

Relationship of Macrophage Function and Phenotype in COPD

Jessica Dorsie Tilman

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**National Heart and Lung Institute
Imperial College London
Dovehouse Street
London, SW3 6LY, UK**

Abstract

Chronic obstructive pulmonary disease (COPD) is associated with elevated inflammatory signals, protease activity and reduced clearance of pathogens. Macrophages are key in driving inflammatory signals that are subsequently followed by resolution of inflammation and return to steady state. In pathologies such as COPD, macrophages may drive development and progression of disease by loss of regulation of inflammatory signals, which, in turn, may be due to changes in phenotype and loss of cell plasticity.

This thesis investigated the ability of macrophages to adapt to changes in microenvironment and establish whether cell plasticity is lost in COPD cells leading to a 'fixed' inflammatory state. Monocyte-derived macrophages (MDM) and human lung tissue macrophages were cultured in either GM-CSF (pro-inflammatory) or M-CSF (resolving) to evaluate the effect on cell phenotype, function and plasticity between non-smokers, smokers and COPD patients, with the hypothesis that COPD cells would remain inflammatory regardless of environment.

COPD cells released higher levels of pro-inflammatory cytokines following LPS stimulation compared to control cells, and this could be further enhanced when cultured in GM-CSF compared to M-CSF. Non-smoker and smoker cells could be driven to become less inflammatory in the presence of M-CSF, while COPD cells could not. COPD cells also demonstrated elevated protease activity and defects in phagocytosis compared to controls, but these were not affected by growth factors.

Analysis of macrophage subsets based on cell density highlighted morphological and functional differences in both MDM and tissue macrophages, suggesting that phenotype may be predetermined in monocytes, and not purely influenced by environment. Furthermore, macrophage phenotype in COPD may be influenced by epigenetic modifications, driving changes in gene expression and cell function. *KLF4* and *CSF1R*, which have a role in dampening inflammatory response, were downregulated in COPD and may be one mechanism driving inflammation. COPD macrophages show several defects compared to smokers and non-smokers, in both MDM and tissue macrophages, and demonstrated unilateral plasticity to become more inflammatory. Targeting these processes may therefore allow development of novel anti-inflammatory therapies for this disease.

Declaration of Originality

I confirm that I performed all experiments and analysis reported in this thesis, unless otherwise stated.

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Abbreviations

Abbreviation	Meaning
A1AT	A1-Antitrypsin
Akt	Protein Kinase B
AM	Alveolar Macrophage
ANOVA	Analysis of Variance
AP-1	Activator Protein -1
APC	Allophycocyanine
BAL	Bronchoalveolar Lavage
Bcl-X	B-Cell Lymphoma Extra-Large
BLF	British Lung Foundation
BSA	Bovine Serum Albumin
c-Maf	c-Musculoaponeurotic Fibrosarcoma
CCR	C-C Chemokine Receptor
CD	Cluster of Differentiation
CFU	Colony Forming Units
COPD	Chronic Obstructive Pulmonary Disease
CXC	Chemokine (C-X-C motif)
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribose Nucleic Acid
D-PBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal Regulated Kinase
FACS	Fluorescent Activated Cell Sorting
FSC	Forward Scatter
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FEV ₁	Forced Expiratory Volume in One Second
FSC	Forward Side Scatter
FVC	Forced Vital Capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GRO- α	Growth Regulated Oncogene – Alpha
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
H ₂ O ₂	Hydrogen Peroxide
HAT	Histone Acetylase
HBSS	Hank's Balanced Salt Solution
HDAC	Histone Deacetylase
HI	<i>Haemophilus influenzae</i>
HLA-DR	Human Leukocyte Antigen DR-1
HO-1	Haem Oxygenase -1
HRP	Horseradish Peroxidase
I-CAM	Intracellular Adhesion Molecule
IFN	Interferon
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IRAK	Interleukin-1 Receptor-Associated Kinase

IRF	Interferon Regulatory Factor
JAK	Janus Kinase
JMML	Juvenile Myelomonocytic Leukemia
KLF	Kruppel Like Factor
LABA	Long Acting Beta Agonist
LAMA	Long Acting Muscarinic Antagonists
LPS	Lipopolysaccharide
LT-B ₄	Leukotriene – B ₄
M-CSF	Macrophage-Colony Stimulating Factor
Mac-1	Macrophage-1 Antigen
MAPK	Mitogen Activated Protein Kinase
MCP	Monocyte Chemoattractant Protein
MDM	Monocyte Derived Macrophage
MEK	Mitogen Activated Protein Kinase Kinase
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloprotease
MTT	(3-(4,5-Dimethylthiazol-2-yl))-2,5-diphenyltetrazolium bromide
MyD88	Myeloid Differentiation Primary Response 88
NF κ B	Nuclear Factor Kappa Beta
NHS	National Health Service
OD	Optical Density
PAMP	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PDE4	Phosphodiesterase 4
PI3K	Phosphoinositide 3-Kinase
PPAR	Peroxisome Proliferator-Activated Receptor
RA	Rheumatoid Arthritis
Ras	Sat sarcoma
RELA	Reticuloendotheliosis Viral Oncogene Homolog A
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SEM	Standard Error of the Mean
SP	<i>Streptococcus pneumoniae</i>
STAT	Signal Transducer and Activator of Transcription
SSC	Side Scatter
TAM	Tumour Associated Macrophage
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Metalloproteases
TLR	Toll-Like Receptor
Tm ϕ	Tissue Macrophage
TMB	5,5' Tetramethylbenzidine
TNF	Tumour Necrosis Factor
TRIF	TRI (Toll/Interleukin 1 Receptor) Domain-Containing Adapter-Inducing Interferon- β
v/v	Volume by Volume
w/v	Weight by Volume
WHO	World Health Organisation
λ	Wavelength

Conference Abstracts

Tilman J.D., Barnes P.J., Donnelly L.E., 2017, Reduced expression of the transcription factor KLF4 in COPD macrophages is associated with dysfunctional differentiation, ERS Annual Congress, Milan, Italy

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Tilman J.D., Day, A.M.W., Madge, N.J., Barnes, P.J., Donnelly, L.E., 2015, Macrophage phenotype does not affect protease anti-protease imbalance in COPD. ERS Annual Congress, Amsterdam.

Tilman J.D., Day A.M.W., Madge N.J., Barnes P.J., Donnelly L.E., 2015, Macrophage phenotype does not affect protease anti-protease imbalance in COPD, BLF Conference, London.

Tilman J.D., Day A.M.W., Madge N.J., Barnes P.J., Donnelly L.E., 2015, Effect of macrophage phenotype on MMP9 expression and activity in COPD, ERS Lung Science Conference, Portugal

Conference Talks

Tilman J.D., Barnes, P.J., Donnelly L.E., 2017, Do COPD macrophages fail to differentiate correctly?, British Association of Lung Research, Belfast, UK.

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Tilman J.D., Barnes, P.J., Donnelly L.E., 2016, Defective phagocytoses in COPD human lung Tissue macrophages cannot be manipulated by growth factors, British Association of Lung Research, Sheffield, UK.

CHAPTER 1: INTRODUCTION

1.1 Introduction

The innate immune system plays a pivotal role as a first line of defence against invading pathogens, viruses and toxins (Warrington et al., 2011). Elie Metchnikoff first established the theory that cells, such as phagocytes, were key in host defence (Metchnikoff, 1905). This theory was proposed alongside that of Paul Ehrlich, who suggested that adaptive immunity was key to mounting a sustained response to insult. As such, they were both awarded the Nobel Prize over a century ago for their work on immunology and reconciliation of the two theories which are now known as innate and adaptive immunity (Gordon, 2008, Metchnikoff, 1905).

Several leukocytes are involved in the innate immune response, including monocytes, macrophages, neutrophils, basophils, eosinophils, dendritic cells, natural killer cells, mast cells and lymphocytes. The main role of these cells include recruitment of other immune cells to sites of infection and inflammation, through the release of cytokines and chemokines, antigen presentation, as well as the removal of foreign pathogens and apoptotic cells through the process of phagocytosis and efferocytosis (Warrington et al., 2011). Macrophages and neutrophils are the predominant cell types responsible for phagocytosis. As such, these cells have evolved to recognise conserved molecular patterns originating from pathogens and microbes. Examples of these molecular patterns include lipopolysaccharide (LPS), which is found on the surface of gram-negative bacteria, and pathogen associated molecular patterns (PAMPs), found on the surface of invading microbes (Medzhitov and Janeway, 1997). Receptors on the surface of phagocytes recognise these foreign patterns leading to the engulfment of invading material that is subsequently degraded through the process of phagocytosis.

The second arm of the immune system is the adaptive immune response, comprised of T-cells and B-cells, which can target invading material and lead to its degradation. These are recruited by signals released from cells of the innate immune system, and following an initial encounter with foreign

material, form a memory of the foreign pattern, which leads to a more rapid response should the same pattern be encountered again (Warrington et al., 2011).

1.2 Macrophage Classification

Macrophages perform a wide array of functions, ranging from homeostatic tissue maintenance to host defence, and are key players in the innate response. For many years, the prevailing concept has been that tissue macrophages originate from the bone marrow via circulating monocytes, which infiltrate tissues and differentiate into macrophages (van Furth and Cohn, 1968, Sieweke and Allen, 2013). This dogma has recently come into question, with several studies proposing an embryonic origin for tissue macrophages which are capable of self-renewal, under steady-state, throughout adulthood, as well as following insult (Davies et al., 2013, Patel et al., 2017). However, most work in this field has been performed using mouse models, and fate mapping techniques of receptors which do not translate to human biology (Hashimoto et al., 2013, Sieweke and Allen, 2013, Jenkins et al., 2011). Nevertheless, there is some evidence from human observations to support this phenomenon, where patients suffering from monocytopenia, and thus have low numbers of circulating monocytes, maintain normal macrophage numbers in tissue (Hashimoto et al., 2013). Evidence in this field is still developing, and additional human studies are required to support the theory of embryonic vs. monocyte macrophage origin.

1.3 Tissue Macrophages

Tissue macrophages are a heterogeneous population and classification is dependent on many factors, including location, origin, receptor expression and response to stimuli (Davies et al., 2013). The development of macrophages is driven by a series of lineage-determining cytokines and growth factors including macrophage-colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF), as well as key transcriptional regulators such as PU.1 (Saeed et al., 2014). Once monocytes migrate to specific tissues, local environment and interactions with neighbouring cells provide signals for tissue-specific differentiation (Gordon, 2003a).

Macrophages are found in almost all tissues, and have specialised names and functions dependent on the tissue in which they reside (Warrington et al., 2011). Some macrophages have a role in homeostatic maintenance of tissue, such as osteoclasts in the bone which are essential for bone resorption and repair (Vaananen et al., 2000); microglia in the brain which are required for normal development by regulating gliogenesis (Thomas, 1992); Kupffer cells in the liver which are responsible for iron homeostasis (Frantz and Nahrendorf, 2014). On the other hand, there is a large subset of macrophages, which are specialised in surveillance and host defence, with associated key roles as part of the innate response. These are found in the skin, lung, spleen and gut which are more likely to come in contact with pathogens and thus require specialised immune surveillance (Wardle, 1987). Ontogeny of cells as well as environmental cues are thought to drive macrophage heterogeneity allowing for the highly specialised, tissue-specific functions that these cells perform, and may be driven by epigenetic control (Lavin et al., 2014).

1.4 Mononuclear Phagocyte System

Circulating blood monocytes may be exposed to homing factors such as chemokines, which attract them to specific tissues where they differentiate into macrophages. While location and tissue of residence are known to influence macrophage phenotype, it is also possible that distinct monocyte populations give rise to distinct macrophage phenotypes (Passlick et al., 1989, Grage-Griebenow et al., 2001). Monocytes are a heterogeneous population with distinct phenotype and function (Passlick et al., 1989). Classically, monocytes were originally described based on the expression levels of cluster of differentiation (CD)14 (pattern recognition receptor, toll-like receptor (TLR)4 co-receptor) and CD16 (low affinity Fc receptor for immunoglobulin (Ig)G) and defined as CD14⁺⁺CD16⁻. In a healthy individual, this population makes up 70-90% of all monocytes. A second subgroup was termed non-classical or pro-inflammatory and defined as CD14⁺CD16⁺⁺, with higher levels of tumour necrosis factor (TNF) α release and lower interleukin (IL)-10 production upon activation. These make up around 5-10% of

circulating monocytes (Strauss-Ayali et al., 2007). A third, less common, intermediate group has been identified with the profile CD14⁺⁺CD16⁺ (Passlick et al., 1989).

Functional differences have been identified between monocyte subsets, with intermediate monocytes showing overlap with both classical and non-classical subtypes (Stansfield and Ingram, 2015). Classical and intermediate monocytes have greater phagocytic capabilities than non-classical monocytes, however, intermediate and non-classical CD16⁺ monocytes produce significantly more pro-inflammatory cytokines, such as TNF α , compared to non-classical CD16⁻ monocytes (Passlick et al., 1989, Stansfield and Ingram, 2015).

The current hypothesis is that CD14⁺⁺CD16⁻ cells arise from the bone marrow and give rise to CD16⁺ subgroups which are activated during inflammation. This notion is supported by that fact that classical monocytes produce monocyte chemoattractant protein-1 (MCP-1/C-C motif chemokine ligand (CCL)2) and express the corresponding receptor, C-C motif chemokine receptor (CCR)2, which are required for monocyte migration away from bone marrow (Stansfield and Ingram, 2015). In addition, recent work by Patel *et al.* investigated human monocyte kinetics using deuterium-labelled glucose and demonstrated a sequential change from classical monocytes to non-classical monocytes over a period of 7 days. Furthermore, this study showed that classical monocytes have the potential to sequentially become intermediate and non-classical monocytes, providing further evidence to support this hypothesis (Patel et al., 2017).

1.5 Macrophage Heterogeneity

Knowledge regarding macrophage heterogeneity has been expanding over the past few decades, and the complexity within this field has made consistency in definitions and nomenclature difficult to achieve (Martinez and Gordon, 2014). Macrophage phenotype and function are influenced by a plethora of factors, and response to stimuli *in vitro* varies depending on what factors are being used. Initial attempts to classify macrophages into distinct populations were based on observations that

macrophages from Th1 or Th2 mouse strains (C57BL/6, B10D2 or BALB/c, DBA/2 respectively) responded differently to stimulation with interferon (IFN) γ (Mills et al., 2000). The Th1 response was associated with IFN γ production which in turn activates macrophages to release pro-inflammatory cytokines such as TNF α and IL-1 β . Stein *et al.* later identified a distinct macrophage activation state where activation by Th2 cytokines, IL-4 and IL-13, leads to upregulation of the mannose receptor (CD206) in a murine model (Stein et al., 1992). This macrophage activation state was associated with a resolving, anti-inflammatory phenotype with increased release of IL-10 and dampening of inflammatory signals (Stein et al., 1992, Martinez and Gordon, 2014). These data were reconciled into the M1/M2 model of macrophage polarisation with LPS and IFN γ driving pro-inflammatory M1 macrophages and IL-4 and IL-13 activating anti-inflammatory, or resolving M2 macrophages (Gordon, 2003b) (Figure 1.1A).

The M1/M2 terminology has been widely used to describe macrophage polarisation as a linear event with two distinct phenotypes existing on opposing sides, defined by cytokine output and cell surface receptor expression. However, it has become increasingly apparent that this is an oversimplification of a much more complex system in which diversity and plasticity are essential for the wide array of functions that macrophages perform. Mosser *et al* attempted to update the M1/M2 model by proposing a continuum of macrophage phenotypes, analogous to a colour wheel, with the addition of M2 subsets (M2a, M2b and M2c) which are activated by distinct stimuli but retain the ability to produce IL-10 (Mantovani et al., 2002, Mosser, 2003, Mantovani et al., 2004) (Figure 1.1B).

Additional macrophage phenotypes have since been described including tumour-associated macrophages (TAMs) which suppress the immune response and thus promote tumour growth and metastases, as well as several atherosclerosis specific macrophages, which cannot be described as either M1 or M2. Macrophages associated with atherosclerotic lesions include Mhem, Mox, M(Hb) and M4 macrophages which are present at different stages of disease (Tabas and Bornfeldt, 2016). Exposure to oxidized phospholipids results in the development of Mox macrophages which are

different in terms of gene profile, have reduced phagocytic and chemotactic potential and are regulated by nuclear factor erythroid 2-related factors (Nrf)2 signalling (Kadl et al., 2010). M(Hb) cells differentiate as a result of haemorrhage, in the presence of haemoglobin, and are characterised by increased ferroportin and ABC transporter expression as well as reduced intracellular iron and ROS (Finn et al., 2012). The Mhem phenotype is driven by haem, and is characterised by increased expression of haem oxygenase 1 (HO-1) which has a protective role against oxidative stress and lipid accumulation (Boyle et al., 2011). M4 macrophages have been described in the context of atherosclerosis and are induced by C-X-C motif ligand (CXCL)4. While there are several similarities between M4, M1 and M2 macrophages, M4 show a distinct transcriptional profile to either and almost completely lose the ability to phagocytose (Gleissner et al., 2010) (Figure 1.2).

It is evident that macrophages cannot be subdivided into a finite number of isolated populations differing in receptor profile, gene expression and cytokine output. These highly complex cells often share overlapping phenotypes and are able to adapt to changes in microenvironment and stimuli as a result of cell plasticity. The M1/M2 nomenclature is therefore no longer relevant and in order to standardise *in vitro* definitions of macrophages, it has been proposed that macrophage names should include information regarding differentiation medium as well as stimuli used. Thus macrophages cultured in GM-CSF and stimulated with LPS would be termed GM-M ϕ -LPS (Martinez and Gordon, 2014).

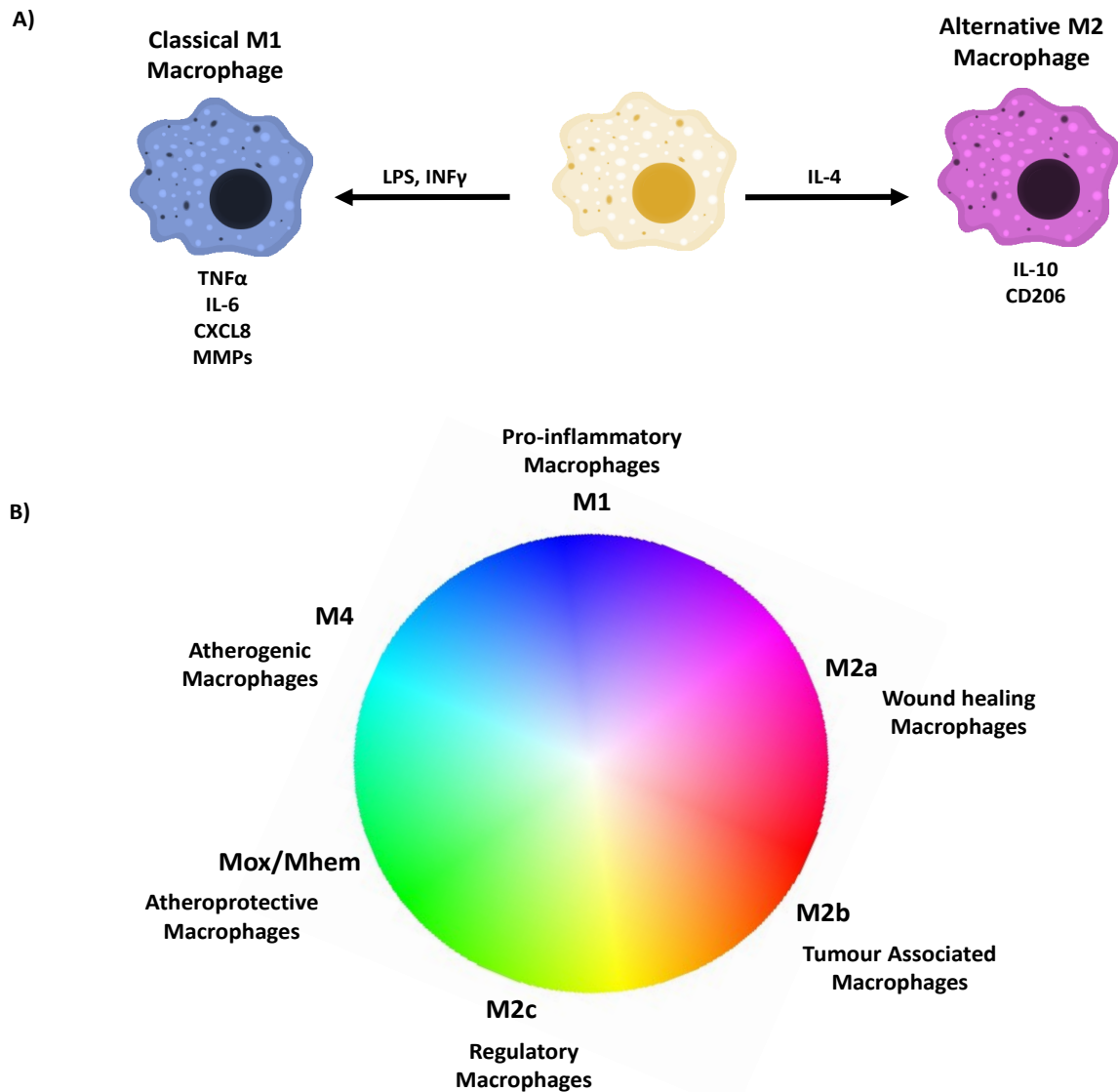


Figure 1. 1 Evolution of macrophage polarisation, linear vs. spectral representation. A) Linear schematic of macrophage polarisation to either M1 or M2 phenotype based on stimuli used *in vitro*. **B)** Adaptation by Mosser and Edwards, 2008, to include additional macrophage phenotypes, represented as a spectrum of overlapping phenotypes. (Mosser and Edwards, 2008). IFN: interferon, LPS: lipopolysaccharide, IL: interleukin, TNF: tumour necrosis factors, MMP: matrix metalloprotease, CD: cluster of differentiation.

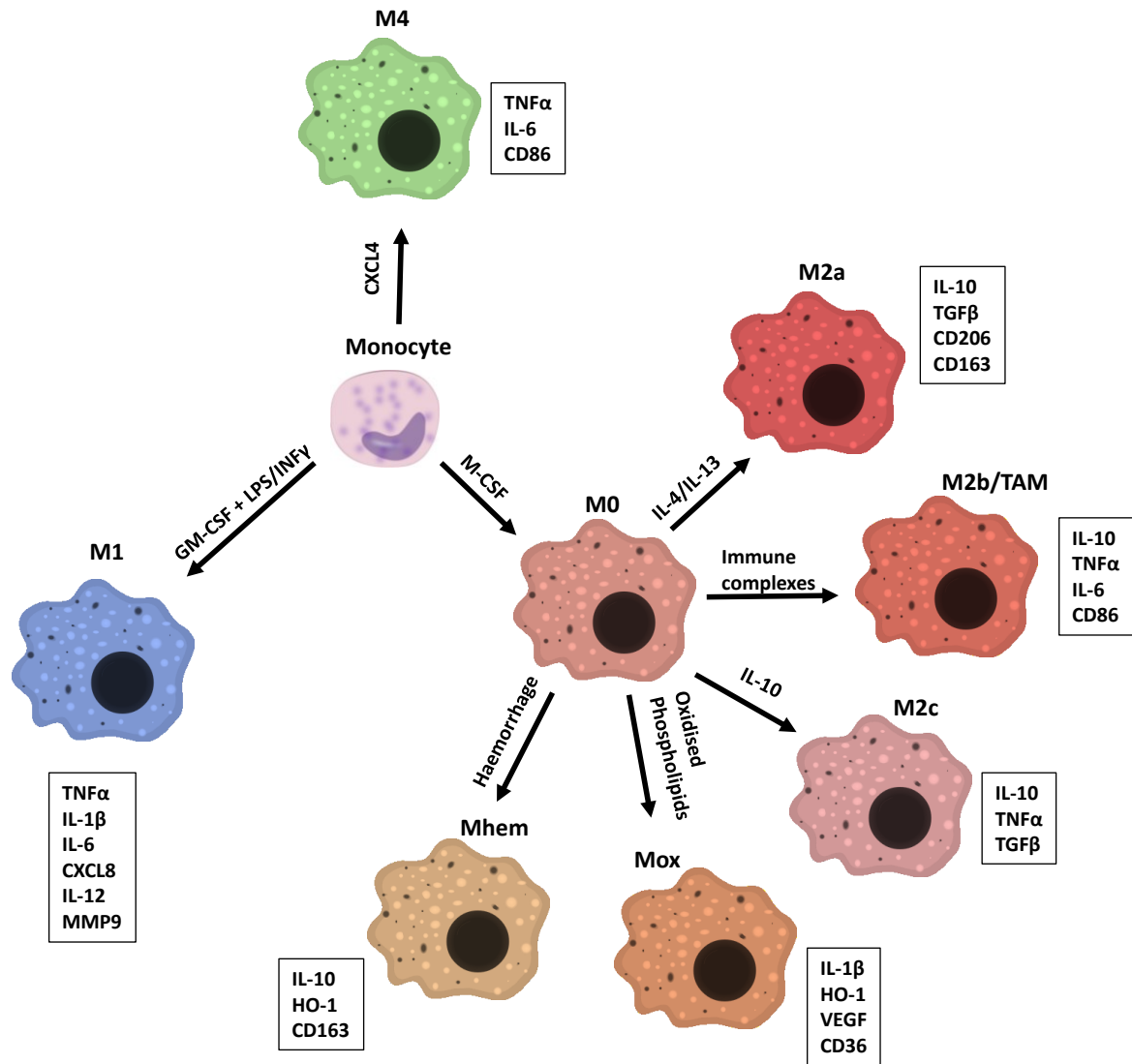


Figure 1. 2 Macrophage phenotypes based on stimuli, cytokine output and cell surface receptors.

GM-CSF: granulocyte macrophage-colony stimulating factors, LPS: lipopolysaccharide, IFN: interferon, TNF: tumour necrosis factor, IL: interleukin, CXCL: C-X-C motif ligand, HO-1: haem oxygenase-1, CD: cluster of differentiation, VEGF: vascular endothelial growth factor, TGF: transforming growth factor. Adapted from (Araujo et al., 2012).

1.6 Macrophage Markers

Investigating macrophage phenotype and plasticity in humans has been challenging due to lack of markers which translate from murine to human cells, although it is generally accepted that macrophage polarisation can be distinguished based on differences in cytokine output, gene transcription profile and receptor expression (Mosser and Edwards, 2008).

Pro-inflammatory macrophages (termed M1 in several studies) are activated by IFN γ , LPS and GM-CSF and secrete high levels of pro-inflammatory cytokines including TNF α , IL-6, IL-12, IL-23 and IL-1 β with low levels of the anti-inflammatory cytokine IL-10 (Wang et al., 2014, Martinez et al., 2006). The pro-inflammatory phenotype is associated with activation of the transcription factors nuclear factor (NF) κ B and activator protein (AP)-1 which drive transcription of pro-inflammatory cytokines and mediators. Signal transducer and activator of transcription (STAT1), STAT5, myeloid differentiation primary response (MyD)88, TRI (Toll/interleukin 1 receptor) domain-containing adapter-inducing interferon- β (TRIF) and Interleukin-1 receptor-associated kinase (IRAK)4 are essential for these signalling cascades which drive inflammation (Fleetwood et al., 2009). Cell surface receptor expression of pro-inflammatory macrophages is characterised in mice by high major histocompatibility complex (MHC) class II, TLR2, TLR4 and CD16 with low CD163 and CD206 (Porcheray et al., 2005).

On the other hand, anti-inflammatory (or M2) cells are activated by IL-4, IL-13, IL-10, M-CSF or glucocorticosteroids and are associated with transcription of IL-10, transforming growth factor (TGF) β , interferon regulatory factors (IRF)4, Kruppel-like factor (KLF)4 and peroxisome proliferator-activated receptor (PPAR) γ (Jaguin et al., 2013, Ghaleb and Yang, 2017). These signalling pathways are associated with STAT6 and c-musculoaponeuronic fibrosarcoma (Maf). Cell surface expression studies performed on mice also demonstrated higher expression of scavenger receptor CD163, mannose receptor CD206, and CD14, which contribute to their ability to uptake pathogens (Stein et al., 1992, Högger et al., 1998).

While there is extensive evidence defining markers of macrophage phenotype in murine studies, similar studies are limited in humans with further work needed to validate characterisation of human macrophage phenotype. There is evidence to suggest, for example, that CD206 is expressed equally in all human macrophages regardless of polarisation (Jaguin et al., 2013) and HLA-DR is expressed by most human macrophages. Furthermore, inducible nitric oxide synthase (iNOS;NOS2), which is extensively used as a marker of pro-inflammatory macrophages in mice, is not elevated in human cells (Rey-Giraud et al., 2012).

1.7 Macrophage Plasticity

Macrophages perform a wide array of functions ranging from homeostatic maintenance to host defence and repair and thus must be capable of rapid adaptation in response to changes in the microenvironments. One relevant example is the response of macrophages to infection; an early pro-inflammatory response is initiated by release of pro-inflammatory cytokines and tissue destructive proteases in order to combat infection. However, this must be followed by resolution of inflammation and repair in order to restore steady state and prevent chronic inflammation and tissue damage (Porcheray et al., 2005). There have been several murine studies demonstrating phenotypic plasticity of macrophages. One experiment treated cells with cytokine prior to LPS stimulation and recorded functional outputs, however if cells treated with cytokine were washed and maintained in the absence of the cytokine prior to LPS stimulation, macrophages showed an identical response to cells which had never been treated with cytokine (Stout and Suttles, 2004, Khallou-Laschet et al., 2010, Arnold et al., 2007). Furthermore, *in vivo* work in mice has demonstrated that pro-inflammatory cells were initially recruited to sites of muscle injury, and once within the tissue switched to an anti-inflammatory activation state which promoted tissue repair (Arnold et al., 2007). Subsequent studies have corroborated the notion that type 1 and type 2 cytokines do not 'fix' cells into a permanent phenotype, but rather elicit a transient signal cascade, and once stimulus is removed, cells revert to basal

phenotype (Stout et al., 2009). Porcheray *et al* provided further evidence that this phenomena occurred in human macrophages, and demonstrated that MDM could be sequentially stimulated towards distinct phenotypes based on expression of CD163, CD206 and production of CCL18 and CCL3 (Porcheray et al., 2005). Macrophage plasticity has also been evaluated by comparing the response of murine macrophages taken from aged and young mice. These different macrophages demonstrated differential responses to LPS, however, when aged macrophages were removed from the aged environment and allowed to reset, cells showed a similar response to LPS stimulation to that observed in the younger cells (Stout and Suttles, 2004).

1.8 Human Lung Macrophage Models

The use of human samples in understanding macrophage function and phenotype is essential for gaining insight into their role in health and disease (Davies et al., 2013). However, due to difficulties in obtaining samples and ethical considerations, murine studies have been used extensively to further understand macrophage biology. The use of knock-out models and fate mapping techniques has aided in better understanding lineage tracing and macrophage phenotypes (Jenkins et al., 2011, Arnold et al., 2007). Unfortunately, there are many differences in the physiology of mouse biology compared to humans, and markers used in mice often do not translate to human macrophage biology (Murray and Wynn, 2011, Lacey et al., 2012). As such, several human macrophage models have been developed to investigate these either directly from lung samples, or from peripheral blood. The monocyte-derived macrophage (MDM) model has been instrumental in terms of understanding macrophage phenotype and function in health and disease (Rey-Giraud et al., 2012). This model is based on isolating monocytes from peripheral blood and thus allows for a relatively non-invasive method of obtaining human samples. Monocytes are selected by their ability to adhere to plastic or by negative selection, and differentiated towards macrophages by culture in foetal calf serum (FCS) and growth factors (Vecchiarelli et al., 1991). GM-CSF and M-CSF are known to be essential for differentiation,

proliferation and survival of macrophages and are thus commonly used in the MDM model (Martinez et al., 2006, Rey-Giraud et al., 2012). GM-CSF is present in humans at low levels under steady state, but has been shown to be elevated in inflammation. M-CSF on the other hand, is ubiquitously expressed by many tissues, and thus is thought to have a role in homeostatic maintenance (Lacey et al., 2012). As such, these growth factors are used to polarise cells towards distinct phenotypes *in vitro*; GM-CSF primes cells toward a pro-inflammatory phenotype with elevated TNF α and CXCL8 production, while M-CSF primes cells towards a resolving macrophage which releases higher levels of IL-10 (Gordon and Taylor, 2005). This model allows the study of the role of phenotype in disease as well as for investigating plasticity by sequentially culturing cells in distinct conditions and assessing phenotype (Porcheray et al., 2005, Lacey et al., 2012).

Bronchoalveolar lavage (BAL), sputum and lung tissue samples also provide an invaluable source to investigate macrophages that are tissue resident and arguably more representative of macrophage function *in vivo*. BAL fluid allows for investigations of alveolar macrophages, and can provide insight into protein and cytokine output, as well as changes in cell numbers between healthy and diseased individuals (Wedzicha and Donaldson, 2003, Budd et al., 2014). Sputum allows for similar studies, although a much more heterogeneous population of immune and epithelial cells is retrieved using this method, thus investigating macrophages in isolation is not optimal (Culpitt et al., 2005).

The most invasive method is to use tissue macrophages isolated from resected lung or transplanted lung tissue. These provide a source of alveolar and interstitial macrophages which can be separated into distinct populations based on cell density using discontinuous Percoll gradients (Chana et al., 2014). Differences in morphology and phenotype have been shown between these pulmonary macrophage subpopulations and early studies suggested that these represent cells at distinct stages of differentiation or maturation, although no definitive data has been published to confirm this (Nakstad et al., 1989, Murphy and Herscowitz, 1984, Chana et al., 2014).

1.9 Role of GM-CSF and M-CSF on Macrophage Function

GM-CSF and M-CSF are commonly used in the MDM model to drive macrophages toward distinct phenotypes and their role in normal homeostasis and disease is highlighted by the defects observed when either pathway is disturbed in both mice and humans (Stanley et al., 1994). GM-CSF is important in macrophage, T-cell and dendritic cell function and is now believed to be relevant following infection and inflammation rather than in maintaining steady state. The role of GM-CSF in pathology has been demonstrated in cases where dysfunction in the GM-CSF pathway result in detrimental effects as in the case of juvenile myelomonocytic leukemia (JMML) and pulmonary alveolar proteinosis. JMML is characterised by increased numbers of leukocytes and monocytes and a diagnostic feature is hypersensitivity to GM-CSF. In addition, macrophages in these patients are unable to combat infection effectively suggesting defects in both cell numbers and function (Hercus et al., 2009). In contrast, alveolar proteinosis is the result of insufficient GM-CSF signalling, either due to autoantibodies against itself or due to mutations in the *CSF2Rα* gene resulting in low macrophage numbers with a concurrent defect in phagocytosis and inflammatory response (Shibata et al., 2001, Trapnell et al., 2009).

GM-CSF signals via its receptor, which is a heterodimer comprised of a binding GMR α subunit and a signalling βc subunit. The GMR α subunit confers specificity for GM-CSF while the βc subunit is shared by closely related IL-3 and IL-5 pathways (Hercus et al., 2009). GM-CSF increases levels of the transcription factor PU.1 in alveolar macrophages. PU.1 regulates myeloid cell lineage and in its absence there is complete ablation of macrophages, and defective neutrophil and T-lymphocyte production (Bonfield et al., 2003) (Figure 1.3). Studies on GM-CSF knock out murine macrophages have shown that PU.1 is completely lost in the absence of GM-CSF, and whilst M-CSF is elevated in these macrophages, it does not rescue PU.1 expression. Furthermore, loss of function in GM-CSF^{-/-} macrophages was rescued following PU.1 transfection, indicating that it is essential in macrophage function and associated with GM-CSF signalling (Shibata et al., 2001).

GM-CSF binding to GM-R α chain leads to association with β chain and a series of phosphorylation events via janus kinase (JAK)2 and receptor β chain leading to phosphorylation of STAT5 and gene transcription of factors such as PU.1. PU.1 is responsible for further transcription of genes associated with macrophage function including GM-CSF-R β , CD32, CD206 and M-CSF-R (Bonfield et al., 2003, Notarangelo and Pessach, 2008). At low concentrations, GM-CSF signalling can also occur via phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt) to promote cell survival and activation of inflammatory response via NF κ B (Vlahos et al., 2006, Notarangelo and Pessach, 2008) (Figure 1.3).

M-CSF acts via a tyrosine kinase receptor, CSF-1R, with roles in macrophage differentiation, proliferation and survival. Most information regarding M-CSF signalling has come from murine macrophages that show that binding of M-CSF to its receptor, results in oligomerisation followed by transphosphorylation of the receptor, leading to signal transduction. Signalling can occur via several pathways including extracellular signal regulated kinase (ERK)1/2, PI3K, mitogen activated protein (MAP) and Akt (Achkova and Maher, 2016). M-CSF is also present in three distinct isoforms, a secreted glycoprotein and proteoglycan and a membrane-spanning cell-surface glycoprotein, all of which are believed to have distinct but overlapping roles (Pixley and Stanley, 2004) (Figure 1.4).

Furthermore, IL-34 was recently discovered to act via the same receptor, although there is no known overlap in localisation of both ligands which suggests distinct functions (Hume and MacDonald, 2012). In addition, loss of M-CSF or M-CSFR in murine studies demonstrated that this is not a redundant signalling pathway, but is required for adequate macrophage function (Strachan et al., 2013). Loss of M-CSF resulted in reduced macrophage numbers in tissues, with significant defects in osteoclast function (Wiktor-Jedrzejczak et al., 1990). M-CSF has been implicated in several cancers, with evidence from breast, ovarian, cervical and other cancers demonstrating elevated serum M-CSF in patients, as well as an upregulation of both M-CSF and its receptor within tissues of interest. M-CSF signalling is believed to drive TAM function and dampen anti-tumour immunity, allowing for cancer progression (Wiktor-Jedrzejczak et al., 1990).

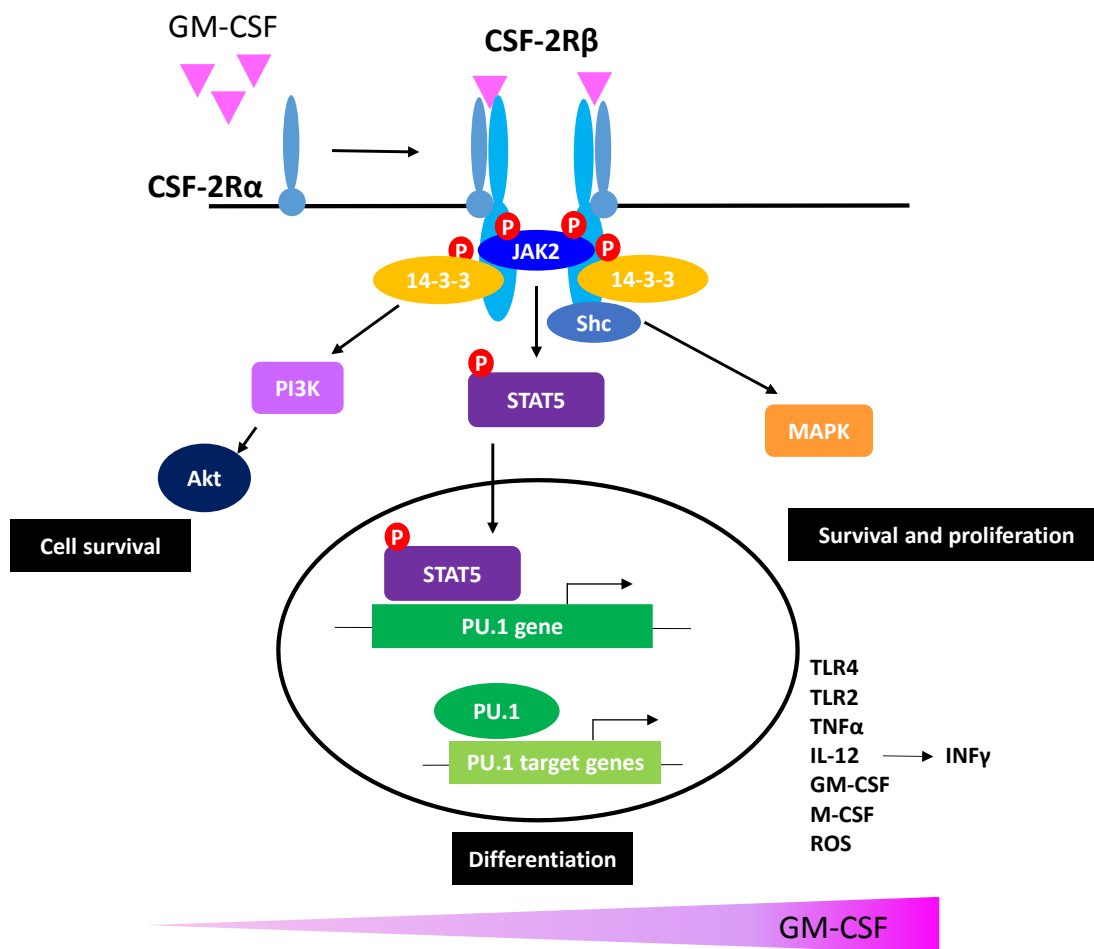


Figure 1. 3 GM-CSF signalling pathway driving survival, differentiation and proliferation of macrophages. GM-CSF initiates signalling by binding to the α chain which can then associates with the β chain. JAK2 is constitutively bound to the β chain, and is activated by phosphorylation. At low GM-CSF levels, signalling occurs via 14-3-3, PI3K and Akt to promote cell survival. At higher concentrations GM-CSF activates STAT5 to induce PU.1 transcription and Shc to promote cell survival, proliferation and inflammatory signals (Trapnell et al., 2009, Hercus et al., 2009, Notarangelo and Pessach, 2008). GM-CSF: granulocyte colony-macrophage colony stimulating factor, Jak: janus kinase, Shc: Src homology 2 domain-containing, STAT: signal transducer and activator of transcription, Akt: protein kinase B, PI3K: phosphoinositide 3 kinase, MAPK: mitogen activated protein kinase, TLR: toll-like receptor, TNF: tumour necrosis factor, IL: interleukin, IFN: interferon, ROS: reactive oxygen species.

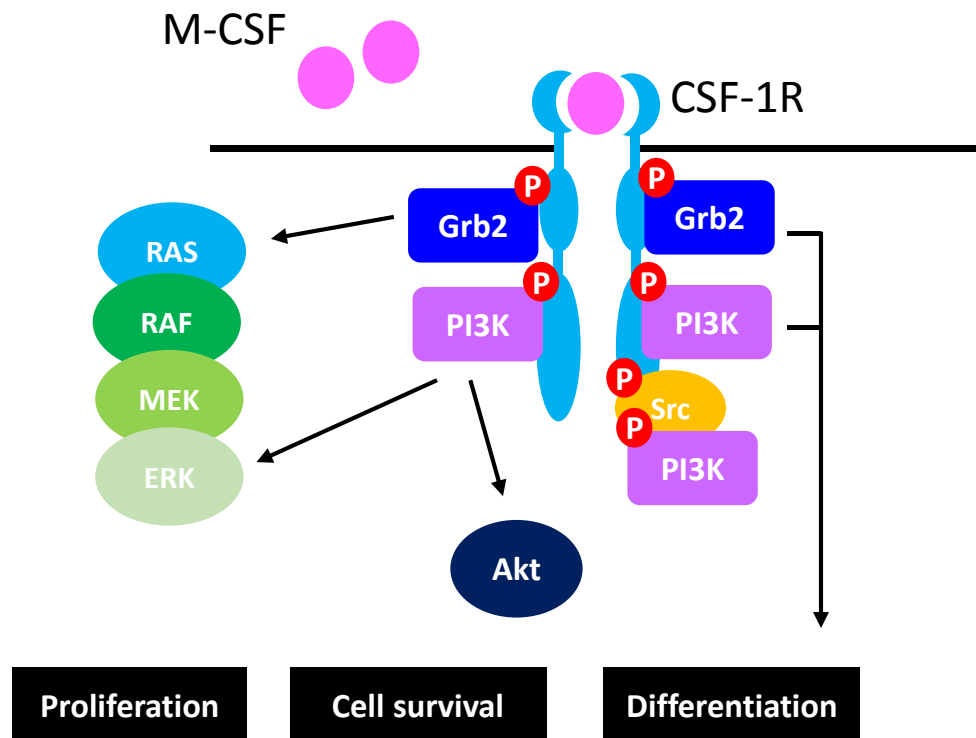


Figure 1. 4 M-CSF signalling for survival, differentiation and proliferation of macrophages. M-CSF binds to the CSF-R1 on the cell surface which leads to dimerization and phosphorylation of the receptor. Phosphorylated sites act as a docking site for adaptor proteins such as PI3K and Grb2 which drive signalling via ERK and Akt signalling (Achkova and Maher, 2016, Pixley and Stanley, 2004, Hercus et al., 2009). M-CSF: macrophage-colony stimulating factor, Grb: growth factor receptor-bound protein, Akt: protein kinase B, PI3K: phosphoinositide 3 kinase, RAS: rat sarcoma, RAF: rapidly accelerated fibrosarcoma, MEK: mitogen activated protein kinase kinase, ERK: extracellular signal regulated kinase, Src: proto-oncogene tyrosine-protein kinase.

1.10 Macrophages in Disease

Macrophages have a crucial role in innate immunity and inflammation and as such play an important role in disease. Tight regulation of macrophage responses and cell plasticity allow for initial inflammatory responses required to combat injury or infection to be rapidly resolved and followed by a resolution phase (Wynn et al., 2013). While the inflammatory response is required, it also leads to tissue damage due to release of ROS and proteases, and if this remains uncontrolled, can contribute

to disease progression. This effect is highlighted in inflammatory diseases such as atherosclerosis, inflammatory bowel disease, rheumatoid arthritis (RA), idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD), where macrophages secrete elevated inflammatory signals which contribute to disease pathology (Murray and Wynn, 2011, Hamilton et al., 2012). Macrophages have also been investigated in the field of cancer, where TAMs have an initial pro-inflammatory role allowing tumour development and later acquire an immuno-suppressive role by which tumour growth can remain undetected by host immune system, while at the same time promoting angiogenesis and cell migration (Wynn et al., 2013, Sica and Mantovani, 2012). Chronic inflammatory and autoimmune conditions are associated with macrophages, which remain pro-inflammatory and fail to return to a regulatory state. This can be observed in Crohn's disease where a subset of pro-inflammatory macrophages have been shown to be distinct from normal resident intestinal macrophages, and secrete high levels of IL-23 and TNF α (Kamada et al., 2008). Another disease in which macrophages are believed to be key orchestrators, is in COPD, where continued insult from cigarette smoking results in exaggerated inflammation and tissue destruction within the lung (Barnes et al., 2003).

1.11 Chronic Obstructive Pulmonary Disease

1.11.1 Definition and Overview

COPD is an umbrella term encompassing several pathophysiologies which includes chronic bronchitis, small airway disease and emphysema (Figure 1.5) and accounted for 26% of all lung disease related deaths, and was the 5th leading cause of death in the UK in 2016 (BLF, 2016). COPD is characterised by persistent respiratory symptoms, including dyspnoea, coughing and increased sputum production and results in poorly-reversible and progressive airflow limitations (Barnes et al., 2003).

Diagnosis is made by evaluating symptoms, risk factors and requires spirometry to determine lung function, where a post-bronchodilator forced expiratory volume in 1 second/forced vital capacity (FEV₁/FVC) ratio of <0.7 confirms diagnosis (Vogelmeier et al., 2017). The incidence of COPD is rising,

with a 27% increase in the past decade and an estimated 115,000 individuals diagnosed every year in the UK (BLF, 2016). The World Health Organisation has predicted an increase in incidence in the coming years, due to an increasing smoking population in developing countries as well as a growing aging population worldwide (WHO, 2016).

COPD patients present a heterogeneous population, and many attempts have been made to stratify patients into relevant groups to aid better treatment and prognosis. The revised 2017 GOLD guidelines utilises the ABCD grading system whereby symptoms and exacerbation rate and frequency are used to classify patients (Vogelmeier et al., 2017). Exacerbations are defined as acute worsening of symptoms that require additional medication and are often due to viral or bacterial infection as well as environmental factors (Barnes et al., 2003). COPD exacerbations account for a major cause of morbidity and mortality and is associated with a concomitant decline in lung function. In addition to decline in patient standards of life, there is an associated healthcare burden with over 140,000 hospital admissions per year in the UK (BLF, 2016).

1.11.2 Risk Factors and Pre-dispositions

Cigarette smoking is the most common risk factor for developing COPD and approximately 90% of all COPD cases in the UK can be attributed to cigarette use. Furthermore, there is evidence to suggest a correlation between smoking history, measured by pack years (1 pack year = 20 cigarettes a day for a year), and decline in lung function (Barnes et al., 2003). Never-smokers who develop COPD also present with milder symptoms and better prognosis compared to smokers. In addition to smoking, other environmental factors have been implicated in the development of COPD, including air pollution and occupational exposure to dust and fumes. Use of biomass fuels for indoor cooking, which is particularly prevalent in developing nations such as India, is believed to account for 35% of COPD cases in low and middle income nations (Mannino and Buist, 2007, Salvi and Barnes, 2009).

The role of gender in COPD predisposition has been controversial with some evidence to suggest women are more susceptible (Buist et al., 2007). Until recently, COPD has been predominantly male-

dominated, which reflects smoking patterns as well as occupational exposure of men compared to women in the past 60 years. However, the gender divide is no longer observed as a result of increasing prevalence of female smokers (Mannino and Buist, 2007, Salvi and Barnes, 2009).

However, while cigarette smoking is prevalent among COPD patients, only about 15-20% of smokers develop COPD, highlighting that other factors are involved (Mannino and Buist, 2007). Individuals who develop COPD are believed to have a genetic pre-disposition, which, in combination with environmental factors such as smoking, drives the development of COPD (Malhotra and Olsson, 2015). The most striking genetic association is that of alpha-1-antitrypsin deficiency, an inherited condition where individuals lack the alpha-1-antitrypsin protein, resulting in early-onset emphysema and COPD (Mannino and Buist, 2007). Additional genetic polymorphisms have been identified in linkage studies which are believed to predispose individuals to COPD, including genes associated with TGF β signalling, matrix metalloproteases (MMPs) and iron metabolism (Tesfaigzi et al., 2006, Malhotra and Olsson, 2015). However, many of these have remained unverified, or contradicting results found when conducted in different populations, thus the significance of these genetic polymorphisms in COPD remains to be proven.

Epigenetic modifications are also thought to play a role in the progression of COPD, influencing transcription of inflammatory genes which drive pathophysiology. Inflammatory pathways such as those leading to activation of NF κ B are often regulated by modifications of histone residues (Malhotra and Olsson, 2015). Histone acetylation can modify chromatin and is regulated by levels of histone acetyl transferases (HATs) and histone deacetylases (HDACs). Acetylated residues are associated with open chromatin and thus allow for gene transcription to occur, and in COPD there are reports of increased histone acetylation in lung biopsies, with a concomitant decrease in key HDACs such as HDAC2 and sirtuin (SIRT)1. (Yang et al., 2007, Rajendrasozhan et al., 2009). Loss of HDAC activity has been shown to result in elevated pro-inflammatory signalling in alveolar macrophages and contribute to inflammation in COPD (Ito et al., 2005).

1.11.3 Pathophysiology of COPD

1.11.3.1 Chronic Bronchitis

Chronic bronchitis is defined by persistent cough and production of sputum for at least three months of the year and for two consecutive years, and can result in accelerated decline in lung function, airway obstruction and increased incidence of infections and exacerbations (Kim and Criner, 2013). Chronic bronchitis is caused by mucus hypersecretion due to goblet cell hyperplasia which occurs as a result of exposure to noxious fumes such as cigarette smoke, chronic infections and exaggerated inflammatory response (Kim and Criner, 2013, Barnes et al., 2003). Increased number of goblet cells has been reported in both smokers and COPD patients providing further evidence that cigarette smoking may result in goblet cell hyperplasia (Pauwels et al., 2001). Decrease in ciliary clearance in combination with elevated number of goblet cells results in accumulation of mucus in the airways and is correlated with elevated incidence of viral and bacterial infections, which in turn have a detrimental effect on lung function (Ebert and Terracio, 1975).

