DOCTOR OF PHILOSOPHY

The role of apoptotic cell clearance in a high-lipid environment

links to ageing

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Thesis Summary

Individuals within the aged population show an increased susceptibility to infection, implying a decline in immune function, a phenomenon known as immunosenescence. Paradoxically, an increase in autoimmune disease, such as rheumatoid arthritis, is also associated with ageing, therefore some aspects of the immune system appear to be inappropriately active in the elderly. The above evidence suggests inappropriate control of the immune system as we age.

Macrophages, and their precursors monocytes, play a key role in control of the immune system. They play an important role in host defence in the form of phagocytosis, and also link the innate and adaptive immune system via antigen presentation. Macrophages also have a reparative role, as professional phagocytes of dead and dying cells. Clearance of apoptotic cells by macrophages has also been shown to directly influence immune responses in an anti-inflammatory manner. Inappropriate control of macrophage function with regards to dead cell clearance may contribute to pathology as we age.

The aims of this study were to assess the impact of lipid treatment, as a model of the aged environment, on the ability of macrophages to interact with, and respond to, apoptotic cells. Using a series of *in vitro* cell models, responses of macrophages (normal and lipid-loaded) to apoptotic macrophages (normal and lipid-loaded) were investigated. Monocyte recruitment to apoptotic cells, a key process in resolving inflammation, was assessed in addition to cytokine responses. Data here shows, for the first time, that apoptotic macrophages (normal and lipid-loaded) induce inflammation in human monocyte-derived macrophages, a response that could drive inflammation in age-associated pathology e.g. atherosclerosis. Monoclonal antibody inhibition studies suggest the classical chemokine CX3CL1 may be involved in monocyte recruitment to apoptotic macrophages, but not apoptotic foam cells, therefore differential clearance strategies may be employed following lipid-loading. CD14, an important apoptotic cell tethering receptor, was not found to have a prominent role in this process, whilst the role for ICAM-3 remains unclear.

Additionally, a small pilot study using macrophages from young (<25) and mid-life (>40) donors was undertaken. Preliminary data was gathered to assess the ability of primary human monocyte-derived macrophages, from young and mid-life donors, to interact with, and respond to, apoptotic cells. MØ from mid-life individuals showed no significant differences in their ability to respond to immune modulation by apoptotic cells compared to MØ from young donors. Larger cohorts would be required to investigate whether immune modulation of MØ by apoptotic cells contribute to inflammatory pathology throughout ageing.

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Abbreviations

aJK	Apoptotic Jurkat
aMØ	Apoptotic macrophage
aFC	Apoptotic foam cell
acLDL	Acetylated low-density lipoprotein
ABCA	ATP-binding cassette transporter
AC	Apoptotic cell
ACAMP	Apoptotic cell-associated molecular pattern
ATP	Adenosine triphosphate
BAI1	Brain-specific angiogenesis inhibitor 1
BL	Burkitt Lymphoma
BMDM	Bone marrow-derived macrophage
CFS	Cell-free supernatant
DIT	Diffuse intimal thickening
EC	Endothelial cell
EDTA	Ethylenediaminetetra-acetic acid disodium salt
ELISA	Enzyme-linked immunosorbent assay
ESL-1	Endothelial selectin ligand 1
FC	Foam cell
FMI	Forward Migration Index
GPCR	G protein-coupled receptor
HMDM	Human monocyte-derived macrophage
HMGB1	High-mobility group box-1 protein
HRP	Horse radish peroxidase
HUVEC	Human umbilical vein endothelial cell
ICAM-3	Intercellular adhesion molecule 1

IFN	Interferon
IgM/G	Immunoglobulin M/G
IL	Interleukin
JK	Jurkat
LDL	Low-density lipoprotein
LDL-R	Low-density lipoprotein receptor
LFA-1	Leukocyte function-associated antigen 1
LOX-1	Lectin-like oxidised LDL receptor 1
LPC	Lysophosphatidylcholine LPS
LPS	Lipopolysaccharide
LRP1	Low-density lipoprotein receptor-related protein 1/CD91
MØ	Macrophage
MARCO	MAcrophage Receptor with COllagenous structure
MBL	Mannan-binding lectin
M-CSF	Macrophage colony-stimulating factor
MCP-1	Monocyte chemotactic protein 1
MFG-E8	Milk fat globule epidermal growth factor 8
MMP	Matrix metalloproteinase
MP	Microparticle
MyD88	Myeloid differentiatin primary response gene 88
NF-κB	Nuclear factor Kappa-light-chain-enhancer of activated B cells
oxLDL	oxidised low-density lipoprotein
PAMP	Pathogen-associated molecular pattern
PECAM-1	Platelet endothelial cell adhesion molecule 1
PGE2	Prostaglandin-E2
PKC	Protein kinase C

PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PTX	Pentraxin
RAGE	Receptor advanced glycation end-products
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted/CCL5
S1P	Sphingosine-1-phosphate
SAP	Serum amyloid protein
SDoA	Standard Deviation of Angle
SEM	Standard error of mean
SIRPα	Signal regulatory protein-alpha
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cell
SP-A/D	Surfactant protein-A/D
SR (A/B)	Scavenger receptor-A/D
TACE	TNF-α converting enzyme
TGF	Transforming growth factor
TIM-1/4	T cell immunoglobulin mucin-1/ 4
TLR	Toll-like receptor
TNF	Tumour-necrosis factor
TSP-1	Thrombospondin-1
UTP	Uridine 5'-triphosphate
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
WC	Whole cell culture

Contents

Introduction

1.1 What is ageing?

Although the concept of ageing is familiar, it is a complex term to define. In a 1952 lecture on ageing entitled 'An Unresolved Problem of Biology', ageing was described as a collection of changes that render human beings progressively more likely to die[1]. This unresolved problem of biology refers to the inextricable link of ill health with ageing, however current research aims to dissociate the two and ensure that healthy ageing and a greater quality of life come hand in hand with increased lifespan. It could be argued that science is much closer to solving the problem[2], or at least gaining a better understanding of the underlying mechanisms that result in decline of health with advancing age, and therefore can look at ways to counteract the origins of ill health in the elderly.

Improving the quality of life of the elderly is an area of increasing focus as both the relative and absolute number of elderly individuals in the population has not only increased over the last century but is set to increase further, due to improved social and economic conditions[3]. This leads to a huge economic burden, with a reduced working population, and increasing social and economic burden with regards to age-related disease[3].

Theories on the mechanisms behind ageing vary widely, from psychological and sociological theories based on reduced social engagement and inactivity, down to the cellular and molecular level such as shortening of telomeres, the protective sequences of DNA at the end of genes which protect DNA from damage during replication[4].

Biological theories cover evolutionary, molecular, cellular and systemic concepts[4]. Popular biological theories of ageing include wear and tear, accumulative waste, somatic mutation, error accumulation, telomere shortening, oxidative stress, mitochondrial damage, free-radical, cross-linkage, autoimmune, reproductive cell cycle, DNA damage, and mTOR theories[4]. In reality many of these are backed by scientific evidence and also have considerable crossover with each other. No single theory can explain all phenomena associated with ageing, with the likely conclusion that ageing is a complex process with contributing factors from across the range of ageing theories[5].

1.2. Immunosenescence and inflammaging

Immunosenescence, the ageing of the immune system, results in an increased susceptibility to infection in the aged population, implying a decline in immune function[6-8]. Paradoxically, an increase in autoimmune disease is also associated with ageing, a result of inappropriately active components of the immune system[9,10]. Ageing is an established risk factor in

autoimmune diseases such as rheumatoid arthritis, and is also associated with increased autoantibody titre[11,12]. The above evidence suggests not just a decline in overall immune function, but inappropriate control of the immune system with advanced age, with the net effect of chronic low-grade inflammation. This chronic low-grade inflammation has been associated with cardiovascular disease, diabetes, frailty and cognitive decline/dementia[13].

Inflammaging is a recently coined term to describe the low-grade chronic inflammatory status associated with many elderly individuals[14,15]. Franceschi *et al*. argue that many phenotypes of ageing can be explained by an imbalance of pro- and anti-inflammatory networks, which drive frailty and other common age-related pathologies[15].

Ageing of the immune system is associated with phenotypes that include thymic involution[5], reduced population of naïve T lymphocytes, increased population of memory T and B cells with reduced antigen recognition[16]. Higher circulating levels of inflammatory mediators are also a feature of inflammaging, with consistently higher levels of Tumor Necrosis Factor-alpha (TNF-α), interleukin-6 (IL-6) and C-reactive Protein (CRP) across studies[13]. Evidence also suggests reduced levels of circulating anti-inflammatory mediator IL-10 in the elderly, which could exacerbate the inflammatory environment[17]. The increase in inflammatory mediators such as IL-6 and TNF-α has been attributed to accumulated oxidative damage, increased adiposity and declining levels of sex hormones as we age[13].

Ageing of the innate immune system is multifaceted and still poorly understood. Research into age-related changes in innate immunity is less advanced than that of adaptive immunity, and methodology varies widely, often resulting in conflicting results[7]. In order to establish the cause of immunosenescence and inflammaging, studies must be undertaken at the cellular level to understand which components of the immune system are under or over active as we age.

1.2.1. Senescence of monocytes and macrophages

The innate immune system is not only the first line of defence following a breach of physical barriers, but is also vital in maintaining homeostasis in the body, clearing dead and dying cells or cells flagged as harmful or unwanted. Cells within the innate immune system are also link the innate and adaptive immune system, priming this second line of defence and working synergistically. When dysregulation of the innate immune system occurs consequences can be wide-reaching.

1.2.1.1. Monocyte and macrophage function

Macrophages, and their precursors monocytes, play an important role in host defence in the form of phagocytosis, and also link the innate and adaptive immune system via antigen presentation[18]. Classically-activated ‘M1’ macrophages are pro-inflammatory, which can be

induced by encountering pathogenic material or pro-inflammatory mediators. Classically activated macrophages have been attributed to play a pathological role in inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease asthma and atherosclerosis[19]. Alternatively activated 'M2' macrophages have a largely reparative role, including clearance of apoptotic bodies and debris from tissues [20].

1.2.1.2. Monocyte and macrophage senescence

It is important to understand the root causes of monocyte and macrophage senescence in order to improve innate immunity in ageing. This could include whether changes in innate immune function are a result of changes in the overall populations of innate immune cells such as monocytes or macrophages, as seen in the adaptive immune system with naïve T cells and thymic involution[16], or the reduced ability of an aged cell to function. The effects of an aged environment may also be driving the effects of immunosenescence, rather than the cells.

A decrease in CD68-positive macrophage-lineage cells has been observed in human bone marrow in aged subjects[21] which may account for the impact ageing has on innate immunity as monocytes/macrophages are one of the first cell types to respond to invading microorganisms. However this was not replicated in a murine model, important to note due to the frequent use of mice in ageing research[22].

In murine studies to address immune cell function in ageing, peritoneal macrophage function was impaired in aged mice with regard to adherence, opsonization, phagocytosis, superoxide production and antibody-dependent cell cytotoxicity[23-25]. Also, a decrease in phagocytic ability of kupffer cells, specialised tissue macrophages of the liver, was found in aged mice by Videla *et al.* [25]. This would ultimately reduce the ability of macrophages to interact with and destroy pathogens and unwanted self, e.g. tumour cells, seemingly impaired functions in the elderly population with regard to increased risk of infection and cancer[24].

Another mechanism for infection control elicited by macrophages is pro-inflammatory cytokine production via toll-like receptor (TLR) stimulation to elicit an immune response. Interestingly, some impairment in TLR function has been observed with ageing[26], which could be predicted with increased incidence of infection[16], however this does not explain the systemic increase in inflammatory cytokines in the elderly population[14].

Renshaw *et al.* observed impaired TLR expression, including reduction in TLR4 expression, and impaired TLR function in peritoneal and splenic macrophages from aged female C57BL/6 mice[26]. In their study, function of TLRs was measured as secretion of cytokines IL-6 and TNF-α following stimulation of TLRs with known ligands, further possible explanations for the increased incidence of infection in the elderly [26]. Conversely, Boehmer *et al.* found no

change in TLR4 expression in peritoneal macrophages between young and aged female BALB/c mice, but results supported a decrease in TNF-α and IL-6 secretion following LPS stimulation [27]. This was attributed to a decrease in basal levels of p38 and c-jun NH2-terminal kinase (JNK) MAPK and resulting reduction in MAPK activation, also found by Ding *et al.* [24,27]. Despite an apparent inability of aged macrophages to release this cytokine, TNF-α has been found in elevated levels in the circulation of elderly subjects, a characteristic of inflammaging[28,29].

CD14 expression, another key pattern recognition receptor (PRR) important in LPS response, was reduced in aged C57BL/6 (B6) mice[30], however expression in monocytes and macrophages with ageing has not been extensively studied. CD14 expression and function would also be of interest with regards to AC clearance[6,31].

Research on human primary cells is one way of comparing young and aged individuals and has been carried out using peripheral blood mononuclear cells (PBMC), PBMC-derived macrophages (HMDM) and bone marrow-derived macrophages (BMDM). As found in the murine models, monocytes isolated from aged individuals display a decrease in IL-6 and TNF production following LPS activation, which was attributed to deficient PKC and MAPK activation[32]. Whether PBMC derived macrophages are comparable to tissue macrophages is a question which is yet to be answered, a simpler conundrum in murine models, where tissue macrophages can be harvested.

1.2.1.3. Apoptotic cell clearance in ageing

Very few studies have addressed the role of apoptotic cell clearance in ageing, and the association of systemic inflammatory markers in ageing. Whether there are defects in AC clearance through ageing, or subsequent immune-modulation following interaction with AC, could be a result of an aged cell failing to function, the effect on an aged environment on cell function, or likely a combination of both. The levels of redundancy shown in AC clearance may imply that even if cells become less efficient as we age, there may be other factors present that subsidise these effects e.g. presence of AC:phagocyte bridging molecules.

A study in mice found reduced apoptotic keratinocyte clearance *in vivo* in aged animals, and a reduction in AC interaction with thioglycollate-elicited peritoneal MØ from old mice *in vitro*[33]. Aged mice also displayed an increased anti-nuclear antibody titre, as associated with ageing in humans, and seen in autoimmune disease[12,33,34]. AC interaction was also reduced in MØ from young mice in the presence of donor serum from aged mice in the same study, showing interesting effects of both cell ageing and an aged environment on MØ ability to clear AC[33]. This is an accessible method of assessing the influence of an aged environment on MØ function, which to date has not been replicated in human studies.

1.3. The macrophage – the professional scavenger of AC

1.3.1. Apoptotic cell clearance

Apoptosis, or programmed cell death, allows removal of aged, damaged, infected or unwanted cells in a controlled manner, an essential process for maintenance of homeostasis in multicellular organisms. Apoptosis is an immunologically silent method of cell death, and would be functionally ineffective without subsequent clearance of apoptotic cells (AC), or efferocytosis (fig. 1). Failed AC clearance can result in secondary necrotic bodies and potential inflammation[35], and has been implicated in a number of pathologies. Defects in genes associated with AC clearance are well established mechanisms behind autoimmune disease, such as deficient C1q expression, which has been show to drive systemic lupus erythmatomous (SLE)[36]. Other examples include age-associated pathologies, including the dubbed 'inflammaging'[37], and a number of age-related conditions, including arthritis, neuropathy and atherosclerosis (reviewed in[35]).

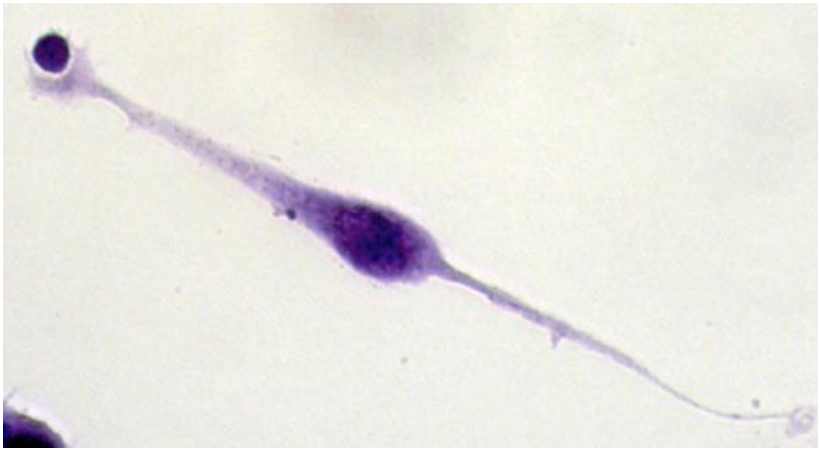


Figure 1. Macrophage phagocytosis of apoptotic cells *Differentiated THP-1 macrophage interacting with apoptotic Jurkat cell.*

In contrast, *in vivo* murine studies have found persistent apoptotic cells, without the associated inflammatory effects[38,39]. Necrotic cell clearance has also been found to be non-inflammatory in some models[40], highlighting gaps in the current understanding of clearance mechanisms and subsequent immune modulation.

ACs are rarely observed *in vivo* due to clearance efficiency, which is the result of a complex, multi-stage process[41]. Neighbouring cells, termed 'amateur phagocytes' are thought to assist in the removal of apoptotic cells before classical morphological features associated with

apoptosis are displayed[42,43], resulting in the need for *in vitro* AC clearance models to gain a full understanding of the process. The recruitment of 'professional phagocytes', such as macrophages (MØ) occurs at sites, often pathological, with high apoptosis levels, e.g. the atherosclerotic plaque[35,44-46].

1.3.2. Macrophages; janitors of cell death

Macrophages are inherently plastic; they have the ability to respond to varying microenvironments and alter their phenotype accordingly, in a reversible manner[47]. The activation state, or phenotype, of macrophages depends greatly on the balance of extracellular stimuli encountered[48]. There are several classification systems of macrophage phenotypes, e.g. M1 v. M2, or classical v. alternative, however the polar classifications used to define macrophage phenotype is likely to be a simplistic view, and therefore MØ phenotypes are difficult to truly classify. Researchers have tried breaking down MØ phenotypes into further subgroups and allocate reliable markers for identification, however a spectrum of varying characteristics depending on local stimuli is likely to be a more physiologically relevant model[49].

Monocytes and MØ both have designated subpopulations, though the link between monocyte subpopulations, and subsequent MØ subpopulations remain ill-defined. Murine monocyte subpopulations are well characterised according to surface receptor expression, and are designated as $CD62L^{+}CCR2^{+}CX3CR1^{lo}Ly6^{hi}$ (inflammatory monocytes), or $CD62L^{-}CCR2^{-}CX3CR1^{hi}Ly6^{lo}$ (resident monocytes)[50,51] . Murine monocyte subpopulations do not appear to directly correlate functionally with human monocyte subpopulations[52], which is important to consider in investigations into monocyte recruitment from the blood, e.g. in atherosclerosis, where murine models are prominent. Human monocyte subpopulations consist of a majority of classical $CD14^{hi}CD16^{-}$ monocytes, and a second $CD14^{lo}CD16^{+}$ ($CX3CR1^{hi}CCR2^{-}$) non-classical subpopulation[50,53].

Classification of MØ activation was initially based on a linear M1-M2 scale, with classically-activated MØ at one end of the spectrum (M1), and alternatively-activated MØ at the other extreme (M2), with different degrees of activation in between (fig. 2; left). This was largely based on whether cells were exposed to Th1 or Th2 cytokines. Mosser and Edwards (2008) have since proposed a more physiologically relevant model, based on a colour wheel[49] (fig. 2; right), whereby prominent groups may preside (colour blocks), but MØ could have any combination of characteristics according to the surrounding milieu, therefore activation status is less easily categorised[49]. This would explain why, unlike monocyte subgroups, specific 'M1' and 'M2' markers have been difficult to define.

Figure 2. Schematic depicting models of macrophage activation *based on Mosser and Edwards (2008)[49]. A MØ activation spectrum based on a colour wheel (right) has been proposed to replace the linear M1-M2 scale of activation (left). Rather than distinct M1 and M2 phenotypes on either end of a linear scale of MØ activation (left), prominent groups may preside (right), but MØ could still have any combination of characteristics according to the surrounding milieu.*

Some phenotypes of MØ activation are well defined, such as the response to the classical pro-inflammatory Gram-negative bacterial wall component lipopolysaccharide (LPS). This classical subpopulation is associated with a cell-mediated immune response to invading pathogenic material, and is inflammation is maintained by Th1 cells[49]. Classical/M1-like activation is induced by tumour necrosis factor (TNF), or toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS), and interferon-γ (IFN-γ)[49]. This results in a pro-inflammatory phenotype. Markers include TNF-α, interleukin-1 (IL-1), IL-6 and IL-10 release, and increased microbicidal activity via superoxide and free radical production[49].

Alternatively-activated macrophages have reparative roles, and encompass a broader spectrum of MØ activation, such as wound-healing and regulatory MØ[49]. Wound healing phenotypes occur at sites of tissue damage, following granulocyte release of IL-4, stimulating arginase activity in mice[49]. Arginase converts arginine to ornithine, a precursor required for extracellular matrix production. *In vitro*, MØ treated with Th2-associated cytokines IL-4 and/or IL-13 are less efficient at dealing with pathogens, as determined by significantly reduced pro-inflammatory cytokine release and reduced intracellular microbicidal activity[49]. Paracrine mediators released by these MØ also seem to control inflammation in the surrounding microenvironment. Regulatory macrophages, which are often grouped in with alternatively-activated MØ, are less well defined. They can be observed in response to AC clearance, and have also been shown to dampen an inflammatory response, e.g. by increased IL-10 release[49]. Other inducers of regulatory MØ phenotype include glucocorticoids, prostaglandins and IL-10[49].

In the case of interaction with ACs, it is thought a reparative role predominates in the surrounding MØs, promoting clearing up of debris and dampening/resolving inflammation via

anti-inflammatory cytokine secretion and subsequent suppression of pro-inflammatory mediator release[54-56]. The alternative/M2 phenotype is associated with more efficient AC interaction than their M1 counterparts[57]. This phenotype may be less well defined in the plaque environment, where a large quantity of ACs are located, due to the large variety of pro-inflammatory and anti-inflammatory stimuli in the immediate microenvironment of the MØ[46,58].

1.3.3. Phagocyte recruitment – a balance of 'find-me' versus 'keep-out' signals

In the case of professional phagocyte recruitment, e.g. MØ, ACs release 'find-me' signals, which attract phagocytes from the surrounding areas. Phagocytes detect these 'find-me' mediators, which could be soluble or microparticle-associated[59,60], and move along a concentration gradient towards the cell requiring clearance. This area of research has been largely neglected by current *in vitro* methods of assessing AC removal, therefore there is large potential for discovery of further 'find-me' mediators.

A wide variety of mediator classes have been identified as 'find-me' signals, including chemokines, lipids and nucleotides[61-63]. CX3CL1, also known as CX3CL1 or neurotactin, was the first and, until recently, the only chemokine to be discovered with a role in phagocyte recruitment to apoptotic cells[61]. CX3CL1 release has been shown in Burkitt's lymphoma (BL) cells in association with microparticles (MPs), which are released following zeiosis, or blebbing, of the AC membrane[61]. CX3CL1 ligates to the phagocyte surface receptor CX3CR1, stimulating phagocyte migration[61,64]. The release of CX3CL1 from apoptotic cells within the plaque may also play a direct role in monocyte recruitment, or be conferred to endothelial cells via MPs. CX3CL1 can also be cleaved from the surface of cells as a soluble mediator, which has previously been implicated in pathologies associated with failed AC clearance and inflammation[65-67]. A subset of $CX3CR1^{+}$ monocytes have been identified[50], and CX3CL1 has previously been shown to have a role in recruitment of monocytes to the atherosclerotic plaque via interaction of monocytic CX3CR1 ligation to membrane bound CX3CL1 on the endothelial cell surface[64,68]. CX3CL1 also increases the expression of phagocyte:AC bridging molecule MFG-E8 (milk fat globule-endothelial growth factor 8), thus contributing to successful clearance[69]. A recent study has implicated a wider range of chemokines as 'find-me' signals released by cells induced to apoptosis via Fas/CD95 ligation[59]. Monocyte chemotactic protein-1 (MCP-1/CCL2) and IL-8 (CXCL8) were shown to attract THP-1 monocytes and primary human neutrophils respectively[59], which could have inflammatory consequences[70].

Intercellular adhesion molecule-3 (ICAM-3/CD50) has recently been identified as a mediator of phagocyte recruitment to apoptotic B cells, in association with MPs[71]. Whether ICAM-3, or

CX3CL1, is released in association with MPs by apoptotic MØ, which occur in atheroma, is currently unknown, and may have implications in atherosclerosis progression, a pathology with a high proportion of localised ACs, including apoptotic MØ[46].

Nucleotide chemoattractants adenosine triphosphate (ATP) and uridine-5'-triphosphate (UTP) were shown to be released in early apoptosis, and interact with purinoreceptor-2 (P2Y2) on the phagocyte surface[63], and have been suggested to act as short-range effectors in phagocyte recruitment. This highlights potential differences in roles between 'find-me' signals that have yet to be fully investigated.

Lysophosphatidylcholine (LPC) and sphingosine-1-phosphate (S1P) are lipid mediators shown to have a role in phagocyte attraction to dead cells [62,72,73], which also have additional autocrine functions. LPC interacts with G-protein-coupled receptor (GPCR) G2A[72], and has been shown to induce expression of monocyte, neutrophil and lymphocyte chemoattractants, MCP-1, IL-8 and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted/CCL5) respectively[74]. S1P also has autocrine functions, inducing phagocytic IL-8 and IL-10 secretion, and inhibiting TNF-α and IL-12p70 release[75-77].

In order to maintain immunologically silent removal of ACs, recruitment of phagocytes must be controlled in terms of phagocyte lineage. 'Find-me' mediators have to compete with 'keep-out' signals such as lactoferrin, which has been shown to selectively inhibit migration of granulocytes, but not mononuclear phagocytes[78,79]. This is important given the evidence of AC-induced IL-8 release, a neutrophil chemoattractant[59], and the possible detrimental effects of such recruitment with regards to inflammation[70]. As this is a relatively new area of research, more 'keep-out' signals are likely to be discovered.

1.3.4. Recognition and tethering – a balance of 'eat-me' versus 'don't eat-me' signals

Following phagocyte recruitment, ACs must be identified as phagocytic targets. This occurs via upregulation of 'eat-me' flags, balanced with the downregulation of 'don't eat-me' markers, usually associated with viable cells[80]. Identified 'eat-me' markers are gained from a variety of mechanisms, including exposure of intracellular molecules (e.g. phosphatidylserine[81,82] and annexin I[83]), modification, redistribution and removal of cellular proteins, lipids and glycoproteins (e.g. modification of ICAM-3[84], phosphatidylserine (PS) oxidation[85], removal of sialic acid[86]) and binding of opsonins (e.g. mannan-binding lectin (MBL), complement component C1q[87,88]).

Loss of PS asymmetry, resulting in extracellular exposure, is a well-established 'eat-me' signal expressed during apoptosis[81,82,89]. PS is normally restricted to the inner leaflet of the cell membrane[89], and redistribution is thought to be required for AC clearance[81,82]. PS exposure alone, however, is not enough for successful execution of AC clearance,

demonstrated by a lack of clearance of viable cells induced to express PS constitutively[90]. It may be that further 'eat-me' markers are required, and also downregulation of 'don't eat-me' markers is likely to be essential for engulfment[80]. Identified 'don't-eat-me' flags CD300a[91], CD31[92] and CD47[93] inhibit phagocytosis of viable cells, and have been manipulated by cancer cells to evade the immune system[94]. Mechanisms of action vary; CD300a competitively binds AC surface phospholipids, including PS[91,95]; CD31 on viable cells binds homophilically to phagocytic CD31, preventing ingestion, but becomes functionally altered following induction of apoptosis, enabling phagocyte tethering[92]; CD47 inhibits engulfment via phagocytic receptor SIRPα (SIgnal Regulatory Protein α), with successful engulfment only achieved following CD47 disruption[93].

The phagocytic receptor for PS proved elusive for a significant period of time, however several receptors have recently been identified, T cell immunoglobulin mucins 1 and 4 (TIM-1, TIM-4)[96], brain-specific angiogenesis inhibitor-1 (BAI1)[97], stabilin-2[98] and the Receptor for Advanced Glycation Endproducts (RAGE)[99]. Numerous bridging molecules have also been identified as facilitators of phagocyte binding via AC expressed PS, including growth arrest-specific 6 (Gas6), protein S and MFG-E8. Gas6 and protein S bridge to Mer on the phagocyte membrane, whereas MFG-E8 bridges to α_v integrins[100]. The phagocyte receptor LRP1 (low-density lipoprotein [LDL] receptor-related protein 1 or CD91) has been shown to bind PS when colocalized with redistributed calreticulin, which is upregulated during apoptosis[93]. Calreticulin can also associate with bridging molecules C1q and MBL for recognition by LRP1[88]. Oxidative modification of PS results in recognition by phagocytic scavenger receptors such as CD36[101]. Scavenger receptors (SRs) CD68, SRA-1, SRB-1 and lectin-like oxidised LDL receptor-1 (LOX-1) also have roles in apoptotic cell recognition[102-105]. The importance of the role of PS in AC removal becomes clear with genetic alteration of PS recognition mechanisms, resulting in pathology, including atherosclerosis and Alzheimer's disease[106-110].

ICAM-3 is a cell surface receptor that is expressed on human, but not murine, leukocytes, resulting in it being overlooked in a lot of cell clearance models[80]. Functional modification of ICAM-3 during apoptosis allows phagocyte recognition[71,84]

Alongside Gas6, protein S and MFG-E8, collectins (MBL, surfactant protein-A (SP-A), SP-D), complement components (C1q, C3b), thrombospondins (TSP-1) and pentraxins (C-reactive protein (CRP), serum amyloid P (SAP), pentraxin 3 (PTX3)) have all been shown to facilitate phagocyte:AC bridging[111-119]. Immunoglobulin M (IgM) and IgG antibodies also have a role in opsonising ACs[120-122]. Soluble CD14 has also been observed to bind PS and AC, but a role in AC clearance has not yet been established[38].

Phagocyte interaction with AC clearance is largely associated with a dominant non- or anti-inflammatory response[55,56]. Interestingly, many receptors implicated in clearance of AC are also involved in an inflammatory innate immune response. This has resulted in the hypothesis that certain 'eat-me' flags on the AC surface are the equivalent to pathogen-associated molecular patterns (PAMPs) on invading pathogens, and have been termed ACAMPs (apoptotic cell-associated molecular patterns)[123]. In support of this, Tennant *et al.* observed that monoclonal antibodies targeting LPS also bind apoptotic cells, indicating structural similarities between PAMPs and ACAMPs[124].

1.3.5. Tickling/Signalling and engulfment

Phagocytic downstream signalling pathways, activated following AC interaction, vary according to the ligand:receptor combinations activated within the phagocytic synapse. This is likely to vary between different phagocyte and AC cell types/lineages, however these cell-specific interactions are yet to be characterised.

Two downstream signalling pathways have been characterised, with further pathways so far restricted to research in *Caenorhabditis elegans*[125]. One pathway ensues following interaction of TAM family receptor Mer[126], BAI1[97], or α_v integrins[127], with PS or PS bridging molecules. This activates the CrkII-Dock180-ELMO complex, initiating Rac activation via GDP-GTP exchange[128]. This initiates Scar/WAVE mediated cytoskeletal rearrangement, and subsequent AC engulfment[129,130].

A second pathway is initiated via phagocytic LRP-1 or stabilin-2 ligation via adaptor protein GULP (enGULfment adaPter protein), activating ABCA1 (ATP-binding cassette transporter 1) and/or ABCA7[131-133]. The full signalling pathway is yet to be established, however evidence indicates that the two pathways converge at the equivalent of Rac activation[134]. Following cytoskeletal rearrangement and AC phagocytosis, phagosomes acidify, fuse with lysosomes[135], and ACs are digested. This process can regulate future events, including further engulfment potential[136], cytokine release[55,56] and self-antigen presentation. This is important in relation to atherosclerosis research, where ingestion of lipid-laden apoptotic cells may impact engulfment potential and cytokine release, given persistent ACs and inflammation within the atherosclerotic plaque[46,58].

1.3.6. What next for the phagocyte? Immune modulation following apoptotic cell clearance

Cytokines are secreted proteins vital for intercellular communication, with an important role in regulating numerous physiological processes, including growth, adiposity and haematopoiesis[137]. The term encompasses interleukins (IL), tumour necrosis factors (TNF), interferons (IFN), colony stimulating factors (CSF), transforming growth factors (TGF) and

chemokines[137]. Cytokines play a key role in regulation of innate and adaptive immune responses[137,138].

AC ingestion has been shown to induce TGF-β1, IL-10, prostaglandin E2 (PGE2), platelet-activating factor (PAF), and suppress mediators associated with inflammation, including TNF-α, IL-1, IL-12 and IL-8[55,56]. Results also showed apoptotic cells were able to dampen a pro-inflammatory response to LPS[56]. TGF-β production could have particular significance, as it has been shown to regulate eicosanoid and nitric oxide synthesis in the favour of an anti-inflammatory phenotype in murine MØ[139].

Resolution of inflammation is an active process in which regulatory MØ release of anti-inflammatory mediators, and autocrine and paracrine suppression of pro-inflammatory mediator release, plays an important part. Following resolution of inflammation, aided by lipoxins, resolvins and protectins[140,141], immune cells migrate into the lymphatic system, and accumulate in local lymph nodes[142].

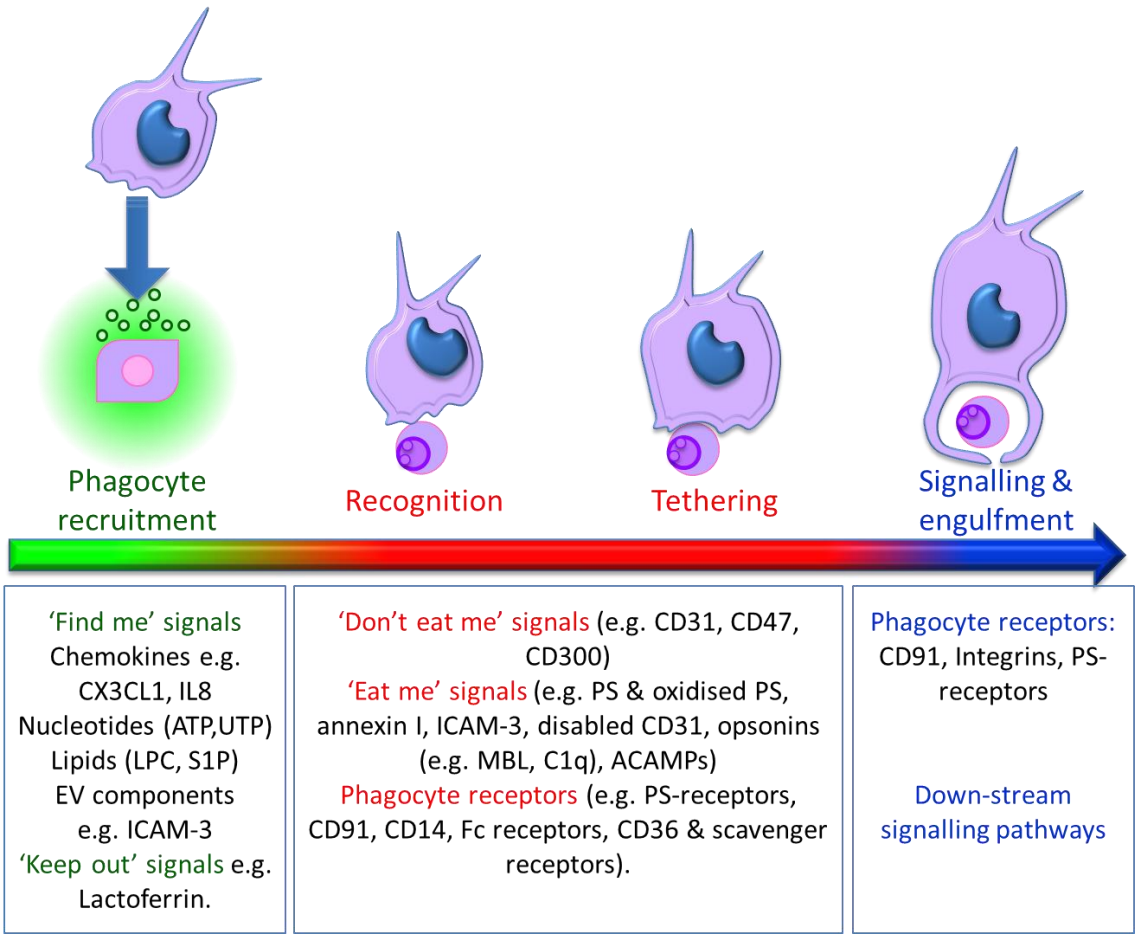


Figure 3. Summary of key stages and mediators of apoptotic cell clearance *Apoptotic cell clearance is initiated by 'find-me' mediator release (if professional phagocytes are recruited), followed by recognition and tethering given the correct balance of 'eat-me' and 'don't eat-me' flags on the apoptotic cell surface. 'Tickling' or signalling results in engulfment*

of the apoptotic cell, and subsequent immune-modulation. Figure taken from Hawkins & Devitt[80].

1.4. Atherosclerosis as an example of defective AC clearance in ageing

Cardiovascular disease (CVD) has been described as the largest single contributor to global mortality, and is predicted to continue to dominate in the future[143]. Risk factors include high blood pressure, high circulating levels of LDL, low circulating levels of HDL, physical inactivity, smoking and a genetic predisposition to hypercholesterolemia[144]. Low socioeconomic status, psychological factors e.g. stress and depression and inflammation, amongst other factors, are also thought to predispose individuals to CVD[144].

Age, obesity and diabetes are also risk factors, and with an ageing population, and increasing incidence of obesity and obesity-induced diabetes, the incidence of CVD is set to rise[145]. CVD is more prevalent in men, with death rates higher in areas of greatest deprivation[146]. With such high death rates, and a cost of £19 billion to the UK economy[146], greater understanding of the drivers of this disease is required.

CVD is one of the leading causes of death in the UK, with the common underlying cause of atherosclerosis. In 2010, 180,000 people died of CVD in the UK, including 46,000 premature deaths[146]. Atherosclerosis is caused by the gradual build-up of lipid deposits, live and apoptotic immune cells and fibrous material in the sub-endothelial space of arteries[147,148]. This occurs in major arteries at branching points, where the endothelial barrier is under stress and becomes breached or activated. The result of plaque formation is narrowing of the artery lumen, and should the plaque progress, necrotic core formation[149]. The necrotic core forms as cells trapped within the plaque die by apoptosis, however the usual mechanisms that result in the clearance of dead cells fail, resulting in the accumulation of secondary necrotic cells[150]. This is despite the continual infiltration of macrophages, professional scavengers of apoptotic cells[150]. Formation of a necrotic core occurs in advanced plaques and is associated with plaque instability and rupture, leading to blockage of the artery lumen and subsequent cardiovascular events e.g. heart attack or stroke[149].

The failed clearance of apoptotic cells is viewed as a key driver of atherosclerosis[151], and the mechanisms behind this could give insights into failed clearance in other pathologies, or indeed ageing.

1.4.1. The inflammatory background of Atherosclerosis

It is now widely accepted that atherosclerosis has a largely inflammatory background, with involvement of immune cells and inflammatory mediators from the initial stages of plaque formation, through progression of the plaque, destabilisation and rupture[46,58].

Cellularity of advanced plaques is a crowded milieu of endothelial cells (ECs), smooth muscle cells (SMCs), monocytes, MØ, foam cells (MØ- and SMC- derived), mast cells, T lymphocytes, including live, apoptotic and necrotic cells[147,148]. Subsequently the extracellular milieu within the plaque is multifarious, containing oxidised lipoproteins, Th1 and Th2 cytokines, matrix metalloproteinases (MMPs), fibrin, collagen, proteoglycans and cellular debris[58,147].

Atherosclerotic plaque formation is initiated by sub-endothelial lipoprotein retention, inflammation, LDL modification including EC-mediated oxidation, and EC dysfunction[152-155]. This causes EC adhesion receptor upregulation, recruiting monocytes from the blood stream[156-160]. Monocytes differentiate to macrophages and accumulate in the arterial intima, gorging on oxidised lipoproteins, inducing foam cell (FC) formation and death by apoptosis[45,46,153,161]. Apoptotic cell accumulation attracts further monocytes into the arterial intima, exacerbating plaque formation, and the process continues in a cycle[162], accumulating further immune cells and inflammatory mediators, as listed above. This process can eventually lead to weakening of the artery wall, due to factors such as MMP accumulation, plaque rupture and thrombus formation[46,58,150,163].

Studies have found inflammatory effects of oxLDL on endothelial cells and MØs, which can account for the origins of monocyte recruitment to the subendothelial space and atherogenesis[164-166]. Why inflammation remains unresolved is unknown, and despite the prominence of MØ-derived foam cells in the plaque, the inflammatory status of these cells, either live or apoptotic, is unclear.

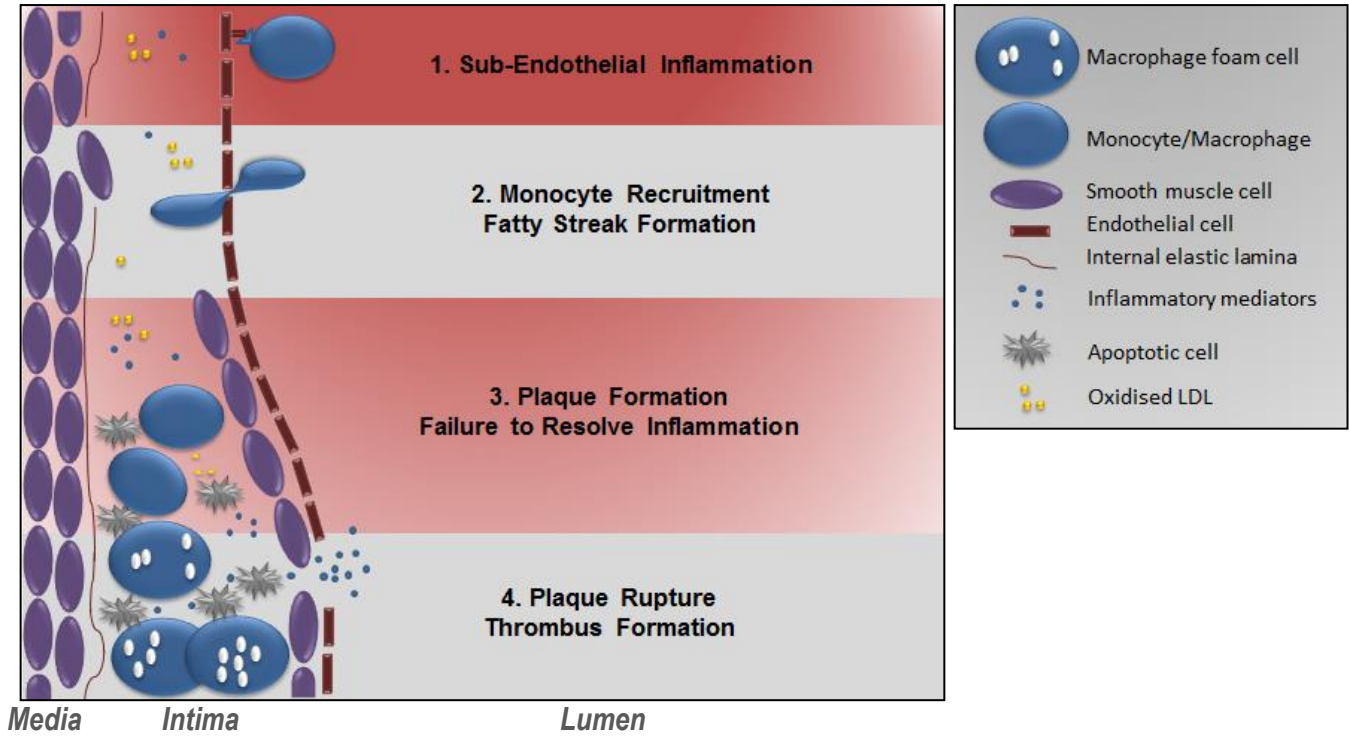


Figure 4. The role of monocytes and macrophages in key stages of atherosclerotic plaque formation *1. Lipoprotein retention and resulting sub-endothelial inflammation cause upregulation of adhesion molecules on the endothelial cell surface 2. Following firm adhesion, monocytes are recruited to the subendothelial space via diapedesis, differentiating into MØ and gorging on lipoproteins 3. Lipid laden MØ-derived foam cells accumulate in the plaque, die by apoptosis and recruit further monocytes from the circulation, exacerbating plaque progression and inflammation 4. Non-resolving inflammatory milieu can cause plaque progression to arterial wall weakening, plaque rupture and thrombus formation*

1.4.2. Interplay between LDL and monocytes/macrophages in atherogenesis

Low-density lipoprotein (LDL) modification, monocyte recruitment and foam cell formation are well characterised as the initiating events of atherosclerotic plaque formation. LDL is the main carrier of serum cholesterol in humans, and although cholesterol has vital physiological roles, including maintenance of cell membrane fluidity and signal transduction, high levels of LDL in the blood is associated with increased risk of atherosclerosis[147,167]. The single initiating event that leads to the formation of fatty streaks, leading on to plaque formation is still debated, however strong evidence supports the Response-to-Retention theory[152,168-170]. Williams and Tabas argue that although many pathological processes contribute to early atherogenesis, they are generally normal physiological responses that would occur in healthy tissue as a result of retained subendothelial lipoproteins. Without this retention, the other defining features of atherogenesis and plaque progression, such as EC dysfunction and FC formation, would not ensue. This theory is strongly supported in study by Nakashima *et al.*

(2007)[45], which found that diffuse intimal thickening (DIT) and lipoprotein accumulation preceded macrophage infiltration in autopsy samples of human coronary arteries. Lipoproteins retained within the sub-endothelium may then become modified, including oxidative modification[154,155]. Research has shown that ECs themselves may be primarily responsible for LDL oxidation[153].

1.4.2.1. LDL oxidation and the effect on recognition by macrophages

LDL particles can be described as heterogeneous in nature due to variances in size, composition and structure[171], and because of this, and factors such as diet, LDL samples from different individuals can vary greatly in the state of and susceptibility to oxidation[172].

The LDL particle consists of an outer layer of phospholipid and unesterified cholesterol, with a single apolipoprotein B-100 (apoB100) molecule at the surface[171]. The core of the LDL particle contains more unesterified cholesterol, and also triglycerides and cholesteryl ester[171]. Hevonoja *et al.* (2000)[171] suggested a three-layer model for LDL particle structure, consisting of an outer surface layer, an interfacial layer and the core. Native LDL (nLDL) is bound by the LDL receptor, however modification of the LDL particle will result in recognition by alternative receptors, including scavenger receptors CD36, SRA-1 and LOX-1. Although scavenger receptors recognise various modified LDL particles, e.g. acetylated LDL, it is oxidised LDL particles that are thought to be the most physiologically relevant[173].

Sufficient oxidation of LDL particles must occur for recognition by scavenger receptors, as opposed to the LDL-R; for example, at least 60 lysine residues on the apo-B100 moiety must be substituted with aldehydes for recognition by SRA-1[174]. Despite a common affinity toward anionic phospholipids, scavenger receptors vary in the binding site recognised on the oxLDL molecule[175-177]. CD36 binds to the lipid moiety of oxLDL, whereas SRA-1 binds the oxidised apo-B100 molecule[178,179]. Much attention has been given to the role of scavenger receptor in atherogenesis, so as to determine whether specific receptors can be targeted to prevent oxLDL uptake by MØ and subsequent plaque formation.

1.4.2.2. LDL-R

Many MØ cell surface receptors have been implicated in modified LDL uptake. The LDL receptor (LDL-R) is responsible for uptake of native LDL particles and is regulated homeostatically by levels of intracellular cholesterol, preventing excess intracellular lipid accumulation[180]. Following oxidation of LDL, the LDL-R is no longer able to bind the LDL particle, which is now a recognisable ligand for receptors including scavenger receptors type A and B SRA/SRB), CD68, MARCO (MAcrophage Receptor with COllagenous structure) and LOX-1, many of which have also been shown to have a role in apoptotic cell clearance (see fig. 1)[181].

1.4.2.3. SRA-1

Scavenger receptor A-1 (SRA-1) has been described as a principal receptor in modified LDL uptake[182]. Initially identified as an acetylated LDL receptor, known ligands include oxidised LDL (oxLDL), LPS, PS and apoptotic cells[103,177,179,183,184]. It is a trimeric transmembrane glycoprotein with 6 domains, including the collagen-like domain; positive residues in this domain form a site for modified lipoprotein interaction[177,185]. SRA-1 expression is confined mainly to MØ, and has roles in adhesion, cell-cell interaction and innate immunity, therefore could have multiple roles in the development of an atherosclerotic lesion[177,186]. A role for SRA in clearance of apoptotic thymocytes has also been shown[103]. Foam cell formation has in part been attributed to SRA-1 mediated uptake of oxLDL, with a 30% reduction in macrophage oxLDL uptake in SRAI/II knockout mice[187].

1.4.2.4. CD36

CD36 is another oxLDL receptor with a central role in foam cell formation. Mice lacking both CD36 and SRA were found to have a reduction in oxLDL degradation of 70-90%, with no apparent foam cell formation[182]. Studies with CD36 blocking antibodies show that approximately 50% oxLDL binding is mediated by CD36[178]. CD36 is a class B scavenger receptor expressed by monocytes, MØ, platelets, ECs and adipose tissue[175]. CD36 has been shown to bind oxLDL and LDL modified by monocyte-generated reactive nitrogen species[188,189]. Apoptotic cells and native lipoproteins have also been shown as CD36 ligands[190,191].

1.4.2.5. LOX-1

LOX-1 is a novel oxLDL receptor which is primarily found on ECs[192]. Like SRA-1 and CD36, LOX-1 has been shown to have multiple ligands, including PS, bacteria and apoptotic cells[176]. The type II transmembrane protein receptor is shown to be expressed by MØ, and expression is thought to be upregulated in pro-atherogenic conditions[176,193]. The primary role for LOX-1 in atherogenesis is thought to be endothelial dysfunction and apoptosis following oxLDL uptake[194].

These key receptors play an important role in oxLDL uptake, foam cell formation and subsequent atherogenesis.

1.4.3. Apoptotic cell clearance in a lipid-rich environment

The atherosclerotic plaque is lipid-rich and contains persistent apoptotic cells. The established plaque is a complex, dynamic microenvironment consisting of numerous different cell types and inflammatory mediators, including ECs, SMCs, monocytes, MØ, FCs, mast cells, T lymphocytes, oxidised lipoproteins, Th1 and Th2 cytokines, MMPs, fibrin, collagen,

proteoglycans and cellular debris[58,147,148]. This results in high rates of apoptosis and secondary necrosis within the plaque[195]. Although all cell types play a role in plaque progression, monocytes, MØ and MØ-derived FCs play a central role in formation and progression of the plaque, including plaque rupture, making these cells a desirable target in controlling inflammation in this disease.

Failed AC clearance in atherosclerosis has become widely accepted[150], largely due to the presence of ACs in the plaque[46], despite a poor understanding of the mechanisms behind this. ACs largely go unseen *in vivo*, due to efficient clearance by neighbouring non-professional and recruited professional phagocytes[41,195]. At sites of high apoptosis, e.g. the thymus, some free ACs can be detected, and free AC numbers are increased when AC clearance mechanisms are interfered with, e.g. as seen in $CD14^{-/-}$ mice[38]. In the atherosclerotic plaque, ACs persist to the point of secondary necrosis, resulting in a necrotic core in advanced atheromas, leading to the hypothesis of defective AC clearance[196]. Whilst mechanisms of MØ cell death in the plaque are largely studied, clearance mechanisms post apoptosis are less well understood, and a small number of studies into foam cell clearance, and the presence of ACs in the plaque, has led to the conclusion that clearance is defective[46,195,197].

Previous studies looked into the causes of such a high number of AC in a localised area, and have shown oxLDL as an inducer of apoptosis in foam cells; this was attributed to lysosomal damage, with lysosomal membrane disruption causing leaking of contents into the cytosol[161,198,199]. Excess intracellular cholesterol, via acetylated LDL (acLDL) loading, has also been demonstrated to induce apoptosis after trafficking to the endoplasmic reticulum (ER), where calcium stores are depleted, resulting in death effector expression[200]. However other studies, have not found prominent oxLDL induced toxicity of MØ, demonstrating a need for further study[197,201].

Competition between oxLDL or oxidised red blood cells was observed to affect AC binding using murine peritoneal MØ, at oxLDL concentrations of 250µg/ml[195]. Studies have also found that oxLDL competes with apoptotic thymocytes for the same receptors[202]. It is well established that scavenger receptors, such as those present in this model, CD36, SRA-1 and LOX-1, recognise both AC and oxLDL[175-177]. The oxLDL receptor CD36 is thought to play an essential role in AC clearance *in vivo* via recognition of oxidised phosphatidylserine molecules on the AC surface, a molecule that may not be recognised if oxLDL remains bound to the receptor[101,203].

Varying approaches exist to investigate AC clearance within the plaque, and clarity is vital when defining models, to enable comparison of results across studies. The use of human

endartectomy samples gives a real example of the plaque environment, however the observation of live cellular processes is not possible, only the outcomes of such processes can be investigated. Sample numbers are often low depending on availability and results can vary greatly due to differences in genetic backgrounds and lifestyle. Murine endartectomy samples are popular as genetic backgrounds can be controlled and also the environment and diet of the mice can be controlled. The significant drawback using this method, along with the inability to observe live cell processes, is the comparison of results across species. The same drawback occurs with the use of *ex vivo* murine macrophages (e.g. peritoneal), though live cell processes can be observed, and *ex vivo* human macrophages are not available. The absence of true human macrophage cell lines also makes the use of murine macrophage cell lines a popular method to study macrophage behaviour. For those wanting maintain a human focus to research, monocyte-like cell lines can be induced to macrophage-like cells, though the method of induction and similarity of the cells to macrophages does raise questions of their own. Human monocyte-derived macrophages (HM-DMØ) from peripheral blood monocytes are arguably the closest fit to a true human monocyte/macrophage model, however even within this model, methods of monocyte isolation vary greatly, and the chosen method of differentiation is also inconsistent between research groups.

