Macrophage Plasticity in Chronic Obstructive Pulmonary Disease

Amy Day

Imperial College London

National Heart and Lung Institute

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Abstract

Macrophages respond to their microenvironment by altering phenotype. In response to chronic pro-inflammatory signals, such as those in chronic obstructive pulmonary disease (COPD) macrophages can become skewed towards a pro-inflammatory phenotype. However, macrophages are important in the resolution of inflammation and consequently need to alter their phenotype to maintain homeostasis. COPD is a chronic inflammatory condition and therefore the hypothesis of this thesis is that macrophages from COPD patients have lost their ability to alter phenotype and lack plasticity.

This thesis used monocyte derived macrophages (MDM) from non-smokers, smokers and COPD patients differentiated in either GM-CSF (GM-MDM) or M-CSF (M-MDM). It also explored plasticity of these cells through manipulation of culture environment.

The response of these cells to LPS and IL-4 stimulation showed GM-MDM were more pro-inflammatory and less able to phagocytose bacteria compared with M-MDM in non-smokers. It also showed that MDM from COPD patients were more pro-inflammatory and less phagocytic regardless of environment. However, cell surface receptors alone were insufficient at defining phenotype. While MDM from non-smokers were able to alter phenotype in response to environmental change, MDM from COPD patients only became more pro-inflammatory, demonstrating unidirectional plasticity.

Analysis of monocyte subsets within whole blood showed no significant differences between subject groups, suggesting differences in MDM phenotype occur during differentiation. MDM from COPD patients expressed higher levels of pro-inflammatory-linked transcription factors compared with non-smokers and were less able to alter this expression in response to environmental changes, again demonstrating a lack in plasticity in these cells.

MDM from COPD patients lack ability to alter phenotype in response to environmental change, demonstrating a lack of plasticity which accentuates the pro-inflammatory COPD phenotype.

Declaration of Originality

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

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Abbreviations

Abbreviation	Meaning
AAT	A1-Antitrypsin
ADORA2A	Adenosine A_{2A} Receptor
ANOVA	Analysis Of Variance
AP	Activator Protein
APC	Allophycocyanine
Arg1	Arginase1
ASK	Apoptosis Signal-Regulating Kinase
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
BHI	Brain Heart Infusion
BL/6	Black 6
BLF	British Lung Foundation
BSA	Bovine Serum Albumin
CCL	C-C Motif Chemokine Ligand
CD	Cluster of Differentiation
CFU	Colony Forming Units
c-Maf	C-Musculoaponeurotic Fibrosarcoma
COPD	Chronic Obstructive Pulmonary Disease
CSE	Cigarette Smoke Extract
CXC	Chemokine (C-X-C Motif)
CXCL8	Interleukin-8
Cy	Cyanine
DAPI	4',6-Diamidino-2-Phenylindole
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
D-PBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
FBS	Foetal Bovine Serum
FcR	Fc Receptor
FEV_1	Forced Expiratory Volume in One Second
FITC	Fluorescein Isothiocyanate
FIZZ1	Resistin-Like Molecule Alpha
FSC	Forward Scatter
FVC	Forced Vital Capacity
GC	Glucocorticoids
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor

GOLD	Global Initiative For Chronic Obstructive Lung Disease
H_2O_2	Hydrogen Peroxide
HBSS	Hanks Balanced Salt Solution
HDAC	Histone Deacetylase
HI	*Haemophilus Influenzae*
HLA-DR	Human Leukocyte Antigen DR-1
HO-1	Haemoxygenase-1
HRP	Horseradish Peroxidase
IFN	Interferon
Ig	Immunoglobulin
IKKi	Inducible IκB Kinase
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IRF	Interferon Regulatory Factor
JAK	Janus Activated Kinase
LOX1	Lectin-Like Ox-LDL Receptor-1
LPS	Lipopolysaccharide
LTB	Leukotriene
MAF	Musculoaponeurotic Avian Fibrosarcoma
MAP	Mitogen Activated Protein
MCP	Monocyte Chemoattractant Protein
M-CSF	Macrophage-Colony Stimulating Factor
MDA	Malondialdehyde
MDM	Monocyte Derived Macrophage
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteases
MTT	(3-(4,5-Dimethylthiazol-2-Yl))-2,5-Diphenyltetrazolium Bromide
MyD88	Myeloid Differentiation Primary Response 88
NFκB	Nuclear Factor Kappa B
NO	Nitric Oxide
OD	Optical Density
ox LDL	Oxidised Low Density Lipoprotein
PacBlue	Pacific Blue
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein Complex
PPARγ	Peroxisome Proliferator-Activated Receptor-Γ
PS	Phosphatidylserine
RELA	Reticuloendotheliosis Viral Oncogene Homolog A
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SEM	Standard Error of the Mean

SOCS	Suppressor of Cytokine Signalling
SP	*Streptococcus Pneumoniae*
STAT	Signal Transducer and Activator of Transcription
TAM	Tumor Associated Macrophage
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitors of Metalloproteinases
TIRAP	Toll-Interleukin 1 Receptor Domain Adaptor Protein
TLR	Toll-Like Receptor
TMB	5,5' Tetramethylbenzidine
TNFα	Tumour Necrosis Factor-Alpha
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR-Domain Adapter-Inducing Interferon-B
TKB	Tyrosine kinase binding
$^{v}/_{v}$	Volume by Volume
VEGF	Vascular Endothelial Growth Factor
$^{w}/_{v}$	Weight by Volume
WHO	World Health Organisation

Publications

Day AMW, Barnes PJ, Donnelly LE (2014) Lack of plasticity in macrophages from chronic obstructive pulmonary disease (COPD) patients, *Immunology*, 143, pp.150.

Day AMW, Barnes PJ, Donnelly LE (2014) Elevated GM-CSF in COPD may drive defective macrophage phenotype, *European Respiratory Journal*, 44(58), pp.1482.

Day AMW, Barnes PJ, Donnelly LE (2013) COPD Monocytes Differentiate into Pro-inflammatory Macrophages regardless of Environment, *European Respiratory Journal* 42(57), pp.3873.

Abstracts

Day AMW, Barnes PJ, Donnelly LE, 2015, Monocyte sub-populations do not reflect differences in COPD macrophages in COPD macrophage phenotype, European Respiratory Society Annual Congress, Amsterdam.

Day AMW, Barnes PJ, Donnelly LE, 2015, Differential macrophage functions in COPD are not reflected by cell surface markers, European Respiratory Society Lung Science Conference, Portugal.

Day AMW, Barnes PJ, Donnelly LE, Barnes PJ, Donnelly LE, 2014, Elevated GM-CSF in COPD may drive defective macrophage phenotype, European Respiratory Society Annual Congress, Munich.

> €1000 European Respiratory Society Travel Grant for the Best Abstracts on COPD 2014

Day AMW, Barnes PJ, Donnelly LE, 2013, COPD Monocytes Differentiate into Pro-inflammatory Macrophages Irrespective of Their Environment, British Association for Lung Research Summer Conference, Nottingham.

> £100 Travel award British Association for Lung Research Young Scientist Competition

Chana KK, Day AMW, Ward AJN, Barnes PJ, Donnelly LE, 2012, Lack of Macrophage Plasticity in Chronic Obstructive Pulmonary Disease, European Respiratory Society Annual Congress, Vienna.

CHAPTER 1 INTRODUCTION

1.1 Macrophage Function and Signalling

Over 100 years ago, in 1908, Élie Metchnikoff and Paul Ehrlich were awarded the Nobel Prize for their description of cellular immunity and the discovery of phagocytes (Metchnikoff 1905).

Phagocytes make up part of the innate immune system which forms the first line of defence and protects the body from illness caused by foreign micro-organisms, viruses and toxins (Warrington et al. 2011). The innate system comprises several types of leukocytes including monocytes, macrophages, neutrophils, dendritic cells, mast cells, NK cells, eosinophils, and some T-cell subsets. Structural cells, such as epithelial cells, also have a role in the innate immune response.

This thesis will aim to look at the role of macrophages, focusing on their plasticity and their role in chronic obstructive lung disease. The main roles of the macrophages are immune signalling through release of cytokines, phagocytosis and antigen presentation.

1.1.1 Cytokine Production

The release of cytokines and chemokines from macrophages is triggered by the binding of foreign material to the macrophage surface. Lipopolysaccharide (LPS) a component of bacterial cell membranes, binds to a lipid binding protein (LBP) shuttle protein which allows it to connect with cluster of differentiation (CD)14 (the co-receptor for LPS). Upon binding, CD14 subsequently forms a complex with toll-like receptor (TLR)4 which stimulates transcription factor activation and subsequent upregulation of inflammatory cytokines and chemokines such as tumor necrosis factor alpha (TNFα)

and chemokines such as interleukin 8 (CXCL8). Once produced these cytokines go on to stimulate pro-inflammatory processes. CXCL8 binds to the CXCR1 or CXCR2 receptors, (G-protein coupled receptors) on neutrophils (Raghuwanshi et al. 2012). Once activated, this causes upregulation of integrin within the neutrophils which adhere, migrate and degranulate, releasing digestive enzymes and reactive oxygen species (ROS) which kill the invading pathogen (Segal 2005). TNFα has several functions within the immune system (Parameswaran & Patial 2010). Once produced it can stimulate further pro-inflammatory cytokine release via activation of the nuclear factor-kappa B (NF-κB) pathway (Wajant et al. 2002).

1.1.2 Phagocytosis

Phagocytosis describes recognition and engulfment of foreign or non-self material by phagocytes (Mogensen 1979; Janeway et al. 2001). Scavenger receptors (such as the mannose receptor, CD206) and toll-like receptors (such as TLR4), on the surface of macrophages can recognise and bind to bacterial membrane proteins. Once activated, these receptors trigger receptor clustering and Rho-kinase activation which causes actin remodelling leading to the pseudopod extension around the invading material. Once entirely engulfed the material is enclosed within a vesicle known as the phagosome. Lysosomes containing digestive enzymes then fuse with the phagosomes leading to the digestion of ingested material which is then discharged from the cell or presented via MHCII to the adaptive immune cells (Aderem & Underhill 1999). Macrophages can also engulf apoptotic cells in a process known as efferocytosis. Efferocytosis is mediated by recognition of exposed phosphatidylserine (PS) on apoptotic cells by distinct receptors such as CD36 or CD91 (Martin et al. 2014). The engulfing process in efferocytosis is initiated by Rac1 kinase which

stimulates actin polymerisation (Martin et al. 2014). The resulting vesicle (or efferosome) then fuses with lysosomes to destroy the apoptotic material. The adaptive system, consisting of other T-cell subsets and B cells, will then react by targeting the invading material and forming a “memory” against the specific material, allowing for a faster response should the same target be confronted again.

1.2 Macrophage Lineage and Classification

One of the most important cells in the innate immune system is the macrophage. Macrophages can derive from circulating blood monocytes, which, upon activation, migrate into the tissue and differentiate into tissue resident macrophages (Wynn et al. 2013). There is also research indicating some innate tissue-derived macrophage populations which may provide a source of tissue-derived proliferating macrophages which remain in the tissue and replicate upon activation of innate immune responses (Epelman et al. 2014; Czernielewski & Demarchez 1987). However, much of this work has been performed in mice models using fate mapping receptors which do not translate to human macrophage biology, therefore, this phenomenon cannot yet be generalised to humans (Davies et al. 2013).

Macrophages are usually classified based on several factors including lineage, location, receptor expression, cytokine expression and role in disease.

1.3 Tissue Macrophage Locations

Macrophages within different tissues are known to perform different functions; the microglia in the brain are important in brain development and adult physiology (Tremblay et al. 2011), the pancreatic and renal macrophages are essential for organ development (Calderon et al. 2015; Weisheit et al. 2015), osteoclasts (bone macrophages) allow for bone turnover and repair (Cho 2015) while cardiac macrophages are crucial for repair after infarction (Visan 2014) (Figure 1.1). Other macrophages, such as those in the skin, liver, gut, spleen and lung are more specialised for immune surveillance. These organs are more likely to come into contact with foreign material, and so the macrophages here play essential roles in the innate immune response.

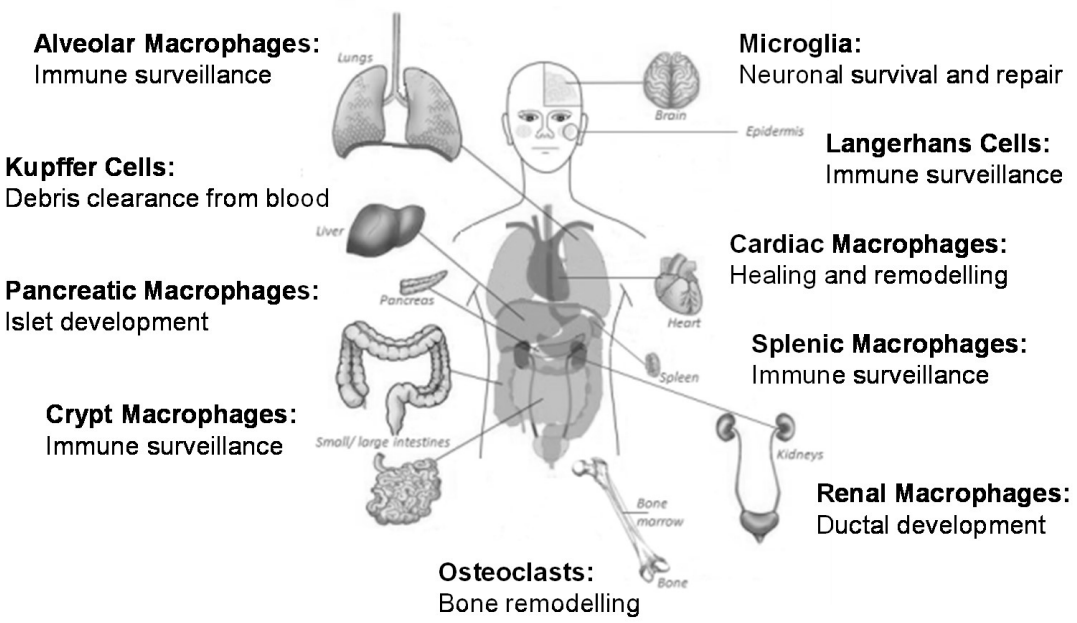


Figure 1.1: Location and classification of human macrophages. Schematic showing the types and locations of macrophages in human tissues. Adapted from (Murray & Wynn 2011).

1.4 Monocyte Derived Macrophages

As well as location defining macrophage classification and function, it has been hypothesised that different macrophage populations may develop from different monocyte populations. Monocytes can be classified into three subtypes based on their relative expression of CD14 and CD16 (low affinity immunoglobulin (Ig)G Fc receptor) (Passlick et al. 1989). The first subtype are classical monocytes which are $CD14^{++}CD16^{-}$. The less common, intermediate monocytes display a $CD14^{++}CD16^{+}$ cell surface expression while finally the non-classical monocytes exhibit a $CD14^{+}CD16^{++}$ profile. Classical monocytes are the most common subtype, making up approximately 90% of the total in a healthy person (Strauss-Ayali et al. 2007). They have been shown to be critical in innate immunity due to their enhanced ability to recognise LPS on the surface of bacteria (Stansfield & Ingram 2015). Non-classical monocytes, on the other hand, have been associated with the onset of infection and pro-inflammatory processes based on their high TNFα cytokine expression, and low interleukin-10 (IL-10) ($TNF\alpha^{High}$ $IL\text{-}10^{Low}$) (Frankenberger et al. 1996) and their enhanced ability to present antigens (Passlick et al. 1989). The expression of non-classical monocytes is also reportedly increased in rheumatoid arthritis (Hepburn et al. 2004), atherosclerosis (Rothe et al. 1996) and bacterial sepsis (Fingerle et al. 1993) which indicates they may exhibit a pro-inflammatory nature.

Intermediate monocytes, as a percentage of total, make up the smallest of the three subsets (1-3% in a healthy person) (Passlick et al. 1989) and may represent the transitional phenotype between the classical and non-classical subsets or they may be the progenitors of an entirely different population altogether. Unfortunately, this is not yet clear due to the lack of a translational lineage markers in human monocytes (Schneemann & Schoeden 2007). Indeed, most of the lineage and fate-mapping data

accumulated thus far has been from murine studies using the mouse lineage macrophage marker Ly6C (Rose et al. 2012), which has no current human equivalent.

Conversely, the theory of different macrophage phenotypes developing from different monocyte populations has been challenged with the indication of inherent tissue resident macrophage populations. Some studies have suggested that the macrophages found commonly in tissues are established before birth (Crofton et al. 1978; Yona et al. 2013). These studies suggest that the “normal” steady state adult macrophage population is self-renewing (Tarling et al. 1987) and only during infection or chronic inflammation is there an influx of monocyte derived cells (Jenkins et al. 2011).

1.5 Cell Surface and Cytokine Classification

The availability of monoclonal antibody technology, along with multi-colour flow cytometric advances, have enabled the ability to classify cells into subtypes based on receptor expression. The idea of classifying macrophages based on their cell surface repertoire first emerged in 1992 when Stein et al. discovered that IL-4 stimulation led to the generation of “alternatively activated macrophages” with increased levels of mannose receptor expression (CD206) compared with the “classic” interferon (IFN)γ stimulated cells (Stein et al. 1992).

After the discovery that Th1 mouse macrophages (C57BL/6, B10D2) stimulated with IFNγ and LPS released significantly more pro-inflammatory nitric oxide (NO) compared with Th2 strains (BALB/c, DBA/2), the terms M1 and M2 were derived to describe the pro- and anti-inflammatory macrophage populations respectively (Mills et al. 2000). These terms were widely accepted and various research began describing the linear polarisation of these cells into the specific M1 or M2 phenotypes based on

their cytokine and cell surface profiles (Alberto Mantovani et al. 2002) (Figure 1.2A). Mantovani et al, went on to describe an additional three distinct M2 macrophage subsets (M2a, M2b, M2c) which, although activated by differing stimuli, all produced the anti-inflammatory cytokine IL-10 (Mantovani et al. 2004). Since these macrophage phenotypes were proposed, several other functionally distinct macrophages have been discovered which do not ascribe to the M1 or M2 phenotype (Table 1.1).

Tumour-associated macrophages (TAMs) are expressed in tumour tissues and are thought to promote tumour development and progression through their anti-inflammatory, immune suppressive phenotype (Lin & Pollard 2007; Mantovani et al. 2004). M2d macrophages are a type of TAM which are activated by IL-6 and utilise macrophage-colony stimulating factor (M-CSF) to create an anti-inflammatory immunosuppressive phenotype (Duluc et al. 2007).

Several populations of macrophages, specific to atherosclerosis, have now been identified with the development of knowledge in this disease. M4 macrophages, activated by platelet factor 4 (chemokine (C-X-C motif) ligand 4 (CXCL4)), express a different transcriptome compared with M1 and M2 macrophages (Gleissner 2012). They also demonstrate a reduced ability to phagocytose and a loss of atheroprotective haemoxygenase-1 (HO-1) (Colin et al. 2014). Expression of these M4 macrophages are also significantly increased in atherosclerotic plaques (Erbel et al. 2015) and they are thought to contribute to plaque rupture (Braganza & Bennett 2001) making them leading to a subsequent pro-atherosclerotic phenotype.

The term Mhem was coined by Boyle et al 2012, when they described another separate macrophage phenotype present in atherosclerotic plaques. These macrophages were activated by haem which lead to induction of the anti-oxidant gene,

HO-1 and promoted cholesterol efflux, giving the cells a distinct atheroprotective phenotype (Boyle et al. 2009). Another phenotype of macrophages related to atherosclerosis are the Mox macrophages. Originally described by Kadl et al in 2010, these cells are found in atherosclerotic lesions, and are activated by oxidised low density lipoprotein (ox LDL) (Kadl et al. 2010). They have a distinct pro-inflammatory genetic signature which, again differs from M1 and M2 macrophages and are thought to be pro-atherogeneic in nature (Kadl et al. 2010).

	M1 (Classic)	M2a (Wound	M2b (TAM)	M2c (Regulatory)	M2d (TAM)	M4	MHem	Mox
Activation	IFNγ LPS GM-CSF	IL-4 IL-13 M-CSF	IL-1R	IL10 TGFβ GC	IL-6 Adenosine M-CSF	CXCL4	Heme	oxLDL
Cell Surface Receptor Expression	CD80 CD86 CD16 MHC II TLR4 TLR2	CD163 CD206 CD14 *Mouse:* Ym1/2, Fizz1 Arg1	CD86 MHC II	CD163 TLR1 TLR8	ADORA2A	CD86 MHC II	CD163	LOX1 CD36
Cytokine Expression	TNFα IL-6 IL-12	TGFβ IL-10	TNFα IL-6 IL-10	TGFβ IL-10	TNFα TGFβ IL-10	TNFα IL-6	HO-1 IL-10	HO-1 VEGF IL-1β

Table 1.1 Summary of defined macrophage phenotypes. IFNγ – Interferon gamma, LPS – Lipopolysaccharide, GM-CSF – Granulocyte macrophage colony stimulating factor, IL – Interleukin, TGFβ – Transforming growth factor beta, GC – glucocorticoids, CXCL4 – Platelet factor 4, oxLDL – oxidised low density lipoprotein, MHC – Major histocompatibility complex, FIZZ1 – Resistin-like molecule alpha, Arg1 – Arginase1, ADORA2A – adenosine A_{2A} receptor, LOX1 - Lectin-like oxLDL receptor-1, TNFα – Tumour necrosis factor alpha, iNOS – inducible Nitric oxide synthase, HO-1 – heme oxygenase 1, VEGF – vascular endothelial growth factor, TAM – Tumour associated macrophage. Adapted from (Boyle et al. 2009; Martinez et al. 2009; Mantovani et al. 2004; Anders & Ryu 2011; Rőszer 2015)

1.6 Macrophage Heterogeneity and Plasticity

Mosser and Edwards further reviewed the concept of macrophage activation in 2008 and proposed a spectral system of classification whereby, rather than being called M1, M2a/b/c etc., macrophages are classified based on their homeostatic function within tissue environments, namely “classically activated”, “wound healing” and “regulatory macrophages” (Mosser & Edwards 2008). This was also the first comprehensive description of macrophage plasticity. This is the idea that macrophage phenotypes, rather than maintaining a singular function, actually dynamically change based on cues in their environment, indicating a high level of plasticity in these cells (Figure 1.2B). Therefore, theoretically, once a classically activated, pro-inflammatory macrophage has fulfilled its task of attracting other immune cells and killing invading material, it may subsequently transform into an anti-inflammatory resolving, highly phagocytic macrophage in order to remove any debris and effectively resolve the immune response.

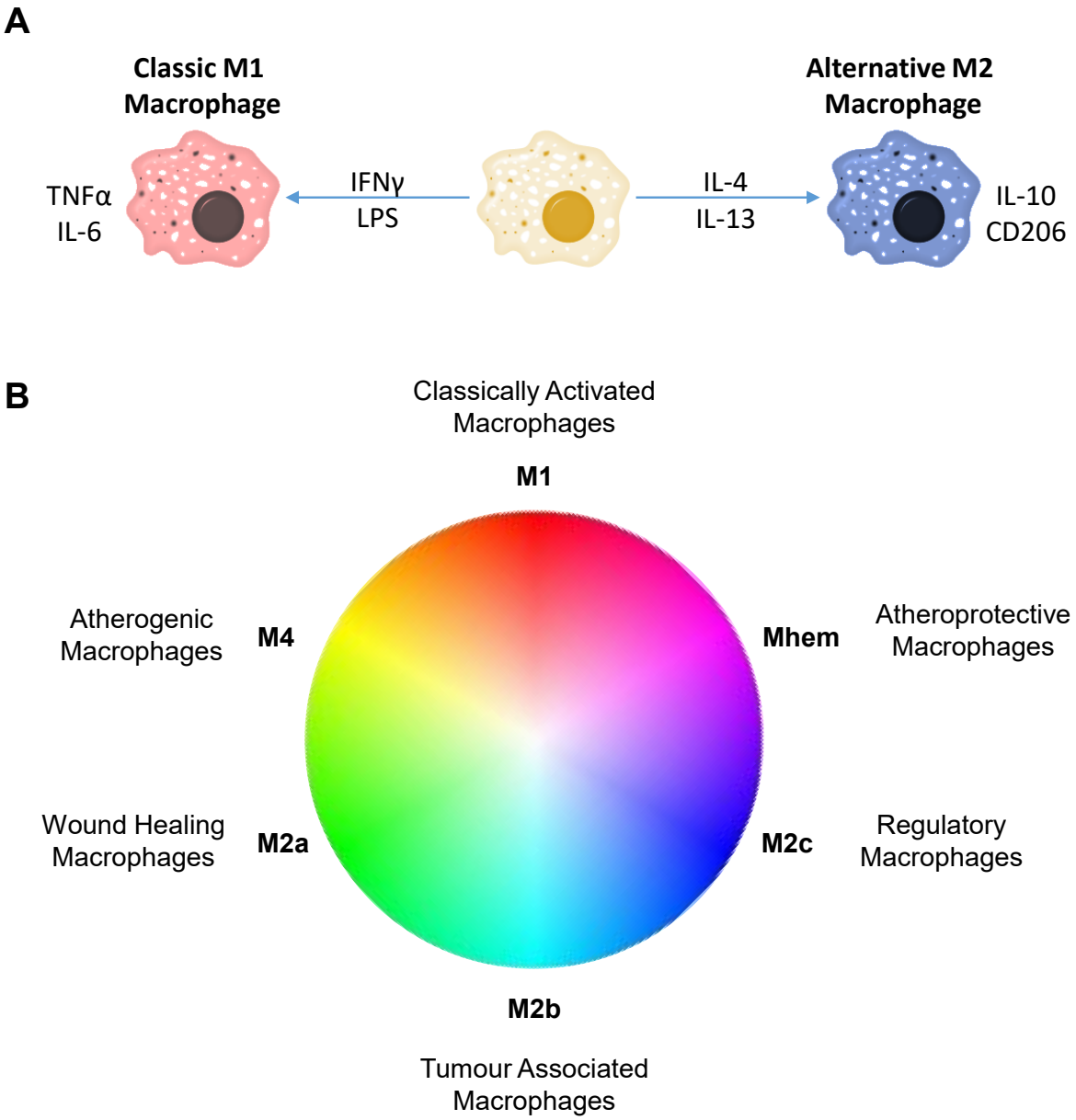


Figure 1.2: Linear *vs*. Spectral polarisation of macrophages. Schematic detailing the previously accepted linear model of macrophage polarisation into either pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages (**A**) against the more spectral model of macrophage phenotype which incorporates several subsets of macrophage and suggests macrophages can adapt to their environment and can change phenotype accordingly (**B**). Adapted from Mosser and Edwards 2008. More recently discovered macrophage phenotypes added to the spectrum according to overlap in activation markers/cell surface receptor descriptions. (Mosser & Edwards 2008)

Phenotypic plasticity of macrophages has been demonstrated in various murine macrophage models. A notexin induced muscle injury/regeneration model in the mouse showed, through measuring changes in cytokine levels and cell surface markers over time, different subsets of macrophages sequentially dominate during different stages of muscle repair (Arnold et al. 2007). During muscle injury, macrophages were pro-inflammatory, producing high levels of TNFα and IL-1β, then during the muscle regeneration stage, this population shifted to an anti-inflammatory phenotype, producing high levels of IL-10 and transforming growth factor beta (TGFβ) (Arnold et al. 2007). Another study showed “M2 like” murine TAMs which were exposed to IL-12, switched to a more pro-inflammatory, pro-immunogenic macrophage population (producing high levels of TNFα, IL-15 and IL-18) (Watkins et al. 2007).

Demonstrating macrophage plasticity in human tissue macrophages is far more elusive due partly to lack in translational murine markers and availability of these cells without invasive procedures. However, one study indicated macrophage plasticity using a model of human monocyte-derived-macrophages (MDM). Using MDM isolated from healthy human peripheral blood, Porcheray *et al.,* examined the ability of MDM to switch phenotype after a change in stimuli (Porcheray et al. 2005). This study demonstrated healthy human MDM had a high degree of plasticity, and were able to switch from a pro-inflammatory phenotype ($CD163^{low}CD206^{low}CCL3^{high}$) to an anti-inflammatory ($CD163^{high}CD206^{high}CCL18^{high}$) phenotype and *vice versa*. These studies also indicate a possible therapeutic initiative, allowing phenotypic manipulation of macrophages which could be applied to various macrophage-associated immune diseases (Fleming & Mosser 2011; Buhtoiarov et al. 2006).

1.7 Factors Affecting Macrophage Phenotype

1.7.1 GM-CSF and M-CSF

There are many factors which influence the phenotype of macrophages, the most important being their local environment. Studies have shown that the colony stimulating factors Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and Macrophage-Colony Stimulating Factor (M-CSF) are crucial in determining macrophage phenotype and function (Bergamini et al. 2000; Hamilton 2008). GM-CSF cultured cells had increased pro-inflammatory gene expression (TNFα and CXCL8), compared with the increased anti-inflammatory gene expression (IL-10) elicited by the M-CSF cultured cells (Lacey et al. 2012) leading to their association with M1-like and M2-like macrophage phenotypes, respectively.

1.7.2 Transcriptional Influences on Macrophage Phenotype

Various transcription factors are known to be important in both macrophage differentiation and polarization (Martinez et al. 2006). Some transcription factors are associated with a pro-inflammatory phenotype (AP1, STAT1, IRF3 and IRF7) (Figure 1.3). *Jun* is the gene which encodes for c-Jun. In response to Th1 type cytokine activation, and downstream of several kinases, c-Jun is phosphorylated and dimerises with c-Fos to form the transcription factor activator protein (AP)1 (Czaja et al. 2003). AP1 then migrates to the nucleus where it binds and upregulates pro-inflammatory gene expression in macrophages (Biswas & Mantovani 2010).

Signal transducer and activator of transcription (STAT)1, another pro-inflammatory associated transcription factor is encoded by the gene *STAT1* and is known to regulate interferon stimulated pro-inflammatory gene expression in macrophages (Sica &

Mantovani 2012). Upon IFNγ stimulation, STAT1 in the cytoplasm is phosphorylated by Janus activated kinase (JAK) and forms homodimers or can also dimerise with other STAT1 molecules before translocating to the nucleus and upregulating pro-inflammatory gene expression (Khodarev et al. 2004). The interferon regulatory factor (*IRF)3* gene encodes for the transcription factor IRF3, which is phosphorylated by the paired inducible IκB kinase (IKKi)-tyrosine kinase binding 1 (TKB1) compound, before dimerising and translocating to the nucleus where it upregulates *TNFα* and another of the interferon regulatory factors, *IRF7* (Biswas et al. 2006). The *IRF7* gene encodes for the transcription factor IRF7 which is important in virally induced, pro-inflammatory interferon signalling. Like IRF3, IRF7 is also phosphorylated by the IKKI-TKB1 compound. It then forms homodimers before translocating to the nucleus where it upregulates pro-inflammatory IFNγ in macrophages (Günthner & Anders 2013). V-rel avian reticuloendotheliosis viral oncogene homolog A (*RELA)* is the gene which encodes for the p65 sub unit of nuclear factor-kappa B (NF-κB). Upon LPS stimulation of Toll-like receptor 4 (TLR4), and subsequent signalling through toll-interleukin 1 receptor domain adaptor protein (TIRAP) and myeloid differentiation primary response 88 (MyD88) which removes the IκB from the NF-κB complex. The uninhibited p65 (RELA) /p50 NF-κB complex can then translocate and trigger upregulation of inflammatory genes such as TNFα, CXCL8 and IL-12 in macrophages (Lawrence & Natoli 2011).

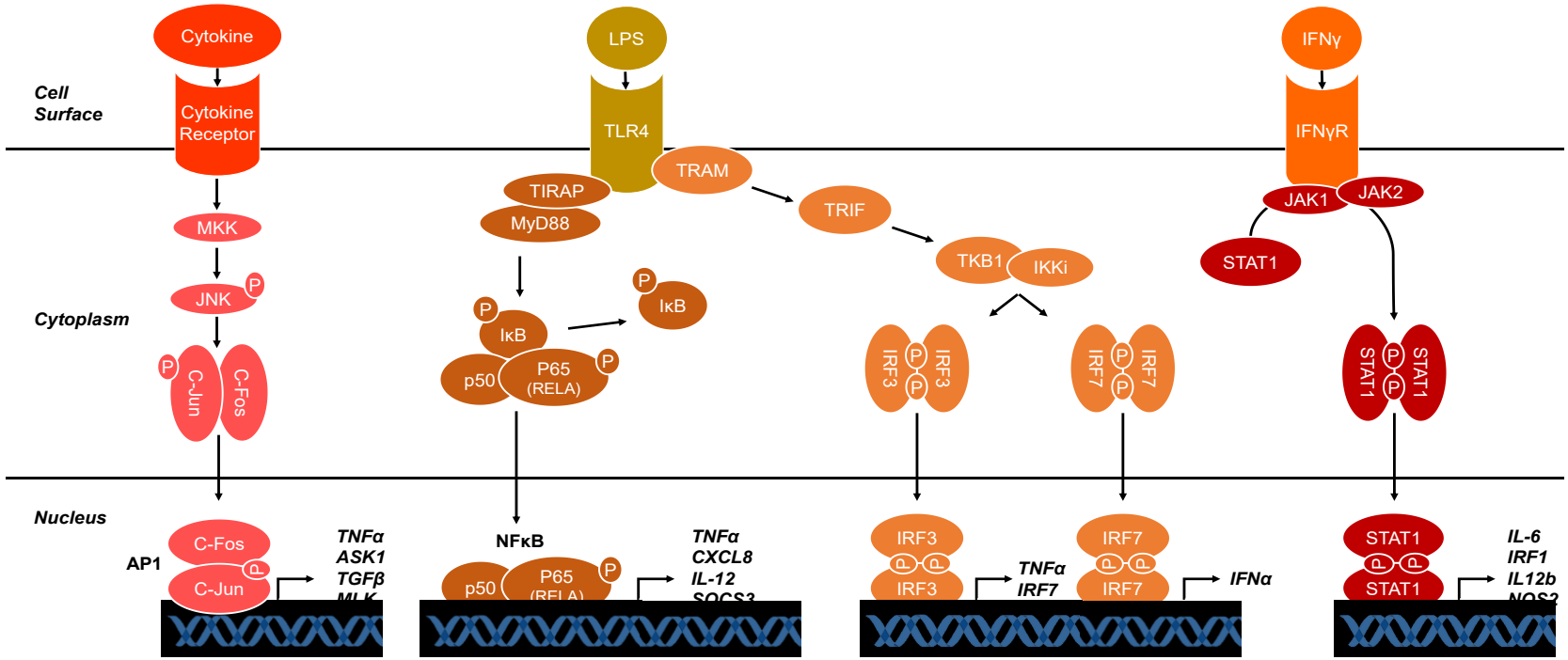


Figure 1.3: Transcriptional regulation of pro-inflammatory macrophage phenotype. Schematic diagram of transcriptional signalling in pro-inflammatory macrophages. MSK: mitogen stress activated kinase, TLR: toll-like receptor, LPS: lipopolysaccharide, TIRAP: toll-interleukin 1 receptor domain adaptor protein, MyD88: myeloid differentiation primary response 88, RELA: reticuloendotheliosis homolog A, NF-κB: nuclear factor-κB, TRIF: TIR-domain adapter-inducing interferon-β, TRAM: TRIF-related adaptor molecule, TKB: tyrosine kinase binding, IRF: interferon-regulatory factor, STAT: signal transducer and activator of transcription, IL: interleukin, IFNγ: interferon-γ, JAK: Janus kinase, ASK: Apoptosis signal-regulating kinase, TGFβ: Transforming growth factor beta, SOCS: Suppressor of cytokine signalling, TNF: tumour necrosis factor, NOS: nitric oxide synthase, Adapted from: (Lawrence & Natoli 2011; Martinez et al. 2006; Chawla 2010)

While these transcription factors drive pro-inflammatory processes in macrophages, other transcription factors, such as STAT6 and c-Maf, are associated with an anti-inflammatory phenotype (Figure 1.4). STAT6 is encoded by the gene *STAT6.* Upon IL-4 binding to the IL-4 receptor (IL-4Rα) STAT6 is phosphorylated by JAK and forms homodimers which translocate to the nucleus (Wurster et al. 2000). Binding of STAT6 is known to regulate IL-4 mediated anti-inflammatory gene expression in macrophages and induces upregulation of anti-inflammatory genes including arginase, peroxisome proliferator-activated receptor-γ (PPARγ) and IL-10 (Sica & Mantovani 2012). Another anti-inflammatory linked transcription factor is c-Maf. c-Maf is encoded by the gene *MAF*. Upon IL-4 activation, c-Maf translocates to the nucleus where it binds to the promoter of IL-10 and upregulates expression and is essential for the production of IL-10 by macrophages (Cao et al. 2005).

All of these transcription factors have been shown to influence macrophage phenotype but as of yet, the levels and roles of these factors in human macrophage plasticity is yet to be defined.

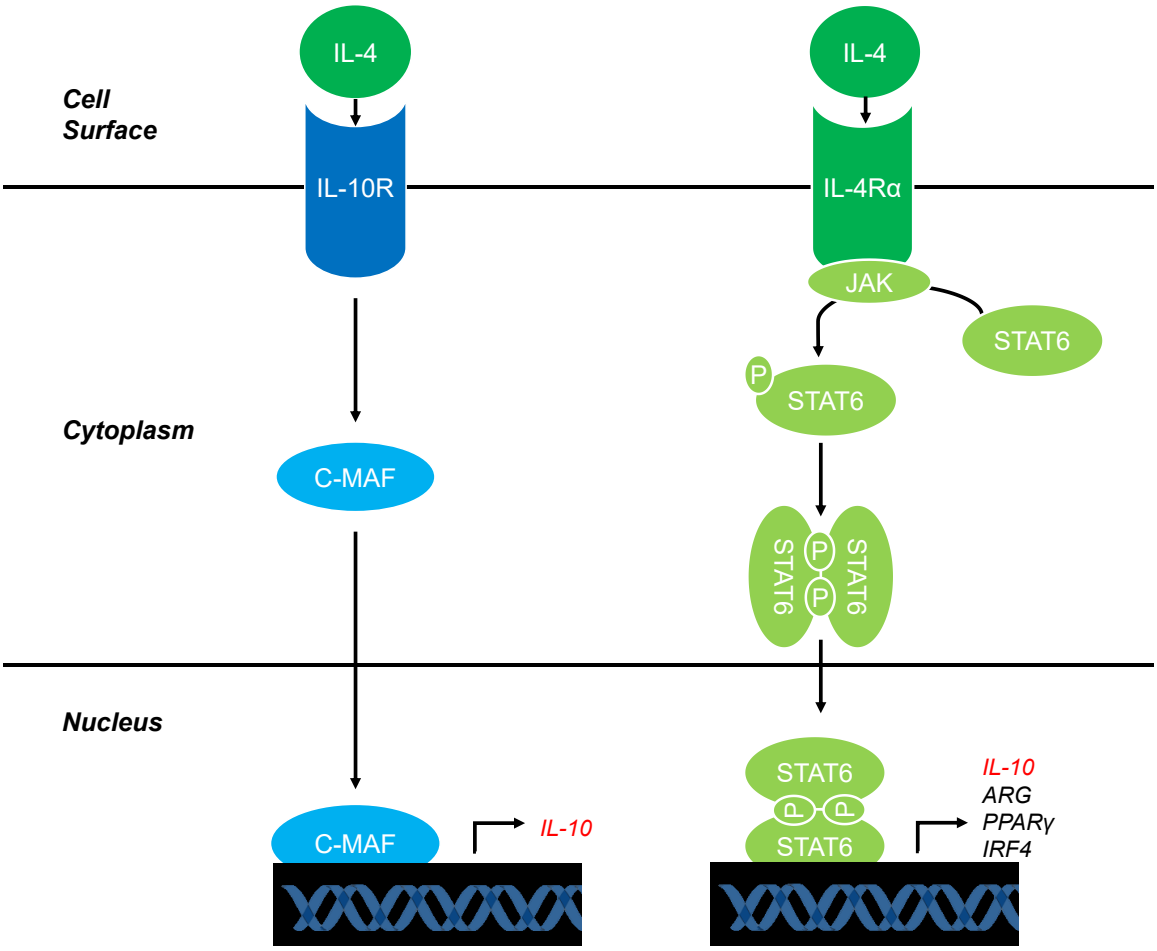


Figure 1.4: Transcriptional regulation of anti-inflammatory macrophage phenotype. Schematic diagram of transcriptional signalling in anti-inflammatory macrophages. IL: interleukin, STAT: signal transducer and activator of transcription, JAK: Janus kinase, MAF: musculoaponeurotic avian fibrosarcoma, ARG: arginase, PPARγ: peroxisome proliferator-activated receptor-γ, IRF: interferon-regulatory factor. Adapted from: (Wurster et al. 2000; Cao et al. 2005).

1.8 Macrophages Plasticity in Disease

The ability of macrophages to change phenotype is important since the response to bacterial or viral infection requires strict regulation of the necessary pro- and anti-inflammatory processes in order to combat pathogens, whilst also avoiding damage to the healthy tissue.

However, in some immune mediated conditions and diseases, macrophages can become skewed towards a particular phenotype and, with an exaggerated response, can subsequently drive disease pathogenesis (Sica & Mantovani 2012). One example of this is during sepsis, where macrophages are exposed to high levels of endotoxin and become desensitised and skew towards a highly immunosuppressive 'M2-like' phenotype which can ultimately lead to mortality (Biswas & Lopez-Collazo 2009). Another example is in atherosclerosis in which 'M1-like' polarised macrophages have been more heavily associated with Ox-LDL uptake (Hirose et al. 2011) and foam cell formation (Dushkin 2012). These cells also localise to atherosclerotic plaques (Stöger et al. 2012) where they then contribute to rupture and thrombosis associated with atherosclerosis mortality (Hasan et al. 2012).

Tumour associated macrophages represent a cell which, because of their skewed phenotype, drive pathogenesis of some cancers. Their excessive tissue remodelling and proangiogenic nature coupled with their immunosuppressive phenotype, allow for the development and progression of the cancer (Lin & Pollard 2007; Mantovani et al. 2004). Another disease in which macrophage phenotypic skewing becomes detrimental is chronic obstructive pulmonary disease (COPD).

1.9 Chronic Obstructive Pulmonary Disease

1.9.1 Definition and Statistics

COPD is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines as:

"*a common preventable and treatable disease, characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients.*" (GOLD 2016).

As of January 1st 2013 there were 1,201,685 diagnosed cases of COPD in the UK and the incidence of new disease is approximately 114,219 new cases per year. This figure is equal to one new case every 5 minutes, and incidence is rising (British Lung Foundation, The Battle for Breath Report, (British Lung Foundation 2016)).

The burden on health services equates roughly to 140,000 admissions (1.7% of total admissions) each year, most of which are to emergency care. The mortality rate is also increasing, and COPD is now the UK's fifth largest cause of death with around 30,000 people dying per year from the disease (British Lung Foundation 2016).

Interestingly, London has one of the highest recorded hospital admissions for COPD, compared to similar regions in the UK, although a direct cause for this higher incidence has yet to be distinguished.

1.9.2 Risk Factors

1.9.2.1 Cigarette Smoking

Smoking is by far the greatest cause of COPD with at least 80% of cases in the UK attributed to cigarette smoking (NICE 2010). Studies have shown direct correlations between high pack year history (1 pack year = 20 cigarettes per day for 1 year) and decline in lung function (Willemse et al. 2004). Smoking cessation is also associated with an improvement in lung function after 5 years in COPD patients (Scanlon et al. 2000). Although the incidence COPD patients who smoke (or have smoked previously) is high, the overall percentage of smokers who develop COPD is relatively low, approximately 15% (Stratelis et al. 2004), which suggests that smoking may be one of a multitude of triggers required for development of the disease, and hints at an inherent defect in those who go on to develop COPD.

1.9.2.2 Genetics

Alpha-1-antitrypsin (AAT) is a protease inhibitor which is important for maintaining the protease/anti-protease balance in the lung. AAT deficiency, caused by a mutation on chromosome 14, is a rare genetic disease which currently affects approximately 1 in every 3000 people in the UK (BLF 2016) and can lead to an imbalance of the protease/anti-protease balance in the lung. Severity is related to the number of alleles affected; if one allele is deficient (PI*MZ) the resultant phenotype is mild compared with the double allele deficiency (PI*ZZ) which is associated with a marked decrease in serum AAT and increased risk of emphysema (Hersh et al. 2004). Lack of this anti-protease leads to increased and uncontrolled protease activity that causes damage to the pulmonary tissue and leads to development of emphysema (Tetley et al. 1987; Mittman & Taylor 1985).

1.9.2.3 Air Pollution

In contrast to high-income countries where tobacco smoking is the largest risk factor for COPD, in middle and low-income countries, there is a high incidence of non-smoking related COPD which can be attributed to exposure to air pollution and inhalation of biomass. There is a high incidence in these countries of non-smoking female COPD in women who burn biomass indoors as fuel for cooking (Salvi & Barnes 2009). Current research is working to understand if the underlying phenotype of the disease is similar to that of smoking-related COPD (Ghosh et al. 2015; Holloway, Guney, et al. 2014; Salvi & Barnes 2009).

1.9.2.4 Age

COPD is associated with an increase in age with estimates that globally, COPD afflicts 10% of the population over the age of 40 (Buist et al. 2007). The statistics for the UK indicate that as of 2012 approximately 9% of people over the age of 71 in the UK had been diagnosed with COPD (Figure 1.5).

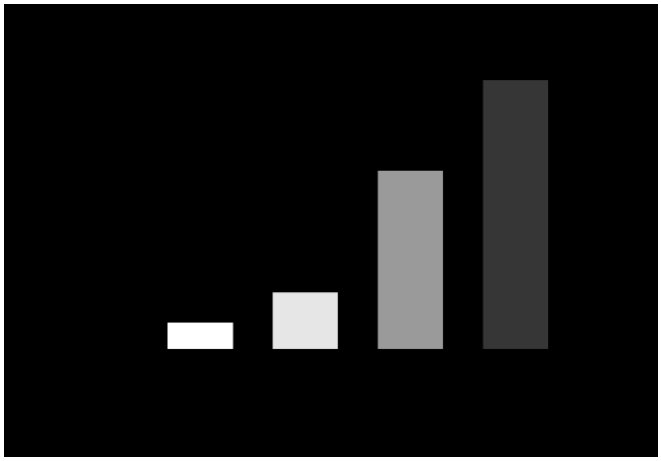


Figure 1.5: Percentage of diagnosed COPD cases increases with increasing age. Data taken from BLF report of 2012 WHO figures for percentage of diagnosed COPD cases in the UK within specific age groups (British Lung Foundation 2016).

1.9.2.5 Gender

Men are statistically 10% more likely to be diagnosed, and die from COPD compared with women. This is probably due to the historical prevalence for male smoking compared with females. Interestingly, this pattern may change to reflect the increasing number of female smokers in the population (ASH Report 2007), but as COPD is a long term illness in which the symptoms do not develop until later life, this may require longitudinal studies.

1.9.3 Pathology

There are three underlying pathophysiologies associated with COPD; chronic bronchitis, small airway disease and emphysema (Abboud & Vimalanathan 2008; Roth 2008) (Figure 1.6).

1.9.3.1 Chronic Bronchitis

Chronic bronchitis is a condition characterised by mucous hypersecretion and poor mucus clearance in the airways (Reid 1954). This, coupled with immune cell infiltration (Hogg 2004a), gives rise to the symptoms of cough and increased sputum production (Burton & Dixon 1969). Mucus hypersecretion is caused by goblet cell hyperplasia and hypertrophy which can be in response to smoking (Innes et al. 2006), bacterial infection or immune cell mediated activation of mucin genes (Rubin 2002). Studies indicate that both healthy smokers and smoking COPD patients have increased numbers of airway goblet cells (Innes et al. 2006), possibly as a response to chronic exposure to harmful chemicals in cigarette smoke (Saetta 1999). The layer of lingering mucus then provides ample environment for bacterial growth (Sethi et al. 2002)

including *Haemophilus influenzae* and *Streptococcus pneumoniae* which contribute to exacerbations in COPD symptoms (Erkan et al. 2008).

1.9.3.2 Small Airway Disease

Small airway disease is characterised by marked airway remodelling and obstruction in the small airways of the lung (those that are non-cartilaginous and are less than 2mm diameter) (Hogg et al. 2013). The obstruction is caused by airway wall thickening in smokers and patients with COPD (Deveci et al. 2004) and an increase in fibrosis in response to cell damage caused by cigarette smoke (Hogg 2004a). The extracellular matrix deposition is altered in COPD patients (Bidan et al. 2015) with reduced expression of peptidoglycans and elastins (Black et al. 2008). There is some overlap with chronic bronchitis, in that there is increased immune cell infiltration in small airway disease (Hogg 2004a) and increased mucus secretion which can lead to complete airway occlusion (Cosio et al. 1980).

1.9.3.3 Emphysema

Emphysema is defined as the damage and enlargement of alveolar sacs in the lung (Thurlbeck 1984). The driver of this destruction is exaggerated and unregulated elastase and other proteases (Demkow & van Overveld 2010). Increased CXCL8 in the lungs of smokers and non-smokers (Blidberg et al. 2012) leads to increased infiltration of neutrophils which release neutrophil elastase (Sandhaus & Turino 2013). There is also an increase in macrophage infiltration into the lung (Grashoff et al. 1997) which produce matrix metalloproteases (MMP) (Shapiro 2002). The chronic imbalance of proteases to anti-proteases leads to the destruction of the alveolar epithelium (Tetley et al. 1987) and correlates directly with the decline in lung function (Montaño et al. 2014).

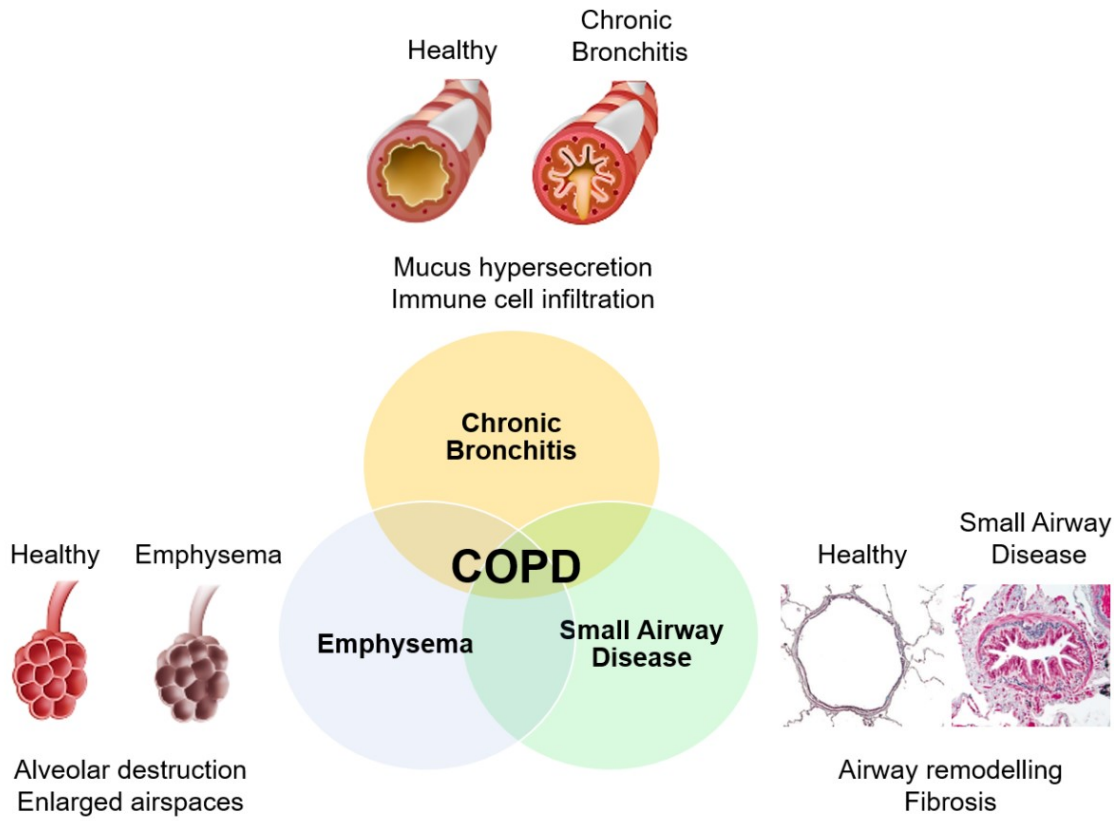


Figure 1.6: COPD pathophysiology. COPD encompasses three pathophysiologies; chronic bronchitis, emphysema and small airway disease. The contribution of each pathophysiology may vary between individuals. Combination leads to symptoms expressed by COPD patients. Adapted from (Tubby et al. 2011; Hogg 2004a; Barnes 2004).

1.9.4 Current Treatment

There are currently no medications which are disease modifying or stop progression of COPD (Barnes 2003). Medication including bronchodilators, glucocorticosteroids and oxygen therapy are commonly used to treat symptoms and prevent exacerbations (Sonetti et al. 2010; Menezes et al. 2011) although the efficacy of these therapies is poor (Barnes 2013). Most medications work by either trying to supress the immune response or act as bronchodilators, for example, β2-agonists which work to reduce pro-inflammatory GM-CSF production (Kaur et al. 2008). Other medications aim to

reduce smooth muscle contraction, e.g. anticholinergics which work to try and reduce air resistance by promoting relaxation in airway muscle (Cazzola & Hanania 2006; Scullion 2007). Oral and inhaled steroids are also commonly given to patients, especially during exacerbation (Singh et al. 2002), however there is conflicting evidence on the ability of steroids to improve lung function (McIvor et al. 2011; Highland et al. 2003) and long term steroid use can lead to considerable side effects (Niewoehner et al. 1999). Inhaled corticosteroids work by binding to the glucocorticoid receptor which leads to the downregulation of NFκB driven inflammatory gene expression (Ito et al. 2005). This process is dependent on a histone deacetylate (HDAC), specifically HDAC2, which has been shown to be significantly reduced in the alveolar macrophages from COPD patients (Cosio et al. 2004). The reduction in HDAC2 observed in COPD, is thought to be linked to oxidative stress induced nitrosylation of the cysteine residues.

The transcription factor Nuclear factor erythroid 2–related factor 2 (Nrf2), which induces various antioxidant genes (Malhotra et al. 2008), is also reduced in COPD alveolar macrophages (Suzuki et al. 2008). One study, by Malhotra et al, showed that restoration of NRF2 activation with sulforaphane was able to restore HDAC activity and subsequent steroid sensitivity in alveolar macrophages from COPD suggesting that tis pathway may be worth targeting with regards to COPD treatment. Although a recent clinical trial of oral sulforaphane showed no effect on NRF2 activity in alveolar macrophages from COPD patients (Wise et al. 2016). Unfortunately, these many COPD treatments work by suppressing immune responses, which leaves the patient vulnerable to secondary bacterial infections (King et al. 2013) that ultimately contribute to progression of the disease (Sethi et al. 2002) and increased exacerbation frequency (Monsó et al. 1995; Wedzicha & Donaldson 2003).

1.9.5 Immune Cells in COPD

1.9.5.1 Neutrophils and T-cells

Studies show increased levels of neutrophils in COPD sputum (Stănescu et al. 1996) bronchoalveolar lavage (Capelli et al. 1999) and lung tissue (Baraldo et al. 2004) which correlate with severity of COPD (MacNee et al. 1989). This is thought to be caused by the increased levels of the potent neutrophil chemoattractant CXCL8 in the lungs of COPD patients (Beeh et al. 2003; Tanino et al. 2002), but other neutrophil chemoattractants are also increased including leukotriene B_4 (Beeh et al. 2003), CXCL1, CXCL5 and CXCL7 (Traves et al. 2002; Kaur & Singh 2013; de Boer et al. 2000). Studies have shown that these neutrophils are highly pro-inflammatory (Yamamoto et al. 1997; Stockley 2002; Noguera et al. 2004) and produce higher levels of damaging proteases compared with cells from non-smokers (Kamal et al. 2002) leading to the degradation of lung tissue and emphysema.

There is also a higher number of CD8+ cytotoxic T-cells in both sputum (Chrysofakis et al. 2004; Tzanakis et al. 2004) biopsies (Zhu et al. 2001) and lung paranchyma (Saetta 1999; Cosio et al. 2002) from COPD patients compared with healthy controls. Their increased infiltration has been correlated with decline in lung function in COPD patients (Freeman et al. 2010; O'Shaughnessy et al. 1997) and is thought to be caused by the release of cytotoxic granules from these cells which drive apoptosis of type II pneunocytes leading to an emphysematous phenotype in the lung (Chrysofakis et al. 2004).

1.9.5.2 Epithelial Cells in COPD

The upper airway epithelium is one of the first barriers to come into contact with inhaled cigarette smoke. Toxic agents in cigarette smoke are thought to drive epithelial turnover and promote fibroblast driven fibrosis leading to increased airway obstruction and resistance (Vlahovic et al. 1999). This significant remodelling of the airways is a characteristic of small airway disease seen in COPD (Aoshiba et al. 2003). In addition to fibrosis, goblet cell hyperplasia and reduced mucociliary clearance within the epithelial layer both contribute to the mucus accumulation associated with chronic bronchitis (Hogg 2004b). The alveolar epithelium is made up of type 1 cells (AT1), and type 2 cells (AT2). AT1 cells have a large surface area to allow for gaseous exchange while AT2 cells secrete surfactant to reduce surface tension in the lung. AT2 cells also secrete catalase which is vital in hydrogen peroxide clearance (Farioli-Vecchioli et al. 2001) and protects against hydrogen peroxide epithelial damage (Mulier et al. 1998). Catalase mRNA expression in AT2 cells is markedly decreased in lung tissue from COPD patients and significantly correlates with the degree of airflow limitation (Tomaki et al. 2007) indicating poor function of AT2 cells during COPD. AT2 cells also secrete protease inhibitors elafin and secretory leukocyte proteinase inhibitor (SLPI). SLPI has been shown to be reduced in COPD exacerbation and at sites of epithelial damage which may account for the damage caused by exaggerated neutrophil elastase (Tetley 2006). In addition, AT2 cells in COPD tissue samples produce more MMP2 and MMP9 (Segura-Valdez et al. 2000), which is thought to be linked to increased MAPK signalling activated by cigarette smoke exposure (Mercer & D'Armiento 2006).

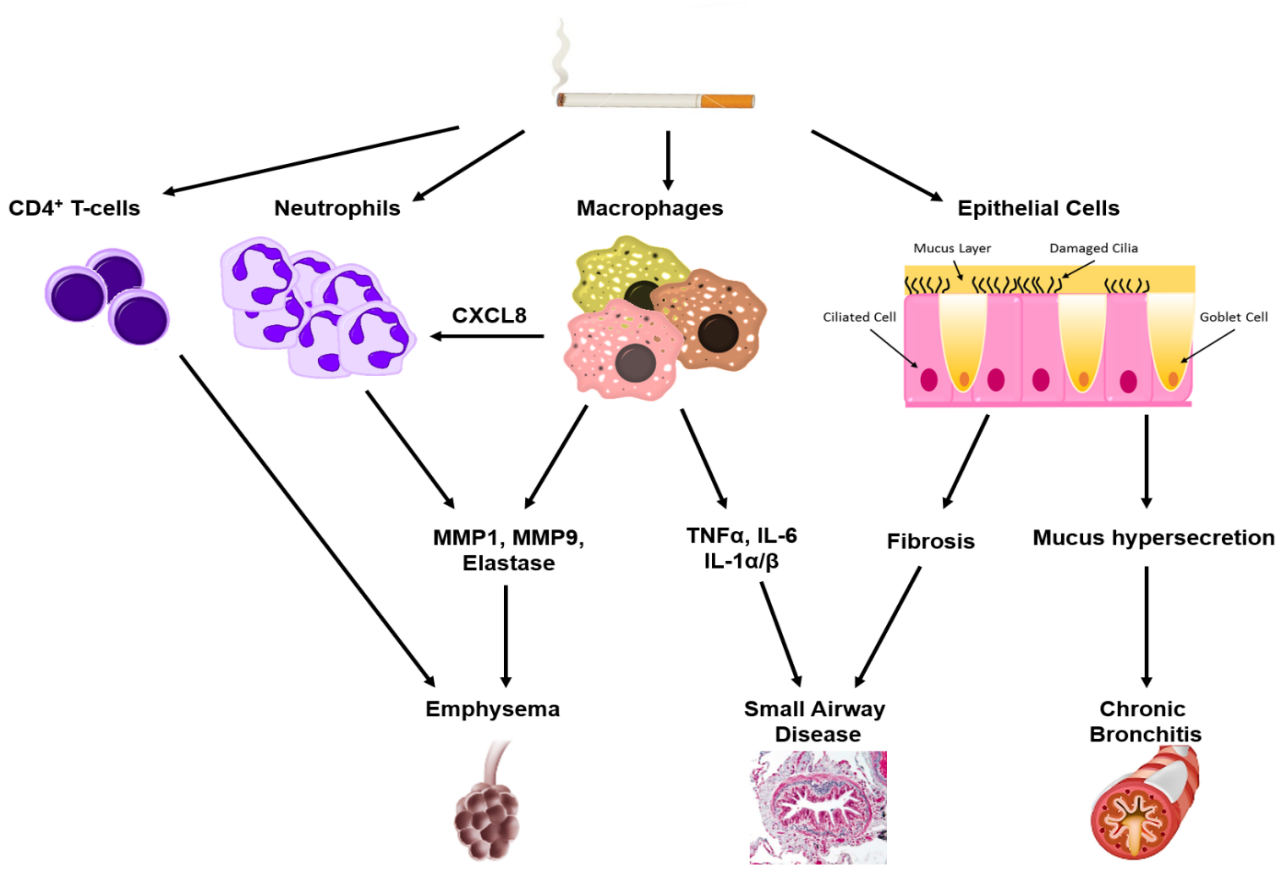


Figure 1.7: Immune cells in COPD pathophysiology. Cigarette smoke stimulates airway epithelial and immune cells to produce highly damaging levels of pro-inflammatory and proteases leading to the common pathophysiology seen in COPD: emphysema, small airway disease and chronic bronchitis. CXCL8: CXC-chemokine ligand 8, MMP: matrix metalloproteases, TNFα: tumour necrosis factor alpha, IL: Interleukin. Adapted from Barnes 2008. (Barnes 2008)

1.9.5.3 Macrophages in COPD

In a healthy lung, macrophages play essential roles in the immune response and are important for the removal of harmful or foreign products from the lung. However, in a chronic inflammatory situation, such as continued exposure to cigarette smoke, tissue environment is altered and the subsequent macrophage population is skewed.

1.9.5.3.1 Increased Macrophage Infiltration in COPD

Studies have shown that the number of macrophages in the airway lumen and epithelium is increased in COPD (Grashoff et al. 1997). Elevated macrophage infiltration has also been seen in BAL from COPD patients (Pesci et al. 1998) and positively correlated with disease severity (Di Stefano et al. 1998). The potent monocyte chemoattractants CXCL1 and CCL2 are increased in sputum samples from patients with COPD patients (Traves et al. 2002) and blockage of the CCL2 receptor, CCR2, significantly decreases migration (Mirzadegan et al. 2000). CCR2 inhibitors present a possible treatment for COPD and were recently trialled in rheumatoid arthritis (Haringman et al. 2006) but due to complex chemokine cross-talk and compensation mechanisms, these therapies are sometimes ineffective (Lebre et al. 2011).

1.9.5.3.2 Macrophages and the Protease/Anti-protease Imbalance

Alveolar macrophages from COPD patients have been shown to produce increased levels of MMPs. Both MMP-9 and MMP-2 levels are higher in BAL from COPD patients (Finlay et al. 1997) and MMP9 is higher in parenchyma from patients with emphysema (Ohnishi et al. 1998). Another study also indicated that MMP-9 was more active in patients with COPD compared to smokers and non-smokers (Russell et al. 2002),

possibly due to an increased oxidative stress activation in these cells (Rajagopalan et al. 1996). This same study also showed reduced levels of tissue inhibitor of metalloproteinase 1 (TIMP1), which usually inhibits and regulates MMP (Russell et al. 2002). This, therefore, indicates a loss of the protease/anti-protease balance in cells from COPD patients, leading to the emphysematous destruction in COPD. Another study by Culpitt et al showed an increased expression of TIMP1 in sputum of COPD patients (Culpitt et al. 2005). The same paper theorised that although the levels of TIMP1 were increased this was not sufficient enough to combat the overriding increase in proteases and oxidants and thus brings about the protease/anti-protease imbalance patients (Culpitt et al. 2005).

1.9.5.3.3 Pro-inflammatory Macrophage Cytokines in COPD

Not only do these cells increase in number and proteolytic capacity, alveolar macrophages from COPD patients also express an excessive amount of pro-inflammatory cytokine mediators (Barnes 2008). Studies have indicated higher levels of TNFα in sputum and serum from COPD patients (Keatings et al. 1996; Pitsiou et al. 2002). TNFα activates NFκB leading to increased pro-inflammatory gene expression in macrophages and drives a self-promoting feedback loop of inflammation in these cells. COPD macrophages also express higher levels of pro-inflammatory IL-1α in bronchial biopsies compared to healthy controls (Botelho et al. 2011) and BAL macrophages from COPD patients produce more IL-1β and IL-12 in comparison with those from healthy non-smoking human patients (S. Hodge et al. 2007). IL-6 has also been implicated in the progression of COPD (Cosio et al. 2009) and is expressed by COPD macrophages (Soler et al. 1999) and is elevated in serum during exacerbations (Ferrari et al. 2013). A recent study has shown that smoking cessation can promote a

more anti-inflammatory macrophage phenotype characterised by expression of higher levels of CD163 compared to current smokers with COPD (Kunz et al. 2011). However, this study also demonstrated that the levels of pro-inflammatory cytokines CXCL8 and IL-6 were not changed by smoking cessation, which may indicate that, although cell surface markers have altered, these macrophages have already become dysfunctional and are less likely to revert back to the resolving 'M2' like phenotype (Kunz et al. 2011).

1.9.5.3.4 Reduced Phagocytosis by COPD Macrophages

Macrophages in COPD have been linked to ineffective resolution of inflammation (Robb et al. 2016; Korns et al. 2011). This may be due to dysfunctional phagocytosis and efferocytosis. The lungs of COPD patients are known to be colonised with bacteria such as *Haemophilus influenzae* and *Streptococcus pneumoniae* (Sethi et al. 2006). Cigarette smoke impairs alveolar macrophage phagocytosis of *H. influenzae* (Martí-Lliteras et al. 2009) and efferocytosis of apoptotic cells (S. Hodge et al. 2007) which may lead to accumulation of bacteria and elastase-producing neutrophils in the airways (Vlahos & Bozinovski 2014). A study by Taylor *et al* in 2010 used a monocyte-derived macrophage (MDM) model to show dysfunctional phagocytosis of *H. influenzae* and *S. pneumoniae* in cells derived from COPD patients (Taylor et al. 2010) that was not due to changes in scavenger receptor expression or physical ability of the cell to phagocytose as cells from non-smokers and COPD patients were both equally able to effectively phagocytose inert beads (Taylor et al. 2010). This suggests that the defective phagocytosis is specific to bacteria, or apoptotic cells, leading to ineffective bacterial clearance and subsequent bacterial colonisation of the airways present in COPD.

1.9.5.3.5 Macrophage Polarisation and Plasticity in COPD

While no direct assessment has yet been made of plasticity of human alveolar macrophages in disease, there is some evidence that these macrophages may become skewed towards a particular phenotype. It is proposed that macrophages in COPD become dysfunctional, skewing towards a pro-inflammatory (Keatings et al. 1996; S. Hodge et al. 2007) and anti-resolving (Taylor et al. 2010; Takanashi et al. 1999) phenotype thought to contribute heavily to the pathophysiology of COPD (Frankenberger et al. 2011). Other studies have indicated a smoking-dependent reprogramming of alveolar macrophages towards a more “M2-like” phenotype with significantly up-regulated CD36 (scavenger receptor) gene and protein expression (Shaykhiev et al. 2009). The same study also showed down regulation of “M1-associated” genes, such as those encoding CD80 and TNF-related proteins, in COPD BAL macrophages (Shaykhiev et al. 2009). This increased ‘M2’ signalling may be indicative of a failed compensatory mechanism whereby the macrophages from COPD patients have lost their ability to become pro-resolving, anti-inflammatory homeostatic cells. It is crucial, therefore, to study the effect of macrophage phenotype and plasticity in the pathophysiology of COPD.

1.10 Human macrophage models

There are many challenges involved in studying macrophages *in vivo*. Murine models have made progress with mapping and labelling alveolar macrophages (Yona et al. 2013) and through various genetic knock-out studies have identified clear roles for these cells in both health and disease (Zhang et al. 2008). Unfortunately, there is little crossover of these markers and functions into human cells (Schneemann & Schoeden 2007; Mestas & Hughes 2004).

Studying mature, human alveolar macrophages involves invasive methods such as tissue resection, BAL and sputum induction. These methods are difficult and have an increased risk of infection in patients with COPD who are already immuno-compromised (Barnes & Celli 2009). Furthermore, it is not possible to sample patients with more severe disease or during exacerbations.

In order to study these cells in humans, *in vitro* models can be utilised. The most commonly used model is the monocyte-derived macrophage or MDM, whereby monocytes from the peripheral blood are isolated and differentiated into macrophages. The most frequently used MDM model involves the use of adherent cells cultured in the presence of foetal calf serum (FCS). Both adherence and FCS, however, have now been shown to alter subsequent macrophage phenotype. Adhering macrophages to plastic culture plates has been shown to induce pro-inflammatory gene expression (Standiford et al. 1991) and enhance STAT1 activation (Coccia et al. 1999), whist the presence of FCS improves macrophage phagocytosis and boosts cytokine production in human MDM (Rey-Giraud et al. 2012).

1.11 Nomenclature

Varying results from different studies into macrophage phenotypes, may arise, due to the multitude of different culture and stimulation techniques. Indeed, it is important to distinguish between differentiation, activation and polarisation. A large number of papers still only refer to macrophages as being either M1 or M2. Some have argued that a new nomenclature should be brought in to define the subsets based on their source, activators and markers (Murray et al. 2014). Martinez and Gordon, two of the key researchers involved in defining the macrophage subsets agreed there is need for contextualisation and proposed that rather than grouping macrophages by phenotype, they should be considered as dynamic cells which respond to their environment, forming a mixed phenotype (Martinez & Gordon 2014). Prior research has classified healthy macrophage subsets, however, the markers, activation, function and phenotype of macrophages in disease may be entirely different to those pre-defined subsets, making nomenclature incredibly complex.

This thesis will use MDM from non-smokers, smokers and COPD patients and differentiate them in the presence of either GM-CSF (forming GM-MDM) or M-CSF (forming M-MDM). It will examine different differentiation conditions, different activation, and functionality and identify differences between subject groups. This study will then go on to assess macrophage plasticity by examining ability of the cells to alter phenotype once culture environment is altered.

1.12 Hypothesis

Macrophages from COPD patients will show a lack of plasticity and will not be able to revert to a pro-resolving, anti-inflammatory phenotype in comparison with cells from non-smoking subjects.

1.13 Aims

- To characterise the affect of adherence and FCS on phenotype of MDM isolated from non-smokers, smokers and COPD patients.
- To characterise the phenotype of MDM from non-smokers, smokers and COPD patients cultured in either GM-CSF or M-CSF by measuring functional outputs.
- Alter the culture environment of MDM cultured in either GM-CSF or M-CSF to measure the plasticity of differentiated cells from non-smokers, smokers and COPD patients.
- Identify possible reasons for lack in plasticity including monocyte sub-populations, sputum environment and transcriptional regulation.
- Analyse the cell surface receptor expression of several “M1” and “M2” linked markers in MDM.
- Analyse the affect of changing culture environment on cell surface marker expression.
- Compare these results, where possible to macrophages isolated from resected human lung tissue

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

Material	Supplier
4',6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich, Dorset, UK
Amphotericin-B	Gibco, Invitrogen, Paisley, UK
Anti-c-Maf antibody	Abcam, Cambridge, UK
Anti-IRF3 (phospho S386) antibody	Abcam, Cambridge, UK
Anti-rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor® 594 Conjugate)	Cell Signaling Technology, Schuttersveld, The Netherlands
BD Biosciences FACS Canto II machine	Beckton Dickinson, Oxford, UK
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Dorset, UK
Brain Heart Infusion broth	Sigma-Aldrich, Dorset, UK
Cell dissociation solution	Sigma-Aldrich, Dorset, UK
Cell Fix	Invitrogen, Paisley, UK
Cell TrackerTM Green	Invitrogen, Paisley, UK
Compensation Beads Particle Set	Beckton Dickinson, Oxford, UK
Corning® Costar® Ultra-Low attachment multiwell plates	Sigma-Aldrich, Dorset, UK
Costar 96-well tissue culture treated microwell plates	Fisher, Loughborough, UK
CXCL8 capture, detection, standard antibody	R&D Systems, Abingdon, UK
Dextran	Sigma-Aldrich, Dorset, UK
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich, Dorset, UK
dithiothreitol (DTT)	Sigma-Aldrich, Dorset, UK
Dulbecco's Phosphate Buffered Saline (D-PBS)	Sigma-Aldrich, Dorset, UK
Enhanced Chemiluminescence substrate (ECL)	GE Healthcare, Buckinghamshire, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Dorset, UK
FACS Clean	Beckton Dickinson, Oxford, UK
FACS Flow	Beckton Dickinson, Oxford, UK
FACS Flow	Beckton Dickinson, Oxford, UK
FACS Rinse	Beckton Dickinson, Oxford, UK
Fluorescent microspheres (2µm, yellowgreen)	Invitrogen, Paisley, UK
FLUOstar Optima plate reader	BMG Labtech Ltd., Bucks, UK
Foetal bovine serum (FBS)	Invitrogen, Paisley, UK
Formaldehyde (10% v/v)	Sigma-Aldrich, Dorset, UK
Granulocyte macrophage-colony stimulating factor (GM-CSF)	R&D Systems, Abingdon, UK

GraphPad Prism v 5.0	GraphPad Software Inc., CA, USA
G-Storm Thermal Cycler	G-Storm, Somerset, UK
Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich, Dorset, UK
Human Monocyte Isolation kit II	Miltenyii biotech, Surrey, UK
IL-10 capture, detection, standard antibody	R&D Systems, Abingdon, UK
L-Glutamine	Sigma-Aldrich, Dorset, UK
Lipopolysaccharide (LPS)	Sigma-Aldrich, Dorset, UK
Live/Dead® Fixable Dead cell stain kit (aqua)	Invitrogen, Paisley, UK
Macrophage colony-stimulating factor (M-CSF)	R&D Systems, Abingdon, UK
MACS LS Column	Miltenyii biotech, Surrey, UK
Methanol	Sigma-Aldrich, Dorset, UK
Methylthiazolyldiphenyl-tetrazolium bromide (MTT)	Sigma-Aldrich, Dorset, UK
Mouse anti-human CD11b Pacific Blue	Beckton Dickinson, Oxford, UK
Mouse anti-human CD14 APC-Cy7	Beckton Dickinson, Oxford, UK
Mouse anti-human CD14 FITC	Beckton Dickinson, Oxford, UK
Mouse anti-human CD16 Pacific Blue	Beckton Dickinson, Oxford, UK
Mouse anti-human CD16 PerCP-Cy-5.5	Beckton Dickinson, Oxford, UK
Mouse anti-human CD163 APC	BioSciences, Cambridge, UK
Mouse anti-human CD206 PE	Beckton Dickinson, Oxford, UK
Mouse anti-human CD226 FITC	Beckton Dickinson, Oxford, UK
Mouse anti-human CD282 AlexaFluor647	Beckton Dickinson, Oxford, UK
Mouse anti-human CD80 PE	Beckton Dickinson, Oxford, UK
Mouse anti-human CD93 FITC	Beckton Dickinson, Oxford, UK
Mouse anti-human HLA-DR-APC	BioSciences, Cambridge, UK
Mouse Anti-phosphor-Stat6 (pY641) PE	Beckton Dickinson, Oxford, UK
Mouse Anti-Stat1 (pY701) APC	Beckton Dickinson, Oxford, UK
Mouse BALB/c IgG1κ FITC	Beckton Dickinson, Oxford, UK
Mouse BALB/c IgG1κ Pacific Blue	Beckton Dickinson, Oxford, UK
Mouse IgG1κ AlexaFluor647	Beckton Dickinson, Oxford, UK
Mouse IgG1κ APC	BioSciences, Cambridge, UK
Mouse IgG1κ Pacific Blue	Beckton Dickinson, Oxford, UK
Mouse IgG1κ PE	Beckton Dickinson, Oxford, UK
Mouse IgG1κ PerCP-Cy-5.5	Beckton Dickinson, Oxford, UK
Mouse IgG2ακ FITC	Beckton Dickinson, Oxford, UK
Mouse IgG2β APC-Cy7	Beckton Dickinson, Oxford, UK
Mouse IgG2βκ FITC	Beckton Dickinson, Oxford, UK
NUNC Maxisorp plates	Sigma-Aldrich, Dorset, UK
Penicillin Gibco	Invitrogen, Paisley, UK

Percoll	GE Healthcare, Buckinghamshire, UK
Phosflow Perm Buffer III	Beckton Dickinson, Oxford, UK
Phosphate buffered saline tablets (PBS)	Sigma-Aldrich, Dorset, UK
Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb	Cell Signaling Technology, Schuttersveld, The Netherlands
Phospho-Stat6 (Tyr641) Antibody	Cell Signaling Technology, Schuttersveld, The Netherlands
Polyoxyethylene-sorbitan monolaurate (Tween-20)	Sigma-Aldrich, Dorset, UK
Polystyrene round bottomed 5ml FACS tubes	Beckton Dickinson, Oxford, UK
Rat anti-human CD120b PE	Beckton Dickinson, Oxford, UK
Rat IgG2βκ APC	BioSciences, Cambridge, UK
Rat IgG2βκ PE	Beckton Dickinson, Oxford, UK
Real Time PCR System 7500	Applied Biosystems, USA
Recombinant human IL-4	R&D Systems, Abingdon, UK
RLT buffer	Qiagen, Manchester, UK
RNAse free water	Qiagen, Manchester, UK
Rneasy kit	Qiagen, Manchester, UK
Roswell Park Memorial Institute media (RPMI)-1640	Sigma-Aldrich, Dorset, UK
SDS software 7500 version 2.0.6	Applied Biosystems, USA
Sodium Azide	Sigma-Aldrich, Dorset, UK
Sodium Chloride (0.9% w/v)	Baxter, Norfolk, UK
Sodium Chloride (9% w/v)	Sigma-Aldrich, Dorset, UK
Spectramax Plus 384 plate reader	Molecular Devices Ltd., Berkshire, UK
Streptavidin-Cy3 HRP Conjugated Antibody	Invitrogen, Paisley, UK
Streptomycin	Invitrogen, Paisley, UK
Sucrose	Sigma-Aldrich, Dorset, UK
Syringes	3S Healthcare, London, UK
TaqMan master mix	Fisher, Loughborough, UK
TaqMan primers and probes for STAT1, STAT6, IRF3, IRF7, RELA, c-MAF, JUN and HPRT1	Fisher, Loughborough, UK
Tetramethylbenzine solution (TMB)	Sigma-Aldrich, Dorset, UK
TNF-a capture, detection, standard antibody	R&D Systems, Abingdon, UK
Triton™ X-100	Sigma-Aldrich, Dorset, UK
Trypan Blue	Sigma-Aldrich, Dorset, UK
Tween-20	Sigma-Aldrich, Dorset, UK
Zeiss LSM-510 inverted confocal microscope	Zeiss, Cambridge, UK
β-mercaptoethanol	Sigma-Aldrich, Dorset, UK

2.2 Methods

2.2.1 Subject Selection: Monocyte Derived Macrophage Experiments

Volunteers were recruited by the Royal Brompton Hospital under ethics approved by the NRES Ethics Committee London - Brent (13/LO/0354). All subjects gave full informed written consent. All subjects were subjected to skin prick allergy tests for common airway allergens (house dust mite, *Aspergillus fumigatus*, cat hair and grass pollen), to test for atopy. Any atopic subjects were excluded. Spirometry was performed by the research nurses at the Respiratory Biomedical Research Unit (BRU). All subjects underwent spirometry to measure lung capacity and function.

