Characterization of the orphan chemokines CCL18 and CXCL4: potential players in atherosclerosis

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Declaration of Originality

The work in this thesis is my own. Where others have directly contributed to the work presented here, this is acknowledged or referenced in the text.

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Abstract

Atherosclerosis is an inflammatory reaction of the arteries, dependent upon monocyte recruitment into the sub-endothelial space. There, monocytes differentiate or polarize into macrophages which play pivotal roles in disease progression. Differentiation is a hallmark of monocytes and is driven by cytokines signalling. Chemokines are a family of cytokines released during inflammation, chiefly to recruit leukocytes from the circulation. CCL18 and CXCL4 are two poorly characterised chemokines present in atherosclerotic lesions. CXCL4 enhances the survival of monocytes and drives their differentiation into so-called "M4" macrophages with a unique transcriptome. CCL18 is reported to be amongst the genes actively transcribed in M4 macrophages. We hypothesized that CCL18 may play a role in the development of atherosclerosis and set out to further characterise the effects of CCL18 on monocyte function. A main experimental aim was to identify the CCL18 receptor, which despite conflicting reports in the literature, remains undiscovered.

As studies commenced, a brief report was published identifying the chemokine receptor CCR8 as a functional CCL18 receptor. We therefore initially focussed on CCR8 to determine its role in CCL18 signalling. Several CCR8-expressing cell lines were used to probe CCL18 responses by a variety of different assays, including ligand binding, chemotaxis and receptor endocytosis. Although robust responses to CCL1, the principal CCR8 ligand were seen, no significant responses to CCL18 were observed. We therefore conclude that the specific receptor for CCL18 remains unidentified.

We subsequently examined the responses of monocytes to CCL18. Specifically, we examined the ability of freshly isolated monocytes to bind CCL18 on their surface and

to migrate in response to CCL18. Although we found that CCL18 could bind specifically to monocytes, the cells were unable to navigate CCL18 gradients, suggesting that the chemokine may not recruit monocytes *in vivo*. However, we did find that CCL18 was an efficacious monocyte survival factor, enhancing monocyte survival in serum-free media.

To better understand the relationship between M4 differentiation and CCL18 production, we examined the properties of M4 macrophages, directly comparing them to M0 macrophages cultured in the presence of macrophage colony-stimulating factor. M4 macrophages rapidly increased in size and were significantly bigger than M0 macrophages following 7 days of culture. Profiling of cellular supernatants found that enhanced secretion of CCL22 but not CCL18 was a characteristic early event in M4 polarization. We also observed that M4 macrophages scavenged significantly lower levels of oxidised low density lipoprotein (oxLDL) than M0 macrophages throughout polarization, which correlated with lower expression of CD36 mRNA, a well-known oxLDL scavenger. To further illuminate the M4 polarization process, we also examined the expression of 84 transcriptional factors by quantitative polymerase chain reaction (qPCR) and identified two transcription factors as being significantly upregulated during M4 polarization.

In summary, the work described here has enhanced our understanding of the effects of CCL18 and CXCL4 on monocyte function, with potential relevance to the atherosclerotic process. Translating these findings into novel therapeutic approaches should be a key goal of future research.

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Abbreviations

μM	Micrmolar
Ab	Antibody
ACAT1	Acetyl-CoA acetyltransferase, mitochondrial
ABCA1	ATP-binding cassette transporter
AF-CCL18	Alexa Fluor® 647-CCL18
AMAC-1	Alternative macrophage activation-associated CC chemokine-1
ANOVA	Analysis of Variance
APC	Allophycocyanin
ApoB-LP	Apoliprotein B-containing lipoprotein
ATF-1	Activating transcription factor 1
ATF3	Cyclic AMP-dependent transcription factor
ATP	Adenosine Triphosphaste
BAFF	B-cell activating factor
BSA	Bovine serum albumin
BAL	Bronchoalveolar lavage
CASMC	Coronary artery smooth muscle cells
CC	cysteine-cysteine
CCL	Chemokine with a CC motif
CCR	CC chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic acid
CHD	Coronary heart disease
CI	Chemotactic Index
CVD	Cardiovascular disease
C_T	cycle threshold
CXC	Cysteine-X-cysteine
CXCL	chemokine with a CXC motif

CXCR	CXC chemokine receptor
CYP1A1	Human cytochrome P450 1A1
DC	Dendritic Cell
DC-CK1	Dendritic cell-chemokine 1
Dil	fluorescent lipophilic cationic indocarbocyanine dye
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EBV	Epstein–Barr virus
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EGR1	Early growth response protein 1
ELISA	enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESR1	Estrogen receptor alpha
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein
FOXA2	Forkhead box protein A2
FOXG1	Forkhead box protein G1
G418	Geneticin
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenases
GO	Gene ontology
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HA	Human influenza haemaglutanin
HAND2	Heart and neural crest derivatives expressed 2
HDL	High density lipoprotein

HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HIF-1a	Hypoxia-inducible factor 1-alpha
HLA-DR	Human Leukocyte Antigen – antigen D Related
HNF1A	Hepatocyte nuclear factor 1 homeobox A
HO-1	Heme oxygenase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HRP	Horseradish peroxidase
ICAM1	Intracellular adhesion molecule 1
IFN-ɣ	Interferon gamma
IL	Interleukin
iNOS	Nitric oxide synthases
JUN	Jun proto-oncogene, AP-1 transcription factor subunit
kDa	kilodalton
LDL	Low-density lipoprotein
LDLR	Low-Density Lipoprotein (LDL) Receptor
LFA1	Lymphocyte function-associated antigen 1
LOX1	Lectin-like oxidised LDL receptor 1
LXR-beta	Liver X receptor beta
mAb	monoclonal Antibody
MFI	Median / Mean fluorescence intensity
MC148	Molluscum contagiosum
MCP	Monocyte Chemoattractant Protein
M-CSF	Macrophage colony-stimulating factor
MDC	Macrophage-Derived Chemokine
$MgCl_2$	Magnesium Chloride
MHCII	Major histocompatibility complex class II
MIP-1α	Macrophage Inflammatory Proteins-1α
MIP4	Macrophage inflammatory protein-4

MMP	Matrix metalloproteinase
mRNA	Messenger Ribonucleic Acid
NaN_3	Sodium Azide
NFATC2	Nuclear factor of activated T-cells 2
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer cells
Nrf2	Nuclear factor (erythroid-derived 2)-like 2,
OD	Optical density
oxLDL	Oxidised low-density lipoprotein
PARC	Pulmonary and activation-regulated chemokine
PAX6	Paired box protein Pax-6
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	R-phycoerythrin
Pen/Strep	Penicillin/Streptomycin
PerCP	Peridinin-chlorphyll
PH	Pleckstrin-homology
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-biphosphate 3- kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PITPNM3	Membrane-associated phosphatidylinositol transfer protein 3
PRR	Pattern recognition receptor
P-selectin	Platelet selectin
PSGL-1	P-selectin glycoprotein ligand-1
PSI	Plexin-semaphorin-intergrin
PTEN	Phosphatase and tensin homologue deleted on chromosome 10

qPCR	Quantitative polymerase chain reaction
RANKL	Receptor activator of nuclear factor kappa-B ligand
RANTES	Regulated on activation, normal T cell-expressed and secreted
RIN	RNA integrity number
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute media
RT	Real-Time
RT-PCR	Real-Time Poly-chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard errors of the mean
Sema3A	Semaphorin 3A
SMC	Smooth muscle cell
SR-A1	Scavenger receptor A1
SR-B1	Scavenger receptor B1
SREC1	Scavenger receptor expressed by endothelial cells 1
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
TCF21	Transcription factor 21
TCM	Tissue culture media
TFA	Trifuoroacetic acid
Th1	T helper 1 Lymphocyte cells
Th2	T helper 2 Lymphocyte cells
TLR	Toll-like receptor
TLR	Toll-like receptor
TMB	Tetramethylbenzene
TNFα	Tumour Necrosis Factor
Treg	T regulatory 2 Lymphocyte cells
tRNA	Transfer ribonucleic acid
Txnrd	Thioredoxin reductase 1

VCAM	Vascular cell adhesion molecule 1
VLA4	Very late antigen 4
VLDL	Very low density lipoproteins
xFMI	Forward migration index (along the y-axis)
yFMI	Forward migration index (along the y-axis)

1 Introduction

1.1 Atherosclerosis

Atherosclerosis is the medical name for hardening and restricting of the arteries, which is a chronic immune response and one of the leading causes of cardiovascular disease (CVD). Atherosclerosis develops over time, as it is a result of a prolonged process that might start during childhood and progress during adulthood and is characterised by the accumulation of lipids and fibrous elements in the large arteries in what is known as an atherosclerotic plaque. Most adults, especially over the age of 40, are likely to have some degree of atherosclerosis. Atherosclerosis is common for people with a family history of atherosclerosis and cardiovascular disease (CVD). Other factors that increase the risk of developing atherosclerosis are diabetes, high blood pressure, high plasma cholesterol levels, smoking, being overweight or obese, and lack of exercise. A major biggest problem in developing atherosclerosis that it usually causes no visible symptoms until the plaque ruptures, when it lead to several serious conditions such as heart attacks, angina, or strokes (British Heart Foundation, www.bhf.org.uk)

The progression of the atherosclerosis plaque is well documented with the immune system known to play a principal role (Figure 1.1). The earliest stage of the disease is when the endothelium coating the inner layer of the artery is damaged or injured. This can be triggered by a variety of stimuli, including high cholesterol and triglyceride levels, and toxic substances in cigarette smoke. The arterial endothelial cells generally resist attachment of the circulating leukocytes on their surface (Libby et al. 2011). However, when the endothelial cells are injured, they become activated and induce

adhesion molecules such as Vascular Cell Adhesion molecule-1 (VCAM-1) as an inflammatory response to capture leukocytes and direct the migration of the bound leukocytes into the intima to initiate repair (Libby et al. 2002). Parallel changes in endothelial permeability also emerge. This increase in vascular permeability allows leukocytes such as monocytes and T cells to migrate further into the intima. Cholesterol-containing low-density lipoprotein (LDL) particles also flow into the artery wall. (Libby et al. 2011). LDL within the arterial wall can then undergo oxidation in a complex process, with both the ApoB protein component and the lipids of the particle being oxidised by the actions of metal ions such as copper and iron and enzymes such as peroxidases and lipoxygenases (Parthasarathy et al. 2010). LDL damaged by such processes is termed oxidised-LDL (oxLDL) and is readily scavenged by macrophages using a variety of cell surface receptors that recognise the particles. Endocytosed oxLDL can accumulate within the cytoplasm of these cells and these lipid-engorged cells are often referred to as foam cells, which are prone to undergo apoptosis or necrosis.

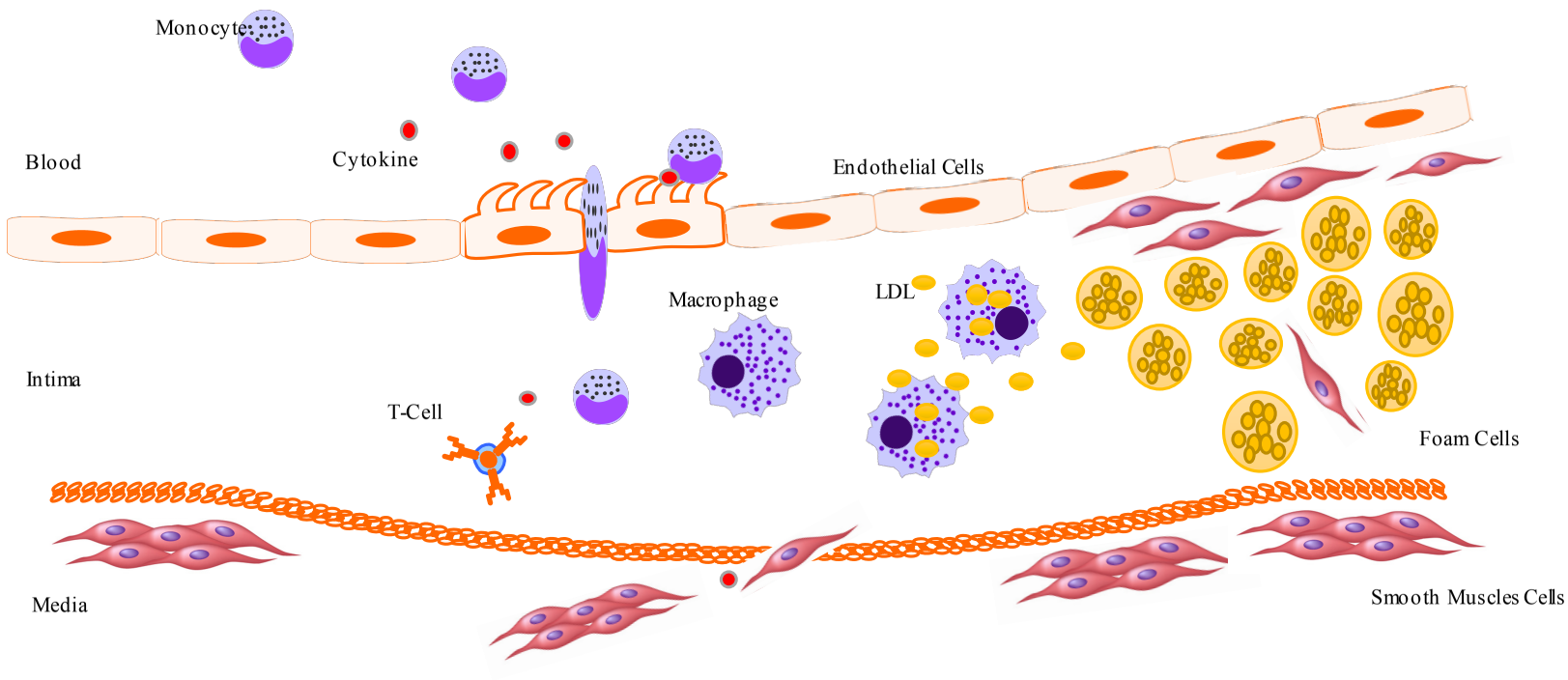


Figure 1.1 Diagram showing atherosclerotic plaque progression.
Migration of leukocytes such as T cells, monocytes, and smooth muscle cells to the injured artery and foam cell formation & the accumulation of foam cells and growth of the plaque, leading to rupture and thrombus formation. Digram prepared with the use of images from motifolio.com.

The accumulation of those foam cells gives rise to a fatty lump or atheroma within the vessel. In a repair process, arterial smooth muscle cells (ASMs) are recruited from the middle layer of the artery wall (tunica media) into the intima where they produce extracellular matrix molecules such as collagen. This process results in the formation of a fibrous cap over the plaque. If this process is imperfect, then the fibrous cap of the plaque can become thinner and unstable, leading it prone to rupture. Once ruptured, the inner necrotic plaque material can induce thrombosis that can interrupt local blood flow or embolize and lodge in other vessels leading to myocardial infarction or stroke (Libby et al. 2011). Plaque stability and plaque regression are therefore clinically desirable.

1.2 Monocytes & Macrophages

When examining all risk factors for developing atherosclerosis, high cholesterol levels in the serum have been found to be uniquely sufficient to encourage atherosclerosis in both humans and animal models (Glass and Witztum 2001). The trafficking of leukocytes to and from atherosclerosis lesions is an essential part of the disease process, both in the induction and maintainance of the atherosclerotic plaque. With increasing evidence for the involvement of monocyte-derived macrophages in the pathogenesis of atherosclerosis, considering the mechanism by which monocytes entering plaques and differentiate adaptively to clear lipid may therefore, be a critical transcriptional decision in the atherosclerotic process (Boyle et al. 2012).

Adult haemopoetic stem cells (HSC) within the bone marrow are the source of peripheral blood monocytes. From these HSC, monocytes can develop which are

released into the circulation, where these cells can constitute 5–10% of the total peripheral-blood leukocytes. In humans, circulating monocytes can be classified into different populations based upon their cell surface expression of CD14 and CD16. Classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{+}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$). Studies over half a century ago identified bone-marrow derived monocytes as the source of tissue macrophages (van Furth et al. 1972) with local growth factors, cytokines, and microbial products driving their differentiation into specialised cells such as dendritic cells (DCs) and osteoclasts (Gordon and Taylor 2005). Recent work, however, has challenged this simple paradigm with fate-mapping studies in the mouse showing that macrophages in the liver and brain originate from fetal liver or yolk sac precursors respectively (Ginhoux et al. 2010) (Yona et al. 2013) (Schulz et al. 2012) .

Macrophages play a leading role as the first line of defence against microbial pathogens and are key cells in the coordination and resolution of inflammatory responses. Their name derives from the Greek for "Big Eater" with phagocytosis a key function. They are well-equipped with a variety of cell surface receptors which allows them to scavenge pathogens and apoptotic cells.

Interestingly, studies of the mononuclear-phagocyte system have shown that there is substantial heterogeneity of macrophage phenotypes, reflecting the specialisation of individual macrophage populations within their microenvironments. (Gordon and Taylor 2005) Monocyte- derived macrophages have been acknowledged to be critical contributors to inflammatory diseases such as atherosclerosis(Moore and Tabas 2011).

1.3 Monocyte–Macrophage Differentiation & Polarization

Macrophages are very adaptable cells, and depending on the local microenvironment they find themselves, can go through substantial changes in gene expression as they differentiate into different phenotypes. In the atherosclerosis setting, circulating monocytes becomes activated following their tethering to the vascular endothelium by adhesion molecules. Subsequently, the cells traverse the endothelial layer and enter the arterial wall where they differentiate, transforming from free rounded floating cells into adherent cells of irregular shape that take up antigen and migrate within the arterial wall (Gleissner 2012).Perhaps the best studied inducer of monocyte–macrophage differentiation is the cytokine Macrophage Colony-Stimulating Factor (M-CSF) (Stanley et al., 1978). M-CSF is continuously present in the circulation and helps to maintain the survival of circulating monocytes (Tushinski et al. 1982, Hanamura et al. 1988). In *in vitro* studies, M-CSF has become the growth factor of choice used to generate monocyte-derived human macrophages. For this reason, the functional and transcriptional level of monocyte macrophage differentiation induced by M-CSF has been closely studied (Martinez et al. 2006, Cho et al. 2007).

When macrophages respond to external stimuli such as cytokines, they polarise by changing their functional characteristics and phenotypic. Polarised macrophages were originally classified as being either an M1 or M2 type. Classically activated macrophages, so called "M1" macrophages are considered pro-inflammatory, as they reflect the Th1 response seen in T cells. The M1 phenotype can be induced by exposure

to lipopolysaccharide (LPS) or interferon- gamma (IFN-γ), and the cells typically respond by expressing IL-1β, IL-6, CXCL8, and tumour necrosis factor-alpha (TNF-α). Alternatively activated or "M2" macrophages were described for the first time by the laboratory of Siamon Gordon in Oxford in1992 (Stein et al. 1992). M2 macrophages are considered anti-inflammatory, as they reflect the Th2 response. M2 macrophages can be further divided into 3 categories M2a, M2b and M2c (Mantovani et al. 2004). Those induced by exposure to IL-4 (M2a), immune complexes (M2b), or IL-13/IL-10 (M2c), and they express IL-10, CD36, scavenger receptor-A, or mannose receptor. (Gordon and Taylor 2005, Martinez et al. 2006, Sica and Mantovani 2009). The paradigm of M1/M2a–c has been extensively studied, resulting in the finding of different phenotypes of polarised macrophages that are not necessarily classified as M1 or M2 macrophages.

Bouhlel et al. (2007) were the first to find evidence of macrophage heterogeneity within human atherosclerotic plaques. They identified that the cell populations were not consistent with plaques containing several macrophage subsets with distinct phenotypic and functional characteristics (Bouhlel et al. 2007). Notably, they detected expression of both the M2 marker mannose receptor and the M1 marker monocyte chemotactic protein-1 (MCP-1) within plaques. Since then several other distinct macrophage phenotypes have been described in human atherosclerotic lesions which will be described later in the chapter. It is likely that our current knowledge regarding macrophage heterogeneity will be significantly expanded by studying the factors that induce macrophage differentiation and polarisation.

1.4 Recruitment of monocytes to the atherosclerotic plaque

1.4.1 Adhesion Molecules

The first step in the recruitment of circulating monocytes to the site of injured endothelium occurs when the monocyte physically interacts with the activated endothelium and forms a loose tether. This is achieved by the expression of molecules such as P-selectin which forms weak transient interactions with its ligand P-selectin glycoprotein ligand-1 (PSGL-1) expressed on the monocyte surface. The forward flow of the blood within the vessel gives rise to rolling of the monocyte along the endothelial surface. Interactions between P-selectin and PSGL-1 also activate integrins and induce monocyte activation and arrest on the endothelial surface (Mestas and Ley 2008). The importance of P-selectin in the recruitment of monocytes to atherosclerotic plaques was highlighted by a study in apo E–deficient mice also lacking the gene for P selectin. These mice showed significantly decreased monocyte to the plaque when compared to P-selectin sufficient mice(Dong et al. 1998).

For the firm adhesion of monocytes to the luminal surface of the endothelium, vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1) binds to very late antigen 4 (VLA4) and lymphocyte function-associated antigen 1 (LFA1), respectively (Moore et al. 2013). Then monocyte transmigrates across the endothelium in response to chemokines secreted by endothelial cells, intimal macrophages, and smooth muscle cells (SMCs). Also of important in the adhesion process is the atypical chemokine CX3CL1 which is expressed on the surface of

activated endothelial cells (Bazan et al. 1997). CX3CL1 acts to induce the firm adhesion of monocytes expressing the receptor CX3CR1(Imai et al. 1997) and its importance to atherosclerosis is highlighted by the CX3CR1-M280A mutation which impairs monocyte adhesion to endothelium and renders homozygotes with a significantly lower risk of CVD (McDermott et al. 2003).

1.4.2 Chemokines & Chemokine receptors

Chemokines are important molecules involved in recruiting leukocytes to distinct tissue locations in both homeostasis and inflammatory disease such as atherosclerosis. Their name is derived from the description chemotactic cytokine which reflects what is perceived to be their principal role, namely directing leukocyte trafficking from the blood to tissues by the process of directed migration or chemotaxis (Zlotnik and Yoshie 2000). Chemokines are typically small proteins, approximately 8 to 11 kilodaltons (kD) in molecular weight, and are often positively charged or basic at physiological pH. Around 40 chemokines have been identified in humans which shared conserved structural characteristics(Martins-Green et al. 2013). In addition to directing leukocyte trafficking, chemokines also play essential roles in other different biological processes, such as haematopoiesis, growth regulation, embryologic development, and angiogenesis (Pease and Horuk 2009a).

The first naming system for chemokines was based on their function, which led to some confusion, with laboratories often giving the same chemokine several different names. In 2000, a new classification system was adopted, with naming system based on the spacing of the first two conserved cysteine residues at the chemokine N-terminus

(Zlotnik and Yoshie 2000). This allows the chemokines to be divided into two main groups. The first group are known as CXC chemokines (CXC ligands or CXCL) and these chemokines have two N-terminal cysteine residues separated by another amino acid (X). The second group, known as CC chemokines (CC ligands or CCL) have two N-terminal cysteine residues adjacent to each other (Zlotnik and Yoshie 2000). Two much smaller classes are the CX3C chemokines which have two N-terminal cysteine residues separated by three amino and the XC chemokines with a single N-terminal cysteine residue. Two XC chemokines have been identified, XCL1 and CXCL2, whilst only one CX3C chemokine is known to exist (CX3CL1). Chemokines function by binding to seven-transmembrane G protein-coupled receptors (GPCRs) found on the surface of target cells(Bachelerie et al. 2014). which typically signal by activating heterotrimeric G-proteins notably G proteins of the Gαi class (Mortier et al., 2012). Chemokine receptors are also grouped into two main families such as CXCR and CCR (CC and CXC receptor), based on the chemokine family they bind to, for example, CXCR1 binds several CXC chemokines, notably CXCL8. A list of human chemokines and their receptors is shown in Table 1.1.

Systematic Name	Receptor usage		Systematic Name	Receptor usage
CCL1	CCR8		CXCL1	CXCR2
CCL2	CCR2		CXCL2	CXCR2
CCL3	CCR1, 5		CXCL3	CXCR2
CCL3L1	CCR1, 5		CXCL4	CXCR3B
CCL4	CCR5		CXCL5	CXCR2
CCL4L1	CCR5		CXCL6	CXCR1, 2
CCL5	CCR1,3,5		CXCL7	CXCR2
CCL7	CCR1,2,3 (CCR5)		CXCL8	CXCR1,2
CCL8	CCR3		CXCL9	CXCR3, (CCR3)
CCL11	CCR3, (CCR2)		CXCL10	CXCR3, (CCR3)
CCL13	CCR2, 3		CXCL11	CXCR3, (CCR3)
CCL14	CCR1		CXCL12	CXCR4
CCL15	CCR1, 3		CXCL13	CXCR5
CCL16	CCR1		CXCL14	
CCL17	CCR4		CXCL16	CXCR6
CCL18	(CCR3)			
CCL19	CCR7		XCL1	XCR1
CCL20	CCR6		XCL2	XCR1
CCL21	CCR7			
CCL22	CCR4		CX3CL1	CX3CR1
CCL23	CCR1			
CCL24	CCR3			
CCL25	CCR9			
CCL26	CCR3, (CCR1,2,5)			
CCL27	CCR10			
CCL28	CCR10, 3			

Table 1.1 Human chemokines and their receptors.

The systematic names, together with the eceptor agonist activity are shown. Antagonist activity at other chemokine receptors is shown in parenthesis. Some human chemokines appear to be missing from the list, e.g. CCL6. In such instances, whilst a chemokine of that name has been identified in the mouse, no human orthologue has been documented.

With respect to atherosclerosis, we know that chemokines play a critical role in the initial recruitment of monocytes to atherosclerotic lesions. The activated endothelium, intimal macrophages, and smooth muscle cells express elevated levels of adhesion molecules and secrete chemokines that direct the transmigration of monocyte across the endothelium into the arterial intima by the leukocyte adhesion cascade. CCL2 is thought to be the main chemotactic factor for monocytes and together with its receptor CCR2, has probably been the most studied chemokine/receptor pair in the context of atherogenesis (Moore et al. 2013). However, chemokines work cooperatively and other chemokines likely play roles alongside CCL2 in monocyte recruitment, including CX3CL1 and CCL5, binding to their respective receptors CX3CR1 and CCR5. When all three axes were abolished by deletion in a murine model of atherosclerosis ($ApoE^{-/-}$ background), a 90% reduction in atherosclerotic lesion size was reported (Combadiere et al. 2008).

1.4.3 Macrophages and foam cell formation in atherosclerosis

As detailed above, monocytes are recruited into the arterial intima where they can differentiate into macrophages and phagocytose a variety of materials including lipoproteins. Uptake of lipoproteins is one of the earliest pathogenic events in the developing plaque. However, persistent uptake of modified forms of LDL via so-called scavenger receptors can result in cholesterol accumulation within the monocyte and the development of foam cells (Yamada 1998, Glass and Witztum 2001, Moore et al. 2013). Foam cells contain free cholesterol as well as cholesterol esters that were formed by hydrolysis in lysosomes. Free cholesterol has several potential metabolic fates,

including esterification in the endoplasmic reticulum (ER), by acetyl-coenzyme A: cholesterol acetyltransferase 1 (ACAT1) which gives rise to the lipid droplets that characterise foam cells (Glass and Witztum 2001, Moore and Tabas 2011). Several receptors may contribute to the scavenging of modified LDL, but the scavenger receptors A (SR-A) and CD36 have been demonstrated to play perhaps the most significant roles in foam cell formation. SR-A1 and CD36 have been shown to mediate between 75-90% of oxLDL degradation *in vitro* (Glass and Witztum 2001, Kunjathoor et al. 2002). Comparisons between Apo E–deficient mice lacking the SR-A or CD36 scavenger receptors and control apo E–deficient mice revealed that lacking SR-A or CD36 receptors had significantly reduced levels of atherosclerosis (Suzuki et al. 1997).

Well-developed foam cells containing relatively large amounts of cholesterol often induce dysregulation in the lipid metabolism of the cell, promoting its apoptosis. Apoptotic cells induce pro-inflammatory signals such as cytokines, reactive oxygen species (ROS), and matrix metalloproteinases (MMPs). This process induces the recruitment of smooth muscles cells to the arterial intima where they can proliferate and secrete collagen, elastin, and proteoglycans, forming a fibrous cap around the dying foam cells (Glass and Witztum 2001). Understanding the mechanisms of monocyte recruitment into plaques and key events in macrophage differentiation and foam cell formation may help to identify a strategy to reduce or delay plaque progression. Moreover, investigating chemokines and their receptors has become a new therapeutic option for atherosclerosis (Graziano and Valeriana 2012).

1.5 Macrophage plasticity in atherosclerosis.

In the atherosclerotic plaque, the two extreme macrophage phenotypes are represented by M1 and M2. However, some macrophages within plaques differentiate into distinct phenotypes which do not fit either the M1 or M2 categories In addition, it has become apparent that depending on the microenvironment, macrophages can change their phenotype and biological functions. The chronic inflammation in atherosclerosis caused by the oxidative tissue damage has consequently led to the finding of several other macrophage phenotypes in the atherosclerotic plaque. However, macrophage phenotypic changes in response to various stimuli have revealed several new macrophage phenotypes distant from M1 & M2 such as Mox, Mhem and M4 macrophages.

In Mice, $Ly6C^{+}$ monocytes in mice are proposed to be precursors of M1 and M2 macrophages subsets in human. These can be divided into $Ly6C^{hi}$ cells which represent 50% of monocytes, and which also express high levels of CCR2 (Swirski et al. 2007, Tacke et al. 2007). Because of their preferential recruitment to sites of inflammation, including atherosclerotic plaques, they are considered to be the M1 macrophage precursors as they correspond to the $CD14^{+}CD16^{-}$ monocyte subset in humans. $Ly6C^{low}$ monocytes express high levels of CX3CR1 and are thought to patrol the vasculature in a homeostatic function and correspond to $CD14^{low}CD16^{+}$ monocyte subset in humans proposed to be precursors of M2 macrophages (Swirski et al. 2007, Tacke et al. 2007, Moore et al. 2013).

In Humans, the classically activated macrophages M1, induced by exposure to LPS, or

IFN-γ show potent pro-inflammatory properties involved in lesion progression. These macrophages express a high level of inducible nitric oxide synthase (iNOS), MHC class II molecules (MHCII), co-stimulatory molecules CD80 and CD86 and pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα. M1 macrophages express the pro-inflammatory transcription factors nuclear factor-κB, activator protein 1 (AP1) and hypoxia-inducible factor 1α (HIF-1α) (Adamson and Leitinger 2011).

The alternatively activated M2 macrophages induced by exposure to IL-4, and IL-13 show strong anti-inflammatory properties involved in lesion regression and are involved in tissue repair and remodelling. These macrophages have a high endocytic activity as they take up and oxidise fatty acids. M2 macrophages express high levels of arginase 1 and have increased secretion of collagen, which promotes tissue repair. They also secret anti-inflammatory molecules such as IL-10 and IL-1 receptor antagonist, and can be distinguished by expression of CD163, mannose receptor 1 (also known as CD206) and FIZZ1. The transcription factors Krüppel-like factor 4, peroxisome proliferator activated receptor-γ and signal transducer and activator of transcription 6 (STAT6) are upregulated in M2(Chinetti-Gbaguidi et al. 2011).

1.5.1 Mox macrophages

After taking up oxidised phospholipids, Kadl et al. found that monocytes can undergo phenotypic changes , resulting in a novel macrophage phenotype distinct from the established M1 and M2 macrophage phenotypes which they called Mox (Kadl et al. 2010). Mox macrophages carry out different biological functions as they have a decreased phagocytotic and chemotactic capacity. Treatment with oxidised

phospholipids induces both M1 and M2 macrophages to switch to the Mox phenotype, highlighting the plasticity of macrophage polarisation. When they compared the differences in gene expression pattern between M1, M2 and Mox macrophages, Kadl et al. established several Mox marker genes, whose expression is mainly mediated by the redox-sensitive transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2). Mox macrophages show anti-oxidant properties, as they express genes involved in the antioxidant responses such as haem oxygenase-1 (HO-1), Thioredoxin reductase-1 (Rxnrd1) and Sulfiredoxin-1 (Srxn1). The Nrf2 target gene HO-1 was shown to be a major survival factor because of its anti-apoptotic and anti-inflammatory effects. Additionally, Kadl et al. found that 30% of all macrophages in advanced atherosclerotic lesions of low-density lipoprotein receptor knockout (LDLR-/-) mice are Mox macrophages. Mox macrophages biological properties suggest this phenotype may play an important role in atherosclerotic lesion development; however, their potentially atheroprotective effect still needs to be demonstrated (Kadl et al. 2010).

1.5.2 Mhem macrophages

Boyle et al. identified a unique state of macrophages within intraplaque haemorrhage (IPH), which they called haemorrhage-specialist macrophages (Mhem). IPH is a common feature of atherosclerotic plaque damage which involves the rupture of neovessels causing blood to escape into the surrounding tissues, leading to cholesterol and haem/iron loading. The monocytes that enter the plaques and differentiate to macrophages to clear haemorrhage-related iron or lipid are known as Mhem macrophages to discriminate them from the classic lipid-laden macrophages (foam

cells) (Boyle et al. 2010).Boyle et al showed that haem induces a set of Mhem specific genes distinct from those that contribute to M1, M2, or Mox cell differentiation, which act to suppress HLA-DR and increase surface CD163 expression. In this paper, they used microarray analysis of human blood-derived monocytes stimulated with haem to identify the mechanism that regulates this functional specialisation. Activating transcription factor 1(ATF-1) was one of the most upregulated genes immediately after stimulation, but subsequently, there was upregulation of effectors genes such as haem oxygenase 1 (HO-1). Dr Boyle's team conducted further transcriptional analysis that also highlighted the transcription factor liver X receptor beta (LXR-β), a "master regulator" of lipid metabolism. They, therefore, hypothesize that ATF-1 directs the production of both LXR-β and HO-1, and expression of a network of genes responsible for lipid and iron handling.

Lastly, they examined serial sections of human plaques to see if they could discriminate Mhem macrophages from foam cells. Mhem were smaller than foam cells, suggesting that they were resistant to becoming foam cells. They also found colocalization of p-ATF-1 with HO-1 and ABCA1 (adenosine-triphosphate-binding-cassette-transporter (ABC) proteins-A1) in Mhem cells but not in foam cells. ABCA1 is a key cholesterol exporter for HDL, indicating that the Haem/ATF-1 pathway drives lipid export and protects cells from becoming foam cells. In IPH macrophages differentiation is a key pathophysiological mechanism dependent on specific signalling with the plaque. Mhem-specific gene expression could explain the functional mechanisms of how cells handle iron and lipid and become atheroprotective macrophages (Boyle et al. 2012).

1.5.3 M4 Macrophages

As previously outlined, in atherosclerotic arteries, blood monocytes likely differentiate into macrophages in the presence of a variety of growth factors and molecules. M-CSF and CXCL4 (also known as Platelet Factor Four) are two growth factors that have been shown *in vitro* to promoter monocyte to macrophage differentiation. The transcriptomes of M-CSF–induced macrophages (M0) have been thoroughly studied and compared to the M1 or M2 polarisation phenotypes, with M0 inducing a transcriptome that is similar to that of M2 macrophages (Martinez et al. 2006). Different studies performed using knockout mice deficient in M-CSF (CSF1) or its receptor (CSF1R) showed that mice were protected from atherogenesis when compared to WT littermates, suggesting that this ligand:receptor axis might drive atherosclerosis (Yoshida et al. 1990, Orekhov et al. 1998). By contrast, the role of the platelet chemokine CXCL4 in macrophage polarisation is a little less clear. The earliest study by Scheuerer et al. on macrophages cultured with CXCL4 reported that the cells were induced to express CD86, but not HLA-DR on the cell surface. Similar to M-CSF, CXCL4 was also demonstrated in the same study to prevent monocyte apoptosis in the absence of serum (Scheuerer et al. 2000).

Gleissner et al. subsequently reported a comprehensive analysis of the transcriptome of monocyte-derived macrophages induced by the chemokine CXCL4 and compared ot with the transcriptome of macrophages induced by M-CSF, defining in the process a new macrophage phenotype they called M4 which was distinct from that induced by M-CSF (Gleissner et al. 2010). The CXCL4-induced transcriptome shares some similarities, with some M1 and M2 genes and the corresponding cytokines expressed

at the protein level; however, their transcriptome clustered with neither M1 nor M2 transcriptomes. Gleissner et al. used different analysis approaches such as gene set enrichment, modified principal component, and hierarchical clustering analysis, and all confirmed the uniqueness of the CXCL4- induced macrophage transcriptome. Most significantly, in M4 macrophages, the chemokines CCL18 and CCL22 were overexpressed compared with M0 macrophages. Assessing the cytokine release in the cell culture supernatant by ELISA confirmed the pattern was seen at the protein level. M4 macrophages IL-6, TNF (both M1), CCL18, and CCL22 (both M2) levels being higher, while IL-10 (M2) levels being higher in M0 macrophages.

