The role of macrophage migration inhibitory factor in airways disease

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A Thesis submitted to Imperial College London for the degree of Doctor of Philosophy by

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Abstract

Chronic obstructive pulmonary disease (COPD) and severe asthma are progressive chronic inflammatory diseases of the airways. Both diseases are characterised by airflow limitation and share some pulmonary symptoms. However they have distinct inflammatory cell signatures and differ in response to corticosteroid (CS) treatment. Most asthmatic patients control their disease with CS, with a few showing a relative CS resistance; however COPD patients show little or no improvement with CS and are CS insensitive. Macrophage migration inhibitory factor (MIF) is a pleiotropic pro-inflammatory mediator whose function is yet to be fully elucidated. MIF has been shown to counter-act the immunosuppressive action of CS. MIF is elevated in chronic diseases such as asthma and atherosclerosis. The role of MIF in COPD has not been investigated and its role in asthma is not fully understood.

MIF inhibition attenuated ozone-induced airway inflammation and lung function *in vivo* but did not affect CS sensitivity. MIF expression did not vary between stable COPD patients and controls. Pro-inflammatory effects of MIF were investigated in THP-1 monocytes and primary cells. There was no clear role for MIF in LPS-induced inflammation. MIF modulated the transactivation functions of CS in THP-1 cells. Finally I took an unbiased approach to generate new hypotheses for MIF function using proteomic and transcriptomic techniques. The RIG-I-like pathway was identified by proteomics as a novel target pathway and was investigated in THP-1 cells and human BAL macrophage samples following viral infection.

The role of MIF in airway inflammation remains unclear and results demonstrated here show MIF function does not necessarily translate from mouse to humans. MIF does not seem to have a role in the inflammation of stable disease. The proteomic data suggests that the association between viral infection, MIF and CS in regulating CS sensitivity in COPD and severe asthma should be investigated.

Declaration of originality

I, Kirsty Russell declare that I wrote this thesis and the experiments and work described herein, except where appropriately referenced, was performed by myself. Information derived from the published and unpublished work of others has been acknowledged in the text and full references are given.

Copyright declaration

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Table of Contents

List of Figures

List of Tables

Abbreviations

AP-1	Activating protein-1
ARDS	Acute respiratory distress syndrome
Arg	Arginine
BALF	Bronchoalveolar lavage fluid
Bcl2	B-cell lymphoma-2
BRD4	Bromodomain containing 4 gene
CD	Cluster of differentiation
Cdk	Cyclin-dependent kinases
ChIP	Chromatin immuno-precipitation
CLF	Chemokine-like function
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase 2
CREB	cAMP response element-binding protein
CS	Corticosteroid
CSE	Cigarette smoke extract
CVD	Cardiovascular disease
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DNA	Deoxyribonucleic acid
DUSP	Dual-specificity phosphoprotein phosphatase
ERK	Extracellular-signal-regulated kinases
FDR	False discovery rate
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GILZ	Glucocorticoid-induced leucine zipper
GINA	Global Initiative for Asthma
GITR	Glucocorticoid-induced TNFR family related gene
GOLD	Global Initiative for chronic Obstructive Lung Disease
GR	Glucocorticoid receptor
HAT	Histone acetyltransferase
HDAC	Histone deacetylases
HDM	House dust mite
HO-1	Hemeoxygenase-1
i.p.	Intraperitoneally
IFN	Interferon
IKK	IκB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IRAK	Interleukin-1 receptor-associated kinase
IRF3	Interferon regulatory factor-3
ISO-1	((S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester)
IκB	Inhibitor of NF-κB
JAB1	c-Jun activation domain-binding protein
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
KC	Keratinocyte-derived chemokine

lncRNA	Long non-coding RNA
LPS	Lipopolysaccharide
Lys	Lysine
MAPK	Mitogen activating protein kinase
MDA5	Melanoma differentiation-associated protein 5
MIF	Macrophage migration inhibitory factor
miRNA	microRNA
MKP-1	Mitogen activating protein kinase phosphatase-1
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary-response protein 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T-cells
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PI3K	Phosphatidylinositide 3-kinase
PGE_2	Prostaglandin E_2
ppm	Parts per million
PRR	Pattern recognition receptor
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modifications
RA	Rheumatoid arthritis
RIG-1	Retinoic acid-inducible gene 1
R_L	Pulmonary resistance
RLR	RIG-I-like pathway
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SILAC	Stable isotope labeling by amino acids in cell culture
siRNA	Small interfering ribonucleic acid
SIRT2	NAD-dependent deacetylase sirtuin-2 gene
SOCS	Suppressor of cytokine signalling proteins
STAT	Signal Transducer and Activator of Transcription
TAK1	TGF-β activated kinase-1
T_C	T cytotoxic
TGF	Tumour growth factor
T_H	T Helper
Thr	Threonine
TIR	Toll/interleukin-1 receptor
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor associated factor
TRX	Thioredoxin
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Chronic obstructive pulmonary disease (COPD)

1.1.1 Definition, risk factors and prevalence

COPD is a common, preventable and treatable disease of the lung airways, it is characterised by persistent and usually progressive, airflow limitation (GOLD, 2013). COPD is associated with an enhanced inflammatory response of the airways to noxious particles or gases, especially cigarette smoking, with some extra-pulmonary effects. An acute exacerbation, which is a sudden worsening of symptoms and COPD comorbidities contribute to the severity of disease in individual patients (GOLD, 2013). COPD is a major cause of morbidity and mortality worldwide (GOLD, 2013) and is currently the 4th leading cause of death worldwide, it is predicted to become the 3rd leading cause of death by 2030 (Mathers and Loncar, 2006). Comorbidities with COPD include cardiovascular disease (Burrows et al., 1972) and diabetes mellitus (Rana et al., 2004).

COPD is clinically diagnosed using lung function spirometry tests. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) states a post-bronchodilator forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) ratio of <0.70 and a FEV_1 of ≤70% confirms the presence of persistent airflow limitation (Table 1.1) and thus a diagnosis of COPD (Rabe et al., 2007, GOLD, 2013). Disease severity can also be classified into four stages of COPD (GOLD stage A-D) incorporating symptoms and risk of exacerbations in a combined COPD assessment (Figure 1.1). The impact of the patients symptoms are assessed using the COPD assessment test (CAT) that gives a score between 0-40; a score ≥10 indicates a high level of symptoms (Jones et al., 2009). The exacerbation risk can be calculated from spirometry and FEV_1 values or based on individuals' patient history of exacerbations. Two or more exacerbations in the preceding year indicate high risk (Hurst et al., 2010). The presence of COPD symptoms such as dyspnoea, chronic cough and chronic sputum production along with exercise level (measured by 6-min walk test) and exposure to risk factors must also be taken into account (Mannino and Buist, 2007). Exacerbations increase the rate of lung function decline and increase disease severity (Donaldson et al., 2002).

Table 1.1: Spirometry classifications of COPD airflow limitation

GOLD Classification	FEV_1 % predicted
Stage 1: Mild	≥80%
Stage 2: Moderate	50% ≤ FEV1 < 80%
Stage 3: Severe	30% ≤ FEV1 < 50%
Stage 4: very severe	<30% or <50%+chronic respiratory failure

Disease severity can be classified by FEV_1 % predicted in combination with a FVC ratio of <0.70 and presence of COPD symptoms such as chronic cough and sputum production.

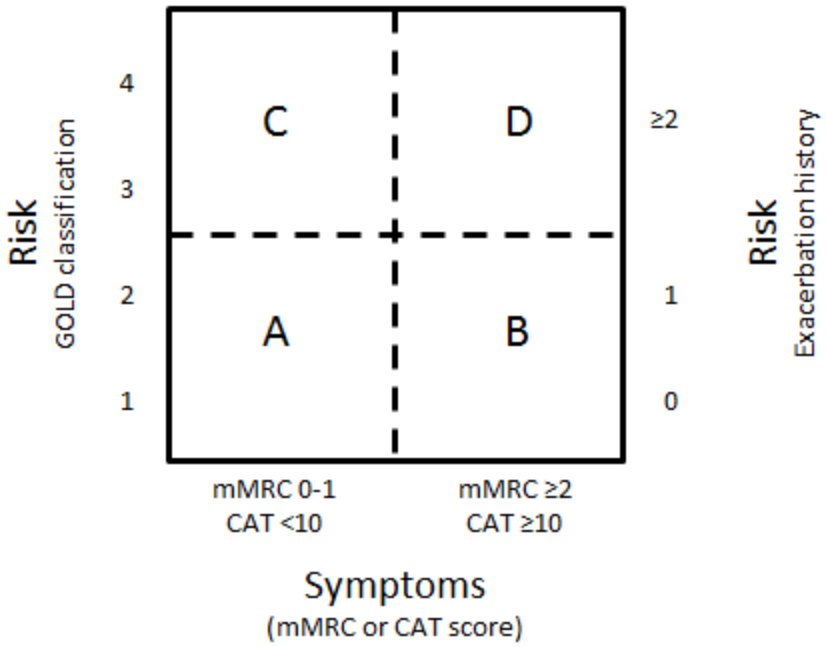


Figure 1.1: Combined COPD assessment
A combined COPD assessment is used to understand the impact of COPD on individuals. This method takes into account the symptomatic assessment (CAT test) with patient's spirometry (GOLD classification) and risk of exacerbations (adapted from (GOLD, 2013)).

Tobacco smoke is the main aetiological factor in COPD development but other factors have been shown to be associated with disease prevalence, such as outdoor, occupational and indoor pollutants e.g. burning of biofuels for heat and cooking and a genetic predisposition (Mannino and Buist, 2007, Barnes and Adcock, 2009).

COPD was previously more common in men compared to women. However, as more women smoke and are given equal exposures the gender difference has reduced and COPD is now equally prevalent between genders (Mannino and Buist, 2007). COPD is generally accepted as a disease of the elderly and associated with accelerated ageing of the lung (Ito and Barnes, 2009). COPD is associated with

significant economic burdens, in the European Union, 6% of total health care costs are spent on respiratory care, 56% of which accounts for spending on COPD. The worldwide burden of COPD is predicted to increase due to continued exposure to COPD risk factors and ageing of the population (Lopez et al., 2006).

1.1.2 **Pathophysiology**

Varying contributions of small airways disease and parenchymal destruction cause the characteristic airflow limitation seen in COPD patients (Barnes, 2000a). Small airways disease is the result of compression and narrowing of bronchioles due to chronic inflammation and structural changes such as fibrosis and scarring (airway remodelling) (Saetta et al., 1998). The narrowing of the airways increases resistance and reduces airflow. Parenchymal destruction is the permanent destruction of the lung parenchyma by chronic inflammation. This destruction results in the enlargement of airspaces distal to the terminal bronchioles and loss of alveolar attachments, lung elastic recoil (lung compliance) (Kim et al., 1991) and leads to airflow limitation, as there is an inability for the airways to remain open during expiration (Figure 1.2).

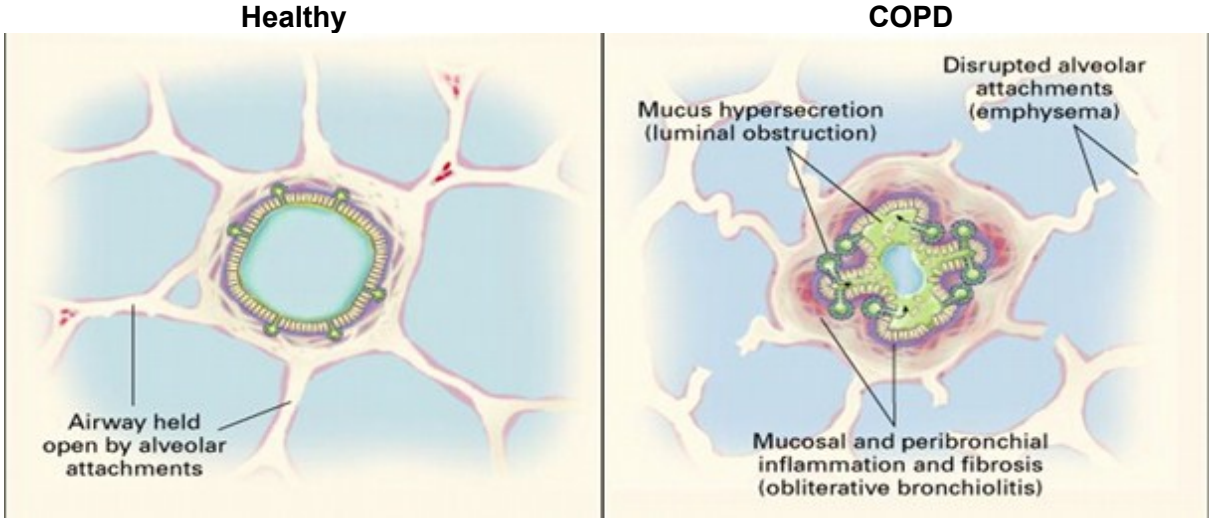


Figure 1.2: Illustration of healthy and COPD airways
COPD presents with the characteristic airflow limitation due to chronic inflammation and fibrosis of the small airways narrowing the lumen of the airway and increasing the airflow resistance. The airways are also obstructed due to increased mucus production again contributing to the reduced airflow. Another feature that leads to airflow limitation is the destruction of lung parenchyma and the reduced ability of keeping the airway open. Reproduced with permission from (Barnes, 2000a), Copyright Massachusetts Medical Society.

Previous definitions of COPD have included emphysema and chronic bronchitis however these clinical terms are no longer used (GOLD, 2013). Emphysema or the destruction of alveoli only describes a single structural abnormality seen in patients and therefore unsuitable for such a complex disease. Chronic bronchitis or the presence of a persistent cough and sputum production (for at least 3 months in 2 consecutive years) remains clinically important. However, chronic bronchitis is not a pathophysiological feature of the characteristic airflow limitation as the cough and sputum production may precede the development of airflow limitation due to increased mucus production in the upper airways. Although, there is also reduced mucociliary clearance with chronic bronchitis and this has been shown to contribute towards increased bacterial colonisation and infections leading to COPD exacerbations (Patel et al., 2002, Sethi et al., 2000).

The decline in FEV_1 and severity of disease correlates only with narrowing of small airways and not with chronic bronchitis or emphysema, suggesting a predominant role of airway remodelling in airflow limitation and COPD development (Hogg et al., 2004).

1.2 **Asthma**

1.2.1 **Definition, risk factors and prevalence**

Asthma is one of the most common chronic diseases in the world, with 235 million people worldwide being treated for the disease (WHO, 2013) and its prevalence is increasing, especially among children (GINA, 2012). Asthma is associated with persistent inflammation, bronchoconstriction and airway hyper-responsiveness that presents clinically as recurrent episodes of wheezing, breathlessness, chest tightness and coughing (GINA, 2012). However, unlike COPD, asthma is characterised by reversible expiratory airflow limitation.

Asthma is a significant burden on healthcare costs with increased hospital admissions and care visits costing an estimated £2.5 billion in the UK and resulting in about 20 million working days lost annually (Masoli et al., 2004). The quality of life of patients is also compromised by disease-associated comorbidities (Wenzel, 2006).

Asthma is an umbrella term for a heterogeneous disease that is divided into phenotypic subgroups with multiple physiological and clinical characteristics (Bel, 2013, Wenzel, 2006). However these phenotypes are not fully characterised and the distinction between the groups is continually evolving and remains controversial (Wenzel, 2012).

Due to the complexity of the disease, risk factors can be categorised into asthma development risks and asthma triggers. Developmental risks include genetic predisposition (Holgate, 1999, Holloway et al., 1999), sensitisation to indoor allergens such as house dust mite and fungi (Gelber et al., 1993), tobacco smoking (Lange et al., 1998) and respiratory infections (Zambrano et al., 2003). The triggers that can induce an exacerbation or 'asthma attack' include exposure to allergens e.g. house dust mites (Sporik et al., 1990), exercise (Parsons and Mastronarde, 2005), stress and smoke (GINA, 2012).

1.2.2 **Pathophysiology**

The characteristic airway narrowing (bronchoconstriction) in asthma is a result of persistent inflammation; airway hyper-responsiveness and airway remodelling in asthma and can become relatively irreversible in some patients (Figure 1.3).

In asthmatic patients the inflammation is more pronounced in the medium-sized bronchi within the lung and causes thickening of the airway wall (GINA, 2012). It is also important to note that the airway inflammation is persistent even though the symptoms are episodic. During the inflammatory response bronchoconstrictor mediators such as histamine and prostaglandin D_2 are released from mast cells and stimulate the constriction of airway smooth muscle cells (ASMCs) resulting in airway narrowing; this is largely reversed by bronchodilators (Manning and O'Byrne, 1988).

Airway hyper-responsiveness is the functional characteristic of asthma and results in airway narrowing after an innocuous stimulus for a non-asthmatic individual (GINA, 2012). Airway hyper-responsiveness is associated with both inflammation and repair mechanisms of the airway; however its mechanisms are not fully understood. Constriction stimulated by the release of bronchoconstrictor mediators may result in excessive narrowing of the airways presenting asthmatic symptoms of dyspnoea (Wang et al., 2003). Another contributing feature to airway hyper-responsiveness is

epithelial shedding, which is the loss of barrier function to prevent allergen penetration and the initiation of an allergic response (Montefort et al., 1992).

Airway remodelling is a characteristic structural feature of asthma and corresponds to the severity of disease and the relative irreversibility of airway narrowing (Chetta et al., 1997). Contributing towards airway remodeling is sub-epithelial fibrosis, which is the thickening of airway walls and a reduction in the airway lumen (Bousquet et al., 2000).

Other pathophysiological changes that may give rise to the airway remodelling and ultimately the airway narrowing of asthma include, hypertrophy and hyperplasia of ASMCs by increasing the thickness of the airway walls and mucus hypersecretion leading to 'mucus plugging' and increased resistance within the airways (Bousquet et al., 2000, James and Wenzel, 2007). Also, mucosal oedema due to vascular leakage during the inflammatory response is associated with asthmatic airway narrowing (Wenzel, 2012, Chung et al., 1990, Bousquet et al., 2000).

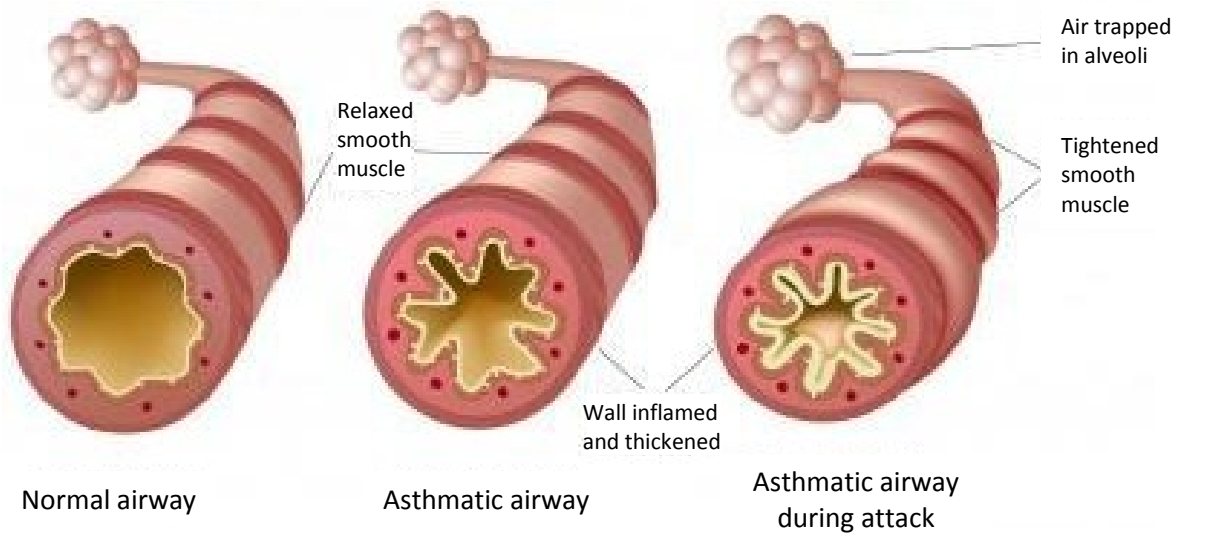


Figure 1.3: Illustration of normal and asthmatic airway
The airways of asthmatic patients are characteristically narrowed with a smaller airway lumen and increased resistance to airflow. The wall of the airway in asthmatics is thicker due to inflammation and hyperplasia and hypertrophy of ASMCs. Airway hyper-responsiveness increases the sensitivity to allergens and stimuli, resulting in excessive airway narrowing on bronchoconstriction. Adapted from: (NIH, 2013).

1.3 Inflammation

1.3.1 Inflammation in health

The inflammatory response is a protective mechanism against tissue injury by restricting the tissue damage at an affected site. The hallmarks of inflammation were first described over 2000 years ago by the Roman medical writer Aulus Cornelius Celsus (30BC-38AD) and are calor (heat), rubor (redness), dolor (pain) and tumor (swelling): functio laesa (loss of function) was only included as a cardinal sign of inflammation by Galen years later (Kindt et al., 2007). Within minutes of the injury, there is vasodilation resulting in increased blood volume but reduced blood flow at the site of injury. This increase in blood, heats up the surrounding tissue and causes the area to redden; this inflammatory sign is only visible at injury sites near to the skin (Kindt et al., 2007). Increased vascular permeability is another trait of the inflammatory response that leads to leakage of fluid from the blood vessel into tissue. This accumulation of fluid contributes to the swelling/oedema and redness of the injury site. Pain is felt after the release of mediators stimulating nerve endings and pain receptors; again this sign is only apparent where nerves and receptors are present (Kindt et al., 2007). Histamine a potent mediator released from activated cells causes further vasodilation and smooth muscle contraction (Manning and O'Byrne, 1988).

Part of the inflammatory response is the activation of the immune response, which is split between two systems, the innate and the adaptive systems. Importantly, these two systems do not act independently but collaboratively. Inflammation is also characterised by cells within these systems with recruitment of leukocytes, circulating monocytes and tissue macrophages releasing inflammatory mediators (Ryan and Majno, 1977) and lymphocytes amplifying inflammation, killing cells or producing antibodies (Ennis et al., 1977, Verbonitz et al., 1978).

1.3.1.1 *Innate immunity*

The innate response is the first line of defence against infection and is activated within a few hours of infection; however it is less specific than adaptive immunity. Initially pathogens have to overcome the hosts' barriers, which include the skin and mucosal membranes (Abbas and Lichtman, 2006). Phagocytic cells such as tissue macrophages and neutrophils are front-line cells of the innate response and reside

close to the barriers; some pathogens get phagocytosed and destroyed. These resident innate cells also have pattern recognition receptors (PRR) and recognise specific molecules, pathogen-associated molecular patterns (PAMPS) that are unique to the microbes (Mogensen, 2009). Toll-like receptors (TLRs) are a type of PRR and are found on the surface and within the cytoplasm of innate immune cells such as macrophages.

One way the innate response is initiated is by the detection of PAMPs by the TLRs leading to the release of pro-inflammatory mediators such as cytokines, chemokines and lipid mediators and the resulting activation, recruitment and/or migration of cells to the site of infection (Akira et al., 2001). TLRs belong to the IL-1 receptor (IL-1R) family, however the TLR and IL-1R have very different extracellular domains and distinct ligands but have a conserved intracellular domain that is known as the Toll/IL-1R (TIR) domain (Slack et al., 2000). TLR activation leads to the recruitment of adapter proteins (e.g. MyD88) and kinases (e.g. IRAK) (Wesche et al., 1997, Akira and Takeda, 2004) (Figure 1.4).

Directly or indirectly, transcription factors (AP-1, CREB, NF-κB and IRF3) are activated and translocate to the nucleus, where they bind to promoters of pro-inflammatory genes and induce gene transcription (Karin, 1995, Ghosh and Karin, 2002, Park et al., 2005). Target genes for these pro-inflammatory transcription factors include the common inflammatory cytokines IL-1 and TNFα that act in a feed forward loop to drive and amplify the inflammatory response as well as more specific cytokines such as IL-6, IL-12 and type 1 interferon (IFN) and their receptors (Hiscott et al., 1993, Collart et al., 1990, Murphy et al., 1995).

The inflammatory response is not only driven and maintained by the control of gene transcription but also by post-transcriptional modifications including modulating mRNA turnover, transport and translation (Winzen et al., 2004, Dean et al., 2004).

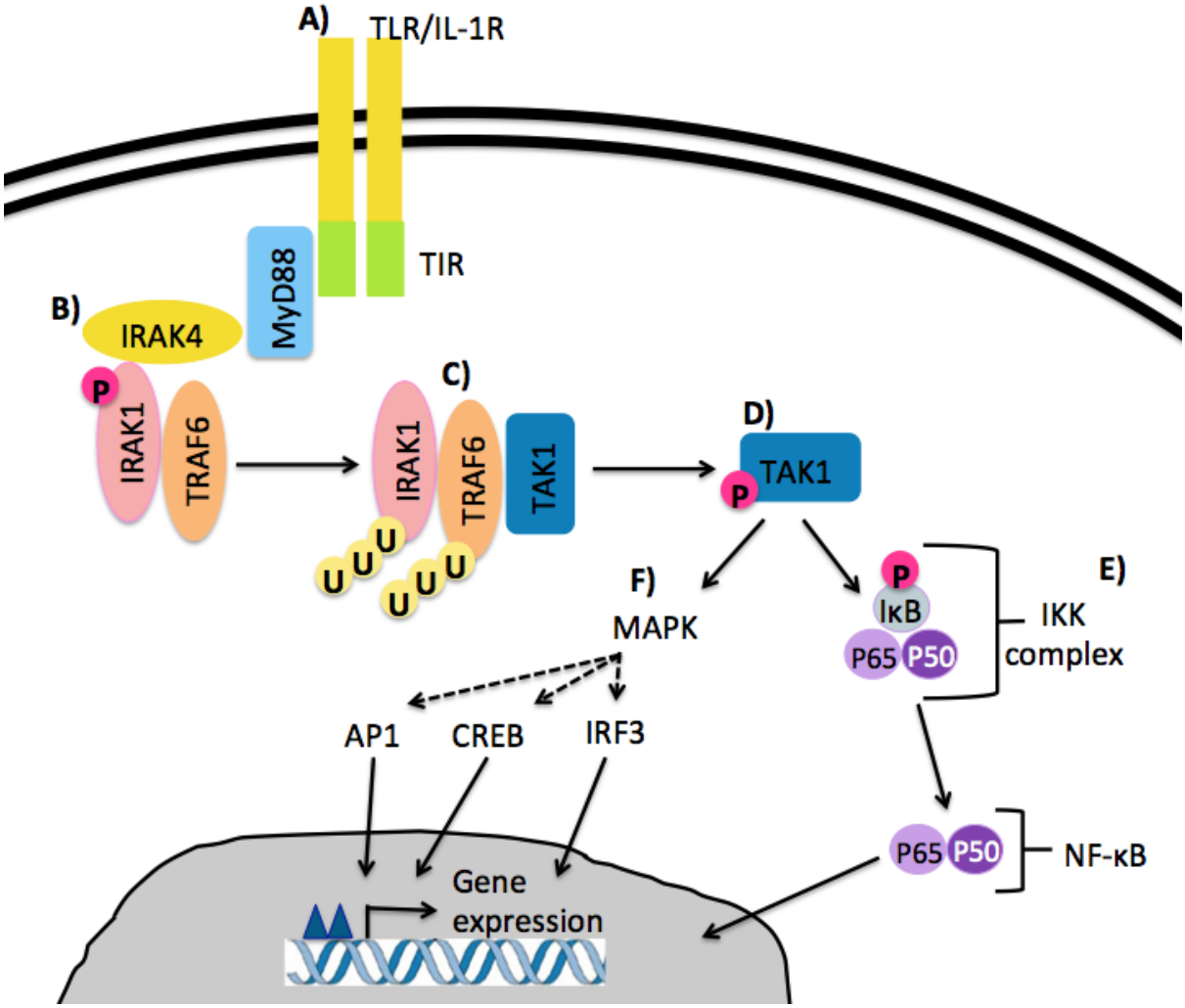


Figure 1.4: Schematic diagram for toll-like receptor (TLR) signalling to initiate inflammatory response

A) Cells with pattern recognition capability identify non-self antigens and these; in turn bind to pattern recognition receptors (PRR) such as Toll-like receptors (TLR) to initiate an immune response. The Toll/IL-1R (TIR) domain recruits adapter proteins MyD88 and members of the IL-1R-associated kinase (IRAK) family (Suzuki et al., 2002). B) Phosphorylation (P) of IRAK1 by IRAK4 recruits TNF receptor-associated factor-6 (TRAF6). C) The IRAK1-TRAF6 complex dissociates and binds transforming growth factor-β-activated kinase (TAK1) (Arch et al., 1998, Baud and Karin, 2001, Takeda et al., 2003); both IRAK1 and TRAF6 are then degraded by ubiquitination (UUU). D) TAK1 auto-phosphorylates (Takeda et al., 2003) and activates either E) IKK (Ghosh and Karin, 2002) or F) MAPK (Chang and Karin, 2001) and pathways leading to changes in the transcriptional activity and potentiating the inflammatory response. (Schematic adapted (Akira and Takeda, 2004)).

1.3.1.2 *Adaptive immunity*

The adaptive immune response is a stronger, more effective and highly specific mechanism for clearing pathogens and, importantly, has memory compared to the innate immune system (Alberts et al., 2002b). Nonetheless, this immunity can take days to develop after the initial exposure. The specificity of the adaptive immunity allows for subtle differences among antigens to be detected and the memory ability allows for a much quicker and heightened response to occur on a second challenge (Kindt et al., 2007).

Lymphocytes are antigen-presenting cells (APC) and are the main cell type in adaptive immunity. APCs present antigens on the cell surface that are recognised by the T cell receptor on T lymphocytes (Abbas and Lichtman, 2006, Kindt et al., 2007).

There are two types of adaptive immunity that are mediated by different lymphocytes and have different methods to defend against different types of infection. Humoural immunity is mediated by B-lymphocytes that respond by secreting antibodies to neutralise and eliminate microbes in the blood and lumen of organs such as the respiratory tract (extracellular) (Abbas and Lichtman, 2006, Kindt et al., 2007, Alberts et al., 2002b). In contrast, when cells have taken up microbes intracellularly by either phagocytosis or are infected i.e. viruses are dividing within cells, then T-lymphocytes mediate a cell-mediated response. T-lymphocytes sub-divide into T helper (T_H) cells and T cytotoxic (T_C) cells, the former are involved in activating macrophages to kill the phagocytosed microbes and the latter induce cell death of infected cells (Abbas and Lichtman, 2006, Kindt et al., 2007, Alberts et al., 2002b). This is summarised in Figure 1.5.

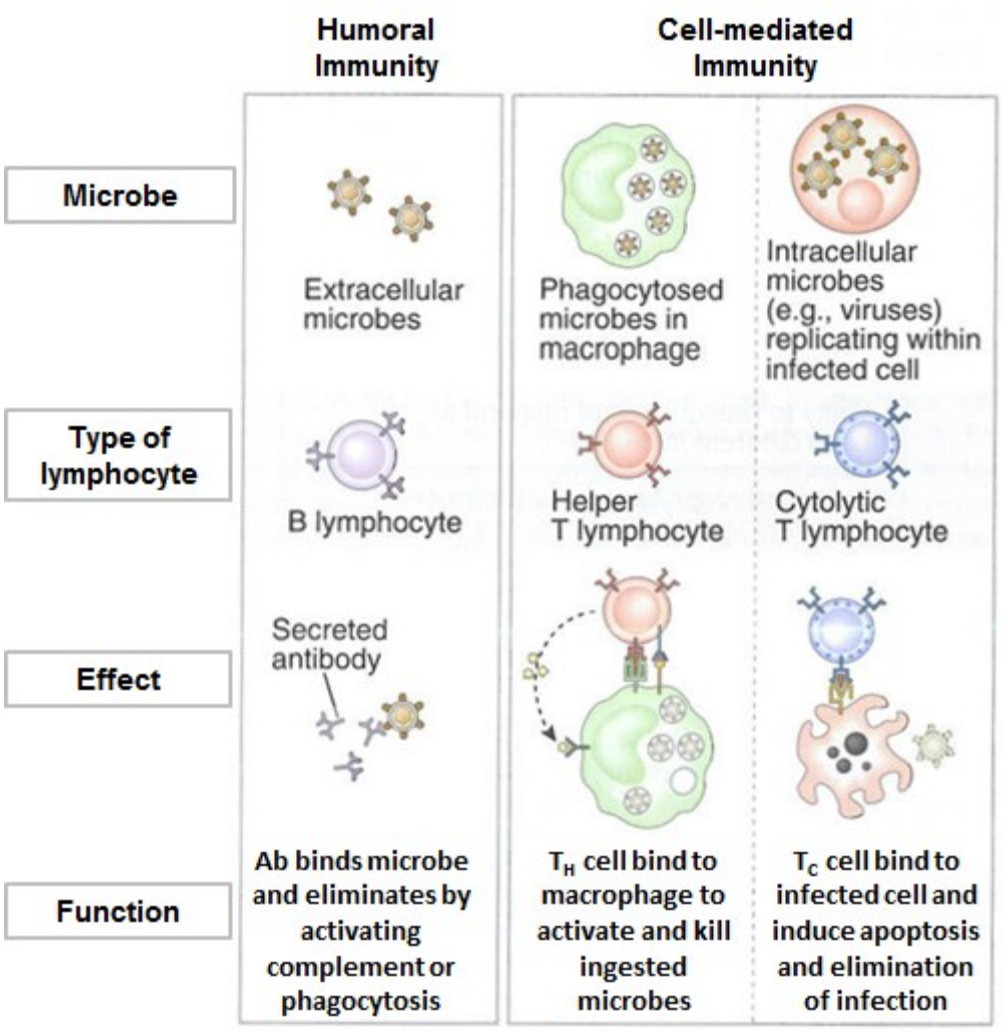


Figure 1.5: Types of adaptive immunity
Adaptive immunity is split into two types, humoural and cell-mediated. The latter is further divided into different cell types. Each has specific cell types that mediate the immunity and each has different effects and functions to eliminate and remove the microbes from the body. (Abbas and Lichtman, 2006).

1.3.2 **Resolution of inflammation**

Once an inflammatory process and the resulting immune response have been initiated the response requires resolution to reduce excessive tissue damage and restore tissue function (Lawrence and Gilroy, 2007). Historically, the resolution of inflammation was understood to be achieved by macrophage phagocytosis of neutrophils (Savill et al., 1989). However, resolution has more recently been accepted to be an active, complex and coordinated process that has many mechanisms to resolve the inflammation quickly and efficiently with minimal damage (Gilroy et al., 2004, Serhan, 2011).

Chemokines are crucial in recruiting cells such as neutrophils and macrophages to the inflamed site and augmenting the inflammatory response. To resolve the inflammation, cells must be cleared and numbers must be returned to basal levels. There are many mechanisms to achieve this such as reducing cellular recruitment by chemokine truncation (McQuibban et al., 2000, Dean et al., 2008), removal of cells without spilling cytotoxic content by neutrophil apoptosis (van den Berg et al., 2001) and macrophage re-programming from pro- to anti-inflammatory phenotypes (Fadok et al., 1998). Other mechanisms demonstrated include efferocytosis, where macrophages phagocytose cells, such as neutrophils (Henson, 2003), macrophage regression via the lymphatic drainage system (Llodra et al., 2004) and induction of tissue repair to reduce fibrosis for example by tumour growth factor-β (TGF-β) (Rappolee et al., 1988).

Mechanisms for the negative regulation of the pro-inflammatory mediators include mitogen activated protein kinase phosphatases (MKP) that inhibit mitogen activated protein kinase (MAPK) signalling (Clark, 2003) and inhibiting the degradation of IκB that sequesters Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in the cytoplasm and prevent transcription (Baeuerle and Baltimore, 1988). Binding of TRAF3 instead of TRAF6 to IRAK1 leads to the induction of IL-10 gene expression, which is an anti-inflammatory cytokine (Hacker et al., 2006).

Alternatively, there is an increasing literature regarding the role of anti-inflammatory lipids such as resolvins (Serhan, 2011). These were not measured in this thesis and so are not discussed any further.

1.3.3 **Immune dysfunction and the consequences**

Failure to resolve inflammation leads to immune dysfunction and pathogenesis of inflammatory diseases (Lawrence and Gilroy, 2007). Disorders due to excessive or aberrant immune responses to an antigen challenge are collectively known as hypersensitivity diseases (Abbas and Lichtman, 2006, Kindt et al., 2007). Chronic inflammation can also develop when an antigen or stimulus persists and this is common in autoimmune diseases in which self-antigens continually activate the immune response.

1.3.3.1 *Chronic inflammation*

Continually activated immune cells will constantly release pro-inflammatory mediators and will result in a persistent and chronic inflammatory response. Continual stimulation of fibroblast proliferation and collagen production will promote fibrosis and induce the development of scarring, which can impede normal tissue function (Diegelmann and Evans, 2004). Chronic inflammation can result in numerous diseases effecting different tissues and organs and either being systemic or localised, such as COPD, asthma, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).

1.3.3.1.1 Chronic inflammation in COPD and asthma

Asthma and COPD are chronic inflammatory diseases of the airways, however the characteristics of the chronic inflammation differ, this is summarised in Table 1.2.

Elevated numbers of neutrophils have been found in bronchoalveolar lavage fluid (BALF) (Thompson et al., 1989) and induced sputum samples from COPD patients (Keatings et al., 1996, Hill et al., 1999, Barnes, 2000a). At sites of destroyed parenchyma a predominance of macrophages and $CD8^+$ (T_C) lymphocytes have been observed (Barnes, 2000a). These COPD-associated cells release cytokines and chemokines to drive and amplify inflammation; elevated cytokine and chemokine levels have been detected in BALF and induced sputum from COPD patients compared to healthy controls, reviewed in (Barnes, 2008a).

Levels of the chemokines CCL2 and CCR2 correlate with number of neutrophils, macrophages and mast cells in the lung (de Boer et al., 2000). During exacerbations, chemokines are further induced, resulting in more macrophages recruited to the inflammation site and augmenting the immune response (Norzila et al., 2000).

Elevated levels of TNFα have been reported especially during exacerbations and in patients with COPD-associated weight loss (Eagan et al., 2012). In addition, TNFα has been shown to act on ASMCs to increase contractibility and have a role in airway hyper-responsiveness (Kips et al., 1993). Inhibition of TNFα has been shown to have therapeutic benefits in other chronic inflammatory diseases but has limited efficacy in asthma despite improvement in lung function and reduced exacerbations

being demonstrated in early small studies (Berry et al., 2006). Indeed, blocking TNFα is not now considered therapeutically beneficial in asthma since there is no clinical benefit of golimumab and its use is associated with severe adverse side effects including death (Wenzel et al., 2009). A similar lack of efficacy associated with detrimental effects is seen in COPD (Barnes, 2008a).

Allergic asthma is immunoglobulin E (IgE)-dependent and the binding of sensitised-IgE to mast cells orchestrates a rapid asthmatic inflammatory response on second exposure of the specific allergen (Gould and Sutton, 2008). Allergic asthma is also accepted to have a predominant T_H2 response and levels of eosinophils are increased in the sputum (Barnes, 2008a), however asthma is a multifactorial disease and other subsets of asthma have been described (Wenzel, 2006). For example, severe asthmatics have neutrophil-mediated inflammation compared to the conventional eosinophil-rich inflammation in allergic asthmatics, also a mix of T_H1/T_H2 or principally T_H17 phenotypes of asthma have been described (Cosmi et al., 2011). The characteristic bronchoconstriction seen in asthmatic patients is a result of histamines and mediators released from activated mast cells and eosinophils.

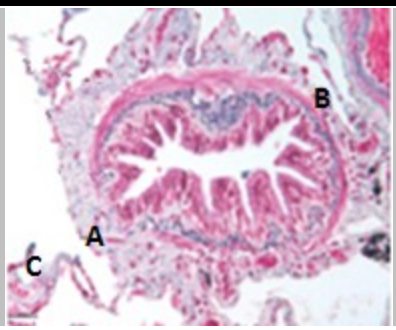
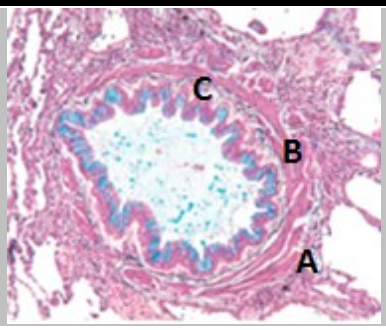
Numbers of T_H17 cells have been shown to be increased in the airways of asthmatic patients compared to healthy controls and these cells release IL-17A (Bullens et al., 2006, Pene et al., 2008). The expression of IL-17 is also increased in the epithelium of asthmatic patients (Chang et al., 2011). The T_H2 response is further amplified by increased release of IL-33 and activity of GATA3 (Smithgall et al., 2008), a transcription factor that controls the transcription of typical T_H2-associated cytokines. Increased IL-17F release, a mediator secreted from T_H17 cells, is also associated with neutrophil-mediated inflammation and induces further chemokine release; this is also seen in severe asthmatic patients (Schmidt-Weber et al., 2007).

The levels of CXCL8 correlate with the number of neutrophils in the sputum of severe asthmatics (Norzila et al., 2000) and have been shown to be elevated during a viral exacerbation (Fujimoto et al., 2005, Spruit et al., 2003). IL-10, an anti-inflammatory cytokine has significantly reduced transcription and secretion levels; again, this is common between COPD and asthmatic patients (Takanashi et al., 1999, John et al., 1998).

Growth factors promote the differentiation, survival and proliferation of neutrophils, eosinophils, macrophages and fibroblasts, augmenting the inflammation and instigating fibrosis of the small airways in COPD (Minshall et al., 1997). Increased levels of growth factors, such as TGF-β from eosinophils have been associated with the characteristic sub-epithelial fibrosis seen in the airways of asthmatic patients (Minshall et al., 1997).

The inflammation in COPD and asthma is maintained due to the induction of cytokine signalling pathways, which induce the production of further pro-inflammatory mediators from activated cells in a feed-forward mechanism. This results in chronic inflammation as the acute inflammation is not resolved due to mechanisms not fully elucidated. The inflammatory cycle therefore continues and the phenotypes of disease develop.

Table 1.2: Inflammatory characteristics of COPD and asthma

	COPD	Asthma
Histopathology		
Structural changes	↑↑↑ Fibrosis (A) ↑ Airway smooth muscle Loss of alveolar attachments (emphysema) (B) Enlargement of airspaces (loss of lung compliance) (C)	↑ Fibrosis (A) ↑↑↑ Airway smooth muscle (B) ↑↑ Thickened basement membrane (C)
Inflammation location	Small airways Lung parenchyma	Larger airways
Cells types	Neutrophils Alveolar macrophages Epithelial cells T_H1 and T_C lymphocytes	Eosinophils Epithelial cells Activated mast cells T_H2 and T_H17 lymphocytes Airway smooth muscle
Associated-cytokines (elevated in disease patients vs. healthy controls)	TNFα, IL-6, IL-1β, IFN-γ, IL-17F	TNFα, IL-1β, IL-6, IL-4, IL-5, IL-9, IL-13, IL-17A and IL-33
Associated chemokines	CXCL8, CCL2	CXCL8
Associated growth factors	GM-CSF, TGF-β	TGF-β

Airways from a COPD patient and asthmatic patient show overall difference in histology between diseases, locations of structural changes are shown on histology image. The major cell types that are involved in orchestrating and driving inflammation in the diseases differ. There are some common but also disease specific cytokines, chemokines and growth factors that are released (Keatings and Barnes, 1997, Saetta et al., 1993, Aaron et al., 2001, Gajewska et al., 2003, Traves et al., 2002, Schmidt-Weber et al., 2007, de Boer et al., 2000). Reproduced with permission from (Hogg et al., 2004), Copyright Massachusetts Medical Society.

1.3.4 **Macrophages**

Macrophages play an important role in the inflammatory response of COPD (Shapiro, 1999) and asthma (Peters-Golden, 2004) and are the major cell type in recognising infection or injury in the hosts' innate response. The numbers of macrophages are far greater in COPD than in asthma (Saetta et al., 1993). Once the macrophages are activated, for example by cigarette smoke or other inhaled

irritants; they release reactive oxygen species (ROS) and neutrophillic chemotactic factors along with prostaglandins, cytokines and extracellular matrix proteins orchestrating the inflammatory response (Shapiro and Senior, 1999). In addition, macrophages are detected in higher numbers in COPD BALF samples compared to healthy controls (5-10 fold increase) and the numbers in the bronchial submucosa increase with disease severity (Barnes, 2000a, Hogg et al., 2004, Saetta et al., 1993). The presence of monocyte-attracting chemokines such as MCP-1 (monocyte chemotactic protein or CCL2) is elevated in sputum and BALF of patients with COPD (de Boer et al., 2000) and may account, in part, for the increased recruitment of circulating monocytes into the airways.

Increased proliferation of cells, including alveolar macrophages and fibroblasts, has been associated with fibrosis of the small airways and the progression of COPD (Barnes, 2008b). Similarly, large clusters of macrophages in the small airways may be associated with the emphysema observed in some COPD patients (Fraig et al., 2002). In asthmatics, pulmonary macrophages have long been recognised to induce histamine release from mast cells and basophils to modulate the contraction of ASMCs (Schulman et al., 1985) and they are therefore intimately involved in the airway hyper-responsiveness seen in asthma.

Macrophages are also phagocytic and there is a reduction in the ability of macrophages to clear apoptotic and dying cells in COPD and asthma (Hodge et al., 2003). This may result in secondary necrosis and exacerbation of the inflammatory process (Vandivier et al., 2006, Huynh et al., 2005). Alveolar macrophages show impaired phagocytosis of neutrophils if exposed to cigarette smoke (Kirkham et al., 2004) and express reduced levels of recognition receptors such as CD44 and CD31 (Hodge et al., 2007). The clearance of apoptotic cells by macrophages is termed efferocytosis and this function has been reported to be defective in clearing epithelial cells in COPD (Hodge et al., 2003).

Alveolar macrophages also release the anti-inflammatory cytokine IL-10 in response to inflammatory stimuli. However there are markedly decreased in levels of sputum IL-10 in COPD patients compared to healthy controls (Takanashi et al., 1999).

1.3.5 Inflammatory signalling

1.3.5.1 *MAPK signalling cascade*

MAPKs are a family of signal transduction enzymes and one of the major signalling systems used by cells to initiate a response. There are three classes of MAPK, p38, extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK). MAPKs are organised into three hierarchal levels and they all operate via the phosphorylation of both threonine (Thr) and tyrosine (Tyr) residues converting them into the activated form and finally resulting in the interaction with cytoplasmic substrates and the translocation into the nucleus (Ferrell and Bhatt, 1997). All p38, ERK and JNK signalling pathways have been implicated in inflammatory processes; ERK MAPK is involved in cell proliferation, differentiation and survival, whereas p38 and JNK MAPKs are mainly involved in mediating pro-inflammatory responses and apoptosis (Herlaar and Brown, 1999). Disease initiation and progression has been shown to correlate with the activation status of p38 MAPK in severe asthmatics (Bhavsar et al., 2008) and smokers with COPD have increased activated p38 MAPK in lung alveolar spaces and walls (Renda et al., 2008). Recent evidence from a small placebo-controlled clinical trial indicates that the orally available p38 MAPK inhibitor PH-797804 is well tolerated by COPD patients and has some beneficial effects on lung function and dyspnoea (MacNee et al., 2013). Alternatively, p38 MAPK inhibition in combination with an inhaled CS (ICS) has been proposed to be of potential benefit in severe asthma and COPD (Armstrong et al., 2011).

1.3.5.2 *Proliferation and p53*

Proliferation of alveolar macrophages and their role in activating surrounding tissues and cells has been implicated in the development of fibrosis of the small airways and overall progression of COPD (Barnes, 2004). In asthma, a pathophysiological feature of disease is the hypertrophy of ASMCs; this may be the result of the cells being stimulated by growth factors secreted by activated inflammatory cells (Barnes, 1996).

The p53 gene (*TP53*) is a tumour-suppressor gene; its fundamental role is to protect against cellular damage by controlling cell cycle or inducing apoptosis (Alberts et al., 2002a). Baseline levels of p53 are kept low until its activation when the levels are

dramatically increased and its functional targets, such as $p21^{WAF1}$ are induced (Mirzayans et al., 2012).

Proliferation, cell growth and apoptosis are not only driven via p53-dependent pathways but through the pro-inflammatory MAPK pathways (Tamemoto et al., 1992, Pedram et al., 1998, Takenaka et al., 1998, Zhang and Liu, 2002).

1.4 Corticosteroids

Corticosteroids (CSs) are potent anti-inflammatory substances used as therapeutics in many inflammatory diseases including inflammatory bowel disease, RA and autoimmune diseases. CSs are used to control asthma and COPD and although the majority of asthmatic patients are relatively well controlled with CS, a small percentage of severe asthmatics do not see any therapeutic benefits even with high doses of oral CS (Wenzel et al., 1999). Most COPD patients show little or no benefit to CSs (Barnes, 2000b). Severe asthmatics and COPD patients are said to be relatively steroid insensitive with respect to their anti-inflammatory properties. Understanding the mechanisms in which CS function has aided in elucidating the mechanisms of steroid insensitivity.

CSs diffuse across the plasma membrane and bind to the glucocorticoid receptor (GR) complex in the cytoplasm (Rhen and Cidlowski, 2005). GR has two isoforms; GRα and GRβ. In its inactivated form GR is chaperoned by a number of proteins including heat shock protein 90 (HSP90). CS bind to GR resulting in conformational changes and release from HSP90 and chaperone proteins (Kang et al., 1999). The GR-CS complex translocates into the nucleus, where two distinct pathways are activated, transrepression and transactivation, reviewed in (Barnes and Adcock, 2003).

Transrepression results in the suppression of inflammation by turning off activated genes that encode for cytokines, chemokines, adhesion molecules and receptors (Barnes and Adcock, 2003) via the regulation of transcription factors AP-1 and NF-κB (Barnes and Adcock, 1998). Activated GR monomers bind to the activated DNA-bound pro-inflammatory transcription factors and results in changes of the chromatin structure through the recruitment of co-repressors with histone deacetylase (HDAC) activity (Ito et al., 2000). HDAC proteins prevent DNA acetylation and reduce

transcriptional activity. More recently the concept of assisted loading whereby the previous presence of other transcription factors can prime chromatin and direct the activated GR or other transcription factors to select regions in the genome has been proposed as key to GR function (Biddie et al., 2011, Voss et al., 2011).

Transactivation is DNA-dependent and induces the expression of anti-inflammatory genes such as MKP-1 (Kassel et al., 2001), IκBα (Heck et al., 1997) and the anti-inflammatory cytokine IL-10 (Rea et al., 2000). MKP-1 de-phosphorylates MAPK proteins and therefore suppresses the inflammatory response (Lasa et al., 2002) and IκBα is a protein that retains the NF-κB complex in the cytoplasm preventing its translocation into the nucleus (Verma et al., 1995). Mechanistically, initial data suggested that binding of the GR-CS complex to the GR response element (GRE) within the promoter of GR-inducible genes leads to the recruitment of co-activators with histone acetyltransferase (HAT) activity and changes in the chromatin structure, enabling increased gene transcription (Barnes and Adcock, 2003). More recent evidence has indicated that the assisted loading process may also occur at GREs with preloading of AP-1 allowing a more amenable chromatin structure for GR activation to form (Voss et al., 2011).

Transcriptional activity is also affected by epigenetic regulators and unrecognised regulatory proteins suggesting that GR may not directly participate in the ultimate regulation of gene transcription (Uhlenhaut et al., 2013).

1.4.1 **Corticosteroid insensitivity**

Severe asthma and COPD are diseases that are uncontrolled and show poor treatment responses due to lack of novel therapeutics and steroid insensitivity. Disease-associated burdens include major health care costs but also lost productivity, reduced participation in family life and CS side effects (GINA, 2012, GOLD, 2013).

Persistent asthma in both adults and children is successfully treated in the majority of cases with CSs and people lead normal lives with improved lung function and quality of life and reduced frequency of exacerbations (Barnes, 1996, O'Byrne and Parameswaran, 2006). However about 10% of asthmatic patients require the maximum inhaled CS dose and 1% of these patients require oral CSs (Barnes,

2013) despite evidence for compliance with treatment. These patients are described as CS insensitive and present with poor disease control and no reduction in the inflammation (Adcock and Lane, 2003). Smoking asthmatics also show CS insensitivity and require higher doses (Thomson and Spears, 2005, Ahmad et al., 2008); biopsies of steroid insensitive asthmatics have revealed the typical eosinophilic inflammation of asthma (Szefler and Leung, 1997).

COPD patients prescribed with high doses of ICS or orally available CSs show little improvement in lung function or reduction in numbers of inflammatory cells or mediators within the lung (Adcock et al., 2006, Keatings et al., 1997). However, a 25% reduction in COPD exacerbation rate was seen with inhaled CSs (ICS) (Yang et al., 2007). There is small clinical benefit of CS monotherapy and the increasing evidence that high doses are potentially harmful (Walters et al., 2009).

Increasing doses of CSs also result in deleterious side-effects despite failure to inhibit the pro-inflammatory cytokine release (Keatings and Barnes, 1997) leaving no treatment available for these patients. Steroid insensitivity has been reported in many other inflammatory diseases such as RA and cystic fibrosis (Barnes and Adcock, 2009).

There are many mechanisms implicated in causing steroid insensitivity in COPD and severe asthma. These mechanisms are also reported in other chronic inflammatory diseases indicating common impairments causing CS insensitivity and not specific disease traits.

1.4.1.1 ***Mechanisms of CS insensitivity***

1.4.1.1.1 Genetics

It was first reported that “CS-resistant asthma” was more common within families, indicating a strong genetic role in the development of CS insensitivity (Carmichael et al., 1981). Genetic studies using microarrays and genome wide association studies have identified polymorphisms within GR and differentially expressed genes in CS-insensitive/resistant asthmatics compared to healthy controls (Donn et al., 2007, van den Akker et al., 2006). One gene, bone morphogenetic protein receptor type II (*BMPRII*) was found to enhance the CS-effect when transfected into CS insensitive cells (Donn et al., 2007). To date, a link between COPD patients and genetic

susceptibility has not been established however there is evidence that genetics may play a role in COPD development.

1.4.1.1.2 GRβ

Another proposed mechanism leading to CS-insensitivity is the elevated expression of GRβ, the dominant negative isoform of GR (Webster et al., 2001), which has been reported in steroid-resistant asthmatic patients (Hamid et al., 1999, Sousa et al., 2000). There is no evidence of increased expression of GRβ in COPD but GRα is reduced (Gagliardo et al., 2000). This change in GRβ to GRα ratio within the inflammatory cells may impair the CS function (Marwick and Chung, 2010), although there have been no studies into the functional effects of an increased GRβ ratio. Moreover, knockdown of GRβ in alveolar macrophages resulted in enhanced nuclear translocation of GRα and increased CS sensitivity (Goleva et al., 2006).

1.4.1.1.3 HDAC2

Removal of acetyl groups on core histones or on non-histone proteins by HDACs results in the suppression of gene transcription and a 'closed' chromatin structure. Reports have shown steroid insensitive COPD patients have significantly reduced HDAC expression by more than 95% compared to non-smokers and have enhanced inflammatory response (Ito et al., 2005). HDAC is essential for GRα transrepression of some inflammatory genes and therefore reduced HDAC expression and activity may impair CS function either through altering GR acetylation status or modifying histone or other non-histone proteins (Ito et al., 2006). Restoring HDAC function correlates with improved CS ability to suppress the expression of select inflammatory genes (Cosio et al., 2004).

1.4.1.1.4 p38 MAPK

Kinase signalling amplifies the inflammatory response and p38 MAPK has been shown to be elevated in peripheral lungs and alveolar macrophages from COPD patients compared to smokers (Renda et al., 2008) and in patients with severe asthma compared to non-severe disease (Bhavsar et al., 2008). In support of this enhanced pro-inflammatory response, reduced expression of MKP-1 was reported in severe asthmatics (Bhavsar et al., 2008). However, few reports have shown CS-induced MKP-1 or related genes in asthmatics or COPD patients (Kelly et al., 2012). Therefore, it is unknown whether the CS insensitivity is due to impaired MKP-1

production and activity or altered CS-signalling in disease. IL-2, IL-5 and IL-4 expression is increased in the airways of patients with CS-insensitive asthma (Leung et al., 1995) and *in vitro* the combination of IL-2 and IL-4 induces steroid insensitivity by enhancing p38 MAPK activation (Irusen et al., 2002). The increased p38 MAPK activation leads to the phosphorylation of GR and reduces CS affinity binding within nucleus (Irusen et al., 2002). More recently direct phosphorylation of GR by p38 MAPK induced by stress responses and/or prolonged inflammation has been shown (Galliher-Beckley et al., 2011).

1.4.1.1.5 Oxidative stress

COPD patients have increased oxidative stress in their lungs and airways compared to healthy patients, (Brindicci et al., 2005, Montuschi et al., 2000, Rahman et al., 2002) which may be augmented by exogenous cigarette smoke or endogenous sources such as respiratory burst of cells (Park et al., 2009). Both the cigarette smoke and increased oxidative stress have an inhibitory effect on HDAC function and reduces HDAC expression (Adcock et al., 2005, Ito et al., 2001, Ito et al., 2004, Yang et al., 2006) and this, in part, may explain the reduced action of CSs (Barnes, 2000b). In COPD patients, steroid sensitivity is not restored upon smoking cessation as once instigated ROS stress self-perpetuates for many years or even decades (Keatings and Barnes, 1997, Rahman et al., 2002). Studies investigating the production of antioxidants have demonstrated that COPD patients have reduced capacity to induce antioxidant defences (Smolonska et al., 2009). Furthermore, the oxidative stress caused by cigarette smoking enhances the need for efficient antioxidant defences and if due to genetic reasons the production is altered then the development of COPD is more likely (Mak et al., 2007).

1.5 Macrophage migration inhibitory factor (MIF)

MIF was one of the first cytokines to be discovered; it was originally described in 1966 as a soluble mediator released from activated T-lymphocytes that inhibited the random migration of macrophages, in the delayed-type hypersensitivity (DTH) reaction (Bloom and Bennett, 1966, David, 1966). However MIF remained unstudied until the cloning of human MIF complementary DNA (cDNA) in 1989 (Weiser et al., 1989) and its rediscovery as a pituitary-derived peptide released in response to endotoxin in mice in 1993 (Bernhagen et al., 1993).

Subsequently, MIF has been shown to be constitutively expressed by an extensive variety of cells (Calandra et al., 1994, Donnelly et al., 1997). Although previously thought of as a MIF target, monocytes/macrophages have been shown to be the predominant source of MIF both constitutively and in response to LPS (Calandra et al., 1994). Other immune cells secreting MIF include; lymphocytes (Bacher et al., 1996) neutrophils (Daryadel et al., 2006), eosinophils (Rossi et al., 1998) and structural cells include; ASMCs (Verschuren et al., 2005), epithelial (Imamura et al., 1996) and endothelial cells (Nishihira et al., 1998). MIF is also expressed in many tissues such as the brain, lung, heart, kidney and liver (Calandra and Roger, 2003).

MIF is pre-formed and stored within vesicles in the cytoplasm of cells, allowing for rapid release, in contrast to other pro-inflammatory mediators like IL-1β that require *de novo* synthesis before being secreted (Calandra et al., 1994). The MIF gene does not encode for a N-terminal signal sequence and therefore the MIF protein cannot translocate to the endoplasmic reticulum (ER) for the classical pathway of secretion (Flieger et al., 2003). Similarly to IL-1β but conversely to other pro-inflammatory mediators like CXCL8 and TNFα, MIF is released from cells via a non-classical pathway involving ATP-binding cassette transporters (ABCA1) (Flieger et al., 2003). After secretion MIF stimulates and activates many target cells in an autocrine and paracrine fashion such as macrophages, lymphocytes, epithelial and ASMCs.

1.5.1 **From genome to protein**

The *MIF* gene is located on chromosome 22q11.2 of the human genome (Paralkar and Wistow, 1994, Budarf et al., 1997). *MIF* mRNA encodes a 115-amino acid non-glycosylated protein with a molecular weight of 12.5kDa. The *MIF* gene consists of 3 exons, 205bp, 173bp and 183bp in length and 2 introns (189bp and 95bp) (Weiser et al., 1989). There is 90% homology between the human *MIF* and mouse *Mif* gene at the amino acid level and MIF is a highly conserved protein across many mammalian species (human, rat, mouse and cattle), despite this, genetic deletion of *Mif* in mice results in no immunological or physiological abnormalities (Bozza et al., 1999).

The control of *MIF* expression is not fully understood but the *MIF* gene is known to contain several putative DNA-binding sequences for transcription factors including,

AP-1, NF-κB, GATA and cyclic adenosine 3',5'-monophosphate (cAMP)-responsive element binding (CREB) protein (Calandra and Roger, 2003).

Three-dimensional X-ray crystallography studies of human and rat showed that MIF exists as a homotrimer (Figure 1.6) (Suzuki et al., 1996, Sun et al., 1996a). Each monomer consists of 2 anti-parallel α-helices and six β-strands, the trimer is a barrel structure with a solvent-accessible channel running through the centre of the protein (Suzuki et al., 1996, Sun et al., 1996a). It has been suggested that MIF action is either via the monomer or dimer form of the protein, dependent upon its concentration however this is controversial with no clear evidence either way (Tomura et al., 1999, Mischke et al., 1998).