Forced expiratory volume in one second (FEV_1) describes the volume of air expelled (after maximum inhalation) in the first second of maximum exhalation. Forced vital capacity (FVC) defines the total volume of air exhaled forcibly after maximum inhalation. FEV_1 and FVC were recorded for all subjects before and after they had inhaled a bronchodilator (2.5mg Salbutamol). An FEV_1/FVC ratio was calculated to allow categorisation of patients into groups based on their lung function. Lung function results were recorded along with age, height, weight and smoking and medical history.

2.2.1.1 Non-Smokers

Non-smokers were aged between 45 and 80 and had normal lung function (FEV_1:FVC ratio of >0.7 and post-bronchodilator FEV_1% predicted >85%. Subjects had no history of allergic or respiratory disease, no smoking history and were not on any regular medication. Subjects also had no upper respiratory tract infection within the 4 weeks prior to sampling.

2.2.1.2 Smokers

Smokers were aged between 45 and 80 had normal lung function (FEV_1:FVC ratio of >0.7 and post-bronchodilator FEV_1% predicted >85%. Subjects had no history of allergic or respiratory disease and were not on any regular medication. Subjects also had no upper respiratory tract infection within the 4 weeks prior to sampling. All subjects had a smoking history >10 pack years (where 1 pack year = 20 cigarettes/day for 1 year).

2.2.1.3 COPD Patients

COPD patients were aged between 45 and 80 and were categorised as having COPD based on the GOLD definition:

“Chronic Obstructive Pulmonary Disease (COPD), a common preventable and treatable disease is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients. Spirometry is required to make diagnosis in the clinical context; the presence of a post-bronchodilator FEV_1:FVC ratio of <0.70 confirms the presence of persistent airflow limitation and thus COPD.” (GOLD 2016).

All COPD patients had a post-bronchodilator FEV_1:FVC ratio of <0.7 and FEV_1% predicted < 85%. Subjects had no upper respiratory tract infection within the 4 weeks prior to sampling. All subjects had a smoking history >10 pack years (where 1 pack year = 20 cigarettes per day for 1 year).

2.2.2. Monocyte-Derived Macrophage Isolation

2.2.2.1 Dextran Sedimentation and Percoll Gradient Centrifugation

60ml of human peripheral whole blood was taken by venepuncture in the Respiratory BRU at the Royal Brompton Hospital, London, by either research nurses or clinical research staff. Blood was taken using three 20ml syringes containing 0.5ml EDTA (0.5M) and a butterfly needle. In a sterile culture hood, 30ml of 6% ($^{w}/_{v}$) dextran was divided into three 50ml Falcon centrifuge tubes (10ml in each). 20ml whole blood was added to each tube then 20ml Hanks' balanced salt solution (HBSS) was added per tube. Blood was incubated at room temperature for 20 min to allow erythrocytes to sediment out and leaving the leukocyte rich layer on top. The leukocyte layer was taken up into three new 50ml tubes using a plastic Pasteur pipette before addition of 20ml HBSS. The mixture was then centrifuged at 300x*g* for 10 min (room temperature, maximum acceleration and deceleration) to pellet the cells.

In order to separate out the peripheral blood mononuclear cells (PBMC) from the granulocytes, 55%, 68% and 81% ($^{v}/_{v}$) Percoll gradients were made up using Percoll and 0.9% ($^{v}/_{v}$) saline. 4ml of the 81% ($^{v}/_{v}$) Percoll gradient was added to two 15ml tubes. 4ml of the 68% ($^{v}/_{v}$) Percoll gradient was then layered on top using a 5ml plunger free syringe barrel and a 21-gauge needle. Tubes containing the pelleted samples were removed carefully from the centrifuge into the culture hood. Supernatant was aspirated and pellet resuspended in the 55% ($^{v}/_{v}$) Percoll before layering into gradient filled tubes (Figure 2.1). Cells were centrifuged at 1000x*g* for 30 min (room temperature, low acceleration and deceleration in order to preserve the layering of buffy coats). The top layer (made up of mostly dead cells and cell fragments) was aspirated. The PBMC which formed a layer at the 55%-68% interface were taken up

into a fresh 50ml tube. 40ml of HBSS was added before cells were centrifuged at 300x*g* for 5 min to pellet (room temperature, maximum acceleration and deceleration).

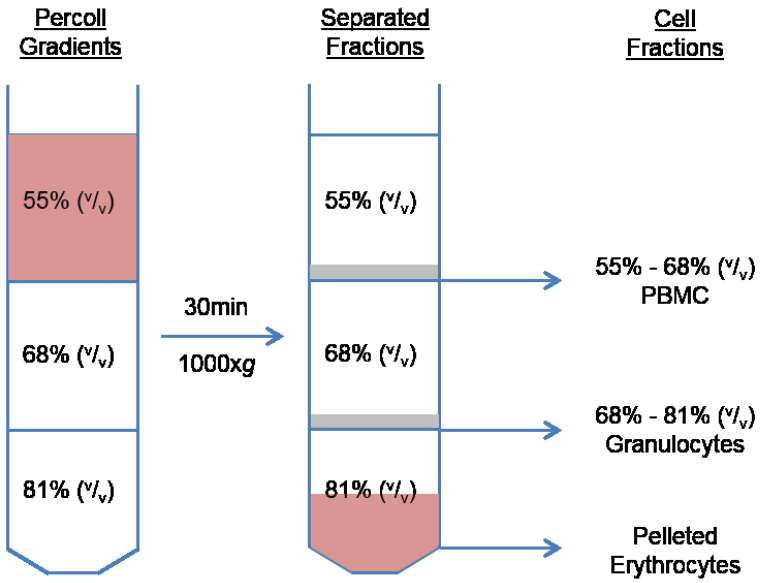


Figure 2.1: Diagram showing discontinuous Percoll gradient isolation of PBMC from human peripheral blood. 68% (v/v) and 81% (v/v) Percoll gradients were layered into a 15ml tube. Leukocyte rich cell pellet was resuspended in 55% (v/v) Percoll before being layered on top then centrifuged at 1000x*g* for 30min. PBMC which formed a layer at the 55% - 68% (v/v) interface were extracted into a fresh tube, washed and used for further experiments.

Supernatant was aspirated and pellet resuspended in 1ml complete MDM media (RPMI 1640 media supplemented with 10% (v/v) Foetal Bovine Serum, 10mg/ml Penicillin/Streptomycin, 2mM L-glutamine). 10µl cells were used for counting using a 1:100 dilution with Kimura stain (0.05% (v/v) toluidine blue, 0.03% (v/v) light green, 10% (v/v) saponin and 0.07M phosphate buffer). Cells were counted on a haemocytometer and standard light microscope.

PBMC were seeded at $4x10^5$ cells/well in 96 well plates and monocytes allowed to adhere for 2h at 37°C in 5% ($^{v}/_{v}$) CO_2. Media containing non-adherent PBMC was removed, leaving the adherent monocytes which were then cultured in complete MDM media supplemented with either 2ng/ml GM-CSF or 100ng/ml M-CSF. Cells were allowed to differentiate for 12 days with media replaced on days 4 and 8. Cells cultured for 12 days in GM-CSF were termed GM-MDM and cells cultured in M-CSF were termed M-MDM.

2.2.2.2 Manipulation of Cell Culture Environment

In order to assess plasticity of MDM, experiments were devised to assess macrophage phenotype before and after change of culture environment. At day 12, after cells had fully differentiated, some MDM were used in functional and phenotyping experiments (ELISA: LPS/IL-4 stimulated cytokine release, fluorimetry: bacterial phagocytosis and flow cytometry: cell surface markers). The remainder then had their culture environment changed. Cells that were cultured in GM-CSF for 12 days (GM-MDM) had all GM-CSF media removed and replaced with M-CSF media (100ng/ml). Similarly, cells cultured in M-CSF for 12 days (M-MDM) were switched to media containing GM-CSF (1ng/ml) for a further 6 days with measurements taken at baseline (before any switching of media) then on days 2, 4 and 6 post switching of media (Figure 2.2).

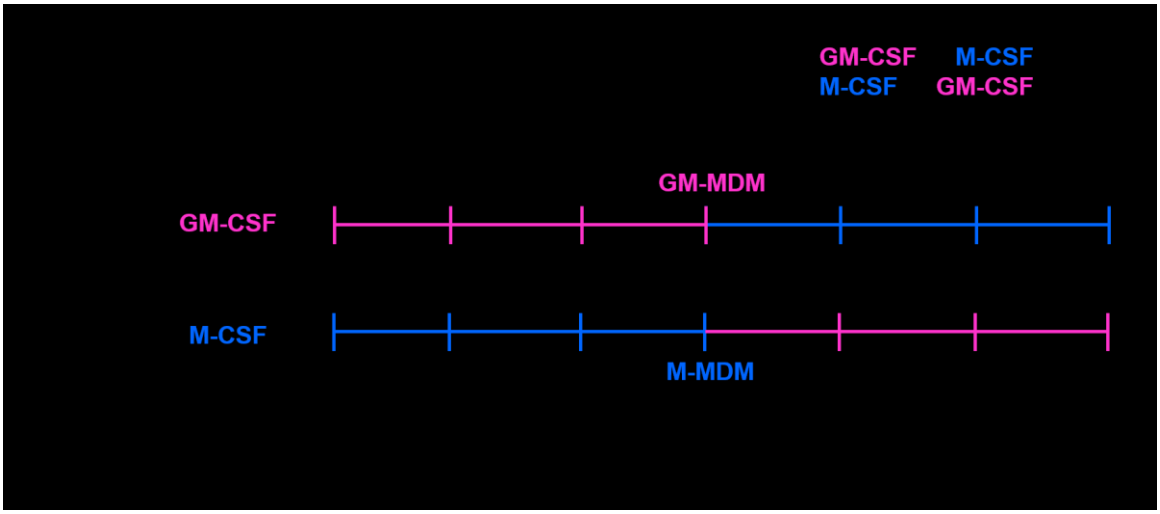


Figure 2.2: Manipulation of cell culture environment to analyse MDM plasticity. Monocytes were isolated from human peripheral blood and cultured in either GM-CSF or M-CSF for 12 days. Baseline measurements were done at day 12 while remaining MDM culture media was the switched over a 6 day time course. Functional outputs were measured then at days 2, 4 and 6 post-switch.

2.2.3 Lung Tissue Derived Macrophage Experiments

Resected tissue deemed to be macroscopically normal was taken by the Respiratory BRU at the Royal Brompton and Harefield hospital (Ethics number: 10/H0504/9). Medical records for the patients undergoing resection were used to categorise patients into non-smokers, smokers and COPD patients using the same criteria as in section 2.2.1.

2.2.3.1 Macrophage Isolation from Resected Lung Tissue

Tissue was placed into a sieve and flushed with 120ml macrophage wash buffer (RPMI 1640 + 0.5M (1% $^{v}/_{v}$) EDTA + 10µg/ml Penicillin/Streptomycin/Amphotericin (1% $^{v}/_{v}$) + 2mM (1% $^{v}/_{v}$) L-glutamine) using a 50ml syringe and 21-gauge needle into a sterile 120ml sterilin tube. Tissue exudate was then transferred into three 50ml Falcon tubes and centrifuged at 300x*g* for 5 min (room temperature, max acceleration and deceleration). 10%, 20%, 30%, 40%, 50% and 60% ($^{v}/_{v}$) Percoll gradients were made up using Percoll and 0.9% ($^{v}/_{v}$) saline. 2ml of each Percoll gradient was layered into two 15ml tubes (60% at bottom followed by then 50%, then 40%, then 30%, then 20%) using a 2ml plunger free syringe barrel and a 21gauge needle. Tissue exudate was aspirated and the pellet resuspended in 10% ($^{v}/_{v}$) Percoll before layering into gradient filled tube. Cells were then centrifuged at 1000x*g* for 30 min (room temperature, low acceleration and deceleration) (Figure 2.3). Tubes were removed from centrifuge and top two layers (10% and 20% Percoll) were aspirated as these contained only dead cells (Chana et al. 2013).

Macrophage gradients were defined by their interface level which corresponded to the following densities: 30% - 40% ($^{v}/_{v}$): 1.036 to 1.048 g/mL, 40% - 50% ($^{v}/_{v}$): 1.048 to 1.06 g/mL and 50% - 60% ($^{v}/_{v}$): 1.061 to 1.073 g/mL. Each fraction was extracted into

separate 50ml tubes and washed in 40ml 1x Dulbecco's Phosphate Buffered Saline (D-PBS) by centrifuging at 300x*g* for 5 min (room temperature, maximum acceleration and deceleration). Each fraction was counted using Trypan blue (0.13% ($^{w}/_{v}$) Trypan Blue powder in D-PBS) and resuspended at a concentration of $1x10^{6}$ cells/ml. Cells were then pooled and divided into specific culture conditions, centrifuged at 300x*g* for 5 min (room temperature) and resuspended in complete culture media. Cells were then seeded at $1x10^{5}$/well in 96 well plates and kept at 37°C in 5% ($^{v}/_{v}$) CO_2 until used for experiments.

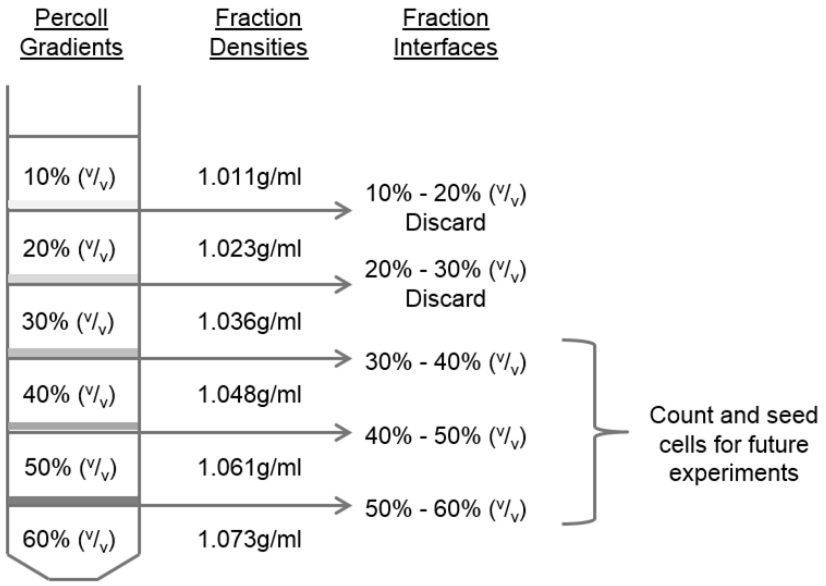


Figure 2.3: Diagram showing cell fractions of human lung tissue macrophages following Percoll gradient isolation. Percoll gradients were layered into a 15ml tube. Cell pellet was resuspended in 10% ($^{v}/_{v}$) Percoll before being layered onto gradients and centrifuged at 1000x*g* for 30 min. Cells were separated out and formed layers based on their relative densities at the Percoll gradient interfaces. Lung tissue macrophages at interfaces between 10% - 20% ($^{v}/_{v}$) and 20% - 30% ($^{v}/_{v}$) layers were discarded. Lung tissue macrophages at interfaces between 30% - 40% ($^{v}/_{v}$), 40% - 50% ($^{v}/_{v}$) and 50% - 60% ($^{v}/_{v}$) layers were extracted into separate tubes, washed in D-PBS and plated for further experiments.

2.2.4 Induced Sputum

2.2.4.1 Induced Sputum Collection

Subjects were selected as in section 2.2.1. Sputum was induced by the research nurses at the Royal Brompton hospital using a 3% ($^{w}/_{v}$) inhaled saline solution via a nebulizer. Expectorated sputum was collected into a sterile 50ml tube.

2.2.4.2 Sputum Processing

Sputum plugs were separated from saliva using a sterile Pasteur pipette and aspirated into a fresh 50ml tube. An equal volume of D-PBS was added to the sputum plugs before addition of 0.05 ($^{v}/_{v}$) dithiothreitol (DTT) (a reducing mucolytic agent). Sputum was incubated in DTT at room temperature for 40 min on a bench top shaker. Cells were strained through a single cell 100µm filter into a fresh 50ml tube and then centrifuged at 300x*g* for 5 min (room temperature). Supernatant was taken into sterile Eppendorf tubes and stored at -80°C until use. Cells were resuspended in 1ml sterile D-PBS and counted using Kimura stain and a haemocytometer as in section 2.2.2.1. If squamous cells accounted for more than 40% of the total cell count, the sample was deemed to be unsuitable and was discarded.

2.2.4.3 Measurement of Sputum GM-CSF and M-CSF by ELISA

NUNC Maxisorp plates were coated with 100µl/well (2µg/ml) monoclonal capture antibody for either GM-CSF or M-CSF. Plates were incubated at room temperature for 12 h and then capture antibody removed by tipping off and blotting onto tissue. Plates were washed three times with ELISA wash buffer (0.05% ($^{v}/_{v}$) Tween20 in D-PBS) and blocking buffer (0.05% ($^{w}/_{v}$) sodium azide + 1% ($^{w}/_{v}$) bovine serum albumin (BSA) + 5% ($^{w}/_{v}$) sucrose) was added at 125µl/well for 2 h at room temperature. Blocking buffer

was aspirated and plates were washed three times in ELISA wash buffer and blotted onto tissue. Sputum supernatants were added neat to the plates at 100µl/well in quadruplicate. Standard solutions of 2000pg/ml to 31.25pg/ml of recombinant GM-CSF or M-CSF were reconstituted in ELISA buffer containing 0.05 ($^{v}/_{v}$) DTT and added to the plate. Plates were incubated for 2 h at room temperature before samples and standards were aspirated and plates were washed three times in ELISA wash buffer and blotted onto tissue. Detection antibodies for GM-CSF or M-CSF were reconstituted in ELISA buffer and added to the plate at 100µl/well (1µg/ml). Plates were incubated for 2 h at room temperature before detection antibody was aspirated and plates were washed three times in ELISA wash buffer and blotted onto tissue. Horseradish peroxidase (HRP) conjugated to streptavidin was reconstituted (0.1mg/ml) in ELISA buffer and added to the plates at 100µl/well. Plates were incubated for 25 min at room temperature before streptavidin-HRP was aspirated and plates were washed five times in ELISA wash buffer and blotted onto tissue. Substrate solution was reconstituted using equal parts of Reagent A (H_2O_2) and Reagent B (Tetramethylbenzidine) then added to the plates at 100µl/well. This induced a colourless to blue colour change. Reaction was stopped by adding H_2SO_4 (1M) at 100µl/well to whole plate. Absorbance was read at λ450nm and λ550nm wavelengths using a spectrophotometer (Spectramax Plus, Molecular Devices). The 550 λnm value (background noise value) was subtracted from the λ450nm value (sample value) and concentration of GM-CSF or M-CSF in sputum supernatant were determined by interpolation from standard curves (Figure 2.4) using GraphPad Prism software (version 5.03). Lower limit of detection (sensitivity) of all assays was 31.25pg/ml.

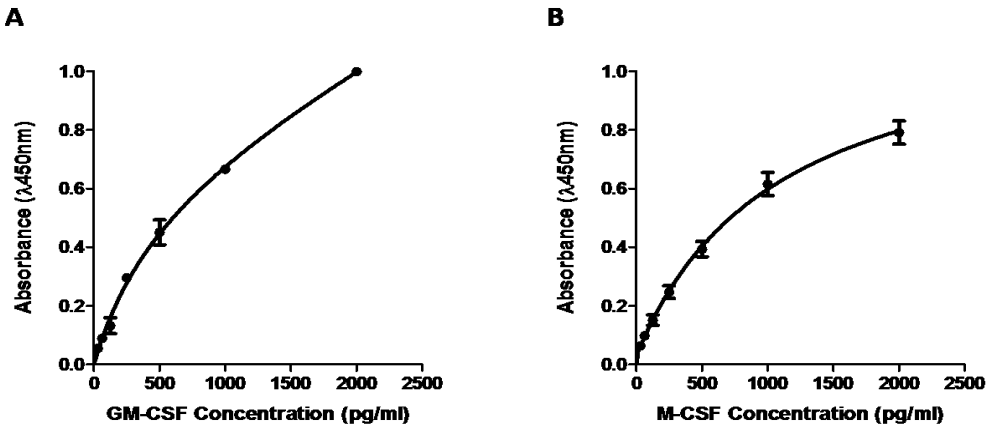


Figure 2.4: GM-CSF/M-CSF standard curves. A standard curve of recombinant GM-CSF (A) or M-CSF (B) ranging from 2000pg/ml to 31.25pg/ml and containing 0.05 ($^{v}/_{v}$) DTT, was run alongside sputum samples to allow for interpolation of unknown concentrations of GM-CSF or M-CSF. Absorbance was read at λ450nm.

2.2.5 MDM Stimulation

Lipopolysaccharides (LPS) (from *Salmonella enterica* serotype enteritidis) or IL-4 were reconstituted in RPMI at a concentration of 10ng/ml. This concentration was based on pervious optimization of this protocol and subsequent measureable cytokine concentrations in the Cell Biology – Airway Disease laboratory. MDM or lung tissue macrophages had all culture media aspirated before stimulation with LPS or IL-4 in quadruplicate at concentrations indicated for 24h before supernatant was harvested and stored at -80°C until used for analysis.

2.2.6 Measurement of TNFα, CXCL8 and IL-10 by ELISA

Levels of TNFα, CXCL8 and IL-10 in cell media from stimulated cells were analysed by ELISA according to manufacturer's instructions. NUNC Maxisorp plates were coated with 100µl/well (2µg/ml) of appropriate monoclonal capture antibody diluted in sterile D-PBS. Plates were incubated at room temperature for 12 h before antibody was aspirated, plates washed three times in ELISA wash buffer (0.05% ($^{v}/_{v}$) Tween20 in D-PBS) and blotted onto tissue. Blocking buffer (0.05% ($^{w}/_{v}$) sodium azide + 1% ($^{w}/_{v}$) BSA + 5% ($^{w}/_{v}$) sucrose) was added at 125µl/well for 2 h at room temperature. Blocking buffer was aspirated and plates were washed three times in ELISA wash buffer and blotted onto tissue. Media from stimulated cells was diluted in ELISA buffer (0.5% ($^{w}/_{v}$) BSA in D-PBS) to appropriate concentrations (TNFα - 1:40, CXCL8 - 1:300 and IL-10 - 1:2) and added to the plates at 100µl/well. A standard concentration (2000pg/ml) of recombinant TNFα, CXCL8 or IL-10 was diluted in ELISA buffer and added to the plate. A standard concentration curve ranging from 2000pg/ml-31.25pg/ml was constructed using serial dilutions. Plates were incubated for 2 h at room temperature before samples and standards were aspirated and plates were washed three times in ELISA wash buffer and blotted onto tissue. Detection antibody for TNFα, CXCL8 and IL-10 were reconstituted in ELISA buffer and added to the plate at 100µl/well (0.4µg/ml). Plates were incubated for 2 h at room temperature before detection antibody was aspirated and plates were washed three times in ELISA wash buffer and blotted onto tissue. Horseradish peroxidase (HRP) conjugated to streptavidin was reconstituted (0.1mg/ml) in ELISA buffer and added to the plates at 100µl/well. Plates were incubated for 25 min at room temperature before streptavidin-HRP was aspirated and plates were washed five times in ELISA wash buffer and blotted onto tissue.

Substrate solution was then added to the plates before reaction was stopped by adding H_2SO_4 (1N) at 100µl/well to whole plate.

Absorbance was read at λ450nm and λ550nm wavelengths using a spectrophotometer (Spectramax Plus, Molecular Devices). The λ550nm value (background noise value) was subtracted from the λ450nm value (sample value) and concentrations of TNFα, CXCL8 or IL-10 in supernatant were determined by interpolation from standard curves (Figure 2.5) using GraphPad Prism software (version 5.03). Lower limit of detection (sensitivity) of all assays was 31.25pg/ml.

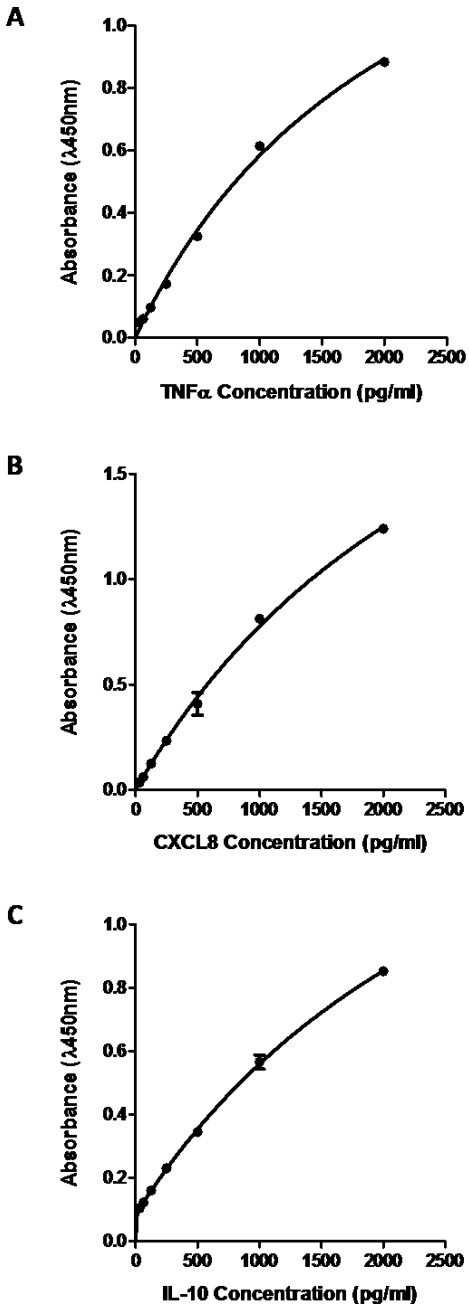


Figure 2.5: TNFα, CXCL8 and IL-10 standard curves. A standard curve of recombinant TNFα, CXCL8 or IL-10 ranging from 2000pg/ml to 31.25pg/ml, was run alongside samples to allow for interpolation of unknown concentrations of cytokines in the cell supernatant. Absorbance was read at λ450nm.

2.2.7 Phagocytosis Assay

2.2.7.1 Bacterial Culture and Heat Killing

Streptococcus pneumoniae serotype 9V, strain 10692 and non-typeable *Haemophilus influenzae,* strain 1479 (both isolated from an exacerbating COPD patient) were plated onto iso-sensitest agar plates for 24 h at 37°C in a shaker incubator. Colonies were inoculated in 20ml Brain Heart Infusion broth and placed in a shaker incubator at 37°C overnight. Growth rates were determined by spectrophotometer using optical density (OD) measurements every hour at λ600nm and bacteria were harvested once an OD of 1.2 was reached. Colony forming units (CFU) were counted on 12 h cultured agar plates (CFU = (colony number x dilution factor) / volume plated)). Bacteria were heat killed at 90° for 2 h and washed before fluorescent labelling with 1g/ml Alexafluor 488. Bacteria were washed and resuspended in D-PBS before being aliquoted and stored at -20°C until use in experiments.

2.2.7.2 Phagocytosis Assay

Monocytes were isolated as described in section 2.2.2. All cells used for phagocytosis experiments were cultured on black 96 well plates to prevent light interference from neighbouring wells. Prey were sonicated in a water bath for 2 min before reconstitution to allow for even suspension of particles. Prey consisted of either inert yellow-green fluorescent 2µm diameter FluoSphere Carboxylate-modified microspheres (50 $x10^6$ beads/ml), Gram-negative *H. influenza* or Gram-positive *S.pneumoniae* (both conjugated to Alexafluor 488 and reconstituted RPMI only media (1:10)). Culture media was aspirated from wells before addition of either control media (RPMI media only) or prey in quadruplicate. Prey were then added to plates at 100µl/well. Plates were incubated for 4 h at 37°C in 5% ($^v/_v$) CO_2. Prey were aspirated from wells and

cells were washed with D-PBS at 100µl/well for 1 min to remove any unbound prey. D-PBS was aspirated and then 1% ($^{v}/_{v}$) Trypan Blue dye added at 100µl/well for 5 min to quench any extracellular fluorescence. Trypan Blue was aspirated and plate blotted onto tissue to remove any excess liquid. Plates were then placed into a fluorescent plate reader (FLUOstar OPTIMA, BMG) at an excitation of λ480nm and an emission of λ520nm (gain value 1100 for beads and 1900 for bacteria).

2.2.8 Flow Cytometry

2.2.8.1 Whole Blood Monocyte Sub-population Marker Analysis by Flow Cytometry

200µl of blood, collected as in section 2.2.2, was pipetted into an Eppendorf tube before addition of 200µl of cold FACS FIX (30ml BD FACS flow + 1ml BD Cellfix + 9ml dH_2O). This was mixed gently by pipetting for 1 min before 50µl was transferred into FACS tubes containing appropriate antibodies (Table 2.1). CD14, CD16 and CD11b antibodies were added concurrently to allow for simultaneous multi-colour staining of the same sample.

Receptor	Concentration µg/ml	Conjugated to
CD14	12	Fluorescein isothiocyanate (FITC)
CD16	10	Pacific Blue
CD11b	10	Allophycocyanin (APC)
CD14 Isotype	12	FITC
CD16 Isotype	10	Pacific Blue
CD11b Isotype	10	APC
Live/Dead	1	Pacific Blue

Table 2.1: Flow Cytometry Panel 1 Details. Table detailing concentrations and colours of antibodies used in experiments looking at monocyte sub-population markers in human whole blood.

Cells were incubated for 30 min at 4°C in the dark before hypotonic shock lysis of red blood cells by addition of 1ml ice cold dH_2O for 30 s. Cells were then washed by addition of 2ml FACS buffer (0.5% ($^{w}/_{v}$) BSA in D-PBS) and centrifugation at 300x*g* for 5 min (room temperature, max acceleration and deceleration). Excess liquid was aspirated and blotted on tissue before pellet was resuspended in 300µl FACS Fix (0.5% ($^{v}/_{v}$) formaldehyde in D-PBS). Cells were then acquired on a BD Biosciences

FACS Canto II machine at a medium speed. A minimum of 5000 events was collected per sample and FCS files were analysed using FlowJo (software version 10).

2.2.8.2 Phenotypic Marker Analysis by Flow Cytometry

Monocytes were then cultured in either GM-CSF or M-CSF as detailed in section 2.2.1. In order to assess cell surface markers during environmental switch, flow cytometry was performed at day 12 of differentiation and also on days 2, 4 and 6 post environmental switch (Figure 2.6).

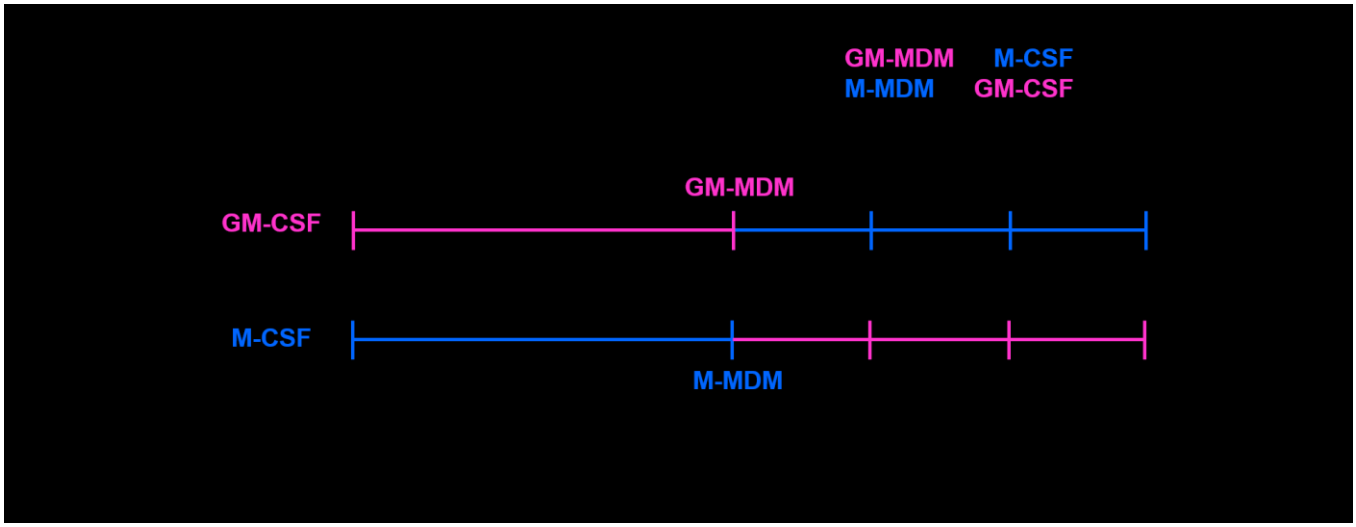


Figure 2.6: MDM Flow Cytometry Timeline. Monocytes were isolated from human peripheral blood by dextran sedimentation and Percoll gradient centrifugation. Monocytes were then cultured in either GMCSF or M-CSF for 12 days before media was switched (GM-CSF to M-CSF and M-CSF to GM-CSF. Cells were harvested on day 12, and then at days 2, 4 and post-switch for use in flow cytometry experiments.

To harvest cells, 300µl cell dissociation solution was added to each well and plates were incubated for 20 min at 37°C in 5% ($^{v}/_{v}$) CO_2. Detached cells were then removed from the plate into Eppendorf tubes (1 per condition) and centrifuged at 300x*g* for 5 min. Cells were then blocked with 1% ($^{v}/_{v}$) human serum for 15 min at room temperature before being washed in 1ml FACS buffer and centrifuged at 300x*g* for 5 min to pellet. Excess liquid was aspirated and pellet resuspended at 1 $x10^5$ cells in 100µl FACS buffer. 100µl of cells were then transferred into FACS tubes containing appropriate antibodies as detailed in table 2.2.

Receptor	**Concentration µg/ml**	**Conjugated to**
CD14	12	APC-Cyanine7 (APC-Cy7)
CD16	10	Peridinin Chlorophyll Protein Complex-Cyanine-5.5 (PerCP-Cy-5.5)
CD11b	10	Pacific Blue
CD206	10	Phycoerythrin (PE)
CD226	10	Fluorescein isothiocyanate (FITC)
CD282	10	AlexaFluor647
CD93	10	FITC
CD120b	10	PE
CD80	10	PE
CD163	10	APC
CD14 Isotype	12	APC-Cy7
CD16 Isotype	10	PerCP-Cy-5.5
CD11b Isotype	10	Pacific Blue
CD206 Isotype	10	PE
CD226 Isotype	10	FITC
CD282 Isotype	10	AlexaFluor647
CD93 Isotype	10	FITC
CD120b Isotype	10	PE
CD80 Isotype	10	PE
CD163 Isotype	10	APC
Live/Dead	1	Pacific Blue

Table 2.2: Flow Cytometry Panel 2 Details. Table detailing concentrations, volumes and colours of antibodies used in experiments looking at macrophage phenotypic markers in MDM.

Cells were incubated for 30 min at 4°C in the dark before washing by addition of 2ml FACS buffer (0.5% ($^{w}/_{v}$) BSA in D-PBS) and centrifugation at 300x*g* for 5 min. Excess liquid was aspirated and blotted on tissue before pellet was resuspended in 300µl FACS Fix (0.5% ($^{v}/_{v}$) formaldehyde in D-PBS). Cells were then acquired on a BD Biosciences FACS Canto II machine at a medium speed. A minimum of 5000 events was collected per sample and FCS files were analysed using FlowJo (software version 10).

2.2.8.3 Identification of STAT1 and STAT6 phosphorylation by Phosflow

Monocytes were isolated from peripheral blood, plated onto 24 well plates at a density of 5 x 10^5 cells per well and cultured in either GM-CSF or M-CSF as detailed in section 2.2.1. At day 12 of differentiation, cells were stimulated with either IFNγ, LPS or IL-4 (10ng/ml) in serum free RPMI. After a 30 min stimulation, media was removed and cells were detached from the plate using cell dissociation solution. Cells were placed into Eppendorf tubes and fixed in 4% ($^{v}/_{v}$) formaldehyde in D-PBS for 10 min. Cells were then washed once in D-PBS and permeabilised with Phosflow Perm Buffer III for 30 mins on ice. Cells were pelleted and resuspended in FACS buffer containing 0.1µg of either APC-conjugated phospho-Stat1 (pY701) antibody or PE-conjugated phosphor-Stat6 (pY641). Cells were incubated for 30min at room temperature then washed in D-PBS and resuspended in FACS buffer for analysis on a FACS Canto II at a medium speed. A minimum of 5000 events was collected per sample and FCS files were analysed using FlowJo (software version 10).

2.2.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Monocytes were isolated from peripheral blood as detailed in section 2.2.2.1. Some monocytes were harvested for PCR experiments while the remainder were cultured in either GM-CSF or M-CSF as detailed in section 2.2.1. In order to assess the affect of environmental switch on gene expression, PCR was performed at days 0, 12 and day 6 post switch. Cells were washed 2x with 1ml sterile D-PBS before 200µl lysis buffer (Qiagen buffer RLT + 10% ($^{v}/_{v}$) β-mercaptoethanol) was added /well. Cell lysates were put into sterile Eppendorf tubes (1 per condition) and kept at -80°c until use.

2.2.9.1 RNA Isolation

RNA was isolated using a Qiagen RNeasy® mini kit as per manufactures instructions. Lysate was homogenised by centrifuging through a QIAshredder™ placed in a collection tube at 10,000x*g* for 3 min. The resultant filtrate was then transferred to a gDNA eliminator spin column and centrifuged at 8000x*g* for 30 s. An equal volume of 70% ($^{v}/_{v}$) ethanol was added to flow through. This was mixed by pipetting for 1 min and then transferred into an RNeasy spin column. Tubes were centrifuged at 8000x*g* for 30 s to bind any RNA to the column. Flow through was discarded and 700µl of RW1 buffer was added to the column which was then centrifuged at 8000x*g* for 30 s. Flow through was discarded and 500µl RPE buffer was added to the column. Tubes were then centrifuged at 8000x*g* for 30 s, flow through was discarded and another 500µl RPE buffer was added to the column. Tubes were centrifuged again at 8000x*g* for 30 s and flow through discarded. Columns were placed into a new 1.5ml collection tube and 30µl RNAse-free water was added directly to the column membrane. Tubes were centrifuged at 8000x*g* for 1 min to elute RNA. Elution step was repeated with the

flow through. RNA concentration and purity was identified by Nanodrop® ND-1000 spectrophotometer and samples were kept at -80°C until use.

2.2.9.2 cDNA Synthesis

1µg of RNA was used per condition; this was diluted in RNAse free water in a final volume of 10µl, allowing a 100µg/µl RNA concentration. A 10µl master mix containing 2µl 10x RT buffer, 0.8µl dNTP mix, 2µl random primers and 4.2µl of RNAse free water was then added to each sample so that the final volume was 20µl. PCR tubes were then placed into a G-STORM GS1 thermal cycler and run on the programme detailed in table 2.13 using G-STORM software (version 3.0).

Time	Temperature
10 min	25°C
120 min	37°C
5 min	85°C
Infinity	4°C

Table 2.3: PCR cycle. RNA was isolated from cells and 1ug was converted into cDNA using the G-STORM PCR machine set to the above protocol.

2.2.9.3 Quantitative PCR using TaqMan

TaqMan primers and probes were purchased for STAT1, STAT6, IRF3, IRF7, RELA, c-MAF, and JUN. cDNA was diluted in RNAse free water to a concentration of 5ng/µl. 2µl of cDNA (10ng) was added to the qPCR plate in duplicate. A standard of known cDNA concentrations ranging from 10,000pg/µl to 16pg/µl was also run in duplicate on every plate for each probe. A master mix containing 5µl TaqMan master mix, 0.5µl of the specific gene probe or the endogenous housekeeping probe for HPRT1 and 2.5µl of RNAse free water was added to each sample to make a final volume of 10µl per well. Plates were covered and centrifuged at 300x*g* for 30 s to mix and then run on a 7500 Real Time PCR System (Applied Biosystems, USA) using the 7500 SDS software (version 2.0.6).

2.2.10 Confocal Microscopy

Monocytes were isolated from peripheral blood, plated onto 24 well plates containing a coverslip at a density of 5 x 10^5 cells per well and cultured in either GM-CSF or M-CSF as detailed in section 2.2.1. At day 12 of differentiation, cells were stimulated with either IFNγ, LPS or IL-4 (10ng/ml) in serum free RPMI. After a 15 min incubation, cells had all media aspirated and were fixed with 4% ($^v/_v$) formaldehyde in D-PBS for 10 min. Cells were then washed three times in D-PBS before permeabilisation in 100% methanol for 10 min at 4°C. Cells were washed three times with D-PBS and blocked with 5% ($^v/_v$) human serum in D-PBS. Cells were then incubated in Rabbit-anti-human mAb for either phospho-Stat1 (Tyr701) (58D6), phospho-Stat6 (Tyr641), c-Maf or phospho-IRF3 (S386). Cells were incubated for 2 hours at room temperature before being washed three times in D-PBS. Secondary antibody, anti-rabbit IgG (H+L), F (ab') 2 Fragment (Alexa Fluor® 594 Conjugate), was reconstituted in D-PBS containing 1%

($^{w}/_{v}$) BSA (1:500 dilution), and added to the cells. Cells were incubated in the dark for 2 hours at room temperature before washing twice in D-PBS. CellTracker Green dye (12.5μM) was diluted 1:1000 in D-PBS containing 1% ($^{w}/_{v}$) BSA and added to cells. Cells were incubated for 30 min at 37°C before all media was removed. Cells were washed three times with D-PBS before being stained with nuclear 4′,6-diamidino-2-phenylindole (DAPI) stain for 5 min. Cells were washed three times with D-PBS before coverslip was removed from the well and mounted onto microscope slides using mounting media. Slides were stored in the dark until viewing on a Zeiss LSM-510 inverted confocal microscope.

2.2.11 Cell Metabolic Activity Assay – Cell Viability

All cells that were adherent after assays were performed underwent methylthiazoyldiphenyl-tetrazolium bromide (MTT) assay. Metabolic activity was used to compare treated cells to untreated cells and also different culture conditions as a measure of cell viability. All media was aspirated from wells and cells were incubated in MTT in HBSS (1mg/ml) for 30 min at 37°C. MTT was then aspirated before 100μl of DMSO was added to each well. The DMSO induced a reduction of MTT (yellow) to formazan (purple). Absorbance was read at λ550nm using a spectrophotometer (Spectramax Plus, Molecular Devices). A viability of 100% was assigned to non-treated cells and all data reported as a percentage of this control.

2.2.12 Statistical analysis

Data were discussed with a medical statistician who advised on the best statistical tests depending on the design of the experiment. Comparisons between subject groups were conducted using either Kruskal-Wallace or Mann-Whitney tests with Dunn's post-correctional tests. Comparisons between GM-CSF cultured cells and M-CSF cultured cells from the same patient group were also done using Mann-Whitney tests with Dunn's post-correctional tests. Comparisons between different treatments were conducted using Wilcoxon signed rank tests. Data were expressed as mean ± SEM and analysed using GraphPad Prism software GraphPad Prism software (version 5.03). A p value of <0.05 was considered statistically significant.

CHAPTER 3 EXAMINING THE AFFECTS OF DIFFERENT CULTURE CONDITIONS ON MONOCYTE-DERIVED-MACROPHAGES PHENOTYPE

3.1 Introduction

Many researchers use MDM as a model to study macrophage phenotype and function (Lacey et al. 2012; Erbel et al. 2013). This is mostly due to MDM being far easier and less invasive to obtain from subjects compared to either sputum induction or bronchoalveolar lavage, especially when studying diseased patient groups or collecting samples at exacerbations.

However, there is currently no universally agreed method for culturing MDM. Most groups use adherent cells cultured in RPMI supplemented with FCS for 7-12 days, sometimes with addition of various growth factors and/or cytokines (Davies & Gordon 2005). The addition of growth factors GM-CSF and/or M-CSF is very common as both enhance cell survival and direct monocytes into a macrophage lineage (Bailey et al. 2011; Akagawa 2002). Both GM-CSF and M-CSF are measurable in the BAL and sputum (Saha et al. 2009; Kunz et al. 2011) and changes in levels of these growth factors have been associated with COPD (Saha et al. 2009; Kunz et al. 2010).

It has also been demonstrated that macrophages differentiated *in vitro* using GM-CSF differ compared with macrophages differentiated in M-CSF, both at transcriptional and phenotypic levels (Akagawa et al. 2006; Lacey et al. 2012). Therefore, it is important to define any culture system when reporting outcomes from MDM models.

Other factors that could contribute to the heterogeneity of MDM include differences in culture conditions such as adherence *vs.* non-adherence and the addition of FCS to the culture medium (both of which have now been shown to alter macrophage

phenotype). Adhering macrophages to plastic culture plates can alter pro-inflammatory gene expression (Standiford et al. 1991) and enhance STAT1 activation (Coccia et al. 1999), whist the presence of FCS has been shown to improve macrophage phagocytosis and boost cytokine production in human MDM (Rey-Giraud et al. 2012). Therefore, it is important to classify the affect of these various cell culture conditions on subsequent macrophage phenotype, not only in 'normal' cells from healthy individuals but also in macrophages from disease patients where macrophage function is altered.

Macrophages from COPD patients have a pro-inflammatory, poorly resolving, steroid resistant, dysfunctional phenotype which may contribute to the disease pathophysiology (Chana et al. 2016; Taylor et al. 2010). It is important therefore to understand macrophage function in COPD, however, invasive mechanisms such as BAL and sputum induction are not always possible in more severe patients, especially during exacerbations. Therefore, the MDM model presents a good way of studying macrophage phenotype and possible therapeutic options.

Therefore, in order to carry out experiments to examine the various factors possibly affecting macrophages in COPD, a functional, standardised MDM model must be fully characterised. In order to do this, preliminary experiments were devised to examine MDM phenotype under various culture conditions including adherent *vs* non-adherent conditions, FCS *vs* serum-free media conditions and GM-CSF *vs* M-CSF conditions and to compare the responsiveness of the resultant cells with macrophages isolated from lung tissue from both healthy and diseased lung.

3.1.1 Hypothesis

Monocytes from COPD patients will differentiate into a pro-inflammatory MDM phenotype regardless of culture environment and these reflect the phenotypic changes observed in lung macrophages from COPD patients

3.1.2 Aims

- Characterise the MDM model by examining the affect of adherence and FCS on phenotype of MDM isolated from non-smokers, smokers and COPD patients.
- Assess phenotype based on morphology, LPS-stimulated cytokine release and IL-4-stimulated cytokine release.
- Comparing responses of MDM under these conditions with macrophages isolated from lung tissue.

3.2 Methods

3.2.1 Subject Selection

Subjects were selected as in section 2.2.1. Smokers had at least a 10 pack-year history and COPD patients had an FEV_1: FVC ratio <0.7 and post-bronchodilator FEV_1 % predicted <85%.

3.2.2 Monocyte Isolation by Magnetic Labelling

As some cells would need to be cultured non-adherently, the previously described method of adherence monocyte separation would not be appropriate. Therefore, monocytes were further isolated using negative selection. Firstly, PBMC were obtained by dextran sedimentation and discontinuous Percoll gradient as described in section 2.2.2. Cells were washed and counted then centrifuged at 300x*g* for 5 min before being resuspended in 30µl of sterile HBSS per 10^7 cells. Monocytes were isolated using the Miltenyii Human Monocyte Isolation kit II as per the manufacturer's instructions. Briefly, an Fc blocking agent and antibody cocktail was added at 10µl per 10^7 cells and cells left at 4°C for 10 minutes. Cells were gently mixed before addition of another in 30µl of sterile HBSS per 10^7 cells and then 20µl of biotin conjugated microbeads per 10^7 cells were added for 15 min at 4°C. This cell mixture was adjusted to 20ml with HBSS and centrifuged at 300x*g* for 5 min. Supernatant was aspirated and pellet suspended in 2ml HBSS. A MACS LS Column was set up in a MACS magnet stand and flushed through with 5ml HBSS (which was then discarded). Resuspended cells were then added to the column and allowed to pass through magnet to be collected into a fresh 15ml falcon tube. All biotin labelled cells (non-monocytes) stuck to magnet and unlabeled monocytes flowed through into a 15ml collection tube (Figure 3.1).

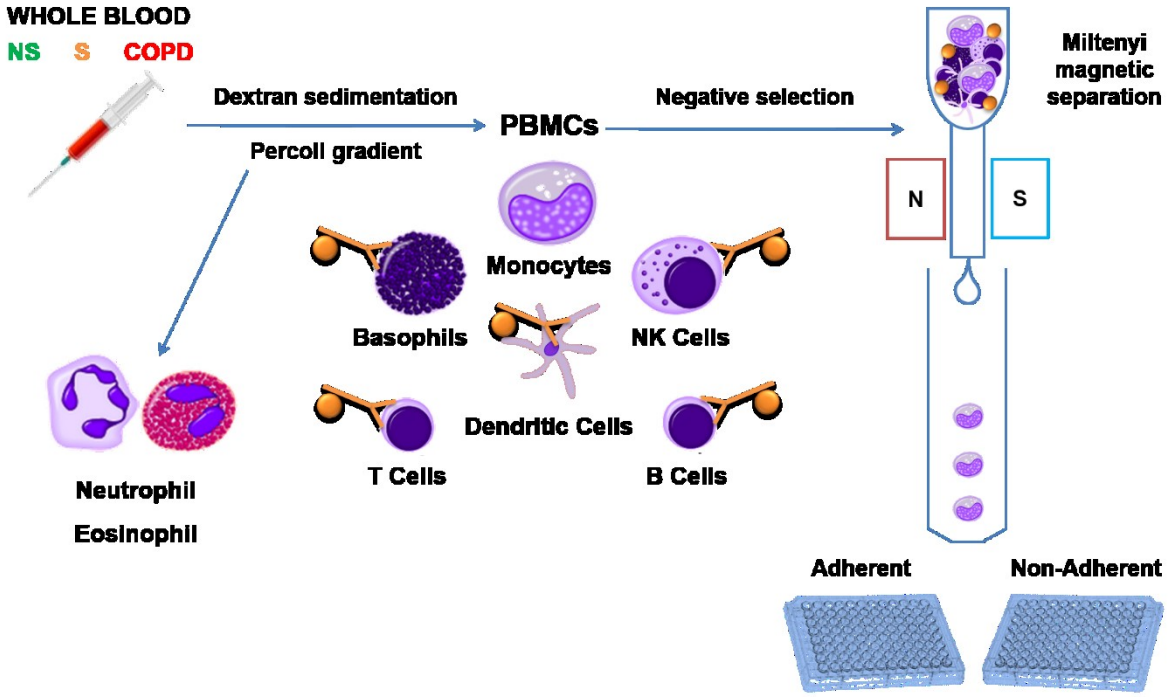


Figure 3.1: Negative selection of human monocytes from peripheral blood. PBMC from non-smokers (NS), smokers (S) or COPD patients (COPD) were first isolated from peripheral blood using dextran sedimentation followed by a discontinuous Percoll gradient. Following this, the Miltenyii Human Monocyte Isolation kit II was used to negatively select monocytes using magnetic labelling and MACS column separation.

The column was washed through 3 times with 3ml of HBSS to elute all monocytes which were then centrifuged at 300x*g* for 5min (room temperature, maximum acceleration and deceleration) and resuspended in 1ml HBSS. Monocytes were then counted as before in section 2.2.2 and resuspended in HBSS to a concentration of $1x10^6$ cells/ml. Cells were then divided into eight 15ml falcon tubes so that each tube contained 2.5 $x10^6$ monocytes. Cells were then centrifuged at 300x*g* for 5 min and resuspended at 1 $x10^6$/ml in media relevant to their subsequent culture conditions. Monocytes were seeded at 1 $x10^5$ cells per well on either adherent or ultra-low

attachment plates, with or without addition of 10% (v/v) FCS media and with addition of either GM-CSF (2ng/ml) or M-CSF (100ng/ml) as appropriate (Figure 3.2).

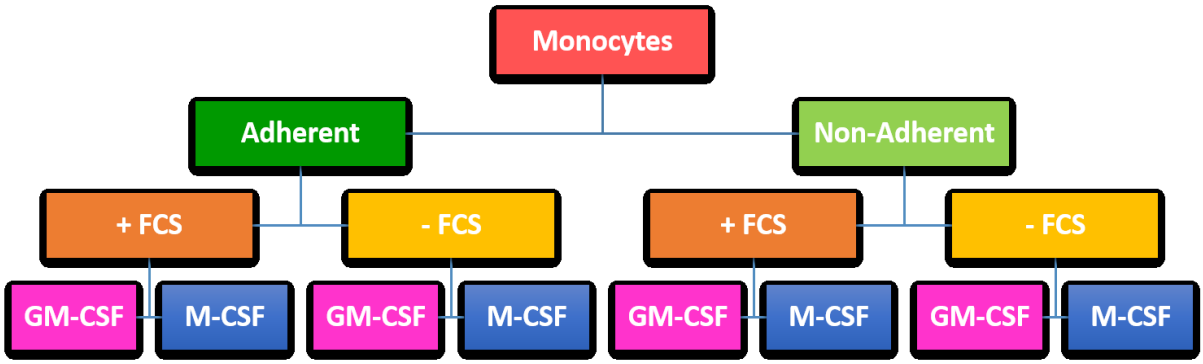


Figure 3.2: Monocyte-derived macrophage model culture conditions. Human monocytes from non-smokers, smokers and COPD patients were isolated from peripheral blood and cultured in differing conditions for 12 days to create monocyte derived macrophages. Cells were then plated onto either adherent or ultra-low attachment plates, with or without addition of FCS and with addition of either GM-CSF or M-CSF for 12 days.

Cells were allowed to differentiate for 12 days at 37°C in 5% (v/v) CO_2 with media replaced on days 4 and 8. Non-adherent plates were centrifuged at 300x*g* for 5 min and then resuspended in fresh media on days 4 and 8.

3.2.4 Morphology of Macrophages Cultured in Different Conditions

Morphology of cells was analysed using light microscopy. Cells cultured under the various conditions were placed on an inverted microscope attached to a Canon digital DSLR camera. Photographs of cells were taken at 20x magnification.

3.2.5 Tissue Macrophage Isolation

Lung tissue macrophages from non-smokers, smokers of COPD patients were isolated from resectioned lung tissue as in section 2.2.3.1. They were then pooled and plated onto on either adherent or ultra-low attachment plates, with or without addition of 10% ($^{v}/_{v}$) FCS media. Cells were placed in an incubator at 37°C in 5% ($^{v}/_{v}$) CO_2 until stimulation.

3.2.6 Measurement of LPS and IL-4-Stimulated Cytokine Release

Cells were treated for 24h at 37°C in 5% ($^{v}/_{v}$) CO_2 in quadruplicate with either control media, 10ng/ml LPS or 10ng/ml IL-4 as in section 2.2.5. LPS and IL-4 stimulated cytokine release was measured by ELISA as detailed in section 2.2.6. Cell supernatant was collected from cells and diluted in ELISA buffer (0.5% ($^{w}/_{v}$) BSA) for specific cytokine analysis (TNFα 1:40, CXCL8 1:300 and IL-10 1:2). All samples were run in quadruplicate against a known concentration standard curve. Plates were read at 450nm and 550nm wavelengths using a spectrophotometer, the 550nm value subtracted from the 450nm value and data interpolated using GraphPad Prism software.

3.2.7 Measurement GM-CSF and M-CSF in Sputum Supernatant

Induced sputum was gained from patients as in section 2.2.4.1. Sputum was processed as in section 2.2.4.2 and concentration of GM-CSF and M-CSF in sputum supernatant was analysed by ELISA as in section 2.2.4.3. Samples were run in triplicate against a standard curve containing DTT. Plates were read at 450nm-550nm using a spectrophotometer, and data interpolated using GraphPad Prism software.

3.2.8 Statistical analysis

All data were expressed as mean ± SEM and analysed using Graphpad Prism software. As there were more than two groups and data were non-parametric and non-paired, comparisons between groups were performed by Kruskall-Wallis tests followed by Dunns' post correctional tests comparing all columns. Comparisons between specific treatments (e.g. GM-CSF *vs* M-CSF in non-smokers) were conducted using Wilcoxon signed rank tests.

3.3 Results

3.3.1 Subject Demographics (MDM)

Subject characteristics and spirometry results are shown in Table 3.1. All groups had equal numbers of subjects and were age matched. There was no significant difference in pack years between smokers and COPD patients. COPD patients had significantly lower FEV_1 % predicted and FEV_1:FVC ratio compared with non-smokers and smokers.

	Non-Smokers (n=4)	**Smokers (n=4)**	**COPD Patients (n=4)**
Age (years)	58 ± 4	59 ± 2	63 ± 1
Sex (M:F)	2:2	2:2	0:4
Smoking History (pack years)	0 ± 0	34 ± 3	43 ± 8
FEV_1 (l)	2.8 ± 0.6	3.1 ± 0.2	2.1 ± 0.5
FEV_1 % Predicted	97 ± 0.1	98 ± 0.1	70 ± 0.1*
FVC (l)	4 ± 0.7	4 ± 0.4	3.5 ± 0.4
FVC % Predicted	104 ± 9.9	115 ± 5.1	94 ± 5.3
FEV_1:FVC	0.8 ± 0.02	0.7 ± 0.04	0.5 ± 0.1*

Table 3.1: Subject demographics for MDM experiments. All participants underwent spirometry testing. FEV_1: Forced Expiratory Volume exhaled at the end of the first second of forced expiration FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for 1 year. Data are expressed as mean ± SEM. Kruskall-Wallis test with Dunn's multiple comparison test. * $p<0.05$ (COPD *vs.* non-smokers).

3.3.2 Affect of Different Culture Conditions on MDM Morphology

Monocytes isolated from human peripheral blood were differentiated for 12 days under 8 different culture conditions (as detailed in section 3.2.2) and photographs were taken on day 12 to compare morphology (Figure 3.3). MDM cultured in GM-CSF (GM-MDM) (left panels) displayed the typical 'fried egg', rounded morphology compared to cells cultured in M-CSF (M-MDM) (right panels) which showed a more spindle like appearance. MDM cultured in FCS media showed no apparent differences compared with those cultured without FCS although, cells cultured without FCS tended to differentiate faster than their FCS counterparts and were fully differentiated by day 8 according to changes in morphology. This was similar in all subject groups.

MDM cultured in non-adherent conditions tended to form a loosely associated monolayer within the wells which dispersed on agitation of the plate. A small number of cells formed attachments onto the plate surface but would easily detach when the plate was agitated. Contrary to the adherent MDM, which tended to form a constant monolayer across the wells, non-adherent MDM tended to aggregate, particularly around the edges of the wells, and send out long dendritic-like extensions to other aggregates close by. This was mostly evident in non-adherent M-MDM cultured without FCS although was also seen in M-MDM cultured in FCS (Figure 3.3).

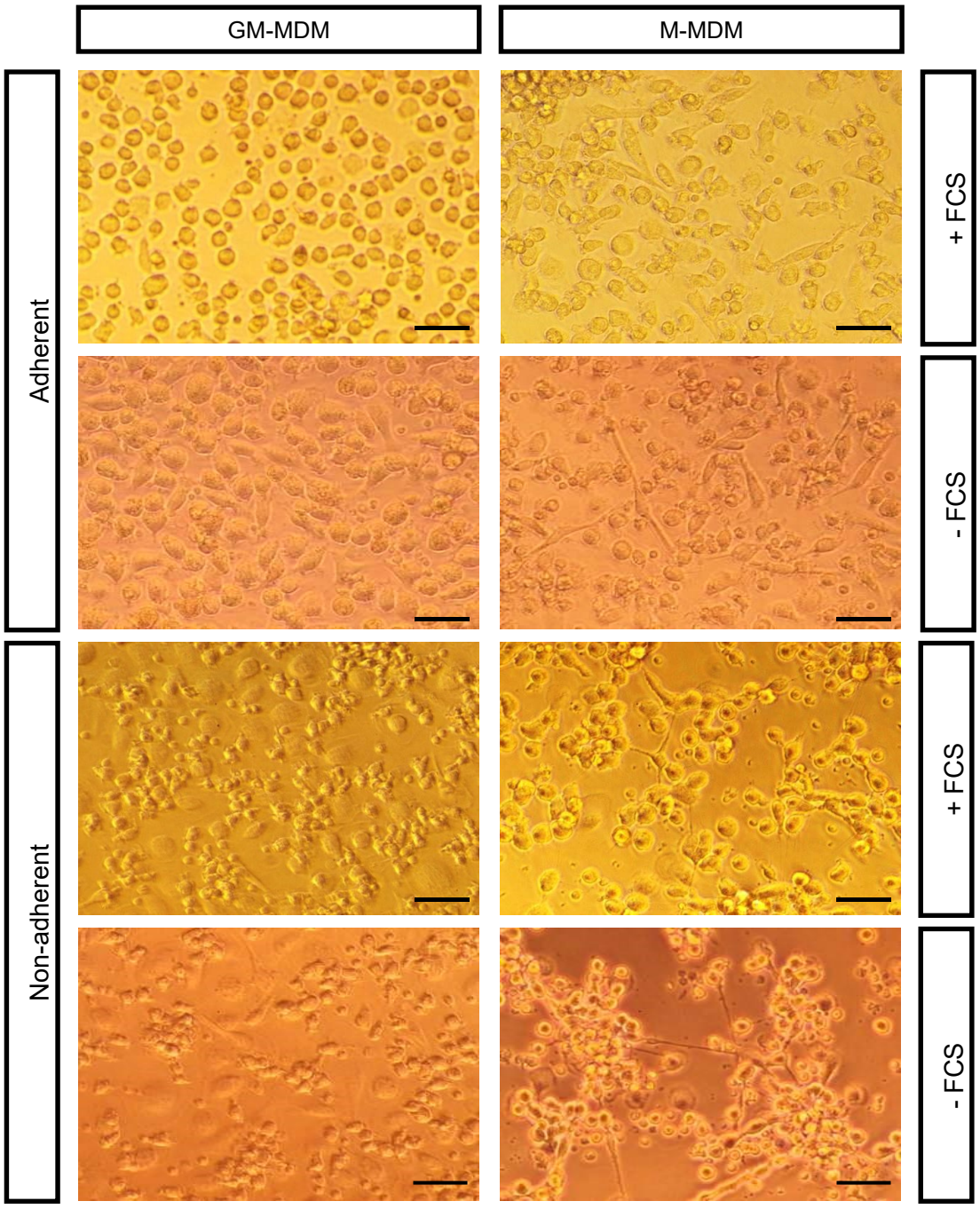


Figure 3.3: Morphology of MDM cultured in different conditions. Human monocytes were isolated from peripheral blood and cultured in either FCS media or serum-free media, on either adherent or non-adherent plates and with addition of either GM-CSF or M-CSF for 12 days to create monocyte derived macrophages (MDM). Photographs show MDM from a healthy participant on day 12, representative of 4 experiments. Photographs taken using an inverted microscope and Optimus E420 digital camera at x40 magnification. Scale bar = 50μm.

3.3.3 LPS-stimulated Cytokine Release from MDM Cultured in Different Conditions

To establish the affect of the different culture conditions on macrophage phenotype, MDM were simulated for 24 h with a sub-maximal concentration of LPS (10ng/ml) (Tudhope et al. 2008). Pro-inflammatory CXCL8, TNFα and anti-inflammatory IL-10 were then measured using ELISA.

3.3.3.1 LPS-stimulated CXCL8 Release

Baseline levels of CXCL8 did not significantly differ between subject groups or culture conditions (Figure 3.4).

Following LPS stimulation, there was a trend for adherent GM-MDM from COPD patients that were cultured in FCS, to release more CXCL8 compared with GM-MDM from non-smokers and smokers cultured under the same conditions (Figure 3.4A), although this was not statistically significant ($p<0.05$). GM-MDM from non-smokers and COPD patients also produced significantly more CXCL8 compared with their M-MDM counterparts (Figure 3.4A).

Non-adherent GM-MDM from COPD patients, cultured in FCS, produced twice as much CXCL8 compared with GM-MDM from non-smokers and smokers ($p<0.05$) (Figure 3.4B). GM-MDM from all subject groups also produced significantly more CXCL8 compared with their M-MDM counterparts (Figure 3.4B).

Adherent GM-MDM from COPD patients, cultured without FCS, produced significantly more CXCL8 than GM-MDM from non-smokers and smokers cultured under the same conditions ($p<0.05$) (Figure 3.4C). GM-MDM from non-smokers smokers produced similar levels to M-MDM cultured in the same way but M-MDM from COPD patients

produced significantly less CXCL8 compared with their GM-MDM counterparts ($p<0.05$) (Figure 3.4C).

Non-adherent GM-MDM from COPD patients, cultured without FCS, produced significantly more CXCL8 compared with GM-MDM from non-smokers and smokers cultured in the same way ($p<0.05$) (Figure 3.4D). M-MDM from COPD patients, also produced significantly more CXCL8 compared with M-MDM from non-smokers cultured in the same way ($p<0.05$) (Figure 3.4D).

When comparing the affect of different culture conditions on CXCL8 release from MDM derived from a single subject group, there were no statistically significant differences in MDM cultured in FCS compared with those cultured without FCS. Nor were there any significant differences in any subject group between adherent and non-adherent MDM. There was also no significant change to overall pattern; COPD MDM consistently produced more CXCL8 compared with MDM from non-smokers and smokers regardless of culture condition.

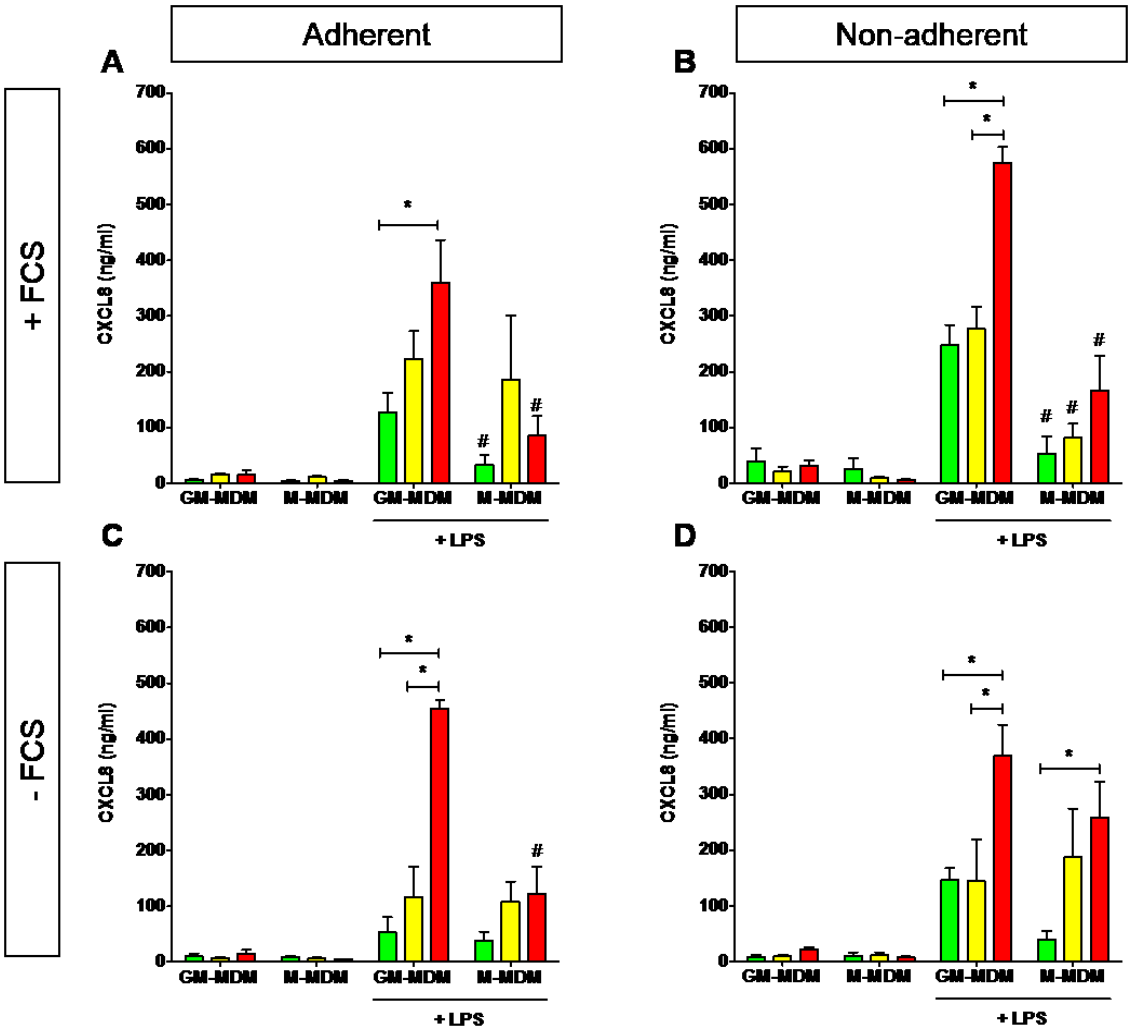


Figure 3.4: LPS-stimulated CXCL8 release under differing culture conditions. Monocytes from non-smokers (green n=4), smokers (yellow n=4) and COPD patients (red n=4) were cultured in either GM-CSF (GM-MDM) or M-CSF (M-MDM). Cells were cultured for 12 days on either adherent plates (A and C) or non-adherent plates (B and D) and either in the presence of 10% ($^{v}/_{v}$) FCS (A and B) or without FCS (C and D). MDM were then stimulated with 10ng/ml LPS for 24 h and CXCL8 in the supernatant was measured using ELISA. Data expressed as mean ± SEM. * $p<0.05$ comparing subject groups, # $p<0.05$ GM-MDM *vs*. M-MDM.

3.3.3.2 LPS-stimulated TNFα Release

Baseline levels of TNFα did not differ significantly between subject groups or different culture conditions (Figure 3.5). Adherent GM-MDM from COPD patients, cultured in FCS, produced significantly more LPS-stimulated TNFα compared with GM-MDM from non-smokers and smokers ($p<0.05$) (Figure 3.4A). GM-MDM from non-smokers and COPD patients also produced significantly more TNFα compared with their M-MDM counterparts ($p<0.05$) (Figure 3.4A). There was a trend for non-adherent GM-MDM from COPD patients, cultured in FCS, to produce more TNFα compared with GM-MDM from non-smokers and smokers, although this did not reach significance ($p>0.05$) (Figure 3.4B). GM-MDM from all subject groups also trended towards more TNFα compared with their M-MDM counterparts, although this did not reach significance ($p>0.05$) (Figure 3.4B). Adherent GM-MDM from COPD patients, cultured without FCS, produced significantly more TNFα than GM-MDM from non-smokers and smokers cultured under the same conditions ($p<0.05$) (Figure 3.4C). GM-MDM from COPD patients also produced significantly more TNFα compared with their M-MDM counterparts (Figure 3.4C).

Non-adherent GM-MDM from COPD patients and smokers, cultured without FCS, produced slightly more TNFα compared with GM-MDM from non-smokers and smokers cultured under the same conditions, although this did not reach significance ($p>0.05$) (Figure 3.4D). GM-MDM from all subject groups produced similar levels of TNFα compared with their M-MDM counterparts (Figure 3.4C). When comparing the affect of different MDM culture conditions on cells form single subject group, there were no statistically significant differences in TNFα production by MDM cultured in FCS compared with those cultured without FCS, although there was a trend for less TNFα by non-smoker GM-MDM in the cells cultured without FCS (p value = 0.08).

There were also no significant differences when comparing adherent MDM with non-adherent MDM (p>0.05).