Gleissner et al. also used gene orthology (GO) analysis to examine the relative expression of genes associated with processes fundamental to atherogenesis in an attempt to speculate upon the potential relevance of M4 macrophages in disease. Most of the gene groups did not display a consistent pattern, indicating that both pro-atherogenic and anti-atherogenic genes are expressed in M4 macrophages. Strikingly, several genes implicated in foam cell formation, showed differential gene expression between the M4 and M0 groups. M4 macrophages showed higher levels of the cholesterol efflux transporters ABCG1 and lower levels of CD36, scavenger receptors necessary for uptake of modified LDL. Furthermore, they confirmed how this finding might be translated into relevant functional differences by showing signficinatly reduced phagocytosis of modified LDL in M4 macrophages compared with M0 macrophages(Gleissner et al. 2010).

The concept that the M4 macrophage phenotype can be identified within human atherosclerotic lesions and might have pathophysiologic relevance in atherosclerosis

provided new insights into the process of macrophage differentiation by chemokines and may provide novel starting points for further atherosclerosis related research.

Macrophage type	Induced by	Markers	TF's	Function	role in atherosclerotic lesion	CK	CKR	Ref.
Classically activated macrophages M1	LPS, IFN-γ	(iNOS), (MHCII), CD80, CD86, IL-1β, IL-6 and TNFα	TNFκB, (AP1), (HIF-1α)	pro-inflammatory properties	involved in lesion progression.	CCL5 CXCL16 CXCL9,10 ,11 CX3XL1	CCR5 CXCR6 CXCR3 CX3CR1	(Adamson and Leitinger 2011) (Mantovani et al. 2004)
Alternatively activated macrophages M2	IL-4, IL-13	arginase 1, IL-10, IL-1R antagonist, CD163, CD206, FIZZ1	Krüppel-like factor 4, peroxisome proliferator activated receptor-γ and (STAT6)	anti-inflammatory properties	involved in lesion regression and tissue repair and remodelling	CCL24 CCL22 CCL17 CCL18 CCL1 CXCL13	CCR3 CCR4 CCR8 CCR1 CXCR5	(Chinetti-Gbaguidi et al. 2011) (Mantovani et al. 2004)
Mox macrophages	oxidized phospholipids and nitrosylated fatty acids	(HO-1), (Rxnrd1) (Srxn1)	(Nrf2).	anti-oxidant properties	involved in lesion progression.	CCL2 CCL5 CCL7 CCL12	CCR2	(Kadl et al. 2010) (Kadl et al. 2009)
Mhem macrophages	Heme-iron	(HO-1), (LXR-β) ABCA1 CD163	(ATF-1)	anti-inflammatory & anti-oxidant properties	involved in intraplaque haemorrhage (IPH). atheroprotective	ND	ND	(Boyle et al. 2010) (Boyle et al. 2012)
M4 macrophages	CXCL4	CD86 ABCG1	ND	anti-inflammatory	atherosclerotic lesions Reduce foam cells formation	CCL18 CCL22	ND	Petersen et al 2000a) (Gleissner et al. 2010) (Gouwy et al. 2016)

Table 1.2 Characteristics of different polarized macrophage populations. Macrophages polarize and in response to environment-derived signals that acquire different functional properties. Abbreviations ND: not determined; TF's: Transcription Factors; CK: Chemokine; CKR: Chemokine Receptor; Ref: References.

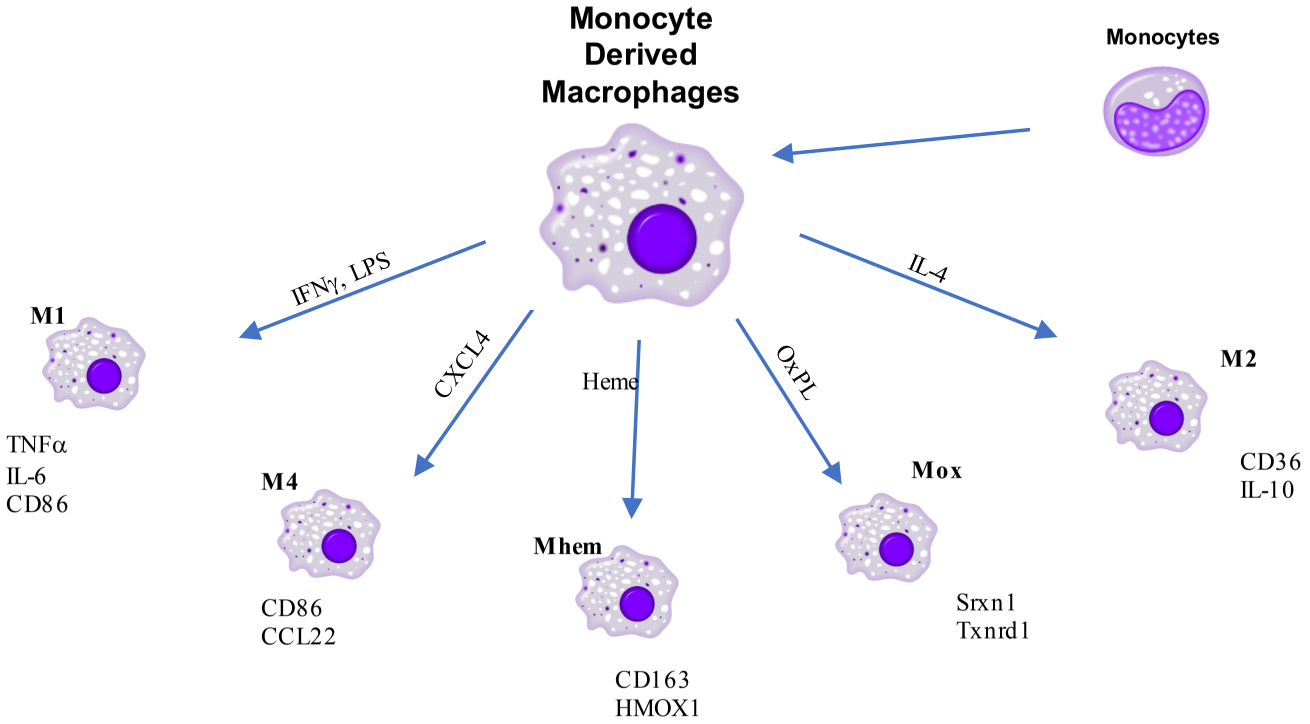


Figure 1.2: Diagram showing macrophage phenotypes described within the atherosclerotic plaque. Depending on the microenvironment, macrophages can change their phenotype and biological functions.

1.6 Atypical chemokines in atherosclerosis

1.6.1 CXCL4 – an atypical platelet-derived chemokine

Platelets, in addition to monocytes and monocyte-derived macrophages, are present within atherosclerotic lesions, and it is now clear that they too contribute to lesion formation. Among the many chemokines released from activated platelets to be identified and sequenced, CXCL4 (formerly known as platelet factor-4) is one of the most abundant. In 1948 Conley noticed that patients with thrombocytopenia had increased sensitivity to heparin (Conley et al. 1948). He found that platelets released a small protein that neutralised the anticoagulant effect of heparin. The protein for this platelet-derived small protein was partially purified in 1957 when Deutsch et al. described it as Platelet Factor 4 (PF4) (Deutsch et al. 1957). It was not until 1977 that the amino acid sequence of CXCL4 was finally discovered following protein sequencing of a heparin-affinity purified protein from platelets (Deuel et al. 1977). Although not appreciated at the time, CXCL4 was actually the first of the family of chemokines to be discovered, with an N-terminal CXC motif making it a member of the CXC family. CXCL4 is preformed and stored within granules of blood platelets and becomes released when platelets degranulate (Deuel et al. 1981). Accordingly, CXCL4 was found to be released in human blood in micromolar concentrations upon platelet activation (Scheuerer et al. 2000).

1.6.2 The biology of CXCL4

CXCL4 has been described as an "enigmatic" chemokine as it lacks the ELR domain seen in many CXC chemokines and its function is not obvious (Gear & Camerini 2003). An early paper suggested that it might be chemotactic for monocytes and neutrophils (Deuel et al. 1981), but this has not been reproduced by others using highly purified chemokine (Pervushina et al. 2004) and it is though that the earlier platelet-preparations of CXCL4 might have been contaminated with other platelet chemokines such as CCL5 and CXCL8. A follow up study by the same group investigating CXCL4-binding sites on PMNs suggested that CXCL4- induced functions were not elicited not through binding to GPCRs, but through an interaction with an chondroitin sulphate proteoglycan expressed on the cell surface of human PMN (Petersen et al. 1999).

CXCL4 has also been shown to mediate the release of histamine by basophils and to play a role in adherence of eosinophils (Brindley et al. 1983). CXCL4 is also involved in long-term differentiation and regulatory processes by enhancing the survival of hematopoietic stem cells as well as of progenitor cells and suppresses the development and maturation of cells from the megakaryopoietic lineage (Han et al. 1990).

The mechanisms by which CXCL4 exerts its effects, notably the inhibition of monocyte apoptosis, and the promotion of monocyte to macrophage differentiation remains poorly understood, with the specific CXCL4 receptor on monocytes as yet unidentified.

1.6.3 CXCL4 in atherosclerosis

During atherogenesis, activated platelets release pro-inflammatory cytokines, such as

IL-1 and chemokines, such as CCL5, CXCL4 and its variant CXCL4L (Gouwy et al. 2016). As outlined above, CXCL4 has been recognised to play an important role in the activation of monocytes and differentiation of monocytes into macrophages which may be important in the development of atherosclerotic lesions. Within atherosclerotic plaques, the presence of CXCL4 was shown to correlate with clinical parameters such as lesion grade or the presence of symptoms, in which high levels of plasma CXCL4 appeared to correlate with lesion severity (Pitsilos et al. 2003). Deletion of the gene encoding CXCL4 in ApoE deficient mice resulted in a 60% reduction in atherosclerotic lesion formation, suggesting a pro-atherogenic role for CXCL4 (Sachais et al. 2007). CXCL4 has also been shown to form heterodimers with the chemokine CCL5 which enhances its capacity to arrest monocytes on activated endothelial cells (Hundelshausen et al. 2005). A small peptide designed to inhibited formation of the CXCL4:CCL4 heterodimer was found to impair the development of atherosclerotic plaques in in ApoE deficient mice, again, suggestive of a pro-atherogenic role for CXCL4 (Koenen et al. 2009).

1.6.4 What is the specific receptor for CXCL4?

CXCL4 takes a specific position within the family of chemotactic cytokines as an atypical chemokine with little evidence for driving leukocyte chemotaxis. CXCL4 has been shown to be capable of binding to the CXCR3 variants CXCR3A and CXCR3B on microvascular endothelial cells (Lasagni et al. 2003) and T lymphocytes (Mueller et al. 2008) although neither receptor has been reported to be expressed by monocytes. CXCL4 has been was reported to induce the down-regulation of CC chemokine

receptors CCR1, CCR2, and CCR5, thereby inhibiting monocyte chemotaxis migration towards several chemokines. The authors of that study suggested that CXCL4 may act as a selective regulator of monocyte migration by stimulating the release of autocrine, receptor-desensitizing chemokine ligands.(Schwartzkopff et al. 2011).

1.6.5 CCL18 – another atypical chemokine implicated in atherosclerosis.

Discovery of CCL18

The chemokine CCL18 was independently discovered by four groups and initially given contrasting and perhaps confusing names. Hieshima et al found CCL18 to be constitutively expressed at high levels in the lung and named it pulmonary and activation-regulated chemokine (PARC)(Hieshima et al. 1997). Adema et al. cloned the CCL18 cDNA from a library generated from DCs and showed that the recombinant chemokine could specifically recruit naive T cells, leading them to name it human dendritic cell CC chemokine or DC-CK1(Adema et al. 1997). Separately, Wells and Peitsch discovered the CCL18 cDNA sequence from an expressed sequence tag (EST) library and named the gene product as macrophage inflammatory protein-4 (MIP-4)(Wells and Peitsch 1997). Finally, CCL18 was also independently cloned from a macrophage cDNA library of macrophages by Kodelja et al. and named alternative macrophage activation-associated CC chemokine-1 (AMAC-1)(Kodelja et al. 1998). Following the new systematical chemokine nomenclature, PARC/MIP-4/DC-CK1/AMAC-1 were renamed CCL18(Zlotnik and Yoshie 2000). Interestingly, analysis of the human and murine genomes suggests there is no direct orthologue of CCL18 in mice (Zlotnik and Yoshie 2012), although CCL8 has been put forward as a functional

orthologue of CCL18 in mice (Islam et al. 2011).

1.6.6 Biology of CCL18

CCL18 has been reported to be expressed by alternatively activated (M2) macrophages (Kodelja et al. 1998) and also by those activated by M4 macrophages(Gleissner et al. 2010). The mature CCL18 protein consists of 69 amino acids and is 63%, 38% and 33% identical to the chemokines CCL3 (MIP-1α), CCL5 (RANTES) and CCL8 (MCP-2), respectively (Figure 1.3). Some chemokines are constitutively expressed at detectable levels in normal human plasma such as CCL14, CCL16, CXCL4, and CXCL7. Interestingly, CCL18 was found to be one of those constitutive plasma chemokines (Schutyser et al. 2005). However, increased CCL18 levels are also found in different inflammatory diseases. For instance, CCL18 levels are selectively enhanced in atherosclerosis (Hägg et al. 2009), pulmonary disorders (Prasse et al. 2006) and dermatitis (Nomura et al. 2003). Most notably, serum CCL18 levels have been reported to be dramatically elevated (an average of 29 fold higher than controls) in patients with Gaucher's disease, a lysosomal storage disorder, leading to its rapid acceptance as a biomarker for diagnosis (Boot et al. 2004).

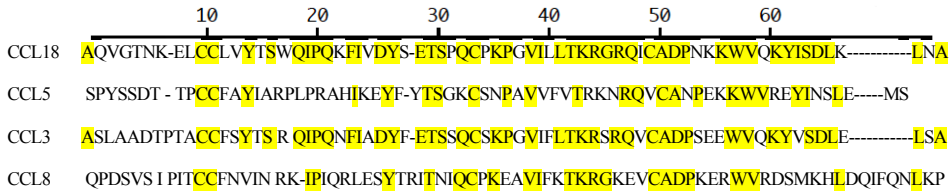


Figure 1.3: Alignment of CCL18 and related CC chemokines.

The mature protein sequences were aligned using the program MegAlign, part of the Lasergene software package (DNASTAR , Madison, WA, USA). CCL18 shares the following identities with each chemokine shown. CCL5 (35.3%), CCL3 (62.3%) and CCL8 31.9%).

Similar to leukocyte responses to many other chemokine, pre-treatment of naïve ($CD45RA^+$) T cells with *pertussis* toxin, a potent inhibitor of Gαi proteins completely abrogated their chemotactic response to CCL18, indicating that the response is via a G protein-coupled receptor (Adema et al. 1997).The authors of that study postulated that CCL18 might, therefore, be used by dendritic cells to preferentially attract naive T cells, which, after recognition of peptide/MHC complexes presented by dendritic cells results in the induction of an adaptive immune response.

Subsequent studies have also found CCL18 to be chemotactic for Th2 cells (de Nadaï et al. 2006), Tregs (Chenivesse et al. 2012) and monocytes (Wimmer et al. 2006) suggesting it has a broad spectrum of targets and may be associated with allergic disease. Another study by Wimmer and colleagues found that when CCL18 was added to serum starved macrophages, they were significantly protected from apoptosis

(Wimmer et al. 2006) which may suggest it may have autocrine effects on macrophages. Notably, freshly isolated monocytes were unresponsive to CCL18 in chemotaxis assays and had to be cultured for 3-4 days before becoming responsive, suggesting unknown factors regulate CCL18 responsiveness.

1.6.7 CCL18 in Atherosclerosis

The role of CCL18 is not at all well studied in atherosclerosis and cardiovascular disease. Since CCL18 is chemotactic for naïve T- and B-cells (Adema et al. 1997, Hieshima et al. 1997), several groups have attempted to studied the potential role of CCL18 in atherogenesis to see if it is also a potential drug target. Two studies Studies have investigated the expression of CCL18 in human atherosclerotic plaques by immunohistochemistry. They concluded that CCL18 is highly expressed in plaques taken from human carotid arteries and it produced by $CD68^+$ macrophages residing in the plaques (Reape et al. 1999, Hägg et al. 2009). Another group have evaluated CCL18 as a circulating biomarker for atherosclerosis and found that high levels of serum CCL18 were an independent predictor of future cardiovascular events such as myocardial infarction (De Sutter et al. 2010).

CCL18 has been associated with the fibrosis process from several studies, both *in vivo* and *in vitro*. CCL18 protein has been shown to be upregulated in the bronchoalveolar lavage (BAL) fluid of patients suffering from idiopathic pulmonary fibrosis, with alveolar macrophages the primary CCL18 source (Prasse et al. 2006). *In vitro*, CCL18 drives the production of collagen by human lung fibroblasts (Atamas et al. 2003), and interestingly, it was shown that collagen could itself induce CCL18 production from

alveolar macrophages, suggesting a positive feedback loop that drives fibrosis(Prasse et al. 2006). As CCL18 is expressed by alternatively activated (M2) macrophages (Kodelja et al. 1998), it would appear to be part of a remodelling process and in the atherosclerotic setting, might be postulated to play a role in the formation of the fibrous cap in the developing lesion.

1.6.8 Identification of a specific receptor for CCL18

In the last few years, three different receptors have been put forward as CCL18 receptors, and the area therefore remains controversial. The chemokine receptor CCR6 was the first to be presented in a conference abstract as a human CCL18 receptor, although notably, no full paper was subsequently published (Zissel et al. 2011). Soon after, Luzina and colleagues showed that intratracheal administration of CCL18 in wild type mice, resulted in T cell recruitment no different from that observed in CCR6 deficient mice, suggesting that CCR6 was not the CCL18 receptor (Luzina and Atamas 2012). These experiments have to be interpreted carefully, as there is no direct homologue of CCL18 in the mouse (Zlotnik and Yoshie 2012). Another receptor, PITPNM3 was put forward as a CCL18 receptor by Chen and workers driving the metastasis of breast tumours (Chen et al. 2011). However, subsequent studies have failed to detect PITPNM3 on leukocytes, suggesting they are not responsible for the effects of CCL18 on these cells (Krohn, Garin, et al. 2013).

In my masters' project in 2012, I expressed both CCR6 and PITPNM3 in a mammalian transfection system and found neither receptor functioned as a chemokine receptor, suggesting the existence of an alternative CCL18 receptor (Hussain, 2012).

In September 2013, the group of Andrew Luster at Harvard University published a paper suggesting that the chemokine receptor CCR8 was a functional receptor for CCL18(Islam et al. 2013). CCR8 was initially described as a receptor for the chemokine CCL1(Roos et al. 1997, Tiffany et al. 1997) and has been the focus of earlier research in the Pease Laboratory at Imperial College London (Najarro et al. 2003, Fox et al. 2006)

1.7 Targeting macrophages in advanced atherosclerotic lesions

Risk factors, including physical inactivity, insulin resistance, obesity, and smoking lead to increased morbidity and mortality of atherosclerotic across the globe. Statins were one of the first drugs that validated the hypothesis that lowering plasma cholesterol levels would reduce coronary heart disease morbidity and mortality (Steinberg 2008). Lifestyle changes and drugs that decrease sub-endothelial cholesterol accumulation by lowering serum cholesterol levels are currently the most efficient and direct way to prevent or treat atherosclerosis. Studies in ApoE deficient mice have shown that the number of monocytes within atherosclerotic lesions can be lowered by complimentary strategies such lowering circulating LDL by ApoE complementation (Potteaux et al. 2011) or by encouraging monocyte egress by the induction of CCR7 on the monocyte surface (Feig et al. 2010). This has led to the notion that if prior to the formation of plaques, circulating levels of lipoproteins could be lowered below a threshold level required for subendothelial retention then perhaps atherosclerosis and the resulting complications could be completely prevented (Moore and Tabas 2011).

It is hoped that a greater understanding of the molecular and cellular mechanisms involved in plaque development and advanced plaque progressions may help the development of novel therapeutics which could be used in the treatment of atherosclerosis. Studies in mice have been critical in helping our understanding of the events surrounding the initiation and development of atherosclerotic plaques. Manipulating monocyte entry into lesions by antibody or gene-targeted neutralisation of chemokines, chemokine receptors, or adhesion molecules has been shown to lessen the atherosclerotic burden in mice (Mestas and Ley 2008). However, since human patients present with advanced atherosclerotic lesions, targeting monocyte recruitment to the plaque is unlikely to reverse key elements of plaque progression, such as necrotic core formation (Moore and Tabas 2011). An efficient strategy may therefore be to therapeutically alter those macrophage processes that are involved in advanced plaque progression. For example, HDL/sterol efflux-based therapy or enhancement of the phagocytic clearance of the dead macrophages, which would result not only in decreased necrotic core formation but also in fewer macrophages in advanced lesions (Tabas, 2010a).

CK	Source of expression	Effect	Pathological Involvement	Putative Receptors	Receptor selectivity	ref
CCL18	dendritic cell macrophage (M2,M4) lung alveolar macrophages	recruit naive T cells, Th2, Tregs, & monocytes Protected monocytes from apoptosis induction of an adaptive immune response fibrosis process production of collagen by human lung fibroblasts	atherosclerosis idiopathic pulmonary fibrosis, dermatitis Gaucher's disease	CCR6 PITPNM3 CCR8	*pertussis* toxin abrogated their chemotactic response to CCL18 in T cells	(Hieshima et al. 1997) (Adema et al. 1997) (Wells and Peitsch 1997) (Kodelja et al. 1998) (Schutyser et al. 2005) (Hägg et al. 2009) (Prasse et al. 2006) (Nomura et al. 2003) (Boot et al. 2004) (de Nadaï et al. 2006), (Chenivesse et al. 2012) (Wimmer et al. 2006)
CXCL4	activated platelets activated T cells	mediate the release of histamine by basophils play a role in adherence of eosinophils inhibition of monocyte apoptosis. differentiation of monocytes into macrophages CXCL4 may act as a selective regulator of monocyte	atherosclerosis inflammatory bowel disease and rheumatoid arthritis,	CXCR3A and CXCR3B	not through binding to GPCRs, but through an interaction with an chondroitin sulphate proteoglycan	(Deuel et al. 1981) (Petersen et al. 1999). (Brindley et al. 1983). (Schwartzkopff et al. 2011) (Lasagni et al. 2003) (Mueller et al. 2008) (Schaffner et al. 2005) (Vandercappellen et al. 2007) (Vandercappellen et al. 2011)

Table 1.3 Overview of CXCL4 and CCL18. This table summarizes the sources of expression, pathological effects and putative receptors for CCL18 and CXCL4.

1.8 Hypotheses and Aims

1.8.1 Hypothesis

CCL18 and CXCL4 are poorly characterized chemokines that have been identified as being components of human atherosclerotic plaques. The potential roles of the two chemokines may be related since CCL18 has been shown to be produced by the so-called 'M4' macrophage generated by the differentiation of human monocytes with the chemokine CXCL4. In Consideration of CXCL4-induced macrophages are unique and different from other known polarization types and CCL18 is reported to recruit Th2 and Treg cells, act as a monocyte survival factor and induce fibrosis, we hypothesize that CXCL4-induced macrophages and CCL18 may be a key part of the remodelling response, which is associated with an atheroprotective Th2 phenotype in contrast to the pro-atherogenic Th1 phenotype.

1.8.2 Aims

1) Determine whether CCR8 is a bona fide receptor for CCL18 as recently described in a publication (Islam et al. 2013).
2) Examine the biological effects elicited by exposure of human monocytes to CCL18.
3) Determine whether the enhanced survival seen in CXCL4-treated macrophages is due to the autocrine effect of CCL18 release, in particular, the effects of both

chemokines upon macrophage survival and differentiation.

4) Probe the phenotypic signature of CXCL4-driven monocyte differentiation.
5) Use a transcriptomic approach to examine factors that may be important in the early stages of M4 macrophage differentiation.

2 Materials and Methods

2.1 Materials

Unless otherwise stated, all materials were obtained from ThermoFisher Scientific, Paisley, UK. L1.2 cells were a kind gift of Dr Paul Ponath (Boston, USA) and have previously been described (Vaiedhi et al, 2007). The parental 4DE4 cell line was a kind gift form Dr Louis Staudt, (NIH, Bethesda, MD, USA). Two 4DE4 clonal lines stably expressing human CCR8 (4DE4-CCR8) and human CCR3 (4DE4-CCR3) were generated in the laboratory of Dr Philip Murphy, (NIH, Bethesda, MD, USA) and have previously been described (Tiffany et al. 1997, Pease et al. 1998). HA-tagged human CC chemokine receptor 8 (CCR8) cDNA inserted in the vector pCDNA3 was already available in the lab (Fox et al. 2006). Oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Recombinant chemokines, cytokines and the chemokine antagonist MC148 were from PeproTech EC (London, UK) and R&D Systems. (Abingdon, UK). Human Dil-labelled Oxidized-LDL was from Intracel (Frederick, MD USA).

Penicillin, Streptomycin, BSA, Tween-20 and EDTA were purchased from Sigma-Aldrich (Poole, UK). X-VIVO serum-free culture media was purchased from Lonza, (Basel, Switzerland). The 96-well ChemoTx System chemotaxis plates (101-5, pore size 5 μm) were obtained from Neuroprobe Inc. (Rockville, MD, USA). The Cell Titer-Glo reagent Luminescence Cell Viability Assay was from Promega (Southampton, UK). RNA isolation kits and qPCR materials were purchased from Qiagen (Crawley, UK) unless otherwise stated. Ficoll-HistopaqueTM was from GE, Life Sciences,

(Uppsala, Sweden). The EasySep Monocyte purification kit was purchased from Stem Cell Technologies (Grenoble, France).

Primary Antibody (isotype)	Clone	Conjugate	Source	Secondary Antibody (species)
Anti-HA (IgG_1)	HA.11		Cambridge Biosciences	FITC-labelled Anti mouse $F(ab')_2$ (Goat)
IgG_1 isotype control			Sigma-Aldrich	
Anti-human CCR8 (IgG_{2b})	433H		Professor Dave Cousins, KCL.	
Anti-CCR2 (IgG_{2b})	48607.121		Sigma-Aldrich	
IgG_{2b} isotype control			Sigma-Aldrich	
Anti-human CCR1		PE	R&D Systems	
IgG2b isotype control	53504	PE	R&D Systems	
CD14 (IgG_1)		FITC	Biolegend	
CD86 (IgG_1)		FITC	Biolegend	

Table 2.1 Antibodies

All primary monoclonal antibodies used in the thesis were generated in mice and are listed in Table 2.1, together with the isotype, conjugated fluorophore and source. Abbreviations: HA- epitope of human influenza haemaglutanin used to label hCCR8; FITC, Fluorescein Isothiocyanate; PE, Phycoerythrin.

2.2 Methods

2.2.1 Cell Culture

All procedures were carried out in a Class II Microbiological Safety Cabinet to protect the user and to maintain the sterility of the cell culture.

Maintenance of the L1.2 & 4DE4 Cell lines

L1.2 & 4DE4 cells are murine pre-B cell lymphoma cells. Both cell lines were maintained in RPMI (1640 medium with Glutamax-I, 25mM HEPES) supplemented with 50ml of heat-inactivated Fetal Calf Serum (FCS), 5ml of penicillin/ streptomycin liquid, 5ml of 100X non-essential amino acids, 5ml of 1mM sodium pyruvate and 0.5 ml of 1mM β-mercaptoethanol. This media, referred to as "Complete" culture media was kept at 4°C until needed, and was used to maintain the L1.2 and 4DE4 cell lines at a concentration of 0.5 to 1.0 x 10^6 cells/ml in a 37ºC incubator with 5% CO_2 until required. Transfected cells stably expressing human CCR8, (CCR8-L1.2 & CCR8-4DE4) were cultured in the same complete media as described above, with the addition of G418 at 1 mg/ml to maintain selection.

2.2.2 Peripheral Blood Mononuclear Cell (PBMC) preparation

Blood was taken from healthy normal subjects with informed consent, according to a protocol approved by a local ethics committee. Blood was anti-coagulated with 3.8% citrate, BDH Chemicals Ltd. (Poole, UK), made in water, at a ratio of 45 ml blood: 4.4 ml citrate solution, and centrifuged at 300 g for 20 min without a break. The platelet

rich plasma layer was removed and the remaining cells carefully layered onto 13ml of Ficoll-Plaque PLUS medium (GE Healthcare, Buckinghamshire, UK) for density gradient separation and centrifuged for 20 minutes at 1200g without any braking, to pellet the granulocytes, and separate the PBMCs by their lower density. The PBMC layer seen at the plasma:ficoll interface was removed using a pasteur pipette and the pellet washed twice in MACS buffer consisting of PBS (phosphate buffered saline), 1mM EDTA (ethylenediaminetetraacetic acid) and 2% FBS. PBMCs were processed immediately for monocyte isolation, or were suspended in Freezing medium (90% FBS, 10% DMSO) and underwent cryopreservation in liquid nitrogen until further use.

2.2.3 Isolation of monocytes

Negative Selection from whole blood

Cells were purified by negative selection using a Rosette-Sep monocyte purification kit (StemCell Technologies, Grenoble, France) according to the manufacturer's instructions.

Negative Selection from PBMCs

Monocytes were purified by negative selction from either fresh or frozen PBMC preparations by the use of the EasySep™ Human Monocyte Enrichment Kit (StemCell Technologies).

Both procedures gave untouched and highly purified CD14+CD16- monocytes up to 95% pure. Isolated cells monocytes were immediately used for downstream applications such as flow cytometry, culture, or DNA/RNA extraction by immunomagnetic negative selection. Isolated cells are immediately available for downstream applications such as flow cytometry, culture, or DNA/RNA extraction. The Kit from (StemCell Technologies, Grenoble, France) according to the manufacturer's instructions.

Purification by adhesion to plastic ware

For oxLDL scavenging assays, monocytes were purified via adherence to tissue culture plastic. Frozen PBMC pellets were thawed with 10ml of X-Vivo 15 media and centrifuged at 300g for 5 minutes at 4ºC. The supernatant was discarded and the PBMC pellet was re-suspended with an appropriate volume of X-Vivo 15 to give a

concentration of 5 x 10^6 cells/mL. Cells were left for 30 minutes at room temperature for the monocytes to lightly adhere to the plastic of the wells. Cells were then incubated for 2 hours at 37 ºC, after which gentle washing with 0.5ml of X-Vivo 15 was then carried out for a minimum of 5 washes to remove non-adherent T-cells and B-cells. The remaining adherent monocytes were cultured further, depending upon the nature of the assay.

Microscopic images

Phase-contrast images of macrophage cultures were taken through a DM IRBE microscope (Leica, Wetzlar, Germany) with a Hamamatsu (Hamamatsu City, Japan) camera, taken with a Lumix camera at 40x & 10x, and transferred to Photoshop (Adobe Systems, San Jose, CA) TIFF files.

2.2.4 Electroporation

Transient transfection of L1.2 Cells with plasmids

From a stock flask of L1.2 cells at concentration of approximately 1.0 x 10^6 cells/ml, 10ml of cells were centrifuged for 5 minutes at 300g, RT to pellet the cells. The supernatant was aspirated and the pelleted cells resuspended in 800µl of RPMI. In an electroporation cuvette with 4.0 mm gap size (VWR International, Leicestershire, UK) 50µl of a 10mg/ml solution tRNA (Sigma-Aldrich) to act as a carrier was added. The appropriate amount of plasmid DNA containing the cDNA of interest was also added. For each 1.0 x 10^6 cells to be transfected, 1µg of plasmid DNA was used. The next step was the addition of the resuspended cells to the cuvette and mixing the cells by gently

flicking the cuvette. The cells were then incubated inside for 20 minutes at RT. Next the cells were electroporated using a Biorad Gene-Pulser II Electroporator at 330 volts and 975 μF. Afterwards, cells were rested for 20 minutes at RT after which they were transferred to a T-75 tissue culture flask containing 10ml of complete RPMI. A disposable plastic pipette was used to gently break up any surface cell clumps in the culture by pipetting. Transfected cells were then incubated for 3-5 hour at 37°C, 5% CO_2. Sodium butyrate solution was subsequently added to the cells to a final concentration of 10mM (1:100 dilution). After 18 to 24 hours of culture, the cell surface receptor expression was examined by flow cytometry before using them in functional assays.

2.3 Flow cytometric analyses of chemokine receptor expression and function

2.3.1 Cell Surface Receptor Expression

Flow cytometry staining buffer was prepared by supplementing a 500ml bottle of 1X Dulbecco's phosphate buffered saline (D-PBS) with 1.25g of bovine serum albumin (BSA) and 500μl of a 10% sodium azide solution and kept at 4°C until needed. All incubations were carried out on ice unless otherwise stated.

Firstly, an aliquot of cells was taken, typically containing 0.2 to 0.5 x 10^6 cells and pelleted in an Eppendorf tube by centrifugation for 5 minutes at 300g at RT. The supernatant was aspirated and the cell pellets resuspended by gently pipetting in 100μl

of staining buffer containing either the relevant primary antibody at a concertation of 10µg/ml. Following incubation on ice for up to 15 minutes, cells were washed by adding 500µl of staining buffer and centrifuging for 5 minutes, 300g at RT. The supernatant was aspirated and the cells were resuspended in 100µl of goat anti-mouse FITC labeled $F(ab')_2$ secondary antibody diluted in staining buffer (1:20) and incubated on ice for 15-30 minutes. Cells were then washed again as previously described and the cell pellets were resuspended in 500µl of staining buffer. For dead cell exclusion, TOPRO-3 was added to the staining buffer at a dilution of 1:10000. Cells were transferred to flow cytometry tubes and analysed on a FACScalibur flow cytometer (Becton Dickinson, Oxford, UK) using CellQuest Pro software following the manufacturer's instruction. 10,000 events were typically acquired.

2.3.2 Receptor Internalization assays

Flow cytometry using the 433H CCR8 mAb was used to evaluate CCR8 receptor internalization. Briefly, 5×10^6 cells CCR8- 4DE4 cells were resuspended in 100 µl of ice-cold culture medium in duplicate, followed by the addition of buffer (negative control) or buffer supplemented with chemokines at the following final cocrntrations: CCL1 (100 nM), CCL18 (1 µM), CCL17 (100 nM) and MC148 (500 nM). Duplicated tubes were incubated for 30 minutes either at 37°C or 4°C. After the incubation cells were pelleted by centrifugation at 300g and the pellets were resuspended in a 100µL of FACS buffer containing either the CCR8 mAb or an isotype control, both at 10 µg/mL final concentration. After incubation 5-10 minutes 500 µL of FACS buffer was added to tubes and these were centrifuged at 300g for 5 minutes to wash away unbound mAb.

Cell pellets were resuspended with 300-400 µL of FACS buffer containing TOPRO-3 for dead cell exclusion and transferred into FACS tubes prior to analysis by flow cytrometry, using CellQuest Pro software following the manufacturer's instruction. 10,000 events were typically acquired. The percentage of CCR8 expression was normalized to the expression levels of untreated cells.

Flow cytometry using the PE-conjugated mouse 53504 (anti-hCCR1) and PE mouse IgG2b isotype control was used to evaluate CCR1 receptor internalization on monocytes. 5×10^6 cells freshly isolated monocyte were resuspended in 100 µl of ice-cold culture medium in duplicate, followed by addition of buffers containing chemokines to give the following final concentrations: (50 nM CCL3, 1 µM CXCL4 or buffer alone (negative control). One sample was incubated for 30 min at 37°C, whereas the other was kept at 4°C. After the incubation cells were spun again at 300g and the pellets were resuspended in a 100uL of FACS buffer containing 5 µl per test with either the CCR1 mAb or an isotype control, both at 10 µg/mL final concentration. After incubation for 5-10 minutes 500 µL of FACS buffer was added to tubes and these were centrifuged at 300g for 5 minutes to wash away unbound mAb. The cell pellets wre then analysed by flow cytometry as above. The percentage of CCR1 expression was normalized to the CCR1 expression levels observed on untreated monocytes.

2.3.3 Alexa Fluor (AF_{687}) CCL18 binding assays

AF_{687} labelled CCL18 was purchased from Almac (Craigavon, UK). An aliquot of cells (typically, 0.5 -1 x 10^6 cells) were placed in an Eppendorf tube and centrifuged for 5 minutes at 300g, RT, and the supernatant was aspirated. The cell pellets were then

resuspended by gently pipetting in 100µl of FACS buffer supplemented with varying concentrations of AF-CCL18, and incubated for 30 minutes on ice. Next, cells were washed by adding 500µl of staining buffer and centrifuging for 5 minutes at 300g, RT. The supernatant was aspirated and the cells were resuspended in FACS buffer and transferred to a flow cytometry test tube. Samples were analyzed on the flow cytometer as previously described, with 10,000 events typically acquired. When assessing specificp CCL18 binding to monocytes, cells were incubated with 100µl of FACS buffer containing 100nM AF-CCL18 alone or supplemented with 1µM unlabelled CCL18.