Recent studies have shown there is one gene that is homologous with *MIF* that encodes a protein known as D-dopachrome tautomerase (D-DT). MIF and D-DT are both located on chromosome 22 and also share a structural homology and functional activities (Merk et al., 2012). D-DT recently has been described as a member of the MIF superfamily and designated MIF-2 (Merk et al., 2012) however studies into the role of D-DT in severity and augmentation of disease and its association with MIF will need elucidating.

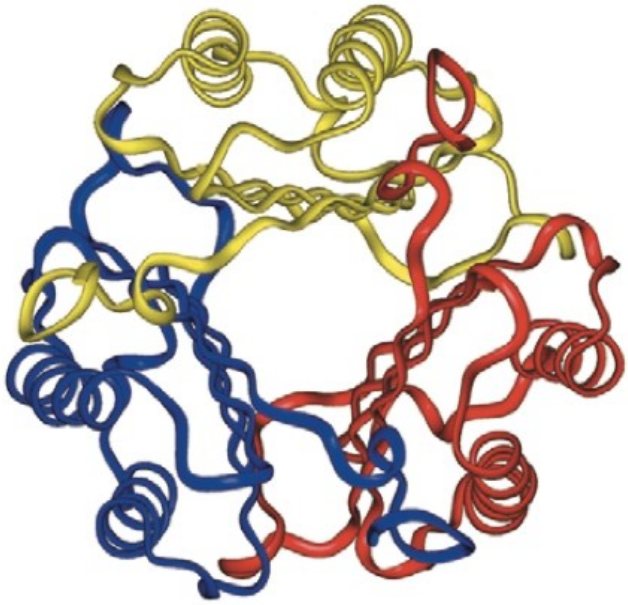


Figure 1.6: Ribbon structure of MIF protein
Ribbon diagram of three-dimensional structure of the MIF trimer. Red, yellow and blue represent the monomers of the trimer. In the monomer, two α-helices pack against the β-sheets. In the trimer, the β-stranded barrel is surrounded by six α-helices. (Lolis, 2001).

1.5.2 **Genetic polymorphisms**

Mutations in the *MIF* gene may predispose affected individuals to altered susceptibility to, or severity of inflammatory/infectious diseases. Polymorphisms within the *MIF* gene have been associated with disease severity and poor clinical manifestations (Plant et al., 2005).

To date, four polymorphisms within the *MIF* gene and promoter have been reported: a 5-8-CATT tetranucleotide repeat at position -794 (-794 $CATT_{(5-8)}$) and 3 single-nucleotide polymorphisms (SNPs) at positions -173 (-173*G/C), +254 (+254*T/C) and +656 (+656*C/G). The +254 and +656 SNPs are positioned in introns and therefore do not affect the coding sequence of *MIF* gene (Baugh and Bucala, 2002, Donn et al., 2001, Donn et al., 2002). Gene reporter assays and human clinical studies have shown that the increased number of the $CATT_{(5-8)}$ repeats in the promoter region is associated with increased *MIF* expression (Baugh et al., 2002). Studies on inflammatory diseases including asthma and RA have shown the presence of more CATT repeats, thus high-expression of MIF, is related to disease severity (Mizue et al., 2005, Radstake et al., 2005).

1.5.3 **Role of MIF in the immune response**

Although MIF was originally discovered as a factor secreted from activated T-lymphocytes in delayed-type hypersensitivity (DTH) reactions, the current view favours a predominant role in innate immunity. The role of MIF in adaptive immunity is not completely defined and most reports are from mouse cells or models.

1.5.3.1 ***MIF in the innate response***

MIF has been shown to act in an autocrine and paracrine fashion, directly and indirectly acting on its own synthesis induction and other pro-inflammatory mediators induction, along with activating and recruiting inflammatory cells and inducing phagocytosis (Bacher et al., 1996, Bozza et al., 1999, Donnelly et al., 1997, Makita et al., 1998, Mitchell et al., 2002, Mitchell et al., 1999). Overall it is understood that MIF initiates the inflammatory response (Calandra et al., 1994, Bernhagen et al., 1993). MIF is rapidly released from various immune cells on exposure to pathogens, microbes, pro-inflammatory cytokines or during antigen-specific activation (Calandra et al., 1998)

In mouse models, MIF treatment augments LPS-induced toxicity and anti-MIF neutralisation protects the mice from lethal endotoxic shock (Bernhagen et al., 1993). Genetically deleted MIF mice are likewise resistant to LPS-lethality (Bozza et al., 1999). The inflammatory role of MIF and the attenuation of toxicity, by anti-MIF antibodies and MIF knockout mice, were also seen with bacterial exotoxin (Calandra et al., 1998, Bacher et al., 1996). The innate response of MIF is not species specific and has also been demonstrated in rats with endotoxemia (Bacher et al., 1997). The importance of MIF in LPS signal transduction and gram-negative bacteria recognition was studied in RAW 264.7 mouse macrophages, where MIF-induced up-regulation of the TLR4 receptor was shown, allowing for quick recognition of gram-negative bacteria and endotoxin activating the innate immune response (Roger et al., 2001).

1.5.3.2 ***MIF in the adaptive response***

Mouse T-cells constitutively express MIF with T_H2 cells producing more MIF than T_H0 and T_H1 cells (Bacher et al., 1996). *In vitro*, MIF is induced from primary mouse T-cells by anti-CD3 and super-antigen and is associated with IL-2 secretion and proliferation, both of which were decreased with anti-MIF antibodies (Bacher et al., 1996).

In vivo mouse studies confirm the role of MIF in DTH reactions, with exogenous MIF potentiating the reaction and MIF-neutralising antibodies resulting in reduced antigen-specific T-cell proliferation and IgG production (Santos et al., 2001, Bernhagen et al., 1996). This reduced DTH reaction was further supported in genetically deleted MIF mouse studies (Santos et al., 2008).

More recently MIF has been associated with other cell-mediated immunity roles including activating T cells in contact hypersensitivity and mixed lymphocyte reactions (Shimizu et al., 2003). MIF is also able to stimulate IL-17 secretion from mouse lymph node cells (Stojanović et al., 2009). A mitogenic effect of MIF on T-cells has not been shown and therefore MIF may exert its role in adaptive immunity as a co-factor.

1.5.4 **Mechanism of action**

MIF is yet to be fully understood functionally and its roles within the immune system are still to be fully elucidated. MIF belongs to no known protein superfamily and has

hence been described as a cell-specific cytokine, chemokine, hormone and/or enzyme to explain its plethora of cellular functions, summarised in Figure 1.7.

1.5.4.1 *Cytokine*

Cytokines are soluble factors that react with receptors on various cell types to exert a signalling cascade to perform functions and coordinate immune responses.

MIF released into the circulation behaves as a classic pro-inflammatory cytokine by activating MAPK and modulating the gene transcription of various mediators including cytokines (TNFα, IFNγ, IL-6 and IL-1β), chemokines (CXCL8 and CXCL2), and prostaglandin and leukotrienes (prostaglandin E_2 (PGE_2) and cyclooxygenase 2 (COX-2)) in mice (Calandra et al., 1994, Bozza et al., 1999, Mitchell et al., 1999, Lue et al., 2006). Furthermore, MIF was shown to directly induce TNFα in human monocytes (THP-1 cells) and mouse macrophages (RAW264.7 cells) (Senter et al., 2002, Calandra et al., 1995) but not in murine peritoneal macrophages (de Jong et al., 2001). Mixed results have been reported regarding MIF induction of IL-6, IL-12, IL-1β and COX-2 as well (Onodera et al., 2004, Santos et al., 2004).

Other MIF cytokine-like functions include the enhancement of proliferation and activation of T-lymphocytes (Calandra et al., 1998) and the suppression of p53-induced apoptosis (Mitchell et al., 1999). MIF was first implicated in cellular proliferation as an essential regulatory mediator in T-lymphocyte and endothelial cell proliferation and modulating the immune response after activation of lymphocytes (Bacher et al., 1996). Association between MIF and p53 was proposed in 1999 (Hudson et al.) and MIF inhibiting p53 activation in mice macrophages was demonstrated a few years later (Mitchell et al., 2002). MIF cysteine residue (Cys^{81}) directly binds to two cysteine residues on p53 (Cys^{242} and Cys^{238}), confirmed by co-imunnoprecipitation and results in inhibition of many p53 targets including $p21^{waf1}$ and the pro-apoptotic BAX gene expression (Jung et al., 2008). MIF has also been suggested to drive inflammation through its proliferative effects as shown in *ex vivo* vascular smooth muscle cells in atherosclerosis mice, causing hypertrophy and artery remodelling (Chen et al., 2004).

MIF is generally accepted to be a pro-inflammatory mediator maintaining the inflammatory response; although some bacterially expressed MIF reagents have

been shown to contain various levels of LPS contamination (Bernhagen et al., 1994, Calandra et al., 1995, Lubetsky et al., 2002) and hence some reports give controversial results.

MIF concentrations in the serum of healthy individuals (0.1-100ng/ml) (Petrovsky et al., 2003) are much higher than those of other pro-inflammatory cytokines (TNFα and IL-6) at baseline that can barely be detected, questioning the potency of MIF. Also the lack of homology between other pro-inflammatory cytokines and the ability of MIF to signal via non-receptor endocytosis and to bind and inactivate c-Jun activation domain-binding protein (JAB1) do not support the cytokine-like function of MIF.

1.5.4.2 ***Chemokine***

Chemokines are low molecular weight proteins that activate and cause leukocytes to migrate to the site of injury or infection. In contrast to its name, reports have demonstrated the direct induction of classical chemokines by MIF, including CXCL8 (Onodera et al., 2004, Santos et al., 2004), CCL5 (RANTES) and CCL2 (MCP-1) (Gregory et al., 2006). MIF has been classified as having a 'chemokine-like function' (CLF) chemokine (Bernhagen et al., 2007, Gregory et al., 2006, Ren et al., 2003), other CLF chemokines include thioredoxin (TRX) (Bertini et al., 1999) and complement factor 5a (C5a) (Sozzani et al., 1995). Much of the current understanding of the chemokine-like functions of MIF have been elucidated through vascular research such as in atherosclerosis (Burger-Kentischer et al., 2002), although this is a major comorbidity of COPD (Burrows et al., 1972).

The involvement of MIF in cellular migration, recruitment and promoting trafficking has been demonstrated in the following *in vivo* models and *in vitro* systems. Intravital microscopy was used to report that exogenous MIF recruited more macrophages via MIF induced-CCL2 (Gregory et al., 2006). MIF-induced CCL2 and MAPK activation was also shown *in vitro* to increase migration of mouse neutrophils and macrophages (Fan et al., 2011, Santos et al., 2011).

Gregory and colleagues have also shown that genetically deleted MIF mice have reduced leukocyte-endothelial cell interactions in response to stimuli (Gregory et al., 2004). In human *in vitro* studies using umbilical vein endothelial cells (HUVECs),

exogenous MIF was shown to increase TNFα-induced leukocyte rolling and adhesion via the up-regulation of p-selectin expression (Cheng et al., 2010, Simons et al., 2011).

Increased numbers of neutrophils present in a mouse model of LPS-induced airway inflammation was shown to be MIF dependent (Makita et al., 1998) and MIF neutralisation of an ovalbumin (OVA)-challenged mouse model resulted in reduced recruitment of neutrophils and eosinophils to the lung (Kobayashi et al., 2006). MIF was found to exert its chemotactic properties on neutrophils, monocytes and T-cells via binding to receptor complexes involving CD74/CXCR2/CXCR4 (Bernhagen et al., 2007).

MIF also contributes to cellular recruitment and migration in non-immune cells, by promoting the migration of rat smooth muscle cells (Okamoto et al., 2008) and up-regulating the migration of mouse fibroblasts in a model of wound healing (Dewor et al., 2007).

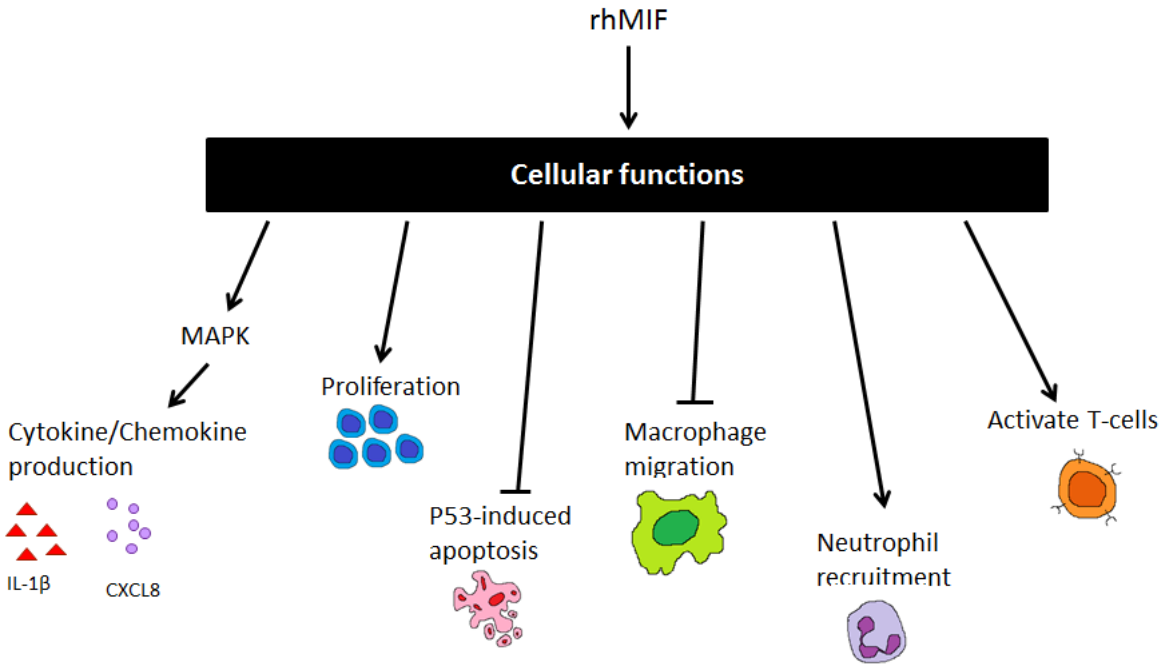


Figure 1.7: Schematic of MIF cellular functions
MIF has been reported to function in many ways including as a cytokine, chemokine, hormone and enzyme. At the cellular level, MIF has been shown to induce cytokine and chemokine production, induce cellular proliferation and recruitment and to inhibit macrophage migration and p53-induced apoptosis.

1.5.4.3 ***Hormone***

Based on rodent models, MIF is considered a mediator of the hypothalamic-pituitary-adrenal (HPA) axis (Bucala, 1996, Bernhagen et al., 1993). MIF is released in a hormone-like fashion from the hypothalamus, pituitary gland and adrenal gland in response to stress or stimuli (Bernhagen et al., 1993, Nishino et al., 1995, Bacher et al., 1998). The levels of MIF have been reported to rise with adrenocorticotropic hormone (ACTH), a hormone released from the pituitary to stimulate the adrenal release of CSs acting to counteract the immunosuppressive function (Calandra et al., 1994, Nishino et al., 1995) (See Section 1.6).

In human studies, MIF release is secreted in a diurnal rhythm corresponding to that of endogenous CSs with a plasma concentration of 0.1-100ng/ml (Petrovsky et al., 2003). However, an effect of exogenous CSs on MIF secretion from pituitary cells and subsequent systemic effects have not been demonstrated (Isidori et al., 2002) although MIF can override the hydrocortisone induced-inhibition of NF-κB activation (Daun and Cannon, 2000). The doses of hydrocortisone used in this latter study were physiological and therefore MIF was shown to antagonise the effect of naturally occurring steroids at circulating levels, enabling an immune response to occur (Daun and Cannon, 2000). Although MIF is released in a hormone-like fashion the function of MIF may be mediated through its cytokine-like or catalytic activity.

1.5.4.4 ***Enzyme***

MIF shares a similarity in protein structure with enzymes such as the human D-DT (Sugimoto et al., 1997) and some prokaryotic enzymes for example the 4-oxalocrotonate tautomerase. Interestingly, MIF not only shows structural similarity to but also functional activity with these enzymes. Reports show different catalytic activities of MIF; including that of a phenylpyruvate keto-enol isomerase (Rosengren et al., 1996), D-DT (Rosengren et al., 1996) and a thiol-mediated oxidoreductase (Kleemann et al., 1998) that may be involved in the biological function of MIF.

Similar to its recently demonstrated homologue D-DT, MIF is capable of tautomerising non-physiological substrates such as D-dopachrome into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Rosengren et al., 1996). Inhibition of the tautomerase activity of MIF with (*S,R*)3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole

acetic acid methyl ester (ISO-1) (Lubetsky et al., 2002) has been reported to attenuate inflammation (See Section 1.5.7). The immune function of MIF regarding mouse endotoxemia and its protective effects towards LPS-lethality is comparable to that of D-DT, although further investigations into the role of D-DT are required (Merk et al., 2012).

MIF possesses a CXXC motif that is also present in thiol-mediated oxidoreductase enzymes, like thioredoxin (TRX). These enzymes function in cellular oxidation/reduction processes acing as anti-oxidants (Sun et al., 1996b). A positive correlation between the anti-oxidant TRX and MIF in plasma of sepsis patients has been reported (Leaver et al., 2010). However the relationship between TRX, MIF and oxidative stress in COPD and severe asthma has not been investigated.

The amino-terminal proline (Pro-1) residue has been shown to be essential for MIF catalytic activity (Bendrat et al., 1997). However it remains to be established if this enzymatic activity is required for the biological function of MIF.

1.5.5 **MIF receptors**

Before 2003, no receptor for MIF was known and the precise MIF/receptor complex profiles are yet to be fully resolved. To date the receptors appear to be cell and function specific and these are being extensively researched.

1.5.5.1 ***Membrane-bound receptors***

Leng and colleagues identified CD74 as a cell surface binding protein for MIF by using an expression cloning strategy in THP-1 monocytes (Leng et al., 2003). CD74 is the cell surface form of the major histocompatibility class II-associated invariant chain, however CD74 has no signal transduction ability (Stumptner-Cuvelette and Benaroch, 2002). Pathways and mechanisms to transduce the signal have been suggested, such as regulated intramembrane cleavage, recruitment of cytosolic proteins and recruitment of membrane signalling proteins (Leng and Bucala, 2006).

A signal transduction molecule that has been shown to complex with CD74 is CD44. CD44 as a co-receptor is needed to elicit a response through Src tyrosine kinase activation (Meyer-Siegler et al., 2004). Activation of CD74-CD44 by MIF leads to subsequent pro-survival signals in B-lymphocytes, for example proliferation and anti-

apoptotic protein production (Gore et al., 2008), activation of the MAPK pathways ERK1/2 (Mitchell et al., 1999, Lue et al., 2006), which is most characterised and p38 MAPK (Santos et al., 2004), inhibition and activation of JNK (Lue et al., 2011) and production of PGE_2 (Mitchell et al., 2002) as well as activation of phosphatidylinositide 3-kinase (PI3K) (Lue et al., 2007, Amin et al., 2003). MIF/CD74 signalling has been associated with both the pro-inflammatory and anti-apoptotic functions of MIF.

CD74 has also been shown to complex with chemokine receptors. CXCR2 and CD74 binding on monocytes, increases the MIF-induced adhesion of monocytes (Bernhagen et al., 2007), whilst CD74/CXCR4 binding activates JNK MAPK via Src/PI3K/JNK/AP1 pathway and induces CXCL8 production (Kleemann et al., 2000, Lue et al., 2011).

It has also been postulated that extracellular MIF binds to chemokine receptors, CXCR4/CXCR2 complexes or CXCR4 and CXCR2 alone (Bernhagen et al., 2007) and more recently CXCR7 (Tarnowski et al., 2010) to elicit its CLF.

MIF and CXCR2 binding has been shown to mediate the CLF and indirectly affect leukocyte adhesion. Inhibitor studies have shown the MIF/CXCR2 binding mediates monocyte and T-lymphocyte arrest and PI3K activation leading to neutrophil adhesion (Lue et al., 2007, Smith et al., 2006) and modulating expression of adhesion proteins via NF-κB and gene transcription (Lee et al., 2012).

CXCR4 is widely expressed on immune cells including T-lymphocytes, monocytes and structural fibroblasts (Murphy et al., 2000) and has been implicated in cell recruitment processes. MIF induces T-lymphocyte recruitment and arrest through CXCR4 binding (Bernhagen et al., 2007). MIF/CXCR4 binding also activates AKT signalling in monocytes and fibroblasts, which was confirmed by inhibiting receptor binding (Schwartz et al., 2009).

1.5.5.2 *Intracellular signalling*

MIF has also been shown to interact and directly bind with c-Jun activation domain-binding protein-1 (JAB1). Both endogenous intracellular MIF and exogenous MIF after endocytosis, bind to JAB1 (Kleemann et al., 2000). MIF antagonises JAB1-stimulated AP-1 transcription and JAB1-induced JNK activity (Kleemann et al.,

2000). JAB1 also binds to $p27^{Kip1}$, stopping the cell cycle, and promoting its degradation. MIF inhibits this function and $p27^{Kip1}$ levels rise, reducing cell proliferation (Hudson et al., 1999). MIF/JAB1 complexes have been detected in atherosclerotic plaque tissue (Burger-Kentischer et al., 2002) and pituitary tumours (Pyle et al., 2003).

JAB1 has also been shown to serve as a 'molecular sink', where MIF/JAB1 complexes prevent MIF secretion (Kleemann et al., 2000) and therefore inhibit MIF-induced PI3K/Akt activation. Genetic inhibition of JAB1 by RNA interference resulted in a significant increase in secreted MIF in human embryonic kidney cells (HEK293) (Lue et al., 2007). MIF-induced PI3K/Akt signalling promotes cell growth and survival and inhibits apoptosis by inactivating pro-apoptotic proteins (BAD and FOXO3a); MIF/JAB1 complexes therefore prevent an excessive pro-survival response of cells (Lue et al., 2007).

It has been suggested that at low MIF concentrations, MIF would signal through membrane bound receptors (CD74) functioning in a pro-inflammatory fashion. However at higher concentrations of MIF it could act via the non-receptor based JAB1-mediated signalling pathway and thus have a negative effect on inflammation and cell growth (Bucala, 2000) (Figure 1.8).

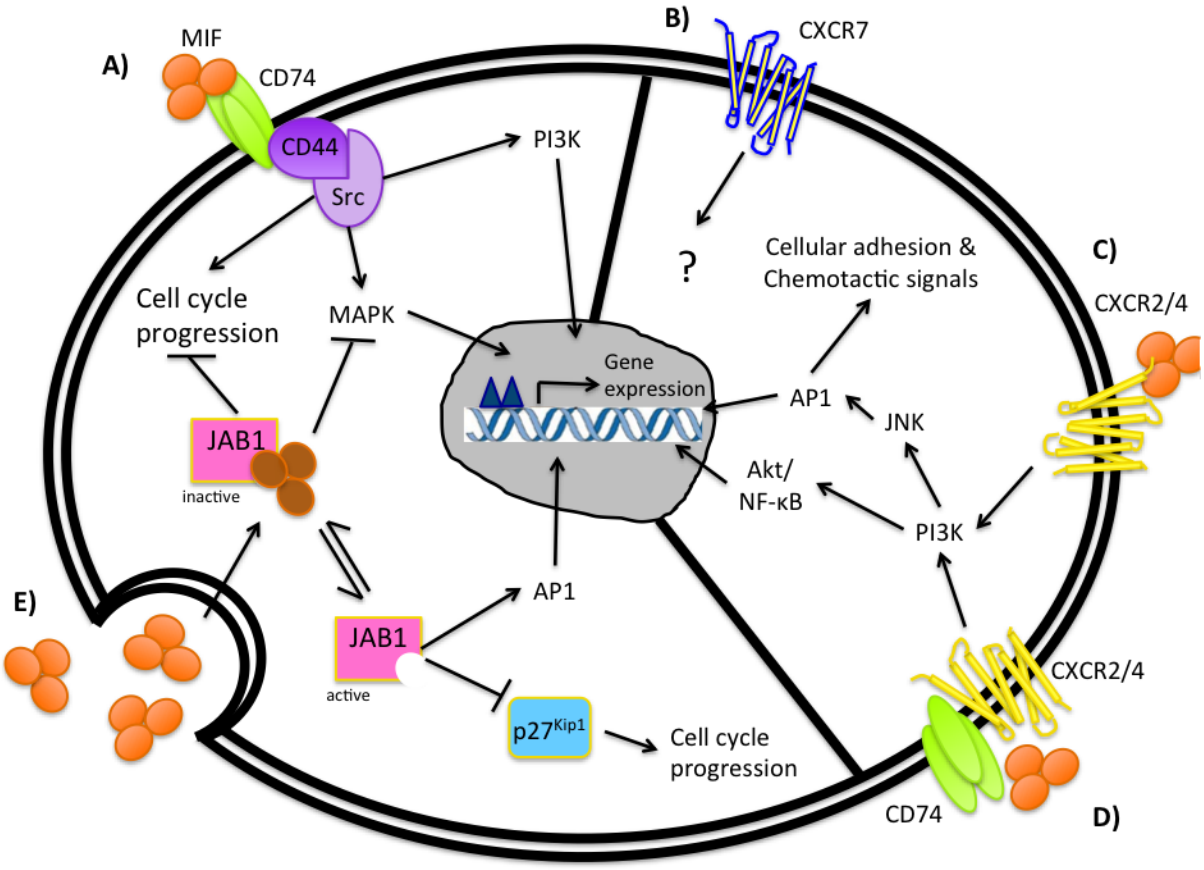


Figure 1.8: Overview of exogenous MIF signalling to control inflammation and cell growth
MIF can initiate signalling cascades via membrane-bound receptor complexes or via non-receptor binding to modulate the pro-inflammatory processes and growth of cells. A) MIF binding to membrane bound CD74 in complex with CD44 leads to activation of MAPK and PI3K signalling pathways and increased production of pro-inflammatory cytokines. MIF-CD74/CD44 binding also enhances cell cycle progression by inhibiting apoptosis. B) MIF signalling through CXCR7 has been stipulated but downstream signalling is not fully elucidated. C) MIF signals via chemokine receptors to initiate chemokine functions such as inducing cellular adhesion and migration to site of injury. D) MIF binds chemokine receptors in complex with CD74 to activate cytokine and chemokine functions. E) At high concentrations of exogenous MIF, the protein is endocytosed into the cytoplasm of the target cell where it binds and inactivates JAB1. Inactive JAB1 inhibits anti-apoptotic processes and results in reduced pro-inflammatory mediator production. Adapted from (Bucala, 2000, Tillmann et al., 2013).

1.5.6 **Molecular tools for MIF blockade**

Neutralising anti-MIF antibodies have been used in various animal models of disease to demonstrate the anti-inflammatory efficacy of inhibiting MIF, for example in endotoxemia and exotoxemia (Bernhagen et al., 1993, Calandra et al., 1998), arthritis (Leech et al., 1998) and cancer (Meyer-Siegler and Hudson, 1996).

However, neutralising antibodies make poor therapeutic agents due to the loss of efficacy over time, the inconvenient administration and the cost of production, therefore small molecule inhibitors are preferred (Chames et al., 2009). The lack of evidence for specific MIF functions and, at the time, no MIF receptor being identified made testing inhibitors challenging. However, the enzymatic activity of MIF and the identification of MIFs catalytic domain allowed the design of selective low molecular weight inhibitors. The dopachrome tautomerisation assay was mainly used to measure potency of potential MIF inhibitors, however functions including MIF-dependent phosphorylation of ERK1/2 in fibroblasts (Mitchell et al., 1999) and MIF overriding CS suppression of TNFα in murine cells (Calandra et al., 1994) was also used to confirm the efficacy of MIF inhibitors.

ISO-1 is a small molecule antagonist of MIF tautomerase activity that binds to the enzymatic active site (Lubetsky et al., 2002) and is the best characterised MIF inhibitor (Figure 1.9). ISO-1 has been shown to inhibit the D-DT conversion and to prevent the CS-mediated suppression to TNFα, PGE_2 and COX-2 release in a concentration-dependent manner *in vitro* (Lubetsky et al., 2002). ISO-1-treated mice have enhanced survival in a model of sepsis following caecal ligation and puncture (Al-Abed et al., 2005). Furthermore, ISO-1 was shown to attenuate OVA-induced lung inflammation in a mouse model of allergic asthma to comparable levels seen with dexamethasone pre-treatment (Chen et al., 2010).

Other compounds have been shown to inhibit MIF function, albeit at differing potencies and acting via different mechanisms, for example irreversible inhibition of MIF by acetaminophen metabolites (Senter et al., 2002) or isothiocyanate-based molecules (Ouertatani-Sakouhi et al., 2009) or reversible inhibition by benzisothiazolones (Jorgensen et al., 2011).

More recently, a newer irreversible inhibitor of MIF was reported, 4-iodo-6-phenylpyrimidine (4-IPP) (Winner et al., 2008) (Figure 1.9). Similarly to ISO-1, the 4-IPP compound was shown the block MIF-dependent tautomerase activity, MIF-dependent cell migration and MIF-dependent cell growth although demonstrating ≈5x to 10x more potency than ISO-1 (Winner et al., 2008). This compound has been proposed as a potential anti-cancer agent (Winner et al., 2008).

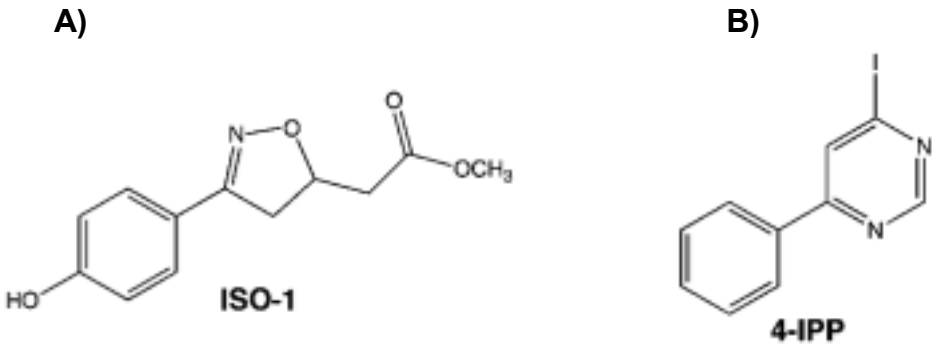


Figure 1.9: MIF inhibitors
Chemical structures of small molecule inhibitors of MIF A) ISO-1 ((*S,R*)3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester) and B) 4-IPP (4-iodo-6-phenylpyrimidine) (Winner et al., 2008)

MIF has also been successfully deleted from the genome of mice by removing exon 3 of the *Mif* gene. Genetically deleted MIF mice present normal growth, fertility and development (Bozza et al., 1999) and have been used to confirm the involvement of MIF in many disease models (Leech et al., 2003, Mizue et al., 2005, Santos et al., 2004).

1.5.7 **MIF association with diseases**

MIF expression is elevated and implicated in the pathophysiology of a plethora of diseases including asthma (Rossi et al., 1998), RA (Leech et al., 1999), atherosclerosis (Burger-Kentischer et al., 2002, Lin et al., 2000), SLE (Rovensky et al., 1975) and many cancers such as prostate, brain and non-small cell lung cancer (Brock et al., 2012, Wang et al., 2011a, Meyer-Siegler et al., 2002). Studies investigating the role of MIF in these diseases have revealed MIF functioning in many of the ways discussed above. Although the role and purpose of MIF in these diseases are not fully understood it remains to be determined if the role of MIF is beneficial or detrimental in a particular disease.

Of major interest to this study, Rossi and colleagues first reported that MIF was significantly elevated in BALF from asthmatic patients compared to healthy controls and eosinophils were a major source of MIF release (Rossi et al., 1998, Yamaguchi et al., 2000). The understanding that MIF is predominantly secreted from T_H2 cells compared to T_H1 cells (Bacher et al., 1996), expressed by both immune and lung structural cells (Donnelly et al., 1997) and in the absence of MIF, an inefficient

control of helminthic infection (Rodriguez-Sosa et al., 2003) supports the suggestion that MIF has a role in the pathogenesis of allergic asthma.

Increased serum levels of MIF in SLE patients compared to healthy controls correlated with SLE-related tissue damage scores and CS exposure (Foote et al., 2004, Mizue et al., 2005). Further evidence of the involvement of MIF in chronic inflammatory diseases was found when a single polymorphism in the MIF promoter region (-173*C) was reported to not only correlate with increased prevalence of SLE (Sanchez et al., 2006) but was also associated with the acceleration of radiological damage and increased disease severity in RA (Radstake et al., 2005). The role of MIF in the pathogenic events of RA is best characterised and well accepted (Morand et al., 2006). There are many reports demonstrating increased MIF in the serum or synovial fluid of RA patients compared to controls and that MIF expression correlates with disease severity (Baugh and Bucala, 2002, Leech et al., 1999, Onodera et al., 1999, Morand et al., 2002). In support of this, antagonism of MIF by an anti-MIF antibody resulted in prolonged survival or reduced disease severity in a rat adjuvant arthritis model (Leech et al., 1998), a mouse antigen-induced arthritis (AIA) model (Santos et al., 2001) and in a mouse collagen-induced arthritis model (Mikulowska et al., 1997). The involvement of MIF in RA was further confirmed in MIF deficient mice in an AIA model (Leech et al., 2003). Many RA-associated cells have been shown to express MIF (macrophages, endothelial cells and fibroblast-like synoviocytes) (Leech et al., 1999, Onodera et al., 1999) and in turn MIF has been shown to stimulate the release of RA-related cytokines and pro-inflammatory mediators (TNFα, IL-1 and CXCL8) (Onodera et al., 2004, Calandra et al., 1995, Santos et al., 2004, Donnelly et al., 1997).

MIF has been shown to be elevated during the progression of atherosclerosis, a chronic inflammatory disease characterised by the chemokine-mediated influx of cells to site of injury in the arterial wall (Burger-Kentischer et al., 2002). In a similar manner to RA, many pathogenic events involved in atherosclerosis can be mediated by or induce the release of MIF. For example MIF is implicated in plaque destabilisation due to MIF-induced matrix metalloproteinase release in the artery wall (Schober et al., 2004, Kong et al., 2005).

Interestingly, cardiovascular disease (CVD) and the risk of atherosclerosis is increased in patients with SLE (Bruce, 2005) and RA (Goodson, 2002). COPD patients have many systemic burdens and comorbidities caused by many factors including chronic inflammation and long-term smoking that increase the risk of further complications. COPD patients also have increased risk of developing CVD (Burrows et al., 1972); however, the role of MIF in either potentiating or causing CVD in COPD patients has not been investigated.

1.6 MIF and CS insensitivity

As previously discussed, relative CS insensitivity is a great burden on patients with severe asthma and COPD, resulting in loss of disease control. The discovery that MIF counter-regulates the anti-inflammatory actions of CS has driven MIF research in several chronic inflammatory diseases, where relative CS insensitivity is a major clinical concern (Calandra et al., 1995). The relationship between CSs and MIF also differs compared to other pro-inflammatory cytokines as MIF expression is induced by low concentrations of CSs (Calandra et al., 1995, Bacher et al., 1996). However this MIF induction was not seen at the gene level and a role for post-translational mechanisms has been proposed (Fingerle-Rowson et al., 2003). It has also been reported that CSs positively regulate the expression and secretion of MIF (Petrovsky et al., 2003).

MIF-mediated CS-antagonistic effects have been shown *in vivo*, where exogenous MIF overrode CS inhibition of lethality by endotoxic shock in mice (Calandra et al., 1995). Similarly, Santos and colleagues demonstrated that exogenous MIF inhibited dexamethasone suppression of inflammation in an allergen-induced arthritis model in MIF deficient mice (Santos et al., 2001). MIF counter-regulation of CSs has also been shown in rat models of RA, where neutralisation of MIF showed reduced joint inflammation and histological severity of diseases was reversed (Leech et al. 2000). In 2006 it was reported that MIF expression levels are raised in colonic mononuclear cells from patients with CS resistant ulcerative colitis, and the anti-inflammatory response of CSs was restored with anti-MIF antibodies (Ishiguro et al., 2006). These data suggest that together MIF and CSs reciprocally control the regulation of inflammation.

The mechanisms behind this reciprocal regulation have not been fully elucidated but include inhibition of MKP-1 (Roger et al., 2005) and induction of IκB (Daun and Cannon, 2000). MKP-1 is up-regulated by CSs and negatively regulates all MAPK pathways (Flaster et al. 2007), addition of MIF inhibits MKP-1 induction in a concentration-dependent manner (Roger et al., 2005). IκB binds and inhibits the translocation of NF-κB into the nucleus, therefore retaining the transcription factor in the cytoplasm and reducing inflammation (Karin, 1999).

In human studies, genetic polymorphisms in the *MIF* gene have been associated with CS insensitivity and reduced clinical response in RA (De Benedetti et al., 2003), nephrotic syndrome in children (Berdeli et al., 2005), SLE (Baugh and Bucala, 2002) and asthma (Rossi et al., 1998). Although this has been disputed (Ayoub et al., 2008), the evidence still warrants investigation of MIF counter-regulating CS function.

There is currently relatively little research into MIF and its associations with asthma and/or COPD compared to the other chronic inflammatory diseases despite advances in MIF biology and the development of newer antagonists. Due to the comparable chronic inflammatory status and the similar pathophysiology of these diseases with COPD and severe asthma, measuring MIF and investigating its effect on CS function in lung diseases may provide a rationale for novel therapies in these diseases.

1.7 **Hypothesis**

Patients with chronic obstructive pulmonary disease (COPD) and severe asthma have elevated levels of macrophage migration inhibitory factor (MIF) that drives the chronic airway inflammation. MIF also counter-regulates the immune-suppressive action of corticosteroids and therefore is a mechanism for the relative corticosteroid insensitivity seen in COPD and asthma.

1.8 **Aims**

i. Investigate the role of MIF in a CS insensitive *in vivo* model of COPD and determine whether MIF antagonism can restore this CS sensitivity.
ii. Establish expression profiles of MIF in COPD patient samples.
iii. Investigate the role of MIF at baseline and in a LPS-induced inflammation model in both primary and immortalised human cells.
iv. Confirm the counter-regulatory role of MIF on CS function and investigate potential mechanisms in human THP-1 cells.
v. Investigate global mRNA and protein expression changes to identify novel pathways and protein interactions involved in human MIF signalling.

Chapter 2

Materials and Methods

2.1 **Materials**

Table 2.1: Cell culture reagents

REAGENT	SUPPLIER
Dialysed foetal bovine serum	Sigma-Aldrich, Dorset, UK
Heat-inactivated, foetal bovine serum (FBS)	Sigma-Aldrich, Dorset, UK
L-Arginine:HCL (U-13C6)	CK gas products, Hampshire, UK
L-Arginine:HCL (U-13C6, U-15N4)	CK gas products, Hampshire, UK
L-Glutamine	Sigma-Aldrich, Dorset, UK
L-Lysine:2HCL (4,4,5,5-D4)	CK gas products, Hampshire, UK
L-Lysine:2HCL (U-13C6, U-15N2)	CK gas products, Hampshire, UK
L-Methionine	Sigma-Aldrich, Dorset, UK
Phosphate-buffered saline (PBS)	Sigma-Aldrich, Dorset, UK
Roswell park memorial institute (RPMI-1640 medium	Sigma-Aldrich, Dorset, UK
RPMI 1640 medium without arginine, leucine, lysine, and phenol red	Sigma-Aldrich, Dorset, UK
Sterile tissue culture grade water	Sigma-Aldrich, Dorset, UK
TIB-202 (THP-1 cells)	ATCC, Rockville, MD, USA
Trypan Blue	Sigma-Aldrich, Dorset, UK

Table 2.2: Laboratory Reagents

REAGENT	SUPPLIER
5x siRNA Buffer	Dharmacon, Colorado, USA
ACCUSPINTM System-Histopaque®-1077	Sigma-Aldrich, Dorset, UK
AMV reverse transcriptase enzyme	Promega, Southampton, UK
AMV reverse transcriptase reaction buffer	Promega, Southampton, UK
BD OptEIA™ TMB substrate reagent	BD sciences, Erembodegem, Belgium
Bovine serum albumin (BSA)	Sigma-Aldrich, Dorset, UK
Bradford solution	Bio-Rad Laboratories, Hertfordshire, UK
Calcium chloride	Sigma-Aldrich, Dorset, UK
Deoxynucleotide triphosphates (dNTPs)	Promega, Southampton, UK
Diff-Quick	Reagena, Toivala, Finland
Dimethylsulphoxide (DMSO)	Sigma-Aldrich, Dorset, UK
ECF substrate	GE Healthcare, Buckinghamshire, UK
Fura-2AM	Invitrogen, Paisley, UK
Hanks buffered salt solution (HBSS)	Sigma-Aldrich, Dorset, UK
Marvel Milk Powder	Waitrose, Berkshire, UK

Methylthiazolydiphenyl-tetrazolium bromide (MTT)	Sigma-Aldrich, Dorset, UK
MIF ON-TARGETplus non-targeting pool siRNA	Dharmacon, Colorado, USA
Novex® Sharp pre-stained protein standard	Invitrogen, Paisley, UK
NuPAGE® LDS sample buffer (4x)	Invitrogen, Paisley, UK
NuPAGE® MES SDS running buffer (20x)	Invitrogen, Paisley, UK
NuPAGE® MOPS SDS running buffer (20x)	Invitrogen, Paisley, UK
NuPAGE® Novex® Bis-Tris 10% mini gels	Invitrogen, Paisley, UK
ON-TARGETplus SMARTpool MIF siRNA	Dharmacon, Colorado, USA
Phosphate-buffer solution with Tween® 20	Sigma-Aldrich, Dorset, UK
Pluronic acid	Invitrogen, Paisley, UK
QIAshredders™ homogenisers	Qiagen, West Sussex, UK
Quantitect SYBR® Green PCR mastermix	Qiagen, West Sussex, UK
Random primers	Promega, Southampton, UK
ReBlot™ Plus antibody stripping solution	Millipore, MA, USA
Recombinant RNasin ribonuclease inhibitor	Promega, Southampton, UK
RNase-free water	Sigma-Aldrich, Dorset, UK
Sodium chloride	Sigma-Aldrich, Dorset, UK
TaqMan® Gene Expression Master Mix	Invitrogen, Paisley, UK
Trizma	Sigma-Aldrich, Dorset, UK
Tween® 20	Sigma-Aldrich, Dorset, UK
β-mercaptoethanol	Sigma-Aldrich, Dorset, UK

Table 2.3: Assay kits

KIT	SUPPLIER
Amaxa® cell line nucleofector® kit V	Lonza, Cologne, Germany
Cell proliferation BrdU kit	Roche diagnostics, West Sussex, UK
DuoSet ELISA kits	R&D Systems, Abingdon, UK
Nuclear extraction kit	Active Motif, Rixensart, Belgium
PhosphoTracer ELISA	Abcam, Cambridge, UK
Proteome Profiler Human Phospho-MAPK Array Kit	R&D Systems, Abingdon, UK
RNeasy mini kit	Qiagen, West Sussex, UK
TransAM™ assays	Active Motif, Rixensart, Belgium

Table 2.4: PCR primers and annealing temperatures

GENE	OLIGO SEQUENCE	ANNEALING TEMPERATURE
MIF forward	5'-CGGCAAGCCCGCACAGTACATC-3'	58ºC
MIF reverse	5'-CACGTTGGCAGCGTTCAT-3'	
18S forward	5'-CTTAGAGGGACAAGTGGCG-3'	60ºC
18S reverse	5'-ACGCTGAGCCAGTCAGTGTA-3'	
DUSP1 forward	5'-ACCACCACCGTGTTCAACTT-3'	60ºC
DUSP1 reverse	5'-GAGGTCGTAATGGGGCTCTG-3'	
IL8 forward	5'-TTTTGCCAAGGAGTGCTAAAG-3'	60ºC
IL8 reverse	5'-TTTCTGTGTTGGCGCAGTGTGG-3'	

The following Taqman® probes were commercially ordered from Applied Biosystems® at Invitrogen, Paisley, UK.

Table 2.5: TaqMan Gene Expression Assaysprobes

GENE	PRODUCT CODE	DYE
18S	4310893E	VIC™ TAMRA™
MIF	Hs00236988	FAM
DUSP1	Hs00610256	FAM
DDX58	Hs00204833	FAM
IFIH1	Hs01070332	FAM
TNFα	Hs01113624	FAM
IL8	Hs00174103	FAM
TP53	Hs01034249	FAM
CD74	Hs00269961	FAM

Table 2.6: Primary antibodies for Western blotting

PRIMARY ANTIBODY	FINAL CONC	SUPPLIER
β-actin Anti-Mouse	1/20000	Abcam, Cambridge, UK
MDA5 Anti-Rabbit	1/1000	Cell signaling, Danvers, MA, USA
MIF Anti-Goat	1/2500	Abcam, Cambridge, UK
Total IRF3 Anti-Rabbit	1/2000	Cell signaling, Danvers, MA, USA
p-IRF3 Anti-Rabbit	1/1000	Cell signaling, Danvers, MA, USA
RIG1 Anti-Rabbit	1/1000	Cell signaling, Danvers, MA, USA

Table 2.7: Secondary alkaline phosphatase conjugated antibodies for Western blotting

SECONDARY ANTIBODY	FINAL CONC	SUPPLIER
Polyclonal goat anti-rabbit	25ng/ml	Millipore, MA, USA
Polyclonal rabbit anti-goat	25ng/ml	Millipore, MA, USA
Polyclonal rabbit anti-mouse	25ng/ml	Millipore, MA, USA

Table 2.8: Compounds

COMPOUND	STOCK SOLUTION	SUPPLIER
Anti-CD74 (c-16)	200µg/ml	Santa Cruz, Heidelburg, Germany
Dexamethasone	5×10^{-2}M in sterile H_2O	Sigma-Aldrich, Dorset, UK
ISO-1	5mg/ml in 10% DMSO	VWR International, Leicestershire, UK
Ketaset (Ketamine)	100mg/ml	Fort Dodge Animal Health, Fort Dodge, IA, USA
Lipopolysaccharide (LPS)	1mg/ml	Sigma-Aldrich, Dorset, UK
Pentobarbital sodium	20% w/v	JM Loveridge, Hampshire, UK
Poly (I:C)	1mg/ml in sterile H_2O	InvivoGen, San Diego, CA, USA
Recombinant human MIF	0.1mg/ml	eBioscience, Hatfield, UK
Xylacare (Xylazine)	2% w/v 20mg	Animalcare, York, UK

Table 2.9: Equipment

EQUIPMENT	SUPPLIER
7500 Real-time PCR systems	Applied Biosystems, Invitrogen, Paisley, UK
96-well MicroWell™ NUNC plates	Fisher Scientific UK, Loughborough, UK
Aeroneb® Lab Micropump Nebulizer	EMMS, Hants, UK
Biotek Instuments microplate reader	Winooski, VT, USA
Corning® clear bottom 96-well plates	Sigma-Aldrich, Dorset, UK
Fluostar Optima Fluorimeter	BMG LabTech, Aylesbury, UK
G-storm Thermocycler	LabTech International, East Sussex, UK
iBlot® dry blotting system	Invitrogen, Paisley, UK
ImageQuant TL Software	GE Healthcare, Buckinghamshire, UK

Inverted light microscope	Leica Microsystems, Buckinghamshire, UK
Microcentrifuge Hettich Mikro 22R	DJB Labcare, Buckinghamshire, UK
NanoDrop™ Lite	LabTech International, East Sussex, UK
Nucleofector® II device	Lonza, Cologne, Germany
Oesophageal catheter	EMMS, Hants, UK
Optical microscope	Olympus Optical Company Ltd., Tokyo, Japan
Ozonizer (model 500)	Sander, Wuppertal, Germany
Pneumotachograph	EMMS, Hants, UK
RotorGene RG3000	Corbett Research, Crawley, UK
Shandon cytospin 4	Thermo Electron Corporation, MA, USA
Sigma 4-16K centrifuge	SciQuip Ltd, Shropshire, UK
SkanWasher 400	Skatron, Molecular Devices, CA, USA
Storm 840 Phosphoimager	Molecular Dynamics, New Jersey, USA
Transducer	EMMS, Hants, UK
Ventilator (MiniVent 845)	Hugo Sach Electronic, Germany

2.2 In Vitro Methods

2.2.1 *In vitro* cell culture

THP-1 cells, a human leukaemia monocytic cell line, were purchased from the American Type Culture Collection (ATCC) and cultured in complete RPMI-1640 growth medium supplemented with 10% FBS and 2mM L-glutamine at a density of 0.3×10^6 cells/ml. Sub-culturing every 2-3 days ensured that the cell density did not exceed 1×10^6 cells/ml. Cells were cultured in a humidified 5% CO_2 atmosphere, kept at 37°C and counted using trypan blue (1:10 dilution) and a haemocytometer. Prior to stimulation and experiments, cells were cultured in starvation media (phenol red-free RPMI-1640 medium supplemented with 0.5% FBS and 2mM L-glutamine) at a cell density of $0.5x10^6$ cells/ml

2.2.2 Isolation of peripheral blood mononuclear cells (PBMCs)

Venous blood (60 ml) was collected into three 20 ml syringes containing 1 ml of acid citrate dextrose (ACD) solution. PBMCs were isolated using the ACCUSPIN™ System-Histopaque®-1077 that utilises a porous high-density polyethylene barrier. Anticoagulated blood (20ml) was poured into each tube and then centrifuged at 1000×*g* for 10 min at room temperature. After centrifugation the plasma layer "upper layer" was carefully removed and the opaque interface, containing mononuclear cells (PBMCs) was transferred into a clean tube. PBMCs were washed with Hank's

buffered saline solution (HBSS) and centrifuged at 250×*g* for 10 min at room temperature this was repeated twice. PBMCs were then resuspended in the appropriate volume of complete RPMI-1640 medium. PBMCs were counted using a cell counting chamber and seeded onto 96-well plates for further experiments to be carried out.

2.2.3 **Cell viability**

2.2.3.1 ***MTT***

Cell viability was determined by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The assay measures the ability of the mitochondria within living cells to reduce the yellow MTT dye to its purple formazan product. This product is then dissolved with DMSO; the absorbance reading of the resulting solution is proportional to the number of viable cells.

Briefly, cells were plated in 96-well tissue culture plates (0.1×10^6 cells/well) and stimulated according to the specific experimental procedure. At the end of the experiment, the plates were spun down and the supernatants were carefully removed. The cells were then incubated in 100µl of 0.5mg/ml MTT solution in serum-free RPMI medium for 3 hours. Confirmation of the purple product within the cells was observed using a light microscope, 100µl DMSO was added to each well to lyse cells and dissolve the formazan product. The plates were placed on a plate shaker for a few minutes to ensure the product is dissolved. The absorbance at 550nm was measured using a microplate reader. The effect of treatment on cell viability was calculated as a percentage of OD relative to the untreated control.

2.2.4 **Transient Transfection**

Knockdown of MIF was achieved using the nucleofection process developed by Amaxa AG Biosystems. The effects of siRNA on the *MIF* gene were temporary and loss of gene expression was reversed as the cells proliferated.

2.2.4.1 ***Nucleofection***

THP-1 cells were transiently transfected with *MIF* siRNA following Amaxa protocols specific for THP-1 cells. Transfection efficiency is reported by Lonza to be ≈70%, measured by transfecting 0.5µg of a plasmid encoding enhanced green fluorescent protein.

Transfection experiments had three controls: Naïve- untransfected cells, Mock transfection- cells which were subjected to nucleofection but in the absence of any siRNA and Scramble- cells which were subjected to nucleofection with functional non-targeting siRNA.

THP-1 cells (2×10^6) were either resuspended in nucleofector solution with supplement (100µl per control and treatment) or 10% FBS complete RPMI media. *MIF* On-target siRNA (200nM) was carefully added to 100µl of cell suspension to each specific electroporation cuvettes; was then added to each cuvette to be electroporated. All samples, apart from the naïve- untransfected control, were electroporated with the pre-set program V-001 on the nucleofector device. Transfected cells were then resuspended in 10% FBS complete RPMI media and reseeded into 6-well, 24-well and 96-well plates for 24, 48 and 72 hours. The seeding density was empirically determined as 750µl for 6-well plates, 200µl for 24-well plates and 50µl (in triplicate) for 96-well plates. The cells from the 6-well and 24-well plates were for protein (Section 2.2.6) and mRNA (Section 2.2.5) extraction respectively to determine the MIF protein knock down, whereas the cells from the 96-well plates were for treatment and stimulant experiments.

2.2.5 **Determination of mRNA expression**

The determination of mRNA expression was achieved by extracting total RNA from the cells, preparing complementary DNA (cDNA) by reverse transcription (RT) and then measuring by real-time quantitative PCR (RT-qPCR)

2.2.5.1 ***RNA extraction***

Total RNA was isolated from THP-1 cells using the RNeasy Mini Kit; all centrifugation was performed at room temperature. Lysis and disruption of cells was achieved by the addition of RLT buffer with 1% β-mercaptoethanol (β-ME), which immediately inactivated RNases and maintained integrity of RNA. Homogenisation of lysate was performed using QIAshredder spin columns and centrifuged for 2 minutes at 12000×g. An equal volume of ethanol (70%) was mixed, with the homogenised lysate and the sample transferred to RNeasy spin columns. The columns were centrifuged for 15s at 12000×g; spin columns were washed with Buffer RW1, and then washed twice with Buffer RPE, after the final wash step the remaining RPE buffer is removed by centrifuge for 2 minutes at 12000×g. Total RNA

was then eluted from the spin column in 50µl RNase-free water by centrifugation for 1 min at 12000×g.

Total RNA was then stored at -80°C until use, quantification and purity was achieved using a Nanodrop™ Lite. RNA concentration was given in ng/µl and purity was determined by ratio of absorbance at 260nm and 280nm (A260/A280), mRNA used had a value ≥1.6 and ≤2.

2.2.5.2 *Reverse transcription*

Total RNA (0.5µg) was resuspended in 10µl of RNase free water and was denatured at 70°C for 5 minutes. Single stranded cDNA was then synthesised by addition of a master mix containing relevant components to complete the RT reaction, shown in Table 2.10. Samples were then incubated at 42°C for 1hr followed by an enzyme inactivation step at 90°C for 4 minutes. After the RT reaction, 40µl of DEPC-treated water was added to the RT-product to dilute magnesium present in sample. cDNA was stored at -20°C until use.

Table 2.10: Master mix components for reverse transcription (RT) reaction

REAGENT	VOLUME/ REACTION (µL)	FINAL CONCENTRATION
5x AMV buffer	4	1x
dNTPs	2	1mM
Random primers	1	1µg
RNasin	1	40U
AMV RTase	1	10U
H_2O-DEPC	1	N/A
cDNA product	Up to 10µl in DEPC-H_2O	

2.2.5.3 *Real time quantitative polymerase chain reaction (qPCR)*

Gene transcript levels of specific genes were quantified by Real Time- qPCR using either QuantitTect SYBR® Green performed on a Rotor-Gene 3000 machine and Rotor-Gene 6 software (Corbett Research; Australia) or TaqMan® performed on an ABI systems model and analysed using 5700 software. The total reaction volume was 20µl for both protocols; see Table 2.11 & 2.12 for reagents and concentrations. To account for variations within the protocol, gene transcript levels of *18S* RNA, a housekeeping gene, was also quantified and used for normalisation. Primer

sequence and optimal annealing temperatures used in the SYBR® green method are in Table 2.4. For the SYBR® green method, the reaction involved an initial heat step of 95°C for 15 minutes to activate the DNA polymerase followed by 30-40 cycles of denaturation (94°C for 15 seconds), annealing (55-60°C for 30 seconds) and extension (72°C for 30 seconds). Similarly, the TaqMan® protocol consisted of an initial 50ºC step for 2 minutes followed by 95ºC for 10 minutes then 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute (acquisition step); the commercially available probes are designed to all anneal at 60ºC.

Table 2.11: Components for Sybr® green RT-qPCR master mix

COMPONENT	VOLUME
2x QuantiTect SYBR Green PCR Master Mix	1X
Forward primer	1µl
Reverse primer	1µl
RNase-free water	3µl
Total master mix reaction volume	15µl
cDNA product	5µl

Table 2.12: Components for TaqMan® RT-qPCR master mix

COMPONENT	VOLUME
TaqMan® Master Mix	10µl
18S Probe	1µl
Gene of interest Probe	1µl
RNase-free water	3µl
Total master mix reaction volume	15µl
cDNA product	5µl

2.2.6 **Determination of intracellular protein expression**

Changes in intracellular protein content of THP-1 cells were determined from protein lysates extracted from the cells. THP-1 cells were seeded into 6-well plates at a density of 0.5×10^6 cells/ml and treated according to the specific experiment being performed. After completion of the experiment the plates were kept on ice and intracellular proteins isolated. Differences between treatment groups were either

measured by Western blotting or quantitatively by sandwich enzyme linked immunosorbent assays (ELISA), see Section 2.7.

2.2.6.1 *Whole cell extraction*

At completion of the experiment the cells were transferred to pre-chilled 15ml centrifuge tubes and pelleted by centrifugation at 14,000×g for 3 minutes at 4°C. Briefly, the cells were washed with ice-cold PBS containing phosphatase inhibitors to maintain the phosphorylation state of the proteins. After the removal of all supernatant the cell pellet was resuspended in complete lysis buffer containing lysis buffer, 10mM DTT and 1X protease inhibitor cocktail, thorough mixing was ensured with a 10-second vortex. Samples were incubated on ice for 10 minutes followed by a 30-second vortex. Finally samples were centrifuged at 14,000×g for 20 minutes in a 4°C pre-chilled microcentrifuge, the supernatant (whole cell extract) was transferred into pre-chilled tube and stored at -80°C.

2.2.6.2 *Cytoplasmic and nuclear extraction*

THP-1 cells were plated in 6-well plates at a seeding density of 0.5×10^6 cells/ml before being subjected to specific conditions. Cells were then collected and transferred to pre-chilled 15ml centrifuge tubes, pelleted and washed in ice-cold phosphatase inhibitors in PBS. After a second spin, the pellet was gently resuspended in 1X hypotonic buffer, transferred to a pre-chilled 1.5ml tube and incubated on ice for 15 minutes. After detergent was added and samples were vortex for 10 seconds, the samples were centrifuged for 30 seconds at 14,000×g at 4°C. The supernatant (cytoplasmic fraction) was collected and transferred into a new tube. The pellet was resuspended in complete lysis buffer, containing 1X lysis buffer, 10mM DTT and 1X protease inhibitor cocktail, the tube was then scraped against a metal tube rack to break down the nuclear membrane. The samples were then incubated for 30 minutes on ice, vortex before centrifugation at 14,000×g for 10 minutes at 4°C; the resulting supernatant (nuclear extract) was transferred into a new pre-chilled tube and stored at -80°C until further analysis.

2.2.6.3 *Bradford protein assay*

Protein concentrations of whole cell, cytoplasmic and nuclear extracts were determined by using a Bradford assay. This assay is based on an absorbance shift of Coomassie brilliant blue dye, the red form of the dye is converted into the blue

form with the addition of proteins. Bradford reagent (200µl/well) was dispensed in a 96-well microtitre plate. A curve, using a stock of 2mg/ml BSA standard solution and 1µl of the unknown samples was added in duplicate to the wells. After 5 minutes, the absorbance was read at 595nm using a microtitre plate reader and a standard curve generated using GraphPad Prism and this was used to determine the concentrations of the extracts.

2.2.6.4 ***Western blotting***

Protein extracts (30-50µg) were transferred into new tubes and gently mixed with the correct volume of 4X NuPAGE® LDS sample buffer containing 4% β-ME. The samples were then boiled for 5 minutes at 95°C to denature the proteins. The samples were then loaded onto a 10% Bis-Tris Novex® pre cast mini-gel along with Novex® Sharp pre-stained protein standard to allow for band size comparisons. The precast gel were placed in electrophoresis gel tanks and filled with either 1X NuPAGE® MES SDS running buffer, for small molecular weight proteins, or 1X NuPAGE® MOPS SDS running buffer, for larger molecular weight proteins. The tank was connected to a power supply and the gel was electrophoresed for 35-55 minutes at 200V.

2.2.6.5 ***Semi-dry transfer***

After electrophoresis, the proteins were transferred from the polyacrylamide gel onto a nitrocellulose membrane by a semi-dry method using the iBlot™ Dry Blotting system. Briefly, the gel cassettes were opened with a gel knife and the gel was transferred to the nitrocellulose membrane on the 'bottom' stack of the iBlot™ gel transfer stacks. This 'bottom' stack contains a copper anode (positive) and a nitrocellulose membrane. Filter paper soaked in deionised water was then placed on top of the gel before a 'top' stack was added, a copper cathode (negative). The sandwich of layers was placed on the iBlot™ device in addition to a sponge and tightly closed. A mini roller was used to remove any bubbles between all the layers. The iBlot™ device was used on program 3 at 20V and run for 7 minutes. After the transfer had completed, the sandwich of layers was dissembled and the nitrocellulose membrane, on which the proteins had now transferred, was cut to size using a scalpel. The membrane was rinsed in TBS-Tween® (0.1% Tween®) before immunodetection.