1.11.3.2 Small Airway Disease

Narrowing of small airways occurs as a result of structural changes and increased mucus and inflammatory cells within the airway (Barnes et al., 2003). Small airways are defined as <2mm in internal diameter, and extent of narrowing is correlated to severity of disease, as is the presence of lymphoid follicles which are present in severe disease (McDonough et al., 2011).

Airway remodelling and fibrosis is thought to occur as a consequence of attempts to repair damage caused by continued inflammatory signalling within the lung, and a continuous damage-repair cycle which results in structural remodelling (Pauwels et al., 2001). Murine studies have demonstrated that overexpression of TGF β leads to remodelling similar to that observed in small airways of COPD patients, and may be a mechanism by which increased collagen deposition occurs (Hogg et al., 2004, Lee et al., 2001). Failure of the innate immune response to clear excess mucus and bacteria from the lower airways results in further narrowing and persistent colonisation by bacterial species. This is

believed to drive increased infiltration of cells of the adaptive response which account for increased lymphocyte presence and formation of lymphoid follicles in small airway disease (Barnes et al., 2003, Hogg et al., 2004).

While there is overlap in the pathologies of small airway disease and chronic bronchitis, mucus hypersecretion and sputum production from chronic bronchitis is thought to be independent of the pathology occurring in the small airways (Hogg et al., 2004).

1.11.3.3 Emphysema

Emphysema is the destruction of lung tissue leading to permanent air space enlargement as a result of elevated levels and activity of proteolytic enzymes within the lung (Abboud and Vimalanathan, 2008). Reduced elastic support can result in small airway collapse and reduced elastolytic recoil, and together contribute to airflow obstruction (Demkow and van Overveld, 2010). The leading hypothesis as to the cause of emphysema is the protease-anti-protease hypothesis by which there is elevated activity of proteases either as a result of increased secretion, or reduced presence of their inhibitors. Secretion of proteases in COPD is thought to be mainly by neutrophils and macrophages which secrete elastase, cathepsins and MMPs and are known to be elevated in COPD lungs (Abboud and Vimalanathan, 2008). Alveolar macrophages from COPD patients have also been shown to secrete higher levels of MMPs with increased activity (Russell et al., 2002). Animal models of emphysema have been developed by instilling proteolytic enzymes in the tracheas of guinea pigs, as well as by continuous cigarette smoke exposure, providing further evidence for the role of both smoking and proteases as drivers of emphysema (Janoff et al., 1976, Senior et al., 1977).

Patients with higher smoking histories have been found to present with more severe emphysema but with no correlation to increased disease progression (Coxson et al., 2013).

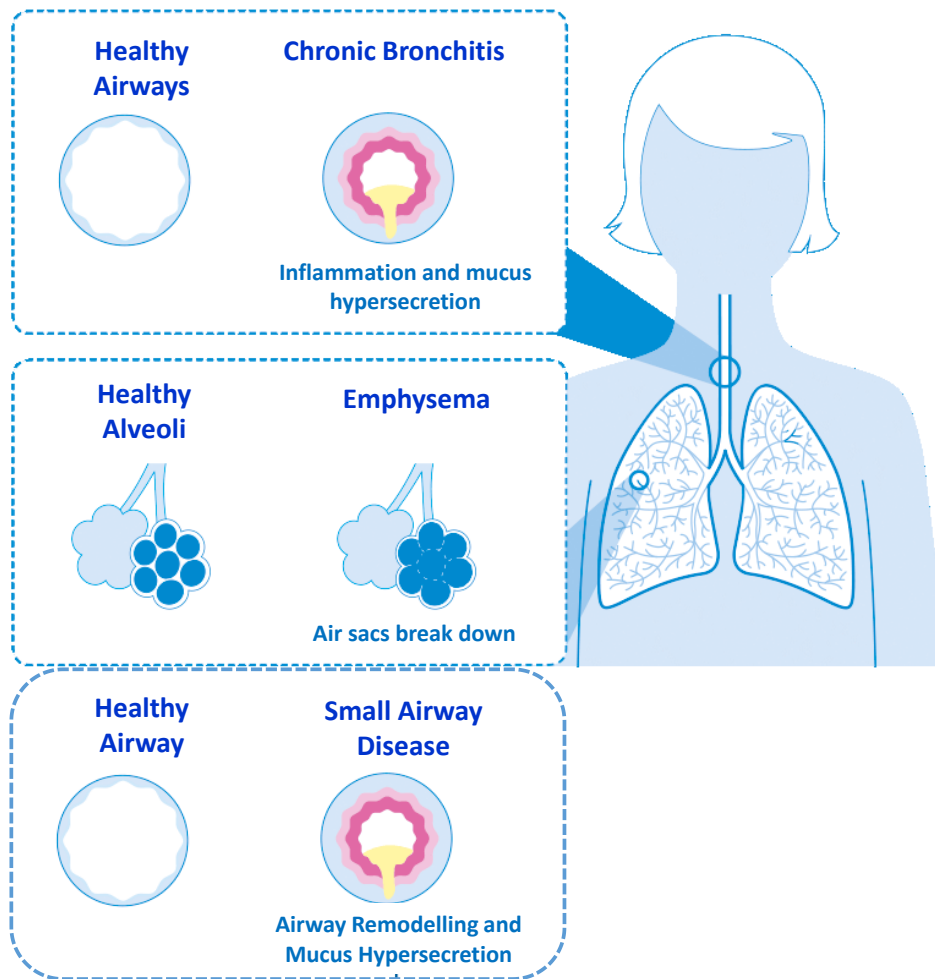


Figure 1. 5 COPD pathophysiology. COPD is an umbrella term, which encompasses several pathologies, including chronic bronchitis, emphysema and small airway disease. Patients do not present with the same degree of severity for each pathology and emphysema and chronic bronchitis are often on opposite spectrums of the disease. Adapted from (BLF, 2016).

1.11.4 Treatment

There is currently no cure for COPD, with little progress in the past few decades in the development of new therapies to treat this condition and ameliorate symptoms and co-morbidities. Smoking cessation is the most effective option in treating and slowing progression of COPD. Pulmonary rehabilitation is another non-pharmacological method of improving COPD symptoms, and has been shown to improve physical exertion potential as well as quality of life (NHS, 2016).

Several medications are commonly prescribed to COPD patients in order to alleviate symptoms, but do not target the underlying problems. Long acting bronchodilators, including long acting beta agonists (LABA) and long acting muscarinic antagonists (LAMA), are most often prescribed for patients with persistent shortness of breath and can be complemented with inhaled corticosteroids and oral phosphodiesterase (PDE)4 inhibitors for patients with exacerbations and severe airflow obstruction (GOLD, 2016, NHS, 2016). Antibiotics are used to treat exacerbations caused by bacterial species, and evidence suggests that continued antibiotic use can reduce the number of exacerbations suffered by patients in a year, although issues of bacterial resistance arise as a result (Vogelmeier et al., 2017).

In extreme cases, where resting hypoxemia is $<88\%$, continuous oxygen is provided. Surgical interventions are used as a last resort, with lung volume resection therapy and lung transplants performed for severe emphysema, both of which have been shown to improve health and function, but with no increase in overall survival (Vogelmeier et al., 2017).

1.12 Inflammatory cells in COPD

1.12.1 Neutrophils

BAL and sputum samples from COPD patients have consistently shown elevated numbers of neutrophils compared to samples from non-smokers, although presence within the parenchyma appears normal (Barnes et al., 2003, Quint and Wedzicha, 2007). This suggests that there is rapid movement through the lung and accumulation within the airway lumen and mucosal surface (Makris et al., 2009).

Neutrophils are recruited to the lung by chemoattractant such as CXCL8, GM-CSF and leukotriene (LT)- B_4 , all of which are elevated in COPD and secreted by macrophages, fibroblasts and epithelial cells (Barnes et al., 2003, Traves et al., 2002). Neutrophils themselves can release CXCL8 and form a positive feedback loop whereby more neutrophils are recruited to the lung (Keatings et al., 1996, Tanino et al., 2002). Neutrophil migration is controlled by interactions with vascular endothelium and selectin mediated rolling and capture. Chemoattractants cause upregulation of macrophage-1 antigen (MAC-

1) (CD11b/CD18) on neutrophils, which interacts with its endothelial counterpart, intracellular adhesion molecule (ICAM)-1, resulting in extravasation into tissue. There is evidence to suggest that COPD neutrophils have greater endothelial interactions and migratory potential (Woolhouse et al., 2005, Quint and Wedzicha, 2007).

Neutrophils are short-lived and thus high turnover occurs during inflammation. Agents such as LPS and GM-CSF prolong survival of cells, which retain their potential to become activated. Furthermore, glucocorticosteroids, which are often prescribed to COPD patients, have been shown to increase survival of neutrophils (Cox, 1995). Increased numbers of neutrophils within the lung, as well as prolonged survival contribute to the continued inflammatory conditions observed in COPD. Release of granules, ROS, as well as proteases such as elastase, cathepsin G, proteinase-3 and MMPs are implicated in tissue destruction, epithelial damage and development of emphysema, but also in the release of mucus, thus driving chronic bronchitis (Abboud and Vimalanathan, 2008). However, chronic airway neutrophilia is observed in other respiratory conditions such as cystic fibrosis and bronchiectasis which are not associated with emphysema, thus suggesting that other cells must be involved in driving this pathophysiology in COPD (Barnes et al., 2003) (Figure 1.6).

1.12.2 T-lymphocytes

The adaptive immune system is also thought to play a role in the pathogenesis of COPD with both CD8⁺ and CD4⁺ T-cells are elevated in the lung parenchyma and small airways of COPD patients (O'donnell et al., 2006). However, it is the CD8⁺ cells which are believed to have a key role in COPD pathogenesis and particularly in emphysema, although there is still considerable speculation regarding the role of these cells in COPD. An important role performed by CD8⁺ cells is to combat viral infection by the destruction of infected cells, or through induction of apoptosis (Kagi et al., 1994). CD8⁺ cells can release lytic substances such as perforin and granzyme B, both of which have been shown to be elevated in COPD sputum samples compared to non-smokers, and may contribute to alveolar cell destruction (Chrysafakis et al., 2004). These cells may also drive apoptosis of alveolar epithelial cells in smokers and COPD lungs, and an association has been made between the number of cells

undergoing apoptosis and the presence of CD8⁺ cells (Majo et al., 2001). T-cells may also contribute to COPD through Th1 type inflammation, with increased expression of CXCR3, CXCL10 and IFN γ in CD8⁺ cells in COPD airways, which can perpetuate further recruitment of Th1 cells and drive a cycle of inflammation (Saetta et al., 2002) (Figure 1.6).

1.12.3 Epithelial cells

The airway epithelium is exposed to continuous insult during day to day life and is one of the first barriers to come into contact with inhaled cigarette smoke (Barnes, 2004). Cigarette smoke can activate epithelial cells driving release of pro-inflammatory mediators such as TNF α , IL-1 β , CXCL8 and GM-CSF, leading to increased recruitment of immune cells such as neutrophils and monocytes which further drive inflammation and injury. Both cigarette smoke and diesel exhaust particles have been shown to increase release of CXCL8 from epithelial cells (Hellermann et al., 2002). Due to cell damage as a result of pathogens or noxious fumes, the epithelium is capable of removing damaged cells and replacing them, either via migration or proliferation through a process which requires TGF β (Puchelle et al., 2006, Chung, 2001). However, continued injury to the epithelium from cigarette smoking can result in aberrant remodelling with elevated release of TGF β , and continued cycles of inflammation and repair result in fibrosis. Furthermore, epithelial cells from COPD patients have been shown to have higher rates of apoptosis and this is not reversed by smoking cessation (Hodge et al., 2005). Continued insult and inflammation, as well as inappropriate cell repair can lead to squamous cell metaplasia and mucus hyperplasia and is one of the driving forces behind the development of small airway disease (Takizawa et al., 2001) (Figure 1.6).

1.12.4 Macrophages

Macrophages are key orchestrators in the pathophysiology of COPD and contribute to most of the features that define this disease. Macrophage numbers are elevated in COPD patients compared to non-smokers in sputum, BAL and tissue samples with reports of 5-25 fold increase, which correlate with the severity of disease (Traves et al., 2002, Hogg et al., 2004, Russell et al., 2002). Increased numbers of macrophages in the lungs of COPD patients could be due to increased recruitment of

circulating monocytes, increased local proliferation of tissue and alveolar macrophages or prolonged survival within the lung (Chung and Adcock, 2008). Indeed, there is evidence to suggest all may play a role in increasing macrophage presence in the lung. CCL2 is a potent monocyte chemoattractant and has been shown to be elevated in COPD sputum samples compared to non-smokers, indicating that monocyte recruitment from peripheral blood is higher than in non-smokers (Traves et al., 2002). Recently, work by several groups have identified self-renewing macrophages within the lungs of mice, which are capable of replenishing the cell population in steady state and following insult (Sieweke and Allen, 2013, Hashimoto et al., 2013, Jenkins et al., 2011). It has been suggested that this population exists in human lungs, which replenish tissue resident cells, while monocyte recruitment only occurs following injury, although evidence to support this is still lacking in humans (Hashimoto et al., 2013). Increased proliferation and survival of macrophages is difficult to prove in human lungs. However, studies on human alveolar macrophages have shown elevated levels of B-cell lymphoma-extra-large (Bcl-X) which is an anti-apoptotic molecule, as well as reduced nuclear p21 which negatively regulates cell cycle progression, and thus suggest increased proliferation and reduced apoptosis in COPD cells (Tomita et al., 2002) (Figure 1.6).

1.12.4.1 Cytokine Production

Macrophages in the lung are able to secrete large amounts of inflammatory mediators, including lipid mediators, cytokines, chemokines, and reactive oxygen and nitrogen species. CXCL8 is the most commonly investigated chemokine in COPD, and is a potent neutrophil recruiter. Alveolar macrophages from COPD patients have been shown to produce higher levels of CXCL8 compared to cells from non-smokers at baseline and following cigarette smoke extract (CSE) or IL-1 β stimulation, and this could not be inhibited by the glucocorticosteroid, dexamethasone (Keatings et al., 1996, Culpitt et al., 2003b).

Several other inflammatory mediators, including TNF α , IL-1, IL-6, IL-1 β and CCL2, are also produced by macrophages and studies on BAL and sputum have demonstrated that these are elevated in COPD (Keatings et al., 1996, Caramori et al., 2003, Churg et al., 2004). The transcription of these and other

molecules involved in the pro-inflammatory response are regulated by transcription factors including NFκB and AP-1 (Di Stefano et al., 2002). NFκB exists as a heterodimer comprising RelA/p65 and p50 subunits, which, upon activation, translocate into the nucleus where they bind target genes, and can be used as an indicator of whether inflammatory mediators are being produced. Several genes regulated by NFκB can, in turn, drive activation of NFκB and form a positive feedback loop to amplify the inflammatory response (Barnes and Karin, 1997). Previous work on sputum macrophages has demonstrated an increase in nuclear RelA during exacerbations in COPD patients compared to steady state which was not observed in neutrophils (Caramori et al., 2003).

Macrophages are also activated by IFNγ to secrete CXCL9, CXCL10 and CXCL11 which act through the CXCR3 receptor found on T-cells. CXCR3 ligands are upregulated in COPD samples (Costa et al., 2016), and in this way T-cells can form a positive feedback loop by initial release of IFNγ, driving recruitment into the lung (Kelsen et al., 2009).

On the other hand, IL-10 is also secreted by macrophages and T-cells, and is essential as an anti-inflammatory mediator, downregulating cytokine release from many cell types including monocytes and neutrophils (Ouyang et al., 2011). Sputum samples from COPD and smokers have shown decreased levels of IL-10 compared to non-smokers, and identified macrophages as the main source of IL-10 (Takanashi et al., 1999).

1.12.4.2 Protease-Anti-Protease Imbalance

In addition to driving inflammation in COPD, macrophages also secrete proteases, which are believed to be crucial in the development of emphysema. An imbalance in protease activity is thought to occur as a result of elevated release and activity of proteases such as cathepsins and MMPs, with a concomitant but insufficient increase in the corresponding inhibitors, which are unable to overcome protease activity (Abboud and Vimalanathan, 2008). Macrophages are known to be an important source of proteases, and several studies have demonstrated that alveolar macrophages and MDM from COPD patients have increased release and activity of MMP9 and MMP12 which are heavily

implicated in the development of emphysema. Furthermore, these cells retained this imbalance even after *in vitro* culture for 3-12 days (Finlay GA, 1997, Russell et al., 2002, Culpitt et al., 2005). There is contradictory evidence as to whether there is a decrease in TIMP1, a key MMP inhibitor, with some studies showing it is downregulated in COPD, and others showing no difference compared to non-smoker cells (Lim et al., 2000, Russell et al., 2002, Mercer et al., 2005). There is also some evidence to suggest that several polymorphisms exist in MMP and TIMP genes, which are associated with susceptibility to emphysema and COPD. Polymorphisms in TIMP2, MMP1, MMP9 and MMP12 have been identified in a population in Helsinki and Texas (Tesfaigzi et al., 2006, Kukkonen et al., 2013). However, these have not been verified in other populations, and the effect of a single polymorphism is yet to be used effectively as a marker of COPD predisposition.

1.12.4.3 Reduced Phagocytosis

The ability of macrophages to uptake bacteria, foreign particulates and apoptotic cells is essential in the homeostatic maintenance of the lung, and sterility of the lower airways (Donnelly and Barnes, 2012). Phagocytosis by macrophages in the lungs occurs mainly via the non-opsonic route as the lungs are not a serum-rich environment. As such, cells make contact with foreign objects directly, including the two most prevalent bacterial species in COPD, *Haemophilus influenza* and *Streptococcus pneumoniae*. These are taken up into the cell resulting in the formation of the phagolysosome where foreign bacterial species are degraded (Donnelly and Barnes, 2012, Arredouani et al., 2005). Defects in the phagocytic system can result in increased infections and exacerbations, as well as accumulation of apoptotic and necrotic cells which can damage healthy tissue. There is evidence to suggest that cigarette smoke and diesel exhaust fumes reduce the ability of macrophages to phagocytose (Lundborg et al., 2006, Hodge et al., 2007). Furthermore, there is extensive evidence demonstrating that alveolar macrophages and MDM from COPD patients have a decreased ability to uptake prey, including *Haemophilus influenza*, *Escherichia coli*, *Streptococcus pneumoniae* and inert beads, thus contributing to the high exacerbation rate of these patients (Berenson et al., 2006, Martí-Llitas et al., 2009, Taylor et al., 2010, Budd et al., 2014).

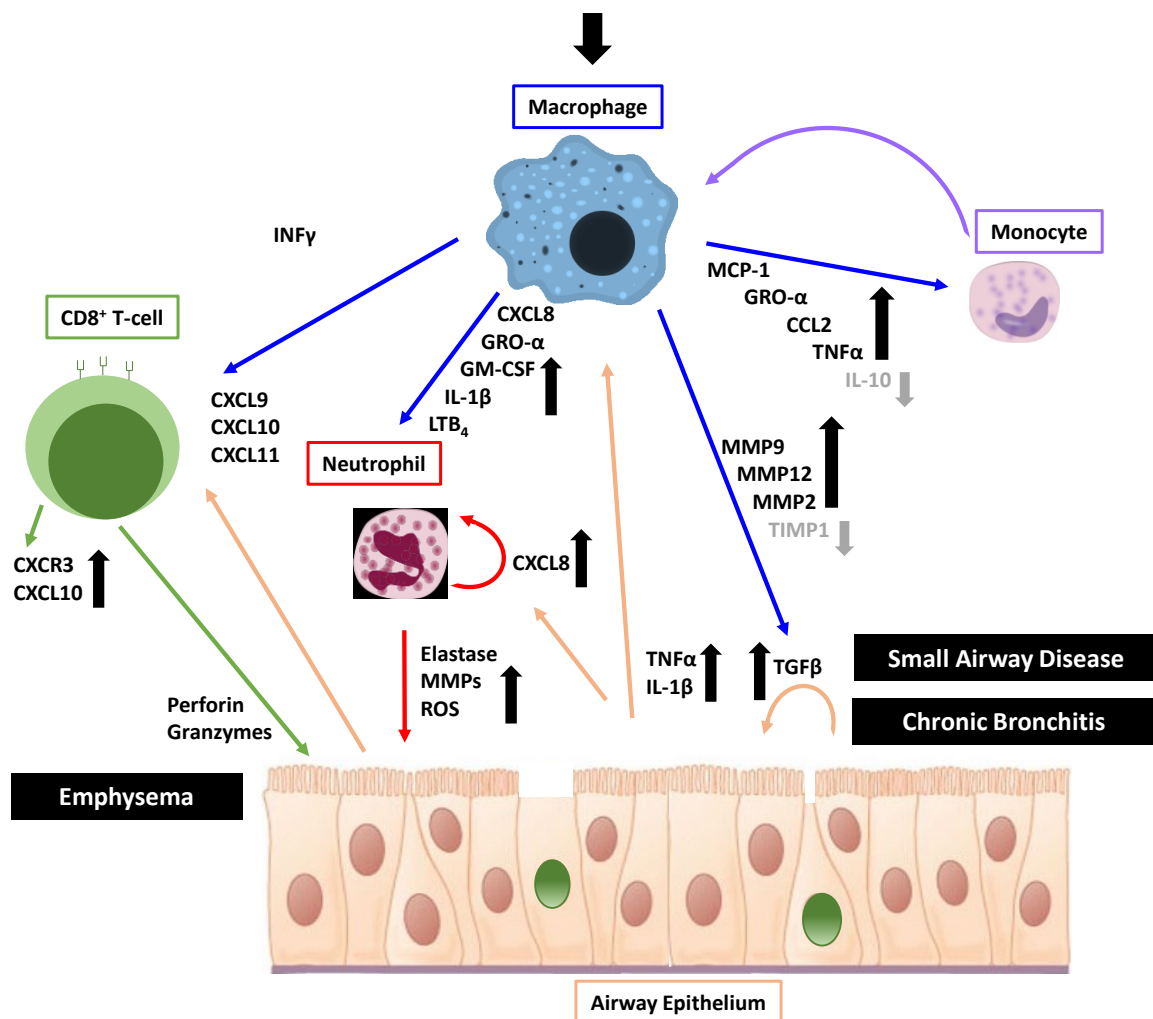


Figure 1. 6 Macrophages as orchestrators of COPD. Cigarette smoke drives macrophages to release higher levels of pro-inflammatory cytokines, which lead to the recruitment of other inflammatory cells including monocytes, neutrophils and T-cells. These can then perpetuate inflammation by further release of cytokines, ROS and proteases and recruitment of additional cells as well as damaging the airway epithelium. Macrophages themselves secrete proteases which drive emphysema, and stimulate secretion of mucus and TGF β which promote airway remodelling and mucus hypersecretion which are hallmarks of small airway disease and chronic bronchitis (Barnes, 2004, Chung, 2001, Notarangelo and Pessach, 2008).

1.13 Hypothesis

Macrophages are critical in the pathophysiology of COPD, driving increased inflammation, tissue destruction and recruitment of other inflammatory cells, although the underlying changes in macrophage dysfunction remain unknown. Phenotype and plasticity are essential for normal macrophage function with growth factors such as GM-CSF and M-CSF needed to drive cells towards the appropriate response. Changes in phenotype, or an imbalance in phenotype and loss of plasticity because of changes in the lung environment, due to oxidative stress and cigarette smoking, may drive dysfunction of these cells in COPD. The hypothesis of this thesis is therefore:

COPD macrophages will demonstrate an inflammatory phenotype, which will be exaggerated by GM-CSF culture, with a loss of plasticity and inability to return to a resolving state, compared to cells from non-smokers and smokers.

1.14 Aims

In order to address this hypothesis, the following aims were explored:

- 1 Comparison of macrophage phenotype and function between lung tissue macrophages and MDM isolated from non-smokers, smokers and COPD patients in terms of cytokine output, protease balance and phagocytosis.
- 2 Investigation of the effect of GM-CSF and M-CSF on mature macrophage phenotype in cells isolated from tissue and using the MDM model.
- 3 Analysis of whether tissue macrophages and MDM models show similar defects in COPD cells compared to non-smokers and smokers.
- 4 Identification of whether MDM subpopulations isolated based on cell density behave in a similar manner to cells isolated using the same method from tissue.
- 5 Analysis of the effect of changing culture environment on macrophage phenotype to establish macrophage plasticity in tissue macrophages and MDM

- 6 Identification of molecular differences in receptor expression and transcription factor gene expression between subject groups.
- 7 Investigation of the role of epigenetic modifications on driving COPD phenotype in macrophages by using a histone demethylase inhibitor to maintain 'closed chromatin' in MDM and tissue macrophages.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

Material	Supplier
2-Mercaptoethanol	Sigma-Aldrich, Dorset, UK
3-(4,5-Dimethylthiazol-2-yl)-tetrazolium bromide (MTT)	Sigma-Aldrich, Dorset, UK
4', 6-diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich, Dorset, UK
μ-Slide 8 well glass bottom plates	Ibidi, Thistle Scientific, Glasgow, UK
Accutase® solution	Sigma-Aldrich, Dorset, UK
Alexa fluor 405 NHS ester	Invitrogen, Paisley, UK
Alexa fluor 488 NHS ester	Invitrogen, Paisley, UK
Amphotericin-B	Gibco, Invitrogen, Paisley, UK
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Dorset, UK
Brain Heart Infusion broth	Sigma-Aldrich, Dorset, UK
cDNA Standard	Clontech, France
Cell dissociation solution	Sigma-Aldrich, Dorset, UK
Cell tracker™ Green	Invitrogen, Paisley, UK
Cell tracker™ Red	Invitrogen, Paisley, UK
Colloidal Blue staining kit	Invitrogen, Paisley, UK
Costar 6-, 24-, 96- well tissue culture plates	Fisher, Loughborough, UK
CXCL8 capture, detection, standard antibody	R&D Systems, Abingdon, UK
Dextran	Sigma-Aldrich, Dorset, UK
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich, Dorset, UK
Dulbecco's Phosphate Buffered Saline (D-PBS)	Sigma-Aldrich, Dorset, UK

Ethylenediaminetetra-acetic acid (EDTA)	Sigma-Aldrich, Dorset, UK
FACS Clean	Beckton Dickinson, Oxford, UK
FACS Flow	Beckton Dickinson, Oxford, UK
FACS Rinse	Beckton Dickinson, Oxford, UK
Foetal Bovine Serum (FBS)	Invitrogen, Paisley, UK
Full range rainbow molecular weight marker	GE Healthcare, Buckinghamshire, UK
Granulocyte macrophage-colony stimulating factor (GM-CSF)	R&D Systems, Abingdon, UK
GM-CSF qPCR primer	Applied Biosystems, Paisley, UK
<i>Haemophilus influenza</i> strain 1479	ATCC, Teddington, UK
HANK's balanced salt Solution	Sigma-Aldrich, Dorset, UK
HPRT1 primer (Hs02800695_m1)	Applied Biosystems, Paisley, UK
Human Active MMP9 Fluorokine E Kit	R&D Systems, Abingdon, UK
Interleukin-10 (IL-10) capture, detection, standard antibody	R&D Systems, Abingdon, UK
KLF4 qPCR primer	Applied Biosystems, Paisley, UK
L-glutamine	Sigma-Aldrich, Dorset, UK
Lipopolysaccharide (LPS)	Sigma-Aldrich, Dorset, UK
Live/dead® Fixable cell stain (aqua)	Invitrogen, Paisley, UK
Live/dead® Fixable cell stain (far red)	Invitrogen, Paisley, UK
Macrophage-colony stimulating factor (M-CSF)	R&D Systems, Abingdon, UK
M-CSF qPCR primer	Applied Biosystems, Paisley, UK
Methanol	Sigma-Aldrich, Dorset, UK
MMP9 Primer (Hs00234579_m1)	Applied Biosystems, Paisley, UK
MMP2 Primer (Hs01548727_m1)	Applied Biosystems, Paisley, UK
Novex 10% Zymogram (Gelatin) protein gels	Invitrogen, Paisley, UK

Novex Tris-Glycine Running Buffer (10X)	Invitrogen, Paisley, UK
Novex Tris-Glycine SDS sample Buffer (2X)	Invitrogen, Paisley, UK
Novex Zymogram Developing Buffer (10X)	Invitrogen, Paisley, UK
Novex Zymogram Renaturing Buffer (10X)	Invitrogen, Paisley, UK
NUNC Maxisorp 96-well plates	Sigma-Aldrich, Dorset, UK
Paraformaldehyde	Sigma-Aldrich, Dorset, UK
Penicillin	Invitrogen, Paisley, UK
Percoll	GE Healthcare, Buckinghamshire, UK
pHrodo dye NHS ester	Invitrogen, Paisley, UK
Phosphate buffered saline tablets	Sigma-Aldrich, Dorset, UK
Poly-L-lysine	Sigma-Aldrich, Dorset, UK
qPCR Reference cDNA	Takara Bio Europe, France
Recombinant human IL-4	R&D Systems, Abingdon, UK
Reverse Transcription kit	Fisher, Loughborough, UK
RNeasy mini Kit	QIAGEN, Manchester, UK
Roswell Park Memorial Institute Media (RPMI) – 1640	Sigma-Aldrich, Dorset, UK
Saline (0.9% w/v)	Baxter Healthcare Ltd, Norfolk, UK
Sodium azide	Sigma-Aldrich, Dorset, UK
Sodium Chloride 0.9% (w/v)	Baxter, Norfolk, UK
Sodium Chloride 9% (w/v)	Sigma-Aldrich, Dorset, UK
<i>Streptococcus pneumoniae</i> strain 9V, strain 10692	Gift from Professor David Dockrell
Streptavidin-Cy3 HRP Conjugated Antibody	Invitrogen, Paisley, UK
Streptomycin	Invitrogen, Paisley, UK
Sucrose	Sigma-Aldrich, Dorset, UK
Sulphuric Acid (H ₂ SO ₄)	Sigma-Aldrich, Dorset, UK

Superscript III	Invitrogen, Paisley, UK
Streptomycin	Invitrogen, Paisley, UK
Syringes	3S Healthcare, London, UK
TaqMan Universal PCR Mix	Roche, Indianapolis, USA
TIMP1 primer (Hs00171558_m1)	Applied Biosystems, Paisley, UK
TIMP2 primer (Hs00234278)	Applied Biosystems, Paisley, UK
TIMP3 primer (Hs00165949)	Applied Biosystems, Paisley, UK
TIMP4 primer (Hs00162784)	Applied Biosystems, Paisley, UK
Tissue necrosis factor (TNF) α capture antibody	R&D Systems, Abingdon, UK
Tissue necrosis factor (TNF) α detection antibody	R&D Systems, Abingdon, UK
TMB substrate reagent set (hydrogen peroxide and tetramethylbenzidine)	BD , Oxford, UK
Tween® 20	Sigma-Aldrich, Dorset, UK
QIAshredder	QIAGEN, Manchester, UK

2.2 Methods

2.2.1 Subject Selection for Whole Blood Samples

Non-smoking and smoking volunteers, and COPD patients were recruited by research nurses from the Royal Brompton Hospital Respiratory Biomedical Research Unit (BRU), London. All volunteers provided informed written consent in accordance with ethical approval obtained from the NHS National Research Service, London-Brent (13/LO/0354). Exclusion criteria included any active lung disease (excluding COPD), pregnancy or any positive allergy tests, or chest infection in the preceding six weeks. Smoking histories were obtained and spirometry performed to measure lung function on all volunteers. Forced expiratory volume in one second (FEV₁) measures the volume of air exhaled in the first second after maximal inhalation while forced vital capacity (FVC) is a measure of total volume

of air exhaled forcibly after maximal inhalation. Lung function was measured before and after treatment with a bronchodilator (2.5mg Salbutamol) to test airway reversibility. Values are shown as absolute or percentage predicted for age, sex and height, and were used to categorise subjects.

2.2.1.1 Non-Smokers

Non-smokers were between the ages of 47 and 76 with normal baseline lung function results predicted for age, sex and height. No current or past medical history of respiratory conditions recorded and negligible (<3 years) smoking history.

2.2.1.2 Smokers

Smokers were between the ages of 51 and 70 with normal baseline lung function results predicted for age, sex and height. No current or past medical history of respiratory conditions recorded. Subjects had a minimum smoking history of at least 10 pack years with one pack year.

2.2.1.3 COPD

COPD subjects were between the age of 56 and 81 and had been diagnosed with COPD in accordance with definitions described by the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD)(GOLD, 2016). Subjects presented with an FEV₁:FVC ratio of <0.7, an FEV₁% predicted of <80% and a smoking history of at least 10 pack years. In addition, subjects did not present with any ongoing respiratory infections or suffered any infection six weeks prior.

2.2.2 Subject Selection for Lung Tissue Samples

Human lung tissue was collected from patients undergoing pulmonary resections by the Respiratory Biomedical Research Unit (BRU) at the Royal Brompton NHS Trust under approved ethics and patient consent. Subjects were categorised using the same criteria as in 2.2.1.

2.2.3 Cell isolation

2.2.1.4 Monocyte Derived Macrophages (MDM)

Fresh whole blood was collected by venepuncture from subjects into three 20ml syringes containing 2% (^w/_v) EDTA. Blood was added to three 50ml Falcon tubes containing 10ml of 6% (^w/_v) dextran and

20ml of Hank's Buffered Salt Solution (HBSS) and sedimented for 20 min. The top layer of leukocyte rich plasma was transferred into new Falcon tubes and centrifuged at 300xg, for 5min, at room temperature and supernatant discarded. Percoll gradients of 81% (v/v), 68% (v/v) and 55% (v/v) were prepared using 100% (v/v) Percoll (21.6ml Percoll, 2.4ml of 9% (w/v) saline) diluted in 0.9% (w/v) saline. The gradients were prepared in two 15ml Falcon tubes by layering 4ml of 68% (v/v) Percoll on top of 4ml of 81% (v/v) Percoll. Cell pellets were resuspended in 8ml of 55% (v/v) Percoll and 4ml added to each gradient prior to centrifuging at 1000xg for 30min. The peripheral blood mononuclear cell (PBMC) fraction was harvested from the 55%/68% (v/v) Percoll interface (Figure 2.1), and was transferred into a new 50ml Falcon tube, washed with Dulbecco's phosphate buffered saline solution (DPBS) and centrifuged at 300xg for 5min. Cell pellets were resuspended in 1ml MDM complete media (RPMI 1640, 10% (v/v) foetal calf serum, 10mg/ml (w/v) penicillin/streptomycin, 2mM (v/v) L-glutamine) and cells counted using a haemocytometer. Cells were diluted to 1×10^6 cells/ml and seeded in, either 96-well plates at a density of 1×10^5 monocytes/well, or in 24-well plates at a density of 5×10^5 monocytes/well. Monocytes adhered to the plate for 2h, incubated at 37°C, 5% (v/v) CO₂, prior to replenishing the cell media with the addition of either granulocyte macrophage-colony stimulating factor (GM-CSF) (2ng/ml) or macrophage-colony stimulating factor (M-CSF) (100ng/ml). Monocytes were differentiated over 12-18 days with media replenished every 4 days. At day 12, media was switched from GM-CSF containing media to M-CSF and M-CSF to GM-CSF for a further 6 days. Control cells were grown in the same media for 18 days. The time-point of 6 day switching of media to observe cell plasticity was based on previous work from the Donnelly laboratory, demonstrating that 6 days were required to observe changes in phenotype and function (Day, 2017; pending publication).

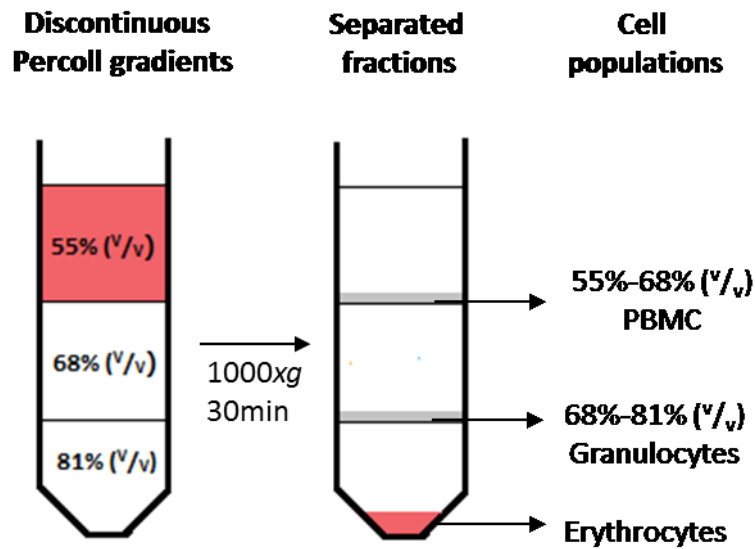


Figure 2.1 Diagram of discontinuous Percoll gradient used to separate PBMC from human peripheral blood samples. Percoll gradients were prepared at 81%, 68% and 55% (v/v) and layered in a 15ml Falcon tube. Leukocyte rich cell pellet was resuspended in 55% (v/v) Percoll before being layered at the top. Cells were centrifuged at 1000xg for 30min and the PBMC layer formed in the 55%-68% (v/v) interface isolated into a new tube.

2.2.1.5 Lung Tissue Macrophage (Tm ϕ) Isolation

Fresh human lung tissue was isolated as described by Chana *et al* (Chana et al., 2014). Briefly, tissue was flushed with 120ml wash buffer (RPMI 1640, 1% (v/v) L-glutamine, 1% (v/v) penicillin/streptomycin, 1% (v/v) EDTA) over a cell strainer and gently massaged. All collected flow through was centrifuged at 300xg for 5min. A discontinuous gradient was prepared using 100% Percoll (25ml Percoll + 2.5ml 10X PBS) diluted with DPBS to make 10, 20, 30, 40, 50 and 60% (v/v) layers. The gradients were prepared by overlaying 2ml of 50% (v/v) Percoll onto 60% (v/v) Percoll in a 15ml Falcon tube, followed by the other layers. The cell pellet was resuspended in 10% (v/v) Percoll and layered at the top prior to centrifuging at 1000xg for 30min. Cells were collected from the 30-40%, 40-50% and 50-60% (v/v) interfaces corresponding to the densities of 1.036-1.048, 1.048-1.06 and 1.061-1.073 g/ml

respectively (Figure 2.2). Tissue macrophages (Tm ϕ) were counted using a haemocytometer, and diluted in tissue media (MDM complete, 1% (v/v) amphotericin) prior to plating at the same density as MDM. Cells adhered for 24h prior to replenishing media with either tissue media alone, or with the

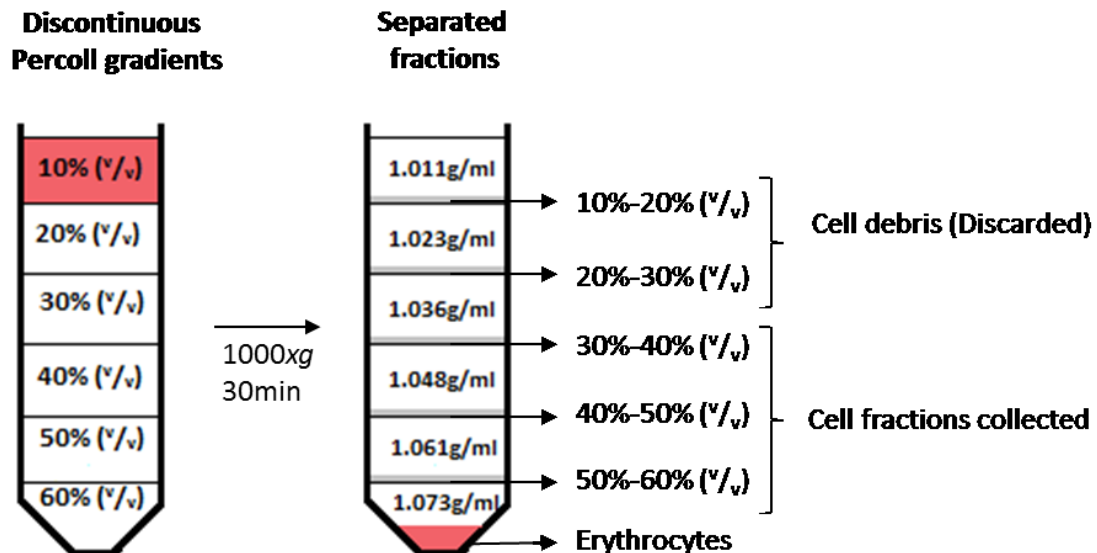


Figure 2. 2 Diagram of discontinuous Percoll gradient used to separate tissue macrophage cell fractions from lung tissue. Percoll gradients were prepared at 10%, 20%, 30%, 40%, 50% and 60% (v/v) and layered in 15ml Falcon tubes. Leukocyte rich cell pellet was resuspended in 10% (v/v) Percoll before being layered at the top. Cells were centrifuged at 1000xg for 30min and the cell fractions in the 50%-60%, 40%-50% and 30%-40% interfaces collected and counted.

addition of GM-CSF or M-CSF, and incubated for 6 days at 37°C, 5% (v/v) CO₂.

2.2.1.6 MDM Density Fraction Cell Isolation

MDM were isolated as described in section 2.2.1.4 and seeded in 12-well plates at a cell density of 1x10⁶ cells/well. Cells were differentiated over 12 days in GM-CSF. At day 12 media was aspirated and cells washed in D-PBS prior to adding 500 μ l of Accutase cell dissociation reagent per well and incubating at RT for 20min. MDM were collected in a 50ml Falcon tube by vigorous pipetting and centrifuged at 300xg for 10min. A discontinuous gradient was prepared using Percoll and layered as described in section 2.2.1.5. Cells were collected from the 20-30%, 30-40%, 40-50% and 50-60% (v/v) interfaces. MDM were counted using a haemocytometer and plated in MDM complete media for 24h

prior to processing in either a 96-well plate (100,000 cells/well) for enzyme-linked immunosorbent assay (ELISA), or 24-well plate (500,000 cells/well) for RNA extraction.

2.2.4 RNA Extraction and cDNA Synthesis

MDM and tissue macrophages (Tm ϕ) were harvested at specific time points and RNA extracted using the Qiagen RNeasy Mini kit following manufacturer's specifications. Media was removed from wells prior to adding 350 μ l of RLT lysis buffer containing 5% (v/v) 2-mercaptoethanol per well and cells were scrapped and transferred into Eppendorf tubes. Samples were stored at -80°C until RNA extraction could be performed.

Lysates were homogenised using QiaShredder spin columns and centrifuged at 800xg for 2min. Flow through was transferred into a gDNA Eliminator spin column and centrifuged at 800xg for 30s. 1 volume of 70% (w/v) ethanol was added to the flow through and mixed by pipetting prior to transferring to an RNeasy spin column and centrifuged at 800xg for 30s. Flow through was discarded and column washed by adding 700 μ l of Buffer RW1 and centrifuged at 800xg for 30s. Two further washes were performed by adding 500 μ l Buffer RPE and centrifuged at 800xg for 30s and then for 2min. Spin columns were placed in new 1.5ml collection tubes and RNA eluted by adding 30 μ l of RNase-free water and centrifuged at 800xg for 1min. Flow through was added to the column once more and centrifuged at 800xg for 1min to ensure maximum RNA yield. RNA was quantified and purity measured using NanoDrop 1000 spectrophotometer (Thermo Scientific) and 2.5-5ng/ μ l of cDNA synthesised. Briefly, RNA was diluted to the desired concentration in 10 μ l using RNase-free water and 10 μ l of mastermix (Buffer RT, dNTP, random primers, water and SuperScript II reverse transcription enzyme) was added to each sample following manufacturer's specifications. Samples were cycled at 25°C for 10 min, 37°C for 2h and 85°C for 5min in G-Storm thermocycler (Kapa Biosystems) prior to being diluted and stored at -20 °C.

2.2.5 Gene Expression Measured by Real Time qPCR

A cDNA standard of known concentration was serially diluted (0-10000 pg/μl) to establish a standard curve for each primer pair (Figure 2.3). Samples and standards were pipetted in duplicates in 96-well reaction plates, and the *HPRT1* gene used as a housekeeper. 2μl of sample or standard was added to each well with 8μl of mastermix (2.5μl RNase free water, 5μl TaqMan DNA polymerase and 0.5μl of the primer of interest). Plates were sealed, centrifuged at 400xg for 1min and RT-qPCR performed on a 7500 real time PCR system (Applied Biosystems) for 40 cycles. C_t values for each gene were normalised to the corresponding standard curve and shown as a percentage ratio of gene expression relative to *HPRT1*.

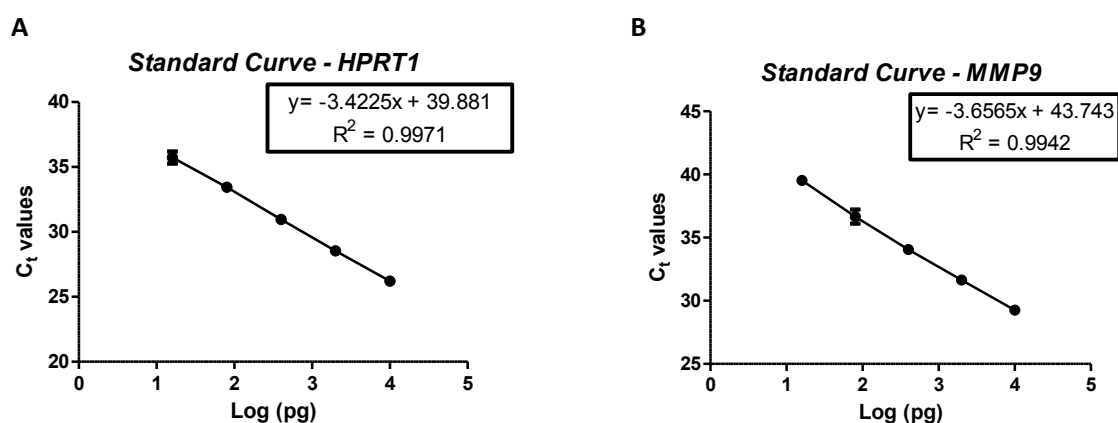


Figure 2. 3 Standard curves for HPRT1 and MMP9. Representative standard curve for (A) HPRT1 and (B) MMP9. Reference cDNA of concentration 10,000 pg/μl was serially diluted 1:5 to create standard curve for each primer used. Unknown sample values were extrapolated from these curves and shown as a percent ratio relative to HPRT1.

2.2.6 Measurement of Cytokine Output from Cell Supernatant; CXCL8, IL-10 and TNFα

ELISA antibodies for CXCL8, IL-10 and TNFα were used according to the manufacturer's specifications. Briefly, 96-well NUNC Maxisorp plates were coated with 100μl/well of anti-human CXCL8 (0.5μg/ml), IL-10 (1μg/ml) or TNFα (1.5μg/ml), diluted in sterile D-PBS, and incubated overnight at room temperature. Capture antibody was removed and plates blocked by adding 120μl/well of blocking buffer (1% (w/v) BSA, 5% (w/v) sucrose, 0.05% (w/v) sodium azide) for 2h at RT. Plates were washed three

times with wash buffer (dPBS, 0.05% (v/v) Tween 20) and excess liquid removed. Standards of recombinant human CXCL8, IL-10 and TNF α were added to the appropriate wells in duplicate. Cell supernatants were diluted 1:400 (CXCL8), 1:2 (IL-10) and 1:10 (TNF α) using sample buffer (D-PBS, 0.5% (w/v) BSA) and 100 μ l/well added to the corresponding plates and incubated for 2h at RT. The plates were washed three times in wash buffer, then 100 μ l/well of detection antibody was added to the corresponding plates at a concentration of 0.4 μ g/ml (CXCL8), 0.1 μ g/ml (IL-10) and 0.2 μ g/ml (TNF α) and incubated for 2h at RT. The plates were washed three times and 100 μ l horseradish peroxidase (HRP)-conjugated streptavidin added to each well (1 μ l/ml) and incubated for 30min at RT. The plates were washed five times and 100 μ l/well of substrate solution was added (by mixing equal volumes of Reagent A (H₂O₂) and Reagent B (Tetramethylbenzidine) directly before use). The reaction was stopped by addition of 100 μ l/well 1M sulphuric acid (H₂SO₄).

A spectrophotometer was used to determine the optical density of each well, read at λ 450nm and also at λ 590nm to account for background noise. The value at λ 590nm was subtracted from the value at λ 450nm. Standard curves were calculated for each cytokine (Figure 2.4) and unknown values derived by interpolation to determine cytokine concentrations. Lower level of detection for each cytokine assayed were calculated by taking the lowest standards and adding two standard deviations: CXCL8 (0.009ng/ml), IL-10 (0.009ng/ml) and TNF α (0.009ng/ml).

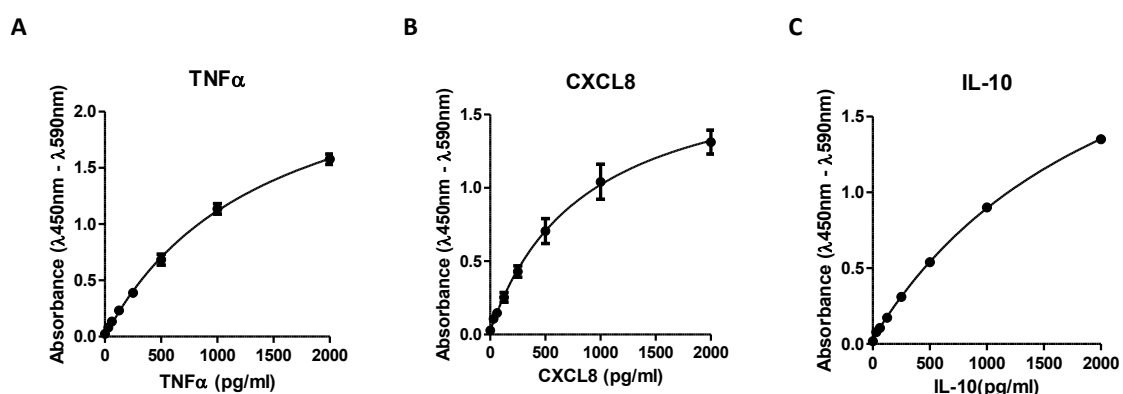


Figure 2. 4 TNF α , CXCL8 and IL-10 ELISA standard curves. A standard curve of recombinant human (A) TNF α , (B) CXCL8 and (C) IL-10. Absorbance was read at λ 450 and λ 590. Values at λ 590 represent background noise, and these values were subtracted from the values obtained at λ 450.

2.2.7 Zymography

Zymography was performed to assess level and activity of the gelatinase MMP9 by performing electrophoresis under non-reducing conditions, to maintain enzymatic activity (Toth et al., 2012). This was performed using a 10% (w/v) SDS-polyacrylamide pre-cast gel, co-polymerised with gelatin (0.1% (w/v)). Samples were prepared by adding 5µl of loading buffer to 10µl of cell supernatant or standard (0.1ng/ml) and loaded onto a gel. The MMP9 standard was used as an internal control for each gel, and all bands from samples normalised to the corresponding band of the standard. Cell supernatants from MDM were diluted 1/20 prior to running on the gel, and Tm ϕ supernatants were used neat. A negative control of RPMI alone was included in each gel. Proteins were separated by electrophoresis using 1X running buffer at 120V for 1.5h prior to incubating the gel in 1X renaturing buffer for 30min on a rocking platform at room temperature to allow for the removal of SDS from the gel. The gel was then incubated for 30min in developing buffer, before refreshing the buffer and incubating overnight at 37°C in order to cause partial renaturing of the enzyme which can then hydrolyze the gelatin found within the gel.

Staining was performed using the colloidal blue staining kit following manufacturer's specifications, by staining the gel for 3h on a rocking platform at room temperature prior to washing in distilled water for a further 3h. After staining, clear bands appeared in areas cleared of gelatin, thus indicating areas of gelatinase (MMP9) activity. Both pro- and active MMP9 were detected using this method. A faint MMP2 band also appeared in some gels, as it is also a gelatinase, but no standard was included for MMP2, therefore it was not analysed (Figure 2.5). Gels were imaged using GelDoc-IT Imaging System (UVP) and analysed to provide semi-quantitative protein analysis.