2.4 Monocyte survival assays

2.4.1 TOPRO-3 exclusion

Monocytes were purified from PBMCs using EasySep™ Human Monocyte Enrichment Kit. 500,000 cells were resuspended in serum-free RPMI in a 24 well plate. Individual cultures consisted of a volume of 350 µL media supplemented with final concentrations of either 100ng/mL M-CSF, 1µM CXCL4 or 1µM CCL18. Cells cultured in serum free media alone were used as a control. Cells were incubated at 37 ºC with 5% CO_2 for 72 hours after which they were washed with 1mL of fresh RPMI, followed by 1mL PBS and were then detached from the plate by incubation with trypsin at 37 ºC for 5 minutes. The cells were dislodged by gentle pipetting and resuspended in FACS buffer (10% sodium azide solution in a 0.25% BSA and PBS solution) containing the fluorescent dye TOPRO-3. Finally, samples were analyzed on the flow cytometer

following the manufacturer's instruction. 10,000 events were typically acquired.

2.4.2 CellTiter Glo® Monocyte Survival Assay

Monocytes were purified from PBMCs using EasySep™ Human Monocyte Enrichment Kit and resuspended at a concentration of 6 x 10^4 cells/mL in simple RPMI (serum free) media. To wells of a 96 well plate, 150 µL of the cell mix was added. Cells were incubated at 37 ºC with 5% CO_2 for 72 hours. After 72h of serum starvation, the media was removed and fresh media was added, supplemented with 100ng/mL of M-CSF or with 1µM CXCL4 or increasing concentrations of CCL18 (100,250,500, and 1000 µM). Cells were incubated at 37 ºC with 5% CO_2 for an additional 24 hours. On the next day, media was removed and cells were washed with 200 µL PBS and then lysed by adding 30µl of the live cell dye CellTiter Glo® (Promega, Southampton, UK) and incubating for 10 minutes with gentle shaking. 30µl of the lystate was transferred into seprate wells of a 96-well white opaque plate and luminescence counts read using a TopCount (Perkin Elmer, Waltham Massachusetts, USA). The average cell survival was calculated at each chemokine concentration andwas divided by the average cell count observed to buffer alone (unstimulated cells, for normalization purposes.

2.5 Chemotaxis assays

2.5.1 Modified Boyden chamber assays

These assays used a modified version of the Boyden chamber (Boyden, 1960) in which

cells migrate through specific pores of a membrane in response to chemoattractants. Firstly, 30µl of blocking buffer (RPMI 1% BSA (w/v)) was pipetted into each well of a 5µm pore size 96-well chemotaxis plate that was to be used and the plate incubated for 30 minutes at RT. Assay buffer (RPMI 0.1% BSA (w/v)) was then prepared by making a 1:10 dilution of the blocking buffer with simple RPMI. From stock concentrations of chemokine (10µM), 8 µl was added to 72 µl of assay buffer and mixed by gentle flicking of the tube to make a 1µM solution. Then serial dilution of the relevant chemokine was prepared using the assay buffer in a dose-response range of 0.1, 1, 5, 10, 50, 100, 500 and 1000 nM (unless otherwise stated).

Cells to be assayed were centrifuged for 5 minutes at 300g at RT to pellet the cells, then resuspended in assay buffer at 1 x 10^7 cells/ml, so that 20 µl buffer contained 2 x 10^5 cells. Then the blocking buffer was removed from each well of the chemotaxis plate by aaspiration. Into duplicate wells, 28 µl of each of the chemokine dilution was pipetted, and into two separate wells 28 µl of assay buffer was added (to measure basal migration). After that the membrane filter was secured on top of the plate making sure that no air bubbles were visible between the plate and the membrane. Onto the top of each filter, 20 µl of the cell suspension was added. The plate lid was then secured on top. Then the plate was put into a humidified chamber and incubated in tissue-culture incubator for 5 hours at 37°C, 5% CO_2.

At the end of the incubation, the cells from the top of the membrane were removed by gentle scraping with a dedicated scaper, taking care to avoid damaging the membrane. Cells migrating into the lower well were pelleted in the wells by centrifugation for 5 minutes at 300g, RT and the membrane removed from the chemotaxis plate. A filter

funnel (Neuroprobe), followed by an opaque white plate was placed on top and the stack inverted and centrifuged for 5 minutes at 300g, RT. This ensured that migrating cells were transferred to a white opaque plate. To each well containing cells, 30 µl of the live cell dye CellTiter Glo® (Promega, Southampton, UK) was added and luminescence counts read using a TopCount. The average cell migration calculated at each chemokine concentration was divided by the average cell migration observed to buffer alone, to generate a chemotactic index (CI).

MC148 & 433H mAb inhibition of CCR8.

In chemotaxis experiments looking at the inhibition of CCR8-4DE4 transfectant migration, the same protocol as detailed above was used following the same protocol stated above except that prior to loading the cells into the chemotaxis plate, a blockade step was performed by incubating the cells in assay buffer supplemented with the anti-CCR8 mAb 433H (10μg/ml) or with the chemokine antagonist MC148 (500μM) for 20 minutes at 37°C

CCL18 inhibition of CCR3-mediated migration.

In chemotaxis experiments looking at the inhibition of the CCR3-4DE4 transfectant migration by CCL18, the same assay was employed with the difference that a fixed 1nM concentration of CCL1 and CCL11 was supplemented with increasing concentrations of CCL18

2.5.2 Imaging of Chemotaxis in real time by TAXIScan

For the real time analysis of migrating monocytes, a 12-channel TAXIScan system (Kanegasaki et al. 2003)was used according to the manufacturer's protocol (Effector Cell Institute, Tokyo. Japan). In this system, cells are allowed to crawl along a glass surface and images taken of their migration from which individual cell tracks are analysed. A chip made of etched silicon substrate is placed on top of the glass to form 12 separate compartments with a 260 μm long, 5 μm-deep horizontal channel in between. The chip was immersed in assay buffer (RPMI 0.1% BSA) and brought up to 37ºC using the instrument plate heater. Monocytes freshly isolated by the Rosette Sep

Kit were resuspended in assay buffer at a density of 5 x10^5 cells/mL and 1 µL aliquots delivered by a microsyringe into one end of each of the 12 available channels. After cell alignment, a chemokine gradient was established by the injection of 1 µL of a chemokine solution into the opposing ends of the channels. These were made up in assay buffer and consisted of 1 µM chemokine CCL2 (positive control) 1 µM CCL18 and 10 µM CCL18. To assess basal migration, some wells had no chemokine injected. The apparatus was then set to record images every minute for 1 hour with a CCD camera located beneath the glass, equipped with a high-performance lens and a coaxial episcopic illumination system.

Sequential image data were generated from individual jpegs processed with ImageJ (National Institutes of Health), equipped with the manual tracking (Fabrice Cordelieres, Institut Curie, Orsay (France) and chemotaxis tool plugins (Ibidi, Martinsried, Germany). Individual experiments consisted of monocyte responses to each stimulus in triplicate and illustrated data are collated from three experiments as highlighted in the figure legend. For each individual cell, the forward migration indices in the y axis (yFMI) and x axis (xFMI) were calculated using the chemotaxis tool plugin. FMI is defined as the distance travelled by the cell in either the x-axis (perpendicular to the chemokine gradient) or the y axis (parallel to the chemokine gradient) divided by the accumulated distance travelled by the cell and is a reliable measure of migration along a chemoattractant gradient. The directionality parameter refers to the Euclidian distance travelled by each cell divided by the accumulated distance travelled and is an alternative measure of directed migration.

2.6 OxLDL Scavenging Assays

Cellular uptake of OxLDL was studied by flow cytometry utilizing dil- labelled oxLDL which is visible in the FL2 channel. Initial experiments compared the uptake of oxLDL in M0 Macrophages (M-CSF cultured) compared with that of M4 macrophages (CXCL4 cultured). Monocytes were cultured in 24 well plates and were polarized to either phenotype by culture in 350 μL of X-Vivo 15 media containing M-CSF or CXCL4 at final concentrations of 100ng/mL and 1μM respectively and the cells incubated at 37 ºC with 5% CO_2 for the duration of the culture. Initial experiments looked at cells polarized for 7 days in culture, whilst subsequent experiments looked at cells polarised for 2, 4 or 7 days.

At the end of the culture period, media was aspirated from the cell surface and cells were washed with 400 μL of X-Vivo 15. Macrophages were subsequently "fed" by replacing the media with 400 μL of X-Vivo 15 containing Dil-OxLDL at a final concentration of 10μg/mL. Macrophages were incubated for 4 hours to allow scavenging, after which they were washed sequentially with 1mL of fresh X-Vivo 15, 1 mL PBS and were then detached from the plate by incubation with trypsin at 37 ºC for 5 minutes. The cells were dislodged by gentle pipetting and resuspended in FACS buffer containing TOPRO-3 for dead cell exclusion. Cells were transferred into FACS tubes prior to analysis by flow cytometry using CellQuest Pro software following the manufacturer's instruction. 10,000 events were typically acquired.

2.7 Measurement gene expression by qRT-PCR

Monocytes obtained from three healthy donors that had undergone 3 days of differentiation with 100ng/mL of M-CSF or with 1μM CXCL4 were washed by adding 500μl 0.1% bovine serum albumin (BSA) The BSA was decanted and the cells were resuspended in 300μl buffer RLT This buffer is a proprietary component of RNeasy Kits. Buffer RLT contains a high concentration of guanidine isothiocycanate, which supports the binding of RNA to the silica membrane. for direct lysis ß-mercaptoethanol should be added to Buffer RLT before use to effectively inactivate RNAses in the lysate (10 μl ß-Mercaptoethanol per 1 ml Buffer RLT). RNA isolation was carried our according to the manufacturer's recommendation using RNeasy Microprep Kit. The integrity of the RNA obtained was analysed using an Agilent 2200 TapeStation system prior to further usage. The standard for the assessment of RNA quality is the RNA Integrity Number (RIN), which is a reliable and robust method to analyse RNA integrity assuring the quality and the quantity of the RNA.

The expression levels of genes of interest were analysed using an Applied Biosystems Viia7 RT-PCR cycler (Life technologies, CA, USA). Firstly, cDNA was synthesised from 100 ng of total RNA using a QuantiTect reverse transcription kit (Qiagen). The purified RNA sample is briefly incubated in gDNA Wipeout Buffer at 42°C for 2 minutes to effectively remove contaminating genomic DNA. After genomic DNA elimination, the RNA sample is ready for reverse transcription using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The entire reaction takes place at 42°C and is then inactivated at 95°C.

Then cDNA was mixed with primers (revers & forword) in combination with SYBR® Green qPCR Mastermix.

Specific primers for the chemokine receptors CCR1, CCR2, CCR3, CCR4 and CCR8, the chemokines CCL18, CCL17, CCL22, the cell surface markers CD86 and CD36 and the house keeping gene β-actin were used as listed in Table 2.2.

Duplicates of samples were run in sealed 384-well PCR plates (Applied Biosystems, MA, USA) useing ViiA™ 7 system (Life Technologies). cDNA samples were subjected to a 5 minute denaturation at 95ºC followed by 40 cycles of 10s at 95ºC followed by 1 minutes at 60ºC.

Finally, the cycle threshold (C_T) in relation to β-actin (the housekeeping gene) was plotted as $1/\Delta C_T$ to show qualitatively mRNA levels.

2.8 Assessing chemokine production using the Proteome Profiler kit

In order to determine the expression of multiple chemokines produced by M4 macrophage s, the Proteome Profiler Human Chemokine Array (R&D Systems) was performed according to manufacturer's instructions. A culture supernatant pooled from M4 macrophages (differentiated for 6 days) and Th2 cells (Kind gift Caroline Anderson and Dawid Swieboda) was used to probe paired blots. Briefly, supernatants (pooled using equal volumes from three separate cultures) were mixed with a cocktail of biotinylated antibodies raised against human chemokines and used to probe a

membrane covered with an array of capture antibodies to different human chemokines. Finally, blots were developed using a biotin-streptavidin reported and visualized by chemiluminescent detection antibodies using a MyECL Imager (Thermo Fisher Scientific, MA, USA).

Table 2.2 Primers used for qRT-PCR

PCR Primer	Sequence (5' – 3')
Chemokine receptors	
CCR1 forward	GACTATGACACGACCACAGAGT
CCR1 reverse	CCAACCAGGCCAATGACAAATA
CCR2 forward	CCACATCTCGTTCTCGGTTTATC
CCR2 reverse	CAGGGAGCACCGTAATCATAATC
CCR3 forward	TGGCATGTGTAAGCTCCTCTC
CCR3 reverse	CCTGTCGATTGTCAGCAGGATTA
CCR4 forward	CCCACGGATATAGCAGACACC
CCR4 reverse	GTGCAAGGCTTGGGGATACT
CCR8 forward	GGTCATCCTGGTCCTTGTGG
CCR8 reverse	CAGGGCCAGGTTCAAGAGG
Chemokines	
CCL17 forward	ACC TGC AAA GCC TTG AGA GGT
CCL17 reverse	CG GTG GAG GTC CCA GGT A
CCL18 forward	GGTGTCATCCTC- CTAACCAAGAGA
CCL18 reverse	GCTGATGTATTTCTGGACCCACTT
CCL22 forward	TAC TCT GAT GAC CGT GGC CTT G
CCL22 reverse	AGA GAG TTG GCA CAG GCT TCT G
Cell surface markers	
CD36	GGCTGTGACCGGAACTGTG
CD36 reverse	AGGTCTCCAACTGGCATTAGAA
CD86	CTGCTCATCTATACACGG
CD86 reverse	GGAAACGTCGTACAGTTCTGTG
Housekeeping Gene	
β-actin forward	ATTGCCGACAGGATGCA
β-actin reverse	GCTGATCCACATCTTGCTG

2.9 Assessing chemokine production via ELISA

Levels of CCL18, CCL17 and CCL22 protein in the supernatants of M0 and M4 macrophages were measured using separate sandwich enzyme-linked immunosorbent assays (ELISAs). All samples were assayed in duplicate. 100 µL of the coating mouse anti-human CCL18, CCL17 or CCL22 at a concentration of 2 µg/mL was added to 96-well flat-bottomed ELISA plates and incubated overnight at RT, covered with am ELISA plate seal. The next day, plates were washed three times using wash buffer (1 x PBS, 0.05% Tween-20, dried and blocked with 100 µL of blocking buffer (1% BSA in PBS). After one hour at RT, plates were washed again, air dried and 100 µL of protein standards (0, 0.25, 0.5, 1, 2, 4, 8, 16 ng/mL) with 100 µL of each sample (n=3, each diluted 1:4 in 1x Reagent Diluent) were added and incubated at RT. After 2 hours, the content was discarded, plates washed and 100 µL of detection antibodies were added (0.2 µg/mL) for 2 hours at RT. The content was discarded, washed as before and 100 µL of 1x Streptavidin-HRP solution was added for 20 minutes. At this stage, wells were aspirated followed by a final wash. To each well, 100 µL of substrate solution was then added and plates were placed in the dark for 20 minutes. The reaction was stopped using 50 µL of stop solution (2N H_2SO4) and the absorbance was measured at 450 nm on a SpectraMaxi3x (Molecular Devices LLC, CA, USA). Data were analysed using SoftMaxPro 6.5 Software (Molecular Devices) with specific chemokine concentrations assessed by interpolation of a standard curve.

2.10 RT^2 Profiler PCR Array analysis of transcription factors produced by M4 macrophages.

Monocytes were purified from the whole blood of three healthy donors by the EasySep® protocol, resuspened in X-vivo15 media and plated in a 24 well tissue culture plate at 5 x 10^5 cells per well. Cells were left to rest for 2 hours afer which they were stimulated by replacing the media with fresh media containing 1μM CXCL4 (test groups) or unsupplemented media (controls groups). At 1 hour and 4 hour time points, the media was removed and the cells washed by adding 500μl bovine serum albumin (BSA) buffer. The BSA buffer was decanted and the cells were lysed in 300μl of RLT buffer. RNA isolation was carried out according to the manufacturer's recommendation using the RNeasy Microprep Kit (Qiagen). RNA quality was determined using Tapestation. 12ng of total RNA was reverse transcribed using the RT^2 First strand kit (Qiagen) and subsequently pre-amplified with the RT^2 Pre-AMP kit, being subjected to a 10 minute denaturation at 95ºC followed by 12 cycles of 15s at 95ºC followed by 2 minutes at 60ºC. The amplified cDNA was immediately used in real-time RT^2 Profiler PCR Array (QIAGEN) in combination with RT^2 SYBR® Green qPCR Mastermix. cDNA samples were subjected to a 10 minute denaturation at 95ºC followed by 45 cycles of 15s at 95ºC followed by 1 minutes at 60ºC.

C_T values were exported to an Excel file to create a table of C_T values. This table was then uploaded on to the data analysis web portal at **http://www.qiagen.com/geneglobe**. Samples were assigned to controls and test groups. C_T values were normalized based on an automatic selection from House Keeping Gene panel of reference genes. The data

analysis web portal calculates fold change/regulation using $\Delta\Delta$ C_T method and plots scatter plots, volcano plots, and heat maps. A data analysis report was exported from the QIAGEN web portal at GeneGlobe.

2.11 Statistical Analysis

All statistical analysis was carried out using Prism 7 for Macintosh (Graphpad software, La Jolla, CA). Statistical results were based on a one-way or two-way analysis of variance (ANOVA) which compares multiple data sets to a control. The significance between two different groups was calculated using an unpaired Student t-test, with Bonferroni's multiple comparisons test, unless otherwise stated. Data reflect mean ± S.E.M. values, unless otherwise stated. Values above $P<0.05$ were considered to be statistically significant.

3 Characterization of CCL18 as a CCR8 agonist

3.1 Introduction

Some chemokines are constitutively expressed at detectable levels in normal human plasma such as CCL14, CCL16, CXCL4, and CXCL7 (Sallusto F et al. 2007,Schulz-Knappe et al. 1996, Files et al. 1981, Scheuerer et al. 2000). Interestingly CCL18 is one such chemokine found constitutively expressed in the plasma at levels of 10-72 ng/ml of healthy individuals (Schutyser et al. 2005). However, increased CCL18 levels have also been reported in a variety of inflammatory diseases. For example, concentrations of 2285 ng/ml of CCL18 have been reported in the plasma of patients suffering from Gaucher's disease, leading to its usage as an alternative biomarker (Boot et al. 2004). Similarly, IHC studies have shown that CCL18 is abundantly found in human atherosclerotic plaques where its expression is associated with macrophages (Hägg et al. 2009). Nevertheless, the precise role of CCL18 in disease progression is still a matter for speculation and is hindered by the lack of knowledge regarding its receptor, despite the original identification of the chemokine back in 1997 (Adema et al. 1997).

CCL18 has been reported to have a variety of activities, including its initial description as a T-cell chemoattractant (Adema et al. 1997), an antagonist of the chemokine receptor CCR3 (Nibbs et al. 2000) and more recently as a monocyte survival factor (Wimmer et al. 2006). This suggests that it may signal via more than one receptor and also be associated with different cell types in different disease settings. It is believed that CCL18 signals via one or more G protein-coupled receptors (GPCR) since in two studies it was reported that pre-treatment of naïve T-cells with pertussis toxin, a potent

inhibitor of Gαi proteins completely abrogated the chemotactic response to CCL18. (Adema et al. 1997, Krohn, Bonvin, et al. 2013).

In the last few years, at least three different receptors have been put forward as CCL18 receptors, and the area remains contentious. The chemokine receptor CCR6 was reported in a conference abstract to function as a human CCL18 receptor, although notably, no full paper was subsequently published (Zissel et al. 2011). Soon after, Luzina and colleagues showed that intratracheal administration of CCL18 in wild-type mice, resulted in T cell recruitment no different from that observed in CCR6 deficient mice, suggesting that CCR6 was unlikely to be a CCL18 receptor(Luzina and Atamas 2012). These experiments must be interpreted carefully, as there is no direct homologue of CCL18 in the mouse (Zlotnik and Yoshie 2012).

Another receptor, PITPNM3 was subsequently put forward as a CCL18 receptor by Chen and co-workers, postulated to drive the metastasis of breast tumours (Chen et al. 2011). However, subsequent studies have failed to detect PITPNM3 on leukocytes, suggesting they are unlikely to be responsible for the effects of CCL18 on these cells (Krohn, Bonvin, et al. 2013). Indeed, in my Masters' project in 2012, I successfully expressed both CCR6 and PITPNM3 using a well-established mammalian transfection system (Vaidehi et al, 2006) and found that neither receptor mediated chemotactic responses to CCL18, suggesting the existence of an alternative CCL18 receptor.

Proudfoot et al., have addressed several questions concerning the biological role of CCL18. Initially, they attempted to identify the CCL18 receptor by expression cloning from leukocyte subsets responsive to CCL18. Interestingly, when they analyzed CCL18

binding to peripheral blood leukocytes (PBLs), they found that the major binding interaction was to glycosaminoglycans (GAGs) (Krohn, Bonvin, et al. 2013). Another study by the same group showed that CCL18 was able to inhibit CCR1, CCR2, CCR4, and CCR5 mediated chemotactic responses to a variety of ligands. As this inhibitory effect of CCL18 on the cellular recruitment of several receptors might result in the disruption of the directional signal for cells to migrate, they have suggested a regulatory or anti-inflammatory role of CCL18 mediated through a GAG binding mechanism. They proposed that this inhibitory effect of CCL18 is not mediated by direct binding to the receptors, but through a GAG binding mechanism since the abrogation of GAG binding by mutagenesis abrogates the inhibitory function (Krohn, Garin, et al. 2013).

In September 2013, as I commenced my PhD studies, another receptor was put forward as a CCL18 receptor by the group of Andrew Luster at Harvard University. They published a paper suggesting that the chemokine receptor CCR8 was a functional receptor for CCL18 (Islam et al. 2013). They reported that CCL18 induced calcium flux response and chemotaxis in both human CCR8-transfectants and also in human Th2 cells. CCR8 was originally described as a receptor for the chemokine CCL1 (Roos et al. 1997, Tiffany et al. 1997) and to date has had no other human ligands had been assigned to it. In this chapter, I used a variety of complimentary assays to independently verify the publication of Islam et al and examine if CCL18 could bind to CCR8 and drive functional responses.

3.2 Results

3.2.1 Molecular characterization of CCL18 as a CCR8 agonist

3.2.2 Assessing CCL18 as a potential CCR8 agonist.

In September 2013, the group of Andrew Luster at Harvard published a brief report identifying human chemokine receptor CCR8 as a CCL18 receptor (Islam et al. 2013). They indicated that CCL18 induced the chemotaxis of human CCR8-transfected 4DE4 cells using a cell line that originated from our group (Tiffany et al. 1997). To validate this finding and confirm whether CCL18 could induce the migration of CCR8 transfectants, the same clone of 4DE4 cells transfected with a plasmid encoding CCR8 were thawed and used in chemotaxis assays. CCL1 the known ligand for CCR8 (Tiffany et al. 1997) was used as a positive control for migration (Figure 3.1). The parental naïve cell line 4DE4, devoid of CCR8 was also used as a negative control.

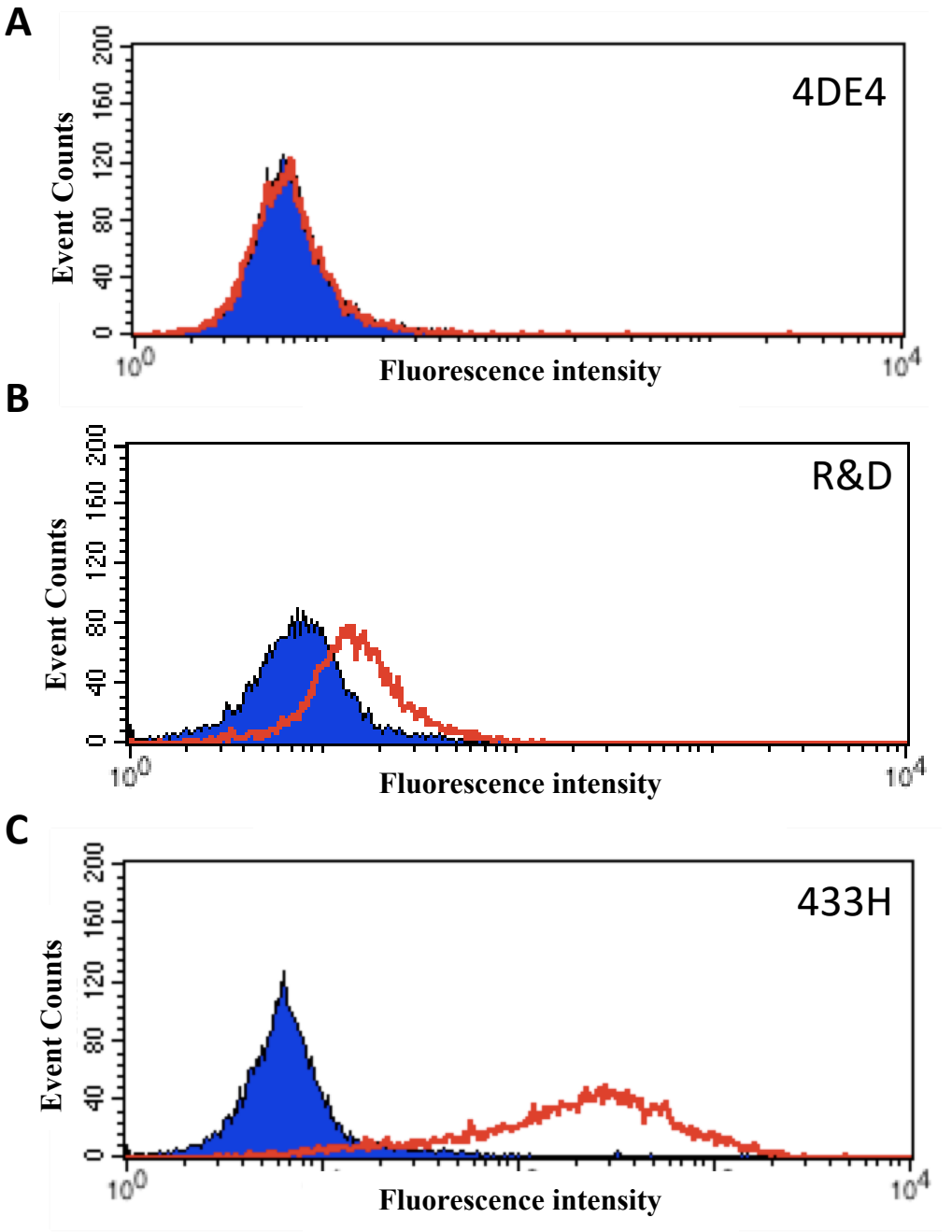


Figure 3.1: Detection of CCR8 on the surface of 4DE4-CCR8 cells. A Representative histogram showing surface expression of CCR8 on naive 4DE4 (A) and hCCR8-transfected 4DE4 (B & C). filled histogram shows isotype control antibody, red line shows CCR8 mAb staining from R&D (B) and 433H (C).

We first evaluated the expression of CCR8 on the surface of the cells by flow cytometry using a receptor-specific antibody. Commercially available CCR8-specific antibodies are known within the chemokine community to be unreliable (Pease and Horuk 2009b). In contrast, ICOS corporation generated a CCR8-specific monoclonal antibody known as 433H that has been validated alongside staining profiles generated with fluorescently labelled CCL1 (Mutalithas et al. 2010). Figure 3.1 shows staining of the 4DE4-CCR8 transfectants with either a clone from R&D systems (clone) or 433H. Significant cell surface expression of CCR8 was observed only with 433H, hence this antibody was used in all future experiments examining CCR8 expression by flow cytometry.

Having verified CCR8 expression, the cells were then used in chemotaxis assays using modified Boyden chambers (Figure 3.2). The data clearly show that the CCR8 construct was functional, as a robust response to CCL1 (known CCR8 agonist) was detected, with peak response seen at around 10nM of ligand. CCL18 appeared to be a full agonist of CCR8 but with significantly lower potency compared with CCL1 (100 nM vs. 10 nM respectively).

Curiously, the naïve 4DE4 cells devoid of CCR8 exhibited a partial response to CCL18 at high nanomolar concentrations, suggesting an additional endogenous CCL18 receptor may exist on this pre-B cell line

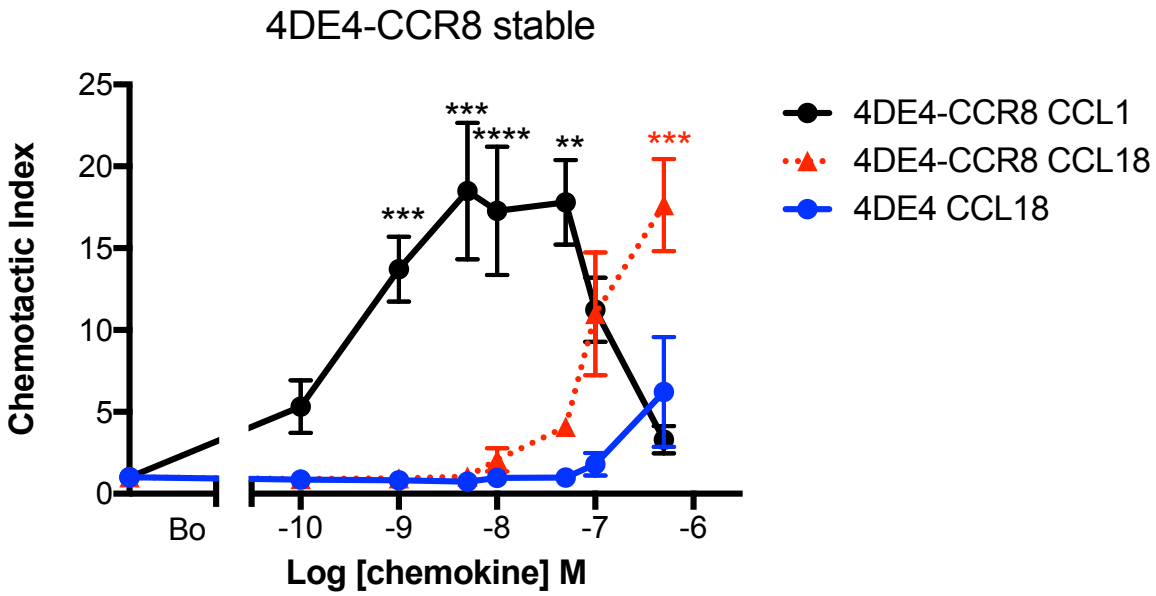


Figure 3.2. The chemotactic activity of CCL18 and CCL1 for CCR8 transfectants. The chemotactic responses of naïve 4DE4 cells to CCL18 (blue) and CCR8-4DE4 cells to a range of concentrations of CCL1 (black) and CCL18 (red). Data shown are the mean ± SEM of 3 experiments. Migration to the positive controls for CCR8 was observed at 10nM. ****= $p<0.0001$, ***= $p<0.001$, **= $p<0.01$ using two way ANOVA. & Bonferroni's multiple comparisons test for CCL1 responses.

3.2.3 Limiting dilution clones of CCR8-4DE4 cell line maintained in low concentrations of G418 responded weakly to CCL18.

The CCR8-4DE4 chemotactic responses to CCL18 were not robust and weakened over a few weeks of culture following the acquisition of the first data set shown in Figure 3.2. The Luster group had advised in their paper the use of clones of our CCR8-4DE4 transfectants that had been weaned off the original Geneticin (G418) concentration of 1 mg/ml, as they found this inhibitory for responses to CCL18 (Islam et al. 2013). They had generated clones cultivated in lower concentrations (0.1–0.4 mg/ml) of G418.

We investigated this, by generating two sub-clones of the parental CCR8-4DE4 line by limiting dilution (named clone 4 and clone 10), which were grown in 200μg/ml G418. In Boyden chamber assays, excellent CCL1 responses, similar or better than those seen on the parental 4DE4-CCR8 cell line, were observed (Figure 3.3), potentially justifying the Luster group's point that the concentration of G418 could affect the function of the cell line. Unfortunately, that was not the case for CCL18 as we found that neither clone responded robustly to increasing concentrations of CCL18. Similar to the initial chemotaxis data observed with our original clone, insignificant responses to CCL18 were seen at the upper concentrations of 1 μM as observed in the parental 4DE4 cell line (Figure 3.1).

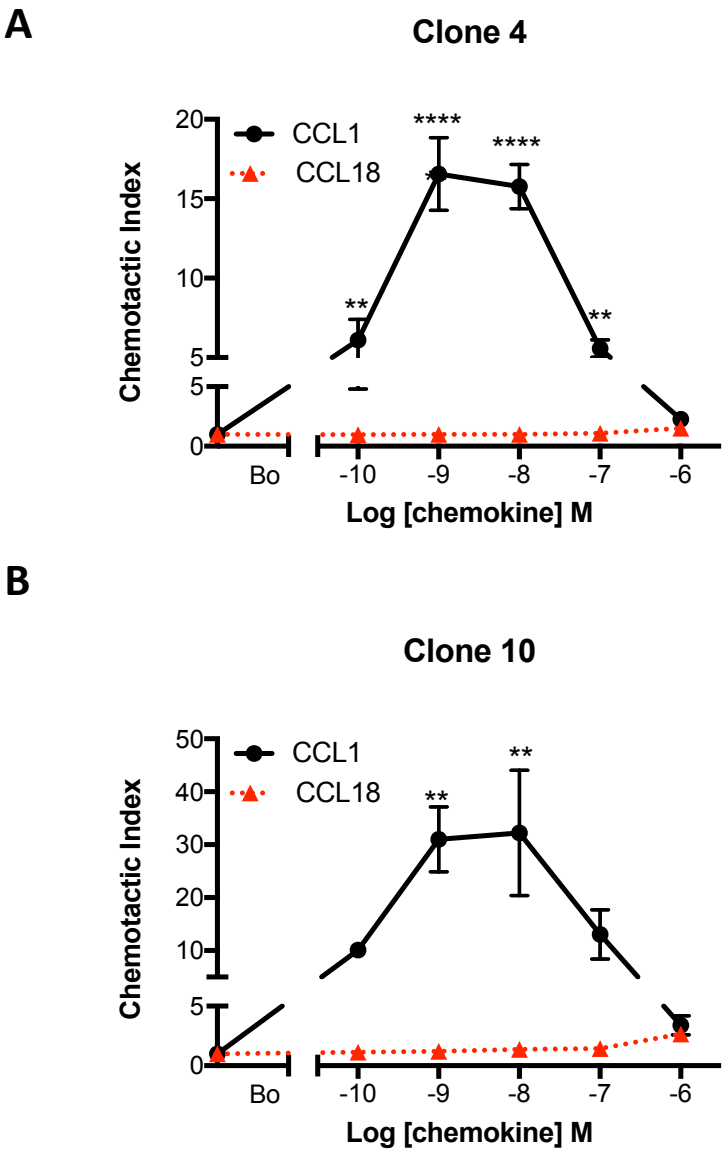


Figure 3.3: Lower G418 Concentrations do not aid CCL18 responses from the 4DE4:CCR8 cell line. Chemotaxis of CCR8-4DE4 transfectants clone 4 (A) and clone 10 (B) to increasing concentrations of CCL18 (red) and CCL1 (black). Data shown are the mean ± SEM of 3 experiments. Significant migration to the positive controls for CCR8 was observed at several concentrations 10nM. ****= $p<0.0001$, **= $p<0.01$ for CCL1 responses using two way ANOVA. & Bonferroni's multiple comparisons test

3.2.4 The L1.2-CCR8 cell line does not migrate in response to CCL18

To further assess the ability of CCR8 to function as a CCL18 receptor, we turned to another murine pre-B cell line, named L1.2 (Berg et al, 1991). Figure 3.4 shows chemotaxis responses to CCL1 and CCL18 from either an existing L1.2 cell line stably transfected with a CCR8 plasmid (maintained in G418) or L1.2 cells transfected with a plasmid encoding CCR8 and transiently expressing CCR8 on the cell surface (Fox et al, 2006). Compared to the excellent CCL1 responses seen in both cell types , CCL18 had little activity, with again a feeble response observed at concentrations approaching 1μM CCL18.