2.2.6.6 *Immunodetection*

Membranes were incubated in blocking buffer, 5% milk in TBS-Tween®, for at least 1 hour at room temperature on a rocker, to prevent non-specific binding of proteins. Membranes were then incubated in a suitable concentration of primary antibody (see Table 2.6) diluted in 5% milk in TBS-Tween® for 1 hour at room temperature or at 4°C overnight on a rocker. After incubation the membranes were washed three times in TBS-Tween® for 5 minutes on rocker, followed by 1 hour incubation with a secondary antibody diluted in 5% milk in TBS-Tween®, the secondary antibodies were conjugated with alkaline phosphatase (AP) (Table 2.7). The membranes were again washed three times in TBS-Tween® for 10 minutes each and then gently blotted to remove excess wash buffer. The blotted dry membranes were placed on Saran wrap, protein side up and ECF substrate solution pipetted to cover them (24µl/cm^2), they were then incubated for 1 minute protected from light; again they were blotted to remove excess substrate and then placed between plastic sheets and scanned on a storm phosphoimager. Membranes can be re-probed for the detection of different proteins by incubating in reBlot™ stripping buffer for 15-20 minutes. The membranes are then re-blocked in 5% milk in TBS-Tween® and the process repeated with the new primary antibody.

2.2.6.7 *Band quantitation*

Band density is quantified using ImageQuant TL software and the rolling ball method was used for background subtraction. Densitometric data of phosphorylated proteins were normalised to levels of total protein and levels of endogenous control β-actin.

2.2.7 **Determination of protein release**

Concentrations of cytokine detected (human- CXCL8, MIF, and TNFα) (murine- KC, MIF and TNFα) in sample or the concentration of MIF protein in extracted protein lysates (Section 2.2.6) were measured by species-specific sandwich ELISA. THP-1 cells were seeded into tissue culture 96-well plates at a final concentration of 0.1×10^6 cells/ml and treated or stimulated according to the specific experiment in a final volume of 200µl/well. After the required time, the plates were spun down (300×g for 5 mins) and supernatants removed and transferred to new 96-well plates where they were stored at -20°C.

DuoSet ELISA kits for particular cytokines or chemokines were used according the manufacturer's instructions, the MIF ELISA kit was cross-reactive with human and mouse unlike the other mediators that were species specific. Briefly, 96-well microtitre plates were coated with 100µl of capture antibody (Table 2.13) at the working concentration in PBS for 16 hours at room temperature. The capture antibody is aspirated off and the plate washed three times with 300µl of wash buffer (PBS-Tween) using an ELISA plate washer. The plate was then blocked with 300µl blocking buffer (1% BSA in PBS) for at least 1 hour followed by a further three washes as described above. Eight recombinant protein standards including a blank were then prepared by a 2-fold serial dilution of the stock protein standard in reagent diluent buffer, 100µl (in duplicate) of these were then dispensed into the wells along with 100µl of diluted sample supernatant in triplicate, for protein lysates 1µg of total protein was diluted into 100µl of reagent diluent. The plates were incubated at room temperature for 2 hours followed by another wash step. Detection antibody diluted to the working concentration (Table 2.13) in reagent diluent (100µl) was dispensed into the wells and incubated for a further 2 hours at room temperature. The plate was again washed and 100µl of Streptavidin-Horseradish peroxidase (HRP) solution, diluted to the working concentration in reagent diluent, was added to the wells and incubated for 20 minutes at room temperature avoiding direct light. A final wash step was performed before 100µl of the substrate solution, prepared by mixing equal volumes of colour reagents A (H_2O_2) and B (Tetramethylbenzidine), were added to wells and incubated for 10 minutes to allow colour to develop. The substrate reaction was stopped by the addition of 50µl of stop solution (2N H_2SO_4). The absorbance of each well was measured at 450nm and 540nm (correction) using a microtitre spectrophotometer. A four-parameter logistic standard curve was generated using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and the cytokine concentrations of each sample calculated from this, the concentrations were corrected for dilution by multiplying by the dilution factor.

Table 2.13: Antibodies, concentrations and standard curves for ELISA

CYTOKINE	ANTIBODY	STOCK CONCENTRATION	STANDARD CURVE
MIF	**Capture**- mouse anti-human MIF **Detection**- goat anti-human MIF (biotinylated) **Standard**- recombinant human MIF	2.0µg/ml 100ng/ml 30ng/ml	2000-7.81pg/ml
CXCL8	**Capture**- mouse anti-human CXCL8 **Detection**- goat anti-human CXCL8 (biotinylated) **Standard**- recombinant human CXCL8	4.0µg/ml 20ng/ml 100ng/ml	2000-15.63pg/ml
TNFα (Human)	**Capture**- mouse anti-human TNFα **Detection**- goat anti-human TNFα (biotinylated) **Standard**- recombinant human TNFα	2.0µg/ml 100ng/ml 370ng/ml	1000-3.91pg/ml
KC	**Capture**- rat anti-mouse KC **Detection**- goat anti-mouse KC (biotinylated) **Standard**- recombinant human KC	360µg/ml 36µg/ml 210ng/ml	1000-3.91pg/ml
TNFα (Mouse)	**Capture**- goat anti-mouse TNFα **Detection**- goat anti-mouse TNFα (biotinylated) **Standard**- recombinant mouse TNFα	144µg/ml 36µg/ml 310ng/ml	2000-15.63pg/ml

Table 2.14: Reagents used in ELISA

CYTOKINE	WASH BUFFER	BLOCK BUFFER	REAGENT DILUENT
MIF	0.05% Tween® 20 in PBS	1% BSA in PBS	1% BSA in PBS
CXCL8	0.05% Tween® 20 in PBS	1% BSA in PBS with 0.05% NaN_3	0.1% BSA, 0.05% Tween® 20 in Tris-buffered saline (20mM Tris HCL pH 7.4, 150mM NaCl)
TNFα (Human)	0.05% Tween® 20 in PBS	1% BSA in PBS	1% BSA in PBS
KC	0.05% Tween® 20 in PBS	1% BSA in PBS	1% BSA in PBS
TNFα (Mouse)	0.05% Tween® 20 in PBS	1% BSA in PBS	1% BSA in PBS

2.2.8 **Cell proliferation Assay**

The proliferation of THP-1 cells was determined by BrdU colorimetric ELISA-based assay. When cells are cultured with labelling medium that contains 5-bromo-2'-deoxyuridine (BrdU), this pyrimidine analog is incorporated in place of thymidine into the newly synthesised DNA of proliferating cells. The assay analyses the incorporation of BrdU into the cellular using an anti-BrdU antibody. Briefly 1×10^4 THP-1 cells/well were seeded into black tissue culture 96-well plate and treated with various dilutions of compounds/stimulants to a final volume of 100µl for 48 hours. BrdU labelling reagent was added to a final concentration of 10µM and re-incubated for 24 hours at 37ºC, after this the plate was spun down (300×g for 5 mins), all supernatant removed and wells gently blotted dry. The cells were fixed with the manufacturer's fix/denaturing solution followed by an incubation of 90 minutes with a working concentration of anti-BrdU-POD at room temperature. The antibody solution was removed and the plate washed three times with 1X wash buffer, finally substrate solution was added and incubated for 3 minutes. Luminescence was read on a plate reader at 460nm. Data was normalised as a percentage of untreated cells.

2.2.9 **TransAM™ Transcription factor ELISAs**

To detect and quantify transcription factor activation the TransAM™ ELISA-based assay was used. The basic protocol was the same for each transcription factor (p53, GR and NFAT) although specific kits were used for each transcription factor, any differences between the kits are detailed in Table 2.15. Each TransAM™ kit contained a 96-well plate on which oligonucleotides that contain the specific transcription factors consensus DNA binding site had been immobilised, any transcription factors present in the sample nuclear extract would specifically bind to these and would be detected.

THP-1 cells were treated with compounds according to specific experiments, at completion of treatments nuclear proteins were extracted (Section 2.2.6.2) and quantified (Section 2.2.6.3). First, binding buffer was added to each well to be used before a set amount of nuclear extract, diluted in lysis buffer, was added; negative and positive controls were included in each assay. The plate was then incubated for 1 hour at room temperature with mild agitation followed by three washes with wash buffer. Primary antibody diluted in antibody binding buffer (Table 2.16) was

dispensed into all wells and incubated for 1 hour at room temperature without agitation; followed by three wash steps. Secondary antibody conjugated with HRP, diluted in antibody binding buffer (Table 2.16) was added to the wells and then incubated for 1 hour at room temperature without agitation. The plate was washed a final time with 4 washes before developing solution was added and incubated for 5-10 minutes, protected from light. The reaction was then stopped with kit supplied stop solution; the absorbance of each well was then read by a plate reader at 450nm with a background reading at 655nm. These absorbance readings were normalised to the background reference and the negative control values were also subtracted. The measurements were then calculated as a percentage of naïve cells.

Table 2.15: DNA consensus sequences for transcription factors in TransAM® kit

	P53	GR	NFAT
DNA consensus motif	5'-GGACATGCCC GGGCATGTCC-3'	5'-GGTACAnnn TGTTCT-3'	5'-AGGAAA-3'
Nuclear extract (µg)	10	20	10
Sample volume (µl)	10	20	10

Table 2.16: TransAM® antibody dilutions

	P53	GR	NFAT
Primary Antibody	1:1000	1:1000	1:500
Secondary Antibody	1:1000	1:1000	1:1000

2.2.10 Proteome profiler™ Array

For the parallel determination of relative levels of phosphorylation of MAPKs and other serine/threonine kinases, a human phospho-MAPK array was used. Nitrocellulose membranes were spotted with 26 capture and control antibodies in duplicate, these were then used to analyse the amount of phosphorylated protein for each corresponding spot detected in the sample.

THP-1 cells were treated with compounds for specific lengths of time at a cell density of 0.5×10^6/ml. Protein lysates were prepared by rinsing cells in PBS followed by lysing in Lysis buffer (1×10^7 cells/ml) and incubating at 2-8ºC for 30 minutes. The suspension was then centrifuged at 14000×g for 5 minutes and supernatant transferred to clean tubes, quantitation of sample protein was completed (Section 2.2.6.3). The membranes, already dotted with specific phospho-antibodies, were blocked in Array buffer 5 for 1 hour at room temperature on a rocking platform shaker, during this time samples were prepared. Samples (300µg protein in a maximum volume of 400µl) were diluted in 1.5ml Array Buffer 1 along with 20µl of detection antibody cocktail; this mixture was incubated for 1 hour at room temperature to form complexes. Array buffer 5 was aspirated from the 4-well multi dish and the prepared sample/antibody mix added to the membranes and incubated overnight at 2-8ºC on a rocking platform shaker. The following day, membranes were washed three times in 1X wash buffer for 10 minutes each and then incubated for 30 minutes at room temperature in streptavidin-HRP diluted in array buffer 5. Membranes were then washed as described above and then blotted dry, to remove excess buffer; finally Chemi Reagent mix was evenly pipetted over the membranes and incubated for 1 minute, protected from light. Again, excess liquid was blotted from the membranes before they were wrapped in plastic, placed into autoradiography films cassettes and then exposed to X-ray films for 1-10 minutes. The developed film showed the phosphorylated kinase signals and the spots could be identified using the transparency overlay provided in the kit. Pixel densitometry was used to quantify the differences in kinase activity. The average density of the duplicate spots was normalised to the negative control within the array and then calculated as a percentage of untreated control.

2.2.11 MAPK PhosphoTracer ELISA

For the semi-quantitative measurement of ERK 1/2 (pT202/Y204), p38 MAPK (pT180/Y182) and JNK 1/2/3 (pT183/Y185) concentrations in cell culture extracts a PhosphoTracer ELISA was used, the all-in-one method was followed.

THP-1 cells (20µl at 10×10^6 cells/ml) were incubated at 37ºC in wells of a kit provided 96-well plate for a few hours to allow handling-mediated pathway activation to subside. rhMIF (2X final concentration) was then dispensed to each well and incubated at 37ºC for specific time points. At completion of experiment the cells

were lysed with 5X lysis mix concentrate and incubated for 10 minutes at room temperature on a plate shaker; 1X lysis mix was used for negative and positive controls. Antibody mix (50µl) was then added onto this extract suspension, the plate sealed and then incubated for 1 hour at room temperature on a plate shaker. The wells were then washed with wash buffer and the plate tapped dry to remove excess fluid. Prior to its use, substrate mix was prepared and then added 100µl/well and incubated for 10 minutes at room temperature, protected from light, on a plate shaker. The reaction was then stopped with the addition of kit supplied stop solution; before the fluorescence signal was read on a plate spectrophotometer (Excitation:530nm / Emission:590nm) the plate was briefly mixed. Fluorescence readings for negative controls were subtracted from sample values and then these were calculated as a percentage of time 0.

2.2.12 Stable Isotope Labelling with Amino acids in cell Culture

Stable isotope labelling with amino acids in cell culture (SILAC) is a global proteomic technique (Ong et al., 2002). THP-1 cells were cultured as normal with standard cell densities but using special designated complete SILAC RPMI-1640 media, see Table 2.17. The cells were cultured for five divisions to ensure that full incorporation of heavy amino acids ($^{13}C_6$-Arg and $^{2}H_4$- Lys) into the proteins was achieved. The cells were then spun down, resuspended in fresh designated SILAC media and seeded into new flasks. The cells were then treated with compounds for 24 hours before whole cell protein extractions were prepared.

Liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) analysis was performed at the University of Bristol. Briefly, cell extracts were run on an SDS-PAGE gel and the equivalent gel lanes were cut into 10 slices. Each slice was subjected to in-gel tryptic digestion using a ProGest automated digestion unit (Digilab UK). The resulting peptides were fractionated using a Dionex Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, MA, USA). In brief, peptides in 1% (vol/vol) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific Dionex, MA, USA). After washing with 0.5% (vol/vol) acetonitrile 0.1% (vol/vol) formic acid peptides were resolved on a 250mm × 75µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific Dionex, MA, USA) over a 150 min organic gradient, using 7 -gradient segments (1-6% solvent B over 1min., 6-15% B over 58min., 15-32% B

over 58min., 32-40% B over 3min., 40-90% B over 1min., held at 90% B for 6min and then reduced to 1% B over 1min.) with a flow rate of 300nlmin^{-1}. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionised by nano-electrospray ionisation at 2.1kV using a stainless steel emitter with an internal diameter of 30µm (Thermo Scientific, MA, USA) and a capillary temperature of 250°C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific, MA, USA) and operated in data-dependent acquisition mode. The Orbitrap was set to analyse the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300 to 2000 and the top six multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30s; exclusion list size, 500) were used. Fragmentation conditions in the LTQ were as follows: normalised collision energy, 40%; activation q, 0.25; activation time 10ms; and minimum ion selection intensity, 500 counts.

Full incorporation of the labelled isotopes to cellular proteins is essential. Results were calculated as a ratio of light media.

Table 2.17: Components for SILAC media

Light: R0K0	**Heavy:** R6K4
$^{12}C_6$ Arginine	$^{13}C_6$ Arginine
$^{12}C_6$ Lysine	$^{2}H_4$ Lysine
10% dialysed FBS	10% dialysed FBS
2mM L-glutamine	2mM L-glutamine
Methionine	Methionine

2.2.13 Calcium signalling

THP-1 cells were resuspended in 1% BSA in phenol red free RPMI-1640 medium at a cell density of 5×10^{6}cells/ml and incubated for 30mins (37ºC, 5% CO_2) with FURA-2 AM and pluronic acid to fluorescently label the intracellular calcium. Cells were

then washed with HBSS and the labelled cells resuspended in calcium buffer (50ml Hanks Buffer, 0.5% BSA, 0.5ml HEPES, 50µl CaCl, titrated with NaOH to pH 7.4). Cells were immediately loaded (100µl/well of cell suspension) into a black 96-well plate. Maximum and minimum calcium release was measured before each experiment using digitonin (5µg/ml final concentration) and EDTA (50mM final concentration), fluorescence was measured at excitation 340-10nm and emission 520-35nm with a gain of 2150. Stimulants and compounds were made up in calcium buffer at 10X final concentration and injected into wells accordingly; the fluorescence was measured every 2.8 seconds. Calcium concentration was calculated using the following the equation, R represents 380/340nm ratio and R_{min} and R_{max} was empirically determined from cells. Sf2 and sb2 represents the averages of the 380nm values at R_{min} and R_{max} respectively.

$$[Ca^{2+}] = (R - R_{min})/(R_{max} - R) \times 224 \times (sf2/sb2)$$

2.2.14 RNA microarrays

Global mRNA expression profiles were determined using the SurePrint G3 Human microarrays V2 (Agilent Technologies, CA, USA). Arrays were run according to manufacturer's instructions at the University of Manchester. Raw data files were returned to Imperial College London for further analysis.

2.3 *In vivo* animal models

Male C57/BL6 mice (Harlan, UK) were purchased and housed in specific pathogen free conditions within the CBS facility at Imperial College London, South Kensington campus. Mice were housed in groups of four for the duration of the experiment and each experiment group had six mice (n=6). All experiments were conducted when the mice were 6-8 weeks old and were performed under a Project License (70/7581) from the British Home Office, UK, under the Animals (Scientific Procedures) Act 1986.

2.3.1 Mouse ozone exposure- COPD model

C57/BL6 mice were exposed to ozone generated from an ozonizer, which was mixed with air to a 3 part per million (ppm) concentration. Mice were exposed for 3 hours twice a week for 6 weeks and anaesthetised 24 hours after the last exposure using ketamine and xylazine for airway hyper-responsiveness (AHR) analysis and

subsequent BALF collection. Control groups were exposed to normal air in an identical experimental setup. Groups were pre-treated with ISO-1 or ISO-1 and dexamethasone in combination intraperitoneally (i.p.) 30 minutes before exposure to ozone/air with a dose of 35mg/kg (ISO-1) and 2mg/kg (dexamethasone).

2.3.2 **Measuring airway hyper-responsiveness**

Twenty-four hours after ozone exposure the mice were anesthetised with Ketamine and xylene to measure airway hyper-response (AHR). Once all reflexes were absent the mice were tracheostomised and ventilated at a rate of 250 breaths/minute and with a tidal volume of 250μl. Mice were continuously monitored in a whole body plethysmograph with a pneumotachograph connected to a transducer. Transpulmonary pressure was assessed via an oesophageal catheter. Pulmonary resistance (R_L) was recorded for 3 minute periods during increasing concentrations of acetylcholine (4-256mg/ml) administered with an Aeroneb® Lab Micropump Nebulizer. R_L was expressed as percentage change from baseline R_L with nebulised PBS. The concentration of acetylcholine required to increase R_L by 200% from baseline was calculated (PC_{200}) and –log PC_{200} was taken as a measure of airway responsiveness.

2.3.3 **Sample collection and preparation**

After AHR measurements the mice were sacrificed by overdose of pentobarbital sodium (500μl).

2.3.3.1 ***Bronchoalveolar lavage fluid***

BALF samples were collected by rinsing the lungs three times with 0.8ml ice-cold PBS via an endotracheal tube and retrieved as BALF. BALF samples were centrifuged to isolate cells from fluid (1500×g for 10 minutes at 4°C); the cell pellets were then resuspended in 200μl PBS and counted on a haemocytometer (total cell count). BALF was frozen at -80°C until analysis. Inflammatory mediators (KC, TNFα, MIF) in BALF were determined by ELISA.

2.3.3.2 ***Differential cell count***

BALF cell samples were centrifuged at 480rpm for 6 minutes, onto glass slides using a cytospin. Cells on glass slides were stained using Diff-Quick kit and differential cell counts obtained using an optical microscope.

2.4 **Statistical analysis**

Results are expressed as mean±standard error of mean (SEM) (n≥3) for *in vitro* work and mean±standard deviation (SD) for *in vivo* studies. EC_{50} values were calculated from concentration response curves, and considered to be the concentration of compound that inhibits or induces the effect by 50% of the maximal response. GraphPad Prism® was used for statistical analysis, analysis of variance (ANOVA) was performed by Kruskal-Wallis analysis (non-parametric) and for significant results; Dunn's post-test was used. The comparisons of individual samples were analysed using Mann-Whitney *t*-test. *p* values less than 0.05 were considered statistically significant.

Chapter 3

Macrophage migration inhibitory factor inhibition in a steroid insensitive ozone-exposed *in vivo* murine model

3.1 Introduction

MIF is a critical regulator of inflammation and has been shown to be involved in various inflammatory conditions such as RA (Bernhagen et al., 1996), sepsis (Bozza et al., 1999), atherosclerosis (Burger-Kentischer et al., 2002), acute respiratory distress syndrome (ARDS) (Donnelly et al., 1997) and asthma (Rossi et al., 1998). Whilst COPD is associated with elevated levels of pro-inflammatory cytokines there have been no reports of MIF associated with human COPD to date. CSs are the gold standard treatment for many of these inflammatory diseases as they control disease progression and relieve symptoms. COPD and a subset of severe asthmatics patients show CS insensitivity and hence have less control of the disease, presenting more severe symptoms and co-morbidities (Keatings and Barnes, 1997). Similarly to other cytokines, MIF is suppressed by CSs at therapeutic concentrations however, unique to MIF; low physiological concentrations of CSs have been shown to induce MIF (Calandra et al., 1994). In addition to the pro-inflammatory functions, MIF has also been shown to counter-regulate the anti-suppressive actions of CSs (Calandra et al., 1995, Bucala, 1996). Anti-MIF and MIF inhibition strategies have therefore been of major interest to restore CS sensitivity and potentially reduce the prescribed doses needed for efficacy.

Animal models are invaluable to research, not only for investigating the effects within a whole living system but also for proof-of-concept and from drug safety aspects. The ozone model used in this study has previously been reported as a model of COPD showing some phenotypes of disease (Triantaphyllopoulos et al., 2011, Wiegman et al., 2013). Cigarette smoking is regarded as a major risk factor of developing COPD and has been shown to be the major cause of emphysema (Barnes, 2007). However, *in vivo* models of cigarette smoke exposure depend upon the dose and timing of cigarette smoke exposure and may result in both pro- and anti-inflammatory outcomes. Cigarette smoke contains >4000 distinct chemical entities along with $>10^{15}$ reactive oxygen species per puff (Pryor and Stone, 1993). Ozone is an oxidant and the inflammatory profile that is induced by ozone is comparable to that induced by cigarette smoke. The ozone-induced lung inflammation model shows enlargement of alveolar space with evidence of emphysema, induced pro-inflammatory cytokines like IL-13, IFNγ and KC and reduced anti-apoptotic proteins such as Bcl-2 reflecting several important COPD

traits (Triantaphyllopoulos et al., 2011). Induced sputum samples from COPD patients have elevated levels of CXCL8 and TNFα (Keatings et al., 1996) and the ozone-induced inflammation model here also shows increased levels of KC (a murine CXCR2 agonist) and TNFα. The elevated cytokine release has been associated with high numbers of neutrophils and macrophages also found in the sputum of COPD patients (Keatings et al., 1996) in support of this, the ozone-induced mouse model shows significantly increased macrophages, neutrophils and lymphocytes detected in the BALF. The advantage of the ozone-induced model compared to the cigarette smoke-induced inflammation model is that the former model induces these COPD-like characteristics more rapidly, 6 weeks of twice weekly exposure compared to possible 6-8 months of daily smoke exposure (Guerassimov et al., 2004).

In addition to the current model showing characteristics of human COPD, the anti-inflammatory functions of dexamethasone are not seen suggesting that this model also demonstrates relative CS insensitivity. Although the mechanism of the CS insensitivity is not fully elucidated, it was demonstrated that the Nrf2-regulated haemeoxygenase-1 (HO-1) gene was suppressed after chronic exposure to ozone (Triantaphyllopoulos et al., 2011), suggesting an ozone-induced loss of anti-oxidant properties.

ISO-1 is the best-characterised MIF inhibitor to date. MIF has tautomerase activity and converts non-physiological substrate D-dopachrome into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Rosengren et al., 1996). ISO-1 binds to the enzymatic active site irreversibly inhibiting MIF (Lubetsky et al., 2002). Furthermore, attenuation of OVA-induced lung inflammation by ISO-1 has been reported in a mouse model of allergic asthma (Chen et al., 2010).

To date, there are no reports on the effects of MIF inhibition on sensitivity to CSs within asthma or a COPD model; in this chapter I will investigate a CS insensitive COPD model and assess whether MIF inhibition by ISO-1, can improve the anti-inflammatory effects of CSs.

3.2 Chapter hypothesis and aims

Ozone exposure induces a steroid insensitive lung inflammation associated with elevated MIF expression. Inhibition of MIF attenuates this ozone-induced inflammation and restores steroid sensitivity.

1. Confirm inflammation and dexamethasone insensitivity induced by ozone-exposure in *in vivo* mouse model
2. Investigate the effects of MIF inhibition by ISO-1 pre-treatment on ozone-induced inflammation in mouse *in vivo* model
3. Investigate the effects of ISO-1 pre-treatment on ozone-induced dexamethasone insensitivity

3.3 Results

3.3.1 Ozone-induced CS insensitive COPD inflammation model

Male C57/BL6 mice were pre-treated with ISO-1 i.p. (35mg/kg) or dexamethasone (2mg/kg) alone or in combination 30 minutes prior to being exposed to ozone (3ppm) or air for 3 hours, twice a week, for 6 weeks. Bodyweights were measured before each exposure to assess the effects of ozone and compounds on overall health of the mice. During the 6 week experiment there was a steady increase in the bodyweights of the mice in all groups, the compounds or ozone had no detrimental effects on overall health (Figure 3.1).

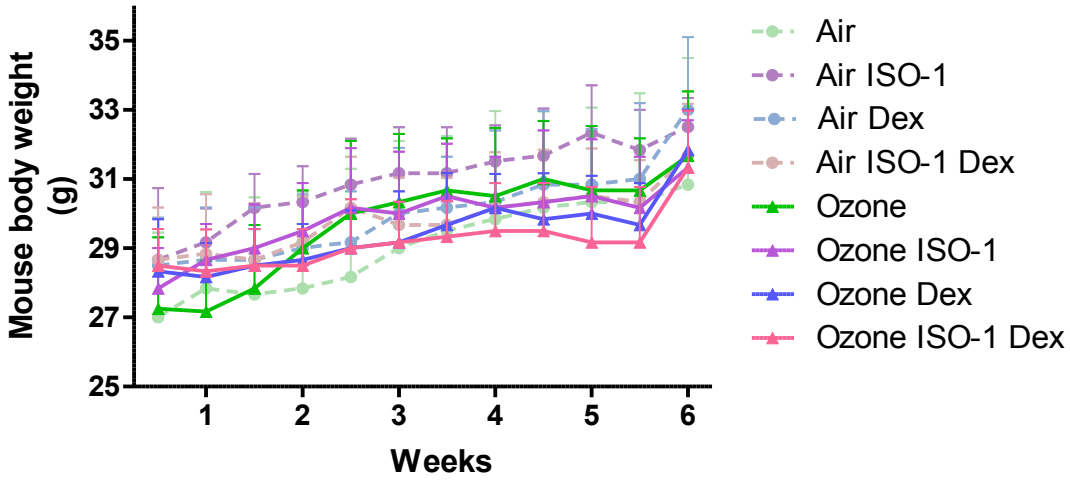


Figure 3.1: Drug and ozone effects on mouse body weights over the experimental time course
C57BL/6 mice were injected i.p. with either ISO-1 (35mg/kg) or dexamethasone (Dex, 2mg/kg) alone or in combination 30 minutes before 3-hour exposure to ozone (3ppm) or air. This occurred twice a week for 6 weeks and mice were weighed before each exposure. The data are presented as mean±SD for 6 animals per group.

Airway hyper-responsiveness was measured 24 hours after the final ozone/air exposure. The mice were anaesthetised and once all reflexes were absent, they were tracheotomised and ventilated. Pulmonary resistance (R_L) was measured with increasing concentrations of acetylcholine (4-256mg/ml). The concentration of acetylcholine required to increase R_L by 200% compared to baseline was calculated (PC_{200}) and –log PC_{200} was taken as a measure of airway hyper-responsiveness. Ozone alone significantly increased the R_L compared to air control (326.5±29.8% vs. 239.0±37.3% at 256mg/ml ACh, p<0.01) but dexamethasone pre-treatment had no effect on this (Figure 3.2A). Ozone exposure significantly elevated the responsiveness of the airway (PC_{200}) to acetylcholine and this was not affected by dexamethasone pre-treatment (2.3±0.2% vs. 2.3±0.2%, p=NS) indicating a relative CS insensitivity (Figure 3.2D).

BALF samples were collected from all mice to assess the effect of ozone on lung inflammation; both total and differential cell counts were calculated. Ozone exposure significantly increased the total number of BALF cells when compared to the air control 3-fold ($4.1\pm1.8\times10^5$ cells vs. $12.3\pm4.0\times10^5$ cells, p<0.01). This increase in cell numbers was seen for all cell-types counted; neutrophils increased by 3.5-fold

($0.6\pm0.2\times10^5$ cells vs. $2.1\pm0.5\times10^5$ cells, $p<0.05$), macrophages by 3.5-fold ($1.0\pm0.4\times10^5$ cells vs. $3.2\pm0.7\times10^5$ cells, $p<0.01$) and lymphocytes increased by 3-fold ($2.9\pm1.8\times10^5$ cells vs. $8.4\pm2.4\times10^5$ cells, $p<0.01$) compared to air control (Figure 3.3).

In order to assess the inflammation occurring in the lungs, the levels of pro-inflammatory mediators (MIF, KC and TNFα) in BALF were measured. BALF MIF levels were elevated 3-fold (2.5±0.9ng/ml vs. 7.0±1.6ng/ml, $p<0.01$) following ozone treatment compared to air control. Ozone also induced BALF KC (46.4±20.2pg/ml vs. 108±26.6pg/ml, $p<0.01$) and TNFα (85.4±45.8pg/ml vs. 236.3±99.5pg/ml, $p<0.01$) levels 2.3-fold and 2.8-fold respectively. Relative dexamethasone insensitivity was seen in this ozone-induced inflammation model. Dexamethasone pre-treatment before ozone-exposure did not attenuate the levels of BALF MIF (7.0±1.6ng/ml vs. 5.6±0.7ng/ml, *p*=NS), KC (108±26.6pg/ml versus 116.3±24.6pg/ml, *p*=NS) or TNFα (236.3±99.5pg/ml vs. 190±28.5pg/ml, *p*=NS) and none were significantly different to levels detected in mice exposed to ozone alone (Figure 3.4).

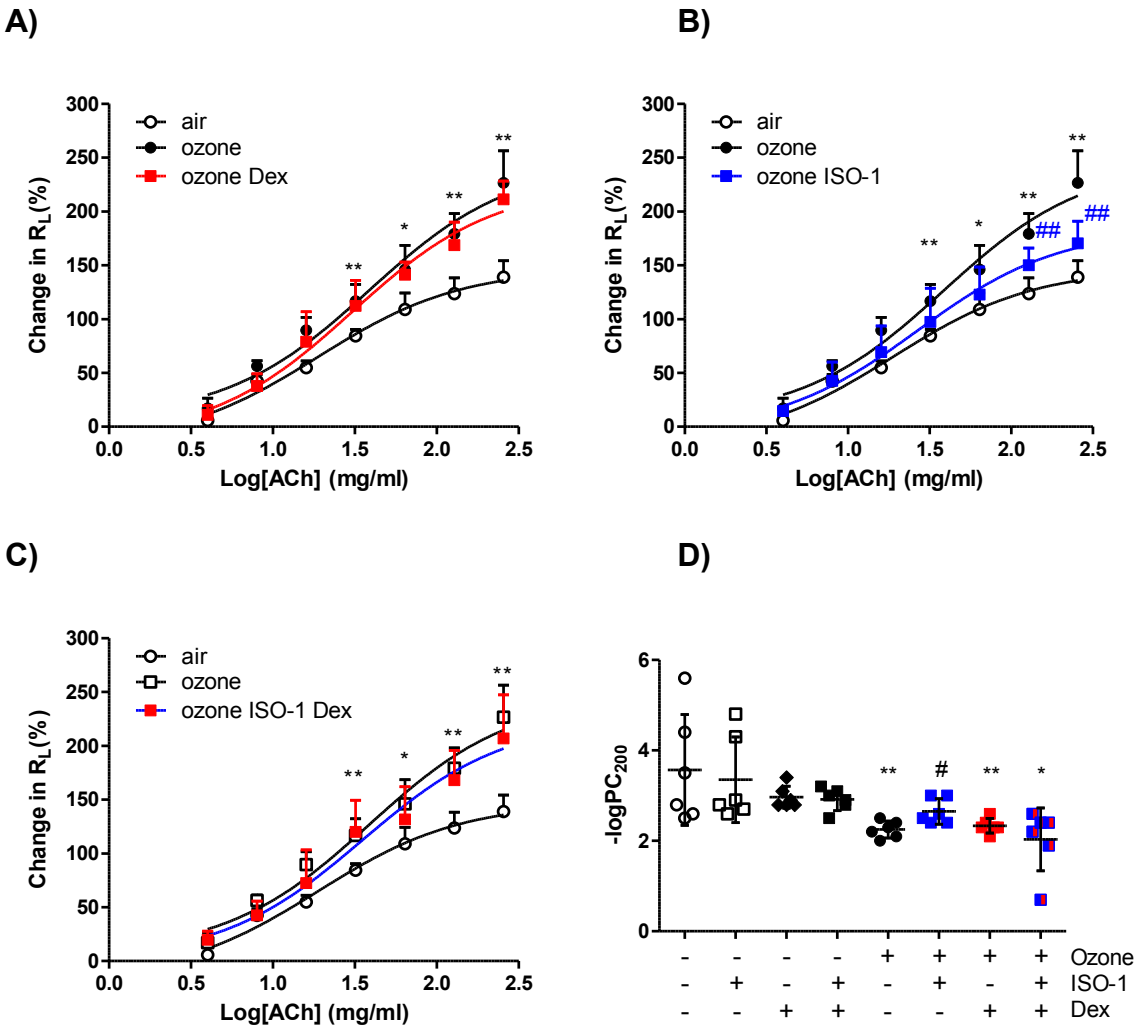


Figure 3.2: Effects of dexamethasone and ISO-1 on ozone affected pulmonary resistance and airway hyper-responsiveness

C57BL/6 mice were injected (i.p.) with ISO-1 (35mg/kg) or dexamethasone (Dex, 2mg/kg) alone or in combination before being exposed to ozone (3ppm) or air for 3 hours, twice a week for 6 weeks. Pulmonary resistance (R_L) to acetylcholine (4-256mg/ml) was measured and presented as percentage change from baseline. The pre-treatment effects on ozone-affected pulmonary resistance were assessed (A) Dexamethasone alone, (B) ISO-1 alone, (C) ISO-1 and dexamethasone in combination. (D) Airway hyper-responsiveness was calculated as –log PC_{200} (PC_{200} is the concentration of acetylcholine required to increase R_L by 100% compared to baseline). Data is presented as mean±SD for 6 animals per group. **p*<0.05 vs. air control, ***p*<0.01 vs. air control, #*p*<0.05 vs. ozone, ##*p*<0.01 vs. ozone as calculated by individual Mann-Whitney t-test for graphs A-C and ANOVA with Dunns post-hoc test for graph D.

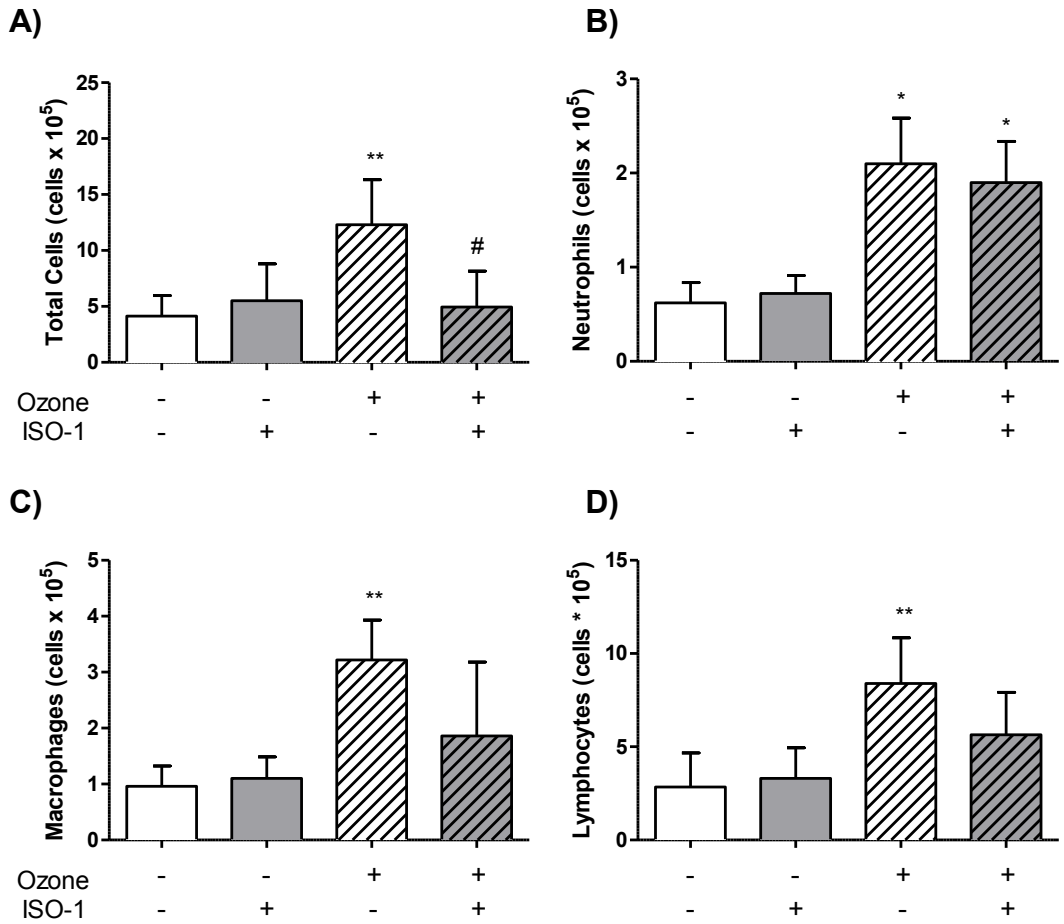


Figure 3.3: Effects of ISO-1 on ozone-induced BALF differential cell counts
C57BL/6 mice were pre-treated with ISO-1 (35mg/kg) 30 minutes before a 3-hour ozone/air exposure twice a week, for 6 weeks. BALF samples were collected and total cell (A), neutrophils (B), macrophages (C) and lymphocytes (D) numbers were counted. Data is presented as mean±SD for 6 animals per group. **p*<0.05 vs. air control, ***p*<0.01 vs. air control and #*p*<0.05 vs. ozone-exposed as calculated by ANOVA with Dunns post-hoc test.

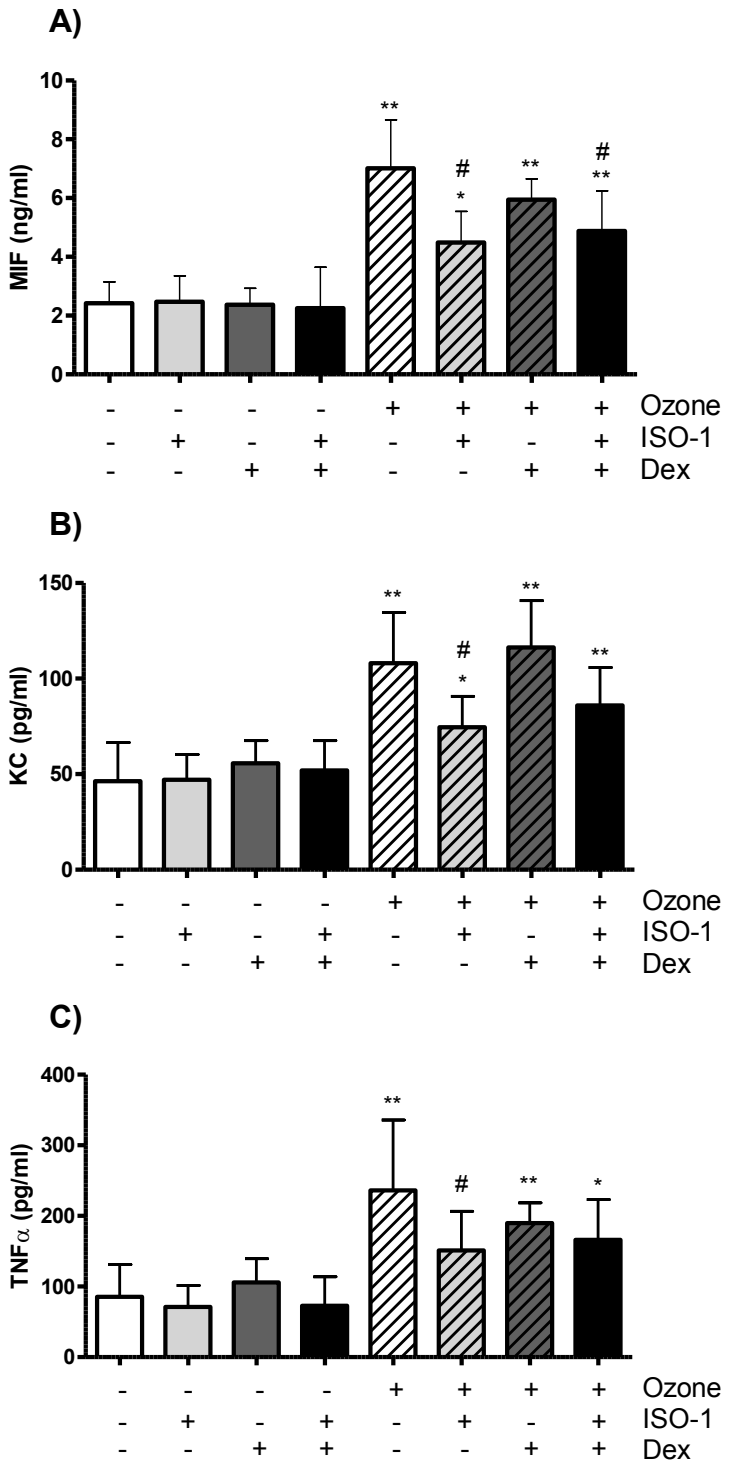


Figure 3.4: Pro-inflammatory mediator levels in BALF supernatants from ozone-exposed and dexamethasone- or ISO-1-treated mice
Male C57BL/6 mice were injected (i.p) with ISO-1 (35mg/kg) or dexamethasone (Dex, 2mg/kg) alone or in combination 30 minutes before being exposed to ozone/air for 3 hours this was repeated twice a week for 6 weeks. BALF samples were collected and (A) MIF, (B) KC and (C) TNFα levels measured. Data is presented as mean±SD for 6 animals per group. **p*<0.05 vs. air control, ***p*<0.01 vs. air control, #*p*<0.05 vs. ozone exposed as calculated by ANOVA with Dunns post-hoc test.

3.3.2 MIF inhibition on CS insensitive ozone-induced inflammation

In this study, relative dexamethasone insensitivity was seen; dexamethasone pre-treatment had no effect on the ozone-induced R_L. Ozone alone increased the R_L with an enhanced acetylcholine concentration-dependent increase from baseline compared to air control. In addition, dexamethasone did not suppress ozone-induced BALF MIF, KC or TNFα levels.

The role of MIF in the ozone model was investigated by pharmacological inhibition using ISO-1. Mice were pre-treated with ISO-1 (35mg/kg) 30 minutes prior to ozone or air-exposure. Pre-treatment with ISO-1 before ozone exposure significantly reduced ozone-induced R_L at the two highest concentrations of acetylcholine used (279.1±18.8% vs. 250±15.9% at 128mg/ml Ach, p<0.01; 326.5±29.8% vs. 270.3±20.7% at 256g/ml Ach, p<0.01) (Figure 3.2B). There was a significant difference seen between ozone alone and ozone plus ISO-1 treated animals with regards to –Log PC_{200} (2.3±0.2% vs. 2.7±0.3%, p<0.05) suggesting that ISO-1 had a protective effect on airway hyper-responsiveness (Figure 3.2D).

ISO-1 pre-treatment attenuated the total BALF cell counts (12.3±4.0×10^5 cells vs. 5.0±3.2×10^5 cells, p<0.05) although there was no significant effect seen on individual cell types. Neutrophil levels were not affected by MIF inhibition as BALF neutrophilia was similar in the ozone-treated compared to ISO-1 pre-treated animals (2.1±0.5×10^5 cells vs. 1.9±0.4×10^5 cells, p=NS) with both levels being significantly greater than that seen in air controls (0.6±0.2×10^5 cells, p<0.05). ISO-1 reduced the BALF numbers of both macrophages (3.2±0.7×10^5 cells vs. 1.86±1.3×10^5 cells, p=NS) and lymphocytes (8.4±2.4×10^5 cells vs. 5.6±2.3×10^5 cells, p=NS) induced by ozone although these differences did not reach significance (Figure 3.3).

Ozone-induced BALF pro-inflammatory mediator expression was also attenuated by ISO-1 pre-treatment. Ozone-induced BALF MIF levels were significantly reduced by 1.6-fold with ISO-1 pre-treatment (7.0±1.6ng/ml vs. 4.5±1.1ng/ml, p<0.05). In addition, KC (108±26.6pg/ml vs. 74.6±16.1pg/ml, p<0.05) and TNFα (236.3±99.5pg/ml vs. 151.1±55.6pg/ml, p<0.05) levels were reduced 1.4-fold and 1.6-fold respectively when compared to ozone alone-exposed. However, the levels of ISO-1 pre-treated ozone-induced MIF and KC remained significantly increased compared to air control (p<0.05 for BALF MIF and p<0.05 for BALF KC) (Figure 3.4).

3.3.3 The effect of MIF inhibition on dexamethasone sensitivity

As previously described, this ozone-induced model of COPD lung inflammation shows a relative CS insensitivity with a failure of dexamethasone to suppress AHR, BALF cell numbers and pro-inflammatory mediator expression. MIF levels are significantly induced by ozone exposure and MIF has been shown to counteract the anti-inflammatory activity of CSs. Therefore, to investigate if the relative dexamethasone insensitivity in this model could be reversed with MIF inhibition; the two compounds (ISO-1 and dexamethasone) were given in combination pre-ozone-exposure.

Ozone-induced R_L was not affected by pre-treatment with ISO-1 and dexamethasone in combination. The previously shown attenuation of R_L with ISO-1 alone was no longer evident when in combination with the CS (326.5±29.8% vs. 306.9±40.3%, *p*=NS) (Figure 3.2C). Ozone-induced airway hyper-responsiveness (PC_{200}) was also not affected by the drugs in combination and remained elevated compared to the air control group (2.0±0.7% vs. 3.6±1.2%, $p<0.05$). MIF inhibition by ISO-1 did not enhance the anti-inflammatory action of dexamethasone nor restore airway hyper-responsiveness to air controls levels (Figure 3.2D).

MIF release induced by ozone-exposure was significantly suppressed by pre-treatment with ISO-1 and dexamethasone in combination (7.0±1.6ng/ml vs. 4.9±1.4ng/ml, $p<0.05$). However, the effect was not enhanced compared to the actions of ISO-1 alone on ozone-induced BALF MIF levels. Levels of MIF detected in the BALF remained significantly increased in the ISO-1 and dexamethasone pre-treated ozone-exposed group compared to air control (4.9±1.4ng/ml vs. 2.5±0.9ng/ml, $p<0.01$). The ozone-induced BALF levels of KC (108.0±26.6pg/ml vs. 86.0±19.9pg/ml, *p*=NS) and TNFα (236.3±99.5pg/ml vs. 166.3±56.8pg/ml, *p*=NS) were also reduced in animals treated with the combination of ISO-1 and dexamethasone but again this did not reach significance and no additive effect was seen compared to that of the individual drugs on ozone induced mediator release. In summary, I was unable to show that MIF inhibition enhanced the dexamethasone effects in this model (Figure 3.4).

3.4 Discussion

3.4.1 ISO-1 attenuates ozone-induced inflammation

There are various reports on the suppression of inflammation by MIF inhibition, in rodent models of inflammatory diseases. Anti-MIF antibodies in rodents have been shown to be protective against endotoxemia (Calandra et al., 1995), arthritis (Leech et al., 1998) and sepsis (Al-Abed et al., 2005). Mice with genetically deleted MIF also showed less inflammation in atopic dermatitis (Yoshihisa et al., 2011) and endotoxemia (Bozza et al., 1999). Most importantly to this study, reports of MIF inhibition on rodent models of allergen-induced asthma, led us to investigate the role of ISO-1, a small molecule antagonist of MIF, in a COPD inflammatory model.

In previous reports eosinophil-rich inflammatory mouse and rat allergic asthma models that were induced by OVA-challenge showed attenuation of the inflammation by anti-MIF antibodies. This was measured by reduced infiltration of inflammatory cells into BALF and by airway hyper-responsiveness (Amano et al., 2007, Kobayashi et al., 2006). More recent studies have explored the association of MIF in mouse OVA-challenged models of inflammation and used ISO-1 (Chen et al., 2010). In the Chen study, ISO-1 suppressed lung inflammation and airway remodelling, reduced BALF cell numbers, airway smooth muscle hypertrophy, sub-epithelial collagen deposition and TGFβ expression (Chen et al., 2010). In support of this anti-inflammatory action of MIF inhibition, I show here that ISO-1 pre-treatment attenuated ozone-induced inflammation with regards to R_L and BALF cells and BALF MIF, KC and TNFα mediator levels. Nevertheless, the ozone-induced airway hyper-responsiveness was not affected by ISO-1 pre-treatment and the ISO-1-suppressed ozone-induced MIF and KC remained significantly elevated compared to air and thus the inflammation was not fully repressed.

In COPD, the inflammation is characterised as neutrophilic and models administrating low doses of LPS i.p. induce a neutrophil-rich lung inflammation (Hirano, 1996). Anti-MIF antibodies attenuated the number of neutrophils recruited to the rat lung following LPS instillation in a model of lung injury/ARDS (Makita et al., 1998). LPS is a component of cigarette smoke, the major aetiological factor of COPD, which supports the potential relevance of this LPS model to human COPD. Ozone is also recognised as inducing a neutrophilic-rich inflammation

(Triantaphyllopoulos et al., 2011) and ISO-1 significantly suppressed the ozone-induced cellular recruitment to the lung. Although the ISO-1 effect on specific cell types was less obvious, macrophages and lymphocytes were reduced but this did not reach statistical significance and there was no effect of ISO-1 on BALF neutrophilia.

In contrast to these studies, Korsgren and colleagues did not show attenuation of inflammation in OVA-challenged or LPS-treated mice models (Korsgren et al., 2000), and in this current study, the infiltration of neutrophils specifically, were not affected by ISO-1 pre-treatment. There was also a disparity between this current study and that of Chen and colleagues who reported that ISO-1 attenuated lung inflammation; this may reflect the different inflammatory profiles induced by OVA and by ozone as the same strain of mice and dose of ISO-1 pre-treatment was used in both studies. OVA-treatment induces an eosinophil-rich inflammation in contrast to ozone, which induces a neutrophil-rich inflammatory response and therefore ISO-1 may have cell specific effects. It is also noteworthy that the inflammation induced by OVA was twice as strong, with total cells reaching ≈30×10^5 with OVA (Chen et al., 2010) compared to the $12.30\pm1.6\times10^5$ with ozone, which may have concealed the ISO-1 effect.

Korsgren and colleagues used anti-MIF serum compared to Makita and colleagues using anti-MIF antibody (Korsgren et al., 2000, Makita et al., 1998), however the 200µl-injected i.p. may not have been potent enough to suppress the inflammatory response. There may also be a species difference seen as MIF inhibition of LPS-induced neutrophillic inflammation has been demonstrated in rats (Makita et al., 1998). On the other hand there was also no suppression of OVA-induced eosinophil inflammation seen with anti-MIF serum (Korsgren et al., 2000). However, this inflammation was milder than other studies and therefore could have masked the possible anti-inflammatory effects of MIF inhibition.

3.4.2 ISO-1 does not restore dexamethasone sensitivity

CS insensitivity is a major issue when treating COPD and severe asthma. The unique function of MIF counter-regulating the function of CSs (Calandra et al., 1995) has driven research in many CS insensitive inflammatory diseases in an attempt to restore CS sensitivity, to improve disease control, to reduce exacerbations and

disease progression and lower the doses of oral CSs prescribed. Anti-MIF studies have very recently started phase 1 studies for cancer treatment (Identifier NCT01765790) (ClinicalTrials, 2013) and pre-clinical studies are continuing for inflammatory and autoimmune diseases to fully elucidate its role within disease.

To date, there is only one other report investigating ISO-1 and dexamethasone in a lung inflammatory model (Chen et al., 2010). The authors report that the anti-inflammatory effects of ISO-1 are comparable to those of dexamethasone in the OVA-induced murine asthma model; however unlike this current study they do not investigate the effects of the compounds in combination. In contrast, ISO-1 and dexamethasone are non-comparable in my study and ISO-1 pre-treatment results in a more potent anti-inflammatory effect than seen with dexamethasone in the ozone model of COPD. This reflects the fact that the ozone model is relatively CS insensitive, whilst OVA-induced inflammation is exquisitely CS sensitive. Also, the dose of dexamethasone used in my study was 5-fold lower than that used by Chen and colleagues (2mg/kg vs. 10mg/kg) in the OVA-challenge model.

ISO-1 and dexamethasone pre-treatment in combination showed no enhanced anti-inflammatory effects on any of the ozone-induced parameters measured, pulmonary resistance, airway hyper-responsiveness or BALF MIF, KC and TNFα levels. The model remained CS insensitive with MIF inhibition. However, it would be worth investigating if other inhibitors such as 4-IPP, a newer and more potent inhibitor of MIF (Winner et al., 2008) would restore the CS sensitivity in this model.

In conclusion, this ozone-induced CS-insensitive murine model of COPD lung inflammation model was associated with increased BALF MIF levels. R_L, total BALF cell counts and BALF mediator levels were attenuated by pre-treatment with ISO-1. This supports a pro-inflammatory role for MIF in driving lung inflammation in this COPD model. However, ISO-1 pre-treatment had no effect on suppressing ozone-induced neutrophilia and did not reverse the CS insensitivity in the model, indicating that MIF is not the primary driver of CS insensitivity in this COPD model. Further investigation into the role of MIF and its CS counter-regulatory function in CS insensitive COPD will be needed to understand if antagonism of MIF could be therapeutically beneficial.

Chapter 4

The effect of MIF on inflammation

4.1 Introduction

COPD and asthma are both characterised by the infiltration of activated inflammatory cells and the subsequent release of cytokines (TNFα) and chemokines (CXCL8) in the respiratory tract. Despite some similar clinical features the underlying pattern of the chronic inflammation are markedly different between the diseases (Barnes, 2008b).

Most cells and organs constitutively express MIF; within the immune system it is released from monocytes/macrophages, lymphocytes and granulocytes and within the lungs, MIF is predominately found in macrophages and epithelial cells (Calandra and Roger, 2003).

MIF is a pro-inflammatory cytokine involved in innate immunity to drive inflammation (Calandra et al., 1994). MIF has been shown to induce TNFα in human monocytes, mouse macrophages, Raw 264.7 cells and human THP-1 monocytes (Calandra et al., 1995, Senter et al., 2002) and also IL-1β, IL-6, CXCL8 and INF-γ (Calandra et al., 1994, Bacher et al., 1996, Donnelly et al., 1997). MIF has also been shown to be involved in other pro-inflammatory functions such as phagocytosis (Juttner et al., 1998), proliferation (Hudson et al., 1999), MAPK signalling (Mitchell et al., 1999) and activating T lymphocytes (Bacher et al., 1996).

Inhibition of MIF attenuates inflammation in murine inflammatory models *in vivo* (Lubetsky et al., 2002) and *in vitro* in mouse macrophages (Al-Abed et al., 2005). Suppression of inflammation by MIF-inhibition has also been shown in peripheral blood mononuclear cells (PBMCs) from healthy subjects (Lubetsky et al., 2002) and PBMCs from patients with cystic fibrosis (Adamali et al., 2012).

MIF has been associated with the pathogenesis of allergic inflammation in mouse models of asthma (Mizue et al., 2005). Differences between MIF levels of asthmatic patients and controls in BALF, serum and sputum samples have been reported (Rossi et al., 1998, Yamaguchi et al., 2000). Whilst, there are correlations between MIF and severity of disease and differences between disease state seen in other chronic inflammatory diseases including RA (Mikulowska et al., 1997), ARDS (Donnelly et al., 1997) and more recently pulmonary hypertension (Zhang et al., 2012). To date there are no MIF expression profiles published from COPD patients.

Much of the knowledge and the functional role of MIF have resulted from mouse models and in murine cell lines with little information in human cells. Accordingly, I have measured MIF in COPD human samples and investigated the function of MIF in human cell types.

4.2 Chapter hypothesis and aims

Macrophage migration inhibition factor released from monocytes/macrophages drives chronic inflammation. Inhibition of MIF attenuates inflammation and stimulation with MIF enhances inflammation by increasing production of pro-inflammatory cytokines and cellular proliferation through MAPK proteins.

1. Compare MIF concentrations in non-smokers, smoker and COPD patient groups
2. Investigate the pro-inflammatory action of MIF
3. Compare the pharmacological (ISO-1) and biochemical (siRNA knockdown) inhibition of intracellular MIF on inflammatory processes in human primary (healthy, COPD and asthmatic patients) and immortalised cells
4. Investigate MIF signalling pathways

4.3 Results

4.3.1 MIF expression profiles in COPD patient cohorts

Concentrations of MIF expression were measured in sputum supernatants, serum, protein lysates from BAL macrophages and lung biopsies from non-smokers, smokers and COPD patients using ELISA and RT-qPCR. The characteristics of the subjects included in these studies are shown in Tables 4.1-4.4. MIF content in sputum supernatants was increased in patients with COPD compared to healthy non-smokers (p=0.007) however this difference between subject groups was not seen in circulating serum. Intracellular MIF levels in proteins extracted from macrophages isolated from BALF did not change between subject groups and *MIF* gene expression levels also did not differ between groups (Figure 4.1).

MIF was measured in BALF supernatants and was also detected in alveolar macrophages from the peripheral lung tissue of healthy smokers and COPD patients

by immunohistochemistry (work carried out by Dr Gaetano Caramori). Characteristics of the subjects recruited for this study are shown in Table 4.5. There was no difference in MIF levels in BALF supernatants between healthy smokers and COPD patients (Figure 4.2). *MIF* expression levels were not altered in COPD peripheral lung macrophages when compared to smokers with normal lung function (Figure 4.3).