2.2.8 Cell Viability Assay

Cell viability was indirectly calculated by measuring cell metabolic activity. This was performed by removing media from cells and adding 50µL of 0.1% (w/v) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to each well. Plates were incubated at 37°C for 30-40min before removing MTT and adding 50µl of dimethyl sulfoxide (DMSO) to lyse the cells. This induced a colour

change from yellow to purple which is representative of metabolic activity. Absorbance was measured at $\lambda 570\text{nm}$ using a Spectramax photometer to quantify cell viability. Each treated well was normalised to the average of the non-treated control which was set to 100% viability.

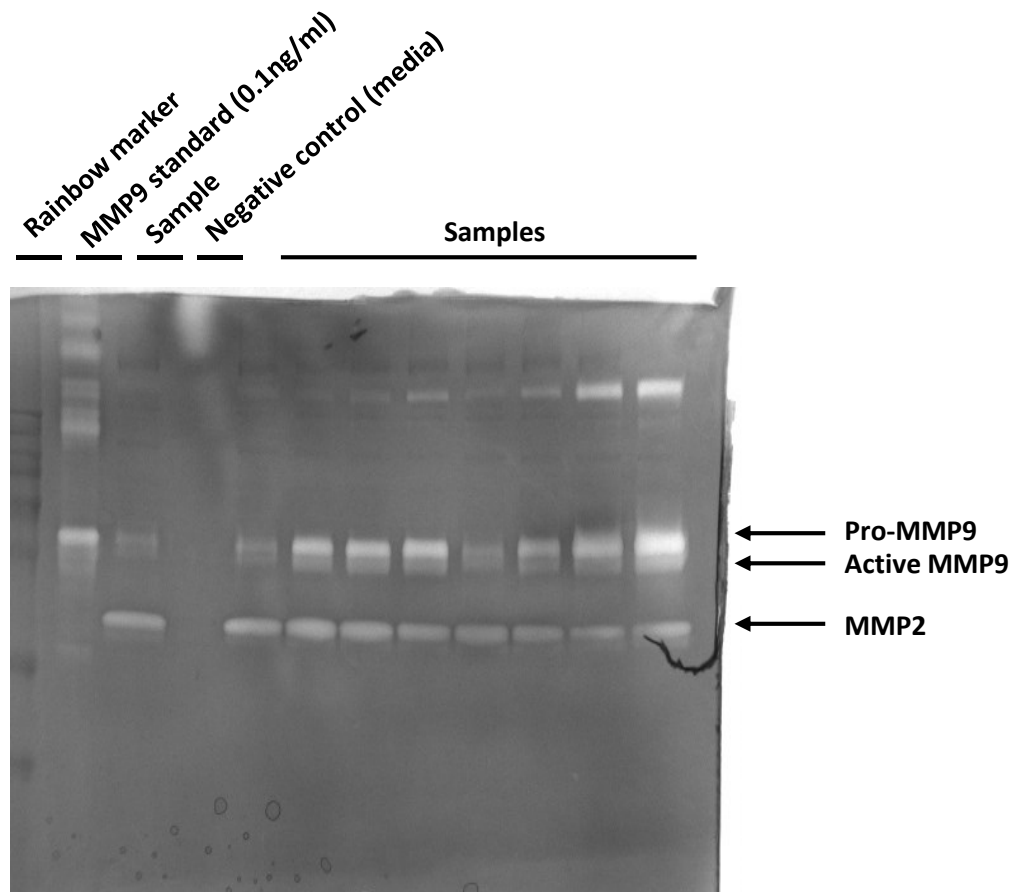


Figure 2. 5 Representative zymography gel for gelatinase activity. Cell supernatant samples were analysed by electrophoresis and standardised for each gel by using an internal control of MMP9 standard (0.1ng/ml). RPMI was used as a negative control. Bands for pro and active MMP9 and MMP2 can be observed. Only MMP9 was analysed as no MMP2 standard was used.

2.2.9 Preparation of Heat Killed Fluorescently Labelled Bacteria

2.2.1.7 Bacteria Culture and Heat Killing

Non-typeable *H. influenza*, strain 1479 and *Streptococcus pneumoniae* serotype 9V strain 10692, isolated from exacerbating COPD patients, were streaked onto agar plates and incubated at 37°C for 24h. Colonies were inoculated in 20ml of Brain Heart Infusion broth and kept overnight in a shaker incubator at 37°C. Optical densities (OD) were used to assess growth rate by measuring at $\lambda 600\text{nm}$ every hour using a spectrophotometer, and bacteria harvested when OD reached 1.2. Bacteria were cultured for 12h on agar plates and the colony forming units (CFU) counted using the following equation:

$$\text{CFU} = \text{colony number} \times \text{dilution factor} / \text{volume plated}$$

In order to heat kill bacteria, bacterial suspensions were incubated at 90°C for 2h and washed a total of four times prior to being fluorescently labelled in 1mg/ml Alexa fluor 405 NHS ester, Alexa fluor 488 NHS ester, or pHrodo red. In order to confirm the efficacy of killing, 10 μl of each bacterial suspension was taken and streaked onto LB nutrient agar plates and incubated overnight at 37°C. Absence of any colonies indicated bacteria were heat killed.

2.2.1.8 Phagocytosis Analysis Using Flow Cytometry

Fluorescently labelled bacteria were added to cells (50×10^7 CFU/well) in RPMI and incubated for 4h at 37°C. After 4h, the media was removed and 300 μl of Accutase was added per well and left for 20min at RT. Cells were dissociated from the plate by vigorous pipetting prior to centrifuging at 300 $\times g$ for 5min in BD polystyrene tubes. Live/dead stain was diluted 1/500 in PBS and 500 μl added to each tube and incubated at RT for 30min. An unstained control was resuspended in PBS alone. Tubes were centrifuged at 300 $\times g$ for 5min, supernatant discarded and cells resuspended in 200 μl of 4% (v/v) PFA for a minimum of 10min to fix the cells prior to analysing samples on a BD FACS Canto II using DIVA software. A minimum of 5000 events were collected per condition and data were analysed using FlowJo software (version 10).

2.2.1.9 Staining of Cells for Confocal Microscopy

MDM were isolated as described in 2.2.3.1 and plated in μ -Slide 8 well glass bottom plates at a cell density of 2×10^5 monocytes per well. Plates were prepared by coating in Poly-L-lysine (100 μ l/well) for 5 min prior to washing twice in D-PBS and plating cells. MDM were differentiated and on day 12 were incubated with fluorescently labelled heat killed bacteria (5×10^7 CFU/well) for a range of time points up to 4h in serum free RPMI. At each time point, cells were fixed by adding 200 μ l/well of 4% (v/v) paraformaldehyde (PFA) and incubating for 10min at RT. PFA was removed and cells permeabilised by adding 100% ice cold methanol for 5min prior to washing twice in D-PBS. Celltracker dye (either celltracker red CMTPX dye or celltracker green CMFDA dye) was added to the cells at 10 μ g/ml in PBS and incubated at 37°C for 30min to allow staining of the cytoplasm. Wells were washed and 300nM DAPI added for 5min at 37°C for nuclear staining prior to washing and leaving in D-PBS. Images were acquired using Zeiss LSM-510 inverted confocal microscope and analysed using ImageJ software.

2.2.10 Statistical Analysis

Data were analysed to determine whether there were statistically significant differences for each experiment. Comparison between MDM subject groups at multiple time points during differentiation or between tissue macrophage subject groups cultured under multiple conditions were performed using a Two-Way ANOVA with Bonferroni post-test. When comparing culture conditions for MDM, comparisons between MDM subject groups at one time-point or between tissue macrophage cell fractions, Kruskal-Wallis followed by a Dunn's post-test were performed. Comparisons on day 18 between MDM where media was changed at day 12 vs. those that remained in the same media was performed using a Wilcoxon matched-pairs signed ranks test. All data were expressed as mean $\pm$ standard error of the mean (SEM) and analysed using GraphPad Prism software (version 5.03). Statistical significance was assigned for p values of <0.05 .

CHAPTER 3: INVESTIGATING THE ROLE OF MACROPHAGE PHENOTYPE ON LPS-STIMULATED CYTOKINE RELEASE

3.1 Introduction

Macrophages play an important role in maintenance of lung homeostasis as well as coordinating an inflammatory response upon activation. These roles are carefully controlled and rely on macrophages adapting to changes in their microenvironment, and as such, this plasticity allows cells to drive pro-inflammatory responses when required, followed by a return to steady-state (Akagawa et al., 2006, Chana et al., 2014). Release of cytokines, reactive oxygen and nitrogen species by macrophages are a key mechanism by which cells initiate a pro-inflammatory response upon activation by a foreign body (Bazzan et al., 2017). In COPD, chronic inflammation is thought to be primarily due to macrophages and neutrophils which are present in higher numbers in COPD lungs compared to healthy individuals (Pesci et al., 1998). Pro-inflammatory signals from macrophages occur via the release of cytokines such as TNF α which further amplifies inflammatory signal by inducing release of CXCL8, a potent neutrophil chemoattractant (Keatings et al., 1996, Chung, 2001). Both these cytokines have been shown to be elevated in BAL and sputum samples from COPD patients (Keatings et al., 1996, Culpitt et al., 2003a). Conversely, IL-10 is important for the resolution phase of inflammation and has been found at lower levels in the sputum and serum of COPD patients compared to non-smokers (Takanashi et al., 1999, Zhang et al., 2013).

The mechanism by which macrophages become altered in COPD has been a topic of interest for many years. However, whether this is due to changes in the balance of macrophage phenotype, or a loss of plasticity remains unclear. The nature of macrophage heterogeneity and presence of multiple subpopulations within the lung further complicates studies in this field. Many studies have used the monocyte-derived macrophage (MDM) model due to the relative ease of obtaining blood samples

from patients and healthy volunteers. Furthermore, the MDM model provides a method to investigate monocyte differentiation and allows for manipulation of resulting macrophage phenotypes (Stout and Suttles, 2004, Lacey et al., 2012). The addition of GM-CSF and M-CSF *in vitro* are commonly used to drive differentiation into a macrophage lineage, either towards a pro-inflammatory or resolving phenotypes respectively (Lacey et al., 2012, Mosser and Edwards, 2008, Porcheray et al., 2005). These phenotypic differences can be seen at the transcriptomic level as well as in functional aspects (Martinez et al., 2006). While this model is essential for examining plasticity of macrophages, the use of human lung tissue macrophages provides a more representative model of how macrophage function is altered within the COPD lung. Studies of this nature have been limited due to the difficulty in obtaining human lung tissue and thus characterisation of these cells has not been widely investigated. Investigating cytokine output and whether cell phenotype can be manipulated by changes in microenvironment could be beneficial in developing new therapeutic strategies for COPD.

A comparison of both MDM and tissue macrophage models is essential in order to determine whether alterations observed in COPD MDM can be observed in tissue cells. Due to the role of GM-CSF and M-CSF in driving monocyte differentiation towards distinct macrophage phenotypes (Gordon, 2003a), it is possible that these growth factors could play a role in altering mature macrophage phenotype and drive plasticity in the lung. The role of macrophage phenotype and plasticity was therefore assessed using both models to establish the role of GM-CSF and M-CSF on cell function and phenotype, and to determine whether cells can be manipulated from a pro-inflammatory COPD phenotype towards a resolving cell type. The use of these growth factors to manipulate MDM and tissue macrophages has not been shown in the literature and may provide new therapeutic options to consider in COPD.

While the MDM model has been extensively used, there is limited data comparing this model to tissue macrophages in COPD. Assessing similarities and differences between the two models is therefore essential. Isolation of tissue macrophages by Percoll gradient separation results in three distinct cell fractions, isolated based on density. These populations of cells are thought to represent macrophages

at distinct levels of maturity, with the more dense cells, 50-60%, believed to represent monocyte-like immature cells, while the less dense population, 30-40%, represent larger mature tissue resident macrophages (Chana et al., 2014). Whether these fractions can be replicated using the MDM model has never been examined, and was investigated to determine how similar MDM differentiation is *in vitro* compared to in the lung. In addition, examining whether there are differences in cytokine output in cell fractions from both tissue macrophages and MDM could aid in characterising pulmonary macrophage populations and whether all cells behave in a similar manner in COPD, or specific subpopulations function differently.

3.1.1 Hypothesis

COPD MDM and tissue macrophages release increased levels of pro-inflammatory cytokines compared to macrophages from non-smokers and healthy smokers, and this will be reversed by culture with M-CSF.

3.1.2 Aims

- Characterise cytokine output from MDM and tissue macrophages from non-smokers, smokers and COPD patients.
- Assess the role of GM-CSF and M-CSF culture on resulting cell phenotypes in MDM and tissue macrophages in terms of cytokine output.
- Examine how MDM density fractions relate to tissue macrophage fractions in cytokine output.

3.2 Methods

3.2.1 LPS-stimulated Cytokine Output

3.2.1.1 Tissue Macrophages

Tissue macrophage fractions isolated as in section 2.2.1.5 were plated in 96-well plates and cultured for 6 days in media alone, GM-CSF or M-CSF. On day 0 and day 6 cells were treated with either control media or LPS (10ng/ml) for 24h at 37°C in 5% (v/v) CO₂ in triplicate. Cytokine output was measured by ELISA as in section 2.2.6. Cell supernatants were collected and diluted in ELISA buffer (D-PBS + 0.5%

(w/v) BSA) for cytokine analysis (TNF α 1:10, CXCL8 1:300 and IL-10 1:2). Standards of known concentration were analysed alongside cell supernatants. Absorbance was read using a spectrophotometer at 450nm and 590nm wavelengths. The standard curve was used to interpolate unknown samples using GraphPad Prism software.

3.2.1.2 Monocyte-derived Macrophages

Monocytes were isolated from whole blood as in section 2.2.3.1 and cultured for 12 days in either GM-CSF or M-CSF. On day 12, cells either remained in the same culture media for a further 6 days (controls) or media was switched from GM-CSF to M-CSF and from M-CSF to GM-CSF for 6 days. Cells were incubated with LPS (10ng/ml) or control medium on day 0, 12 and 18 and ELISA performed to measure cytokine output as described in section 2.2.6.

3.2.1.3 MDM Density Fractions

Monocytes were cultured for 12 days in GM-CSF (2ng/ml) prior to dissociating and processing to isolate cell fractions as in section 2.2.3.3. Cell fractions were then plated and allowed to adhere for 24h prior to stimulating and analysing cytokine output as in section 2.2.6.

3.2.2 Statistical Analysis

All data were expressed as mean $\pm$ SEM, and analyses performed using GraphPad Prism software. Data were non-parametric, and comparisons between subject groups was performed using a Two-Way Anova with Bonferroni corrections. For tissue macrophages a Kruskal-Wallis with Dunn's post-test was used to examine differences between culture conditions, and between cell fractions. In MDM, this test was used to examine differences between GM-CSF and M-CSF culture throughout differentiation as well as to compare differences between days 12 and 18 to day 0. A Wilcoxon matched-pairs signed ranks test was used to test significance between day 18 MDM that had media switched vs. unswitched/control media. Comparisons between MDM density cell fractions were performed using a Two-Way Anova with Bonferroni corrections.

3.3 Results

3.3.1 Subject Demographics

3.3.1.1 Tissue Macrophages

Subject demographics and lung function data for lung tissue samples are shown in Table 3.1. Subjects across all groups were age matched and smokers and COPD patients had significantly higher smoking history compared to non-smokers. COPD patients had significantly lower FEV₁ and FEV₁:FVC compared to non-smokers and smokers, and lower FEV₁% predicted compared to non-smokers.

	Non-smoker	Smoker	COPD
Sex (F:M)	4:1	4:2	5:4
Age	72.4±6.3	67.3±6.7	68.5±2.8
Smoking history (Pack years)	1.0±1.0	37.8±7.6*	38.6±9.8*
FEV ₁ (L)	2.4±0.2	2.2±0.2	1.4±0.2** #
FEV ₁ % predicted	99.4±2.3	98.8±12.7	66.0±7.5*
FVC (L)	3.0±0.1	2.8±0.3	2.8±0.3
FEV ₁ :FVC	0.8±0.0	0.8±0.0	0.5±0.1* ##

Table 3. 1 Subject demographics for tissue macrophages from non-smokers, smokers and COPD patients. All subjects underwent lung function tests. FEV₁: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes smoked per day for one year. Data shown are mean ±SEM. Kruskal-Wallis test with Dunns post-test was used to test significance between non-smokers and smokers or COPD patients *p<0.05, **p<0.01 and between smokers and COPD patients #p<0.05, ##p<0.01.

3.3.1.2 Monocyte-Derived Macrophages

Subject demographics and lung function data for those who donated blood are shown in Table 3.2. Smoking history was significantly higher for smokers and COPD patients compared to non-smokers. In addition, COPD patients had significantly lower FEV₁% predicted and FEV₁:FVC compared to smokers and non-smokers.

	Non-smoker	Smoker	COPD
Sex (F:M)	2:4	3:4	1:4
Age	57.3±2.7	61.0±3.9	68.6±3.6
Smoking history (Pack years)	0.0±0.0	43.0±9.3*	41.5±11.1*
FEV ₁ (L)	3.1±0.3	2.7±0.4	2.0±0.3
FEV ₁ % predicted	100.4±4.7	89.7±7.3	65.2±6.4*
FVC (L)	4.1±0.5	3.7±0.7	3.7±0.5
FEV ₁ :FVC	0.8±0.0	0.7±0.0	0.5±0.0*

Table 3.2 Subject demographics for MDM from non-smokers, smokers and COPD patients. All subjects underwent lung function tests. FEV₁: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes smoked per day for one year. Data shown are mean ±SEM. Kruskal-Wallis test with Dunns post-test was used to test significance between non-smokers and smokers or COPD patients *p<0.05.

3.3.2 Effect of Culture Conditions on Cytokine Release from Tissue Macrophage Cell Fractions

3.3.2.1 Baseline Cytokine Production by Tissue Macrophages

Firstly, experiments were established to examine release of cytokines, both at baseline and following LPS stimulation, from tissue macrophages isolated from COPD patients, smokers and non-smokers. In addition, cell fractions were plated in media alone, GM-CSF or M-CSF and cultured for 6 days to determine whether the response of these mature cells could be altered by their microenvironment to

change phenotype with respect to cytokine release. There were no significant differences in baseline CXCL8, TNF α or IL-10 between the subject groups or between cell fractions. In addition, baseline cytokine production at day 0 was not different from cells cultured in media, GM-CSF or M-CSF for 6 days (Table 3.3).

CXCL8 (ng/ml)		Day 0	Media	GM-CSF	M-CSF
30-40%	Non-smokers	32.4 $\pm$ 18.0	9.7 $\pm$ 4.3	12.8 $\pm$ 3.0	15.1 $\pm$ 4.9
	Smokers	26.3 $\pm$ 15.1	13.2 $\pm$ 8.7	6.6 $\pm$ 1.0	11.3 $\pm$ 6.1
	COPD	36.4 $\pm$ 10.7	9.7 $\pm$ 3.1	19.6 $\pm$ 6.5	13.1 $\pm$ 3.5
40-50%	Non-smokers	43.5 $\pm$ 30.7	11.9 $\pm$ 5.0	14.9 $\pm$ 3.4	22.7 $\pm$ 6.5
	Smokers	21.0 $\pm$ 3.8	12.1 $\pm$ 4.2	11.5 $\pm$ 3.6	9.9 $\pm$ 2.6
	COPD	29.1 $\pm$ 5.6	16.3 $\pm$ 6.4	28.0 $\pm$ 11.9	19.5 $\pm$ 5.6
50-60%	Non-smokers	12.6 $\pm$ 2.8	6.2 $\pm$ 2.4	7.9 $\pm$ 2.7	9.3 $\pm$ 2.3
	Smokers	3.2 $\pm$ 0.5	5.1 $\pm$ 1.5	5.7 $\pm$ 1.0	5.6 $\pm$ 1.1
	COPD	11.7 $\pm$ 4.5	9.5 $\pm$ 3.4	1.4 $\pm$ 2.8	9.7 $\pm$ 2.5
TNFα (ng/ml)		Day 0	Media	GM-CSF	M-CSF
30-40%	Non-smokers	0.4 $\pm$ 0.4	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
	Smokers	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
	COPD	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
40-50%	Non-smokers	0.3 $\pm$ 0.2	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
	Smokers	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
	COPD	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.1 $\pm$ 0.0	0.2 $\pm$ 0.1
50-60%	Non-smokers	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.3 $\pm$ 0.2	0.1 $\pm$ 0.0
	Smokers	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0

	COPD	0.1±0.0	0.1±0.1	0.1±0.0	0.2±0.5
IL-10 (pg/ml)		Day 0	Media	GM-CSF	M-CSF
30-40%	Non-smokers	28.5±11.5	23.3±6.3	17.5±3.8	21.8±4.7
	Smokers	14.3±2.1	20.8±7.1	9.6±4.1	43.7±17.5
	COPD	25.8±7.8	35.0±10.8	17.4±6.9	25.5±9.1
40-50%	Non-smokers	25.7±5.5	24.6±4.8	14.7±3.6	24.3±5.3
	Smokers	20.2±3.7	18.2±6.0	8.7±3.7	14.0±6.1
	COPD	29.5±6.9	40.2±12.1	19.0±8.8	40.8±2.8
50-60%	Non-smokers	24.0±6.4	23.5±6.5	18.9±4.4	26.9±5.4
	Smokers	26.5±7.7	20.1±7.5	8.2±3.0	14.2±5.8
	COPD	37.4±15.3	39.5±1.8	18.8±8.1	45.6±19.5

Table 3.3 Cytokine output at baseline for each tissue macrophage density fraction from non-smokers, smokers and COPD patients. Cytokine production from tissue macrophages isolated from **non-smokers (n=4)**, **smokers (n=6)** and **COPD patients (n=9)** was measured at day 0 and day 6 (media, GM-CSF and M-CSF) for all cell fractions (shown on the left hand column; 30-40%, 40-50% and 50-60%). CXCL8, TNF α and IL-10 were measured using ELISA. Data shown are mean $\pm$ SEM.

3.3.2.2 LPS-Stimulated TNF α Release

LPS was used to stimulate cytokine production over 24h by eliciting a pro-inflammatory response, and led to a significant increase in TNF α release compared to baseline (Table 3.3, Figure 3.1). When comparing subject groups, there were no significant differences in LPS-stimulated TNF α release between the subject groups at day 0, or following 6 days in culture. In addition, there were no differences in TNF α production between the cell fractions for any of the subject groups or culture conditions examined, although denser cells isolated from the 50-60% Percoll interface showed a trend towards reduced TNF α production in all subject groups compared to the other fractions (Figure 3.1).

COPD cells that were cultured in GM-CSF produced significantly more TNF α compared to cells cultured in media alone or in M-CSF. This was observed in the 30-40% and 40-50% cell fractions (Figure 3.1 A,B), while 50-60% tissue macrophages showed a trend towards increased TNF α release when cultured in GM-CSF, but did not reach significance compared to the other culture conditions (Figure 3.1C). Tissue macrophages from smokers cultured in GM-CSF also released significantly higher levels of TNF α in the 40-50% fraction compared to media alone or M-CSF cultured cells. The different culture conditions had no significant effect on LPS-stimulated TNF α release in tissue macrophages from non-smokers for any of the cell fractions (Figure 3.1).

3.3.2.3 LPS-Stimulated CXCL8 Release

Having shown that GM-CSF culture resulted in increased LPS-stimulated TNF α production compared to other culture conditions, it was important to determine whether similar effects occurred on other pro-inflammatory cytokines. Therefore, similar experiments were performed to measure LPS-stimulated CXCL8 release from tissue macrophages. There was a significant increase in CXCL8 release from all cells following LPS stimulation compared to baseline (Table 3.3, Figure 3.2). However, there were no differences in production of CXCL8 between the subject groups or between any of the culture conditions examined. Furthermore, there was a trend of lower CXCL8 release from the 50-60% cell fraction compared to 30-40% and 40-50% for all subject groups but this was only significant for COPD tissue macrophages cultured in GM-CSF (Figure 3.2).

3.3.2.4 LPS-Stimulated IL-10 Release

Tissue macrophages showed cytokine specific changes in production in response to culture conditions. Thus, investigating what effect GM-CSF had on a cytokine associated with the resolving response of macrophages was next performed by investigating production of IL-10. LPS-stimulated production of IL-10 was not different between the cell fractions or culture conditions, although GM-CSF culture appeared to result in lower levels of IL-10 in all subject groups compared to cells cultured in media alone or M-CSF (Figure 3.3). COPD tissue macrophages showed a trend of increased IL-10 production

compared to non-smokers and smokers, and this was significantly higher in the 30-40% fraction for COPD cells cultured in media and M-CSF compared to non-smoker and smoker cells respectively (Figure 3.3).

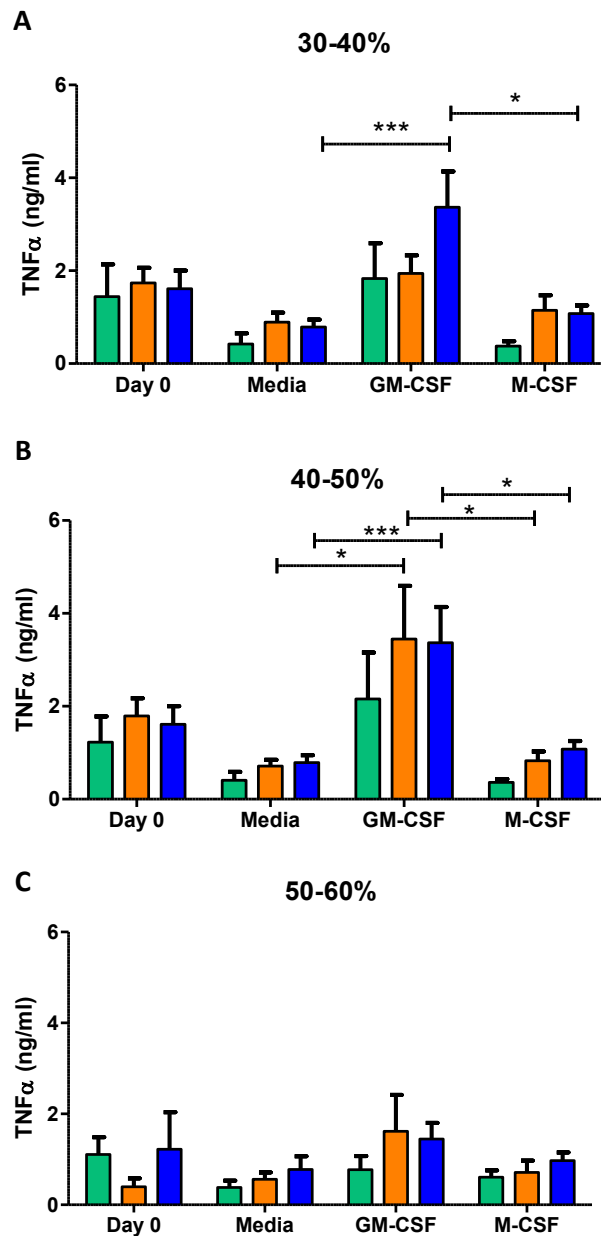


Figure 3.1 TNF α output following LPS-stimulation from tissue macrophages. Tissue macrophages were isolated from resected lung tissue based on cell density fractions **(A)** 30-40%, **(B)** 40-50% and **(C)** 50-60% from **non-smokers (n=4)**, **smokers (n=6)** and **COPD patients (n=9)** and cultured for 6 days in GM-CSF, M-CSF or media alone. Cells were stimulated with LPS (10ng/ml) for 24h and supernatant collected on day 0 and day 6. TNF α was measured using ELISA. Data are expressed as mean $\pm$ SEM. A Kruskal-Wallis with Dunn's post-test was used to test significance between culture conditions * $p < 0.05$, *** $p < 0.001$.

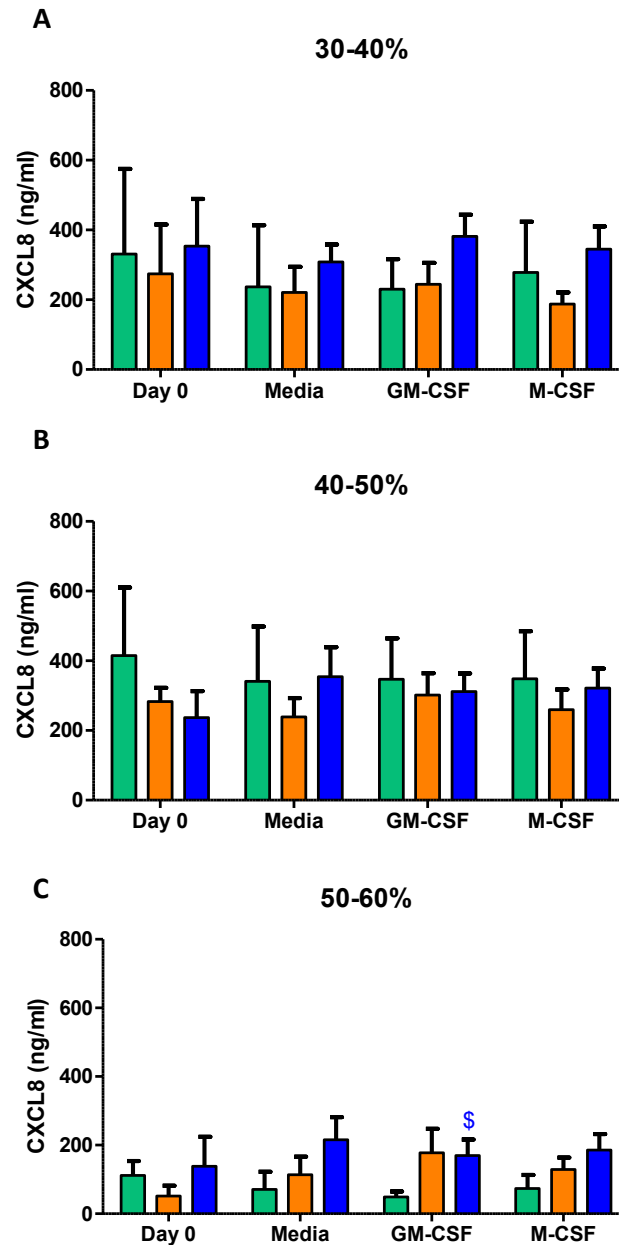


Figure 3.2 CXCL8 output following LPS-stimulation from tissue macrophages. Tissue macrophages were isolated from resected lung tissue based on cell density fractions **(A)** 30-40%, **(B)** 40-50% and **(C)** 50-60% from **non-smokers (n=4)**, **smokers (n=6)** and **COPD patients (n=9)** and cultured for 6 days in GM-CSF, M-CSF or media alone. Cells were stimulated with LPS (10ng/ml) for 24h and supernatant collected on day 0 and day 6. CXCL8 was measured using ELISA. Data are expressed as mean $\pm$ SEM. Kruskal-Wallis test with Dunns post-test was used to test significance between cell fractions, ^{\$}p<0.05 30-40% vs 50-60%.

3.3.3 Cell Viability of Tissue Macrophages

An MTT assay was performed on all cells to assess viability by measuring cell metabolic activity as an indirect measure of cell survival. This was performed to test for differences in cell viability between cell fractions, culture conditions and to compare baseline vs. LPS-stimulation.

Tissue macrophages from non-smokers had a similar viability between cell fractions and for all culture conditions tested (Figure 3.4A). Smoker and COPD cells cultured in GM-CSF had significantly higher viability compared to media or media and M-CSF respectively (Figure 3.4B-C). Furthermore, the 40-50% cell fraction had significantly higher viability in smoker cells compared to the 50-60% fraction when cultured in media and M-CSF. COPD cells also showed a similar trend with significantly higher viability in the 40-50% fraction compared to 50-60% cell fraction when cultured in GM-CSF (Figure 3.4B-C).

The effect of LPS stimulation on cell viability was also examined to account for any effect of stimulation on cell survival. Cell viability remained the same following 24h stimulation with LPS when compared to baseline, which was set to 100% cell viability. This was observed for all subject groups, all culture conditions and all cell fractions (Figure 3.5). Thus, the differences in cytokine output observed could not be attributed to changes in cell viability

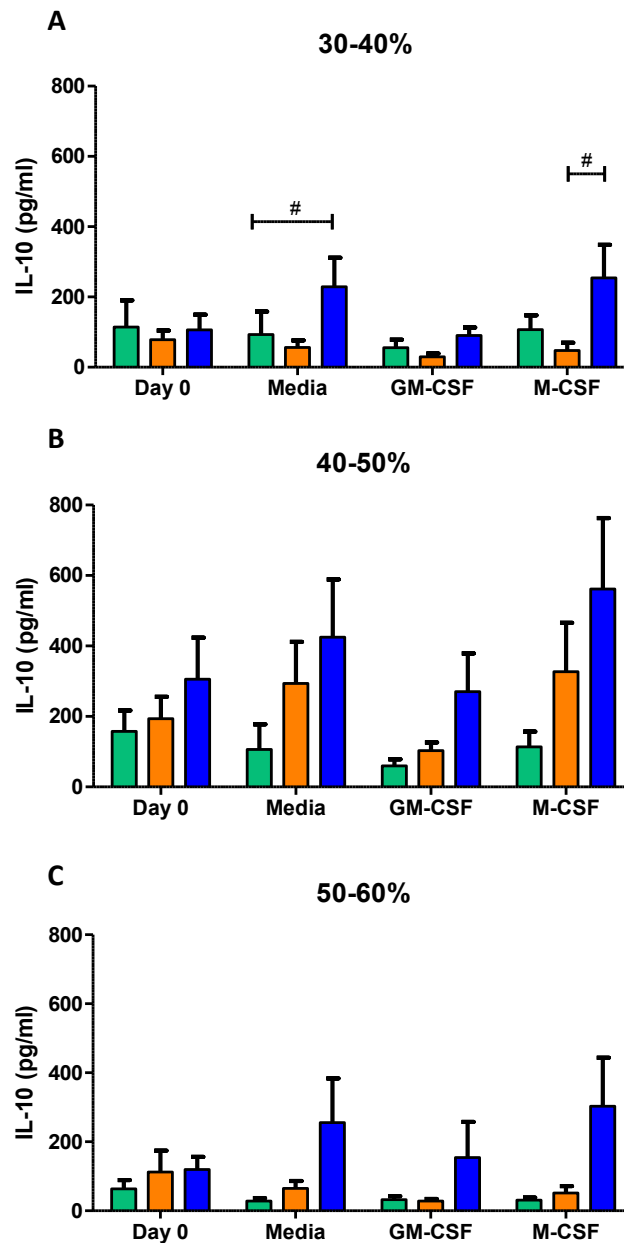


Figure 3.3 IL-10 output following LPS-stimulation from tissue macrophages. Tissue macrophages were isolated from resected lung tissue based on cell density fractions **(A)** 30-40%, **(B)** 40-50% and **(C)** 50-60% from **non-smokers (n=4)**, **smokers (n=6)** and **COPD patients (n=9)** and cultured for 6 days in GM-CSF, M-CSF or media alone. Cells were stimulated with LPS (10ng/ml) for 24h and supernatant collected on day 0 and day 6. IL-10 was measured using ELISA. Data are expressed as mean $\pm$ SEM. A Two-Way Anova with Bonferroni corrections was used to test significance between subject groups [#] $p < 0.05$.

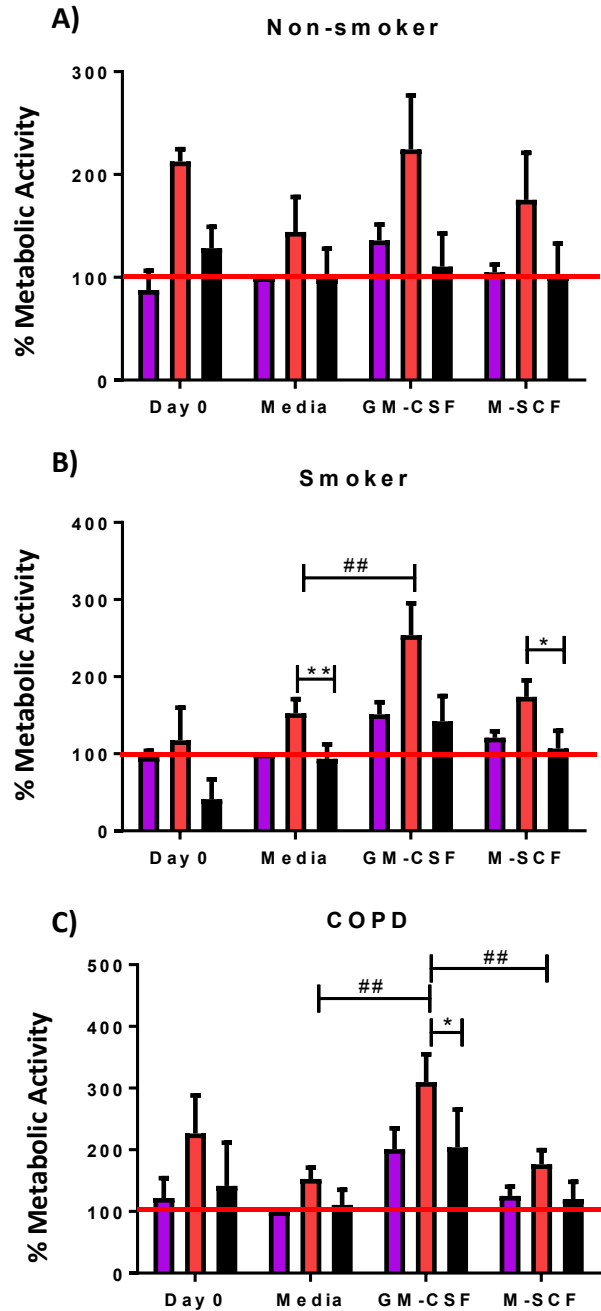


Figure 3.4 Cell viability of tissue macrophage cell fractions. Tissue macrophages from **(A) non-smokers (n=4)**, **(B) smokers (n=6)** and **(C) COPD patients (n=9)** were isolated from resected lung tissue based on cell density fractions and cultured for 6 days in GM-CSF, M-CSF or media alone. Cell metabolic activity was measured using an MTT assay as measure of cell viability for each cell fraction; **30-40%**, **40-50%** and **50-60%**. All values were normalised to 30-40% cell fraction cultured in media for the corresponding days, which was set to 100% cell viability (shown as a red line). Data are expressed as mean $\pm$ SEM. A Two-Way Anova with Boneferroni corrections was used to test differences between cell fractions, * $p < 0.05$, ** $p < 0.01$ and a Kruskal-Wallis with Dunn's post-test to test differences between culture conditions ## $p < 0.01$.

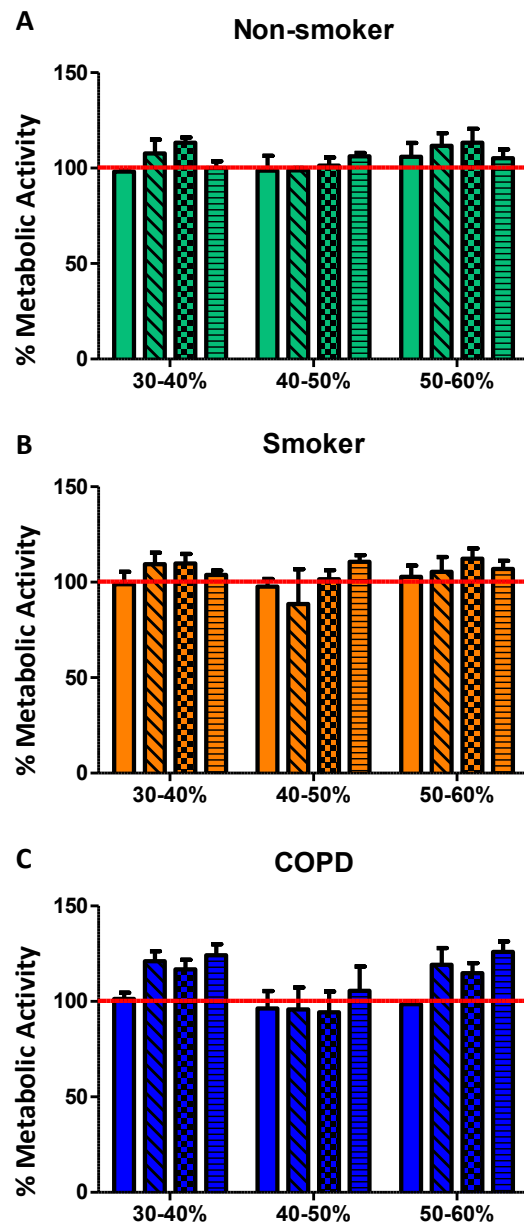


Figure 3.5 Cell viability of tissue macrophage stimulated with LPS vs. baseline. Tissue macrophages from (A) non-smokers (n=4), (B) smokers (n=6) and (C) COPD patients (n=9) were isolated from resected lung tissue based on cell density fractions and examined at day 0 (□) or cultured for 6 days in media (▨), GM-CSF (▩) or M-CSF (■). Cell metabolic activity was measured following 24h LPS stimulation (10ng/ml) using an MTT assay as a measure of cell viability. Each fraction was normalised to the corresponding non-stimulated control, which was set to 100% (shown as a red line). Data are expressed as mean $\pm$ SEM.

3.3.4 Effect of Macrophage Phenotype on Cytokine Output from MDM

As MDM are often used as a surrogate model system for lung macrophages, it was important to determine how relevant this model is with respect to cytokine release. In order to compare the results obtained using tissue macrophages to the MDM model, similar experiments were performed by differentiating MDM in GM-CSF or M-CSF over 18 days towards pro-inflammatory G-MDM or resolving M-MDM phenotypes respectively. Cytokine output was compared between subject groups, culture condition and comparing differentiated MDM (day 12 and 18) to day 0 monocytes. There were no differences in baseline cytokine release between subject groups or between G-MDM and M-MDM for all cytokines tested. Furthermore LPS stimulation caused a significant increase in all cytokines compared to baseline (Figure 3.6).

LPS-stimulated TNF α release was significantly higher in COPD cells compared to non-smokers and smokers at day 0 and in M-MDM at day 12 with a similar trend in G-MDM throughout differentiation (Figure 3.6A). M-MDM cells showed a time dependent decrease in TNF α production during differentiation in all subject groups (Figure 3.6 A) and produced significantly less TNF α on day 12 and 18 compared to G-MDM in non-smokers and smoker but not COPD cells (Table 3.4).

However, there was no difference in LPS-stimulated CXCL8 production between subject groups for any of the days examined (Figure 3.6B). There was no change in output in G-MDM during differentiation, although there was a trend of decreased CXCL8 release in M-MDM by day 12 in non-smokers and smokers, and this decrease was significant in COPD cells at day 12 and 18 compared to day 0 (Figure 3.6B). Furthermore G-MDM produced significantly more CXCL8 at day 12 in non-smokers and day 18 in COPD cells compared to M-MDM (Table 3.4).

Similarly to CXCL8 production, there was no difference in LPS-stimulated IL-10 output between subject groups. However, there was a significant time-dependent decrease throughout differentiation in non-smokers and COPD patients on day 12 and 18 compared to day 0 (Figure 3.6C). G-MDM from non-

smokers and COPD patients released significantly lower levels of IL-10 at day 12 and 18 compared to M-MDM (Table 3.4).

	Non-smoker	Smoker	COPD
TNF α			
Day 0	-	-	-
Day 12	* 	-	-
Day 18	* 	* 	-
CXCL8			
Day 0	-	-	-
Day 12	* 	-	-
Day 18	-	-	* 
IL-10			
Day 0	-	-	-
Day 12	* 	-	* 
Day 18	* 	-	* 

Table 3. 4 Comparison of cytokine output between G-MDM and M-MDM. A Kruskal-Wallis with Dunns post-test was performed to determine any significant differences between cells cultured in GM-CSF or M-CSF for all subject groups and throughout differentiation (day 0, 12 and 18). Stars indicate a significant difference between both culture conditions for each day (* p<0.05), and a dash indicated no difference. Direction of arrow indicates if levels were higher or lower in G-MDM compared to M-MDM.

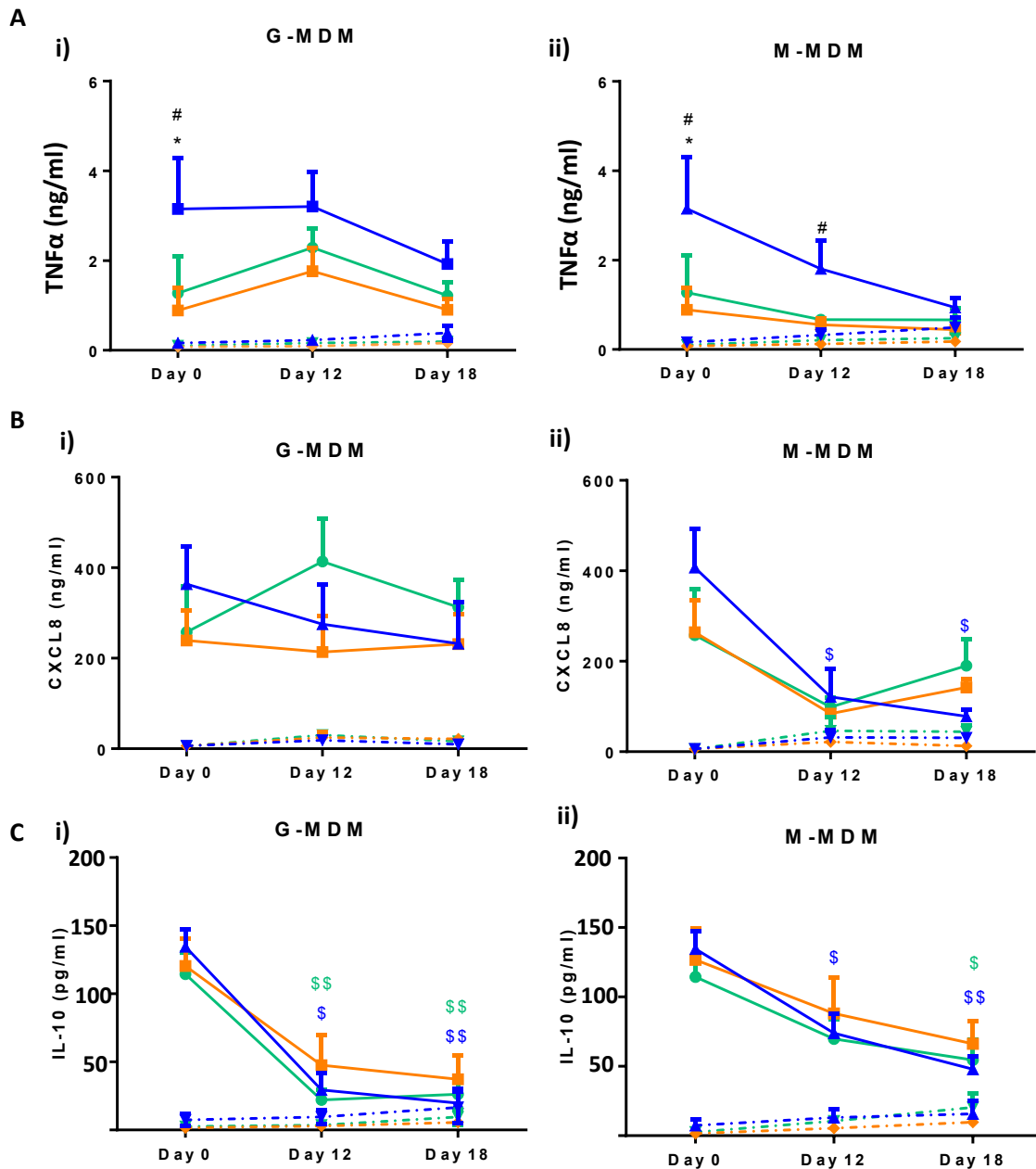


Figure 3.6 Cytokine output of MDM differentiated in GM-CSF or M-CSF over 18 days. Monocytes were isolated from **non-smokers (n=5)**, **smokers (n=6)** and **COPD patients (n=5)** and differentiated over 18 days in (i) GM-CSF (G-MDM) or (ii) M-CSF (M-MDM). Cytokine output was measured by ELISA for **(A) TNFα**, **(B) CXCL8** and **(C) IL-10** at baseline (dashed lines) and following 24h stimulation with LPS (10ng/ml – solid lines). Data are expressed as mean $\pm$ SEM. A Two-Way Anova with Bonferroni corrections was used to test significance between subject groups for each day, NS vs. COPD * $p < 0.05$, S vs COPD # $p < 0.05$ and Kruskal-Wallis with Dunn's post-test for comparing each day to day 0 \$ $p < 0.05$.

3.3.5 MDM plasticity

Having investigated the effect of GM-CSF and M-CSF culture on cytokine output throughout MDM differentiation, the next experiments investigated the plasticity of these cells and their ability to adapt to changes in microenvironment. Day 12 MDM represent mature macrophages and changing environment at this stage was performed to mimic experiments performed using Tm ϕ to assess cell plasticity. LPS-stimulated TNF α release was significantly decreased in G-MDM from smokers when these were switched to M-CSF culture compared to cells that remained in GM-CSF (Figure 3.7Aii). Cells from non-smokers showed a similar trend but did not reach significance while cells from COPD patients had no change in TNF α production following switching of culture (Figure 3.7A i,iii). Similarly, when M-MDM cell media was changed to GM-CSF at day 12, LPS-stimulated TNF α release was significantly increased in smoker cells, showed a trend of increase production in non-smokers but did not change in COPD cells (Figure 3.7B).

LPS-stimulated CXCL8 significantly decreased in G-MDM from non-smokers and smokers when media was changed to M-CSF compared to those that remained in GM-CSF media (Figure 3.8A i,ii). COPD cells demonstrated a similar trend, with 4 of 5 samples showing a decrease in CXCL8 when media was switched to M-CSF, although this did not reach significance (Figure 3.8A iii). Conversely, when the opposite switch was performed, and M-MDM media changed to GM-CSF culture, CXCL8 significantly increased in all subject groups compared to cells which remained in M-CSF media (Figure 3.8B).

There was no change in LPS-stimulated IL-10 production in any subject groups when G-MDM cells were cultured in M-CSF (Figure 3.9A). Similarly, there was no change in IL-10 output in M-MDM cells when these were changed to GM-CSF culture for any of the subject groups (Figure 3.9B).

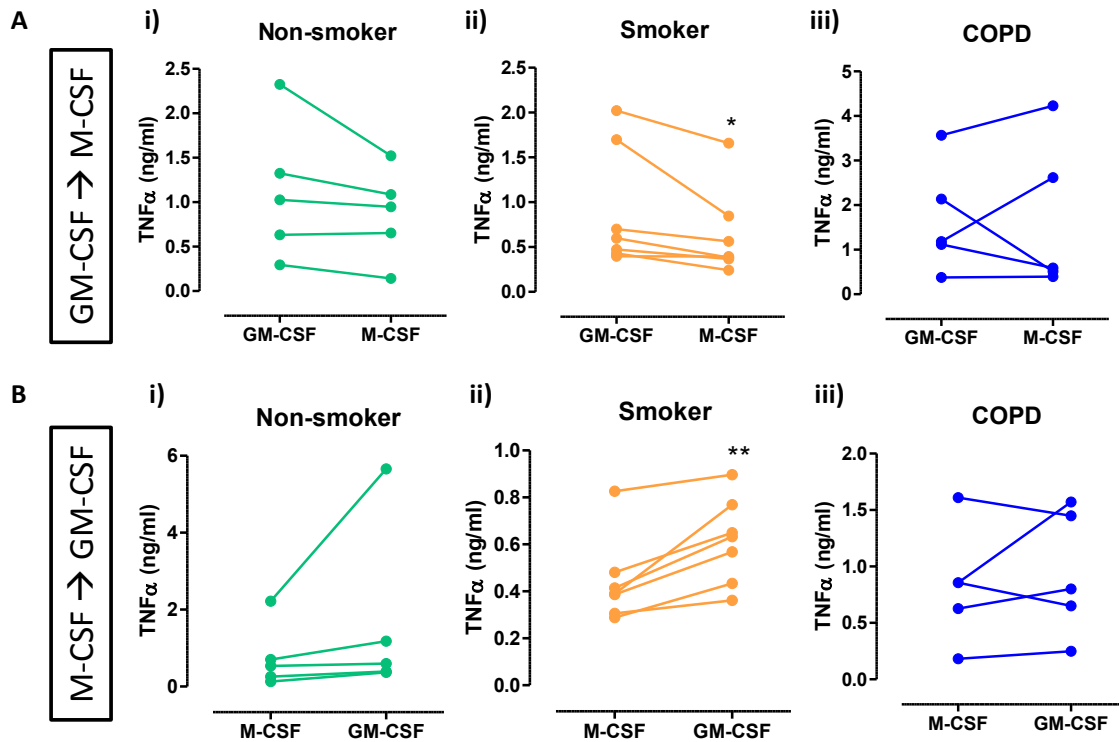


Figure 3. 7 Effect of switching culture conditions on TNF α production. MDM isolated from i) **non-smokers** (n=5), ii) **smokers** (n=7) and iii) **COPD patients** (n=5) were cultured for 12 days in **(A)** GM-CSF or **(B)** M-CSF. On day 12 media was switched from GM-CSF to M-CSF or from M-CSF to GM-CSF for a further 6 days. Control cells remained in the same media for the entire 18 days and are shown on the left side of the graphs. Cells were stimulated with LPS (10ng/ml) for 24h prior to collecting supernatant for ELISA. Data are shown as mean $\pm$ SEM. Wilcoxon matched-pairs signed rank test was used to compare significant differences in TNF α release between cells in unchanged media vs. switched media, * p<0.05, **p<0.005.