It is important to understand the basis of AC accumulation in the plaque[195], especially as an accretion of cells undergoing secondary necrosis exacerbate the pro-inflammatory environment of the plaque, contributing to plaque instability[149,195]. The nature of mechanisms behind inefficient AC clearance in the plaque is unknown, and could be a result of reduced function, competition in response to the local microenvironment, or a physiological change in phagocyte function in aged or damaged cells. The ability of foam cells to eat apoptotic foam cells is also uncharacterised, including the specific ligand-receptor partnering, and whether this compares to 'normal' cell clearance mechanisms. The persistence of inflammation within the plaque, despite immuno-modulatory mechanisms that usually follow AC clearance, has also not been addressed in human cells. Mechanisms of monocyte recruitment into the plaque have been partially characterised, but the impact of plaque-derived AC mediators have not been addressed with regards to possible over-recruitment of monocytes, and lack of macrophage egress in the plaque. Full characterisation of FCs and apoptotic FCs (aFC) in clearance could indicate where a failure in communication or function may arise. Many different AC receptors have been identified on phagocytes, including many of the oxLDL receptors already discussed, and bridging molecules such as thrombospondin and C1q should also be considered (see fig. 1)[204].

1.4.3.1. The role of necrotic cells as inducers of inflammation within the plaque

It is the accepted phenomenon that apoptotic and necrotic (primary and secondary) cell death have opposing immunological outcomes. Apoptosis is associated with immunologically silent cell death and corpse removal, with increasing evidence of a role in dampening inflammation[55,56]. Necrosis is associated with damage or trauma-induced cell death, loss of membrane integrity, and release of inflammatory intracellular contents, such as the much referred to HMGB1[205]. These 'danger signals' have been proposed to contain DAMPs (damage-associated molecular patterns), that like ACAMPs, mimic PAMPs for PRR recognition, and potential inflammation consequences[206]. Secondary necrosis occurs when apoptotic cells remain undisposed and develop features including, most importantly in the case of inciting inflammation, loss of membrane integrity, causing intracellular contents to leak from the dying cell[207].

Evidence now suggests that immune responses induced by these events are not as clear-cut as previously suggested. For example some ACs have been shown to induce an inflammatory response[208-210]. Persistent apoptotic (which can develop into secondary necrotic) or primary necrotic cells have also been shown to be non-inflammatory[38,40,211] Immunogenicity of cell death is not only predicted by the cell death pathway, other parameters must also be taken into account, including the intrinsic antigenicity of the dying cell, previous stress of activation, the cell death inducer and the availability of responding immune cells[212].

Anti-inflammatory cytokine release is an important stage in resolution of inflammation and occurs in response to AC interaction[54-56]. It may be that a foam cell, or an aged cell, is no longer capable of eliciting an anti-inflammatory response in response to AC interaction, contributing to the dominant pro-inflammatory environment within the plaque, and potentially contributing to the systemic inflammatory status of aged individuals and those with atherosclerosis[14,58,213].

Evidence of the inflammatory consequences of secondary necrosis is lacking. Fadok *et al*. observed anti-inflammatory signals could be induced via early apoptotic, late apoptotic or lysed neutrophils, however these signals were overridden by inflammatory proteases released by secondary necrotic cells[207]. Anti-inflammatory effects were overridden, however protease inhibitors abrogated any inflammatory response[207]. In support of the inflammatory consequences of secondary necrotic cells, Shibata *et al.* found that early apoptotic, but not secondary necrotic, neutrophils down-regulate LPS-induced pro-inflammatory cytokine production of murine macrophages via induction of NO production[214]. The study also found that secondary necrotic neutrophils induced TNF-α release, but both apoptotic and

secondary necrotic cells suppressed inflammatory IL-12p40 and IL-6 release[214]. This supports the notion that perceived immune modulation following certain mechanisms of cell death is not as clear-cut as often stated.

Immunogenic factors of primary necrosis, such as HMGB1, have been shown to be released during primary but not secondary necrosis of primary murine cells[205]. In contrast to this, HMGB1 release by apoptotic cell lines Jurkat, HeLa and U937 has been shown, demonstrating the crossover between markers for different cell death mechanisms[215]. The most compelling evidence for inflammatory consequences of uncleared ACs remains to be the presence of persisting AC and inflammation in autoimmune disease[34], but even in these cases, causality is not established. Examples in murine models have also been shown of failed AC clearance, without the expected associated inflammation or autoimmune disease[38,39]. Primary necrosis may also drive inflammatory cytokine expression in the plaque via release of inflammatory intracellular components, and evidence of primary necrosis in atherosclerosis in mice has been found[216]. However not all studies into necrotic cells have observed resulting inflammation described[40].

1.4.4. Cytokines in the atherosclerotic plaque

The cytokine profile in the atherosclerotic plaque is extremely complex given the quantity and variety of immune cells present, and the combination of pro-inflammatory mediators, e.g. oxLDL[217], and anti-inflammatory mediators, e.g. AC[55,56], and the resulting autocrine and paracrine effects on subsequent cytokine release. Sources of cytokine production can be categorised as primary, e.g. cytokine response to retained subendothelial lipoproteins, and secondary, e.g. cytokine response following established subendothelial inflammation and plaque formation, sources.

One of the key features of AC clearance is the non-inflammatory manner in which unwanted ‘self’ cells are disposed. This is not due to a lack of reaction by the phagocyte, but rather the release of a balance of cytokines, which have autocrine and paracrine effects, resulting in an immunologically silent or, even anti-inflammatory, response to self[54-56]. The interesting phenomenon in the atherosclerotic plaque is that despite an excess of ACs and phagocytic MØ, a pro-inflammatory environment persists[46,58].

Just as persistence of ACs is poorly understood, the failure of occurring phagocyte:AC interactions to regulate the inflammatory environment of the atherosclerotic plaque, as seen with other inflammatory responses[56], is also unknown. Cytokines found to be expressed in human atherosclerotic plaques include TNF-α, interleukins 1-3, 6, 8, 10, 12, 15,18, IFN-γ, TGF-β1-3, macrophage colony-stimulating factor (M-CSF) and a variety of chemokines[137].

A selection of cytokines associated with immune modulation following AC clearance, and also found in human atherosclerotic plaques, will be investigated as part of this study. This is based on the hypothesis that inapt FC responses to AC, or inappropriate tickling of MØ or FC by apoptotic FC, may contribute to the inflammatory environment of the plaque.

1.4.4.1. TNF-α in atherosclerosis

TNF-α is a classic pro-inflammatory cytokine of the TNF superfamily, which has 2 identified receptors, TNF-R1 and TNF-R2[137,218]. TNF-α is released by a broad variety of cells, but is primarily produced in monocytes and MØ[218], and is a suggested marker of classical activation. Cleavage of pro-TNF-α by TNF-α converting enzyme (TACE) ADAM17, an MMP, results in an active form of TNF-α; ADAM17 has also been implicated in soluble CX3CL1 shedding[219]. Basal levels of TNF-α production, and the ability of aFCs to suppress TNF-α release, may provide insight into MØ subtype following lipid-loading of a MØ model, giving clues as to the drivers of inflammation in the plaque.

TNF-α has been identified in human atherosclerotic plaques, and has been classed as a pro-atherogenic cytokine[137,220-222]. TNF-α has the ability to drive inflammation in the plaque, but was not shown to be essential for inflammation. TNF-α deficient *apoe*$^{-/-}$ mice have reduced levels of inflammation in the plaque and reduced lesion size, but inflammation was still present[223]. Other studies have found no effect of TNF-α on lesion size in mice. TNF-α has also been shown to reduce scavenger receptor expression in HM-DMØ, which could have athero-protective effects[224]. Increased plasma concentrations of TNF-α have been implicated as a biomarker for risk of recurrent coronary events in stable post-MI patients[225]. A Finnish study also found TNF-α was a predictor of coronary heart disease, cardiovascular disease events and total mortality in men[226].

AC ingestion has been shown to suppress LPS-induced TNF-α release *in vitro*[56]. It is unknown whether aFC have the ability to suppress inflammation in a similar manner to other AC models, e.g. apoptotic neutrophils.

1.4.4.2. IL-10 and IL-12 in atherosclerosis

IL-12 is a cytokine that is also associated with inflammation, and drives a Th1/inflammatory response[227]. IL-12 consists of 2 subunits, p35 and p40, to produce an active heterodimer, and is produced by phagocytes and antigen presenting cells[218]. The IL-12R is also made up of 2 subunits, IL-12Rβ1 and IL-12Rβ2. The ratio of IL-10:IL-12 release has been proposed as a marker for MØ activation, with high IL-10 and low IL-12 release associated with alternative MØ activation, and the opposite profile representing classical MØ activation[49,228]. *In vivo* the plaque milieu may prime or maintain monocytes, MØ and foam cells in a classically activated

state, which is supported by the data that pro-inflammatory Th1 cytokines are prominent within plaques[58].

IL-12 has been identified in human atherosclerotic plaques, and was found to be produced by monocytes *in vitro* in response to oxLDL[228]. It has also been found to drive anti-oxLDL antibody production in murine models of atherosclerosis, driving inflammation[229]. It has a cross-regulatory role with IL-10, and has been shown to be suppressed by AC ingestion[55,228]. The contribution of FC and aFC induced IL-12 production in response to pro- and non-inflammatory stimuli will be investigated in this study to aid understanding of the failed resolution of inflammation in atherosclerosis.

IL-10 is a regulatory, or anti-inflammatory, cytokine, associated with regulatory MØ activation, or Th2 responses[137]. As discussed in the previous section, IL-10:IL-12 ratios can be a marker of classical or regulatory MØ activation[49], and they have cross-regulatory roles[228].

IL-10 has been found in human atherosclerotic plaques, which in murine models was found to correlate with reduced iNOS expression and cell death, showing some degree of immune modulation within the plaque[228,230], and has been designated an anti-atherogenic cytokine[137]. It has been shown to be produced following AC ingestion in some studies[55], however Fadok *et al*. (1998) found suppression of IL-10 in response to AC[56]. Whether aFCs can regulate the IL-10:IL-12 balance in unknown.

Some evidence suggests that a reparative ‘M2’ phenotype may be favoured by processes within the plaque, such as oxLDL mediated induction of PPARγ expression[231,232], however the overall pro-inflammatory environment and lack of AC clearance indicates an ‘M1-like’ phenotype may predominate[58,195,217,233]. This has implications in further cell clearance, as TNF-α has been shown to inhibit clearance mechanisms[57,234]. Further research has manipulated MØ activation to an alternative phenotype to see if pathology is improved. In a rat model of myocardial infarction, a pathological event following atherosclerotic plaque rupture, repair was improved via administration of PS-liposomes, promoting a reparative macrophage phenotype in cardiac MØ[235].

1.5. Role of chemotaxis in clearance and plaque formation

The continual recruitment of monocytes to the atherosclerotic plaque is a key factor in disease progression, and inhibition of recruitment may prove to be athero-protective. MØ apoptosis is thought to be athero-protective in early lesions, with successful AC clearance by infiltrating monocytes dampening inflammation[236]. MØ apoptosis, and the subsequent recruitment of further monocytes from the blood stream, is thought to be a key event in

driving pathology of late atherosclerotic lesions, with persistent ACs and inflammation contributing to necrotic core formation and plaque instability[149].

A lack of MØ egress out of the atherosclerotic plaque via chemotaxis is also a contributing factor to atherosclerosis, which likely doesn't occur due to the failure of resolved inflammation, contributing to leukocyte trapping in the plaque[80,237,238].

Much research into apoptotic cell removal focuses on the interaction and phagocytosis phases of clearance, with increasing interest in subsequent immune-modulation. Research into the 'find-me' stages of AC clearance is lagging, however recent research has identified roles for CX3CL1[61] and ICAM-3[71] in attraction of monocytes to sites of cell death. Interest in these earlier stages of AC clearance is becoming more prominent as the potential for therapeutic targets at sites of inappropriate cell recruitment increases, e.g. in atherosclerosis. To this end, a strong model for investigating the precise mechanisms of cell recruitment employed in different pathological scenarios would be valuable when considering prevention of inappropriate or excessive cell recruitment, as seen in atherosclerosis[156,239].

1.5.1. Recruitment of monocytes to the plaque

Chemotaxis is the directional movement of cells along a concentration gradient of an attractive agent, an active process which is distinct from chemokinesis, the general, non-directional movement of cells[240]. Chemo-attractive agents induce cell movement via promotion of cell motility and upregulation of adhesion molecule expression. Chemotaxis of immune cells to sites of infection or damage is a vital starting point to a robust immune response. Circulating leukocytes continually but transiently interact with the endothelium during immune surveillance, rolling along the endothelium as a result of shear force and low-affinity interaction, which is mediated by adhesion molecules known as selectins on the leukocyte and endothelial cell surfaces[241,242].

1.5.1.1. Selectins and selectin ligands

Selectins are type I transmembrane glycoproteins, a subclass from the lectin family (reviewed in [243]). Selectin expression is restricted to leukocytes, platelets and the vascular system, thus have a specific role in leukocyte-endothelial cell adhesion and leukocyte recruitment to sites infection and inflammation[244]. L-selectin is found on the leukocyte cell surface and is thought to be crucial for leukocyte rolling *in vivo*[245]. L-selectin is able to mediate leukocyte rolling independent of E- and P-selectin, and is important in endothelial cell tethering[246]. Secondary capture via L-selectin can also occur between circulating and adherent leukocytes on the endothelial cell surface, which has been shown to have particular importance in leukocyte tethering in arterial venules and subsequently, atherogenesis[247]. E-selectin, expressed on endothelial cells, is upregulated by inflammatory cytokines including

TNF-α[160,248], which is found in human atherosclerotic plaques[222]. P-selectin is expressed by platelets, megakaryocytes and endothelial cells[249]. It is expressed constitutively in endothelial cells, packaged intracellularly in Weibel-Palade bodies and is trafficked rapidly to the endothelial cell surface following activation by effector molecules including histamine[249].

P-selectin expression is increased in endothelial cells overlying active atherosclerotic plaques, and co-expressed with upregulated ICAM-1, which has low expression levels in healthy arteries[157]. E-selectin and ICAM-1 expression is also increased in human plaque endothelial cells[250]. ICAM-1and E-selectin were constitutively expressed on intimal endothelium of both normal coronary arteries and those overlying fatty streaks, but ICAM-1 is increased only on and around plaque macrophages[251].

Following oxLDL exposure, it was found that ECs bind an increased number of monocytes with a stronger binding force than non-exposed ECs, via mechanisms including increased expression of ICAM-1 and P-selectin[158,252]. Vascular cell adhesion molecule-1 (VCAM-1) was also found to be upregulated indirectly via oxLDL induced cytokine-activated gene expression[159], specifically TNF-α. OxLDL has also been shown to stimulate the release of monocyte chemotactic protein-1 (MCP-1) from endothelial and smooth muscle cells, recruiting circulating monocytes[253].

All selectins recognise sialylated carbohydrates, and other modified carbohydrates and glycoproteins with varying avidities, including fucosylation, sulphation and glycation.[243] On leukocytes, PGSL-1 is the dominant ligand for P- and L-selectin[243,254,255]. E-selectin receptors have been difficult to identify, but include endothelial selectin ligand-1(ESL-1)[243,256,257].

1.5.1.2. Integrins and integrin ligands

Stable interaction is established by activation of integrins. β_2 (e.g. LFA-1) and α_4 integrins (VLA-4) are the integrins that regulate leukocyte trafficking[258]. LFA-1 is expressed constitutively on leukocytes, and interacts with ICAM-1 and 2[258]. VLA-4 is expressed on monocytes and lymphocytes with homing potential, and interacts with a number of receptors, including VCAM-1[258]. PECAM-1 was also found to promote atherogenesis, attributed to its mechanosensitive properties, and the role of shear stress in atherogenesis[258].

Subendothelial lipoprotein retention has been shown to induce VCAM-1 expression[259]. VCAM-1 and ICAM-1 have been found on human coronary arteries[260]. TNF-α also induces VCAM-1 and ICAM-1 expression in human umbilical vein endothelial cells (HUVECs)[261]. ICAM-1 expression was also increased by native LDL in HUVECs, enhancing monocyte recruitment to the endothelium[262].

1.5.1.3. Integrin-independent leukocyte adhesion

CX3CL1 has also been shown to mediate leukocyte adhesion in an integrin-independent manner[68]. CX3CL1 is expressed by endothelial cells in response to inflammatory mediators and acts as an adhesion molecule. CX3CL1 also has a role in NK-mediated endothelium damage[263].

1.5.1.4. Transendothelial monocyte migration

Transendothelial migration of monocytes has been shown to promote monocyte differentiation to macrophages and formation of foam cells in the plaque[153]. Monocytes in the sub-endothelium can be induced to differentiate into macrophages directly via oxLDL and also by oxLDL stimulated release of M-CSF[264,265]. Macrophages then gorge on modified lipoproteins via receptor mediated endocytosis in an unregulated manner, becoming lipid-laden foam cells[177,266]. These events further demonstrate a key role for oxLDL and monocytes/macrophages in plaque progression.

1.5.1.5. Apoptotic cell-derived microparticles

Microparticles have been widely studied in atherosclerosis, but in the context of biomarkers of disease and thrombus formation[267]. The role of MPs in inflammation and immune cell chemotaxis is now becoming of greater focus[268], with the majority of plaque microparticles thought to be macrophage-derived[269].

Microparticles (MPs) are phospholipid and protein rich submicron particles, derived from cell membranes of activated or dying cells[270]. MPs range in size from 0.1-2µm, with particles less than 100nm termed exosomes, and those greater than 1.5µm in diameter referred to as apoptotic bodies[270] MPs are generated following zeiosis, or blebbing, and requires uncoupling of the cell membrane with the underlying cytoskeleton[271 271,272]. This results in shedding of microparticles (MPs), or released blebs, which have been shown to be attractive to monocytes[60]. UV-B has been shown to induce apoptosis via intrinsic and extrinsic pathways, a non-specific inducer of apoptosis, with mechanisms including DNA damage, oxidative stress and death receptor upregulation[273]. These pathways culminate in caspase cascade activation and resultant apoptosis[274]. Loss of cytoskeletal integrity is a key stage of apoptosis that leads to the generation of blebs/MPs, and eventually larger apoptotic bodies[275].

Lauber *et al.*[62], through the use of caspase-3 deficient MCF-7 cells, showed AC-mediated chemotaxis induction is caspase-3 dependent. Caspase-3, a cysteine-aspartic acid protease, is an effector caspase which is present in cell lines used in studies here. It plays a central role in apoptosis, and can activate ROCK-1 or PAK2 to induce membrane blebbing[276,277].

This generates phospholipid rich MPs, which also contain a rich source of cell specific antigens, including proteins and lipids, which could provide a huge source of unrecognised 'find-me' signals for phagocyte recruitment to dying cells[278].

MPs in human atherosclerosis have been studied to the degree of proteomic, metabolomic, and immunomic characterisation[278], but this has been focused largely on thrombotic potential and as biomarkers of underlying disease[269]. Immunogenicity of leukocyte-derived microparticles on endothelial cells has been shown[279,280]. MPs have also been shown to promote monocyte recruitment to the plaque via the transfer of ICAM-1 to endothelial cells[281]. The administration of THP-1-derived MPs was also shown to contribute to atherogenesis in a murine model of atherosclerosis[282]. So overall, the ability of MPs to modulate mechanisms in atherosclerosis has been addressed in many areas, however the influence of MPs on recruitment of monocytes to apoptotic cells in the plaque has not been addressed.

A role for MPs as a 'find-me' mediator in AC clearance is emerging, and is of particular interest in a plaque-like environment as MPs are extremely heterogeneous in nature, and reflect the status of the parent cell. If the parent cell, e.g. a lipid loaded MØ (known as foam cells) has properties that enhance disease progression, MPs can confer this message to surrounding cells, as they bear antigens of the parent cell. MP-derived antigens may confer signals via ligands on the surface of surrounding cells, or MPs may behave as a vector, transferring antigens directly to be incorporated into surrounding cells[281].

Chemoattractants CX3CL1 and ICAM-3, have recently been associated with apoptotic B cell-derived MPs, and blocking action of these attractants was shown to reduce monocyte migration to MP[61,71], however this has not been studied in the context of apoptotic MØ or atherosclerosis, despite the known role of CX3CL1 and its cognate receptor (CX3CL1) in atherogenesis[239,283]. As MPs are a result of membrane blebbing, they contain a high proportion of phospholipids, including PS, and membrane proteins[284]. The receptors for MP-induced chemotaxis are not well defined, and as CD14 has been previously shown to mediate AC recognition in this model, it is a prime candidate for AC-derived MP recognition.

1.5.1.6. Soluble factors

Chemokines are a cytokine subfamily, of mediators that can induce directed migration of cells to points of cell stress, death or disease. They are categorised into 4 families according to the spacing of the cysteine residues (C,CC,CXC and CX_3C) , and CX3CL1 (CX3CL1) is the only identified member of the CX_3C chemokine subclass[238,285]. The unusual transmembrane structure of CX3CL1 consists of an extracellular N-terminal chemokine domain presented on a mucin-like stalk, a transmembrane α helix and a short cytoplasmic tail[285,286] (fig. 4). Endothelial cells express CX3CL1 in response to activation or initiation of

apoptosis, where it acts as an adhesion molecule, initiating firm adhesion of CX3CL1 receptor CX3CR1 positive monocytes[68]. CX3CR1 is a 7 transmembrane domain GPCR expressed predominantly on leukocytes, NK cells and T cells[64,242].

To function as a classic chemokine, CX3CL1 is cleaved via metalloproteinases tumour necrosis factor-alpha converting enzyme (TACE/ADAM17) or ADAM10[219,287] (fig. 4). Following cleavage, from the cell membrane, CX3CL1 is shed from the cell surface and functions as a chemokine to recruit CX3CR1 positive cells[288]. G-protein signalling is required for chemotaxis of CX3CR1 positive cells[64].

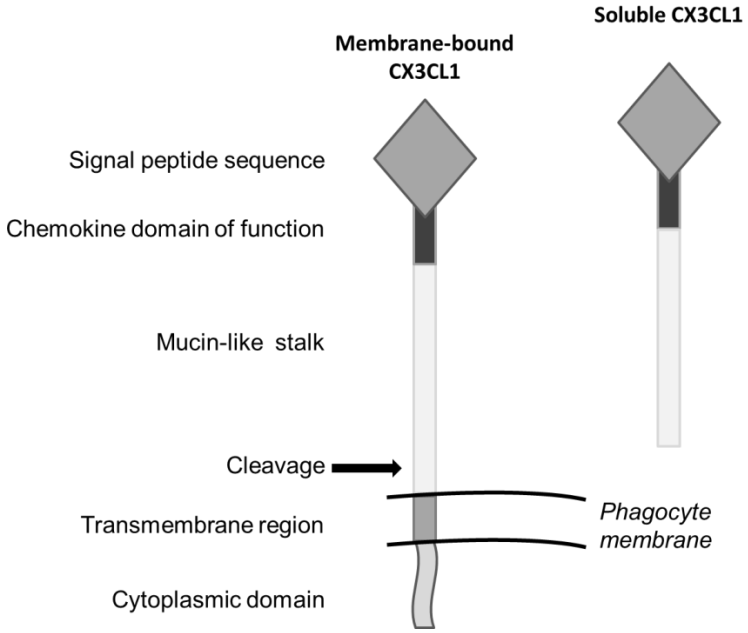


Figure 5. Schematic of membrane bound and soluble CX3CL1 *Membrane bound CX3CL1 acts as an adhesion molecule in monocyte recruitment to the endothelium during atherogenesis. Apoptotic cells have been shown to release soluble and microparticle-associated CX3CL1 to attract phagocytes for clearance. Adapted from Liu* et al.[289].

CX3CL1 was identified by Truman *et al*. on microparticles released by apoptotic B cells, and is recognised by CX3CR1 on the phagocyte surface[64]. CX3CL1 was also found to increase expression of milk fat globule-endothelial growth factor 8 (MFG-E8), an identified bridging molecule between apoptotic cells and phagocytes, thus enhancing clearance[69]. With such a prominent role in recruitment to dead cells, it is only logical that CX3CR1 has a role to play in atherogenesis, especially considering the influence this chemokine has on other processes involved in plaque progression.

CX3CL1 is also a survival signal against apoptosis. This has implications when considering the accumulation of potentially 'sick' lipid-laden cells within the plaque, and may be athero-protective, or atherogenic, according to whether plaques are in the early or late stages[149].

Soluble CX3CL1 also influences monocyte integrin function, enhancing avidity to ICAM-1 and VCAM-1[290,291].

Other soluble factors involved in phagocyte recruitment to AC in the plaque are likely to include other 'find-me' signals discussed previously (see 1.1.2.), including chemokines IL-8 and MCP-1[59], nucleotide chemoattractants ATP and UTP[63] and lipid mediators LPC and S1P[62,72,73]. 'Keep-out' signals such as lactoferrin may also be present within the plaque[78,79].

1.5.2. Modelling chemotaxis to apoptotic cells

As interest in chemotaxis to apoptotic cells develops, modelling mechanisms must equally develop. Previous research into monocyte recruitment to apoptotic cells has focused on vertical systems including transwells and vertical chambers[61-63,71], e.g. modified Boyden chamber,which has become the most widely used method to study mammalian cell chemotaxis *in vitro*[292-294], but whilst the system has advantages, it also has many limitations[292].

In vertical assay systems, the concentration gradient of the attractive agent is at 100% in the lower wells of the vertical chamber, and 0% in the upper wells, above the microporous membrane, which is the starting point of the migrating cells. The exact nature of the gradient around the membrane is unknown and is likely affected by the migrating cells themselves[292], which will settle onto the top of the membrane before moving through, and blocking, the pores. Also the nature of cell movement cannot be distinguished during migration, so conclusions must be made based on the final distribution of migrated cells at the end of the assay[292].

More recently, increasingly precise methods are being sought to enable the mechanisms of chemotaxis to be investigated more thoroughly, with particular emphasis on stable concentration gradients of attractants, greater optical features and greater sensitivity and reproducibility[292,295,296], which traditional methods such as the vertical chambers and under-agarose assays do not provide[292,294,297].

1.5.2.1. Advantages of a horizontal assay system

The Dunn chamber is a horizontal assay system using modified glass slides. The optics using the Dunn chamber are excellent and precise movement of individual cells can be observed. The Dunn chamber also provides the ability to observe in detail the kinetics of cell movement, including velocity and distance, and the precise time frame over which individual cells move, rather than the time frame of the assay. It can also be deduced whether cells within a population are moving uniformly or within subpopulations, a key focus of study of monocyte migration, given the observation of monocytic subpopulations with preferential

migratory properties according to differential surface receptor expression[50,298]. The Dunn chamber also provides a linear concentration gradient of attractant, which is established quickly between inner and outer chambers following assay set up (approx. 10-30 minutes), with a half-life of up to 30 hours depending on the molecular weight of the attractant[292].

There are advantages of using the vertical chamber, particularly the widespread use of the technique in the field, resulting in a well-characterised assay[61,71,293]. The assay also provides a high throughput of assays compared to a many other methods, including the Dunn method, allowing statistical significance to be established in fewer assays. Clear trends can be seen on qualitative plots generated using the Dunn chamber in these studies, but many lack the power of statistical significance, indicating a need for further repeats of assays. Multiple wells in the vertical chamber (48) make it more efficient in this respect, though multiple fields of view can be used in the Dunn assay to improve statistical data.

Dunn chambers have not been used to date to investigate migration of monocytes to apoptotic cells. Whether apoptotic MØ- and apoptotic FC-derived MPs play a role in monocyte recruitment to the atherosclerotic plaque is not known.

1.6. Aims and objectives

The current study aims to better understand the clearance of apoptotic cells by MØ in ageing and age-related conditions, with a particular emphasis on apoptotic cell clearance in atherosclerosis. It aims to address the current gaps in knowledge relating to the mechanisms that underpin apoptotic cell clearance and how these are affected by ageing and by the aged-environment (as may occur within the atheromatous plaque).

The primary hypothesis under consideration is that AC clearance becomes defective in ageing. In addressing this hypothesis, the influence of an aged environment or altered cell function will be assessed.

The specific aims of this project are:

1. <u>To assess the ability of MØ and foam cells to interact with apoptotic cells:</u>

Initial studies will assess the use of human myeloid leukaemia THP-1 cells as a model of monocyte and MØ function, with regards to mechanisms involved in apoptotic cell clearance. This will include relevant processes to the atherosclerotic plaque, with the use of lipid-loaded

(oxLDL) THP-1-derived MØ to create foam cells, a cell phenotype specific to the atherosclerotic plaque.

The ability of MØ and foam cells (FC) to interact with apoptotic cells, including apoptotic MØ and apoptotic FC, will be assessed. This is a process that has not been studied using human MØ-derived apoptotic cells[55,203].

Human monocyte-derived MØ will also be used as a phagocyte model, to compare responses in immortal human cell lines versus human primary cells. The ability of HM-DMØ to interact with THP-1 models of apoptotic MØ and apoptotic FC will be assessed. HM-DMØ interaction with apoptotic MØ or FC has not previously been investigated.

2. <u>To assess the nature of cytokine responses of MØ and foam cells to apoptotic cells:</u>

To further understand the drivers of inflammation in the plaque, TNF-α, IL-10 and IL-12 profiles of THP-1 derived- and HM-DMØ following exposure to a variety of stimuli will be assessed. This includes cytokine release at rest, following long-term lipid loading, to deduce whether foam cells are hyper-inflammatory. The response to LPS will be assessed, to allow comparison of inflammatory responses between lipid- (FC) and non-lipid-loaded (MØ) phagocytes. Cytokine responses to apoptotic MØ and apoptotic foam cells will be investigated, as this has not been studied in human cells, so it is unknown whether a non-inflammatory phenotype, as observed in other apoptotic cell models, is relevant to the plaque environment[54]. The subsequent immune modulation in response to apoptotic cells, will also be assessed. This includes the ability of apoptotic MØ and apoptotic FC to 'switch-off' an inflammatory response, as seen with other apoptotic cell models[55].

Human monocyte-derived MØ will also be used as a phagocyte model, to compare responses in immortal human cell lines versus human primary cells. The ability of HM-DMØ to modulate immune responses following interaction with THP-1 models of apoptotic MØ and apoptotic FC will be assessed. HM-DMØ interaction with apoptotic MØ or FC has not previously been investigated.

3. <u>To assess monocyte recruitment to apoptotic MØ and foam cells:</u>

The chemoattractive properties of apoptotic cells and apoptotic cell-derived supernatant to THP-1 monocytes will also be assessed in a novel manner, with the use of a horizontal chemotaxis chamber. This will include monocyte migration to apoptotic MØ and apoptotic FC-conditioned medium, as 'find-me' signals released by apoptotic cells in the plaque have not previously been studied. Mechanisms of apoptotic-cell induced chemoattraction will be

investigated, as these have not been studied previously using MØ as an apoptotic cell model, by targeting candidate mediators with blocking antibodies.

4. To assess the ability of MØ from aged individuals to interact with and respond to AC:

Finally, a small scale pilot study using young (<25) and mid-life (>40) donors will be carried out to assess the ability of HM-DMØ from each age group to interact with, and modulate inflammation in response to, apoptotic cells.

Donor plasma will also be used to condition THP-1-derived MØ in a 'young' or 'aged' environment, and assess the effect on THP-1-derived MØ clearance of apoptotic cells. This will be carried out in the absence of presence of donor serum from young or mid-life donors, to assess the role of immediate effects, versus the long term effects, of MØ interaction with apoptotic cells, in the presence of a 'young' or 'aged' environment. Similar studies have been done in rodents[33], but this has not previously been assessed in humans. This may also indicate links between failure of control of immune responses in ageing and in atherosclerosis.

Results from this study will advance current knowledge of phagocyte clearance of apoptotic cells in ageing and in an age-related environment. This would then provide future direction on the improvement of control of inflammatory responses in conditions such as atherosclerosis and ageing.

Materials and Methods

2.1. Cell culture

2.1.1. Cell lines

THP-1: A human monocytic leukaemia cell line which grow in suspension. THP-1 cells can be differentiated into macrophage-like cells using various compounds[299,300].
Jurkat: A human T cell leukaemia cell line which grow in suspension[301].
Mutu: EBV-positive Burkitt lymphoma cell line which grow in suspension[302].
HUVEC: Human umbilical vein endothelial cell line, grown according to manufacturer's instruction (ATCC, Virginia, USA) and maintained at P<6.

2.1.2. Cell culture and differentiation

THP-1, Jurkat and Mutu cell lines were cultured in RPMI supplemented with 10% (v/v) foetal call serum (FCS), 2mM L-Glutamine, 100RU/ml Penicillin, and 100µg/ml Streptomycin (cRPMI), all purchased from PAA (Yeovil, UK). Cells were incubated at 37C in a 5% CO_2 humidified incubator and passaged when high levels of confluence were reached, approximately every 2-4 days. This maintained cell densities between 2 $x10^5$ -1 x 10^6 cells/ml.

HUVECs were cultured in Endothelial Cell Growth Medium (PromoCell, Heidelberg, Germany). Cells were incubated at 37C in a 5% CO_2 humidified incubator and passaged when high levels of confluence were reached, approximately every 4-5 days. Cells were maintained at a maximum of 80% confluence and used at a passage number of <6.

THP-1 cells were treated with 100nm VD3 (Enzo Life Sciences, Exeter, UK), 250nm PMA (Sigma-Aldrich, Dorset, UK) or both (double stimulated, DS) and incubated for 72 hours to induce differentiation into macrophage-like cells. Phase contrast images (20x) were taken to observe morphology using a fully motorised Zeiss Axiovert 200 M fluorescence microscope (Carl Zeiss Ltd, Welwyn Garden City, Fradley, Staffordshire, UK) and Hamamatsu Orca camera driven by Velocity (Perkin-Elmer, Cambridge, UK).

2.1.3. Primary cell isolation and culture

Solutions

Dextran solution:	6% w/v Dextran in PBS
Isotonic Percoll stock solution:	10% v/v PBS (10X) in Percoll
Percoll working solution:	64% v/v Isotonic Percoll stock solution in 1X PBS

Up to 50ml of blood was taken from healthy volunteers using 9ml K3EDTA Vacuettes (Greiner Bio-One, Stonehouse, Great Britain), in accordance with ethical guidelines (Aston University Ethics Committee). Anti-coagulated (EDTA) blood samples were centrifuged to separate cells from platelet-rich plasma (350g; 20mins/brake off). The plasma layer was removed and frozen for future use. 6% dextran in Dulbecco's PBS (w/v) (Pharmacosmos, Holbaek, Denmark; PAA), was added to the cell fraction (2:1 total cell volume:dextran solution) to sediment red blood cells (2h; RT), leaving a leukocyte-rich fraction resting on top of the red blood cell fraction. The leukocyte-rich upper layer was harvested and dextran was removed by washing in sfRPMI + 1% (w/v) BSA (PAA) (350g; 4mins), and the leukocyte pellet resuspended in approximately 1ml sfRPMI + 1% BSA. Resuspended leukocytes were layered onto a band of 64% Percoll working solution, and centrifuged (700g; 20mins; brake off), leaving a peripheral blood mononuclear cell (PBMC) band on top of a Percoll layer, and an uppermost layer of RPMI. The PBMC band was extracted with a 1ml Pasteur pipette and washed in sfRPMI + 1% BSA (w/v) (350g; 6mins). PBMCs were resuspended in specialist chemically-defined MØ medium (Life Technologies, Paisley, UK) (serum-free, 2mM L-Glutamine, 100RU/ml Penicillin, and 100µg/ml Streptomycin) and seeded directly into 24 or 96 well plates, or onto glass coverslips, for future assays. Non-adherent cells (lymphocytes) were removed 20-24 hours post-seeding by extensive washing. Adherent cells (monocytes) were allowed to differentiate to MØ for 7-14 days prior to use. Medium was replaced every 3-5 days prior to assay use.

2.2. Foam cell formation

2.2.1. LDL isolation and oxidation

Solutions

Light solution:	15.1046g KBr in 100ml dH_2O
Heavy solution:	33.4218g KBr in 100ml dH_2O
BCA/copper sulphate working solution:	200µl copper (II) sulphate solution (4% w/v)
	19.8mls BCA solution

Human plasma was obtained from healthy volunteers. Native LDL (nLDL) was isolated from the plasma by ultracentrifugation at 32000xg (20h;16C), using light and heavy potassium bromide (KBr) solutions (Sigma-Aldrich) to provide a density gradient. In brief, 50mg sucrose (Sigma-Aldrich) and 770mg Kbr were dissolved in 2mls of plasma. 200µl ethylene glycol (Sigma-Aldrich) was added and 2mls of heavy solution was layered on top of the plasma. 4mls of KBr heavy solution was then layered on top, followed by dH_2O, which was added until 2-3mm space remained at the top of the centrifuge tube. The nLDL band was removed

and desalted by size exclusion chromatography using PD10 columns (GE Healthcare, Buckinghamshire, UK). The nLDL was then filtered through a 0.45µm filter to sterilise. Liquid nitrogen was used to snap freeze samples in 1 ml aliquots, aliquots were then stored at -20C until required.

The BCA assay was used to measure concentration of LDL samples, which was chosen on the principle that there is one protein molecule per LDL particle (apo-B100). Standards of protein from 0-2.5mg/ml were generated using BSA diluted in PBS (PAA). Samples were incubated with BCA/copper sulphate (Sigma-Aldrich) working solution for 30 minutes at 37C before the optical density was read at 570nm using a Biotek EL800 Microplate Reader. A standard curve was generated, based on known concentrations of standard solutions, to estimate the concentration of LDL in the sample.

Oxidised LDL samples (oxLDL) were produced by incubating nLDL with 10µM copper sulphate (1h; 37C). After 1 hour, 10µl (0.5M) of the chelating agent EDTA (Sigma-Aldrich) was added to prevent further oxidation. The oxLDL was then desalted by size exclusion chromatography using PD10 columns and sterile filtered at 0.45µm. Samples were snap frozen using liquid nitrogen and stored in 1ml aliquots at -20C until required.

2.2.2. Carbonyl ELISA to measure protein oxidation

Solutions:

Coating buffer:	1 x carbonate-bicarbinate buffer capsules in 100ml dH_2O
Wash buffer:	0.9% (w/v) saline supplemented with 0.05% (v/v) Tween 20
Block buffer:	1% (v/v) Tween 20 (Sigma-Aldrich) in PBS

LDL samples and BSA standards (provided by Chris Dunston, Aston University, UK) were diluted to 20µg/ml protein and made up to 200µl in coating buffer. 50µl of each sample was added to wells in triplicate and incubated (1h; 37C). Wells were washed 3 times with wash buffer and 50µl 2,4-dinitrophenylhydrazine (DNPH), (1mM in 2M HCl), (Sigma-Aldrich), was added for 1 hour at room temperature to derivatise carbonyl groups. Wells were washed 3 times with wash buffer and 50µl secondary antibody (goat anti-mouse IgE, diluted 1:5000 in blocking buffer) (Sigma-Aldrich) was added and the plate incubated (1h; 37C). Wells were washed 3 times with wash buffer and samples developed with *o*-Phenylenediamine dihydrochloride (OPD) (100µl/well) (Sigma Aldrich). The reaction was stopped with 50µl of 1M HCl (Sigma-Aldrich) per well and the plate read at 490nm using a Biotek EL800 Microplate Reader. A standard curve was generated, based on known concentrations of standard solutions, to estimate the carbonyl content in each sample.

2.2.3. Foam cell generation

THP-1 cells were seeded in 24 well tissue culture plates and differentiated into macrophage-like cells as above (see 2.1.2.). Unstimulated, VD3 stimulated, PMA stimulated and double stimulated (DS) THP-1 cells were then incubated for a further 72 hours at 37C with desired concentrations of nLDL and oxLDL (diluted in cRPMI). Phase contrast images (20x) were taken to observe morphology using a fully motorised Zeiss Axiovert 200 M fluorescence microscope (Carl Zeiss Ltd) and Hamamatsu Orca camera driven by Velocity (Perkin-Elmer).

2.2.4. Visualising lipid droplets with light microscopy – Oil Red O staining

THP-1, VD3 stimulated, PMA stimulated and double stimulated cells were treated with nLDL and oxLDL as above (2.2.3.), fixed with 1% w/v formaldehyde (Sigma-Aldrich) solution (diluted in PBS) and stored at 4C until staining. Immediately prior to staining, Oil Red O working solution (Cayman Chemical, Michigan, USA) was prepared by adding 6 parts stock solution to 4 parts dH_2O and filtered through a 0.4µm filter to remove precipitate prior to staining. Fixative was removed and each well washed with dH_2O. 60% isopropanol (Sigma-Aldrich) was then added to each well for 5 minutes. The isopropanol was removed and Oil Red O working solution was added to each well for 30 minutes at room temperature. Wells were then washed with dH_2O, lipid accumulation observed in cells and phase contrast images (10x, 20x) were taken to observe morphology using a fully motorised Zeiss Axiovert 200 M fluorescence microscope (Carl Zeiss Ltd) and Hamamatsu Orca camera driven by Velocity (Perkin-Elmer).

2.2.5. Visualising lipid droplets with fluorescence microscopy – Nile Red staining

In order to observe lipid accumulation in cells, double stimulated cells (following LDL treatment) were fixed with 1% (w/v) formaldehyde solution (diluted in PBS) and stored at 4C until staining. Oleic acid treatment was used as a positive control for staining (1:5000 dilution in cRPMI). Staining was carried out as per manufacturer's instructions (Lipid Droplets Fluorescence Assay Kit, Cayman Chemical). In brief, cells were washed with provided assay buffer before Nile Red staining solution was added (1:1000 dilution in assay buffer) for 15 minutes at room temperature. Cells were then washed with assay buffer and lipid droplet staining observed via fluorescence microscopy (excitation/emission = 485/535) (40x). Lipid droplets appear as green round spots. Images (20x) were taken to observe morphology using a fully motorised Zeiss Axiovert 200 M fluorescence microscope (Carl Zeiss Ltd) and Hamamatsu Orca camera driven by Velocity (Perkin-Elmer).

2.2.6. Quantifying lipid droplet accumulation – assay optimisation

A black opaque 96 well plate (Cat. No. DPS-134-070R, Fisher Scientific, Loughborough, UK) and a clear 96 well tissue culture plate (Cat. No. CC015, Appleton Woods, Birmingham, UK)

were loaded with varying concentrations of goat anti-mouse-FITC (Sigma-Aldrich) to compare overspill of emitted light between wells. Additionally, cells were cultured in a clear 96 well tissue culture plate, then lysed with 2% SDS (Sigma-Aldrich). Whole cell lysate was then transferred to corresponding wells in a black 96 well plate for Nile Red staining (see 2.2.5.).

2.2.7. Quantifying lipid droplet accumulation – fluorescence plate reader assay

Double stimulated cells were treated with oxLDL as above (2.2.5.), at a density of 5 x 10^3 cells per well, in a black, clear bottomed 96 well tissue culture plate (Cat. No. DPS-130-010N, Fisher Scientific, Loughborough, UK). Oleic acid treatment was used as a positive control for staining (1:5000 dilution in full culture medium). Staining was carried out as per manufacturer's instructions. In brief, cells were washed with provided assay buffer and fixed with fixative solution (diluted 1:10 in assay buffer) for 10 minutes. The plate was centrifuged and washed with assay buffer before Nile Red staining solution was added (1:1000 dilution in assay buffer) for 15 minutes at room temperature. Cells were then washed with assay buffer and lipid droplet staining observed with a Spectramax Gemini EM fluorescence plate reader (Molecular Devices) (excitation/emission = 485/535). Results showed that this protocol was not efficient at predicting foam cell formation (data not shown).

2.3. Assessment of cell viability

Levels of apoptosis were continually monitored across all cell lines using light microscopy to assess the presence of morphological features of apoptosis, such as cell shrinkage, nuclear condensation and blebbing. Cells were also routinely analysed via flow cytometry to assess proportions of the population that were displaying live or dead morphology.

2.3.1. Annexin V/Propidium iodide staining

Solutions

Binding buffer: 150mM NaCl, 10mM HEPES (pH7-7.5), 2.5mM $CaCl_2$ in dH_2O

To assess cell viability, cells were washed in binding buffer and stained with 2µl (1:50 in binding buffer) Annexin V-FITC (AxV-FITC) (Bender MedSystems, Vienna, Austria) for 15 minutes on ice and washed once with, and resuspended in, 1ml binding buffer. Immediately prior to flow cytometric analysis of each sample, 5µl of stock propidium iodide (PI) from the AxV-FITC Apoptosis Detection Kit was added to reveal the proportion of cells with a leaky cell membrane, a marker of cell necrosis. Cells were analysed using a Beckman Coulter Quanta SC flow cytometer. Unstained cells were used as a negative control.

2.3.2. Assessing toxicity induced by native and oxidised LDL

To assess toxicity of LDL treatment on monocyte-like cells, THP-1 were seeded in 6 well tissue culture plates in a total volume of 5mls. Cells were treated with nLDL and oxLDL diluted in cRPMI. Following LDL treatment, 500µl samples were taken from each well daily for up to 7 days to assess levels of apoptosis and necrosis induced by LDL treatment, as in 2.3.1.

To assess toxicity of LDL treatment on macrophage-like cells, THP-1 cells were seeded in 6 well tissue culture plates. Cells were treated with nLDL and oxLDL diluted in cRPMI and one tissue culture plate was sampled each day. Immediately prior to staining, cells in all wells of one plate were incubated in 5mM EDTA in PBS for 1 hour at 37C to bring adherent cells into suspension. Cells were then washed twice in binding buffer before AxV/PI staining as above, as on 2.3.1. Unstained cells were used as a negative control.

2.3.3. Apoptosis induction

To induce apoptosis with minimal secondary necrosis, Jurkat, Mutu, THP-1 macrophages and THP-1 foam cells were treated with 100mJ/cm^2 UV radiation (typically 2-4 minutes treatment), using a UVP UXV Chromato-Vue C-71 light box, and incubated for 18-24 hours at 37C. To confirm induction of apoptosis, cells were stained for flow cytometry according to the protocol in section 2.3.1. UV dose was monitored using a UXP UVX Radiometer. Induction of apoptosis was also confirmed using light microscopy, as discussed above.

To confirm levels of apoptosis in cells for individual assays, a sample of the apoptotic cells were fixed with 1% (w/v) formaldehyde in PBS for flow cytometry and side scatter and forward scatter evaluated. The smaller, granular population appear in the 'dead' zone, whereas the larger, healthy cell population appear in a distinct 'live' zone[303].

<u>All apoptotic cells used in these studies were human cell-line derived.</u>

2.3.4. Isolation of cell-free supernatant

Viable cells were seeded at 2x10^6 cells/ml in chemically-defined macrophage medium + 2mM L-glutamine + 100IU/ml penicillin. Medium was also replaced prior to UV treatment. Apoptosis was induced by UVB irradiation (see 2.3.3.) and supernatants harvested following 18-24 hours incubation at 37C. Large cell debris was removed by centrifugation at 350xg for 6 minutes to generate cell-free supernatant (CFS), which contains microparticles and soluble factors (e.g. cytokines and chemokines).

2.4. Immunofluorescence staining of cell surface proteins

Cells were washed with 0.1% w/v BSA in PBS and 2 x 10^5 cells were added to each tube for staining. Cells were stained for 45 minutes on ice with the relevant primary antibodies, to a final volume of 100µl, using 20% v/v normal human serum (NHS) (PAA) in PBS as a diluent. All primary antibodies were titrated and concentrations chosen according to saturation of receptor staining. Control antibody concentrations were chosen to match maximum antibody concentrations of the same isotype. Following primary antibody staining, cells were washed twice with 5% v/v NHS (in PBS) and tubes were stained with secondary antibody goat anti-mouse-FITC (GAM-FITC) for 30 minutes on ice, including cells with no primary antibody staining (secondary/FITC control). GAM-FITC was diluted 1/2000 in 20% v/v normal goat serum (NGS) (PAA) and 100µl was added per well. Following staining, cells were washed twice with 5% v/v NGS and fixed with 1% w/v formaldehyde. Cells with no primary or secondary antibody staining were also analysed (unstained control). Cells were stored at 4C until analysis via flow cytometry.

2.4.1. Surface receptor expression of THP-1 models

Unstimulated and VD3, PMA and double stimulated THP-1 cells were stained with mouse anti-human monoclonal antibodies targeting CD36 (Abcam, Cambridge, UK), LOX-1 [clone 331212] (R&D Systems, Abingdon, UK), SRA-1 (R&D Systems), CD14 (clones 61D3/63D3, made in house) and ICAM-3 (clone MA4, made in house). THP-1 cells were seeded in 6 well plates and differentiated for 72 hours as above. Following differentiation, PMA and DS cells were treated with 5mM EDTA in PBS (1h; 37C) to bring adherent cells into suspension. Cells were stained with primary antibody as above (2.4.). The following final concentrations were used for each antibody: CD36 (20µg/ml), LOX-1 (50µg/ml), SRA-1 (50µg/ml), CD14 (neat supernatant), ICAM-3 (1/100 dilution), IgG_1 control [clone MOPC21] (20 µg/ml) (Sigma-Aldrich), IgG_{2b} control (50µg/ml) (Abcam). All primary antibodies were titrated and concentrations chosen according to saturation of receptor staining. Receptor saturation could not be reached with LOX-1 and SRA-1 antibodies, so concentrations were chosen with positive receptor staining and low background staining. The staining of these antigens would not be useful for quantifying cell surface protein expression, but can be used for semi-quantitative/qualitative staining. Control antibody concentrations were chosen to match maximum antibody concentrations of the same isotype. Cells were then stained with secondary antibody as above (2.4.) Cells were fixed with 1% w/v formaldehyde and stored at 4C until ELISA analysis.

In order to assess cell surface receptor expression in cells following oxLDL treatment, indirect immunostaining of cells of interest was carried out with monoclonal antibodies

targeting CD36, LOX-1, SRA-1, CD14 and ICAM-3, according to the protocol above. Cells were fixed with 1% w/v formaldehyde and stored at 4C until analysis via flow cytometry.

To support chemotaxis studies, the anti-CX3CL1 [clone 51637] (R&D Systems) and anti-ICAM-3 antibodies were used for indirect immunostaining of cell surface CX3CL1 and ICAM-3 in live and apoptotic MØ and foam cells, to gather evidence for loss of expression via membrane shedding, as in section 2.4. Cells were fixed with 1% w/v formaldehyde and stored at 4C until analysis via flow cytometry.

2.4.2. Endothelial cell phenotyping following treatment with live and apoptotic MØ/FC-conditioned medium

Human umbilical vein endothelial cells (HUVECs) were cultured in 6 well plates and incubated in the presence of live MØ/FC CFS and apoptotic MØ/FC CFS, set up as in 2.3.4., or medium control (24h; 37C). Following incubation, conditioned medium was removed and cells washed with PBS and incubated in trypsin/EDTA (PAA) to lift adherent cells into suspension (20min; 37C). Direct immunostaining was carried out with FITC-labelled anti-CD62E, or FITC-labelled control, and results analysed by flow cytometry (see 2.4.).

2.5. Phagocyte interaction with, and responses, to apoptotic cells and other mediators

2.5.1. Interaction of phagocytes with apoptotic cells

THP-1 cells were seeded at $2x10^4$ cells per well in 24 well tissue culture plates in cRPMI with 250nm PMA or 100nm VD3 + 250nm PMA (DS) (72h; 37C). Following differentiation, cells were treated with (FC) or without (MØ) oxLDL (indicated concentrations) (72h; 37C). Culture medium was removed and 2×10^5 apoptotic jurkat, THP-1 macrophage or THP-1 foam cells were added per well, in 500µl serum free RPMI + 1% P/S + 0.2% BSA. A 10:1 ratio of apoptotic cells: macrophages was chosen to provide excess ACs for interactions. Plates were incubated (1h; 37C) and wells were then washed with 1ml cold PBS to prevent further interactions and remove unbound apoptotic cells. Cells were fixed with 1% formaldehyde (w/v in PBS) and stored at 4C until staining. To stain, 2-3 drops of Diff-Quik (made in house) were added to each well to visualise cells. Macrophages (THP-1 derived) appear as large adherent light-blue cells, and apoptotic cells appear as small dark blue spots. Interactions were counted as number of macrophages interacting with apoptotic cells per 200 cells counted in each well.

Primary human monocytes were seeded as above and allowed to adhere overnight in chemically-defined macrophage medium (CD-MØ medium). The culture medium was replaced, removing non-adherent cells (lymphocytes) and adherent monocytes left to

differentiate for 7-14 days in CD-MØ medium, which was replaced every 3-5 days until the time of the assay. Interaction assays were then carried out as above in chemically defined macrophage medium with apoptotic THP-1 derived macrophages/foam cells.

2.5.2. Interaction of oxLDL treated cells with *E.coli*

THP-1 cells were seeded in 6 well tissue culture plates. Following differentiation and oxLDL treatment, the culture medium was removed and cells washed in complete or serum-free medium. In dark conditions, dilutions of fluorescein conjugated *E. coli* (Life Technologies) in either complete or serum-free medium were added to relevant wells and plates incubated (40min; 37C). Wells were then washed with 1ml cold PBS to prevent further interactions and remove unbound *E. coli*. Differentiated THP-1 cells were then treated with 5mM EDTA in PBS (30min; RT) to bring adherent cells into suspension. Cells were washed in 0.1% BSA in PBS then fixed with1% w/v formaldehyde and stored at 4C until analysis via flow cytometry. Following optimisation, a 1/1000 dilution of fluorescein conjugated *E. coli* in SF RPMI was chosen. After samples were analysed by flow cytometry, trypan blue (Sigma-Aldrich) was used to quench fluorescence of *E. coli* particles bound to the THP-1 macrophage/foam cell surface, and samples re-analysed by flow-cytometry. This enables differentiation between bound and internalised *E.coli*, highlighting any physiological differences between function of THP-1 macrophages and THP-1 foam cells. This may highlight whether any decreases in apoptotic cell binding is a result of altered function, or whether a general decrease in activity may be associated with foam cells as they were 'sick'.

2.5.3. AC inhibition of inflammatory response

THP-1 cells were seeded at $5x10^5$ cells per well in 24 well tissue culture plates in cRPMI with 250nm PMA or 100nm VD3 + 250nm PMA (DS) (72h; 37C). Following differentiation, cells were treated with (FC) or without (MØ) oxLDL at a concentration appropriate to the experiment (72h; 37C). Culture medium was removed and $1.5x10^6$ apoptotic jurkat (see 2.3.3.), THP-1 macrophage, THP-1 foam cells (6h post UV) or medium control were added per well, in 500µl sfRPMI containing 1% P/S + 0.2% BSA, and plates incubated (20h; 37C). 0.5ng/ml LPS (from *E. coli* O111:B4, Sigma-Aldrich), diluted in sfRPMI, or sfRPMI alone was then added to wells with or without the presence of ACs, with normal human serum (10% final conc.) and incubated (4h; 37C). Supernatants were harvested and stored at -20C until ELISA analysis.