Table 4.1: Patient characteristics for sputum samples and protein lysates from BAL macrophages

GROUP	AGE	SEX (M/F)	*N* NUMBER	PACK YEARS	FEV_1 (% PRED)	FEV_1/FVC RATIO (%)
Non smoker	59(46-71)	9/10	19	N/A	106±4	79±1
Smoker	54(41-66)	8/7	15	32(21-51)	99±3	77±2
COPD	59(44-72)	13/5	18	39(25-57)	65±2	58±2

COPD patients are GOLD stage 2 as diagnosed by the ATS guidelines. Normally distributed data is represented as mean±SD and data not normally distributed is represented as median (25-75 quartiles)

Table 4.2: Patient characteristics for serum samples

GROUP	AGE	SEX (M/F)	*N* NUMBER	PACK YEARS	FEV_1 (% PRED)	FEV_1/FVC RATIO (%)
Non smoker	51±2	8/6	14	N/A	105±4	98±3
Smoker	59±2	14/8	22	28±3	86±3	84±3
COPD	70±2	16/9	25	44±11	63±4	58±2

COPD patients are GOLD stage 2 as diagnosed by the ATS guidelines. Normally distributed data is represented as mean±SD

Table 4.3: Patient characteristics for lung biopsy samples

GROUP	AGE	SEX (M/F)	*N* NUMBER	PACK YEARS	FEV_1 (% PRED)	FEV_1/FVC RATIO (%)
Non-smoker	60.5(58-63)	2/0	2	N/A	107.5(99-116)	103.8(97.1-110.5)
Smoker	50.5±6.4	2/6	8	33.3±4.5	93.8±12.5	93.5±12.3
COPD	56.5±6.2	4/6	10	36.1±9.1	80.7±10.4	78.6±4.9

COPD patients are GOLD stage 2 as diagnosed by the ATS guidelines. Normally distributed data is represented as mean±SD and data not normally distributed is represented as median (25-75 quartiles)

Table 4.4: Patient characteristics for BALF supernatants

GROUP	AGE	*N* NUMBER	PACK YEARS	FEV_1 (% PRED)	FEV_1/FVC RATIO (%)
Smoker	63.9±6.3	12	36.8±8.5	91.6±8.0	81.5±6.5
COPD	66.8±5.5	12	36.5 (27-48.9)	61.2±10.8	60.5 (52.6-63.5)

COPD patients are GOLD stage 1 or 2 as diagnosed by the ATS guidelines. Normally distributed data is represented as mean±SD and data not normally distributed is represented as median (25-75 quartiles)

Table 4.5: Patient characteristics for peripheral lung immunohistochemistry

GROUP	AGE	SEX (M/F)	*N* NUMBER	PACK YEARS	FEV_1 (% PRED)	FEV_1/FVC RATIO (%)
Smoker	74.1± 5.9	13/2	15	40 (30-50)	95.1± 17.0	78.1± 4.7
COPD	71.8± 5.7	15/0	15	38.7± 14.1	74.5± 16.2	59.8± 8.7

COPD patients are GOLD stage 1 or 2 as diagnosed by the ATS guidelines. Normally distributed data is represented as mean±SD and data not normally distributed is represented as median (25-75 quartiles)

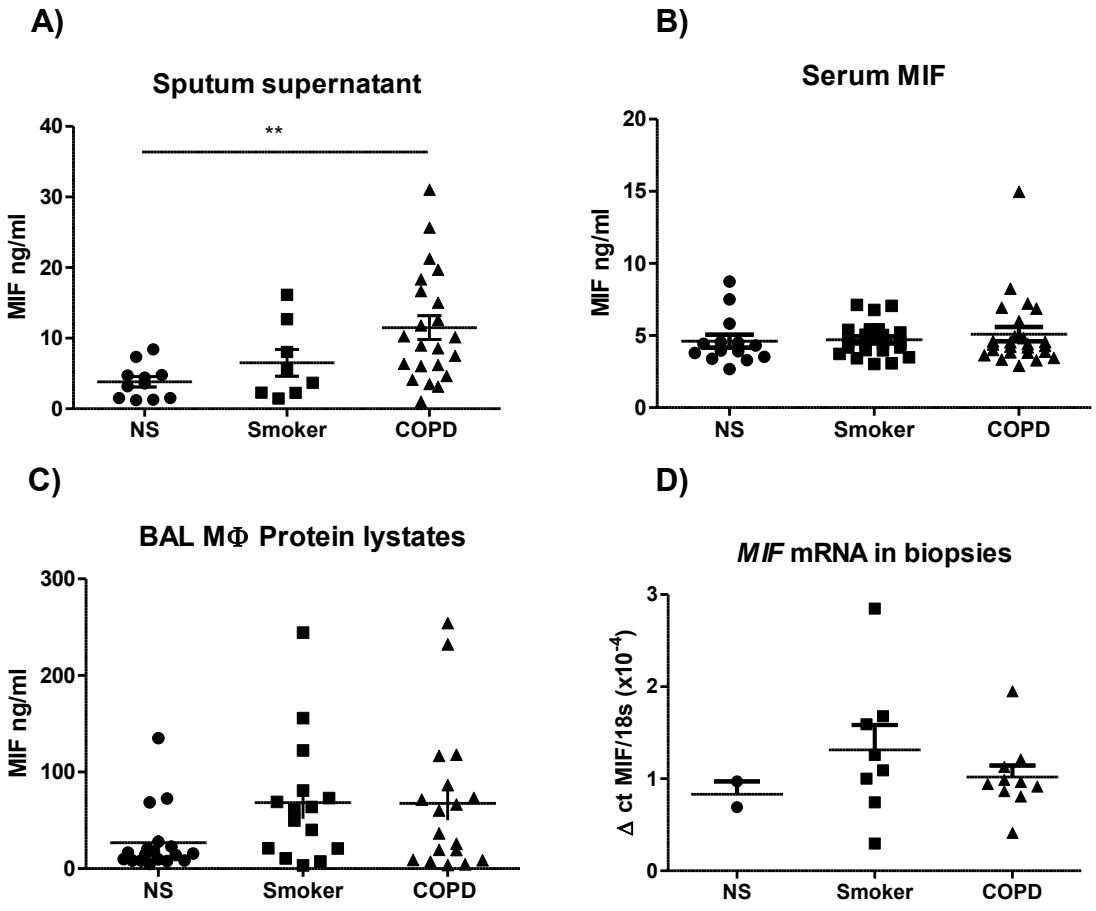


Figure 4.1: MIF expression profiles in human samples from non-smokers, smokers and COPD patients
Concentrations of MIF were measured in various human samples from non-smokers (NS), smokers with normal lung function and patients with COPD. MIF levels detected by ELISA in (A) induced-sputum supernatants, (B) circulating serum, (C) protein lysates extracted from BAL macrophages, (D) *MIF* gene expression measured by RT-qPCR (relative to *18S* mRNA control gene expression) in lung biopsy samples. ***p*<0.01 as calculated by ANOVA with Dunns post-hoc test

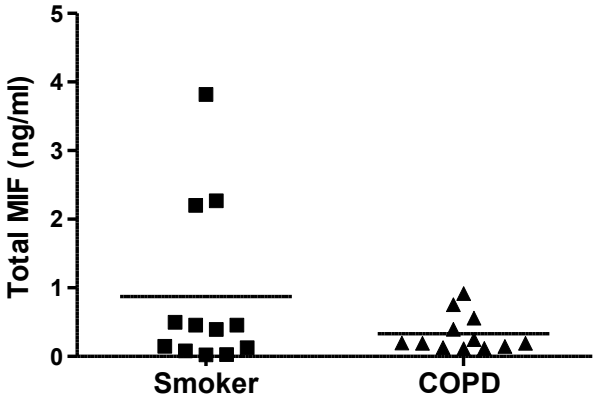


Figure 4.2: MIF expression in BALF supernatants
MIF levels were measured by ELISA, in BALF supernatants from smokers (n=12) with normal lung function and COPD patients (n=12).

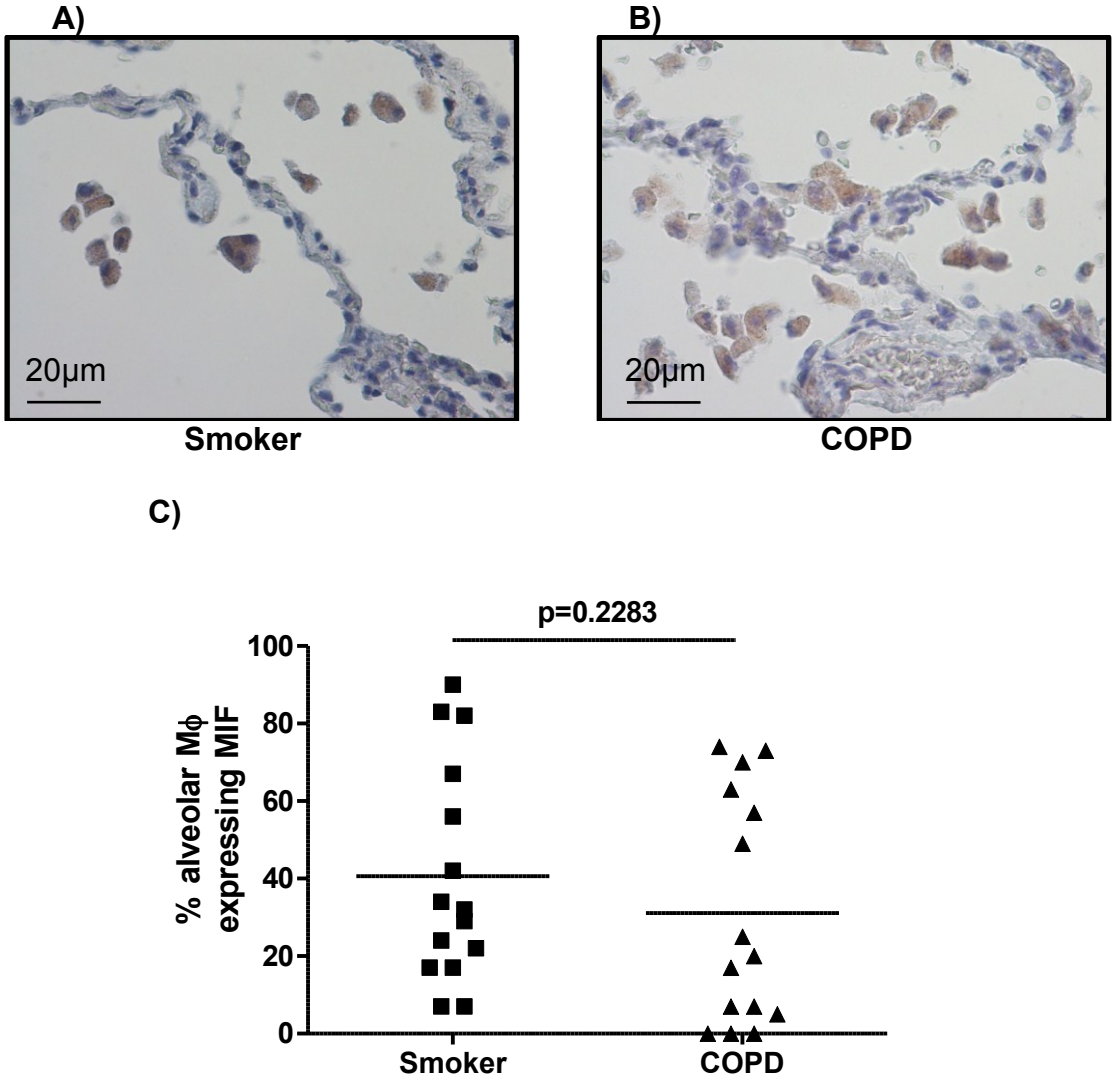


Figure 4.3: MIF expression in alveolar macrophages of peripheral lung from smokers and COPD patients
MIF was detected using immunohistochemistry (magnification x400). MIF expression was detected in alveolar macrophages of peripheral lung tissue sections. Representative images of MIF staining from (A) smokers and (B) COPD patients Haematoxylin and eosin stain was used initially and anti-MIF antibody (brown staining) to highlight MIF protein. (C) Graphical representation of MIF staining in smokers (n=15) and COPD patients (n=15).

4.3.2 MIF regulation by LPS in THP-1 cells

LPS is a pro-inflammatory component of cigarette smoke and was used to model COPD *in vitro*. THP-1 monocytes were stimulated with LPS (1-20µg/ml) for 4 hours before total RNA was extracted and *MIF* gene expression measured by RT-qPCR. LPS significantly inhibited *MIF* expression in a concentration-dependent manner; the maximum suppression was by 71.1±5.6% of naïve with 10µg/ml LPS (Figure 4.4).

A concentration curve of LPS (0.1-20µg/ml) was incubated with THP-1 cells for 24 hours. MIF levels released into the cell-free media were analysed by ELISA. After 24 hours, LPS significantly induced MIF release from THP-1 cells in a concentration-dependent manner to a maximal enhancement of 237.6±23.4% of naïve (Figure 4.5).

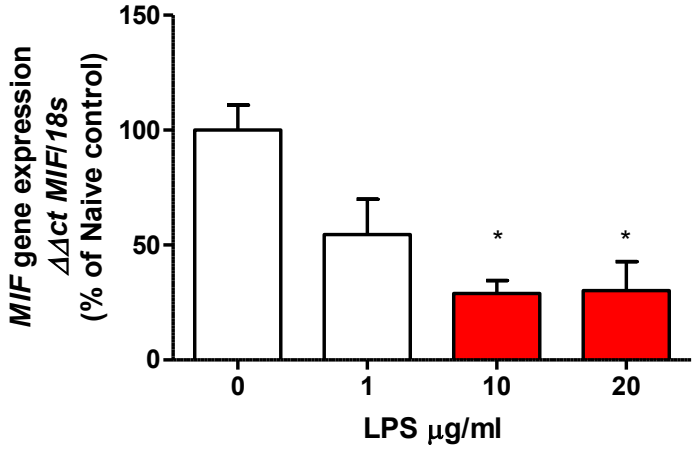


Figure 4.4: LPS stimulation on *MIF* gene expression in THP-1 cells
THP-1 monocytes were stimulated with increasing concentrations of LPS (1-20µg/ml) for 4 hours. Total RNA was extracted, reversed transcribed and analysed for *MIF* expression by RT-qPCR. Data is normalised as a percentage of naïve control and expressed as mean±S.E.M. for four independent experiments. *p<0.05 vs. Naïve control as calculated by ANOVA with Dunns post-hoc test.

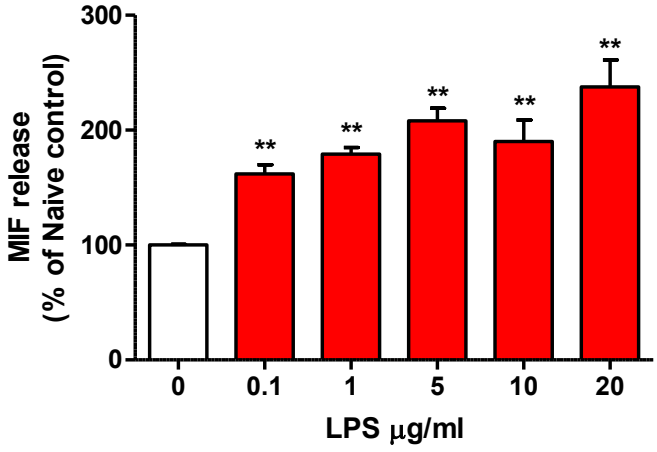


Figure 4.5: LPS-induced MIF release from THP-1 monocytes
THP-1 cells were incubated with LPS (0.1-20µg/ml) for 24 hours. LPS-induced MIF release was measured by ELISA. Data is normalised as a percentage of naïve control and expressed as mean±S.E.M. for four independent experiments. **p<0.01 vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

4.3.3 The pro-inflammatory effects of MIF in THP-1 cells

To investigate the pro-inflammatory function of MIF, I studied the direct effect of human recombinant MIF (rhMIF) on changes in gene expression levels. THP-1 monocytes were treated with increasing concentrations of rhMIF (0.1-1000ng/ml) for 4 hours before total RNA was extracted and RT-qPCR performed to analyse *TNFA*, *IL8* and *MIF* gene expression. Alternatively, in order to measure pro-inflammatory mediator release by ELISA, cells were treated with rhMIF for 1 hour prior to 24 hours LPS stimulation, or left untreated.

After 4 hours treatment there was no significant effect of rhMIF on either *TNFA* (Figure 4.6A) or *IL8* (Figure 4.6B) gene expression; however *MIF* expression levels were significantly inhibited in a concentration-dependent manner to a maximal suppression at 49.5±6.8%, $p<0.001$ of naïve control (Figure 4.6C).

rhMIF-induced CXCL8 and TNFα release in a concentration-dependent manner although this only reached statistical significance at the highest concentration of rhMIF for CXCL8 release (1.7-fold compared to naïve control, $p<0.05$, Figure 4.7A, C). LPS significantly induced the release of both CXCL8 and TNFα release. There was no stimulatory effect seen on LPS-induced CXCL8 (Figure 4.7B). No effect was seen on LPS-induced TNFα by rhMIF either (Figure 4.7D).

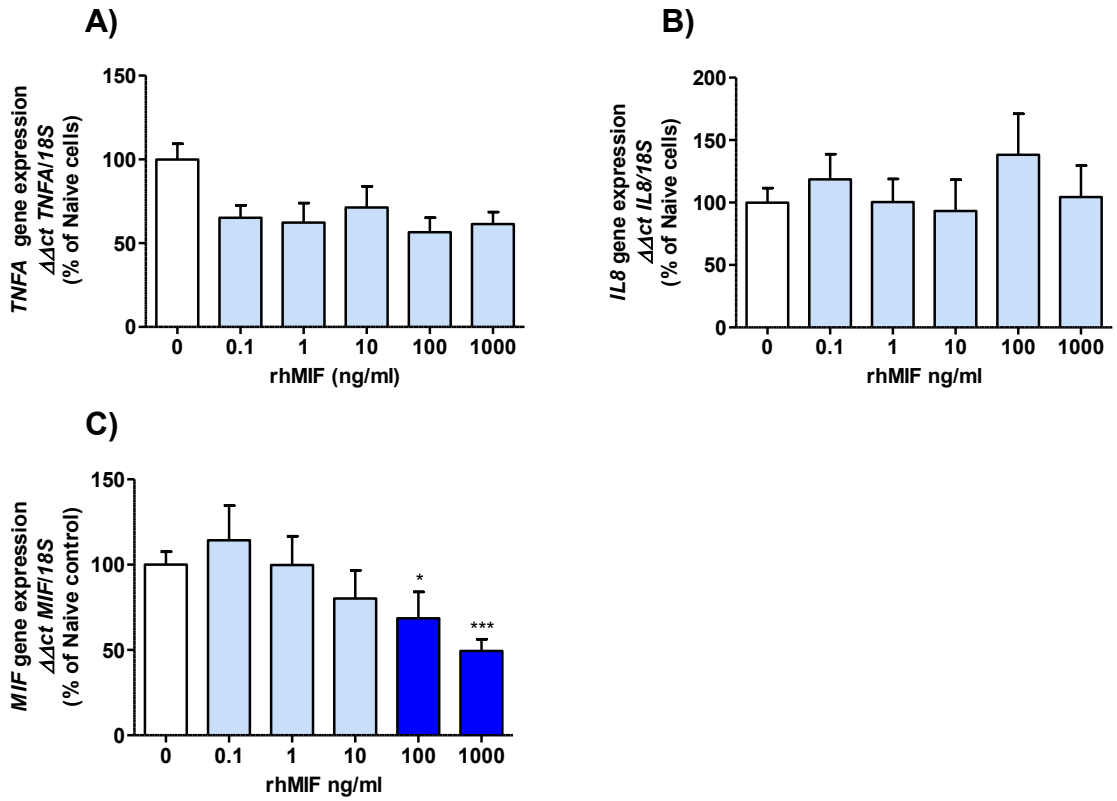


Figure 4.6: rhMIF reduces MIF gene expression levels in THP-1 cells
Cells were incubated with increasing concentrations of rhMIF (0.1-1000ng/ml) for 4 hours. Total RNA was extracted and reverse transcribed for RT-qPCR. Gene expression for *TNFA* (A), *IL8* (B) and *MIF* (C) is normalised to the housekeeping gene *18S* and then represented as a percentage of naïve cells as mean±S.E.M for at least four independent experiments for each gene. * $p<0.05$, *** $p<0.001$ vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

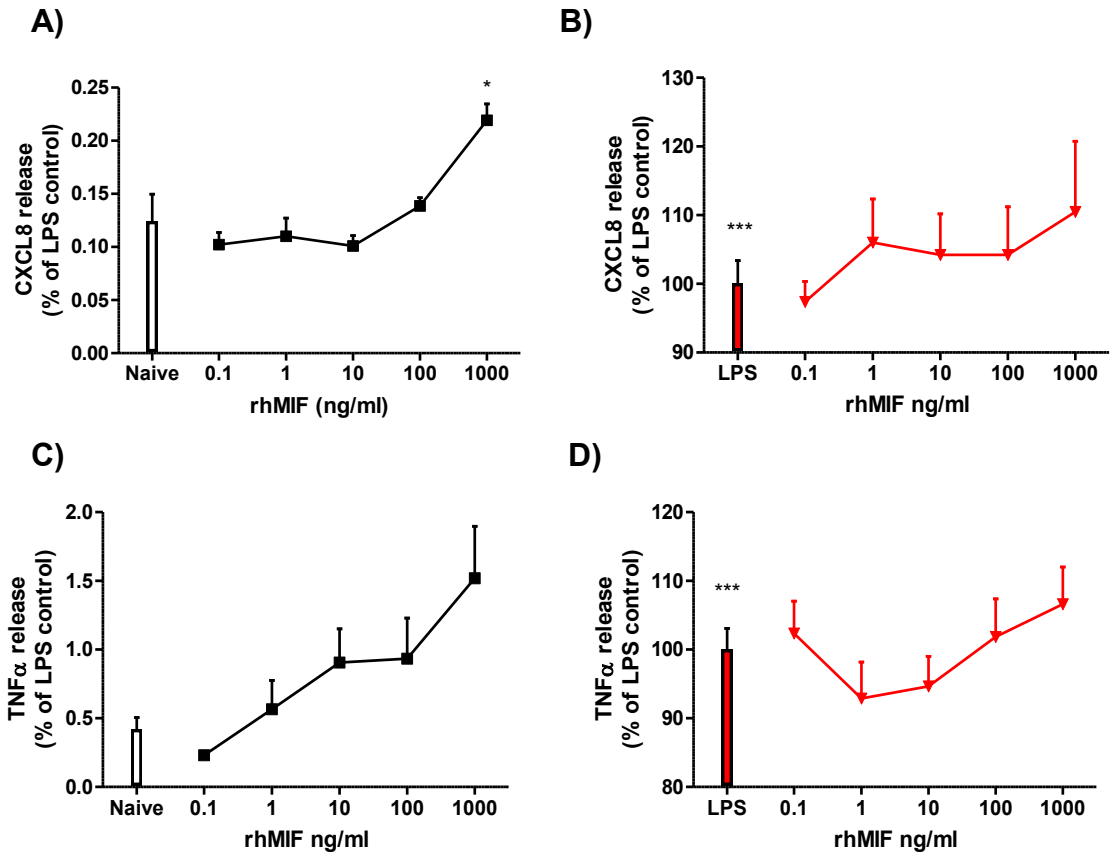


Figure 4.7: MIF activation shows pro-inflammatory mediator release in THP-1 cells
THP-1 monocytes were stimulated with rhMIF (0.1-1000ng/ml) for 1 hour before stimulation with or without LPS (100ng/ml) for 24 hours. Medium was removed and analysed for CXCL8 release (A, B) and TNFα release (C, D). Data is normalised as a percentage of LPS control and expressed as mean±S.E.M. for at least 3 independent experiments. Black line represents no stimulation (media alone) and Red line represents LPS stimulated. *p<0.05 vs. naïve control, ***p<0.001 vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

4.3.4 Inhibition of MIF suppresses pro-inflammatory cytokine release in cells isolated from asthmatic patients

MIF inhibition by ISO-1 has been shown to attenuate inflammation in mouse macrophages (Al-Abed et al., 2005) and also in ozone-induced (Chapter 3) and OVA-challenge mouse inflammation models (Chen et al., 2010). ISO-1-suppressed inflammation has been reported in PBMCs from cystic fibrosis patients (Adamali et al., 2012). I investigated this in LPS-stimulated PBMCs isolated from whole blood taken from non-severe and severe asthmatic patients (Table 4.6). This study was approved by the Ethics Committee of the Royal Brompton & Harefield Hospitals National

Health Service Trust and all patients and healthy volunteers had given informed consent.

Table 4.6 Demographics of asthmatic subjects for PBMCs isolation

GROUP	AGE	SEX (M/F)	*N* NUMBER	FEV_1 (% PRED)	FEV_1/FVC RATIO (%)
Non-Severe	40.0±18.2	2/4	6	77.9±25.1	77.0±9.0
Severe	50.8±13.8	2/7	9	64.5±27.3	62.8±18.3

Normally distributed data is represented as mean±SD and data not normally distributed is represented as median (25-75 quartiles)

4.3.4.1 Treatment of PBMCs from asthmatic patients with ISO-1 was cytotoxic at the highest concentrations

PBMCs isolated from both severe and non-severe asthmatic patients were pre-treated with the MIF inhibitor, ISO-1 (10-500μM) for 30 minutes and then stimulated with LPS (100ng/ml) for 24 hours (Figure 4.8) following this the cell viability was assessed by MTT. The highest concentrations, 200μM and 500μM, caused significant cell death in the severe asthmatic PBMCs, cell viability was reduced by 40.1±6.9%, $p<0.001$ at 200μM and 48.3±5.6%, $p<0.01$ at 500μM (Figure 4.8B). This toxicity was not seen in the non-severe asthmatic PBMCs (Figure 4.8A). The concentrations of ISO-1, that were shown to be cytotoxic for the PBMCs taken from severe asthmatics, were not used in any subsequent experiments.

4.3.4.2 Treatment with ISO-1 inhibits LPS-induced pro-inflammatory cytokine release in PBMCs from asthmatic patients

Pro-inflammatory cytokine release (CXCL8, TNFα and MIF) was measured in the supernatant from PBMCs isolated from both severe and non-severe asthmatics. The cells were pre-treated with ISO-1 (10-100μM) for 30 minutes followed by stimulation with LPS (100ng/ml) for 24 hours (Figure 4.9). CXCL8 and TNFα release were both significantly induced by LPS stimulation in both patient groups. The non-severe asthmatic PBMCs showed no ISO-1 effect on LPS-induced CXCL8 or TNFα release (Figure 4.9A, C). Pre-treatment with ISO-1 inhibited the enhanced cytokine release

in a concentration-dependent manner in PBMCs from the severe asthmatic subjects only. LPS-induced CXCL8 and TNFα release was suppressed to 55.4±11.0% (p<0.001) and 62.1±5.5% (p<0.001) of the LPS control with 50μM of ISO-1 respectively and 36.0±6.3% (p<0.001) and 57.8±9.8% (p<0.001) respectively with 100μM of ISO-1 (Figure 4.9B, D). MIF release from PBMCs was not significantly enhanced by LPS stimulation for either severe or non-severe patient groups. However, LPS-induced MIF release was suppressed by 59.7±7.6%, p<0.001 with 100μM of ISO-1 in non-severe asthmatic PBMCs (Figure 4.9E) and similarly, by 59.4±6.2%, p<0.001 with 100μM of ISO-1 in the severe asthmatic patients (Figure 4.9F).

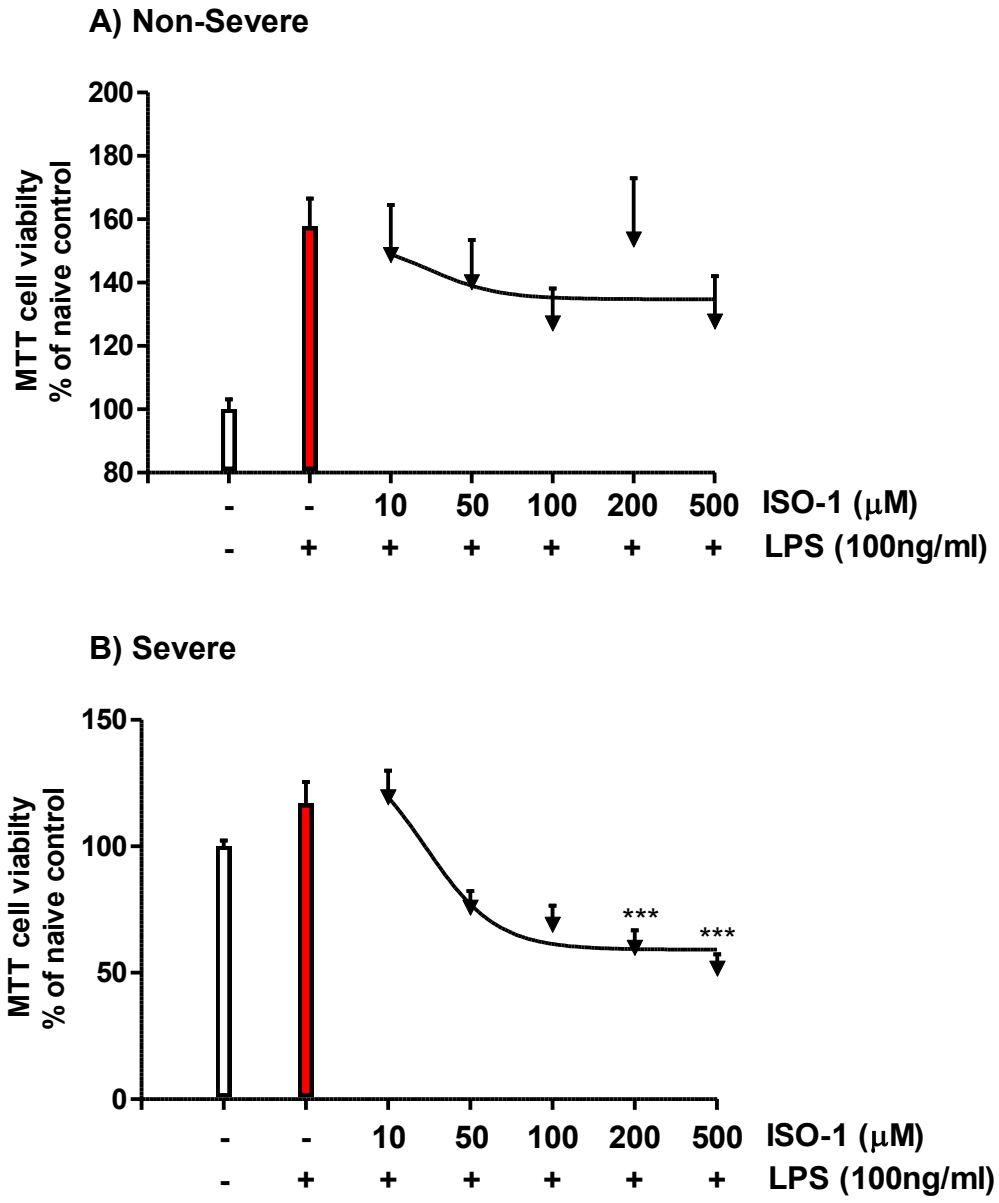


Figure 4.8: The effect of ISO-1 and LPS on non-severe and severe asthmatic PBMC cell viability

PBMCs were isolated from whole blood from non-severe and severe asthmatic patients and pre-treated with ISO-1 at concentrations ranging from 10μM to 500μM before being stimulated with LPS for 24 hours. Cell viability was measured using MTT Assay, (A) non-severe asthmatics (n=4) and (B) severe asthmatics (n=6). Data is normalised as % of naïve untreated cells and represented as mean±S.E.M. for 4-6 independent experiments. ***$p<0.001$ vs. naïve cells as calculated by ANOVA with Dunns post-hoc test.

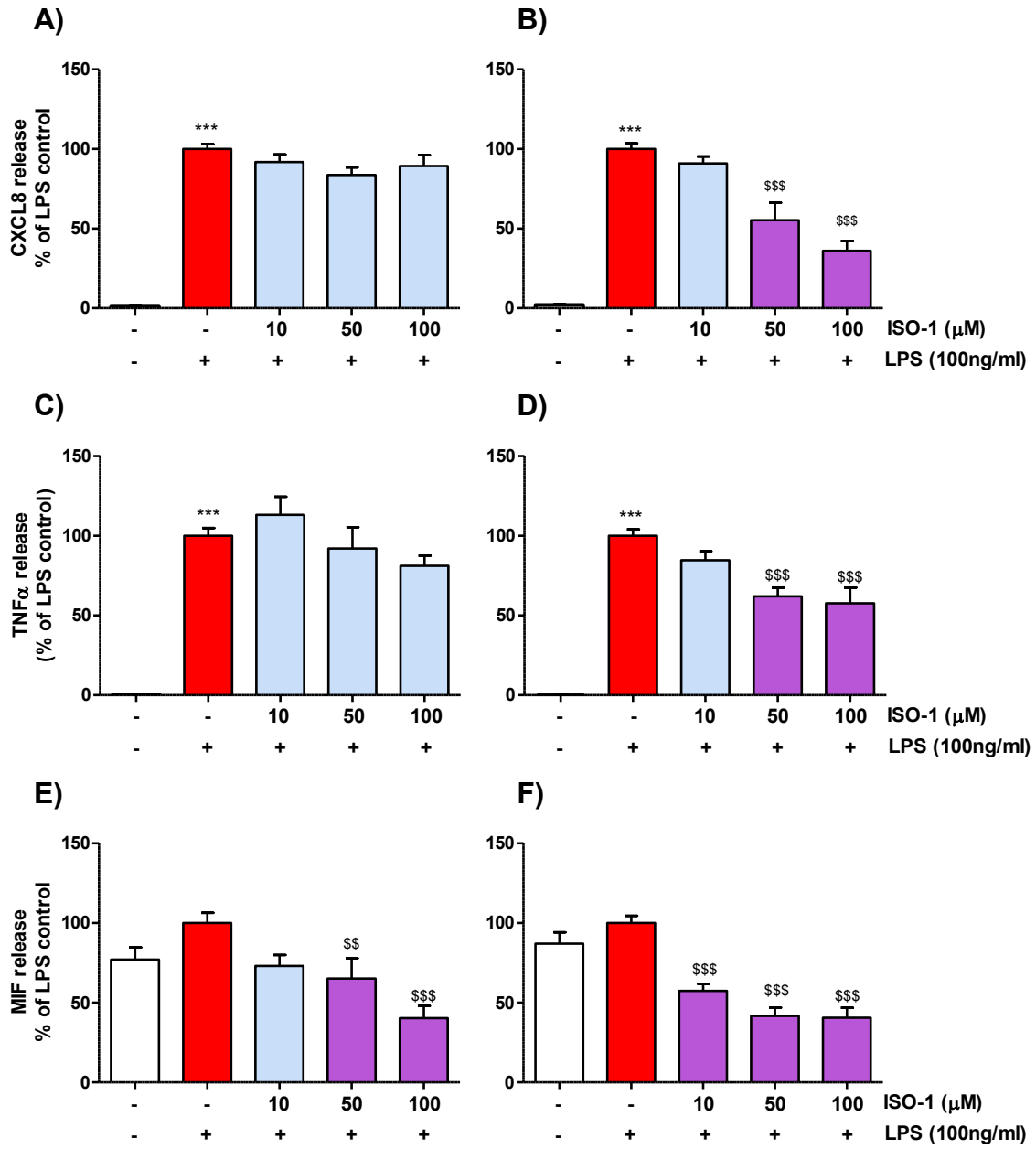


Figure 4.9: The ISO-1 effect on LPS-induced pro-inflammatory mediators in non-severe and severe asthmatic PBMCs

Isolated PBMCs from non-severe (A,C,E) and severe (B,D,F) asthmatics were pre-treated with ISO-1 (10μM-500μM) for 30mins followed by 24 hour LPS stimulation. Pro-inflammatory mediators, CXCL8 (A, B), TNFα (C, D) and MIF (E, F) were measured in supernatants by ELISA. Levels of release are expressed as % of LPS induced and represent mean±S.E.M. of six (non-severe asthmatics) and eight (severe asthmatics) independent experiments. ****p<0.001* vs. naïve control, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs. LPS induced control as calculated by ANOVA with Dunns post-hoc test.

4.3.5 Inhibition of MIF suppresses pro-inflammatory cytokine release in lung tissue macrophages

Lung tissue biopsies were taken and macrophages isolated from non-COPD and COPD patients. Non-COPD patients were cancer patients undergoing lung resection surgery; tissue biopsies were taken away from cancer site.

4.3.5.1 Effect of ISO-1 on lung tissue macrophage cytotoxicity

Tissue macrophages were pre-treated with increasing concentrations of ISO-1 (10-500μM) for 30 minutes followed by a 24hrs LPS (100ng/ml) stimulation. MTT was used to evaluate the cell viability of the macrophages after these treatments in non-COPD (Figure 4.10A) and COPD (Figure 4.10B) macrophages. ISO-1 was less toxic in tissue macrophages compared to PBMCs. The highest concentration of ISO-1 tested (500μM) reduced viability in the non-COPD macrophages by 13.4±7.2% and in the COPD macrophages by 6.3±11.2%. Therefore concentrations up to 500μM were used in subsequent experiments.

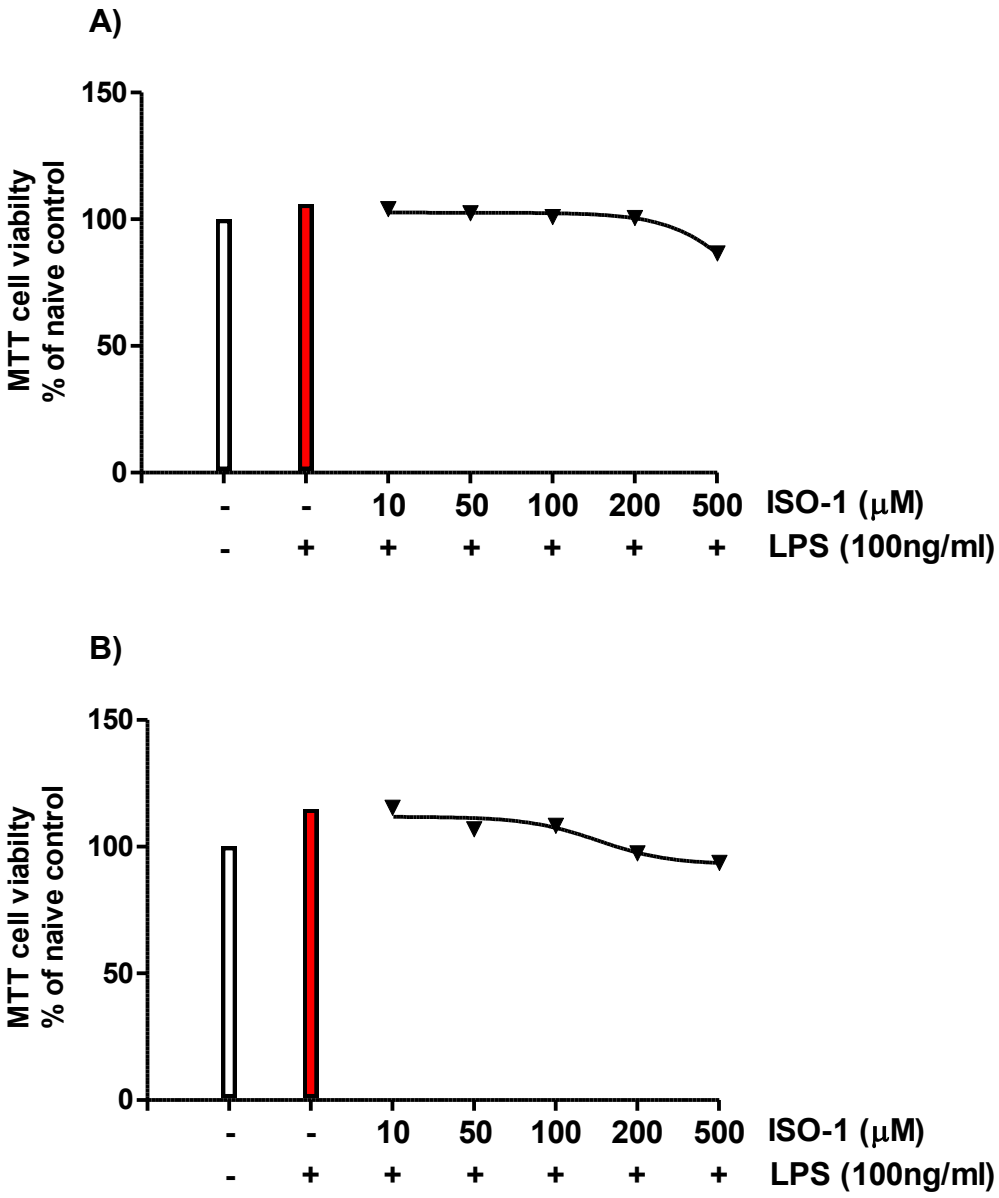


Figure 4.10: Cell viability after ISO-1 pre-treatment for tissue macrophages
'Healthy' non-COPD (A) and COPD (B) lung tissue macrophages were pre-treated with MIF inhibitor, ISO-1 and then stimulated with LPS for 24 hours. Cell viability is represented as % of mean naïve control of 2 independent experiments each.

4.3.5.2 LPS-induced pro-inflammatory mediator release from tissue macrophages is inhibited by ISO-1 pre-treatment

Tissue macrophages isolated from non-COPD and COPD patient lung tissue were pre-treated with ISO-1 for 30 minutes, before being stimulated with LPS for 24 hours. LPS significantly induced CXCL8 in both non-COPD and COPD macrophages. Pre-treatment with ISO-1 inhibited LPS-induced CXCL8 release from COPD macrophages by 30.3%±7.9, $p<0.05$ and 43.4%±8.3, $p<0.01$ at 200μM and 500μM

respectively (Figure 4.11A and B) but ISO-1 had no effect on LPS-induced CXCL8 release from non-COPD macrophages. Similarly, TNFα release was also significantly induced by LPS stimulation in non-COPD and COPD macrophages. However, LPS-induced TNFα release was significantly reduced by ISO-1 at 500μM by 49.7%±5.7, $p<0.001$ (Figure 4.11C) and 47.3%±8.3, $p<0.01$ (Figure 4.11D) in non-COPD and COPD macrophages respectively. LPS had no effect on MIF release from either non-COPD or COPD macrophages, although ISO-1 significantly inhibited LPS-induced MIF release in a concentration-dependent manner for both non-COPD (Figure 4.11E) and COPD (Figure 4.11F) macrophages.

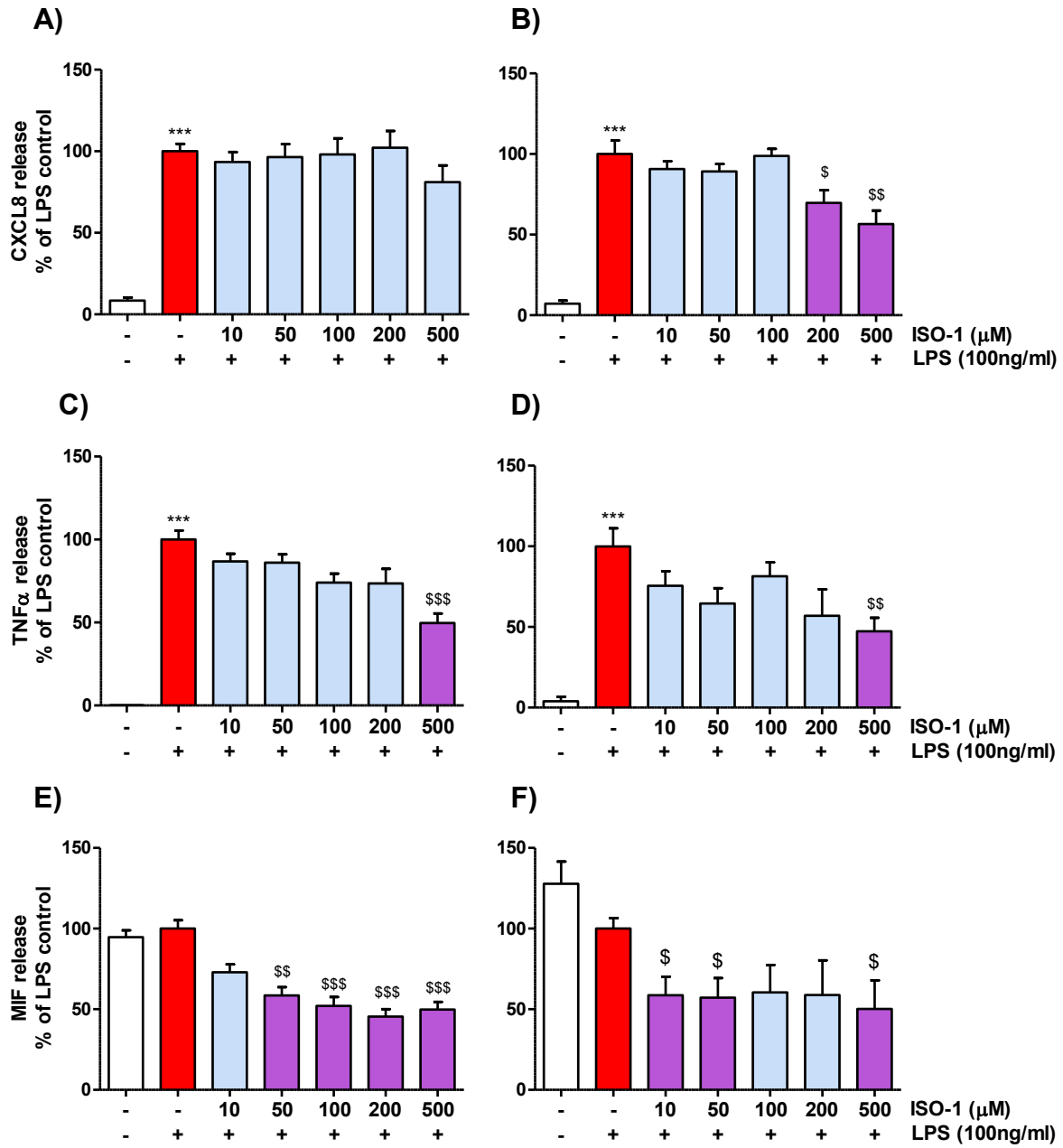


Figure 4.11: Effect of ISO-1 pre-treatment on LPS-induced pro-inflammatory mediators on tissue macrophages from 'healthy' non-COPD and COPD patients

Tissue macrophages from 'healthy' non-COPD and COPD patients were pre-treated with ISO-1 for 30 minutes followed by 24 hours stimulation with LPS (100ng/ml). Supernatants were measured for CXCL8 (A, B), TNFα (C, D) and MIF (E, F) release by ELISA. Levels of cytokine release are expressed as % of LPS-induced control and represent the mean±S.E.M. of four (non-COPD; A, C, E) and three (COPD; B, D, F) independent experiments. ****p<0.001* vs. naïve control, *$p<0.05, $$p<0.01, $$$p<0.001* vs. LPS induced control as calculated by ANOVA with Dunns post-hoc test.

4.3.6 Inhibition of MIF in THP-1 cells

MIF inhibition by ISO-1 reduced LPS-induced inflammatory mediator production in two different primary cell types, PBMCs and lung tissue macrophages, from two inflammatory diseases, asthma and COPD. I went on to investigate the role of MIF inhibition (pharmacological and biochemical) in the LPS-induced inflammation model in THP-1 monocytes as a model of primary cells.

4.3.6.1 Pharmacological inhibition of MIF by ISO-1 in THP-1 cells

4.3.6.1.1 Effect of ISO-1 pre-treatment on THP-1 cell viability

THP-1 cells were pre-treated with the MIF inhibitor, ISO-1, ranging from 10μM to 500μM for 30 minutes and then stimulated with LPS (100ng/ml) for a further 24 hours (Figure 4.12). Cell viability after these conditions was then evaluated using MTT assay. Cell viability was significantly reduced by 82.3±2.7 with 500μM ISO-1 and LPS stimulation; this combination was not used in further experiments.

4.3.6.1.2 Pharmacological inhibition of MIF effects on inflammation

THP-1 cells were pre-treated with ISO-1 for 30 minutes before 24 hours stimulation with LPS (100ng/ml). LPS-induced CXCL8 and TNFα release was analysed using ELISA. The results show that LPS stimulation significantly induced the release of both CXCL8 (Figure 4.13A) and TNFα (Figure 4.13B) by 99.1±1.6% and 84.9±5.8% respectively. Pre-treatment with ISO-1 inhibited LPS-induced CXCL8 at 200μM by 32.7±11.5% but had no effect at lower concentrations. ISO-1 pre-treatment had no effect on LPS-induced TNFα in THP-1 monocytes.

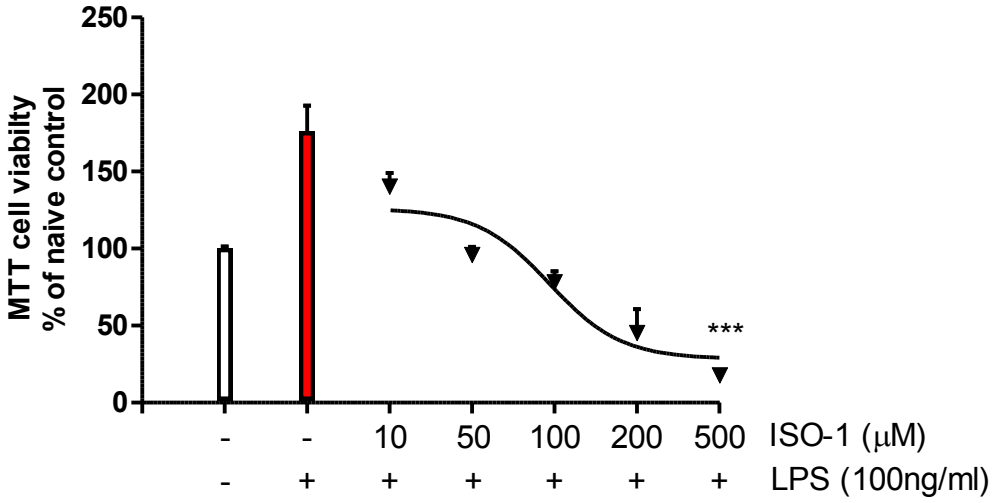


Figure 4.12: Cell viability of THP-1 cells after ISO-1 in combination with LPS treatment
Cells were pre-incubated with ISO-1 (10μM-500μM) for 30 minutes prior to 24 hour stimulation with LPS (100ng/ml). Cell viability was measured by MTT assay and data is represented as % of naïve control as mean±S.E.M. of five independent experiments. ****p<0.001* vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

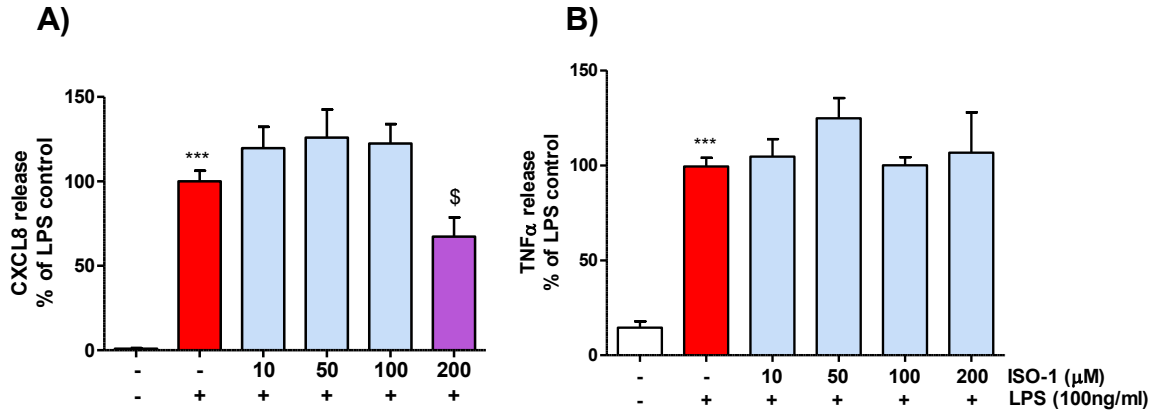


Figure 4.13: ISO-1 reduces the LPS-induced pro-inflammatory mediator release
Monocytes (THP-1) were pre-treated for 30 minutes with ISO-1 (10μM- 200μM) and then stimulated with LPS (100ng/ml) for 24 hours. (A) CXCL8 and (B) TNFα release into the media was analysed by ELISA. The graphs are presented as % of LPS control as mean±S.E.M. for six independent experiments. ****p<0.001* vs. naïve control and $*p<0.05* vs. LPS control as calculated by ANOVA with Dunns post-hoc test.

4.3.6.2 Knockdown of MIF expression by siRNA

In order to confirm the effects of ISO-1 inhibiting MIF and attenuating LPS-induced inflammation I used On-Target MIF siRNA transfection in THP-1 cells.

4.3.6.2.1 Effect of transfection with MIF siRNA on THP-1 cell viability

Electroporation was used to transfer siRNA (200nM) into the nucleus of the THP-1 cells, in the log phase of growth, at a cell density of 0.4×10^6/ml. There were four experimental conditions for these experiments, untransfected (Naïve), transfected with no siRNA (Mock), Scramble siRNA and On-target MIF siRNA. Cell viability was assessed using MTT assay 24 hours (Figure 4.14A), 48 hours (Figure 4.14B) and 72 hours (Figure 4.14C) post-electroporation. Electroporation alone caused significant cell death, around 50%, at all-time points; transfection with scramble and On-target siRNA had no additional effects on this cell viability at any time point.

4.3.6.2.2 Knockdown of MIF gene expression and protein is time dependent

Gene expression of *MIF* and intracellular MIF protein levels were evaluated after transfection as described above by RT-qPCR and Western blotting respectively. Levels of MIF expressed by the THP-1 cells, after the experimental conditions, were normalised to *18S* mRNA for gene expression and to β-actin for protein expression. On-target MIF siRNA significantly reduced *MIF* mRNA gene expression at 24 hours (4-fold) and 48 hours (3-fold) post electroporation compared to scramble control siRNA (Figure 4.15A, C). However, this effect was lost by 72 hours (Figure 4.15E).

On-target siRNA also reduced intracellular MIF protein in a time-dependent manner. MIF protein was significantly reduced at 48 hours (1.75-fold) and 72 hours (1.85-fold) compared to scramble siRNA control post electroporation (Figure 4.15D, E).

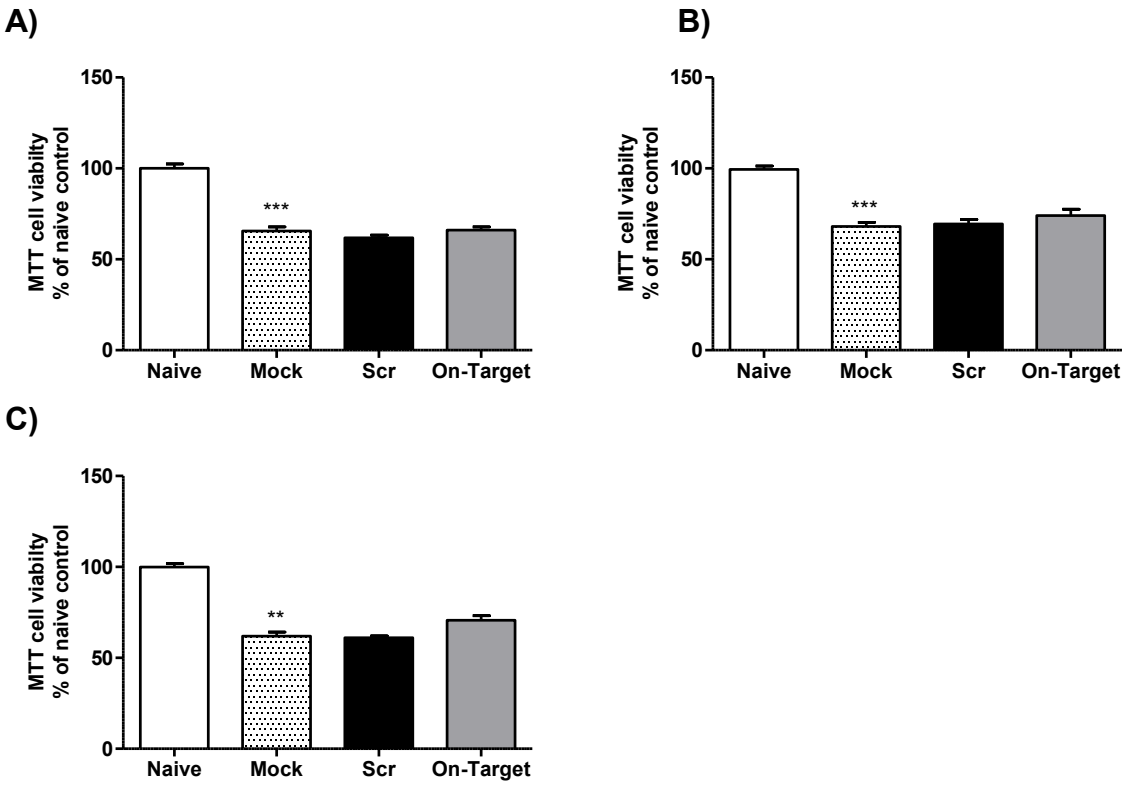


Figure 4.14: Electroporation reduced THP-1 cell viability but siRNA had no additive impact on cell death

THP-1 cells were electroporated without siRNA (Mock), with scramble siRNA (Scr) and On-target MIF siRNA or left untransfected (naïve) and then incubated for (A) 24, (B) 48 and (C) 72 hours. The cell viability post transfection was analysed using MTT assay. Data is represented as mean±S.E.M. as a percentage of naïve (untransfected control) of at least four independent experiments. ** $p<0.01$ vs. naïve control, *** $p<0.001$ vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

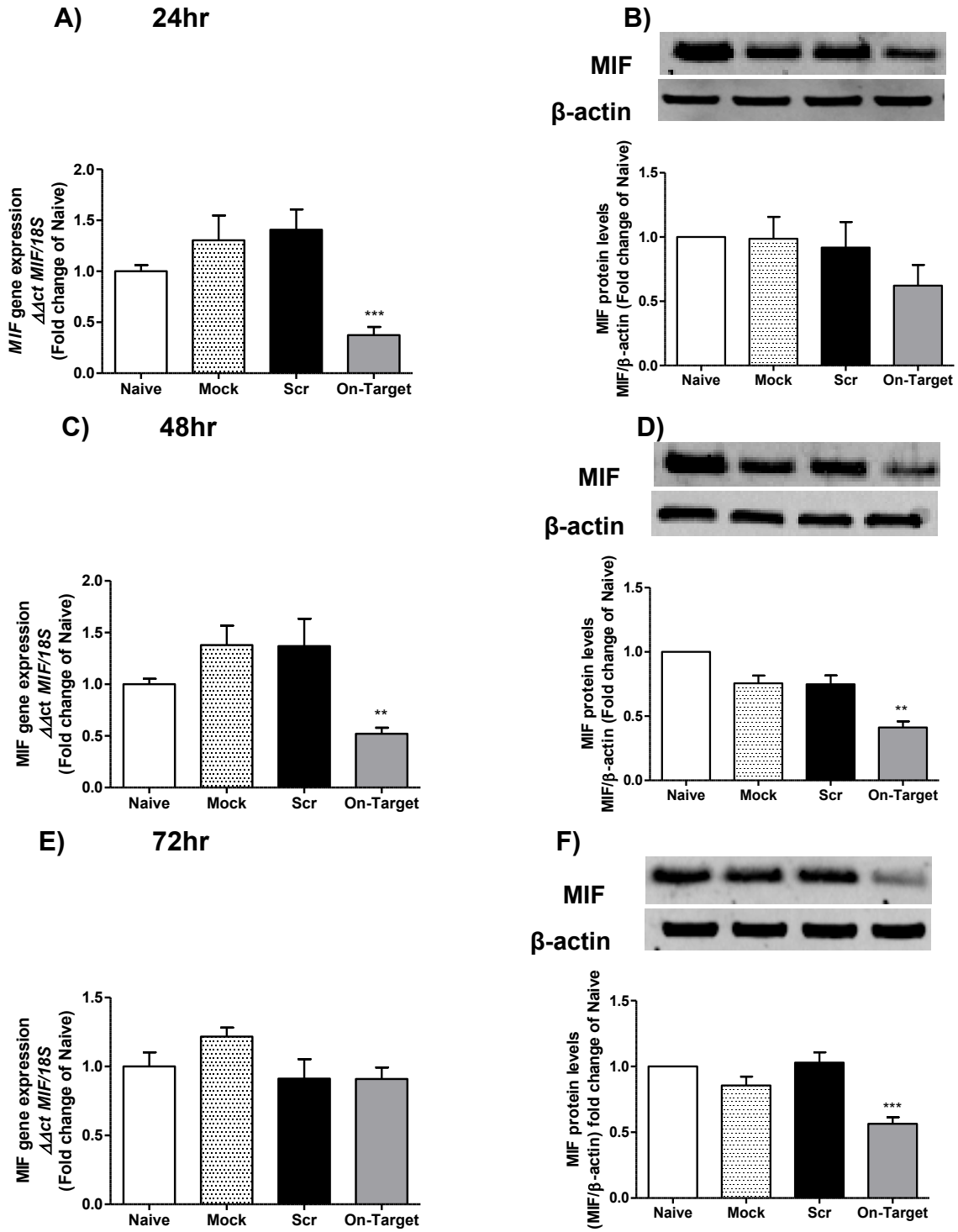


Figure 4.15: Gene expression and protein levels of MIF after siRNA transfection
THP-1 cells either naïve or electroporated alone (Mock), with scramble siRNA (Scr) or On-target MIF siRNA were incubated for 24 hour (A, B), 48 hours (C, D) and 72 hours (E, F). Knockdown of MIF was analysed at gene expression level using RT-qPCR (A, C, E) or by measuring intracellular MIF protein by Western blot (B, D, F). Data is presented as mean±S.E.M. as a fold change of naïve of at least four independent experiments for gene expression analysis and seven independent experiments for protein analysis. Western blot images are representative of one independent experiment for each time point. ***p<0.01* vs. scramble control ****p<0.001* vs. scramble control siRNA as calculated by ANOVA with Dunns post-hoc test.

4.3.6.2.3 Genetic inhibition of MIF on LPS-induced inflammation

At each time point, 24, 48 and 72 hour post electroporation, THP-1 cells were incubated in 1% FBS RPMI media for 3 hours and then stimulated with LPS (1µg/ml) for 24 hours. LPS-induced CXCL8 and TNFα release were not affected by any treatments and the time points tested (Figure 4.16).

