

***THE EFFECTS OF ATHEROGENIC
DYSLIPIDAEMIA ON BLOOD
MONOCYTE FUNCTION***

William Jackson

Division of Immunology and Inflammation

Department of Medicine

Imperial College London

This dissertation is submitted for the degree of Doctor of Philosophy

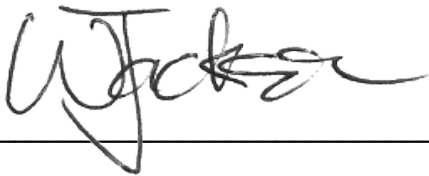
January 2016

“Science is more than a body of knowledge. It is a way of thinking; a way of skeptically interrogating the universe with a fine understanding of human fallibility. If we are not able to ask skeptical questions, to interrogate those who tell us that something is true, to be skeptical of those in authority, then we are up for grabs for the next charlatan who comes rambling along.”

- Carl Sagan

DECLARATION OF ORIGINALITY

This dissertation is the result of my own work and includes nothing that is the outcome of collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university or institution for any degree, diploma, or other qualification.

Signed:  _____

Date: 14/08/2015

William Jackson BSc MSc
Imperial College London

COPYRIGHT DECLARATION

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work.

ABSTRACT

Blood monocytes are heterogeneous effector cells of the innate immune system that can be divided into at least two functionally distinct subpopulations. In circulation these cells are constantly in contact with lipid-rich lipoproteins, yet this interaction is poorly characterized. The aim of this PhD was to examine the functional response of blood monocyte subpopulations to dyslipidaemia, defined as high levels of plasma VLDL and LDL. Initially in the *Ldlr*^{-/-} mouse, I demonstrated that monocytes accumulate cytoplasmic neutral lipid vesicles when exposed to high fat diet induced dyslipidaemia. In this model, lipid loaded monocytes exhibit impaired chemotaxis towards peritonitis due to retention of monocytes in the greater omentum. In vitro assays using human monocytes confirmed neutral lipid vesicle accumulation after exposure to LDL or VLDL, via several receptors including CD36. Neutral lipid accumulation did not inhibit other functions including phagocytosis, endothelial adhesion and transendothelial migration, nor did it lead to an overtly pro-inflammatory phenotype. However, lipid loading led to a migratory defect in both monocyte subsets towards C5a and was accompanied by a disruption of cytoskeletal regulation. These cytoskeletal changes included subset specific differences in cell morphology, and an inhibition of RHOA activation and myosin light chain phosphorylation. These data emphasise the functional consequences of blood monocyte lipid accumulation and reveal important implications for treating inflammation, infection and atherogenesis in the context of dyslipidaemia.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Kevin Woollard for the opportunity to undertake this PhD and for his support and guidance across the past three years. I am also indebted to many others for their help and advice during my project, most notably Dorian Haskard, Charles Pusey and Marina Botto. I have been fortunate to have great colleagues to share in the successes and failures that are part of a PhD, particularly Shenny, Becky, Nicola, Shamim, Tabi and Theresa. Thank you for always helping me to put things into perspective.

A somewhat dubious ‘thank you’ also goes to Lucie Evans, for starting me down this path nearly ten years ago. A little warning would have been nice!

To my family: your unwavering belief that I would succeed has been invaluable, even though most of the time you had no idea what I was doing. To tell you the truth, neither did I.

Finally, the biggest ‘thank you’ must go to my fiancé Lisa, the cleverest person I know. After supporting each other through PhDs, I am sure everything else will seem easy.

CONTENTS

1. INTRODUCTION.....	15
1.1. THE MONONUCLEAR PHAGOCYTE SYSTEM	15
1.1.1. <i>History of the MPS</i>	15
1.1.2. <i>Myelopoeisis and embryonic macrophages</i>	17
1.1.3. <i>Blood monocyte subpopulations and effector functions</i>	22
1.2. LIPID METABOLISM AND LIPOPROTEINS	26
1.2.1. <i>Homeostasis</i>	26
1.2.2. <i>Dyslipidaemia</i>	29
1.3. THE MPS AND ATHEROSCLEROSIS	31
1.3.1. <i>The ‘response to retention’ hypothesis of atherogenesis</i>	31
1.3.2. <i>Macrophage proliferation in the plaque</i>	33
1.4. AIMS	34
1.5. REFERENCES.....	35
2. METHODS	42
2.1. MICE	42
2.2. MONOCYTE PURIFICATION AND FLOW CYTOMETRY	42
2.3. MONOCYTE LDL/ VLDL UPTAKE AND LIPID QUANTIFICATION.....	46
2.3.1. <i>DIO- LDL and DIO- VLDL uptake</i>	46
2.3.2. <i>Neutral lipid staining</i>	46
2.3.3. <i>Membrane cholesterol staining</i>	47
2.4. PERITONITIS MODEL	47
2.5. INTRAVITAL IMAGING	48
2.6. QUANTIFYING LDL/VLDL MODIFICATION.....	48
2.7. BLOCKING LDL/ VLDL UPTAKE	49
2.8. LPL IMMUNOFLUORESCENCE	49

2.9.	APOPTOSIS	49
2.10.	qPCR AND MICROARRAY ANALYSIS	50
2.11.	STATISTICS	50
2.12.	CYTOKINE PRODUCTION:.....	52
2.13.	PHAGOCYTOSIS	52
2.14.	TRANSWELL CHEMOTAXIS ASSAY	52
2.15.	2D REAL-TIME MIGRATION ASSAY	53
2.16.	MONOCYTE ADHESION TO ENDOTHELIUM	53
2.17.	TRANSENDOTHELIAL MIGRATION	53
2.18.	RHOA AND CDC42 ACTIVATION ASSAYS	54
2.19.	WESTERN BLOT.....	54
2.20.	CELL MORPHOLOGY ANALYSIS	55
2.21.	DROSOPHILA HEMOCYTE IMAGING	55
2.22.	REFERENCES	56
3.	THE RESPONSE OF MOUSE MONOCYTES TO DYSLIPIDAEMIA	57
3.1.	INTRODUCTION	57
3.2.	RESULTS	58
3.2.1.	<i>Neutral lipid accumulation in mouse monocytes</i>	58
3.2.2.	<i>The response of lipid-loaded monocytes to sterile inflammation.</i>	64
3.2.3.	<i>Intravital imaging of GR1^{low} monocyte patrolling after HFD</i>	71
3.3.	DISCUSSION	73
3.1.	REFERENCES	77
4.	UPTAKE OF LDL AND VLDL BY MONOCYTES	80
4.1.	INTRODUCTION	80
4.1.1.	LDL AND VLDL MODIFICATION	80
4.1.2.	<i>The LDL Receptor</i>	82

4.1.3. SCAVENGER RECEPTORS	84
4.2. RESULTS	86
4.2.1. <i>Neutral lipid accumulation after LDL/VLDL treatment</i>	86
4.2.2. <i>Monocyte uptake of fluorescent LDL and VLDL</i>	89
4.2.3. <i>Blocking monocyte LDL/ VLDL uptake</i>	92
4.2.4. <i>Blocking monocyte neutral lipid accumulation</i>	96
4.3. DISCUSSION	101
4.4. REFERENCES:.....	105
5. THE EFFECTS OF ATHEROGENIC LIPOPROTEINS ON MONOCYTE FUNCTION	109
5.1. INTRODUCTION.....	109
5.2. RESULTS	111
5.2.1. <i>The effect LDL/VLDL on monocyte apoptosis</i>	111
5.2.2. <i>Modulation of monocyte activation and cytokine production by LDL/ VLDL</i>	112
5.2.3. <i>Monocyte phagocytosis, chemotaxis and chemokinesis following LDL/ VLDL treatment.</i>	117
5.2.4. <i>The effect of LDL/ VLDL treatment on monocyte- endothelial adhesion and transendothelial migration.</i>	123
5.3. DISCUSSION	125
5.1. REFERENCES	130
6. CYTOSKELETAL REGULATION IN LIPID-LOADED MONOCYTES	134
6.1. INTRODUCTION.....	134
6.1.1. <i>Cytoskeletal regulation during cell migration</i>	134
6.1.2. <i>Cell migration in Drosophila hemocytes</i>	138
6.2. RESULTS	139
6.2.1. <i>Cytoskeletal gene expression changes during hypercholesterolemia.</i>	139

6.2.2.	<i>Cytoskeletal GTPase activation levels following VLDL treatment.....</i>	140
6.2.3.	<i>Monocyte morphological changes induced by VLDL or RHOA activation.</i>	145
6.2.4.	<i>Monocyte motility following pharmacological RHOA activation.</i>	148
6.2.5.	<i>Drosophila hemocyte migration following LDL injection.....</i>	150
6.3.	DISCUSSION	152
6.4.	REFERENCES	156
7.	DISCUSSION.....	160
7.1.	SUMMARY OF FINDINGS.....	160
7.2.	THERAPEUTIC IMPLICATIONS	162
7.2.1.	<i>Atherosclerosis</i>	162
7.2.2.	<i>Infection</i>	164
7.2.3.	<i>Chronic inflammatory disease.....</i>	165
7.3.	FUTURE WORK	167
7.3.1.	<i>Lipid uptake.....</i>	167
7.3.2.	<i>Monocyte migration.....</i>	169
7.3.3.	<i>Cytoskeletal regulation.....</i>	172
7.4.	CONCLUSION	173
7.5.	REFERENCES	174

LIST OF FIGURES AND TABLES

FIGURE 1.1 MONOCYTE AND MACROPHAGE ORIGINS DURING HOMEOSTASIS.	21
FIGURE 1.2 GATING STRATEGIES FOR MONOCYTE SUBSET IDENTIFICATION IN THE HUMAN AND MOUSE.....	25
FIGURE 1.3: AN OVERVIEW OF LIPOPROTEIN METABOLISM.	28
FIGURE 2.1 ELIMINATION OF PLATELET CONTAMINATION FROM MONOCYTES USING ANTI-CD61.....	43
FIGURE 2.2 CONFIRMATION OF MONOCYTE PURITY AFTER MACS ISOLATION.	44
TABLE 2-1 ANTIBODIES USED FOR FLOW CYTOMETRY, IMMUNOFLUORESCENCE AND WESTERN BLOTTING.	45
TABLE 2-2 RECEPTOR BLOCKING STRATEGIES FOR LDL/ VLDL UPTAKE	49
TABLE 2-4 HUMAN RT-QPCR PRIMER SEQUENCES	51
FIGURE 3.1: FACS PHENOTYPING OF BLOOD LEUKOCYTES FROM <i>LDLR</i> ^{-/-} MICE.....	59
FIGURE 3.2: BLOOD MONOCYTES FROM <i>LDLR</i> ^{-/-} MICE ARE LIPID LOADED.	60
FIGURE 3.3 EXPRESSION OF CD11c BY MURINE MONOCYTES ON HFD.	63
FIGURE 3.4: STERILE PERITONITIS MODEL IN DYSLIPIDAEMIC <i>LDLR</i> ^{-/-} MICE.	66
FIGURE 3.5: BLOOD MONOCYTE TRACKING DURING PERITONITIS USING LATEX BEADS.....	69
FIGURE 3.6: LEUKOCYTE ACCUMULATION IN THE OMENTUM AFTER THG PERITONITIS.....	70
FIGURE 3.7: INTRAVITAL IMAGING OF MONOCYTES IN CHOW OR HFD FED <i>Cx3CR1</i> ^{GFP/+} MICE.....	72
FIGURE 4.1 MEASUREMENT OF LIPOPROTEIN MODIFICATION BY CONJUGATED DIENE CONTENT.	83
FIGURE 4.2 LIPID ACCUMULATION OVER TIME IN LDL/ VLDL TREATED HUMAN MONOCYTES.	87
FIGURE 4.3 MEMBRANE CHOLESTEROL STAINING IN HUMAN MONOCYTES AFTER LDL/ VLDL TREATMENT.	88
FIGURE 4.4 UPTAKE OF FLUORESCENT LDL AND VLDL BY HUMAN MONOCYTE SUBPOPULATIONS.	90
FIGURE 4.5 UPTAKE OF FLUORESCENT LDL BY MOUSE MONOCYTES IN VIVO.	91
FIGURE 4.6 BLOCKING LDL/ VLDL UPTAKE VIA THE LDLR-FAMILY, CD36 AND CLASS-A SCAVENGER RECEPTORS.	95
FIGURE 4.7 THE EFFECT OF ANTI- CD36 BLOCKING ANTIBODY ON LDL INDUCED NEUTRAL LIPID ACCUMULATION.....	97
FIGURE 4.8 THE EFFECT OF HEPARINASE TREATMENT ON LDL AND VLDL INDUCED NEUTRAL LIPID ACCUMULATION.	98
FIGURE 4.9 CELL SURFACE LPL STAINING AND ORLISTAT TREATMENT OF HUMAN MONOCYTES.	100
FIGURE 5.1 MONOCYTE APOPTOSIS FOLLOWING LDL/ VLDL TREATMENT.....	112
FIGURE 5.2 EFFECT OF LDL/ VLDL TREATMENT ON MONOCYTE SURFACE MARKER EXPRESSION.....	113
FIGURE 5.3 EFFECT OF LDL/ VLDL TREATMENT ON MONOCYTE SURFACE CD11c AND CD36.	114
FIGURE 5.4 EFFECT OF LDL/ VLDL TREATMENT ON MONOCYTE CYTOKINE RESPONSE.....	116
FIGURE 5.5 EFFECT OF LDL/ VLDL TREATMENT ON MONOCYTE PHAGOCYTOSIS.	118
FIGURE 5.6 EFFECT OF LDL/ VLDL TREATMENT ON MONOCYTE CHEMOTAXIS.....	121
FIGURE 5.7 EFFECT OF VLDL TREATMENT ON MONOCYTE CHEMOKINESIS.	122
FIGURE 5.8 EFFECT OF LDL/ VLDL TREATMENT ON MONOCYTE ADHESION TO HUVEC AND TRANSENDOTHELIAL MIGRATION.	124
FIGURE 6.1 SUMMARY OF CYTOSKELETAL REGULATION DURING CELL MIGRATION.....	137
FIGURE 6.2 LEUKOCYTE SMALL GTPASE GENE EXPRESSION DURING DYSLIPIDAEMIA.....	141
FIGURE 6.3 RHOA ACTIVATION LEVELS FOLLOWING VLDL TREATMENT.	142
FIGURE 6.4 MYOSIN LIGHT CHAIN PHOSPHORYLATION IN VLDL TREATED HUMAN MONOCYTES.	144

FIGURE 6.5 CDC42 AND PAK PATHWAY ACTIVITY AFTER MONOCYTE LDL AND VLDL TREATMENT.	145
FIGURE 6.6 THE EFFECTS OF VLDL TREATMENT AND RHOA ACTIVATION ON MONOCYTE MORPHOLOGY.	147
FIGURE 6.7 MONOCYTE CHEMOKINESIS AFTER VLDL AND CN03 TREATMENT.	149
FIGURE 6.8 LDL INDUCED MIGRATORY IMPAIRMENT IN <i>DROSOPHILA</i> EMBRYONIC HEMOCYTES.....	151
FIGURE 7.1 SUMMARY SCHEMATIC OF PROPOSED MIGRATORY DYSFUNCTION IN MONOCYTE LIPOPHAGES.....	161

LIST OF ABBREVIATIONS AND ACRONYMS

Abbreviations are written in full on their first mention in each chapter and standard abbreviations are used where they exist. For clarity, gene names are abbreviated according to the guidelines of the HUGO Gene Nomenclature Committee. Protein names are abbreviated according to the Universal Protein Resource (UniProt).

Abbreviation	Full text
BMDM	Bone marrow derived macrophage
BSA	Bovine serum albumin
cMoP	Common monocyte progenitor
CMP	Common myeloid progenitor
CS	Chondroitin sulfate
CVD	Cardiovascular disease
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DS	Dextran sulphate
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
F-actin	Filamentous actin
FACS	Fluorescence associated cell sorting
FFA	Free fatty acid
FH	Familial hypercholesterolemia
FITC	Fluorescein isothiocyanate
FLIM	Fluorescence-lifetime imaging microscopy
FMT	Fluorescence molecular tomography

FRET	Förster resonance energy transfer
FSC	Forward scatter
G-actin	Globular actin
GAP	Rho GTPase activating protein
GEF	Guanine nucleotide exchange factor
GMP	Granulocyte/ macrophage progenitor
GPCR	G-protein coupled receptor
HDL	High density lipoprotein
HFD	High fat diet
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell
HSPG	Heparan sulphate proteoglycans
HUVEC	Human umbilical vein endothelial cells
IDL	Intermediate density lipoprotein
IP	Intraperitoneal
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MDA	Malondialdehyde
MDP	Macrophage/ dendritic cell progenitor
MPS	Mononuclear phagocyte system
MUFA	Monounsaturated fatty acid
oxLDL	Oxidized low density lipoprotein
PAGE	Polyacrylamide gel electrophoresis

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid arthritis
RES	Reticuloendothelial system
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SFA	Saturated fatty acid
SIV	Simian immunodeficiency virus
SPM	Small peritoneal macrophage
SSC	Side scatter
TBS	Tris buffered saline
TEM	Transendothelial migration
TGRL	Triglyceride rich lipoprotein
THG	Thioglycollate
TRITC	Tetramethylrhodamine
VLDL	Very low density lipoprotein

LIST OF PUBLICATIONS AND AWARDS

DIRECTLY RELATED PUBLICATIONS

Jackson WD; Weinrich T; Woollard KW. Very-low and low-density lipoproteins induce neutral lipid accumulation and impair migration in monocyte subsets. *Scientific Reports*, 2016.

Saja MF; Baudino L; **Jackson WD** et al. Triglyceride-rich lipoproteins modulate the steady-state distribution of blood Gr1^{low} monocytes and orchestrate their extravasation. *Cell Reports*, 2015.

Jackson WD & Woollard KW. Targeting monocyte and macrophage subpopulations for immunotherapy: a patent review (2009 - 2013). *Expert Opin Ther Pat*. 2014.

OTHER PUBLICATIONS

McAdoo SP et al. Spleen Tyrosine Kinase Inhibition Attenuates Autoantibody Production and Reverses Experimental Autoimmune GN. *JASN*, 2014.

CONFERENCES

Oral Presentation, European Atherosclerosis Society Congress 2015.

Poster, IUPS Congress 2013

Poster, British Cardiovascular Society Annual Conference 2013

AWARDS

Young Investigator Oral Presentation Award, European Atherosclerosis Society Congress, 2015.

Young Investigator Fellowship, European Atherosclerosis Society Congress, 2015.

2nd Place, 2015 Three Minute Thesis Competition, Department of Medicine, Imperial College London.

ESM-EVBO Poster Abstract Prize, IUPS Congress 2013.

1. INTRODUCTION

1.1. The Mononuclear Phagocyte System

1.1.1. History of the MPS

In 1908, the Russian zoologist Ilya Metchnikoff was jointly awarded the Nobel Prize for his controversial discovery of motile phagocytes, from the Greek 'phagos' (to eat) and 'cyte' (cell) [1,2]. While taking refuge from political instability in a private lab in 1882, Metchnikoff wounded transparent starfish using splinters from a tangerine tree and observed an accumulation of cells at the site of inflammation, attempting to envelope the foreign objects [3]. In doing so, he was the first to recognise that the phagocyte has key functions not only in development via the engulfment of phagocytic bodies, but also in the defence from invading microbes. Due to this striking 'eating' behaviour, Metchnikoff hypothesized that these cells could not only 'eat', but also 'feed' other cell types due to their high level of motility [3]. While he could not have understood the systemic implications of his theory, the concept that phagocytes are metabolically active cells forms a cornerstone of the work presented here and is a theory as old as the discovery of phagocytes themselves. The impact of this work for subsequent research on innate immunity cannot be overstated, for it directly contradicted the dogma of the time that immunity was driven by the humoral antibody and complement response, for which Paul Ehrlich shared Metchnikoff's Nobel prize [4].

Despite these initial findings, phagocytes received limited scientific attention when compared to adaptive immunity for the following 60 years. The 1920s saw some attempts by Ashcroft and colleagues to classify phagocytes and the creation of the 'reticuloendothelial system' (RES), within which modern macrophages and dendritic cells were classified together based on histological appearance as 'histiocytes' [5]. However, understanding of these cells did not progress much further until the work of

Zanvil Cohn and James Hirsch in the late 1960s. Following substantial work on neutrophil phagocytosis and degranulation, Cohn turned his attention to mouse macrophages. Cohn was the first to describe a culture method for the mouse peritoneal macrophage [6] and to use phase and electron microscopy to characterize the induction of macrophage lysosomes by pinocytic activity and the phagocytosis of exogenous particles [7-10]. At the same time, Cohn also described the in vitro differentiation of blood monocytes to functional macrophages in a series of seminal papers [11], [12]. In later work with his postdoctoral fellow Ralph Steinman, Cohn discovered morphologically abnormal phagocytes in the mouse spleen while studying the induction of primary antibody responses, which were termed 'dendritic cells' (DCs) [13]. Using the lack of Fc-receptor expression by DCs, the pair developed a method of enriching populations of dendritic cells and macrophages for culture, allowing for distinction between the two cell types [14]. Steinman would go on to posthumously receive the Nobel Prize for this discovery and a lifetime of subsequent work on DCs. Cohn continued to generate seminal work in monocyte and macrophage biology until his death in 1993, perhaps most notably observing that monocyte transendothelial migration was dependent on endothelial PECAM-1 in 1989 [15].

Following publications with contemporaries Cohn and Hirsch, Ralph Van Furth proposed a new classification of all cells with high levels of phagocytic activity to other experts in the field at the *1969 Conference on Mononuclear Phagocytes* in Leiden: the 'mononuclear phagocyte system' or MPS [16]. Largely eschewing morphological criteria, this system of phagocyte classification relied on fundamental functional properties in addition to phagocytosis, including adhesion to a glass surface and expression of immunoglobulin receptors [16]. A key distinction was made in Van Furth's system between macrophages and the recently discovered myeloid dendritic cells, with the latter not deemed phagocytic enough to be classified within the MPS [16]. Fundamentally, this is the root of a debate regarding the function and ontology of these two cell types which continues today [17]. Crucially,

consensus at this time was that all cells in the MPS must originate from the blood monocyte, a conclusion that would subsequently be disproven.

1.1.2. Myelopoiesis and embryonic macrophages

The classical model of myelopoiesis is centred on the sequential differentiation of precursor cells in the bone marrow and the original definition of the MPS dictated that all cells in the system must derive from blood monocytes in a linear fashion. Indeed, the primary role of monocytes was seen as a source of 'immature' macrophages to replenish supplies in peripheral tissues, both during homeostasis and inflammation [12]. In the most widely accepted model, monocytes develop from haematopoietic stem cells (HSCs) via series of increasingly committed progenitors, including the common myeloid progenitor (CMP), the granulocyte/macrophage progenitor (GMP), the macrophage/DC progenitor (MDP) and the common monocyte progenitor (cMoP) [18-20], although the existence of a bona-fide MDP is subject to ongoing debate [21], [20]. Major discoveries in the 1990s by Siamon Gordon and others determined that this process is dependent on the growth factor colony stimulating factor (CSF)-1 and the transcription factor PU.1 [22,23]. Crucially, the recent discovery of cMoP and extensive proteomic analysis has determined that monocytes and monocyte-derived macrophages are derived from a distinct clonogenic precursor to DCs, thus largely ending the longstanding debate on the functional independence of these cell types [17,20].

However, as early as the late 1980s it became apparent that the accepted dogma of blood monocytes as obligatory precursors for tissue macrophages during homeostasis was incomplete. In the mouse, primitive macrophages appear on the ninth day of gestation (E9), prior to the development of the bone marrow [24,25]. This led to fate mapping studies to determine the origin of adult tissue macrophage populations. In rodents, there are two major waves of embryonic haematopoiesis. At around E7, primitive nucleated myeloid cells and erythrocytes are generated in the yolk sac, resulting in primitive macrophages appearing in the blood islands of the

yolk sac by E9 without the requirement for a monocytic precursor [24,25]. By E10, the embryonic circulatory system is fully established [26], and these primitive yolk sac macrophages spread throughout the body to seed all tissues [27]. However, in all macrophages that have been studied, with the possible exception of the brain microglia [27], these cells are subsequently replaced by a second wave of haematopoiesis. At E10.5, haematopoietic stem cells (HSCs) are generated in the aorta-gonad-mesonephros (AGM) of the embryo, which then spread to the foetal liver [28]. Primitive foetal liver monocytes are generated from these HSC precursors [28] and from yolk sac- derived erythro-myeloid progenitors (EMPs) at E11.5-12.5 [29], although the relative contribution of these two sources remains unclear and from this point forward the origin of tissue macrophages is still debated. In 2012, Schulz et al were the first to demonstrate that this embryonic macrophage seeding from the foetal liver was widespread and applicable to the majority of tissues including the liver, spleen, lung, pancreas and kidney, although importantly was proposed to be independent of the HSC transcription factor *Myb* [30]. In addition, subsequent work demonstrated that these *Myb* independent tissue-resident macrophages are a separate lineage of MPS to bone marrow derived infiltrating macrophages, that can self maintain via in-situ proliferation with minimal contribution from blood monocytes [31]. However, Ginhoux and colleagues have demonstrated that the contribution of *Myb* independent, yolk sac derived precursors to tissue macrophages is minor [27]. As such, while it is clear that the vast majority of tissue macrophages are seeded before birth and self-renew in situ, their exact developmental origin requires further confirmation. Current understanding of the ontogeny of tissue macrophages is summarized in Figure 1.1 and was recently reviewed by Varol and colleagues [32].

A notable exception to this process is the homeostasis of intestinal macrophages. For many years it was difficult to distinguish between macrophages and dendritic cells in the intestinal lamina propria, as gut macrophages have a phenotype that overlaps heavily with DCs and both express CD11c and high levels of MHCII [33,34]. A key discovery was the expression of CD64 by gut macrophages but not DCs, thus

allowing for distinction between these two populations [35]. While resident and pro-inflammatory macrophages are found in the colon, these cell populations do not proliferate in situ and both require continuous replenishment from Ly6C^{hi} blood monocytes [36,37]. At present the gut is the only tissue thought to require this process during homeostasis. In the case of resident macrophages, infiltrating monocytes differentiate through several intermediate stages over 4-5 days to generate a mature F4/80^{hi}CD64⁺MHCII⁺CD11c⁺CX3CR1^{hi} resident population, while during intestinal inflammation these cells arrest in a CX3CR1^{low} inflammatory state during maturation [36]. Currently there is limited understanding of the signals which mediate renewal of gut macrophages by blood monocytes. Monocyte recruitment is dependent on CCR2, as both CCR2 and CCL2 knockout mice have a substantial decrease in the number of macrophages in the intestinal mucosa [38]. However, the source of intestinal CCL2 driving recruitment is the subject of ongoing work. There have also been suggestions that recruitment is mediated by IL-8 and TGF- β produced by epithelial cells and lamina propria mast cells, although these data derive only from in vitro studies [37].

It is also clear that the tissue microenvironment is the biggest influence on macrophage phenotype and function. Far from the rigid M1/M2, classic vs alternative model of macrophage activation, a complex spectrum of stimuli can program macrophages in a reversible and dynamic manner [39,40]. Recent comparative transcriptional profiling of tissue macrophages from the spleen, liver, lung, peritoneum and brain has revealed that these populations are transcriptionally distinct in their expression of all gene families that have classically characterized macrophage phenotype, including chemokine receptors, Toll-like receptors and scavenger receptors, while sharing expression of common surface markers such as CD64 and MerTK [41]. Incredibly, the tissue microenvironment of the lung is sufficient to program naive macrophages. When fluorescent bone marrow-derived macrophages (BMDM) are transplanted into the alveoli in mice, transplanted cells can be detected proliferating in the lung of the recipient 1-year after transplant and

possess a phenotype that is indistinguishable from host tissue-resident macrophages [42].

Of all tissue macrophages, those in the peritoneal cavity are the best defined in the context of local signals which define their phenotype. Compared to other tissue resident populations, bone marrow-derive macrophages and fetal liver macrophages, resident peritoneal macrophages express high levels of the zinc finger transcription factor GATA6 [41,43,44]. This expression is induced by the action of retinoic acid on macrophages in the 'milky spots' of the omentum, which mature and migrate into the peritoneal cavity to form a resident population, often termed 'large peritoneal macrophages' (LPMs). It is important to note that monocyte-derived macrophages recruited during peritoneal inflammation are not dependent on retinoic acid and do not express GATA6, forming a distinct population of 'small peritoneal macrophages' (SPMs) [43]. As more work is performed to characterize resident populations the specific signals in other tissues will undoubtedly be identified, but at present the peritoneum remains the best described.

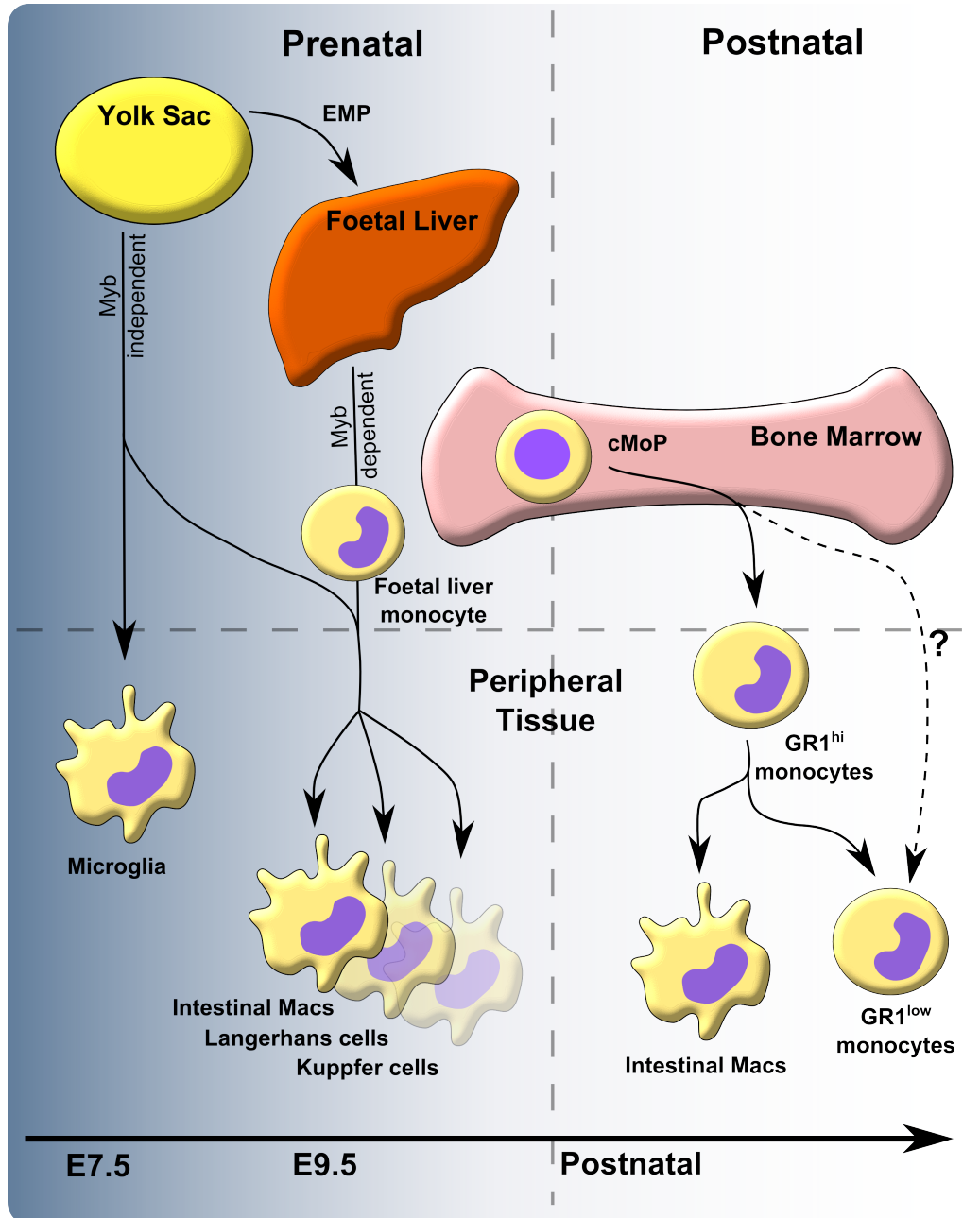


Figure 1.1 Monocyte and macrophage origins during homeostasis.

Tissue macrophages seed during embryogenesis during two waves of haematopoiesis. At E7.5, primitive macrophages appear in the yolk sac and subsequently spread throughout the embryo via the blood in a Myb independent process. From E9.5, progenitor cells from the yolk sac and AGM appear in the foetal liver and give rise to Myb dependent primitive monocytes, which colonize all tissues as macrophages with the possible exception of the brain microglia, progressively replacing yolk sac- derived cells. During adulthood, following development of the bone marrow, canonical haematopoiesis gives rise to GR1^{high} classical monocytes, which constantly replenish intestinal macrophages. In the blood, GR1^{high} monocytes differentiate to a GR1^{low} subpopulation, which are not thought to contribute to the macrophage pool in the steady state.

1.1.3. Blood monocyte subpopulations and effector functions

Far from being inert precursors, it is now known that blood monocytes are a heterogeneous, functionally independent population of innate immune effector cells. At least two blood monocyte subsets exist in the human, mouse, rat, pig and cow, each with independent effector functions [45-49]. In the mouse, two subsets exist based on their expression of GR1: GR1^{high} 'inflammatory' monocytes and GR1^{low} 'patrolling' monocytes [45]. In humans, these can be characterized based on expression of CD16 (FcγRIII) and CD14: 'classical' CD14^{high} CD16^{neg}, 'non-classical' CD14^{low} CD16^{pos} [46] (Figure 1.2). A third 'intermediate' CD14^{high} CD16^{pos} subset has been postulated to exist in humans, although they are poorly characterized and some studies have suggested that they resemble the human 'non-classical' CD14^{low} CD16^{pos} population based on transcriptional and functional analysis [50,51]. We have confirmed these findings in the Woollard lab via hierarchical clustering and principal component analysis of published microarray data (data not shown) and therefore define our human monocytes as CD16^{pos} and CD16^{neg} for all functional work, as previously described [51].

In addition, there is now evidence that classical GR1^{high} monocytes in the mouse are obligatory precursors for the GR1^{low} subset, which represent an older, mature population [52]. Although the stimuli that trigger this conversion are not known, there is evidence that the development of GR1^{low} monocytes is dependent on both Nuclear receptor subfamily 4 group A member 1 (NR4A1)[53] and Sphingosine 1-phosphate receptor 5 (S1PR5)[54]. In *S1pr5*^{-/-} animals there is a complete absence of peripheral GR1^{low} non-classical monocytes, while the bone marrow remains unaffected [54]. The authors hypothesize that S1PR5 may be required for GR1^{low} monocyte egress from the bone marrow; although in the context of newer data on peripheral monocyte conversion this seems unlikely. It is possible that S1PR5 controls GR1^{low} monocyte half-life and clearance from circulation rather than affecting conversion and this possibility requires further investigation. Interestingly, NR4A1 deletion affects both of these compartments, with the few remaining GR1^{low} monocytes in the bone marrow

exhibiting cell cycle arrest and apoptosis [53]. If this data is extrapolated to humans and the continuum of CD14/CD16 expression, any definition of subpopulations is arbitrary due to continuous inter-conversion.

However, while they clearly possess considerable plasticity, these subpopulations have been shown to be functionally distinct in response to a range of stimuli. Based on gene expression, CD14^{high} CD16^{neg} human monocytes resemble the GR1^{high} murine subset and also share similarities in their functional response, whereas GR1^{low} monocytes are the functional homologues of the human CD14^{low} CD16^{pos} subset [46] [50,51]. Both CD14^{high} CD16^{neg} and GR1^{high} subsets display a potent cytokine and chemokine response to LPS stimulation *in vitro*, producing high levels of reactive oxygen species (ROS), IL-8, IL-6, IL-10, RANTES and CCL-2, whereas the GR1^{low}/ CD14^{low} non-classical monocyte LPS response is minimal and characterized by TNF- α production [46,51]. In contrast, GR1^{low}/ CD14^{low} monocytes respond to viruses, nucleic acids and immune complexes, producing IL-1 β , TNF- α and CCL3 via a TLR7/TLR8- MEK pathway, while the CD14^{high} CD16^{neg} response to viral stimuli is characterized almost exclusively by IL-8 production [46]. In addition, human CD14^{high} monocytes display high phagocytic activity regardless of CD16 expression, whereas CD14^{low} CD16^{pos} monocytes are poor phagocytes [46].

In vivo, functional heterogeneity is also becoming clearer in both homeostasis and inflammation. Most obviously, non-classical CD16^{pos}/ GR1^{low} monocytes are unique in their ability to patrol the vasculature in the steady state. Whereas leukocyte rolling on the endothelium is transient and at a velocity of approximately 40 μ m/second [55], 'patrolling' monocytes crawl 100-1000 fold slower, independent of the direction of blood flow and do not extravasate without inflammatory stimuli [46] [56]. This behaviour requires firm adhesion to the vascular endothelium and is mediated by high expression of the chemokine receptor CX3CR1 and the B2-integrin LFA-1, whereas CD16^{neg}/ GR1^{high} monocyte rolling and capture is mediated by the well-characterized classical adhesion cascade [56]. Currently, GR1^{low} monocyte patrolling

has been visualized in small blood vessels in the vascular beds of the dermis, mesentery and cremaster, although preliminary observations from our lab also suggests that this behaviour occurs in larger vessels, including the aorta (Woollard, K. Unpublished data). In the kidney cortex, these patrolling cells monitor for virally infected or necrotic endothelium, simultaneously clearing cellular debris and recruiting neutrophils during vascular inflammation [57]. As expected from in vitro data, during a range of viral infections including hepatitis B and dengue virus CD16^{pos} patrolling monocytes produce a dramatically more pronounced inflammatory response than their CD16^{neg} counterparts [58,59]. In contrast, it has long been appreciated that CCR2 mediated recruitment of GR1^{high} murine monocytes is essential for the control of bacterial infection in the mouse, most markedly in the response to intracellular pathogens such as *Listeria monocytogenes* [60] and *Mycobacterium tuberculosis* [61], although corresponding functional data is yet to be gathered in humans. Strikingly, GR1^{high} monocytes are able enter and survey peripheral tissues under homeostatic conditions, traffic to the lymph nodes and present antigen without differentiating to macrophages or DCs [62].

In conjunction with the new appreciation of embryonic tissue macrophages described above, these data re-enforce a revised paradigm of monocytes, macrophages and DCs as functionally independent populations.

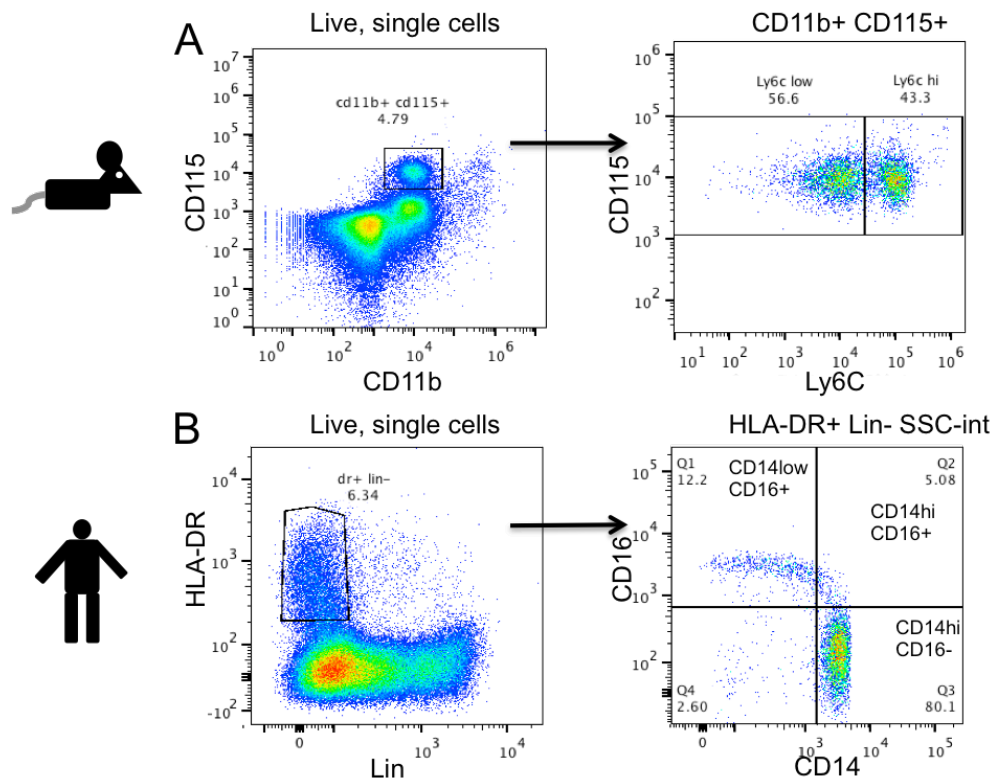


Figure 1.2 Gating strategies for monocyte subset identification in the human and mouse.

Monocytes were gated in a population of whole blood leukocytes using flow cytometry. In the mouse (A), at least two monocyte subsets can be identified from CD11b+ CD115+ leukocytes based on their expression of GR1/ Ly6C. In humans (B) at least two subsets are can be defined from CD16 and CD14 expression patterns, from a population of HLA-DR+, Lineage- (CD2-, CD19-, CD15-, CD56-) cells. Adapted from [63].

1.2. Lipid metabolism and lipoproteins

1.2.1. Homeostasis

Blood lipoproteins act as transport vehicles for cholesterol and triacylglycerol are among the most abundant components of the human plasma proteome [64]. They are therefore in constant contact with blood monocytes and may impact on monocyte function, yet little work has been performed to investigate this hypothesis. Fundamentally, cholesterol is a Janus molecule: under homeostatic conditions, the delivery of circulating cholesterol to all cells is essential for the maintenance of cell membrane fluidity and as a precursor in a range of biosynthetic pathways (e.g. steroid hormones), while inappropriate cholesterol accumulation can be lethal. At the core of this issue is the insolubility of cholesterol in water, necessitating a mechanism of plasma cholesterol transport which was elegantly delineated in the Nobel Prize winning work of Brown and Goldstein [65]. In eukaryotes this mechanism is heavily conserved and involves the packaging of cholesterol and triacylglycerol into lipoprotein molecules with a core of neutral lipids and cholesterol esters surrounded by a hydrophobic surface monolayer of phospholipids and non-esterified cholesterol, stabilized by a protein [65] [66]. The five major classes of lipoproteins, in order of increasing density are chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL) [65].

Lipoprotein metabolism is exceedingly complex and has been reviewed extensively elsewhere [65,67], but an overview will be provided here and summarised in Figure 1.3. Fundamentally, the process can be split into three distinct yet interconnected stages: (1) the packaging and transport of dietary lipid, (2) the hepatic metabolism of endogenous lipid and (3) reverse cholesterol transport from peripheral tissues. Following dietary intake, lipid is packaged into chylomicrons with apolipoprotein B (apoB) by intestinal enterocytes and secreted into the blood. The triacylglycerol in the chylomicrons is hydrolysed in the blood by free lipoprotein lipase (LPL) to form

chylomicron remnants, which are scavenged by the liver via LDL-like receptor protein (LRP). It is at this stage that dietary lipid enters the cycle of endogenous lipid metabolism. Triglyceride- rich VLDL particles are synthesized from the chylomicron remnants and secreted into the blood. In the blood these VLDL particles also undergo hydrolysis by LPL to produce free fatty acids, glycerol and the smaller lipoprotein species IDL. LPL is also highly expressed by highly metabolically active tissues with a large requirement for free-fatty acids (FFAs), such as the heart and skeletal muscle [68]. At this stage, a proportion of the IDL is removed from circulation by the liver via the LDL receptor (LDLR), while the remainder is hydrolysed again by hepatic lipase to generate LDL. In the steady state it is the subsequent internalization of LDL via binding of apolipoprotein B-100 (apoB-100) to the LDLR that is thought to deliver the necessary cholesterol to all peripheral tissues.

Following cholesterol internalization and utilization, the reverse cholesterol transport (RCT) pathway efficiently effluxes cholesterol from peripheral tissues to circulating HDL to be metabolized by the liver. The mechanisms by which this occurs are still not fully elucidated, but considerable progress has been made in the field. In brief, ApoA1 is synthesized by the liver and interacts with serum phospholipids to form nascent discoidal HDL (ndHDL). ndHDL initially binds cholesteryl esters which have already been externalized to the extracellular space by ATP-binding membrane cassette protein A1 (ABCA1) [69]. These larger, mature HDL molecules can then directly induce cholesterol efflux from the plasma membrane via scavenger-receptor B1 (SR-B1) and/or ABCG1 binding [70]. HDL-cholesterol then drains from peripheral tissues through the lymphatic vessels to the plasma [71]. Upon reaching the liver, the selective uptake of cholesteryl esters from mature HDL is mediated largely by SR-B1, which in mice has been shown to be responsible for up to 90% of cholesterol removal from HDL, ultimately leading to fecal excretion of excess cholesterol [72].

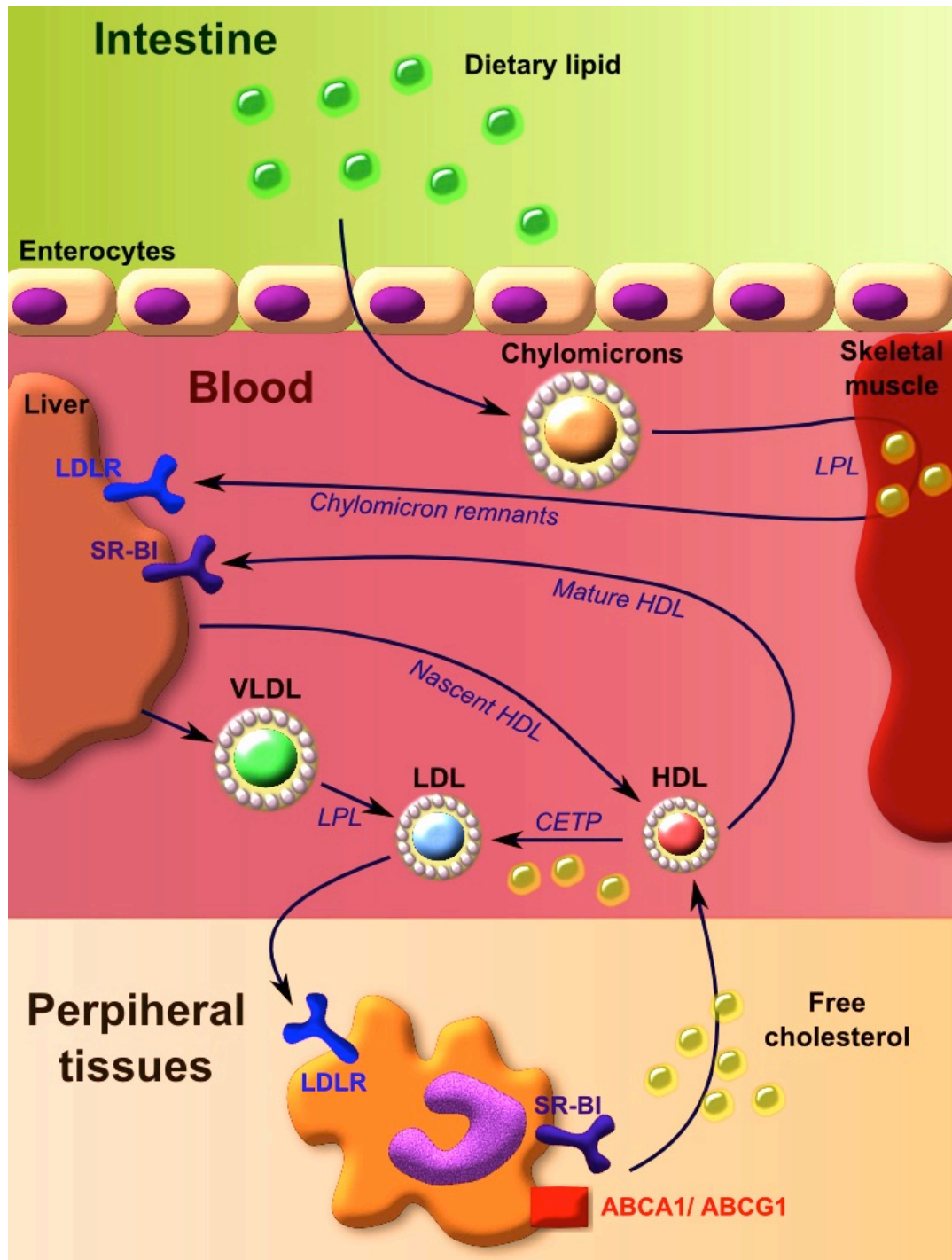


Figure 1.3: An overview of lipoprotein metabolism.

Dietary fat is absorbed by enterocytes in the intestine and packaged into chylomicrons, which are secreted into the blood. Highly metabolically active tissues such as skeletal muscle hydrolyse chylomicrons via LPL and the liver takes up the chylomicron remnants. The liver synthesizes VLDL, which undergoes hydrolysis to LDL in the blood. LDL delivers cholesterol to peripheral tissues via the LDLR and excess cholesterol is exported to HDL via SR-B1, ABCA1 and ABCG1. The cholesterol in HDL is transported to the liver via SR-B1 and transferred to LDL/ VLDL via cholesterol ester transfer protein (CETP).

1.2.2. Dyslipidaemia

1.2.2.1. Monogenic Hypercholesterolemia

There are several monogenic genetic diseases that affect lipid metabolism and lead to severely disruption of one or several of the blood lipoprotein fractions. Of these, familial hypercholesterolemia (FH) is the best characterized. FH is dominantly inherited monogenic genetic disorder affecting LDL uptake and metabolism and was the first genetic disease of lipid metabolism to be clinically characterized [73]. While the clinical presentation of FH in the population is high at around 1:200-500, homozygous FH is much more rare at around 1: 1 million, although accurate estimates of prevalence are confounded by low rates of diagnosis and treatment [74]. FH is characterized by a severe elevation of plasma LDL levels, which presents clinically as cutaneous xanthomas and premature cardiovascular disease. In homozygotes, untreated individuals rarely survive past the age of 20, while heterozygotes often develop severe atherosclerotic plaques before the third decade of life [75]. True FH is caused by mutations in the *LDLR* gene itself, although over 1000 causative mutations in the *LDLR* have been described [75]. Within the homozygote population the severity of the disease can therefore be further characterized based on the degree of loss of LDLR function in their skin fibroblasts: those with less than 2% normal LDLR activity are classed as LDLR negative and have the worst prognosis, while those with 2-25% activity are receptor-defective [73,76].

Among other forms of monogenic hypercholesterolemia, mutations in the LDL protein apoB-100 are the most clinically similar to bona-fide FH. In these cases, a mutation in the LDLR-binding domain of apoB-100 results in a failure to clear LDL from the plasma, thus resulting in a similar but slightly less severe hypercholesterolemia than FH patients [77]. Among Europeans prevalence is around 1:1000 and a molecular distinction is often not established between true FH and apoB-100 mutants [74]. Mutations in the proteinase PCSK9 can also cause a similar clinical phenotype, although the mechanisms are still not entirely understood [78].

In addition to mutations affecting LDL uptake, there are also some individuals with monogenic disorders resulting in extreme elevation of plasma triacylglycerol and triacylglycerol-rich lipoproteins (TGRL) such as chylomicrons and VLDL. These include LPL deficiency and lysosomal acid lipase (LAL) deficiency, both of which are rare with prevalence estimated at 1:1 million and 25: 1 million respectively [79,80]. Due to the rare nature of these disorders, very little is understood about the impact of such high TGRL levels on the function of the innate immune system.

1.2.2.2. Dyslipidaemia of obesity and high fat diet

Obesity is a worldwide epidemic that, in contrast to FH, leads to a dyslipidaemia driven predominantly by elevated TGRLs, free fatty acids (FFAs) and low levels of HDL, in both the fasting and post-prandial state [81]. Obesity is often accompanied by metabolic syndrome, characterized by insulin insensitivity and a chronic peripheral inflammation driven by macrophages and adipocytes [82]. The major contribution of obesity to dyslipidaemia is an inhibition of both LPL expression and activity in skeletal muscle[83] and adipose tissue [84] through poorly defined mechanisms. Ultimately, this leads to impaired FFA and TGRL clearance from the plasma accompanied by an increase in the density of plasma LDL [85]. While TGRL elevation is now implicated in atherosclerosis and will be discussed below, FFAs can be directly cytotoxic and induce a pronounced inflammatory response. In particular, there is strong evidence that saturated fatty acids (SFAs), such as the predominant dietary FA palmitic acid [86], can stimulate nuclear factor kappaB (NFkB) activation and subsequent inflammatory cytokine production, including TNF- α and IL-6 [87]. In some instances, this is due to direct binding of SFAs to TLR4 due to a structural similarity between SFAs and the lipid A moiety of bacterial lipopolysaccharide (LPS) [88,89]. However, despite this wealth of evidence of a tissue inflammatory response to obesity and metabolic syndrome, limited research has been performed on blood monocytes that are constantly bathed in a dynamic pool of TGRLs and FFAs.

1.3. The MPS and atherosclerosis

1.3.1. The 'response to retention' hypothesis of atherogenesis

It is not possible to discuss dyslipidaemia and the MPS without touching upon atherosclerosis and cardiovascular disease (CVD), the leading cause of death among developed nations [90]. Traditionally, atherosclerosis has been thought of as a chronic, inflammatory disorder characterised by the expansion of the arterial intima over a number of decades. Although scientific opinion in the 1980s, driven by Russell Ross and others, was that early atherogenesis was driven by injury to the vascular endothelium [91], the most widely accepted hypothesis is now the 'response to retention' hypothesis, elegantly outlined by Ira Tabas [92]. Since elevated plasma LDL has long been established as the one of the strongest risk factors for atherosclerosis [93], 'response to retention' postulates that the key event in atherogenesis is the deposition of apoB-containing lipoproteins (i.e. LDL and VLDL) in the arterial intima. It is important to note in this context that atherosclerotic lesions occur predominantly at vascular bifurcations and branch points, where alterations in blood rheology such as oscillatory shear stress can alter vascular function; increasing LDL uptake by endothelial cells and increasing vascular permeability [94,95]. In addition, oscillatory or low shear stress environments can activate the endothelium to increase expression of surface adhesion molecules including ICAM-1, VCAM-1 and E-Selectin [96,97].

Endothelial activation at these sites is perpetuated further by the subendothelial deposition of LDL, which can become oxidized once trapped in the vessel intima. Oxidized LDL can then stimulate up-regulation of surface P-Selectin, ICAM-1 and VCAM-1 on endothelial cells, both in the steady state and after TNF- α treatment [98,99], which are all ligands for integrins on circulating monocytes. VCAM-1 expression is strongly localized to atherosclerotic lesion sites and blockade of VCAM-1 using a monoclonal antibody significantly decreases atherosclerosis severity in mouse models [100,101]. Concurrently, activated endothelial and smooth

muscle cells can secrete chemokines that lead to the recruitment of blood monocytes. Mostly notably, endothelial cells produce CCL2 and IL-8 in response to modified LDL [102,103], while platelets can be induced to produce CCL5 that is deposited on the activated endothelium [104]. In vitro perfusion assays have subsequently demonstrated that CCL2 and IL-8 alone are sufficient to trigger firm adhesion of monocytes on vascular endothelium [105]. On classical GR1^{high} monocytes CCR1 and CCR5 are now thought to be the key chemokine receptors mediating recruitment to the plaque. *Ccr1*^{-/-} and *Ccr5*^{-/-} monocytes display significantly impaired infiltration of the plaque following adoptive transfer into atherosclerotic *Apoe*^{-/-} recipients [106]. It has previously been suggested that CCR2 also mediates monocyte recruitment, as *Ccr2*^{-/-} mice have decreased lesion formation [107]. However, adoptively transferred *Ccr2*^{-/-} monocytes do not have a recruitment defect and these data can be explained by a requirement of CCR2 for monocyte egress from the bone marrow [60,106,108]. For non- classical monocytes GR1^{low} monocytes there is less consensus in the literature. While there is some suggestion that these cells do make a minimal contribution to the plaque via CCR5 [109], the majority of evidence in mouse models is that GR1^{low} monocytes do not directly contribute to disease progression in atherosclerosis [106,110]. NR4A1 deletion at either a whole organism or bone marrow level, which prevents the development of blood GR1^{low} monocytes, either increases[111,112] or does not affect[113] atherosclerosis in mice. This would indicate that the recruitment of GR1^{low} monocytes does not play a major part in the progression of the plaque, with the worsening in atherosclerosis proposed to be via increased M1 inflammatory macrophage polarization [111].

Interestingly, there is also considerable evidence that activated platelets facilitate monocyte recruitment to the atherosclerotic plaque [104,114,115]. In the *Apoe*^{-/-} mouse, intravital microscopy has revealed that acute blockade of platelet membrane glycoproteins GPIb α and GPIIb-IIIa reduces platelet adhesion to the endothelium of the common carotid artery [114]. When performed chronically over 12 weeks, this blockade of platelet adhesion attenuates atherosclerosis severity and decreases

leukocyte recruitment to the vascular wall [114], thus implicating platelets in leukocyte recruitment during dyslipidaemia. While mechanistic data on this phenomenon is incomplete, the chemokines CXCL4 and CCL5 derived from activated platelets have been implicated in the process by increasing leukocyte binding to endothelial VCAM-1 [115]. In addition to effects on leukocytes, platelet-derived CCL5 can become immobilized on the inflamed vascular endothelium via an undefined binding site to trigger monocyte arrest [104]. In an in vitro co-culture system, Rainger and colleagues have demonstrated that platelets can form adhesive bridges between monocytes and TGF- β activated endothelial cells, with monocytes bound to platelet P-selectin becoming activated by endothelial CCL2 [116]. As such, platelets may be an under appreciated therapeutic target during early atherogenesis.

1.3.2. Macrophage proliferation in the plaque

In Tabas' original 'response to retention' hypothesis, the recruitment of monocytes by the activated vascular endothelium as outlined above was presumed to be necessary and sufficient to drive the development of early atherosclerosis. Until very recently, strong evidence supported this hypothesis almost to the point of certainty. Hypercholesterolemia causes a pronounced monocytosis of GR1^{high} monocytes via APOE feedback to progenitors in the bone marrow [110,117]. In turn, fate mapping experiments can track these blood monocytes into the vessel wall during atherosclerosis and this recruitment seems to be proportional to the extent of disease even in the mature atheroma [110,118]. By inhibiting CCL2, CX3CR1 and CCR5 this monocytosis can be abrogated and development of atherosclerosis reduced by around 90% [119]. However, the discovery that the majority of tissue macrophage populations are self-maintained by local proliferation has led to new perspectives on the MPS in atherosclerosis. In work that has contradicted decades of research, Robbins et al used the *Apoe*^{-/-} mouse to demonstrate that >90% of macrophages in the atherosclerotic plaque proliferate in situ after 8 weeks of HFD [120]. More strikingly, chimerism experiments using parabiosis demonstrated that while at 4 weeks HFD >70% of aortic macrophages are derived from monocyte recruitment, by 8 weeks this number has dropped to less than 5%, with >95% of

macrophages derived from local proliferation [120]. As such, these data suggest any chemokine-axis inhibition aimed at ameliorating atherosclerosis progression will only be effective if administered at the very earliest stages of atherogenesis. There is some contrary evidence that inhibition of monocyte chemokine receptors during established atherosclerosis and atherosclerotic plaque regression also decreases plaque macrophage content [121]. However these experiments were fairly crude and utilised pertussis toxin to inhibit all G protein-coupled receptors, thus the results must be interpreted with caution. At present, the essential role of macrophage proliferation in the plaque proposed by Robbins et al is supported by convincing data but awaits confirmation from others in the field.

1.4. Aims

While atherosclerosis is the predominant focus in dyslipidaemia research, the impact of dyslipidaemia on other aspects of the innate immunity remains enigmatic. Given the ever-increasing appreciation of monocyte heterogeneity and macrophage independence to blood monocytes, the fundamental aim of this thesis was to characterize the functional response of blood monocyte subpopulations to dyslipidaemia, defined as elevated proportions of plasma VLDL and LDL. This aim centres around three key hypotheses to be investigated:

1. Blood monocytes interact with plasma lipoproteins in circulation and at the endothelial interface.
2. Dyslipidaemia results in an accumulation of intracellular lipid in blood monocyte subpopulations.
3. Monocyte intracellular lipid accumulation affects their function as immune effector cells in a subset- specific manner.

To investigate these hypotheses I planned to utilise a mix of in vitro and in vivo analysis, examining mouse monocytes in the hypercholesterolemic *Ldlr*^{-/-} model and human monocytes after incubation with atherogenic lipoproteins.

1.5. References

- 1 Schmalstieg FC, Goldman AS. Ilya Ilich Metchnikoff (1845–1915) and Paul Ehrlich (1854–1915): the centennial of the 1908 nobel prize in physiology or medicine. *Journal of medical biography* 2008.
- 2 Gordon S. Elie Metchnikoff: father of natural immunity. 2008. doi:10.1002/eji.200838855
- 3 Metchnikoff E, Binnie Fg, L Wt. Immunity In Infective Diseases. *The American Journal of the Medical Sciences* 1906;**132**:924.
- 4 Piro A, Tagarelli A, Tagarelli G, *et al.* *Paul Ehrlich: the Nobel Prize in physiology or medicine* 1908. 2008. doi:10.1080/08830180701848995
- 5 Aschoff L. Das reticulo-endotheliale System. *Ergebnisse der inneren Medizin und Kinderheilkunde* 1924.
- 6 Cohn ZA, BENSON B. The Differentiation Of Mononuclear Phagocytes. Morphology, Cytochemistry, And Biochemistry. *J Exp Med* 1965;**121**:153–70.
- 7 Cohn ZA, Fedorko ME, Hirsch JG. The in vitro differentiation of mononuclear phagocytes. V. The formation of macrophage lysosomes. *J Exp Med* 1966;**123**:757–66.
- 8 Cohn ZA. The regulation of pinocytosis in mouse macrophages. I. Metabolic requirements as defined by the use of inhibitors. *J Exp Med* 1966;**124**:557–71.
- 9 Cohn ZA, Parks E. The regulation of pinocytosis in mouse macrophages. II. Factors inducing vesicle formation. *J Exp Med* 1967;**125**:213–32.
- 10 Cohn ZA, Parks E. The regulation of pinocytosis in mouse macrophages. 3. The induction of vesicle formation by nucleosides and nucleotides. *J Exp Med* 1967;**125**:457–66.
- 11 Bennett WE, Cohn ZA. The isolation and selected properties of blood monocytes. *J Exp Med* 1966;**123**:145–60.
- 12 van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. *J Exp Med* 1968;**128**:415–35.
- 13 Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 1973;**137**:1142–62.
- 14 Steinman RM, Kaplan G, Witmer MD, *et al.* Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. *J Exp Med* 1979;**149**:1–16.
- 15 Muller WA, Ratti CM, McDonnell SL, *et al.* A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. *J Exp Med* 1989;**170**:399–414.
- 16 van Furth R, Cohn ZA, Hirsch JG. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bulletin of the World ...* 1972.
- 17 Hume DA. Macrophages as APC and the dendritic cell myth. *The Journal of Immunology* 2008;**181**:5829–35.
- 18 Fogg DK, Sibon C, Miled C, *et al.* A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 2006;**311**:83–7. doi:10.1126/science.1117729
- 19 Akashi K, Traver D, Miyamoto T, *et al.* A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 2000;**404**:193–7. doi:10.1038/35004599
- 20 Hettinger J, Richards DM, Hansson J, *et al.* Origin of monocytes and macrophages in a committed progenitor. *Nat Immunol* 2013;**14**:821–30. doi:10.1038/ni.2638

- 21 Sathe P, Metcalf D, Vremec D, *et al.* Lymphoid tissue and plasmacytoid dendritic cells and macrophages do not share a common macrophage-dendritic cell-restricted progenitor. *Immunity* 2014;**41**:104–15. doi:10.1016/j.immuni.2014.05.020
- 22 Wiktor-Jedrzejczak W, Gordon S. Cytokine regulation of the macrophage (M phi) system studied using the colony stimulating factor-1-deficient op/op mouse. *Physiological Reviews* 1996;**76**:927–47.
- 23 Anderson KL, Smith KA, Connors K, *et al.* Myeloid development is selectively disrupted in PU.1 null mice. *Blood* 1998;**91**:3702–10.
- 24 Naito M, Yamamura F, Nishikawa S, *et al.* Development, differentiation, and maturation of fetal mouse yolk sac macrophages in cultures. *Journal of Leukocyte Biology* 1989;**46**:1–10.
- 25 Takahashi K, Yamamura F, Naito M. Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: a light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. *Journal of Leukocyte Biology* 1989;**45**:87–96.
- 26 McGrath KE, Koniski AD, Malik J, *et al.* Circulation is established in a stepwise pattern in the mammalian embryo. *Blood* 2003;**101**:1669–76. doi:10.1182/blood-2002-08-2531
- 27 Ginhoux F, Greter M, Leboeuf M, *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 2010;**330**:841–5. doi:10.1126/science.1194637
- 28 Kumaravelu P, Hook L, Morrison AM, *et al.* Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development* 2002;**129**:4891–9.
- 29 Hoeffel G, Chen J, Lavin Y, *et al.* C-myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity* 2015;**42**:665–78. doi:10.1016/j.immuni.2015.03.011
- 30 Schulz C, Gomez Perdiguero E, Chorro L, *et al.* A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 2012;**336**:86–90. doi:10.1126/science.1219179
- 31 Hashimoto D, Chow A, Noizat C, *et al.* Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* 2013;**38**:792–804. doi:10.1016/j.immuni.2013.04.004
- 32 Varol C, Mildner A, Jung S. Macrophages: development and tissue specialization. *Annu Rev Immunol* 2015;**33**:643–75. doi:10.1146/annurev-immunol-032414-112220
- 33 Pavli P, Woodhams CE, Doe WF, *et al.* Isolation and characterization of antigen-presenting dendritic cells from the mouse intestinal lamina propria. *Immunology* 1990;**70**:40–7.
- 34 Rogler G, Hausmann M, Vogl D, *et al.* Isolation and phenotypic characterization of colonic macrophages. *Clinical & Experimental Immunology* 1998;**112**:205–15.
- 35 Tamoutounour S, Henri S, Lelouard H, *et al.* CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur J Immunol* 2012;**42**:3150–66. doi:10.1002/eji.201242847
- 36 Bain CC, Scott CL, Uronen-Hansson H, *et al.* Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol* 2013;**6**:498–510. doi:10.1038/mi.2012.89
- 37 Smythies LE, Maheshwari A, Clements R, *et al.* Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and

- myeloid cells. *Journal of Leukocyte Biology* 2006;**80**:492–9. doi:10.1189/jlb.1005566
- 38 Takada Y, Hisamatsu T, Kamada N, *et al.* Monocyte chemoattractant protein-1 contributes to gut homeostasis and intestinal inflammation by composition of IL-10-producing regulatory macrophage subset. *The Journal of Immunology* 2010;**184**:2671–6. doi:10.4049/jimmunol.0804012
- 39 The M1 and M2 paradigm of macrophage activation: time for reassessment. 2014;**6**:13. doi:10.12703/P6-13
- 40 Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. 2014;**40**:274–88. doi:10.1016/j.immuni.2014.01.006
- 41 Gautier EL, Shay T, Miller J, *et al.* Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol* 2012;**13**:1118–28. doi:10.1038/ni.2419
- 42 Pulmonary macrophage transplantation therapy. 2014;**514**:450–4. doi:10.1038/nature13807
- 43 Okabe Y, Medzhitov R. Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell* 2014;**157**:832–44. doi:10.1016/j.cell.2014.04.016
- 44 Rosas M, Davies LC, Giles PJ, *et al.* The transcription factor Gata6 links tissue macrophage phenotype and proliferative renewal. *Science* 2014;**344**:645–8. doi:10.1126/science.1251414
- 45 Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 2003;**19**:71–82.
- 46 Cros J, Cagnard N, Woollard K, *et al.* Human CD14dim Monocytes Patrol and Sense Nucleic Acids and Viruses via TLR7 and TLR8 Receptors. *Immunity* 2010;**1**:1–12. doi:10.1016/j.immuni.2010.08.012
- 47 Ahuja V, Miller SE, Howell DN. Identification of two subpopulations of rat monocytes expressing disparate molecular forms and quantities of CD43. *Cell Immunol* 1995;**163**:59–69. doi:10.1006/cimm.1995.1099
- 48 Chamorro S, Revilla C, Alvarez B, *et al.* Phenotypic and functional heterogeneity of porcine blood monocytes and its relation with maturation. *Immunology* 2005;**114**:63–71. doi:10.1111/j.1365-2567.2004.01994.x
- 49 Hussen J, Düvel A, Sandra O, *et al.* Phenotypic and functional heterogeneity of bovine blood monocytes. *PLoS ONE* 2013;**8**:e71502. doi:10.1371/journal.pone.0071502
- 50 Ingersoll MA, Spanbroek R, Lottaz C, *et al.* Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood* 2010;**115**:e10–9. doi:10.1182/blood-2009-07-235028
- 51 Wong KL, Tai JJY, Wong WC, *et al.* Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood* 2011;**118**:e16–e31. doi:10.1182/blood-2010-12-326355
- 52 Yona S, Kim KW, Wolf Y, *et al.* Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 2012.
- 53 Hanna RN, Carlin LM, Hubbeling HG, *et al.* The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C[–] monocytes. *Nat Immunol* 2011;**12**:778–85. doi:10.1038/ni.2063
- 54 Debieen E, Mayol K, Biajoux V, *et al.* S1PR5 is pivotal for the homeostasis of patrolling monocytes. *Eur J Immunol* Published Online First: 20 March 2013. doi:10.1002/eji.201343312
- 55 Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994;**76**:301–14.

- 56 Auffray C, Fogg D, Garfa M, *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 2007;**317**:666–70. doi:10.1126/science.1142883
- 57 Carlin LM, Stamatiades EG, Auffray C, *et al.* Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell* 2013;**153**:362–75. doi:10.1016/j.cell.2013.03.010
- 58 Wong KL, Chen W, Balakrishnan T, *et al.* Susceptibility and response of human blood monocyte subsets to primary dengue virus infection. *PLoS ONE* 2012;**7**:e36435. doi:10.1371/journal.pone.0036435
- 59 Zhang J-Y, Zou Z-S, Huang A, *et al.* Hyper-activated pro-inflammatory CD16 monocytes correlate with the severity of liver injury and fibrosis in patients with chronic hepatitis B. *PLoS ONE* 2011;**6**:e17484. doi:10.1371/journal.pone.0017484
- 60 Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol* 2006;**7**:311–7. doi:10.1038/ni1309
- 61 Peters W, Scott HM, Chambers HF, *et al.* Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis. *Proc Natl Acad Sci USA* 2001;**98**:7958–63. doi:10.1073/pnas.131207398
- 62 Jakubzick C, Gautier EL, Gibbings SL, *et al.* Minimal Differentiation of Classical Monocytes as They Survey Steady-State Tissues and Transport Antigen to Lymph Nodes. *Immunity* 2013;**39**:599–610. doi:10.1016/j.immuni.2013.08.007
- 63 Targeting monocyte and macrophage subpopulations for immunotherapy: a patent review (2009 - 2013). 2014;**24**:779–90. doi:10.1517/13543776.2014.914495
- 64 Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics* 2002;**1**:845–67.
- 65 Brown MS, Goldstein JL. A Receptor-Mediated Pathway for Cholesterol Homeostasis. *Science* 1986;**232**:1–14.
- 66 Lindgren FT, Elliot HA, Gofman JW. The Ultracentrifugal Characterization And Isolation Of Human Blood Lipids And Lipoproteins, With Applications To The Study Of Atherosclerosis. *Journal of Physical Chemistry* 1951;**55**:80–93.
- 67 Kwiterovich PO. The metabolic pathways of high-density lipoprotein, low-density lipoprotein, and triglycerides: a current review. *Am J Cardiol* 2000;**86**:5L–10L.
- 68 Goldberg IJ, Eckel RH, Abumrad NA. Regulation of fatty acid uptake into tissues: lipoprotein lipase- and CD36-mediated pathways. *Journal of Lipid Research* 2009;**50 Suppl**:S86–90. doi:10.1194/jlr.R800085-JLR200
- 69 Oram JF. ABCA1 Is the cAMP-inducible Apolipoprotein Receptor That Mediates Cholesterol Secretion from Macrophages. *Journal of Biological Chemistry* 2000;**275**:34508–11. doi:10.1074/jbc.M006738200
- 70 la Llera-Moya de M, Rothblat GH, Connelly MA, *et al.* Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface. *Journal of Lipid Research* 1999;**40**:575–80.
- 71 Martel C, Li W, Fulp B, *et al.* Lymphatic vasculature mediates macrophage reverse cholesterol transport in mice. *J Clin Invest* Published Online First: 25 March 2013. doi:10.1172/JCI63685
- 72 Trigatti B, Rigotti A, Krieger M. The role of the high-density lipoprotein receptor SR-BI in cholesterol metabolism. *Curr Opin Lipidol* 2000;**11**:123–31.
- 73 Goldstein JL, Brown MS. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *Journal of Biological Chemistry* 1974;**249**:5153–62.

- 74 Austin MA, Hutter CM, Zimmern RL, *et al.* Genetic causes of monogenic heterozygous familial hypercholesterolemia: a HuGE prevalence review. *Am J Epidemiol* 2004;**160**:407–20. doi:10.1093/aje/kwh236
- 75 Hill JS, Hayden MR, Frohlich J, *et al.* Genetic and environmental factors affecting the incidence of coronary artery disease in heterozygous familial hypercholesterolemia. *Arterioscler Thromb* 1991;**11**:290–7.
- 76 Goldstein JL, Harrod MJ, Brown MS. Homozygous familial hypercholesterolemia: specificity of the biochemical defect in cultured cells and feasibility of prenatal detection. *Am J Hum Genet* 1974;**26**:199–206.
- 77 Soria LF, Ludwig EH, Clarke HR, *et al.* Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. *Proc Natl Acad Sci USA* 1989;**86**:587–91.
- 78 Abifadel M, Varret M, Rabès J-P, *et al.* Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet* 2003;**34**:154–6. doi:10.1038/ng1161
- 79 Santamarina-Fojo S. The familial chylomicronemia syndrome. *Endocrinol Metab Clin North Am* 1998;**27**:551–67–viii.
- 80 Meikle PJ, Hopwood JJ, Clague AE, *et al.* Prevalence of Lysosomal Storage Disorders. *JAMA* 1999;**281**:249–54. doi:10.1001/jama.281.3.249
- 81 Dyslipidemia in obesity: mechanisms and potential targets. 2013;**5**:1218–40. doi:10.3390/nu5041218
- 82 Gustafson B, Hammarstedt A, Andersson CX, *et al.* Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis. 2007;**27**:2276–83. doi:10.1161/ATVBAHA.107.147835
- 83 Roberts CK, Barnard RJ, Liang KH, *et al.* Effect of diet on adipose tissue and skeletal muscle VLDL receptor and LPL: implications for obesity and hyperlipidemia. *Atherosclerosis* 2002;**161**:133–41.
- 84 Clemente-Postigo M, Queipo-Ortuño MI, Fernandez-Garcia D, *et al.* Adipose tissue gene expression of factors related to lipid processing in obesity. *PLoS ONE* 2011;**6**:e24783. doi:10.1371/journal.pone.0024783
- 85 Packard CJ. Triacylglycerol-rich lipoproteins and the generation of small, dense low-density lipoprotein. *Biochem Soc Trans* 2003;**31**:1066–9.
- 86 Kris-Etherton PM, Griel AE, Psota TL, *et al.* Dietary stearic acid and risk of cardiovascular disease: intake, sources, digestion, and absorption. *Lipids* 2005;**40**:1193–200.
- 87 Ajuwon KM, Spurlock ME. Palmitate activates the NF-kappaB transcription factor and induces IL-6 and TNFalpha expression in 3T3-L1 adipocytes. *J Nutr* 2005;**135**:1841–6.
- 88 Lee JY, Sohn KH, Rhee SH, *et al.* Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *Journal of Biological Chemistry* 2001;**276**:16683–9. doi:10.1074/jbc.M011695200
- 89 Suganami T, Tanimoto-Koyama K, Nishida J, *et al.* Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. 2007;**27**:84–91. doi:10.1161/01.ATV.0000251608.09329.9a
- 90 British Heart Foundation. Coronary Heart Disease Statistics. 2012;;1–211.
- 91 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993;**362**:801–9. doi:10.1038/362801a0
- 92 Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. 1995;**15**:551–61.

- 93 Castelli WP, Doyle JT, Gordon T, *et al.* HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation* 1977;**55**:767–72. doi:10.1161/01.CIR.55.5.767
- 94 Niwa K, Kado T, Sakai J, *et al.* The effects of a shear flow on the uptake of LDL and acetylated LDL by an EC monoculture and an EC-SMC coculture. *Ann Biomed Eng* 2004;**32**:537–43.
- 95 Himburg HA, Grzybowski DM, Hazel AL, *et al.* Spatial comparison between wall shear stress measures and porcine arterial endothelial permeability. *Am J Physiol Heart Circ Physiol* 2004;**286**:H1916–22. doi:10.1152/ajpheart.00897.2003
- 96 Chiu J-J, Lee P-L, Chen C-N, *et al.* Shear stress increases ICAM-1 and decreases VCAM-1 and E-selectin expressions induced by tumor necrosis factor-[alpha] in endothelial cells. 2004;**24**:73–9. doi:10.1161/01.ATV.0000106321.63667.24
- 97 Walpole PL, Gottlieb AI, Cybulsky MI, *et al.* Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress. 1995;**15**:2–10.
- 98 Gebuhrer V, Murphy JF, Bordet JC, *et al.* Oxidized low-density lipoprotein induces the expression of P-selectin (GMP140/PADGEM/CD62) on human endothelial cells. *Biochem J* 1995;**306** (Pt 1):293–8.
- 99 Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. 1995;**95**:1262–70. doi:10.1172/JCI117776
- 100 Nakashima Y, Raines EW, Plump AS, *et al.* Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. 1998;**18**:842–51.
- 101 Cybulsky MI, Iiyama K, Li H, *et al.* A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest* 2001;**107**:1255–62. doi:10.1172/JCI11871
- 102 Claise C, Edeas M, Chalas J, *et al.* Oxidized low-density lipoprotein induces the production of interleukin-8 by endothelial cells. *FEBS Lett* 1996;**398**:223–7.
- 103 Cushing SD, Berliner JA, Valente AJ, *et al.* Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci USA* 1990;**87**:5134–8.
- 104 Hundelshausen von P, Weber KS, Huo Y, *et al.* RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. *Circulation* 2001;**103**:1772–7.
- 105 Gerszten RE, Garcia-Zepeda EA, Lim YC, *et al.* MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* 1999;**398**:718–23. doi:10.1038/19546
- 106 Soehnlein O, Drechsler M, Döring Y, *et al.* Distinct functions of chemokine receptor axes in the atherogenic mobilization and recruitment of classical monocytes. *EMBO Mol Med* 2013;**5**:471–81. doi:10.1002/emmm.201201717
- 107 Decreased lesion formation in CCR2^{-/-} mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* 1998;**394**:894–7. doi:10.1038/29788
- 108 Tsou C-L, Peters W, Si Y, *et al.* Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest* 2007;**117**:902–9. doi:10.1172/JCI29919
- 109 Tacke F, Alvarez D, Kaplan TJ, *et al.* Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J Clin Invest* 2007;**117**:185–94. doi:10.1172/JCI28549
- 110 Swirski FK, Libby P, Aikawa E, *et al.* Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytoysis and give rise to macrophages in atheromata. *J Clin Invest* 2007;**117**:195–205. doi:10.1172/JCI29950

- 111 Hanna RN, Shaked I, Hubbeling HG, *et al.* NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis. *Circulation Research* 2012;**110**:416–27. doi:10.1161/CIRCRESAHA.111.253377
- 112 Hamers AAJ, Vos M, Rassam F, *et al.* Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis. *Circulation Research* 2012;**110**:428–38. doi:10.1161/CIRCRESAHA.111.260760
- 113 Chao LC, Soto E, Hong C, *et al.* Bone marrow NR4A expression is not a dominant factor in the development of atherosclerosis or macrophage polarization in mice. *Journal of lipid ...* 2013.
- 114 Massberg S, Brand K, Grüner S, *et al.* A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *J Exp Med* 2002;**196**:887–96. doi:10.1084/jem.20012044
- 115 Huo Y, Schober A, Forlow SB, *et al.* Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* 2003;**9**:61–7. doi:10.1038/nm810
- 116 Kuckleburg CJ, Yates CM, Kalia N, *et al.* Endothelial cell-borne platelet bridges selectively recruit monocytes in human and mouse models of vascular inflammation. *Cardiovasc Res* 2011;**91**:134–41. doi:10.1093/cvr/cvr040
- 117 ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. 2011;**121**:4138–49. doi:10.1172/JCI57559
- 118 Swirski FK, Pittet MJ, Kircher MF, *et al.* Monocyte accumulation in mouse atherogenesis is progressive and proportional to extent of disease. *Proc Natl Acad Sci USA* 2006;**103**:10340–5. doi:10.1073/pnas.0604260103
- 119 Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C(hi) and Ly6C(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. 2008;**117**:1649–57. doi:10.1161/CIRCULATIONAHA.107.745091
- 120 Robbins CS, Chudnovskiy A, Rauch PJ, *et al.* Extramedullary Hematopoiesis Generates Ly-6Chigh Monocytes That Infiltrate Atherosclerotic Lesions. *Circulation* 2012;**125**:364–74. doi:10.1161/CIRCULATIONAHA.111.061986
- 121 Potteaux S, Gautier EL, Hutchison SB, *et al.* Suppressed monocyte recruitment drives macrophage removal from atherosclerotic plaques of Apoe–/– mice during disease regression. *J Clin Invest* 2011;**121**:2025–36. doi:10.1172/JCI43802DS1

2. METHODS

2.1. Mice

Adult male and female mice on a C57Bl/6 background were used for all animal experiments. C57Bl/6 mice were purchased from Charles River. C57Bl/6.LDLR-deficient mice (*Ldlr*^{-/-}) and B6.129P-Cx3cr1^{tm1Litt}/J (*Cx3cr1*^{gfp/gfp}) were purchased from Jackson Laboratory (Bar Harbor, USA). *Ldlr*^{-/-} mice were maintained as homozygotes for breeding and experiments. *Cx3cr1*^{gfp/gfp} were kept as homozygotes for breeding and male *Cx3cr1*^{gfp/gfp} animals were crossed with female C57Bl/6 mice to generate *Cx3cr1*^{gfp/+} heterozygotes for experiments. All animals were housed in individually ventilated cages and handled in accordance with institutional guidelines and procedures approved by the UK Home Office. Mice were maintained on a high fat (HFD) or standard chow diet for 6-16 weeks, as specified in individual experiments. High fat diet contained 15% cocoa butter and 1% corn oil, resulting in a total fat and cholesterol content of 16% and 0.25% respectively (Arie Blok Animal Nutrition).

2.2. Monocyte purification and flow cytometry

Human peripheral blood from healthy volunteers was collected by venepuncture into ethylenediaminetetraacetic acid (EDTA) coated vacutainers after informed consent with ethical approval: REC reference 14/LO0662 (NRES Committee London). Human peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Lymphoprep (Stem Cell) according to manufacturer instructions. Total monocytes were obtained by magnetic depletion of lineage positive leukocytes with the Monocyte Isolation Kit II. The manufacturer recommends centrifugation at 200xg in order to remove platelets at this stage. However, in our hands this still resulted in considerable platelet contamination, therefore 20µl of an anti-CD61-Biotin antibody (Miltenyi) was added to the depletion antibody cocktail to eliminate platelet contamination (Figure 2.1). Monocytes were separated into CD16^{pos} and CD16^{neg}

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

populations by magnetic positive selection using anti-CD16 MicroBeads (Miltenyi). Population purity was confirmed by flow cytometry, as shown in Figure 2.2. average purity for total monocytes was >90%. N.B: Figure 2.2C does not represent true levels of monocyte CD16 expression, as positive selection using anti-CD16 MicroBeads partially blocks subsequent anti-CD16 antibody binding. See Figure 1.2 for realistic CD16 levels.

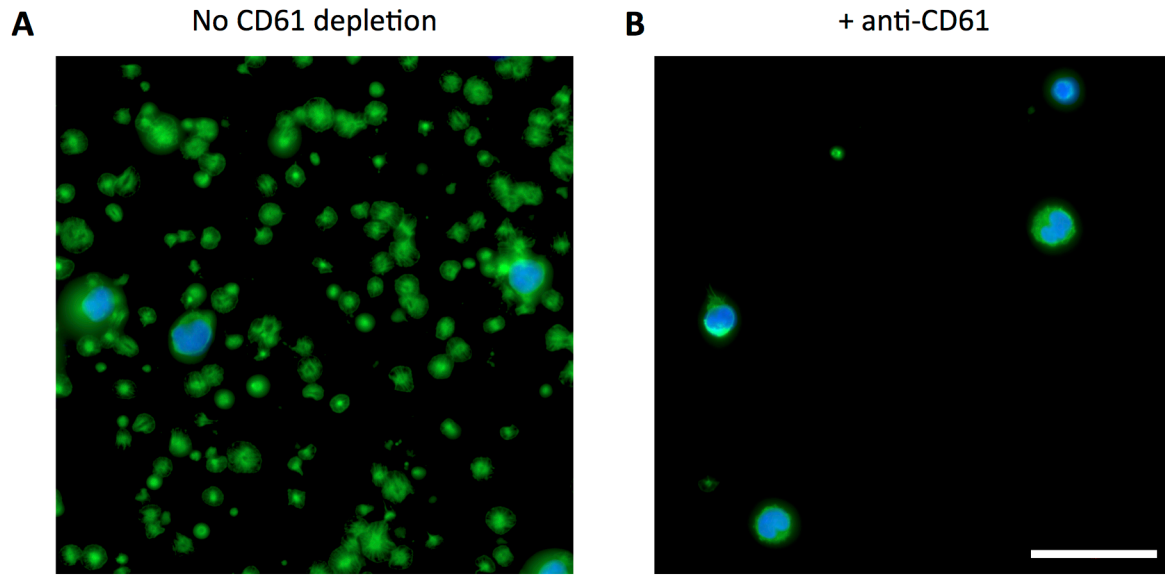


Figure 2.1 Elimination of platelet contamination from monocytes using anti-CD61.

Human monocytes isolated from PBMCs using (A) Monocyte Isolation Kit II as per manufacturer instructions or (B) Monocyte Isolation Kit II with an anti-CD61-Biotin antibody added to the negative depletion cocktail. Cells were allowed to adhere to tissue-culture plastic and stained using fluorescent phalloidin (green) and DAPI (blue) and imaged on a Zeiss AxioObserver fluorescence widefield microscope. Scale bar represents 50µm.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

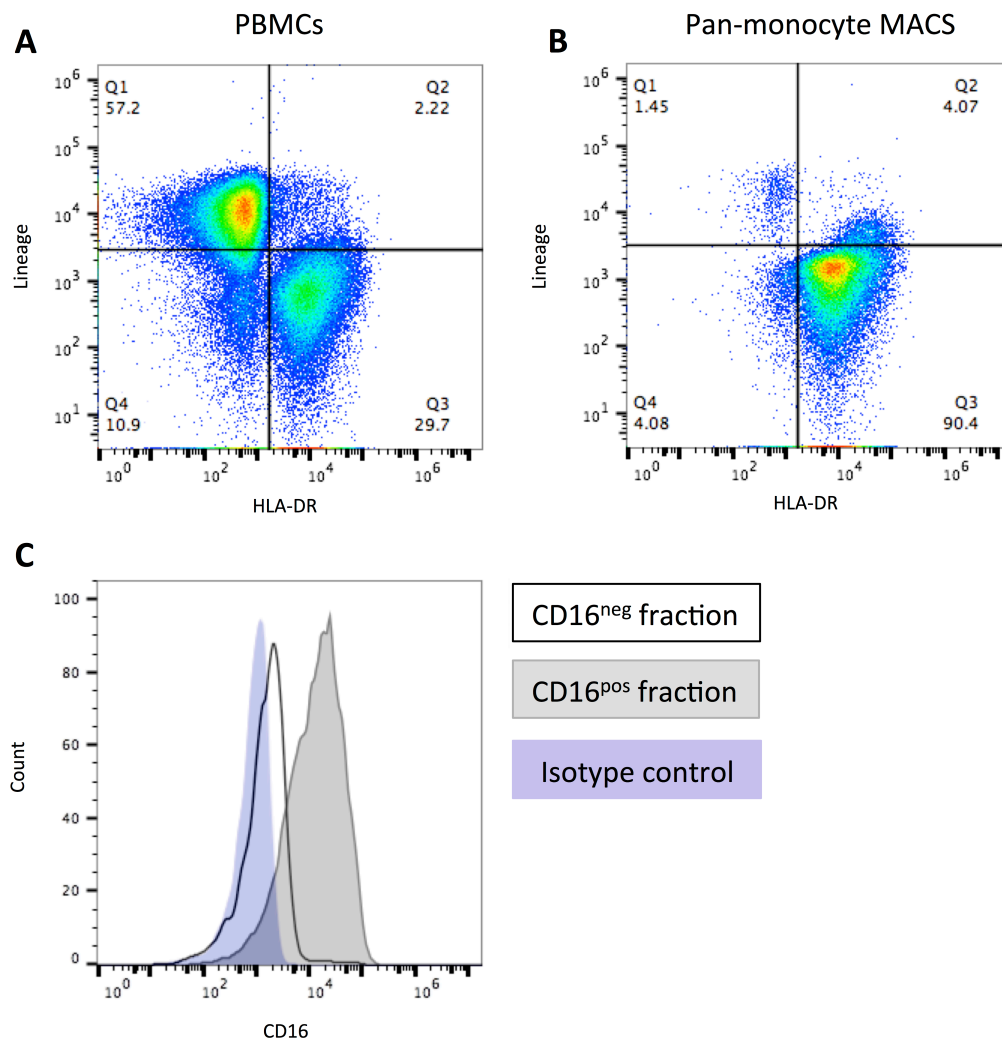


Figure 2.2 Confirmation of monocyte purity after MACS isolation.

Human monocytes were isolated from PBMCs using magnetic depletion of lymphocyte populations with the Monocyte Isolation Kit II (Miltenyi). A flow cytometry scatter plot of lineage (CD2, CD56, NKp46, CD19) versus HLA-DR expression is shown for (A) PBMCs prior to magnetic isolation and (B) monocytes after magnetic isolation. Total monocytes were separated into CD16^{pos} and CD16^{neg} populations by magnetic positive selection using anti-CD16 MicroBeads (Miltenyi). (C) CD16 surface expression in the CD16^{neg} and CD16^{pos} fractions compared to isotype control antibody.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

In some experiments, whole leukocytes were isolated by red blood cell lysis from human peripheral blood using a hypotonic lysis buffer (dH₂O, NH₄Cl, NaHCO₃, EDTA) and monocyte subpopulations were analyzed by flow cytometry. Monocytes were defined as SSC^{int} HLA-DR^{pos} CD2^{neg} CD56^{neg} NKp46^{neg} and subpopulations were gated based on expression of CD14 and CD16 (Figure 1.2). Levels of surface protein expression were assessed by flow cytometry median fluorescence intensity.

Mouse monocytes were phenotyped from peripheral blood samples obtained by tail vein venipuncture or purified from samples obtained by cardiac puncture under terminal anesthesia. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Lymphoprep (Stem Cell) as above and monocyte subpopulations were obtained by FACS according to the gating strategy in Figure 1.2, Surface protein expression was assessed by flow cytometry. All antibodies used in FACS are listed in Table 2-1.

Table 2-1 Antibodies used for flow cytometry, immunofluorescence and Western blotting.

Antigen	Target Species	Clone	Manufacturer
CD14	Human	M5E2	BD Biosciences
CD16	Human	3G8	BD Biosciences
HLA-DR	Human	TU36	BD Biosciences
CD11c	Human	3.9	Biolegend
CD61	Human	Y2/51	Miltenyi
CD88	Human	S5/1	Biolegend
CD2	Human	RPA-2.10	BD Biosciences
CD56	Human	MY31	BD Biosciences
NKp46	Human	9E2	BD Biosciences
LPL	Human	5D2	AbD Serotech
pPAK1/2	Human	Polyclonal	Novus Biologicals
P38	Human	C-20	Santa Cruz Biotech
pMLC (Ser19)	Human	N/A	Cell Signaling Technology
CD115	Mouse	AF598	eBioscience
CD11b	Mouse	M1/70	BD Bioscience
F4/80	Mouse	BM8	BD Bioscience
CD45	Mouse	30-F11	eBioscience
GR1	Mouse	RB6-8C5	BD Bioscience

2.3. Monocyte LDL/ VLDL uptake and lipid quantification

2.3.1. DIO- LDL and DIO- VLDL uptake

Human peripheral blood leukocytes were incubated with 100µg/ml Low-density lipoprotein (LDL) labelled with 3,3'-Diocetadecyloxacarbocyanine Perchlorate (DIO) or Very low-density lipoprotein (VLDL)-DIO (Biomedical Technologies, Inc.) for indicated time points. Cells were washed with PBS and stained for flow cytometry with anti- HLA-DR, CD14, CD16, CD2, CD56 and NKp46. Monocytes were gated as described (Figure 1.2) and percentage of cells LDL/ VLDL positive was determined based on fluorescence-minus-one (FMO) control samples containing no DIO. Data was acquired on a BD LSR-II analyser. In some experiments, intracellular localization of LDL-DIO or VLDL-DIO was confirmed by acquiring cells on an Amnis ImageStream X Mark II using 40x magnification. Fluorescence excitation was achieved with a 125 mW 405nm laser, a variable power solid state 488 nm laser and a 120 mW 658 nm laser and images were collected on a six-channel charge-coupled device (CCD) camera. Data was analysed in Ideas software (Amnis).

2.3.2. Neutral lipid staining

CD16^{pos}/ CD16^{neg} human monocytes were treated with 100µg/ml LDL or VLDL for the indicated time points at 37°C. Cells were seeded (1.5×10^6 /ml) into tissue-culture treated 0.4µm chamber slides (IBIDI) in Dulbecco's Modified Eagle's Medium (DMEM) and allowed to adhere for 30 minutes. Adherent cells were immediately fixed in 4% paraformaldehyde (PFA) for 15 minutes and washed with 0.1M glycine. For neutral lipid staining, LipidTox-Green (Life Technologies) was diluted 1/200 in phosphate buffered saline (PBS). Cells were mounted in 4',6-diamidino-2-phenylindole (DAPI)-Vectashield (Vector Labs) and imaged using either a Zeiss AxioObserver widefield or a Leica SP5 confocal microscope with a 63x/1.4 objective. Images were analysed using Imaris software (Bitplane) or ImageJ, as indicated.

2.3.3. Membrane cholesterol staining

Cells were processed as for neutral lipid staining above, but stained for unesterified cholesterol with 0.05 mg/ml filipin (Sigma) in PBS for 2 hours and imaged on a Leica SP5 confocal microscope with a 63x/1.4 objective. Images were analysed using Imaris software (Bitplane).

2.4. Peritonitis model

Ldlr^{-/-} mice were maintained on HFD or chow for 16 weeks. To induce peritonitis, mice were injected intraperitoneally (IP) with either 1ml sterile 4% thioglycollate medium or 10µg lipopolysaccharide (LPS) in 100µl sterile saline, serotype 055:B5 (Sigma Aldrich). After 72 hours, mice were culled by exposure to rising concentration of CO₂ and the peritoneal cavity was lavaged with 10ml ice-cold PBS. Cells were counted on a hemocytometer and washed in PBS. Approximately 3 x 10⁵ cells were stored in Tri-Reagent (Sigma Aldrich) for RNA extraction, the remainder were stained in PBS-0.5% bovine serum albumin (BSA) for flow cytometric analysis in a saturating concentration of anti-CD16/32 (2.4G2) using combinations of the following antibodies: anti-CD115 (AF598), anti-CD45 (30-F11) (eBioscience), anti-CD11b (M1/70), anti-GR1 (RB6-8C5) anti-F4/80 (BM8) (BD Biosciences). Monocytes were defined as CD115^{pos} CD11b^{pos} SSC^{int} (Figure 3.4).

In some experiments, omental tissue was harvested by blunt dissection into Hank's Buffered Salt Solution (HBSS) and the weight recorded. Tissue was washed and digested using Liver Digestion Media (Gibco) for 15 minutes at 37°C. After washing to obtain a single-cell suspension, cells were counted and stained for flow cytometry as detailed above.

To track monocyte migration from the blood into the peritoneum, mice were injected IV with 100µl 1µm red-fluorescent latex beads at a concentration of 1 x 10¹⁰/ml (Invitrogen). The beads are manufactured from ultraclean, high-quality polystyrene

and were stored without sodium azide preservative to avoid adverse effects in vivo. Beads were injected 4 hours prior to IP thioglycollate injection and the peritoneal lavage was collected at 72 hours for flow cytometry as detailed above.

2.5. Intravital imaging

Cx3cr1^{gfp/+} mice were fed chow or HFD for 6 weeks and were 10-12 weeks of age when used for imaging. Blood lipid profiles were generated by automated analysis using an Abbott Architect ci8200 (Abbott Diagnostics). Intravital imaging of the ear dermis was performed as previously described [1]. Briefly, mice were anesthetized using a cocktail of fentanyl/fluanisone and midazolam injected intraperitoneally and were maintained at 37°C with oxygen supplementation. The ear to be imaged was taped to the center of the coverslip and 80µL of tetramethylrhodamine (TRITC) conjugated 70kDa dextran (70µM) was injected intravenously. Light was generated from 488-nm and 562-nm lasers, and emitted light signal was detected to generate two colour 8-bit images, using a 10x/0.4 objective on a Leica SP5 confocal microscope. Images were analysed using Imaris software (Bitplane). Briefly, dextran signal was used to select only intravascular cells for tracking and cells were automatically selected based on the quality and intensity of their GFP signal. Cells were tracked using the inbuilt autoregressive model based algorithm and tracks were manually verified for accuracy. In order to analyse intravascular patrolling, cells that were present for less than 240 seconds were excluded.

2.6. Quantifying LDL/VLDL modification

Human LDL or VLDL were purchased from Biomedical Technologies, Inc. Vials were opened upon receipt and left for approximately 1 week at 4°C before use. Lipid modification was measured via the absorbance of conjugated dienes at 234nm as previously described [2] using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.7. Blocking LDL/ VLDL uptake

In some experiments, LDL or VLDL uptake mechanism was investigated using receptor-blocking strategies prior to incubation of human monocytes with LDL/ VLDL. The appropriate kinetics and concentrations of the blocking agents varies, therefore these are detailed in Table 2-2 below.

Table 2-2 Receptor blocking strategies for LDL/ VLDL uptake

Blocking agent	Manufacturer	Target	Time	Concentration
RAP	Enzo Life Sciences	LDLR family	60 minutes	1.25µM
Anti- CD36 (SMO)	Novus Biologicals	CD36	60 minutes	10µg/ml
Dextran sulphate	Sigma	Class A Scavenger Receptors	60 minutes	100µg/ml
Heparinase 1/3	Sigma	Heparan sulphate proteoglycans	60 minutes	5UN/ml
Tetrahydrolipstatin (Orlistat)	Sigma	Lipases	60 minutes	50µM

2.8. LPL Immunofluorescence

Human CD16^{pos} and CD16^{neg} primary monocytes were seeded (1.5×10^6 /ml) into tissue-culture treated 0.4µm chamber slides (IBIDI) in DMEM and allowed to adhere for 30 minutes. Adherent cells were immediately fixed in 4% PFA for 15 minutes and washed with 0.1M glycine. Cells were permeabilized with Triton X-100 and actin was stained using fluorescently conjugated phalloidin (Life Technologies). Lipoprotein lipase (LPL) was stained using a biotin-conjugated anti-LPL primary antibody (1/50, AbD Serotech) and a streptavidin-conjugated, PE-labelled secondary antibody (1/500, Biolegend). Cells were mounted in DAPI-Vectashield (Vector Labs) and imaged using either a Zeiss AxioObserver widefield or a Leica SP5 confocal microscope with a 63x/1.4 objective.

2.9. Apoptosis

Purified human peripheral blood mononuclear cells were incubated at 5×10^6 /ml in DMEM for 2 hours at 37°C with or without 100µg/ml LDL or VLDL (Biomedical Technologies, Inc). Cells were washed and stained for apoptosis using an anti-

Annexin V antibody at a concentration of 1/20 according to manufacturer instructions (eBioscience). Cells were washed again and stained for flow cytometry using anti-HLA-DR (TU36), anti- CD16 (3G8) and anti- CD14 (M5E2).

2.10. qPCR and microarray analysis

Following individual experimental treatments, RNA was extracted from cells by acid guanidinium thiocyanate-phenol-chloroform extraction using Tri-Reagent (Sigma Aldrich). cDNA was synthesized by reverse transcription with SuperScript III Reverse Transcriptase (Life Technologies). The qPCR reaction was performed on an Eppendorf Mastercycler Ep system using Sensimix SYBR (Bioline). Input cDNA was quantified using a standard curve generated with serial dilutions of pooled samples and normalized to the mean values of housekeeping GAPDH and 18s-rRNA (mouse) or GAPDH and RPLP0 (human). All primers were ordered as custom oligonucleotides from Sigma Aldrich and are listed below Table 2-3 (mouse) and Table 2-4 (human), with the exception of human CDC42, RAC1 and RPLP0, which were Quantitech pre-validated (Qiagen).

2.11. Statistics

Comparison between two groups was performed using a two-tailed Mann–Whitney U test to avoid assumptions of parametric distribution. Multiple (<2) independent groups were analysed using a mixed ANOVA with a post-test following the Bonferroni method. $P < 0.05$ was considered significantly different. Throughout, p values are represented as follows: $p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function
William Jackson- January 2016

Table 2-3 Mouse RT-qPCR primer sequences

Target	Forward	Reverse
Il1b	CAACCAACAAGTGATATTCTCC	GATCCACACTCTCCAGCTGCA
Tnf	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
Il10	CAGCCGGGAAGACAATAACTG	CCGCAGCTCTAGGAGCATG
Gsn	CTCTGGACCACCACCTCATT	G TTCAGGGCTTTGAGTCGTC
Cdc42	CCCATCGGAATATGTACCAACTG	CCAAGAGTGTATGGCTCTCCAC
Rac1	GAAAGAGATCGGTGCTGTCAA	CAACAGCAGGCATTTTCTCTT
Rhoa	AGCTTGTGGTAAGACATGCTTG	GTGTCCCATAAAGCCAACTCTAC
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
18s	CTGGAGCCTGTTTTGCTTCTG	TGAGATGGACTGTCGTCGGATG
Cx3cr1	GAGTATGACGATTCTGCTGAGG	CAGACCGAACGTGAAGACGAG
Cxcl10	CTCATCCTGCTGGGTCTGAG	CCTATGGCCCTCATTCTCAC

Table 2-4 Human RT-qPCR primer sequences

Target	Forward	Reverse
RHOA	AGCAAGCATGTCTTTCCACA	GAAGAGGCTGGACTCGGATT
GSN	GATCGAACTTCTCCACACGC	ACCCTGCACAGCCTTGTTAG
IL1B	AAGCCCTTGCTGTAGTGGTG	GAAGCTGATGGCCCTAAACA
TNF	GCCAGAGGGCTGATTAGAGA	TCAGCCTCTTCTCCTTCCTG
IL10	CTCATGGCTTTGTAGATGCCT	GCTGTCATCGATTTCTTCCC
IL8	AGCACTCCTTGGCAAACTG	CGGAAGGAACCATCTCACTG
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

2.12. Cytokine production:

MACS isolated CD16^{pos} and CD16^{neg} human monocytes (0.2×10^6) were incubated with 100µg/ml VLDL or PBS for 2 hours at 37°C. Monocytes were then incubated with/ without 2µg/ml R848 (Enzo Life Sciences) and 100ng/ml lipopolysaccharide (LPS) from *Salmonella enterica* serotype typhimurium (Sigma-Aldrich) for 16 hours. The supernatants were collected and stored at -80°C. TNF-α and IL-1β levels were measured using a BD Cytometric Bead Array on a BD Accuri C6 flow cytometer according to manufacturer instructions (BD Biosciences).

2.13. Phagocytosis

Purified human peripheral blood mononuclear cells were incubated at 5×10^6 /ml in DMEM for 2 hours at 37°C with or without 100µg/ml LDL or VLDL (Biomedical Technologies, Inc). To assess phagocytosis, cells were treated 1/100 with 1µm carboxylated fluorescent yellow-green latex beads (Sigma Aldrich) for 1 hour at 37°C. Cells were washed twice and stained for flow cytometry using anti- HLA-DR (TU36), anti- CD16 (3G8) and anti- CD14 (M5E2).

2.14. Transwell chemotaxis assay

Purified human CD16^{pos} and CD16^{neg} blood monocytes were incubated in DMEM for 2 hours at 37°C with or without 100µg/ml LDL or VLDL (Biomedical Technologies, Inc). 1×10^5 cells per well were seeded into 3µm-pore transwell inserts (Corning), using a chemoattractant gradient of 250ng/ml recombinant human C5a (RND Systems) and incubated at 37°C for 2 hours. Non-adherent cells were removed and the transwell inserts were fixed in 4% PFA. Non-migrated cells were removed using a cotton bud before the membranes were excised and mounted on microscope slides in Vectashield mounting media containing DAPI (Vector Labs). Slides were imaged using a 20X/0.7 objective on an Olympus BX51 widefield fluorescence microscope and nuclei were counted using ImageJ analysis software.

2.15. 2D real-time migration assay

Purified human CD16^{pos} and CD16^{neg} blood monocytes were re-suspended at 3×10^6 cells/ml in DMEM-0.5% BSA and seeded into IBIDI 2D chemotaxis chambers (IBIDI) for 1 hour at 37°C with or without 100µg/ml LDL or VLDL (Biomedical Technologies, Inc). CD16^{pos} monocytes were labelled for imaging using an AlexaFluor-647 conjugated anti-CD16 antibody (3G8, Biolegend) 1/25 concentration. For chemotaxis experiments, a gradient of 250ng/ml human recombinant C5a was established on one side of the chamber according to manufacturer instructions. For chemokinesis experiments, C5a was added directly to the cell channel to achieve uniform distribution. Slides were allowed to equilibrate for 30 minutes before imaging on a Leica SP5 confocal microscope using a 10x/0.4 objective and a 37°C environmental chamber.

2.16. Monocyte adhesion to endothelium

Human umbilical venous endothelial cells (HUVECs) were a kind gift from Professor Justin Mason and were isolated and cultured as previously described [3]. Briefly, HUVECs were seeded into collagen-I (Sigma-Aldrich) coated 0.4µm chamber slides (IBIDI) at 2×10^6 /ml and allowed to adhere 30 minutes at 37°C. Cells were washed with complete M199 and left to reach confluence for 24 hours at 37°C. In separate experiments, HUVECs or human monocytes were treated with 100µg/ml LDL or VLDL for 2 hours at 37°C. Cells were washed and monocytes were stained with NucBlue (Life Technologies) and added to the endothelium at 1×10^6 /ml for 30 minutes. Co-cultures were fixed in 4% PFA and washed to remove non-adherent monocytes. Slides were imaged using a 20X/0.7 objective on a Leica SP5 confocal microscope and nuclei were counted using ImageJ analysis software.

2.17. Transendothelial migration

For endothelial transmigration experiments, transwell inserts were coated with Type I collagen (Sigma Aldrich) overnight, seeded with HUVECs at 1×10^5 cells/cm² and

allowed to reach confluence for 24 hours. HUVECs were activated with 2ng/ml TNF- α (PeproTech) for 3 hours. Monocytes were labeled with CFSE for 30 minutes to allow for distinction from endothelial cells, washed and seeded into transwells as described above. Transmigrated monocytes were imaged as described above.

2.18. RHOA and CDC42 activation assays

Human CD16^{pos} and CD16^{neg} primary monocytes (0.5×10^6) were treated with combinations of 100 μ g/mL LDL/ VLDL and 2 μ g/ml RHOA activator CN03 (Cytoskeleton, Inc.) for 2 hours in DMEM at 37°C. Cells were lysed with supplied lysis buffer and lysates clarified by centrifugation at 4°C (14000xg, 3 min). Protein concentration for each sample was determined using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific) and remaining lysates were snap-frozen in liquid nitrogen for storage at -80°C. RHOA activity in human primary monocytes was measured using a luminescence-based G-LISA RHOA activation kit according to manufacturer instructions (Cytoskeleton, Inc.). Chemiluminescence was measured at 445 nm 5 min after the HRP substrate was added, using a FluoStar Galaxy instrument (BMG Lab Technologies). CDC42 activity was assessed in cells treated and processed as above using a colorimetric-based G-LISA kit according to manufacturer instructions (Cytoskeleton, Inc.) and absorbance at 562 nm was measured using a Biotek ELX 800 microplate reader (Bio-Tek Instruments).

2.19. Western blot

Monocytes were lysed on ice in 70 μ L of lysis buffer (50 mM NaCl, 250 mM Tris-HCL, 1% NP-40) supplemented with phosphatase inhibitors (5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄) and protease inhibitor cocktail (Sigma-Aldrich) for 30 minutes. Proteins were separated by 15% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto nylon membranes (GE Healthcare). Membranes were blocked in TBS containing 5% BSA and 0.1% Tween 20 and incubated overnight with anti-phospho-PAK1/2/3 (Novus Biologicals) or anti-p38 (SC535, Santa Cruz Biotechnology). Protein was detected with horseradish

peroxidase-conjugated secondary antibodies (Cell Signaling Technology) in combination with Amersham ECL Western Blotting Reagents (GE Healthcare).

2.20. Cell morphology analysis

Human CD16^{pos} and CD16^{neg} primary monocytes were treated with combinations of 100 µg/mL LDL/ VLDL and 2µg/ml RHOA activator CN03 (Cytoskeleton, Inc.) for 2 hours in DMEM at 37°C. Cells were seeded (1.5x10⁶/ml) into tissue-culture treated 0.4µm chamber slides (IBIDI) in DMEM and allowed to adhere for 30 minutes. Adherent cells were immediately fixed in 4% PFA for 15 minutes and washed with 0.1M glycine. Cells were permeabilized with Triton X-100 and actin was stained using fluorescently conjugated phalloidin (Life Technologies). Cells were mounted in DAPI-Vectashield (Vector Labs) and imaged using either a Zeiss AxioObserver widefield or a Leica SP5 confocal microscope with a 63x/1.4 objective. Cell morphology was analysed in ImageJ using automatic thresholding and masking on the phalloidin channel followed by circularity and area measurement.

2.21. Drosophila hemocyte imaging

Drosophila work was a collaboration with the group of Professor Will Wood at the University of Bristol and Dr Kate Comber performed the experiments. I contributed to experimental design and analysed the videos provided by collaborators in order to generate the data presented here.

Drosophila stocks were maintained under standard conditions at 25°C as previously described [4]. All flies used expressed a UAS-GFP construct driven by the hemocyte-specific promoters *serpent* (*srp*) and *croquemort* (*crq*) and were on a w¹¹⁸ background. Rho1-V14 flies expressed a dominant-negative UAS-Rho1-V14 construct also driven by these two promoters, as previously described [5]. Stage 11-12 embryos were injected with LDL-AlexaFluor-594 (Life Technologies) or PBS using an Eppendorf Microinjection System (Eppendorf). Embryos were allowed to recover for 3 hours prior to imaging at stage 15. Imaging was performed on a Leica SP8

confocal laser scanning microscope (Leica) with a 40x/ 1.3 objective. Hemocyte motility was analyzed using Bitplane Imaris software (Bitplane).

2.22. References

- 1 Carlin LM, Auffray C, Geissmann F. Measuring Intravascular Migration of Mouse Ly6Clow Monocytes In Vivo Using Intravital Microscopy. *Current Protocols in Immunology* 2013;:14.33.1–14.33.16. doi:10.1002/0471142735.im1433s101
- 2 Esterbauer H, Striegl G, Puhl H, *et al.* Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radic Res Commun* 1989;**6**:67–75.
- 3 Mason JC, Yarwood H, Sugars K, *et al.* Induction of decay-accelerating factor by cytokines or the membrane-attack complex protects vascular endothelial cells against complement deposition. *Blood* 1999;**94**:1673–82.
- 4 Wood W, Faria C, Jacinto A. Distinct mechanisms regulate hemocyte chemotaxis during development and wound healing in *Drosophila melanogaster*. *J Cell Biol* 2006;**173**:405–16. doi:10.1083/jcb.200508161
- 5 Kadandale P, Stender JD, Glass CK, *et al.* Conserved role for autophagy in Rho1-mediated cortical remodeling and blood cell recruitment. *PNAS* 2010;**107**:10502–7. doi:10.1073/pnas.0914168107

3. THE RESPONSE OF MOUSE MONOCYTES TO DYSLIPIDAEMIA

3.1. Introduction

The concept of blood leukocytes interacting with lipid in the plasma is long established, yet only sporadically described in leukocyte subpopulations. As early as the 1960s, rats fed a high fat, high cholesterol diet were observed to have lipid-rich blood leukocytes by light microscopy, which were principally monocytes and became known as 'lipophages' [1,2]. In the context of atherosclerosis, these lipid rich monocytes were hypothesized by some to deliver lipid to the plaque and mature to foam cells [2]. In the 1970s, others even went as far as to postulate that infiltrating lipophages were a functionally distinct population in the plaque, and that traditional foam cells derived from smooth muscle cells (SMC) [3]. However, this theory fell out of favour as more evidence arose for the independent subendothelial deposition of LDL and the functional response of lipid-loaded leukocytes was dismissed without further investigation. More recently, evidence for blood monocyte lipid-loading has once again appeared: monocytes from the *Apoe*^{-/-} mouse have increased granularity as measured by flow cytometry side-scatter (SSC) and this appears to be accounted for by intracellular lipid [4]. There is some suggestion that this is specific to GR1^{low} monocytes, however the functional impact of this lipid loading remains unclear, particularly in the context of functionally independent monocyte subpopulations.

I aimed here to confirm and characterize this phenotype using the *Ldlr*^{-/-} mouse. Initially generated in 1993, the *Ldlr*^{-/-} mouse is a well-established model of hypercholesterolemia and atherosclerosis [5,6]. On a normal chow diet, total plasma cholesterol doubles compared to *Ldlr*-competent mice to around 300mg/dl [6]. However, after just 2 weeks of HFD these levels rise to approximately 2500mg/ml, driven primarily by an increase in VLDL and LDL, while wild-type mice remain relatively unaffected [6]. When fed a chronic HFD, these *Ldlr*^{-/-} animals develop advanced atherosclerotic lesions in the aortic sinus after 3 months that extend to the

thoracic aorta by 6 months and the abdominal aorta at 9 months [7]. As such, these animals are a useful model to investigate the effects of extreme dyslipidaemia on blood monocytes.

3.2. Results

3.2.1. Neutral lipid accumulation in mouse monocytes

To examine blood monocytes after short-term high fat diet, *Ldlr*^{-/-} mice were fed a chow or HFD for 6 weeks and the blood sampled for flow cytometric analysis. Monocyte flow cytometry gating strategy is shown in Figure 3.1A: monocytes were defined as CD11b^{pos}, CD115^{pos} and subsets then split into GR1^{low} and GR1^{high}. Other groups have reported monocytosis after HFD in the *Apoe*^{-/-} mouse, which I sought to confirm in the *Ldlr*^{-/-} mouse under these conditions. At 6 weeks HFD in my experiments there was no significant difference in GR1^{low} or GR1^{high} monocyte count between chow and HFD fed *Ldlr*^{-/-} animals (Figure 3.1B). After 6 weeks, monocytes from animals fed HFD had a significantly increased SSC when compared to chow controls, indicating an increase in granularity (Figure 3.1C). I also investigated monocyte activation by assessing expression levels of cell surface receptors. However, contrary to some previous reports in the *Apoe*^{-/-} model [8,9], HFD did not increase monocyte levels of the cell surface integrin CD11b. There was also no increase in surface expression of the colony-stimulating factor 1 (M-CSF) receptor CD115 or of GR1. In addition, neutrophils (defined as CD11b^{pos}, CD115^{neg}, GR1^{pos}, SSC^{hi}) displayed no changes in granularity or surface CD11b levels after HFD, indicating a monocyte specific phenotype (Figure 3.1E-I).

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson- January 2016

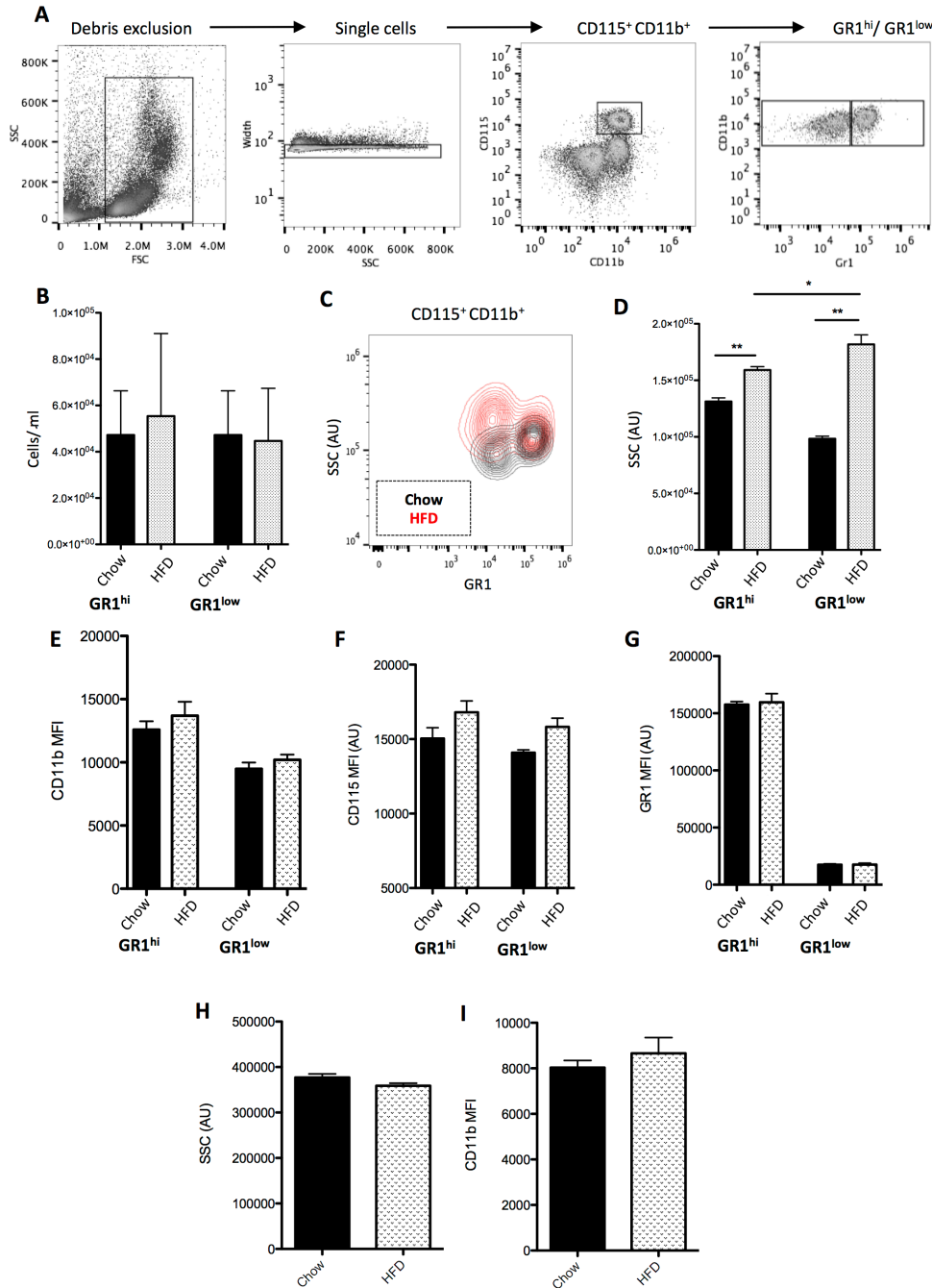


Figure 3.1: FACS phenotyping of blood leukocytes from *Ldlr*^{-/-} mice.

(A) Blood monocyte subpopulations were FACS gated as follows: debris and platelets were excluded using FSC and SSC, representing size and granularity respectively. Single cells were selected using ratio of SSC area to width, and of those CD115⁺ CD11b⁺ cells were gated as monocytes. Monocyte subpopulations were then gated as GR1^{hi} or GR1^{low}. Blood monocytes from *Ldlr*^{-/-} mice fed chow or HFD for 6 weeks were analyzed by FACS. (B) Monocytes per ml of blood, (C) A representative FACS example of monocyte SSC, (D) quantification of monocyte SSC. (E) Monocyte CD11b MFI, (F) CD115 MFI (G) GR1 MFI. Blood neutrophils were analysed for (H) SSC and (I) CD11b MFI. N=5 per group, error bars show the mean ± SEM. Groups were compared using a two-tailed Mann Whitney U test.

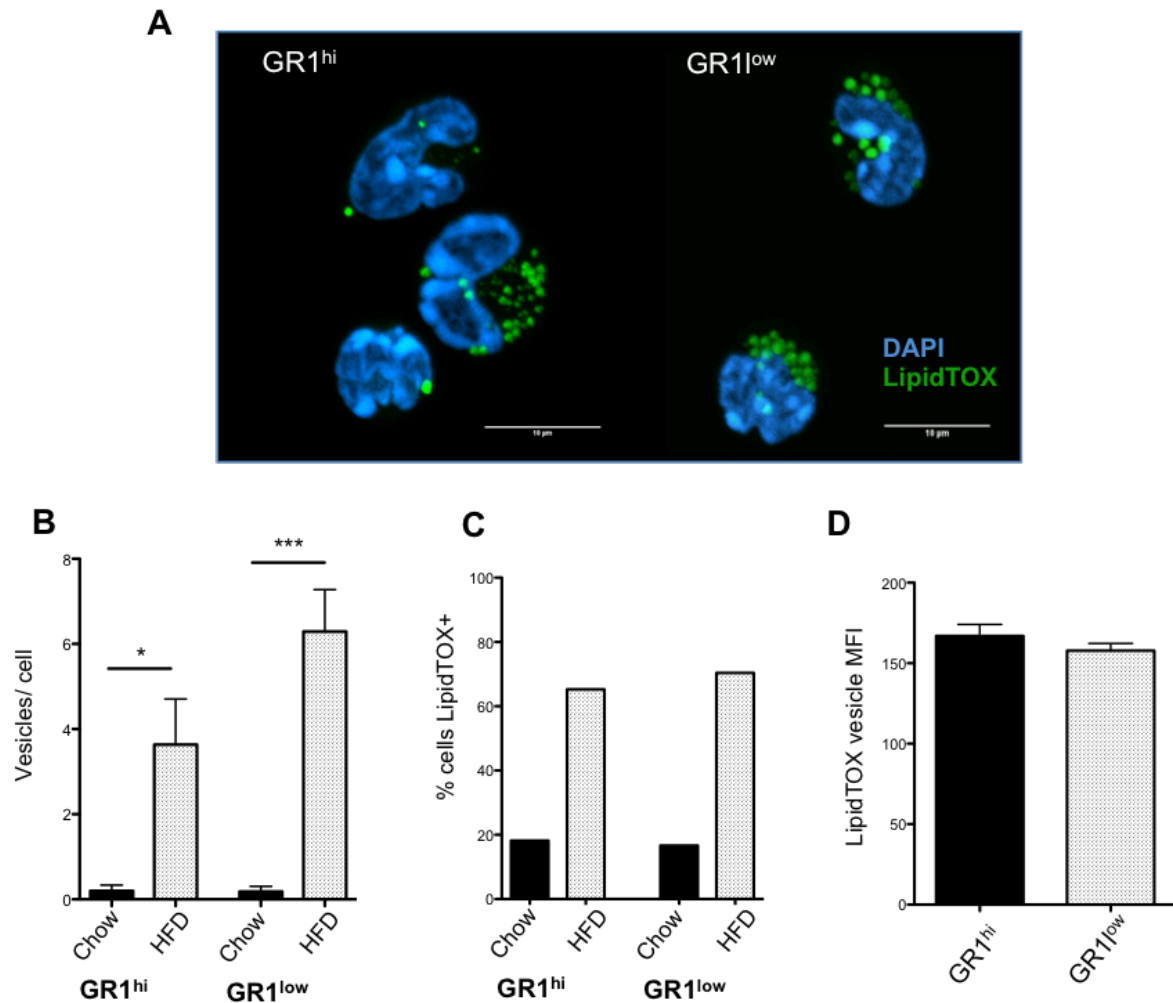


Figure 3.2: Blood monocytes from *Ldlr*^{-/-} mice are lipid loaded.

(A) Neutral lipid staining of GR1^{hi} and GR1^{low} blood monocytes from *Ldlr*^{-/-} mice, 16 weeks HFD. Scale bar represents 10μm. Staining is quantified as (B) vesicles per cell, (C) % of cells containing neutral lipid vesicles and (D) LipidTox vesicle MFI, n=>20 cells per condition, cells were pooled from 3 mice per condition. Error bars show the mean ± SEM. Groups were compared using a two-tailed Mann Whitney U test.

The next step was to investigate the composition of the increased monocyte granularity. At 16 weeks after chow/ HFD, peripheral blood mononuclear cells were isolated and monocytes were sorted into GR1^{high} and GR1^{low} subpopulations for imaging. Cells were adhered to tissue culture plastic and stained for neutral lipid content using LipidTOX-Green (Invitrogen). Confocal microscopy revealed a large number of neutral lipid vesicles in the cytoplasm of the majority of monocytes in both subsets after HFD; representative images are shown in Figure 3.2A. Image analysis revealed that percentage of cells containing lipid rose significantly on HFD and the mean number of neutral lipid vesicles per cell increased by >10-fold compared to chow (Figure 3.2B-C). There was no difference in lipid vesicle intensity between monocyte subsets, although there was a trend towards more intracellular neutral lipid vesicles in the GR1^{low} monocytes (Figure 3.2B). Interestingly, the accumulation of neutral lipid was restricted to cytoplasmic droplets and did not appear to occur in the cell membrane.

While this thesis was being prepared for submission, Wu and colleagues published work in the *Apoe*^{-/-} mouse, demonstrating that HFD causes the development of a population of lipid-loaded, SSC^{high}, CD11c^{pos} 'foamy' monocytes that contribute to lipid trafficking during early atherogenesis [10]. This occurs after as little as 3 days HFD, and peaks in severity between 5 and 10 weeks [10]. To confirm this phenotype in the *Ldlr*^{-/-} model, I analyzed blood leukocytes from animals fed chow or HFD for 8 weeks. As noted in Figure 3.1D, HFD increased the SSC of blood monocytes (gating shown in Figure 3.3A), with the proportion of SSC^{high} monocytes rising from approximately 7% on chow to approximately 20% on HFD (Figure 3.3B). In the *Apoe*^{-/-} animals on HFD for a similar time point, this figure was over 30% [10]. When monocytes were assessed for CD11c expression by flow cytometry, approximately 10% of SSC^{high} cells were CD11c^{pos} on both chow and HFD, while CD11c was only present on 3-4% of the SSC^{low} population (Figure 3.3C-D). Interestingly, the proportion of SSC^{hi} monocytes that were also CD11c^{pos} was not altered by HFD, perhaps indicating that these cells have a short circulating half-life. In addition, this was a much less pronounced phenotype than reported in *Apoe*^{-/-} mice, in which 70-80% of SSC^{high} monocytes express CD11c^{pos} after 8 weeks of HFD [10]. When gated

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

into monocyte subpopulations, over 10% of GR1^{low} monocytes were CD11c^{pos}, while in GR1^{high} cells this population was only 1-2% (Figure 3.3E-F).

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson- January 2016

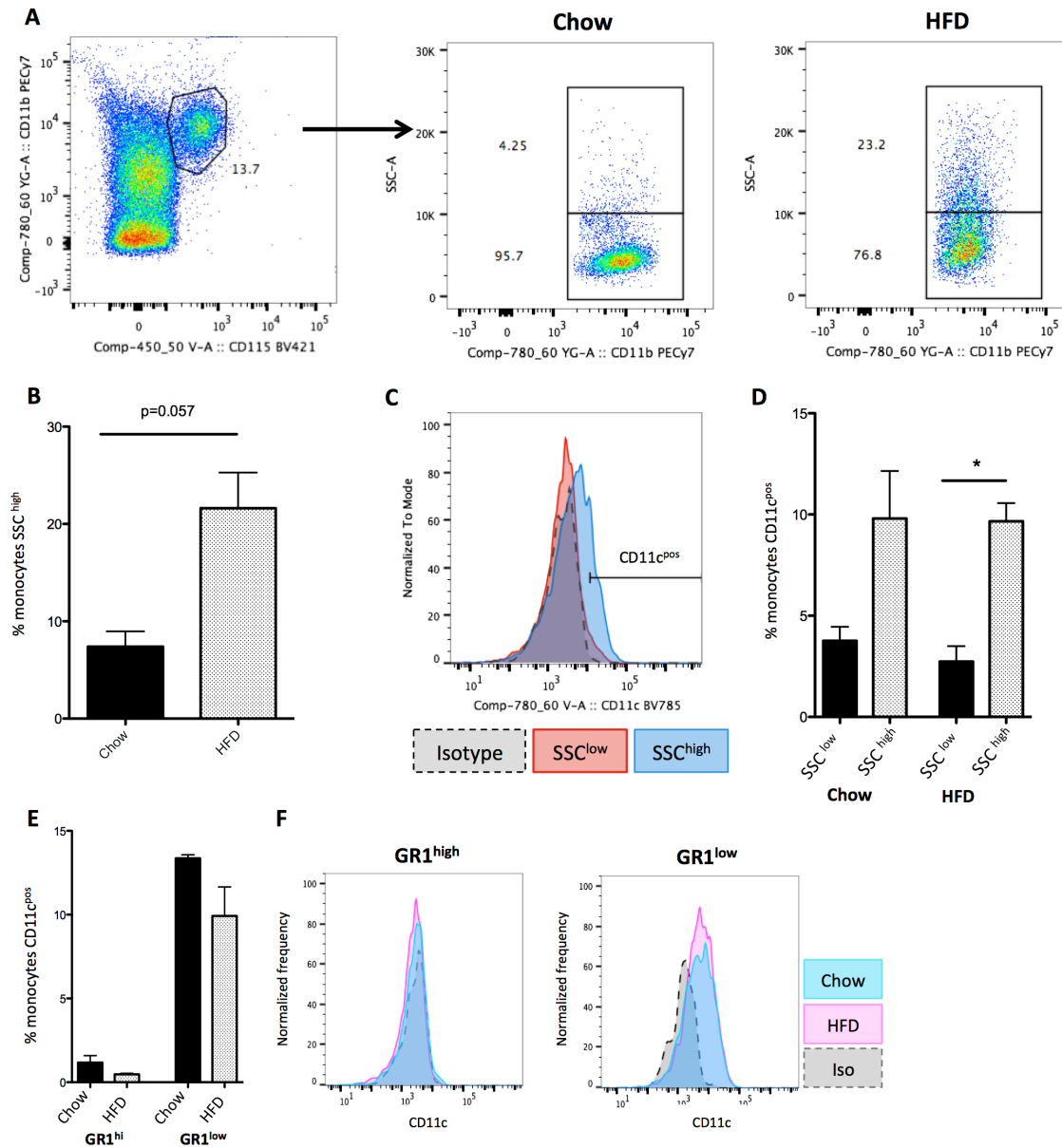


Figure 3.3 Expression of CD11c by murine monocytes on HFD.

Ldlr^{-/-} mice were fed chow or HFD for 8 weeks and blood monocytes were analyzed for SSC and surface CD11c, n=4 per group. (A) Representative flow cytometry plots of SSC^{high}/ SSC^{low} monocyte gating from blood leukocytes. (B) Percentage of monocytes SSC^{high} on chow or HFD. (C) Representative histogram of SSC^{high} and SSC^{low} monocyte CD11c levels after HFD. (D) Percentage of monocytes CD11c positive on chow or HFD. (E) Percentage of GR1^{high} and GR1^{low} monocytes CD11c positive on chow or HFD. (F) Representative histogram of GR1^{high} and GR1^{low} monocyte CD11c levels on chow or HFD. Error bars show mean $\pm$ SEM, data was compared using a two-tailed Mann-Whitney U test.

3.2.2. The response of lipid-loaded monocytes to sterile inflammation.

To investigate the functional impact of blood monocyte lipid loading on their response to sterile inflammation, we performed thioglycollate (THG) peritonitis in the *Ldlr*^{-/-} mouse after 16 weeks chow or HFD and FACS analysed cells from the peritoneal lavage at 72 hours post-injection; a time point well characterized to represent peak monocyte/ macrophage infiltration [11,12]. Originally designed to test aerotolerance of bacteria, thioglycollate broth principally contains sodium thioglycolate, thioglycolic acid and L-cysteine and induces inflammation when injected into mice, although the exact mechanisms of this are not well defined [13]. As expected, the peritoneal lavage 72 hours after THG injection was dominated by CD115^{pos} monocytes and macrophages in our experiments. The FACS gating strategy for peritoneal monocytes/ macrophages is shown in Figure 3.4A. In the steady state, there was no change in peritoneal total cell numbers or monocytes/ macrophages after HFD (Figure 3.4B-C). However, after THG injection there was a significant, approx. 50% decrease in total peritoneal cells, the majority of which was accounted for by a decrease in monocytes/ macrophages (Figure 3.4B-C). Interestingly, there was also a decrease in CD115^{neg} CD11b^{pos} SSC^{hi} granulocytes, although these cells were only a very small proportion of the peritoneal population (Figure 3.4D).

Due to the poorly defined mechanisms of THG induced inflammation I confirmed these findings in a model of endotoxin-induced peritonitis. LPS was injected IP and the peritoneal lavage collected and analysed at 72 hours as described above. While there was no change in total cell number on HFD (Figure 3.4E), peritoneal CD115^{pos} monocytes/ macrophages decreased by approx. 50%, consistent with findings from the THG model (Figure 3.4F). The discrepancy in total cell number and monocyte/ macrophage number appears to be due to an increase in an unidentified population of CD11b^{neg} CD115^{neg} SSC^{low} cells after HFD that would require further investigation to characterize. There was also a small and non-significant decrease in CD115^{neg} CD11b^{pos} SSC^{hi} granulocyte numbers (Figure 3.4G).

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

It has previously been reported that HFD in *Ldlr*^{-/-} results in a 'deactivated' phenotype in peritoneal macrophages via desmosterol-LXR signalling, resulting in decreased inflammatory gene expression [14]. To confirm this observation, I assessed mRNA abundance of targets previously associated with this pathway. In agreement with published work, I observed a trend towards less expression of *IL1B*, *IL10* and *CXCL10* on HFD (Figure 3.4H), although none of these reached statistical significance.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

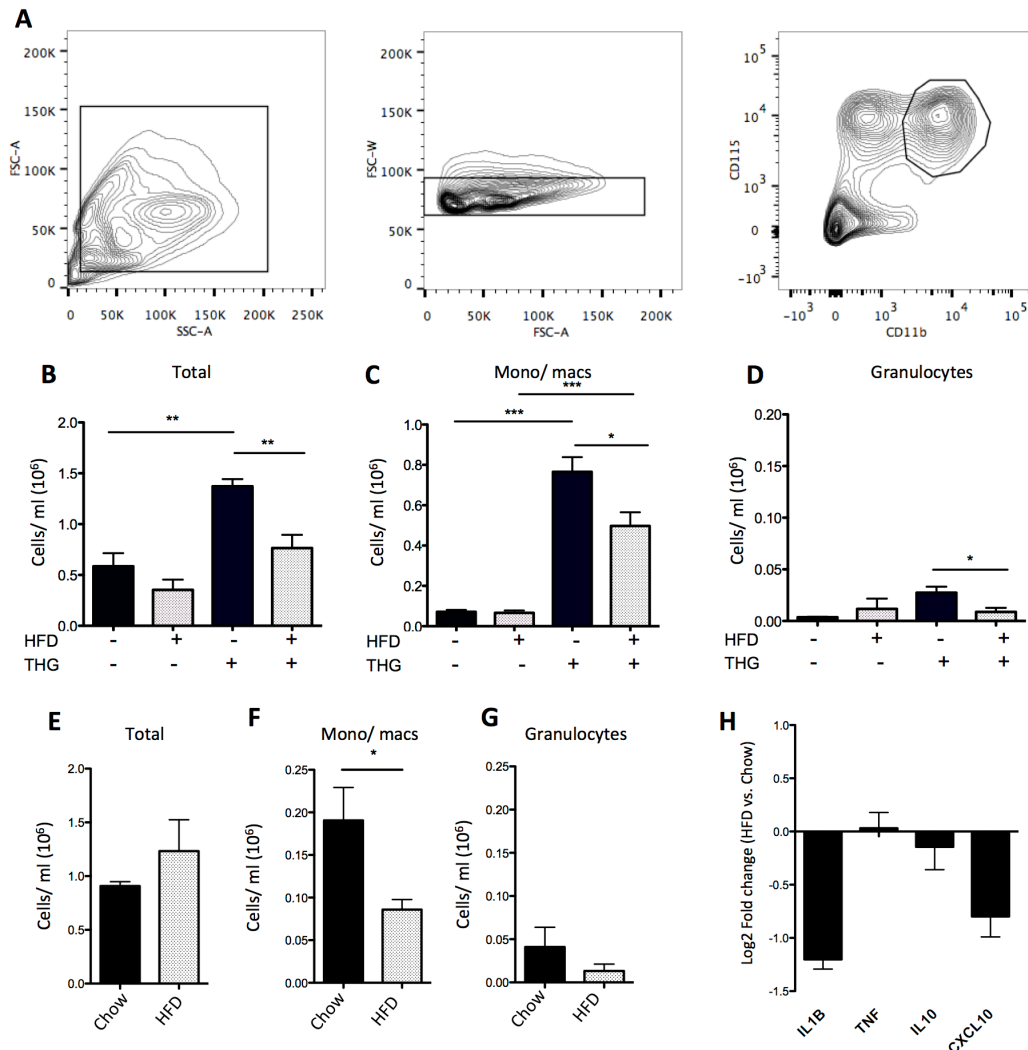


Figure 3.4: Sterile peritonitis model in dyslipidaemic *Ldlr*^{-/-} mice.

Thioglycollate peritonitis (1ml of 4% THG per mouse) was induced in *Ldlr*^{-/-} mice and the peritoneal lavage analysed by FACS at 72 hours post-injection. (A) Representative gating of infiltrating monocytes/ macrophages, (B) Total cells/ ml, (C) CD115^{pos} CD11b^{pos}/ ml (D) CD11b^{pos} SSC^{hi} CD115^{neg}/ml peritoneal lavage from *Ldlr*^{-/-} mice on chow/ HFD, non-injected or 72 hours post- THG IP, n=5 per group. (E-G) Cells/ ml is shown for the same populations are shown after 72 hours LPS-induced peritonitis (10 μ g per mouse), n=4 per group. (H) Peritoneal cells after THG were analyzed for inflammatory gene expression by RT-qPCR, data shown as HFD log2 fold change versus chow. Error bars show the mean $\pm$ SEM, groups were compared using a two-tailed Mann Whitney U test.

These data potentially indicated that blood monocyte migration to the peritoneal cavity during inflammation is impaired by HFD. To test this hypothesis I utilized a strategy previously employed by the Randolph group [15], where blood monocytes can be labelled using intravenous (IV) injection of fluorescent latex beads. This utilises the high phagocytic ability of blood monocytes and allows them to be tracked out of the blood. As expected, injection of 1µm carboxylated latex beads predominantly labelled the GR1^{low} subpopulation, but also labelled some GR1^{high} monocytes (Figure 3.5A). Peak labelling occurred at 30 minutes post- injection, with approx. 10% GR1^{high} bead-positive (bead^{pos}) and 25% GR1^{low} bead^{pos}. By 24 hours post-injection these proportions had fallen to approx. 12% and 3% respectively, but labelling was still present and detectable. For peritonitis experiments, beads were injected IV 3 hours prior to IP THG injection. When the peritoneal lavage was collected at 72 hours post THG, the number of bead^{pos} CD115^{pos} CD11b^{pos} cells in the peritoneum was decreased almost ten-fold by HFD (Figure 3.5B), confirming that the decrease in peritoneal monocytes during HFD is a defect in extravasation to the peritoneal cavity. Monocyte/ macrophage mean bead fluorescence intensity was not altered between diets, indicating that phagocytosis of beads was not impaired by HFD (Figure 3.5C).

I then sought to investigate the fate of blood monocytes on HFD after peritonitis if they were failing to reach the peritoneal cavity. Previous work in rodent models of peritonitis has indicated that the greater omentum is the major site of leukocyte extravasation from the blood into the peritoneal cavity [16,17]. The greater omentum is a large fold of visceral tissue that hangs down from anterior stomach and creates a physical barrier to the spread of infection and hernia. As discussed earlier, omental 'milky spots' also contain a reserve of maturing tissue resident peritoneal macrophages [18,19]. Again using THG peritonitis as a model, I harvested the greater omentum at 72 hours post-injection and analysed the leukocyte populations in the tissue using flow cytometry. CD45 staining was used to distinguish leukocytes from other cells, and representative plots of CD45^{pos} cells stained for CD115 and the macrophage antigen F4/80 are shown in Figure 3.6A. Peritonitis decreases the total

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

leukocyte number in the omentum on chow and HFD, as resident macrophages migrate out from the omentum into the peritoneal cavity. However, on HFD there is a striking approximately 10 fold increase in the number of CD115^{pos} F4/80^{low} monocytes present in the inflamed omentum (Figure 3.6C), accompanied by a trend towards less CD115^{neg} F4/80^{high} mature tissue macrophages (Figure 3.6D). This mirrors the approx. 10-fold decrease seen in blood monocyte migration into the peritoneum on HFD using latex-bead labelling, suggesting a HFD- induced migratory defect.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson- January 2016

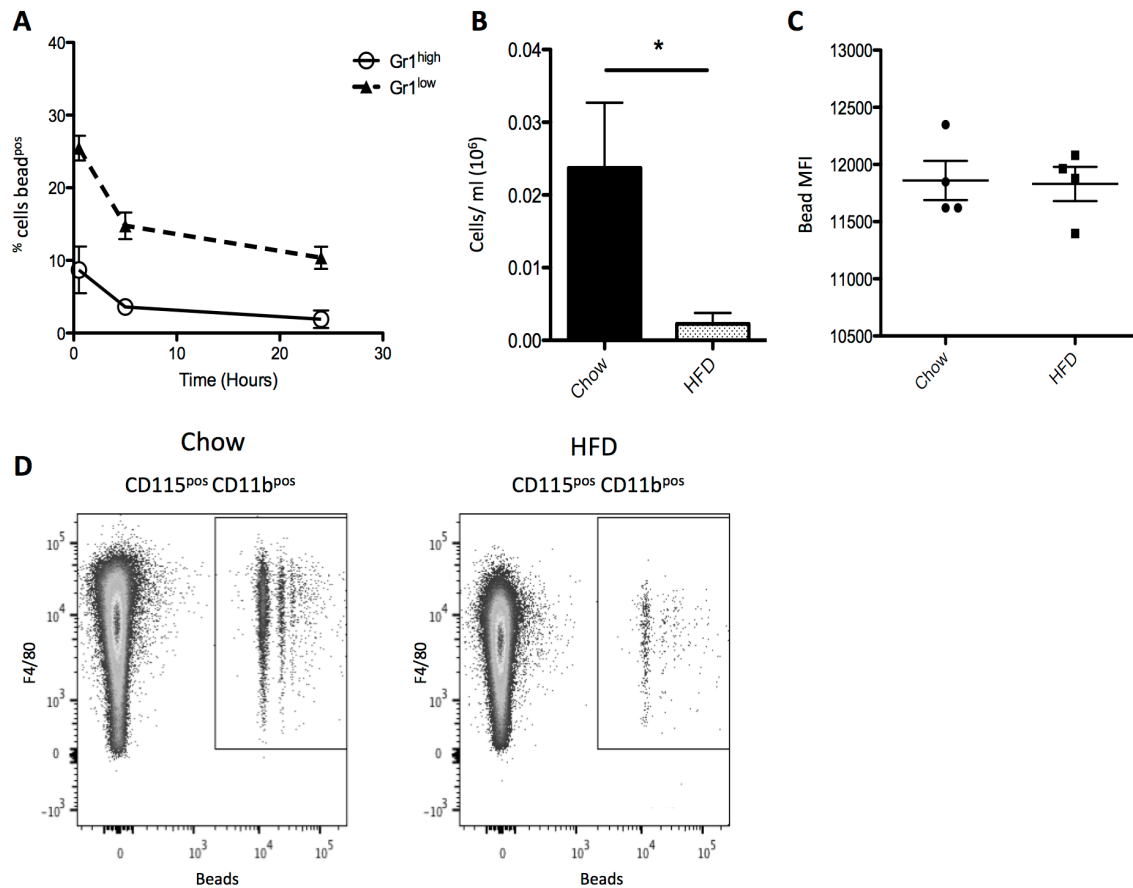


Figure 3.5: Blood monocyte tracking during peritonitis using latex beads.

IV injection of 1µm latex beads was used to track monocyte migration out of the blood during peritonitis in *Ldlr*^{-/-} mice with/without HFD. (A) Percentage of blood Gr1^{hi} or Gr1^{low} monocytes that were bead^{pos} post- bead injection. (B) Representative FACS plots showing latex bead positive populations gated from peritoneal CD115⁺ CD11b⁺ monocytes/ macrophages after 72 hours thioglycollate peritonitis. (C) Median bead fluorescence intensity of bead^{pos} peritoneal monocytes/ macrophages. (D) Representative FACS plots of bead uptake by CD115^{pos} CD11b^{pos} monocytes/ macrophages. n=4 per group, error bars show the mean ± SEM, groups were compared using a two-tailed Mann Whitney U test.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson - January 2016

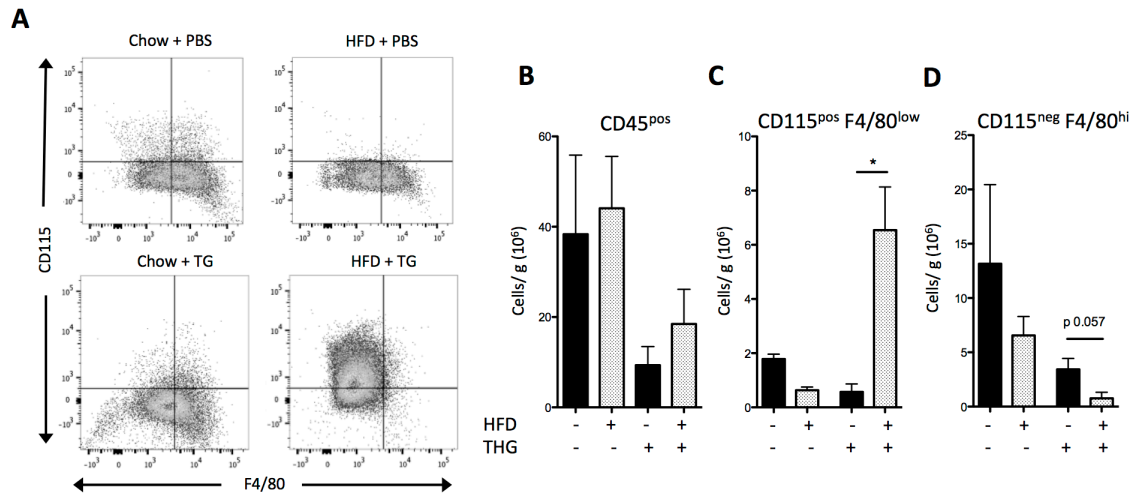


Figure 3.6: Leukocyte accumulation in the omentum after THG peritonitis.

THG peritonitis was induced in *Ldlr*^{-/-} mice and cell populations in the omentum were analysed by flow cytometry. (A) Representative FACS plots of CD115 and F4/80 staining in the omentum of *Ldlr*^{-/-} mice 72 hours post-THG IP. Gated on CD45⁺ cells. (B-D) Leukocyte populations per gram of tissue from this experiment: (B) CD45⁺, (C) CD115⁺ F4/80^{low}, (D) CD115^{neg} F4/80^{hi}, n=4-5 per group. Error bars show the mean ± SEM, groups were compared using a two-tailed Mann Whitney U test.

3.2.3. Intravital imaging of GR1^{low} monocyte patrolling after HFD

Interested in whether HFD was causing a migratory defect in monocytes, I attempted to visualize GR1^{low} monocyte locomotion in real time using the *Cx3cr1^{GFP/+}* mouse. Created by Jung and colleagues [20], this mouse has been well characterized as a model to study the long- range intravascular patrolling behaviour of non-classical monocytes, which express high levels of the fractalkine- receptor *Cx3cr1* [21,22]. C57Bl/6 *Cx3cr1^{GFP/+}* mice were fed a chow or HFD for 6 weeks in order to generate a mild hypercholesterolemia without genetic alteration of lipid metabolism. At 6 weeks, animals on HFD had a significantly increased plasma total cholesterol and LDL, as has been previously reported in wild-type mice [23] (Figure 3.7A). To examine whether this mild hypercholesterolemia was sufficient to cause cytoplasmic neutral lipid loading blood monocytes were sorted into GR1^{high} and GR1^{low} subpopulations for confocal imaging. Cells were adhered to tissue culture plastic and staining for neutral lipid content using LipidTOX-Red (Invitrogen). Although not as pronounced as in the *Ldlr^{-/-}* animals, some blood monocytes from the HFD group did contain clear neutral lipid accumulation, which was not present in monocytes from mice fed chow (Figure 3.7B). In general, the lipid punctae in monocytes from the *Cx3cr1^{GFP/+}* animals on HFD were smaller and less numerous than those present in the *Ldlr^{-/-}*.

Given that 6 weeks of HFD affects plasma cholesterol levels and monocyte neutral lipid content, I proceeded to intravital imaging of GR1^{low} monocytes in these animals. The vasculature in the dermis of the ear is an ideal site for non-invasive confocal microscopy of anaesthetised animals and fluorescently labelled 70kDa-dextran was injected IV to delineate vascular structure. A representative still image from intravital imaging is shown in Figure 3.7C to demonstrate the quality of imaging achievable. After 45 minutes imaging, there was a subtle increase in monocyte track speed on HFD, however this did not translate to a significant difference in track displacement, track length, track duration or confinement ratio (Figure 3.7D-H). In addition, tracks plots were visually similar with no clear defects after HFD (Figure 3.7I), leading to the

conclusion that modest hypercholesterolemia does not affect intravascular monocyte patrolling.

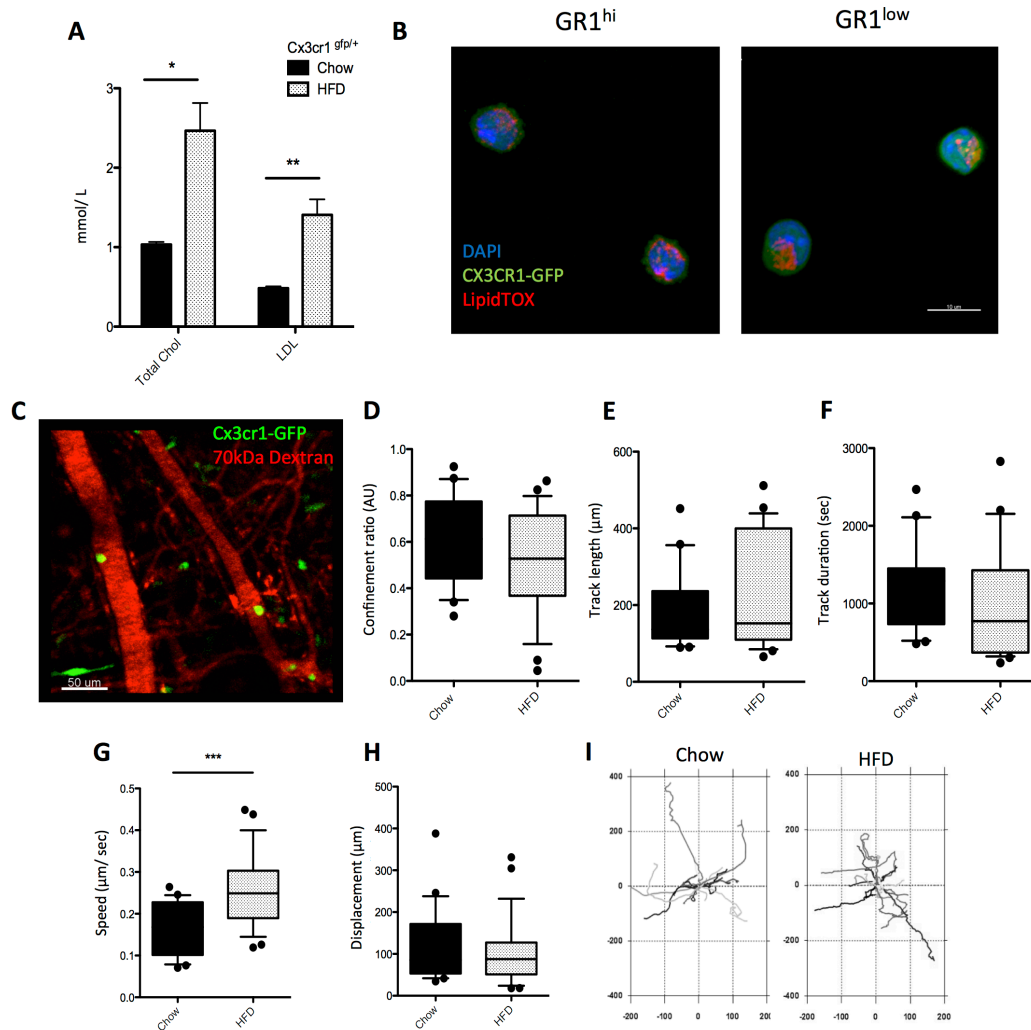


Figure 3.7: Intravital imaging of monocytes in chow or HFD fed *Cx3cr1*^{gfp/+} mice. *Cx3cr1*^{gfp/+} mice were fed chow/ HFD for 6 weeks (A) Blood total and LDL cholesterol levels of chow vs HFD mice, n=4 per group, error bars show the mean ± SEM. (B) Neutral lipid staining of GR1^{hi}/ GR1^{low} blood monocytes from *Cx3cr1*^{gfp/+} mice on HFD. Scale bar represents 10µm. Gr1^{low} monocyte vascular crawling was then assessed by intravital microscopy. (C) Representative field of view from dermis vasculature, (D) Track straightness (E) Track length (µm) (F) track duration (seconds) (G) track speed (µm/ sec) and (H) track displacement (µm) length of intravascular monocytes during approximately 45 minutes of imaging. (I) track projections of patrolling monocytes from *Cx3cr1*^{GFP/+} mice fed HFD or chow during ~45 minutes intravital imaging, 4 mice per diet, n=>20 cells analysed per condition. Whiskers display the 10th and 90th percentile; groups were compared using a two-tailed Mann Whitney U test.

3.3. Discussion

In this chapter I present data strongly suggesting that intracellular neutral lipid accumulation impairs the migration of blood monocytes towards an inflammatory stimulus, in this case peritonitis. The strongest data to support this conclusion is the failure of bead-labelled blood monocytes to accumulate in the peritoneal cavity during inflammation on HFD, clearly indicating less migration of leukocytes from the blood. In addition, it appears that this is due to monocytes being retained in the greater omentum while trafficking to the peritoneal cavity, during or after extravasation from the blood. However, the mechanism of this migratory impairment remains unresolved.

One possibility that is not addressed here is that monocytes are undergoing cell death in the hypercholesterolemic environment. Previous data have demonstrated that oxidised LDL (oxLDL) treatment can cause apoptosis in macrophages and endothelial cells [24,25]. However, contradictory reports show that oxLDL can act as a survival factor for macrophages, inhibiting apoptosis [26]. This paradox is mostly likely due to different LDL modifications and species mediating distinct effects; while heavily oxidised LDL can cause apoptosis, minimally modified LDL acts as a growth factor [27]. In addition, no evidence of apoptosis or necrosis was seen by flow cytometry in lipid-loaded blood monocytes. While HFD caused monocytes to increase their SSC due to cytoplasmic lipid droplets, there was no decrease in forward-scatter (FSC), as would be expected if cells were shrinking and becoming apoptotic [28]. Furthermore, late-stage cell death and necrosis causes a dramatic drop in SSC as cells leak their cytoplasmic constituents, the opposite to what is observed in blood monocytes after HFD [28]. However, peritoneal macrophages are predominantly cleared via local apoptosis rather than emigration to the lymph nodes during thioglycollate peritonitis [29] and it is plausible that HFD accelerates this process, thus contributing to the decreased number of CD115+ cells in the peritoneum following HFD. In addition, monocyte/ macrophage egress from the peritoneum for splenic clearance could be accelerated by HFD and these

possibilities cannot be excluded without further cell tracking and kinetic studies using this model.

Alternatively, monocytes may be retained in the omentum during peritonitis simply due to increased adhesion. As discussed previously, oxLDL activates the endothelium to increase expression of numerous adhesion molecules, including P-Selectin, ICAM-1 and VCAM-1 [30,31], which could retain monocytes at their site of extravasation. In addition, monocytes from FH patients have significantly elevated cell surface expression of the integrins CD11c [32] and CD11b [33], accompanied by increased adhesion to an endothelial monolayer when compared to healthy controls [33]. Others have also shown monocyte CD11b [34] and CD11c [35] expression to be increased acutely during post-prandial lipaemia following a high fat meal or after incubation with TGRL. In our *Ldlr*^{-/-} model I did not see any significant increase in monocyte CD11b expression on HFD, although CD11c was up regulated in a SSC^{high} population. However, this was a subtle phenotype, as SSC^{high} CD11c^{pos} cells represented only 2% of total monocytes on HFD. Moreover, integrin function is also mediated by spatial localization and clustering [36], as well as total surface levels, which remain poorly characterized in response to plasma lipoproteins. Taken together, it seems unlikely from these data that increased surface integrin expression is a key mechanism of monocyte retention in the omentum. Further work in this thesis using human monocytes to explore monocyte- endothelial adhesion in vitro may help to clarify this issue (Chapter 5.2.4).

It is also interesting that the emergence of CD11c^{pos} SSC^{high} monocytes in *Apoe*^{-/-} mice on HFD described by Xu et al [10] is much more pronounced than observed here in the *Ldlr*^{-/-} model. While 70-80% of SSC^{high} monocytes were reported as CD11c^{pos} in the *Apoe*^{-/-} after HFD [10], this figure was closer to 10% in the *Ldlr*^{-/-}. There are several potential explanations for this, one being the severity and composition of dyslipidaemia in the *Apoe*^{-/-} animals versus the *Ldlr*^{-/-}. While in the *Ldlr*^{-/-} on HFD TGRLs peak at approximately 1500mg/dl and LDL at 1100mg/dl, these figures for the *Apoe*^{-/-} are 2300mg/dl and 1000mg/dl respectively [6]. Caution is

required in interpreting these data, as it is exceedingly rare for lipid values achieved in the *Apoe*^{-/-} on HFD to occur in a human. Even in patients with a homozygous lipoprotein lipase (LPL) deficiency characterized by very severe hypertriglyceridemia, plasma triglyceride has been reported as approximately 1500mg/dl [37], although other cases have been recorded with levels above 2000mg/dl [38]. As such, the development of CD11c^{pos} monocytes may be dose dependent on triglyceride present in plasma chylomicrons and/or VLDL.

Another possibility is that the LDLR itself is required for monocyte up-regulation of CD11c in response to HFD. While traditionally thought of as simple endocytic carrier receptors, the LDLR family are now appreciated to participate in signal transduction [39]. For example, low-density lipoprotein receptor-related protein 1 (LRP1) has been implicated in a range of signaling pathways in response to ligand binding [39], via adaptor proteins including Shc, JIP1 and JIP2 [40]. While signalling downstream of the LDLR itself is less well established, the modular adaptor protein autosomal recessive hypercholesterolemia (ARH) binds the cytoplasmic tail of the LDLR and promotes its localization in clathrin coated pits [41], and therefore may also affect other downstream targets. Conversely, APOE is involved in monocyte homeostasis via the modulation of bone marrow progenitor populations [8] and it is also possible that reconstitution of APOE would reduce the proportion of CD11c^{pos} monocytes on HFD.

Although the monocyte migratory defect described here is present in response both TG and endotoxin induced inflammation, it is possible that this phenotype is specific to the peritoneum. Since there are less CD115^{neg} F4/80^{high} mature tissue macrophages in the inflamed omentum after HFD, it may be the case that HFD is affecting the GATA-6 dependent maturation of infiltrating monocytes to peritoneal macrophages in the omentum (Figure 3.6D). The binding activity of GATA transcription factors can be modulated by LDL in endothelial cells [42], while the cholesterol, biosynthesis intermediate desmosterol induces a vast phenotypic shift in TG elicited peritoneal macrophages [14]. However, other groups have reported an in

vitro defect in macrophage chemotaxis after free- cholesterol loading, mediated by the cytoskeletal small- GTPases RHOA and RAC1 [43,44], indicating a more basic and universal mechanism may be responsible.

Interestingly, HFD in the metabolically normal *Cx3cr1^{gfp/+}* animals did not impair GR1^{low} monocyte intravascular locomotion. One simple explanation for this is that the very mild dyslipidaemia in these animals was not pronounced enough to cause a defect. While *Ldlr^{-/-}* have total cholesterol levels of around 6mmol/L on chow and up to 70mmol/L following HFD [6], we were only able to achieve a modest doubling of baseline total cholesterol from 1mmol/L to ~2.5mmol/L using HFD in the *Cx3cr1^{gfp/+}* mice. Although cytoplasmic neutral lipid was increased in monocytes following HFD, it did not take the form of the large, pronounced lipid droplets that are visible in the *Ldlr^{-/-}* monocytes on HFD. In addition, neutral lipid droplets were rarely present in the *Ldlr^{-/-}* on chow, despite total cholesterol levels which are double those found in a wild-type mouse fed HFD [6]. As such, it may be that chylomicrons and/or VLDL are driving monocyte neutral lipid accumulation, as these TGRL species are elevated in wild-type mice on HFD, but not in *Ldlr^{-/-}* fed chow [6]. The *Apoe^{-/-}* mouse would provide an interesting comparison in this context, as TGRL levels are high even when fed a chow diet [6]. Neutral lipid content has previously been reported in monocytes of *Apoe^{-/-}* mice on HFD, however this has always been compared to *Apoe^{-/-}* competent animals and not to *Apoe^{-/-}* mice on chow [4,15].

Crucially, intravascular patrolling by GR1^{low} monocytes is thought to happen during homeostasis, rather than as a response to injury or inflammation [21]. This represents another key difference to peritonitis; GR1^{low} monocytes crawl in a seemingly random, chemokinetic manner, with and against the blood flow and are not obviously polarized towards a chemotactic stimulus. Indeed, the track projections presented here (Figure 3.7I) confirm previous findings that there is no preferential migration in one direction. In contrast, TG peritonitis is a strong inflammatory stimulus that recruits monocytes predominantly through a MCP-1/ CCR2 chemokine axis, as *Mcp1^{-/-}* mice have substantially decreased monocyte recruitment to the

inflamed peritoneum [45]. Interestingly, since GR1^{low} monocytes do not express CCR2 [22] and contribute minimally to TG peritonitis [20], it is possible that the HFD-induced migratory defect is specific to classical GR1^{high} monocytes. While monocyte subpopulation tracking remains extremely challenging in vivo due to cellular plasticity and a lack of reliable reporters, these questions and others raised by these data may be addressed more easily by characterizing monocyte uptake of LDL/ VLDL and its functional impact in vitro.

3.1. References

- 1 SIMON RC, STILL WJ, O'Neal RM. The circulating lipophage and experimental atherosclerosis. *J Atheroscler Res* 1961;**1**:395–400.
- 2 Marshall JR, O'Neal RM. The lipophage in hyperlipemic rats: an electron microscopic study. *Exp Mol Pathol* 1966;**5**:1–11.
- 3 Bálint A, Veress B, Nagy Z, *et al.* Role of lipophages in the development of rat atheroma. *Atherosclerosis* 1972;**15**:7–15.
- 4 Wu H, Gower RM, Wang H, *et al.* Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. *Circulation* 2009;**119**:2708–17. doi:10.1161/CIRCULATIONAHA.108.823740
- 5 Ishibashi S, Brown MS, Goldstein JL, *et al.* Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* 1993;**92**:883–93. doi:10.1172/JCI116663
- 6 Ishibashi S, Herz J, Maeda N, *et al.* The two-receptor model of lipoprotein clearance: tests of the hypothesis in 'knockout' mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc Natl Acad Sci USA* 1994;**91**:4431–5.
- 7 Ma Y, Wang W, Zhang J, *et al.* Hyperlipidemia and Atherosclerotic Lesion Development in Ldlr-Deficient Mice on a Long-Term High-Fat Diet. *PLoS ONE* 2012;**7**:e35835. doi:10.1371/journal.pone.0035835.t002
- 8 ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. 2011;**121**:4138–49. doi:10.1172/JCI57559
- 9 Swirski FK, Libby P, Aikawa E, *et al.* Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J Clin Invest* 2007;**117**:195–205. doi:10.1172/JCI29950
- 10 Xu L, Dai Perrard X, Perrard JL, *et al.* Foamy Monocytes Form Early and Contribute to Nascent Atherosclerosis in Mice With Hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 2015;:ATVBAHA.115.305609. doi:10.1161/ATVBAHA.115.305609
- 11 Potter PK, Cortes-Hernandez J, Quartier P, *et al.* Lupus-prone mice have an abnormal response to thioglycolate and an impaired clearance of apoptotic cells. *J Immunol* 2003;**170**:3223–32.
- 12 Ploplis VA, French EL, Carmeliet P, *et al.* Plasminogen deficiency differentially affects recruitment of inflammatory cell populations in mice. *Blood* 1998;**91**:2005–9.
- 13 Konat GW. Signaling by toll-like receptors. 2008.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

- 14 Spann NJ, Garmire LX, McDonald JG, *et al.* Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell* 2012;**151**:138–52. doi:10.1016/j.cell.2012.06.054
- 15 Potteaux S, Gautier EL, Hutchison SB, *et al.* Suppressed monocyte recruitment drives macrophage removal from atherosclerotic plaques of Apoe^{-/-} mice during disease regression. *J Clin Invest* 2011;**121**:2025–36. doi:10.1172/JCI43802
- 16 Doherty NS, Griffiths RJ, Hakkinen JP, *et al.* Post-capillary venules in the ‘milky spots’ of the greater omentum are the major site of plasma protein and leukocyte extravasation in rodent models of peritonitis. *Inflamm Res* 1995;**44**:169–77.
- 17 Fukatsu K, Saito H, Han I, *et al.* The greater omentum is the primary site of neutrophil exudation in peritonitis. *J Am Coll Surg* 1996;**183**:450–6.
- 18 Okabe Y, Medzhitov R. Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell* 2014;**157**:832–44. doi:10.1016/j.cell.2014.04.016
- 19 Rosas M, Davies LC, Giles PJ, *et al.* The transcription factor Gata6 links tissue macrophage phenotype and proliferative renewal. *Science* 2014;**344**:645–8. doi:10.1126/science.1251414
- 20 Jung S, Aliberti J, Graemmel P, *et al.* Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol* 2000;**20**:4106–14.
- 21 Auffray C, Fogg D, Garfa M, *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 2007;**317**:666–70. doi:10.1126/science.1142883
- 22 Cros J, Cagnard N, Woollard K, *et al.* Human CD14dim Monocytes Patrol and Sense Nucleic Acids and Viruses via TLR7 and TLR8 Receptors. *Immunity* 2010;**32**:1–12. doi:10.1016/j.immuni.2010.08.012
- 23 Schreyer SA, Wilson DL, LeBoeuf RC. C57BL/6 mice fed high fat diets as models for diabetes-accelerated atherosclerosis. *Atherosclerosis* 1998;**136**:17–24.
- 24 HARDWICK SJ, HEGYI L, CLARE K, *et al.* Apoptosis In Human Monocyte - Macrophages Exposed To Oxidized Low Density Lipoprotein. *The Journal of Pathology* 1996;**179**:294–302. doi:10.1002/(SICI)1096-9896(199607)179:3<294::AID-PATH590>3.0.CO;2-X
- 25 M Sata KW. Oxidized LDL activates fas-mediated endothelial cell apoptosis. *J Clin Invest* 1998;**102**:1682–9. doi:10.1172/JCI3531
- 26 Hundal RS, Gómez-Muñoz A, Kong JY, *et al.* Oxidized low density lipoprotein inhibits macrophage apoptosis by blocking ceramide generation, thereby maintaining protein kinase B activation and Bcl-XL levels. *Journal of Biological Chemistry* 2003;**278**:24399–408. doi:10.1074/jbc.M209179200
- 27 Björkerud B, Björkerud S. Contrary effects of lightly and strongly oxidized LDL with potent promotion of growth versus apoptosis on arterial smooth muscle cells, macrophages, and fibroblasts. 1996;**16**:416–24. doi:10.1161/01.ATV.16.3.416
- 28 Darzynkiewicz Z, Juan G, Li X, *et al.* Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 1997.
- 29 Gautier EL, Ivanov S, Lesnik P, *et al.* Local apoptosis mediates clearance of macrophages from resolving inflammation in mice. *Blood* 2013;**122**:2714–22. doi:10.1182/blood-2013-01-478206
- 30 Gebuhrer V, Murphy JF, Bordet JC, *et al.* Oxidized low-density lipoprotein induces the expression of P-selectin (GMP140/PADGEM/CD62) on human endothelial cells. *Biochem J* 1995;**306** (Pt 1):293–8.
- 31 Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. 1995;**95**:1262–70. doi:10.1172/JCI117776

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

- 32 Mosig S, Rennert K, Krause S, *et al.* Different functions of monocyte subsets in familial hypercholesterolemia: potential function of CD14+CD16+ monocytes in detoxification of oxidized LDL. *The FASEB Journal* 2009;**23**:866–74. doi:10.1096/fj.08-118240
- 33 Weber C, Erl W, Weber KSC, *et al.* HMG-CoA Reductase Inhibitors Decrease CD11b Expression and CD11b-Dependent Adhesion of Monocytes to Endothelium and Reduce Increased Adhesiveness of Monocytes Isolated From Patients With Hypercholesterolemia. *JAC* 1997;**30**:1212–7. doi:10.1016/S0735-1097(97)00324-0
- 34 Alipour A, van Oostrom AJHHM, Izraeljan A, *et al.* Leukocyte activation by triglyceride-rich lipoproteins. 2008;**28**:792–7. doi:10.1161/ATVBAHA.107.159749
- 35 Gower RM, Wu H, Foster GA, *et al.* CD11c/CD18 Expression Is Upregulated on Blood Monocytes During Hypertriglyceridemia and Enhances Adhesion to Vascular Cell Adhesion Molecule-1. 2010;**31**:160–6. doi:10.1161/ATVBAHA.110.215434
- 36 Roca-Cusachs P, Gauthier NC, Del Rio A, *et al.* Clustering of alpha(5)beta(1) integrins determines adhesion strength whereas alpha(v)beta(3) and talin enable mechanotransduction. *PNAS* 2009;**106**:16245–50. doi:10.1073/pnas.0902818106
- 37 Sugandhan S, Khandpur S, Sharma VK. Familial chylomicronemia syndrome. *Pediatr Dermatol* 2007;**24**:323–5. doi:10.1111/j.1525-1470.2007.00415.x
- 38 Benlian P, De Gennes JL, Foubert L, *et al.* Premature atherosclerosis in patients with familial chylomicronemia caused by mutations in the lipoprotein lipase gene. *N Engl J Med* 1996;**335**:848–54. doi:10.1056/NEJM199609193351203
- 39 May P, Bock HH, Herz J. Integration of endocytosis and signal transduction by lipoprotein receptors. *Sci STKE* 2003;**2003**:PE12–pe12. doi:10.1126/stke.2003.176.pe12
- 40 Gotthardt M, Trommsdorff M, Nevitt MF, *et al.* Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. *Journal of Biological Chemistry* 2000;**275**:25616–24. doi:10.1074/jbc.M000955200
- 41 Garuti R, Jones C, Li W-P, *et al.* The modular adaptor protein autosomal recessive hypercholesterolemia (ARH) promotes low density lipoprotein receptor clustering into clathrin-coated pits. *Journal of Biological Chemistry* 2005;**280**:40996–1004. doi:10.1074/jbc.M509394200
- 42 Lin JH, Zhu Y, Liao HL, *et al.* Induction of vascular cell adhesion molecule-1 by low-density lipoprotein. *Atherosclerosis* 1996;**127**:185–94.
- 43 Nagao T, Qin C, Grosheva I, *et al.* Elevated cholesterol levels in the plasma membranes of macrophages inhibit migration by disrupting RhoA regulation. 2007;**27**:1596–602. doi:10.1161/ATVBAHA.107.145086
- 44 Pagler TA, Wang M, Mondal M, *et al.* Deletion of ABCA1 and ABCG1 impairs macrophage migration because of increased Rac1 signaling. *Circulation Research* 2011;**108**:194–200. doi:10.1161/CIRCRESAHA.110.228619
- 45 Takahashi M, Galligan C, Tessarollo L, *et al.* Monocyte chemoattractant protein-1 (MCP-1), not MCP-3, is the primary chemokine required for monocyte recruitment in mouse peritonitis induced with thioglycollate or zymosan A. *The Journal of Immunology* 2009;**183**:3463–71. doi:10.4049/jimmunol.0802812

4. UPTAKE OF LDL AND VLDL BY MONOCYTES

4.1. Introduction

Although mechanism and functional impact have been only loosely described, there are previous descriptions of human monocyte lipid loading. In some instances, this is concerning the post-prandial accumulation of what is presumed to be free fatty acids or chylomicrons following a high fat meal [1]. In a similar study, carbon-13 (^{13}C) labelled palmitic acid was used to track dietary fatty acids into blood leukocytes in the post-prandial state, confirming at least in part that intracellular neutral lipid accumulation is due to uptake and not a biosynthetic pathway [2]. In addition, monocytes from FH patients have been shown to have impaired phagocytosis of oxLDL [3]. In the mouse, there is also some evidence that neutral lipid accumulation in monocytes from *Ldlr*^{-/-} animals is via TLR-4 dependent pinocytosis [4]. However, very little work has been performed in vitro on monocyte LDL and VLDL uptake and the mechanisms of cell entry remain largely undetermined in monocyte subpopulations. In addition, there is little evidence that the pronounced neutral lipid accumulation described here in the *Ldlr*^{-/-} mouse in Chapter 3 occurs in human monocytes, or indeed whether this accumulation is dependent on endocytosis or on an intracellular biosynthetic pathway. Based on previous work in macrophages there are several candidate pathways for this phenotype that could be explored.

4.1.1. LDL and VLDL modification

While LDL exists in circulation in its native, unmodified form (nLDL), it is also able to undergo a spectrum of oxidative modification to generate a variety of LDL species that are often collectively termed 'oxidised LDL' (oxLDL). While there are numerous groups who have isolated LDL with a negative shift in electrophoretic mobility from human and mouse plasma [5-7], and associated this presence with the progression and severity of CVD [8,9] the mechanistic details of LDL oxidation in vivo remain largely the subject of speculation and conjecture. Heavy oxidative modification of

LDL was initially modelled in vitro using acetylation, however these LDL species have never been proven to exist in vivo. Furthermore, it is clear that the mechanism of uptake of oxLDL in macrophages depends on the extent of its oxidation [10]. While minimal oxidative modification of LDL (mmLDL) can lead to fatty acid peroxidation, cholesterol conversion to oxysterols and phospholipid oxidation, more extensive copper-mediated oxidation often utilized in vitro can extensively alter APOB protein structure via the production of reactive aldehydes including malondialdehyde (MDA) [11]. There is clear evidence that mmLDL is present in the plasma, however the presence of heavily oxidised LDL in the blood seems unlikely. There is a high concentration of anti-oxidants such as vitamin C and uric acid in the plasma that may prevent any extensive oxidation and oxLDL has an extremely short half-life in the blood when injected into both rats and rabbits [12]. As such, evidence suggests that heavily oxidised LDL is irrelevant to any phenotype seen in blood monocytes and will not be discussed further.

VLDL is less well described, but can also go undergo oxidative modification although contradictory reports exist regarding the dependency on VLDL modification or oxidation for its uptake by macrophages [13-15]. In our hands LDL undergoes rapid minimal oxidative modification during in-vitro use and storage in air (autoxidation), as assessed by conjugated diene formation and previously described [11], although interestingly this does not happen to VLDL (Figure 4.1). As such, all references to 'LDL' henceforth refer to LDL that has undergone mild oxidative modification. The oxidation of LDL has been clearly demonstrated to be a lipid peroxidation process, where polyunsaturated fatty acids undergo peroxidation and decomposition to a complex mix of reactive aldehyde species such as malondialdehyde and other related by-products [16,17]. During autoxidation of LDL, polyunsaturated linoleic and arachidonic acid are the main targets of this process, while other saturated and monounsaturated species are relatively resistant to peroxidation [17]. Since I do not observe autoxidation of VLDL in this manner, it is possible that our VLDL species have already been oxidized during isolation, or that differences in the fatty-acid composition of VLDL compared to LDL makes it less susceptible to this process.

4.1.2. The LDL Receptor

Discovered in Nobel Prize winning work by Brown and Goldstein, the LDLR is the canonical pathway of nLDL uptake into peripheral tissues during homeostasis, most notably the liver [18]. LDL and IDL bind cell surface LDLR and are internalized on average within 10 minutes via receptor-mediated endocytosis into clathrin-coated pits [18]. Once internalized, LDL is trafficked to lysosomes for degradation and hydrolysis of cholesterol esters, while the receptor itself is recycled to the cell surface [18]. Deficiency of the LDLR, as found in *Ldlr*^{-/-} mouse and in FH patients, elevates LDL both by delaying its removal from the blood directly and by delaying the removal of its precursor IDL, which leads to increased LDL synthesis [18]. In the context of moderate, VLDL-driven dyslipidaemia in metabolically normal mice fed a HFD after being reconstituted with *Ldlr*^{-/-} bone marrow, the macrophage LDLR is crucial in the formation of foam cells and the atherosclerotic plaque [19]. In addition, the LDLR facilitates VLDL uptake in macrophages and trafficking to distinct intracellular compartments to LDL [13,20]. However, given that *Ldlr*^{-/-} animals succumb to rapid and severe atherosclerosis characterized by macrophage foam cell formation when fed HFD, clearly this is not the case in extreme hypercholesterolemia [21]. In addition, most FH patients with homozygous *LDLR* defects do not survive into their second decade due to advanced cardiovascular disease [22]. As such, the role of the LDLR in macrophage foam cell formation remains unclear.

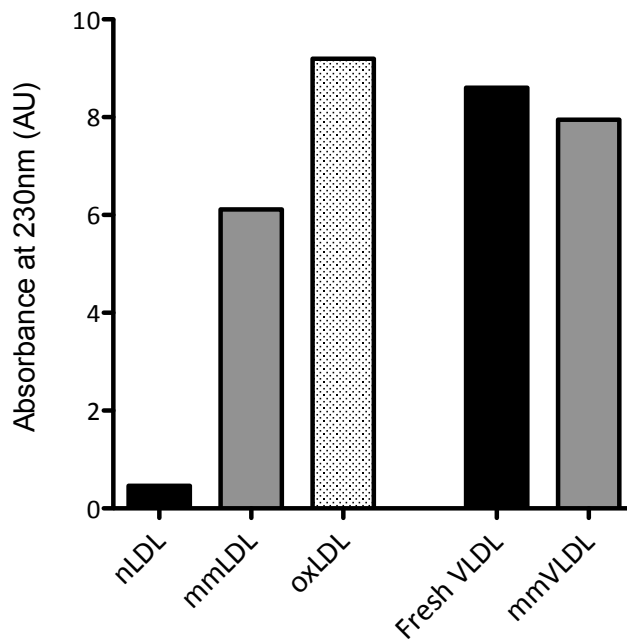


Figure 4.1 Measurement of lipoprotein modification by conjugated diene content.

Conjugated diene content was measured by absorbance at 230nm in fresh, native-LDL (nLDL), LDL modified by storage at 4°C in air (mmLDL), copper-oxidised LDL (oxLDL), fresh VLDL and VLDL modified by storage at 4°C in air (mmVLDL). One representative experiment is shown of two.

4.1.3. Scavenger receptors

Once LDL has undergone a degree of oxidative modification, as discussed above, high- affinity binding and endocytosis becomes possible via scavenger receptors including Macrophage scavenger receptor 1 (MSR1)[23] and the thrombospondin receptor Cluster of differentiation 36 (CD36)[24]; indeed MSR1 was first isolated and characterized due to its ability to bind modified LDL [23]. Scavenger receptors are an extremely heterogeneous and functionally versatile family, consisting of 8 classes with little homology between them [25]. Their ligands are numerous and can generally be classes as danger-associated molecular patterns (DAMPs) which require clearance for the maintenance of homeostasis or the elimination of disease, including lipopolysaccharide (LPS) and lipoteichoic acid (LTA) on the surface of bacteria and phosphatidylserine on apoptotic cells [25].

In the context of oxLDL, binding to CD36 or MSR1 on macrophages leads to unregulated uptake. Internalization of oxLDL activates the transcription factor Peroxisome proliferator-activated receptor gamma (PPAR- γ) via accumulation of the fatty acids 9-HODE and 13-HODE, consequently increasing the expression of CD36 and thus perpetuating the cycle of oxLDL uptake [26,27]. Interestingly, CD36 shares significant sequential homology with myelin fatty acid binding protein (MFABP), which is involved in the intracellular transport of fatty acids, leading to postulation that CD36 possesses a fatty acid binding site at residues 127 and 279[28,29], although multiple sites have subsequently been suggested [30]. In vitro, CD36 will bind long chain, but not short chain FAs and facilitate their translocation across the plasma membrane [29]. However, it was subsequently discovered that this was not due to direct fatty acid translocase activity, but instead resulting from the facilitation of intracellular esterification of FAs into triacylglycerols, thus increasing the rate of FFA uptake [31]. In addition FA binding also increases the binding and uptake of oxLDL via CD36, potentially via altering CD36 conformation [30]. Interestingly this is particularly true for elaidic acid [30], which is a trans-fatty acid highly abundant in the atherosclerotic plaque, although the role of elevated free fatty acids as a risk factor for atherosclerosis remains largely unsupported by epidemiological evidence [32]. In both familial hypercholesterolemia patients [3] and *Apoe*^{-/-} mice [33], dyslipidaemia

has also been reported to up-regulate monocyte surface levels of CD36, potentially perpetuating a feedback loop of further lipid uptake.

Scavenger receptors have also been implicated in cellular uptake and metabolism of VLDL. Scavenger receptor BI (SR-BI) binds to VLDL in vitro [34] and its in vivo deletion significantly delays plasma clearance of injected VLDL, predominantly by the liver [35]. In addition, SR-BI is required for hepatic VLDL production, potentially via the uptake of HDL cholesterol esters that contribute to a pool from which VLDL can be synthesised [36]. In addition, SR-BI may act synergistically with CD36, heparan sulphate proteoglycans and cell surface lipases to mediate the hydrolysis and uptake of FFAs from TGRLs [37,38], although the tissue specificity and exact mechanisms of this pathway are still being explored. Following the observation that hypercholesterolemia leads to an accumulation of neutral lipid droplets in mouse monocytes, I then aimed to determine whether this occurs in vitro in human and mouse monocytes and examine some of the likely candidates for neutral lipid entry discussed above.

4.2. Results

4.2.1. Neutral lipid accumulation after LDL/VLDL treatment

Initially I sought to confirm that LDL or VLDL treatment causes neutral lipid droplet accumulation in human monocytes, similar to that seen in the *Ldlr*^{-/-} mouse after HFD. Human monocytes were isolated, separated into CD16^{pos} and CD16^{neg} subpopulations and treated with 100µg/ml LDL or VLDL for 30 - 150 minutes. When incubated with 100 µg/mL LDL or VLDL for the longest time point of 150 minutes, both monocyte subsets accumulated cytoplasmic neutral lipid vesicles. In VLDL treated cells, vesicles were present in approximately 70% of CD16^{neg} cells and 40% of CD16^{pos} (Figure 4.2A), while in LDL treated cells this was slightly increased to 100% and 50% respectively (Figure 4.2B). When quantified as the more sensitive metric of number of lipid vesicles per cell over time, peak accumulation was at 30 minutes in VLDL treated cells (Figure 4.2C). CD16^{neg} monocytes containing approximately 15 lipid vesicles per cell and CD16^{pos} approximately half this number. A similar phenotype was seen with LDL treatment and although peak lipid accumulation was delayed to 60 minutes the number of vesicles per cell was largely identical to VLDL treatment (Figure 4.2D). Representative images are shown in Figure 4.2E, and appear to be visually similar to blood monocytes from the *Ldlr*^{-/-} mouse on HFD. In addition, the relationship between LDL/ VLDL treatment and neutral lipid accumulation was investigated further using a dose curve of LDL and VLDL, including 0, 20, 100 and 500µg/ml of lipoprotein for 1 hour. As observed in the time course in Figure 4.2A-D, CD16^{neg} monocytes have approximately double the number of lipid droplets of CD16^{pos} cells after 60 minutes of treatment with VLDL (Figure 4.2F) and LDL (Figure 4.2G). An increasing concentration of both lipoproteins increased intracellular neutral lipid and this was most pronounced up to a concentration of 100µg/ml (Figure 4.2F-G). In CD16^{neg} monocytes treated with LDL, no additional effect of 500µg/ml was seen compared to 100µg/ml, suggesting that 100µg/ml LDL represents a saturating dose of lipid in these cells (Figure 4.2G). Given these data, 100µg/ml was chosen as an appropriate dose of LDL and VLDL for future experiments.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson- January 2016

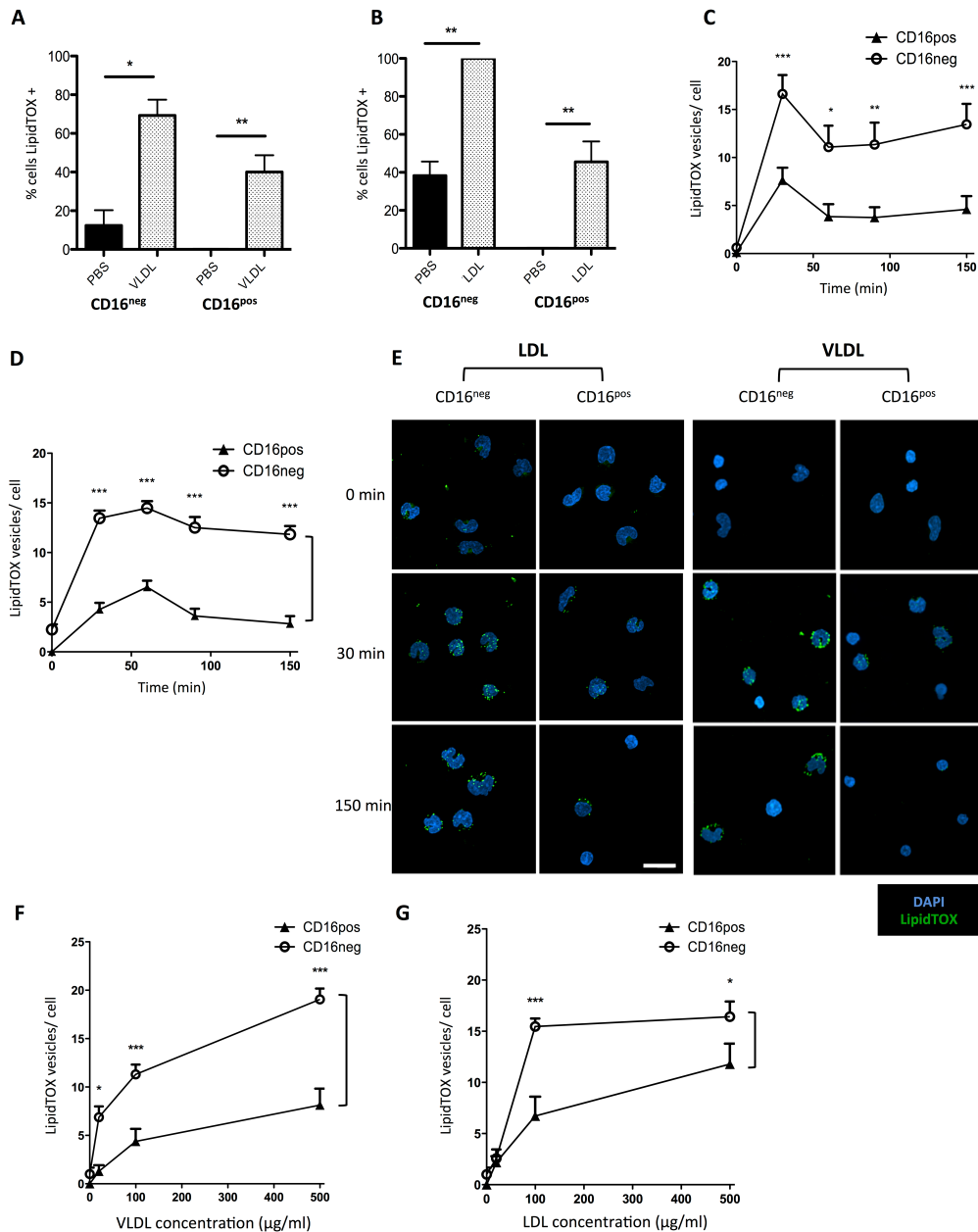


Figure 4.2 Lipid accumulation over time in LDL/ VLDL treated human monocytes.

Human CD16^{pos} and CD16^{neg} monocytes were purified and treated with LDL or VLDL (100μg/ml) and intracellular neutral lipid was quantified using LipidTOX by confocal microscopy. (A) Percentage cells neutral lipid positive after 150mins VLDL treatment (n=10 fields). (B) Number of neutral lipid vesicles per cell over time after VLDL treatment (n=25-50 cells). (C-D) Same as A-B respectively with LDL treatment. (E) Representative examples of neutral lipid staining, scale bar represents 20μm. (F-G) CD16^{pos}/CD16^{neg} monocytes were treated with 0, 20, 100 or 500μg/ml of (F) VLDL or (G) LDL for 1 hour and number of neutral lipid vesicles per cell was quantified using LipidTOX by confocal microscopy, n=30-35 cells per condition. One representative experiment is shown from two. Error bars show the mean±SEM. In A-B, groups were compared using a two-tailed Mann Whitney U test. In C-D and F-G, groups were compared using a mixed ANOVA with a post-test following the Bonferroni method.

ABCA1/ ABCG1 knockout in macrophages has previously been shown to increase membrane cholesterol content and cause functional defects, although this was not observed in LDL treated cells [39]. To investigate this possibility I used filipin staining to analyse non-esterified membrane cholesterol content by confocal microscopy. LDL and VLDL treatment at 100µg/ml for 120 minutes did indeed cause a modest increase in membrane cholesterol measured by filipin staining, however this only occurred in CD16^{neg} monocytes (Figure 4.3A). Interestingly, it was VLDL that caused a more pronounced phenotype than LDL, although both were subtle. Representative images of filipin staining are shown in Figure 4.3B and unlike the neutral lipid vesicle accumulation the phenotype is not visually obvious.

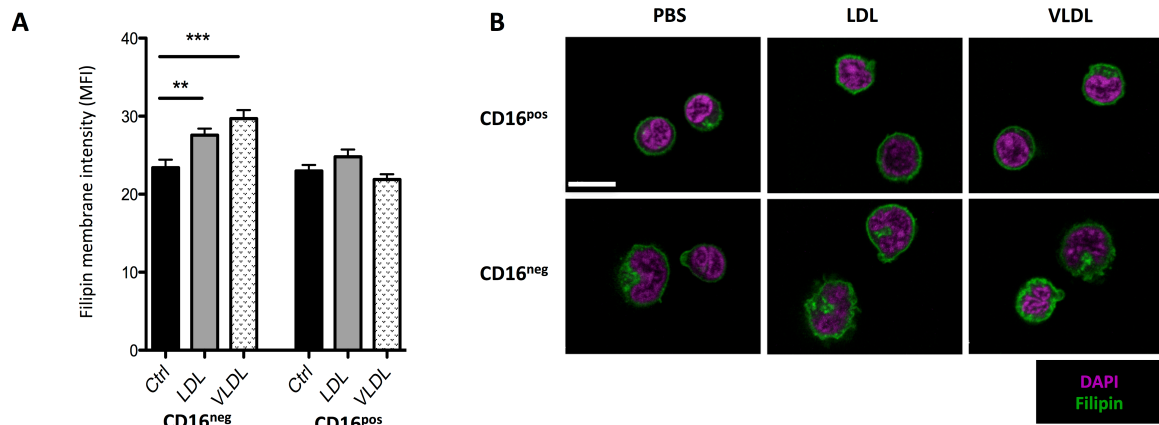


Figure 4.3 Membrane cholesterol staining in human monocytes after LDL/ VLDL treatment.

Human CD16^{pos} and CD16^{neg} monocytes were purified and treated with LDL or VLDL (100µg/ml). (A) Membrane cholesterol content in monocytes was quantified by filipin staining intensity. (B) Representative examples of filipin cholesterol staining, scale bar represents 10µm. n=45-60 cells from two pooled experiments, error bars show the mean±SEM. Groups were compared using a two-tailed Mann Whitney U test.

4.2.2. Monocyte uptake of fluorescent LDL and VLDL

Given that neutral accumulation occurs in human monocytes after LDL and VLDL treatment, I then investigated whether these lipid droplets could be due to uptake of complete lipoprotein particles or whether LDL/ VLDL exposure was stimulating mobilization of neutral lipid from another source. To do this, I utilized fluorescently labelled LDL and VLDL species and attempted to detect uptake in monocytes using flow cytometry. Whole leukocytes were isolated from human blood from healthy donors and incubated for 30-120 minutes with or without 100µg/ml DIO-labelled LDL or VLDL. Monocyte subpopulations were distinguished by fluorescent antibody staining for surface antigen expression, with monocytes defined as SSC^{int}, HLA-DR+, Lineage^{neg}, as shown in Figure 1.2. In LDL and VLDL treated cells, the percentage of cells containing fluorescence signal from the DIO-labelled lipoproteins did rise over time, potentially indicating uptake was taking place. Following LDL treatment approx. 25% of CD16^{neg} and 15% of CD16^{pos} monocytes were LDL-positive (LDL+) after 120 minutes (Figure 4.4A). Of these cells, the LDL median fluorescence intensity (MFI) was not significantly different between the two subpopulations, although there was a trend towards less LDL uptake in CD16^{pos} cells (Figure 4.4B), consistent with previous neutral lipid accumulation data (Figure 4.2C). For VLDL, uptake levels were lower and the kinetics appeared to be delayed; at 120 minutes incubation approx. 15% of CD16^{pos} and 10% of CD16^{neg} monocytes were VLDL+, although this difference was not significant (Figure 4.4C). There was also a concurrent trend towards slightly less VLDL in CD16^{pos} monocytes at 120 minutes, as measured by MFI (Figure 4.4D). Representative histograms of LDL or VLDL- DIO signal from monocyte subpopulations are shown in Figure 4.4E and Figure 4.4F respectively, which display a positive shift in LDL/VLDL signal after incubation without clear positive or negative populations.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson - January 2016

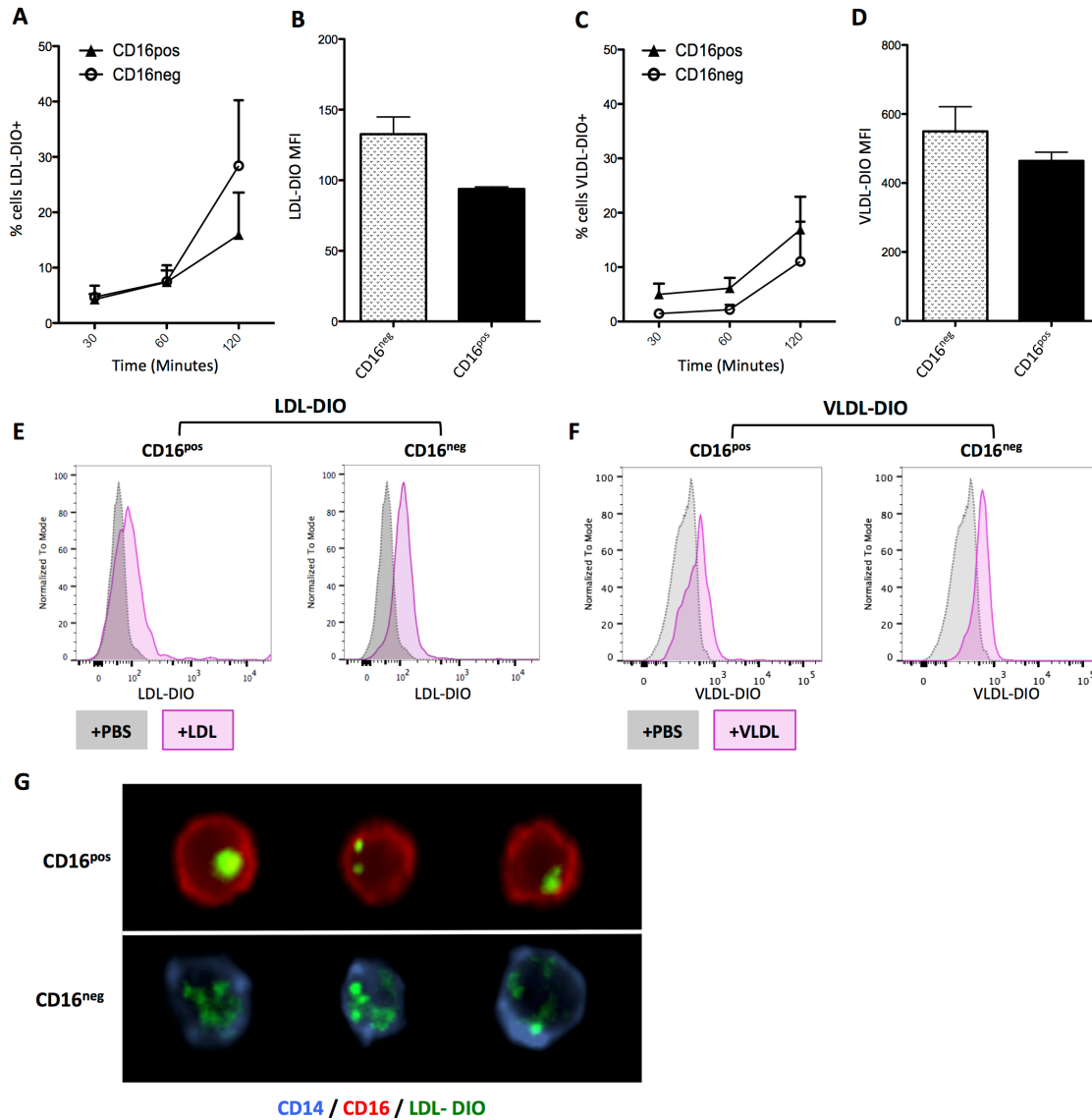


Figure 4.4 Uptake of fluorescent LDL and VLDL by human monocyte subpopulations. Human blood leukocytes were purified and treated with DIO-labeled LDL or VLDL (100µg/ml). Lipoprotein uptake was assessed in CD16^{pos} and CD16^{neg} monocytes using fluorescent antibody staining and flow cytometry, with monocytes defined as SSC^{int}, HLA-DR+, Lineage^{neg}, n=3 donors. (A) Percentage of CD16^{pos} and CD16^{neg} monocytes that were LDL+ over time. (B) LDL-DIO median fluorescence intensity (MFI) of LDL+ monocytes after 120 minutes incubation. (C) and (D) represent the same data for VLDL-DIO treatment. (E-F) Representative histograms of (E) LDL-DIO and (F) VLDL-DIO signal intensity in untreated total leukocytes (grey) or monocyte subpopulations after 120 minutes LDL/VLDL treatment (magenta). (G) Representative ImageStream micrograph from 3 independent experiments of CD16^{pos} and CD16^{neg} monocytes after 120 minutes treatment with LDL-DIO. Error bars show the mean±SEM.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson- January 2016

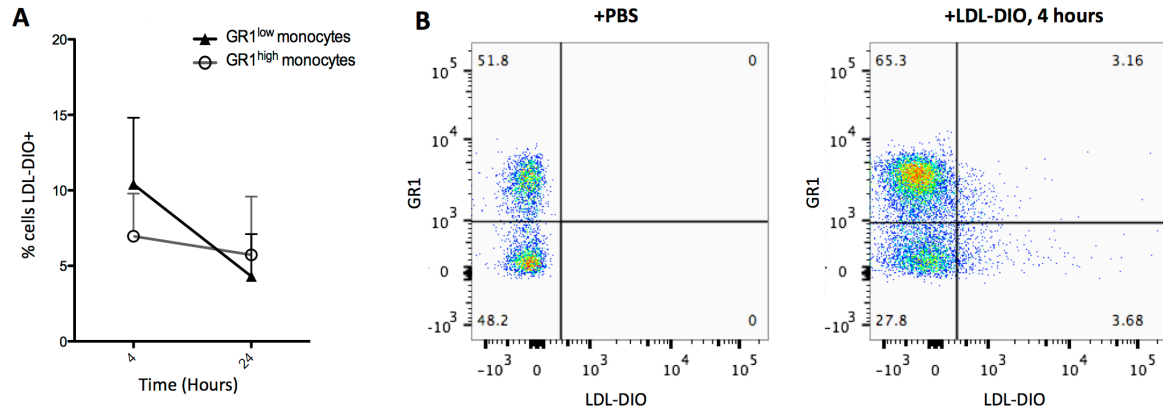


Figure 4.5 Uptake of fluorescent LDL by mouse monocytes in vivo.

C57Bl/6 mice were injected IV with 25µg LDL-DIO per mouse. Blood GR1^{high} and GR1^{low} monocytes were analyzed for LDL uptake at 4 and 24 hours post-injection, n=3 mice per group. (A) Percentage of cells LDL-positive. (B) Representative flow cytometry plots of LDL signal in GR1^{high} and GR1^{low} monocytes (Gated as CD11b^{pos} CD115^{pos}). Error bars show the mean±SEM.

Due to the subtle nature of the uptake as measured by flow cytometry, I decided it was important to try and confirm that: 1) the LDL and VLDL were entering the cell and not stuck to the outside of the cell membrane and 2) the signal from the DIO channel was not due to non-specific labelling of the cells due to free dye in the lipoprotein preparations. To eliminate some of this possibility I utilized Amnis ImageStream technology to analyse LDL-DIO uptake, which allows for high-resolution fluorescence microscopy during flow cytometry. Cells were treated the same as for conventional flow cytometry and representative images of LDL+ monocyte subpopulations are shown in Figure 4.4G. Clear intracellular punctae of DIO signal were seen in LDL⁺ cells and the signal was not outside of the cell membrane. In addition CD16^{pos} monocytes seemed to contain less LDL punctae than CD16^{neg}, which is in agreement with conventional flow cytometry LDL-MFI data (Figure 4.4B).

To confirm that fluorescent LDL uptake also occurred in mouse monocytes *in vivo*, wild-type C57Bl/6 mice were intravenously injected with 25µg of LDL-DIO. Blood was taken at 4 and 24 hours post-injection in order to assess GR1^{high} and GR1^{low} monocyte (CD115^{pos} CD11b^{pos} SSC^{int}) LDL uptake by flow cytometry. Approximately 7% of GR1^{high} and 10% of GR1^{low} monocytes had internalized LDL after 4 hours, and there was a subtle fall in these proportions in both subsets by 24 hours (Figure 4.5A-B). Therefore, even at this low dose, LDL uptake can be seen in mouse monocytes within 4 hours of exposure.

4.2.3. Blocking monocyte LDL/ VLDL uptake

While neutral lipid accumulation in the *Ldlr*^{-/-} mouse would suggest that the LDLR is not involved in LDL/ VLDL uptake in mouse monocytes, at this stage there was no clear evidence regarding the LDL or VLDL uptake mechanism in human monocyte subpopulations. As such, I attempted to utilize receptor-blocking strategies to determine the uptake pathways for these lipoproteins. Initially, I targeted the LDLR family using Low density lipoprotein receptor-related protein-associated protein 1

(RAP). RAP is an endoplasmic reticulum- resident protein that acts as a chaperone, preventing ligand binding and facilitating receptor maturation during the intracellular transport of the LDLR, low-density lipoprotein receptor-related protein (LRP) and other members of the LDLR family [40,41]. An excess of exogenous RAP binds to LDLR family members and antagonizes lipoprotein binding in an allosteric manner [40]. Therefore, cells were incubated with 1.25 μ M RAP for 1 hour, which is greater than the previously reported saturating concentration for blocking LDLR family members [42]. Monocytes were then treated with LDL-DIO and VLDL-DIO and assessed for uptake by flow cytometry as described above. RAP treatment resulted in an approx. 50% decrease in percentage of CD16^{neg} monocytes that were LDL+, and a trend towards less CD16^{pos} LDL+ cells (Figure 4.6A). However, this was only accompanied by a slight decrease in LDL signal intensity in monocytes when assessed by histogram (Figure 4.6B). Similar results were seen for VLDL, with modest decreases in uptake following RAP treatment in both CD16^{neg} and CD16^{pos} subpopulations (Figure 4.6C-D).

Another likely candidate for LDL and VLDL uptake in monocytes was the class-B scavenger receptor CD36. Antagonism of CD36 binding was performed using a blocking antibody as previously described [43], with monocytes treated at a concentration of 10 μ g/ml for 1 hour prior to 2 hours of LDL-DIO or VLDL-DIO treatment. CD36 blockade led to a greater than 5-fold decrease in the percentage of monocytes LDL+ in both subpopulations, although this change was only significant in CD16^{neg} cells (Figure 4.6E). This was accompanied by a notable shift in LDL-DIO histogram peak in all monocytes (Figure 4.6F). However, anti-CD36 treatment had no significant effect on percentage of monocytes to uptake VLDL, with a trend towards less uptake in the CD16^{neg} subset (Figure 4.6G-H). Given that neither the LDLR family nor CD36 seemed to be major contributors to VLDL uptake; I proceeded to examine other candidate pathways. Previously there has been some suggestion that class-A scavenger receptors are involved in VLDL uptake [44] and this group of receptors can be competitively blocked using dextran sulphate (DS) treatment [45]. Using chondroitin sulphate (CS) as a control glycosaminoglycan (GAG), monocytes were treated with/ without 100 μ g/ml CS or DS for 1 hour before

LDL or VLDL treatment and analysis by flow cytometry as above. However, this methodology had two major technological problems: 1) the CS control increased the percentage of monocytes which uptake VLDL compared to PBS (Figure 4.6I) and 2) CS abolished any CD16 staining in monocytes (Figure 4.6J-K). It was thus impossible to make any conclusions regarding the ability of DS to block VLDL uptake, or to make any comparisons between monocyte subpopulations. This issue existed on every occasion the experiment was performed and resulted in no further investigation of the class-A scavenger receptors in this context.

To additionally confirm that the LDLR played on a minor role in LDL and VLDL uptake in monocytes, murine *Ldlr*^{-/-} total blood monocytes were purified and treated with 100µg/ml LDL or VLDL-DIO for 2 hours as in previous experiments. By flow cytometry, loss of the LDLR did not significantly decrease the percentage of monocytes positive for LDL or VLDL uptake (Figure 4.6L).

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

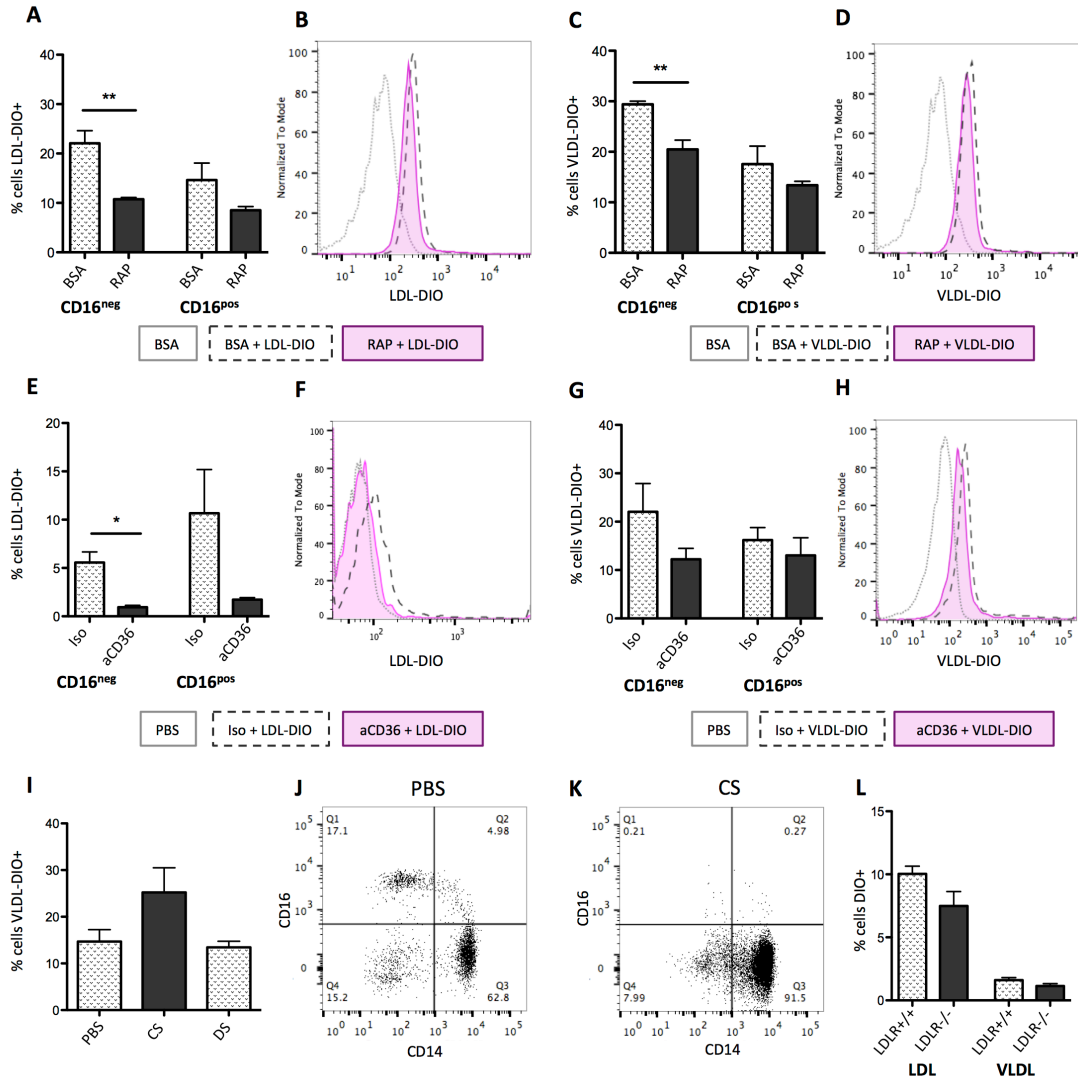


Figure 4.6 Blocking LDL/ VLDL uptake via the LDLR-family, CD36 and Class-A Scavenger Receptors.

In all experiments, human blood leukocytes were purified and lipoprotein uptake was assessed in CD16^{pos} and CD16^{neg} monocytes using fluorescent antibody staining and flow cytometry, n=3 donors. To block the LDLR family, cells were pre-treated with 50µg/ml RAP or BSA control, 30 minute and then treated with DIO-LDL or VLDL (100µg/ml), 2 hours. (A) Percentage of cells LDL-positive. (B) Representative histogram of LDL uptake with/ without RAP pre-treatment. (C) and (D) show the same experiment with VLDL. To block CD36, cells were pre-treated with 10µg/ml anti-CD36 antibody for 1 hour and then treated with DIO-LDL or VLDL (100µg/ml), 2 hours. (E) Percentage of cells LDL-positive. (F) Representative histogram of LDL uptake with/ without RAP pre-treatment. (G) and (H) show the same data for VLDL. To block class-A scavenger receptor, cells were treated with 100µg/ml DS or CS control. (I) Percentage monocytes VLDL- positive, (J-K) Representative flow cytometry plots of monocyte (SSC^{int}, HLA-DR+) CD14 and CD16 staining after (J) PBS treatment or (K) CS treatment. Monocytes from wild-type or *Ldlr*^{-/-} mice were purified and treated with DIO-LDL or VLDL (100µg/ml), 2 hours, n=4 pooled technical replicates. (L) Percentage of cells monocytes LDL or VLDL positive. Error shows the mean ± SEM, groups were compared using a two-tailed Mann Whitney U test.

4.2.4. Blocking monocyte neutral lipid accumulation

Given the relatively low percentage of monocytes which uptake exogenous LDL/VLDL by flow cytometry (10-20%) and the generally weak signal obtained from internalized LDL or VLDL-DIO using this method, I decided to investigate uptake mechanisms further using neutral-lipid staining and confocal microscopy following LDL or VLDL treatment. As shown in Figure 4.2, 40-100% of monocytes are lipid-positive following treatment using this metric, and the exact number, size and localization of lipid vesicles can be accurately assessed. Initially, I sought to confirm the observation made by flow cytometry that CD36-blocking impairs LDL uptake in monocytes. Monocyte subpopulations were treated with an IgM isotype control antibody or an anti-CD36 antibody for 1 hour as described above, followed by 2 hours of LDL treatment. Cells were then stained for neutral lipid and analysed by confocal microscopy. Intriguingly, although LDL significantly increased neutral lipid vesicle number in both monocyte subpopulations as expected, anti-CD36 treatment did not impair this lipid accumulation (Figure 4.7A-B). This raised the interesting possibility that the neutral lipid vesicles observed on microscopy after LDL or VLDL exposure do not represent complete endocytosed lipoprotein particles, but are in fact generated via a separate uptake or biosynthetic pathway.

For VLDL, others have previously described a pathway in smooth muscle cells and macrophages where the lipoprotein binds to heparan sulphate proteoglycans (HSPG) on the cell surface, which facilitates the hydrolysis of triacylglycerol to free fatty acids by membrane bound lipases, most notably lipoprotein lipase (LPL) [37]. This process could occur independently of scavenger receptors and could explain the discrepancy between LDL endocytosis and neutral lipid accumulation. To test this possibility monocyte subsets were pre-treated with 5UN/ ml mixed heparinase-I and heparinase-III for 1 hour in order to cleave cell surface HSPGs, as previously described [46]. Subsequently monocytes were exposed to LDL or VLDL and imaged as in previous experiments. Heparinase treatment resulted in an almost complete inhibition of VLDL- induced neutral lipid vesicle formation in CD16^{pos} monocytes,

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

while no effect was seen in the CD16^{neg} subset (Figure 4.8A-B). A similar effect was also seen following LDL treatment, restricted to CD16^{pos} monocytes (Figure 4.8C-D).

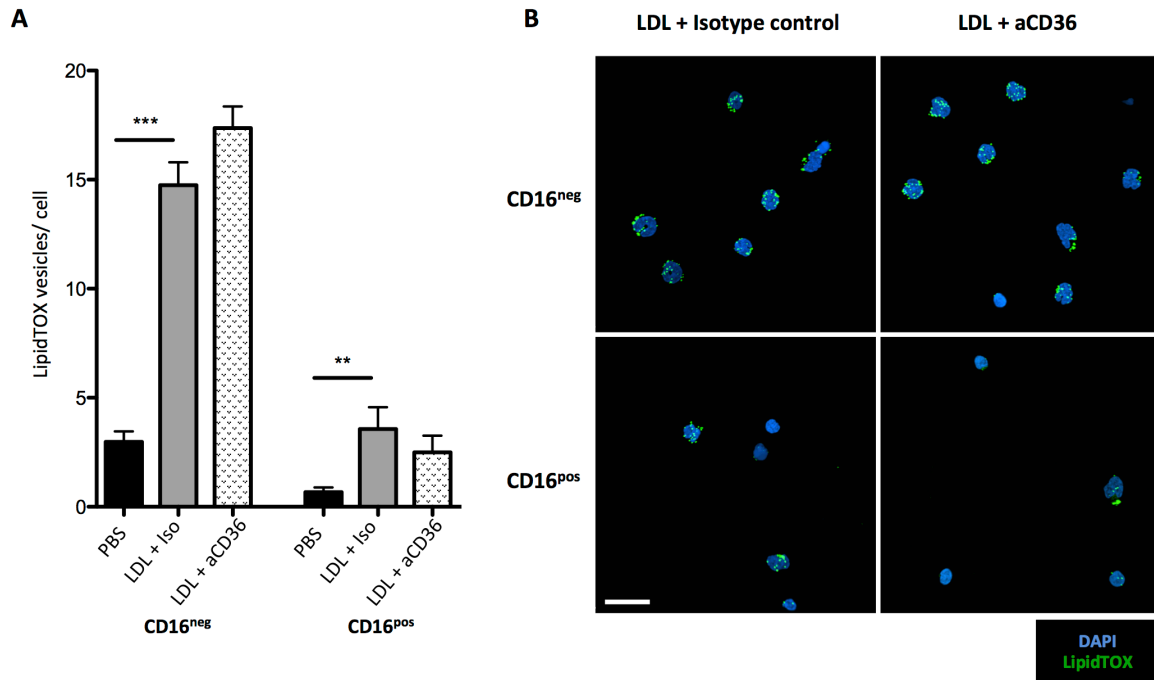


Figure 4.7 The effect of anti- CD36 blocking antibody on LDL induced neutral lipid accumulation.

Human CD16^{pos} and CD16^{neg} monocytes were purified and treated with 10µg/ml anti-CD36 or IgM isotype control antibody for 1 hour. LDL or VLDL (100µg/ml) was then added for 2 hours and intracellular neutral lipid was quantified using LipidTOX by confocal microscopy, n=30-45 cells. (A) Number of neutral lipid vesicles per cell. (B) Representative examples of neutral lipid staining, scale bar represents 20µm. Error bars show the mean±SEM, groups were compared using a two-tailed Mann Whitney U test.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson - January 2016

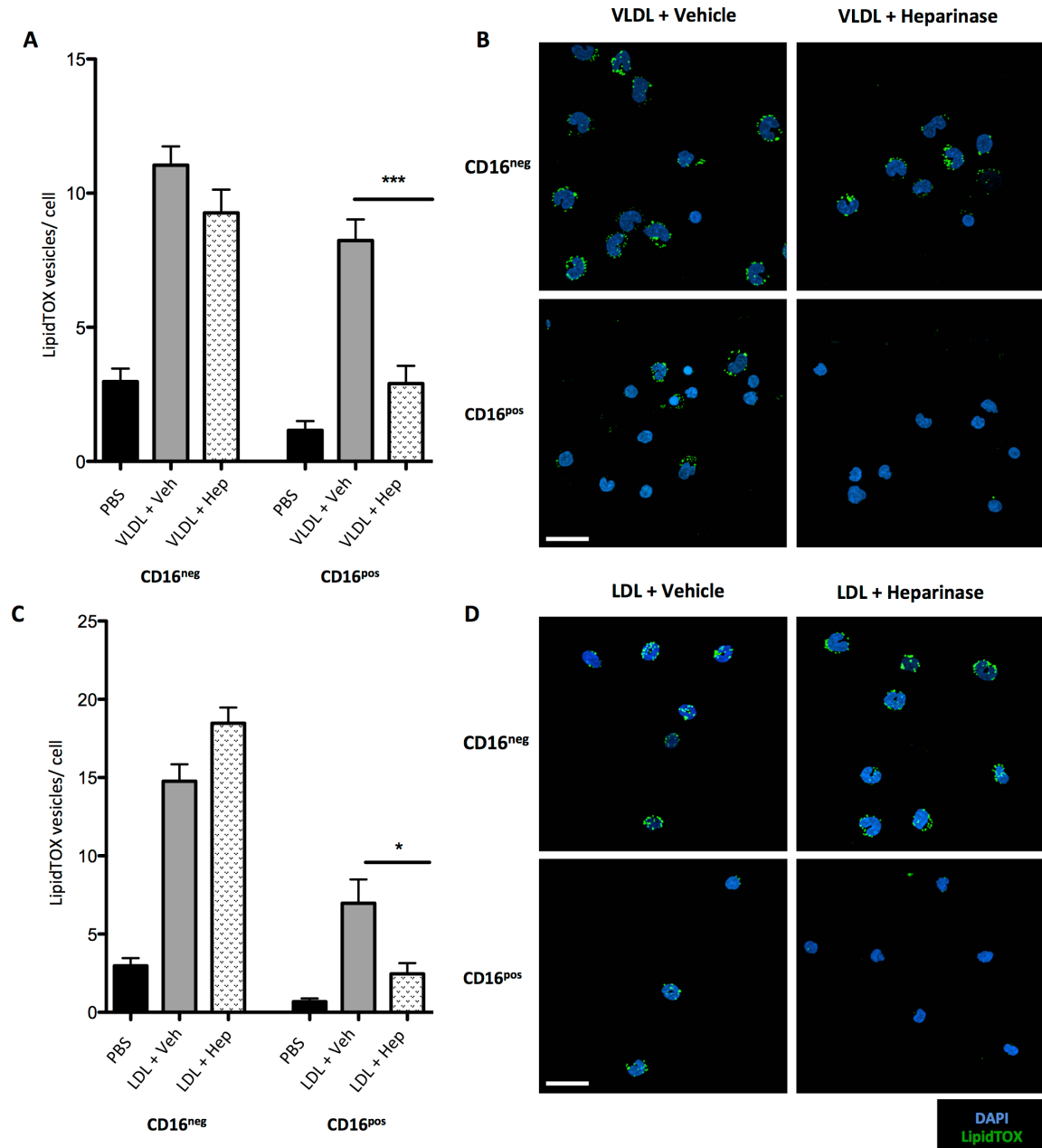


Figure 4.8 The effect of heparinase treatment on LDL and VLDL induced neutral lipid accumulation.

Human CD16^{pos} and CD16^{neg} monocytes were purified and treated with 5UN/ml heparinase or vehicle control for 1 hour. LDL or VLDL (100µg/ml) was then added for 2 hours and intracellular neutral lipid was quantified using LipidTOX by confocal microscopy, n=35-50 cells, data shows 1 representative experiment of 3. (A) Number of neutral lipid vesicles per cell after VLDL treatment. (B) Representative examples of neutral lipid staining in VLDL treated cells. The same data is shown for LDL treatment in (C) and (D). Scale bars represents 20µm. Error bars show the mean±SEM, groups were compared using a two-tailed Mann Whitney U test.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

These data suggested that proteoglycan binding and cell surface lipase might indeed be involved to some degree in neutral lipid uptake. As such, I used immunofluorescent staining of monocytes to determine whether LPL is present on the cell membrane. As shown in Figure 4.9A, punctae of LPL staining can be seen in monocytes from healthy donors by confocal microscopy and appears to localize to the cell surface, although this staining pattern was present in both monocyte subpopulations. In these cells, fluorescent actin staining with phalloidin was also used to delineate cell structure. To assess the role of LPL in neutral lipid uptake monocytes were treated with 50 μ M tetrahydrolipstatin (orlistat), a commercially available lipase inhibitor that is used clinically to prevent lipid absorption by intestinal enterocytes [47]. However, prior to the addition of LDL or VLDL orlistat increased neutral lipid vesicle number in both CD16^{neg} and CD16^{pos} monocytes, although most notably in CD16^{pos} (Figure 4.9B), making its use as an inhibitor of LDL or VLDL induced lipid accumulation challenging to interpret.

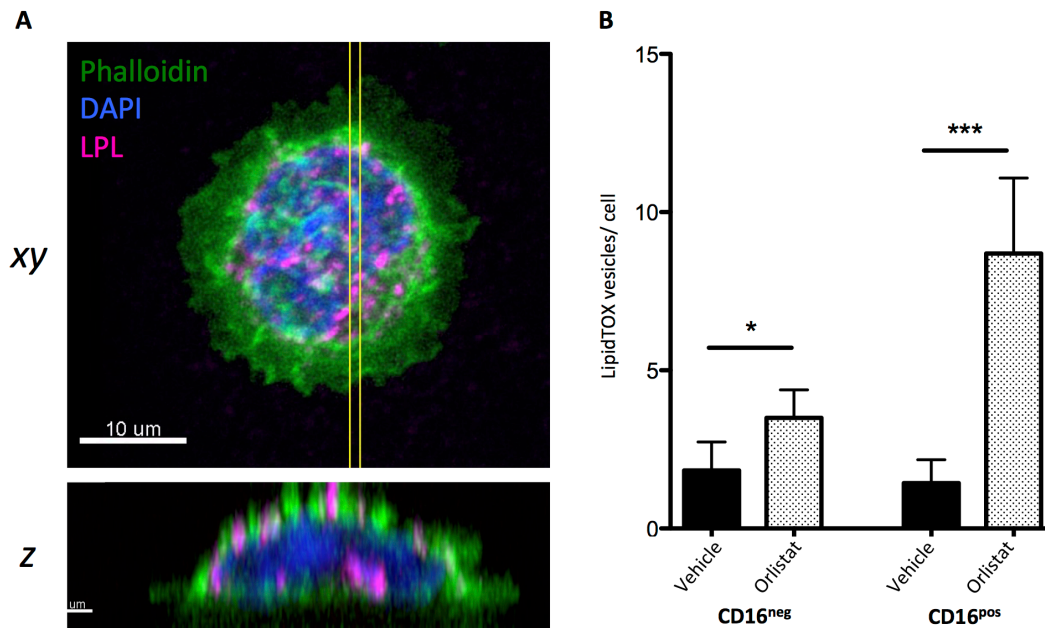


Figure 4.9 Cell surface LPL staining and orlistat treatment of human monocytes.

Human monocytes were purified and stained for the presence of lipoprotein lipase (LPL) and actin using immunofluorescence. (A) A representative confocal micrograph of monocyte LPL and actin staining, scale bar represents 10 μm . Monocytes were treated with 50 $\mu\text{g}/\text{ml}$ orlistat or vehicle control for 1 hour and intracellular neutral lipid was quantified using LipidTOX by confocal microscopy, $n=20-25$ cells. (B) Number of neutral lipid vesicles per cell after orlistat or vehicle treatment. Error bars show the mean $\pm$ SEM, groups were compared using a two-tailed Mann Whitney U test.

4.3. Discussion

The data presented here clearly demonstrate that human monocytes will undergo cytoplasmic neutral lipid accumulation in response to LDL or VLDL, similar to that observed in the *Ldlr*^{-/-} fed HFD. Monocytes also internalize fluorescently labeled LDL and VLDL as assessed by flow cytometry, although it is unclear how related these two phenotypes are. The kinetics of uptake would suggest that neutral lipid accumulation is not a direct metric of lipoprotein internalization, as while neutral lipid content peaks at 30 minutes incubation and is lower at subsequent time points, LDL and VLDL uptake increases over time in a fairly linear fashion. However, it is important to note that quantifying amount of LDL or VLDL internalized by flow cytometry was challenging and hampered by low sensitivity, limiting quantification of lipoprotein uptake to percentage of cells LDL or VLDL positive. The low proportion of LDL/ VLDL positive cells by flow cytometry also suggests that sensitivity may be an issue in this assay. While DIO- signal did increase over time, generally only around 15% of monocytes had detectable uptake after 2 hours; considerably lower than neutral lipid staining that in CD16^{neg} LDL treated monocytes reached 100% at 150 minutes incubation.

The specificity of LipidTOX used for neutral lipid staining and DIO for LDL/VLDL labeling may also be useful in understanding differences in uptake patterns. Although the structure of LipidTOX remains unpublished, it is reported to have high specificity for neutral lipid droplets. Neutral lipids are a broad group of lipid species encompassing triacylglycerols, sterol esters and wax esters, which acts as storage molecules for sterols and FFAs and cannot be incorporated into membrane bilayers due their lack of charged groups [48]. It is well known that there are triacylglycerol and cholesterol ester specific lipid droplets in many cell types for lipid storage, although these are distinct organelles surrounded by a phospholipid monolayer and originating from the endoplasmic reticulum (ER), not as a direct result of endocytosis. Once LDL or VLDL undergoes endocytosis it is delivered to lysosomes where cholesterol esters are hydrolysed forming unesterified cholesterol. Lipoprotein derived cholesterol is then delivered to the ER, where excess is re-esterified by ACAT and is subsequently packaged into neutral lipid droplets for storage [18,20].

Previous work in macrophages has demonstrated that internalized VLDL associates with ribosomal, ACAT-rich structures after only 10 minutes of incubation [49], emphasizing the rapid kinetics of this process. As such, it is conceivable that the initial increase in neutral lipid staining at 30 minutes in Figure 4.2B represents staining of cholesterol esters in endocytosed VLDL, which subsequently decreases at 60 minutes as the cholesterol esters are hydrolysed and begins to increase again at 150 minutes as the cholesterol is re-esterified by ACAT. In contrast to LipidTOX, DIO is a lipophilic carbocyanine dye that predominantly labels the cell membrane, thus implying specificity for polar lipids such as non-esterified sterols and phospholipids. It is also possible that low rates of LDL and VLDL uptake by DIO labeling are due to dye degradation, as the acidic pH of the lysosome and lysosomal hydrolases result in a complete digestion of LDL proteins to their constituent amino acids within 60 minutes of endocytosis [18].

While the work presented here is primarily focused on lipid uptake, impaired cholesterol efflux is also a key pathway in the formation of macrophage foam cells (Reviewed in [50]). Notably, myeloid knockout of the efflux-associated cholesterol transporters ABCA1 and ABCG1 promotes macrophage foam cell formation and atherosclerosis progression in mice [51] and these macrophages develop lipid droplet accumulation in response to cholesterol in vitro [39]. In the context of monocytes, the non-classical subpopulation has been shown to have increased cholesterol efflux in vitro compared to classical monocytes [52]. However, monocytes from the *Ldlr*^{-/-} mouse are theoretically efflux-competent, as they possess the necessary transporter proteins and are in the presence of cholesterol acceptors such as HDL. Given that these monocytes still accumulate neutral lipid droplets on HFD and that this is most pronounced in the GR1^{low} non-classical population, the data suggest that a lack of effective cholesterol efflux is not a major contributor to this phenotype. In addition, a low plasma level of cholesterol acceptors (i.e. HDL) is a key feature of the dyslipidemia that drives cardiovascular disease [52,53]. Given these data, cholesterol acceptors were not added to in vitro cultures used in the experiments presented here.

Intriguingly, receptor-blocking experiments also suggest that neutral lipid accumulation and LDL/ VLDL endocytosis may be mediated via separate pathways. Using DIO labeling it appears that the LDLR and its associated family has only a modest contribution to in vitro LDL and VLDL uptake in these experiments, as suggested by in vivo data. This is fairly unsurprising as LDLR mediated uptake is thought to be a regulated, homeostatic process that should not result in excessive intracellular lipid accumulation. In addition, the LDL used in these experiments has undergone a degree of oxidative modification as measured by conjugated diene formation, thus presumably generating electronegative LDL that would be targeted to scavenger receptors. Consistent with this hypothesis, LDL-DIO uptake can be significantly impaired by blockade of CD36, although this is not true to VLDL. Although VLDL has high conjugated diene content in our experiments, this does not increase after storage under air and the role of oxidative modification in VLDL uptake is still subject to debate [13,14]. When an attempt was made to examine the potential for MSRA mediated VLDL uptake, experiments were hampered by clear off target effects of the inhibition, particularly for the control treatment with chondroitin sulphate (CS). CS both increased VLDL uptake and completely prevented the detection of any CD16^{pos} monocytes by flow cytometry. The increase in uptake may be due to CS stimulating a compensatory increase in membrane receptor turnover, while the lack of CD16 staining on monocytes is more difficult to explain. One possibility is that CS is competing for CD16 binding with the anti-CD16 antibody, although more work would be required to investigate this hypothesis.

However, unexpectedly CD36 blocking did not alter significantly neutral lipid accumulation after LDL treatment. This was further confounded by the finding that heparinase removal of HSPGs almost completely abolishes neutral lipid vesicle formation after LDL and VLDL treatment, but only in CD16^{pos} monocytes. One possibility is that while CD16^{neg} monocyte lipoprotein uptake is mediated by endocytosis through an undetermined receptor, CD16^{pos} cells also utilize the pathway proposed by Ira Goldberg, where lipoproteins bind to HSPGs and their triacylglycerols are hydrolysed to free fatty acids, allowing passage through the cell membrane [37]. Alternatively, CD16^{neg} cells could undergo faster proteoglycan

synthesis than the rate of removal by heparinase, or utilize alternative proteoglycan species such as chondroitin sulphate for LDL binding as has been reported in macrophages [54]. While all interesting possibilities, at this stage there is little evidence for any of these hypotheses. Investigation of this pathway was also impaired by the rapid formation of neutral lipid vesicles in monocytes treated with the lipase inhibitor orlistat, independent of lipoprotein treatment. In retrospect, this is unsurprising: cholesterol esters in macrophages have been reported to undergo a continuous cycle of hydrolysis and re-esterification by various lipases (cholesteryl ester hydrolases) and ACAT respectively [55]. By inhibiting lipase-mediated hydrolysis, one presumably causes cholesterol in the cell to become retained in an esterified, neutral state, resulting in more LipidTOX staining.

Clearly, there are multiple receptors and uptake pathways utilized by monocytes in the internalization of LDL and VLDL. While CD36 dependent uptake is important in LDL endocytosis, it does not appear to be involved in VLDL uptake, nor does it mediate the neutral lipid accumulation seen after LDL/ VLDL treatment. Many studies of uptake mechanisms in primary cells are hampered by a lack of appropriate tools, as receptor blocking strategies can have unexpected off- target effects. A key conclusion can be drawn from these data: LDL and VLDL uptake occurs in human and mouse monocytes, leading to intracellular lipid accumulation. The exact mechanisms of this await discovery and require more detailed investigation, possibly in a more genetically tractable system such as an immortalized monocyte cell-line.

4.4. References:

- 1 Gower RM, Wu H, Foster GA, *et al.* CD11c/CD18 Expression Is Upregulated on Blood Monocytes During Hypertriglyceridemia and Enhances Adhesion to Vascular Cell Adhesion Molecule-1. 2010;**31**:160–6. doi:10.1161/ATVBAHA.110.215434
- 2 Alipour A, van Oostrom AJHM, Izraeljan A, *et al.* Leukocyte activation by triglyceride-rich lipoproteins. 2008;**28**:792–7. doi:10.1161/ATVBAHA.107.159749
- 3 Mosig S, Rennert K, Krause S, *et al.* Different functions of monocyte subsets in familial hypercholesterolemia: potential function of CD14+CD16+ monocytes in detoxification of oxidized LDL. *The FASEB Journal* 2009;**23**:866–74. doi:10.1096/fj.08-118240
- 4 Choi SH, Harkewicz R, Lee JH, *et al.* Lipoprotein Accumulation in Macrophages via Toll-Like Receptor-4-Dependent Fluid Phase Uptake. *Circulation Research* 2009;**104**:1355–63. doi:10.1161/CIRCRESAHA.108.192880
- 5 Sánchez-Quesada JL, Camacho M, Antón R, *et al.* Electronegative LDL of FH subjects: chemical characterization and induction of chemokine release from human endothelial cells. *Atherosclerosis* 2003;**166**:261–70.
- 6 Demuth K, Myara I, Chappey B, *et al.* A cytotoxic electronegative LDL subfraction is present in human plasma. 1996;**16**:773–83.
- 7 Barros MRAC, Bertolami MC, Abdalla DSP, *et al.* Identification of mildly oxidized low-density lipoprotein (electronegative LDL) and its auto-antibodies IgG in children and adolescents hypercholesterolemic offsprings. *Atherosclerosis* 2006;**184**:103–7. doi:10.1016/j.atherosclerosis.2004.11.027
- 8 Matsuo Y, Kubo T, Okumoto Y, *et al.* Circulating malondialdehyde-modified low-density lipoprotein levels are associated with the presence of thin-cap fibroatheromas determined by optical coherence tomography in coronary artery disease. *Eur Heart J Cardiovasc Imaging* 2013;**14**:43–50. doi:10.1093/ehjci/jes094
- 9 Holvoet P, Vanhaecke J, Janssens S, *et al.* Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation* 1998;**98**:1487–94.
- 10 Loughheed M, Steinbrecher UP. Mechanism of uptake of copper-oxidized low density lipoprotein in macrophages is dependent on its extent of oxidation. *Journal of Biological Chemistry* 1996;**271**:11798–805.
- 11 Picard S, Parthasarathy S, Fruebis J, *et al.* Aminoguanidine inhibits oxidative modification of low density lipoprotein protein and the subsequent increase in uptake by macrophage scavenger receptors. *Proc Natl Acad Sci USA* 1992;**89**:6876–80.
- 12 Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. *Journal of Biological Chemistry* 1997;**272**:20963–6.
- 13 Perrey S, Ishibashi S, Kitamine T, *et al.* The LDL receptor is the major pathway for beta-VLDL uptake by mouse peritoneal macrophages. *Atherosclerosis* 2001;**154**:51–60.
- 14 Whitman SC, Miller DB, Wolfe BM, *et al.* Uptake of type III hypertriglyceridemic VLDL by macrophages is enhanced by oxidation, especially after remnant formation. 1997;**17**:1707–15.
- 15 Whitman SC, Argmann CA, Sawyez CG, *et al.* Uptake of type IV hypertriglyceridemic VLDL by cultured macrophages is enhanced by interferon-gamma. *Journal of Lipid Research* 1999;**40**:1017–28.
- 16 Steinbrecher UP, Parthasarathy S, Leake DS, *et al.* Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci USA* 1984;**81**:3883–7.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

- 17 Esterbauer H, Jürgens G, Quehenberger O, *et al.* Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *Journal of Lipid Research* 1987;**28**:495–509.
- 18 Brown MS, Goldstein JL. A Receptor-Mediated Pathway for Cholesterol Homeostasis. *Science* 1986;**232**:1–14.
- 19 Herijgers N, Van Eck M, Groot PH, *et al.* Low density lipoprotein receptor of macrophages facilitates atherosclerotic lesion formation in C57Bl/6 mice. 2000;**20**:1961–7.
- 20 Tabas I, Lim S, Xu XX, *et al.* Endocytosed beta-VLDL and LDL are delivered to different intracellular vesicles in mouse peritoneal macrophages. *J Cell Biol* 1990;**111**:929–40.
- 21 Ishibashi S, Herz J, Maeda N, *et al.* The two-receptor model of lipoprotein clearance: tests of the hypothesis in ‘knockout’ mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc Natl Acad Sci USA* 1994;**91**:4431–5.
- 22 Goldstein JL, Harrod MJ, Brown MS. Homozygous familial hypercholesterolemia: specificity of the biochemical defect in cultured cells and feasibility of prenatal detection. *Am J Hum Genet* 1974;**26**:199–206.
- 23 Brown MS, Basu SK, Falck JR, *et al.* The scavenger cell pathway for lipoprotein degradation: specificity of the binding site that mediates the uptake of negatively-charged LDL by macrophages. *J Supramol Struct* 1980;**13**:67–81. doi:10.1002/jss.400130107
- 24 Endemann G, Stanton LW, Madden KS. CD36 is a receptor for oxidized low density lipoprotein. *Journal of Biological ...* 1993.
- 25 Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. *Nature Reviews Immunology* 2013;**13**:621–34. doi:10.1038/nri3515
- 26 Tontonoz P, Nagy L, Alvarez JG, *et al.* PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* 1998;**93**:241–52.
- 27 Nagy L, Tontonoz P, Alvarez JG, *et al.* Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell* 1998;**93**:229–40.
- 28 Spitsberg VL, Matitashvili E, Gorewit RC. Association and coexpression of fatty-acid-binding protein and glycoprotein CD36 in the bovine mammary gland. *Eur J Biochem* 1995;**230**:872–8.
- 29 Baillie AG, Coburn CT, Abumrad NA. Reversible binding of long-chain fatty acids to purified FAT, the adipose CD36 homolog. *J Membr Biol* 1996;**153**:75–81.
- 30 Jay AG, Chen AN, Paz MA, *et al.* CD36 binds oxidized low density lipoprotein (LDL) in a mechanism dependent upon fatty acid binding. *J Biol Chem* 2015;**290**:4590–603. doi:10.1074/jbc.M114.627026
- 31 Xu S, Jay A, Brunaldi K, *et al.* CD36 enhances fatty acid uptake by increasing the rate of intracellular esterification but not transport across the plasma membrane. *Biochemistry* 2013;**52**:7254–61. doi:10.1021/bi400914c
- 32 Virtanen JK, Mursu J, Tuomainen T-P, *et al.* Dietary fatty acids and risk of coronary heart disease in men: the Kuopio Ischemic Heart Disease Risk Factor Study. 2014;**34**:2679–87. doi:10.1161/ATVBAHA.114.304082
- 33 Xu L, Dai Perrard X, Perrard JL, *et al.* Foamy Monocytes Form Early and Contribute to Nascent Atherosclerosis in Mice With Hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 2015;:ATVBAHA.115.305609. doi:10.1161/ATVBAHA.115.305609
- 34 Calvo D, Gómez-Coronado D, Suárez Y, *et al.* Human CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, and VLDL. *Journal of Lipid Research* 1998;**39**:777–88.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

- 35 Van Eck M, Hoekstra M, Out R, *et al.* Scavenger receptor BI facilitates the metabolism of VLDL lipoproteins in vivo. *Journal of Lipid Research* 2008;**49**:136–46. doi:10.1194/jlr.M700355-JLR200
- 36 Wiersma H, Nijstad N, Gautier T, *et al.* Scavenger receptor BI facilitates hepatic very low density lipoprotein production in mice. *Journal of Lipid Research* 2010;**51**:544–53. doi:10.1194/jlr.M000844
- 37 Goldberg IJ, Eckel RH, Abumrad NA. Regulation of fatty acid uptake into tissues: lipoprotein lipase- and CD36-mediated pathways. *Journal of Lipid Research* 2009;**50** Suppl:S86–90. doi:10.1194/jlr.R800085-JLR200
- 38 Hu L, van der Hoogt CC, Espirito Santo SMS, *et al.* The hepatic uptake of VLDL in *Irp-ldlr*^{-/-}*vldlr*^{-/-} mice is regulated by LPL activity and involves proteoglycans and SR-BI. *Journal of Lipid Research* 2008;**49**:1553–61. doi:10.1194/jlr.M800130-JLR200
- 39 Pagler TA, Wang M, Mondal M, *et al.* Deletion of ABCA1 and ABCG1 impairs macrophage migration because of increased Rac1 signaling. *Circulation Research* 2011;**108**:194–200. doi:10.1161/CIRCRESAHA.110.228619
- 40 Migliorini MM, Migliorini MM, Behre EH, *et al.* Allosteric modulation of ligand binding to low density lipoprotein receptor-related protein by the receptor-associated protein requires critical lysine residues within its carboxyl-terminal domain. *Journal of Biological Chemistry* 2003;**278**:17986–92. doi:10.1074/jbc.M212592200
- 41 Li Y, Lu W, Schwartz AL, *et al.* Receptor-associated protein facilitates proper folding and maturation of the low-density lipoprotein receptor and its class 2 mutants. *Biochemistry* 2002;**41**:4921–8. doi:10.1021/bi011894i
- 42 Shan L, Pang L, Zhang R, *et al.* PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide. *Biochemical and Biophysical Research Communications* 2008;**375**:69–73. doi:10.1016/j.bbrc.2008.07.106
- 43 Wintergerst ES, Jelk J, Rahner C, *et al.* Apoptosis induced by oxidized low density lipoprotein in human monocyte-derived macrophages involves CD36 and activation of caspase-3. *Eur J Biochem* 2000;**267**:6050–9.
- 44 Mazzone T, Lopez C, Bergstraesser L. Modification of very low density lipoproteins leads to macrophage scavenger receptor uptake and cholesteryl ester deposition. *Arteriosclerosis* 1987;**7**:191–6.
- 45 Tsubamoto Y, Yamada N, Watanabe Y, *et al.* Dextran sulfate, a competitive inhibitor for scavenger receptor, prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Atherosclerosis* 1994;**106**:43–50.
- 46 ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. 2011;**121**:4138–49. doi:10.1172/JCI57559
- 47 Guercioli R. Mode of action of orlistat. *Int J Obes Relat Metab Disord* 1997;**21** Suppl 3:S12–23.
- 48 Athenstaedt K, Daum G. The life cycle of neutral lipids: synthesis, storage and degradation. *Cell Mol Life Sci* 2006;**63**:1355–69. doi:10.1007/s00018-006-6016-8
- 49 Khelef N, Buton X, Beatini N, *et al.* Immunolocalization of acyl-coenzyme A:cholesterol O-acyltransferase in macrophages. *Journal of Biological Chemistry* 1998;**273**:11218–24.
- 50 Ouimet M, Marcel YL. Regulation of lipid droplet cholesterol efflux from macrophage foam cells. *Arterioscler Thromb Vasc Biol* 2012;**32**:575–81. doi:10.1161/ATVBAHA.111.240705
- 51 Yvan-Charvet L, Ranalletta M, Wang N, *et al.* Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. *J Clin Invest* 2007;**117**:3900–8. doi:10.1172/JCI33372
- 52 Rogacev KS, Zawada AM, Emrich I, *et al.* Lower Apo A-I and lower HDL-C levels are associated with higher intermediate CD14⁺⁺CD16⁺ monocyte counts that

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

- predict cardiovascular events in chronic kidney disease. 2014;**34**:2120–7. doi:10.1161/ATVBAHA.114.304172
- 53 Nissen SE, Tsunoda T, Tuzcu EM, *et al.* Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA* 2003;**290**:2292–300. doi:10.1001/jama.290.17.2292
- 54 Kaplan M, Aviram M. Macrophage plasma membrane chondroitin sulfate proteoglycan binds oxidized low-density lipoprotein. *Atherosclerosis* 2000;**149**:5–17.
- 55 Brown MS, Ho YK, Goldstein JL. The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters. *Journal of Biological Chemistry* 1980;**255**:9344–52.

5. THE EFFECTS OF ATHEROGENIC LIPOPROTEINS ON MONOCYTE FUNCTION

5.1. Introduction

Traditionally, any work performed assessing monocyte function in response to dyslipidaemia has focused on one of two contexts: 1) hypercholesterolemia characterized by elevated LDL and VLDL or 2) post-prandial lipaemia, characterized by raised chylomicrons, free-fatty acids (FFAs) and VLDL. In both contexts, much of the past literature is compromised by an assumption that monocytes are a homogenous population that must differentiate to tissue macrophages in order to have immune effector functions. Nonetheless, some interesting work exists on the monocyte response to atherogenic lipoproteins.

In the context of LDL and VLDL, studies have been performed on monocytes from patients with familial hypercholesterolemia (FH). Initially, Mosig et al noted that a greater proportion of monocytes from FH homozygotes contain neutral lipid than healthy controls [1]. A concurrent gene expression microarray of monocytes healthy controls, FH heterozygotes and FH homozygotes demonstrated that monocytes from FH patients have alterations in cholesterol metabolism that increase in severity in homozygotes [1]. Interestingly, an increase in *CD36* and *LRP1* expression is accompanied by down-regulation of *ABCA1* in FH monocytes, which is hypothesized to increase uptake of oxidized LDL and decrease cholesterol efflux leading to an intracellular lipid overload [1]. This increased *CD36* expression was subsequently confirmed by others and associated with atherosclerosis severity in FH heterozygotes [2]. Functional analysis in monocyte subsets from FH patients demonstrated increased surface integrin expression in both $CD16^{pos}$ and $CD16^{neg}$ subpopulations accompanied by altered adhesion to an endothelial monolayer, although this phenotype was most pronounced in $CD16^{pos}$ monocytes [3].

Phagocytosis of latex beads was also impaired in FH monocytes in both subsets, both at baseline and following LDL stimulation [4]. Although this study did not assess any further functional impact of hypercholesterolemia on monocytes in the context of inflammation, in vitro oxLDL treatment has been shown to both increase[5] and inhibit[6] inflammatory cytokine production both alone and in response to TLR stimulation in human monocytes, highlighting the on-going confusion in this field.

A high fat meal significantly elevates plasma chylomicrons, VLDL and free fatty acids, peaking at 3 to 4 hours post-prandially [7]. As such, post-prandial analysis of monocytes can be used to study the acute effects of triglyceride-rich lipoproteins (TGRL) on these cells. ^{13}C –labelled palmitic acid has been tracked into leukocytes following a high fat meal [8], indicating that this stimulus could have a functional impact. Analysis of monocyte postprandial gene expression has revealed increased expression of integrins, including CD11b (*ITGAM*) and CD11c (*ITGAX*) and alterations in lipid handling genes including *ABCA1* and *APOB48R* [9,10]. Using a mixed high fat meal, Simon et al demonstrate that monocytes can acutely accumulate intracellular lipid, resulting in an up-regulation of surface CD11c protein and increased adhesion to a VCAM-1 layer during perfusion [11], which has subsequently been hypothesized to lead to increased recruitment to the atherosclerotic plaque [12]. Postprandial VLDL lipolysis products have also been shown to increase monocyte surface integrins and adhesion to an endothelial monolayer in vitro, via an NF- κ B/ ERK2 dependent pathway [13]. Interestingly, the transcriptional effect of post-prandial lipaemia effect appears to be specific to saturated fatty acids (SFAs) and not polyunsaturated fatty acids (PUFAs), consistent with a role for PUFAs as protective in cardiovascular disease [10]. In addition, dietary supplementation with PUFA-rich fish oil inhibits the expression of monocyte adhesion molecules [14], decreasing monocyte adhesion to an endothelium and subsequent neutrophil recruitment [15]. However, this is not accompanied by a decrease in inflammatory cytokine production [15] and the mechanisms of this phenotype are poorly described. Despite this intriguing literature there is very little data on the functional impact of TGRLs on monocyte subpopulations and postprandial studies are often confounded by effects concurrent to lipaemia,

including elevated plasma glucose and insulin [7]. As such, I planned to further investigate the functional response of LDL and VLDL treated monocyte subpopulations in vitro, given the pronounced uptake of these lipoprotein species exhibited by both human and mouse monocytes.

5.2. Results

5.2.1. The effect LDL/VLDL on monocyte apoptosis

Given previous literature demonstrating the ability of oxLDL to induce macrophage apoptosis [16,17], the first logical stage of functional analysis in LDL and VLDL treated human monocytes was to confirm that in vitro lipoprotein exposure was not inducing cell death. Total monocytes were isolated from human peripheral blood and treated with 100µg/ml LDL or VLDL for two hours, as in previous experiments. At this time point, cells were stained for CD14, CD16 and HLA-DR expression to distinguish monocyte subpopulations by flow cytometry and for apoptosis using a fluorescently labelled Annexin V. Annexin V is a cellular protein of unknown function that binds to phosphatidylserine (PS), which becomes exposed on the surface of cells undergoing programmed cell death [18]. It is important to note that activated platelets also express PS, making exclusion of platelet contamination important to obtain an accurate result. (See Chapter 2.2 for details). Using this metric approximately 5% of monocytes were apoptotic in both subpopulations and this number was unchanged by LDL or VLDL treatment (Figure 5.1A). A representative flow cytometry histogram for Annexin-V staining of total monocytes is shown in Figure 5.1B. As such, I concluded that LDL and VLDL treatment is not affecting monocyte cell death.

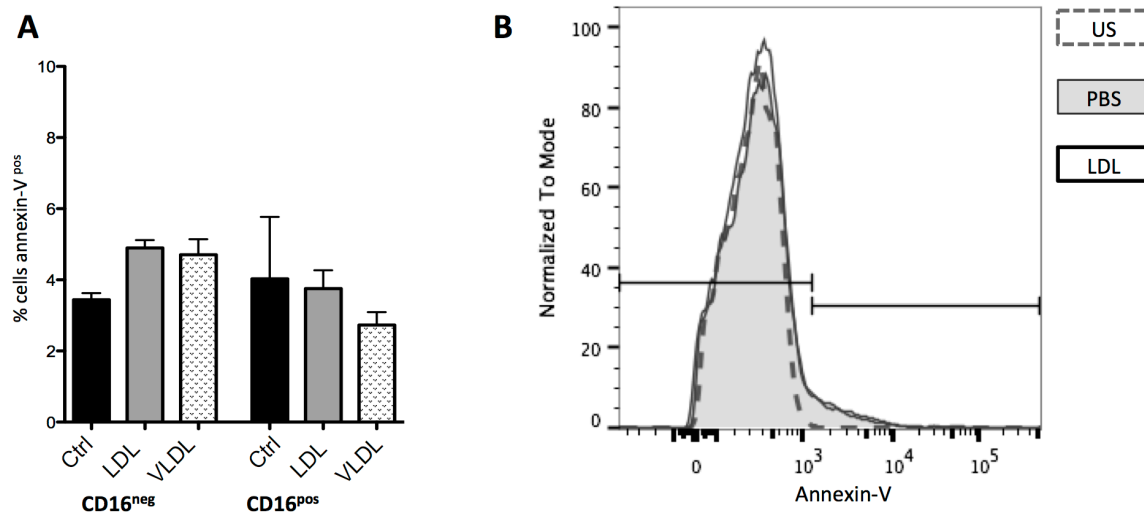


Figure 5.1 Monocyte apoptosis following LDL/ VLDL treatment. CD16^{pos} and CD16^{neg} human monocytes were first incubated with and without 100µg/ml LDL or VLDL for 2 hours and analysed by flow cytometry, n= 3 donors. (A) Percentage of CD16^{pos}/CD16^{neg} monocytes which are apoptotic as assessed by annexin-V staining. (B) Representative Annexin-V intensity histogram of total monocytes treated with PBS or LDL and stained with Annexin-V or unstained (US). Error bars show the mean ± SEM.

5.2.2. Modulation of monocyte activation and cytokine production by LDL/ VLDL

Given that apoptosis is unaffected, I then investigated whether lipoprotein treatment causes an ‘activated’ phenotype in human monocytes. Monocyte activation following TLR stimulation and the process of monocyte to macrophage differentiation are both accompanied by a modulation of CD16, CD14 and HLA-DR expression, which are also used to distinguish monocyte subsets [16,17]. As such, I utilised flow cytometry of human PBMCs following 2 hours of LDL or VLDL treatment in order to assess cell surface levels of these antigens. HLA-DR, CD14 and CD16 were all unchanged compared to PBS stimulation by both LDL (Figure 5.2A-C) and VLDL (Figure 5.2E-G) at this time in both CD16^{pos} and CD16^{neg} monocytes. As discussed previously, TGRs can also cause up-regulation of surface integrin expression in murine monocytes, most notably CD11c [11]. In addition, Wu and colleagues recently reported that a population of CD11c^{pos} CD36^{pos} lipid-loaded monocytes appears when *Apoe*^{-/-} mice are fed HFD. However, CD11c (Figure 5.3A-B) and CD36 (Figure 5.3C-D) surface expression was unchanged following 2 hours LDL and VLDL

treatment in both monocyte subpopulations. While both monocyte subpopulations express CD11c and CD36, CD11c was elevated in CD16^{pos} monocytes and CD36 was enriched in the CD16^{neg} population, consistent with previously published work [18]. These data therefore suggest that in vitro treatment with these lipoproteins at 2 hours does not cause overt monocyte activation.

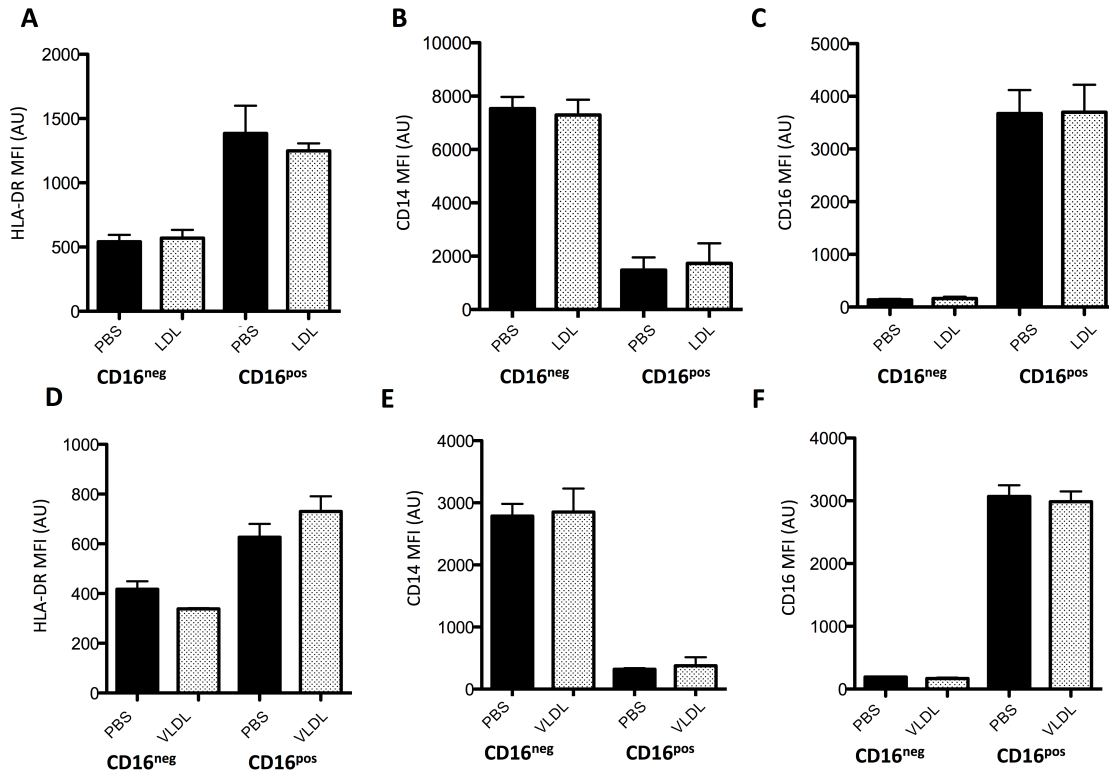


Figure 5.2 Effect of LDL/ VLDL treatment on monocyte surface marker expression.

For all experiments, CD16^{pos} and CD16^{neg} human monocytes were first incubated with and without 100µg/ml LDL or VLDL for 2 hours, n=3-4 donors. After LDL treatment: cell surface (A) HLA-DR MFI, (B) CD14 MFI and (C) CD16 MFI.(D-F) Corresponding data after VLDL treatment. Error bars show the mean $\pm$ SEM.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson - January 2016

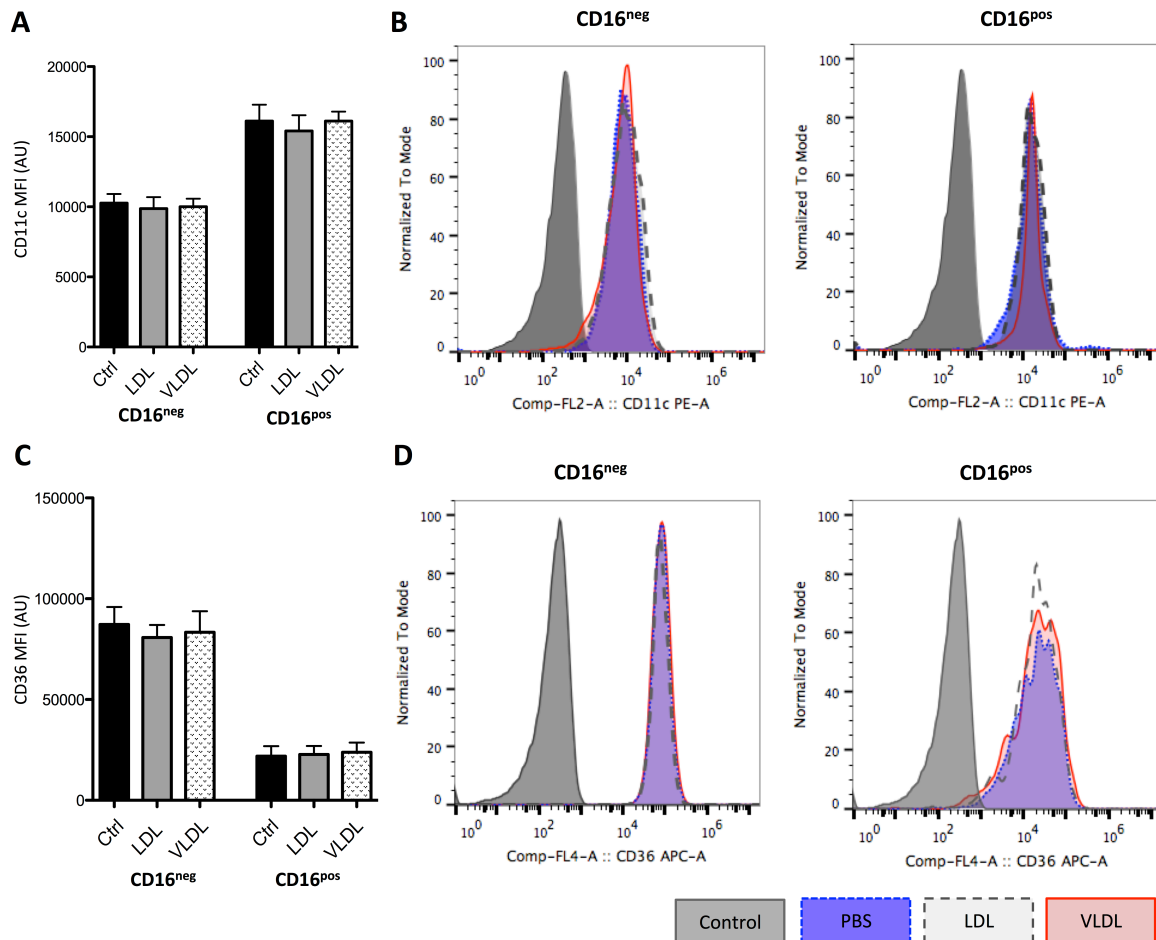


Figure 5.3 Effect of LDL/ VLDL treatment on monocyte surface CD11c and CD36.
For all experiments, CD16^{pos} and CD16^{neg} human monocytes were first incubated with and without 100µg/ml LDL or VLDL for 2 hours, n=3-4 donors. Cell surface protein was assessed by flow cytometry (A) CD11c MFI, (B) Representative CD11c histograms. (C-D) Same data is shown for CD36. Error bars show the mean ± SEM.

The role of exogenous lipid in the response to inflammation by monocytes and macrophages remains controversial, and a lack of changes in surface marker expression led me to investigate this further by assessing the monocyte cytokine response. When mRNA abundance was measuring using qPCR following 2 hours of LDL (Figure 5.4A) or VLDL (Figure 5.4B) stimulation there was no change in transcription of *IL1B*, *TNF* or *IL8* in either CD16^{pos} or CD16^{neg} monocytes, indicating that inflammatory gene expression was not affected. Although there are subtle changes such as an increase in IL-8 transcription in CD16^{neg} monocytes, these data are presented on a log2 ratio scale and the changes observed are extremely limited. However, at this early time point in macrophages some cytokines (i.e. TNF- α) are not transcriptionally regulated, but instead derived from a constitutively transcribed pool of mRNA that is rapidly stabilized and translated following stimulation [19]. In addition, lipid loading has been shown to both inhibit and perpetuate TLR- driven inflammatory responses in monocytes and macrophages. As such, I examined the effect of LDL and VLDL pre-treatment on monocyte IL-1 β and TNF- α production in response to 16 hours LPS and R848 stimulation using a multiplex bead array. Using a mixed inflammatory stimulus of LPS and R848 allows direct comparison of CD16^{neg} and CD16^{pos} monocytes, since these subpopulations exhibit an optimal cytokine response to TLR4 and TLR7/8 agonists respectively [19]. While LPS and R848 exposure induced a robust cytokine response as expected, LDL (Figure 5.4C-D) or VLDL (Figure 5.4E-F) treatment had no significant effect on the IL-1 β or TNF- α production induced by TLR activation. However, VLDL alone did induce a modest decrease in CD16^{neg} monocyte TNF- α levels (Figure 5.4F). Taken together, these data confirm that this in vitro lipoprotein treatment in human monocytes is definitively not an inflammatory stimulus and may in fact cause a slight inhibition of TNF- α production under homeostatic conditions, as has been previously proposed for the cholesterol biosynthesis intermediate desmosterol in macrophages [20].

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson - January 2016

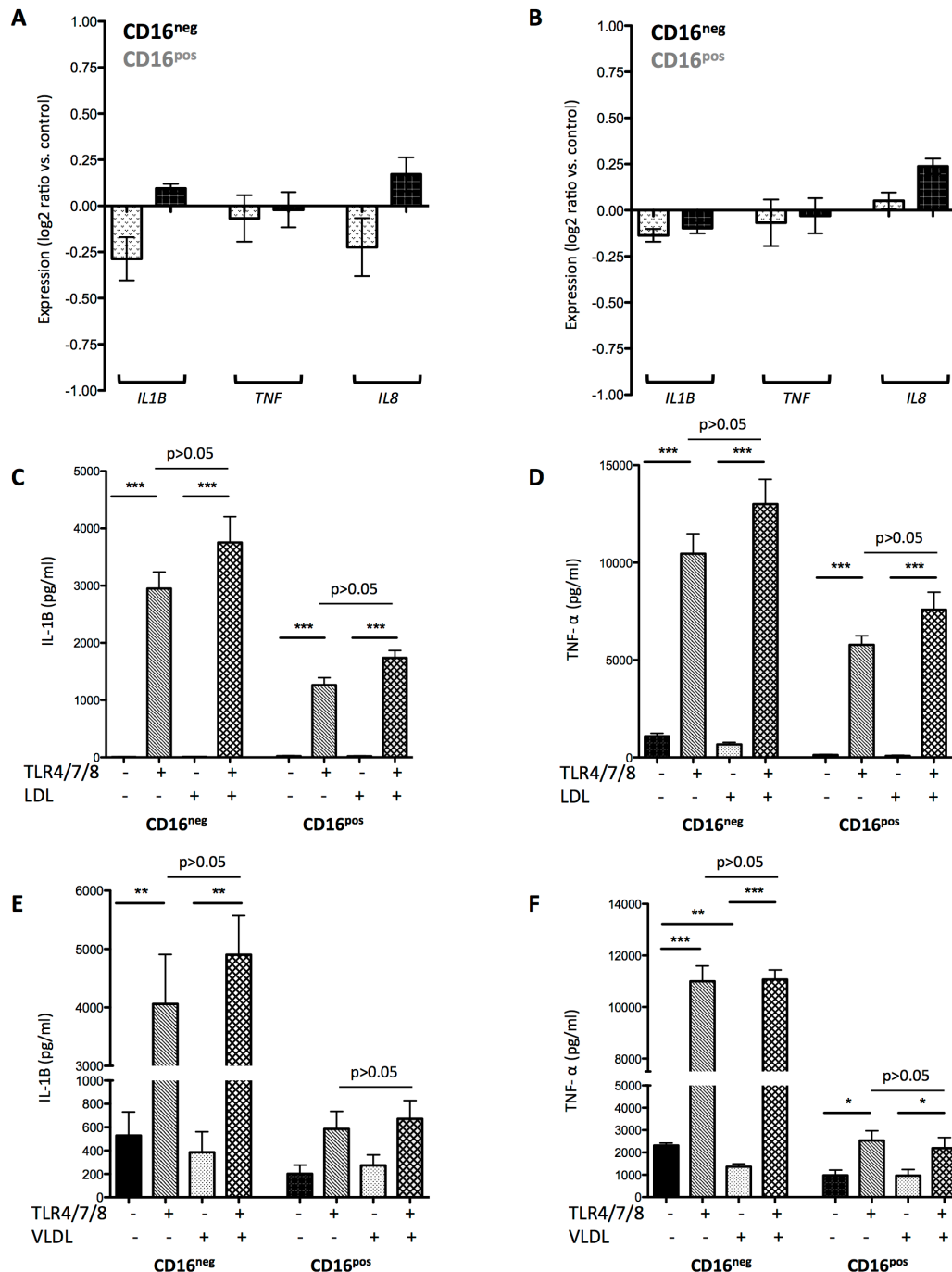


Figure 5.4 Effect of LDL/ VLDL treatment on monocyte cytokine response.

Human CD16^{pos} and CD16^{neg} monocytes were purified and treated (2hrs) with LDL or VLDL (100 μ g/ml). Cytokine gene expression was analysed by qPCR after (A) LDL or (B) VLDL treatment (n=3 donors in duplicate). Following LDL/ VLDL treatment, monocytes were incubated for 16 hours with or without LPS (100ng/ml) and R848 (2 μ g/ml). Supernatants were collected for cytokine analysis (n=3-4 donors in duplicate). (C) IL-1 β and (D) TNF- α secretion after LDL pre-treatment. (E-F) Same as C-D except with VLDL treatment. Error bars show the mean $\pm$ SEM, groups were compared using a two-tailed Mann-Whitney U test.

5.2.3. Monocyte phagocytosis, chemotaxis and chemokinesis following LDL/ VLDL treatment.

Given that HFD reduces monocyte accumulation in the peritoneum of the mouse during inflammation, I hypothesized that neutral lipid loaded monocytes may have an intrinsic migratory defect. In addition, migration and phagocytosis of exogenous particles are intrinsically linked processes and monocytes from FH patients have been previously reported to have a defect in phagocytosis [4]. As such, I initially examined phagocytosis of fluorescent latex beads by flow cytometry in monocyte subsets treated with LDL or VLDL for 2 hours. Number of internalized latex beads can clearly be gated in monocytes using peaks in fluorescence intensity, as shown in Figure 5.5A. After 1 hour, only approx. 5-10% of CD16^{neg} monocytes and 25-30% CD16^{pos} monocytes remained negative for bead uptake (Figure 5.5B), with >80% CD16^{neg} and >35% CD16^{pos} cells having phagocytosed more than 4 beads (Figure 5.5C). These proportions were largely unaffected by LDL or VLDL treatment, with a slight trend towards more phagocytosis following lipoprotein incubation. This was supported by the median bead fluorescence intensity in the bead-positive population, which does not change significantly between the control, LDL and VLDL groups, indicating that the amount of intracellular beads is equal (Figure 5.5D). As such, it appears that acute lipoprotein exposure does not mediate the same phagocytic defect reported in FH patient monocytes and in general there is a trend towards the opposite phenotype.

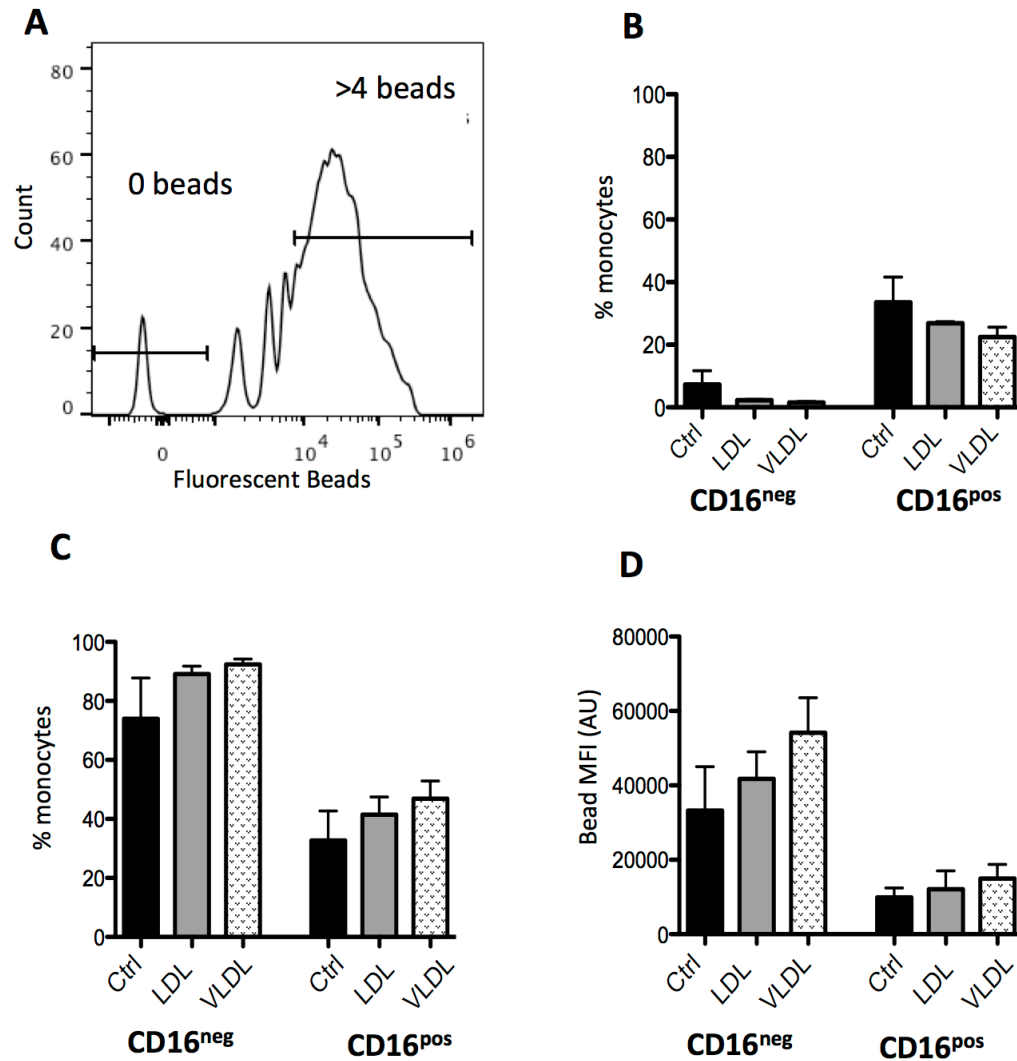


Figure 5.5 Effect of LDL/ VLDL treatment on monocyte phagocytosis.

CD16^{pos} and CD16^{neg} human monocytes were first incubated with or without 100µg/ml LDL or VLDL for 2 hours. Cells were then incubated with carboxylated 1µm latex beads to assess phagocytosis. (A) Representative histogram of latex bead gating. (B) % of cells which are bead^{neg} or (C) which have phagocytosed >4 beads after 1 hour incubation. (D) Bead median fluorescence intensity (MFI) in bead-positive cells. n=3 donors in duplicate. Error bars show the mean±SEM.

Cholesterol loading has also been reported to impair macrophage chemotaxis in *ABCA1/ABCG1*^{-/-} cells [21] and as such I then investigated the ability of monocytes to migrate towards a chemoattractant, in this case C5a, after treatment with 100µg/ml LDL or VLDL for two hours. C5a is a well-characterized chemoattractant for monocytes [22] and transgenic mice lacking C5 have a strongly impaired response to thioglycollate peritonitis [23]. Initially, I utilized a simple transwell chamber assay where treated monocytes are seeded onto the top of a porous, polycarbonate membrane and migrate through the membrane towards a chemoattractant gradient in the bottom of the chamber. Cells on the underside of the membrane can then be stained and counted by microscopy. Using this approach, VLDL treatment caused an approx. 50% reduction in the number of CD16^{pos} monocytes that had migrated and a subtle, non-significant decrease in CD16^{neg} monocyte migration (Figure 5.6A), representative images of the nuclei of migrated cells for counting are shown in Figure 5.6B. LDL treatment also caused a decrease in monocyte migration although in this case only in CD16^{neg} monocytes (Figure 5.6C), potentially highlighting subset specific effects of the two lipoprotein species.

However, in transwell assays it is difficult to determine whether true chemotaxis is occurring towards a stimulus, or to make any accurate assessment of effects on cell kinematics. As such, I used a 2D chemotaxis chamber slide in order to assess monocyte migration towards a C5a gradient in real time following 2 hours of VLDL treatment. From this point onwards, I chose to focus predominantly on VLDL, since this is the lipoprotein most elevated in the *Ldlr*^{-/-} mouse on HFD [24] and causes a monocyte migratory defect in transwells. Unlike the transwell assay, total monocytes were seeded and an anti-CD16 fluorescent antibody was used to distinguish CD16^{pos} and CD16^{neg} subsets. Cells were tracked in real time over approx. 90 minutes and representative track projections are shown in Figure 5.6D. The track projections demonstrate an almost complete inhibition of monocyte chemotaxis by VLDL and this is confirmed by track analysis: both monocyte subpopulations have a decreased track confinement ratio (Figure 5.6E) and displacement (Figure 5.6F) in the presence of VLDL and CD16^{neg} monocytes also display a significant decrease in track speed (Figure 5.6G). Interestingly and agreement with previous in vivo data [19,25], the

migratory behaviour of CD16^{pos} non-classical monocytes is slower than the CD16^{neg} population. One obvious explanation for this VLDL induced phenotype would be that lipid-loaded monocytes are down-regulating expression of the C5a receptor CD88. To exclude this possibility, LDL or VLDL treated monocytes were analysed for cell surface CD88 expression using flow cytometry. While monocytes expressed CD88 as expected, neither lipoprotein species caused any change in CD88 levels in either CD16^{pos} or CD16^{neg} monocytes (Figure 5.6H) and a representative histogram of total monocyte CD88 staining is shown in Figure 5.6I. As such, lipoprotein treated monocytes should still be capable of responding to C5a at a receptor level.

In addition to polarising and migrating towards a chemoattractant, monocytes will also undergo random, chemokinetic movement both with and without stimulation [26]. In the *Cx3cr1^{gfp/+}* animals utilized previously, HFD did not affect non-classical monocyte intravascular patrolling, during which monocytes appear to have random directionality[25] (Figure 3.7). I therefore hypothesized that VLDL treatment may only affect monocyte polarization towards a stimulus, rather than random chemokinetic migration. To test this hypothesis in human cells, experiments were performed as above using 2D chemotaxis chambers with a diffuse concentration of C5a rather than a gradient. As expected, track projections reveal cells migrating in all directions (Figure 5.7A). However, this migration was impaired in both CD16^{pos} and CD16^{neg} monocytes by VLDL pre-treatment, as quantified by displacement (Figure 5.7B), track speed (Figure 5.7C) and confinement ratio (Figure 5.7D). As such, VLDL does not require monocyte polarization towards a chemoattractant in order to inhibit cell migration in vitro.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson- January 2016

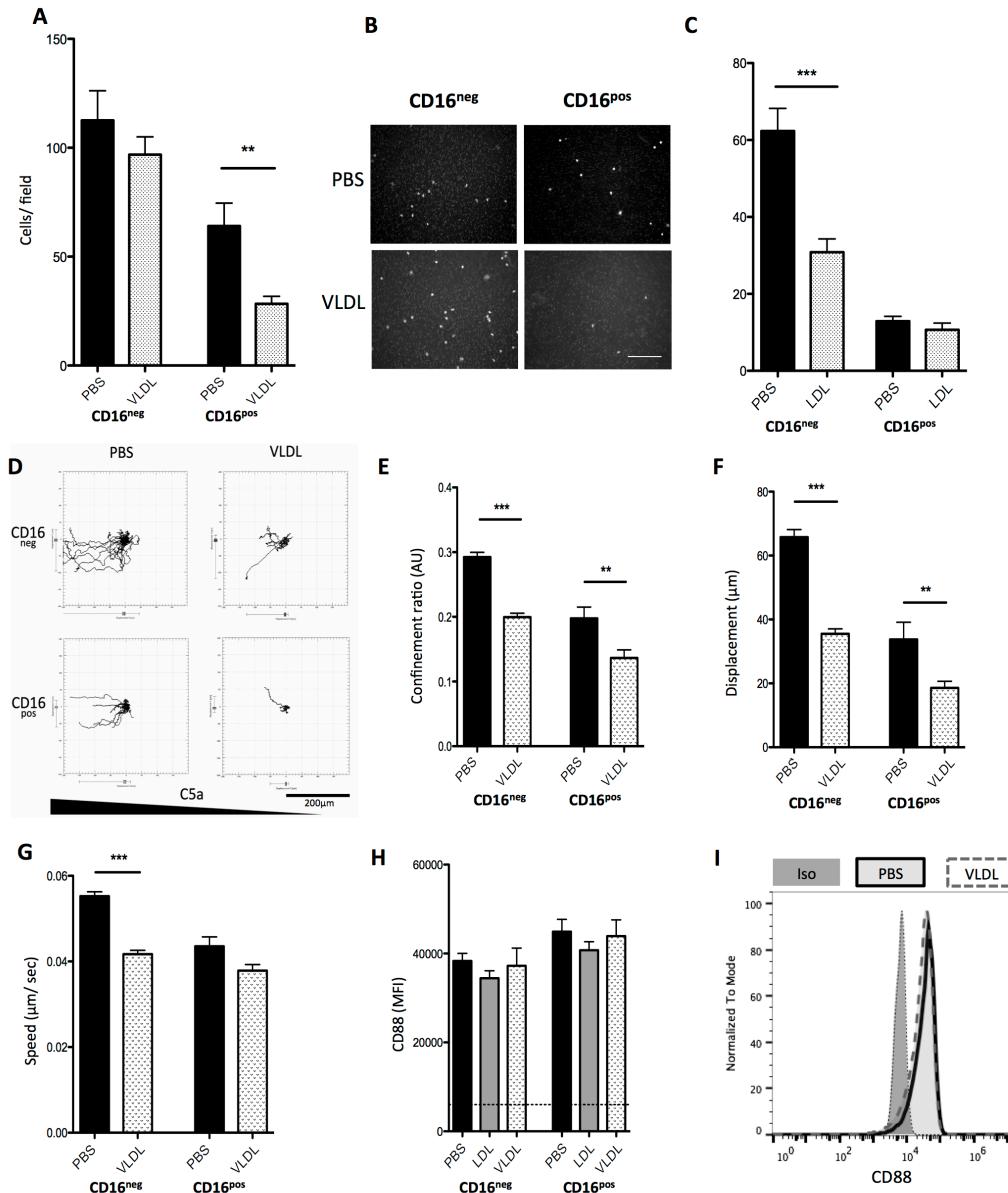


Figure 5.6 Effect of LDL/ VLDL treatment on monocyte chemotaxis.

(A) Transwell migration of CD16^{pos} and CD16^{neg} monocytes to C5a (250ng/ml) with or without VLDL pre-treatment (2hrs; 100µg/ml). Data represents 12 fields of view from 3 independent experiments. (B) Representative cells/field migrated shown in (A) (scale bar = 100µm). (C) Transwell migration as in (A), but following LDL treatment. (D) Representative track projections from CD16^{pos} and CD16^{neg} monocytes with or without VLDL pre-treatment (2hrs; 100ug/ml) in a 2D real-time chemotaxis assay towards a C5a gradient (n=3 independent experiments). Tracks were analysed for (E) confinement ratio (F) displacement (µm) and (G) speed (µm/sec) (n=120-800 cells) (H) CD16^{pos} and CD16^{neg} monocyte cell surface expression of CD88 after 2 hours LDL/VLDL treatment, assessed by flow cytometry (n = 2 donors in duplicate). (I) Representative histogram of CD88 expression or isotype control signal (Iso) on total monocytes. Error bars show the mean±SEM, groups were compared using a two-tailed Mann-Whitney U test

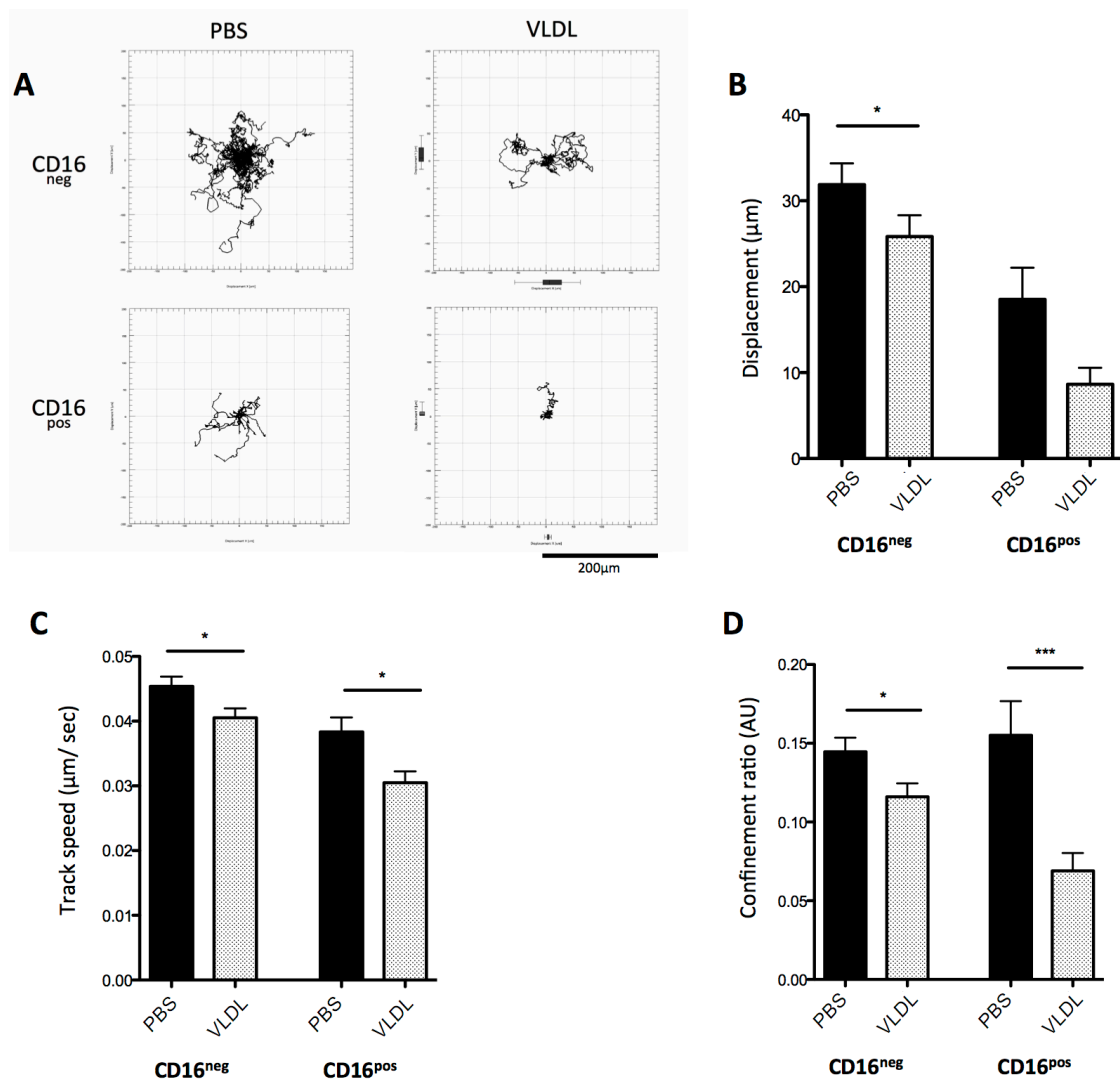


Figure 5.7 Effect of VLDL treatment on monocyte chemokinesis.

(A) Track projections from CD16^{pos} and CD16^{neg} monocytes with or without VLDL pre-treatment (2hrs; 100µg/ml) in a 2D real-time chemokinesis assay with diffuse C5a stimulation. Tracks were analyzed for (B) displacement (µm) (C) speed (µm/sec) and (D) confinement ratio (AU). n=50-160 cells per condition, 1 representative experiment of 2. Error bars show the mean±SEM, groups were compared using a two-tailed Mann-Whitney U test

5.2.4. The effect of LDL/ VLDL treatment on monocyte- endothelial adhesion and transendothelial migration.

A key factor in the regulation of monocyte chemotaxis and cell polarity is integrin-mediated adhesion [27]. Since LDL and VLDL treatment has previously been reported to increase monocyte adhesion [13,28], I investigated the effects of lipoprotein treatment on CD16^{pos} and CD16^{neg} monocyte static adhesion to an endothelial layer. Human venous endothelial cells (HUVECs) were cultured in chamber slides to confluence and either CD16^{pos}/CD16^{neg} human monocytes or the HUVECs were treated with LDL or VLDL for 2 hours. Following treatment, monocytes were stained with a fluorescent nuclear dye for imaging and incubated with the HUVEC for 30 minutes, before paraformaldehyde (PFA) fixation of adherent monocytes and quantification by confocal microscopy. LDL treatment of monocytes caused an increase in the number of adherent CD16^{neg} monocytes per field and a trend towards more adherent CD16^{pos} cells (Figure 5.8A). Interestingly, LDL treatment of the endothelium did not have the same effect, as there was no significant change in the adherence of either subpopulation (Figure 5.8B). The same experiment with VLDL treatment did not produce any changes in adhesion when either the monocytes (Figure 5.8C) or the endothelial cells (Figure 5.8D) were treated. A representative image of fluorescently labelled monocytes adhered to a HUVEC monolayer for analysis is shown in Figure 5.8E.

However, given the chemotaxis and chemokinesis impairment seen in VLDL treated monocytes in chamber slides, I hypothesized that LDL or VLDL treatment would impair monocyte transendothelial migration (TEM) in transwell chambers. HUVECs were grown on transwell membranes to confluence and activated with TNF- α in order to induce monocyte TEM. CFSE- labelled monocytes were LDL/VLDL pre-treated as above and seeded onto the endothelium for 2 hours, before analysis by fluorescence microscopy. Somewhat unexpectedly, both LDL (Figure 5.8F) and VLDL (Figure 5.8G) treatment significantly increased monocyte TEM selectively in the CD16^{neg} subpopulation, raising the interesting possibility that monocyte

chemotaxis is impaired by LDL/ VLDL following TEM exclusively in the extravascular compartment.

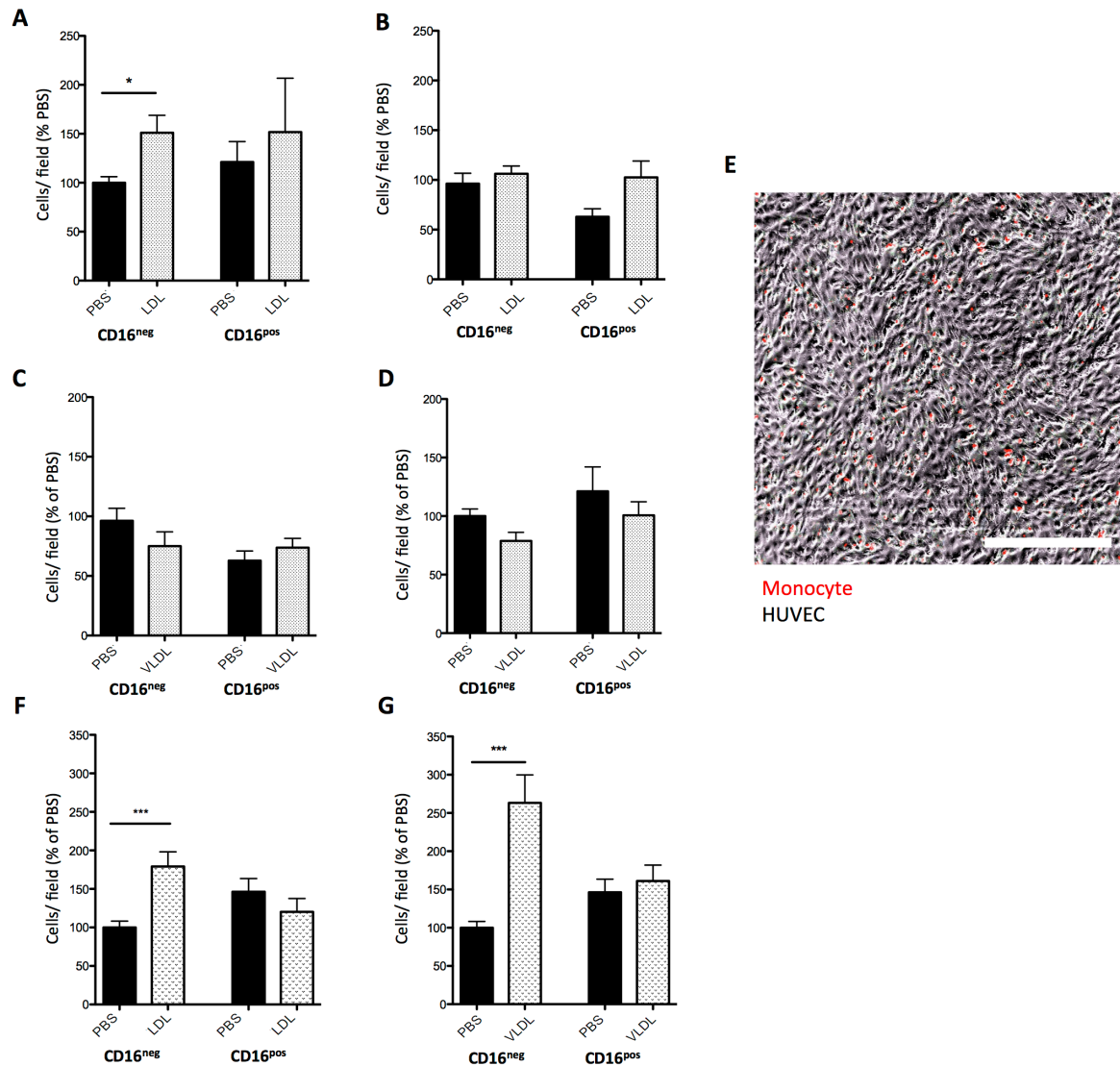


Figure 5.8 Effect of LDL/ VLDL treatment on monocyte adhesion to HUVEC and transendothelial migration.

(A-B) Adhesion of CD16^{pos} and CD16^{neg} monocytes to HUVECS, after (A) Monocyte pre-treatment with VLDL (2hrs; 100 µg/ml), normalised to CD16^{neg} PBS treated (n= 4 donors). (B) Same as (A) with only HUVECS treated with VLDL. (C-D) Corresponding data for LDL treatment. (E) Representative image of fluorescently labelled monocytes adhered to a HUVEC monolayer, scale= 500µm. (F-G) Transmigration of CD16^{pos} and CD16^{neg} monocytes pre-treated with or without (F) LDL, or (G) VLDL (2hrs, 100 µg/ml) through TNF activated HUVEC: cells per field, normalised to CD16^{neg} PBS treated (n=4 donors). Error bars show the mean±SEM, groups were compared using a two-tailed Mann-Whitney U test

5.3. Discussion

In previous literature, exposure to TGRLs or LDL is reported to have a profound inflammatory or anti-inflammatory effect in macrophages, as evidenced by cytokine transcription and release [5,6,29,30]. Largely through TLR signalling, modified LDL has been shown to modulate inflammation [5,6,29,30], while cholesterol crystals can activate the inflammasome [31]. Interestingly, Spann et al have subsequently suggested that these effects will be counter regulated via LXR-signalling during chronic dyslipidaemia, since accumulation of the cholesterol anabolic intermediate desmosterol leads to a deactivated phenotype in macrophage foam cells [20]. It is therefore somewhat surprising that the LDL and VLDL treatments used here did not modulate the transcription or release of the cytokines tested, both during homeostasis and after mixed TLR stimulation.

However, given that most previous work in this field focuses on macrophages, it is important to emphasize that monocytes and macrophages are metabolically distinct cells that may differ in their lipid handling and inflammatory response to lipid signals [32-34]. Metabolic profiling during human monocyte to macrophage differentiation *in vitro* reveals a dramatic up-regulation of fatty acid synthesis, which is essential for normal macrophage function [34]. This is accompanied by a down-regulation of SREBP2 target genes involved in cholesterol synthesis and results in a shift in cellular lipid composition from predominantly free cholesterol in monocytes to phosphatidylcholine in macrophages [34]. In addition, the polarization signals received by monocyte-derived macrophages also affect their metabolic program [35,36]. While LPS/ IFN γ stimulated 'M1' macrophages rely on aerobic glycolysis, IL-4 stimulated 'M2' macrophages undergo extensive fatty acid oxidation, inhibition of which can prevent M2 differentiation [35]. Interestingly in the context of the work presented here, this metabolic program results in the presence of cytoplasmic neutral lipid droplets in M1, but not M2 polarized macrophages [35], suggesting that monocytes may not be utilizing fatty acid oxidation as a primary energy source. There is also some evidence that non-classical monocytes have increased mitochondrial oxygen consumption versus the classical subpopulation [37], which

may contribute to the lower levels of neutral lipid accumulation observed here in non-classical CD16^{pos} monocytes.

Regarding cytokine transcription, it is possible 2 hours of LDL/VLDL stimulation may be too short a time frame for cytokines to be regulated at this level. In particular this is true for TNF- α , which is regulated largely by post-transcriptional mRNA processing and stored as large reserves of immature transcript [33]. However IL-8, which was also unchanged by LDL/VLDL exposure, is rapidly transcriptionally regulated in response to appropriate stimuli. For example, IL-1 can cause RNA-polymerase II recruitment and binding to the IL-8 (*CXCL8*) promoter within less than 30 minutes [38]. In addition, *CXCL8* gene induction can be extremely dramatic, with some stimuli such as IL-1 causing a >100-fold up-regulation of expression [38]. Similarly rapid transcription kinetics have also been reported for *IL1B*, which can be induced by LPS stimulation in human monocytes and U937 monocytic cells within 1 hour [39]. As such, it appears physiologically possible that LDL and VLDL could induce cytokine transcription within 2 hours in monocytes, although this clearly does not occur in the experiments performed here.

In addition, a lack of change in IL-1 β release following LDL or VLDL stimulation with or without LPS and R848 suggests that the inflammasome is not being modulated in this context. Following *IL1B* mRNA induction, pro- IL-1 β protein is synthesized and requires a second signal to be cleaved into its biologically active and secreted form. This occurs through caspase-1, which is activated via inflammasome complexes such as the NLRP3 inflammasome [40]. Cholesterol crystals have been reported to induce activation of this process in both monocytes and macrophages [31]. Given that no such phenotype is observed here, this effect may be mediated by the crystalline structure itself of the cholesterol rather than via the storage of cholesterol esters. Indeed, other crystals such as asbestos and silica have also been demonstrated to activate the NLRP3 inflammasome [37], suggesting that this process may be independent of cholesterol.

Surface integrin expression levels have previously been shown to be a marker of monocyte activation[38] and receptors such as CD16 and HLA-DR are modulated during early monocyte- macrophage differentiation [17]. In keeping with the above observations that LDL and VLDL are not inflammatory in our experiments, I observed no changes in surface CD11b *in vivo*, or in CD14, CD16, HLA-DR, CD36 and CD11c levels during *in vitro* stimulation. Given that monocyte activation and increased integrin expression is strongly linked to monocyte adhesion, it is fairly unsurprising that I also observed limited differences in monocyte adherence to an endothelial monolayer. In my experiments, only CD16^{neg} monocytes exhibited any change in adherence, and this was exclusively after LDL treatment. This subset specific effect may be due to different adhesion molecules utilized by CD16^{neg} and CD16^{pos} monocytes in their interactions with the endothelium: while CD16^{neg} cells utilize the classical adhesion cascade, CD16^{pos} monocytes depend on the B2 integrin LFA-1 and the chemokine receptor CX3CR1[41]. In addition, many studies prior to knowledge of monocyte heterogeneity used enrichment of CD14^{high} cells to isolate monocytes, which excluded the CD14^{low} CD16^{pos} non-classical population.

However, one must reconcile the data here with previous studies that have shown monocyte activation after VLDL or TGRL exposure [8,11,13]. Ultimately, nuances in the lipoprotein species used may explain differing the phenotypes. While the data presented here uses incubation with commercially available VLDL isolated from blood-bank plasma, both Gower et al and Alipour et al utilise either leukocytes from subjects who have ingested a high fat meal, or *in vitro* incubation with TGRLs isolated from post-prandial plasma [8,11]. Although not addressed in this study, the fatty acid composition of TRGLs including VLDL is known to change in the postprandial period depending on dietary intake [42]. As such, mixed commercial VLDL may not be comparable to VLDL isolated from subjects undergoing postprandial lipaemia from a high fat meal. It is unclear which of these stimuli would best represent what occurs in chronic elevation of TGRLs, such as in LPL-deficient patients [43].

Most strikingly in these experiments, VLDL caused a pronounced inhibition of both monocyte chemotaxis and chemokinesis. In transwell experiments, VLDL impairs migration in CD16^{pos} monocytes with little effect on the CD16^{neg} subpopulation, while LDL selectively affects CD16^{neg} cells. Although when a real time chemotaxis assay is used migration is clearly impaired in both subpopulations, the transwell data may indicate a subset specific response of monocytes to these different lipoprotein species. CD16^{pos} non-classical monocytes have been shown to undergo increased fatty acid oxidation compared to CD16^{neg} cells [37], which may relate to their specific response to fatty-acid rich VLDL in this assay. In addition, CD16^{pos} monocytes have been hypothesized to be functionally similar to 'M2-like' alternatively- activated macrophages [44], which also preferentially utilize fatty acid oxidation rather than aerobic glycolysis in comparison to 'M1-like' cells [35]. Moreover, monocyte subsets have differential express of adhesion molecules which result in distinct migration kinetics, with non-classical monocytes exhibiting higher levels of the chemokine receptor CX3CR1 and the B2-integrin LFA-1[25]. As such, it is possible that the lipoproteins are affecting the expression or organization of these monocyte subset-specific receptors. Further work is required to determine the effect of LDL in a real-time chemotaxis assay. While there may indeed be monocyte subset specific signalling, another possibility is that the crude transwell method of assessing chemotaxis does not give an entirely accurate result. Although still widely utilized transwell assays can cause difficulties due to an inability to quantify or maintain a gradient of chemoattractant, an inability to accurately count migrated cells and vast experimental variability between labs and transwell membrane manufacturers [45]. As observed in macrophages, cells may also translocate their nucleus through transwell pores and therefore stain as having migrated, while the remainder of the cell body stays un-retracted and dysfunctional within the transwell membrane [46].

Moreover, it is interesting that monocyte TEM does not appear to be impaired by LDL or VLDL. Indeed, CD16^{neg} monocyte TEM is increased by both LDL and VLDL treatment, seemingly the opposite of what would be expected from the 2D real-time migration assay. However, if a similar phenotype is occurring in our peritonitis model on HFD, monocyte retention in the omentum en route to the peritoneal cavity may

occur subsequent to TEM. At this stage, it is only possible to speculate what may promote this increase, although clearly TEM is a much more elaborate process than crawling on a 2D surface. One potential explanation may be found in the method by which monocytes cross the endothelium; leukocytes can undergo canonical, paracellular migration at endothelial cell junctions or in some circumstances can pass directly through the endothelial cell itself in a process known as transcellular migration [47]. Some have hypothesized that transcellular migration can increase when leukocytes are unable to execute the locomotion events required reach an appropriate endothelial junction. For example, CD11b deficient leukocytes [48] or leukocytes with cytoskeletal disruption [49] tend to preferentially use the transcellular route of TEM. Strikingly, disrupting the cytoskeletal GTPase RAP1B in neutrophils increased the rate of neutrophil TEM exclusively via the transcellular route both in vitro and in vivo [50]. Among others, this observation then led me to further investigate the role of the cytoskeleton in regulating the monocyte migratory phenotype described here.

However, one consideration when interpreting these data is that all adhesion and TEM experiments performed here were under static conditions, which may not represent the effects of physiological rheology. Arterial shear stress is known to have a pronounced affect on endothelial adhesion molecule expression (i.e. ICAM-1) and to alter monocyte-endothelial interactions [51-53]. This may be particularly important in the context of dyslipidaemia, since atherosclerotic plaques occur preferentially at sites of disrupted rheology such as vascular bifurcations [54]. While static leukocyte-endothelial co-cultures have been published on extensively due to their ease of use, they do not allow for the assessment of the transition from leukocyte rolling to initial firm adhesion. As such, future work would be required to confirm the observations on monocyte adhesion and TEM under physiological rheology, either in an in vitro perfusion system or via further intravital imaging.

5.1. References

- 1 Mosig S, Rennert K, Büttner P, *et al.* Monocytes of patients with familial hypercholesterolemia show alterations in cholesterol metabolism. *BMC Med Genomics* 2008;**1**:60. doi:10.1186/1755-8794-1-60
- 2 Hjuler Nielsen M, Irvine H, Vedel S, *et al.* Elevated atherosclerosis-related gene expression, monocyte activation and microparticle-release are related to increased lipoprotein-associated oxidative stress in familial hypercholesterolemia. *PLoS ONE* 2015;**10**:e0121516. doi:10.1371/journal.pone.0121516
- 3 Mosig S, Rennert K, Krause S, *et al.* Different functions of monocyte subsets in familial hypercholesterolemia: potential function of CD14+CD16+ monocytes in detoxification of oxidized LDL. *The FASEB Journal* 2009;**23**:866–74. doi:10.1096/fj.08-118240
- 4 Mosig S, Rennert K, Krause S, *et al.* Different functions of monocyte subsets in familial hypercholesterolemia: potential function of CD14+CD16+ monocytes in detoxification of oxidized LDL. *The FASEB Journal* 2009;**23**:866–74. doi:10.1096/fj.08-118240
- 5 Chávez-Sánchez L, Chávez-Rueda K, Legorreta-Haquet MV, *et al.* The activation of CD14, TLR4, and TLR2 by mmLDL induces IL-1 β , IL-6, and IL-10 secretion in human monocytes and macrophages. *Lipids Health Dis* 2010;**9**:117. doi:10.1186/1476-511X-9-117
- 6 Kannan Y, Sundaram K, Aluganti Narasimhulu C, *et al.* Oxidatively modified low density lipoprotein (LDL) inhibits TLR2 and TLR4 cytokine responses in human monocytes but not in macrophages. *J Biol Chem* 2012;**287**:23479–88. doi:10.1074/jbc.M111.320960
- 7 Rifai N, Merrill JR, Holly RG. Postprandial effect of a high fat meal on plasma lipid, lipoprotein cholesterol and apolipoprotein measurements. *Ann Clin Biochem* 1990;**27** (Pt 5):489–93.
- 8 Alipour A, van Oostrom AJHHM, Izraeljan A, *et al.* Leukocyte activation by triglyceride-rich lipoproteins. 2008;**28**:792–7. doi:10.1161/ATVBAHA.107.159749
- 9 Varela LM, Ortega A, Bermudez B, *et al.* A high-fat meal promotes lipid-load and apolipoprotein B-48 receptor transcriptional activity in circulating monocytes. *Am J Clin Nutr* 2011;**93**:918–25. doi:10.3945/ajcn.110.007765
- 10 Bouwens M, Grootte Bromhaar M, Jansen J, *et al.* Postprandial dietary lipid-specific effects on human peripheral blood mononuclear cell gene expression profiles. *Am J Clin Nutr* 2010;**91**:208–17. doi:10.3945/ajcn.2009.28586
- 11 Gower RM, Wu H, Foster GA, *et al.* CD11c/CD18 Expression Is Upregulated on Blood Monocytes During Hypertriglyceridemia and Enhances Adhesion to Vascular Cell Adhesion Molecule-1. 2010;**31**:160–6. doi:10.1161/ATVBAHA.110.215434
- 12 Wu H, Gower RM, Wang H, *et al.* Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. *Circulation* 2009;**119**:2708–17. doi:10.1161/CIRCULATIONAHA.108.823740
- 13 Hartigh den LJ, Altman R, Norman JE, *et al.* Postprandial VLDL lipolysis products increase monocyte adhesion and lipid droplet formation via activation of ERK2 and NF κ B. *Am J Physiol Heart Circ Physiol* 2014;**306**:H109–20. doi:10.1152/ajpheart.00137.2013
- 14 Hughes DA, Pinder AC, Piper Z, *et al.* Fish oil supplementation inhibits the expression of major histocompatibility complex class II molecules and adhesion molecules on human monocytes. *Am J Clin Nutr* 1996;**63**:267–72.
- 15 Luu N-T, Madden J, Calder PC, *et al.* Dietary supplementation with fish oil modifies the ability of human monocytes to induce an inflammatory response. *J Nutr* 2007;**137**:2769–74.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

- 16 Landmann R, Knopf HP, Link S, *et al.* Human monocyte CD14 is upregulated by lipopolysaccharide. *Infection and Immunity* 1996;**64**:1762–9.
- 17 Liu H, Shi B, Huang C-C, *et al.* Transcriptional diversity during monocyte to macrophage differentiation. *Immunol Lett* 2008;**117**:70–80. doi:10.1016/j.imlet.2007.12.012
- 18 Ancuta P, Liu K-Y, Misra V, *et al.* Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16+ and CD16- monocyte subsets. *BMC Genomics* 2009;**10**:403. doi:10.1186/1471-2164-10-403
- 19 Cros J, Cagnard N, Woollard K, *et al.* Human CD14dim Monocytes Patrol and Sense Nucleic Acids and Viruses via TLR7 and TLR8 Receptors. *Immunity* 2010;**31**:1–12. doi:10.1016/j.immuni.2010.08.012
- 20 Spann NJ, Garmire LX, McDonald JG, *et al.* Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell* 2012;**151**:138–52. doi:10.1016/j.cell.2012.06.054
- 21 Pagler TA, Wang M, Mondal M, *et al.* Deletion of ABCA1 and ABCG1 impairs macrophage migration because of increased Rac1 signaling. *Circulation Research* 2011;**108**:194–200. doi:10.1161/CIRCRESAHA.110.228619
- 22 Pieters WR, Houben LA, Koenderman L, *et al.* C5a-induced migration of human monocytes is primed by dexamethasone. *Am J Respir Cell Mol Biol* 1995;**12**:691–6. doi:10.1165/ajrcmb.12.6.7766432
- 23 Segal BH, Kuhns DB, Ding L, *et al.* Thioglycollate peritonitis in mice lacking C5, 5-lipoxygenase, or p47(phox): complement, leukotrienes, and reactive oxidants in acute inflammation. *Journal of Leukocyte Biology* 2002;**71**:410–6.
- 24 Ishibashi S, Herz J, Maeda N, *et al.* The two-receptor model of lipoprotein clearance: tests of the hypothesis in 'knockout' mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc Natl Acad Sci USA* 1994;**91**:4431–5.
- 25 Auffray C, Fogg D, Garfa M, *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 2007;**317**:666–70. doi:10.1126/science.1142883
- 26 Weber C, Springer TA. Interaction of very late antigen-4 with VCAM-1 supports transendothelial chemotaxis of monocytes by facilitating lateral migration. *J Immunol* 1998;**161**:6825–34.
- 27 Rzeniewicz K, Neue A, Rey Gallardo A, *et al.* L-selectin shedding is activated specifically within transmigrating pseudopods of monocytes to regulate cell polarity in vitro. *PNAS* 2015;**112**:E1461–70. doi:10.1073/pnas.1417100112
- 28 Weber C, Erl W, Weber PC. Enhancement of monocyte adhesion to endothelial cells by oxidatively modified low-density lipoprotein is mediated by activation of CD11b. *Biochemical and Biophysical Research Communications* 1995;**206**:621–8. doi:10.1006/bbrc.1995.1088
- 29 Miller YI, Viriyakosol S, Worrall DS, *et al.* Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. 2005;**25**:1213–9. doi:10.1161/01.ATV.0000159891.73193.31
- 30 Bzowska M, Nogiec A, Skrzeczyńska-Moncznik J, *et al.* Oxidized LDLs Inhibit TLR-induced IL-10 Production by Monocytes: A New Aspect of Pathogen-Accelerated Atherosclerosis. *Inflammation* 2012;**35**:1567–84. doi:10.1007/s10753-012-9472-3
- 31 Rajamäki K, Lappalainen J, Oörni K, *et al.* Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PLoS ONE* 2010;**5**:e11765. doi:10.1371/journal.pone.0011765
- 32 Tuomisto TT, Riekkinen MS, Viita H, *et al.* Analysis of gene and protein expression during monocyte-macrophage differentiation and cholesterol loading--cDNA and

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

- protein array study. *Atherosclerosis* 2005;**180**:283–91. doi:10.1016/j.atherosclerosis.2004.12.023
- 33 Martinez FO, Gordon S, Locati M, *et al.* Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 2006;**177**:7303–11.
- 34 Ecker J, Liebisch G, Englmaier M, *et al.* Induction of fatty acid synthesis is a key requirement for phagocytic differentiation of human monocytes. *PNAS* 2010;**107**:7817–22. doi:10.1073/pnas.0912059107
- 35 Huang SC-C, Everts B, Ivanova Y, *et al.* Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat Immunol* 2014;**15**:846–55. doi:10.1038/ni.2956
- 36 Rodríguez-Prados J-C, Través PG, Cuenca J, *et al.* Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. *The Journal of Immunology* 2010;**185**:605–14. doi:10.4049/jimmunol.0901698
- 37 Schmidl C, Renner K, Peter K, *et al.* Transcription and enhancer profiling in human monocyte subsets. *Blood* 2014;**123**:e90–9. doi:10.1182/blood-2013-02-484188
- 38 Hoffmann E, Dittrich-Breiholz O, Holtmann H, *et al.* Multiple control of interleukin-8 gene expression. *Journal of Leukocyte Biology* 2002;**72**:847–55.
- 39 Fenton MJ, Vermeulen MW, Clark BD, *et al.* Human pro-IL-1 beta gene expression in monocytic cells is regulated by two distinct pathways. *J Immunol* 1988;**140**:2267–73.
- 40 Black RA, Kronheim SR, Cantrell M, *et al.* Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. *Journal of Biological Chemistry* 1988;**263**:9437–42.
- 41 Woollard KJ, Geissmann F. Monocytes in atherosclerosis: subsets and functions. *Nat Rev Cardiol* 2010;**7**:77–86. doi:10.1038/nrcardio.2009.228
- 42 Heath RB, Karpe F, Milne RW, *et al.* Selective partitioning of dietary fatty acids into the VLDL TG pool in the early postprandial period. *Journal of Lipid Research* 2003;**44**:2065–72. doi:10.1194/jlr.M300167-JLR200
- 43 Santamarina-Fojo S. The familial chylomicronemia syndrome. *Endocrinol Metab Clin North Am* 1998;**27**:551–67–viii.
- 44 Italiani P, Boraschi D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. *Front Immunol* 2014;**5**:514. doi:10.3389/fimmu.2014.00514
- 45 Bignold LP. Measurement of chemotaxis of polymorphonuclear leukocytes in vitro. The problems of the control of gradients of chemotactic factors, of the control of the cells and of the separation of chemotaxis from chemokinesis. *Journal of Immunological Methods* 1988;**108**:1–18.
- 46 Nagao T, Qin C, Grosheva I, *et al.* Elevated cholesterol levels in the plasma membranes of macrophages inhibit migration by disrupting RhoA regulation. 2007;**27**:1596–602. doi:10.1161/ATVBAHA.107.145086
- 47 Muller WA. Mechanisms of leukocyte transendothelial migration. *Annu Rev Pathol Mech Dis* 2011;**6**:323–44. doi:10.1146/annurev-pathol-011110-130224
- 48 Schenkel AR, Mamdouh Z, Muller WA. Locomotion of monocytes on endothelium is a critical step during extravasation. *Nat Immunol* 2004;**5**:393–400. doi:10.1038/ni1051
- 49 Gérard A, van der Kammen RA, Janssen H, *et al.* The Rac activator Tiam1 controls efficient T-cell trafficking and route of transendothelial migration. *Blood* 2009;**113**:6138–47. doi:10.1182/blood-2008-07-167668
- 50 Kumar S, Xu J, Kumar RS, *et al.* The small GTPase Rap1b negatively regulates neutrophil chemotaxis and transcellular diapedesis by inhibiting Akt activation. *Journal of Experimental Medicine* 2014;**211**:1741–58. doi:10.1084/jem.20131706

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

- 51 Chiu J-J, Lee P-L, Chen C-N, *et al.* Shear stress increases ICAM-1 and decreases VCAM-1 and E-selectin expressions induced by tumor necrosis factor-[alpha] in endothelial cells. 2004;**24**:73–9. doi:10.1161/01.ATV.0000106321.63667.24
- 52 Expression of ICAM-1 and VCAM-1 and Monocyte Adherence in Arteries Exposed to Altered Shear Stress. 1995;**15**:2–10. doi:10.1161/01.ATV.15.1.2
- 53 Tsao PS, Lewis NP, Alpert S, *et al.* Exposure to shear stress alters endothelial adhesiveness. Role of nitric oxide. *Circulation* 1995;**92**:3513–9.
- 54 Himburg HA, Grzybowski DM, Hazel AL, *et al.* Spatial comparison between wall shear stress measures and porcine arterial endothelial permeability. *Am J Physiol Heart Circ Physiol* 2004;**286**:H1916–22. doi:10.1152/ajpheart.00897.2003

6. CYTOSKELETAL REGULATION IN LIPID-LOADED MONOCYTES

6.1. Introduction

6.1.1. Cytoskeletal regulation during cell migration

Cell migration is a complex, tightly regulated process that is fundamental to development, tissue homeostasis and disease. In the context of immunology, efficient leukocyte migration is required to mount an appropriate inflammatory response. While processes such as embryogenesis or wound healing require the collective migration of tightly associated layers of cells, the migration of leukocytes occurs almost exclusively as individual cells in an amoeboid manner [1]. Genes involved in cytoskeletal regulation compose 4.2% of all open reading frames in the human genome [2] and have spawned an entire field of ongoing research, thus it is challenging to present any more than a basic summary here. Nonetheless, some basic mechanisms of cell motility span most cell types and migratory processes.

In leukocytes, chemotaxis is initiated by attachment and firm adhesion to the endothelium or substratum, mediated largely by selectins and integrins respectively. This process is specific to leukocyte subpopulations and has been reviewed extensively elsewhere [3,4]. Regardless of this initial step, once adhered there are a number of basic processes that must occur in order for a leukocyte to undergo polarization and locomotion towards a stimulus, all of which are actin- dependent. Actin accounts for 20-30% of total protein content in highly motile cells such as leukocytes and exists in monomeric, globular subunits (G-actin), or polymerized filamentous F-actin [5]. Transition between G- and F- actin is catalysed by ATP hydrolysis and allows for the extension and retraction of actin microfilaments, which are the key regulator of both morphology and motility in all cells [6]. Microfilaments are polar in structure and consist of a 'pointed' origin end and a 'barbed'

protrusion that grows away from the origin [7]. In addition, actin filaments also undergo lateral cross-linking, thus forming either parallel or anti-parallel bundles via a number of adaptor proteins [8].

Initially, a migrating cell will extend protrusions generally in one of two morphologies: 1) lamellipodia or 2) filopodia. A lamellipodium is an F-actin rich fan-shaped structure at the leading edge of motile cells moving on a flat substratum, which protrude and retract in a transient manner. When long, thin protrusions extend beyond the lamellipodia, these are termed filopodia and are formed of unbranched, parallel actin bundles. Generally, these filopodia are thought to allow a cell to explore its extended environment prior to commitment to migration [9]. However, it is important to note that in some situations leukocytes appear to be able to migrate by membrane blebbing independent of lamellipodia [10,11], although the significance of this process remains controversial. Regardless, in presence of a chemoattractant gradient the cell then senses the migratory cue, often via asymmetric ligation of G-protein-coupled receptors (GPCR) [12] and begins the process of front-back polarization towards the stimulus.

Critical effectors in polarization are Rho family of GTP hydrolases (GTPase), which fluctuate between a GDP-bound 'inactive' and a GTP-bound 'active' state to act as molecular switches for cytoskeletal rearrangement [13]. The transition between these two states is controlled by guanine-nucleotide exchange factors (GEFs), which cause GTPase activation via the exchange GDP to GTP and GAPs, which promote GTP hydrolysis [14]. Upon GPCR binding, activation of phosphoinositide 3-kinase (PI3K) generates a gradient of the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the newly established leading edge of the cell, thus leading to GEF recruitment [12]. This in turn sequentially activates the GTPases CDC42 and RAC to promote rapid actin polymerization and lamellipodia protrusion [14]. Despite CDC42 and RAC acting in a cooperative manner to form lamellipodia, filopodia formation is promoted by CDC42 activity via N-WASP and the ARP2/3 complex, but may conversely inhibited by RAC [15], thus highlighting the complexity

of this process. In addition, other less well-characterized GTPases can also promote filopodia extension, including RIF [16].

Once polarity and forward protrusion have been established via RAC and CDC42, the cell must contract and detach the rear of the cell (or uropod) in order to move forward in a process dependent on RHOA and Myosin-II [17]. Key to this process is an inverse spatial correlation of RHOA and RAC activities to maintain cell polarity through a poorly characterized negative feedback loop [17,18]. Myosin-II is an actin-binding motor protein with one pair of heavy chain peptides (MHC) and two pairs of light chains (MLC), with phosphorylation of one of the MLC pairs regulating Myosin-II activity [17]. It is Myosin-II that can induce tension or propel the movement of actin filaments via ATPase activity. When GTP-bound, RHOA activates Myosin-II via Rho Kinase (ROCK) by directly phosphorylating MLC and inhibiting MLC phosphatase, thus causing actomyosin contraction [17]. MLC can also be activated directly by increased intracellular calcium via myosin light chain kinase (MYLK) and this process may be important for contraction at the cell periphery [19]. As well as stimulating actomyosin contractility, ROCK also stabilizes actin filaments via inactivation of the actin-binding protein cofilin [20]. Finally, in some cells the tension generated on the rear of the cell during cytoskeletal contraction can be sufficient to mechanically open calcium channels, which may signal via calcium-activated proteases and phosphatases to detach adhesions in the uropod from the substratum [21]. This process is summarised in Figure 6.1.

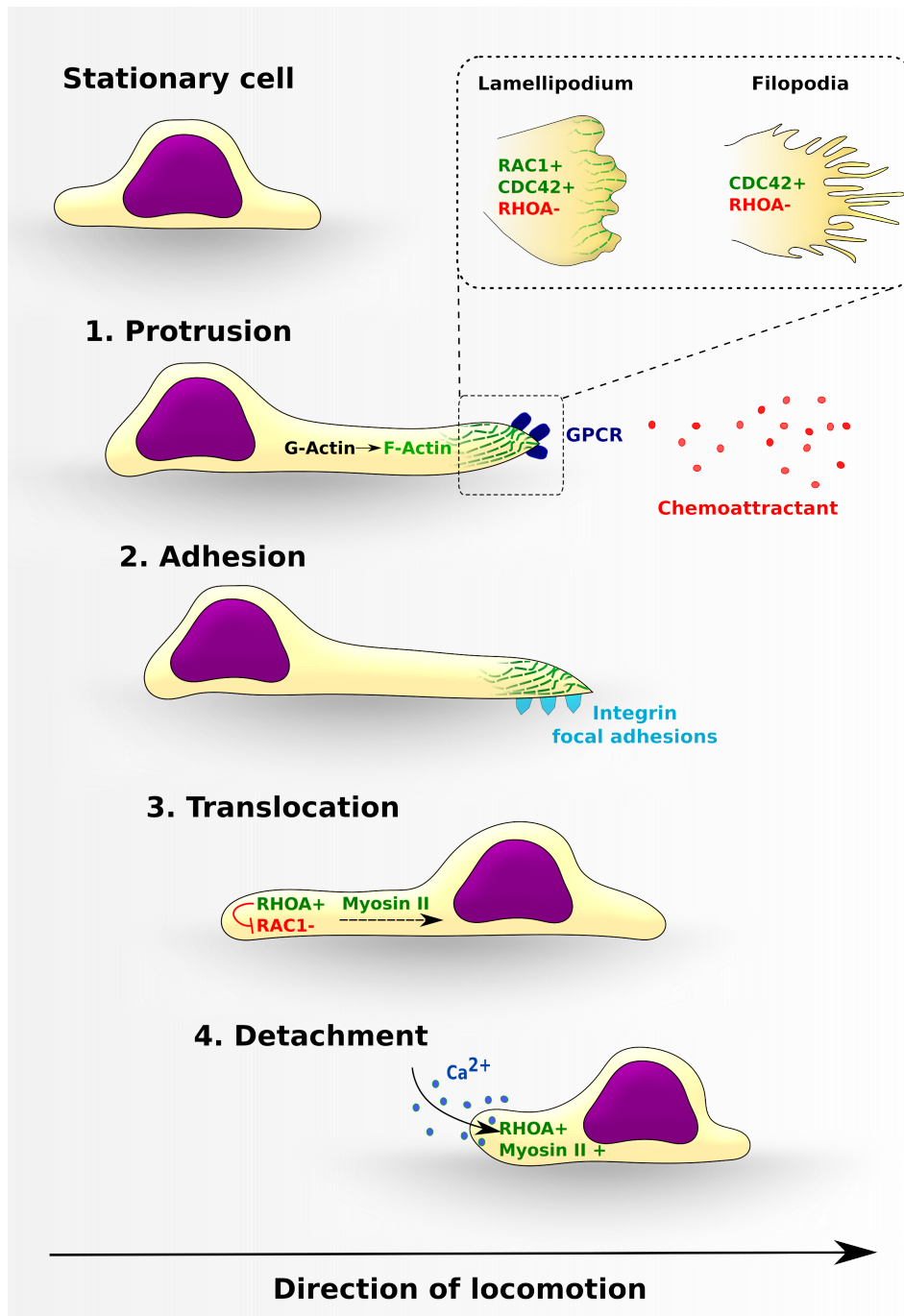


Figure 6.1 Summary of cytoskeletal regulation during cell migration.

(1) In the presence of a migratory stimulus, asymmetrical ligation of GPCRs leads to extension of an F-actin rich lamellipodia or filopodia towards the stimulus, mediated by RAC1/CDC42 and CDC42 respectively. (2) Clusters of integrins at the leading edge form focal adhesions between the cell and the extracellular matrix. (3) Activation of RHOA and inactivation of RAC1 at the rear of the cell activates myosin-II, which induces contraction of actin filaments to propel the rear of the cell, or uropod, forward. (4) Tension generated at the rear of the cell opens mechanosensitive calcium channels, activating effectors including proteases and myosin-II in order to detach the uropod from the substratum.

6.1.2. Cell migration in *Drosophila* hemocytes

The mechanisms of cytoskeletal regulation during single cell migration are heavily evolutionarily conserved across billions of years from the simple eukaryote *Dictyostelium discoideum* to humans [8]. The fruit fly *Drosophila melanogaster* also has professional, monocyte/ macrophage- like phagocytes termed hemocytes. Strikingly, *Drosophila* hematopoiesis is controlled by many homologous transcription factors and signaling pathways to those involved in the mammalian process i.e. GATA and RUNX families and JAK/STAT signaling. Embryonic hemocytes are highly motile cells that migrate along fixed pathways during embryogenesis. An initial dispersal from the head mesoderm along the ventral midline is followed by rapid lateral migration facilitated by cytoskeletal small GTPases, the chemoattractants Pvf2/3 and contact inhibition of locomotion[22,23]. Since hemocytes are relatively superficial to the embryo surface, migration can be imaged at high spatio-temporal resolution in vivo [23]. Using the GAL4-UAS system, gene expression in *Drosophila* can be manipulated in a highly tissue-specific manner, thus making *Drosophila* hemocytes an elegant system to elucidate phagocyte cytoskeletal dynamics in vivo. Interestingly, *Drosophila* hemocytes become dysfunctional when flies are fed a high fat diet, and efficiently phagocytose human LDL [24].

While delivery of both LDL and cyclodextrin-bound free cholesterol has been shown to disrupt the cytoskeleton in macrophages via modulation of Rac[25,26] and Rho[27] activity, the mechanism and specificity of this interaction remains unclear. In addition, no work has been performed in either total monocytes or monocyte subsets. Since it has long been appreciated that monocyte differentiation to macrophage leads to profound changes in the cytoskeleton [28], I attempted to determine whether similar mechanisms might be governing the migratory inhibition observed here in LDL and VLDL treated monocytes.

6.2. Results

6.2.1. Cytoskeletal gene expression changes during hypercholesterolemia.

During peritonitis experiments using the *Ldlr*^{-/-} mouse on HFD (Chapter 3.2.2), RNA from peritoneal cells after thioglycollate was stored to subsequently analyse gene expression by RT-qPCR. In the context of impaired monocyte migration into the peritoneum after HFD and after LDL/VLDL treatment in vitro, I assessed mRNA abundance of the cytoskeletal small GTPases *CDC42*, *RHOA* and *RAC1*. While there was no pronounced change in *CDC42* or *RAC1* expression, there was a significant down-regulation of *RHOA* (Figure 6.2A). However, the composition of the peritoneal infiltrate is clearly altered after HFD, thus these data could be the result of RNA harvested different proportions of cells. To assess this further, I analysed publicly available microarray data from monocytes of patients with familial hypercholesterolemia (GEO Accession GDS3668, [29]). Strikingly, expression of *CDC42*, *RAC1* and *RAC2* was up regulated in FH homozygotes compared to healthy controls, while *RHOA* expression was decreased (Figure 6.2B) as observed in the *Ldlr*^{-/-} mouse on HFD. This opposite regulation of *RHOA* versus *CDC42* and *RAC1/2* is consistent with previous data demonstrating that these proteins are functionally antagonistic in regulating cell morphology and migration [17,18]. To confirm whether this was a monocyte specific phenotype or applied to all leukocytes in a hypercholesterolemic environment, I analysed another microarray of blood T-cell gene expression from the same group (GEO Accession GDS3669, [29]). While *CDC42* was also up regulated in FH homozygotes, there was no significant change in *RAC1*, *RAC2* or *RHOA* (Figure 6.2C), suggesting a monocyte specific phenotype. To investigate small GTPase transcriptional regulation following LDL/ VLDL in vitro treatment, monocytes were incubated with lipoproteins for 2 hours as described in previous experiments and transcript abundance analysed by RT-qPCR. There was no significant change in *CDC42*, *RHOA* or *RAC1* expression versus PBS after LDL (Figure 6.2D) or VLDL (Figure 6.2E) treatment, although taken overall there was a general trend towards the down-regulation of these genes in both monocyte subpopulations. However, as discussed in the introduction, changes in Rho-GTPase

transcript abundance do not necessarily translate to an increase in active GTP-bound protein and this was the next obvious pathway to investigate.

6.2.2. Cytoskeletal GTPase activation levels following VLDL treatment.

As discussed previously, Rho-family GTPases can exist in either a GDP-bound inactive form or a GTP-bound active form (illustrated in Figure 6.3A), facilitating the phosphorylation of downstream effector proteins such as ROCK for RHOA or PAK1 for RAC1 and CDC42 [17]. Given the gene expression changes observed in the *Ldlr*^{-/-} mouse and in FH patients, I hypothesized that activation of these proteins, particularly RHOA, would be altered by acute neutral lipid loading of human monocytes. CD16^{pos} or CD16^{neg} human monocytes from healthy donors were therefore incubated for 2 hours with 100µg/ml VLDL and the cell lysates were analysed for active RHOA using ELISA. However, VLDL alone produced no change in levels of RHOA activity in either monocyte subpopulations (Figure 6.3B). Following this observation, I postulated that VLDL might not affect RHOA under homeostatic conditions, but may instead impair the spatio-temporally regulated activation required during polarization and migration. As such, I utilized the RHOA activator CN03 concurrent with VLDL incubation, which is based on the catalytic domain of bacterial cytotoxic necrotizing factor toxins and causes RHOA to become constitutively GTP-bound [30]. When CN03 was added at 2µg/ml for 2 hours as previously reported [31], both CD16^{pos} and CD16^{neg} cells displayed a marked increase in RHOA activation as expected (Figure 6.3C). However, this effect was significantly abrogated by simultaneous treatment with 100µg/ml VLDL, suggesting that neutral lipid accumulation is directly inhibiting the GTP-binding of RHOA (Figure 6.3C).

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson- January 2016

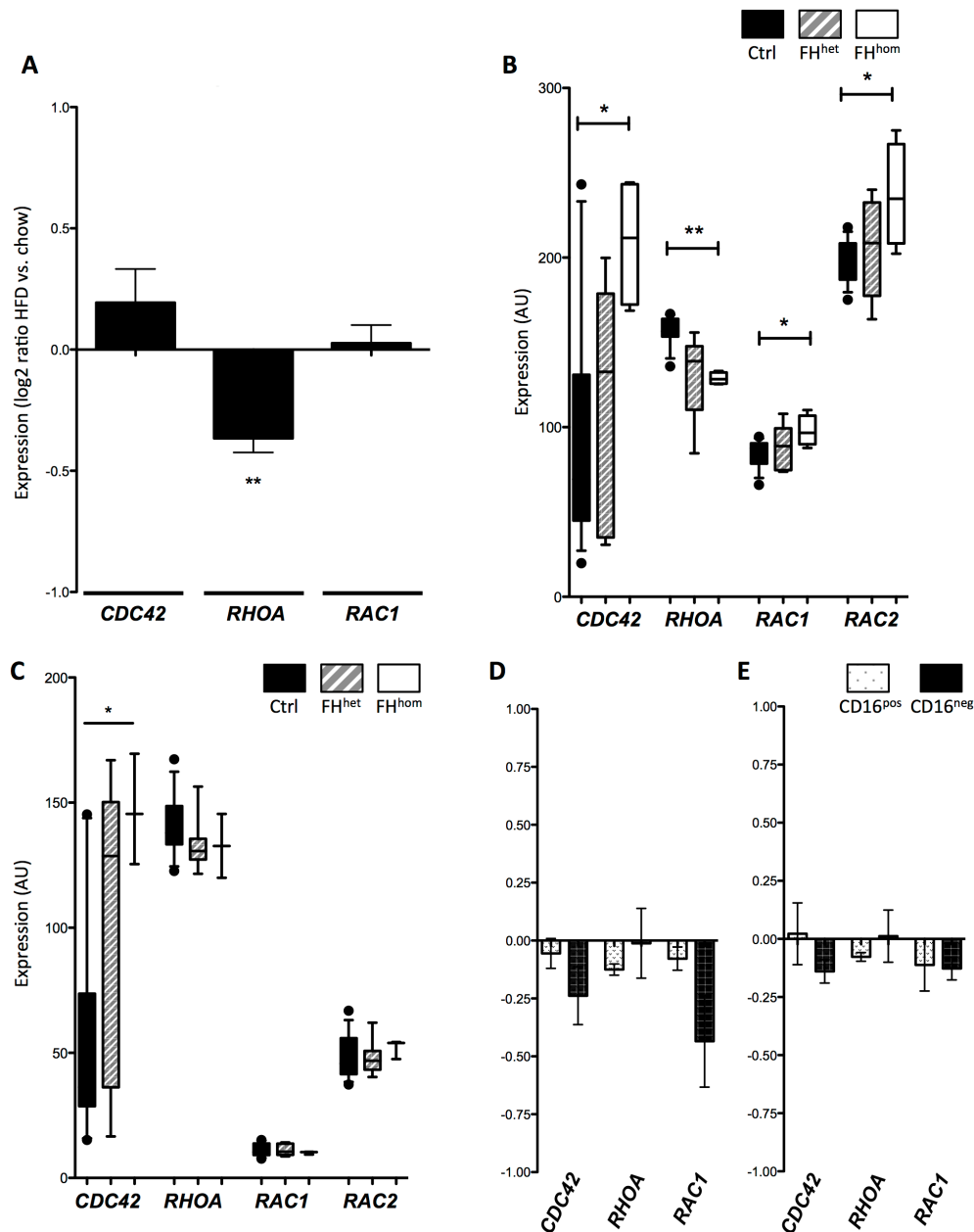


Figure 6.2 Leukocyte small GTPase gene expression during dyslipidaemia.

(A) Cytoskeletal small GTPase gene expression assessed by RT-qPCR in peritoneal cells from *Ldlr*^{-/-} mice on chow or HFD following thioglycollate peritonitis (See also Figure 3.4), n=4 per group. Error shows the mean±SEM. (B) Small GTPase gene expression in monocytes from heterozygous FH patients (FH^{het}), homozygous FH patients (FH^{hom}) or healthy controls (Ctrl), as presented in GEO microarray GDS668 [29], n= 4-13. (C) As (B), but data for blood T-lymphocytes, GEO microarray GDS669, n= 3-13. Boxes display 10th-90th percentile. (D) Cytoskeletal small GTPase gene expression assessed by RT-qPCR in human CD16^{neg}CD16^{pos} monocytes incubated with (D) LDL, or (E) VLDL. Data shown as log2 ratio versus PBS, n=3. Error shows the mean±SEM, groups were compared using a two-tailed Mann-Whitney U test.

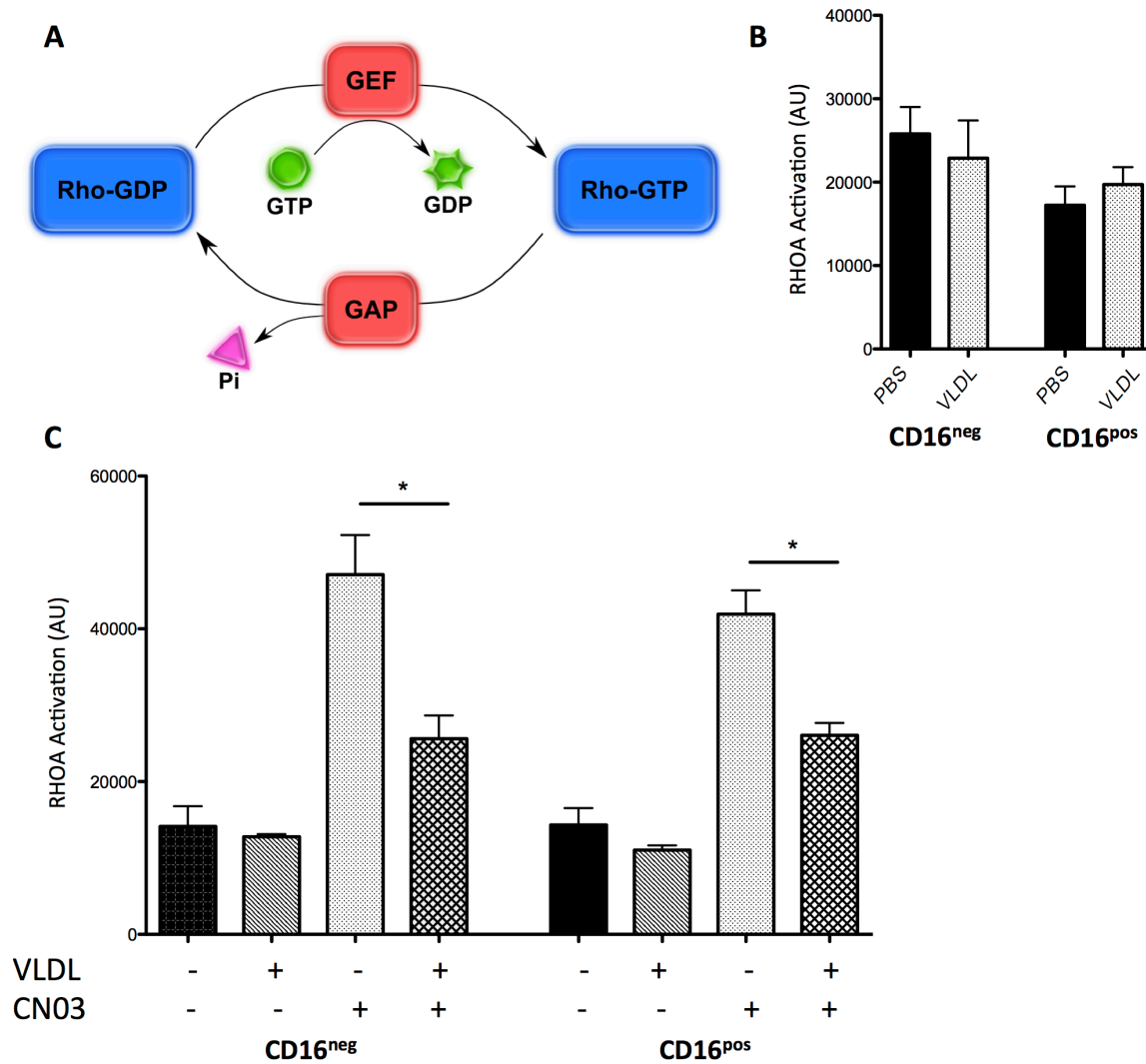


Figure 6.3 RHOA activation levels following VLDL treatment.

(A) Schematic of Rho-family GTPase activation, demonstrating shuttling between active (Rho-GTP) and inactive (Rho-GDP) forms. (B) RHOA activation in human CD16^{pos}/CD16^{neg} monocytes after VLDL treatment (2 hours, 100µg/ml), n=3 donors in duplicate. (C) As in (B), except monocytes were additionally treated with or without CN03 (2µg/ml), n=3 donors in duplicate. Error bars show the mean±SEM. groups were compared using a two-tailed Mann-Whitney U test.

To investigate effects on the RHOA pathway further, I tested the hypothesis that MLC phosphorylation downstream of RHOA would also be affected by VLDL treatment. When CD16^{pos} and CD16^{neg} monocytes were treated with VLDL alone, no significant effect was observed on levels of pMLC as assessed by Western blot (Figure 6.4A-B). However, as in RHOA it was possible that a phenotype might only be visible when cells were required to activate MLC signaling. Calcium (Ca²⁺) influx causes RHOA dependent and independent MLC phosphorylation (pMLC) [32,33] and can occur due to mechanical forces during cell migration [21]. As such, the calcium ionophore A23187 was used following 2 hours of VLDL pre-treatment. As expected, A23187 caused an increase in pMLC in both monocyte subpopulations, but surprisingly VLDL only caused a significant impairment of MLC phosphorylation in CD16^{pos} monocytes, with a subtle trend towards less pMLC in the CD16^{neg} population (Figure 6.4A-B). Contrary to RHOA activation data, this implies that VLDL may have a monocyte subset-specific effect on the cytoskeleton.

Furthermore, separate to the RHOA/ MLC pathway others have previously demonstrated increased cell membrane cholesterol in macrophages modulating the activity of other cytoskeletal GTPases, including RAC1 [25,26]. To simultaneously screen for activity of multiple cytoskeletal GTPases, I assessed phosphorylation of PAK1 and PAK2, effector kinases downstream of both CDC42 and the RAC family, which are antagonistic to RHOA [34]. Using Western blot, there was no apparent change in either PAK1 or PAK2 phosphorylation after 2 hours of VLDL treatment in both monocyte subpopulations (Figure 6.5A). This was further confirmed at the level of CDC42, which also had unchanged activity in response to both LDL and VLDL as assessed by ELISA (Figure 6.5B). It was therefore inferred that the RAC/ CDC42/ PAK pathway is not altered by neutral lipid loading in blood monocytes.

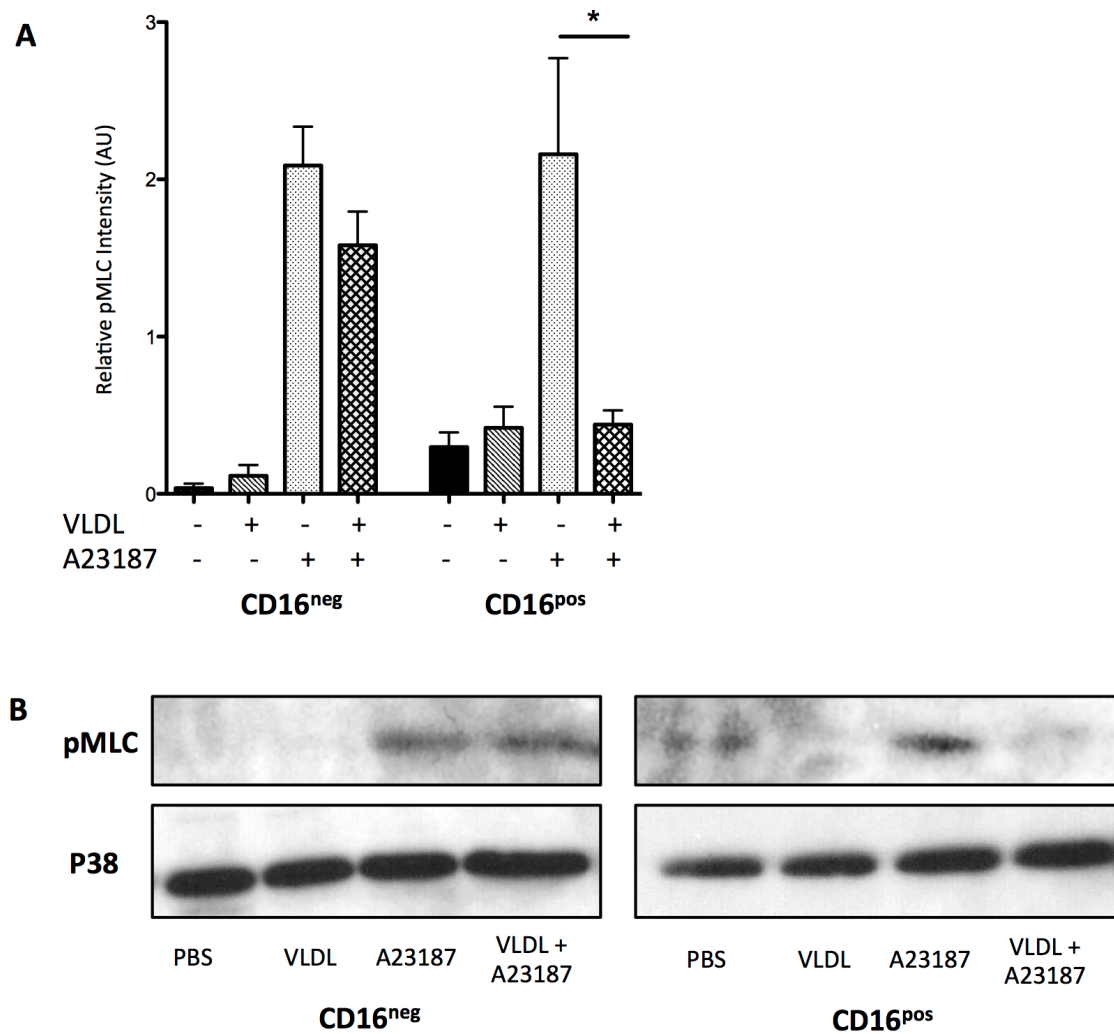


Figure 6.4 Myosin Light Chain phosphorylation in VLDL treated human monocytes.

CD16^{neg} and CD16^{pos} human monocytes were treated with PBS or VLDL (2 hours, 100µg/ml) followed by PBS or A23187 for 15 minutes and MLC phosphorylation was assessed by Western blot. (A) Relative pMLC levels measured by band densitometry, normalized to total P38, n=4 donors from 3 independent experiments. (B) Representative Western blot example of data displayed in (A). Error bars show the mean±SEM, groups were compared using a two-tailed Mann-Whitney U test.

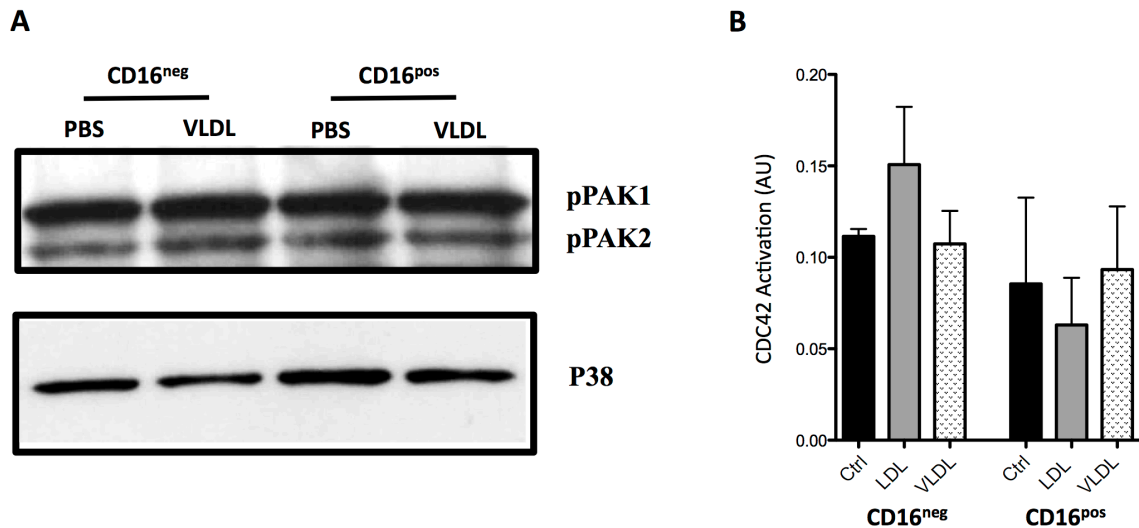


Figure 6.5 CDC42 and PAK pathway activity after monocyte LDL and VLDL treatment. Human CD16^{pos} and CD16^{neg} monocytes treated with PBS, LDL or VLDL (100ug/ml) for 2 hours. (A) PAK1/2 phosphorylation following PBS or VLDL treatment using Western blot, a representative blot of two is shown. (B) CDC42 activity measured using ELISA after PBS/ LDL/ VLDL, n=3 donors. Error bars show the mean±SEM.

6.2.3. Monocyte morphological changes induced by VLDL or RHOA activation.

While the data presented thus far strongly suggest that RHOA/ MLC inactivation may be responsible for the altered motility observed in VLDL treated monocytes, this hypothesis required support with functional data. Since the cytoskeleton is composed primarily of actin and controls cell shape [8] and VLDL seems to prevent RHOA activation (Figure 6.3A), I reasoned that VLDL and CN03 should promote distinct and antagonistic effects on monocyte morphology. To test this, human monocyte subpopulations were treated with PBS or VLDL in conjunction with/ without CN03 for 2 hours, before being allowed to adhere to tissue culture treated plastic for 45 minutes. Cells were then fixed and actin was fluorescently stained using phalloidin in order to visualize the cytoskeleton by fluorescence confocal microscopy. Representative images of CD16^{neg} and CD16^{pos} cell morphology after these treatments are shown in Figure 6.6A. Cell morphology was then assessed using automated analysis for cell area, where a higher value indicates increased cell spreading, and circularity, where a lower value generally indicates the presence of

more protrusions such as filopodia. After control PBS treatment, there was already subset-specific morphology evident in monocytes. While CD16^{neg} monocytes will spread on a plastic surface and form lamella-like structures without stimulation, CD16^{pos} monocytes remain smaller and highly circular (Figure 6.6B). VLDL treatment reverses this phenotype; CD16^{neg} monocytes no longer spread, evidenced by lower cell area and higher circularity, while CD16^{pos} monocytes become significantly less circular and extend filopodia-like protrusions at what appears to be the rear of the cell (Figure 6.6A-C). After CN03 treatment, both cell populations spread, form pronounced lamella and have increased cell area with no change in circularity, as would be expected from previous data in fibroblasts [35]. Strikingly, concurrent treatment with VLDL and CN03 completely reverses the CN03-induced spreading and this can be seen in both subpopulations as a significant decrease in their cell area (Figure 6.6B). While the circularity of CD16^{neg} cells again remained unchanged, CD16^{pos} monocytes had substantially decreased circularity due to the extension of long (>20µm), thin protrusions from the cell in a manner not seen in any of the other treatments (Figure 6.6C). Evidently, there is clearly some degree of cytoskeletal signalling that is specific to monocyte subpopulations that requires further exploration. However, VLDL obviously reversed cell spreading and lamella extension induced by CN03, confirming the previous RHOA activation data (Figure 6.3A) in a more functional setting.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function William Jackson- January 2016

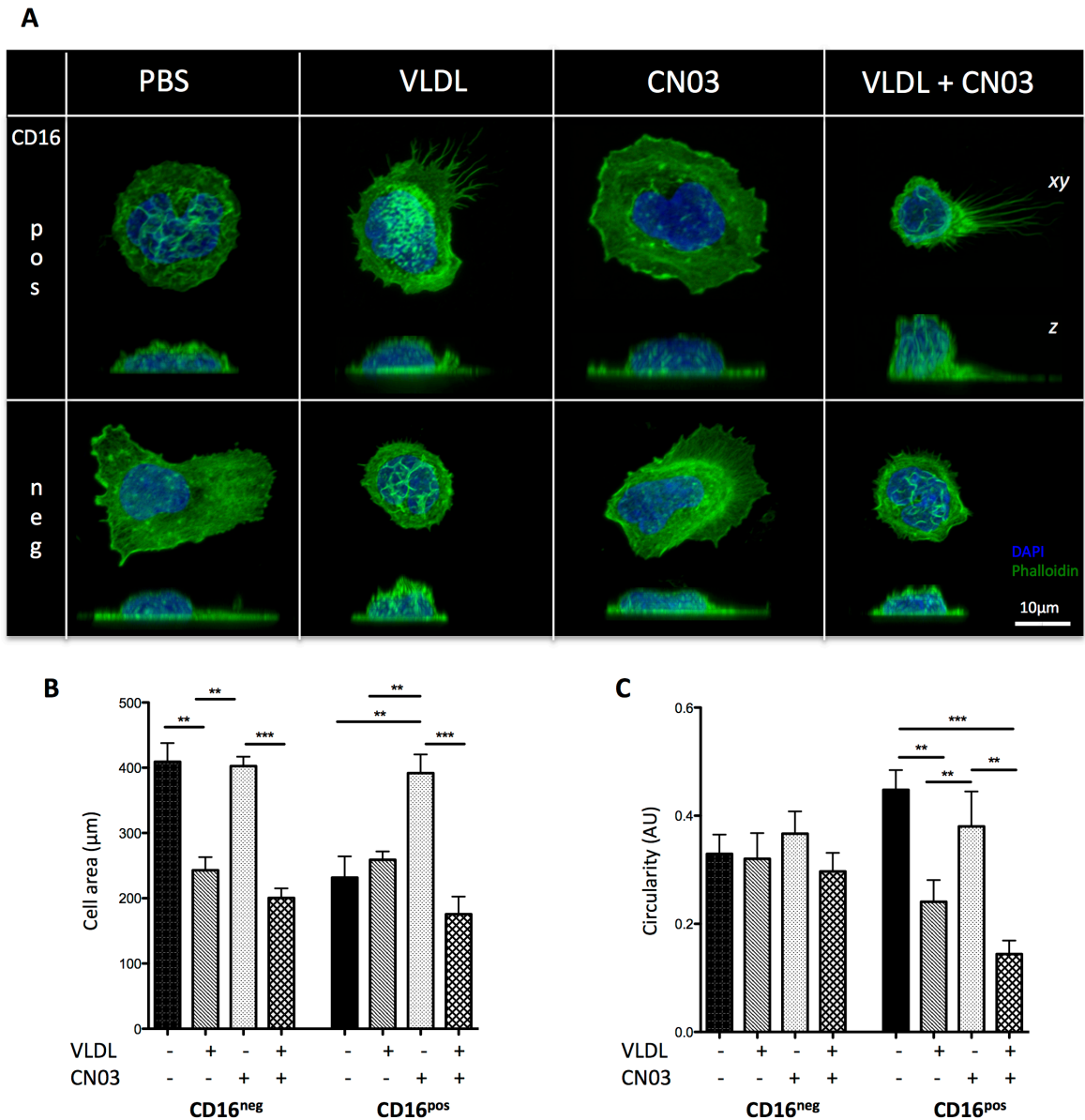


Figure 6.6 The effects of VLDL treatment and RHOA activation on monocyte morphology.

Human CD16^{neg} and CD16^{pos} monocytes were treated with PBS or VLDL (2 hours, 100µg/ml), with or without CN03 (2µg/ml). Cells were adhered to tissue culture plastic and actin stained with fluorescent phalloidin. (A) Representative examples of cell morphology (green= phalloidin, blue= DAPI). Images were analyzed for (B) Cell area (µm) and (C) Circularity. n= 20-25 cells per condition, data shows one representative experiment from two. Error bars show the mean±SEM, groups were compared using a two-tailed Mann-Whitney U test.

6.2.4. Monocyte motility following pharmacological RHOA activation.

With the knowledge that VLDL incubation was able to CN03-induced monocyte spreading, I hypothesized that RHOA activation using CN03 might also improve the migratory impairment in neutral-lipid loaded monocytes. To test this, I used a simple real-time chemokinesis assay (as in Figure 5.7) in total monocytes that had been treated with PBS or VLDL (2 hours, 100µg/ml), with or without CN03. After 90 minutes of imaging, as seen previously (Figure 5.7), VLDL caused a subtle yet significant decrease in monocyte displacement (Figure 6.7B), speed (Figure 6.7C) and confinement ratio (Figure 6.7D). However, in this experiment CN03 caused a similar inhibition of migration that was more pronounced than the effects of VLDL when measured as displacement (Figure 6.7B) and confinement ratio (Figure 6.7D). In addition, CN03 did not reverse the VLDL migratory inhibition as hypothesized, but instead the CN03 and VLDL combination caused a more severe impairment of migration than either of these treatments alone (Figure 6.7A-D). As such, pharmacological activation of RHOA is not able to restore migration in VLDL treated monocytes, which may concur with morphological data showing that treatment with a CN03/VLDL combination prevents cell spreading in both monocyte subpopulations (Figure 6.6).

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

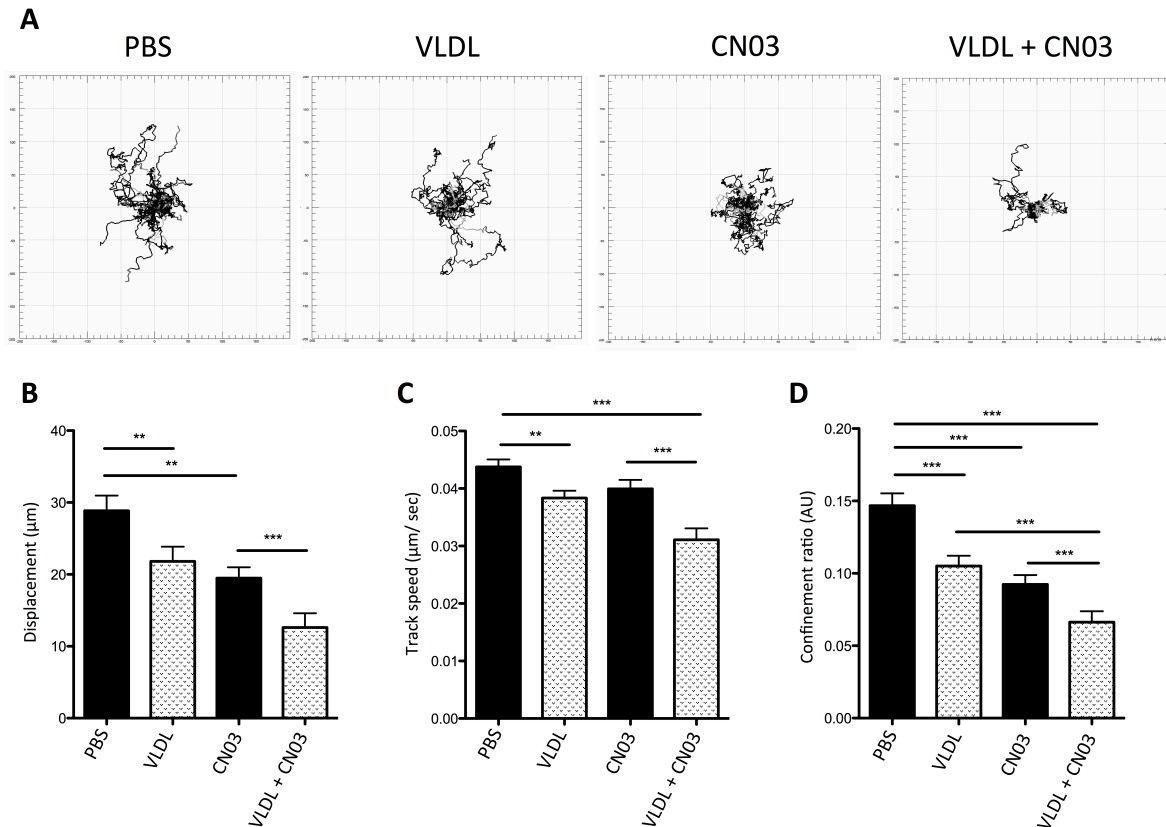


Figure 6.7 Monocyte chemokinesis after VLDL and CN03 treatment.

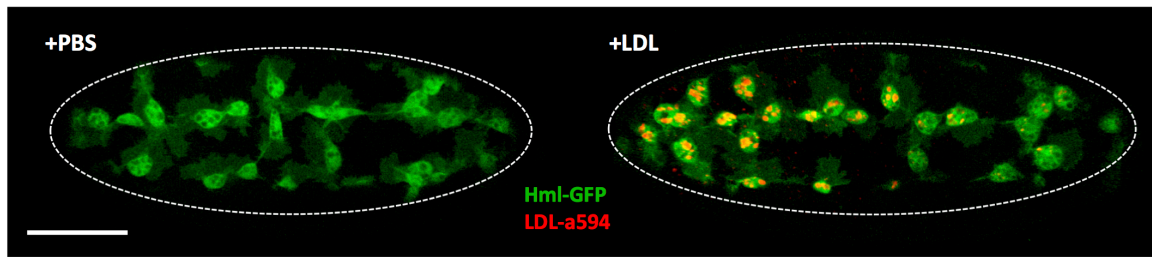
(A) Track projections from total human monocytes treated with PBS or VLDL (2 hours, 100 $\mu\text{g}/\text{ml}$), with or without CN03 (2 $\mu\text{g}/\text{ml}$) in a 2D real-time chemokinesis assay with diffuse C5a stimulation. Tracks were analyzed for (B) displacement (μm) (C) speed ($\mu\text{m}/\text{sec}$) and (D) confinement ratio (AU). $n=75\text{-}205$ cells per condition. Error bars show the mean $\pm$ SEM, groups were compared using a two-tailed Mann-Whitney U test.

6.2.5. *Drosophila* hemocyte migration following LDL injection.

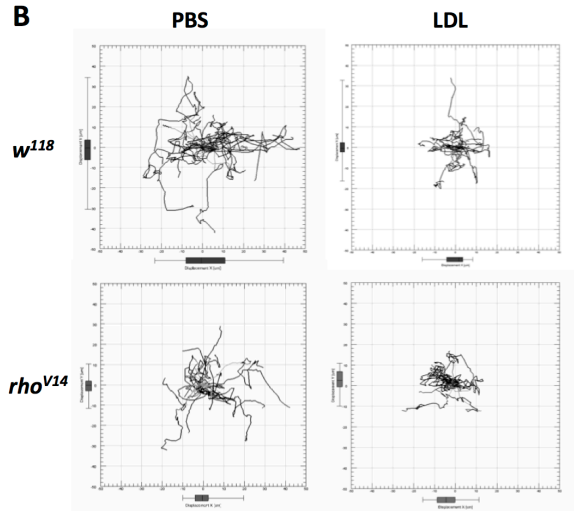
Given the inconclusive data obtained with CN03 and lack of appropriate reagents for studying primary monocyte GTPase activity during cell migration in vitro, I sought an appropriate in vivo model to validate my hypotheses. *Drosophila* hemocytes have previously been shown to efficiently phagocytose human LDL and respond to a high fat diet [24]. The genetic tractability of *Drosophila* also allows the generation of flies that express a constitutively active Rho (Rho^{V14}) exclusively in hemocytes, as previously described [36]. Using flies that express a UAS-GFP construct under the control of the hemocyte-specific promoters *croquemort* (*crq*) and *serpent* (*srp*), I investigated the effects of LDL on hemocyte migration during embryogenesis in collaboration with the group of Prof Will Wood at University of Bristol. The aims of this work were two-fold: 1) To establish whether the migratory inhibition of mononuclear phagocytes by lipoproteins was evolutionarily conserved in *Drosophila*, and 2) to determine whether expression of a constitutively active Rho could be used to reverse any LDL-induced migratory impairment.

When *Crq/ Srp-Gal4; UAS-GFP Drosophila* embryos were injected with fluorescent LDL, it rapidly (<2 hours) localized inside hemocytes (Figure 6.8A), confirming previously published data [24]. In agreement with data from human monocytes, LDL injection appeared to visually decrease migration in hemocytes (Figure 6.8B). This could be quantified as a significant decrease in cell speed (Figure 6.8C) and displacement (Figure 6.8D), although this was less pronounced than in VLDL incubated monocytes and no significant change was seen in confinement ratio (Figure 6.8E). However, expression of Rho^{V14} caused no detectable alteration in any metric used to assess cell migration when compared to wild type, both after PBS and LDL injection (Figure 6.8C-E). No gross defects in cell morphology were observed in any condition, although this was challenging to analyze formally without higher resolution imaging or a fluorescent cytoskeletal reporter such as actin-GFP. As such, it was concluded that while LDL-induced migratory impairment appears to also occur in *Drosophila* hemocytes, expression of constitutively active Rho is unable to reverse this phenotype.

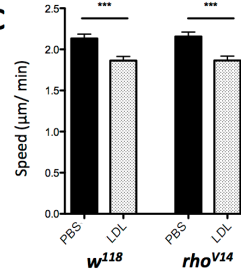
A



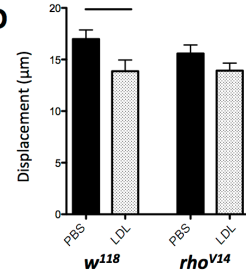
B



C



D



E

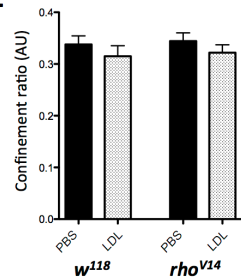


Figure 6.8 LDL induced migratory impairment in *Drosophila* embryonic hemocytes.

Hml-GFP *Drosophila* embryos expressing either wild-type (w^{118}) or constitutively active (Rho^{V14}) Rho1 were injected with PBS or LDL-AlexaFluor-594 and imaged by confocal microscopy 2 hours post-injection. (A) Representative examples of hemocyte and LDL localization. Scale bar= 50 μ m. (B) Track projections from migrating hemocytes. Hemocyte locomotion was analysed and quantified as (C) speed (μ m/ sec), (D) displacement (μ m) and (E) confinement ratio. n=115-145 hemocytes per group, from 5-6 flies per group. Error bars show the mean $\pm$ SEM, groups were compared using a two-tailed Mann-Whitney U test.

6.3. Discussion

These data clearly demonstrate that the monocyte cytoskeleton is perturbed by exposure to either in vivo dyslipidaemia or in vitro VLDL exposure, most notably through regulation of the small GTPase *RHOA*. Interestingly, this occurs not only at the level of *RHOA* activation, but also through transcriptional modulation of *Rhoa* in *Ldlr*^{-/-} mice and of *RHOA*, *CDC42*, *RAC1* and *RAC2* in FH patients. However, the same transcriptional changes are not present at monocytes treated acutely with VLDL in vitro, indicating that transcriptional regulation of these cytoskeletal proteins may be a delayed response during chronic exposure to dyslipidaemia. The mechanism linking transcriptional regulation to reduced *RHOA* activity is unclear, but may represent a negative feedback loop orchestrated by *RHOA* itself. *RHOA* is known to activate several transcription factors and therefore modulate a range of inflammatory and metabolic targets via NFκβ, GATA-4, C/EBPβ, MRTF and c-MYC [37-41]. While inhibition of the *RHOA* effector kinase ROCK decreases levels of *MYC* expression [41], knockdown or deletion of *MYC* also conversely decreases levels of *RHOA* transcript and active protein in cancer cells [42], and thus is the most likely candidate to participate in a negative- or positive-feedback loop. In the currently proposed model, c-MYC co-operates with the E3-ligase SKP2 to bind a non-canonical E-box motif in the *RHOA* promoter, recruiting the *MYC* co-activators MIZ1 and EP300 in order to induce *RHOA* transcription [42].

This complex feedback loop linking Rho-family GTPase activity to gene expression also highlights the difficulties in interpreting functional data Rho-GTPase-null animals or cell lines, due to the vast number of transcriptional targets that will presumably also be modulated in this case. For example, data from *Rhoa/Rhob*^{-/-} mouse macrophages shows that knockout of this pair causes morphological defects, but in fact increases speed of chemotaxis both in vitro and during peritonitis, the opposite effect to what would be hypothesized from the data presented here [43]. Conversely, pharmacological inhibition of *RHOA* signaling in both leukocytes and fibroblasts greatly decreases chemotaxis [35,44]. In addition, while termed 'inactive' there is evidence that Rho-family GTPases may in reality have effector functions while GDP-bound. For example, *RHOA*-GDP binding decreases the stability of

RHOB protein, which is therefore strongly up regulated when RHOA is silenced [45]. This may explain functional discrepancies between experiments employing GTPase inactivation versus genetic suppression or deletion.

Under homeostatic conditions, VLDL treatment had no effect on monocyte levels of RHOA-GTP. However, a strong inhibition of activation was seen in both CD16^{pos} and CD16^{neg} monocytes after addition of the RHOA activator CN03. CN03 prevents RHOA from dissociating from a GTP-bound to a GDP-bound inactive state and it is via this mechanism that its derivative bacterial toxin cytotoxic necrotizing factor 1 facilitates *Escherichia coli* invasion across the blood-brain barrier endothelium [30]. Since RHOA activation is required for cell migration as discussed above, the inhibition of monocyte motility by VLDL may only become evident following an inflammatory or activatory stimulus, such as C5a. Given the mode of action of CN03, VLDL is most probably acting directly on RHOA to prevent its association with GTP, or on the guanine exchange factors (GEFs) that catalyze this process.

One likely mechanistic candidate for neutral lipid modification of this process is RHOA prenylation. Prenylation involves the addition of a hydrophobic farnesyl or geranylgeranyl isoprenyl group to a protein by the enzymes farnesyl transferase, Caax protease or geranylgeranyl transferase I [46] and is crucial for RHOA biological activity [47]. Most notably, prenylation is essential for the interaction of the Rho-family with RHO-GDI, which acts as a chaperone during membrane delivery and recycling of Rho-GTPases [48]. This interaction is key for the subcellular localization and membrane binding of Rho-GTPases [49], which in turn is essential for their activation and function [50,51]. Cholesterol is itself an isoprenoid, meaning that cholesterol metabolism and protein prenylation are intricately linked. Both geranylgeranyl pyrophosphate and farnesyl pyrophosphate are synthesized by the HMG-CoA reductase pathway and the combination of two farnesyl pyrophosphate molecules forms squalene, a direct precursor for cholesterol via lanosterol [52]. Unsurprisingly in this context, the inhibition of HMG-CoA reductase with statins to treat hypercholesterolemia has also been shown to inhibit Rho-family prenylation in

endothelial cells [53]. As such, it is highly conceivable that an excess of intracellular cholesterol ester could alter Rho-family prenylation and activation, although currently it is less clear why this effect would be RHOA-specific at early time points. Further work would be required to decipher the exact subcellular localization of these GTPases during monocyte migration, which may reveal why VLDL is inhibiting RHOA, while RAC1 and CDC42 seem unaffected. In addition, it is possible that while PAK1/2 phosphorylation is unchanged by VLDL, Rac signaling may be bypassing this pathway. RAC2 is expressed in myeloid cells and can signal independently of PAK1/2 to mediate chemotaxis [54], and therefore may be affected in VLDL treated monocytes.

While RHOA activation is impaired by VLDL treatment in both monocyte subsets, inhibition of MLC phosphorylation in response to a calcium ionophore (A23187) appears to be specific to CD16^{pos} cells. Calcium influx can cause MLC phosphorylation in a RHOA-dependent manner or independent of RHOA via MYLK [17,19,32]. Analysis of published microarrays on human monocyte subpopulations (GEO Accessions GSE18565[55], GSE16836[56]) shown that *MYLK* transcript is expressed at much higher levels in CD16^{neg} monocytes than in CD16^{pos}. As such, CD16^{neg} monocytes may utilize the MYLK pathway to continue to phosphorylate MLC in response to calcium in the absence of sufficient RHOA activity.

However, migration and cell morphology are disrupted by VLDL in both CD16^{pos} and CD16^{neg} monocytes, so this continued ability to phosphorylate MLC is clearly not sufficient to rescue the functional impact of RHOA inhibition. In addition, activation of RHOA using CN03 does not restore normal morphology or chemokinesis in VLDL treated monocytes. While this may indicate that these phenotypes are not RHOA mediated, it is more likely that the use of pharmacological activation does not restore the fine balance of spatio-temporal Rho-GTPase regulation that is required for proper cytoskeletal function [50,51]. Indeed, in the chemokinesis assay a combination of VLDL and CN03 actually caused a more severe migratory inhibition than either alone, emphasizing the complexity and subtlety of the signals required for

correct cell migration and the challenging nature of targeting cytoskeletal GTPases therapeutically.

In the time remaining of this PhD, it was not feasible to generate *Rhoa* mutant mice, whether over expression or constitutive activation. In addition, for reasons outlined above, pharmacological manipulation of Rho-GTPases is challenging and can generate data that is difficult to interpret. The high level of spatial and temporal resolution possible during in vivo imaging and ease of genetic manipulation made *Drosophila* embryos an interesting model to study mononuclear phagocyte migration in response to lipoproteins. It is also useful for data interpretation purposes that *Drosophila* possess only one GTPase of the Rho subfamily [57], while in mammals RHOA, RHOB and RHOC have a degree of functional redundancy and can compensate in cases where the function of one family member is disrupted [43]. Interestingly, LDL injection also causes migratory impairment in *Drosophila* hemocytes, suggesting that the phenotype presented here may be via an evolutionarily conserved mechanism. Constitutive activation of *Rho1* was unable to reverse this phenotype, but crucially, did also not cause any obvious migratory or morphological defect after PBS injection, potentially suggesting that hemocyte migration is Rho-independent. Some functional discrepancies between human RHOA and *Drosophila Rho1* are inevitable, given that the pair share only 73% cDNA sequence homology (Ensembl 2015[58]). In addition, while *Drosophila* possess several apoB-lipoproteins for lipid transport including *apolpp* and *apoltp*, these are structurally distinct from human LDL and no apoA orthologue has been found in the fly genome[59]. As such, this may represent a response of hemocytes to the phagocytosis of any exogenous material via scavenger receptors, rather than a lipid-specific phenotype. These data warrant further investigation, as the high-resolution, real-time imaging of actin dynamics that is possible in *Drosophila* hemocytes [23] may present an elegant in vivo system to study the effects of lipoproteins on the cytoskeleton.

6.4. References

- 1 Friedl P, Wolf K. Plasticity of cell migration: a multiscale tuning model. *J Cell Biol* 2010;**188**:11–9. doi:10.1083/jcb.200909003
- 2 Mi H, Muruganujan A, Thomas PD. PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. *Nucleic Acids Res* 2013;**41**:D377–86. doi:10.1093/nar/gks1118
- 3 Ley K, Laudanna C, Cybulsky MI, *et al.* Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature Reviews Immunology* 2007;**7**:678–89. doi:10.1038/nri2156
- 4 Muller WA. Mechanisms of leukocyte transendothelial migration. *Annu Rev Pathol Mech Dis* 2011;**6**:323–44. doi:10.1146/annurev-pathol-011110-130224
- 5 Korn ED. Biochemistry of actomyosin-dependent cell motility (a review). *Proc Natl Acad Sci USA* 1978;**75**:588–99.
- 6 Loisel TP, Boujemaa R, Pantaloni D, *et al.* Reconstitution of actin-based motility of *Listeria* and *Shigella* using pure proteins. *Nature* 1999;**401**:613–6. doi:10.1038/44183
- 7 Moore PB, HUXLEY HE, DeRosier DJ. Three-dimensional reconstruction of F-actin, thin filaments and decorated thin filaments. *J Mol Biol* 1970;**50**:279–95.
- 8 Pollard TD, Borisy GG. Cellular motility driven by assembly and disassembly of actin filaments. *Cell* 2003;**112**:453–65.
- 9 Mejillano MR, Kojima S-I, Applewhite DA, *et al.* Lamellipodial versus filopodial mode of the actin nanomachinery: pivotal role of the filament barbed end. *Cell* 2004;**118**:363–73. doi:10.1016/j.cell.2004.07.019
- 10 Charras G, Paluch E. Blebs lead the way: how to migrate without lamellipodia. *Nat Rev Mol Cell Biol* 2008;**9**:730–6. doi:10.1038/nrm2453
- 11 Lämmermann T, Bader BL, Monkley SJ, *et al.* Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature* 2008;**453**:51–5. doi:10.1038/nature06887
- 12 Siehler S. Regulation of RhoGEF proteins by G12/13-coupled receptors. *British Journal of Pharmacology* 2009;**158**:41–9. doi:10.1111/j.1476-5381.2009.00121.x
- 13 Nobes CD, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 1995;**81**:53–62.
- 14 Bustelo XR, Sauzeau V, Berenjeno IM. GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *Bioessays* 2007;**29**:356–70. doi:10.1002/bies.20558
- 15 Rohatgi R, Ma L, Miki H, *et al.* The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* 1999;**97**:221–31.
- 16 Pellegrin S, Mellor H. The Rho family GTPase Rif induces filopodia through mDia2. *Curr Biol* 2005;**15**:129–33. doi:10.1016/j.cub.2005.01.011
- 17 Hall A. Rho family GTPases. *Biochem Soc Trans* 2012;**40**:1378–82. doi:10.1042/BST20120103
- 18 Chauhan BK, Lou M, Zheng Y, *et al.* Balanced Rac1 and RhoA activities regulate cell shape and drive invagination morphogenesis in epithelia. *PNAS* 2011;**108**:18289–94. doi:10.1073/pnas.1108993108
- 19 Hansen SH, Zegers MM, Woodrow M, *et al.* Induced expression of Rnd3 is associated with transformation of polarized epithelial cells by the Raf-MEK-extracellular signal-regulated kinase pathway. *Mol Cell Biol* 2000;**20**:9364–75.
- 20 Maekawa M, Ishizaki T, Boku S, *et al.* Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 1999;**285**:895–8.
- 21 Lee J, Ishihara A, Oxford G, *et al.* Regulation of cell movement is mediated by stretch-activated calcium channels. *Nature* 1999;**400**:382–6. doi:10.1038/22578

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

- 22 Wood W, Faria C, Jacinto A. Distinct mechanisms regulate hemocyte chemotaxis during development and wound healing in *Drosophila melanogaster*. *J Cell Biol* 2006;**173**:405–16. doi:10.1083/jcb.200508161
- 23 Davis JR, Luchici A, Mosis F, *et al.* Inter-cellular forces orchestrate contact inhibition of locomotion. *Cell* 2015;**161**:361–73. doi:10.1016/j.cell.2015.02.015
- 24 Woodcock KJ, Kierdorf K, Pouchelon CA, *et al.* Macrophage-derived upd3 cytokine causes impaired glucose homeostasis and reduced lifespan in *Drosophila* fed a lipid-rich diet. *Immunity* 2014.
- 25 Pagler TA, Wang M, Mondal M, *et al.* Deletion of ABCA1 and ABCG1 impairs macrophage migration because of increased Rac1 signaling. *Circulation Research* 2011;**108**:194–200. doi:10.1161/CIRCRESAHA.110.228619
- 26 Park YM, Drazba JA, Vasanthi A, *et al.* Oxidized LDL/CD36 interaction induces loss of cell polarity and inhibits macrophage locomotion. *Mol Biol Cell* 2012;**23**:3057–68. doi:10.1091/mbc.E11-12-1051
- 27 Nagao T, Qin C, Grosheva I, *et al.* Elevated cholesterol levels in the plasma membranes of macrophages inhibit migration by disrupting RhoA regulation. 2007;**27**:1596–602. doi:10.1161/ATVBAHA.107.145086
- 28 Lehto VP, Hovi T, Vartio T, *et al.* Reorganization of cytoskeletal and contractile elements during transition of human monocytes into adherent macrophages. *Lab Invest* 1982;**47**:391–9.
- 29 Mosig S, Rennert K, Büttner P, *et al.* Monocytes of patients with familial hypercholesterolemia show alterations in cholesterol metabolism. *BMC Med Genomics* 2008;**1**:60. doi:10.1186/1755-8794-1-60
- 30 Flatau G, Lemichez E, Gauthier M, *et al.* Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature* 1997;**387**:729–33. doi:10.1038/42743
- 31 Herr MJ, Mabry SE, Jennings LK. Tetraspanin CD9 regulates cell contraction and actin arrangement via RhoA in human vascular smooth muscle cells. *PLoS ONE* 2014;**9**:e106999. doi:10.1371/journal.pone.0106999
- 32 Masiero L, Lapidos KA, Ambudkar I, *et al.* Regulation of the RhoA pathway in human endothelial cell spreading on type IV collagen: role of calcium influx. *Journal of Cell Science* 1999;**112 (Pt 19)**:3205–13.
- 33 Ludowyke RI, Scurr LL, McNally CM. Calcium ionophore-induced secretion from mast cells correlates with myosin light chain phosphorylation by protein kinase C. *J Immunol* 1996;**157**:5130–8.
- 34 Manser E, Leung T, Salihuddin H, *et al.* A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* 1994;**367**:40–6. doi:10.1038/367040a0
- 35 Arthur WT, Burridge K. RhoA inactivation by p190RhoGAP regulates cell spreading and migration by promoting membrane protrusion and polarity. *Mol Biol Cell* 2001;**12**:2711–20.
- 36 Kadandale P, Stender JD, Glass CK, *et al.* Conserved role for autophagy in Rho1-mediated cortical remodeling and blood cell recruitment. *PNAS* 2010;**107**:10502–7. doi:10.1073/pnas.0914168107
- 37 Perona R, Montaner S, Saniger L, *et al.* Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. *Genes Dev* 1997;**11**:463–75.
- 38 Charron F, Tsimiklis G, Arcand M, *et al.* Tissue-specific GATA factors are transcriptional effectors of the small GTPase RhoA. *Genes Dev* 2001;**15**:2702–19. doi:10.1101/gad.915701
- 39 Montaner S, Perona R, Saniger L, *et al.* Activation of serum response factor by RhoA is mediated by the nuclear factor-kappaB and C/EBP transcription factors. *Journal of Biological Chemistry* 1999;**274**:8506–15.
- 40 Staus DP, Weise-Cross L, Mangum KD, *et al.* Nuclear RhoA signaling regulates MRTF-dependent SMC-specific transcription. *Am J Physiol Heart Circ Physiol* 2014;**307**:H379–90. doi:10.1152/ajpheart.01002.2013

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

- 41 Sauzeau V, Berenjeno IM, Citterio C, *et al.* A transcriptional cross-talk between RhoA and c-Myc inhibits the RhoA/Rock-dependent cytoskeleton. *Oncogene* 2010;**29**:3781–92. doi:10.1038/onc.2010.134
- 42 Chan C-H, Lee S-W, Li C-F, *et al.* Deciphering the transcriptional complex critical for RhoA gene expression and cancer metastasis. *Nat Cell Biol* 2010;**12**:457–67. doi:10.1038/ncb2047
- 43 Königs V, Jennings R, Vogl T, *et al.* Mouse macrophages completely lacking Rho subfamily GTPases (RhoA, RhoB, and RhoC) have severe lamellipodial retraction defects, but robust chemotactic navigation and altered motility. *J Biol Chem* 2014;**289**:30772–84. doi:10.1074/jbc.M114.563270
- 44 Worthylake RA, Burridge K. RhoA and ROCK promote migration by limiting membrane protrusions. *Journal of Biological Chemistry* 2003;**278**:13578–84. doi:10.1074/jbc.M211584200
- 45 Ho TTG, Merajver SD, Lapière CM, *et al.* RhoA-GDP regulates RhoB protein stability. Potential involvement of RhoGDI α . *Journal of Biological Chemistry* 2008;**283**:21588–98. doi:10.1074/jbc.M710033200
- 46 Casey PJ, Seabra MC. Protein prenyltransferases. *Journal of Biological Chemistry* 1996;**271**:5289–92.
- 47 Solski PA, Helms W, Keely PJ, *et al.* RhoA biological activity is dependent on prenylation but independent of specific isoprenoid modification. *Cell Growth Differ* 2002;**13**:363–73. doi:10.1074/jbc.M114.563270
- 48 Tnimov Z, Guo Z, Gambin Y, *et al.* Quantitative analysis of prenylated RhoA interaction with its chaperone, RhoGDI. *J Biol Chem* 2012;**287**:26549–62. doi:10.1074/jbc.M112.371294
- 49 Bilodeau D, Lamy S, Desrosiers RR, *et al.* Regulation of Rho protein binding to membranes by rhoGDI: inhibition of releasing activity by physiological ionic conditions. *Biochem Cell Biol* 1999;**77**:59–69.
- 50 Kurokawa K, Matsuda M. Localized RhoA activation as a requirement for the induction of membrane ruffling. *Mol Biol Cell* 2005;**16**:4294–303. doi:10.1091/mbc.E04-12-1076
- 51 Michaelson D, Silletti J, Murphy G, *et al.* Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. *J Cell Biol* 2001;**152**:111–26.
- 52 Laufs U, Liao JK. Isoprenoid metabolism and the pleiotropic effects of statins. *Curr Atheroscler Rep* 2003;**5**:372–8.
- 53 Greenwood J, Walters CE, Pryce G, *et al.* Lovastatin inhibits brain endothelial cell Rho-mediated lymphocyte migration and attenuates experimental autoimmune encephalomyelitis. *The FASEB Journal* 2003;**17**:905–7. doi:10.1096/fj.02-1014fje
- 54 Carstanjen D, Yamauchi A, Koornneef A, *et al.* Rac2 regulates neutrophil chemotaxis, superoxide production, and myeloid colony formation through multiple distinct effector pathways. *J Immunol* 2005;**174**:4613–20.
- 55 Ingersoll MA, Spanbroek R, Lottaz C, *et al.* Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood* 2010;**115**:e10–9. doi:10.1182/blood-2009-07-235028
- 56 Ancuta P, Liu K-Y, Misra V, *et al.* Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16⁺ and CD16⁻ monocyte subsets. *BMC Genomics* 2009;**10**:403. doi:10.1186/1471-2164-10-403
- 57 Magie CR, Meyer MR, Gorsuch MS, *et al.* Mutations in the Rho1 small GTPase disrupt morphogenesis and segmentation during early Drosophila development. *Development* 1999;**126**:5353–64.
- 58 Cunningham F, Amode MR, Barrell D, *et al.* Ensembl 2015. *Nucleic Acids Res* 2015;**43**:D662–9. doi:10.1093/nar/gku1010

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

- 59 Palm W, Sampaio JL, Brankatschk M, *et al.* Lipoproteins in *Drosophila melanogaster*--assembly, function, and influence on tissue lipid composition. *PLoS Genet* 2012;**8**:e1002828. doi:10.1371/journal.pgen.1002828

7. DISCUSSION

7.1. Summary of findings

Taken together, these data add to the relatively limited pool of published knowledge on the response of monocyte subpopulations to dyslipidaemia. Since the 1960s, the circulating lipophage has been an enigma that is often ignored [1]. This work confirms that these cells are neutral lipid- laden monocytes and that there is a functional impact of this lipid uptake. Fundamentally there are a number of key conclusions that can be drawn from this work:

1. Monocytes from the dyslipidaemic *Ldlr*^{-/-} mouse on HFD accumulate cytoplasmic neutral lipid via a mechanism independent of the LDLR and this occurs to a lesser extent in C56Bl/6 wild-type animals.
2. Following an appropriate stimulus, such as peritonitis, monocytes from a dyslipidaemic mouse transmigrate appropriately from the blood, but are then less able to move to the site of inflammation. In wild-type animals on HFD, non-classical monocyte patrolling remains largely unaffected.
3. LDL or VLDL is internalized by human monocytes and also leads to cytoplasmic neutral lipid accumulation via an undetermined mechanism. Although kinetics are similar, levels of neutral lipid are higher in CD16^{neg} than CD16^{pos} monocytes.
4. There is no pronounced affect of LDL or VLDL treatment on monocyte apoptosis, cytokine transcription or release, surface marker expression or phagocytosis. Subtle, subset specific effects were observed on adhesion and transmigration on an endothelial layer.
5. VLDL causes a strong pronounced inhibition of monocyte chemotaxis and chemokinesis in both subpopulations, accompanied by modulation of cytoskeletal regulation via RHOA and MLC. This mechanism may be responsible for an impaired monocyte response to peritonitis on HFD.

These conclusions are summarised in Figure 7.1. These conclusions have implications in a wide range of pathophysiological contexts, including

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

atherosclerosis, infection and inflammatory disease. These implications will be discussed further in turn below.

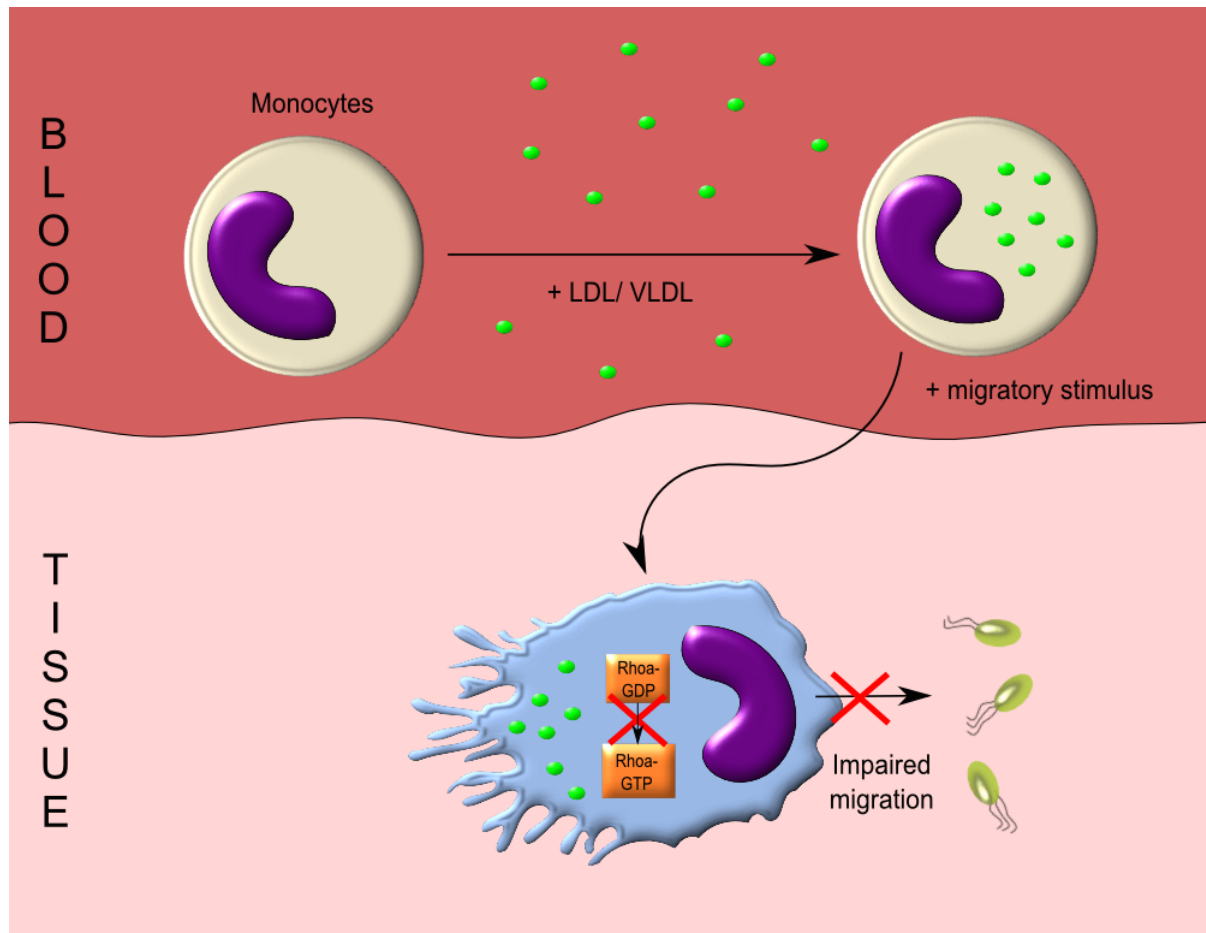


Figure 7.1 Summary schematic of proposed migratory dysfunction in monocyte lipophages.

Monocytes of both subsets accumulate neutral lipid vesicles during elevated VLDL or LDL exposure. These lipophages can adhere to, crawl on and transmigrate through the endothelium, but have impaired chemotaxis and chemokinesis towards in the extravascular environment. This is accompanied by adherent cytoskeletal regulation via RHOA inactivation.

7.2. Therapeutic implications

7.2.1. Atherosclerosis

It seems reasonable to presume that neutral lipid- loaded blood monocytes occur in dyslipidaemic patients at high risk of cardiovascular disease, although this hypothesis is not directly tested here. Based on previous data, extreme plasma dyslipidaemia in familial hypercholesterolemic (FH) patients does cause increased intracellular lipid in monocytes [2], although more moderate hyperlipoproteinemia has been reported to cause no detectable accumulation [3]. To directly relate these findings to atherosclerosis, more work is required to determine the plasma lipid threshold at which monocyte dysfunction begins to occur. However, even if conclusions are restricted to FH patients there are clearly implications of these data for cardiovascular disease.

When the vascular endothelium becomes activated by deposition of modified LDL, monocytes undergo transendothelial migration (TEM) into the arterial intima [4]. In CD16^{neg} classical monocytes, the prime contributors to the atherosclerotic plaque in mouse models [5], LDL treatment increases TEM (Figure 5.8). As such, classical monocytes that are already lipid laden in circulation could have a greater propensity to accumulate in the arterial intima and contribute to atherosclerotic plaque formation. As discussed previously, I hypothesize that this is due to an increased propensity of dysfunctional, lipid loaded monocytes to undergo transcellular migration, although this has never been tested either in vitro or in atherosclerosis. Further, I find that both monocyte subpopulations have impaired migration and cytoskeletal regulation after lipoprotein exposure. If these data are extrapolated to atherosclerosis, initial monocyte accumulation in the plaque due to increased TEM may then be perpetuated by a failure of monocytes to leave the intima due to cytoskeletal- related migratory defects. In the past similar hypotheses have been made for macrophages that phagocytose modified apoB-lipoproteins within the plaque [6,7] and more recently it has been demonstrated that lipid loaded, CD11c^{pos} blood monocytes contribute to early atherogenesis [8]. However, this may only be applicable to cases of extreme dyslipidaemia, since the monocyte phenotype

reported in the *Apoe*^{-/-} mouse on HFD[8] is much less pronounced in experiments performed here utilizing the *Ldlr*^{-/-}.

Fundamentally, the importance of these findings for atherosclerosis hinges upon an on-going debate in the field regarding the relative importance of monocyte recruitment and resident macrophage proliferation in atherogenesis. While some data argue that macrophage proliferation is the key contributor to the plaque following early monocyte recruitment [9], this is contradictory to decades of research, including their own, demonstrating that progressive monocyte recruitment is proportional to plaque burden and disease severity [5,10,11]. These data offer no further insight into that debate and it awaits resolution by further research. Regardless, it is possible that these two processes are linked by lipid loading of blood monocytes affecting the phenotype of macrophage that monocytes differentiate to once within the vessel wall. While monocytes do not proliferate in the blood [12], lipid loading could drive subsequent proliferation and inflammation once monocytes have differentiated to macrophages following extravasation. Although this is a bold hypothesis, recent work on monocyte epigenetic memory supports this theory. When monocytes are exposed to oxLDL acutely and subsequently differentiated, the resulting macrophages have an inflammatory phenotype in response to TLR stimulation via modulation of histone methylation at relevant promoter sites [13]. This also occurs following initial inflammatory priming with β -glucan prior to differentiation, affecting over 3000 genomic distal regulatory elements [14]. This phenomenon, termed 'trained innate immunity', may be a greatly underestimated factor in the contribution of environmental stimuli to inflammatory disease.

In addition, atherosclerosis is largely thought of as a chronic, inflammatory disease, yet contradictory data on the inflammatory effects of VLDL and LDL on monocytes and macrophages make it unclear exactly how subpopulations of these cells contribute to this process. It is certain that multiple macrophage phenotypes exist in the plaque, with >30% estimated exhibit 'M2-like' anti-inflammatory function in the lesions of *Ldlr*^{-/-} mice [15]. Related to this, HFD exposed peritoneal macrophages

from the same mice exhibit a striking, deactivated phenotype characterized by decreased expression of inflammatory response genes and resulting from modification of LXR- signalling by the cholesterol intermediate desmosterol [16]. In confirmation of this, I found expression of *Il1b* and *Cxcl10* were significantly down regulated in the inflamed peritoneum of *Ldlr*^{-/-} mice on HFD versus chow. However, I find no clear effect of LDL or VLDL on the in vitro inflammatory response of monocytes at both a transcriptional and protein level. It is possible that embryonically- derived local resident macrophages and infiltrating monocyte- derived macrophages have distinct functions and phenotypes within the plaque, which may help to explain the range of effects of LDL that have been observed on mononuclear phagocytes. In addition, caution must be taken when interpreting data using bone marrow derived macrophages culture in vitro, as the complex contribution of the tissue microenvironment in determining macrophage function means that traditional culture methods rarely generate anything resembling cells found in vivo [17]. For example, bone marrow derived dendritic cells (DCs) are generally produced using GM-CSF treatment, however in reality this generates a mixed population of DCs and CD11c^{pos} macrophages [18]. In this sense, freshly isolated monocytes that have undergone limited manipulation could be argued to better represent the physiological response.

7.2.2. Infection

While atherosclerosis is the most widely studied disease process in the context of dyslipidaemia, the impairment of monocyte chemotaxis and chemokinesis presented here has broad implications for a range of inflammatory processes. Currently, greater than 6% of patients in England contract an infection resulting from treatment in a healthcare setting, amounting to over 1 million patients per year [19]. Patients in the 95th percentile of stratified total cholesterol levels are 4-fold more likely to develop a nosocomial infection following general surgery and in the UK in 2011, 50% of adults had a total plasma cholesterol above the target value of 5mmol/L [20]. As such, the scale of potential impact for monocyte dysfunction in dyslipidaemia is immense. Interestingly, the use of statins to lower plasma cholesterol levels has been repeatedly reported to reduce severity of bacterial infections [21] [22], although

some meta-analyses have failed to support this trend [23]. Somewhat confusingly, it is suggested that this difference in infection severity and mortality is due to inhibition of HMG-CoA by statins and a subsequent impairment of Rho-GTPase prenylation and activation [24], as discussed earlier. Since it has also been demonstrated here that hypercholesterolemia interferes with this process, the precise relationship between intracellular neutral lipid and the cytoskeleton remains enigmatic.

In addition, infection can result in dyslipidaemia in what may in some instances represent a host defence mechanism and in others a destructive feedback loop evolved and initiated by the invading pathogen. Perhaps the most well studied infection in this context is human immunodeficiency virus (HIV). While dyslipidaemia in HIV is a well-established phenomenon, it was thought to derive primarily as a side effect of the protease inhibitors used to treat the infection via poorly characterized mechanisms [25]. However, there is also a direct role for HIV itself, since the HIV protein Nef markedly elevates plasma total cholesterol and triglyceride levels in mice, leading to accelerated atherosclerosis [26]. The same effect has also been demonstrated for simian immunodeficiency virus (SIV) Nef in macaques, through a mechanism that appears to involve inhibition of ABCA1-dependent reverse cholesterol transport [27]. Currently, around 29% of HIV mortality is due to secondary infections related to AIDS. Although this is primarily thought to be a result of T-lymphocyte deficiency, dyslipidemia-related monocyte immunosuppression may also be contributing to this problem and require further therapeutic attention [28].

7.2.3. Chronic inflammatory disease

Modulation of the innate immune system by dyslipidemia may also have implications for other chronic inflammatory processes, including autoimmune diseases such as rheumatoid arthritis (RA). Compared to the healthy population, RA patients have a twofold increase in morbidity from cardiovascular disease [29], although there is ongoing debate as to whether this is driven by conventional dyslipidemia [30]. However, while dyslipidemia is often thought of as a consequence of anti-TNF α therapy in RA patients [31], data from the last decade also highlight it as a potential risk factor for the disease. In a retrospective study of 1078 RA patients, elevated

plasma levels of total cholesterol, apoB lipoproteins, triglyceride and low levels of HDL were present at least 10 years prior to disease onset [32]. In addition, factors that improve these metrics of plasma lipid profile such as omega-3-fatty acid consumption [33,34] and statin treatment [35,36] have been associated with reduced onset and amelioration of clinical symptoms in RA.

Massive infiltration of the synovial membrane by monocytes is a major contributor to RA pathophysiology [37], most notably by CD14^{high} classical and intermediate monocytes [38]. Once within the inflamed joint, these monocytes are activated by TNF- α and IL-1 β to produce intracellular IL-17 and activate a Th17 response via a cell-contact dependent mechanism [37]. In some instances lipid-laden macrophage foam cells similar to those in the atherosclerotic plaque have also been reported to be present in the synovial membrane of RA patients, emphasizing some analogous pathogenic features between the two diseases [39]. Much like in the plaque, monocyte and macrophage retention in the synovia clearly escalates disease and a decrease of monocyte and macrophage numbers in the synovia are strongly associated with positive response of RA to treatment and clinical improvement [40]. As such, impaired monocyte motility due to dyslipidemia in RA may perpetuate joint inflammation and destruction.

Another notable chronic inflammatory pathology accompanied by dyslipidemia is inflammatory bowel disease (IBD), which can be split primarily into ulcerative colitis and Crohn's disease [41]. Much like RA, IBD patients are prone to atherosclerosis and are over 4 times more likely to suffer a cardiovascular event than the general population [42]. Although variation has been observed in different patient populations, the dyslipidemia of IBD is predominantly an elevation of plasma triglyceride accompanied by lower levels of HDL [43,44]. The exact pathological initiating mechanisms of IBD are not entirely understood, but Crohn's disease is characterized by macrophage aggregation in mucosal lesions to form granulomas, where they perpetuate the inflammation via the production of TNF- α and IL-1 β [41,45]. As reviewed in Chapter 1.1.2, gut macrophages require continuous

replenishment from GR1^{high} blood monocytes [46]. During IBD, infiltrating blood monocytes arrest in a CX3CR1^{low} inflammatory state during maturation to macrophages, thus simultaneously increasing the production of inflammatory cytokines and failing to replenish the IL-10 producing regulatory tissue population [46]. It has been hypothesized for dendritic cells that a chemokine mediated retention traps mature myeloid DC at the site of inflammation during IBD maintaining a cycle of autoimmune inflammation, although it is unclear whether these DC are monocyte derived [47]. It is possible that a similar retention could be occurring in infiltrating CX3CR1^{low} inflammatory monocyte- derived macrophages, perpetuated by dyslipidemia impairing macrophage egress from mucosal lesions.

7.3. Future work

While providing insight into monocyte subset biology in dyslipidaemia, there are a number of key questions that arise from these data that require future investigation.

7.3.1. Lipid uptake

On a basic level, it is important to understand the origin and composition of the neutral lipid vesicles observed in monocytes after HFD and LDL or VLDL exposure. A lipidomic analysis of monocytes using mass spectrometry may yield further insight into the precise lipid molecular species that are enriched in monocytes following lipoprotein exposure, as has been reported during monocyte activation [48]. There is some indication from mechanistic work in Chapter 4 that these neutral lipid punctae are not simply internalized lipoprotein particles, but may be derived from a biosynthetic pathway. As such, co-localization of exogenously stained lipoproteins with neutral lipid stain over time may provide insight into how LDL and VLDL are trafficked and contribute to this neutral lipid pool. This could be performed both in vitro using human monocytes and in vivo using IV injection of fluorescent lipoproteins into mice. The potential for dissociation of the lipid and protein portions of LDL and VLDL once internalized, as occurs in the LDLR pathway [49], is also a source of confusion. This could be usefully addressed by distinct labeling of these two subfractions i.e. with both a lipophilic dye such as DIO or BODIPY and amine labeling of APOB protein using a fluorochrome such as the AlexaFluor family. High

speed live cell imaging of vesicle trafficking may also be useful for deciphering this relationship, as has been previously described for LDLR-dependent cholesterol trafficking [50].

Similar strategies could also be utilized to further investigate the mechanism of LDL and VLDL uptake in monocytes subsets. It is clear that CD36 is important for LDL uptake in both subpopulations (Figure 4.6), but does not seem to be involved in neutral lipid accumulation (Figure 4.7), indicating disparate pathways. There is also an effect of CD36 knockout on lipid- loaded 'foamy monocyte' formation in the *Apoe*^{-/-} mouse, although the subtle nature of the phenotype implies that other pathways are also involved. A different lipid accumulation mechanism may exist in these mice, since APOE reconstitution reduces neutral lipid load in blood monocytes by promoting cholesterol efflux [51]. In this context, it would be interesting to investigate whether over expression of *Apoe* in *Ldlr*^{-/-} mice is able to exert a similar effect and examining monocytes from the *Cd36*^{-/-} *Ldlr*^{-/-} mouse on HFD could also provide further insight [52].

Moreover, it is unclear whether elevated levels of LDL and VLDL are sufficient to drive monocyte lipid loading, or if this is also affected by the structure and composition of these lipoprotein species. Studies in humans have found both acute [53,54] and chronic [55,56] changes in LDL and VLDL composition in response to different dietary fats, as measured by protein to lipid ratio, levels of cholesterol species and triacylglycerol content. Interestingly, supplementation with short chain poly-unsaturated fatty acids from fish oils has been demonstrated to be atheroprotective in *Ldlr*^{-/-} mice [57]. However, while moderate consumption appears to be beneficial, current results from human trials using fish oil supplementation are contradictory and inconclusive [58]. One suggested mechanism for fish oil- mediated cardiovascular benefits is an increase in the size of LDL particles [59], thus reducing the presence of small, dense LDL that has been associated with atherosclerosis progression and cardiovascular events [60,61]. Although research into fish oil in atherosclerosis in humans is still on-going, it may be informative to change the fatty

acid profile of the HFD fed *Ldlr*^{-/-} and determine whether a HFD rich in MUFA or PUFA yielded the same functional effect on monocytes.

In addition, it is possible that post-prandial changes in plasma lipid profile also mediate the same effect on monocyte function acutely. It is broadly recognised that a meal high in saturated fats will elevate VLDL levels within 4 hours of eating [62] [63]. Dietary fatty acids are incorporated into these lipoproteins within 6 hours [53] and have subsequently been tracked into leukocytes using isotope labelling [64]. As discussed above, dietary fat intake can alter the structure, composition and metabolic properties of both LDL and VLDL [53,63]. As such, perhaps the discrepancies seen between this work and others on monocyte activation and adhesion after LDL and VLDL exposure [65,66] are due to the precise lipid profile of the lipoproteins used in vitro. Chylomicrons and free fatty acids are also elevated post-prandially [67] and both represent another avenue for investigation into monocyte function and are currently being investigated in the Woollard lab.

7.3.2. Monocyte migration

It is clear from both thioglycolate and LPS peritonitis experiments that less monocytes and macrophages accumulate in the peritoneal cavity at 72 hours when *Ldlr*^{-/-} mice are fed a HFD. Intravenous bead labelling confirms that this is at least in part accounted for by impaired blood monocyte migration, while it seems that blood monocytes are retained in the omentum en route to the peritoneum. Although technically challenging and time consuming, adoptive transfer of monocytes from *Ldlr*^{-/-} mice into wild-type animals would confirm that this phenomenon is driven by monocyte lipid loading and not by effects on tissues such as the endothelium, as I hypothesize from in vitro data. Cell phenotypes in the omentum after peritonitis suggest that HFD delays monocyte-macrophage differentiation, since the number of CD115^{low} F4/80^{hi} resident macrophages is decreased on HFD (Figure 3.4). While flow cytometry could be used to track fluorescently labelled monocytes into the peritoneum and omentum in these experiments, this would require many animals and would intrinsically limit the number of time points that could be studied. A more elegant solution may be the use of far-red fluorescent monocyte labelling and

fluorescence molecular tomography (FMT) as previously described in myocardial infarction [68]. When combined with micro-CT, this provides a non-invasive method of tracking cells in vivo over time to known organs and tissues [68]. However, preliminary work in the Woollard lab using FMT indicates that the majority of adoptively transfer monocytes rapidly pool in the liver, spleen and lungs, as previously reported [67], which can hamper detection of cells in the peritoneal cavity (Data not shown).

In addition, it would be important to confirm these findings from the *Ldlr*^{-/-} mouse in a clinically relevant human in vivo or ex vivo setting. The obvious candidate for this is the use of monocytes from patients with familial hypercholesterolemia, that have been previously reported to be lipid loaded [2]. Options for studying leukocyte migration in vivo in humans are limited, but one possibility is the use of a cantharidin blister model [69]. Cantharidin is a defensive compound of the beetle *Meloidae coleopteran* that disrupts cell-cell junctions in the skin epidermis when applied topically, leading to blistering and leukocyte recruitment [70]. Its validity as an in vivo model of inflammation have been validated in recent years, with anti-TNF treatment shown to produce a pronounced inhibition of neutrophil recruitment [71]. Alternatively, comparison of monocytes from FH with those from healthy donors in the established 2D chemotaxis assay would also confirm the physiological relevance of the described migratory phenotype described in VLDL treated cells.

Of the observations made here, the discrepancy between lipid treated monocyte migration in 2D in vitro assays and peritonitis versus those during TEM is one of the most difficult to reconcile and warrants further experiments. While CD16^{neg} monocytes have a pronounced migratory inhibition in transwells after LDL exposure and in real-time migration assays after VLDL, both of these treatments promote increased TEM. As alluded to previously, this may be due to a preference for monocytes with cytoskeletal dysfunction to utilise transcellular migration, thus leading a total increase in TEM despite inhibition of locomotion. In order to test this, future experiments would require real-time, high-resolution imaging of TEM

accompanied by staining of endothelial cell-cell junctions to determine the route of transmigration. However, in vivo endothelial cells are constantly subject to haemodynamic mechanical forces resulting from the shear stress of the blood flow. In turn this triggers a variety of signalling events that alter endothelial cell function via a mechanosensory complex of PECAM-1 and VE-cadherin [72,73]. In the current model, blood flow triggers cytoskeletal signalling through an unidentified mechanosensor in endothelial cells, reducing myosin-dependent tension in VE-cadherin and simultaneously increasing tension in junctional PECAM-1, triggering intracellular signalling events [72,73]. In areas of disturbed flow, PECAM-1 signalling in this manner can activate the transcription factor NFkB and the subsequent expression of inflammatory genes [73]. There is also some evidence that low or oscillatory shear stress alters monocyte adhesion to the endothelium, and may be responsible for preferential atherogenesis at vascular bifurcations [74,75]. As such, while TEM experiments performed here were under static conditions, these conclusions should be repeated and confirmed in real time under physiological perfusion rates.

Alternatively, Nourshargh and colleagues have recently pioneered the use of intravital microscopy to study leukocyte TEM in vivo in real time [76] and this may also be possible if an appropriate fluorescent reporter mouse was created on an *Ldlr*^{-/-} background. During this project I utilised the available *Cx3cr1*-GFP mouse, which allows for delineation of CX3CR1^{high} GR1^{low} intravascular patrolling monocytes by intravital confocal microscopy [77]. However, this mouse is of limited use to study TEM for several reasons. Firstly, GR1^{low} monocytes have never been observed leaving the blood in the steady state [77] and rarely transmigrate during inflammation [78,79]. Moreover, when GR1^{high} monocytes transmigrate in response to sterile injury they up-regulate *Cx3cr1* expression [78], thus making classical and non-classical monocytes indistinguishable by imaging in this mouse. A more suitable option for studying monocyte TEM in vivo would be the recently created CD68-GFP mouse, as it allows for universal stable monocyte labelling that persists following transmigration into the tissue and monocyte- macrophage differentiation [80]. Alternatively, a dual colour approach to label both monocyte subsets, such as a combination of *Cx3cr1*-

GFP and *Ccr2*-RFP may also provide more information on monocyte dynamics prior to and following TEM [78].

7.3.3. Cytoskeletal regulation

While decreased RHOA activation and transcription in lipid-loaded monocytes has been observed here concurrent with a migratory defect, it has not been possible to definitively prove that these two observations are functionally linked. This is clearly a key area of future work before these findings can be translated to therapeutic use. Investigating the subcellular localization of active Rho-GTPases could provide insight into the underlying mechanisms of lipid- cytoskeleton interactions. The development of the Raichu fluorescent resonance energy transfer (FRET) biosensors for RHOA, RAC1 and CDC42 activity in the last decade now allows for this to be visualized in real time in migrating cells [81,82]. However, these probes would require transfection in order to achieve stable expression in primary cells, something that is challenging in freshly- isolated monocytes and may result in TLR activation, particularly since non-classical monocytes mount a robust inflammatory response to nucleic acids via TLR7/8 [83]. As such, quantification of FRET by fluorescence lifetime imaging microscopy (FLIM) in this context may require the use of a genetically tractable monocytic cell line, as has been described for the analysis of L-Selectin shedding in THP-1 monocyte-like cells [84]. Such an approach would also hopefully shed light on the precise effects of dyslipidaemia on other GTPases such as RAC1 and clarify discrepancies between this work and that in cholesterol-loaded macrophages [85].

Another potential future option is the use of the available constitutively active RHOA mouse [86,87], which could be used to reconstitute bone marrow in the *Ldlr*^{-/-} HFD model prior to peritonitis experiments, thus providing selective activation of RHOA in myeloid cells. However, for the same reasons discussed for pharmacological activation of RHOA using CN03, it is unlikely that this experiment would yield meaningful data. Over-activation of Rho-GTPases can be as detrimental as under-activation during cell migration, and simply constitutively activating RHOA would not provide the fine spatio-temporal balance required for chemotaxis [88,89]. However, further investigation into Rho-GTPase prenylation in lipid treated cells may provide

more opportunities for therapeutic intervention. If dyslipidaemia causes adherent RHOA prenylation and trafficking, there are several pharmacological options to target this pathway including the use of statins to reduce HMG-CoA reductase activity and the synthesis of isoprenoids [90]. This could be a more elegant method of restoring appropriate levels of RHOA activation in lipid-loaded cells with drugs that are already in clinical use.

7.4. Conclusion

In conclusion, the work presented in this thesis details the interactions of monocyte subsets with lipoproteins during dyslipidaemia. In a more detailed confirmation of previous observations, I demonstrate that monocytes accumulate neutral lipid in response to LDL and VLDL in a potentially subset specific manner, via various receptors including CD36, but independent of the LDL receptor *in vivo*. While this does not impair many aspects of their response to inflammation, including cytokine production, there is a clear effect of dyslipidaemia on monocyte locomotion and cytoskeletal regulation in both CD16^{pos} and CD16^{neg} subpopulations. In the setting of infection, this phenotype may impair monocyte recruitment and clearance of invading pathogens. Conversely, in chronic inflammatory disease such as atherosclerosis and rheumatoid arthritis dyslipidaemia may perpetuate inappropriate retention of monocytes at the site of inflammation. These findings underscore the functional independence of monocytes from macrophages and their importance as immune effector cells prior to monocyte- macrophage differentiation. Further work is now required to continue to decipher the interactions of environmental and dietary stimuli with the innate immune system, in the context of ever-expanding knowledge on the functional role of monocyte subpopulations.

7.5. References

- 1 Suzuki M, O'Neal RM. Accumulation of lipids in the leukocytes of rats fed atherogenic diets. *Journal of Lipid Research* 1964;**5**:624–7.
- 2 Mosig S, Rennert K, Büttner P, *et al.* Monocytes of patients with familial hypercholesterolemia show alterations in cholesterol metabolism. *BMC Med Genomics* 2008;**1**:60. doi:10.1186/1755-8794-1-60
- 3 Querfeld U, Wendtland J, Hodenberg von E, *et al.* Lipid levels in monocytes of patients with moderate hyperlipoproteinemia. *Atherosclerosis* 1992;**94**:129–34. doi:10.1016/0021-9150(92)90237-B
- 4 Quinn MT, Parthasarathy S, Fong LG, *et al.* Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc Natl Acad Sci USA* 1987;**84**:2995–8.
- 5 Potteaux S, Gautier EL, Hutchison SB, *et al.* Suppressed monocyte recruitment drives macrophage removal from atherosclerotic plaques of Apoe^{-/-} mice during disease regression. *J Clin Invest* 2011;**121**:2025–36. doi:10.1172/JCI43802
- 6 Nagao T, Qin C, Grosheva I, *et al.* Elevated cholesterol levels in the plasma membranes of macrophages inhibit migration by disrupting RhoA regulation. 2007;**27**:1596–602. doi:10.1161/ATVBAHA.107.145086
- 7 Llodra J, Angeli V, Liu J, *et al.* Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci USA* 2004;**101**:11779–84. doi:10.1073/pnas.0403259101
- 8 Xu L, Dai Perrard X, Perrard JL, *et al.* Foamy Monocytes Form Early and Contribute to Nascent Atherosclerosis in Mice With Hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 2015;:ATVBAHA.115.305609. doi:10.1161/ATVBAHA.115.305609
- 9 Robbins CS, Hilgendorf I, Weber GF, *et al.* Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat Med* 2013;**19**:1166–72. doi:10.1038/nm.3258
- 10 Swirski FK, Pittet MJ, Kircher MF, *et al.* Monocyte accumulation in mouse atherogenesis is progressive and proportional to extent of disease. *Proc Natl Acad Sci USA* 2006;**103**:10340–5. doi:10.1073/pnas.0604260103
- 11 Soehnlein O, Drechsler M, Döring Y, *et al.* Distinct functions of chemokine receptor axes in the atherogenic mobilization and recruitment of classical monocytes. *EMBO Mol Med* 2013;**5**:471–81. doi:10.1002/emmm.201201717
- 12 Hettinger J, Richards DM, Hansson J, *et al.* Origin of monocytes and macrophages in a committed progenitor. *Nat Immunol* 2013;**14**:821–30. doi:10.1038/ni.2638
- 13 Bekkering S, Quintin J, Joosten LAB, *et al.* Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes. *Arterioscler Thromb Vasc Biol* 2014;**34**:1731–8. doi:10.1161/ATVBAHA.114.303887
- 14 Saeed S, Quintin J, Kerstens HHD, *et al.* Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science* 2014;**345**:1251086–6. doi:10.1126/science.1251086
- 15 Kadl A, Meher AK, Sharma PR, *et al.* Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. *Circulation Research* 2010;**107**:737–46. doi:10.1161/CIRCRESAHA.109.215715
- 16 Spann NJ, Garmire LX, McDonald JG, *et al.* Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell* 2012;**151**:138–52. doi:10.1016/j.cell.2012.06.054
- 17 Gautier EL, Shay T, Miller J, *et al.* Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol* 2012;**13**:1118–28. doi:10.1038/ni.2419

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

- 18 Helft J, Böttcher J, Chakravarty P, *et al.* GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. *Immunity* 2015;**42**:1197–211. doi:10.1016/j.immuni.2015.05.018
- 19 Weston D. Fundamentals of Infection Prevention and Control. John Wiley & Sons 2013.
- 20 Mindell J, Centre GBNHSI. *Health Survey for England 2011*. 2012.
- 21 Sever PS, Chang CL, Gupta AK, *et al.* The Anglo-Scandinavian Cardiac Outcomes Trial: 11-year mortality follow-up of the lipid-lowering arm in the U.K. *Eur Heart J* 2011;**32**:2525–32. doi:10.1093/eurheartj/ehr333
- 22 Björkhem-Bergman L, Bergman P, Andersson J, *et al.* Statin treatment and mortality in bacterial infections--a systematic review and meta-analysis. *PLoS ONE* 2010;**5**:e10702. doi:10.1371/journal.pone.0010702
- 23 van den Hoek HL, Bos WJW, de Boer A, *et al.* Statins and prevention of infections: systematic review and meta-analysis of data from large randomised placebo controlled trials. *BMJ* 2011;**343**:d7281–1. doi:10.1136/bmj.d7281
- 24 Cordle A, Koenigsknecht-Talboo J, Wilkinson B, *et al.* Mechanisms of statin-mediated inhibition of small G-protein function. *Journal of Biological Chemistry* 2005;**280**:34202–9. doi:10.1074/jbc.M505268200
- 25 Lee GA, Rao MN, Grunfeld C. The effects of HIV protease inhibitors on carbohydrate and lipid metabolism. *Curr HIV/AIDS Rep* 2005;**2**:39–50.
- 26 Cui HL, Ditiatkovski M, Kesani R, *et al.* HIV protein Nef causes dyslipidemia and formation of foam cells in mouse models of atherosclerosis. *The FASEB Journal* 2014;**28**:2828–39. doi:10.1096/fj.13-246876
- 27 Asztalos BF, Mujawar Z, Morrow MP, *et al.* Circulating Nef induces dyslipidemia in simian immunodeficiency virus-infected macaques by suppressing cholesterol efflux. *J Infect Dis* 2010;**202**:614–23. doi:10.1086/654817
- 28 Smith CJ, Ryom L, Weber R, *et al.* Trends in underlying causes of death in people with HIV from 1999 to 2011 (D:A:D): a multicohort collaboration. *Lancet* 2014;**384**:241–8. doi:10.1016/S0140-6736(14)60604-8
- 29 Maradit-Kremers H, Crowson CS, Nicola PJ, *et al.* Increased unrecognized coronary heart disease and sudden deaths in rheumatoid arthritis: a population-based cohort study. *Arthritis Rheum* 2005;**52**:402–11. doi:10.1002/art.20853
- 30 Dessein PH, Christian BF, Solomon A. Which are the determinants of dyslipidemia in rheumatoid arthritis and does socioeconomic status matter in this context? *J Rheumatol* 2009;**36**:1357–61. doi:10.3899/jrheum.090288
- 31 Curtis JR, John A, Baser O. Dyslipidemia and changes in lipid profiles associated with rheumatoid arthritis and initiation of anti-tumor necrosis factor therapy. *Arthritis Care Res (Hoboken)* 2012;**64**:1282–91. doi:10.1002/acr.21693
- 32 van Halm VP, Nielen MMJ, Nurmohamed MT, *et al.* Lipids and inflammation: serial measurements of the lipid profile of blood donors who later developed rheumatoid arthritis. *Ann Rheum Dis* 2007;**66**:184–8. doi:10.1136/ard.2006.051672
- 33 Nielsen GL, Faarvang KL, Thomsen BS, *et al.* The effects of dietary supplementation with n-3 polyunsaturated fatty acids in patients with rheumatoid arthritis: a randomized, double blind trial. *Eur J Clin Invest* 1992;**22**:687–91.
- 34 Volker D, Fitzgerald P, Major G, *et al.* Efficacy of fish oil concentrate in the treatment of rheumatoid arthritis. *J Rheumatol* 2000;**27**:2343–6.
- 35 Jick SS, Choi H, Li L, *et al.* Hyperlipidaemia, statin use and the risk of developing rheumatoid arthritis. *Ann Rheum Dis* 2009;**68**:546–51. doi:10.1136/ard.2008.091967
- 36 Chodick G, Amital H, Shalem Y, *et al.* Persistence with statins and onset of rheumatoid arthritis: a population-based cohort study. *PLoS Med* 2010;**7**:e1000336. doi:10.1371/journal.pmed.1000336

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

- 37 Evans HG, Gullick NJ, Kelly S, *et al.* In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. *PNAS* 2009;**106**:6232–7. doi:10.1073/pnas.0808144106
- 38 Yoon BR, Yoo S-J, Choi YH, *et al.* Functional phenotype of synovial monocytes modulating inflammatory T-cell responses in rheumatoid arthritis (RA). *PLoS ONE* 2014;**9**:e109775. doi:10.1371/journal.pone.0109775
- 39 Winyard PG, Tatzber F, Esterbauer H, *et al.* Presence of foam cells containing oxidised low density lipoprotein in the synovial membrane from patients with rheumatoid arthritis. *Ann Rheum Dis* 1993;**52**:677–80.
- 40 Haringman JJ, Gerlag DM, Zwinderman AH, *et al.* Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis. *Ann Rheum Dis* 2005;**64**:834–8. doi:10.1136/ard.2004.029751
- 41 Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007;**448**:427–34. doi:10.1038/nature06005
- 42 Yarur AJ, Deshpande AR, Pechman DM, *et al.* Inflammatory bowel disease is associated with an increased incidence of cardiovascular events. *The American Journal of Gastroenterology* 2011;**106**:741–7. doi:10.1038/ajg.2011.63
- 43 Ripollés Piquer B, Nazih H, Bourreille A, *et al.* Altered lipid, apolipoprotein, and lipoprotein profiles in inflammatory bowel disease: consequences on the cholesterol efflux capacity of serum using Fu5AH cell system. *Metab Clin Exp* 2006;**55**:980–8. doi:10.1016/j.metabol.2006.03.006
- 44 Sappati Biyyani RSR, Putka BS, Mullen KD. Dyslipidemia and lipoprotein profiles in patients with inflammatory bowel disease. *J Clin Lipidol* 2010;**4**:478–82. doi:10.1016/j.jacl.2010.08.021
- 45 Rugtveit J, Nilsen EM, Bakka A, *et al.* Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. *Gastroenterology* 1997;**112**:1493–505.
- 46 Bain CC, Scott CL, Uronen-Hansson H, *et al.* Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol* 2013;**6**:498–510. doi:10.1038/mi.2012.89
- 47 Middel P, Raddatz D, Gunawan B, *et al.* Increased number of mature dendritic cells in Crohn's disease: evidence for a chemokine mediated retention mechanism. *Gut* 2006;**55**:220–7. doi:10.1136/gut.2004.063008
- 48 Balgoma D, Astudillo AM, Pérez-Chacón G, *et al.* Markers of monocyte activation revealed by lipidomic profiling of arachidonic acid-containing phospholipids. *The Journal of Immunology* 2010;**184**:3857–65. doi:10.4049/jimmunol.0902883
- 49 Brown MS, Goldstein JL. A Receptor-Mediated Pathway for Cholesterol Homeostasis. *Science* 1986;**232**:1–14.
- 50 Kanerva K, Uronen R-L, Blom T, *et al.* LDL cholesterol recycles to the plasma membrane via a Rab8a-Myosin5b-actin-dependent membrane transport route. *Dev Cell* 2013;**27**:249–62. doi:10.1016/j.devcel.2013.09.016
- 51 Gaudreault N, Kumar N, Posada JM, *et al.* ApoE Suppresses Atherosclerosis by Reducing Lipid Accumulation in Circulating Monocytes and the Expression of Inflammatory Molecules on Monocytes and Vascular Endothelium. 2012;**32**:264–72. doi:10.1161/ATVBAHA.111.238964
- 52 Kennedy DJ, Kuchibhotla SD, Guy E, *et al.* Dietary cholesterol plays a role in CD36-mediated atherogenesis in LDLR-knockout mice. *Arterioscler Thromb Vasc Biol* 2009;**29**:1481–7. doi:10.1161/ATVBAHA.109.191940
- 53 Heath RB, Karpe F, Milne RW, *et al.* Selective partitioning of dietary fatty acids into the VLDL TG pool in the early postprandial period. *Journal of Lipid Research* 2003;**44**:2065–72. doi:10.1194/jlr.M300167-JLR200

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

- 54 Callow J, Summers LKM, Bradshaw H, *et al.* Changes in LDL particle composition after the consumption of meals containing different amounts and types of fat. *Am J Clin Nutr* 2002;**76**:345–50.
- 55 Baudet MF, Dachet C, Lasserre M, *et al.* Modification in the composition and metabolic properties of human low density and high density lipoproteins by different dietary fats. *Journal of Lipid Research* 1984;**25**:456–68.
- 56 Kratz M, Cullen P, Kannenberg F, *et al.* Effects of dietary fatty acids on the composition and oxidizability of low-density lipoprotein. *Eur J Clin Nutr* 2002;**56**:72–81. doi:10.1038/sj.ejcn.1601288
- 57 Zampolli A, Bysted A, Leth T, *et al.* Contrasting effect of fish oil supplementation on the development of atherosclerosis in murine models. *Atherosclerosis* 2006;**184**:78–85. doi:10.1016/j.atherosclerosis.2005.04.018
- 58 Weitz D, Weintraub H, Fisher E, *et al.* Fish oil for the treatment of cardiovascular disease. *Cardiol Rev* 2010;**18**:258–63. doi:10.1097/CRD.0b013e3181ea0de0
- 59 Suzukawa M, Abbey M, Howe PR, *et al.* Effects of fish oil fatty acids on low density lipoprotein size, oxidizability, and uptake by macrophages. *Journal of Lipid Research* 1995;**36**:473–84.
- 60 Watts GF, Mandalia S, Brunt JN, *et al.* Independent associations between plasma lipoprotein subfraction levels and the course of coronary artery disease in the St. Thomas' Atherosclerosis Regression Study (STARS). *Metab Clin Exp* 1993;**42**:1461–7.
- 61 Zambon A, Hokanson JE, Brown BG, *et al.* Evidence for a new pathophysiological mechanism for coronary artery disease regression: hepatic lipase-mediated changes in LDL density. *Circulation* 1999;**99**:1959–64.
- 62 Rifai N, Merrill JR, Holly RG. Postprandial effect of a high fat meal on plasma lipid, lipoprotein cholesterol and apolipoprotein measurements. *Ann Clin Biochem* 1990;**27 (Pt 5)**:489–93.
- 63 Wojczynski MK, Glasser SP, Oberman A, *et al.* High-fat meal effect on LDL, HDL, and VLDL particle size and number in the Genetics of Lipid-Lowering Drugs and Diet Network (GOLDN): an interventional study. *Lipids Health Dis* 2011;**10**:181. doi:10.1186/1476-511X-10-181
- 64 Alipour A, van Oostrom AJHHM, Izraeljan A, *et al.* Leukocyte activation by triglyceride-rich lipoproteins. 2008;**28**:792–7. doi:10.1161/ATVBAHA.107.159749
- 65 Hartigh den LJ, Altman R, Norman JE, *et al.* Postprandial VLDL lipolysis products increase monocyte adhesion and lipid droplet formation via activation of ERK2 and NFκB. *Am J Physiol Heart Circ Physiol* 2014;**306**:H109–20. doi:10.1152/ajpheart.00137.2013
- 66 Gower RM, Wu H, Foster GA, *et al.* CD11c/CD18 Expression Is Upregulated on Blood Monocytes During Hypertriglyceridemia and Enhances Adhesion to Vascular Cell Adhesion Molecule-1. 2010;**31**:160–6. doi:10.1161/ATVBAHA.110.215434
- 67 Bonham MP, Linderborg KM, Dordevic A, *et al.* Lipidomic profiling of chylomicron triacylglycerols in response to high fat meals. *Lipids* 2013;**48**:39–50. doi:10.1007/s11745-012-3735-5
- 68 Leuschner F, Rauch PJ, Ueno T, *et al.* Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. *Journal of Experimental Medicine* 2012;**209**:123–37. doi:10.1084/jem.20111009
- 69 Day RM, Harbord M, Forbes A, *et al.* Cantharidin blisters: a technique for investigating leukocyte trafficking and cytokine production at sites of inflammation in humans. *Journal of Immunological Methods* 2001;**257**:213–20.
- 70 Bertaux B, Prost C, Heslan M, *et al.* Cantharide acantholysis: endogenous protease activation leading to desmosomal plaque dissolution. *Br J Dermatol* 1988;**118**:157–65.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson - January 2016

- 71 Dinh PHD, Corraza F, Mestdagh K, *et al.* Validation of the cantharidin-induced skin blister as an in vivo model of inflammation. *Br J Clin Pharmacol* 2011;**72**:912–20. doi:10.1111/j.1365-2125.2011.04020.x
- 72 Conway DE, Breckenridge MT, Hinde E, *et al.* Fluid shear stress on endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1. *Curr Biol* 2013;**23**:1024–30. doi:10.1016/j.cub.2013.04.049
- 73 Tzima E, Irani-Tehrani M, Kiosses WB, *et al.* A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature* 2005;**437**:426–31. doi:10.1038/nature03952
- 74 Tsao PS, Lewis NP, Alpert S, *et al.* Exposure to shear stress alters endothelial adhesiveness. Role of nitric oxide. *Circulation* 1995;**92**:3513–9.
- 75 Hsiai TK, Cho SK, Wong PK, *et al.* Monocyte recruitment to endothelial cells in response to oscillatory shear stress. *The FASEB Journal* 2003;**17**:1648–57. doi:10.1096/fj.02-1064com
- 76 Colom B, Bodkin JV, Beyrau M, *et al.* Leukotriene B4-Neutrophil Elastase Axis Drives Neutrophil Reverse Transendothelial Cell Migration In Vivo. *Immunity* 2015;**42**:1075–86. doi:10.1016/j.immuni.2015.05.010
- 77 Auffray C, Fogg D, Garfa M, *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 2007;**317**:666–70. doi:10.1126/science.1142883
- 78 Dal-Secco D, Wang J, Zeng Z, *et al.* A dynamic spectrum of monocytes arising from the in situ reprogramming of CCR2+ monocytes at a site of sterile injury. *Journal of Experimental Medicine* 2015;**212**:447–56. doi:10.1084/jem.20141539
- 79 Carlin LM, Stamatiades EG, Auffray C, *et al.* Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell* 2013;**153**:362–75. doi:10.1016/j.cell.2013.03.010
- 80 Iqbal AJ, McNeill E, Kapellos TS, *et al.* Human CD68 promoter GFP transgenic mice allow analysis of monocyte to macrophage differentiation in vivo. *Blood* 2014;**124**:e33–44. doi:10.1182/blood-2014-04-568691
- 81 Itoh RE, Kurokawa K, Ohba Y, *et al.* Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. *Mol Cell Biol* 2002;**22**:6582–91. doi:10.1128/MCB.22.18.6582-6591.2002
- 82 Pertz O, Hodgson L, Klemke RL, *et al.* Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature* 2006;**440**:1069–72. doi:10.1038/nature04665
- 83 Cros J, Cagnard N, Woollard K, *et al.* Human CD14dim Monocytes Patrol and Sense Nucleic Acids and Viruses via TLR7 and TLR8 Receptors. *Immunity* 2010;**1**:1–12. doi:10.1016/j.immuni.2010.08.012
- 84 Rzeniewicz K, Neue A, Rey Gallardo A, *et al.* L-selectin shedding is activated specifically within transmigrating pseudopods of monocytes to regulate cell polarity in vitro. *PNAS* 2015;**112**:E1461–70. doi:10.1073/pnas.1417100112
- 85 Pagler TA, Wang M, Mondal M, *et al.* Deletion of ABCA1 and ABCG1 impairs macrophage migration because of increased Rac1 signaling. *Circulation Research* 2011;**108**:194–200. doi:10.1161/CIRCRESAHA.110.228619
- 86 Zhu L, Jiang R, Aoudjit L, *et al.* Activation of RhoA in podocytes induces focal segmental glomerulosclerosis. *Journal of the American Society of Nephrology* 2011;**22**:1621–30. doi:10.1681/ASN.2010111146
- 87 Sah VP, Minamisawa S, Tam SP, *et al.* Cardiac-specific overexpression of RhoA results in sinus and atrioventricular nodal dysfunction and contractile failure. *J Clin Invest* 1999;**103**:1627–34. doi:10.1172/JCI6842
- 88 Michaelson D, Silletti J, Murphy G, *et al.* Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. *J Cell Biol* 2001;**152**:111–26.

The Effects of Atherogenic Dyslipidaemia on Blood Monocyte Function

William Jackson- January 2016

- 89 Kurokawa K, Matsuda M. Localized RhoA activation as a requirement for the induction of membrane ruffling. *Mol Biol Cell* 2005;**16**:4294–303. doi:10.1091/mbc.E04-12-1076
- 90 Greenwood J, Walters CE, Pryce G, *et al.* Lovastatin inhibits brain endothelial cell Rho-mediated lymphocyte migration and attenuates experimental autoimmune encephalomyelitis. *The FASEB Journal* 2003;**17**:905–7. doi:10.1096/fj.02-1014fje