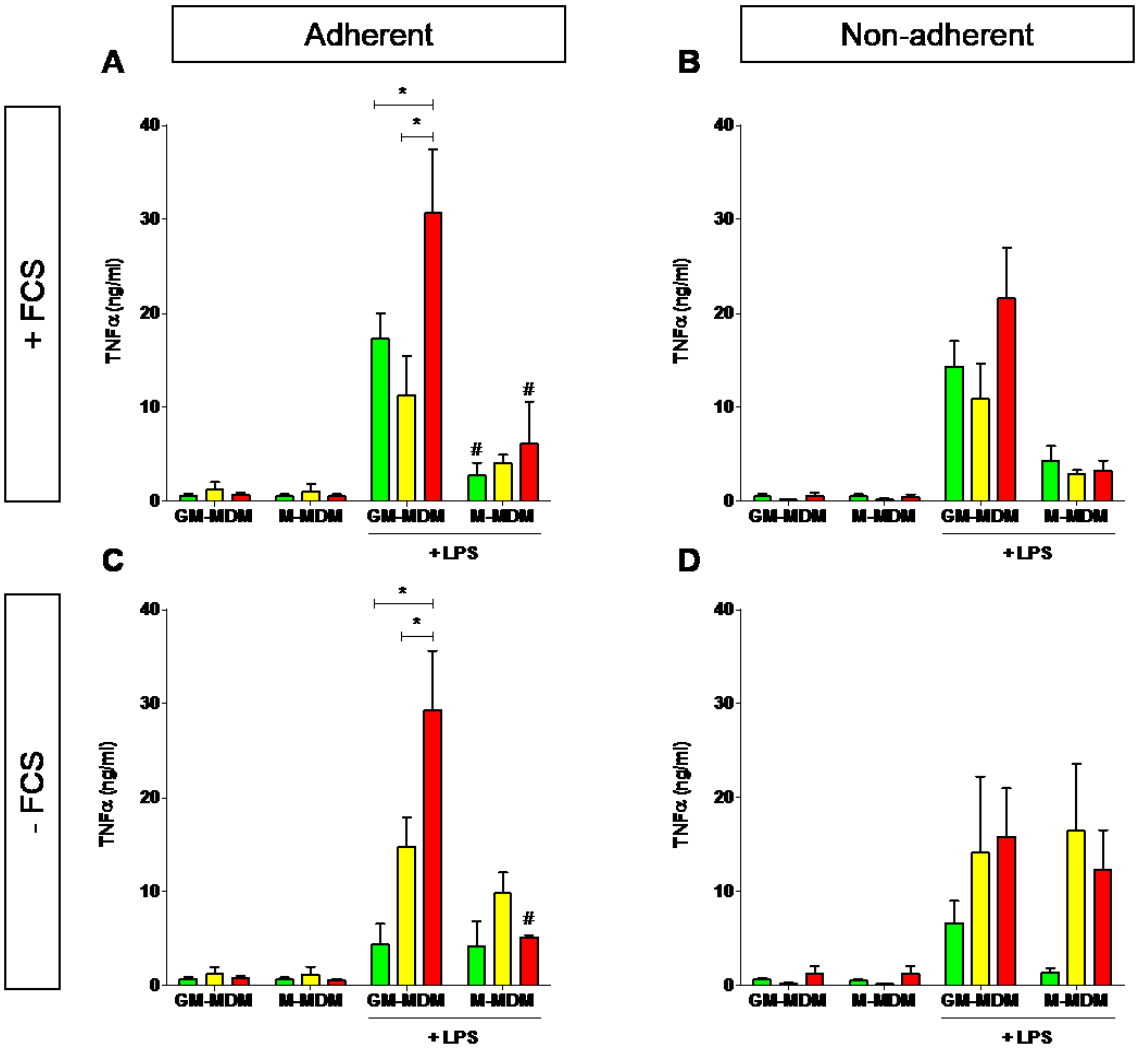


Figure 3.5: LPS-stimulated TNFα release under differing culture conditions. Monocytes from non-smokers (green n=4), smokers (yellow n=4) and COPD patients (red n=4) were cultured in either GM-CSF (GM-MDM) or M-CSF (M-MDM). Cells were cultured for 12 days on either adherent plates (A and C) or non-adherent plates (B and D) and either in the presence of 10% ($^{v}/_{v}$) FCS (A and B) or without FCS (C and D). MDM were then stimulated with 10ng/ml LPS for 24 h and TNFα in the supernatant was measured using ELISA. Data expressed as mean ± SEM. * p<0.05 comparing subject groups, # p<0.05 GM-MDM *vs*. M-MDM.

3.3.3.3 LPS-stimulated IL-10 Release

Baseline levels of IL-10 did not differ significantly between subject groups or different culture conditions (Figure 3.6).

Following LPS stimulation, adherent M-MDM from non-smokers, cultured in FCS, released significantly more IL-10 compared with M-MDM from smokers and COPD patients cultured under the same condition ($p<0.05$) (Figure 3.5A). There was also a trend for non-smoker GM-MDM to produce less IL-10 compared with their M-MDM counterparts cultured under the same conditions, although this did not reach significance ($p>0.05$) (Figure 3.5A).

There was no significant difference when comparing non-adherent M-MDM cultured in FCS (Figure 3.5B). However, there was a trend for GM-MDM from all subject groups to produce less IL-10 compared with their M-MDM counterparts cultured under the same conditions, although this did not reach significance ($p>0.05$) (Figure 3.4A).

There was no significant increase in LPS-stimulated-IL-10 production in the adherent MDM cultured without FCS in any subject group (Figure 3.5C). This was also the case for non-adherent MDM cultured without FCS, regardless of subject group (Figure 3.5D).

When comparing the affect of different culture conditions on a single subject group there were significant differences in the amount of IL-10 produced by M-MDM from non-smokers. The levels of IL-10 produced were reduced slightly in the non-adherent condition ($p=0.09$) and reduced significantly in the cells cultured without FCS ($p<0.05$).

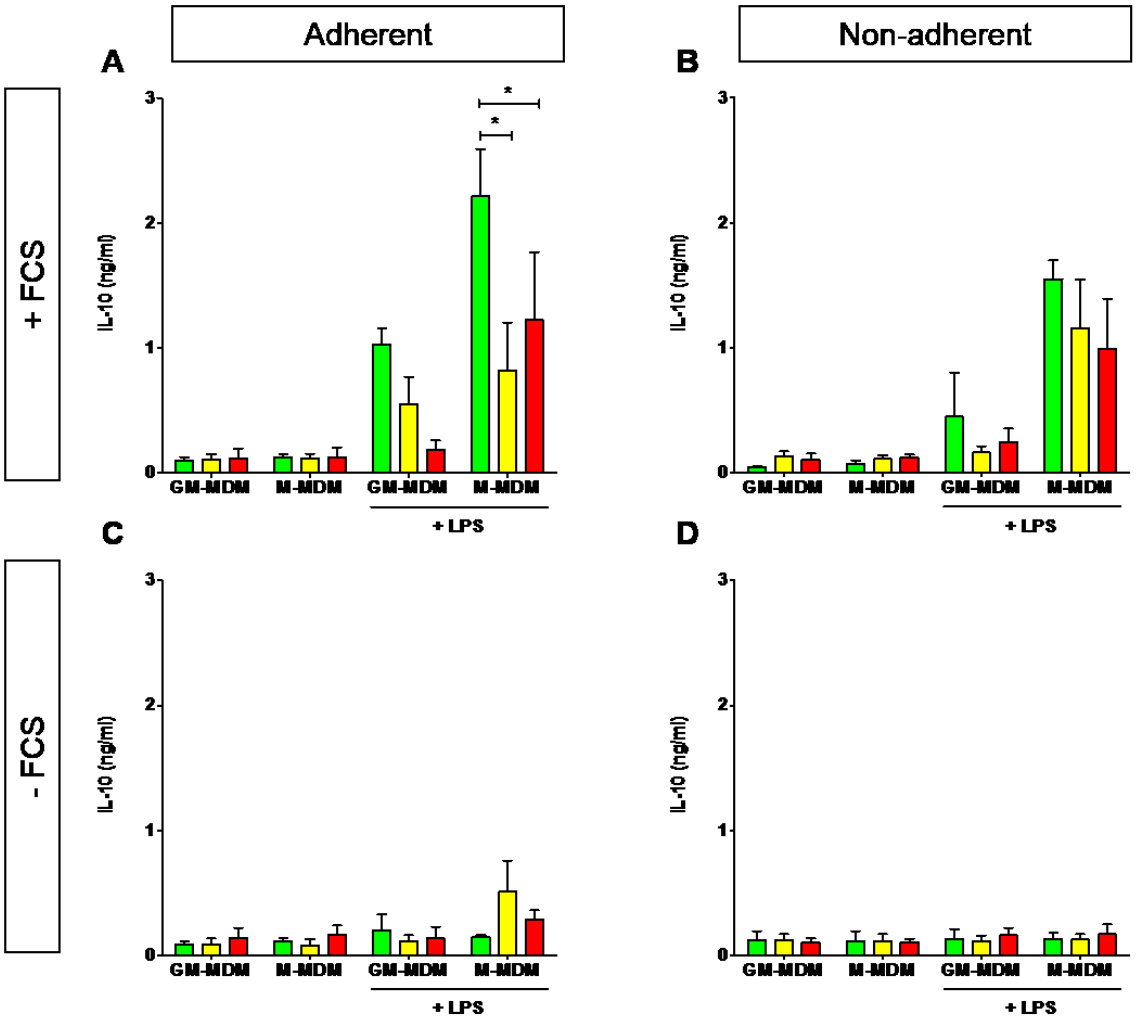


Figure 3.6: LPS-stimulated IL-10 release under differing culture conditions. Monocytes from non-smokers (green n=4), smokers (yellow n=4) and COPD patients (red n=4) were cultured in either GM-CSF (GM-MDM) or M-CSF (M-MDM). Cells were cultured for 12 days on either adherent plates (A and C) or non-adherent plates (B and D) and either in the presence of 10% ($^{v}/_{v}$) FCS (A and B) or without FCS (C and D). MDM were then stimulated with 10ng/ml LPS for 24 h and IL-10 in the supernatant was measured using ELISA. Data expressed as mean ± SEM. * $p<0.05$ comparing subject groups.

3.3.4 IL-4-stimulated Cytokine Release from MDM Cultured in Different Conditions

In order to investigate the affect of IL-4 activation, MDM were simulated for 24 h with 10ng/ml IL-4, then CXCL8, TNFα and IL-10 were measured using ELISA. IL-4 did not stimulate above baseline release of either CXCL8 or TNFα in any of the subject groups under any of the culture conditions, nor were there any significant differences between subject groups.

3.3.4.3 IL-4-stimulated IL-10 Release

Baseline levels of IL-10 did not differ between subject groups or different culture conditions (Figure 3.7). Following IL-4 stimulation, adherent M-MDM from non-smokers, cultured in FCS, released significantly more IL-10 compared with M-MDM from smokers and COPD patients cultured under the same condition ($p<0.05$) (Figure 3.6A). M-MDM from non-smokers also produced significantly more IL-10 compared with non-smoker GM-MDM cultured under the same conditions (Figure 3.6A). Non-adherent M-MDM from non-smokers, cultured in FCS, also produced significantly more IL-10 compared with M-MDM from smokers and COPD patients ($p<0.05$) (Figure 3.6B). M-MDM from non-smokers also produced significantly more IL-10 compared with non-smoker GM-MDM cultured under the same conditions (Figure 3.6B). There was no significant increase in IL-4-stimulated-IL-10 production in the adherent MDM cultured without FCS in any subject group (Figure 3.6C). This was also the case for non-adherent MDM cultured without FCS, regardless of subject group (Figure 3.6D). When comparing the affect of different culture conditions on a single subject group there were again significant differences in the amount of IL-10 produced by non-smokers when comparing cells cultured either with or without FCS ($p<0.05$). There

was no above baseline release of IL-10 when cells were stimulated following differentiation without FCS regardless of whether they were adherent of non-adherent.

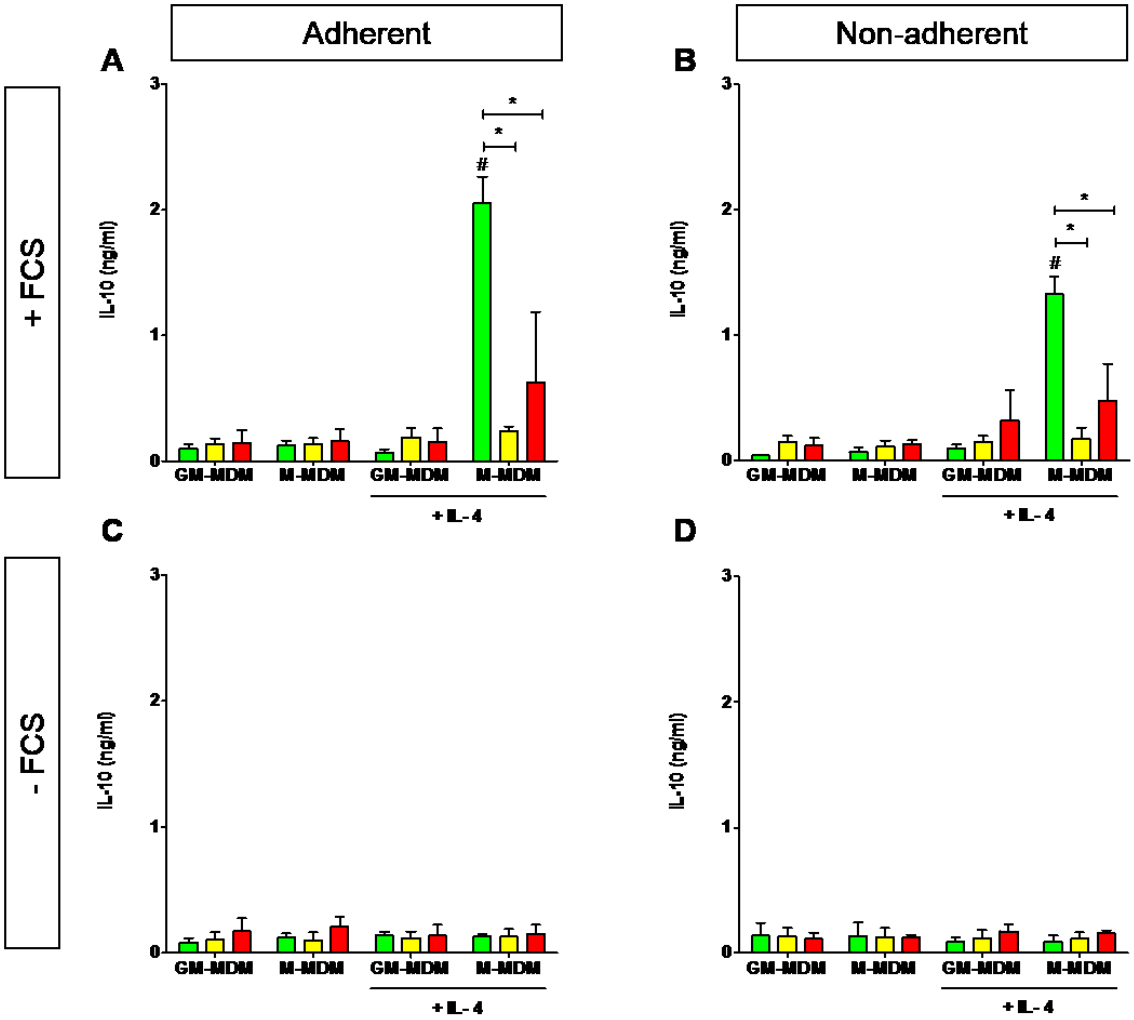


Figure 3.7: IL-4-stimulated IL-10 release under differing culture conditions. Monocytes from non-smokers (green n=4), smokers (yellow n=4) and COPD patients (red n=4) were cultured in either GM-CSF (GM-MDM) or M-CSF (M-MDM). Cells were cultured for 12 days on either adherent plates (A and C) or non-adherent plates (B and D) and either in the presence of 10% ($^{v}/_{v}$) FCS (A and B) or without FCS (C and D). MDM were then stimulated with 10ng/ml IL-4 for 24 h and IL-10 in the supernatant was measured using ELISA. Data expressed as mean ± SEM. * $p<0.05$ (comparing subject groups), # $p<0.05$ (GM-MDM *vs*. M-MDM).

3.3.5 Subject Demographics (Lung Tissue Macrophages)

Subject characteristics and spirometry results are shown in Table 3.2. Non-smokers were younger than smokers and COPD patients. There was no significant difference in pack years between smokers and COPD patients although there was a trend for higher pack years in the smokers. COPD patients also had significantly reduced FEV_1 % predicted, and FEV_1:FVC ratio compared with non-smokers and smokers.

	Non-Smokers (n=3)	**Smokers (n=3)**	**COPD Patients (n=4)**
Age (years)	43 ± 3	73 ± 6	70 ± 6
Sex (M:F)	1:2	1:2	2:2
Smoking History (pack years)	0 ± 0	58 ± 17	34 ± 9
FEV_1 (l)	2.7 ± 0.4	3.2 ± 0.1	2 ± 0.5
FEV_1% Predicted	92 ± 3	86 ± 5	35.± 1*
FVC (l)	3.5 ± 0.3	4 ± 0.4	3.9 ± 0.9
FVC % Predicted	92 ± 3	99 ± 9	50 ± 11*
FEV_1:FVC	0.85 ± 0.04	0.88 ± 0.02	0.44 ± 0.6*

Table 3.2: Subject demographics for lung tissue experiments. All participants underwent spirometry testing. FEV_1: Forced Expiratory Volume exhaled at the end of the first second of forced expiration FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for 1 year. Data are expressed as mean ± SEM. Kruskall-Wallis test with Dunn's multiple comparison test. * $p<0.05$ COPD *vs.* non-smokers.

3.3.6 LPS and IL-4-stimulated Cytokine Release from Lung Tissue Macrophages Cultured in Different Conditions

Having shown differences in expression some pro and anti-inflammatory cytokine release in MDM, it was important to compare this with macrophages isolated from lung tissue. Therefore, similar experiments were devised using tissue macrophages and culturing under adherent or non-adherent conditions in the presence or absence of 10% ($^{v}/_{v}$) FCS.

3.3.6.1 Lung Tissue Macrophage CXCL8

There was a consistent trend for macrophages from COPD lung tissue to produce more CXCL8 following stimulation with LPS or IL-4 compared with macrophages from smokers or non-smokers, regardless of cell culture method. Within each subject group, levels of CXCL8 release were not significantly altered by adherence or the presence of FCS which remained consistent regardless of cell culture method (Figure 3.8). LPS-stimulated macrophages produced more CXCL8 compared with IL-4 stimulated cells.

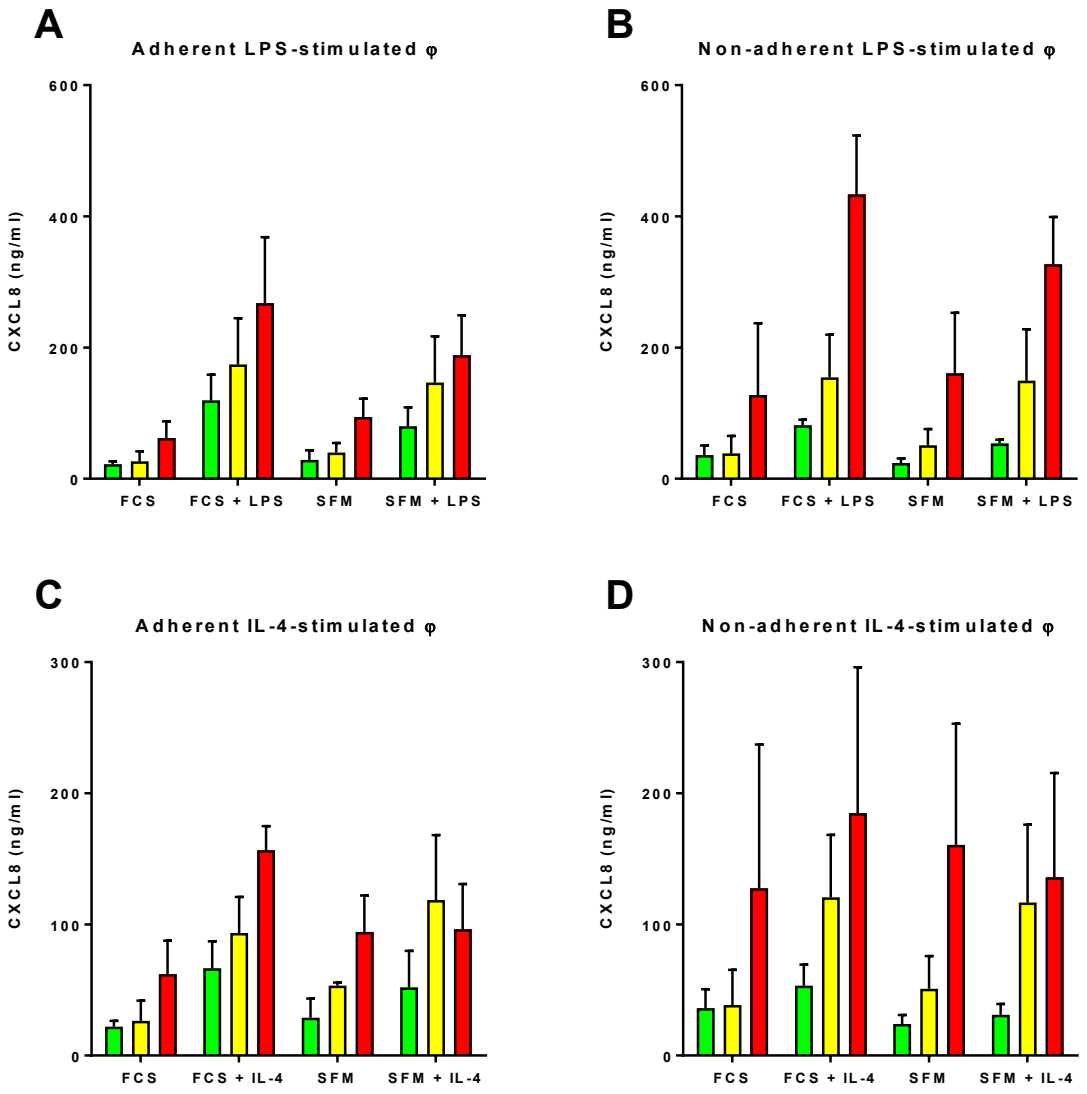


Figure 3.8 LPS and IL-4-stimulated CXCL8 release under differing culture conditions. Lung tissue macrophages (φ) were isolated from non-smokers (green n=4), smokers (yellow n=4) and COPD patients (red n=4) and were plated on either adherent plates (A and C) or non-adherent plates (B and D) and either in the presence of 10% ($^{v}/_{v}$) FCS or under serum free conditions (SFM). Cells were then stimulated with 10ng/ml LPS or IL-4 for 24h and CXCL8 in the supernatant was measured using ELISA. Data expressed as mean ± SEM.

3.3.6.1 Lung Tissue Macrophage TNFα

There was also a trend for macrophages from COPD lung to produce more LPS-stimulated TNFα compared with macrophages from smokers or non-smokers, regardless of cell culture method, although this did not reach significance ($p>0.05$) (Figure 3.9).

Within each subject group, LPS-stimulated TNFα levels were not significantly altered by adherence ($p>0.05$) or the presence of FCS ($p>0.05$) which remained consistent regardless of cell culture method (Figure 3.9).

There was no significant increase in TNFα release with IL-4 stimulation in any group.

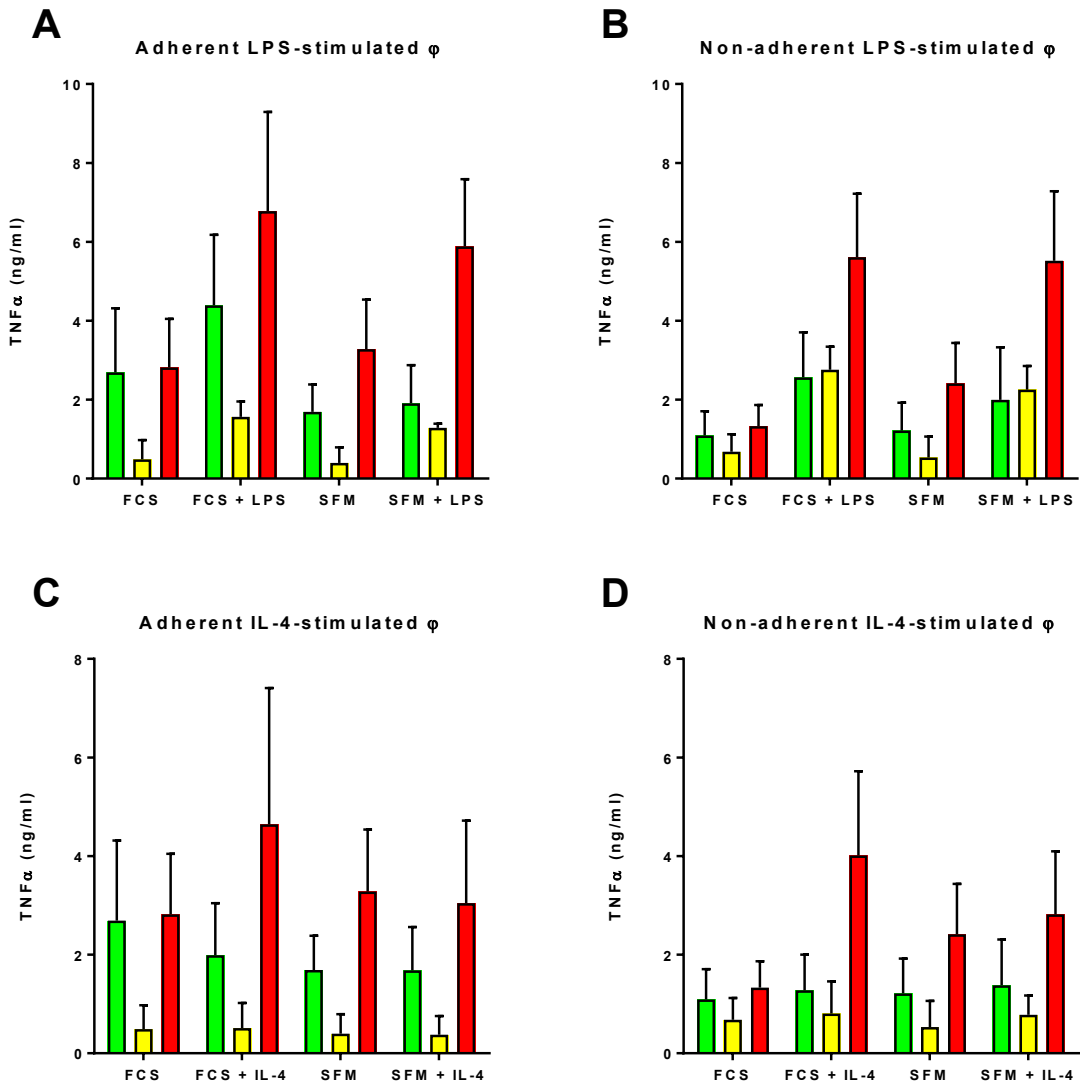


Figure 3.9 LPS and IL-4-stimulated TNFα release under differing culture conditions. Lung tissue macrophages (φ) were isolated from non-smokers (green n=4), smokers (yellow n=4) and COPD patients (red n=4) and were plated on either adherent plates (A and C) or non-adherent plates (B and D) and either in the presence of 10% ($^{v}/_{v}$) FCS or under serum free conditions (SFM). Cells were then stimulated with 10ng/ml LPS or IL-4 for 24h and TNFα in the supernatant was measured using ELISA. Data expressed as mean ± SEM.

3.3.6.1 Lung Tissue Macrophage IL-10

There was no significant increase in IL-10 release in the adherently cultured macrophages stimulated with LPS and there was also no significant difference between subject groups under this condition (Figure 3.10A).

In the non-adherent macrophages, there was a trend for higher baseline IL-10 release in cells from COPD patients compared with smokers and non-smokers which was slightly increased with LPS stimulation, but this did not reach significance, regardless of the presence of FCS ($p>0.05$) (Figure 3.10B).

There was a trend for macrophages from COPD patients stimulated by IL-4 to produce higher levels of IL-10 compared with non-smokers and smokers, although there was large variance between patient samples leading to large error ($p>0.05$) (Figure 3.10C).

Again non-adherent macrophages from COPD patients, stimulated with IL-4, released more IL-10 compared with non-smokers, although this was not statistically significant ($p>0.05$).

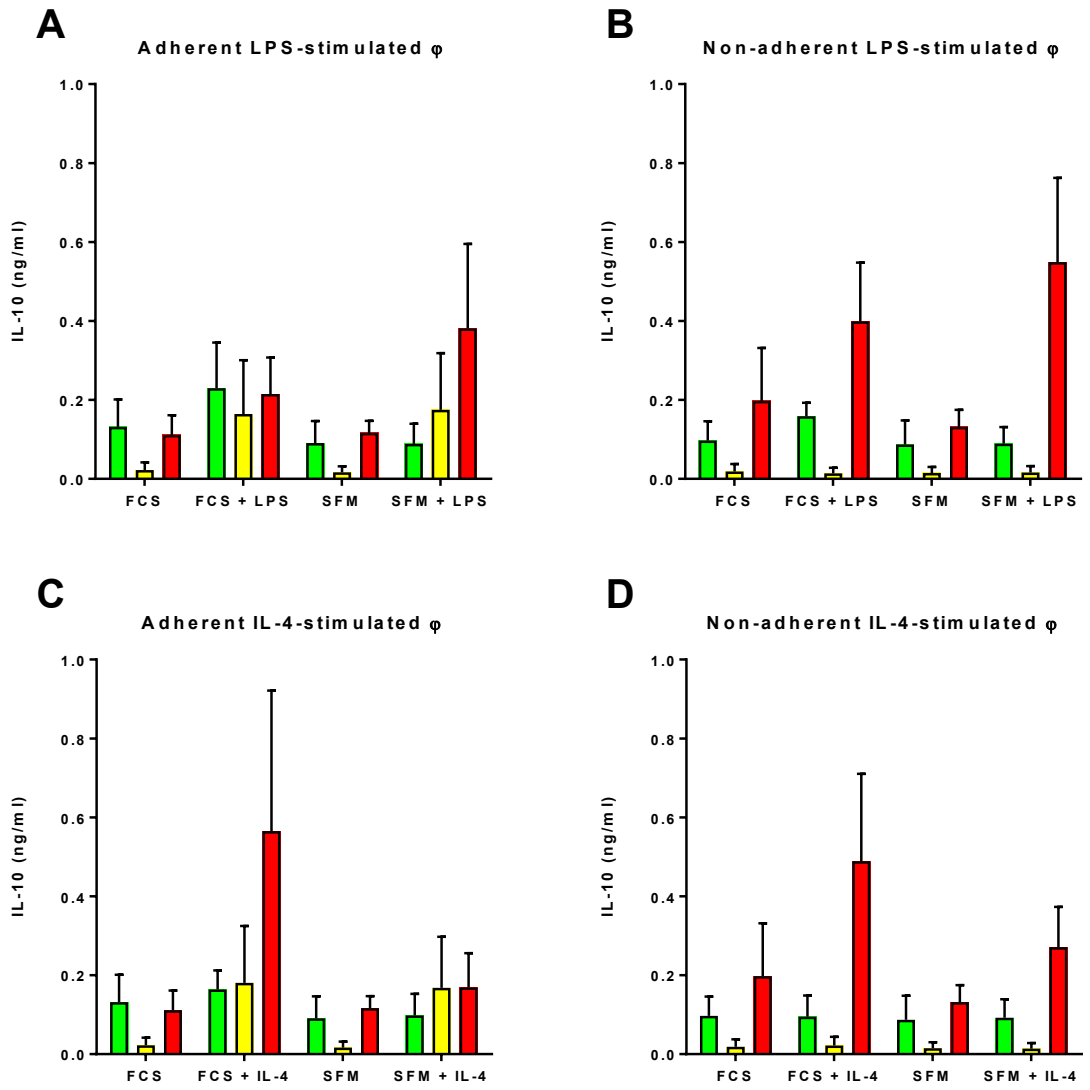


Figure 3.10 LPS and IL-4-stimulated IL-10 release under differing culture conditions. Lung tissue macrophages (φ) were isolated from non-smokers (green n=4), smokers (yellow n=4) and COPD patients (red n=4) and were plated on either adherent plates (A and C) or non-adherent plates (B and D) and either in the presence of 10% ($^{v}/_{v}$) FCS or under serum free conditions (SFM). Cells were then stimulated with 10ng/ml LPS or IL-4 for 24h and IL-10 in the supernatant was measured using ELISA. Data expressed as mean ± SEM.

3.3.7 GM-CSF and M-CSF Concentrations in Sputum

Having shown some differences in cytokine expression from GM-CSF and M-CSF cultured cells, it was important to measure levels of these cytokines, known to be important in macrophage differentiation and whether they differ in COPD. Therefore, levels of GM-CSF and M-CSF in induced sputum samples collected from non-smokers, smokers and COPD were measured by ELISA.

3.3.7.1 Subject Demographics Sputum

Subject characteristics and spirometry results are shown in Table 3.3. COPD patients had significantly higher pack year history compared with smokers. COPD patients had significantly lower FEV_1, FEV_1 % predicted and FEV_1:FVC ratio compared with non-smokers and smokers.

	Non-Smokers (n=8)	Smokers (n=8)	COPD Patients (n=9)
Age (years)	50 ± 2	52 ± 2	62 ± 7
Sex (M:F)	6:2	3:5	7:2
Smoking History (pack years)	0 ± 0	26 ± 3	52 ± 8*
FEV_1 (l)	3.4 ± 0.4	2.8 ± 0.3	1.4 ± 0.2*#
FEV_1 % Predicted	104 ± 3	94 ± 5	55 ± 7*#
FVC (l)	4.3 ± 0.6	3.6 ± 0.4	3.2 ± 0.2
FVC % Predicted	113 ± 4	97 ± 9	73 ± 7
FEV_1:FVC	0.8 ± 0.02	0.8 ± 0.02	0.4 ± 0.04*#

Table 3.3: Subject demographics for sputum experiments. All participants underwent spirometry testing. FEV_1: Forced Expiratory Volume exhaled at the end of the first second of forced expiration FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for 1 year. Data are expressed as mean ± SEM. Kruskall-Wallis test with Dunn's multiple comparison test. * $p<0.05$ COPD *vs.* non-smokers.

3.3.7.2 GM-CSF and M-CSF in Sputum Supernatant

Sputum supernatant from COPD patients had significantly more GM-CSF compared with sputum supernatant from smokers ($p<0.05$) and non-smokers ($p<0.05$) (Figure 3.11A). Non-smokers had a significantly higher concentrtion of M-CSF in sputum supernatant compared with smokers ($p<0.05$) (Figure 3.10B). There was also a trend for samples from COPD patients to have less M-CSF compared with non-smokers although this did not reach statistical significance (Figure 3.11B).

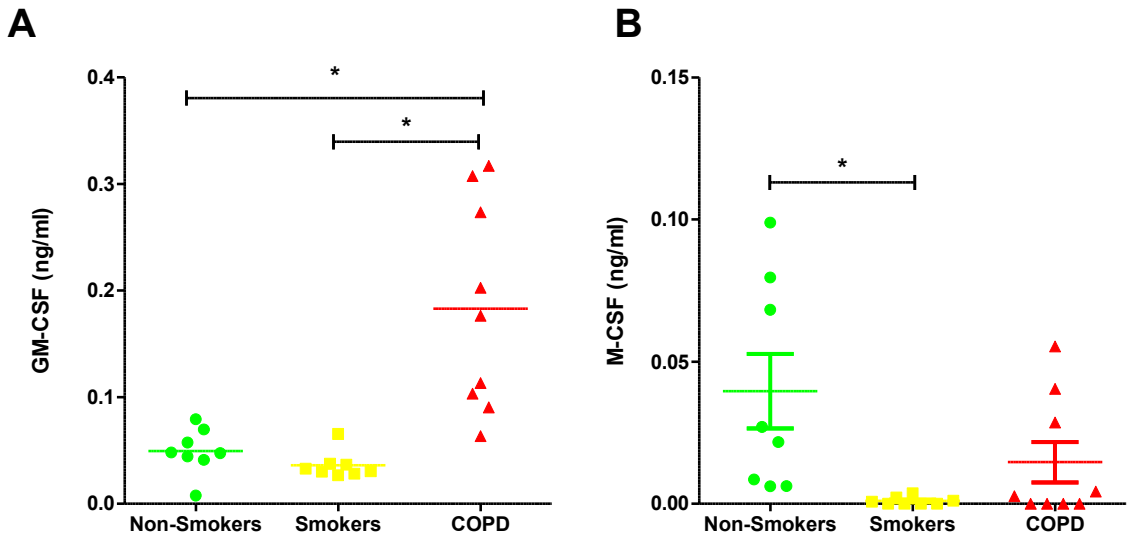


Figure 3.11 GM-CSF and M-CSF concentration in sputum samples. Induced sputum samples were obtained from non-smokers (green n=8), smokers (yellow n=8) and COPD patients (red n=9). ELISA was used to identify concentrations of (A) GM-CSF and (B) M-CSF in sputum supernatant. Data expressed as mean ± SEM. *$p<0.05$ comparing subject groups

3.4 Discussion

Before investigating macrophage phenotype and plasticity in COPD, the model used for these studies needed to be fully characterised in order to ensure any effects observed were not simply artefacts of the culture system. The affect of adherence and addition of FCS were assessed in MDM from non-smokers, smokers and COPD patients together with the addition of growth factors.

3.4.1 Morphology

Analysis of the morphology of the differently cultured MDM showed distinct morphological features of the GM-CSF or M-CSF models in line with other studies showing the divergent morphological properties of the two cell types (Akagawa et al. 2006; Waldo et al. 2008). GM-MDM exhibited a spherical morphology compared with the more spindle like morphology of the M-MDM. This is a widely reported feature of the model (Lemaire & Yang 1996; Bailey et al. 2011; Lacey et al. 2012; Ploeger et al. 2013). There were no apparent morphological differences in cells cultured with FCS compared with cells cultured without FCS suggesting serum does not affect the morphology of the cells.

3.4.2 Affect of Adherence

The process of adherence has long been known as an integral step of the peripheral monocyte to tissue macrophage transition. Although, adherence to plastic, may not represent a physiologically relevant model. Adherence of human monocytes to plastic, such as in a cell culture model, has been shown to influence pro-inflammatory gene expression of human monocytes (Standiford et al. 1991; Eierman et al. 1989) and influence their phagocytic ability in comparison with non-adherent cells (Sköld et al.

1995; Gross 2010; Selvarajan 2009). However, no studies have yet directly compared the cytokine production of differently cultured cells within a disease model. Data from this chapter demonstrate that adherence to plastic does not significantly affect the LPS-stimulated pro-inflammatory cytokine profile of MDM from any subject group when compared with non-adherent cultured cells in the MDM model; the levels of cytokines released from cells were comparable under each of the conditions examined.

Conversely, adherence appeared to have an affect on LPS-induced IL-10 production from non-smoker MDM. This is in line with another study, by Petit-Bertron *et al,* showing adherence directly affected IL-10 signalling and anti-inflammatory gene expression in macrophages from healthy human donors (Petit-Bertron et al. 2005). Another study by the same group also showed receptor expression and TNFα inhibition were dependent on adherence and that when cells were not adhered to plastic, the affects of IL-10 were reduced (Petit-Bertron et al. 2003). They also suggested that adherence may act as a "priming mechanism" which subsequently affects LPS-induced IL-10 signalling in human PBMC (Petit-Bertron et al. 2003).

3.4.3 Affect of Serum

The results from this chapter showed that the presence of FCS during differentiation did not significantly affect the pro-inflammatory cytokine profile in any of the subject groups in the MDM model compared with MDM cultured without FCS. However, this chapter also showed that the addition of FCS into the culture media was necessary in order to generate measureable differences in IL-10 release after stimulation. FCS is high in bovine serum albumin (BSA) and also contains integrin beta-1, alpha-1- anti-proteinase and TGF-β (Zheng et al. 2006). The lung liquid interface also contains high

levels of serum albumin, complement factors and angiotensin (Adkins et al. 2002) as well as several other constituents including lipids, surfactants and enzymes (Moliva et al. 2014) which are important for lung heath (Notter 2000). However, the lung is not normally considered a serum rich environment, although, serum proteins including those mentioned above, are made by many cells lining the airways. An increase in serum proteins would indicate vascular leakage or damage in the lung. Nevertheless, serum containing cell culture media has long been used to culture many cells types including macrophages (Andreesen et al. 1983; Rios et al. 2017). Although this is not a physiological environment, there are some areas where the culture system is comparable such as the presence of albumin, as albumin composes approximately 50% of the proteins in human serum (Artemyev et al. 2016). Another limitation of the cell culture model is that airway macrophages would not be submerged under ~1cm of liquid however, there are no systems currently used that can be described as truly mimicking the physiological environment and this is a caveat for interpretation of many studies including the present thesis.

It could be possible that factors in FCS-containing media may 'prime' the cells so that, when stimulated, they release higher levels of IL-10 compared with those cultured without FCS. It is thought that macrophages can be 'primed' so that when stimulated, they may subsequently modulate cytokine output and respond more effectively to insults (Partrick et al. 1997). These 'primed' cells have also been associated with a 'not yet activated' phenotype which does not produce pro-inflammatory mediators until a second stimuli is introduced (Ma et al. 2003). This is in line with the data presented herein showing that only MDM cultured in FCS media and adhered to the plate increased IL-10 production above baseline levels when stimulated with either LPS or IL-4. It is possible that the MDM in our study were 'primed' by components in FCS

such as growth factors, so they reacted to a secondary stimulus, leading to a higher IL-10 response.

3.4.4 MDM from COPD patients are consistently pro-inflammatory

It was noted in all cytokine profiling that MDM from COPD patients remained consistently more pro-inflammatory when stimulated with LPS, regardless of culture condition, compared with cells from non-smokers and smokers. In addition, MDM from both smokers and COPD patients released lower levels of LPS and IL-4-stimulated IL-10 compared with MDM from non-smokers showing that they also were less 'anti-inflammatory', regardless of culture environment. This suggests that the peripheral monocytes from COPD patients, and to some extent smokers, are already programmed to be pro-inflammatory, prior to any influence of the lung environment on macrophage phenotype.

3.4.5 Comparisons to Lung Tissue Macrophages

There were similarities in the overall pattern of expression of pro-inflammatory cytokines between MDM and lung tissue macrophages. Although, there was more subject variability in the tissue macrophage samples compared with the MDM model, most likely as a result of an individual and heterogeneous population of subjects and the small number of samples used. Similarly to the MDM model, COPD macrophages were consistently more pro-inflammatory compared with non-smokers. This is in agreement with various other studies showing high levels of pro-inflammatory cytokine release from COPD tissue macrophages (Hackett et al. 2008) and from macrophages isolated from COPD patient sputum (Frankenberger et al. 2011).

The higher levels of pro-inflammatory cytokine release in COPD tissue macrophages may also explain the higher levels of IL-10 in COPD tissue macrophages. While there is evidence of low IL-10 expression in sputum macrophages from COPD patients compared with non-smoking controls (Takanashi et al. 1999). This is the first demonstration of IL-10 production from human lung tissue macrophages indicating increased IL-10 release by cells from COPD patients. A reason as to why the levels were higher in COPD compared to non-smoker samples may be a compensatory mechanism to account for the highly pro-inflammatory lung environment from which they were isolated. When comparing IL-10 expression to the MDM model, the COPD MDM produced far less IL-10, this may be explained by the lack of contact with the lung environment by the MDM. It is also worth noting, however, the large error and small sample size, indicating more work would need to be done into these cells before direct conclusions can be made.

3.4.6 Sputum GM-CSF and M-CSF

Results from this chapter showed that levels of GM-CSF were higher in sputum supernatants from COPD patients compared with non-smokers. This is in agreement with previous research by Saha et al who used ELISA and histochemical staining of endobronchial biopsies to show increased GM-CSF in COPD patients compared with controls (Saha et al. 2009). This is the first instance of M-CSF levels being measured in sputum and many of the samples from smokers and COPD patients failed to reach the limit of detection.

3.4.7 Conclusions

- Different culture conditions had no significant affect on LPS-stimulated TNFα or CXCL8 release by MDM.
- MDM from COPD patients produced more pro-inflammatory cytokines and less anti-inflammatory cytokines regardless of culture environment.
- Addition of serum may be 'priming' MDM to produce more IL-10 when stimulated and this result is similar in lung tissue macrophages.
- Results from lung tissue macrophages showed similar patterns to MDM in terms of their pro-inflammatory cytokine expression.

Therefore, the hypothesis of "Monocytes from COPD patients will differentiate into a pro-inflammatory MDM phenotype regardless of culture environment" can be accepted. From these data a model of adherent MDM cultured in the presence of FCS was taken forward for future experiments.

Following on from these results, it was decided to use cells which had been cultured adherently and in the presence of FCS. The reasoning behind this was so that we would have a measureable anti-inflammatory response with which to compare the GM-CSF and M-CSF cultured cells and also to identify changes to the plasticity of these cells with altered cell culture.

CHAPTER 4 ALTERING CULTURE ENVIRONMENT TO EXAMINE MDM PHENOTYPIC PLASTICITY

The previous chapter showed differences between GM-MDM and M-MDM phenotypes and confirmed a culture method to take forward. This chapter will use adherent MDM cultured in the presence of FCS to examine how altering environment affects phenotypic plasticity in these cells.

4.1 Introduction

Macrophages have two main basic functions within the innate immune system. These are to phagocytose and remove foreign organisms or particles, and to produce cytokines (Sasmono & Hume 2004). These functions are part of a dynamic process which allows macrophages to change phenotype based on changes in their microenvironment. For example, if the macrophage encounters a pro-inflammatory environment (high levels of IFNγ, or LPS on the surface of bacteria) then it will respond by producing pro-inflammatory cytokines (e.g. TNFα to further induce inflammatory gene expression (Maini et al. 1995) and CXCL8 to trigger a neutrophilic response (Ludwig et al. 1997). Alternatively, if the macrophage encounters a more TH2-type environment (high levels of IL-13 or IL-4) or if the pro-inflammatory response is complete, then they will produce anti-inflammatory cytokines (e.g. IL-10) which will help dampen the immune response; preventing tissue damage and promoting homeostasis (Hackett et al. 2008). They will also increase phagocytosis of dead or foreign material and presentation to the adaptive immune system (Sasmono & Hume 2004).

The ability of macrophages to alter phenotype depending on changing environment/stimuli has been demonstrated in both murine macrophage models (Arnold et al. 2007) and healthy human macrophages (Porcheray et al. 2005). However, a skewing of this delicate balance of plasticity has also been implicated in several macrophage-driven diseases such as sepsis (Biswas & Lopez-Collazo 2009) and atherosclerosis (Johnson & Newby 2009; Erbel et al. 2015; Boyle 2005; Gleissner 2012).

4.1.1 Macrophage Phenotype in COPD

Dysfunctional macrophages are thought to play an important role in COPD (Barnes 2004). In a healthy lung, alveolar macrophages are essential in the innate immune response and are important for the removal of harmful or foreign materials. However, in a chronic inflammatory situation, such as continued exposure to cigarette smoke, macrophage regulation and function may become skewed towards a particular pro-inflammatory phenotype. It has been proposed that, in COPD, macrophage skew to a more pro-inflammatory macrophage phenotype (Byers & Holtzman 2011) producing elevated pro-inflammatory cytokines in comparison with cells from healthy non-smokers (Hodge et al. 2007). It has also been shown that macrophages from COPD patients are defective in their ability to phagocytose bacteria (Taylor et al. 2010) and apoptotic cells (Hodge et al. 2003) indicating again that they have become skewed in their phenotype.

Exploring changes in macrophage phenotype and how these are affected by changes in microenvironment may be a beneficial tool in preventing inflammation and tissue damage in COPD. Therefore, experiments were designed using the MDM model to

identify differences in macrophage phenotype and plasticity between non-smokers, smokers and COPD patients.

4.1.2 Hypothesis

MDM from COPD patients will retain a pro-inflammatory phenotype that will lack plasticity when environment is altered.

4.1.3 Aims

- Characterise the phenotype of MDM cultured in either GM-CSF or M-CSF by measuring functional outputs (LPS or IL-4 stimulated cytokine production and bacterial phagocytosis).
- Alter the culture environment of MDM cultured in either GM-CSF or M-CSF to measure the plasticity of cells from non-smokers, smokers and COPD patients.

4.2 Methods

4.2.1 Subject Selection

Subjects were selected as in section 2.2.1. Smokers had at least a 10 pack-year history and COPD patients had an FEV_1: FVC ratio <0.7 and post-bronchodilator FEV_1 % predicted <85%.

4.2.2 Measurement of LPS or IL-4 stimulated TNFα, CXCL8 and IL-10 by ELISA

Monocytes were isolated and plated as detailed in section 2.2.2, and cultured on adherent plates in the presence of 10% ($^{v}/_{v}$) FCS. Cells were differentiated for 12 days in either GM-CSF or M-CSF supplemented media, before undergoing cell culture switching as indicated in section 2.2.2.2.

At day 12, then 2, 4 and 6 post switch, cells were left untreated (media replaced with fresh complete media) or stimulated with 10ng/ml LPS or 10ng/ml IL-4 in quadruplicate as detailed in section 2.2.5. Cells were left for 24 h before supernatant was harvested and levels of TNFα, CXCL8 and IL-10 in cell supernatant were analysed by ELISA as detailed in section 2.2.6. A standard curve of recombinant TNFα, CXCL8 or IL-10 ranging from 2000pg/ml to 31.25pg/ml, was included alongside samples to allow for interpolation of unknown concentrations of cytokines in the cell supernatant using GraphPad Prism software (version 5.03).

4.2.3 Measurement of Phagocytosis by Fluorimetry

Cells were isolated and plated as detailed in section 2.2.2, allowed to differentiate for 12 days then underwent cell culture changes as indicated in section 2.2.2.2.

At day 12, then 2, 4 and 6 post switch, cells were left untreated (media replaced with fresh RPMI) or incubated with fluorescently labelled prey (inert beads, *S.pneumoniae* or *H influenzae*) for 4 h as detailed in section 2.2.7. After incubation, cells were washed, extracellular fluorescence quenched and plates were read on a fluorescent plate reader.

6.2.2 Statistical Analysis

Comparisons between subject groups were conducted using either Kruskal-Wallace or Mann-Whitney tests with Dunn's post-correctional tests. Comparisons between GM-CSF cultured cells and M-CSF cultured cells from the same patient group were also done using Mann-Whitney tests with Dunn's post-correctional tests. Comparisons between different time points were conducted using Wilcoxon signed rank tests. Data were expressed as mean ± SEM and analysed using GraphPad Prism software GraphPad Prism software (version 5.03). A p value of <0.05 was considered statistically significant.

4.3 Affect of Changing MDM Environment on Cytokine Release

4.3.1 Subject Demographics

Subject characteristics and spirometry results are shown in Table 4.1. There was no significant difference in smoking history between smokers and COPD patients and no significant difference in age between any subject groups. COPD patients had significantly lower FEV_1 % predicted and FEV_1:FVC ratio compared with non-smokers and smokers.

	Non Smokers (n=14)	Smokers (n=8)	COPD Patients (n=9)
Age (years)	59 ± 3	61 ± 2	68 ± 5
Sex (M:F)	6:8	3:5	5:4
Smoking History (pack years)	0 ± 0	37 ± 3	39 ± 5
FEV_1 (l)	2.8 ± 0.4	2.5 ± 0.7	1.3 ± 0.2*
FEV_1 % Predicted	96 ± 3	98 ± 6	58.± 4*
FVC (l)	3.8 ± 0.5	3.5 ± 0.7	2.1 ± 0.3*
FVC % Predicted	100 ± 5	98 ± 5	89 ± 4*
FEV_1:FVC	0.9 ± 0.02	0.8 ± 0.04	0.5 ± 0.1*

Table 4.1: Subject demographics for MDM experiments. All participants underwent spirometry testing. FEV_1: Forced Expiratory Volume exhaled at the end of the first second of forced expiration FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for 1 year. Data are expressed as mean ± SEM. Kruskall-Wallis test with Dunn's multiple comparison test. * $p<0.05$ COPD *vs.* non-smokers.

4.3.2 LPS-stimulated CXCL8 Release

The initial experiments examined whether altering the growth factors present in cell culture media, would lead to a phenotypic change in LPS responsiveness with respect to release of CXCL8 from these cells. When comparing individual subject groups and examining the differences between GM-MDM and M-MDM, at day 12, GM-MDM from non-smokers released significantly more LPS-stimulated CXCL8 compared with M-MDM ($p<0.05$) (Figure 4.1A). When GM-MDM were switched to media containing M-CSF, there was a significant decrease in LPS-stimulated CXCL8 release by day 2 ($p<0.05$) that was sustained over time. When M-MDM were switched to media containing GM-CSF, there was a significant increase in CXCL8 release by day 2 that was slightly reduced by days 4 and 6 but still significantly higher than day 12 M-MDM ($p<0.05$).

At Day 12, GM-MDM from smokers released significantly more LPS-stimulated CXCL8 compared with M-MDM from smokers ($p<0.05$) (Figure 4.1B). When GM-MDM were switched to media containing M-CSF, there was a trend for lower CXCL8 release by day 6 (which did not reach significance). When M-MDM were switched to media containing GM-CSF, there was a trend for increased CXCL8 release by day 6 (although this did not reach significance).

At day 12, there was a trend for GM-MDM from COPD patients to release higher levels of LPS-stimulated CXCL8 release compared with M-MDM from COPD patients (although this did not reach significance) (Figure 4.1C). When GM-MDM were switched to media containing M-CSF there was no significant change in CXCL8 levels by day 6. When M-MDM were switched to media containing GM-CSF there was no significant change in CXCL8 levels by day 6.

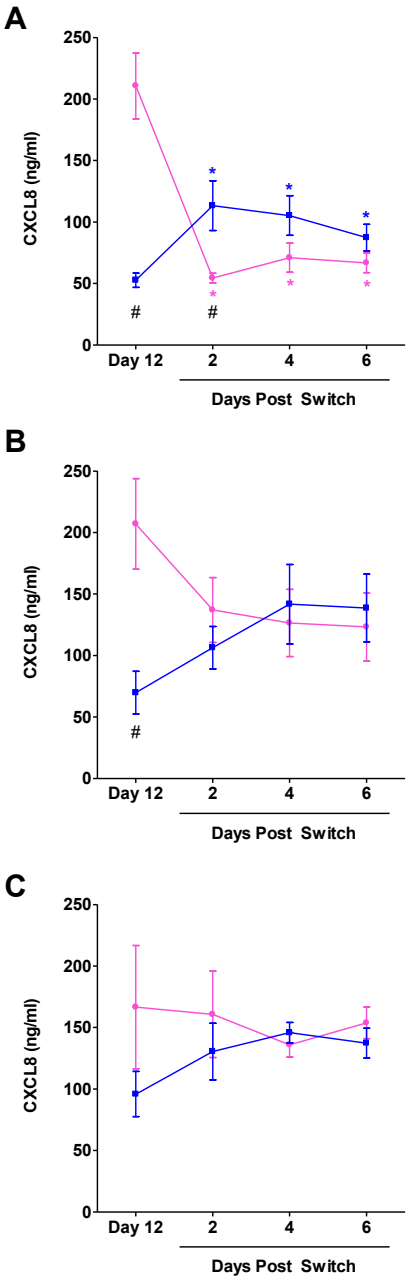


Figure 4.1: LPS-stimulated CXCL8 release by GM-MDM and M-MDM after differentiation and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=7 (**B**) smokers and n=7 (**C**) COPD patients n=6, were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml LPS for 24h before CXCL8 in supernatant was measured by ELISA. Data expressed as mean ± SEM. # $p<0.05$ GM-CSF cultured cells *vs.* M-CSF cultured cells. * $p<0.05$ *vs.* Day12.

Taking these data and comparing the responsiveness of MDM between groups showed that at day 12, GM-MDM from non-smokers and smokers trended towards increased production of CXCL8 compared with GM-MDM from COPD patients but did not reach significance (Figure 4.2A). Changing media to that containing M-CSF showed a significant decrease in LPS-induced CXCL8 in non-smokers ($p<0.05$), a trend towards a decrease in cells from smokers and no significant decrease in cells from COPD patients which continued to produce similar levels of CXCL8 (significantly more than MDM from non-smokers, $p<0.05$) regardless of environment.

At day 12, there was a trend for M-MDM from non-smokers to produced less CXCL8 compared with M-MDM from smokers and COPD patients, although this did not reach significance (Figure 4.2B). When M-MDM switched to media containing GM-CSF there was a trend towards a time-dependent increase in CXCL8 release from cells from all subject groups although, this was only statistically significant in cells from non-smokers. There was also a trend for MDM from smokers and COPD patients to produce more CXCL8 compared to MDM from non-smokers at days 4 and 6 post-switch, although this was not statistically significant.

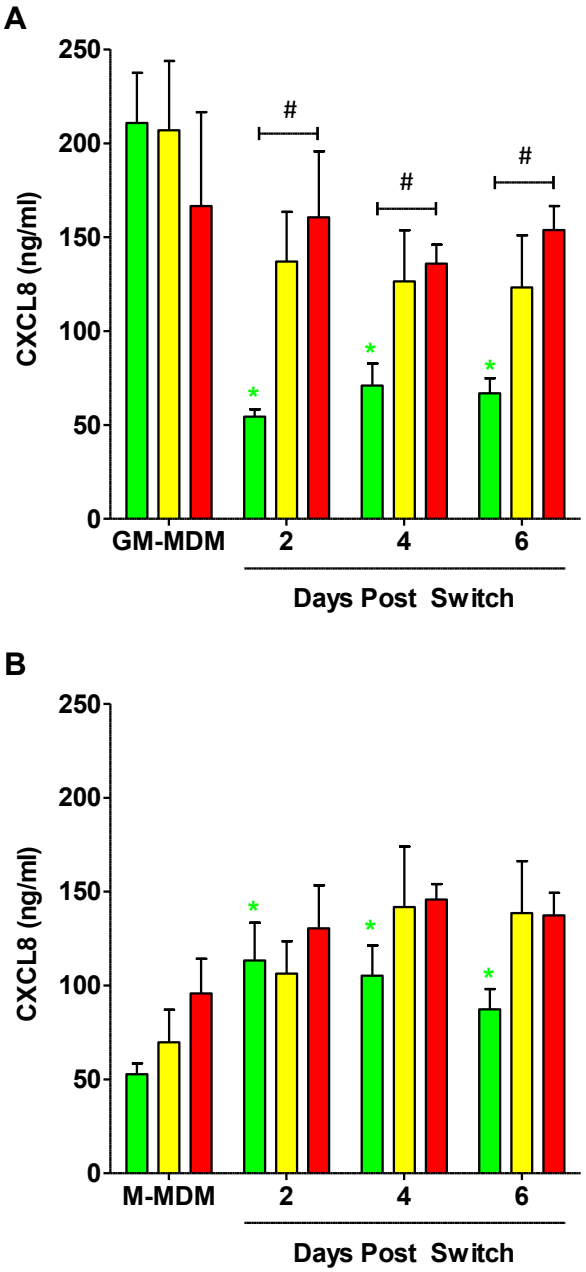


Figure 4.2: LPS-stimulated CXCL8 release by GM-MDM and M-MDM after differentiation and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml LPS for 24h before CXCL8 in supernatant was measured by ELISA. Data expressed as mean ± SEM. # $p<0.05$ non-smokers *vs.* COPD patients. * $p<0.05$ *vs.* Day12.

4.3.3 IL-4-stimulated CXCL8 Release

Having shown a differential affect of changing media post-differentiation on LPS-stimulated CXCL8 release, it was important to determine whether similar affects could be observed when a different activation stimulus was used. Therefore, similar experiments were performed using IL-4. Firstly, individual subject groups were compared to examine the differences between GM-MDM and M-MDM. In non-smokers at day 12, GM-MDM produced significantly more IL-4-stimulated-CXCL8 compared with M-MDM ($p<0.05$) (Figure 4.3A). When GM-MDM were switched to media containing M-CSF, there was a significant decrease in CXCL8 by day 2 that was sustained at days 4 and 6 ($p<0.05$). When M-MDM were switched to media containing GM-CSF, there was a significant increase in CXCL8 by day 2 and day 6 when compared with day 12 M-MDM ($p<0.05$).

In smokers, at day 12, there was a trend for GM-MDM from smokers to release more IL-4-stimulated-CXCL8 compared with M-MDM but this did not reach significance (Figure 4.3B). When GM-MDM were switched to media containing M-CSF, there was a trend towards a decrease in CXCL8 by day 4 and 6 post-switch but again, this did not reach significance when compared to levels released from day 12 GM-MDM. When M-MDM were switched to media containing GM-CSF, there was a trend towards an increase in CXCL8 levels by day 6 but again, these were not significantly different to levels released from day 12 M-MDM.

In COPD patients, at day 12, (and day 2 post-switch) GM-MDM from COPD patients released significantly higher levels of IL-4-stimulated CXCL8 compared with M-MDM from COPD patients ($p<0.05$) (Figure 4.3C). When GM-MDM were switched to M-CSF media there was a trend for decreased CXCL8 levels by day 2 that remained similar

at days 4 and 6, although, was not significantly different from CXCL8 levels released on day 12. When M-MDM were switched to GM-CSF media there was a trend towards an increase in CXCL8 by days 2, 4 and 6, although again, this was not significantly different to CXCL8 produced on day 12.

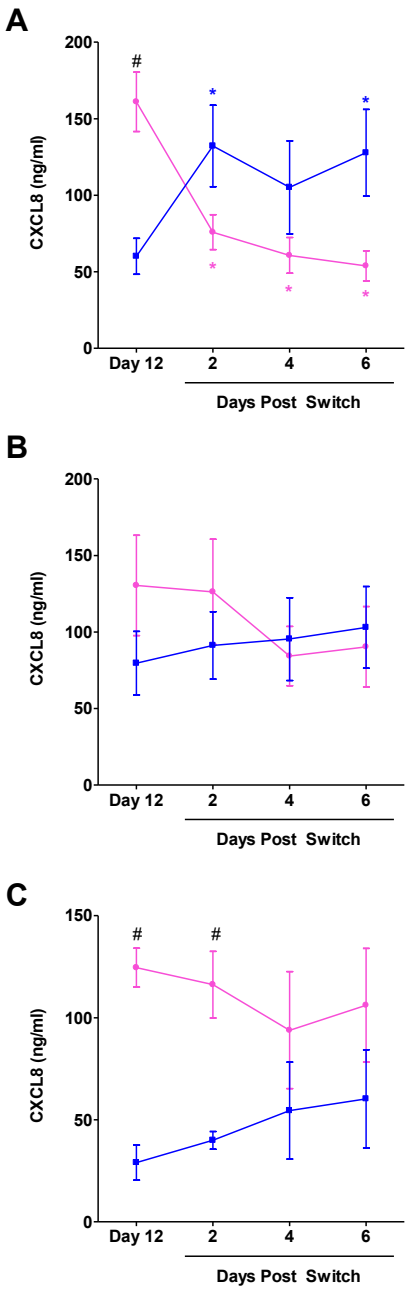


Figure 4.3: IL-4-stimulated CXCL8 release by GM-MDM and M-MDM after differentiation and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=7 (**B**) smokers n=7 and (**C**) COPD patients n=6 were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml IL-4 for 24h before CXCL8 in supernatant was measured by ELISA. Data expressed as mean ± SEM. # $p<0.05$ GM-CSF cultured cells *vs.* M-CSF cultured cells. * $p<0.05$ *vs.* Day12.

Taking these data together and comparing the responsiveness of MDM between groups showed that at day 12, there was a trend for GM-MDM from non-smokers to produce more IL-4-stimulated CXCL8 release compared with GM-MDM from smokers and COPD patients, although this was not statistically significant (Figure 4.4A). Changing media to that containing M-CSF showed a significant decrease in IL-4 induced CXCL8 in non-smokers ($p<0.05$), a trend towards a decrease in the MDM from smokers and no significant decrease in the CXCL8 from COPD MDM which continued to produce similar levels of CXCL8 regardless of environmental change.

At day 12, there was a trend for M-MDM from non-smokers and smokers to produce more CXCL8 compared with M-MDM from COPD patients although this was not statistically significant (Figure 4.4B). When M-MDM were switched to media containing GM-CSF there was an increase in IL-4-induced CXCL8 release by non-smoker M-MDM by day 2. This was significantly higher compared with CXCL8 produced by MDM from COPD patients at day 2 post-switch ($p<0.05$). There was also a trend for MDM from smokers and COPD patients to produce less CXCL8 compared to MDM from non-smokers at days 4 and 6 post-switch, although, this was not statistically significant.

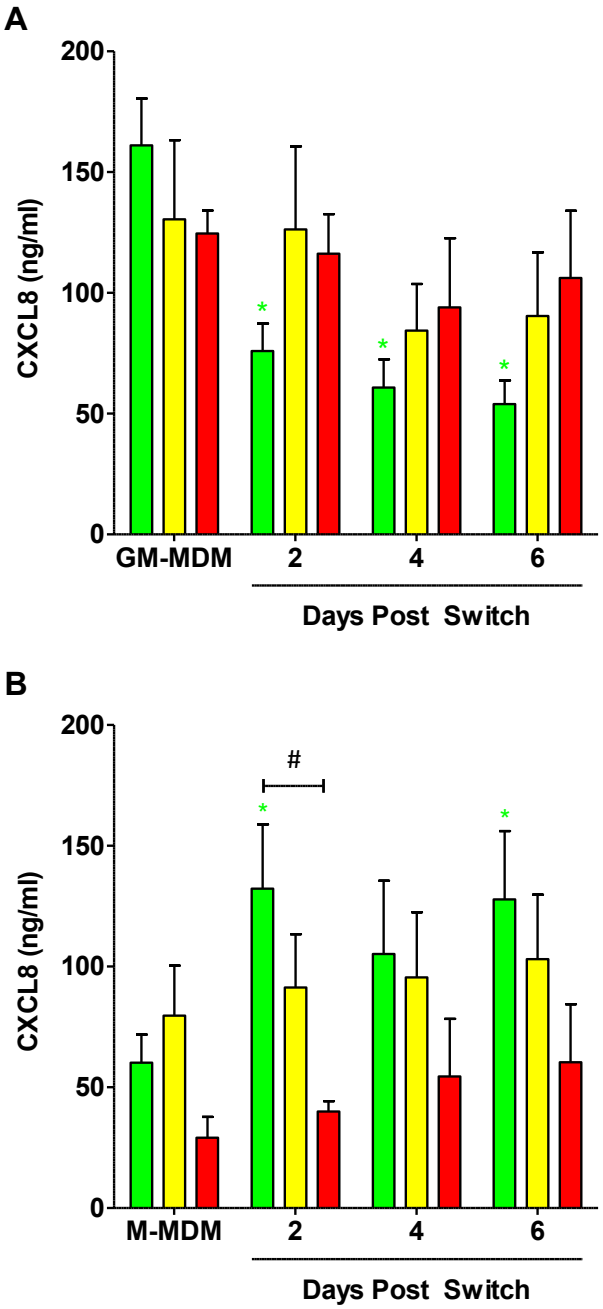


Figure 4.4: IL-4-stimulated CXCL8 release by GM-MDM and M-MDM after differentiation and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml IL-4 for 24h before CXCL8 in supernatant was measured by ELISA. Data expressed as mean ± SEM. # $p<0.05$ non-smokers *vs.* COPD patients. * $p<0.05$ *vs.* Day12.

4.3.4 LPS-stimulated TNFα Release

Having shown a differential affect on CXCL8 release by COPD MDM, it was next decided to determine whether this affect was CXCL8 specific or whether other inflammatory protein expression was similarly affected. Therefore, the affect of altering culture environment on the release of LPS-stimulated TNFα was examined. Firstly, individual subject groups were compared to examine the differences between GM-MDM and M-MDM. In non-smokers at day 12, GM-MDM from non-smokers produced significantly more TNFα compared with M-MDM ($p<0.05$) (Figure 4.5A). When GM-MDM were switched to media containing M-CSF, there was a significant decrease in LPS-stimulated TNFα by day 2 ($p<0.05$) that was sustained at days 4 and 6 ($p<0.05$). When M-MDM were switched to media containing GM-CSF, there was a significant increase in TNFα by day 4 that was sustained at day 6 ($p<0.05$).

At day 12, GM-MDM from smokers released significantly more TNFα compared with M-MDM ($p<0.05$) (Figure 4.5B). When GM-MDM were switched to M-CSF, there was a non-significant decrease in LPS-stimulated TNFα levels by day 2 which was sustained over time. When M-MDM were switched to media containing GM-CSF, there was a trend towards an increase in TNFα levels by day 2, although this was not statistically significant at day 6.

There was no significant difference in TNFα release from GM-MDM compared with M-MDM from COPD patients, at day 12, (Figure 4.5C). When GM-MDM were switched to M-CSF there was a no significant change in TNFα levels by day 6. When M-MDM were switched to media containing GM-CSF there was a trend towards an increase in TNFα by which was sustained to day 6, although, this was not significantly different to levels released from M-MDM on day 12.

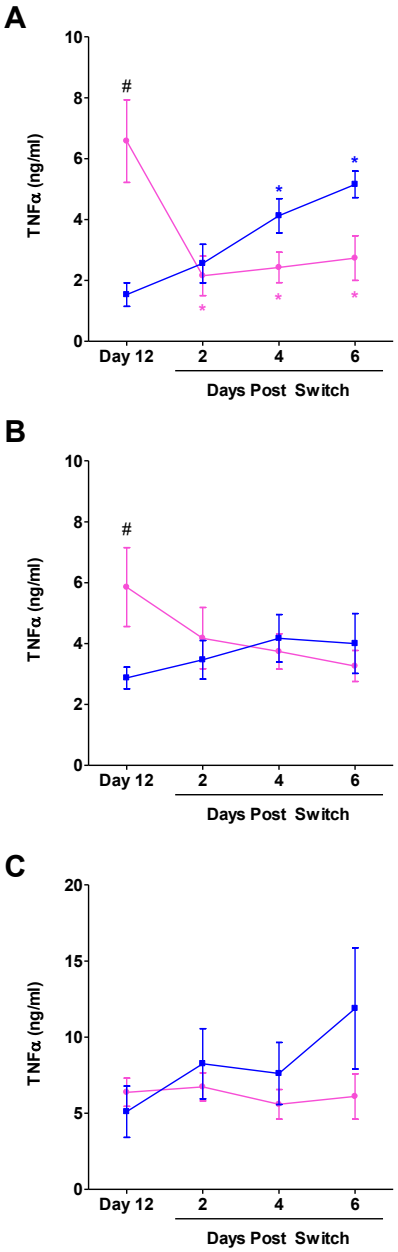


Figure 4.5: LPS-stimulated TNFα release by GM-MDM and M-MDM after differentiation and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=7 (**B**) smokers n=7 and (**C**) COPD patients n=6 were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml LPS for 24h before TNFα in supernatant was measured by ELISA. Data expressed as mean ± SEM. # $p<0.05$ GM-CSF cultured cells *vs.* M-CSF cultured cells. * $p<0.05$ *vs.* Day12.

Combining data from the different groups and comparing the responsiveness of MDM showed that at day 12, GM-MDM from all groups produced similar levels of LPS-stimulated TNFα (Figure 4.6A). However, changing media to that containing M-CSF showed a significant decrease in TNFα in non-smokers ($p<0.05$), a non-significant trend towards the same pattern in the smokers, but no significant decrease in the cells from COPD patients which continued to produce higher levels of TNFα compared with cells from non-smokers and smokers, regardless of environmental change.

At day 12, there was a trend for M-MDM from non-smokers to produce less TNFα compared with MDM from smokers and COPD patients, although this was not statistically significant (Figure 4.6B). When M-MDM were switched to media containing GM-CSF there was a significant increase in TNFα in non-smokers ($p<0.05$), but no significant change in TNFα from smokers and COPD patients. MDM from COPD patients constantly produced more TNFα compared to MDM from non-smokers ($p<0.05$ at days 2 and 6) and smokers ($p<0.05$ at day 6 post-switch).

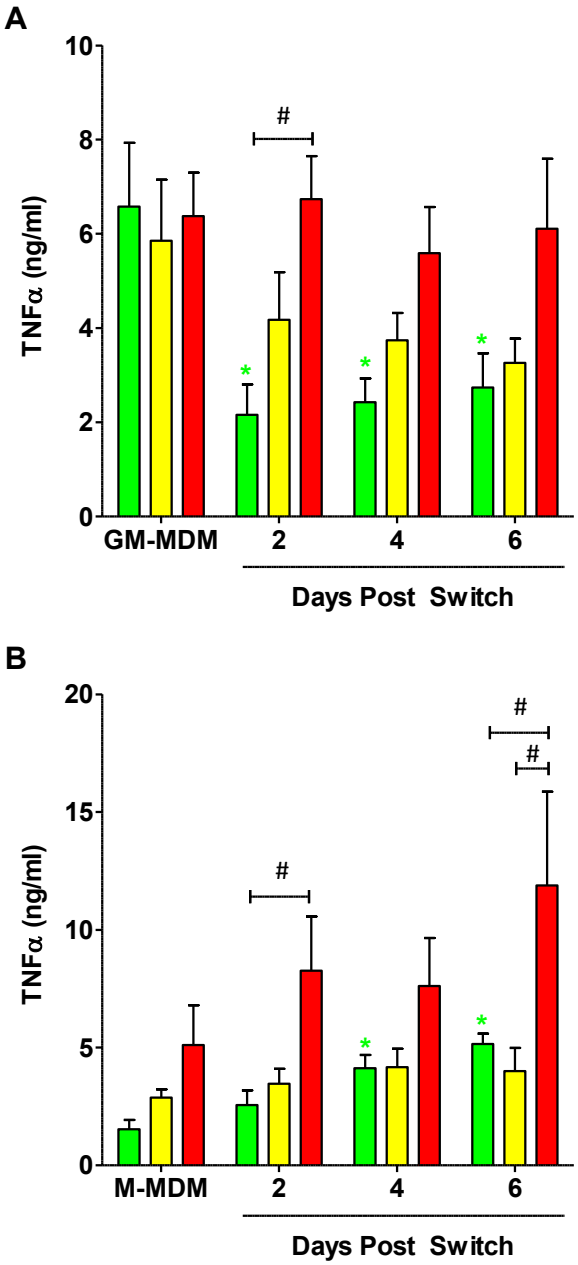


Figure 4.6: LPS-stimulated TNFα release by GM-MDM and M-MDM after differentiation and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml LPS for 24h before TNFα in supernatant was measured by ELISA. Data expressed as mean ± SEM. # $p<0.05$ non-smokers *vs.* COPD patients. * $p<0.05$ *vs.* Day12.

4.3.5 IL-4-stimulated TNFα Release

Having shown a differential affect of changing media in TNFα release from COPD MDM, it was again important to determine whether similar affects could be observed when using a different activation stimulus (IL-4). Firstly, individual subject groups were compared to examine the differences between GM-MDM and M-MDM. In non-smokers at day 12, there was a trend for GM-MDM to produce more IL-4-stimulated TNFα compared with M-MDM, although this did not reach significance (Figure 4.7A). When GM-MDM were switched to media containing M-CSF, there was a trend towards a decrease in TNFα by day 6, although this did not reach significance. When M-MDM were switched to media containing GM-CSF, there was a time-dependent increase in TNFα, but again, this was not significant.

At day 12, there was a trend for GM-MDM from smokers to release more IL-4-stimulated-TNFα compared with M-MDM although this did not reach significance (Figure 4.7B). When GM-MDM were switched to media containing M-CSF, there was a trend towards a decrease in TNFα, although TNFα levels at day 6 were not significantly different to that released on day 12. When M-MDM were switched to media containing GM-CSF, there was a trend towards an increase in TNFα levels by day 6, which again were not significantly different to that released on day 12. In MDM from COPD, at day 12, there was a trend for GM-MDM to release higher levels of IL-4-stimulated TNFα compared with M-MDM, however, this did not reach significance (Figure 4.7C). When GM-MDM were switched to media containing M-CSF there was a trend towards a decrease in TNFα levels that was not significantly different to that of day 12. When M-MDM were switched to GM-CSF media there was a trend towards an increase in TNFα by day 6, although this was not significantly different to that of day 12.

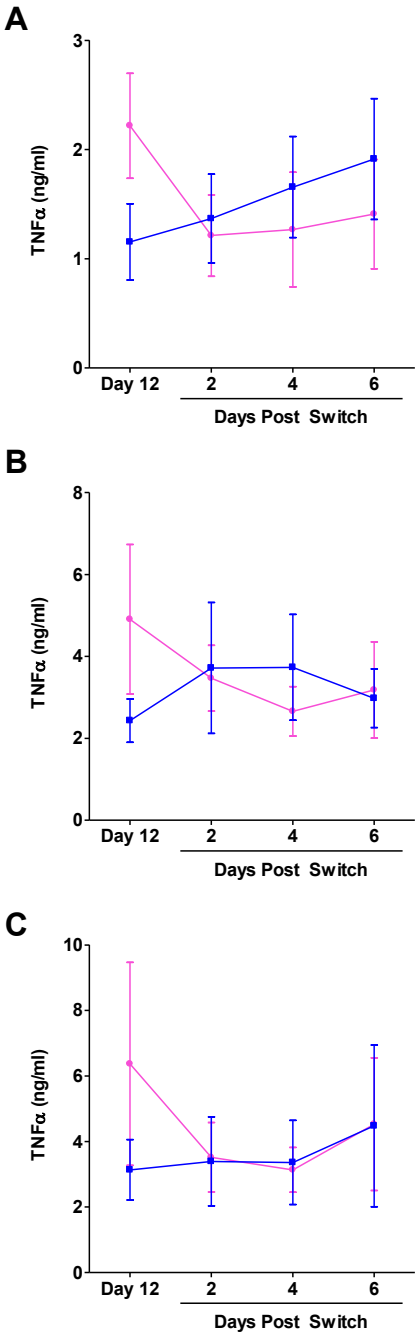


Figure 4.7: IL-4-stimulated TNFα release by GM-MDM and M-MDM after differentiation and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=7 (**B**) smokers n=7 and (**C**) COPD patients n=6 were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml IL-4 for 24h before TNFα in supernatant was measured by ELISA. Data expressed as mean ± SEM.