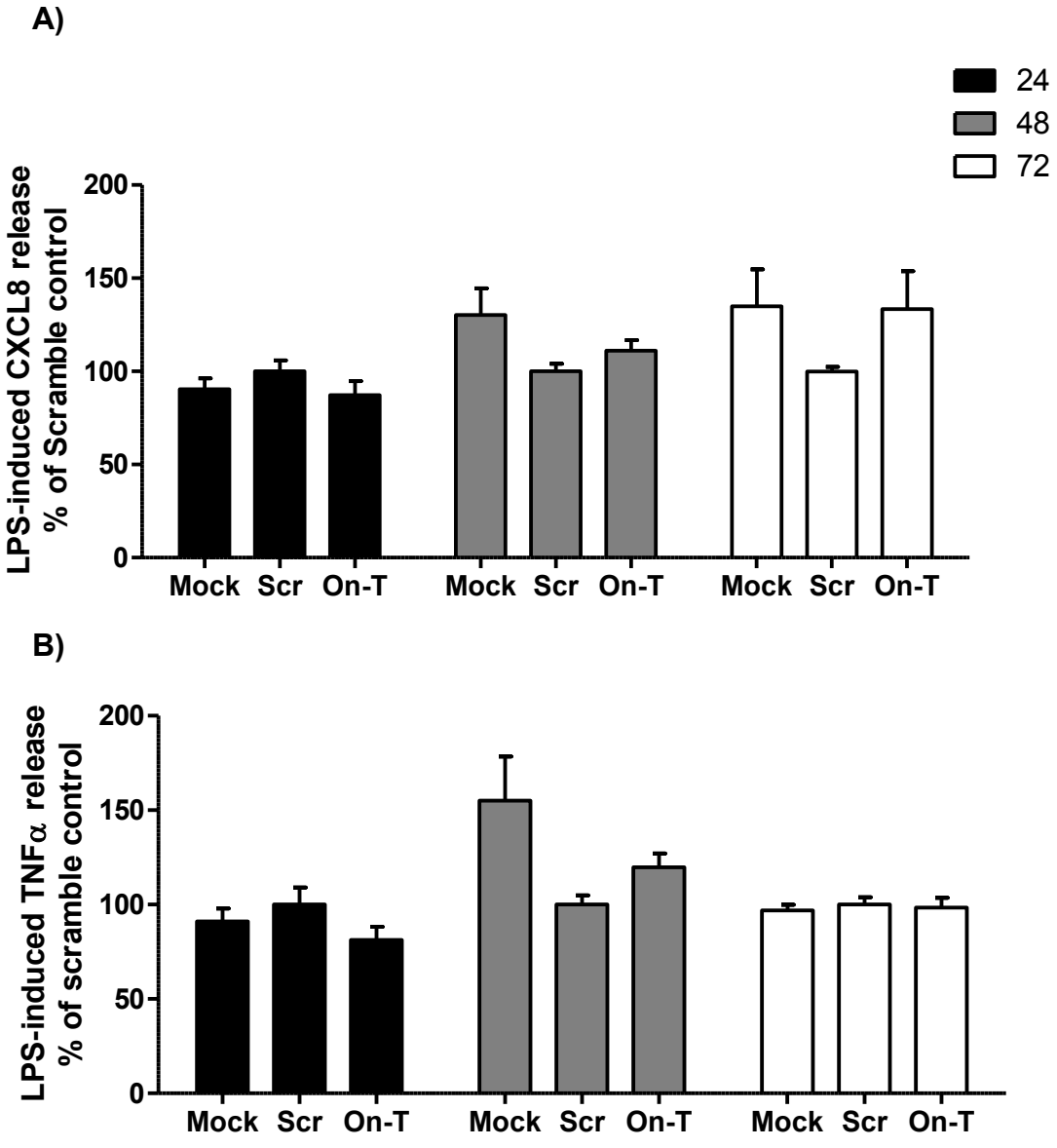


Figure 4.16: Reduced intracellular MIF has no effect on LPS-induced inflammation
THP-1 cells were electroporated alone (Mock), with scramble siRNA (Scr) or On-target MIF siRNA (On-T) and incubated for 24 hours (black bars), 48 hours (grey bars) and 72 hours (white bars), following this the cells were stimulated with LPS (1µg/ml) and incubated for a further 24 hours. LPS-induced CXCL8 (A) and TNFα (B) secreted was analysed by ELISA. Data is normalised as mean±S.E.M. as a percentage of scramble siRNA control for each time point. Each condition at each time point is representative of three independent experiments.

4.3.7 MIF signalling

4.3.7.1 CD74 activation

Inhibition of MIF, in human primary cells and THP-1 cell lines, by pharmacological or genetical methods gave incompatible results in THP-1 cells. MIF is thought to signal via binding to the membrane bound CD74 receptor (Leng et al., 2003). I proceeded to elucidate the pro-inflammatory signalling of MIF in THP-1 cells with anti-CD74 (C-16), an agonist for the putative MIF receptor. THP-1 monocytes were treated with a concentration range of anti-CD74 (0.1-5μg/ml) for 4 hours before total RNA was extracted and RT-qPCR performed to analyse *TNFA*, *IL8* and *MIF* gene expression. For pro-inflammatory mediator release measured by ELISA, cells were treated with the agonist for 1 hour prior to 24 hours LPS stimulation.

In contrast to Section 4.3.3, activation of the putative MIF receptor, CD74, resulted in different gene expression profiles compared to stimulating with rhMIF. *TNFA* gene expression was not affected by CD74 activation (Figure 4.17A) and *IL8* and *MIF* gene expression increased in a concentration-dependent manner however did not reach statistical significance (Figure 4.17B, D).

Activation of CD74 on pro-inflammatory mediator release evoked a similar response to that seen with rhMIF stimulation. The CD74 agonist directly enhanced the release of CXCL8 3-fold at the highest concentration tested (5μg/ml, $p<0.01$) (Figure 4.18A). There was no effect on baseline TNFα release with CD74 activation (Figure 4.18C). LPS significantly induced the release of both CXCL8 and TNFα release. Furthermore, the CD74 agonist did not significantly enhance LPS-induced CXCL8 or TNFα release (Figure 4.18B, D).

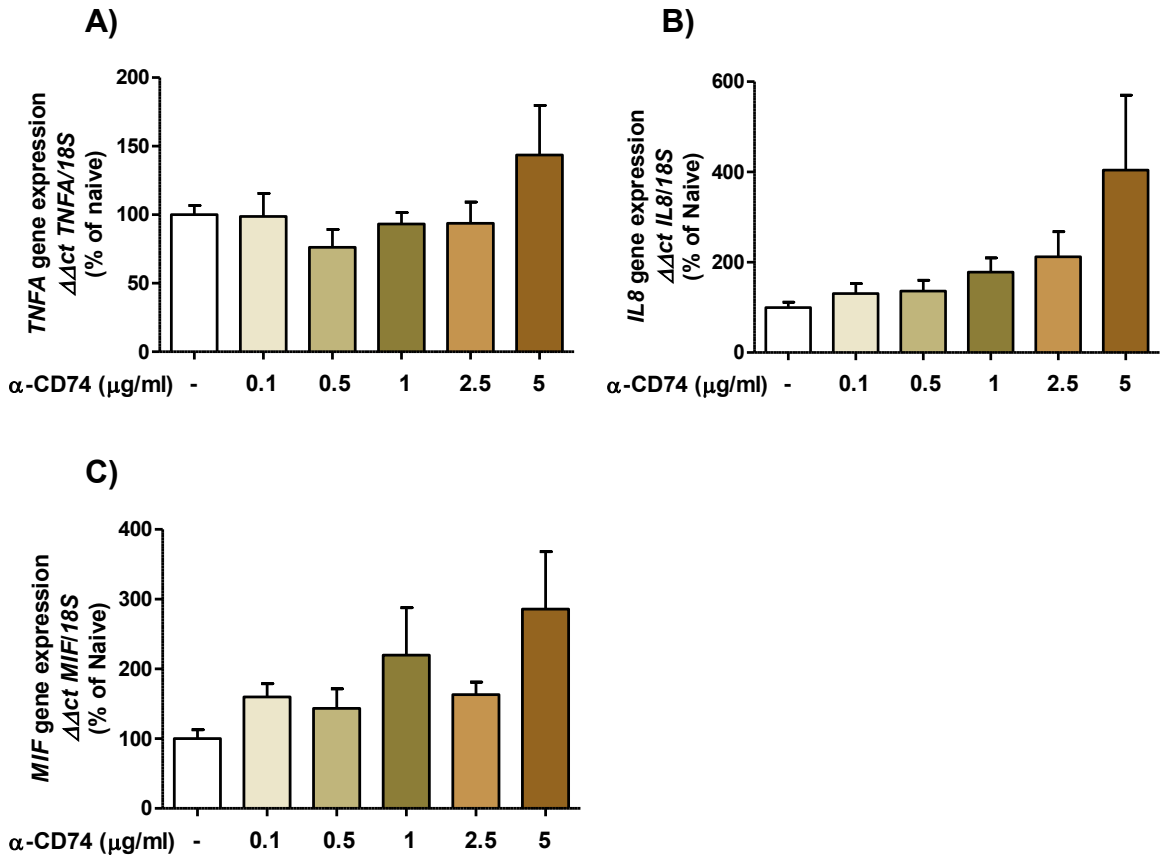


Figure 4.17: Activation of CD74 receptor affects gene expression levels in THP-1 cells
Cells were incubated with a range of concentrations of CD74 agonist (0.1μg/ml to 5μg/ml) for 4 hours. Total RNA was extracted and gene expression was quantified by RT-qPCR. Gene expression for *TNFA* (A), *IL8* (B), *MIF* (C) is normalised to the *18S* mRNA internal control and then represented as a percentage of naïve cells as mean±S.E.M for at least four independent experiments for each gene.

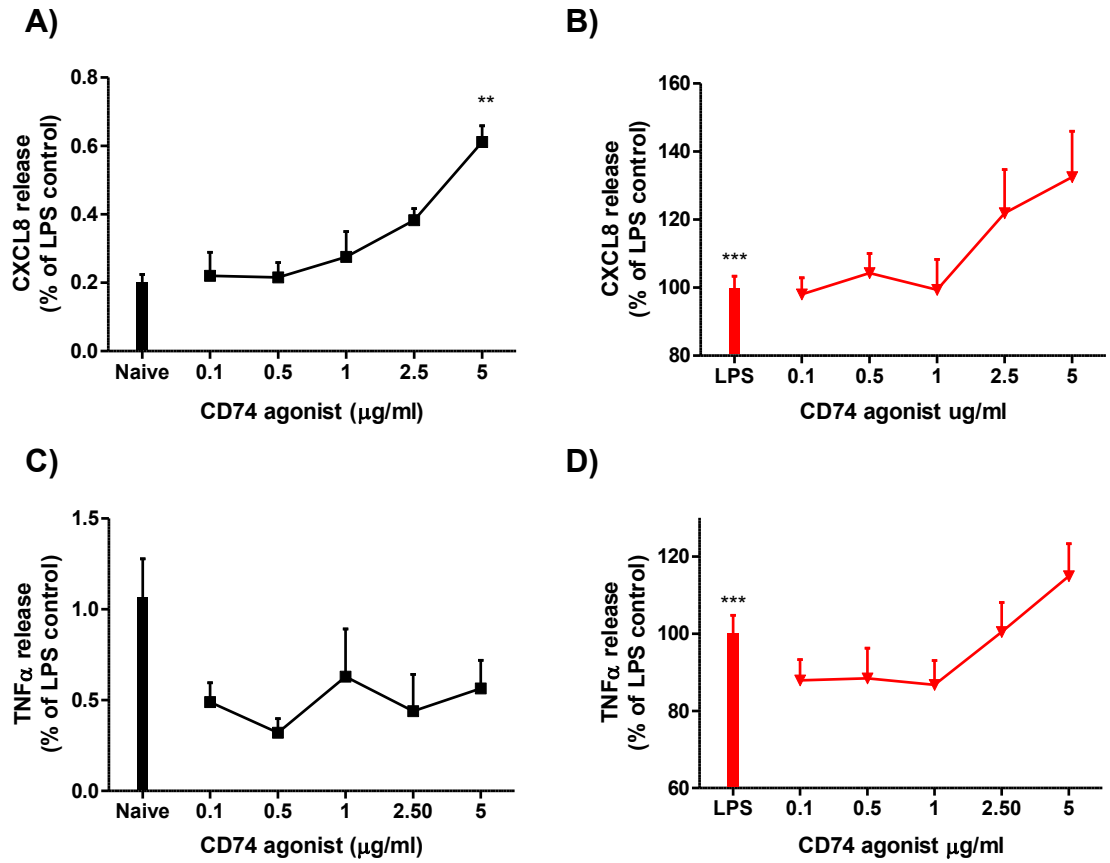


Figure 4.18: CD74 activation shows pro-inflammatory mediator release in THP-1 cells
THP-1 monocytes were stimulated with CD74 agonist (0.1-5µg/ml) for 1 hour before stimulation with or without LPS (100ng/ml) for 24 hours. Medium was removed and analysed for CXCL8 release (A, B) and TNFα release (C, D). Data is normalised as a percentage of LPS control and expressed as mean±S.E.M. for at least 3 independent experiments. Black line represents no stimulation (media alone) and Red line represents LPS stimulated. ***p*<0.01 vs. naïve control, ****p<0.001* vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

4.3.7.2 Mitogen-activated protein kinases (MAPK)

4.3.7.2.1 Transient induction of MAPK by MIF

The ability of rhMIF to directly affect the phosphorylation states of p38, ERK 1/2 and JNK MAPKs was investigated in THP-1 monocytes, MIF signalling through MAPK has been shown in mouse and human fibroblasts cells via ERK1/2 (Lue et al., 2006), p38 (Santos et al., 2004) and JNK (Lue et al., 2011). rhMIF at a concentration of 100ng/ml was incubated with the cells for specified times up to 60 minutes and the phosphorylation of the MAPK proteins were analysed using an ELISA-based PhosphoTracer. Phosphorylation of p38 MAPK on Thr180 and Tyr182, ERK1/2

(p44/42) at Thr202 and Tyr204 and JNK MAPK at Thr183 and Tyr185 phosphorylation sites signify the activation of these kinases. MIF reduced p-p38 and p-JNK MAPK at all time points studied. This effect was significant at 60 minutes for p-p38 MAPK (60.1±8.6%, p<0.01) and at 40 minutes for p-JNK (38.4±11.0%, p<0.05) (Figure 4.19A, B). There was no significant effect detected on the activation of ERK1/2 MAPK at any time point within the hour (Figure 4.19C).

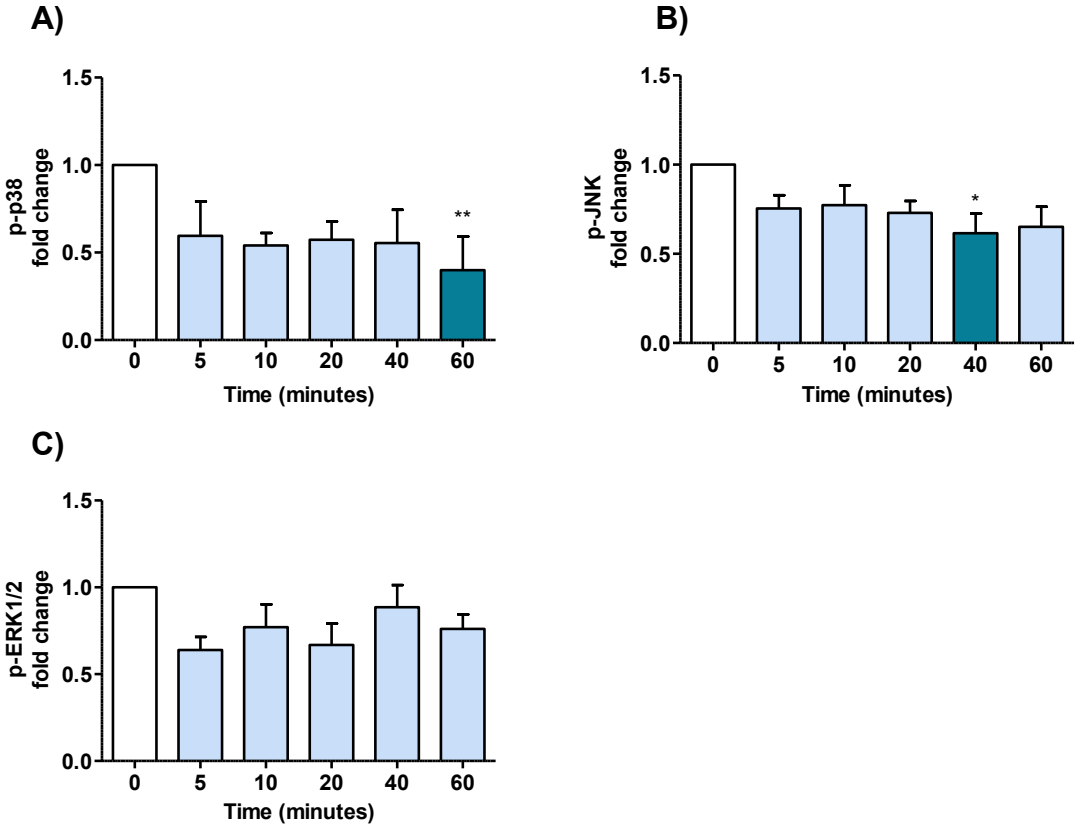


Figure 4.19: rhMIF directly inhibits the baseline activation of p38 and JNK MAPK
Monocyte THP-1 cells were treated with rhMIF (100ng/ml) for specified times over an hour period. Phosphorylation of p38, JNK and ERK1/2, as a marker of activation, was analysed using an ELISA-based PhosphoTracer. Data is represented as fold change of phosphorylation status from 0 minutes from five independent experiments. *p<0.05, **p<0.01 vs. 0 minutes as calculated by ANOVA with Dunns post-hoc test.

4.3.7.2.2 Sustained induction of MAPK by MIF

In order to examine whether rhMIF induced or supressed kinase activity over a sustained period, THP-1 cells were incubated with rhMIF (100ng/ml) for 24 hours. Whole cell lysates were probed for phosphorylated kinase proteins using a kinase array; an increase in phosphorylated kinase is a marker of its activation. The antibodies for the phosphorylated kinases were spotted in duplicate and pixel

densitometry was used to quantify changes in phosphorylation status. The results were normalised as a percentage of naïve control. Various kinases showed an increase in the phosphorylated state after 24hr treatment of rhMIF when compared to the relevant untreated (naïve) control, although none were significantly affected (Figure 4.20).

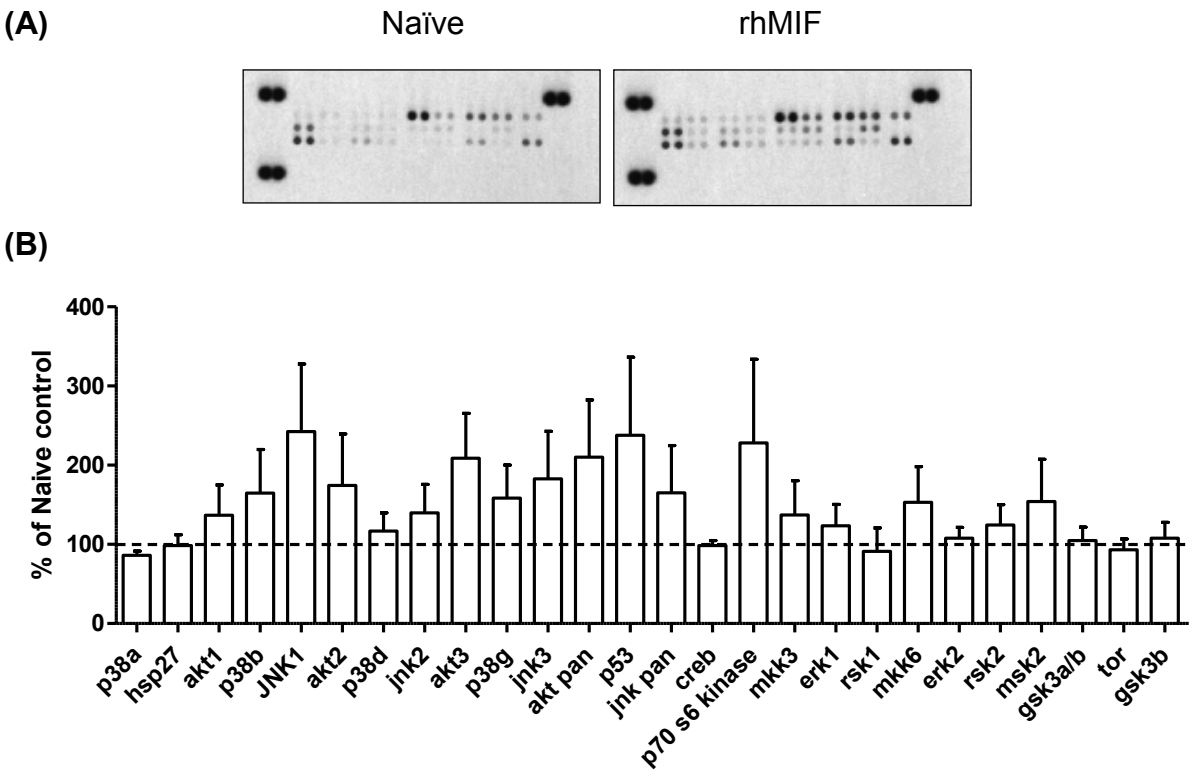


Figure 4.20: rhMIF has no direct sustained effect on MAPKs in THP-1 cells
rhMIF was incubated with THP-1 cells for 24 hours, before whole cell protein lysates were prepared. The lysates were used to identify the activated kinases by presence of the phosphorylated proteins; this is depicted by the dark spots on the kinase array (A). The spots were used for pixel densitometry to graph activation status of the kinases (B). The graph represents the average pixel densitometry signal (duplicate spots) normalised as a percentage of the same kinase on the naïve array (100% dotted line for naïve kinase). Data represents the mean±S.E.M of five independent experiments. Data was non significant as calculated by individual Mann-Whitney t-tests against naïve control.

4.3.7.3 Proliferation

Proliferation of THP-1 cells was measured using a BrdU assay. THP-1 cells (1×10^4 total) were plated into 96-well plates and incubated with increasing concentrations of rhMIF (0.1-1000ng/ml) or with the MIF inhibitor ISO-1 (10-200μM) in 0.5% FBS media for 48 hours. BrdU labelling reagent was added and allowed to incorporate into the DNA for 24 hours before the cells were fixed and analysed. Cell proliferation

was determined by comparing the treatment with BrdU incorporation in THP-1 cells grown in 0.5% FBS. rhMIF enhanced cell proliferation in a concentration-dependent manner, reaching a maximal increase of 123.6±5.3%, $p<0.05$ with 100ng/ml rhMIF compared to naïve control (Figure 4.21A). ISO-1 inhibited cell proliferation in a concentration-dependent manner with a maximal suppression of 90.7±2.1%, $p<0.001$ of naïve control with 200μM ISO-1 (Figure 4.21B).

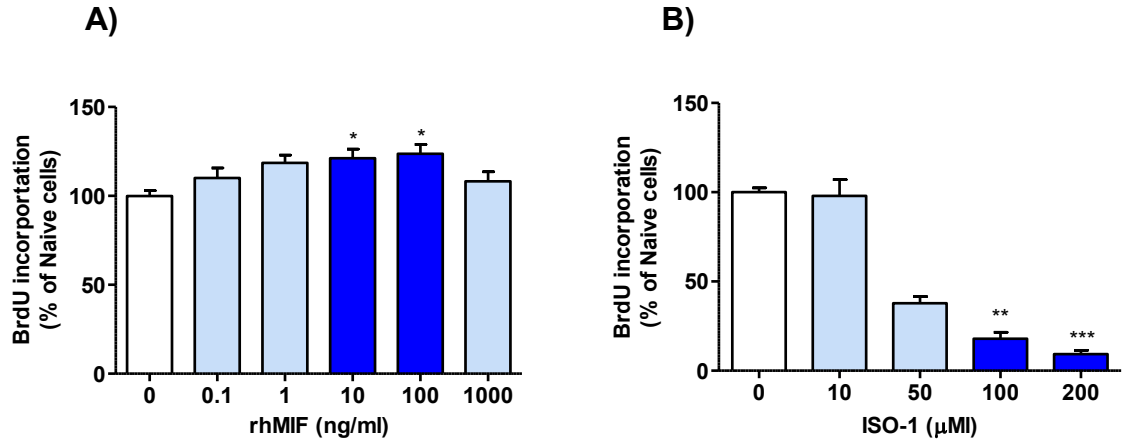


Figure 4.21: MIF is proliferative and essential for maintaining cell growth of THP-1 cells
Cells were incubated with increasing concentrations of rhMIF (0.1-1000ng/ml) (A) or increasing concentrations of ISO-1 (10-200μM) (B) for 48 hours before the cells were incubated with BrdU labelling reagent for 24 hours. All media contained 0.5% FBS as a positive control for normal cell growth. Proliferation of THP-1 cells was analysed using a chemiluminesence BrdU ELISA. Data is represented as a percentage of the untreated naïve cells plotted as mean±S.E.M. of four independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. naïve as calculated by ANOVA with Dunns post-hoc test.

4.3.7.3.1 Proliferation control: p53

The tumour suppressor protein p53 is known to control cell cycle arrest, apoptosis and DNA repair (Alberts et al., 2002a). MIF and p53 were first shown to be associated in 1999 and since then much of the research into this relationship has been in animal models and cell systems (Hudson et al., 1999, Mitchell et al., 2002, Jung et al., 2008). To further validate these findings I investigated MIF's inhibitory effects on p53 in human cells, THP-1. Monocytes were treated with rhMIF (0.1-1000ng/ml) for 4 hours, total RNA was then extracted and the gene expression of the p53 protein, *TP53* was determined. The expression of *TP53* mRNA was inhibited by rhMIF in a concentration-dependent manner at this time point; *TP53* gene

expression was inhibited to 55.8±8.8%, $p<0.05$ and 50.3±6.6%, $p<0.01$ of the untreated control with 100ng/ml and 1000ng/ml respectively (Figure 4.22).

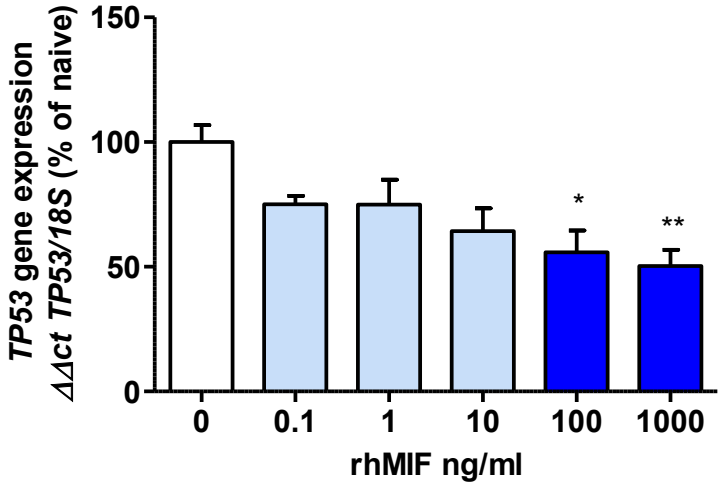


Figure 4.22: *TP53* gene expression is inhibited by rhMIF
THP-1 cells were incubated with a range of rhMIF (0.1ng/ml to 1000ng/ml) for 4 hours. Total RNA was extracted and 0.5µg RNA was reverse transcribed before qPCR was performed. Data is normalised to the internal control *18S* gene expression and then represented as a percentage of the naïve control as mean±S.E.M. for four independent experiments. **p<0.05, **p<0.01* vs. naïve control as calculated by ANOVA with Dunns post-hoc test..

In addition to measuring *TP53* gene expression levels I examined whether MIF directly affected p53 activity. THP-1 cells were treated with rhMIF (100ng/ml) for a time course up to 24 hours. Nuclear proteins were extracted and p53 DNA binding capacity measured by TransAM®. rhMIF had no effect on the binding capacity of p53 to its promoter region (Figure 4.23).

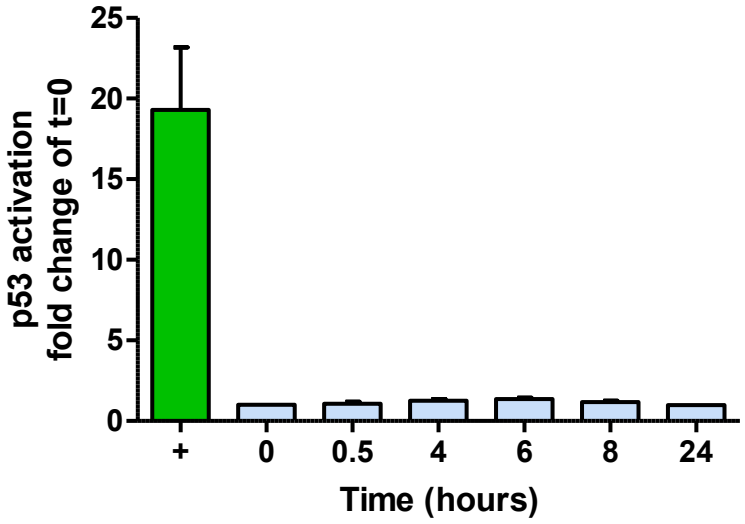


Figure 4.23: p53 binding capacity to the response element over time
THP-1 cells were incubated with 100ng/ml rhMIF for specified times during 24 hour period. Nuclear proteins were isolated and the amount of p53 translocated to the nucleus and able to bind the response element was measured, positive control (+; green bar) was included in kit (H_2O_2 -treated MCF-7 cells). Data is represented as fold change compared to 0 hours (mean±S.E.M.) for four independent experiments.

4.3.7.3.2 Proliferation control: MAPK

Proliferation and cell cycle control is not only modulated through p53 but also p38 MAPK. Activity of p38 may exert both stimulatory and inhibitory effects at certain stages of the cell cycle (Ono and Han, 2000). Therefore the proliferative action of rhMIF was further investigated by the addition of a p38 MAPK inhibitor. The experimental design was the same as before, however the THP-1 cells were pre-treated with a p38 inhibitor (VX-745) (0.1nM-10μM) for 1 hour before being stimulated with rhMIF at a concentration of 100ng/ml. As previously shown, rhMIF enhanced proliferation of THP-1 cells by ≈20% and pre-treatment with VX-745 shows a concentration dependent inhibition of this rhMIF-induced proliferation. The inhibitor, at concentrations greater than 10nM, suppressed proliferation to basal levels and the highest concentration (10μM) suppressed growth that THP-1 proliferation was less than the naïve control (77.0±7.1% of naïve control, p<0.001) (Figure 4.24).

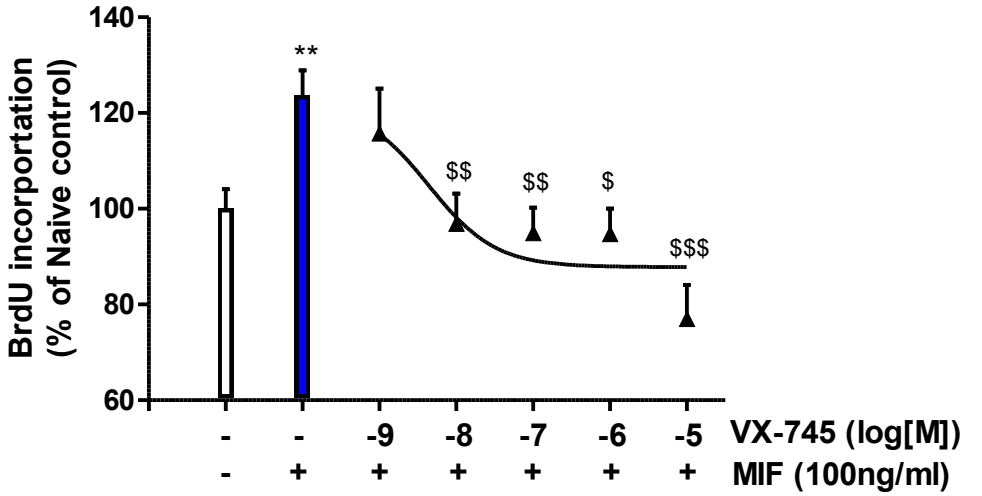


Figure 4.24: MIF-induced proliferation is suppressed with VX-745, a p38 inhibitor
THP-1 cells were pre-treated with increasing concentrations of VX-745 (10^{-9}-10^{-5}M), before being stimulated with 100ng/ml rhMIF for 48 hours. BrdU labelling reagent was added for 24 hours. The amount of BrdU incorporated into the DNA on the cells was measured by ELISA. Data is displayed as a percentage of naïve cells as mean±S.E.M for four independent experiments. $^{**}p<0.01$ vs. naïve control, $^{\$}p<0.05$, $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ vs. LPS control as calculated by ANOVA with Dunns post-hoc test.

4.4 Discussion

4.4.1 MIF expression profile in COPD

In contrast to other chronic inflammatory diseases and the correlation between serum MIF and disease severity such as asthma (Rossi et al., 1998), RA (Morand et al., 2002) and atherosclerosis/cardiovascular disease (Burger-Kentischer et al., 2002), systemic MIF measured in blood serum showed no difference between the COPD and control patient groups tested. It is also worthy to note that serum MIF is present in relatively high concentration in normal individuals (0.1-100ng/ml) (Lubetsky et al., 2002, Kudrin and Ray, 2008), so large *n* numbers would be required for statistical power to detect small differences in expression.

MIF detected in sputum supernatants was increased in COPD patients compared to healthy non-smokers. The levels were non-significantly increased compared to smokers, suggesting that smoking may affect MIF levels released from cells in sputum. This smoke-induced MIF increased is also seen in protein lysates from BAL macrophages, however there was no further enhancement with COPD compared to

smokers. Correspondingly, there was also no significant difference in the MIF released from smokers and COPD cells into the BALF supernatant but there was no healthy non-smoking group to investigate the effect of smoking on MIF. From this speculation, further investigation into the induction of MIF by LPS, a cigarette smoke component, was completed in THP-1 monocytes.

MIF protein is stored within the cytoplasm of the cells (Calandra et al., 1994), however, there was no difference in MIF cellular content detected by immunohistochemistry in alveolar macrophages from smokers or COPD patients. Again, there was no healthy non-smoker group to investigate the effect of smoking. There was also no difference seen in the production of MIF at gene expression level between healthy non-smokers, smokers or COPD patients, this supports the lack of difference seen in the storage of intracellular MIF but the patient numbers are small and inconclusive.

Having no healthy non-smoker group in some of the studies investigated has limited the understanding of the MIF profile within COPD patients. Smoking is a significant risk factor in COPD development and therefore could induce or alter MIF function driving the inflammation and disease progression. The effect of smoking on MIF in COPD should be further investigated.

It is also noteworthy that the samples used in this study were collected from stable COPD patients that had relative control of the disease. MIF may play a role during acute inflammation in disease, for example during an exacerbation. There are complications with studying exacerbations as it can be difficult to collect samples from patients, to get comparable timings within the exacerbation and there are also the ethical considerations into patients' well-being.

4.4.2 Pro-inflammatory effects of MIF

MIF signalling is unique among pro-inflammatory cytokines; the molecular mechanisms are complex, broad, cell type specific and have roles established in both innate and adaptive immunity. MIF is understood to be pro-inflammatory although several reports give controversial results and this could be due to LPS contamination in bacterially expressed rhMIF (Kudrin et al., 2006) or cell specific functions.

At the gene expression level, MIF stimulation had no overall effect on baseline *IL8* production, however this did not correspond to protein release as MIF induced the baseline release of CXCL8 in a concentration-dependent manner from THP-1 cells. This is in support of the pro-inflammatory properties known for MIF (Bernhagen et al., 1994). In contrast, MIF was shown to induce *IL8* gene expression and CXCL8 release from human gastric epithelial cells (Beswick et al., 2005) and in chronic lymphocytic leukaemia B-lymphocytes (Binsky et al., 2007).

Similarly, MIF had no significant effects on *TNFA* gene expression although there was a non-significant concentration-dependent trend for MIF to enhance baseline TNFα release from THP-1 cells. This data is not in agreement with reports suggesting that MIF and TNFα exist in a positive feedback loop and that MIF is essential for maximal induction of TNFα in mouse *in vivo* and *in vitro* systems (Calandra et al., 1994). However, my data is in support of results published by Kudrin, who also report the lack of TNFα induction by MIF in THP-1 cells (Kudrin et al., 2006).

The lack of gene production modulation by MIF seen in this study could be a time issue and a range of time points investigated would be needed to establish whether *IL8* and *TNFA* gene expression was induced by MIF in THP-1 cells. The small MIF stimulatory effects were not strong enough to significantly enhance the release of LPS-induced CXCL8 or TNFα.

rhMIF inhibited its own gene expression in a concentration-dependent manner. Interestingly, LPS induced MIF release from THP-1 cells in a concentration-dependent manner; however, LPS also resulted in a concentration-dependent inhibition of MIF gene expression. Suggesting that MIF is involved in a negative feedback loop and it may act in an autocrine fashion in these cells.

MIF does not show potent pro-inflammatory functions in THP-1 cells as MIF-induced CXCL8 and TNFα release is only seen at baseline and not in synergy with LPS and no pro-inflammatory action is seen at gene expression level. The effects of MIF on cellular recruitment and phagocytosis may show more potent pro-inflammatory signals.

4.4.3 Inhibition of MIF on LPS-induced CXCL8 and TNFα release

As previously shown and discussed (Chapter 3), *in vivo* studies have shown the involvement of MIF in inflammation by using MIF knockout mice, anti-MIF antibodies, anti-MIF serum or ISO-1 in different inflammation models (Chen et al., 2010, Korsgren et al., 2000, Makita et al., 1998). Different stimuli are used to model different aspects of disease, for asthma and COPD stimuli such as allergen-challenge (OVA or house dust mite HDM), LPS instillation, smoke inhalation or ozone are used. LPS is a component of cigarette smoke and induces inflammation. To translate this to humans I investigated the effects of ISO-1 on LPS-induced inflammation in human cells, firstly *ex vivo* and then *in vitro* to establish an inflammatory model.

PBMCs isolated from severe asthmatic subjects showed an ISO-1 concentration-dependent inhibition of LPS-induced CXCL8 and TNFα but had no effect on PBMCs from non-severe asthmatics. ISO-1 also had an inhibitory effect on LPS-induced MIF release from non-severe and severe asthmatic PBMCs although LPS alone had no effect on MIF release in either subjects' cells. This attenuation of LPS-induced inflammation by ISO-1 was also seen with regards to lung tissue macrophages. Macrophages isolated from both, non-COPD and COPD subjects showed a concentration-dependent inhibition by ISO-1 on LPS-induced TNFα release, however the ISO-1 inhibition was only seen in COPD patient lung macrophages for LPS-induced CXCL8 release. This data is in support of current findings; that ISO-1 pre-treatment shows similar attenuation of inflammation with regards to LPS-induced TNFα in PBMCs isolated from cystic fibrosis patients (Adamali et al., 2012).

Overall this reduction in inflammation was more pronounced in the diseased cells, either severe asthmatic PBMCs or COPD macrophages compared to the controls. This difference supports the hypothesis that MIF is involved in the chronic inflammation, although with no clear difference in expression levels, MIF function or activity may be altered giving the distinct responses.

In order to further examine this function of MIF on inflammation the immortalised monocyte THP-1 cell line was used. THP-1 cells did not give comparable results to the lung tissue macrophages and asthmatic PBMCs and ISO-1 treatment showed no

attenuation of LPS-induced TNFα release. Nonetheless, the highest non-toxic concentration of ISO-1 (200μM) did reduce LPS-induced CXCL8 release. Amano and colleagues also found that ISO-1 did not affect cytokine release associated inflammation but validated further the ISO-1 effect on recruitment of cells into the BALF in an allergic asthma mouse model (Amano et al., 2007).

Due to this disparity of ISO-1 effect in THP-1 cells compared to primary cells, I wanted to confirm the lack of ISO-1 inhibition on LPS-induced inflammation in THP-1 cells by using siRNA to genetically inhibit MIF. At 24 hours post-electroporation, *MIF* gene expression was already reduced by 3-fold compared to scramble control; however this was not translated into the intracellular protein level. At 48 hours, *MIF* gene expression remained suppressed and the intracellular protein level was also reduced (1.75-fold). However at 72 hours post electroporation the reduced *MIF* gene expression was lost but the protein level was at its greatest reduction, 2-fold less compared to scramble control. Due to the controversy in MIF function and roles within the cell, I continued to look at all time points to get a complete overview of the role of MIF in LPS-induced inflammation. In support of the ISO-1 experiments in THP-1, siRNA-mediated reduction of MIF showed no modulation of LPS-induced CXCL8 or TNFα release at any of the time points tested.

The cell type specificity of ISO-1 has been reported; one study has shown LPS-induced cytokine inhibition with ISO-1 in primary monocytes however not shown the same response in monocyte-derived macrophages (MDMs) (West et al., 2008). Primary cells would be a better model for human and disease state and showed a MIF inhibitory response, however due to the intensity of investigations needed to elucidate the function of MIF and its inhibition under specific conditions the cell line would be more appropriate at this stage. The variations in responses between patients may hide/enhance any MIF modulatory effects when investigating its role and therefore result in difficulty in detecting functional outputs. For these reasons, I continued with THP-1 cells and explored the direct modulatory effects of human recombinant MIF (rhMIF) on pro-inflammatory processes.

4.4.4 MIF signalling

In order to confirm whether MIF signals through the putative receptor, CD74, an agonist for the receptor was used and the results compared with the direct stimulation with rhMIF.

CD74 activation had a concentration-dependent trend towards increasing *IL8* gene expression; however this did not reach statistical significance. This corresponds with protein release as the CD74 agonist induced the baseline release of CXCL8 in a concentration-dependent manner in THP-1 cells. This release of CXCL8 after CD74 activation is comparable with the MIF stimulation seen in this study and others (Beswick et al., 2005, Binsky et al., 2007).

CD74 activation had no significant effects on *TNFA* gene expression or baseline and LPS-induced TNFα release from THP-1 cells. This data is not in agreement with reports (Calandra et al., 1994).

In contrast, MIF and CD74 activation had opposing effects on *MIF* expression in THP-1 cells; rhMIF inhibited its own gene expression whilst CD74 activation seemed to enhance *MIF* expression. This data suggests that MIF may not be acting through CD74 in these cells or that the CD74 agonist has additional targets.

In support of the published data on MIF and p53 (Bacher et al., 1996, Hudson et al., 1999), MIF treatment induced THP-1 monocyte proliferation and inhibition of MIF-suppressed cellular growth when compared to the positive control (0.5% FBS media). rhMIF (100ng/ml) suppressed the baseline levels of *TP53* gene expression, as p53 is a key regulator of proliferation, reduction of its levels would help drive the cell cycle and enhance proliferation. I can speculate that the difference between the small induction of proliferation by exogenous MIF and large inhibition of proliferation by ISO-1 shows the importance of the intracellular MIF stores and constitutive production on maintaining regular cell growth and division. This inhibition of p53 production was not translated into reduced p53 transcriptional activity, as measured by TransAM® assay showing although MIF reduced expression levels it did not affect p53 DNA binding. More selective p53 binding to specific target genes could be measured by chromatin immunoprecipitation (ChIP) assays.

A recent study noted that p53 was significantly higher in the lung of patients with emphysema secondary to smoking compared with tissue from smokers without emphysema (Morissette et al., 2008). Another group investigated the association of p53 and MIF with cigarette smoke extract (CSE) and demonstrated that in human endothelial cells, CSE-induced MIF protein expression and the CSE-induced apoptosis was p53 dependent (Damico et al., 2011). The authors also showed exogenous MIF prevented CSE-induced apoptosis. It was therefore speculated that chronic smoking altered MIF function or expression in a way that prevented its cytoprotective function causing enhanced p53-dependent endothelial death and emphysema (Damico et al., 2011), my data supports this hypothesis.

MIF is present in most cells, both structural and immune (Calandra and Roger, 2003) and therefore extending these investigations to other cell types, for example epithelial cells, may elucidate if MIF, within the lung, can contribute to the p53-inhibited proliferation driven fibrosis and/or p53-dependent cell death driven emphysema. One study also investigated the p53-dependent effects of MIF on cell growth/proliferation and demonstrated a direct link between MIF-mediated PI3K/Akt signalling and cell survival/growth and inhibition of apoptosis in mouse primary and immortalised fibroblasts (Lue et al., 2007). In this study, rhMIF had no effect on the phosphorylation and hence activation status of Akt protein after 24 hours stimulation.

Cell cycle control is critical for proliferation of cells and therefore further studies in to the effects of MIF on cyclins and cyclin-dependent kinases (Cdk) expressions and the stimulatory or inhibitory effects on MAPK activation may reveal the role of MIF in reducing apoptosis or enhancing proliferation and driving chronic inflammation.

Proliferation, cell growth and apoptosis are not only driven via p53-dependent pathways but through the pro-inflammatory MAPK pathways. MIF has also been shown to signal through MAPK pathways; the direct modulation of the ERK 1/2 pathway by MIF is most characterised (Lue et al., 2006, Mitchell et al., 1999). Although p38 activation and JNK activation by MIF has also been reported (Lue et al., 2011, Veillat et al., 2010). Again most of these reports have been studied in rodent cells. The proliferative effects of MIF via MAPK signalling have not been reported in THP-1 human monocytes.

As previously discussed, MIF enhanced cell growth in a concentration-dependent manner and this was abrogated by ISO-1. This MIF-induced proliferation was also suppressed with pre-treatment of VX-745, again in a concentration-dependent manner, suggesting that the proliferative action of MIF may signal through p38 MAPK cascade. Although, it could be possible that VX745 reduces the basal proliferation and thus too potent for MIF-induced proliferation to be seen.

It has been demonstrated that MIF stimulates ERK1/2 MAPK in a sustained manner, (Mitchell et al., 1999) and in a transient manner (Lue et al., 2006) in murine fibroblasts and macrophages. The effect of MIF on MAPK signalling in THP-1 cells was in contrast to these findings. MIF did not directly affect ERK 1/2 activation within 1 hour (transient) or at 24 hours (sustained) as measured by change in phosphorylation status of the kinase. A dual role for MIF has been described, whereby MIF at physiological concentrations in an inflammatory context (50-100ng/ml) stimulates resting cells, enhances proliferation and initiates an inflammatory response. However, in an acute exacerbation, higher concentrations of MIF, in the upper ng/ml to µg/ml range, inhibits ERK1/2 MAPK and may activate a 'switch off' mechanism (Lue et al., 2006). Therefore, 100ng/ml rhMIF, as used in these experiments may be too high a concentration to activate MAPKs for prolonged periods and hence 'switched off' the activation of MAPKs detected in the array; there are a few MAPKs that have non-significant increases when compared to the naïve treated kinases, suggesting that the concentration of MIF is critical in initiating its signalling and functions.

MIF directly inhibited serum-activated p38 and JNK MAPK in THP-1 cells, with the maximal suppression at 60 minutes for p38 MAPK and 40 minutes for JNK MAPK. This was slightly unexpected with regards to p38 MAPK, as VX-745 pre-treatment had suppressed the MIF-induced proliferation. On the other hand, the BrdU assay, to analyse proliferation, was a 72-hour experiment and MIF-induced p38 MAPK inhibition was only seen transiently at 60 minutes and not sustained to 24 hours.

The involvement of MIF in the chronic inflammation of COPD and severe asthma remains to be fully elucidated. I have shown here, no distinct difference in MIF expression was seen between COPD patients and healthy smoking controls. Also, that MIF signalling may not be through membrane-bound CD74, but that rhMIF

stimulation leads to inflammation and proliferation, reduced *TP53* gene expression levels and inhibitory effects on MAPKs. MIF signalling and its association with p53 to either increase proliferation in airway fibrosis or to induce apoptosis and instigate emphysema and the relationship between MIF and smoking needs further investigation.

Furthermore, MIF has been reported to override the immunosuppressive action of CSs (Calandra et al., 1995). COPD and severe asthma patients show CS insensitivity and therefore in addition to the inflammatory effects of MIF, the counter-regulatory effect of MIF on THP-1 cells as a model for COPD and asthma and its potential to be a therapeutic target is investigated in the next chapter. Ideally these studies would have been conducted in primary cells from patients with COPD or severe asthma but limited cell availability prevented this approach.

Chapter 5

The effect of rhMIF on dexamethasone function in monocytes

5.1 Introduction

The mainstay treatment for inflammatory diseases is CSs; they effectively suppress inflammation by reducing the recruitment of inflammatory cells, inhibiting the production of chemotactic mediators and adhesion molecules, inducing apoptosis of inflammatory cells and reducing the release of pro-inflammatory cytokines (Barnes, 2006). However, in some inflammatory diseases subsets of patients are relatively CS resistant whilst in other diseases like COPD, patients are generally CS resistant (Barnes and Adcock, 2009). CS insensitivity leads to poor disease management and is a huge burden on NHS healthcare costs (Wenzel, 2006). The precise mechanisms causing CS insensitivity are not completely understood, although many mechanisms have been suggested including genetic susceptibility (Carmichael et al., 1981), defective GR binding and translocation (Matthews et al., 2004) and excessive activation of pro-inflammatory transcription factors such as AP-1 (Adcock et al., 1995).

The function of MIF within the immune system and inflammatory diseases is not fully understood (Bucala, 2000). However, one action of MIF that potentially highlights its role in CS insensitivity is its potent anti-CS effects. MIF has been shown to counteract the immunosuppressive effects of CSs (Calandra et al., 1995).

The mechanisms by which MIF counteract CS actions are not fully elucidated but MIF has been shown to interact with some aspects of GR signalling pathways, such as via MKP-1 (Aeberli et al., 2006b, Roger et al., 2005) and via the inhibitor of NF-κB (IκB) (Daun and Cannon, 2000). However the majority of reports into MIF counteracting the immunosuppressive action of CSs are in rodent models and systems and therefore I aimed to confirm these responses in human cells. I have chosen to use a human monocytic cell line THP-1 as a model to investigate the ability of MIF to interfere with dexamethasone actions as monocytes are a major cell type that responds to MIF. A cell line rather than primary was selected as the number of cells required for some of the mechanistic studies would not be achievable in primary blood monocytes or BAL macrophages.

5.2 Chapter hypothesis and aims

I hypothesise that MIF not only drives the inflammatory response in COPD but also reduces the effectiveness of steroids in monocytes/macrophages.

1. Confirm that rhMIF counter-regulates the ability of dexamethasone to enhance a number of CS functions in human monocytic THP-1 cells.
2. Investigate the possible mechanisms by which MIF modifies CS actions.

5.3 Results

5.3.1 Dexamethasone suppression of inflammation

5.3.1.1 The anti-inflammatory function of dexamethasone

Monocytic THP-1 cells were treated with dexamethasone (10^{-10}-10^{-6}M) and incubated for 2 hours (Figure 5.1) and inflammatory readouts either measured at baseline or following stimulation with LPS (1µg/ml) (Figure 5.2). Total RNA was extracted and gene expression levels for *IL8*, *MIF* and *DUSP1* measured. Dexamethasone significantly inhibited baseline levels of *IL8* expression (Figure 5.1A) in a concentration-dependent manner with a plateau at 10^{-7}M (66.0±7.0% suppression) and 10^{-6}M (73.2±7.0% suppression). The EC_{50} for dexamethasone-suppression of basal *IL8* mRNA expression was 5.6nM. In contrast, *DUSP1* gene expression (Figure 5.1B) was significantly induced by dexamethasone in a concentration-dependent manner with a 6-fold induction (653.2±111.2%) at the highest concentration (10^{-6}M) tested. The EC_{50} for *DUSP1* induction was 12nM. Dexamethasone had no effect on baseline levels of *MIF* gene expression (Figure 5.1C).

LPS stimulated both *IL8* (Figure 5.2A) and *DUSP1* (Figure 5.2B) expression although the latter did not reach statistical significance; LPS had no effect on *MIF* (Figure 5.2C) gene expression levels. Dexamethasone significantly inhibited LPS-induced *IL8* gene expression with a maximal effect of 55.4±8.0% at 10^{-6}M (EC_{50} 7.6nM). In contrast, dexamethasone significantly further enhanced LPS-induced *DUSP1* expression 4-fold (395.4±26.8%) at the highest concentration tested (10^{-6}M)

with an induction EC_{50} of 15nM (Figure 5.2B). *MIF* gene expression was not affected by dexamethasone in combination with LPS.

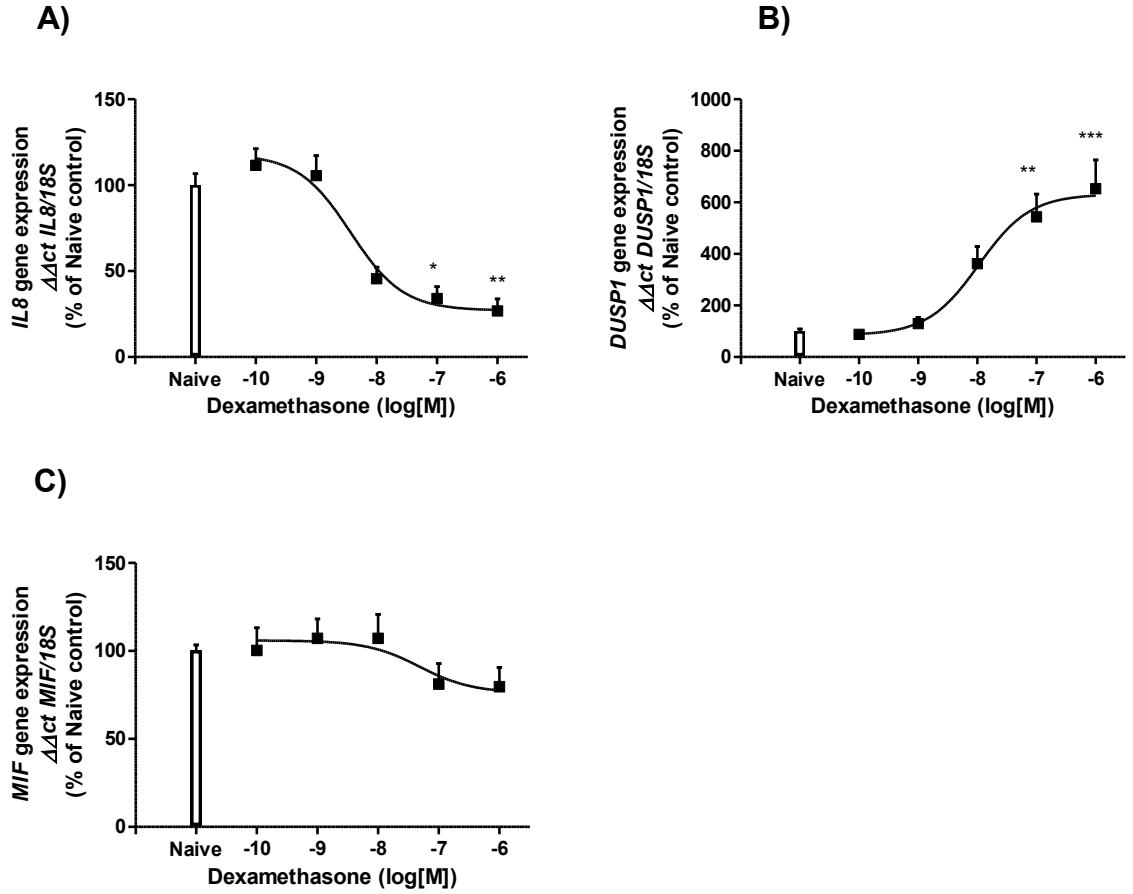


Figure 5.1: Effect of dexamethasone on *IL8*, *MIF* and *DUSP1* baseline gene expression levels

THP-1 cells were incubated with dexamethasone (10^{-10}-10^{-6}M) for 2 hours before RNA was extracted and gene expression of *IL8* (A), *DUSP1* (B) and *MIF* (C) were evaluated by RT-qPCR. Data is normalised to the *18S* internal control and calculated as a percentage of naïve control. Results are presented as mean±S.E.M. for four independent experiments. **p<0.05*, ***p<0.01* and ****p<0.001* vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

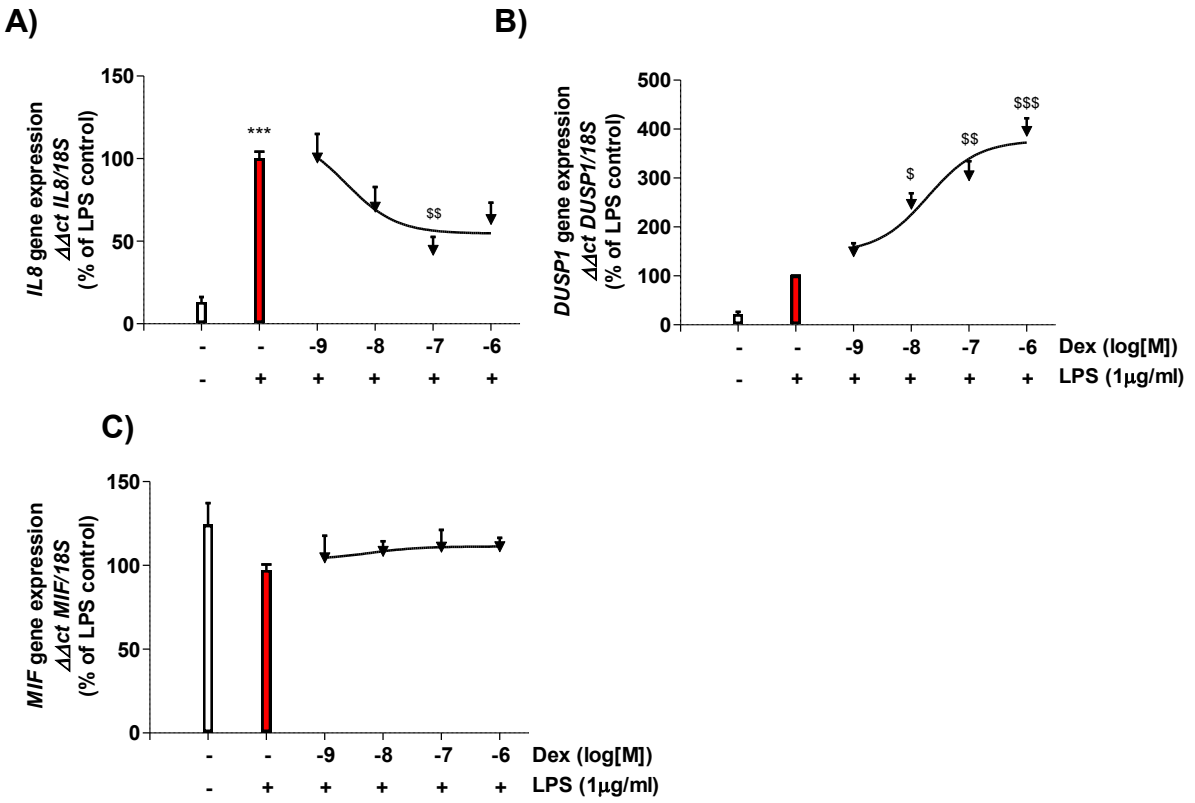


Figure 5.2: Effect of dexamethasone on LPS-induced *IL8*, *MIF* and *DUSP1* gene expression levels
THP-1 cells were pre-treated with dexamethasone (Dex) for 2 hours before being stimulated with LPS (1µg/ml) for 30 minutes. Total RNA was extracted from the cells and then reverse transcribed before qPCR was performed. *18S* gene expression was used as an internal control to normalise the gene expression of *IL8* (A), *DUSP1* (B) and *MIF* (C). The data is presented as mean±S.E.M. of the percentage of LPS control (set at 100%) for three independent experiments. ****p<0.001* vs. naïve control, $*p<0.05*, $$*p<0.01* and $$$*p<0.001* vs. LPS control as calculated by ANOVA with Dunns post-hoc test.

CSs have been shown to affect MIF release in a bell-shaped concentration response manner, with low levels inducing MIF release but high concentrations suppressing its secretion in mouse macrophages (Calandra et al., 1995). In order to determine whether this may have affected earlier results I extended the dexamethasone concentration range. Dexamethasone (10^{-13}-10^{-6}M) had no effect on MIF released from THP-1 cells (Figure 5.3).

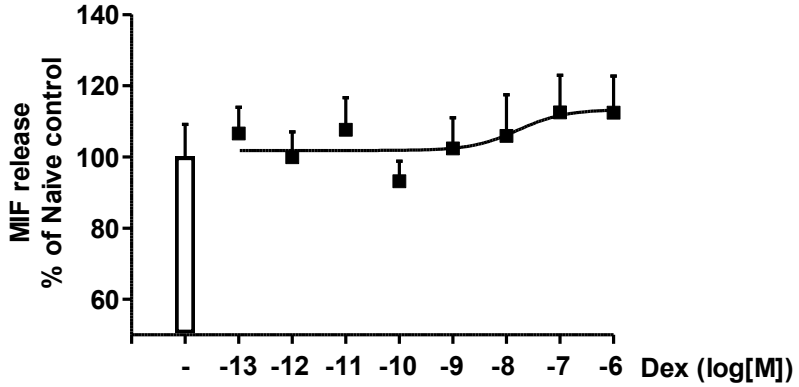


Figure 5.3: Dexamethasone does not enhance MIF release in THP-1 cells
Monocytic THP-1 cells were treated with increasing concentrations of dexamethasone (Dex, 10^{-13}-10^{-6}M) for 24 hours before MIF secretion was analysed by ELISA. Data is normalised as a percentage of naïve control and presented as the mean±S.E.M. for four independent experiments.

Dexamethasone suppression of LPS (1µg/ml)-induced cytokine release was investigated in THP-1 cells following 1 hour pre-incubation and overnight stimulation. Supernatants were removed and the levels of inflammatory mediators measured by ELISA, measuring for pro-inflammatory mediator CXCL8 (Figure 5.4). LPS significantly induced CXCL8 release 6-fold (653.3±97.3%, p<0.001) compared to naïve control cells. Dexamethasone pre-treatment significantly inhibited LPS-induced CXCL8 release in a concentration-dependent manner with the highest concentrations of dexamethasone (10^{-7} & 10^{-6}M) reducing CXCL8 levels to basal levels (134.9±10.6, p<0.01 and 139.3±4.5, p<0.05) respectively (Figure 5.4). Dexamethasone suppression of CXCL8 release had an EC_{50} value of 6.4nM.