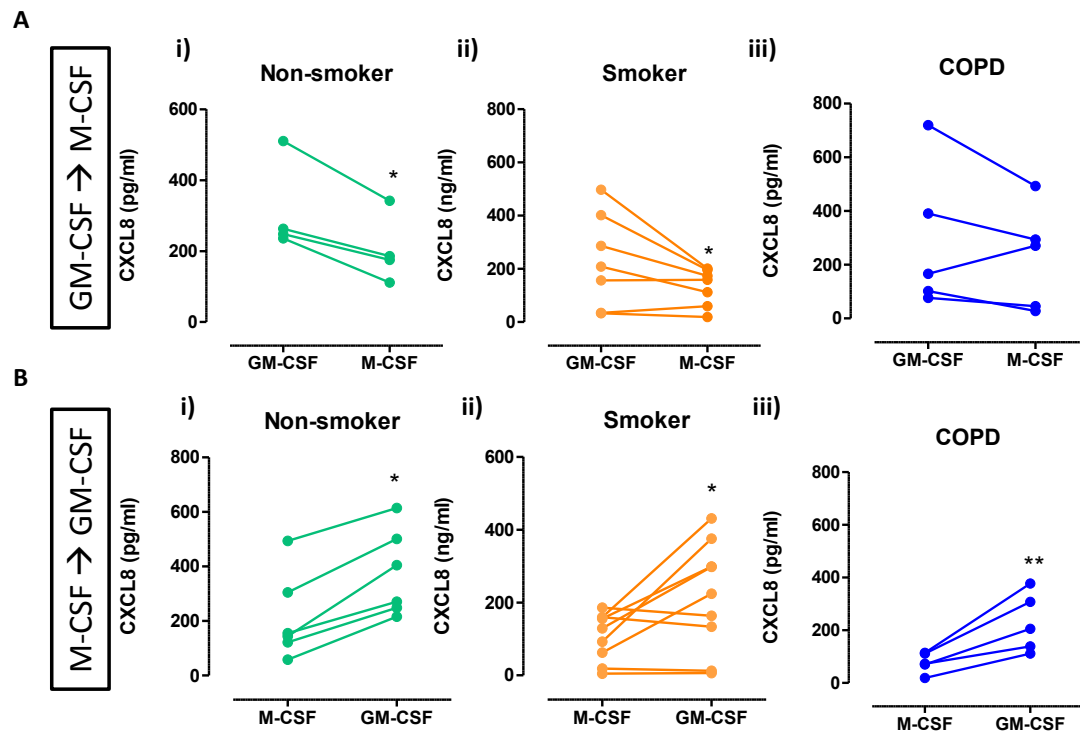


Figure 3.8 Effect of switching culture conditions on CXCL8 production. MDM isolated from i) **non-smokers (n=4-6)**, ii) **smokers (n=7)** and iii) **COPD patients (n=5)** were cultured for 12 days in **(A)** GM-CSF or **(B)** M-CSF. On day 12 media was switched from GM-CSF to M-CSF or from M-CSF to GM-CSF for a further 6 days. Control cells remained in the same media for the entire 18 days and are shown on the left of the graph. Cells were stimulated with LPS (10ng/ml) for 24h prior to collecting supernatant for ELISA. Data are shown as mean $\pm$ SEM. Wilcoxon matched-pairs signed rank test was used to compare significant differences in CXCL8 release between cells in unchanged media vs. switched media, * $p < 0.05$, ** $p < 0.005$.

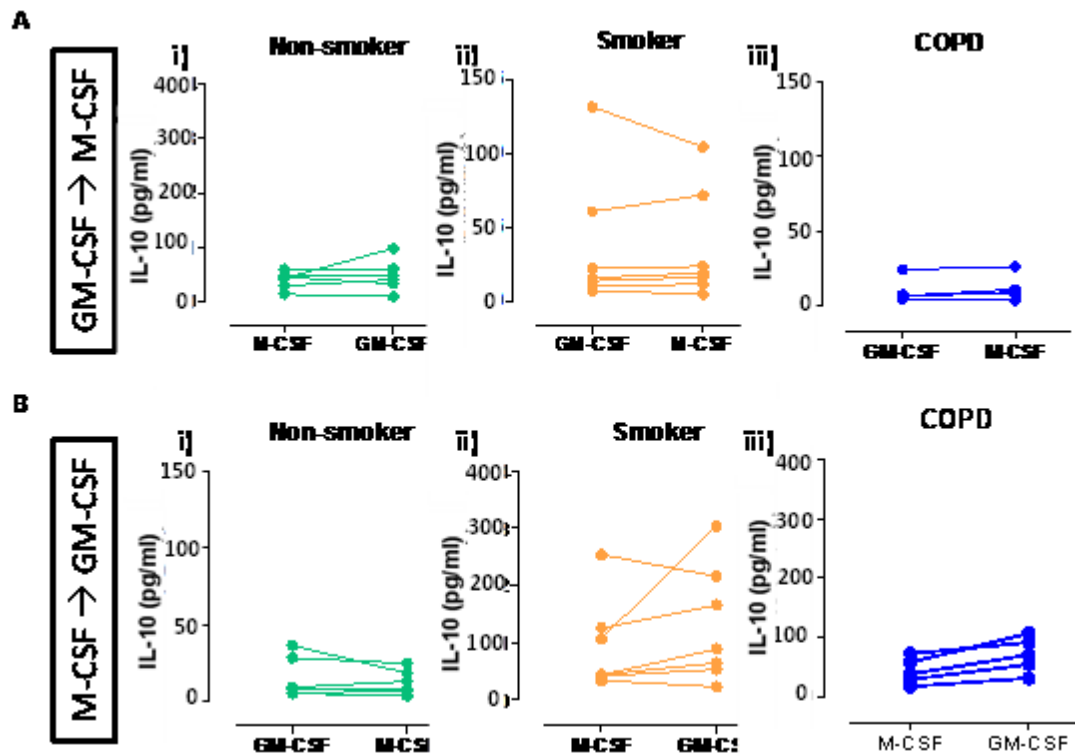


Figure 3.9 Effect of switching culture conditions on IL-10 production. MDM isolated from i) **non-smokers (n=6)**, ii) **smokers (n=7)** and iii) **COPD patients (n=5)** were cultured for 12 days in **(A)** GM-CSF or **(B)** M-CSF. On day 12 media was switched from GM-CSF to M-CSF or from M-CSF to GM-CSF for a further 6 days. Control cells remained in the same media for the entire 18 days and are shown on the left of the graph. Cells were stimulated with LPS (10ng/ml) for 24h prior to collecting supernatant for ELISA. Data are shown as mean $\pm$ SEM. Wilcoxon matched-pairs signed rank test was used to compare significant differences in IL-10 release between cells in unchanged media vs. switched media, * $p < 0.05$.

3.3.6 MDM Cell Viability Following LPS Stimulation

Cell viability was measured using an MTT assay following 24h stimulation with LPS to ensure that any changes in cytokine output were not the result of altered cell survival. There was no change in cell viability following LPS stimulation compared to baseline in any of the subject groups in both G-MDM and M-MDM for any of the days tested (Figure 3.10A,B).

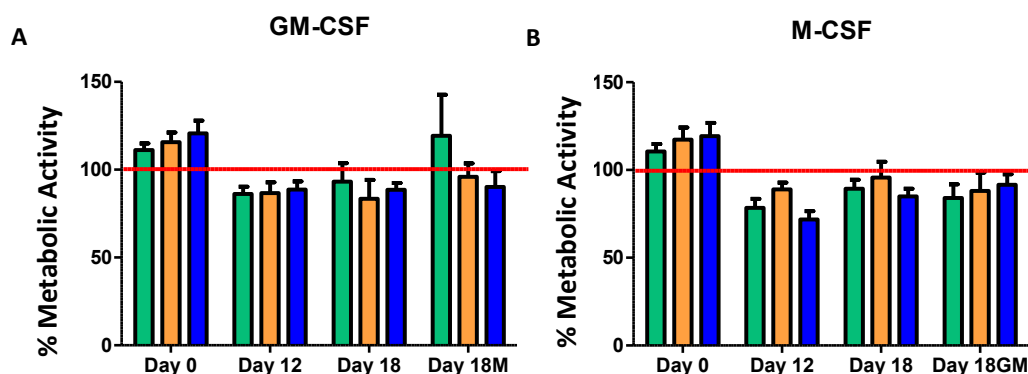


Figure 3.10 Cell viability of LPS-stimulated MDM during differentiation. MDM isolated from **non-smokers (n=6)**, **smokers (n=7)** and **COPD patients (n=5)** were cultured for 18 days in **(A) GM-CSF** or **(B) M-CSF**. On day 12 media was switched from GM-CSF to M-CSF or from M-CSF to GM-CSF for a further 6 days, shown as 18M and 18GM respectively. Cells were stimulated with LPS (10ng/ml) for 24h prior to removing supernatant and performing an MTT assay. Data were normalised to the unstimulated controls which were set to 100% cell viability (red line) and are shown as mean $\pm$ SEM.

3.3.7 Release of Cytokines from MDM Density Fractions

Finally, MDM from non-smokers were cultured for 12 days in GM-CSF prior to being processed and cells separated based on density into corresponding fractions to those obtained using tissue. This allowed further comparison of the MDM model with macrophages isolated from tissue, and distinct cell populations analysed. An additional layer was included, the 20-30% cell fraction, as these were shown to be viable cells, unlike in tissue macrophages (Chana et al., 2014). LPS and IL-4 were used to stimulate the cells in order to gain a more comprehensive characterisation of the cell fractions. Due to time restraints and sample availability, all experiments were only performed in non-smokers.

Cytokine output was measured following 24h stimulation with LPS or IL-4 and at baseline. There was no difference between cell fractions in TNF α or CXCL8 production at baseline or following IL-4 stimulation. However, LPS-stimulation lead to a significantly greater release of both cytokines from 20-30% and 30-40% cell fractions compared to 50-60%. Furthermore, there was a trend of lower cytokine output in the 40-50% cell fraction compared to 20-30% and 30-40%, and this was significant

for TNF α output compared to 30-40% cells (Figure 3.11A,B). In contrast, IL-10 release remained the same following either stimulation compared to baseline, and there were no significant differences between cell fractions (Figure 3.11C).

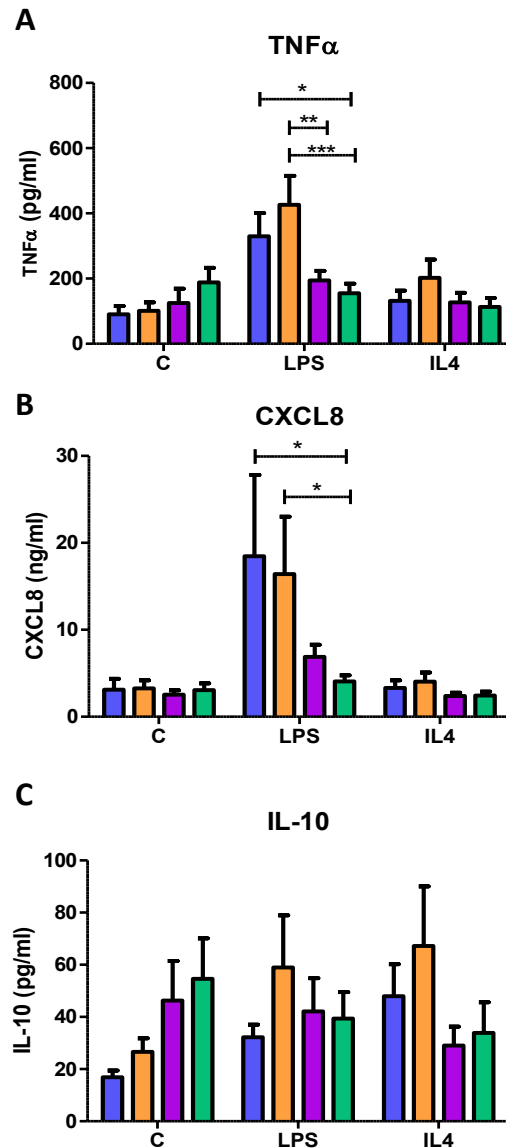


Figure 3. 11 Cytokine output from MDM density cell fractions. MDM from **non-smokers (n=4)** were cultured for 12 days in GM-CSF, re-suspended in discontinuous Percoll gradients and separated based on density by centrifugation. The resulting cell fractions correspond to densities used to separate tissue macrophages; **20-30%**, **30-40%**, **40-50%** and **50-60%**. Cells were plated overnight and then stimulated with LPS, IL-4 (10ng/ml) or control (C) for 24h and supernatant collected for ELISA of **(A)** TNF α , **(B)** CXCL8 and **(C)** IL-10. Data shown are mean $\pm$ SEM. Two-way ANOVA was performed to test differences between cell fractions, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3.8 Cell Viability of MDM Cell Fractions

Cell viability was measured following 24h stimulation using an MTT assay. All results were normalised to the 20-30% cell fraction with no stimulation. There were no differences between any of the cell fractions or following stimulation with either LPS or IL-4 (Figure 3.12).

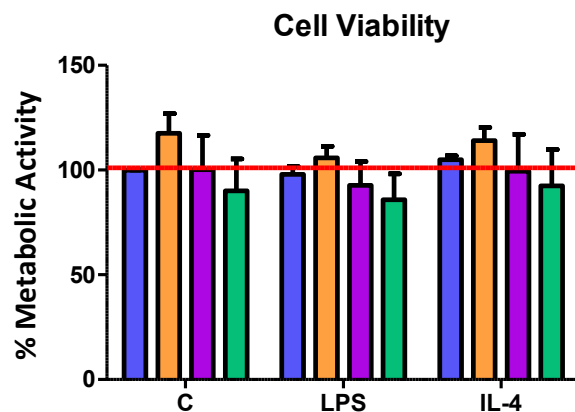


Figure 3.12 Cell metabolic activity of MDM density cell fractions. MDM were cultured for 12 days in GM-CSF, re-suspended in discontinuous Percoll gradients and separated based on density by centrifugation. The resulting cell fractions correspond to densities used to separate tissue macrophage cell populations, 20-30%, 30-40%, 40-50% and 50-60%. Cells were plated overnight and then stimulated with LPS, IL-4 (10ng/ml), or media as a control (C) for 24h and cell viability assessed by an MTT assay. All results were normalised to 20-30% control cells, which was set to 100% (red line). Data shown are mean $\pm$ SEM.

3.4 Discussion

3.4.1 The Role of GM-CSF and M-CSF on Cytokine Output Throughout Monocyte Differentiation

The growth factors GM-CSF and M-CSF have been used extensively in monocyte differentiation *in vitro*, in order to drive cells towards pro-inflammatory and resolving phenotypes respectively (Mosser, 2003, Akagawa et al., 2006, Lacey et al., 2012). In order to investigate the ability of these growth factors to influence macrophage plasticity, it was important to establish how cytokine output was influenced by GM-CSF and M-CSF during differentiation. Results obtained show that COPD MDM release significantly higher levels of the pro-inflammatory cytokine TNF α at day 0 and throughout differentiation and that G-MDM from all subject groups secrete higher levels compared to M-MDM. These results are in keeping with other studies which have shown higher levels of TNF α gene expression in COPD MDM (Ward et al., 2011, Lacey et al., 2012) in GM-CSF culture compared to M-CSF, as well as being elevated in patient sputum and serum samples compared to healthy controls (Keatings et al., 1996). Furthermore, polymorphisms in the TNF α promoter region, resulting in higher release, has been linked to COPD in a Taiwanese population (Huang et al., 1997), although these findings have not been replicated.

In addition, high levels of TNF α at day 0 indicates that circulating monocytes from COPD patients have an altered cytokine profile without having been present in the lung where tissue macrophages are exposed to high levels of oxidative stress from smoking. This supports the theory that systemic effects are present in peripheral cells in COPD patients but not in cells from healthy smokers, and therefore that a pro-inflammatory environment is not restricted to the lung.

CXCL8 levels were also higher in G-MDM compared to M-MDM, once again supporting data which indicated that GM-CSF promotes a pro-inflammatory phenotype (Akagawa et al., 2006, Lacey et al., 2012). Reports of CXCL8 production from COPD MDM have been varied, with indications that *mRNA* levels are elevated in COPD (Lacey et al., 2012) while other studies reported no difference in LPS-

stimulated CXCL8 release between non-smoker and COPD cells (Ward et al., 2011). Similarly, there is no consensus from studies on alveolar macrophages regarding CXCL8 output, with one study showing elevated CXCL8 in BAL (Culpitt et al., 2003a), while others have shown no difference between COPD and non-smokers in both BAL and sputum (Kunz et al., 2011). Data obtained here showed no difference in CXCL8 between subject groups. TNF α release leads to activation of NF κ B, which then activates transcription of *CXCL8* and subsequent release from other cell types including neutrophils and epithelial cells. It is possible that distinct isolation methods, and presence of other cell types may account for discrepancies in reports of CXCL8 release from human samples, and perhaps a longer time-point between stimulation and collection is required for CXCL8 output to change in macrophages.

Finally, data presented in this chapter indicate that there is no difference in IL-10 release from MDM from the different subject groups with a trend of higher output from M-MDM compared to G-MDM. Once again, these data are in keeping with findings that M-CSF primes cells towards a resolving phenotype (Akagawa et al., 2006, Martinez et al., 2006, Lacey et al., 2012). IL-10 is known to promote anti-inflammatory responses, and promote homeostasis by dampening release of pro-inflammatory cytokines, MMPs and promoting removal of dysfunctional mitochondria (Chung, 2001, Ip et al., 2017). Regardless of increases in pro-inflammatory cytokines, there is no concurrent increase in IL-10, which could explain in part, a lack of control from macrophages to reduce inflammatory signals, and contribute to the pathophysiology of COPD. Whether this is one of the causes, or a result of macrophage dysfunction is yet to be determined.

3.4.2 The Role of GM-CSF and M-CSF on Cell Plasticity in Tissue Macrophages and MDM

Having shown that GM-CSF and M-CSF can drive monocytes towards distinct macrophage phenotypes, the role of these growth factors in manipulating cell phenotype and plasticity was investigated using both tissue macrophage and MDM models. Tissue macrophages represent differentiated cells and were cultured *in vitro* in either media alone, GM-CSF or M-CSF for 6 days, while day 12 MDM (G-MDM and M-MDM), also representing differentiated macrophages, were cultured for a further 6 days in

either the same media as the initial 12 days, or media was switched for 6 days (GM-CSF→M-CSF or from M-CSF →GM-CSF). Analysis of cytokine output from tissue macrophages revealed that GM-CSF could drive cells toward a more pro-inflammatory phenotype with increased release of TNF α , but this was limited to cells from smokers and COPD patients and not observed in non-smoker cells. This effect was seen only in the less dense cells taken from 30-40% and 40-50% interfaces, but not 50-60%. This is in keeping with studies that suggest the 50-60% cell population represents a more monocyte-like cell and thus has not been resident in the lung for long (Holian et al., 1983, Chana et al., 2014). In contrast, the less dense cells may be older, mature cells and therefore could have been in the lung for several years, exposed to continuous oxidative stress and inflammation from cigarette smoking, leading to exaggerated release of pro-inflammatory signals. These cell populations have also previously been shown to be glucocorticosteroid insensitive in COPD samples (Chana et al., 2014), supporting the theory that these cells may have been exposed to stress for a longer period of time. Cells in the 50-60% cell fraction also produce significantly lower levels of CXCL8 compared to the less dense fractions. It is also possible that rather than representing distinct levels of maturity, these subpopulations represent functionally distinct cells, which would also explain differences in cytokine output. However, additional data is required to support this.

GM-CSF has been shown to be elevated in COPD lungs and is known to induce TNF α (Kunz et al., 2011). Cells from COPD lungs may therefore have an exaggerated response to this growth factor and thus may explain why tissue macrophage phenotype becomes more inflammatory than cells cultured in media alone or M-CSF, while cells from non-smokers do not show the same response. In the MDM model, changing media to GM-CSF resulted in a trend of increased release of TNF α from non-smoker cells, and a significant increase in smoker MDM. CXCL8 was significantly higher in cells switched to GM-CSF in all subject groups. In contrast, while M-CSF had no effect on tissue macrophage cytokine output, MDM cells showed a decrease in both pro-inflammatory cytokines in non-smokers and smokers but not COPD cells.

Data obtained in this study suggests that macrophages can be manipulated towards a more pro-inflammatory phenotype, which is enhanced in COPD cells, but only MDM from non-smokers and smokers can be manipulated towards a resolving phenotype, indicating unilateral plasticity in COPD MDM. Furthermore, while both pro-inflammatory cytokines could be manipulated by one or both growth factors, IL-10 remained the same in both models, suggesting differences in signalling pathways involved in activation of these cytokines. Several inflammatory diseases, including inflammatory bowel disease and psoriasis have implicated dysfunctional IL-10 signalling as one of the mechanisms for disease progression. Therefore, while targeting GM-CSF may aid in reducing inflammatory phenotype of COPD macrophages, it may also be beneficial to further investigate how IL-10 suppression and activation is involved in this disease.

Cell metabolic activity was measured by MTT assay as an indirect measurement for cell viability. There were some differences in MTT results for tissue macrophages, with the 40-50% cell fraction appearing to have increased metabolic activity. However, experiments were performed to assess the effect of GM-CSF and M-CSF on cytokine output for each cell fraction, and thus this did not affect analysis. However, GM-CSF also appears to result in increased cell viability. However, GM-MDM have consistently higher MTT results compared to M-MDM, which is likely the result of changes to cell metabolism rather than actually representing a decrease in cell survival. Therefore, although changes in cell viability between cell fractions and culture conditions were observed, these are likely due to the nature of the assay used, and should not affect the interpretation of cytokine output performed. Additional viability techniques would be beneficial to confirm whether these growth factors affect cell survival, or change metabolic activity.

3.4.3 Comparison of Cytokine Output from Tissue Macrophage and MDM Density Fractions

Understanding how the MDM model represents tissue macrophage phenotype and function is essential as this model is used extensively to represent lung macrophages. To date, there have been no studies investigating whether subpopulations exist within MDM and whether these are similar to those obtained from tissue. Data obtained from tissue macrophage and MDM fractions demonstrate that the less dense cells fractions release higher levels of pro-inflammatory cytokines compared to the denser cells in both models. This may represent a level of maturity within the cells as has previously been suggested, with the 'older' cells displaying an inflammatory phenotype (Holian et al., 1983, Chana et al., 2014), or could simply distinguish subpopulations of macrophages, with distinct functional outputs. In both tissue macrophages and MDM, there is no difference in IL-10 production between the cell fractions. These data indicate that cell fractions obtained from lung tissue, and those from MDM, have similar cytokine outputs, and since MDM have never been resident in the lung, it also suggests that the presence of subpopulations occurs regardless of the lung microenvironment. It may be that monocytes differentiate at different rates, creating multiple populations, or that different monocyte subpopulations drive differentiation of distinct macrophages, which may be phenotypically and functionally different.

3.4.4 Conclusions

Taken together, the data from this chapter indicate that GM-CSF can influence macrophage phenotype both during differentiation, and also that of mature cells, and should thus be considered as a potential therapeutic target in COPD. GM-CSF promoted a pro-inflammatory phenotype during monocyte differentiation and could manipulate mature macrophages towards an even more pro-inflammatory phenotype. G-MDM and tissue macrophages cultured in GM-CSF released increased levels of TNF α with no concurrent increase in IL-10, and this was even greater in cells from COPD cells.

Furthermore, M-MDM switched to GM-CSF resulted in increased release of pro-inflammatory cytokines in all subject groups, but when switched in the other direction only cells from non-smoker and smokers could be manipulated towards a resolving phenotype. This lack of plasticity in COPD cells may indicate a key difference between smokers who develop COPD and those who do not, where healthy smoker cells retain the ability to respond to anti-inflammatory signals. Levels of GM-CSF have been shown to be elevated in COPD patients, and may be driving cells towards a more inflammatory phenotype which become 'fixed' in that state, and are unable to revert back to resolving or steady state phenotype.

CHAPTER 4: EXAMINING CHANGES IN THE PROTEASE-ANTI-PROTEASE BALANCE IN COPD MACROPHAGES

4.1 Introduction

The previous chapter indicated that GM-CSF could drive increased release of the pro-inflammatory cytokine, TNF α , from tissue macrophages and MDM, resulting in a more pro-inflammatory phenotype. Inflammatory cytokines, including TNF α , IL-1 β and CXCL8, as well as LPS and cigarette smoke extract stimulation have been shown to induce release of MMPs from macrophages (Russell et al., 2002, Steenport et al., 2009). MMPs have been widely implicated in the development of emphysema in COPD (Churg et al., 2012, Lowrey et al., 2008, Finlay GA, 1997), thus this chapter will focus on identifying changes in proteases and anti-proteases expressed by macrophages from non-smokers, smokers and COPD patients, and to determine whether GM-CSF or M-CSF have any effect on this balance. In particular, MMP9 has been implicated in human studies of COPD, with several reports of elevated MMP9 in BAL from COPD patients as well as from alveolar macrophages and MDM compared to non-smokers (Russell et al., 2002, Mercer et al., 2005). Furthermore, MMP2 has been suggested to play a role in the regulation of MMP9, therefore both of these proteases were investigated (Mercer et al., 2005, Culpitt et al., 2005).

One of the defining pathophysiology's associated with COPD is emphysema, which is the result of proteolytic degradation within the lung, leading to damaged alveoli, increased airspaces and reduced lung function (Churg et al., 2007, Barnes et al., 2003). The hypothesis that proteolytic enzymes are the cause of emphysema originated from the observation that people with the genetic disorder alpha-1-antitrypsin deficiency (A1AT) develop early onset emphysema. A1AT is the main inhibitor of neutrophil elastase, an important proteolytic enzyme in the degradation of extracellular matrix. Patients with A1AT are therefore unable to regulate neutrophil elastase, resulting in the development of emphysema in early adulthood, and this can be further exacerbated by cigarette smoking (Churg et

al., 2007, Russell et al., 2002). Additional support for this theory came from animal studies that showed that instillation of proteolytic enzymes such as neutrophil elastase into the trachea of mammals induced the development of emphysema (Senior et al., 1977, Janoff et al., 1976).

Increased proteolytic activity is thought to occur due to upregulation of proteases as well as a dampening of their inhibitors, resulting in degradation of the extracellular matrix and further induction of inflammatory signals (Mercer et al., 2005). There is increasing evidence from murine and human studies that matrix metalloproteases (MMPs) are major contributors to the protease imbalance observed in emphysema, and in particular MMP2, MMP9 and MMP12 have been investigated in the context of COPD in humans (Finlay GA, 1997, Mercer et al., 2005, Culpitt et al., 2005).

MMPs are calcium dependent, zinc containing endopeptidases with key roles in homeostasis, cell migration, tissue remodelling and wound healing. They are tightly regulated through various mechanisms, including regulation at the gene transcription level, activation by other proteases through cleavage of the pro-peptide, and via inhibition by tissue inhibitor of metalloproteases (TIMPs). Over twenty MMPs have been identified to date, classified based on specificity and substrate, and four TIMPs (1-4) all of which can bind in a 1:1 complex and inhibit MMPs with no known specificity between individual MMPs and TIMPs (Snoek and Von den Hoff, 2005, Lowrey et al., 2008).

Macrophages and neutrophils are the main source of MMPs in the lungs, and studies using immunohistochemical and morphometric analysis of resected human lung tissue have shown a correlation between the number of macrophages present and extent of emphysema, which was not observed with neutrophils (Finkelstein et al., 1995). In addition, multiple studies have demonstrated elevated macrophage numbers in BAL and sputum samples from COPD patients compared to non-smoking and healthy smoking controls. Many of these studies went on to demonstrate elevated quantities of MMP9 in those samples as well as from cultured alveolar macrophages from COPD patients, supporting the theory that macrophages in the lung are the predominant drivers of MMP9

production (Russell et al., 2002, Mercer et al., 2005, Lowrey et al., 2008). Whether this increase in MMPs is counteracted by a concomitant increase in TIMPs has remained controversial, with some studies showing elevated TIMP1 in COPD samples while others report no difference between subject groups (Russell et al., 2002, Lowrey et al., 2008). Regardless, it is clear that MMPs play an important role in COPD pathophysiology, are induced by pro-inflammatory signals and cigarette smoking, and thus further understanding macrophage dysfunction in this context could aid in developing new therapies.

This chapter will focus on investigating MMP and TIMP gene expression from lung tissue macrophages and MDM as well as MMP9 protein and activity levels. Most studies investigating MMPs in COPD have used heterogeneous cell populations found in sputum and BAL, with some investigating alveolar macrophages in culture (Russell et al., 2002, Mercer et al., 2005). The present study will determine the role of lung tissue macrophages in the protease anti-protease balance in COPD, and whether GM-CSF could be driving increased production, possibly as a result of elevated TNF α in COPD macrophages. In addition, this chapter will investigate whether specific macrophage subpopulations are key sources of MMPs by investigating tissue and MDM cell fractions.

4.1.1 Hypothesis

Proteases and protease activity will be elevated in COPD macrophages compared to those from non-smokers and smokers, and this will be greater in GM-CSF cultured cells.

4.1.2 Aims

- Examine changes in gene expression of proteases and anti-proteases in MDM and tissue macrophages from non-smokers, smokers and COPD patients.
- Determine protein level and activity of MMP9 in cells from non-smokers, smokers and COPD patients.
- Assess the role of GM-CSF and M-CSF on changing the balance between proteases and anti-proteases in macrophages.

4.2 Methods

4.2.1 RNA extraction and qPCR

4.2.1.1 Tissue Macrophages

Macrophage fractions isolated as described in 2.2.3.2 were seeded on 24-well plates and cultured for 6 days, in either media alone, or in the presence of GM-CSF or M-CSF. Samples were collected on day 0 and day 6 and RNA extracted as described previously in 2.2.4. Gene expression of *MMP9*, *MMP2* and *TIMPs 1-4* was assessed.

4.2.1.2 Monocyte-Derived Macrophages

Monocytes were seeded in 24-well plates and cultured in GM-CSF or M-CSF for 12 days. At day 12 media was switched from GM-CSF to M-CSF, or M-CSF to GM-CSF for a further 6 days, while control cells remained in the same media for 18 days as described in section 2.2.3.1. Samples were collected on day 0, 12 and 18 and RNA extracted as described in section 2.2.4. Gene expression was assessed for *MMP9*, *MMP2* and *TIMPs 1-4*.

4.2.1.3 MDM Density Fractions

Monocytes were cultured for 12 days in GM-CSF (2ng/ml) prior to dissociating and separating cell fractions based on density as described previously 2.2.3.3. Resulting cell fractions were adhered to plastic overnight and samples collected for RNA extraction as in section 2.2.4. Gene expression was assessed for *MMP9*, *MMP2* and *TIMPs 1-4*.

4.2.2 Zymographic Measurement of MMP Activity

4.2.2.1 Tissue macrophages

Cells were cultured in GM-CSF, M-CSF or media alone for 6 days in 96-well plates. On day 0 and day 6 cell supernatants were collected and zymography performed as described in section 2.2.7. All samples

from the same patient were placed on the same gel and all gels, alongside a known standard, and were imaged for semi-quantitative analysis performed using GelDoC-IT system.

4.2.2.2 Monocyte-Derived Macrophages

Cells were cultured in GM-CSF or M-CSF for 12 days. At day 12 media was switched from GM-CSF to M-CSF or M-CSF to GM-CSF for a further 6 days. Supernatants were collected on day 0, 12 and 18 and zymography performed as stated in section 2.2.7. All gels were imaged and semi-quantitative analysis performed using GelDoC-IT system.

4.2.3 Statistical Analysis

All data were expressed as mean $\pm$ SEM, and analyses performed using GraphPad Prism software. Data were non-parametric, and a Two-Way Anova with Bonferroni post-test was used to examine differences between subject groups. Comparisons between culture conditions for tissue macrophages was performed using a Kruskal-Wallis with Dunn's post-test. This was also used to evaluate differences between different days of differentiation of MDM. A Wilcoxon matched-pairs signed rank test was used to assess differences in MDM on day 18 between cells where media was switched on day 12 compared to those that remained in the same media.

4.3 Results

4.3.1 Subject Demographics

4.3.1.1 Tissue Macrophages

Subject demographics and lung function data for lung tissue samples are shown in table 4.1. All subjects were aged matched and smokers and COPD patients had a significantly higher smoking history compared to non-smokers but were not significantly different from each other. In addition, COPD patients had significantly lower FEV₁ and FEV₁:FVC ratio compared to non-smokers.

	Non-smoker	Smoker	COPD
Sex (F:M)	5:1	4:3	8:3
Age	68.3±7.3	68.0±6.1	68.0±2.7
Smoking history (Pack years)	3.8±2.4	40.0±7.2 [#]	40.0±7.1*
FEV ₁ (L)	2.4±0.3	2.1±0.3	1.3±0.2*
FEV ₁ % predicted	89.1±8.0	98.5±12.7	62.1±8.4
FVC (L)	2.9±0.4	2.6±0.3	2.5±0.2
FEV ₁ :FVC	0.9±0.0	0.8±0.0	0.5±0.0**

Table 4. 1 Subject demographics for tissue macrophages from non-smokers, smokers and COPD patients. All subjects underwent lung function tests. FEV₁: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for one year. Kruskal-Wallis with Dunn's post-test was performed to test for significant differences between subject groups, COPD vs. NS *p<0.05, **p<0.01 and smoker vs. NS [#]p<0.05.

4.3.1.2 Monocyte-Derived Macrophages

Subject demographics and lung function data for volunteers who donated blood are shown in table 4.2. Smokers and COPD patients had a significantly higher smoking history compared to non-smokers, but were not statistically different from each other. Furthermore, COPD patients had lower lung

function as shown by significantly lower FEV₁, FEV₁% predicted and FEV₁:FVC ratio when compared to non-smokers.

	Non-smoker	Smoker	COPD
Sex (F:M)	4:8	3:5	4:4
Age	59.3±1.9	58.3±2.6	69.6±2.5
Smoking history (Pack years)	1.3±1.3	30.0±6.8 [#]	39.0±7.1*
FEV ₁ (L)	3.1±0.2	2.6±0.2	1.7±0.2*
FEV ₁ % predicted	99.4±11.4	85.2±5.3	33.7±4.3**
FVC (L)	4.1±0.4	3.6±0.4	3.2±0.4
FEV ₁ :FVC	0.8±0.0	0.7±0.0	0.6±0.1**

Table 4. 2 Subject demographics for MDM from non-smokers, smokers and COPD patients. All subjects underwent lung function tests. FEV₁: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for one year. Kruskal-Wallis with Dunn's post-test was performed to test for significant differences between subject groups, COPD vs. NS *p<0.05, **p<0.01 and smoker vs. NS [#]p<0.05.

4.3.2 Effect of Cell Culture on Gene Expression of Proteases and Anti-proteases in Cell Fractions from Tissue and MDM

4.3.2.1 Tissue Macrophages

Proteases play an important role in driving emphysema as a result of elevated activity or inadequate inhibition. In order to investigate changes in MMP9 and MMP2, and their inhibitors, TIMP 1-4, gene expression was analysed using qPCR in tissue cell fractions cultured in GM-CSF, M-CSF or media alone for 6 days. There were no significant differences in *MMP9* or *MMP2* gene expression in any of the tissue macrophage cell fractions between subject groups or culture conditions tested (Figure 4.1). However, in the 50-60% fraction there appeared to be increased expression of *MMP9* and *MMP2* in

smoker and COPD tissue macrophages compared to non-smokers with significantly higher expression of *MMP2* in smoker cells compared to non-smokers cultured in media (Figure 4.1 iii). Furthermore, while there were no differences in *MMP9* expression between day 0 and day 6 (media, GM-CSF or M-CSF) (Figure 4.1A), *MMP2* was significantly lower on day 0 compared to day 6 in smoker cells in all density fractions. This pattern was also observed for non-smoker cells in 30-40% and 40-50% cell fractions and COPD tissue macrophages in the 30-40% fraction (Figure 4.1B).

MMP activity is inhibited by TIMPs (1-4), thus it was important to assess whether there were concomitant changes in *TIMP* gene expression between subjects, culture conditions and cell fractions. Cells from non-smokers appeared to express higher *TIMP1* than cells from smokers and COPD patients in all cell fractions and regardless of culture, although this did not reach significance (Figure 4.2A). COPD cells expressed significantly higher *TIMP1* on day 0 compared to day 6 when cultured in GM-CSF or media alone in the 40-50% and 50-60% fractions respectively (Figure 4.2A). A similar pattern was observed for *TIMP4* gene expression, with a trend of higher expression in non-smokers compared to smokers and COPD patients (Figure 4.2D). Furthermore, this difference was significant in 30-40% cell fraction between GM-CSF non-smokers and smokers and M-CSF non-smokers and COPD cells, as well as in 50-60% cell fraction between GM-CSF non-smokers and smokers. *TIMP4* gene expression was significantly lower on day 0 compared to day 6 GM-CSF cultured cells from smokers in the 30-40% cell fraction and in COPD cells in the 50-60% fraction (Figure 4.2D). Conversely, *TIMP3* gene expression appeared higher in COPD cells compared to non-smokers and smokers in all cell fractions and under all culture conditions (Figure 4.2C). There was no difference in *TIMP2* expression between subjects or culture conditions (Figure 4.2B).

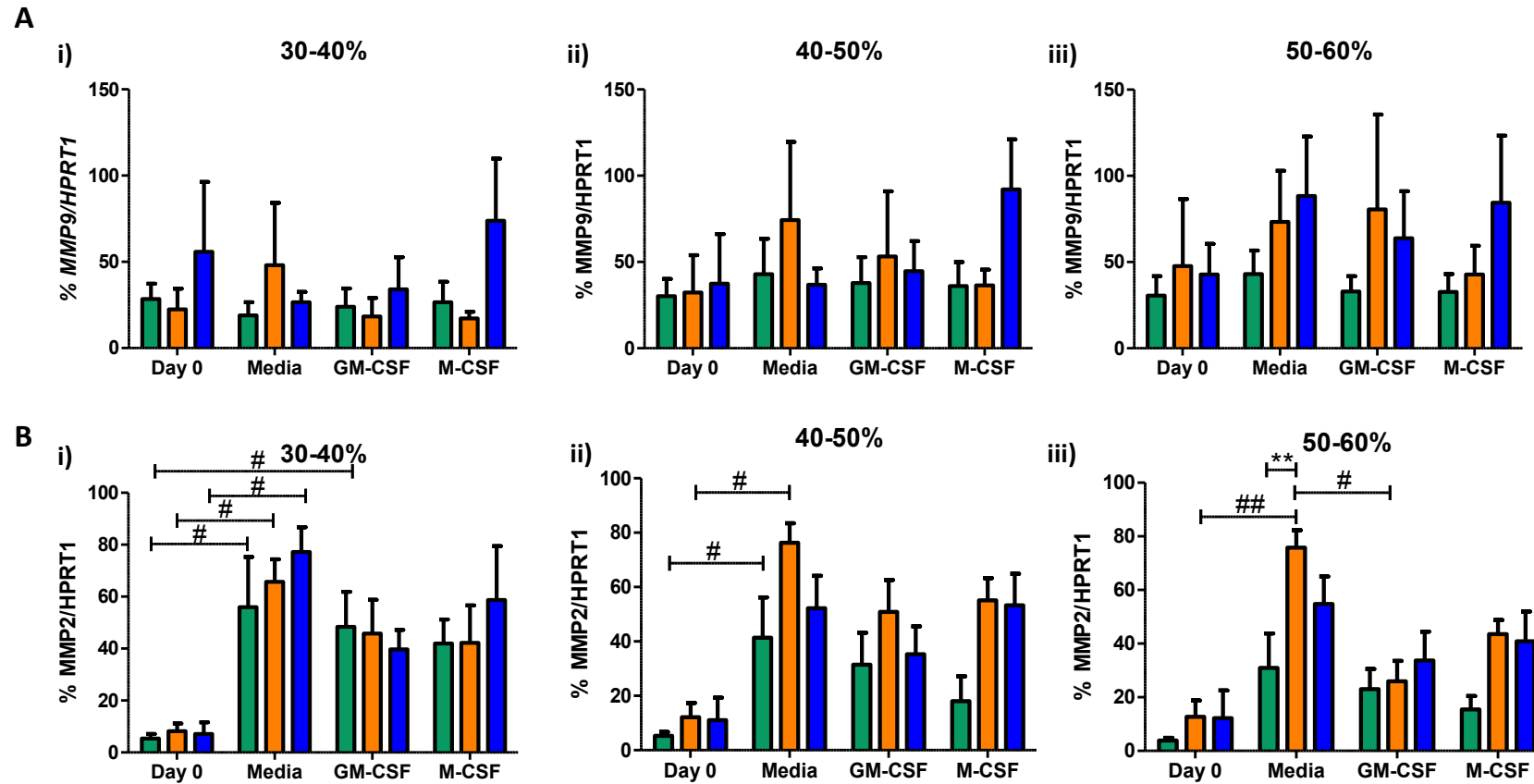


Figure 4. 1 Effect of different culture conditions on MMP gene expression from tissue macrophages. Cells from **non-smokers (n=5)**, **smokers (n=5)** and **COPD patients (n=6)** were cultured for 6 days in GM-CSF, M-CSF or media alone. Samples were collected on day 0 and day 6 for RNA extraction and gene expression of **(A)** MMP9 and **(B)** MMP2 was analysed using RT-qPCR for tissue macrophage cell fractions (i) 30-40%, (ii) 40-50% and (iii) 50-60%. Data shown represent mean $\pm$ SEM. Comparisons between subject groups was performed using a two-way Anova with Bonferroni corrections, * $p<0.05$, and a Kruskal-Wallis with Dunn's post-test to examine differences between culture conditions # $p<0.05$, ## $p<0.01$.

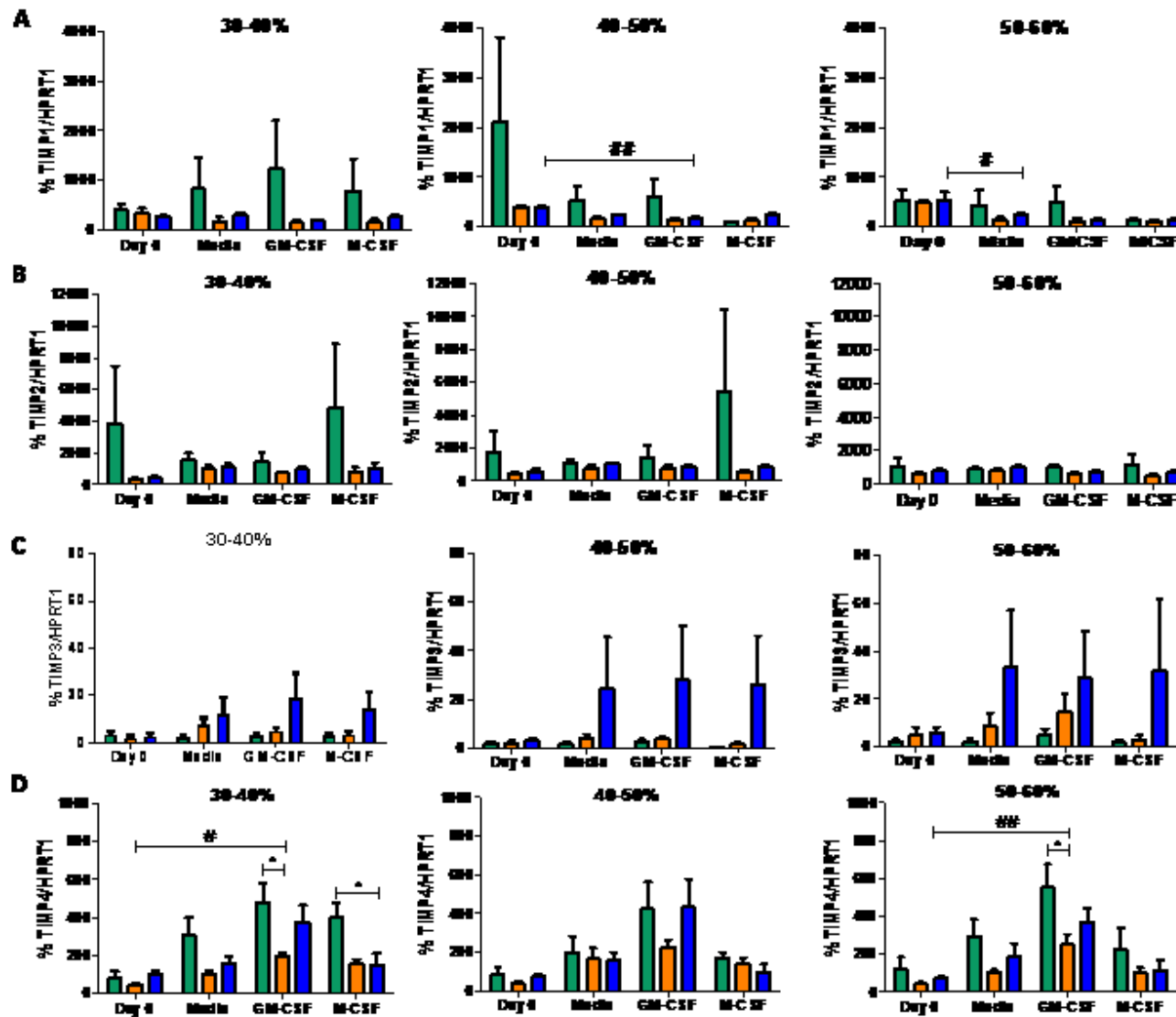


Figure 4. 2 Effect of different culture conditions on TIMP gene expression from tissue macrophages. Cells from **non-smokers** (n=4), **smokers** (n=4) and **COPD patients** (n=5) were cultured for 6 days in GM-CSF, M-CSF or media alone. Samples were collected on day 0 and day 6 for RNA extraction and gene expression of (A) TIMP1, (B) TIMP2, (C) TIMP3 and (D) TIMP4 was analysed using RT-qPCR. Data shown represent mean $\pm$ SEM. Two-way Anova with Bonferroni corrections was performed to test differences between subject groups, * $p < 0.05$ and a Kruskal-wallis with Dunn's post-test performed between culture conditions # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$.

4.3.2.2 MDM Density Fractions

Having investigated changes in gene expression of proteases and anti-proteases in tissue macrophage cell fractions, it was important to establish whether similar results could be observed in MDM cell fractions. Day 12 MDM from non-smokers were therefore processed and cell fractions isolated based on density, corresponding to fractions obtained from tissue. Results suggest that there are subpopulations within MDM, with distinct gene expression profiles. Both *MMP9* and *MMP2* were significantly lower in the denser cell fractions (40-50% and 50-60%) compared to the lower density fractions (20-30% and 30-40%) (Figure 4.3 A-B). There were no differences in *TIMP 1-4* gene expression between any of the MDM fractions (Figure 4.3 C-F).

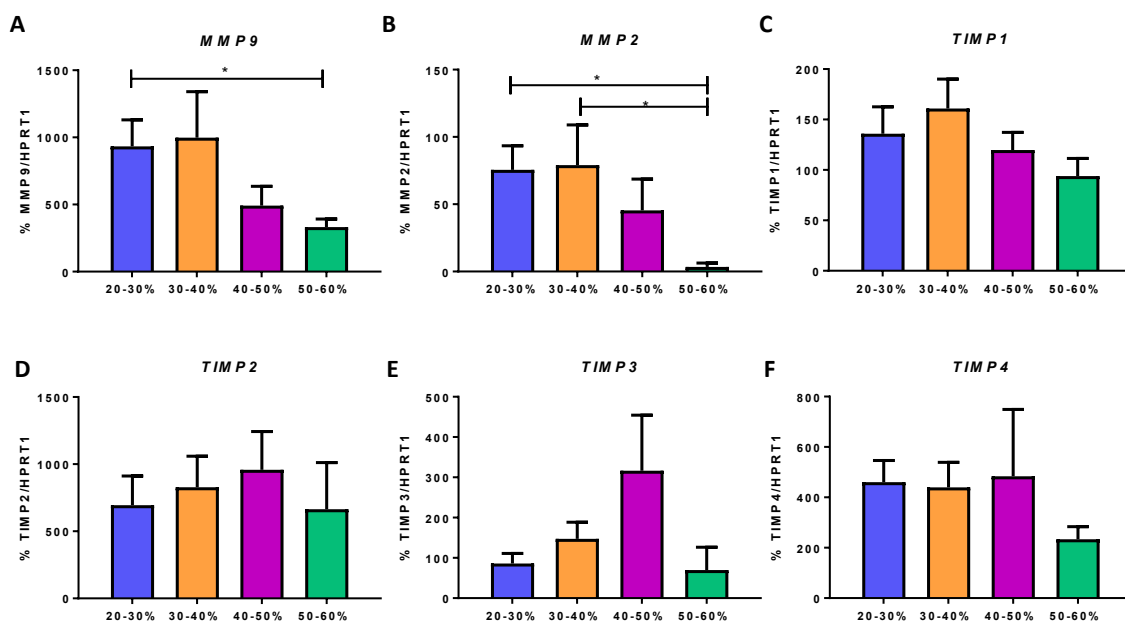


Figure 4. 3 Gene expression of MMPs and TIMPs from MDM density fractions. MDM from **non-smokers (n=5)** were cultured for 12 days in GM-CSF, re-suspended in discontinuous Percoll gradients and separated based on density by centrifugation. The resulting cell fractions correspond to densities used to separate tissue macrophage populations. Cells were plated overnight prior to lysing cells for RNA extraction. Gene expression was analysed using RT-qPCR for **(A-B)** MMPs and **(C-F)** TIMPs. Data shown represent mean $\pm$ SEM and a Kruskal-Wallis with Dunn's post-test was used to test significance between cell fractions * $p < 0.05$.

4.3.3 Gene Expression of Proteases and Anti-proteases during MDM Differentiation and After Changing Culture Conditions.

4.3.3.1 Gene Expression During MDM Differentiation

In order to compare changes in proteases and anti-proteases between both models, gene expression of *MMP9*, *MMP2* and *TIMP1-4* were also measured in the MDM model during differentiation between day 0 to day 18 in either GM-CSF or M-CSF culture.

MMP9 expression levels increased significantly from monocytes on day 0 to MDM on day 12 and 18 in non-smokers and COPD cells but not smokers (Table 4.3, Figure 4.4A). Furthermore, *MMP9* was significantly higher in COPD cells cultured in M-CSF on day 12, compared to GM-CSF cultured cells. MDM from smokers had significantly lower *MMP9* gene expression compared to non-smokers and COPD cells regardless of culture condition (Figure 4.4A). Similarly, *MMP2* gene expression increased significantly from day 0 to day 12 in non-smokers and from day 0 to days 12 and 18 in COPD cells (Table 4.3), but not cells from smokers. However, there were no differences in expression of *MMP2* between subject groups or culture conditions (Figure 4.4B).