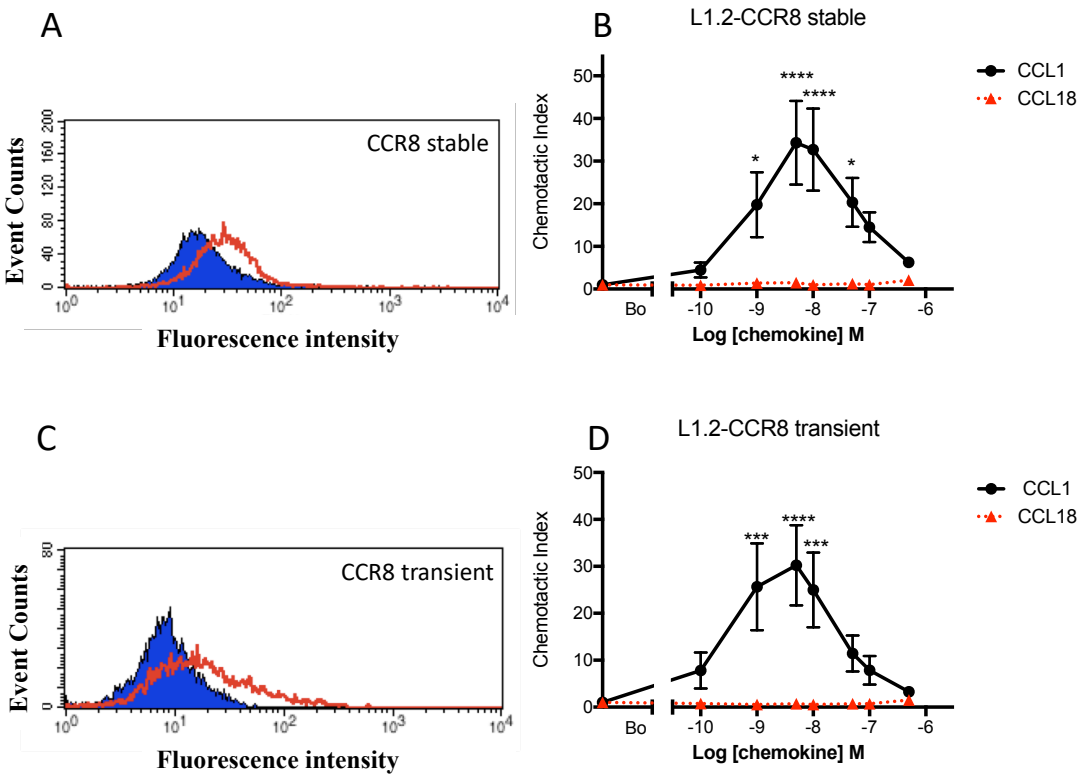


Figure 3.4 Weak chemotactic responses to CCL18 are observed from L1.2 cells expressing CCR8. The chemotactic responses of L1.2-CCR8 transfectants stably (A) or transiently (B) expressing CCR8 to increasing concentrations of CCL18 (red) and CCL1 (black) are shown (C & D). Data shown are the mean ± SEM of 3 experiments. Significant migration to the positive controls for CCR8 was observed between at 1-10nM **** shows $p<0.0001$, *** shows $p<0.001$ and * indicates $p<0.05$ for CCL1 responses using two way ANOVA. & Bonferroni's multiple comparisons test.

3.2.5 The virally encoded CCR8 antagonist MC148 efficiently blocks chemotactic responses of the 4DE4-CCR8 cell.

The poxvirus *Molluscum contagiosum* virus produces a protein named MC148 which has homology to CC chemokines and is a blocker of CCR8 function (Damon et al. 1998). We subsequently extended our studies to assess the efficacy of MC148 as a blocking agent. Monoclonal antibody 433H reported to be a CCR8 antagonist (Mutalithas et al. 2010) was also evaluated. Cells were pretreated with either MC148 or 433H prior to assessing their chemotactic responses to increasing concentrations of CCL1. As controls, cells were pretreated with PBS (MC148 treatment) or an isotype control (433H treatment). The data are shown in Figure 3.5. Interestingly, 433H treatment (Figure 3.5 Panel A) showed significant inhibition of responses to low concentrations of CCL1, but at the optimal CCL1 concentration of 10nM, was barely effective. In contrast, MC148 inhibited behaviour typical of a competitive antagonist of CCR8, right-shifting the CCL1signficantly (Figure 3.5 Panel B). Notably, approximately 90% of 4DE4-CCR8 migration to CCL1 at the optimum concentration was inhibited by MC148. We consequently pursued all our functional studies using MC148 as a CCR8 antagonist.

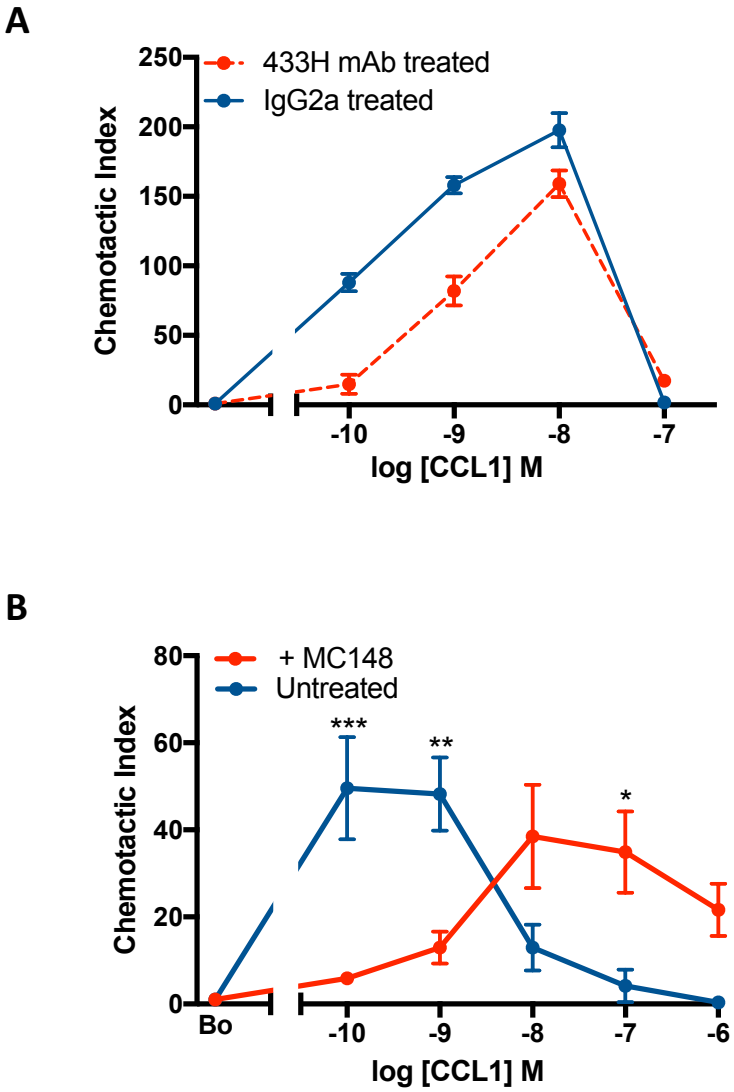


Figure 3.5 MC148 is an effective inhibitor of CCR8. Chemotaxis of CCR8-4DE4 transfectants following blockade with 433H mAb (Panel A) and MC148 (Panel B) to increasing concentrations of CCL1. Data shown are the mean ± SEM of 5 experiments (433H mAb) and 7 experiments (MC148). Significant inhibition of the CCR8 responses to CCL1 was observed at 10nM with 433H mAb and (0.1 nM) with MC148. *** shows $p<0.001$ using two way ANOVA. & Bonferroni's multiple comparisons test.

3.2.6 CCL18 is unable to induce CCR8 internalization

Following incubation with their cognate ligands, chemokine receptors typically undergo endocytosis (Neel et al. 2005). To further clarify if CCL18 is a bona fide ligand of CCR8, we used the CCR8-4DE4 cell line to look at CCR8 endocytosis in response to both CCL18 and CCL1. CCR8 expression was monitored by flow cytometric analysis using the 433H monoclonal antibody or a relevant isotype control, following incubation with ligand for 30 minutes. As anticipated from previous work (Fox et al. 2006) when the 4DE4-CCR8 cells were incubated with 1nM CCL1 for 30 minutes at 37°C, CCR8 was readily endocytosed as observed by the loss of 433H staining. 4DE4-CCR8 cells were subsequently incubated with CCL1 (100 nM) CCL17 (100 nM) , CCL18 (1 μM) or MC148 (500 nM) for 30 minutes at 37°C and CCR8 levels assessed by 433H staining. Cells identically treated but incubated at 4°C, (no endocytosis) were used to calculation of the percentage of CCR8 internalisation (Figure 3.6 B). Whilst CCL1 significantly reduced CCR8 expression by ~80%, CCL18 and CCL17 (negative control) had no effect on CCR8 cell surface levels. Likewise, MC148 did not reduced CCR8 expression as might be expected of an antagonist.

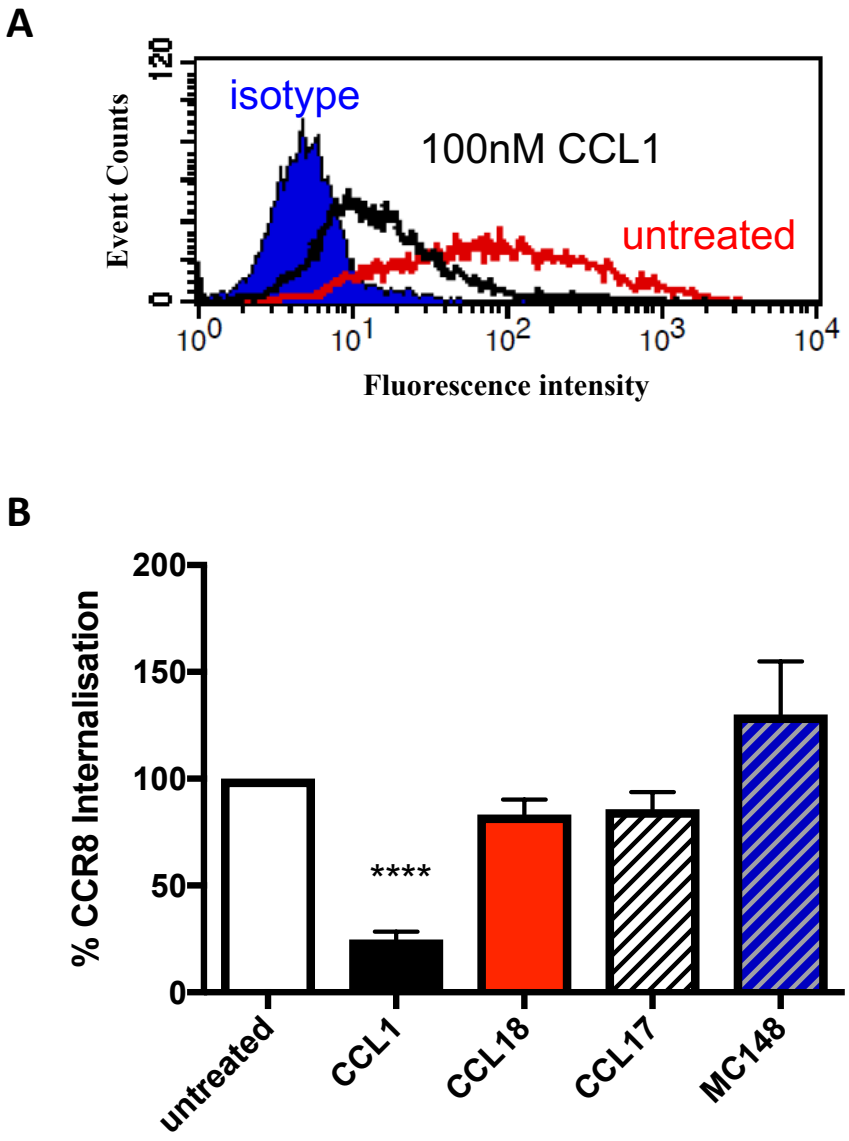


Figure 3.6 Internalization of CCR8 in CCR8-4DE4 cells is not driven by CCL18 stimulation. (A) Representative histogram showing surface expression of CCR8 on 4DE4-CCR8 transfectants incubated for 30 minutes at 37°C with 100 nM CCL1 (black line), or media (red line), isotype control antibody (filled histogram). (B) Surface CCR8 expression on 4DE4-CCR8 transfectants following incubation for 30 minutes at 37°C with ligands. 4DE4-CCR8 cells were left unstimulated or incubated with 100nM CCL1, 1μM CCL18, 100 nM CCL17 or 500nM MC148 for 30 minutes at 37°C. CCR8

expression was normalized to expression levels of untreated cells. CCL1-treatment significantly induced CCR8 internalisation compared with untreated condition $p<0.0001$**** using one way ANOVA. & Bonferroni's multiple comparisons test. Data are expressed as the mean ± SEM, from six separate experiments.

3.2.6 CCL18 is a competitive ligand, binding to CCR3 but not CCR8.

Thus far, all the data generated with CCL18 pointed to a lack of function at CCR8. It was therefore important that we confirmed that our supply of CCL18 was functional, to rule out the possibility that we had generated falsely negative data. Nibbs and workers previous described that CCL18 is an antagonist of the related chemokine receptor CCR3 and could effectively inhibit CCR3 responses in a dose-dependent manner (Nibbs et al. 2000). Therefore, we tested the ability of our CCL18 stocks to inhibit the activity of CCR3 transfectants in chemotaxis assays. 4DE4-CCR3 transfectants previously generated by the group (Pease et al. 1998) were thawed and the levels of CCR3 examined by flow cytometry to ensure robust expression levels (Figure 3.7 B). Being satisfied that the cells were expressing CCR3, chemotactic responses to an optimum concentration of CCL11 (1 nM) were examined in the absence and presence of increasing concentrations of CCL18 (Figure 3.7 A). CCL18 inhibited CCL11 in a dose-dependent manner, with responses completely inhibited when 500 nM CCL18 was used. In contrast, an identical concentration range of CCL18 had little or no effect on the migration of CCR8 transfectants migration towards an optimum concentration of 1 nM CCL1 (Figure 3.7 C). Thus, we conclude that our CCL18 stocks were functional, with respect to inhibition of CCR3 as previously reported.

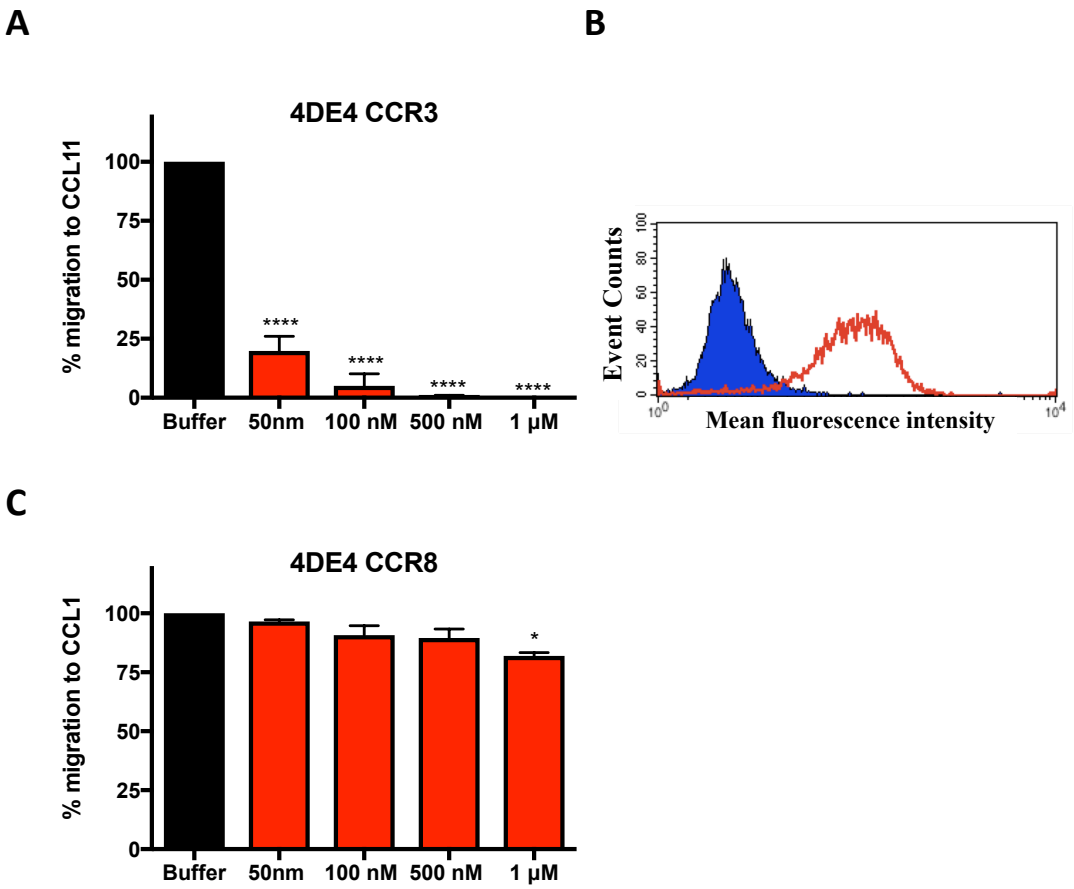


Figure 3.7 CCL18 inhibits CCR3-mediated migration. (B) a Representative histogram of surface expression of CCR3 on 4DE4-CCR3 transfectants. (A & C) Chemotaxis assays were used to assess the inhibitory effects of increasing concentrations of CCL18 on the migration of 4DE4-CCR3 cells to 1 nM CCL11(A), or the migration of 4DE4-CCR8 cells to 1 nM CCL1 (C), 100% migration was calculated from cells responding to the fixed concentration from the relative chemokine without CCL18. A representative of three experiments (**** shows $p<0.0001$ for 4DE4-CCR3 and * indicates $p<0.05$ for 4DE4-CCR8 treated with CCL18 using one-way ANOVA & Bonferroni's multiple comparisons test.

3.2.7 CCL18 binding to 4DE4-CCR8 cells is undetectable.

Recombinant CCL18 with an Alexa Fluor 647 tag at the C-terminus (AF-CCL18), was used as a tool to determine direct interactions CCR8 in flow cytometry ligand binding assays (Figure 3.8). Again, 4DE4-CCR3 cells were used as a positive control. Cells were incubated with a range of concentrations of AF-CCL18 for 30 minutes at 37°C, and then the relative efficacies of binding were examined by flow cytometry. AF-CCL18 was able to specifically stain 4DE4-CCR3 transfectants when used at a concentration of 100 nM, in keeping with the earlier chemotaxis data. However, no significant staining of 4DE4-CCR8 trasnfectants above the level of staining of the parental 4DE4 line was observed We therefore conclude that at best, CCL18 is a low-affinity ligand for CCR8, beyond the limits of detection in our assay.

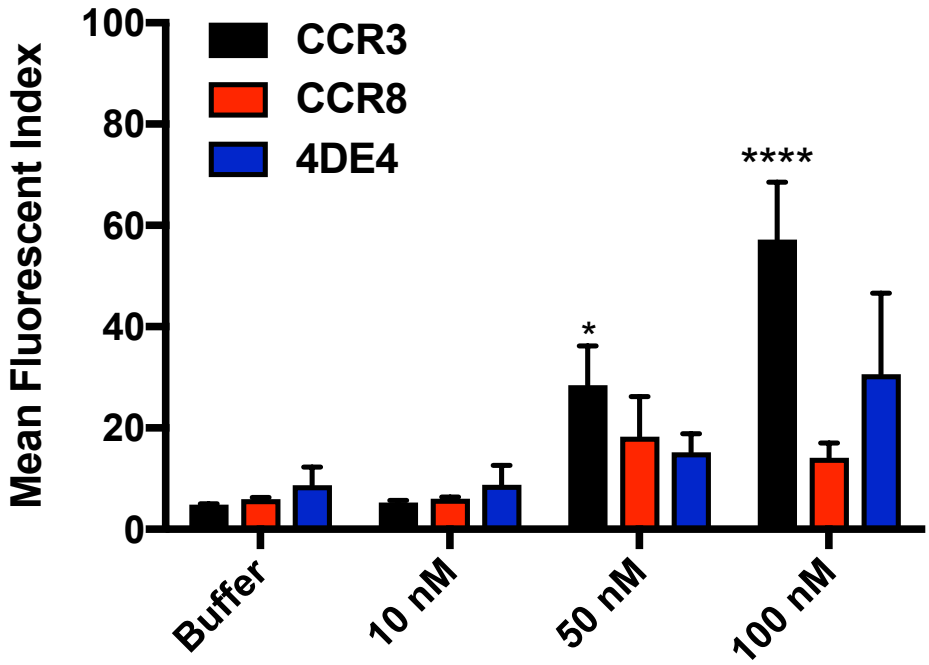


Figure 3.8: Alexa Fluor 647-CCL18 is unable to bind to CCR8 transfectants. – 4DE4-CCR3, 4DE4-CCR8 and naïve 4DE4 cells were incubated with PBS or with increasing concentrations of AF-CCL18 (10, 50, and100 nM). After washing steps, cells were analysed by flow cytometry. Data shown are the mean ± SEM of 6 experiments (**** shows $p<0.0001$ between CCR3 at 100 nM treated and Mock treated, and CCR3 at 50 nM showed * shows $p<0.01$ between treated and Mock treated for CCL18 binding). using two way ANOVA. & Bonferroni's multiple comparisons test.

3.2.8 Evidence for a CCL18 receptor on 4DE4 cells distinct from CCR8.

As described earlier in the chapter, the initial chemotactic responses of the same cells to CCL18 seen in Figure 3.9 were fragile and faded as cells were cultured over a comparatively short period of time, even when the concentration range of CCL18 was increased to 2 μM (Figure 3.9 A). Remarkably, the naïve 4DE4 cells devoid of CCR8 still showed a feeble chemotactic response at micromolar concentrations of CCL18 (Figure 3.9 B). However, responses to the primary CCR8 ligand CCL1, remained healthy in 4DE4-CCR8 cells (Figure 3.9 C) suggesting that the loss of CCL18 responses was not simply due to a loss of CCR8 expression.

We therefore hypothesized that the small chemotactic response to CCL18 shown by 4DE4 cells was not a CCR8 response and was likely coming from a low affinity CCL18 receptor endogenously expressed by the parental 4DE4 cell line. We used MC148 which we have previous shown to be an effective antagonist of CCR8 (Figure 3.5) to test this theory. Naive 4DE4 and 4DE4-CCR8 cells were incubated with a concentration of 500 nM MC148 or PBS control for 15 minutes before examining their chemotactic response to fixed concentrations of 1μM CCL18 or 10nM CCL1 (Figure 3.9 D). Incubation with MC148 was seen to ablate the responses of CCR8-4DE4 transfectants to CCL1 but had no effect on CCL18 responses from 4DE4. This supports our hypothesis that 4DE4 cells express a low affinity CCL18 receptor distinct from CCR8.

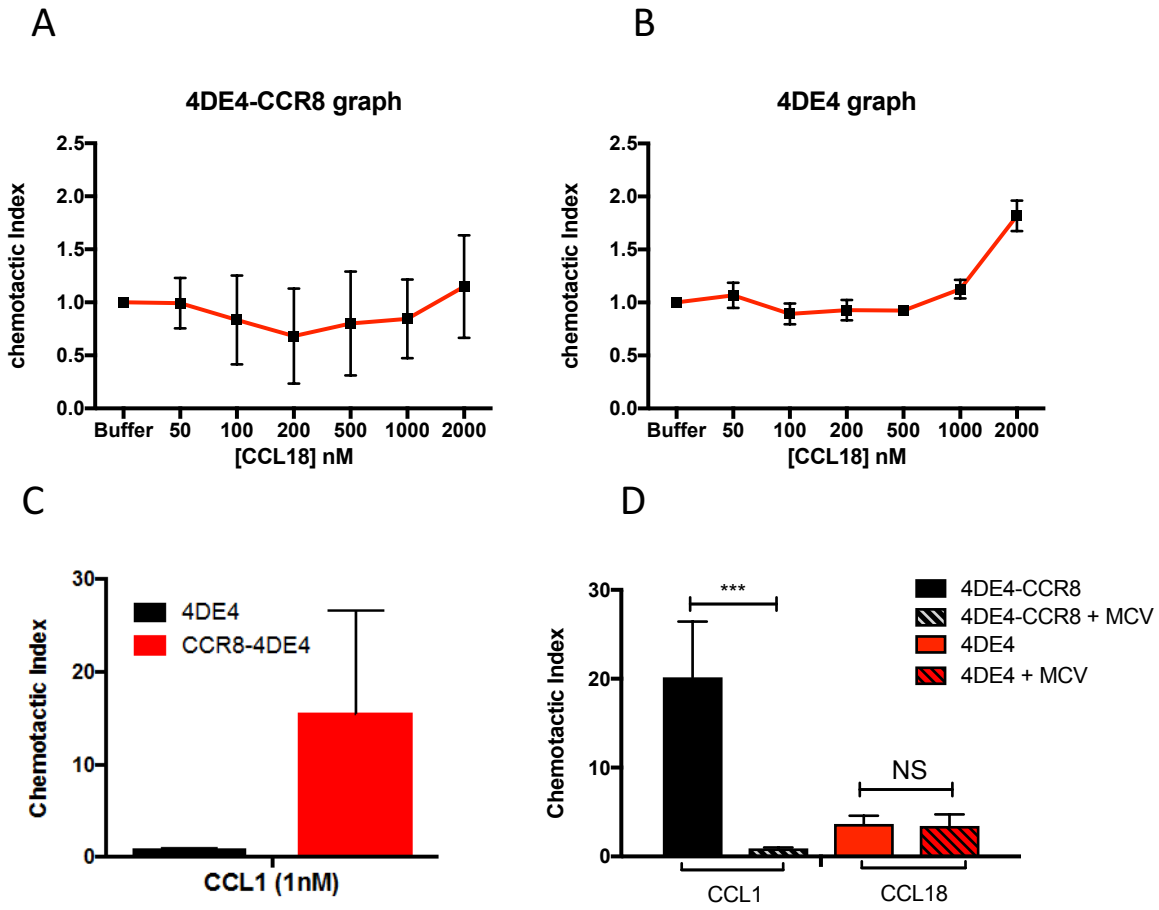


Figure 3.9: Endogenous responses of the 4DE4 cells line are not blocked by the CCR8 antagonist MC148. Chemotactic response to CCL18 from 4DE4-CCR8 cells (A) and 4DE4 cells (B). The data are representative of two independent experiments. (C) Responses to 1 nM CCL1 from 4DE4-CCR8 and 4DE4, data are representative of two independent experiments. (D) The migration of 4DE4-CCR8 and 4DE4 cells pre-treated with MC148 or PBS control to 1 nM CCL1or 1 μM CCL18 . Data are shown as mean ± SEM from three experiments (P = NS for 4DE4 treated with CCL18, *** indicates $p<0.001$ between treated and untreated conditions for 4DE4-CCR8 response to CCL1 using paired t test analysis).

3.3 Discussion

A fundamental problem in understanding the complexities of CCL18 biology has been the failure to identify its receptor, almost twenty years since the discovery of the chemokine (Adema et al. 1997). Identification of the CCL18 receptor will offer new insights into the role of this important human chemokine and it might prove to be a potential target for therapeutic intervention (Pease and Horuk 2009a, 2009b). As the CCL18 gene only exists in primates and does not appear to have a rodent orthologue, CCL18 is also difficult to study in animal models.

The identification of a receptor for CCL18 has been suggested several times in the literature with CCR6 and PITPNM3 described as CCL18 receptors (Chen et al. 2011), which was subsequently disputed by others (Hussain et al, 2011)(Krohn, Bonvin, et al. 2013, Krohn, Garin, et al. 2013).

The description of CCR8 as a CCL18 receptor (Islam et al. 2013) appeared to be more authoritative, with several lines of functional data including ligand binding, chemotaxis and endocytosis assays in both transfectants and in Th2 cells. Moreover, the group postulated that CCL18 was a functional analogue of the mouse CCL8 which lacks a human orthologue which they had previously shown to be a valid CCR8 ligand (Islam et al, 2011). Several lines of evidence from studies of lung inflammation are supportive of this postulate. Firstly, CCL18 and mouse CCL8 are highly induced in alveolar macrophages by stimuli such as IL-4 and IL-13 (Kodelja et al. 1998, Schutyser et al. 2005). Secondly, CCL18 is the most highly expressed chemokine in the lung disease idiopathic pulmonary fibrosis or IPF (Prasse et al., 2009). Thirdly, mouse CCL8 was

reported to be the most highly induced chemokine in a bleomycin-induced model of pulmonary fibrosis (Liu et al., 2011).

Our initial aims then, were to replicate some of the Luster group's findings. We performed a series of controlled experiments, initially to confirm whether CCL18 can induce CCR8 transfectant migration, using the same 4DE4-CCR8 cell line that was shipped from London to Boston to be utilized by the Luster group. This line expresses detectable levels of CCR8 on the cell surface and gives a robust, reliable chemotactic response to CCL1 the principal ligand for CCR8(Roos et al. 1997, Tiffany et al. 1997).

In our hands, CCL18 was a curiously weak chemoattractant for CCR8. The preliminary experiments showed that CCL18 was a full agonist in chemotaxis assays compared to CCL1 but with a significantly lower potency when compared with the known ligand CCL1. However, it was notable that the naïve 4DE4 cells showed a similar chemotactic response towards high concentrations of CCL18. The response from the naïve 4DE4 cells line was similar in efficacy to the response reported for the 4DE4-CCR8 cell line by Islam et al (Islam et al. 2013). This observation was the first hint that the responses we observed may not mediated by CCR8 but perhaps by endogenous receptors on the cell surface. Tellingly, a small chemotactic index (CI) was observed by the Luster group report for CCL18 responses (~ 4) which is more typical of endogenous receptors that the inflated CI of ~ 20 that they reported for CCL1 which is typical of expression systems (Islam et al. 2013). Alternatively, it could be that CCL18 is a partial agonist in this assay when compared with CCL1.

Of concern to us, the chemotactic responses to CCL18 were lost in this cell line over a few weeks in culture, despite the cell surface levels of CCR8 and the robust responses

to CCL1 being maintained. To confirm that our supply of CCL18 is functional, we used the only other receptor that CCL18 has been reported to bind to, which is CCR3, where it acts as an antagonist of CCL11 responses (Nibbs et al. 2000). We observed detectable binding of CCL18-AF to CCR3 transfectants although we were unable to show significant binding of CCL18-AF to CCR8 transfectants under identical conditions, in agreement with Krohn and colleagues (Krohn, Bonvin, et al. 2013, Krohn, Garin, et al. 2013). Moreover, CCL18 was unable to significantly inhibit the responses of CCR8 transfectants to CCL1, whereas CCL18 significantly inhibited the responses to CCL11 of 4DE4-CCR3 transfectants in a dose-dependent manner. CCL18 was also without activity in assays of CCR8 endocytosis in which CCL1 was again an efficacious agonist.

Returning to the paper by Islam and colleagues, we were mindful of their observation that the selection agent G418 was inhibitory to CCL18 responses. To circumvent this, they subcloned our original 4DE4-CCR8 line, weaning it off the original 1mg/ml G418 to grow in media with 200µg/ml of G418. (Islam et al. 2013). We performed identical subcloning experiments generating two clones which grew in 200ug/ml of G418. While a robust response to CCL1 was maintained in these lines which was similar or better than those seen with the parental 4DE4-CCR8 cell line, we were unable to detect any response to CCL18. Identical datasets were generated in both a transient expression system utilising L1.2 cells and a stable L1.2 CCR8 cell line, namely in the presence of robust CCL1 responses, no chemotaxis was observed to CCL18. This is in agreement with Islam et al who saw no chemotaxis to CCL18 in this cell background. These findings could be explained by the L1.2 cell line missing a signalling component, present in the 4DE4 line, which is permissive for CCL18-mediated chemotaxis.

Alternatively, in keeping with our earlier data, 4DE4 cells, but not L1.2 cells, express an endogenous receptor which is able to mediate responses to CCL18. Perhaps supportive of this latter observation, the signal observed in the binding of CCL18-AF to naïve 4DE4 cells was a little more intense than that observed for the CCR8-4DE4 cells. We attempted to characterize this potential endogenous CCL18 receptor further. Chemotaxis assays using the anti-CCR8 mAb 433 and the *Molluscum* protein MC418 validated the latter agent as an effective inhibitor of CCR8-mediated chemotaxis. However, pre-treatment of the naïve 4DE4 cells with MC148 was unable to block the weak chemotactic response to CCL18 suggesting that the endogenous CCL18 receptor was distinct from human CCR8. Since MC418 has been reported to have little or no antagonistic activity at murine CCR8 (Lüttichau et al. 2001) we cannot rule out the possibility that the murine orthologue of CCR8 was responsible for the weak CCL18 responses we observed in 4DE4 cells. However, since mCCR8 is responsive to human CCL1 (Lüttichau et al. 2001) and the parental 4DE4 cell has been reported to be unresponsive to human CCL1 (Tiffany et al. 1997) this would appear to be unlikely.

In summary, we were unable to independently verify that CCR8 functions as a bona fide CCL18 receptor, using a variety of well-controlled chemotaxis, endocytosis and ligand binding assays. The insignificant response from a naïve murine pre-B cell line suggests the existence of an unidentified CCL18 receptor, distinct from CCR8.

4 The effects of CCL18 on monocyte function

4.1 Introduction

Experimental assessment of the role of CCL18 in atherosclerosis and cardiovascular disease has been challenging because of the lack of a known CCL18 receptor and the fact that CCL18 is present in primates only, obviating the use of rodent models. CCL18 is present at relatively high concentrations in human plasma, but in some disease states, levels of the chemokine have been reported to be elevated. Several studies, both *in vivo* and *in vitro* clearly link CCL18 to the fibrotic process. Investigations of alveolar macrophages recovered from the bronchoalvelolar lavage (BAL) of patients suffering from idiopathic pulmonary fibrosis revealed that CCL18 protein was significantly upregulated (Prasse et al. 2006).

In vitro, CCL18 has been shown to drive the production of collagen by human lung fibroblasts (Atamas et al. 2003), and interestingly, it was shown that collagen could itself induce CCL18 production from alveolar macrophages, suggesting a positive feedback loop that drives fibrosis (Prasse et al. 2006). In the atherogenesis setting, Hagg et al. investigated the expression of CCL18 in atherosclerotic plaques by immunohistochemistry. They concluded that CCL18 is highly expressed in human carotid plaques and it is the CD68+ macrophages situated in the plaques that express the chemokine CCL18 (Hägg et al. 2009)(Reape et al. 2010). De Sutter et al. subsequently evaluated CCL18 as a circulating biomarker for atherosclerosis and found that high levels of serum CCL18 were an independent predictor of future cardiovascular events such as myocardial infarction (De Sutter et al. 2010). Since CCL18 is expressed

by alternatively activated (M2) macrophages (Kodelja et al. 1998), it would appear to be part of a remodeling process and likely to play a role in the formation of the fibrous cap in the developing atherosclerotic lesion.

Since CCL18 is predominantly produced by dendritic cells and monocytes/macrophages, we sought to study the effect of CCL18 on these producer cells in an effort to understand various activation programs and functional phenotypes induced by CCL18 which could possibly be translated to the disease setting to give a better insight in the potential pro or anti-inflammatory roles of this CC chemokine.

In vitro findings show that CCL18 is known to be chemotactic for naïve T- and B-cells (Adema et al. 1997, Hieshima et al. 1997) Although freshly isolated monocytes do not respond to CCL18, maturing monocytes/macrophages cultured for 3 to 4 days develop transient responsiveness to CCL18 (Schraufstatter 2004).

To understand the biological function for CCL18 Schraufstatter et al., screened various leukocyte populations for CCL18 expression and response to CCL18, with the idea that the cellular source may link CCL18 to disease states in which it may be involved. They found that peripheral blood monocyte/macrophages in culture for 3–4 days responded to CCL18 and induced calcium mobilization, directed migration, and actin polymerization. Freshly isolated monocytes did not show this activity in agreement with the previous report by Hieshema and colleagues (Hieshima et al. 1997).

A study by Wimmer and colleges established that CCL18 stimulates the hematopoiesis-supportive function of the hematopoietic stem/progenitor cell (HSPC) microenvironment indirectly by regulating gene expression and cytokine release in monocytes. Wimmer et al. revealed that CCL18 had an anti-apoptotic effect on

monocyte and described a new regulatory function for CCL18 in hematopoiesis. They presented gene expression profiling and found that enhanced survival seen in Monocyte stimulated with CCL18 lead to the down-regulation of several pro-apoptotic proteins, including caspase-8 and programmed cell death 11 at the mRNA level. Enhancing cell survival, which is not a typical activity for chemokines has been reported for some other family members (e.g., CXCL12 and CCL2 and CXCL4) (Scheuerer rt al. 2000, Eugenin et al. 2003, Kortesidis et al. 2005) .

Our main aim was to elucidate the role of monocyte-derived CCL18 in atherosclerosis, since monocyte/macrophages play a prominent role in the development of atherosclerotic plaques. For this reason, these cells were the primary focus of our investigation. We assessed the ability of CCL18 to bind to freshly isolated monocytes, to act as a monocyte recruiting molecule in chemotaxis assays and also the ability of CCL18 to promote monocyte survival

4.2 Results

4.2.1 The effects of CCL18 on Monocyte Survival and Function

4.2.2 Gating of Monocytes and relative CD14 Expression

PBMCs were freshly isolated from human whole blood donated from healthy volunteers by Ficoll gradient centrifugation. The peripheral blood mononuclear cells (PBMCs) were then further purified followed using a Human Monocyte Isolation Kit (EasySep™) which is designed to isolate CD14+CD16- monocytes by negative selection from fresh or previously frozen PBMCs. Flow cytometry analysis was used to determine the purity of the isolated cells purity and to assess contamination with different lymphocytes. First, we evaluated the cells' viability by looking at membrane integrity using exclusion of a fluorescence dye (TOPRO-3) in the FL-4 channel. Then staining with a FITC-isotype control mAb or FITC-anti-CD14 mAb was used to measure the purity of the isolated cells. A representative example is shown in Figure 4.1. Typically, the percentage of CD14-positive cells in the purified monocytes was in the order of 98% of cells.

4.2.3 CCL18-AF647 binding to freshly isolated monocytes varies from donor to donor.

We first set out to examine the ability of CCL18 to specficially bind to monocytes. For this, we used a recombinant CCL18 with an Alexa Fluor 647 tag at the C-terminus (AF-CCL18). as a tool in flow cytometry ligand binding assays. We examined the binding

of 100nM of AF-CCL18 to monocytes in the presence or absence of a 10-fold excess (1μM) of unlabelled CCL18 (Figure 4.2). CCL18 binding to monocytes was detectable although quite variable in intensity. For Donors 1 & 2 the unlabelled CCL18 was seen to reduce the binding of the AF-CCL18 to monocytes whereas for Donor 3, binding appeared largely unaffected.

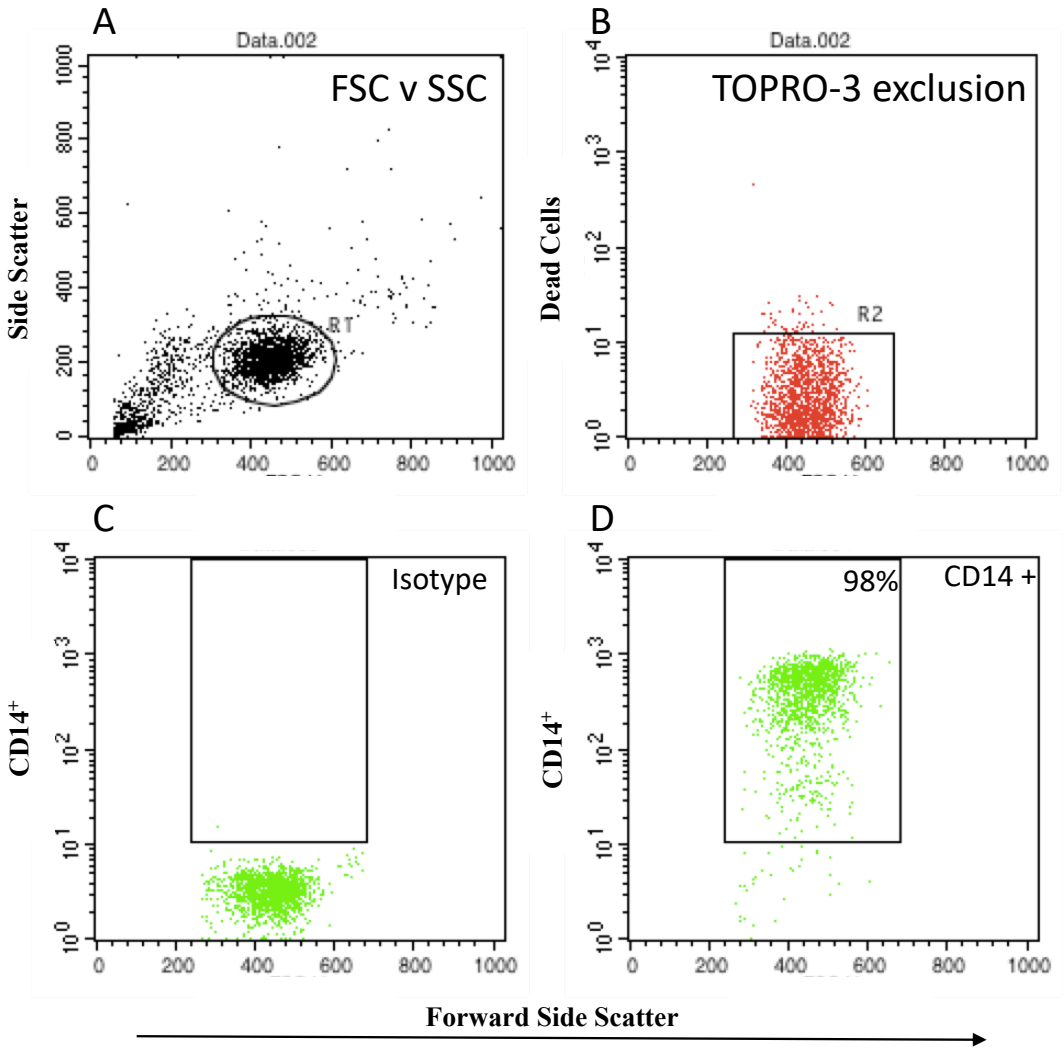


Figure 4.1. CD14 Expression on freshly isolated monocytes. Purified human monocytes were gated on FFSc v SSC (Panel A), gated against TOPRO-3 uptake to gate live cells (R2, Panel B). A FITC labelled isotype control mAb (Panel C) or FITC-anti-CD14 mouse IgG (Panel D) was used to ascertain the purity of the preparation. FACS data is from a single preaparation, representative of several purifications.

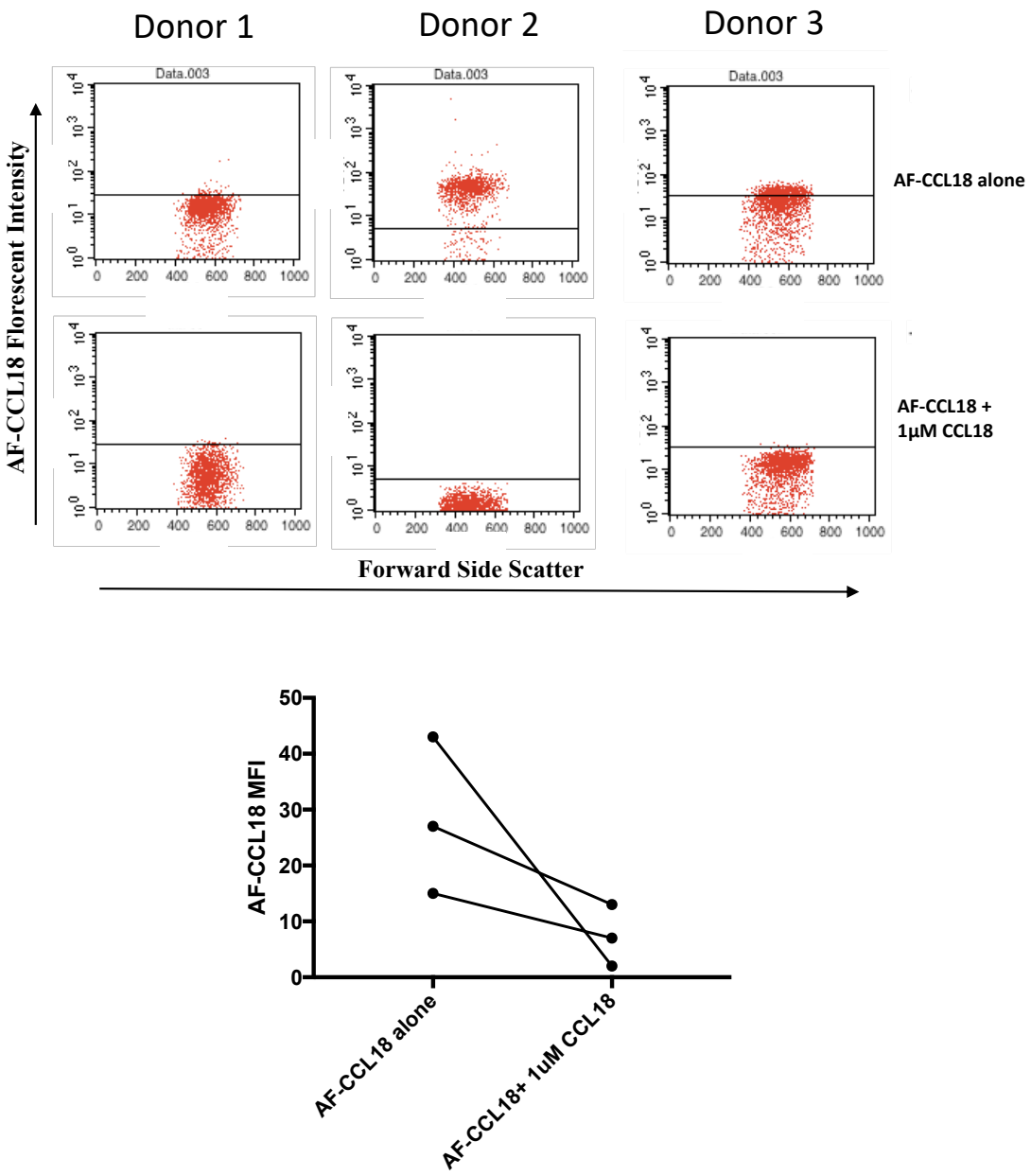


Figure 4.2. CCL18 Binding to Monocytes.

Analysis by flow cytometry of the fluorescent intensity of 100 nM AF-CCL18 binding to freshly isolated monocyte in the presence or absence of 1µM unlabelled CCL18, after incubation for 30 minutes at 37C°. Data shown are from single experiments using monocytes purified from 3 different donors

4.2.4 Chemokine Receptor Expression on freshly isolated monocytes

Since in the previous previous chapter we had generated data which questioned the ability of CCR8 to act as a CCL18 recptor, we first sought to determine if CCR8 was expressed at all by monocytes. We used the same 433H CCR8-specific mAb used previously to stain CCR8 transefctants. A MAb specific for CCR2 was used as a control (Figure 4.3). Although CCR2 was reaily detectable, little specific staining for CCR8 was observed, suggesting that any function data acquired using CCL18 on freshly isolated monocytes is likely through a different receptor.

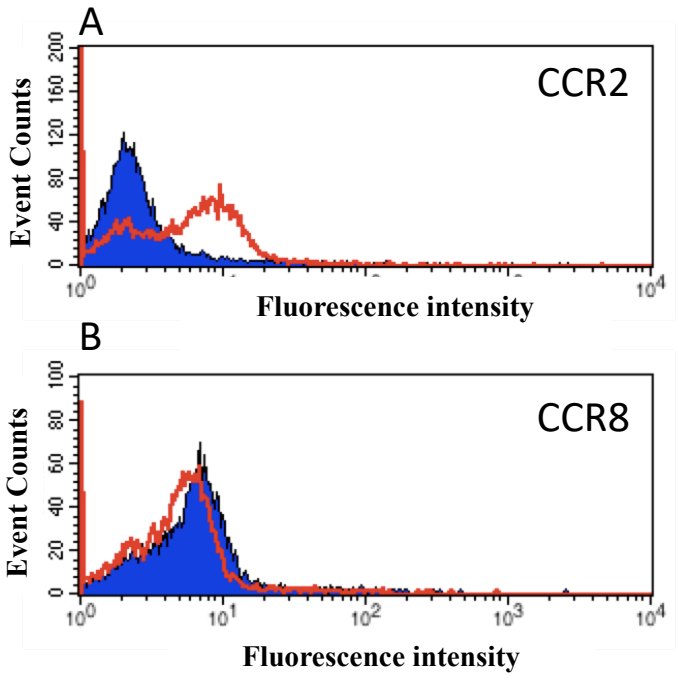


Figure 4.3. CCR2 and CCR8 expression on on freshly isolated monocytes. Purified human monocytes were stained with a monoclonal antibody directed against CCR2 (Panel A) or CCR8 (Panel B). Dead cells were excluded form alanlysis by the use of

TOPRO-3. Staining with the relevant isotype control is shown in blue. Data are representative of at least 3 seperate experiments.

4.2.5 Chemotaxis of monocytes to CCL18 visualized via TAXIScan.

Having established that CCL18 was bound by monocytes we assessed whether it drove the directed migration of monocytes, a property typical of chemokines. For this we used real-time chemotaxis assays (TAXIScan) in which monocytes were allowed to migrate along a BSA-coated glass slide in response to gradients of chemokine. Gradients of CCL18 were formed by the addition of 1µl of 1µM or 10 µM to the system. A gradient formed from 1µM CCL2 was sued as positive control, whilst basal migration was assessed in the absence of a chemokine gradient. Images of migrating monocytes were captured over a 1hr period and subjected to manual tracking.

Although CCL2 was clearly able to stimulate monocyte recruitment, in contrast the migration observed in the presence of CCL18 was undistinguishable from that seen in the absence of a chemokine gradient (Figure 4.4). Analysis of the tracks revealed migration of cells when CCL2 was the stimulus but no difference between the buffer (negative control) and CCL18 (Figure 4.5). Noticeably, Monocyte cells were found to be poorly responsive to 1 µM CCL18 but at 10 µM more cells responded and showed a trend of more directional migration towered CCL18. only 33 cells seen migrating and some migrating away from the source of chemokine at 1 µM, where 58 cells migrated to 10 µM (Figure 4.4 C&D).

Analysis of the tracks revealed significant directional migration of cells when CCL2 was the stimulus but no significant difference between the unstimulated cells and CCL18 (Figure 4.5 B). Assessment of the yFMI parameter also showed CCL2 was the

only significant migration along the chemokine gradient, with CCL18 being no better than no stimulus at 1 μM but at 10 μM slight increase was detected.

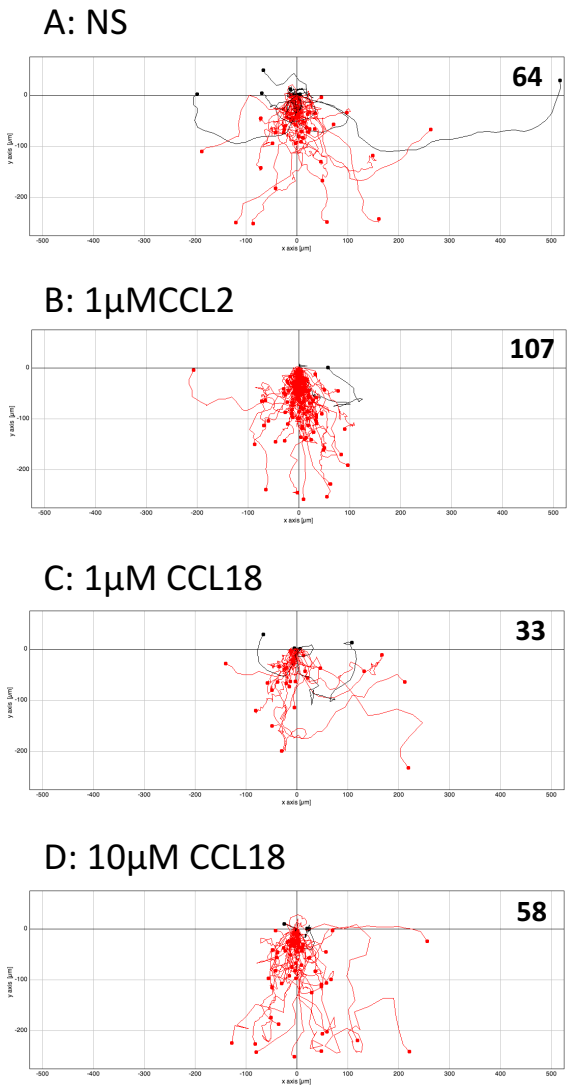


Figure 4.4. Chemotaxis of monocytes to gradients of chemokine

XY migration plots of monocyte migration in the absence of stimulus (A) or gradient of chemokine 1 uM CCL2 (B) 1 uM CCL18 (C) or 10uM CCL18 (D). plots were generated from combining corresponding tracked data from 3 different experiments pooling data from 9 movies assayed by TAXIScan for 60 minutes at 37 C°. Panels A to D represent each cell with a red dot and its migration path with a red line. The total number of migrating cells for each panel is shown in the upper right hand corner.

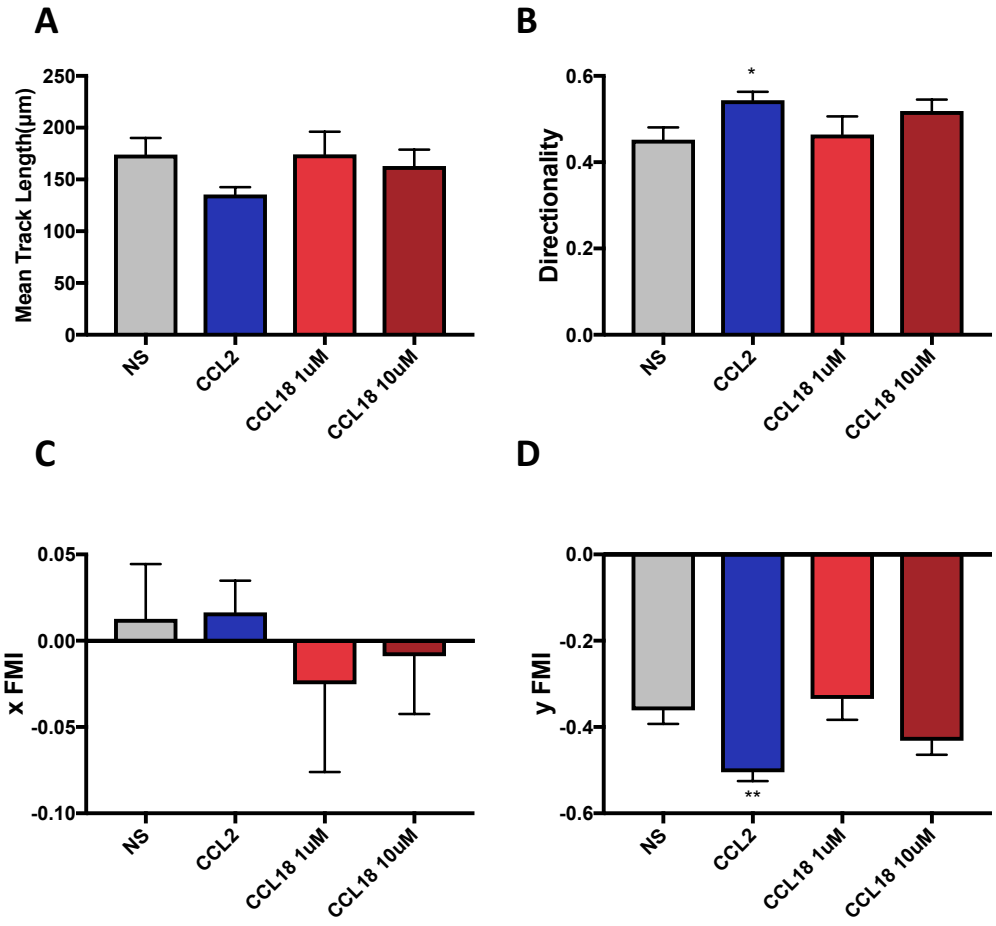


Figure 4.5 Analysis of monocyte migration

Data in figure 4.4 were analysed using the Chemotaxis tool, generating several tracking parameters, (A) shows the mean track length, (B) shows the directionality, (C) shows the forward migration in the x axis (xFMI) and (D) shows the forward migration in the y axis (yFMI). Data shown are the mean ± SEM and significance was determined by one-Way ANOVA with Bonferroni's Multiple Comparisons test was performed.

4.2.6 CCL18 acts as a monocyte survival factor

Since CCL18 appears to be an atypical chemokine in terms of being unable to recruit monocytes we assessed other potential functions induced by CCL18 that might be relevant to the process of atherosclerosis. Since Wimmer and colleagues have previously reported that CCL18 can function as a monocyte survival factor (Wimmer et al. 2006) we assessed this using a variety of methods. In the first instance, monocytes were freshly isolated from PBMCs by negative selection as before and cultured in serum-free RPMI for 72h. After this, media was rempoved and replaced with serum-free RPMI alone or serum-free RPMI containing increasing concentrations of CCL18. 100ng/ml of M-CSF (Hashimoto et al. 1997) was also used as positive controls. Cells were returned to the incubator for a further 24h after which viability was assessed by measuring ATP levels using the Promega Cell Titer Glo kit (Figure 4.6).

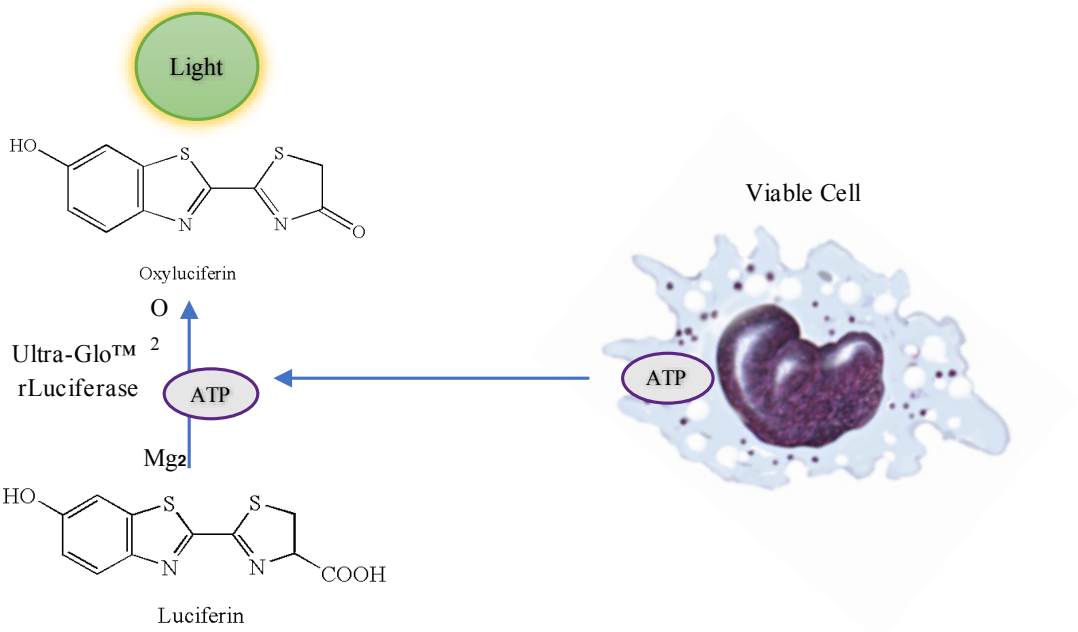


Figure 4.6. Schematic showing the science behind the Cell Titer Glo assay. ATP is released by cell lysis which drives the generation of Oxyluciferin from Luciferin by means of a luciferase. Emitted light is directly proportional to the number of viable cells.

The CellTiter-Glo system uses luciferase reaction that require ATP for the generation of a luminescent signal. viable cells, which are metabolically active cells, are the source for the ATP in this reaction. When CellTiter-Glo is added to cell culture cell lysis and generation of a luminescent signal relative to the amount of ATP present.

Using this assay of cell survival, there was a slight reduction in ATP levels when the M-CSF control treated cells were compared to untreated cells, although this was not significant (Figure 4.7A). There was a trend for increasing concentrations of CCL18 to rescue this phenotype, but again the effects were not signficant. The assay was subsequently varied to compare the effects of a fixed concentration of CCL18 on cell survival with the positive controls M-CSF and CXCL4 (Scheuerer et al. 2000). Monocytes were cultured for 72h in the presence or absence of 100 ng/ml of M-CSF, or 1μM CXCL4 after which they were subjected to lysis with the Cell Titer Glo reagent and asssessement of relative ATP levels by luminsence.

There was a more pronounced reduction of ATP levels when the M-CSF control treated cells were compared to untreated cells, although again this was not significant (Figure 4.7B). The fixed concntrations of CXCL4 and CCL18 trended towards rescuing the untreated phenotype but once more, this was not significant.

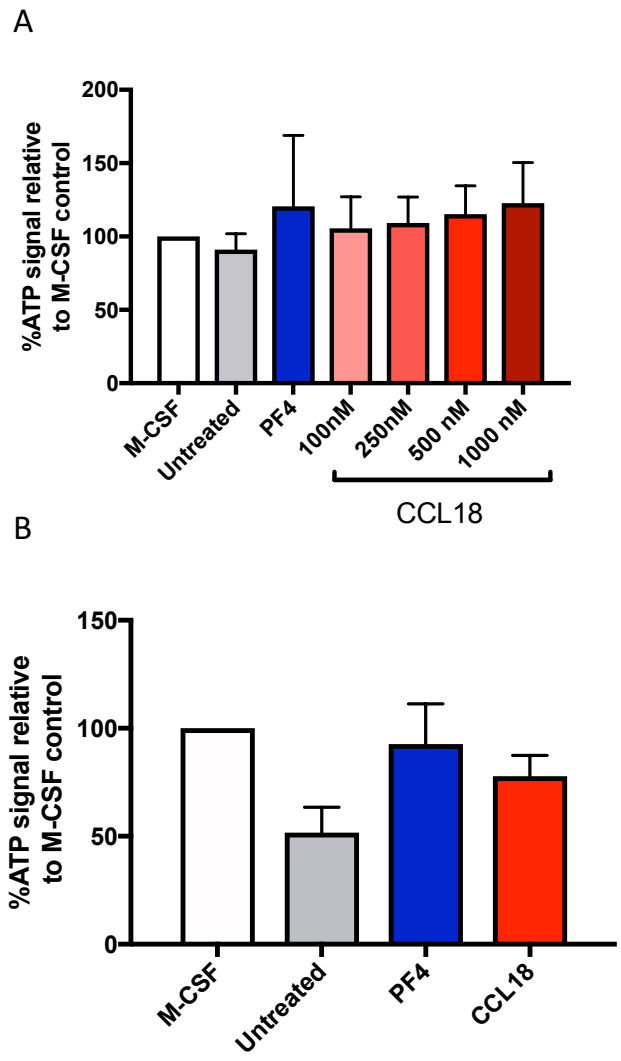


Figure 4.7. CCL18 Protects monocytes from apoptosis as assessed by intracellular ATP. (A) After 3 days in culture, monocytes/macrophages (6 10^4 cells/condition) were incubated in serum-free RPMI for 24 hours in the presence of 100ng/ml M-CSF (positive control), Untreated (buffer control) or 100, 250, 500, 1000 nM CCL18. Data shown are from 7 different experiments. (B) Purified human monocytes (5 10^5 cells/condition) were cultured in serum-free RPMI for 72 hours in the presence of 100ng/ml M-CSF (positive control) or 1μM CCL18, or 1μM CXCL4 or left untreated. The ATP luminescent signal was relative to the positive control as percentage. Data shown the mean ± SEM from 5 different experiments.

4.2.7 CCR8 blockade suggests an alternative receptor for CCL18

Despite there being little evidence of CCR8 on freshly isolated monocytes, it remained feasible that low levels of CCR8 near the limits of detection may mediate the CCL18 survival signal. We therefore examined if this survival effect was mediated through CCR8 in the presence of the CCR8 blocking mAb (433H) which we had shown in Chapter Three to be effective at inhibiting CCR8. Monocytes were freshly isolated from PBMCs by negative selection and after cultured for 72h in serum-free media with or without CCL18 and in the preence or absence of the anti-CCR8 mAb or an IgG_{2a} isotype control (Figure 4.8A).

The Increased ATP levels observed in CCL18-treated monocytes compared with the serum-starved human monocytes were not adversely affected by the CCR8-blocking mAb 433H. This data suggest that as expected, the pro-survival effects of CCL18 on monocytes effects are independent of CCR8 and that most likely, there exists an as yet unidentified receptor for CCL18 on monocytes. In an attempt to assess the CCL18 blocking monoclonal antibody, we repeated the same experiment using CCL18 mAb or IgG1 isotype control in the presence or absence of 1 μM CCL18. We could not detect any significant difference in the ATP levels observed after CCL18 treatment in both conditions (mAb and the isotype control) on the monocyte (Figure 4.8B). Unexpectedly the survival effect was decreased in both conditions.

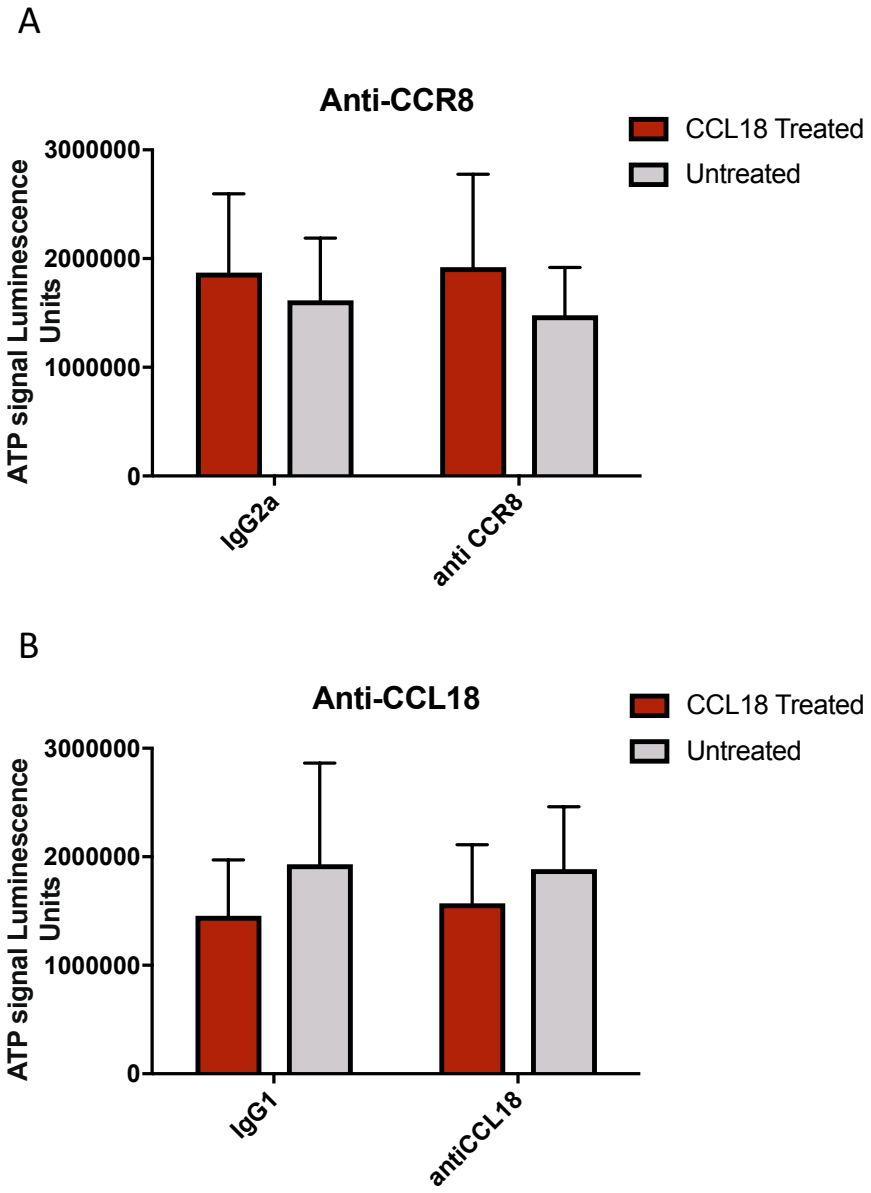


Figure 4.8. The pro-survival effects of CCL18 on monocytes are independent of CCR8. After 3 days in culture, monocytes/macrophages (6 10^4 cells/condition) were incubated in serum-free RPMI for 24 hours in the presence of 1μM CCL18 with CCR8 mAb (433H) or isotype control (IgG2a) or left unstimulated with CCR8 mAb or isotype control. Data shown are the mean ± SEM from 2 experiments.

4.2.8 Assessing monocyte viability by an alternative method

Cell viability can also be measured by examining cell membrane integrity, which is easily measure by flow cytometry, using exclusion of a fluorescence molecule e.g. propodium iodide (PI) which is impermeable to healthy cells but taken up by dead or dying cells. We subsequently used such an assay to examine the anti-apoptotic effect of CCL18 treatment of monocytes, using the molecule TOPRO-3 which is highly fluorescent in the FL-4 channel but unlike PI is non-toxic. Worried that in the absence of serum-contianing medium, the chemokine might stick to the plasticware and be unavailable to the monoyctes, we included BSA in RPMI medium at a final concentration of 1% (w/v). Freshly isolated monocytes were cultured for 3 days in the presence or absence of 1μM CCL18 or 1μM CXCL4 or 100 ng/ml M-CSF, after which cells were recoveded from the plasticware and resuspended in a buffer containing TOPRO-3 after which cell viability was assessed by flow cytometry. Data from a representative experiment is shown in Figure 4.9.

In the absence of cytokine, monocytes failed to thrive, with only 9% of the plated cells failing to uptake TOPRO-3 (Panel A). In contrast, all three cytokines were shown to preserve monocyte viability, with 4-6 times more cells surviving than in the absence of treatment (Panels B-D). When the data from cummulative expreiments were analysed, CXCL4 and CCL18 were seen to significantly increase the survival of monoyctes in culture, whilst M-CSF trended towards a pro-survival activity (Figure 4.10).

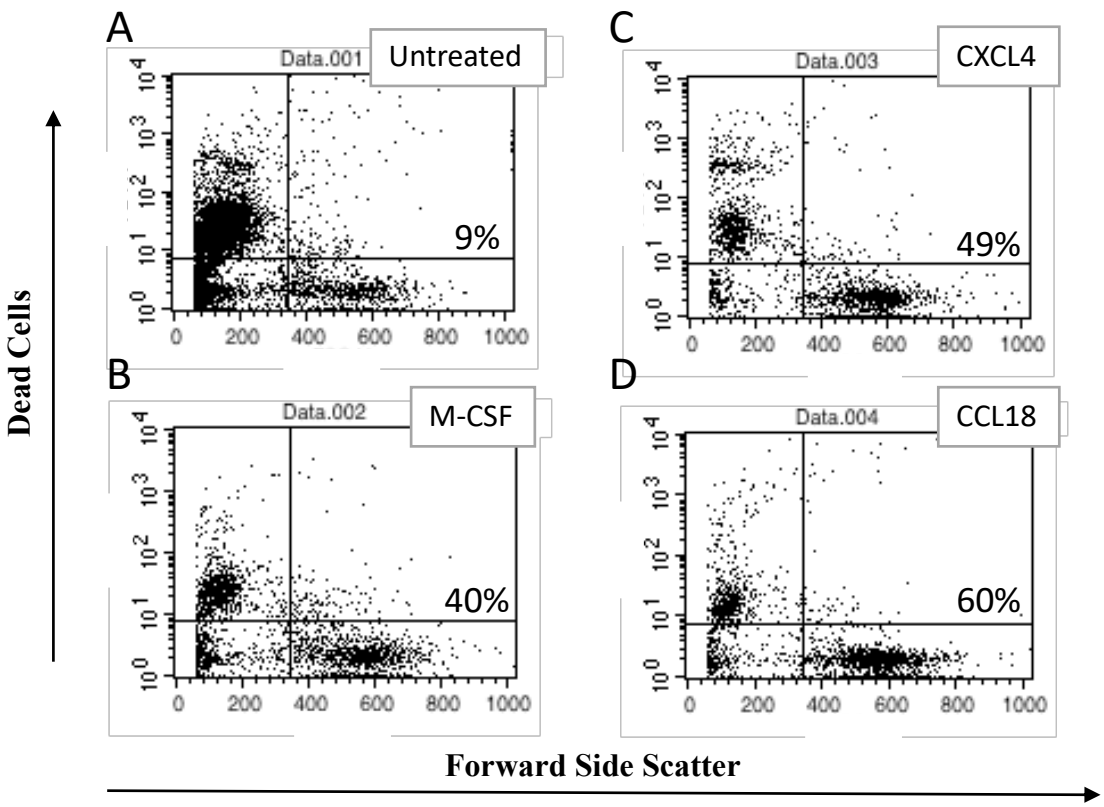


Figure 4.9. CCL18 is a monocyte pro-survival factor as assessed by flow cytometry. Purified human monocytes (5 x 10^5 cells/condition) were cultured in serum-free RPMI with 1% (w/v) BSA for 72 hours in the presence of 100ng/ml M-CSF (positive control) or 1μM CCL18, or 1μM CXCL4 or left untreated. Apoptosis was determined by TOPRO-3 exclusion which is highly fluorescent in the FL-4 channel was used in which viable cells remained un- stained in the lower right quadrant of a FACS dot plot. Data shown are representative from 7 different experiments.

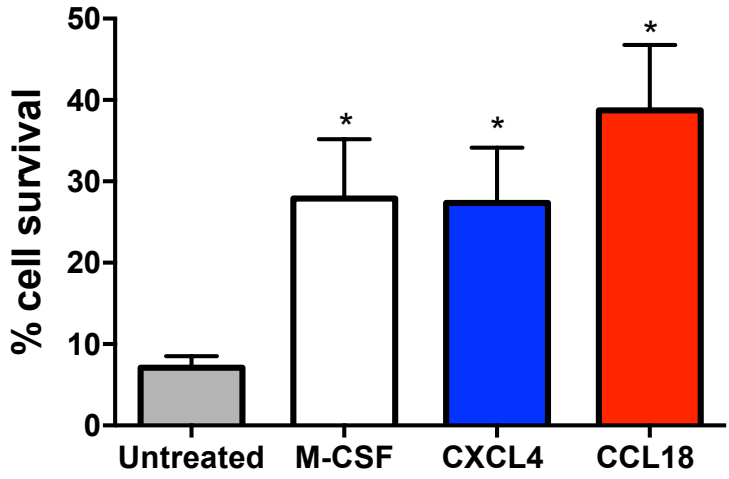


Figure 4.10 Both CCL18 and CXCL4 are significantly active pro-survival factors for monocytes.

Purified human monocytes (5 10^5 cells/condition) were cultured in serum-free RPMI with 1% (w/v) BSA for 72 hours in the presence of 100ng/ml M-CSF (positive control) or 1μM CCL18, or 1μM CXCL4 or left untreated. Apoptosis was determined by TOPRO-3 exclusion. Data shown are the mean ± SEM from 7 different experiments. Statistical significance was evaluated by unpaired Student's t-test for each treatment compared to untreated cells.

4.3 Discussion

Investigating the phenotypic responses of monocytes to CCL18 is hypothesized to give a better insight into the role of this CC chemokine in atherosclerosis since monocytes play a significant role in the development of foam cells and subsequent establishment of human atherosclerotic plaques. We examined in this chapter, the responses of cultured monocytes and freshly isolated monocytes to CCL18. We initially assessed the ability of freshly isolated monocytes to specifically bind CCL18 to detect the presence or absence of a putative CCL18 receptor. Interestingly we found that CCL18 could bind specifically to monocytes, but there was some heterogeneity between cells isolated from the three donors we examined. These data suggest that monocytes express a receptor for CCL18 and the variation in CCL18 binding between donors may be explained by variable levels of expression of the receptor between donors.

Another way of interpreting this data is agreeing with what was suggested before by others (Krohn, Bonvin, et al. 2013) namely that the primary binding interaction between CCL18 and responsive cells such as PBLs is mediated by glycosaminoglycans (GAGs). In those two studies, the authors confirmed the binding of CCL18 to heparin using heparin coated binding plates and compared the binding between intact CCL18 and a mutant variant of CCL18 that been designed to abrogate interactions with GAGs. These studies demonstrated that replacement of the basic residues by alanine in the 40s cluster of the chemokine abolished approximately 75% of the binding capacity to PBLs, suggesting either an involvement of the 40s cluster in receptor binding or that much of

binding to the cells is mediated by GAGs. Taking into consideration our data from the previous chapter, we suggest that the indicated binding of CCL18 to monocyte is likely via a receptor other than CCR8 as CCR8 was not expressed on the surface of freshly isolated monocytes.

To assess the chemotactic ability of CCL18 to potentially recruit monocytes to atherosclerotic plaques, we examined monocyte chemotaxis along CCL18 gradients in real-time chemotaxis assays (TAXIScan). Although the monocytes responded robustly to CCL2 gradients, we found that CCL18 gradients do not drive the recruitment of freshly isolated monocytes, in agreement with (Wimmer et al. 2006) and (Schraufstatter 2004). This result suggested that CCL18 could be binding to atypical chemokine receptor (ACKRs) as this type of receptor could bind to chemokine but fail to induce classical signaling and downstream cellular responses typical for chemokine receptors (Ulvmar et al. 2011). Schraufstatter et al. explained the freshly isolated monocyte unresponsiveness to CCL18 could be due either to the lack of CCL18 receptors at this point or desensitization of CCL18 receptors from previous stimulation with cell-derived CCL18. Krohn et al. found that CCL18 is a remarkably weak chemoattractant of leukocytes and they suggested that either that the CCL18 receptor is uniformly expressed at low levels among peripheral blood leukocyte populations or that only a minor subpopulation of cells is responsive to CCL18 (Krohn, Bonvin, et al. 2013).

Nevertheless, when we stimulated three days old monocytes cultured in serum-free RPMI with increasing concentration of CCL18, there was a trend towards an increase in the number of surviving cells, suggesting that CCL18 signalling could rescue monocytes from apoptosis. When we switched to an alternative protocol in which

monocytes were cultured continuously with CCL18, it was clear that CCL18 could act as a survival factor for in agreement with (Wimmer et al. 2006). Attempting to block this CCL18 survival effect on maturing monocyte (3 days old) using the CCR8 mAb (433H) was unsuccessful, suggesting once more, that CCR8 is not the receptor that CCL18 binds to and delivers this function in monocytes.

Notably, in the study by Wimmer et al. preliminary gene array anaysis suggested that some key pro-apoptotic genes were downregulated in monocytes following culture with CCL18 (e.g. caspase 8 and BAD) while the anti-apoptotic signaling molecule PI3K-alpha was upregulated. Although the fact that the survival effect for chemokines is unusual, this has been noted for other monocyte-specific chemokines. The platelet-derived chemokine CXCL4 is also able to protect monocytes from spontaneous apoptosis (Scheuerer et al. 2000). Remarkably, when we compared the effect of identical concentrations of CCL18 and CXCL4 on monocyte survival, we found that CCL18 appeared to be more efficacious than CXCL4. In a study performed by Gliessner et al., comparing the gene expression signature of CXCL4-induced macrophages (M4 macrophages) with M-CSF–induced macrophages (M0 macrophages), CCL18 expression was found to be induced in M4 macrophages following 6 days of culture (Gleissner et al. 2010). Since cultured monocytes responded to both CCL18 and CXCL4 in our survival assay, the question arose whether CCL18 might regulate the survival function of monocytes/macrophges through the autocrine release of CCL18 from CXCL4-stimulated monocyte/macrophages.

Therefore, in the next chapter we turned our attention to CXCL4, examining the effects of CXCL4 on CCL18 release in monocytes and other associated monocyte functions.

5 M4 macrophages and the production of CCL18

5.1 Introduction

In the previous chapter, data were presented which showed confirmed previous studies associating CXCL4 and CCL18 signalling with the promotion of monocyte survival (Scheuerer et al. 2000, Wimmer et al. 2006). Gleissner et al. have previously reported that monocytes cultured for 6 days in the presence of the platelet-derived CXC chemokine known as CXCL4/Platelet Factor Four polarized into macrophages with a unique transcriptome (Gleissner 2012). These macrophages which they dubbed "M4" macrophages, were shown to express significantly elevated levels of CCL18 compared to M0 macrophages cultured in the presence of M-SCF. We hypothesised that the induction of CCL18 by CXCL4 may be responsible for their enhanced survival. In this chapter, we therefore set about further characterizing the M4 macrophage phenotype.

5.2. Results

5.2.1 CXCL4 induces changes in monocytes morphology during culture

To investigate the M4 phenotype, a standardized protocol was established in which monocytes were purified as previously described and cultured in the presence or absence of additional growth factors. The serum-free medium X-vivo 15 was chosen to remove any confounding issues with serum-derived growth factors. Cells were cultured for up to 7 days in either media alone (unstimulated) or media supplemented with either 10ng/ml M-CSF or 1μM CXCL4 (Figure 5.1).

M-CSF is a well-characterized growth factor known to promote *in vitro* differentiation of monocytes into M0 macrophages (Metcalf 1986) . Similarly, the 1μM concentration of CXCL4 that used by Gleissner and colleagues in their transcriptome study and one well known to be sufficient to induce macrophage differentiation from monocytes (Scheuerer et al. 2000). In some studies, the effects of a combination of CXCL4 and M-CSF were examined.

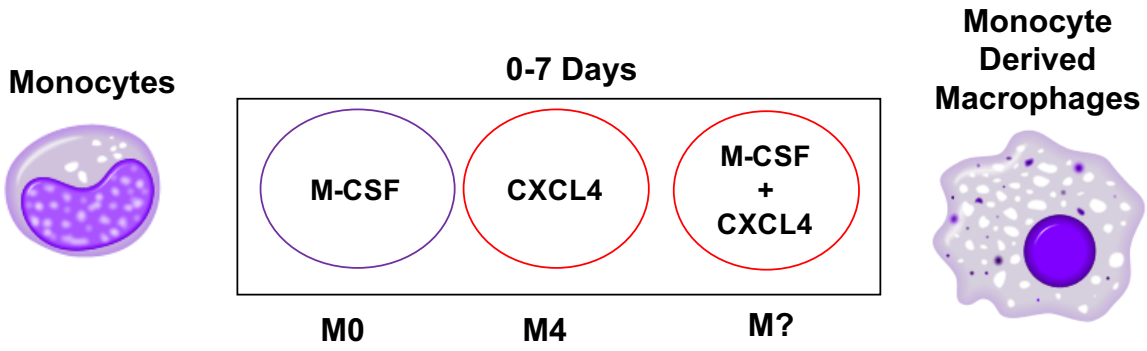


Figure 5.1 Schematic diagram showing the culture conditions used to drive macrophage polarisation in this chapter.

When we performed a microscopical examination after three days of monocyte culture, we noticed that relatively few of the unstimulated cells were adherent and acquired a macrophage-like morphology, whereas wells in which cells were stimulated with either M-CSF or CXCL4 looked more confluent, with a higher number of cells adhered to the plastic surface. Moreover, these cells looked substantially bigger. To confirm the increase in cell size, cells were detached from the plastic and flow cytometric analysis used to compare the forward scatter signal. Data for both cell treatments was normalized to that of the untreated cells. A significant increase in size was observed for CXCL4 treated cells, which were around 10% bigger than M-CSF treated cells. (Fig. 5.2).

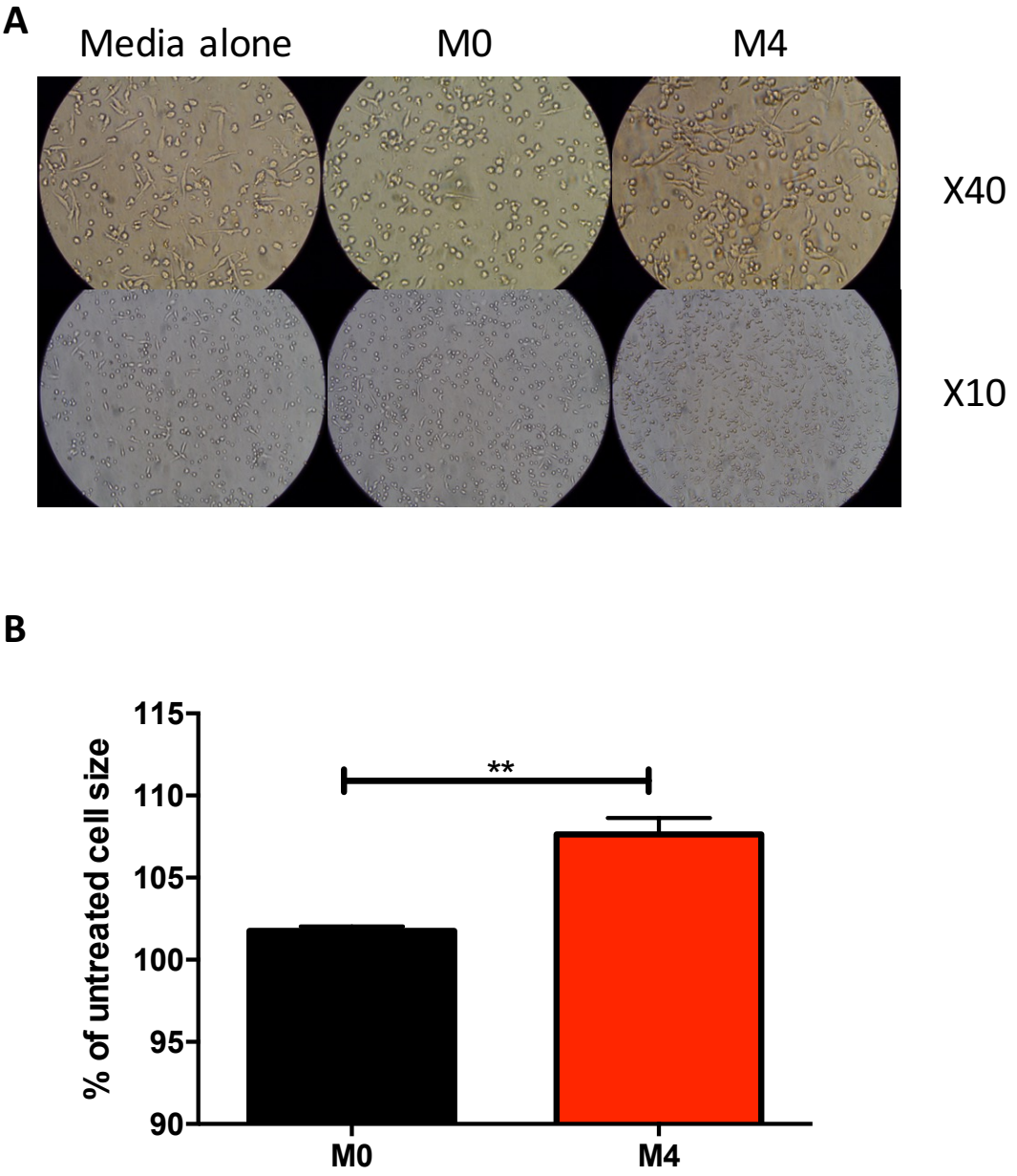


Figure 5.2. CXCL4 induced changes in monocyte morphology. Purified human monocytes were cultured for 72 hours in the presence of 1 μM PF4 or 10ng/ml M-CSF or in media alone. Panel A shows standard microscope images taken at the X10 (lower panel) and X40 (upper panel). Panel B shows cell size compared using forward scatter signal in flow cytometry analysis normalized to the % of untreated cell size. Data shown are the mean ± SEM from 4 experiments and were analysed by a paired, two-tailed t-test.

5.1.2 CXCL4 induced changes in cell-surface markers of the cultured monocytes

Cell surface markers are often regulated during monocyte differentiation in response to stimuli. For example, CD86, a costimulatory molecule required for the activation of T cells by monocytes was reported to be upregulated in CXCL4-treated cells compared to untreated (Scheuerer et al. 2000). To further characterize the phenotype of CXCL4-treated monocytes and verify that the changes in the cell size were due to cellular differentiation towards a macrophage (M4) different from the M-CSF stimulated cells (M0), we examined CD86 expression. Following 3 days of culture in media alone or supplemented with CXCL4 or M-CSF, cells were stained for CD86 expression and analysed by flow cytometry (Figure 5.3). The expression of CD86 was normalized to that of untreated cells and the differences between the two treatments examined. Significantly increased CD86 expression was observed on the CXCL4-treated cells compared with M-CSF treated cells, which might indicate a distant role for these macrophages.

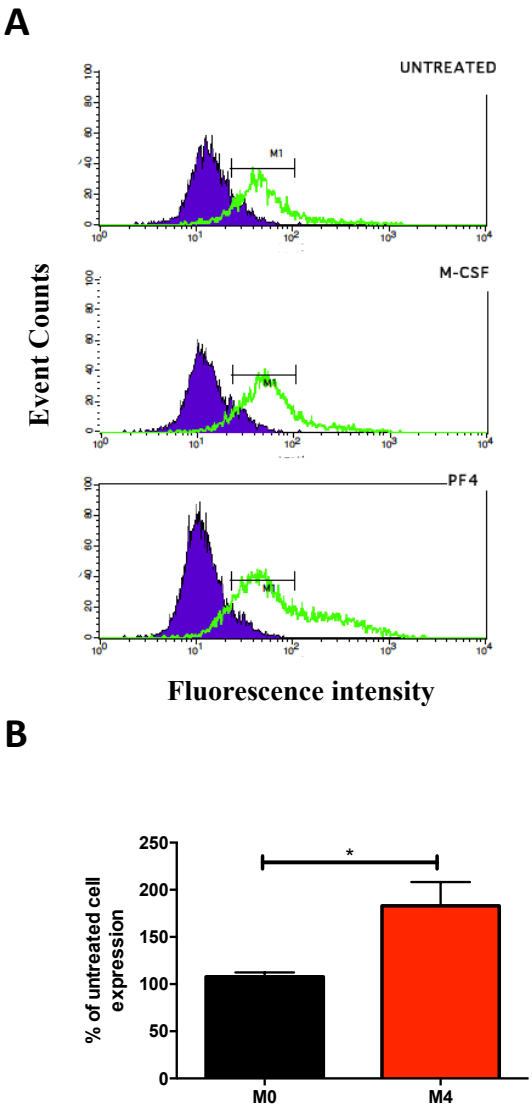


Figure 5.3. The effects of CXCL4 on expression of the differentiation marker CD86. Human monocytes were stained with monoclonal antibodies directed against CD86. Panel A shows a representative histogram of CD86 surface expression (red histograms) or isotype controls (blue histograms) on untreated cells or those cultured with 10ng/ml M-CSF or 1μM CXCL4. Data are representative of 6 experiments. Panel B shows analysis by flow cytometry of the mean fluorescent intensity of CD86 expression normalized to that of the untreated cells. Data shown are the mean ± SEM of 4 experiments and were analysed by a paired, two-tailed t-test.

5.2.1. Are the pro-survival effects of CXCL4 due to CCL18 release by M4 macrophages?

Gleissner et al. have previously performed transcriptome analysis of the mM4 macrophages cultured over a 6 day period. They showed that the transcriptome of CXCL4-induced macrophages is unique and different from M-CSF or other known polarization types. Interestingly for us, one of the genes that they reported to be upregulated in the M4 macrophages was CCL18, which was upregulated at both the mRNA and protein levels (Gleissner et al. 2010). We hypothesized therefore, that the previously characterized survival effects of CXCL4 (Scheuerer et al. 2000) may be due to autocrine effect of CCL18 induction in CXCL4-stimulated cells.

To test this hypothesis, cells were polarized as before the the M0 or M4 phenotype for up to 5 days. As a positive control, some monocytes underwent IL-4 stimulation which is known to induce CCL18 production by monocytes(Kodelja et al. 1998). Untreated monocytes were used as a negative control for normalisation purposes. At the end of the culture period, the supernatant was removed and reserved for ELISA, whilst mRNA was isolated from the cells for q-PCR studies using specific primers for CCL18 (Islam et al. 2013) and the house keeping gene β-actin. M4 macrophages cultured for 72 hr were seen to have similar levels of CCL18 mRNA compared to the untreated control, whilst M0 macrophages trend towards a small increase in CCL18 mRNA expression. In contrast, as expected, IL-4 treatment induced a significant induction CCL18 mRNA expression at levels approaching 300 fold those of untreated cells (Figure 5.4A).

We next measured CCL18 production by ELISA in the supernatant from monocytes treated with M-CSF or CXCL4 for 7 days, sampling the cells at day 2 and day 7 of

culture (Figure 5.4B). On day 2 the levels of CCL18 in all three supernatants were barely above the lower level of detection of the ELISA. By day 7, there was a trend towards M0 macrophages producing more CCL18 than either M4 cells or untreated cells, in the order of 300 pg/ml CCL18. In contrast, M4 macrophages did not secrete significantly more CCL18 than untreated cells alone. Collectively, these data suggest that the survival effect of CXCL4 observed in Chapter 4 is unlikely to be due to CXCL4-induced release of CCL18.

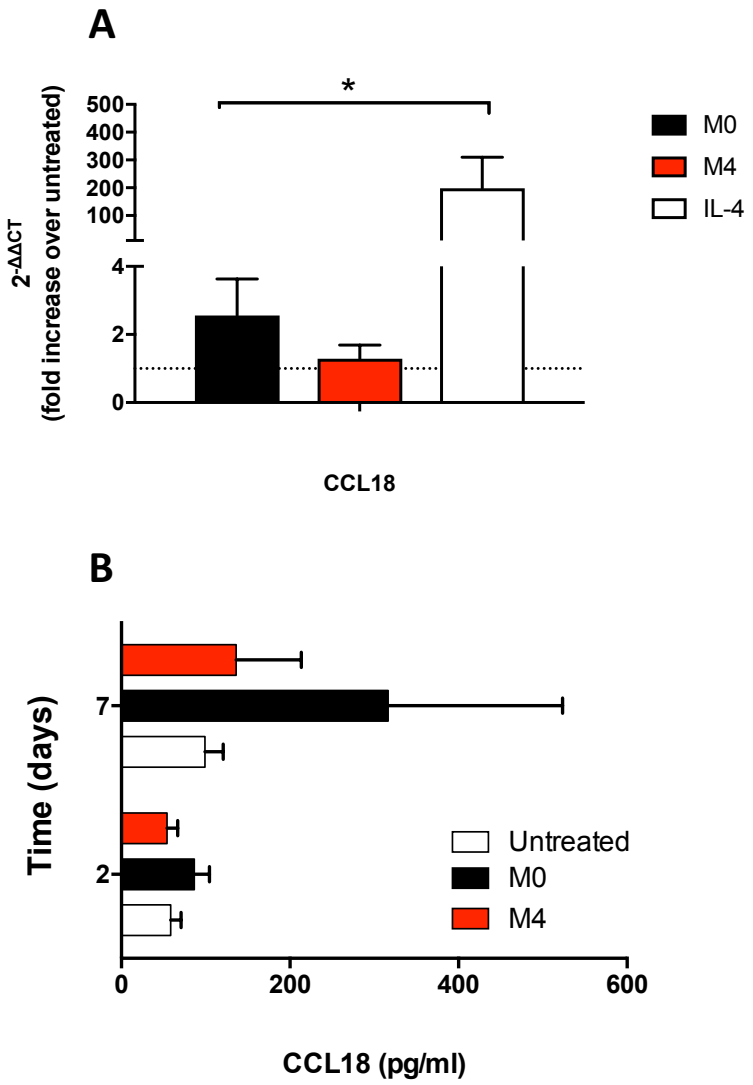


Figure 5.4. CCL18 is not produced at significant levels by M4 macrophages Monocytes were differentiated in the presence of 10 ng/ml IL-4, 10 ng/ml M-CSF or 1μM CXCL4 for up to 72 hours after which CCL18 mRNA and protein were measured by q-PCR and ELISA. Panel A shows the fold increase in relative gene expression for CCL18 mRNA normalized to β -actin (housekeeping gene). Data are representative of 4 experiments and were analysed by a paired, two-tailed t-test. Panel B shows the levels of CCL18 protein secreted by monocytes cultured in media alone or with M-CSF or CXCL4. The data shown are the mean ± SEM three experiments and were analysed by one-way ANOVA.

5.2.2. CCR1 is a potential receptor for the orphan chemokine CXCL4

Scheuerer et al. have previously suggested that CXCL4 actively accelerates the differentiation process of monocyte since upregulation of differentiation markers such as CD86 can be detected within 3 days (Scheuerer et al. 2000). We continued to investigate monocytes cultured for 3 days in an attempt to which chemokine receptors might be transducing the CXCL4 signal. We hypothesized that CXCL4 treatment might induce the desensitization and down regulation of its principal receptor on monocytes. We therefore examined the expression of mRNA for CCR1, CCR2, CCR3, CCR4, CCR8 after 72 hours of stimulation with either M-CSF or CXCL4. The only significant difference between treatment groups was a down-regulation in CCR1 mRNA levels following CXCL4-treatment. (Fig. 5.5A). We subsequently confirmed that CCR1 was expressed on the surface of freshly isolated monocyte (Fig. 5.5B).

To further assess the ability of CXCL4 to directly down-regulate CCR1 we carried out endocytosis assays on freshly isolated monocytes using CCL3 as a positive control (Figure 5.6). Cells were incubated for 30 minutes at 37°C or 4°C with either ligand after which CCR1 staining was used to assess endocytosis. Untreated monocytes that remained on ice were used as a control to which data were normalized. As expected, CCL3 was able to induce the endocytosis of CCR1 at 37°C, which was significantly different from cells incubated at 4°C. Likewise, CXCL4 was also able to induce the endocytosis of CCR1 at 37°C, which was significantly different from cells incubated at 4°C. Together, these data suggest that CXCL4 might exert its biological function on monocytes via CCR1.

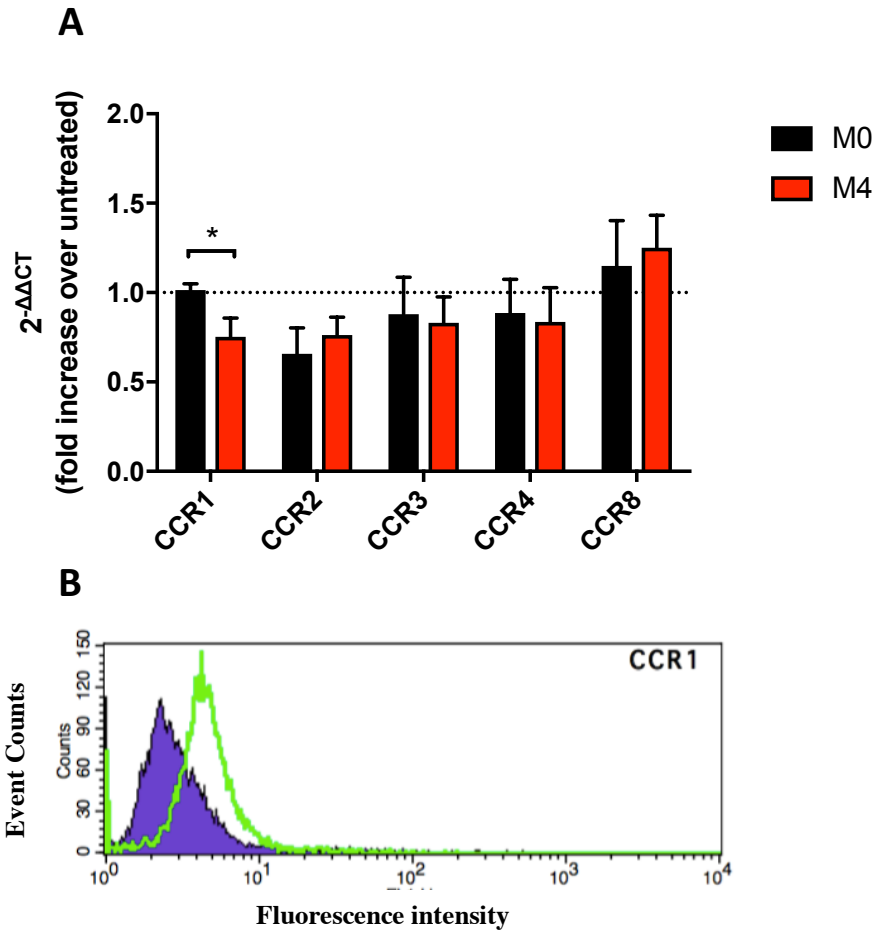


Figure 5.5. CCR1 is downregulated in M4 macrophages. Panel A shows the fold increase in gene relative expression for CCR1, CCR2, CCR3, CCR4 and CCR8 mRNA normalized to β-actin in monocytes cultured for 3 days. Data are shown are from 5 different experiments and were analysed by a paired, two-tailed t-test. Panel B shows a representative plots of CCR1 surface expression on freshly isolated human monocyte (red histogram) compared with isotype control staining (blue histogram). Data shown are the mean ± SEM of 3 experiments.

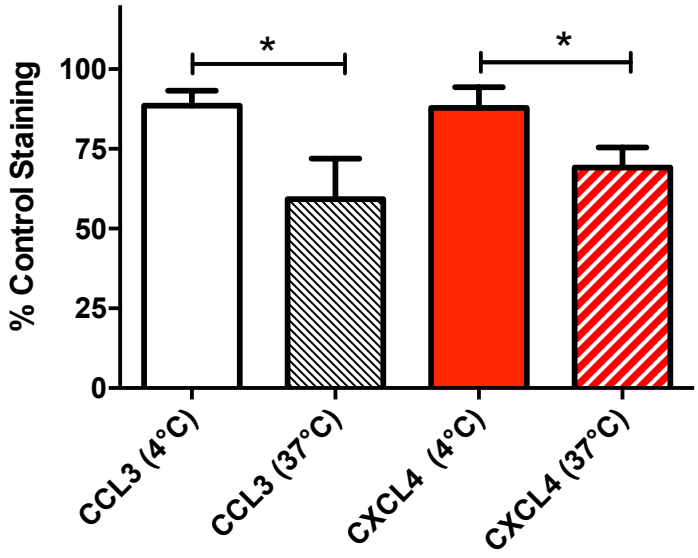


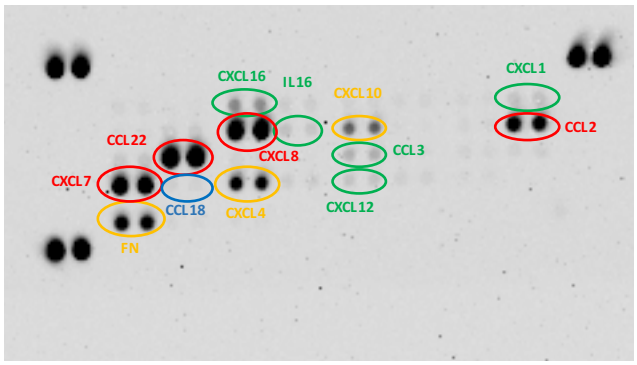
Figure 5.6 CXCL4 induces significant internalization of CCR1 on freshly isolated monocytes.

Monocytes were stimulated with 10nM CCL3 or 1μM CXCL4 for 30 minutes at 37°C or 4°C (control condition) after which staining for CCR1 was carried out. Unstimulated cells incubated at 4°C, (no endocytosis) were used as 100% to calculate the percentage of CCR1 internalisation. Data shown are the mean ± SEM from 5 different experiments and were analysed by a paired, two-tailed t-test.

5.2.3. Assessment of chemokine production by M4 macrophages

Since M4 polarization did not appear to enhance CCL18 production, we asked the question which, if any chemokines are secreted by M4 macrophages. Culture supernatants from M4 macrophages that had cultured for 6 days with CXCL4 were pooled (three different donors) and the supernatant examined for chemokine expression by a Proteosome Profiler array (R & D Systems) to detect the level of 31 chemokines in a single assay. As a comparator, an equal volume of tissue culture supernatant from a 13 day Th2 cell culture (again pooled from three donors) was also assessed. Data are shown in Figure 5.7. Unsurprisingly, CXCL4 was detected on the M4 array which had been added to the culture medium to drive M4 polairzation. An additional six chemokine spots were highlighted, namely CXCL10, CXCL16, CXCL17, CXCL8, CCL2, and CCL22. Comparing the spots intensities, CXCL17, CCL22 and CXCL8 appeared to be the most highly expressed chemokines. No staining of CCL18 was observed, in keeping with our earlier ELISA data. With the exception of CXCL8, none of these chemokines appeared to be robustly expressed by Th2 cells.

A: M4 Macrophages



B: Th2 cells

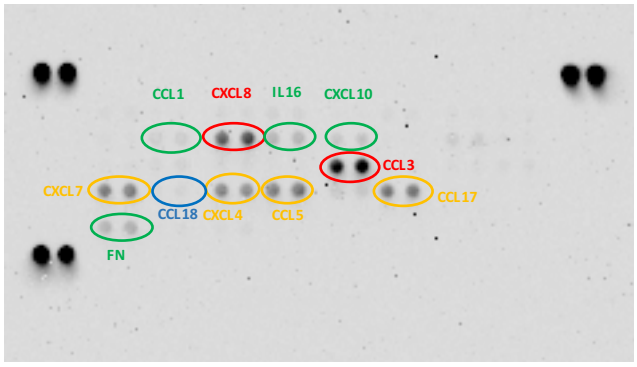


Figure 5.7. Chemokine secretion by M4 macrophages and Th2 cells

Human monocytes were differentiated in the presence of 1μM CXCL4 for 7 days, whilst T cells were polarized to the Th2 phenotype for 13 days. The cell supernatants from three different donors were pooled and applied to a chemokine protein array. Data shown are from a single experiment, with chemokines of interest highlighted.

5.2.4. CXCL4 treatment enhances CCL22 production in M4 macrophages

It has been extensively reported that cytokines and chemokines secreted by macrophages polarized to the M1 and M2 phenotype are quite distinct (Mantovani et al. 2004).

Interestingly, CCL22 a ligand for the chemokine receptor CCR4 was reported to be the most highly upregulated gene in M4 macrophages cultured for 6 days when compared to M0 macrophages, which was confirmed at the protein level (Gleissner et al. 2010). In agreement with that study, we found that culture of monocytes with CXCL4 enhanced the production of CCL22 (Figure 5.8A). Monocytes were cultured for 3 days in the presence or absence of 10 ng/ml M-CSF or 1μM CXCL4 after which they lysed, mRNA isolated, cDNA generated and the levels of CCL22 mRNA expression assessed by qPCR. As a positive control, monocytes were also cultured with 10 ng/ml IL-4 which is known to upregulated CCL22 expression (Kodelja et al. 1998). As a comparator, expression of the other known CCR4 ligand, CCL17 was also examined.

M0 macrophages produced little if any in increase in CCL17 or CCL22 mRNA expression when compared to untreated cells. In contrast, M4 macrophages trended towards producing a 4-fold increase in the mRNA levels of both cytokines. IL-4 treatment induced the expression of both CCL17 and CCL22 mRNA with 2000-fold and 20-fold increases respectively, although this was not significant due to donor variations. We next measured the protein production by ELISA in the supernatants of IL-4 treated monocytes (Figure 5.8B). In contradiction to the mRNA analysis, following 72hr of culture, CCL22 protein was found to be expressed at significantly greater levels than either CCL17 or CCL18, suggesting that the time point chosen for

mRNA analysis is likely to be critical

We therefore attempted to compare the kinetics of CCL17 and CCL22 production in M0 and M4 macrophages, looking at the day2, day 4 and day7 time points by ELISA (Fig. 5.9). Following 2 days of culture, CCL17 was expressed at low levels (50pg/ml) under all three culture conditions. By by day 7, untreated cells and M4 macrophages produced similar amounts of CCL17, with M0 macrophages trending towards a 2-fold increase in CCL17 in comparison. In stark contrast, CCL22 was found to be secreted by cells at high levels under all three culture conditions, with levels approaching 10,000 pg/ml in the supernatant. There was a trend for greater secretion of CCL22 at day 2 in M4 macrophages compared with M0 or untreated cells, but by day 7, these cell types had appeared to have caught up with the M4 cells, with little difference in CCL22 production at this time point. These findings suggest that M4 polarisation is accompanied by an increase in the kinetics of CCL22 secretion but not secretion of the related ligand CCL17.

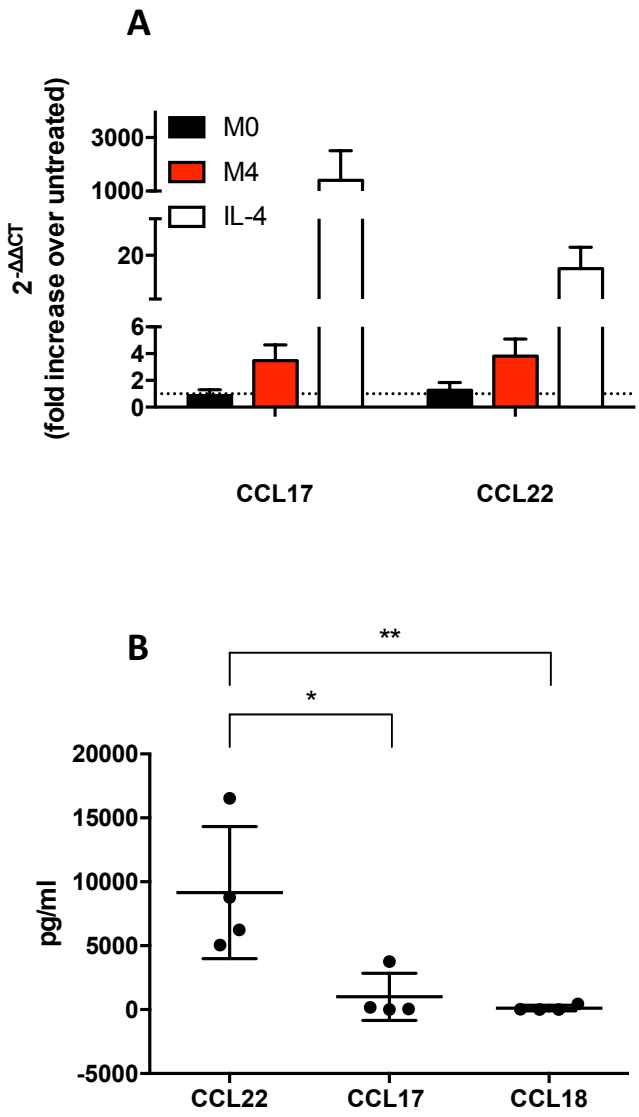


Figure 5.8. Induction of CCL17, CCL18 and CCL22 mRNA and protein in macrophages. Monocytes were differentiated in the presence of 10 ng/ml IL-4, 10 ng/ml M-CSF, or 1μM CXCL4 for 72 hours. Total RNA was isolated, reverse transcribed and the fold increase in gene for CCL22 and CCL17 mRNA normalized to β-actin. Panel A shows the relative gene expression as the fold increase of the mRNA levels observed in untreated cells. Data shown are the mean ± SEM of 4 independent

experiments with monocytes isolated from four different donors. No significant differences between data sets were observed using one-way ANOVA. Panel B shows the levels of CCL17, CCL18, or CCL22 detected in supernatants from monocytes stimulated with IL-4 for 3 days as analysed by ELISA. Data shown are the mean ± SEM using macrophages prepared from 3 different donors. Analysis was by one-way ANOVA.

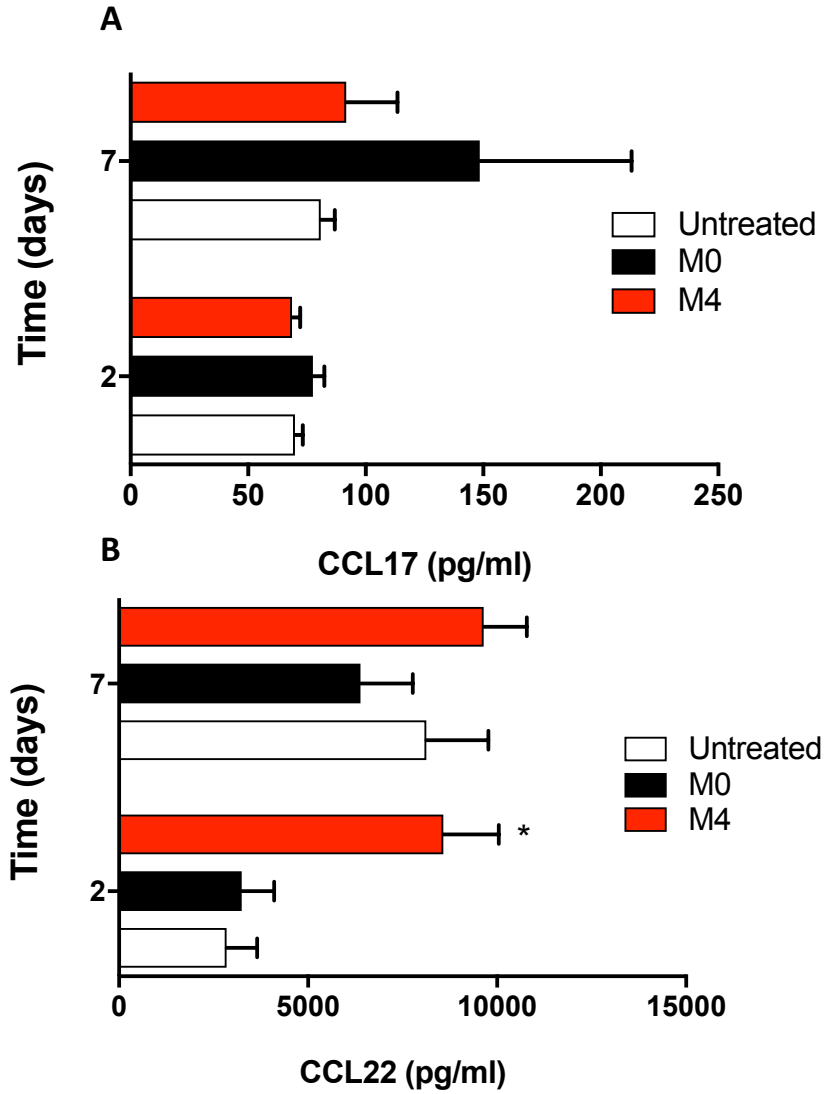


Fig 5.9. The kinetics of CCL17 and CCL22 secretion in M0 v M4 Macrophages

Monocytes were differentiated in the presence of media alone or media supplemented with either 10 ng/ml M-CSF, or 1μM CXCL4 for 7 days. Cell supernatants were taken at day 2 and day 7 of culture and were analyzed by ELISA to determine the amount of CCL22 and CCL17 secreted at each time point. Data shown are the mean ± SEM of 3 independent experiments with monocytes isolated from three different donors. No

significant differences between data sets were observed using one-way ANOVA.

5.2.5. The influence of M4 polarisation on the scavenging of oxLDL

Since we had confirmed that CXCL4 could induce a difference in the monocyte phenotype as measured by morphological changes, cell surface markers expression and cytokine secretion, we turned out attention to another key macrophages function, namely the ability to to phagocytose pathogens and foreign materials (Gordon 2007). Previously, Gleissner and colleagues had reported that following day 6 of culture, M4 macrophages were less able to scavenge modified low density lipoprotein (LDL) (Gleissner et al. 2010). This correlated with a significant reduction in the expression of CD36 at the mRNA level, a key scavenger of oxidised LDL (oxLDL) which is implicated in foam cell formation during atherogenesis (Kunjathoor et al. 2002).

We therefore investigated the differential expression of CD36 mRNA in M0 and M4 macrophages at an earlier time point of 72 hours in culture. We confirmed by qPCR analysis that CD36 mRNA was expressed at significantly lower levels in M4 macrophages compared to M0 macrophages (Fig. 5.10). As CD36 is a principal scavenger of modified LDL we hypothesized that the M4 macrophages would be less able to scavenge modified LDL than their M0 counterparts.

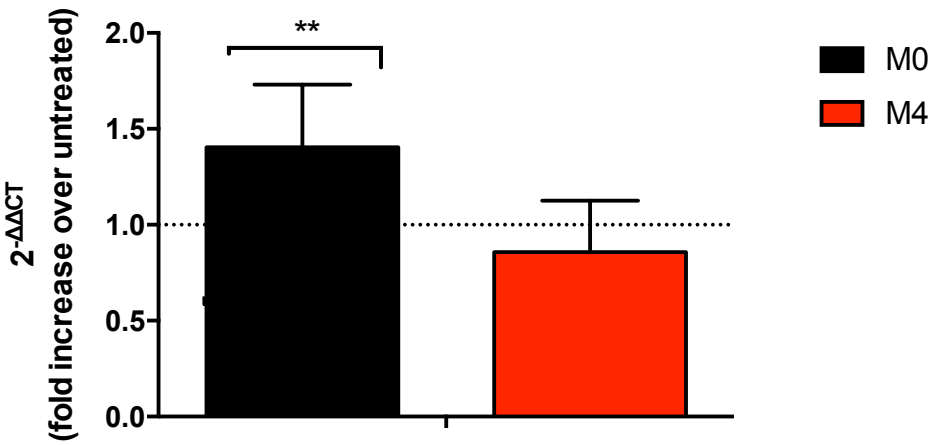


Figure 5.10. M4 macrophages express significantly less CD36 mRNA than M0 macrophages

Total RNA was isolated from monocytes differentiated in either media alone or in the presence of 10 ng/ml M-CSF or 1μM CXCL4 for 72 hours. mRNA was reverse transcribed and the fold increase in CD36 mRNA expression was normalized to β-actin then expressed as a fold induction over the expression of untreated cells. Data shown are the mean ± SEM of 6 independent experiments with monocytes isolated from six different donors. Significant differences between data sets were observed using a two-tailed t-test.

5.2.6. Phagocytotic function

To assess the functional relevance of the reduced CD36 mRNA expression in M4 macrophages, we tested the ability of both M0 and M4 macrophages to uptake oxLDL, examining macrophages that had been allowed to differentiate for 2, 4 and 7 days in culture. At each time point, cells from the same donor were incubated with 10 mg/ml Dil-labelled ox-LDL for 4 hours and the scavenging assessed by flow cytometric analysis following cell washing. At 7 days of culture, M0 macrophages exhibited a robust ability to take up oxLDL with the vast majority of cells staining positive (Fig. 5.11A). In contrast, only around 50% of the M4 macrophages appeared to have taken up detectable amounts of oxLDL. The mean fluorescence values for oxLDL scavenging were subsequently examined over the 7 day differentiation period. M4 macrophages were significantly poorer at scavenging oxLDL compared with M0 macrophages as early as 2 days in culture, which was confirmed over the entire differentiation period (Figure 5.11B). The apparent M4 gene signature thus translates into a distinct cellular function.

Looking at individual cytokines in isolation *in vitro* is obviously far removed from the situation *in vivo*, where macrophages are exposed to multiple stimuli. To assess the potential effects of CXCL4 signalling on the responses to other stimuli, we polarized cells to the M0 or M4 phenotype as before by culture in media supplemented with M-CSF or CXCL4 respectively. As a comparator, monocytes were cultured in parallel which were exposed to both stimuli. After 7 days in culture, we assessed the ability of the macrophages to scavenge oxLDL (Figure 5.12). As before, M0 macrophages robustly scavenged significantly more oxLDL than M4 macrophages. The scavenging

capacity of macrophages cultured in a combination of both CXCL4 and M-CSF was indistinguishable from that of the M4 macrophages, suggesting that the CXCL4-induced signalling events induced in macrophages are able to override those of M-CSF.

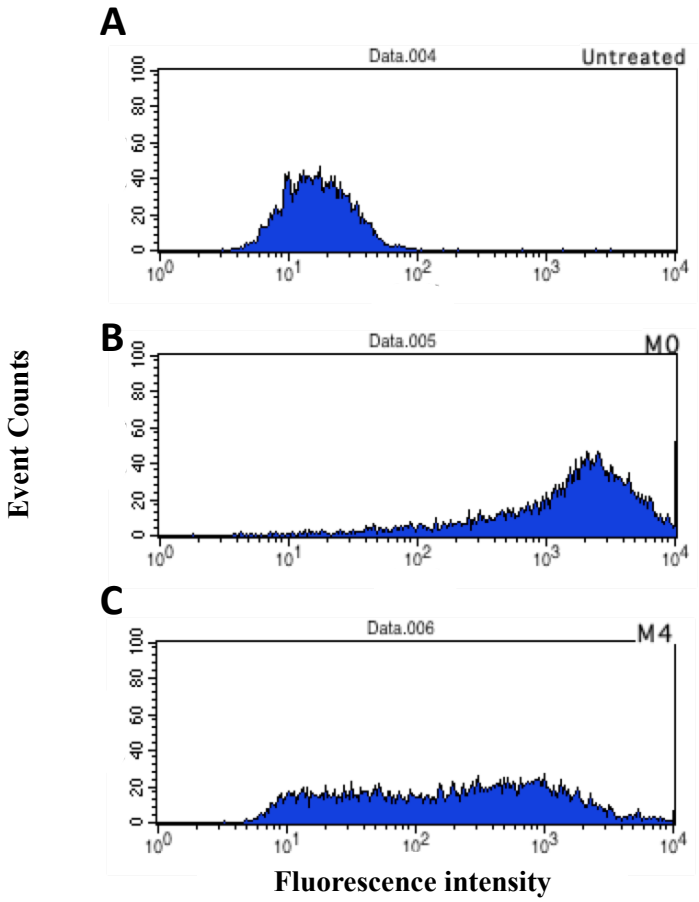


Figure 5.11 ox-LDL scavenging capacity is reduced in M4 macrophages - I. Human monocytes were differentiated in either media alone or in the presence of 10 ng/ml M-CSF or 1μM CXCL4 for up to 7 days, after which the scavenging of dil-labelled oxLDL was assessed by flow cytometry. Panels show representative FACS plots of oxLDL uptake in M0 (B) and M4 (C) macrophages cultured for 7 days, with untreated (unfed) macrophages (A) serving as a negative control. Data shown are typical of at least four independent experiments using monocytes isolated from different donors.

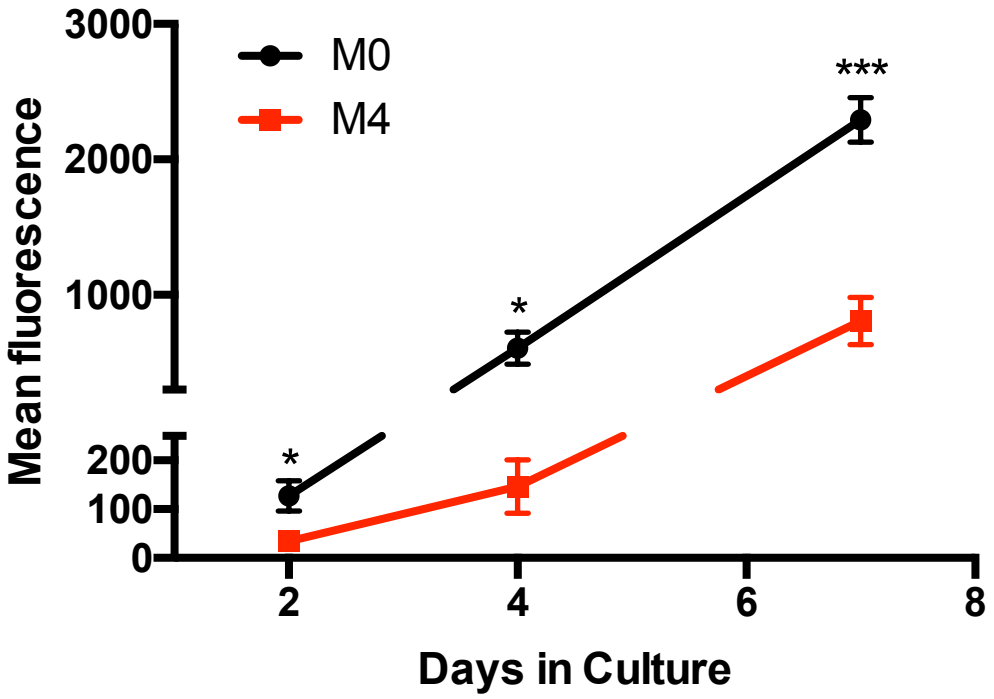


Figure 5.12 ox-LDL scavenging capacity is reduced in M4 macrophages - II.

Data shows the mean fluorescence intensity (MFI) of oxLDL uptake by M0 and M4 macrophages at day 2, day 4, and day 7 of culture. Data shown are the mean ± SEM of four independent experiments using monocytes isolated from different donors. Significant differences were assessed by a two-tailed paired t-test.

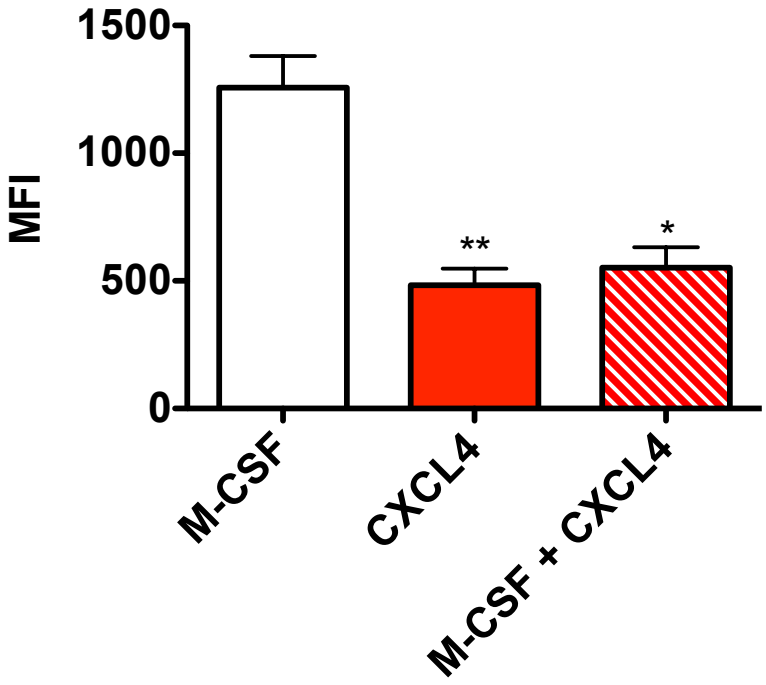


Figure 5. 13 CXCL4 signalling can override the oxLDL scavenging of M0 macrophages. Human monocytes were cultured for 7 days in the presence of 10 ng/ml M-CSF, 1μM CXCL4, or a combination of both cytokines after which the scavenging of dil-labelled oxLDL was assessed by flow cytometry. Data shown are the mean ± SEM of five independent experiments using monocytes isolated from different donors. Significant differences were assessed by one-way ANOVA and corrected for multiple comparisons by Bonferroni's multiple comparisons test.

5.3 Discussion

The simple M1 and M2 macrophage classification has been changed by the discovery of heterogeneous macrophage types that have distinct phenotypic and functional characteristics similar or distinct to both M1 & M2 types. Chemokines are named with reference to their ability to induce the directional migration of cells whereas growth factors are known for their ability to initiate monocyte differentiation and polarization into macrophages. However, CXCL4, released from activated platelets has been shown to not only prevent monocyte apoptosis but also induce monocyte differentiation into distant macrophages subtype called M4 (Scheuerer et al. 2000). The biological role for CXCL4 is not entirely understood as little is known about the receptor that CXCL4 signals through on monocytes.

In this chapter, we wanted to explore the signature of CXCL4-driven monocyte differentiation and its relation to CCL18. Freshly isolated monocytes were stimulated with 1μM CXCL4 which is a concentration that has been shown previously to induce M4 macrophages(Gleissner et al. 2010) and compared with M0 macrophages, induced by stimulattionwith macrophage colony stimulating Factor M-CSF (Metcalf 1986). The CXCL4 treated macrophages showed some significant changes in their morphology after only three days of stimulation. The M4 cells became significantly larger than the M0 cells and the expression of cell surface markers associated with differentiation was also different compared to M0 cells. For example, a costimulatory molecule required for the activation of T cells by monocytes was significantly up-regulated in the M4 cells, as was previously reported by Scheuerer et al. who found that CD86 expression was upregulated compared to untreated monocytes. The authors of

that study suggested that CXCL4 actively accelerated the differentiation process of monocytes, inducing the upregulation of differentiation markers (Scheuerer et al. 2000).

CXCL4 treatment might induce the desensitization and downregulation of its principal receptor on monocytes so that differences in the level of cell surface receptors between the M0 & M4 cells was another marker we needed to consider. To probe which chemokine receptors might be transducing the CXCL4 signal we looked at the mRNA expression levels in M0 & M4 macrophages for CCR1, CCR2, CCR3, CCR4, & CCR8 after three days of culture. Interestingly, a downregulation in CCR1 mRNA was the only significant difference between treatment groups. CCR1 is currently being proposed by Dr. James Pease as a potential receptor for the orphan chemokine CXCL4 (manuscript in submission). In assessing further the ability of CXCL4 to directly downregulate CCR1, we also found that CXCL4 was able to induce the endocytosis of CCR1 which is suggestive that CXCL4 might exert its biological function on monocytes via CCR1.

Macrophages are a known source of chemokines and cytokines as they exert their biological functions. Nevertheless, we established that the survival effect on monocyte induced by CXCL4 was not mediated by CCL18 autocrine release, since CCL18 levels at both protein and mRNA were not enhanced by CXCL4 treatment. This is in disagreement with Gleissner et al., who had previously reported that after six days of culture with CXCL4, M4 macrophages produced significantly more CCL18 than M0 macrophages. (Gleissner et al. 2010). In a study by Gouwy et al., comparing the CXCL4 survival effect on monocytes with its variant CXCL4L1, the authors also found that,

similar to our observation, CCL18 was released in the CXCL4-treated cells at levels significantly lower than cells M-CSF treated cells (Gouwy et al. 2016). In that study, although CXCL4 and CXCL4L1 differentially affected monocyte survival and dendritic cell differentiation and phagocytosis, no differences in the production of CCL18 between treatments was observed.

Then we asked the question which chemokines do M4 macrophages preferentially express? In a single proteomic screen, we scanned for 31 chemokines, and found in agreement with a previous report, that CXCL4 increased the production of CCL22 (Gleissner et al. 2010) (Gouwy et al. 2016). Subsequently, in a kinetic study, we found that CXCL4 significantly enhanced the production of CCL22 as early as 48 hours after stimulation. CCL22 and CCL17 are ligands for the chemokine receptor CCR4 and both CCL22 and CCL17 are known to preferentially attract Th2 cells and regulatory T cells via CCR4 (Imai et al. 1999). As a comparator, we therefore also examined the expression of CCL17 by M4 macrophages. At a single 72h time point, we found increased production of both CCL17 and CCL22 mRNA by M4 macrophages, compared to M0 macrophages. In contradiction to the mRNA analysis, following 48 h of culture, CCL22 protein was produced by M4 macrophages at significantly greater levels than M0 macrophages, a difference which was lost by day 7 of culture. In contrast, CCL17 production was barely above the level of detection by the ELISA, in contrast to the ng/ml concentrations of CCL22 produced by M4 cells. It is likely, therefore that the time point chosen for mRNA analysis is of critical importance. Collectively, the findings indicate that M4 polarization of macrophages is accompanied by an increase in CCL22 secretion but not secretion of the related ligand CCL17.

Phagocytosis of oxLDL was used to assess the M4 atherosclerosis related function, since it is a crucial step in foam cell formation. It was previously reported by Gleissner and colleagues (Gleissner et al. 2010) that M4 macrophages are likely to be more prone to foam cell formation induced by modified (acetylated or oxidized) low-density lipoprotein (LDL) as this could represent one mechanism by which CXCL4 may moderate atherosclerosis. Notably, CXCL4 has been shown to inhibit the binding and uptake of LDL which might enhance oxidation of LDL to about 10-fold increase compared to the amount of esterified ox-LDL in macrophages (Nassar et al. 2003, Sachais et al. 2007).

We tested the kinetics of oxLDL uptake and established that the M4 macrophages take up significantly less ox-LDL (~50% less) compared to M0 macrophages at all stages of culture examined. Comparing mRNA expression of CD36, a principle scavenging receptor for modified LDL we found that M4 macrophages expressed significantly lower levels of CD36 mRNA than M0 cells, which may account for this modified LDL uptake (Kunjathoor et al. 2002, Gleissner et al. 2010). When the cells were stimulated together with CXCL4 and M-CSF, it was observed that the dual treatment resulted in cells with an oxLDL uptake identical to that of the M4 macrophage, leading us to postulate that M4 polarization induces a sufficiently strong signal to override the M0 polarization signal. We can speculate that CXCL4 most likely does not promote foam cell formation in atherosclerotic lesions, and it may indeed have an atheroprotective effect in.

Having that in mind, in the final chapter of this thesis we asked the question which transcription factors (TFs) control M4 polarization?

6 Analysis of Transcription factors involved in M4 differentiation

6.1 Introduction

As previously stated, atherosclerosis is an inflammatory disease of the coronary arterial walls, with a major causes driving the development of atherosclerotic plaques being the lipid peroxidant stress from oxidized low-density lipoprotein (OxLDL) (Parthasarathy et al. 2010). If unchecked, the ensuing inflammation can drive the progression of plaques, resulting in unstable plaques prone to rupture or intraplaque haemorrhage (IPH). In response to the stress, the arterial wall enhances the recruitment of monocytes in an attempt to resolve the inflammatory process (Glass and Witztum 2001). Monocytes initially have to firmly adhere to the endothelial cells lining the vessel lumen which is achieved by the expression of cell adhesion molecules under the control of inflammatory cytokines. Adherent monocytes subsequently emigrate into the subendothelial space where they differentiate into macrophages, again under the control of multiple cytokines (Glass and Witztum 2001). Initially, the macrophages play a protective function, by removing cytotoxic and pro-inflammatory ox-LDL particles and apoptotic cells which is facilitated by scavenger receptors on the macrophage surface. Progressive accumulation of ox-LDL via the continued scavenging leads to the macrophage taking on a “foam cell” appearance, which are a hallmark of atherosclerotic lesions (Glass and Witztum 2001).

Many studies have considered the mechanisms by which monocytes enter plaques and the stimuli that drive them to differentiate to macrophages in an attempt to clear lipids. A previous report by Boyle et al. focused on macrophages that clear haemorrhage-related iron (Mhem macrophages) in IPH (Boyle et al. 2010). The authors were particularly interested in what transcription factors drove the differentiation of recruited macrophages to the Mhem phenotype. To study this, they examined the the kinetics of Mhem macrophage differentiation by microarray analysis and found that at an early time point (1h) the primary response gene set was enriched for genes encoding transcription factors, and proteins involved in signaling, and nucleotide metabolism. Notable amongst these this gene set was the transcription factor activating transcription factor 1 (ATF-1). Four hours after stimulation with haemoglobin, the response gene set was enriched in genes coding for lipid handling, including lipid export and -oxidation pathways, although the largest single category was genes that are still not annotated (Boyle et al. 2012). Notably, the gene encoding haem oxygenase-1 (HO-1), which can break down the reactive haem, was significantly upregulated which was shown by knockdown studies to be directly under the control of ATF-1.

Interestingly, they found that the Mhem macrophages do not exhibit the lipid accumulation of the type that defines foam cells. Mhem macrophages are therefore likely to be atheroprotective, as they increase the uptake of intracellular iron (reducing the potential for the generation of hydroxyl radicals by so-called “Fenton chemistry”) (Sadrzadeh et al. 1984) and also inhibit inflammation via the release of the anti-inflammatory cytokine IL-10 (Boyle et al. 2010). Consequently, an understanding of

the molecular mechanisms underpinning macrophage differentiation in atherogenesis may point to novel therapeutic approaches in the treatment of the disorder.

Here, we applied a similar approach to Boyle et al. using a focussed PCR array to examine which transcription factors might be driving the differentiation of monocytes into M4 macrophages.

6.2 Results

A volume of 30ml of peripheral blood was taken from healthy volunteers with informed consent. Monocytes were freshly isolated from PBMCs by EasySep™ Human monocyte isolation kit and then cells were left to rest in serum free media for 2 hours at 37ºC with 5% CO_2. Then cells were either stimulated with 1µM CXCL4 or left unstimulated under the same culture conditions. At 1hr and 4hr time points, media was removed from the wells, and the cells were lysed for RNA extraction. A schematic of the approach is shown in Figure 6.1.

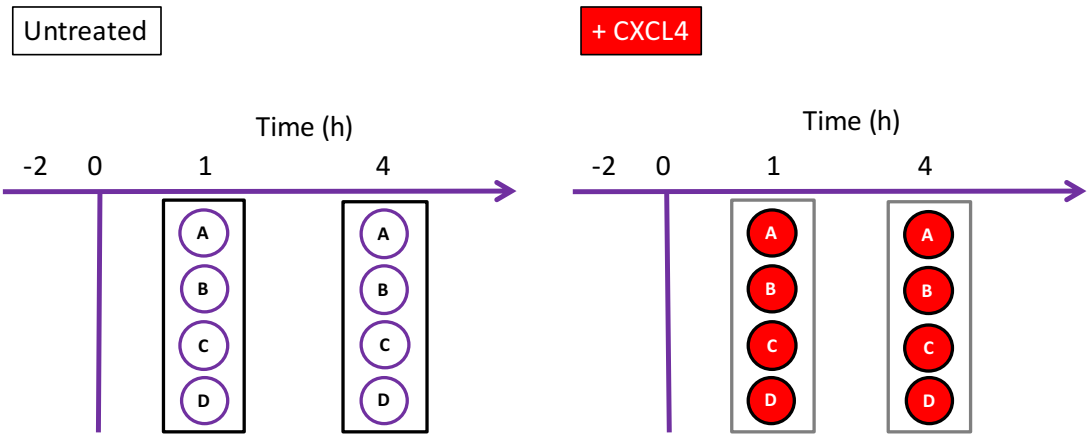


Figure 6.1 Schematic showing the treatment of cells prior to their array by RT² PCR Array. Monocytes were isolated from four different donors (A-D) and plated on plastic in serum-free medium (-2 timepoint). Cells were cultured at 37C with 5% CO_2. After 2 hours of culture, media was replaced with the presence or absence of 1µM CXCL4 and the plates returned to the incubator. At 1hr and 4hr time points, media was removed from the wells and the cells lysed prior to RNA extraction.

The integrity of the RNA obtained was analysed using an Agilent 2200 TapeStation system prior to further usage. The standard for the assessment of RNA quality is the RNA Integrity Number (RIN), which is a reliable and robust method to analyse RNA integrity assuring the quality and the quantity of the RNA that would subsequently be used to generate a cDNA probe for use in the RT² PCR Array.

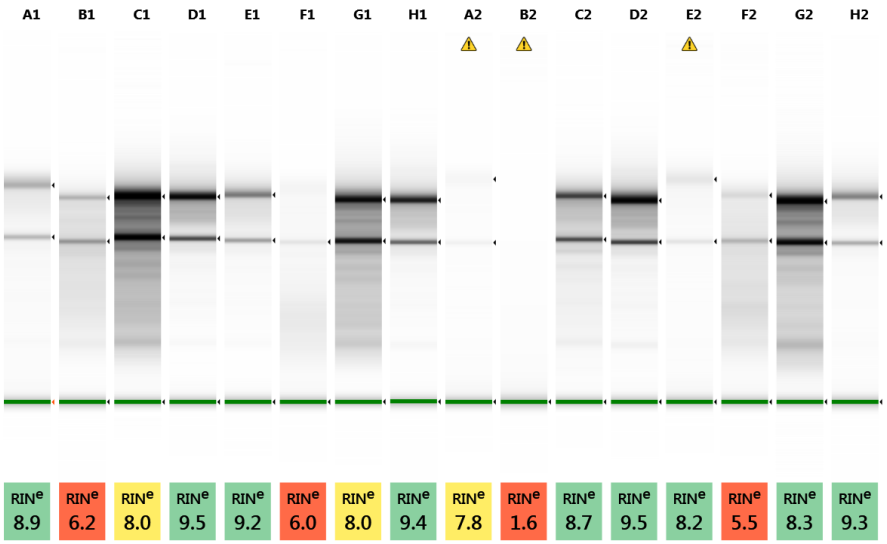
Results are presented as a gel image showing the separation profile for each sample using the 2200 TapeStation software (Figure 6.2). RIN values are cited as between 1 and 10, where 10 represents the highest quality RNA sample. In this study, we compared the RIN quality score obtained from all 16 samples and eliminated donor B from further analysis, since the RNA isolated at the 1hr time point from untreated cells was of poor quality, with a low RIN score of 1.6.

To examine potential transcriptional factors involved in M4 macrophage differentiation we used a RT2 Profiler PCR Array (Qiagen, Crawley, UK) to examine the RNAs isolated from the macrophages at the 1htr time point. The RT2 Profiler PCR Array protocol is an easy-to-use profiling technology allowing quick sample loading and data analysis. A schematic of the work flow is shown in Figure 6.3. In brief, RNA was reverse transcribed using a cDNA conversion kit and applied to each well of the real-time RT^2 Profiler PCR Array in combination with RT^2 SYBR® Green qPCR mastermix. The plate was loaded onto an ABI Viia7 cycler with a programme of 1 cycle of 10 minutes at 95ºC followed by 40 cycles of 15s at 95ºC followed by 60s at 60ºC.

Baselines and thresholds were set according to the manufacturer's instructions to give a threshold value above the background signal but within the lower third to one-half of the linear phase of the amplification plot. These settings were applied to all 6 arrays

used in the analysis. Samples were assigned to controls (untreated cells) and test groups (CXCL4 treatment). Cycle threshold (C_T) values for each array were exported to an Excel file to create a table of C_T values. This table was then uploaded on to the data analysis web portal at http://www.qiagen.com/geneglobe.

A



B

Lane	RIN	28S/18S (Area)	RNA [pg/µl]
A1 Untreated 1hr Donor A	8.9	2.2	1470
B1 Untreated 1hr Donor B	6.2	0.7	2770
C1 Untreated 1hr Donor C	8.0	1.6	5600
D1 Untreated 1hr Donor D	9.5	3.0	4960
E1 PF4 1h Donor A	9.2	2.6	1930
F1 PF4 4h Donor B	6.0	-	1200
G1 PF4 1h Donor C	8.0	1.4	10500
H1 PF4 1h Donor D	9.4	3.1	3850
A2 Untreated 4hr Donor A	7.8	1.7	355
B2 Untreated 4hr Donor B	1.6	-	183
C2 Untreated 4hr Donor C	8.7	1.8	4550
D2 Untreated 4hr Donor D	9.5	3.6	5980
E2 PF4 4h Donor A	8.2	2.2	665
F2 PF4 1h Donor B	5.5	0.7	2670
G2 PF4 4h Donor C	8.3	1.7	11200
H2 PF4 4h Donor D	9.3	2.9	1650

Figure 6.2 Quality Control of total RNA isolated from monocytes. RNA analysis carried out using the Agilent 2200 TapeStation system. Panel A shows a gel image showing different RNA samples. Panel B shows table for all samples conditions and concentration in pg/um and the RIN for RNA quality analysis (RINe is presented as a value between 1 and 10, where 10 represent the highest quality RNA sample).

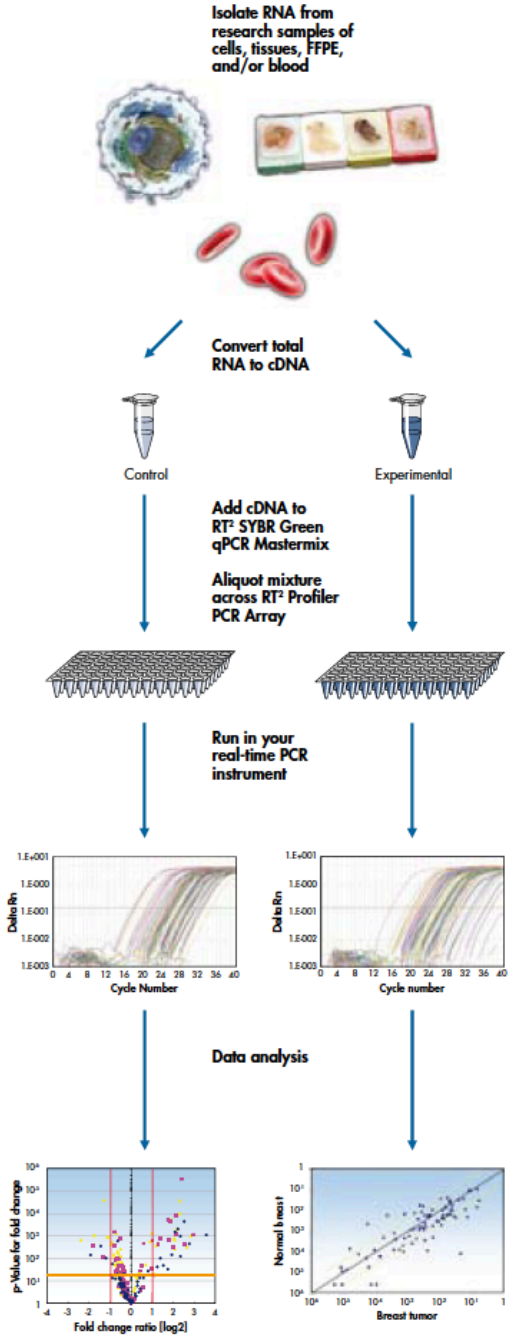


Figure 6.3 Schematic of the workflow with the RT² PCR Array. RNA is isolated from cells of interest, cDNA generated and probed for relative expression of genes of interest (GOI) in a convenient plate-format using a series of 84 gene-specific PCR primers.

Each array contains a set of primers to measure for PCR array reproducibility, RT efficiency and genomic DNA contamination. Also included on each array is an internal set of primers specific for five housekeeping genes, namely beta-actin, beta-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase-1 (HPRT1) and the large Ribosomal protein, P0. Following amplification of the arrays on a ABI, the data for all six samples was uploaded to the server. All samples were deemed by the software to have passed the internal tests PCR array reproducibility, RT efficiency and genomic DNA contamination.

The dedicated software measured and identified HRPT1 as the housekeeping gene with the smallest change in expression across all six of the different samples via a non-normalized calculation. These values were then used to normalize the C_T values for the transcription factor genes which were geometrically averaged and used for the $\Delta\Delta C_T$ calculations (ΔC_T (Test Group)- Δ C_T (Control Group)). Fold Change was then calculated using the 2^ ($-\Delta\Delta C_T$) formula. The data analysis web portal also plotted the data in the form of scatter plots, volcano plots, and heat maps for ease of analysis.

Figure 6.4 shows the relative numbers of genes from each of the six arrays with their range of C_T values. Of the84 genes that were arrayed in this study, the majority had a C_T value of less than 25 when either untreated monocytes or CXCL4-treated monocytes were studied, suggesting that most of the genes detected by the array were well expressed by both untreated and CXCL4 treated cells.

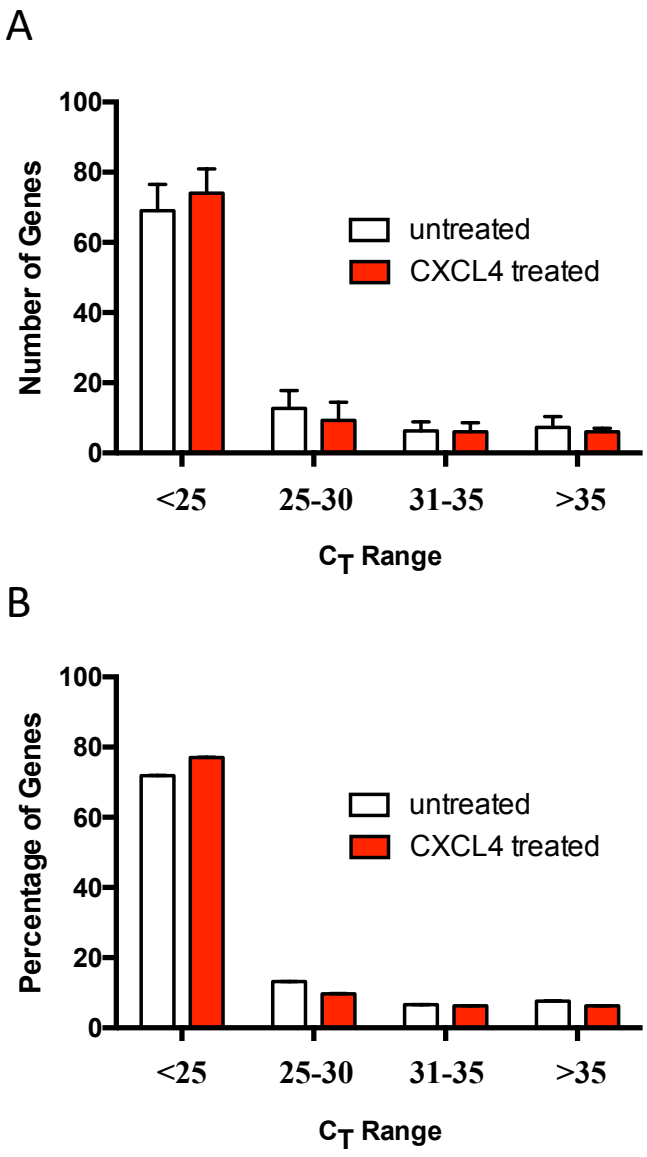
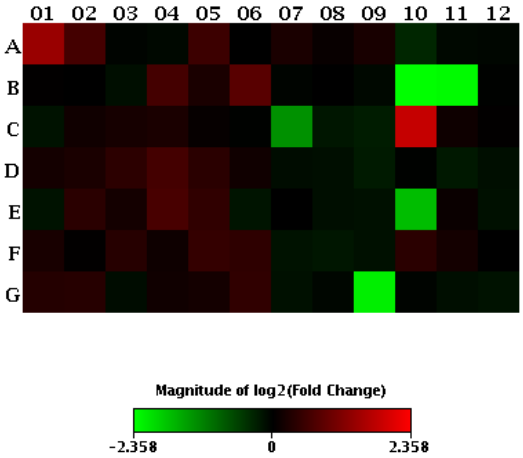


Figure 6.4. Primary analysis of Gene expression by reference to C_T range. Six arrays (3x untreated cells and 3 x CXCL4 treated cells) were initially ranked by reference to C_T range. The number (A) or percentage (B) of the genes within each C_T range are shown.

Preliminary analysis of gene expression was undertaken to highlight the fold changes in expression between the selected groups for each of the 84 genes in the array (Figure 6.5). A 1.5-fold change was used initially as a cut off to identify genes that were potentially upregulated or downregulated after one-hour of stimulation with 1 uM CXCL4.

A



B

LAYOUT	1	2	3	4	5	6	7	8	9	10	11	12
A	AR 2.7	ARNT 1.54	ATF1 -1.03	ATF2 -1.06	ATF3 1.46	ATF4 1	CEBPA 1.19	CEBPB 1.05	CEBPG 1.16	CREB1 -1.28	CREBBP -1.05	CTNNB1 -1.04
B	DR1 1.01	E2F1 -1	E2F6 -1.09	EGR1 1.55	ELK1 1.19	ESR1 1.75	ETS1 -1.03	ETS2 1	FOS -1.05	FOXA2 -5.13	FOXG1 -5.02	FOXO1 -1.01
C	GATA1 -1.12	GATA2 1.1	GATA3 1.15	GTF2B 1.18	GTF2F1 1.04	HAND1 -1.01	HAND2 -2.56	HDAC1 -1.16	HIF1A -1.19	HNF1A 3.55	HNF4A 1.09	HOXA5 1.01
D	HSF1 1.13	ID1 1.18	IRF1 1.32	JUN 1.55	JUNB 1.3	JUND 1.1	MAX -1.08	MEF2A -1.09	MEF2C -1.19	MYB -1.01	MYC -1.17	MYF5 -1.09
E	MYOD1 -1.13	NFAT5 1.3	NFATC1 1.13	NFATC2 1.59	NFATC3 1.36	NFATC4 -1.14	NFKB1 -1.01	NFYB -1.09	NR3C1 -1.1	PAX6 -3.38	POU2AF1 1.06	PPARA -1.11
F	PPARG 1.17	RB1 1.01	REL 1.27	RELA 1.1	RELB 1.4	SMAD1 1.35	SMAD4 -1.12	SMAD5 -1.15	SMAD9 -1.1	SP1 1.31	SP3 1.14	STAT1 -1
G	STAT2 1.26	STAT3 1.28	STAT4 -1.08	STAT5A 1.11	STA5B 1.13	STAT6 1.37	TBP -1.11	TCF7L2 -1.05	TFAP2A -4.69	TGIF1 -1.03	TP53 -1.09	YY1 -1.12

C

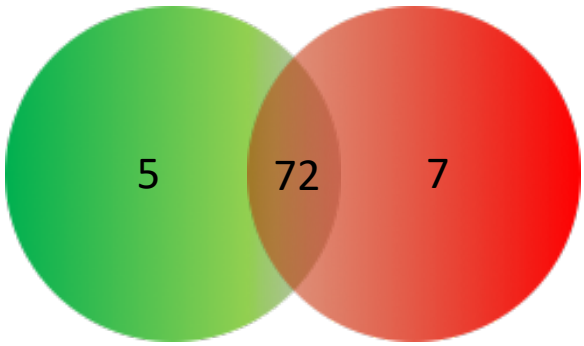


Figure 6.5 Preliminary analysis of gene expression. Panel A shows a Heat Map which highlights the fold changes in expression between the selected groups for every gene in the array. Panel B is a table showing the fold regulation data used to construct

the heat map. Genes upregulated 1.5-fold or more are in red whilst those downregulated 1.5 fold or more are in green. Panel C shows the data as a Venn diagram, with the number of up regulated, down regulated or unchanged genes shown.

Of the genes that were screened in this assay, we found that four genes were downregulated and seven genes were upregulated. The four down-regulated genes in order of decreasing down-regulation were Forkhead box A2 (FOXA2), Forkhead box G1 (FOXG1), Transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha (TFAP2A), Paired box 6 (PAX6) and Heart and neural crest derivatives expressed 2 (HAND2). The seven upregulated genes in order of decreasing upregulation were HNF1 homeobox A (HNF1A), Androgen receptor (AR), Estrogen receptor 1 (ESR1), Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATC2), Early growth response 1 (EGR1), Jun proto-oncogene (JUN) and Aryl hydrocarbon receptor nuclear translocator (ARNT).

The entire gene set was subsequently reanalysed using p values calculated using a Student's t-test of the replicate 2^ ($-\Delta\Delta C_T$) values for each gene in the control and treatment groups. Following analysis, it was found that only the genes encoding ARNT and V-rel reticuloendotheliosis viral oncogene homolog B (RELB) were significantly upregulated in M4 macrophages (Figure 6.6A). Of the genes downregulated in M4 macrophages, none were downregulated at statistically significant levels (Figure 6.6B). Scatter plot analysis was undertaken to indicate how far the relative gene expression, was downregulated or upregulated, from the unchanged genes relevant to the control group data (untreated cells). The highlighted genes in this analysis (Figure 6.7) were FOXA2, FOXG1, TFAP2A, PAX6, HAND2, cAMP responsive element binding

protein 1(CREB1), (all downregulated) and RELB, ARNT, JUN, ESR1, NATC2, Signal transducer and activator of transcription 6, interleukin-4 induced (STAT6), AR, HNF1A, and Activating transcription factor 3 (ATF3), (all upregulated).

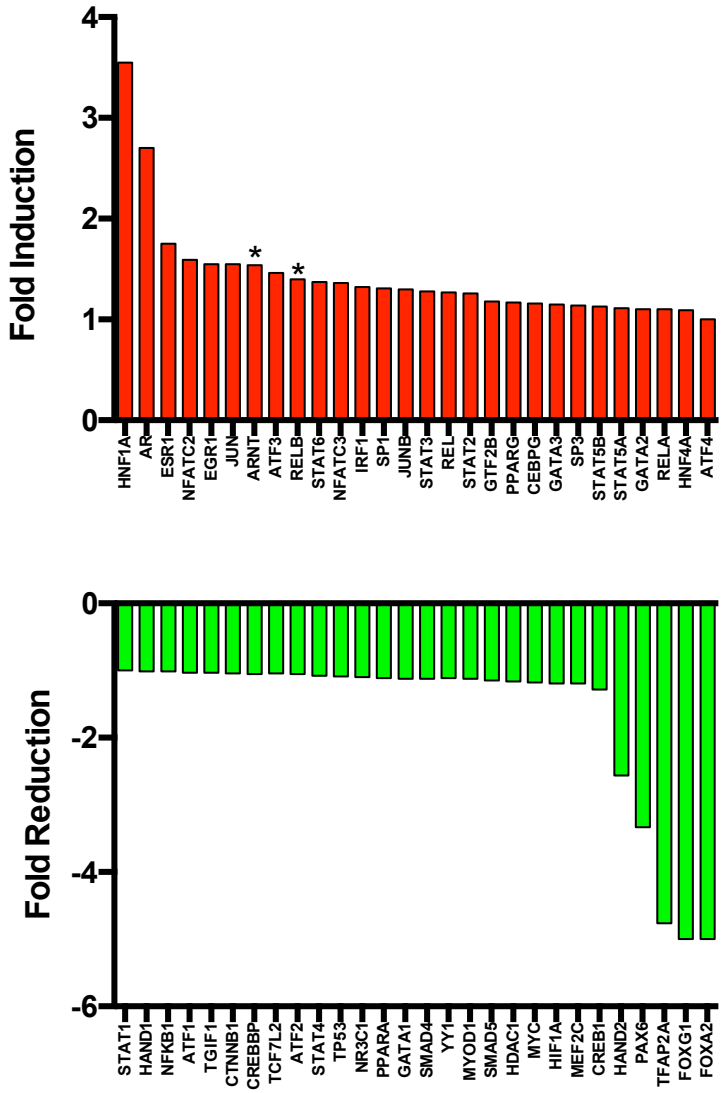


Figure 6.6 Fold Induction and Fold Reduction of GOI. Panel A and B show a selected group of transcription factors whose expression levels in monocytes were increased (A) or decreased (B) following treatment with CXCL4 for 1 hr. A level of 1 refers to identical expression of the GOI in untreated monocytes following normalization to the housekeeping gene HPRT1. * The significance of the change in gene expression between the treated and untreated cells was evaluated by unpaired Student's t-test for each gene. The level of statistical significance refer to a p value of <0.05.

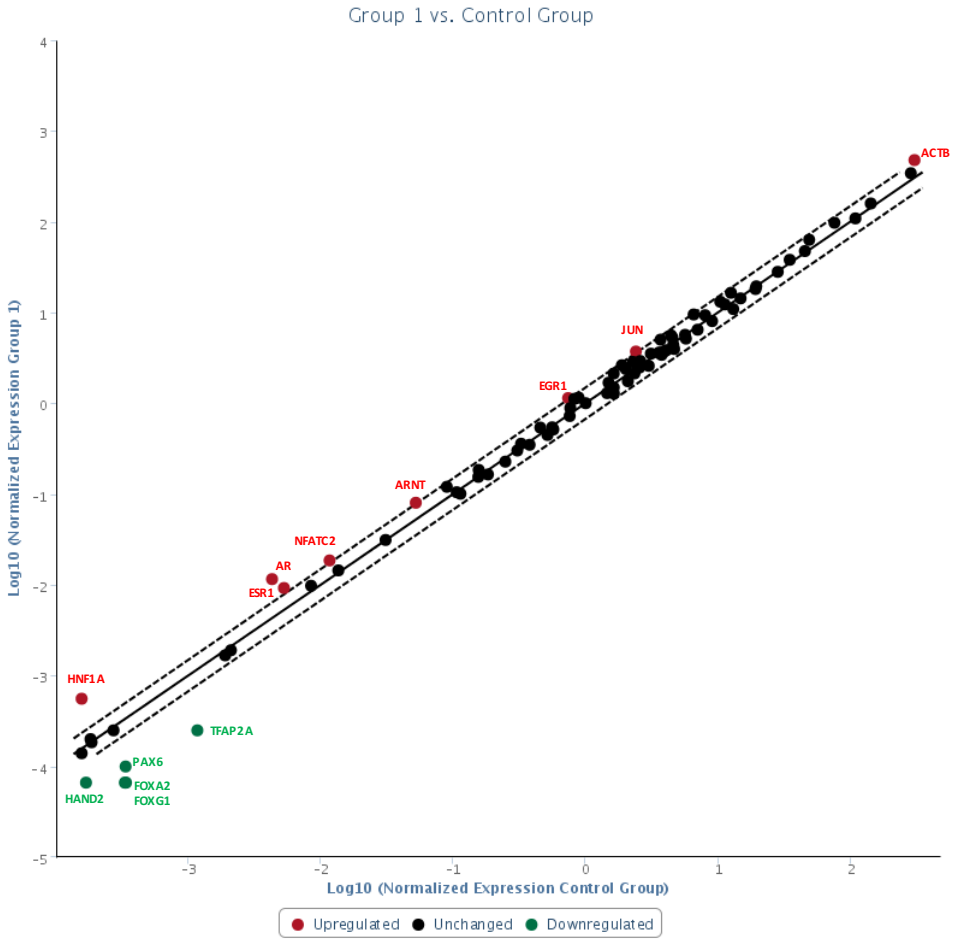


Figure 6.7 Scatter plot of Gene Induction. Plot shows the normalized expression of every gene on the array with with the control group data (untreated cells) plotted against the treatment Group 1 (CXCL4 treatment). The central line indicates unchanged gene expression and the dotted lines a fold regulation threshold of 1.5. GOI identified in the heat map as upregulated by CXCL4 treatment are depicted in red whilst those downregulated following treatment with CXCL4 are depicted in green.

The Volcano plot of Gene Induction is another way of showing the statistical significance of gene induction versus the fold-change. using such analysis, the induction of RelB and ARNT were the only genes with statistically significant difference seen in this study (Figure 6.8)

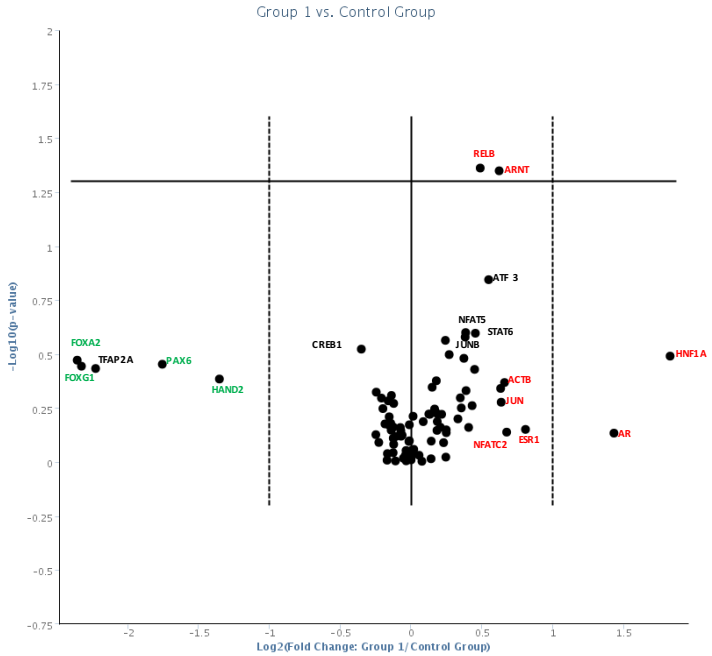


Figure 6.8 Volcano plot of Gene Induction. Plot shows p-values versus fold-change on the y and x axes, respectively. GOI identified in the heat map as upregulated by CXCL4 treatment are depicted in red whilst those downregulated following treatment with CXCL4 are depicted in green. Induction of RelB and ARNT can be seen to be statistically significant. * The significance of the change in gene expression between the treated and untreated cells was evaluated by unpaired Student's t-test for each gene. The level of statistical significance (horizontal line) refer to a p value of <0.05.

6.3 Discussion

In this chapter, a PCR array was used to screen a total of 84 genes simultaneously for their expression levels in M4 macrophages. Although two other studies have carried out analysis of gene expression in monocytes stimulated with CXCL4 or its variant CXCL4, both studies examined gene expression levels at day 6 of culture (Gleissner et al. 2010, Gouwy et al. 2016). Since in the previous chapter, we showed that an M4 phenotype is clearly distinct after only 2 days of culture, we designed a small study to examine what transcription factors might be involved in M4 polarisation. Basing our efforts on a previous study, we chose a time point of 1hr following CXCL4 stimulation as this had previously been used to identify the activating transcription factor 1 (ATF-1) as being key for the generation of Mhem macrophages (Boyle et al. 2012).

Interestingly, we were able to highlight a handful of genes which appeared to be down regulated, namely FOXA2, FOXG1, TFAP2A, PAX6, HAND2, CREB1 and Ra slightly larger group of genes which appeared to be upregulated: ELB, ARNT, JUN, ESR1, NATC2, STAT6, AR, HNF1A, and ATF3. A major limitations of the study is that it was performed using RNA from only 3 different donors and may be underpowered to see quite small differences in gene expression. For this small number of replicates, we observed statistically significant differences in the upregulation of only two genes namely RELB and ARNT.

The human ARNT (aryl hydrocarbon receptor nuclear translocator, also known as Hypoxia-inducible factor (HIF)-1β) protein forms a complex with ligand bound AHR (aryl hydrocarbon receptor) and facilitates the movement of this complex to the

nucleus, where it promotes the expression of genes involved in xenobiotic metabolism such as cytochrome P450 (Denison and Nagy 2003) The AHR is a member of the basic-helix-loop-basic family of transcription factors and binds both endogenous ligands such as flavonoids and also synthetic poly cyclic hydrocarbons and dioxins. At first glance it is unclear what relevance this gene has in M4 polarization, although a recent study observed that the activation of AHR by endogenous ligands inhibited the maturation of monocyte-derived DCs and induced IL-10 production, supportive of an atheroprotective role for this receptor(Wang et al. 2014). A recent study performed by Kim and colleagues, found that Transcription factor 21 (TCF21) and the AHR cooperate to activate a pro-inflammatory gene expression program in coronary artery smooth muscle cells (CASMC), notably the upregulation of IL1A, MMP1, and CYP1A1 (Kim et al. 2017). They also showed that oxLDL could activate the AHR to induce IL1A, supportive of a role for this receptor in atherogenesis.

The second significantly upregulated gene we observed in this PCR screen was RelB, which is known to interact with the p100 and p52 subunits of the NFκB signaling pathway. The NF-κB family consists of 5 members: NF-κB1 (p105/p50), NF-κB2 (p100/ p52), RelA (p65), RelB, and c-Rel which may form different homo- and heterodimers associated with the differential regulation of target genes including many of the genes involved in immunity and inflammation (Bonizzi G et al..2004). Studies of RelB and p52-deficient mice have established a major role for these proteins in dendritic cell (DC) function and the generation of cell-mediated immunity (Caamaño et al. 1998, Franzoso et al. 1998, Weih et al. 2001, Speirs et al. 2004).

The activation of RelB/p52 complexes is observed in the alternative NF-κB pathway

which is activated by LT, CD40L, BAFF, and RANKL, but not TNF-α.(Lawrence 2009). Of potential interest is a previous report from Nakayama and co-workers detailing the selective secretion of ng/ml concentrations of CCL22 in human B cells immortalized with Epstein Barr virus (EBV) with little CCL17 production (Nakayama et al. 2004). Induction of CCL22 mRNA was shown to be sensitive to an inhibitor of the NF-κB pathway suggestive of a role for NF-κB in CCL22 induction. Analysis of minimal promoters for CCL22 and CCL17 shows both to contain NF-κB binding sites (Figure 6.9) and deletion of these sites was shown to significantly reduce the activity of the CCL22 promoter in response to the EBV membrane protein LMP1(Nakayama et al. 2004). It is tempting to speculate that the induction of RelB in monocytes soon after exposure to CXCL4 is responsible for the selective induction of CCL22 in M4 macrophages observed in the previous chapter.

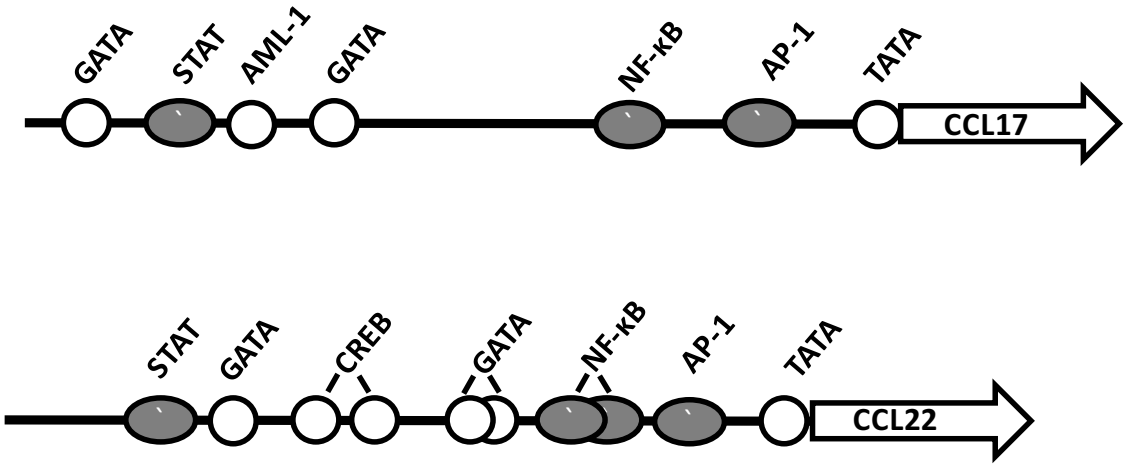


Figure 6.9 Schematic showing potential transcriptional elements in the minimal promoter regions of CCL17 and CCL22, adapted from Nakayama, et al, 2004

Although the upregulation of STAT6 in M4 macrophages fell short of statistical significance, Shen and co-workers have previously shown that phosphorylated NFκB can directly bind to STAT6 and drive an IL-4 reporter construct *in vitro*, suggesting that the two transcription factors may co-operate to induce the transcription of IL-4 induced genes such as CCL22 (Shen and Stavnezer 1998).

Alternatively, RelB may interfere with NF-kB activity in the nucleus through protein – protein interactions with RelA (Jacque et al. 2005). Other work has described the reciprocal recruitment of RelA and RelB to NF-kB target gene promoters and showed that the replacement of RelA-containing dimers with RelB complexes results in the down-regulation of individual NF-kB target genes (Saccani et al. 2003). The physiological significance of these putative mechanisms has not yet been established *in vivo*.

In summary, we have shown that two transcription factors, RelB and ARNT are significantly upregulated in human monocytes following 1hr of stimulation with CXCL4 when compared to untreated monocytes . We speculate that both these transcription factors play roles in M4 polarization although further experimental work is needed to confirm such speculation.

7 General Discussion

Atherosclerosis is a complex inflammatory reaction involving multiple cell types. The accumulation and activation of monocytes, macrophages and T lymphocytes in the inflamed arterial wall is known to play a significant role in the development of atherosclerosis. In this work, we attempted to validate the potential role, if any, of two orphan chemokines, CCL18 and CXCL4, in the process of atherogenesis. Both chemokines are highly upregulated in atherosclerotic plaques and substantial evidence has been generated by others, suggesting that they may be involved as either atherogenic or atheroprotective factors. Initially, we formulated the hypothesis that CXCL4 and CCL18 are likely to have atheroprotective qualities. Following culture with CXCL4, monocytes become polarized to the M4 macrophage phenotype which has been previously been shown to result in CCL18 secretion (Gleissner et al. 2010). Given that another study has reported that CCL18 can protect monocytes from spontaneous apoptosis, (Wimmer et al. 2006) we hypothesized that CXCL4 induction of CCL18 might therefore enhance the stability of atherosclerotic plaques. In addition, CCL18 is known to drive fibrosis in the lung (Atamas et al. 2003) so it was also plausible that that CCL18 could be one of the factors that driving collagen production within atherosclerotic lesions, helping to stabilize the fibrous cap surrounding the plaques.

The identification of a receptor for CCL18 has been suggested several times in the literature with CCR6 (Zissel et al. 2011)and PITPNM3 previously described as CCL18 receptors (Chen et al. 2011), which was subsequently disputed by others (Hussain, 2012;(Krohn, Bonvin, et al. 2013, Krohn, Garin, et al. 2013)). Not knowing the functional receptor for CCL18 makes it difficult to dissect the downstream signalling

pathways underlying the cellular responses. For this reason, one of our original aims was to identify the functional receptor for CCL18 on monocytes by probing a gene expression library generated from responding cells. Just before we commenced this project, the group of Andrew Luster published a brief report identifying the chemokine receptor CCR8 as a functional CCL18 receptor (Islam et al. 2013). In response, we modified our hypothesis to specifically investigate the potential for the effects of CCL18 in promoting monocyte survival thought to be mediated through CCR8.

Initial studies were aimed at reproducing some of the CCR8 data produced by the Luster group, since we had supplied the same exact cell line used in their publication, namely the mouse pre-B cell line 4DE4 stably transfected with human CCR8 cDNA (4DE4-CCR8). Comparing the 4DE4-CCR8 migration to CCL1 the known ligand for CCR8 (Roos et al. 1997, Tiffany et al. 1997) we were surprised to find that CCL18 did not drive the migration of the CCR8 transfectants but produced a small response from the naïve 4DE4 cell line. We confirmed the lack of CCR8-mediated migration using another pre-B cell line, L1.2, in which CCR8 was expressed both stably and transiently. The lack of chemotactic activity prompted us to wonder if the CCL18 we had obtained was functional and specific. To examine this, we used the only chemokine receptor that CCL18 is undisputedly known to bind to, namely CCR3, where it antagonises CCR3 function (Nibbs et al. 2000).

CCL18 binding to CCR3 and antagonism of CCR3-mediated chemotaxis was readily detectable although once again, we observed some binding of CCL18 to the naïve 4DE4 cell line. CCL18 was completely ineffective in inhibiting CCR8 transfectant responses to CCL1, suggesting that CCL18 is unlikely to bind to CCR8. Taking into consideration

all the approaches that we used to look at the interaction between CCL18 and CCR8, we must therefore conclude that CCR8 is unlikely to be a functional receptor for CCL18 and the response was seen by the Luster group, may well be coming from an endogenous receptor on the 4DE4 cell line.

An alternative proposal by the group of Amanda Proudfoot is that the binding of CCL18 is predominantly to GAGs on the cell surface (Krohn, Bonvin, et al. 2013, Krohn, Garin, et al. 2013). Even though some other receptors have been reported to be CCL18 receptors such as CCR6, PITPNM3, and CCR8, the binding of CCL18 to GAGs could be the best way to explain the finding that CCL18 can bind to naïve rodent transfectants which which are lacking human chemokine receptors. Indeed, as was the case with our study, Proudfoot et al. observed that CCL18 could displace the binding of a variety of chemokines to heparin, and at high concentrations (100nM and above) could inhibit the chemotactic responses of a variety of CCR-expressing transfectants (Krohn, Bonvin, et al. 2013). This appeared to require the BBXB loop in the "40s region" of the CCL18, since a mutant in which these basic residues were replaced by alanine was without antagonist effect. Thus, in both our experiences, CCL18 appears to remain an orphan chemokine, currently without a signalling chemokine receptor ascribed to it.

Investigating the phenotypic responses of monocytes to CCL18 is hypothesized to give us a better insight into the role of this CC chemokine in atherosclerosis, since monocytes play a significant role in the development of foam cells and the subsequent establishment of atherosclerosis. We have shown that fluorescently labelled CCL18 (AF-CCL18) was able to bind to the surface of monocytes. This binding was partially competed with a 100-fold excess of unlabelled CCL18, suggesting that monocytes

express a receptor for CCL18. The variations in CCL18 binding between the monocytes from different donors may be explained by variable levels of expression of the putative CCL18 receptor between donors. Another way of interpreting this data is agreeing with what was suggested before by others (Krohn, Bonvin, et al. 2013, Krohn, Garin, et al. 2013) namely that the primary binding interaction between CCL18 and responsive cells such as monocytes is mediated by glycosaminoglycans.

We showed that freshly isolated monocytes were unable to migrate in response to CCL18 in real-time chemotaxis assays (TAXIScan). in agreement with Wimmer et al. (Wimmer et al. 2006) and Schraufstatter et al., (Schraufstatter 2004). One interpretation of our findings is that CCL18 could be binding to an atypical chemokine receptor (ACKR) as this class of receptor are known to bind to chemokine but fail to induce classical signalling and downstream cellular responses typical for chemokine receptors (Ulvmar et al. 2011). Alternatively, the freshly isolated monocytes may need to upregulate a signalling component required by the CCL18 receptor for effective chemotaxis. Supportive of this theory, a previous study from the group showed that murine mast cell progenitors cultured in the absence of IL-3 were unable to respond to CCL2 despite expressing good levels of CCR2 on their surface as detected by ligand binding assays (Collington et al. 2010). Addition of IL-3 to the cultures was able to restore functionality, suggesting that chemokine responsiveness can be regulated independently of receptor expression.

In experiments culturing monocytes in the absence of serum, we found that micromolar concentrations of CCL18 had the ability to rescue monocyte from apoptosis as previously reported (Wimmer et al. 2006). This appeared not to be attributable to

CCR8 binding, since we were unable to detect CCR8 on the surface of monocytes. Although this anti-apoptotic effect is unusual for chemokines, it has been noted for other monocyte-specific chemokines. For example, CXCL4 shares this function with CCL18 (Scheuerer et al. 2000). CCL18 was reported by Gleissner to be one of the significantly upregulated genes during M4 polarization, which was confirmed at the protein level by ELISA on culture supernatants (Gleissner et al. 2010). We therefore subsequently compared the survival effects of identical concentrations of CCL18 and CXCL4 on monocyte over a three-day culture period in serum-free media. To our, surprise, CCL18 was as efficacious as CXCL4 in promoting monocyte survival.

Macrophages are a known source of chemokines and cytokines, which made us wonder if the survival effect previously attributed to CXCL4 was the autocrine release of CCL18, particularly since CCL18 has been reported to be induced by polarization of monocytes to M4 macrophages (Gleissner et al. 2010). To test this hypothesis, we examined the gene expression for CCL18 in CXCL4-derived macrophages. Disappointingly, CCL18 message was not detected in mRNA extracted from monocyte-derived macrophages stimulated with CXCL4, nor was CCL18 protein detected in the supernatants of those cultures. This data therefore fundamentally disagrees with that of Gleissner et al., who had previously reported that following six days of culture with CXCL4, M4 macrophages produced significantly more CCL18 than M0 macrophages. (Gleissner et al. 2010). In agreement with our observations, Gouwy et al. have recently reported that rather than enhance CCL18 secretion by monocytes, CXCL4 treatment of monocytes results in a reduction of CCL18 secretion when compared to cells cultured in the presence of M-CSF (Gouwy et al. 2016). Thus, it is unlikely that the survival effects of CXCL4 we observed are attributable to the

autocrine release of CCL18.

We therefore became curious as to which chemokines does the M4 macrophage might secrete. In a single proteomic screen, we assessed the expression of 31 chemokines in the supernatants of pooled M4 cultures. We confirmed a lack of induction of CCL18 but observed that CCL22 was one of the chemokines that was significantly expressed at the protein level. This was subsequently confirmed at the mRNA level. This is in agreement with previous reports (Gleissner et al. 2010, Gouwy et al. 2016).

CCL22, together with CCL17 are ligands for the chemokine receptor CCR4 and both CCL22 and CCL17 are known to preferentially attract Th2 cells and regulatory T cells via CCR4 signalling (Imai et al. 1999, Iellem et al. 2001). At a single 72h time point, we found increased production of both CCL17 and CCL22 mRNA by M4 macrophages, compared to M0 macrophages. Subsequently, in a kinetic study, we investigated the secretion of CCL22 and CCL17 during M4 polarization. Secretion of CCL22 by M4 macrophages at nanomolar concentrations was observed after only 48 h of culture, whilst CCL17 was secreted at levels barely above the detection of the ELISA.

CCL22 and CCL17 have been reported to be biased agonists of CCR4 (Anderson et al. 2016), with unpublished data from our group suggesting that CCL22 but not CCL17 is able to recruit Th2 cells *in vitro* (C. Anderson, personal communication). In line with our hypothesis that CXCL4 has atheroprotective properties, the secretion of CCL22 could be postulated to recruit both Tregs and Th2 cells to the plaque, providing either an anti-inflammatory response or skewing the inflammation from the M1 phenotype.

One of the crucial steps in atherosclerosis is foam cell formation, which is mainly a macrophage function derived from the ability to phagocytose modified (acetylated or oxidized) LDL. It was previously reported by Gleissner and colleagues that M4 macrophages have a reduced phagocytic capacity for modified LDL, again in keeping with an atheroprotective function for CXCL4 (Gleissner et al. 2010). When we examined these finding we were in agreement with their previously published data that M4 take up considerably less oxLDL than M0 cells, and were able to extend these findings to show that this was evident as early as 48 hours into the polarization protocol. Excitingly, we also showed that the M4 "signature" endowed on the macrophages was strong enough to override the M-CSF signal, when cells were cultured in the presence of both cytokines. We found that at the mRNA level, CD36, a principle scavenging receptor for modified LDL, was downregulated in M4 after 72 hrs of culture, suggesting one potential mechanism for our observations.

The early events in M4 polarization subsequently became our focus, in particular, which transcription factors might be responsible for the M4 "signature". Although two other studies have carried out analysis of gene expression in monocytes stimulated with CXCL4 or its variant CXCL4, both studies examined gene expression levels at day 6 of culture, when polarization is well established. Taking a 1hr time point as a likely point at which the levels of transcription factors in polarizing monocytes are at their peak of modulation (Boyle et al. 2012) we designed asmall study (using monocytes from three donors) to examine what transcription factors might be involved in M4 polarisation and subsequently to the induction of genes responsible for the M4 phenotype.

Interestingly, we were able to highlight the transcription factors RELB and ARNT as being significantly upregulated in M4 macrophages. Further experimental work is needed to confirm our supposition that both these transcription factors play a role in M4 polarization. RelB is known to form signalling heterodimers with the p100 and p52 subunits of the NFκB signalling pathway and the sensitivity of CCL22 induction in B-cells to the NFκB inhibitor BAY 11-7082 provides circumstantial evidence that RelB may play a potential role in CCL22 production in M4 macrophages (Nakayama et al. 2004). The potential role for ARNT in M4 polarization was less simple to formulate, although AHR (which forms a signalling complex with ARNT) has been previously shown to sense LDL that has been modified by vascular sheer stress or oxidation(McMillan and Bradfield 2007). Thus, it may be that induction of ARHT expression by CXCL4 primes the M4 macrophages to respond to oxLDL by AHR signalling. In this respect, it might be informative to also assess the induction of AHR expression in M4 macrophages, since this gene was not present in the PCR array used.

To summarize, in this thesis, I have shown that CCL18 and CXCL4 act as cell survival factors, promoting the survival and differentiation of monocytes into distinct phenotypes with potential roles in the process of atherogenesis. Key to the elucidation of those roles is the identification of their monocyte receptors.

Future Work

The molecular-cellular mechanisms and *in vivo* relevance of processes involved in plaque development and advanced plaque progression has been studied extensively throughout the years in order to find the best therapeutic approaches for treating atherosclerosis. Consequently, translational researchers in this area have considered the concept that therapy directed at the arterial wall in general and specifically at macrophages, could be improved with realistic goals. One strategy could be therapeutically modifying macrophage processes that are involved in advanced plaque progression. For example, enhancing either the survival of the plaque macrophages or the phagocytic clearance of dead macrophages, both of which would result in decreased necrotic core formation with benefits for plaque stability (Tabas 2010). The data generated in this thesis suggest that if the atheroprotective functions of CCL18 and CXCL4 could be harnessed in a form that could be delivered to atherosclerotic plaques, then plaque stability might be achieved. This would likely have to be in a form that did not have an atherogenic function. However, small molecule mimetics, which bind to the CCL18 and CXCL4 receptors and induce atheroprotective signalling would be a viable solution. A key step in this approach would be the identification of the monocyte receptors for CCL18 and CXCL4 which have so far remained elusive.

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