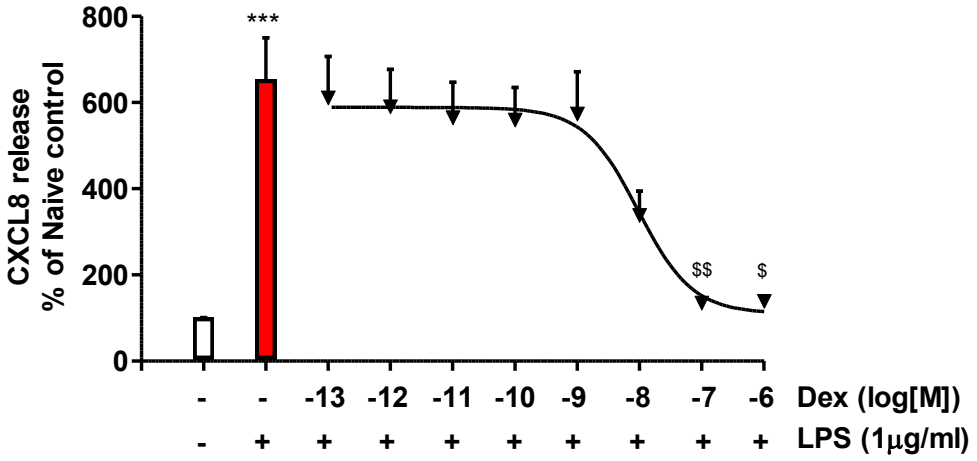


Figure 5.4: Dexamethasone inhibits LPS-induced CXCL8 release from THP-1 cells
Cells were pre-incubated with dexamethasone (Dex, 10^{-13}M to 10^{-6}M) for 1 hour before being stimulated with 1μg/ml LPS for 24 hours. CXCL8 release into the supernatant was measured by ELISA. Data is presented as mean±S.E.M. of the percentage of naïve control for four independent experiments. ***$p<0.001$ vs. naïve control, $^{\$}p<0.05$, $^{\$\$}p<0.01$ vs. LPS control as calculated by ANOVA with Dunns post-hoc test.

5.3.2 MIF effects on dexamethasone-induced gene expression

THP-1 cells were stimulated with rhMIF (0.1-1000ng/ml) for 4 hours before total RNA was extracted and RT-qPCR used to analyse *DUSP1* gene expression. *DUSP1* gene expression levels were significantly inhibited in a concentration-dependent manner with a maximum suppression down to 35.84±6.8% of naïve control levels at 1000ng/ml MIF (Figure 5.5). The direct effect of MIF on pro-inflammatory *IL8* gene expression was analysed previously in Chapter 4, Figure 4.6B.

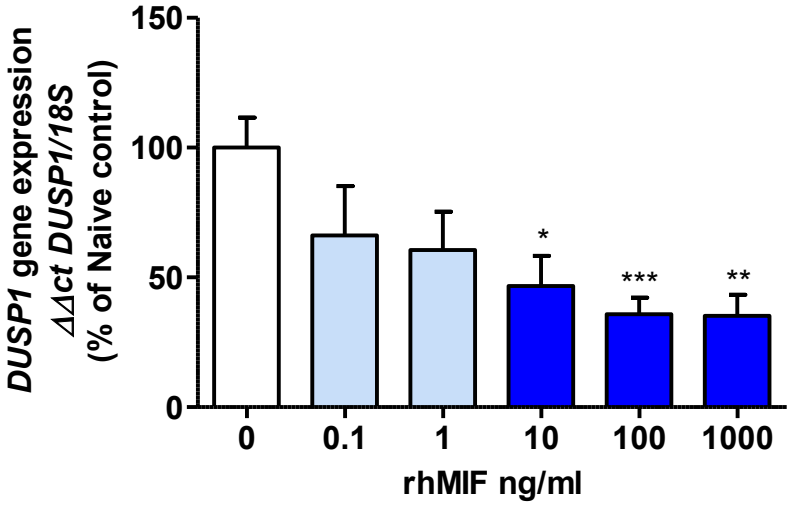


Figure 5.5: MIF inhibits the expression of the anti-inflammatory *DUSP1* gene in THP-1 cells
THP-1 monocytes were incubated with rhMIF (0.1-1000ng/ml) for 4 hours. Total RNA was extracted and *DUSP1* gene expression measured by RT-qPCR before being normalised to *18S* gene expression. Data is presented as mean±S.E.M. of the percentage of naïve cells from at least four independent experiments **p*<0.05 vs. naïve, ***p*<0.01 vs. naïve and ****p*<0.001 vs. naïve as calculated by ANOVA with Dunns post-hoc test.

5.3.3 MIF counter regulation of dexamethasone actions

The effect of dexamethasone, rhMIF and LPS on cell viability was measured. THP-1 cells were treated with rhMIF for 30 minutes and dexamethasone for a further 1 hour. Cells were subsequently stimulated with LPS (1μg/ml) overnight and cytotoxicity measured using an MTT assay. There was no significant effect on cell viability by any treatment alone or in any combination (Figure 5.6).

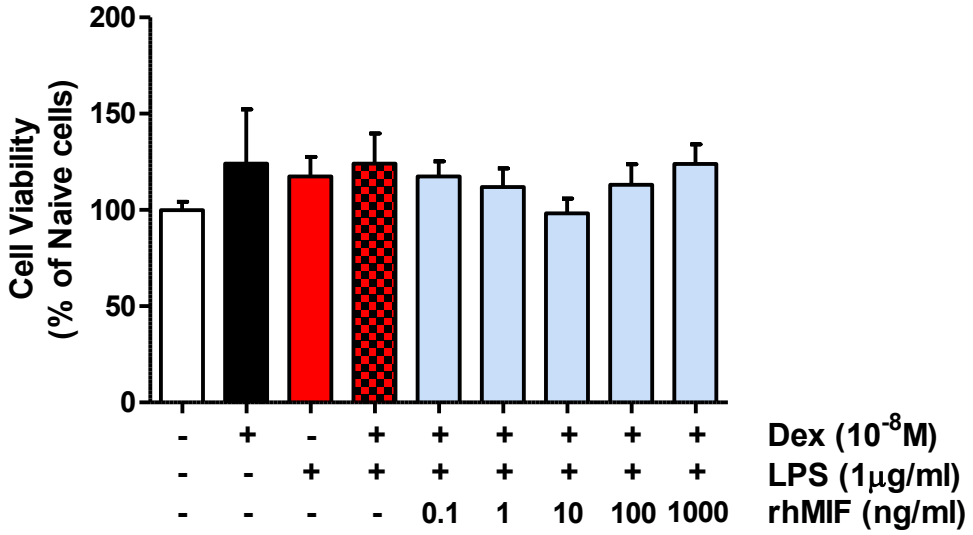


Figure 5.6: Dexamethasone and rhMIF have no effect on viability of LPS-stimulated THP-1 cells
Cells were incubated with rhMIF (0.1-1000ng/ml) for 30 minutes followed by 1 hour pre-treatment with dexamethasone (10^{-8}M). Cells were then stimulated with LPS (1μg/ml) for 16 hours and cytotoxicity measured by MTT assay. Data is presented as the percentage of the naïve value and plotted as the mean±S.E.M of three independent experiments.

5.3.3.1 rhMIF counter-regulates dexamethasone-induced DUSP1 gene expression at baseline

To study whether rhMIF counteracts the action of dexamethasone at the gene expression level, THP-1 cells were pre-treated with rhMIF (0.1-1000ng/ml) for 1 hour and then incubated with dexamethasone (10^{-8}M) for 2 hours. *DUSP1* and *IL8* gene expression levels were measured. Dexamethasone suppression of baseline *IL8* gene expression was not affected by rhMIF (Figure 5.7A). In contrast, rhMIF significantly inhibited dexamethasone-induced *DUSP1* gene expression in a concentration-dependent manner (Figure 5.7B). The maximal inhibition reached 50.9±11.9% of the dexamethasone control at 100ng/ml rhMIF.

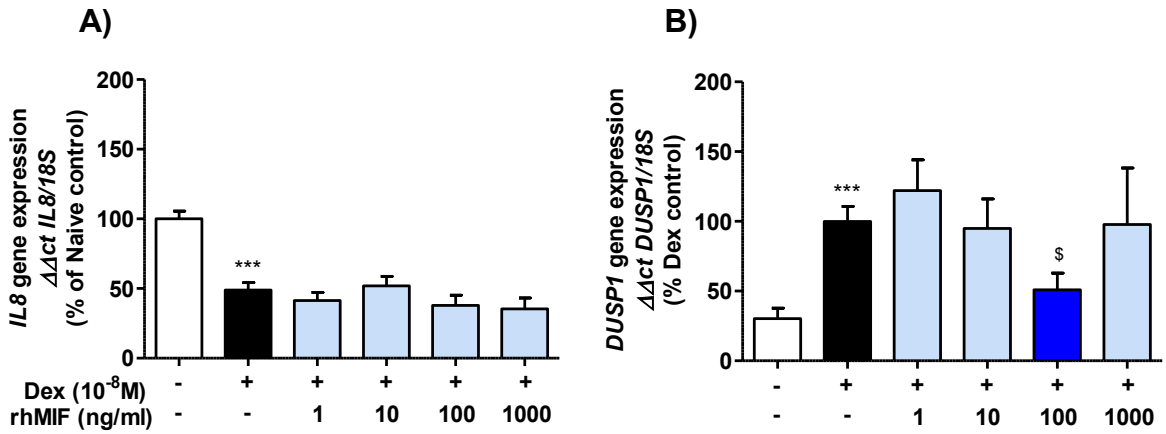


Figure 5.7: Effect of rhMIF on dexamethasone-controlled gene expression levels in THP-1 cells
Cells were pre-incubated with rhMIF (0.1ng/ml to 1000ng/ml) for 30 minutes before stimulation for 2 hours with dexamethasone (Dex; 10^{-8}M). Total RNA was extracted, reverse transcribed and *IL8* (A) and *DUSP1* (B) gene expression levels measured by qPCR. *18S* gene expression was used as an internal control and for normalisation of the gene expression. Data is presented as the percentage of naïve control values and plotted as the mean±S.E.M. for four independent experiments. ***$p<0.001$ vs. naïve control, $^{\$}p<0.05$ vs. dexamethasone control as calculated by ANOVA with Dunns post-hoc test.

5.3.3.2 rhMIF does not reverse dexamethasone function in the presence of LPS

The opposing effects of rhMIF on dexamethasone function after LPS stimulation was investigated. THP-1 cells were pre-treated with rhMIF for 30 minutes and dexamethasone (10^{-8}M) for an additional 2 hours. LPS was then used to induce an inflammatory response for 30 minutes and *IL8* and *DUSP1* gene expression was measured. The release of TNFα and CXCL8 into the culture medium was also investigated under essentially the same conditions as described above except that cells were stimulated overnight with LPS (1µg/ml) after an 1 hour pre-incubation with dexamethasone (10^{-8}M).

IL8 gene expression was significantly inhibited by dexamethasone after LPS stimulation (67.1±10.2% of LPS control, $p<0.01$) (Figure 5.8A). The addition of rhMIF had no significant effect on dexamethasone inhibition of LPS-induced *IL8* gene expression (Figure 5.8A).

DUSP1 expression was significantly enhanced by dexamethasone and LPS (261.9±26.7%, $p<0.001$) compared to the levels seen with LPS stimulation alone.

rhMIF had no effect on dexamethasone and LPS-induced *DUSP1* gene expression (Figure 5.8B).

LPS significantly induced the release of both CXCL8 (8-fold) and TNFα (7-fold) and this induction was significantly inhibited by 10^{-8}M dexamethasone in both cases. CXCL8 release inhibited by 54.0±2.9%, $p<0.001$ and TNFα release was suppressed by 16.8±2.1%, $p<0.001$. The addition of rhMIF had no significant effect on dexamethasone suppression of LPS-induced CXCL8 (Figure 5.9A) or TNFα release (Figure 5.9B) in THP-1 cells.

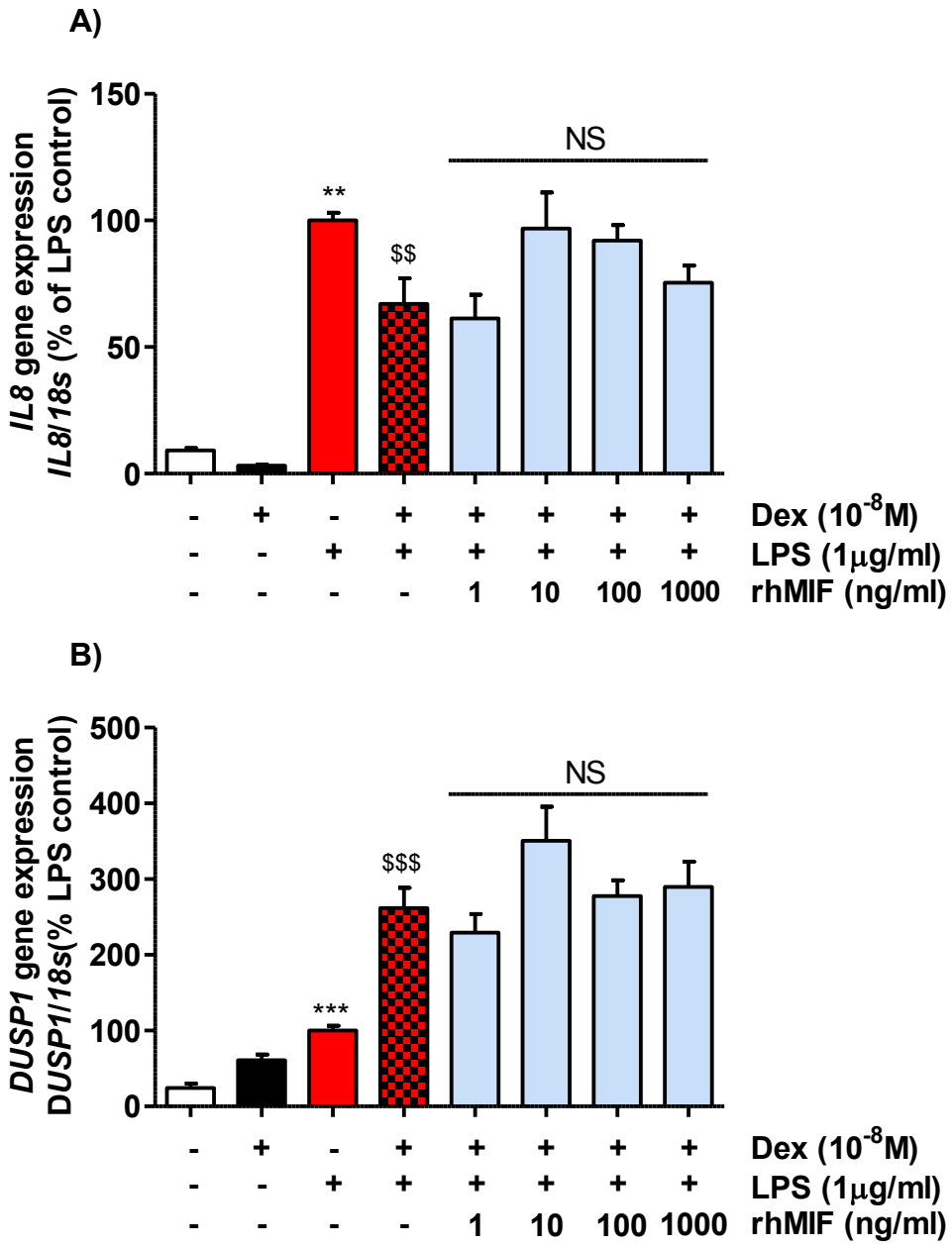


Figure 5.8: rhMIF has no effect on dexamethasone modulation of LPS-induced *IL8* and *DUSP1* gene expression in THP-1 cells

THP-1 cells were pre-treated with rhMIF (0.1-1000ng/ml) for 1 hour, followed by 2 hour incubation with dexamethasone (Dex; 10^{-8}M) and 30 minutes stimulation with 1μg/ml LPS. RNA was extracted from the cells, reverse transcribed and *IL8* (A) and *DUSP1* (B) gene expression measured by qPCR. *18S* gene expression was used as an internal control. Data is normalised as a percentage of LPS control and plotted as the mean±S.E.M. for four independent experiments. ***p<0.01, ***p<0.001* vs. naïve control, $^{\$\$}$*p<0.01,* $^{\$\$\$}$*p<0.001* vs. LPS control as calculated by ANOVA with Dunns post-hoc test.

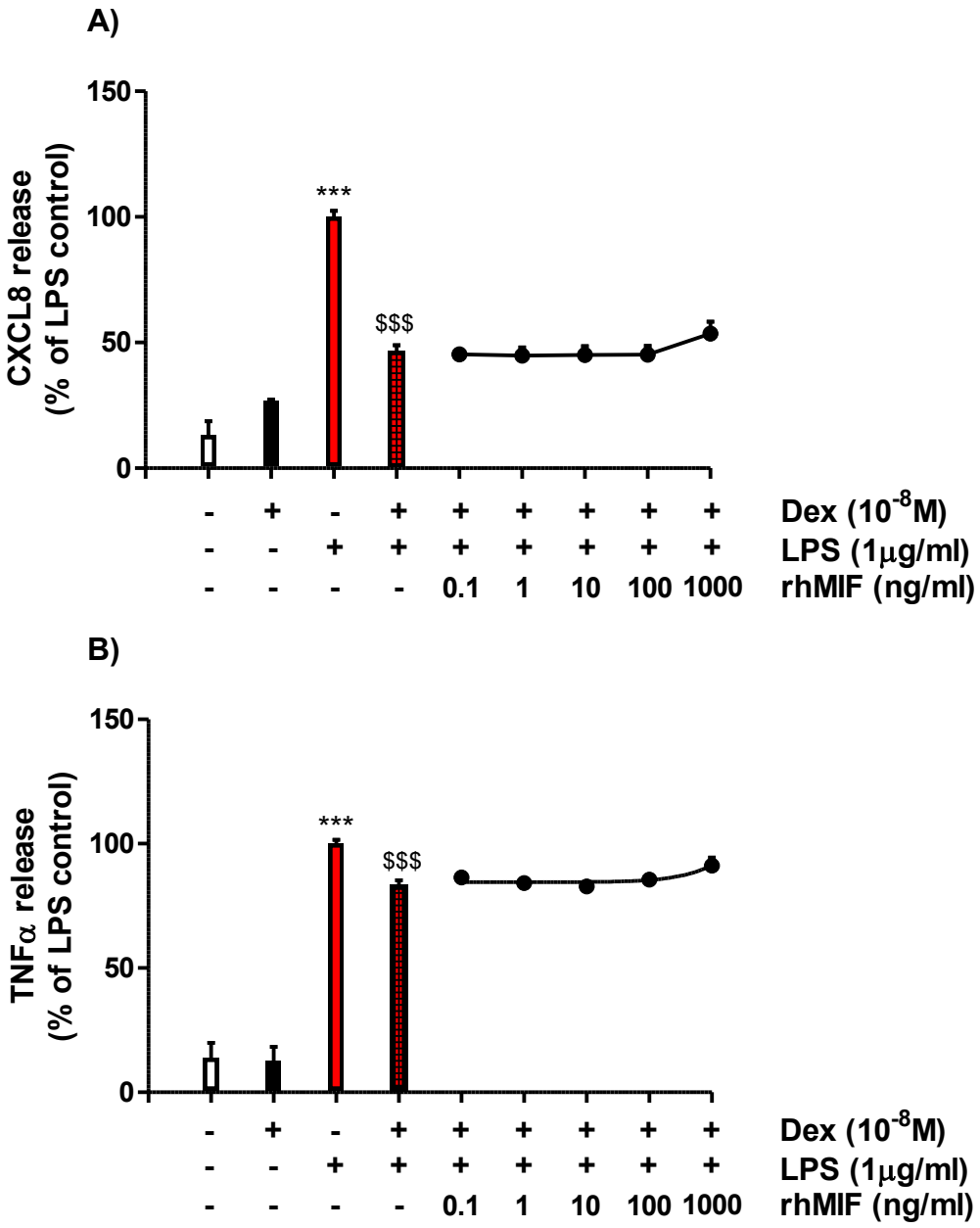


Figure 5.9: The effect of rhMIF on dexamethasone suppression of LPS-induced TNFα and CXCL8 release from THP-1 cells
THP-1 cells were pre-treated with rhMIF (0.1-1000ng/ml) for 30 minutes followed by 1 hour exposure to dexamethasone (Dex; 10^{-8}M) before stimulation with LPS (1μg/ml) for 16 hours. CXCL8 (A) and TNFα (B) release was measured by ELISA. Data is presented as the percentage of LPS control and plotted as the mean±S.E.M. of six independent experiments. ***$p<0.001$ vs. naïve control, $^{\$\$\$}p<0.001$ vs. LPS control as calculated by ANOVA with Dunns post-hoc test.

5.3.4 Inhibition of MIF affects dexamethasone function in unstimulated THP-1 cells

rhMIF inhibits dexamethasone-induced *DUSP1* gene expression but not LPS and dexamethasone-induced *DUSP1*. In order to confirm this MIF suppression I inhibited MIF with ISO-1 and siRNA.

5.3.4.1 Effect of pharmacological inhibition of MIF by ISO-1 on dexamethasone-regulated gene expression

THP-1 monocytes were pre-treated with rhMIF (100ng/ml) or the inhibitor ISO-1 (100μM) for 30 minutes and then stimulated with dexamethasone (10^{-8}M) for 2 hours. Total RNA was then isolated and gene expression for *IL8* and *DUSP1* evaluated by qPCR. Dexamethasone inhibited baseline *IL8* gene expression by 49.3±5.5% (p<0.001), whereas rhMIF and ISO-1 alone had no effect on basal *IL8* expression. In addition, neither rhMIF (55.5±6.5%, p=NS) nor ISO-1 (57.3±10.4%, p=NS) had any effect on dexamethasone-suppression of *IL8* gene expression (Figure 5.10A).

DUSP1 gene expression was induced by dexamethasone (10^{-8}M) by 70.9±6.0% above baseline (p<0.001). In contrast to the previous set of experiments, rhMIF did not inhibit the baseline expression of *DUSP1* (35.9±4.0% vs. 31.1±4.0%, p=NS); however pre-treatment with ISO-1 resulted in a 5.5-fold increase in *DUSP1* gene expression compared to naïve levels (172.8±27.9% vs. 31.1±4.0%, p<0.001). In support of previous results, rhMIF inhibited the dexamethasone-induced *DUSP1* gene expression by 44.0±9.9% (p<0.01) and ISO-1 enhanced dexamethasone-induced *DUSP1* gene expression by 22.3±20.5% although this did not reach statistical significance (Figure 5.10B).

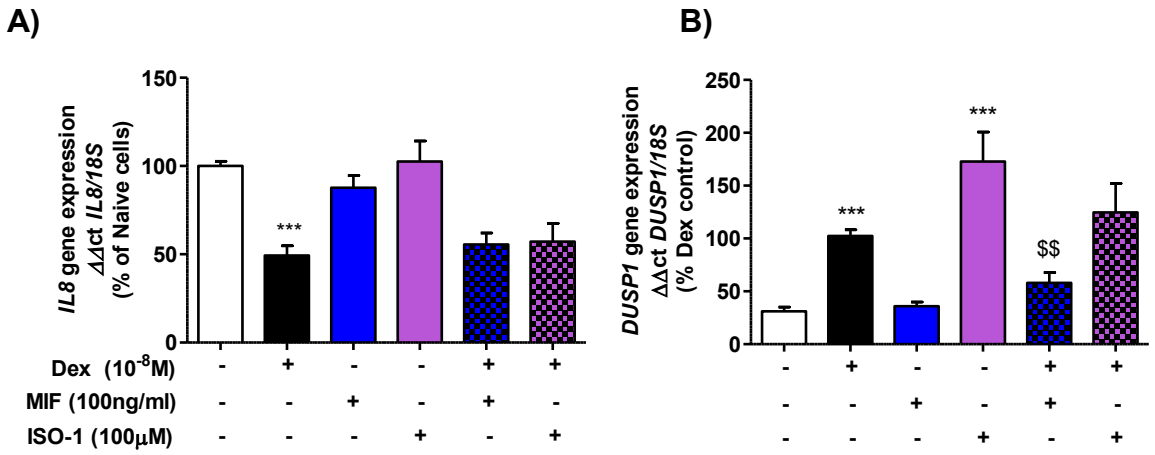


Figure 5.10: ISO-1 attenuates MIF inhibition of dexamethasone-regulated gene expression
THP-1 cells were pre-treated with ISO-1 (100μM) or rhMIF (100ng/ml) for 30 minutes followed by 2 hour stimulation with dexamethasone (Dex; 10^{-8}M). RNA was extracted and *IL8* (A) and *DUSP1* (B) gene expression evaluated using qPCR. Data is normalised to *18S* gene expression and plotted as a percentage of the naïve levels for *IL8* expression and as a percentage of dexamethasone control for *DUSP1* expression. Data represent the mean±S.E.M. of four independent experiments. ****p<0.001* vs. naïve control and $$ *p<0.05* vs. dexamethasone control as calculated by ANOVA with Dunns post-hoc test.

5.3.4.2 Effect of MIF knockdown on dexamethasone-regulated gene expression

On-target MIF siRNA was used to knockdown MIF in THP-1 cells. After electroporation the cells were incubated for 24, 48 and 72 hours and were then serum deprived using 1% FBS in phenol red free RPMI-1640 medium for 3 hours followed by treatment with dexamethasone (10^{-8}M) for 2 hours. Total RNA was extracted and *IL8* and *DUSP1* gene expression measured by RT-qPCR. MIF knockdown using On-target MIF siRNA had no effect on the basal levels of *IL8* expression at any of the time points studied. As seen in Figure 5.11A, dexamethasone inhibited the expression of *IL8* in cells electroporated with scramble control siRNA at 24 hours (58.6±5.9% of scramble control, $p<0.05$). However, this suppressive effect of dexamethasone on *IL8* gene expression was lost in cells treated with On-target MIF siRNA (139.5±46.0% of scramble control, *p*=NS) at 24 hours. As the incubation times were extended the ability of dexamethasone to inhibit basal *IL8* expression was reduced (41.4±5.9% at 24 hours compared to 12.9±9.8% at 72 hours). Dexamethasone did not significantly affect *IL8* gene expression in cells transfected with On-target MIF siRNA at any of the time points tested (Figure 5.11A).

Dexamethasone was able to induce *DUSP1* gene expression at all-time points studied irrespective of whether cells were electroporated with scramble control siRNA or On-target MIF siRNA. Reducing MIF levels in THP-1 cells had no effect on the baseline levels of *DUSP1* gene expression when compared to scramble siRNA at any time point (Figure 5.11B). However dexamethasone-induced *DUSP1* was significantly greater in cells transfected with On-target siRNA compared to dexamethasone-treated scramble control siRNA at 24 hours (181.4±33.0% of scramble control, $p<0.05$), 48 hours (165.8±29.9% of scramble control, $p<0.05$) and 72 hours (176.0±23.5% of scramble control, $p<0.01$) (Figure 5.11B).

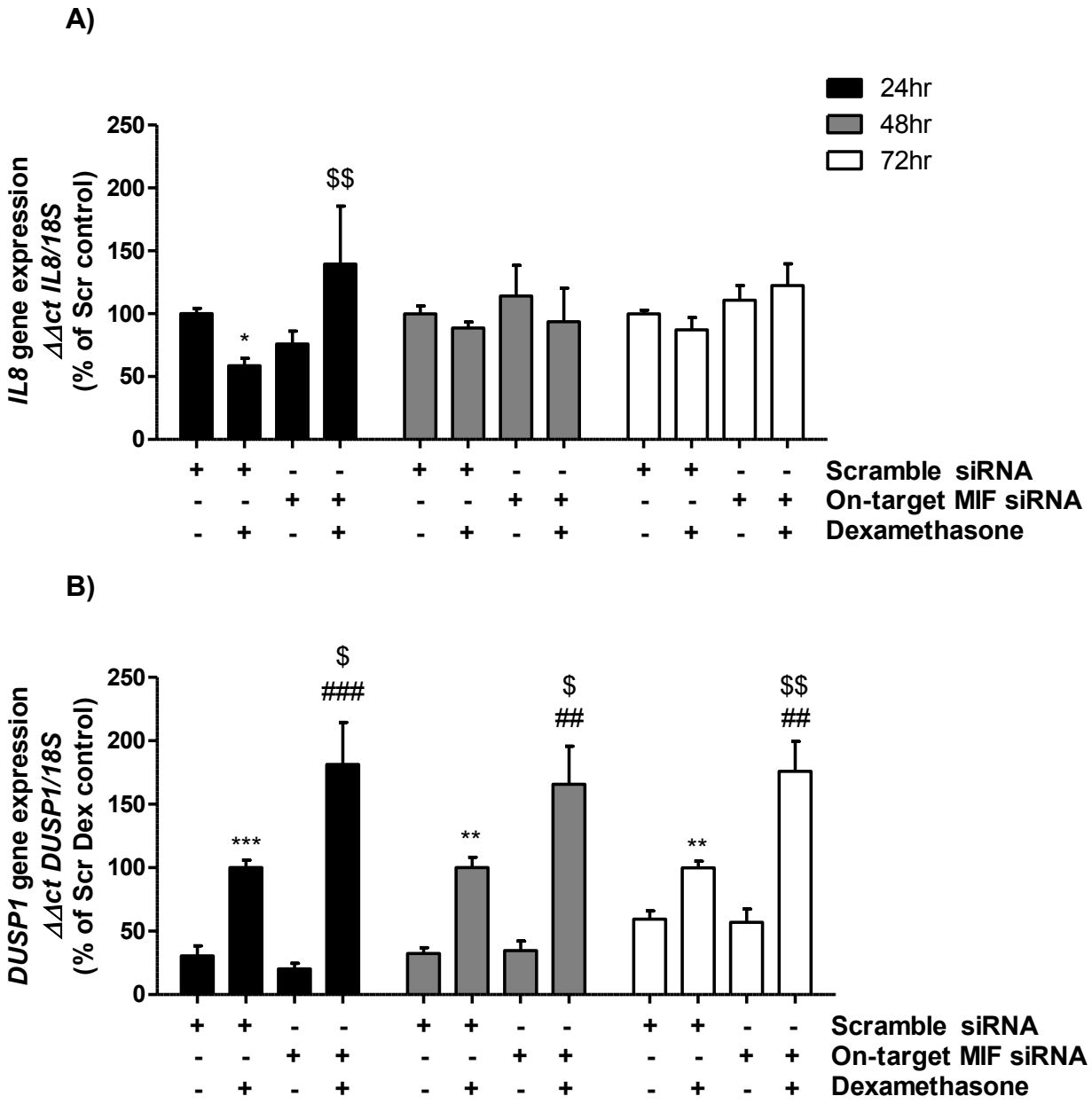


Figure 5.11: The effect of MIF siRNA on dexamethasone regulation of *IL8* and *DUSP1* gene expression at 24, 48 and 72 hours

THP-1 monocytes were electroporated with scramble or On-target MIF siRNA and then incubated for 24, 48 or 72 hours. Cells were then treated with or without dexamethasone (Dex; 10^{-8}M) for 2 hours before total RNA was extracted and *IL8* (A) and *DUSP1* (B) mRNA measured by qPCR. Results are normalised to *18S* gene expression and presented as the percentage of scramble control for *IL8* and of dexamethasone-treated scramble control for *DUSP1* gene expression at each time point. Data is presented as mean±S.E.M. of three independent experiments. **p<0.05* vs. scramble control, ***p<0.01* vs. scramble control, ****p<0.001* vs. scramble control, ##*p<0.01* vs. On-target MIF siRNA, ###*p<0.001* vs. On-target MIF siRNA, $*p<0.05* vs. dexamethasone-treated scramble control, $$*p<0.01* vs. dexamethasone-treated scramble control as calculated by ANOVA with Dunns post-hoc test.

5.3.4.3 Effect of MIF knockdown using siRNA on dexamethasone suppression of LPS-induced cytokine release

In addition to gene expression cytokine release was also measured by ELISA. At each time point post electroporation, THP-1 cells were incubated in 1% FBS/RPMI media for 3 hours followed by pre-treatment with dexamethasone (10^{-8}M) for 1 hour and then stimulated with LPS (1µg/ml) for 24 hours. Dexamethasone significantly suppressed LPS-induced CXCL8 release at 24 hours (25.0±2.4%, p<0.01), 48 hours (55.1±11.9%, p<0.01) and 72 hours (34.6±2.8%, p<0.001) in scramble siRNA transfected cells (Figure 5.12A). Dexamethasone was still able to fully suppress LPS-induced CXCL8 at 24 hours (25.9±1.6% vs. 87.3±7.4%, p<0.01), 48 hours (57.9±12.7% vs. 111.1±5.8%, p<0.01) and 72 hours (47.8±4.6% vs. 164.4±25.5%, p<0.001) following transfection of cells with On-target MIF siRNA (Figure 5.12A).

Similar trends are seen with LPS-induced TNFα release with MIF knockdown having no significant effect on the ability of dexamethasone to inhibit TNFα levels at any of the time points studied when compared to scrambled control siRNA (Figure 5.12B). However, the ability of dexamethasone to suppress LPS-induced TNFα expression was reduced over time with scramble siRNA from 24 hours (49.2±3.7%, p<0.001) through 48 hours (19.0±8.1%, p<0.05) to 72 hours (14.1±2.3%, p=NS) (Figure 5.12B). This suggests that electroporation alone affects the efficacy of dexamethasone to regulate the expression of subsets of inflammatory genes.

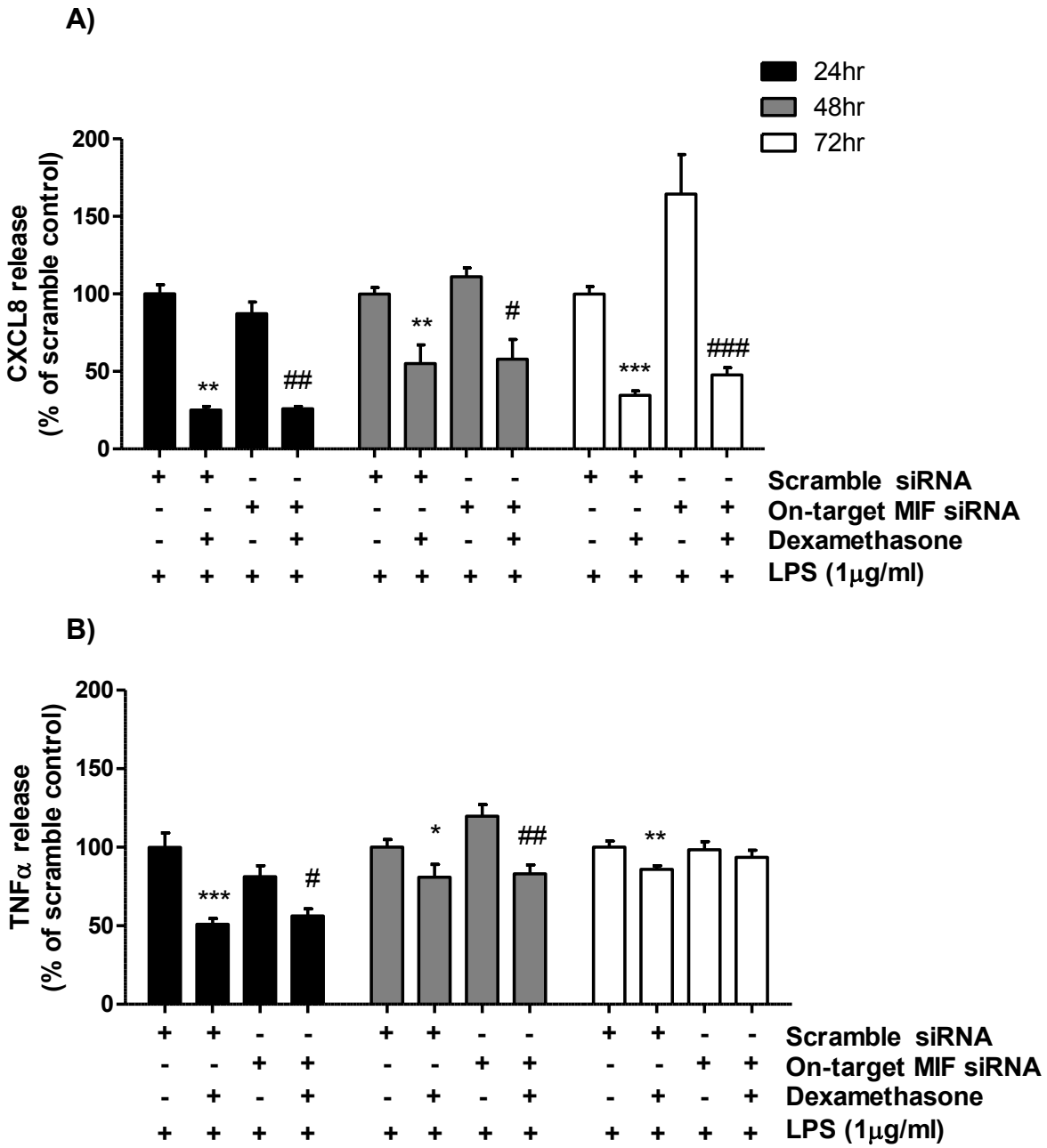


Figure 5.12: MIF knockdown has no effect on dexamethasone suppression of LPS-induced CXCL8 or TNFα release

THP-1 cells were electroporated with either scramble siRNA or On-target MIF siRNA and incubated for 24 (black bars), 48 (grey bars) and 72 hours (white bars). The cells were pre-treated with dexamethasone (10^{-8}M) for 30 minutes and then stimulated with LPS (1μg/ml) for a further 24 hours. CXCL8 (A) and TNFα (B) released were measured by ELISA. Data is normalised to the effect of scramble siRNA at each time point and plotted as the mean±S.E.M. of four independent experiments. *$p<0.05$ vs. scramble control, **$p<0.01$ vs. scramble control, ***$p<0.001$ vs. scramble control, # $p<0.05$ vs. On-target MIF siRNA, ## $p<0.01$ vs. On-target MIF siRNA, ### $p<0.001$ vs. On-target MIF siRNA as calculated by ANOVA with Dunns post-hoc test.

5.3.5 The effect of rhMIF on dexamethasone suppression of LPS-induced p38 MAPK phosphorylation

MIF has been reported to modulate p38 MAPK activity (Santos et al., 2004), a known target of dexamethasone actions (Clark and Lasa, 2003). THP-1 cells were pre-treated with rhMIF (100ng/ml) for 1 hour and then with dexamethasone (10^{-8}M) for 2 hours. The cells were subsequently stimulated with LPS (1μg/ml) for 30 minutes and p38 MAPK activation measured by ELISA-based PhosphoTracer analysis. Total p38 MAPK levels were not affected by any treatment conditions. Phosphorylated p38 MAPK (p-p38) was non-significantly enhanced by LPS (937.7±58.6% of naïve control, p<0.06) and neither dexamethasone (950.0±68.4%, p=NS) or dexamethasone and rhMIF (1147.0±109.4%, p=NS) significantly affected LPS-induced p38 MAPK activation (Figure 5.13).

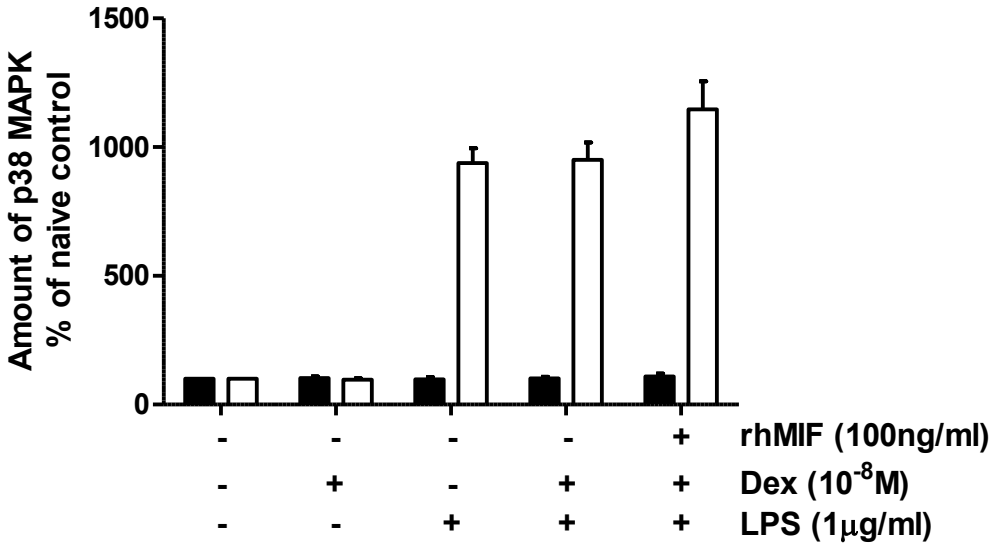


Figure 5.13: Neither dexamethasone nor rhMIF modulate LPS-induced p38 MAPK phosphorylation.
Cells were pre-treated with 100ng/ml rhMIF for 1 hour followed by 2 hour treatment with dexamethasone (Dex;10^{-8}M) and a final stimulation with LPS (1μg/ml) for 30 minutes. Total p38 MAPK (black bars) and p-p38 MAPK (white bars) were measured using an ELISA-based PhosphoTracer. Data is plotted as fluorescent units normalised to naïve control samples. Results for both total and p-p38 MAPK are reported as the mean±S.E.M for five independent experiments.

5.3.6 rhMIF on dexamethasone-induced glucocorticoid receptor-glucocorticoid response element (GR-GRE) DNA binding

THP-1 cells were pre-treated with rhMIF (1-1000ng/ml) for 1 hour before stimulation with dexamethasone (10^{-8}M) for 30 minutes. Nuclear proteins were isolated and GR DNA binding measured. The ability of activated GR to bind to its consensus GRE sequence (5'-GGTACAnnnTGTTCT-3') was determined by TransAM® assay. The results showed that dexamethasone induced GR-GRE binding 4-fold (28.0±3.7% vs. 100%, *p*=NS) but rhMIF had no effect on dexamethasone induced GR binding (Figure 5.14).

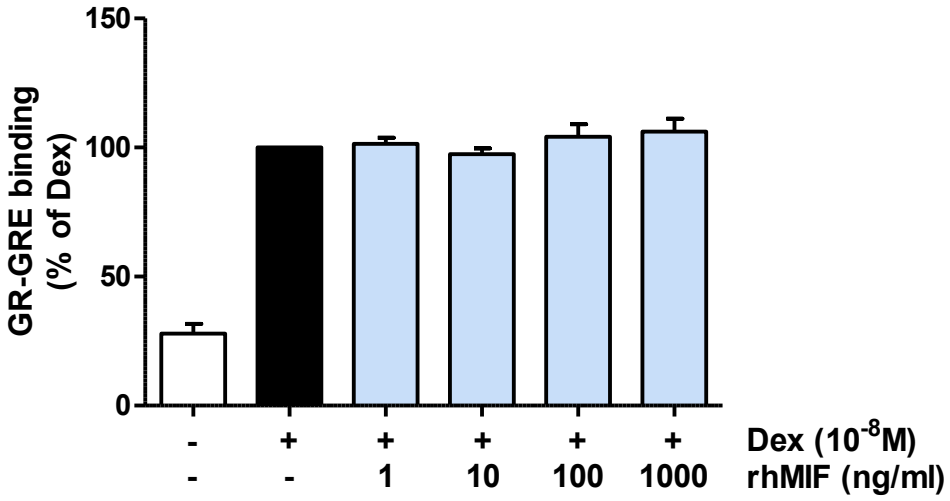


Figure 5.14: rhMIF has no effect on dexamethasone-induced glucocorticoid receptor binding to the glucocorticoid response element
THP-1 cells were pre-treated with rhMIF (1-1000ng/ml) for 1 hour followed by 30 minutes stimulation with dexamethasone (Dex; 10^{-8}M). Nuclear proteins were immediately extracted and the binding capacity of GR to GRE was analysed using a TransAM® ELISA based assay. Data is plotted as the mean±S.E.M. of the percentage of dexamethasone control for four independent experiments.

5.4 Discussion

5.4.1 Counteracting the immunosuppressive actions of CSs

As expected dexamethasone exerted anti-inflammatory effects in THP1 cells indicated by both suppressing *IL8* and inducing *DUSP1* gene expression, both at baseline and after LPS-induction. Dexamethasone also suppressed LPS-induced CXCL8 and TNFα release. However, dexamethasone had no effect on MIF expression in these cells in contrast to the data of Calandra and colleagues who first demonstrated the biphasic control of CS on MIF release (Calandra et al., 1995). These authors suggested that CSs directly inhibit pro-inflammatory cytokine release at high concentrations of dexamethasone (10^{-8} to 10^{-6}M) but at low steroid concentrations (10^{-14} to 10^{-12}M), CSs are able to induce MIF release (Calandra et al., 1995). This results in a bell-shaped concentration response curve that has been reported in mouse macrophages (Calandra et al., 1995), mouse T lymphocytes (Bacher et al., 1996) and human fibroblasts (Leech et al., 1999). This is in contrast to the data reported here where dexamethasone had no effect on MIF expression or release. This conflicting result on MIF induction might be due to an immortalised cell line effect on MIF, as dexamethasone suppressed LPS-induced CXCL8 release at the concentrations expected.

The relationship between MIF and CSs gave rise to new understandings of how endogenous CSs maintain a homeostatic environment in the healthy immune system. Stress or inflammatory stimuli initiate an immune response and this up-regulates the release of endogenous CSs to suppress this response. To prevent the potential over-suppression of the immune system, MIF release is simultaneously induced by CSs to act as a 'brake' and to maintain a healthy immune response (Morand and Leech, 1999). More recent studies also report that the presence of MIF can reduce CS responsiveness. For example, the loss of MIF expression in a murine knockout model (Aeberli et al., 2006b) and *in vitro* using transfection of an anti-sense MIF plasmid in mouse RAW264.7 macrophages (Roger et al., 2005) resulted in increased CS sensitivity. Correlations have also been shown between elevated MIF expression and the corresponding CS insensitivity in different diseases including juvenile RA (De Benedetti et al., 2003), children's nephrotic syndrome (Berdeli et al., 2005), and ulcerative colitis (Ishiguro et al., 2006).

MIF counteracts the immunosuppressive effects of CSs on LPS-induced cytokine release *in vitro* and *in vivo* in a mouse model of endotoxic shock. Importantly, rhMIF blocked the protective effect of CSs on LPS lethality (Calandra et al., 1995). To date the association between MIF and CS insensitive COPD has not been explored. In this thesis, the effects of MIF on dexamethasone regulation of cytokine production was investigated in THP-1 cells; to investigate transrepression, dexamethasone inhibition of *IL8* (CXCL8) and *TNFA* (TNFα) were evaluated and for transactivation, dexamethasone induction of *DUSP1* (MKP-1) was analysed. Baseline *IL8* gene expression was suppressed by dexamethasone as expected and increasing concentrations of rhMIF had no effect on this inhibitory response. Furthermore, MIF inhibition by ISO-1 had no effect on basal *IL8* gene expression or on dexamethasone-suppressed *IL8* gene expression. This failure of ISO-1 to affect basal *IL8* gene expression was supported, in part, by siRNA-mediated knockdown of MIF, whereby although MIF knockdown reversed dexamethasone-induced suppression of *IL8* gene expression at 24 hours, this effect was lost at 48 and 72 hours when MIF knockdown was greatest. This result is in stark contrast to results demonstrated in mice peritoneal macrophages, whereby loss of MIF increased the sensitivity to dexamethasone and enhanced its suppressive action (Aeberli et al., 2006b).

Dexamethasone also suppressed LPS-induced *IL8* production, which was not affected by the presence of rhMIF. Similarly, rhMIF did not affect LPS-induced release of CXCL8 and TNFα or the ability of dexamethasone to suppress this release. The effects of reducing MIF protein content using siRNA was also investigated and again failed to show a clear modulation of basal or LPS-induced CXCL8 and TNFα release or on the ability of dexamethasone to suppress LPS-induced CXCL8 and TNFα release. However, with extended incubation times the ability of dexamethasone to suppress LPS-induced CXCL8 and TNFα release was reduced suggesting that it was not the loss of MIF content within the cell that effected CS function but the electroporation method itself. Although this lack of MIF ability to modulate dexamethasone function is in line with results seen at the gene expression level and with regards to reports on THP-1 monocytes (Kudrin et al., 2006), these data are in conflict with results published in other cells types (Calandra et al., 1995, Santos et al., 2001, Aeberli et al., 2006b, Bacher et al., 1996, Kudrin et

al., 2006). Another immortalised monocyte cell line, U937 cells were also investigated however these results were similar to those seen with THP-1 cells (data not shown). It is also noteworthy that Kudrin and others used a lower concentration of LPS to stimulate CXCL8 and TNFα release from cells (Kudrin et al., 2006) and that 1µg/ml LPS may have produced an overpowering response and masked the anti-CS effects of MIF.

MIF reversed the dexamethasone induction of *DUSP1* gene expression in this study; however this effect was not seen following stimulation of the cells with LPS. The ability of dexamethasone to induce *DUSP1* expression was significantly reversed by 100ng/ml MIF and, although this is a fairly high concentration, it is possible to detect these levels in human samples from inflamed conditions. The sensitivity of the cells to dexamethasone was also enhanced with MIF inhibition, resulting in a greater stimulation of *DUSP1* gene expression with On-target MIF siRNA compared to scramble siRNA at all time points studied. A similar result was also observed with ISO-1 pre-treatment however it did not reach statistical significance.

MIF inhibition by siRNA transfection alone had no effect on baseline levels of *DUSP1* gene expression yet inhibition with ISO-1 alone, stimulated *DUSP1* gene expression levels to a significantly greater extent than seen with dexamethasone. This disparity between the effects seen with MIF inhibition using two different approaches is difficult to explain; I have shown that rhMIF inhibits the baseline levels of *DUSP1* and that ISO-1 can enhance *DUSP1* gene expression. Nonetheless, this could be an ISO-1-specific effect as siRNA transfection is known to be highly specific and the reduced levels of intracellular MIF did not support the *DUSP1* induction. Alternatively, only a small amount of intracellular MIF may be needed to 'control' the baseline expression levels of *DUSP1.* Transfection with siRNA did not abolish MIF levels completely with the maximal level of MIF knockdown achieved being 54% of the scrambled control level at 72 hours. This opposing effect on the transactivation pathway of CS signalling could be further investigated by examining mRNA production and the release of the anti-inflammatory cytokine IL-10 (Rea et al., 2000) or on the expression of other CS-inducible genes such as GILZ (D'Adamio et al., 1997) and GITR (Nocentini et al., 1997).

Further experiments will be required to fully elucidate all CS functions that MIF counteracts and to study this within COPD or asthma; primary cells such as PBMCs, bronchial epithelial cells or alveolar macrophages may give more disease relevant results.

5.4.2 Mechanism of MIF counteraction of CS actions

The mechanisms by which MIF counteracts the CSs' action are not fully elucidated but MIF has been implicated to impact upon both transrepression and transactivation pathways of GR signalling. However, MIF seems to have more dominant effects on GR transactivation functions.

The ability of MIF to affect the function of MKP-1 was investigated in this study by analysing the ability of rhMIF to modify the actions of dexamethasone on LPS-induced p-p38 MAPK. MKP-1 is induced by dexamethasone and in turn deactivates MAPKs, preventing the signalling cascade to activate NF-κB and AP-1 gene transcription. However, I was unable to show an effect of dexamethasone on LPS-induced p-p38 MAPK. This may reflect the single time-point investigated here and therefore the timings for this specific event need to be further investigated. LPS stimulates the activation of p38 MAPK within 30 minutes but the 2 hours pre-treatment with the CS may be too long and the effect may have been missed. This longer incubation time is needed to see a difference in *DUSP1* gene transcription and therefore due to MAPK signalling occurring very transiently and quickly any effect may have been missed. In other experiments I have shown that rhMIF directly inhibits the *DUSP1* gene expression at 4 hours and this experiment had rhMIF in culture media for at least 3.5 hours. In this case, MKP-1 levels will be low and dephosphorylation of the p-p38 will not occur. Interestingly, there was a small non-significant increase in p-p38 MAPK in my study, supporting the concept that lower MKP-1 levels may augment p38 MAPK-dependent inflammation due to hyper-activation (Wu and Bennett, 2005).

Alveolar macrophages from COPD and severe asthmatic patients have been shown to have higher levels of activated p38 MAPK compared to controls and p-p38 MAPK has been shown to correlate with CS insensitivity (Bhavsar et al., 2008, Renda et al., 2008), these patients have also been shown to have hyper-activated NF-κB and AP-1 transcription factors (Barnes and Adcock, 1998). MKP-1 can de-activate other

MAPK signalling pathways, JNK and ERK1/2 MAPK each of which can activate other transcription factors. MIF, acting via MKP-1, has also been reported to affect other downstream targets such as JNK MAPK and AP-1 (Roger et al., 2005) and future studies may examine the effect of MIF on their activity.

Another mechanism by which MIF can counteract CS transactivation function is via IκBα (Daun and Cannon, 2000, Roger et al., 2005). CSs induce the synthesis of IκBα and it, in turn, inhibits NF-κB translocation into the nucleus, preventing DNA binding and transcription of pro-inflammatory genes (Verma et al., 1995). There is great controversy on this mechanism and no clear evidence for MIF effecting NF-κB activation (Kleemann et al., 2000, Lacey et al., 2003). In this study, IκBα was not evaluated but the downstream NF-κB (p65 subunit) DNA binding activity was measured. I was unable to show any effect of MIF on p65 DNA binding either on its own or in combination with dexamethasone. To confirm that MIF had no effect on the NF-κB pathway, further experiments including reporter gene assays and Western blotting for IκBα protein levels would be needed.

CSs up-regulate the expression of the anti-inflammatory cytokine IL-10 via transactivation (Rea et al., 2000). Interestingly IL-10 has been shown to inhibit MIF release (Wu et al., 1993) and conversely MIF can inhibit IL-10 (Stavitsky and Xianli, 2002). To date there are no reports on the effect of MIF on CS-induced IL-10.

It is unlikely, that CS insensitivity with respect to the control of the inflammation seen in COPD and severe asthmatic patients will be wholly down to MIF interacting with the GR transactivation pathway and reducing MKP-1 levels. GR transrepression mechanisms may also be important targets for MIF in these diseases. Pro-inflammatory gene expression is under the control of transcription factors such as NF-κB in complexes with transcriptional co-activators, which have HAT activity. This results in enhanced local histone acetylation and chromatin remodelling enabling greater transcriptional activity (Biddie et al., 2011). This change in local chromatin structure can be reversed by CSs in a process that involves activated GR (transrepression) and AP-1 (Biddie et al., 2011). For some genes, recruitment of GR to this remodelled chromatin may be affected by recruitment of co-repressor proteins that contain HDAC activity. This may affect GR acetylation status and further interaction with NF-κB and co-repressor complexes or play a role in altering the local

chromatin environment (Ito et al., 2006). There are no reports on MIF modulation of GR-associated HAT activity or on the composition of GR-transactivation complexes or on local GR-associated chromatin complexes. Although in this study, no modulation was seen by rhMIF on CS-suppressed LPS-induced pro-inflammatory mediator release it would be of interest to investigate this potential mechanism in other cells lines or PBMCs or alveolar macrophages to fully elucidate the CS sparing functions of MIF.

CS insensitivity is a major issue in treating and controlling COPD and severe asthma. Novel treatments are needed to either re-sensitise patients to the anti-inflammatory effects of CSs or new anti-inflammatory drugs are required that differ mechanistically to CSs. I have shown here that MIF interacts with CS function via the transrepression pathway. Inhibiting the counter-regulatory effect of MIF may improve CS sensitivity however the potential immunosuppression and increased susceptibility to infections may be a greater risk as seen in MIF deficient mouse models (Rodriguez-Sosa et al., 2003, Satoskar et al., 2001). Inhibiting the downstream functions of MIF, for example inducing MKP-1 expression and increasing its stability, would alleviate the infection risk and also increase the anti-inflammatory effects of CSs.

Chapter 6

The effect of MIF on global protein and gene expression

6.1 Introduction

As discussed in previous chapters, I have seen no clear functional output of rhMIF or MIF activation of pathways investigated in THP-1 monocytes. In this chapter, I used two methods to investigate the global effect of MIF stimulation on protein and RNA expression. The "Stable Isotope Labelled Amino acids in Cell culture" (SILAC) method (Ong et al., 2002) was performed to assess global changes in protein levels after MIF stimulation in THP-1 cells. Furthermore, differentially regulated genes by MIF at the mRNA level in THP-1 cells were analysed by RNA microarray.

SILAC is a novel quantitative proteomic approach for identifying changes in protein content (Ong et al., 2002). Briefly, isotopically labelled arginine and lysine ($^{13}C_6$-Arg and $^{2}H_4$-Lys) are added to amino acid deficient cell culture media and are incorporated into all synthesised proteins during regular cell proliferation/growth, producing 'heavy' proteins. Control cells are cultured with non-labelled amino acids for 'light' proteins. The labelled amino acids do not affect protein function in any way other than the protein mass. Protein lysates from all samples are mixed together as the amino acids labels are incorporated directly into the amino acid sequence of the protein and the exact ratio of heavy to light amino acids will indicate differentially expressed or tagged proteins. Where ratios do not alter it reflects a lack of protein differences. Finally the peptides are analysed by liquid chromatography-mass spectrometry (LC-MS) and further quantitated by MS, Figure 6.1.

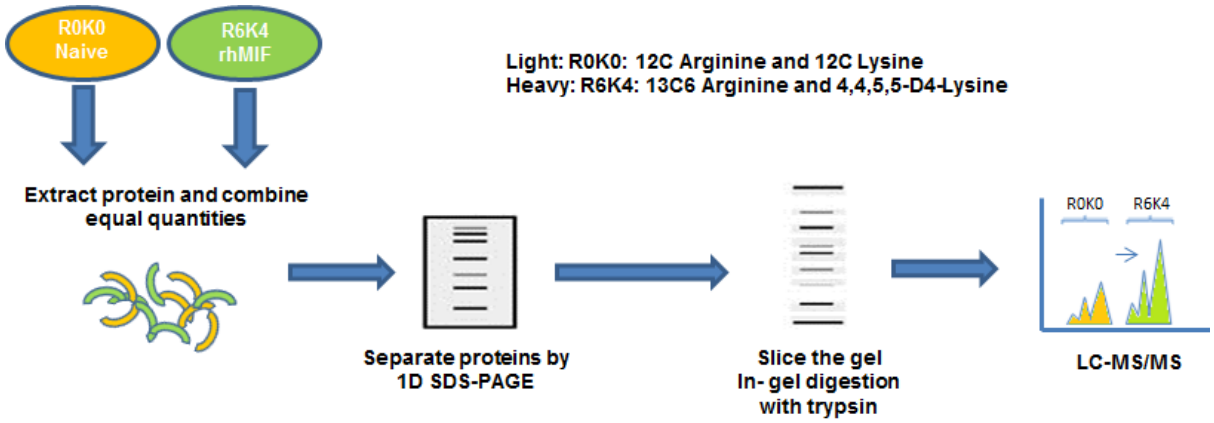


Figure 6:1: Schematic of SILAC protocol
THP-1 cells were grown in SILAC 'light' or 'heavy' media for five doublings. Protein extracts from naïve (light-labelled) and rhMIF-treated (heavy-labelled) cells were mixed, separated by SDS-PAGE and digested using trypsin. The peptides were analysed by LC-MS/MS. A shift to the right for corresponding peptides shows incorporation of the 'heavier' amino acids and hence proteins from the 'heavy' cells and treatment. The relative height of the protein/peptide peaks indicates a relative change in protein expression levels.

Genome-wide analysis of mRNA expression will provide a global analysis of gene expression changes following cell treatment with drug or between cells from different patient groups (Wheelock et al., 2013). It is the most well described and validated 'omic' technology currently used (Wheelock et al., 2013). Briefly, the RNA samples were prepared and quality assessed before being reverse transcribed and labelled with cyanine fluorophores Cy3 or Cy5. The labelled cDNA samples were then hybridised to the pre-defined probes captured onto the array (Agilent SurePrint G3 Human Gene Expression microarrays). The microarray was finally scanned and image analysis performed and subsequently the raw data was extracted from the microarray image files (Agilent Feature Extraction Image Analysis Software (Version 10.7.3)).

6.2 Chapter Aims

1. Identify novel pathways, associations and targets that are involved in MIF signalling
    a. At the protein expression level using Stable Isotope Labelled Amino acids in Cell culture (SILAC) method
    b. At the gene expression level (mRNA) using RNA microarray
2. Validate any findings from (1) above to confirm MIF involvement

6.3 Chapter data analysis

6.3.1 SILAC analysis

Trypsin digest, LC-MS/MS and the following initial protein analysis was carried out by Dr K. Heesom from University of Bristol. Raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Scientific, MA, USA). Briefly, data was searched against UniProt/SwissProt Human database release version 57.3 using the SEQUEST (ver.28 Rev. 13) algorithm. Peptide precursor mass tolerance was set at 10ppm and MS/MS tolerance at 0.8Da. Searches were performed with full tryptic digestion and a maximum of one missed cleavage was allowed. Search criteria included fixed modifications (carbamidomethylation of cysteine) and variable modifications including oxidation of methionine and appropriate SILAC labels $^{2}H_{4}$-Lys and $^{13}C_{6}$-Arg. The reverse database search option

was enabled and all peptide data was filtered to satisfy a false discovery rate (FDR) of 5%. The minimum cross-correlation factor filter was readjusted for each individual charge state separately to optimally meet the predetermined target FDR of 5% based on the number of random false positive matches, thus each data set has its own passing parameters. Quantitation was performed using a mass precision of 2ppm. After extracting each ion chromatogram the Proteome Discoverer software runs several filters to check for interfering peaks and the presence of the expected isotope pattern amongst other parameters. Peptides, which do not meet these criteria, are not used in calculating the final ratio for each protein.

Protein values were then represented as the median of the raw measured peptide ratios of naïve (light media) for the corresponding protein. I performed this second analysis. Proteins that had a 2-fold increase or decrease in expression $(x \leq 0.5)$ or $(x \geq 2)$ were identified as significantly affected by the treatment and were included in the pathway analysis. The significant protein set was transferred to the Database for Annotation, Visualisation and Integrated Discovery (DAVID) version 6.7 (http://david.abcc.ncifcrf.gov/). Pathway analysis was performed by KEGG within DAVID.

6.3.2 Microarray analysis

The array design was a direct two-colour experiment where Cy3 and Cy5 labelled signals were compared within the array. Raw two-colour microarray data were quality assessed, normalised and log transformed using the limma (ver. 2.12) and MArray packages as implemented in RStudio (ver. 0.97.551) (RStudio, MA, USA). MA-plots were viewed to assess the quality of the data (Figure 6.2) and hence pre-processing of the two-colour array data was performed. Pre-processing included background correction and data normalisation, both within the same array and across different arrays (Figure 6.3 and 6.4).

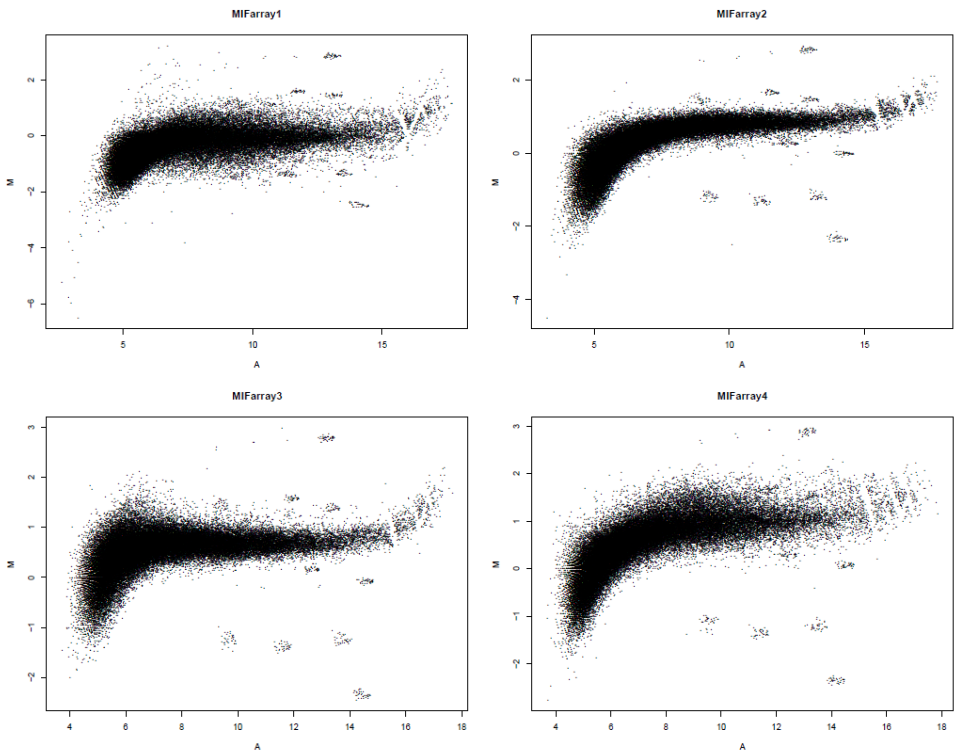


Figure 6:2: MA-plots for two-colour array data
An MA-plot is a plot of log 2-intensity ratios (M-values) versus log 2-intensity averages (A-values). The quality of the data and the necessity of data normalisation are assessed from these plots, in this experiment the data (n=4) required normalisation.

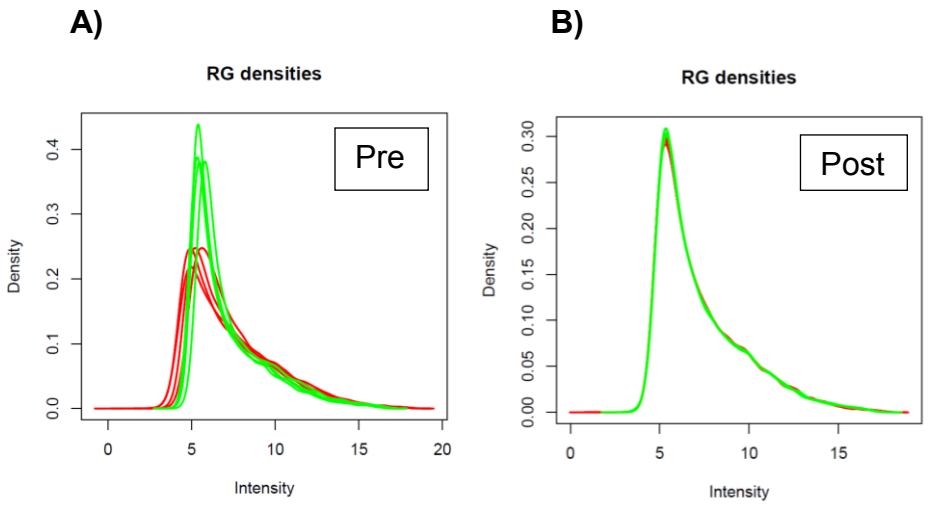


Figure 6:3: Colour densities pre and post normalisation
Before normalisation there is considerable variation between red and green channels (A). Post-normalisation within (M-values) and between (A-values) arrays makes them more comparable and equally distributed (B).

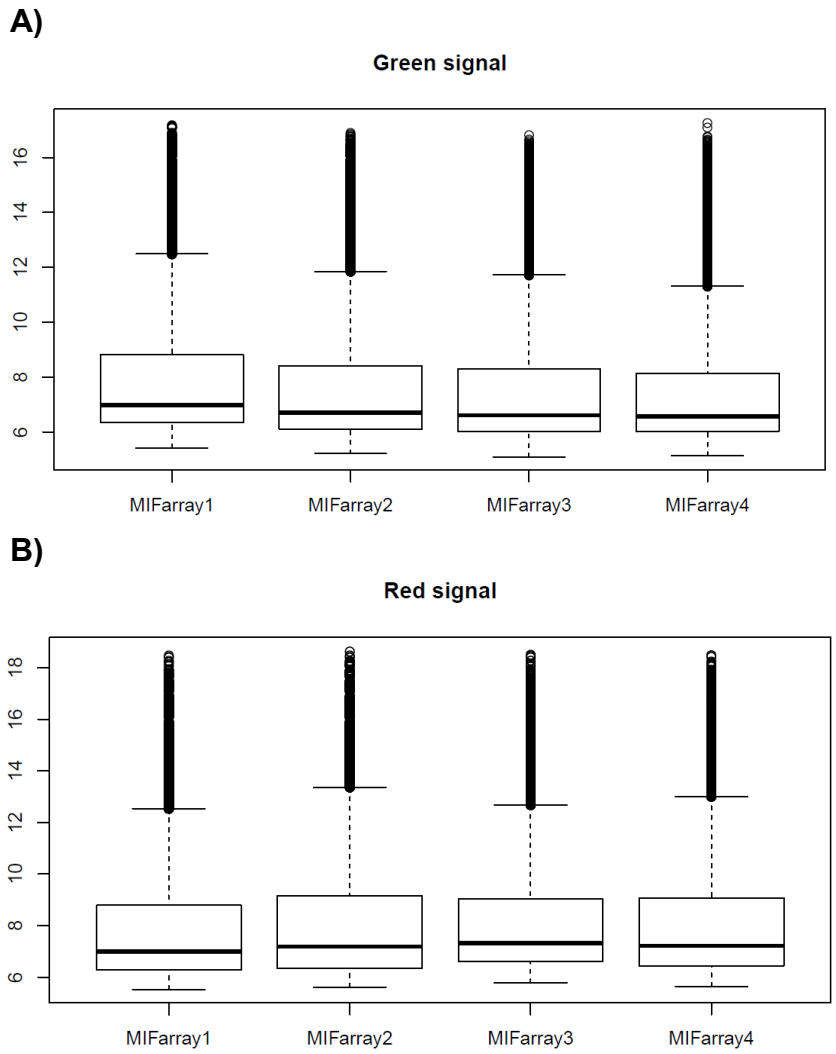


Figure 6:4: Post normalisation within arrays and between arrays
Post-normalisation box and whisker plots for (A) green signal and (B) red signal provide a graphical view of the median, quartiles, maximum, and minimum for each experimental replicate (n=4). MIFarray1-4 each represents a replicate. Outliers for each box and whisker plot are shown. This data has normalised showing equal distribution between the arrays for both colour signals.

6.4 Results

6.4.1 Global protein expression

THP-1 cells were stimulated with rhMIF (100ng/ml) for 24 hours, after which the proteins were isolated and sent for LC-MS/MS analysis. Figure 6.1 depicts the SILAC protocol.

From a 10-slice in-gel tryptic digestion 6508 total proteins could have been detected, MIF treatment on THP-1 cells identified 3767 of these total proteins at varying expression levels. A threshold of $(x \leq 0.5)$ and $(x \geq 2)$ was set and 235 proteins reached this criteria; 70 being up-regulated and 165 being down-regulated (see Appendix 1 for full differential protein lists and Figshare for full SILAC results (http://figshare.com/articles/SILAC_data_/840470). Specific proteins of interest that were identified as being up-regulated in response to MIF stimulation included T-lymphocyte surface glycoprotein CD4 and MAP kinase kinase kinase 1 (MAP3K). NAD^+-dependent protein de-acetylase sirtuin-2 (SIRT2), bromodomain-containing protein-4 (Brd4), Melanoma Differentiation Associated Protein-5 (MDA5) and Retinoic Acid-Inducible Gene 1 Protein-1 (RIG-1) were significantly down-regulated.

Pathway analysis, using the DAVID database, of the differentially expressed proteins in response to MIF stimulation identified the RIG-I-like receptor (RLR) signalling pathway as the biggest hit (Figure 6.5). In addition, calcium (Ca^{2+}) signalling was also identified as being regulated by MIF.

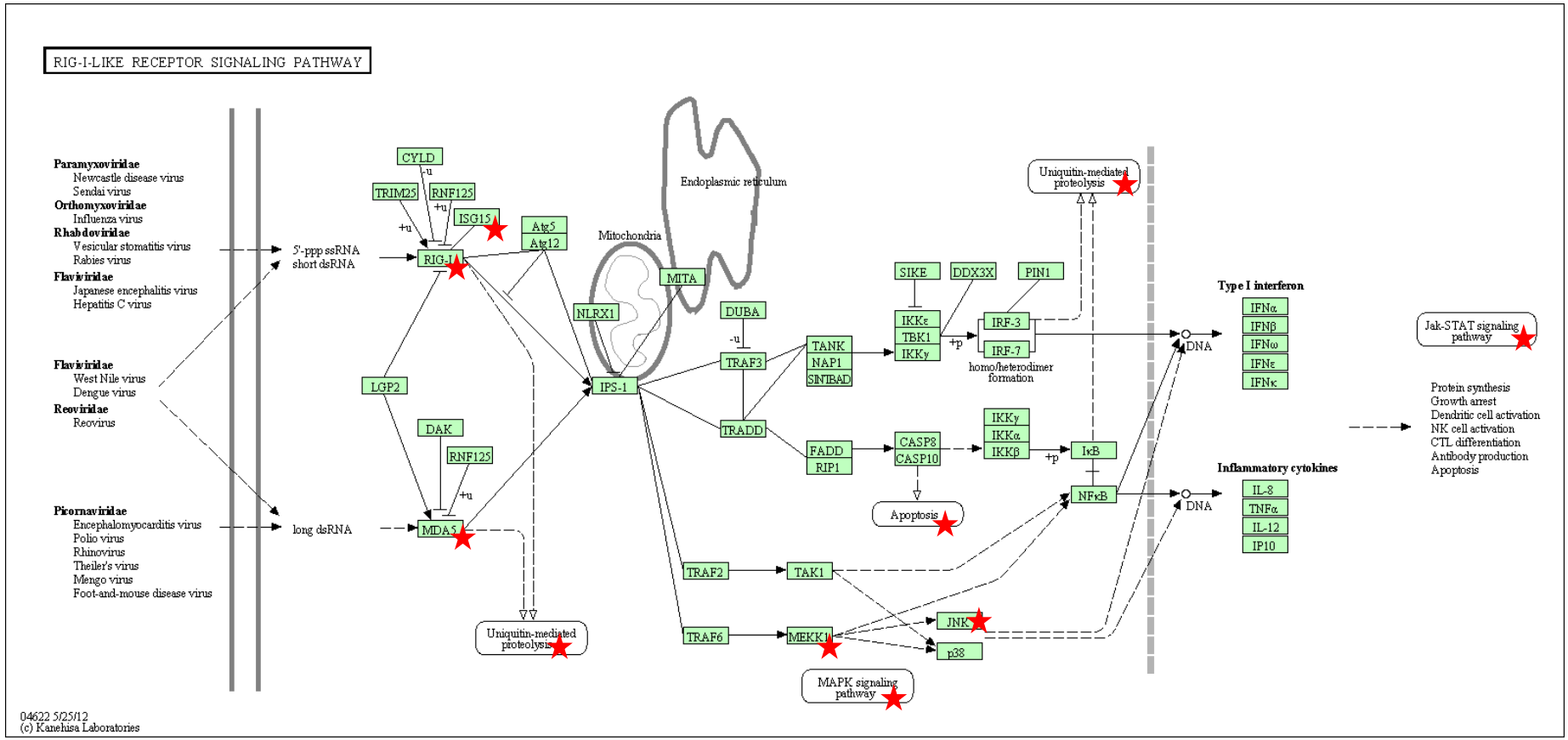


Figure 6:5: Schematic of RIG-I-like receptor (RLR) signalling pathway

THP-1 cells were treated with rhMIF (100ng/ml) for 24 hours in specific SILAC media, intracellular proteins were extracted and the lysates were analysed by LC-MS-MS. Five proteins and four processes were differentially expressed or affected by MIF stimulation as illustrated by the red stars. Modified from KEGG pathway (www.genome.jp/kegg/pathway).

6.4.1.1 RLR signalling pathway validation

The direct effect of MIF on in the RLR pathway was first investigated with regards to gene expression of proteins identified in the SILAC experiment. THP-1 cells were stimulated with rhMIF (0.1-1000ng/ml) for 4 hours before total RNA was extracted and *IFH1* (MDA5) and *DDX88* (RIG-1) gene expression levels were analysed. The gene expression of both *IFH1* and *DDX88* were not affected by MIF stimulation at the time point investigated (Figure 6.6).

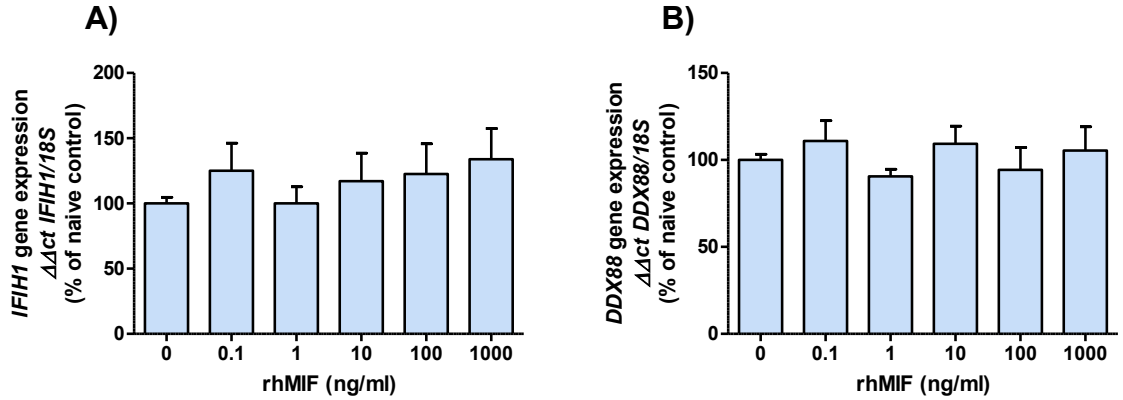


Figure 6:6: MIF has no direct effect on RLR-associated gene expression
THP-1 cells were stimulated with rhMIF (0.1-1000ng/ml) for 4 hours. Total RNA was extracted and analysed by RT-qPCR. Gene expression for (A) *IFIH1* (MDA5) and (B) *DDX88* (RIG-1) is normalised to *18S* internal control and presented as the percentage of naïve control. Results represent the mean±S.E.M. of four independent experiments.

The protein levels of MDA5 and RIG-1 proteins were assessed in monocytic THP-1 cells stimulated with rhMIF (0.1-1000ng/ml). After 24 hours incubation MDA5 and RIG-1 proteins were measured in the whole cell protein lysates by Western blotting. Neither MDA5 nor RIG-1 protein content was directly affected by MIF stimulation (Figure 6.7).

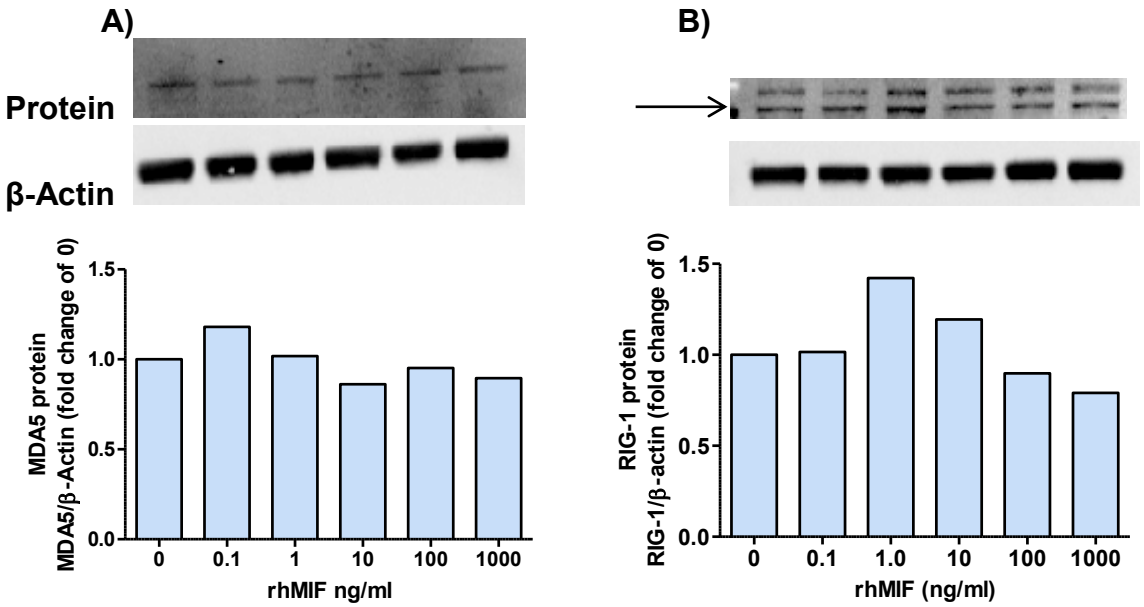


Figure 6:7: MIF has no effect on RLR-associated protein content
THP-1 monocytes were incubated with rhMIF (0.1-1000ng/ml) for 24 hours. Total intracellular proteins were extracted and Western blotting and densitometry was used to quantify (A) MDA5 and (B) RIG-1 protein expression. Each protein is normalised to β-actin as an internal control and the data is presented as fold change from naïve control. Results represent the mean of two independent experiments.

The involvement of MIF in the RLR pathway was further investigated using the RLR pathway activator, poly (I:C), a synthetic analogue of double-stranded RNA (dsRNA). THP-1 cells were stimulated with poly (I:C) (1-20μg/ml) for 4 hours, total RNA extracted and gene expression levels evaluated using qPCR. IFNβ, a type 1 interferon (*IFNB1*) was used as a positive control for the RLR pathway. As expected, poly (I:C) stimulated the expression of IFNβ in a concentration-dependent manner (287.3±35.2%, $p<0.05$ at 10μg/ml and 417.8±133.0%, $p<0.05$ at 20μg/ml) (Figure 6.8A). *MIF* gene expression was also increased in a concentration-dependent manner; however this did not reach statistical significance (Figure 6.8B).

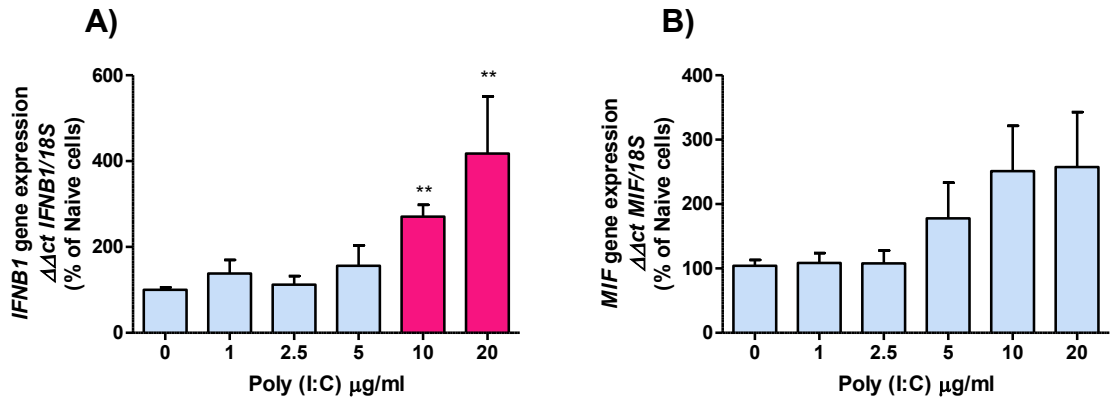


Figure 6:8: Poly (I:C) induces pro-inflammatory gene expression
THP-1 cells were stimulated with poly (I:C) (1-20μg/ml) for 4 hours. Total RNA was extracted and gene expression for (A) *IFNB1* and (B) *MIF* was analysed by qPCR. Gene of interest expression is normalised to *18S* internal control and is presented as the percentage of naïve control. Results represent the mean±S.E.M. of at least three independent experiments for each gene., ***p*<0.01 vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

MIF secretion from THP-1 cells was evaluated by ELISA after 24 hour poly (I:C) (1-20μg/ml) stimulation. MIF release was induced in a concentration-dependent manner, the two highest concentrations tested induced MIF release significantly, 219.9±28.8%, $p<0.05$ at 10μg/ml and 226.9±24.3%, $p<0.01$ at 20μg/ml, (Figure 6.9).

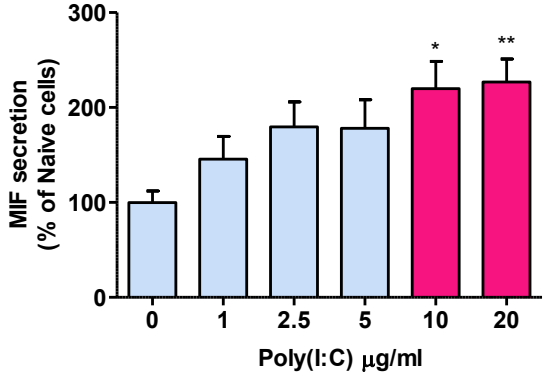


Figure 6:9: Poly (I:C) induces MIF secretion
THP-1 monocytes were treated with poly (I:C) (1-20μg/ml) for 24 hours. Conditioned medium was removed and MIF secretion was evaluated by ELISA. Data is presented as mean±S.E.M of percentage of the naïve control for six independent experiments. **p*<0.05, ***p*<0.01 vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

After demonstrating the production and secretion of MIF by synthetic viral activation the effect of RV16 infection was evaluated in THP-1 cells. Monocytic THP-1 cells were either incubated in media or infected with RV16 virus for a time course of up to 96 hours. At each time point, total RNA was extracted and *MIF* gene expression was analysed by qPCR. *MIF* gene expression did not significantly change during the 96 hour time course for the media control (Figure 6.10A); however RV16 infection significantly increased *MIF* gene expression in a time-dependent manner (Figure 6.10B). After 96 hours incubation time with RV16, *MIF* gene expression was significantly induced 4-fold (397.3±79.6%, $p<0.01$).

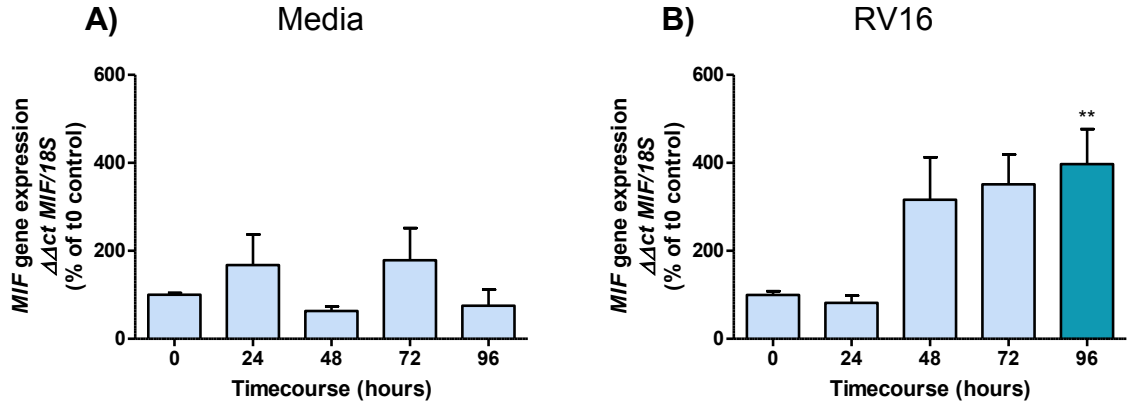


Figure 6:10: RV16 infection induces *MIF* expression
THP-1 monocytes were infected with RV16 and incubated for up to 96 hours. Total RNA was extracted and MIF expression analysed by qPCR from (A) media control and (B) RV16-infected cells. Data is presented as mean±S.E.M of the percentage of naïve control for each condition (n=3). $^{**}p<0.01$ vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

To further explore the secretion and production of MIF induced by viral activation an *in vivo* approach was taken. BALF was obtained by bronchoscopy as previously described (Mallia et al., 2013) at baseline and 7 days post experimental-induced RV16 infection of age-matched healthy non-smokers (n=18), healthy smokers (n=15) and COPD patients (n=17) (Table 6.1), the samples were provided by Dr. J. Footit. BAL macrophages were isolated from BALF and subsequently whole cell proteins were extracted and analysed for MIF content by ELISA. Intracellular BAL macrophage MIF levels were not affected by RV16 infection in non-smokers. However, in smokers and COPD patients, there was a significant decrease in the

amount of intracellular MIF detected in BAL macrophages post-RV16 infection. The average MIF content at baseline was 68.3±16.7ng/ml and 70.0±18.3ng/ml for smokers and COPD patients respectively compared to post-RV16 infection values of 40.8±8.3ng/ml and 26.4±6.7ng/ml for smokers and COPD patients respectively, $p<0.05$ for both groups (Figure 6.11).

Table 6:1: Patient demographics for RV16 infection

GROUP	AGE	SEX (M/F)	*N* NUMBER	PACK YEARS	FEV_1 (% PRED)	FEV_1/FVC RATIO (%)
Non smoker	59(46-71)	9/10	19	N/A	106± 4	79± 1
Smoker	54(41-66)	8/7	15	32(21-51)	99± 3	77± 2
COPD	59(44-72)	13/5	18	39(25-57)	65± 2	58± 2

COPD patients are GOLD Stage 2 as diagnosed by ATS guidelines. Normally distributed data is represented as mean±SD and data not normally distributed is represented as median (25-75 quartiles)

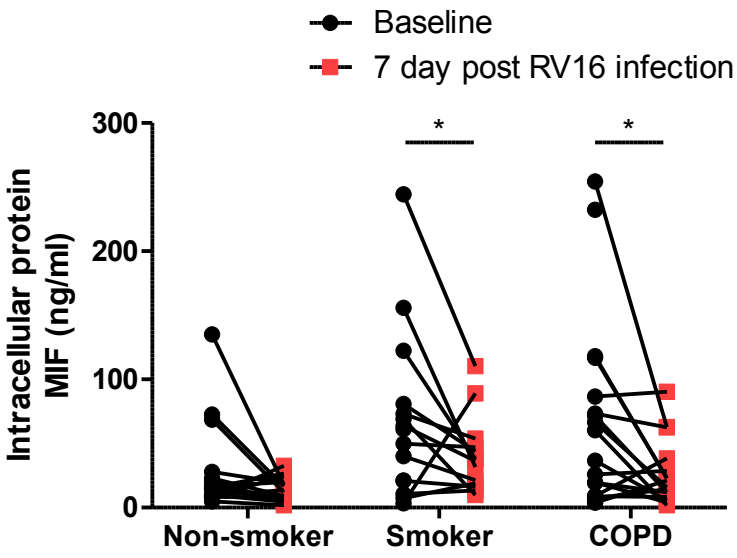


Figure 6:11: RV16 infection reduces intracellular MIF content in BAL macrophages
BAL macrophages were isolated from BALF taken from non-smokers (n=18), smokers (n=15) and COPD patients (n=17) at baseline and 7 days post RV16 infection. Intracellular MIF was measured by ELISA in protein lysates extracted from the BAL macrophages. Data is presented as intracellular MIF protein levels (ng/ml) for each subject, pre- and post RV16 infection. *$p<0.05$ as calculated by individual Mann-Whitney t-test.

6.4.1.2 Calcium signalling validation

The association between calcium (Ca^{2+}) signalling and MIF identified by the SILAC experiment was also assessed. THP-1 cells were incubated with FURA-2 AM to fluorescently label intracellular Ca^{2+}. Cells were stimulated with rhMIF (0-1000ng/ml) and the Ca^{2+} released from intracellular stores was measured. Ca^{2+} release from intracellular stores was concentration-dependent (Figure 6.12A and B). To confirm the concentration-dependent induction of Ca^{2+} release I extended the concentration curve to include non-physiological concentrations of MIF (0-5000ng/ml). This is because the MIF-induction of Ca^{2+} release was relatively small and it was possible that the diluent was having a small effect. However, after controlling for an increase in diluent the rhMIF even at supra-physiological levels produced a concentration-dependent induction of Ca^{2+} release (Figure 6.12C and D).

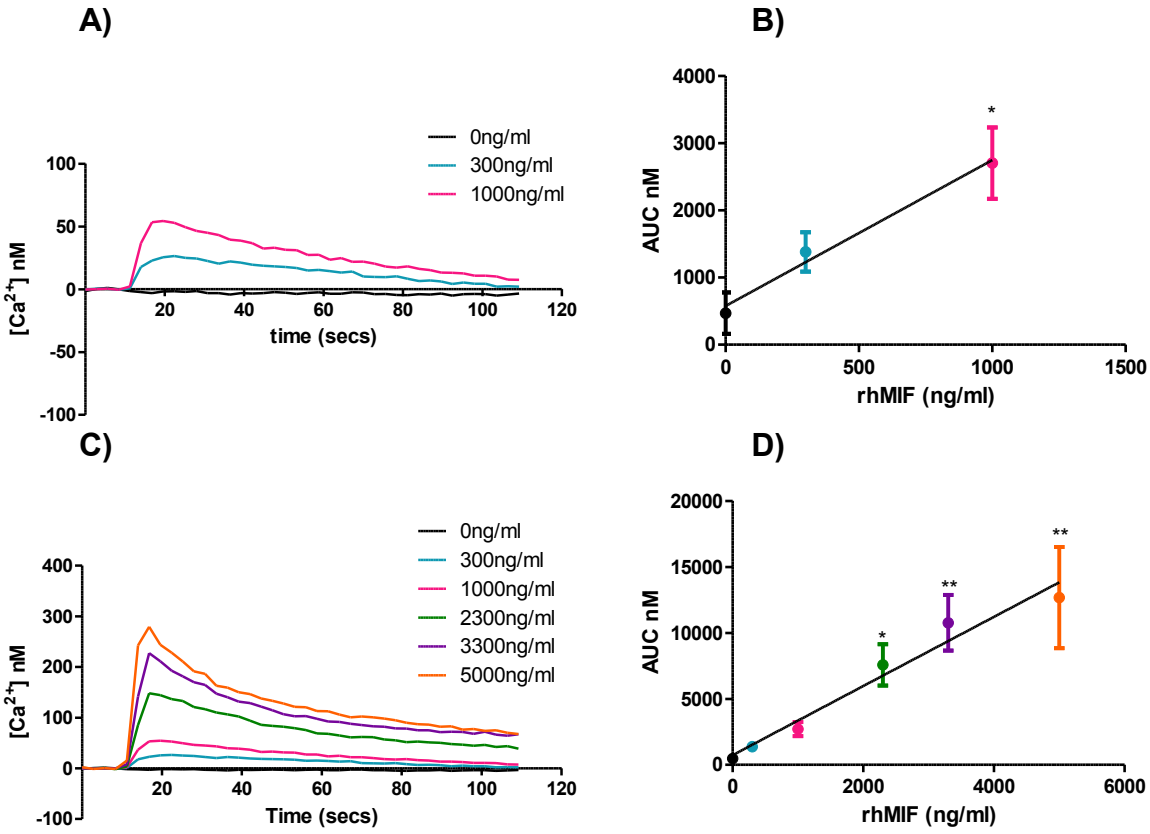


Figure 6:12: Calcium signalling is induced by MIF stimulation
Intracellular calcium was fluorescently labelled in THP-1 cells. rhMIF-induced calcium release was measured by detection of the fluorescence signal every 2.8 seconds. Physiological MIF concentrations (300-1000ng/ml) induced a time-dependent induction of Ca2+ release (A) which was quantified using the area under the curve (AUC) (B). The time course results for all MIF concentrations (0-5000ng/ml) are shown in (C) and AUC quantification reported in (D). *p<0.05, **p<0.01 vs. naïve control as calculated by ANOVA with Dunns post-hoc test.

In resting cells, nuclear factor of activated T cells (NFAT) is phosphorylated and resides in the cytoplasm (Beals et al., 1997, Okamura et al., 2000). An elevation of Ca^{2+} activates phosphatase calcineurin, which in turn dephosphorylates NFAT (Shaw et al., 1995). NFAT then translocates to the nucleus to bind to its gene promoter region (Shibasaki and McKeon, 1995). The expression/activation of the calcium dependent transcription factor NFAT was assessed to investigate the downstream effects of MIF-induced calcium release. THP-1 monocytes were stimulated with rhMIF (100ng/ml) for up to 60 minutes. Nuclear proteins were extracted and the binding capacity of NFAT to the canonical DNA response element was evaluated by TransAM® assay. rhMIF had no effect on the binding capacity of NFAT to its DNA consensus motif (Figure 6.13).

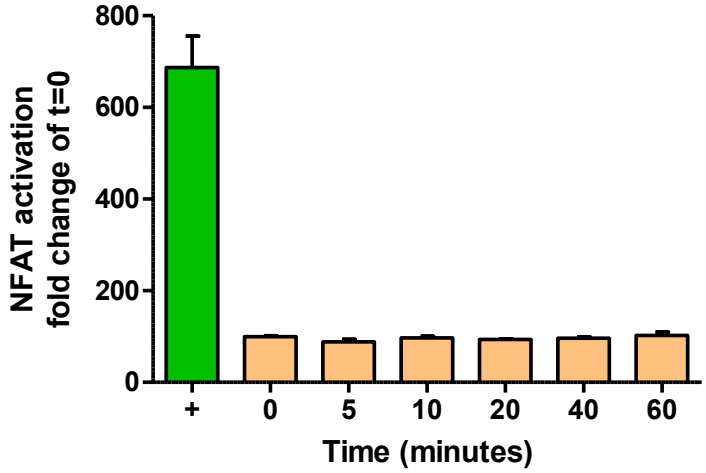


Figure 6:13: NFAT binding capacity to response element over time
THP-1 cells were incubated with 100ng/ml rhMIF for up to 60 minutes. Nuclear proteins were isolated and the capacity of NFAT to bind to DNA was measured, positive control (+; green bar) was included in kit (PHA-treated Jurkat cells). Data is reported as the fold change compared to 0 hours and presented as the mean±S.E.M. of four independent experiments.

6.4.2 Global RNA expression

THP-1 monocytes were stimulated with or without rhMIF (100ng/ml) for 4 hours (n=4), after this time total RNA was extracted and quantified. A concentration of 17ng/µl total RNA for both naïve and MIF treated samples were sent for microarray analysis. Raw data was pre-processed and normalised as previously described; differential gene expression by MIF stimulation was assessed using an Empirical

Bayes linear model approach. The default method to adjust *p*-values for multiple testing was the Benjamini-Hochberg (BH) method, the BH method controls for the FDR (Benjamini and Hochberg, 1995). FDR was set to 5% in this array experiment. The results for this array experiment indicated that no genes were directly affected by 4 hours stimulation of MIF. A table of the top 20 genes with the lowest adjusted *p*-value (FDR) are shown in Table 6.2. As no significant hits were seen I did not perform qPCR validation on these samples.

Non-significant genes and long non-coding RNA (lncRNA) with fold change $(x \geq 1.5)$ or $(x \leq 0.7)$ were extracted from the array results to generate new hypotheses. This resulted in 27 up-regulated and 210 down-regulated genes and lncRNAs (Appendix 2); the top 10 hits for up and down regulated genes are shown in Table 6.3. A full list of genes and lncRNAs detected from the RNA array are available on Figshare (http://figshare.com/articles/RNA_microarray_results/840472). Even though the expression changes were non-significant pathway analysis was performed on these biggest hits to identify new areas of research that could warrant further investigations with regards to MIF stimulation. The pathway analysis program (DAVID) recognised 191 genes and identified glycolysis/gluconeogenesis, vascular endothelial growth factor (VEGF) signalling and B-cell receptor signalling as pathways involving the biggest hit genes. All 23 non-significant lncRNAs were searched against the Ensembl database (http://www.ensembl.org/index.html) and any known lncRNAs were then further investigated by internet searches and LNCipedia (http://lncipedia.org/).

Table 6:2: Top 20 non-significant genes with lowest adjusted *p*-value

UP-REG GENE ID	GENE NAME	GENE	*P*-VALUE	ADJ. *P*-VALUE	FOLD CHANGE
NR_024244	Small ILF3/NF90-associated RNA G2 small nuclear RNA	SNAR-G2	0.032	0.479	1.39
A_33_P3402773	Uncharacterised		0.027	0.479	1.36
NM_014977	Apoptotic chromatin condensation inducer 1 transcript variant 1	ACIN1	0.023	0.479	1.24
NM_006887	ZFP36 ring finger protein-like 2	ZFP36L2	0.015	0.479	1.23
NR_003016	Small nucleolar RNA, H/ACA box 26	SNORA26	0.031	0.479	1.28
XM_003119593	Hypothetical protein		0.011	0.479	1.47
NM_001031847	Carnitine palmitoyltransferase 1A	CPT1A	0.029	0.479	1.24
NM_138459	Nuclear undecaprenyl pyrophosphate synthase 1 homolog	NUS1	0.041	0.479	1.21
NM_003760	Eukaryotic translation initiation factor 4 gamma, 3	EIF4G3	0.017	0.479	1.21
DOWN-REG GENE ID	**GENE NAME**	**GENE**	***P*-VALUE**	**ADJ. *P*-VALUE**	**FOLD CHANGE**
NM_021177	LSM2 homolog, U6 small nuclear RNA associated	LSM2	0.008	0.479	0.78
NM_002661	Phospholipase C, gamma 2	PLCG2	0.042	0.479	0.85
A_24_P401090	Uncharacterised		0.031	0.479	0.85
NM_001127320	Ubiquitin associated protein 2-like transcript variant 2	UBAP2L	0.023	0.479	0.81
NM_003104	Sorbitol dehydrogenase transcript variant 1	SORD	0.005	0.479	0.75
NM_014845	FIG4 homolog	FIG4	0.010	0.479	0.72
NR_003267	Gamma-glutamyltransferase 3 pseudogene	GGT3P	0.027	0.479	0.83
NM_021130	Peptidylprolyl isomerase A	PPIA	0.027	0.479	0.81
NM_005692	ATP-binding cassette, sub-family F member 2	ABCF2	0.011	0.479	0.66
NM_052916	Ring finger protein 157	RNF157	0.006	0.479	0.78
NM_001042416	Zinc finger protein 596	ZNF596	0.027	0.479	0.83

Up-regulated (top) and down-regulated (bottom) genes

Table 6:3: Top 10 non-significant greatest fold change

Up-reg Gene ID	Gene Name	Gene	*P*-value	Adj. *P*-value	Fold Change
NR_003271	Small nucleolar RNA, C/D box 3B-1	SNORD3B-1	0.001	0.479	2.00
NM_020535	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 5A	KIR2DL5A	0.091	0.522	1.92
NR_040108	Uncharacterized LOC728175 non-coding		0.066	0.500	1.89
NR_004400	RNA variant U1 small nuclear 18	RNVU1-18	0.001	0.479	1.79
NM_004113	Fibroblast growth factor 12	FGF12	0.010	0.479	1.68
NR_034143	Cancer susceptibility candidate 14 non-protein coding	CASC14	0.114	0.543	1.60
ENST00000419440	^^	Lnc-C11orf39-3	0.010	0.479	1.60
TCONS_00009863	^^	Lnc-ADCY2-4	0.105	0.535	1.59
NR_027030	Uncharacterized protein MGC34034		0.058	0.490	1.57
NM_153267	MAM domain containing 2	MAMDC2	0.001	0.479	1.55
Down-reg Gene ID	**Gene Name**	**Gene**	***P*-value**	**Adj. *P*-value**	**Fold Change**
NM_016536	Zinc finger protein 571	ZNF571	0.062	0.493	0.37
AJ276510	Partial mRNA for hypothetical protein	ORF1	0.066	0.500	0.38
TCONS_00029565	^^	Lnc-CRYBA4-8	0.076	0.509	0.40
ENST00000441356	^^	Lnc-RND3-1	0.193	0.614	0.40
NM_001822	Chimerin 1	CHN1	0.040	0.479	0.41
NM_004797	Adiponectin, C1Q and collagen domain containing	ADIPOQ	0.166	0.590	0.42
TCONS_00022613	^^	Lnc-RP11-1070N10.3.1-1	0.100	0.531	0.43
TCONS_00019700	^^	Lnc-STARD10-1	0.112	0.541	0.43
XR_109045	Hypothetical LOC100507282		0.044	0.479	0.45
NM_018711	SV2 related protein homolog	SVOP	0.110	0.540	0.45

Up-regulated (top) and down-regulated (bottom) genes (≥1.5 or ≤0.7)
^^ Represents long non-coding (lnc) RNAs

6.5 Discussion

6.5.1 SILAC proteomic analysis

There were 70 up-regulated proteins and 165 down-regulated proteins that were identified through the SILAC experiment. Differentially expressed proteins that were of interest with regards to COPD research include the up-regulation of $CD4^+$ T-lymphocytes (Hogg et al., 2004) and MAP3K involvement in chronic pro-inflammatory cytokine release (Tudhope et al., 2008) and down-regulation of the SIRT2, a protein that has been associated with aging (Wang et al., 2007, North et al., 2003) and BRD4, a protein involved in the recruitment and function of transcription factors (e.g. NF-κB) on chromatin (Huang et al., 2009).

6.5.1.1 RIG-I-Like Receptor (RLR) pathway

RIG-1, melanoma differentiation-associated protein 5 (MDA5) and RIG-I -like receptor LGP2 (LGP2) belong to the RLR family of intracellular pattern recognition receptors (PRR) (Yoneyama et al., 2005, Yoneyama et al., 2004, Andrejeva et al., 2004). Both RIG-1 and MDA5 were reduced by MIF stimulation as detected by the SILAC experiment. These particular RLRs are involved in the recognition of viral double-stranded RNA (dsRNA) and contain DExD/H box RNA helicase domains. However only RIG-1 and MDA5 possess two caspase recruiting domain (CARD)-like domains and these induce the cellular response (Saito et al., 2007, Yoneyama et al., 2005, Rothenfusser et al., 2005). The recognition of viral dsRNA by RLRs is cell-type and viral-specific (Wang et al., 2009). Initiating the RLR pathway leads to the production of Type 1 IFNs including IFNα and subtypes, IFNβ, IFNω and IFNε as well as pro-inflammatory cytokines IL-6 and TNFα via NF-κB or IRF3 transcription factors (Honda et al., 2006). RLR activation also results in natural killer cell activation, the production of antibodies and the stimulation of viral-specific cytotoxic T-lymphocytes, which leads to the activation of the adaptive immune response (Longhi et al., 2009). Mice deficient in RIG-1 or MDA5 are more susceptible to viral infections compared to wild type mice (Kato et al., 2006, Errett et al., 2013). However, in some strains of mice RIG-1 deficiency is not viable and they show embryonic lethality due to liver degeneration (Kato et al., 2005).

The SILAC experiment identified five proteins and four processes within the RLR pathway as being differentially expressed and hence affected by MIF stimulation. MIF stimulation had no direct effect on the gene expression or protein levels of *IFIH1*/MDA5 or *DDX88*/RIG-1 in THP-1 cells as measured by qPCR and Western blotting respectively. The discrepancy between the Western blotting and SILAC experiments could be due to the mass spectrometry being more sensitive and thus detecting smaller changes that would not be seen with the Western blotting technique. Alternatively, the SILAC approach may be detecting protein hits due to alterations in post-translational modifications (PTMs) and changes in protein-protein interactions (PPI). Further investigations of the effects of MIF on PPI in the RIG-I pathway may yield additional interesting areas of research.

Similar to RLRs, TLR3 is a PRR that recognises viral dsRNA and induces the activation of transcription factors (NF-κB and IRF3) to produce type 1 IFNs. In contrast to the RLRs, TLR3 can be expressed on the cell surface (Matsumoto et al., 2003) or as an endosomal receptor (Alexopoulou et al., 2001). Poly (I:C) is a synthetic dsRNA analogue that activates TLR3 and induces the production of type 1 IFNs, if transfected into cells, poly (I:C) can also be recognised by intracellular sensors RIG-1 and MDA5 (Gitlin et al., 2006, Kato et al., 2005). In support of previous reports showing poly (I:C)-induced MIF production in uterine epithelial cells (Schaefer et al., 2005), stimulating THP-1 monocytes with poly (I:C) also induced MIF secretion and increased *MIF* gene production. Further supporting the association with MIF, RLR and TLR stimulation and viral infection. Future experiments examining the ability of MIF to modify the poly (I:C)-induced RIG-I pathway may also give insight as to how MIF affects cellular function.

There is a growing body of evidence that the lungs are not sterile as previously thought and characterisation of the lung microbiome has generated great interest (Erb-Downward et al., 2011, Sze et al., 2012). Viral nucleic acid has been detected in COPD lung tissue, BALF and sputum samples in stable and exacerbating (episodes of worsening symptoms and may require the patient to seek medical help) COPD patients (Seemungal et al., 2001, Rohde et al., 2003, Wedzicha, 2004, Macek et al., 1999, Papi et al., 2006, Utokaparch et al., 2013). Viral infection is an associated factor for initiating an exacerbation in COPD patients, with rhinovirus

(RV) being the most common and accounting for 58% of the viruses detected during an exacerbation (Seemungal et al., 2001, McManus et al., 2008).

To date there are no publications reporting on MIF and RV infection however there have been reports on the association between MIF and Influenza virus. Elevated levels of MIF induced by infection with H5N1 influenza virus were reported in a mouse model (Hou et al., 2009). MIF content in primary NHBE cells reduced in a time-dependent manner post infection with Influenza Virus A, this was paralleled with an increase in secreted MIF (Arndt et al., 2002).

RV16 infection can be recognised by RIG-1, MDA5 or TLR3 (Slater et al., 2010) and THP-1 cells infected with RV16 showed a time-dependent induction of *MIF* gene expression. Furthermore, RV16 infection reduced the intracellular MIF levels in BAL macrophages isolated from smokers and COPD patients and I propose that the MIF has been secreted from the macrophages in response to the viral infection. However, MIF levels in the BALF supernatant were not measured in these patients due to no sample remaining for analysis and so this hypothesis needs to be confirmed in a separate study. It is not known whether the RV16-induced MIF production is via RIG-1, MDA5, TLR3 or all of them and this would need further investigations to elucidate. It would also be of interest to look at the direct effects of MIF on TLR3 production, when stimulating cells with bacterial LPS, there is a positive loop of cytokine production and production of TLR4 (Roger et al., 2001).

From these studies, I would speculate that MIF is involved in viral-induced exacerbations instead of in a stable disease state. RV16 infection induces MIF release which may in turn drive a pro-inflammatory response and effect CS sensitivity. Patients with stable COPD do not have elevated levels of MIF and so a direct effect of MIF on the enhanced sensitivity of COPD bronchial epithelial cells to respond to viral infection compared to controls (Professor Donna Davies, Southampton University, personal communication) is unlikely to reflect an effect of these MIF levels. Further investigations will be needed to elucidate this exciting novel area of MIF function within COPD research.

6.5.1.2 Calcium signalling

Ca^{2+} is major intracellular secondary messenger that can regulate many cellular functions including cell proliferation (Lu and Means, 1993, Berridge, 1995), gene transcription (Crabtree, 1999) and apoptosis (Kass and Orrenius, 1999). It was previously understood that Ca^{2+} signalling was a rapid response such as during neurotransmission (Douglas and Rubin, 1961) yet it is now understood to have functions that occur after some substantial delay, for example the regulation of gene transcription (Bading et al., 1997). In this study, MIF induces Ca^{2+} release in THP-1 cells in a concentration-dependent manner, which is supported by data published in rat testicular peritubular cells (Wennemuth et al., 2000). In this latter study however, the concentrations of MIF-induced Ca^{2+} reached ≈1100nM compared to 25nM with 50ng/ml rhMIF, this difference may be cell-specific, species-specific or the rhMIF used. Wennemuth and colleagues produced their own rhMIF (Wennemuth et al., 2000) but previous studies have shown LPS contamination in some bacterially expressed rhMIF (Kudrin et al., 2006, Wennemuth et al., 2000) whereas a commercially available rhMIF was used in this study. Moreover, an earlier publication had shown that MIF did not alter Ca^{2+} uptake in guinea pig macrophages (Block et al., 1978).

Ca^{2+} cannot transduce a transcriptional signal in its own right and therefore requires intermediate messenger proteins. These act by transducing the Ca^{2+} activation signal into a protein cascade to signal to transcription factors and co-activators such as CREB, NFAT and NF-κB and cause increased gene expression. There are three main Ca^{2+} signalling pathways each involving different kinase proteins, 1) calmodulin-dependent kinases 2) MAPK proteins specifically Ras (Cullen and Lockyer, 2002) lead to transcription regulated by CREB and NF-κB (Hardingham et al., 2001, Lilienbaum and Israël, 2003) and 3) phosphatase calcineurin leads to NFAT regulation (Feske et al., 2003, Hogan et al., 2003). I further investigated the THP-1 MIF-induced Ca^{2+} release by assessing the downstream transcription factor NFAT and its DNA binding capacity to consensus region. However at the times studied, there was no MIF-enhanced DNA binding of NFAT.

The limitations with this study include the restricted number of time-points studied; future work could include ChIP analysis to detect specific NFAT binding to inducible

genes. NFAT is expressed and activated in monocytes and myeloid cells, however, its expression in these cells is low and it often requires a strong inducer such as RANKL, a TNF family member, to activate NFAT (Yarilina et al., 2011). NFAT function is also much better understood in T-lymphocytes (Hogan et al., 2003). The other Ca^{2+} signalling pathways will need to be investigated to fully understand and confirm the MIF-induced Ca^{2+} release described.

It is possible that several other pathways may be important in MIF function in these cells. The pathway analysis database identifies pathways containing known proteins and therefore any unknown or undiscovered proteins/peptides detected during the mass spectrometry analysis will not be recognised and will fail to flag a potential MIF-modulated pathway.

6.5.2 RNA microarray- mRNA analysis

In this study, there were no genes that were significantly differentially expressed after 4 hour MIF stimulation. This is in contrast to MIF-modulated genes assessed by qPCR at the same time point, such as *DUSP1*, *TP53* and *MIF* itself, as shown in previous chapters. If the MIF-induced gene expression modulations are small then these may not be detected in an array analysing ≈62000 genes and lncRNAs and therefore using a more focussed array might identify smaller but significant gene changes induced by MIF. It is also noteworthy that significant gene modulations may not have been detected by the microarray due to a lack of statistical power and therefore increasing the *n* number of experiments would alleviate this issue. Gene expression changes occur at different times after stimulation for different genes and investigating one time point (4 hours) will only snapshot a specific moment. Investigating different time points using a time course will give a more accurate result into the role of MIF regulating genes.

Even though no significant genes were detected within the FDR cut-off, microarrays can still be used as a hypothesis-generating tool. Usually a cut off of fold change (>1.5) is the final criteria to identify differentially expressed genes. For hypothesis-generating microarray analysis the FDR cut-offs can also be changed, either to a less restrictive value or a more restrictive value if many genes are identified for example 0.01 or 1%. The genes that are detected using these less restrictive analyses can then be further analysed using pathway analysis to identify potential

signalling pathways. In this experiment, non-significant genes with a fold change $(x \geq 1.5)$ or $(x \leq 0.7)$ were investigated and pathway analysis competed to identify potential new research areas.

To date, specific lncRNAs have not been associated with COPD or severe asthma and their functions still remain to be fully understood. Many mechanisms of lncRNAs functions have been described, including acting as an RNA decoy by preventing transcription factors binging to promoter regions (Kino et al., 2010) and recruiting chromatin-modifying complexes to their DNA target (Wang et al., 2011b). Another suggested role for lncRNAs is acting as a microRNA sponge and preventing the microRNAs binding to their mRNA targets (Cesana et al., 2011). The expression of a number of lncRNAs were modulated by MIF, including the down regulation of Lnc-RND3-1. Information on Lnc-RND3-1 is very limited however hsa-miR-3148, hsa-miR-211-3p and hsa-miR-629-3p are known to be targeting microRNAs and could be mopped up by the lncRNAs. Investigating this microRNA further may elucidate a function for Lnc-RND3-1 and association with MIF. Interestingly hsa-miR-3148 has been shown to negatively regulate TLR7 expression in SLE patients (Deng et al., 2013). In this case, MIF-reduced expression of Lnc-RND3-1 would result in more available hsa-miR-3148 and hence lower TLR7 expression. Viral infection is recognised by TLR7 (Lund et al., 2004). This is in support of the new viral and MIF hypothesis I have speculated in this thesis as viral susceptibly may be increased due to reduced expression of TLR7 in a similar fashion to reduced RLRs. Further investigations would be needed to confirm the MIF-modulated lncRNAs and viral susceptibility.

From the ‘biggest hit’ genes, the glycolysis and gluconeogenesis pathway was identified as ‘active’ during MIF stimulation; interestingly this is a major area of research for COPD patients. COPD severity has been associated with increased muscle loss and weakness (Maltais et al., 2000) and alterations in macronutrient metabolism (Franssen et al., 2011). Alterations to insulin sensitivity and diabetes are well-established clinical implications for increased risk of CVD that in turn is a major co-morbidity of COPD (Sin et al., 2005) there has been no reports on the involvement of MIF. Another pathway that was suggested through pathway analysis was the VEGF pathway. Both MIF and VEGF are understood to be monocytes’ chemo-attractants and their expression is positively correlated in human

glioblastomas (Munaut et al., 2002). It has been reported that MIF directly induces VEGF gene expression in a concentration- and time-dependent manner in human endometrial cells (Bondza et al., 2008). VEGF is induced in hypoxic conditions caused by cigarette smoke, a risk factor of COPD, and has been shown to be elevated in COPD patients (Pavlisa et al., 2010).

However, it must be strongly emphasised that these gene and lncRNA changes were not statistically significant and the pathways identified by the proteomic and transcriptomic approaches are not comparable. Therefore the significance of the RNA microarray results, including the gene and lncRNA expression changes are uncertain.

Even though there were proteins that had enhanced or reduced levels after MIF stimulation, as detected by the SILAC technique, this was not paralleled by an increase or decrease in the corresponding gene production measured by the array. However this discrepancy may be due to the presence of PTMs. PTMs are chemical modifications of a protein after it has been translated, there are many different PTMs such as phosphorylation, glycosylation, ubiquitination, methylation and proteolysis; there are ≈200 different types of PTMs (Krishna and Wold, 1993).

A PTM that may explain the discrepancy between the SILAC experiment and the RNA microarray could be ubiquitination. Proteins tagged with the 8kDa polypeptide ubiquitin are targeted for degradation (Ciechanover et al., 1980, Hershko et al., 1980, Goldstein et al., 1975). If transcription levels are constant but there is reduced ubiquitination (degradation rate) this would lead to an increase in protein content and hence give the results shown here but this would need to be confirmed.

Studies have shown that PTMs including methylation and acetylation occur on histones and subsequently controlling gene transcription (Adcock et al., 2006). Regulation of gene transcription involves the acetylation of histones by enzymes with HAT activity. HDAC enzymes reverse the acetylation and increase chromosomal condensation preventing gene transcription. Oxidative and nitrative stress for example from cigarette smoking can also cause PTMs, effecting protein levels and activity, for example reducing HDAC2 by increasing proteasomal degradation (Ito et al., 2005, Osoata et al., 2009). COPD has also been associated with PTMs on transcription factors GR (Bodwell et al., 1998, Matthews et al., 2011) and NF-κB

(Gloire and Piette, 2009) that are either in response to oxidants from cigarette smoking or effect sensitivity to CSs. To date, the regulation of PTMs on HDAC or HAT proteins by MIF has not been investigated. However it must be emphasised that from the RNA array data, MIF did not affect the transcription of any genes or lncRNAs and so these PTMs are not likely to be MIF mechanisms of action. The disconnect between the proteomic and transcriptomic data maybe due to the times chosen and the effects of MIF may need longer than 4 hours to show any transcriptional control. The optimal time at which MIF may control transcription will need extensive investigations.

Phosphorylation is a common PTM that controls and regulates the activity of the protein, for example kinases during signal transduction. MIF is not a kinase, as it does not have phosphorylation ability however MIF may have a PTM role via its enzymatic function. Although to date no physiological substrate is known. PTMs would need to be investigated to elucidate if MIF has a role in this mechanism. Furthermore, the potential of MIF to regulate proteins by PTMs in viral exacerbations of COPD and other chronic inflammatory diseases should be investigated.

Proteomic and transcriptomic studies were an effective way to identify potential new interactions and pathways modulated by MIF stimulation. The downstream functions of MIF have yet to be fully elucidated; however the methods described here have identified new targets of MIF. The involvement of MIF in the viral RLR pathway and the Ca^{2+} signalling needs further confirmation although the results are encouraging.

Chapter 7

General Discussion

7.1 General Discussion

The hypothesis, that elevated MIF drives chronic inflammation and CS insensitivity in COPD, was first tested *in vivo*, in a 6-week chronic ozone-induced mouse model of COPD airway inflammation (Triantaphyllopoulos et al., 2011, Wiegman et al., 2013). The model used here was CS insensitive and showed chronic lung inflammation with increased recruitment of immune cells, increased pro-inflammatory mediators in BALF and increased pulmonary resistance and airway hyper-responsiveness. The levels of MIF in BALF were elevated in the COPD model compared to controls. Concordant with the literature and in support of the hypothesis, inhibition of MIF does reduce and prevent inflammation in *in vivo* models of inflammation (Chen et al., 2010, Korsgren et al., 2000, Makita et al., 1998). Overall, cellular recruitment to the lung was significantly reduced after ISO-1 pre-treatment as was airway hyper reactivity; further supporting the hypothesis that MIF is a key driver of inflammation in COPD. However, in contrast to the attenuation of inflammation, CS sensitivity was not restored by MIF inhibition in the ozone-induced model. To date, the restoration of CS sensitivity by MIF inhibition has not been reported in this or any other *in vivo* COPD model. Different doses of dexamethasone will need to be assessed in the model to determine whether the dose of CS used here was too powerful for the MIF effect to be detected. In addition, another MIF inhibitor such as the more potent 4-IPP (Winner et al., 2008), should be used to confirm the findings shown with ISO-1. The results demonstrated here were in support of the hypothesis (*in vivo* results summarised in Figure 7.1) and I went on to investigate and translate the expression of MIF in human patient samples with MIF function in human cells.

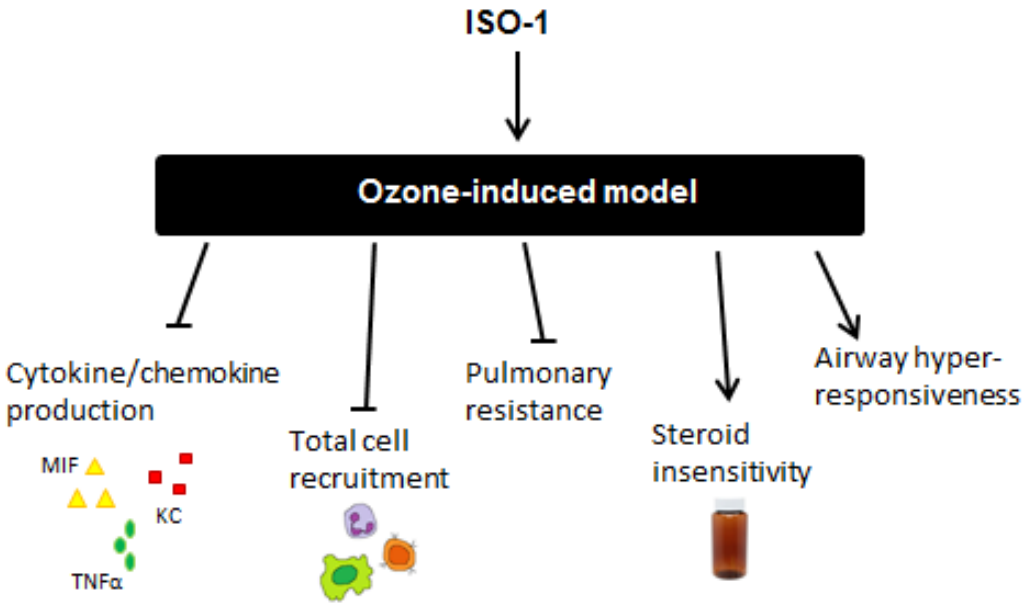


Figure 7.1: Illustration summarising the results from *in vivo* ISO-1 pre-treated ozone-induced inflammatory model (chapter 3)
Inhibition of MIF by ISO-1 resulted in attenuation of some aspects of inflammation in an ozone-induced mouse model. Cytokine and chemokine release in BALF, total cellular recruitment and pulmonary resistance was reduced after pre-treatment with ISO-1 compared to no ISO-1 treatment before ozone exposure. CS insensitivity and airway hyper-responsiveness was not affected by ISO-1 pre-treatment before ozone exposure.

Results in the human experiments were less supportive of the hypothesis that MIF is a key driver of inflammation in the airway. MIF was characterised in samples taken from age-matched healthy non-smokers, healthy smokers and stable COPD patients. There was no clear difference in MIF expression between healthy controls and patient groups in serum and lung biopsy samples or protein lysates isolated from BAL macrophages. This was in contrast to the reported differences in serum MIF expression in a disease-severity dependent manner for asthmatics (Rossi et al., 1998) and RA patients (Morand et al., 2002). MIF expression increased in a stepwise fashion in induced sputum samples from age-matched non-smokers, smokers and COPD. However, only the difference between non-smokers and COPD patients was significant. Unfortunately, due to the lack of BALF and immunohistochemistry samples from healthy non-smoker controls it is not possible to conclude whether MIF was elevated due to smoking or other COPD-associated factors.

As MIF was elevated in the mouse model of COPD and is frequently described in literature as a potent pro-inflammatory cytokine the role of MIF driving inflammation was investigated. The immortalised cell line THP-1 and PBMCs and tissue macrophages from age-matched non-severe and severe asthmatics, COPD patients and controls were used in a LPS-induced inflammatory model. Initial experiments in the primary cells showed an attenuation of LPS-inflammation by MIF inhibition. This was more pronounced in the diseased cells and therefore may indicate an alteration in the MIF function or activity. The ISO-1 attenuation of LPS-induced cytokine release was supported by previous reports by Adamali and colleagues in PBMCs isolated from cystic fibrosis patients (Adamali et al., 2012). In stark contrast, MIF inhibition had no effect on LPS-induced CXCL8 or TNFα release in THP-1 cells. Stimulation with rhMIF confirmed the lack of effect in the LPS-stimulated inflammatory model. MIF stimulation did not further enhance the release of LPS-induced CXCL8 or TNFα, which is supported by earlier studies (Amano et al., 2007). As previously discussed, continuing the experiments in primary cells would have been advantageous, but with the intensity of the investigations needed to elucidate the role of MIF in COPD and the numbers of patients to reduce the natural variation, THP-1 cells were used. Nevertheless MIF stimulation and the inhibition of MIF on LPS-induced mediators should be investigated in different human primary cell types such as epithelial or ASMCs as CD74 and MIF staining was also detected in these cells (data not shown). MIF functions seem to vary between cells and are dependent on the role of that specific cell-type in the immune system.

Activation of CD74, the putative MIF receptor (Leng et al., 2003), was investigated to act as a positive control for rhMIF activity. Stimulation with rhMIF and α-CD74 antibodies both induced the baseline levels of CXCL8 but not TNFα release in THP-1 cells, which is comparable to current literature (Beswick et al., 2005, Binsky et al., 2007), supporting the notion that the rhMIF was biologically active and signals via CD74 activation. However the slight pro-inflammatory function on CXCL8 release was not investigated in primary cells and due to the disconnect between primary and THP-1 cells seen previously, future work will need to be completed in order to confirm this finding. It is also worth noting that rhMIF, ISO-1 and α-CD74 gave inconsistent results on the expression and release of MIF itself. Further investigation into baseline rhMIF-induced cytokine release would confirm whether ISO-1

specifically inhibits MIF and/or signals through the CD74 receptor. Moreover, these effects were not seen in the LPS model and therefore the importance of the modulations at baseline is questionable when investigating the role of MIF in inflammatory diseases.

My original hypothesis also stated that MIF modulates the CS sensitivity seen in COPD patients and severe asthmatics. The lack of modulation of CS sensitivity in the murine model is comparable to the results found in THP-1 cells, showing no MIF modulation of the CS transrepression mechanism with regards to *IL8* and *TNFA* gene expression or CXCL8 or TNFα release in THP-1 cells. However with regards to the CS transactivation mechanisms, MIF counter-acts the CS-induced *DUSP1* gene expression, which is in agreement with other reports (Aeberli et al., 2006a, Aeberli et al., 2006b, Roger et al., 2005). Moreover, MIF inhibition by ISO-1 and MIF siRNA knockdown increased the sensitivity of THP-1 cells to dexamethasone shown by an enhanced induction of *DUSP1* gene expression at baseline and in the LPS-stimulated model. This finding supports the hypothesis of MIF potentially being a mechanism of inducing CS insensitivity. To assess whether the LPS concentration used here was too high and hence overpowered the MIF counteraction, lower concentrations of LPS should be investigated. Further evidence to support the assumption that MIF counteracts CSs via the GR-transactivation pathway instead of the GR-transrepression pathway is also required. For example, additional investigation into CS-inducible genes (*IL10* and *GILZ*) (Nocentini et al., 1997, D'Adamio et al., 1997) and GR transcriptional activity is needed to validate the role of MIF in the transactivation mechanism. Investigations on MIF-modulation of the GR-transrepression pathway are also needed to confirm that MIF is not involved in this mechanism. I also demonstrated that MIF enhanced THP-1 proliferation through an effect on *TP53* expression and activation of the p38 MAPK pathway. Further experimental work is needed to confirm this data in primary cells and potentially see if this could interfere with CS actions on cell proliferation.

Studying the control of CSs by MIF in other cell types will also be essential as MIF will not only effect monocytes but other cell types for example epithelial or T-lymphocytes. Finally, investigating whether MIF can counteract different clinically relevant CSs such as fluticasone propionate or budesonide would further confirm this unique role of MIF and confirm that it is not a dexamethasone-specific effect. MIF

seems to be a very cell-specific cytokine that may have differing roles at higher and lower concentrations. Future work to enable this would include, investigating the modulation of CS-transactivation by MIF in animal models of COPD and in primary cell experiments. Much more research is required to understand the outcomes of MIF signalling, how MIF drives inflammation and how MIF can restore CS sensitivity (MIF-modulated inflammatory results are summarised in Figure 7.2).

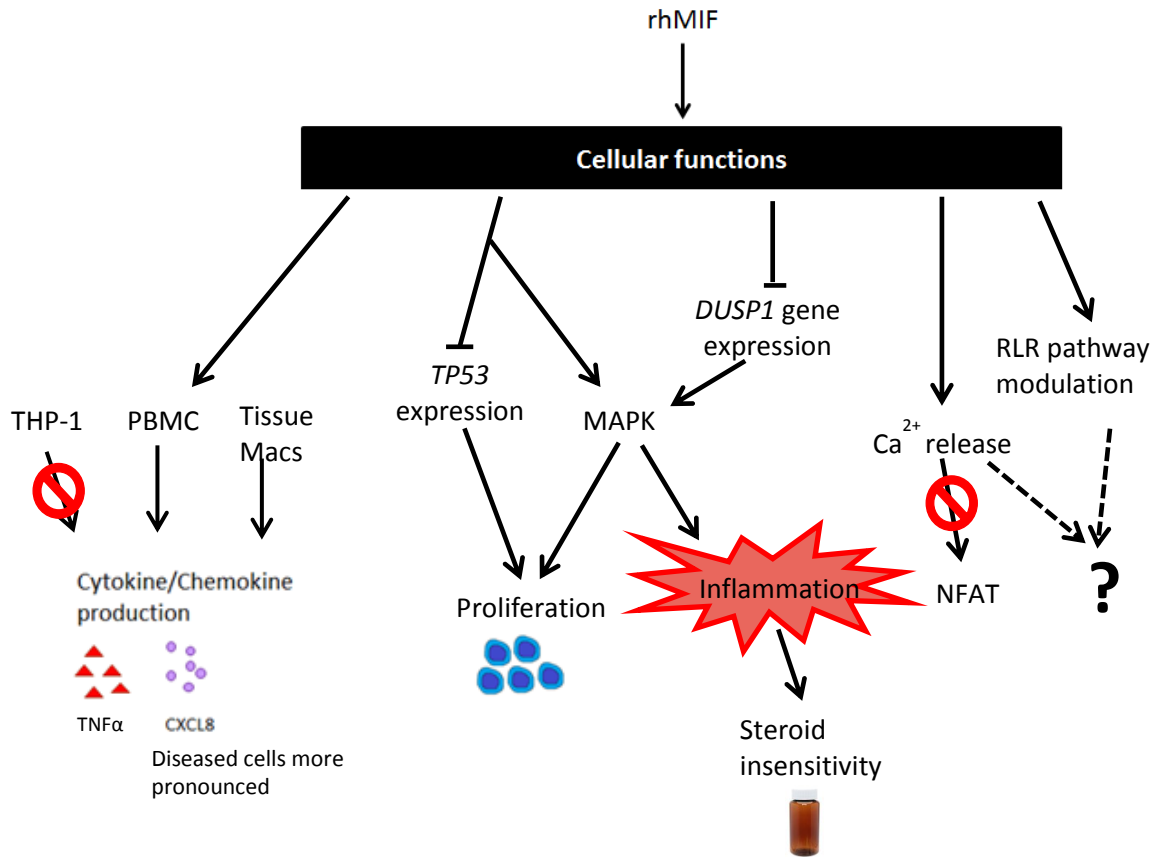


Figure 7.2: Illustration summarising main results from MIF stimulation (chapters 4-6) MIF stimulation resulted in cell-type specific cytokine release. THP-1 cells released no CXCL8 or TNFα in response to MIF stimulation. However cells from severe asthmatics and COPD patients showed a more pronounced ISO-1-suppressed LPS-induced cytokine release than in cells from non-severe asthmatics and non-COPD patients respectively. MIF-induced proliferation could be via MIF-suppressed *TP53* expression or through p38 MAPK activation. MIF reverses the dexamethasone-induced *DUSP1* production and in turn increases MAPK signalling, this could be a potential mechanism for CS insensitivity. MIF induced Ca^{2+} release however did not signal through NFAT. RV16 Infection induced the production and release of MIF in THP-1 and RV16 infection of healthy smokers and COPD patients resulted in reduced intracellular MIF in BAL macrophages.

Overall, from the data presented here I am rejecting my original hypothesis. In contrast to the murine models MIF is not generally elevated in human COPD patients and MIF is not a potent inflammatory mediator in the human cells studied here. Even though I have demonstrated that MIF reverses the transactivation mechanism of CSs in THP-1 cells. It is unlikely that sensitivity of CSs is modulated by MIF enough to cause the characteristic CS insensitivity in COPD.

One important result from this thesis is the discrepancy between the *in vivo* murine ozone-induced inflammatory model and the subsequent translation into humans. Interestingly much of the inflammatory and mechanistic MIF research has been completed *in vivo* or in rodent cells *ex vivo* and therefore whether the same mechanisms and functions occur in human cells and models will need to be investigated. Rodent but especially mouse models are the basis of biomedical research and aid in identifying candidate agents for clinical trials and investigating the effects of drugs in an organism. However, there are many examples were drugs work well in mice but fail to show an efficacious effect once tested in humans for sepsis (Hotchkiss and Opal, 2010, Mitka, 2011).

Animal models of disease differ greatly to that of the human disease. Firstly they have to be induced 'artificially' and are usually modelled on a disease phenotype. Therefore, the animal model of disease may not reproduce the same disease at the molecular level. Ultimately, this could be a different disease being modelled. MIF may have a role in chronic inflammation, as elevated levels have been shown in many chronic inflammatory diseases including, RA (Morand et al., 2002), atherosclerosis (Burger-Kentischer et al., 2002), SLE (Rovensky et al., 1975) and asthma (Rossi et al., 1998), however the mechanism and stimulus may not be precisely the same to the mouse model of disease. Ozone-induced inflammation shows COPD-associated features such as inflammatory markers and CS insensitivity (Triantaphyllopoulos et al., 2011, Wiegman et al., 2013). However compared to COPD patients the diseases differ in complexity and no model has all the characteristics of the disease. COPD is not caused by the presence of one single stimulus or inducer alone (ozone, cigarette smoke or pollutants) but is a complex disease associated with many risk factors (Mannino and Buist, 2007); therefore it is very difficult to model in animals. The genomic sequence of MIF differs between mice and human, there is 90% homology between the human *MIF* gene

and the mouse *mif* gene (Bozza et al., 1999). This could affect the function, the activity and/or the binding ability of MIF. For these reasons, the discrepancy between the ozone-induced COPD model and the MIF profiles in human samples can be explained. Different *in vivo* models of COPD, for example cigarette-induced inflammation or LPS-instillation will need to be investigated to confirm that MIF drives airway inflammation. It would also be of interest to examine ISO-1 attenuation in asthma mouse models using either OVA or HDM/CFA challenge, this would also assess whether the stimulus/challenge-method directly effects MIF induction or if MIF release is controlled by the immune response. It is also possible that ISO-1 is having off-target effects in the mouse and therefore showing a different response to that in humans.

Single-cell *in vitro* assays are also difficult to compare to *in vivo* models that contain multiple cells types, tissues and organs. Cells signal to activate different cells/cell types and to initiate multiple responses and/or signalling cascades. The presence of the liver, that metabolises drugs and agents into more or less active metabolites, may have a greater or lesser effect on attenuating or augmenting a response is also potentially crucial in affecting drug efficacy and drug interactions. These changes would not be seen *in vitro* and would be difficult to assess without animal models. In single-cell experiments these interactions between cells and tissues are lost due to the simplicity of the model and therefore are not taken into account. However the effects on the single cell type and the signalling pathways activated by the stimuli can be investigated much easier without these complications in simplified models. Monocytes were chosen as the single cell to investigate in this thesis as macrophages have been shown to predominantly express MIF (Calandra et al., 1994), however investigating other cell types, both immune and structural for example T-lymphocytes and epithelial cells, is essential and future work could include co-culturing different cell types to assess the role of MIF in a multiple cellular model. An alternative approach would be to culture biopsy samples with ISO-1 to examine its effects in a complex disease-relevant tissue.

Human primary cells (PBMCs and tissue macrophages) and the human immortalised cell line (THP-1) gave different responses to stimuli and MIF inhibition. There are advantages and disadvantages to both primary and immortalised cells however mechanisms and functions should be confirmed in both to validate the finding.

Primary cells are a better model for humans and disease state, however getting the samples and appropriate cell numbers can be difficult, especially in severe subgroups of disease. Primary cells also have a short life span and can change phenotype as they divide. Whereas immortalised cell lines are better characterised with less variation and do not change with passages. However different immortalised cells of the same cell type can give different responses, therefore most mechanistic work is initially done in immortalised cells and then confirmed in primary cells. Much of the data demonstrated here in the THP-1 cells, especially the counteraction of MIF on CS-transactivation mechanisms should be confirmed in primary cells such as tissue or BAL macrophages or PBMCs.

Proteomic and transcriptomic methods were used to generate new hypotheses and identify novel associations between MIF and COPD and severe asthma. The SILAC experiment reported numerous proteins that had been significantly affected by MIF stimulation however the RNA microarray gave no significant differentially expressed genes. Although, there were no MIF-modulated gene changes detected by my RNA microarray, pathway analysis identified glycolysis/gluconeogenesis, VEGF signalling and B-cell receptor signalling as pathways involved. Pilot experiments could be performed to establish any modulation by MIF, as these pathways are associated with COPD. Nonetheless, it must be noted that the changes were not significant in the array and therefore the relevance is uncertain. Repeating the experiment with greater *n* numbers would increase the statistical power of the experiment and may detect changes. MIF-induced no differential gene expression changes after multiple testing. Using more focussed arrays or using RNA-seq as a more sensitive technique (Sirbu et al., 2012) may detect smaller but significant changes induced by MIF stimulation. The MIF effects after multiple time points should also be assessed as MIF could affect gene expression very transiently or in a delayed response.

One main issue with the proteomic and transcriptomic studies was the discrepancy between the results. Changes detected at the protein level did not correlated with corresponding gene changes; this could be due to PTMs, most likely to be caused by changes in the stability or degradation of the proteins. Confirmation of this speculation would be needed and could include investigating PTMs by MIF on associated proteins and assess the functional effect on either protein activity or ability to bind and complex. Furthermore, modulation of PTMs present on

transcriptional co-activators and co-repressors associated with GR function and on GR itself by MIF could affect CSs functions and should be investigated.

The SILAC method identified the RLR pathway as interacting with MIF, which was further investigated in THP-1 cells as well as human samples. Whilst MIF did not seem to play a role in LPS driven inflammation or stable COPD, the role of MIF during viral infection and associated pathways should be further examined. Western blotting, pharmacological inhibitors, siRNA and co-immunoprecipitation could be used to assess the specific roles and activity status of MDA5 and RIG-1 proteins and the respective associations with MIF. This would elucidate the role these proteins play in the viral-induced MIF pathway. There are very few published reports on MIF and viral infection, however in a mouse model of alphavirus-induced arthritic inflammation, deficiency of the MIF receptor CD74, leads to attenuation of tissue inflammation and disease severity (Herrero et al., 2013). Studying the attenuation of inflammation, the CS action and the potential restoration of CS sensitivity by MIF inhibition in an *in vivo* viral-infected model of COPD would confirm the role of MIF in exacerbating COPD-associated inflammation. COPD patients can have many exacerbations per year and during any stage of disease, from mild to severe, however they become more frequent as the disease severity increases (Hurst et al., 2010). Exacerbations lead to a sudden decline in health and an increase in disease severity (Donaldson et al., 2002, Burge and Wedzicha, 2003) and either early detection or preventative treatment would help in controlling COPD progression. Measuring MIF in patients' samples collected during an acute viral exacerbation would confirm this viral induction of MIF. Future experiments to investigate this viral-MIF association could be to assess a viral exacerbation on top of an inflammatory-induced model *in vivo*. Moreover, the effect of MIF inhibition (ISO-1 and 4-IPP) or MIF knockout mice in this exacerbating COPD inflammatory model would further validate the previous findings, and again validate findings and further investigations in primary cells. The proteomic approach of SILAC was effective and integral in identifying a novel pathway associated to MIF.

The aim of this thesis was to investigate whether MIF has a role in COPD and severe asthma inflammation and to assess if MIF was a mechanism for inducing the disease characteristic CS insensitivity. The *in vivo* murine model supported the hypothesis however this was not true in LPS-induced human cell models. MIF was

shown to modulate the CS transactivation mechanism by counteracting dexamethasone-induced *DUSP1* gene expression but had no effect on genes regulated via the CS transrepression mechanism. Overall I have rejected my original hypothesis; MIF has no role in stable COPD. However, the SILAC results indicate that MIF may be involved in response to viral infection. Therefore, I propose a new hypothesis that viral-induced MIF and not bacterial-induced MIF may drive the inflammation during exacerbations and/or heighten the immune response to the viral infection; this may lead to or worsen the CS insensitivity seen in COPD patients.

Chapter 8

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Chapter 9

Appendix

Appendix 1

** Unknown proteins

SILAC: Up-regulated proteins in response to MIF stimulation

Entry name	Protein names	Gene names	Length	Ratio
A6NCQ5	Methionine sulfoxide reductase B2	MSRB2	159	2.173
A6NGR9	Maestro heat-like repeat-containing protein family member 6	MROH6	719	100
A6NMY6	Putative annexin A2-like protein	ANXA2P2	339	100
A8MVM3	**			100
A8MY34	**			8.215
A8MYY3	**			100
B3KSZ7	cDNA FLJ37361		697	100
B3KVZ3	Centromere protein H	CENPH	228	100
B3KY83	Retinoic acid receptor RXR-alpha	RXRA	365	9.076
B4DEU5	L-fucose kinase	FUK	180	100
B7Z3B9	GRB2-associated-binding protein 1	GAB1	591	100
B7ZKT6	Son of sevenless homolog 2	SOS2	1,299	100
C9IYU0	**			100
C9J1C1	Cysteine protease ATG4B	ATG4B	66	100
C9J6U6	**			100
C9J7Y4	Cytoskeleton-associated protein 2	CKAP2	97	100
C9JID5	Transmembrane protein 40	TMEM40	157	100
C9JYM0	Ribonuclease P protein subunit p20	POP7	137	100
C9K0J2	Uridine phosphorylase 1	UPP1	29	100
D6RB81	Alpha-methylacyl-CoA racemase	AMACR	367	100
E2QRG7	4-hydroxybenzoate polyprenyltransferase, mitochondrial	COQ2	328	100
E7ETX8	**			100
E7EUC5	**			100
E9PE41	**			100
E9PEZ1	Cullin-9	CUL9	2,489	100
F1D8N3	Estrogen nuclear receptor beta variant b	NR3A2	495	49.843

F5GWQ7	**			11.065
F5H3Y6	**			83.851
F5H480	T-cell surface glycoprotein CD4	CD4	148	100
O60486	Plexin-C1	PLXNC1	1,568	100
O94888	UBX domain-containing protein 7	UBXN7	489	100
O95394	Phosphoacetylglucosamine mutase (PAGM)	PGM3	542	100
O95801	Tetratricopeptide repeat protein 4	TTC4	387	3.258
P12931	Proto-oncogene tyrosine-protein kinase Src	SRC	536	81.132
P13196	5-aminolevulinate synthase, nonspecific, mitochondrial	ALAS1	640	100
P28749	Retinoblastoma-like protein 1	RBL1	1,068	10.077
Q13233	Mitogen-activated protein kinase kinase kinase 1	MAP3K1	1,512	9.443
Q13362	Serine/threonine-protein phosphatase 2A 56kDa regulatory subunit gamma isoform	PPP2R5C	524	100
Q14814	Myocyte-specific enhancer factor 2D	MEF2D	521	100
Q15818	Neuronal pentraxin-1	NPTX1	432	9.619
Q2TAM9	Tumor suppressor candidate gene 1 protein	TUSC1	212	100
Q4G0J3	La-related protein 7	LARP7	582	8.955
Q5HYC2	Uncharacterized protein KIAA2026	KIAA2026	2,103	100
Q5I2W7	1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial	CYP24	372	100
Q5T0N5	Formin-binding protein 1-like	FNBP1L	605	100
Q702N8	Xin actin-binding repeat-containing protein 1	XIRP1	1,843	100
Q86WS4	Uncharacterised protein C12orf40	C12orf40	652	100
Q8IVG5	Sterile alpha motif domain-containing protein 9-like	SAMD9L	1,584	7.949
Q8IWR0	Zinc finger CCCH domain-containing protein 7A	ZC3H7A	971	19.816
Q8N655	Uncharacterised protein C10orf12	C10orf12	1,247	100
Q8NFC6	Biorientation of chromosomes in cell division protein 1-like 1	BOD1L1	3,051	10.325
Q96JG9	Zinc finger protein 469	ZNF469	3,925	100
Q96L73	Histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific	NSD1	2,696	100
Q99797	Mitochondrial intermediate peptidase	MIPEP	713	100
Q99961	Endophilin-A2	SH3GL1	368	9.119
Q9BQE5	Apolipoprotein L2	APOL2	337	100
Q9BRH9	Zinc finger protein 251	ZNF251	671	100
Q9H5Q4	Dimethyladenosine transferase 2, mitochondrial	TFB2M	396	8.013

Q9NPD8	Ubiquitin-conjugating enzyme E2 T	UBE2T	197	7.832
Q9NQP4	Prefoldin subunit 4	PFDN4	134	8.430
Q9NTZ6	RNA-binding protein 12	RBM12	932	100
Q9NUQ2	1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	AGPAT5	364	9.544
Q9UIS9	Methyl-CpG-binding domain protein 1	MBD1	605	10.580
Q9UJ71	C-type lectin domain family 4 member K)	CD207	328	100
Q9UPW5	Cytosolic carboxypeptidase 1	AGTPBP1	1,226	5.344
Q9Y2U9	Kelch domain-containing protein 2	KLHDC2	406	100
Q9Y512	Sorting and assembly machinery component 50 homolog	SAMM50	469	7.386
Q9Y6I9	Testis-expressed sequence 264 protein	TEX264	313	9.750

SILAC: Down-regulated proteins in response to MIF stimulation

Entry name	Protein names	Gene names	Length	Ratio
A2A3F3	Transient receptor potential cation channel subfamily M member 3	TRPM3	1,566	0.01
A2AB27	Guanine nucleotide-binding protein-like 1	GNL1	264	0.418
A6NHL2	Tubulin alpha chain-like 3	TUBAL3	446	0.094
A6QL64	Ankyrin repeat domain-containing protein 36A	ANKRD36	1,941	0.01
A8K968	cDNA FLJ77757		756	0.049
A8MWV3	Osteoclast-associated immunoglobulin-like receptor	OSCAR	161	0.01
B0QY86	MICAL-like protein 1	MICALL1	210	0.093
B1AH77	Ras-related C3 botulinum toxin substrate 2	RAC2	148	0.01
B4DE86	Protein NDRG2	NDRG2	367	0.01
B4DH53	MAP1S light chain	MAP1S	1,033	0.420
B4DI70	cDNA FLJ53509, highly similar to Galectin-3-binding protein		413	0.496
B4DJX9	Syntaxin 16	STX16	139	0.01
B4DK85	Glutaryl-CoA dehydrogenase, mitochondrial	GCDH	394	0.359
B4DP52	HCG2005638	DDX39B	350	0.01
B4DPV4	Graves disease carrier protein	SLC25A16	234	0.01
B4DSZ9	E3 ubiquitin-protein ligase TTC3	TTC3	612	0.01
B4DUI8	cDNA FLJ52761, highly similar to Actin, aortic smooth muscle		332	0.01

B4E2Q6	Regulation of nuclear pre-mRNA domain-containing protein 2	RPRD2	934	0.075
B4E2X3	Methyltransferase-like protein 13 (cDNA FLJ56024)	METTL13	698	0.086
B5MDE0	Protein RFT1 homolog	RFT1	502	0.432
B7Z207	3-phosphoinositide-dependent protein kinase 1	PDK1	281	0.01
B7Z6M1	Plastin-3	PLS3	585	0.01
B7Z8H8	ADP-ribosylation factor GTPase-activating protein 1	ARFGAP1	293	0.01
B7ZM37	PDE3B protein	PDE3B	1,061	0.01
C9JGW8	**			0.092
C9JKR2	Albumin, isoform CRA_k	ALB	417	0.01
C9JN15	Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	PPIG	246	0.390
C9JR33	NAD-dependent protein deacetylase sirtuin-2	SIRT2	73	0.171
C9JRE4	Tudor domain-containing protein 1	TDRD1	717	0.01
C9JS68	Thromboxane-A synthase	TBXAS1	150	0.01
C9JSP5	**			0.1
C9JUG1	5'-AMP-activated protein kinase subunit gamma-2	PRKAG2	163	0.474
D6RBS5	ELMO domain-containing protein 2	ELMOD2	168	0.455
E5RJV7	Serine/threonine-protein kinase Sgk3	SGK3	109	0.01
E7EMU3	**			0.01
E7EMV0	Protein diaphanous homolog 1	DIAPH1	863	0.01
E7ENF8	RalA-binding protein 1	RALBP1	207	0.01
E7EPR3	Gamma-interferon-inducible protein 16	IFI16	733	0.085
E7EQS7	**			0.01
E7ERA4	**			0.01
E7ESA8	U4/U6 small nuclear ribonucleoprotein Prp31	PRPF31	450	0.097
E7ETY2	Treacle protein	TCOF1	1,488	0.429
E7EU54	**			0.064
E7EWJ5	**			0.01
E9PBK4	Protein NRDE2 homolog	NRDE2	933	0.491
E9PE69	**			0.368
E9PEW8	Haemoglobin subunit delta	HBD	104	0.01
E9PGY2	HEAT repeat-containing protein 2	HEATR2	280	0.079
E9PS71	Peroxisomal biogenesis factor 19	PEX19	166	0.01

F2Z2C3	Bcl-2-like protein 13	BCL2L13	78	0.083
F2Z2M1	Solute carrier organic anion transporter family member 1B1	LST1	66	0.425
F5GWB8	**			0.01
F5H196	**			0.01
F5H2U6	**			0.01
F5H456	RRP12-like protein	RRP12	1,015	0.094
F5H4N7	**			0.01
F5H696	**			0.01
F5H721	WW domain-binding protein 11	WBP11	607	0.01
F5H7W4	Sterol O-acyltransferase 2	SOAT2	310	0.01
O14879	Interferon-induced protein with tetratricopeptide repeats 3	IFIT3	490	0.152
O15381	Nuclear valosin-containing protein-like	NVL	856	0.098
O60885	Bromodomain-containing protein 4	BRD4	1,362	0.01
O76039	Cyclin-dependent kinase-like 5	CDKL5	1,030	0.01
O94822	E3 ubiquitin-protein ligase listerin	LTN1	1,766	0.01
O95716	Ras-related protein Rab-3D	RAB3D	219	0.486
O95758	Polypyrimidine tract-binding protein 3	PTBP3	552	0.086
O95786	Retinoic Acid-Inducible Gene 1 Protein-1 (RIG-1)	DDX58	925	0.01
P02533	Keratin, type I cytoskeletal 14	KRT14	472	0.01
P02647	Apolipoprotein A-I	APOA1	267	0.01
P02765	Alpha-2-HS-glycoprotein	AHSG	367	0.01
P02788	Lactotransferrin	LTF	710	0.01
P04004	Vitronectin	VTN	478	0.01
P04264	Keratin, type II cytoskeletal 1	KRT1	644	0.01
P05161	Ubiquitin-like protein ISG15	ISG15	165	0.08
P05787	Keratin, type II cytoskeletal 8	KRT8	483	0.01
P08779	Keratin, type I cytoskeletal 16	KRT16	473	0.01
P09601	Heme oxygenase 1	HMOX1	288	0.244
P09913	Interferon-induced protein with tetratricopeptide repeats 2	IFIT2	472	0.01
P0CG38	POTE ankyrin domain family member I	POTEI	1,075	0.01
P10606	Cytochrome c oxidase subunit 5B, mitochondrial	COX5B	129	0.498
P11678	Eosinophil peroxidase	EPX	715	0.01

P13645	Keratin, type I cytoskeletal 10	KRT10	584	0.01
P13647	Keratin, type II cytoskeletal 5	KRT5	590	0.01
P16455	Methylated-DNA--protein-cysteine methyltransferase	MGMT	207	0.450
P20591	Interferon-induced GTP-binding protein Mx1	MX1	662	0.01
P20592	Interferon-induced GTP-binding protein Mx2	MX2	715	0.01
P29400	Collagen alpha-5(IV) chain	COL4A5	1,685	0.01
P34931	Heat shock 70kDa protein 1-like	HSPA1L	641	0.466
P35527	Keratin, type I cytoskeletal 9	KRT9	623	0.01
P35908	Keratin, type II cytoskeletal 2 epidermal	KRT2	639	0.01
P42858	Huntingtin	HTT	3,142	0.342
P47914	60S ribosomal protein L29	RPL29	159	0.078
P48200	Iron-responsive element-binding protein 2	IREB2	963	0.092
P53674	Beta-crystallin B1	CRYBB1	252	0.487
P53779	Mitogen-activated protein kinase 10	MAPK10	464	0.087
P57105	Synaptojanin-2-binding protein	SYNJ2BP	145	0.490
P62699	Protein yippee-like 5	YPEL5	121	0.133
P69905	Hemoglobin subunit alpha	HBA1	142	0.01
Q00653	Nuclear factor NF-kappa-B p100 subunit	NFKB2	900	0.489
Q13393	Phospholipase D1	PLD1	1,074	0.477
Q14213	Interleukin-27 subunit beta	EBI3	229	0.01
Q14344	Guanine nucleotide-binding protein subunit alpha-13	GNA13	377	0.490
Q15417	Calponin-3	CNN3	329	0.01
Q2TAL8	Glutamine-rich protein 1	QRICH1	776	0.09
Q32N00	DNA polymerase delta subunit 3	POLD3	360	0.081
Q4KMP7	TBC1 domain family member 10B	TBC1D10B	808	0.099
Q4W5H2	Putative uncharacterized protein BMP2K	BMP2K	662	0.01
Q53EL6	Programmed cell death protein 4	PDCD4	469	0.098
Q58FF6	Putative heat shock protein HSP 90-beta 4	HSP90AB4P	505	0.01
Q5JV73	FERM and PDZ domain-containing protein 3	FRMPD3	1,810	0.01
Q5SNV9	Uncharacterised protein C1orf167	C1orf167	1,468	0.01
Q5T0A6	Dehydrodolichyl diphosphate synthase	DHDDS	169	0.342
Q5TH58	Uncharacterised protein C6orf106	C6orf106	224	0.477

Q68CZ6	HAUS augmin-like complex subunit 3	HAUS3	603	0.01
Q6ZNK6	TRAF-interacting protein with FHA domain-containing protein B	TIFAB	161	0.01
Q7Z2Y8	Interferon-induced very large GTPase 1	GVINP1	2,422	0.01
Q86TB9	Protein PAT1 homolog 1	PATL1	770	0.098
Q86U10	60kDa lysophospholipase	ASPG	573	0.01
Q8IXQ6	Poly [ADP-ribose] polymerase 9	PARP9	854	0.01
Q8IY21	Probable ATP-dependent RNA helicase DDX60	DDX60	1,712	0.01
Q8N4A0	Polypeptide N-acetylgalactosaminyltransferase 4	GALNT4	578	0.01
Q8N6G6	ADAMTS-like protein 1	ADAMTSL1	1,762	0.01
Q8NBT2	Kinetochore protein Spc24	SPC24	197	0.092
Q8NGU2	Olfactory receptor 9A4	OR9A4	314	0.01
Q8NHZ6	Mannose phosphate isomerase isoform		267	0.315
Q8NI60	Chaperone activity of bc1 complex-like, mitochondrial	ADCK3	647	0.01
Q8TAD4	Zinc transporter 5	SLC30A5	765	0.01
Q8WTU0	Protein DDI1 homolog 1	DDI1	396	0.01
Q8WVD3	E3 ubiquitin-protein ligase RNF138	RNF138	245	0.01
Q8WWI1	LIM domain only protein 7	LMO7	1,683	0.122
Q96AH8	Ras-related protein Rab-7b	RAB7B	199	0.033
Q96AZ6	Interferon-stimulated gene 20kDa protein	ISG20	181	0.265
Q96JB5	CDK5 regulatory subunit-associated protein 3	CDK5RAP3	506	0.422
Q96KN8	Ca^{2+}-independent N-acyltransferase	HRASLS5	279	0.124
Q96PP9	Guanylate-binding protein 4	GBP4	640	0.450
Q96SI9	Spermatid perinuclear RNA-binding protein	STRBP	672	0.01
Q9BRX2	Protein pelota homolog	PELO	385	0.08
Q9BSL1	Ubiquitin-associated domain-containing protein 1	UBAC1	405	0.081
Q9BV86	N-terminal Xaa-Pro-Lys N-methyltransferase 1	NTMT1	223	0.092
Q9BVP2	Guanine nucleotide-binding protein-like 3	GNL3	549	0.096
Q9BVS4	Serine/threonine-protein kinase RIO2	RIOK2	552	0.498
Q9BYF1	Angiotensin-converting enzyme 2	ACE2	805	0.01
Q9BYK8	Helicase with zinc finger domain 2	HELZ2	2,649	0.401
Q9BYX4	Melanoma Differentiation Associated Protein-5 (MDA5)	IFIH1	1,025	0.497
Q9BZE2	tRNA pseudouridine(38/39) synthase	PUS3	481	0.01

Q9H1K6	Mesoderm development candidate 1	MESDC1	362	0.01
Q9H444	Charged multivesicular body protein 4b	CHMP4B	224	0.484
Q9H4L7	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1	SMARCAD1	1,026	0.01
Q9H974	Queuine tRNA-ribosyltransferase subunit QTRTD1	QTRTD1	415	0.089
Q9H9L3	Interferon-stimulated 20kDa exonuclease-like 2	ISG20L2	353	0.01
Q9HC10	Otoferlin	OTOF	1,997	0.01
Q9NQX4	Unconventional myosin-Vc	MYO5C	1,742	0.078
Q9NRK6	ATP-binding cassette sub-family B member 10, mitochondrial	ABCB10	738	0.180
Q9NRX1	RNA-binding protein PNO1	PNO1	252	0.086
Q9NS37	CREB/ATF bZIP transcription factor	CREBZF	354	0.01
Q9NYL9	Tropomodulin-3	TMOD3	352	0.403
Q9UDY2	Tight junction protein ZO-2	TJP2	1,190	0.098
Q9UID3	Vacuolar protein sorting-associated protein 51 homolog	VPS51	782	0.01
Q9UII4	E3 ISG15--protein ligase HERC5	HERC5	1	0.01
Q9UK61	Protein FAM208A	FAM208A	1,670	0.481
Q9UN19	Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide	DAPP1	280	0.450
Q9Y3I1	F-box only protein 7	FBXO7	522	0.084
Q9Y3Q8	TSC22 domain family protein 4	TSC22D4	395	0.01
Q9Y6N7	Roundabout homolog 1	ROBO1	1,651	0.420

Appendix 2

^^ LncRNAs
** Unknown Transcript

RNA microarray: Up-regulated genes in response to 4 hour MIF stimulation (fold change ≥1.5)

Entry Name	Gene Name	Gene ID	Fold change
NR_003271	Small nucleolar RNA, C/D box 3B-1	SNORD3B-1	2.00
NM_020535	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 5A	KIR2DL5A	1.92
NR_040108	Uncharacterised LOC728175 non-coding		1.89
NR_004400	RNA variant U1 small nuclear 18	RNVU1-18	1.79
NM_004113	Fibroblast growth factor 12	FGF12	1.68
NR_034143	Cancer susceptibility candidate 14 non-protein coding	CASC14	1.60
ENST00000419440	^^	LncC11orf39-3	1.60
TCONS_00009863	^^	Lnc-ADCY2-4	1.59
NR_027030	Uncharacterised protein MGC34034		1.57
NM_153267	MAM domain containing 2	MAMDC2	1.55
NR_023343	RNA, U4atac small nuclear U12-dependent splicing	RNU4ATAC	1.55
NM_000230	Leptin	LEP	1.55
TCONS_00006780	^^	Lnc-THUMPD3-1	1.54
NR_003064	Peptide YY, 2 (pseudogene) non coding	PYY2	1.54
NM_178466	BPI fold containing family A, member 3	BPIFA3	1.54
TCONS_00004035	^^	Lnc-SLC4A3-8	1.53
NM_175868	Melanoma antigen family A, 6 (MAGEA6)		1.53
NM_001004695	Olfactory receptor, family 2, subfamily T, member 33 (OR2T33		1.53
NR_000020	Small nucleolar RNA, C/D box 33 (SNORD33		1.52
TCONS_00023084	^^	Lnc-STXBP6-1	1.52
NR_030737	Tetratricopeptide repeat domain 3 pseudogene 1 (TTC3P1		1.52
NM_183062	Protease, serine, 38 (PRSS38		1.52
XM_001719518	Hypothetical protein		1.51
NR_002575	Nucleolar RNA, H/ACA box 27 (SNORA27		1.51
TCONS_00022841	^^	Lnc-SERPINA12-1	1.51

ENST00000428157	^^	Lnc-ZAP70-2-2	1.51
XR_132564	Hypothetical LOC100652913		1.51

RNA microarray: Down-regulated genes in response to 4 hour MIF stimulation (fold change ≤0.7)

Entry Name	Gene Name	Gene ID	Fold change
NM_016536	Zinc finger protein 571	ZNF571	0.37
AJ276510	Partial mRNA for hypothetical protein	ORF1	0.38
TCONS_00029565	^^	Lnc-CRYBA4-8	0.40
ENST00000441356	^^	Lnc-RND3-1	0.40
NM_001822	Chimerin 1	CHN1	0.41
NM_004797	Adiponectin, C1Q and collagen domain containing	ADIPOQ	0.42
TCONS_00022613	^^	Lnc-RP11-1070N10.3.1-1	0.43
TCONS_00019700	^^	Lnc-STARD10-1	0.43
XR_109045	Hypothetical LOC100507282		0.45
NM_018711	SV2 related protein homolog	SVOP	0.45
BC047507	Solute carrier family 2 (facilitated glucose transporter), member 13, mRNA		0.47
BC034958	Hypothetical protein LOC283665		0.48
NM_001080451	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 11	SERPINA11	0.49
XR_109658	Uncharacterised LOC100505973		0.51
TCONS_l2_00012321	^^		0.51
ENST00000430672	^^	Lnc-HDDC2-2	0.51
NM_001024227	ADP-ribosylation factor 1	ARF1	0.51
NM_005566	Lactate dehydrogenase A	LDHA	0.53
NM_032704	Tubulin, alpha 1c	TUBA1C	0.53
NM_006793	Peroxiredoxin 3	PRDX3	0.54
ENST00000458017	^^	Lnc-SLC35D3-1	0.54
NM_032551	KISS1 receptor	KISS1R	0.55
NM_001950	E2F transcription factor 4, p107/p130-binding	E2F4	0.55
NM_001402	Eukaryotic translation elongation factor 1 alpha 1	EEF1A1	0.55
NM_014214	Inositol(myo)-1(or 4)-monophosphatase 2	IMPA2	0.55

NM_002629	Phosphoglycerate mutase 1	PGAM1	0.56
NM_206956	Preferentially expressed antigen in melanoma	PRAME	0.56
TCONS_00026344	^^	Lnc-MALT1-1	0.56
NM_001042465	Prosaposin	PSAP	0.56
NM_033198	Phosphatidylinositol glycan anchor biosynthesis, class S	PIGS	0.56
NM_014045	Low density lipoprotein receptor-related protein 10	LRP10	0.57
NM_001006617	Mitogen-activated protein kinase associated protein 1	MAPKAP1	0.57
NM_021932	RIC8 guanine nucleotide exchange factor A	RIC8A	0.57
NM_032985	Sec23 homolog B	SEC23B	0.57
NM_006009	Tubulin, alpha 1a	TUBA1A	0.57
NM_000687	Adenosylhomocysteinase	AHCY	0.57
NM_006839	Inner membrane protein, mitochondrial	IMMT	0.57
NM_020679	MIF4G domain containing	MIF4GD	0.57
NM_030752	T-complex 1	TCP1	0.57
NM_020531	Adipocyte plasma membrane associated protein	APMAP	0.58
NM_000820	Growth arrest-specific 6	GAS6	0.58
NM_006029	Paraneoplastic Ma antigen 1	PNMA1	0.58
NM_153824	Pyrroline-5-carboxylate reductase 1	PYCR1	0.58
NM_001012732	dCMP deaminase	DCTD	0.58
ENST00000417260	^^	Lnc-RP3-377D14.1.1-1	0.58
NM_005265	Gamma-glutamyltransferase 1	GGT1	0.58
NM_015171	Exportin 6	XPO6	0.58
NM_004550	NADH-coenzyme Q reductase	NDUFS2	0.58
NM_001012662	Solute carrier family 3 (amino acid transporter heavy chain), member 2	SLC3A2	0.58
NM_001004356	Fibroblast growth factor receptor-like 1	FGFRL1	0.59
NM_032539	SLIT and NTRK-like family, member 2	SLITRK2	0.59
NM_003331	Tyrosine kinase 2	TYK2	0.59
NM_023075	Metallophosphoesterase 1	MPPE1	0.59
NM_000532	Propionyl CoA carboxylase, beta polypeptide	PCCB	0.59
NM_002631	Phosphogluconate dehydrogenase	PGD	0.59
NM_002755	Mitogen-activated protein kinase kinase 1	MAP2K1	0.60

NM_020414	DEAD (Asp-Glu-Ala-Asp) box helicase 24	DDX24	0.60
NM_001067	Topoisomerase (DNA) II alpha 170kDa	TOP2A	0.60
NM_201222	Melanoma antigen family D, 2	MAGED2	0.60
NM_001039619	Protein arginine methyltransferase 5	PRMT5	0.60
NM_053045	Transmembrane protein 203	TMEM203	0.60
NM_014003	DEAH (Asp-Glu-Ala-His) box polypeptide 38	DHX38	0.60
NM_002194	Inositol polyphosphate-1-phosphatase	INPP1	0.60
NM_014230	Signal recognition particle 68kDa	SRP68	0.60
ENST00000466304	^^		0.60
NM_014669	Nucleoporin 93kDa	NUP93	0.60
NM_198976	Negative elongation factor complex member C/D	NELFCD	0.60
NM_001184768	Armadillo repeat containing, X-linked 6	ARMCX6	0.61
NM_014889	Pitrilysin metallopeptidase 1	PITRM1	0.61
NM_144699	ATPase, Na+/K+ transporting, alpha 4 polypeptide	ATP1A4	0.61
ENST00000490216	^^		0.61
NR_001562	Annexin A2 pseudogene 1	ANXA2P1	0.61
NM_012401	Plexin B2	PLXNB2	0.61
NM_153201	Heat shock 70kDa protein 8	HSPA8	0.61
NM_003575	Zinc finger protein 282	ZNF282	0.61
NM_053052	Synaptosomal-associated protein, 47kDa	SNAP47	0.61
NM_002115	Hexokinase 3	HK3	0.61
NM_014063	Drebrin-like	DBNL	0.61
NM_005120	Mediator complex subunit 12	MED12	0.61
NM_006677	Ubiquitin specific peptidase 19	USP19	0.61
NM_021095	Solute carrier family 5 (sodium/multivitamin and iodide cotransporter), member 6	SLC5A6	0.61
NM_001206710	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	PRKAG1	0.61
NM_005850	Splicing factor 3b, subunit 4, 49kDa	SF3B4	0.61
NM_001017405	Macrophage erythroblast attacher	MAEA	0.61
TCONS_l2_00014935	^^		0.62
NM_014845	FIG4 homolog	FIG4	0.62
NM_003626	Protein tyrosine phosphatase, receptor type, f polypeptide	PTPRF	0.62

NM_001686	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	ATP5B	0.62
NM_004581	Rab geranylgeranyltransferase, alpha subunit	RABGGTA	0.62
NM_017617	Notch 1	NOTCH1	0.62
NM_003730	Ribonuclease T2	RNASET2	0.62
ENST00000375713	^^		0.62
NM_020944	Glucosidase, beta (bile acid) 2	GBA2	0.62
NM_004946	Dedicator of cytokinesis 2	DOCK2	0.63
NM_004281	BCL2-associated athanogene 3	BAG3	0.63
NM_005186	Calpain 1, (mu/I) large subunit	CAPN1	0.63
NM_006453	Transducin (beta)-like 3	TBL3	0.63
NM_003680	Tyrosyl-tRNA synthetase	YARS	0.63
NM_002110	Hemopoietic cell kinase	HCK	0.63
ENST00000380291	^^		0.63
NM_004879	Etoposide induced 2.4	EI24	0.63
NM_173717	ElaC ribonuclease Z 2	ELAC2	0.63
A_24_P246963	**		0.63
A_33_P3357227	**		0.63
NM_052853	aarF domain containing kinase 2	ADCK2	0.63
NM_007011	Abhydrolase domain containing 2	ABHD2	0.63
NM_031229	RanBP-type and C3HC4-type zinc finger containing 1	RBCK1	0.63
NM_002601	Phosphodiesterase 6D	PDE6D	0.63
NM_001693	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2	ATP6V1B2	0.64
NM_001127	Adaptor-related protein complex 1, beta 1 subunit	AP1B1	0.64
NM_025204	TraB domain containing	TRABD	0.64
NM_005174	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	ATP5C1	0.64
NM_145805	ISL LIM homeobox 2	ISL2	0.64
NM_033296	Morf4 family associated protein 1	MRFAP1	0.64
NM_004793	lon peptidase 1, mitochondrial	LONP1	0.64
NM_006357	Ubiquitin-conjugating enzyme E2E 3	UBE2E3	0.64
NM_017547	FAD-dependent oxidoreductase domain containing 1	FOXRED1	0.64
NM_014230	Signal recognition particle 68kDa	SRP68	0.64

NM_001428	Enolase 1, (alpha)	ENO1	0.64
NM_015160	Peptidase (mitochondrial processing) alpha	PMPCA	0.64
ENST00000435049	^^	Lnc-WRAP73-1	0.64
XR_110047	Uncharacterised LOC389102		0.64
NM_024068	Nucleic acid binding protein 2	NABP2	0.64
NM_130475	MAP-kinase activating death domain	MADD	0.64
NM_138793	Calcium activated nucleotidase 1	CANT1	0.64
NM_015374	Sad1 and UNC84 domain containing 2	SUN2	0.64
NM_001077351	RNA binding motif protein 23	RBM23	0.64
NM_173165	Nuclear factor of activated T-cells,	NFATC3	0.65
NM_015440	Methylenetetrahydrofolate dehydrogenase1-like	MTHFD1L	0.65
NM_005337	NCK-associated protein 1-like	NCKAP1L	0.65
TCONS_00012632	^^	Lnc-FAM120B-6	0.65
NM_000026	Adenylosuccinate lyase	ADSL	0.65
NM_058004	Phosphatidylinositol 4-kinase, catalytic, alpha	PI4KA	0.65
NM_015913	Thioredoxin domain containing 12	TXNDC12	0.65
NM_007024	Transmembrane protein 115	TMEM115	0.65
NM_000938	Polymerase (RNA) II (DNA directed) polypeptide B, 140kDa	POLR2B	0.65
NM_006230	Polymerase (DNA directed), delta 2, accessory subunit	POLD2	0.65
NM_001042388	Protein phosphatase 4, regulatory subunit 1	PPP4R1	0.65
NM_005098	Musculin	MSC	0.65
NM_020362	PITH domain containing 1	PITHD1	0.65
NM_006325	RAN, member RAS oncogene family	RAN	0.65
NM_002738	Protein kinase C, beta	PRKCB	0.65
NM_001002878	THO complex 5	THOC5	0.65
NM_016176	Stromal cell derived factor 4	SDF4	0.65
NM_020190	Olfactomedin-like 3	OLFML3	0.65
NM_005701	Snurportin 1	SNUPN	0.65
NM_005015	Oxidase (cytochrome c) assembly 1-like	OXA1L	0.65
NM_001099668	HIG1 hypoxia inducible domain family, member 1A	HIGD1A	0.65
NR_015434	Uncharacterized LOC148413		0.65
NM_013335	GDP-mannose pyrophosphorylase A	GMPPA	0.65

NM_022765	Microtubule associated monooxygenase, calponin and LIM domain containing 1	MICAL1	0.65
NM_002086	Growth factor receptor-bound protein 2	GRB2	0.65
NM_033296	Morf4 family associated protein 1	MRFAP1	0.65
NM_153611	Cytochrome b561 family, member A3	CYB561A3	0.65
NM_001114618	Mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase	MGAT1	0.65
NM_015941	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H	ATP6V1H	0.65
A_33_P3358347	**		0.65
NM_002953	Ribosomal protein S6 kinase, 90kDa, polypeptide 1	RPS6KA1	0.65
NM_014287	NODAL modulator 1	NOMO1	0.65
NM_000969	Ribosomal protein L5	RPL5	0.65
NM_014338	Phosphatidylserine decarboxylase	PISD	0.65
NM_017719	SNF related kinase	SNRK	0.65
NM_001087	Angio-associated, migratory cell protein	AAMP	0.65
NM_032508	Transmembrane protein 185A	TMEM185A	0.65
NM_001127695	Cathepsin A	CTSA	0.66
NM_006819	Stress-induced-phosphoprotein 1	STIP1	0.66
NM_005390	Pyruvate dehydrogenase (lipoamide) alpha 2	PDHA2	0.66
NM_002872	Ras-related C3 botulinum toxin substrate 2	RAC2	0.66
NM_006369	Leucine rich repeat containing 41	LRRC41	0.66
NM_000294	Phosphorylase kinase, gamma 2 (testis)	PHKG2	0.66
NM_176096	CDK5 regulatory subunit associated protein 3	CDK5RAP3	0.66
NM_005788	Protein arginine methyltransferase 3	PRMT3	0.66
NM_152293	Transcriptional adaptor 2B	TADA2B	0.66
NM_139279	Multiple coagulation factor deficiency 2	MCFD2	0.66
NM_001040033	CD53 molecule	CD53	0.66
NM_000183	Hydroxyacyl-CoA dehydrogenase hydratase beta subunit	HADHB	0.66
NM_007065	Cell division cycle 37	CDC37	0.66
A_33_P3283167	**		0.66
NM_002168	Isocitrate dehydrogenase 2 mitochondrial	IDH2	0.66
NM_005692	ATP-binding cassette, sub-family F member 2	ABCF2	0.66

NM_007001	Solute carrier family 35 member D2	SLC35D2	0.66
NM_130803	Multiple endocrine neoplasia I	MEN1	0.66
NM_001077351	RNA binding motif protein 23	RBM23	0.66
NM_006357	Ubiquitin-conjugating enzyme E2E 3	UBE2E3	0.66
NM_000918	Prolyl 4-hydroxylase, beta polypeptide	P4HB	0.66
NM_178819	1-acylglycerol-3-phosphate O-acyltransferase 6	AGPAT6	0.66
NM_032985	Sec23 homolog B	SEC23B	0.66
NM_021822	Apolipoprotein B mRNA enzyme, catalytic polypeptide-like 3G	APOBEC3G	0.66
A_24_P603890	**		0.66
NM_019015	Chondroitin polymerizing factor 2	CHPF2	0.66
NM_007067	K(lysine) acetyltransferase 7	KAT7	0.66
NR_003226	AFG3 ATPase family member 3-like 1 pseudogene	AFG3L1P	0.66
NM_014771	Ring finger protein 40, E3 ubiquitin protein ligase	RNF40	0.66
NM_006831	Cleavage and polyadenylation factor I subunit 1	CLP1	0.66
NM_032272	MAF1 homolog	MAF1	0.66
NM_001243646	CD2 (cytoplasmic tail) binding protein 2	CD2BP2	0.66
NM_000918	Prolyl 4-hydroxylase, beta polypeptide	P4HB	0.66
NM_002969	Mitogen-activated protein kinase 12	MAPK12	0.66
NM_024419	Phosphatidylglycerophosphate synthase 1	PGS1	0.66
NM_001961	Eukaryotic translation elongation factor 2	EEF2	0.66
TCONS_l2_00005546	^^		0.66
NM_006066	Aldo-keto reductase family 1, member A1	AKR1A1	0.66
NM_033446	Multivesicular body subunit 12B	MVB12B	0.66
NM_016238	Anaphase promoting complex subunit 7	ANAPC7	0.67
NM_004393	Dystroglycan 1	DAG1	0.67
NM_015274	Mannosidase, alpha, class 2B, member 2	MAN2B2	0.67
NM_032564	Diacylglycerol O-acyltransferase 2	DGAT2	0.67
NM_004168	Succinate dehydrogenase complex, subunit A	SDHA	0.67
A_33_P3361817	**		0.67
NM_024833	Zinc finger protein 671	ZNF671	0.67

Russell, Kirsty E

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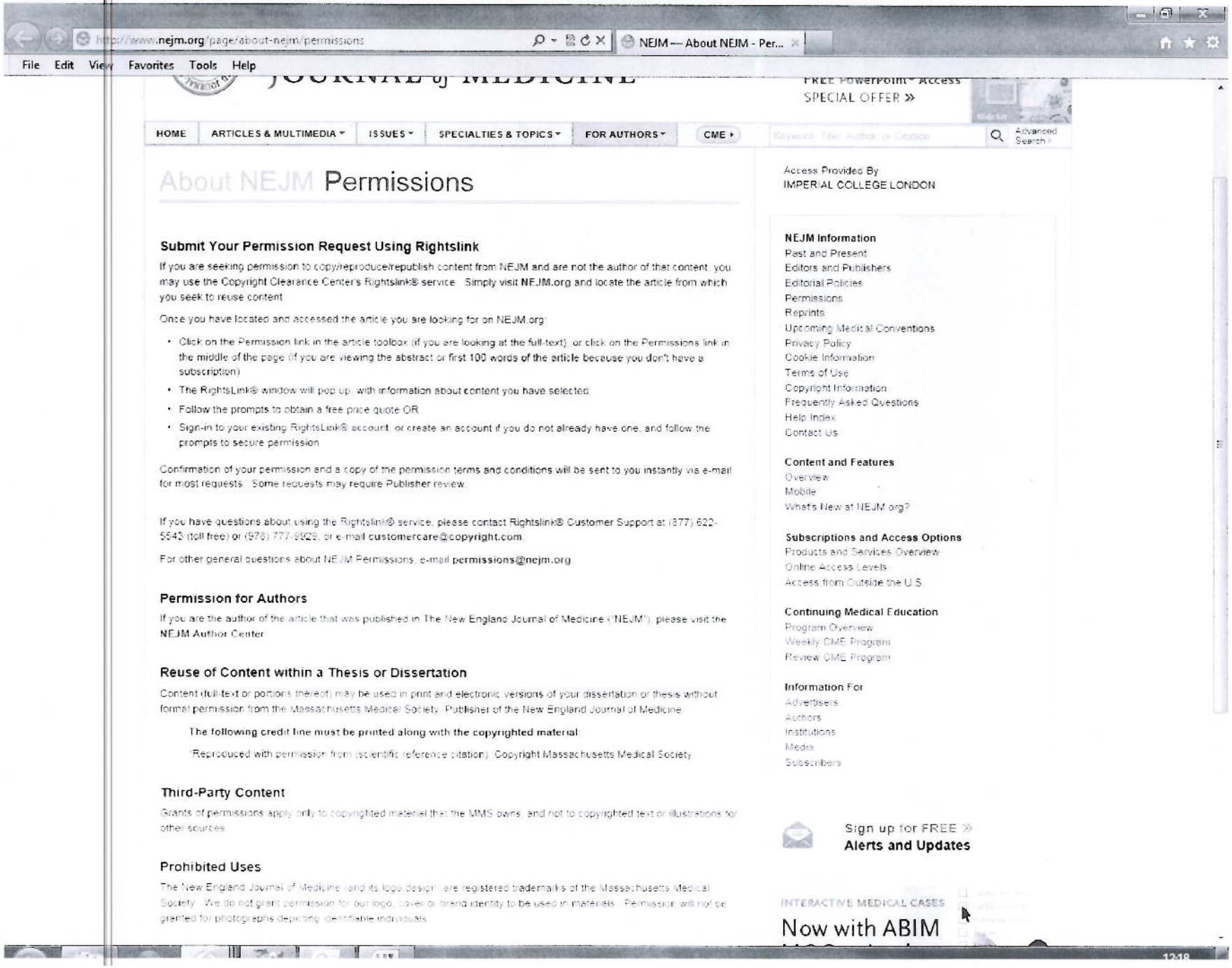
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Russell, Kirsty E

From: Suzanne Hurd <hurd1839@comcast.net>
Sent: 21 October 2013 19:12
To: Russell, Kirsty E
Subject: Re: New Application For GOLD Materials Submission

Follow Up Flag: Flag for follow up
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Dear Ms. Russell:

Thank you for your email message; we are very pleased to learn of your interest in the use of materials from the "Global Strategy for the Diagnosis, Management, and Prevention of COPD, 2013."

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If you have questions, or need more information, please let us know.

Best regards,
Suzanne S. Hurd, PhD
Scientific Director, GOLD
http://www.goldcopd.org

On Oct 19, 2013, at 5:40 AM, Global initiative for chronic Obstructive Lung Disease <hurd1839@comcast.net> wrote:

> Application For GOLD Materials Submission October 19, 2013 05:40 AM 1.
> Applicant Name:
> Kirsty Russell
>
> 2. Applicant Address:
> Flat 35 Kingfisher Court, 15 Queensmere road
>
> 3. Email Address:
> k.russell09@imperial.ac.uk
>
> 4. Organization Represented: (if any)
> Imperial College London
>
> 5. Is commercial support involved directly or indirectly in the
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>
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> Kirsty Russell
>
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> 2013-10-19
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Russell, Kirsty E

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Subject: permissions

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