TIMP1 was significantly higher in COPD cells compared to non-smokers and smoker cells on day 0, and significantly decreased during differentiation (Table 4.3). MDM from non-smokers and smokers showed consistent gene expression levels throughout differentiation. There were no differences between GM-CSF or M-CSF culture (Figure 4.5A). Conversely, *TIMP2* significantly increased during differentiation in all subject groups, and was significantly higher in COPD MDM on day 18 compared to smoker MDM. Similarly, *TIMP3* and *TIMP4* gene expression appeared to increase during differentiation for all subject groups, although no difference was observed between subject groups or culture conditions (Table 4.3, Figure 4.5B-D).

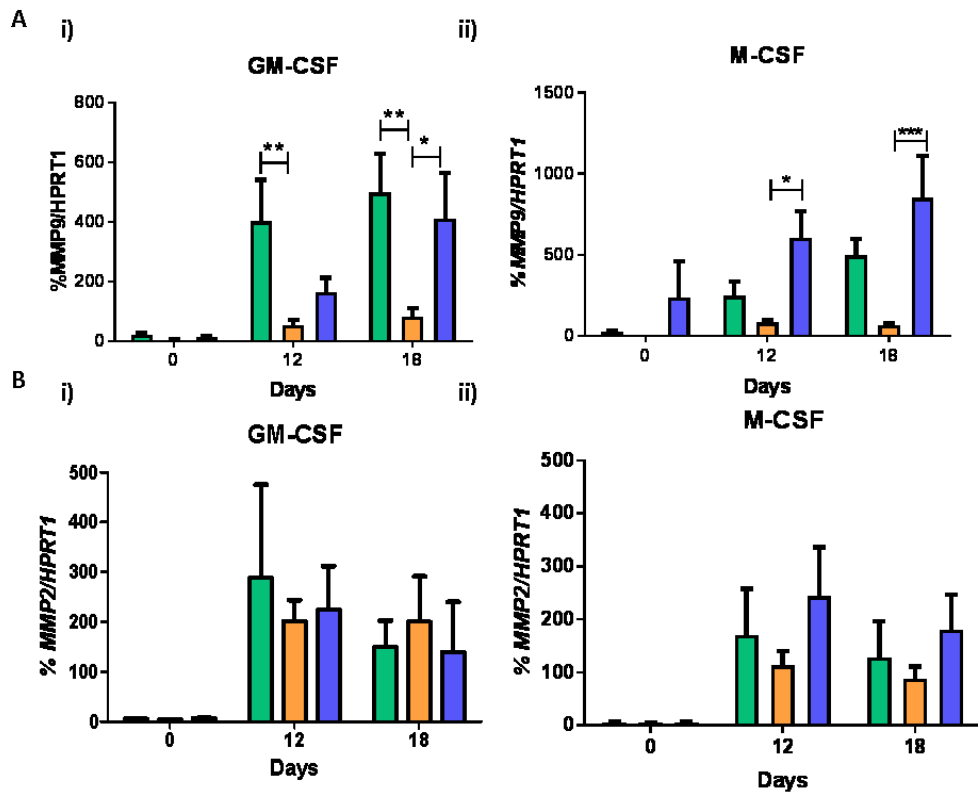


Figure 4. 4 Expression of *MMP9* and *MMP2* during MDM differentiation. MDM isolated from **non-smokers (n=5)**, **smokers (n=6)** and **COPD patients (n=5)** were cultured for 18 days in (i) GM-CSF or (ii) M-CSF. RNA was extracted on day 0, 12, 16 and 18 and gene expression analysed using RT-qPCR for **(A) MMP9** and **(B) MMP2**. Data are shown as mean $\pm$ SEM. Two-way Anova with Bonferroni post-test was used to test significance between subject groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

GM-CSF		<i>MMP9</i>	<i>MMP2</i>	<i>TIMP1</i>	<i>TIMP2</i>	<i>TIMP3</i>	<i>TIMP4</i>
Non-smoker	0 vs. 12	**	*		***		**
	0 vs. 18	***			**		
Smoker	0 vs. 12						
	0 vs. 18				*		
COPD	0 vs. 12			***	***		
	0 vs. 18	***		***	***	**	**
M-CSF		<i>MMP9</i>	<i>MMP2</i>	<i>TIMP1</i>	<i>TIMP2</i>	<i>TIMP3</i>	<i>TIMP4</i>
Non-smoker	0 vs. 12		*	*	*	*	
	0 vs. 18	***					
Smoker	0 vs. 12				**		
	0 vs. 18						
COPD	0 vs. 12	**	**	***	***		*
	0 vs. 18	***	*	***	***		

Table 4. 3 Comparison of gene expression between day 0 and day 12 or 18. A Kruskal-Wallis with Dunn's post-test was performed to assess differences in gene expression between day 0 and day 12 or 18 for MDM cultures in GM-CSF or M-CSF from all subject groups, *p<0.01, **p<0.01, ***p<0.001.

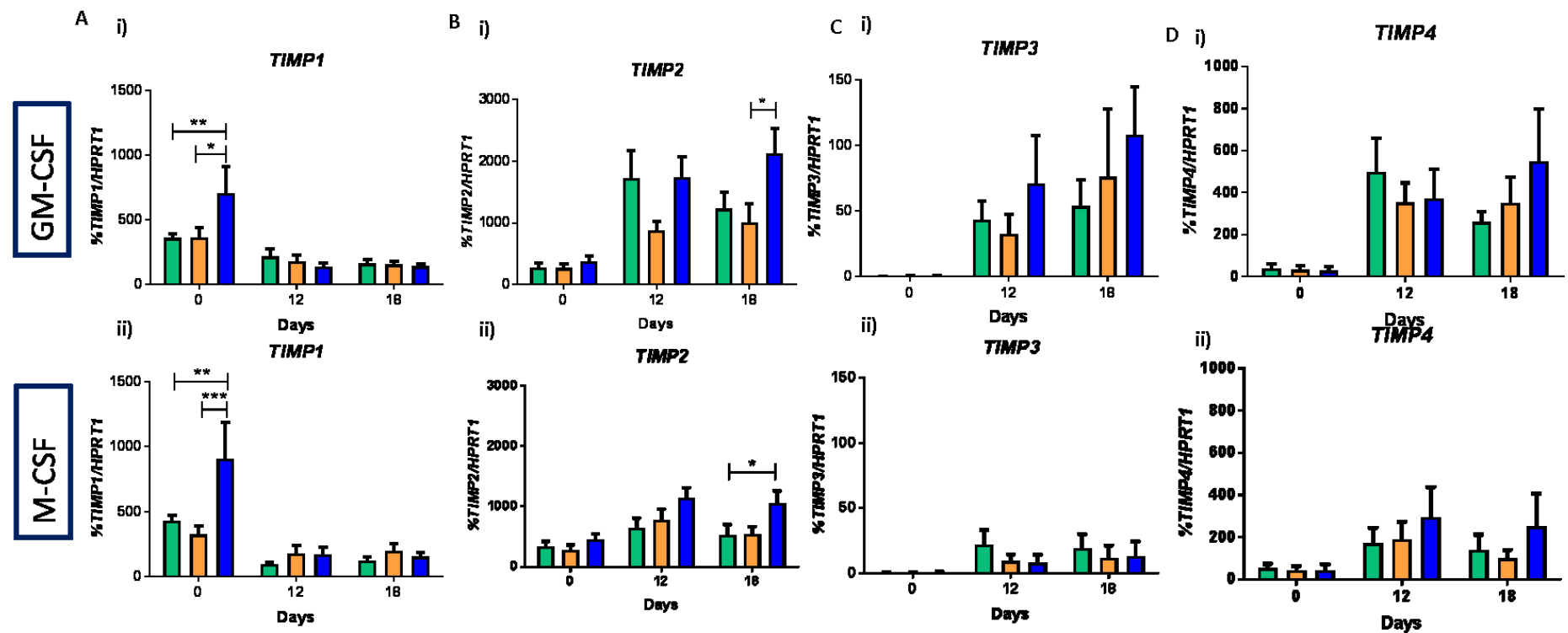


Figure 4. 5 Expression of Tissue Inhibitor of Matrix Metalloproteases (TIMP) during MDM differentiation. MDM isolated from **non-smokers (n=5)**, **smokers (n=6)** and **COPD patients (n=6)** were cultured for 18 days in (i) GM-CSF or (ii) M-CSF. RNA was extracted on day 0, 12, 16 and 18 and gene expression analysed using RT-qPCR for (A) *TIMP1*, (B) *TIMP2*, (C) *TIMP3* and (D) *TIMP4*. Data are shown as mean $\pm$ SEM. Two-way Anova with Bonferroni post-test was used to test significance between subject groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3.3.2 Gene Expression Following Changes in Culture Conditions

Macrophage plasticity was assessed in order to determine whether gene expression could be manipulated by changing the local environment. Day 12 MDM cultured in GM-CSF were switched to M-CSF culture, and M-CSF cultured cells switched to GM-CSF for a further 6 days. Control cells were maintained in the same media from day 0 to day 18.

There was no effect on *MMP9* or *MMP2* expression following changing the culture conditions, irrespective of any of the subject groups. However, there was a trend showing that switching media to GM-CSF lead to an increase in *MMP2* in all subject groups, but this failed to reach significance (Figure 4.6). In addition, smoker MDM had significantly lower *MMP9* expression compared to non-smokers and COPD cells as described previously (Figure 4.4A), but changing of culture had no effect (Figure 4.6A). When assessing the effect of switching on *TIMP* gene expression, there was no difference between control and switched cells for all subject groups, regardless of which media was switched (Figure 4.7).

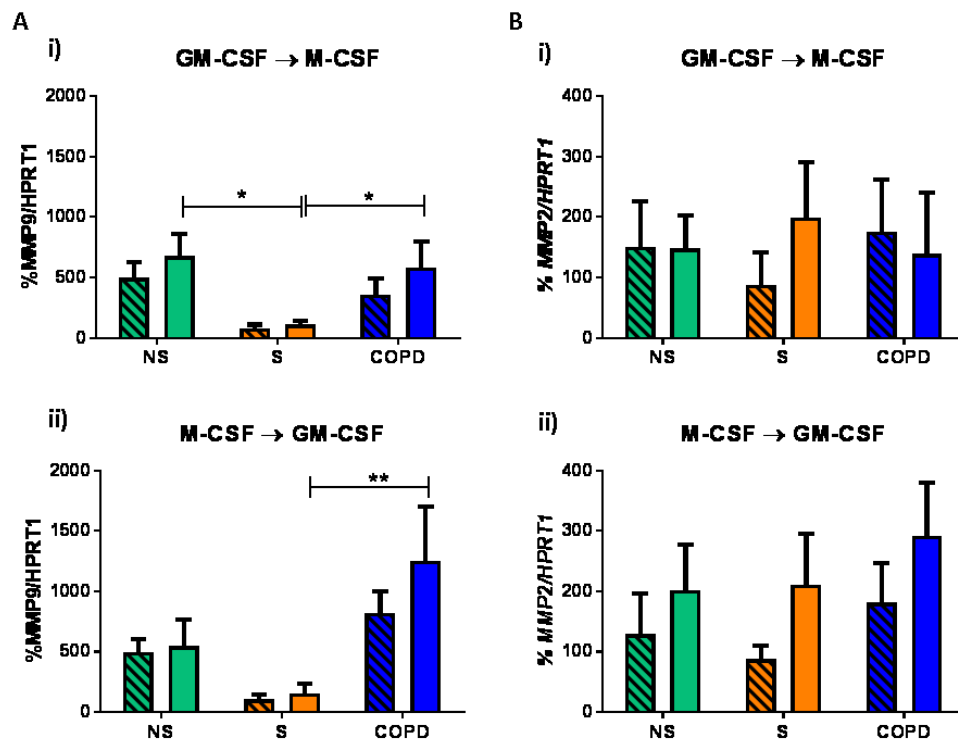


Figure 4. 6 MMP gene expression on day 18 following switching of media. MDM isolated from **non-smokers (n=5)**, **smokers (n=6)** and **COPD patients (n=6)** were cultured for 18 days in (i) GM-CSF or (ii) M-CSF. RNA was extracted on day 18 from control cells (dashed bars) or cells where media was switched on day 12 (clear bars) and gene expression analysed using RT-qPCR for **(A)** MMP9 and **(B)** MMP2. Data are shown as mean $\pm$ SEM. Two-way Anova with Bonferroni post-test was used to test significance between subject groups, * $p < 0.05$.

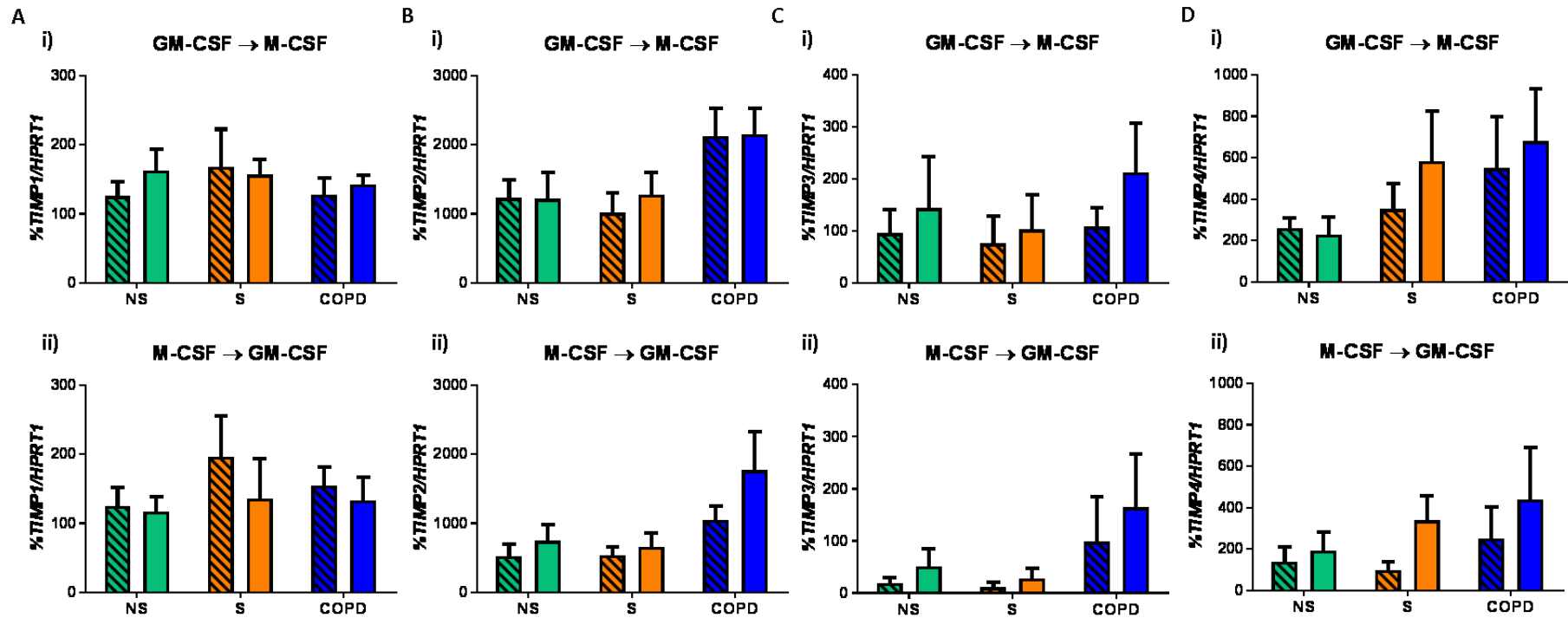


Figure 4. 7 TIMP gene expression on day 18 following switching of media. MDM isolated from **non-smokers (n=5)**, **smokers (n=6)** and **COPD patients (n=6)** were cultured for 18 days in (i) GM-CSF or (ii) M-CSF. RNA was extracted on day 18 from control cells (dashed bars) or cells where media was switched on day 12 (clear bars) and gene expression analysed using RT-qPCR for **(A) MMP9** and **(B) MMP2**. Data are shown as mean $\pm$ SEM. Two-way Anova with Bonferroni post-test was used to test significance between subject groups, * $p < 0.05$.

4.3.4 Characterising the Effect of Culture on Total Protein and Activity of MMP9 in Macrophages

4.3.4.1 Tissue Macrophages

Gene expression data does not always translate to a corresponding pattern in protein level or activity due to the presence of post-translational inhibitors such as microRNAs as well as inhibition by other proteins. It was therefore important to assess expression of proteases at the protein level and evaluate activity to determine whether this was elevated in COPD cells and whether it could be modified by GM-CSF or M-CSF culture.

MMP9 protein level and activity were measured at baseline by performing zymography on cell supernatants. Pro-MMP9 protein appeared higher in tissue macrophages from smokers and COPD patients compared to non-smokers although this did not reach significance (Figure 4.8A). Similarly, MMP9 activity showed the same trend, with higher activity in smokers and COPD cells compared to non-smokers, although this did not reach significance (Figure 4.8B). There were no differences in pro- or active MMP9 between cell fractions or culture.

4.3.4.2 Monocyte-derived Macrophages

Having investigated pro and active MMP9 levels in tissue macrophages, similar experiments were performed using the MDM model. Monocytes were cultured for 12 days in either GM-CSF or M-CSF. At day 12 media was switched from GM-CSF to M-CSF or M-CSF to GM-CSF for a further six days.

Pro and active MMP9 was significantly higher on day 12 compared to day 0 for all subjects in both GM-CSF and M-CSF culture (Table 4.2). Pro-MMP9 appeared higher in GM-CSF COPD cells and was significantly higher in smoker MDM compared to non-smokers on days 12 and 18 (Figure 4.9 Ai). Similarly, on day 12 and 18, M-CSF MDM from smokers and COPD patients release significantly higher pro-MMP9 compared to non-smokers (Figure 4.9 Aii). Levels of active MMP9 appeared greater in GM-CSF smoker and COPD MDM compared to non-smokers, reaching significance at day 18 between non-smoker and COPD cells (Figure 4.9Bi). M-CSF MDM from smokers and COPD patients released

significantly higher active MMP9 compared to non-smokers by day 12 and 18 for smokers and day 18 for COPD (Figure 4.7Bii). Switching media at day 12 had no effect on pro or active MMP9 levels (Figure 4.9).

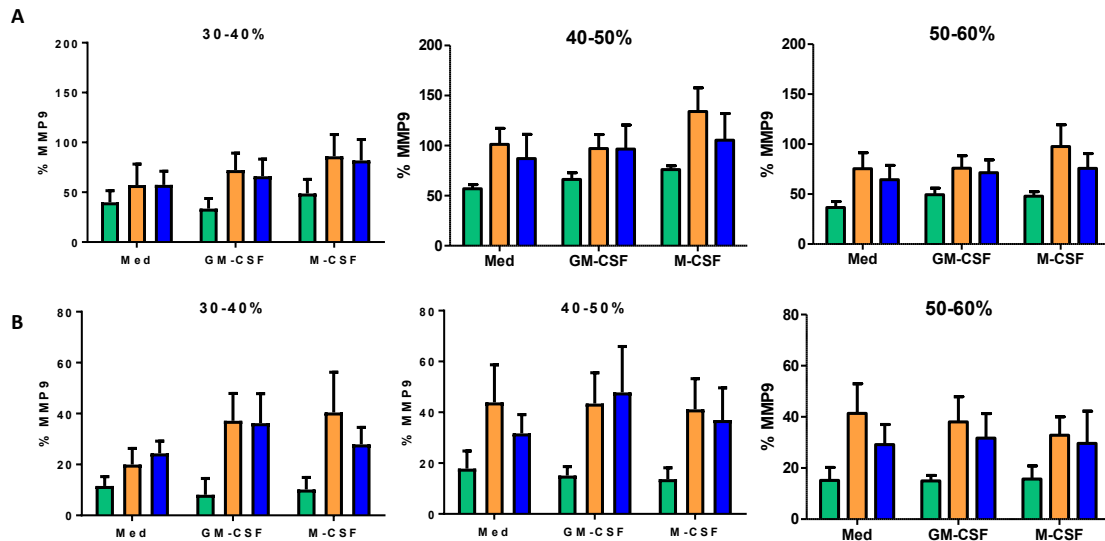


Figure 4. 8 Total and active MMP9 from tissue macrophages cultured in media, GM-CSF and M-CSF. Tm ϕ from **non-smokers (n=4)**, **smokers (n=4)** and **COPD patients (n=5)** were cultured for 6 days in GM-CSF, M-CSF or media alone. Cell supernatants were collected and **(A)** pro-MMP9 and **(B)** MMP9 activity measured using zymography and quantified by densitometry. Data shown represent mean $\pm$ SEM.

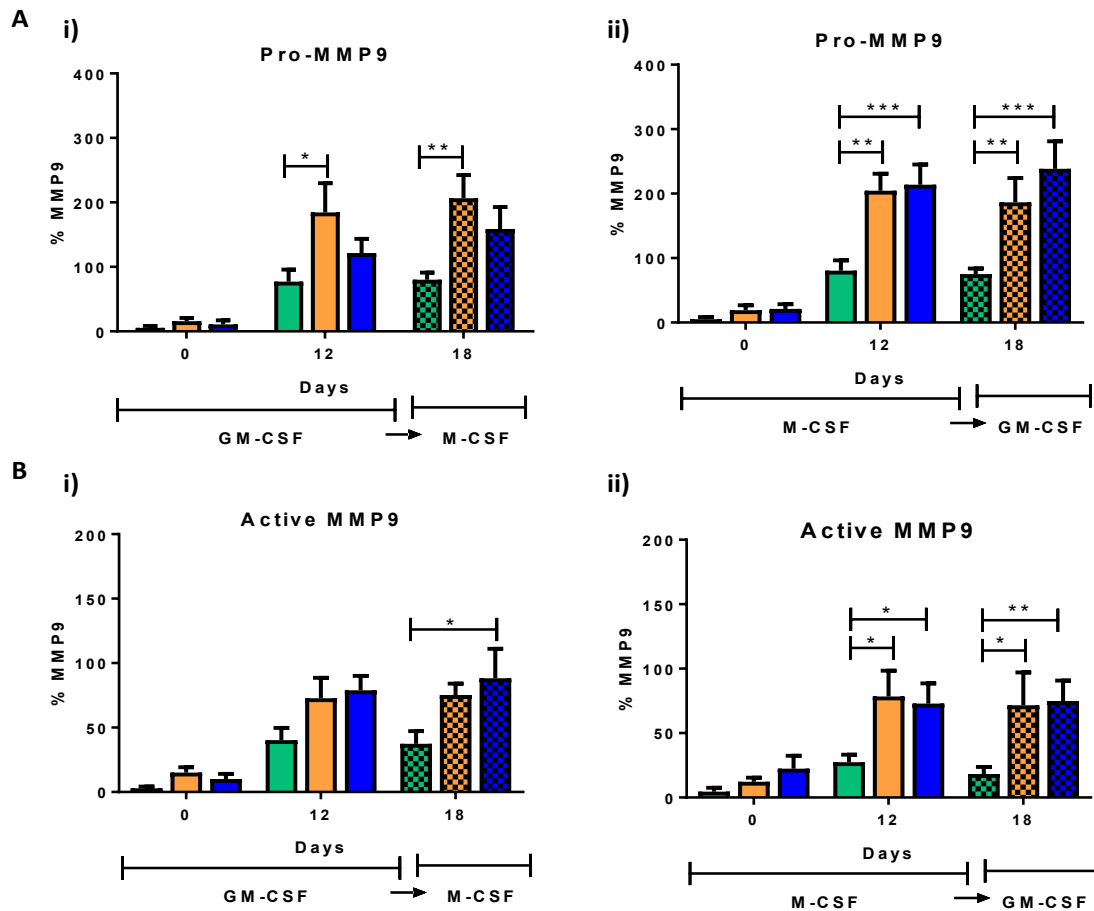


Figure 4. 9 Total MMP9 and activity measured by zymography. MDM isolated from **non-smoker (n=6)**, **smoker (n=5)** and **COPD patients (n=5)** were cultured for 12 days in **(i) GM-CSF** or **(ii) M-CSF**. On day 12, media was switched from GM-CSF to M-CSF or M-CSF to GM-CSF for a further 6 days. Cell supernatants were collected on day 0, 12 and 18 and MMP9 **(A)** total protein and **(B)** activity measured by zymography. Semi-quantitative analysis was performed by densitometry using GelDoC-IT system. Data are shown as mean $\pm$ SEM. Two-way Anova with Bonferroni corrections was performed to test differences between subject groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

		GM-CSF		M-CSF	
		Pro-MMP9	Active MMP9	Pro-MMP9	Active MMP9
Non-smoker	0 vs. 12	*	*	**	
	0 vs. 18	*		**	
Smoker	0 vs. 12	**	**	***	***
	0 vs. 18		***	***	***
COPD	0 vs. 12	***	***	***	***
	0 vs. 18	***	***	***	***

Table 4. 4 Comparison of pro and active MMP9 between day 0 and day 12 or 18. A Kruskal-Wallis with Dunn's post-test was performed to assess differences for each subject group. *p<0.05, **p<0.01, ***p<0.001

4.4 Discussion

4.4.1 The Protease-Anti-Protease Hypothesis of Emphysema in Tissue and Monocyte-Derived Macrophages

Changes in the pulmonary protease and anti-protease balance have been widely accepted as the driving force in the development of emphysema in COPD, and there is increasing evidence that MMPs and their inhibitors are key orchestrators of this imbalance (Mercer et al., 2005, Russell et al., 2002). Most studies have used sputum and BAL samples to measure MMP levels in the lungs as well as investigating alveolar macrophages *in vitro* (Li et al., 2016, Russell et al., 2002). This chapter aimed to investigate changes in MMPs and TIMPs using the MDM and human lung tissue macrophage models, to gain a better understanding of the underlying cause of this imbalance and whether similar expression levels occur between tissue resident and peripheral cells.

Data obtained from both tissue macrophages and MDMs suggests that pro and active MMP9 are elevated in smokers and COPD patients compared to non-smokers, with no significant change in gene expression. These results are in keeping with previous studies where MMP9 was elevated in sputum and BAL samples from smokers and patients with COPD and/or emphysema (Finlay GA, 1997, Russell et al., 2002, Lowrey et al., 2008, Mercer et al., 2005, Chana et al., 2014). Macrophages in the lung may become dysfunctional due to continued exposure to oxidative stress and elevated levels of pro-inflammatory signals, including TNF α (Russell et al., 2002); the data presented in this chapter suggests that additional, intrinsic, mechanisms may be responsible for changes in monocytes, resulting in dysfunctional MDM.

MDM in this study were cultured for 18 days, during which time they were not exposed to oxidative stress or inflammatory signals, yet COPD and smoker cells had significantly higher pro and active MMP9 compared to cells from non-smokers at baseline. Several polymorphisms in MMPs and specifically MMP9 have been shown to increase the risk of developing COPD, and may be one of the mechanisms driving elevated MMP activity in macrophages (Haq et al., 2010, Tesfaigzi et al., 2006).

Together, the data obtained using the MDM model and tissue macrophages further show that protease levels are elevated in COPD, and that a combination of environmental, intrinsic and potentially epigenetic changes may be driving the development of emphysema.

While several studies have investigated changes in TIMP1 gene expression and protein level in COPD, there has been no comprehensive analysis of the role of other TIMPs (Mercer et al., 2005, Culpitt et al., 2005). TIMP1 is suggested to be the primary inhibitor of MMP9, however, all TIMPs are capable of inhibition of all MMPs, and whether there is preferred selectivity remains unclear. In this thesis, TIMP1-4 gene expression was investigated in both MDM and tissue macrophages. There was no clear pattern of differences in gene expression between subject groups, and between the two models investigated. Previous studies have failed to reach a consensus regarding changes in TIMP1 levels in COPD, with some reporting a concomitant increase along with MMP9 in COPD, while other show similar levels compared to healthy controls (Finlay GA, 1997, Russell et al., 2002, Culpitt et al., 2005). It is possible that protein analysis would provide a more thorough understanding of whether reduced TIMPs drive an increase in MMP activity than gene expression as several post-translational modifications may be involved, thus further work in this area is required.

4.4.2 Comparing Gene Expression to Protein Level and Activity

While there is considerable data showing elevated MMP9 protein and activity in COPD, few studies have investigated changes in gene expression in combination with protein analysis, thereby failing to elucidate the mechanism by which proteases may be elevated in COPD (Finlay GA, 1997). In this study, both gene expression, protein levels and activity were measured in tissue macrophages and MDM in order to further understand whether elevated MMP9 is the result of *de novo* transcription or via protein regulatory mechanisms. The results from this study indicate that there is no difference in *MMP9* gene expression between non-smokers and COPD patients in both MDM and tissue macrophages. However, MDM from smokers consistently express lower levels of *MMP9* than non-smokers and COPD cells. This difference may separate disease from a 'normal' stress response

observed in smoker cells but not those from COPD MDM, and these intrinsic differences in peripheral monocytes may represent a mechanism by which some smokers develop COPD and others do not. However, protein analysis using zymography demonstrated that pro- and active MMP9 are elevated in smokers and COPD cells compared to non-smokers.

The discrepancy observed between gene expression and protein data may be due to post-translational modifications and/or inhibition by microRNAs which lead to mRNA degradation (Toth et al., 2003, Yan et al., 2011). Activation of latent MMP9 occurs as a result of cleavage of the pro-domain and other MMPs have been shown to be essential for this process (Toth et al., 2003). MMP2 in the presence of TIMP2 has been shown to activate MMP9 and lead to increased activity. Furthermore, MMP3 and membrane-type MMPs (MT-MMPs) have also been demonstrated to play a role in the activation of MMP9 (Fridman et al., 1995, Toth et al., 2003). The observed increase in MMP9 protein but not gene expression therefore suggests that the increased activity is due to insufficient regulation or increased activation of MMP9, rather than increased *MMP9* gene transcription, and further investigation into changes in the levels of other proteases involved in this pathway may provide additional therapeutic targets that may aid in slowing the progression of emphysema in COPD.

In addition to protein regulation, several microRNAs have been identified to target and degrade *MMP9* and *MMP2* directly and lead to post-transcriptional repression (Yan et al., 2011). Most of these microRNAs have been identified due to their role in tumour invasion, with miR-125b, miR451 and miR-7 showing repression of both MMP9 and MMP2 in glioblastoma and hepatocellular carcinoma (Yan et al., 2011, Gabriely et al., 2008, Li and Li, 2013). Additional microRNAs have been shown to repress these proteases in the context of tumour invasion and metastases in various cancers, including miR206, miR-340, miR-9 and miR196b (Targetscan, 2010, Liu et al., 2012, Liao et al., 2012). Investigating the role of specific microRNAs in MMP regulation in COPD may also lead to better understanding of MMP regulation.

Investigating gene expression and protein analysis has allowed for additional understanding of how MMP9 regulation is different in COPD cells with similar results obtained from both models used. Further investigations into the pathways involved in MMP regulation and changes in other proteases and microRNAs responsible for regulating MMP9 may lead to the development of new therapeutic targets.

4.4.3 Effect of GM-CSF and M-CSF on MMP and TIMP Gene Expression and Protein Levels

The inflammatory phenotype of macrophages is associated with increased release of cytokines such as TNF α as well as proteases like MMPs (Fridman et al., 1995, Li et al., 2016). *In vitro* the growth factor GM-CSF is commonly used to drive cells towards an inflammatory phenotype (Akagawa et al., 2006), and this chapter explored whether exposure to distinct growth factors, including GM-CSF, could manipulate cell phenotype in terms of MMP9 release.

MDM were differentiated in GM-CSF and M-CSF and gene expression and protein analysis performed to determine functional differences in terms of the protease/anti-protease balance. Throughout differentiation, gene expression of both MMP9 and MMP2 increased, and there was a concomitant increase in MMP9 protein and activity from day 0 to day 12. There was no difference between cells cultured in GM-CSF or M-CSF. In addition, when media was switched at day 12, there was no change in MMP or TIMP gene expression or MMP9 protein level after 6 days. These data suggest that while GM-CSF and M-CSF prime cells towards distinct phenotypes, MMP9 and MMP2 levels are similar between both, and therefore switching media does not lead to changes in the levels of these proteases. MMP9 is induced by pro-inflammatory signals such as TNF α and LPS and thus is associated with pro-inflammatory phenotype (Tudhope et al., 2008). However, no studies have investigated the effect of macrophage phenotype on MMP9 expression, and this is the first showing that regardless of subject group, GM-CSF and M-CSF differentiated macrophages release similar levels of MMP9. While gene expression was assessed for all TIMPs, investigating changes at the protein level may further elucidate whether differences exist between distinct macrophage phenotypes.

To further investigate the role of growth factors on MMP9, mature tissue macrophages were isolated and cultured for 6 days in GM-CSF, M-CSF or media alone, and gene expression and protein level analysed. Similar to results obtained from MDM, GM-CSF and M-CSF had no effect on MMP or TIMP gene expression or MMP9 protein level. However, significant differences were observed in *MMP2* gene expression between day 0 and day 6 (GM-CSF, M-CSF and media alone). This difference was not specific for either growth factor, but instead is observed for all day 6 conditions and is likely an effect of *in vitro* cell culture rather than growth factors. This is further supported by the observation that culture of cells for 6 days in media alone showed similar changes. These data are in keeping with previous studies in tissue macrophages which showed elevated levels of MMP9 protein in COPD patients (Chana et al., 2014), and is the first to show that growth factors do not influence MMP9 levels in either MDM or tissue macrophages. Therefore, it appears that MMP9 production is not associated with any particular macrophage phenotype, but rather is a common functional output regardless of phenotype, and is elevated in COPD cells compared to non-smokers.

4.4.4 Role of Cell Density Fractions on Protease-Anti-Protease Balance

In addition to investigating the role of macrophage phenotype and plasticity on altering the protease-anti-protease balance, sub-populations identified by cell density were also assessed. Published data on tissue macrophages is limited, with only one previous study from this laboratory investigating the significance of subpopulations isolated based on density. Chana *et al.* demonstrated few differences between the cell fractions in terms of cell surface receptors and in the levels of active MMP9 (Chana et al., 2014). Results from this chapter are in agreement with previous data with no differences observed in MMP or TIMP gene expression or MMP9 protein and activity between the cell fractions isolated from tissue macrophages.

However, when MDM were processed and subsets of cells isolated by cell density, there were significant differences in MMP9 and MMP2 gene expression between the cell fractions. These data

highlight the presence of macrophage subpopulations within the MDM model, and shows that the least dense cells (20-30%, 30-40%) release significantly higher levels of MMPs than the denser cells (40-50%, 50-60%). There have been no other studies investigating macrophage heterogeneity within the MDM model, thus further characterisation of how these cells differ is needed. These fractions may represent cells, which are intrinsically different, perhaps through epigenetic changes, and thus differentiate in a distinct manner with different functional roles. Another theory may be that the denser cells represent monocytes that failed to differentiate completely, or take longer to do so and as such represent a monocyte phenotype rather than a distinct macrophage subpopulation. Data from this chapter has shown monocytes express lower levels of MMPs than mature MDM and thus supports the idea that the 40-50% and 50-60% cell fractions represent un-differentiated or under-differentiated monocytes in the MDM model. What is clear is that cell fractions from MDM and those from tissue macrophages are distinct.

Additional studies into MDM cell fractions, such as functional activity, will elucidate cellular differences and whether they are comparable to tissue macrophage cell fractions. However, due to the difficulty in obtaining sufficient cells after processing, limited studies could be performed to evaluate MDM cell fractions in this chapter.

4.4.5 Conclusions

Experiments designed in this chapter were aimed to determine whether an imbalance between proteases and anti-proteases could be observed in COPD cells using the MDM and lung tissue macrophage models and by investigating both gene expression and protein levels to elucidate whether this was the result of gene expression or post-transcriptional mechanisms. Results obtained indicate that differences between subject groups were only observed at the protein level, with smoker and COPD cells releasing significantly higher levels of pro and active MMP9. These results may indicate that protease/anti-protease imbalance in COPD could be the result of changes in post-transcriptional mechanisms, such as microRNA, as well as alterations in TIMP-mediated inhibition. Furthermore,

MDM from smokers expressed significantly less *MMP9* throughout differentiation compared to non-smokers and COPD cells, and may represent a 'normal' response to stress which is lost in COPD cells. While GM-CSF was previously shown to drive cells to become more pro-inflammatory, both GM-CSF and M-CSF differentiated cells expressed similar levels of *MMP9* and *MMP2*, and thus had no additional effect on switching the cells.

Both peripheral cells and lung tissue-resident macrophages demonstrate the same increase in *MMP9* at the protein level, in accordance with several other studies (Russell et al., 2002, Culpitt et al., 2005), and suggests that changes in protease activity in COPD cells may be through dysfunctional inhibitory mechanisms, including microRNA, other MMPs and TIMPs.

CHAPTER 5: INVESTIGATING THE EFFECT OF GROWTH FACTORS ON MACROPHAGE PHAGOCYTOSIS OF BACTERIA

5.1 Introduction

Macrophages have many roles within the lung including removal of particulates and pathogenic microorganisms in order to maintain the sterility of the lower airways and prevent infections (Sethi et al., 2006, Donnelly and Barnes, 2012). However, there is increasing evidence that strongly suggests that in COPD patients, macrophages are defective in their ability to clear bacteria, resulting in increased exacerbation frequency in COPD patients (Taylor et al., 2010, Harvey et al., 2011). Exacerbations often result in hospital admission and account for a large percentage of healthcare costs as well as severe morbidity and mortality in COPD patients, and has been linked to an irreversible decline in lung function (Wedzicha and Donaldson, 2003, Celli and Barnes, 2007). Approximately half of all exacerbations are caused by bacterial infections, with the most common species responsible being *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* (Berenson et al., 2006, Sethi et al., 2006, Donnelly and Barnes, 2012). The underlying mechanism leading to macrophage phagocytic dysfunction is yet to be determined, and there are several ongoing investigations into this field that have the potential to identify novel therapeutic targets in the aid of preventing and treating COPD exacerbations.

Phagocytosis relies on the initial recognition of foreign and invading particles and microorganisms and can occur through two main pathways. One common pathway involves coating foreign particles and pathogens with proteins found in serum, which allows cells such as macrophages to recognise these via specific receptors; this is the opsonising method. Following recognition, cells engulf any foreign body, leading to the formation of a phagosome, which then fuses with a lysosome to produce a phagolysosome. The acidification within the phagolysosome results in the destruction of any foreign particles (Donnelly and Barnes, 2012). However, the lung is not a serum-rich environment and therefore non-opsonised phagocytosis is considered predominant. This process involves a plethora of

scavenger receptors, which allow macrophages to directly recognise and bind foreign particles (Arredouani et al., 2005, Taylor et al., 2010). This method is not fully understood, and makes identifying specific defects in COPD difficult. Several scavenger receptors have been investigated in the context of COPD, and macrophage receptor with collagenous structure (MARCO) has been suggested to be key in the recognition and ingestion of non-opsonised bacteria and inert particles (Arredouani et al., 2005). However, it is unlikely that a single receptor is responsible for phagocytic defects in COPD, particularly considering the wide variety of prey that are not cleared appropriately by COPD macrophages including both gram positive and negative bacteria, apoptotic cells and fungal conidia (Taylor et al., 2010, Harvey et al., 2011, Wrench et al., 2017).

Oxidative stress and cigarette smoke extract have also been shown to decrease the ability of macrophages to phagocytose, without reducing the levels of pro-inflammatory signals (Martí-Llitas et al., 2009, Harvey et al., 2011, Pang et al., 2014). In addition, Lundborg *et al.* demonstrated that alveolar macrophages exposed to ultrafine carbon particles or diesel exhaust particles had reduced ability to ingest microorganisms and silica particles (Lundborg et al., 2006). There is also evidence that household air pollution leads to alveolar macrophages becoming loaded with particulates and the level of loading correlates with a decrease in phagocytic potential (Rylance et al., 2014). These data indicate that in addition to air pollution, which has a negative effect on phagocytosis, cigarette smoking could be driving additional defects in macrophages which may result in increased bacterial colonisation and exacerbation rates in COPD patients. Investigating phagocytosis in COPD cells is essential in understanding disease progression, as studies on BAL have shown a correlation between increased bacterial load and elevated pro-inflammatory cytokines and MMP9 in COPD patients, as well as a decrease in FEV₁ (Wilkinson et al., 2003, Sethi et al., 2006).

This chapter aims to demonstrate defects in phagocytosis in tissue macrophages, as well as MDM, in order to better understand whether an inability to engulf pathogenic bacteria is the result of environmental factors within the lung or an inherent defect in monocytes. In addition, experiments

were established to investigate the role of macrophage phenotype in phagocytosis and to determine whether cells could be manipulated in order to improve phagocytosis of bacteria in COPD.

5.1.1 Hypothesis

COPD macrophages from lung tissue and blood will have reduced phagocytic ability compared to cells from non-smokers and culture in M-CSF will result in increased phagocytosis of bacteria.

5.1.2 Aims

1. Compare phagocytosis of bacteria in macrophages from non-smokers, smokers and COPD patients in tissue macrophages and MDM
2. Investigate the effect on phagocytosis of GM-CSF and M-CSF culture during monocyte differentiation
3. Assess the ability of GM-CSF and M-CSF to manipulate cell phenotype on tissue macrophages and MDM

5.2 Methods

All phagocytosis experiments were performed using heat-killed fluorescently labelled *Haemophilus influenzae* and *Streptococcus pneumoniae*, and 50×10^7 CFU/well of a 24-well plate, as described in 2.2.9.

5.2.1 Confocal microscopy of Tissue macrophages

Tissue macrophages were isolated and plated in chamber-slide wells in either GM-CSF, M-CSF or media alone as described in section 2.2.9.3. Cells were then incubated for up to 4h with *H. influenzae* or *S. pneumoniae* prior to being washed in PBS to remove any free labelled bacteria which was not taken up by cells. Cells were fixed and stained as described (2.2.9.3). Bacteria were heat killed and fluorescently labelled with Alexa fluor 488. Images were acquired using a Zeiss LSM-510 inverted confocal microscope.

5.2.2 Flow cytometry

5.2.2.1 Tissue macrophages

Tissue macrophages were isolated and cultured in GM-CSF, M-CSF or media alone for 6 days and then incubated with bacteria for 4h prior to washing in PBS to remove any free label, as described in section 2.2.9.2. Bacteria were heat killed and fluorescently labelled with Alexa fluor 405 (pacific blue channel). Cells were then incubated with live dead stain (APC channel) and fixed prior to analysing samples on the BD FACS Canto II. Cells were gated to exclude dead cells, and from the live cell population, phagocytosis was gated using a negative control. Gating strategy is shown in Figure 5.1.

5.2.2.2 MDM

Monocytes were isolated and cultured for 12 days in GM-CSF or M-CSF. On day 12, media was switched from GM-CSF to M-CSF and M-CSF to GM-CSF for a further 6 days. Control cells were cultured in the same media for the entire 18 days. Cells were analysed on days 0, 12 and 18 by incubating with bacteria for 4h, prior to incubation with live dead stain and fixed in 4% PFA. Samples were analysed on the BD FACS Canto II as described in section 2.2.9.2. Gating strategy is similar to tissue macrophages but with pHrodo-labelled bacteria (PE channel) and aqua live/dead stain (Pacific blue channel), and is shown in Figure 5.2.

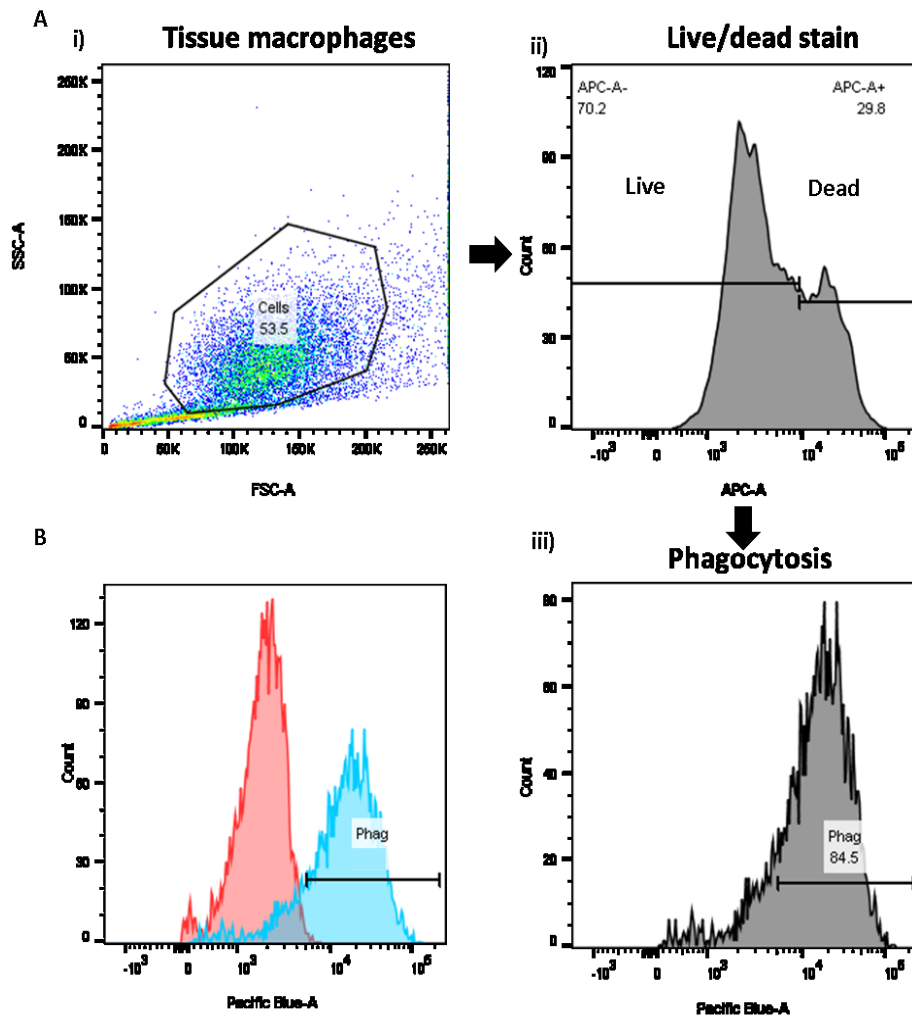


Figure 5. 1 Flow cytometry gating strategy for tissue macrophage phagocytosis. Cell population was gated based on forward and side scatter (**A i**), and any dead/apoptotic cells excluded based on uptake of APC - live/dead stain (**A ii**) which can only enter cells which have disrupted membranes. From the live populations, a control with no bacteria was used to set the gate for phagocytosis + cells (**A iii, B**). **B**) A representative image of **negative control** and **phagocytosis+** sample.

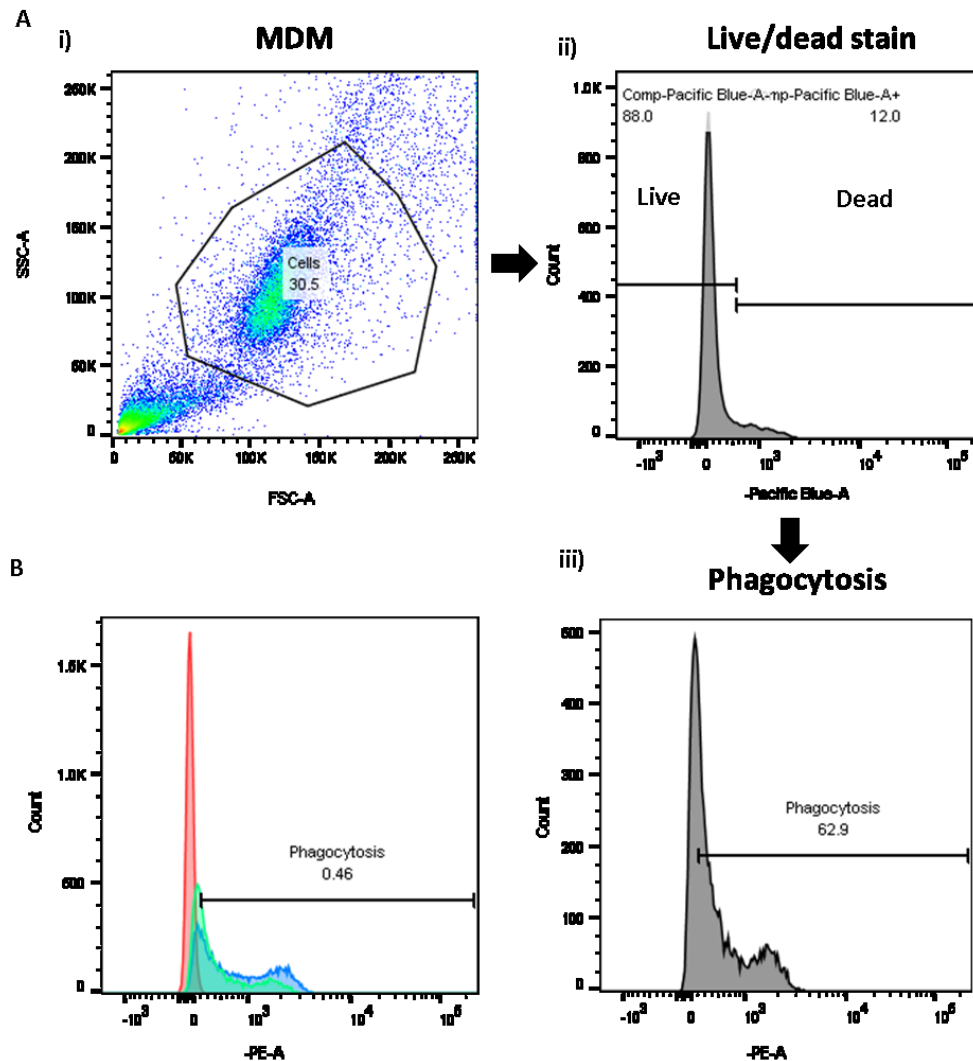


Figure 5. 2 Flow cytometry gating strategy for MDM phagocytosis. (A i) Cell population was gated based on forward and side scatter and **(A ii)** any dead/apoptotic cells excluded based on uptake of Alexa-fluor 405 - live/dead stain. From the live populations, **(A iii, B)** a control with no bacteria was used to set the gate for phagocytosis+ cells. **B)** A representative image of **negative control** and phagocytosis+ samples for *H. influenzae* and *S. pneumoniae*.

5.2.3 Statistical Analysis

All data were expressed as mean $\pm$ SEM, and analysis performed using GraphPad Prism software. Data were non-parametric, and comparisons between subject groups was performed using a two-way Anova with Bonferroni corrections. For tissue macrophages a Kruskal-Wallis with Dunn's post-test

was used to examine differences between culture conditions. In MDM, this test was used to examine differences between GM-CSF and M-CSF culture throughout differentiation as well as to compare differences between days 12 and 18 to day 0. A Wilcoxon matched-pairs signed ranks test was used to test significance between day 18 MDM that had media switched vs. unswitched media.

5.3 Results

5.3.1 Subject Demographics

5.3.1.1 Tissue Macrophages

Subject demographics for human lung tissue samples are shown in Table 5.1. All subjects were age matched and smokers and COPD patients had a significantly higher smoking history compared to non-smokers. In addition, COPD patients had significantly lower lung function results, measured by FEV₁, FEV₁ % predicted and FEV₁:FVC, compared to smokers and non-smokers.

	Non-smoker	Smoker	COPD
Sex (F:M)	4:0	2:3	6:5
Age	48.0±8.8	65.6±7.5	69.8±2.5
Smoking history (Pack years)	0.25±0.25	52.0±13.8 [#]	43.5±8.3 [*]
FEV ₁ (L)	2.7±0.2	2.6±0.3 ^{\$\$}	1.2±.2 [*]
FEV ₁ % predicted	94.4±3.0	101.0±8.4 ^{\$}	54.4±8.7
FVC (L)	3.1±0.2	3.5±0.4	2.4±0.2
FEV ₁ :FVC	0.9±0.0	0.7±0.0 ^{\$}	0.5±0.0 ^{**}

Table 5. 1 Subject demographics for tissue samples from non-smokers, smokers and COPD patients. All subjects underwent lung function tests. FEV₁: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes smoked per day for one year. Data shown are mean ±SEM. Kruskal-Wallis test with Dunn's post-test was used to test significance between subject groups, non-smokers vs. COPD ^{*}p<0.05, ^{**}p<0.01, non-smokers vs. smokers [#]p<0.05, and smokers vs. COPD patients ^{\$}p<0.05, ^{\$\$}p<0.01.