Primary human monocytes were seeded at $8.3x10^4$ in 96 well tissue culture plates and allowed to adhere overnight in chemically-defined macrophage medium (CD-MØ medium). The culture medium was replaced, removing non-adherent cells (lymphocytes) and adherent

monocytes left to differentiate for 7-14 days in CD-MØ medium, which was replaced every 3-5 days until the time of the assay. Interaction assays were then carried out as above in chemically defined macrophage medium with apoptotic THP-1 derived macrophages/foam cells.

2.5.4. Measurement of cytokine release via indirect Enzyme-Linked Immuno-Sorbent Assay (ELISA)

Solutions

ELISA diluent: 0.05% (v/v) Tween 20 (Sigma-Aldrich), 0.1% (w/v) BSA in PBS
ELISA wash buffer: 0.9% (w/v) sodium chloride (Sigma-Aldrich), 0.05% (v/v) Tween 20
ELISA block buffer: 1% (w/v) BSA, 5% (w/v) sucrose in PBS

To detect cytokine release by THP-1-derived MØ (PMA) and HMDM at basal levels, and in response to ACs and LPS, stored supernatants from section 2.5.3. were analysed via indirect ELISA.

TNF-α, IL-10, IL-12 (Peprotech, New Jersey, USA) and CX3CL1 (R&D Systems) ELISAs were carried out according to manufacturer's instructions (PeproTech). Briefly, ELISA plates were coated in 1µg/ml (or otherwise recommended) relevant capture antibodies in PBS (100µl/well) and stored at room temperature overnight. ELISA plates were washed (wash buffer; three times) and 200µl block buffer added per well (1h; RT). ELISA plates were washed and serial dilutions (in ELISA diluent) of recombinant TNF-α, IL-10, IL-12 or CX3CL1 added, or harvested supernatants (100µl/well) (neat or diluted in sfRPMI according to the individual experiment) (2h; RT). ELISA plates were washed and 0.5µg/ml (or otherwise recommended) TNF-α, IL-10, IL-12 and CX3CL1 detection antibody (in ELISA diluent) added (100µl/well) (2h; RT). ELISA plates were washed and streptavidin-HRP added at 1:2000 dilution in ELISA diluent (100µl/well) (30m; RT). ELISA plates were washed and 100µl OPD added per well. Following a colour change, the reaction was stopped with 1M HCl (50µl/well) and ELISA plates read at 490nm. A standard curve was generated for each plate, based on known concentrations of recombinant proteins, to estimate the cytokine concentration in each sample.

2.5.5. Effect of PMA on inflammatory response of human monocyte-derived macrophages

Primary human monocyte-derived macrophages were seeded in 24 well tissue culture plates and allowed to adhere overnight in chemically defined macrophage medium (CD-MØ medium). The culture medium was replaced, removing non-adherent cells (lymphocytes) and

adherent monocytes left to differentiate for 7-14 days in CD-MØ medium, which was replaced every 3-5 days until the time of the assay. HMDM were co-cultured with apoptotic Jurkat (10:1 AC:MØ), (see 2.3.3.), (24h; 37C), in the presence or absence of indicated PMA concentrations.

2.6. Models of phagocyte chemotaxis

2.6.1. Vertical monocyte chemotaxis model - the Boyden Chamber

Initial experiments were carried out using the modified Boyden Chamber method, utilising a NeuroProbe 48 well vertical chemotaxis chamber (Fig. 9). This was to ascertain chemotactic properties of apoptotic cell-derived microparticles and soluble factors.

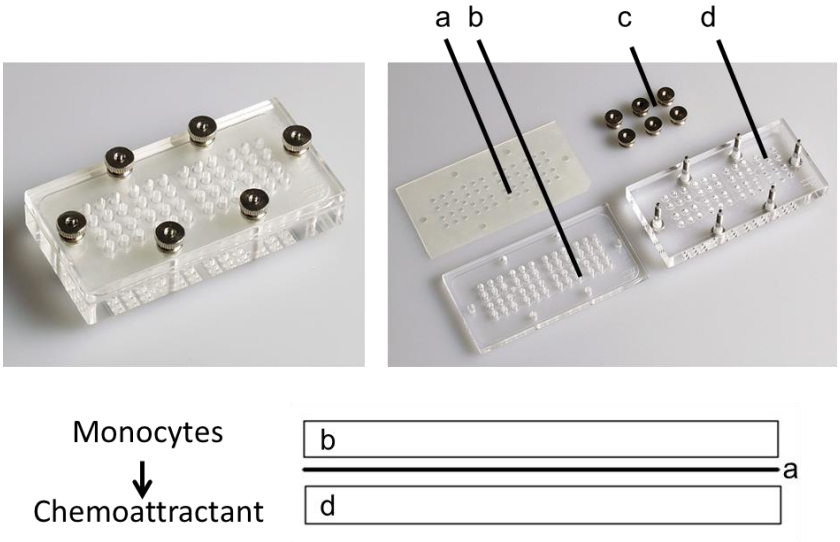


Figure 6. Assembly of NeuroProbe 48-well vertical migration chamber *Image of an assembled vertical migration chamber (top left). Chamber design (top right) consists of lower wells for chemoattractant loading (d), sealing gasket (a) which supports a polycarbonate membrane, upper wells for monocyte loading (b) and metal fittings to ensure minimal spaces between components (c). Bottom figure depicts direction of monocyte migration from upper wells (b) to chemoattractant in lower wells (d), through a polycarbonate membrane which is supported by a gasket (a). Images adapted from supplier website (NeuroProbe.com).*

A 48 well vertical chemotaxis chamber was used according to manufacturer's instructions (NeuroProbe Inc., Gaithersburg, Madison, WI, USA). In brief, 25µl control or chemoattractant was loaded into the lower chambers (Fig. 9; d.), followed by a PVP-free polycarbonate membrane (pore size: 5µm; pore density: 4 x 10^3 pores/mm^2) and assembly of the upper chamber. VD3 stimulated THP-1 cells were suspended in assay medium (RPMI + 0.2% BSA + 2mM ʟ-glutamine + 100IU/ml penicillin) at 2x10^6 cells/ml. 50µl per well of THP-1 suspension was added to the upper chambers (1x10^5 cells/well) (Fig. 9; b.). Chambers were incubated for 4 hours at 37C before cell migration was assessed. Membranes were washed

and stained with Diff Quik solution; migrated monocytes remained stuck to the underside of the membrane or embedded in the microporous membrane, whilst monocytes that had settled on top of the membrane were removed by washing and scraping on a proprietary blade. Total migrated cells (5 fields of view per well) were counted using light microscopy (40X).

2.6.2. Horizontal monocyte chemotaxis model optimisation - Dunn Chamber

A true chemoattractant gradient is not established in the vertical models of chemotaxis, rather a step gradient of chemoattractant is located immediately below a microporous membrane. In order to establish a true chemotactic gradient, without having to factor in the effects of gravity on chemotactic activity, a horizontal assay system was established using the Dunn chemotaxis chamber.

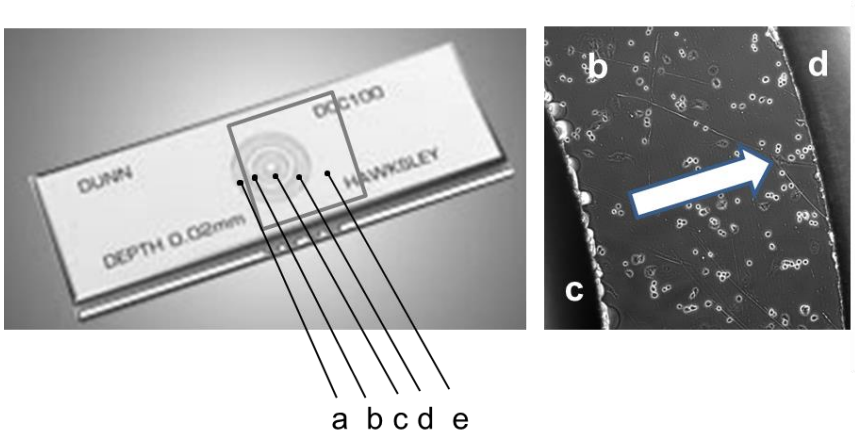


Figure 7. Schematic of the Dunn chemotaxis chamber *Image (left) depicts chemoattractant loading area (a), viewing bridge (gradient) (b), inner well (medium) (c), outer well (chemoattractant) (d), and placement of coverslip with adherent monocytes (underside) (e). Right image shows direction of monocyte migration towards chemoattractant (arrow), across the viewing bridge (b), following establishment of a chemoattractive gradient from the outer well (d) to the inner well (c).*

MCP-1, first isolated from THP-1-conditioned medium, is an established chemoattractant for monocytes[304-306]. MCP-1 was used as an attractant to establish a horizontal chemotaxis assay for this model, using the Dunn chemotaxis chamber, adapted from methods outlined by Zicha *et al.*[292]. This group deduced that proteins with a molecular weight of 350-750Da will produce a stable linear gradient within 10 minutes and that decay to half will take 10 hours[292]. Large proteins (10-20kDa) form a linear gradient within 30 minutes, with decay to half in 30 hours[292]. At 8-9kDa, an MCP-1

gradient should be established at an early time point in the assay, and be stable throughout the time course of the assay.

THP-1, VD3, PMA and DS cells were cultured on glass coverslips (24mmx24mm) in cRPMI, with or without differentiating agents, in 6 well plates. Human PBMCs, human peripheral blood monocytes and human monocyte-derived MØ were also cultured on glass and plastic coverslips to assess migration. sfRPMI or CD-MØ medium was added to both wells of the Dunn chemotaxis chamber. Using sterile forceps, coverslips with cultured cells were placed face down (cell-side down) on top of the wells, leaving a gap for addition of chemoattractant in the outer well (Fig. 10). Edges and the top of the coverslip were dried using filter paper, and 3 edges were sealed with hot paraffin wax using a small paintbrush. The gap left on the fourth edge for chemoattractant loading was used to drain the outer well of medium using filter paper, with care taken not to make contact with the underside of the coverslip, where cells should be present. 100ng/ml MCP-1 in either sfRPMI or CD-MØ medium was promptly added to the outer well and the edge sealed with hot wax using a small paintbrush. Cells were observed over the bridge area of the chamber, where the gradient between wells will be established.

Assays had to be optimised to provide an area with optimal cell density, which needed to be chosen as too many cells made tracking difficult, and too few gave less powerful statistics. Tutorials by Ibidi on use of bespoke chemotaxis tracking software Chemotaxis and Migration Tool v.2.0 recommended 40 cells per assay should be tracked for powerful statistical data. Seeding densities had to be adjusted per cell differentiation method due to varying adherence levels. Initial assays were observed for 48 hours, however following observation they were cut down to 2 hours, which was the time at which monocyte migration had ceased. It was interesting to note that apoptotic cell-derived CFS proved a more robust chemoattractant than the positive control MCP-1.

Quantitative assessment was carried out using Image J and the Ibidi Chemotaxis and Migration Tool (version 2.0, standalone software), which provided measures of directness (Euclidean/accumulated distance), distance, velocity, forward migration index (FMI) and the Rayleigh test. A further assessment was utilised using the provided measurement of angle of the end points, and quantifying the average standard deviation of angle endpoints (SDoA). This should indicate a level of uniformity of migration of the monocyte population.

2.6.3. Monocyte chemotaxis to apoptotic cells - modelling in the Dunn chamber

To assess the chemoattractive properties of apoptotic cells and apoptotic cell-derived mediators, whole cell cultures, and cell free supernatants, were harvested from live and UV-induced apoptotic cells. Apoptotic cells were generated as in 2.3.3. Live cell cultures were prepared under the same conditions, with the exception of UV exposure. Assays were carried out as above (2.6.2.), with CD-MØ medium in the inner well, and apoptotic whole cell culture (WC) (2.3.3.) or cell-free supernatant (2.3.4.) (CFS) in the outer well.

The addition of blocking antibodies targeting CX3CL1 (5ng/ml), ICAM-3 (1/10 dilution of tissue culture supernatant) and CD14 (1/10 dilution of tissue culture supernatant) were added to WC or CFS preparations immediately prior to loading.

2.7. Function of aged macrophages; a pilot study

Up to 50ml of blood was harvested from an age range of healthy donors in accordance with Aston University Ethics Committee, and age and gender recorded alongside anonymised sample reference numbers. PBMCs were isolated (as in section 2.1.3.) and seeded in 24 well plates (AC interactions assays; see 2.5.1.) or 96 well plates (cytokine responses to AC; see 2.5.3.) in CD-MØ medium. Non-adherent cells (lymphocytes) were removed after 24 hours culture, and adherent cells (monocytes) allowed to differentiate to MØ over a period of 7-14 days. AC interaction assays with UV-induced apoptotic Jurkat cells (see 2.3.3.) were carried out as previously (see 2.5.1.), with the use of CD-MØ medium instead of RPMI. Cytokine responses following co-culture with UV-induced apoptotic Jurkat cells were carried out as previously (see 2.5.3.), with the use of CD-MØ medium instead of RPMI. TNF-α ELISA was carried out as per manufacturer's instructions (PeproTech) (2.5.4.).

For plasma-conditioning assays, THP-1 cells were seeded at $2x10^4$ cells per well in 24 well tissue culture plates in cRPMI with 250nm PMA + 100nm VD3 (DS) (72h; 37C). Following differentiation, cells were treated with plasma derived from young or mid-life donors (10%v/v in sfRPMI) (72h; 37C). Culture medium was removed and 2×10^5 apoptotic jurkat added per well, in the presence or absence of serum from the same donor. This assay was designed to show functional differences in the effects of long term exposure of cell line MØ to an 'aged' environment, and the impact of the 'aged' environment on functions occurring in the immediate local microenvironment, i.e. AC clearance.

Modelling apoptotic foam cell clearance *in vitro*

3.1. Introduction

The atherosclerotic plaque continues to provide a unique and interesting area of investigation in the field of apoptotic cell (AC) clearance. Research has shown that a highly inflammatory environment persists within advanced atherosclerotic lesions[58]. At these sites, monocytes are recruited to the arterial sub-endothelium, where they differentiate into macrophages (MØ) and gorge on modified lipoproteins, resulting in the formation of lipid-laden 'foam cells'[162]. These foam cells undergo apoptosis, recruiting further monocytes and the process repeats[46]. Given the high number of localised ACs being cleared by macrophages, the plaque microenvironment contradicts evidence that AC clearance dampens inflammatory responses[55,56].

In this chapter, a THP-1 MØ-derived foam cell model is developed and characterised. Data is presented that addresses phenotype and function of macrophages within an environment with plaque-like features. Viability is assessed, and expression of receptors relevant to modified lipid uptake and apoptotic cell clearance is shown. The ability of foam cells to recognise and interact with apoptotic cells is investigated, as is the ability of apoptotic foam cells to be recognised by 'normal' non-foam cell phagocytes.

Current research suggests defective AC clearance, or efferocytosis, may be a leading cause of plaque progression, leading to plaque instability and pathological outcomes[149,150,195,197]. This has been attributed to loss of function of lipid-laden MØ, known as foam cells[195,197], however the contribution of apoptotic foam cells to defective clearance has had very little focus. Indeed, the clearance of apoptotic MØ has attracted little attention.

The hypothesis for the work in this chapter is that MØ from a lipid-laden environment may be defective in their capacity to interact with apoptotic cells, and thus promote resolution of inflammation. In order to address this, the THP-1 model established in this chapter will be used to answer the research questions listed in the following table.

	Phagocyte	
Apoptotic cell	**Macrophage (MØ)**	**Foam cell (FC)**
Apoptotic MØ (aMØ)	How well do non-lipid-laden MØ and non-lipid-laden aMØ interact?	Do FC have the ability to recognise, interact with and phagocytose aMØ?
Apoptotic FC (aFC)	Do aFC upregulate 'eat-me' and downregulate 'don't-eat-me' signals to MØ, enabling recognition, interaction and phagocytosis?	Does the combination of FC/aFC result in defective clearance in this model?

Table 1. Summary of key research questions addressed in Chapter 3

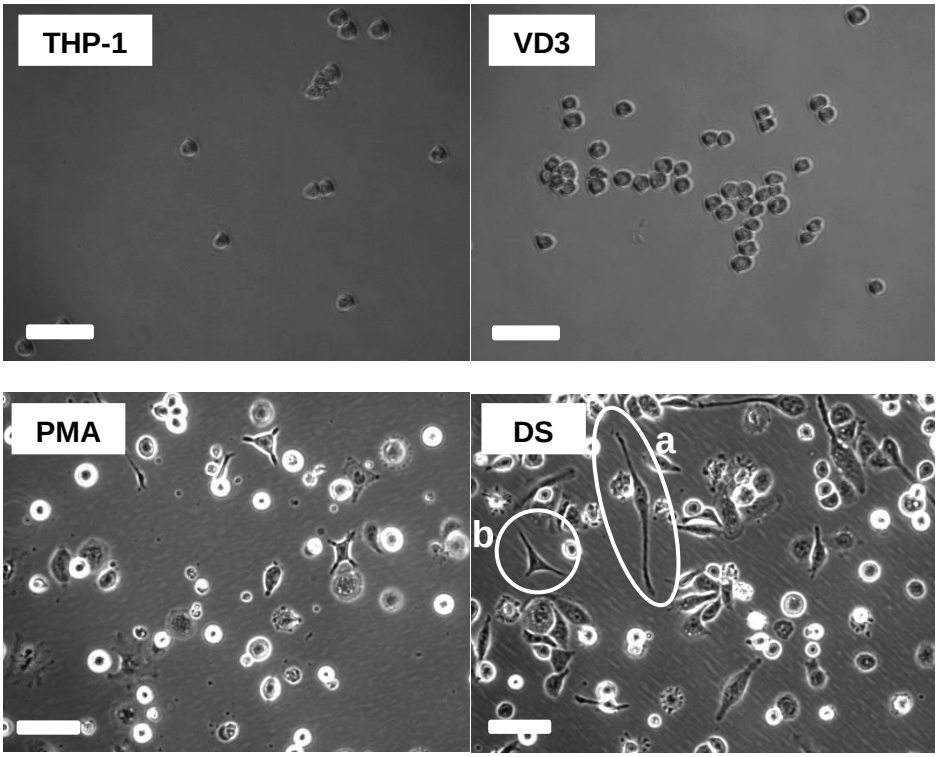
3.2. Results

3.2.1. Morphology of monocyte and macrophage models

In order to assess macrophage (MØ) function, it was necessary to establish a MØ model for analysis. A number of models are available ranging from primary human and mouse MØ, through to cell lines and their stimulated derivatives. THP-1 cells have been used extensively as multiple monocyte-MØ models can be obtained from the same originating cell line [307-311]. These cells and their stimulated derivatives are functional for apoptotic cell clearance, inflammatory responses, and show representative changes in lipid metabolism-gene expression throughout differentiation, important for a representative foam cell model[300,307,312]. THP-1 cells can be differentiated into more MØ-like cells using various differentiation strategies; no treatment (THP-1), vitamin D3 (VD3) treatment, PMA treatment or double stimulation (DS) with both agents[310,311]. In order to establish successful differentiation strategies for use in the foam cell model, morphology was examined following 72 hours differentiation.

In order to follow the differentiating effects of VD3 and PMA, THP-1 cells were stimulated to differentiate with VD3, PMA or both agents. Using light microscopy and flow cytometry,

morphological changes were assessed relative to unstimulated THP-1 cells (Fig. 8). Morphologically, VD3 cells remain similar to THP-1 cells in their monocytic appearance. Both cell types show weak adherence to tissue culture plastic (i.e. they are easily removed with washing) and spreading is not apparent. They remain homogeneous in nature, with a defined population observed on flow cytometry histograms, with equivalent cell size (forward scatter) and granularity (side scatter) to unstimulated THP-1 cells, indicating a monocytic phenotype. PMA and DS cells differ greatly in morphology in comparison to THP-1 and VD3 cells, with visible spreading and strong adherence to tissue culture plastic (i.e. few cells are removed by washing). PMA and DS cells are also larger in appearance and more granular than THP-1 and VD3 cells, as observed when comparing forward scatter (FS) and side scatter (SS) by flow cytometry (Fig. 8). Of the adherent cells, two phenotypes became apparent, MØ that spread in an elongated manner (fig. 5, circled cell a), and rounder MØ (fig. 5, circled cell b). THP-1 and VD3 cells were both observed to proliferate, which was apparent when counting cells for antibody staining, as supported by Thomas *et al.* (2013)[312]. PMA and DS cells decreased in number, suggesting terminal differentiation and some cell death or cell fusion. Results suggest unstimulated THP-1 and VD3 stimulated cells may be a suitable monocyte model, whereas PMA and DS stimulated cells could better represent a MØ model.



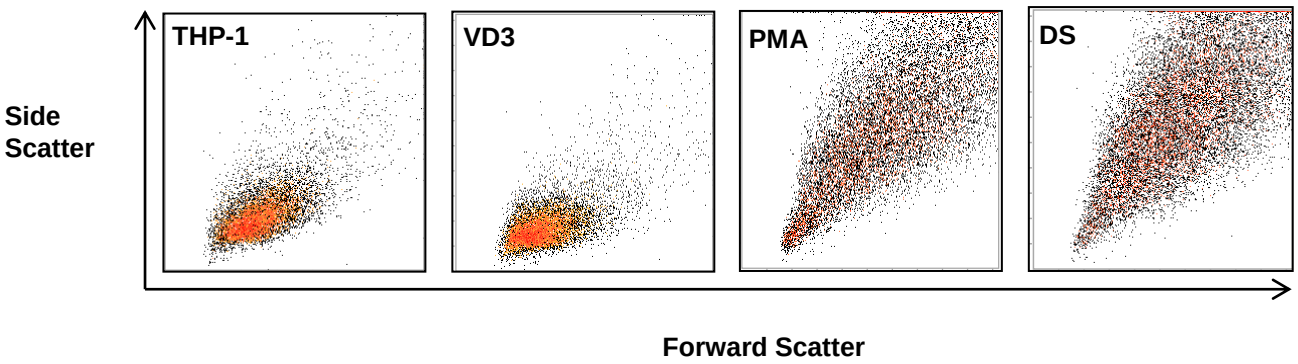


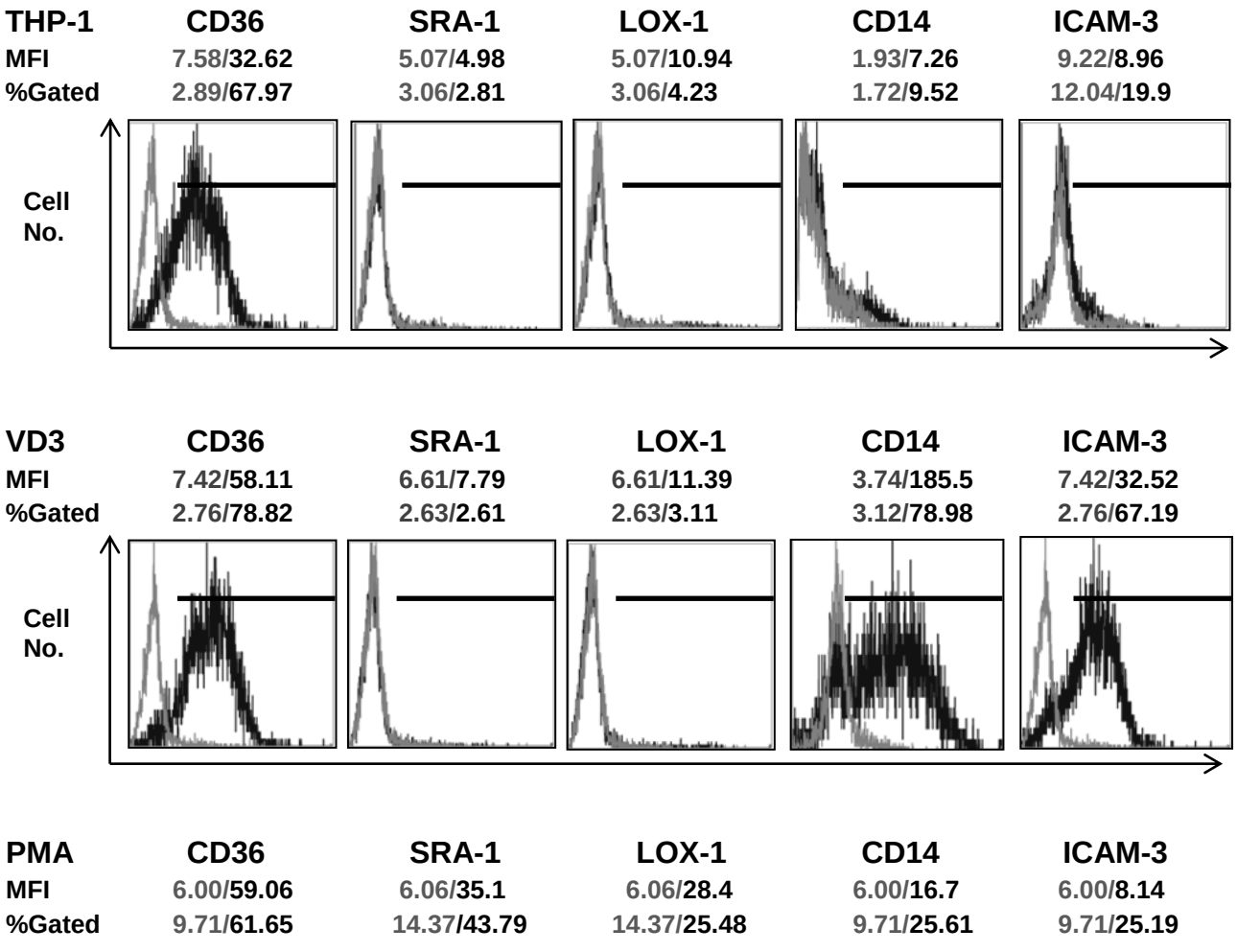
Figure 8. Alternative differentiation methods produce varying phenotypes in THP-1 cells *THP-1 cells were either undifferentiated or differentiated with VD3, PMA or both VD3 and PMA (DS) as indicated (72h; 37C), and examined by light microscopy and flow cytometry to compare morphology. Scale bars represent 100µm in THP-1 and VD3, 200µm in PMA and DS.*

3.2.2. Surface receptor expression of monocyte and macrophage models

The surface receptor expression profiles for oxLDL receptors CD36, SRA-1 and LOX-1, for the multifunctional receptor CD14 and the adhesion molecule ICAM-3 (a leukocyte-restricted IgSF member) were investigated using indirect immune-fluorescence staining and flow cytometry (Fig. 9) CD36, SRA-1 and LOX-1 are key receptors in oxLDL uptake and foam cell formation and are also known to bind apoptotic cells[103,176,182,190,192]. By immunophenotyping each model for expression of these receptors, the likelihood of each THP-1 model to produce foam cells may be predicted. CD14 expression is also investigated in order to predict the ability of each model to tether apoptotic cells, a function which is potentially defective in foam cells as part of defective AC clearance[31,46]. ICAM-3 has also been implicated in AC clearance[71,84].

In THP-1 cells, CD36 expression was high, and CD14 and ICAM-3 expression present but low (Fig. 9). This would indicate the ability to uptake oxLDL, and theoretically some ability to bind AC. SRA-1 and LOX-1 expression appears undetectable on frequency histogram plots, however a small increase in mean fluorescence intensity was detected with LOX-1 staining, compared to isotype control, should be noted. What levels of oxLDL receptor expression, and the required combination of various oxLDL receptors, are necessary for function should also be considered, as very low levels may still be relevant and functional. In VD3 differentiated cells, CD36 and ICAM-3 expression was high, with very high expression of CD14 also present, suggesting comparable likelihood of THP-1 and VD3 cells to form foam cells in the presence of oxLDL, but perhaps an increased ability in AC binding following VD3 differentiation (Fig. 9). SRA-1 and LOX-1 expression was undetectable, with a small increase in MFI with LOX-1 staining, as in THP-1 cells.

PMA and DS cells show expression of all receptors, indicating oxLDL uptake and subsequent susceptibility to foam cell formation, and also improved AC interaction efficiency (Fig. 9). PMA and DS cells show increased SRA-1 and LOX-1 expression compared to THP-1 and VD3 cells, however VD3 cells show the highest expression levels of CD14. Staining of LOX-1, and to some extent SRA-1, in PMA and DS cells, revealed dual populations within these models. This suggests upregulation of receptors only in a subpopulation of cells as 2 distinct peaks were observed on frequency histogram plots, whereas CD36 was expressed across the whole cell population. ICAM-3 expression is also upregulated in PMA and DS cells compared to THP-1, however VD3 cells showed the highest levels of ICAM-3 expression. Results point towards PMA and DS differentiated THP-1 cells as strong candidates for foam cell modelling.



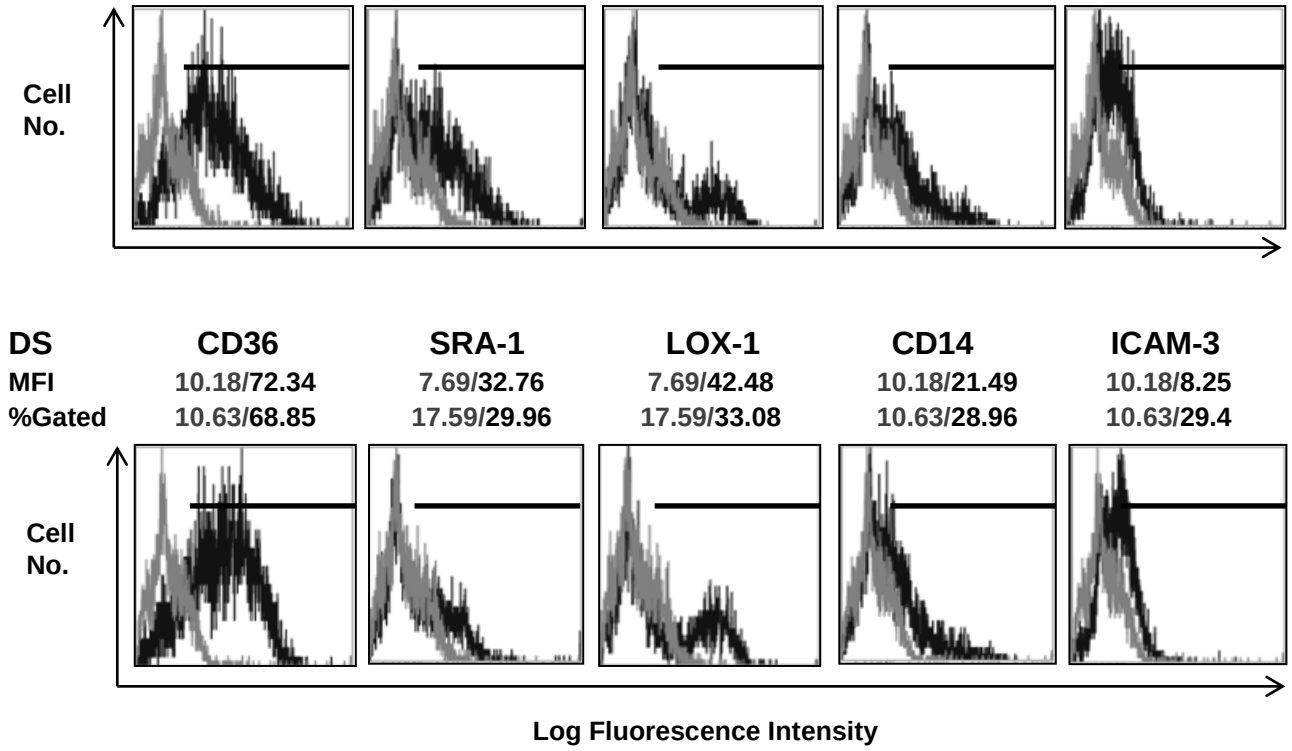


Figure 9. Phenotyping THP-1 models for key oxidised LDL and apoptotic cell receptors *Undifferentiated or VD3, PMA or DS (VD3+PMA) differentiated THP-1 cells were stained with primary monoclonal antibodies targeting CD36, SRA-1, LOX-1, CD14 and ICAM-3 (black) or isotype matched control antibodies (grey), and displayed as log fluorescence intensity. Expression levels were compared using flow cytometry. Results are also shown as mean fluorescence intensity and % gated (solid black line represents positive gating). A representative example of at least 3 experiments is shown.*

3.2.3. Oxidation of LDL

Subendothelial retention of modified LDL, including oxidised LDL (oxLDL), is the origin of vascular cell damage and foam cell formation, therefore plays a key role in atherogenesis[45,169,170]. In order to create foam cells for the model, LDL must first be isolated from donors and oxidised. It is important to be aware of the variance in levels of oxidation in native LDL (nLDL), as this can be influenced by LDL particle size and diet[171]. LDL from different donors is also susceptible to oxidation at different levels, so it is also important to establish differences in oxidation levels between samples following the same oxidation method[172]. Various methods can be employed to oxidise and measure oxidation levels of LDL. Carbonyl ELISA measurement is popular as carbonylated proteins remain stable and the ELISA method provides sensitivity[313].

Carbonyl ELISA results (tab. 3) demonstrate an increase in carbonyl content, and therefore protein oxidation, following exposure to copper sulphate (tab. 3). Also to note, is that the fold increase in carbonyl content is much greater in LDL#1, which had lower basal carbonyl

content, and therefore oxidation. Results also show how basal levels of LDL oxidation can vary between donors, as seen in previous research[314]. Also to note, the fold increase in carbonyl content is much greater in the LDL#1, which had lower levels of basal oxidation (tab. 3). It is therefore important that steps should be taken to minimise variation of LDL oxidation levels between assays.

	Carbonyl content (nmol/mg protein)	
	Native	**Oxidised**
LDL #1	0.31	4.65
LDL #2	2.69	6.91

Table 2. Copper sulphate treatment results in LDL oxidation *Native LDL (nLDL) samples from 2 donors (#1 and #2) were oxidised with 10μM copper sulphate (1hr; 37C). The reaction was stopped with 10mM EDTA and samples desalted. Carbonyl content before and after copper sulphate treatment was estimated using an ELISA to indicate basal levels of protein oxidation (native) and oxidation levels following exposure to oxidant copper sulphate (oxidised). n=2.*

3.2.4. Generation of foam cells

3.2.4.1. Oil Red O staining of foam cells

In order to establish the best differentiation method for foam cell formation, THP-1, VD3, PMA and DS cells were all treated with oxLDL and observed after 24, 48 and 72 hours treatment to assess intracellular lipid accumulation, a key morphological marker of foam cell generation. It can then be investigated whether there is any correlation between receptor expression on each cell type and subsequent lipid uptake and accumulation.

Droplet formation within PMA and DS cells, but not THP-1 or VD3 cells, was observed following 72 hours of native LDL (nLDL) or oxidised LDL (oxLDL) treatment (0-50μg/ml), however droplet formation was much more robust, and seen at lower concentrations, with the use of oxLDL. Following 24 hours of oxLDL exposure, droplet formation was observed from concentrations as low as 5μg/ml oxLDL, and increased numbers of droplets were observed after 48 hours. After 72 hours, droplet formation seemed to have stabilised. Various qualitative methods can be used to assess foam cell formation, however Oil Red O, a neutral lipid dye, is commonly chosen as a cheap qualitative method, and results can be seen easily using light microscopy[195,315-317]. Nile Red staining can be used to assess the amount of

intracellular neutral lipid qualitatively and quantitatively, however it is a more expensive method.

Following 72 hours treatment of cells with oxLDL, foam cell formation was confirmed by staining for intracellular neutral lipid with Oil Red O (Fig. 10). Lipid accumulation is not detectable in monocytic THP-1 and VD3 cells, although red areas can be seen due to extracellular precipitation of the dye. Intracellular staining of lipid droplets is much more defined in the PMA and DS models, showing intracellular lipid has been accumulated in these macrophage-like cell types, as shown by arrows.

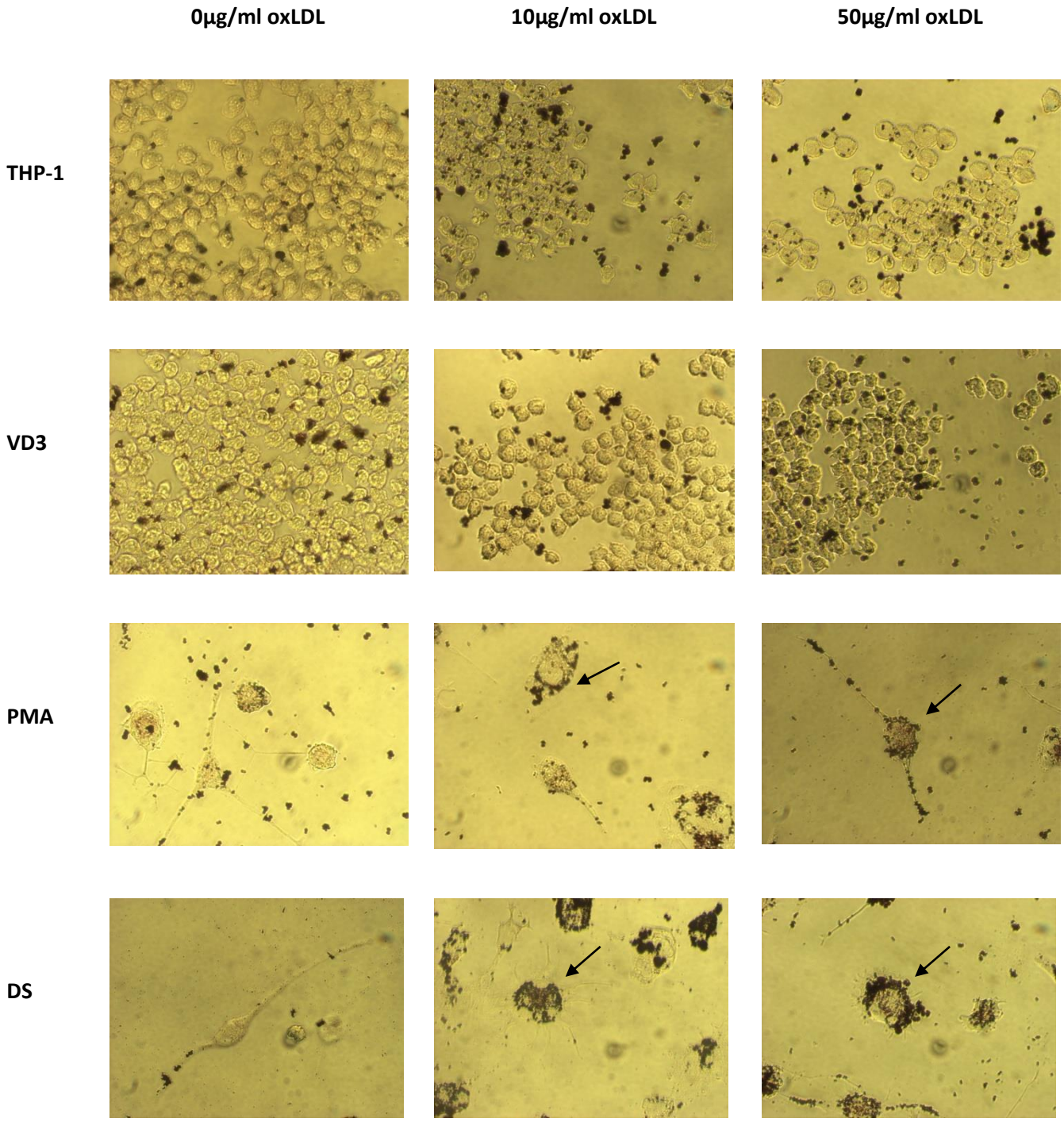


Figure 10. Oil Red O staining shows intracellular lipid accumulation in macrophages *Undifferentiated or VD3, PMA or VD3+PMA (DS) differentiated THP-1 cells were treated with 0, 10 or 50µg/ml oxLDL (72h; 37C). Cells were stained with Oil Red O for intracellular lipid accumulation (seen as red/darkly stained regions) and observed via light microscopy. Phase contrast images (20x) are shown. Images are representative of 3 independent experiments.*

3.2.4.2. Nile Red staining of foam cells for fluorescence microscopy

Foam cell formation was confirmed using a second assay of lipid accumulation, staining for intracellular neutral lipid with the fluorescent stain Nile Red (Fig. 11). This method confirms previous results that lipid has not accumulated in monocytic THP-1 cells when compared to basal levels of fluorescence. Fluorescence appears slightly increased following oxLDL treatment in VD3 cells, representing possible neutral lipid accumulation, though it is much less apparent than in PMA and DS cells, where formed droplets can be distinguished. Intracellular staining of lipid droplets can again be seen in the PMA and DS models, showing intracellular lipid has been accumulated in these more MØ-like cell types, as seen with Oil Red O staining. Basal levels of fluorescence are also higher in the PMA and DS models, which may be improved if lipid levels in growth medium can be reduced, however increased levels of autofluorescence have been shown previously in macrophages compared to monocytes[311].

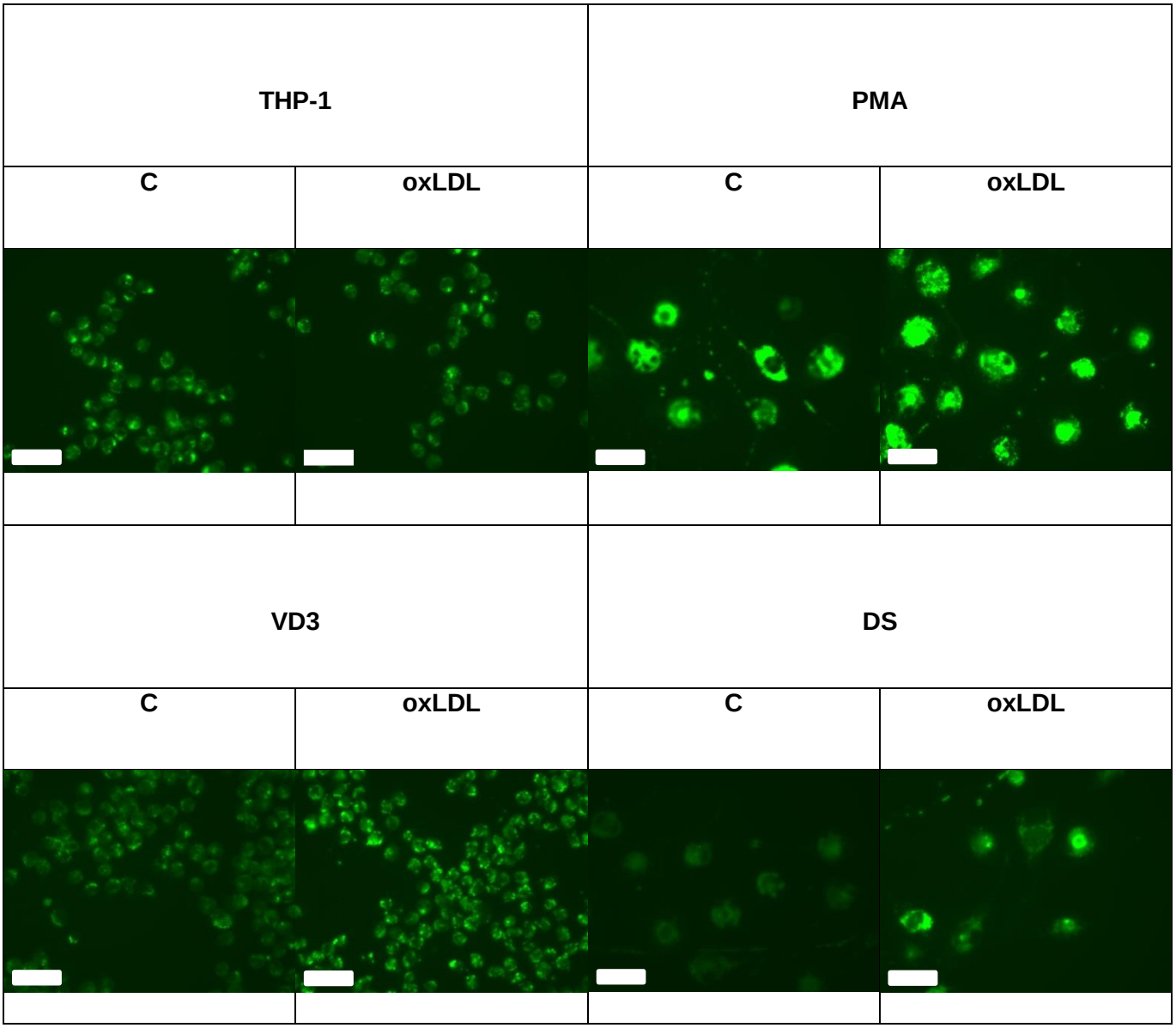


Figure 11. Nile Red staining shows intracellular lipid accumulation in MØ *Undifferentiated or VD3, PMA or VD3+PMA (DS) differentiated THP-1 cells were treated with 0 or 10µg/ml oxLDL (72h; 37C). Cells were stained with Nile Red for intracellular lipid accumulation (bright green vesicles) and observed via fluorescence microscopy (40x). Scale bars (white) represent 100µm. Images are representative of 2 independent experiments.*

3.2.5. Assessing toxicity of nLDL and oxLDL

It is important to establish a balance between sufficient LDL concentrations to induce foam cell formation, whilst having minimal effect on viability of the cells, for successful investigation of foam cell function. Cell death would be the final phase of LDL induced toxicity, so it is logical to use this end point to determine any overall toxic effects of LDL treatment. This will also provide a useful indicator of whether it is possible to induce apoptosis throughout the monocyte or MØ population using nLDL or oxLDL alone, as this would provide a source of apoptotic foam cells for future experiments, in a physiologically relevant manner.

THP-1 cells and DS cells were used to represent monocytes and macrophages MØ respectively. Following oxLDL exposure, levels of apoptosis and necrosis were low in the monocyte model, with fluctuations at 4 days likely to be due to experimental design (Fig. 12). Higher apoptosis and necrosis levels were found in MØ after 5 days, however this was not higher than control cells.

Control over cell death is required to maintain experimental feasibility, therefore for further experiments, cell death was induced by UV following foam cell generation, so more synchronous cell death is achieved when comparing foam cell and non-lipid loaded cell models.

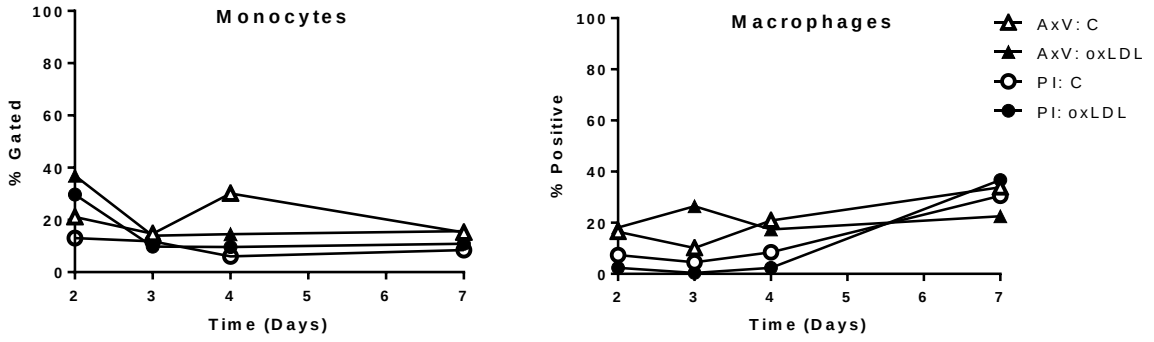


Figure 12. Oxidised LDL does not induce significant apoptosis or necrosis in monocyte and macrophage models *THP-1 cells (monocytes) were treated with or without (C) 150µg/ml oxLDL, and sampled over 7 days to monitor levels of apoptosis (AxV positive*

cells) and necrosis (PI positive cells) using flow cytometry. This was repeated with DS cells (MØ) which were treated with or without 50µg/ml oxLDL following differentiation. Results are representative of 3 independent experiments.

3.2.6. Effect of oxidised LDL treatment on surface receptor expression

Changes in surface receptor expression for CD36, SRA-1, LOX-1, CD14 and ICAM-3 were investigated using flow cytometry following 0, 10 and 50µg/ml oxLDL exposure (Fig. 13). CD36, SRA-1 and LOX-1 are key receptors in oxLDL uptake and foam cell formation, and are also known to bind apoptotic cells[103,176,182,190,192]. By phenotyping each model for expression of these receptors following oxLDL exposure, the implications of changes in receptor expression in apoptotic cell removal and therefore plaque progression may be assessed. CD14 expression is also investigated in order to predict changes in the ability of each model to tether apoptotic cells within the plaque environment, a function which is potentially defective[31,46].

An increase in CD36 expression was found in THP-1, VD3 and PMA cells, but not DS cells (Fig. 13). CD14 expression was increased in VD3 cells following oxLDL treatment. No overall increase in LOX-1 and SRA-1 expression is observed following oxLDL treatment, and ICAM-3 levels remain consistent apart from a potential increase in expression in DS cells. CD14 expression is upregulated following 50µg/ml oxLDL treatment in VD3 cells. Results in THP-1, VD3 and PMA models indicate the presence of a positive feedback loop of CD36 expression following oxLDL exposure, supporting previous research[318,319]. This was not found with SRA-1 and LOX-1 following exposure to oxLDL over 72 hours, therefore increased SRA-1 and LOX-1 expression following exposure to oxLDL may not have a prominent role in foam cell formation in these models, as found previously with LOX-1[318].

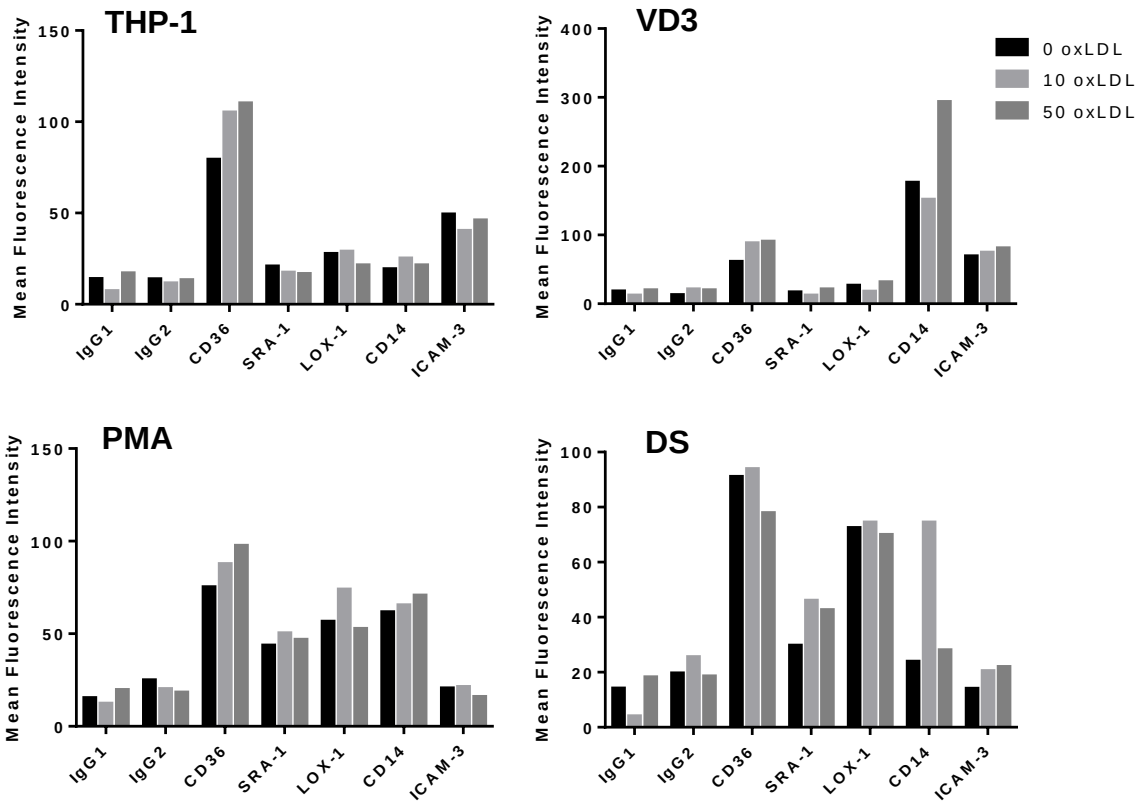


Figure 13. Modest changes in surface receptor expression of some monocyte/macrophage models following oxidised LDL treatment *THP-1, VD3, PMA and DS cells were treated with 0, 10 or 50µg/ml oxLDL (72h; 37C) and were stained with primary antibodies targeting CD36, SRA-1, LOX-1, CD14 and ICAM-3. Expression levels were compared with indirect immunostaining, followed by flow cytometry. Results are shown as mean fluorescence of FITC secondary antibody. Results are representative of 3 independent experiments.*

3.2.7. Foam cell interaction with apoptotic cells

Despite a high proportion of localised monocytes and macrophages, the atherosclerotic plaque contains a high proportion of apoptotic and necrotic cells, including apoptotic macrophages and T cells, suggesting impaired clearance mechanisms[46,195]. To investigate whether oxLDL treatment affects the ability of macrophages to interact with apoptotic cells (ACs), PMA and DS cells were treated with indicated concentrations of oxLDL for 72 hours prior to co-culture with AC to permit lipid-loading, and foam cell formation (Fig. 14). UV-induced apoptotic Jurkat (aJK) were used as they are a well-established AC model, often used in such studies[54,210]. In the DS MØ model oxLDL did not significantly reduce MØ ability to interact with aJK compared to untreated control. The PMA model shows a significant reduction in AC interaction following oxLDL treatment when compared to untreated control, with reductions in interaction with aJK apparent from as low as 5µg/ml oxLDL treatment ($P<0.01$), and becoming more significant at concentrations of 10µg/ml oxLDL and above

(P<0.001). Results suggest that PMA differentiated THP-1 cells may be a useful tool in modelling defective clearance within the atherosclerotic plaque.

