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An Investigation of the Immunometabolic Interactions of Human Obesity

Submitted to the National University of Ireland, Galway
for the degree
Doctor of Philosophy (PhD)

By

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April, 2012

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Declaration

This thesis describes work that I undertook between 2009 and 2012 at the Regenerative Medicine Institute, National University of Ireland, Galway. This work was supervised and mentored by Professors Matthew Griffin (Transplant Biology), Timothy O'Brien (Medicine) and Rhodri Ceredig (Immunology).

I declare that the results presented herein are from original experimental work which has been carried out by me for the purpose of this thesis. The work described within this thesis has not been submitted for degree, diploma or other qualification at any other University.

I have no conflict of interest pertaining to the subject matter of this work.

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Summary of Contents

Human monocytes are classified as classical, intermediate and non-classical according to their surface expression of CD14 and CD16. They also represent precursors to atherogenic foam cells and macrophages in metabolically adverse crown-like structures. Monocyte distribution, phenotype and function were investigated in obese individuals with and without type 2 diabetes in relation to clinical parameters of metabolic and cardiovascular risk.

Peripheral monocytes obtained from obese and non-obese subjects were enumerated and their phenotype investigated using flow cytometry and q-RT-PCR. Multivariate analyses were performed against validated clinical markers of cardiovascular and metabolic risk. When compared to their non-obese counterparts, obese individuals demonstrated decreased total and classical monocyte numbers with proportionately increased intermediate monocytes and unchanged non-classical monocytes. Surface staining of intermediate monocytes for HLA-DR identified two distinct subpopulations with mid (DR^{mid}) and high (DR^{hi}) level expression respectively. Expansion of the intermediate subset of monocytes in obese individuals occurred specifically within the DR^{mid} subpopulation and this correlated with key indices of lipid metabolism and glycemia. DR^{mid} intermediate monocytes displayed higher surface expression of pro-inflammatory and lipid receptors and higher uptake of acetylated-LDL.

A distinct subpopulation of intermediate monocytes sharing phenotypic features with atherogenic foam cells and macrophages in crown-like structures is expanded in the blood of obese individuals with and without type 2 diabetes. Direct interactions between modified lipoproteins and circulating monocyte subpopulations may represent an early pathogenic driver of obesity-associated atherosclerosis and adipose tissue inflammation.

Section 1: Introduction

Chapter 1

Basic Monocyte Biology

1.0 Basic Monocyte Biology

Monocytes are bone-marrow derived, phagocytic cells of innate immunity, which circulate in the bloodstream and migrate, in response to inflammatory stimuli, across the endothelial barrier and into the tissues, where they carry out subtype-specific effector functions in addition to undergoing tissue specific terminal differentiation to either macrophages or dendritic cells (DCs).[1]

Most research describing monocyte biology derives from mice, but considerable human data also exist. Numerous models of tissue injury have been used to investigate monocyte response and function namely, lipopolysaccharide (LPS) stimulation, bacterial infection with organisms such as *Listeria (L) Monocytogenes,* myocardial infarction (MI) and atherosclerosis.[1-3] Recent years have seen an improved understanding of the monocyte lifecycle. This includes better knowledge relating to the transcriptional regulation of bone marrow monocytopoesis,[1, 4] sequestration within the spleen,[5, 6] extramedullary monocytopoesis,[7, 8] in addition to monocyte trafficking and their fate within the tissues.[1, 9-11] Monocytes are reliably identified according to surface phenotype and functional characteristics, which predict their effector role in inflammation as well as their eventual maturation to macrophages and dendritic cells.[3] It is now apparent that monocytes represent a complex family of immune cells which demonstrate considerable plasticity as multifunctional progenitor cells, in addition to playing their principal role as accessory and effector cells. Moreover, monocytes are abundantly and widely distributed throughout the organism *via* the bloodstream, from which they have the ability to selectively migrate into tissues in response to specific chemotactic signals.[3, 9, 12-14] While the primary monocyte response provides essential protection against

infection and tissue injury, a dysfunctional or unregulated monocyte response can often contribute to tissue injury and this may be associated with considerable and broad-reaching pathology.[3, 15-17]

This introduction outlines, in detail a description of basic monocyte biology (Chapter 1) and describes their influence and known immunometabolic effects in human obesity and metabolic disease (Chapter 2).

1.1 Monocyte Morphology and Classification

Morphologically, monocytes are irregular cells, with lobular nuclei and abundant intracellular vesicles.[18] (Figure 1.1) They show considerable heterogeneity of size, shape and granularity.[19, 20] Accurate monocyte characterisation relies upon the use of multi-parameter flow cytometry, which measures surface molecules labelled with fluorochrome conjugated monoclonal antibodies (MAb). Using a combination of light-scatter characteristics and fluorescence, monocytes can be distinguished from other elements of the macrophage / dendritic cell system (MDS) and sub-classified according to a combination of their relative surface expression of specific proteins.[6, 20]

Human monocytes universally show high expression of CD45 (a non-specific leukocyte marker), HLA-DR (class II major histocompatibility antigen) and demonstrate variable expression of CD14 [part of the lipopolysaccharide (LPS) receptor complex] and CD16 (FCγ Receptor).[19] Monocytes from humans and mice share the expression of a number of surface molecules, which suggest interspecies homology between individual monocyte subpopulations. Both species highly express various other pattern recognition and toll like receptors (TLRs) including TLR-1, 2, 3, 4, 6, 7, 8 & 9. Additionally numerous chemokine receptors including CCR2, CCR5,

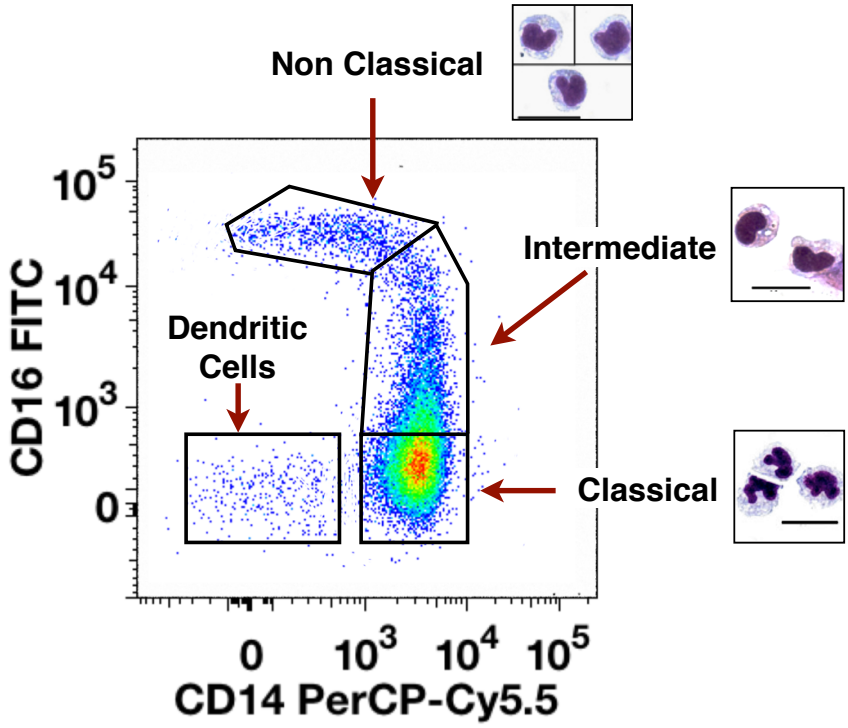
CXCR4 and CX3CR1, are expressed at different levels by individual monocyte subpopulations and relative patterns of expression for these chemokine receptors are used to distinguish monocyte subpopulations phenotypically.[18, 21, 22] Chemokine receptor - ligand interactions mediate monocyte trafficking to injured tissues, where they then initiate an acute injury response.[4, 23] Monocytes also participate in the reparative process through efferocytosis of apoptotic cells, toxins and extracellular debris.[4, 18] This is highlighted by their expression of a wide array of scavenger receptors including the lipoprotein scavengers, namely CD36, scavenger receptor (SR) -A, SR-B, and the haem scavenger, CD163.[24-27] Monocytes also universally bear the mark of their bone marrow origins through the expression of the M-CSF receptor, CD115.[1, 11] Table 1.1 shows a detailed outline of the phenotypic characteristics of monocytes according to their relative cell surface expression of specific protein markers.

1.1.1 Monocyte Sub-classification

Monocytes are defined on the basis of their size, morphology and distribution within the organism. They have traditionally been described as circulating mononuclear cells of myeloid origin, with an irregular horseshoe-shaped nucleus and abundant granular cytoplasm found. They differ from macrophages whereby macrophages are located within the tissues and have a larger cytoplasmic volume. Dendritic cells on the other hand differ in shape from monocytes, having numerous elongated cellular projections, from which they earn their name. Monocytes also produce higher quantities of cytokines and humoral mediators when compared to their macrophage counterparts.[6, 28]

Using characteristics of surface phenotype, discrete monocyte subsets are described

Figure 1.1: Human Monocyte Subclassification



Human monocytes are sub-classified, using flow cytometry according to their surface expression of CD14 and CD16 as Classical ($CD14^{hi}$,$CD16^{-}$), Intermediate ($CD14^{hi}$, $CD16^{+}$) and Non-Classical ($CD14^{lo}$, $CD16^{+}$)

Accompanying light micrographs *(adapted from Cros et al, 2010)* show characteristic lobular nuclei and granular cytoplasm.

which have differing functional roles in the pathophysiology of inflammation and the pathology of disease.[4, 18, 22] While best understood in mouse and humans, functionally distinct monocyte subsets are also evident across other species including primates, pigs and rats. In humans, the most recent consensus on monocyte classification

Table 1.1: Human Monocyte Phenotype

	Classical	Intermediate	Non-Classical
Proportion	70 - 80%	15 - 20%	<10%
Universal Monocyte Markers			
HLADR	++	++	++
CD86	+	+	++
CD115	++	++	++
CD11b	++	++	+
CD11c	++	++	++
Adhesion Molecules / Chemokine Receptors			
CD62L	++	++	lo
CX3CR1	lo	+	++
CCR-2	++	++	lo
CCR-5	++	++	lo
LFA-1	+	?	++
VLA-4	++	?	+
PSGL-1	++	?	+
Apolipoprotein & Scavenger Receptors			
CD163	++	++	lo
CD36	++	++	lo
LRP-1	++	?	++
SR-A	++	++	+
LDL-R	++	?	++
Pattern Recognition Receptors			
TLR-4	++	++	+
TLR-2	++	++	+
TLR-7	+	+	++
TLR-9	+	+	++

Abbreviations

CD: Cluster of Differentiation
HLA: Human Leukocyte Antigen
CCR: C-C Chemokine Receptor
LFA: Lymphocyte Function Associated Antigen
VLA: Very Late Antigen
PSGL: P-Selectin Glycoprotein Ligand
LRP: Low density lipoprotein receptor-related protein
SR: Scavenger Receptor
LDL-R: Low Density Lipoprotein Receptor
TLR: Toll like Receptor

Monocyte subsets differ broadly in their surface expression of numerous markers relating to cellular adhesion / migration, in addition to functions such as scavenging and response to infection.

recommends categorisation based on relative surface expression of CD14 and CD16 into three subsets, namely classical ($CD14^{hi}$,$CD16^{-}$), non-classical ($CD14^{lo}$, $CD16^{+}$) and intermediate ($CD14^{hi}$, $CD16^{+}$) monocytes (Figure 1.1).[19] In mice two subsets are described according to their common surface expression of CD115 and their differing expression of Ly6C, CCR2, $CX3CR_1$ and L-selectin (CD62L), namely $Ly6C^{hi}$ ($CX_3CR_1^{lo}$, $CCR2^{hi}$, $CD62L^{hi}$) and $Ly6C^{lo}$ ($CX_3CR_1^{hi}$, $CCR2^{lo}$, $CD62L^{lo}$) monocytes.[6]

Functionally, mouse and human monocyte subsets are not direct homologues. Human classical monocytes share many characteristics with their putative mouse equivalents $Ly6C^{hi}$, while non-classical and $Ly6C^{lo}$ monocytes also share phenotypic and functional similarities.[18, 29] The role of human intermediate monocytes, in addition to their homology with mouse monocyte subclasses, is less well defined.[18, 21]

1.2 Monocyte Function

1.2.1 Functional Characteristics of Mouse Monocyte Subsets

Robust *in vivo* and *in vitro* studies of monocyte function have described their properties in accordance with phenotype in mice. Numerous stimuli have been used to investigate the monocyte inflammatory response, including viruses and bacteria such as *E. Coli, L. Monocytogenes*, *S Pneumoniae and M. Tuberculosis.* Additionally lipopolysaccharide / bacterial endotoxin at varying doses has been used to simulate bacterial infection.[4] Models of non-infective inflammation and tissue injury, such as atherosclerosis and induced myocardial infarction are also used to investigate specific monocyte responses.[30, 31]

1.2.1.1 Ly6C^{hi} Monocytes

The two monocyte subclasses in mice demonstrate dichotomy of function. Ly6C^{hi} monocytes rapidly adopt a pro-inflammatory phenotype and abundantly, but specifically, migrate to injured tissue [1, 4]. Ly6C^{hi} migration occurs in a chemokine dependent manner, predominantly *via* CCL2 and CCL7 through the receptors, CCR2 and CCR5.[32, 33] These monocytes demonstrate high expression of L-selectin (CD62L), through which they bind and interact with endothelium prior to migration.[4] Following transfer across the endothelial barrier, to the tissues, they differentiate readily into TNFα / iNOS producing DCs (TipDCs) and pro-inflammatory macrophages (often termed M1 polarised macrophages) which also release high quantities of TNFα and IL-1β.[1] However, Ly6C^{hi} monocytes represent a powerful effector cell in their own right.[34] Moreover, the acute inflammatory response instituted by Ly6C^{hi} monocytes probably occurs prior to first contact with the

endothelial barrier, before migration, and may involve complex humoral cross-talk with their Ly6C^{lo} counterparts.[35]

Undifferentiated, effector Ly6C^{hi} monocytes possess a vast cellular machinery which equips them to respond quickly to inflammatory stimuli, arising from tissue injury. This is reflected by their activity whereby, once activated, they readily phagocytose microbial elements and cellular debris. They contain high intracellular concentrations of lysosomal enzymes responsible for digesting the contents of phagocytic vesicles, namely myeloperoxidase and lysozyme, albeit that these lysosomal enzymes are expressed in higher quantities by tissue macrophages.[11, 28] This monocyte subpopulation also produces reactive oxygen species and independently releases pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 in response to bacterial stimuli and lipopolysaccharide (LPS).[36] Numerous *in vivo* studies clearly show that the circulating fraction of Ly6C^{hi} monocytes is expanded in response to bacterial infection[37] as well as in conditions of sterile inflammation such as rheumatoid arthritis, MI and atherosclerosis.[4, 32, 38] These features point towards a pivotal role in the defence against infection but also in the pathogenesis of inflammatory conditions.

1.2.1.2 Ly6C^{lo} monocytes

Ly6C^{lo} monocytes are ascribed a policing role, whereby they remain in the circulation and crawl along the luminal surface of the vascular endothelium, bound to the lymphocyte function associated antigen (LFA)-1 / β_2 integrin adhesion molecule complex.[38, 39] In steady-state conditions, Ly6C^{lo} have a prolonged circulating half-life, when compared to Ly6C^{hi} monocytes.[30] Many hypotheses have been proposed to explain their endothelial patrolling behaviour, including a scavenging role whereby they remove oxidised lipids, dead cells and pathogens from the endothelial surface

and present these back to CD4 lymphocytes within the spleen or lymph nodes.[40] Cross-talk between patrolling Ly6C^{lo} monocytes and the subendothelial microenvironment may also mediate a process whereby they selectively migrate into the tissues and replenish tissue fixed, resident and M2 polarised macrophages.[41, 42] However, recent data describes an interesting role for Ly6C^{lo} monocytes as very early, pro-inflammatory responders to tissue injury.[39]

Patrolling Ly6C^{lo} monocytes can mount a rapid, but transient, pro-inflammatory response to *L Monocytogenes* and LPS, and may represent the earliest tissue-infiltrating monocyte subtype.[1, 32, 39] Under high LPS conditions, they release TNFα, IL-1β and CCL2. Moreover they recruit and activate other effector cells of innate immunity, such as granulocytes and NK cells, in addition to recruiting their Ly6C^{hi} counterparts. During this initial pro-inflammatory response, Ly6C^{lo} monocytes upregulate their expression of key surface markers responsible for migration of myeloid immune cells, inflammatory signalling, scavenger receptors and molecules which modulate cross-talk between NK cells and monocytes, namely TLR-1, TLR-2, CD36, SR-A, dectin-2, Myeloid DAP12-associating lectin (MDL)-1 as well as complement and defensins.[1, 38] Within eight hours of the initial stimulus, Ly6C^{lo} monocytes have completely egressed from the site of tissue injury and are replaced by a Ly6C^{hi} predominant infiltrate. The underlying reason for this change in phenotypic dominance is unknown as is the eventual fate of Ly6C^{lo} monocytes following their early and robust pro-inflammatory response.[38] An interesting observation demonstrates a gradual phenotypic switchback to a Ly6C^{lo} predominant inflammatory infiltrate between days 4 and 7 post-tissue injury, which has regressed to normal pre-injury repertoire by day 16 (See Section 1.3.1.3 *"Phases of Monocyte Infiltration"* below).[38, 43, 44]

It is increasingly acknowledged that the majority of monocytes probably undergo apoptosis and efferocytosis after they fulfill an effector function. Evidence from models of sterile inflammation (MI) and infection, shows that following an initial pro-inflammatory response, Ly6C^{lo} monocytes not undergoing apoptosis or egressing from the site of tissue injury, adopt a differentiated anti-inflammatory / reparative macrophage (termed M2) profile by increasing their expression of *Arginase-1, IL-4Rα, cMaf and MafB*.[1] The M2 profile adopted by Ly6C^{lo} monocytes contrasts with that of Ly6C^{hi} monocytes which undergo a *PU.1* transcription factor driven differentiation to M1 macrophages and hence propagate the pro-inflammatory response.[1, 4, 39]

Overall, Ly6C^{lo} monocytes appear to represent a complex, versatile and mature monocyte subtype in their own right, whereby they demonstrate autonomy of function in addition to expressing high levels of anti-proliferative transcription factors *cMaf* and *MafB,* associated with mature phenotypes.[1]

1.2.1.3 Phases of Monocyte Infiltration

Investigating the monocyte response to acute myocardial infarction (MI), Nahrendorff *et al.* identified 2 distinct phases of monocyte infiltration, termed Phase I and Phase II respectively. Phase I occurred in the first four days following MI and was characterised by a Ly6C^{hi} predominant inflammatory infiltrate.[38] The Ly6C^{hi} proportion in the inflammatory infiltrate during phase I was modulated by a 4.5 fold increase in circulating Ly6C^{hi} monocytes, which were drawn to the site of injury in a CCL2 / CCR2 dependent manner. During phase I, Ly6C^{hi} monocytes had high phagocytic activity and released high quantities of proteinase and pro-inflammatory cytokines. Hence, this phase was dominated by proteolysis, phagocytosis,

inflammation and scavenging of cellular debris mediated by the monocyte infiltrate. The Ly6C^{hi} predominant infiltrate of phase I was replaced by a Ly6C^{lo} monocyte infiltrate of phase II between day 4 and 7. During phase II, Ly6C^{lo} monocytes were drawn to the injured tissue through an increase in CX_3CL1 release from injured tissue as well as an associated higher VCAM-1 expression on associated endothelium. The absolute circulating Ly6C^{lo} monocyte count did not change during the acute / subacute inflammatory process. This phase was also dominated by an increase in vascular endothelial growth factor (VEGF) -1 release, accompanied by an accumulation of myofibroblasts, along with angiogenesis and collagen deposition.[38]

These interesting data indicate a physiological "*clean-up*" role for monocytes whereby Ly6C^{hi} monocytes scavenge and Ly6C^{lo} monocytes repair following the initial damage caused by the inflammatory stimulus and ensuing neutrophil response.

1.2.2 Functional Characteristics of Human Monocyte Subpopulations

Studies of human monocytes are limited by a lack of robust *in vivo* functional assays. The majority of data relating to function derive either from *in vitro* assays or from studies of phenotype which draw inference from mouse models.[18, 45] Moreover it is difficult to safely investigate human monocyte distribution between the bone marrow, bloodstream, tissues and spleen. Consequently most studies focus on the circulating monocyte fraction. While broad interspecies comparisons can be made between mouse and human monocyte subpopulations, drawing direct interspecies inference is limited by the fact that human monocytes are currently classed into three subpopulations *versus* two in mice.[19, 29] Hence the human repertoire appears to possess greater complexity. Nonetheless, current data suggest that Ly6C^{hi} and Ly6C^{lo} mouse monocytes represent respective interspecies homologues of classical and non-

classical human monocytes[18, 29, 45]. The established role of human intermediate monocytes is not fully appreciated.

1.2.2.1 Non-Classical Monocytes

While non-classical monocytes represent a lower circulating monocyte fraction than their classical counterparts, they are well characterised in terms of their homology with mouse monocytes. Human non-classical and mouse Ly6C^{lo} monocytes share the greatest interspecies phenotypic and functional homology. Both share high expression levels for CX_3CR1 in addition to absent or low expression of CCR2 and CD163.[11] These monocyte subsets show interspecies preservation of function whereby both demonstrate endothelial crawling activity.[18, 39] Using *intra-vital* microscopy in a humanised mouse model, Cros *et al* demonstrated crawling activity in non-classical monocytes along the endothelial surface in a LFA-1 dependent manner.[18] Transcription factors including *Vav2* and *ArhGef18* are also highly expressed in non-classical monocytes. These act through *RhoGTPases* to mediate cytoskeletal re-arrangement and plasticity which is probably related to endothelial crawling activity. In line with the *"policing"* role inferred by endothelial crawling, non-classical monocytes may also possess selective scavenging functions, and highly express scavenger receptors for Apolipoprotein E (ApoE) and ApoA but express the lipoprotein scavenger receptors SR-A and CD36 at very low levels.[11, 45]

Non-classical monocytes adopt an anti-inflammatory profile in their resting state. Similar to Ly6C^{lo} monocytes, they highly express M2 associated transcription factors namely *cMaf* and *MafB,* and exhibit relatively poorer phagocytic activity when compared to their classical and intermediate counterparts.[1] They also have a characteristically anti-inflammatory chemokine profile and express the IL-10

receptor at high concentrations as well as producing large quantities of the IL-1 receptor antagonist (RA).[18, 45, 46] Recent studies have also shown a pro-inflammatory profile for non-classical monocytes. Cros *et al* have shown that, when exposed to viral particles, normally quiescent, non-classical monocytes produce a robust, pro-inflammatory response, whereby they secrete TNFα, IL-1β, IL-6 and IL-8. This response is largely mediated through TLR-7 and TLR-8 *via* MyD88 signalling. Viral replication is not necessary to produce the non-classical monocyte pro-inflammatory response, which initiates immediately upon viral particle uptake. Gene cluster analyses have demonstrated a high expression of genes associated with FcR mediated phagocytosis in non-classical monocytes, in addition to abundant expression of several complement components, including C1qA, C1qB, C2 and C3. These data further support a putative role for non-classical monocytes in the response to viral infection.[18]

While there is compelling evidence for an early pro-inflammatory response mediated by Ly6C^{lo} monocytes in response to bacterial infection,[39] the evidence relating to a bacterially mediated pro-inflammatory response for non-classical monocytes is conflicted. Results demonstrating high TNFα production in response to LPS have not been reproduced across different studies. Inconsistent results have been attributed to differences in cell isolation techniques and culture conditions. Further studies are required to clarify this aspect of non-classical monocyte function.[18, 45, 47]

1.2.2.2 Classical Monocytes

Classical monocytes highly express CD14 and have low or absent expression of CD16. Under normal conditions, they represent the largest proportion of circulating monocytes and show high surface expression of TLR-4, CCR-2, CXCR4, CD62L in

addition to the scavenger receptors CD36 and SR-A.[18, 45] This monocyte subpopulation exhibits high phagocytic activity and shares many characteristics with pro-inflammatory macrophages and DCs, for which it acts as a precursor. Classical monocytes may also represent immature monocyte precursors for their intermediate and non-classical counterparts.[45] While supported by large studies of monocyte genotype and phenotype, direct *in vitro* or *in vivo* evidence has not yet been produced to support this maturation theory.

Studies which have compared mouse and human monocytes suggest the greatest interspecies homology between classical and Ly6C^{hi} monocytes.[1, 29] Classical monocytes highly express several markers associated with angiogenesis, inflammation, coagulation and wound healing.[47-49] These characteristics indicate an ability to respond to various external cues and produce widely varying responses, from a destructive pro-inflammatory response, to anti-inflammatory mediated tissue repair. It also suggests that of all circulating subsets, classical monocytes represent a well equipped and versatile subset which is primed to adapt and differentiate readily in response to pro-inflammatory stimuli. These cells respond readily to LPS but interestingly, they do not produce high quantities of TNFα or IL-1β *in vitro*. Rather they produce IL-6, IL-8 and IL-10.[18, 45] This peculiarity of classical monocyte function contrasts with that of Ly6C^{hi} monocytes in mice.

In fact in studies of monocyte function, performed prior to introduction of the current classification system, CD16^{+} monocytes were consistently shown to produce the highest concentrations of pro-inflammatory cytokines.[46, 50] It is not clear whether this is a function of intermediate monocytes or non-classical monocytes and this is an area for which current published evidence also conflicts.

1.2.2.3 Intermediate Monocytes

The intermediate subpopulation comprises monocytes which highly express CD14 and demonstrate a broad ranging, mid level expression for CD16, when classified using flow cytometry. On scatter plots of CD14 *versus* CD16, this subpopulation is continuous below with classical, and above with non-classical monocytes. Hence, objective isolation of the intermediate monocyte subpopulation requires a careful gating strategy, whereby they are selected using flow minus one (FMO) negative controls for both CD14 and CD16.[51]

Intermediate monocytes share various aspects of their phenotype with the other monocyte subpopulations, eponymously demonstrating intermediate-level surface expression of markers for which classical and non-classical monocytes demonstrate polar expression. In a study by Wong *et al.*, a total of 1,554 genes were common to all three monocyte subpopulations. However, whereas monocytes within the classical subpopulation most highly expressed 862 of these and non-classical 557, intermediate monocytes demonstrated the highest expression for only 135 and demonstrated mid-level expression for the remainder.[45] Intermediate monocytes appear to represent the most heterogenous of the monocyte subpopulations and their finer phenotypic and functional details remain unclear. Two large reports which addressed monocyte phenotype across all subpopulations have reached very different conclusions regarding the relationship between intermediate monocytes and their non-classical and classical counterparts.[18, 45]

In a study by Cros *et al.*, it was proposed that intermediate monocytes represent an activated form of classical monocyte which share many functional characteristics with mouse Ly6C^{hi} monocytes.[18] Following principal component analysis (PCA) of a

range of genomic markers, measured using multi-array, intermediate monocytes clustered with classical monocytes. Further selective analysis of phenotype demonstrated that intermediate monocytes shared high surface expression of CD163, CD11b with classical monocytes, but they also shared some phenotypic characteristics with non-classical monocytes i.e. higher expression of CX_3CR1 and CD11c, and lower expression of CD62L. Functionally, intermediate monocytes produced a strong pro-inflammatory response to LPS stimulation, in which they uniquely released high amounts of TNFα and IL-β.[18] Additionally, similar to classical monocytes, they released high concentrations of IL-8, IL-6, IL-10 and CCL-3, but differed in that they did not express high levels of reactive oxygen species (ROS).[18]

Conversely, Wong *et al.* described intermediate monocytes as a subpopulation more closely associated with non-classical monocytes . They proposed a model in which intermediate monocytes are presented as a truly transitional monocyte subpopulation which progressively matures, with increasing CD16 expression, from the classical to the non-classical phenotype. In contrast to the work of Cros *et al*, multi-array data from this study clustered intermediate with non-classical, rather than classical, monocytes.[45] However, detailed phenotypic analysis using flow cytometry and functional assays of cytokine production concurred between both studies. Wong *et al.*, furthered their analysis by demonstrating that across the intermediate subpopulation, expression levels for each typically *"non-classical monocyte marker"* increased proportionately with CD16 expression. The authors hypothesised that these characteristics may signify a phenotypic ripening of intermediate monocytes, whereby they mature in the direction of CD16 expression towards the non-classical phenotype.[45]

This paradigm somewhat challenges *in vivo* evidence from mouse models which does not demonstrate appreciable phenotypic maturation of Ly6C^{hi} to Ly6C^{lo} monocytes either in the circulation or within the tissues.[52] Therefore convincing functional evidence to support a *"maturation" hypothesis* for human monocytes is required. Phenotypic characteristics suggestive of monocyte maturation may in fact indicate the heterogeneity of the wider intermediate subpopulation of monocytes currently classified within a single group. While differing in their conclusions from respective microarray data, both studies concur regarding a pro-inflammatory function of the intermediate monocyte subpopulation.[11, 18, 45, 47] Nonetheless, the differing conclusions regarding their phenotype may indicate that intermediate monocytes are heterogenous in their own right and that investigations to identify specific subsets within the intermediate population are necessary to clarify their roles in health and disease.

1.2.2.4 Phases of Monocyte Infiltration

Investigation of time-dependent patterns of circulating human monocyte repertoire following tissue injury suggests phasic tissue infiltration in a manner similar to that seen in mice. In the immediate aftermath of cardiovascular events in humans, the numbers of classical and intermediate monocyte subpopulations increase rapidly and remains elevated for the first 4 days. Following which the number of non-classical monocytes peak. This pattern is reminiscent of the biphasic monocyte response seen in mice following acute myocardial infarction.[53]

1.3 The Monocyte Life-Cycle: Origin, Development & Distribution

The role of monocytes as early responders of innate immunity requires that they migrate quickly, and preferentially, to inflammed tissues. Equally as important is that the consequently depleted circulating monocyte pool must be replenished efficiently. Reduced proliferative capacity of monocytes, once differentiated from myeloid progenitor cells, requires that the process of monocyte replenishment is mediated through *de novo* monocyte synthesis either within the bone marrow or resultant to extra-medullary monocytopoesis.[1, 38, 52, 54] Sequestered monocyte pools within the spleen also provide a large store which can be mobilised in response to tissue injury.[52, 55] Cross-talk within the bone-marrow between mesenchymal stem cells (MSCs) and endothelial cells is central to efficient monocyte replenishment through migration of both monocytes and monocyte precursors from the bone marrow into the blood-stream. The response of bone marrow and splenic monocyte reservoirs to tissue derived humoral factors is also key to monocyte homeostasis and sufficient replenishment.[56]

1.3.1 Bone Marrow Synthesis of Monocytes

Monocytes develop within the bone marrow from macrophage / DC progenitor cells (MDPs) and share common lineage with granulocyte / macrophage progenitor cells (GMPs). Both MDPs and GMPs are products of haemopoeitic stem cell (HSCs) derived common myeloid progenitors (CMPs). The rate limiting step upon the MDP differentiation pathway is catalysed by monocyte / macrophage colony stimulating factor (M-CSF or CSF-1) through its receptor CSF-1R (CD115).[40, 57] The overall process governing monocyte / macrophage lineage determination is under tight

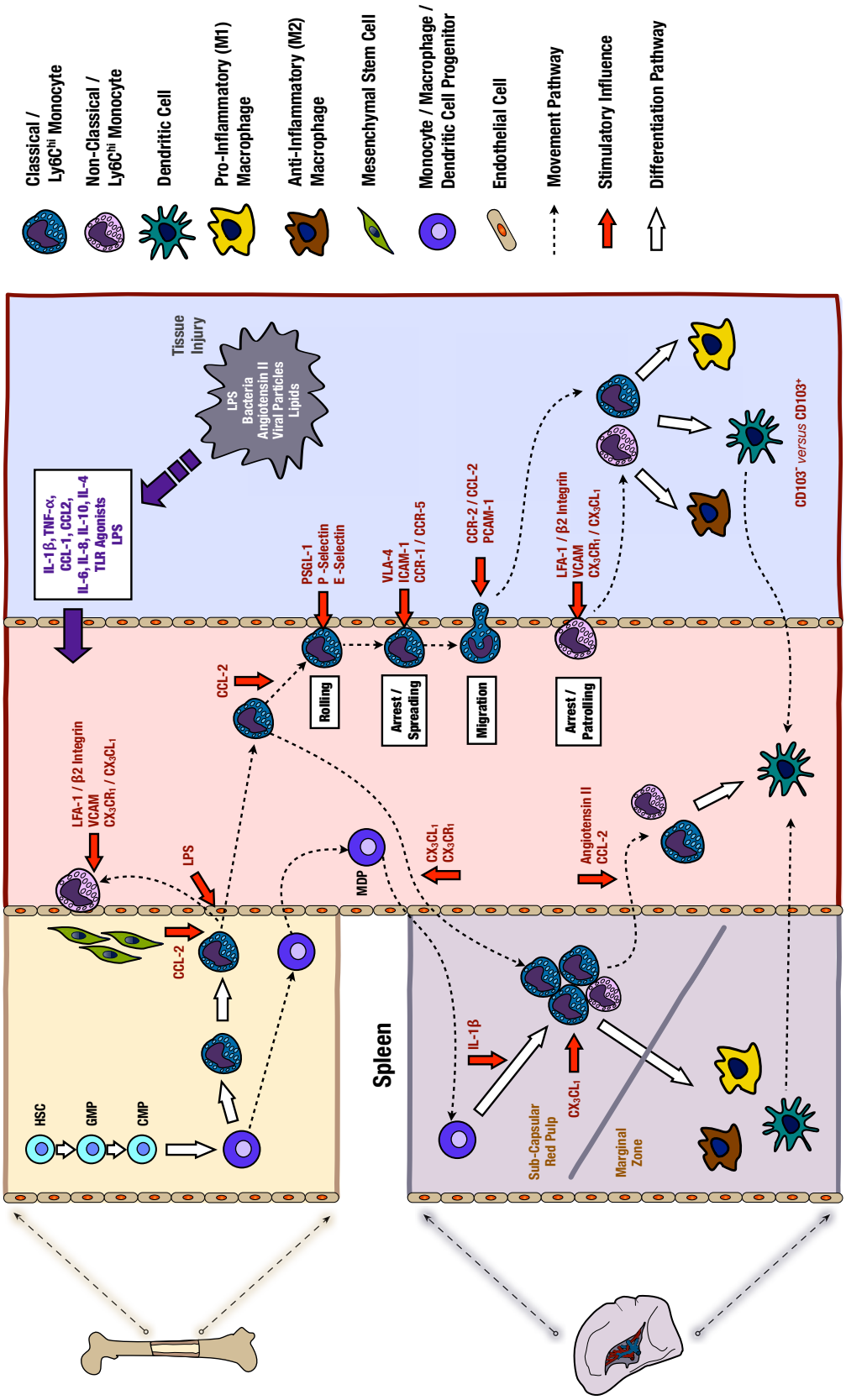


Figure 1.2: Schema of Monocyte Development, Migration, Function and Fate

transcriptional regulation.[1, 11] The governing transcription factor for MDP determination PU.1, produces its effects in a concentration dependent manner and mediates numerous steps within the monocyte differentiation pathway. At the early stages of monocyte determination PU.1 diverts HSCs away from erythroid and megakaryocyte lineage by inhibiting the transcription factors GATA1 & 2. PU.1 directly transcribes production of CSF-1R, necessary both for differentiation and survival of cells of the MDP pathway. At later stages, PU.1 facilitates the action of downstream transcription factors such as *Egr.Nab* and *Kruppel-like Factor-4* (KLF-4), both of which promote monocyte lineage differentiation while simultaneously inhibiting CCAAT-enhancer-binding protein (C/EBP)-α, necessary for granulocyte lineage commitment. The transcription factors, *MafB and c-Maf* are central to later stages of monocyte lineage determination. Both affect the inflammatory fate of monocytes and are themselves regulated by PU.1 in a concentration dependent manner. At high expression levels, PU.1 inhibits both *MafB* and *c-Maf* and favours a pro-inflammatory macrophage or DC fate in circulating monocytes, while at moderate expression levels of PU.1, their expression is stimulated to favour an anti-inflammatory fate.[1]

The process of monocyte development within the bone marrow takes approximately 1 week to complete. PU.1 and *MafB* confer an anti-proliferative signal which inhibits cell division in MDPs , prior to their differentiation into monocytes. This produces a legacy effect whereby circulating and tissue infiltrating monocytes have a significantly reduced proliferative capacity once differentiated from common myeloid progenitor cells, even in response to inflammation and tissue injury. A robust mechanism for monocyte mobilisation in times of need, which is independent of proliferation, is therefore required.[1] Compensatory mechanisms for monocyte sequestration, storage

in addition to extra-medullary monocytopoeisis ensures sufficient monocyte supply to fulfil their role in innate immunity.[52, 54, 58]

There is considerable debate as to whether or not a phenotypic shift, or switch, occurs whereby Ly6C^{hi} and classical monocytes mature towards a Ly6C^{lo} or non-classical monocytes. While there are data to support this hypothesis, more robust developmental data indicate Ly6C^{hi} and Ly6C^{lo} monocyte determination occurs during bone marrow monocytopoeisis.[59] Each subpopulation follows separate differentiation pathways, mediated by activation of different transcription factors. The combination of PU.1 and KLR-4 activation promotes the differentiation of monocytes with a pro-inflammatory phenotype, namely Ly6C^{hi} monocytes. Separately, the activation of another transcription factor, namely *Nur77*, in association with PU.1 promotes differentiation of Ly6C^{lo} monocytes.[1, 11]

Figure 1.2 demonstrates a representative schema for monocyte determination in the bone marrow, distribution within the circulation and terminal differentiation within the tissues.

1.3.2 Bone Marrow-Derived Monocyte Release

The mechanisms governing human monocyte migration from bone marrow to the circulation are not known and hence, we rely upon inference from mouse models of monocyte biology.

Emigration of monocytes from the bone marrow into the circulation is reliant upon the interaction of CCR2, expressed on the monocyte surface, with its ligand CCL2. Differentiated Ly6C^{hi} monocytes are drawn in a CCR2 dependent manner to the peripheral zones of the marrow and localise with vascular endothelial (VE) cadherin

positive cells, adjacent to the sinusoids. In extra-medullary tissues, endothelial cells are the principal producers of CCL2. However MSCs, rather than endothelium represent the source of CCL2 within the bone marrow marrow. Hence, MSCs are key cells regulating the release of monocytes into the circulation from the peripheral zones of the marrow.[56] During steady-state homeostasis, the bone marrow provides a consistent source of monocytes which are recruited to the tissues or sequestered within the splenic reservoir. Efficient monocyte kinetics in the case of tissue injury are supported by this process of synthesis, slow release, sequestration and partitioning of monocytes in the periphery.[4, 54]

During the acute phase of tissue injury, the marrow responds by increasing its release of monocytes in response to CCL2. High circulating concentrations of CCL2 originate from endothelium, connective tissue or immune elements of the injured peripheral tissues.[4, 32] During the very early stages of infection the marrow itself produces CCL2 in response to low circulating concentrations of bacterial products, which promotes an increased rate of monocyte release. Within the first two hours of inoculation with either *L. monocytogenes* or LPS, CCL2 is secreted by bone marrow mesenchymal stem cells (BM-MSCs) and CXCL-12 abundant reticular (CAR) cells, situated adjacent to VE-cadherin positive endothelial cells. In response to resultant high intra-marrow concentrations CCL-2, Ly6C^{hi} monocytes are recruited to the peripheral zones of the marrow and released into the bloodstream, where they then circulate to the source of infection.[32, 35, 60] Interestingly, bone marrow CCL-2 secretion and consequent monocyte release occurs only in response to a low inoculum of LPS, while higher LPS concentrations produce a dose-dependent inhibition of this process. [56] Hence bone marrow derived Ly6C^{hi} monocytes may provide the initial monocyte response to infection, which is then supported by a secondary splenic source if the

marrow response becomes overwhelmed.

1.3.4 The Role of the Spleen

1.3.4.1 Sequestration, Storage and Release

The ability to provide a continual source of monocytes during the prolonged process of tissue injury and repair is dependent upon the spleen. The spleen sequesters monocytes from the circulation and provides a unique micro-environment where they rest, without differentiating, and egress back to the bloodstream in response to an inflammatory stimulus.[54]

CX3CR1 mediates the recruitment of circulating Ly6C^{hi} and Ly6C^{lo} monocytes to the spleen. The membrane bound ligand for CX3CR1, namely CX3CL1 (fractalkine), is abundantly expressed in the splenic marginal zone. The absolute number of monocytes stored within the spleen exceeds the circulating monocyte fraction and sequestered monocytes gather in clusters in the sub-capsular red pulp. CX3CL1, responsible for recruiting circulating monocytes, confers a survival signal which prolongs the lifespan of monocytes once sequestered within the spleen.[54] The constant and dynamic trafficking of monocytes between the circulation and splenic compartments is reflected by the preservation of the ratio of Ly6C^{hi} to Ly6C^{lo} monocyte subpopulations between both compartments. All monocyte subtypes, whether of splenic or blood-borne origin share similar characteristics. They have a similar phenotype. Functionally, there appears to be no difference between monocytes derived from spleen or circulating blood monocytes. When exposed to humoral factors, such as GM-CSF, M-CSF and IL-4, both blood borne and splenic monocytes also have similar differentiation characteristics.[4, 9, 11, 54, 59] Hence the spleen

and circulation represent continuous functional compartments in the monocyte lifecycle.

The mechanism whereby monocytes sequestered in the spleen enter the bloodstream before being recruited to sites of inflammation and tissue injury is not fully understood. Following myocardial infarction in mice, angiotensin (AT) II provides a signal to sequestered splenic monocytes to enter the bloodstream. In this model, the process of recruitment from spleen occurred in a CCR2 independent manner, while migration of splenic derived Ly6C^{hi} monocytes to the injured myocardium was mediated through the usual CCR2 dependent mechanism.[52, 55] The fate of splenic Ly6C^{lo} monocytes remains unknown.

1.3.4.2 Extramedullary Splenic Monocytopoesis

The paradigm whereby the spleen acts only as reservoir for bone-marrow derived monocytes, sequestered from the bloodstream has expanded to include extra-medullary splenic monocytopoesis.

Following sterile tissue injury induced by MI, splenic monocytes account for the bulk of infiltrating monocytes in mouse myocardium where they dominate the inflammatory infiltrate for up to 6 days. Leuschner et al demonstrated that during this period of sustained splenic egress, monocyte repletion was mediated by recruiting GMP from bone marrow which upon entering the red pulp, committed to MDP lineage. Splenic monocytopoesis, under these conditions, occurs with equal efficiency to that of bone marrow but is mediated by IL-1β during acute tissue injury, rather than through the M-CSF-dependent mechanism which mediates marrow monocytopoeisis.[52]

Hence, the spleen not only provides an environment for storage and survival of bone marrow derived monocytes but also acts to replenish its own monocyte stores when depleted.

1.4 Monocyte Fate

It is clear that monocytes are principally distributed through four locations within the organism, namely bone marrow, bloodstream, spleen and within the tissues. In the steady state, the spleen and blood contain the highest quantities of monocytes. While the role of the marrow and spleen in production and supply of monocytes is central to homeostasis and the circulation provides the obvious role of distribution, a key question relates to monocyte fate within the tissues.[4]

The traditional view that monocytes acted only as precursors to macrophages or DCs within the tissues has been replaced by a more complex model of monocyte biology. In fact the majority of monocytes do not in fact differentiate to either macrophages of DCs. Circulating monocytes act as pro-inflammatory effector cells in their own right. Once released from haemopoeitic tissues, namely bone marrow and spleen, Ly6C^{hi} monocytes have a short life-span (circa 20 hours) within the bloodstream or the tissues to which they migrate. Following completion of their effector function, approximately 80% are removed by apoptosis and efferocytosis. The remaining 20% either egress from the tissues to liver and lymphoid organs or alternatively they differentiate into macrophages or DCs.[52, 61, 62]

The more detailed elements of the monocyte lifecycle have not yet been confirmed in humans. However, phenotypic and functional investigation based upon the current classification system, suggests that human monocytes possess considerable

complexity above and beyond that of their mouse equivalents.[18, 45] Nonetheless functional homogeneity of human classical and non-classical subpopulations with mouse Ly6C^{hi} and Ly6C^{lo} subpopulations respectively, reasonably supports inference between mouse and human models of monocyte biology.

1.4.1 Trans-Endothelial Migration - The Role of Adhesion Molecules

Monocyte recruitment to injured tissue follows the general paradigm of leukocyte migration and involves the sequential processes of rolling, arrest, spreading and endothelial transmigration. Each step is regulated by intercellular interactions between surface expressed adhesion molecules on monocytes and endothelial cells.

The surface molecule P-Selectin Glycoprotein (PSGL) -1 is ubiquitously expressed across all monocyte subsets and binds to endothelial P and E-Selectin molecules. This interaction initiates the process of rolling. Monocyte arrest and spreading is mediated differently between monocyte subpopulations. Very Late Antigen (VLA) -4, highly expressed on classical and Ly6C^{hi} monocytes binds to endothelial intercellular adhesion molecule (ICAM) -1 and this mediates their arrest and spread over endothelium adjacent to areas of tissue injury. In circumstances of turbulent blood flow (shear flow), arrest of classical and Ly6C^{hi} monocyte subpopulations is additionally facilitated through CCR1, while CCR5 mediates monocyte spreading under these conditions.[4, 63, 64] In non-classical and Ly6C^{lo} monocytes, arrest is principally mediated through Lymphocyte Function-associated Antigen (LFA) -1 which binds to endothelial Vascular Cell Adhesion Molecule (VCAM) -1. The LFA-1 / VCAM-1 interaction produces a tight bond and results in slow rolling which is proposed to produce the vascular endothelial patrolling activity observed in non-classical and Ly6C^{lo} monocytes.[18, 39] The eventual process of trans-endothelial

migration is not fully understood but is thought to involve a series of interactions involving Platelet Endothelial Cell Adhesion (PECAM) -1 and CCL2 / CCR2.[65, 66]

The interaction between the ubiquitously expressed chemokine, CCL2 and its receptor, CCR2 facilitates transendothelial monocyte migration *via* two mechanisms. The chemotaxis model proposes a direct chemokine / receptor interaction, whereby a downstream signal initiates monocyte cytoskeletal rearrangement and consequent diapedesis.[4] The chemokinesis model on the other hand, proposes that CCL2 secreted under circumstances of tissue injury, initially binds with extracellular matrix glycosaminoglycans (GAGs) also released by injured cells. The CCL2 / GAG complexes then migrate to the endothelial surface and once exposed at the endothelial interface bind to monocyte CCR2 and draw arrested monocytes across the endothelial barrier.[32, 67]

1.4.3 Apoptosis & Efferocytosis versus Recirculation

Detailed *in vivo* studies of monocyte kinetics in mice have provided unique insights into monocyte fate, demonstrating that the majority of monocytes are removed from the tissues after providing an effector function without undergoing terminal differentiation. Overall, 80% of monocytes are removed from the tissues by apoptosis. Approximately 70% of infiltrating Ly6C^{hi} monocytes undergo cell death within 24 hours of entering the tissues and at any time-point between 2 - 6% of tissue-infiltrating Ly6C^{hi} monocytes are undergoing apoptosis. Cellular debris resultant to monocyte apoptosis is thought to be cleared through the action of resident M2 polarised macrophages and Ly6C^{lo} monocytes.[9, 38, 52, 68]

Of the remaining 20% of monocytes, not undergoing cell death, 5% - 13% of these

(i.e. 1-3% of al infiltrating monocytes) leave the tissues by egressing through the lymph and venous system to liver and spleen. Interestingly, egressing monocytes are not F4/80 positive which indicates that they have not differentiated prior to egress. Monocytes entering the liver are removed from the circulation by Kuppfer cells. The fate of those entering to the lymphatic system and spleen is not known although it is hypothesised that they interact with T cells in the lymph and undergo differentiation as "memory monocytes" in the spleen.[9, 52, 68] The chemokine messengers that govern monocyte egress are not known and are the subject of further investigation. Based on these studies, it can be concluded that only an approximate 10% of tissue-infiltrating monocytes undergo differentiation to macrophages and dendritic cells.

1.4.3 Terminal Differentiation of Monocytes to Macrophages

While macrophages and monocytes are phenotypically distinct cell types, it is widely accepted that monocytes act as precursor cells for macrophages.[4, 69, 70] To date, studies of human monocyte differentiation to macrophages have relied upon *in vitro* models of differentiation. Moreover, the majority of these studies have investigated differentiation of whole monocyte populations rather than individual subpopulations. While large studies have addressed transcriptional changes during the differentiation process, precise information about monocyte differentiation to macrophages is still lacking.

Macrophages reside in the peripheral tissues where they characteristically consume little oxygen and steadily produce modest amounts of proteins and cytokines. Resident macrophages are thought to enter the tissues as early as during embryogenesis and may last for the lifetime of the organism as a primary innate host immune response.[71] A large proportion of macrophages also derives from monocytes

and, following differentiation, is broadly categorised based on cytokine production as either M1 or M2.[9, 44] M1 macrophages represent classically activated, pro-inflammatory macrophages which are induced by monocyte exposure to LPS, IFNγ, TNFα or GM-CSF *in vitro*. They express higher amounts of IL-23 and IL-12 and low levels of IL-10.[9, 41, 72] M1 polarised macrophages readily produce reactive oxygen species and the pro-inflammatory cytokines IL-1β, TNFα and IL-6. However, monocytes are the principal producers of the pro-inflammatory cytokine response and produce these mediators at higher concentrations than their macrophage counterparts. M1 macrophages are also inducers of the Th1 lymphocyte response and mediate resistance against intracellular parasites, tumours and bacterial infection. So-called M2 macrophages represent an heterogenous family of macrophages which have broadly reparative functions. The M2 polarised subpopulation has been proposed to represent three subpopulations of tissue resident macrophages, which have primary functions in wound healing, as regulatory macrophages and in relation to modulating the host response to tumour cells.[71] Wound healing M2 macrophages are produced in the presence of IL-4, produced by Th2 lymphocytes. They upregulate production of arginase-1, they do not readily produce pro-inflammatory cytokines or ROS and they have poor antigen presenting ability. However, they have been shown to produce higher quantities of extracellular matrix components. Regulatory macrophages are produced by the Th2 lymphocyte response, TGFβ and are responsive to systemic glucocorticoids. They are responsible for dampening the immune response through the production of IL-10. In contrast to their M1 counterparts, all M2 macrophages commonly demonstrate low expression of IL-23 and IL-12 while expressing high quantities of IL-10. They are referred to as alternatively activated and generated *in vitro* by monocyte exposure to IL-4, IL-10, IL-13, immune complexes, glucocorticoids

and Vitamin D_3.[73, 74]

The transition from monocyte to macrophage occurs over a 16 to 168 hours time-course, under humoral influence. During normal homeostasis, the natural direction of monocyte to macrophage differentiation occurs towards an M2 phenotype under the predominant influence of M-CSF, which is normally present at high physiologic concentrations. Differentiation to the M1 phenotype occurs under pathological conditions and under the influence of GM-CSF, in addition to pro-inflammatory cytokines.[41, 71, 75] The maturation process from monocytes to macrophage involves complex regulation of a number of genes involved in diverse cellular processes. Global transcriptome analysis performed by Martinez *et al,* compared monocytes with differentiated macrophages and demonstrated a 90% variation of transcriptional profile between circulating whole monocyte fractions and M1 polarised macrophages. However, when monocytes and M2 polarised macrophage preparations were compared, relative transcriptomal expression levels between both populations differed only by 8%. In this study, macrophage differentiation was also shown to induce a global reduction in CCR2 and CX_3CR1 expression, the latter down-regulating at a slower rate. Interestingly M1 polarisation was associated with significant up-regulation of molecules associated with lipid transportation and atherosclerosis while M2 polarisation was associated with an up-regulation of molecules involved in cellular trafficking.[41]

1.5 Summary

Monocytes represent a diverse and responsive family of innate immune cells which demonstrate considerable heterogeneity. They have distinct functions as effector cells

and demonstrate a broad range of fates from apoptosis to egress to differentiation. In their differentiated form monocytes demonstrate diverse forms from macrophages to dendritic cells, and have polar functions from pro-inflammatory to anti-inflammatory. While this family of cells and their wide ranging functions are essential in the normal immune response, they may also become maladaptive in response to the toxic challenges presented by modern lifestyle, particularly in response to obesity. Chapter 2 addresses how obesity and excessive lipid metabolism affect the many facets of monocyte biology to result in two hallmark pathologies of the Western lifestyle namely type 2 diabetes and atherosclerosis.

Section 1: Introduction

Chapter 2

Monocytes and Obesity

2.0 Monocytes and Obesity

During the innate immune response to tissue injury, monocyte responses typically follow an initial granulocyte infiltration. Monocyte subpopulations then enter affected tissues in a sequential and phasic manner over an eight day period, following which they either die through apoptosis / efferocytosis, egress to draining lymph nodes or differentiate into macrophages and DCs.[4, 52, 76] Dysregulation of this pathway precipitates an abnormal response whereby chronic and ongoing monocyte infiltration continues after acute tissue injury has ceased.[76] Prolonged phases of monocyte infiltration, together with a consequent higher M1 macrophage population, propagate a low-grade, self-perpetuating, pro-inflammatory response, causing unchecked tissue destruction and fibrosis with consequent health implications.[30, 38, 76] Obesity represents one such morbid state in which dysregulated monocyte / macrophage activity plays a central role in mediating the related complications of type 2 diabetes, the metabolic syndrome and cardiovascular disease.[16]

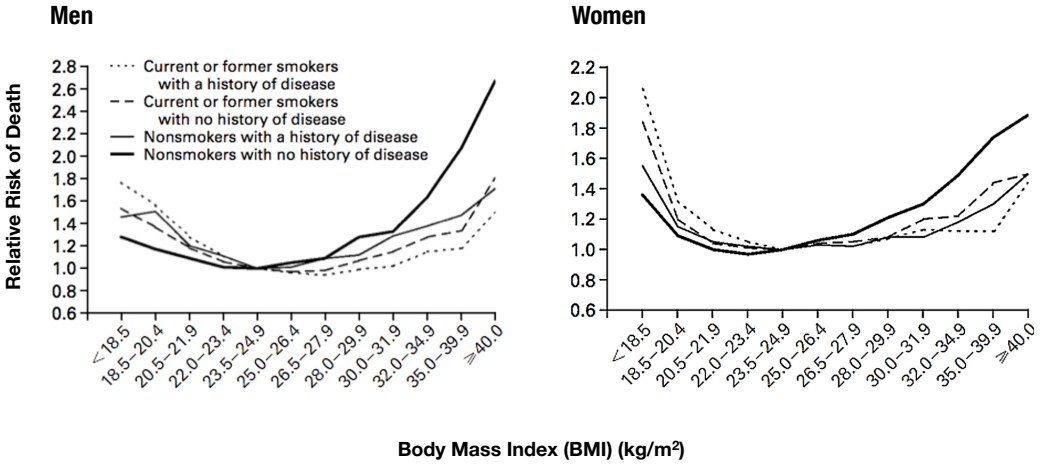
2.1 Epidemiology of Obesity

Obesity presents one of the greatest challenges to the practicing clinician, across all disciplines. It is a chronic disease of paradoxical overfed malnutrition and represents the single greatest modifiable risk factor for cardiovascular disease and the metabolic syndrome worldwide. Obesity is defined according to body mass index (BMI) [weight in kilograms / (height in metres)^2; (kg/m^2)] which is categorised as underweight (<18kg/m^2), normal (18 - 24.9kg/m^2), overweight (25 - 29.9kg/m^2), and obese (≥ 30kg/m^2). BMI criteria for overweight and obesity represent imposed cut-off values, based on theoretical points of inflection from a continuous curve of BMI versus

mortality.[77-79] The relationship curve relating to morbidities such as type 2 diabetes and cardiovascular disease lies to the left of the mortality curve. (Figure 2.1).[80, 81] The use of BMI in the classification of obesity has numerous limitations, including over-estimating the prevalence of obesity in tall individuals and the converse for short individuals. Measures of abdominal obesity or "fatness", such as waist circumference represent more accurate clinical techniques for estimation of obesity associated risks of morbidity and mortality.[78]

Observational studies demonstrate a rightward shift in the worldwide population

Figure 2.1: Mortality Curves for BMI in Men and Women



Multivariate analyses were performed on a cohort of 1,184,657 men and women to determine the relative risk of death, adjusted for disease and smoking status. Based on the relative risk of death, points of inflection at 25 and 30 kg/m2 were chosen as cut-off points to define overweight and obese body weights respectively.

Adapted from Calle et al, 1999

distribution of BMI over the past 30 years. Using the accepted definition, the incidence of obesity has risen to pandemic proportions over the past 20 years. In 2005, the World Health Organisation (WHO) estimated that approximately 1.7 billion adults worldwide were overweight (BMI > 25kg/m2) and 400 million were obese, forecasting an increase to 700 million obese individuals worldwide by 2015.[82, 83] Alarmingly, 10% of the world's children, under the age of 15 years, are obese (>95th percentile for age specific growth curves). In the U.S. the rate of childhood obesity has tripled in the period since 1980 and carries a current prevalence of 17-19%. Figures from the U.K. and Ireland show similar prevalence.[84] In the developing world the incidence of obesity has increased threefold over the past 20 years. Figures from developed countries estimate that between 24 and 35% of the general adult population are obese and a further 40% are overweight.[83] It is interesting that adults of normal BMI are now in the minority and that the prevalence of obesity in adult women is greater than that of adult males.[82, 83]

2.2 Aetiology & Pathogenesis of Obesity

Higher rates of obesity worldwide have coincided with the widespread adoption of the so-called Westernised lifestyle of lower physical activity and a diet of highly processed nutrient poor foods, rich in saturated fats and simple carbohydrates.

Within the general population, genetic susceptibility accounts for an estimated 40% variance in body mass. Genetic influence mainly produces an obesigenic effect through an interplay with environmental factors. Striking examples of the interaction between genetic susceptibility and environmental factors are seen in the epidemic of obesity and type 2 diabetes in Pima Indians living in Arizona, which occurred after

switching from the indigenous low fat diet (<15% total energy) to a diet rich in saturated fats (>50% total energy intake).[85-87] Similar statistics are demonstrated for urbanised Australian Aboriginals when compared to their outback dwelling, hunter-gatherer counterparts.[88]

2.2.1 Genetics

Numerous candidate genes for obesity have been identified. Most are associated with rare monogenic forms of obesity. Most of these mutations are associated with an hypothalamic orexigenic effect, hyperphagia and increased energy intake. Many genetic mutations occur as part of complex syndromes involving other endocrine abnormalities such as hypogonadism. Moreover monogenic obesity is not always associated with the same magnitude of risk for type 2 diabetes and cardiovascular disease as polygenic or idiopathic obesity, more common in the general population. Genetic candidates for monogenic obesity include mutations of the leptin gene or receptor, mutations in the pro-opiomelanocortin (POMC) gene and mutations in the melanocortin-4 receptor (MC4R).[89, 90]

In the general population, genetic susceptibility for obesity is likely to be conferred by polygenic mutations in numerous candidate genes, the modest contribution of each adding to a greater cumulative risk over time in association with environmental influences. One such candidate gene has been identified as the fat-mass and obesity associated gene (FTO).[91-93] Numerous different mutations of FTO contribute variously to the risk of obesity and type 2 diabetes. However, robust direct interactions between genetic factors and the immune system have not been demonstrated for genetic causes of obesity.

2.2.2 Energy Intake versus Energy Expenditure

Whether through genetic or environmental influences, obesity is underpinned by an imbalance between energy expenditure and energy intake. The rate of resting energy expenditure (REE) does not differ appreciably between lean and obese individuals and hence this balance is probably governed by physical activity rates and the magnitude of calorific intake.[94] The absolute calorific intake may play a secondary role to the constituent elements of dietary intake in the pathogenesis of cardiovascular and metabolic complications of obesity. For instance, a high dietary inoculum of certain carbohydrates and lipids, which enrich the Western diet, are toxic to adipose tissue, liver and vascular endothelium. Many lipids such as saturated long-chain free fatty acids (FFA) are not only obesigenic, but at high concentrations also appear to underlie the pathogenesis of obesity related cardiovascular disease and type 2 diabetes mellitus.[95-97] The lipotoxic effects of saturated long-chain FFA on individual elements of the innate immune system present an interaction common to the pathogenesis of insulin resistance, underlying the metabolic syndrome, and atherosclerosis underlying cardiovascular disease.[95]

2.3 Complications of Obesity

Obesity is associated with metabolic, cardiovascular, musculoskeletal, pulmonary and neoplastic complications.[78] This work focuses on the metabolic and cardiovascular complications of obesity and how these may be linked through immunometabolic interactions of obesity, an adverse metabolic profile and the innate immune system.

The metabolic syndrome is an epidemiological cluster of disease, characterised clinically by impaired glucose homeostasis, hypertriglyceridaemia, reduced high

density lipoprotein (HDL-C) and hypertension.[98] The incidence of the metabolic syndrome increases linearly with increasing BMI. Its pathogenesis is underpinned by insulin resistance with associated impaired glucose and lipid disposal. Insulin resistance associated with the metabolic syndrome and obesity results in a higher incidence of overt type 2 diabetes. The NHANES III study showed a linear increase in the prevalence of type 2 diabetes from 2% in men of BMI < 25 kg/m^2 to 13% in men of BMI >35 kg/m^2.[99] The nurses heart study showed similar statistics for women.[100] The metabolic syndrome is also linked with cardiovascular risk factors which include high serum levels of apolipoprotein (Apo) B, small dense low density lipoprotein (LDL) particles and high serum lipoprotein (LP) -(a) concentrations.[98, 101]

It is clear that the cardiovascular and metabolic complications of obesity share the characteristics of high circulating concentrations of lipids and cholesterol in addition to their carrier molecules, namely lipoproteins. Both conditions are also causally linked with persistent activation of the innate immune system through the action of monocytes and macrophages.[16, 76]

2.4 Cholesterol, Lipoproteins and the Innate Immune System

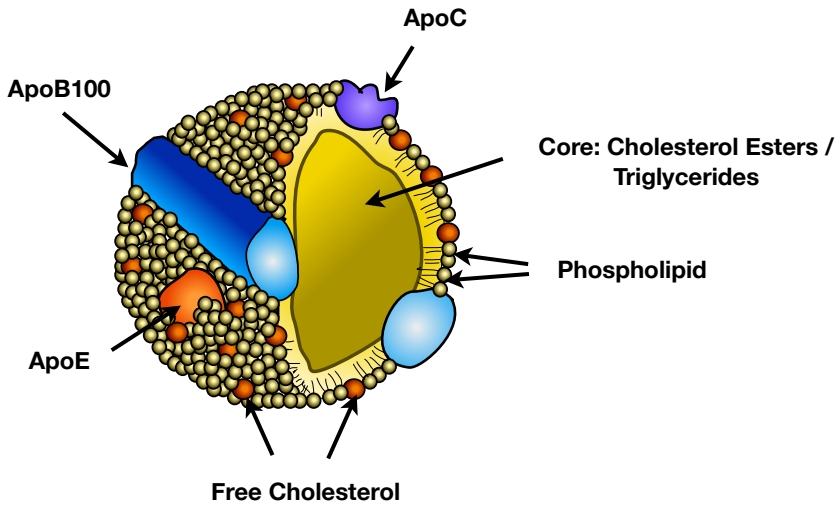
The innate immune system represents a primary biological interface between the environment and the organism. It is therefore not surprising that obesity, which is underpinned by environmental factors such as a high cholesterol diet, is associated with changes in the activation state of the innate immune system.[16] Increased inflammatory macrophages in omental and visceral fat are causally related to insulin resistance in obesity. Moreover the role of cellular and chemokine elements of innate immunity have long been associated with atherosclerosis and vascular disease.[14, 16, 76,

[102] Hence, the role of the monocyte / macrophage phagocytic system and its interaction with plasma lipoproteins may represent a common mechanism underlying the dual pathogeneses of insulin resistance and atherosclerosis.

2.4.1 Triglyceride, Cholesterol and Lipoprotein Metabolism

Plasma lipoproteins comprise neutral lipids, polar lipids and specialised proteins, namely, apolipoproteins (Apos) (Figure 2.2).

Figure 2.2: Lipoprotein Structure



Lipoproteins are specialised molecules adapted to trafficking lipids and cholesterol. They contain an outer lipophilic phospholipid layer which envelops the hydrophobic core, comprising triglycerides and cholesterol esters. Apolipoproteins on the lipoprotein surface act as ligands for native lipoprotein and scavenger receptors on hepatic cells, monocytes and adipocytes.

The major neutral lipids are cholesterol and triglyceride and these lie within the core of the lipoprotein. The neutral core is surrounded by a polar lipid coat, mainly containing phospholipids, phosphatidylcholine, sphingomyelin as well as free cholesterol. According to their density, maturity and apolipoprotein composition, circulating lipoproteins are classified as chylomicrons, very low density lipoproteins (VLDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL) (Table 2.1).

Table 2.1: Physical Characteristics of the Major Lipoprotein Subclasses

		Composition (%)					
		Core			Surface		
Class	**Density (g/mL)**	**TG**	**CE**	**FC**	**PL**	**Pro**	**Major Apolipoproteins**
Chylomicrons	<0.93	86	3	2	7	2	B48, E, AI, AII, AIV, C
VLDL	0.95 - 1.006	55	12	7	18	8	B100, CI, CII, CIII, E
LDL	1.019 - 1.063	6	42	8	22	22	B100
IDL	1.006 - 1.019	23	29	9	19	19	B100, E
HDL_2	1.063 - 1.125	5	17	5	33	40	AI, AII
HDL_3	1.125 - 1.210	3	13	4	25	55	AI, AII
LP(a)	1.055 - 1.085	3	33	9	22	33	B100, Apo(a)

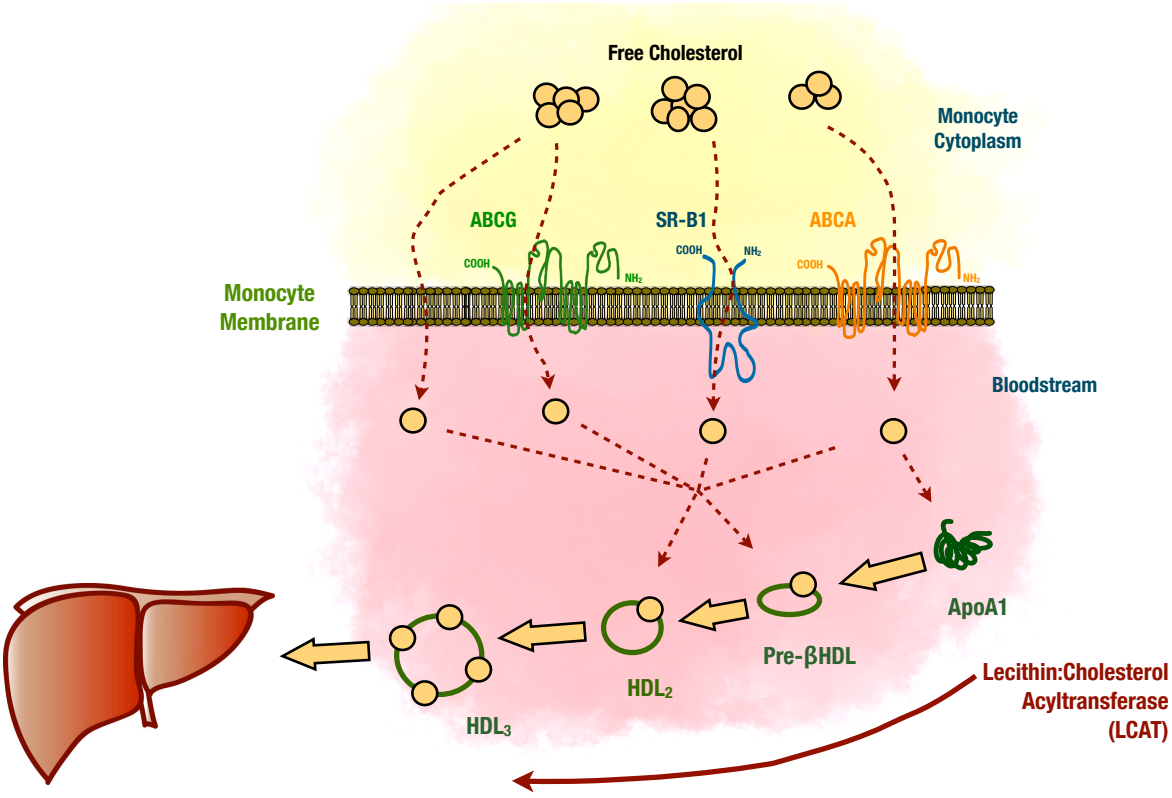
VLDL: Very Low Density Lipoprotein; **IDL:** Intermediate Density Lipoprotein;
LDL: Low Density Lipoprotein; **HDL:** High Density Lipoprotein;
Lp(a): Lipoprotein (a); **TG:** Triglycerides; **CE:** Cholesterol Esters; **FC:** Free Cholesterol;
PL: Phospholipids; **Pro:** Protein / Apolipoprotein.

Lipoproteins can be separated from plasma by density gradient ultra-centrifugation or according to their electrophoretic shift patterns. Based on the latter, HDL molecules are further classed as HDL_{1-3}, according to their maturity and their cholesterol ester content. LDL molecules have a second subclass known as small

dense LDL molecules, which have potentially higher atherogenicity in addition to a strong association with insulin resistance. Another lipoprotein, namely lipoprotein-(a) [Lp(a)] shares homology with thrombin and possesses both pro-thrombotic and atherogenic properties.[103, 104]

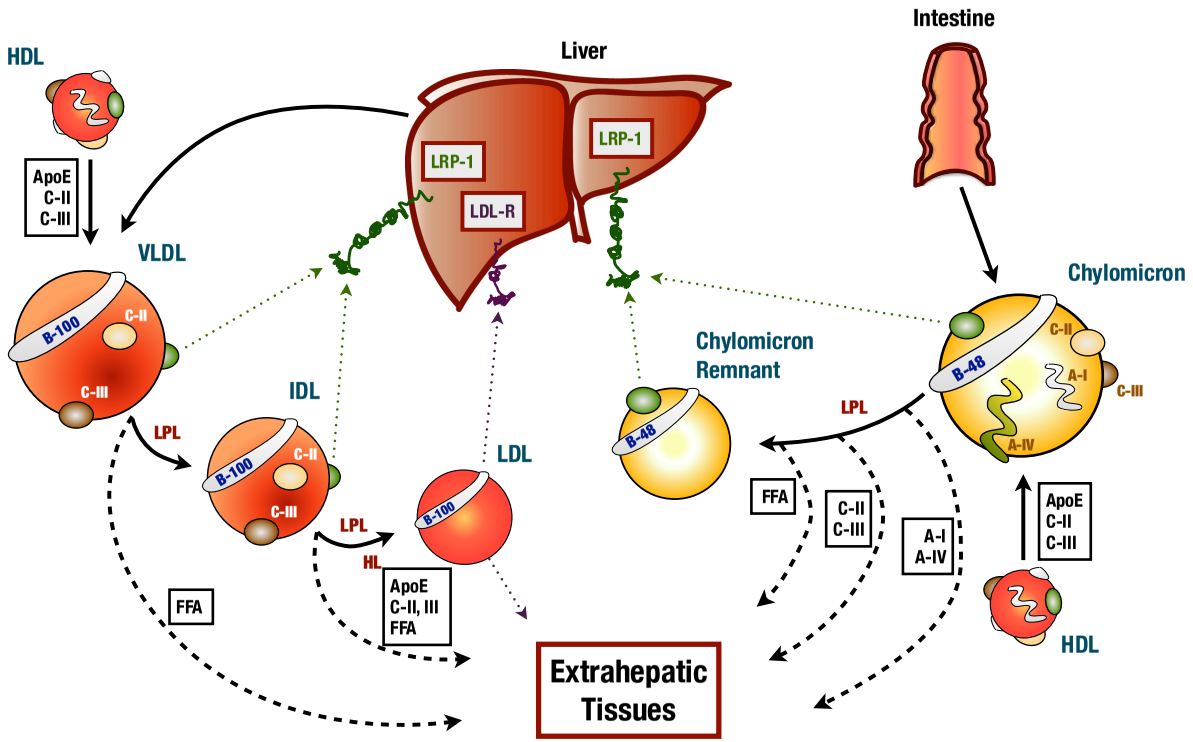
Chylomicrons, chylomicron remnants, VLDL and IDL are the main carriers of triglyceride within the circulation. Chylomicrons package triglycerides absorbed from the intestine and traffic these through the lymph to the liver. The entire chylomicron is taken up by hepatocytes and the constituent triglycerides are liberated through endosomal transport before being re-packaged as VLDL. VLDL is released into the circulation, where much of it is transported to adipose tissue depots throughout the organism, depositing its contents as structural or stored fat. LDL and HDL are cholesterol dominant lipoproteins. LDL is synthesised in the liver and carries cholesterol to the tissues, where it is used in the production of steroid hormones and cell membranes. HDL, on the other hand, transports cholesterol in the opposite direction. HDL accumulates excess cholesterol from peripheral tissues and transport this back to the liver for recycling or for excretion in the bile. This process is known as reverse cholesterol transport (RCT) (Figures 2.3). It has recently been shown that passage of HDL molecules through the lymphatic system is a key element responsible for effective RCT.[103, 105] The various lipoproteins and their role in the process of cholesterol transportation are represented in Figure 2.4.

Figure 2.3: Reverse Cholesterol Transport (RCT)



Free cholesterol is liberated from ApoB containing lipoproteins which have been taken up by cells of the monocyte / macrophage system. This can then pass to Apo A1, nascent HDL or HDL_2 molecules via efflux proteins in the monocyte membrane, namely ATP-binding cassette transporter (ABCA)-1, ATP-binding cassette sub-family G member (ABCG)-1 and Scavenger Receptor B1 (SR-B1).

Figure 2.4: Apolipoprotein Expression and Lipoprotein Transport



Apo: Apolipoprotein; **LPL**: Lipoprotein Lipase; **HL:** Hepatic Lipase; **HDL:** High Density Lipoprtein; **VLDL:** Very Low Density Lipoprotein; **LDL:** Low Density Lipoprotein; **IDL:** Intermediate Density Lipoprotein; **LRP-1:** Lipoprotein Related Peptide Type 1; **LDL-R:** LDL Receptor.

Cholesterol and fatty acids absorbed from the intestine are packaged as ApoB48 containing chylomicrons and are then transported to the liver. Chylomicrons and chylomicron remnants bind via ApoE to the LRP-1 receptor on hepatocytes. Intra-hepatic processing of triglycerides and cholesterol results in formation of nascent VLDL molecules, which are released into the circulation. Circulating VLDL matures within the circulating forming IDL and LDL. Under normal circumstances, these molecules deliver necessary fats and cholesterol to the periphery for structural and hormonal synthesis. Many are also recycled through the liver to bile acids.

Physiologic homeostasis of lipoproteins is difficult to maintain in the setting of dietary fat overload. Beyond their function in providing the essential building blocks for energy and hormonal metabolism, excessive production of VLDL, IDL and LDL occurs consequent to the high fat challenge of the Western Diet. In excess, these lipoproteins are converted to modified forms, such as oxidised (ox) LDL, which in turn play a central role in the pathogenesis of atherosclerosis, insulin resistance and the development of both cardiovascular disease and type 2 diabetes.[76, 106]

Lipoprotein function is defined by their surface protein composition. So-called apolipoproteins provide the interface through which the cholesterol and lipid components of the lipoprotein molecule interact with their environment.[103]

2.4.1.1 Apolipoproteins

Apolipoproteins are effector molecules on the lipoprotein surface which interact with lipoprotein receptors expressed on epithelial and endothelial surfaces of the gut, liver, adipose tissue.[107] Apolipoproteins mediate the interaction between lipoproteins and innate immune cells via native lipoprotein receptors and scavenger receptors, which are abundantly expressed on the surface of monocytes and macrophages.[76, 108-110] They also act as co-factors and facilitators in the enzymatic processing of lipids, fatty acids and cholesterol necessary for maturation of lipoprotein molecules. Apolipoproteins, expressed on the lipoprotein surface provide an interface whereby lipoproteins can deliver lipids and cholesterol between the various tissues throughout the organism. Apolipoproteins are classified A - E and each class is expressed in differing densities on lipoprotein molecules of varying origin, density and maturity. Three principal classes of apolipoprotein are involved in the interactions between innate immune cells and lipoproteins, namely ApoA, ApoB and ApoE.[107]

ApoB48 and ApoB100 make up the B class of apolipoproteins and play a central role in lipid and cholesterol uptake by the tissues and immune cells through interaction with native lipoprotein receptors. ApoB100 is found on VLDL, LDL, IDL and Lp(a) and represents the principal ligand for the LDL-Receptor (LDL-R) which is expressed in the liver, peripheral tissues and on immune cells.[107, 111] Synthesis of ApoB100 occurs within hepatocytes, where it is immediately packaged with triglycerides, within the smooth endoplasmic reticulum (ER) to form VLDL through a reaction requiring *Microsomal Triglyceride Transfer Protein* (MTP). Following synthesis, VLDL is released from hepatocytes into the *Space of Disse.* At this point exposure of the ApoB100 molecule to LDL-R is obscured by high surface concentrations of phosphatidyl-ethanolamine on the nascent lipoprotein, which protects against immediate VLDL reabsorption by hepatocytes. Within the circulation, VLDL molecules lose triglyceride and increase their relative cholesterol concentration to form LDL. ApoB100 is retained on the surface of LDL at a ratio of 1:1 (Table 2.1).[112]

The other major class B lipoprotein, ApoB48, is closely related to ApoB100. Both apolipoproteins are encoded by the same gene on chromosome 2. Through post-translational RNA editing, ApoB48 loses its C-terminus and retains only 48% the size of ApoB100. During this post-translational editing, ApoB48 loses its LDL-R binding domain. It does however act as a ligand for an ApoB48 receptor expressed on hepatocytes and on innate immune cells of the monocyte / macrophage family. ApoB48 is synthesised within the GI tract and packaged exclusively with chylomicrons. Uptake of chylomicrons and chylomicron remnants in non-hepatic and non-immune cells is reliant upon their surface expression of ApoE (see below) which binds with the native lipoprotein receptor, *Lipoprotein Related Peptide (LRP)-1* (Figure 2.4).[113]

At high serum concentrations, ApoB100 is associated with cardiovascular risk but this relationship is not entirely causal. Rather, this association reflects the expression of ApoB on LDL and VLDL.[110] The presence of normally functioning ApoB100 is essential for cholesterol homeostasis, whereby cholesterol molecules are delivered to the tissues as structural components or as precursors for steroid hormone synthesis. In the absence of normal ApoB100 - LDL-R binding, cholesterol uptake into the tissues occurs through the dysregulated process of lipoprotein scavenging which increases cardiovascular risk. *Familial Defective ApoB100* (FDB) involves a somatic mutation of ApoB100 that inhibits normal ApoB100 - LDL-R binding and is associated with higher circulating LDL cholesterol concentrations in addition to carrying a higher risk for cardiovascular disease.[107, 114]

Five classes of ApoA have been described. Of these, ApoA1 is the most abundant apolipoprotein on HDL and is the major regulator of RCT. Free cholesterol which effluxes from cells of the MDS through ATP-binding cassette transporter (ABC) A-1, ABCG-1 and scavenger receptor (SR)B-1 is delivered directly to ApoA1 and lipid poor HDL_2. Not only does ApoA1 accept free cholesterol to form HDL, but it also activates Leichtin Cholesterol Acetyl Transferase (LCAT), which catalyses the formation of cholesterol esters within the HDL molecule. This results in the formation of mature HDL.[107] ApoA1 is considered anti-atherogenic, although mutations which block the interactions between ApoA1 and either free cholesterol or LCAT, do not result in a higher incidence of atherogenesis. Hence it is thought that the protective effect conveyed by this molecule takes effect only in the setting of an atherosclerotic environment.[115]

ApoE is present on all circulating lipoproteins except for LDL. This apolipoprotein

interacts with both LDL-R and LRP-1. Due to the absence of ApoB100 on chylomicrons and chlyomicron remnants, ApoE represents the single-most important apolipoprotein for the clearance of intestinal derived lipoproteins, through its interaction with LRP-1. ApoE is present at high concentrations in the liver and brain. Interestingly, this molecule can be synthesised by macrophages, in addition to hepatocytes.[116]

2.4.2 Atherosclerosis, Vascular & Cardiovascular Disease

Atherosclerosis is the pathological process underlying vascular disease in obesity and the metabolic syndrome. The broad reaching clinical sequelae of atherosclerosis include MI, peripheral vascular disease, cerebrovascular disease, hypertension and chronic kidney disease (CKD). The principal cellular mediators of atherosclerosis are monocytes, which abnormally accumulate lipids and differentiate into so-called "foam cells" which have a macrophage-like phenotype.[76]

In humans, atherosclerosis involves a decades long expansion of the vascular intima and media, which occurs at sites of disturbed laminar flow, such as arterial branch-points.[96, 117] The key underlying process is that of subendothelial accumulation of apo B containing lipoproteins, namely chylomicrons, very low density lipoprotein (VLDL), LDL and Lipoprotein (a). Apo B containing lipoproteins initially enter the subendothelial space *de-novo* by an unknown mechanism. An inflammatory cascade is then initiated which involves two key elements; activation of the overlying endothelial cells and monocyte recruitment to the subendothelial space. The physiological role of this inflammatory response is to remove abnormal apo-B lipoprotein deposits and recycle excess cholesterol back to the liver. Chronic over-stimulation, driven by hypercholesterolaemia and lipoprotein oxidation, leads to

defective regulation of this system whereby, cholesterol containing monocytes are retained within the subendothelial space and contribute to the lesion.[26, 118] Consequently, a self-propagating process occurs which culminates in formation and enlargement of the atherosclerotic plaque.[76, 119]

2.4.2.1 Monocytes and Atherosclerosis

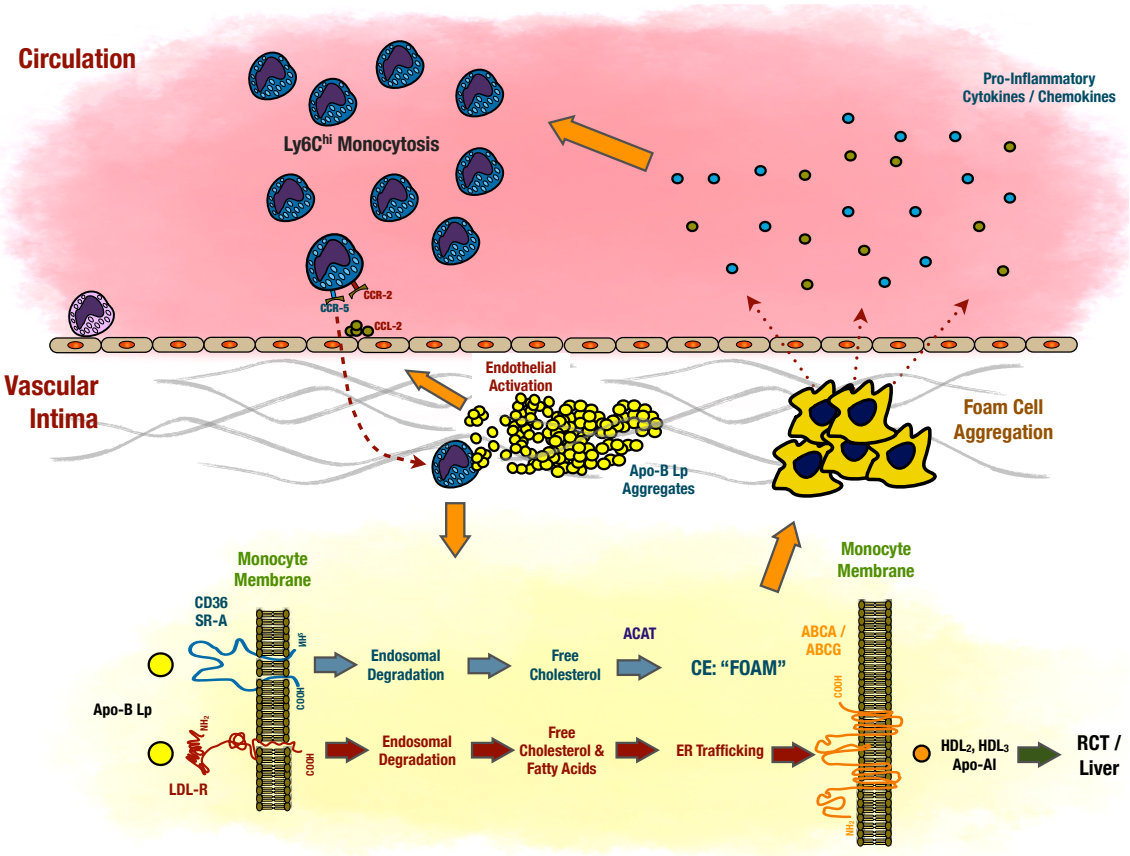
CCL-2 released by activated endothelium overlying apo-B lipoprotein deposits mediates chemokinesis of monocytes through CCR-2 and CCR-5 dependent mechanisms. Interactions between PSGL-1 / P-Selectin and VLA-4 / ICAM-1 then draw monocytes into the subendothelial space. Following migration, recruited monocytes remove aggregated apo-B lipoproteins largely through uptake *via* native lipoprotein receptors (LDL-R) and to a lesser extent through the action of scavenger receptors, including SR-A and CD36.[76] In mice, this process is mediated by $Ly6C^{hi}$ monocytes, found in the highest abundance of all cells within the plaque.[30] In humans, it is not known which monocyte subpopulation predominate in this process. The role of $Ly6C^{lo}$ monocytes in atherosclerosis is also unknown. As the atherosclerotic plaque matures, they may be recruited in higher numbers where they deposit matrix proteins and GAGs as part of the reparative process. However, GAG deposition by $Ly6C^{lo}$ monocytes in atherosclerosis may worsen the process by recruiting further apo-B containing lipoproteins to the subendothelial space.[38, 59]

Under normal conditions, intracellular processing of ingested lipoproteins results in their breakdown within endosomes. Liberated cholesterol esters are then further broken down to free cholesterol and fatty acids. Free cholesterol passes through the (ER), following which it is delivered by efflux proteins, namely ABCA-1 and ABCG-1 to Apo-A1 containing HDL particles. These in turn traffic back to the liver, where

the cholesterol is delivered for processing. During the normal monocyte response, delivery of cholesterol to the ER down-regulates LDL-R, in addition to switching off Sterol Regulatory Element-Binding Protein (SREBP)-mediated intracellular cholesterol formation.[115] However, high concentrations of cholesterol, in addition to native and modified apo-B lipoproteins in nascent atherosclerotic lesions overwhelm the normal processes of monocyte cholesterol uptake and efflux. During atherosclerosis, as LDL-R is down-regulated, scavenger receptors undergo compensatory up-regulation and become predominant in the process of cholesterol and lipoprotein uptake.[24, 76, 120] As a consequence, subendothelial monocytes undergo an unregulated and uncontrolled influx of cholesterol containing lipoproteins. The ER responds to the high influx of free cholesterol by up-regulating the activity of Acetyl-Coenzyme A acetyltransferase (ACAT), which re-esterifies free cholesterol. These newly formed cholesterol esters cannot efflux efficiently from the monocytes and become trapped intracellularly representing the "*foam*" in *foam cells* (Figure 2.4 & 2.5).[121]

Cholesterol loading of monocytes induces a compensatory response whereby, pro-inflammatory cytokines are released, as well as chemokines, such as CCL2. The cholesterol containing monocytes, which should egress from the plaque, then differentiate into tissue resident macrophage-like cells, so-called *foam cells*.[76, 110, 122] In fact, more monocytes are recruited to the site of the nascent plaque and this perpetuates the cycle of foam cell formation which drives plaque maturation. Biologically active *foam cells,* in addition to releasing high quantities of pro-inflammatory humoral mediators, also form the structural back-bone of the atherosclerotic plaque.[76]

Figure 2.5: Monocytes and Atherogenesis



Hypercholesterolaemia associated monocytosis (HAMS) occurs in the primed, pro-inflammatory setting of atherosclerosis. Sub-endothelial deposits of ApoB containing lipoproteins activate overlying endothelium, which then attracts circulating monocytes to the sub-intimal space by releasing Chemokine (C-C motif) ligand (CCL)-2, the ligand for C-C chemokine receptor (CCR)-2 and CCR-5 on pro-inflammatory monocyte membranes.

Under normal homeostatic conditions, monocytes remove excessive ApoB lipoproteins by absorbing them via a highly regulated LDL-R and unregulated scavenger receptor (CD36) mediated mechanism. Free cholesterol is liberated from the lioprotein molecules and delivered via efflux proteins to ApoA1 and HDL for reverse cholesterol transport to the liver. This system becomes overwhelmed by high concentrations of lipoproteins and the unregulated scavenger receptor mediated mechanism predominates. High intracellular cholesterol is esterified by ACAT and resultant cholesterol esters become trapped within monocytes, forming foam cells. Foam cells gather within the intima, forming the nascent atherosclerotic lesion and feeding forward via pro-inflammatory mediators to propagate the plaque.

The monocyte response to hypercholesterolaemia may occur prior to the deposition of apo-B lipoproteins in the nascent plaque. Blood-borne monocytes respond to hypercholesterolaemia by increasing their circulating numbers (Figure 2.5). Hypercholesterolaemia associated monocytosis (HAMS) is described in mice fed with high-fat diet (HFD) and in mouse models of atherosclerosis, namely LDL-R $^{-/-}$ and Apo-E $^{-/-}$. The process of HAMS was shown to be mediated by a Ly6C^{hi} monocyte expansion, rather than Ly6C^{lo}.[30, 123] It is not known whether spleen or bone marrow represents the principal sites from which these monocytes are recruited. In mouse models of MI, the spleen dominated as the main site from which monocytes were recruited and this has been shown to occur in an angiotensin II dependent manner. Recent data have shown that immediately following induced-MI in mice, release of high concentrations of angiotensin II, resultant to myocardial injury causes coronary plaques to enlarge and worsened ischaemia in the acute phase.[31, 55] Considerable data are also available regarding the contribution of circulating monocytosis in human atherosclerosis.

2.4.2.2 Human Studies of Monocytes and Atherosclerosis

In humans, monocytes also represent the driving cellular element of atherosclerosis. However, it has proven challenging to use this knowledge in the clinical setting. The total absolute monocyte count has not been shown to be of prognostic value for progression of atherosclerotic plaques or acute coronary events.[124] The relative contribution of each monocyte subpopulation to the pathogenesis of atherosclerosis is also unknown. Published data regarding monocyte counts, fractional distribution and subpopulation repertoire have not been consistent.[125, 126] In advance of the current human monocyte classification system, expansion of the CD16^{+} monocyte

fraction had been reported to be associated with atherosclerosis and coronary artery disease. Atherosclerosis and poor cardiovascular outcomes has been associated with higher circulating fractions of so-called $CD16^+$ monocytes in individuals with human immunodeficiency virus (HIV) infection. Additionally markers of monocyte migration and endothelial activation including CCL-2, P-Selectin, E-Selectin, ICAM-1 and VCAM-1. are increased in these individuals.[127, 128] Therefore there are significant data which associate higher circulating $CD16^+$ monocytes as a marker of atherosclerosis and cardiovascular disease. However it remains unclear how either of the CD16+ intermediate or non-classical monocyte subpopulations contribute to the observed associations with cardiovascular disease.

Studies applying the updated classification system have not reached consensus regarding which $CD16^+$ subpopulation is increased in response to vascular risk.[125] Univariate analyses have shown weak associations for non-classical monocyte numbers and proportions with parameters of vascular risk, such as triglycerides, low HDL cholesterol and intimal medial thickness (IMT). These associations however were not significant when multivariate analyses were used.[129] Longitudinal studies have shown an association between intermediate monocytosis, obesity and increased vascular risk. Followed up over five years, higher intermediate monocyte counts in patients with chronic kidney disease were associated with lower event free survival and higher mortality rates . Interestingly, higher angiotensin receptor expression on intermediate monocytes was associated with a poorer prognosis in the cohort of patients with CKD.[130] Higher intermediate monocyte counts on sequential sampling, in the aftermath of myocardial infarction were described as a predictor of in-stent restenosis following coronary stenting. In this case, the direct renin inhibitor aliskiren reduced the intermediate monocyte count and was associated with improved

prognosis. The latter observations concur with the mechanistic findings of Swirski and colleagues, relating to angiotensin II mediated splenic monocyte release worsening atherosclerotic plaque progression in response to acute myocardial infarction in mouse.[31, 55]

2.4.3 Type 2 Diabetes and Inflammation

Type 2 diabetes is a state of chronic hyperglycaemia, which is associated with obesity and the metabolic syndrome. The earliest step in the pathogenesis of type 2 diabetes involves insulin resistance, whereby insulin receptors in liver, muscle and fat couple less efficiently with downstream effectors.[131] However, hyperglycaemia does not immediately occur in response to insulin resistance alone. Normoglycaemia is initially maintained by a compensatory hyperinsulinaemia, mediated by pancreatic β-cells. This combination of insulin resistance and compensatory hyperinsulinaemia is seen as the hallmark feature of the metabolic syndrome. As the metabolic syndrome progresses, pancreatic β-cell failure occurs and compensatory hyperinsulinaemia can no longer be sustained. Hyperglycaemia and overt type 2 diabetes mellitus ensue. It is thought that the duration from the time of onset for insulin resistance to overt type 2 diabetes may take as long as 15 years.[132] During this time, many of those who have developed insulin resistance will also have manifested additional complications of the metabolic syndrome, such as hypertension and hyperlipidaemia.[132, 133]

Chronic activation of innate immunity has been causally associated with the development of insulin resistance, as well as β cell failure and progressive insulin deficiency. Hence, the role of innate immune cells, such as monocytes may present a unifying pathogenic influence for the metabolic syndrome, cardiovascular disease and type 2 diabetes mellitus.[16, 17, 102]

2.4.3.1 Adipose Tissue Inflammation

White adipose tissue plays a central role in energy balance and is under a constant state of flux and remodelling. Constituent adipocytes can undergo hypertrophy, hyperplasia or can atrophy to fulfil their role in energy homeostasis. Adipose tissue remodelling refers to the overall process whereby white adipose tissue adapts to changes in nutrient and energy intake. The remodelling process involves recruitment of adipocyte precursors, so-called pre-adipocytes, which gradually accumulate triglycerides and cholesterol while differentiating into mature adipocytes. The expansion of adipocytes is accompanied by angiogenesis, which prevents hypoxia providing a drainage system for removal of toxic lipids and metabolites.[134]

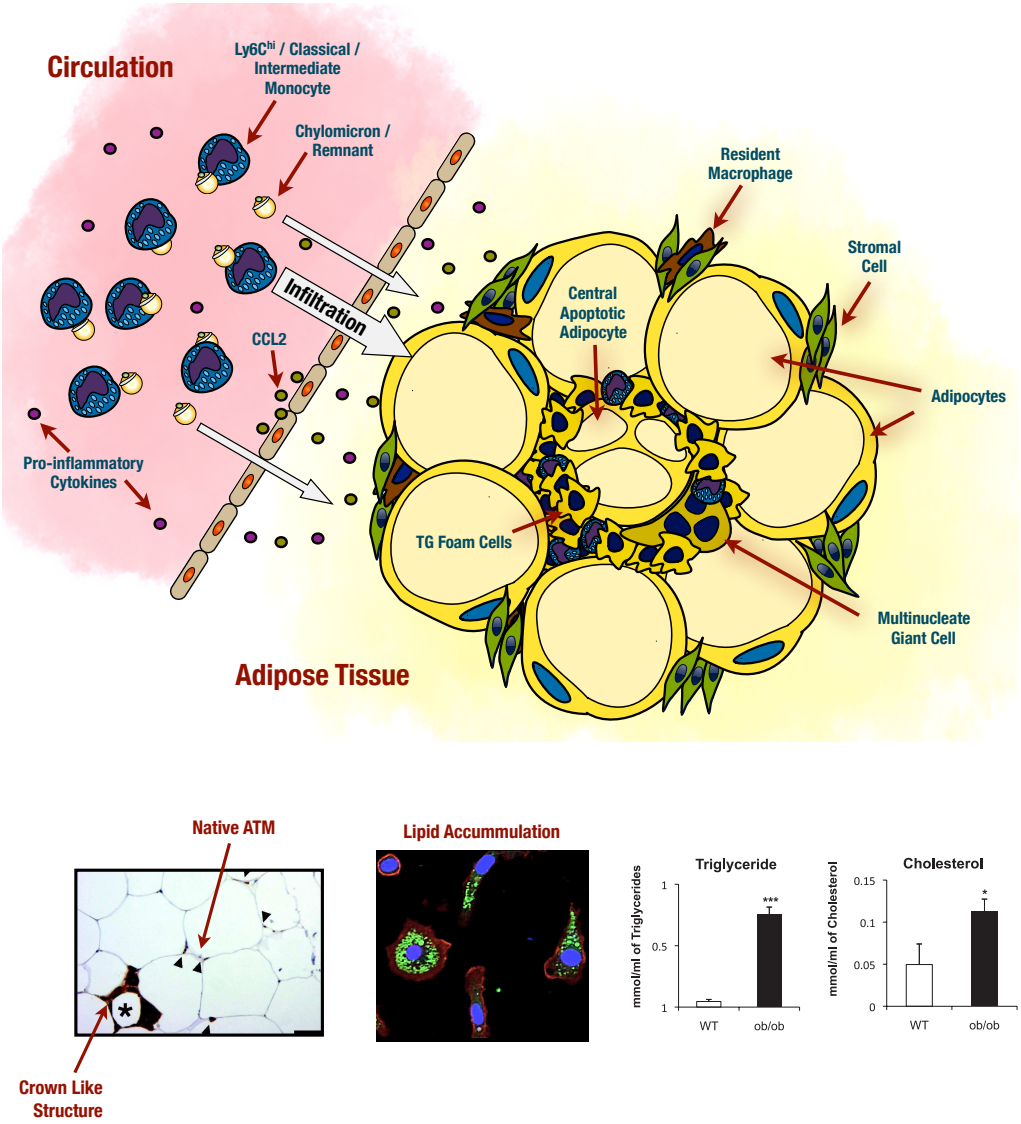
Obesity pathologically accelerates adipose tissue remodelling whereby pre-adipocyte recruitment is not sufficient to manage the calorific overload. Already mature adipocytes attempt to compensate by accumulating the excessive lipid. As a result, adipocyte hypertrophy occurs rather than adipocyte hyperplasia. Adipocyte hypertrophy initially acts as a protective mechanism against the lipotoxic effects of excessive lipid exposure.[135] However, within the sustained environment of over-nutrition, adipocyte hypertrophy is accompanied by an over-production of extracellular matrix and angiogenesis is reduced.[134] Eventually, through the combination of hypoxia, adipocyte overload and reduced clearance of extracellular toxic metabolites, adipocyte integrity is compromised. The ensuing widespread adipocyte apoptosis is accompanied by an innate immune cell infiltrate in an effort to clear the resultant cellular and lipid debris. The net result is that of a chronically inflamed, hypoxic, fibrotic environment which is worsened by the sequential pathological maladaptation of mechanisms designed to protect the organism from

lipotoxicity (Figure 2.6)[135, 136].

The onset and progression of diet induced obesity produces a number of pathological effects within the adipose tissue: (i) with increasing BMI, adipocyte size increases, (ii) with increasing size, adipocyte apoptosis increases, (iii) with increasing adipocyte apoptosis, the monocyte/macrophage population within the adipose tissue expands and becomes more pro-inflammatory in phenotype.[14, 16, 137] The net effect is known as the "*Obesity Driven Phenotypic Switch in Macrophage Polarisation*" whereby a prominent population of M2 polarised resident adipose tissue macrophages (ATM) is replaced by a pro-inflammatory infiltrate of M1 polarised ATMs. These so-called M1 polarised ATMs aggregate in distinct formations which surround apoptotic adipocytes, termed *Crown-Like Structures* (CLS) (Figure 2.6).[16, 138-140]

Two mechanisms have been proposed to explain the phenotypic switch in ATMs. The first hypothesis proposes a mechanism through which usually quiescent resident ATMs, respond to adipocyte apoptosis by "switching" to a so-called M1 phenotype and migrating to areas of adipocyte death, where they organise as CLSs.[138, 139] A number of studies have demonstrated the proliferative capacity of tissue macrophages as part of the innate immune response, independent of monocyte infiltration.[71, 141, 142] Therefore the hypothesis of a phenotypic switch involving local proliferation of tissue macrophages is plausible and likely. A second hypothesis, proposes CCR-2 dependent infiltration of circulating monocytes as the primary source of M1 CLS-ATMs. This has been supported by data showing that (i) $CCR2^{-/-}$ mice are resistant to the obesity mediated phenotypic switch in ATMs [14, 60] and (ii) CLS-ATMs demonstrate high surface expression of CCR2 and low expression of CX_3CR1, which reflects their monocyte origin.[14, 73, 143] Numerous other chemokines, including CXCL14, MIP-1α,

Figure 2.6: Adipose Crown Like Structures



(A) Pro-inflammatory, triglyceride-laden cells of monocyte / macrophage lineage surround apoptotic adipocytes to form crown structures. Many monocyte / macrophages coalesce, forming large multinucleate cells. The resulting pro-inflammatory microenvironment, whereby high concentrations of chemokines (CCL-2) and adipocytokines are released stimulates further infiltration of pro-inflammatory monocytes.

(B) Light micrograph demonstrating the microscopic appearance of crown like structures in comparison to resident macrophages. Bodipy staining for lipid content shows the high lipid content of crown like structure foam cells, which are predominantly triglycerides on lipidomic analysis.

Micrographs adapted from Harman Boehm et al, Prieur et al.

MCP-2 and RANTES have also been associated with ATM population expansion in response to obesity which also supports a significant contribution from an infiltrative process involving circulating monocytes entering the adipose tissue.[17, 133, 144, 145]

In keeping with their M1 phenotype, several studies have shown that ATMs in CLSs have higher expression of CD86, CD14, CD11c and iNOS; whereas resident ATMs show higher expression of markers such as CD206 and Arginase I.[17, 144, 146] Crown like structure ATMs also produce higher quantities of the pro-inflammatory cytokines IL-6 and IL-1β. However, the true driver for the formation of CLS is likely to resemble the process of atherogenesis.[17]

The energy burden of obesity is stored predominantly as triglyceride within white adipose tissue. White adipocytes contain a single lipid droplet which occupies >98% of the cell. The main byproduct of adipocyte apoptosis is that of the residual, large, inert lipid droplet which neither contains the protein coat associated with the intracellular droplet, nor the necessary lipases to mediate hydrolysis of the constituent triglycerides.[95, 139] The homeostatic mechanism for clearance of these toxic lipid products naturally involves cells of the monocyte / macrophage system. In support of this, several studies have shown that CLS-ATMs contain high quantities of intracellular lipids, predominantly triglycerides. However, normal homeostasis also dictates that infiltrating ATMs should enzymatically process and transfer phagocytosed triglycerides appropriately to lipoproteins for further metabolism, rather than accumulating intracellular lipids.[139] Furthermore, rather than residing within the adipose tissue, these cells should more appropriately egress once they have completed the process of removing excessive apoptotic debris. Hence, the formation of sequestered, syncitial, lipid-laden CLS-ATMs represents an abnormal process,

similar to foam cell formation within the vascular media (Figure 2.6).[147]

Studies examining the specific lipid content of CLS-ATMs have shown high quantities of cholesterol but a predominant triglyceride composition. The two most abundant triglyceride species, triglyceride (52:2) and triglyceride (54:3), are found at the highest intracellular concentrations in response to obesity, whereas levels of polyunsaturated triglyceride (54:6), is decreased by 40%. The triglyceride rich, intracellular content of CLS-ATMs also demonstrates high concentrations of ApoB48 and ApoB100, which reflect chylomicron, VLDL and LDL uptake. Transcriptional regulation of elements of lipid scavenging including CD36 and FATP1 are up-regulated in CLS-ATMs in rodent models of obesity, while expression of mRNA for ApoE is reduced. Overall there is an up-regulation of unchecked lipoprotein scavenging, with a simultaneous down-regulation of efflux mechanisms necessary for effective re-packaging of intracellular FFA from CLS-ATMs.[139] These molecular changes, mimic the changes of atherogenesis, whereby the normal process of monocyte lipid clearance becomes overwhelmed and leads to the pathological formation of adipose tissue foam cells. Hence CLS development within the visceral adipose tissue may well represent a form of atherogenesis occurring in the omental adipose tissue. Whereas atherogenesis of the vasculature leads to cardiovascular disease, plaque rupture and myocardial infarction, atherogenesis of the omentum manifests as insulin resistance and type 2 diabetes.[95, 147]

2.4.3.2 Chronic Inflammation and Insulin Homeostasis

Chronic, low-grade inflammation provides a molecular pathway that links the environment and obesity with insulin resistance, and in turn links insulin resistance with its sequelae, namely type 2 diabetes and atherosclerosis. Innate immune cells are

anatomically juxtaposed with metabolically active cells in the liver, gut and adipose tissue.[133] Hence these cells are poised to react to the physiologic responses involved in nutrient absorption, assimilation and processing.

Since the link between insulin resistance and inflammation was proposed in 2003 by Weisberg et al, numerous studies have investigated the molecular links between inflammation and insulin resistance.[16] These studies have invariably highlighted the up-regulation of two molecular pathways which relate insulin resistance to inflammation, namely the IKKB /NFκB and the JNK pathways. Both pathways are activated through respective upstream receptor dependent and receptor independent mechanisms. Receptor dependent mechanisms include (i) the direct activation of cytokine receptors by their respective pro-inflammatory ligands; (ii) activation of pattern recognition receptors / TLRs by saturated FFA or by high circulating concentrations of bacterial LPS.[17, 97, 148, 149] The main receptor independent mechanisms are mediated through the effects of lipid accumulation within innate immune cells. Unregulated cellular accumulation of lipid species contributes directly to lipotoxicity, causing oxidative and ER stress, which culminates in the production of reactive oxygen species (ROS) and the unfolded protein response (UPR). Both ROS production and the UPR directly activate the IKKB /NFκB and JNK pathways. The consequent production of pro-inflammatory cytokines results in a feed-forward loop, which under persistent stimulation, leads to chronic activation of these pathways.[95, 96] Intracellular lipid excess also activates NFκB directly via stimulation of protein kinase C (PKC) isoforms, while an excess of ceramides has been shown to directly activate both IKKB /NFκB and JNK pathways. (Figure 2.7).[133, 137]

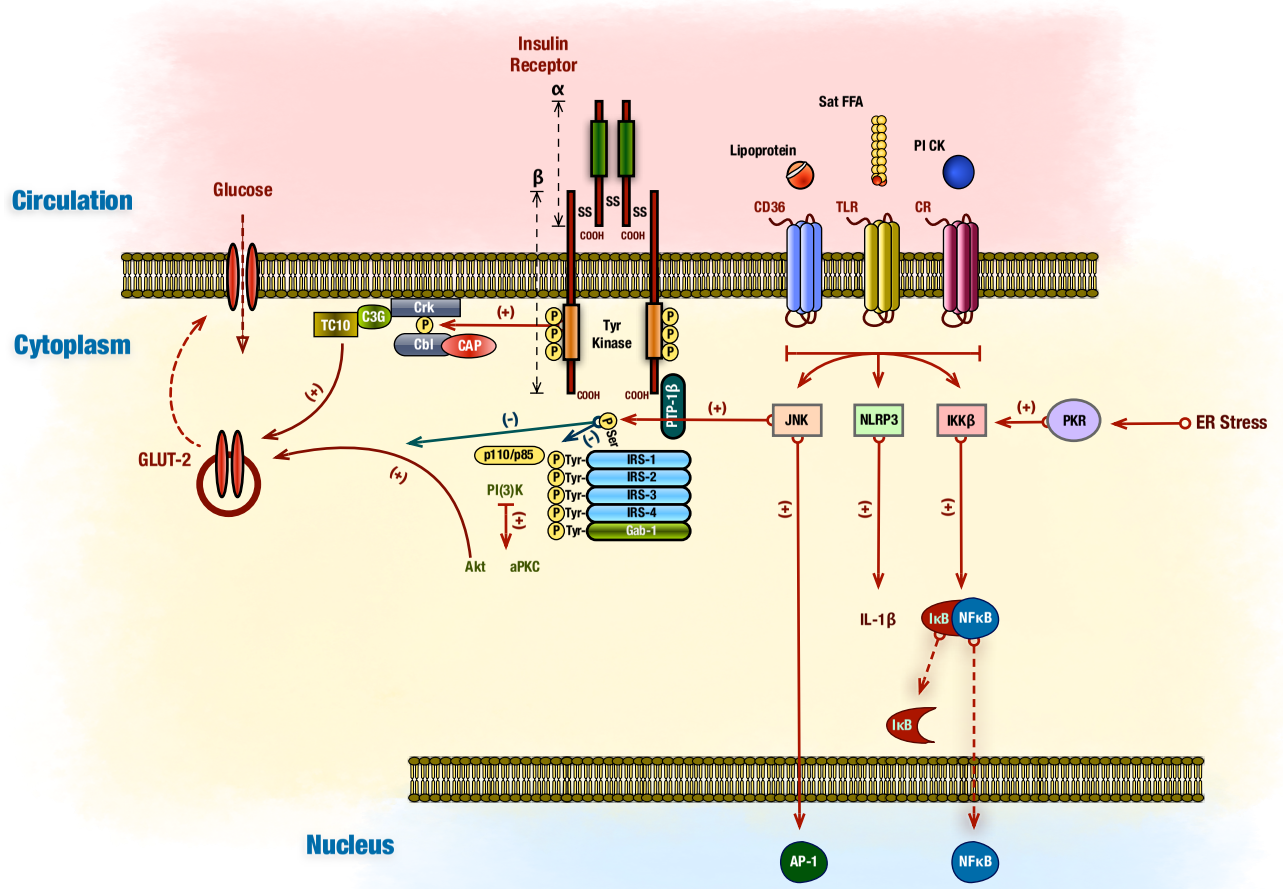
JNK and IKKB /NFκB have diverse roles in intracellular signalling. Both pathways

regulate transcription as well influencing intracellular signalling cascades through phosphorylation of amino acid residues. JNK is a stress kinase and can produce insulin resistance through a direct effect on the insulin receptor.[17] Normal insulin signalling involves sequential phosphorylation of a series of insulin receptor substrates (IRS), namely IRS 1-4. The IRS phosphorylation cascade is catalysed by tyrosine kinases. JNK inhibits the action of tyrosine kinases by abnormally phosphorylating serine / threonine residues on IRS-1 which halts progression of the phosphorylation cascade and thereby inhibits insulin signalling.[17, 133] Insulin resistance ensues due to fewer effective functioning insulin receptor units. While JNK directly interferes with insulin signalling, NFκB causes insulin resistance through transcriptional regulation. During transcriptional quiescence, NFκB is prevented from translocating to the nucleus by its interaction with the un-phosphorylated molecule IκB, to which it remains bound within the cytoplasm. IκB is the physiological substrate for the kinase, IKKB. Once phosphorylated, IκB liberates NFκB, which translocates to the nucleus where it promotes the production of a host of receptors and humoral mediators, associated with insulin resistance and atherosclerosis.[17, 133, 137]

Figure 2.7: Insulin Signaling and Inflammation

Lipoproteins, saturated free fatty acids and pro-inflammatory cytokines act upon their respective receptors to induce insulin resistance through the NFκB / IKKB pathway. Endoplasmic reticular stress due to high intracellular lipids also directly activates this pathway.

Insulin receptor activation liberates the glucose channel, GLUT-2, to the cell membrane thereby allowing intracellular passage of glucose. This is mediated predominantly through the Crk and the Akt pathways, both of which are dependent upon effective phosphorylation of the IRS. The Akt element of this pathway is inhibited by activation of the IKKB / NFKB pathway, resulting in insulin resistance.



FFA: Free Fatty Acid; PI CK: Pro-inflammatory cytokine
TLR: Toll Like Receptor; CR: Cytokine Receptor;
JNK: cJun NH_2-terminal kinase; **AP-1:** Activator Protein 1 **NFKκB:** Nuclear Factor κ B
PKR: Double-stranded RNA-dependent protein kinase **NLRP3:** NOD-like receptor family, pyrin domain containing 3
IKKB: IκB Kinase β; **IRS:** Insulin Receptor Substrate; **PTP-1β:** protein-tyrosine phosphatase 1B;
PI(3)K: Phosphoinositide 3-kinase; **aPKC:** Atypical Protein Kinase C; **GLUT:** Glucose Transporter.

2.5 Summary

The Westernised, hypercalorific diet, rich in dietary saturated fats produces monocyte activation, resulting in a predominantly pro-inflammatory response which culminates in a feed forward, self-perpetuating cycle. The consequences are those of insulin resistance and type 2 diabetes mellitus, and atherosclerosis, causing cardiovascular and peripheral vascular disease.

Results of large scale randomised controlled clinical trials analysing the effects of tight glycaemic control in type 2 diabetes have not shown an improvement in macrovascular outcomes.[150, 151] These data challenge the traditional paradigm whereby poor glycaemic control is considered as the causative influence for macrovascular complications. Perhaps these two pathologies share a common pathogenesis?

2.6 Knowledge Gaps at the time of Project Design

At the time of study design, the following knowledge gaps existed in the published literature.

(i) There were no data describing the distribution of the individual monocyte subpopulations in relation to obesity, the metabolic syndrome or type 2 diabetes mellitus. Moreover data which described monocyte repertoire in human disease largely related to proportional distribution of the individual subpopulations, without providing demonstrating absolute monocyte counts.

(ii) There were no robust, multivariate data describing whether or not associations existed between parameters of human monocyte repertoire and metabolic risk, particularly in relation to individual monocyte subpopulations.

(iii) The finer phenotypic and functional details relating to intermediate and non-classical monocytes had not been described. It was also not known which of these shared phenotypic homology with each of the $Ly6C^{hi}$ and $Ly6C^{lo}$ monocyte subpopulations in mice, on which inference to human cardiovascular and metabolic disease is based.

(iv) There were no data relating to the uptake of lipoproteins in human monocyte subpopulations in the setting of obesity and metabolic disease.

2.7 Overarching Hypothesis

In metabolically unhealthy obese individuals, alterations in the repertoire of specific subpopulations of circulating monocytes are associated to higher metabolic and cardiovascular risk, which is mediated through their interactions with circulating lipoproteins.

2.8 Objectives

The experimental work carried out for this thesis was designed to investigate human monocyte repertoire across a cohort of obese individuals, with and without a diagnosis of type 2 diabetes mellitus. The objectives of these experiments were as follows:

(i) To develop robust and standardised assays for the investigation of circulating counts, subpopulation distribution and phenotype of human monocytes isolated from peripheral blood.

(ii) To investigate monocyte characteristics in obese individuals with and without a diagnosis of type 2 diabetes.

(iii) To characterise the potential contribution of monocyte subpopulations to the pathogeneses of insulin resistance and cardiovascular disease by:

  (a) analysing monocyte repertoire in relation to clinically established parameters by measuring cardiovascular risk and assessing for diagnostic features of the metabolic syndrome;
  (b) establishing monocyte phenotype across each subpopulation;
  (c) investigating the response of individual monocyte subpopulations when

exposed to high concentrations of lipoprotein molecules.

Section 2: Methods

Chapter 3

Methodology & Statistical Analysis

3.0 Methodology and Statistical Analysis

3.1 Study Enrolment, Data and Sample Collection

Obese study subjects were enrolled under informed consent from general diabetes and weight management outpatient clinics at Galway University Hospital (GUH). Samples from healthy volunteers were collected at GUH and at the adjacent campus of the National University of Ireland, Galway. Sample collection took place between March 2010 and June 2012 following approval of the protocol by the GUH Human Research Ethics Committee.

Clinical, demographic and laboratory data for each subject were collected prospectively and entered into a secure, password-protected database (Filemaker Pro® Advanced, Version 12; Santa Clara, CA, USA). At the time of study enrollment, body mass index (BMI, calculated from weight and height measurement) and blood pressure (average of two readings) were recorded and blood was drawn for measurement of glycosylated hemoglobin (HbA1c), fasting serum glucose, triglycerides, high density lipoprotein (HDL) and calculated low density lipoprotein (LDL) by standard clinical laboratory methodology. For experimental studies, an additional 8 - 30mL of blood was drawn into Vacutainer® ethylene-diamine-tetra-acetic acid (EDTA)-containing tubes (BD Medical Supplies, Crawley, U.K.) which were maintained at room temperature for a maximum of 4 hours before processing at the research laboratory.

Study subjects were grouped as follows: Control: BMI < 25 kg/m2 with no history of diabetes mellitus (DM); Obese (OB): BMI ≥ 30 kg/m2, with no history of DM; Obese Diabetic (OBDIAB): BMI ≥ 30 kg/m2, with a documented history of type 2 diabetes

diagnosed according to American Diabetes Association (ADA) Guidelines (fasting glucose ≥ 7.0 mmol/L (greater than two occasions), or 2 hour post 75g oral glucose load (2h pp) ≥ 11.1 mmol/L, or HbA1c > 6.5%).[152] Exclusion criteria were as follows: current smoker; history of inflammatory, hematologic, oncologic, infectious or chronic kidney disease (> Stage 1); current foot or leg ulcers; current or prior regular use of systemic glucocorticoid, biologic agents, anti-inflammatory / disease modifying anti-rheumatic drugs (DMARD) or chemotherapeutic agents.

3.1.2 Participant Demographic Details

128 subjects were enrolled: 33 Control (15 female), 42 OB (24 female) and 53 OBDIAB (22 female). In study participants who are obese without diabetes, our data show a prevalence of the metabolic syndrome of 56% for women and 43% for men. Relevant demographic and metabolic indices for the 3 groups are summarised in Table 4.1 and Figure 4.1. Amongst the OBDIAB group 80% were receiving statins, 74% were receiving either an angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) and 70% were receiving aspirin. The proportions of OBDIAB receiving anti-hyperglycaemic therapy were as follows: 74% on metformin, 24% sulphonylureas, 34% insulin, 8% PPARγ agonists, 14% a GLP-1 receptor agonist and 14% a DPP IV antagonist. None of the Control or OB group was receiving anti-hyperglycaemic or secondary prevention therapy (i.e. aspirin therapy, statin therapy or angiotensin II inhibition). OBDIAB subjects were older and had higher HbA1c values compared both to control and OB study participants while additionally having higher triglyceride and lower HDL levels when compared to the Control group. OB study participants also had higher triglycerides and LDL and lower HDL when compared to Controls (Table 3.1; Figure 3.1).

Table 3.1: Demographic Details and Clinical Characteristics

	Participant Group		
	Control	*OB*	*OBDIAB*
Number	**33**	**42**	**53**
Age (years)	**34.7**	**39.0**	**55.8**** ‡
p(1,2)<0.0001; F=33.42	(27.6, 41.8)	(34.2, 43.9)	(53.0, 58.6)
Mean Difference v Control	-	**4.3 (-4.3, 13.1)**	**21.1 (13.2, 29.0)**
Mean Difference v OB	-	-	**16.8 (10.5, 23.1)**
Body Mass Index (kg/m²)	**21.93**	**46.93****	**35.44**** ‡
p(1,2)<0.0001; F=33.89	(20.25, 23.62)	(41.18, 52.68)	(33.44, 37.43)
Mean Difference v Control	-	**25.0 (17.62, 32.38)**	**13.5 (7.01, 20.0)**
Mean Difference v OB	-	-	**11.5 (6.31, 16.68)**
Systolic Blood Pressure (mmHg)	**124.7**	**123.3**	**136.7**
p(1,2)=0.03; F=3.60	(122.5, 126.8)	(122.5, 126.8)	(129.8, 143.6)
Mean Difference v Control	-	**-1.3 (-19.6, 16.9)**	**12.01 (-2.7, 26.7)**
Mean Difference v OB	-	-	**13.24 (-1.3, 28.0)**
Low Density Lipoprotein (mmol/L)	**2.18**	**3.01***	**2.37 †**
p(1,2)=0.02; F=4.0	(1.81, 2.54)	(2.54, 3.47)	(2.08, 2.67)
Mean Difference v Control	-	**0.8 (0.05, 1.72)**	**0.2 (-0.61, 1.01)**
Mean Difference v OB	-	-	**0.63 (0.04, 1.23)**
High Density Lipoprotein (mmol/L)	**2.12**	**1.24 ****	**1.13****
p(1,2)<0.0001; F=57.65	(1.91, 2.34)	(1.13, 1.36)	(1.04, 1.21)
Mean Difference v Control	-	**-0.88 (-1.13, -0.63)**	**-0.99 (-1.21, -0.77)**
Mean Difference v OB	-	-	**0.11 (-0.06, 0.29)**
Triglycerides (mmol/L)	**0.74**	**1.60**	**2.05****
p(1,2)=0.0009; F=7.76	(0.63, 0.85)	(1.23, 1.99)	(1.73, 2.37)
Mean Difference v Control	-	**0.85 (-0.04, 1.75)**	**1.3 (0.50, 2.12)**
Mean Difference v OB	-	-	**0.44 (-0.15, 1.04)**
HbA1c (%)	**4.8**	**5.2**	**7.9**** ‡
p(1,2)<0.0001; F=32.75	(4.6, 5.0)	(5.1, 5.4)	(7.4, 8.5)
Mean Difference v Control	-	**0.6 (-0.7, 1.9)**	**3.1 (1.8, 4.3)**
Mean Difference v OB	-	-	**2.5 (1.6, 3.3)**

Control: Healthy Individuals, BMI < 25kg/m^2.

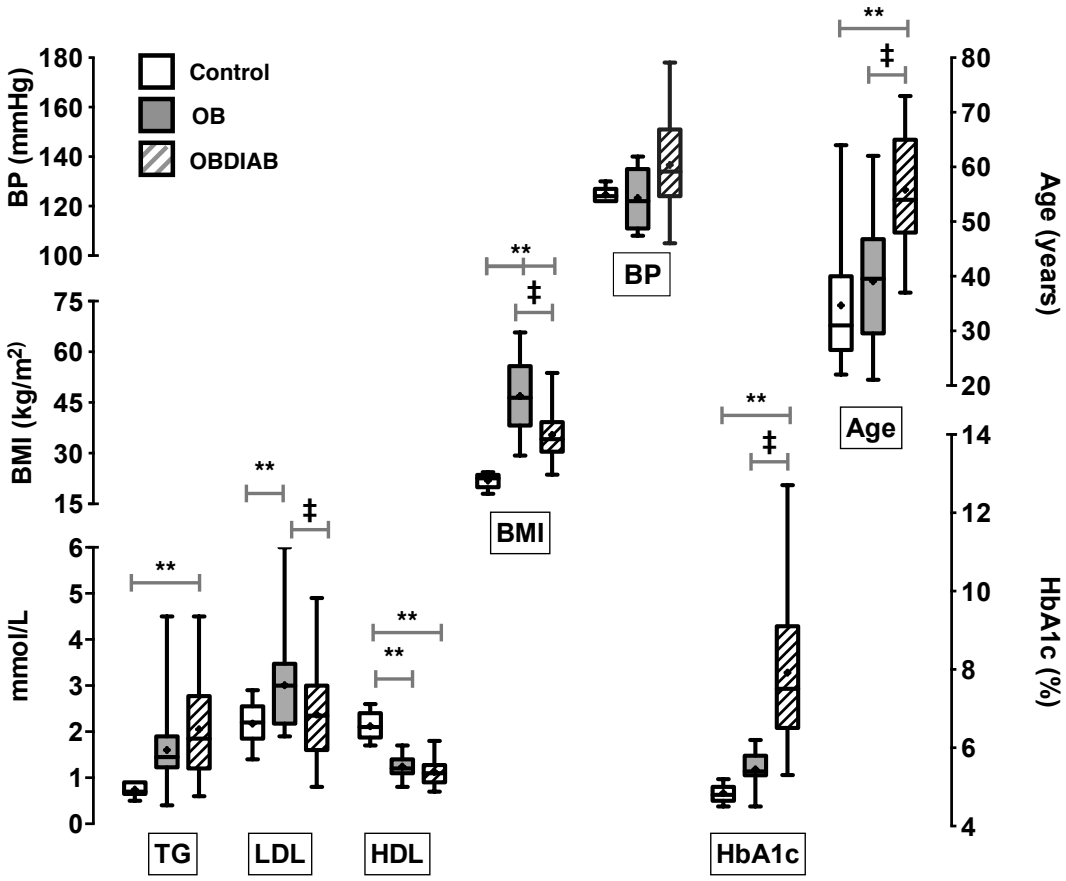
OB: Obese Individuals, BMI ≥ 30kg/m^2 without type 2 diabetes.

OBDIAB: Obese Individuals, BMI ≥ 30kg/m^2 with type 2 diabetes.

Multiple Group Comparisons - Analysis of Variance (MANOVA).
Post hoc analysis for individual between group comparisons: Tukey's test.

Data expressed as mean with 95% Confidence Intervals
** p<0·05; ** p<0.01 versus Control; † p< 0·05 versus Obese; ‡ p<0.01 versus Obese*

Figure 3.1 Demographic Details and Clinical Characteristics



Graphical representation of patient characteristics shown in Table 1. Serum triglycerides (TG) (mmol/L), low density lipoprotein (LDL) (mmol/L), high density lipoprotein (HDL) (mmol/L), body mass index (BMI) (kg/m2), systolic blood pressure (Systolic BP) (mmHg), glycosylated haemoglobin (HbA1C) (%) and age (years)

Box limits = interquartile range; median line = median; + = mean
Statistical Analysis: Analysis of Variance (ANOVA) with Tukey's post hoc test
** P<0.05; ** P<0.01 versus Control*
† P<0.05; ‡ P<0.01 versus Obese

3.2 Study Design

A schematic overview for the study design is presented in Figure 3.2. In addition to recording demographic, anthropometric and clinical data on study participants, blood samples were drawn for monocyte analysis.

Peripheral blood mononuclear cells were initially isolated from each blood sample for further analysis of monocyte phenotype using flow cytometry and qRT-PCR, or quantification of lipoprotein uptake using a novel flow-cytometry assay, measuring the uptake of bodipy conjugated ApoB lipoproteins. The individual methodological aspects of study design are described in more detail below.

Figure 3.2: Experimental Design

3.3 Isolation and Flow Cytometry Analysis of PBMCs

3.3.1 Isolation of PBMCs

Peripheral blood mononuclear cells (PBMC) were isolated from anti-coagulated whole blood by gradient centrifugation using endotoxin-free Ficoll-Paque Plus® (1.077 g/mL) (GE Healthcare, Bucks, U.K.) by aseptic technique as described by Yang et al.[153] Following removal of the PBMC containing "buffy-coat", samples were washed twice in staining buffer [Phosphate Buffered Saline (PBS) (Sigma-Aldrich, Dublin, IRL) with 1% Fetal Calf Serum (FCS) (Lonza, Walkersville, MD, USA)] prior to incubation with optimized dilutions of fluorochrome-coupled monoclonal antibodies (MAb) in staining buffer.

Processing of blood samples and isolation of PBMCs were performed within 4 hours of collection. This time point was chosen on the basis of preliminary experimentation which demonstrated a change in scatter and expression characteristics of monocytes when stored for > 4 hours (Figure 3.3). In other preliminary experiments not presented in this thesis for reasons of space, it was shown that these changes were unaffected by storage at room temperature *versus* 4°C.

3.3.2 Enumeration of Circulating Monocytes

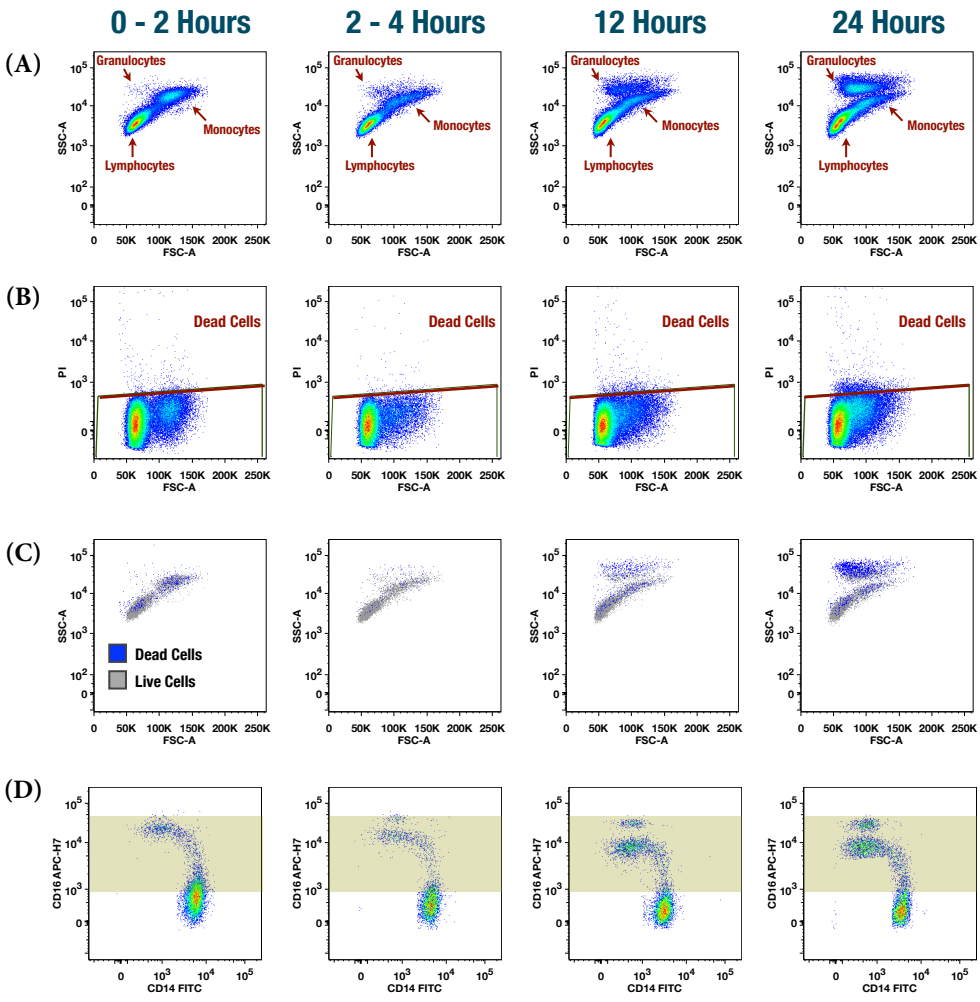
To calculate total monocyte counts, 500 µL aliquots of anti-coagulated whole blood were stained with anti-CD45, anti-CD14, anti-HLA-DR and anti-CD16 followed by lysis of erythrocytes with RBC Lysis Buffer® (Biolegend, San Diego, CA, USA), washing and re-constitution in 500 µL staining buffer and enumeration of cells within the monocyte gates from three 100µL acquisition cycles on an Accuri® C6 flow cytometer (BD Biosciences).

3.3.3 Monocyte Staining with Fluorochrome Conjugated Monoclonal Antibodies

Flow cytometry was used to enumerate, analyse and phenotype human monocyte subpopulations. Flow cytometry was performed on a FACS Canto I Cytometer (BD Biosciences; Oxford, U.K.), using FACS DiVa (Version 6.0) acquisition software (BD Biosciences). A five colour staining matrix was applied in the investigation of monocytes, using the following fluorchromes: allophycocyanin (APC), APC-Cyanine Tandem (APC.H7), Peridinin Chlorophyll Protein Complex- Cyanine (PerCp-Cy5.5), Fluorescein Thiocyanate (FITC) and Phycoerythrin (PE). Emission spectra for each fluorchrome are shown in Figure 3.4. Single staining for compensation of spectral overlap was performed using mouse MAb compensation beads (Invitrogen, Life Sciences, Dublin, IRL) and human cells. Non-specific MAb binding was controlled for by using staining buffer containing 1% fetal calf serum (Lonza). CD16/CD32 specific blocking agents were not used as these interfere with CD16 binding, necessary to identify monocyte subsets.[51]

All samples were stained with antibodies against CD45, HLA-DR, CD14 and CD16 to identify human monocyte subpopulations according to a validated gating strategy

Figure 3.3: Effects of Storage on Monocyte Characteristics



Flow cytometry scatter plots showing the changes induced by delay in processing of monocyte samples over time. These plots are representative of blood samples were stored in EDTA containing Vacutainer ® bottles at room temperature. PBMCs were isolated using Ficoll Paque ® **(A)** demonstrates the increase in the appearance of granulocytes within the scatter gate over time. When a viability gate using propidium iodide (PI) staining was used **(B)** there was an increase in events emerging within the dead cell gate, which when backgated **(C)** onto the scatter gate, comprising predominantly granulocytes. **(D)** demonstrates the progressive decline in CD16 fluorescence for monocytes which have not been immediately processed and stained. All samples were subjected to an identical staining matrix, using pre-mixed MAb, at the same optimised concentrations. The area shaded in green is an arbitrarily picked range of CD16 fluorescence. Note also the emergence of granulocytes, high CD16 fluorescence and the artifactual expansion of non-classical monocytes.

(Figure 3.5).[51] To each fully-stained sample, an additional, "phenotyping" MAb (anti-CD163, anti-CCR2, anti-TLR-4, anti-PSGL-1, anti-CD36, anti-LRP-1) was added at optimised dilution. Dilutions of fluorochrome conjugated MAb were optimised by measuring the fluorescence of sequentially titrated concentrations of MAb and choosing the optimal MAb concentration from a dilution curve, plotting MAb concentration against MFI. Fluorescence minus one (FMO) controls were used to set analysis gates. Staining combinations and concentrations of monoclonal antibodies are shown for specific experiments in Chapter 5, Table 5.1. Details of individual monoclonal antibodies used for all experiments are included in the appendix.

Table 3.1: Monoclonal Antibodies - Clone, Isotype and Format

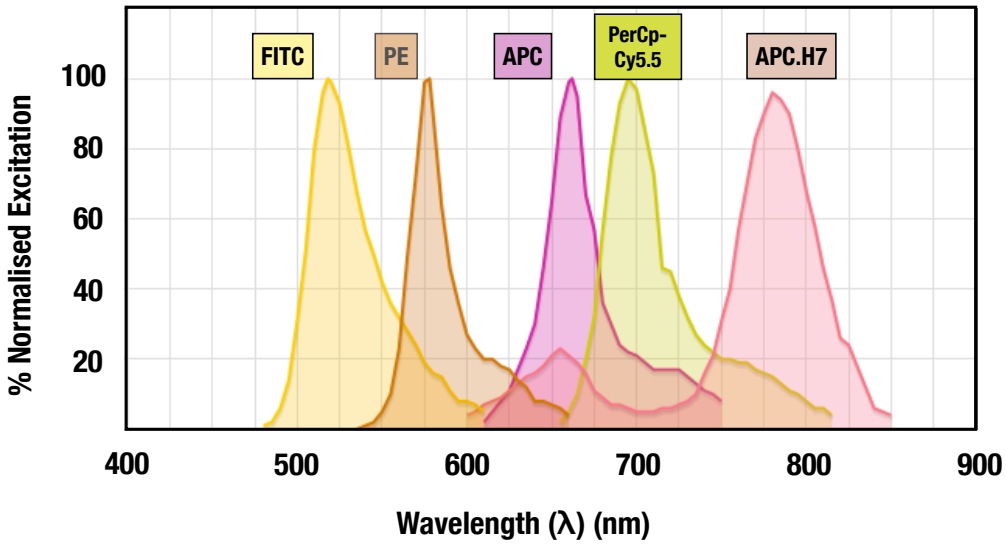
Antibody	Clone	Isotype	Format
CD14	M5E2	Ms IgG2a, κ	PerCP.Cy5.5; FITC
CD16	3G8	Ms IgG1, κ	FITC; APC.H7
CD45	HI30	Ms IgG1, κ	APC, PE
HLA-DR	G46-6	Ms IgG2a, κ	APC-H7, PE
CD86	2331 (FUN-1)	Ms IgG1, κ	PE, APC
CD163	GHI/61	Ms IgG1, κ	PE
CD36	CB38	Ms IgM, κ	PE
CD91	A2MR-α2	Ms IgG1, κ	PE
CD162	KPL-1	Ms IgG1, κ	PE
TLR-4	HTA125	Ms IgG2a, κ	Biotin
CCR2	48607	Ms IgG2b, κ	Alexa Flour 647

All antibodies were sourced from BD Biosciences® , Ireland

3.3.4 Assay Standardization, Data Acquisition and Analysis

Data acquisition was performed on a FACSCanto® flow cytometer with pre-analysis set-up using Cytometer Setup and Tracking (CS&T) beads and FACS DiVa V 6.0® software (BD Biosciences, Oxford, U.K.). Day-to-day assay variability was adjusted for by calculating the molecules of equivalent soluble fluorophore (MESF) using Spherotech® Rainbow Beads (Spherotech Inc, Chicago, IL, USA) as described by Wang et al.[154] Calculation of MESF was performed by constructing a standard curve, using median fluorescence intensity values for each fluorochrome, for each of the eight peaks produced using Rainbow Calibration Beads®. The median fluorescence intensity for the events of interest were then measured from the standard curve and

Figure 3.4: Fluorochrome Emission Spectra



***APC**: Allophycocyanin; **APC.H7**: APC-Cyanine Tandem;*
***PerCp**: Peridinin Chlorophyll Protein Complex; **FITC**: Fluorescein Thiocyanate;*
***PE**: Phycoerythrin*

MESF calculated (Figure 3.6). Compensation for spectral overlap and data interrogation were performed using FlowJo® V 9.5 software (Tree Star Inc; Ashland, OR, USA).

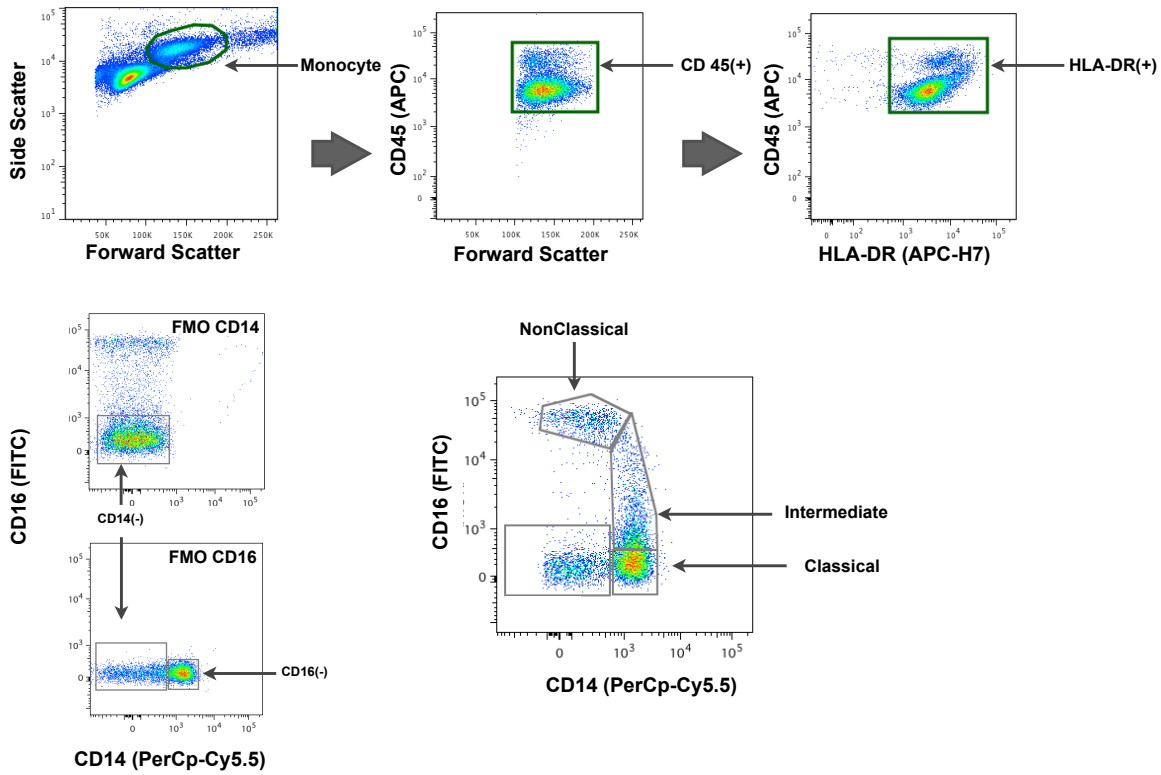
For individual analyses, the parameters of measurement employed included: 1. The percentage of cells falling within positive staining gates (as determined using FMO control). 2. The resolution metric / staining index (R_D) calculated to correct for background fluorescence by the following formula as described by Ortyn et al [155]

Resolution Metric (R_D):

$$MESF_{(stained)} - MESF_{(FMO)} / rSD_{(stained)} + rSD_{(FMO)}$$

MESF = Molecules of Equivalent Molecular Fluorescence; FMO = Flow minus one control; rSD = Robust Standard Deviation

Figure 3.5: Gating Strategy for Identification of Human Monocyte Subsets



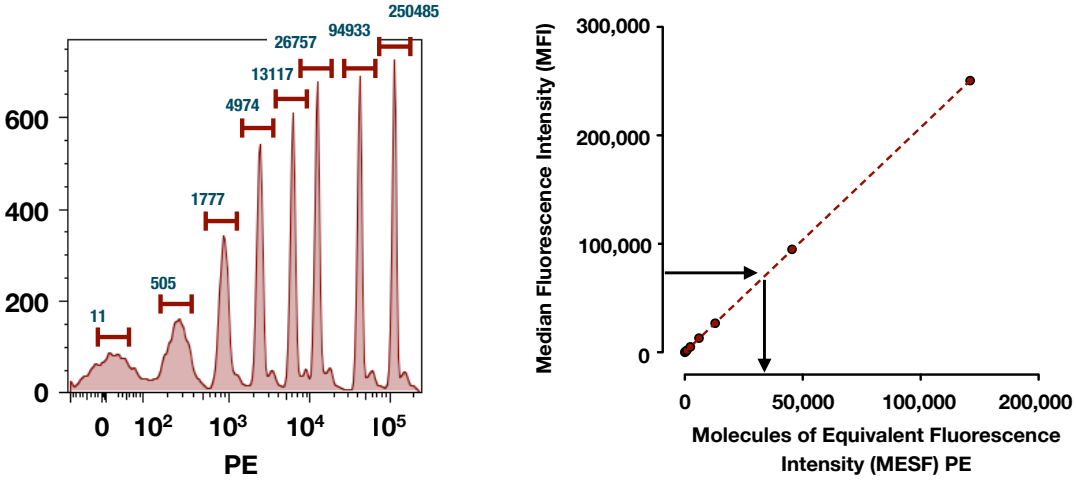
Singlet events only were selected by plotting forward scatter height against forward scatter area. Monocytes were selected from scatter plots of forward versus side scatter. CD45 (+) HLA-DR(+) events were then selected as described by Heimbech et al.[51] Using appropriate flow minus one (FMO) control samples, gates were applied to identify each monocyte subpopulation.

3.4 Fluorescence-Activated Cell Sorting

Individual monocyte subpopulations were identified and sorted from total PBMC fractions. A total of 30 - 50 million PBMCs were isolated for each sort and stained with the following monoclonal antibody combination: CD45 APC, HLA-DR APC-H7, CD16 FITC in staining buffer prior to reconstitution in sorting buffer [PBS (Sigma-Aldrich), 1% FCS (Lonza), 25mM HEPES, 1mM EDTA, 0.1% Xanthan Gum] at a concentration $5x10^6$ cells / mL. In order to maintain monocytes in suspension during each sort, sorting buffer density was increased by adding inert 0.1% xanthan gum. FACS sorting was performed using a 70μM nozzle under low flow pressure. Flow rate was adjusted to permit a maximal event rate of 5000 events/s. Sorting was performed on a FACS Aria II Flow Cytometer (BD Biosciences). Monocytes were sorted under sterile conditions to > 90% purity into RPMI 1640 medium (Sigma-Aldrich) containing 1% FCS (Lonza) using a FACS ARIA II® flow cytometer (BD Biosciences). Following sorting, monocyte sub-populations were immediately pelleted by centrifugation for 8 minutes at 450g then re-suspended in RNAlater® (Qiagen, Life Technologies, Crawley, U.K.) prior to storage at -80°C for subsequent RNA isolation.

Experiment-specific details relating to FACS sorting are described in Chapter 5 (Section 5.3.1.3). Samples were sorted into four subpopulations and purity of cellular isolation for each monocyte subpopulation was checked following each sort (Figure 5.1).

Figure 3.6: Measurement of Molecules of Equivalent Soluble Fluorophore



Calibration beads were used with each flow cytometric assay which produced eight fluorescence peaks of known, measured molecules of equivalent soluble fluorophore (MESF). The median fluorescence intensity (MFI) was measured for each peak as demonstrated in the flow cytometry histogram. Plotting these MFI values on the *y-axis* against the *true* fluorescence for each peak on the x-axis, a standard curve is constructed. The MESF is then calculated by plotting the measured MFI for the target population on this standard curve.

Assays were standardised for day to day variability in by calculating MESF from the standard curve using MFI values for the cellular population of interest.

3.5 Low Density Lipoprotein (LDL) Uptake

Bodipy® (4,4-difluoro-3a,4a- diaza-s-indacene) is an intrinsically lipophilic fluorophore and its fluorescence can be readily measured using flow cytometry. Consequently bodipy® can be used to quantify lipid uptake by cells using flow cytometry and fluorescence microscopy. Probes which incorporate bodipy® also closely mimic the properties of natural lipids. Hence fatty acids, phospholipids and cholesteryl ester preparations conjugated to bodipy® are useful tools to evaluate the transit and metabolism of various lipids and lipoproteins within the cellular microenvironment. They are therefore effective tracers of lipid trafficking.

Native and acetylated (ac) LDL uptake was investigated using bodipy®-conjugated LDL and ac-LDL respectively (Invitrogen; Life Sciences). Whole-fraction PBMCs, prepared as described above, were re-suspended in RPMI 1640 (Sigma-Aldrich) supplemented with 2% FCS (Lonza) and either 10μg/mL LDL-bodipy® or 5μg/mL acLDL-bodipy® (Invitrogen; Life Sciences). Monocytes in static culture readily activate, adhere to plastic or differentiate with a consequent loss of CD16 expression. To prevent monocyte activation and/or differentiation, samples were kept under conditions of continuous motion during incubation with bodipy® conjugated lipoproteins. An optimised incubation time of 1 hour was chosen based on preliminary uptake experiments (See: Chapter 6; Figure 6.1) . Samples were incubated for 1 hour at 37°C in a shaking incubator at 50 rpm then immediately cooled to 4°C, stained with the following fluorochrome-coupled monoclonal antibodies: CD45 APC, CD14 PerCP-Cy5.5, CD16 APC-H7 and HLA-DR PE and then analysed by flow cytometry with fluorescence on the FL-1 (FITC) channel used as an indicator of bodipy®-LDL uptake (See Chapter 6; Table 6.1).

3.6 Reverse Transcription and Quantitative Polymerase Chain Reaction

RNA was extracted and first strand complimentary DNA (cDNA) synthesized from sorted monocytes using High Pure® PCR Template Kit (Roche Applied Science; Burgess Hill, U.K.) according to manufacturer's instruction (www.roche-applied-science.com "Light Cycler® 480 SYBR Green I Master). cDNA purity, quality and concentration was analysed using a Nanodrop® spectrophotometer. Stock concentrations of cDNA were diluted to a concentration of 300ng/mL prior to performing qRT-PCR (See Chapter 5; Figure 5.2).

qRT-PCR was performed on a LightCycler® 480 II using SYBR Green I Master Mix (Roche Applied Science). Samples were run in triplicate on a 96 well plate with a final reaction volume of 20mcL per well as follows: SYBR Green I Master Mix 10mcL, PCR-grade water 3mcL, PCR primers 2mcL (1mcL Forward, 1mcL Reverse) and cDNA (300ng/mL) 5mcL. The following custom-designed primers were used (Primer3 software, www.simgene.com):

PPARγ:	Forward:	AGAAGCCTGCATTTCTGCAT
	Reverse:	TCAAAGGAGTGGGAGTGGTC
DGAT1:	Forward:	CATCCTGAACTGGTGTGTGG
	Reverse:	GCTGGGAAACACAGAATGGT
VEGF-A:	Forward:	AAGGAGGAGGGCAGAATCAT
	Reverse:	ATCTGCATGGTGATGTTGGA.
β-Actin:	Forward:	GGACTTCGAGCAAGAGATGG
	Reverse:	AGCACTGTGTTGGCGTACAG
β-Tubulin:	Forward:	CCTGGCTACTGGATTGGTGT
	Reverse:	AGTGGCTGAGCTAGGAGCTG

The instrument set-up and conditions for qRT-PCR are shown in Table 3.2. Relative quantification was calculated using the $2^{(-\Delta C_T)}$ methodology as follows:

$$2^{-(C_T \text{ Target} - C_T \text{ Calibrator})}$$

The final result of the transcript relative concentration was calculated from the crossing threshold (C_T) values calculated for target and housekeeping genes (Figure 3.7).[156-158] Three target genes were investigated; PPARγ, DGAT-1 and VEGF-A, as well as two internal control (housekeeping genes); β-Actin and β-Tubulin. The geometric mean for the C_T of both housekeeping genes was calculated and used as the calibrator for calculation of the Δ^{CT} value for each target gene.

3.7 Data Analysis & Statistics

Multiple-group comparisons were made using a single factor analysis of variance (ANOVA) or multivariate analysis of variance (MANOVA) for independent samples. Paired sample and repeated measures multiple-group comparisons were made using the repeated measures ANOVA. Multiple regression analysis was modeled to predict associations between monocyte subpopulation statistics and validated clinical markers of metabolic and cardiovascular risk (LDL, triglycerides, HDL, BMI and HbA1c). Multivariate analyses were adjusted for the following covariates: age, sex and secondary prevention therapy (aspirin, statin, ACEi and ARB) and took three formats:

(i) principal component analysis was used for factor reduction and to investigate broad associations between covariates within the model;

(ii) categorical dependent variables were modelled using binary logistic regression analysis;

(iii) continuous dependent variables were modelled using either multivariate linear regression for continuous independent variables or the analysis of covariance (ANCOVA) for categorical dependent variables.

All statistical comparisons were performed using Statistics Package for the Social Sciences (SPSS)® V 20 (IBM, Armonk, NY, USA) and GraphPad® Prism V 6.0 (GraphPad Software, San Diego, CA, USA). Detailed descriptions of analysis and statistical design for each experiment are given in Chapters 4, 5 and 6. Biostatistics support was provided for this set of experiments by the clinical research facility at National University of Ireland, Galway.

Section 3: Results

Chapter 4

Monocyte Subpopulations in Obesity

4.0 Monocyte Subpopulations in Obesity

4.1 Introduction

Monocytes have been classified according to their relative expression of CD14 and CD16 into classical, intermediate and non-classical subpopulations (Figure 4.2).[19] The functional properties of human monocytes are not fully known. Classical and intermediate monocytes share many characteristics with inflammatory Ly6C^{hi} monocytes in mice. Specifically intermediate and Ly6C^{hi} monocytes share pro-inflammatory characteristics, such as the release of the cytokines TNFα and IL-6, when stimulated by LPS or saturated FFA.[18, 29] Non-classical and Ly6C^{lo} monocyte subpopulations share cross-species characteristics of patrolling and repair.[18, 39]

Mouse models of obesity, type 2 diabetes and atherosclerosis have demonstrated higher numbers and proportions of circulating, and splenic, Ly6C^{hi} monocytes.[76, 133] This monocyte repertoire has been associated with a pro-inflammatory profile in these animals. Moreover, Ly6C^{hi} monocytosis has been causally related to development of cardiovascular complications associated with obesity. Similar to obesity in mice, obese humans show features consistent with a higher pro-inflammatory profile including higher circulating pro-inflammatory cytokines, inflammation within adipose tissue and higher serum C-reactive protein concentrations.[30, 138] However, in humans the pattern of monocyte subpopulation distribution is less clear within the settings of obesity, type 2 diabetes mellitus and cardiovascular disease.

It is well established that CD16^{+} monocytes are expanded in association with cardiovascular disease and clinical parameters of poor metabolic health.[46] However,

the current literature pertaining to whether intermediate or non-classical monocytes predominate this $CD16^+$ monocyte expansion is conflicting.[125, 129] Data from two research groups have demonstrated an expansion of the non classical monocyte subpopulation in association with obesity.[126, 129] In these studies, higher numbers and proportions of non-classical monocytes were associated, using univariate analysis only, with parameters of adverse metabolic health, such as higher serum triglycerides and lower HDL. However, these associations were offset by increasing BMI when multivariate analysis was applied.[129] Intermediate monocyte subpopulation expansion has also been described in obese individuals with chronic kidney disease. Higher intermediate monocytes in this cohort of patients showed more robust associations with parameters of cardiovascular risk such as coronary plaque instability and higher levels of ApoB containing lipoproteins.[125] Moreover, the overall odds for survival, over a six year period, were lower in individuals with higher intermediate monocyte counts.[125] Weight loss following bariatric surgery has also been associated with a reduction in the circulating proportion of intermediate monocytes.[129]

4.2 Hypothesis and Objectives

4.1.1 Hypothesis

The adverse metabolic milieu of human obesity is associated with high circulating numbers of intermediate monocytes.

4.1.2 Objectives

In this set of experiments, the updated classification of human monocytes was applied to achieve two principal objectives:

(i) to characterise the monocyte repertoire in human obesity and type 2 diabetes, using a robust flow cytometry gating strategy which enumerates monocytes in terms of both their circulating counts in addition to the proportions of each subpopulation

(ii) to investigate whether or not there is an association between the circulating distribution of monocyte subpopulations and clinical measurements of poor metabolic health, using multivariate analysis.

4.3 Experimental Design and Statistical Analysis

4.3.1 Experimental Design

Peripheral blood was collected from Control, OB and OBDIAB subjects in EDTA-containing tubes, for monocyte analysis, or Serum-Gel tubes (Vacutainer®) for analysis of serum biochemical parameters (Chapter 3; Figure 3.2). Circulating monocytes were enumerated as described (Chapter 3; Section 3.3.2). PBMCs were isolated and stained for the expression of common monocyte markers (Chapter 3; Section 3.3.3) and monocyte subpopulations were identified using a standardised gating strategy (Chapter 3; Figure 3.5), with CD45 (APC), HLA-DR (APC.H7), CD14 (PerCp-Cy5.5) and CD16 (FITC). Compensation matrices were calculated and analysis of monocyte phenotype was performed using FlowJo (Version 9.4) software (TreeStar Inc).

Clinical data were collected prospectively on all subjects entering the study (Chapter 3; Figure 3.2). Anthropometric measurements such as weight, height and calculated BMI were recorded, in addition to biochemical parameters of metabolic health, namely HbA1c, LDL, Triglycerides and HDL. All clinical, biochemical and experimental data were entered into an electronic database, matched to each study participant. Patient characteristics and demographic details are shown in Chapter 3; Section 3.1.2; Table 3.1; Figure 3.1.

4.3.2 Statistical Analysis

The general methodology applied to statistical analysis is detailed in Chapter 4 (Section 4.7). This study was designed to (i) identify differences in monocyte subpopulation distribution between the three participant groups namely Control, OB, OBDIAB; (ii) identify associations between summary statistics describing monocyte repertoire and those of clinically validated markers of metabolic and cardiovascular risk. Hence the statistical strategy involved (i) analysis for multiple group comparisons; (ii) adjusted multiple regression analysis.

Multiple-group comparisons were made using a single factor or multivariate analysis of variance (ANOVA/MANOVA) for independent samples. Matched sample and repeated measures comparisons were made using repeated measures ANOVA.

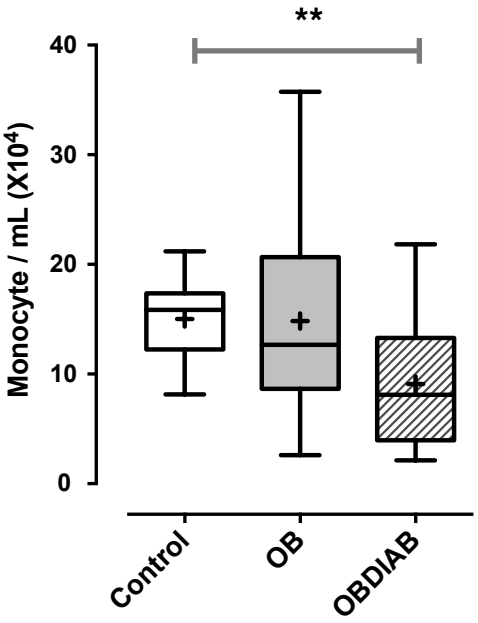
Multiple regression analyses of the entire study sample were modelled to predict associations between statistics of monocyte subpopulation and validated clinical markers of metabolic and cardiovascular risk (LDL, triglycerides, HDL, BMI and HbA1c) and were adjusted for age, sex and medications (aspirin, statin, ACEi and ARB). This took three formats: (i) principal component analysis to investigate broad associations between variables; (ii) categorical dependent variables were analysed using binary logistic regression analysis; (iii) continuous dependent variables were analyzed using multivariate linear regression or the generalised linear model where appropriate.

4.4 Results

4.4.1 Total Circulating Monocyte Count

Expressed as circulating monocytes / mL of peripheral blood, the mean total circulating monocyte count differed across the study groups [ANOVA: $p(1,2)=0.02$; $F=6.92$] (Figure 5.1; Table 5.1). Using *Tukey's post hoc* intergroup comparison, OBDIAB individuals had a lower mean total monocyte count [9.85 (7.19, 12.51) x 10^4] when compared to control [17.01 (14.08, 19.95) x 10^4] ($p<0.01$) but not OB [14.69 (11.63, 17.76) x 10^4] individuals. There was also no difference in total monocyte count between OB and control individuals

Figure 4.1



Boxplots comparing the calculated number of monocytes (monocytes / mL) across the study groups and demonstrating a reduced circulating monocyte count in OBDIAB

Summary statistics:

box limits = interquartile range;

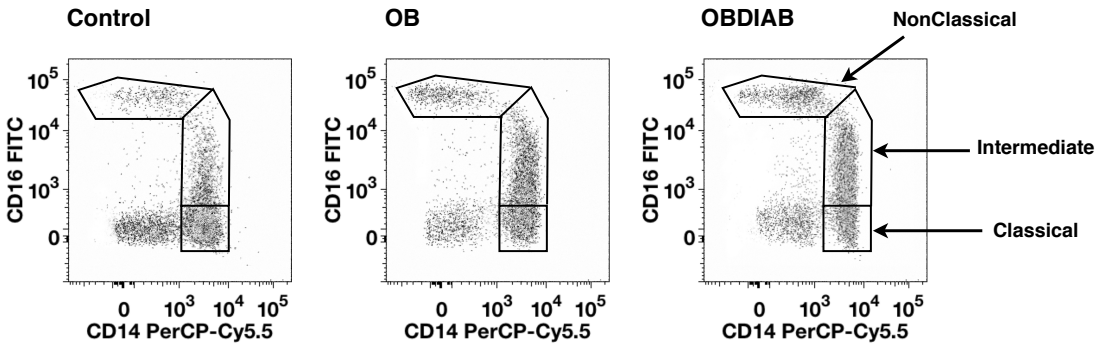
median line = median; + = mean

Statistical Analysis:

Analysis of Variance (ANOVA) Tukey's post hoc

*** P<0.01 versus Control*

Figure 5.2



Representative two-dimensional flow cytometry scatter plots demonstrating the relative expansion of intermediate monocytes in OB and OBDIAB individuals.

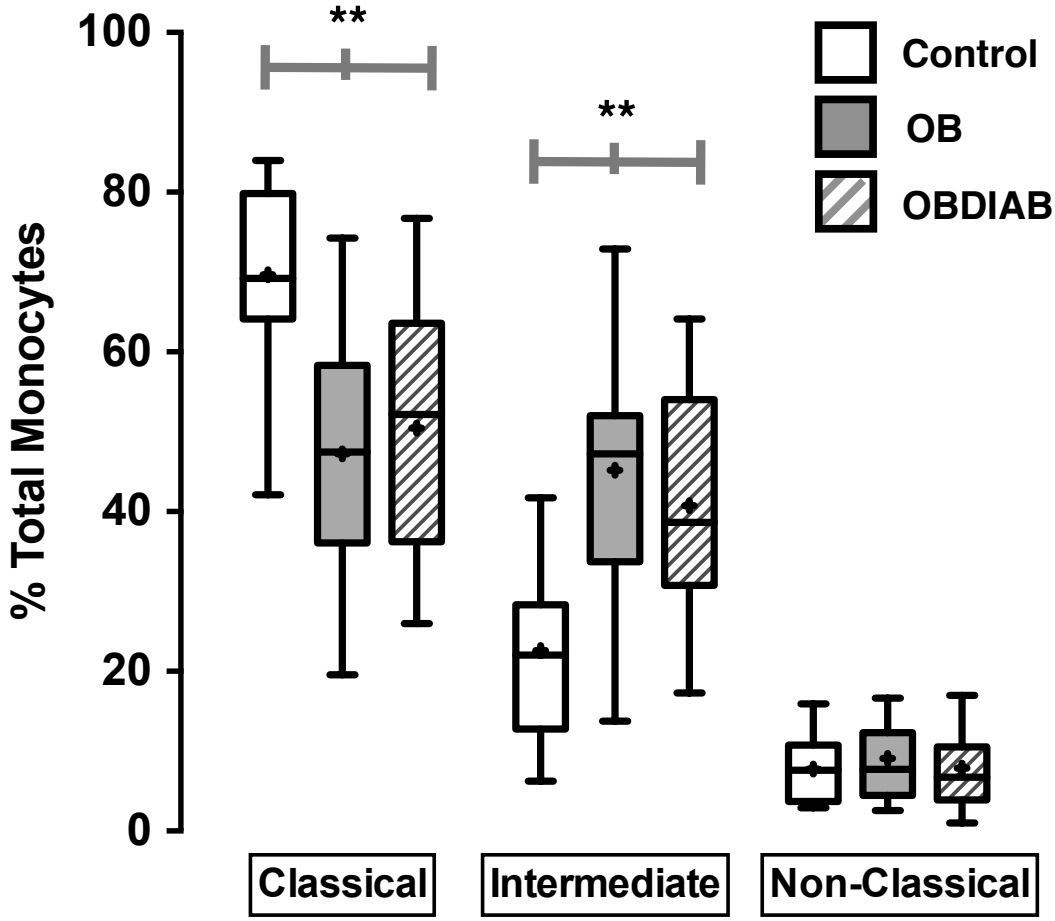
Gating was performed on $CD45^+$ and $HLA\text{-}DR^+$ events. CD14 is plotted against CD16 to describe monocyte subpopulations as previously described. (Reference to methodology section).

4.4.2 Distribution of Monocyte Subpopulations

The distribution statistics for both circulating counts and proportions differed significantly across the study groups for classical [ANOVA: $p(1,2)<0.0001$; $F=17.61$], intermediate [ANOVA: $p(1,2)<0.0001$; $F=19.32$] and non-classical [ANOVA: $p(1,2)=0.03$; $F=3.71$] monocyte subpopulations. (Figures 4.2 & 4.3; Table 4.1). The percentage of classical monocytes was significantly lower and that of intermediate monocytes higher both in OB [*Classical:* 44.36% (37.73, 50.99) ($p<0.01$); *Intermediate:* 46.0% (39.87, 52.13)($p<0.01$)] individuals OBDIAB [*Classical:* 50.89% (45.15, 56.64) ($P<0.01$); *Intermediate:* 40.47% (35.16, 45.78)($p<0.01$)] individuals when compared to their control counterparts [*Classical:* 70.21% (63.87, 76.54); *Intermediate:* 21.24% (15.38, 27.13)]. There was no proportionate difference for non-classical monocytes across the study groups.

In terms of absolute counts of each respective subpopulation. There was a strikingly lower circulating number of classical monocytes in both the OB [7.33 (5.07, 9.59) x 10^4 ($p<0.01$)] and OBDIAB [5.14 (3.14, 7.10) x 10^4 ($p<0.01$)] groups when compared to control [12.17 (10.01, 14.33) x 10^4]. The intermediate monocyte count was higher in OB individuals [6.01 (4.87, 7.14) x 10^4] when compared to control [3.41 (2.33, 4.48) x 10^4 ($p<0.01$)] and OBDIAB study participants [3.87 (2.89, 4.84) x 10^4 ($p<0.05$)]. The circulating number of non-classical monocytes did not differ between control and OB study participants, but there was a lower non-classical count in OBDIAB [0.82 (0.48, 1.15) x 10^4 ($p<0.05$)] individuals when compared to controls [1.45 (1.08, 1.82) x 10^4] (Figure 4.4; Table 4.1).

Figure 4.3: Monocyte Proportions



Boxplots comparing monocyte subpopulation proportions (%) across the study groups. The classical monocyte proportion is reduced and the intermediate monocyte proportion increased in OB and OBDIAB study participants.

Summary statistics:

box limits = interquartile range;

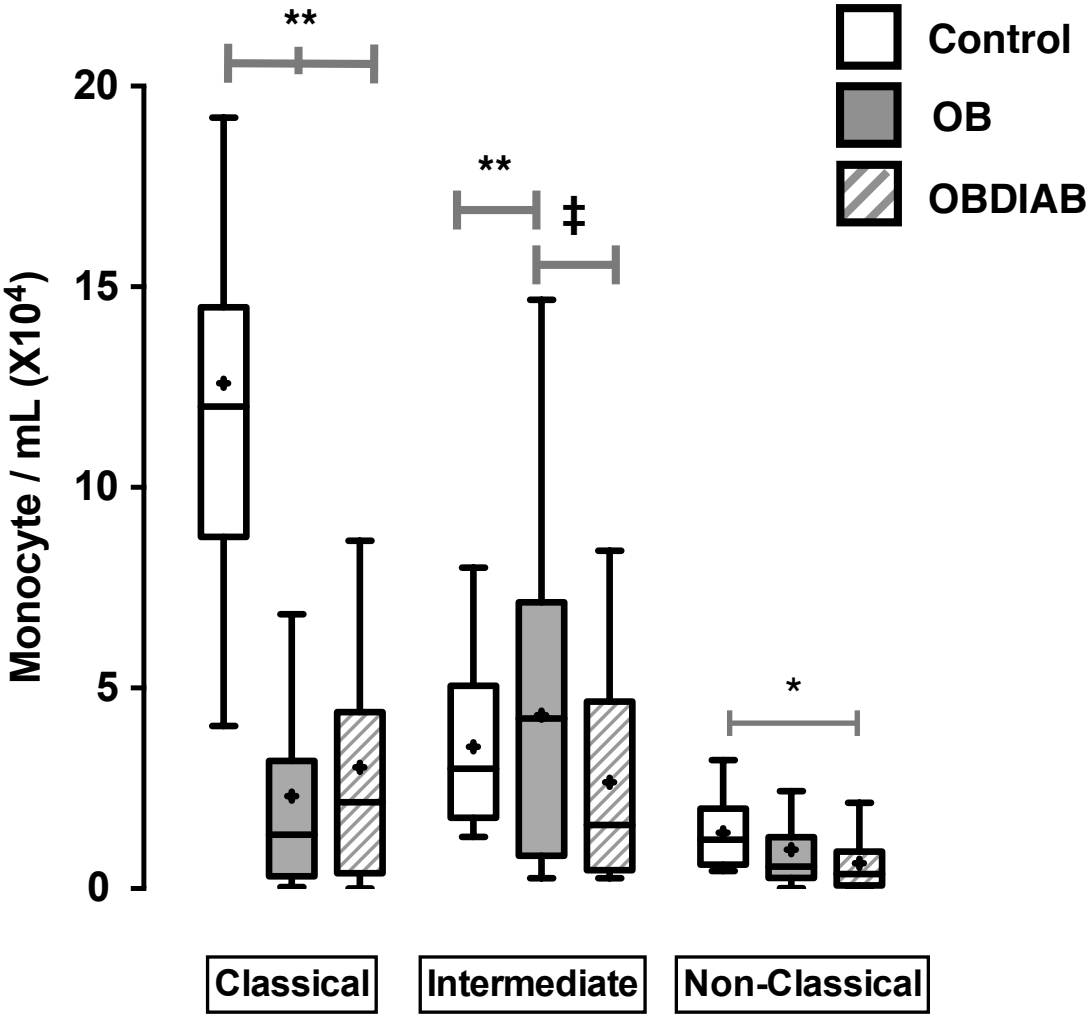
median line = median; + = mean

Statistical Analysis:

Analysis of Variance (ANOVA) Tukey's post hoc

*** P<0.01 versus Control*

Figure 4.4: Monocyte Counts



Boxplots comparing monocyte subpopulation counts (monocyte / mL x 10^4) across the study groups. The classical monocyte count was significantly reduced in OB and OBDIAB groups while the intermediate monocyte count was increased in OB but not the OBDIAB study participants.

Summary statistics:

box limits = interquartile range;

median line = median; + = mean

Statistical Analysis:

Analysis of Variance (ANOVA) Tukey's post hoc

**P<0.05, ** P<0.01 versus Control*

Table 4.1 Monocyte counts and proportions in Control and Obese Study Participants

Monocyte Subset	Proportion of Total Monocytes (%)			Monocyte Count (Monocyte / mL x 10^4)		
	Control	*OB*	*OBDIAB*	*Control*	*OB*	*OBDIAB*
Total Monocytes **p(1,2)=0.02; F=6.92**	-	-	-	**17.01** *(14.08, 19.95)*	**14.69** *(11.63, 17.76)*	**9.85**** *(7.19, 12.51)*
Mean Difference v Control				-	***-2.32 (-7.41, 2.78)***	***-7.17 (-11.92, -2.42)***
Classical **p(1,2)<0.0001; F=17.61**	**70.21** *(63.87, 76.54)*	**44.36**** *(37.73, 50.99)*	**50.89**** *(45.15, 56.64)*	**12.17** *(10.01, 14.33)*	**7.33**** *(5.07, 9.59)*	**5.14**** *(3.18, 7.10)*
Mean Difference v Control	-	***-25.85 (-38.86, -14.84)***	***-19.31 (-29.58, -9.05)***	-	***-4.84 (-8.60, -1.09)***	***-7.03 (-10.53, -3.52)***
Intermediate **p(1,2)<0.0001; F=19.32**	**21.24** *(15.38, 27.13)*	**46.00**** *(39.87, 52.13)*	**40.47**** *(35.16, 45.78)*	**3.41** *(2.33, 4.48)*	**6.01**** *(4.87, 7.14)*	**3.87†** *(2.89, 4.84)*
Mean Difference v Control	-	***24.76 (14.57, 34.94)***	***19.23 (9.73, 28.73)***	-	***2.60 (0.74, 4.47)***	***0.46 (-1.29, 2.20)***
Non-Classical **p(1,2)=0.03; F=3.71**	**8.58** *(6.52, 10.65)*	**9.01** *(6.85, 11.17)*	**8.30** *(6.43, 10.17)*	**1.45** *(1.08, 1.82)*	**1.34** *(0.95, 17.24)*	**0.82*** *(0.48, 1.15)*
Mean Difference v Control	-	***0.41 (-3.17, 4.01)***	***0.29 (-3.63, 3.06)***	-	***-0.11 (-0.75, 0.53)***	***-0.63 (-1.23, -0.03)***

Control: Healthy Individuals, BMI < 25kg/m².

OB: Obese Individuals, BMI ≧ 30kg/m² without type 2 diabetes.

OBDIAB: Obese Individuals, BMI ≧ 30kg/m² with type 2 diabetes.

Multiple Group Comparisons - Multiparameter Analysis of Variance (MANOVA). Post hoc analysis for individual between group comparisons: Tukey's test.

Data expressed as mean with 95% Confidence Intervals

** p<0·05; ** p<0.01 versus Control; † p< 0·05 versus Obese*

4.4.3 Multivariate Analysis of Monocyte Repertoire: Intermediate Monocyte Expansion is related to Parameters of Poor Metabolic Health.

We further explored the relationships between circulating monocyte distribution and clinical parameters related to metabolic health (weight, BMI, HbA1c, triglycerides, LDL and HDL), using stepwise multiple linear regression analyses for the entire study cohort (Control, OB & OBDIAB; n=128). These analyses were modelled to identify the individual contribution of each variable to the measured outcomes. Hence we present results which were adjusted for the heterogeneity of individual patient characteristics, which included all variables of interest and which corrected for interactions between individual variables.

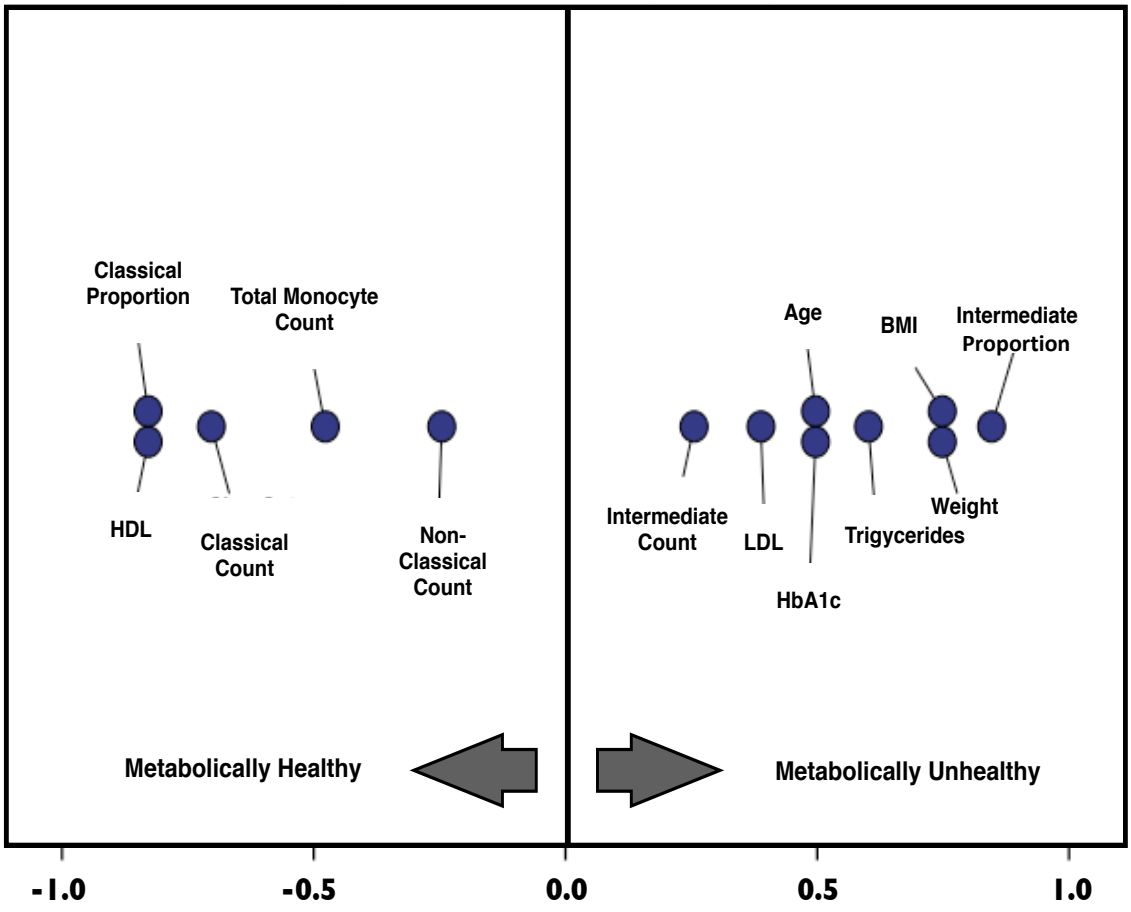
Factor reduction was performed using principal component analysis and showed that a higher proportion and count for intermediate monocytes clustered unfavourably with parameters of poor metabolic health including LDL, HbA1c, Triglycerides and BMI. Higher classical monocyte counts and proportions interestingly clustered with HDL, a marker of metabolic health (Figure 4.5). Adjusted multivariate linear regression analysis was then performed to investigate the associations between each clinical covariate and monocyte related dependent variables modelled as total monocyte count as well as counts and proportions of each monocyte subpopulation.

The total monocyte count was inversely associated with HbA1c [Regression Coefficient (B): -1.8 (-2.8, -0.07) x 10^4; p=0.001] but not with other parameters of metabolic health. The classical monocyte count was also inversely associated with HbA1c [B: -0.9 (-1.8, -0.09) x 10^4; p=0.03] and directly associated with serum HDL concentrations [B: 3.5 (1.4, 5.6) x 10^4; p=0.001]. The intermediate monocyte count was directly associated with serum calculated LDL concentrations [B: 0.8 (0.1, 1.6) x

10^4; p=0.026]. Non-classical monocyte counts were not associated with measured parameters of metabolic health. The proportion of classical monocytes was associated with serum HDL [B: 9.2 (2.5, 15.9); p=0.008] and inversely related to BMI [B: -0.4 (-0.7, -0.01); p=0.044]. Conversely the intermediate monocyte proportion was inversely associated with serum HDL [B: -9.7 (-15.7, -3.6)%; p=0.002] (Table 4.2).

Reduced monocyte counts had not previously been described in association with type 2 diabetes. Given the heterogeneity of this study group particularly in terms of therapeutic intervention, individuals from this group were selected and multivariate analysis applied using generalised linear model to account for the effects of medications on observed outcomes. Interestingly, the observed reduction in monocyte count amongst the OBDIAB group could be attributed predominantly to an effect of combined secondary prevention therapy (aspirin, statin and ACEi/ARB). After adjusting for all other variables, secondary prevention accounted for an absolute reduction in monocyte count compared with Control and OB of 6.1 x 104 (-10.2 x 104, -1.9 x 104) monocytes/mL; F = 8.7, p = 0.004.

Figure 4.5: Multivariate Principal Component Analysis / Factor Reduction



Principal component analysis plot demonstrating the relationship between statistics of monocyte repertoire with clinical and biochemical measurements of metabolic health. Each variable is represented by a dot. Associations between variables are represented by their distance from the median and their proximity to each other.

Table 4.2 The relationship between monocyte repertoire and clinical characteristics across the study cohort

	BMI	LDL	HDL	Triglycerides	HbA1c	Age
Monocyte Counts ***(Monocyte / mL)***						
Total	37.9 (-1688, 1764) p=0.96	1.0 (-0.9, 2.9) x 10^4 p=0.31	2.26 (-0.7, 5.2) x 10^4 p=0.13	-0.7 (-3.4, 1.9) x 10^4 p=0.57	***-1.7 (-2.7, -0.7) x 10^4 p=0.02****	88.2 (-1433, 1609) p=0.91
Classical	-142 (-1369, 1083) p=0.82	-0.03 (-1.5, 1.5) x 10^4 p=0.98	***3.5 (1.4, 5.6) x 10^4 p=0.001****	-0.6 (-2.4, 11.2) x 10^4 p=0.47	***-0.9 (-1.7, -0.09) x 10^4 p=0.03****	162 (-914, 1249) p=0.78
Intermediate	94.0 (-559, 747) p=0.77	***0.84 (0.1, 1.58) x 10^4 p=0.03****	-0.98 (-2.1, 0.16) x 10^4 p=0.09	-0.14 (-1.1, 0.79) x 10^4 p=0.75	-0.4 (-0.8, 0.05) x 10^4 p=0.09	-10.6 (-596, 574) p=0.97
Monocyte Proportion ***(%)***						
Classical	***-0.36 (-0.7, -0.1) p=0.04****	-3.5 (-7.5, 0.48) p=0.08	***9.2 (2.5, 15.9) p=0.008****	-2.37 (-7.06, 2.3) p=0.31	-1.39 (-3.68, 0.90) p=0.15	0.07 (-0.239, 0.381) p=0.65
Intermediate	0.29 (-0.02, 0.60) p=0.07	3.06 (-0.51, 6.63) p=0.09	***-9.71 (-15.75, -3.67) p=0.02****	1.05 (-3.24, 5.34) p=0.57	0.74 (-1.29, 2.78) p=0.47	-0.05 (-0.33, 0.24) p=0.75

Statistical analysis: Backward stepwise multivariate linear regression analysis.

Data expressed as Regression Coefficients (B) and 95% Confidence Intervals (CI)

Statistically significant associations are stressed in Semibold Italic and highlighted by an asterix (*)

4.5 Discussion

In this set of experiments, obesity, the metabolic syndrome and type 2 diabetes were associated with significant changes in the number of circulating human monocytes and the relative distribution of their subpopulations. Total circulating monocyte numbers were reduced significantly in obese individuals with type 2 diabetes. There was also a marked expansion of the intermediate monocyte subpopulation and a corresponding decrease in the classical monocyte subpopulation in all obese individuals, irrespective of a diagnosis of diabetes. In contrast to previous studies, there were no changes observed for the non-classical monocyte subpopulation in the setting of obesity or type 2 diabetes. Multivariate analyses demonstrated that the pattern of distribution for monocytes, whereby there was a decrease in classical monocyte subpopulations and an increase in intermediate monocytes, was related to individual parameters of metabolic health, such as LDL, HDL and HbA1c, within the entire study cohort.

4.5.1 Altered Monocyte Subpopulation Distribution

Before causally relating the observed change in monocyte distribution of metabolic disease, one must consider the role that monocytes play in delivering cholesterol and triglycerides from Apo-B containing lipoproteins to Apo-AI containing HDL, in advance of hepatic cholesterol disposal and recycling.[107] In mice, this process is mediated predominantly by Ly6C^{hi} monocytes and the innate response to hypercholesterolaemia is principally mediated through the action of monocytes recruited from the spleen, rather than bone-marrow derived monocytes.[54, 76] In humans the subpopulation-specific contribution of monocytes to Apo-B lipoprotein

uptake and atherosclerosis is not known. Inference from mouse models suggests that the classical and intermediate monocytes represent Ly6C^{hi} homologues and therefore represent likely candidates for the removal and processing of excess Apo-B lipoproteins.[30]

The results presented herein conform with this hypothesis and suggest that classical and intermediate monocytes respond in a dynamic and co-ordinated fashion to circulating lipoproteins. In the entire study cohort, higher numbers of intermediate and lower numbers of classical monocytes were associated with higher LDL levels, lower HDL concentrations. Hence the pattern of lower classical and higher intermediate monocytes observed in obesity coincides with an adverse metabolic profile and higher cardiovascular risk.

In the currently accepted paradigm for atherosclerosis, pro-inflammatory foam cells are formed by the down-regulation of native LDL receptors and dysregulation of cholesterol efflux proteins in favour of scavenger receptor up-regulation, which is then followed by cholesterol ester sequestration within the monocyte.[76] Data presented from the current set of experiments may be interpreted in a number of ways and hence raise a number of areas for further investigation regarding the the interactions between circulating classical monocytes, intermediate monocytes and lipoproteins.

- It is possible that higher classical monocyte populations confer a protective effect and are causally associated with higher serum HDL and lower LDL. This may result from higher efficiency at delivering cholesterol to Apo-AI lipoproteins when compared to their intermediate counterparts.
- It is also possible, that classical monocyte populations are reduced in response

to relatively higher circulating concentrations of Apo-B lipoproteins and the lipotoxic effects of their constituent cholesterols and FFA.

- Classical monocytes may be leaving the circulation and migrating to liver, adipose tissue and the vasculature in response to increasing tissue deposits of Apo-B lipoproteins.
- Lipotoxicity and the consequent pro-inflammatory environment may "activate" immature classical monocytes, causing them to adopt pro-inflammatory characteristics and "mature" towards an intermediate phenotype while still circulating.
- Expansion of the intermediate subpopulation may alternatively result from the release of pre-activated intermediate monocytes from bone marrow or spleen in response to lipotoxicity, a pro-inflammatory environment or due to a humoral signal from classical monocytes which have migrated to the tissues.

4.5.2 Lower Circulating Monocyte Counts

Another interesting finding relates to the overall reduction in total monocyte counts in obese individuals with type 2 diabetes. The lower total number of circulating monocytes occurred primarily at the cost of the classical monocyte subpopulation. Nonetheless, the proportionate expansion of intermediate monocytes remained unaffected in the obese group with diabetes. On multivariate analysis, secondary prevention therapy emerged as the principal covariate of influence on total circulating monocyte count, offsetting the effects of HbA1c and age within this diabetic sub-cohort. This was the first time that a reduced monocyte count had been described in individuals with diabetes and associated with secondary prevention therapy, namely

angiotensin II inhibition, statin therapy and aspirin.

These observational findings may be explained by studies in mice. Swirski *et al*, demonstrated that the release of splenic monocytes, in response to tissue injury occurred in an angiotensin II dependent fashion, via the angiotensin II type 1 receptor (AGTR-1). Knock-down of the AT-1 receptor, the receptor target for angiotensin II and ARBs, reduced total circulating monocyte counts by inhibiting monocyte release from the spleen.[55] Other data have shown an inhibitory effect of statin therapy on CCR2 mediated monocyte migration from the bone marrow, which may account for lower circulating monocytes.[159] Aspirin has long established anti-inflammatory effects through the cyclo-oxygenase pathway and can also directly suppress myelopoeisis. The mechanisms underlying the individual pharmacological contributions of elements of secondary prevention therapy have not been investigated in this set of experiments and further studies are necessary to elucidate the individual effects of these drugs in human monocyte biology. Moreover, the potential effects of unmeasured covariates, associated with secondary prevention therapy can not be definitively out-ruled without prospectively and longitudinally analysing the individual contributions of each drug class to monocyte counts and distribution.

These data demonstrate interesting observations which associate monocytes, obesity, diabetes and cardiovascular risk in humans. However, additional phenotypic and functional studies were necessary to further investigate the potential effects of the intermediate monocyte subpopulation in obesity.

Section 3: Results

Chapter 5

DR^{mid} and DR^{hi} Intermediate Monocyte: A Novel Subclassification System for Intermediate Monocytes

5.0 DR^{mid} and DR^{hi} Intermediate Monocytes: A Novel Subclassification System for Intermediate Monocytes

5.1 Introduction

In the previous chapter, the results of detailed analyses of human monocyte repertoire were shown from non-diabetic, obese individuals compared to healthy, non-obese subjects as well as obese individuals with actively managed type 2 diabetes mellitus. The results indicated striking alterations to the circulating monocyte repertoire in non-diabetic, obese compared to non-obese individuals consisting of decreased total and classical monocyte numbers with proportionately increased intermediate monocytes and unchanged non-classical monocytes. Higher intermediate monocytes also correlated with clinical and biochemical parameters of adverse metabolic health. From these observations, we concluded that the intermediate monocyte subpopulation may play a direct role in the pathogenesis of adverse metabolic health, which may be explained through its interaction with circulating lipoproteins.

Our current knowledge of the intermediate monocyte subpopulation is limited by conflicting published data. In spite of two detailed studies in humans, the precise phenotype and functional roles of the intermediate monocyte subpopulation remains unclear. Studies by Cros *et al* and Wong *et al* have variably described intermediate monocytes as possessing phenotypic characteristics more closely related to classical and non-classical monocyte subpopulations respectively. Functional aspects of both studies concur, both demonstrating that intermediate monocytes release high concentrations of pro-inflammatory cytokines under conditions of LPS stimulation.[18, 45] Based on their findings, Wong *et al* have proposed a mechanism whereby

intermediate monocytes represent a transitional subpopulation which matures from a classical to a non-classical phenotype. They supported this assertion by demonstrating a phenotypic shift of intermediate monocytes from a classical-like to a non-classical phenotype as CD16 expression increased across the subpopulation. While this maturation hypothesis for intermediate monocytes is reasonable, there are no robust, supporting longitudinal data which demonstrate intermediate monocyte differentiation.[45] It is therefore possible that the phenotypic differences observed with higher expression of CD16 may reflect an heterogeneity of the intermediate subpopulation which can be explained by the existence of more that one subset.

This study tested the hypothesis that intermediate monocyte heterogeneity can be explained by the existence of more than one subset and identified two intermediate monocyte subsets, which differed in phenotype and which could be identified based upon their surface expression of HLA-DR. These monocyte subsets were called DR^{mid} and DR^{hi} intermediate monocytes based on their respective high and mid-level expression of HLA-DR.

5.2 Hypothesis and Objectives

5.2.1 Hypothesis

Variability in phenotype attributed to intermediate monocytes is reflective of heterogeneity within the intermediate subpopulation which may be explained by the presence of more than one intermediate monocyte subset.

5.2.2 Objectives

(i) To address the unresolved issue of intermediate monocyte heterogeneity, by investigating the $CD14^{hi}$ $CD16^{+}$ subpopulation, analysing size, granularity and expression levels for surface molecules ubiquitously and highly expressed on human monocytes including CD45 and HLA-DR.

(ii) Focusing on phenotypic heterogeneity within the intermediate subpopulation, to perform a detailed phenotypic analysis of each monocyte subpopulation; selecting markers specific for aspects of monocyte migration, inflammation, lipoprotein metabolism / atherogenesis.

(iii) To investigate the contribution of intermediate monocyte heterogeneity to metabolic disease in obesity, by examining intermediate monocytes in detail across the study groups and in terms of clinical parameters of metabolic disease.

5.3 Experimental Design and Statistical Analysis

5.3.1 Experimental Design

5.3.1.1 Sample Collection

Peripheral blood was collected from Control, OB and OBDIAB subjects in EDTA-containing tubes, for monocyte analysis, or Serum-Gel tubes (Vacutainer®) for analysis of serum biochemical parameters (Chapter 3; Figure 3.2). Circulating monocytes were enumerated as described (Chapter 3; Section 3.3.2). PBMCs were isolated and stained for the expression of common monocyte markers (Chapter 3; Section 3.3.3) and monocyte subpopulations were identified using a modified gating strategy based upon the phenotypic heterogeneity of the intermediate monocyte subpopulation.

5.3.1.2 Data Collection

Clinical data were collected prospectively on all subjects entering the study (Chapter 3; Figure 3.2). Anthropometric measurements such as weight, height and calculated BMI were recorded, in addition to biochemical parameters of metabolic health, namely HbA1c, LDL, Triglycerides and HDL. All clinical, biochemical and experimental data were entered into an electronic database, matched to each study participant. Patient characteristics and demographic details are shown in Chapter 3; Section 3.1.2; Table 3.1; Figure 3.1).

5.3.1.2 Flow Cytometry Analysis of Monocyte Phenotype

A five colour flow cytometric staining matrix was used to identify and phenotype human monocytes. A detailed description of the staining matrix is shown in Table

5.1, and the emission spectra for each fluorochrome are shown in Chapter 3; Figure 3.4. As described previously monocytes were identified based on their expression of CD45, HLA-DR, CD14 and CD16 (Chapter 4; Figure 4.5). An additional *"phenotyping"* stain was applied to the matrix to investigate specific markers relating to monocyte migration (CCR-2; PSGL-1); lipoprotein uptake (CD36; LRP-1) and inflammation / FFA uptake (TLR-4). Staining was also performed for the ubiquitous monocyte marker and haem scavenger receptor, CD163. A stopping gate for 10,000 events was applied to CD45(+), HLADR(+) events, satisfying scatter characteristics for monocytes. A single tube containing Countbright Rainbow ® calibration beads was also run as a standardised control for each flow cytometry session and this was used to calculate the MESF (Chapter 3; Figure 3.5; Section 3.3.4).[154] Compensation matrices were calculated and analysis of monocyte phenotype was performed using FlowJo (Version 9.4) software (TreeStar Inc; Portland, OR). The resolution metric was calculated and used to adjust for background fluorescence and for multiple comparisons between monocyte subpopulations.[155]

Table 5.1: Flow Cytometry Staining Matrix to Identify Monocyte Phenotype

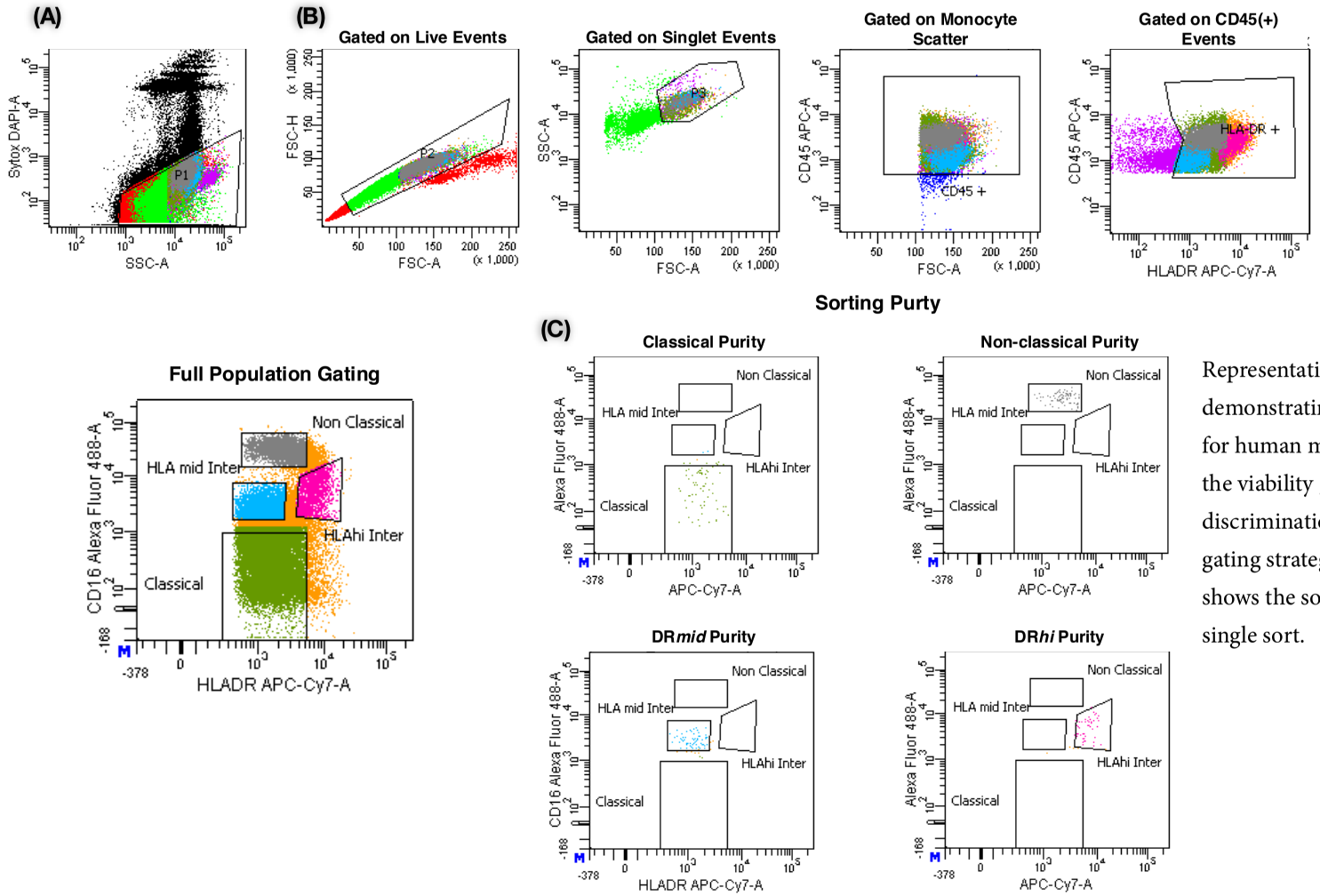
Stain	Cells / Beads	APC	APC.H7	PerCp-Cy5.5	FITC	PE
SS APC	Beads	CD45	-	-	-	-
SS APC.H7	Beads	-	CD45	-	-	-
SS PerCp-Cy5.5	Beads	-	-	CD14	-	-
SS FITC	Beads	-	-	-	CD45	-
SS PE	Beads	-	-	-	-	CD45
FMO APC	Cells 500,000/mL	-	HLA-DR	CD14	CD16	CD45
FMO APC.H7	Cells 500,000/mL	CD45	-	CD14	CD16	CD45
FMO PerCp-Cy5.5	Cells 500,000/mL	CD45	HLA-DR	-	CD16	CD45
FMO FITC	Cells 500,000/mL	CD45	HLA-DR	CD14	-	CD45
FMO PE	Cells 500,000/mL	CD45	HLA-DR	CD14	CD16	CD45
CCR-2	Cells 500,000/mL	CD45	HLA-DR	CD14	CD16	CCR-2
TLR-4	Cells 500,000/mL	CD45	HLA-DR	CD14	CD16	TLR-4
CD36	Cells 500,000/mL	CD45	HLA-DR	CD14	CD16	CD36
LRP-1 (CD91)	Cells 500,000/mL	CD45	HLA-DR	CD14	CD16	LRP-1
PSGL-1 (CD162)	Cells 500,000/mL	CD45	HLA-DR	CD14	CD16	PSGL-1
CD163	Cells 500,000/mL	CD45	HLA-DR	CD14	CD16	CD163
CD86	Cells 500,000/mL	CD45	HLA-DR	CD14	CD16	CD86

***SS**: Single Stain; **FMO**: Flow Minus One Control; **CD**: Cluster of Differentiation;*
***CCR**: C-C chemokine receptor; **LRP**: Low density lipoprotein receptor-related protein;*
***TLR**: Toll-Like Receptor; **PSGL**: P-Selectin Glycoprotein Ligand; **HLA**: Human Leukocyte Antigen;*
***APC**: Allophycocyanin; **APC.H7**: APC-Cyanine Tandem;*
***PerCp**: Peridinin Chlorophyll Protein Complex; **FITC**: Fluorescein Thiocyanate;*
***PE**: Phycoerythrin*

5.3.1.3 Fluorescence Activated Cell Sorting

Monocytes were sorted into four groups, using fluorescence activated cell sorting. All groups were sorted based on their relative cell surface expression of HLA-DR, CD14 and CD16 (Figure 6.1) into Classical ($CD14^{hi}$, $CD16^{-}$, $HLA\text{-}DR^{+}$) and Non-Classical ($CD14^{lo}$, $CD16^{hi}$, $HLA\text{-}DR^{+}$) subpopulations, as well as 2 Intermediate Subsets, namely DR^{mid} Intermediate ($CD14^{hi}$, $CD16^{mid}$, $HLA\text{-}DR^{mid}$) and DR^{hi} Intermediate ($CD14^{hi}$, $CD16^{mid}$, $HLA\text{-}DR^{hi}$) (Figure 5.3). Based on a series of optimisation sorts, MAb on the following fluorochromes were chosen CD45 (APC), HLA-DR (PE), CD14 (PerCp-Cy5.5), CD16 (APC.H7). Live / dead event discrimination was performed by placing a gate on events which stained negatively for the nuclear stain Sytox® (blue fluorescent DAPI) (Figure 5.1). Optimised technical conditions, under which FACS was performed are described in Chapter 3 (Section 3.4). Sort purity was checked after each run and demonstrated a consistent purity >90% (Figure 5.1). Monocytes were sorted into RPMI containing 1% serum at 4°C. Following each sort, cells were pelleted, re-suspended in RNAlater® and stored at -80°C prior to RNA extraction for q-RT-PCR.

Figure 5.1: Fluorescence Activated Sorting; Gating Strategy and Sort Purity

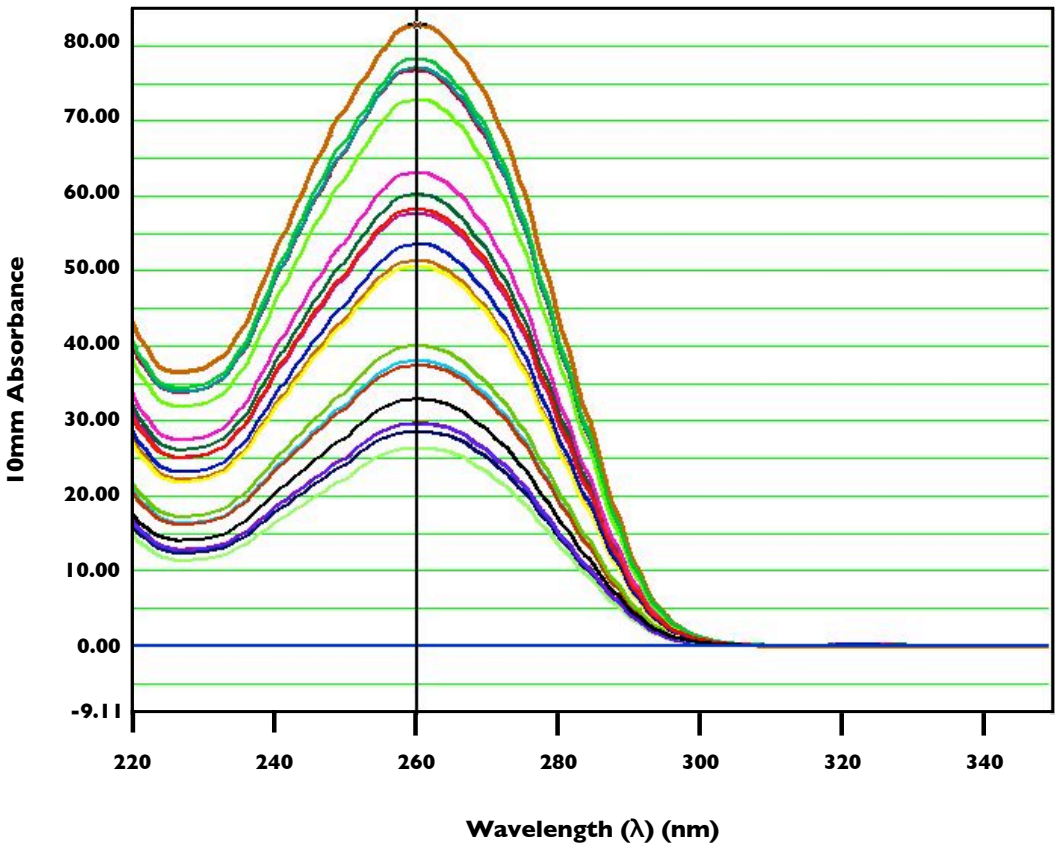


Representative flow cytometry dot demonstrating the sorting strategy for human monocytes. (A) shows the viability gate for live / dead discrimination; (B) demonstrates the gating strategy employed and(C) shows the sorting purity after a single sort.

5.3.1.4 Quantitative RT-PCR

Total RNA was extracted and cDNA synthesised from each monocyte subpopulation as described in Chapter 3 (Section 3.6). Complimentary DNA was checked for quality, purity and concentration using a Nanodrop® Spectrophotometer (Thermo Scientific)(Figure 5.2). cDNA was accepted as reaching sufficient purity when the 260/280 ratio achieved a value >1.80. cDNA was stored at -20°C prior to performing qRT-PCR. Quantitative RT-PCR was performed in triplicate for each subpopulation using optimised primers for detection of PPARγ, DGAT-1 and VEGF-A as described in Chapter 4 (Section 4.4).Two housekeeping genes were used as internal controls, namely β-Actin and β-Tubulin. The geometric mean for both of these was used as the calibrator for relative quantification using the $2^{-(\Delta C_T)}$ method (Chapter 3; Section.3.6).[157, 158]

Figure 5.2: Purity of cDNA Isolation for Sorted Monocyte Subpopulations



Electropherograms of wavelength against absorbance for complimentary DNA isolated from sorted monocyte subpopulations. Absorbance was measured for each peak at λ 260nm and 280nm. A ratio of absorbance at λ 260nm/280nm >1.80 was used as a benchmark for acceptable purity of cDNA.

5.3.2 Statistical Analysis

Statistical design for this study was similar to that of Chapter 5. This study was more specifically designed to (i) identify phenotypic differences between monocyte subpopulations, focusing on DR^{mid} and DR^{hi} Intermediate monocytes and to (ii) assess the distribution of DR^{mid} and DR^{hi} Intermediate monocytes between the three participant groups namely Control, OB, OBDIAB. Multivariate analyses were also used to investigate associations between intermediate monocyte subgroups and validated clinical and biochemical parameters of metabolic and cardiovascular risk. A similar statistical strategy involving (i) multivariate analysis for multiple group comparisons; (ii) repeated measures analysis for inter-individual comparisons, (iii) adjusted multiple regression analysis was also used for this study.

Multiple-group comparisons were made using a single factor or multivariate analysis of variance (ANOVA/MANOVA) for independent samples. Matched sample (e.g. inter-individual comparisons of monocyte subpopulations) and repeated measures comparisons were made using repeated measures (RM)-ANOVA. To ensure meaningful interpretation, assumptions for the ANOVA statistical model require that the data is parametrically distributed and also that the data is not widely distributed. Where these assumptions were not met, data transformation was performed (log_{10} or Square Root). Results and confidence intervals were afterwards appropriately back-transformed for accuracy.

Multiple regression analysis of the entire study sample was modelled to predict associations between statistics of DR^{mid} and DR^{hi} intermediate monocyte subgroups and validated clinical markers of metabolic and cardiovascular risk (LDL, triglycerides, HDL, BMI and HbA1c). These were adjusted for age, sex and

medications (aspirin, statin, ACEi and ARB). This took three formats: (i) principal component analysis to investigate broad associations between variables; (ii) categorical dependent variables were analysed using binary logistic regression analysis; (iii) continuous dependent variables were analysed using multivariate linear regression or the generalised linear model (GLM) where appropriate.

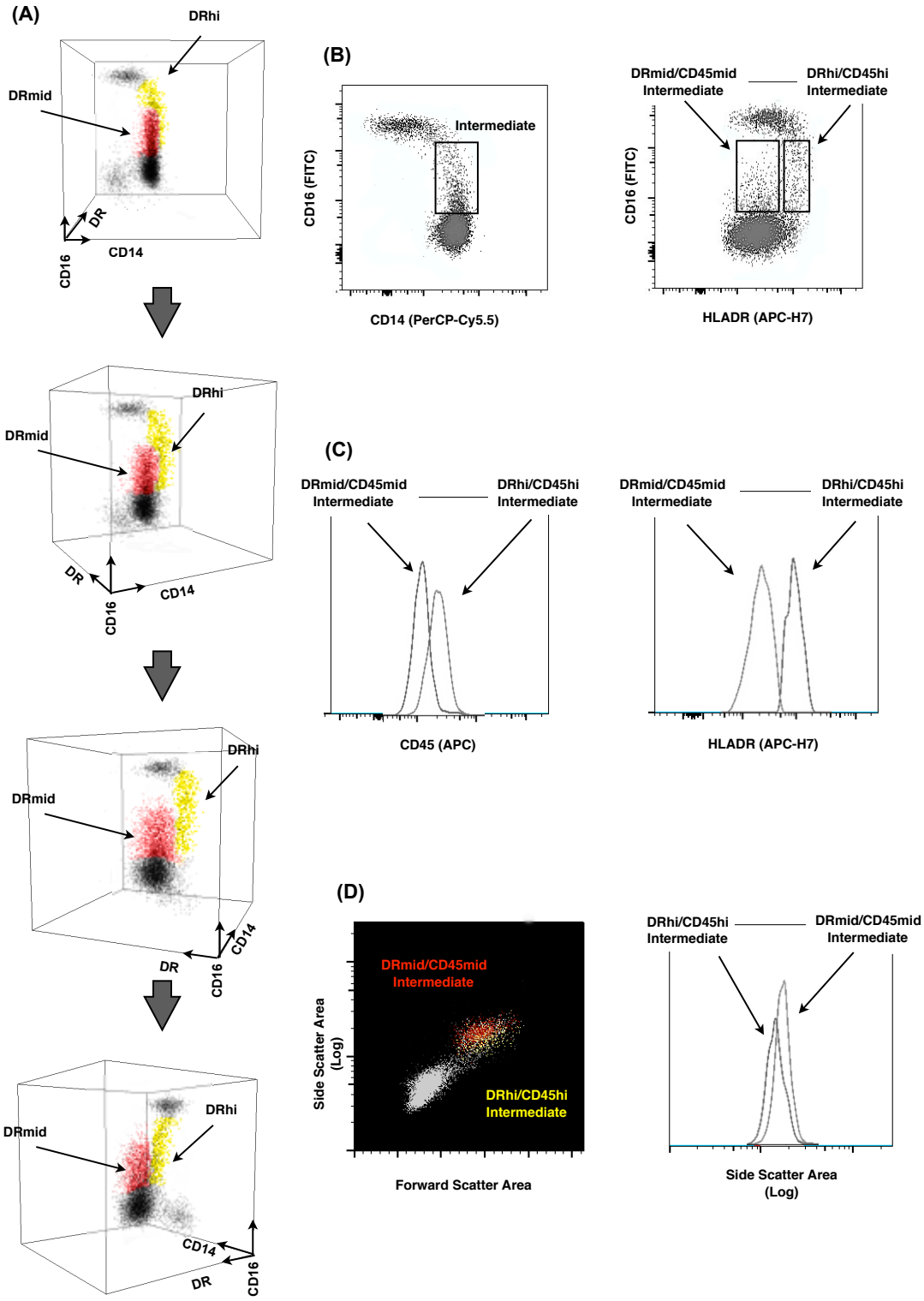
5.4 Results

5.4.1 A Novel Sub-classification of Intermediate Monocytes: Phenotypic Heterogeneity between DR^{hi} and DR^{mid} Intermediate Monocytes

5.4.1.1 Intermediate Monocyte Sub-classification: DR^{mid} and DR^{hi}

To address the unresolved issue of intermediate monocyte heterogeneity, the CD14^{hi} CD16^{+} intermediate monocyte subpopulation was analysed for size, granularity and expression levels of surface molecules ubiquitously and highly expressed on human monocytes including CD45 and HLA-DR. As shown in Figures 5.3 and 5.6, these analyses identified two discreet subsets of intermediate monocytes, which could be distinguished on the basis of surface staining for HLA-DR. In subsequent analyses, these two subsets were designated DR^{mid} (HLA-DR^{mid}) and DR^{hi} (HLA- DR^{hi}) intermediate monocytes respectively. DR^{mid} and DR^{hi} intermediate subsets also differed in their expression of CD45 (higher on DR^{hi}) and granularity, as reflected by side scatter (higher on DR^{mid}) (Figure 5.3). Using this subdivision, the DR^{mid} monocyte subpopulation consistently accounted for the larger proportion of intermediate monocytes (See Section 5.4.2; Figure 5.5).

Figure 5.3: DR^{mid} and DR^{hi} Intermediate Monocyte Subsets



Accompanying Figure Legend Overleaf

(A) Representative, three dimensional, flow cytometry dot plots of CD16 (y-axis), CD14 (z-axis) and HLA-DR (x-axis). Demonstrating the spatial separation of the intermediate monocyte subpopulation, HLA-DR^{mid}, CD45^{mid} (red) and HLA-DR^{hi}, CD45^{hi} (yellow) upon co-staining with fluorochrome associated monoclonal antibodies directed against HLA-DR versus CD16.

(B) Representative, two dimensional, flow cytometry dot plots of CD16 (y-axis) versus CD14; and CD16 (y-axis) versus HLA-DR (x-axis). Demonstrating the a two dimensional gating strategy which separates intermediate monocytes into HLA-DR^{mid}, CD45^{mid} and HLA-DR^{hi}, CD45^{hi} intermediate subsets.

(C) Representative, histograms showing the demonstrating the difference in fluorescence for HLA-DRmid, CD45^{mid} and HLA-DR^{hi}, CD45^{hi} intermediate monocytes, when labelled with APC conjugated monoclonal antibodies directed against CD45 and APC-H7 conjugated monoclonal antibodies against HLA-DR.

(D) Backgate scatter analysis of HLA-DR^{mid}, CD45^{mid} (red) and HLA-DR^{hi}, CD45^{hi} (yellow) intermediate monocytes, with representative histograms of side scatter for each intermediate subset

Compensation for fluorescence spectral overlap was performed using fluorchrome labelled compensation beads and a stopping gate of 10,000 events was set on the "Monocyte Gate"

5.4.1.2 Phenotypic Heterogeneity between DR^{mid} and DR^{hi} Intermediate Monocytes

In order to further distinguish DR^{mid} and DR^{hi} intermediate monocytes, a detailed phenotypic analysis was undertaken to investigate their expression of key markers reported to be highly expressed on foam cells associated with atherosclerosis and macrophages associated with inflammation of visceral fat.[76, 147] Surface markers selected for flow cytometric analysis included CD36, Lipoprotein receptor associated receptor (LRP) -1, Toll-like receptor (TLR)-4, chemokine-chemokine (C-C motif) receptor (CCR)-2 and P-Selectin Glycoprotein Ligand (PSGL) -1.

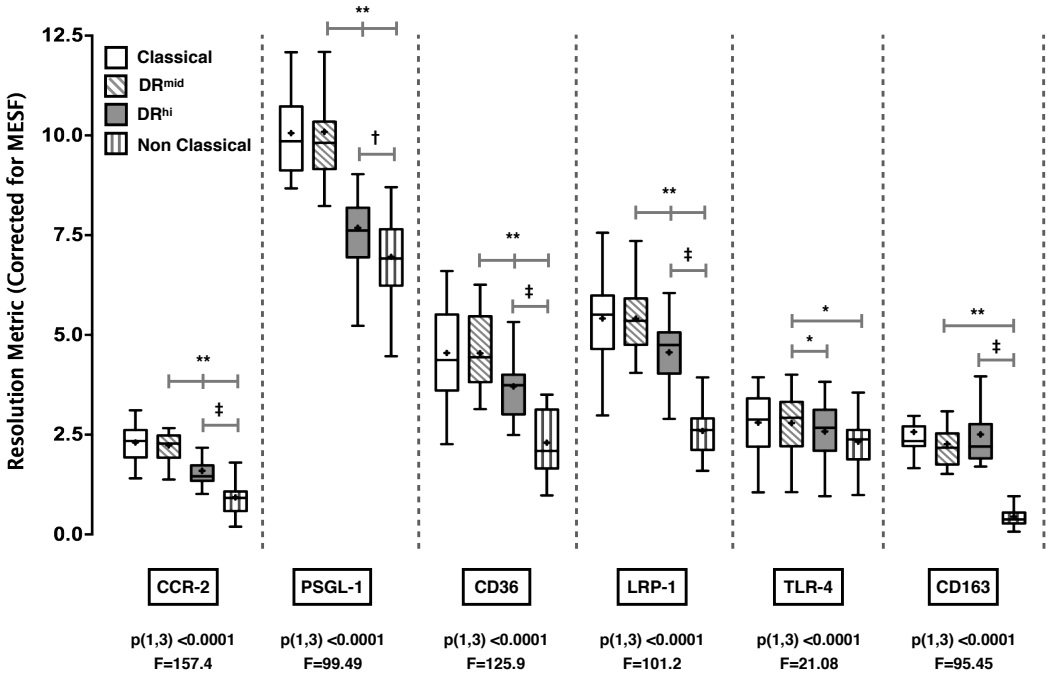
In addition to flow cytometric analysis of surface markers, expression of the nuclear factor, peroxisome proliferator-activated receptor γ (PPARγ), the lipogenic enzyme DGAT-1 and the vasculogenic growth factor, Vascular Endothelial Growth Factor (VEGF)-A were analysed by q-RT-PCR on cDNA of RNA isolated from monocyte subpopulations purified using fluorescence-activated cell sorting (FACS) (Section 5.3.1.3 & 5.3.1.4).

There were clear phenotypic differences across the intermediate monocyte subpopulation between DR^{mid} and DR^{hi} intermediate monocytes (n=27) (Table 5.2; Figure 5.4). When compared to DR^{hi} intermediate monocytes, the DR^{mid} subset demonstrated a higher surface expression of CCR2 [2.23 (2.06, 2.41) *versus* 1.59 (1.41, 1.78); p<0.0001]; PSGL-1 [10.09 (9.26, 10.92) *versus* 7.68 (7.05, 8.32); p<0.0001]; CD36 [4.54 (4.15, 4.93) *versus* 3.71 (3.35, 4.08); p<0.0001]; LRP-1 [5.41 (4.94, 5.88) *versus* 4.56 (4.21, 4.91); p=0.0008] and TLR-4 [2.79 (2.48, 3.09) *versus* 2.58 (2.30, 2.86); p=0.0008]. Overall, the DR^{mid} intermediate and classical monocytes did not differ in their expression of migration, lipoprotein uptake or inflammatory markers, while the DR^{hi} intermediate subset was closer, but not identical, in phenotype to non-

classical monocytes. Detailed data relating to the RM-ANOVA and *post hoc* analysis for surface marker expression of monocyte phenotype are presented in Table 5.2.

Quantitative RT-PCR analysis (n=8 per group) demonstrated a statistically significant difference of expression between DR^{hi} *versus* DR^{mid} intermediate monocytes for CCR-2 [Mean Difference: -0.24 (-0.46, -0.02); $p<0.05$], PPARγ [Mean Difference: 0.08 (0.01, 0.22); $p<0.05$] and DGAT-1 [Mean Difference: -0.10 (-0.27, -0.05); $p<0.05$]. The two intermediate monocyte subsets did not differ in their expression of VEGF-A (Figure 5.5).

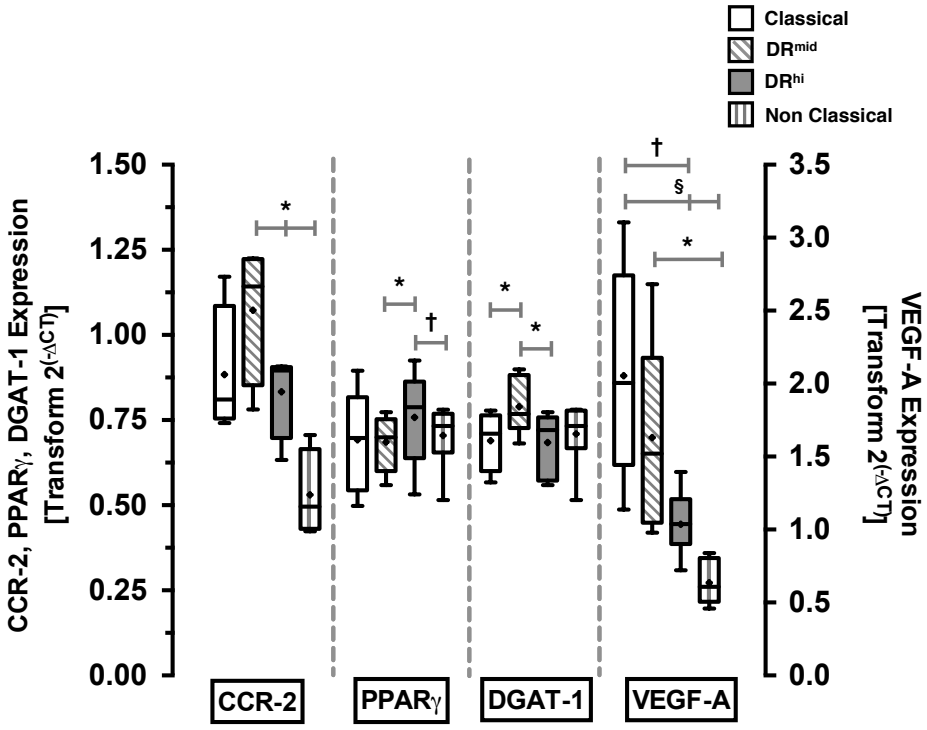
Figure 5.4: Surface Expression of Migration, Atherogenic and Inflammatory Markers



Boxplots demonstrating comparisons for surface expression on monocyte subpopulations namely Classical (clear), DR^{mid} intermediate (diagonal lines), DR^{hi} intermediate (shaded) and Non-Classical (vertical lines). of chemokine-chemokine (C-C motif) receptor -2 (CCR-2); P-selectin glycoprotein ligand -1 (PSGL-1); CD36; lipoprotein receptor associated protein -1 (LRP-1); toll-like receptor -4 (TLR-4) and CD163. DR^{mid} intermediate and Classical monocytes show phenotypic similarity across all markers and demonstrate higher expression of CCR-2, PSGL-1, CD36, LRP-1 and TLR-4 when compared to DR^{hi} intermediate monocytes.

Measurement of surface expression was performed using compensated, multi-color flow cytometry (n=42). Assay standardization was performed by calculating the molecules of equivalent soluble fluorescence (MESF). Correction for background fluorescence was performed by calculating the Resolution Metric (R_D). Data compared for healthy individuals only (n=27).

Figure 5.5: qRT-PCR for Markers of Inflammation, Metabolism, Lipogenesis and Vasculogenesis



Boxplots demonstrating the expression of CCR-2,Peroxisome Proliferator-activated Receptor (PPAR) -γ and Diacylglycerol Acetyl Transferase (DGAT) -1 across four monocyte subpopulations namely, Classical (clear), DR^{mid} intermediate (diagonal lines), DR^{hi} intermediate (shaded) and Non-Classical (vertical lines). DR^{hi} intermediate monocytes demonstrated the highest expression of PPARγ, while highest expression of DGAT-1 and CCR-2 was evident in DR^{mid} intermediate monocytes.
Cellular expression was measured by qRT-PCR on cDNA manufactured from RNA extracted from monocytes sorted using flow cytometry . $2^{-(\Delta CT)}$ values were calculated using β-tubulin and β-actin as reference genes and calculating the geometric mean C_T for both as the calibrator value.

Summary statistics are represented as follows:
box limits = interquartile range; median line = median; + = mean
Data transformation was performed for normality using $(-1/\log_{10}x)$ *where* $x = 2^{(-\Delta CT)}$ *value*
Statistical Analysis: Repeated Measures Analysis of Variance (RM-ANOVA) with Tukey's post hoc. Data compared for healthy individuals only (n=8).

** P<0.05 versus DR^{mid} Intermediate; † P<0.05 versus DR^{hi} Intermediate;*
§ P<0.05 versus Classical

Table 5.2: Surface Expression of Migration, Atherogenic and Inflammatory Markers

	Resolution Metric (R_D) Calculated from Molecules of Equivalent Soluble Fluorescence (MESF)			
	Classical	**DR^{mid}**	**DR^{hi}**	**Non-Classical**
CCR2 p(1,3) <0.0001; F=157.4	**2.30** *(2.11, 2.49)*	**2.23** *(2.06, 2.41)*	**1.59** *(1.41, 1.78)*	**0.94** *(0.77, 1.11)*
versus DR^{mid}	*0.06 (-0.05, 0.19); p=0.41*	-	*-0.64 (-0.84, -0.44); p<0.0001*	*-1.29 (-1.49, -1.10); p<0.0001*
versus DR^{hi}	*0.71 (0.52, 0.90); p<0.0001*	*0.64 (0.44, 0.84); p<0.0001*	-	*-0.65 (-0.88, -0.42); p<0.0001*
PSGL-1 p(1,3) <0.0001; F=99.49	**10.06** *(9.28, 10.84)*	**10.09** *(9.26, 10.92)*	**7.68** *(7.05, 8.32)*	**7.06** *(6.42, 7.71)*
versus DR^{mid}	*-0.03 (-0.52, 0.46); p=0.99*	-	*-2.41 (-2.86, -1.86); p<0.0001*	*-3.02 (-3.83, -2.22); p<0.0001*
versus DR^{hi}	*2.38 (1.86, 2.89); p<0.0001*	*2.41 (1.86, 2.86); p<0.0001*	-	*-0.62 (-1.21, -0.02); p=0.038*
CD36 p(1,3) <0.0001; F=125.9	**4.55** *(4.10, 5.02)*	**4.54** *(4.15, 4.93)*	**3.71** *(3.35, 4.08)*	**2.37** *(2.04, 2.69)*
versus DR^{mid}	*0.01 (-0.30, 0.31); p=0.99*	-	*-0.82 (-1.17, -0.48); p<0.0001*	*-2.17 (-2.46, -1.89); p<0.0001*
versus DR^{hi}	*0.83 (0.40, 1.27); p=0.0001*	*0.82 (0.48, 1.17); p<0.0001*	-	*-1.34 (-1.69, -0.99)*
LRP-1 p(1,3) <0.0001; F=101.2	**5.41** *(4.97, 5.85)*	**5.41** *(4.94, 5.88)*	**4.56** *(4.21, 4.91)*	**2.55** *(2.19, 2.89)*
versus DR^{mid}	*0.002 (-0.48, 0.48); p=0.99*	-	*-0.88 (-1.37, -0.33); p=0.0008*	*-2.17 (-2.46, -1.89); p<0.0001*
versus DR^{hi}	*0.85 (0.37, 1.33); p<0.0001*	*0.88 (0.33, 1.37); p=0.0008*	-	*-1.34 (-1.69, -0.99); p<0.0001*
TLR-4 p(1,3) <0.0001; F=21.08	**2.79** *(2.49, 3.10)*	**2.79** *(2.48, 3.09)*	**2.58** *(2.30, 2.86)*	**2.47** *(2.20, 2.73)*
versus DR^{mid}	*0.01 (-0.07, 0.88); p=0.99*	-	*-0.21 (-0.33, -0.08); p=0.0008*	*-0.32 (-0.48, -0.15); p=0.0001*
versus DR^{hi}	*0.21 (0.08, 0.35); p=0.0012*	*0.21 (0.08, 0.34); p=0.0009*	-	*-0.11 (-0.24, 0.02); p=0.13*
CD163 p(1,3) <0.0001; F=21.08	**2.57** *(2.23, 2.91)*	**2.27** *(1.98, 2.55)*	**2.50** *(2.08, 2.92)*	**0.40** *(0.31, 0.51)*
versus DR^{mid}	*0.30 (0.002, 0.6); p=0.04*	-	*0.24 (-0.15, 0.63); p=0.34*	*-1.86 (-2.24, -1.48); p<0.0001*
versus DR^{hi}	*0.06 (-0.27, 0.40); p=0.95*	*-0.24 (-0.63, 0.15); p=0.34*	-	*-2.10 (-2.65, -1.48); p<0.0001*

Data expressed as mean, or mean difference, with 95% Confidence Intervals.
Statistical Analysis for multiple group comparisons - Repeated Measures (RM) ANOVA with Tukey's Post hoc test.

Resolution Metric (R_D):
[MESF(stained) - MESF(FMO)] / [rSD (stained) + rSD(FMO)]

MESF = Molecules of Equivalent Molecular Fluorescence;
FMO = Flow minus one control;
rSD = Robust Standard Deviation

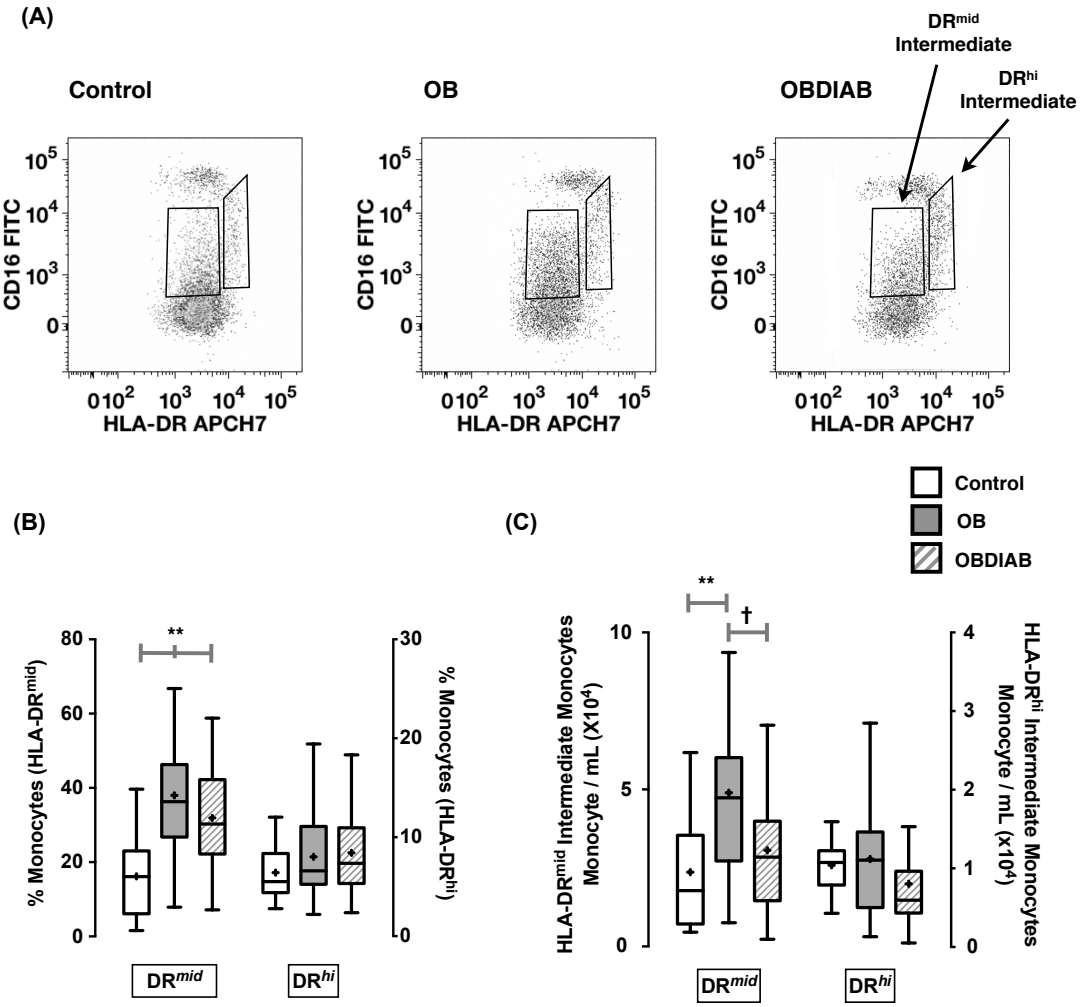
5.4.2 DR^{mid} Intermediate Monocytes are Expanded in Obesity

Sub-analyses of intermediate monocytes in peripheral blood indicated that proportions and absolute numbers of the DR^{mid} [ANOVA: p(1,2)<0.0001; F=18.35] but not the DR^{hi} [ANOVA: p(1,2)=0.31; F=1.94] subpopulations differed across the study groups. *Post hoc* analyses demonstrated that the proportions of DR^{mid} intermediate monocytes were higher in OB [37.98% (32.06, 43.89)] and in OBDIAB [32.01 (26.88, 37.13)] compared to controls [14.43 (8.78, 20.09); *Mean Difference:* 23.54 (13.72, 33.36) p<0.01 (*versus OB)* and 17.57 (8.41, 26.73) p<0.01 (*versus OBDIAB)*] (Figure 5.6: A-C). While the absolute circulating number of DR^{mid} intermediate monocytes differed between OB [4.89 (3.89, 5.89) x 10^4 monocytes/mL] and control individuals[2.37 (1.41, 3.33) x 10^4 monocytes/mL; *Mean Difference:* 2.52 (0.86, 4.19) x 10^4 monocytes/mL p<0.01], there was no difference in circulating DR^{mid} intermediate monocyte count between OBDIAB and control individuals. This was reflective of the lower total circulating monocyte count in the OBDIAB group (Chapter 4; Section 4.4.1; Figure 4.1). Hence in the case of OB and OBDIAB individuals, the expanded intermediate monocyte subpopulation could be explained by an increase in a discrete subset of intermediate monocytes, namely the DR^{mid} subset. Summary statistics and multiple group comparisons of data relating to intermediate monocyte subsets amongst the three study groups is presented in Table 5.3.

Sub-analyses of the expression of CCR-2, PSGL-1, CD36, LRP-1 and TLR-4 on the DR^{mid} intermediate monocyte subset were performed across the patient groups. There was a significant intergroup difference for the expression of CCR-2, PSGL-1, CD36 and LRP-1 across all participant groups. OB individual demonstrated higher

expression for each surface marker when compared to their control counterparts. OB individuals also showed higher expression of CCR-2 and PSGL-1, when compared to their OBDIAB counterparts. However, no significant difference was seen for the surface expression of these markers when comparisons were made between control and OBDIAB individuals (Figure 5.7).

Figure 5.6: DR^{mid} and DR^{hi} Intermediate Monocyte Subsets in Obesity & Type 2 Diabetes



Comparisons of monocyte distribution in which the classification of the intermediate monocyte subpopulation is refined based on DR^{mid} and DR^{hi} intermediate monocytes from from Control (Clear; n=33) OB (Shaded; n=42) and OBDIAB (Patterned; n=53) groups. **(A)** Representative two-dimensional flow cytometry scatter plots demonstrating the predominant expansion of DR^{mid} intermediate monocytes in OB and OBDIAB individuals; **(B)** Boxplots comparing proportional changes of the DR^{mid} and DR^{hi} intermediate subsets across study groups and demonstrating a significant increase in DR^{mid} but not the DR^{hi} subset in OB and OBDIAB; **(C)** Boxplots comparing the circulating monocyte counts for DR^{mid} and DR^{hi} intermediate monocyte subsets which show that the OB group had a higher circulating DR^{mid} intermediate monocyte count than Control or OBDIAB groups.

Summary statistics are represented as follows:

box limits = interquartile range; median line = median; + = mean

Statistical Analysis: Multivariate Analysis of Variance (MANOVA) with Tukey's posthoc

** P<0.05; ** P<0.01 versus Control*

† P<0.05; ‡ P<0.01 versus Obese

Table 5.3: Intermediate Monocyte Sub-classification: Distribution, Proportions and Counts

Monocyte Subset	Proportion of Total Monocytes (%)			Monocyte Count (Monocyte / mL x 10^4)		
	Control	*OB*	*OBDIAB*	*Control*	*OB*	*OBDIAB*
Intermediate	**21.24**	**46.00****	**40.47****	**3.41**	**6.01****	**3.87†**
p(1,2)<0.0001; F=19.32	*(15.38, 27.13)*	*(39.87, 52.13)*	*(35.16, 45.78)*	*(2.33, 4.48)*	*(4.87, 7.14)*	*(2.89, 4.84)*
Mean Difference v Control	-	***24.76 (14.57, 34.94)***	***19.23 (9.73, 28.73)***	-	***2.60 (0.74, 4.47)***	***0.46 (-1.29, 2.20)***
DR*mid* Intermediate	**14.43**	**37.98****	**32.01****	**2.37**	**4.89****	**3.06**
p(1,2)<0.0001; F=18.35	*(8.78, 20.09)*	*(32.06, 43.89)*	*(26.88, 37.13)*	*(1.41, 3.33)*	*(3.89, 5.89)*	*(2.20, 3.93)*
Mean Difference v Control	-	***23.54 (13.72, 33.36)***	***17.57 (8.41, 26.73)***	-	***2.52 (0.86, 4.19)***	***0.70 (-0.86, 2.25)***
DR*hi* Intermediate	**6.81**	**8.02**	**8.47**	**1.04**	**1.12**	**0.80**
p(1,2)=0.31; F=1.94	*(5.19, 8.41)*	*(6.34, 9.71)*	*(7.01, 9.93)*	*(0.79, 1.29)*	*(0.86, 1.38)*	*(0.58, 1.03)*
Mean Difference v Control	-	***1.22 (-1.59, 4.02)***	***1.65 (0.96, 4.27)***	-	***0.08 (-0.35, 0.51)***	***-0.24 (-6.38, 0.16)***

Control: Healthy Individuals, BMI < 25kg/m^2.

OB: Obese Individuals, BMI ≧ 30kg/m^2 without type 2 diabetes.

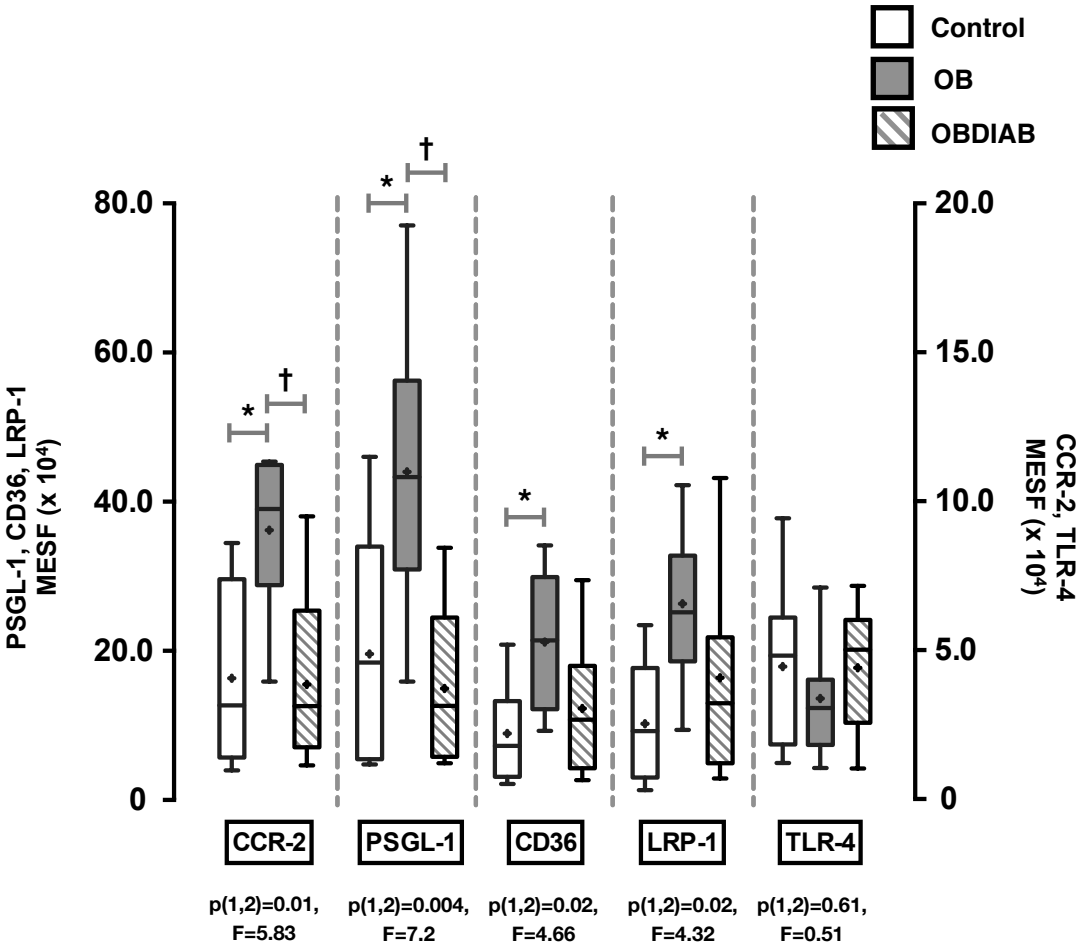
OBDIAB: Obese Individuals, BMI ≧ 30kg/m^2 with type 2 diabetes.

Multiple Group Comparisons - Multiparameter Analysis of Variance (MANOVA). Post hoc analysis for individual between group comparisons: Tukey's test.

Data expressed as mean with 95% Confidence Intervals

** p<0·05; ** p<0.01 versus Control; † p< 0·05 versus Obese*

Figure 5.7: Surface Expression of Inflammatory and Migration Markers in DR^{mid} Intermediate Monocytes



Boxplots demonstrating comparisons for surface molecule expression on DR^{mid} intermediate monocytes between study participant groups namely Control (clear) (n=10), Obese (OB) without diabetes (shaded) (n=10) and obese with type 2 diabetes (OBDIAB) (diagonal lines) (n=10) for chemokine-chemokine (C-C motif) receptor -2 (CCR-2); P-selectin glycoproetin ligand -1 (PSGL-1); CD36; lipoprotein receptor associated protein -1 (LRP-1) and toll-like receptor -4 (TLR-4). OB individuals demonstrated higher expression for CCR-2, PSGL-1, CD36 and LRP-1.

Summary statistics are represented as follows:

box limits = interquartile range; median line = median; + = mean

Statistical Analysis: Multivariate Analysis of Variance (MANOVA) with Tukey's post-hoc

** P<0.05 versus Control*

† P<0.05 versus Obese

5.4.3 HLA-DR^{mid} intermediate monocytes are associated with clinical parameters of poor metabolic health.

Expansion of the total intermediate monocyte population was associated with clinical parameters of poor metabolic health (weight, BMI, HbA1c, triglycerides, LDL and HDL) (Chapter 4: Section 4.4.3, Figure 4.5, Table 4.2). However, this analysis did not take account of intermediate monocyte sub-classification according to their expression of HLA-DR (Figure 5.3). Considering the heterogeneity of DR^{mid} and DR^{hi} intermediate monocytes and the predominant expansion of DR^{mid} subset in obesity, their association with parameters of poor metabolic health was investigated using adjusted multivariate analysis.

Principal component analysis demonstrated that DR^{mid} intermediate monocyte counts and proportions clustered with unfavourable metabolic indices such as serum HbA1c, LDL and BMI. The relationships with clinical markers of poor metabolic health applied predominantly to DR^{mid} intermediate monocytes and were generally stronger for DR^{mid} intermediate monocytes than for the total intermediate population (Tables 4.2 and 5.4; Figure 5.8).

The relationships between DR^{mid} intermediate monocytes and individual metabolic indices within the entire study cohort are shown in Table 5.4. Following adjustment for interactions and the influence of covariates, DR^{mid} intermediate monocyte count showed a strong association with higher LDL levels while DR^{mid} proportions directly correlated with BMI, HbA1c and inversely correlated with HDL. The relationship between DR^{mid} intermediate monocytes and adverse indices of metabolic health persisted when analyses were performed selecting only OB and Control individuals, excluding OBDIAB individuals.

Table 5.4: The relationship between intermediate monocytes and clinical characteristics across the study cohort

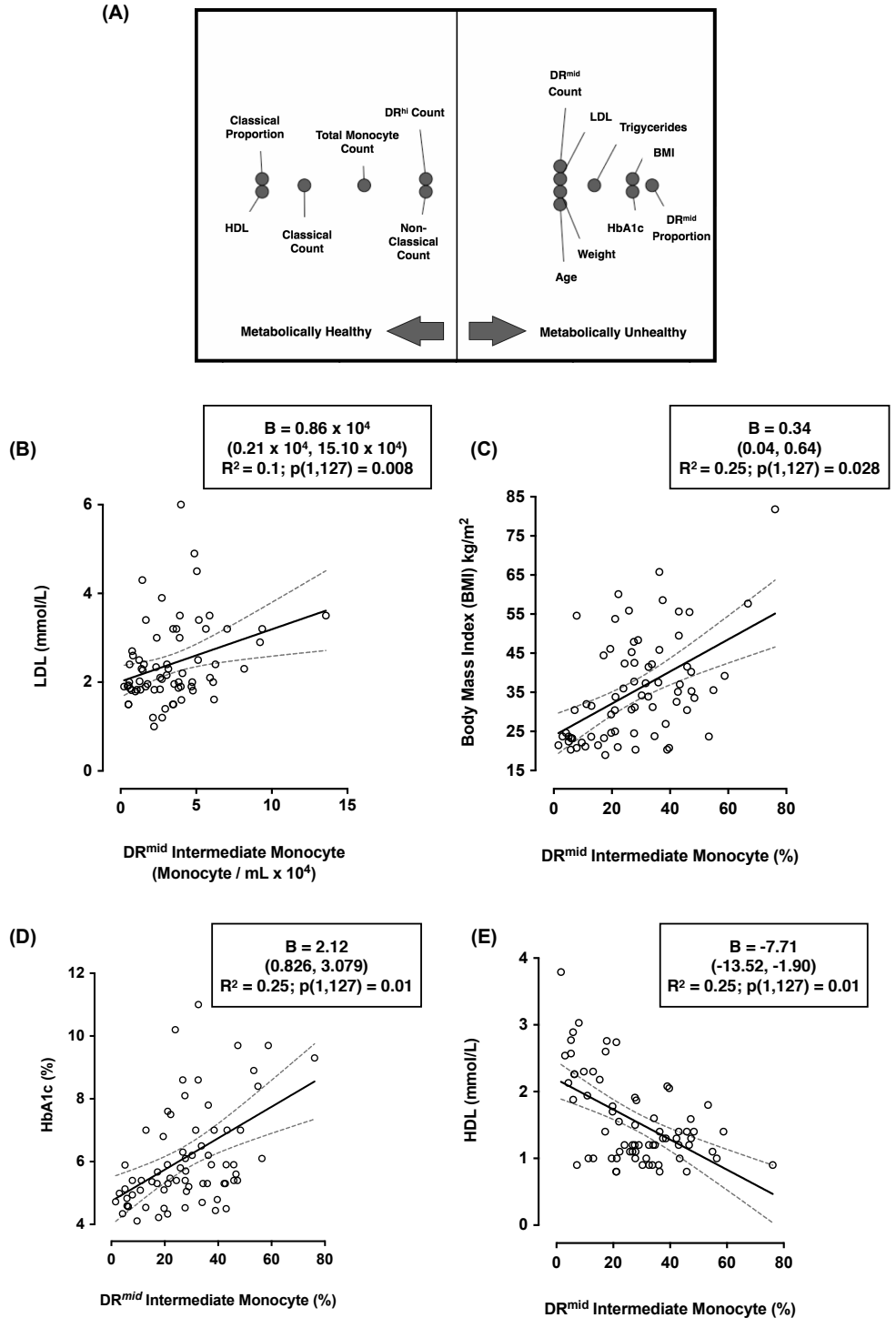
	BMI	LDL	HDL	Triglycerides	HbA1c	Age
Monocyte Counts ***(Monocyte / mL)***						
Intermediate	94.0 (-559, 747) p(1,127)=0.77	***0.84 (0.1, 1.58) x 10^4 p(1,127)=0.03****	-0.98 (-2.1, 0.16) x 10^4 p(1,127)=0.09	-0.14 (-1.1, 0.79) x 10^4 p(1,127)=0.75	-0.4 (-0.8, 0.05) x 10^4 p(1,127)=0.09	-10.6 (-596, 574) p(1,127)=0.97
DR^{mid} Intermediate	0.34 (0.04, 0.64) p(1,127)=0.028	***0.86 (0.21, 15.10) x 10^4 p(1,127)=0.008****	-0.63 (-1.5, 0.2) x 10^4 p(1,127)=0.16	-0.13 (-0.9, 0.7) x 10^4 p(1,127)=0.75	-0.2 (-0.6, 0.2) x 10^4 p(1,127)=0.26	43.5 (-479, 566) p(1,127)=0.86
DR^{hi} Intermediate	-54.2 (-198, 72) p(1,127)=0.39	0.9 (-0.6, 2.5) x 10^4 p(1,127)=0.38	-0.70 (-13.3, 1.9) x 10^4 p(1,127)=0.59	-23.2 (-2246, 2199) p(1,127)=0.98	***-1.2 (-2.1, -0.4) x 10^4 p(1,127)=0.05****	-49 (-160, 61) p(1,127)=0.37
Monocyte Proportion (%)						
Intermediate	0.29 (-0.02, 0.60) p(1,127)=0.07	3.06 (-0.51, 6.63) p(1,127)=0.09	***-9.71 (-15.75, -3.67) p(1,127)=0.02****	1.05 (-3.24, 5.34) p(1,127)=0.57	0.74 (-1.29, 2.78) p(1,127)=0.47	-0.05 (-0.33, 0.24) p(1,127)=0.75
DR^{mid} Intermediate	***0.34 (0.04, 0.64) p(1,127)=0.03****	***2.97 (0.47, 6.4) p(1,127)=0.05****	***-7.71 (-13.52, -1.89) p(1,127)=0.01****	0.26 (-4.23, 4.75) p(1,127)=0.91	1.05 (-1.08, 3.18) p(1,127)=0.90	-0.02 (-0.25, 0.29) p(1,127)=0.90
DR^{hi} Intermediate	-0.05 (-014, 0.04) p(1,127)=0.27	-0.14 (-1.2, 0.94) p(1,127)=0.79	***-1.5 (-2.92, -0.09) p(1,127)=0.04****	0.92 (-0.37, 2.23) p(1,127)=0.16	-0.31 (-0.94, 0.33) p(1,127)=0.33	-0.05 (-0.12, 0.02) p(1,127)=0.16

Statistical analysis: Backward stepwise multivariate linear regression analysis.

Data expressed as Regression Coefficients (B) and 95% Confidence Intervals (CI)

Statistically significant associations are stressed in Semibold Italic and highlighted by an asterix (*)

Figure 5.8: Principal Component Analysis and Simple Linear Regression for DR^{mid} Intermediate Monocytes and Clinical Parameters of Metabolic Health



Accompanying figure legend overleaf.

Multivariate analysis for all study participants (n=128) which models monocyte distribution as a predictor for clinical markers of metabolic health. **(A)** Principal component analysis (PCA) plot which shows the close clustering of clinical markers associated with adverse metabolic health such as BMI, HbA1c, LDL and triglycerides with the DR^{mid} intermediate subset. Linear regression plots show the direct correlation between DR^{mid} intermediate monocytes with LDL **(B)**, BMI **(C)**, HbA1c **(D)**. The inverse correlation between DR^{mid} intermediate monocytes and the positive marker of metabolic health, HDL, is shown in (E). Regression coefficients (B) for adjusted stepwise multiple regression analysis, which included all covariates are shown in boxes above each graph.

Serum triglycerides (TG) (mmol/L), low density lipoprotein (LDL) (mmol/L), high density lipoprotein (HDL) (mmol/L), body mass index (BMI) (kg/m2), systolic blood pressure (Systolic BP) (mmHg), glycosylated haemoglobin (HbA1C) (%) and age (years).

Summary statistics are represented as follows:

Best fit line + 95% Confidence Interval (CI) Limits

Statistical Analysis: Multivariate Linear Regression Analysis with Tukey's post hoc

B = Regression Coefficient; (x, y) = 95% CI;

R^2 = Coefficient of Determination

5.5 Discussion

This set of experiments has demonstrated for the first time that the intermediate monocyte subpopulation can be divided into two phenotypically distinct subsets, designated DR^{mid} and DR^{hi} intermediate monocytes. Both intermediate subsets are readily identifiable by flow cytometry and have differing relationships with accepted parameters of metabolic health and cardiovascular risk factors.[160, 161] DR^{mid} intermediate monocytes were closer in phenotype to classical monocytes and displayed higher surface expression of molecular markers associated with monocyte migration, lipogenesis and lipoprotein uptake. Interestingly, these markers are also highly expressed on foam cells in atherosclerosis and on metabolically adverse, crown-like structure omental macrophages in obesity.[30, 76, 147] The DR^{mid} subset accounted for the intermediate monocyte expansion apparent in association with obesity as demonstrated in Chapter 5. Moreover, the DR^{mid} subset accounted predominantly for the association between higher intermediate monocyte subpopulations and parameters of both poor metabolic health and cardiovascular risk. Finally, on investigation of monocyte phenotype across the study groups, DR^{mid}, but not DR^{hi} intermediate monocytes demonstrated higher expression of surface markers associated with atherogenesis and inflammation in obese individuals. These data suggest that DR^{mid} intermediate monocytes represent a distinct monocyte subpopulation which drive a chronic inflammatory process underlying atherosclerosis and insulin resistance.

5.5.1 Distinct Intermediate Monocyte Subsets - DR^{mid} versus DR^{hi}

Analysis of human monocytes has evolved significantly in the last number of years

but extrapolation of results between mouse and humans remains challenging due to interspecies differences of subclass, particularly due to the absence of a mouse intermediate subpopulation equivalent.[29] Moreover, consensus is currently lacking with regard to the phenotype relating to intermediate monocytes.[18, 45] In the current study it is clear that intermediate monocyte heterogeneity can be explained, at least in part, by the existence of distinct DR^{mid} and DR^{hi} subsets which differ in size and granularity and which also differ across a range of phenotypic markers. Intermediate monocyte subtype was further analysed focusing on surface expression of molecular markers known to be associated with atherogenesis and insulin resistance namely, TLR-4, CCR-2, CD36, LRP-1 and PSGL-1.[14, 16, 76] Surface expression for each marker was highest on DR^{mid} intermediate and classical monocytes and lowest on DR^{hi} intermediate and non-classical monocytes. Quantitative RT-PCR also demonstrated lower expression of PPARγ on DR^{mid} intermediate monocytes, suggesting that they represent a pro-inflammatory population of monocytes.[72, 102] Expression of DGAT-1 and VEGF-A, which play roles in intracellular lipogenesis and atherogenesis respectively were increased on DR^{mid} intermediate monocytes, while decreased on their DR^{hi} counterparts.[95, 162]

The consistent differences between the two intermediate monocyte subsets indicates that they likely represent distinct subtypes, with the DR^{mid} population alone having a profile similar to that of foam cells, macrophages with known atherogenic properties and omental macrophages associated with crown like structures in obesity and insulin resistance.[76, 143] It is also noteworthy that DR^{mid} intermediate and classical monocytes shared many phenotypic attributes. Thus DR^{mid}, but not DR^{hi}, intermediate monocytes share a potentially metabolically adverse phenotype with classical monocytes.

5.5.2 DR^{mid} Intermediate Monocytes are Expanded in Obesity and Type 2 Diabetes

Two striking features of monocyte distribution were apparent in all obese individuals. There was a marked depletion of classical monocytes associated with a directly proportional expansion of the DR^{mid} , but not the DR^{hi}, intermediate subset and this pattern of monocyte distribution was robustly associated with clinical parameters of poor metabolic health, whereby higher proportions and counts of DR^{mid} intermediate monocytes were associated with higher LDL, HBA1c and BMI but lower HDL measurements. Additionally, surface expression for each of the markers CCR2, PSGL-1, CD36 and LRP-1, respectively associated with inflammation, monocyte migration and lipoprotein uptake,[17, 76] was higher on DR^{mid} intermediate monocytes isolated from obese individuals without a diagnosis of diabetes. This was not apparent for individuals with a diagnosis of diabetes. Two factors should be considered when interpreting these data.

Firstly, the majority of individuals with a diagnosis of type 2 diabetes were medicated with statins, ACEi/ARBs and aspirin, whereas obese non-diabetic individuals were not. Each of these medications has previously been associated with reduced expression of receptors associated with inflammation and lipoprotein uptake in macrophages.[130, 163, 164] Obese study participants, without diabetes, also had higher circulating levels of LDL and lower HDL when compared to their diabetic counterparts, which reflects statin therapy within this study group (Chapter 3). Overall, these data suggest that circulating pools of classical and DR^{mid} intermediate monocytes respond dynamically and in coordinated fashion to a lipid and glucose rich plasma.

Poitou et al recently described higher CD16+ intermediate and non-classical

proportions in obese individuals.[129] Observations from the set of experiments presented herein, relating to the intermediate subpopulation, are consistent with the report of Poitou *et al*. However, there was no expansion observed for non-classical monocytes. Additionally, by applying a modified flow cytometric gating technique and multivariate analysis, this experimental work has extended the findings of Poitou *et al* describing the specific association of the phenotypically distinct DR^{mid} monocytes to obesity and metabolic disease. The calculation of circulating monocyte numbers for each subpopulation, has added a more comprehensive interpretation of monocyte repertoire in human metabolic and cardiovascular disease. There was also a robust association between DR^{mid} intermediate monocytes and cardiovascular risk factors, using an adjusted multivariate analysis. Prior studies have shown only univariate associations between non-classical or intermediate monocyte subpopulations, which were off-set by the effects of covariates such as obesity.[125, 129]

The data from this set of experiments points significantly towards a role for DR^{mid} intermediate monocytes as pro-atherogenic cells which are involved in the processes of insulin resistance and cardiovascular complications of type 2 diabetes. In Chapter 6 the functional role of these monocytes in the uptake of ApoB containing lipoproteins is explored further.

Section 3: Results

Chapter 6

The Effects of Lipoprotein Exposure on Human Monocytes

6.0 The Effects of Lipoprotein Exposure on Human Monocytes

6.1 Introduction

Intermediate monocytes, specifically the DR^{mid} subset, demonstrate phenotypic properties which suggests that they contribute significantly to metabolic and cardiovascular disease. Experimental work, described in Chapter 5, showed that DR^{mid} intermediate monocytes highly express the native lipoprotein receptor, LRP-1 in addition to the scavenger receptor CD36, suggestive of a higher propensity for lipoprotein uptake amongst these cells.

The uptake of ApoB containing lipoproteins by cells of monocyte / macrophage lineage represents the central pathogenic element to foam cell formation in the atherosclerotic plaque. Monocytes are initially recruited to subendothelial spaces in which deposits of ApoB lipoproteins have accumulated, where they remove the excess cholesterol esters. This occurs through a regulated process involving lipoprotein uptake via LDL-R, LRP-1 and scavenger receptors, followed by the intracellular processing of cholesterol esters and eventual delivery of free cholesterol to Apo-A1 and HDL cholesterol for reverse cholesterol transport to the liver (Chapter 2; Section 2.4.2; Figures 2.4 & 2.5).[76, 110] Unsustainable and continued assault of infiltrating monocytes by high concentrations of ApoB lipoproteins, switches on an up-regulated system, driven by scavenger receptor predominant uptake of lipoproteins which lacks the appropriate regulatory safeguards of the native lipoprotein receptor mediated system.[24, 118] The native LDL receptors become down-regulated and free cholesterol becomes re-esterified within the cells, resulting in foam cell formation (Figure 2.5). This process becomes compounded by the presence of modified forms of LDL in the

subendothelial space, namely oxidised (ox) LDL and acetylated (ac) LDL, both of which are formed by excessive oxidative activity of the inflammatory process. Both oxLDL and acLDL are absorbed solely through a scavenger receptor mediated mechanism, each taken up by both CD36 and SR-A.[26] Hence, while scavenger receptor up-regulation is ongoing, the production of modified forms of LDL is increasing and the process of atherogenesis is magnified exponentially.

There is significant evidence that adipocyte apoptosis in the obese omentum, similarly liberates an excess of ApoB lipoproteins and triglycerides, which overwhelm infiltrating cells of monocyte / macrophage lineage and result in formation of crown like structures (Figure 2.6).[95, 139, 147] High circulating concentrations of LDL are causally associated with atherogenesis and metabolic disease.

While higher expression of CD36 and LRP-1 on DR^{mid} intermediate monocytes suggested greater potential for lipoprotein uptake on these cells, the functional aspects of lipoprotein uptake had not been formally evaluated. The experimental data presented in this chapter demonstrate (i) the quantitative uptake of lipoproteins across each of the human monocyte subpopulations and (ii) the regulation of native LDL and scavenger receptors in response to native and modified forms of LDL.

6.2 Hypothesis and Objectives

6.2.1 Hypothesis

DR^{mid} intermediate monocytes represent an atherogenic monocyte subpopulation, primed to readily scavenge lipoproteins in the setting of atherosclerosis.

6.2.2 Objectives

(i) To quantitatively evaluate the uptake of native and acLDL in each of the four monocyte subpopulations, inclusive of the DR^{hi} and DR^{mid} intermediate subsets.

(ii) To investigate the regulation of surface expression of the lipoprotein receptor, LRP-1 and the scavenger receptor, CD36, in response to a high lipoprotein environment.

6.3 Experimental Design and Statistical Analysis

6.3.1 Experimental Design

6.3.1.1 Sample Collection

Peripheral blood was collected from control individuals of normal BMI and without a diagnosis of type 2 diabetes in EDTA-containing tubes (Vacutainer®), for monocyte analysis. PBMCs were isolated using gradient centrifugation (Chapter 3; Section 3.3.2) and resuspended in RPMI 1640 (Sigma-Aldrich) containing 2% fetal calf serum (Lonza).

6.3.1.2 Culture Conditions

Lipoprotein uptake was quantified using the bodipy® conjugated ApoB lipoproteins LDL and acLDL respectively (Invitrogen®) (Chapter 3; Section 3.5). Following isolation of the PBMC fraction, monocytes were enumerated as described previously (Chapter 3; Section 3.3.2) and seeded in 96 well, round bottomed plates (Nunc®) at a cell concentration of 250,000 monocytes in 125μL culture medium. PBMCs were cultured under constant motion in a shaking incubator at 50rpm in the presence or absence of LDL (10μg/mL) or acLDL (5μg/mL) for 1 hour. This timepoint was chosen based on time-course experimentation as shown in Figures 6.1. Conditions for cell culture are outlined in Table 6.1.

Table 6.1: Culture Conditions for Lipoprotein Uptake in Human Monocytes

	Conditions				
Volume (µL)	**1**	**2**	**3**	**4**	**Control**
Cells	100	100	100	100	100
RPMI 1640	22.5	22.5	22.5	22.5	22.5
Fetal Calf Serum	1.25	1.25	1.25	1.25	1.25
Bodipy-LDL (1.0mg/mL Stock)	1.25	-	-	-	-
Bodipy-acLDL (0.5mg/mL Stock)	-	1.25	-	-	-
acLDL (0.5mg/mL Stock)	-	-	1.25	-	-
LDL (1.0mg/mL Stock)	-	-	-	1.25	-
FACS Buffer	-	-	-	-	1.25

Cells were added at a concentration of 250,000 monocytes per 100mcL to a total volume of 125mcL. Cells were incubated in the presence or absence of lipoprotein for 1 hour in a shaking incubator at 50rpm.

Bodipy conjugated lipoprotein was used to quantify lipoprotein absorption. Unlabelled lipoprotein was used to investigate the regulation of surface molecules, LRP-1 and CD36 in response to a high lipoprotein environment.

6.3.1.3 Measurement of Lipoprotein Uptake

Following an incubation period of 1 hour, culture plates containing monocytes, in the presence of lipoprotein were immediately placed on ice, following which they were centrifuged (400g at 4°C) and the supernatant removed. Samples were then washed twice and afterwards stained for flow cytometric analysis as demonstrated in Table 6.2. Each monocyte subpopulation was identified using the gating strategies highlighted in Chapter 5 (Section 5.4.1.1; Figure 5.3) into the following subpopulations: classical, non-classical, DR^{mid} intermediate and DR^{hi} intermediate. An optimised time point for lipoprotein uptake was selected following preliminary experimentation examining lipoprotein uptake rates against time (Figure 6.1).

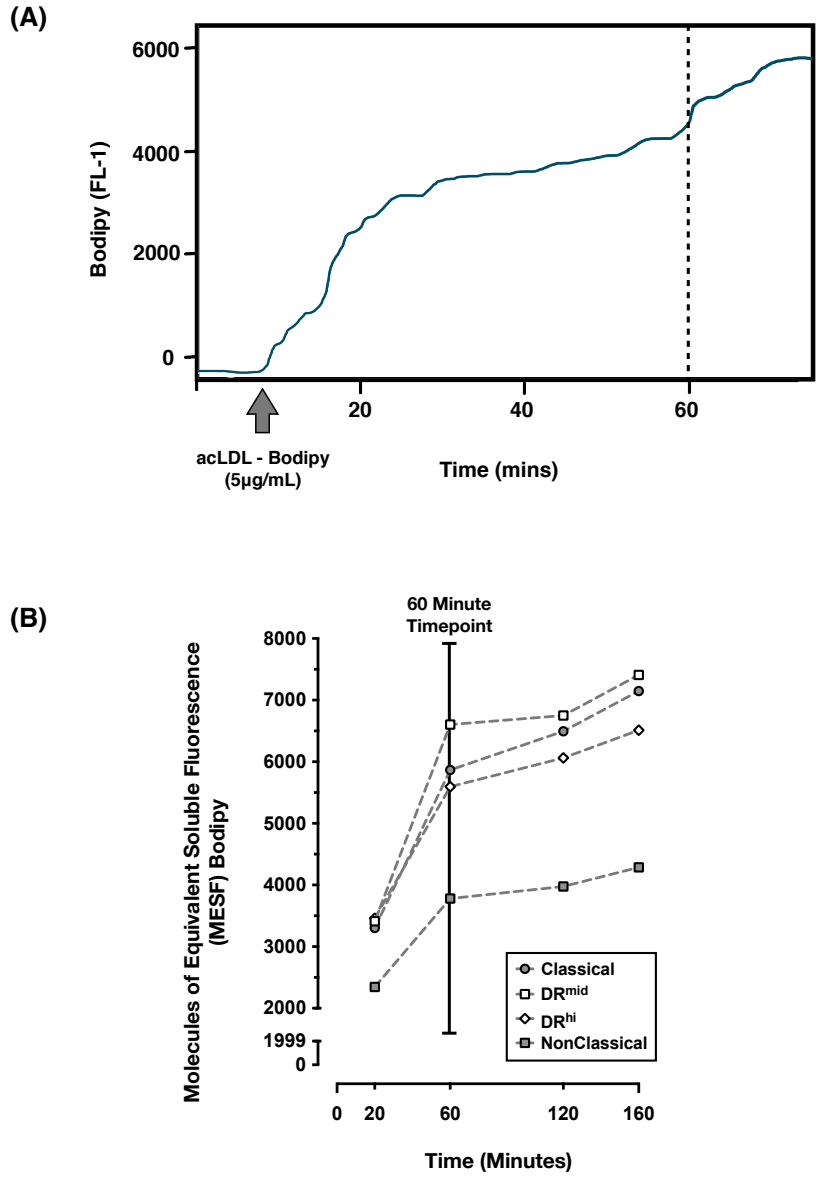
In order to evaluate the effects of a high lipoprotein environment on scavenger and lipoprotein receptors on each monocyte subpopulation, Samples were also stained to identify CD36 and LRP-1 before and after incubation with unlabelled acLDL or LDL.

Table 6.2: **Flow Cytometry Staining Matrix**

Stain	Cells / Beads	APC	APC.H7	PerCp-Cy5.5	FITC	PE
SS APC	Beads	CD45	-	-	-	-
SS APC.H7	Beads	-	CD45	-	-	-
SS PerCp-Cy5.5	Beads	-	-	CD14	-	-
SS FITC	Beads	-	-	-	CD45	-
SS PE	Beads	-	-	-	-	CD45
SS Bodipy	Cells	-	-	-	Bodipy	-
FMO APC	Cells 250,000	-	HLA-DR	CD14	CD16	CD45
FMO APC.H7	Cells 250,000	CD45	-	CD14	CD16	CD45
FMO PerCp-Cy5.5	Cells 250,000	CD45	HLA-DR	-	CD16	CD45
FMO FITC	Cells 250,000	CD45	HLA-DR	CD14	-	CD45
FMO PE	Cells 250,000	CD45	HLA-DR	CD14	CD16	CD45
CD36	Cells 250,000	CD45	HLA-DR	CD14	CD16	CD36
LRP-1 (CD91)	Cells 250,000	CD45	HLA-DR	CD14	CD16	LRP-1
acLDL-Bodipy	Cells 250,000	CD45	CD16	CD14	Bodipy	HLA-DR
LDL-Bodipy	Cells 250,000	CD45	CD16	CD14	Bodipy	HLA-DR

***SS**: Single Stain; **FMO**: Flow Minus One Control; **CD**: Cluster of Differentiation;*
***LRP**: Low density lipoprotein receptor-related protein;*
***HLA**: Human Leukocyte Antigen; **APC**: Allophycocyanin; **APC.H7**: APC-Cyanine Tandem;*
***PerCp**: Peridinin Chlorophyll Protein Complex; **FITC**: Fluorescein Thiocyanate;*
***PE**: Phycoerythrin*

Figure 6.1: Lipoprotein Uptake Kinetics in Human Monocytes



(A) Bodipy® conjugated acLDL (5µg/mL) was added to PBMCs isolated from whole blood. Fluorescence (FL-1 channel) was continuously measured over an 80 minute period on a selected monocyte gate as a measure of acLDL uptake using an Accuri C6® Flow Cytometer.

(B) Demonstrates relative uptake of acLDL (5µg/mL) across individual monocyte subpopulations. Monocytes were incubated with bodipy® conjugated acLDL for 20, 60, 120 and 160 minutes respectively. Monocytes were then gated according to a modified gating strategy (Figure 6.3) into classical, non-classical, DR^{mid} intermediate and DR^{hi} intermediate subpopulations. 60 minute was chosen as an optimal time-point for acLDL and LDL uptake on the basis of its position on the upstroke of the curve, prior to plateau.

6.3.2 Statistical Analysis

This study was designed to characterise the uptake of both LDL and acLDL across monocyte subpopulations specifically to detect inter-individual variations. Multiple group comparisons for each subpopulation were made using a repeated measures (RM) ANOVA for matched samples. The regulation of LRP-1 and CD36 in response to lipoprotein exposure was also analysed using a RM-ANOVA to account for the passage of time and hence to account for "before" *versus* "after" results in matched samples. RM-ANOVA was chosen rather than the paired *Student's t-test*, as this account for the multiple monocyte subpopulations within each sample and hence prevented the *type I error*.

To ensure meaningful interpretation, assumptions for the ANOVA statistical model require that the data is parametrically distributed and also that the data is not widely distributed. Where these assumptions were not met, data transformation was performed ($\log_{10}$ or Square Root). Results and confidence intervals were then appropriately back- transformed.

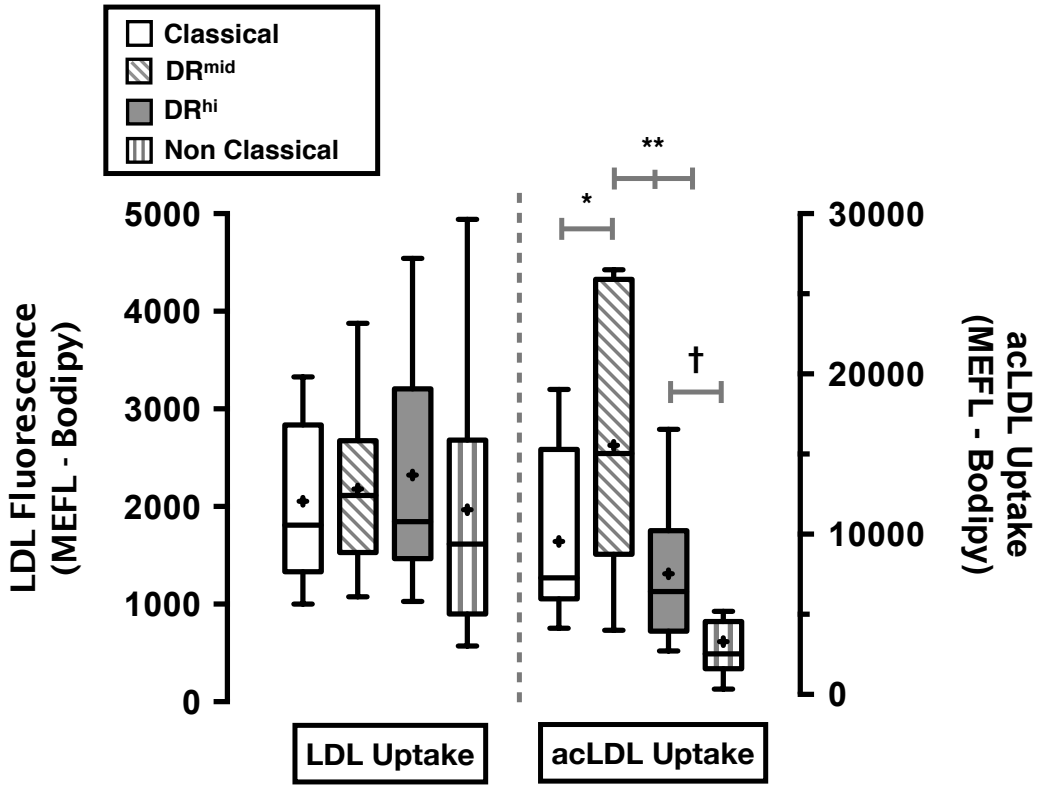
6.4 Results

6.4.1 DR^{mid} Intermediate Monocytes Demonstrate High Lipoprotein Uptake

Each monocyte subpopulation was identified by flow cytometry based on surface marker expression and uptake of LDL and acLDL was then represented for each, according to the fluorescence of bodipy® for each subpopulation.

Uptake of acLDL (n=13 per group) differed across the monocyte subpopulations [p(1,3)<0.0001; F=16.33] and was higher for DR^{mid} when compared to DR^{hi} intermediate monocytes [1.55 (1.05, 2.06) x 10^4 *versus* 0.75 (0.49, 1.01) x10^4; ***Mean Difference:*** 0.80 (0.11, 1.49) x 10^4; p=0.02] (Figure 6.2). Indeed, when compared to all other monocyte subpopulations, DR^{mid} intermediate monocytes demonstrated the highest uptake of acLDL while uptake of unmodified LDL did not differ between the monocyte subpopulations [p(1,3)=0.32; F=1.18] (Figure 6.2). The findings were consistent with the observed higher expression of receptors responsible for lipoprotein uptake, CD36 and LRP-1 on the DR^{mid} subset (Figure 5.5).

Figure 6.2: Lipoprotein uptake across Monocyte Subpopulations



Lipoprotein uptake was measured across all monocyte subpopulations using flow cytometry following incubation with either unmodified low density lipoprotein (LDL) or acetylated LDL (acLDL) conjugated to the fluorescent molecule bodipy (n=13). Quantitative analysis is represented for LDL and acLDL uptake across all monocyte subpopulations, namely Classical (clear), DR^{mid} intermediate (diagonal lines), DR^{hi} intermediate (shaded) and Non-Classical (vertical lines). Whilst the uptake of unmodified LDL was not altered across the subpopulations, DR^{mid} intermediate monocytes showed higher acLDL uptake when compared both to their classical and DR^{hi} counterparts.

Summary statistics are presented as follows:

box limits = interquartile range; median line = median; "+" = mean

Statistical Analysis: Repeated Measures Analysis of Variance (RM-ANOVA) with Tukey's post hoc

** P<0.05; ** P<0.01 versus DRmid Intermediate*

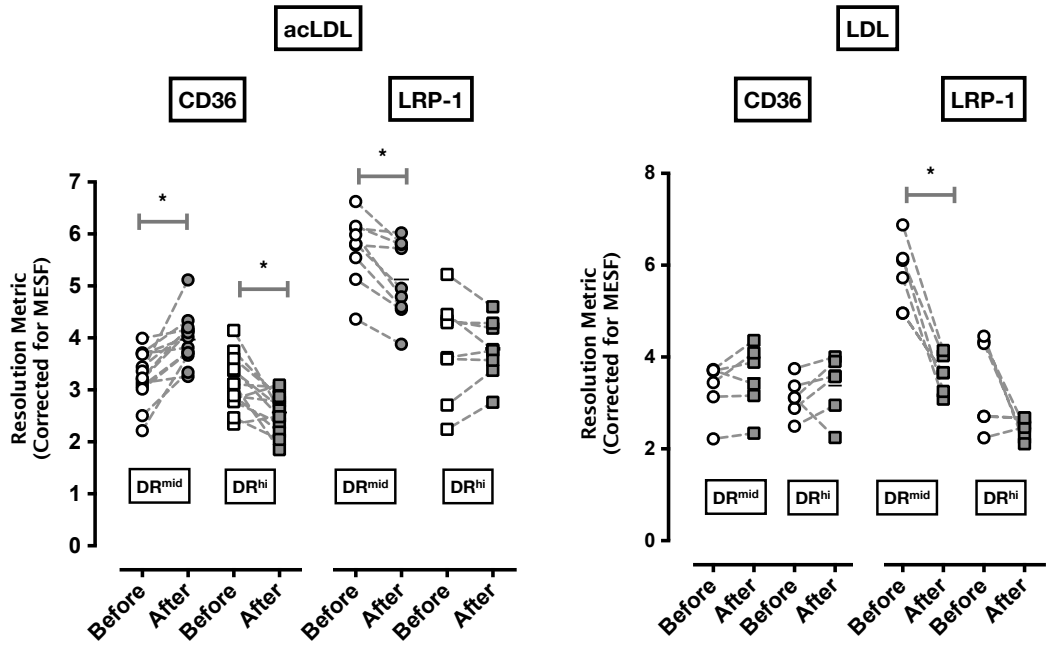
† P<0.05; ‡ P<0.01 versus DRhi Intermediate

6.4.2 Lipoprotein Uptake is Associated with Longitudinal Changes in Scavenger Receptor and Native Lipoprotein Receptors in DR^{mid} Intermediate Monocytes

Surface expression of CD36 on intermediate monocytes was altered in the presence of a high acLDL environment (n=13) [p(1,3)<0.0001; F=29.62]. DR^{mid} expression levels for CD36 expression was higher following incubation with acLDL [***Mean Difference (Pre- versus Post-):*** 0.72 (0.36, 1.07); p(1,3)<0.001], suggesting up-regulation of this receptor on DR^{mid} intermediate monocytes in the presence of modified lipoprotein. Interestingly surface CD36 expression was reduced on DR^{hi} intermediate monocytes in the presence of a high concentration of acLDL [***Mean Difference (Pre- versus Post-):*** -0.58 (-0.94, -0.18); p(1,3)<0.01] The expression of CD36 remained unchanged on both DR^{mid} and DR^{hi} intermediate monocytes in response to high concentrations of native LDL (n=6) [p(1,3)=0.30; F=1.34] (Figure 6.3)

LRP-1 surface expression levels also changed across the intermediate monocyte subsets in response to acLDL and LDL exposure [p(1,3)<0.0001; F=91.79 and p(1,3)<0.0002; F=23.28 respectively]. In contrast to CD36, the expression of LRP-1 was reduced on DR^{mid} intermediate monocytes in response to both high concentrations of acLDL (n=13) [***Mean Difference (Pre- versus Post-):*** -0.59 (-0.95, -0.24); p(1,3)<0.01] and LDL (n=6) [***Mean Difference (Pre- versus Post-):*** -2.16 (-3.09, -1.23); p(1,3)<0.01] while its expression remained unchanged in the DR^{hi} subset of intermediate monocytes (Figure 6.3).

Figure 6.3: Lipoprotein and Scavenger Receptor Regulation in Response to Lipoproteins



Surface expression of the scavenger receptor, CD36 and the native lipoprotein receptor, LRP-1 was measured in response to lipoprotein uptake in each of the DR^{mid} and DR^{hi} intermediate monocyte subsets following exposure to either acLDL (n=13) or LDL (n=6). Repeated measures analyses are represented for each subset prior to (open circles) and following (shaded squares) lipoprotein exposure. CD36 expression was increased on DR^{mid} and decreased on DR^{hi} intermediate monocytes in response to acLDL, while remaining unchanged in response to LDL. LRP-1 expression was decreased on DR^{mid} intermediate monocytes in response to acLDL and LDL.

Summary statistics are presented as follows:

aligned "before/after" dot-plots representing pre-exposure and post-exposure to acLDL

Statistical Analysis: Repeated Measures Analysis of Variance (RM-ANOVA) with Tukey's post hoc

** P<0.05 versus pre-incubation with acLDL or LDL*

6.5 Discussion

The experimental work presented in this chapter demonstrates increased uptake of modified LDL, but not native LDL, by the DR^{mid} intermediate monocyte subpopulation. Increased uptake of acLDL in DR^{mid} intermediate monocytes was accompanied by higher scavenger receptor expression, while expression of the native lipoprotein receptor LRP-1 was decreased. The pattern of acLDL uptake in DR^{hi} monocytes differed from that of their DR^{mid} counterparts whereby they exhibited comparatively lower acLDL uptake, which was conversely associated with reduced scavenger receptor expression and unchanged LRP-1 expression. While LRP-1 receptor expression was reduced in response to native LDL exposure, scavenger receptor expression remained unaffected.

These results demonstrate a number of interesting aspects relating to lipoprotein uptake in intermediate monocytes. DR^{mid} subpopulation of intermediate monocytes, while similar in phenotype to classical monocytes (Chapter 6; Section 6.4.1.2, Figures 6.5 & 6.5), can be distinguished functionally from all other monocyte subpopulations by its higher propensity for uptake of modified LDL. The phenotypic differences between DR^{mid} and DR^{hi} intermediate monocytes demonstrated in Chapter 6 clearly have a functional significance, whereby higher baseline expression of CD36 and LRP-1 in the DR^{mid} subset corresponds with higher uptake of modified lipoprotein. However, the regulation of lipoprotein receptors in response to the presence of high concentrations of acLDL and unmodified LDL respectively suggests that higher acLDL uptake in DR^{mid} intermediate monocytes is modulated by a scavenger receptor mediated mechanism. Unmodified LDL uptake, which does not differ across the monocyte groups is not accompanied by scavenger receptor up-regulation. whereas

the higher acLDL uptake in DR^{mid} intermediate monocytes does. These data also suggest that DR^{hi} intermediate monocytes are protected from excessive acLDL uptake, and possibly foam accumulation, as a result of scavenger receptor down-regulation.

Scavenger receptor mediated lipoprotein uptake modulates the uptake of modified forms of LDL such as acLDL and oxLDL.[26] Moreover, scavenger receptor mediated uptake of modified LDL is associated with increased monocyte ACAT expression, higher intracellular esterification of liberated cholesterol and consequent reduced delivery of free cholesterol to Apo-A1 via cholesterol efflux receptors.[165] Hence the scavenger receptor mediated mechanism of modified lipoprotein uptake, demonstrated by these experiments, occurring predominantly amongst DR^{mid} intermediate monocytes, is likely to underpin the pathogenesis of foam cell formation. It is therefore reasonable to infer that the DR^{mid} intermediate subpopulation of monocytes represents a dominant monocyte precursor candidate for the formation of foam cells in atherosclerosis and possibly the formation of crown like structure macrophages in omental adipose tissue.

Section 4: Conclusion

Chapter 7

Discussion and Future Direction

7.0 Discussion and Future Direction

7.1 Discussion

The experimental data, which are presented in this thesis provide a detailed profile of human monocytes in relation to obesity and treated type 2 diabetes. Arising from these data, it is clear that the intermediate monocyte subpopulation can be further sub-classified into two subsets, namely DR^{mid} and DR^{hi} intermediate monocytes, which differ phenotypically. These intermediate monocyte subsets also differ functionally in relation to metabolic disease and lipoprotein uptake. Specifically, the DR^{mid} subset demonstrates a higher propensity for the uptake of modified, atherogenic lipoproteins through an apparent scavenger receptor mediated mechanism. Consequently, it may be concluded that DR^{mid} intermediate monocytes represent a likely precursor to foam cell formation, in addition to pathogenic macrophage formation in crown like structures.

When adjusted multivariate analysis was applied, higher circulating proportions and counts for DR^{mid} intermediate monocytes were associated with clinically validated markers of metabolic disease and increased cardiovascular risk, namely higher calculated LDL, lower circulating HDL, higher HbA1c and higher BMI. Moreover the circulating proportion of DR^{mid} intermediate monocytes was increased in association with obesity and type 2 diabetes, while the absolute counts for this subpopulation were increased in obese individuals without diabetes. Surface expression of lipoprotein and scavenger receptors, in addition to adhesion molecules was higher on DR^{mid} intermediate monocytes in obese individuals when compared to all other study groups. Given their capacity for uptake of lipoprotein, one may conclude that these

cells are responding in a dynamic manner to a high lipid, high glycaemic and adverse metabolic environment.

These data also provide a novel methodology and a refined approach to accurately profiling circulating monocytes in humans which may be useful for diagnostics and clinical surveillance. Robust, standardised flow cytometry assays were developed to measure parameters of monocyte phenotype and to specifically sort each of the four monocyte subpopulations with a high degree of purity, prior to further analysing each subpopulation using q-RT-PCR. Careful clinical data collection, involved use of an optimised database and these were combined with scientific findings to model robust multivariate analyses of monocyte repertoire in relation to the clinical scenario of obesity in addition to metabolic and cardiovascular risk.

Using these techniques a number of interesting features emerged relating to the individual study groups. It is apparent that, while the metabolic syndrome had not been diagnosed at the time of study recruitment, the majority of individuals within the OB study group had manifested an adverse metabolic profile. The data relating to this study cohort are broadly in line with the findings of NHANES which demonstrated an overall prevalence for the metabolic syndrome of 65% in obese men [aOR 31.92 (20.06–50.78)] and 55% in obese women [aOR=17.14 (12.54–23.44)].[99] Individuals within the OB study group were drug naïve and hence, their lipid and cardiovascular risk profiles were poorer than those within the OBDIAB group who were receiving secondary prevention therapy. The effects of secondary prevention therapy correlated with a lower circulating monocyte count in individuals with a diagnosis of diabetes. Moreover, while their proportions of DR^{mid} intermediate monocytes were higher than lean control individuals, their absolute counts were

lower than obese individuals without diabetes. These interesting data support a direct anti-inflammatory effect of statin or aspirin therapy on the overall monocyte population, or may suggest an effect of these medications on monocytopoesis. It may also reflect a possible effect of angiotensin II inhibition in preventing the release of monocytes from the spleen, which has been shown to be a key factor influencing the development of atherosclerosis and the pathogenesis of myocardial infarction in mouse models.[55] It is possible that we are already treating the effects of altered monocyte repertoire in the setting of metabolic disease using current standards of therapy. These data also present the possibility that specific targeting of pathogenic expansion of monocyte subpopulations, such as the DR^{mid} intermediate monocytes will present a useful therapeutic target for the future management of cardiovascular and metabolic disease.

In summary I present the following key novel findings within this thesis:

(i) these experiments demonstrate reduced total circulating monocyte numbers in obese individuals with type 2 diabetes which, using a multivariate model of analysis, was shown to be associated with secondary prevention therapy.

(ii) a distinct alteration in the distribution of circulating monocyte subpopulations has been shown in obese individuals (both non-diabetic and diabetic) characterised by expansion of intermediate monocytes in comparison to healthy, normal body mass index individuals. We demonstrate these results uniquely as circulating monocyte numbers in addition to proportions.

(iii) strong associations have also been shown between the expansion of the intermediate monocyte population and clinical/biochemical markers of adverse metabolic control.

(iv) this work defines, for the first time, a sub-categorisation of intermediate monocytes according to their surface expression of HLA-DR and CD45 which we have designated DR^{hi} and DR^{mid}, both of which are phenotypically and functionally distinct.

(v) changes to the novel DR^{mid} subpopulation specifically explain the observed intermediate monocyte expansion and its correlation with parameters of poor metabolic health in obesity. Additionally, subpopulation expansion of DR^{mid} intermediate monocytes is robustly and independently associated with parameters of poor metabolic health on multivariate analysis.

(vi) Through detailed phenotypic and functional analyses across all monocyte subpopulations, this work provides additional evidence that DR^{mid} intermediate monocytes possess characteristics consistent with higher pro-atherogenic potential than their counterparts.

Overall, these results add to the growing body of evidence demonstrating that a close relationship exists between the circulating monocyte repertoire and metabolic abnormalities associated with unhealthy lifestyle.[125, 129, 133, 166] By more clearly characterising monocyte derangements in obesity and treated type 2 diabetes and by identifying a novel intermediate monocyte subpopulation that is more specifically associated with adverse cardiovascular and metabolic risk, the experimental work presented herein represents an important step toward defining the pathogenic links between lifestyle, inflammation and metabolic/cardiovascular disease. As a model for

future investigation of the pathobiology of monocyte subpopulations, I propose that the DR^{mid} intermediate monocyte represents a distinct circulating cell type which has high atherogenic potential.

7.2 Future Direction

The overarching hypothesis for this work proposed that ***"in metabolically unhealthy obese individuals, alterations in the repertoire of specific subpopulations of circulating monocytes are associated to higher metabolic and cardiovascular risk, which is modulated through their interactions with circulating lipoproteins"***. The data presented herein demonstrates significant and strong phenotypic, functional and observational data to support this hypothesis. The experimental work presented in this thesis is also hypothesis generating and suggests that in metabolically unhealthy obese individuals, specific monocyte subpopulations drive a predominantly lipid mediated pro-atherogenic and pro-inflammatory pathogenic role common to the progression of insulin resistance, type 2 diabetes and cardiovascular disease.

There are limitations in interpreting the results of this work within the context of how representative the circulating fraction of monocytes is to the behaviour of monocyte/macrophages and dendritic cells at the level of the peripheral tissues. Circulating monocytes have various roles within the circulation including cytokine / chemokine release, surveillance, patrolling, scavenging and antigen presentation. Within the environment of the circulation, monocytes interact with numerous cellular, humoral and hormonal influences which modulate their behaviour and propensity to migrate. Importantly, the circulation also functions as a transportation system for passage of monocytes between the organs where they are synthesised and stored, to the organs where they produce effects and differentiate.[1] This work has shown that the circulating monocyte fraction, while under dynamic conditions, has a consistent pattern in obese, metabolically unhealthy humans. The next set of research questions relate to monocytes in the metabolically adverse environment of obesity within

organs of monocytopoesis and within tissues such as liver, muscle, adipose tissue and the vaculature. Moreover the interaction between human monocytes and other immune cell populations within lymphatic and splenic tissues, that may influence their recruitment to the circulation and into peripheral tissues must be explored in the context of human obesity and poor metabolic health.

Further studies of human monocyte biology are necessary to address these questions. However such human studies of monocyte biology are limited by difficult access to monocyte fractions from tissues such as spleen and bone marrow. Moreover, in the absence of clear subpopulation homology between animal and human models, *in vitro* studies are required to investigate the functional aspects of the monocyte / macrophage / dendritic cell system.

A number of aspects of the current experimental work will be expanded upon for future investigation in addition to addressing questions which have arisen in the course of completing this thesis. Future studies will address the following areas:

(i) ***Endothelial / Monocyte Interactions***. Cross-talk between monocytes and endothelial surfaces represent an important step in recruiting monocytes to sites of tissue injury in addition to eventual monocyte interaction.[4] There is also significant evidence to support differential interactions between individual monocyte subpopulations in animal models and in humans.[18, 167] A significant body of evidence proposes close relationships between so-called endothelial progenitor cells and monocytes.[49, 162, 168] Experimental data are necessary to investigate these interactions further in human monocytes under experimental conditions which more closely mimic the physiological behaviour of these cells.

(ii) ***Lipoprotein Uptake and Lipidomic Profiles of Human Monocytes.*** The experimental work presented in this thesis has developed robust techniques for measuring lipoprotein uptake in human monocytes. Additionally, interesting aspects of modified lipoprotein uptake have been highlighted. Further interesting aspects of lipoprotein uptake have yet to be addressed. These include whether monocytes take up lipoproteins while still circulating, or whether this occurs only within the sub-endothelial space. Further investigation of downstream signalling of CD36 and SRA is required to identify their effects on aspects of cholesterol esterification i.e. the ACAT system, in addition to their effects on regulation of cholesterol efflux through LXR and ABCA / ABCG.

(iii) ***Lipidomic Analyses.*** Another interesting aspect of lipoprotein metabolism which requires investigation is the direct analysis of the lipid, cholesterol and phospholipid composition of lipoproteins drawn from metabolically obese humans.

(iv) M***onocyte Infiltration of Human Omentum.*** Application of flow cytometry techniques developed for this study can be used to analyse in further detail the monocyte / macrophage composition of human omentum, in addition to their lipid composition.

(v) ***Secondary Prevention Therapy.*** These data have demonstrated observational evidence which supports an effect of secondary prevention on circulating human monocyte counts. Further investigation of the specific effects of individual aspects of secondary prevention therapy are necessary to further establish the true aetiology of these observations. Future planned work will investigate the renin, angiotensin, aldosterone system in relation to human

monocyte biology, focusing on subpopulation specific effects using clinical and *in vitro* models.

(vi) ***Monocytes as Prognostic Indicators.*** Prospective longitudinal collection of human samples from the clinical setting, using a semi-automated system of monocyte analysis will be used to assess monocyte repertoire as a predictor of adverse metabolic and cardiovascular outcomes in individuals carrying high clinical risk.

Section 5: References

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Appendix

Appendix

Consent and Patient Information Leaflets

Galway University Hospitals - Information Leaflet

Dear Sir/Madam

We would like to invite you to participate in a scientific research study at Galway University Hospital and National University of Ireland, Galway. The purpose of this study is to investigate the mechanisms underlying the development of complications of obesity such as Diabetes Mellitus, high blood pressure and raised cholesterol. The mechanism by which obesity can contribute to these complications is currently poorly understood. By furthering our knowledge as to these mechanisms, it will be possible in the future to develop newer treatments for obesity and its metabolic complications.

It is now recognized that cells from the immune system can negatively influence fat tissues around the abdomen (belly) by causing inflammation of this fat. This inflammatory response is likely to contribute to the development of diabetes mellitus, high blood pressure, high cholesterol, fatty infiltration of the liver and perhaps even cancer. We aim to investigate the role of immune cells called macrophages in contributing to this inflammatory response, and also to develop ways in which we can stop this process from contributing to the adverse outcomes associated with obesity, i.e. to develop new treatments.

We ask that you consent to donating a small blood sample today, and if you are undergoing a surgical procedure for the treatment of obesity, removal of a gallbladder, or elective gynecological surgical procedure, that you consent to a small piece of fat to be taken during the operation, for the purpose of investigating immune cells and stem cells in your blood and tissue. You are under no obligation to participate, and should you prefer to decline, we will accept your decision without question.

Should you consent to participate in the study, you are free to withdraw at any time that you wish. All blood and tissue samples, in addition to your clinical information will be coded numerically for storage in the laboratory at the Dept. of Surgery or at the Regenerative Medicine Institute at NUI, Galway. In both of these sites the strictest confidentiality is employed through encrypted and password protected systems.

It is hoped that our understanding of obesity will be improved through research. Galway University Hospital and NUI, Galway are actively involved in research that aims to identify markers that will predict how diseases such as obesity develop, progress and respond to a variety of treatments. We aim to develop, through our research, treatments which improve the outcomes of those who are obese and suffer as a result of its metabolic complications. Although this study may not have a direct benefit to you, the results obtained from it may benefit patients like you in the future. Thank you for your time and participation so far.

Yours,

Dr. Michael C Dennedy,
Specialist Registrar / NSAFP Fellow,
Department of Endocrinology, Galway University Hospital,
REMEDI, NUI, Galway

Prof Timothy O'Brien,
Professor of Medicine & Director of REMEDI,
Dept of Endocrinology, Galway University Hospital,
REMEDI, NUI, Galway

Galway University Hospitals - BioBank Informed Consent

Participant Declaration

I have read, or had read to me, this consent form and patient information leaflet. I had the opportunity to ask questions and these questions have been answered to my complete satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I understand that I may withdraw from the study at any time.

Participant's Name: _____

Contact Details: _____

_____

Participant's Signature: _____

Date: _____

Where the participant is incapable of comprehending the nature, significance or scope of the consent required, this form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake, or conduct, the study). If the participant is a minor (under 18 years old) the signature of parent or guardian must be obtained.

Name of Consenter, Parent or Guardian:

_____

Signature: _____

Relationship to Participant: _____

Declaration of Investigator's Responsibility

I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and have fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Name: _____

Signature: _____

Outputs Arising from this Work

Presentations / Abstracts

1. Michael C Dennedy, Stephanie Slevin, Eanna Connaughton, Donal O'Shea, Timothy O'Brien, Rhodri Ceredig & Matthew Griffin.
   Monocyte Subsets in Diabetes and Obesity. A Flow Cytometric Analysis of Phenotype.
   Immunology Society of Ireland, Annual Meeting, National University of Ireland, Galway, August 2010. *Poster Presentation*.

2. Michael C Dennedy, Stephanie Slevin, Eanna Connaughton, Donal O'Shea, Timothy O'Brien, Rhodri Ceredig & Matthew Griffin.
   Circulating Monocytes Differ in Phenotype According to Metabolic Milieu in Obesity.
   Professor T.J. McKenna Research Medal Meeting, Royal College of Physicians in Ireland, Kildare St., Dublin; May 2011. Oral Presentation.
   First Prize Award

3. Michael Conall Dennedy, Sonia Prado Lopez, Shirley Hanley, Eanna Connaughton, Stephanie Slevin, Rhodri Ceredig, Donal O'Shea, Tim O'Brien, Matthew Griffin.
   Circulating Human Monocytes differ in Phenotype and Function According to Metabolic Profile in Obesity and Type 2 Diabetes Mellitus.
   Annual Meeting of the Irish Endocrine Society, Malahide, Dublin; November 2012. *Oral Presentation.*

4. Michael Conall Dennedy, Eanna Connaughton, Stephanie Slevin, Shirley A. Hanley, Timothy O'Brien, Rhodri Ceredig, and Matthew D Griffin.
   The human intermediate monocyte subset can be further subdivided on the basis of HLA-DR expression revealing a distinct abnormality in obesity and diabetes.
   15th International Congress of Immunology; Milan, Italy. August 2013. *Poster Presentation.*

Submission in Progress

Michael Conall Dennedy, Eanna Connaughton, Shirley Hanley, Stephanie Slevin, Sonia Prado Lopez, Rhodri Ceredig, Donal O'Shea, Tim O'Brien, Matthew Griffin.
A novel subset of intermediate monocytes is expanded in obesity and is associated with adverse metabolic risk. Running Title: Novel Intermediate Monocytes and Obesity.
Arteriosclerosis Thrombosis and Vascular Biology - 2013.

Student Projects Supervised / Student Mentorship

1. Ms Jennifer Culligan, BSc Biotechnology, Final Year Project, August - Dec 2010
   An Investigation of the Response of Peripheral Blood Monocytes to Stimulation with Saturated Free Fatty Acid (FFA)
   Winner Best Presentation Final Year BSc Project, 2011/2012.

2. Ms Stephanie Slevin, MSc Regenerative Medicine, March - August 2011
   Differential Phenotype of Human Monocyte Subsets in Obesity and Diabetes Mellitus

Winner: MMI PhD Translational Medicine Bursary.

3. Mr Eanna Connaughton, MSc Regenerative Medicine, March - August 2011

   Differential Phenotype of Human Monocyte Subsets in Preterm Labour

   Winner: MMI PhD Translational Medicine Bursary.

4. Mr Eanna Connaughton, MMI PhD Translational Medicine, Sept - December 2011

   Monocyte / Endothelial Interactions in Response to Saturated Free Fatty Acid.

Fellowships Arising from this Work

Postdoctoral Research Fellowship in Lipidomics and Monocyte Biology

Metabolic Research Laboratories, Institute of Metabolic Sciences, Addenbrooke's Hospital & Cambridge University, Cambridge, U.K.

Supervisor:

Prof Antonio Vidal Puig, Professor of Nutritional Medicine and Lipid Biology,

Sept 2012 - Dec 2013