5.3.1.2 MDM

Table 5.2 shows subject demographics for all volunteers and patients who donated blood samples. All subjects were age matched, with smokers and COPD patients having a significantly higher smoking history compared to non-smokers. Furthermore, lung function from COPD patients was significantly lower compared to smokers and non-smokers, as seen by the FEV₁% predicted and FEV₁:FVC ratio.

	Non-smoker	Smoker	COPD
Sex (F:M)	3:3	2:4	2:3
Age	62.6±4.1	61.8±3.5	64.2±1.7
Smoking history (Pack years)	1.3±1.3	29.2±5.5 [#]	29.0±11.7*
FEV ₁ (L)	2.7±0.4	2.8±0.3	1.9±0.3
FEV ₁ % predicted	99.6±10.1	90.3±7.2	61.6±6.3*
FVC (L)	3.7±0.5	3.8±0.5	3.3±0.4
FEV ₁ :FVC	0.7±0.0	0.7±0.0	0.6±0.0*

Table 5. 2 Subject demographics for blood samples from non-smokers, smokers and COPD patients. All subjects underwent lung function tests. FEV₁: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes smoked per day for one year. Data shown are mean ±SEM. Kruskal-Wallis test with Dunn's post-test was used to test significance between subject groups, non-smokers vs. COPD *p<0.05, **p<0.01, smokers vs. non-smokers [#]p<0.05.

5.3.2 Phagocytosis of Bacteria by Tissue macrophages

5.3.2.1 Confocal microscopy

Confocal microscopy was performed to visually confirm that bacteria were taken up by macrophages and internalisation was occurring by using 3D Z-stacks. Tissue macrophages were incubated with bacteria, either *H. influenzae* or *S. pneumoniae*, for 30min, 1h or 4h. Internalisation of bacteria was observed after 30min and continued up to 4h for both bacterial species (Figures 5.3, 5.4). In addition, tissue macrophages cultured in GM-CSF, M-CSF or media were all capable of phagocytosis with some

cells becoming elongated and extending out in order to identify/capture bacteria. This elongation was not observed in control cells which were not exposed to prey, and therefore suggests these morphological changes are due to the presence of bacteria. Furthermore, although some cells appeared to take up several bacteria, others showed no fluorescence, indicating lack of uptake of bacteria (Figures 5.3, 5.4). In order to confirm internalisation of bacteria, 3D Z-stacks were performed, and images obtained demonstrate that bacteria were indeed taken inside the cells rather than remaining on the outside (Figure 5.5).

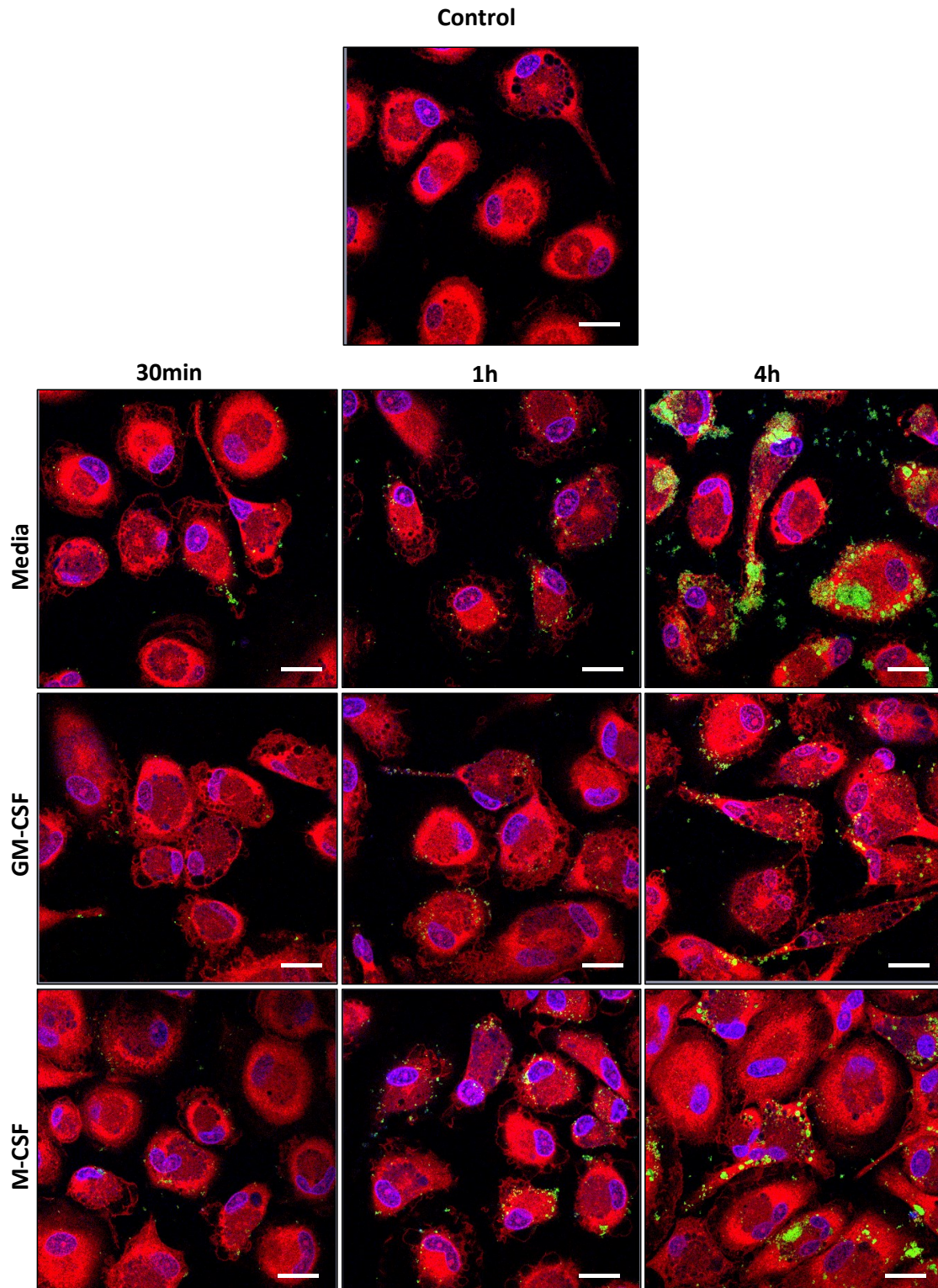


Figure 5. 3 Confocal images of tissue macrophages incubated with fluorescently labelled *H. influenzae*. Tissue macrophages were cultured in GM-CSF, M-CSF or media for 6 days and then incubated with *H. influenzae* for 30min, 1h or 4h. Cell tracker red (RED) was used to stain **cytoplasm**, DAPI (BLUE) to stain the **nucleus** and **bacteria** were labelled with Alexa fluor 488 (GREEN). Scale bar = 24µm. Representative images from 2 independent experiments.

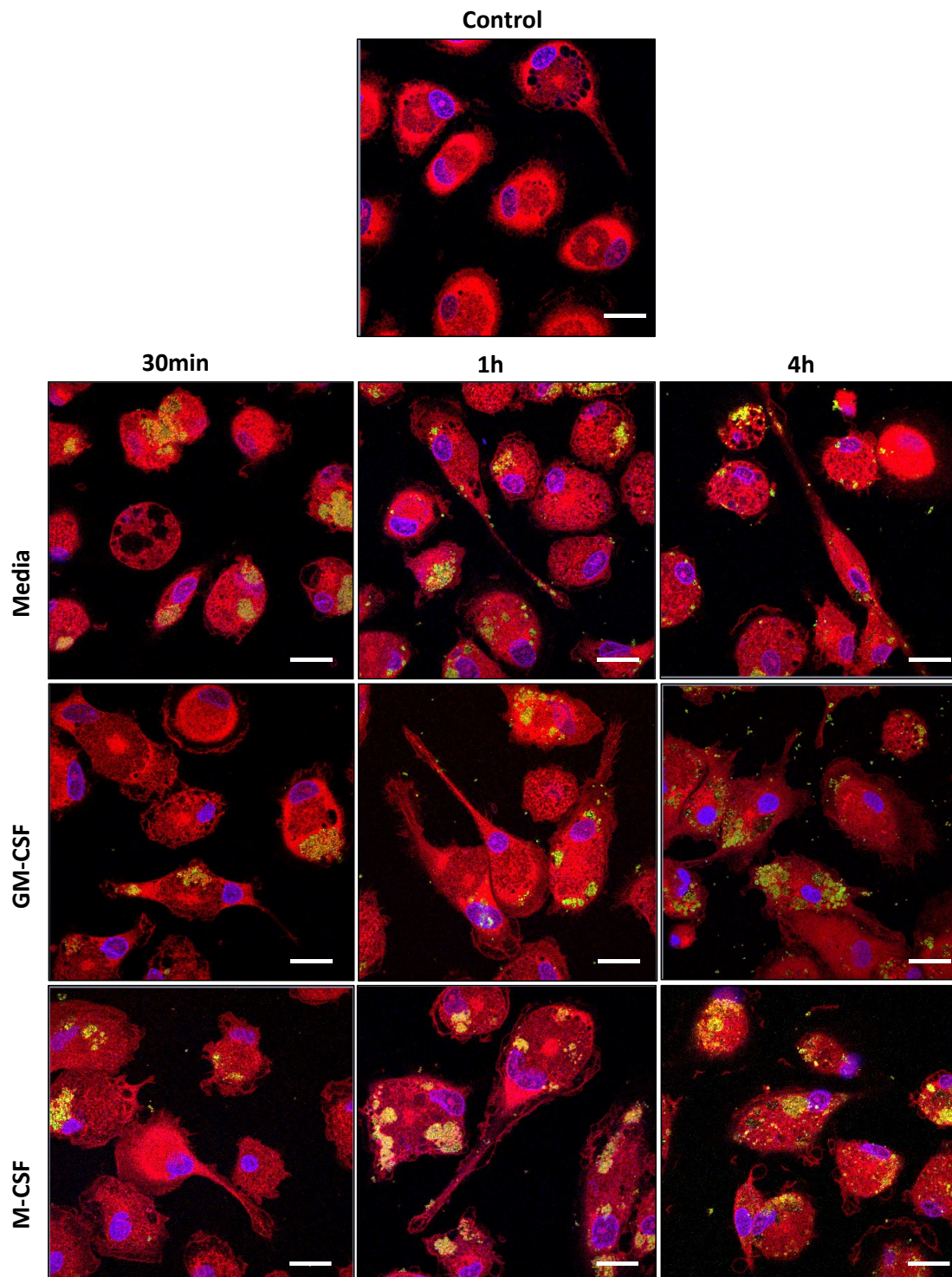


Figure 5. 4 Confocal images of tissue macrophages incubated with fluorescently labelled *S. pneumoniae*. Tissue macrophages were cultured in GM-CSF, M-CSF or media for 6 days and then incubated with *S. pneumoniae* for 30min, 1h or 4h. Cell tracker red (RED) was used to stain **cytoplasm**, DAPI (BLUE) to stain the **nucleus** and **bacteria** were labelled with Alexa fluor 488 (GREEN). Scale bar = 24µm. Representative images from 2 independent experiments.

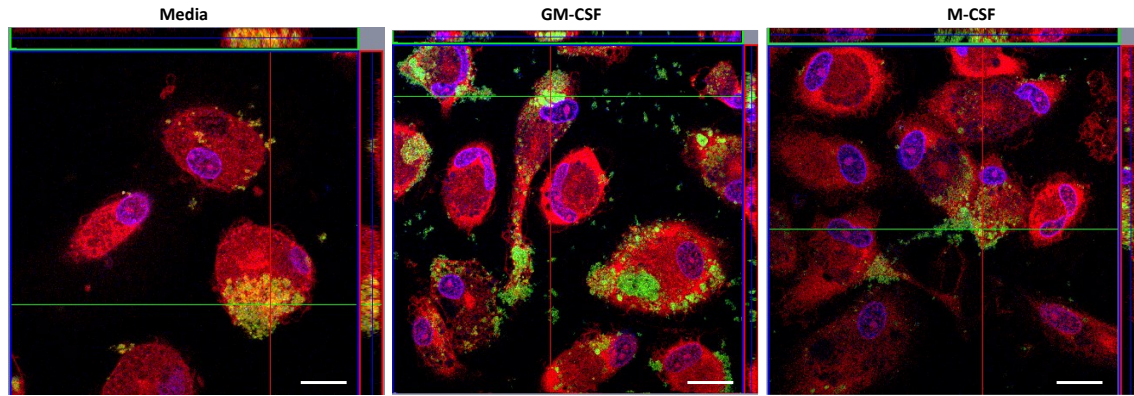


Figure 5.5 3D Z stacks of tissue macrophage phagocytosis. Z-Stacks taken at 4h incubation with *H. influenzae* for each of the culture condition. Cell tracker red was used to stain **cytoplasm**, **DAPI** to stain the **nucleus** and *H. influenzae* was labelled with Alexa fluor 488. Scale bar = 24µm. Representative images from 2 independent experiments.

5.3.2.2 Flow cytometry

Having visually observed phagocytosis of bacteria by tissue macrophages, and confirmed that bacteria were internalised by the cells, it was important to quantify phagocytosis and compare between subject groups, and between culture conditions, and this was performed using flow cytometry. In order to investigate any defects in COPD phagocytosis, two parameters were measured; percent live phagocytosis to measure how many cells were capable of phagocytosis and median fluorescence intensity (MFI) as a measure of how many bacteria were taken up per cell. When analysing the percentage of live cells able to phagocytose bacteria, there were no significant differences between subject groups or culture conditions for either bacterial species (Figure 5.6A). However, significantly fewer non-smoker cells phagocytosed *S. pneumoniae* on day 0 compared to smoker and COPD cells (Figure 5.6A ii), although this result may be due to the low number of non-smoker samples used. MFI was used as an indicator of how many bacteria were taken up per cells, and the data indicate that non-smoker cells could phagocytose a higher number of bacteria than smoker and COPD cells. This trend was observed on day 6 phagocytosis of *H. influenzae* and was significantly higher in non-smokers compared to smoker and COPD cells for phagocytosis of *S.pneumoniae* (Figure 5.6B). In addition, cells

from non-smokers were able to take up more bacteria on day 6 compared to day 0, and this was significantly higher for phagocytosis of *S. pneumoniae* (Figure 5.6B).

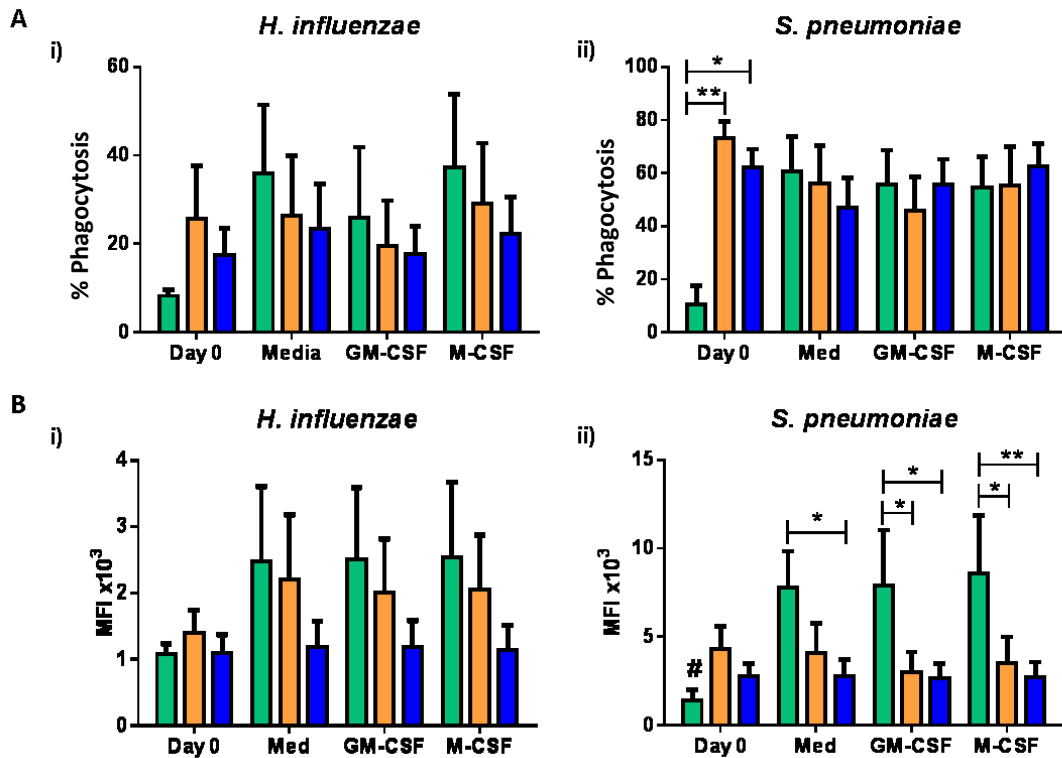


Figure 5. 6 Phagocytosis of bacteria by tissue macrophages. Tissue macrophages were isolated from **non-smokers (n=3)**, **smokers (n=5)** and **COPD patients (n=9)** and cultured for 6 days in media, GM-CSF or M-CSF. Cells were incubated for 4h with either i) *H. influenzae* or ii) *S. pneumoniae*, and phagocytosis assessed using flow cytometry to measure **A**) % phagocytosis and **B**) MFI. Data are shown as mean $\pm$ SEM. A two-way Anova with Bonferoni post-test was used to test significance between subject groups * $p < 0.05$, ** $p < 0.01$ and a Kruskal-Wallis with Dunn's post-test to compare significance between day 0 and day 6 # $p < 0.05$.

5.3.3 Phagocytosis of bacteria by MDM

5.3.3.1 Phagocytosis Throughout Differentiation

In order to compare tissue macrophages with the MDM model, phagocytosis was analysed throughout differentiation, by incubating cells with bacteria on days 0, 12 and 18 and measuring phagocytosis

using flow cytometry. Bacteria used for the MDM model were labelled with pHrodo dye which is non-fluorescent at neutral pH but fluoresces brightly under acidic conditions, such as that of a phagolysosome and thus ensures only internalised bacteria were measured. In a similar process as with tissue macrophages, MDM phagocytosis was measured as percent phagocytosis and MFI. Analysis demonstrated a trend showing fewer COPD cells capable of phagocytosis (measured as % phagocytosis) compared to non-smokers and smokers, although this difference did not reach significance. This trend could be observed for cells culture in GM-CSF and M-CSF throughout differentiation, and for both bacteria used (Figure 5.7). MFI was also measured to determine differences in the number of bacteria taken up per cell. When incubated with *H. influenzae*, both GM-MDM and M-MDM from COPD patients took up significantly more bacteria on day 18 compared to non-smokers and smokers (Figure 5.8 Ai, Bi). Furthermore, COPD MDM phagocytosed significantly more *H. influenzae* on day 18 compared to day 0, while there was no difference in MFI between days 12 and 18 compared to day 0 in non-smoker and smoker cells (Figure 5.8 Ai, Bi). In addition, when cells were incubated with *S. pneumoniae*, there were no differences in MFI between subject groups or between days 12 or 18 compared to day 0 (Figure 5.8 Aii, Bii). When comparing GM-MDM to M-MDM, there was no difference in % phagocytosis or MFI for either bacteria investigated.

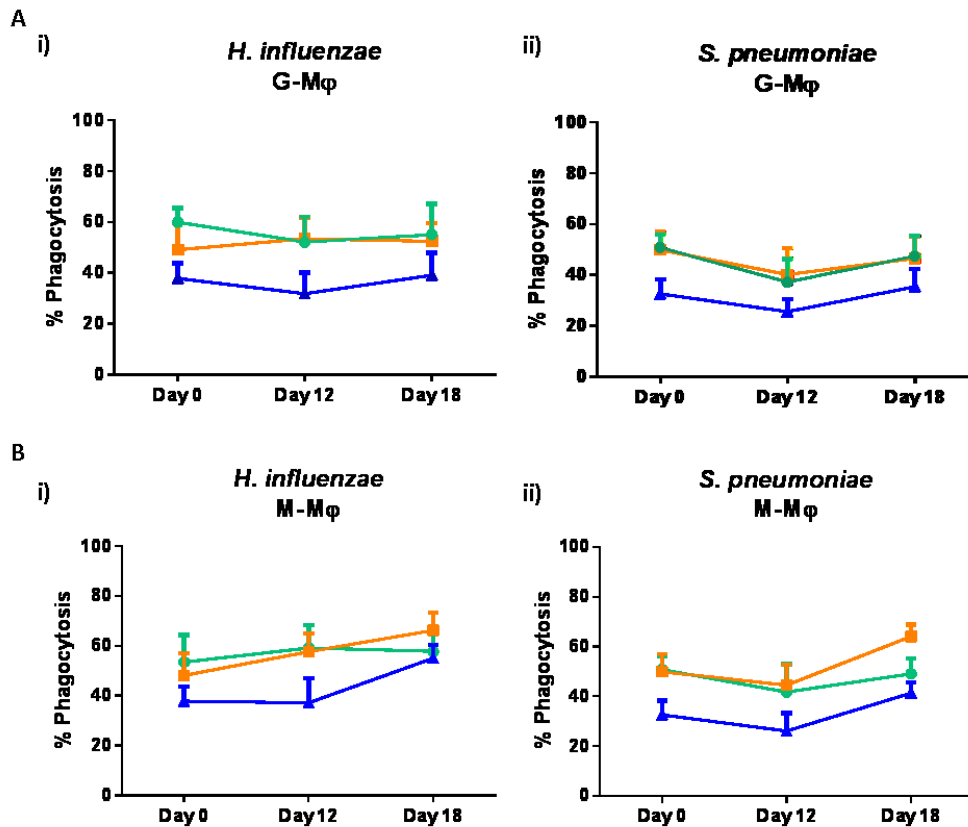


Figure 5.7 % Phagocytosis of bacteria by MDM throughout differentiation. Monocytes were isolated from **non-smokers (n=5)**, **smokers (n=5)** and **COPD patients (n=5)** and cultured for 18 days in **A)** GM-CSF or **B)** M-CSF. Cells were incubated for 4h on days 0, 12 and 18 with either i) *H. influenzae* or ii) *S. pneumoniae*, and % phagocytosis assessed using flow cytometry. Data are shown as mean $\pm$ SEM.

5.3.3.2 Effect of GM-CSF and M-CSF on phagocytosis

In order to investigate the effect of culture conditions on phagocytosis, cells were cultured in either GM-CSF or M-CSF for 12 days and then media switched for a further 6 days. Control cells were maintained in the same media throughout the 18 days to establish differences between control and switched cells. Phagocytosis was assessed using flow cytometry on day 18 by measuring percent phagocytosis and MFI (Figures 5.9 and 5.10). There were no differences in percent phagocytosis or

MFI following switching in any of the subject groups or with either bacteria tested. This was regardless of which media was switched, GM-CSF → M-CSF or M-CSF → GM-CSF.

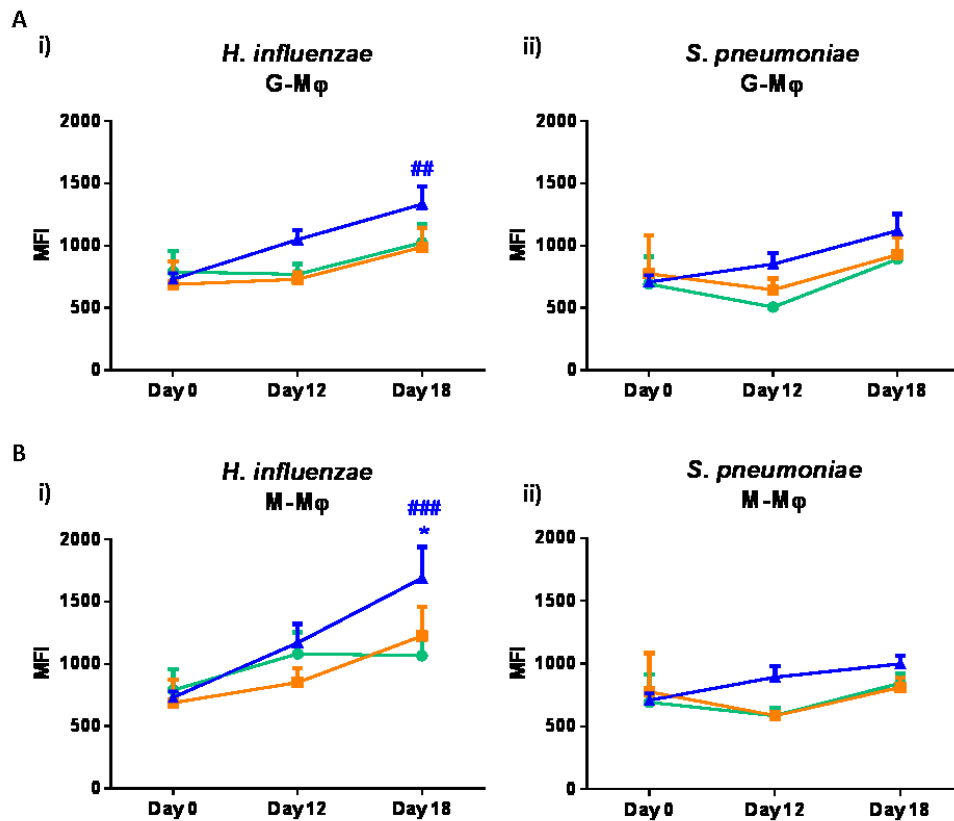


Figure 5. 8 Phagocytosis of bacteria by MDM throughout differentiation. Monocytes were isolated from **non-smokers (n=5)**, **smokers (n=5)** and **COPD patients (n=5)** and cultured for 18 days **in A)** GM-CSF or **B)** M-CSF. Cells were incubated for 4h on days 0, 12 and 18 with either i) *H. influenzae* or ii) *S. pneumoniae*, and MFI measured using flow cytometry. Data are shown as mean ±SEM. Two-way Anova with Bonferonni post-test was used to determine significant differences between subject groups *p<0.05 (vs non-smoker), and a Kruskal-wallis with Dunn's post-test to determine differences between day 12 and 18 compared to day 0, ^{##}p<0.01, ^{###}p<0.001.

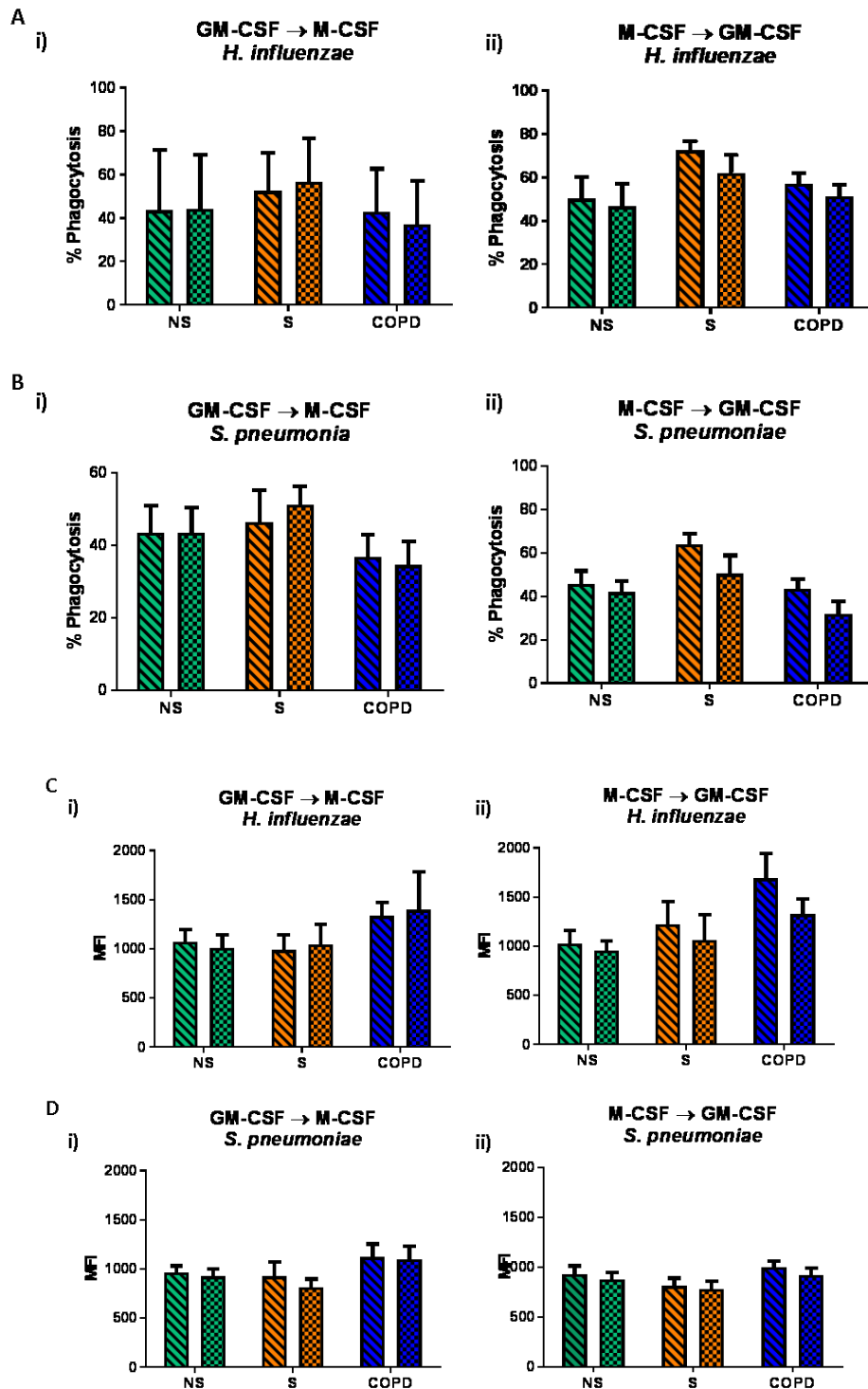


Figure 5. 9 Effect of switching media on MDM phagocytosis of bacteria. Monocytes were isolated from **non-smokers (n=5)**, **smokers (n=5)** and **COPD patients (n=5)** and cultured for 12 days in i) GM-CSF or ii) M-CSF, prior to switching media for a further 6 days. Control cells remained in the same media for 18d. Control cells are shown with dashes and switched cells are chequered. Cells were incubated for 4h on day 18 with either **A, C)** *H. influenzae* or **B, D)** *S. pneumoniae*, and **A, B)** % phagocytosis and **C, D)** MFI measured using flow cytometry. Data are shown as mean $\pm$ SEM.

5.4 Discussion

5.4.1 Defects in Phagocytosis by COPD Macrophages

The ability of macrophages to clear particulates and pathogenic microorganisms from the airways is essential as a first line of defence in preventing infections. Macrophages act as sentinels, detecting foreign objects which are subsequently engulfed and destroyed (Vecchiarelli et al., 1991, Taylor et al., 2010). This role is not limited to foreign objects, but also to the uptake of apoptotic cells, which is termed efferocytosis. This process relies on similar pathways as phagocytosis and is required to prevent the build-up of necrotic material (Donnelly and Barnes, 2012). Cigarette smoking leads to continued influx of particulates and tar into the lung, and has been shown to have a detrimental effect on the ability of macrophages to perform phagocytosis and efferocytosis (Hodge et al., 2007). There is increasing evidence that COPD macrophages are dysfunctional in their ability to engulf microorganisms including *C. albicans*, *H. influenzae*, *S. pneumoniae*, *E.coli* as well as inert particles (Arredouani et al., 2005, Berenson et al., 2006, Pang et al., 2014, Taylor et al., 2010). While most studies performed in COPD alveolar macrophages have shown a defect in phagocytosis, those performed on MDM have not consistently shown differences between COPD and healthy macrophages. Whether the defect observed is the result of environmental insult within the lung, or an inherent defect in monocytes therefore remains to be shown.

Results obtained in this study, demonstrate a clear defect in tissue macrophages in their ability to phagocytose heat-killed bacteria, regardless of whether these were gram negative or gram positive. This is supported by previous work by Taylor *et al.* who demonstrated similar results using a fluorimeter to detect levels of phagocytosis in tissue macrophages (Taylor et al., 2010), as well as by several other studies performed on alveolar macrophages (Vecchiarelli et al., 1991, Arredouani et al., 2005). In order to further understand how macrophages are defective, flow cytometry was used to measure the percentage of cells able to phagocytose, and also the relative number of bacteria taken up per cell, measured by MFI. While there was no difference in the number of cells able to phagocytose

between the subject groups for either bacteria, significantly fewer non-smoker cells phagocytosed *S. pneumoniae* on day 0 compared to smoker and COPD cells. This may be due to a low number of non-smoker samples used in the experiments, or could represent an effect of the cell isolation process, and a lack of recovery by non-smoker cells, as phagocytosis levels were significantly higher on day 6. Cells isolated from smokers and COPD patients may not require as much recovery time as those isolated from non-smokers as they are in a constant state of activation due to continued smoking and high levels of oxidative stress found in the lungs. Median fluorescence intensity was used as an indirect measure of how many bacteria could be engulfed by one cell, and showed that COPD and smoker cells appeared to take up fewer *H. influenzae* per cell, and significantly fewer *S. pneumoniae* compared to non-smokers. This difference was apparent on day 6 but not day 0. Again, this may be due to cells not fully recovering from the process of cell isolation, and thus not performing at their optimal ability on day 0. In addition, since this pattern was observed for both gram positive, and gram negative bacteria, it suggests that this defect is not pathway specific, or the results of dysfunction in one type of receptor. Confocal microscopy was also used to observe phagocytosis, and confirm internalisation of bacteria, and could be used to validate data obtained by flow cytometry. However, due to low number of repeats, images were not quantified.

These data suggest that there is no defect in the ability of tissue macrophages to initially recognise and engulf bacteria, but rather there is a defect in the number of bacteria they can take up. This may be due to an inability of cells to recycle scavenger receptors or due to early satiety caused by previous uptake of other particulate matter as a result of air pollution and cigarette smoking. This is supported by evidence that cells from current smokers exhibit a similar decline in MFI as COPD cells compared to non-smokers, suggesting environmental factors within the lung may, in part, drive a phagocytic defect. Furthermore, there is evidence to suggest that COPD patients who are current smokers have worse phagocytic ability compared to ex-smokers (Hodge et al., 2007).

In order to determine whether this phagocytic defect was purely the result of environmental factors within the lung, or additional inherent defects in COPD cells are important, the MDM model was used to analyse phagocytosis. Literature on this topic is controversial with some results indicating reduced phagocytosis in COPD MDM (Vecchiarelli et al., 1991, Taylor et al., 2010) and others finding no difference between subject groups (Berenson et al., 2006). Lack of consensus may be due to different techniques used to culture MDM and perform phagocytosis assays. In order to compare results obtained from MDM to those from tissue macrophages, phagocytosis was assessed using flow cytometry. In addition, the novel pH-dependent fluorescence label, pHrodo, was used to prevent measuring extracellular bound bacteria. Due to limited tissue samples, it was not possible to perform phagocytosis using pHrodo labelled bacteria on tissue macrophages.

While there was no significant difference in the percentage of MDM performing phagocytosis between subject groups, there was a trend showing decreased number of COPD GM-MDM and M-MDM capable of performing phagocytosis compared to non-smoker and smoker cells. In contrast to results obtained from tissue macrophages, COPD cells had significantly greater MFI when incubated with *H. influenza* on day 18 compared to day 0 as well as compared to non-smoker cells. These data suggest that fewer COPD MDM were able to perform phagocytosis, which may lead to the remaining cells, which are actively taking up bacteria, to take up more bacteria per cells as a compensation mechanisms.

Taken together, these data indicate that there may be a dual mechanism of dysfunction occurring in COPD macrophages. Data obtained from tissue macrophages clearly demonstrated that COPD and smoker cells were less able to take up as many bacteria as cells from non-smokers, and this may be because they are already full of other particulate matter and pathogens which are known to be elevated in the lungs of COPD patients. It is also possible that scavenger receptors, such as MARCO which has been implicated in COPD, are expressed at lower levels, or are not recycled as efficiently and therefore only few bacteria are taken in per cell (Arredouani et al., 2005). On the other hand,

MDM cells show a distinct defect, with fewer COPD cells capable of phagocytosis, but those which are actively engulfing are able to take up as many bacteria, if not more, as non-smoker cells. This suggests that monocytes may be affected by the systemic effects of smoking, leading to inherent defects as a result of potential epigenetic changes which prevents some cells from being able to recognise/uptake bacteria. MDM from smokers behaved similarly to those from non-smokers suggesting that these inherent defects in COPD cells may separate smokers who remain healthy from those that develop COPD.

5.4.2 Manipulating Phagocytosis by GM-CSF and M-CSF Culture

Environmental cues and stimuli have been shown to drive monocyte differentiation and also to alter macrophage responses (Stout and Suttles, 2004). GM-CSF drives cells to become pro-inflammatory and release higher levels of TNF α in response to LPS stimulation which is used as a surrogate for bacterial stimulus, and recruits additional macrophages and neutrophils to combat infection (Porcheray et al., 2005, Day et al., 2014). As such, GM-CSF is crucial for the correct response to infection. On the other hand, M-CSF cultured cells are characterised as resolving, and thus implicated in the clearance of apoptotic cells, bacteria and particulate matter (Taylor and Gordon, 2003), and thus may represent a more phagocytic population of macrophages. This chapter investigated the effect of GM-CSF and M-CSF culture on both tissue macrophages and MDM to determine whether 1) mature cells could be manipulated to improve phagocytosis in COPD and 2) to establish whether GM-MDM or M-MDM were associated with greater phagocytic ability.

There was no effect of culture on phagocytosis of either bacteria by tissue macrophages. However, non-smokers consistently showed lower levels of phagocytosis and MFI on day 0 compared to day 6, whereas smokers and COPD cells had similar levels between both days. This suggests that non-smoker cells are more sensitive to the process of cell isolation and therefore require longer to recover and regain phagocytic function. However, GM-CSF or M-CSF did not have any effect on phagocytosis compared to cells cultured in media alone, which may indicate a shared functional phenotype

between both cell populations in terms of phagocytosis. In a similar manner, monocytes cultured in GM-CSF or M-CSF for 12 days showed similar levels of phagocytosis and MFI throughout differentiation which remained the same following switching of media for 6 days. Taking into consideration the fact that phagocytosis levels were similar between GM-MDM and M-MDM on day 12, it is reasonable that switching of media would have no effect on phagocytosis.

5.4.3 Conclusions

COPD macrophages consistently show a defect in phagocytosis, in both tissue macrophages and MDM which suggests that multiple factors are driving decreased phagocytosis in COPD. In tissue macrophages, this appears to be due to cells taking up fewer bacteria, whereas in MDM fewer cells are capable of phagocytosis compared to non-smokers. This combination of environmental factors within the lung, and systemic effects on monocytes represents dual mechanisms to be targeted in order to prevent and treat COPD exacerbations. Investigating expression levels of scavenger receptors in tissue macrophages as well as potential epigenetic changes which may be driving inherent defects in monocytes could help to elucidate why COPD cells are less able to phagocytose bacteria, and other microorganisms which are detrimental to the health of COPD patients.

CHAPTER 6: MOLECULAR MECHANISMS UNDERLYING MACROPHAGE PHENOTYPE IN COPD

6.1 Introduction

Previous chapters have investigated functional differences between non-smoker, smoker and COPD macrophages, and demonstrated that COPD cells show increased release of pro-inflammatory cytokines, MMP9 activity as well as an inability to phagocytose as effectively as cells from non-smokers. Furthermore, the effects of growth factors such as GM-CSF and M-CSF highlighted that cell plasticity is selective, and while cytokine output could be manipulated, MMP9 activity and phagocytosis remained unchanged. While MDM from non-smokers and smokers became less inflammatory in M-CSF culture, with decreased TNF α and CXCL8 release, COPD MDM did not change. On the other hand, GM-CSF culture drove cells towards a more pro-inflammatory phenotype in MDM and tissue macrophages from all subjects.

Recent studies have focused on understanding molecular, transcriptional and epigenetic changes responsible for macrophage polarisation and dysfunction, but data remains lacking in a disease setting (Davies et al., 2013). A recent study by Xue *et al.* performed extensive analysis on human macrophage activation states, and demonstrated that using 28 stimuli resulted in a spectrum of macrophage activation states, each with distinct transcriptional profiles. Furthermore, transcriptional profiles of alveolar macrophages isolated from non-smokers, smokers and COPD patients showed enrichment of distinct pathways, highlighting differences in cell plasticity between health and disease (Xue et al., 2014). This chapter therefore investigated expression levels of the receptors for GM-CSF and M-CSF as well as transcription factors implicated in determining macrophage lineage, in order to better understand drivers of cell phenotype, and their role in disease.

GM-CSF and M-CSF are key growth factors in the differentiation of macrophages, and are used extensively *in vitro* to drive cell polarisation (Porcheray et al., 2005, Stout and Suttles, 2004). However,

the corresponding receptors, CSF2R α and CSF1R respectively, and their expression levels on macrophages have not been fully investigated in the context of either health or disease. Considering the importance of these growth factors in macrophage differentiation and function, it is imperative to determine whether changes in receptor expression could be behind some of the phenotypic differences observed in macrophages, as well as their importance in COPD. In addition to growth factors, several transcription factors have been shown to play a role in macrophage differentiation, with expression patterns changing during monocyte-macrophage transition, including *KLF4*, *RELA*, *RFX1* and *SREBF1* (Wallner et al., 2016). PU.1 is a key transcription factor implicated in macrophage lineage, and recently *KLF4* has been shown to be under its control, and to play an important role in macrophage differentiation as well as in driving resolving cell phenotype (Liao et al., 2011, Saeed et al., 2014).

The ability of macrophages to adapt to changes in their environment has been linked to rapid transcriptional changes and epigenetic modifications (Alder et al., 2008, Gosselin and Glass, 2014). Therefore, understanding which genes are selectively activated or inhibited during macrophage differentiation, as well as in response to stimuli and whether these respond differently in COPD could aid in identifying key therapeutic targets (Wallner et al., 2016).

In order to investigate the role of epigenetic modifications on macrophage phenotype and in health and disease, the demethylase inhibitor, GSK-J4 was used (Kruidenier et al., 2012). GSK-J4 is a selective inhibitor of demethylases Jumanji (JMJD)-3 and UTX which are members of the KDM6 family responsible for demethylation of H3K27, resulting in 'opening' of chromatin and allowing gene transcription (Kruidenier et al., 2012, Heinemann et al., 2014). GSK-J4 was shown to inhibit TNF α release from human primary macrophages with an IC₅₀ of 9 μ M, with no significant effect on other histone demethylases (Kruidenier et al., 2012). However, a subsequent study by Heinemann *et al* demonstrated that while GSK-J4 preferentially inhibited KDM6 demethylases, it was also capable of some inhibition of the KDM5 family (Heinemann et al., 2014).

Histone methylation results in 'closing' of chromatin, and therefore inhibits gene transcription. Macrophages have been shown to have more 'open' chromatin compared to monocytes, with lower levels of H3K27 methylation (Wallner et al., 2016). Inhibition of histone demethylases should result in preventing *de novo* gene transcription of genes under the control of JMJD3 and UTX, many of which are important in the inflammatory response. JMJD3 is upregulated in activated macrophages via NFκB signalling, and is recruited to the start sites of over 70% of all genes induced by LPS stimulation (Kruidenier et al., 2012). Thus, treating macrophages with GSK-J4 may result in a decrease in inflammatory response, and alter monocyte differentiation to a more anti-inflammatory phenotype.

6.1.1 Hypothesis

1. GM-CSF and M-CSF differentiated cells will show differential expression of receptors and transcription factors that drive cell polarisation.
2. Treatment of macrophages with GSK-J4 will reduce inflammatory phenotype in tissue macrophages and alter MDM phenotype when cells are differentiated in the presence of inhibitor.

6.1.2 Aims

1. Establish whether specific receptors and/or transcription factors are important in monocyte to macrophage differentiation, and whether they are associated with certain phenotype.
2. Investigate expression levels of *GSFR2α*, *CSFR1*, *KLF4*, *RELA*, *RFX1* and *SREBF1* between subject groups in tissue macrophages and MDM.
3. Treat tissue macrophages and MDM with GSK-J4 to determine the effect on cell plasticity and monocyte differentiation.

6.2 Methods

6.2.1 Gene Expression

6.2.1.1 Tissue Macrophages

Tissue macrophages were isolated and cultured in media, GM-CSF or M-CSF for 6 days. Samples were collected on day 0 and 6 for RNA extraction. Gene expression was analysed using RT-qPCR as described in section 2.2.4.

6.2.1.2 MDM

Monocytes were isolated and cultured for 12 days in GM-CSF or M-CSF. On day 12, media was switched from GM-CSF to M-CSF or M-CSF to GM-CSF for a further 6 days. Control cells remained in the same conditions for 18 days. Samples were collected on days 0, 12 and 18 to assess gene expression of transcription factors, as described in section 2.2.4.

6.2.2 ELISA Concentration Response to Histone Demethylase Inhibitors

6.2.2.1 GSK-J4 Concentration Response in MDM

Monocytes from non-smokers were isolated and cultured for 12 days in GM-CSF prior to 24h incubation with GSK-J4 (1-100 μ M). Following 24h incubation, cells were stimulated with LPS (10ng/ml) in the presence of GSK-J4 for a further 24h. An untreated control and LPS stimulation control were included as well as a vehicle control (0.01% DMSO) to ensure any effect observed was due to GSK-J4. After 24h, supernatant was collected and cytokines measured using ELISA.

6.2.2.2 GSK-J4 Concentration Response in Tissue Macrophages

Tissue macrophages were isolated and cultured in media, GM-CSF and M-CSF for 6 days. Cells were treated with GSK-J4 (1-25 μ M) on day 0 and day 3. Untreated controls were maintained in parallel. On day 6, cells were stimulated with LPS (10ng/ml) in the presence of GSK-J4 for 24h, prior to collection of supernatant for the measurement of cytokines using ELISA.

6.2.2.3 Cell toxicity assay

Cell viability was assessed for all samples used for ELISA. Following collection of supernatant, cells were incubated with MTT and cell viability assessed as described in section 2.2.8.

6.2.3 GSK-J4 Treatment of MDM

Monocytes were cultured for 12 days in either GM-CSF alone or in the presence of GSK-J4. GSK-J4 was added to monocytes on day 0, day 4 or day 8, and every subsequent media change was replenished with fresh GSK-J4. All samples were collected on day 12, so the cells had been in the presence of GSK-J4 for 12 days, 8 days or 4 days (Figure 6.1). An untreated control was cultured in GM-CSF alone and collected on day 12.

6.2.3.1 Gene expression

Following treatment with GSK-J4, all samples were collected on day 12 and RNA extracted for gene expression as described in section 2.2.4.

6.2.3.2 Phagocytosis

Phagocytosis was assessed on day 12 by incubating cells with heat-killed, pHrodo labelled *S. pneumoniae* for 4h prior to staining cells with live/dead stain and assessing phagocytosis using flow cytometry as described in section 2.2.9.2.

6.2.4 GSK-J4 Treatment of Tissue Macrophages

Tissue macrophages were cultured for 6 days in media, GM-CSF or M-CSF either in the presence or absence of GSK-J4. GSK-J4 was added to cells on day 0 and day 3 and samples collected on day 6 (Figure 6.1).

6.2.4.1 Gene Expression

Samples were collected on day 0, and day 6 following treatment with GSK-J4, and RNA extracted for gene expression analysis as described in section 2.2.4.

6.2.4.2 Phagocytosis

Following treatment with GSK-J4 for 6 days, cells were incubated with Alexa fluor-405 labelled *S. pneumoniae* for 4h prior to staining with live/dead stain and analysing phagocytosis using flow cytometry as described in section 2.2.9.2.

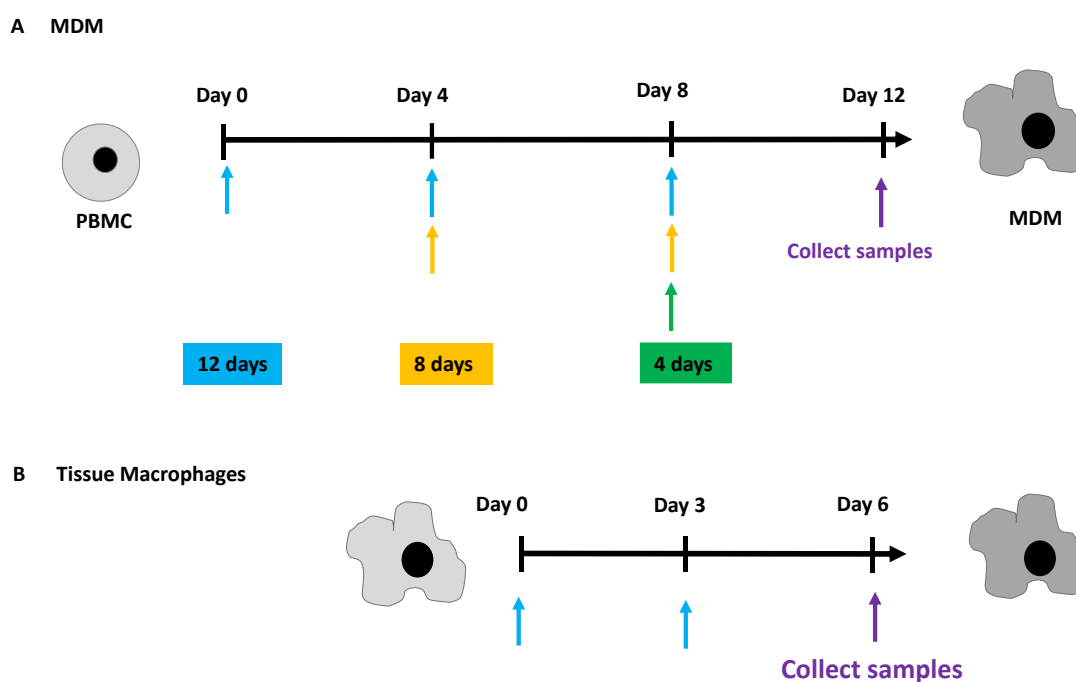


Figure 6. 1 Timeline of GSK-J4 treatment on MDM and tissue macrophages. A) Monocytes from non-smokers were cultured for 12 days in GM-CSF. GSK-J4 was added to cells on day 0 (and replenished on day 4 and 8). Non-treatment control was replenished with GM-CSF and all samples collected on day 12. Samples collected are shown by the number of days they were exposed to GSK-J4 (12, 8, 4 or control). **B)** Tissue macrophages were cultured in media, GM-CSF or M-CSF for 6 days in the presence or absence of GSK-J4, which was added on day 0 and 3, and samples collected on day 6.

6.3 Results

6.3.1 Subject Demographics

6.3.1.1 Tissue Macrophages

Subject demographics for tissue samples are shown in table 6.1. Due to low number of samples available, no statistical differences were found between smokers and COPD patients. However, both groups have over 20 pack year history, and COPD patients have lung function data corresponding to GOLD classification of COPD (FEV_1 % predicted <80% and $FEV_1:FVC$ ratio <0.7) with significantly lower lung function, measured by $FEV_1:FVC$, compared to smokers (GOLD, 2016).

	Smoker	COPD
Sex (F:M)	2:2	4:1
Age	66.5±3.3	65.8±4.1
Smoking history (Pack years)	43.5±14.8	59.3±13.4
FEV_1 (L)	2.3±1.7	1.7±0.3
FEV_1 % predicted	98.3±9.0	67.5±14.5
FVC (L)	3.4±0.2	3.1±0.6
$FEV_1:FVC$	0.7±0.0	0.5±0.0*

Table 6. 1 Subject demographics and lung function for tissue samples from non-smokers, smokers and COPD patients. All subjects underwent lung function tests. FEV_1 : Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for one year. Mann-Whitney test was performed to test for differences between subject groups, * $p < 0.05$.