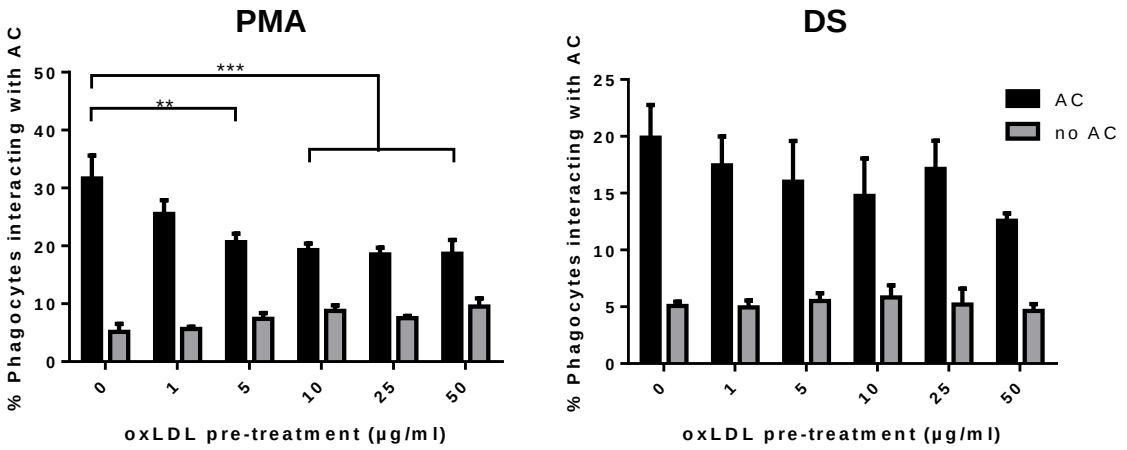


Figure 14. Oxidised LDL treatment reduces macrophage interaction with apoptotic Jurkat cells *PMA or DS differentiated THP-1 were treated with oxLDL (72h; 37C) to promote lipid loading of MØ and to drive foam cell formation. aJK were added to each well (10:1 aJK:MØ ratio) and unbound aJK removed after co-culture (1h; 37C). The number of MØ interacting with aJK was counted out of 200 cells per well. Data shown are mean % interaction ± S.E of 4 independent experiments. ** P<0.01, *** P<0.001 2way ANOVA with Tukey's* post-hoc *test.*

One simple explanation of reduced AC (aJK) binding by lipid-loaded PMA cells is that MØ pre-treatment with oxLDL results in persistent blocking of receptors, by oxLDL, that are required in AC tethering or engulfment, post oxLDL removal. If oxLDL-receptor binding persisted following removal of unbound oxLDL, and addition of ACs for co-culture, receptors required for AC interaction would be blocked, e.g. SRA-1, LOX-1 and CD36. Also, if residual unbound oxLDL remained in the assay system, competition for the same receptors may occur, reducing binding in those models with higher concentrations of oxLDL pre-treatment. To investigate whether straight forward competition between oxLDL and ACs for shared phagocytic receptors contributes to reduced aJK binding in the PMA model, PMA MØ were treated with indicated concentrations of oxLDL for 1 hour, to allow interactions to take place. After 1 hour, supernatants were replaced with medium with or without aJK. This was either in the presence or absence of oxLDL at indicated concentrations, to account for any impact of unbound oxLDL competing for receptors during co-culture of phagocytes with aJK (Fig. 15). No significant decrease in percent of MØ interacting with aJK was found following 1hr oxLDL treatment of PMA MØ cells compared to untreated control, either in the presence or absence of oxLDL during co-culture. This supports the hypothesis that physiological changes following

lipid-loading, not just receptor competition, are at play in defective interaction of lipid-laden MØ and aJK.

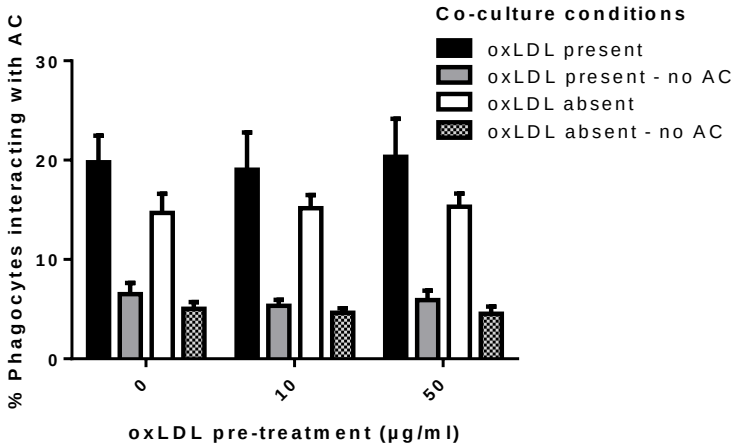


Figure 15. Reduction in apoptotic Jurkat cell interaction is not due to receptor competition with oxidised LDL *PMA differentiated THP-1 were pre-treated with oxLDL (1h; 37C), medium was removed and aJK were added to each well, in the presence or absence of indicated oxLDL concentrations. Cells were co-cultured (1h; 37C) and unbound aJK removed by washing. The number of MØ interacting with aJK was counted out of 200 cells per well. Data shown are mean % interaction ± S.E of 3 independent experiments. 2way ANOVA with Tukey's* post-hoc *test showed no statistical significance between oxLDL treatment groups.*

To investigate whether reduced interaction of PMA-derived MØ with aJK is a result of general reduced binding/phagocytic ability of the phagocyte following lipid-loading, an alternative phagocytic target was utilised. Following differentiation with PMA, MØ were treated with (FC) or without (MØ) 50µg/ml oxLDL (72h; 37C) for MØ/FC generation. The ability of MØ and FC to bind and phagocytose dead fluorescently labelled *Escherichia coli* (K-12 strain) was compared (Fig. 16). MØ and FC were exposed to fluorescent *E. coli* (or medium control) for 40 minutes at room temperature, unbound *E. coli* removed by washing, and cells analysed via flow cytometry. Mean fluorescence intensity was compared before (black bars) and after (grey bars) quenching of fluorescence of surface bound *E. coli* to determine whether differences in binding or uptake of *E. coli* could be observed between lipid-loaded and non-lipid-loaded cells. Results show no significant differences between MØ and FC ability to bind and phagocytose fluorescently labelled *E. coli*. Following quenching with trypan blue, a similar, but not significant, decrease in overall fluorescence of cells exposed to fluorescently labelled *E. coli* was observed. This supports the hypothesis that there may be a change in physiological function following lipid-loading when comparing ability to clear some apoptotic cells, and not solely an overall decline in health of cells, or overall binding/phagocytic ability.

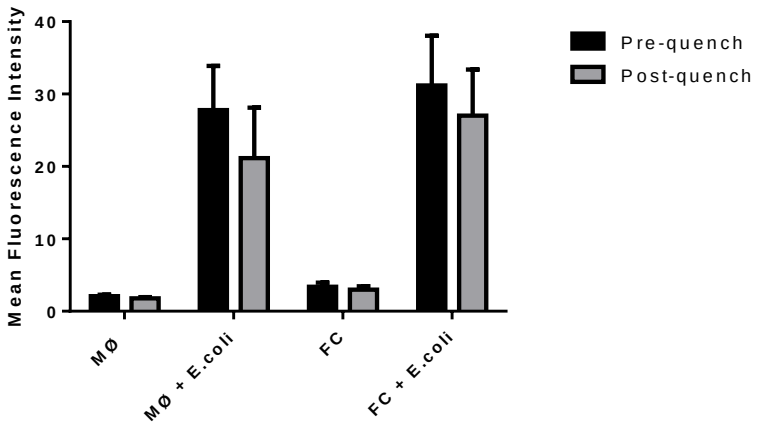


Figure 16. Reduction in apoptotic Jurkat cell interaction is not due to reduced phagocytic ability *MØ and FC were incubated with fluorescently labelled E. coli, or cRPMI control (40min;37C) and MØ/FC washed to remove unbound E. coli. MØ/FC were detached, fixed and samples stored at 4C until flow cytometry (black bars). Following analysis via flow cytometry, trypan blue was added to quench fluorescence of surface bound E. coli, MØ/FC washed and samples re-analysed (grey bars). Data shown are mean % interaction ± S.E of 3 independent experiments. 2way ANOVA with Tukey's* post-hoc *test showed no statistical significance between oxLDL treatment groups.*

3.2.8. Interaction with apoptotic foam cells

It is not fully understood whether perceived defects in AC clearance within the atherosclerotic plaque[150,195,197] originate from failed functionality of phagocytic cells to interact with AC, or whether apoptotic foam cells fail to attract/elicit the correct response from phagocytes entering the plaque. It was investigated whether oxLDL treatment affects the ability of phagocytes to interact with lipid-loaded apoptotic cells (aFC) in this model. Also shown was the effect of lipid-loading on the ability of MØ to interact with aMØ and aFC. PMA MØ were treated with (FC) or without (MØ) oxLDL for MØ/FC generation, and subsequently co-cultured with MØ/FC induced to apoptosis by UV exposure (aMØ/aFC) (Fig. 17a). HMDM were also used as a phagocytic cell (no oxLDL exposure) (Fig. 17a). Data show no significant differences in the ability of all phagocyte types to interact with aMØ compared to aFC. Interestingly, lipid-loaded MØ (i.e. foam cells) show no reduction in ability to interact with aMØ or aFC when compared to non-lipid-loaded MØ, differing from results found using aJK as an apoptotic cell model. This may be a result of cell type variations in phagocyte:AC interaction, with regards to mediating ligands/receptors. Both AC models represent cell types found in the plaque[46], so are relevant to atherosclerotic plaque research.

When comparing phagocyte interaction levels with aJK (Fig. 14), versus aMØ and aFC (Fig. 17a), combined data show a significant reduction in MØ interaction with aMØ and aFC when compared to aJK (Fig. 17b). A significant reduction in FC interaction with aFC compared to

aJK was also observed, but the reduction was not significant when comparing FC interaction with aMØ. A general reduction in clearance rates of apoptotic foam cells compared to other AC types could have implications in plaque progression and further investigation into the mechanisms of AC clearance between differing phagocyte and AC types would be of benefit.

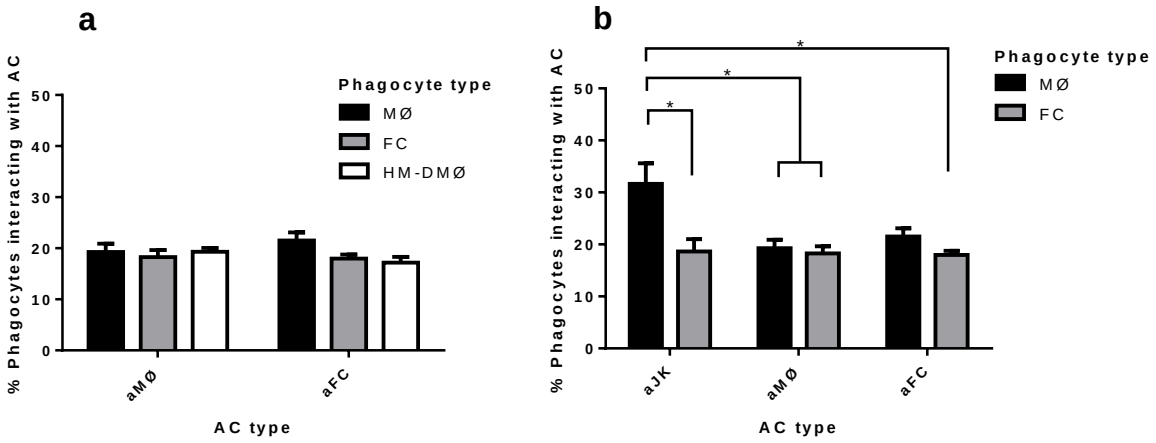


Figure 17. Recognition of apoptotic macrophages and apoptotic foam cells is not affected by lipid loading in THP-1 or primary cell model *MØ and FC were induced to apoptosis (100mJ/cm^2) to generate apoptotic MØ and FC (aMØ/aFC). MØ, FC and HMDM were co-cultured with a 10:1 ratio of AC:phagocyte. Unbound aMØ/aFC were removed by washing and samples fixed. The number of phagocytes interacting with AC was counted out of 200 cells per well. Data shown are mean % interaction ± S.E of 3 independent experiments. * P<0.05 2way ANOVA with Tukey's* post-hoc *test.*

3.3. Discussion

The ageing population, especially in the 'oldest old', will cause an increase in the incidence of age-related disease[320]. As the average age of the population increases, the range of 'healthy ageing' lags behind. Research into age-related disease is becoming ever prominent as researchers endeavours to broaden the healthy age-range and therefore improve quality of life for the oldest old.

Atherosclerosis becomes more prominent as we age[321], so improving understanding of the disease is a key research target given the ageing population[322]. Atherosclerosis also provides a paradoxical environment of great interest to the field of AC clearance, given the inflammatory environment despite the presence of localised monocytes and MØ and dead cells[46], a combination largely thought to dampen inflammation[55,56].

Results in this chapter show that oxLDL treatment of MØ, but not monocytes, induces intracellular lipid droplet accumulation and foam cell formation. This does not lead to constitutive upregulation of scavenger receptor expression in this foam cell model.

Comparison of non-lipid-loaded MØ and foam cell function showed equal ability to interact with bacteria, aMØ and aFC. Interestingly, foam cells showed a reduced capacity to interact with apoptotic Jurkat cells compared to MØ. Optimal rates of interaction with aJK by MØ were not reached with MØ or FC interaction with aMØ or aFC. This result could ***mean*** that even under optimal conditions, aMØ/aFC are cleared at sub-optimal rates when compared to maximum possible rates of AC clearance. The consequence of this at sites with large numbers of aMØ/aFC could go some way to explain the persistence of AC within the plaque.

	Phagocyte	
Apoptotic cell	**Macrophage (MØ)**	**Foam cell (FC)**
Apoptotic MØ (aMØ)	Non-lipid-laden MØ and non-lipid-laden aMØ were found to interact but rates of interaction were 1/3 lower than rates of MØ interaction with aJK	FC have the ability to recognise, interact with and phagocytose aMØ and rates of interaction were comparable to the rates of MØ interaction with aMØ
Apoptotic FC (aFC)	aFC were recognised by MØ to the same degree as aMØ therefore it is likely that aFC upregulate 'eat-me' and downregulate 'don't-eat-me' signals to MØ, enabling recognition, interaction and phagocytosis	The combination of FC/aFC does not result in defective clearance in this model compared to the MØ/aMØ interaction, however interaction is more successful with aJK interaction

Table 3. Summary of results from key research questions addressed in Chapter 3

3.3.1. Oxidised LDL in the foam cell model

It is important to be aware of how differences in oxidation levels of LDL particles can affect the method of oxLDL binding and internalisation to ensure experimental consistency when setting up a foam cell model. The use of varying methods of LDL modification, e.g. acetylated or aggregated LDL, or varying methods of LDL oxidation, are likely to add difficulty to interpretation and comparison of results across studies and research groups[195,199,200].

Sufficient oxidation of LDL particles must occur for recognition by scavenger receptors, as opposed to the LDL-R; Mild oxidation produces minimally modified LDL (mmLDL) which may still be recognised by the LDL-R, but is not sufficiently modified to be recognised by scavenger receptors[201]. Further characterisation of modified LDL used in this study would be required to define whether it is minimally or extensively oxidised. A more recent study (2010) used LDL oxidised in the same manner, and for the same length of time, as the present study, and defined it as mmLDL[323], however other studies have cultured native LDL with 15-lipoxygenase expressing fibroblast cell lines[201,324]. mmLDL induced MØ spreading, via CD14, was found to inhibit phagocytosis of ACs[201] . In this model, despite presence of CD14 (figs. 5/6/7), spreading is not observed in THP-1 and VD3 models. Spreading is already induced by PMA and DS differentiation (Fig. 8), and phagocytic ability is present (Fig. 14/12/14). Whilst it is important to be aware of the implications of different oxidation states of LDL and the biological effects they can elicit, it must also be noted that terms such as mmLDL are subjective. In order to allow for comparison of results with oxLDL across studies, it is important to be explicit regarding oxidation methods used and to clearly define terms such as mmLDL.

Various methods can be employed to oxidise and measure oxidation levels of LDL. Physiologically relevant methods can be used to mildly oxidise LDL, however oxidation by metal ions is a common method employed to oxidise LDL and produces similar results in *in vivo* oxidation[201,325,326], and was the method of choice in this study. Carbonyl formation as a result of protein oxidation was used to confirm LDL oxidation via the chosen method of metal ion oxidation. Carbonyls are a popular choice of marker for oxidation measurement due to the stability of carbonylated proteins[313]. The ELISA method employed is also highly sensitive and minimal protein is required to complete the assay[313]. Results demonstrate the aforementioned donor variability in oxidation levels of basal and modified LDL samples (tab. 3). It is therefore important to minimise variability of protein oxidation levels to ensure differences in LDL oxidation status between experiments does not skew results and impact conclusions formed. One method that can be used to combat variation in oxidation levels between samples collected on different days or from different donors is to pool LDL samples from a number of donors and use the pooled LDL across experiments, a method adopted for the rest of these studies.

3.3.2. Morphology, surface receptor expression and foam cell formation of THP-1 models

Human cell lines are an important tool in atherosclerotic plaque research, due to the limited availability of human plaque samples, and the species variability of the often used murine and lapine models.

The advantages of using the monocytic THP-1 cell line is that various methods can be employed to differentiate cells into a MØ-like phenotype, resulting in cells that appear to be at different stages of differentiation[310,311]; VD3 cells appear more monocytic than PMA and DS cells, which show MØ-like characteristics. This can be used as a tool to determine at which point during monocyte-MØ differentiation do cells become susceptible to oxLDL induced changes to phenotype and function. It also allows modelling of the plaque *in vitro*, from infiltrating monocytes through to lipid-loaded foam cells. This provides a comprehensive, straightforward model, without the need to cross over between species, e.g. as in Schrijvers *et al*., an important paper in the field, which drew conclusions based on human endarterectomy samples, rabbit endarterectomy samples, murine thioglycollate elicited peritoneal MØ (phagocyte model in AC clearance assays), murine MØ cell line J774 (foam cell model), human monocyte cell line U937 (AC model), and human platelets and RBC from fresh blood[195]. Also to note is the use of *apoe-/-* murine models, knocking out Apolipoprotein E (ApoE), resulting in alterations in lipid trafficking, and resulting atherosclerosis, which is not readily found in mice[327]. However care should be taken to draw conclusions on AC clearance in the plaque of these experimental models as ApoE has been identified as having a role in AC clearance, and knockout of this molecule results in a persistence of ACs and an inflammatory state *in vivo*[328].

Monocyte-like THP-1 cells show some adherence but do not spread and are easily removed from tissue culture plastic by washing (Fig. 8). Presence of CD36 on THP-1 cells has been shown in previous studies[329], however despite high levels of CD36 in this model (Fig. 9), evidence of oxLDL uptake in the form of intracellular accumulation of lipid droplets, and therefore foam cell formation, is not observed via Oil Red O or Nile Red staining (figs. 7/8). An explanation for this could be the comparatively low expression levels of oxLDL scavenger receptors SRA-1 and LOX-1 compared to PMA and DS models (Fig. 9), however CD36 is thought to account for at least 50% of oxLDL binding[178]. Previous research has found low levels of SRA mRNA expression in isolated human monocytes, which was upregulated during differentiation[177]. Undetectable levels of LOX-1 mRNA expression was observed previously in THP-1 MØ and human PBMCs, and LOX-1 mRNA expression was upregulated with differentiation[330], as seen in the THP-1 model (Fig. 9). Priming or activation of monocytes may be required before active endocytosis of oxLDL[266]. Monocyte-like THP-1 phenotypes (unstimulated/VD3) may also have more efficient mechanisms of cholesterol efflux compared to MØ-like models, PMA/DS THP-1 cells, so although oxLDL uptake could be occurring, it is not being accumulated in the form of neutral lipid droplets. This could be achieved by measuring MØ uptake of DiI (1,1'-dioctadecyl-3,3,3'3'-tetra-methylindocyanide percholorate) labelled oxLDL, or measuring labelled oxLDL concentration in media before

and after exposure to cells[317]. Despite a lack of foam cell formation, the presence of monocytes in the atherosclerotic plaque may make unstimulated THP-1 cells a relevant part of the model being established[331].

Vitamin D3 (VD3) has been described as having a functional role in the hemopoietic system[332], and induces differentiation of cells via the vitamin D receptor (VDR)[333]. Morphological changes following VD3 induced differentiation are minimal; cells continue to proliferate, show some adherence but little spreading and are easily removed from tissue culture plastic by washing (Fig. 8). Previous studies have shown that VD3 treated cells are a more representative monocyte model than unstimulated THP-1 cells, with upregulation of CD14 expression, as confirmed in the present study, enhanced phagocytic activity and superoxide production[310,312,332,334]. Terminal differentiation is not reached following VD3 stimulation and upon removal of the stimulant, cells may revert to the THP-1 phenotype[310,334]. Calcium priming by VD3 may explain why functionally, VD3 stimulated cells appear further along the differentiation pathway than THP-1 cells, despite still phenotypically appearing THP-1 like[334], as shown by FS and SS on flow cytometry histograms (Fig. 8). Expression of CD36 is comparable to THP-1 cells (summarised in table 4). LOX-1 and SRA-1 expression levels are also low, and given that CD36 and SRA-1 are thought to account for up to 80% of total oxLDL binding, it could be predicted that VD3 stimulated cells would not be the most efficient model for active oxLDL uptake[335]. This was confirmed by Oil Red O and Nile Red staining in figures 7 and 8 respectively. Expression of CD14 is at its highest in this model, which may predict enhanced phagocytic ability in response to ACs compared to both unstimulated and PMA stimulated cells, however the monocytic nature of VD3 cells makes this difficult to test in our system. It may also suggest that any reduction in oxLDL uptake is not due to insufficient oxidation of LDL as mmLDL would bind CD14[201]. Miller *et al.* (2003) found reduced apoptotic thymocyte uptake by murine J774 MØ following mmLDL induced MØ spreading[201].

Treatment with the phorbol ester PMA results in a more prominent MØ-like phenotype; cells become much more adherent and spreading can be observed (Fig. 8). PMA is a potent activator of protein kinase C (PKC) and is thought to mimic the second messenger signalling lipid diacyl-glycerol (DAG)[310]. Basal expression of SRA-1 and LOX-1 was higher than the THP-1 and VD3 models (Fig. 9; summarised in table 4). Histograms revealed two populations of cells following PMA differentiation in terms of LOX-1, and to some extent SRA-1, expression, with 2 peaks observed on the histograms, unlike CD36 expression, which is upregulated in all cells when comparing MFI to that in the THP-1 model (Fig. 9). This supports previous findings in PBMCs that PMA treatment upregulates CD36 mRNA

expression, and that PMA also stimulates upregulation of LOX-1 mRNA in THP-1 cells[330,336]. As CD36 and SRA-1 receptors account for up to 80% of total oxLDL binding and degradation, it is reasonable to anticipate that use of this model will result in foam cell formation[178,335]. Cell surface CD36 protein expression was high in all models, however the combined receptor profile following PMA stimulation correlated with high uptake of oxLDL, as detected by Oil Red O and Nile Red staining of accumulated neutral lipid (figs. 7/8). CD14 levels were also increased in this model compared to unstimulated THP-1, though did not reach levels of VD3 stimulated cells (summarised in table 4). This supports previous studies[312], and would indicate good potential ability to uptake ACs, especially considering the ability of scavenger receptors in AC interaction[102-105].

DS cells have a similar level of CD14 to that of cells treated with PMA alone, despite also being treated with VD3, therefore the pathway activated by PMA stimulated differentiation may override that of VD3 (fig 6; summarised in table 4). Like cells treated with PMA alone, DS cells have a typically MØ-like phenotype, with strong adherence and spreading (Fig. 8). Thomas *et al.* reported differences in LPS response in these THP-1 models that were not directly linked to CD14 expression, with high TNF-α release in response to LPS in DS cells, and to a lesser extent, PMA cells. LPS stimulation resulted in a very small amount of TNF-α release by comparison, and unstimulated THP-1 response was negligible[312]. DS cells also had similar levels of oxLDL receptor expression when compared to the PMA model, with high levels of CD36 and lower levels of SRA-1 and LOX-1, however SRA-1 and LOX-1 expression was higher than expression observed in the THP-1 model. CD14 expression was also detected (Fig. 9; summarised in table 4). Uptake of both oxLDL and apoptotic cells should be apparent in this model, and foam cell formation as indicated by lipid accumulation was shown by Oil Red O and Nile Red staining (figs. 7/8).

In summary, despite the prominent role of CD36 in oxLDL uptake[178], expression of this receptor alone is not predictive of foam cell formation. It may be that SRA-1 and LOX-1 presence is also essential, or upregulation of signalling molecules is required alongside differentiation to MØ.

THP-1 models	Surface Receptors					Foam cell formation
	CD36	SRA-1	LOX-1	CD14	ICAM-3	
THP-1	++	+/-	+/-	+	+	**N**
VD3	+++	+/-	+/-	++++	+++	**N**
PMA	+++	++	++	++	++	**Y**
DS	+++	++	++	++	++	**Y**

Table 4. Summary of expression of key surface receptors and foam cell formation in THP-1 models *Summary of surface receptor expression of each THP-1 model as analysed by flow cytometry – Mean fluorescence intensity was examined and expression scored as follows: +/- little or no detectable expression, + low expression, ++ medium expression, +++ high expression, ++++ very high expression. Foam cell formation was also analysed by Oil Red O staining and scored as either presence (Y) or absence (N) of foam cells after 72hr oxLDL exposure at 50µg/ml.*

3.3.3. Effects of oxidised LDL treatment on THP-1 models

3.4.3.1. Toxicity

The upregulation of scavenger receptors in PMA and DS models, and the accumulation of lipids in these models, implies that PMA and DS cells may be more susceptible to toxic effects of oxLDL, however once cells are of a foam cell phenotype, induction of apoptosis may be altered. For example, expression of SRA-1 was found to reduce oxLDL induced apoptosis, a mechanism which may contribute to prolonged life of MØ in the plaque and the production of foam cells[337].

Previous studies have found oxLDL induces apoptosis in foam cells, however other studies, including the present work (Fig. 12), have not found prominent oxLDL induced toxicity of MØ[197,201]. Miller *et al.* (2003)[201] found no difference in murine peritoneal or cell-line MØ function between 10-50µg/ml oxLDL treatment when investigating actin response. Khan *et al.* also found that oxLDL at 100µg/ml did not induce MØ cell death in rat MØ[197]. Conversely, significant levels of apoptosis in PBMC-derived MØ (PBMC-DMØ) were found from 50µg/ml oxLDL treatment[198], however LDL samples were extensively oxidised (24 hours copper sulphate oxidation, versus 1 hour in this study). Contradictory results are likely due to the

varying extent of LDL oxidation across studies, with results by Bjorkerud & Bjorkerud (1996) showing oxLDL can induce cell growth or apoptosis depending on oxidation levels and cell types studied[199].

Minimal toxicity of oxLDL was found in both monocyte-like THP-1 cells and MØ-like DS cells (Fig. 12). As monocytes seem less susceptible to the effects of oxLDL, higher concentrations were tested than in MØ models. The lack of observed toxicity allows use of a broad range of oxLDL concentrations without the concern of toxicity, however further investigation will need to be carried out to make sure cells are still fully functional following oxLDL treatment (e.g. standard cell viability assays such as the MTT assay, or functional assays such as *E.coli* phagocytosis, e.g. Fig. 16, and cytokine responses to basic stimuli such as lipopolysaccharide, e.g. Fig. 21; Chapter 4).

As oxLDL induced apoptosis is not readily detected in this model using annexin V versus PI staining, an alternative way of apoptosis induction was chosen in order to further investigate the full spectrum of AC clearance within the plaque. UV induction was chosen as a well-established method of apoptosis induction, with a comparable outcome to other apoptosis induction methods[62]. This also allows a good level of control over apoptosis induction across assays.

3.4.3.2. Receptor expression in THP-1 models following oxLDL exposure

Expression of the LDL receptor (LDL-R) is regulated homeostatically by the presence of free intracellular cholesterol[266]. Unlike the LDL-R, scavenger receptor expression is independent of the level of free intracellular cholesterol, accounting for the failure of cellular homeostasis mechanisms within the atherosclerotic plaque, and the subsequent production of foam cells[177]. The LDL-R becomes downregulated when free intracellular cholesterol levels are high as a result of high quantities of available ligand[266]. Conversely, expression of the scavenger receptor CD36, in response to the ligand oxLDL, is increased in the THP-1, VD3 and PMA models (Fig. 13), as found in previous studies with murine J774 and RAW 264.7 MØ[319,338]. CD36 upregulation has been shown to occur via oxLDL induced PPAR-γ expression[232,339]. In contrast to the effect on CD36, PPAR-γ activation has been found to suppress SRA-1 expression[340]. This may explain why an upregulation of SRA-1 following oxLDL treatment was not found in any of the models used in this study (Fig. 13).

OxLDL also upregulates CD14 expression in VD3 cells (Fig. 13), which *in vivo* may exacerbate the pro-inflammatory environment found within the plaque. Pasini *et al*., (2007)[341] found that enhanced levels of oxLDL upregulated CD14 and toll-like receptor 4 (TLR4) expression in circulating monocytes. This was then suggested to cause cytokine

overproduction due to the resulting priming of monocytes[341]. The upregulation of CD14 and TLR4 is comparable to that of pro-inflammatory ligands such as LPS[342]. It is possible that oxLDL treatment primes monocytes to skew towards the pro-inflammatory M1, rather than reparative M2, phenotype. A study by Nagornev and Maltseva (1996)[233] found a proportion of MØ within human atherosclerotic plaques had not transformed into foam cells. These MØ were associated with pro-inflammatory cytokine interleukin-1 (IL-1) and TNF-α production, which is unusual due to the close proximity of ACs[233]. Priming of MØ into an M1-like phenotype may also cause inefficient AC clearance within the plaque, or a lack of anti-inflammatory cytokine release in response to ACs[56].

Human monocyte and macrophage interaction with mmLDL via CD14, TLR4 and TLR2 was also found to exacerbate an inflammatory response via increased IL-1β, IL-6 and IL-10 release. A combination of CD14 upregulation and mmLDL could therefore exacerbate the inflammatory environment in the plaque[323]. Cytokine profiling of MØ pre-treated with (FC) or without (MØ) oxLDL will be investigated in the next chapter.

3.4.3.3. Interaction with apoptotic cells

It is generally perceived based on a small number of studies, and the presence of uncleared ACs in the plaque, that AC clearance is defective within the plaque via unknown mechanisms[46,195,197]. This has been demonstrated in this differentiated THP-1 model with regards to foam cell interaction with apoptotic T lymphocytes (Fig. 14), which are a major component of plaque ACs[46]. However foam cell interaction with apoptotic macrophages (aMØ) or apoptotic foam cells (aFC), the prominent AC type in the plaque[46], was not impaired when compared to interaction rates of the same AC types with MØ (Fig. 17a).

A straightforward explanation for decreased aJK binding in the THP-1 model could be that oxLDL treatment is toxic to PMA and DS MØ, however, toxicity testing (see section 3.4.3.1.) revealed little apoptosis or necrosis. Schrijvers *et al.* (2006)[195] and Khan *at al*. (2003)[197] also ruled out toxicity as a mechanism behind reduced AC phagocytosis. This does not mean that the cells are healthy, as this is a very late marker of toxicity. However further research into cytokine responses in this model (see Chapter 4) seems to show comparable ability to produce cytokines between MØ and FC. This implies that rather than all functions of foam cells being affected due to oxLDL induced toxicity, only some functions are affected due to an alteration in MØ phenotype following foam cell formation.

Another reported mechanism of defective AC clearance is direct receptor competition between ACs and oxLDL. Looking at PMA and DS cells, the ability to bind aJK, although still functional, appears to be impaired following oxLDL treatment (Fig. 14). This occurs despite

an upregulation of receptors which have been shown to recognise ACs, such as CD36 (Fig. 13). Conversely, in the current study, using a short time period of oxLDL exposure, direct competition between oxLDL and aJK was not found to affect aJK binding by a human cell line (Fig. 12). The method employed in the THP-1 system involves replacing medium containing oxLDL with fresh medium before adding ACs, decreasing the likelihood that oxLDL is competing directly with ACs for particular receptors in this model. Additionally, Khan *et al.* (2003)[197] found no effect on AC interaction in the presence of oxLDL in a Balb/c mouse (thioglycollate elicited) peritoneal MØ model. The system used in Khan's study used LDL that was oxidised by the same method as in the THP-1 model, but LDL was exposed to the oxidising agent for 24 hours, compared to 1 hour in the THP-1 model, which will significantly alter oxidation levels, and potentially dictate whether ACs and oxLDL are competing for the same binding site on MØ surface receptors. Miller *et al.* (2003)[201] found reduced phagocytosis of ACs by resident peritoneal MØ following exposure to mmLDL, supporting results found using aJK in the THP-1 model. It is unclear whether oxLDL was removed from the medium before addition of ACs, therefore direct competition for CD14 between mmLDL and ACs may be the cause of reduced phagocytosis[31,201]. Alternatively, relevant to defective clearance in the plaque, but not the THP-1 model, monoclonal antibodies targeting oxLDL have been found to bind to ACs, inhibiting phagocytosis[343]. However, the same antibodies have been found to facilitate clearance of ACs in another study[344].

Following binding, ACs are engulfed by phagocytes. In the presence of oxLDL, it was previously found that rather than reduced binding, engulfment of ACs was delayed, which may exacerbate the pro-inflammatory environment found within the plaque[197]. The assay used here did not distinguish between binding and engulfment of ACs as it was carried out at 37C, and both bound cells, and cells being engulfed, are observed. However, methods in Khan's study did not involve pre-incubation of macrophages with oxLDL and subsequent foam cell formation. OxLDL was present in the model system and introduced at the same time as the ACs, and interactions observed over 72 hours. Thus, it is unclear whether delayed engulfment was due to the altered phenotype of the MØs over time or the influence of the environment on MØ or AC behaviour, e.g. competition for binding sites. In contrast, Li *et al.* (2006)[210] found no change in ability of peritoneal MØ from female C57BL6/J mice to engulf ACs when monitored over 60 minutes.

If physiological changes in phagocyte or AC function following lipid loading are the cause of defective clearance, rather than an inflammatory or competitive local microenvironment as found in the plaque, then any of the multiple stages of AC clearance could be affected[43]. Effective clearance of dying cells requires the orchestration of a complex multi-stage

process, and it is important to note that any defects could occur on the side of the phagocyte, the target AC, or a combination of both. This could include changes at the level of phagocyte recruitment to AC, AC recognition, tethering (binding), tickling (signalling) and AC engulfment[43]. Immune modulation following clearance is also a key research focus due to the paradoxical inflammatory environment found in the plaque[58]. The model of AC interaction used in these studies focuses on the recognition, interaction and phagocytosis of apoptotic cells, as with many models of clearance.

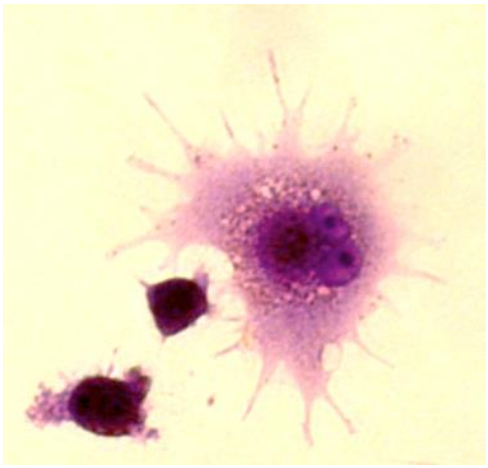


Figure 18. Foam cell phagocytosis of apoptotic cells *Differentiated THP-1-derived foam cell interacting with apoptotic Jurkat cell. Macrophages were treated with 10 x ACs and incubated for 1 hour at 37C. Unbound ACs were removed by washing with PBS and the cells stained with Diff Quik. ACs appear as small, dark, round cells.*

If phagocyte cell death or direct receptor competition do not account for reductions in aJK binding following lipid loading of MØ, as with aJK binding (Fig. 14) a physiological change in phagocyte function may be the cause. Schrijvers *et al.* (2005)[195] reported defective clearance in the plaque following observation of a 19-fold increase in the ratio of free versus phagocytosed ACs in human endarterectomy samples, compared with human tonsils. This could initially be attributed to overwhelming AC numbers in the plaque compared to the tonsil, or insufficient phagocyte numbers, however comparable rates of apoptosis in both environments were reported, and a greater number of MØ in plaques versus tonsils. This method gives a snapshot of clearance in both environments, and a physiological change in MØ function was investigated. Further investigations into mechanisms and kinetics of defective clearance are done in an alternative murine model, using platelets or aggregated LDL to produce foam cells. Using separate *in vitro* studies, lipid loading with platelets or aggregated LDL did not affect uptake of apoptotic monocytes (U937 human monocytic cell line), only preloading of inert beads or induction of oxidative stress reduced AC uptake. This did not affect uptake of further beads or *E.coli*. This supports data in this study that function

between MØ and FC to clear cells of a myeloid lineage, and to phagocytose E.*coli*, was comparable (figs. 13/14a).

Also to consider is whether aMØ and aFC are a favourable substrate for ingestion compared to popular AC models such as aJK. This study found that interaction with aMØ and aFC was functional, but levels of interaction by MØ were reduced compared to aJK interaction (Fig. 17b). When observing MØ interaction rates, a healthy phagocyte, with aJK, a standard AC model, and comparing with aMØ or aFC interaction, interaction could be deemed less efficient with the latter AC types (Fig. 17b). It may therefore not just be lipid loading phagocytes, or clearing lipid-loaded ACs, that is the fundamental issue, but a less effective clearance process when MØ have to clear up after themselves. Li *et al.* found similar results, with the percentage of mouse-peritoneal MØ ingesting mouse peritoneal-derived aFCs at 20–30%, compared with 40–50% for UV irradiated Jurkat cells[210]. In the Schrijvers *et al.* model of AC clearance, MØ clearing apoptotic B cells in tonsil specimens contained more ACs than MØ clearing dead cells (prominently aMØ) within the plaque[195]. Given that high densities of aMØ requiring clearance is not common, and it is not a straightforward cell type to study due to adherence, research into aMØ clearance is limited.

As clearance mechanisms become better elucidated, they are being linked with defective clearance mechanisms in the plaque. For example the role of the TAM receptor Mer, which recognises PS on the AC surface, both directly and indirectly, is gradually being elucidated, with roles found in both AC clearance and atherosclerosis[108,109,126]. In a murine model of atherosclerosis, Thorp *et al*. (2008) found Mer receptor mutation reduces efficiency of AC clearance, promoting AC accumulation and plaque necrosis[108]. Defects in Mer are also associated with reduced AC clearance and development of autoimmunity in mice[109,110]. Soluble Mer, cleaved from the cell surface under inflammatory conditions, has also been shown to inhibit AC clearance by competition with ACs for bridging molecule Gas6, an interesting result if you consider the inflammatory environment of the plaque[345]. A role for Mer has also been found in immune modulation following 'tickling' by apoptotic foam cell membranes[210]. Further research into Mer in human cell lines and primary human cells would be beneficial to elucidating failed clearance mechanisms in human atherosclerotic lesions.

Cell stress may also alter phagocytic efficiency. For example, autophagy, induced in stressed cells, in macrophages undergoing apoptosis was found to enhance their recognition by phagocytes[346]. Oxidative stress was also found to inhibit phagocytosis of apoptotic cells, including apoptotic Jurkat[347]. All AC models in the current study were induced to apoptosis using the same method, so if oxidative stress levels are enhanced at this point, it is likely that they are equally exacerbated in all AC models.

Subsequent immune-modulation following AC interaction may also have knock-on effects on further AC uptake in the plaque as differing signals may enhance or reduce further uptake ability. Defective immune-modulation could originate from the phagocyte, if lipid loading alters the ability of MØ to switch between M1 and M2 phenotypes. Also to consider is the role of the apoptotic cell, and whether it has the ability to confer the correct signal to the phagocyte. This will be investigated further in Chapter 4.

Another consideration is whether *in vivo* chemotactic factors are still released by apoptotic foam cells, and whether phagocytic foam cells have the ability to recognise and migrate towards apoptotic cells within the plaque. This may not be relevant in this model of AC interaction (figs. 11/12/14) due to the direct and excess placement of ACs on phagocytes. It also may not be relevant within the plaque itself if there is such a concentration of ACs in the plaque that movement would not be required to contact the AC, however when considering the continuous recruitment of monocytes into the plaque, exacerbating the pathological features atherosclerosis, chemotactic factors may play a key role in plaque progression and are an important area of study. Mechanisms of monocyte chemotaxis to apoptotic cells will be further studies in Chapter 5.

Mediators in the local environment may also play a pivotal role in clearance in the plaque, at which point the effects of an aged environment may come into play. For example, established phagocyte:AC bridging molecules MFG-E8[348], C1q[349] and Gas6[107] knockout models have recently been shown to impact atherosclerosis progression in mice. Similar trends are found in autoimmune disease and ageing, where links have been found between reduced function of bridging molecules, and persistent apoptotic cells or inflammation[34,350]. Interestingly murine models of SLE, an autoimmune disease driven by defective AC clearance, show accelerated atherosclerosis[351]. This also demonstrates a pivotal role for bridging molecules, including Gas-6, levels of which could alter in ageing and pathology. Local inflammation is also likely to exacerbate is also likely to drive pathology. TNF-α, a classic inflammatory cytokine, has been shown to inhibits AC clearance by MØ, via cytosolic phospholipase A2 and oxidant-dependent mechanisms[57]. The effects of ageing on AC clearance will be further explored in a Pilot Study, detailed in Chapter 6.

Understanding the underlying molecular mechanisms behind defective clearance of, or response to, ACs would be an important development for research into atherosclerosis due to the consequences of secondary necrosis of uncleared cells and subsequent plaque instability[150].

3.4. Conclusions and future work

Results demonstrate THP-1, a well-established human monocytic cell line, is a useful tool for investigating all stages of apoptotic cell clearance, which can be applied to research into potentially defective mechanisms of apoptotic cell clearance in the atherosclerotic plaque. VD3 differentiated THP-1 will be used in further studies into chemotaxis, as a monocyte model with adherent properties. PMA differentiated THP-1 cells have proved robust in macrophage and foam cell formation, and will be taken forward to investigate further aspects of AC clearance relevant to the plaque environment.

Further characterisation of oxLDL utilised in these studies would help define levels of oxidation, and whether these are comparable to minimally modified LDL, as this has implications on receptor interaction, phagocytic activity and immunological outcome[201,323]. As mmLDL has been reported to be inflammatory, studies detailed in Chapter 4 may provide evidence to support minimal or extensive oxidation levels. Although different physiological outcomes have been reported following treatment of MØ with mmLDL and oxLDL, set parameters for oxidation levels have never been established.

Results in this study support previous research that foam cells are in some cases defective in clearance of some AC lineages[195,197]. Further study into the mechanisms of overall reduction in phagocyte interaction with aMØ and aFC, compared to aJK, could be of importance, given the composition of ACs within the plaque.

The involvement of Mer in AC clearance in a plaque environment in human cells would also bolster current research in the area, and could be done using the THP-1 model, which has been shown to express Mer[352].

Research into other phases of AC clearance relevant to the plaque environment will be investigated in the following chapters.

Cytokine profiling of foam cell model

4.1. Introduction

It is the accepted theory that efficient AC clearance is necessary, before secondary necrosis and subsequent inflammation ensues[35], though contradictory evidence illustrates that mechanisms are poorly understood[38,40]. Clearance of apoptotic cells has also been shown to directly influence immune modulation in an anti-inflammatory manner[55,56].

Macrophage plasticity is well recognised, with phenotype altering between pro-inflammatory 'M1-like' MØ, and 'M2-like' MØ which have more of a reparative/janitorial role[353]. MØ phenotypes vary according to the local microenvironment, and inflammatory responses, associated with 'M1'-like MØ, have been shown to be dampened in the presence of ACs[56]. AC interaction is associated with an 'M2'-like phenotype, and has been shown to induce anti-inflammatory mediators release, including TGF-β1, IL10, PGE2, PAF[55,56]. The release of pro-inflammatory mediators has also been shown to be supressed following AC interaction, including TNF-α, IL-1, IL-12 and IL-8[55,56]

Following phagocyte recruitment, AC clearance has been proposed to comprise of 4 stages; recognition of the AC by the phagocyte, tethering of the AC (binding), tickling of the phagocyte by the AC (signalling) and phagocytosis. Subsequent modulation of immune responses in the phagocyte follows on from this. The previous chapter investigated recognition, tethering and phagocytosis of apoptotic foam cells by non-lipid laden and lipid laden MØ. Whilst defective clearance of AC has been proposed to promote plaque progression[150,195], the immunological responses of lipid-laden phagocytes to human lipid-laden ACs is unknown and altered phagocyte responses to apoptotic cells within the plaque environment may also help drive disease. This chapter will focus on the tickling/signalling phase, and post phagocytosis responses, by investigating the immunological outcome of lipid-loading MØ, and the ability of these MØ to respond to ACs in a non- or anti-inflammatory manner. The ability of foam cells to switch between an 'M1' and 'M2' output will be investigated, and also the ability of apoptotic foam cells to tickle lipid- and non-lipid laden MØ into a non- or anti-inflammatory phenotype.

There remain a number of unanswered questions addressing issues fundamental to the inflammatory environment within the plaque, including those relating to the immunological response post AC ingestion within a plaque environment.

The hypothesis underlying this chapter is that control of regulating appropriate immune responses is lost by MØ in an aged (e.g. lipid-laden) environment, resulting in failure to

resolve inflammation. Some of the research questions used to address this are listed in the table below.

	Phagocyte	
Apoptotic cell	**Macrophage (MØ)**	**Foam cell (FC)**
Apoptotic MØ (aMØ)	What is the immunological outcome of interaction between non-lipid laden MØ and non-lipid laden aMØ?	Can FC respond to aMØ in a non- or anti-inflammatory manner?
Apoptotic FC (aFC)	Can aFC tickle MØ into a non- or anti-inflammatory phenotype?	Does the combination of FC/aFC cause/exacerbate defective immune response?

Table 5. Summary of key research questions addressed in Chapter 4

4.2. Results

4.2.1. THP-1-derived macrophage and foam cell cytokine responses to apoptotic T cells

To establish whether foam cells (lipid-laden MØ) respond to apoptotic cells in a comparable manner to non-lipid-laden MØ, we used an established apoptotic cell model, UV-induced apoptotic Jurkat (aJK).

TNF-α is a key driver of inflammation and had been found in human atherosclerotic plaques[221,222]. TNF-α release by phagocytes has also been shown to be dampened following AC interaction[55,56]. TNF-α release was measured at basal levels (i.e. without treatment of macrophages) to examine whether lipid-loading of MØ induces an inflammatory cytokine output. Cytokine production in response to aJK was examined to establish whether MØ and FC both respond to aJK in a non- or anti-inflammatory manner. The addition of LPS allowed observation of a classical activation, and aJK-mediated switch off of an immune response, indicating a switch of a classical to a regulatory phenotype in MØ.

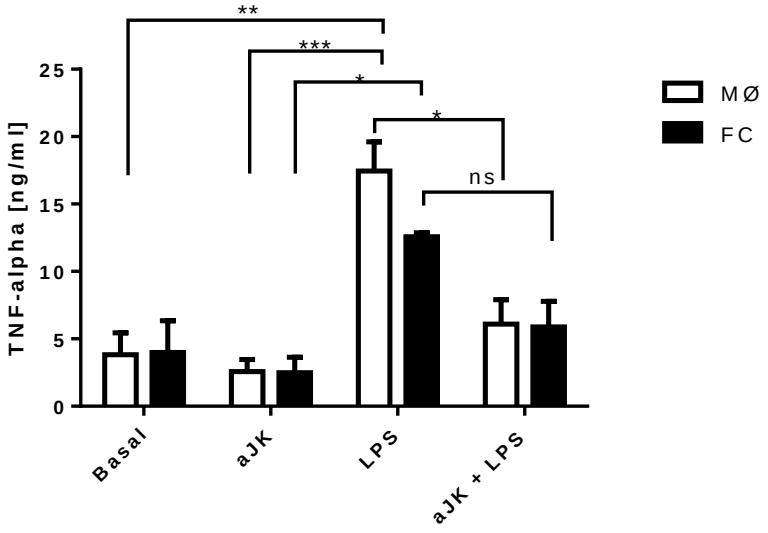


Figure 19. Apoptotic Jurkat reduce LPS-induced TNF-α production from both macrophages and foam cells *MØ and FC were co-cultured with aJK (harvested 6h post UV) or sfRPMI control (20h; 37C). 0.5ng/ml LPS or sfRPMI control was added to relevant wells with normal human serum (10% v/v final conc.) and incubated (4h; 37C). TNF-α ELISA was carried out according to manufacturer's instructions (PeproTech Ltd). Data shown is mean ± SEM of 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, two-way ANOVA with Tukey's* post-hoc *analysis.*

Figure 19 shows MØ (no oxLDL treatment) and FC (50µg/ml oxLDL, 72h) released low and comparable basal levels of TNF-α, and similarly low levels following co-culture with aJK. Cytokine production in response to LPS, an established pro-inflammatory mediator, was also measured to compare the ability of MØ and FC to produce an inflammatory/'M1-like' response, and whether this can be inhibited by co-culture with aJK. TNF-α secretion was significantly increased in MØ following LPS treatment ($P<0.01$), and was increased, but not significantly so, in FC, compared to basal levels. FC appeared to show reduced TNF-α release compared to MØ in response to LPS, but this difference was not significant. TNF-α release in response to LPS was significantly increased in MØ and FC compared to aJK alone ($P<0.001$ and $P<0.05$ respectively). TNF-α release in response to LPS was inhibited significantly in MØ following co-culture with aJK ($P<0.05$), and in FC levels were reduced but not significantly so. Results show FC are perhaps less inflammatory than their non-lipid-laden counterparts, and that increased basal levels of TNF-α release may not be responsible for the inflammatory environment within the plaque.

IL-12 can also drive inflammation, and had been found in human atherosclerotic plaques[228]. IL-12 and anti-inflammatory cytokine IL-10, have been found to cross-regulate, and the secreted IL-10: IL-12 ratio has been proposed as a marker for classical versus regulatory MØ

activation[49,228]. IL-12 release by phagocytes has also been shown to be dampened following AC interaction, however IL-10 release in response to ACs is less clear, with some studies finding upregulation, and others downregulation, of this cytokine[55,56]. IL-10 and IL-12 release was measured at basal levels (i.e. without treatment of macrophages) to examine the effect of lipid-loading MØ on cytokine output. IL-10 and IL-12 production in response to aJK was examined to establish whether MØ and FC both respond to aJK in a non- or anti-inflammatory manner. The addition of LPS allowed observation of a classical activation, and aJK-mediated switch off of an immune response, indicating a switch of a classical to a regulatory phenotype in MØ.

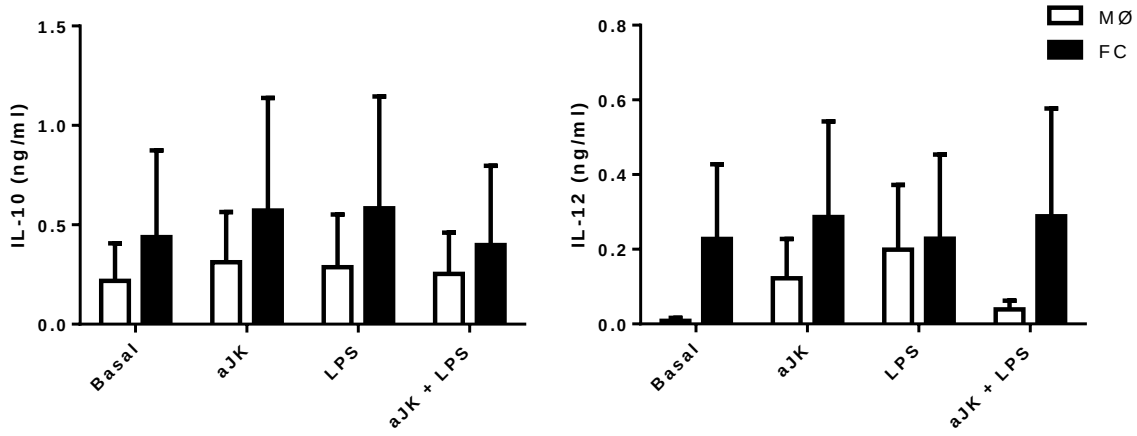


Figure 20. IL-10 and IL-12 response to apoptotic Jurkat by live macrophages and live foam cells *MØ and FC were co-cultured with aJK, LPS or sfRPMI as in Fig. 19. Samples were diluted 1/5 in sfRPMI prior to ELISA analysis. IL-10 and IL-12 ELISAs were carried out according to manufacturer's instructions (PeproTech Ltd). Data shown is mean ± SEM of 3 independent experiments. Data analysed using two-way ANOVA with Tukey's* post-hoc *analysis.*

Results show that despite the same methods being employed as in investigation of TNF-α responses, IL-10 and IL-12 responses are much more variable, and the trends associated with LPS response and AC clearance are not apparent (Fig. 20).

4.2.2. THP-1-derived macrophage and foam cell cytokine responses to apoptotic macrophages and apoptotic foam cells

MØ and FC models were used to investigate the ability of foam cells, compared to MØ, to respond to non-lipid-laden apoptotic macrophages (aMØ) and apoptotic foam cells (aFC). This also would address whether aMØ and aFC have the ability to 'tickle' MØ and FC into the appropriate regulatory phenotype. Figure 21 shows that both MØ and FC release low basal levels TNF-α, consistent with data in Figure 19. Figure 21 also shows low TNF-α release by MØ and FC when co-cultured with aMØ or aFC, at comparable levels to basal TNF-α release. This would suggest that foam cells are not hyper-inflammatory, and that neither

aMØ nor aFC elicit an inflammatory response. This is despite co-culture up to 24 hours post UV treatment, by which time cells induced to apoptosis may be secondarily necrotic. The TNF-α response to LPS is identical in MØ and FC, and significantly higher than both basal and aMØ/aFC co-cultured treatment groups ($P<0.0001$). This indicates comparable functionality, or 'health' of FC compared to MØ, given similar levels of cell death shown in the previous chapter (Chapter 3; Fig. 12).

MØ co-culture with aMØ significantly inhibited LPS-induced TNF-α release ($P<0.05$). MØ co-culture with aFC also significantly reduced LPS-induced TNF-α release ($P<0.01$), indicating aFC are equally capable of switching off an inflammatory response by THP-1 MØ, compared to aMØ (Fig. 21).