Looking at these data together and comparing the responsiveness of MDM between groups showed that at day 12, there was a trend for GM-MDM from non-smokers to produce less IL-4-stimulated-TNFα compared with GM-MDM from smokers and COPD patients but this was not statistically significant (Figure 4.8A). This trend continued over the time course with MDM from COPD patients and smokers MDM constantly producing more TNFα compared with MDM from non-smokers.

At day 12, there was a trend for M-MDM from non-smokers to produce less TNFα compared with M-MDM from COPD patients, although this was not statistically significant (Figure 4.8B). Again this trend continued over the time course but was not statistically significant.

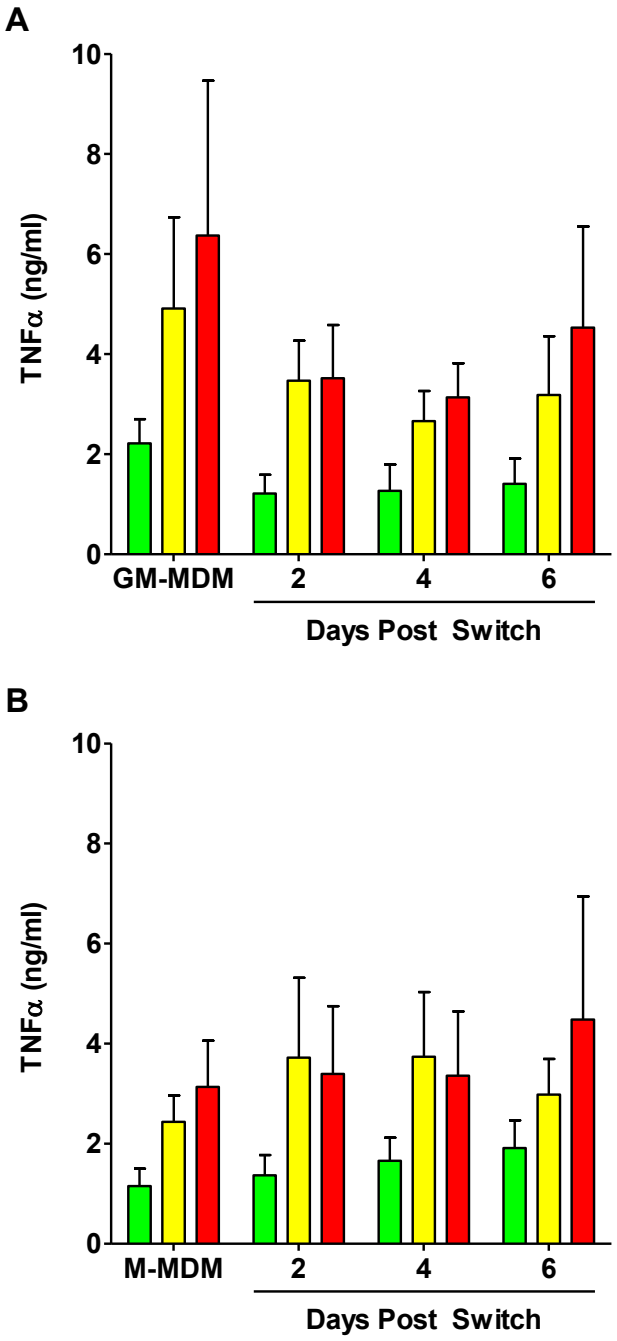


Figure 4.8: IL-4-stimulated TNFα release by GM-MDM and M-MDM after differentiation and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml IL-4 for 24h before TNFα in supernatant was measured by ELISA. Data expressed as mean ± SEM.

4.3.6 LPS-stimulated IL-10 Release

Having established that changing growth factors in the media could alter macrophage responsiveness to release pro-inflammatory mediators, it was important to determine whether this was the case with an anti-inflammatory mediator. Therefore, the release of IL-10 was next examined.

When comparing individual subject groups and looking at the differences between GM-MDM and M-MDM, at day 12, M-MDM from non-smokers produced significantly more IL-10 compared with GM-MDM ($p<0.05$) (Figure 4.9A). When GM-MDM were switched to media containing M-CSF, there was an increase in LPS-stimulated IL-10 by day 2 that was increased further at days 4 and 6 ($p<0.05$). When M-MDM were switched to media containing GM-CSF, there was a significant decrease in IL-10 by day 2 that was maintained to day 6 post-switch ($p<0.05$).

At Day 12, M-MDM from smokers released significantly more IL-10 compared with GM-MDM from smokers ($p<0.05$) (Figure 4.9B). When GM-MDM were switched to media containing M-CSF, there was a trend towards an increase in LPS-stimulated IL-10 levels by day 6, although this was not significant. When M-MDM were switched to media containing GM-CSF, there was a significant decrease in IL-10 levels by day 2 which was similar on days 4 and 6 post-switch ($p<0.05$).

At day 12, M-MDM from COPD patients released significantly higher levels of IL-10 compared with GM-MDM from COPD patients ($p<0.05$) (Figure 4.9C). When GM-MDM were switched to media containing M-CSF there was a non-significant increase in LPS-stimulated IL-10 levels by day 2 which was sustained over the time course. When M-MDM were switched to GM-CSF there was a significant decrease in IL-10 by day 6 compared with that released on day 12 M-MDM ($p<0.05$).

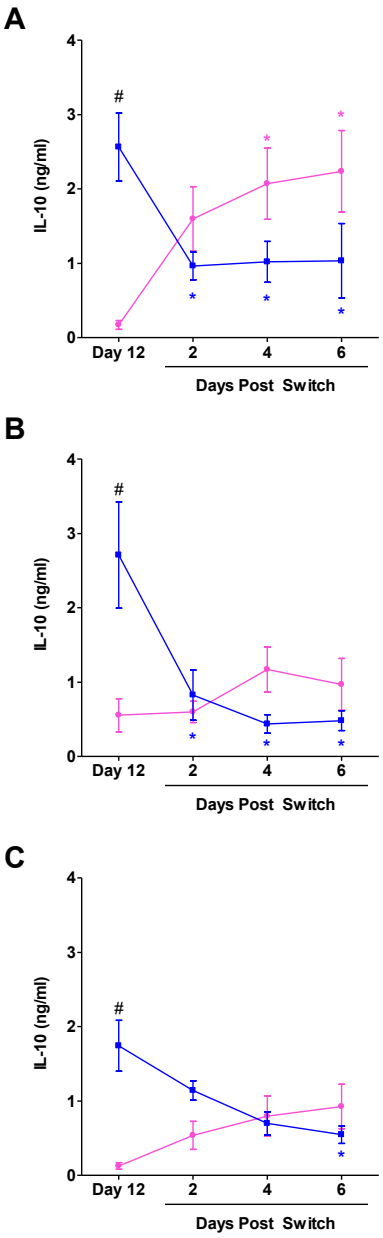


Figure 4.9: LPS-stimulated IL-10 release by GM-MDM and M-MDM after differentiation and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=7 (**B**) smokers n=7 and (**C**) COPD patients n=6 were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml LPS for 24h before IL-10 in supernatant was measured by ELISA. Data expressed as mean ± SEM. # $p<0.05$ GM-CSF cultured cells *vs.* M-CSF cultured cells. * $p<0.05$ *vs.* Day12.

Combining data from the different groups and comparing the responsiveness of MDM showed that at day 12, GM-MDM from all groups produced similar levels of IL-10 (Figure 4.10A). However, changing media to that containing M-CSF led to a significant increase in LPS-induced IL-10 from cells from non-smokers ($p<0.05$). This pattern was not mimicked by cells from smokers or COPD patients which continued to produce significantly less IL-10 compared with non-smokers ($p<0.05$).

At day 12, there was a trend for M-MDM from non-smokers and smokers to produce more IL-10 compared with M-MDM from COPD patients, but this was not statistically significant (Figure 4.10B). When M-MDM were switched to media containing GM-CSF there was a decrease in IL-10 in all subject groups and there was no significant difference between subject groups over the time course.

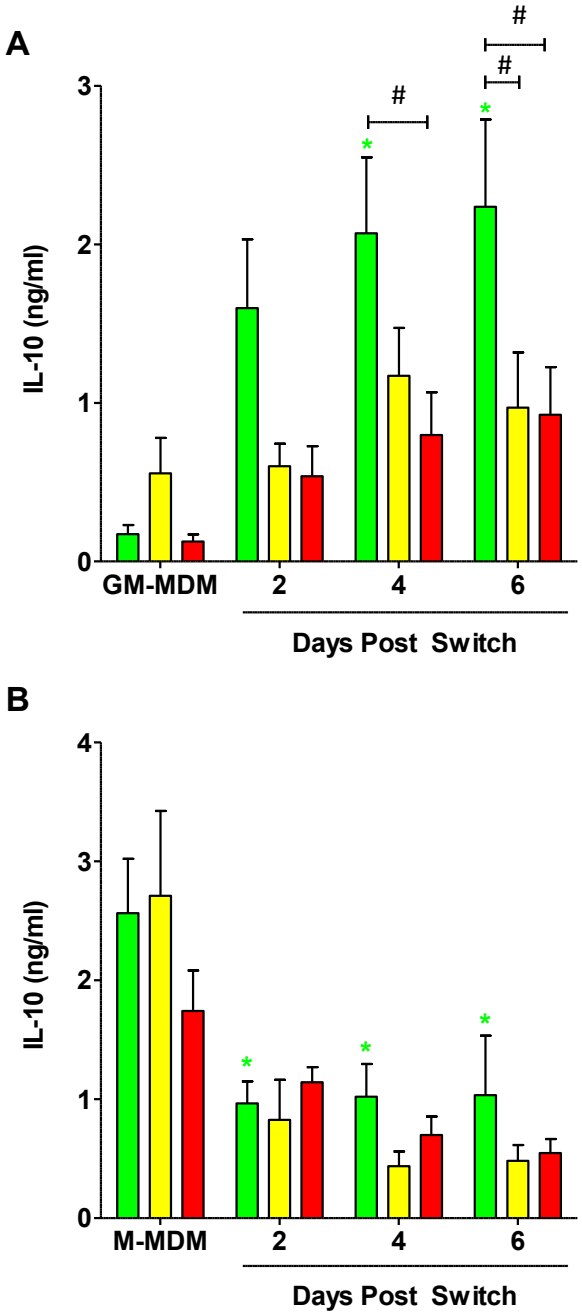


Figure 4.10: LPS-stimulated IL-10 release by GM-MDM and M- after differentiation and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml LPS for 24h before IL-10 in supernatant was measured by ELISA. Data expressed as mean ± SEM. # $p<0.05$ non-smokers *vs.* COPD patients. * $p<0.05$ *vs.* Day12.

4.3.7 IL-4 -stimulated IL-10 Release

Having shown differential LPS-stimulated-IL-10 release from COPD MDM, it was again important to determine whether similar affects could be observed when IL-4 was used as a different activation stimulus.

When comparing individual subject groups and looking at the differences between GM-MDM and M-MDM, at day 12, M-MDM from non-smokers produced significantly more IL-4-stimulated- IL-10 compared with M-MDM ($p<0.05$) (Figure 4.11A). When GM-MDM were switched to media containing M-CSF, there was a significant increase in IL-10 by day 2 that was sustained at days 4 and 6 ($p<0.05$). When M-MDM were switched to media containing GM-CSF, there was a significant decrease in IL-10 by day 2 that was sustained at days 4 and 6 ($p<0.05$).

At day 12, M-MDM from smokers released significantly more IL-4-stimulated- IL-10 compared with GM-MDM ($p<0.05$) (Figure 4.11B). When GM-MDM were switched to media containing M-CSF, there was a trend towards an increase in IL-10 by days 4 and 6 which was not significantly different to that released on day 12. When M-MDM were switched to media containing GM-CSF, there was a trend towards a decrease in IL-10 levels by day 2 which continued to day 4 but again, was not significantly different to the levels released on day 12 by M-MDM. At day 12, M-MDM from COPD patients released significantly higher levels of IL-4-stimulated IL-10 compared with GM-MDM from COPD patients ($p<0.05$) (Figure 4.11C). When GM-MDM were switched to media containing M-CSF there was no significant change in IL-10 release which remained similar over the time course. When M-MDM were switched to GM-CSF media there was a trend towards a decrease in IL-10 by day 6 that was not significantly different to that released on day 12.

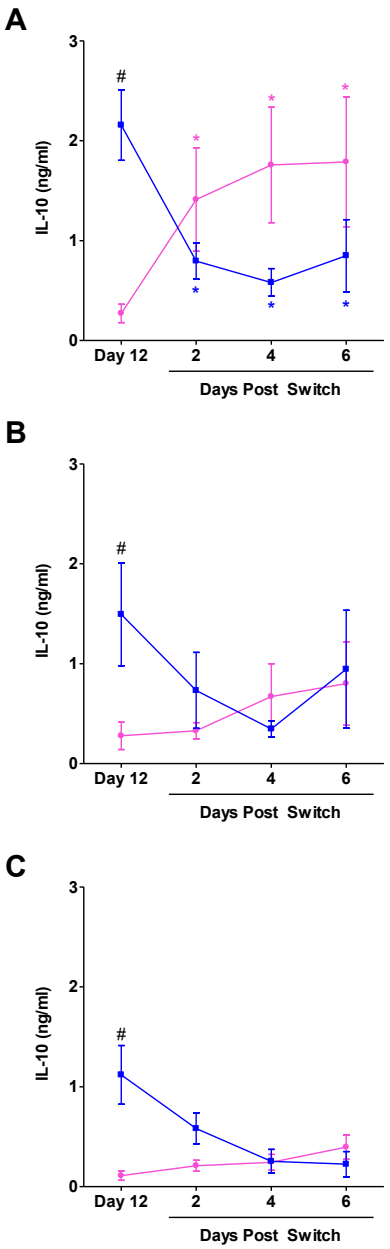


Figure 4.11: IL-4-stimulated IL-10 release by GM-MDM and M-MDM after differentiation and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=7 (**B**) smokers n=7 and (**C**) COPD patients n=6 were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml Il-4 for 24h before IL-10 in supernatant was measured by ELISA. Data expressed as mean ± SEM. # $p<0.05$ GM-CSF cultured cells *vs.* M-CSF cultured cells. * $p<0.05$ *vs.* Day12.

Combining these data comparing the responsiveness of MDM between groups showed that at day 12, there was a trend for GM-MDM from non-smokers and smokers to produce more IL-10 compared with GM-MDM from COPD patients (Figure 4.12A). However, changing media to that containing M-CSF led to a significant increase in IL-4-induced IL-10 release from cells from non-smokers, which was not mimicked by cells from COPD patients which continued to produce significantly less IL-10 throughout the time course ($p<0.05$ at day 4 and 6 post-switch).

At day 12, there was a trend for M-MDM from non-smokers to produce slightly more IL-10 compared with M-MDM from smokers and COPD patients although this was not statistically significant (Figure 4.12A). When M-MDM were switched to media containing GM-CSF there was a decrease in IL-10 in all subject groups but there was no significant difference between subject groups over the time course.

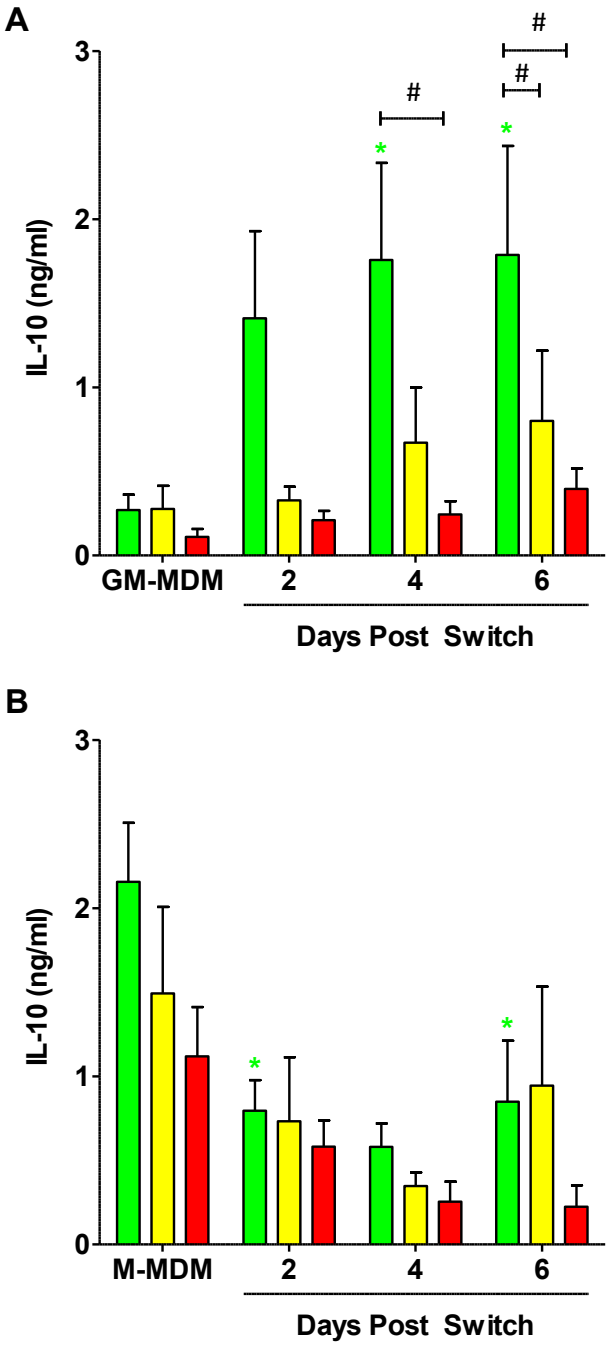


Figure 4.12: IL-4-stimulated IL-10 release by GM-MDM and M-MDM after differentiation and during environmental switch. Monocytes from non-smokers (green) n=7, smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days with measurements on days 2, 4 and 6 post-switch. Cells were stimulated with 10ng/ml Il-4 for 24h before IL-10 in supernatant was measured by ELISA. Data expressed as mean ± SEM. # $p<0.05$ non-smokers *vs.* COPD patients. * $p<0.05$ *vs.* Day12.

4.4 Affect of Changing MDM Environment on Phagocytosis

Having seen a clear affect of culture changes on cytokine production, it was important to also look at another functional output of MDM. Phagocytosis is a crucial macrophage function which has been shown to be affected by phenotype. Experiments were performed to identify how culture environment, and subsequent changes to environment, affect the ability of MDM to phagocytose and compare this between the subject groups.

4.4.1. Phagocytosis of Inert Beads

Firstly, experiments were performed to assess the affects of changing culture conditions on the phagocytosis of inert beads. GM-MDM from non-smokers, smokers and COPD patients were all able to phagocytose inert beads with no significant difference in the amount engulfed (Figure 4.13). Changing culture media, to that containing M-CSF, did not alter phagocytosis of beads by MDM from any subject group.

Similarly, M-MDM from all subject groups showed no significant difference in phagocytosis of inert beads (Figure 4.14A). Again, changing culture media, this time to GM-CSF, did not lead to a change in levels of phagocytosis in any subject group beads (Figure 4.14A). There was also no significant difference in phagocytosis of beads when comparing GM-MDM with M-MDM from any subject group. Taken together, this data shows that MDM phagocytosed inert beads equally regardless of cell culture environment.

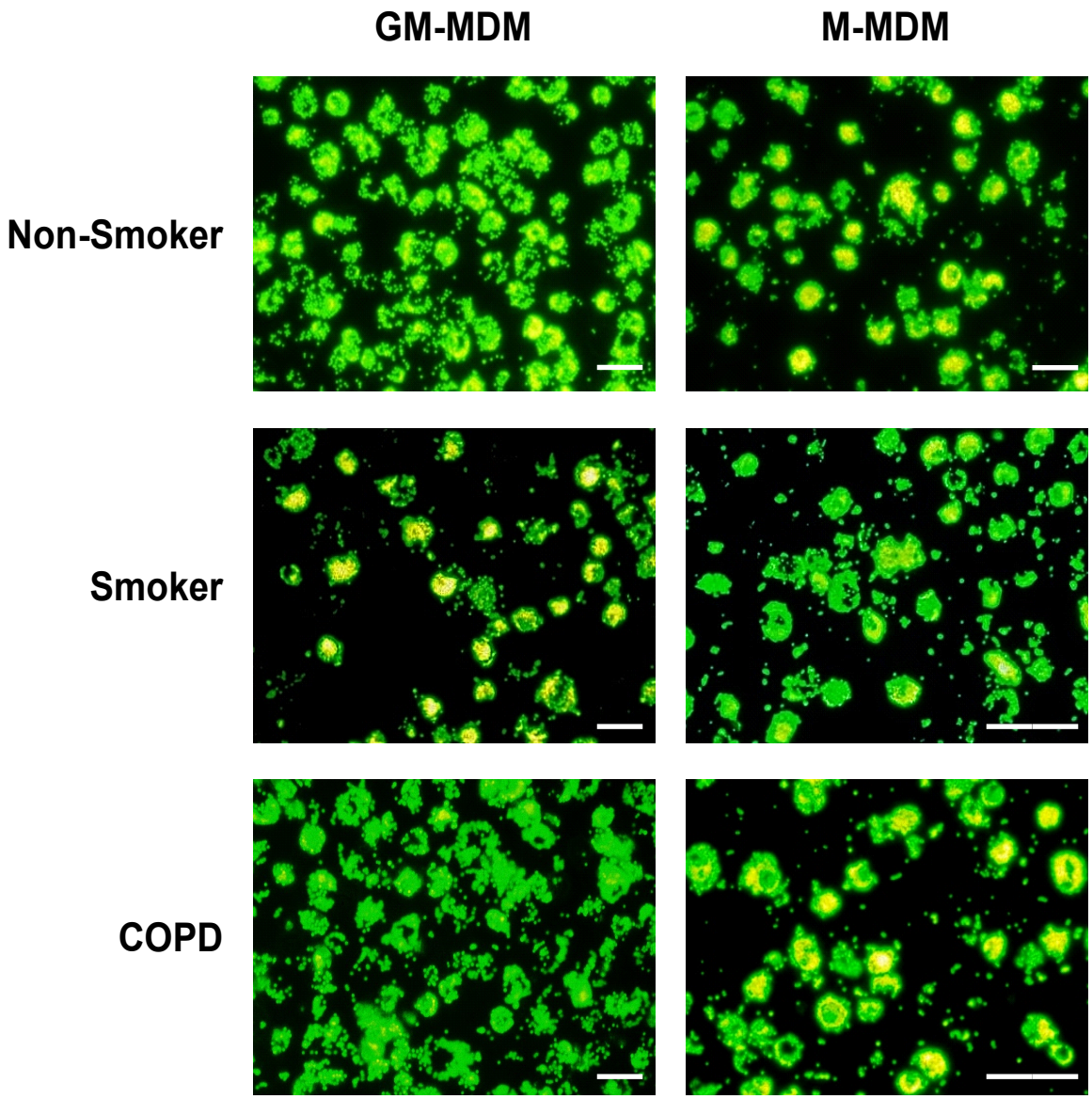


Figure 4.13: Phagocytosis of inert beads by GM-MDM and M-MDM from non-smoker, smoker and COPD patient. Representative photographs taken of GM-MDM (left) or M-MDM (right) from a non-smoker (top), smoker (middle) and COPD patient (bottom) after 4h exposure to fluorescent inert beads. Photographs taken using an inverted microscope and digital Olympus SLR Camera, after quenching extracellular fluorescence with trypan blue dye. Scale bar = 50μm.

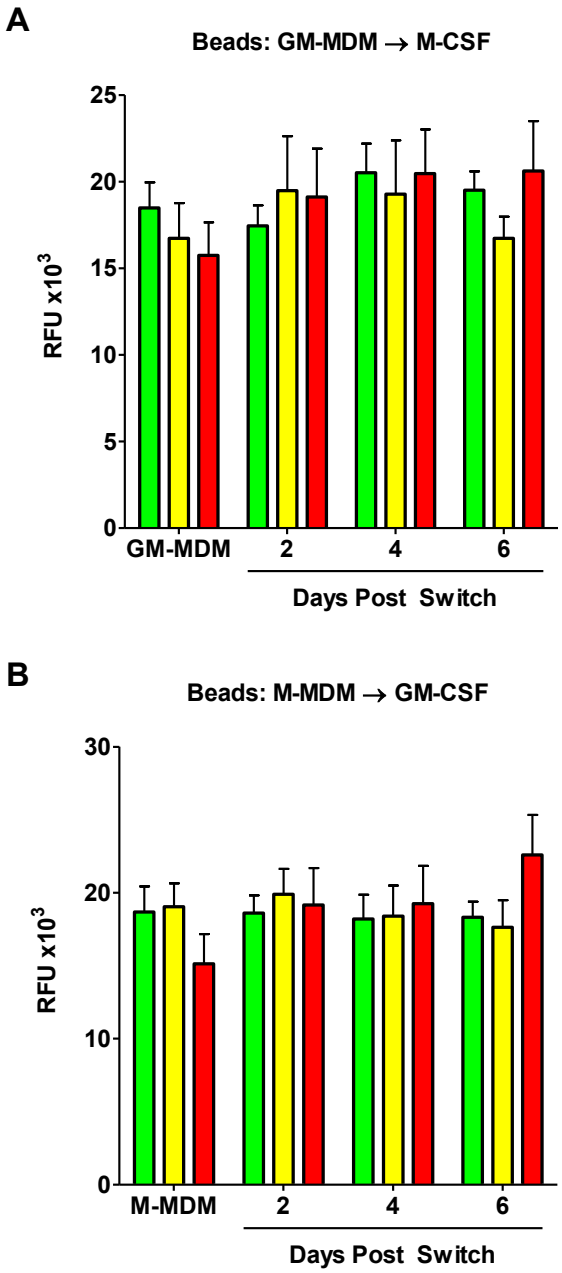


Figure 4.14: Phagocytosis of inert beads by GM-MDM and M-MDM after differentiation and during environmental switch. Monocytes from non-smokers (green n=14), smokers (yellow n=8) and COPD patients (red n=9) were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days with measurements on days 2, 4 and 6 post switch. Cells were exposed to fluorescently labelled inert beads for 4 hours before being quenched and RFU (relative fluorescent units) read via fluorimetry. Data are expressed as mean ± SEM.

4.4.2 Phagocytosis of *Streptococcus pneumoniae*

Having shown that culture conditions did not affect phagocytosis of inert beads, it was important to determine whether this was similar for phagocytosis of pathogenic bacteria that colonise the COPD airways. Therefore, similar experiments were performed using the Gram positive bacteria *S. pneumoniae*. These data showed that GM-MDM from non-smokers phagocytosed approximately 75% less *S. pneumoniae* compared with M-MDM from non-smokers ($p<0.05$) (Figure 4.15A). Changing GM-MDM to media containing M-CSF led to an increase in phagocytosis of *S. pneumoniae* by day 2 that was sustained over time and was significantly increased by day 6 ($p<0.05$). Inversely, when M-MDM were changed to media containing GM-CSF, there was a decrease in phagocytosis of *S. pneumoniae* by day 2 which was sustained and was significant by day 6 post switch ($p<0.05$) (Figure 4.15A).

Having established that MDM from non-smokers showed plasticity in their phagocytic response to *S. pneumoniae*, it was important to determine whether similar levels of plasticity could be observed in cells derived from smokers and patients with COPD. Differentiated GM-MDM from smokers phagocytosed less *S. pneumoniae* compared with M-MDM from smokers although this was not statistically significant (Figure 4.15B). When GM-MDM were changed to media containing M-CSF, there was a trend for more phagocytosis of *S. pneumoniae* by day 6 compared with day 12. M-MDM from smokers changed to media containing GM-CSF showed was a slight, non-significant, decrease in phagocytosis of *S. pneumoniae* by day 2 which continued over time but was not significantly reduced compared to day 12 M-MDM (Figure 4.15B).

The differences in plasticity of MDM was then further explored in cells from COPD patients. Differentiated GM-MDM from COPD patients phagocytosed similar levels of

S. pneumoniae compared with M-MDM from COPD patients (Figure 4.15C). These levels did not alter when media was changed to that containing M-CSF (Fig 4.15C). Similarly, when M-MDM from COPD patients were changed to media containing GM-CSF, there were no significant differences in phagocytosis of *S. pneumoniae* indicating a lack of plasticity in this response.

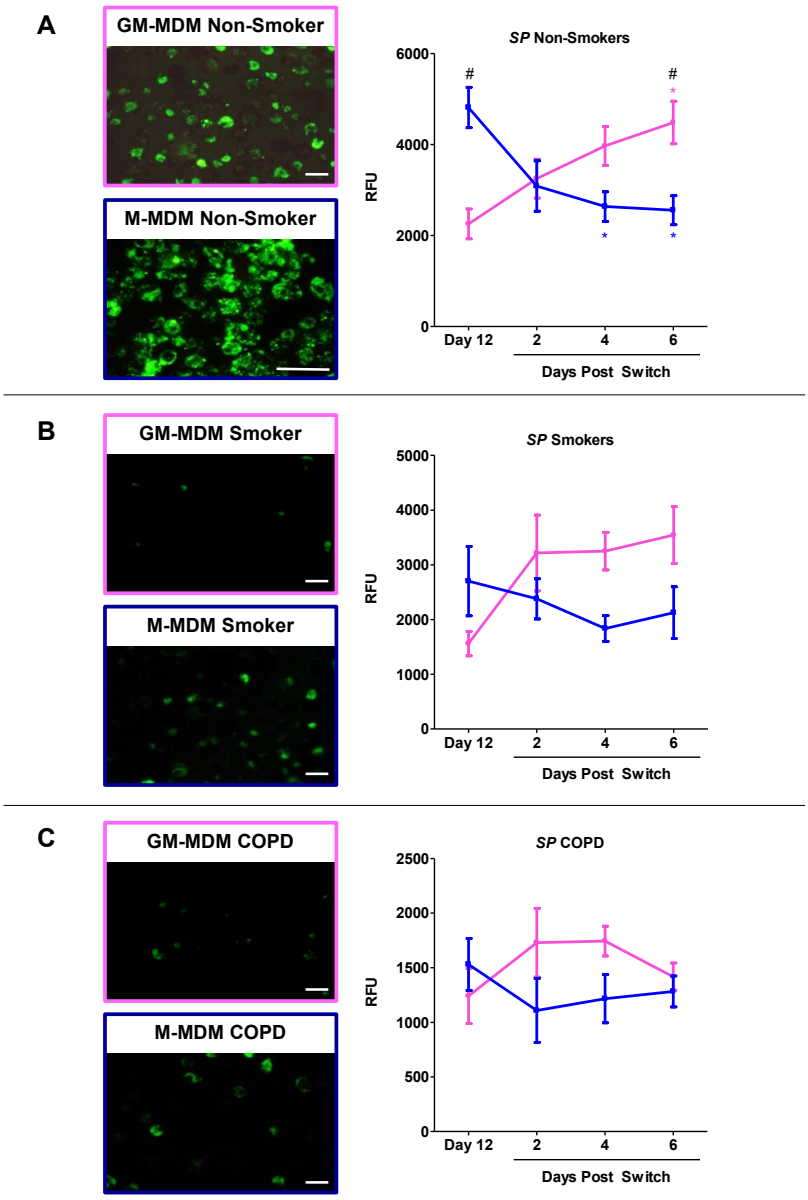


Figure 4.15: Phagocytosis of *Streptococcus pneumoniae* by GM-MDM and M-MDM after differentiation and following change of environment. Monocytes from (**A**) non-smokers (n=14) (**B**) smokers (n=8) and (**C**) COPD patients (n=9) were cultured in either GM-CSF for 12 days then changed to M-CSF (pink) or M-CSF for 12 days then changed to GM-CSF (blue). Phagocytosis was measured at day 12 then 2, 4 and 6 post-switch. Cells were exposed to fluorescently labelled *Streptococcus pneumoniae* (*SP*) for 4h before being quenched and RFU (relative fluorescent units) determined via fluorimetry. Data are expressed as mean ± SEM. # $p<0.05$ GM-CSF cultured cells *vs.* M-CSF. * $p<0.05$ *vs.* Day12. Representative photographs of GM-MDM or M-MDM from a non-smoker, smoker and COPD patient at day 12. Scale bar = 50μm.

Taking these data and comparing the responsiveness of MDM between groups showed that, at day 12, there was a trend for GM-MDM from non-smokers to phagocytose more *S. pneumoniae* compared with GM-MDM from smokers and COPD patients (Figure 4.16A) but this did not reach significance. Changing media, to that containing M-CSF, showed a significant time-dependent increase in phagocytosis of *S. pneumoniae* in cells from non-smokers and smokers but not COPD patients ($p<0.05$ non-smokers *vs.* COPD at days 2, 4 and 6 and $p<0.05$ smokers *vs.* COPD at day 6).

M-MDM from non-smokers phagocytosed significantly more *S. pneumoniae* compared with M-MDM from smokers ($p<0.05$) and both phagocytosed significantly more *S. pneumoniae* compared with COPD patients ($p<0.05$) (Figure 4.16B). When M-MDM were changed to media containing GM-CSF, there was a significant, time-dependent decrease in phagocytosis of *S. pneumoniae* by MDM from non-smokers but not in cells from smokers or COPD patients.

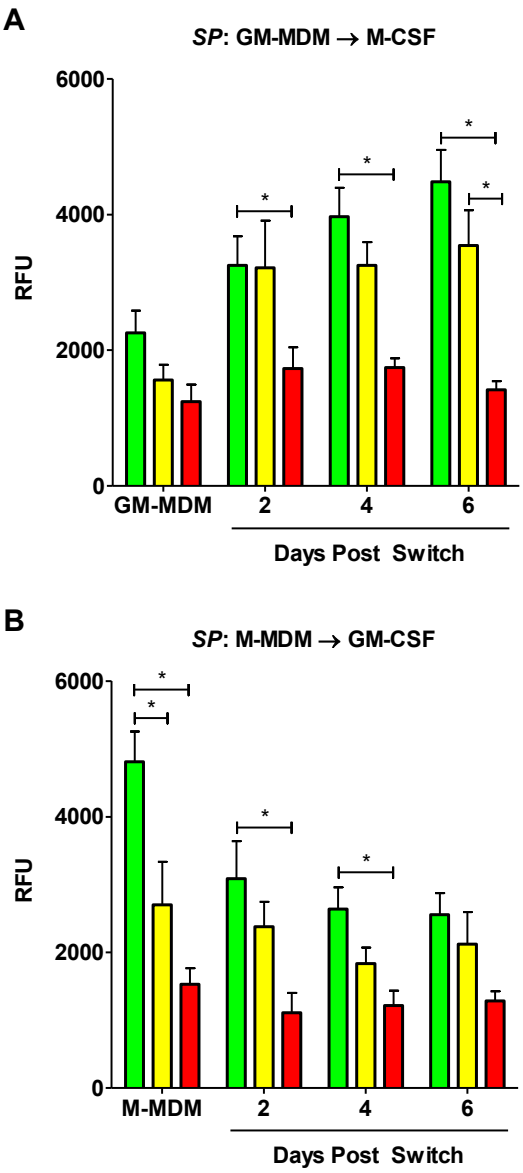


Figure 4.16: Phagocytosis of *Streptococcus pneumoniae* by GM-MDM and M-MDM after differentiation and during environmental switch (by culture environment). Monocytes from non-smokers (green) (n=14) smokers (yellow) (n=8) and COPD patients (red) (n=9) were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF for 6 days or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days with measurements on days 2, 4 and 6 post switch. Cells were exposed to fluorescently labelled *Streptococcus pneumoniae* for 4 hours before being quenched and RFU (relative fluorescent units) read via fluorimetry. Data expressed as mean ± SEM. * $p<0.05$ non-smokers *vs.* smokers, non-smokers *vs.* COPD and smokers *vs.* COPD.

4.4.3 Phagocytosis of *Haemophilus influenzae*

Since phagocytosis of *S. pneumoniae* was altered by macrophage culture conditions, it was important to investigate whether this effect was also apparent when another pathologically relevant strain of bacteria was used. Therefore, similar experiments were performed with Gram negative bacteria *H. influenzae*. These data showed GM-MDM from non-smokers phagocytosed approximately 60% less *H. influenzae* compared with M-MDM from non-smokers ($p<0.05$) (Figure 4.17A).

Changing GM-MDM to media containing M-CSF led to an increase in phagocytosis of *H. influenzae* by day 2 that was sustained over time and was significantly increased by days 4 and 6 ($p<0.05$). Conversely, when M-MDM were changed to media containing GM-CSF, there was a decrease in phagocytosis of *H. influenzae* by day 2 which was sustained and was significant by days 4 and 6 post switch ($p<0.05$) (Figure 4.17A).

Having again established that MDM from non-smokers showed plasticity in their phagocytic response to *H. influenzae*, it was important to determine whether similar levels of plasticity could be observed in cells derived from smokers and patients with COPD.

GM-MDM from smokers phagocytosed slightly less *S. pneumoniae* compared with M-MDM from smokers but this was not significant (Figure 4.17B). When GM-MDM were changed to media containing M-CSF, phagocytosis of *H. influenzae* again increased by day 2 but did not increase further by day 6 and was not significantly different to the day 12 GM-MDM. M-MDM from smokers changed to media containing GM-CSF showed was a slight, non-significant, decrease in phagocytosis of *S. pneumoniae* by

day 2 which continued over time till day 6 but was not significantly reduced compared to M-MDM (Figure 4.17B).

It was then important to observe any differences in cells from COPD patients. GM-MDM from COPD patients phagocytosed similar levels of *H. influenzae* compared with M-MDM from COPD patients (Figure 4.17C). In GM-MDM, there was a trend for increased phagocytosis when media was changed to M-CSF, but this was not statistically significantly over the time course (Figure 4.17C). When M-MDM from COPD patients were changed to media containing GM-CSF, there was no significant change in phagocytosis compared to day 12 M-MDM, again indicating a lack of plasticity in the MDM response of COPD patients.

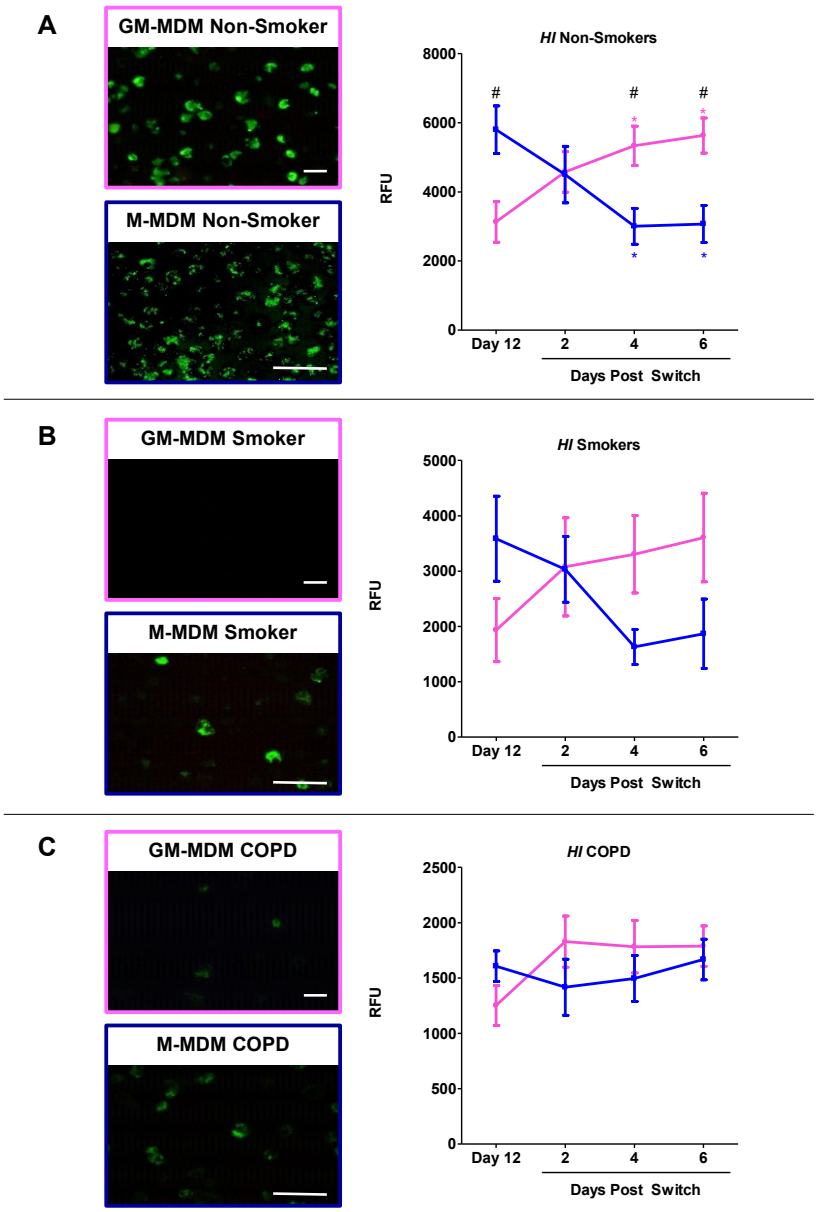


Figure 4.17: Phagocytosis of *Haemophilus influenzae* by GM-MDM and M-MDM after differentiation and following change of environment. Monocytes from (**A**) non-smokers (n=14) (**B**) smokers (n=8) and (**C**) COPD patients (n=9) were cultured in either GM-CSF for 12 days then changed to M-CSF (pink) or M-CSF for 12 days then changed to GM-CSF (blue). Phagocytosis was measured at day 12 then 2, 4 and 6 post-switch. Cells were exposed to fluorescently labelled *Haemophilus influenzae* for 4h before being quenched and RFU (relative fluorescent units) determined via fluorimetry. Data are expressed as mean ± SEM. # $p<0.05$ GM-CSF cultured cells *vs.* M-CSF. * $p<0.05$ *vs.* Day12. Representative photographs of GM-MDM or M-MDM from a non-smoker, smoker and COPD patient at day 12. Scale bar = 50μm.

Taking these data and comparing the responsiveness of MDM between groups showed that there was again a trend for GM-MDM from non-smokers to phagocytose more *H. influenzae* compared with GM-MDM from smokers and COPD patients (Figure 4.18A) but this did not reach significance. Changing media, to that containing M-CSF, showed a time-dependent increase in phagocytosis of *H. influenzae* in cells from non-smokers and smokers (to a lesser extent compared with non-smokers), but not in COPD patients which remained at similar levels regardless of environmental change ($p<0.05$ non-smokers *vs.* COPD at days 2, 4 and 6).

M-MDM from non-smokers phagocytosed significantly more *H. influenzae* compared with M-MDM from smokers ($p<0.05$) and again, both phagocytosed significantly more *H. influenzae* compared with COPD patients ($p<0.05$) (Figure 4.18B). When M-MDM were changed to media containing GM-CSF, there was again, a significant, time-dependent decrease in phagocytosis of *S. pneumoniae* by MDM from non-smokers. There was also a time dependent decrease in phagocytosis by MDM from smokers. And again, there was no change in phagocytosis of COPD patients suggesting a lack of plasticity in these cells.

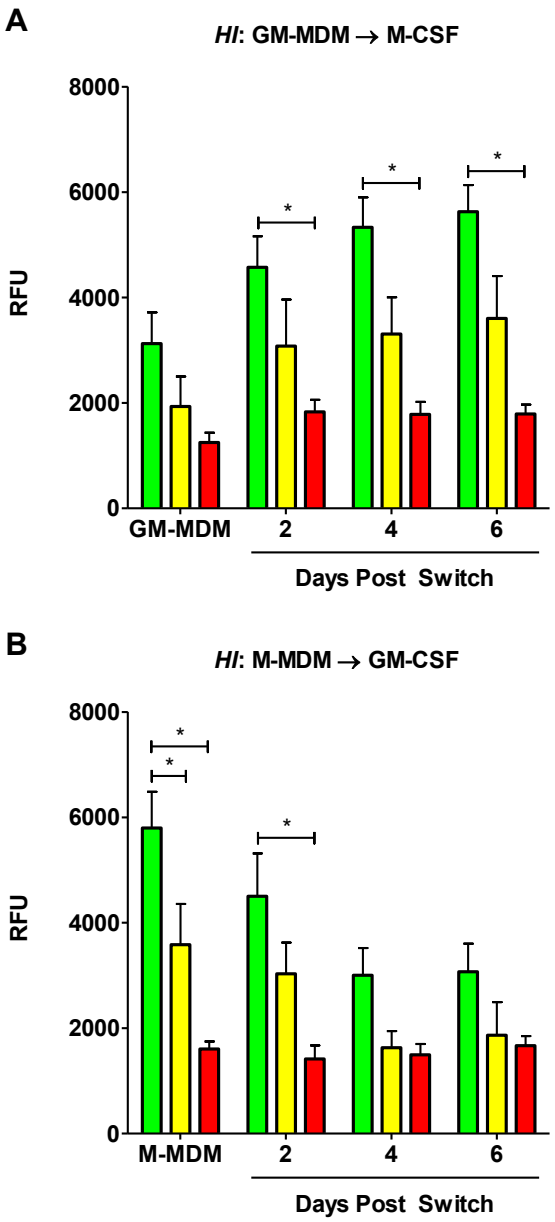


Figure 4.18: Phagocytosis of *Haemophilus influenzae* by GM-MDM and M-MDM after differentiation and during environmental switch (by culture environment). Monocytes from non-smokers (green) (n=14) smokers (yellow) (n=8) and COPD patients (red) (n=9) were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days with measurements on days 2, 4 and 6 post switch. Cells were exposed to fluorescently labelled *Haemophilus influenzae* for 4 hours before being quenched and RFU (relative fluorescent units) read via fluorimetry. Data expressed as mean ± SEM. * $p<0.05$ non-smokers *vs.* smokers or non-smokers *vs.* COPD.

4.5 Discussion

4.5.1 Comparison of MDM cytokine output at day 12

MDM from non-smokers, smokers and COPD patients were cultured in either GM-CSF or M-CSF before functional outputs were measured. Analysis of the cytokine output showed that, overall, GM-MDM were more pro-inflammatory, producing a higher concentration of LPS and IL-4 -stimulated CXCL8 and TNFα, than M-MDM from non-smokers. This is in line with studies showing increased pro-inflammatory gene expression when healthy human MDM are cultured in GM-CSF compared with those cultured in M-CSF (Lacey et al. 2012; Akagawa et al. 2006; Fleetwood et al. 2009).

Results from smokers at day 12, showed GM-MDM to be more pro-inflammatory compared with the M-MDM. Although, smoking did not alter this response when compared with non-smoking controls. It has been shown *in vitro* that cigarette smoke extract can modify healthy macrophage function, causing an increase in TNFα release (Pessina et al. 1993; Demirjian et al. 2006), and also IL-8 mRNA levels (Kent et al. 2008). However, the data reported herein did not show any difference. This could be due to differentiation for 12 days *ex-vivo* in the absence of a cigarette smoke insult, leading to a possible alteration of the cell phenotype in culture. Although, MDM were cultured similarly to the method described in the paper by Kent et al 2008, the addition of 25% cigarette smoke extract before functional analysis in the paper may account for the dissimilar results (Kent et al. 2008). The results from this chapter also showed that COPD MDM released similar amounts of TNFα regardless of whether they were differentiated in GM-CSF or M-CSF and that LPS and IL-4-stimulated TNFα release was higher in the cells from COPD patients compared with MDM from non-smokers and smokers. This is in agreement with various studies indicating the exaggerated

pro-inflammatory nature of alveolar macrophages from COPD patients (Culpitt et al. 2003) which remains unaltered by smoking cessation (Kunz et al. 2011). Results from an anti-inflammatory cytokine examination showed M-MDM were more anti-inflammatory than GM-MDM from non-smokers and smokers, again in line with previous studies indicating higher levels of IL-10 in M-CSF cultured monocytes from healthy subjects (Martinez et al. 2006).

There was a trend for M-MDM from smokers to produce less IL-4-stimulated-IL-10 compared with non-smokers although this was not statistically significant. This may be due to low numbers of subjects; a power calculation showed that there would be a $p<0.05$ significant difference if numbers were increased from 7 to 16. Other studies have shown higher IL-10 mRNA in BAL macrophages isolated from smokers compared with non-smokers, however, this did not translate to stimulated protein levels which were slightly lower in smoker macrophages, agreeing with results presented in this chapter (Chen et al. 2007).

IL-10 production by COPD M-MDM was significantly less than that of M-MDM from non-smokers. Various studies have shown that while COPD increases pro-inflammatory cytokine output, it also decreases anti-inflammatory IL-10 cytokine production in alveolar macrophages (Takanashi et al. 1999; Hackett et al. 2008) supporting the data herein and indicating that this reduced anti-inflammatory effect is not only a characteristic of alveolar macrophages but that newly recruited cells may also show similar responses.

4.5.2 Comparison of MDM phagocytosis at day 12

Having shown differences in cytokine output of GM-MDM and M-MDM, it was important to identify further functional differences caused by culture condition or subject group. The ability of macrophages to phagocytose inert beads did not differ between GM-MDM and M-MDM, or between subject groups, suggesting that neither culture condition, disease state, nor smoking status affected the ability of the macrophages to phagocytose inert prey. This agreed with findings published previously showing identical results in alveolar macrophages and MDM from non-smokers, smokers and COPD patients (Taylor et al. 2010; Hodge et al. 2003).

Only when MDM were challenged with bacteria were differences observed. MDM from smokers and COPD patients consistently phagocytosed less bacteria compared with non-smokers (regardless of culture condition). These results are similar to previous findings showing decreased bacterial phagocytic capacity of alveolar macrophages from smokers (Green & Carolin 1967; Thomas et al. 1978) and dysfunctional bacterial phagocytosis in alveolar macrophages from COPD patients compared with healthy controls (Berenson et al. 2006; Taylor et al. 2010; Martí-Lliteras et al. 2009). This decrease in phagocytic capacity has also been demonstrated in patients with severe asthma (Liang et al. 2014) suggesting that chronic lung disease may be associated with dysfunctional phagocytosis, however whether the mechanisms are similar to that in COPD remains unanswered.

Results from this chapter showed that, mechanistically, all MDM could phagocytose inert prey, yet bacteria were less readily phagocytosed in smokers and COPD patients. This indicates a difference in the pathways involved in phagocytosing inert and pathogenic prey and also highlights that it is the bacteria driven pathways that are

dysfunctional in COPD patients and smokers. This may be due to altered recognition receptors in these cells. For example, the macrophage receptor with collagenous structure (MARCO), a macrophage scavenger receptor involved in clearance of microorganisms, is decreased on the surface of monocytic cell line-derived macrophages (THP-1 cells) in response to cigarette smoke (Baqir et al. 2016). Another study on alveolar macrophages from smokers and COPD patients showed downregulation of several apoptotic associated recognition receptors, including CD31, CD44, CD71 and CD91 compared with non-smokers (Hodge et al. 2007). However, a study by Taylor et al in 2010 showed there was no significant difference in the levels of expression of TLR2, TLR4, MARCO, CD163, CD36 or the mannose receptor (Taylor et al. 2010), suggesting that the receptors involved with recognising bacteria are not altered in human cells and that the driving mechanism behind the defective phagocytosis in COPD remains unknown.

4.5.3 Comparison of MDM Phenotype after Environmental Change

Environmental changes were applied to the cells and functional assays were carried out to measure plasticity. MDM were cultured for 12 days in either GM-CSF or M-CSF then had media switched (GM-MDM to M-CSF, M-MDM to M-CSF) over a 6-day time course. Main results are summarised in table 4.2.

	Non-Smoker	Smoker	COPD
CXCL8 GM-MDM → M-CSF	Decrease *	Trend to decrease	No significant change
CXCL8 M-MDM → GM-CSF	Increase *	Trend to Increase	Trend to Increase
TNFα GM-MDM → M-CSF	Decrease *	Trend to decrease	No significant change
TNFα M-MDM → GM-CSF	Increase *	Trend to Increase	Trend to Increase
IL-10 GM-MDM → M-CSF	Increase *	Trend to Increase	No significant change
IL-10 M-MDM → GM-CSF	Decrease *	Decrease *	Trend to decrease
SP Phagocytosis GM-MDM → M-CSF	Increase *	Trend to Increase	No significant change
SP Phagocytosis M-MDM → GM-CSF	Decrease *	Trend to decrease	No significant change
HI Phagocytosis GM-MDM → M-CSF	Increase *	Trend to Increase	No significant change
HI Phagocytosis M-MDM → GM-CSF	Decrease *	Trend to decrease	No significant change

Table 4.2: Summary of altering culture environment to examine MDM phenotypic plasticity. Table showing results of LPS-stimulated cytokine release and *S.pneumoniae* (*SP*) or *H.influenzae* (*HI*) phagocytosis experiments. * indicates significance of $p<0.05$ when comparing day 12 MDM to day 6 post switch.

As indicated in table 4.2, MDM from healthy subjects were able to completely switch their phenotype when environment was altered. This was the case for both cytokine expression and also bacterial phagocytosis, indicating complete functional plasticity in MDM from non-smokers. This in agreement with previous papers demonstrating phenotypic plasticity in healthy human macrophages (Porcheray et al. 2005).

4.5.3.1 Cytokine Expression in MDM Undergoing Environmental Change

MDM from smokers also displayed some plasticity, although this was not always statistically significant and it took cells longer to respond to environmental changes (4 days as opposed to 2 days for the non-smoker MDM). MDM from smokers also remained more pro-inflammatory compared with non-smokers when MDM were switched to M-CSF media, indicating a lesser plasticity in the smoker MDM. This may be similar to the paper by Kunz et al, which showed that even after smoking cessation (e.g. removal of the pro-inflammatory environment) macrophages retained a pro-inflammatory phenotype (Kunz et al. 2011). Slower transition times in the smoker cells may also be due to a desensitisation effect caused by downregulation of M-CSF receptors on the surface of the cells. van Leeuwen et al showed that even small amounts of cigarette smoke condensate could significantly downregulate expression of CSF1-R (the M-CSF receptor) in human PBMC (van Leeuwen et al. 2005). This effect is yet to be determined in smoker MDM, but may explain their reduced plasticity compared with non-smokers.

The results in this chapter also showed that MDM from COPD patients produced consistently high levels of LPS and IL-4-stimulated-CXCL8 and TNFα which did not change when cells were switched to an M-CSF rich environment. However, when M-MDM were switched to GM-CSF containing media, there was an increase in LPS and

IL-4-stimulated-CXCL8 and TNFα cells from COPD patients. This could be indicative of a priming effect in MDM from COPD patients whereby they have been fixed with a pro-inflammatory phenotype and display a unidirectional plasticity indicating that they could change their phenotype to become more pro-inflammatory but could not become less inflammatory. Corresponding with this concept, *ex vivo* models of acute lung inflammation have shown an 'exaggerated' TNFα response in lung tissue from COPD patients, in comparison to healthy volunteers, in response to LPS stimulation (Hackett et al. 2008).

Analysing IL-10 levels in response to changing environments again showed that MDM from non-smokers had the ability to effectively reverse their phenotype depending on their environment. This plasticity was not replicated to the same extent in cells from smokers and COPD patients, again indicative of a lack in plasticity when comparing the subject groups. The lack of IL-10 response may be due to dysfunctional IL-4 receptor signalling in MDM from smokers and COPD patients. This is yet to be established in human MDM but a paper by Wei et al, in 2013, showed that STIP1 homology and U-Box containing protein1 (STUB1a) chaperone-dependent E3 ubiquitin ligase which is crucial in regulating IL-4R signalling is upregulated in the lung tissue of COPD patients (Wei et al. 2014). This upregulation of STUB1 is associated with a decrease in IL-4R signalling (Wei et al. 2014) which may explain the subsequent lack in IL-10 signalling in these cells. It could also be indicative of a loss of signalling in the IL-10 signalling pathway of these cells. Other studies that have shown lower IL-10 expression and fewer IL-10 positive macrophages in COPD patients and smokers compared to non-smokers (Takanashi et al. 1999). Takanashi et al. also indicated that low levels of IL-10 were not due to clearance mechanisms such as proteases,

suggesting instead that the low levels were a result of the overall pro-inflammatory nature brought about by smoking (Takanashi et al. 1999).

4.5.3.2 Phagocytosis in MDM Undergoing Environmental Change

It was important to examine phagocytosis as another readout of macrophage function in response to changing environment. Again, MDM from non-smokers were fully able to switch phenotype and increased or decreased phagocytosis when switched to M-CSF or GM-CSF respectively for both *S.pneumoniae and H.influenzae*. MDM from smokers also showed similar switching patterns but were not significantly changed when compared with day 12 MDM. This may be because of small numbers and large variations in the cohort. The group comprised half current smokers and half ex-smokers; however, more detailed analysis showed a spread of results was not indicative of smoking status. It may be that if the cells had been left longer they would have eventually reached significance, alternatively lack of significance may have been due to lack of power. For example, a power calculation showed that we would need 16 patients to gain significance in phagocytosis of *S. pneumoniae* when switching to M-CSF for 6 days.

COPD patients consistently phagocytosed less bacteria compared with non-smokers and smokers regardless of culture or environmental change indicating again that they are stuck in a pro-inflammatory, non-phagocytic phenotype which could be related to poor bacterial clearance and bacterial overload in the COPD lung (Hodge et al. 2003; Berenson et al. 2006; Taylor et al. 2010).

4.5.4 Comparing Cohorts - Cytokine Results

Comparing the results in this chapter (cohort 2) to cytokine results in the previous chapter (cohort 1) showed that similar values were gained for all M-MDM samples in terms of their LPS-stimulated TNFα, CXCL8 and IL-10 concentrations and IL-4-stimulated IL-10.

There was a trend in cohort 1 for GM-MDM from COPD patients to produce more LPS-stimulated CXCL8 and TNFα compared with cohort 2, although this did not reach statistical significance. Differences may be due to variation in subjects. Cohort 1 consisted of groups of 4 patients while cohort 2 had 6 COPD subjects. Age was not significantly different between cohorts. Gender was different in the COPD cohort 1 having only females and cohort 2 consisting mostly of males (5 males, 1 female). Smoking history was not significantly different between cohorts. There was a trend for lung function parameters to be lower in cohort 2 (FEV1, FEV_1% predicted and FEV_1:FVC ratio) although these were not statistically significant differences. Differences may also be a result of batch variation in additional serum or growth factors.

4.5.5 Conclusions

These data taken together have shown:

- GM-MDM were more pro-inflammatory (produced more CXCL8 and TNFα) and phagocytosed less bacteria compared with M-MDM
- M-MDM were more anti-inflammatory (produced more IL-10) and phagocytosed more bacteria compared with GM-MDM
- While MDM from non-smokers were able to change phenotype depending on environmental changes, MDM from COPD patients remained pro-inflammatory regardless of environmental change.
- MDM from COPD patients were less able to switch to an anti-inflammatory, highly phagocytic phenotype compared with MDM from non-smokers and COPD patients.

Therefore, the hypothesis that MDM from COPD patients will retain a pro-inflammatory phenotype that lack plasticity when environment is altered can be accepted.

CHAPTER 5 FLOW CYTOMETRIC ANALYSIS OF MONOCYTE, MDM AND LUNG TISSUE MACROPHAGES

5.1 Introduction

The previous chapter showed that MDM from COPD patients have a different functional phenotype to MDM from non-smokers, and that plasticity of COPD MDM appears to be unidirectional towards a pro-inflammatory phenotype, it was decided to attempt to identify phenotypic specific cell surface markers that could be used to distinguish COPD cells using flow cytometry.

Macrophages have long been classified into ‘M1’ and ‘M2’ phenotypes based on their cell surface receptor expression (Mantovani et al. 2002; Martinez et al. 2006; Martinez & Gordon 2014). Classically activated human macrophages have been shown to express high levels of CD80, CD16, CD86 and MHCII while alternatively activated ‘M2’ macrophages are thought to express high levels of Fizz1, Ym1, arginase-1 (murine markers) and CD206, CD14 and CD163 (human markers) (Mantovani et al. 2002).

The majority of surface marker and macrophage lineage studies however, have been performed in murine macrophages. Unfortunately, these markers rarely translate into human research. For example, the murine macrophage marker F4/80 has limited use in human studies as its homologue EMR1 is not found on human monocytes, but is expressed by eosinophils (Hamann et al. 2007). There are also no known human macrophage homologs of Fizz1, Ym1 or arginase-1; all established ‘M2’ markers in mice. Human studies have also shown varying results in MDM cell surface marker expression. This is partly due, as mentioned previously, to differing differentiation protocols (Murray et al. 2014) and no clear nomenclature describing stimuli and phenotype (Martinez & Gordon 2014). Therefore, results can only be attributed to the

models used in specific papers, which can vary dramatically. A study by Beyer *et al.,* in 2012 relayed some promising transcriptomic data, showing transcriptional and protein upregulation of several 'M1' and 'M2'-like specific human markers (Beyer et al. 2012). Their study used either GM-CSF or M-CSF culture followed by classic or alternative activation and analysed over 475 differently expressed surfaceome genes. By doing this they were able to show that CD120b and CD282 were increased in 'M1' macrophages, while CD93 and CD226 were increased in 'M2-like' macrophages (Beyer et al. 2012). The main functions of these markers are summarised in table 5.1.

Receptor	Other Names	Function
CD14	LPS-Receptor	Co-receptor for LPS
CD16	IgG Receptor	Antibody-dependent cytotoxicity
CD11b	Integrin αM	Activation and adhesion of monocytes and macrophages
CD80	BB1	T cell activation and survival
CD163	M130	Haemoglobin scavenger receptor
CD206	Mannose receptor	Mediates endocytosis and phagocytosis
CD282	TLR2	Recognises Gram-negative and Gram-positive bacteria
CD120b	TNFRII	TNF receptor which induces recruitment of anti-apoptotic molecules
CD226	PTA1	Activation and adhesion of monocytes/macrophages
CD93	C1QR1	Cell adhesion and clearance of apoptotic cells

Table 5.1: Human monocyte and macrophage receptors and functions. Table detailing the functions of documented monocyte and macrophage cell surface receptors. Those highlighted in pink are thought to be highly expressed in 'M1' macrophages compared with those expressed in blue which have been linked to 'M2' macrophage phenotypes (A Mantovani et al. 2002; Beyer et al. 2012).

More recently, a paper by Desch et al in 2016 used whole lung tissue and differential labelling of hematopoietic cells to fully describe cells from the mononuclear phagocyte system based on their surface markers (Desch et al. 2016). They were able to distinguish blood derived monocytes/macrophages from lung derived alveolar macrophages based on their CD14 and CD16 expression and showed that all cells (aside from dendritic cells) constitutively expressed CD206 (Desch et al. 2016).

If clear macrophage phenotype markers exist and can be identified in MDM from humans, this may present a way of targeting specific cells, which enter into the lung environment during COPD, and which may subsequently contribute to the pro-inflammatory skewed COPD phenotype indicated in previous chapters.

5.1.1 Differences in Monocyte Sub-populations

Phenotypical differences seen in the COPD MDM experiments could also be due to a different monocyte starting population in COPD patients. Monocytes can be classified into sub-populations based on their relative expression of CD14 and CD16. Classical monocytes have a high CD14 expression and lack CD16 (CD14++CD16-) and have been linked with pro-inflammatory roles (Geissmann et al. 2003). Intermediate monocytes have a high CD14 and low CD16 expression ($CD14^{++}CD16^{+}$) and non-classical monocytes have a low CD14 high CD16 expression ($CD14^{+}CD16^{++}$) which have been associated with cell immune-surveillance and viral responses (Cros et al. 2010). It may be that monocytes in COPD consist of a different sub-population to those in non-smokers, leading to the differences in MDM phenotype.

Experiments were therefore devised to analyse monocyte sub-populations in the peripheral whole blood of non-smokers, smokers and COPD patients. It could also be possible that monocytes from COPD patients are more “active” than monocytes from COPD patients. In order to examine monocyte activation, CD11b was used as a marker and experiments were designed to examine expression levels of CD11b within the monocyte sub-populations.

5.1.2 Hypothesis

Monocytes from COPD patients will consist of a different sub-population compared with non-smokers.

GM-MDM from non-smokers will express more CD80 and less CD163 compared with M-MDM

MDM from COPD patients will express more CD80 and less CD163 compared with MDM from non-smokers

M-MDM and GM-MDM will differentially express selective phenotypical cell surface markers.

5.1.3 Aims

- Identify monocyte sub-population distribution in whole blood from non-smokers, smokers and COPD patients.
- Analyse the cell surface receptor expression of several ‘M1’ and ‘M2’-linked markers in MDM.
- Analyse the affect of changing culture environment on cell surface marker expression.

5.2 Methods

5.2.1 Subject Selection

Subjects were selected as in section 2.2.1. Smokers had at least a 10 pack-year history and COPD patients had an FEV_1: FVC ratio <0.7 and post-bronchodilator FEV_1 % predicted <85%.

5.2.2 Multicolour Flow Cytometry Controls

Several controls were used in all multi-colour flow cytometry experiments to reduce confounding factors and produce accurate ways of measuring cell surface markers.

5.2.1.1 Compensation Controls

Fluorochromes emit light over a range of wavelengths. When several fluorochromes are used in the same sample, the wavelengths at which they emit may overlap, producing false positive results. In order to combat this, compensation was applied to all samples. Positive and negative beads compensation beads were prepared in 100µl PBS together with each single fluorochrome at the concentration to be used in the further experiments. The beads were incubated in the dark for 10 min at room temperature before addition of 2ml FACS buffer and centrifugation at 300x*g* for 5 min. Supernatant was tipped off and pellet resuspended in 400µl FACS buffer. Each fluorochrome was then run through a BD Biosciences FACS Canto II machine which compared the wavelengths applied automatic compensation thereby lowering the chance of any false positive results.

5.2.1.2 Fluorochrome Minus One Controls

Some cells, including macrophages, are inherently fluorescent and can “leak” into the wavelengths of certain fluorochromes. This can be corrected for when performing single staining but can create false results when performing multi-colour staining. To combat this, a fluorochrome minus one control was used for all experiments using multiple colours. All fluorochromes except the one of interest were pipetted into a FACS tube containing $1x10^6$ cells resuspended in 200µl PBS. The sample was acquired using a FACS Canto II. Cells were then correctly gated to exclude signal produced by the cells themselves. This control allowed the removal of any spread into the unlabeled channel and was used in setting gates for all multi-colour experiments.

5.2.1.3 Isotype Controls

Antibodies can bind either specifically to the antigen, or to the specific FcR on the cell surface, or non-specifically, to a nonspecific target on the cell. Isotype controls were used in order to gate out any non-specific binding and avoid false negative results.

5.2.1.4 Doublet Exclusion

Cells sometimes clump together which can affect the way the laser light is refracted meaning the subsequent signal picked up on the detectors is not a true reflection of the cells being analysed. In order to combat this, all cells were assessed by plotting forward scatter width against forward scatter height. This allowed the detection of doublet cells which produced a wider signal. Single cells were gated and any further analysis done only on the single cell population.

5.2.1.5 Live Dead

Stimulation, processing and staining of cells can sometimes cause cell death. Dead cells can non-specifically bind fluorochromes leading to false positive results. In order to combat this, cells were stained with a Live/Dead stain conjugated to a specific fluorochrome. This stain can penetrate the permeable membrane of dead cells, allowing for distinction of dead cells (which emit a strong signal) and live cells (which emit a much weaker signal).

5.2.2 Whole Blood Monocyte Sub-population Staining

Blood was collected as in section 2.2.2 and stained for CD14, CD16 and CD11b as in section 2.2.8.1. Monocytes were gated out using forward and side scatter (Figure 5.1A). Compensation, doublet exclusion and live/dead exclusion were applied, before cells were plotted based on their expression of CD14 and CD16. Gates were set using isotype controls (Figure 5.1B). The percentage of cells in each population which expressed CD11b was then identified using appropriate isotype controls.

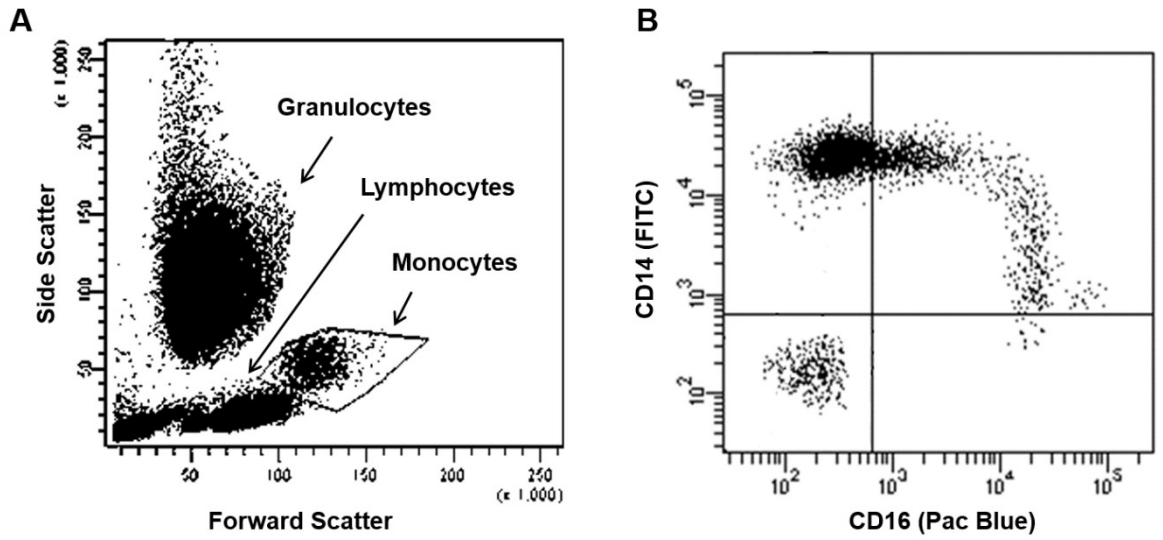


Figure 5.1: Flow cytometry gating strategy for whole blood experiments. Whole blood was fixed and stained with antibodies for CD14 and CD16. Monocytes were gated out using size (forward scatter) and granularity (side scatter) (A) then assessed for expression of CD14 and CD16 (B) allowing for separation into monocyte sub-populations; classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$).

5.2.3 MDM Staining

Monocytes were isolated and plated as detailed in section 2.2.2, and cultured on adherent plates in the presence of 10% ($^{v}/_{v}$) FCS. Cells were differentiated for 12 days in either GM-CSF or M-CSF supplemented media, before undergoing cell culture switching as indicated in section 2.2.2.2. On day 12 and then at days 2, 4 and 6 post-switch cells were harvested and stained as in section 2.2.8.2 (Figure 5.2). Cells were then fixed and acquired on a FACS Canto II and results analysed using FlowJo Version 10.

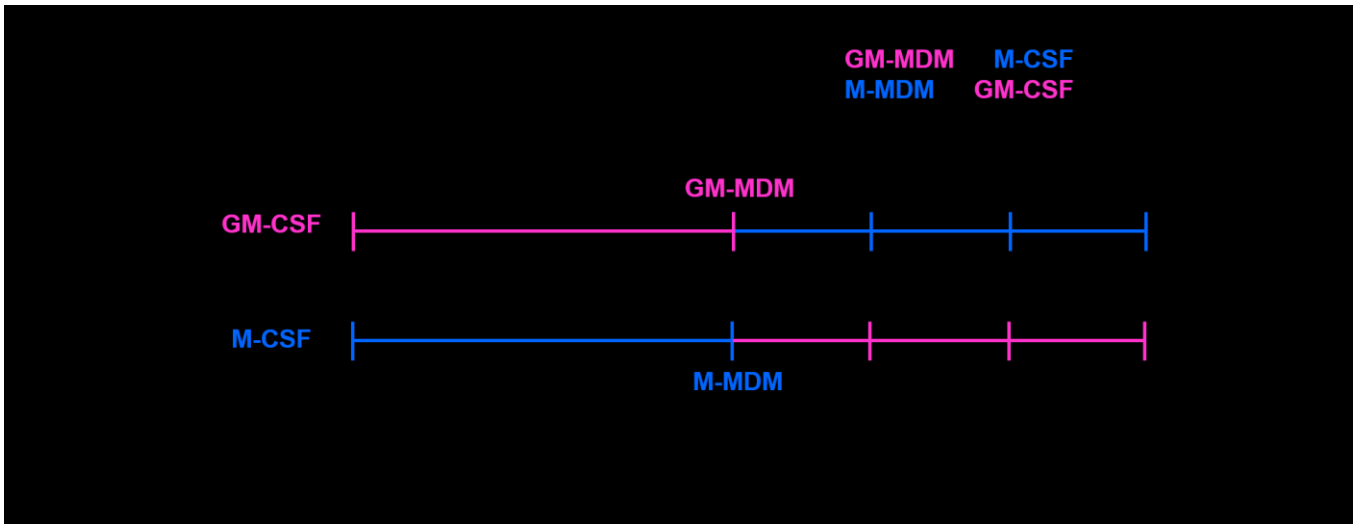


Figure 5.2: MDM flow cytometry timeline. Monocytes were isolated from human peripheral blood by dextran sedimentation and Percoll gradient centrifugation. Monocytes were then cultured in either GMCSF or M-CSF for 12 days before media was switched (GM-CSF to M-CSF and M-CSF to GM-CSF. Cells were harvested on day 12, and then at days 2, 4 and post-switch for use in flow cytometry experiments.

5.2.4 Statistical Analysis

Comparison between subject groups was performed using Kruskall-Wallis (ANOVA) with Dunn's post-correctional tests. Comparisons between GM-MDM and M-MDM from the same patient group were done using Mann-Whitney tests with Dunn's post-correctional tests. Comparisons between different time points were conducted using Wilcoxon signed rank tests. Data were expressed as mean ± SEM and analysed using GraphPad Prism software GraphPad Prism software (version 5.03). A p value of <0.05 was considered statistically significant.

5.3 Results

5.3.1 Whole Blood FACS

5.3.1.1 Subject Demographics

Subject characteristics and spirometry results are shown in Table 5.2. There was no significant difference in smoking history between smokers and COPD patients and no significant difference in age between all subject groups. COPD patients had significantly lower FEV_1, FEV_1 % predicted FVC % predicted and FEV_1:FVC ratio compared with non-smokers.

	Non Smokers (n=10)	Smokers (n=7)	COPD Patients (n=8)
Age (years)	60 ± 4	59 ± 5	67 ± 3
Sex (M:F)	5:5	4:3	3:5
Smoking History (pack years)	0 ± 0	33 ± 5	36 ± 6
FEV_1 (l)	2.8 ± 0.3	3.1 ± 0.4	1.4 ± 0.2*
FEV_1 % Predicted	96 ± 5	86 ± 5	55.± 9*
FVC (l)	3.6 ± 0.4	4.4 ± 0.5	2.5 ± 0.2
FVC % Predicted	108 ± 3	106 ± 11	76 ± 11*
FEV_1:FVC	0.8 ± 0.02	0.7 ± 0.02	0.5 ± 0.5*

Table 5.2: Subject demographics for whole blood experiments. All participants underwent spirometry testing. FEV_1: Forced Expiratory Volume exhaled at the end of the first second of forced expiration FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for 1 year. Data are expressed as mean ± SEM. Kruskall-Wallis test with Dunn's multiple comparison test. * $p<0.05$ COPD *vs.* non-smokers.

5.3.1.2 Monocyte CD14 CD16 Quadrants

Grouping monocytes into sub-populations revealed that most of the cells had a classical monocyte cell surface expression (CD14++CD16-) (approximately 75%) (Figure 5.3). This was the same across all subject groups with approximately 10% of cells being intermediate monocytes (CD14++CD16+) and around 5% non-classical (CD14+CD16++). The other 10% of cells were negative for both CD14 and CD16.

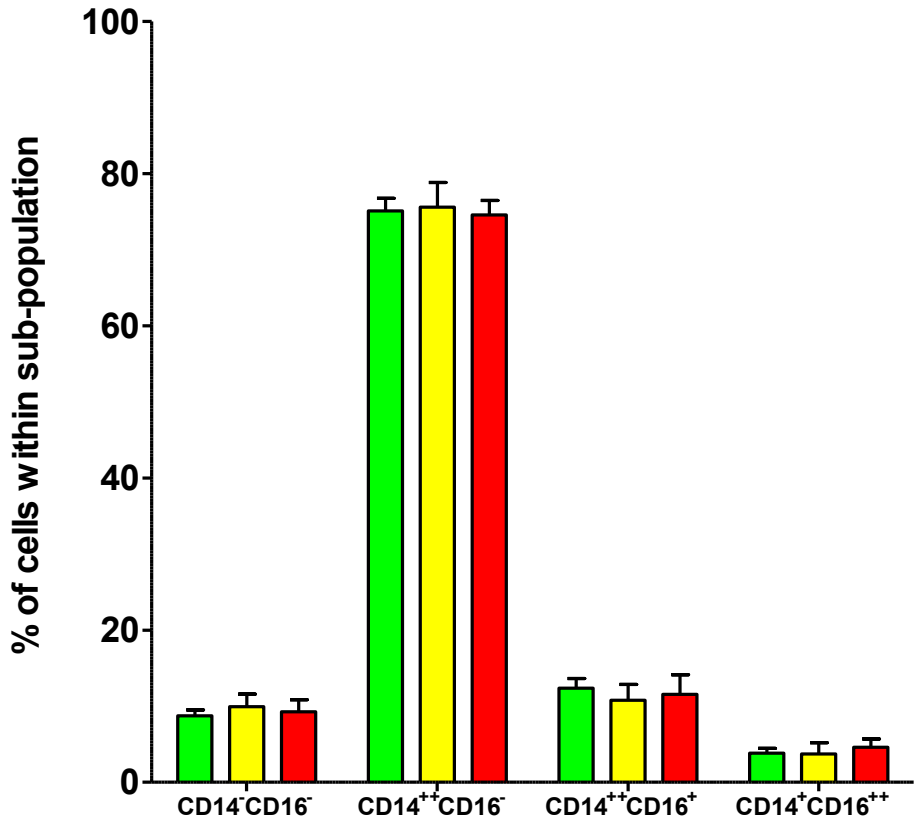


Figure 5.3: Monocyte sub-populations within whole blood. Whole blood from non-smokers (green n=10), smokers (yellow n=7) and COPD patients (red n=8) was fixed and stained using CD14 (FITC) and CD16 (Pac Blue) conjugated antibodies. Cells were acquired using a FACS Canto II and gated using appropriate controls. Data presented as percentage of cells within each sub population; negative ($CD14^{-}CD16^{-}$), classic ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$). Data expressed as mean ± SEM.

5.3.1.3 Monocyte CD11b Quadrants

As there were no discernible differences in monocyte sub-population distribution, it was decided to examine CD11b levels within the sub-populations as a marker of activity.

Overall results showed that a higher percentage of classical monocytes expressed CD11b, compared with intermediate and non-classical monocytes, in all subject groups (Figure 5.4A). This was statistically significant in non-smokers and smokers but did not reach statistical significance in COPD patient monocytes. Within the monocyte subsets, it was shown that monocytes from COPD patients and smokers consistently expressed more CD11b on their surface compared to those from non-smokers in all sub-populations, although this did not reach statistical significance (Figure 5.4A).

Classical monocytes expressed significantly more CD11b per cell, compared with non-classical monocytes, in all subject groups (Figure 5.4B). Examining MFI of CD11b within the sub-populations showed that classical monocytes from COPD patients expressed less CD11b per cell compared with classical and intermediate monocytes from non-smokers, although this did not reach statistical significance (Figure 5.4B).

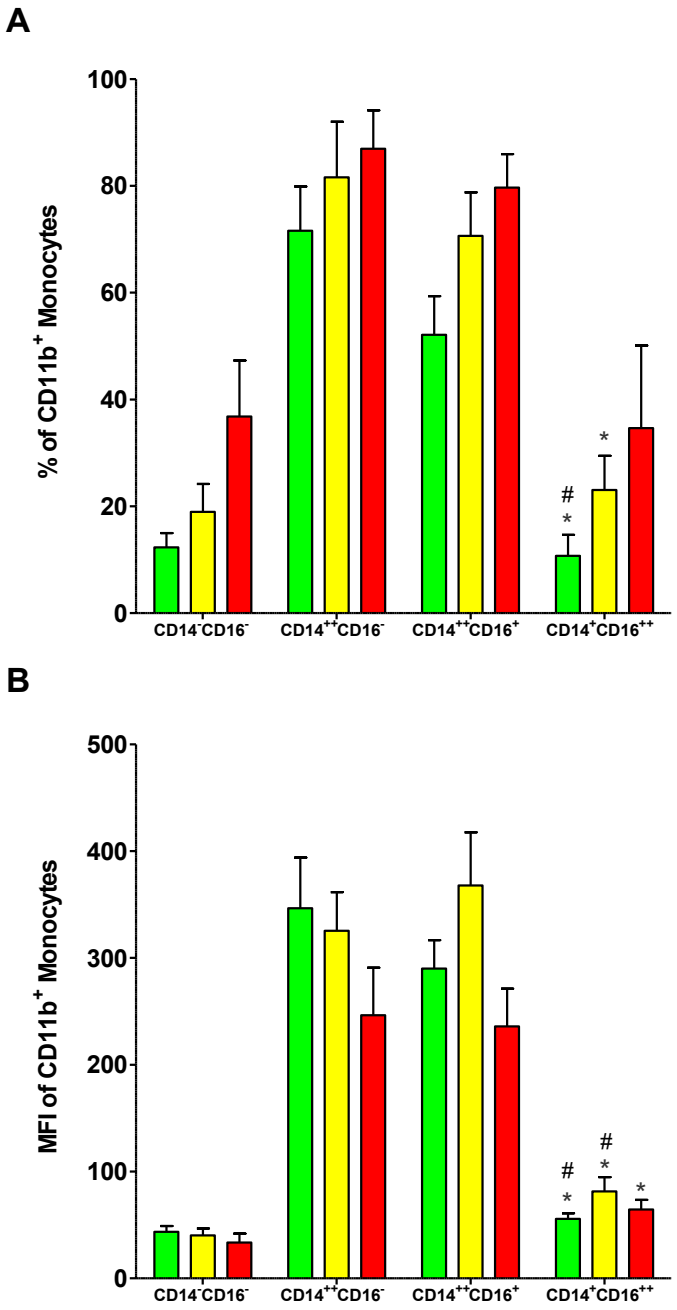


Figure 5.4: CD11b expression within monocyte sub-populations. Whole blood from non-smokers (green n=10), smokers (yellow n=7) and COPD patients (red n=8) was fixed and stained using CD14 (FITC), CD16 (Pac Blue) and CD11b (APC) conjugated antibodies. Cells were acquired using a FACS Canto II and gated using appropriate controls. Data presented as (A) percentage of cells within each sub population that are $CD11b^+$ or (B) median fluorescence intensity (MFI) of $CD11b^+$ cells within each sub-population. Data expressed as mean ± SEM. * $p<0.05$ classical vs. non-classical. # $p<0.05$ intermediate vs. non-classical.

5.3.2 Known Phenotypic Markers: CD80 and CD163

Many studies have classified macrophages as 'M1' and 'M2' based upon their CD80 and CD163 expression (Mantovani et al 2002, Martinez et al 2006). Experiments were therefore devised to identify the expression of these markers in the MDM models used in this thesis and compare expression in non-smokers, smokers and COPD patients.

5.3.2.1 Subject Demographics

Subject characteristics and spirometry results are shown in Table 5.3. There was no significant difference in smoking history between smokers and COPD patients and no significant difference in age between all subject groups. COPD patients had significantly lower FEV_1, FEV_1 % predicted FVC, FVC % predicted and FEV_1:FVC ratio compared with non-smokers.

	Non Smokers (n=15)	Smokers (n=5)	COPD Patients (n=11)
Age (years)	62 ± 3	58 ± 3	67 ± 2
Sex (M:F)	10:5	4:1	5:6
Smoking History (pack years)	0 ± 0	34 ± 7	49 ± 8
FEV_1 (l)	3 ± 0.3	3 ± 0.3	1.2 ± 0.2*
FEV_1 % Predicted	98 ± 6	84 ± 5	48.± 6*
FVC (l)	3.9 ± 0.4	4.6 ± 0.5	2.4 ± 0.2*
FVC % Predicted	107 ± 7	89 ± 9	73 ± 8*
FEV_1:FVC	0.8 ± 0.01	0.7 ± 0.03	0.4 ± 0.05*

Table 5.3: Subject demographics for CD80 CD163 MDM experiments. All participants underwent spirometry testing. FEV_1: Forced Expiratory Volume exhaled at the end of the first second of forced expiration FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for 1 year. Data are expressed as mean ± SEM. Kruskall-Wallis test with Dunn's multiple comparison test. * $p<0.05$ COPD *vs.* non-smokers.

5.3.2.2 CD80 and CD163 expression in Day 12 MDM

A significantly higher percentage of GM-MDM from non-smokers expressed CD80 on their surface (78±8%) compared with M-MDM from non-smokers (45±6%) (Figure 5.5A) ($p<0.05$). There was also a trend for GM-MDM from non-smokers to express more CD80 per cell compared with M-MDM from non-smokers although this did not reach significance (Figure 5.5B). There was no significant difference in either percentage of cells expressing CD80 or level of CD80 expressed when comparing GM-MDM and M-MDM from smokers or COPD patients.

The percentage of M-MDM from non-smokers expressing CD163 was significantly higher (70±10%) compared with GM-MDM from non-smokers (20±5%) (Figure 5.5C) ($p<0.05$). The same pattern was seen in both smokers and COPD with more M-MDM expressing CD163 compared with GM-MDM although this did not reach statistical significance. There were also significantly less CD163 expressing M-MDM in COPD patients compared with M-MDM from non-smokers ($p<0.05$) (Figure 5.5D). Similarly, when analysing MFI, M-MDM from non-smokers expressed significantly higher levels of CD163 compared with GM-MDM from non-smokers as well as M-MDM from smokers and COPD patients ($p<0.05$).

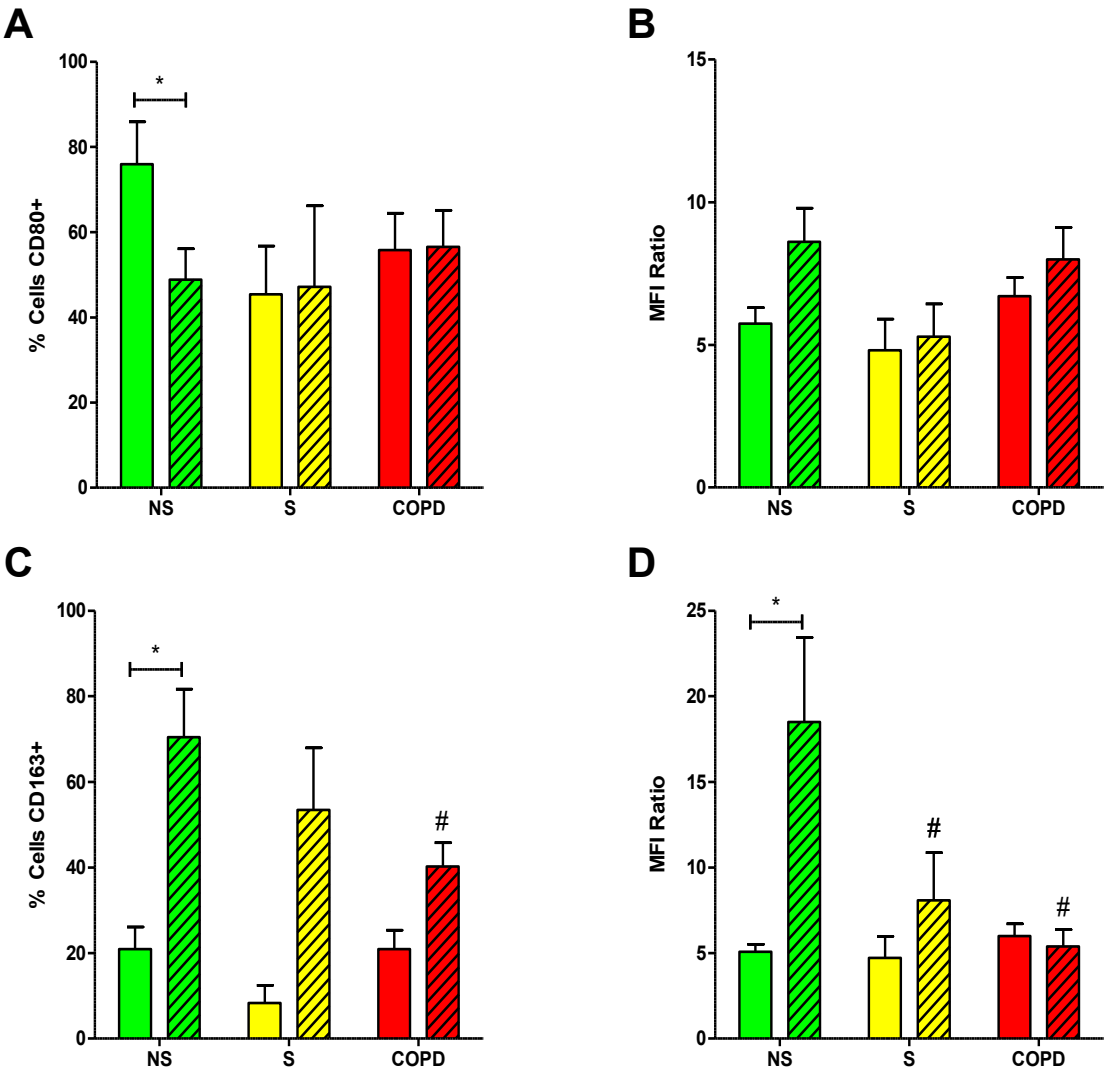


Figure 5.5: CD80 and CD163 expression in Day 12 MDM. MDM from non-smokers (green n=11), smokers (yellow n=5) and COPD patients (red n=10) were cultured in either GM-CSF (filled bars) or M-CSF (hatched bars) for 12 days. At day 12, cells were stained with antibodies for CD80 (PE) and CD163 (FITC) and acquired on a FACS Canto II. (A) % of cells which are $CD80^+$ (B) CD80 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control) (C) % of cells which are $CD163^+$ (D) CD163 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). * $p<0.05$ GM-MDM vs M-MDM. # $p<0.05$ vs. non-smoker M-MDM.

5.3.2.3 Affect of switching MDM culture conditions on expression of CD80 and CD163

Having seen differences in CD80 and CD163 expression in differentiated MDM from non-smokers compared with cells from COPD patients, it was decided to examine the affect of changing environmental culture on cell surface marker expression and compare with MDM from smokers and COPD patients. Again, a higher percentage of GM-MDM from non-smokers expressed CD80 compared with smokers and COPD patients at day 12 ($p=0.06$) (Figure 5.6A). When GM-MDM were switched to media containing M-CSF there was a trend towards a decrease in the percentage of cells expressing CD80 in non-smokers, although this was not significant after 6 days. Percentage of cells from COPD patients expressing CD80 was slightly increased when media was switched to M-CSF, but not significantly. MFI was not affected by switching media (Figure 5.6C. Neither percentage of cells expressing CD80 or amount of CD80 expressed was significantly altered in cells from in smokers during environmental switch (Figure 5.6B).

When M-MDM were switched to media containing GM-CSF there was a trend towards an increase in the percentage of cells expressing CD80 in non-smokers, although this was not significant after 6 days (Figure 5.6B). There was also a slight increase in percentage of cells from smokers and COPD patients which expressed CD80 but this was again not significantly different to results at day 12. All cells had a slight increase in CD80 MFI when M-MDM were switched to media containing GM-CSF (Figure 5.XD).

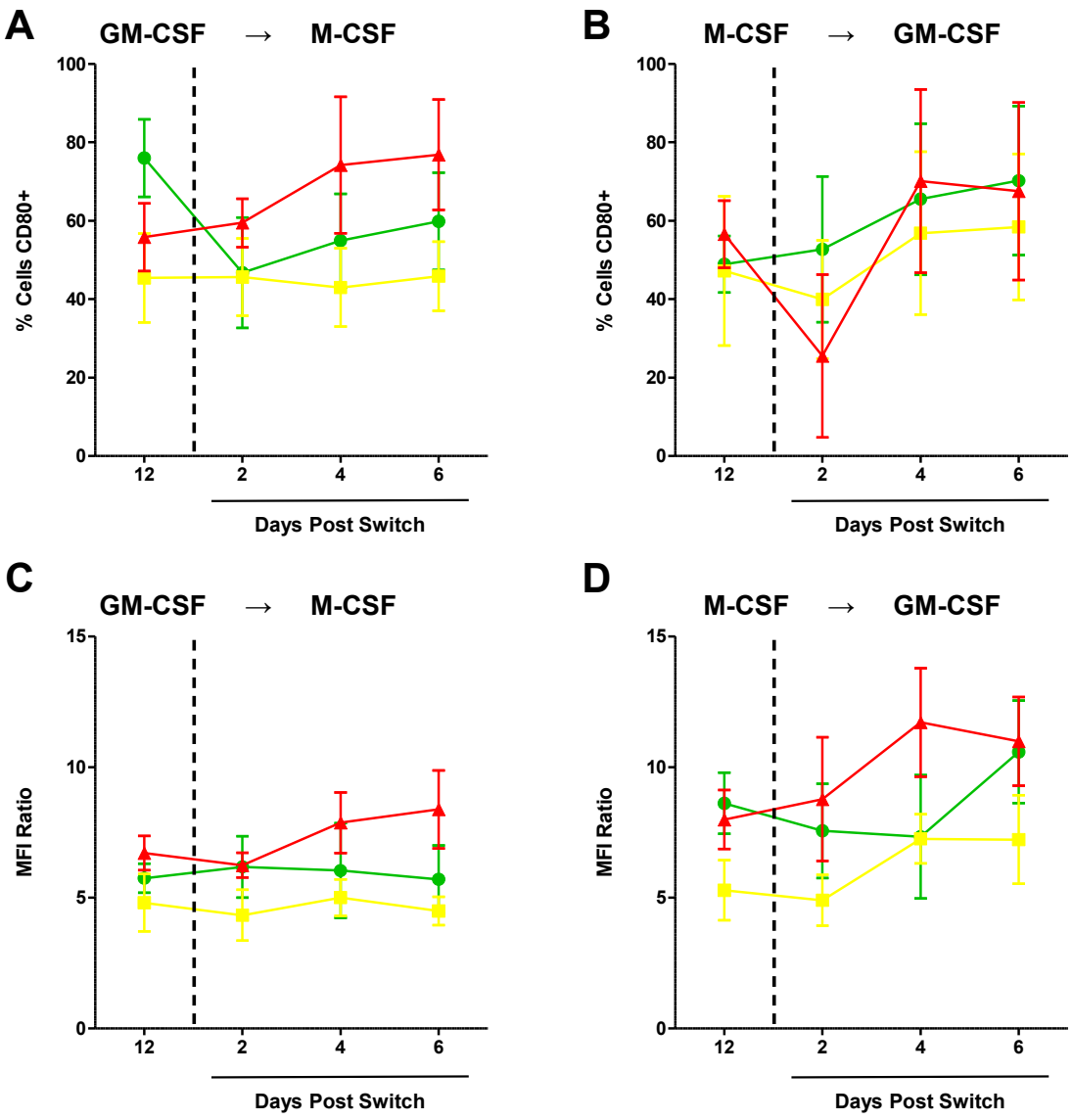


Figure 5.6: Effect of changing culture environment on CD80 expression. MDM from non-smokers (green n=5), smokers (yellow n=5) and COPD patients (red n=4) were cultured in either GM-CSF or M-CSF for 12 days then had media switched to either M-CSF or GM-CSF respectively. At days 12, then 2, 4 and 6 post switch, cells were stained for CD80 (PE) and acquired on a FACS Canto II. (A) GM-MDM → M-CSF, % of cells which are $CD80^+$ (B) M-MDM → GM-CSF, % of cells which are $CD80^+$ (C) GM-MDM → M-CSF, CD80 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (D) M-MDM → GM-CSF, CD80 MFI ratio. Data expressed as mean ± SEM.

There was a trend for a higher percentage of GM-MDM from COPD patients to express CD163 compared with smokers and non-smokers at day 12 although this was not statistically significant (Figure 5.7A). There was no significant change in percentage of cells expressing CD163 when media was changed to that containing M-CSF. There was also no significant change in MFI over the 6 days in cells from smokers and COPD patients (Figure 5.7C).

When M-MDM were switched to media containing GM-CSF there was a trend towards a decrease in the percentage of cells expressing CD163 in non-smokers, although this was not significant after 6 days. Percentage of cells from COPD patients expressing CD163 was increased when media was switched to M-CSF, but not significantly.

CD163 MFI was significantly higher in M-MDM from non-smokers compared with smokers and COPD patients at day 12 and day 6 post switch ($p<0.05$) but MFI was not significantly altered during environmental switch in any subject group (Figure 5.7D).

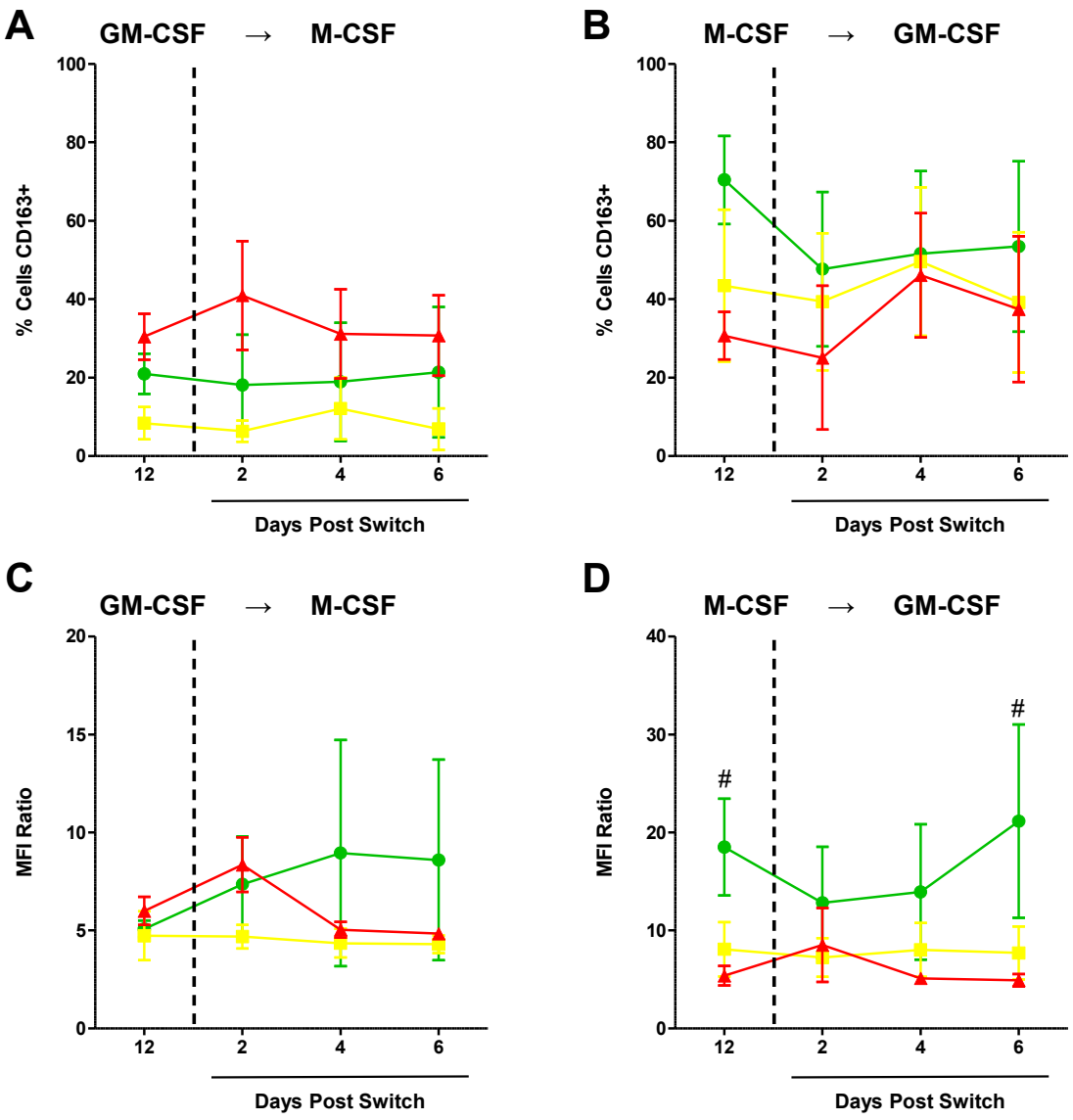


Figure 5.7: Effect of changing culture environment on CD163 expression. MDM from non-smokers (green n=5), smokers (yellow n=5) and COPD patients (red n=4) were cultured in either GM-CSF or M-CSF for 12 days then had media switched to either M-CSF or GM-CSF respectively. At days 12, then 2, 4 and 6 post switch, cells were stained for CD163 (FITC) and acquired on a FACS Canto II. (A) GM-MDM → M-CSF, % of cells which are $CD163^+$ (B) M-MDM → GM-CSF, % of cells which are $CD163^+$ (C) GM-MDM → M-CSF, CD163 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (D) M-MDM → GM-CSF, CD163 MFI ratio. Data expressed as mean ± SEM. # $p<0.05$ non-smokers *vs*. COPD patients.

5.3.2.4 CD80 and CD163 expression in lung tissue macrophages

Having identified differences in CD80 and CD163 expression in MDM it was important to see if these differences could also be observed in lung tissue macrophages. Therefore, similar experiments were set up to look at expression of cell surface CD80 and CD163 in lung tissue macrophages on the day of isolation.

5.3.2.4.1 Subject Demographics

Subject characteristics and spirometry results are shown in Table 5.4. There was no significant difference in smoking history between smokers and COPD patients and no significant difference in age between all subject groups. COPD patients had lower lung function results compared with non-smokers but this was not statistically significant.

	Non Smokers (n=3)	Smokers (n=3)	COPD Patients (n=3)
Age (years)	56 ± 13	68 ± 7	69 ± 5
Sex (M:F)	1:2	1:2	3:0
Smoking History (pack years)	0 ± 0	25 ± 15	24 ± 3
FEV_1 (l)	2.7 ± 0.5	2.1 ± 0.5	1.9 ± 0.3
FEV_1 % Predicted	86 ± 4	99 ± 5	76.± 19
FVC (l)	4 ± 0.9	3.1 ± 0.1	3.5 ± 0.3
FVC % Predicted	108 ± 2	127 ± 29	106 ± 18
FEV_1:FVC	0.7 ± 0.04	0.7 ± 0.2	0.5 ± 0.04

Table 5.4: Subject demographics for tissue macrophage experiments. All participants underwent spirometry testing. FEV_1: Forced Expiratory Volume exhaled at the end of the first second of forced expiration FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for 1 year. Data are expressed as mean ± SEM.

5.3.2.4.2 Lung Macrophage Receptor Expression

When examining macrophages from non-smokers, a higher percentage of the macrophages in the 30-40% and 40-50% fraction expressed CD80 compared with macrophages from the 50-60% fraction although this was not statistically significant (Figure 5.8A). The percentage of macrophages from COPD patients that expressed CD80 did not differ between fractions. The amount of CD80 expressed per cell did not differ between fractions but there was a trend for higher MFI in macrophages from non-smokers compared with macrophages from smokers or COPD patients, although this did not reach significance (Figure 5.8B).

A similar percentage of macrophages from non-smokers in the 30-40 and 40-50% fractions expressed CD163 compared with lung macrophages from COPD patients (Figure 5.8C). There was a trend in the 50-60% fraction for a higher percentage of cells from COPD patients to express CD163, although there was considerable variation in these results resulting in a large standard error. Again, the amount of CD163 expressed per cell did not differ significantly between fractions, but there was a trend for higher MFI in macrophages from non-smokers compared with macrophages from smokers or COPD patients although this did not reach significance (Figure 5.8D).

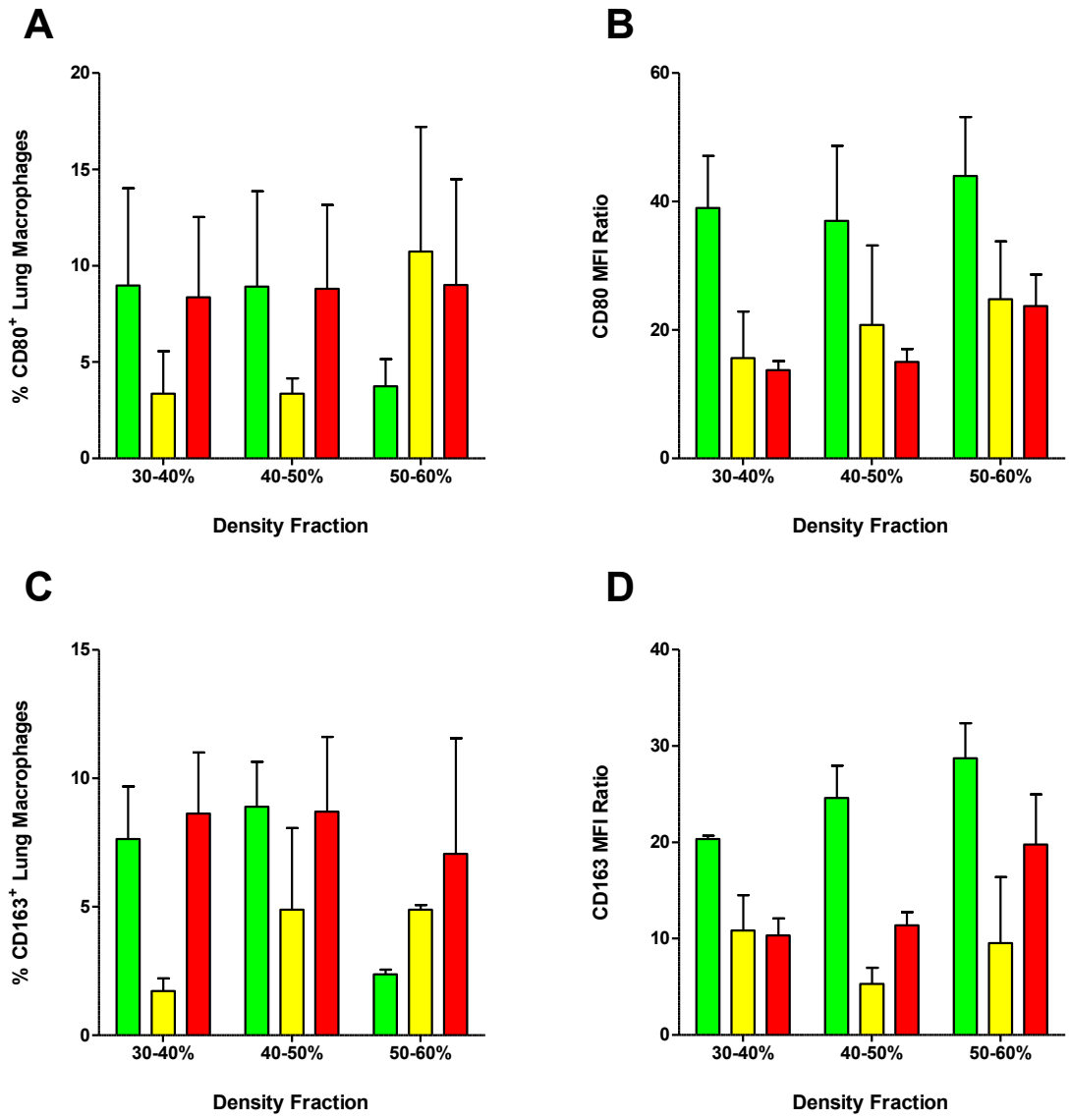


Figure 5.8: CD80 and CD163 expression in fractionated lung tissue macrophages. Lung tissue macrophages were isolated from resection tissue from non-smokers (green n=3), smokers (yellow n=2) and COPD patients (red n=3). Macrophages were then separated based on density into 3 fractions (30-40%, 40-50% and 50-60%). Fractionated macrophages were stained with antibodies for CD80 (PE) and CD163 (FITC) and acquired on a FACS Canto II. (A) % of cells which are $CD80^+$ (B) CD80 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (C) % of cells which are $CD163^+$ (B) CD163 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control).

5.3.3 New MDM Phenotypic Markers: Panel 2

Having shown that culture environment affected cell surface receptor expression on MDM, experiments were devised to test affect of different differentiation culture and also change in culture condition on several other 'M1' and 'M2' associated cell surface markers.

5.3.3.1 Subject Demographics

Subject characteristics and spirometry results are shown in Table 5.5. There was no significant difference in smoking history between smokers and COPD patients and no significant difference in age between all subject groups. COPD patients had significantly lower FEV_1, FEV_1 % predicted, FVC % predicted and FEV_1:FVC ratio compared with non-smokers.

	Non Smokers (n=4)	Smokers (n=3)	COPD Patients (n=3)
Age (years)	65 ± 3	62 ± 7	74 ± 4
Sex (M:F)	2:2	3:0	1:2
Smoking History (pack years)	0 ± 0	44 ± 23	48 ± 1
FEV_1 (l)	3 ± 0.4	3 ± 0.2	0.9 ± 0.2
FEV_1 % Predicted	96 ± 10	91 ± 3	42.± 10*
FVC (l)	3 ± 0.5	5 ± 0.6	2 ± 0.2
FVC % Predicted	104 ± 8	107 ± 6	63 ± 1*
FEV_1:FVC	0.8 ± 0.02	0.7 ± 0.05	0.5 ± 0.1*

Table 5.5: Subject demographics for MDM experiments – Panel 2. All participants underwent spirometry testing. FEV_1: Forced Expiratory Volume exhaled at the end of the first second of forced expiration FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for 1 year. Data are expressed as mean ± SEM. Kruskall-Wallis test with Dunn's multiple comparison test. * $p<0.05$ COPD *vs.* non-smokers.

5.3.3.2 MDM Cell Surface Markers

5.3.3.2.1 CD14

There was no significant difference in percentage of MDM expressing CD14 at day 12 between subject groups (Figure 5.9A). There was also no significant difference when comparing GM-MDM with M-MDM. Switching culture conditions had no affect on the percentage of cells expressing CD14 in any subject group (Figure 5.9A and B).

The amount of CD14 expressed per cell in GM-MDM from non-smokers was higher (50±20MFI) compared with GM-MDM from smokers (16±3MFI) and COPD patients (10±6MFI) although this was not statistically significant (Figure 5.9C). When media was switched to M-CSF there was a non-significant decrease in CD14 MFI in cells from non-smokers by day 2 that was continued to day 6. There was no significant change in MFI of cells from smokers or COPD patients when media was switched to that containing M-CSF.

When M-MDM were switched to media containing GM-CSF there was an increase in CD14 MFI in non-smokers over the time course which did not reach significance (Figure 5.9D). Again, there was no significant change in MFI of cells from smokers or COPD patients when media was switched to that containing GM-CSF.

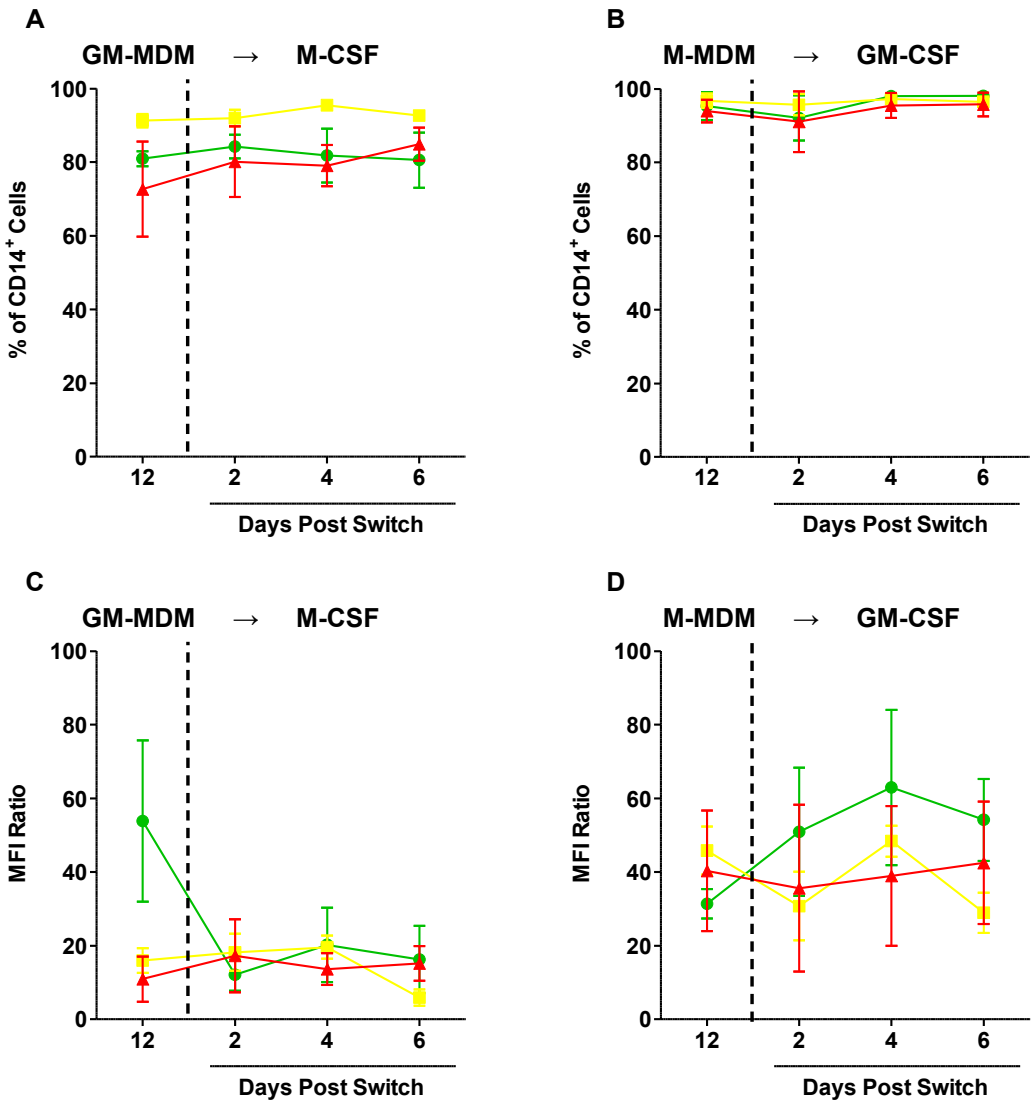


Figure 5.9: Effect of changing culture environment on CD14 expression. MDM from non-smokers (green n=4), smokers (yellow n=3) and COPD patients (red n=3) were cultured in either GM-CSF or M-CSF for 12 days then had media switched to either M-CSF or GM-CSF respectively. At days 12, then 2, 4 and 6 post switch, cells were stained for CD14 (APC-Cy5.5) and acquired on a FACS Canto II. (A) GM-MDM → M-CSF, % of cells which are $CD14^+$ (B) M-MDM → GM-CSF, % of cells which are $CD14^+$ (C) GM-MDM → M-CSF, CD14 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (D) M-MDM → GM-CSF, CD14 MFI ratio. Data expressed as mean ± SEM.

5.3.3.2.2 CD16

There was no significant difference in percentage of GM-MDM expressing CD16 at day 12 between subject groups (Figure 5.10A). When media was switched to M-CSF there was a non-significant decrease in percentage of cells from non-smokers expressing CD16 that was not mimicked by cells from smokers or COPD patients which remained consistent regardless switching to M-CSF media.

At day 12 there was a significantly higher percentage of M-MDM from smokers expressing CD16 (80±5%) compared with non-smokers (20±6%) and COPD patients (40±17%) (Figure 5.10B). When M-MDM were switched to media containing GM-CSF there was a significant increase in CD16 by day 2 which was sustained over the time course ($p<0.05$). There was also a trend towards an increase in the percentage of cells from COPD patients expressing CD16 when switched to GM-CSF media, although this was not significant.

Switching culture conditions had no significant affect on the MFI of CD16 in any subject group (Figure 5.10C and D).

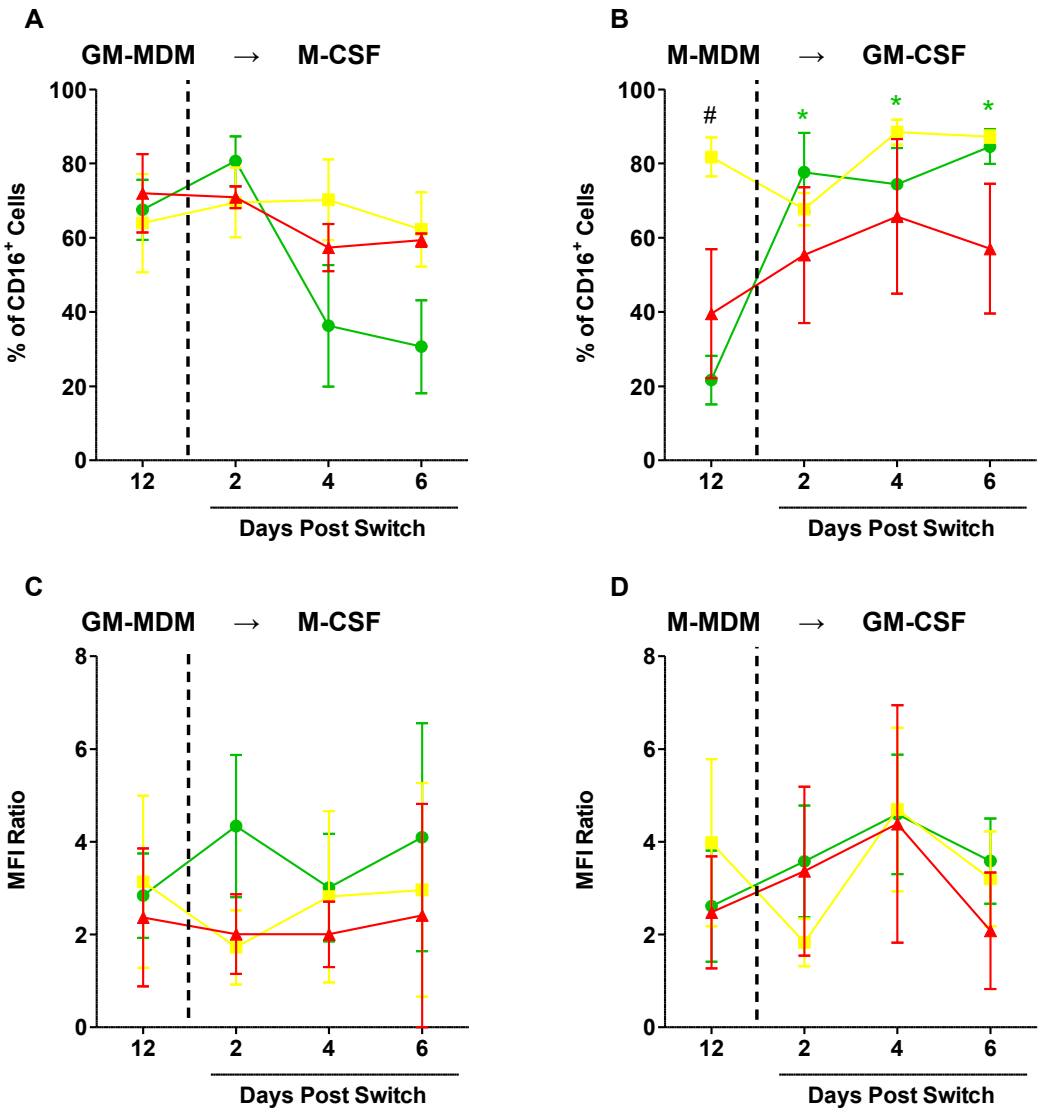


Figure 5.10: Effect of changing culture environment on CD16 expression. MDM from non-smokers (green n=4), smokers (yellow n=3) and COPD patients (red n=3) were cultured in either GM-CSF or M-CSF for 12 days then had media switched to either M-CSF or GM-CSF respectively. At days 12, then 2, 4 and 6 post switch, cells were stained for CD16 (PerCP-Cy5.5) and acquired on a FACS Canto II. (A) GM-MDM → M-CSF, % of cells which are $CD16^+$ (B) M-MDM → GM-CSF, % of cells which are $CD16^+$ (C) GM-MDM → M-CSF, CD16 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (D) M-MDM → GM-CSF, CD16 MFI ratio. Data expressed as mean ± SEM. * $p<0.05$ *vs.* day 12 cells.

5.3.3.2.3 CD11b

There was no significant difference in percentage of MDM expressing CD11b at day 12 between subject groups (Figure 5.11A). There was also no significant difference when comparing GM-MDM with M-MDM. Switching culture conditions had no significant affect on the percentage of cells expressing CD11b in non-smokers and smokers (Figures 5.11A and B), but did show a trend towards a decrease in percentage of cells from COPD patients expressing CD11b when media was switched to M-CSF (Figure 5.11A).

Amount of CD11b expressed per cell was not significantly different between subject groups on day 12. Switching to M-CSF media had no significant affect on the MFI of CD11b in any non-smokers of smokers (Figure 5.11C) but did show a trend towards a decrease in CD11b MFI in MDM from COPD patients when media was switched to M-CSF (Figure 5.11A). Switching to GM-CSF media also had no significant affect on the MFI of CD11b in any subject group (Figure 5.11D).

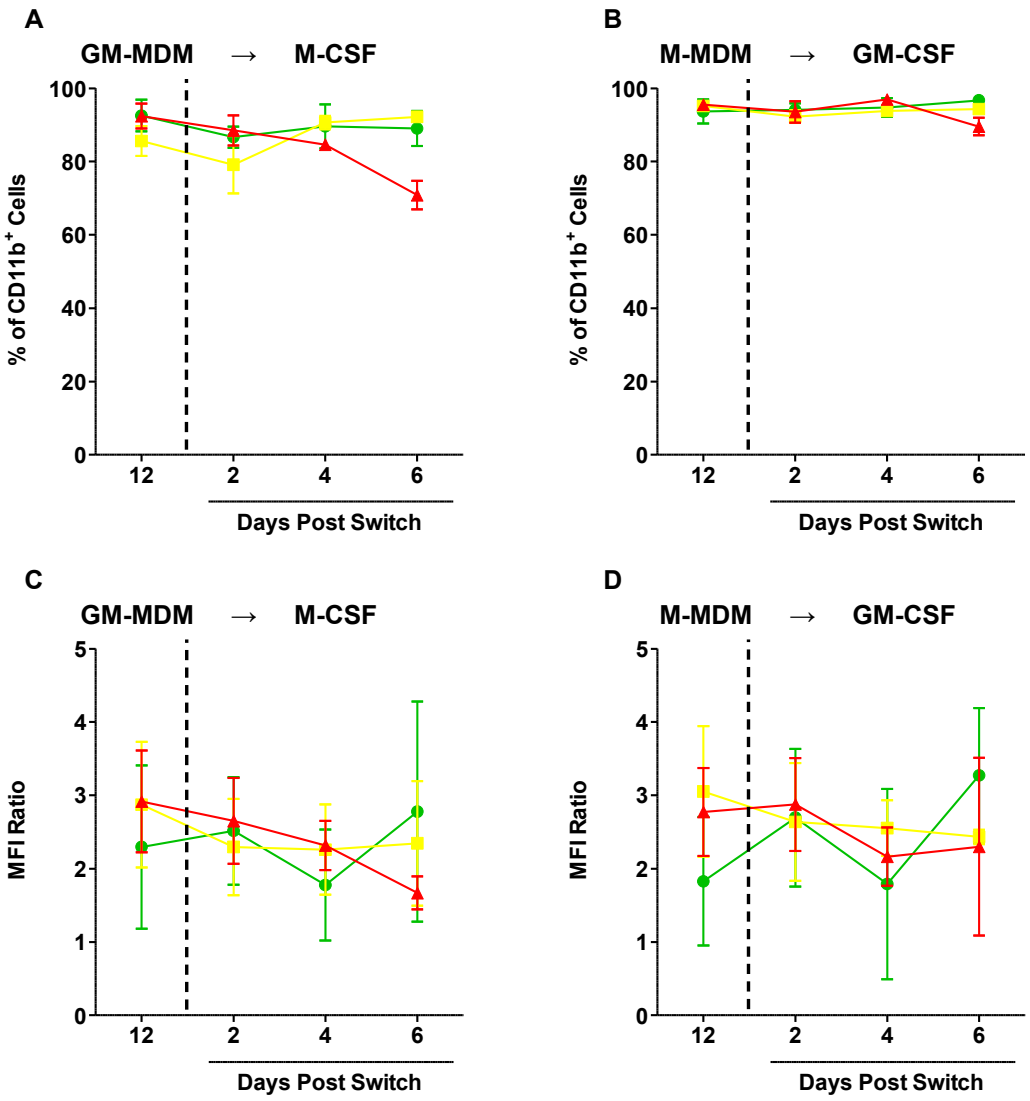


Figure 5.11: Effect of changing culture environment on CD11b expression. MDM from non-smokers (green n=4), smokers (yellow n=3) and COPD patients (red n=3) were cultured in either GM-CSF or M-CSF for 12 days then had media switched to either M-CSF or GM-CSF respectively. At days 12, then 2, 4 and 6 post switch, cells were stained for CD11b (Pac Blue) and acquired on a FACS Canto II. (A) GM-MDM → M-CSF, % of cells which are $CD11b^+$ (B) M-MDM → GM-CSF, % of cells which are $CD11b^+$ (C) GM-MDM → M-CSF, CD11b median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (D) M-MDM → GM-CSF, CD11b MFI ratio. Data expressed as mean ± SEM.

5.3.3.2.4 CD206

There was no significant difference in percentage of MDM expressing CD206 at day 12 between subject groups (Figures 5.12A and B) which accounted for approximately 80-90% of cells. There was also no significant difference when comparing GM-MDM with M-MDM. Switching culture conditions had no significant affect on the percentage of cells expressing CD206 in any subject group (Figures 5.12A and B).

Amount of CD206 expressed per cell was not significantly different between subject groups on day 12 in either GM-MDM or M-MDM. Switching to M-CSF media had no significant affect on the MFI of CD206 in any subject group (Figure 5.12C). Switching to GM-CSF media also had no significant affect on the MFI of CD206 in any subject group (Figure 5.12D).

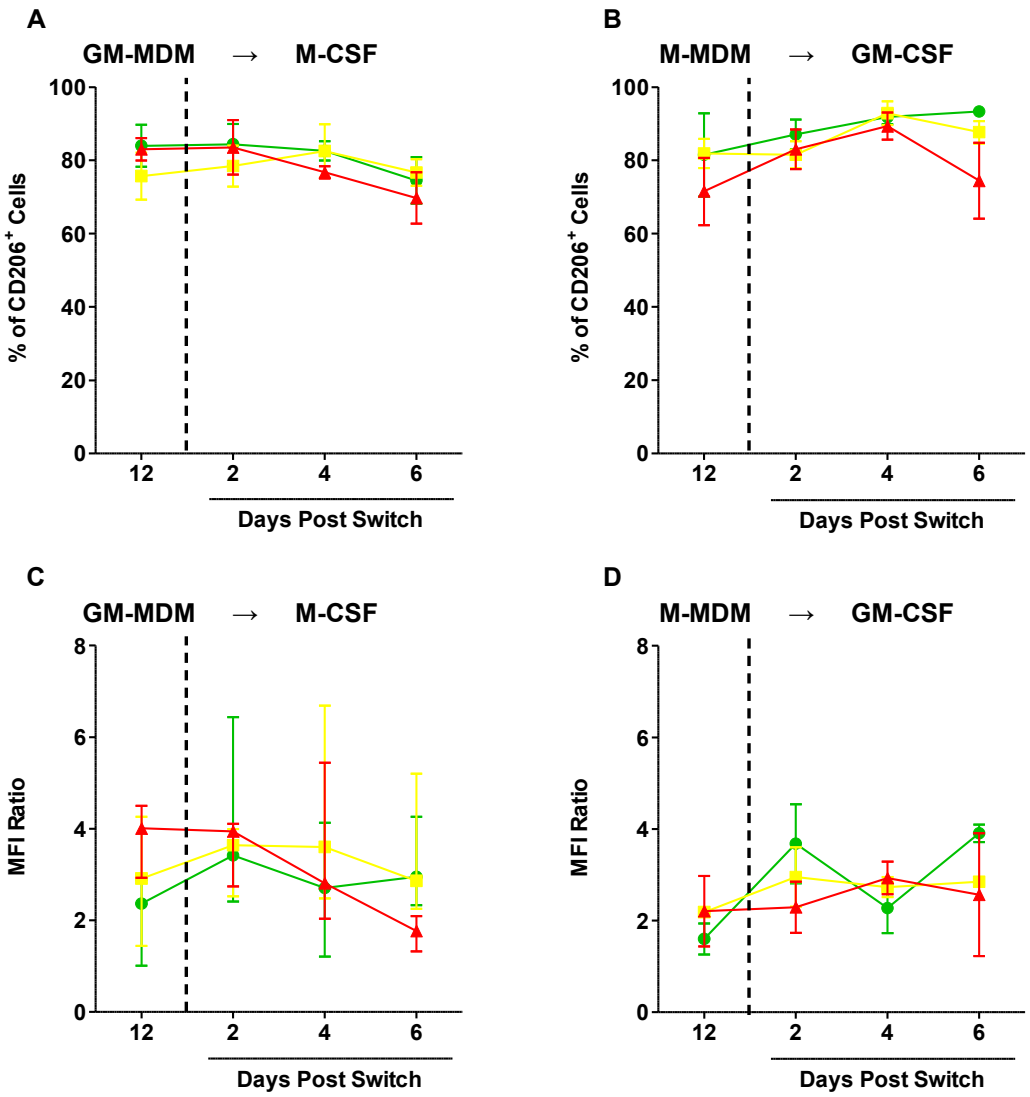


Figure 5.12: Effect of changing culture environment on CD206 expression. MDM from non-smokers (green n=4), smokers (yellow n=3) and COPD patients (red n=3) were cultured in either GM-CSF or M-CSF for 12 days then had media switched to either M-CSF or GM-CSF respectively. At days 12, then 2, 4 and 6 post switch, cells were stained for CD206 (PE) and acquired on a FACS Canto II. (A) GM-MDM → M-CSF, % of cells which are $CD206^+$ (B) M-MDM → GM-CSF, % of cells which are $CD206^+$ (C) GM-MDM → M-CSF, CD206 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (D) M-MDM → GM-CSF, CD206 MFI ratio. Data expressed as mean ± SEM.

5.3.3.2.5 CD93

There was no significant difference in percentage of MDM expressing CD93 at day 12 between subject groups (Figures 5.13A and B). There was also no significant difference when comparing GM-MDM with M-MDM. Switching media to that containing M-CSF had no significant affect on the percentage of cells expressing CD93 any subject group (Figure 5.13A). Switching to media containing GM-CSF induced a time dependent increase in the percentage of cells from non-smokers expressing CD93 (Figure 5.13B) which was not significant by day 6 (due to large variation between patients) and was not mimicked by smokers or COPD patients which produced similar results at day 6 compared to day 12 cells.

Amount of CD93 expressed per cell was not significantly different between subject groups on day 12. Switching to M-CSF media had no significant affect on the MFI of CD93 in any subject group by day 6 (Figure 5.13C). Switching to GM-CSF media also showed no significant affect on the MFI of CD93 in any subject group by day 6 (Figure 5.13D).

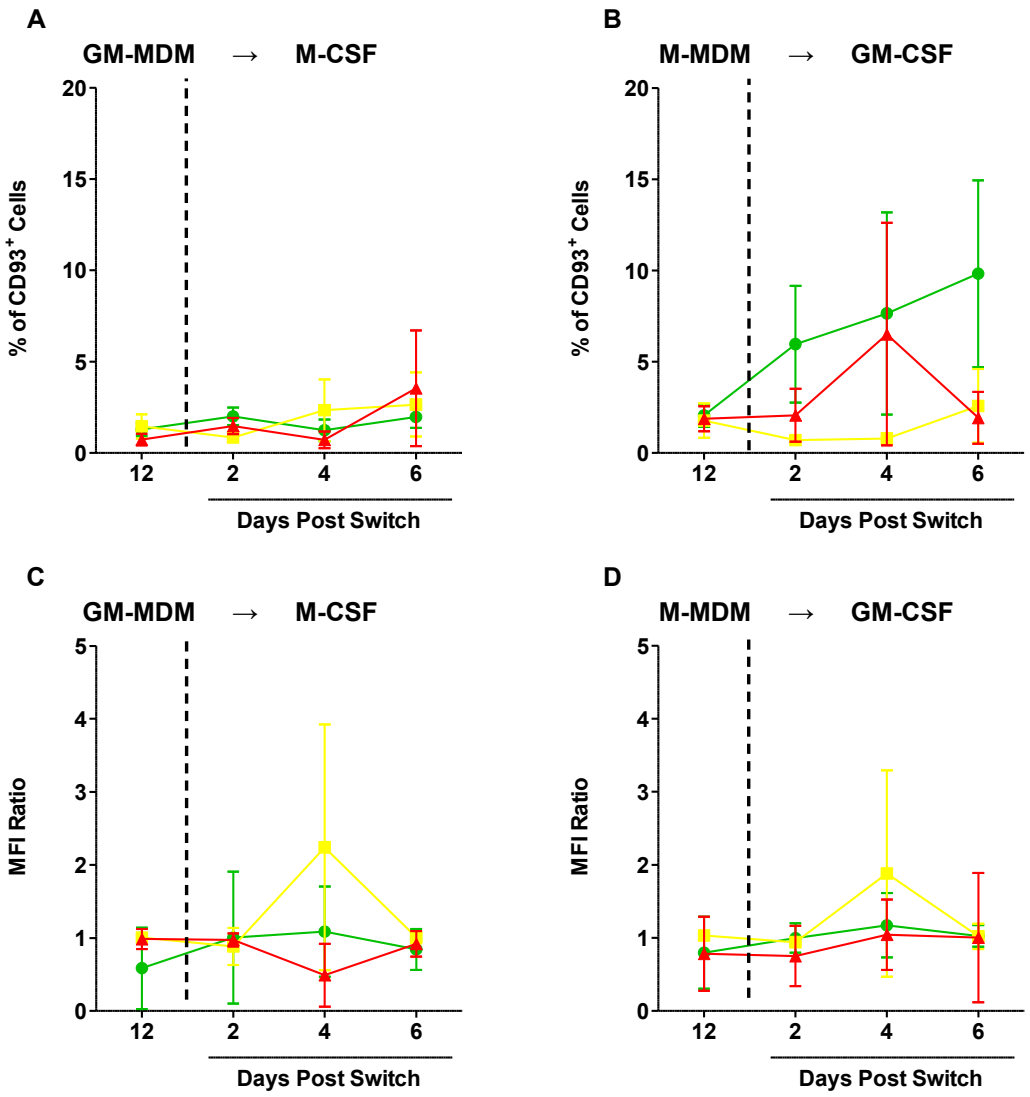


Figure 5.13: Effect of changing culture environment on CD93 expression. MDM from non-smokers (green n=4), smokers (yellow n=3) and COPD patients (red n=3) were cultured in either GM-CSF or M-CSF for 12 days then had media switched to either M-CSF or GM-CSF respectively. At days 12, then 2, 4 and 6 post switch, cells were stained for CD93 (FITC) and acquired on a FACS Canto II. (A) GM-MDM → M-CSF, % of cells which are $CD93^+$ (B) M-MDM → GM-CSF, % of cells which are $CD93^+$ (C) GM-MDM → M-CSF, CD93 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (D) M-MDM → GM-CSF, CD93 MFI ratio. Data expressed as mean ± SEM.

5.3.3.2.6 CD226

There was no significant difference in percentage of MDM expressing CD226 at day 12 between subject groups or between GM-MDM and M-MDM (Figures 5.14A and B).

Switching media to that containing M-CSF had no significant affect on the percentage of cells expressing CD226 any subject group (Figure 5.14A). Switching to media containing GM-CSF induced a time dependent decrease in the percentage of cells from all subjects expressing CD226 (Figure 5.14B).

Amount of CD226 expressed per cell was not significantly different between subject groups on day 12. Nor did it differ between GM-MDM and M-MDM in any subject group (Figures 5.14C and D). Switching to M-CSF media had no significant affect on the MFI of CD226 in any subject group by day 6 (Figure 5.14C). Switching to GM-CSF media also showed no significant affect on the MFI of CD226 in any subject group by day 6 (Figure 5.14D).

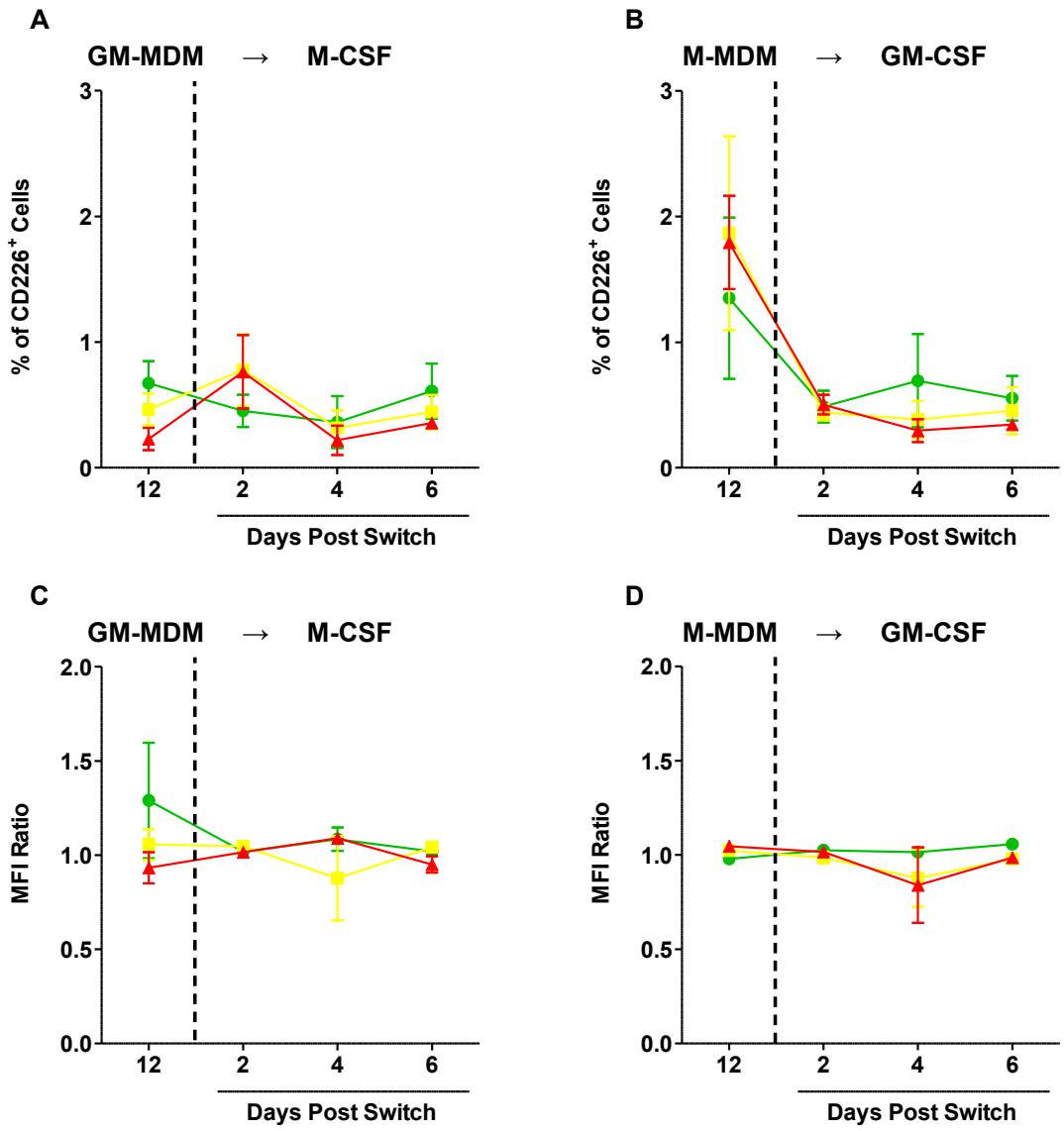


Figure 5.14: Effect of changing culture environment on CD226 expression. MDM from non-smokers (green n=4), smokers (yellow n=3) and COPD patients (red n=3) were cultured in either GM-CSF or M-CSF for 12 days then had media switched to either M-CSF or GM-CSF respectively. At days 12, then 2, 4 and 6 post switch, cells were stained for CD226 (FITC) and acquired on a FACS Canto II. (A) GM-MDM → M-CSF, % of cells which are $CD226^+$ (B) M-MDM → GM-CSF, % of cells which are $CD226^+$ (C) GM-MDM → M-CSF, CD226 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (D) M-MDM → GM-CSF, CD226 MFI ratio. Data expressed as mean ± SEM.

5.3.3.2.7 CD282

There was a significantly higher percentage of MDM from COPD patients expressing CD282 at day 12 (78±10%) compared with MDM from non-smokers (18±8%) and smokers (10±5%) (Figures 5.15A and B). There was no significant difference in percentage of cells expressing CD282 comparing individual subject group GM-MDM to M-MDM at day 12 (Figures 5.15A and B). When media was switched to M-CSF there was a significant decrease in percentage of cells from COPD patients expressing CD282 that was not mimicked by cells from non-smokers or smokers which remained consistent regardless switching to M-CSF media (Figure 5.15A). When media was switched to that containing GM-CSF, there was a slight, non-significant decrease in percentage of cells from COPD patients expressing CD282. Conversely, there was a slight, non-significant increase in percentage of cells from non-smokers and smokers expressing CD282 when media was switched to GM-CSF (Figure 5.15B).

Amount of CD282 expressed per cell was not significantly different between subject groups on day 12. Nor did it differ between GM-MDM and M-MDM in any subject group (Figures 5.15C and D). Switching to M-CSF media had no significant affect on the MFI of CD282 in any subject group by day 6 (Figure 5.15C). Switching to GM-CSF media also showed no significant affect on the MFI of CD282 in any subject group by day 6 (Figure 5.15D).

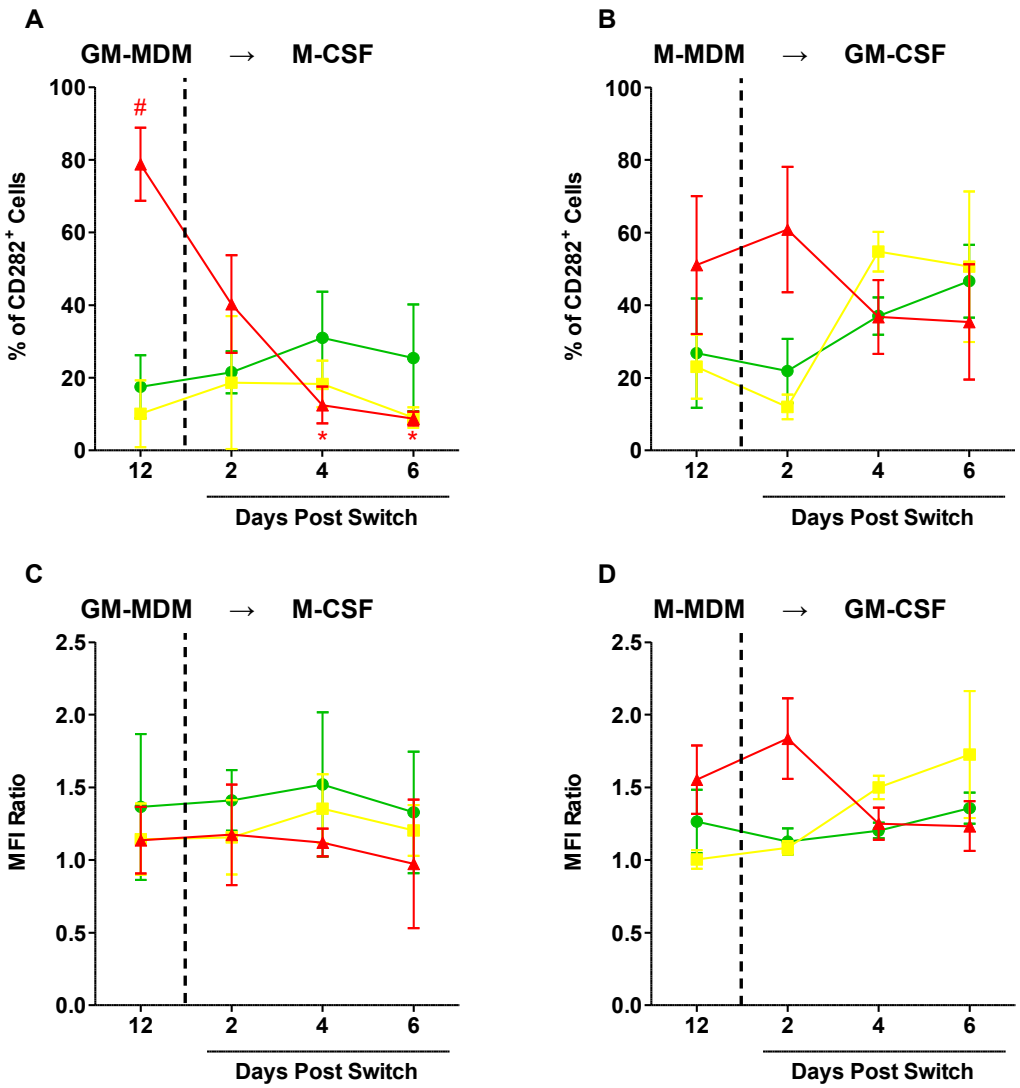


Figure 5.15: Effect of changing culture environment on CD282 expression. MDM from non-smokers (green n=4), smokers (yellow n=3) and COPD patients (red n=3) were cultured in either GM-CSF or M-CSF for 12 days then had media switched to either M-CSF or GM-CSF respectively. At days 12, then 2, 4 and 6 post switch, cells were stained for CD282 (APC) and acquired on a FACS Canto II. (A) GM-MDM → M-CSF, % of cells which are CD282$^+$ (B) M-MDM → GM-CSF, % of cells which are CD282$^+$ (C) GM-MDM → M-CSF, CD282 median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (D) M-MDM → GM-CSF, CD282 MFI ratio. Data expressed as mean ± SEM. * $p<0.05$ *vs*. day 12 cells. # $p<0.05$ *vs*. non-smoker GM-MDM.

5.3.3.2.8 CD120b

There was a significantly higher percentage of MDM from COPD patients expressing CD120b at day 12 (53±15%) compared with MDM from non-smokers (2±1%) and smokers (2±1%) (Figures 5.16A and B) ($p<0.05$). There was no significant difference in percentage of cells expressing CD120b comparing individual subject group GM-MDM to M-MDM at day 12 (Figures 5.16A and B). When media was switched to M-CSF there was a non-significant decrease in percentage of cells from COPD patients expressing CD120b that was not mimicked by cells from non-smokers which remained consistent regardless switching to M-CSF media or smokers which showed a slight non-significant increase in expression (Figure 5.16A). When media was switched to that containing GM-CSF, there was no significant change in percentage of cells from any subject group expressing CD120b by day 6 (Figure 5.16B).

Amount of CD120b expressed per cell was not significantly different between subject groups or between GM-MDM and M-MDM on day 12 (Figures 5.16C and D). Switching to M-CSF media had no significant affect on the MFI of CD120b in any subject group by day 6 (Figure 5.16C). Switching to GM-CSF media showed a non-significant decrease in CD120b MFI in cells from non-smokers while there was no significant affect on the MFI of CD120b of cells from smokers or COPD patients by day 6 (Figure 5.16D).

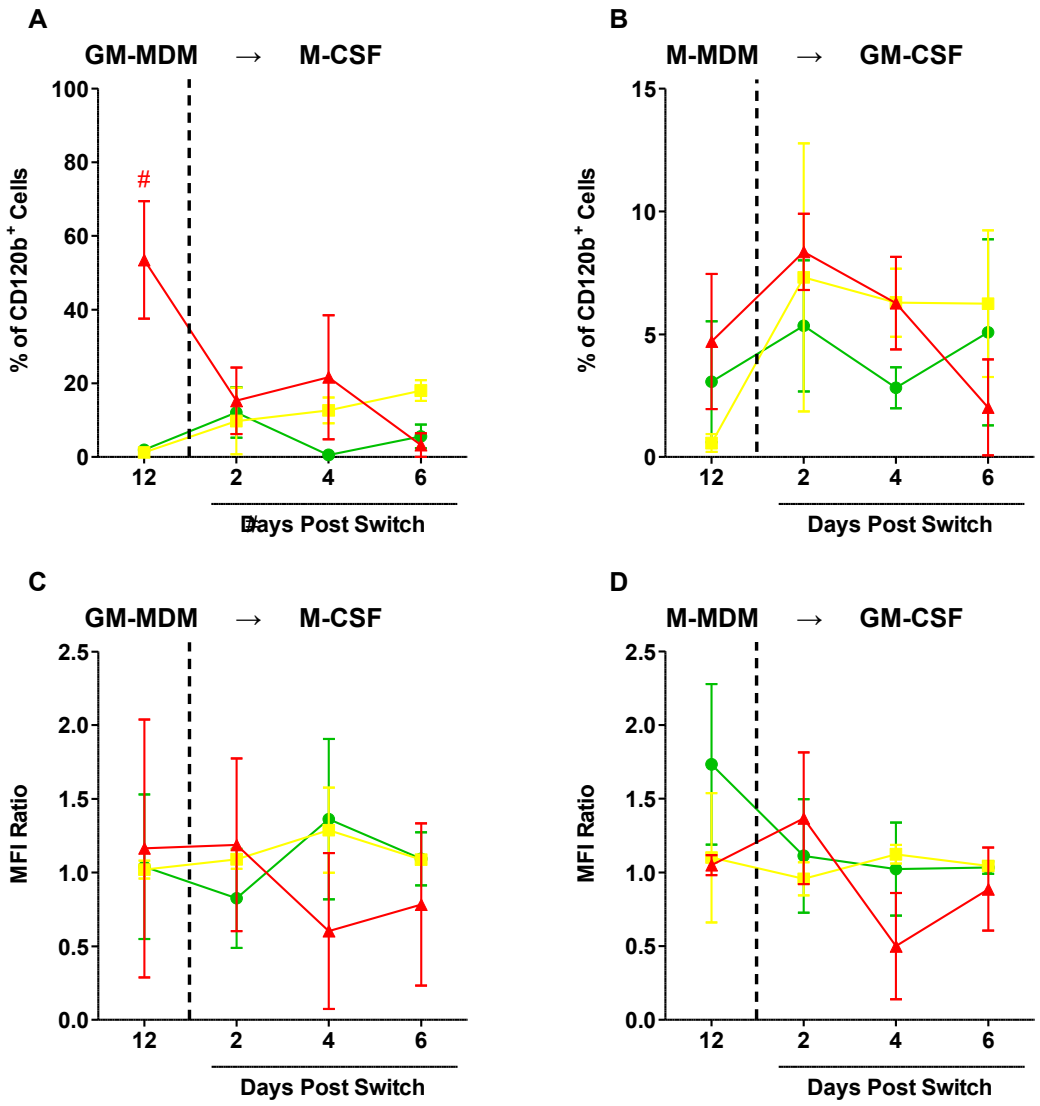


Figure 5.16: Effect of changing culture environment on CD120b expression. MDM from non-smokers (green n=4), smokers (yellow n=3) and COPD patients (red n=3) were cultured in either GM-CSF or M-CSF for 12 days then had media switched to either M-CSF or GM-CSF respectively. At days 12, then 2, 4 and 6 post switch, cells were stained for CD120b (PE) and acquired on a FACS Canto II. (A) GM-MDM → M-CSF, % of cells which are CD120b$^+$ (B) M-MDM → GM-CSF, % of cells which are CD120b$^+$ (C) GM-MDM → M-CSF, CD120b median fluorescence intensity (MFI) ratio (MFI of sample/MFI of isotype control). (D) M-MDM → GM-CSF, CD120b MFI ratio. Data expressed as mean ± SEM. # $p<0.05$ *vs*. non-smoker GM-MDM.

5.4 Discussion

5.4.1 Monocyte Sub-Populations

The majority of monocytes analysed in this study displayed a classical phenotype. This is in agreement with other research showing the majority of monocytes found in human blood are classical monocytes (Tolouei Semnani et al. 2014; Strauss-Ayali et al. 2007). While some studies have suggested that there are a higher percentage of classical and intermediate monocytes in blood from COPD patients (Melgert et al. 2013), there were no significant differences in the distribution of monocyte sub-populations between the patient groups in the present study. When analysing the data from the study by Melgert et al. in 2013 in more detail, it was found that after results were corrected for age there was also no significant difference in monocyte subset distribution (Melgert et al. 2013). This may explain the lack of difference between subject groups in the current study, as all patients were age matched, although, it does raise an important question about changing monocyte sub-populations with advanced age.

Results in this chapter indicated a slightly higher percentage of cells from COPD patients expressed CD11b. This could indicate an increased baseline activity level in these cells compared with non-smokers. A study by Ray et al in 2006 showed that monocytes isolated from women who were chronically exposed to biomass smoke had an increased CD11b expression compared with cells from controls (Ray et al. 2006). It may be that the chronic smoke stimuli could lead to constant activation of peripheral blood monocytes which may in turn affect their subsequent phenotype.

5.4.2 Cell Surface Markers in in MDM

Cell surface receptor expression is a commonly used tool to classify macrophages into phenotypes. However, due to lack of translational mouse studies and the absence of a properly defined method of cell culture and stimulation, these markers are constantly changing and up for debate, even by those who coined the initial phenotypic nomenclature (Martinez & Gordon 2014).

	Non-smokers		Smokers		COPD	
	GM-MDM	M-MDM	GM-MDM	M-MDM	GM-MDM	M-MDM
CD80	78*	45	42	45	58	59
CD163	20*	70	8	50	22	41
CD14	81*	97	90	98	75	97
CD16	68*	21	63	40	70	81
CD11b	93	97	85	96	92	97
CD206	83	81	77	81	81	70
CD282	20	27	10	25	80	50
CD120b	1	3	1	1	55*	4
CD226	0.7	1.4	0.5	1.9	0.2	1.8
CD93	2	2	2	2	1	2

Table 5.6: MDM cell surface marker expression at day 12. MDM from non-smokers (n=4), smokers (n=3) and COPD patients (n=3) were cultured in either GM-CSF or M-CSF for 12 days before being stained for antibodies to identify cell surface receptor expression. Data are expressed as mean of % of MDM positive for expression. Conditional formatting using colour scales within each receptor comparing high (dark red) to low expression (white) within subject groups. * $p<0.05$ in comparison with M-MDM.

Results from this chapter showed CD80 and CD163 differed in expression between GM-MDM and M-MDM from non-smokers (Table 5.6). This concurs with previous studies showing higher CD80 expression in ‘M1-like’ macrophages and a higher CD163 expression in ‘M2-like’ macrophages (Mantovani et al. 2002). In the current study both MDM and tissue macrophages from non-smokers expressed higher levels of CD80 compared with cells from COPD patients. This is in agreement with various studies showing low CD80 expression in COPD BAL and tissue macrophages compared with non-smokers (Pons et al. 2005; Löfdahl et al. 2006). CD80 is a co-receptor for LPS and therefore having lower expression of this receptor in COPD may account for dysfunctional immune function and subsequent colonisation by bacteria (Pons et al. 2005; Droemann et al. 2005). However, the data indicated in this thesis and in various other research (Hackett et al. 2008; Frankenberger et al. 2011) suggests increase in LPS-stimulated pro-inflammatory cytokine release from COPD MDM. This therefore suggests that either this receptor is not important in LPS signalling in COPD patients or the pro-inflammatory cytokines examined are being stimulated through a different pathway.

There was a trend for lower CD163 expression in M-MDM from COPD patients. This is in agreement with research by Chana et al who showed significantly less CD163 expression in COPD tissue macrophages compared with non-smoker cells (Chana et al. 2016) and Kunz et al who showed reduced CD163 expression in BAL macrophages from COPD compared with current smokers (Kunz et al. 2011). A reduction in CD163 may account for loss of phagocytic ability noted in previous chapters, however, Taylor et al showed that this was not the case in MDM which showed no significant difference in CD163 expression between subject groups (Taylor et al. 2010).

Another phagocytic receptor, CD206, the mannose receptor, is commonly described as an ‘M2-like’ marker (Stein et al. 1992; Goerdt & Orfanos 2016; Högger et al. 1998; Gordon 2003), however, this was not the case in this study. Both GM-CSF and M-CSF derived MDM expressed CD206 and there were no significant differences in expression between patient groups. Some studies have found low expression of CD206 in sputum from COPD macrophages which may be linked to protease removal of the cell surface marker in the lungs of COPD patients (Wright et al. 2009). It may be that because cells were taken from the peripheral blood that these differences were not identified because the cells are not maintained in a protease rich environment. However, other studies have also shown CD206 to be constitutively expressed by lung tissue macrophages from COPD patients (Chana et al. 2016; Kaku et al. 2014), agreeing with the data presented herein and indicating that CD206 may not be a generic ‘M2’ marker.

While data from this chapter mimics work showing higher CD16 expression in ‘M1’like GM-CSF cultured cells (Zhang et al. 2013), contrasting studies have shown a decreased expression of CD16 in macrophages from COPD patients compared with cells from non-smokers (Löfdahl et al. 2006). Differences in results may be due to the differences in cell type as the Löfdah study used BAL macrophages as opposed to MDM in the present study. As CD16 is involved in immune signal transduction and T-cell activation, low CD16 expression in COPD may be connected with poor immune signalling in the disease, although more work needs to be done to examine contrasting results in the different cell types.

There was a significantly higher percentage of GM-MDM from COPD expressing CD282 and CD120b. As both of these were indicated as being upregulated in the M1-like cells in the Beyer paper, this could indicate that these cells express more of an M1

phenotype prior to activation that could contribute to their subsequent pro-inflammatory phenotype (Beyer et al. 2012). However, large variation and low subject numbers mean this would need further verification.

There were also no significant differences in any of the ‘M2’ associated receptors identified by the Beyer paper, CD226 and CD93 (Beyer et al. 2012) between GM-MDM and M-MDM and the results showed that less than 2% of MDM stained with these markers suggesting these receptors are not expressed on these cells. The contrasting results may be a result of differently cultured MDM. In the current study, cells were cultured for 12 days in either GM-CSF or M-CSF while in the Beyer study, cells were cultured for 3 days in either GM-CSF or M-CSF then stimulated for 3 days with either IFNγ, LPS or IL-4. It may be that upon activation, these receptors are increased and differences in cell surface expression are unmasked.

These results also highlight the redundancy of using cell surface markers alone as a means of identifying cell phenotype. Cell surface receptor expression is dependent on environment and disease type, therefore results may vary depending on culture and subject.

5.4.3 Change in surface marker expression after environmental change

	Non-smokers		Smokers		COPD	
	GM-MDM → M-CSF	M-MDM → GM-CSF	GM-MDM → M-CSF	M-MDM → GM-CSF	GM-MDM → M-CSF	M-MDM → GM-CSF
CD80	Decrease	Increase	No change	No change	Increase	Increase
CD163	No change	Decrease	No change	No change	No change	No change
CD14	No change	No change	No change	No change	No change	No change
CD16	Decrease	Increase*	No change	No change	No change	Increase
CD11b	No change	No change	No change	No change	No change	No change
CD206	No change	No change	No change	No change	No change	No change
CD282	No change	Increase	No change	Increase	Decrease*	Decrease
CD120b	No change	No change	No change	No change	Decrease	No change
CD226	No change	Decrease	No change	Decrease	No change	Decrease
CD93	No change	Increase	No change	No change	No change	No change

Table 5.7: Summary of cell surface marker expression changes after environmental change for 6 days. Monocytes from non-smokers (n=4), smokers (n=3) and COPD patients (n=3) were cultured in either GM-CSF or M-CSF for 12 days generating (GM-MDM and M-MDM respectively). After differentiation cells had media switched (GM-MDM switched to M-CSF and M-MDM switched to GM-CSF) for 6 days. At day 12 of differentiation and then at day 6 post switch, MDM were stained for antibodies to identify cell surface receptor expression. Results indicate if and how % receptor expression was effected by changing environment. * $p<0.05$ day 12 *vs.* day 6 post-switch. Those highlighted in pink are thought to be highly expressed in 'M1'like macrophages compared with those expressed in blue which have been linked to 'M2'like macrophage phenotypes (Alberto Mantovani et al. 2002; Beyer et al. 2012).

Results examining receptor expression under changing environment showed that changing culture to GM-CSF media led to a slight decrease in CD163 in M-MDM from non-smoker and a slight decrease in CD80 when non-smoker GM-MDM were switched to M-CSF media. This was not seen in cells from either the smokers or COPD subjects, suggesting that the plasticity of these markers is affected by disease and may not be useful in classifying macrophage phenotype alone in COPD.

Results examining other macrophage markers also showed differences between cells from non-smokers and COPD subjects in terms of their plasticity (Table 5.7). Both CD14 MFI and percentage of cells expressing CD16 increased when media was switched to GM-CSF and decreased when switched to M-CSF in cells from non-smokers, although changes to CD14 MFI were not statistically significant and there was high sample variability. Again this was not mimicked by the cells from COPD patients or smokers and highlights the differences in plasticity between subject groups and the difficulty in using these receptors as markers of cell phenotype in disease.

5.4.4 Conclusions

Taken together these results have shown:

- Monocytes from COPD patients did not consist of a different sub-population using the markers examined herein compared with non-smokers (hypothesis 1 rejected).
- GM-MDM from non-smokers did express more CD80 and less CD163 compared with M-MDM (hypothesis 2 accepted)
- MDM from COPD patients did not express more CD80 compared with MDM from non-smokers (hypothesis 3 rejected)
- MDM from COPD patients expressed less CD163 compared with MDM from non-smokers (hypothesis 4 accepted)
- M-MDM and GM-MDM differentially express phenotypical cell surface markers. This was the case for CD14 (MFI) CD16 (%) for cells from non-smokers and also in CD282 and CD120b in cells from COPD patients (hypothesis 4 partly accepted).
- Cell surface expression was altered by changing environment. This was the case for CD16 for cells from non-smokers (hypothesis 4 partly accepted)

Since non-smoker MDM macrophages show functional plasticity that is not always reflected by changes in their cell surface markers, it is likely that using these cell surface markers may not be a good way of classifying macrophages.

Taking these data together, it is clear that while MDM cell surface markers can be a useful tool but should not be used solely to determine phenotype. They should be accompanied by functional output (such as cytokine expression or phagocytic capability) in order to provide a more holistic overview of macrophage phenotype.

CHAPTER 6 TRANSCRIPTIONAL DIFFERENCES WITHIN MDM FROM COPD PATIENTS

6.1 Introduction

It is important to consider that the cells used in this present study were isolated from peripheral blood monocytes and have therefore, not yet been exposed to the lung environment. Despite this, clear phenotypical and functional differences can be seen after 12 days in culture between MDM derived from different subject groups. However, the present study was unable to show significant differences in monocyte populations, suggesting that the differences in MDM phenotype and plasticity may derive after the monocyte to macrophage differentiation and further polarisation.

It is a possibility that the 'normal' macrophage polarisation pathways may be altered in cells from COPD patients. This process is complex and involves the action of various transcription factors within the cells which bind to their cognate DNA sequence and regulate transcription of genes which play a role in phenotype and function of macrophages.

Several transcription factors have been shown to be involved in macrophage phenotype. As previously described, STAT1, AP1, NFκB, IRF3 and IRF7 have all been implicated in macrophage pro-inflammatory gene regulation while STAT6 and c-MAF have been indicated in anti-inflammatory gene induction (Figure 6.1) (Lawrence & Natoli 2011; Natoli et al. 2011; Sica & Mantovani 2012; Baccala et al. 2007; Chawla 2010). It is important, therefore, to understand whether these transcription factors are affected by different culture environments and whether these are altered in COPD cells.

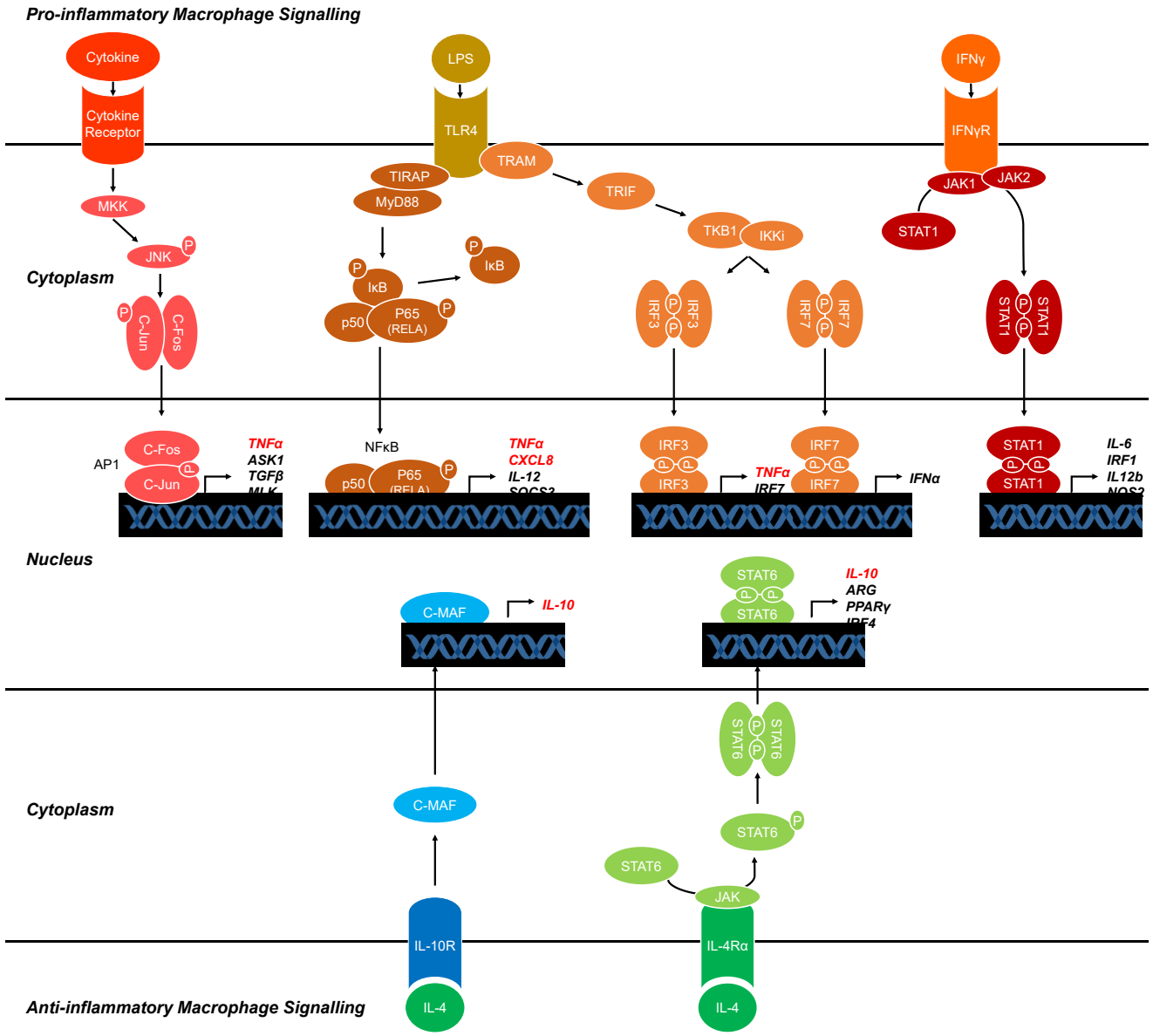


Figure 6.1: Transcriptional regulation of macrophage phenotype. Schematic diagram of transcriptional signalling in macrophages. MSK: mitogen and stress activated kinase, TLR: toll-like receptor, LPS: lipopolysaccharide, TIRAP: toll-interleukin 1 receptor domain containing adaptor protein, MyD88: myeloid differentiation primary response 88, RELA: reticuloendotheliosis homolog A, NFκB: nuclear factor-κB, TRIF: TIR-domain-containing adapter-inducing interferon-β, TRAM: TRIF-related adaptor molecule, TKB: tyrosine kinase binding, IRF: interferon-regulatory factor, STAT: signal transducer and activator of transcription, IL: interleukin, IFNγ: interferon-γ, JAK: Janus kinase, MAF: musculoaponeurotic avian fibrosarcoma, ASK: Apoptosis signal-regulating kinase, TGFβ: Transforming growth factor beta, SOCS: Suppressor of cytokine signalling, TNF: tumour necrosis factor, NOS: nitric oxide synthase, ARG: arginase, PPARγ: peroxisome proliferator-activated receptor-γ. Adapted from: (Lawrence & Natoli 2011; Natoli et al. 2011; Sica & Mantovani 2012; Baccala et al. 2007; Chawla 2010).

6.1.1 Hypotheses

GM-MDM express higher levels of pro-inflammatory-linked transcription factors and lower levels of anti-inflammatory-linked transcription factors compared with M-MDM.

MDM from COPD patients express higher levels of pro-inflammatory-linked transcription factors and lower levels of anti-inflammatory-linked transcription factors compared with MDM from non-smokers.

6.1.2 Aims

- Examine levels of pro and anti-inflammatory-linked transcription factors in GM-MDM and M-MDM from non-smokers, smokers and COPD patients.
- Analyse the affect of changing culture environment on transcription factor expression.
- Identify levels of phosphorylated transcription factors and identify differences due to cell culture conditions and whether COPD cells are altered. Use confocal microscopy to visualise transcription factor location in stimulated MDM and compare subject groups.

6.2 Methods

6.2.1 Subject Selection

Subjects were selected as in section 2.2.1. Smokers had at least a 10 pack-year history and COPD patients had an FEV_1: FVC ratio <0.7 and post-bronchodilator FEV_1 % predicted <85%.

6.2.2 qRT-PCR

Monocytes were isolated and plated as detailed in section 2.2.2, and cultured on adherent plates in the presence of 10% ($^{v}/_{v}$) FCS. Cells were differentiated for 12 days in either GM-CSF or M-CSF supplemented media, before undergoing cell culture switching as indicated in section 2.2.2.2. On day 12 of differentiation and day 6 post switch, cells were harvested for RNA and converted to cDNA as detailed in section 2.2.9. Expression of STAT1, STAT6, IRF3, IRF7, RELA, c-MAF, and JUN was then measured by qRT-PCR using TaqMan primers and probes as detailed in section 2.2.9.3. Expression was analysed relative to HPRT1 (housekeeping gene) expression and each condition had its own non-stimulated controls.

6.2.3 Confocal Microscopy

Cells were isolated as detailed in section 2.2 and plated onto 24 well plates containing a coverslip. At day 12 of differentiation, cells were stimulated for 15 min with either IFNγ, LPS or IL-4 (10ng/ml) in serum free RPMI. Cells were then fixed, washed and stained with fluorescent markers as detailed in section 2.2.10 before mounting onto slides. Images were taken using Zeiss LSM-510 inverted confocal microscope in the Facility for Imaging by Light Microscopy (FILM) at Imperial College London.

6.2.4 PhosFLOW

Cells were isolated and plated as detailed in section 2.2.2. At day 12 of differentiation, cells were stimulated for 30 min with either IFNγ, LPS or IL-4 (10ng/ml) in serum free RPMI. Cells were then washed, detached and fixed in 4% ($^{v}/_{v}$) formaldehyde. Cells were then washed, permeabilised and stained for 30 min using PhosFLOW APC-conjugated antibodies against pSTAT1 and pSTAT6 as in section 2.2.8.3. Cells were then washed, resuspended and analysed on a FACS Canto II. FCS files were analysed using FlowJo (software version 10).

6.2.5 Statistical Analysis

Comparisons between subject groups were done using Kruskall-Wallis (ANOVA) with Dunn's post-correctional tests. Comparisons between GM-MDM and M-MDM from the same patient group were done using Mann-Whitney tests with Dunn's post-correctional tests. Comparisons between time points were conducted using Wilcoxon signed rank tests. Data were expressed as mean ± SEM and analysed using GraphPad Prism software GraphPad Prism software (version 5.03). A p value of <0.05 was considered statistically significant.

6.3 Results

6.3.1 Subject Demographics

Subject characteristics and spirometry results are shown in Table 6.2. There was no significant difference in smoking history between smokers and COPD patients and no significant difference in age between all subject groups. COPD patients had significantly lower FEV_1, FEV_1 % predicted and FEV_1:FVC ratio compared with non-smokers.

	Non Smokers (n=4)	**Smokers (n=4)**	**COPD Patients (n=6)**
Age (years)	65 ± 5	59 ± 4	67 ± 3
Sex (M:F)	1:3	3:1	3:3
Smoking History (pack years)	0 ± 0	22 ± 4	34 ± 6
FEV_1 (l)	3.4 ± 0.3	2.9 ± 0.4	1.8 ± 0.2*
FEV_1 % Predicted	161 ± 19	92 ± 3	64.± 5*
FVC (l)	2.5 ± 0.2	4.3 ± 0.7	1.7 ± 0.2
FVC % Predicted	91 ± 3	93 ± 12	101 ± 6
FEV_1:FVC	0.8 ± 0.02	0.7 ± 0.03	0.5 ± 0.04*

Table 6.1: Subject demographics for MDM experiments. All participants underwent spirometry testing. FEV_1: Forced Expiratory Volume exhaled at the end of the first second of forced expiration FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for 1 year. Data are expressed as mean ± SEM. Kruskall-Wallis test with Dunn's multiple comparison test. *p<0.05 COPD *vs.* non-smokers.

6.3.2 Transcription Factor analysis by qRT-PCR

In order to study transcriptional expression in MDM, firstly, monocytes were cultured in either GM-CSF or M-CSF for 12 days and baseline expression levels of transcription factors were identified using qRT-PCR. Expression was also measured after cell culture media was switched for 6 days (GM-MDM switched to M-CSF and M-MDM switched to GM-CSF) to identify if expression of transcription factors altered when environment was changed.

6.3.2.1 *JUN*

Jun is the gene which encodes for c-Jun. Together c-Jun and c-Fos form the transcription factor, AP1, which activates pro-inflammatory gene expression in macrophages. In order to determine the affect of differentiation conditions and whether disease altered expression, *Jun* was measured in GM-MDM and M-MDM from non-smokers, smokers and COPD patients at day 12 then six days post switch (day 18).

At day 12 of differentiation there was a trend for GM-MDM from non-smokers to express higher levels of *Jun* compared with M-MDM from non-smokers, although this did not reach statistical significance (Figure 6.2A). When GM-MDM were switched to media containing M-CSF for 6 days there was a trend towards a decrease in *Jun* ($p=0.3$). Contrastingly, when M-MDM were switched to media containing GM-CSF for 6 days, there was an increase in *Jun*, albeit non-significant ($p=0.12$).

Jun expression in MDM from smokers, showed no significant difference between GM-MDM and M-MDM at day 12 (Figure 6.2B), although there was a trend for higher *Jun* expression in GM-MDM. Switching GM-MDM to media containing M-CSF reduced *Jun* expression while switching M-MDM to media containing GM-CSF increased *Jun* expression but again, this did not reach significance ($p= 0.12$). With COPD MDM, there

was a trend for *Jun* expression to be higher in M-MDM compared with GM-MDM at day 12 (not statistically significant) (Figure 6.2C). Switching M-MDM to media containing GM-CSF for 6 days reduced *Jun* expression and switching GM-MDM to media containing M-CSF increased *Jun* expression but again was not significant ($p=0.62$).

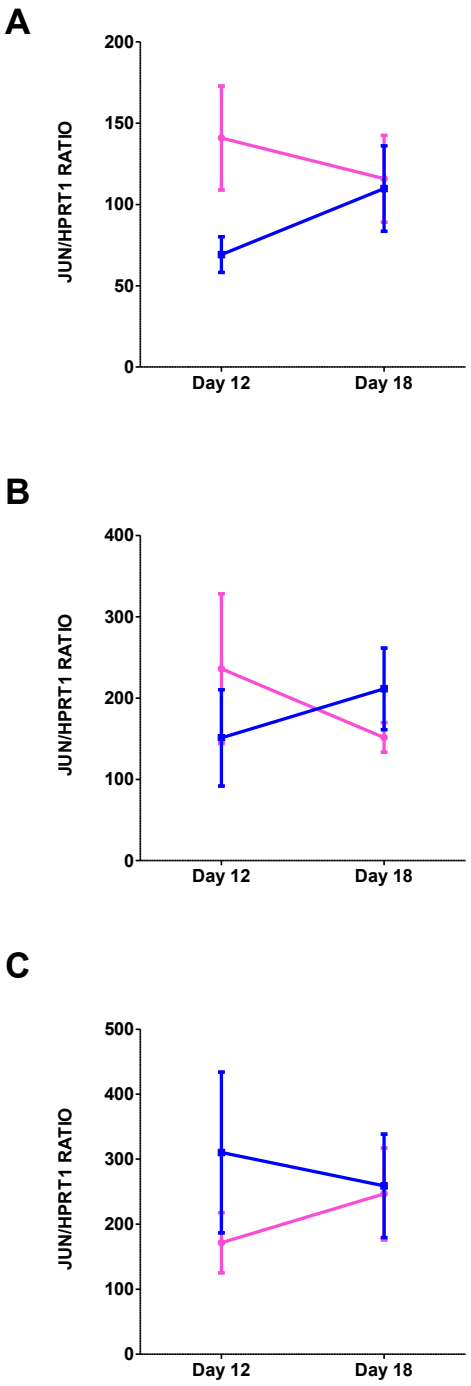


Figure 6.2: *Jun* expression in GM-MDM and M-MDM at baseline and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=4 (**B**) smokers n=4 and (**C**) COPD patients n=6, were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days. *Jun* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

Taking these data together and comparing *Jun* expression in MDM between groups, GM-MDM from all subject groups expressed similar levels of *Jun* at day 12 (Figure 6.3A) and by day 6 of being switched to media containing M-CSF, there was a trend for cells from COPD to express higher levels of *Jun* compared with non-smokers and smokers although this did not reach significance ($p=0.2$).

These data also showed that at day 12, M-MDM from COPD patients expressed significantly higher levels of *Jun* compared with non-smokers ($p<0.05$) (Figure 6.3B). When media was switched to GM-MCF for 6 days, the trend for cells from COPD patients to express higher levels of *Jun* compared with non-smokers and smokers remained consistent after 6 days, although did not reach significance ($p= 0.3$).

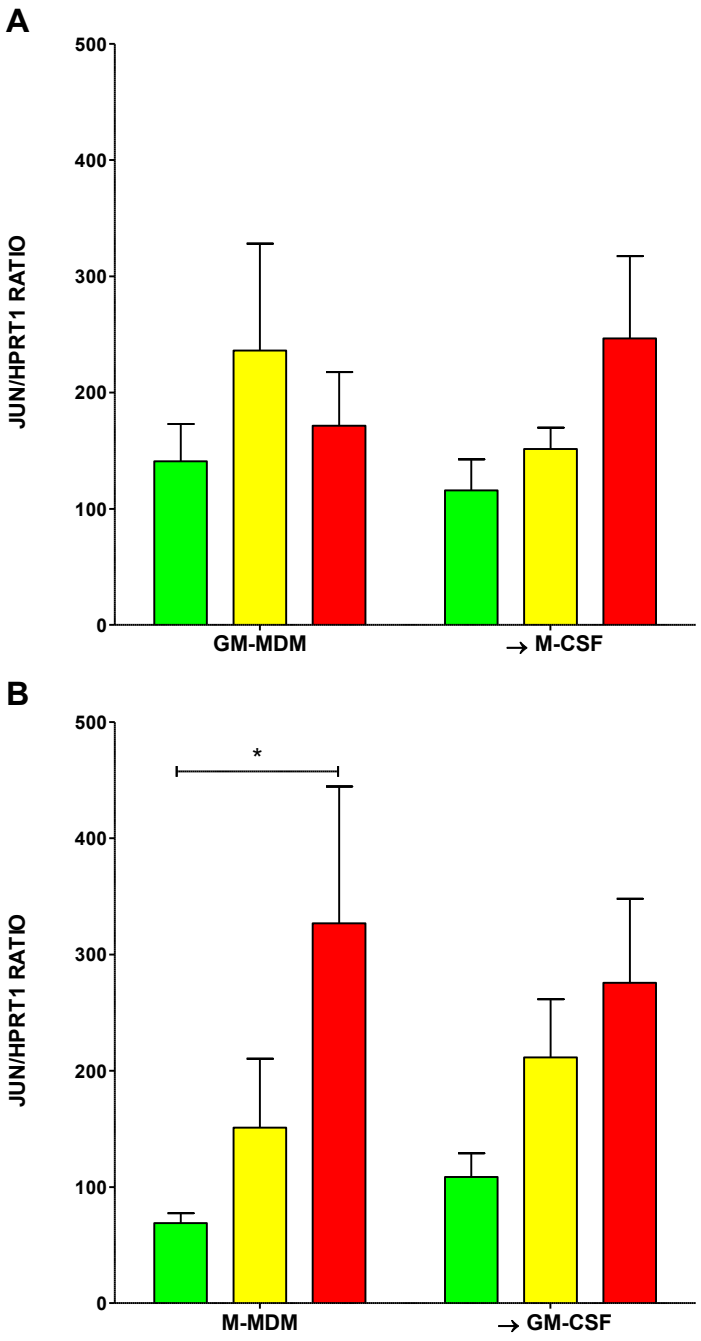


Figure 6.3: *Jun* expression in GM-MDM and M-MDM at baseline and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days. *Jun* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM. * $p<0.05$ Non-smokers *vs.* COPD.

6.3.2.2 STAT1

After showing differences in *Jun* expression in MDM from COPD, it was important to look at other transcription factors involved in macrophage phenotype to identify if these also differed in MDM from COPD patients. STAT1 protein is encoded by the gene *STAT1* and is known to regulate interferon stimulated pro-inflammatory gene expression in macrophages. *STAT1* expression was measured in GM-MDM and M-MDM from non-smokers, smokers and COPD patients at day 12 then six days post switch (day 18).

At day 12 of differentiation GM-MDM from non-smokers expressed a trend towards higher levels of *STAT1* compared with M-MDM from non-smokers, although this did not reach statistical significance (Figure 6.4A). When GM-MDM were switched to media containing M-CSF there was a decrease in *STAT1* expression by day 18 but this was not significant ($p=0.06$). When M-MDM were switched to media containing GM-CSF there was a non-significant increase in *STAT1*. There was also a trend for higher *STAT1* expression in GM-MDM from smokers at day 12 compared with M-MDM from smokers, although this did not reach statistical significance (Figure 6.4B). Switching GM-MDM to media containing M-CSF reduced *STAT1* expression while switching M-MDM to media containing GM-CSF increased *STAT1* expression but was not significant ($p=0.15$). Similarly, there was a trend for *STAT1* expression to be higher in GM-MDM from COPD patients compared with M-MDM at day 12 but this was not significant (Figure 6.4C). Switching M-MDM to media containing GM-CSF for 6 days significantly increased *STAT1* expression in cells from COPD patients ($p<0.05$) while switching GM-MDM to media containing M-CSF had no significant affect on *STAT1* expression in cells from COPD patients ($p=0.37$).

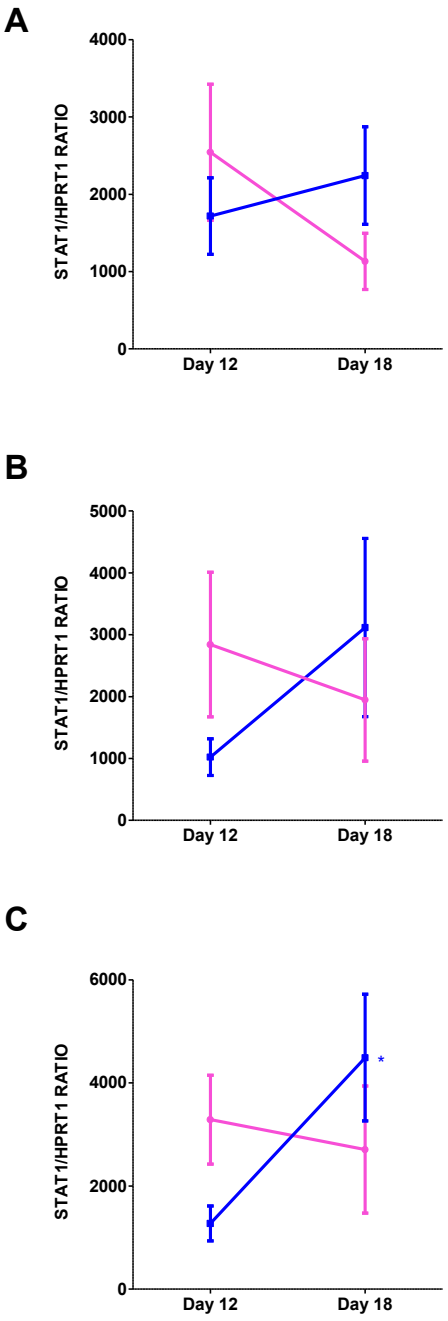


Figure 6.4: *STAT1* expression in GM-MDM and M-MDM at baseline and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=4 (**B**) smokers n=4 and (**C**) COPD patients n=6, were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days. *STAT1* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM. # $p<0.05$ GM-CSF cultured cells *vs.* M-CSF cultured cells. * $p<0.05$ *vs.* Day12.

Taking these data and comparing *STAT1* expression in MDM between groups showed there was no significant difference in *STAT1* expression in GM-MDM from non-smokers, smokers and COPD patients (Figure 6.5A). When these cells were switched to media containing M-CSF there was a trend for cells from COPD patients to express more *STAT1* compared with smokers and non-smokers, although this was not significant (p=0.3).

Again, when comparing M-MDM at day 12, there were no significant differences in *STAT1* expression between subject groups (Figure 6.5B). And when cells were switched to media containing GM-CSF there was a trend for higher *STAT1* expression in cells from COPD patients compared with smokers and non-smokers but was not significant (p=0.25).

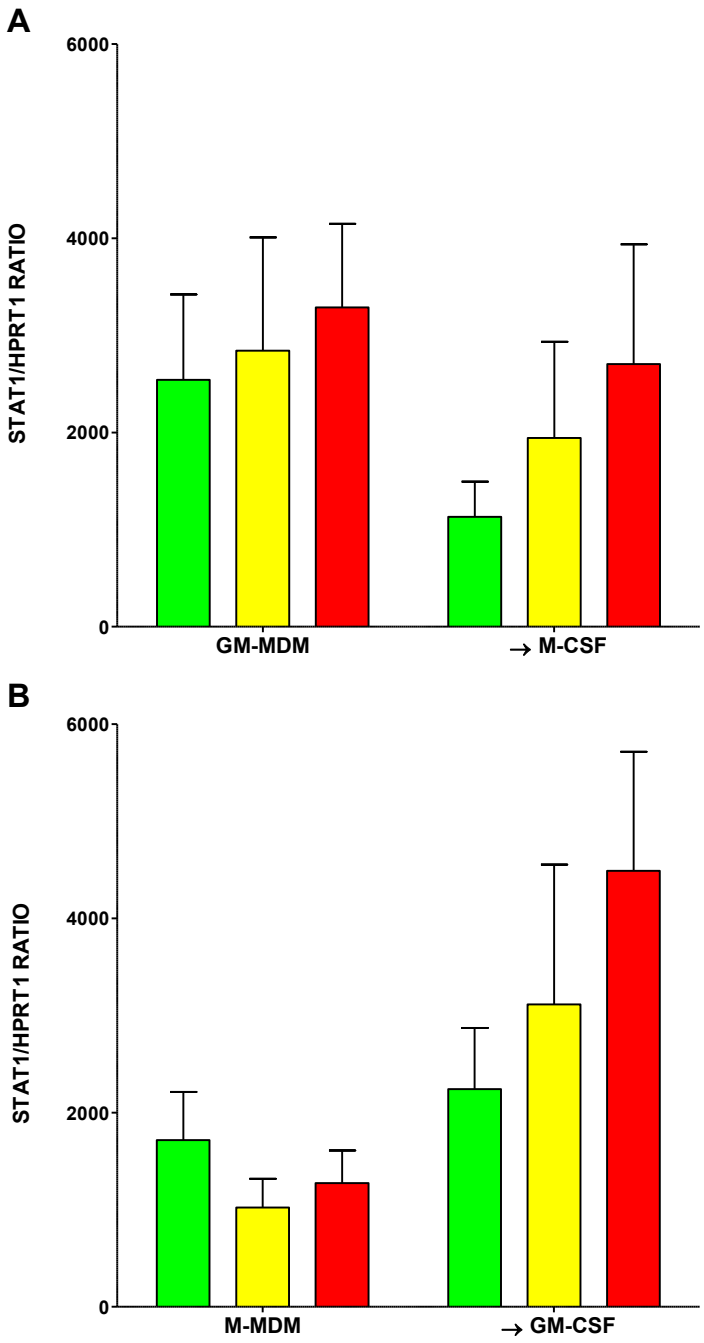


Figure 6.5: *STAT1* expression in GM-MDM and M-MDM at baseline and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days. *STAT1* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

6.3.2.3 *IRF3*

Having shown no significant difference in *STAT1* expression between subject groups it was decided to examine other pro-inflammatory transcription factor expression. The *IRF3* gene encodes for the transcription factor IRF3, which, upon phosphorylation, binds with CREB/BP to stimulate transcription of pro-inflammatory IFNα and IFNβ. *IRF3* expression was measured in GM-MDM and M-MDM from non-smokers, smokers and COPD patients at day 12 then six days post switch (day 18).

At day 12 of differentiation there was no significant difference in *IRF3* expression between GM-MDM and M-MDM from non-smokers (Figure 6.6A). When GM-MDM were switched to media containing M-CSF there was a non-significant decrease in *IRF3* expression by day 18 ($p=0.37$). When M-MDM were switched to media containing GM-CSF there was a trend towards increase in *IRF3* which did not reach statistical significance ($p=0.1$). Examining cells from smokers showed a trend for higher *IRF3* expression in GM-MDM at day 12 compared with M-MDM but was not significant (Figure 6.6B). Switching GM-MDM to media containing M-CSF had a trend to reduce *IRF3* expression but was not significant ($p=0.3$), while switching M-MDM to media containing GM-CSF increased *IRF3* expression but again this was not significant ($p=0.32$). When examining the results from COPD MDM, there was a trend for *IRF3* expression to be higher in M-MDM from COPD patients compared with GM-MDM at day 12, although this did not reach statistical significance (Figure 6.6C). However, conversely to cells from non-smokers and smokers, switching media for 6 days, (both in M-MDM switched to GM-CSF and GM-MDM switched to M-CSF) decreased *IRF3* expression, although again this was not significant compared with day 12 cells ($p=0.31$).

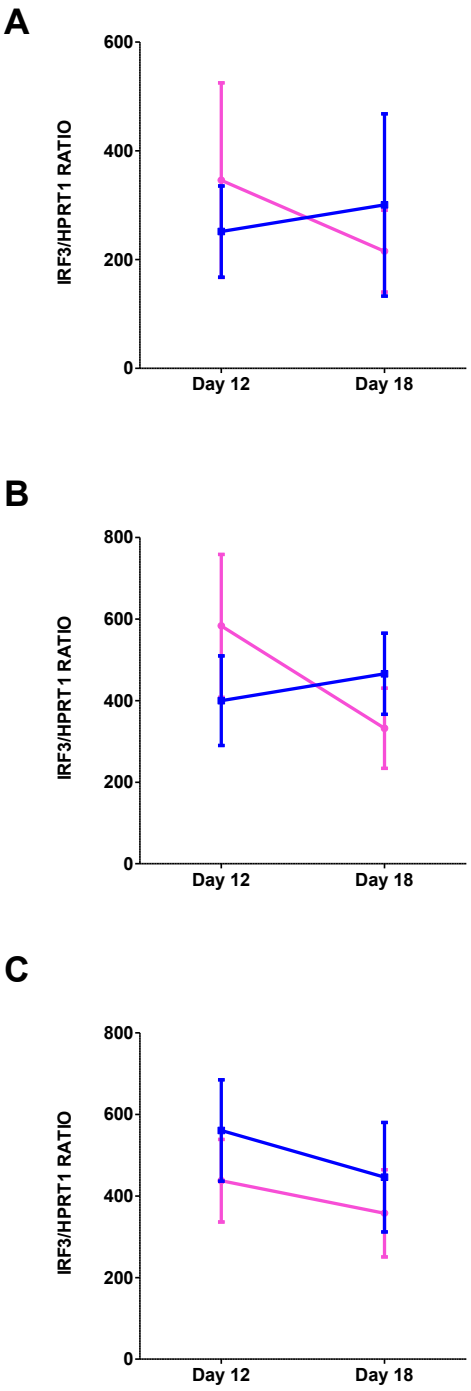


Figure 6.6: *IRF3* expression in GM-MDM and M-MDM at baseline and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=4 (**B**) smokers n=4 and (**C**) COPD patients n=6, were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days. *IRF3* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

Taking these data and comparing *IRF3* expression in MDM between groups showed there was no significant difference in *IRF3* expression in GM-MDM from non-smokers, smokers and COPD patients although there was a trend for higher *IRF3* expression in GM-MDM from smokers (Figure 6.7A). When these cells were switched to media containing M-CSF there was a trend for cells from COPD patients and smokers to express more *IRF3* compared non-smokers, although this was not significant (p=0.7).

When comparing M-MDM at day 12, there was a trend for higher *IRF3* expression in M-MDM from COPD patients compared with smokers and non-smokers but was not significant (Figure 6.7A). When M-MDM were switched to media containing GM-CSF there was a trend for higher *IRF3* expression in cells from COPD patients and smokers compared with non-smokers, although this was not statistically significant (p=0.9).

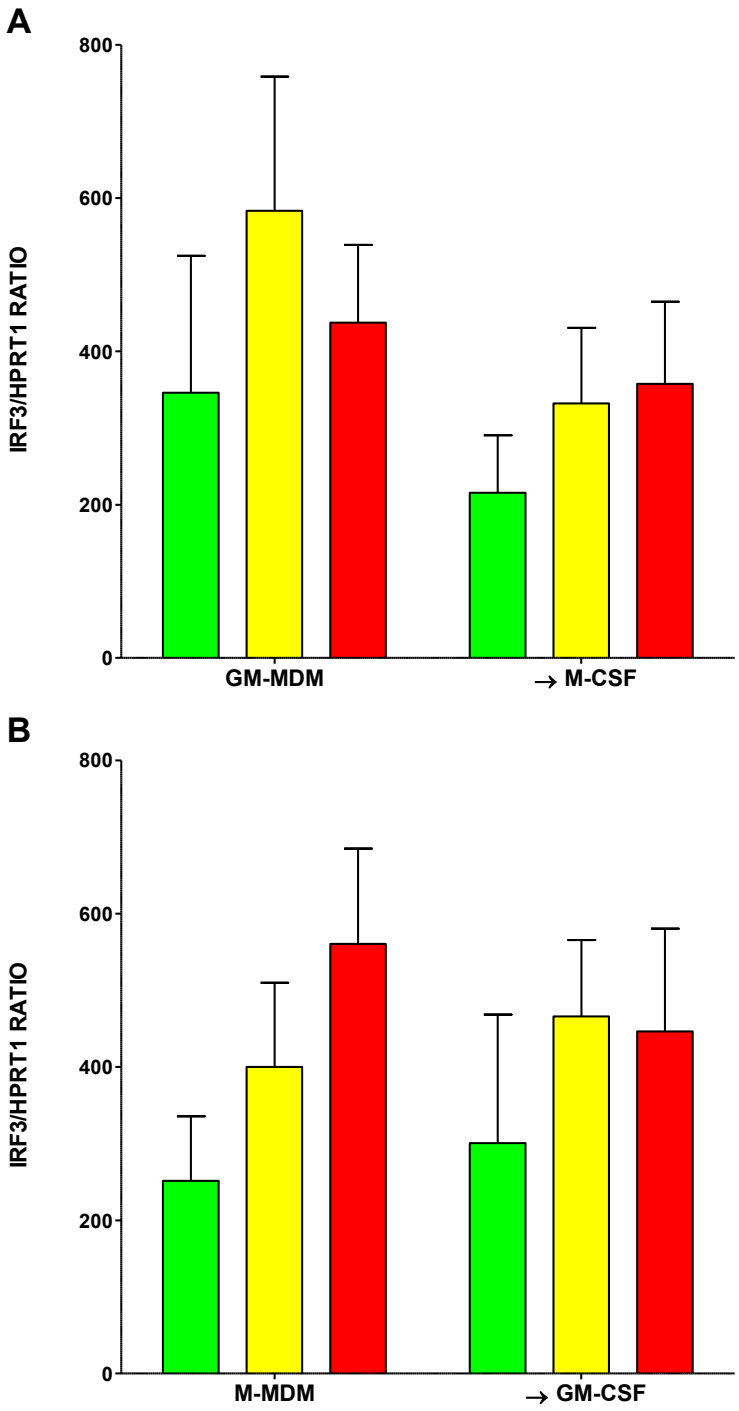


Figure 6.7: *IRF3* expression in GM-MDM and M-MDM at baseline and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days. *IRF3* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

6.3.2.4 *IRF7*

Having shown limited differences in IRF3 expression in cells from COPD patients during environmental change, it was decided to examine another of the interferon regulatory factor genes, IRF7. The *IRF7* gene encodes for the transcription factor IRF7 which is important in virus-induced, pro-inflammatory interferon signalling. *IRF7* expression was measured in GM-MDM and M-MDM from non-smokers, smokers and COPD patients at day 12 then six days post switch (day 18).

At day 12 of differentiation GM-MDM from non-smokers expressed significantly higher levels of *IRF7* compared with M-MDM from non-smokers (Figure 6.8A) ($p<0.05$). When non-smoker GM-MDM were switched to media containing M-CSF there was a decrease in *IRF7* expression by day 18 but this was not significant ($p=0.12$), while switching M-MDM to GM-CSF caused a significant increase in *IRF7* by day 18 ($p<0.05$) indicating the plasticity in these cells reacting to changes in their culture environment. Examining this response in cells from smokers, there was a similar trend for higher *IRF7* expression in GM-MDM at day 12 compared with M-MDM from smokers but this was not statistically significant (Figure 6.8B). Switching GM-MDM to media containing M-CSF showed a trend to reduced *IRF7* expression ($p=0.2$) while switching M-MDM to media containing GM-CSF did not significantly affect *IRF7* expression in these cells ($p=0.7$). When examining the results from COPD MDM, there was no significant difference in *IRF7* expression between GM-MDM and M-MDM at day 12 (Figure 6.8C). There was also no significant change in *IRF7* expression from COPD MDM when media was switched to either GM-CSF or M-CSF ($p=0.7$).

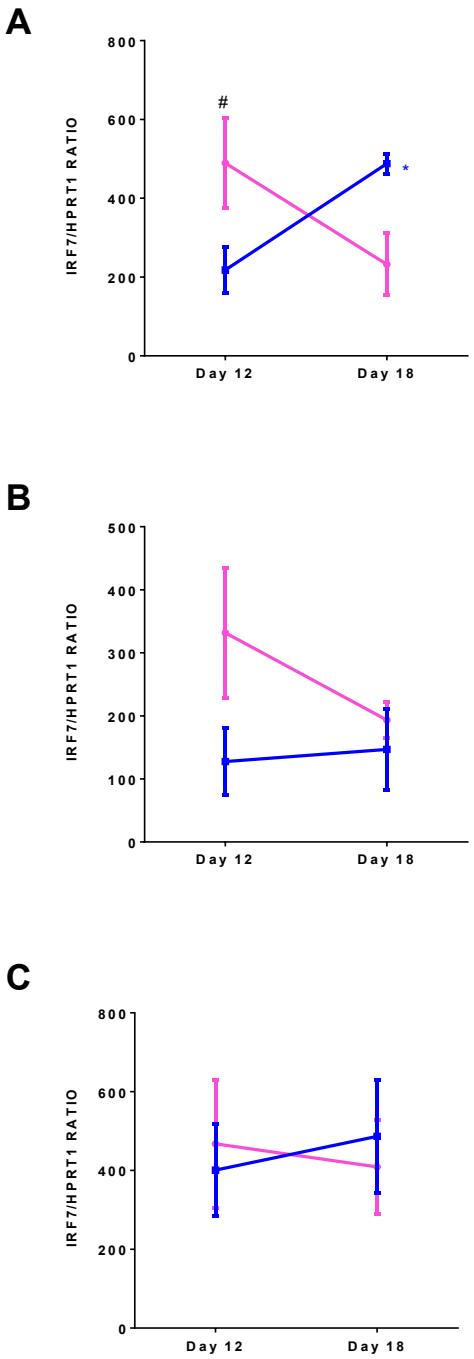


Figure 6.8: *IRF7* expression in GM-MDM and M-MDM at baseline and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=4 (**B**) smokers n=4 and (**C**) COPD patients n=6, were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days. *IRF7* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM. # p<0.05 GM-CSF cultured cells *vs.* M-CSF cultured cells. * p<0.05 *vs.* Day12.

Taking these data and comparing *IRF7* expression in MDM between groups showed a non-significant trend for higher *IRF7* expression in GM-MDM from COPD patients compared with non-smokers and smokers (Figure 6.9A). When these cells were switched to media containing M-CSF, this trend was still evident after 6 days, although this was not statistically significant (p=0.3).

When comparing M-MDM at day 12, there was again a trend for higher *IRF7* expression in M-MDM from COPD patients compared with smokers and non-smokers (Figure 6.9A). When M-MDM were switched to media containing GM-CSF there was a trend for lower *IRF7* expression in cells from smokers compared with non-smokers and COPD patients, although this was not statistically significant.

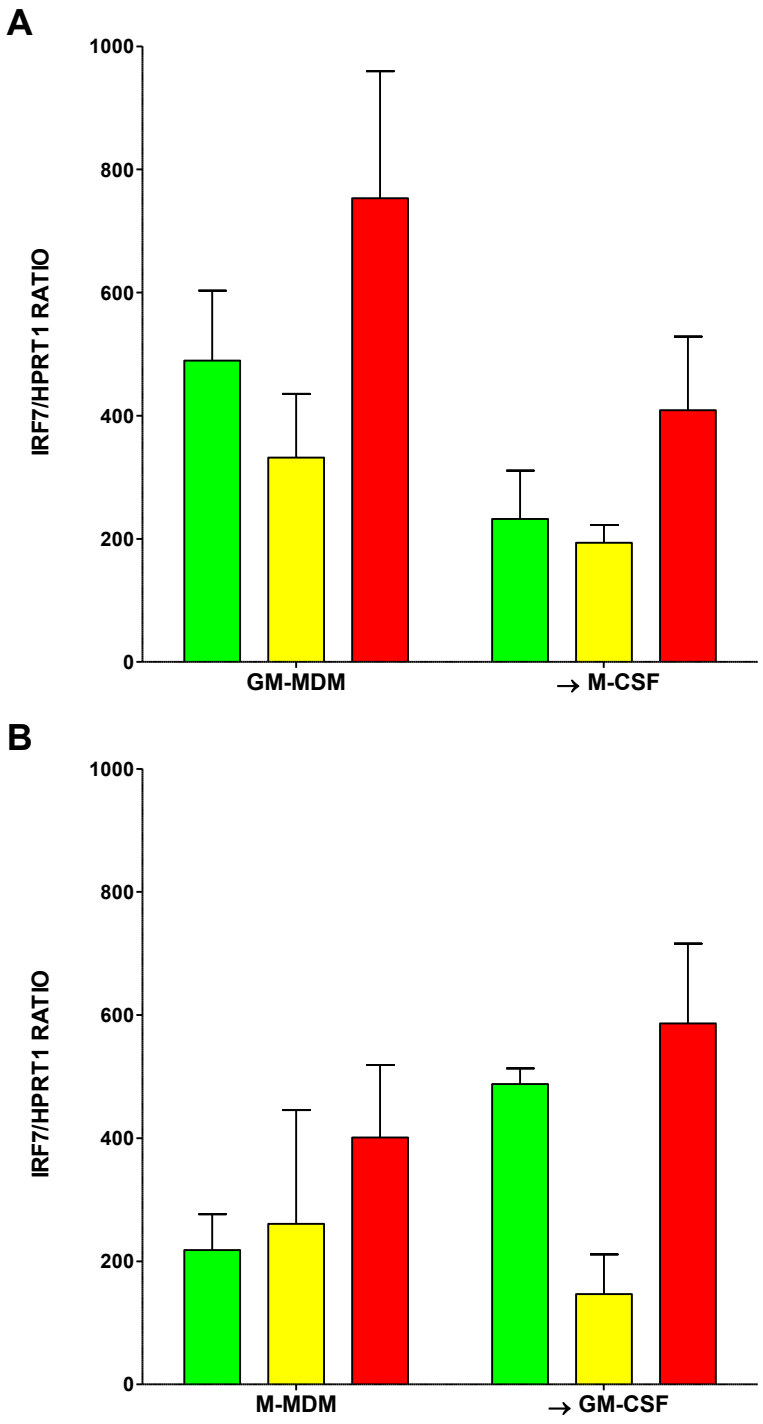


Figure 6.9: *IRF7* expression in GM-MDM and M-MDM at baseline and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days. *IRF7* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

6.3.2.5 *RELA*

RELA is the gene which encodes for the p65 sub unit of NFκB. Together with p50 the p65/p50 NFκB complex triggers up-regulation of inflammatory genes. *RELA* expression was measured in GM-MDM and M-MDM from non-smokers, smokers and COPD patients at day 12 then six days post switch (day 18).

At day 12 of differentiation GM-MDM from non-smokers expressed similar levels of *RELA* compared with M-MDM from non-smokers (Figure 6.10A) with a trend towards higher expression in GM-MDM. When GM-MDM were switched to media containing M-CSF there was a non-significant decrease in *RELA* expression by day 18 (p=0.4) while switching to media containing GM-CSF caused a non-significant increase in *RELA* by day 18 (p=0.6). Examining the results from smokers showed no significant difference in *RELA* expression in GM-MDM compared with M-MDM at day 12 (Figure 6.10B). Switching media in MDM from smokers for 6 days, both in M-MDM switched to GM-CSF, and GM-MDM switched to M-CSF, increased *IRF7* expression, although again this was not significant compared with day 12 cells (p=0.15). Going on to examine *RELA* expression in cells from COPD patients, results showed a trend for higher *RELA* expression in M-MDM compared with GM-MDM at day 12 (Figure 6.10C), although this did not reach statistical significance. Switching M-MDM to media containing GM-CSF for 6 days showed a non-significant decrease in *RELA* expression in cells from COPD patients (p=0.9), while switching GM-MDM to media containing M-CSF did not significantly change *RELA* expression compared with day 12 cells p=0.1).

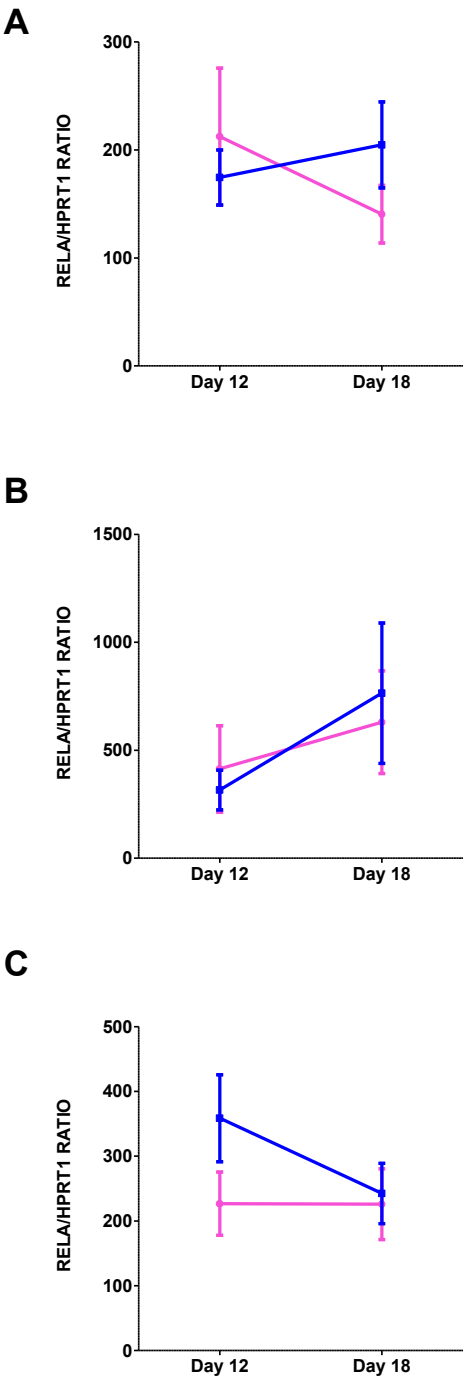


Figure 6.10: *RELA* expression in GM-MDM and M-MDM at baseline and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=4 (**B**) smokers n=4 and (**C**) COPD patients n=6, were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days. *RELA* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

Taking these data and comparing *RELA* expression in MDM between groups showed a non-significant, trend for higher *RELA* expression in GM-MDM from smokers at day 12 (Figure 6.11A). When these cells were switched to media containing M-CSF there was still a trend for cells from smokers to express more *RELA* compared with non-smokers and COPD patients, although, again, this was not statistically significant (P value 0.4)

When comparing M-MDM at day 12, there was a trend for higher *RELA* expression in M-MDM from COPD patients and smokers compared with non-smokers, although this did not reach statistical significance (Figure 6.11B). When M-MDM were switched to media containing GM-CSF there was a trend for higher *RELA* expression in cells from smokers compared with non-smokers and COPD patients, although this was not statistically significant (p=0.5).

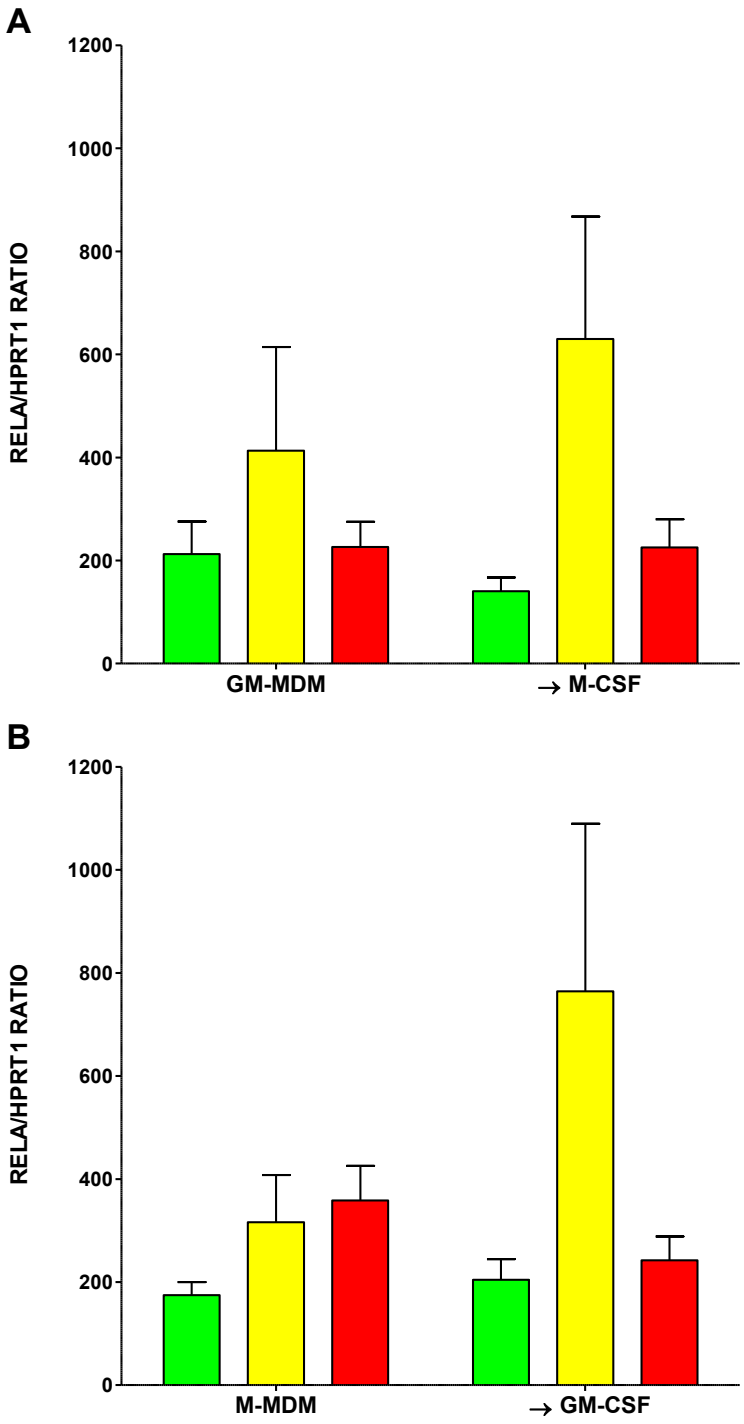


Figure 6.11: *RELA* expression in GM-MDM and M-MDM at baseline and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days. *RELA* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

6.3.2.6 *STAT6*

STAT6 protein is encoded by the gene *STAT6* and is known to regulate IL-4 mediated anti-inflammatory gene expression in macrophages. *STAT6* expression was measured in GM-MDM and M-MDM from non-smokers, smokers and COPD patients at day 12 then six days post switch (day 18).

At day 12 of differentiation M-MDM from non-smokers expressed higher levels of *STAT6* compared with GM-MDM from non-smokers, although this did not reach statistical significance (Figure 6.12A). Switching M-MDM to media containing GM-CSF decreased *STAT6* expression by day 18 but this was not significant (p=0.06), while switching to M-CSF led to a trend towards an increase in *STAT6* expression although this did not reach statistical significance (p=0.06). When examining cells from smokers, there was a trend for higher *STAT6* expression in M-MDM compared with GM-MDM at day 12 which did not reach significance (Figure 6.12B). Switching M-MDM to media containing GM-CSF slightly reduced *STAT6* expression but this was not significant (p=0.6), while switching GM-MDM to media containing M-CSF appeared to increase *STAT6* expression by day 18 but was not significant (p=0.09). Finally, looking at results from COPD patients showed a non-significant trend for higher *STAT6* expression in M-MDM compared with GM-MDM at day 12 (Figure 6.12C). Switching M-MDM to media containing GM-CSF for 6 days appeared to decrease *STAT6* expression in cells from COPD patients but was not significant (P value 0.07), while switching GM-MDM to media containing M-CSF showed a trend to increase *STAT6* expression but was not significant (P value 0.7).

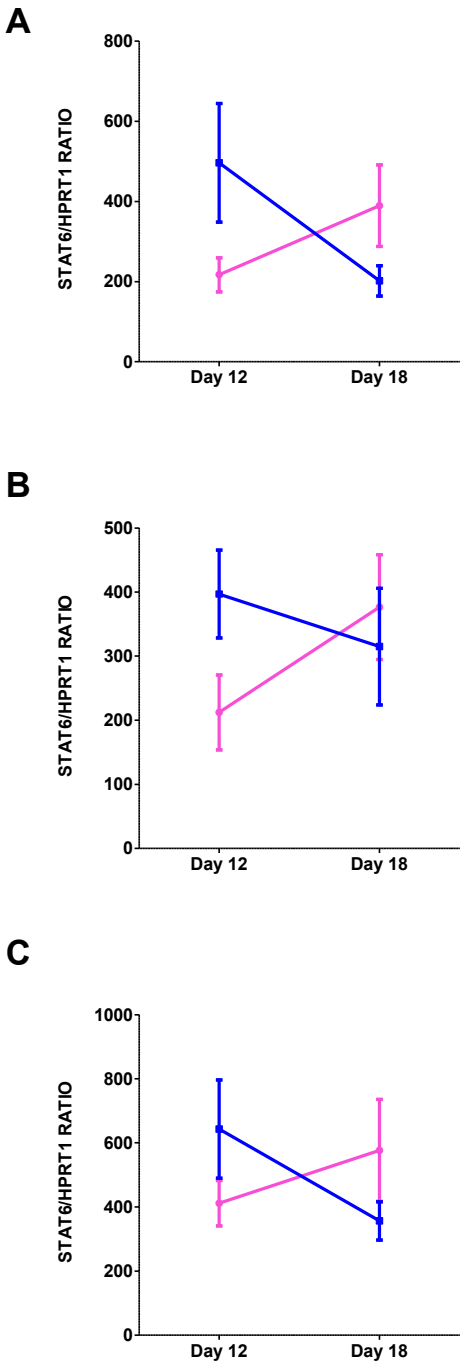


Figure 6.12: *STAT6* expression in GM-MDM and M-MDM at baseline and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=4 (**B**) smokers n=4 and (**C**) COPD patients n=6, were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days. *STAT6* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

Taking these data and comparing *STAT6* expression in MDM between groups showed a trend for higher *STAT6* expression in GM-MDM from COPD patients compared with non-smokers and smokers, although this did not reach statistical significance (Figure 6.13A). When these cells were switched to media containing M-CSF there was again a trend for cells from COPD patients to express more *STAT6* compared with non-smokers and COPD patients, although this was not significant (p=0.8)

When comparing M-MDM at day 12, there was a trend for higher *STAT6* expression in M-MDM from COPD patients compared with non-smokers and smokers but was not significant (Figure 6.13B). When M-MDM were switched to media containing GM-CSF there was a trend for higher *STAT6* expression in cells from COPD patients compared with non-smokers and smokers, although this was not statistically significant (p=0.7)

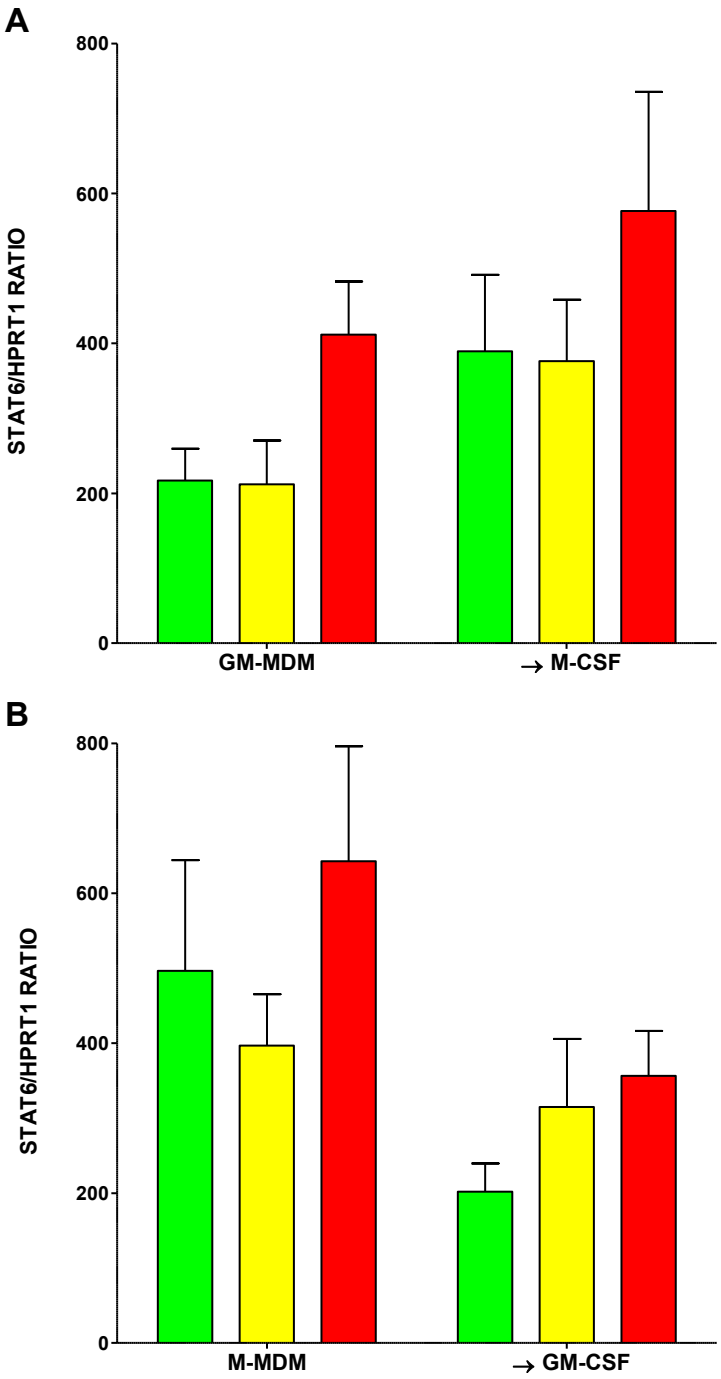


Figure 6.13: *STAT6* expression in GM-MDM and M-MDM at baseline and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days. *STAT6* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

6.3.2.7 *MAF*

Having shown no significant difference in *STAT6* expression between subject groups, it was decided to examine another anti-inflammatory linked transcription factor, c-Maf. c-Maf is encoded by the gene *MAF* and has been shown to regulate anti-inflammatory IL-10 gene expression in macrophages. *MAF* expression was measured in GM-MDM and M-MDM from non-smokers, smokers and COPD patients at day 12 then six days post switch (day 18).

At day 12 of differentiation there was a trend for M-MDM from non-smokers to express higher levels of *MAF* compared with GM-MDM from non-smokers, although this did not reach statistical significance (Figure 6.14A). Switching M-MDM to media containing GM-CSF led to a small decrease in *MAF* expression by day 18 which was not statistically significant ($p=0.3$) while switching GM-MDM to media containing M-CSF caused trend towards an increase in *MAF* by day 18 ($p=0.06$). Examining *MAF* expression in cells from smokers showed significantly higher expression in M-MDM compared with GM-MDM at day 12 ($p<0.05$) (Figure 6.14B). Switching M-MDM to media containing GM-CSF reduced *MAF* expression but was not significant ($p=0.3$), while switching GM-MDM to media containing M-CSF caused a small, con-significant increase in *MAF* expression by day 18 ($p=0.1$). Finally, examining results from COPD MDM showed no significant difference in *MAF* expression when comparing GM-MDM and M-MDM at day 12 (Figure 6.14C). Conversely to cells from non-smokers and smokers, there was no significant change to *MAF* expression when media was switched both in M-MDM switched to GM-CSF and GM-MDM switched to M-CSF ($p=1$).

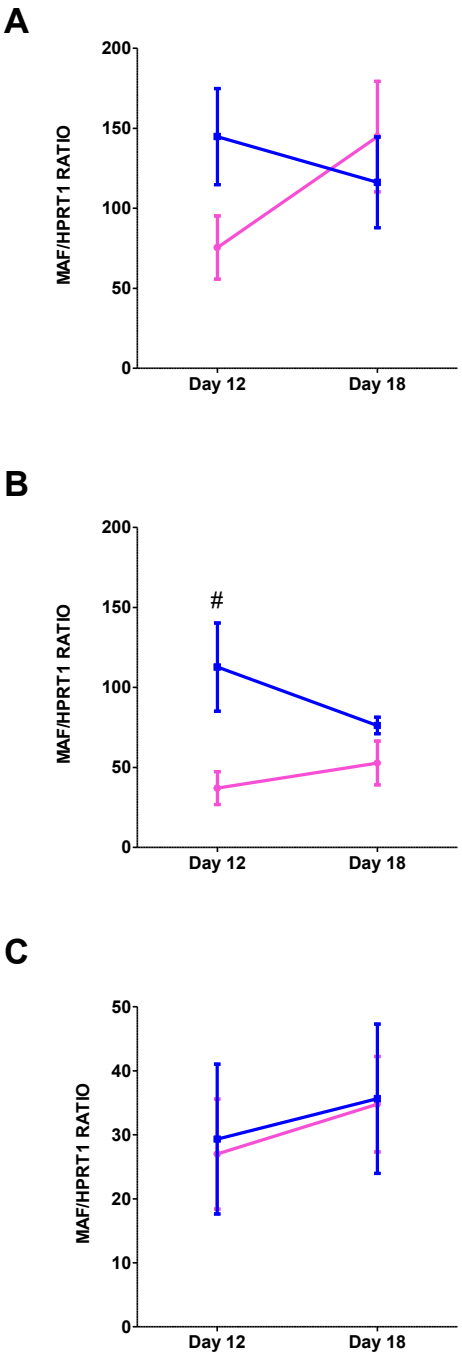


Figure 6.14: *MAF* expression in GM-MDM and M-MDM at baseline and during environmental switch (by patient type). Monocytes from (**A**) non-smokers n=4 (**B**) smokers n=4 and (**C**) COPD patients n=6, were cultured in either GM-CSF for 12 days then switched to M-CSF (pink) or M-CSF for 12 days then switched to GM-CSF (blue) for 6 days. *MAF* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM. # $p<0.05$ GM-CSF cultured cells *vs.* M-CSF cultured cells.

Taking these data and comparing *MAF* expression in MDM between groups showed a trend for higher *MAF* expression in GM-MDM from non-smokers compared with COPD patients and smokers but this was not statistically significant (Figure 6.15A). When these cells were switched to media containing M-CSF there was an increase in *MAF* expression in non-smokers which was not mimicked to the same extent by smokers or COPD patients, which expressed lower levels of *MAF* by day 18, although this did not reach statistical significance (p=0.06).

When comparing M-MDM at day 12, there was a trend for higher *MAF* expression in M-MDM from non-smokers compared with COPD patients and smokers but this was not significant (Figure 6.15B). When M-MDM were switched to media containing GM-CSF this trend continued, with higher *MAF* expression in M-MDM from non-smokers compared with COPD patients and smokers but was not statistically significant (p=0.08).

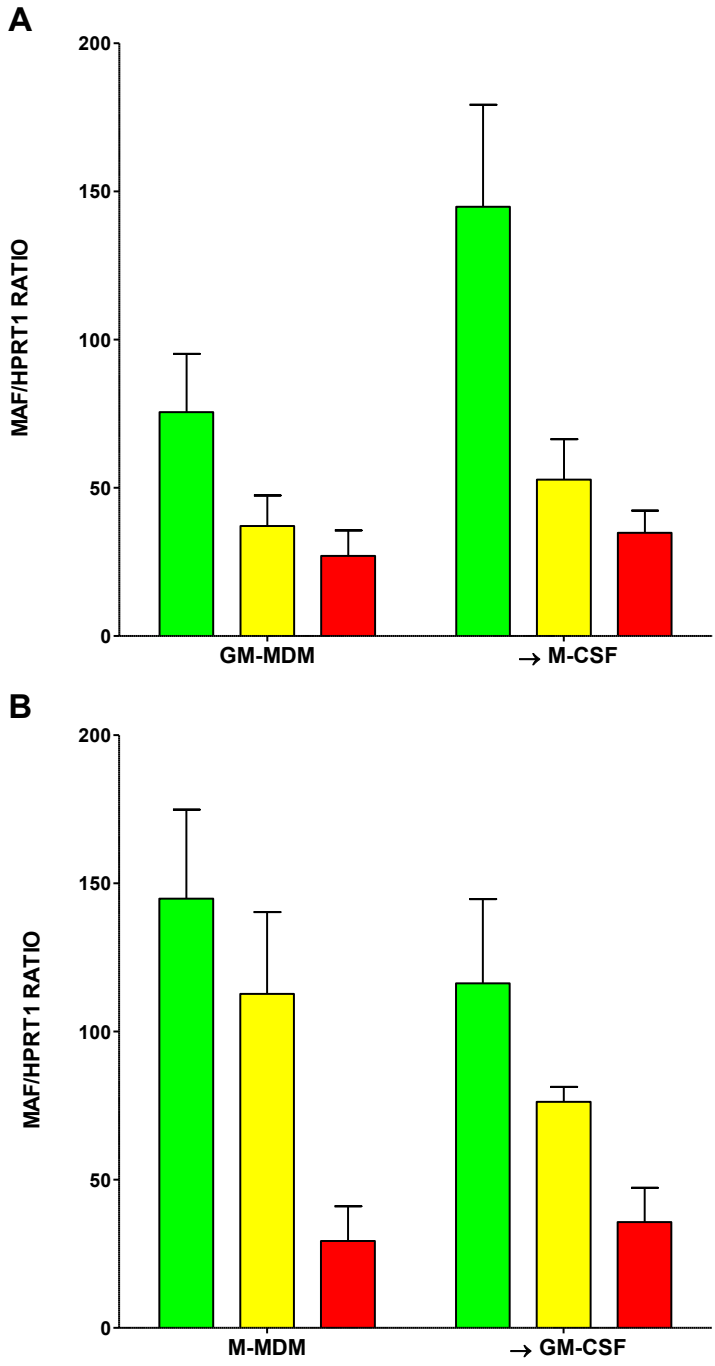


Figure 6.15: *MAF* expression in GM-MDM and M-MDM at baseline and during environmental switch. Monocytes from non-smokers (green) n=7 smokers (yellow) n=7 and COPD patients (red) n=6 were cultured in either (**A**) GM-CSF for 12 days then switched to M-CSF or (**B**) M-CSF for 12 days then switched to GM-CSF for 6 days with measurements on days 2, 4 and 6 post-switch. *MAF* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

6.3.2.8 Lung Tissue Macrophages

Having shown some differences in transcriptional expression in MDM it was important to compare results to that of lung tissue macrophages. Lung tissue macrophages were isolated as in section 2.2.3.

Macrophages were pooled and transcription factor expression was analysed by qRT-PCR as in section 2.2.9.

6.3.2.8.1 Subject Demographics

Subject characteristics and spirometry results are shown in Table 6.2. There was no significant difference in smoking history between smokers and COPD patients and no significant difference in age between all subject groups. COPD patients had significantly lower FEV_1, FEV_1 % predicted and FEV_1:FVC ratio compared with non-smokers.

	Non Smokers (n=4)	**Smokers (n=4)**	**COPD Patients (n=7)**
Age (years)	58 ± 10	65 ± 8	75 ± 2
Sex (M:F)	2:2	3:1	4:3
Smoking History (pack years)	0 ± 0	38 ± 7	35 ± 5
FEV_1 (l)	2.5 ± 0.3	2.5 ± 0.3	1.8 ± 0.2*
FEV_1 % Predicted	103 ± 14	90 ± 10	77.± 10*
FVC (l)	3.2 ± 0.3	3.2 ± 0.4	3.6 ± 0.5
FVC % Predicted	127 ± 6	102 ± 36	107 ± 15
FEV_1:FVC	0.8 ± 0.05	0.8 ± 0.03	0.5 ± 0.05*

Table 6.2: Subject demographics for lung tissue experiments. All participants underwent spirometry testing. FEV_1: Forced Expiratory Volume exhaled at the end of the first second of forced expiration FVC: Forced Vital Capacity. 1 pack year: 20 cigarettes a day for 1 year. Data are expressed as mean ± SEM. Kruskall-Wallis test with Dunn's multiple comparison test. * $p<0.05$ (COPD *vs.* non-smokers).

6.3.2.8.2 Tissue transcription factor expression

There was no significant difference in lung tissue macrophage expression of *STAT1* (*STAT1*:*HPRT1* - NS: 342 ± 138, S: 330 ± 41, COPD: 276 ± 65), *IRF3* (*IRF3*:*HPRT1* - NS: 281 ± 25, S: 230 ± 14, COPD: 244 ± 21) or *Jun* (*Jun*:*HPRT1* - NS: 435 ± 73, S: 330 ± 40, COPD: 365 ± 20) between subject groups (Fig. 6. 16).

There was a trend for *RELA* expression to be higher in non-smoker lung macrophages compared with smokers and COPD (*RELA*:*HPRT1* - NS: 467 ± 120, S: 226 ± 50, COPD: 392 ± 25) although this was not statistically significant (Fig. 6.16).

There was also a trend for higher *STAT6* expression in non-smoker lung tissue macrophages compared with lung tissue macrophages from smokers and COPD patients (*STAT6*:*HPRT1* - NS: 440 ± 141, S: 270 ± 25, COPD: 340 ± 18) although again, this was not statistically significant. (Fig. 6.16).

MAF expression was also higher in non-smoker lung tissue macrophages compared with cells from COPD patients (*MAF*:*HPRT1* - NS: 101 ± 6, S: 126 ± 14, COPD: 47 ± 8), although again, this was not statistically significant (P value 0.07) (Fig. 6.16).

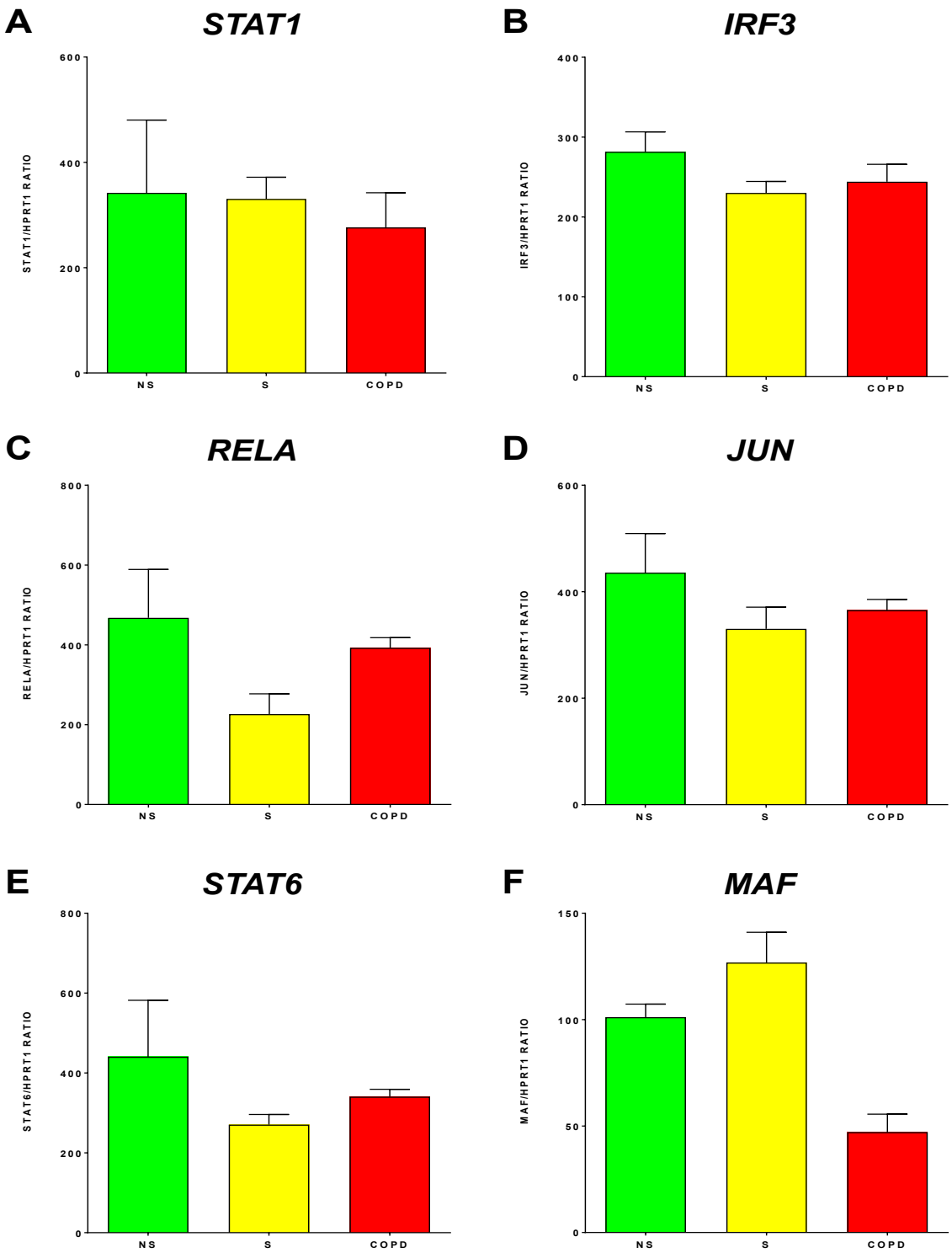


Figure 6.16: Transcription factor expression in lung tissue macrophages. Macrophages from non-smokers (green) n=4 smokers (yellow) n=4 and COPD patients (red) n=7 were isolated from resected lung tissue. *STAT1*, *IRF3*, *RELA*, *Jun*, *STAT6* and *MAF* expression was measured by qRT-PCR. Data expressed as a ratio of the housekeeping HPRT1 gene. Data are mean ± SEM.

6.3.3 Confocal Microscopy

Having shown via qRT-PCR that some transcription factor expression was altered in MDM from COPD patients, it was important to confirm whether these differences were translated to protein expression. It was also important to understand if and how transcription factor expression could be altered by stimulation. Confocal microscopy experiments were devised to examine the affects of IFNγ, LPS and IL-4 on STAT1, STAT6, IRF3 and MAF expression in cells from a non-smoker and COPD patient. Cells were cultured, stimulated, fixed and stained using fluorescent markers to identify nucleus (DAPI blue) and the transcription factor of interest (red) as detailed in section 2.2.10.

6.3.3.1 STAT1

As previous qRT-PCR experiments showed no significant differences in *STAT1* expression between subject groups, it was important to identify any differences at protein level and examine the affects of various stimuli on both expression and cellular location. Non-stimulated MDM expressed STAT1 in the cytoplasm and there were no discernible differences in GM-MDM and M-MDM but the COPD MDM appeared to have higher baseline expression of cytoplasmic STAT1 compared with non-smoker MDM (Figure 6.17). When cells were stimulated with IFNγ there was clear nuclear translocation of STAT1 in both GM-MDM and M-MDM from both subjects; more so in the GM-MDM compared with the M-MDM. LPS stimulation again led to translocation of STAT1 to the nucleus in non-smoker MDM. This contrasted to the cells from the COPD patient which showed more STAT1 translocation in GM-MDM compared with M-MDM. IL-4 stimulation caused some nuclear translocation of STAT1 but only in non-

smoker M-MDM. GM-MDM from both subject groups showed only baseline levels of cytoplasmic STAT1.

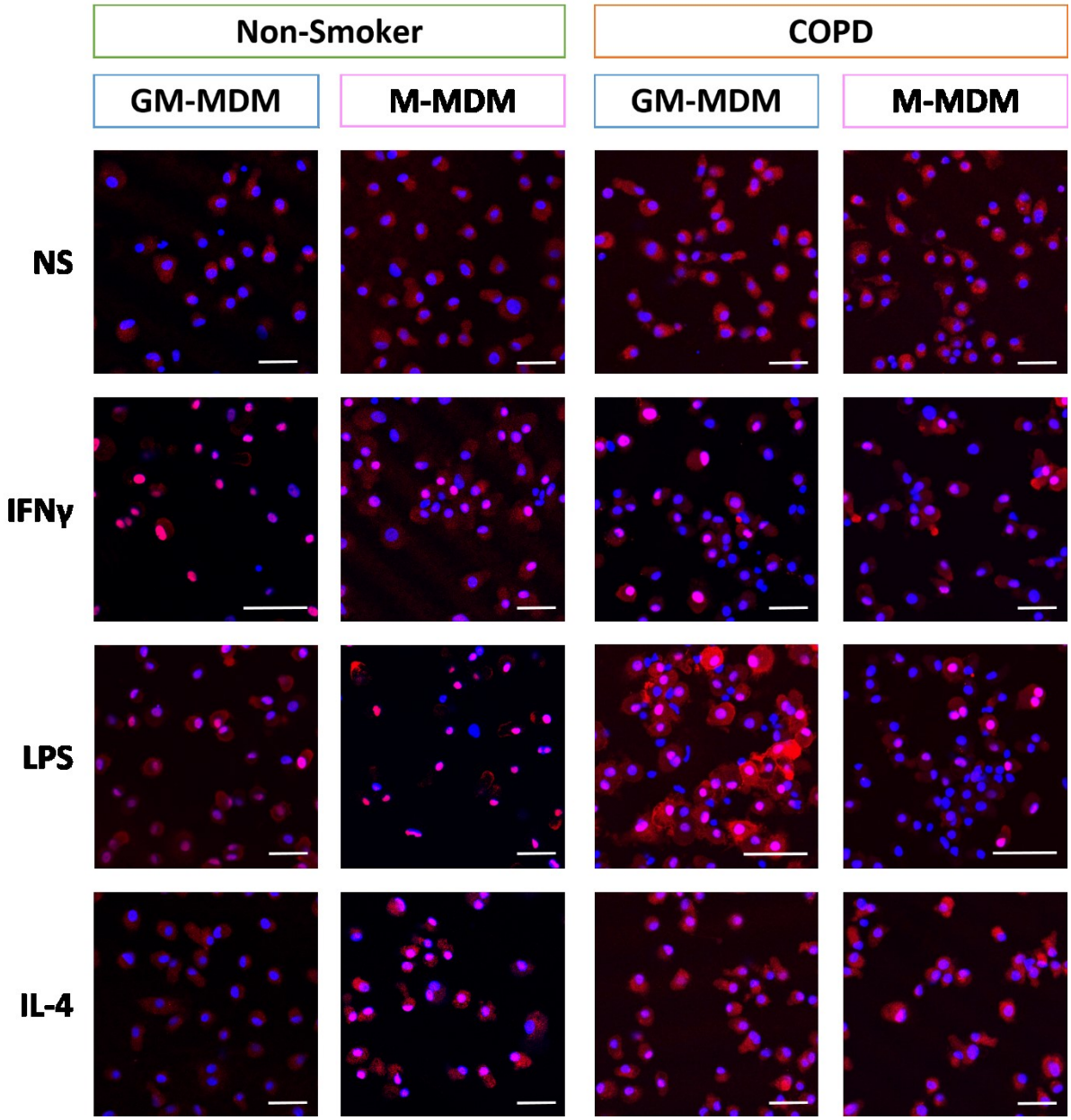


Figure 6.17: STAT1 confocal microscopy of GM-MDM and M-MDM from a non-smoker and COPD patient. Representative photographs taken of GM-MDM or M-MDM from a non-smoker (left) and COPD patient (right). Cells were non-stimulated (NS) or stimulated with either 10ng/ml IFNγ, LPS or IL-4 for 10min then fixed and stained with nuclear DAPI (blue) and pSTAT1 (red) before visualisation on a confocal microscope. Scale bar = 50μm.

6.3.3.2 IRF3

Having shown in qRT-PCR experiments that IRF3 expression in cells from COPD patients differed from non-smokers, it was important to identify if these differences existed at the protein level.

GM-MDM and M-MDM from both subjects expressed IRF3 in the cytoplasm (Figure 6.18). There were no discernible differences when comparing GM-MDM and M-MDM in cells from the non-smoker. Again, MDM from the COPD patient appeared to have higher baseline expression of cytoplasmic IRF3 compared with MDM from the non-smoker.

Cells stimulated with IFNγ showed nuclear translocation of IRF3 in both GM-MDM and M-MDM from both subjects; more so in the M-MDM compared with the GM-MDM. LPS stimulation also led to translocation of IRF3 to the nucleus in GM-MDM and M-MDM from the non-smoker and GM-MDM from the COPD patient only. IL-4 stimulation had no affect on IRF3 expression or location in GM-MDM from either subject but appeared to increase cytoplasmic expression in M-MDM from the non-smoker.

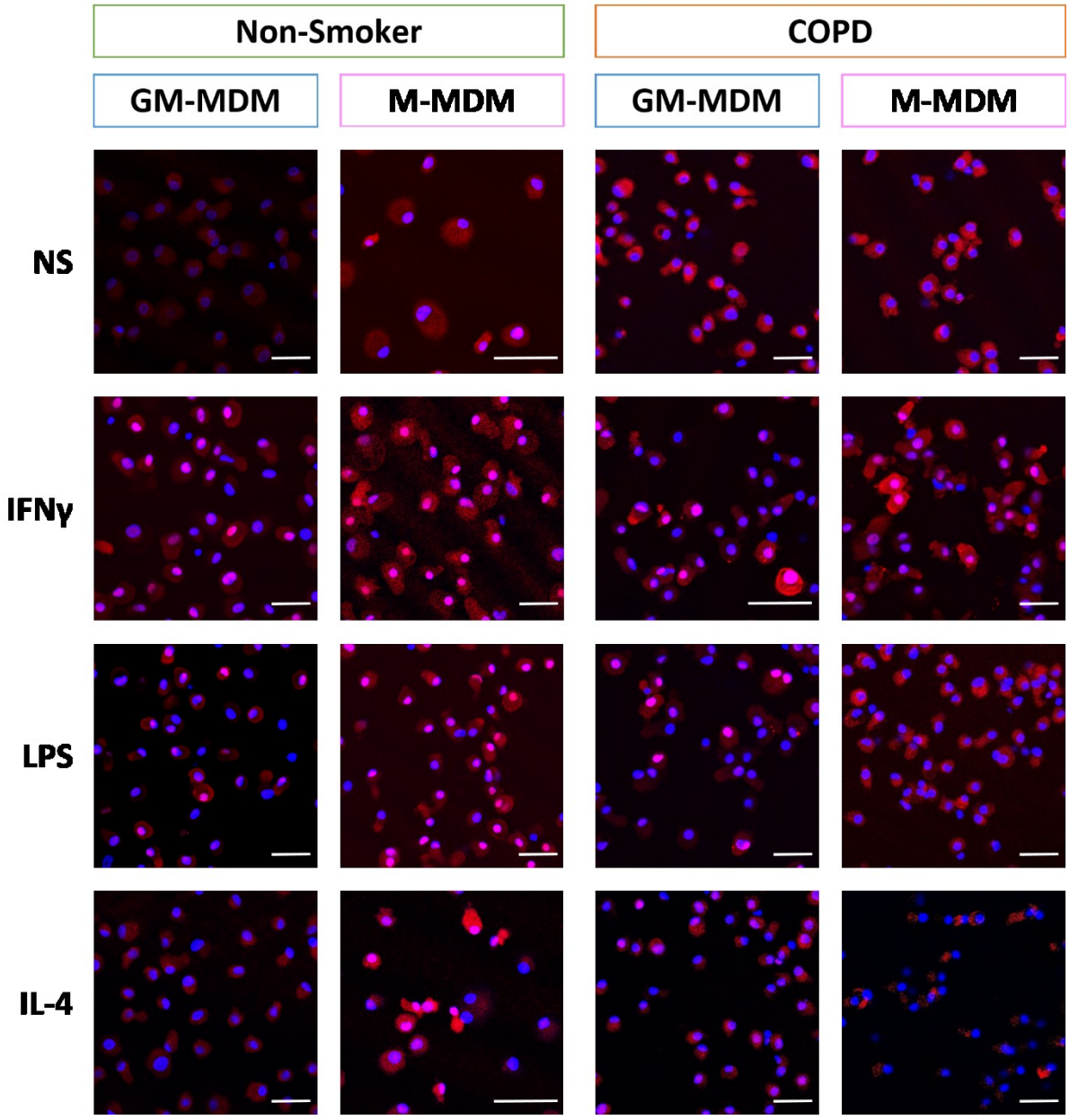


Figure 6.18: IRF3 confocal microscopy of GM-MDM and M-MDM from a non-smoker and COPD patient. Representative photographs taken of GM-MDM or M-MDM from a non-smoker (left) and COPD patient (right). Cells were left non-stimulated (NS) or stimulated with either 10ng/ml IFNγ, LPS or IL-4 for 10min then fixed and stained with nuclear DAPI (blue) and IRAF3 (red) before visualisation on a confocal microscope. Scale bar = 50μm.

6.3.3.3 STAT6

qRT-PCR experiments showed no statistical differences in *STAT6* expression between subject groups, although there was a trend for higher expression in COPD patients. It was therefore important to use different methodology to identify these differences at a protein level and under different cell stimulation.

GM-MDM and M-MDM from both subjects expressed STAT6 in the cytoplasm (Figure 6.19). There were no discernible differences when comparing GM-MDM and M-MDM although, similar to the qRT-PCR results, MDM from the COPD patient appeared to have higher baseline expression of cytoplasmic STAT6 compared with MDM from the non-smoker.

Cells stimulated with IFNγ showed nuclear translocation of STAT6 in both GM-MDM and M-MDM from the non-smoker. Again this was more evident in the M-MDM compared with the GM-MDM. MDM from the COPD patient also appeared to express less STAT6 compared with the non-smoker cells. LPS stimulation appeared to have no affect on STAT6 expression, which remained similar to baseline cytoplasmic samples. IL-4 stimulation led to nuclear translocation in both GM-MDM and M-MDM from the non-smoker; more so in the M-MDM compared with the GM-MDM, while there was little or no affect of IL-4 stimulation on COPD cells which remained similar to the baseline cytoplasmic expression.

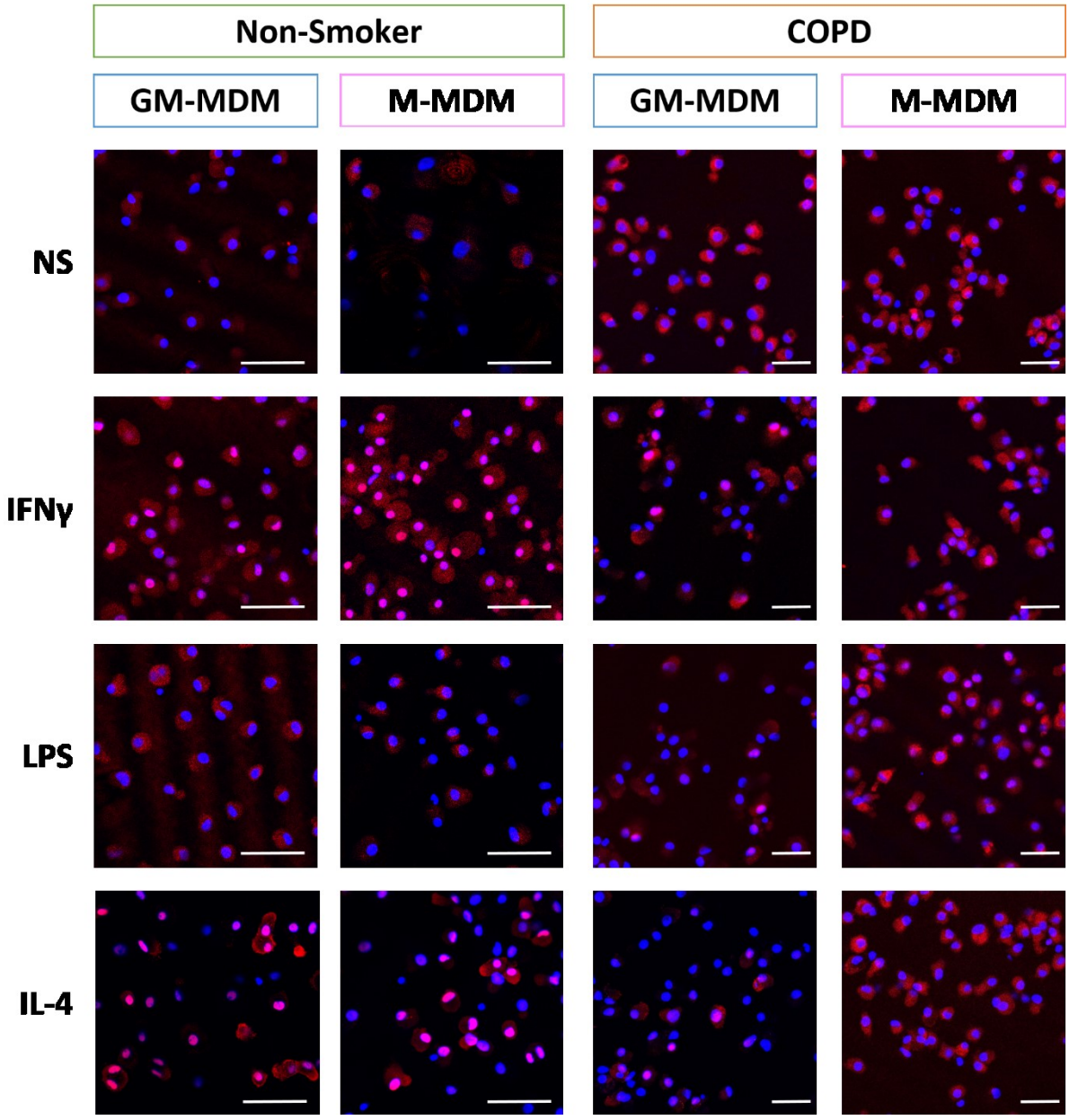


Figure 6.19: STAT6 confocal microscopy of GM-MDM and M-MDM from a non-smoker and COPD patient. Representative photographs taken of GM-MDM or M-MDM from a non-smoker (left) and COPD patient (right). Cells were left non-stimulated (NS) or stimulated with either 10ng/ml IFNγ, LPS or IL-4 for 10min then fixed and stained with nuclear DAPI (blue) and STAT6 (red) before visualisation on a confocal microscope. Scale bar = 50μm.

6.3.3.4 c-MAF

Having demonstrated clear differences in *MAF* expression in COPD MDM, it was important to see if these differences translated to protein level and identify the affect of various stimuli.

GM-MDM and M-MDM from both subjects expressed c-MAF in the cytoplasm (Figure 6.20). Unlike at mRNA level, there were no differences when comparing GM-MDM and M-MDM and MDM from the COPD patient also appeared to have higher baseline expression of cytoplasmic c-MAF compared with MDM from the non-smoker.

Cells stimulated with IFNγ showed nuclear translocation of c-MAF in M-MDM from the non-smoker and both GM-MDM and M-MDM from the COPD patient; again more so in the M-MDM compared with the GM-MDM.

LPS stimulation appeared to increase both cytoplasmic and nuclear c-MAF expression in M-MDM from the non-smoker and COPD patient while GM-MDM from both subjects expressed similar to baseline cytoplasmic levels of c-MAF.

IL-4 stimulation led to clear nuclear translocation in both GM-MDM and M-MDM from the non-smoker; more so in the M-MDM compared with the GM-MDM, while there was no affect of IL-4 stimulation on COPD cells which remained similar to the baseline cytoplasmic expression.

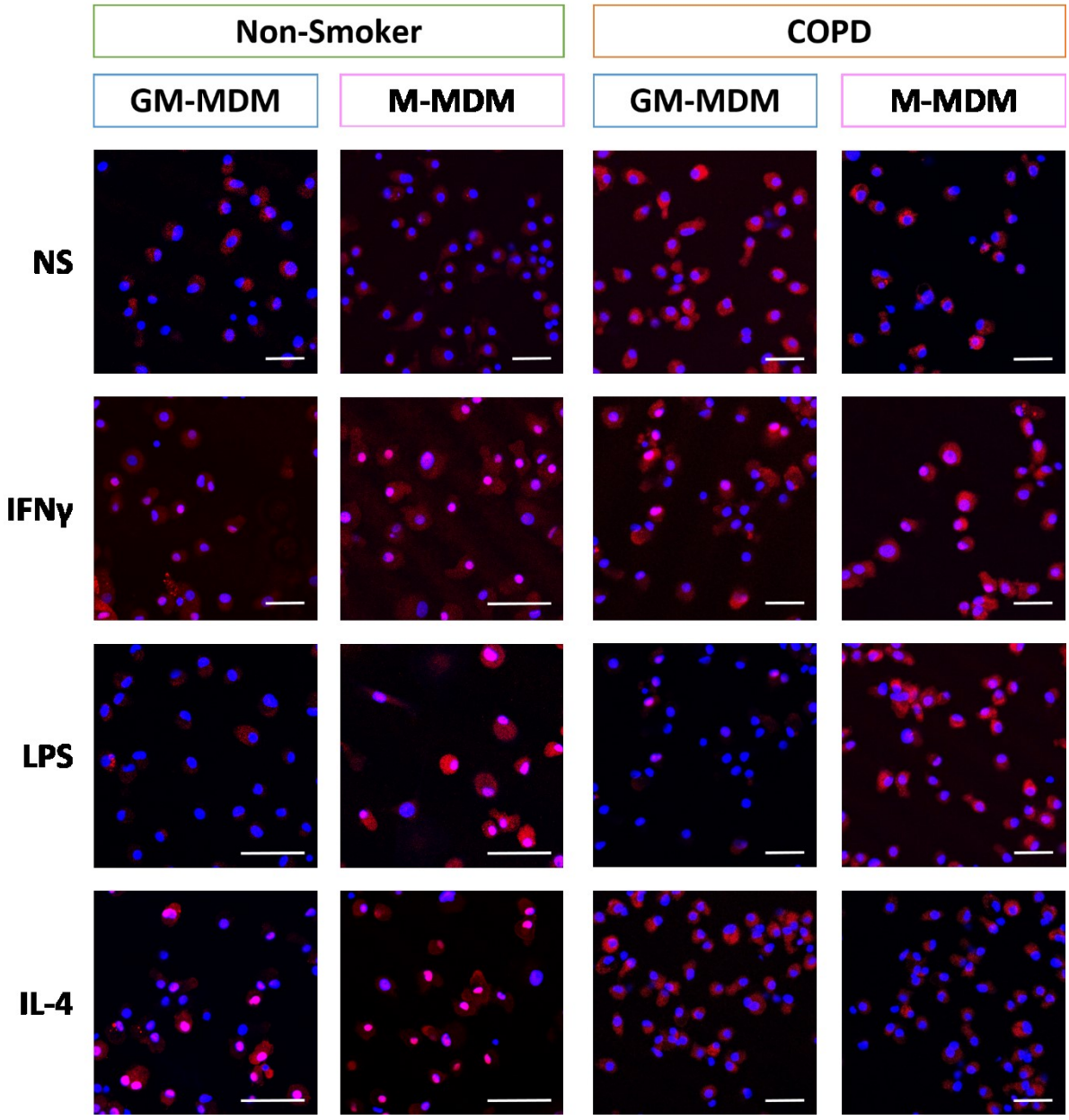


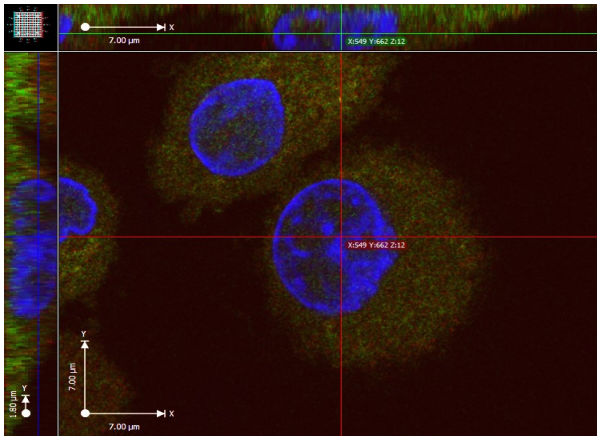
Figure 6.20: MAF confocal microscopy of GM-MDM and M-MDM from a non-smoker and COPD patient. Representative photographs taken of GM-MDM or M-MDM from a non-smoker (left) and COPD patient (right). Cells were left non-stimulated (NS) or stimulated with either 10ng/ml IFNγ, LPS or IL-4 for 10min then fixed and stained with nuclear DAPI (blue) and MAF (red) before visualisation on a confocal microscope. Scale bar = 50μm.

In order to confirm nuclear relocation, Z-stacks were performed using non-smoker cells both at baseline and after stimulation. This technique allowed for cross sectional analysis and more clearly indicated location of the fluorescent signal within the cell.

Cross sectional analysis showed that, in stimulated cells, the red signal was clearly located in the nuclear section of the cell compared with non-stimulated cells in which the red signal was situated around the nucleus, in the cell cytoplasm (Figure 6.21).

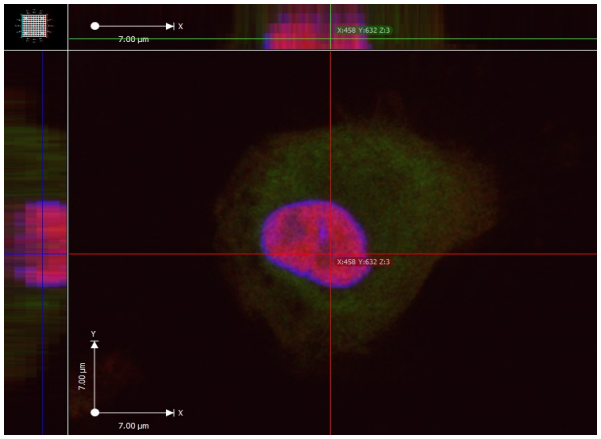
A

GM-MDM
STAT1
Non-stimulated



B

GM-MDM
STAT1
IFNγ-stimulated



C

M-MDM
c-MAF
IL-4-stimulated

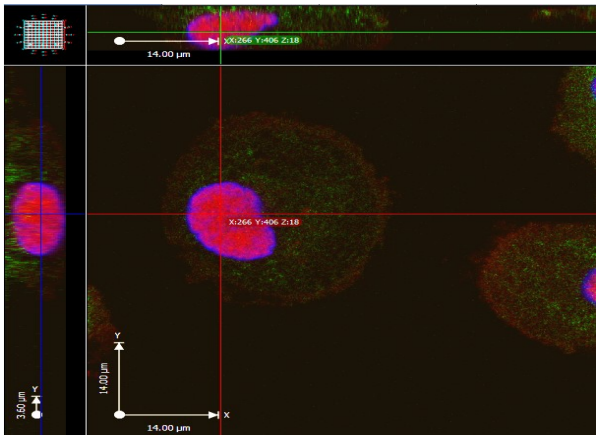


Figure 6.21: Z-Stack confocal microscopy of GM-MDM and M-MDM from a non-smoker. MDM from a non-smoker left unstimulated (A), stimulated with 10ng/ml IFNγ (B) or stimulated with 10ng/ml IL-4 (C). Cells were treated for 10min then fixed and stained with nuclear DAPI (blue) and either STAT1 (A/B) or MAF (C) (red) before visualisation on a confocal microscope. Z-stack photographs were taken of different focal points in the cell to allow visualisation of cellular compartments.

6.3.4 PhosFLOW

Having shown some differences in STAT1 and STAT6 expression at the protein level, it was important to examine the levels of phosphorylation in these proteins and examine if there were differences between subject groups and with stimulation.

Cells were cultured, stimulated, fixed and stained as in section 2.2.8.3.

6.3.4.1 pSTAT1

There was no difference in either the number of cells expressing pSTAT1 (% positive) or the amount of pSTAT1 expressed per cell (MFI) at baseline in any subject group or between culture conditions (GM-CSF or M-CSF) (Figure 6.22). After LPS stimulation, there was a significant increase in both the % of pSTAT1 positive cells and pSTAT1 MFI in GM-MDM from non-smokers ($p<0.05$) (Figure 6.22A and B). pSTAT1 expression was also significantly higher in GM-MDM from non-smokers compared with GM-MDM from smokers and COPD patients ($p<0.05$), in which pSTAT1 expression did not significantly alter from baseline levels.

pSTAT1 levels were also significantly higher in GM-MDM compared with M-MDM from non-smokers ($p<0.05$). The % of pSTAT1 positive LPS-stimulated M-MDM from smokers was similar to that of non-smokers. There were less pSTAT1 positive M-MDM in COPD patients compared with non-smokers and smokers, although this was not statistically significant, and pSTAT1 MFI was similar in M-MDM from smokers and COPD patients and was trended to be higher in non-smokers, although this was not statistically significant (Figure 6.22D).

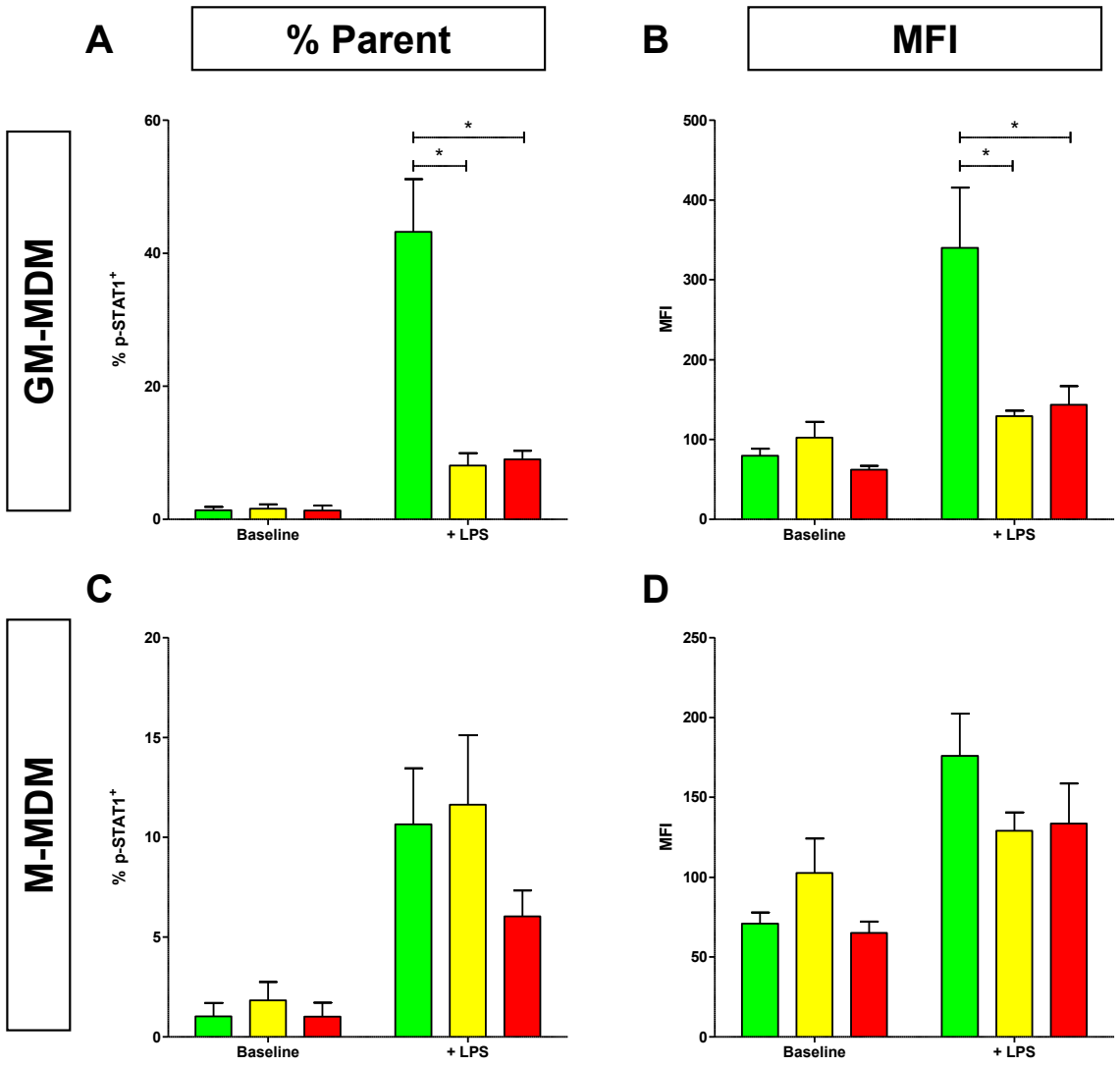


Figure 6.22: pSTAT1 expression in GM-MDM and M-MDM after stimulation. Monocytes from non-smokers (green) n=4 smokers (yellow) n=4 and COPD patients (red) n=3 were cultured in either GM-CSF (A/B) or M-CSF (C/D) for 12 days then stimulated with 10ng/ml LPS for 30 min before pSTAT1 was measured by flow cytometry. Data expressed as % parent - % of cells positive for pSTAT1 or MFI – Mean Fluorescence Intensity – correlating to the amount of pSTAT1 expressed within each cell. Data are expressed as mean ± SEM.

6.3.4.2 pSTAT6

There was no difference in either MFI or % pSTAT6 positive cells at baseline in any subject group or between culture conditions (GM-CSF or M-CSF) (Figure 6.23). After IL-4 stimulation, there was a significant increase in the % of pSTAT6 positive cells and a trend for higher pSTAT1 MFI in GM-MDM from non-smokers ($p<0.05$) (Figure 6.23A and B). pSTAT1 expression was also significantly higher in GM-MDM from non-smokers compared with GM-MDM from smokers and COPD patients ($p<0.05$), in which pSTAT1 expression did not significantly alter from baseline levels.

This was similar to results in M-MDM. After IL-4 stimulation there was a significant increase in pSTAT6 positive cells in the non-smokers ($p<0.05$) which was not seen in the cells from smokers or COPD patients. There was no significant difference when comparing GM-MDM and M-MDM from non-smokers but there was a trend for higher pSTAT6 expression in M-MDM, although this was not statistically significant.

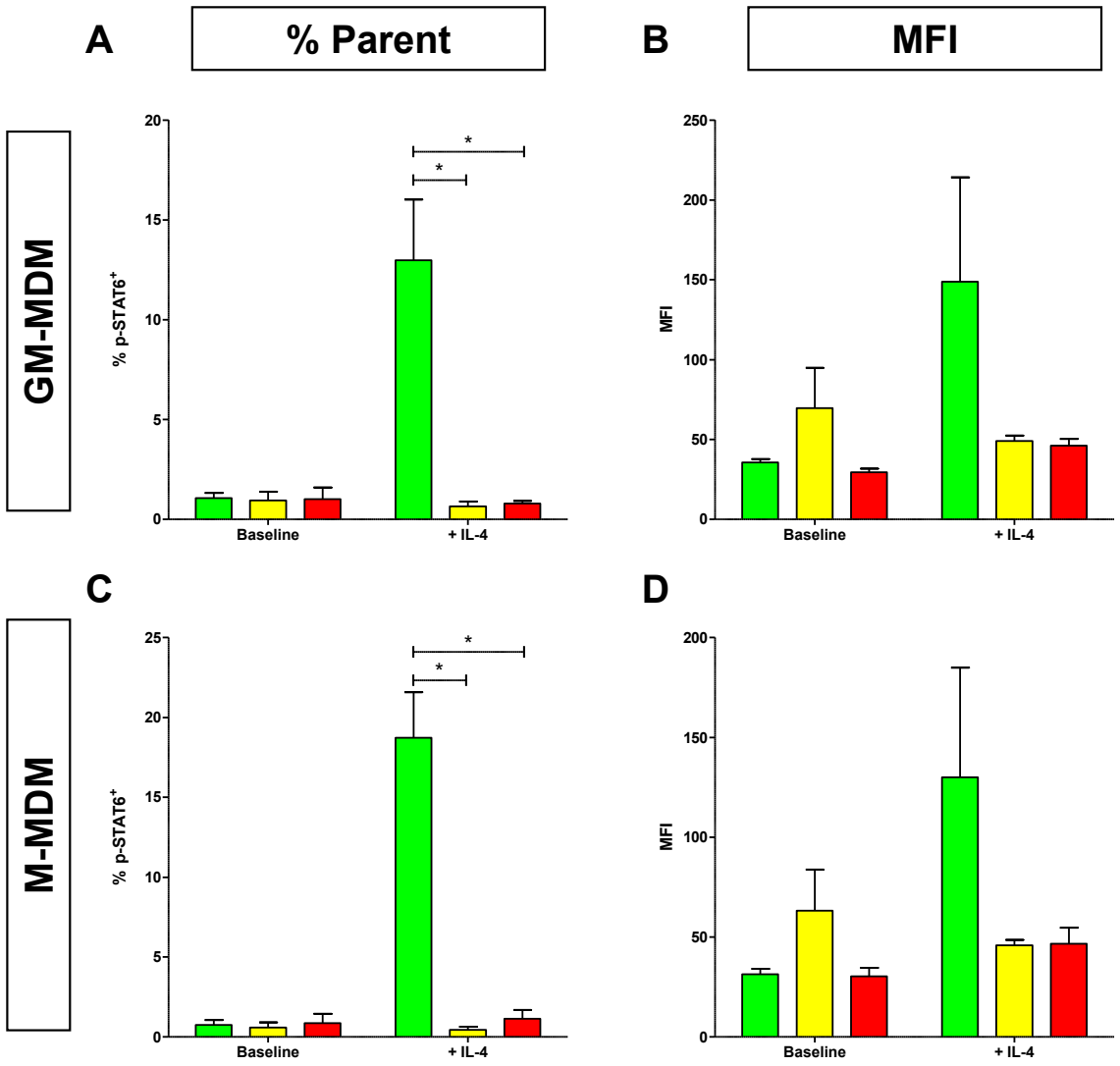


Figure 6.23: pSTAT6 expression in GM-MDM and M-MDM after stimulation. Monocytes from non-smokers (green) n=4 smokers (yellow) n=4 and COPD patients (red) n=3 were cultured in either GM-CSF (A/B) or M-CSF (C/D) for 12 days then stimulated with 10ng/ml IL-for 30 min before pSTAT6 was measured by flow cytometry. Data expressed as % parent - % of cells positive for pSTAT1 or MFI – Mean Fluorescence Intensity – correlating to the amount of pSTAT6 expressed within each cell. Data are expressed as mean ± SEM.

6.4 Discussion

6.4.1 GM-MDM express more pro-inflammatory transcription factors than M-MDM

The first aim of this chapter was to identify differences in transcriptional control of between cells cultured in GM-CSF and M-CSF. The results showed that cells cultured in GM-CSF for 12 days expressed more pro-inflammatory transcription factors (*STAT1, IRF3, IRF7* and *RELA*) compared with cells cultured in M-CSF, which expressed higher levels of anti-inflammatory transcription factors (*STAT6* and *MAF*). This is in agreement with previous studies indicating a more pro-inflammatory gene profiling of GM-CSF cultured cells compared with M-CSF cultured cells in both murine (Fleetwood et al. 2009) and human macrophage models (Lacey et al. 2012; Beyer et al. 2012).

6.4.2 Pro-inflammatory transcription factor expression is altered in MDM from COPD patients

The second aim was to identify differences in transcriptional control between subject groups. The results in this chapter showed trends indicating pro-inflammatory transcription factor expression levels were higher in COPD MDM compared with MDM from non-smokers although many were not statistically significant and would require a larger cohort in order to fully identify significant differences.

RELA expression was higher in smokers compared with non-smokers and COPD patients. This concurs with studies showing increases in NFκB in cigarette smoke-stimulated rat lungs (Marwick et al. 2004), sputum macrophages from human smokers (Caramori et al. 2003), PBMC from smokers and COPD patients (Bahr et al. 2013)

and bronchial biopsies from COPD patients (Di Stefano et al. 2002). To elucidate the possible mechanism behind smoking and increased NFκB expression, one study exposed a human macrophage-like cell line MonoMac6 to cigarette smoke extract (CSE) and showed increased NFκB activation (Yang et al. 2006) possibly caused by the interruption of the histone family that regulates RELA acetylation (Yang et al. 2007). Interestingly, in the present study, there was lower *RELA* expression in smokers compared with COPD patients. This may be due to the fact that the COPD subjects used in these experiments were all ex-smokers (6/6) and the majority of smokers were current smokers (3/4). It could be that the smokers were experiencing higher levels of oxidative stress from current cigarette smoke exposure, which could account for the higher *RELA* expression in those samples.

The results of this chapter also showed that *STAT1* gene expression was increased in both GM-MDM and M-MDM from COPD patients compared with non-smoker samples. This is in line with previous studies which demonstrated higher *STAT1* expression in COPD sputum macrophages compared with non-smokers (Holloway et al. 2014) and higher expression of *STAT1* in lung parenchymal samples taken from COPD patients compared with smokers and non-smokers (Yew-Booth et al. 2015).

The same study also demonstrated increased phosphorylation of STAT1 in smokers and COPD patients which was not supported by the current study. This may be due to differential methods; the study of Yew-Booth *et al* utilised western blotting and immunohistochemistry (Yew-Booth et al. 2015) while this study used flow cytometry. Notably, Yew-Booth *et al* used whole cell protein extracted from frozen cells while the current study used fresh cells. It is possible that the COPD samples used in the flow cytometry experiments were not fixed with formaldehyde at the most appropriate time point for the detection of phosphorylation as they were cultured on adherent plates

and therefore had to be detached after stimulation prior to fixation. Use of non-adherent plates and a time course of stimulation would be needed to confirm this. Another possible reason was non-permeabilisation of the COPD sample, although this was unlikely, as another internal dye control was used which showed permeabilisation had occurred in all samples.

The current study showed baseline *IRF3* expression was slightly increased in patients with COPD (both M-MDM and GM-MDM) compared with non-smoker samples. Previously, IRF3 activation has been linked to both pro- and anti-inflammatory processes. One study showed GM-CSF-primed “M1-like” macrophages express higher levels of IRF3 compared with M-CSF cultured cells (Fleetwood et al. 2009), while a different study showed IRF3 transduced human foetal microglia displayed a completely anti-inflammatory phenotype (Tarassishin et al. 2011). While no such studies have examined *IRF3* expression specifically in MDM from COPD patients, the high expression seen in the current study may be linked with the abnormal NFκB signalling in these cells. The IRF3 promoter has an NFκB binding site and therefore it may be possible that the high levels of NFκB in cells from COPD and smokers (Brown et al. 2009) may be acting on the IRF3 promoter, further increasing its transcription in these cells.

IRF7 expression in the current study was higher in GM-MDM compared with M-MDM and also higher in cells from COPD patients. This is in agreement with papers showing healthy GM-MDM had higher *IRF7* expression compared with M-MDM (Lacey et al. 2012) but is in contrast to another study showing reduced *IRF7* expression in COPD compared with non-smoker bronchoalveolar lavage samples (Shaykhiev et al. 2009). This result may be a limitation of the MDM model or may be a phenomenon only seen in monocyte-derived cells compared to mature tissue macrophages.

6.4.3 Anti-inflammatory transcription factor expression is altered in MDM from COPD patients

It was also important to examine the factors important in anti-inflammatory linked transcriptional control as this also influences macrophage phenotype.

The current study showed baseline expression of STAT6 was higher in cells cultured in M-CSF which agrees with previous research (Lacey et al. 2012) but, baseline STAT6 expression was also higher in cells from COPD patients compared with non-smokers; confirmed by both qRT-PCR and confocal microscopy of day 12 samples. As this is the first study comparing STAT6 levels in COPD MDM to non-smokers the mechanism behind this is currently unknown but could be a possible compensatory mechanism in the COPD cells which may be trying to increase STAT6 production. This may be in response to increased AP-1 or NFκB signalling, both of which have binding sites upstream of the STAT6 promotor region (Shen & Stavnezer 1998). Confocal microscopy also showed lack of nuclear translocation of STAT6 after IL-4 stimulation. IL-4 binding to IL-4R1 is a crucial activator of STAT6 nuclear translocation (Wurster et al. 2000), therefore the lack of translocation may be due to a lack of IL-4 signalling in COPD cells.

Another anti-inflammatory transcription factor, c-MAF, has previously been shown to play a crucial part in the signalling and transcription of IL-4 in Th2 cells (Kim et al. 1999). The deletion of *c-MAF* in a murine model showed a significant detrimental affect on IL-4 gene expression (Kim et al. 1999). More recently c-MAF was shown to be constitutively expressed in human macrophages and is now known to have a vital role in the transcription of IL-10 (Cao et al. 2005). The study by Cao et al demonstrated

how IL-4 stimulation led to a significant increase in c-MAF expression and binding of c-MAF to the IL-10 promoter in human MDM (Cao et al. 2005).

The results from this chapter showed higher levels of *MAF* expression in M-MDM compared with GM-MDM, again agreeing with previous papers indicating a more anti-inflammatory role for MDM cultured in M-CSF (Lacey et al. 2012; Fleetwood et al. 2009). The current study also showed significantly more *MAF* is expressed in MDM from non-smokers compared with smokers and COPD patients (there was also a trend for higher *MAF* expression in lung tissue from non-smokers compared with COPD patients). This is the first instance of this transcription factor being compared in MDM from non-smoking and COPD subjects, therefore a mechanism is yet to be fully elucidated. One possible mechanism could be skewed IFNγ signalling. One study showed how high IFNγ levels, such as those seen in smokers and COPD patients (Xue et al. 2016), can decrease the expression of the IL-4 receptor, IL-4R1 (So et al. 2000), potentially leading to a reduction in c-MAF transcriptional activity.

Taken together these results show higher levels of pro-inflammatory linked transcription factors and lower levels of c-MAF in MDM from COPD patients, which may explain the exaggerated pro-inflammatory functional phenotype seen in previous chapters (Figure 6.24).

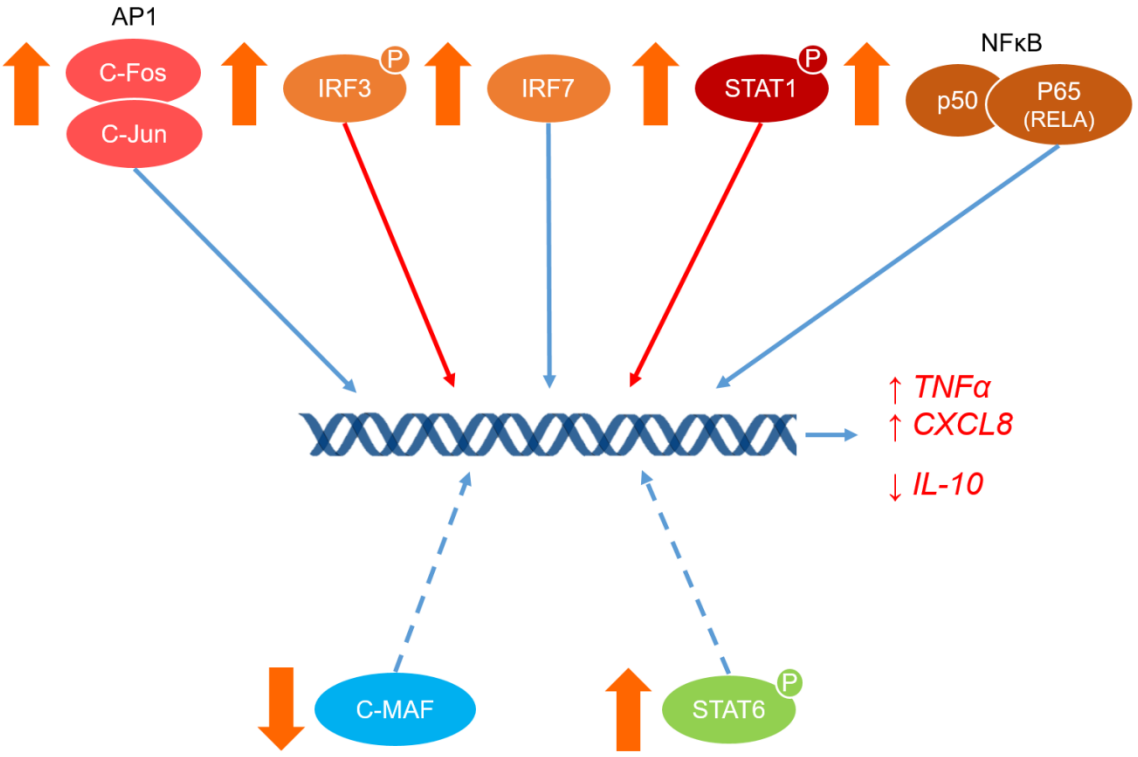


Figure 6.24: Schematic of COPD transcriptional regulation. Diagram summarising the findings of the qRT-PCR results in this chapter. Large arrows indicate higher or lower expression of the specific transcription factor in relation to non-smoker. Smaller arrows denote translocation of the transcription factor into the nucleus – red indicates high levels of nuclear translocation and dotted line shows less or no translocation.

6.4.4 Transcription factor expression differs in COPD MDM undergoing environmental change

Another aim of the chapter was to analyse whether changing culture environment would lead to a change in expression of selective transcription factors. While in some cases, MDM reacted to change in culture environment, this was not always the case. MDM from non-smokers regularly differed compared with cells from smokers and COPD patients. Results are summarised in Table 6.3. Only three out of the seven transcription factors studied were altered in response to changing environment in COPD MDM compared with six out of seven in the non-smokers and five out of seven in the smokers. This data suggests that in some instances the transcriptional regulation of these cells, and their subsequent plasticity, may be affected by smoking and COPD.

	Non Smokers	Smokers	COPD
STAT1	Yes (trend)	Yes (trend)	Yes *
STAT6	Yes (trend)	Yes (trend)	Yes (trend)
JUN	No	No	No
RELA	Yes (trend)	No	No
IRF3	Yes (trend)	Yes (trend)	No
IRF7	Yes*	No	No
MAF	Yes (trend)	No	No

Table 6.3: Summary of ability of MDM to alter transcription factor expression in response to culture changes. Table indicates if levels of transcription factors indicated changed expression in response to altering cell culture media. * indicates significant change in expression.

The current study also showed an inability of COPD and smoker GM-MDM to restore MAF levels once they were switched to M-CSF media. If this is the case and cells have become permanently skewed into a pro-inflammatory phenotype, with dysfunctional MAF signalling, this may explain previous chapter results indicating a lack of IL-4 stiulated-IL-10 signalling in COPD patients and smokers and also a lack in ability to switch phenotype.

If IL-4 signalling and MAF expression is altered in COPD MDM, it could be that the subsequent IL-10 release will be altered. Interestingly, confocal experiments clearly showed IL-4-stimulated translocation of MAF into the nucleus, while present in the sample from the non-smoker, was not observed in the COPD sample. This may indicate a possible defect in the translocation pathway in cells from COPD.

It is worth noting that the confocal experiments represent a single subject sample and therefore no general conclusions can be drawn before this work is replicated. However, the sample was treated on the same day and with the same reagents as the non-smoker sample in order to limit slight variations in protocol that are common in slide staining and microscopy work (St Croix et al. 2005). Similarly, many of the results in this chapter showed trends rather than statistically significant differences, therefore more power is needed to fully draw any conclusions.

One reason behind lack in ability of MDM to switch phenotype may rest in the epigenetic control of these cells. The transcription factors discussed in this chapter are, on another level, regulated by histone modifications. Histone deacetylase (HDAC) 1, 2 and 3 have all been shown to be required for IFNγ-stimulated STAT1 transcription (Klampfer et al 2004) and STAT6 has been shown to be regulated by HDAC4 (Yang et al. 2015). While no specific HDAC activity has yet been associated with c-MAF

signalling, specific histone deacetylases have been shown to be altered in peripheral lung of COPD patients (Ito et al. 2005; Barnes 2006; Barnes 2009) and may lead to exaggeration and sustainment of the pro-inflammatory nature of the COPD cells.

6.4.5 Conclusions

The hypothesis of this chapter was that GM-MDM will express higher levels of pro-inflammatory-linked transcription factors and lower levels of anti-inflammatory-linked transcription factors compared with M-MDM. This was the case so this hypothesis can be accepted. The second hypothesis of this chapter was that MDM from COPD patients will express higher levels of pro-inflammatory-linked transcription factors and lower levels of anti-inflammatory-linked transcription factors compared with M-MDM. Again this was the case with the pro-inflammatory transcription factors but was also observed in relation to STAT6, therefore this hypothesis is partly accepted.

The final conclusion of this chapter is that MDM from COPD patients appear to show unidirectional plasticity in terms of their functional phenotype and this may be due to some differential transcriptional regulation in these cells.

CHAPTER 7 GENERAL DISCUSSION

7.1 Key findings

The hypothesis of this thesis was that macrophages from COPD patients will show a lack of plasticity and will not be able to revert to a pro-resolving, anti-inflammatory phenotype in comparison with cells from non-smoking subjects.

Using the adherent, 10% serum-cultured monocyte-derived macrophage model, this thesis was able to show that MDM from non-smokers were able to display both pro and anti-inflammatory phenotypes depending on in the presence of either GM-CSF or M-CSF respectively. Results presented herein demonstrated that cells from non-smokers were able to switch phenotype; pro-inflammatory cells which were less able to phagocytose bacteria were able to revert to anti-inflammatory cells which readily phagocytosed both gram positive and gram negative bacteria simply by changing culture environment; they displayed high levels of dynamic phenotypic plasticity (Figure 7.1). This is in agreement with the paper by Porcheray et al showing plasticity in healthy human MDM (Porcheray et al. 2005).

The present study also demonstrated that MDM from COPD patients were less able to alter their phenotype to become pro-resolving anti-inflammatory macrophages and that they retained a uni-directional, pro-inflammatory phenotype, regardless of changes to their environment (Figure 7.1). By switching M-MDM to a GM-CSF environment, this study was able to demonstrate that MDM from COPD patients could become more pro-inflammatory and less phagocytic (releasing higher levels of LPS-induced TNFα and CXCL8 and phagocytosing less bacterial prey). However, when switching GM-MDM to M-CSF, the cells from COPD patients were less able, compared with cells from non-smokers, to switch to anti-inflammatory, pro-phagocytic cell type

(they produced low levels of IL-4-induced IL-10 and phagocytosed less bacterial prey). This phenotypical skewing has been demonstrated in COPD (Shapiro 1999; Barnes 2004; Hiemstra 2013; Vlahos & Bozinovski 2014).

The thesis showed that while no statistical differences could be identified in monocyte phenotype, there were slightly higher levels of CD11b expression in monocytes from COPD patients suggesting they may be more activated in the circulation. This could be a reaction to systemic oxidative stress. There is evidence to suggest that COPD and smoking may not only be driven by oxidative stress responses in the lung, but that this affect may be more systemic (MacNee 2005). Peripheral blood neutrophils from COPD patients release more reactive oxygen species (ROS) compared with samples from healthy subjects (Rahman et al. 1996). Zeng *et al.* showed increased MDA, an established marker of oxidative stress, is increased in induce sputum and serum of COPD patients (Zeng et al. 2013). The systemic affects of smoking are well documented and include the increase in many oxidative stress markers such as serum MDA (Isik et al. 2007). Also important is the evidence suggesting cigarette smoke causes decreased levels of anti-oxidants, for example, paraoxonase (PON1) which was found to be significantly decreased in serum samples of smokers compared with healthy controls (Isik et al. 2007). It may be that the systemic oxidative stress, generated by cigarette smoke, alters MDM phenotype, although this needs more examination *in vivo*.

This thesis also showed that c-MAF, the transcription factor which regulates IL-10 production in human macrophages (Cao et al. 2005) was reduced in MDM from COPD patients in comparison to non-smokers. IL-10 is important role in the immune response as it signals a change to the resolving stage and dampens pro-inflammatory signals (Hackett et al. 2008). It may be that while macrophages from COPD patients can

become pro-inflammatory, they have lost the ability to self-regulate and therefore skew towards a pro-inflammatory phenotype.

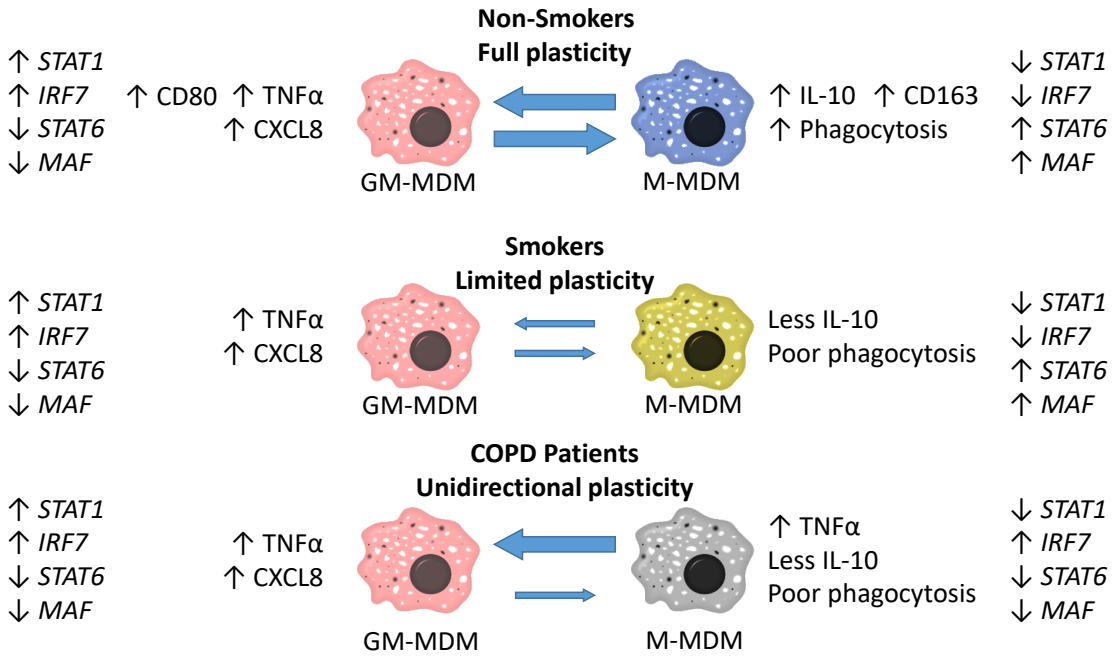


Figure 7.1: Summary of main findings. Monocyte derived macrophages from non-smokers, smokers and COPD patients differentiate using either GM-CSF or M-CSF underwent functional testing, flow cytometric analysis and transcriptional profiling. The schematic details which markers were up or downregulate in either GM-MDM or M-MDM and indicates that MDM from COPD patients display skewed phenotypic plasticity, possible due to differences in transcriptional regulation.

7.2 Macrophage plasticity in other diseases

Understanding macrophage plasticity by studying macrophage phenotype in other diseases may be a useful tool to identify alternative reasons as to the differential plasticity in MDM from COPD patients.

Macrophage skewing has been shown in atherosclerosis. Much of the research suggests the predominance of an ‘M1-like’ macrophage phenotype contributes to the pathogenesis of atherosclerosis (Khallou-Laschet et al. 2010; Moore et al. 2013). A study by Loke et al. showed that in the absence of IL-4 producing T-cells, ‘M2-like’ macrophages will switch to the ‘M1-like’ phenotype (Loke et al. 2007). It may be that, in COPD, other immune cells such as T-cells, which (in a healthy subject) would contribute to macrophage phenotype, are altered, allowing for a skewing of macrophage phenotype due to lack of regulation. Similarly, IL-4 producing CD4(+) T lymphocytes have been shown to skew tumour associated macrophage phenotype towards that of an ‘M2-like’ macrophage, promoting growth and spread of cancerous cells in a breast cancer model (DeNardo et al. 2009).

Studies into chronic venous ulcers have demonstrated that infiltrating macrophages fail to switch from an ‘M1’ phenotype to an ‘M2’ phenotype (Sindrilaru et al. 2011). The study went on to describe the ‘M1’ skewed macrophages as having dysfunctional iron metabolism which prevented the cells from becoming ‘M2-like’ (Sindrilaru et al. 2011). In line with this, a recent study by Philippot et al showed increased iron sequestration in alveolar macrophages in chronic obstructive pulmonary disease which positively correlated with disease severity (Philippot et al. 2014). It is possible that macrophages in COPD have altered iron metabolism which prevents their plasticity.

7.3 Possible clinical implications

The ability to manipulate or re-programme macrophage phenotype towards a more anti-inflammatory, pro-resolving type presents a possible therapeutic target in COPD caused by overactive pro-inflammatory cells. This is limited, of course, to disease type and severity. COPD encompasses emphysema, small airway disease and chronic bronchitis in which macrophages have differential influence. In addition to this, severity is classified into 4 stages.

GOLD Stage	Type	Post-Bronchodilator FEV_1
1	Mild	$FEV_1 \geq$ 80% predicted
2	Moderate	50% $\leq FEV_1 <$ 80% predicted
3	Severe	30% $\leq FEV_1 <$ 50% predicted
4	Very severe	$FEV_1 <$ 50% predicted

Table 7.1: GOLD stages of COPD. COPD severity is split into stages 1 (mild) to stage 4 (very severe) based on severity of airflow limitation as measured by post-bronchodilator FEV_1.

Manipulation of macrophage phenotype may only be useful at a specific stages of disease. For example, in a patient classified to have GOLD stage 4 and marked emphysema, diagnosed by CT imaging, changing macrophage phenotype is less likely to impact the patient's symptoms as it will not change the marked alveolar destruction or reduce breathlessness. In contrast, manipulating macrophage phenotype in a mild GOLD stage 1 patient with predominant chronic bronchitis may alleviate some symptoms caused by the overactive pro-inflammatory cells.

Not only is the stage of the patient important, getting the correct balance of phenotype is difficult. Both pro-and anti-inflammatory cells have their place; if phenotype skews too much towards an ‘M1-like’ phenotype, there will be exaggerated inflammation and tissue destruction but skewing too much towards an ‘M2-like’ phenotype may also have negative implications with regards to infection and tumour progression. Pro-inflammatory cells are necessary for defending against pathogens and signalling to other parts of the immune system. To wipe them out completely would be to increase the risk of opportunistic bacterial and viral infections. To skew then too much towards an ‘M2-like’ phenotype could also trigger tumour growth and metastasis as with tumour associated macrophages (Biswas & Mantovani 2010).

Hormone receptor PPARgamma (PPARγ) agonists have been shown to promote ‘M2-like’ macrophage phenotype in adipose tissue macrophages (Charo 2007) and are currently under investigation in atherosclerosis (Chawla et al. 2016). CCR5 blockade has also been shown to promote ‘M2-like‘ macrophage phenotype *in vitro* (Li et al. 2015). Using an adoptive transfer murine model of anti-CCR5-treated macrophages, Li et al were able to show a decrease in IFNγ and TNFα release and suggested that this was mediated by increased levels of PPARγ (Li et al. 2015). Therefore, PPARγ may represent a potential therapeutic target in COPD macrophages.

The results of this thesis show defective function and plasticity in cells derived from the peripheral blood of COPD patients, therefore suggestive of an inherent defect in the circulating monocyte population in these patients. Although, no significant differences were identified in monocyte sub-population in these experiments, it may be that the correct distinctive target was not identified or that the defects only appear once the cells have differentiated into macrophages. If the latter is true, then it would be logical to target the end stage macrophage environment rather than the monocyte

population. Targeting the lung environment, where monocytes will differentiate into macrophages and therefore the environment which dictates subsequent phenotype, may represent a more affective long term solution to macrophage pathophysiology. Although this approach again may depend on several factors, such as smoking status and history. While research has shown smoking cessation to be associated with improvement in COPD lung function (Pride 2001), it was also demonstrated that macrophages from ex-smokers retain their pro-inflammatory phenotype (Kunz et al. 2011).

The longevity of these cells also represents a significant problem in the management and therapeutics of macrophage phenotype. Macrophages can stay in the lung for many years, and with the lack of a human macrophage marker of age, it is difficult to determine if it is newly differentiated or mature macrophages which are causing the problems in COPD lungs.

7.4 Conclusion

MDM from COPD patients are skewed towards a pro-inflammatory, anti-resolving phenotype regardless of environmental alteration. This may be due to differences in transcriptional regulation of anti-inflammatory mechanisms.

7.5 Limitations

One of the limitations of this thesis was the use of the MDM model to demonstrate macrophage phenotype. This thesis showed that the addition of FCS into the culture media was necessary in order to generate measureable differences in IL-10 release after stimulation. FCS is high in bovine serum albumin (BSA), integrin beta-1, alpha-1-anti-proteinase and TGF-β (Zheng et al. 2006) compared to human serum which contains high levels of albumin, complement factors and angiotensin (Adkins et al. 2002). The affect of FCS or human serum culture on MDM has been demonstrated before (Schmidt et al. 2011) and demonstrates the variability caused by different culture methods. Therefore, it is difficult to generalise results and compare them with those of lung tissue macrophages. Although, the MDM does provide a far less invasive avenue of study and allows manipulation of differentiation environment not capable in tissue macrophages.

Another limitation of this model, is that the MDM are effectively “used up” at the different time points in order to assess phenotype. In order to fully study and confirm plasticity, it would be necessary to culture and stimulate the cells, measure their function and viability in real time, without disturbing them, and then change the environment of those exact same cells to see if they could change phenotype. Unfortunately, in order to carry out many functional tests, the macrophages need to be “used up” in the process, especially to assess viability. The functional tests used herein included plate based ELISAs and fluorimetry to assess functionality and MTT assay to assess viability. Labelling, addition of antibodies and lysis of the cells all mean that the same cells cannot be used for subsequent experiments, and therefore, the assumption is that that those cells which have responded to an environmental switch, were functionally the same cells at day 12 as those being used at later time points.

In addition to these culture limitations are the limitations brought on by the dynamic plasticity and heterogeneity of the cells themselves. Any study examining macrophage phenotype would have limitations due to the plastic nature of the cells, changing phenotype to suit their environment. Most research in this area represents, at best, a snapshot in a consistently changing environment, however through comparing snapshots, and refining techniques, macrophage phenotyping is becoming better understood (Martinez & Gordon 2014).

7.6 Future Work

7.6.1 Culture MDM using human serum

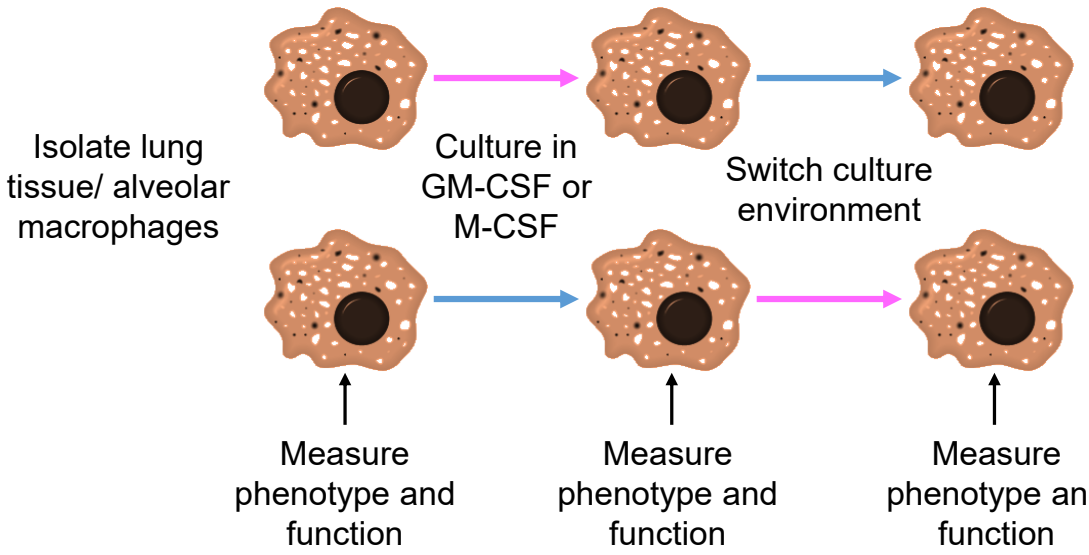
It is possible that there is a circulating factor in the serum of COPD patients that is priming monocytes towards a skewed MDM. To address this, MDM from healthy non-smokers could be cultured in serum of COPD patients to see if their function is altered. Alternatively, MDM from COPD patients could be differentiated in the presence of healthy human serum any alteration in phenotype assessed.

7.6.2 Separate monocyte phenotypes to see if they differentiate into different macrophage populations

It is possible that the different monocyte populations give rise to differential MDM subsets. To address this, flow assisted cell sorting (FACS) could sort monocytes into the different subsets (Classical CD14++CD16-, intermediate CD14++CD16+ and non-classical CD14+CD16+) which could be differentiated and the resulting GM-CSF and M-CSF derived MDM phenotype compared. CD16+ derived monocytes have previously been shown to have different functions in HIV, therefore applying this method to COPD monocytes would be interesting. Measurement of other markers of activation such as calcium kinetics or chemotaxis, previously shown to be affected by COPD (Traves et al. 2004; Costa et al. 2016), may elucidate possible differences in monocyte phenotypes.

7.6.3 Assess plasticity in lung macrophages from non-smokers and COPD patients

It would be of interest to repeat the plasticity experiments using cells isolated from lung tissue macrophages to see if changing cell culture environment affects the cells in the same way as MDM.



7.6.4 Identify IL-4R (CD124), TLR4, CSF-1 and GMCSFR (CD116) expression in MDM

It would be of note to examine the expression of the IL-4, LPS, M-CSF and GM-CSF receptors in the MDM model to see if cell surface expression levels are different between GM-MDM and M-MDM and also between subject groups.

7.6.5 Measure ROS and Iron Metabolism in MDM

Iron metabolism may affect macrophage phenotype (Sindrilaru et al. 2011). Therefore, measurement of iron metabolism in MDM using either immunohistochemical staining

for transferrin or ferritin or Particle-induced X-ray emission (PIXE) would elucidate if this is also the case for MDM or is a phenomenon only observed in lung macrophages.

It would also be good to measure ROS levels from monocytes and MDM isolated from non-smokers, smokers and COPD patients using an Acridan Lumigen PS-3 assay which generates ROS-specific chemiluminescence and detects ROS released into cell culture media.

7.6.6 Analyse transcription factor expression with LPS, IFNγ or IL-4 activated cells

This thesis has highlighted differences in transcription factor expression using qRT-PCR and confocal microscopy transcriptional regulation in COPD MDM. The use of Luminex or microarray techniques to analyse transcriptional regulation of stimulated cells, may show further differential transcriptional regulation of activated macrophages. These experiments would need optimisation of timing, stimulation and fixation in order to determine the signalling pathways. It would also be interesting to compare these results to stimulated lung tissue macrophages.

REFERENCES

Abboud, R.T. & Vimalanathan, S., 2008. Pathogenesis of COPD. Part I. The role of protease-antiprotease imbalance in emphysema. *International Journal of Tuberculosis and Lung Disease*, 12(4), pp.361–367.

Aderem, A. & Underhill, D.M., 1999. MECHANISMS OF PHAGOCYTOSIS IN MACROPHAGES. *Annual Review of Immunology*, 17(1), pp.593–623. Available at: http://dx.doi.org/10.1146/annurev.immunol.17.1.593.

Adkins, J.N. et al., 2002. Toward a Human Blood Serum Proteome: Analysis By Multidimensional Separation Coupled With Mass Spectrometry . *Molecular & Cellular Proteomics* , 1(12), pp.947–955. Available at: http://www.mcponline.org/content/1/12/947.abstract.

Akagawa, K.S. et al., 2006. Functional heterogeneity of colony-stimulating factor-induced human monocyte-derived macrophages. *Respirology*, 11, pp.32–36.

Akagawa, K.S., 2002. Functional Heterogeneity of Colony-Stimulating Factor-Induced Human Nonocyte-Derived Macrophages. *International Journal of Hematology*, 76(1), pp.27–34. Available at: http://dx.doi.org/10.1007/BF02982715.

Anders, H.-J. & Ryu, M., 2011. Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. *Kidney International*, 80(9), pp.915–925.

Andreesen, R., Picht, J. & Löhr, G.W., 1983. Primary cultures of human blood-born macrophages grown on hydrophobic teflon membranes. *Journal of Immunological Methods*, 56(3), pp.295–304. Available at: file://www.sciencedirect.com/science/article/pii/S0022175983800192.

Aoshiba, K., Yokohori, N. & Nagai, A., 2003. Alveolar Wall Apoptosis Causes Lung Destruction and Emphysematous Changes. *American Journal of Respiratory Cell and Molecular Biology*, 28(5), pp.555–562. Available at: http://dx.doi.org/10.1165/rcmb.2002-0090OC.

Arnold, L. et al., 2007. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *The Journal of Experimental Medicine*, 204(5), pp.1057–69.

Artemyev, D.N. et al., 2016. Measurement of human serum albumin concentration using Raman spectroscopy setup. *Optical and Quantum Electronics*, 48(6), p.337. Available at: http://dx.doi.org/10.1007/s11082-016-0610-2.

ASH Report, 2007. Action on Smoking and Health is a campaigning public health charity that works to eliminate the harm caused by tobacco. , pp.1–12.

Baccala, R. et al., 2007. TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. *Nat Med*, 13(5), pp.543–551. Available at: http://dx.doi.org/10.1038/nm1590.

Bahr, T.M. et al., 2013. Peripheral Blood Mononuclear Cell Gene Expression in Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory Cell and Molecular Biology*, 49(2), pp.316–323. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3824029/.

Bailey, M.J. et al., 2011. Extracellular proteomes of M-CSF (CSF-1) and GM-CSF-dependent macrophages. *Immunology and Cell Biology*, 89(2), pp.283–93.

Baqir, M. et al., 2016. Cigarette smoke decreases MARCO expression in macrophages:

Implication in Mycoplasma pneumoniae infection. *Respiratory Medicine*, 102(11), pp.1604–1610. Available at: http://dx.doi.org/10.1016/j.rmed.2008.05.002.

Baraldo, S. et al., 2004. Neutrophilic infiltration within the airway smooth muscle in patients with COPD. *Thorax* , 59(4), pp.308–312. Available at: http://thorax.bmj.com/content/59/4/308.abstract.

Barnes, P.J., 2004. Alveolar macrophages as orchestrators of COPD. *COPD*, 1(1), pp.59–70.

Barnes, P.J., 2003. Chronic obstructive pulmonary disease: New treatments for COPD. *Thorax*, 58(9), pp.803–808. Available at: http://thorax.bmj.com/content/58/9/803.short.

Barnes, P.J., 2009. Histone deacetylase-2 and airway disease. *Therapeutic Advances in Respiratory Disease* . Available at: http://tar.sagepub.com/content/early/2009/10/07/1753465809348648.abstract.

Barnes, P.J., 2008. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol*, 8(3), pp.183–192. Available at: http://dx.doi.org/10.1038/nri2254.

Barnes, P.J., 2013. New anti-inflammatory targets for chronic obstructive pulmonary disease. *Nat Rev Drug Discov*, 12(7), pp.543–559. Available at: http://dx.doi.org/10.1038/nrd4025.

Barnes, P.J., 2006. Reduced histone deacetylase in copd*: Clinical implications. *Chest*, 129(1), pp.151–155. Available at: http://dx.doi.org/10.1378/chest.129.1.151.

Barnes, P.J. & Celli, B.R., 2009. Systemic manifestations and comorbidities of COPD. *European Respiratory Journal*, 33(5), p.1165 LP-1185. Available at: http://erj.ersjournals.com/content/33/5/1165.abstract.

Beeh, K.M. et al., 2003. Neutrophil Chemotactic Activity of Sputum From Patients With COPD: Role of Interleukin 8 and Leukotriene B4. *Chest*, 123(4), pp.1240–1247.

Berenson, C.S. et al., 2006. Impaired Phagocytosis of Nontypeable Haemophilus influenzae by Human Alveolar Macrophages in Chronic Obstructive Pulmonary Disease. *Journal of Infectious Diseases* , 194(10), pp.1375–1384. Available at: http://jid.oxfordjournals.org/content/194/10/1375.abstract.

Bergamini, A. et al., 2000. Granulocyte–macrophage colony-stimulating factor regulates cytokine production in cultured macrophages through CD14-dependent and -independent mechanisms. *Immunology*, 101(2), pp.254–261. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2327074/.

Beyer, M. et al., 2012. High-Resolution Transcriptome of Human Macrophages A. Zirlik, ed. *PLoS ONE*, 7(9), p.e45466. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3448669/.

Bidan, C.M. et al., 2015. Airway and Extracellular Matrix Mechanics in COPD. *Frontiers in Physiology*, 6, p.346. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4667091/.

Biswas, S.K. et al., 2006. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). *Blood*, 107(5), p.2112 LP-2122. Available at: http://www.bloodjournal.org/content/107/5/2112.abstract.

Biswas, S.K. & Lopez-Collazo, E., 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in Immunology*, 30(10), pp.475–487.

Biswas, S.K. & Mantovani, A., 2010. Macrophage plasticity and interaction with lymphocyte

subsets: cancer as a paradigm. *Nat Immunol*, 11(10), pp.889–896. Available at: http://dx.doi.org/10.1038/ni.1937.

Black, P.N. et al., 2008. Changes in elastic fibres in the small airways and alveoli in COPD. *European Respiratory Journal*, 31(5), p.998 LP-1004. Available at: http://erj.ersjournals.com/content/31/5/998.abstract.

Blidberg, K. et al., 2012. Increased neutrophil migration in smokers with or without chronic obstructive pulmonary disease. *Respirology (Carlton, Vic.)*, 17(5), pp.854–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22509802 [Accessed September 2, 2016].

de Boer, W.I. et al., 2000. Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *The Journal of Pathology*, 190(5), pp.619–626. Available at: http://dx.doi.org/10.1002/(SICI)1096-9896(200004)190:5<619::AID-PATH555>3.0.CO.

Botelho, F.M. et al., 2011. IL-1α/IL-1R1 Expression in Chronic Obstructive Pulmonary Disease and Mechanistic Relevance to Smoke-Induced Neutrophilia in Mice. *PLoS ONE*, 6(12), p.e28457. Available at: http://dx.doi.org/10.1371%2Fjournal.pone.0028457.

Boyle, J.J. et al., 2009. Coronary Intraplaque Hemorrhage Evokes a Novel Atheroprotective Macrophage Phenotype. *The American Journal of Pathology*, 174(3), pp.1097–1108.

Boyle, J.J., 2005. Macrophage activation in atherosclerosis: pathogenesis and pharmacology of plaque rupture. *Curr Vasc Pharmacol*, 3. Available at: http://dx.doi.org/10.2174/1570161052773861.

Braganza, D. & Bennett, M., 2001. New insights into atherosclerotic plaque rupture. *Postgraduate Medical Journal*, 77(904), pp.94–98. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1741926/.

British Lung Foundation, 2016. The Battle for Breath Report.

Brown, V. et al., 2009. Dysregulated apoptosis and NFκB expression in COPD subjects. *Respiratory Research*, 10(1), p.24. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2667166/.

Buhtoiarov, I.N. et al., 2006. Synergistic Activation of Macrophages via CD40 and TLR9 Results in T Cell Independent Antitumor Effects. *The Journal of Immunology* , 176(1), pp.309–318. Available at: http://www.jimmunol.org/content/176/1/309.abstract.

Buist, A.S. et al., 2007. International variation in the prevalence of COPD (The BOLD Study): a population-based prevalence study. *The Lancet*, 370(9589), pp.741–750.

Burton, P.A. & Dixon, M.F., 1969. A comparison of changes in the mucous glands and goblet cells of nasal, sinus, and bronchial mucosa. *Thorax*, 24(2), pp.180–185. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC471938/.

Byers, D.E. & Holtzman, M.J., 2011. Alternatively Activated Macrophages and Airway Disease. *Chest*, 140(3), pp.768–774. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3168852/.

Calderon, B. et al., 2015. The pancreas anatomy conditions the origin and properties of resident macrophages. *The Journal of Experimental Medicine* , 212(10), pp.1497–1512. Available at: http://jem.rupress.org/content/212/10/1497.abstract.

Cao, S. et al., 2005. The Protooncogene c-Maf Is an Essential Transcription Factor for IL-10 Gene Expression in Macrophages. *Journal of immunology (Baltimore, Md. : 1950)*, 174(6), pp.3484–3492. Available at:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2955976/.

Capelli, A. et al., 1999. Increased MCP-1 and MIP-1beta in bronchoalveolar lavage fluid of chronic bronchitics. *European Respiratory Journal*, 14(1), p.160 LP-165. Available at: http://erj.ersjournals.com/content/14/1/160.abstract.

Caramori, G. et al., 2003. Nuclear localisation of p65 in sputum macrophages but not in sputum neutrophils during COPD exacerbations. *Thorax*, 58(4), pp.348–351. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1746629/.

Cazzola, M. & Hanania, N.A., 2006. The role of combination therapy with corticosteroids and long-acting β(2)-agonists in the prevention of exacerbations in COPD. *International Journal of Chronic Obstructive Pulmonary Disease*, 1(4), pp.345–354. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2707808/.

Chana, K.K. et al., 2016. Identification of a distinct glucocorticosteroid-insensitive pulmonary macrophage phenotype in patients with chronic obstructive pulmonary disease. *Journal of Allergy and Clinical Immunology*, 133(1), p.207–216.e11. Available at: http://dx.doi.org/10.1016/j.jaci.2013.08.044.

Charo, I.F., 2007. Macrophage Polarization and Insulin Resistance: PPARγ in Control. *Cell Metabolism*, 6(2), pp.96–98.

Chawla, A. et al., 2016. A PPARγ-LXR-ABCA1 Pathway in Macrophages Is Involved in Cholesterol Efflux and Atherogenesis. *Molecular Cell*, 7(1), pp.161–171. Available at: http://dx.doi.org/10.1016/S1097-2765(01)00164-2.

Chawla, A., 2010. Control of macrophage activation and function by PPARs. *Circulation research*, 106(10), pp.1559–1569. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2897247/.

Chen, H. et al., 2007. Tobacco Smoking Inhibits Expression of Proinflammatory Cytokines and Activation of IL-1R-Associated Kinase, p38, and NF-κB in Alveolar Macrophages Stimulated with TLR2 and TLR4 Agonists. *The Journal of Immunology* , 179(9), pp.6097–6106. Available at: http://www.jimmunol.org/content/179/9/6097.abstract.

Cho, S.W., 2015. Role of Osteal Macrophages in Bone Metabolism. *Journal of Pathology and Translational Medicine*, 49(2), pp.102–104. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4367104/.

Chrysofakis, G. et al., 2004. Perforin Expression and Cytotoxic Activity of Sputum CD8+ Lymphocytes in Patients With COPD. *Chest*, 125(1), pp.71–76.

Coccia, E.M. et al., 1999. STAT1 activation during monocyte to macrophage maturation: role of adhesion molecules. *International Immunology* , 11(7), pp.1075–1083. Available at: http://intimm.oxfordjournals.org/content/11/7/1075.abstract.

Colin, S., Chinetti-Gbaguidi, G. & Staels, B., 2014. Macrophage phenotypes in atherosclerosis. *Immunological reviews*, 262(1), pp.153–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25319333 [Accessed September 2, 2016].

Cosio, B.G. et al., 2004. Theophylline Restores Histone Deacetylase Activity and Steroid Responses in COPD Macrophages. *The Journal of Experimental Medicine*, 200(5), pp.689–695. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2212744/.

Cosio, M.G. et al., 1980. Morphologic and Morphometric Effects of Prolonged Cigarette Smoking on the Small Airways. *American Review of Respiratory Disease*, 122(2), pp.265–271. Available at: http://www.atsjournals.org/doi/abs/10.1164/arrd.1980.122.2.265.

Cosio, M.G., Majo, J. & Cosio, M.G., 2002. Inflammation of the Airways and Lung Parenchyma in COPD: Role of T Cells. *Chest*, 121(5), p.160S–165S.

Cosio, M.G., Saetta, M. & Agusti, A., 2009. Immunologic aspects of chronic obstructive pulmonary disease. *N Engl J Med*, 360.

Costa, C. et al., 2016. Enhanced monocyte migration to CXCR3 and CCR5 chemokines in COPD. *Eur Respir J*, 47, pp.1093–1102. Available at: http://dx.doi.org/10.1183/13993003.01642-2015.

Crofton, R.W., Diesselhoff-Den Dulk, M.M.C. & Furth, R. V, 1978. The origin, kinetics, and characteristics of the kupffer cells in the normal steady state. *The Journal of Experimental Medicine*, 148(1), pp.1–17. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2184923/.

Cros, J. et al., 2010. Human CD14(dim) Monocytes Patrol and Sense Nucleic Acids and Viruses via TLR7 and TLR8 Receptors. *Immunity*, 33(3), pp.375–386. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3063338/.

Culpitt, S. V et al., 2003. Inhibition by red wine extract, resveratrol, of cytokine release by alveolar macrophages in COPD. *Thorax* , 58(11), pp.942–946. Available at: http://thorax.bmj.com/content/58/11/942.abstract.

Culpitt, S.V. et al., 2005. Sputum matrix metalloproteases: comparison between chronic obstructive pulmonary disease and asthma. *Respiratory Medicine*, 99(6), pp.703–710.

Czaja, M.J., Liu, H. & Wang, Y., 2003. Oxidant-induced hepatocyte injury from menadione is regulated by ERK and AP-1 signaling. *Hepatology (Baltimore, Md.)*, 37(6), pp.1405–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12774020 [Accessed September 2, 2016].

Czernielewski, J.M. & Demarchez, M., 1987. Further Evidence for the Self-Reproducing Capacity of Langerhans Cells in Human Skin. *Journal of Investigative Dermatology*, 88(1), pp.17–20. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0022202X87902107 [Accessed September 2, 2016].

Davies, J.Q. & Gordon, S., 2005. Isolation and Culture of Murine Macrophages. In C. D. Helgason & C. L. Miller, eds. *Basic Cell Culture Protocols*. Totowa, NJ: Humana Press, pp. 91–103. Available at: http://dx.doi.org/10.1385/1-59259-838-2:091.

Davies, L.C. et al., 2013. Tissue-resident macrophages. *Nat Immunol*, 14(10), pp.986–995. Available at: http://dx.doi.org/10.1038/ni.2705.

Demirjian, L. et al., 2006. Acute effect of cigarette smoke on TNF-α release by macrophages mediated through the erk1/2 pathway. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1762(6), pp.592–597.

Demkow, U. & van Overveld, F.J., 2010. Role of elastases in the pathogenesis of chronic obstructive pulmonary disease: Implications for treatment. *European Journal of Medical Research*, 15(Suppl 2), pp.27–35. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4360323/.

DeNardo, D.G. et al., 2009. CD4(+) T Cells Regulate Pulmonary Metastasis of Mammary Carcinomas by Enhancing Protumor Properties of Macrophages. *Cancer cell*, 16(2), pp.91–102. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2778576/.

Desch, A.N. et al., 2016. Flow Cytometric Analysis of Mononuclear Phagocytes in Nondiseased Human Lung and Lung-Draining Lymph Nodes. *American Journal of Respiratory and Critical Care Medicine*, 193(6), pp.614–626. Available at:

http://dx.doi.org/10.1164/rccm.201507-1376OC.

Deveci, F. et al., 2004. Airway Wall Thickness in Patients with COPD and Healthy Current Smokers and Healthy Non-Smokers: Assessment with High Resolution Computed Tomographic Scanning. *Respiration*, 71(6), pp.602–610. Available at: http://www.karger.com/DOI/10.1159/000081761.

Droemann, D. et al., 2005. Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients. *Respiratory Research*, 6(1), p.68. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1187924/.

Duluc, D. et al., 2007. Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. *Blood*, 110(13), p.4319 LP-4330. Available at: http://www.bloodjournal.org/content/110/13/4319.abstract.

Dushkin, M.I., 2012. Macrophage/foam cell is an attribute of inflammation: Mechanisms of formation and functional role. *Biochemistry (Moscow)*, 77(4), pp.327–338. Available at: http://dx.doi.org/10.1134/S0006297912040025.

Eierman, D.F., Johnson, C.E. & Haskill, J.S., 1989. Human monocyte inflammatory mediator gene expression is selectively regulated by adherence substrates. *The Journal of Immunology* , 142(6), pp.1970–1976. Available at: http://www.jimmunol.org/content/142/6/1970.abstract.

Epelman, S., Lavine, K.J. & Randolph, G.J., 2014. Origin and Functions of Tissue Macrophages. *Immunity*, 41(1), pp.21–35. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4470379/.

Erbel, C. et al., 2013. An In vitro Model to Study Heterogeneity of Human Macrophage Differentiation and Polarization. *Journal of Visualized Experiments : JoVE*, (76), p.50332. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3727313/.

Erbel, C. et al., 2015. Prevalence of M4 macrophages within human coronary atherosclerotic plaques is associated with features of plaque instability. *International Journal of Cardiology*, 186, pp.219–225.

Erkan, L. et al., 2008. Role of bacteria in acute exacerbations of chronic obstructive pulmonary disease. *International Journal of Chronic Obstructive Pulmonary Disease*, 3(3), pp.463–467. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2629984/.

Farioli-Vecchioli, S. et al., 2001. Catalase immunocytochemistry allows automatic detection of lung type II alveolar cells. *Histochemistry and Cell Biology*, 115(4), pp.333–339. Available at: http://dx.doi.org/10.1007/s004180100259.

Ferrari, R. et al., 2013. Three-year follow-up of Interleukin 6 and C-reactive protein in chronic obstructive pulmonary disease. *Respiratory Research*, 14(1), pp.1–7. Available at: http://dx.doi.org/10.1186/1465-9921-14-24.

Fingerle, G. et al., 1993. The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. *Blood*, 82(10), p.3170 LP-3176. Available at: http://www.bloodjournal.org/content/82/10/3170.abstract.

Finlay, G.A. et al., 1997. Elevated levels of matrix metalloproteinases in bronchoalveolar lavage fluid of emphysematous patients. *Thorax* , 52(6), pp.502–506. Available at: http://thorax.bmj.com/content/52/6/502.abstract.

Fleetwood, A.J. et al., 2009. GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on Type I interferon signaling. *Journal of Leukocyte Biology* , 86(2), pp.411–421. Available at: http://www.jleukbio.org/content/86/2/411.abstract.

Fleming, B.D. & Mosser, D.M., 2011. Regulatory macrophages: setting the threshold for therapy. *European journal of immunology*, 41(9), pp.2498–2502. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4299459/.

Frankenberger, M. et al., 2011. Chemokine Expression by Small Sputum Macrophages in COPD. *Molecular Medicine*, 17(7–8), pp.762–770. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3146610/.

Frankenberger, M. et al., 1996. Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. *Blood*, 87(1), p.373 LP-377. Available at: http://www.bloodjournal.org/content/87/1/373.abstract.

Freeman, C.M. et al., 2010. Cytotoxic potential of lung CD8+ T cells increases with COPD severity and with in vitro stimulation by IL-18 or IL-15. *Journal of immunology (Baltimore, Md. : 1950)*, 184(11), pp.6504–6513. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4098931/.

Geissmann, F., Jung, S. & Littman, D.R., 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*, 19. Available at: http://dx.doi.org/10.1016/S1074-7613(03)00174-2.

Ghosh, B. et al., 2015. Monocyte-derived macrophages from non-smoker COPD subjects exhibit defective bacterial phagocytosis. *European Respiratory Journal*, 46(suppl 59). Available at: http://erj.ersjournals.com/content/46/suppl_59/PA380.abstract.

Gleissner, C.A., 2012. Macrophage Phenotype Modulation by CXCL4 in Atherosclerosis. *Frontiers in Physiology*, 3, p.1. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3257836/.

Goerdt, S. & Orfanos, C.E., 2016. Other Functions, Other Genes: alternative activation of antigen-presenting cells. *Immunity*, 10(2), pp.137–142. Available at: http://dx.doi.org/10.1016/S1074-7613(00)80014-X.

GOLD, 2016. Global Strategy for The Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease. *Global Initiative for Chronic Obstructive Lung Disease. Inc*, pp.1–111.

Gordon, S., 2003. Alternative activation of macrophages. *Nat Rev Immunol*, 3(1), pp.23–35. Available at: http://dx.doi.org/10.1038/nri978.

Grashoff, W.F. et al., 1997. Chronic obstructive pulmonary disease: role of bronchiolar mast cells and macrophages. *The American Journal of Pathology*, 151(6), pp.1785–1790. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1858350/.

Green, G.M. & Carolin, D., 1967. The Depressant Effect of Cigarette Smoke on the in Vitro Antibacterial Activity of Alveolar Macrophages. *New England Journal of Medicine*, 276(8), pp.421–427. Available at: http://dx.doi.org/10.1056/NEJM196702232760801.

Gross, T., 2010. Effects of M-CSF and Adherence on Human Monocyte to Macrophage Differentiation. *University of Regensburg, Dissertation*.

Günthner, R. & Anders, H.-J., 2013. Interferon-Regulatory Factors Determine Macrophage Phenotype Polarization. *Mediators of Inflammation*, 2013, p.731023. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3863528/.

Hackett, T.-L. et al., 2008. Dynamics of pro-inflammatory and anti-inflammatory cytokine release during acute inflammation in chronic obstructive pulmonary disease: an ex vivo study. *Respiratory Research*, 9(1), pp.1–14. Available at: http://dx.doi.org/10.1186/1465-9921-9-47.

Hamann, J. et al., 2007. EMR1, the human homolog of F4/80, is an eosinophil-specific receptor. *European journal of immunology*, 37(10), pp.2797–2802.

Hamilton, J.A., 2008. Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol*, 8(7), pp.533–544. Available at: http://dx.doi.org/10.1038/nri2356.

Haringman, J.J. et al., 2006. A randomized controlled trial with an anti-CCL2 (anti-monocyte chemotactic protein 1) monoclonal antibody in patients with rheumatoid arthritis. *Arthritis and rheumatism*, 54(8), pp.2387–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16869001 [Accessed September 2, 2016].

Hasan, D. et al., 2012. Macrophage imbalance (M1 vs. M2) and upregulation of mast cells in wall of ruptured human cerebral aneurysms: preliminary results. *Journal of Neuroinflammation*, 9(1), pp.1–7. Available at: http://dx.doi.org/10.1186/1742-2094-9-222.

Hepburn, A.L., Mason, J.C. & Davies, K.A., 2004. Expression of Fcγ and complement receptors on peripheral blood monocytes in systemic lupus erythematosus and rheumatoid arthritis. *Rheumatology* , 43(5), pp.547–554. Available at: http://rheumatology.oxfordjournals.org/content/43/5/547.abstract.

Hersh, C.P. et al., 2004. PRedictors of survival in severe, early onset copd*. *Chest*, 126(5), pp.1443–1451. Available at: http://dx.doi.org/10.1378/chest.126.5.1443.

Hiemstra, P.S., 2013. Altered Macrophage Function in Chronic Obstructive Pulmonary Disease. *Annals of the American Thoracic Society*, 10(Supplement), pp.S180–S185. Available at: http://dx.doi.org/10.1513/AnnalsATS.201305-123AW.

Highland, K.B., Strange, C. & Heffner, J.E., 2003. Long-Term Effects of Inhaled Corticosteroids on FEV1 in Patients with Chronic Obstructive Pulmonary DiseaseA Meta-Analysis. *Annals of Internal Medicine*, 138(12), pp.969–973. Available at: http://dx.doi.org/10.7326/0003-4819-138-12-200306170-00008.

Hirose, K. et al., 2011. Different responses to oxidized low-density lipoproteins in human polarized macrophages. *Lipids in Health and Disease*, 10, p.1. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3022593/.

Hodge, G. et al., 2007. Increased intracellular T helper 1 proinflammatory cytokine production in peripheral blood, bronchoalveolar lavage and intraepithelial T cells of COPD subjects. *Clinical and Experimental Immunology*, 150(1), pp.22–9.

Hodge, S. et al., 2003. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. *Immunol Cell Biol*, 81(4), pp.289–296. Available at: http://dx.doi.org/10.1046/j.1440-1711.2003.t01-1-01170.x.

Hodge, S. et al., 2007. Smoking Alters Alveolar Macrophage Recognition and Phagocytic Ability. *American Journal of Respiratory Cell and Molecular Biology*, 37(6), pp.748–755. Available at: http://dx.doi.org/10.1165/rcmb.2007-0025OC.

Hogg, J.C., 2004a. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *The Lancet*, 364(9435), pp.709–721.

Hogg, J.C., 2004b. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet*, 364.

Hogg, J.C., McDonough, J.E. & Suzuki, M., 2013. Small Airway Obstruction in COPD: New Insights Based on Micro-CT Imaging and MRI Imaging. *Chest*, 143(5), pp.1436–1443. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3653349/.

Högger, P. et al., 1998. Identification of the Integral Membrane Protein RM3/1 on Human Monocytes as a Glucocorticoid-Inducible Member of the Scavenger Receptor Cysteine-Rich Family (CD163). *The Journal of Immunology* , 161(4), pp.1883–1890. Available at: http://www.jimmunol.org/content/161/4/1883.abstract.

Holloway, R., Guney, T., et al., 2014. Biomass smoke particles induce cytokines and reduce phagocytosis by monocyte-derived macrophages. *European Respiratory Journal*, 44(Suppl 58). Available at: http://erj.ersjournals.com/content/44/Suppl_58/P1469.abstract.

Holloway, R., Nicholson, G., et al., 2014. Detection of STAT1 phosphorylation in COPD sputum by flow cytometry. *European Respiratory Journal*, 42(Suppl 57). Available at: http://erj.ersjournals.com/content/42/Suppl_57/P625.abstract.

Innes, H.E. et al., 2006. Significance of the metastasis-inducing protein AGR2 for outcome in hormonally treated breast cancer patients. *British Journal of Cancer*, 94(7), pp.1057–1065. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2361240/.

Isik, B., Ceylan, A. & Isik, R., 2007. Oxidative Stress in Smokers and Non-smokers. *Inhalation Toxicology*, 19(9), pp.767–769. Available at: http://dx.doi.org/10.1080/08958370701401418.

Ito, K. et al., 2005. Decreased Histone Deacetylase Activity in Chronic Obstructive Pulmonary Disease. *New England Journal of Medicine*, 352(19), pp.1967–1976. Available at: http://dx.doi.org/10.1056/NEJMoa041892.

Janeway, C. et al., 2001. *Immunobiology, The Immune System in Health and Disease*,

Jenkins, S.J. et al., 2011. Local macrophage proliferation, rather than recruitment from the blood, is a signature of Th2 inflammation. *Science (New York, N.Y.)*, 332(6035), pp.1284–1288. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3128495/.

Johnson, J.L. & Newby, A.C., 2009. Macrophage heterogeneity in atherosclerotic plaques. *Curr Opin Lipidol*, 20. Available at: http://dx.doi.org/10.1097/MOL.0b013e3283309848.

Kadl, A. et al., 2010. Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. *Circulation research*, 107(6), pp.737–746. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2941538/.

Kaku, Y. et al., 2014. Overexpression of CD163, CD204 and CD206 on Alveolar Macrophages in the Lungs of Patients with Severe Chronic Obstructive Pulmonary Disease. *PLoS ONE*, 9(1), p.e87400. Available at: http://dx.doi.org/10.1371%2Fjournal.pone.0087400.

Kamal, A.M. et al., 2002. Effect of fluticasone on the elastase:antielastase profile of the normal lung. *European Journal of Clinical Investigation*, 32(9), pp.713–719. Available at: http://doi.wiley.com/10.1046/j.1365-2362.2002.01060.x [Accessed September 2, 2016].

Kaur, M. et al., 2008. Long-Acting β2-Adrenoceptor Agonists Synergistically Enhance Glucocorticoid-Dependent Transcription in Human Airway Epithelial and Smooth Muscle Cells. *Molecular Pharmacology* , 73(1), pp.203–214. Available at: http://molpharm.aspetjournals.org/content/73/1/203.abstract.

Kaur, M. & Singh, D., 2013. Neutrophil Chemotaxis Caused by Chronic Obstructive Pulmonary Disease Alveolar Macrophages: The Role of CXCL8 and the Receptors CXCR1/CXCR2. *Journal of Pharmacology and Experimental Therapeutics* , 347(1), pp.173–180. Available at: http://jpet.aspetjournals.org/content/347/1/173.abstract.

Keatings, V.M. et al., 1996. Differences in interleukin-8 and tumor necrosis factor-alpha in

induced sputum from patients with chronic obstructive pulmonary disease or asthma. *American Journal of Respiratory and Critical Care Medicine*, 153(2), pp.530–534. Available at: http://dx.doi.org/10.1164/ajrccm.153.2.8564092.

Kent, L. et al., 2008. Cigarette smoke extract induced cytokine and chemokine gene expression changes in COPD macrophages. *Cytokine*, 42(2), pp.205–216.

Khallou-Laschet, J. et al., 2010. Macrophage Plasticity in Experimental Atherosclerosis. *PLoS ONE*, 5(1), p.e8852. Available at: http://dx.doi.org/10.1371%2Fjournal.pone.0008852.

Khodarev, N.N. et al., 2004. STAT1 is overexpressed in tumors selected for radioresistance and confers protection from radiation in transduced sensitive cells. *Proceedings of the National Academy of Sciences of the United States of America*, 101(6), pp.1714–1719. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC341831/.

Kim, J.I. et al., 1999. The Transcription Factor c-Maf Controls the Production of Interleukin-4 but Not Other Th2 Cytokines. *Immunity*, 10(6), pp.745–751.

King, P.T., MacDonald, M. & Bardin, P.G., 2013. Bacteria in COPD; their potential role and treatment. *Translational Respiratory Medicine*, 1(1), pp.1–9. Available at: http://dx.doi.org/10.1186/2213-0802-1-13.

Korns, D. et al., 2011. Modulation of Macrophage Efferocytosis in Inflammation. *Frontiers in Immunology*, 2, p.57. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3342042/.

Kunz, L.I. et al., 2010. Macrophage Heterogeneity and Soluble Mediators in Sputum And Bronchoalveolar Lavage from Current Smokers And Ex-smokers With COPD. In *B105. CHRONIC OBSTRUCTIVE PULMONARY DISEASE: PATHOGENESIS AND MECHANISMS OF DISEASE*. American Thoracic Society International Conference Abstracts. American Thoracic Society, pp. A3867–A3867. Available at: http://dx.doi.org/10.1164/ajrccm-conference.2010.181.1_MeetingAbstracts.A3867.

Kunz, L.I.Z. et al., 2011. Smoking status and anti-inflammatory macrophages in bronchoalveolar lavage and induced sputum in COPD. *Respiratory Research*, 12(1), p.34. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3072953/.

Lacey, D.C. et al., 2012. Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models. *Journal of Immunology*, 188(11), pp.5752–65.

Lawrence, T. & Natoli, G., 2011. Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat Rev Immunol*, 11(11), pp.750–761. Available at: http://dx.doi.org/10.1038/nri3088.

Lebre, M.C. et al., 2011. Why CCR2 and CCR5 Blockade Failed and Why CCR1 Blockade Might Still Be Effective in the Treatment of Rheumatoid Arthritis. *PLoS ONE*, 6(7), p.e21772. Available at: http://dx.doi.org/10.1371%2Fjournal.pone.0021772.

van Leeuwen, D.M. et al., 2005. Differential Gene Expression in Human Peripheral Blood Mononuclear Cells Induced by Cigarette Smoke and Its Constituents. *Toxicological Sciences* , 86(1), pp.200–210. Available at: http://toxsci.oxfordjournals.org/content/86/1/200.abstract.

Lemaire, I. & Yang, H., 1996. M-CSF and GM-CSF promote alveolar macrophage differentiation into multinucleated giant cells with distinct phenotypes. *Journal of Leukocyte Biology*, 60(October).

Li, F. et al., 2015. CCR5 Blockade Promotes M2 Macrophage Activation and Improves Locomotor Recovery After Spinal Cord Injury in Mice. *Inflammation*, 38(1), pp.126–133.

Available at: http://dx.doi.org/10.1007/s10753-014-0014-z.

Liang, Z. et al., 2014. Impaired macrophage phagocytosis of bacteria in severe asthma. *Respiratory Research*, 15(1), p.72. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4086996/.

Lin, E.Y. & Pollard, J.W., 2007. Tumor-Associated Macrophages Press the Angiogenic Switch in Breast Cancer. *Cancer Research*, 67(11), p.5064 LP-5066. Available at: http://cancerres.aacrjournals.org/content/67/11/5064.abstract.

Löfdahl, J.M., Wahlström, J. & Sköld, C.M., 2006. Different inflammatory cell pattern and macrophage phenotype in chronic obstructive pulmonary disease patients, smokers and non-smokers. *Clinical and Experimental Immunology*, 145(3), pp.428–437. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1809704/.

Loke, P. et al., 2007. Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. *Journal of immunology (Baltimore, Md. : 1950)*, 179(6), pp.3926–3936.

Ludwig, A. et al., 1997. The CXC-Chemokine Neutrophil-Activating Peptide-2 Induces Two Distinct Optima of Neutrophil Chemotaxis by Differential Interaction With Interleukin-8 Receptors CXCR-1 and CXCR-2. *Blood*, 90(11), p.4588 LP-4597. Available at: http://www.bloodjournal.org/content/90/11/4588.abstract.

Ma, J. et al., 2003. Regulation of macrophage activation. *Cellular and molecular life sciences : CMLS*, 60(11), pp.2334–46.

MacNee, W., 2005. Pulmonary and Systemic Oxidant/Antioxidant Imbalance in Chronic Obstructive Pulmonary Disease. *Proceedings of the American Thoracic Society*, 2(1), pp.50–60. Available at: http://www.atsjournals.org/doi/abs/10.1513/pats.200411-056SF.

MacNee, W. et al., 1989. The Effect of Cigarette Smoking on Neutrophil Kinetics in Human Lungs. *New England Journal of Medicine*, 321(14), pp.924–928. Available at: http://dx.doi.org/10.1056/NEJM198910053211402.

Maini, R.N. et al., 1995. Beneficial effects of tumour necrosis factor-alpha (TNF-alpha) blockade in rheumatoid arthritis (RA). *Clinical and Experimental Immunology*, 101(2), pp.207–212. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1553280/.

Malhotra, D. et al., 2008. Decline in NRF2-regulated Antioxidants in Chronic Obstructive Pulmonary Disease Lungs Due to Loss of Its Positive Regulator, DJ-1. *American Journal of Respiratory and Critical Care Medicine*, 178(6), pp.592–604. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2542433/.

Mantovani, A. et al., 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in Immunology*, 23(11), pp.549–555.

Mantovani, A. et al., 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*, 23. Available at: http://dx.doi.org/10.1016/S1471-4906(02)02302-5.

Mantovani, A. et al., 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology*, 25(12), pp.677–686.

Martí-Lliteras, P. et al., 2009. Nontypeable Haemophilus influenzae Clearance by Alveolar Macrophages Is Impaired by Exposure to Cigarette Smoke . *Infection and Immunity*, 77(10), pp.4232–4242. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2747942/.

Martin, C.J., Peters, K.N. & Behar, S.M., 2014. Macrophages Clean Up: Efferocytosis and Microbial Control. *Current opinion in microbiology*, 0, pp.17–23. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3942671/.

Martinez, F.O. et al., 2006. Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression. *The Journal of Immunology* , 177(10), pp.7303–7311. Available at: http://www.jimmunol.org/content/177/10/7303.abstract.

Martinez, F.O. & Gordon, S., 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Reports*, 6, p.13. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3944738/.

Martinez, F.O., Helming, L. & Gordon, S., 2009. Alternative Activation of Macrophages: An Immunologic Functional Perspective. *Annual Review of Immunology*, 27(1), pp.451–483. Available at: http://dx.doi.org/10.1146/annurev.immunol.021908.132532.

Marwick, J.A. et al., 2004. Cigarette Smoke Alters Chromatin Remodeling and Induces Proinflammatory Genes in Rat Lungs. *American Journal of Respiratory Cell and Molecular Biology*, 31(6), pp.633–642. Available at: http://dx.doi.org/10.1165/rcmb.2004-0006OC.

McIvor, R.A., Tunks, M. & Todd, D.C., 2011. COPD. *BMJ Clinical Evidence*, 2011, p.1502. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3275305/.

Melgert, B.N. et al., 2013. Shifts In Monocyte Subsets In COPD: Age Or Disease-Related? In *A23. CHRONIC OBSTRUCTIVE PULMONARY DISEASE: PATHOGENESIS*. American Thoracic Society International Conference Abstracts. American Thoracic Society, pp. A1107–A1107. Available at: http://dx.doi.org/10.1164/ajrccm-conference.2013.187.1_MeetingAbstracts.A1107.

Menezes, A.M.B. et al., 2011. Tratamento farmacológico da DPOC. *Jornal Brasileiro de Pneumologia*, 37, pp.527–543.

Mercer, B.A. & D'Armiento, J.M., 2006. Emerging role of MAP kinase pathways as therapeutic targets in COPD. *International Journal of Chronic Obstructive Pulmonary Disease*, 1(2), pp.137–150. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2706609/.

Mestas, J. & Hughes, C.C.W., 2004. Of Mice and Not Men: Differences between Mouse and Human Immunology. *The Journal of Immunology* , 172(5), pp.2731–2738. Available at: http://www.jimmunol.org/content/172/5/2731.abstract.

Metchnikoff, É., 1905. Immunity in Infective Diseases (translated from the French by Frances G Binnie). *Cambridge University Press*.

Mills, C.D. et al., 2000. M-1/M-2 Macrophages and the Th1/Th2 Paradigm. *The Journal of Immunology* , 164(12), pp.6166–6173. Available at: http://www.jimmunol.org/content/164/12/6166.abstract.

Mirzadegan, T. et al., 2000. Identification of the Binding Site for a Novel Class of CCR2b Chemokine Receptor Antagonists: BINDING TO A COMMON CHEMOKINE RECEPTOR MOTIF WITHIN THE HELICAL BUNDLE . *Journal of Biological Chemistry* , 275(33), pp.25562–25571. Available at: http://www.jbc.org/content/275/33/25562.abstract.

Mittman, C. & Taylor, J.C., 1985. Pulmonary Emphysema and Proteolysis. *American Review of Respiratory Disease*, 132(2), p.205. Available at: http://www.atsjournals.org/doi/abs/10.1164/arrd.1985.132.2.205.

Mogensen, S.C., 1979. Role of macrophages in natural resistance to virus infections. *Microbiological Reviews*, 43(1), pp.1–26. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC281459/.

Moliva, J.I. et al., 2014. Molecular composition of the alveolar lining fluid in the aging lung. *Age*, 36(3), p.9633. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4082594/.

Monsó, E. et al., 1995. Bacterial infection in chronic obstructive pulmonary disease. A study of stable and exacerbated outpatients using the protected specimen brush. *American Journal of Respiratory and Critical Care Medicine*, 152(4), pp.1316–1320. Available at: http://dx.doi.org/10.1164/ajrccm.152.4.7551388.

Montaño, M. et al., 2014. FEV1 inversely correlates with metalloproteinases 1, 7, 9 and CRP in COPD by biomass smoke exposure. *Respiratory Research*, 15(1), pp.1–7. Available at: http://dx.doi.org/10.1186/1465-9921-15-74.

Moore, K., Sheedy, F. & Fisher, E., 2013. Macrophages in atherosclerosis: a dynamic balance. *Nature reviews. Immunology*, 13(10), pp.709–721. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4357520/.

Mosser, D.M. & Edwards, J.P., 2008. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology*, 8(12), pp.958–969. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2724991/.

Mulier, B. et al., 1998. Hydrogen peroxide-induced epithelial injury: the protective role of intracellular nonprotein thiols (NPSH). *European Respiratory Journal*, 11(2), p.384 LP-391. Available at: http://erj.ersjournals.com/content/11/2/384.abstract.

Murray, P.J. et al., 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*, 41(1), pp.14–20. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4123412/.

Murray, P.J. & Wynn, T.A., 2011. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*, 11(11), pp.723–737. Available at: http://dx.doi.org/10.1038/nri3073.

Natoli, G., Ghisletti, S. & Barozzi, I., 2011. The genomic landscapes of inflammation. *Genes & Development*, 25(2), pp.101–106. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3022255/.

NICE, 2010. Chronic obstructiv obstructive e pulmonary disease in o v er 16s : diagnosis and management. , (June), pp.1–62.

Niewoehner, D.E. et al., 1999. Effect of Systemic Glucocorticoids on Exacerbations of Chronic Obstructive Pulmonary Disease. *New England Journal of Medicine*, 340(25), pp.1941–1947. Available at: http://dx.doi.org/10.1056/NEJM199906243402502.

Noguera, A. et al., 2004. Expression of Adhesion Molecules During Apoptosis of Circulating Neutrophils in COPD. *Chest*, 125(5), pp.1837–1842.

Notter, R.H., 2000. *Lung Surfactants: Basic Science and Clinical Applications*,

O'Shaughnessy, T.C. et al., 1997. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *American Journal of Respiratory and Critical Care Medicine*, 155(3), pp.852–857. Available at: http://dx.doi.org/10.1164/ajrccm.155.3.9117016.

Ohnishi, K. et al., 1998. Matrix metalloproteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema. *Laboratory investigation; a journal of*

technical methods and pathology, 78(9), pp.1077–1087. Available at: http://europepmc.org/abstract/MED/9759652.

Parameswaran, N. & Patial, S., 2010. Tumor Necrosis Factor-α Signaling in Macrophages. *Critical reviews in eukaryotic gene expression*, 20(2), pp.87–103. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3066460/.

Partrick, D.A., Banerjee, A. & Harken, A.H., 1997. Adaptive and Maladaptive Cellular Priming. *Annals of Surgery*, 226(5), pp.587–598.

Passlick, B., Flieger, D. & Ziegler-Heitbrock, H.W., 1989. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood*, 74(7), p.2527 LP-2534. Available at: http://www.bloodjournal.org/content/74/7/2527.abstract.

Pesci, A. et al., 1998. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *European Respiratory Journal*, 12(2), p.380 LP-386. Available at: http://erj.ersjournals.com/content/12/2/380.abstract.

Pessina, G.P. et al., 1993. Chronic cigarette smoking enhances spontaneous release of tumour necrosis factor-α from alveolar macrophages of rats. *Mediators of Inflammation*, 2(6), pp.423–428. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2365436/.

Petit-Bertron, A. et al., 2003. Adherence influences monocyte responsiveness to interleukin-10. *Journal of leukocyte …*, 73(January).

Petit-Bertron, A.-F. et al., 2005. Adherence modifies the regulation of gene expression induced by interleukin-10. *Cytokine*, 29(1), pp.1–12.

Philippot, Q. et al., 2014. Increased Iron Sequestration in Alveolar Macrophages in Chronic Obtructive Pulmonary Disease. *PLoS ONE*, 9(5), p.e96285. Available at: http://dx.doi.org/10.1371%2Fjournal.pone.0096285.

Pitsiou, G. et al., 2002. Tumor necrosis factor—alpha serum levels, weight loss and tissue oxygenation in chronic obstructive pulmonary disease. *Respiratory Medicine*, 96(8), pp.594–598. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0954611102913223 [Accessed September 3, 2016].

Ploeger, D.T. et al., 2013. Cell plasticity in wound healing: paracrine factors of M1/ M2 polarized macrophages influence the phenotypical state of dermal fibroblasts. *Cell Communication and Signaling*, 11(1), p.29.

Pons, A.R. et al., 2005. Phenotypic characterisation of alveolar macrophages and peripheral blood monocytes in COPD. *European Respiratory Journal*, 25(4), p.647 LP-652. Available at: http://erj.ersjournals.com/content/25/4/647.abstract.

Porcheray, F. et al., 2005. Macrophage activation switching: an asset for the resolution of inflammation. *Clinical and Experimental Immunology*, 142(3), pp.481–9.

Pride, N., 2001. Smoking cessation: effects on symptoms, spirometry and future trends in COPD. *Thorax*, 56(Suppl 2), p.ii7-ii10. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1765983/.

Raghuwanshi, S.K. et al., 2012. The chemokine receptors CXCR1 and CXCR2 couple to distinct G protein-coupled receptor kinases to mediate and regulate leukocyte functions. *Journal of immunology (Baltimore, Md. : 1950)*, 189(6), pp.2824–2832. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3436986/.

Rahman, I. et al., 1996. Systemic oxidative stress in asthma, COPD, and smokers. *American Journal of Respiratory and Critical Care Medicine*, 154(4), pp.1055–1060. Available at:

http://dx.doi.org/10.1164/ajrccm.154.4.8887607.

Rajagopalan, S. et al., 1996. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *Journal of Clinical Investigation*, 97(8), pp.1916–1923. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC507261/.

Ray, M.R. et al., 2006. Platelet activation, upregulation of CD11b/CD18 expression on leukocytes and increase in circulating leukocyte-platelet aggregates in Indian women chronically exposed to biomass smoke. *Human & Experimental Toxicology* , 25(11), pp.627–635. Available at: http://het.sagepub.com/content/25/11/627.abstract.

Reid, 1954. Pathology of chronic bronchitis. *Lancet*, pp.275–278.

Rey-Giraud, F., Hafner, M. & Ries, C.H., 2012. <italic>In Vitro</italic> Generation of Monocyte-Derived Macrophages under Serum-Free Conditions Improves Their Tumor Promoting Functions. *PLoS ONE*, 7(8), p.e42656. Available at: http://dx.doi.org/10.1371%2Fjournal.pone.0042656.

Rios, F.J., Touyz, R.M. & Montezano, A.C., 2017. Isolation and Differentiation of Human Macrophages. In R. M. Touyz & E. L. Schiffrin, eds. *Hypertension: Methods and Protocols*. New York, NY: Springer New York, pp. 311–320. Available at: http://dx.doi.org/10.1007/978-1-4939-6625-7_24.

Robb, C.T. et al., 2016. Key mechanisms governing resolution of lung inflammation. *Seminars in Immunopathology*, 38, pp.425–448. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4896979/.

Rose, S., Misharin, A. & Perlman, H., 2012. A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, 81(4), pp.343–350. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3987771/.

Rőszer, T., 2015. Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators of Inflammation*, 2015, pp.1–16. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4452191&tool=pmcentrez&rendertype=abstract\nhttp://www.hindawi.com/journals/mi/2015/816460/.

Roth, M., 2008. Pathogenesis of COPD. Part III. Inflammation in COPD. *International Journal of Tuberculosis and Lung Disease*, 12(4), pp.375–380.

Rothe, G. et al., 1996. Peripheral Blood Mononuclear Phagocyte Subpopulations as Cellular Markers in Hypercholesterolemia. *Arteriosclerosis, Thrombosis, and Vascular Biology* , 16(12), pp.1437–1447. Available at: http://atvb.ahajournals.org/content/16/12/1437.abstract.

Rubin, B.K., 2002. Physiology of airway mucus clearance. *Respiratory care*, 47(7), pp.761–768. Available at: http://europepmc.org/abstract/MED/12088546.

Russell, R.E.K. et al., 2002. Release and Activity of Matrix Metalloproteinase-9 and Tissue Inhibitor of Metalloproteinase-1 by Alveolar Macrophages from Patients with Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory Cell and Molecular Biology*, 26(5), pp.602–609. Available at: http://dx.doi.org/10.1165/ajrcmb.26.5.4685.

Saetta, M., 1999. Airway Inflammation in Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine*, 160, pp.S17–S20. Available at: http://dx.doi.org/10.1164/ajrccm.160.supplement_1.6.

Saha, S. et al., 2009. Granulocyte-macrophage colony-stimulating factor expression in induced sputum and bronchial mucosa in asthma and COPD. *Thorax*, 64(8), pp.671–6.

Available at: http://thorax.bmj.com/content/64/8/671.

Salvi, S.S. & Barnes, P.J., 2009. Chronic obstructive pulmonary disease in non-smokers. *The Lancet*, 374(9691), pp.733–743.

Sandhaus, R.A. & Turino, G., 2013. Neutrophil Elastase-Mediated Lung Disease. *COPD: Journal of Chronic Obstructive Pulmonary Disease*, 10(sup1), pp.60–63. Available at: http://dx.doi.org/10.3109/15412555.2013.764403.

Sasmono, R.T. & Hume, D.A., 2004. The Biology of Macrophages. In *The Innate Immune Response to Infection*. American Society of Microbiology, pp. 71–94. Available at: http://www.asmscience.org/content/book/10.1128/9781555817671.chap4.

Scanlon, P.D. et al., 2000. Smoking Cessation and Lung Function in Mild-to-Moderate Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine*, 161(2), pp.381–390. Available at: http://dx.doi.org/10.1164/ajrccm.161.2.9901044.

Schmidt, D., Joyce, E.J. & Kao, W.J., 2011. Fetal bovine serum xenoproteins modulate human monocyte adhesion and protein release on biomaterials in vitro. *Acta biomaterialia*, 7(2), pp.515–525. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3014429/.

Schneemann, M. & Schoeden, G., 2007. Macrophage biology and immunology: man is not a mouse. *Journal of Leukocyte Biology* , 81(3), p.579. Available at: http://www.jleukbio.org/content/81/3/579.short.

Scullion, J.E., 2007. The development of anticholinergics in the management of COPD. *International Journal of Chronic Obstructive Pulmonary Disease*, 2(1), pp.33–40. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2692120/.

Segal, A.W., 2005. How Neutrophils Kill Microbes. *Annual review of immunology*, 23, pp.197–223. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2092448/.

Segura-Valdez, L. et al., 2000. Upregulation of Gelatinases A and B, Collagenases 1 and 2, and Increased Parenchymal Cell Death in COPD. *Chest*, 117(3), pp.684–694.

Selvarajan, K., 2009. Adherent and Non Adherent Macrophages in Cell Survival and Death. *The Ohio State University, Dissertation*.

Sethi, S. et al., 2006. Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*, 173.

Sethi, S. et al., 2002. New Strains of Bacteria and Exacerbations of Chronic Obstructive Pulmonary Disease. *New England Journal of Medicine*, 347(7), pp.465–471. Available at: http://dx.doi.org/10.1056/NEJMoa012561.

Shapiro, S.D., 2002. Proteinases in chronic obstructive pulmonary disease. *Biochemical Society Transactions*, 30(2), p.98 LP-102. Available at: http://www.biochemsoctrans.org/content/30/2/98.abstract.

Shapiro, S.D., 1999. The Macrophage in Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine*, 160(supplement_1), pp.S29–S32. Available at: http://dx.doi.org/10.1164/ajrccm.160.supplement_1.9.

Shaykhiev, R. et al., 2009. Smoking-dependent Reprogramming of Alveolar Macrophage Polarization: Implication for Pathogenesis of COPD. *Journal of immunology (Baltimore, Md. : 1950)*, 183(4), pp.2867–2883. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2873685/.

Shen, C.-H. & Stavnezer, J., 1998. Interaction of Stat6 and NF-κB: Direct Association and

Synergistic Activation of Interleukin-4-Induced Transcription. *Molecular and Cellular Biology*, 18(6), pp.3395–3404. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC108921/.

Sica, A. & Mantovani, A., 2012. Macrophage plasticity and polarization: in vivo veritas. *The Journal of Clinical Investigation*, 122(3), pp.787–795. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3287223/.

Sindrilaru, A. et al., 2011. An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *The Journal of Clinical Investigation*, 121(3), pp.985–997. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3049372/.

Singh, J. et al., 2002. Corticosteroid therapy for patients with acute exacerbations of chronic obstructive pulmonary disease: A systematic review. *Archives of Internal Medicine*, 162(22), pp.2527–2536. Available at: http://dx.doi.org/10.1001/archinte.162.22.2527.

Sköld, C.M. et al., 1995. Different functional and morphological characteristics in a nonadherent subpopulation of human macrophages recovered by bronchoalveolar lavage. *European Respiratory Journal*, 8(10), pp.1719–1724.

So, E.-Y., Park, H.-H. & Lee, C.-E., 2000. IFN-γ and IFN-α Posttranscriptionally Down-Regulate the IL-4-Induced IL-4 Receptor Gene Expression. *The Journal of Immunology* , 165(10), pp.5472–5479. Available at: http://www.jimmunol.org/content/165/10/5472.abstract.

Soler, N. et al., 1999. Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease. *Eur Respir J*, 14.

Sonetti, D.A., Hospenthal, A.C. & Adams, S.G., 2010. Integrated management strategies for chronic obstructive pulmonary disease. *Journal of multidisciplinary healthcare*, 3, pp.181–188. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3004609/.

St Croix, C.M., Shand, S.H. & Watkins, S.C., 2005. Confocal microscopy: comparisons, applications, and problems. *BioTechniques*, 39(6 Suppl).

Standiford, T.J. et al., 1991. Interleukin-8 Gene Expression from Human Alveolar Macrophages: The Role of Adherence. *American Journal of Respiratory Cell and Molecular Biology*, 5(6), pp.579–585. Available at: http://dx.doi.org/10.1165/ajrcmb/5.6.579.

Stănescu, D. et al., 1996. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax* , 51(3), pp.267–271. Available at: http://thorax.bmj.com/content/51/3/267.abstract.

Stansfield, B.K. & Ingram, D.A., 2015. Clinical significance of monocyte heterogeneity. *Clinical and Translational Medicine*, 4, p.5. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4384980/.

Di Stefano, A. et al., 2002. Increased expression of nuclear factor-κB in bronchial biopsies from smokers and patients with COPD. *European Respiratory Journal*, 20(3), p.556 LP-563. Available at: http://erj.ersjournals.com/content/20/3/556.abstract.

Di Stefano, A. et al., 1998. Severity of Airflow Limitation Is Associated with Severity of Airway Inflammation in Smokers. *American Journal of Respiratory and Critical Care Medicine*, 158(4), pp.1277–1285. Available at: http://dx.doi.org/10.1164/ajrccm.158.4.9802078.

Stein, M. et al., 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The*

Journal of Experimental Medicine, 176(1), pp.287–292. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2119288/.

Stockley, R.A., 2002. Neutrophils and the Pathogenesis of COPD. *Chest*, 121(5), p.151S–155S.

Stöger, J.L. et al., 2012. Distribution of macrophage polarization markers in human atherosclerosis. *Atherosclerosis*, 225(2), pp.461–468.

Stratelis, G. et al., 2004. Early detection of COPD in primary care: screening by invitation of smokers aged 40 to 55 years. *The British Journal of General Practice*, 54(500), pp.201–206. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1314831/.

Strauss-Ayali, D., Conrad, S.M. & Mosser, D.M., 2007. Monocyte subpopulations and their differentiation patterns during infection. *Journal of Leukocyte Biology* , 82(2), pp.244–252. Available at: http://www.jleukbio.org/content/82/2/244.abstract.

Suzuki, M. et al., 2008. Down-Regulated NF-E2–Related Factor 2 in Pulmonary Macrophages of Aged Smokers and Patients with Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory Cell and Molecular Biology*, 39(6), pp.673–682. Available at: http://dx.doi.org/10.1165/rcmb.2007-0424OC.

Takanashi, S. et al., 1999. Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers. *European Respiratory Journal*, 14(2), p.309 LP-314. Available at: http://erj.ersjournals.com/content/14/2/309.abstract.

Tanino, M. et al., 2002. Increased levels of interleukin-8 in BAL fluid from smokers susceptible to pulmonary emphysema. *Thorax* , 57(5), pp.405–411. Available at: http://thorax.bmj.com/content/57/5/405.abstract.

Tarassishin, L., Suh, H.-S. & Lee, S.C., 2011. Interferon regulatory factor 3 plays an anti-inflammatory role in microglia by activating the PI3K/Akt pathway. *Journal of Neuroinflammation*, 8, p.187. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3259120/.

Tarling, J.D., Lin, H.S. & Hsu, S., 1987. Self-renewal of pulmonary alveolar macrophages: evidence from radiation chimera studies. *Journal of Leukocyte Biology* , 42(5), pp.443–446. Available at: http://www.jleukbio.org/content/42/5/443.abstract.

Taylor, A.E. et al., 2010. Defective macrophage phagocytosis of bacteria in COPD. *European Respiratory Journal*, 35(5), p.1039 LP-1047. Available at: http://erj.ersjournals.com/content/35/5/1039.abstract.

Tetley, T. et al., 1987. Effects of cigarette smoking and drugs on respiratory tract proteases and antiproteases. *European Journal of Respiratory Diseases*, 153, pp.93–102.

Tetley, T.D., 2006. Proteinase inhibitors: Secretory Leukoprotease Inhibitor and Elafin. In Elsevier.

Thomas, W.R., Holt, P.G. & Keast, D., 1978. Cigarette smoke and phagocyte function: effect of chronic exposure in vivo and acute exposure in vitro. *Infection and Immunity*, 20(2), pp.468–475. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC421879/.

Thurlbeck, W.M., 1984. The pathobiology and epidemiology of human emphysema. *Journal of Toxicology and Environmental Health*, 13(2–3), pp.323–343. Available at: http://dx.doi.org/10.1080/15287398409530501.

Tolouei Semnani, R. et al., 2014. Human Monocyte Subsets at Homeostasis and Their Perturbation in Numbers and Function in Filarial Infection J. H. Adams, ed. *Infection and Immunity*, 82(11), pp.4438–4446. Available at:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4249311/.

Tomaki, M. et al., 2007. Decreased expression of antioxidant enzymes and increased expression of chemokines in COPD lung. *Pulmonary Pharmacology & Therapeutics*, 20(5), pp.596–605.

Traves, S.L. et al., 2002. Increased levels of the chemokines GROα and MCP-1 in sputum samples from patients with COPD. *Thorax* , 57(7), pp.590–595. Available at: http://thorax.bmj.com/content/57/7/590.abstract.

Traves, S.L. et al., 2004. Specific CXC but not CC chemokines cause elevated monocyte migration in COPD: a role for CXCR2. *Journal of leukocyte biology*, 76(2), pp.441–450.

Tremblay, M.-È. et al., 2011. The Role of Microglia in the Healthy Brain. *The Journal of Neuroscience* , 31(45), pp.16064–16069. Available at: http://www.jneurosci.org/content/31/45/16064.abstract.

Tubby, C. et al., 2011. Immunological basis of reversible and fixed airways disease. *Clinical Science*, 121(7), p.285 LP-296. Available at: http://www.clinsci.org/content/121/7/285.abstract.

Tzanakis, N. et al., 2004. Induced sputum CD8+ T-lymphocyte subpopulations in chronic obstructive pulmonary disease. *Respiratory Medicine*, 98(1), pp.57–65.

Visan, I., 2014. Heart macrophages. *Nat Immunol*, 15(3), p.222. Available at: http://dx.doi.org/10.1038/ni.2837.

Vlahos, R. & Bozinovski, S., 2014. Role of Alveolar Macrophages in Chronic Obstructive Pulmonary Disease. *Frontiers in Immunology*, 5, p.435. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4160089/.

Vlahovic, G. et al., 1999. Cellular and Connective Tissue Changes in Alveolar Septal Walls in Emphysema. *American Journal of Respiratory and Critical Care Medicine*, 160(6), pp.2086–2092. Available at: http://dx.doi.org/10.1164/ajrccm.160.6.9706031.

Wajant, H., Pfizenmaier, K. & Scheurich, P., 2002. Tumor necrosis factor signaling. *Cell Death Differ*, 10(1), pp.45–65. Available at: http://dx.doi.org/10.1038/sj.cdd.4401189.

Waldo, S.W. et al., 2008. Heterogeneity of human macrophages in culture and in atherosclerotic plaques. *The American journal of Pathology*, 172(4), pp.1112–26.

Warrington, R. et al., 2011. An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology*, 7(Suppl 1), p.S1.

Watkins, S.K. et al., 2007. IL-12 Rapidly Alters the Functional Profile of Tumor-Associated and Tumor-Infiltrating Macrophages In Vitro and In Vivo. *The Journal of Immunology* , 178(3), pp.1357–1362. Available at: http://www.jimmunol.org/content/178/3/1357.abstract.

Wedzicha, J.A. & Donaldson, G.C., 2003. Exacerbations of Chronic Obstructive Pulmonary Disease. *Respiratory Care* , 48(12), pp.1204–1215. Available at: http://rc.rcjournal.com/content/48/12/1204.abstract.

Wei, Q. et al., 2014. Regulation of IL-4 Receptor Signaling by STUB1 in Lung Inflammation. *American Journal of Respiratory and Critical Care Medicine*, 189(1), pp.16–29. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3919125/.

Weisheit, C.K., Engel, D.R. & Kurts, C., 2015. Dendritic cells and macrophages: Sentinels in the kidney. *Clinical Journal of the American Society of Nephrology*, 10(10), pp.1841–1851.

Willemse, B.W.M. et al., 2004. The impact of smoking cessation on respiratory symptoms, lung function, airway hyperresponsiveness and inflammation. *European Respiratory Journal*, 23(3), p.464 LP-476. Available at: http://erj.ersjournals.com/content/23/3/464.abstract.

Wise, R.A. et al., 2016. Lack of Effect of Oral Sulforaphane Administration on Nrf2 Expression in COPD: A Randomized, Double-Blind, Placebo Controlled Trial N. Vij, ed. *PLoS ONE*, 11(11), p.e0163716. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5104323/.

Wright, A.K.A. et al., 2009. Pivotal Advance: Expansion of small sputum macrophages in CF: failure to express MARCO and mannose receptors. *Journal of Leukocyte Biology* , 86(3), pp.479–489. Available at: http://www.jleukbio.org/content/86/3/479.abstract.

Wurster, A.L., Tanaka, T. & Grusby, M.J., 2000. The biology of Stat4 and Stat6. *Oncogene*, 19(2000), pp.2577–2584.

Wynn, T.A., Chawla, A. & Pollard, J.W., 2013. Macrophage biology in development, homeostasis and disease. *Nature*, 496(7446), pp.445–455. Available at: http://dx.doi.org/10.1038/nature12034.

Xue, J. et al., 2016. Transcriptome-Based Network Analysis Reveals a Spectrum Model of Human Macrophage Activation. *Immunity*, 40(2), pp.274–288. Available at: http://dx.doi.org/10.1016/j.immuni.2014.01.006.

Yamamoto, C. et al., 1997. Airway Inflammation in COPD Assessed by Sputum Levels of Interleukin-8*. *Chest*, 112(2), pp.505–510.

Yang, Q. et al., 2015. Cross Talk between Histone Deacetylase 4 and STAT6 in the Transcriptional Regulation of Arginase 1 during Mouse Dendritic Cell Differentiation. *Molecular and Cellular Biology*, 35(1), pp.63–75. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4295380/.

Yang, S.-R. et al., 2006. Cigarette smoke induces proinflammatory cytokine release by activation of NF-κB and posttranslational modifications of histone deacetylase in macrophages. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 291(1), p.L46 LP-L57. Available at: http://ajplung.physiology.org/content/291/1/L46.abstract.

Yang, S.-R. et al., 2007. Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-κB in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 292(2), p.L567 LP-L576. Available at: http://ajplung.physiology.org/content/292/2/L567.abstract.

Yew-Booth, L. et al., 2015. JAK–STAT pathway activation in COPD. *European Respiratory Journal*, 46(3), p.843 LP-845. Available at: http://erj.ersjournals.com/content/46/3/843.abstract.

Yona, S. et al., 2013. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*, 38(1), pp.79–91. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3908543/.

Zeng, M. et al., 2013. Local and systemic oxidative stress and glucocorticoid receptor levels in chronic obstructive pulmonary disease patients. *Canadian Respiratory Journal : Journal of the Canadian Thoracic Society*, 20(1), pp.35–41. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3628645/.

Zhang, X., Goncalves, R. & Mosser, D.M., 2008. The Isolation and Characterization of

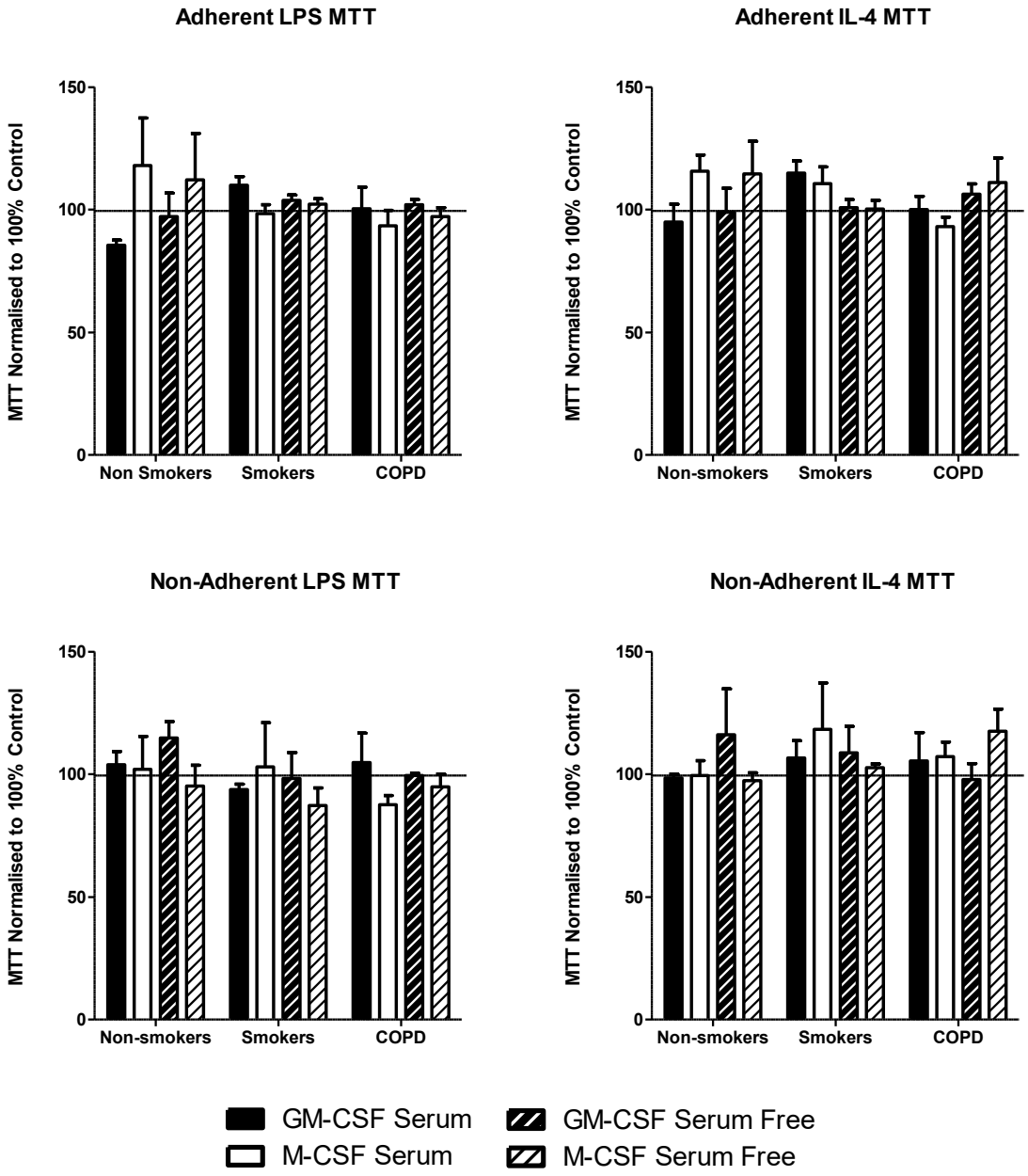
Murine Macrophages. *Current protocols in immunology / edited by John E. Coligan ... [et al.]*, CHAPTER, p.Unit-14.1. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2834554/.

Zhang, Y. et al., 2013. ROS play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages. *Cell Research*, 23(7), pp.898–914. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3698641/.

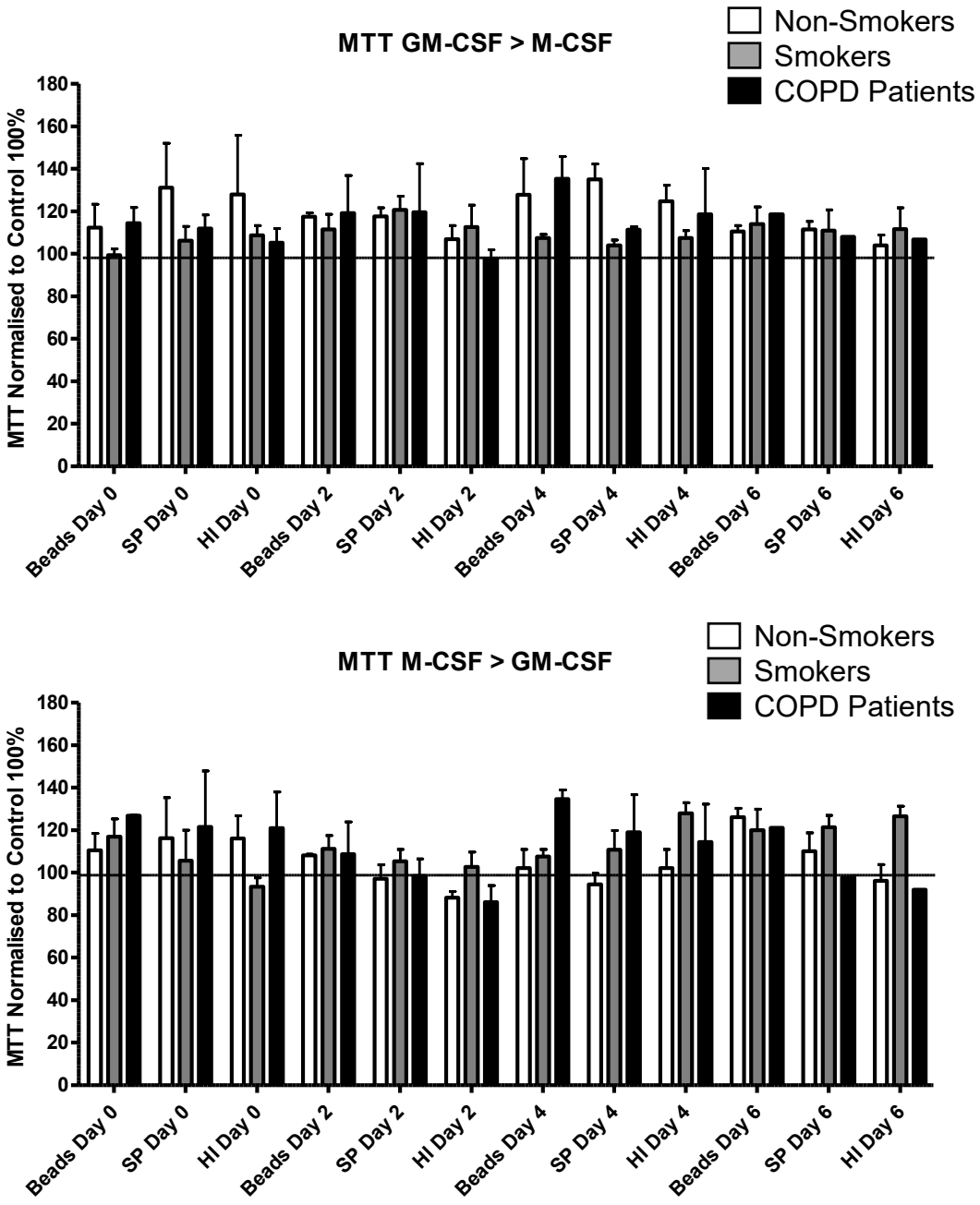
Zheng, X. et al., 2006. Proteomic analysis for the assessment of different lots of fetal bovine serum as a raw material for cell culture. Part IV. Application of proteomics to the manufacture of biological drugs. *Biotechnology progress*, 22(5), pp.1294–1300.

Zhu, J.I.E. et al., 2001. Exacerbations of Bronchitis. *American Journal of Respiratory and Critical Care Medicine*, 164(1), pp.109–116. Available at: http://dx.doi.org/10.1164/ajrccm.164.1.2007050.

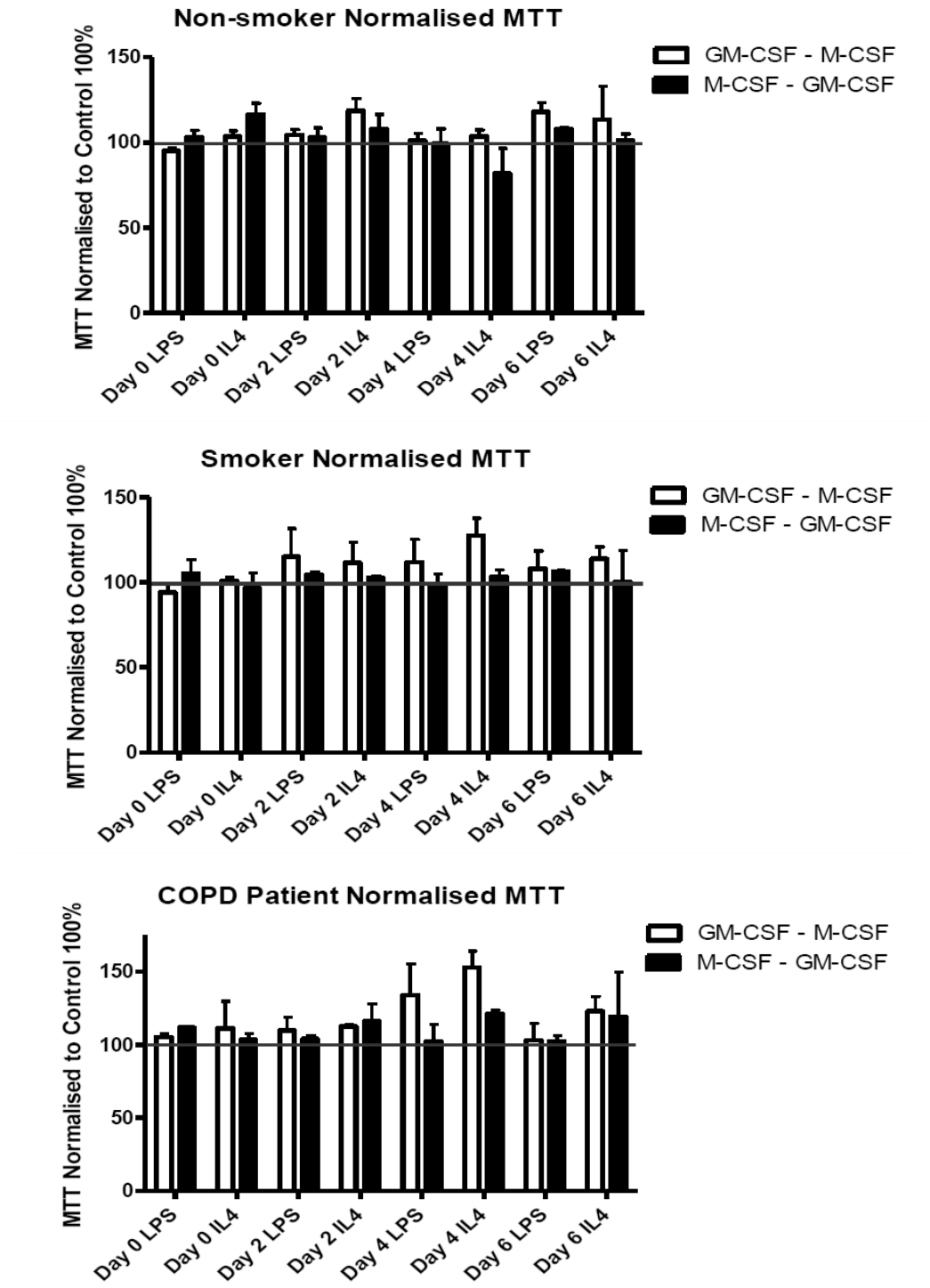
APPENDIX



Appendix Figure 1: LPS and IL-4 Stimulation MDM Condition MTT. LPS or IL-4 stimulated cells used for ELISA experiments in chapter 3 underwent MTT assay to assess cell metabolic activity. Metabolic activity was used to compare treated cells to untreated cells and also different culture conditions as a measure of cell viability. A viability of 100% was assigned to non-stimulated cells and all data reported as a percentage of each specific environmentally matched control. Data presented are mean ± SEM.



Appendix Figure 2: Phagocytosis assay MDM MTT. Cells used for phagocytosis experiments in chapter 4 underwent MTT assay to assess cell metabolic activity. Metabolic activity was used to compare treated cells to untreated cells and also different culture conditions as a measure of cell viability. A viability of 100% was assigned to non-stimulated cells and all data reported as a percentage of each specific environmentally matched control. Data presented are mean ± SEM.



Appendix Figure 3: LPS and IL-4 Stimulation MDM Switch MTT. LPS or IL-4 stimulated cells used for ELISA experiments in chapter 4 underwent MTT assay to assess cell metabolic activity. Metabolic activity was used to compare treated cells to untreated cells and also different culture conditions as a measure of cell viability. A viability of 100% was assigned to non-stimulated cells and all data reported as a percentage of each specific environmentally matched control. Data presented are mean ± SEM.