6.3.1.2 MDM

Blood samples used to investigate gene expression levels are shown in table 6.2. All subjects were age matched, with smokers and COPD patients having significantly higher smoking history than non-

smokers. In addition, lung function of COPD patients was significantly lower compared to non-smokers and show a decline compared to healthy smokers (Table 6.2). All studies investigating the effect of histone demethylase inhibitors were performed on non-smoker samples, and demographics are shown in table 6.3. All donors had normal lung function and had no history of smoking.

	Non-smoker	Smoker	COPD
Sex (F:M)	3:3	1:4	1:5
Age	59.0±1.4	57.8±2.1	67.3±3.5
Smoking history (Pack years)	0.0±0.0	40.5±10.7*	48.3±6.9**
FEV₁ (L)	3.0±0.4	3.1±0.4	2.0±0.2
FEV₁ % predicted	106.4±4.4	94.9±5.8	66.5±3.4**
FVC (L)	4.0±0.6	4.4±0.6	3.7±0.5
FEV₁:FVC	0.8±0.0	0.7±0.0	0.6±0.1*

Table 6. 2 Subject demographics and lung function for blood samples from non-smokers, smokers and COPD patients. All subjects underwent lung function tests. FEV₁: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for one year. Kruskal-wallis with Dunn's post-test was performed to measure differences between subject groups compared to non-smokers *p<0.05, **p<0.01.

	Non-smoker
Sex (F:M)	8:2
Age	64±3.1
Smoking history (Pack years)	0.0±0.0
FEV₁ (L)	2.5±0.2
FEV₁ % predicted	97.8±3.7
FVC (L)	3.4±0.2
FEV₁:FVC	0.7±0.0

Table 6. 3 Subject demographics and lung function for blood samples from non-smokers used for GSK-J4 experiments. All subjects underwent lung function tests. FEV₁: Forced Expiratory Volume in 1 second. FVC: Forced Vital Capacity.

6.3.2 Gene Expression of GM-CSF and M-CSF receptors

6.3.2.1 MDM Differentiation and Effect of Changing Culture Conditions

In order to understand whether expression of the receptors for growth factors known to regulate macrophage phenotype and plasticity, were altered in COPD, gene expression of the GM-CSF and M-CSF receptors, *CSF2Rα* and *CSF1R* respectively, were assessed in MDM from all subjects, differentiated in either GM-CSF or M-CSF. Expression of both *CSF2Rα* and *CSF1R* appeared to decrease from monocytes on day 0 to MDM on days 12 and 18, and expression levels were significantly reduced in cells from smokers on day 12 and 18 compared to day 0 (Figure 6.2). In addition, there was no difference between cells cultured in GM-CSF compared to M-CSF and no differences in *CSF2Rα* expression between subject groups. However, *CSF1R* expression was significantly higher in monocytes from smokers compared to those from non-smokers or COPD patients on day 0, although this

difference was lost in MDM on days 12 and 18, with no differences between subject groups (Figure 6.2B). MDM media was switched on day 12 from GM-CSF to M-CSF or M-CSF to GM-CSF for a further 6 days and gene expression of the receptors analysed. Data from these experiments indicate that changing culture conditions had no effect on *CSF2Rα* or *CSF1R* gene expression in any of the subject groups (Figure 6.3).

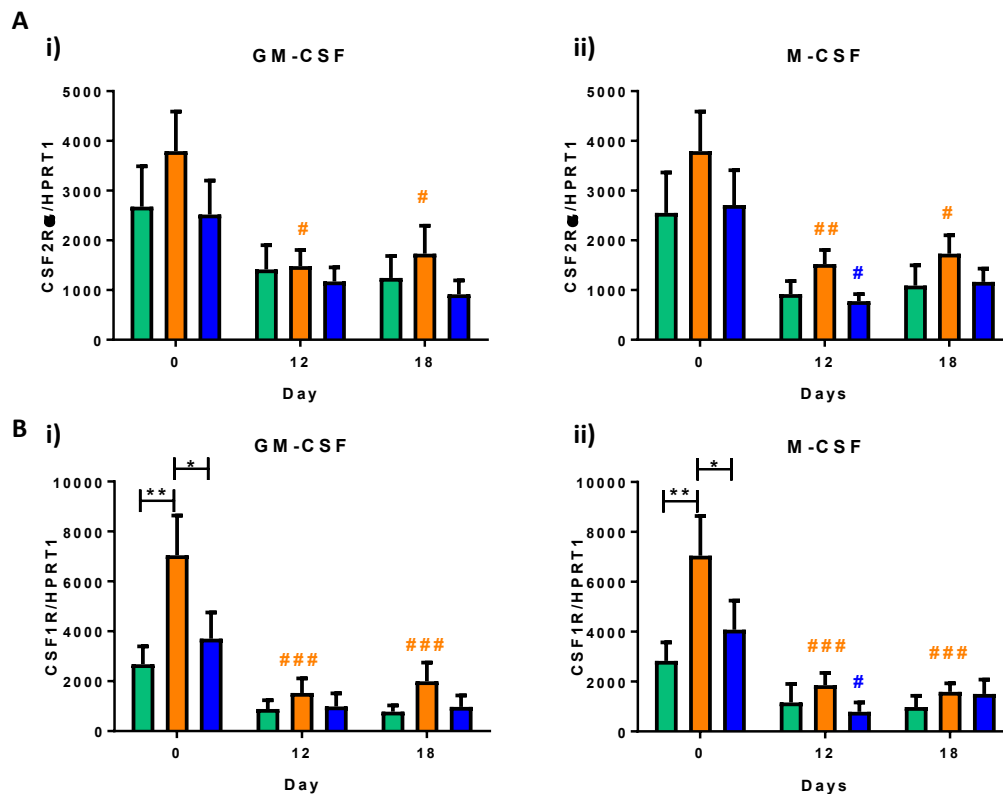


Figure 6. 2 Gene expression of *CSF2Rα* and *CSF1R* throughout macrophage differentiation. Monocytes were isolated from **non-smokers (n=5)**, **smokers (n=5)** and **COPD patients (n=5)** and cultured for 18 days in **i)** GM-CSF or **ii)** M-CSF and samples collected on days 0, 12 and 18 for RNA extraction. Gene expression was analysed using qPCR for **A)** *CSF2Rα* and **B)** *CSF1R*. A two-way Anova with Bonferroni post-test was used to test differences between days 12 and 18 compared to day 0, #p<0.05, ##p<0.01, ###p<0.001 and a Kruskal-Wallis with Dunn's post-test used to compare subject groups *p<0.05, **p<0.01.

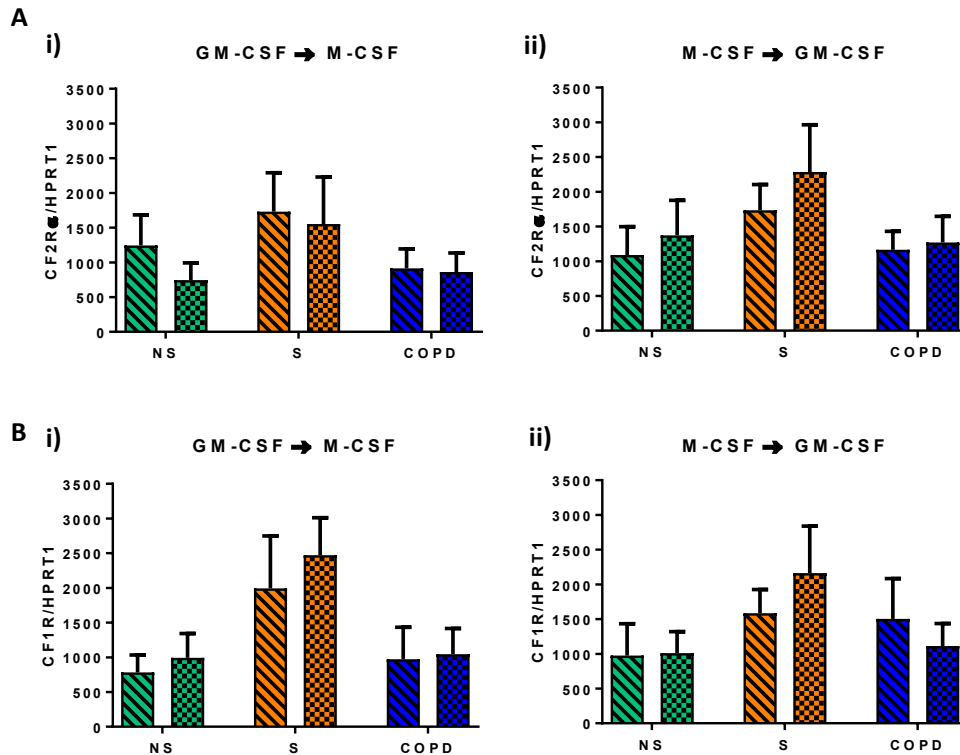


Figure 6. 3 Gene expression of *CSF2Rα* and *CSF1R* in MDM following switching of media. Monocytes from **non-smokers (n=5)**, **smokers (n=5)** and **COPD patients (n=5)** were cultured in GM-CSF or M-CSF for 12 days prior to switching media from **i) GM-CSF to M-CSF** or **ii) M-CSF to GM-CSF** for a further 6 days. Samples were collected on day 18 and gene expression analysed using qPCR for **A) *CSF2Rα*** and **B) *CSF1R***. Control cells were cultured in the same media throughout the 18 days (dashed lines) and compared to cells which had had media switched (chequered bars).

6.3.2.2 Tissue macrophages

Having investigated gene expression of the receptors *CSF2Rα* and *CSF1R* in MDM during differentiation and following switching of media, it was important to establish gene expression of freshly isolated tissue macrophages on day 0 and any changes occurring as a result of *in vitro* culture for 6 days in either media alone, or in the presence of GM-CSF or M-CSF. There was a pronounced reduction in expression of both receptors between day 0 and day 6, regardless of which media the cells were cultured in (Figure 6.4). While there were no significant changes in *CSF2Rα* between day 0

and day 6, *CSF1R* significantly decreased from day 0 to day 6 when cells were cultured in GM-CSF, suggesting that culture conditions may be altering receptor gene expression.

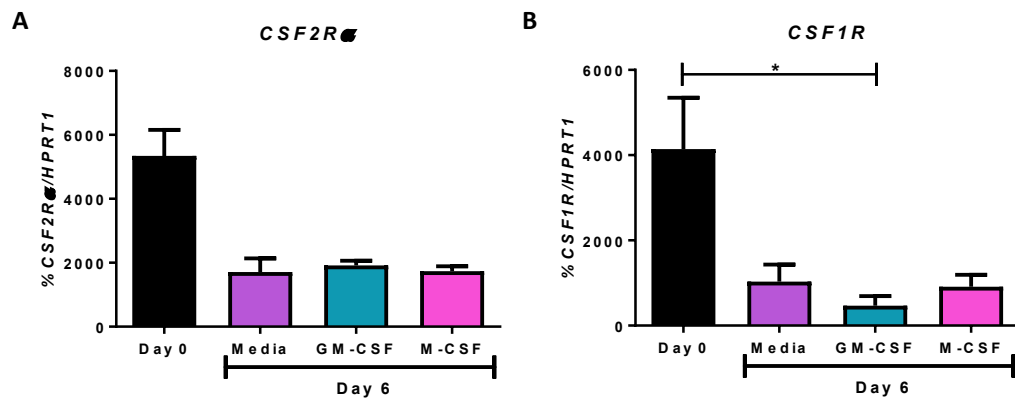


Figure 6. 4 Gene expression of *CSF2Rα* and *CSF1R* in tissue macrophages on days 0 and 6. Tissue macrophages were isolated from COPD patients (n=3) and cultured in media alone or in the presence of GM-CSF or M-CSF for 6 days. Samples were collected on day 0 and day 6 and gene expression analysed using qPCR for **A)** *CSF2Rα* and **B)** *CSF1R*. A Kruskal-Wallis with Dunn's post-test was used to compare differences between day 0 and day 6 for each of the culture conditions tested *p<0.05.

6.3.3 Gene expression of Transcription Factors Involved in Macrophage Lineage

6.3.3.1 MDM Differentiation and Changing of Culture Conditions

To further understand molecular mechanisms underlying macrophage phenotype and plasticity, this study investigated gene expression profiles of several transcription factors that have been implicated in the differentiation of macrophages. Expression of *KLF4* significantly increased in non-smoker MDM from day 0 to day 12 in both GM-CSF and M-CSF culture, with a slight decrease by day 18 (Figure 6.5A). MDM from smokers showed a similar trend, although *KLF4* expression was not significantly higher by day 12, while COPD cells had consistently low levels of *KLF4* gene expression throughout differentiation. Expression of *KLF4* was significantly lower in COPD cells on day 12 compared to non-smokers. *KLF4* gene expression appeared greater in GM-MDM compared to M-MDM and this difference was significantly higher on day 18.

Gene expression of *RELA* and *SREBF1* did not change throughout differentiation, and there was no difference between GM-CSF or M-CSF culture (Figure 6.5B,D). In addition, there was no significant difference in either transcription factor between subject groups, although a trend is present, suggesting that COPD cells have lower levels of *RELA* expression compared to non-smoker and smoker MDM. Expression of *RFX1* was analysed, and results demonstrated that there were no differences between subject groups, or between GM-CSF or M-CSF culture. Furthermore, there was no significant difference observed in non-smoker and smokers cells between day 0 and days 12 and 18, but COPD M-MDM had significantly lower levels of *RFX* expression on days 12 and 18 compared to day 0 (Figure 6.5C).

Having established expression levels of these transcription factors throughout macrophage differentiation, it was important to assess whether there were changes in expression following switching of media, and therefore whether they play a role in cell plasticity. To investigate this, on day 12 of MDM differentiation, cell media was switched from GM-CSF to M-CSF and M-CSF to GM-CSF for a further 6 days and gene expression analysed.

KLF4 expression appeared to change following switching of media in non-smokers, decreasing when media was switched from GM-CSF to M-CSF compared to cells cultured in GM-CSF alone, and increasing when media was changed in the opposite way; M-CSF to GM-CSF (Figure 6.6A). These trends were significantly different in cells from COPD patients, with no differences observed in smoker MDM. The remaining three transcription factors, *RELA*, *RFX1* and *SREBF1* showed no difference in gene expression following switching of media compared to the un-switched controls (Figure 6.6B-D)

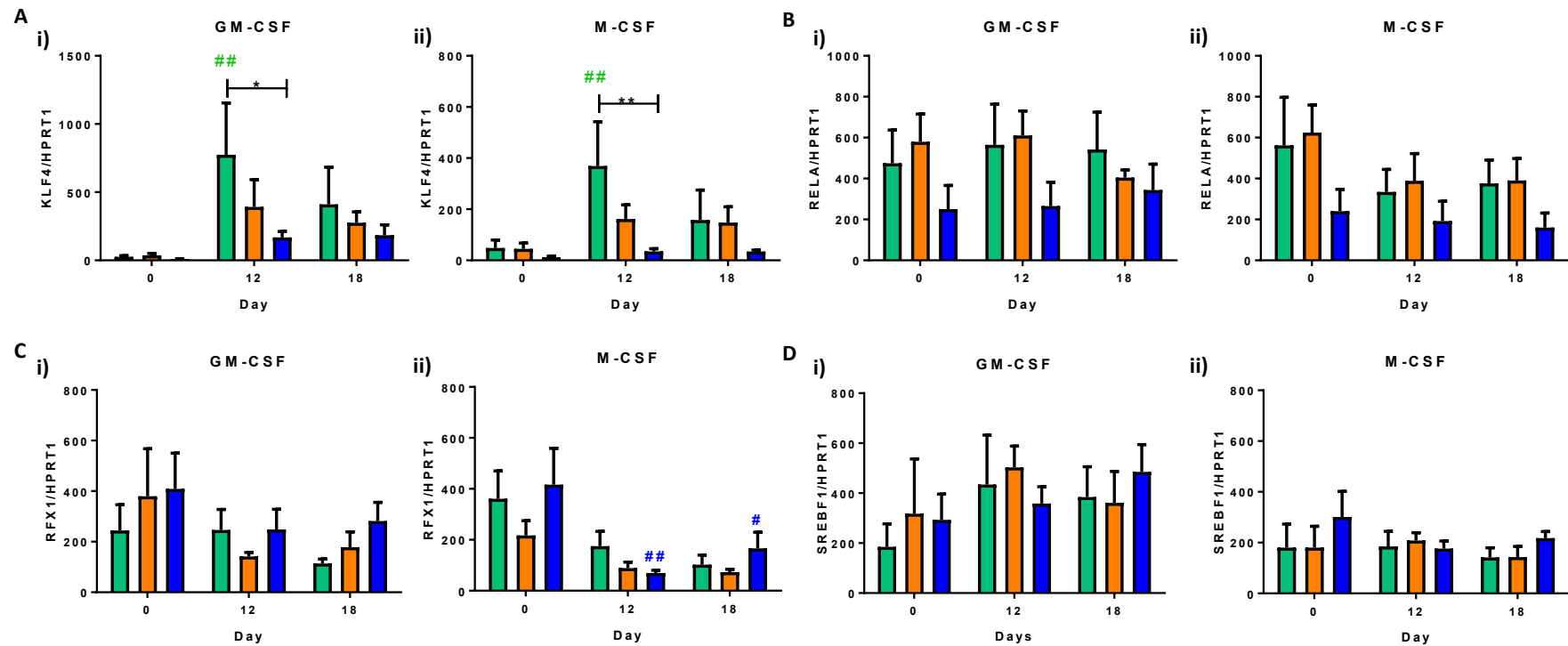


Figure 6. 5 Gene expression of transcription factors throughout macrophage differentiation. Monocytes from **non-smokers (n=5)**, **smokers (n=4)** and **COPD patients (n=6)** were cultured in **i) GM-CSF** or **ii) M-CSF** for 18 days and samples collected on days 0, 12 and 18 for RNA extraction. Gene expression of **A) KLF4**, **B) RELA**, **C) RFX1** and **D) SREBF1** was analysed using qPCR. A two-way Anova with Bonferroni correction was used to test differences between day 0 and days 12 or 18 #p<0.05, ##p<0.01 and a Kruskal-wallis with Dunn's post-test to determine differences between subject groups *p<0.05, **p<0.01.

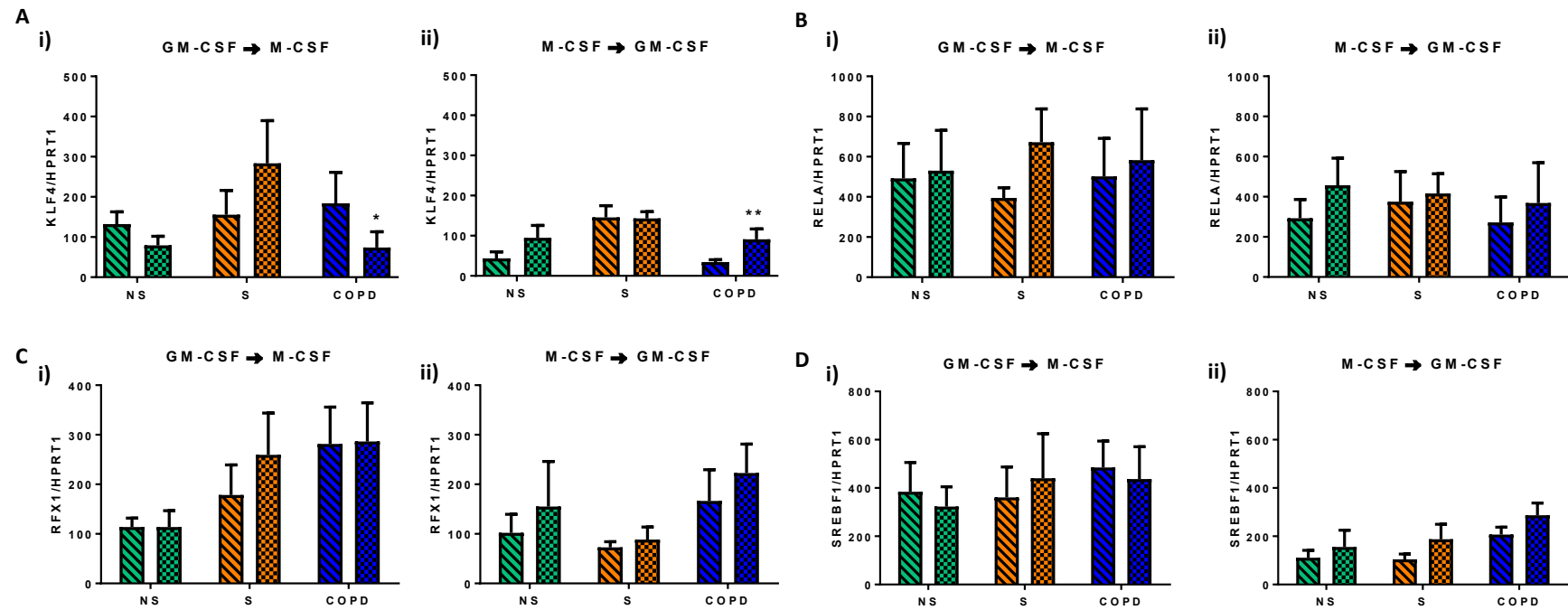


Figure 6. 6 Gene expression of transcription factors in MDM following switching of media. Monocytes from **non-smokers (n=5)**, **smokers (n=4)** and **COPD patients (n=6)** were cultured in GM-CSF or M-CSF for 12 days prior to switching media from **i)** GM-CSF to M-CSF or **ii)** M-CSF to GM-CSF and samples collected on day 18 for RNA extraction. Gene expression of **A) KLF4**, **B) RELA**, **C) RFX1** and **D) SREBF1** was analysed using qPCR. A Mann-Whitney test was used to compare differences between switched cells and control cells *p<0.05, **p<0.01.

6.3.3.2 Tissue Macrophages

In order to further understand the role of transcription factors in macrophage phenotype and plasticity, gene expression was measured in tissue macrophages immediately after isolation and after 6 days in culture. In this way, phenotype and plasticity could be comparable to the MDM model. Unfortunately, due to availability of lung tissue samples, these experiments were only performed on COPD patient samples, and thus comparison between subject groups was not possible.

Expression of *KLF4* appeared to increase for all conditions on day 6 compared to day 0, and was significantly higher for cells cultured in media alone and GM-CSF (Figure 6.7A). Furthermore, GM-CSF cultured cells expressed significantly more *KLF4* than cells cultured in M-CSF. In contrast, *RELA* gene expression appeared to decrease from day 0 to day 6 for all conditions, although this did not reach significance (Figure 6.7B). *RFX1* and *SREBF1* gene expression remained similar between day 0 and day 6, and none of the culture conditions had any effect on altering expression levels (Figure 6.7C-D).

6.3.4 GSK-J4 Concentration Response on Cytokine Release and Cell Viability

Having investigated expression levels of GM-CSF and M-CSF receptor as well as transcription factors *KLF4*, *RELA*, *RFX* and *SREBF1* throughout macrophage differentiation and following environmental change, experiments were performed to determine whether these were under the control of histone modifications. The histone demethylase inhibitor, GSK-J4, was used to selectively inhibit histone H3K27me2-3 demethylation, and thus prevent gene transcription of genes associated with this histone.

An initial concentration response was performed to measure cytokine output following 24h incubation with subsequent LPS stimulation for a further 24h in the presence of GSK-J4. MDM were treated with 1-100 μ M GSK-J4, with a vehicle control (DMSO 0.01% (v/v)) and no treatment control used as comparisons. Vehicle had no effect on any of the cytokines compared to LPS stimulation alone, and

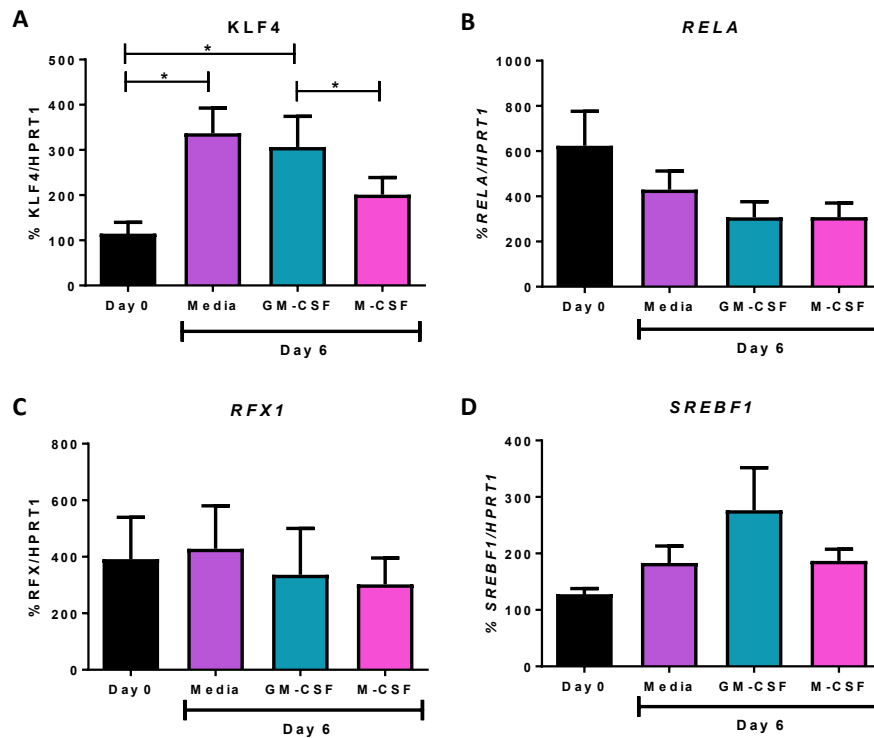


Figure 6. 7 Gene expression of transcription factors in tissue macrophages. Tissue macrophages were isolated from COPD patients (n=4) and cultured in media alone or in the presence of GM-CSF or M-CSF for 6 days. Gene expression was analysed on day 0 and day 6 using qPCR for **A) KLF4**, **B) RELA**, **C) RFX1** and **D) SREBF1**. A Kruskal-Wallis with Dunn's post-test was used to test differences between day 0 and all day 6 conditions (media, GM-CSF or M-CSF), *p<0.05.

LPS resulted in a significant increase in TNF α and CXCL8 release, but not IL-10 (Figure 6.8). GSK-J4 had a concentration-dependent inhibitory effect on TNF α release starting at 10 μ M, although this trend did not reach significance (Figure 6.8A). There was no effect on CXCL8 or IL-10 release from MDM following GSK-J4 incubation (Figure 6.8B-C), and none of the concentrations used had a significant effect on cell viability, which was assessed using an MTT assay (Figure 6.8D).

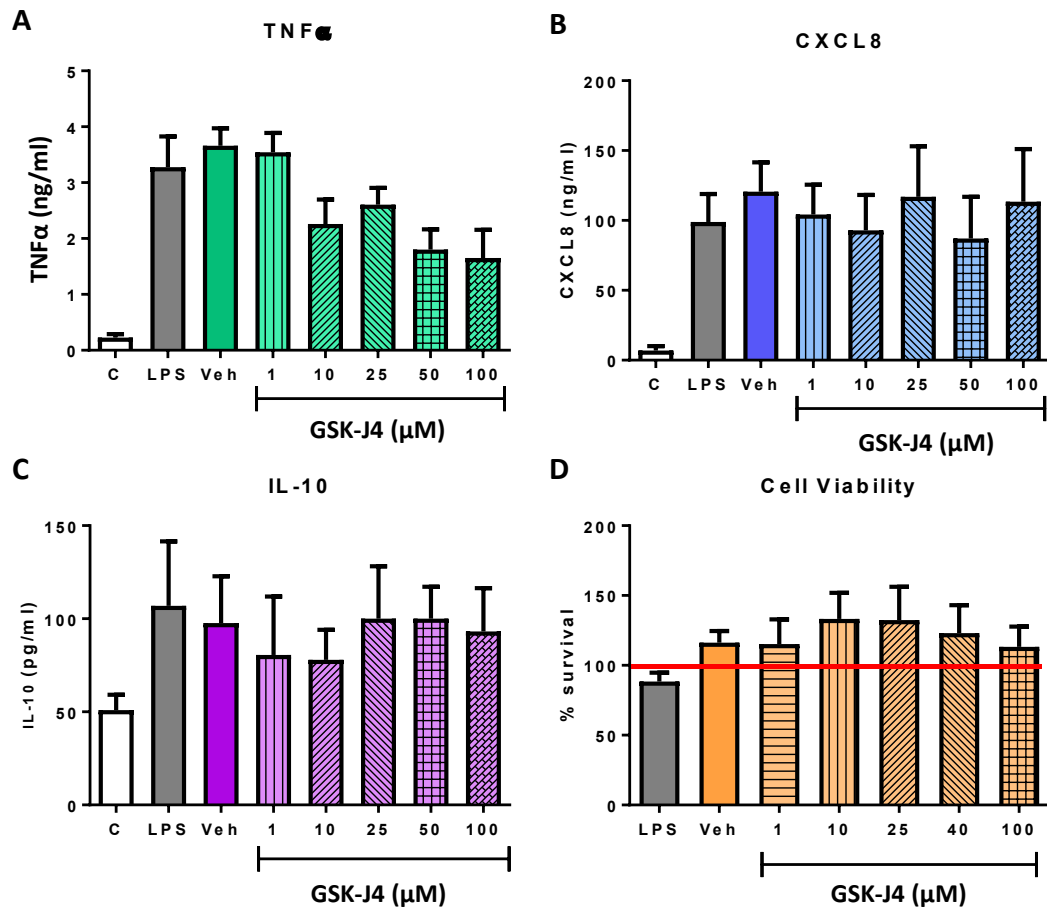


Figure 6. 8 GSK-J4 concentration response on MDM cytokine output. Monocytes were isolated from **non-smokers (n=4)** and cultured for 12 days in GM-CSF. On day 12 cells were inhibited with GSK-J4 (1-100μM), as well as a vehicle control (DMSO 0.01%) or no treatment control for 24h. MDM were then stimulated with LPS (10ng/ml) in the presence of GSK-J4 or vehicle for a further 24h. Cell supernatants were collected and cytokine output measured using ELISA for **A)** TNFα, **B)** CXCL8 or **C)** IL-10. **D)** Cell viability was assessed using an MTT assay and all values normalised to untreated control which was set to 100% (represented by red line on the graph).

Similar experiments were performed on tissue macrophages to establish whether similar concentrations had an effect in cytokine output. Tissue macrophages were cultured for 6 days in media alone or in the presence of GM-CSF or M-CSF. Cells were then cultured with GSK-J4 for 24h prior to LPS stimulation in the presence of GSK-J4 for a further 24h. Vehicle control had no effect on cytokine output, and LPS stimulation resulted in a significant increase in TNFα and CXCL8 release, but not IL-10. In accordance to results obtained using the MDM model, GSK-J4 had a concentration

dependent effect on $\text{TNF}\alpha$, leading to a decrease in cytokine levels following incubation with 10 μM GSK-J4 (Figure 6.9A). A similar effect was observed for CXCL8 release, although these trends did not reach significance. GSK-J4 had no effect on IL-10 release from tissue macrophages. In addition, culture conditions had no effect on any of the cytokines (Figure 6.9). Cell viability was assessed using MTT assay, and results demonstrated no significant effect of GSK-J4, although 25 μM appears to result in a slight decrease in cell viability (Figure 6.10). Tissue macrophages appeared to have increased sensitivity to GSK-J4 compared to MDM, therefore a concentration of 10 μM was chosen to be used in all subsequent experiments in order to prevent any effect on cell viability.

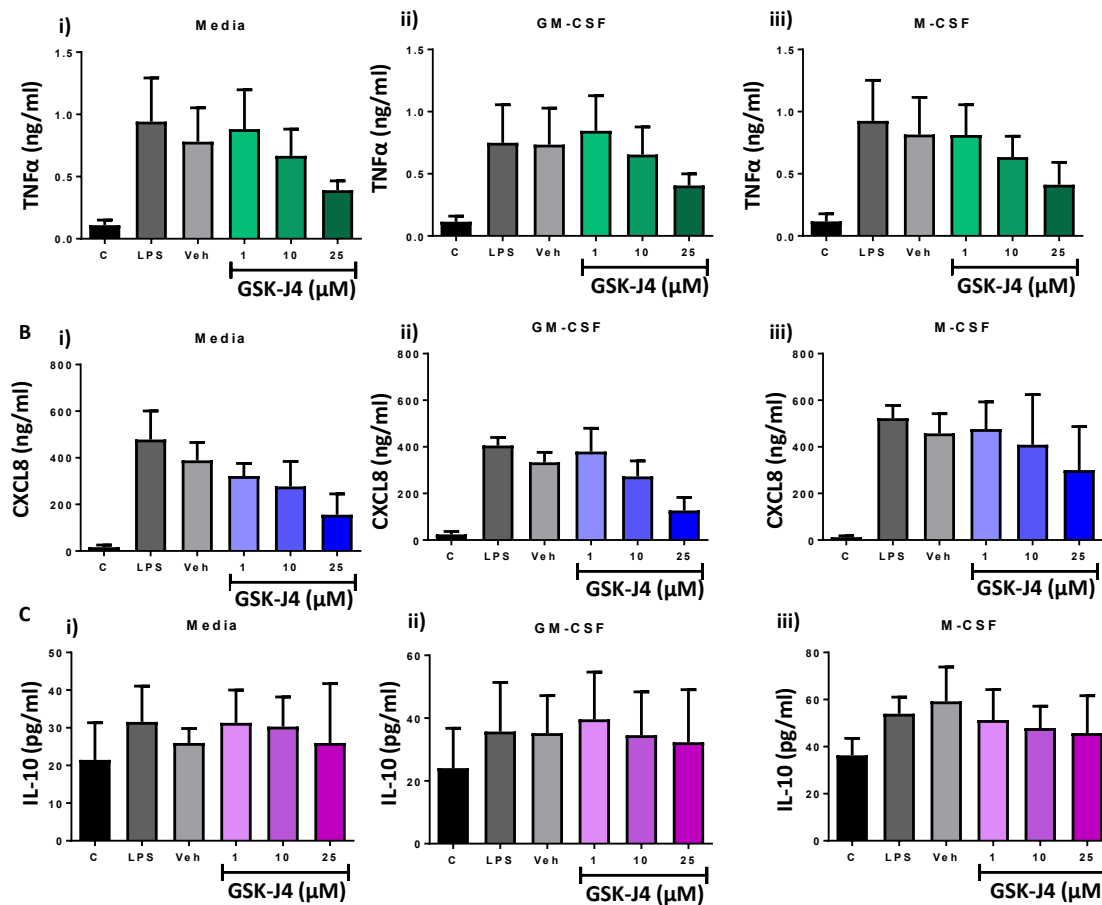


Figure 6. 9 GSK-J4 concentration response on tissue macrophage cytokine output. Tissue macrophages were isolated from COPD patients (n=3) and cultured in i) media alone or in the presence of ii) GM-CSF or iii) M-CSF for 6 days. On day 6, cells were inhibited with GSK-J4 (1-25 μ M), as well as a vehicle control (DMSO 0.01%) or no treatment control for 24h prior to stimulation with LPS (10ng/ml) in the presence of inhibitor or vehicle for a further 24h. Cell supernatants were collected and cytokine output measured using ELISA for A) TNF α , B) CXCL8 or C) IL-10.

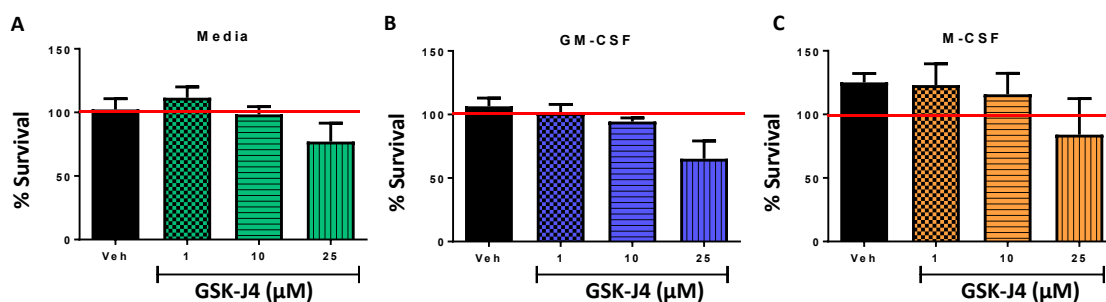


Figure 6. 10 GSK-J4 concentration response on tissue macrophage cytokine output. Tissue macrophages were isolated from **COPD patients (n=3)** and cultured in **A)** media alone or in the presence of **B)** GM-CSF or **C)** M-CSF for 6 days. On day 6, cells were inhibited with GSK-J4 (1-25μM), as well as a vehicle control (DMSO 0.01%) or no treatment control for 24h prior to stimulation with LPS (10ng/ml) in the presence of inhibitor or vehicle for a further 24h. Cell viability was assessed using an MTT assay and all values normalised to untreated control which was set to 100% (represented by red line on the graph).

6.3.5 Effect of GSK-J4 on MDM Differentiation

Monocytes were isolated and cultured in GM-CSF for 12 days in the presence or absence of GSK-J4. Based on data obtained from concentration responses in MDM and tissue macrophages, the 10μM concentration was selected for all further experiments, as it was non-toxic to both MDM and tissue cells. GSK-J4 was added to cells at various points during differentiation to examine the effect it had on monocyte differentiation. GSK-J4 was therefore added to cells on either day 0, day 4 or day 8 so that by day 12 the cells had been exposed to GSK-J4 for either 12, 8 or 4 days respectively. All experiments were performed on day 12 and samples compared to an untreated control.

6.3.5.1 Changes in Cell Morphology

Images of control MDM appear fully differentiated by day 12, with a large rounded shape characteristic of GM-MDM, while monocytes on day 0 appear much smaller (Figure 6.11). In comparison, day 12 MDM cultured in the presence of GSK-J4 appeared much more heterogeneous,

with some differentiated cells but many monocyte-like smaller cells also present. This was most apparent in cells which had been cultured with GSK-J4 from day 0 (total of 12 days) (Figure 6.11).

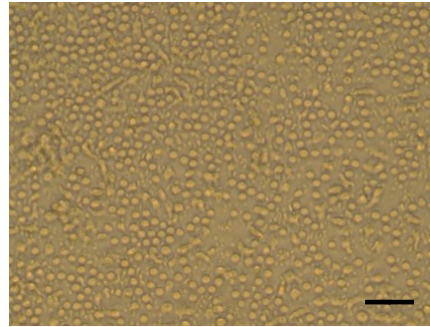
6.3.5.2 Changes in Gene expression

In order to determine whether GSK-J4 had any functional effect on macrophages which had been differentiated in the presence of GSK-J4, gene expression was analysed. *KLF4* and *MMP9* were selected as both are implicated in COPD and previous chapters showed that gene expression changed during MDM differentiation. Results demonstrate that GSK-J4 had no significant effect on gene expression of either gene investigated (Figure 6.12). However, there was a trend suggesting a decrease in *KLF4* when cells were cultured in the presence of GSK-J4 compared to control cells (Figure 6.12A). Furthermore, *MMP9*, appeared to increase in a step wise manner the longer the cells were cultured with GSK-J4, with the highest expression observed for cells cultured in GSK-J4 for 12 days (Figure 6.12B).

6.3.5.3 Effect on Phagocytosis of Bacteria

The effect of GSK-J4 inhibition on phagocytosis of bacteria by MDM was also investigated as defects in this function are known to occur in COPD cells. Live/dead stain was used to exclude any dead cells, and to determine any toxic effect GSK-J4 may have had on cells. There was no significant effect of GSK-J4 on % live cells and addition of bacteria (*S. pneumoniae*) also had no effect on number of live cells (Figure 6.13A). In order to analyse phagocytosis, dead cells were excluded and phagocytosis of *S. pneumoniae* was measured as percentage of cells phagocytosing and also median fluorescence intensity, and data showed that GSK-J4 had no effect on either measurement of phagocytosis (Figure 6.13B-C).

Day 0



Day 12

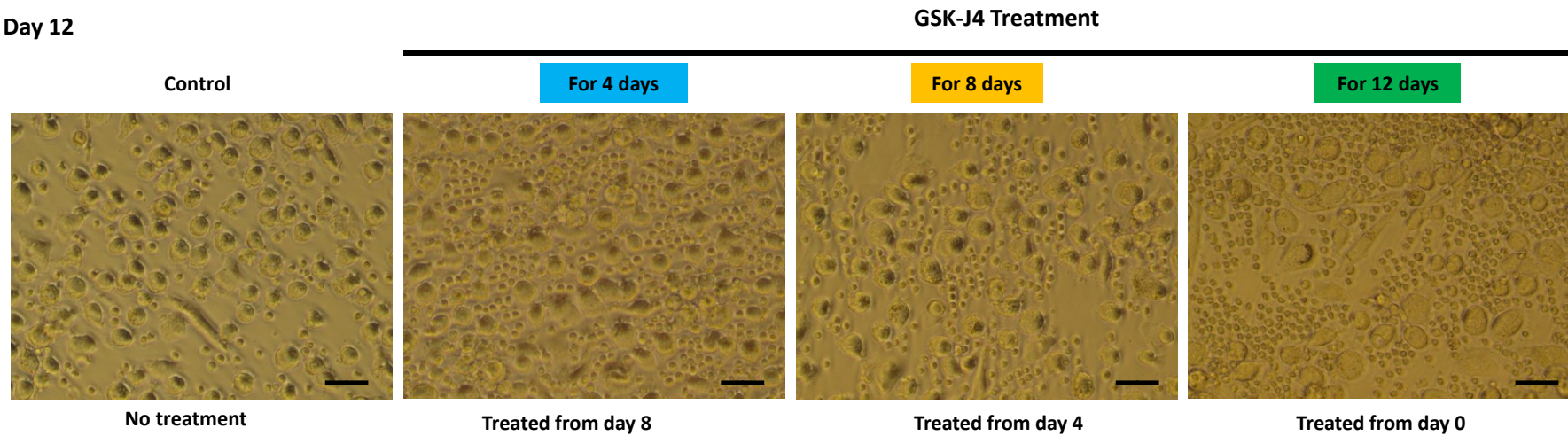


Figure 6. 11 Effect of GSK-J4 on MDM morphology. Monocytes were isolated from **non-smokers** and cultured in GM-CSF for 12 days. GSK-J4 (10 μ M) was added on day 0, 4 and 8 so that cells will have been in the presence of inhibitor for 12, 8 and 4 days respectively. GSK-J4 was added every time cell media was replenished. Control cells remained in GM-CSF alone. Images were taken on day 0 and day 12 (control and GSK-J4-treated cells). Scale bar = 100 μ m. Representative image of three independent experiments.

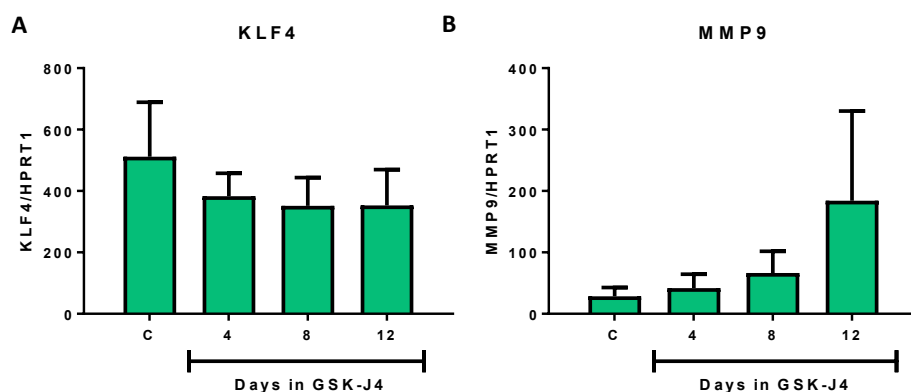


Figure 6. 12 Effect of GSK-J4 on gene expression in MDM. Monocytes were isolated from **non-smokers (n=5)** and cultured in GM-CSF in the presence or absence of GSK-J4 (10 μ M) for either 12, 8 or 4 days. Samples were collected on day 12 and gene expression analysed using qPCR for **A) KLF4** and **B) MMP9**.

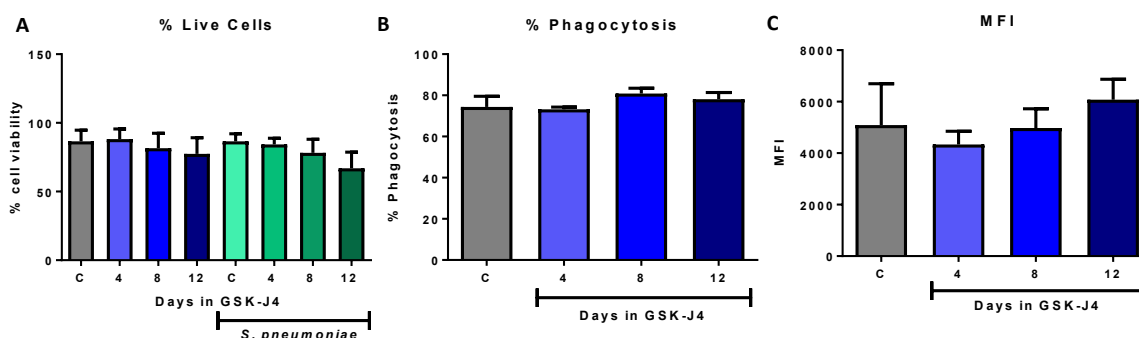


Figure 6. 13 Effect of GSK-J4 on phagocytosis of bacteria by MDM. Monocytes were isolated from non-smokers (n=3) and cultured in GM-CSF in the presence or absence of GSK-J4 (10 μ M) for either 12, 8 or 4 days. On day 12, cells were incubated with heat-killed, pHrodo labelled *S. pneumoniae* for 4h and phagocytosis assessed by flow cytometry. Live/dead stain was used to exclude dead cells, and **A) % live cells** quantified with and without bacteria and GSK-J4. Phagocytosis was measured as **B) % phagocytosis** and **C) MFI**.

6.3.6 Effect of GSK-J4 on Tissue Macrophages and Cell Plasticity

In order to further investigate the effects of GSK-J4 on macrophages, similar experiments were performed on tissue macrophages, as those performed on MDM. As such, lung tissue macrophages were isolated and cultured for 6 days in media alone, GM-CSF or M-CSF in the presence or absence of GSK-J4 (10 μ M). Cells were cultured in GSK-J4 from day 0 (immediately after isolation) and added when media was replenished. All samples were collected on day 6 and compared to untreated controls.

6.3.6.1 Gene Expression

In concordance with data obtained from MDM model, expression levels of *KLF4* did not change in response to culture in GSK-J4 in tissue macrophages (Figure 6.14A). In addition, *MMP9* expression appeared to decrease in cells which had been cultured with GSK-J4 compared to controls, and this was the same for all culture conditions, although this trend did not reach significance (Figure 6.14B).

6.3.6.2 Phagocytosis of Bacteria

Phagocytosis of *S. pneumoniae* by tissue macrophages was investigated following culture in GSK-J4 in comparison to control cells. Cell viability remained unchanged following GSK-J4 treatment, and presence of bacteria had no detrimental effect on cells (Figure 6.15A). Phagocytosis of bacteria was measured as percentage of cells phagocytosing and MFI, and similarly to MDM, GSK-J4 had no effect on either measurement (Figure 6.15B-C).

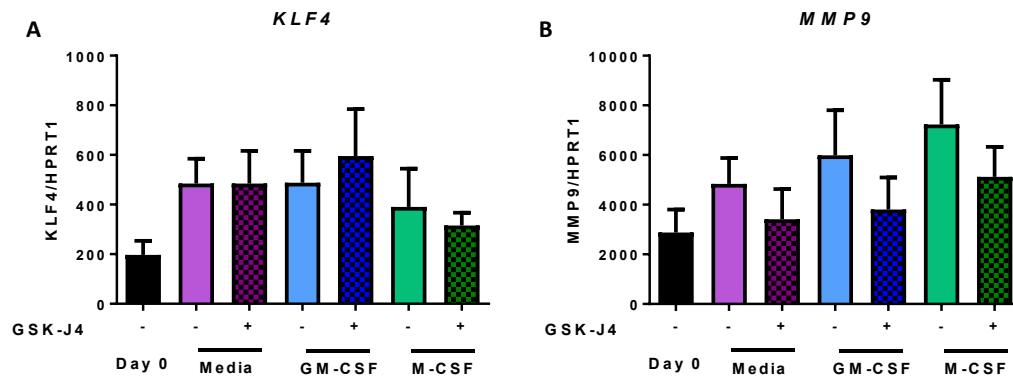


Figure 6. 14 Effect of GSK-J4 on gene expression in tissue macrophages. Cells were isolated from **smokers (n=2)** and **COPD patients (n=3)** and cultured for 6 days in media, GM-CSF or M-CSF in the presence or absence of GSK-J4 (10 μ M – chequered bars). Samples were collected on day 0 and day 6 and gene expression analysed using qPCR for **A) *KLF4*** and **B) *MMP9***. Data shown represent pooled data from smokers and COPD patients.

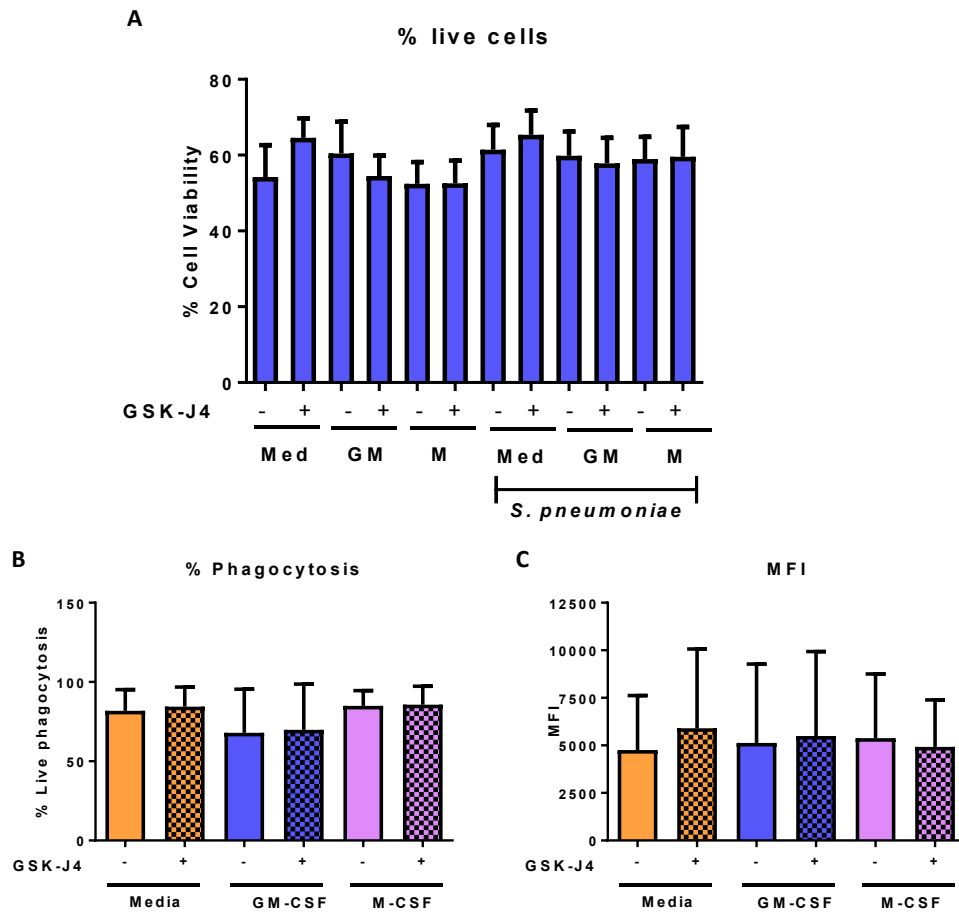


Figure 6. 15 Effect of GSK-J4 on gene expression in tissue macrophages. Cells were isolated from smokers (n=3) and COPD patients (n=3) and cultured for 6 days in media, GM-CSF or M-CSF in the presence or absence of GSK-J4 (10 μ M – chequered bars). On day 6 cells were incubated with *S. pneumoniae* for 4h and phagocytosis assessed using flow cytometry. Live/dead stain was used to exclude dead cells and **A)** % live cells are shown for the different conditions tested. Phagocytosis was measured by **B)** % phagocytosis and **C)** MFI. Data shown represent pooled data from smokers and COPD patients.

6.4 Discussion

6.4.1 *CSF2Rα* and *CSF1R* Expression in MDM and Tissue Macrophages

The role of GM-CSF and M-CSF in maintaining myeloid lineage as well as in macrophage differentiation and survival is well established, and is highlighted by the detrimental effects observed following defects in either pathway (Lacey et al., 2012, Louis et al., 2015). One example demonstrating the importance of GM-CSF is pulmonary alveolar proteinosis, which can occur as the result of autoimmune inhibition of GM-CSF or due to mutations in *CSF2Rα*. This condition is characterised by macrophage dysfunction, with decreased phagocytosis and inflammatory responses (Trapnell et al., 2009). These observations have also been confirmed in *CSF2Rα* knock out mice, which, in addition to demonstrating lack of macrophage function, also highlighted the importance of GM-CSF in regulating the master transcription factor involved in macrophage lineage, PU.1 (Stanley et al., 1994, (Shibata et al., 2001, Trapnell et al., 2009, Gosselin and Glass, 2014). On the other hand, while the role of M-CSF in macrophage maintenance is well established, there is less data investigating the importance of this pathway in human disease (Davies et al., 2013, Stanley et al., 1994). M-CSF has been implicated in driving tumour macrophage phenotype, and thus the progression of cancer by preventing detection of tumorigenic cells by the immune system. There have been attempts to inhibit CSF1R in breast and cervical tumours, in order to attenuate TAM function, which is thought to prevent immune surveillance of tumours and thus promote tumour growth. Results demonstrated a severe reduction in macrophages present within the tumour environment, and further work is underway to determine the validity of this method in cancer therapy (Strachan et al., 2013).

Considering the importance of these growth factors in macrophage differentiation and function, data is lacking regarding how the respective receptors may play a role in macrophage phenotype and changes occurring in COPD. This chapter investigated levels of *CSF2Rα* and *CSF1R* throughout macrophage differentiation as well as following changes in cell culture to assess the role of the respective ligands, GM-CSF and M-CSF, on receptor expression. Data obtained here show a decrease

in both receptors during differentiation, irrespective of health status, suggesting that once initial signalling occurs in the presence of either growth factors there is downregulation of receptor expression. This may be due to receptor recycling following initial contact with growth factors, and thus *de novo* gene transcription is not required. Investigating levels of these receptors at the protein level may therefore aid in further understanding differences between phenotypes and whether COPD cells show differential levels (Shibata et al., 2001). However, due to rapid turnover of these receptors, attempts at measuring cell surface receptor levels were not successful (data not shown) (Shibata et al., 2001, Wicks and Roberts, 2016).

Monocytes isolated from smokers expressed significantly higher levels of *CSF1R* mRNA than non-smokers and COPD cells, and may represent a normal response to stress that is lost in COPD cells. M-CSF drives cells towards resolving phenotype, and therefore increased signalling in this pathway may dampen inflammatory responses in smokers. The difference in cells from smokers and COPD may therefore indicate a potential difference between those patients that do and do not develop disease, with an inability to reduce inflammatory signals within the lung. A similar pattern of gene expression is observed in cells isolated from lung tissue, with expression of both receptors decreasing from day 0 to day 6, which may represent an effect of removing cells from pro-inflammatory environment within the lung. Gene expression of *CSF2Rα* was similar between all day 6 culture conditions, however *CSF1R* mRNA levels were significantly lower in tissue macrophages cultured in GM-CSF compared to day 0. Once again, this data supports the theory that inflammatory signals within the lung result in elevated GM-CSF production (Vlahos et al., 2006), and a dampening of M-CSF signalling which leads to fewer resolving macrophages within the lung. Although his data provide valuable information about receptor gene expression in COPD cells, these experiments need to be repeated in cells from non-smokers and smokers to determine differences between subject groups and gain a more comprehensive understanding of their role in disease.

6.4.2 Role of Transcription Factors During Cell Differentiation and Plasticity

Mechanisms driving macrophage differentiation and phenotype have been an area of increasing interest in the past few years. Identifying cell subpopulations based on function as well as molecular markers could aid in further understanding the role of macrophages in health and disease. In addition, epigenetic modifications have been implicated in driving cell phenotype as well as rapid changes in cell function in response to stimuli. Wallner *et al.* recently investigated changes in gene expression and chromatin modification during monocyte to macrophage differentiation and identified several transcription factor binding sites which became enriched in macrophages, including *KLF4*, *RELA*, *RFX1* and *SREBF1* (Wallner *et al.*, 2016). In addition, major changes in histone modifications were identified, with macrophages having more accessible chromatin compared to monocytes, and specifically reduced levels of H3K27me3 which results in inhibition of *de novo* transcription (Liao *et al.*, 2012, Kapoor *et al.*, 2015).

In order to investigate differences between GM-CSF and M-CSF differentiated macrophages as well as molecular changes in response to environmental cues, this chapter investigated gene expression of the transcription factors identified by Wallner *et al.* There is increasing evidence that *KLF4* is crucial for macrophage maturation and specifically for anti-inflammatory functions (Kapoor *et al.*, 2015, Alder *et al.*, 2008). IL-4 has been shown to drive elevated *KLF4* via STAT6 and expression is decreased in response to LPS. Whereas, on the other hand, increased *KLF4* expression reduces LPS inflammatory response (Liao *et al.*, 2011, Liao *et al.*, 2012). In addition, inflammatory macrophages found in adipose tissue of obese individuals have been shown to express lower levels of *KLF4* compared to lean counterparts, which is thought to be driving inflammation in those individuals (Liao *et al.*, 2011). SIRT1 is a class III HDAC which was recently shown to drive self-renewal capabilities of macrophages by activating *KLF4*, and this protein is known to be decreased in COPD (Imperatore *et al.*, 2017, Soucie *et al.*, 2016). Results obtained in this study suggest that *KLF4* is upregulated during differentiation of MDM from non-smokers, reaching maximum expression by day 12, and then decreasing by day 18.

This indicates that *KLF4* may be important in cell maturation, and once macrophage phenotype is established, expression decreases in steady state. In contrast, COPD MDM expressed significantly lower levels of *KLF4*, and may represent a key defect in COPD cells resulting in aberrant function.

In order to investigate the role of *KLF4* in macrophage plasticity, expression levels were analysed in MDM and tissue macrophages following changes in culture media. When media was switched from GM-CSF to M-CSF, COPD MDM expressed significantly less *KLF4*, and when cells were switched from M-CSF to GM-CSF, expression significantly increased. Similarly, COPD tissue macrophages had significantly higher expression on day 0 compared to day 6 when cultured in GM-CSF or media alone, but not M-CSF. Indeed, M-CSF cultured tissue macrophages expressed significantly less *KLF4* than those cultured in GM-CSF. These data suggest that GM-CSF drives cells to produce more *KLF4* in COPD cells, but does not appear to have an effect on cells from smokers or non-smokers. One explanation may be that GM-CSF drives pro-inflammatory signals, and *KLF4* is expressed as a counter-measure, in order to return cells to a homeostatic state through a feedback loop. Therefore, this increase in *KLF4*, which has previously been associated with a resolving phenotype, may be a compensatory mechanism in COPD cells to attempt to reduce inflammatory conditions within the lung. In addition, COPD cells appeared to show plasticity in terms of *KLF4* gene expression and may provide a valuable target to consider in order to dampen inflammation in these cells. However, additional studies are required to investigate expression levels in tissue macrophages from non-smokers to conclude whether the same is observed as in MDM, as well as investigating *KLF4* protein levels in both models.

Expression of *RELA*, *RFX1* and *SREBF1* were also examined, as these were previously shown to have differential expression during monocyte to macrophage differentiation (Wallner et al., 2016). *RelA*, together with p50, comprise the protein complex of NFκB, which is a key mediator of pro-inflammatory signals that is activated by LPS and CSE (Yang et al., 2007). While *p50* has been shown to be constitutively expressed in monocytes and MDM, *RELA* expression was significantly greater in MDM (Conti et al., 1997). However, data obtained in this study suggests that *RELA* does not change

during differentiation, with similar levels of expression in both monocytes and MDM. Discrepancies between both studies may be due to distinct culture methods as well as techniques used for RNA isolation and gene expression analysis. Additional experiments assessing protein level may be beneficial in determining changes in RELA and NFκB signalling in monocytes and macrophages, as well as investigating changes in cytoplasmic and nuclear localisation, as total protein may not change, but nuclear localisation would indicate activity. Tissue macrophages appear to have lower expression of *RELA* on day 6 compared to day 0, which may occur due to the removal of cells from an inflammatory environment, such as the lungs of smokers and COPD patient, and therefore leads to reduction in inflammatory signals.

RFX1 is essential for the expression of MHC class II genes and *SREBF1* is essential for fatty acid biosynthesis (Ecker et al., 2010). Data from this study indicate that *RFX1* decreases during differentiation of COPD MDM and is significantly decreased in M-MDM on day 12 and 18 compared to day 0. This pattern is also observed in cells from non-smokers and smokers with no differences between G-MDM and M-MDM. Furthermore, there was no change in *RFX1* in tissue macrophages between day 0 and day 6, for any condition tested. Therefore, it is likely that *RFX1* is not driving cell phenotype or dysfunction in COPD. *SREBF1* has been previously shown to drive fatty acid biosynthesis and as such to be essential for macrophage differentiation. Studies have shown that inhibition of this transcription factor resulted in abnormal MDM morphology, organelle development, as well as impaired phagocytosis (Ecker et al., 2010). While previous work on *SREBF1* showed an increase in expression between monocytes and macrophages, results from this study suggest that expression remains at similar levels throughout differentiation. However, in this thesis, measurements were performed on day 12, while work by Ecker *et al.* measured expression at day 6, and these differences may account for discrepancies observed between both studies. It is possible that gene expression increases by day 6 and returns to baseline by day 12, which would support results obtained from both studies. Furthermore, GM-CSF culture appeared to result in elevated *SREBF1* in both MDM and tissue

macrophages, which may represent a molecular difference between cell phenotypes, and could thus be used as a potential marker to identify macrophage subpopulations.

Changes during monocyte to macrophage differentiation highlight important pathways driving correct cell function. Furthermore, cell activation is known to drive a spectrum of transcriptional changes defined by the type of stimuli the cells receive (Xue et al., 2014). Therefore investigating changes in transcription factors and receptors following stimulation may provide more relevant information regarding the role of these genes on cell function and how it may change in COPD cells.

6.4.3 Epigenetics During Macrophage Differentiation

Epigenetic mechanisms are known to control macrophage differentiation and allow for rapid changes in response to stimuli (Gosselin and Glass, 2014). However, this field is relatively novel, and data on chromatin modifications in macrophage phenotype as well as in disease has yet to be explored. PU.1 is an essential lineage determining transcription factor thought to set epigenetic marks in macrophages with additional signal specific transcription factors recruited in response to growth factors and stimuli (Alder et al., 2008, Gosselin and Glass, 2014). KLF4 is downstream of PU.1 and suppresses inflammatory signals, as well as preventing changes in histone modifications to H3K27 (Kapoor et al., 2015, Soucie et al., 2016). Many inflammatory responses, including LPS-induced TLR4 activation, are driven by NF- κ B signalling, leading to expression of pro-inflammatory genes such as *TNF α* , *IL-6*, *cox-2* and *IL-1 β* . This occurs as the result of changes in chromatin, including activation by histone modifications to H3K27 (Kapoor et al., 2015). H3K27 modifications are heavily implicated in macrophage differentiation and activation upon inflammatory stimuli, with a decrease in methylation and increase acetylation.

In order to investigate whether these modifications are important in macrophage differentiation and plasticity, as well as to determine whether epigenetic changes drive COPD phenotype, the selective H3K27met2-3 inhibitor GSK-J4 was used to treat cells and assess effects on gene expression, cytokine output and phagocytosis (Heinemann et al., 2014). GSK-J4 is a derivative of GSK-J1 with increased cell

permeability potential, shown to selectively inhibit the Fe^{2+} - α -ketoglutarate-dependent histone demethylases JMJD1 and UTX which demethylate H3K27 (Kruidenier et al., 2012). Cells treated with GSK-J4 therefore have no *de novo* transcription of genes with promoters and enhancers repressed by H3K27met2-3, which includes several NF κ B regulated inflammatory genes. Previous work on primary macrophages has shown that GSK-J4 inhibits up to 50% of all LPS-induced cytokines, including TNF α (Kruidenier et al., 2012). Data from this study is in accordance with previous work, showing a concentration dependent effect of GSK-J4 on LPS-stimulated TNF α release from MDM and tissue macrophages. In addition, tissue macrophages showed a similar trend, with decreasing levels of TNF α and CXCL8 release in response to GSK-J4, although no change in CXCL8 was observed in MDM. Tissue macrophages appeared to be more sensitive to higher concentrations of GSK-J4, with cell viability affected at 25 μ M, while MDM viability remained stable up to 100 μ M. A concentration of 10 μ M GSK-J4 was used in both MDM and tissue macrophages in order to mitigate any effect on cell viability while maintaining an effective concentration used on both tissue macrophages and MDM. This may explain the effect observed on CXCL8, and perhaps higher concentrations are required in MDM to observe an effect. TNF α is dependent on the NF κ B pathway, which in turn binds to DNA enhancer sites under the control of H3K27. By inhibiting removal of methyl groups from these enhancers, 'opening' of chromatin at these sites is prevented, and therefore transcription is inhibited. It is therefore possible that COPD macrophages contain higher levels of 'open' chromatin allowing for excessive transcription of inflammatory signals. In addition, TNF α is constitutively expressed at low levels, with mRNA degraded unless cells receive stimulus resulting in the stabilising of the mRNA and subsequent translation to protein (Sullivan et al., 2007). This suggests that TNF α chromatin is 'open' to some degree at all times, thus GSK-J4 will have little effect. Any changes in TNF α protein level observed in this chapter may therefore be due to inhibition of other molecules required for stability and translation of TNF α .

MDM were differentiated in the presence or absence of GSK-J4, and results demonstrate a dramatic effect on cell morphology, indicating that monocytes were unable to differentiate appropriately in the

presence of GSK-J4. Changes in genome wide expression patterns are essential for macrophage differentiation, and several of these will be under the control of H3K27me3. In order to investigate molecular changes more in-depth, gene expression of *MMP9* and *KLF4* were investigated following GSK-J4 treatment. *MMP9* promoter contains binding sites for AP-1, NF- κ B and Sp-1, and thus GSK-J4 was expected to inhibit *de novo* transcription (Labrie and St-Pierre, 2013). Results obtained in this study demonstrate that neither *KLF4* nor *MMP9* expression were altered in MDM or tissue macrophages differentiated or cultured in GSK-J4. Under the assumption that GSK-J4 was not ineffective, as changes in cytokine output were observed, these data suggests that *KLF4* and *MMP9* genes are not under the repression of H3K27 and that redundancy in transcription factors allows for normal gene expression of these genes. Additional work is needed in order to confirm activity of GSK-J4 by investigating chromatin binding and gene expression of known targets to confirm that results obtained are not due to inactivity of GSK-J4. The addition of a positive control would allow for definitive evidence that the GSK-J4 compound is effective, and therefore any negative results would be proven to be a lack of selectivity of GSK-J4 for the target of interest, rather than due to inactive compound. Further experiments were performed to determine the effect of GSK-J4 on phagocytosis. However, data from both MDM and lung tissue macrophages indicated that phagocytosis was unaffected by GSK-J4 treatment in terms of percent phagocytosis and MFI. This may be explained by the fact that phagocytosis is a key function of macrophages, and not under the control of a single gene, therefore redundancy in this process allows for correct function regardless of alterations to a single histone modification.

6.4.4 Conclusions

Changes in gene transcription and chromatin modifications are essential for macrophage maturation and function, but insight into specific pathways is still lacking. This chapter aimed to investigate the role of *CSF2R α* , *CSF1R* and expression of several transcription factors implicated in macrophage lineage. Expression of both receptors decrease during differentiation, suggesting that following initial

contact with growth factors, expression of the receptors may be downregulated and control occurs at the protein level via receptor recycling. *KLF4* was the only transcription factor elevated in MDM and tissue macrophages, suggesting it may play an important role in cell maturity and plasticity. Furthermore, *KLF4* was significantly lower in COPD MDM, and may provide a potential mechanism by which macrophages are dysfunctional in COPD. In an attempt to better understand the role of epigenetic changes, the histone demethylase inhibitor, GSK-J4 was used on MDM and tissue macrophages to assess the effect this may have on several macrophage functions. Data demonstrate that while certain inflammatory signals, including TNF α and CXCL8 were downregulated by GSK-J4, gene expression of *MMP9* and *KLF4*, as well as phagocytosis were unaffected. Based on previous work in this study, *MMP9* appeared to be regulated at the protein level, and thus the effect of GSK-J4 is not observed as this only affects *de novo* gene expression. A more comprehensive study of the effects of GSK-J4 is required in regards to how epigenetic changes drive macrophage phenotype and the implications for disease. However, this brief insight, suggest that pro-inflammatory signals can be manipulated throughout epigenetic changes, and that perhaps multiple histone modifying targets need to be inhibited in order to observe significant changes in cell function, such as phagocytosis.

CHAPTER 7: GENERAL DISCUSSION

7.1 Key Findings

The hypothesis of this thesis was that COPD macrophages would become more pro-inflammatory in the presence of GM-CSF but would not become resolving in the presence of M-CSF, while cells from non-smokers and smokers would be able to switch between both phenotypes.

Using macrophages isolated from resected human lung tissue, and peripheral cells isolated from blood, this study showed that cells cultured in GM-CSF released higher levels of the pro-inflammatory cytokine, TNF α , following LPS stimulation compared to M-CSF cultured cells, and although this trend was observed for all subject groups, COPD cells released significantly more TNF α compared to non-smoker and smoker cells. Furthermore, GM-CSF culture appeared to drive a decrease in the release of the anti-inflammatory cytokine IL-10 across all subject groups. COPD macrophages demonstrated reduced plasticity compared to those isolated from non-smokers and smokers. These latter macrophages could be driven towards a less inflammatory phenotype by switching media from GM-CSF to M-CSF. This resulted in decreased release of LPS-stimulated TNF α and CXCL8, which was not observed in COPD cells. When media was switched from M-CSF to GM-CSF, cells from all subjects could be driven towards a more inflammatory phenotype. While previous studies have shown evidence of macrophage plasticity (Porcheray et al., 2005, Stout et al., 2009, Nakamaru et al., 2009), this is the first to investigate the effect of growth factors on tissue macrophages and demonstrate that these growth factors can influence cell phenotype (Figure 7.1).

This thesis also reports decreased cell plasticity in COPD MDM which can only be driven to become more pro-inflammatory while cells from non-smokers and smokers are capable of returning to a resolving state, which is in accordance with previous work (Day et al., 2014). This may be due to, in part, a decrease in M-CSF signalling in COPD cells, with lower *CSF1R* mRNA expression in COPD MDM compared to those from smokers. Both smokers and COPD macrophages are exposed to oxidative stress, and elevated *CSF1R* expression in smokers may represent a 'healthy' response to oxidative

stress, which is lost in COPD cells, thus decreasing macrophage plasticity, and preventing cells from returning to a less inflammatory phenotype. In addition, the transcription factor, KLF4, is implicated in dampening the inflammatory response (Liao et al., 2011), and this study is the first to report a decrease in *KLF4* expression in COPD MDM compared to smokers and non-smokers, suggestive of a role for this transcription factor in macrophage dysfunction in COPD. Both KLF4 and M-CSF are important for anti-inflammatory responses, and downregulation of these signalling pathways in COPD may drive elevated GM-CSF signalling and chronic inflammation, although the interactions between these pathways requires further understanding.

While cytokine output could be manipulated by growth factors, this study also showed that other functional outputs such as MMP9 activity and phagocytosis remained unchanged by GM-CSF or M-CSF in both tissue macrophages and MDM. It is therefore possible that GM-CSF and M-CSF play an important role in control of cytokine output, and when macrophages are unable to respond to anti-inflammatory signals they 'switch' from a healthy response to chronic inflammation that is likely to drive elevated MMP9 activity and defective phagocytosis, which was observed in both tissue and MDM from COPD cells in this thesis.

Furthermore, in evaluating defects in phagocytosis between tissue macrophages and MDM, this thesis also demonstrated the important interplay between microenvironment and inherent defect which may be the result of epigenetic changes. Tissue macrophages appeared to have a defect in the number of bacteria each cell could uptake, but there was no difference in the number of cells capable of phagocytosis compared to non-smokers. MDM on the other hand showed the opposite, with more bacteria taken up per cell, but less macrophages capable of phagocytosis. These results suggest a combined effect of environmental factors within the lung driving 'early satiety' in macrophages, and inherent defects in circulating monocytes, perhaps due to faulty receptor expression and/or recycling, which together drive defective phagocytosis in COPD. This data is in keeping with previous studies which have shown phagocytic defects in alveolar macrophages and MDM with various prey, but also

provides additional information defining the phagocytic defect in two macrophage models (Hodge et al., 2007, Martí-Lliteras et al., 2009, Taylor et al., 2010).

Finally, this thesis attempted to characterise macrophage subpopulations by separating tissue macrophages and MDM by cell density. Results showed that subpopulations are present in MDM as well as in cells isolated from lung tissue, and these demonstrate distinct functional outputs, including cytokine output and gene transcription, which suggests that distinct monocyte phenotypes within the blood, differentiate into different macrophage phenotypes. A previous study by Chana *et al* attempted to characterise lung tissue macrophage subpopulations and demonstrated no difference in cell surface receptor expression of CD14, CD16, CD163, CD40 and CD206, but identified that the least dense cells were glucocorticosteroid insensitive compared to the other two fractions investigated (Chana et al., 2014). However, the theory that monocyte subpopulations dictate macrophage phenotypes requires additional investigation, and it would be beneficial to separate monocytes by flow cytometry, based on cell surface receptor expression, and assess the resulting differentiated macrophages from each monocyte population.

7.2 Macrophage Models of COPD

The MDM model has been used extensively to characterise macrophages in health and disease, and several studies have demonstrated defects in these cells when isolated from COPD patients compared to healthy smokers and non-smokers (Pons et al., 2005, Rey-Giraud et al., 2012, Pang et al., 2014). Alveolar macrophages have also been used to address the role of macrophages in COPD using both BAL and sputum samples, although these processes are more invasive (Russell et al., 2002, Culpitt et al., 2003b). Few studies have used tissue macrophages due to the difficulty in obtaining them. This study aimed to use both MDM and lung tissue macrophage models in an attempt to investigate the effect of cigarette smoking on cells due to environmental stress within the lung, as well as from the systemic effects which could drive inherent changes in circulating monocytes. Furthermore, the MDM

population has never been investigated as a heterogeneous population, and thus this thesis addressed this issue by separating MDM by density, and comparing these cells to the counterparts isolated from tissue.

Both models demonstrated clear differences between cells isolated from COPD patients compared to non-smokers, and this highlights both the systemic and local effects of cigarette smoking. Several aspects of cell function were assessed, and the results are summarised in Figure 7.2. While it is important to compare COPD cells to those from non-smokers, identifying differences between COPD and smoker cells may also be essential in determining how cells under the same environmental stress behave differently and thus drive the difference between developing COPD or not. Key differences identified in this study show that macrophages from smokers tend to show an intermediate phenotype between non-smokers and COPD macrophages, and this was observed for phagocytosis,

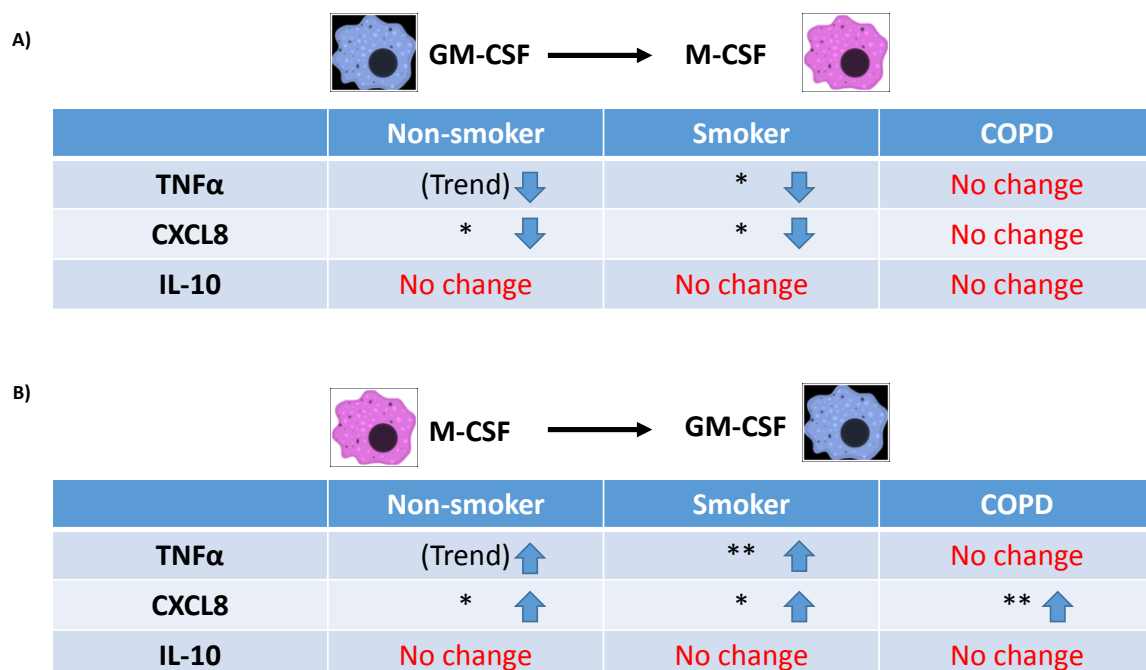


Figure 7. 1 Summary of the effect of switching culture on cytokine output from MDM. Monocytes from non-smokers, smokers and COPD patients were cultured for 12 days in GM-CSF or M-CSF prior to switching media from **A)** GM-CSF to M-CSF or **B)** M-CSF to GM-CSF for a further 6 days. Cytokine output was measured on day 18. Mann-Whitney test was used to test significant changes following switching, *p<0.05, **p<0.01.

cytokine output and *KLF4* gene expression. The response observed in smoker cells may therefore represent a 'healthy' response to stress which is lost in COPD cells. The most dramatic difference was the ability of MDM from smokers to switch from a pro-inflammatory phenotype to resolving, in terms of cytokine output, when cultured in M-CSF, which was completely lost in COPD cells (Figure 7.2).

This study also attempted to further characterise the MDM model by identifying subpopulations of cells which differentiate from the same pool of monocytes and were cultured under the same conditions. This is the first study to isolate distinct macrophage populations from MDM based on density, and results demonstrated that these cells are functionally distinct. The denser cells, which are monocyte-like in morphology, secrete less pro-inflammatory cytokines and express less *MMP9* and *MMP2* than the less dense cell fractions. Work on lung tissue macrophages showed a similar trend with respect to cytokine output, although there were no differences in protease gene expression or activity between cell fractions. Whether the cell subpopulations isolated from tissue and blood represent similar functional groups, or are distinct in different ways remains to be investigated. These subpopulations may represent discrete maturation state or phenotypes of cells which perform diverse functions within the lung. Either way, the fact that MDM are cultured for 12 days under the same conditions and still show distinct subpopulations demonstrates that phenotypic and/or differentiation potential is to some degree predetermined within the cells, and may be under genetic or epigenetic control, rather than driven purely by environmental stimuli. More work is needed in this area to better characterise cell subpopulations and to identify in what way these cells are different, and whether epigenetic control is driving those differences. Applying these studies to COPD cells could thus aid in determining whether specific subgroups are more prevalent in disease, and what mechanisms are driving this inflammatory phenotype.

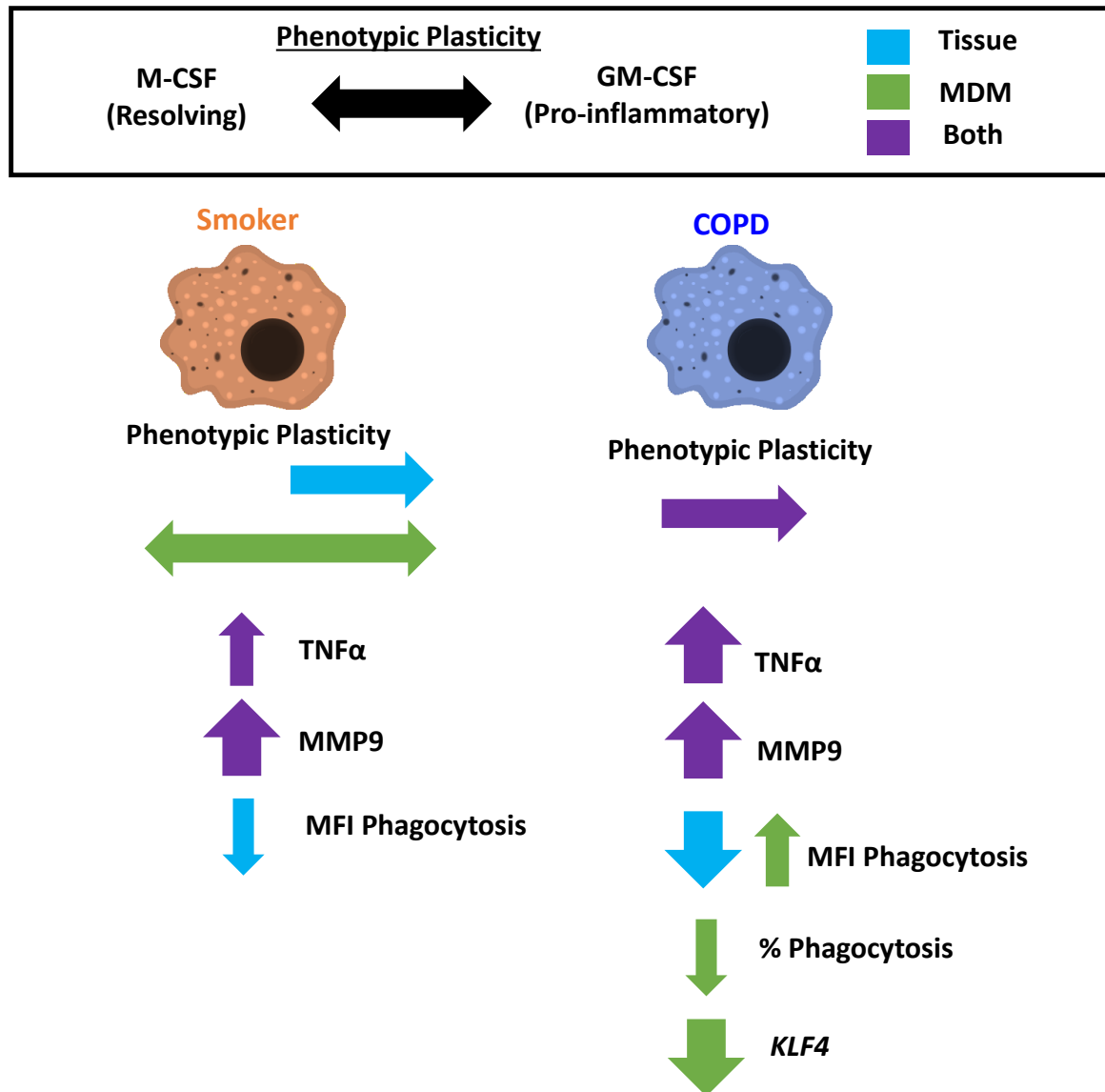


Figure 7. 2 Summary of key differences between smoker and COPD cells compared to non-smokers. Ability of cells to switch phenotype is shown for smoker and COPD cells, and changes in TNF α production, MMP9 activity, phagocytic potential and *KLF4* expression are shown for cells from smokers and COPD patients relative to results from non-smokers. Colours represent whether these results were observed in tissue macrophages, MDM or both. Size of arrows is representative

7.3 Implications for Therapeutic Targeting of Growth Factors

This thesis investigated the effect of growth factors GM-CSF and M-CSF on driving macrophage phenotype and plasticity. Both have been used extensively *in vitro* for differentiation of monocytes and polarisation of macrophages towards pro-inflammatory and resolving phenotypes respectively

(Mosser and Edwards, 2008). Work performed in this study aimed to further understand whether these growth factors could drive mature macrophages to switch phenotype in response to changes in culture, and to investigate whether COPD cells behaved differently from smoker and non-smoker cells.

GM-CSF and M-CSF are essential for macrophage differentiation and function, and several murine studies where either pathway is disrupted have highlighted the importance of these for macrophages in steady state and under stress (Louis et al., 2015). GM-CSF is known to play a role in inflammation by driving myeloproliferation and activation of several cell types and has been implicated in several pathologies including COPD, multiple sclerosis, and rheumatoid arthritis (RA) where GM-CSF levels are elevated in the blood and in synovial fluid (Wicks and Roberts, 2016). Genetic variants in the *CSF2* gene which encodes GM-CSF have also been shown to contribute to RA susceptibility, and thus targeting this pathway has been considered in order to ameliorate inflammation (Okada et al., 2014). Results from this study further demonstrate that this growth factor is likely contributing to the inflammatory pathology of COPD by driving macrophages to become more pro-inflammatory. Higher levels of GM-CSF within the COPD lung may also contribute to cells becoming fixed in an inflammatory phenotype and drive epigenetic modifications in these cells.

GM-CSF blockade has been used in phase I and II clinical trials for RA with significant success and no side effects or decline in lung function. One of the most promising studies was with Mavrilimumab which is a human monoclonal antibody which inhibits GM-CSFR α (Burmester et al., 2011, Takeuchi et al., 2015). Experiments in this thesis found no difference in the expression of *CSF2R α* between subject groups, although investigating cell surface levels and receptor recycling may provide a better understanding of differences between health and disease. The potential benefits of treating COPD patients with anti-GM-CSF therapy are clear, although to date, there are no clinical trials to address this issue. Mouse models of CSE exposure in combination with flu as a model of exacerbations have been used to show anti-GM-CSF therapy proved beneficial, with reduced BALF inflammatory markers and reduced proteases, although there was no effect on weight loss or viral clearance (Vlahos et al.,

2006, Vlahos et al., 2014). It remains to be seen whether similar beneficial effects could be possible in COPD patients.

This thesis demonstrated that M-CSF could drive MDM from non-smokers and smokers to reduce release of pro-inflammatory cytokines, with no effect on COPD MDM or tissue macrophages from any subject. These data suggest that administering M-CSF with the aim of reducing inflammatory signals would not be successful, as macrophages from COPD patients do not appear to respond to this growth factor, and instead appear fixed in a pro-inflammatory state. Understanding molecular changes in these cells, driving inflammation, may therefore provide more viable therapeutic targets.

7.4 Molecular and Epigenetic Mechanisms of COPD

COPD is a complex disease with an interplay of environmental and genetic factors driving development and progression. Changes at the molecular level as well as epigenetic modifications are therefore likely to play a role in driving changes in COPD cells (Kabesch and Adcock, 2012). This thesis attempted to better understand what these changes may be, and identified KLF4 as a potentially important transcription factor regulating macrophage differentiation and inflammation.

KLF4 has a range of roles in different cell types and is one of the key factors which drives pluripotency in the development of iPSCs (Ghaleb and Yang, 2017). Recently, a series of murine studies have shown a role for KLF4 in macrophage biology and specifically as a marker of resolving phenotype. Several studies confirmed that KLF4 acts downstream of IL-4 signalling in a STAT6-dependent manner to drive transcription of anti-inflammatory genes such as MCP1 and IL-10 (Liao et al., 2011). Furthermore, KLF4 is essential in inhibiting NF κ B signalling by preventing recruitment of co-factors p300 and PCAF and thus attenuating inflammatory gene transcription (Figure 7.3) (Liao et al., 2011, Kapoor et al., 2015). KLF4 therefore has the potential to be key in preventing inflammation, and this study is the first to implicate KLF4 in COPD, with data showing decreased *KLF4* mRNA expression in COPD MDM compared to non-smoker and smoker cells. KLF4 and M-CSF signalling are both downregulated in COPD, and it is possible that interplay between these pathways is crucial in dampening inflammatory

signalling, and that one may be driving the other. Additional work is required to better understand how these pathways may be connected, and how dysregulation of either may drive inflammation.

In addition to the STAT6-mediated role of KLF4 induction, deacetylation by SIRT1 is known to drive increased KLF4 levels and activity (Zhang et al., 2017). SIRT1 is an HDAC which, along with HDAC2, has been shown to be decreased in COPD samples and implicated in the pathology of ageing and inflammation (Nakamaru et al., 2009, Rajendrasozhan et al., 2009) (Figure 7.3). As such, it is possible that decreased SIRT1 drives a reduction in *KLF4* expression in COPD macrophages, and this may in part drive decreased IL-4 signalling as well as a concomitant increase in NFκB mediated inflammatory signalling. Further work is required in this area to better understand the role of KLF4 in COPD and in macrophage function and phenotype.

The role of epigenetic modifications in disease has been an area of increasing interest, and is thought to play a role in the development of complex diseases such as COPD and asthma as well as in the development and differentiation of macrophages (Kabesch and Adcock, 2012, Gosselin and Glass, 2014). Histone modifications and DNA methylation are influenced by environmental cues, and are known to be modified during chronic inflammation (Su et al., 2009, Liu et al., 2010). In COPD, cigarette smoke may be driving permanent changes in epigenetic patterns, and thus contributing to disease development. Identifying these patterns and how they differ from non-smokers and smokers without COPD could help in developing new therapies as well as in identifying patterns which characterise individuals who are predisposed and thus at higher risk of developing COPD.

In COPD, the chromatin modification that has been most studied is histone acetylation which promotes gene transcription. An imbalance is thought to exist in the enzymes responsible for adding and removing acetyl groups, HATs and HDACs, resulting in increased transcription of inflammatory genes. Most work has focused on SIRT1 and HDAC2 which have been shown to be decreased in COPD samples and this is thought to be one of the mechanisms behind glucocorticosteroid insensitivity in COPD (Ito et al., 2005). Histone methylation has been investigated to a lesser extent due to the

complexity of roles this can have depending on the site of methylation, although changes in HDM and HMT balance has the potential to change transcription patterns in a similar manner as acetylation (Kabesch and Adcock, 2012) (Figure 7.4). Methylation at H3K27 is associated with transcriptional repression, and this site is known to regulate NFκB-mediated transcription (Kruidenier et al., 2012).

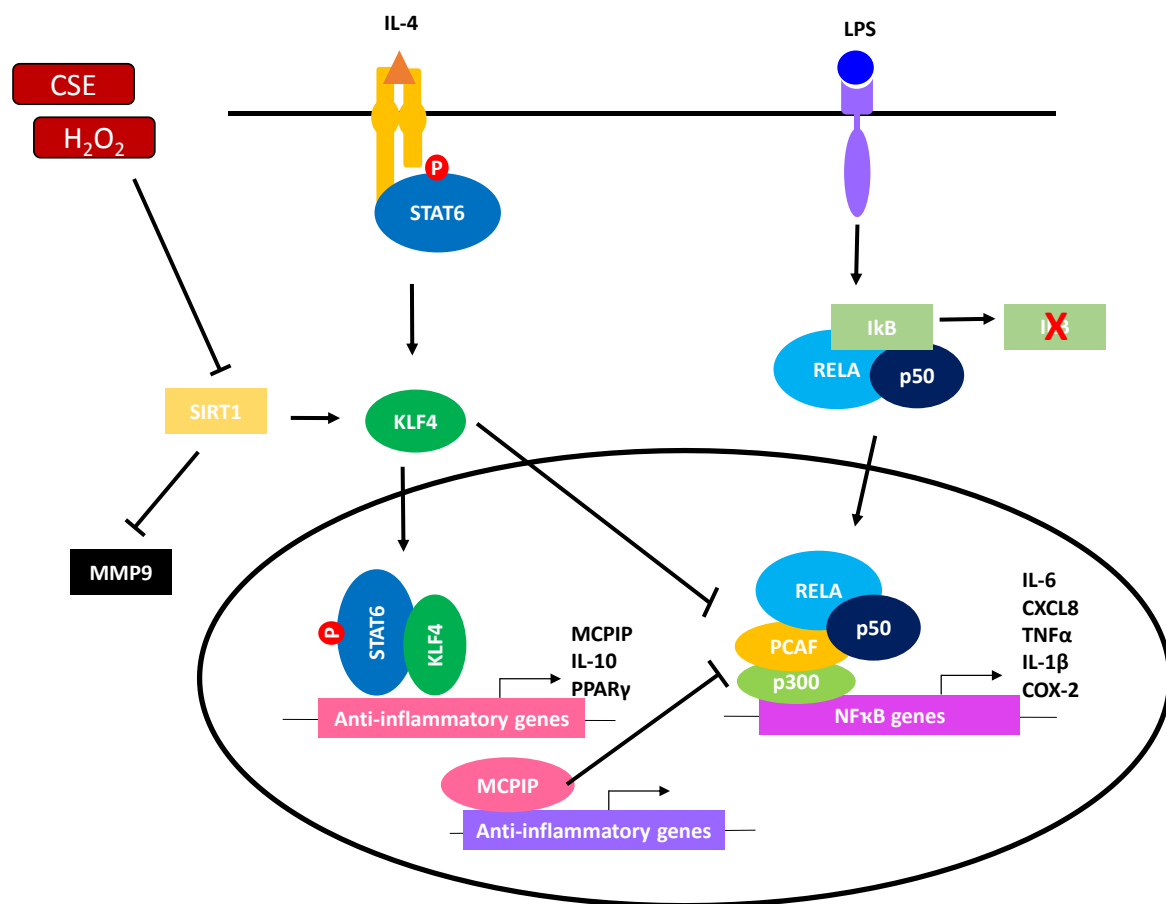


Figure 7. 3 Role of KLF4 in promoting resolving macrophage phenotype and inhibiting NFκB activity. IL-4 signalling leads to phosphorylation of STAT6 which activated KLF4 and together translocate into the nucleus to drive expression of anti-inflammatory genes such as MCPIP. KLF4 and MCPIP can inhibit NFκB activity by preventing recruitment of key co-factors p300 and PCAF. CSE: cigarette smoke extract, SIRT1: sirtuin1, STAT6: signal transducer and activator of transcription 6, KLF4: Kruppel-like factor4, MCPIP: MCP-1 inducible protein, IL: interleukin, LPS: lipopolysaccharide, NFκB: nuclear factor kappa beta, TLR4: toll-like receptor 4. CXCL: chemokine (C-X-C) ligand. (Liao et al., 2011, Kapoor et al., 2015, Zhang et al., 2017).

This study performed a series of experiments using the selective H3K27 methyltransferase inhibitor, GSK-J4, to better understand the role of histone methylation in macrophage function and inflammatory signalling. Macrophages treated with GSK-J4 trended towards reduced pro-inflammatory cytokine production, confirming the role of histone demethylation in promoting transcription of inflammatory mediators. However, GSK-J4 did not affect *KLF4* transcription or the ability of macrophages to phagocytose, likely due to these being under the control of different chromatin modifications. Furthermore, phagocytosis is controlled by several mechanisms, and *de novo* gene transcription may not be required, thus explaining a lack of effect of histone demethylase inhibition. Furthermore, demethylase inhibitors maintain closed chromatin, however these would have no effect on chromatin which is already open. Macrophages are known to increase open chromatin during differentiation (Lavin et al., 2014), which may explain why there is a lack of effect of GSK-J4 on key macrophage functions such as phagocytosis, as well as on gene expression of *KLF4* and *MMP9* which have been shown to be expressed at baseline in macrophages.

The field of epigenetics is vast, and gaining a better understanding of the role this may have in disease could be beneficial in not only developing new treatments, but also in identifying individuals who may be at risk.

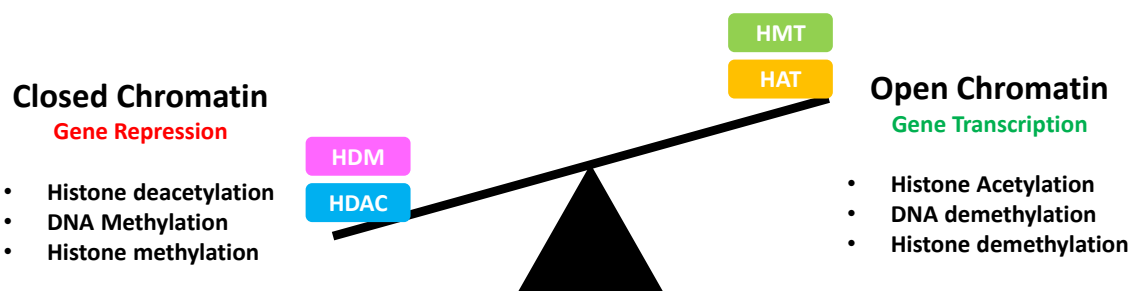


Figure 7. 4 Summary of histone modifications and enzymes involved in driving gene repression and gene transcription. HDM: histone demethylase, HDAC: histone deacetylase, HMT: histone methyltransferase, HAT: histone acetylase. Figure adapted from (Kabesch and Adcock, 2012).

7.5 Conclusion

COPD MDM and tissue macrophages could only be driven towards a more pro-inflammatory phenotype following GM-CSF culture while smoker and non-smoker MDM could revert back to a resolving phenotype following M-CSF culture. Differences between smoker and COPD cells in function, molecular and epigenetic patterns may highlight key information regarding why some individuals develop COPD and some smokers remain healthy. Focusing on these areas may be essential for identifying viable targets to treat COPD patients.

7.6 Limitations

COPD is a heterogeneous disease encompassing several pathologies which can present to different degrees and severities in patients (Barnes, 2004). It is therefore likely that samples obtained from patients will not be representative of all patients, as some skew towards a stronger emphysema pathology while others present with predominant chronic bronchitis (McDonough et al., 2011). When attempting to characterise both MDM and tissue macrophages it is likely that disease phenotype will lead to high variability between samples, and may prevent identification of clear patterns. It may therefore be beneficial to separate patients based on clinical aspects rather than purely lung function, to determine whether additional differences can be identified, and whether certain treatments are more beneficial for some patients than others.

Another limitation is the *in vitro* culture of cells in FCS which itself contains cytokines which are not representative of human serum, and may therefore influence cell phenotype (Zheng et al., 2006). While all samples were maintained in the same medium, except for the addition of GM-CSF or M-CSF, the role of any background factors in the media should therefore not play a role in differences observed in cell phenotype between GM-CSF and M-CSF cultured cells. However, whether these phenotypes are representative of *in vivo* macrophage function has not been determined, and perhaps culturing cells in human serum vs FCS could aid in identifying some of these differences although the use of autologous serum will complicate these pathways further.

One of the major limitations of the lung tissue macrophage model is that patients undergoing surgery were predominantly doing so for tumour resections (Chana et al., 2014). While the tissue used for experiments was taken from non-cancerous areas of the lung, it may still not be representative of 'healthy' tissue as there are likely to be systemic effects from cells within the tumour. The effect of these on the immune cells and inflammatory responses are not known, and thus changes in macrophage phenotype and function due to cancer cannot be defined. However, even with this major limitation, this model still provides a valuable source of information and studies on macrophages within the lung is essential for better understanding how macrophages function *in vivo*. In addition, it is possible that not all macrophages were liberated from the tissue using this method, and thus a truly representative view of the whole macrophage lung population may not have been achieved. Using alternative methods, such as tissue digestion followed by cell sorting with macrophage markers could provide additional information about the macrophage populations within the lung.

Several experiments investigated gene expression in macrophages, including *MMP2*, *CSF1R* and *CSF2-R α* but with no concurrent measurement of protein level. This is an important limitation as mRNA expression is not always representative of protein level or activity due to post-transcriptional modifications. While MMP9 activity was measured using zymography, MMP2 bands could also be visualised on the gel, but no standard for this protein was included on the gel, and thus analysis for this protein was not performed. Investigating GM-CSF and M-CSF receptor protein level using flow cytometry was unsuccessful due to rapid protein turnover, but understanding how levels of these receptors change in disease would provide valuable information, and alternative methods such as western blot may prove more effective.

Finally, the last limitation is the fact that all data obtained provide only a snapshot of cell phenotype and function, when in reality macrophages are continuously changing and adapting to respond to new environments (Murray *et al.* 2014). Assumptions made in the thesis and other studies therefore

continue to provide further evidence for macrophage phenotyping and in understanding changes which occur in disease.

7.7 Future Work

7.7.1 Further Characterise Cell Fractions Isolated by Density in Tissue and MDM

This thesis has demonstrated that macrophage subpopulations can be isolated from both lung tissue and MDM based on cell density. While there are some differences in gene expression and cytokine output, and other work has shown differences in steroid insensitivity (Chana et al., 2014), it is not yet clear whether these cells represent distinct phenotypes or levels of maturity (Janoff et al., 1976). Further characterisation of cell populations could therefore be useful to better understand macrophage diversity, and to establish whether these populations are skewed in COPD. A wide range of experiments could be performed in order to characterise these cells, including mitochondrial function and ROS production using mitoSOX, measurement of scavenger receptors and phagocytic ability using flow cytometry and confocal microscopy and measuring levels of gene expression of factors such as SIRT1, TNF α , CXCL8, IL-10 and KLF4. Separating these cells using fluorescence-activated cell sorting (FACS) and culturing subpopulations separately may also aid in characterising these cells.

7.7.2 Measure CSF2-R α and CSF1R Gene Expression and Protein Level Following Stimulation

In order to better understand the role of these two growth factors in macrophage phenotype and in driving inflammation, it is crucial to fully understand receptor expression. Results from this study showed that there was no difference in receptor expression at the mRNA level between subject groups, although these did decrease throughout macrophage differentiation. However, these experiments were performed at baseline, and assessing levels following LPS and IL-4 stimulation may yield evidence of differential expression between subject groups. Use of multiple stimuli and measuring expression on multiple time points may be best achieved using Luminex or microarray technologies, to analyse transcriptional changes of these receptors. It is also possible that cell surface

expression is the result of receptor recycling rather than *de novo* gene synthesis, thus investigating protein levels would be of interest. Due to rapid receptor recycling, it has proven difficult to measure receptor expression using flow cytometry, and thus it may be necessary to inhibit protein synthesis with compounds such as cyclohexamide in order to observe differences in receptors at baseline and following stimulation. Additional techniques such as Western blot and confocal analysis should also be considered in an attempt to better understand whether differences exist between cells from different subject groups.

7.7.3 Investigate KLF4 in Macrophages and COPD

The role of KLF4 in macrophage differentiation and as a regulator of inflammation has only recently been uncovered, and more work is needed to further understand its role in this complex pathway. This study is the first to investigate KLF4 in the context of COPD, and to show it may play a role in macrophage differentiation in healthy cells. Additional experiments to measure KLF4 protein level and cellular localisation could be performed using Western Blot and nuclear-cytoplasmic fractionation to determine whether there are differences between non-smoker, smoker and COPD cells. It is also important to assess KLF4 levels in tissue macrophages between subject groups to confirm what was observed in MDM. Lastly, investigating KLF4 interactions and signalling pathways would further highlight the mechanism by which signalling occurs and how it is involved in regulating inflammation and in driving macrophage phenotypes towards resolution.

7.7.4 Analyse Role of Epigenetic Patterns in COPD

This thesis attempted to investigate the importance of histone methylation in macrophage differentiation and function by targeting H3K27met specifically. While these studies showed an important role for this chromatin modification in pro-inflammatory cytokine output, it is only a small scratch into a vast field. Gaining a better understanding of epigenetic changes in macrophages, and how these may drive chronic inflammation in COPD should be further investigated. Many differences have already been documented in murine studies investigating tissue resident macrophages within

different tissue types as well as during differentiation, but further work is needed in human cells, as well as between health and disease to better understand the role of epigenetics in cell phenotype and function (Lavin et al., 2014, Saeed et al., 2014). HDACs have already been shown to be decreased in COPD with potential implications for glucocorticosteroid insensitivity, and other epigenetic changes could also be essential in COPD pathology. Chromatin immunoprecipitation studies and use of chromatin modifying drugs should therefore be considered in an attempt to characterise changes between healthy and COPD macrophages.

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