Similar trends were observed when measuring FC TNF-α responses to LPS in combination with aMØ and aFC co-culture (Fig. 21). FC co-culture with aMØ significantly inhibited LPS-induced TNF-α release ($P<0.01$). LPS-induced TNF-α release by FC was inhibited even more significantly by aFC ($P<0.0001$), indicating, as above, that aFC are equally, if not more, capable of switching off an inflammatory response by FC, compared to aMØ. Interestingly, the FC inflammatory response appears to be switched off more effectively by aMØ and aFC than the MØ inflammatory response, although these differences are not significant (ns; Fig. 21).

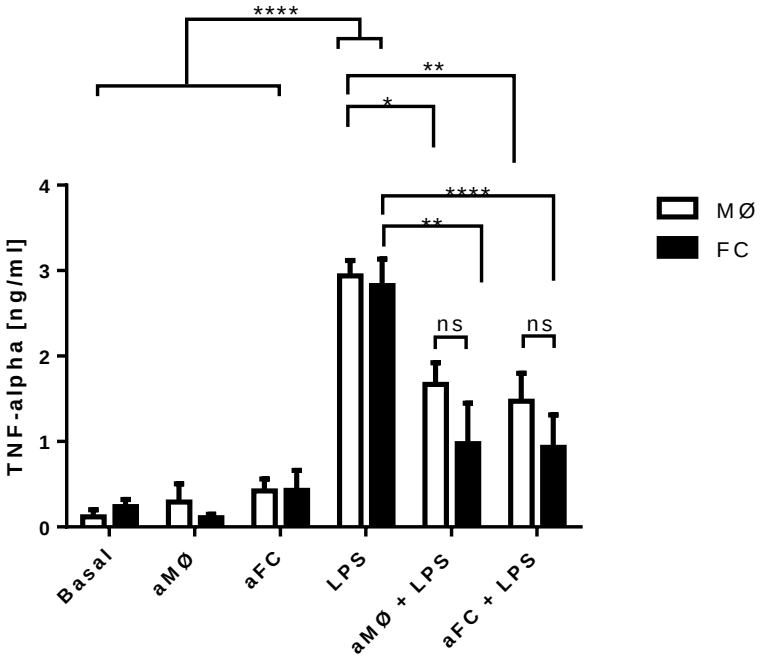


Figure 21. Apoptotic macrophages and apoptotic foam cells reduce LPS-induced TNF-α production from both MØ and FC *MØ and FC were co-cultured with aMØ/aFC (harvested 6h post UV) or sfRPMI control (20h; 37C). 0.5ng/ml LPS or sfRPMI control was*

*added to relevant wells with normal human serum (10% v/v final conc.) and incubated (4h; 37C). Supernatants were diluted 1/5 in sfRPMI prior to ELISA analysis. TNF-α ELISA was carried out according to manufacturer's instructions (PeproTech Ltd). Data shown is mean ± SEM of 4 independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, two-way ANOVA with Tukey's* post-hoc *analysis.*

MØ and FC IL-10 and IL-12 responses were again variable, therefore any trends in response to AC and LPS were not exposed (Fig. 22).

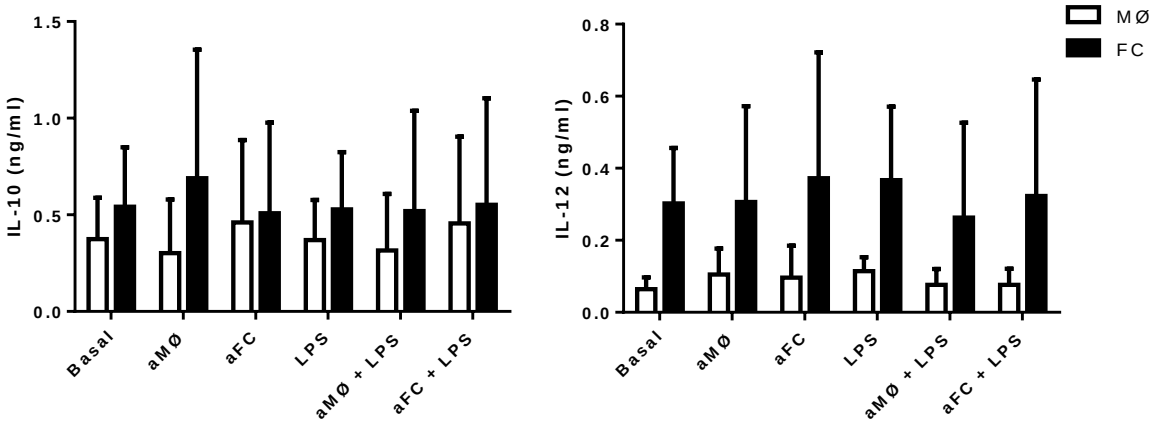


Figure 22. IL-10 and IL-12 response to apoptotic macrophages and apoptotic foam cells by live macrophages and live foam cells *MØ and FC were co-cultured with aMØ/aFC, LPS or sfRPMI as in Fig. 21. Supernatants were diluted 1/5 in sfRPMI prior to ELISA analysis. IL-10 and IL-12 ELISAs was carried out according to manufacturer's instructions (PeproTech Ltd). Data shown is mean ± SEM of 3 independent experiments. Data analysed using two-way ANOVA with Tukey's* post-hoc *analysis.*

4.2.3. Human monocyte-derived macrophage cytokine response to apoptotic cells

THP-1 cells have proved to be a valuable model MØ system for assaying MØ function with regards to AC clearance[312]. In order to establish the relevance of cytokine responses noted above in THP-1-derived MØ and FC models, human monocyte-derived macrophages (HMDM) were employed as a primary human cell system and TNF-α responses to the same stimuli as in THP-1-derived MØ and FC were measured.

Results in HMDM show low TNF-α release at basal levels, and comparably low TNF-α release when with aJK co-culture. This is in direct agreement with the results obtained with THP-1-derived MØ/FC models. However, in contrast to results in THP-1-derived MØ/FC models, HMDM showed significantly higher levels of TNF-α release in response to co-culture with THP-1 derived aMØ and aFC when compared to basal levels and aJK co-culture ($P<0.01$). TNF-α release was higher in response to aMØ/aFC than in response to LPS, but not significantly so. TNF-α response to LPS appeared to be inhibited a small amount with aJK co-culture, but not to a significant degree. TNF-α release in response to LPS was not

inhibited by co-culture with aMØ/aFC, in contrast to the THP-1 MØ/FC model. The TNF-α response to LPS in the presence of aMØ/aFC was significantly higher than basal levels (P<0.0001) and levels in LPS alone treatment group (P<0.01). Levels of TNF-α release by HMDM (Fig. 23) are lower than in the THP-1 model (Fig. 21), which is likely due to differences in live cell numbers following culture.

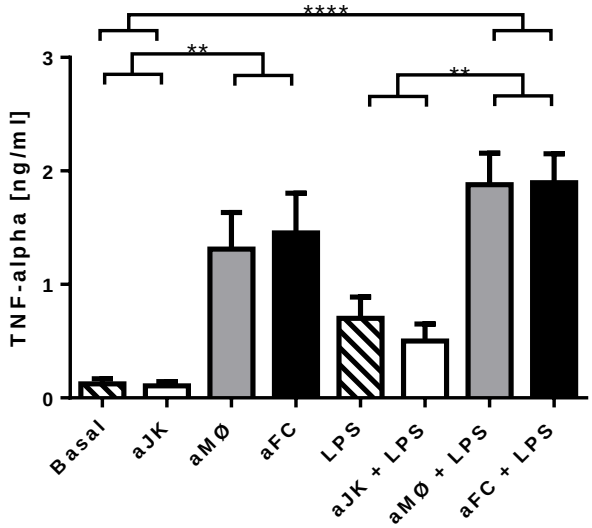


Figure 23. Primary human monocyte-derived macrophages produce TNF-α in response to apoptotic macrophages and apoptotic foam cells *HMDM were co-cultured with aJK/aMØ/aFC (6h post UV) or CDMØ-medium control (20h; 37C). 5ng/ml LPS or CDMØ-medium control was added to relevant wells and incubated (4h; 37C). Samples were diluted 1/5 in sfRPMI prior to ELISA analysis. TNF-α ELISA was carried out as per manufacturer's instructions (PeproTech). Data shown is mean ± SEM of multiple independent experiments. Basal n=20, LPS n=20, aJK (+/-LPS) n=17, aMØ (+/-LPS) n=17, aFC (+/-LPS) n=14. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 one way ANOVA with Tukey's* post-hoc *analysis.*

One possible trivial explanation of these divergent results in HMDM could be the presence TNF-α in AC preparations. This could be derived from intracellular cytokine release induced passively via membrane disruption, e.g. secondary necrosis, or actively via contamination prior to apoptosis induction, e.g. bacterial contamination, which is not considered to be likely. All other assay components are present in basal wells, including NHS, apart from LPS, which is expected to be inflammatory. It is unlikely to be oxidised LDL contamination as aMØ elicit a TNF-α response as well as aFC. To ensure detected TNF-α was not already present in assay preparations, aMØ, or NHS and LPS, preparations alone were assayed for TNF-α following full incubation times, in the absence of phagocytes (Fig. 24). Wells did not contain HMDM, but contained the same working solutions of NHS, LPS and aMØ in CD-MØ medium

as used in the HMD-MØ assays. Results showed very low/negligible levels of TNF-α in control assays.

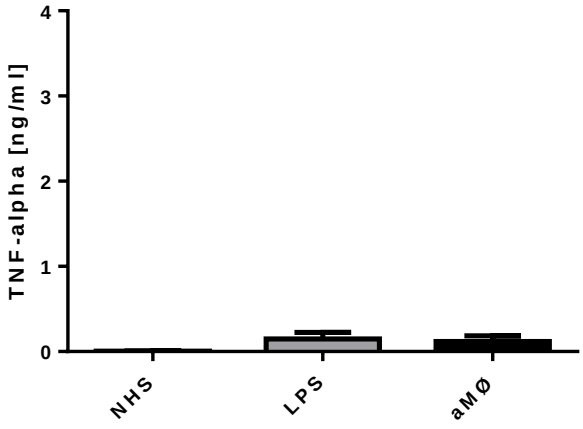


Figure 24. Measured TNF-α is not NHS, LPS or AC-derived *aMØ (6h post UV) were resuspended in CDMØ-medium, and either CDMØ-medium or AC added to relevant wells and incubated (20h; 37C). 10% NHS (v/v, final conc.), 5ng/ml LPS or CDMØ-medium was added to relevant wells and incubated (4hours; 37C). Samples were diluted 1/5 in sfRPMI prior to ELISA analysis. TNF-α ELISA was carried out as per manufacturer's instructions (PeproTech Ltd). Data shown is mean ± SEM of 3 independent experiments.*

One of the variables between the generation of apoptotic Jurkat cultures and apoptotic MØ/FC cultures is the use of PMA to differentiate monocyte-like THP-1 to MØ-like cells prior to inducing apoptosis. Although PMA containing medium is replaced at 96 hours pre-UV exposure, it possible that residual PMA in preparations of THP-1 derived apoptotic cells (aMØ/aFC) may be the cause of increased TNF-α release following HMDM exposure to aMØ or aFC. HMDM were cultured in the presence of PMA at 250nm (concentration used to generate MØ), 25nm and 2.5nm. In case PMA in the presence of AC induces TNF-α release, HMDM were cultured in the presence of PMA at 250nm, 25nm and 2.5nm and non-inflammatory aJK. Preliminary results (Fig. 25) suggest that assay relevant concentrations of PMA do not cause TNF-α release in HMDM in the presence or absence of aJK, and thus suggest that the pro-inflammatory responses of HMDM to aMØ or aFC are the result of HMDM:AC interaction.

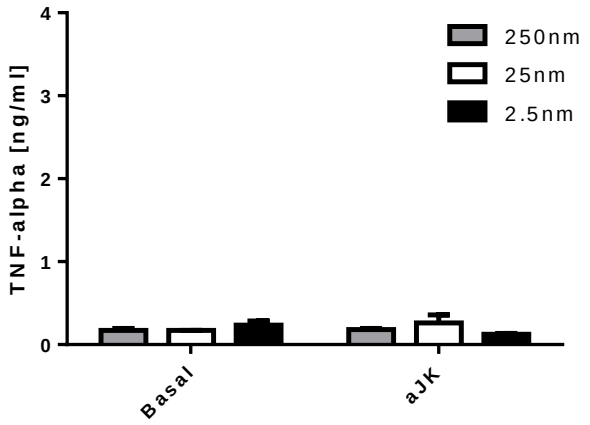


Figure 25. <u>Preliminary data</u>: TNF-α response to apoptotic macrophages and foam cells is not due to residual PMA exposure *HMDM generation were co-cultured with CDMØ-medium + PMA (indicated concs.) or aJK (6h post UV) + PMA (indicated concs.) (20h; 37C). 10% v/v NHS (final conc.) was added to relevant wells and incubated (4h; 37C). Samples were diluted 1/5 in sfRPMI prior to ELISA analysis. TNF-α ELISA was carried out as per manufacturer's instructions (PeproTech Ltd). Data shown is mean ± SD of 2 independent experiments.*

To dissect the source of increased HMDM TNF-α release in response to aMØ/aFC, preliminary work investigated whether apoptotic cells, or released microparticles/soluble factors induced TNF-α release. Culture medium was replaced at the point of UV exposure and HMDM were treated with either AC-derived cell-free supernatants (CFS) or ACs 16-20 hours post UV. Preliminary data show that both AC and EVs or soluble factors in AC supernatants are capable of inducing TNF-α release by HMDM (Fig. 26). Further study is required to confirm initial findings.

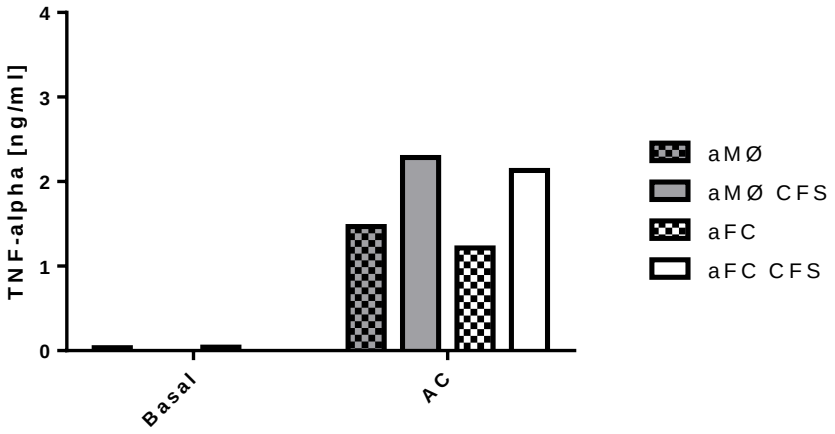


Figure 26. <u>Preliminary data</u>: HMD-MØ TNF-α response to apoptotic cell-derived CFS *HMDM were co-cultured with CDMØ-medium, aMØ and aFC whole culture or aMØ and FC cell-free supernatant (CFS) (centrifugation 350g; 6m) (16-20h; 37C). 10% v/v NHS (final conc.) was added to relevant wells and incubated (4h; 37C). Samples were diluted 1/5 in sfRPMI prior to ELISA analysis. TNF-α ELISA was carried out as per manufacturer's instructions (PeproTech Ltd). Data shown represents 1 experiment.*

4.3. Discussion

Trying to establish the main drivers of inflammation in atherosclerosis is a key target when considering future therapy and disease management. Inflammation within the plaque is now an established component of atherosclerosis, and evidence also points towards systemic inflammatory markers as indicators of disease[58,354,355]. Persistence of apoptotic cells could be exacerbating inflammation, as uncleared cells can become secondary necrotic[110,356], causing autolysis and outflow of inflammatory intracellular components[207].

The results in this chapter show that in the THP-1 model, basal levels of pro-inflammatory cytokine production (TNF-α) are the same in MØ and FC, indicating that FC are not constitutively pro-inflammatory. Responses to pro-inflammatory stimulus LPS were also the same in MØ and FC, indicating that FC have functional cytokine responses. Neither aMØ nor aFC initiated an inflammatory response when interacting with THP-1 MØ or FC, and both AC types had the ability to switch of an inflammatory response. MØ and FC retained the ability to switch from an LPS-induced pro-inflammatory phenotype, to an anti-inflammatory phenotype following AC interaction.

Conversely, HMDM responded in an inflammatory manner to aMØ and aFC, but not to aJK. aMØ and aFC also exacerbated LPS-induced TNF-α release in HMDM, whereas this response was dampened with aJK interaction. Preliminary studies suggest that both interaction with aMØ/aFC and aMØ/aFC-derived MPs and soluble material can induce inflammatory responses in MØ/FC. This could be an important inducer and driver of inflammation in the atherosclerotic plaque, where aFC and aMØ are abundant.

	Phagocyte		
Apoptotic cell	**THP-1 macrophage (MØ)**	**THP-1 foam cell (FC)**	**HMDM**

Apoptotic MØ (aMØ)	aMØ do not induce an inflammatory response in MØ and aMØ can switch off a TNF-α response in the THP-1 model	aMØ do not induce an inflammatory response in FC and aMØ can switch off a TNF-α response in the THP-1 model	aMØ strongly induce TNF-α release in HMDM, and exacerbate LPS-induced TNF-α release
Apoptotic FC (aFC)	aFC do not induce an inflammatory response in MØ and aFC can switch off a TNF-α response in the THP-1 model	aFC do not induce an inflammatory response in FC and aFC can switch off a TNF-α response in the THP-1 model	aFC strongly induce TNF-α release in HMDM, and exacerbate LPS-induced TNF-α release

Table 6. Summary of results from key research questions addressed in Chapter 4

4.3.1. Inflammatory status of foam cells

Few studies have addressed mechanisms behind the persistence of inflammation from the point of the macrophage/foam cell, either live or apoptotic, despite the heavy presence of apoptotic cells and phagocytes, a combination shown to dampen inflammation in a non-plaque environment[54-56].

Secretion of pro-inflammatory cytokines TNF-α and IL-12, markers of classical MØ activation, and IL-10, a regulatory cytokine and proposed marker for alternative MØ activation, was investigated at basal levels in the THP-1 model, with and without oxLDL pre-treatment[49]. This was to determine whether lipid-loading MØ induces an inflammatory phenotype in the THP-1-derived MØ model. Inflammatory cytokines drive macrophages to an 'M1-like' phenotype, which can increase pro-inflammatory cytokine output and reduce the rate of apoptotic cell uptake, or efferocytosis[234,357]. TNF-α has the ability to drive inflammation, and has been found in human atherosclerotic lesions[137,220-222]. Results shown here (figs. 16/18) indicate that macrophages and foam cells secrete comparable basal levels of cytokine TNF-α, suggesting similar basal levels of inflammation when comparing macrophages before and after lipid loading.

IL-12 is another inflammatory cytokine and is a suggested marker for classical MØ activation in conjunction with IL-10, which is a regulatory cytokine and suggested marker of alternative MØ activation[49]. Trends in IL-10 and IL-12 release could not be determined due to variability in results (figs. 17/19). IL-12: IL-10 dysregulation in foam cells could be a point of further study. Studies in primary human monocytes found oxLDL could induce IL-10 and IL-12 release, and that these cytokines were cross-regulatory[228]. Studies with murine bone marrow-derived MØ showed apoptotic neutrophils could inhibit LPS-induced IL-10 and IL-12

release, however secretion was not detected after 4 hours LPS exposure, which is the time period used in these studies[358].

A further hypothesis is that foam cells are hyper-inflammatory in the presence of an inducer of inflammation (i.e. a pro-inflammatory stimulus, e.g. LPS), when compared to non-lipid-laden MØ, as previous studies have implicated infection as a contributor of inflammation in atherosclerosis[359]. LPS and minimally modified LDL (mmLDL) have also been shown to cooperatively activate MØ to produce pro-inflammatory cytokines in murine models[360]. Experiments undertaken here to address this hypothesis reveal TNF-α secretion in response to LPS was comparable (Fig. 21) or lower (not significant; Fig. 19) in FC compared to MØ, suggesting FC are not hyper-inflammatory in the presence of immunogenic stimuli. IL-10 and IL-12 secretion was very low in comparison, and variability of results was high (figs. 17/19). A larger sample size or more sensitive detection method may be required. In conclusion, the mechanism driving the inflammatory status within the atherosclerotic plaque is either not the result of inappropriate phagocyte stimulation or it cannot be modelled in this THP-1-derived phagocyte model.

Interestingly, minimally modified LDL (mmLDL), often referring to mild oxidation methods including short-term copper sulphate oxidation, or fibroblast co-culture, is a potent inducer of inflammation via TLR2/4 activation, resulting in IL-1β, IL-6 and IL-10[323,324,360]. mmLDL is an subjective term that has not been given a solid definition, however it would be interesting to question whether mmLDL is a more or less physiologically relevant model of subendothelial LDL accumulation, or whether is a likely to be a combination of various LDL modifications present. oxLDL in this model could be better defined, however a lack of modified LDL-induced inflammation would suggest the oxLDL in this model is truly oxidised, and not ‘minimally modified’, LDL.

At a technical level, it is possible that differences in live cell numbers between macrophages and foam cells could mask true differences in cytokine output, but taken together with oxLDL toxicity results (Chapter 3; Fig. 12) and microscopic observation this is not thought the be the case in this model.

4.3.2. Cytokine responses by THP-1-derived macrophage and foam cell models to apoptotic cells

An unanswered question is whether foam cells and non-lipid-laden MØ respond to apoptotic cells in the same manner. Experiments in this chapter aim to address whether lipid-loading of phagocytes affects the resulting immune modulation seen in response to apoptotic cells.

In this model, results have shown that MØ-derived THP-1, a human immortal cell line, respond in a non-inflammatory nature to UV-induced apoptotic Jurkat, a well-established model for apoptotic cell clearance[54], with regards to TNF-α release (Fig. 19). aJK also had the ability to dampen an LPS induced TNF-α release (Fig. 19). This supports data by Voll *et al.*[55], which demonstrated reduced TNF-α, IL-12 and IL-1β secretion by peripheral blood monocytes in response to LPS in the presence of apoptotic peripheral blood lymphocytes, and an increase in IL-10 secretion, a regulatory chemokine. Fadok *et al.*[56] also found reduced inflammatory mediator release in response to apoptotic neutrophils, including IL-1β, IL-8, GM-CSF, leukotriene C_4, thromboxane B2 and TNF-α, but conversely found a reduction in IL-10 secretion by human monocyte-derived macrophages. Upregulated secretion of TGF-β, PGE2 and PAF were also found[56].

Foam cells also responded in a non-inflammatory manner to apoptotic Jurkat cells, as seen with MØ, when observing TNF-α release, indicating a 'normal' phagocyte response to apoptotic Jurkat (Fig. 19). Interestingly, FC-mediated TNF-α release in response to LPS appeared to be switched off less effectively than in MØ when co-cultured with apoptotic Jurkat (Fig. 19), however only non-lipid-laden macrophages showed a significant reduction in TNF-α secretion in response to LPS with AC pre-treatment. This could be to the apparent reduction in TNF-α secretion in response to LPS by FC, a difference which was not significant. Increased sample size may clarify these results. FCs appear to be switched off more easily than MØ by aMØ and aFC with regards to LPS-induced TNF-α response.

Little research has focused on the role of the apoptotic cell in studies on defective AC clearance, and a variety of AC models have been utilised. It is unclear whether aMØ and aFC are both able to induce an anti-inflammatory response in phagocytes, or if lipid-loading prior to apoptosis affects subsequent modulation of immune responses. The THP-1 cell line provides a good human MØ model, which can be lipid-loaded and induced to apoptosis to investigate cytokine responses as may occur within the plaque environment.

Using the THP-1 model, MØ and FC TNF-α release is low, and comparable to responses with aJK co-culture, following co-culture with apoptotic MØ (aMØ) and apoptotic FC (aFC) (Fig. 21). aMØ and aFC were both able to suppress LPS-mediated TNF-α release on MØ and FC. This suggests that FC are able to switch between classical and alternative activation, and equally that aFC are capable of switching off an inflammatory response, as observed with aJK interaction (Fig. 19). Li *et al.* [210] observed TNF-α and IL-1β production, but not IL-10 or TGF-β, by murine peritoneal MØ following interaction with free cholesterol-induced apoptotic macrophages (acetylated LDL + ACAT inhibitor), rather than the anti-inflammatory response associated with AC clearance. The same study investigated

interaction and immune modulation following ox-LDL induced aMØ and non-lipid-loaded UV-induced aMØ; comparable interaction was reported with all 3 AC types, however anti-inflammatory responses were observed with oxLDL and UV-induced aMØ, but data was not shown[210].

Results in the current study were variable with regards to MØ and FC IL-10 and IL-12 release, so clear trends could not be distinguished (figs 17/19).

4.3.3. Cytokine responses by primary human macrophages to apoptotic cells

Previous studies have used primary human monocytes to investigate the effects of oxLDL on inflammatory cytokine output[228], however studies of human cytokine responses to apoptotic cells, particularly apoptotic foam cells, are lacking. To investigate whether results are applicable to a primary human cell model, monocytes were isolated from peripheral blood from healthy volunteers and allowed to differentiate to human monocyte-derived MØ (HMDM) over a period of 7-14 days. HMDM were co-cultured with or without AC models used in the THP-1 model, and stimulated with LPS to look at AC-induced knockdown of an inflammatory response.

Results showed the same non-inflammatory response observed in primary MØ as in the THP-1 model with aJK co-culture (Fig. 23), and a reduction in LPS-induced TNF-α release following co-culture with aJK. However, primary MØ interaction with aMØ and aFC resulted in TNF-α release much greater than LPS-induced responses (Fig. 23). This also meant a lack of knockdown, only exacerbation, of an LPS-induced TNF-α response. This supports aspects of the study by Li *et al.,* discussed briefly above, which observed TNF-α and IL-1β release from murine peritoneal MØ following interaction with cholesterol-induced apoptotic macrophages, rather than the anti-inflammatory response associated with AC clearance[210]. A murine study also found that apoptotic T cells release TGF-β, which may account for modulation of immune responses here[361]. In contrast, anti-inflammatory effects were reported with oxLDL-induced and UV-induced murine peritoneal MØ (data was not shown)[210]. This further demonstrates a potential role for the AC-induced inflammation within plaque, and also the role of persistent ACs in driving inflammation should plaque ACs prove to be inflammatory. Khan *et al.*[197] also found pro-inflammatory cytokine release by murine peritoneal MØ following exposure to UV-induced apoptotic vascular smooth muscle cells (VSMCs) in the presence of oxLDL, so a combination of oxLDL and ACs may trigger a pro-inflammatory response in MØ, however the mechanisms are yet to be elucidated. Studies in this chapter only involve oxLDL pre-incubation, without oxLDL presence during phagocyte:AC co-culture. Studies on primary murine cells and human coronary artery endothelial cells (HCAECs) found that ACs with oxidation-specific epitopes were

inflammatory[209]. Thus the results presented here are consistent with previous work that lipid-laden apoptotic cells may represent a pro-inflammatory stimulus to primary macrophages and that this may contribute to the inflammatory nature of the plaque.

Further studies were undertaken to try and dissect the true physiological response of HMDM to aMØ and aFC, and to attempt to rule out other influencing factors. The obvious variable in Jurkat culture and THP-1-derived aMØ generation is the use of PMA. To ensure PMA is not the driver of inflammatory responses observed with HMDM: aMØ/aFC co-culture, HMDM were co-cultured in the presence or absence of non-inflammatory aJK, with relevant dilutions of PMA (Fig. 25). Preliminary data shows PMA does not induce TNF-α release in HMDM in the presence or absence of non-inflammatory ACs. Another possible explanation of these divergent results, compared to the THP-1 model, in HMDM could be the presence TNF-α in AC preparations. This could be derived from intracellular cytokine release induced passively via membrane disruption, e.g. secondary necrosis, or actively via contamination prior to apoptosis induction, e.g. bacterial or PAMP contamination. It is unlikely to be oxidised LDL contamination as aMØ elicit a TNF-α in HMDM to the same extent as aFC, and limulus assay testing was negative on a random selected oxLDL sample (data not shown). Contamination is also less likely if you consider non-inflammatory results in THP-1 MØ in response to the same preparations (Fig. 21). To ensure detected TNF-α was not already present in assay preparations, aMØ, or NHS and LPS, preparations were assayed for TNF-α following full incubation times, in the absence of HMDM, and presence of TNF-α was not at significant levels (Fig. 24).

The presence of secondary necrosis has not been ruled out in this model of aMØ and aFC, however an inflammatory reaction was not seen in co-culture with THP-1-derived MØ. Simple studies on membrane integrity would provide supporting evidence on the presence or absence of secondary necrosis in this model.

The opposing results found when comparing THP-1 MØ and HMDM TNF-α response to THP-1 derived aMØ and aFC models shows the care that needs to be taken when defining models and comparing results not only across species, but between immortal cell lines and primary cells of the same species. Models of atherosclerosis vary widely, without much consensus on the best way to observe processes within the plaque. It could be argued that the use of human primary cells may be the closest model we can use when trying to understand failures in this disease.

It is of interest to note that as such an effect is not seen with THP-1-derived MØ/FC, suggesting that comparison of THP-1-derived and primary macrophages may identify key mediators that underlie the undesirable pro-inflammatory responses. Such mediators may

represent attractive targets for potential therapeutic intervention in atherosclerosis. A drawback of the primary cell research in this chapter, is that cell line-derived ACs were used due to the large numbers of ACs required for assays compared to phagocytes (10X). However it is a key starting point in human cell-orientated research into aFC modulation of immune responses in the atherosclerotic plaque, as current research has only been shown in murine models.

4.3.4. Further considerations

To further dissect the source of increased HMDM TNF-α release in response to aMØ/aFC, preliminary work investigated whether apoptotic cells, or released microparticles/soluble factors, induced TNF-α release. HMDM were co-cultured with whole aMØ/aFC cultures, or cell free supernatants from the same cultures (CFS), over the same time course as previous assays. Preliminary results (Fig. 26) suggest that aMØ/aFC cell free supernatant (CFS) induces more TNF-α release than aMØ/aFC whole cell culture in HMDM. This could suggest than aMØ/aFC could still have some anti-inflammatory properties, as the presence of AC in the culture reduces TNF-α release, but mediators in cell-free supernatant cause an inflammatory response (Fig. 26). Further research is required to confirm these observations, and to dissect which mediators are causing an inflammatory response. The effects of AC-derived microparticles (MPs), or blebs, on immune-modulation in the plaque would be an interesting angle, as MPs they are individual to the originating cell, and can confer messages, e.g. inflammatory/anti-inflammatory mediators, to surrounding cells[268]. MPs have been found in human atherosclerotic plaques, and are thought to be primarily derived from leukocytes[269]. MPs also have a role in monocyte chemoattraction, another pathological feature in plaque progression[60,61,71]. It is unknown whether aFCs for example, confer 'find-me' signals, for phagocytic clearance. This may not be relevant in the models of interaction used in this chapter as a large excess of ACs are loaded onto phagocytes, however consequences in the plaque environment should be considered. If aFCs are inflammatory, persistence in the plaque would exacerbate inflammation.

It is important to note that although the plaque contains many MØ and MØ-derived foam cells, as is the definition of foam cells in this model, the complex plaque environment includes a variety of cell types including endothelial cells (ECs), smooth muscle cells (SMCs), monocytes, mast cells, T lymphocytes and SMC-derived foam cells, including live, apoptotic and necrotic cells[56,57]. It is also important to consider if apoptotic foam cells elicit the same response in phagocytes as do non-lipid-loaded ACs. Do foam cells release comparable find-me signals? This may not be relevant in the model of interaction used in this chapter as a large excess of ACs are loaded onto phagocytes.

4.4. Conclusions and future work

In conclusion, evidence in this chapter shows that lipid-loading does not induce TNF-α release in THP-1-derived MØ, but this needs to be confirmed by generation of foam cells using HMDM, especially given the opposing results observed following AC interaction. Foam cells may be hyper-inflammatory with regards to IL-10 and IL-12 release, however this requires further study with an increased sample size or more sensitive detection method, e.g. via multiplex assays. This would allow a full spectrum of cytokine release to be established in cell line and primary cell models of foam cells. Broader cytokine profiling may also provide further evidence as to whether alternative or classical MØ activation is favoured in these conditions, including TGF-β, which was the aim with the range of cytokines chosen in these studies.

That aMØ can induce inflammation in HMDM is a key observation that, with further investigation, could advance understanding of the persistent inflammatory environment in human atheroma. As clearance of a high density apoptotic MØ is not a common occurrence, and MØ are not an easy AC type to model due to adherence, they are an understudied AC type. It would be interesting to further dissect the differences in THP-1-derived MØ and HMDM responses to apoptotic MØ and apoptotic FC. Generation of human monocyte-derived FC, aMØ and aFC would further complete the model. It could then be established whether immortal cell line-derived AC models are physiologically relevant, or whether it is just a quirk of the assay in question.

Profiling of aMØ/aFC supernatants could also advance knowledge in the area, to further dissect the roles of human cytokine and MP release by apoptotic foam cells. THP-1 derived microparticles were recently shown to promote inflammation and atherogenesis in a murine model of atherosclerosis[282].

Mechanisms of chemotaxis to apoptotic cells

5.1. Introduction

Chemotaxis is the directional movement of cells up a concentration gradient of an attractive agent[240]. Chemotaxis is important in recruitment of immune cells to points of damage, inflammation and, of particular interest to this project, pathological sites such as the atherosclerotic plaque[362,363]. Here, circulating monocytes are recruited from the blood stream by upregulated expression of adhesion molecules, such as selectins, on endothelial cells overlying the plaque[157,250]. The plaque is a highly inflammatory environment[58] with a lot of cell death[46], both factors which are known to result in monocyte recruitment[43,362]. Torr *et al.* showed a role for find-me signal ICAM-3 in recruitment of THP-1-derived monocytes to apoptotic cells, with evidence strongly suggesting ICAM-3 release via shedding of AC-derived MPs[71]. Another find-me signal, CX3CL1, has been demonstrated to recruit HMDM to apoptotic cells, with MP association also shown[61].

The role of apoptotic cell-derived ICAM-3 in recruitment of phagocytes to apoptotic cells has only recently been shown[71], and was initially identified as a mediator in intercellular adhesion between viable cells[364]. In AC clearance, ICAM-3 is thought to be modified, in as yet an undefined manner, to act as an 'eat-me' flag on the surface of apoptotic cells[84]. As a murine homologue has never been identified, studies of ICAM-3 are restricted to human cells, hampering the understanding of the functions of this molecule[80]. This chapter aims to further dissect the role of ICAM-3, which has currently been shown in monocyte recruitment to apoptotic lymphocytes (i.e. B and T cells)[71], in the recruitment stages of apoptotic cell clearance. It is not currently known whether ICAM-3 plays a role in recruitment to other leukocytes undergoing cell death, or in other pathological situations.

Since identification of the role of CX3CL1 in monocyte capture, adhesion to endothelium and activation under flow conditions[68], and discovery of expression in the plaque[365], this chemokine has been implicated heavily in development and progression of atherosclerosis. CX3CL1 expression has been found in atherosclerotic plaques[365,366] and has been implicated in CX3CR1-positive monocyte recruitment to the plaque[239]. Recruitment of monocytes is one of the key pathological events in plaque progression, with blockade of recruitment shown to improve disease state[156]. This chapter will further investigate the role apoptotic cell-derived CX3CL1 in monocyte recruitment to dying cells, with emphasis on CX3CL1 derived from 'plaque relevant' cells, apoptotic macrophages (aMØ) and apoptotic foam cells (aFC).

CD14 is a tethering receptor shown to mediate AC clearance[31], however its role in 'find-me' signal detection and subsequent monocyte migration towards apoptotic cells has not been assessed. CD14, the prototypic LPS receptor[367], has also been implicated in AC clearance[31],

suggesting it has the ability to produce opposing ligand-dependent immunological outcomes. Alongside signalling partner TLR4[368], CD14 interaction with LPS results in a pro-inflammatory immune response, whereas interaction of CD14 with ACs results in a non- or anti-inflammatory outcome[312]. MPs are important in monocyte/MØ recruitment to dying cells, but whether effects are exerted via binding to known tethering receptor CD14 is not known**.** Tethering of MPs via CD14 may result in MØ activation and migration toward dying cells, therefore the role of CD14 in plaque-associated monocyte recruitment will be investigated in this chapter.

This chapter will address the establishment of a horizontal chemotaxis model to be used to observe chemotaxis towards agents including AC-derived MPs and soluble factors. Blocking antibodies against target molecules with known roles in AC clearance will be utilised to establish some of the key molecular players in aMØ and aFC clearance. In previous studies, vertical chamber models have been used[61,71], but this project seeks to establish a model that is not influenced by gravity, and that measures chemotaxis along a true gradient rather than migration down a step gradient to a pool of attractant. Horizontal models of chemotaxis could therefore be more physiologically relevant.

In order to develop the model further from a physiological perspective, a vertical transwell system was used but with the additional barrier of endothelial cells. HUVECs were used as the model endothelial cells of choice. The downstream effects AC-derived MPs exert on endothelial cells in the form of selectin expression was also investigated.

The hypothesis covering this chapter is that in an aged environment (e.g. lipid-laden), monocytes are inappropriately recruited to sites of cell death. Research questions posed in the table below will be addressed in order to investigate this hypothesis.

	Phagocyte
Apoptotic cell	**Monocyte**
Apoptotic MØ **(aMØ)**	How well do non-lipid laden MØ attract monocytes via 'find-me' signals? Are these mechanisms, CD14, ICAM-3 or CX3CL1

	mediated?
Apoptotic FC **(aFC)**	Does lipid-loading enhance or reduce attractiveness of 'find-me' signals to monocytes? Are these mechanisms, CD14, ICAM-3 or CX3CL1 mediated? Does lipid-loading alter effector molecules?

Table 7. Summary of key research questions addressed in Chapter 5

5.2. Results

5.2.1. Modelling chemotaxis using a vertical chamber system

To establish whether microparticles (MPs) from plaque-relevant apoptotic cells (AC) induced monocyte migration, a vertical 48 well chemotaxis chamber (Neuroprobe Inc., Gaithersburg, Madison, WI, USA) was used in preliminary experiments (Fig. 27). In this assay, monocytic VD3 stimulated THP-1 migration was measured from an upper chamber, towards a pool of sfRPMI (Control), apoptotic macrophage (aMØ)-derived cell-free supernatant (CFS), or apoptotic foam cell (aFC)-derived cell-free supernatant (CFS). CFS is generated by centrifugation of whole cell supernatants for 6 minutes at 350xg, pelleting any cell bodies or large cell debris.

Figure 27 shows minimal migration towards medium alone (control), and comparatively increased migration towards aMØ-derived CFS, however this was not significant when compared to control wells ($P>0.05$). THP-1 cells migrated toward aFC-derived CFS in a significant manner when compared to migration toward control and aMØ-derived CFS ($P<0.05$).

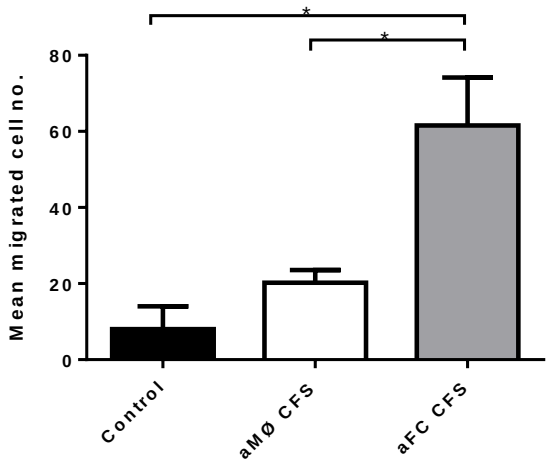


Figure 27. Apoptotic foam cell-derived cell-free supernatants induce monocyte migration *VD3 stimulated THP-1 in sfRPMI were allowed to migrate*

*to aMØ and aFC cell-free supernatants (CFS) or sfRPMI control (4h; 37C). Following migration, the polycarbonate membrane was stained and migrated cells per high power field (HPF)(40X) on the underside of the membrane quantified by light microscopy. Four replicate wells and five HPFs of view per well were assessed. Data shown are mean migrated cell number per HPF ± S.E. of 3 independent experiments. *P<0.05 one way ANOVA with Tukey's* post-hoc *test.*

5.2.2. Dunn chamber optimisation

aFC-derived CFS was shown to induce significant monocyte migration in a vertical assay system. Next, a horizontal system was established to observe monocyte migration along a true chemotactic gradient, based on methods outlined by Zicha *et al.*[292].

THP-1, VD3, PMA and DS cells were cultured on glass coverslips at a range of cell densities and mounted onto Dunn chambers containing sfRPMI or chemically defined (CD)-MØ medium in the inner well, and 100ng/ml MCP-1 diluted in sfRPMI or CD-MØ medium subsequently added to the outer well. This concentration was chosen as VD3 stimulated monocyte chemotaxis to 100ng/ml MCP-1 was observed in preliminary studies using the vertical assay system. Dunn chambers were sealed and placed in a humidified chamber at 37C, and cell migration visualised using time-lapse video microscopy. Images were taken every 10 minutes over a 48 hour time period.

Results showed that although THP-1 adhered to coverslips, adherence was weak and few, if any, THP-1 cells remained on coverslips following mounting onto Dunn chambers, despite increasing seeding density. More VD3 cells remained on coverslips following transfer onto Dunn chambers and some migration was observed in the presence of protein-free CD-MØ medium, though cell numbers were very low. Chemotaxis was not observed when sfRPMI was used in the assay and as a diluent for MCP-1. PMA and DS differentiated THP-1, and human peripheral blood monocytes, are strongly adherent and remained on coverslips following transfer onto Dunn chambers, however migration was not observed in either cell type. Interestingly, PMA and DS cells appeared to show signs of attempted migration towards MCP-1, but appeared to be anchored too firmly to enable migration (Fig. 28). PMA and DS cells were cultured on plastic coverslips to investigate whether this would result in weaker adherence but the same results were observed. Peripheral blood monocytes were mounted on coverslips and used in the Dunn chambers between 20 minutes and 24 hours post isolation but were also too strongly adherent for migration.

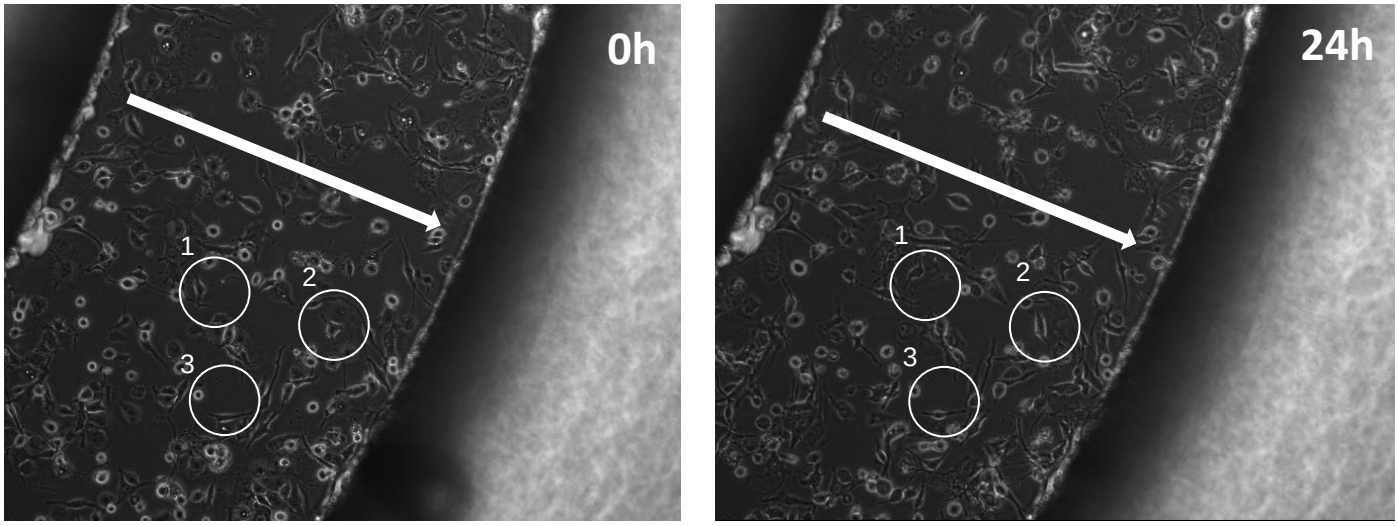


Figure 28. Migration of PMA cells towards MCP-1 *THP-1 monocytes were cultured with PMA on glass coverslips (72h; 37C). Chemically-defined (CD)-MØ medium was placed in the inner well of the Dunn chamber and 100ng/ml MCP-1 in CDMØ-medium was placed in the outer well, providing an MCP-1 gradient between the wells. Coverslips were mounted over the wells and monocyte migration monitored using time-lapse video microscopy. Arrows represent predictive direction of movement based on MCP-1 gradient (low to high). Individual cells are circled for comparison at 1h and 24h.*

Taking these results forward, the VD3 model was chosen for future migration studies due to their monocytic phenotype and ability to adhere and migrate, and adherent cell numbers were increased by raising the seeding density of cells and coating the coverslips in poly-D-lysine (PDL) prior to seeding of cells. Preliminary studies were carried out to investigate a range of concentrations of MCP-1 to promote maximal migration by VD3 stimulated THP-1 cells and 100ng/ml was found to induce robust monocyte migration (data not shown).

5.2.3. Modelling horizontal THP-1 chemotaxis to MCP-1

VD3 stimulated monocytes cultured on PDL-coated glass coverslips were visualised moving across a bridge between the inner well (no attractant) and outer well (attractant) of a Dunn chemotaxis chamber. Migration was recorded using the manual tracking plugin in Image J. This plugin records coordinates from each cell and data can be imported into the Ibidi Chemotaxis and Migration Tool (V2.0) to obtain normalised plots. This tool normalises cells to the same starting point (centre of plot), and allows mapping of cell migration from this point. All axis on qualitative migration plots in this chapter are set to the same units (470x470µm). The Chemotaxis and Migration Tool generates plots and also extracts statistical data, which include directness, distance, velocity, angle, and a bespoke measure of migration termed forward migration index (FMI)[369].

The Chemotaxis and Migration Tool also incorporates the Rayleigh Test as a statistical test, which measures uniformity of a circular distribution of points, in this case the cell endpoints,

and takes into account distance from the origin[369]. The null hypothesis, that cells move in a uniform manner, is rejected when P>0.05.

Figure 29 shows non-directional movement to medium alone (C), also referred to as chemokinesis, whereas assays with MCP-1 (indicated plots) show directional migration towards MCP-1. Rayleigh test results (P values on plots) show cells move in a uniform manner toward MCP-1 in all assays. One control assay shows uniform movement, but in this case distance of cell migration was low. P values (on plots) using the Rayleigh test were extremely low in MCP-1 assays compared to control.

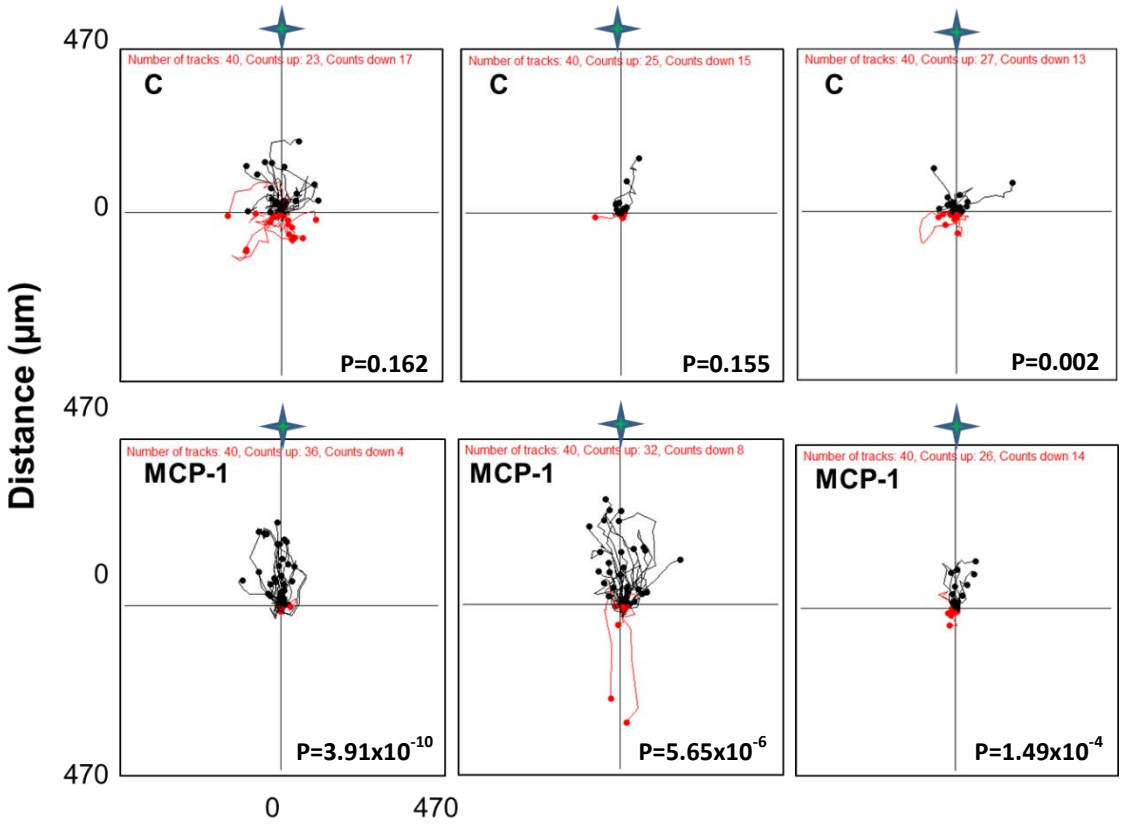


Figure 29. Monocytes migrate towards MCP-1 in the Dunn chamber chemotaxis model *VD3 stimulated THP-1 in chemically-defined (CD)-MØ medium were allowed to migrate to 100ng/ml MCP-1 or CD-MØ medium control (2h; 37C), with plots labelled MCP-1 and C respectively. Monocyte migration was monitored using time-lapse video microscopy. Migration of 40 cells per assay was measured using Image J and analysed using the Ibidi Chemotaxis and Migration Tool (V2.0). Data shown are representative of multiple independent experiments. Control n=3, MCP-1 n=4. P values for individual assays were obtained using the Rayleigh test, with a null hypothesis of non-uniform movement rejected at P<0.05.*

In order to assess statistical significance of movement across multiple assays, rather than individually, basic statistical data of assays (i.e. Euclidian distance, total/accumulated

distance, velocity and directness) shown in Figure 29 was extracted using the Chemotaxis and Migration Tool (Fig. 30). This data failed to show significant quantitative differences between control and MCP-1 directed migration, despite clear differences seen qualitatively (Fig. 29). This is because some monocytes moved in a kinetically similar manner in MCP-1 and control studies, however the direction the cells were travelling in was markedly different.

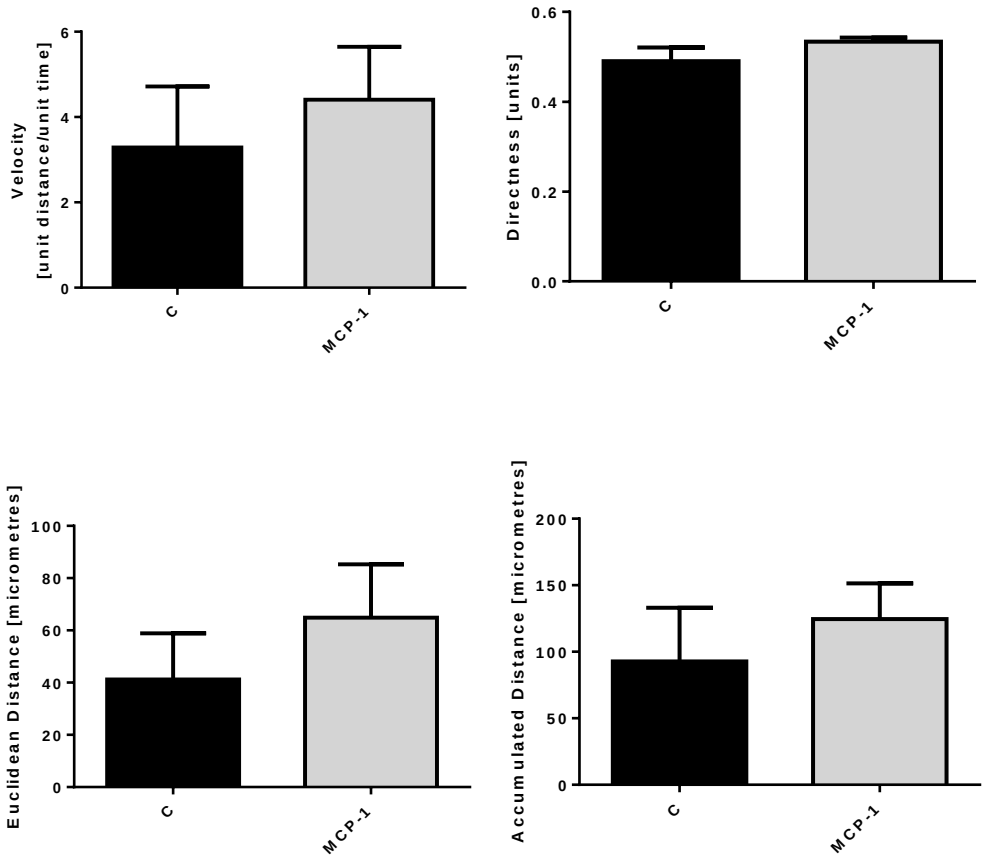


Figure 30. Quantitative measurement of monocyte migration *Extraction of quantitative data relating to monocyte directness, velocity, accumulated distance and Euclidean distance of assays shown in Fig. 29. Migration of 40 cells per assay was measured using Image J and analysed using the Ibidi Chemotaxis and Migration Tool (V2.0). Data shown mean ± SEM and are representative of multiple independent experiments. Control n=3, MCP-1 n=4. Data analysed using unpaired t test. P>0.05 in all parameters.*

5.2.4. Defining directionality

The measure of directness above (Fig. 30) shows how directly cells move between two points, i.e. from A to B, regardless of the location of 'B' in relation to the attractant. To show directionality towards an attractant, forward migration index (FMI) can be utilised.

FMI, an index provided in the Chemotaxis and Migration Tool, represents the efficiency of the forward migration of cells parallel (in this case the y axis; yFMI) and perpendicular (x axis;

xFMI) to the direction of the attractive gradient, according to variables including the distance travelled from the starting point and the location of the endpoint in relation to each axis (Fig. 31). Data is always set up so the chemoattractive gradient runs along the y axis. A high yFMI value (movement parallel to the attractive gradient), and a low xFMI value (perpendicular to the attractive gradient) indicates a powerful chemotactic effect[369]. This measurement takes into account positive and negative migration.

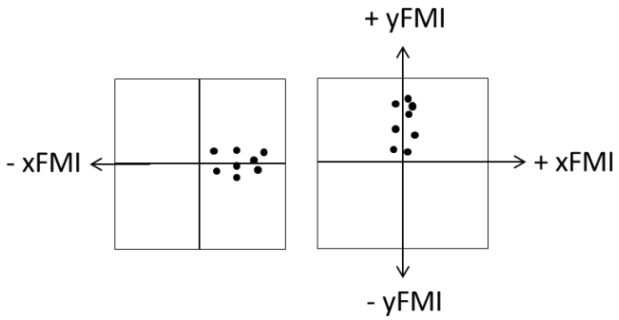


Figure 31. Schematic of monocyte migration plots showing cell endpoints in relation to forward migration index (FMI) *Cell endpoints are allocated an xFMI and a yFMI value according to variables including distance from starting point and location in relation to each axis. The left plot represents a high xFMI value and a low yFMI value (poor chemotaxis). The right plot represents a low xFMI value and a high yFMI value, indicating strong chemotaxis movement, parallel to the concentration gradient.*

An alternative method has also been utilised to extrapolate how well cells move toward an attractant. This method measures uniformity of movement of the monocyte population based on the location of the endpoint. This was calculated by extracting the statistical data from the Chemotaxis and Migration Tool based on the angle measurement. The angle measurement gives the location of each end point of each tracked migrating cell, where the attractant is placed at 0 degrees (top), and cells are normalised as migrating from the centre of the circle (Fig. 32). The standard deviation of the angle was used to show variability in each assay of the final destination of each migrating cell (SDoA). The more the end point of individual cells deviate from the other end points, the higher the SD value (Fig. 32a. and 29c.). This was used as a measure of uniform migration, as the more closely the monocytes move toward an attractant as a population, the lower the standard deviation will be of the angle of the end points. This was a means of showing quantitatively that cells move toward MCP-1 in a significant ($P<0.05$) uniform and directional manner (Fig. 33), corresponding with qualitative data (Fig. 29). Data show mean SD of angle measurements across at least 3 assays (± SEM), where a high value indicates more variability, and therefore less uniformity.

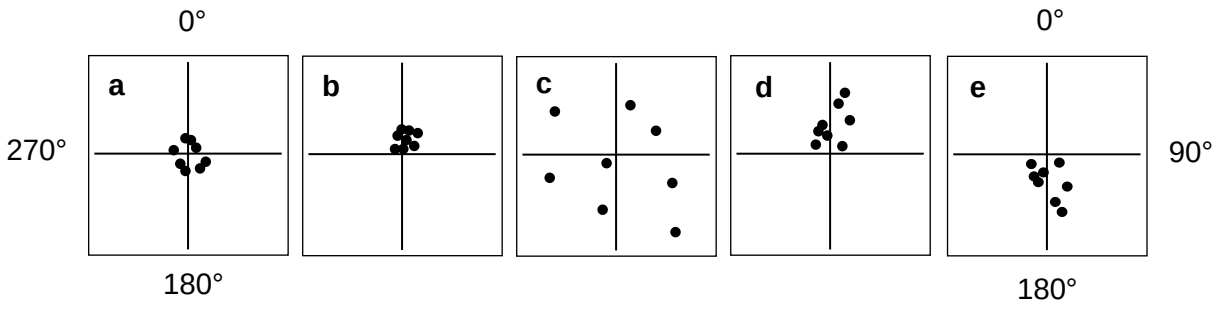


Figure 32. Schematic of monocyte migration plots showing cell endpoints in relation to angle values *Plots a) and b) show small and similar distances travelled, however based on angle measurements, plot a) will have high SD of angle (SDoA) measurement, and plot b) a low SDoA, as cell end points are less variable. Plot c) will have a comparable SDoA to plot a), and plot d) will have a comparable SDoA value to plot b). Plot e) will have the same SDoA value as plot d), as variability of endpoint values, rather than endpoint values themselves, are measured. This measure is a powerful tool to extrapolate uniformity of movement via variability of cell end points, but it does not take into account distance moved or whether direction is positive or negative, so measurements must be observed in conjunction with qualitative plots.*

Figure 33 shows extrapolated statistical data from plots shown in Figure 29. Using the forward migration index (FMI) (left hand graph), xFMI shows that the amount of directional cell migration perpendicular to the attractive gradient is comparable between control (no attractive gradient) and MCP-1 assays. yFMI values, the amount of directional migration parallel to the attractive gradient, are higher in MCP-1 assays, however this was not shown to be significant compared to control ($P>0.05$, 2 way ANOVA with Tukey's *post hoc* test for multiple comparisons).

When investigating variability of movement using SDoA of end points of cells (Fig. 33; right hand graph), results show statistically significant differences in variability between control and MCP-1 assays. SDoA is more variable in control assays, showing less uniform, or more direct, migration of cells. In assays with MCP-1, variability according to SD of angle is significantly lower ($P<0.05$), indicating more uniform migration (unpaired student's t test).

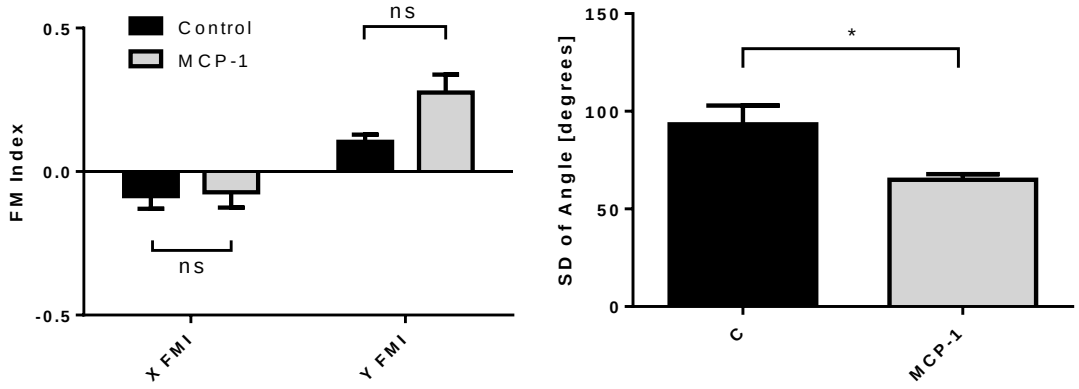


Figure 33. Monocytes migrate directionally towards MCP-1 *Extrapolating data from plots in Fig. 29, the left hand graph shows FMI values perpendicular (xFMI) and parallel (yFMI) to the attractive gradient, where present. FMI values were analysed using 2 way ANOVA with Tukey's* post hoc *test. The angle of each end point of migrating cells was assessed using an unpaired student's t test, P<0.05. Data shown are mean ± SEM of the deviation of migrations of multiple independent experiments. Control n=3, MCP-1 n=4.*

5.2.5. Characterising chemotaxis to apoptotic cell models

5.2.5.1. Chemotaxis to apoptotic Jurkat cells

The Dunn Chamber was used to assess horizontal migration of monocytes (VD3 stimulated THP-1) along a concentration gradient toward plaque-relevant (MØ and FC) conditioned medium. Initially, assays were carried out using an established model of cell death, UV-induced apoptotic Jurkat cells. Apoptotic Jurkat cells are an established inducer of monocyte attraction[54,63], therefore were used to set-up, and ensure robustness of, the assay.

Figure 34 shows representative plots of monocyte migration toward medium conditioned (16-20h) with live jurkat (whole culture; JK WC), live JK conditioned medium with cell bodies removed, leaving MPs and soluble factors (jurkat cell-free supernatant; JK CFS), UV-induced apoptotic jurkat conditioned medium (aJK WC), and UV-induced apoptotic jurkat conditioned medium with cell bodies removed (aJK CFS). All four plots show directional movement towards an attractive factor.

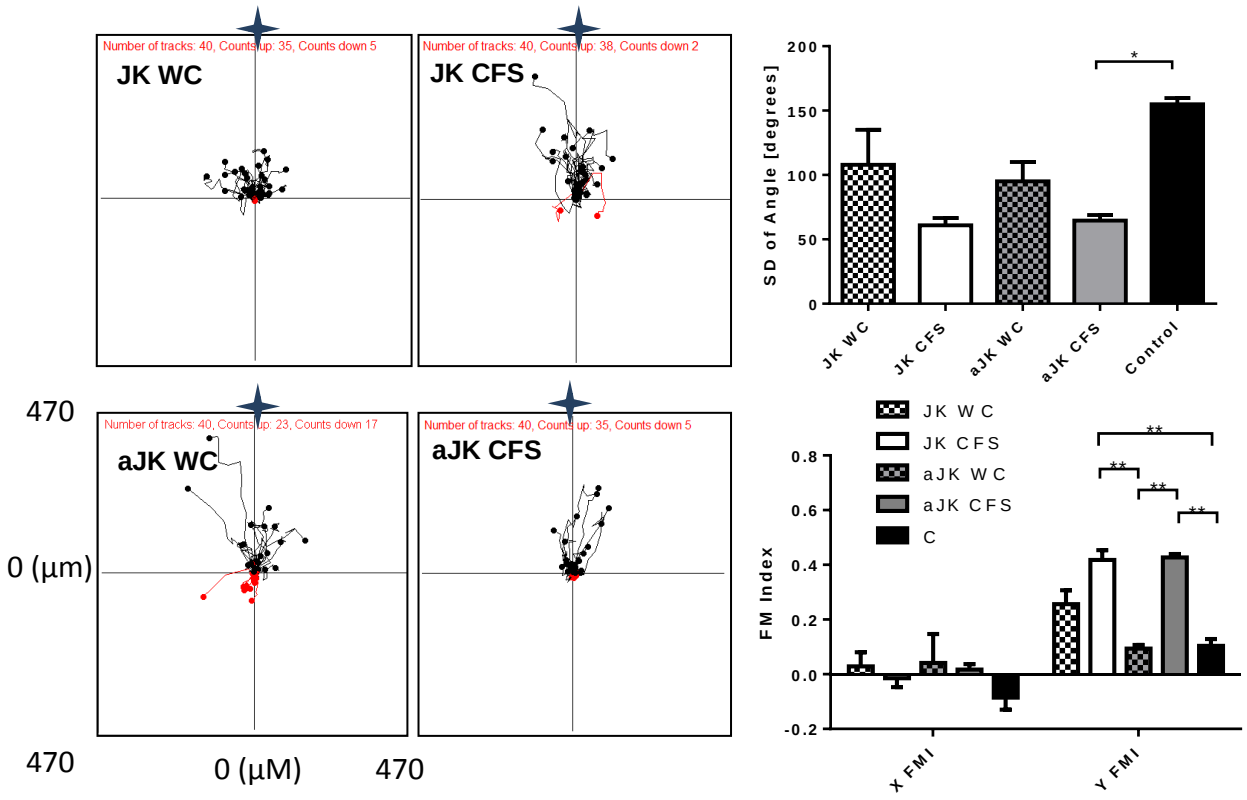


Figure 34. Monocytes migrate towards medium conditioned with live or apoptotic Jurkat cells *VD3 stimulated THP-1 in chemically-defined (CD)-MØ medium were allowed to migrate to live JK or aJK whole cell (WC) or cell-free supernatant (CFS) (2h; 37C). Monocyte migration was monitored using time-lapse video microscopy. Migration of 40 cells per assay was measured using Image J and analysed using the Ibidi Chemotaxis and Migration Tool (V2.0). Qualitative plots and FMI and SD of angle measurements are also shown. Quantitative data shows mean values ± SEM of 3 independent experiments. *P<0.05, **P<0.01 one way (SD of angle) or two way (FMI) ANOVA with Tukey's* post hoc *test.*

To look at directional migration quantitatively, forward migration index (FMI) and SD of angle measurements are compared using the Chemotaxis and Migration Tool (Fig. 34). When comparing migration perpendicular to the gradient of conditioned medium (xFMI), no significant differences in forward migration were found between conditions or compared to medium control (two way ANOVA with Tukey's *post hoc* test, P<0.05). Forward migration parallel to the conditioned medium gradient (yFMI) is significantly increased (P<0.01) toward both live and dead CFS compared to control. Forward migration to JK CFS and aJK CFS was also significantly increased compared to aJK whole culture (P<0.01).

When looking at SD of angle (Fig. 34), significant directional migration was only observed toward aJK CFS compared to control (P<0.05), and no significant differences were found between conditioned medium groups (one way ANOVA with Tukey's *post hoc* test). Overall,

the two approaches to assess directionality, FMI and SD of angle, broadly agree in their analysis of data.

5.2.5.2. Chemotaxis to apoptotic THP-1-derived macrophages

The Dunn Chamber was then used to assess horizontal migration of monocytes to MØ-conditioned medium. Monocyte infiltration into the arterial intima and differentiation to MØ is one of the initiating events in plaque formation[45], and further understanding of how and when MØ recruit further inflammatory cells to the plaque would be beneficial in understanding disease progression.

Figure 35 shows representative plots of monocyte migration toward medium conditioned with live MØ (MØ whole culture/ MØ WC), live MØ conditioned medium with cell bodies removed, leaving MPs and soluble factors (MØ cell-free supernatant/ MØ CFS), UV-induced apoptotic MØ conditioned medium (aMØ WC), and UV-induced apoptotic MØ conditioned medium with cell bodies removed (aMØ CFS). All four plots show directional movement towards an attractive factor.

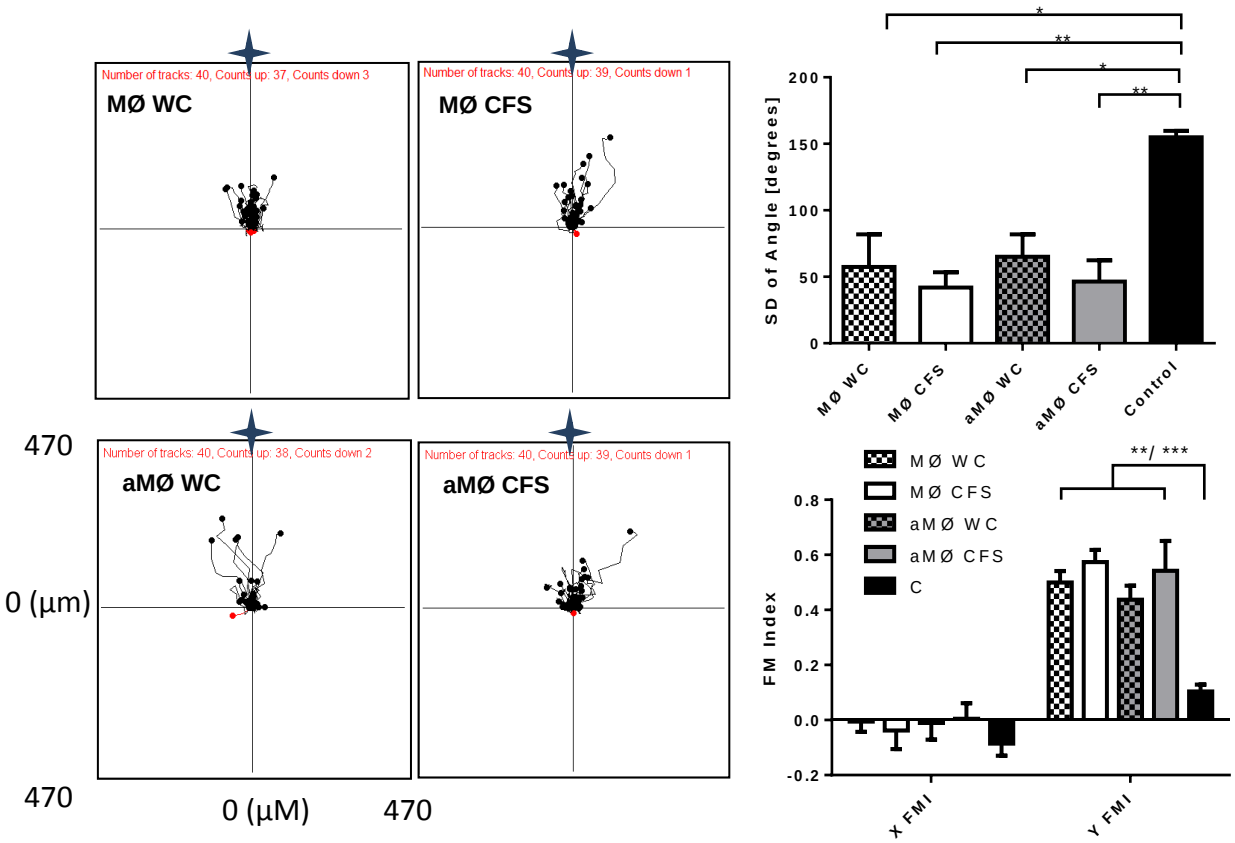


Figure 35. Monocytes migrate towards medium conditioned with live or apoptotic THP-1-derived macrophage cells *VD3 stimulated THP-1 in chemically-defined (CD)-MØ medium were allowed to migrate to live MØ or aMØ whole cell (WC) or cell-free supernatant (CFS)*

*(2h; 37C). Monocyte migration was monitored using time-lapse video microscopy. Migration of 40 cells per assay was measured using Image J and analysed using the Ibidi Chemotaxis and Migration Tool (V2.0). Qualitative plots and FMI and SD of angle measurements are also shown. Quantitative data shows mean values ± SEM of 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001 one way (SD of angle) or two way (FMI) ANOVA with Tukey's* post hoc *test.*

To look at directional migration quantitatively, forward migration index (FMI) and SD of angle measurements are compared using the Chemotaxis and Migration Tool. When comparing migration perpendicular to the gradient of conditioned medium (xFMI), no significant differences in forward migration were found between conditions or compared to medium alone control (P>0.05, two way ANOVA with Tukey's *post hoc* test). All MØ conditioned medium groups showed significant increases in forward migration parallel to the conditioned medium gradient (yFMI), compared to medium alone control, with aMØ WC at P<0.01, and MØ WC, MØ CFS and aMØ CFS at P<0.001 (two way ANOVA with Tukey's *post hoc* test). There were no significant differences in forward migration between conditioned medium groups.

When looking at SD of angle (Fig. 35), significantly decreased variation is seen when migration is toward MØ WC and aMØ WC compared to control (P<0.05), however variation when migration is toward MØ CFS and aMØ CFS show even greater significance when compared to control (P<0.01) (one way ANOVA with Tukey's *post hoc* test). Significant differences were not seen when comparing directional migration toward each different conditioned medium (P<0.05). Overall, the two approaches to assess directionality, FMI and SD of angle, broadly agree in their analysis of data.

Trends in yFMI and SDoA data indicate that soluble factors or MPs within CFS, from both live and dead MØ, are chemoattractive, and that the presence of cells dampens monocyte attraction by comparison.

5.2.5.3. Chemotaxis to apoptotic THP-1-derived foam cells

Next, the Dunn Chamber was used to show horizontal migration of monocytes to FC conditioned medium. MØ infiltration to the plaque is followed by lipid uptake and foam cell formation, leaving questions on the effects of lipid-loading on MØ function[162], many of which remain unanswered, including effects on further monocyte recruitment to the plaque and phagocyte recruitment to apoptotic foam cells.

Figure 36 shows representative plots of monocyte migration toward medium conditioned with live FC (FC whole culture/ FC WC), live FC conditioned medium with cell bodies removed, leaving MPs and soluble factors (FC cell-free supernatant/ FC CFS), UV-induced apoptotic

FC conditioned medium (aFC WC), and UV-induced apoptotic FC conditioned medium with cell bodies removed (aFC CFS). All four plots show directional movement towards an attractive factor.

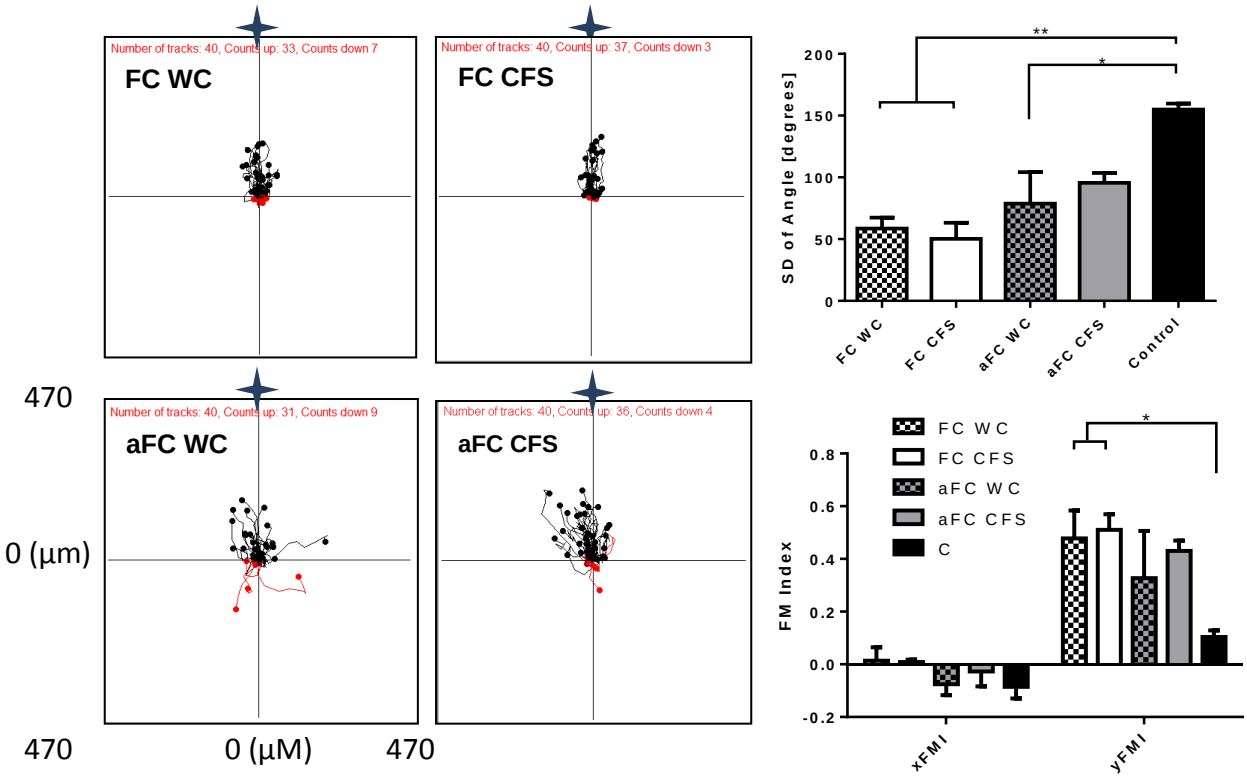


Figure 36. Monocytes migrate towards medium conditioned with live or apoptotic THP-1 foam cells *VD3 stimulated THP-1 in chemically-defined (CD)-MØ medium were allowed to migrate to live FC or aFC whole cell (WC) or cell-free supernatant (CFS) (2h; 37C). Monocyte migration was monitored using time-lapse video microscopy. Migration of 40 cells per assay was measured using Image J and analysed using the Ibidi Chemotaxis and Migration Tool (V2.0). Qualitative plots and FMI and SD of angle measurements are also shown. Quantitative data shows mean values ± SEM of 3 independent experiments. *P<0.05, **P<0.01, one way (SD of angle) or two way (FMI) ANOVA with Tukey's* post hoc *test.*

To look at directional migration quantitatively, forward migration index (FMI) and SD of angle measurements are compared using the Chemotaxis and Migration Tool (Fig. 36). When comparing migration perpendicular to the gradient of conditioned medium (xFMI), no significant differences in forward migration were found between conditions or compared to medium alone control (two way ANOVA with Tukey's *post hoc* test, $P<0.05$). Surprisingly, only migration toward FC WC and FC CFS showed significant increases in forward migration parallel to the conditioned medium gradient (yFMI), ($P<0.05$). There were no significant differences in forward migration between live or dead FC conditioned medium groups.

When looking at SD of angle (Fig. 36), significant directness was observed toward FC WC and FC CFS ($P<0.01$), and aFC WC ($P<0.05$), compared to control (one way ANOVA with Tukey's *post hoc* test). Significant differences were not seen when comparing directional migration toward each different conditioned medium ($P>0.05$). Overall, the two approaches to assess directionality, FMI and SD of angle, broadly agree in their analysis of data.

Trends in SDoA values, and yFMI results, indicate that soluble factors or MPs within CFS from live FC are chemoattractive, however unlike live MØ WC, the presence of FC in the supernatant does not appear to inhibit monocyte recruitment. Monocyte chemoattraction to aFC WC/CFS (SDoA) is also less uniform, indicating a potential reduction in functional 'find-me' mediator release in aFC, or upregulated mechanisms of monocyte chemoattraction in live FC, which could both have pathological implications.

5.2.5.4. Combined data

Comparison of SDoA values towards live and apoptotic cell-conditioned medium showed largely comparable uniformity of monocyte migration between cell types apart from 2 noticeable differences. Firstly, whole live MØ and FC cultures induced significant uniform monocyte migration compared to control ($P<0.05$), whereas live JK WC did not induce significant migration (Fig. 37). Secondly, aFC CFS induced significantly less uniform migration of monocytes compared to aMØ CFS, which could have implications in defective cell clearance (Fig. 37).

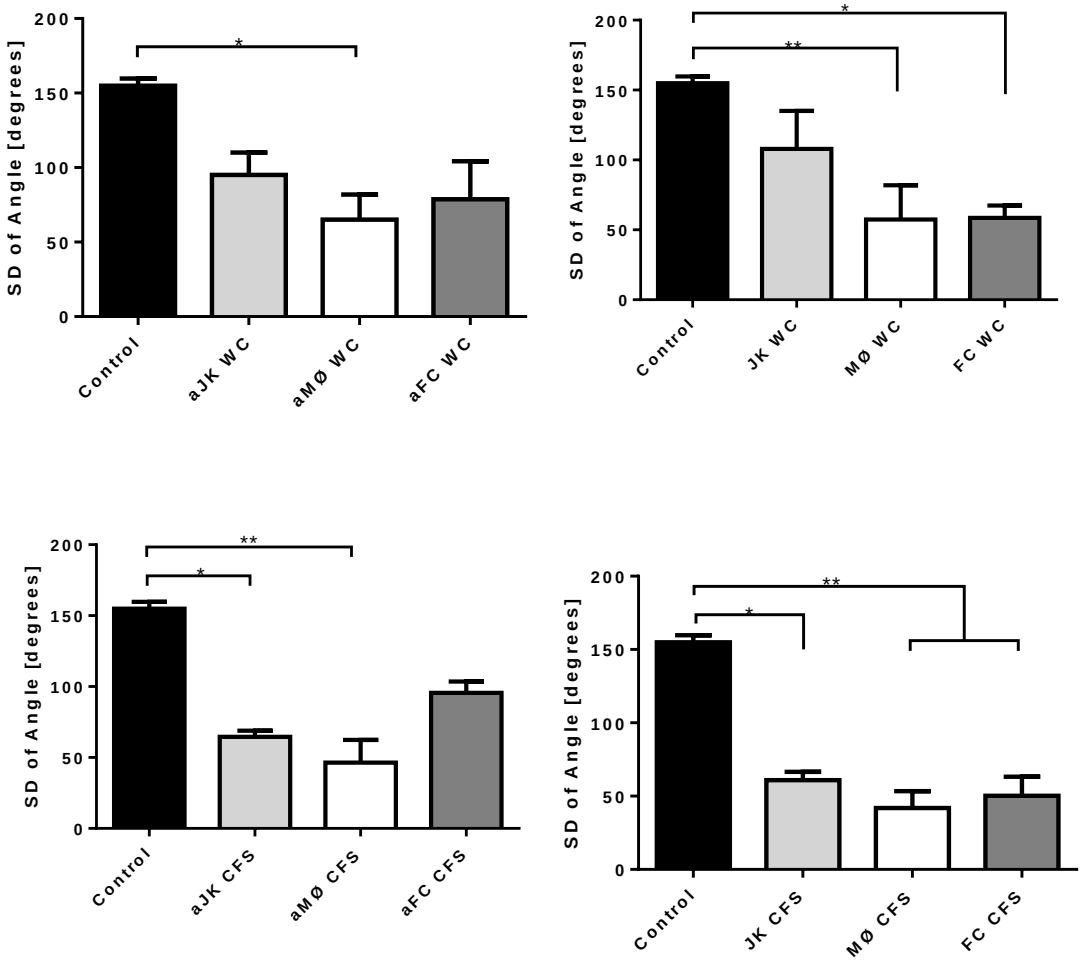


Figure 37. Chemoattractive properties of medium conditioned with Jurkat cells and THP-1-derived macrophages and foam cells *Combined SDoA values from figures 31-33 to compare chemoattraction properties across cell types. Data shows mean values ± SEM of 3 independent experiments. *P<0.05, **P<0.01 one way ANOVA with Tukey's* post hoc *test.*

Distance measures were not sensitive enough to establish significant differences in monocyte migration in these chemotaxis assays (Fig. 38).

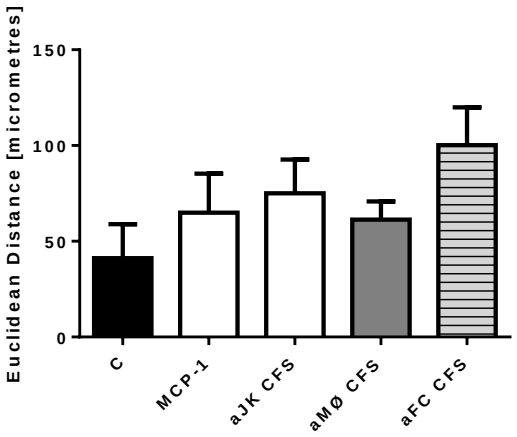


Figure 38. Distance of monocyte migration toward apoptotic cell conditioned cell-free supernatant *Data shows mean values ± SEM of at least 3 independent experiments. Control © n=3, MCP-1 n=4, aJK CFS n=3, aMØ CFS n=6, aFC CFS n=6.*

5.2.6. Mechanisms of monocyte chemotaxis toward apoptotic cell-derived MPs and soluble factors

In order to establish the molecular mechanisms that could be enlisted in monocyte attraction to apoptotic cells, in particular plaque-relevant ACs, roles of attractants ICAM-3 and CX3CL1, previously found in AC-derived MPs[61,71], were investigated. The role of CD14, an LPS receptor also implicated in tethering of apoptotic cells[31], was also investigated.

Whilst it has been shown that CD14 acts minimally as a tethering receptor for AC, it may also have the ability to be stimulated by AC and AC-derived material, such as MPs, as is true when it is ligated by LPS[368]. It is unknown whether CD14 recognition of ACAMPs on MPs may activate monocytes and trigger migration[123,124,370].

Initially, cell surface expression of CD14 in VD3 stimulated THP-1 cells was confirmed using indirect immunofluorescence staining, followed by flow cytometric analysis (Fig. 39). Results showed positive CD14 staining in VD3 stimulated THP-1 monocytes, with a mean 7 fold increase in mean fluorescence intensity (MFI) following CD14 staining, compared to isotype matched control staining, in agreement with Thomas *et al.*[312].

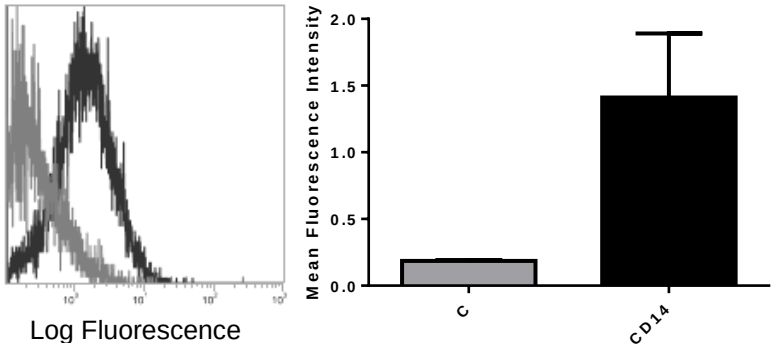


Figure 39. Presence of CD14 on monocyte model *VD3 differentiated THP-1 cells were stained with 61D3, a primary monoclonal antibody targeting CD14 (black plot on frequency histogram diagram) or isotype matched control antibody MOPC21 (grey plot on frequency histogram), followed by secondary antibody FITC, and fixed with 1% w/v formaldehyde in PBS. Expression levels were compared using flow cytometry and plots displayed as log fluorescence. Frequency histogram (left) is a representative example of 2 independent experiments. Data shown is mean fluorescence intensity (MFI) of the cell population ± SD.*

Following confirmation of CD14 expression on the monocyte cell surface, migration toward aMØ- and aFC-conditioned medium was assessed in the absence and presence of the CD14-blocking antibody, 61D3 (Fig. 40). Cell-free supernatant (CFS) was used as an attractant in antibody-blocking studies as previous results have shown some reduction in

variability of monocyte migration toward CFS compared to whole-cell culture (figs. 31-33), and MPs, which are of particular interest to these studies, would remain in CFS[71]. As we are investigating the effects of blocking migration, Euclidean distances travelled by monocytes will be compared.

Plots shown in Fig. 40 do not show any significant reduction in migration in the presence of a CD14-blocking antibody, either toward aMØ CFS or aFC CFS. When comparing mean Euclidean distance of migrated monocytes toward aMØ CFS and aFC CFS, no significant differences in distance migrated were found in the presence of CD14-blocking antibody 61D3 (unpaired t test). A small reduction in distance migrated toward aFC CFS in the presence of 61D3 is shown, but this is not significant.

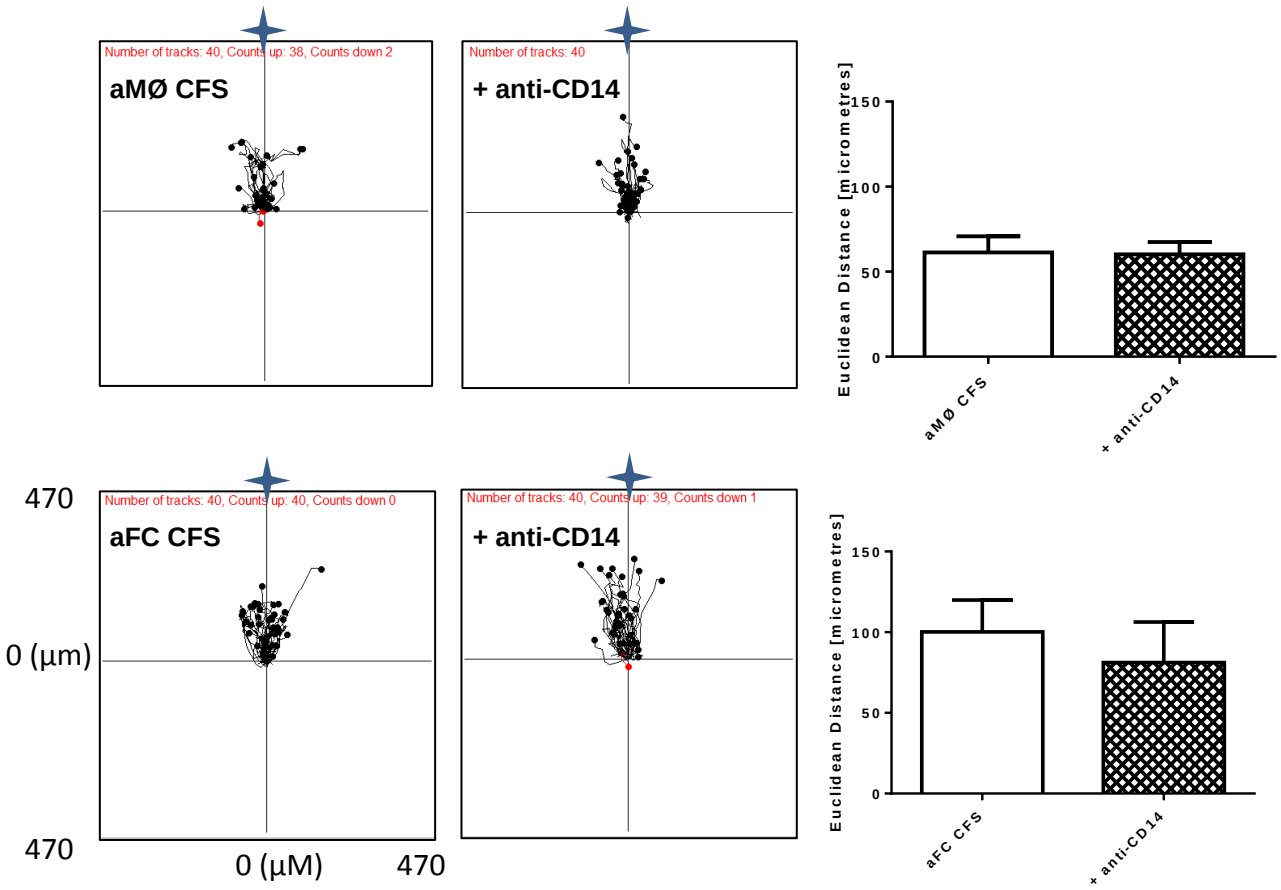


Figure 40. CD14 does not mediate monocyte migration toward apoptotic MØ and apoptotic FC-derived MPs and soluble factors *VD3 stimulated THP-1 in chemically-defined (CD)-MØ medium were allowed to migrate to aMØ or aFC CFS (2h; 37C) in the presence or absence of excess 61D3, a CD14 blocking antibody. Monocyte migration was monitored using time-lapse video microscopy. Migration of 40 cells per assay was measured using Image J and analysed using the Ibidi Chemotaxis and Migration Tool (V2.0). Graphs show mean distance of cell migration ± SEM of multiple independent experiments. CFS n=6, CFS + anti-CD14 n=3. Results show no significant differences (unpaired t test).*

Previous studies using a vertical migration chamber have found a role for ICAM-3 in the clearance of apoptotic cells, in both the phagocytic recruitment and tethering stages[71]. It is suggested that surface-bound ICAM-3 is lost during the apoptosis programme via shedding of ICAM-3 on microparticles (MPs), during a process known as zeiosis, or blebbing[71]. In these studies, monocyte migration toward aMØ- and aFC-CFS was assessed in the presence of the monoclonal ICAM-3-blocking antibody, MA4 (Fig. 41).

Comparing plots in Figure 41, migration to aMØ CFS in the presence and absence of an ICAM-3 blocking antibody are comparable, which is supported by mean Euclidean distance values of migrated cells. Distances are comparable when looking at migration toward aMØ CFS in both the presence and absence of an ICAM-3 blocking antibody. Comparison of plots toward aFC CFS appears to show a visible reduction in monocyte migration in the presence of the ICAM-3 blocking antibody MA4. Mean Euclidean distances were compared, but although distance of monocyte migration was reduced in the presence of MA4, this was not found to be statistically significant. (P=0.12, unpaired t test).

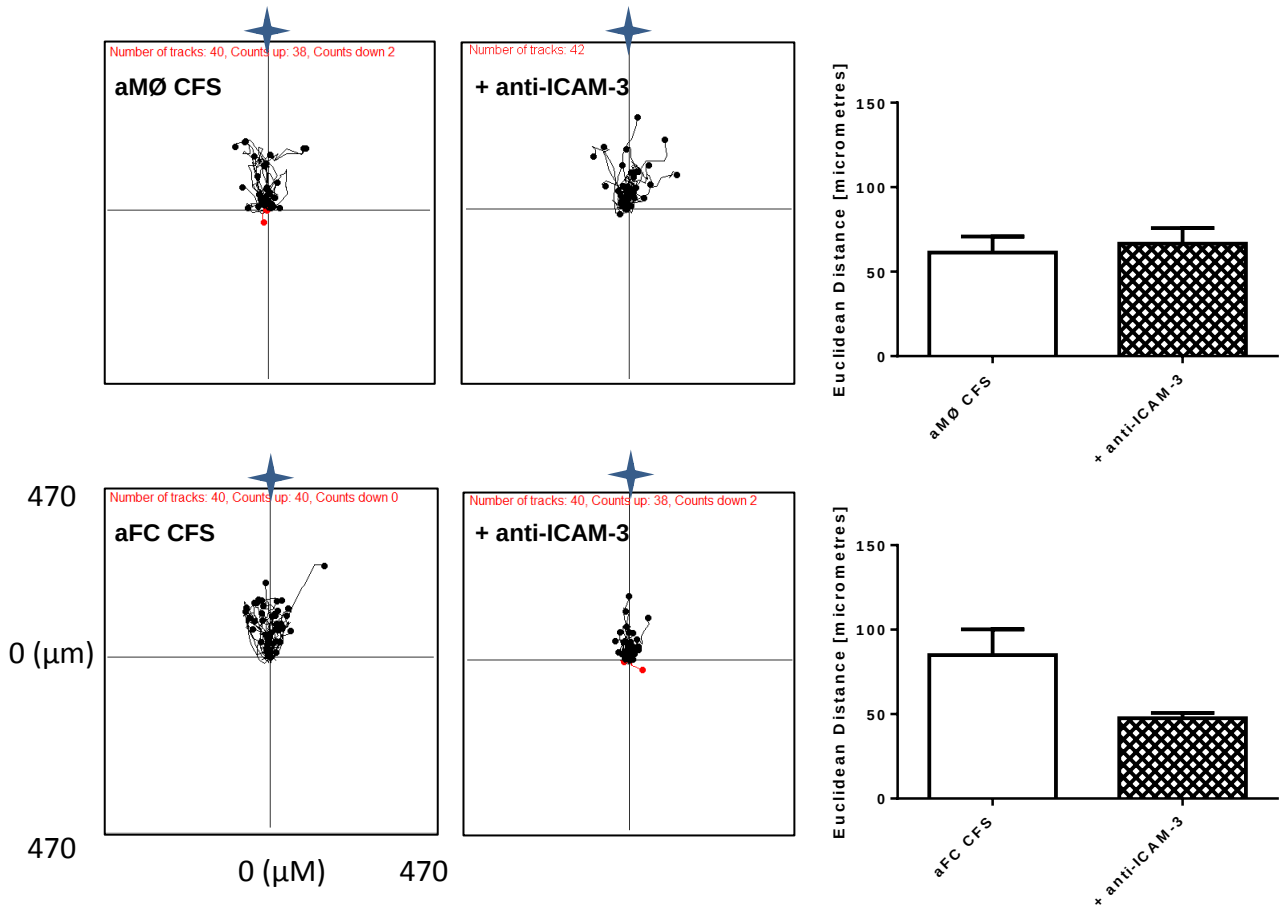


Figure 41. ICAM-3 mediated monocyte migration toward apoptotic foam cell-derived MPs and soluble factors *Dunn chemotaxis assays were set up as in Figure 40, with the exception of the antibody used, which in this figure was an ICAM-3-blocking antibody. Plots*

shown are representative of 3 independent experiments. Graphs show mean distance of cell migration ± SEM of multiple independent experiments. CFS n=6, CFS + anti-ICAM-3 n=3. Results show no significant differences (unpaired t test).

To investigate expression levels of ICAM-3, before and after induction of apoptosis, MØ and FC were stained with MA4, a primary monoclonal antibody targeting ICAM-3. If expression levels reduce following induction of apoptosis, it could be an indication that ICAM-3 is shed on MPs via blebbing.

Histograms show very low levels of ICAM-3 surface expression (Fig. 42), however further analysis of mean fluorescence intensity shows a small decrease in ICAM-3 staining in apoptotic FC, and to a small extent apoptotic MØ, though differences were not significant.

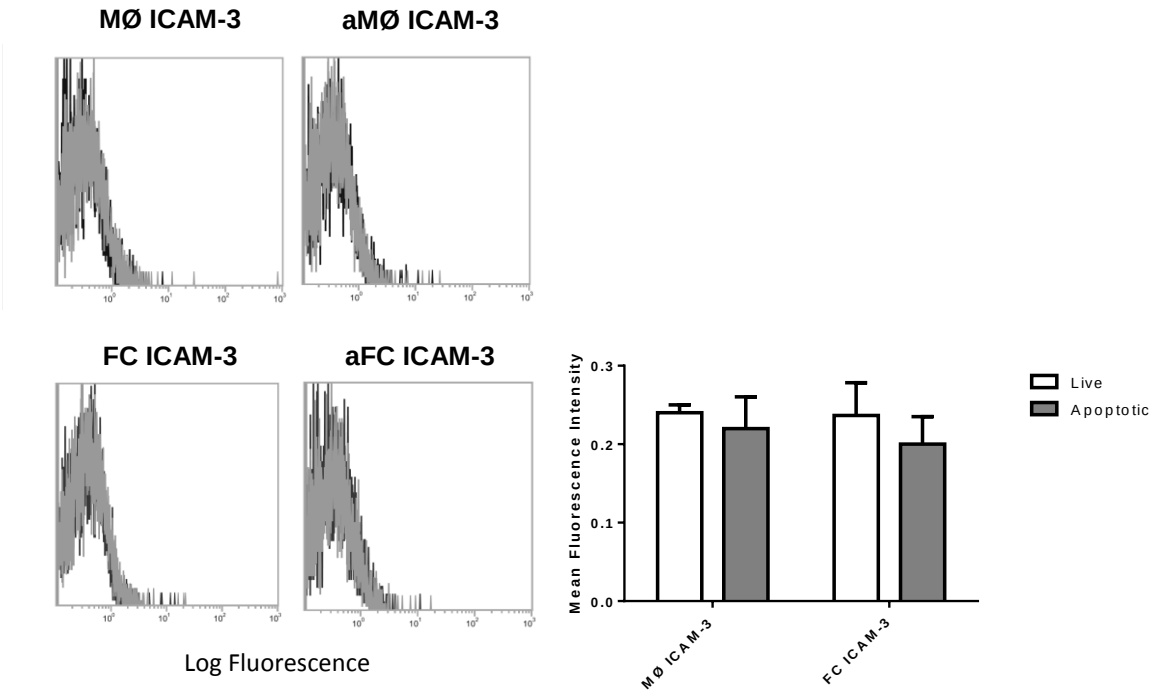


Figure 42. Macrophage and foam cell surface ICAM-3 expression before and after apoptosis induction *Live and apoptotic MØ/FC were stained with an anti-ICAM-3 antibody (black plot on frequency histogram) or isotype matched control antibody MOPC21 (grey plot on frequency histogram), followed by secondary antibody FITC. Expression levels were compared using flow cytometry and plots displayed as log fluorescence. Data shown are mean fluorescence intensity (MFI) of cell population ± SEM from 3 independent experiments. Data analysed by two way ANOVA with Tukey's* post hoc *test.*

Through the use of a vertical migration assay system, previous studies have shown CX3CL1 to act as an attractant to apoptotic cells, including CX3CL1 derived from UV-induced apoptotic BL cells[61]. Using UV-induced apoptotic Mutu, a BL cell line, chemotaxis to AC-derived supernatant in a horizontal assay system was observed (Fig. 43). Plots showing monocyte migration to aMutu CFS appeared to show more migration than control plots,

however whether this was direct migration towards aMutu CFS is unclear. When comparing SD of angle, results shows less variable, therefore more direct, movement towards aMutu CFS compared to control ($P<0.01$, unpaired t test) (Fig. 43, bottom left). When comparing mean Euclidean distance of migrating monocytes (Fig. 43, bottom right), distance migrated was not significantly different toward aMutu CFS in the presence or absence of CX3CL1-blocking antibody ($P>0.05$, unpaired t test). This is in contrast to previous studies, which observed robust blocking to apoptotic B cell supernatant with a CX3CL1-blocking antibody, however there were differences in experimental conditions[61].

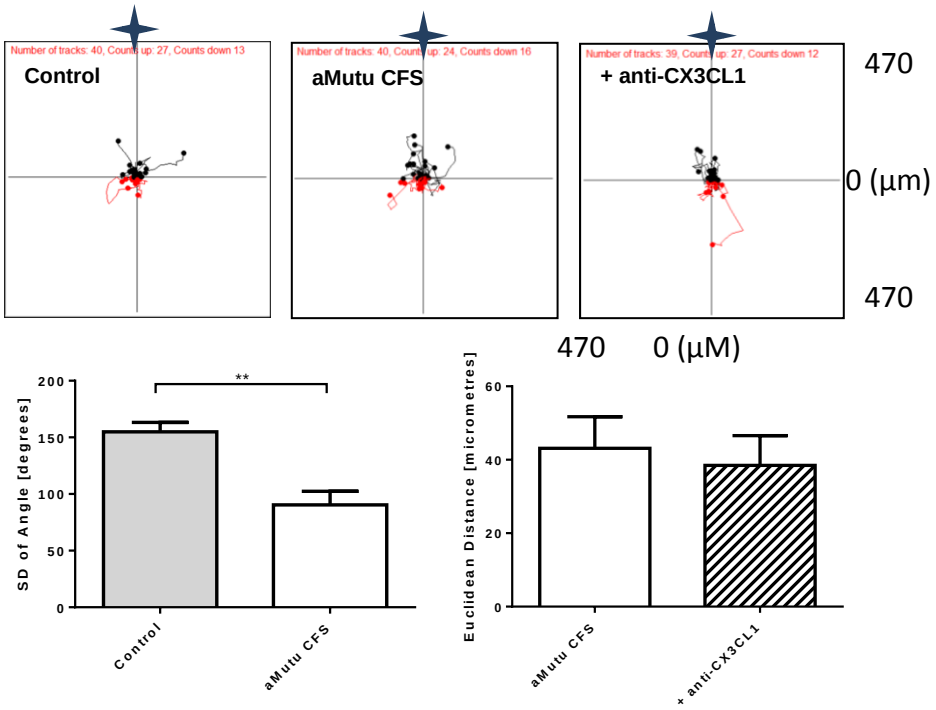


Figure 43. CX3CL1 mediated monocyte migration to apoptotic mutu-derived MPs and soluble factors *VD3 stimulated THP-1 were allowed to migrate to aMutu CFS in the presence or absence of 5ng/ml anti-CX3CL1. Monocyte migration was monitored using time-lapse video microscopy. Migration of 40 cells per assay was measured using Image J and analysed using the Ibidi Chemotaxis and Migration Tool (V2.0). Graphs show SD of angle (left) and mean Euclidean distance of cell migration (right) ± SEM of 3 independent experiments.*

CX3CL1 has been heavily implicated in the development and progression of atherosclerosis[289]. The role of endothelial cell-derived CX3CL1 in the recruitment of monocytes to the arterial intima has previously been studied[68,290,298]. CX3CL1 has also been detected within human plaque MØ using reverse transcription-polymerase chain reaction (RT-PCR)[371], and was found to be associated with MØ-derived FCs in the plaque[366]. The role

of aMØ- and aFC-derived CX3CL1 in monocyte recruitment to ACs in the plaque, and retention within the plaque, has not been characterised.

Figure 44 shows monocyte migration to aMØ and aFC CFS in the presence or absence of a CX3CL1 blocking antibody. Plots showing migration toward aMØ CFS appear to show a significant reduction in migration in the presence of an anti-CX3CL1 antibody. This was shown to be a significant reduction ($P<0.05$, unpaired t test) when comparing mean Euclidean distance of monocyte migration. Interestingly monocyte migration toward aFC CFS does not appear reduced in the presence of anti-CX3CL1, which is confirmed by data showing mean Euclidean distances migrated. Distance migrated is not significantly different toward aFC CFS in the presence or absence of anti-CX3CL1 ($P<0.05$ unpaired t test).

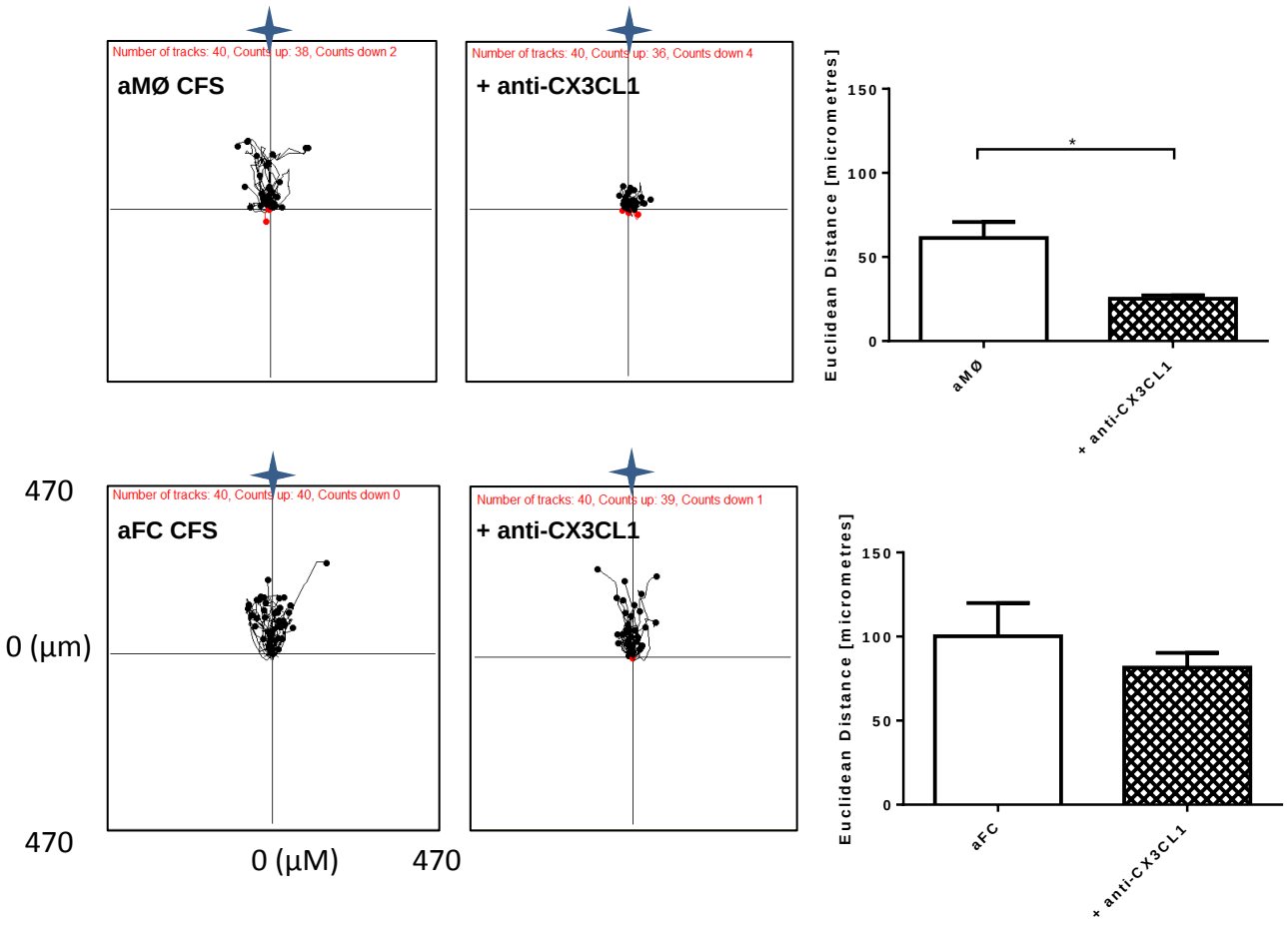


Figure 44. CX3CL1 mediated monocyte migration toward apoptotic macrophage-derived MPs and soluble factors *Dunn chemotaxis assays were set up as in Figure 40, with the exception of the antibody used, which in this figure was a CX3CL1 blocking antibody. Graphs show mean distance of cell migration ± SEM of at least 3 independent experiments, P<0.05, unpaired t test.*

To investigate expression levels of CX3CL1, before and after induction of apoptosis, MØ and FC were stained with a primary monoclonal antibody targeting CX3CL1. If expression levels reduce following induction of apoptosis, it would support evidence that CX3CL1 is shed in the supernatant, as soluble CX3CL1 or bound to the surface of MPs via blebbing, as indicated by Truman *et al.*[61].

Histogram plots in Figure 45 show positive CX3CL1 surface staining of MØ (black plots on frequency histograms) compared to isotype control (grey plots). Staining of aMØ is also positive but to a lesser extent, supporting a loss of membrane bound CX3CL1 (mCX3CL1) following induction of apoptosis (16-20h post UV). This is also shown by the mean fluorescence intensity (MFI) of the stained cell population, with a clear reduction in MFI values shown in aMØ, when compared to live MØ, however this reduction was not shown to be statistically significant (two way ANOVA with Tukey's *post hoc* test).

Expression of mCX3CL1 on FC can also be seen on histogram plots (black plots on frequency histograms), with a reduction of CX3CL1 expression following induction of apoptosis (aFC). This was also reflected in the MFI values, however the reduction was not shown to be statistically significant (two way ANOVA with Tukey's *post hoc* test).

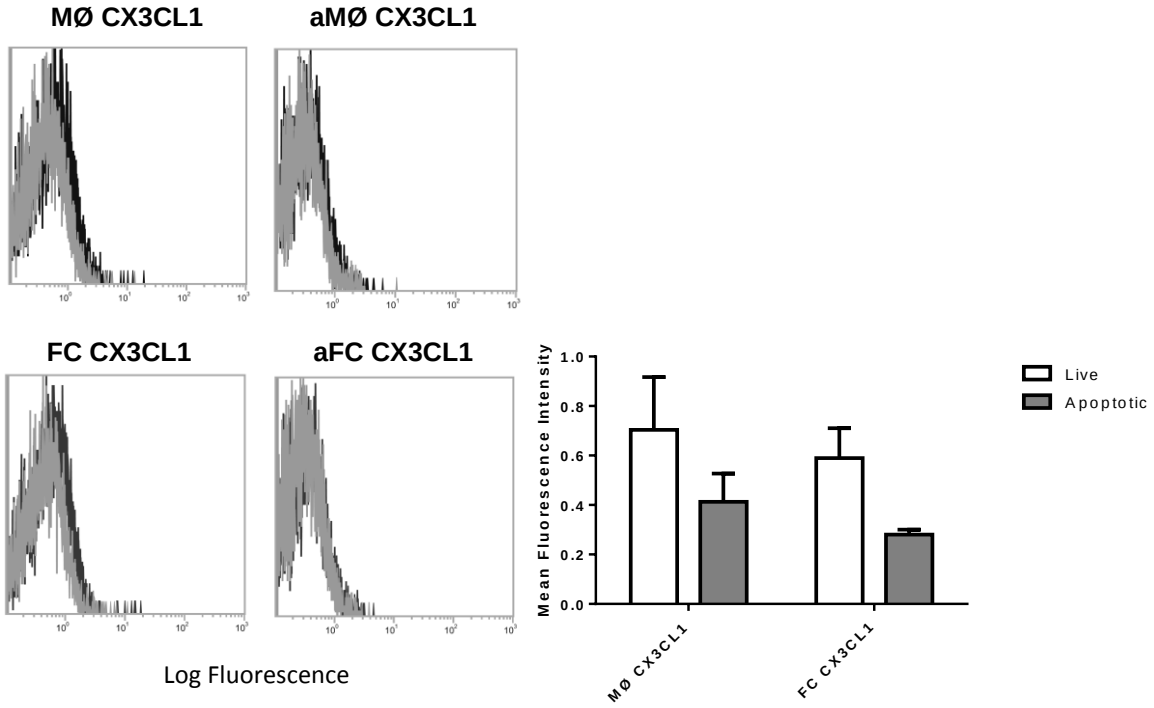


Figure 45. Macrophage and foam cell surface CX3CL1 expression before and after apoptosis induction *Live and apoptotic MØ/FC were stained with an anti-CX3CL1 antibody (black plot on frequency histogram) or isotype matched control antibody MOPC21 (grey plot on frequency histogram), followed by secondary antibody FITC. Expression levels were compared using flow cytometry and plots displayed as log fluorescence. Data shown are*

mean fluorescence intensity (MFI) of cell population ± SEM from 3 independent experiments. Data analysed by two way ANOVA with Tukey's post hoc *test.*

Reduction in detected membrane bound CX3CL1 expression could be occurring for a number or reasons, including internalisation or epitope-masking. In order to further support the hypothesis that CX3CL1 is shed from the surface of dying cells, either by blebbing or cleavage, an aMØ CFS sample was analysed for CX3CL1 (preliminary data). Presence of CX3CL1 in CFS would support the hypothesis that apoptosis induction results in CX3CL1 shedding from the cell surface, where it may exert its effects as an attractant. Preliminary studies showed CX3CL1 presence in aMØ CFS, but levels were not detectable (nd) in medium control (C). This supports the proposed hypothesis, however more work needs to be done, with the appropriate controls, to determine CX3CL1 presence in CFS, and to elucidate whether it is MP-associated or soluble.

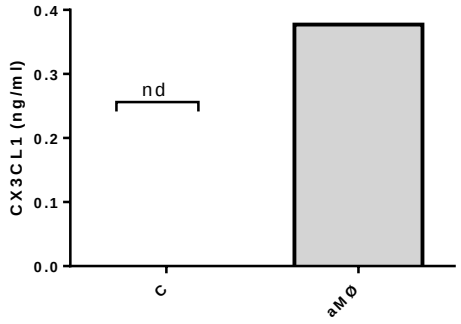


Figure 46. <u>Preliminary data.</u> Presence of CX3CL1 in apoptotic macrophage-conditioned medium *CX3CL1 concentrations in harvested aMØ CFS, or CD-MØ medium control (C), were assessed with a CX3CL1 antibody DuoSet according to manufacturers' instructions (R&D Systems, Abingdon, UK). n=1.*

5.2.7. Effects of apoptotic macrophage and apoptotic foam cell-derived cell-free supernatant on endothelial cells

To add an additional level of understanding to this model as may be observed in a physiological setting, the effects of intra-plaque mediator release by aMØ and aFC on endothelial cells (ECs) will be modelled. ECs are the natural barrier between the blood stream and the atherosclerotic plaque, mediating monocyte recruitment to the plaque. Inflammatory mediators or MPs released by phagocytes or ACs within the plaque may effect EC activation and exacerbate monocyte recruitment, a pro-atherogenic effect. The effects of live and apoptotic MØ/FC mediators on ECs will be modelled by culturing human umbilical vein endothelial cells (HUVECs) in CFS from each AC type (16-20h), and measuring changes in cell surface expression of E-selectin (CD62E), which mediates monocyte adhesion to the artery wall, promoting diapedesis[372]. Results in Figure 47 show very low

levels of E-selectin expression following culture with medium alone (control), however the percent of the cell population staining positive for E-selectin was significantly increased by aFC CFS (P<0.05), and even more so by MØ, aMØ and aFC CFS (P<0.01).

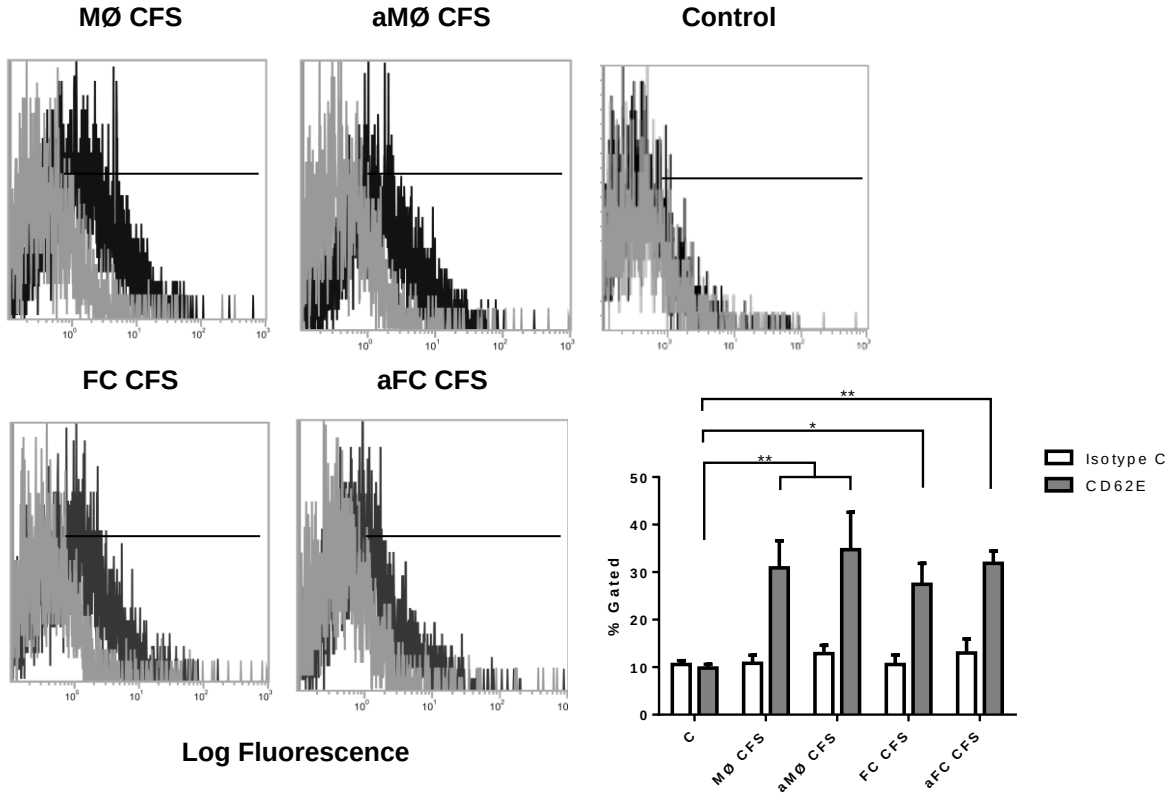


Figure 47. Apoptotic MØ and apoptotic FC cell-free supernatant upregulate E-selectin expression in HUVECs *Live and apoptotic MØ/FC CFS or medium control (C) were placed neat onto cultured human umbilical vein endothelial cells (HUVECs) (16-20h; 37C). HUVECs were harvested and stained with FITC labelled anti-CD62E (black plot on frequency histogram) or control antibody (grey plot on frequency histogram diagram) and expression levels were compared using flow cytometry. Data shown are mean ± SEM from 3 independent experiments. *P<0.05, **P<0.01, two way ANOVA with Tukey's* post hoc *test.*

5.3. Discussion

The aims of these studies were to establish a horizontal assay system to investigate phagocyte chemotaxis to apoptotic cells. This could then be used to investigate chemotaxis to models of apoptotic cell types found in the atherosclerotic plaque, and elucidate possible mechanisms in the 'find-me' stage of apoptotic cell clearance, in particular, the roles of CD14, ICAM-3 and CX3CL1.

Defective AC clearance and consequent failure to resolve inflammation, have been implicated in atherosclerotic plaque progression[58,195,197], however research into the CX3CL1 receptor (CX3CR1) has shown that knocking down key mechanisms of monocyte recruitment

to the arterial intima greatly reduces formation of atherosclerotic plaques[239]. As excessive monocyte recruitment is not beneficial to established plaque resolution, reduction in monocyte recruitment to the plaque could be a key aim for future therapies. To develop these, full understanding of the mechanisms and kinetics of monocyte recruitment to the plaque, and to apoptotic cells once within the plaque, is required.

Results in this chapter show that aMØ and aFC are both strong inducers of chemotaxis in monocytes, however chemotaxis mechanisms vary which could alter the monocyte subtype attracted to the atherosclerotic plaque. Results also show that both aMØ- and aFC-derived MPs/soluble factors have the ability to upregulate monocyte capture molecule E-selectin on the surface of endothelial cells.

	Phagocyte
Apoptotic cell	**Monocyte**
Apoptotic MØ (aMØ)	non-lipid laden MØ and aMØ attract monocytes, which was not significantly inhibited via blocking antibodies targeting CX3CL1, but not targeting CD14 and ICAM-3
Apoptotic FC (aFC)	FC and aFC attract monocytes, in a stronger but more disordered manner than aMØ, which was not significantly inhibited via blocking antibodies targeting CD14, ICAM-3 and CX3CL1

Table 8. Summary of results from key research questions addressed in Chapter 5

5.3.1. Use of the horizontal system

The vertical chamber showed significantly increased ($P<0.05$) mean number of monocytes per HPF migrating towards apoptotic foam cell-derived cell-free supernatant (aFC CFS), compared to aMØ CFS or medium control (Fig. 27). The methods of analysis used for the Dunn assay does not account for total number of monocytes migrated, as a set number of cells are sampled within each assay, so differences in total number of migrated cells would not be teased out using the Dunn method. The different methods of measuring monocyte migration can therefore show discrete data, and give different insights into mechanisms of chemotaxis. This was highlighted by Wilkinson (1998), who suggested multiple assays be

used to understand full locomotive properties of cells[373]. Dunn chemotaxis assays allow visualisation of each stage of chemotaxis, including cell morphology, whereas vertical assays use excess monocytes. This allows total counts of monocyte migration to be established, giving another dimension to understanding of chemoattractive properties of the mediator being tested. However, if you consider overall *in vivo* relevance, vertical chambers are not the strongest assay. Meyvantsson *et al*. developed an assay that combines good optics and high throughput, which was successful in the investigation of neutrophil chemotaxis, however the method is lengthier and more complex than more established assays, and requires the use of an automated liquid handling system (96-tip CyBiWell, CyBio, Jena, Germany)[293].

The effects of gravity in a vertical assay system, must not be ignored. Migrating cells, e.g. monocytes, settle onto the porous polycarbonate membrane. The effect of this on the activation state of monocytes and subsequent migration, following contact with the membrane or other monocytes, is unknown. Using a horizontal assay, such as the Dunn chamber, negates the effects of gravity on monocyte migration. Growth on glass coverslips, in the presence of PDL, may affect migration characteristics, however all tracked monocytes are in contact with coverslips, providing a homogenous state of activation. Migrating cells are also packed into upper chambers in excess in the vertical chamber, which will also have untold effects on activation state of monocytes, an issue not present using the Dunn method.

5.3.2. Optimisation of the Dunn chamber assay and defining 'directness'

5.3.2.1. Selection of migrating cell model

Initially a phagocyte model proficient in chemotaxis that worked in the Dunn chamber had to be identified. Unstimulated THP-1 cells did not have the necessary characteristics to be used in the Dunn chamber as adherence to glass coverslips is required. Differentiation with PMA, or PMA and VD3 (DS), resulted in a phenotype that appeared overly adherent for migration assays. Despite small amounts of visible movement by these cells in the presence of MCP-1 in the Dunn chamber assay, cells appeared to be anchored too strongly to migrate (Fig. 28). This was found using both glass and plastic coverslips. Human peripheral blood monocytes were also too adherent for short-term migration assays using plastic or glass coverslips.

Differentiation of THP-1 cells with VD3 results in a monocyte like phenotype that appeared to be further down the differentiation pathway than unstimulated THP-1 cells, but still with monocyte-like characteristics, rather than MØ-like characteristics, e.g. as seen with PMA/DS stimulated THP-1. This conclusion is based on features including maintenance of small, round morphology (fig. 5: Chapter 3) with a lot of homogeneity, a weakly adherent phenotype, maintenance of a low cytoplasmic: nuclear ratio, high expression of monocyte marker CD14 (Fig. 9: Chapter 3), and continued proliferation. This agreed with observations

made by Schwende *et al.*[310], Daigneault *et al.*[311], and Thomas *et al.*[312]. Daigneault *et al*. found VD3 stimulated THP-1 were most similar to human primary monocytes in terms of CD14 expression, compared to undifferentiated or PMA differentiated THP-1 cells[311]. Schwende *et* al., and Thomas *et al*. found high CD14 expression, low nuclear:cytoplasmic ratio, low LPS response and continued proliferation in VD3 stimulated THP-1 cells, all indications of a monocytic phenotype[310,312]. VD3 stimulated THP-1 could be a better representation of monocytes than unstimulated THP-1, which have been likened to monoblast/immature monocyte-like cells[374]. Studies in murine embryonic stem cell-derived macrophages (ESDM) found increased migration of immature macrophages toward apoptotic BL cell supernatant, compared to migration of mature ESDMs, whereas mature ESDMs were more proficient at phagocytosis of ACs compared to their immature counterparts, seen in these studies when comparing monocytic VD3 and macrophage-like PMA/DS cells[375]. VD3 stimulated THP-1 were also found to be a successful model of monocyte migration in vertical assays by Torr *et al.*[71], as seen in these studies (Fig. 27).

5.3.2.2. Optimisation of control assays

Monocyte chemotactic protein -1 (MCP-1), or CCL2, was originally identified as a novel monocyte chemotactic and activating factor produced following THP-1 culture at high cell density[304]. It is a CC chemokine family member, and is a recognised inducer of monocyte migration[376], and was the chemokine of choice to optimise and characterise VD3 stimulated THP-1 monocyte migration in the Dunn chamber. MCP-1 has a molecular weight of approximately 13kDa[376], which will form a linear concentration gradient between the inner and outer wells of the Dunn chamber approximately 30 minutes following assay set up, with a half-life of around 30 hours[292]. This is well within the parameters of methods used here, in which monocyte migration is assessed over a 2 hour time period. Assaying monocyte migration to MCP-1 as a positive control, a known attractive agent to monocytes[304,306,377], also allowed for the evaluation of a variety of qualitative and quantitative analysis tools to assess the various ways in which the data could be interpreted, depending on analysis methods employed.

5.3.2.3. Qualitative analysis methods for the Dunn assay

Cells are tracked using Image J and qualitative plots are created using the Ibidi Chemotaxis and Migration Tool. As chemotaxis chambers are circular, and monocytes are monitored from any chosen fixed position of the microscope over the circumference of the circle, the positive direction of movement towards MCP-1 could be at any angle over 360 degrees. To make comparison of qualitative data easier, all plots are rotated so direct positive migration would show at 0/360 degrees. Results show clear directional monocyte migration towards MCP-1 (Fig. 29), which is also reflected in P values obtained using the integrated Rayleigh

Test for unimodal clustering. This test measures uniformity of a circular distribution of points, in this case the cell endpoints on a plot, and takes into account distance from the origin based on X and Y axis coordinates, and is often employed in conjunction with qualitative plots, as part of Dunn chamber assay analysis[369,377-379]. The null hypothesis of uniformity is rejected when P>0.05. This gives a good indication of direct migration on an individual assay basis. Rayleigh test results show much higher levels of significance of migration towards MCP-1 than medium control (see P values in Fig. 29), with all MCP-1 assays showing significance levels of at least P<0.001. The most significant P value toward medium control was P=0.002, with the other assays both at P>0.05, rejecting the null hypothesis of non-uniform migration. Studies using murine bone marrow-derived macrophages have successfully modelled chemotaxis to MCP-1 using the Dunn chamber over 24 hours[377]. Many studies use rose diagrams to display data[377-379], however raw data plots were chosen in this instance for a clear representation of results.

5.3.2.4. Quantitative analysis methods for the Dunn assay

5.3.2.4.1. Distance, velocity and directness

The chemotaxis and migration tool also provides quantitative outputs including Euclidean distance, accumulated distance, velocity and directness. Interestingly, despite clear qualitative differences between migration toward MCP-1 compared to CD-MØ medium control, differences in directness, velocity, Euclidean distance and accumulated distance were not significantly different, even with the recommended number of cells tracked to allow for statistical significance. Although cells in control experiments do not migrate in a directional manner, they do show varying levels of chemokinesis, non-directional movement[240]. This means that unless quantitative measures take into account the direction of migration, differences may not be highlighted. The provided measure of directness in this software measures how efficiently cells move from the starting point (A) to the endpoint (B), rather than taking into account the location of B according to the gradient of the attractive agent, and whether cell migration is along the established gradient of attractant[369].

5.3.2.4.2. Forward Migration Index (FMI)

To combat this, another measure is provided, the forward migration index (FMI). FMI values take into account positive and negative migration, distance migrated, and provides values for endpoints of cells both parallel (yFMI) and perpendicular (xFMI) to the attractive gradient. If both yFMI and xFMI values are close to zero, this corresponds to no chemotactic effect. A yFMI value nearer to 1, and an xFMI value close to zero, indicates a strong chemoattractive effect[369].

5.3.2.4.2. Standard Deviation of Angle (SDoA)

Looking at the variability of the cell end points is a useful method to distinguish whether the cell population has moved in a uniform manner, and this can be done by taking the angle (A) measurement of each cell, and calculating the mean standard deviation (SD) of these end points (SDoA). Using the SDoA of cell endpoints also shows statistically significant differences between data sets that show qualitative differences in cell migration of the overall population. Trends of SDoA reflect trends shown by FMI (figs. 31-33). A drawback of this method of analysis is that perfect negative migration would give the same value as perfect positive migration, however negative migration of the cell population was not seen in any of these studies. With attractants present, e.g. Fig. 29, overall migration of the cell population toward the attractant was observed, whereas directional migration of the cell population was not seen in control assays, giving higher SD values when looking at angle measurements. Also, this method fails to take into account distance migrated, unlike the Rayleigh test, which takes into account both distribution of endpoints and distance migrated.

Each method provides useful information on the migration of monocytes. If complex measures of migration are not reflecting the apparent qualitative differences observed in monocyte migration to different attractants, comparing more basic methods such as distance migrated and SDoA may tease out differences, as less variables come into play.

5.3.3. Modelling monocyte chemotaxis towards apoptotic cell models

Whole cell supernatants and cell-free supernatants (supernatants centrifuged at 350xg, 6 mins) were compared to establish differences in attractive properties of live or apoptotic cell bodies and large cell detritus, versus the attractive properties of cell-free supernatant, which would contain microparticles and soluble factors[71]. As crude supernatants were used in these studies, a plethora of 'find-me' signals will be present that have been released by apoptotic cells to attract phagocytes for clearance.

5.3.3.1. Monocyte migration to live cell-conditioned medium

Live cell supernatants were investigated as a control for apoptotic cell supernatants, and were not expected to have strong attractive properties, however monocyte chemotaxis to live Jurkat, MØ and FC whole cell culture (WC) and CFS was observed qualitatively on raw data plots (figs. 31-33). Plots show directional migration of monocytes towards all Jurkat-conditioned medium (Fig. 34), however SDoA data shows only migration toward aJK CFS was significantly less variable than assays towards medium control. yFMI values show comparable forward migration toward live and dead Jurkat CFS, which was significantly more directional than medium control ($P<0.01$). Forward migration to JK CFS and aJK CFS was also significantly increased compared to aJK whole culture ($P<0.01$). Results failed to identify

differences in migration toward whole cell cultures compared to CFS, which could explain differences in the presence or absence of 'keep-out' signals. To date, only granulocyte 'keep-out' signals have been characterised[78,79], but it is likely that more will be discovered in the pursuit of therapies for conditions where cell-specific dampening of immune responses would be of benefit, e.g. atherosclerosis or asthma[70,79].

Monocyte migration towards MØ-conditioned medium (Fig. 35) was directional under all conditions, with significant migration according to SDoA and yFMI values to both live and dead, whole cell culture or cell-free supernatant, compared to control. Although there were no significant differences in migration between conditioned medium groups, CFS from both live and dead MØ showed greater significant differences compared to control than whole cell supernatants (Fig. 35). Interestingly, trends in migration to FC-conditioned medium were slightly different, with migration to all conditioned medium observed in plots as with JK and MØ, but qualitative plots appear to show increased migration to aFC whole cell culture and cell-free supernatants compared, to FC counterparts (Fig. 36). Quantitative data however indicates the opposite trend, as SDoA showed significantly less variability (i.e. more direct migration) in live FC supernatants, compared control ($P<0.01$), and migration to aFC whole-cell supernatant was not as significantly different compared to control ($P<0.05$), and no significant differences were observed between monocyte migration to medium control and aFC CFS. This is in contrast to results shown with Jurkat supernatants, in which significant directness in monocyte migration was observed only between aJK CFS and medium control, when comparing SDoA. yFMI data shows significant forward migration in live FC supernatant groups only, compared to medium control ($P<0.05$). Data indicates that apoptotic foam cells strongly induce migration (Fig. 27), but in a variable or disordered manner (Fig. 36), which could be a result of mixed messages dispatched by the aFC, e.g. similar to lactoferrin, a 'keep-out' signal which keeps granulocytes away from sites of cell death[78,79].

Live cells would not be expected to be attractive to monocytes, indicative of an early stage of an immunological response, whether that be silent or inflammatory. Chemotaxis to live cell-conditioned medium was observed in this study to the same extent as apoptotic cell-conditioned medium, and, in the case of FC-conditioning, was even more attractive to monocytes than aFC-conditioning. This was particularly true in the case of live cell CFS. This was attributed to high cell density culture conditions, which could result in cells becoming stressed or activated, and in the case of immune cells as studied here (T lymphocytes, MØ and MØ-derived FCs), could result in mediator release that would result in activation or attraction of monocytes, e.g. MCP-1[304]. Recent work has also found that oxidative stress, even without cell death, mediates selective recruitment of monocytes[380]. Further investigation also showed high basal levels of apoptosis in live cell cultures, which was most likely due to

the culture conditions used to sequester high MP concentrations. PMA differentiation also results in terminal differentiation, and can alter THP-1 susceptibility to apoptosis[309,311].

Lauber *et al*.[62] investigated THP-1, Mono Mac 6 (human leukaemia cell line), and HMDM migration to a variety of cell-free supernatants (CFS) derived from tumour cell lines, either from live cells or following induction to apoptosis via UV irradiation, using a vertical transwell system. Results saw that whilst almost all apoptotic cell CFS attracted all monocyte models, some monocyte models, but not others, migrated to live cell CFS. For example, THP-1 migration toward live L929 CFS (murine fibroblast cell line) was observed to at least the same extent as other apoptotic cell CFS tested, but not to as great an extent as migration toward apoptotic L929 CFS. This supports data presented here where live cell cultures induce monocyte chemotaxis (figs. 31-33). Mono Mac 6 and HMDM both migrated to live MCF7 CFS (human breast carcinoma cell line) to the same extent as apoptotic MCF7 cells, but THP-1 migration was induced to a greater extent by apoptotic MCF7 CFS. This could be explained by low levels of migration toward both live and dead MCF7 CFS (<5% transmigration). Comparatively high levels of migration toward live Cos7 CFS (African green monkey kidney cell line) by HMDM was also observed (approx. 12% transmigration), but migration to apoptotic Cos7 CFS was greater (approx. 22%). Some HMDM transmigration was also seen toward live HT29 CFS (human colorectal carcinoma cell line). This study highlights that differences in phagocyte and target call type, live or apoptotic, can vary outcome with regards to chemotaxis induction. In contrast to data shown here (Fig. 34), Elliott *et al*.[63] found live Jurkat CFS, harvested from Jurkat cells cultured at the same cell density as cultures used here, were not chemoattractive to undifferentiated THP-1 cells, in a vertical transwell assay.

Every stage of apoptotic cell clearance requires a balance of 'pro-clearance' and 'anti-clearance' mediators, e.g. "find-me" versus "keep-out" molecules in the chemotaxis phase, or "eat-me" versus "don't-eat-me" ligands in the recognition phase[80]. It is likely that the correct balance of pro- and anti-clearance signals is key to successful clearance[90,94]. Given the plethora of effector molecules recognised as having a role in AC clearance, and the levels of redundancy observed[381], it is quite possible that different combinations are required for successful clearance of varying cell types, and in different physiological and pathophysiological scenarios. Work by Lauber *et al*. covered a variety of human monocyte/MØ cell models often used in studies of monocyte and MØ function[62]. In combination with the variety of cell lines used to generate CFS as attractants, it provides a useful insight to the different migration patterns observed when comparing cell models derived from the same species, i.e. cell line versus primary cell[62]. Results become more difficult to interpret and compare when considering cell lines to model ACs are derived from

human, murine and simian origin, however the presence of caspase-3 in all cell lines apart from MCF-7 gave some clues into mechanistic data[62]. Care was taken in the current chemotaxis studies to select a cell model to represent MØ and FCs, and appropriate phagocytes to complete the model. THP-1 cells are a useful tool in this respect as they can be induced to varying degrees of differentiation, so were used to model the migrating monocyte (+VD3), the MØ (+ PMA) and the FC (+ PMA + oxLDL).

5.3.3.2. Monocyte migration to apoptotic cell-conditioned medium

Qualitative plots (figs. 31/32) show robust monocyte migration (VD3 stimulated THP-1) to apoptotic JK and apoptotic MØ-conditioned medium. Monocyte migration to apoptotic FC-conditioned medium was less robust (Fig. 37), with quantitative data suggesting more directional migration was seen toward live FC culture and CFS (Fig. 35). This could be due to increased expression of 'find-me' signals and decreased 'keep-out' mediator release in live FC in response to lipid-loading, or a failure of FC to upregulate 'find-me' mediators, or downregulate 'keep-out' mediators, following lipid-loading.

Previous studies into monocyte chemotaxis towards apoptotic B cells found a role for AC-derived MPs in monocyte migration to sites of cell death[60,61,71]. AC-derived MPs were identified by Segundo *et al*. via centrifugation, filtration, fluorescence microscopy, electron microscopy, and flow cytometry[60]. Recent research suggests that chemoattractants, such as CX3CL1 and ICAM-3, are associated with apoptotic B cell-derived MPs, and blocking action of these attractants reduces monocyte migration to MP[61,71] (discussed further in 5.3.4.). In direct support of this, monocyte migration to apoptotic B cell CFS is observed in this model (Fig. 43).

In contrast, Lauber *et al*. found no loss of chemotaxis induction following removal of microparticles (ultracentrifugation or 0.2µm filtration) from MCF-7 caspase-3 replete cells[62]. The authors concluded that chemotactic ability is not mediated by membrane-derived MPs in this instance as chemotaxis induction was not lost, and was subsequently found to be lipid mediated, however the chemotactic nature of removed MPs were not tested. These results may demonstrate a level of redundancy in 'find-me' mediator function, either in this model or as a universal feature of 'find-me' mechanisms, as seen with 'eat-me' stages of AC clearance[381]. Further clarification of MP versus soluble factor-mediated monocyte recruitment in MØ and FC models would be gained by employing similar methods to Lauber *et al.*, such as ultracentrifugation of CFS, followed by pellet resuspension in fresh medium, to investigate whether chemotactic activity maintained.

Alongside the MPs in cell-free supernatants, there will be a wide range of soluble factors, including cytokines and chemokines, especially as the apoptotic cells in question have high

cytokine outputs upon activation. These may include identified 'find-me' signals such as CX3CL1[61],LPC[62], LPC induced MCP-1, IL-8 and RANTES[74], S1P[75], S1P induced IL-8 and IL-10[76], ICAM-3[71], ATP/UTP[62]. CX3CL1 was, until very recently, the only chemokine to be implicated in phagocyte recruitment to dying cells[61], however a new study has shown release of an array of cytokines and chemokines, including MCP-1 and IL-8, following induction to apoptosis via Fas/CD95 ligation[59]. It is important to understand mechanisms of recruitment, and perhaps over-recruitment, of monocytes to the atherosclerotic plaque, especially as research in murine models suggests reducing recruitment, rather than promoting egress, is beneficial in limiting plaque progression[156].

5.3.4. Mechanisms of monocyte chemotaxis towards apoptotic macrophages and foam cells

CX3CL1 release from apoptotic human B cells has been demonstrated[61], both as a soluble mediator and in association with MPs, and was, until recently, the only classical chemokine implicated in the recruitment of phagocytes to dying cells. This was supported by results in Figure 43, which showed significant ($P<0.01$) uniform monocyte migration to UV-induced apoptotic B cell-derived CFS. CX3CL1 ligates the receptor CX3CR1 on the phagocyte surface to promote directional migration[61,64]. CX3CL1 also enhances clearance via MFG-E8 upregulation, a bridging molecule between apoptotic cells and phagocytes[69]. Further chemokines have recently been implicated in leukocyte attraction following induction to apoptosis through Fas/CD95 ligation. Release of numerous cytokines and chemokines were shown, including MCP-1 and IL-8, which were demonstrated to attract THP-1 monocytes and primary human neutrophils respectively[59].

Truman *et al*. demonstrated that apoptotic human B cell-CFS induced HMDM and murine bone marrow-derived MØ (BMDM) chemotaxis using a vertical transwell system[61]. Migration was then inhibited by addition of an anti-CX3CL1 blocking antibody (50µg/ml) in lower wells containing CFS, or by the addition of recombinant CX3CL1 (100ng/ml) to upper wells, where monocytes are loaded. Results here did not show a significant reduction in VD3 monocyte migration to apoptotic B cell-derived CFS in the presence of CX3CL1 neutralising antibody, also loaded in the CFS chamber (Fig. 43). However, antibody concentration used by Truman *et al.* was ten times the concentration used in this study[61], therefore reduced migration could be replicated in this system with further optimisation. MP generation is also unspecified with regard to cell culture densities[61], and a vertical transwell assay is employed, both of which will impact optimal neutralising antibody concentrations across studies. The use of CX3CL1-neutralising antibodies in aMØ-derived CFS and significantly reduced monocyte migration ($P<0.05$), an effect which was not observed using aFC CFS (Fig. 44). This shows interesting differences in 'find-me' mediator release following lipid-loading, however further investigation

is required. The basis of these results could be an increased (non-saturating) presence of CX3CL1 in CFS or redundancy in CX3CL1 driven monocyte recruitment by aFC following upregulation of other recruitment mechanisms.

In contrast, Elliot *et al*.[63] found purified CX3CL1 did not induce THP-1 chemotaxis, nor did an anti-CX3CL1 depleting antibody inhibit migration, however data was not shown so exact assays, concentrations of antibodies and purified CX3CL1 concentration are not known. LPC was also shown not to mediate THP-1 chemotaxis in transwell assays, in contrast with data shown by Lauber *et al.*, potentially showing differences in AC models[62]. A possible explanation is a lack of expressed CX3CR1 on human THP-1 cells, however expression has been shown in other studies[382], and CX3CL1 mediated-monocyte migration was shown here.

Following apoptosis induction in studies here, significant reduction in cell surface CX3CL1 protein expression was not observed (aMØ/aFC; Fig. 45). In contrast, studies by Truman *et al.*[61] (apoptotic B cell) observed significantly reduced levels of cell surface expression, supporting the notion that CX3CL1 may be lost from the surface via MP shedding, though mechanisms such as epitope masking or internalisation could be at play. To explore this notion further, preliminary assays were undertaken to assess the presence of CX3CL1 in harvested CFS. Initial data suggests CX3CL1 is present in aMØ-derived CFS (Fig. 46). This assay could be used further to compare quantities of released CX3CL1 in both live and apoptotic MØ and FC CFS. This would shed light on whether reduction in CX3CL1-mediated monocyte recruitment by aMØ CFS, and not aFC CFS, was a mechanistic/functional shift, or due to differences in quantities of shed CX3CL1 (Fig. 44). Presence of CX3CL1 in live cell-derived CFS would also explain observed monocyte migration where it wasn't necessarily expected (figs. 31-33), as CX3CL1 has been shown to be constitutively shed in live B cells[61]. Studies here do not differentiate between soluble CX3CL1 (TACE/ADAM17 cleaved[219]) and MP-associated CX3CL1, which could be elucidated initially using ultracentrifugation to separate MPs and soluble factors. Subsequent immunoblotting could then be carried out to assess protein levels, or resuspension in fresh medium could text for function in further chemotaxis assays. Truman *et al*. found soluble and MP-associated CX3CL1 was shed by B cells[61].

CX3CL1-CX3CR1 interaction also provides a survival signal against apoptosis[383]. This has implications when considering the accumulation of potentially 'sick' lipid-laden cells within the plaque. Inefficient clearance of dead cells in the plaque is the main pathological event in atherosclerosis, resulting in a core of cells undergoing secondary necrosis and contributing to plaque instability and rupture[162]. It could be argued that survival signals from CX3CL1 are beneficial to the plaque, keeping macrophages and foam cells alive to engulf cells already

undergoing apoptosis. On the other hand it may also be considered that macrophages are being kept alive for too long, enabling foam cell production and further recruitment of monocytes to the plaque. Macrophage apoptosis is considered to be anti-atherogenic in early lesions, but as lesions progress apoptosis is suggested to be pro-atherogenic[149,236].

Considering further 'find-me' mediators, ICAM-3 is of interest as it has only very recently been shown to act as an inducer of monocyte migration to apoptotic cells[71], and has had little study as it is absent in rodents[384]. Torr *et al.* showed ICAM-3 was MP-associated via the use of GFP-tagged ICAM-3 and fluorescence microscopy, observing locality of ICAM-3 following induction to apoptosis[71]. ICAM-3 shedding via MPs was further evidenced by the reduction in ICAM-3 surface staining following apoptosis induction (flow cytometry), and the presence of ICAM-3 (western blot) in association with supernatant harvested MPs[71].

Cell surface expression of ICAM-3 was assessed here using flow cytometry (Fig. 42), and MFI values were extremely low. Despite this, a very modest reduction in ICAM-3 surface expression was observed following induction to apoptosis, as seen by Torr *et al.*[71]. When adding ICAM-3-blocking antibody MA4 to CFS in chemotaxis assays, monocyte migration toward aMØ CFS was not affected (Fig. 41). There appeared to be decreased migration toward aFC CFS in the presence of an ICAM-3-blocking antibody, which may be proved significant with further repeats, however it was not shown to be significant here. It could indicate an interesting switch in aMØ and aFC phagocyte recruitment mechanisms upon induction of apoptosis. The mechanism behind ICAM-3 induced monocyte migration remains to be elucidated.

Also investigated was the role of CD14 in monocyte migration to AC-derived mediators. CD14 has been shown to have contributing roles in atherogenesis, including pro-atherogenic responses to mmLDL[201,323,324], and polymorphisms in the promoter of CD14 gene were found to be associated with an altered risk of atherosclerosis[385]. CD14 also interacts with ACs, from which MPs may contain some comparable structures, such as PS or ACAMPs[31,284]. Figure 39 shows high expression of CD14 in the VD3 stimulated THP-1 monocyte model, however addition of a CD14-blocking antibody had no effect on monocyte migration toward aMØ or aFC CFS. This supports earlier work by Truman *et al.* (2004), which showed migration toward apoptotic B cells was not impaired in BMDM from $CD14^{-/-}$ mice[386].

5.3.5. Effects of apoptotic cell mediators on endothelial cell adhesion molecule expression

The majority of plaque microparticles are thought to be macrophage-derived[269], however the role of these AC-derived MPs on endothelial cell (EC) function has not been fully established.

A murine LDLR-/- model combined with P- and E-selectin double deficiency ($P/E^{-/-}$) were found to develop 40% smaller and less calcified lesions, confirming the role for E-selectin in plaque development, and re-affirming the importance of inflammation in atherosclerosis, which mediates E-selectin expression[372]. Figure 47 shows the ability of MØ and FCs to induce E-selectin expression in HUVECs, an accepted endothelial cell model. This could indicate an un-modulated mechanism of monocyte recruitment, beyond the point of which recruitment is athero-protective. Further aspects of apoptotic MØ and apoptotic FC-derived MP-modulation on monocyte recruitment and inflammation in the plaque would be of interest.

Human atherosclerotic plaque-derived MPs were shown to promote endothelial ICAM-1-dependent adhesion and transendothelial migration by monocytes, by increased ICAM-1 expression on ECs in a concentration-dependent manner[281]. This was attributed to transfer of ICAM-1 from plaque MPs to ECs, as an increase in ICAM-1 mRNA expression was not observed[281]. Whether AC-derived MPs can confer CX3CL1 in the same manner, contributing to circulating monocyte trapping in the arterial lumen, would be an interesting point of investigation.

Mesri *et al*. found that primary human polymorphonuclear leukocyte (PMN)-derived MPs induced IL-6 production in HUVECs. Human T cell-derived MPs were also shown to induce pro-inflammatory cytokine production and apoptosis in bronchial epithelial cells, in which phagocytosis of MPs was suggested to be required[387]. Cytokines such as TNF-α do not regulate P-selectin expression in humans, but can induce P-selectin expression in mice, which has been attributed to species-specific transcriptional regulation[388]. This highlights the care that needs to be taken in experimental design.

In summary, MPs could have a variety of roles in atherogenesis and plaque progression, many of which are yet to be investigated.

5.4. Conclusions and Future work

In conclusion, this is very early, but much needed work on mechanisms of monocyte attraction to sites of cell death, including those that may be encountered in the human atherosclerotic plaque.

Dissecting out the roles of MPs versus soluble factors would be a logical next step to see if CX3CL1 and ICAM-3 are soluble or MP-associated in this model. Ultracentrifugation of cell-free supernatants, and reconstitution of pellets in fresh medium, should be able to remove and then replace chemoattractive properties to monocytes respectively. Western blotting of pellets for CX3CL1 and ICAM-3 would further support MP-association, as seen in work with

apoptotic B cells[61,71]. Further profiling of MPs could also be carried out with immuno-staining and flow cytometry analysis.

Fluorescently labelled MP could be used to assess binding of MP to MØ in the presence of the CX3CL1, ICAM-3 and CD14 blocking antibodies. This would allow analysis of binding mechanisms and mechanisms of immune modulation to be compared.

The use of an endothelial cell barrier between monocytes and AC-conditioned medium would add an additional level of understanding. This could be carried out using a transwell-based assay, and could be used to assess whether microparticles with chemoattractive properties could attract cells from the other side of an endothelial cell barrier. A further development could then seek to use such a system under conditions of flow, so as to more closely model the interaction of blood monocytes with inflamed endothelium at sites of cell death.

Transwell assays could also be used to assess preferential recruitment of primary human monocyte subtypes to apoptotic MØ and apoptotic FC MPs, as migrated cells can be collected and stained from lower chambers, for analysis by microscopy or flow cytometry. This would be interesting as CX3CR1 monocytes have been hypothesised to be preferentially recruited to the atherosclerotic plaque[389].

Utilising the Dunn chamber method to assess potential mechanisms, or failed mechanisms, from a plaque-like environment may also advance understanding of failed resolution of inflammation.

Macrophage function throughout ageing : A Pilot Study

6.1. Introduction

Individuals within the aged population show an increased susceptibility to infection, implying a decline in immune function, a phenomenon known as immunosenescence[8]. Paradoxically, an increase in autoimmune disease, such as rheumatoid arthritis, is also associated with ageing, therefore some aspects of the immune system appear to be inappropriately active in the elderly[9-11]. The above evidence suggests inappropriate control of the immune system as we age.

Macrophages, and their precursors monocytes, play an important role in host defence in the form of phagocytosis, and also link the innate and adaptive immune system via antigen presentation [18]. Inappropriate control of macrophage function has been a continuing theme throughout this thesis. The following small-scale pilot study aimed to address whether changes in primary monocyte-derived macrophage function could be observed between young and mid-life adults.

Despite immunosenescence now being a widely accepted consequence of ageing, it has proven difficult to reproduce at the cellular level, and the approach to research in this area varies widely [390]. Research into age-related changes in innate immunity is less advanced than that of adaptive immunity, and research into function of mononuclear phagocytes as we age demonstrates the variability in research methodology towards one aspect of innate immunity, and consequently the conflicting results [7]. It could be argued that the use of human cells *ex vivo*, from an age-range of donors, would provide the closest insight into functional changes in cells in human ageing.

The following studies used macrophages derived from human peripheral blood mononuclear cell isolations from an age-range of healthy donors (BMDM). The ability of BMDM to interact with, and respond to, apoptotic cells was investigated. This was to assess the hypothesis that reduced capacity for MØ from aged individuals to be turned off effectively by dying cells may contribute to inappropriate control of the immune system as we age. To investigate the influence of age-related environments on apoptotic cell clearance was also studied, by co-culture of THP-1-derived MØ with apoptotic cells following pre-treatment with plasma samples from individuals of different ages. Investigation into apoptotic cell clearance mechanisms as a result of an aged cell, or an aged environment, may provide clues as to at what point control is lost in the atherosclerotic plaque.

Given the inflammatory environment in the atherosclerotic plaque, despite high levels of cell death, it is hypothesised that the inflammatory environment may result from apoptotic

MØ and apoptotic foam cells exerting an inflammatory effect by aged MØ. Research questions covered in this chapter are shown in the following table.

	Phagocyte	
Function	**Aged HMDM**	**THP-1 derived MØ + aged plasma**
Interaction with AC	Do MØ from mid-life donors interact with AC as efficiently as MØ from young donors? Is CD14-dependent interaction the same in MØ from young and mid-life MØ?	Does an aged environment impair MØ ability to interact with AC?
Immune modulation	Do MØ from mid-life donors respond to stimuli in the same manner as MØ from young donors? Can an inflammatory response be switched off effectively in MØ from mid-life donors?	

Table 9. Summary of key research questions addressed in Chapter 6

6.2. Results

6.2.1. Participant statistics; age and gender groups

Recruitment of local healthy donors was carried out for preliminary experiments under existing ethical approvals. Preliminary data gathered in this pilot study would then be used for study design for larger cohorts recruited through the Aston Research Centre for Healthy Ageing (ARCHA) following ethical approval for a larger scale study, where individuals could be recruited externally from a broader age-range.

Data in Figure 48 show that 44 participants were recruited, from an age range of 18-58 years. This data is the total number of participants recruited, and encompasses successful blood donations, and also donations or monocyte isolations that were not successful. The number of monocytes isolated per participant also varied greatly, so some participants were

used in individual studies only, and some were used across all studies. Interesting to note is the skew of gender groups. Males are the more prominent gender in younger age groups (18-26yrs), whereas females are the more frequent gender in the older age groups (46-57yrs), which should be noted when interpreting some of the following data. As participants from older age groups were not readily available, young (age <25 years) and mid-life age (age >40 years) age groups were compared, excluding results from some participants. This is a limiting factor of this cohort, as access to the 'healthy aged' population was lacking.

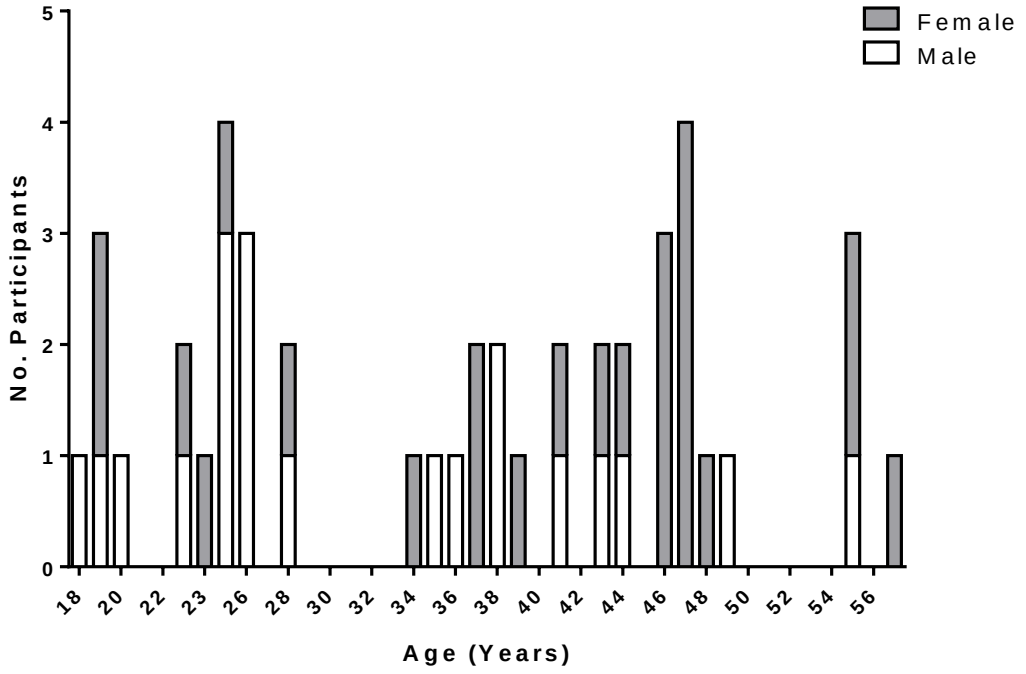


Figure 48. Summary of participants; age and gender *Results show the cohort of participants in this pilot study, revealing an age range of 18-57 years, and a skew towards males in younger age groups (age<25 years) and women in older age groups (age>40 years). n=44.*

6.2.2. Comparison of function in young versus mid-life primary human monocyte-derived macrophages

Both persistence of ACs, and increased autoimmune disease in the elderly has been observed[11,33]. These processes may also contribute to increased systemic inflammation in aged individuals, as they have been shown to have roles in immune modulation[14,391]. This is supported by data on C1q deficiency, where persistence of AC has been shown to drive autoimmunity[392]. CD14 is the prototypic pattern recognition receptor, with roles in inflammatory responses and AC clearance[31]. Whether CD14 contributes to reduced clearance by MØ in aged individuals is unknown, however reduction of CD14 in aged murine splenic MØ has been reported[393,394].

The ability of HMDM to interact with UV-induced apoptotic Jurkat cells was assessed in the presence or absence of the monoclonal CD14 blocking antibody (mAb) 61D3, and compared to the non-blocking mAb 63D3[31] (Fig. 49). Results show no significant differences between AC interaction between age groups, either in the presence or absence of a CD14 blocking antibody, however both groups displayed a trend in reduced AC interaction in the presence of 61D3.

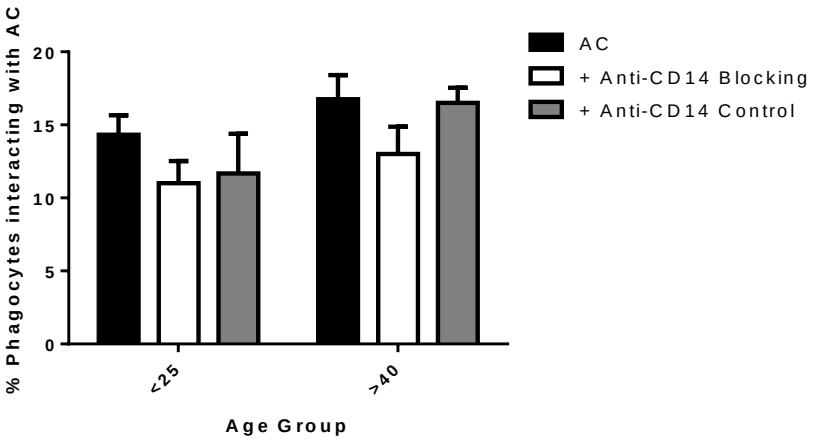


Figure 49. CD14 dependent and independent apoptotic cell interaction by young and mid-life primary human monocyte-derived macrophages *Human monocyte-derived MØ (HMDM) were co-cultured with aJK, in the presence or absence of CD14 blocking antibody 61D3, or non-blocking CD14 binding antibody control (63D3) (1h; 37C). Unbound AC were removed by washing, cells stained with Diff-Quik and the number of MØ interacting with AC was counted out of 200 cells per well. Data shown are mean % interaction ± SEM of 3 (age<25) or 4 (age>40) independent experiments. Data analysed using two way ANOVA with Tukey's* post hoc *test.*

As the ability of MØ to interact with AC did not appear to be impaired between young and mid-life age groups, it was then investigated whether immune-modulation was comparable between age groups following stimulation with pro- or non-inflammatory mediators, given the link between inflammation and ageing, and the hypothesis that a lack of appropriate MØ control is the source[14].

The levels of TNF-α release was assessed without MØ stimulation to investigate whether basal levels of inflammatory cytokine release were associated with either age group (Fig. 50). Results showed no significant differences in TNF-α release between age groups in unstimulated MØ. TNF-α response to apoptotic Jurkat is the same by MØ from young and mid-life age groups. Trends showed increased TNF-α release in response to LPS, compared to basal and AC co-culture, by MØ from both young and mid-life age groups, as seen with THP-1-derived MØ (Fig. 19; Chapter 4). These results were not found to be significantly

different, however an increased sample size would boost statistical power of these assays. Trends suggest an increased TNF-α response by MØ from mid-life donors, compared to young donors, in response to LPS.

To investigate the ability of apoptotic Jurkat to 'switch-off' an inflammatory response by MØ, as seen in THP-1-derived and primary MØ previously (figs. 16/20; Chapter 4), MØ were co-cultured with apoptotic Jurkat prior to addition of LPS (Fig. 50). Interestingly, whilst trends showed AC-induced reduction in LPS-induced TNF-α release in young MØ (as seen in Fig. 23), ACs did not appear to switch off the TNF-α release in mid-life MØ, potentially implicating inappropriate control of immune responses by MØ as we age.

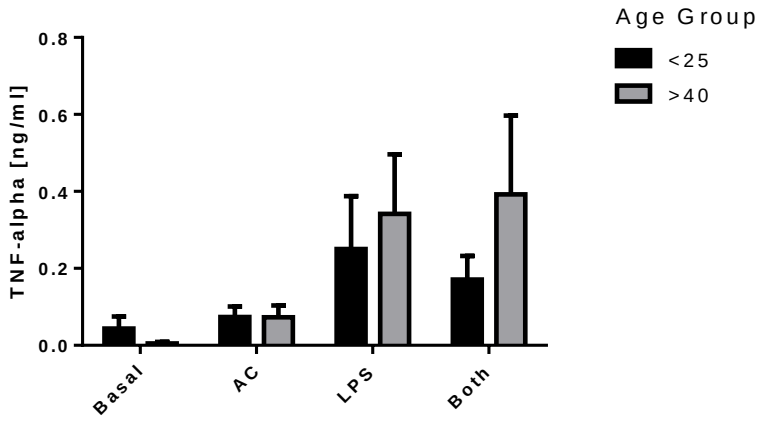


Figure 50. Immune modulation of young and mid-life primary human monocyte-derived macrophages by apoptotic cells *HMDM were co-cultured with aJK or CDMØ-medium control (20h; 37C). 5ng/ml LPS or CDMØ-medium control was added to relevant wells with normal human serum (10% v/v final conc.) and incubated (4h; 37C). Samples were diluted 1/5 in sfRPMI prior to ELISA analysis. TNF-α ELISA was carried out according to manufacturer's instructions (PeproTech Ltd). Data shown is mean ± SEM of 5 independent experiments per age group. Data analysed using two-way ANOVA with Tukey's* post-hoc *analysis.*

6.2.3. Effect of young versus mid-life plasma on THP-1 macrophage ability to interact with apoptotic cells

As human ageing studies are based largely on observation of end-points, such as cytokine profile in the blood, or presence of apoptotic cells *in situ*, it is unknown whether apparent defects in immune modulation are a result of altered functional ability of ageing cells, or the effect of an aged environment on what would be otherwise fully functional cells. Results above (Fig. 49) suggest no change in cell function, with regards to AC clearance, however failed clearance may be impaired *in vivo* if cells are affected by an age-related environment. This could have particular relevance with regards to AC clearance due to the necessity for

bridging molecules in efficient clearance. This area is currently understudied in humans, though some data suggests an increase in anti-C1q autoantibodies in aged individuals[350]. An inflammatory environment can also reduce AC clearance, an environment associated with ageing, as demonstrated by Aprahamian *et al.* in mice[6,14,57].

To this end, the effect of plasma on the ability of THP-1-derived MØ to interact with apoptotic Jurkat cells was investigated. THP-1 MØ provide a consistent model system with a baseline ability for AC interaction. Differentiated THP-1 MØ were cultured in sfRPMI with 10% (v/v) plasma (72h; 37C) to investigate any long-term effects of young or mid-life plasma on function. AC (UV-induced Jurkat) interaction assays were then carried out with pre-treated THP-1 MØ, in the presence of plasma from the same donor as pre-treatment (10% v/v), or in the absence of plasma. This will differentiate between the effects of young versus mid-life plasma conditioning on THP-1 MØ function, and the immediate effects of donor plasma presence on assisting phagocyte:AC interaction, e.g. via bridging molecules or complement components.

Results showed that THP-1 MØ conditioning with young versus mid-life plasma did not significantly alter THP-1 MØ ability to interact with apoptotic Jurkat cells (Fig. 51), though a very small decrease in AC interaction following mid-life plasma conditioning could prove interesting given a larger cohort or older participants (grey bars). This effect was abrogated in the presence of plasma, which appeared to restore full capacity for AC interaction (black bars). These results are not significant, but given a larger cohort, it would be interesting to pursue the hypothesis that an age-related environment may decrease macrophage function.

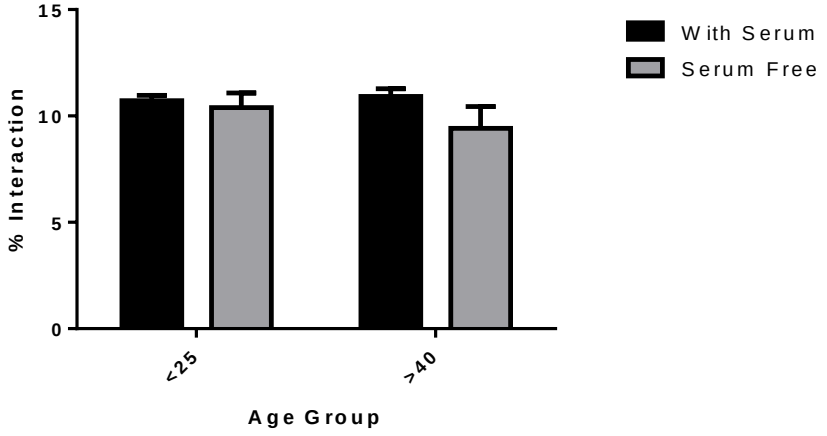


Figure 51. Effects of young versus mid-life plasma samples on THP-1-derived MØ function *DS differentiated THP-1, seeded at 2x10^4 cells/well in 24 well plates, were treated with young or mid-life donor plasma (72h; 37C). 16-20 hours post UV-induction, apoptotic Jurkat were resuspended in fresh sfRPMI, medium in all wells also replaced, and phagocytes*

co-cultured with a 10:1 ratio of AC:MØ , in the presence or absence of the same donor plasma (1h; 37C). Unbound AC were removed by washing and samples fixed. To count, cells were stained with Diff-Quik and the number of MØ interacting with AC was counted out of 200 cells per well No significant differences were found (two way ANOVA with Tukey's post hoc *test). Data shown are mean % interaction ± S.E of 3 (age<25) or 4 (age>40) independent experiments.*

6.3. Discussion

Increased susceptibility to infection implying loss of immune function and, conversely, an increase in immune cell activation, leading to autoimmune disease, have been linked with ageing[1,7,14,9,10]. Autoimmune diseases, e.g. rheumatoid arthritis, and increased autoantibody titre have been associated with ageing as a risk factor [11,12], suggesting inappropriate control of the immune system as we age.

Results from this chapter show no significant differences between the ability of MØ from young and mid-life donors to interact with or respond to apoptotic cells. The use of plasma from mid-life donors does not affect the ability of MØ to interact with AC and vice versa. It is likely that much bigger sample sizes would be required to establish significant differences.

	Phagocyte	
Function	**Aged HMDM**	**THP-1 derived MØ** **+ aged plasma**
Interaction with AC	MØ from mid-life donors interact with AC as efficiently as MØ from young donors. CD14-dependent interaction is the same in MØ from young and mid-life MØ.	An aged environment does not significantly impair MØ ability to interact with AC.
Immune modulation	MØ from mid-life donors respond to stimuli in the same manner as MØ from young donors.	

	Inflammatory responses can be switched off effectively in MØ from mid-life donors.	

Table 10. Summary of results from key research questions addressed in Chapter 6

Adaptive immune responses are more widely studied than innate immunity in ageing research, and even within monocyte and macrophage research on ageing, responses to pathogenic challenge have held the focus[395]. Of the MØ roles that have been studied, primarily in murine models, defective MØ function is implied. Impaired peritoneal macrophage function was found with ageing in BALB/c mice with regard to adherence, opsonisation, phagocytosis and antibody-dependent cell cytotoxicity[23].

PBMC-derived monocytes can be differentiated into human monocyte-derived MØ (HMDM), as was used in this pilot study. Previous studies from monocytes isolated from aged individuals display a decrease in IL-6 and TNF production following LPS activation, as observed in murine models, which was attributed to deficient PKC and MAPK activation[32]. A drawback of using PBMC-derived macrophages is the comparability with tissue macrophages, another advantage of murine models, where tissue macrophages can be used. Because of this, human monocytic cell lines are often used instead of PBMCs due to accessibility and ease of use and comparable disadvantages.

Some evidence suggests reduction in apoptotic cell (AC) clearance with ageing in mice[6]. Aprahamian *et al*. found more apoptotic keratinocytes in skin samples harvested 24 hours post UV-irradiation in skin from aged mice, in comparison to young mice[33]. This could also be explained by increased susceptibility to UV-induced cell death by aged mice, as it is only a correlative result. Further study by the same authors found that aged (thioglycollate) elicited peritoneal MØ were less efficient at clearing injected apoptotic (human) Jurkat cells than their young counterparts[33]. This study found no significant differences in the ability of aged murine MØ to clear AC *in vitro*, however addition of aged murine serum, on young murine MØ, resulted in a reduction in AC clearance[33]. This was supported by trends in data shown here, where no significant differences were observed, between MØ from young and mid-life donors, to interact with apoptotic Jurkat cells (Fig. 49). A very modest trend in the ability aged plasma to reduce THP-1 derived-MØ interaction with AC was observed, and may show significant differences with the use of a larger cohort (Fig. 51).

Research into this area in human cells is sparse, and the relationship between failed AC clearance and inflammation in ageing tends to be correlative rather than causative in nature[6,36]. For example the presence of autoantibodies in ageing is linked to failed AC

clearance[6,12]. Studies on macrophage phagocytosis have largely focused on that of microbial material or beads, whereas studies into AC uptake in aged cells are absent.

The initial aims of this pilot study were to identify mechanisms behind suggested defective clearance of ACs, and subsequent immune modulation, with ageing and age-related disease[33,195]. Given time constraints and challenges in recruiting aged individuals, these studies could not be undertaken as originally planned, however future work could focus on several areas of AC clearance. The release of 'find-me signals by AC to attract phagocytes precedes AC-phagocyte contact, e.g. ICAM-3 or CX3CL1[61,71]. Whether these 'find-me' signals are recognised by phagocytes, or released by ACs, in ageing and age-related disease is unknown. Work presented here indicates a role CX3CL1 in THP-monocyte chemotaxis to apoptotic MØ (Fig. 44; Chapter 5), a mechanism that could contribute to progression of atherosclerosis, a prominent condition in older adults[321]. The ability of phagocytes to migrate towards ACs throughout ageing and age-related disease is yet to be fully established, and could be investigated using chemotaxis assay methods outlined in Chapter 5. Immune cells from young and aged donors could be used as the phagocyte and/or the apoptotic cell, to establish at which point defects in phagocyte recruitment may, if relevant, come about.

Expression of receptors responsible for AC recognition may also be downregulated, or impaired, in phagocytes as age progresses. For example, CD14 expression was found to be reduced in peritoneal and splenic MØ from aged C57BL/6 (B6) mice[393,394], and splenic MØ from BALB/c mice[393], however expression in human monocytes and MØ with ageing has not been extensively studied. As CD14 has often been used as a marker for monocytes, it is likely that the data already exists, but requires analysis in conjunction with ages of participants. Data presented here (Fig. 49) does not show any reduction in MØ interaction with AC from young or mid-life adults, nor any increased or decreased dependence on CD14 mediated recognition of AC. This does not mean the same can be said for older age groups, and the sample size is small, but data of this nature is lacking and could be studied with larger cohorts.

ACs must present 'eat-me' signals, in conjunction with downregulation of 'don't-eat-me' signals found on viable cells[204]. Effective upregulation of 'eat-me' signals, such as phosphatidylserine (PS), or downregulation of 'don't-eat-me' signals, such as CD31 and CD47, may not be occurring in the aged or diseased environment, or recognition may be impaired by phagocytes under the same conditions. Alteration of existing antigens, such as intercellular adhesion molecule-3 (ICAM-3) has also been shown to have a role in AC clearance[84]. It is unclear whether surface expression in aged PBMCs is altered, but any

changes in expression could affect ability of phagocytes to clear apoptotic cells[391]. Investigation into surface expression of these antigens could provide further insight into defective apoptotic cell clearance in the elderly. Figure 49 did not find any defects in young versus mid-life adult MØ ability to recognise AC, however the ability of primary ACs from young and older age ranges were not studied, a cell line, apoptotic Jurkat cells, was favoured instead. The use of primary neutrophils, which undergo spontaneous apoptosis, are often used as models in AC clearance studies, and could also be utilised in the application of clearance of old versus young donor-derived ACs. This leads to the discussion however of the definition of an 'old' cell. The short half-life of neutrophils makes them popular AC models in clearance studies, however is a cell with a short life-span perpetually 'old', e.g. close to death, or 'young', e.g. new born? Will this effect apoptotic cell clearance mechanisms, and therefore skew *in vitro* studies?

If engulfment is inefficient this would also delay AC clearance; this is supported by previous research into defective clearance in the plaque[197,201]. Delayed clearance has also been linked with autoimmune disease, e.g. SLE[396]. As atherosclerosis is prominent in the aged population, it can be questioned whether increased lipid uptake in MØ and impairment of AC clearance in the plaque is due to impaired function of aged MØ, or a direct result of the high-lipid local microenvironment[195]. For example, would a lipid-laden foam cell show impaired clearance in a non-lipid environment? And would a MØ function fully in a high lipid environment? Do lipid-laden apoptotic cells elicit a different response in MØ than untreated apoptotic MØ? It may also be questioned whether, within the aged population, the incidence of atherosclerosis is increasing, with increases in disorders where CVD is exacerbated, such as obesity and diabetes[320,322]. These are also relevant questions to other MØ functions throughout ageing, including AC clearance. If clearance is defective in ageing, as studies by Aprahamian *et al*. suggest[33], is this a primary effect of reduced cell function through ageing, or the secondary effect of an aged environment, e.g. an inflammatory or high lipid environment.

Some preliminary work in this chapter has aimed to address this with the use of plasma from donors of different age groups (Fig. 51). Mediators found in aged environments could have primary effects on cell function, e.g. a loss of necessary bridging molecules in phagocyte:AC bridging molecules, or competition for AC receptors in a high lipid environment, as seen with oxLDL in the plaque[195]. Long term exposure to aged environments could also affect cell activity, especially if the inflammatory status associated with ageing, and the potential knock-on effects on MØ activation, are considered[29,37]. In this study, THP-1 MØ were conditioned with plasma isolated from young and mid-life donors, for 3 days (grey bars) (Fig. 51). Interaction with apoptotic Jurkat cells was then assessed, and although significant

differences were not found, a modest trend in reduced AC interaction was observed in THP-1 MØ following culture with mid-life plasma. Interaction rates were restored in the presence of plasma from the same donors, which may imply that chronic exposure to an aged environment, rather than the gain or loss of mediators required for AC interaction, may affect clearance in older adults. Gomez *et al*. carried out a study in rats, which showed pre-incubation with serum from aged rats impaired the ability of young rat MØ to release TNF-α, in response to LPS[397]. Aprahamian observed similar results trends with young mouse MØ, which showed reduced AC uptake following incubation with serum from old mice[6].

Following successful interaction with apoptotic cells, immune modulation occurs. This work, and work by others, has shown that apoptotic cells have the capacity to reduce inflammation following interaction with MØ[54-56] (fig .16; Chapter 4; Fig. 50). Results also shown here using HMDM, have shown that under certain conditions, immune-modulation by ACs may favour inflammation (Fig. 23; Chapter 4; Fig. 50). Figure 23 shows HMDM released TNF-α in response to THP-1-derived apoptotic MØ and apoptotic foam cells, a cell type induced by a high-lipid environment. Results in this chapter (Fig. 50) also implied that HMDM from mid-life donors released TNF-α in response to apoptotic Jurkat cells, a cell line not usually found to induce inflammation, though statistical significance was not reached (Fig. 23). These results have interesting implications on the roles of lipid-rich/aged environments (Fig. 23) or aged cells, in a failure to modulate immune responses appropriately.

The differences in recruitment of each gender group and the association with age groups should also be considered (Fig. 48), as disparities are present in inflammatory markers in man and women through ageing. For example Gale *et al.* found links between incident frailty risk and inflammatory markers in the blood, namely fibrinogen and CRP, were different between man and women[398].

It is important to note that reduced innate immune function could also be a result of a decrease in the population of innate immune cells. A decrease in CD68-positive macrophage-lineage cells has been observed in human bone marrow in aged subjects [21] which may account for the impact ageing has on innate immunity as macrophages are one of the first cells to respond to invading microorganisms. Wang *et al.* found an increase in Mac-1 positive cells, a murine macrophage antigen, in aged mice, representing the differences between species [22]. This is important to note as murine models are popular in ageing research due to the short lifespan and viable source of tissue macrophages, allowing direct comparison of young and aged organisms from the same genetic background which have been kept under the same environmental conditions.

Overall, these studies provide some interesting clues as to where macrophage control may fail in ageing, however a much larger cohort, or alternative study design is required to achieve significant results, especially if additional markers such as cell surface expression want to be investigated (requiring more cells). Using whole blood or PBMC preparations rather than isolated monocytes may provide the cell numbers required for more powerful data, however this is that expense of investigating the effects of ageing on specific cell types.

6.4. Conclusions and future work

In conclusion, these studies imply some functions may be impaired as we age, starting as early as mid-life (>40 years). Significant differences were not observed due to small sample sizes, however trends suggest immune-modulation following AC clearance may not promote resolution of inflammation in macrophages from mid-life donors. This would be interesting research to take forward to a larger cohort, given the hypothesis that ageing results in loss of control in macrophages. Bigger differences in young and aged MØ function may be observed with inclusion of younger and older participants, or of those with pathology such as atherosclerosis.

Further research into the effects of an aged environment, as used here with plasma conditioning, would provide interesting insights into which aspects of 'inflammaging' are a result of loss of cell function, rather than a direct influence of an aged environment.

As such little research in this area has been done in human cells, there is much scope for expansion of this study.

Discussion

As discussed throughout this thesis, the atherosclerotic plaque is a highly inflammatory site[58], despite the presence of large numbers of MØ and ACs[46], which, when interacting, should dampen inflammation[54-56]. To resolve inflammation, recruitment of inflammatory cells must cease, apoptotic cells must be cleared and egress of live inflammatory cells must take place[151]. It is apparent that control of one or all of these mechanisms must be functionally defective in the atherosclerotic plaque if resolution of inflammation is not achieved, however whether that is a result of the local microenvironment or a loss in cell function, or both, is not known.

A similar loss of immunological control has been observed with ageing. A reduced response to pathogenic challenge (e.g. cytokine), a systemic increase in inflammatory markers and an increase in autoantibodies in the presence or absence of overt pathology, all evidence that as we age, control of immune responses is becoming less tightly regulated[3,12].

Monocyte recruitment to the plaque is a very early stage of plaque formation[45] Research on recruitment of monocytes to the plaque, a process that also drives plaque progression, has centred around the role of inflammatory mediators on monocyte recruitment to the artery wall, via modulation of endothelial cell (EC) responses, and chemoattractant and adhesion molecule expression by ECs. Recruitment to apoptotic cells following diapedesis through the EC layer has not been investigated, and is largely overlooked by popular methods of assaying AC clearance, where ACs are loaded directly onto adherent phagocytes. An emerging role of MPs in the attraction of monocytes to sites of cell death is also of interest, as although MPs have been studied in atherosclerosis, it is in the context of biomarkers to deduce pathology and risk of acute events[399], and as mediators of inflammation, particularly in endothelial cells[268]. The role of MPs in phagocyte recruitment to apoptotic cells in the plaque has not been assessed.

Despite much research into the immunological background of atherosclerosis over the last two decades, research around apoptotic cells in the plaque has focused on dissecting out the inflammatory potential of individual components of modified LDL, the mechanisms of apoptosis induction in MØ, and whether MØ apoptosis is beneficial or detrimental to plaque pathology. Clearance of apoptotic cells is likely to have a multifactorial impact on atherogenesis and plaque progression, and only a handful of papers have investigated this in detail. Across one key paper on defective apoptotic cell clearance in atherosclerosis, observations, and investigated subsequent mechanisms behind these, were spread across a variety of models. These included human endarterectomy samples, rabbit endarterectomy

samples, murine thioglycollate-elicited peritoneal MØ (phagocyte), murine J774 macrophage cell line (phagocyte) and human U937 monocyte cell line (AC)[195]. Persistence of AC and inflammation are contributing factors to unstable plaque formation and subsequent rupture. If mechanisms of AC clearance in an environment that mimics features of the atherosclerotic plaque can be better understood, means of clearing persistent AC and dampening inflammation may be elucidated.

Data is virtually non-existent on modulation of inflammatory responses following corpse clearance in the plaque, and data on human cells is completely absent. The effect on apoptotic cell-elicited immune-responses, and the impact on this following lipid-loading is unknown in humans, and only partially explored in mice, as is the immuno-modulatory effects of apoptotic cells on aged MØ. A more complete understanding of apoptotic cell clearance mechanisms, including phagocyte recruitment and immune responses, in both ageing and atherosclerosis, would contribute to, and likely link further, both research areas.

7.1. Modelling clearance mechanisms in human atherosclerosis

From an immunological perspective, the atherosclerotic plaque is extremely complex. The full interplay between live and dead resident cells, live and dead recruited inflammatory cells, lipid-loaded cells, modified lipids and inflammatory mediators may be impossible to fully characterise *in vitro*, making assays on excised endarterectomy samples attractive[58,195]. However, taking into account individual variation on genetic background and metabolic age, and the fact that data is correlative, full insights into the mechanisms driving inflammation and atherosclerosis would remain ill-defined without broader methods of investigation. With evidence gained from endarterectomy samples, a comprehensive collective of cell types, cell status (i.e. live, stressed or dead), and inflammatory mediators have been reported[137,147], which can allow more accessible *ex vivo* and *in vitro* methods to be utilised whilst still being relevant to mechanisms within the plaque.

In a bid to model potential clearance mechanisms akin to those observed in the atherosclerotic plaque, with a consistent model of phagocytes and AC, the monocytic cell line THP-1 was chosen, and model systems developed to utilise in the investigation of multiple stages of apoptotic cell clearance. Much research into AC clearance uses accessible cell types that are easy to manipulate, rather than use the most physiologically relevant model which, in this case, is apoptotic MØ[46]. Often research uses a multitude of cell lines that span across cell lineages and species[195]. Some additional human AC models (e.g. Mutu and Jurkat) were brought in as controls and to enable comparison of results across studies, the key aim of these studies was to use a representative human cell line to investigate the roles

of monocytes and macrophages in mechanisms that may be of key importance to atherogenesis and subsequent pathology.

The THP-1 model approach used here was then broadened to include human monocyte-derived MØ for comparison between human cell lines and primary human cells. This yielded some interesting results, and highlighted strengths and limitations of THP-1 as a monocyte/MØ model, which could have been overlooked in other functional monocyte/MØ research.

Overall, models used here show that the ability of FC, compared to MØ, to interact with apoptotic cells is decreased in some, but not all cases, and that results were comparable across cell line and primary cell studies.

Figure	Function	MØ	FC	HMDM
13	Receptor expression		No significant changes	
14	Interaction with aJK	30% interaction	20% interaction (P<0.005)	
15	oxLDL blocking of AJK binding	Not found	Not found	
16	Interaction with *E. coli*	Functional	Functional	
16	Phagocytosis of *E. coli*	Functional	Functional	
17	Interaction with aMØ	20%	20%	20%
17	Interaction with aFC	20%	20%	20%
19/23	Basal TNF-α release	Lo	Lo	Lo
19/23	TNF-α response to LPS	Hi	Hi	Med
19/23	TNF-α response to aJK	Lo	Lo	Lo
19/23	TNF-α response to LPS + aJK	Knocked down (P<0.05)	Knocked down (ns)	Knocked down (ns)
21/23	TNF-α response to aMØ	Lo	Lo	Hi
21/23	TNF-α response to LPS + aMØ	50% Knock Down (P<0.05)	60% Knock Down (P<0.01)	Hi
21/23	TNF-α response to aFC	Lo	Lo	Hi
21/23	TNF-α response to LPS + aFC	50% Knock Down (P<0.01)	60% Knock Down (P<0.0001)	Hi
26	Inducer of TNF-α response	Cause of TNF-α response cell-derived and soluble/MP derived	Cause of TNF-α response cell-derived and soluble/MP derived	

27	Apoptotic CFS as an inducer of vertical chemotaxis	No significant induction of monocyte chemotaxis	Significant induction of monocyte chemotaxis P<0.05	
35/36	Monocyte migration to live WC	Directional +	Directional ++	
35/36	Monocyte migration to live CFS	Directional ++	Directional ++	
35/36	Monocyte migration to apoptotic WC	Directional +	Directional +	
35/36	Monocyte migration to apoptotic CFS	Directional ++	ns	
37	Summary of monocyte migration	Directional	Directional/disordered	
40	Monocyte migration to aCFS w/ CD14 blocking	Migration not inhibited	Migration not inhibited	
41	Monocyte migration to aCFS w/ ICAM-3 blocking	Migration not inhibited	Reduced migration (ns)	
44	Monocyte migration to aCFS w/ CX3CL1 blocking	Reduced migration P<0.05	Reduced migration (ns)	
47	HUVEC E-selectin expression following live CFS treatment	Upregulation of E-selectin expression (P<0.01)	Upregulation of E-selectin expression (P<0.05)	
47	HUVEC E-selectin expression following apoptotic CFS treatment	Upregulation of E-selectin expression (P<0.01)	Upregulation of E-selectin expression (P<0.01)	
		Young	***Old***	
49	CD14 dependence for interaction	Comparable	Comparable	
50	TNF-α responses to AC and LPS	Comparable	Comparable	
51	THP-1 MØ rates of interaction following serum conditioning	Comparable	Comparable	

Table 11. Summary of macrophage versus foam cell function and young vs. mid-life function

7.2. Role of monocyte recruitment, apoptotic cell clearance, modulation of immune response, inflammation and ageing in atherosclerotic plaque progression

Previous research indicates a reduced phagocytic efficiency of MØ in atherosclerosis[195,197]. Data presented here reveal that THP-1-derived MØ, following lipid-loading, have a decreased ability to clear apoptotic Jurkat cells (Figure 14), but interaction with apoptotic MØ/FC was unchanged (figs. 14/15), indicating a case for clear definitions of AC models. Despite efficient interaction of THP-1-derived MØ with apoptotic MØ/FC, and non-inflammatory immune responses to these cells by interactions, primary human monocyte-derived MØ (HMDM) behaved differently. Data presented here show, for the first time in human cells, primary HMDM release TNF-α in response to THP-1-derived apoptotic MØ and apoptotic FC, but not apoptotic Jurkat (Fig. 23). This supports research by Li *et al.* in murine peritoneal MØ, which demonstrated an inflammatory response to free-cholesterol loaded apoptotic MØ[210], however these comparisons have not been done before in human cells.

Apoptotic Jurkat dampened LPS-induced TNF-α release by HMDM (Fig. 23), indicating a possible classical to alternative phenotype switch, however inflammation was not dampened, but enhanced, by apoptotic MØ and apoptotic FC. Studies by Lucas *et al*. found that apoptotic neutrophils can boost an early LPS-induced TNF-α response (0-2h post LPS exposure), and that this is dampened in the presence of ACs after 8 hours co-culture, so timings may be crucial[358]. However, apoptotic neutrophils in the absence of LPS were non-inflammatory[358]. Whilst data here indicate apoptotic MØ and apoptotic FC are strongly pro-inflammatory, future work is now required to assess the inflammatory effect of apoptotic primary HMDM and their apoptotic foam cell counterparts.

Lucas *et al*. also showed that apoptotic neutrophils do not switch off a TNF-α response in the presence of IFN-γ, suggesting that IFN-γ overrides AC-mediated resolution of inflammation[358]. IFN-γ stimulates a pro-inflammatory, or classical, phenotype of MØ activation, and has been found in human atherosclerotic plaques[49,58,400]. Classically activated MØ have also been associated with less efficient clearance of apoptotic cells, which is perpetuated in an autologous manner by TNF-α[357]. Studies in specialised MØ (microglia) in mice have also found reduced responsiveness to alternative activating inducer IL-4 in aged cells[401]. Aged peritoneal MØ were also found to produce increased oxygen free radicals, compared to their young counterparts, following treatment with latex and zymosan[402], which could have implications when considering oxidative modification of retained lipoprotein in the sub-endothelium, and the overall pro-inflammatory milieu.

Taken together these data suggest that monocyte entry into early plaques is likely to be athero-protective, but following entry into an inflammatory environment, with possible

contribution from TNF-α-inducing apoptotic MØ and apoptotic FC, a classically-activated MØ phenotype may be induced. Classical activation of MØ in the plaque is not a new theory, but the contribution of apoptotic MØ and apoptotic FC to inflammation has not been shown previously *in vitro* in HMDM. Adopting an inflammatory phenotype reduces clearance efficiency by MØ[57]. The contribution of failed phenotype switching to an alternative phenotype by aged MØ may also contribute to plaque formation in the aged population[401], however this needs further study (Fig. 50). Reduction in monocyte recruitment, inflammation or promotion of reparative MØ phenotypes could be key areas for targeted research into atherosclerosis therapies.

Monocyte recruitment is an established initiating factor of plaque formation, following subendothelial lipid retention[45]. It could be debated whether monocyte recruitment following plaque formation is pro- or anti-atherogenic. It has been suggested that apoptosis of plaque MØ, and therefore recruitment of phagocytes for clearance, is beneficial to early atherosclerotic lesions, due to immuno-modulatory effects of engulfment and the clearance of corpses prior to secondary necrosis[149]. As phagocytic efficiency appears to be lost in more developed lesions, apoptotic cells persist and continual recruitment of monocytes is not likely to be of benefit[149]. The role of apoptotic MØ/FC 'find-me' mediator release has not been investigated, either in monocyte recruitment from the blood stream, or in directed monocyte recruitment to apoptotic MØ/FC following diapedesis. Results here suggest roles for ICAM-3 and CX3CL1 in monocyte recruitment to aMØ/aFC (figs. 38/40).

Results in these studies show that apoptotic MØ and apoptotic FC may use alternative mechanisms to recruit phagocytes for clearance. Monocyte migration to apoptotic MØ is significantly reduced by blocking CX3CL1, but significant reduction is not seen in apoptotic FC-induced monocyte migration (fig.41). This could result from an upregulation of alternative mechanisms, e.g. release other 'find-me' mediators, by apoptotic FC, or non-saturated blocking of CX3CL1 in FC, due to excess secretion or shedding via MPs. This could be further investigated by assessing concentrations of 'find-me' mediator release in conditioned medium, as shown in preliminary data (Fig. 46). In contrast, ICAM-3 blocking showed a trend in reduction of monocyte recruitment to apoptotic FC, but not in recruitment to apoptotic MØ. Whether these finding are a result of upregulation use of some 'find-me' mediators one AC type, or downregulation of 'find-me' mediators in other cell types, remains to be elucidated. The interesting part of this research is that phagocyte recruitment mechanisms by AC vary following culture in an aged environment. This could imply a loss of control of monocyte recruitment by cells in an aged-environment, especially if you consider that data together suggest that apoptotic FCs are potent but less direct inducers of monocyte migration. Trends suggest that more monocytes migrate (Fig. 27), and they may migrate further in distance

(Fig. 38), towards apoptotic FC-derived CFS, but that this is less ordered than recruitment of other AC types (Fig. 37). Investigating a wider spectrum of chemokine release by these cells, including 'keep-out' signals, and expanding the model to primary human cells, would provide further insight on disordered monocyte recruitment to the plaque, and whether mediators such as ICAM-3 and CX3CL1 could be targets for future intervention. CD14 was not shown to play a role in AC-induced chemotaxis in either model, so future work should encompass alternative receptors for 'find-me' mediator interaction, as this is poorly defined if you consider the role of microparticles in chemotaxis induction.

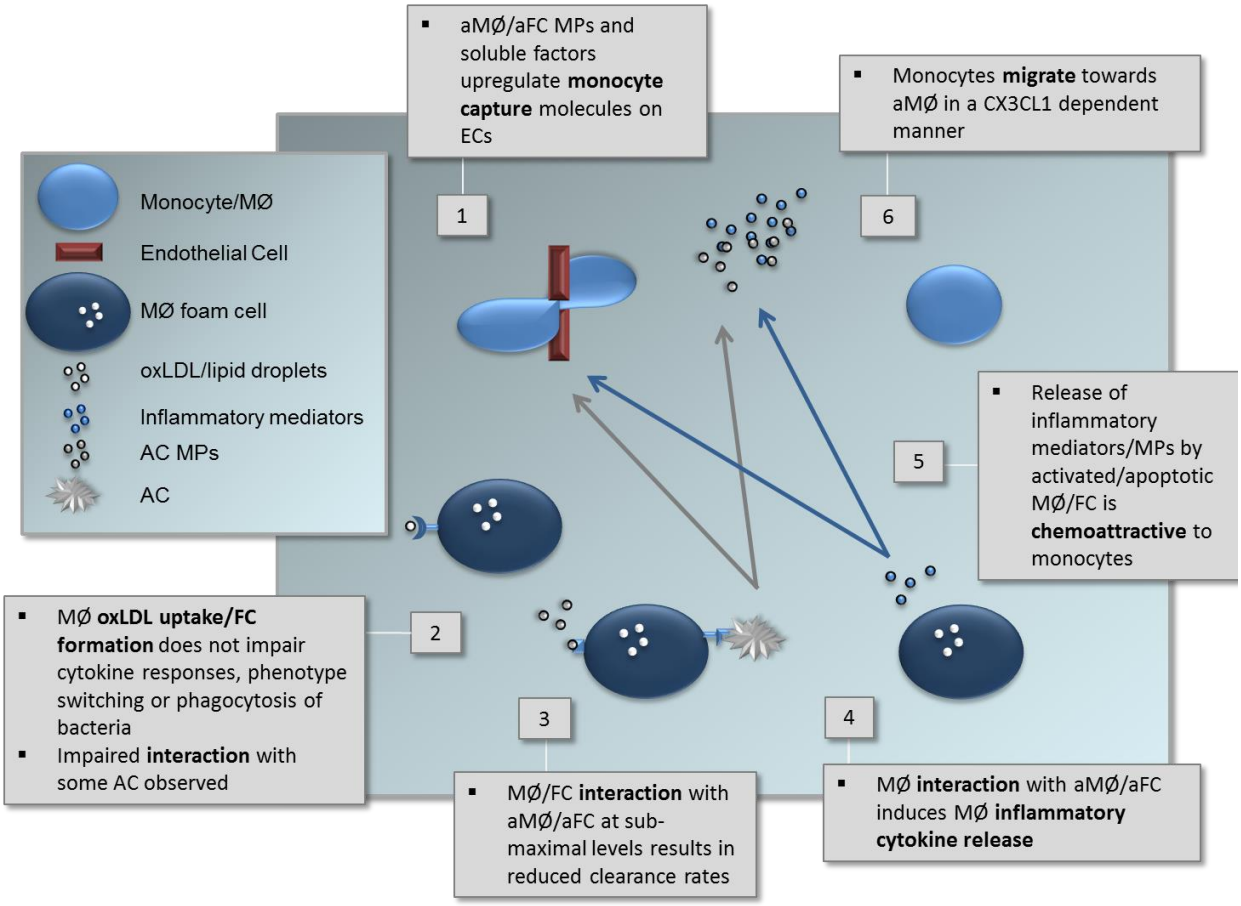


Figure 52. The role of monocytes/macrophages in plaque progression - schematic of results

Pilot study data did not show any differences in the capacity of HMDM from young and mid-life donors to interact with AC. However, it would be interesting to investigate the ability of AC to 'switch-off' inflammation in MØ from mid-life donors, versus those from young donors, further, and with a bigger sample size (Fig. 50). This could mean that as we age, resolving inflammation may become less controlled. Also, if you combine this with foam cell data from the THP-1 model, ACs in the plaque may induce inflammation and, in conjunction with this effect, aged monocyte-derived MØ may not respond to mediators that resolve inflammation.

The modest trend in reduced THP-1 MØ:AC interaction following culture in an 'aged' environment, using plasma samples from mid-life donors, may also contribute to understanding of pathology in ageing and atherosclerosis (Fig. 51). The use of serum from young and old donors is an accessible method of studying MØ function in an aged environment, and whether ageing at the cellular level, or in the local microenvironment, has a greater effect on modulation of immune responses, and the potential loss of control as we age.

Overall, work here has established a flexible and robust *in vitro* model for the derivation of apoptotic MØ/FC, and assays of MØ attraction to, and clearance of, AC. Coupled to this, the responses of phagocytes, in terms of migration and inflammatory mediator release, have been assessed. This shows data that suggest apoptotic MØ and apoptotic FC are strongly pro-inflammatory to HMDM. A potential role for AC-derived CX3CL1 in recruitment of monocytes to apoptotic MØ and apoptotic FC has also been shown for the first time. CD14 was not found to have a prominent role in recruitment of monocytes to apoptotic MØ or apoptotic FC, but there is a trend that implicates AC-derived ICAM-3 in the recruitment of monocytes to apoptotic FC, but possibly not to apoptotic MØ (fig 38). This raises the possibility that ICAM-3 may be a potential target for therapy in atherosclerosis. Whilst no single therapy may be effective, combined therapies as used in cancer can often provide excellent results.

MØ from mid-life individuals have also be shown to be less responsive to resolution of inflammation by apoptotic cells so, if trends are reproduced in larger studies, these factors would come together to drive the inflammatory pathology. Taken together, data also suggests that CX3CL1 might be an ideal target to help modulate this complex pathology, by modulating monocyte recruitment to the atherosclerotic plaque.

References

1. Medawar PB. An Unresolved Problem of Biology. H.K. Lewis, London1952.
2. Holliday R. Aging is No Longer an Unsolved Problem in Biology. *Annals of the New York Academy of Sciences.* 2006;1067(1):1-9.
3. Solana R, Tarazona R, Gayoso I, Lesur O, Dupuis G, Fulop T. Innate immunosenescence: Effect of aging on cells and receptors of the innate immune system in humans. *Seminars in Immunology.* 2012;24(5):331-341.
4. Weinert BT, Timiras PS. Invited Review: Theories of aging. *Journal of Applied Physiology.* 2003;95(4):1706-1716.
5. Nancy JS. Inflammation in aging: cause, effect, or both? *Discov Med.* 2012;13(73):451-460.
6. Aprahamian T, Takemura, Y., Goukassian, D. and Walsh, K. . Ageing is associated with diminished apoptotic cell clearance in vivo. *Clinical & Experimental Immunology.* 2008;152:448-455.
7. Linton PJ, Dorshkind K. Age-related changes in lymphocyte development and function. *Nat Immunol.* 2004;5(2):133-139.
8. Gardner ID. The effect of aging on susceptibility to infection. *Rev Infect Dis.* 1980;2(5):801-810.
9. Sebastián C, Espia M, Serra M, Celada A, Lloberas J. MacrophAging: A cellular and molecular review. *Immunobiology.* 2005;210(2-4):121-126.
10. Prelog M. Aging of the immune system: A risk factor for autoimmunity? *Autoimmunity Reviews.* 2006;5(2):136-139.
11. Tutuncu Z, Kavanaugh A. Rheumatic Disease in the Elderly: Rheumatoid Arthritis. *Rheumatic Disease Clinics of North America.* 2007;33(1):57-70.
12. Hijmans W, Radil J, Bottazzo GF, Doniach D. Autoantibodies in highly aged humans. *Mechanisms of ageing and development.* 1984;26(1):83-89.
13. Singh T, Newman AB. Inflammatory markers in population studies of aging. *Ageing Res Rev.* 2011;10(3):319-329.
14. Franceschi C, Bonafe M, Valensin S, et al. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci.* 2000;908:244-254.
15. Franceschi C, Capri M, Monti D, et al. Inflammaging and anti-inflammaging: A systemic perspective on aging and longevity emerged from studies in humans. *Mechanisms of Ageing and Development.* 2007;128(1):92-105.
16. Weng NP. Aging of the immune system: how much can the adaptive immune system adapt? *Immunity.* 2006;24(5):495-499.
17. Njemini R, Bautmans I, Onyema O, Van Puyvelde K, Demanet C, Mets T. Circulating Heat Shock Protein 70 in Health, Aging and Disease. *BMC Immunology.* 2011;12(1):1-8.
18. Lloberas J, Celada A. Effect of aging on macrophage function. *Experimental Gerontology.* 2002;37(12):1325-1331.
19. Zhang X, Mosser D. Macrophage activation by endogenous danger signals. *The Journal of Pathology.* 2008;214(2):161-178.
20. Gordon S. Macrophage heterogeneity and tissue lipids. *The Journal of Clinical Investigation.* 2007;117(1):89-93.
21. Ogawa T, Kitagawa M, Hirokawa K. Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages. *Mechanisms of Ageing and Development.* 2000;117(1-3):57-68.
22. Wang CQ, Udupa KB, Xiao H, Lipschitz DA. Effect of age on marrow macrophage number and function. *Aging (Milano).* 1995;7(5):379-384.

23. De La Fuente M. Changes in the macrophage function with aging. *Comparative Biochemistry and Physiology Part A: Physiology.* 1985;81(4):935-938.
24. Ding A, Hwang S, Schwab R. Effect of aging on murine macrophages. Diminished response to IFN-gamma for enhanced oxidative metabolism. *J Immunol.* 1994;153(5):2146-2152.
25. Videla LA, Tapia G, Fernandez V. Influence of aging on Kupffer cell respiratory activity in relation to particle phagocytosis and oxidative stress parameters in mouse liver. *Redox Rep.* 2001;6(3):155-159.
26. Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, Sambhara S. Cutting edge: impaired Toll-like receptor expression and function in aging. *J Immunol.* 2002;169(9):4697-4701.
27. Boehmer ED, Goral J, Faunce DE, Kovacs EJ. Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. *J Leukoc Biol.* 2004;75(2):342-349.
28. de Gonzalo-Calvo D, Neitzert K, Fernández M, et al. Differential inflammatory responses in aging and disease: TNF-[alpha] and IL-6 as possible biomarkers. *Free Radical Biology and Medicine.* 2010;49(5):733-737.
29. Franceschi C, BonafÈ M, Valensin S, et al. Inflamm-aging: An Evolutionary Perspective on Immunosenescence. *Annals of the New York Academy of Sciences.* 2000;908(1):244-254.
30. Vega VL, de Cabo R, De Maio A. Age and Caloric Restriction Diets Are Confounding Factors That Modify the Response To Lipopolysaccharide By Peritoneal Macrophages in C57bl/6 Mice. *Shock.* 2004;22(3):248-253.
31. Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL, Gregory CD. Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature.* 1998;392(6675):505-509.
32. Delpedro AD, Barjavel MJ, Mamdouh Z, Faure S, Bakouche O. Signal transduction in LPS-activated aged and young monocytes. *J Interferon Cytokine Res.* 1998;18(6):429-437.
33. Aprahamian T, Takemura Y, Goukassian D, Walsh K. Ageing is associated with diminished apoptotic cell clearance in vivo. *Clinical and experimental immunology.* 2008;152(3):448-455.
34. Munoz LE, Janko C, Grossmayer GE, et al. Remnants of secondarily necrotic cells fuel inflammation in systemic lupus erythematosus. *Arthritis and rheumatism.* 2009;60(6):1733-1742.
35. Elliott MR, Ravichandran KS. Clearance of apoptotic cells: implications in health and disease. *The Journal of cell biology.* 2010;189(7):1059-1070.
36. Botto M. Links between complement deficiency and apoptosis. *Arthritis Res.* 2001;3(4):1-4.
37. Salminen A, Huuskonen J, Ojala J, Kauppinen A, Kaarniranta K, Suuronen T. Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging. *Ageing Research Reviews.* 2008;7(2):83-105.
38. Devitt A, Parker KG, Ogden CA, et al. Persistence of apoptotic cells without autoimmune disease or inflammation in CD14-/- mice. *The Journal of cell biology.* 2004;167(6):1161-1170.
39. Stuart LM, Takahashi K, Shi L, Savill J, Ezekowitz RA. Mannose-binding lectin-deficient mice display defective apoptotic cell clearance but no autoimmune phenotype. *Journal of immunology (Baltimore, Md. : 1950).* 2005;174(6):3220-3226.
40. Brouckaert G, Kalai M, Krysko DV, et al. Phagocytosis of necrotic cells by macrophages is phosphatidylserine dependent and does not induce inflammatory cytokine production. *Molecular biology of the cell.* 2004;15(3):1089-1100.
41. Surh CD, Sprent J. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature.* 1994;372(6501):100-103.
42. Monks J, Rosner D, Geske FJ, et al. Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. *Cell death and differentiation.* 2005;12(2):107-114.
43. Gregory CD, Devitt A. The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? *Immunology.* 2004;113(1):1-14.

44. Hopkinson-Woolley J, Hughes D, Gordon S, Martin P. Macrophage recruitment during limb development and wound healing in the embryonic and foetal mouse. *Journal of cell science.* 1994;107 (Pt 5):1159-1167.
45. Nakashima Y, Fujii H, Sumiyoshi S, Wight TN, Sueishi K. Early human atherosclerosis: accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration. *Arterioscler Thromb Vasc Biol.* 2007;27(5):1159-1165.
46. Bjorkerud S, Bjorkerud B. Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *The American journal of pathology.* 1996;149(2):367-380.
47. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest.* 2012;122(3):787-795.
48. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *Journal of leukocyte biology.* 2004;76(3):509-513.
49. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology.* 2008;8(12):958-969.
50. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity.* 2003;19(1):71-82.
51. Yona S, Jung S. Monocytes: subsets, origins, fates and functions. *Current opinion in hematology.* 2010;17(1):53-59.
52. Strauss-Ayali D, Conrad SM, Mosser DM. Monocyte subpopulations and their differentiation patterns during infection. *Journal of leukocyte biology.* 2007;82(2):244-252.
53. Grage-Griebenow E, Flad H-D, Ernst M. Heterogeneity of human peripheral blood monocyte subsets. *Journal of leukocyte biology.* 2001;69(1):11-20.
54. Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J Clin Invest.* 2002;109(1):41-50.
55. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. *Nature.* 1997;390(6658):350-351.
56. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest.* 1998;101(4):890-898.
57. McPhillips K, Janssen WJ, Ghosh M, et al. TNF-alpha inhibits macrophage clearance of apoptotic cells via cytosolic phospholipase A2 and oxidant-dependent mechanisms. *Journal of immunology (Baltimore, Md. : 1950).* 2007;178(12):8117-8126.
58. Frostegard J, Ulfgren AK, Nyberg P, et al. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis.* 1999;145(1):33-43.
59. Cullen Sean P, Henry Conor M, Kearney Conor J, et al. Fas/CD95-Induced Chemokines Can Serve as “Find-Me” Signals for Apoptotic Cells. *Molecular Cell.* 2013;49(6):1034-1048.
60. Segundo C, Medina F, Rodriguez C, Martinez-Palencia R, Leyva-Cobian F, Brieva JA. Surface molecule loss and bleb formation by human germinal center B cells undergoing apoptosis: role of apoptotic blebs in monocyte chemotaxis. *Blood.* 1999;94(3):1012-1020.
61. Truman LA, Ford CA, Pasikowska M, et al. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood.* 2008;112(13):5026-5036.
62. Lauber K, Bohn E, Kröber SM, et al. Apoptotic Cells Induce Migration of Phagocytes via Caspase-3-Mediated Release of a Lipid Attraction Signal. *Cell.* 2003;113(6):717-730.
63. Elliott MR, Chekeni FB, Trampont PC, et al. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature.* 2009;461(7261):282-286.

64. Imai T, Hieshima K, Haskell C, et al. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell.* 1997;91(4):521-530.
65. Matsunawa M, Isozaki T, Odai T, et al. Increased serum levels of soluble fractalkine (CX3CL1) correlate with disease activity in rheumatoid vasculitis. *Arthritis and rheumatism.* 2006;54(11):3408-3416.
66. Yajima N, Kasama T, Isozaki T, et al. Elevated levels of soluble fractalkine in active systemic lupus erythematosus: Potential involvement in neuropsychiatric manifestations. *Arthritis & Rheumatism.* 2005;52(6):1670-1675.
67. Gaipl U, Franz S, Voll R, Sheriff A, Kalden J, Herrmann M. Defects in the disposal of dying cells lead to autoimmunity. *Curr Rheumatol Rep.* 2004;6(6):401-407.
68. Fong AM, Robinson LA, Steeber DA, et al. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *The Journal of experimental medicine.* 1998;188(8):1413-1419.
69. Miksa M, Amin D, Wu R, Ravikumar TS, Wang P. Fractalkine-induced MFG-E8 leads to enhanced apoptotic cell clearance by macrophages. *Molecular medicine (Cambridge, Mass.).* 2007;13(11-12):553-560.
70. Weiss SJ. Tissue destruction by neutrophils. *The New England journal of medicine.* 1989;320(6):365-376.
71. Torr EE, Gardner DH, Thomas L, et al. Apoptotic cell-derived ICAM-3 promotes both macrophage chemoattraction to and tethering of apoptotic cells. *Cell death and differentiation.* 2012;19(4):671-679.
72. Peter C, Waibel M, Radu CG, et al. Migration to apoptotic "find-me" signals is mediated via the phagocyte receptor G2A. *The Journal of biological chemistry.* 2008;283(9):5296-5305.
73. Nagata S, Hanayama R, Kawane K. Autoimmunity and the Clearance of Dead Cells. *Cell.* 2010;140(5):619-630.
74. Murugesan G, Sandhya Rani MR, Gerber CE, et al. Lysophosphatidylcholine regulates human microvascular endothelial cell expression of chemokines. *Journal of molecular and cellular cardiology.* 2003;35(11):1375-1384.
75. Gude DR, Alvarez SE, Paugh SW, et al. Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2008;22(8):2629-2638.
76. Weigert A, Tzieply N, von Knethen A, et al. Tumor cell apoptosis polarizes macrophages role of sphingosine-1-phosphate. *Molecular biology of the cell.* 2007;18(10):3810-3819.
77. Kluk MJ, Hla T. Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. *Biochimica et biophysica acta.* 2002;1582(1-3):72-80.
78. Bournazou I, Pound JD, Duffin R, et al. Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. *The Journal of Clinical Investigation.* 2009;119(1):20-32.
79. Bournazou I, Mackenzie KJ, Duffin R, Rossi AG, Gregory CD. Inhibition of eosinophil migration by lactoferrin. *Immunology and cell biology.* 2010;88(2):220-223.
80. Devitt A, Hawkins L. Current understanding of the mechanisms for clearance of apoptotic cells - a fine balance. *Journal of Cell Death - In Press.* 2013.
81. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *Journal of immunology (Baltimore, Md. : 1950).* 1992;148(7):2207-2216.
82. Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of

apoptotic cells by macrophages and fibroblasts. *The Journal of biological chemistry.* 2001;276(2):1071-1077.
83. Arur S, Uche UE, Rezaul K, et al. Annexin I Is an Endogenous Ligand that Mediates Apoptotic Cell Engulfment. *Developmental Cell.* 2003;4(4):587-598.
84. Moffatt OD, Devitt A, Bell ED, Simmons DL, Gregory CD. Macrophage recognition of ICAM-3 on apoptotic leukocytes. *Journal of immunology (Baltimore, Md. : 1950).* 1999;162(11):6800-6810.
85. Kagan VE, Gleiss B, Tyurina YY, et al. A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. *Journal of immunology (Baltimore, Md. : 1950).* 2002;169(1):487-499.
86. Meesmann HM, Fehr EM, Kierschke S, et al. Decrease of sialic acid residues as an eat-me signal on the surface of apoptotic lymphocytes. *Journal of cell science.* 2010;123(Pt 19):3347-3356.
87. Nauta AJ, Trouw LA, Daha MR, et al. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. *European journal of immunology.* 2002;32(6):1726-1736.
88. Ogden CA, deCathelineau A, Hoffmann PR, et al. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *The Journal of experimental medicine.* 2001;194(6):781-795.
89. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *The Journal of experimental medicine.* 1995;182(5):1545-1556.
90. Segawa K, Suzuki J, Nagata S. Constitutive exposure of phosphatidylserine on viable cells. *Proceedings of the National Academy of Sciences.* 2011;108(48):19246-19251.
91. Simhadri VR, Andersen JF, Calvo E, Choi SC, Coligan JE, Borrego F. Human CD300a binds to phosphatidylethanolamine and phosphatidylserine, and modulates the phagocytosis of dead cells. *Blood.* 2012;119(12):2799-2809.
92. Brown S, Heinisch I, Ross E, Shaw K, Buckley CD, Savill J. Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. *Nature.* 2002;418(6894):200-203.
93. Gardai SJ, McPhillips KA, Frasch SC, et al. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell.* 2005;123(2):321-334.
94. Jaiswal S, Jamieson CH, Pang WW, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell.* 2009;138(2):271-285.
95. Nakahashi-Oda C, Tahara-Hanaoka S, Honda S, Shibuya K, Shibuya A. Identification of phosphatidylserine as a ligand for the CD300a immunoreceptor. *Biochemical and biophysical research communications.* 2012;417(1):646-650.
96. Kobayashi N, Karisola P, Peña-Cruz V, et al. TIM-1 and TIM-4 Glycoproteins Bind Phosphatidylserine and Mediate Uptake of Apoptotic Cells. *Immunity.* 2007;27(6):927-940.
97. Park D, Tosello-Trampont AC, Elliott MR, et al. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature.* 2007;450(7168):430-434.
98. Park SY, Jung MY, Kim HJ, et al. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. *Cell death and differentiation.* 2008;15(1):192-201.
99. He M, Kubo H, Morimoto K, et al. Receptor for advanced glycation end products binds to phosphatidylserine and assists in the clearance of apoptotic cells. *EMBO reports.* 2011;12(4):358-364.
100. Bratton DL, Henson PM. Apoptotic cell recognition: will the real phosphatidylserine receptor(s) please stand up? *Current biology : CB.* 2008;18(2):R76-79.

101. Greenberg ME, Sun M, Zhang R, Febbraio M, Silverstein R, Hazen SL. Oxidized phosphatidylserine-CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells. *The Journal of experimental medicine.* 2006;203(12):2613-2625.
102. Ramprasad MP, Fischer W, Witztum JL, Sambrano GR, Quehenberger O, Steinberg D. The 94- to 97-kDa mouse macrophage membrane protein that recognizes oxidized low density lipoprotein and phosphatidylserine-rich liposomes is identical to macrosialin, the mouse homologue of human CD68. *Proceedings of the National Academy of Sciences.* 1995;92(21):9580-9584.
103. Platt N, Suzuki H, Kurihara Y, Kodama T, Gordon S. Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. *Proceedings of the National Academy of Sciences.* 1996;93(22):12456-12460.
104. Imachi H, Murao K, Hiramine C, et al. Human scavenger receptor B1 is involved in recognition of apoptotic thymocytes by thymic nurse cells. *Laboratory investigation; a journal of technical methods and pathology.* 2000;80(2):263-270.
105. Murphy JE, Tacon D, Tedbury PR, et al. LOX-1 scavenger receptor mediates calcium-dependent recognition of phosphatidylserine and apoptotic cells. *The Biochemical journal.* 2006;393(Pt 1):107-115.
106. Boddaert J, Kinugawa K, Lambert JC, et al. Evidence of a role for lactadherin in Alzheimer's disease. *The American journal of pathology.* 2007;170(3):921-929.
107. Lutgens E, Tjwa M, Garcia de Frutos P, et al. Genetic loss of Gas6 induces plaque stability in experimental atherosclerosis. *The Journal of pathology.* 2008;216(1):55-63.
108. