolated COPD blood and sputum neutrophils (n=7) following incubation with SB731445 (10–1000nM) for 24 hours. Supernatant levels of TNFα and CXCL8 were measured by ELISA. Data are presented as mean ± SEM. One-Way ANOVA followed by Bonferroni's post-test were performed comparing sputum neutrophils and unstimulated and LPS stimulated PMN; a significantly reduced effect of SB731445 on sputum neutrophils compared with LPS stimulated PMNs is denoted by * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ and compared with unstimulated PMNs denoted by # $p<0.05$, ## $p<0.01$ and ### $p<0.0001$.

4.4 Discussion

This chapter describes the cell-specific presence of phospho-p38 MAPK in the lungs of patients with COPD compared with controls. There were increased numbers of phospho-p38-positive CD20+ and CD8+ lymphocytes within inflammatory follicles of COPD lung tissue compared with both smoking and non-smoking control samples. In addition, there were increased numbers of phospho-p38-positive sputum and alveolar macrophages and small airway epithelial cells in COPD lung tissue compared with control tissue. In contrast, phospho-p38 MAPK was not present in lung neutrophils in any of the tissue samples analysed. This demonstrates that p38 MAPK signalling pathway is activated in specific cell types in the lungs of patients with COPD. For these cell types, the data suggest that cigarette smoking increases the number of cells phospho-p38-positive cells in the lungs and the development of COPD induces a further increase in cell numbers positive for phospho-p38 MAPK.

Increased numbers of lymphoid follicles in the small airways of patients with COPD are associated with more severe disease (Hogg et al, 2004). These follicles may be important sites of antigen presentation to lymphocytes (Van der Strate et al, 2006; Lambers et al, 2009). There was an increased number of phospho-p38-positive CD20+ B cells in COPD lungs compared with smoking and non-smoking controls. The p38 MAPK pathway is known to be involved in a variety of B cell functions, including B cell maturation (Petro et al, 2002), and activation via ligation of CD40 (Brady et al, 2001; Petro et al, 2002), suggesting that pharmacological inhibitors of phospho-p38 MAPK may target B cell functions within the lungs of patients with COPD.

Similarly, there were increased numbers of phospho-p38-positive CD8+ T cells in COPD lungs. Previous studies have shown that the number of CD8+ T cells in the lung parenchyma and small airways increases with disease severity (Saetta et al, 1998; Saetta et al, 1999; O'Shaughnessy et al, 1997; Hogg et al, 2004). *Ex-vivo* stimulation of CD8+ T cells with IL-12 and IL-2 (Gollob et al, 1999), and with CD3/CD28 with and without PMA (Leung-Theung-Long et al, 2009) have both been shown to induce activation of the p38 MAPK pathway in these cells. The p38 MAPK pathway is known to play important roles in the regulation of CD8+ T cell function, such as the production of IFNγ (Merritt et al, 2000), and pharmacological inhibition of p38 MAPK has been shown to reduce IL-2 release from these cells (Tham et al, 2001). Interestingly, there was no increase in numbers of phospho-p38-positive CD20+ B cells and CD8+ T cells within the sub-epithelial regions. This suggests that the lymphocytes within the follicles have a different physiological function compared with other lung lymphocytes, which may be a result of follicular lymphocytes lying within an environment that resembles lymph nodes rather than normal tissue.

Inflammatory follicles within the lung also contain CD4+ T lymphocytes, which are typically located around the periphery of the lymphoid follicle (Hogg et al, 2004; Plumb et al, 2009). *Ex vivo* stimulation of CD4+ T lymphocytes has been shown to induce activation of the p38 MAPK pathway (Wong et al, 2007) and release of IL-4, IL-5, IL-10 and IL-13 by these cells is inhibited by a pharmacological p38 MAPK inhibitor (Dodeller et al, 2005). Numbers of phospho-p38-positive CD4+ T cells was similar in all patient groups. The percentage of phospho-p38-positive CD4+ T cells within these follicles was much lower than that observed for both CD8+ T cells and CD20+ B cells, suggesting that p38 MAPK pathway is less important in the functions

of CD4+ T cells within lung inflammatory follicles. p38 MAPK signalling has been demonstrated to be involved in CD4+ T lymphocyte development (Hsu et al, 2003) and cytokine production (Rincon et al, 1998; Koprak et al, 1999; Dodeller et al, 2005) but these findings suggest that the p38 MAPK pathway does not play a critical role in lung CD4+ T cell physiology. A greater understanding of CD4+ T cell biology in COPD is required to fully understand the role they play in disease progression and to identify alternative inflammatory pathways to target novel anti-inflammatory therapeutics against.

Increased numbers of macrophages are found in the lung parenchyma and alveolar space of smoker and COPD lungs (Finkelstein et al, 1995; Shapiro, 1999; Armstrong et al, 2009). Renda et al, (2008), found an increase in phospho-p38-positive alveolar macrophages in COPD lungs compared with controls (Renda et al, 2008). In the current chapter these findings have been confirmed in alveolar macrophages, and also extended to show the same phenomenon in sputum macrophages. The data suggest that cigarette smoking increases the number of phospho-p38 MAPK-positive alveolar macrophages, and numbers are further increased by the onset of COPD. Exposure to cigarette smoke has been shown to increase p38 MAPK phosphorylation in rat lungs (Marwick et al, 2004), which demonstrates that cigarette smoke is a key orchestrator of p38 MAPK activation in the lung. Alveolar macrophages are well known to play a central role in COPD pathophysiology through the release of a variety of pro-inflammatory mediators such as TNFα and CXCL8. Previous studies have shown that pharmacological inhibition of p38 MAPK can reduce pro-inflammatory cytokine production from macrophages (Smith et al,

2006; Kent et al, 2009; Armstrong et al, 2009), indicating that this cell type is a target for p38 MAPK inhibitors that are currently in clinical development.

Cigarette smoke can induce the release of a variety of pro-inflammatory mediators from bronchial epithelial cells *in vitro ,* including TNFα, TGFβ, IL-1β, IL-6 and CXCL8 (Mio et al, 1997; Takizawa et al, 2001; Hellermann et al, 2002; Beisswenger et al, 2004). Cigarette smoke has also been shown to induce phospho-p38 MAPK in a bronchial epithelial cell line (Nasreen et al, 2012). Together, these data implicate the bronchial epithelium in the pathogenesis of COPD. In the present study, the percentage of phospho-p38-positive small airway epithelial cells was significantly higher in COPD lungs. Increased phospho-p38 MAPK expression in airway epithelial cells has also been found in severe asthma (Liu et al, 2008). COPD and severe asthma are associated with persistently elevated levels of pro-inflammatory mediators in the airways. Activation of the p38 MAPK pathway is induced through a range of inflammatory stimuli, and it appears that bronchial epithelial cells in both severe asthma and COPD lungs respond to these stimuli through phosphorylation of p38 MAPK.

Phospho-p38 MAPK was absent in all lung neutrophils. This was not a disease-specific phenomenon as neutrophils in both smoking and non-smoking lungs were also found to be devoid of activated p38 MAPK. Stimulation of lung neutrophils with LPS, which is known to induce phosphorylated p38 MAPK in a number of different cell types, did not induce phospho-p38 MAPK. The data suggest that the pro-inflammatory activity of lung neutrophils is not dependent on phospho-p38 MAPK signalling. This was confirmed by the lack of effect of the p38 MAPK inhibitor on pro-inflammatory mediator production from lung neutrophils. In contrast, activated p38

MAPK was observed in blood neutrophils, and cytokine production from these cells was inhibited by the p38 MAPK inhibitor. As discussed previously in Chapter 3 this highlights a potential pitfall of using blood neutrophils as a model for lung neutrophils, as there appear to be differences in the signalling mechanisms responsible for pro-inflammatory mediator production.

Neutrophils are key players in the innate immune response, releasing pro-inflammatory cytokines and tissue destructive proteases. The number of neutrophils is increased in the lungs of patients with COPD (Lacoste et al, 1993; Keatings et al, 1996; Keatings and Barnes, 1997), but the data presented herein indicate that p38 MAPK inhibitors that may be used in clinical trials will have little effect on pro-inflammatory mediator production from these cells. The data also indicates that neutrophils leaving the bloodstream and entering the lung undergo phenotypic changes, altering the activity of intracellular signalling pathways required for important cell functions. Previous studies have shown that pharmacological inhibition of p38 MAPK in blood neutrophils inhibits other neutrophil effector functions such as the release of superoxide (Suzuki et al, 2001), and chemotaxis (Cara et al, 2001; Nick et al, 1997; Nick et al, 2002; Fujita et al, 2005). This study demonstrates that p38 MAPK inhibition as a therapeutic strategy in COPD is unlikely to target these actions in lung neutrophils, although further work would be required to confirm this.

Activation of the p38 MAPK pathway may change the response to GC in times of oxidative stress. Indeed, as discussed, increased p38 MAPK activation has been shown in macrophages from patients with COPD and severe asthma and in severe asthma epithelial cells (Renda et al, 2008; Bhavsar et al, 2008; Lui et al, 2008), and patients with asthma and COPD both show limited responses to GC, which further

supports this hypothesis. Previous data has shown that GC insensitivity in IL-2 and IL-4 stimulated peripheral blood mononuclear cells can be reversed through the use of a pharmacological p38 MAPK inhibitor (Irusen et al, 2002). In addition, IL-13, TNFα and IL-1α can inhibit the normal activity of the GR by activating p38 MAPK, possibly via the 211 or 226 phosphorylation sites on the GR (Szatmary et al, 2004; Spahn et al, 1996; Wang et al, 2004). Expression of the negative regulator of p38 MAPK MKP-1 has also been shown to be lower in severe asthma alveolar macrophages (Bhavsar et al, 2008) and dexamethasone has been shown to induce MKP-1 expression (Abraham et al, 2006), thereby regulating p38 MAPK. Taken together, this suggests that cell-specific activation of the p38 MAPK pathway in patients with COPD may be involved in the reduced effectiveness of GC in the management of disease, and therefore, that p38 MAPK inhibition may, in part, restore GC insensitivity in patients with COPD. In addition, activation of the p38 MAPK pathway induces pro-inflammatory mediator release from a variety of cells, which means that increased numbers of cells positive for activated p38 MAPK are likely to be releasing pro-inflammatory mediators, activating other cells, thus perpetuating the pro-inflammatory environment present in patients with COPD. Targeting the p38 MAPK pathway in COPD pathway may be a useful therapeutic strategy to dampen down the inflammation in the lungs. In addition, dual therapy comprising GC and a p38 MAPK inhibitor may offer further clinical benefits in suppressing activated p38 MAPK, restoring GC sensitivity and attenuating the pro-inflammatory environment within COPD lungs.

In conclusion, increased percentages of phospho-p38-positive B cells, CD8+ T cells, macrophages and bronchial epithelial cells were found in COPD lungs compared

with controls. This contrasts with CD4+ T cells and neutrophils, where no increase in numbers was observed. In neutrophils, there was a complete lack of p38 MAPK activation, and these results indicate that p38 MAPK inhibitors in clinical development for the treatment of COPD will have no effect on cytokine production from these cells. Targeting the p38 MAPK pathway may have therapeutic benefits in the treatment of COPD, and these drugs are likely to exert their anti-inflammatory effects through the cell types identified in this study, which are positive for activated p38 MAPK expression.

Chapter 5

The sensitivity of bronchial epithelial cells to GC and p38 MAPK inhibition

5.1 Introduction

The anatomical position of the bronchial airway epithelium allows direct contact with inhaled noxious particles such as cigarette smoke and therefore represents a viable target for anti-inflammatory therapies in COPD. *In vitro* studies show that cigarette smoke activates epithelial cells to produce a variety of pro-inflammatory mediators and proteases, including TNFα, TGFβ, IL-1β, IL-6 and CXCL8 (Hellerman et al, 2002, Mio et al, 1997, Takizawa et al, 2001, Beisswenger et al, 2004) via activation of the NFκB transcription factor pathway (Hellermann et al, 2002, Beisswenger et al, 2004). Primary epithelial cells from patients with COPD produce elevated levels of CXCL8 compared with control cells (Schulz et al, 2003), which may be a result of NFκB (Tasi et al, 2006) and the p65 subunit of NFκB being over-expressed (Di Stefano et al, 2002). Together these data suggest that bronchial epithelial cells are a key cell type involved in the development of COPD.

GC exert their anti-inflammatory affects in two ways; transactivation whereby the receptor/ligand binds to GRES found in the promoter regions of responsive genes; and transrepression whereby the complex binds to and represses other transcription factors, thus inhibiting expression of pro-inflammatory genes. GC are able to mediate responses in bronchial epithelial cells (Wang et al, 1997, Korn et al, 2001, Gallelli et al, 2010) by down-regulating pro-inflammatory cytokine gene expression (Kwon et al, 1995), inducing MKP-1 expression (Newton et al, 2010) and destabilising mRNA molecules (Chang et al, 2001). Although GC are widely used in the treatment of COPD, the effects on disease progression and symptom control are somewhat

limited. GC insensitivity has been shown to be a cytokine and cell-specific phenomenon in alveolar macrophages, which is independent of disease and/or smoking status (Hew et al, 2006, Bhavsar et al, 2008, Armstrong et al, 2009, Kent et al, 2009). Understanding the mechanisms underlying the bronchial epithelial cell response to GC would be beneficial in understanding the contribution of these cells to the overall sensitivity of patients with COPD to GC treatment.

Activation of the p38 MAPK pathway can enhance the function of transcription factors such as NFκB and ATF-2, which further amplify gene expression of pro-inflammatory mediators including IL-6, CXCL8 and TNFα. Post-transcriptional roles for p38 MAPK include the stabilisation of cellular mRNAs involved in the immune response including IL-6 and CXCL8 (Winzen et al, 1999). Inhibitors of p38 MAPK have been shown to attenuate pro-inflammatory mediator production from alveolar macrophages in a concentration-dependant manner (Birrell et al, 2006, Smith et al, 2006, Kent et al, 2009) and as such may be a useful anti-inflammatory treatment in COPD. Pharmacological p38 MAPK inhibitors are in clinical development for the treatment of COPD (Singh et al, 2010).

There is limited published information regarding the p38 MAPK pathway in COPD bronchial epithelial cells, although cigarette smoke has been shown to induce activation of the p38 MAPK pathway in a dose-dependent manner in a bronchial epithelial cell line (Nasreen et al, 2012). Increased phospho-p38 MAPK expression in airway epithelial cells has also been shown in severe asthma (Liu et al, 2008). Like COPD, severe asthma is associated with continually elevated levels of pro-inflammatory mediators in the airways. Bronchial epithelial cells in severe asthma respond to pro-inflammatory stimuli, such as pro-inflammatory cytokines, through

activation of the p38 MAPK pathway. In the previous chapter, increased numbers of bronchial epithelial cells were found to have activated p38 MAPK in COPD lungs compared with controls, suggesting that a similar response to pro-inflammatory stimuli occurs in COPD. Understanding the contribution of the p38 MAPK pathway in bronchial epithelial cell functions would enable a greater understanding of how targeting this pathway in these cells may be useful in COPD treatment.

The aim of this chapter was to characterise the responsiveness of a bronchial epithelial cell line, namely 16HBEs, to GC, the pharmacological p38 MAPK inhibitor birb 796, and combination treatment with GC and birb 796. Previous studies have shown the IC_{50} values (i.e. the concentration of dexamethasone that inhibits 50% of the maximal response) of dexamethasone in LPS-stimulated alveolar macrophages to be 29.5nM, 8.1nM and 13.2nM for CXCL8, IL-6, and RANTES, respectively (Armstrong et al, 2011). The IC_{50} of birb 796 has been reported as 0.1mM (Product information leaflet, Selleck Chemicals). As such, concentrations of 0.1, 1, 10, 100, and 1000nM were chosen to investigate the effects of dexamethasone and birb 796 on pro-inflammatory mediator production in bronchial epithelial cells. The effects of LPS, TNFα and Poly (I:C), as *in vitro* representations of bacterial infection, a pro-inflammatory cytokine and viral infection, on activation of the p38 MAPK pathway was also evaluated.

5.2 Methods

5.2.1 16HBE cell culture

16HBE are an immortalized, but non-transformed cell line, derived from ciliated human bronchial epithelial cells that line the airways of the lung (Cozens et al 1994). They have been demonstrated to retain differentiated epithelial morphology and functions, and have intact tight junctions and cilia. They were chosen as a representative of bronchial epithelial cells due to their wide usage in other studies investigating the p38 MAPK pathway in bronchial epithelial cells.

16HBE were maintained in supplemented MEM. Confluent cells grown in tissue culture plates were placed in fresh supplemented media and stimulated with LPS (1µg/ml), TNFα (10ng/ml) or the synthetic analogue of double-stranded RNA, and as such a TLR3 ligand, Poly (I:C) (100µg/ml). For some experiments cells were pre-incubated for 2 hours with dexamethasone (0.1–1000nM) and/or the p38 MAPK inhibitor birb 796 (0.1–1000nM) and incubated for various time points in 5% CO_2 at 37°C. Plates were centrifuged at 2000rpm for 10 minutes at 4°C and cell free supernatants removed and stored at -80°C for cytokine analysis

5.2.2 Protein expression assay

For western blots, cells were lysed in RIPA buffer (10mM Tris-HCL, PH 7.4, 150mM NaCl, 1mM EDTA, 1%Nonidet P-40, 0.25%) containing phosphatase and protease inhibitors. Cell lysates were stored at -80°C until analysis.

5.2.2.1 Bradford Assay

Protein concentrations from whole cell extracts were determined using Bradford Reagent as described earlier.

5.2.2.2 Polyacrylamide gel electrophoresis

Cell lysates were diluted in sample buffer (62.5MM Tris, 10% glycerol, 1% SDS, 1% beta-mercaptoethanol, and 0.1% bromphenol blue, pH 6.8) and boiled for 10 minutes prior to electrophoresis on SDS-polyacrylamide gels (10%). Proteins were then transferred to Hy-Bond ECL nitrocellulose membranes. Membranes were incubated in blocking buffer (5% dried milk power in TBST) followed by primary antibody diluted in blocking buffer overnight at 4°C. Primary antibodies used were phospho-p38 MAPK, and total p38 MAPK. After washing, the membranes were incubated with a peroxidise-conjugated secondary antibody followed by detection using lumigen TMA-6 enhanced chemiluminescent. Densitometric analysis was performed by normalising band density to that for total antibody using Quantity One v4.6.1 software.

5.2.3 Cytokine release analysis

ELISA was used to determine the supernatant level of CXCL8, IL-6 and RANTES according to the manufacturer's instructions. Lower limits of detection were 32.5pg/ml for CXCL8 and RANTES and 9.375pg/ml for IL-6.

5.2.4 Statistical Analysis

Absolute levels of cytokine and per cent inhibition at each dexamethasone and birb 796 concentration were normally distributed and compared using ANOVA followed by Bonferroni's multiple comparisons tests. All statistical analysis was performed using Graphpad InStat version 3.00 for Windows 95.

To determine whether a combination of dexamethasone and birb 796 exhibited synergy, two additional analyses were carried out by Chris Harbron at Astrazeneca, Macclesfield, UK. The first was a dose-sparing analysis, which determined whether equivalent responses could be achieved at lower doses of drug than expected given the monotherapy responses of the two drugs. This calculates a combination index with confidence intervals, as described previously (Harbron, 2010). The second was an efficacy-enhancing analysis. This determined whether the combination of the two drugs resulted in a significantly greater maximal effect than either compound alone. Hill dose-response curves were fitted to the monotherapy and combination results using both common and separate parameters for maximal response and tested for the improvement-in-fit from allowing the parameter to vary by using an F test. Both analyses were performed assuming a slope parameter in the Hill-dose response equation equal to one. Robustness analyses were also performed estimating the slope parameters and found to give the same conclusions.

5.3 Results

5.3.1 Phosphorylated p38 MAPK expression by 16HBEs

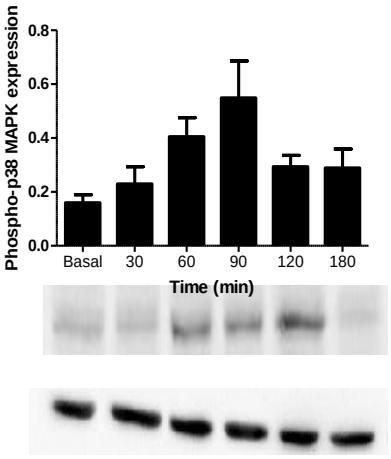
A time course of the phosphorylation of p38 MAPK was conducted using three stimulants; LPS, TNFα and poly (I:C), using previously published data in the literature as an indicator of the time periods to be used. 16HBEs were cultured in the presence of each stimulant; for LPS and poly (I:C) stimulation a time course from 0 to 180 minutes was conducted, for TNFα a time course up to 60 minutes was conducted.

LPS stimulation induced the activation of p38 MAPK after around 60 minutes of stimulation (**Figure 5.1.1**). Phosphorylated p38 MAPK was still present up to 120 minutes after stimulation and had returned to basal levels by 180 minutes.

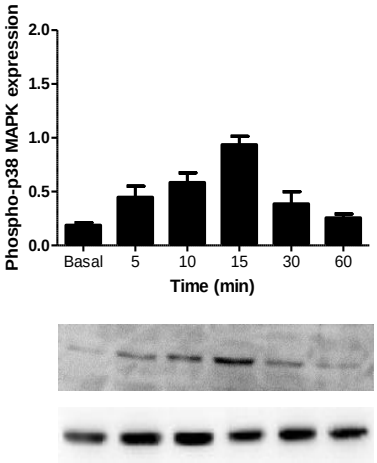
TNFα stimulation induced activation of p38 MAPK much quicker than LPS stimulation, with an increase in phosphorylated p38 MAPK seen after just 5 minutes of stimulation (**Figure 5.1.2**). Phosphorylation peaked at around 15 minutes, returning to basal levels by 60 minutes.

Stimulation of phosphorylated p38 MAPK by poly (I:C) had a time course similar to that of LPS stimulation, with increased levels being seen after 30 minutes, but peaking at around 90 minutes (**Figure 5.1.3**). Levels were returning to basal by 180 minutes.

5.1.1. LPS stimulated phospho-p38 MAPK



5.1.2. TNFα stimulated phospho-p38 MAPK expression



5.1.3. Poly (I:C) stimulated phospho-p38 MAPK expression

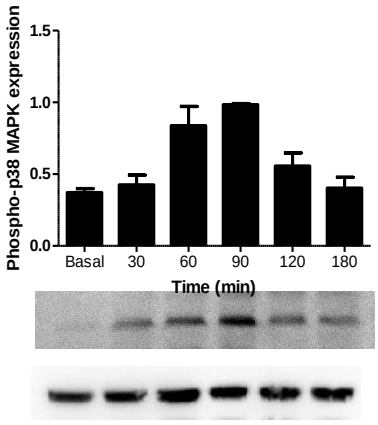


Figure 5.1 The activation of p38 MAPK in 16HBEs
16HBEs were stimulated with (5.1.1) LPS (1µg/ml), (5.1.2) TNFα (10ng/ml) or (5.1.3) Poly (I:C) for various times up to 180 minutes. Data are shown as mean ± SEM. Protein band density normalised to total p38 MAPK. Representatives of 3 blots are shown for each stimulus.

5.3.2 Pro-inflammatory mediator production from human bronchial epithelial cells (16HBEs)

16HBEs were cultured in the presence of one of three stimulants; LPS, TNFα and Poly (I:C), for 24 hours. Supernatant levels of CXCL8, IL-6 and RANTES were measured by ELISA. Mean unstimulated levels of CXCL8, IL-6 and RANTES were 159.92, 350.92 and 459.33pg/ml, respectively.

LPS stimulation increased production of CXCL8, IL-6 and RANTES, with mean levels of 982.08, 1719.86 and 847.47pg/ml measured, respectively. TNFα stimulation also increased the release of all three pro-inflammatory mediators, with mean levels of 3937.58, 5366.23 and 833.12pg/ml of CXCL8, IL-6 and RANTES, respectively. 16HBEs were less responsive to Poly (I:C) stimulation, as 100µg/ml of Poly (I:C) induced 286.03pg/ml of CXCL8 and 1106.81pg/ml of IL-6. Poly (I:C) stimulation did cause higher release of RANTES secretion from 16HBEs, with mean levels of 1829.56pg/ml observed.

5.3.3 Dexamethasone-mediated inhibition of stimulated pro-inflammatory mediator release

5.3.3.1 Stimulated CXCL8 release

Dexamethasone significantly reduced absolute levels of LPS-, TNFα- and Poly (I:C)-stimulated CXCL8 release (ANOVA $p<0.0001$, $p<0.0001$, $p=0.0002$, respectively, **Figure 5.2.1–5.2.3**). Bonferroni multiple comparison post test showed that dexamethasone concentrations of 1–1000nM significantly reduced absolute levels of

CXCL8 release ($p<0.001$ for all comparisons). Dexamethasone significantly reduced TNFα-induced CXCL8 release at 10nM ($p<0.05$) and at 100nM and 1000nM ($p<0.001$ for both comparisons) and Poly (I:C)-induced CXCL8 release at 100nM ($p<0.05$) and 1000nM ($p<0.01$). The mean maximum per cent inhibition of LPS, TNFα and Poly (I:C) induced CXCL8 release were 60.8, 62.7 and 75.3%, respectively.

5.3.3.2 Stimulated IL-6 release

Dexamethasone induced a dose-dependent inhibition of LPS-, TNFα- and Poly (I:C)-stimulated IL-6 release, with mean maximum per cent inhibitions of 81.7, 80.1 and 79.4% seen, respectively (**Figure 5.2.4–5.2.6**).

Dexamethasone significantly inhibited LPS-, TNFα- and Poly (I:C)-stimulated IL-6 release (ANOVA $p<0.0001$ for all comparisons). Bonferroni multiple comparison post-test analysis showed significantly lower levels of LPS-stimulated IL-6 from cells treated with 1nM ($p<0.05$) and 10–1000nM ($p<0.001$ for all comparisons) dexamethasone, significantly lower absolute levels of TNFα-induced release of IL-6 at concentrations of 1nM ($p<0.01$), and 10–1000nM ($p<0.001$ for all comparisons) and concentrations of 10–1000nM ($p<0.001$ for all comparisons) induced significantly lower levels of Poly (I:C)-induced IL-6.

5.3.3.3 Stimulated RANTES release

Stimulated RANTES release was dose-dependently inhibited by dexamethasone treatment, with mean maximum per cent inhibitions of 56.9, 42.5 and 69.1% for LPS-, TNFα- and Poly (I:C)-stimulated release, respectively (**Figure 5.2.7–5.2.9**).

Dexamethasone significantly inhibited LPS-, TNFα- and Poly (I:C)-stimulated RANTES release (ANOVA $p=0.0003$, $p<0.0001$, $p<0.0001$, respectively). Bonferroni multiple comparison post-test analysis showed that concentrations of 1nM ($p<0.05$) and 10nM ($p<0.01$) and 100–1000nM ($p<0.001$ for both comparisons) had the most potent inhibitory effects on LPS-stimulated RANTES release, 10nM ($p<0.05$) and 100–1000nM ($p<0.001$ for both comparisons) significantly inhibiting TNFα-stimulated RANTES release and concentrations of 1nM ($p<0.05$) and 10–1000nM causing significantly lower concentrations of Poly (I:C)-induced RANTES release.

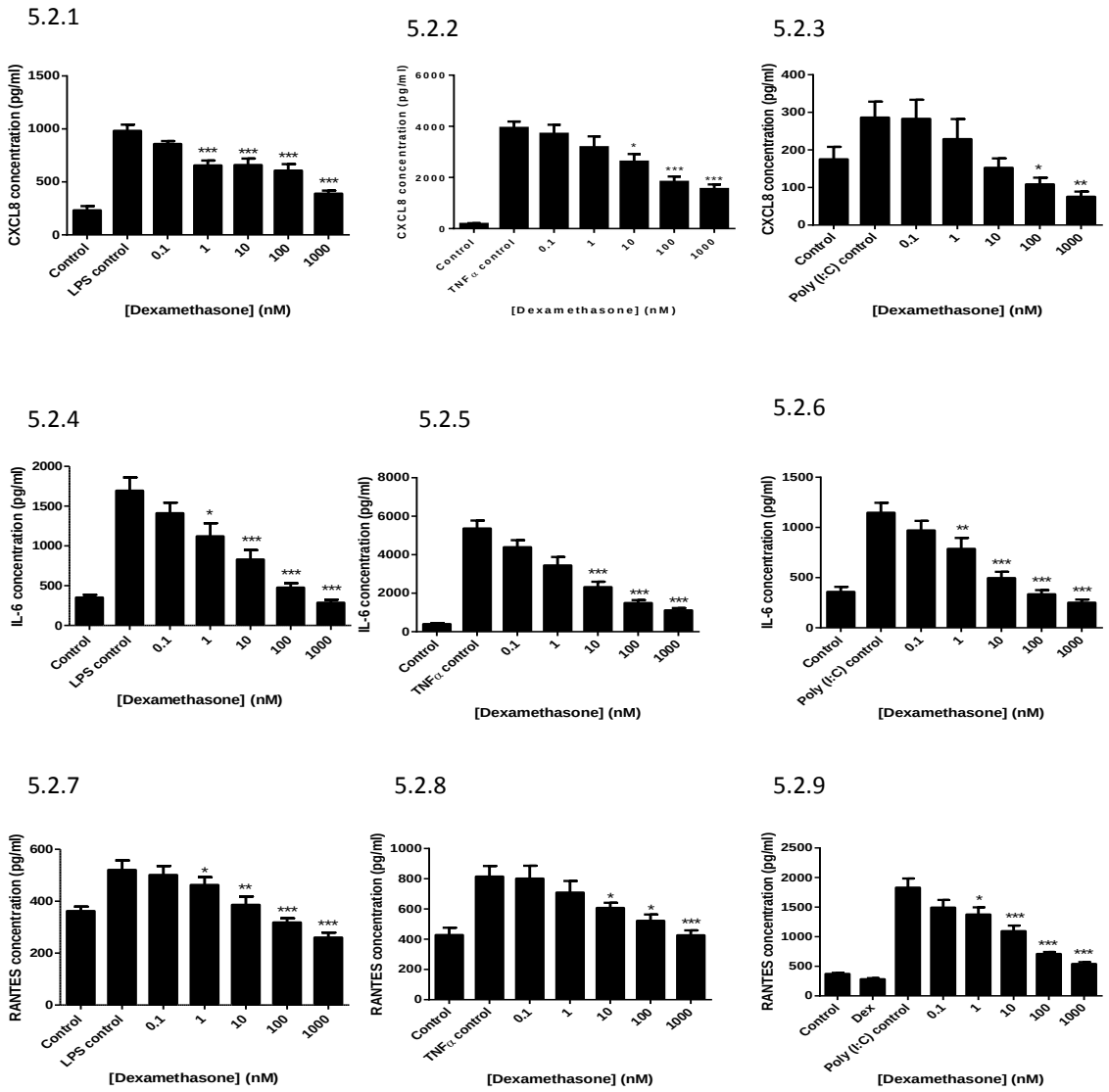


Figure 5.2 The effect of dexamethasone on stimulated pro-inflammatory mediator release

16HBEs were pre-incubated with dexamethasone (0–1000nM) for 2 hours followed by stimulation with (5.2.1, 5.2.4, 5.2.7) LPS (1µg/ml), (5.2.2, 5.2.5, 5.2.8) TNFα (10ng/ml) or (5.2.3, 5.2.6, 5.2.9) Poly (I:C) (100µg/ml) for 24 hours. Supernatant levels of CXCL8, IL-6 and RANTES were measured by ELISA. Data are shown as mean ± SEM of stimulated CXCL8, IL-6 and RANTES release. Data was analysed by ANOVA, followed by bonferroni multiple comparison test for comparing absolute levels of pro-inflammatory mediator release to stimulated control levels. *p<0.05, **p<0.01, ***p<0.001.

5.3.4 Birb 796-*mediated inhibition of stimulated pro-inflammatory mediator release*

5.3.4.1 Stimulated CXCL8 release

Birb796 induced a dose-dependent inhibition of LPS-, TNFα- and Poly (I:C)-stimulated CXCL8 release with mean maximum per cent inhibitions of 47.6, 58.6 and 60.8%, respectively.

Birb 796 significantly inhibited LPS-stimulated CXCL8 release (ANOVA $p<0.0001$; **Figure 5.3.1–5.3.3**), with significantly lower levels of CXCL8 released following incubation with 10–1000nM compared with stimulated release ($p<0.001$ for all comparisons). TNFα-induced CXCL8 release was also significantly inhibited by birb 796 (ANOVA $p<0.0001$). CXCL8 release was significantly following incubation with birb 796 concentrations of 10nM ($p<0.05$) and 100–1000nM ($p<0.001$). Poly (I:C) induced CXCL8 was significantly inhibited by birb 796 (ANOVA $p<0.0001$), with significantly lower levels seen with birb 796 concentrations of 1000nM ($p<0.001$) compared with Poly (I:C)-stimulated release alone.

5.3.4.2 Stimulated IL-6 release

Birb 796 induced a dose-dependent inhibition of stimulated IL-6 release, with mean maximum per cent induced inhibitions of 46.6, 61.8 and 66.2% for LPS-, TNFα- and Poly (I:C)-stimulated release, respectively.

Birb 796 significantly inhibited release of IL-6 from LPS- (ANOVA $p=0.0026$), TNFα- (ANOVA $p<0.0001$) and Poly (I:C)-stimulated (ANOVA $p<0.0001$) IL-6 release

(**Figure 5.3.4–5.3.6**). Bonferroni multiple comparison post-test analyses showed that levels of IL-6 were significantly lower from LPS-stimulated cells treated with 100nM ($p>0.05$) and 1000nM ($p<0.01$) compared with stimulated cells. Levels of TNFα-stimulated IL-6 were also significantly lower in supernatants from cells treated with birb 796 concentrations of 10–1000nM ($p<0.001$) compared with stimulated levels. Absolute levels of IL-6 were significantly lower from Poly (I:C)-stimulated 16HBEs treated with birb 796 at concentrations of 10nM ($p<0.05$) and 100-1000nM ($p<0.001$ for both comparisons) compared with Poly (I:C) stimulated levels.

5.3.4.3 Stimulated RANTES release

Birb 796 induced a dose-dependent inhibition of LPS-, TNFα- and Poly (I:C)-stimulated RANTES release with mean maximum per cent inhibitions of 35, 30.8 and 44.7% observed, respectively.

Birb 796 significantly inhibited LPS-, TNFα- and Poly (I:C)-stimulated RANTES release (ANOVA $p=0.0003$, $p<0.0001$, $p<0.0001$, respectively, **Figure 5.3.7–5.3.9**). Bonferroni multiple comparison post-test analysis of absolute levels of RANTES showed that 10nM ($p<0.01$) and 100–1000nM ($p<0.001$) birb 796 concentrations significantly reduced RANTES levels compared with LPS-stimulated levels alone. Birb 796 was less effective at inhibiting TNFα-induced RANTES release, with no significant differences in absolute levels of RANTES release at any concentration of birb 796 compared with TNFα-stimulated levels. Birb 796 was very effective at inhibiting Poly (I:C)-induced RANTES release with significantly lower absolute levels seen after treatment with 1nM ($p<0.01$) and 10–1000nM ($p<0.001$ for all comparisons) compared with Poly (I:C)-induced levels alone.

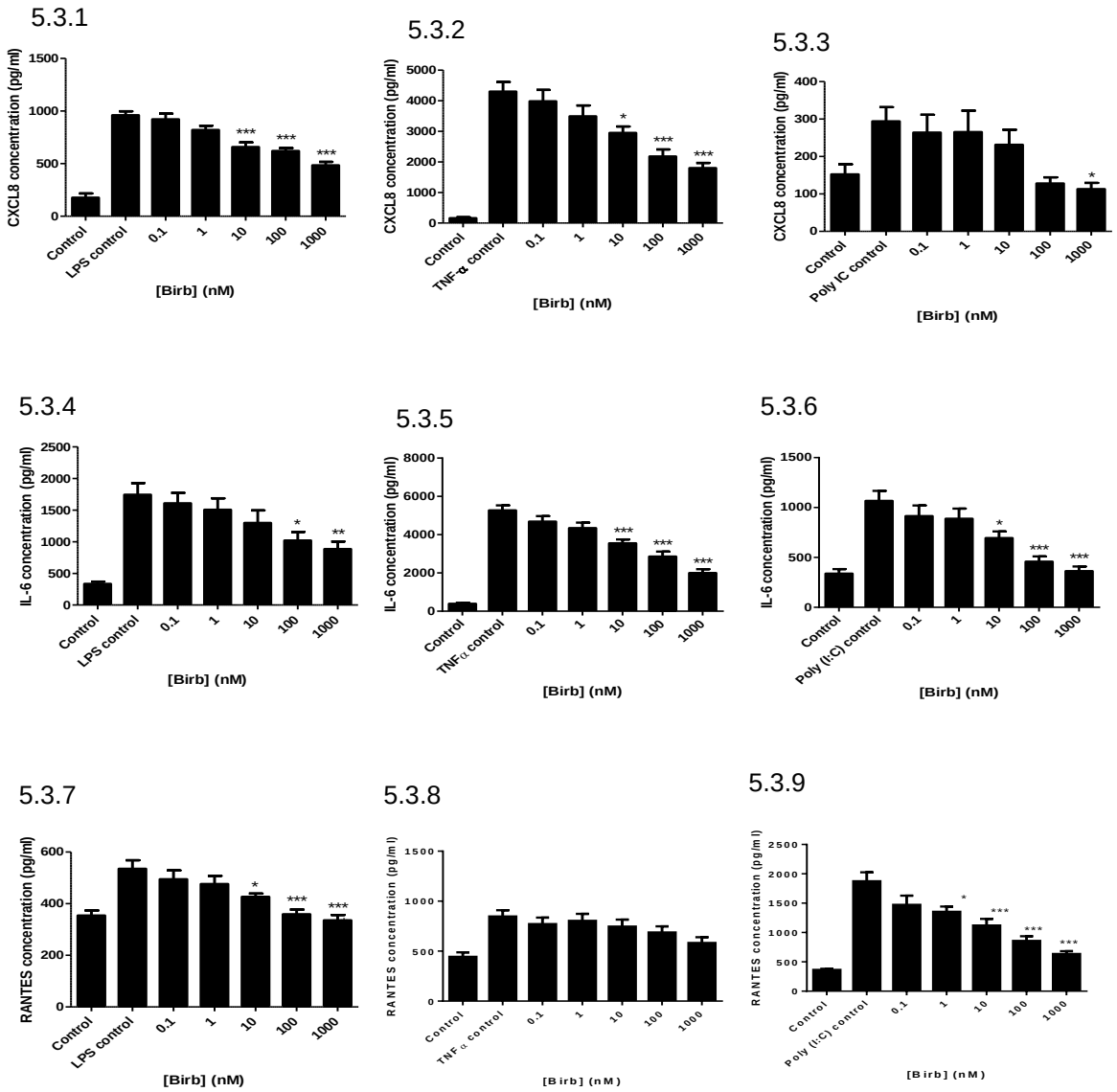


Figure 5.3 The effect of birb 796 on stimulated pro-inflammatory mediator release

16HBEs were pre-incubated with birb 796 (0–1000nM) for 2 hours followed by stimulation with (5.3.1, 5.3.4, 5.3.7) LPS (1µg/ml), (5.3.2, 5.3.5, 5.3.8) TNFα (10ng/ml) or (5.3.3, 5.3.6, 5.3.9) Poly (I:C) (100µg/ml) for 24 hours. Supernatant levels of CXCL8, IL-6 and RANTES were measured by ELISA. Data are shown as mean ± SEM of stimulated CXCL8, IL-6 or RANTES release. Data were analysed by ANOVA, followed by bonferroni multiple comparison test for comparing absolute levels of pro-inflammatory mediator to stimulated control levels. *p<0.05, **p<0.01, ***p<0.001.

5.3.5 Comparison of dexamethasone and birb 796-mediated inhibition on stimulated pro-inflammatory mediator release

5.3.5.1 Stimulated CXCL8 release

Paired t-test analysis showed that dexamethasone had a significantly greater inhibitory effect on LPS-stimulated CXCL8 release at 1nM compared with birb 796 and a significantly greater inhibitor effect on Poly (I:C)-stimulated CXCL8 release at 1nM ($p<0.05$), 10nM ($p<0.001$) and 1000nM ($p<0.05$) compared with birb 796 (**Figure 5.4.1–5.4.3**).

5.3.5.2 Stimulated IL-6 release

Dexamethasone had a significantly greater inhibitor effect on LPS-stimulated IL-6 release at 1nM ($p<0.05$), 10–100nM ($p<0.01$) and 1000nM ($p<0.001$) compared with birb 796 and at 10–1000nM ($p<0.001$ for all comparisons) on TNFα-stimulated IL-6 release. Dexamethasone had a significantly greater effect on Poly (I:C)-induced IL-6 at 1nM ($p<0.05$), and 10–1000nM ($p<0.001$) compared with birb 796 (**Figure 5.4.4–5.4.6**).

5.3.5.3 Stimulated RANTES release

There were no significant differences in the effects of dexamethasone and birb 796 LPS- and TNFα-stimulated RANTES release at any concentrations, except for LPS-stimulated RANTES release where dexamethasone had a greater effect compared with birb 796 at 1000nM ($p<0.05$). Dexamethasone had a significantly greater effect

on Poly (I:C)-stimulated RANTES release at 10nM ($p<0.05$) and 100–1000nM ($p<0.01$) compared with birb 796 (**Figure 5.4.7–5.4.9**).

Table 5.1. **Relative sensitivity of stimulated 16HBE bronchial epithelial cells to dexamethasone and birb 796**

	LPS stimulation	TNFα stimulation	Poly (I:C) stimulation
CXCL8 release	+++ dexamethasone +++ birb 796	+++ dexamethasone +++ birb 796	+++ dexamethasone ++ birb 796
IL-6 release	+++ dexamethasone + birb 796	+++ dexamethasone + birb 796	+++ dexamethasone + birb 796
RANTES release	++ dexamethasone ++ birb 796	++ dexamethasone ++ birb 796	+++ dexamethasone + birb 796

+Indicates relative sensitivity of stimulated pro-inflammatory mediator release to dexamethasone and birb 796. IL, interleukin; RANTES, regulated upon activation, normal T cell expressed and secreted.

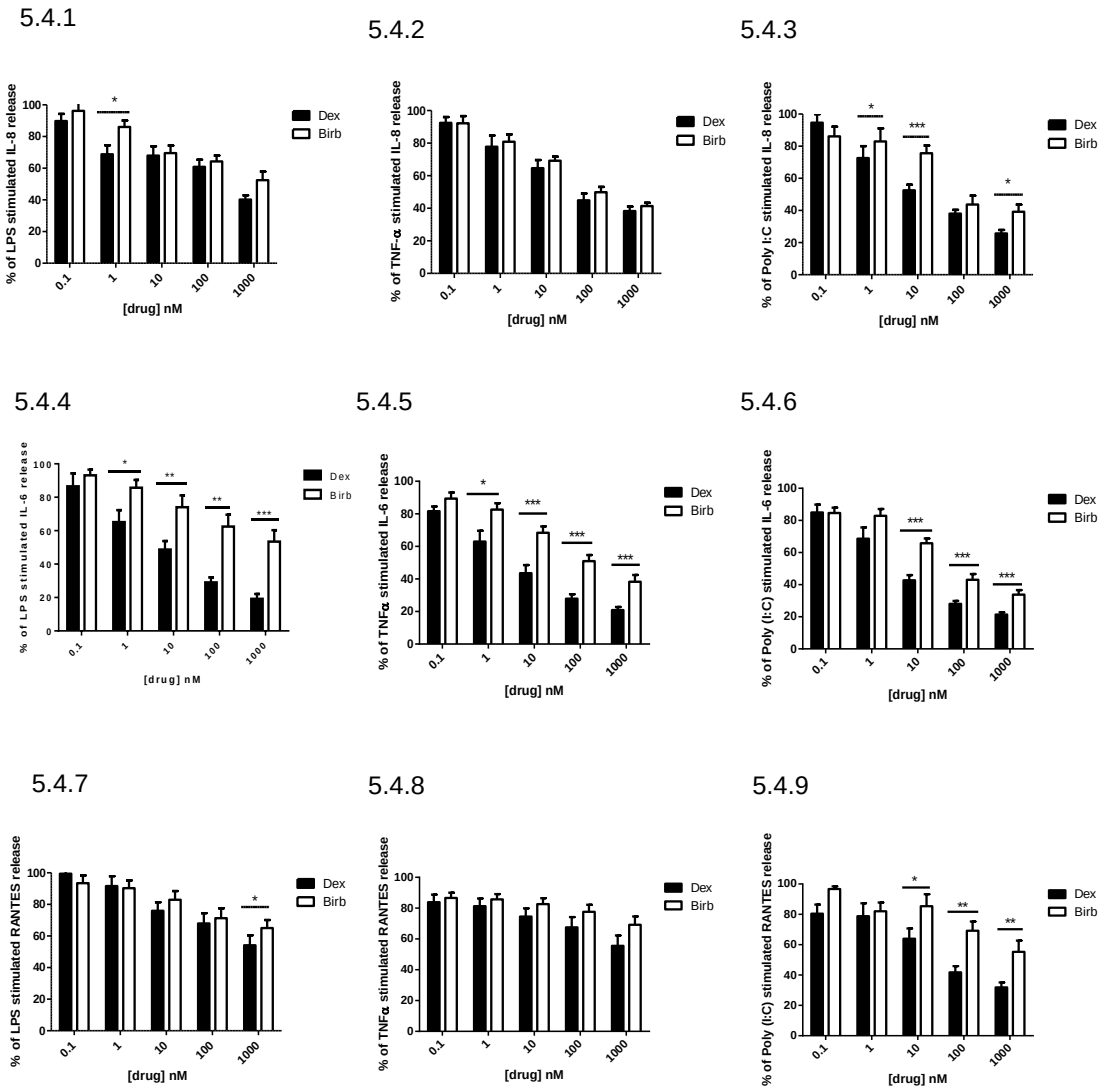


Figure 5.4 Comparison of dexamethasone- and birb 796-mediated inhibition of stimulated pro-inflammatory mediator release

16HBEs were pre-incubated with dexamethasone (0–1000nM) birb (0–1000nM) for 2 hours followed by stimulation with (5.4.1–5.4.3) LPS (1µg/ml), (5.4.4–5.4.6) TNFα (10ng/ml) or (5.4.7–5.4.9) Poly (I:C) (100µg/ml) for 24 hours. Supernatant levels of CXCL8, IL-6 and RANTES were measured by ELISA. Data are shown as % of stimulated CXCL8, IL-6 or RANTES release. Data was analysed by paired t-test comparing percentage of stimulated release between effect of dexamethasone and birb 796. *p<0.05, **p<0.01, ***p<0.001.

5.3.6 Combination effect of dexamethasone and birb 796 on pro-inflammatory mediator production in 16HBEs

5.3.6.1 Stimulated CXCL8 release

Combining 0.1nM birb 796 with increasing concentrations of dexamethasone lead to increased per cent inhibition of LPS-, TNFα- and Poly (I:C)-stimulated CXCL8 release in 16HBEs compared with using birb 796 alone (**Figure 5.5** and **5.8**). Mean maximum per cent inhibition of CXCL8 release for LPS-, TNFα- and Poly (I:C)-stimulated release was 85.3, 79.4 and 89.7%, respectively, which was observed when the maximum concentration of birb 796 (1000nM) was combined with the maximum concentration of dexamethasone (1000nM). For Poly (I:C)- (p=0.004) and TNFα- (p=0.007) stimulated CXCL8 release, there were significant synergistic dose sparing effects for combination therapy of dexamethasone and birb 796 (**Figure 5.11.1**). In addition, significant efficacy enhancement was observed for TNFα-stimulated CXCL8 release (p=0.001) (**Figure 5.11.2**).

5.3.6.2 Stimulated IL-6 release

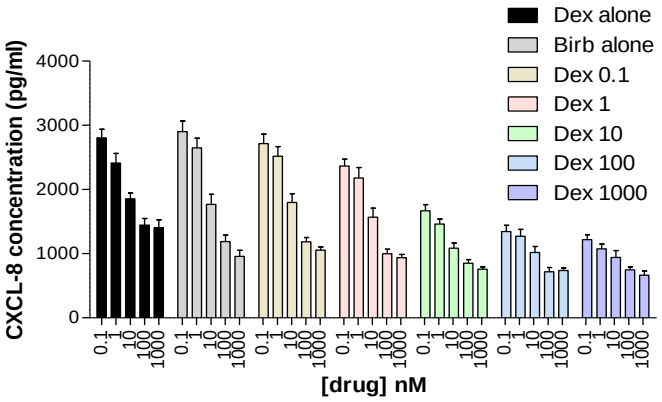
Combining 0.1nM birb 796 with increasing concentrations of dexamethasone induced higher per cent inhibitions of LPS-, TNFα- and Poly (I:C)-stimulated IL-6 release compared with using birb 796 alone (**Figure 5.6 and 5.9**). Combining 0.1nM dexamethasone with increasing concentrations of birb 796 also induced higher per cent inhibitions compared with using dexamethasone alone. At the higher concentrations of dexamethasone, i.e. 10–1000nM there was little added effect of increasing birb 796 concentrations. Maximum mean per cent inhibition of LPS-,

TNFα- and Poly (I:C)-stimulation of IL-6 release from 16HBEs was 88.2, 84.6 and 84.6% respectively, which all correspond to maximum birb 796 and dexamethasone combination treatment (1000nM). For LPS-stimulated IL-6 release, significant synergistic dose-sparing effects were observed for dexamethasone and birb combination therapy (p=0.003) (**Figure 5.11.1**). In addition, significant efficacy enhancements were demonstrated for both LPS- and TNFα-stimulated IL-6 release (p=0.012 and p=0.010 for LPS and TNFα-stimulation, respectively) (**Figure 5.11.2**).

5.3.6.3 Stimulated RANTES release

Combining 0.1nM birb 796 with increasing concentrations of dexamethasone increased per cent inhibition of LPS- and Poly (I:C)-stimulated RANTES release (**Figure 5.7** and **5.10**). Mean maximum per cent inhibitions for LPS- and Poly (I:C)-stimulated RANTES release were 56.04 and 56.4%, respectively, which was achieved by combing the maximum concentrations of both drugs. There was no increased effect of combining dexamethasone and birb on TNFα-stimulated RANTES release compared with using either drug alone. Maximum per cent inhibition of TNFα-stimulated RANTES release was achieved using the maximum concentration of dexamethasone (1000nM). For Poly (I:C)-stimulated RANTES release significant dose-sparing effects were seen when dexamethasone was used in combination with birb 796 (p=0.002) (**Figure 5.11.1**). Significant efficacy enhancements were observed for combination therapy for LPS-stimulated RANTES release (p=0.028) (**Figure 5.11.2**).

5.5.1



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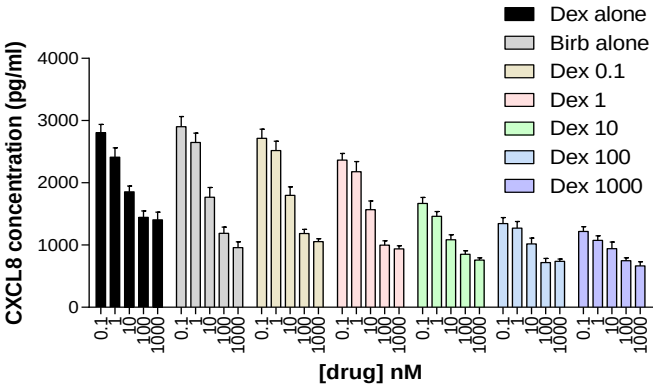

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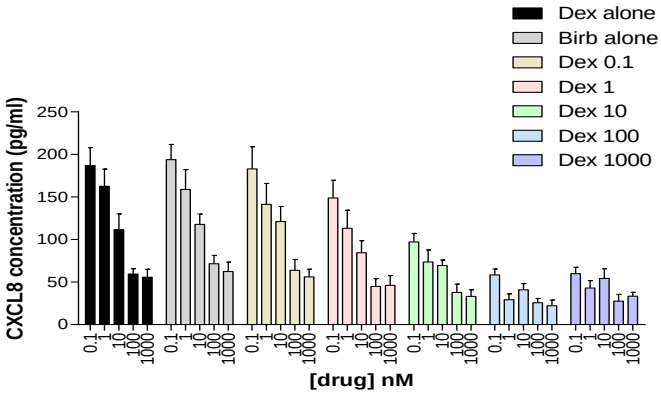
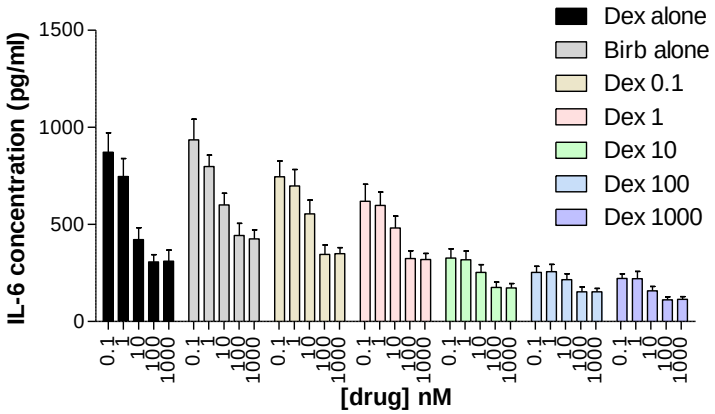


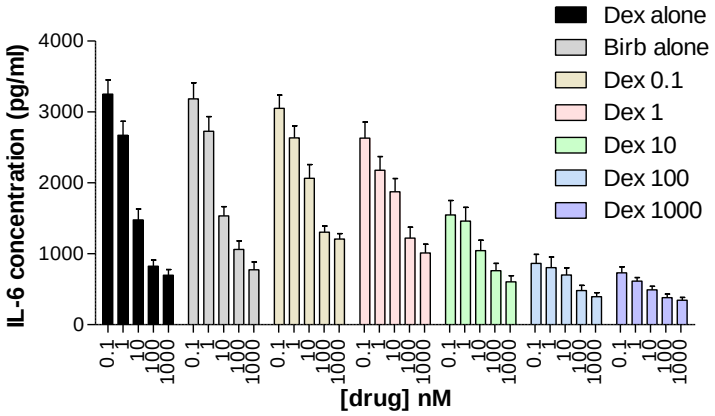
Figure 5.5 The combination effect of dexamethasone and birb 796 on absolute levels of CXCL8 release in 16HBEs

16HBEs were pre-incubated with dexamethasone and/or birb 796 for 2 hours prior to stimulation with 5.5.1) 1µg/ml LPS, 5.5.2) 10ng/ml TNFα or 5.5.3) 100µg/ml Poly (I:C). Supernatant levels of CXCL8 were measured by ELISA. Data shown are mean ± SEM. Each experiment was carried out 4 times with different cell passages.

5.6.1



5.6.2



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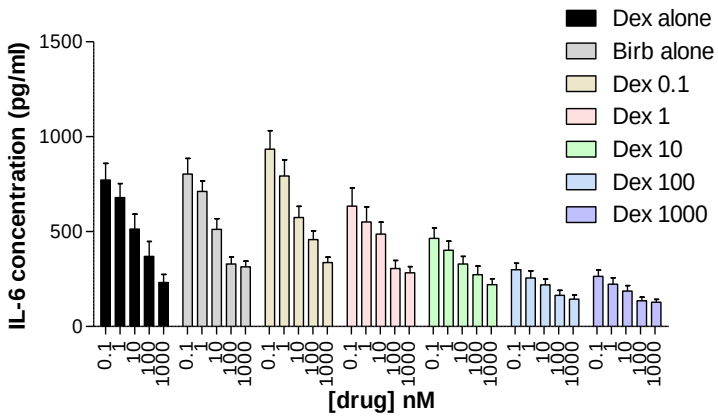
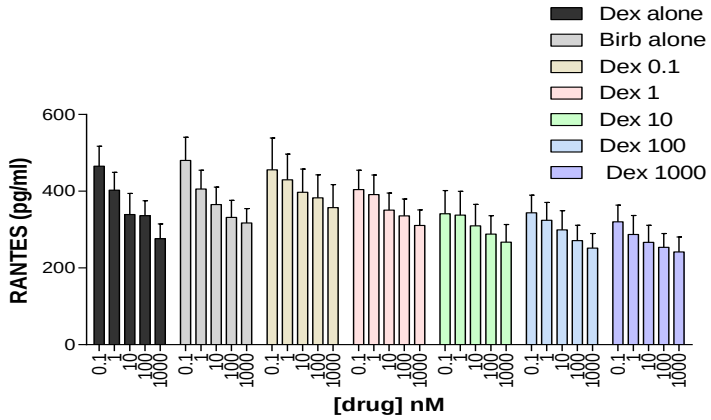


Figure 5.6 The combination effect of dexamethasone and birb 796 on absolute levels of IL-6 release in 16HBEs

16HBEs were pre-incubated with dexamethasone and/or birb 796 for 2 hours prior to stimulation with 5.6.1) 1µg/ml LPS, 5.6.2) 10ng/ml TNFα or 5.6.3) 100µg/ml Poly (I:C). Supernatant levels of IL-6 were measured by ELISA. Data shown are mean ± SEM. Each experiment was carried out 4 times with different cell passages.

5.7.1



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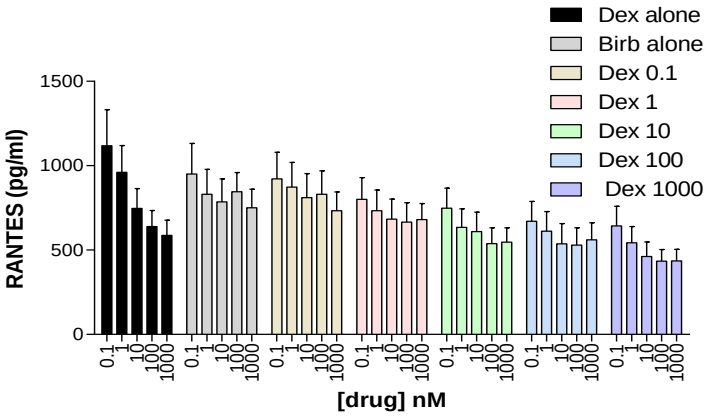

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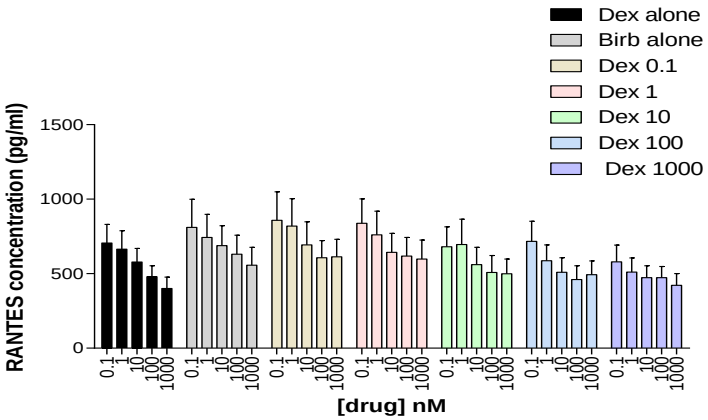
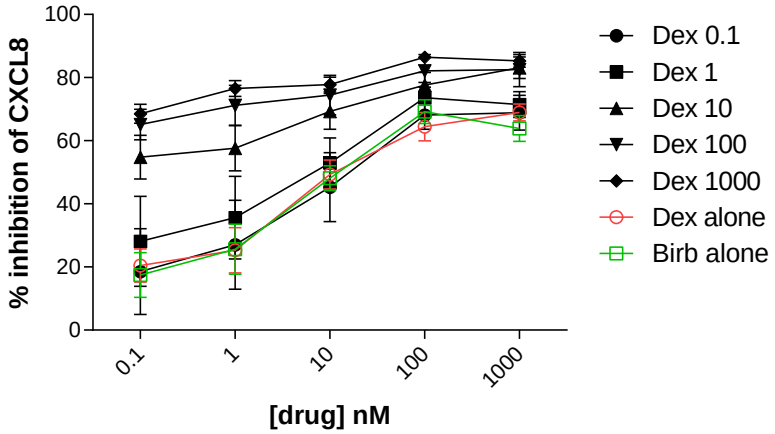


Figure 5.7 The combination effect of dexamethasone and birb 796 on absolute levels of RANTES release in 16HBEs

16HBEs were pre-incubated with dexamethasone and/or birb 796 for 2 hours prior to stimulation with 5.7.1) 1µg/ml LPS, 5.7.2) 10ng/ml TNFα or 5.7.3) 100µg/ml Poly (I:C). Supernatant levels of RANTES were measured by ELISA. Data shown are mean ± SEM. Each experiment was carried out 4 times with different cell passages.

5.8.1



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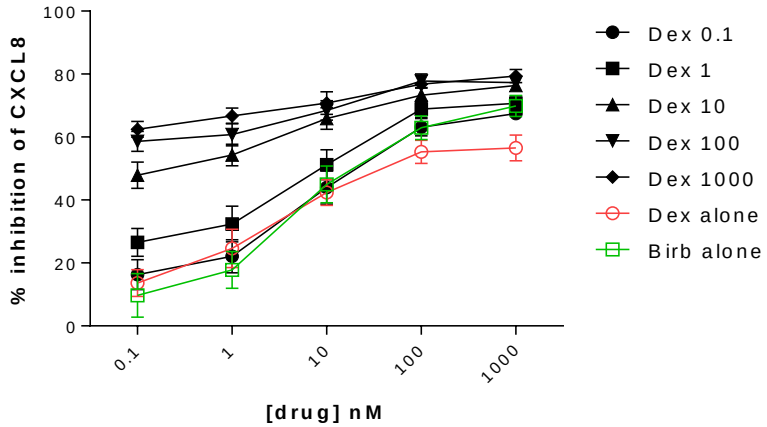

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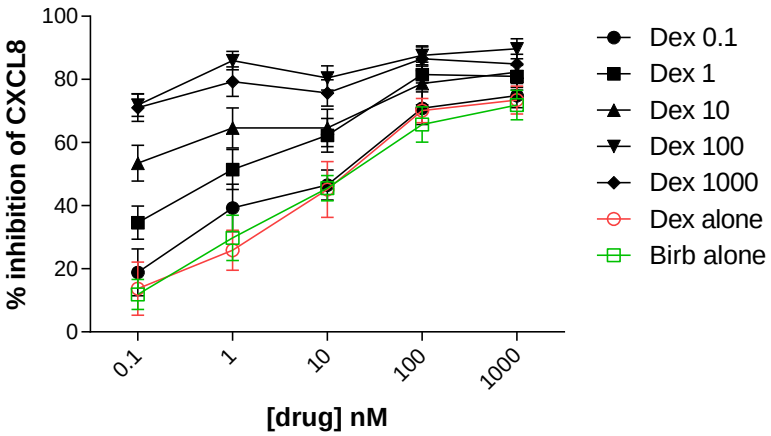
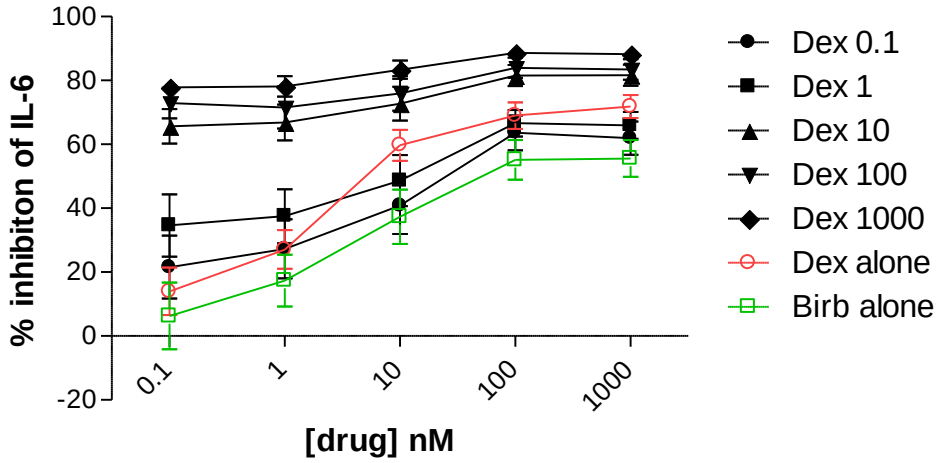


Figure 5.8 The combination effect of dexamethasone and birb 796 on per cent inhibition of CXCL8 release in 16HBEs

16HBEs were pre-incubated with dexamethasone and/or birb 796 for 2 hours prior to stimulation with 5.8.1) 1µg/ml LPS, 5.8.2) 10ng/ml TNFα or 5.8.3) 100µg/ml Poly (I:C). Supernatant levels of CXCL8 were measured by ELISA. Data shown are mean ± SEM.

5.9.1



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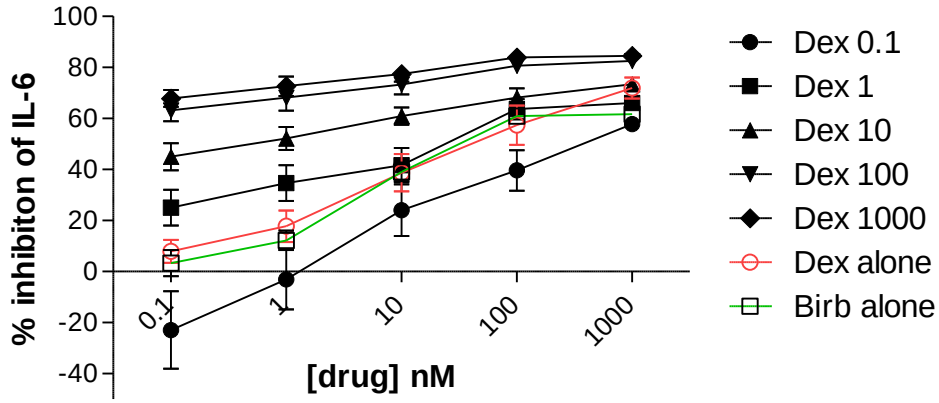

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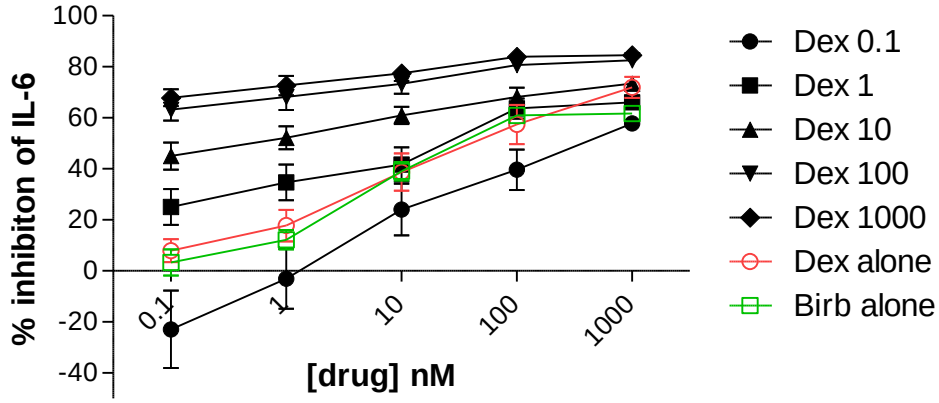
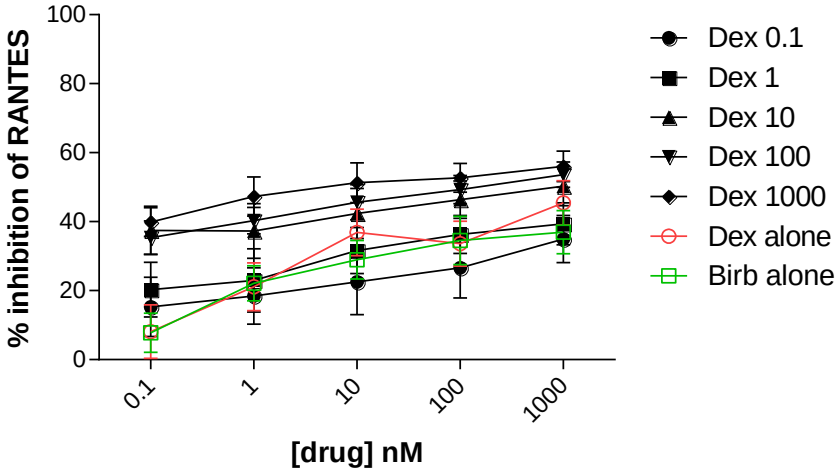


Figure 5.9 The combination effect of dexamethasone and birb 796 on per cent inhibition of IL-6 release

16HBEs were pre-incubated with dexamethasone and/or birb 796 for 2 hours prior to stimulation with 5.9.1) 1µg/ml LPS, 5.9.2) 10ng/ml TNFα or 5.9.3) 100µg/ml Poly (I:C). Supernatant levels of IL-6 were measured by ELISA. Data shown are mean ± SEM.

5.10.1



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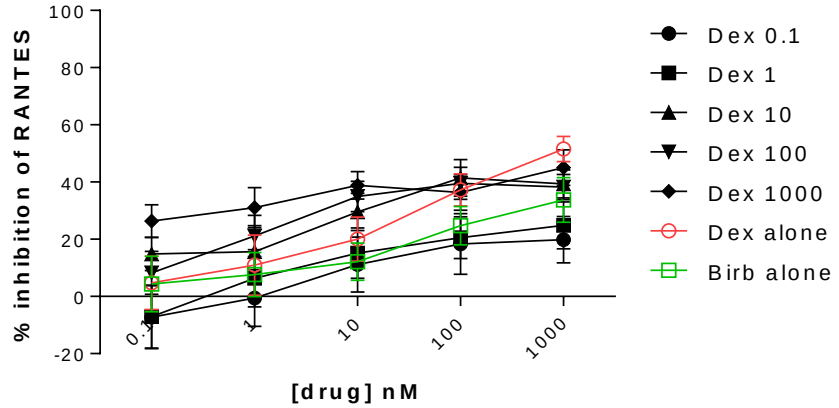

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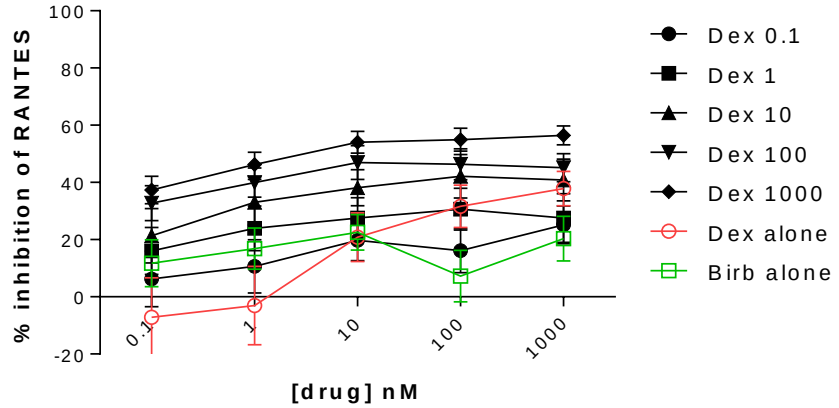
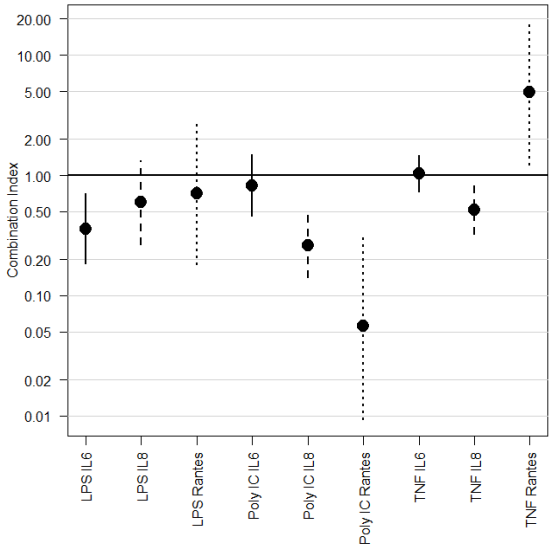


Figure 5.10 The combination effect of dexamethasone and birb 796 on per cent inhibition of RANTES release in 16HBEs

16HBE cells were pre-incubated with dexamethasone and/or birb 796 for 2 hours prior to stimulation with 5.10.1) 1µg/ml LPS, 5.10.2) 10ng/ml TNFα or 5.10.3) 100µg/ml Poly (I:C). Supernatant levels of RANTES were measured by ELISA. Data shown are mean ± SEM. percentage inhibition for IL-6.

5.11.1



5.11.2

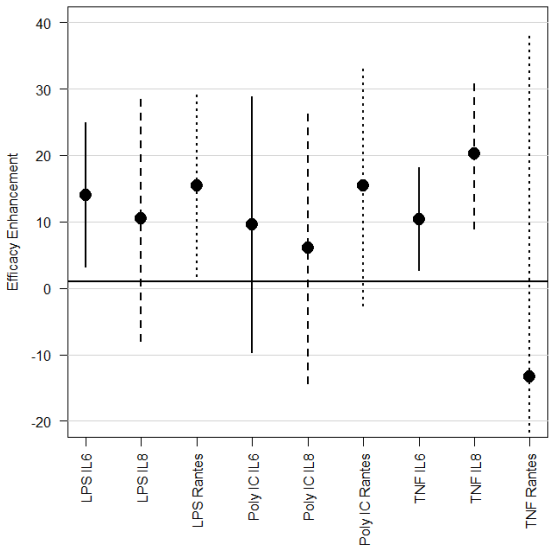


Figure 5.11 Dose-sparing effect and efficacy enhancing benefit of dexamethasone in combination with birb 796

5.11.1 Dose-sparing effect of dexamethasone in combination with birb 796. Estimates of the combination index are plotted with 95% confidence intervals calculated for each pro-inflammatory mediator and each stimulus. A combination index of one corresponds to additivity; values less than one represent synergy. Endpoints where the 95% confidence interval lies completely below one show statistically significant synergy. Missing lines indicate it was not possible to fit the model. 5.1.2 Efficacy-enhancing benefit of dexamethasone in combination with birb 796. Estimates of the efficacy enhancing benefit are plotted with 95% confidence intervals calculated for each cytokine and each stimulus. An efficacy enhancing benefit of zero corresponds to additivity; values greater than zero represent synergy. Endpoints where the 95% confidence interval lies completely above zero show statistically significant synergy. Analysis carried out by Chris Harbron at AstraZeneca, UK.

5.4 Discussion

This chapter shows that stimulation of bronchial epithelial cells with the pro-inflammatory stimuli LPS, TNFα or Poly (I:C) induce the release of CXCL8, IL-6 and RANTES, which is mediated through the activation of the p38 MAPK pathway. CXCL8-, TNFα- and Poly (I:C)-induced release of these pro-inflammatory mediators can be suppressed by a pharmacological p38 MAPK inhibitor and by the GC dexamethasone. The data also shows that the sensitivity of bronchial epithelial cells to either the p38 MAPK inhibitor or to dexamethasone is dependent on both the stimulus used and the pro-inflammatory mediator measured. LPS- and TNFα-stimulated RANTES demonstrate decreased sensitivity to dexamethasone compared with LPS- and TNFα-stimulated IL-6 and CXCL8 release. In contrast, Poly (I:C)-stimulated RANTES release shows a much greater sensitivity to dexamethasone. In general, GC was more effective at inhibiting pro-inflammatory mediator release compared with pharmacological inhibition of the p38 MAPK pathway, thus combining a GC and p38 MAPK inhibitor may be efficacious overall. Indeed, combination treatment using dexamethasone and birb796 was more effective in inhibiting stimulated pro-inflammatory mediator release compared with either drug alone.

LPS signalling is also known to upregulate the p38 MAPK pathway in a number of cell types, in particular in bronchial epithelial cell lines (Wu et al, 2010, Blau et al, 2007, Li et al, 2007). In addition, inhalation of LPS by non-smoking subjects has also shown that upon exposure to LPS, bronchial epithelial cells have enhanced expression of phospho-p38 MAPK (Roos-Engstrand et al, 2005). The data presented in this chapter show that LPS stimulation of bronchial epithelial cells leads to

increased phosphorylation of p38 MAPK in a time-dependent manner, with phosphorylation levels peaking between 60 and 90 minutes. This is in agreement with previous studies (Wu et al, 2010, Blau et al, 2007, Li et al, 2007). Bacterial colonisation of the airways in patients with COPD may be involved in the increased activation of phospho-p38 MAPK in COPD lungs, as shown in chapter 4, through the recognition of bacterial LPS by TLR 4. This may lead to increased production of pro-inflammatory mediator release from key cell types implicated in COPD, including alveolar macrophages, neutrophils, bronchial epithelial cells and lymphocytes. In addition, enhanced recruitment of these cell types into the lung as a result of enhanced pro-inflammatory chemokine release may also be occurring.

In addition to bacterial infections, respiratory viral infections are also a frequent cause of COPD exacerbations with human respiratory syncytial virus, influenza and rhinovirus being the most frequently identified viruses (Dimopoulos et al, 2012). Poly (I:C), a synthetic double-stranded RNA analogue, stimulates TLR3, which recognises double-stranded RNA present in viral genomes or generated during viral replication (Matsumoto and Seya, 2008). TLR3-mediated recognition of double-stranded RNA leads to transmission of signals via the adaptor protein Toll-IL-1 receptor (TIR) domain containing adaptor molecule 1 (TICAM1) (also called TIR domain–containing adaptor inducing IFN-β [TRIF]). This activates a number of transcription factors including IRF3, NFκB, and AP-1, leading to the induction of type 1 IFNs, cytokine and chemokine production and dendritic cell maturation (Matsumoto and Seya, 2008). Poly (I:C) has been shown to activate the p38 MAPK pathway in a number of bronchial epithelial cell lines (Berube et al, 2009; Lam et al, 2011), which is in agreement with the data presented herein. In the current chapter, Poly (I:C)-induced

stimulation of bronchial epithelial cells induced activation of p38 MAPK in a time-dependent manner, with levels peaking between 60 and 90 minutes. The stimulation of TLR3 by viral double-stranded RNA in patients with COPD may therefore be involved in the increased numbers of cells positive for activated p38 MAPK in COPD lungs shown in Chapter 4. As the data in the present chapter suggest, this may lead to enhanced release of pro-inflammatory mediator release from bronchial epithelial cells, and other pro-inflammatory cells, leading to further recruitment of pro-inflammatory cells into the lungs and further secretion of cytokines and chemokines, thus driving the inflammatory environment present in COPD lungs.

Smoking leads to the increased production of the pro-inflammatory cytokine TNFα *in vitro* and *in vivo* (Churg et al, 2002) and is implicated in COPD progression. TNFα can bind two distinct receptors known as TNFα receptor type 1 (TNFR1) and TNFR2. The binding of TNFα to TNFR1 initiates a cascade of events involving activation of the MAPK pathways, leading to the phosphorylation of NFκB and other downstream substrates. In addition, binding to TNFR1 also induces activation of NFκB. This occurs through the activation of inhibitor of nuclear factor Kb (IκB) kinase kinase (IKK) via TNFR1 –associated death domain –containing protein (TRADD). Activated IKK phosphorylates IκB in the NF-κb-IκBα complex, which releases activated NFκB from the complex. NFκB then translocates to the nucleus and binds to target genes, such as cytokines, chemokines and proteases, in a sequence-specific manner. The ability of TNFα signalling to activate the p38 MAPK pathway in bronchial epithelial cells is well established (Matsumoto et al, 1998; Li et al, 2002, Reibman et al, 2006, Ulanova et al, 2006, Ekstrand-Hammarstrom et al, 2006, Liu et al, 2008, King et al,

2009) and the data in the current chapter shows that TNFα-induced stimulation of bronchial epithelial cells induces phospho-p38 MAPK expression in a time-dependent manner. In contrast to LPS- and Poly (I:C)-induced phospho-p38 MAPK expression, TNFα-induced phospho-p38 MAPK expression occurs much quicker and for a shorter time period. TNFα levels are increased in patients with COPD (Culpitt et al, 2003, Di Stefano et al, 2004) and are also associated with COPD exacerbations (Aaron et al, 2001) implicating the cytokine in COPD pathogenesis. Increased levels of TNFα in patients with COPD may contribute to the increased phospho-p38 MAPK in COPD lungs as discussed in chapter 4, thus driving the pro-inflammatory environment present in the lungs of patients with COPD.

The sensitivity of the bronchial epithelial cells to birb 796 and dexamethasone was stimulus- and pro-inflammatory mediator-dependent. Poly (I:C)-induced CXCL8 is more sensitive to dexamethasone than birb 796, whereas the sensitivity of LPS- and TNFα-stimulated CXCL8 release is similar for both birb 796 and dexamethasone. LPS-, TNFα- and Poly (I:C)-stimulated IL-6 release is more sensitive to dexamethasone than birb 796 at all concentrations examined. Only Poly (I:C)-induced RANTES release showed a difference in sensitivity between birb and dexamethasone, with dexamethasone having a much greater effect in suppressing Poly (I:C)-induced RANTES production. Dexamethasone and birb 796 had similar suppressive effects on LPS- and TNFα-stimulated RANTES release. The reason for these stimuli- and pro-inflammatory mediator-dependent differences in sensitivity to dexamethasone and birb 796 is unknown. It may be due to different intracellular signalling pathways being activated by the different stimuli used, thus resulting in differential effects of anti-inflammatory therapies. For example, LPS- and TNFα-

stimulated CXCL8 release was sensitive to birb 796, indicating that the release of CXCL8 via LPS-stimulated TLR4 and TNFα-stimulated TNFR1 and TNFR2 is p38 MAPK-dependent. Poly (I:C)-stimulated CXCL8 release was less sensitive to birb 796, indicating that intracellular signalling pathways other than the p38 MAPK pathway may be involved in Poly (I:C)-stimulated CXCL8 release in bronchial epithelial cells. Previous studies have demonstrated that certain pro-inflammatory mediators are more GC-sensitive than others, for example CXCL8 release from alveolar macrophages is GC-insensitive in both control and disease cells (Standiford et al, 1992, Bhavsar et al, 2008; Armstrong et al, 2009). However, in the current study dexamethasone inhibited CXCL8 release, indicating that the GC sensitivity of CXCL8 release is cell-specific. The data presented here also indicates that the GC sensitivity of certain pro-inflammatory mediators is also stimulus-dependent, thus detailing the complexities underlying GC sensitivity in patients with COPD. Further work investigating the stimulus-, pro-inflammatory mediator-, and cell-specific GC sensitivity are needed in COPD to fully understand each of their relative contributions in patients.

The data also shows that GC suppression of pro-inflammatory mediator production is more effective than birb 796, confirming previous studies (Kent et al, 2009, Bhavsar et al, 2010, Armstrong et al, 2011). Dexamethasone was also found to have a greater suppressive effect on COPD stimulated whole blood cytokine production compared with a p38 MAPK inhibitor (Singh et al, 2010). Clinically, p38 MAPK inhibitors may have different potencies and maximal effects and as such further research into the effects of a pharmacological p38 MAPK inhibitor in COPD is

needed. This presents a rationale for combining a p38 MAPK inhibitor and GC as a therapeutic strategy in COPD.

Previous studies have shown that GC in combination with a p38 MAPK inhibitor have a greater effect on pro-inflammatory mediator release compared with using either drug alone (Kent, 2009, Bhavsar et al, 2010, Armstrong et al, 2011, Rebeyrol et al, 2012). Armstrong and colleagues evaluated full-dose response curves for both drugs in order to evaluate additive and synergistic interactions in LPS-stimulated alveolar macrophages (Armstrong et al, 2011). They demonstrated significant efficacy-enhancing benefits and synergistic dose-sparing effects. In the current study this was extended to investigate the phenomenon in bronchial epithelial cells, which are also likely to be targeted therapeutically in COPD. In agreement with Armstrong et al, increasing the concentration of birb 796 in combination with dexamethasone induced progressively greater inhibition of pro-inflammatory mediator production than using either drug alone. The maximal effect of dexamethasone was increased when used in combination with birb 796. The rationale for evaluating the effects of GC in combination with a p38 MAPK inhibitor is that early clinical trials investigating the use of p38 MAPK inhibitors alone have demonstrated considerable hepatotoxicity. High doses of p38 MAPK inhibitors have also been shown to inhibit several non-p38 MAPKs. The use of p38 MAPK inhibitors as single therapeutic agents may also have other adverse effects, for example; like cytokine networks, signalling cascades are highly redundant and complex. Blocking p38 MAPK may lead to compensatory effects in other kinases that can regulate other genes. Indeed, kinases upstream from p38 MAPK such as MKK3, MKK6, and TAK1 can regulate NFκB and redirect

the signalling flow (Fukushima et al, 2009; Inoue et al, 2006). Humans may also have a physiological escape from p38 MAPK inhibition, as demonstrated by the finding that p38 MAPK inhibitor-induced reductions in C-reactive protein levels returned to baseline after 8 weeks of therapy in a trail investigating the effects of birb 796 in patients with Chrohn's disease (Schreiber et al, 2006). There is also the possibility that as p38 MAPK participates in a negative feedback loop, thus blocking p38 MAPK may divert the signalling flux to other MAPKS such as JNK or ERK. In addition, the long-term use of high-dose GC is linked to a number of unwanted side effects including osteoporosis, adrenocortical suppression, bruising and skin thinning, cataracts and glaucoma (Roland et al, 2004). Using lower doses of GC in combination with a low dose of p38 MAPK inhibitor would reduce the development of these GC-associated side effects, and potentially limit some of the real and hypothesised effects of p38 MAPK inhibition-induced adverse effects. Therefore combining low-dose GC with a p38 MAPK inhibitor may be a more effective therapeutic strategy in the management of COPD. GC inhibit the activity of transcription factors at the promoter regions of pro-inflammatory genes (Adcock et al, 2004) and p38 MAPK inhibitors reduce transcription factor phosphorylation (Rolli et al, 1999, Zhu and Lobie, 2000, Wiggin et al, 2002). As such, both pharmacological agents inhibit the transcription of pro-inflammatory genes. In addition, GC (Lasa et al, 2002, Smoak and Cidlowski, 2006, Quante et al, 2008) and p38 MAPK inhibitors (Winzen et al, 1999) act post-transcriptionally and on protein translation. The data presented in this chapter suggest more than an additive effect of these molecular mechanisms when combination treatment was used. The mechanism under-lying these synergistic effects are unknown. Possible hypotheses are that the synergism

may be a result of inhibition of p38 MAPK-mediated GR phosphorylation (Irusen et al, 2002) enabling effective binding or translocation of GR to the nucleus (Itoh et al, 2002). Alternatively, increased pro-inflammatory mRNA transcript instability may be an alternative explanation. Further work investigating the synergistic mechanisms underlying GC and p38 MAPK inhibitor combination therapy is required.

To conclude, the data presented in this chapter show that the p38 MAPK pathway is activated by pro-inflammatory stimuli including bacterial LPS, the synthetic viral TLR3 ligand Poly (I:C) and the pro-inflammatory cytokine TNFα. GC sensitivity in these cells appears to be stimuli and pro-inflammatory mediator-dependent. Birb 796 was less effective in inhibiting pro-inflammatory mediator release compared with dexamethasone. Combination therapy of dexamethasone and birb 796 has synergistic effects on pro-inflammatory mediator production from a bronchial epithelial cell line. Further work examining the use of combination therapy on primary bronchial epithelial cells is needed to confirm these findings *in vivo*. These data suggest that combination therapy may be a more effective anti-inflammatory therapeutic strategy in the management of COPD.

Chapter 6

Synergistic interactions between the p38 MAPK and glucocorticoid receptor pathways

6.1 Introduction

In the previous chapter the use of a p38 MAPK inhibitor in combination with dexamethasone had a greater effect on suppressing pro-inflammatory mediator production in 16HBEs compared with either drug alone, in agreement with previous studies (Kent et al, 2009, Bhavsar et al, 2010, Armstrong et al, 2011, Rebeyrol et al, 2011). The mechanisms underlying these synergistic/additive between GC and the p38 MAPK pathway effects remain to be fully elucidated.

One potential mechanism may be inhibition of p38 MAPK-induced GR phosphorylation, which may enhance GR functions. Although p38 MAPK phosphorylates GR at serine 211 both directly (Miller et al, 2005) and indirectly (Nader et al, 2010), this is thought to play an important role in the transcriptional activity of GR (Wang et al, 2002), possibly by inducing a functionally active folded conformation (Garza et al, 2010). Indeed, lack of serine 211 phosphorylation has been linked to GC resistance in human lymphoid cells (Miller et al, 2007). p38 MAPK has been shown to inhibit GR transcriptional activity through an indirect action on the AF-2/LBD region (Szatmary et al, 2004). In addition, p38 MAPK-induced phosphorylation of GR is associated with reduced ligand-binding affinity and reduced GC-dependent repression of GM-CSF production (Irusen et al, 2002). The use of a p38 MAPK inhibitor reversed phosphorylation at serine 226, suggesting this site may be a target for p38-induced inhibition of GR function (Irusen et al, 2002). Activation of p38 MAPK by IL-1α administration or over-expression reduces GR transactivation function and GR-GRE binding (Bantel et al, 2002; Szatmary et al, 2004; Wang et al,

2004), implicating cytokine-induced p38 MAPK activation as a mechanism for altered GR function. Indeed, a recent study has shown that increased p38 MAPK-induced GR phosphorylation in severe asthma PBMCs is associated with altered GR nuclear localisation (Mercado et al, 2011). Therefore, inhibiting p38 MAPK may inhibit GR phosphorylation, thus enhancing GR-mediated anti-inflammatory effects through increased GR nuclear localisation. Further work is required to confirm these observations in other cell types, as well as in other inflammatory diseases such as COPD.

GC are involved in the destabilisation of mRNA molecules, including IL-6 (Quante et al, 2008), TNFα (Smoak and Cidlowski, 2006) and COX-2 mRNA (Lasa et al, 2002). This may be through the induction of TTP, an RNA binding protein involved in regulating inflammation-associated transcripts (Smoak and Cidlowski, 2006), or through the induction of MKP-1, a negative regulator of p38 MAPK (Lasa et al, 2001). p38 MAPK stabilises a number of pro-inflammatory mRNA constructs including CXCL8 and IL-6 (Winzen et al, 1999). A number of studies have also shown p38 MAPK phosphorylates TTP (Christensen et al, 2004, Stoeklin et al, 2004), although there is conflicting evidence regarding how this p38 MAPK-induced phosphorylation of TTP effects mRNA decay. Some studies show that unphosphorylated TTP has increased affinity for mRNA decay (Carbello et al, 2001. Cao, 2004, Hitti et al, 2006), but others show that TTP activity is not affected by p38 MAPK-induced phosphorylation (Chrestensen et al, 2004, Cae et al, 2003, Stoeklin et al, 2004). More recent data demonstrates that TTP phosphorylation by p38 MAPK prevents deadenylase recruitment thus blocking TTP-mediated decay (Clements et

al, 2011). In addition, GC are able to induce MKP-1 expression (Lasa et al, 2001) thus inhibiting phospho-p38 MAPK expression (King et al, 2009). Taken together these data indicate that combination therapy may act to enhance destabilisation of pro-inflammatory mRNA transcripts; GC by inducing TTP expression, and a p38 MAPK inhibitor by preventing p38 MAPK-induced TTP phosphorylation. Furthermore, GC-induced MKP-1 expression may also inhibit p38 MAPK-induced TTP phosphorylation and GR phosphorylation, which is another potential mechanism of additivity/synergy between GC and p38 MAPK inhibition. However further work is needed to confirm these interactions.

Therefore, the aim of this chapter was to investigate the mechanisms by which a p38 MAPK inhibitor used in combination with a GC exerts synergistic effects on pro-inflammatory mediator suppression. Specifically, the effects of combination therapy on phosphorylated p38 MAPK expression, CXCL8 mRNA stability, GR translocation and GR phosphorylation were examined in 16HBEs.

6.2 Methods

6.2.1 16HBE cell culture

16HBEs were maintained in supplemented MEM. Confluent cells grown in tissue culture plates or chamber slides were placed in fresh supplemented media and stimulated with LPS (1µg/ml), TNFα (10ng/ml) or Poly (I:C) (100µg/ml). For some experiments cells were pre-incubated with dexamethasone (0.1–1000nM) and/or the p38 MAPK inhibitor birb 796 (0.1–1000nM) and incubated for various time points in 5% CO_2 at 37°C. Plates were centrifuged at 2000rpm for 10 minutes at 4°C and cell free supernatants removed and stored at -80°C for cytokine analysis

6.2.2 Protein expression assay

For western blots, cells were lysed in RIPA buffer (10mM Tris-HCL, PH 7.4, 150mM NaCl, 1mM EDTA, 1% Nonidet P-40, 0.25%) containing phosphatase and protease inhibitors. Cell lysates were stored at -80°C until analysis.

6.2.2.1 Bradford Assay

Protein concentrations from whole cell extracts were determined using Bradford Reagent.

6.2.2.2 Polyacrylamide gel electrophoresis

Cell lysates were diluted in sample buffer (62.5MM Tris, 10% glycerol, 1% SDS, 1% beta-mercaptoethanol, and 0.1% bromphenol blue, pH 6.8) and boiled for 10 minutes prior to electrophoresis on SDS-polyacrylamide gels (10%). Proteins were then

transferred to Hy-Bond ECL membranes. Membranes were incubated in blocking buffer followed by primary antibody diluted in blocking buffer overnight at 4°C. Primary antibodies used were phospho-p38 MAPK, total p38 MAPK, phospho-GR serine 203, phospho-GR serine 226, phospho-GR serine 211 and β actin. After washing, the membranes were incubated with a peroxidise-conjugated secondary antibody followed by detection using lumigen TMA-6 enhanced chemiluminescent. Densitometric analysis was performed by normalising band density to that for total antibody using Quantity One v4.6.1 software.

6.2.3 Cytokine release analysis

ELISA was used to determine the supernatant level of CXCL8, IL-6 and RANTES according to the manufacturer's instructions. Lower limits of detection were 32.5pg/ml for CXCL8 and RANTES and 9.375pg/ml for IL-6.

6.2.4 Polymerase Chain Reaction

6.2.4.1 RNA extraction

16HBEs were harvested for total RNA isolation. A commercially available kit was used to extract total RNA according to the manufacturer's instructions. The resultant RNA concentration was measured with a spectrophotometer at 260nm wavelength, with samples being diluted 1:50 in RNase-free water. The RNA sample was then stored at -80°C until further analysis.

6.2.4.2 Reverse Transcription

To synthesise cDNA from whole RNA reverse transcription was performed on 50ng of RNA from 16HBEs using TaqMan reverse transcription PCR with the Verso-2-step QRT-PCR kit according to the manufacturer's instructions. cDNA was synthesised and subsequently used for qPCR.

6.2.4.3 Real time quantitative polymerase chain reaction (qPCR)

Gene transcript level of CXCL8 and the house-keeping gene GAPDH were quantified by real-time PCR using ABsolute blue qPCR mix on a Stratagene MX3005P. Premade ABI Taqman gene expression assays for CXCL8 and GAPDH were purchased from Thermo Scientific as per the manufacturer's instructions. The PCR cycle was as follows; 50°C for 2 minutes, 95°C for 10 minutes then 40 to 50 cycles of denaturing at 95°C for 15 seconds and 60°C for 60 seconds for annealing and extension. Each transcript was analysed by delta (Δ) CT method and variations in cDNA concentrations between different samples were corrected using GAPDH. Controls without RT-enzyme showed there was no genomic DNA amplification.

6.2.5 Glucocorticoid receptor translocation assay

Cells grown on chamber slides were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Cells were washed in PBS, followed by incubation in 1.5% goat serum in PBS for 30 minutes at room temperature. Cells were then incubated in primary antibody (anti-GR clone 41) 1 in 200 diluted in PBS containing 0.05% triton X overnight at 4°C. After washing, cells were incubated in Alexa 568 conjugated goat anti-mouse antibody 1 in 200 in PBS for 90 minutes at 37°C. Finally cells were

incubated in DAPI, 1 in 50,000 in TBS, for 5 minutes in the dark. Sections were mounted using immunofluorescent mounting medium and stored in the dark at 4°C until analysis.

Bronchial epithelial cells were analysed by assessing the location of the GR. Location was classed as either being all cytoplasmic, cytoplasmic and nuclear; or nuclear. 400 cells were counted per condition.

Digital micrographs were obtained using a Nikon Eclipse 80i microscope equipped with a QImagining digital camera and ImagePro Plus 5.1 software.

6.3 Results

6.3.1 Effect of dexamethasone on phosphorylated p38 MAPK

The effect of dexamethasone on stimulated phosphorylated p38 MAPK expression was analysed by western blot. 16HBEs were pre-incubated for 60 minutes with dexamethasone (1000nM) followed by stimulation with LPS, TNFα or Poly (I:C) for optimum times as determined from time course experiments described in Chapter 5. All three stimulants induced a significant increase in phospho-p38 MAPK expression (Bonferroni multiple comparisons test $p<0.001$ for TNFα-, $P<0.05$ for LPS- and Poly (I:C)-stimulated phospho-p38 MAPK expression, **Figure 6.1**). For all three stimulants, dexamethasone had no effect on expression of phosphorylated p38 MAPK (ANOVA $p>0.05$ for all comparisons).

6.3.2 Effect of Birb 796 on phosphorylated p38 MAPK

16HBEs were also pre-incubated with Birb (1000nM) for 60 minutes followed by stimulation with LPS, TNFα or Poly (I:C). This concentration of birb 796 inhibited phosphorylated p38 MAPK completely. For further experiments, a lower concentration of birb 796 (10nM) was used on LPS-, TNFα- and Poly (I:C)-stimulated 16HBEs; this inhibited phosphorylated p38 MAPK, albeit to a lesser extent than the higher concentration of birb 796 (**Figure 6.1**). Statistical analysis using Bonferroni multiple comparison test showed that birb 796 significantly inhibited TNFα-stimulated ($p<0.001$), LPS-stimulated ($p<0.05$) and Poly (I:C)-stimulated ($p<0.05$) phospho-p38 MAPK expression.

6.3.3 Combination effect of dexamethasone and birb 796 on phosphorylated p38 MAPK expression

The effects of combination treatment on phosphorylated p38 MAPK was analysed using a combination of dexamethasone (1000nM) and birb 796 (10nM). The maximum concentration of dexamethasone was chosen to ensure any effects on phospho-p38 MAPK expression would be observed. The concentration of Birb 796 was chosen as this was shown to have a modest effect on phospho-p38 MAPK expression and would therefore allow any additional effects of combination therapy to be observed. Combination treatment did not have any greater inhibitory effect on phosphorylated p38 MAPK expression compared with the effect of using birb 796 (10nM) alone (ANOVA $p>0.05$ for all comparisons, **Figure 6.1**).

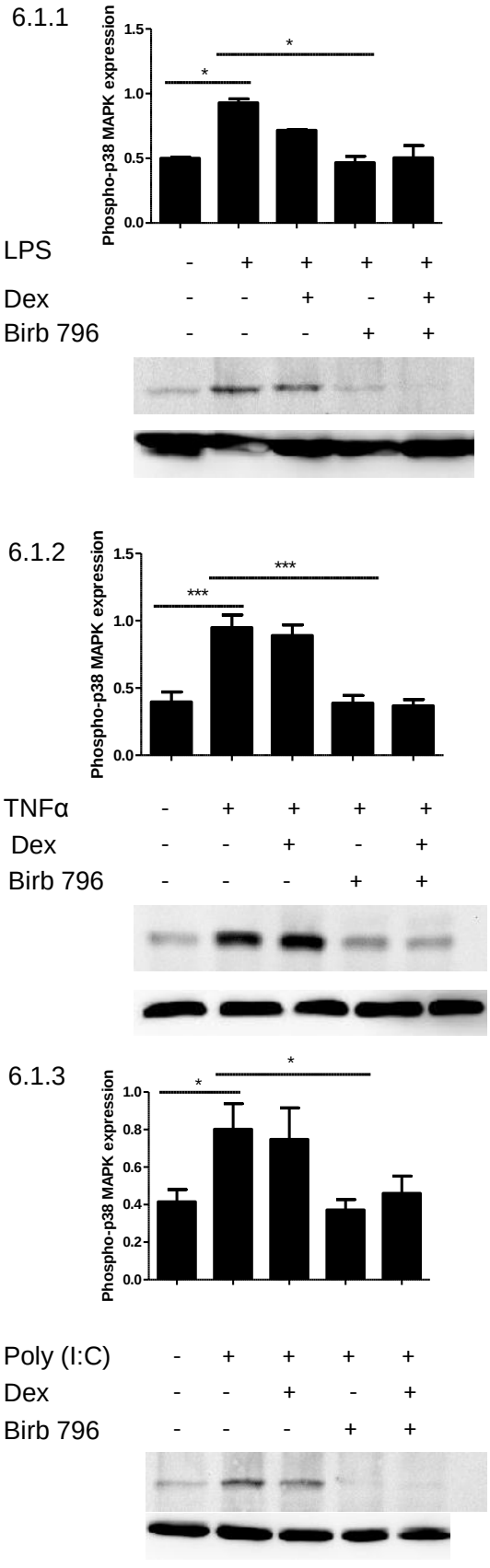


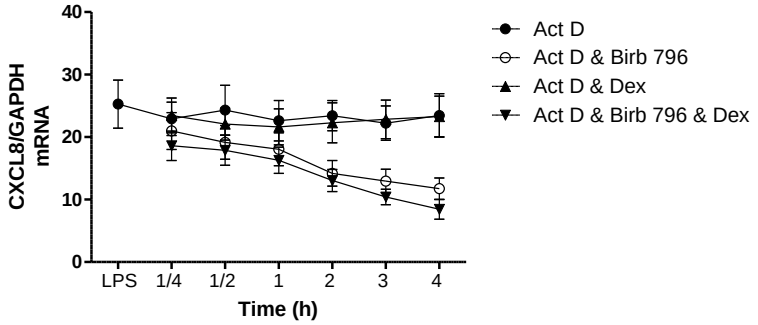
Figure 6.1 The effect of dexamethasone and birb 796 on phosphorylated p38 MAPK expression in 16HBEs

16HBEs were pre-incubated with dexamethasone and/or birb 796 for 1 hour prior to stimulation with 6.1.1) LPS (1µg/ml) for 60 minutes, 6.1.2) TNFα (10ng/ml) for 15 minutes or 6.1.3) Poly (I:C) (100µg/ml) for 60 minutes. Data shown are mean + SEM. of phospho-p38 MAPK normalised to total p38 MAPK. Western blot image is a representative image of 3 separate experiments carried out on a minimum of three passages. Data was analysed by ANOVA followed by Bonferroni multiple comparisons test to compare phospho-p38 MAPK expression under different conditions. *$p<0.05$, ***$p<0.001$.

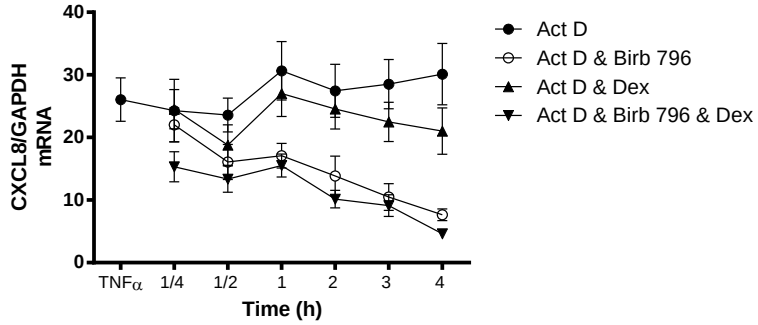
6.3.4 Effects of dexamethasone and birb 796 on CXCL8 mRNA stability

To examine the effect of birb 796, dexamethasone and combination treatment on CXCL8 mRNA stability, 16HBEs were stimulated for 4 hours with LPS, TNFα or Poly (I:C). The transcriptional inhibitor actinomycin D was added to the cultures, and then a time course examining the effect of each treatment was carried out, up to a maximum time point of 4 hours. Following actinomycin D treatment, LPS-, TNFα- and Poly (I:C)-induced CXCL8 mRNA levels remained relatively stable over time (**Figure 6.2**). Following treatment with dexamethasone, LPS-, TNFα- and Poly (I:C)-induced CXCL8 mRNA levels were similar to those seen for stimulant alone, suggesting that dexamethasone has no effect on the stability of CXCL8 mRNA induced by LPS, TNFα and Poly (I:C). In contrast, birb 796 treatment resulted in a time-dependent decrease in the level of, LPS-, TNFα- and Poly (I:C)-induced CXCL8 mRNA, suggesting that the p38 MAPK pathway is involved in the stabilisation of CXCL8 mRNA in stimulated 16HBEs. Combination treatment of dexamethasone and birb 796 had no greater effect on LPS-, TNFα- and Poly (I:C)-induced CXCL8 mRNA levels compared with birb 796 treatment alone.

6.2.1



6.2.2



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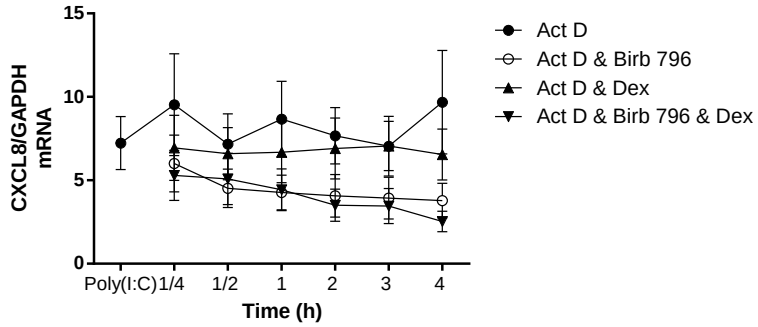


Figure 6.2 The effect of dexamethasone and birb 796 on stimulated CXCL8 mRNA stability in 16HBEs

The effect of dexamethasone, birb 796 and combination treatment on 6.2.1) LPS- (1µg/ml), 6.2.2) TNFα- (10ng/ml) and 6.2.3) Poly (I:C)- (100µg/ml) induced CXCL8 stability in 16HBEs. Cells were stimulated for 4 hours prior to incubation with actinomycin D with or without dexamethasone (1000nM), birb 796 (1000nM) and/or combination treatment for up to 4 hours. At various time points cells were harvested for PCR analysis of CXCL8 mRNA levels. Data are presented as mean ± SEM. Each experiment was repeated 4 times with different cell passages.

6.3.5 Glucocorticoid receptor phosphorylation

The effect of dexamethasone, LPS, TNFα and Poly (I:C) on GR phosphorylation at serine 226, serine 211 and serine 203 residues was examined. 16HBEs were cultured in each of the four conditions for up to 180 mins, then cells were harvested for western blot analysis.

Dexamethasone, LPS-, TNFα- and Poly (I:C)-induced phosphorylation at each serine reside in a similar time-dependent manner (**Figure 6.3**). Phosphorylation at serine 226 and 211 appeared to be maximal at around 30 minutes and had returned to basal levels by 180 mins.

Dexamethasone, LPS, TNFα and Poly (I:C) also had similar effects on phosphorylation at the serine 203 reside, although phosphorylation appeared to peak later, at around 60 minutes, and this also appeared to remain for a longer period of time, with increased phosphorylation still present at 180 mins (**Figure 6.3**).

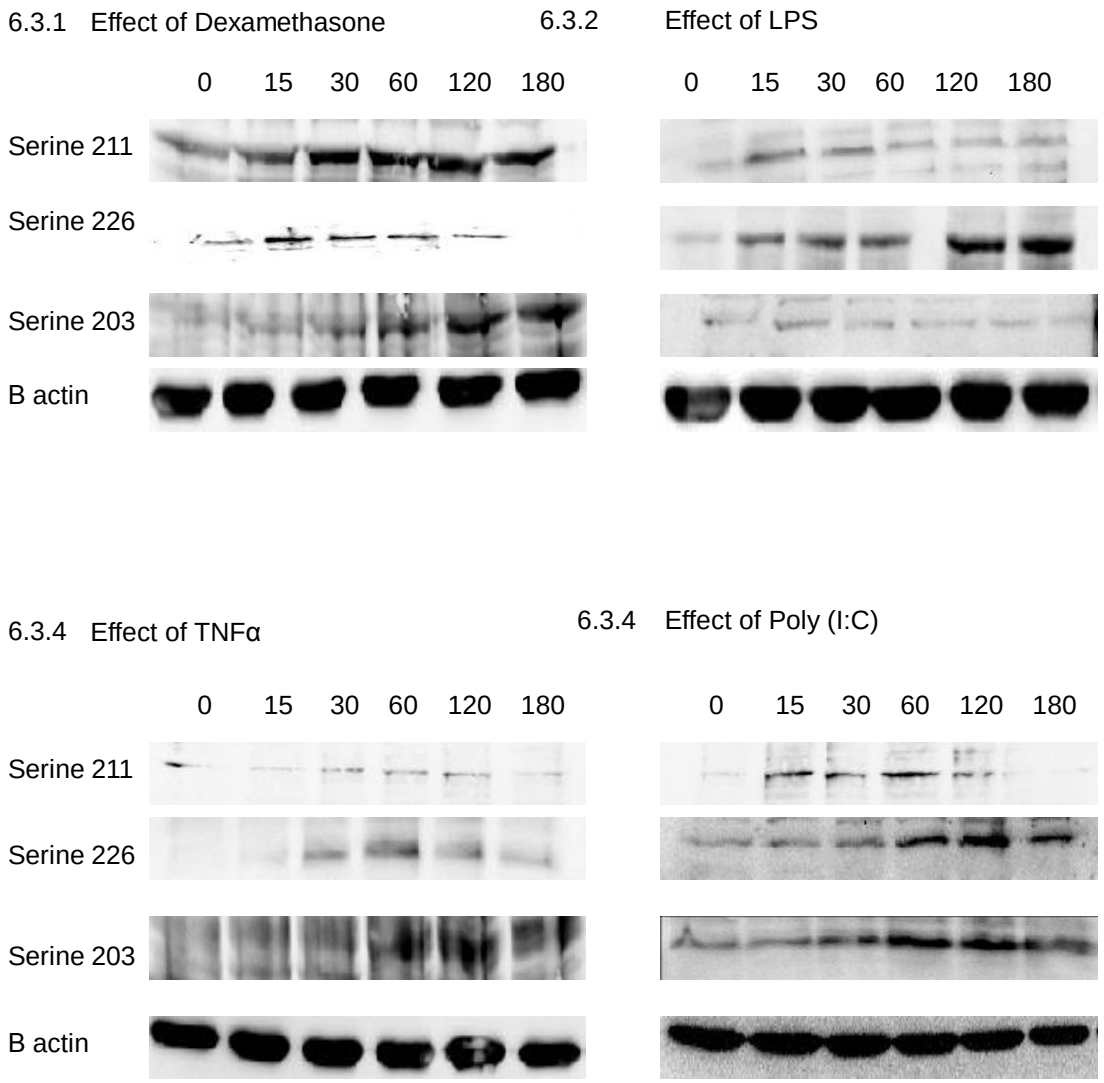


Figure 6.3 Dexamethasone and pro-inflammatory stimuli-induced glucocorticoid receptor phosphorylation in 16HBEs

16HBEs were incubated with 6.3.1) dexamethasone (1000nM), 6.3.2) LPS (1μg/ml), 6.3.3) TNFα (10ng/ml), or 6.3.4) Poly (I:C) (100μg/ml) for up to 180 minutes. Cells were harvested and western blotting analysis for GR phosphorylation at serine 211, serine 226 and serine 203 carried out. β actin levels were also assessed for loading controls. Western blot images are representative images of at least 3 experiments carried out with 3 difference cell passages.

6.3.5.1 Effect of Birb 796 on glucocorticoid receptor phosphorylation

For serine 226 phosphorylation and serine 211 phosphorylation a time point of 30 minutes was chosen to examine the effect of Birb 796. Cells were pre-incubated with Birb 796 (final concentration 1000nM) prior to incubation with dexamethasone, LPS, TNFα or Poly (I:C) for 30 minutes. Western blot analysis showed that Birb 796 inhibited dexamethasone-, LPS-, TNFα- and Poly (I:C)-stimulated GR phosphorylation at serine 211, with levels of phosphorylated serine 211 returning to near basal levels for each condition (**Figure 6.4**). In contrast, Birb 796 appeared to have no effect on dexamethasone-, LPS-, TNFα- or Poly (I:C)-induced GR phosphorylation at serine 226.

To examine the effect of Birb 796 on GR phosphorylation at serine 203, a time point of 60 minutes was chosen. Pre-incubation with Birb 796 had no effect on dexamethasone-, LPS-, TNFα- or Poly (I:C)-induced GR phosphorylation at serine 203 (**Figure 6.4**).

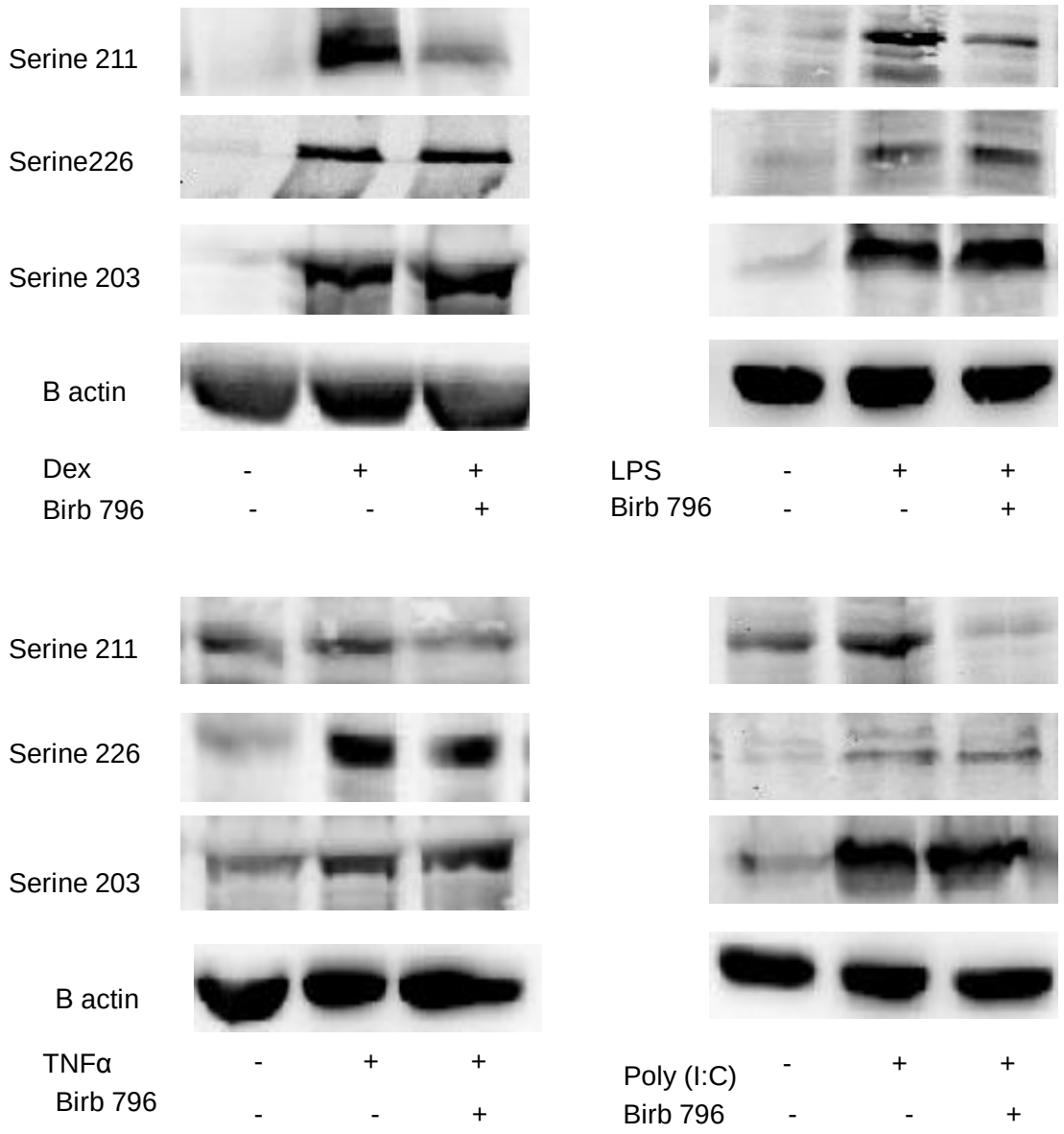


Figure 6.4 The effect of birb 796 on glucocorticoid receptor phosphorylation
16HBEs were incubated with top left panel) dexamethasone (1000nM), top right panel) LPS (1μg/ml), bottom left panel) TNFα (10ng/ml) and bottom right panel) Poly (I:C) (100μg/ml) for various time periods. For some experiments cells were pre-incubated with birb 796 (1000nM) for 1 hour. Cells were harvested and western blotting analysis for GR phosphorylation at serine 211, serine 226 and serine 203 carried out. β actin levels were also assessed for loading controls. Western blot images are representative images of at least 3 experiments carried out with 3 difference cell passages.

6.3.6 Glucocorticoid receptor translocation

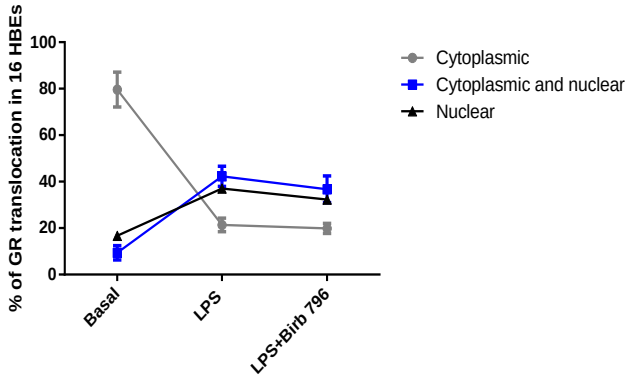
6.3.6.1 Effect of cell stimulation on glucocorticoid receptor translocation

The effects of pro-inflammatory stimuli on GR translocation in 16HBEs was assessed by incubating cells grown on chamber slides for 30 minutes with either LPS, TNFα or Poly (I:C). Translocation was assessed using immunofluorescent detection of GR. In unstimulated cells cultured for 30 minutes, GR was localised in the cytoplasm in around 80% of all cells (**Figure 6.5** and **6.1.1**). Stimulation with LPS, TNFα and Poly (I:C) all had similar effects on GR translocation, with all three stimulants inducing nuclear GR translocation in around half of all cells. (**Figure 6.5.1–6.6.6**).

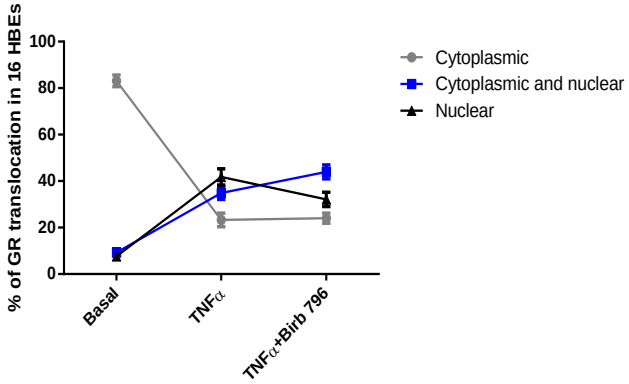
*6.3.6.2 Effect of Birb 796 on stimuli-induced glucocorticoid receptor translocatio*n

To determine whether the effect of LPS, TNFα and Poly IC on GR translocation was p38 MAPK-dependent, the effect of Birb 796 (1000nM) on pro-inflammatory stimuli-induced nuclear translocation was assessed. 16HBEs cultured on chamber slides were pre-incubated with Birb for 60 mins prior to stimulation with either LPS, TNFα or Poly (I:C) for 30 minutes. Birb 796 had no effect on stimuli-induced GR translocation, suggesting that the mechanism by which pro-inflammatory stimuli induces GR translocation into the nucleus is p38 MAPK-dependent (**Figure 6.5** and **6.6.3**, **6.6.5** and **6.6.7**). As a result, all future GR translocation experiments were carried out on unstimulated cells.

6.5.1



6.5.2



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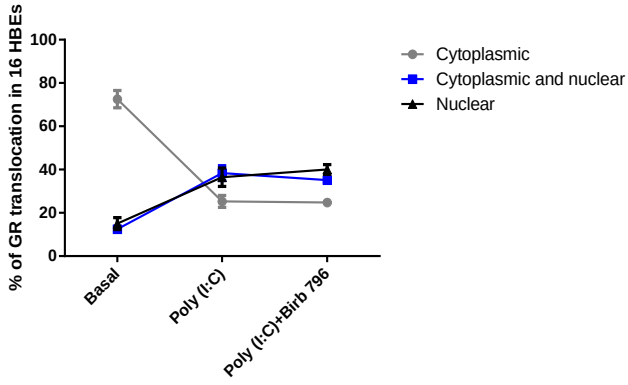


Figure 6.5 The effect of pro-inflammatory stimuli on the localisation of glucocorticoid receptor in 16HBEs

16HBEs were stimulated with 6.5.1) LPS (1µg/ml), 6.5.2) TNFα (10ng/ml) or 6.5.3) Poly (I:C) (100µg/ml)) for 30 minutes. For some experiments cells were pre-incubated with birb 796 (1000nM) for 1 hour prior to stimulation. GR localisation was assessed by immunofluorescent detection of GR and classified as either cytoplasmic, where all GR was present in the cytoplasm; nuclear, where all GR was localised to the nucleus; or cytoplasmic and nuclear; where GR was localised to both the cytoplasm and the nucleus. A total of 300 cells per condition were analysed. Data are presented as mean ± SEM. Each experiment was repeated a minimum of 4 times with different cell passages.

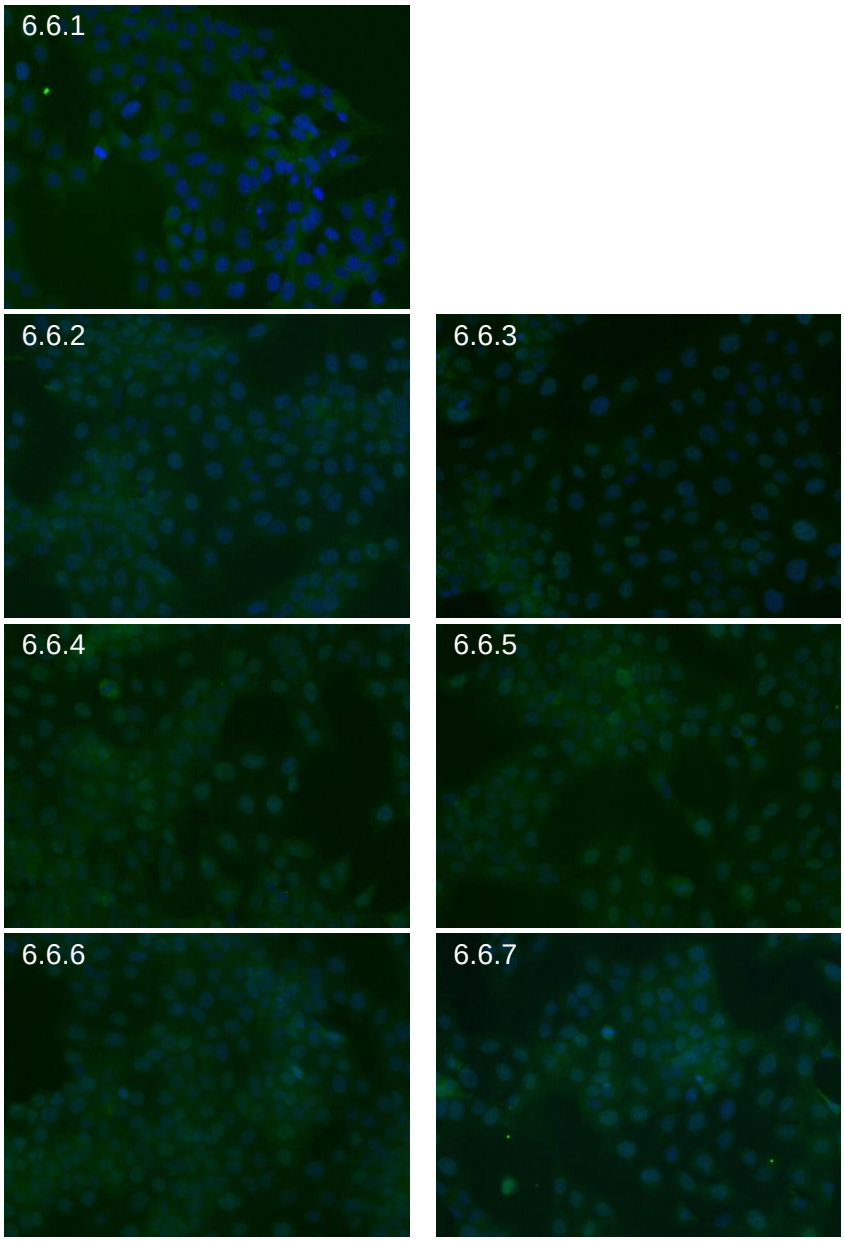


Figure 6.6 The effect of pro-inflammatory stimuli and birb 796 on glucocorticoid receptor translocation in 16HBEs

16HBEs were cultured on chamber slides in the presence of 6.6.2–6.6.3) LPS, (1µg/ml), 6.6.4–6.6.5) TNFα (10ng/ml) or 6.6.6–6.6.7) Poly (I:C) for 30 mins. 6.6.1) Basal (unstimulated) cells are also shown. For some experiments cells were pre-incubated with birb 796 (1000nM) for 30 minutes prior to stimulation (6.6.3, 6.6.5, 6.6.7). Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (blue). GR was detected using an Alexa 468 conjugated goat anti-rabbit secondary antibody (green). Cells were classified according to the location of GR; cytoplasmic, cytoplasmic and nucleic or nucleic.

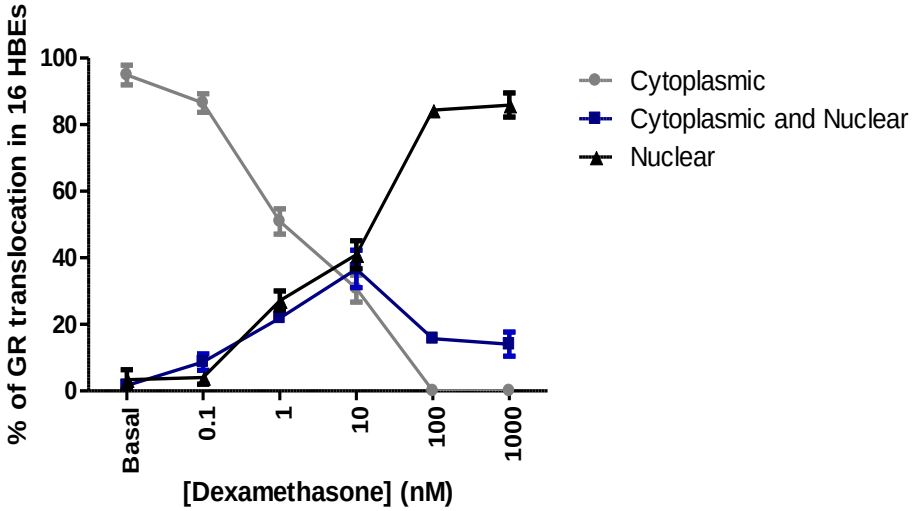
6.3.6.3 Effect of dexamethasone on glucocorticoid receptor translocation

16HBEs were cultured on chamber slides with dexamethasone (0–1000nM) for 30 minutes. Increasing concentrations of dexamethasone induced increased translocation of the GR in to the nucleus, resulting in less GR being localised to the cytoplasm of cells (**Figure 6.7.1** and **6.8**). At the maximum dexamethasone concentration, 85.9% of 16HBEs displayed nuclear GR, with the remaining cells having a combination of cytoplasmic- and nuclear-localised GR. At the lowest concentration of dexamethasone, very little GR had translocated to the nucleus, with 86.5% of cells having cytoplasmic GR. This concentration of dexamethasone was chosen to investigate the effects of birb 796 in future experiments.

6.3.6.4 Effect of birb 796 on glucocorticoid receptor translocation

Maximum concentration of birb 796 alone had no effect on GR translocation. Increasing concentrations of birb 796 used in combination with dexamethasone (0.1nM) increased nuclear GR translocation compared with dexamethasone (0.1nM) alone (**Figure 6.7.2** and **6.9**). Nuclear GR was found in 76.6% of 16HBEs when maximum birb 796 concentration in combination with dexamethasone (0.1nM) was used, which is considerably higher numerically, than using dexamethasone (10nM) alone.

6.7.1



6.7.2

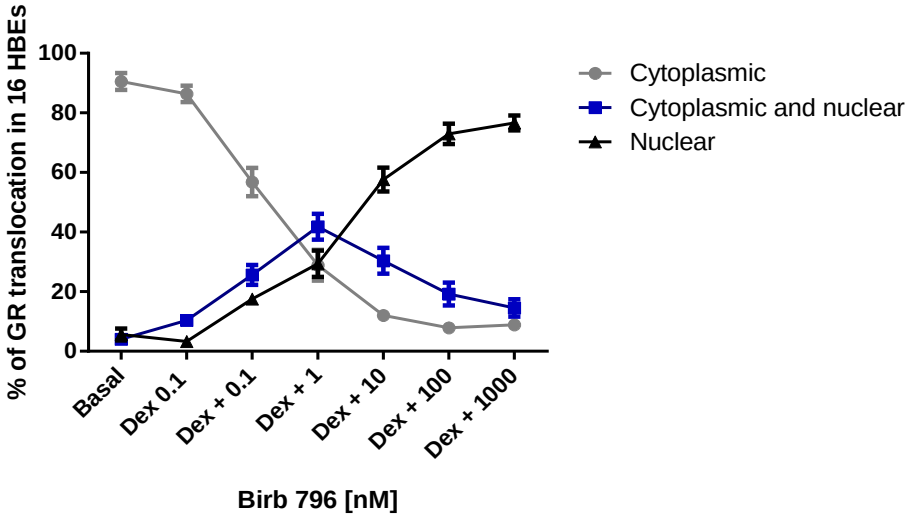


Figure 6.7 The effect of dexamethasone and birb 796 on the localisation of the glucocorticoid receptor in 16HBEs

6.7.1) Cells were incubated with dexamethasone (0–1000nM) for 30 minutes. 6.7.2) Cells were incubated with dexamethasone (0.1nM) and birb 796 (0–1000nM) for 30 minutes, GR localisation was assessed by immunofluorescent detection of GR and classified as either cytoplasmic, where all GR was present in the cytoplasm; nuclear, where all GR IS localised to the nucleus; or cytoplasmic and nuclear; where GR is localised to both the cytoplasm and the nucleus. A total of 300 cells per condition were analysed. Data are presented as mean ± SEM. Each experiment was repeated a minimum of 4 times with different cell passages.

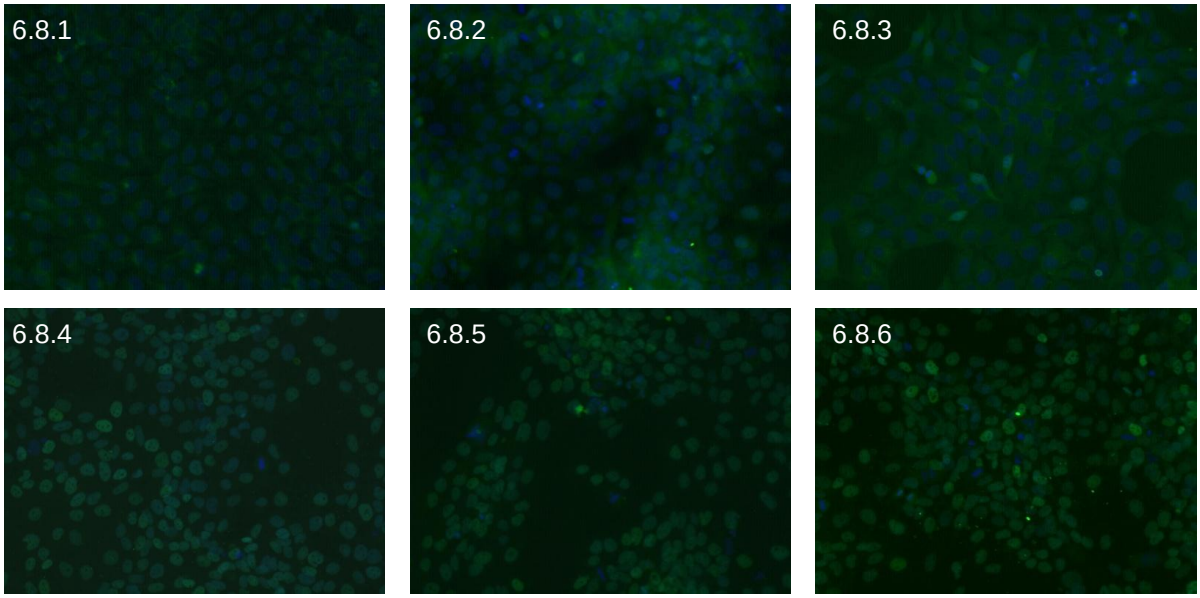


Figure 6.8 The effect of dexamethasone on glucocorticoid receptor translocation in 16HBEs

16HBEs were cultured on chamber slides in the presence of dexamethasone at the following concentrations for 30 minutes; 6.8.2) 0.1nM, 6.8.3) 1nM, 6.8.4) 10nM, 6.8.5) 100nM, 6.8.6) 1000nM. 6.8.1) Basal cells are also shown. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (blue). GR was detected using an Alexa 468 conjugated goat anti-rabbit secondary antibody (green). Cells were classified according to the location of GR; cytoplasmic, cytoplasmic and nucleic or nucleic.

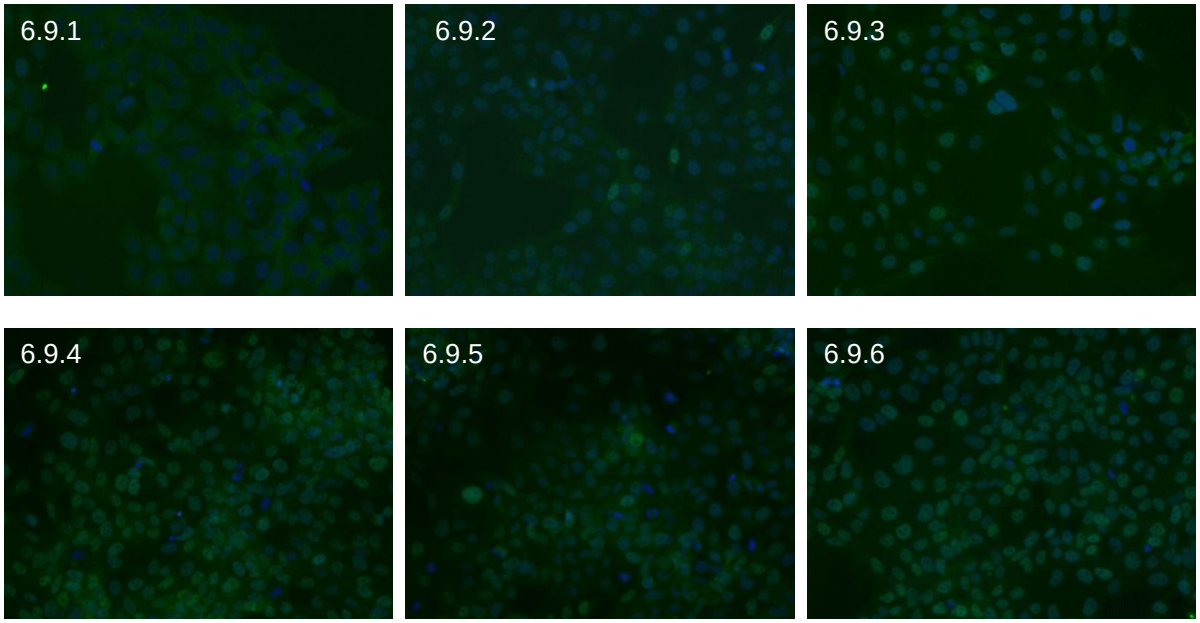


Figure 6.9 The effect of birb 796 on dexamethasone–induced glucocorticoid receptor translocation in 16HBEs

16HBEs were cultured on chamber slides in the presence of 0.1nM dexamethasone in combination with increasing concentrations of birb 796 for 30 minutes; 6.9.2) 0.1nM, 6.9.3) 1nM, 6.9.4) 10nM, 6.9.5) 100nM, 6.9.6) 1000nM. 6.9.1) Basal (unstimulated) cells are also shown. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (blue). GR was detected using an Alexa 468 conjugated goat anti-rabbit secondary antibody (green). Cells were classified according to the location of GR; cytoplasmic, cytoplasmic and nucleic or nucleic.

6.4 Discussion

The aim of this chapter was to examine the mechanisms by which a p38 MAPK inhibitor administered in combination with dexamethasone offers additive and synergistic inhibitory effects on pro-inflammatory mediator production in 16HBEs compared with using either drug alone. Specifically, the effects of combination treatment on phospho-p38 MAPK expression, CXCL8 mRNA stability, GR phosphorylation and GR nuclear translocation were assessed compared with using either drug alone. Dexamethasone had no effect on phospho-p38 MAPK expression. Birb 796 inhibited phospho-p38 MAPK expression, and combination treatment had no greater effect on phospho-p38 MAPK expression compared with using birb 796 alone. Dexamethasone also had no effect on the stability of LPS-, TNFα- or Poly (I:C)-induced CXCL8 mRNA stability, and combination treatment offered no greater benefit over using Birb 796 alone. Dexamethasone treatment induced GR phosphorylation at all three serine residues examined, as did LPS, TNFα and Poly (I:C) stimulation. Birb 796 inhibited dexamethasone, LPS, TNFα and Poly (I:C)-induced phosphorylation at serine 211, but had no effect on phosphorylation at either serine 203 or serine 226. Dexamethasone induced GR translocation into the nucleus of 16HBEs in a dose-dependent manner. Birb 796 also induced GR nuclear translocation in a concentration-dependent manner when used in combination with a low dose of dexamethasone, suggesting that the mechanism by which a p38 MAPK inhibitor and a steroid act synergistically is through increased translocation of GR to the nucleus, thus resulting in increased GR-induced transactivation and transrepression mechanisms, resulting in an overall greater anti-inflammatory effect.

MKPs act to dephosphorylate and inactivate the MAPKs and thus play an important role in MAPK regulation (Owens and Keyse, 2007). MKP-1 can regulate all three MAPKs, but preferentially inactivates p38 MAPK (Franklin and Kraft, 1997). GC are able to induce MKP-1 expression (Lasa et al, 2001) thus inhibiting phospho-p38 MAPK expression (King et al, 2009). In the current study western blotting was used to determine the effect of dexamethasone and/or birb 796 on phospho-p38 MAPK expression. Birb 796 inhibited phospho-p38 MAPK expression, whereas dexamethasone had no effect on phospho-p38 MAPK expression, which is in agreement with previous studies (Armstrong et al, 2011; Dauletbauv et al, 2011). In contrast, dexamethasone has also been shown to reduce phospho-p38 MAPK expression (King et al, 2009). In this study, dexamethasone inhibited phospho-p38 MAPK in TNFα-stimulated A549 pulmonary epithelial cells and BEAS-2B cells after 1 or more hours, whereas in the current study the effect of dexamethasone was only examined at optimal phospho-p38 MAPK time points (15 minutes for TNFα stimulated cells, 60 minutes for LPS- and Poly (I:C)-stimulated cells) which may account for the differences seen. Combination treatment offered no greater benefit compared with using birb 796 alone at the time points examined, suggesting that the mechanism whereby a p38 MPK inhibitor synergistically interacts with dexamethasone is not mediated via dephosphorylation of p38 MAPK. Further work examining the effect of dexamethasone alone or in combination with birb 796 on phosphorylated levels of p38 MAPK at different time points would be helpful in determining the full effect of dexamethasone on phospho-p38 MAPK in 16HBEs.

The p38 MAPK pathway is known to stabilise CXCL8 and IL6 mRNA transcripts (Winzen et al, 1998) thus enhancing the inflammatory micro-environment in specific cell-types. Dexamethasone destabilises IL-6 (Quante et al, 2008), TNFα (Smoak and Cidlowski, 2006) and COX-2 (Lasa et al, 2002) mRNA transcripts as part of its anti-inflammatory actions. Dexamethasone has been shown to induce MKP-1 (Lasa et al, 2002), which is a negative regulator of p38 MAPK, acting to dephosphorylate and thereby inhibit its pro-inflammatory actions (Franklin et al, 1998). TTP is an RNA binding protein involved in the direct regulation of inflammation associated transcripts containing AREs in the 3' UTR region, which targets them for rapid degradation. GC are known to promote TTP transcription (Smoak and Cidlowski, 2006), thus enhancing degradation of pro-inflammatory mRNA transcripts. In addition, TTP is a downstream target of p38 MAPK and recent data suggest that phosphorylation of TTP by p38 MAPK may block TTP-mediated MRNA decay by inhibiting deadenylase recruitment (Clements et al, 2011). In the current study it was hypothesised that the use of a p38 MAPK inhibitor in combination with dexamethasone may act additively/synergistically to destabilise pro-inflammatory mRNA transcripts, thus enhancing the GC- and p38 MAPK inhibitor-induced attenuation of pro-inflammatory mediator production. Dexamethasone had no effect on LPS-, TNFα- or Poly (I:C)-induced CXCL8 mRNA stability in 16HBEs. This is in contrast to a recent study showing dexamethasone-induced CXCL8 destabilisation (Dauletbaev et al, 2011). Dealetbaev and authors also showed that dexamethasone induced MKP-1 expression, which was beyond the scope of the current study. In the current study, 16HBE cells were stimulated for 4 hours prior to incubation with either dexamethasone and/or birb 796 and the transcriptional inhibitor actinomycin D for up

to 4 hours. In the study by Daeletbaev et al, cells were pre-incubated with dexamethasone for half an hour prior to stimulation for 1 hour with IL-1β. The stimulus was then removed and cells were re-incubated with dexamethasone for up to 90 minutes, which may account for the differences seen between the two studies. The differences seen in the two studies may also be due to different regulatory mechanisms being important in different cell types and also due to different stimulants being used, as it was shown in the previous chapter that the effect of pharmacological agents such as birb 796 and dexamethasone can be dependent on different stimulants being used. Inhibiting p38 MAPK with birb 796 lead to a time-dependent reduction in LPS-, TNFα- and Poly (I:C)-induced CXCL8 mRNA, showing that the p38 MAPK pathway is important in the stabilisation of stimulated CXCL8 mRNA in 16HBEs. Combination treatment with dexamethasone and birb 796 offered no greater benefit in the inhibition of CXCL8 mRNA transcript stability compared with using birb 796 alone, suggesting that increased pro-inflammatory mRNA transcript instability is unlikely to be the synergistic mechanism by which p38 inhibitors and steroids interact.

The GR undergoes a number of post-translational modifications including phosphorylation, which plays an important role in modulating its actions. Agonist-dependent phosphorylation occurs at serine 203, 211 and 226 residues present in the human GR transcript. Recently it has also been shown that dexamethasone induces phosphorylation at serine 404, which may be involved in the half-life of the GR molecule (Galliher-Beckley et al, 2008). Phosphorylation at serine 211 is implicated in the transcriptional activity of GR (Miller et al, 2007). Phosphorylation at

serine 226 is associated with increased nuclear export of GR, which prevents GR remaining in the nucleus and enables cytoplasmic GR to be bound by GC when GC enters the cell (Itoh et al, 2002). Serine 203 phosphorylation is also associated with nuclear export of GR (Wang et al, 2002) but this form is believed to be transcriptionally inactive as GR phosphorylated on serine 203 is contained solely in the cytoplasm (Wang et al 2001) and is unable to bind to a number of GRE-containing promoters (Blind and Garabedian, 2008).

A number of MAPKs have been shown to target GR through phosphorylation at specific serine residues. JNK and ERK phosphorylate GR at serine 226 *in vitro* (Rogatsky et al, 1998). Specifically, selective activation of ERK or JNK *in vivo* inhibits GR-mediated transcriptional activation, which depends on phosphorylation at serine 226 by JNK but not by ERK (Rogatsky et al, 1998). JNK inhibits GR transcriptional activation by direct receptor phosphorylation, whereas ERK inhibits indirectly (Rogatsky et al, 1998). Over-expression of JNK has also been shown to induce phosphorylation at serine 226, inducing nuclear export and the termination of GR-mediated transcription (Itoh et al, 2002). ERK is also thought to modulate phosphorylation at serine 203, as the effect of dexamethasone and an ERK inhibitor was shown to dephosphorylate serine 203, inducing translocation of the GR complex from the cytoplasm to the nucleus and subsequent binding of the complex to DNA was enhanced (Takabe et al, 2008). p38 MAPK has been shown to phosphorylate GR at serine 211 (Miller et al, 2008; Nader et al, 2010). p38 MAPK indirectly inhibits GR transcriptional activity through the AF-2/LBD region (Szatmary et al, 2004). Phosphorylation at serine 226 may also occur indirectly through p38 MAPK (Irusen

et al, 2002). The data presented in this chapter show that dexamethasone induces phosphorylation of GR at all three serine resides in a time-dependent manner. The effect of p38 MAPK-inducing pro-inflammatory stimuli on GR phosphorylation was also assessed, and LPS, TNFα and Poly (I:C) were all shown to induce phosphorylation of GR at serine 203, 211, and 226 resides in a time-dependent manner. Time-matched controls were not utilised in this study, so there is the potential that the effects on GR phosphorylation were due to cell culture rather than the effects of dexamethasone and or the stimuli. However, these results are consistent with a large number of previous studies showing GC and pro-inflammatory stimuli-induced GR phosphorylation. In addition, as the cells were plated out and left for 3–4 days prior to experiments being carried out, and since basal levels prior to the experiment starting showed minimal phosphorylation in cells that were not stimulated or treated with dexamethasone, it is unlikely that the effects seen were due to cells being in culture rather than the effect of the drug and/or stimulus. To determine whether the GR phosphorylation observed was p38 MAPK-dependent, the effect of the pharmacological p38 MAPK inhibitor Birb 796 was examined. Birb 796 inhibited dexamethasone-, LPS-, TNFα- and Poly (I:C)-induced phosphorylation at serine 211, suggesting that p38 MAPK directly phosphorylates serine 211, as demonstrated previously (Nader et al, 2011; Miller et al, 2008). In contrast, Birb 796 had no effect on dexamethasone-, LPS-, TNFα- or Poly (I:C)-dependent phosphorylation at serine 203 or 226, suggesting that p38 MAPK does not directly phosphorylate the GR at either of these residues. As LPS, TNFα and Poly (I:C) can also activate the JNK and ERK pathways, experiments assessing the effects of JNK and ERK inhibitors would determine whether this stimuli-induced

phosphorylation was mediated through either of these MAPKs. Since phosphorylation at serine 211 is believed to enhance the transcriptional activity of the GR (Wang et al, 2002) decreased phosphorylation at this residue is unlikely to be the synergistic mechanism by which p38 inhibitors and steroids interact. In addition, as mentioned previously, as there were no time-matched controls in this study, caution must be taken when interpreting these results, as there is no certainty that GR is phosphorylated over time when cells are in culture. Nevertheless, since Birb 796 inhibited phosphorylation at serine 211 but had no effect at 203 or 226, this indicates that regardless of the stimulus, p38 MAPK is not directly involved in phosphorylation at either of these residues. Recently, a study examining the mechanisms of GC insensitivity in severe asthma has shown that PBMCs from these patients have increased cytoplasmic GR phosphorylation, specifically at serine 226, which was attributed to p38 MAPK γ (Mercado et al, 2011). They showed that a selective p38 MAPK α and β inhibitor had no effect on serine 226 phosphorylation, but over-expression of p38 MAPK γ in a cell line lead to increased serine 226 phosphorylation and a similar GC insensitivity and impaired GR nuclear translocation compared with wild-type cells (Mercado et al, 2011). In the current study, the pan-p38 MAPK inhibitor birb 796 was used, which directly inhibits phosphorylation of p38 MAPK, and has been shown to inhibit all phospho-p38 MAPK isoforms (Kuma et al, 2005). No effect of birb 796 on GR phosphorylation at serine 226 was seen, which may be due to birb 796 having a lesser effect on the p38 γ isoform, or it may be due to different mechanisms being present in different cell types. Mercado et al carried used a monocytic cell line, whereas a bronchial epithelial cell line was used in the current study. Further research using quantitative PCR would determine relative

expression levels of different p38 MAPK isoforms, which may be helpful in determining their relative contribution to GR phosphorylation and/or GC insensitivity in other cell types.

After ligand binding, GR nuclear translocation occurs, which is an active process necessary for GC actions. Previously, increased cytoplasmic GR phosphorylation in severe asthma PBMCs has been found to cause impaired GR nuclear localisation compared with disease free cells, contributing to GC insensitivity (Mercado et al, 2011). In the current study, the effect of combination treatment on GR translocation was determined. In basal cells, GR was found to be present in the cytoplasm of nearly all cells, as has been shown previously (Ward and Weigel, 2009). Stimulation of 16HBEs with LPS, TNFα or Poly (I:C), which previously have been shown to activate the p38 MAPK pathway in a time-dependent manner, induced some GR to translocate to the nucleus. To determine whether this translocation was p38 MAPK-dependent, the effect of birb 796 was assessed on GR translocation in stimulated 16HBEs. Birb 796 had no effect on pro-inflammatory stimulator-induced GR translocation. As discussed, both JNK and ERK phosphorylate GR at serine 203 and 226 residues (Rogatsky et al, 1998, Takabe et al, 2008) and many stimuli that specifically activate the p38 MAPK pathway can also activate the JNK and ERK pathways (Cargnello and Roux, 2009). This suggests that pro-inflammatory stimuli-induced translocation of GR from the cytoplasm to the nucleus is mediated through the JNK or the ERK pathways, rather than p38 MAPK pathway. In addition, since birb 796 has no effect on serine 203 and serine 226 GR phosphorylation, this further supports a role for either JNK and/or ERK in pro-inflammatory stimuli–induced GR

translocation. Further research assessing the effect of JNK and/or ERK inhibition on pro-inflammatory stimuli induced GR nuclear translocation would determine a more precise role for these MAPKs in GR nuclear translocation. Dexamethasone treatment induced GR translocation to the nucleus in a concentration-dependent manner. At the highest concentration of dexamethasone (1000nM) GR was present in the nucleus of 85.9% of cells, with the remaining cells having both nuclear and cytoplasmic GR. At the lowest concentration of dexamethasone (0.1nM) there was a small increase in nuclear translocation, but GR was present solely in the cytoplasm of 86.5% of cells treated with this concentration. As such, this sub-optimal concentration of dexamethasone was chosen to examine the effect of birb 796 on GR translocation. Treatment of 16HBEs with 0.1nM dexamethasone in combination with birb 796 lead to increased GR translocation to the nucleus in a concentration-dependent manner. At the higher concentrations of birb 796 (100–1000nM) the percentage of cells with GR located solely in the nucleus was similar to numbers seen with the maximum concentration of dexamethasone. This suggests that combination treatment using a steroid and a pharmacological p38 MAPK inhibitor induces increased GR translocation to the nucleus, thus potentially enabling enhanced GR-mediated transactivation and transrepression mechanisms, resulting in a greater anti-inflammatory effect overall, as demonstrated by the synergistic reduction in pro-inflammatory mediator production in stimulated 16HBEs. Further work assessing the role of p38 MAPK in GR nuclear translocation is required to fully understand the role the p38 MAPK pathway plays. Although the current study shows that inhibiting p38 MAPK can increase dexamethasone-dependent GR nuclear translocation, it does not show how this effect is mediated. It could be suggested that

increased GR translocation into the nucleus leads to enhanced GR-mediated transactivation and transrepression mechanisms, thereby inducing a greater overall anti-inflammatory effect. However, further functional work assessing GR mediated activity would be required to confirm this. For example, ChIP assays could be used to compare GRE-mediated activity in cells treated with a p38 MAPK inhibitor in combination with dexamethasone compared with cells treated with dexamethasone alone. In addition, the effect of combination treatment on GC-induced transactivation and/or transrepression mechanisms could be examined, for example examining GILZ expression or MKP-1 mRNA levels to assess GR transactivation. The increased repression of pro-inflammatory mediator production by dexamethasone and birb 796 combination, as shown in Chapter 5, shows that enhancing GR translocation, as shown in this chapter, enhances GR-mediated transrepression mechanisms. It is also interesting to note that although inhibition of p38 MAPK increases GR nuclear translocation and potentially GR-mediated transrepression and transactivation mechanisms, as discussed earlier inhibition of p38 MAPK also inhibits GR phosphorylation at serine 211. Previous research indicates that serine 211 phosphorylation is required for full transcriptional activity of GR (Wang et al, 2002), possibly by inducing a functionally active folded conformation (Garza et al, 2010). The data presented in this chapter indicate that inhibition of GR phosphorylation at serine 211 by birb 796 enhances GR translocation, which, as shown in Chapter 5, enhances inhibition of pro-inflammatory mediator production from a bronchial epithelial cell line, compared with using either drug alone. Further research investigating the role of GR phosphorylation at serine 211 is required to fully understand its importance in GR activity.

In the previous chapter increased numbers of CD20+ B cells, CD8+ T cells, alveolar and sputum macrophages and bronchial epithelial cells were positive for phospho-p38 MAPK in COPD lungs compared with controls, suggesting that the p38 MAPK pathway is upregulated by COPD disease progression. As discussed earlier increased expression of phospho-p38 MAPK has also been found by other groups in patients with asthma and COPD (Renda et al, 2008; Lui et al, 2008; Bhavsar et al, 2010). Increased numbers of cells with activated phospho-p38 MAPK in COPD lungs may therefore impair their responsiveness to GC, and indeed, inhibition of p38 MAPK has been shown to restore GC responsiveness in asthma PBMCs (Bhavsar et al, 2009). In Chapter 5, combination therapy was shown to be more effective at inhibiting pro-inflammatory mediator release compared with using either drug alone. In the current study, inhibition of p38 MAPK increased dexamethasone-mediated GR nuclear translocation. This data suggest that increased activation of p38 MAPK in patients with COPD may impair normal GR transport from the cytoplasm into the nucleus in these cells, thus altering the GC responsiveness of particular cells. Clinically, the use of a pharmacological p38 MAPK inhibitor in combination with a GC may therefore offer greater anti-inflammatory effects than using either drug alone due to decreasing phospho-p38 MAPK, inducing increased GR- nuclear translocation and as a result, increased GR-mediated transactivation and transrepression mechanisms.

Chapter 7

Conclusion

COPD is a progressive and chronic disease characterised by airflow limitation that is not fully reversible. GC have limited clinical benefit in patients with COPD, and as such, new anti-inflammatory therapies are required. Understanding the mechanisms of GC insensitivity and identifying alternative inflammatory pathways in COPD is crucial in the development of new therapies.

In chapter 3, mixed sputum cell culture was used as a method to examine the functional properties of airway inflammatory cells, mostly macrophages and neutrophils. Mixed sputum cells spontaneously released TNFα and CXCL8, and were unresponsive to LPS stimulation. This is in contrast to alveolar macrophages, which secrete modest amounts of pro-inflammatory mediators without prior stimulation (Armstrong et al, 2009). As discussed previously, this may be due to cells being activated in the airways due to bacterial colonisation, or it may be due to the effect of DTT, which is a mucolytic and may therefore activate the cells during sputum processing. The lack of effect of LPS may be due to a lack of TLR4, which has been shown previously in airway neutrophils (Baines et al, 2009) or due to airway cells being rendered tolerant to LPS due to continual exposure in the airways (Fujihara et al, 2003). More research examining the expression of TRL4 on mixed sputum cells and the effect of DTT on the cells is required to fully interpret their functional properties.

In chapter 3 the GC sensitivity of inflammatory airway cells was also examined, using the same mixed sputum cell culture model. Previous studies have indicated that GC sensitivity is a cell- and cytokine-specific phenomenon irrespective of

disease status (Hew et al, 2006; Bhavsar et al, 2008; Armstrong et al, 2009; Kent et al, 2009). To date, research has examined the GC sensitivity of alveolar macrophages and PBMCs from patients with COPD, but the sensitivity of other cells implicated in COPD has not yet been described. In agreement with previous studies (Hew et al, 2006; Bhavsar et al, 2008; Armstrong et al, 2009; Kent et al, 2009), no differences in the GC sensitivity of COPD mixed sputum cells compared with smoker and non-smoker control cells were observed. This suggests that in COPD, cells do not have an altered response to GC compared with non-disease cells. Instead, GC insensitivity may be an inherent property in certain cells regardless of disease status, and the increased numbers of GC-insensitive macrophages and neutrophils in COPD lungs means that the overall effectiveness of GC is insufficient to inhibit the inflammation present to a reasonable degree in patients with COPD. This is the first study to compare the effects of GC on mixed sputum cells in COPD, smokers and non-smokers, and enhances the existing knowledge regarding cell- and cytokine-specific GC insensitivity irrespective of disease status.

As mixed sputum cells contain macrophages and neutrophils as the major cell types present, these cell types were isolated from sputum and the effect of GC was examined individually on these cell types in order to understand the relative contribution of each cell in terms of pro-inflammatory mediator production and their response to GC. The effect of dexamethasone on sputum macrophages was similar to that seen previously in alveolar macrophages (Armstrong et al, 2010) in that maximum mean per-cent inhibition of the pro-inflammatory mediators TNFα and CXCL8 was around 65%, suggesting a degree of insensitivity in these cells. This is

an important finding since much research so far as focused on the GC sensitivity of alveolar macrophages, monocyte-derived macrophages and PBMCs. This is the first study that shows the effect of GC on sputum macrophages, which are also likely to be involved in the inflammatory environment seen in COPD lungs, and as such represent a viable target to direct new therapies towards. Little is known about the functional properties of sputum macrophages and the role they play in COPD. Sputum macrophages are thought to represent a sub-population of lung macrophages and have been shown to be much smaller in size (Frankenberger et al, 2004) and to express different receptors compared with alveolar macrophages (Lensmar et al, 1998). Nevertheless, they represent an alternative cell type in COPD in which more research is required to fully elucidate their role.

In contrast, dexamethasone had modest effects on pro-inflammatory mediator production from airway neutrophils, which is in agreement with previous literature showing that airway neutrophils from patients with cystic fibrosis (Corvol et al, 2003) and bronchial sepsis (Pang et al, 1997) are largely insensitive to GC, which may be due to low GR expression on these cells (Plumb et al, 2011). GR is expressed on blood neutrophils (Pujols et al, 2002) and the effect of dexamethasone on unstimulated and LPS-stimulated blood neutrophils showed that systemic neutrophils respond to GC, with mean maximum per cent inhibitions of up to 90 per cent seen. This suggests that neutrophils in the blood stream are activated and migrate to the lungs, where they then undergo phenotypic changes, such as reduced GR expression (Plumb et al, 2011), as well reduced TLR4 expression (Baines et al, 2010) and lack of phospho-p38 MAPK expression (as discussed in Chapter 4).

Further research into the functional properties of lung neutrophils is required to fully understand the role they play in COPD and the alternative inflammatory pathways that may be targeted.

In Chapter 4 increased numbers of cells positive for activated p38 MAPK were found in COPD lungs compared with non-smoking controls. For some cell types, there was also an increase in cell numbers positive for phospho-p38 MAPK in smokers compared with non-smokers. This suggests that cigarette smoke increases the activation of p38 MAPK in specific cell types, which is then further enhanced by the development of COPD. Specifically, numbers of phospho-p38 MAPK positive follicular CD20+ B cells and CD8+ T cells, alveolar and sputum macrophages and bronchial epithelial cells were found in COPD lungs. Previously, an increase in activated p38 MAPK in COPD lungs has been reported (Renda et al, 2008), but specific cell types, other than alveolar macrophages, were not identified in this study. Other studies have shown increased p38 MAPK activation in severe asthma macrophages (Bhavsar et al, 2008) and bronchial epithelial cells (Lui et al, 2008). This is the first study to show the presence of cell-specific activated p38 MAPK in COPD lungs compared with controls.

In contrast, lung neutrophils were devoid of activated phospho-p38 MAPK. Functional studies examining the effect of a pharmacological p38 MAPK inhibitor on pro-inflammatory mediator release from these cells showed that LPS did not induce phospho-p38 MAPK and as such, TNFα and CXCL8 release was not inhibited by p38 MAPK inhibition. In blood neutrophils, phospho-p38 MAPK was induced by LPS, and TNFα and CXCL8 release was attenuated by a p38 MAPK inhibitor. This is the

first study to show that lung neutrophils are devoid of activated p38 MAPK and that a pharmacological p38 MAPK inhibitor has limited effects on pro-inflammatory mediator release from these cells. This data further supports previous literature showing lung neutrophils have an altered phenotype compared with systemic blood neutrophils, as discussed above in relation to LPS stimulation and GC sensitivity.

The increase in numbers of cells positive for phospho-p38 MAPK suggests a pharmacological p38 MAPK inhibitor may be useful in the treatment in COPD, by specifically acting on these cells and reducing pro-inflammatory mediator release, as well as other pro-inflammatory functions through the inhibition of activated p38 MAPK, thus reducing overall inflammation present in the lungs. For example, previous research has demonstrated the effects of a p38 MAPK inhibitor in inhibiting lymphocyte pro-inflammatory mediator release (Schafer et al, 1999, Mori et al, 1999, Veiopaulo et al, 2004, Koprak et al, 1999, Rincon et al, 1998), and epithelial cell mucin production (Kim et al, 2002; Song et al, 2003), which are also implicated in COPD pathogenesis. Increased activation of p38 MAPK has also been implicated in GC insensitivity in a number of cells types including macrophages (Bhavsar et al, 2008) and IL-4-stimulated PBMCs (Irusen et al, 2002) and inhibition of p38 MAPK has been demonstrated to restore GC sensitivity in both of these studies. Phosphorylation of GR at serine residue 211 by IL-13-, TNFα- and IL-1α-induced p38 MAPK has been shown to inhibit GR function (Szatmary et al, 2004; Spahn et al, 1996; Wang et al, 2004). Taken together, these data suggest that increased activation of the p38 MAPK pathway may be involved in the inflammatory environment within COPD lungs, as well as playing a role in GC sensitivity of local

lung cells. Targeted inhibition of the p38 MAPK pathway in patients with COPD may attenuate the inflammation and restore GC sensitivity, although further work is needed to confirm this.

The responsiveness of bronchial epithelial cells to GC, p38 MAPK inhibition and combination therapy was investigated in Chapter 5 using three different pro-inflammatory stimuli. The bacterial polysaccharide LPS, the synthetic dose-stranded RNA TLR4 ligand Poly (I:C), and the pro-inflammatory cytokine TNFα were chosen to represent bacterial colonisation, viral infection and pro-inflammatory cytokine, respectively. Sensitivity to GC and p38 MAPK inhibition was dependent on the stimulus and pro-inflammatory mediator measured. In general, GC were more effective than p38 MAPK inhibition in attenuating pro-inflammatory mediator release, as has been demonstrated previously (Kent et al, 2009, Bhavsar et al, 2010, Armstrong et al, 2011). This is the first study to investigate the differential effects of stimuli and pro-inflammatory mediator release on GC and p38 MAPK sensitivity in bronchial epithelial cells. In addition, the effect of combination treatment was also examined by constructing full-dose response curves for both drugs in combination to evaluate additive and synergistic effects. Dexamethasone and birb 796 both reduced stimulated pro-inflammatory mediator release in a dose-dependent manner. In addition, increasing the concentration of birb 796 in combination with dexamethasone induced progressively increased inhibition of pro-inflammatory mediator production compared with either drug alone, which is in agreement with a previous study carried out in alveolar macrophages (Armstrong et al, 2011). These data suggest that combining a p38 MAPK inhibitor and GC may be a more effective

anti-inflammatory treatment in COPD compared with using either drug alone, although further work in other cell types implicated in COPD is needed to confirm these effects.

Combination therapy has a number of advantages over monotherapy. The data shown in chapter 5 indicates that similar inhibition of pro-inflammatory mediator production can be achieved by combining a lower dose of GC and birb 796 compared with either drug alone at a higher concentration. The use of p38 MAPK inhibitors as a monotherapy has demonstrated considerable hepatotoxicity in early clinical trials examining the anti-inflammatory effects. In addition, high doses of p38 MAPK inhibitors have also been shown to inhibit several non-p38 MAPKs, suggesting that the inhibitors are not specific to p38 MAPK, and therefore any beneficial effects cannot be attributed to inhibition of p38 MAPK alone. The use of p38 MAPK inhibitors as single therapeutic agents may also have other adverse effects. Signalling cascades are highly redundant and complex, thus blocking p38 MAPK may lead to compensatory effects in other kinases that can regulate other genes. Kinases upstream from p38 MAPK such as MKK3, MKK6, and TAK1 can regulate NFκB and redirect the signalling flow (Fukushima et al, 2009; Inoue et al, 2006). Humans may also have a physiological escape from p38 MAPK inhibition, as demonstrated by p38 MAPK inhibitor-induced reductions in CRP levels returned to baseline after 8 weeks of therapy in a trial investigating the effects of birb 796 in patients with Chrohn's disease (Schreiber et al, 2006). p38 MAPK may also participate in a negative feedback loop and blocking p38 MAPK with a pharmacological inhibitor may divert the signalling flux to other MAPKS such as JNK

or ERK. Long-term use of high-dose GC is linked to a variety of unwanted side effects including osteoporosis, adrenocortical suppression, bruising and skin thinning, cataracts and glaucoma (Roland et al, 2004). Using lower doses of GC in combination with a low dose of p38 MAPK inhibitor would reduce the development of these GC-associated side effects, and potentially limit some of the real and hypothesised effects of p38 MAPK inhibition-induced adverse effects. Therefore combining low-dose GC with a p38 MAPK inhibitor may be a more effective therapeutic strategy in the management of COPD.

GC inhibit transcription factor activity at the promoter regions of pro-inflammatory genes (Adcock et al, 2004) and p38 MAPK inhibitors attenuate the phosphorylation of transcription factors (Rolli et al, 1999, Zhu and Lobie, 2000, Wiggin et al, 2002). As such, both pharmacological agents inhibit pro-inflammatory gene transcription. In addition, GC (Lasa et al, 2002, Smoak and Cidlowski, 2006, Quante et al, 2008) and p38 MAPK inhibitors (Winzen et al, 1999) act post-transcriptionally and on protein translation. The data presented in Chapter 5 suggest more than an additive effect of these molecular mechanisms when combination treatment was used. The mechanism under-lying these synergistic effects are unknown. Possible hypotheses are that the synergism may be a result of inhibition of p38 MAPK-mediated GR phosphorylation (Irusen et al, 2002) enabling effective binding or translocation of GR to the nucleus (Itoh et al, 2006). Alternatively, increased pro-inflammatory mRNA transcript instability may be an alternative explanation. Further work investigating the synergistic mechanisms underlying GC and p38 MAPK inhibitor combination therapy is required.

In the final results chapter, the synergistic interactions between p38 MAPK inhibitors and GC were investigated by examining phospho-p38 MAPK expression, CXCL8 mRNA stability, GR phosphorylation and GR nuclear translocation. Birb 796 inhibited expression of phospho-p38 MAPK, but dexamethasone had no effect, which is in agreement with previous studies (Armstrong et al, 2011; Dauletbauv et al, 2011). Combination treatment offered no greater effect in inhibiting phospho-p38 MAPK expression compared with using Birb 796 alone. Previous studies have shown that dexamethasone may inhibit phospho-p38 MAPK through the induction of MKP-1 expression (Lasa et al, 2001; King et al, 2009); however this was not shown in the current study. Further research is therefore needed elucidate the effect of dexamethasone on activated p38 MAPK.

Birb 796 also inhibited LPS-, TNFα- and Poly (I:C)-stimulated CXCL8 mRNA transcript stability. Dexamethasone had no effect on CXCL8 mRNA transcript stability, and combination treatment had no greater effect compared with using Birb 796 alone. Dexamethasone has recently been shown to destabilise CXCL8 mRNA (Dauletbaev et al, 2011). These differences in dexamethasone-mediated effects on CXCL8 mRNA transcripts may be due to differences between cell-lines or experimental protocols; therefore further work is needed to fully understand the effect of dexamethasone on CXCL8 stability.

Dexamethasone, LPS, Poly (I:C) and TNFα all induced GR phosphorylation at serine 203, 211 and 226 in a time-dependent manner. Birb 796 inhibited serine 211 phosphorylation, which is in agreement with previous studies (Miller et al, 2008, Nader et al, 2010), but had no effect on serine 203 or 226 phosphorylation.

Previously, inhibition of p38 MAPK has been shown to reduce serine 226 phosphorylation (Irusen et al, 2002) but no such effect was seen in the current study. Previous research has shown that JNK and ERK phosphorylate 226 and 203 serine residues (Rogatsky et al, 1998, Takabe et al, 2008) suggesting that LPS-, Poly (I:C)- and TNFα-induced 226 and 203 phosphorylation is mediated through these MAPK pathways rather than the p38 MAPK pathway, although further work is needed to confirm this.

LPS, Poly (I:C)- and TNFα-induced translocation of GR to the nucleus, which was not dependent on p38 MAPK as birb 796 had no effect. This suggests that LPS-, Poly (I:C)- and TNFα-stimulated GR phosphorylation at serine 203 and 226 and GR nuclear translocation is mediated through the JNK or ERK pathways. Indeed, previous studies have shown that JNK phosphorylates serine 226 (Rogatsky et al, 1998) and ERK phosphorylates serine 203 (Takabe et al, 2008). Dexamethasone induced GR nuclear translocation in a concentration-dependent manner. Birb 796 enhances GR nuclear translocation when used in combination with a sub-optimal dexamethasone concentration. This data suggest that combination therapy increases the translocation of GR to the nucleus, which is necessary for GR-mediated transrepression and transactivation, as shown by enhanced inhibition of pro-inflammatory mediator production in Chapter 5. Therefore, the increased numbers of CD20+ B cells, CD8+ T cells, macrophages and bronchial epithelial cells expressing activated p38 MAPK in COPD lungs may be responsible for altered GR sensitivity in COPD due to impaired GR translocation. Further work examining the role of p38 MAPK in GR nuclear translocation is required.

To conclude, GC sensitivity is a cell- and cytokine-specific phenomenon regardless of disease status. Increased numbers of cells, (including macrophages, both sputum and alveolar, and airway neutrophils) that exhibit only modest responses to GC may, in part, explain why GC have limited clinical benefit in patients with COPD. In addition, there are also increased numbers of specific cell types in COPD lung that have activated p38 MAPK present, which may interfere with GR translocation in these cells, indicating the p38 MAPK as a potential anti-inflammatory target in COPD. A GC administered in combination with a p38 MAPK inhibitor may be a more effective therapeutic strategy in the management of COPD, possibly by enhancing GR-mediated transactivation and transrepression through increased GR-nuclear translocation.

Chapter 8

Future Work

To summarise, this thesis has demonstrated that there are no differences in the GC sensitivity of COPD, smoker and non-smoker mixed sputum cells. It has also shown that in these mixed sputum cultures, the neutrophil population do not respond to LPS stimulation and GC have no effect on pro-inflammatory mediator production from these cells. In addition, increased activated p38 MAPK was present in COPD lungs, specifically localised to follicular CD20+ B cells and CD8+ T cells, in alveolar and sputum macrophages and in bronchial epithelial cells, compared with non-smoking control lungs. For CD20+ B cells and alveolar and sputum macrophages there was also increased activated p38 MAPK in these cells in smoker lungs, compared with non-smoking lungs. In contrast, there was a complete absence of activated p38 MAPK in lung tissue and sputum neutrophils, and as such, a p38 MAPK inhibitor had no effect on pro-inflammatory mediator production from these cells. The final chapters focussed on bronchial epithelial cells and the p38 MAPK pathway. LPS, TNFα and Poly (I:C) induce CXCL8, IL-6 and RANTES release which is dependent on p38 MAPK activation. Indeed, the p38 MAPK inhibitor birb 796 as well as the GC dexamethasone inhibit the release of all three pro-inflammatory mediators, although the degree of inhibition appears to be both stimuli- and pro-inflammatory mediator-dependent. Combination treatment of bronchial epithelial cells with birb 796 and dexamethasone had a greater inhibitor effect on pro-inflammatory mediator production compared with using either drug alone, and for some stimuli and pro-inflammatory mediators, synergistic interaction was demonstrated using statistical analysis. In the final chapter, I sought to understand the mechanisms by which birb 796 and dexamethasone synergistically interact, by examining CXCL8 mRNA stability, GR phosphorylation and GR translocation. Dexamethasone had no effect

on CXCL8 mRNA stability. Birb 796 inhibited CXCL8 mRNA stability in a time-dependent manner, and combination treatment had no greater effect on CXCL8 mRNA stability compared with birb 796 alone. LPS-, TNFα-, Poly (I:C)- and dexamethasone-induced GR phosphorylation at serine's 203, 211 and 226 in a time-dependent manner. Birb 796 inhibited GR phosphorylation at serine 211 but had no effect on phosphorylation at either serine 203 or 226. Finally, birb 796 increased the dexamethasone-mediated nuclear translocation, suggesting that increased p38 MAPK in COPD lungs may interfere with GR-mediated transactivation and transrepression through altering GR translocation to the nucleus. Although a number of questions have been answered within this thesis, there is also much scope for future work in this area, which will be further discussed in the following chapter.

In Chapter 3, mixed sputum cells were found to be unresponsive to LPS stimulation. This was in agreement with a number of other published studies (Pang et al, 1997; Bettiol et al, 2002; Dentener et al, 2006; Scheicher et al, 2007), suggesting that these cells are already activated *in vivo*. In other studies, mixed sputum cells have been shown to responsive to PHA (Lui et al, 2008) and to fMLP (Beeh et al, 2004) suggesting that the unresponsiveness is specific to LPS stimulation. However, further work assessing the effect of other stimulants would confirm whether this un-responsiveness is specific to LPS, or whether it is due to these cells already being pre-activated. It was also shown that isolated sputum neutrophils do not respond to LPS, which is also in agreement with other studies (Baines et al, 2009, Baines et al, 2010). In chapter 4, LPS induced phospho-p38 MAPK in blood neutrophils but not in isolated sputum neutrophils. Previously, isolated sputum neutrophils have been

shown to lack TLR4 (Baines et al, 2010), which the authors suggest is disease specific in severe asthma patients. Further work examining TLR4 expression using PCR in COPD sputum neutrophils would confirm whether the lack of TLR4 expression is common to all sputum neutrophils or whether this is disease-specific phenomenon. In addition, it would also be useful to examine the effects of other stimulants such as TNFα or fMLP on phospho-p38 MAPK in sputum neutrophils to determine whether p38 MAPK activation can be induced in these cells under certain conditions or whether lack of phospho-p38 MAPK expression is a phenotypic change that neutrophils undergo when they leave the bloodstream and enter the lung. In addition, it would also be useful to examine phospho-p38 MAPK in isolated lung neutrophils or BAL neutrophils, although the limited number of cells present in these samples may limit much meaningful analysis. Further work using isolated sputum neutrophils and the effect of p38 MAPK inhibition on other neutrophil functions would also be beneficial. Previous work has shown that in blood neutrophils, inhibition of p38 MAPK inhibits chemotaxis (Cara et al, 2001; Nick et al, 1997; Nick et al, 2002; Fujita et al, 2005, Spisani et al, 2005, Montecucco et al, 2006), and superoxide generation (Zu et al, 1998; Suzuki et al, 1999), and fLMP-induced pro-inflammatory mediator production (Zu et al, 1998; Lal et al, 1999; Sakamoto et al, 2006), which are implicated in COPD pathogenesis. Although the data presented in this thesis suggests that pharmacological p38 MAPK inhibition would have no effect on either chemotaxis or respiratory burst in isolated sputum neutrophils, research to confirm this is needed.

In Chapter 4, increased percentages of CD20+ B cells, CD8+ T cells, alveolar and sputum macrophages and bronchial epithelial cells were positive for phospho-p38 MAPK in COPD lungs compared with smoker and non-smoker lungs, suggesting that this pathway is upregulated by both smoking and by COPD disease development and progression. Previous research has shown that alveolar macrophages express the p38 MAPK delta isoform (Smith et al, 2006) and recently, the p38 MAPK delta isoform has been implicated in GC insensitivity in asthma (Mercado et al, 2011). In addition, clinical development of p38 MAPK inhibitors may focus on specific isoforms of p38 MAPK rather than general p38 MAPK inhibition and as such, examining the expression of different isoforms in COPD lungs would be useful. In this thesis, activated or phosphorylated p38 MAPK was examined. It would not be possible to determine the expression of isoform-specific phosphorylated p38 MAPK as all four isoforms share the same phosphorylation site. However, it would be possible to examine total p38 MAPK isoforms, either using IHC analysis of FFPE lung tissue, or western blot or PCR analysis of lung tissue lysates. Western blot or PCR analysis of specific isoform expression in specific cell types would also be beneficial. For example, CD8+ T cells can be isolated from resected lung tissue taken from patients undergoing surgery for suspected or confirmed lung cancer and the levels of specific p38 MAPK isoforms could be examined between different patient groups. This could also be carried out in primary bronchial epithelial cells using bronchial brushings from patients undergoing a bronchoscopy. Either of these cell types could be used to ascertain whether any differences in isoform expression exist between patient groups.

In Chapters 5 and 6 the bronchial epithelial cell line 16HBE was used to examine the effect of dexamethasone and birb 796 on stimulated pro-inflammatory mediator release, CXCL8 mRNA stability, GR phosphorylation and GR nuclear translocation. It would be useful to repeat these experiments in primary human bronchial epithelial cells to replicate the results and ensure that similar mechanisms also play a role in primary bronchial epithelial cells. The use of birb 796 clinically has been shown to have some undesirable side effects, and as such new p38 MAPK inhibitors are in development. In addition, studies have indicated that birb 796 may also inhibit JNK2, a member of the JNK intracellular signalling family (Gruenbaum et al, 2009; Kuma et al, 2005). These findings raise the question of whether inhibition of JNK2 might contribute to Birb 796-mediated anti-inflammatory effects. Examining the effect of birb 796 on JNK activation in bronchial epithelial cells would determine whether or not the anti-inflammatory effects observed in these chapters was partly due to the effect of birb 796 on JNK inhibition. In addition, repetition of key experiments using an alternative p38 MAPK inhibitor would also be useful to show that the results presented in this thesis are not specific to birb 796.

In Chapter 6 the effect of birb 796, dexamethasone and combination treatment on stimulated CXCL8 mRNA stability was examined. It would be useful to examine the effect of these pharmacological agents on other pro-inflammatory mediators, such as RANTES or IL-6 mRNA to determine whether the results shown are specific to CXCL8 mRNA or to demonstrate that dexamethasone does not affect mRNA stability of other pro-inflammatory transcripts in this cell-line. Previously, dexamethasone has been shown to stabilise IL-6 (Quante et al, 2008), TNFα (Smoak and Cidlowski,

2006), CXCL8 (Dauletbaev et al, 2011) and COX-2 mRNA (Lasa et al, 2002). Further work assessing the effect of dexamethasone on pro-inflammatory transcripts would determine whether this is cell- or pro-inflammatory mediator transcript-specific.

Finally, the effect of birb 796 on GR phosphorylation and dexamethasone-induced GR translocation was also examined in Chapter 6. Levels of GR phosphorylation at serine 203, 211 and 226 were fairly low in 16HBEs and densitometric analysis was not possible due to high background. As such it would be useful to repeat these experiments in an alternative cell line to replicate the results. Birb 796 had no effect on GR phosphorylation at serine 203 or at serine 226 induced by dexamethasone, LPS, TNFα and Poly (I:C). Previous research has shown that JNK (Rogatsky et al, 1998, Itoh et al, 2002) and ERK (Takabe et al, 2008) phosphorylate these serine residues. In addition, LPS, TNFα and Poly (I:C) can also induce activation of both JNK and ERK pathways, thus the use of a JNK and/or an ERK pharmacological inhibitor on LPS-, TNFα- and Poly (I:C)-induced GR phosphorylation at serine 203 and 226 would confirm that this phosphorylation is dependent on ERK and or JNK. Time-matched controls were not carried out to show whether GR phosphorylation occurs under normal physiological conditions during cell culture, therefore it cannot be fully ascertained that the effects on GR phosphorylation were induced by dexamethasone, LPS, TNFα, or Poly (I:C). Although previous studies have shown similar findings with respect to dexamethasone and these stimuli inducing GR phosphorylation, the relative effects of these on GR phosphorylation compared with the effects of cell culture cannot be definitively concluded from this study. Therefore, future experiments would need to be carried out with time-matched controls to fully

demonstrate the effect of dexamethasone, LPS, TNFα and Poly (I:C) on GR phosphorylation. Birb 796 also increased dexamethasone-induced GR nuclear translocation. In these experiments, cells were incubated with dexamethasone for 30 minutes then immunofluorescent detection was used to visualise the localisation of GR in 16HBEs; GR was classified as being present in the cytoplasm, in the nucleus or present in both the cytoplasm and the nucleus. Live cell imaging would enable a more detailed understanding of GR translocation in 16HBEs. In the current study only one time point was examined to look at GR localisation, whereas it would be more useful to visualise the cells over a longer period of time using live cell imaging. Alternatively, confocal microscopy would also be useful in this regard. Further functional work examining the effect of birb 796 on GR-mediated transactivation and or transrepression mechanisms would also show whether birb 796 affects GR function rather than just increasing GR translocation to the nucleus. For example, ChIP assays could be used to examine hormone-dependent transcription in GREs present in GC responsive genes. In addition, LPS, TNFα and Poly (I:C) were also shown to induce GR nuclear translocation, although this was found to be independent of p38 MAPK as birb 796 had no effect. Further work examining the effect of a pharmacological JNK or ERK inhibitor on stimuli-induced GR translocation would show whether this is JNK or ERK dependent.

To summarise, this thesis has demonstrated the importance of the p38 MAPK pathway in COPD, specifically in regards to GC sensitivity. Combination therapy comprising a pharmacological p38 MAPK inhibitor in conjunction with GC may

provide a use anti-inflammatory therapy in patients with COPD, although further work examining the role of p38 MAPK in GC sensitivity is still required.

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Appendix

Publications from this thesis

1. Gaffey, K, Plumb, J, Kaur, M, and Singh, D. (2011). Increased p38 Mitogen Activated Protein Kinase in Chronic Obstructive Pulmonary Disease Lungs. American Thoracic Society, Denver, USA; May 2011. *American Journal of Respiratory and Critical Care Medicine*, **183**; 2011: A2565

2. Gaffey, K, Kaur, M, Plumb, J, Armstrong, J, and Singh, D. (2011). Corticosteroid Insensitivity of Mixed Sputum Cells. American Thoracic Society, Denver, USA; May 2011. *American Journal of Respiratory and Critical Care Medicine*, **183**; 2011: A4458

3. Plumb, J, Gaffey, K, Kane, B, Malia-Milanes, B, Shah, R, Bentley, A, Ray, D, and Singh D. (2012). Reduced glucocorticoid receptor expression and function in airway neutrophils. *International Immunopharmacology*, **12**; 26–33.

4. Gaffey, K, Reynolds, S, Plumb, J, Kaur, M, and Singh, D. (2012). Increased phosphorylated p38 mitogen activated protein kinase in COPD lungs. *European Respiratory Journal*, (EPub ahead of print).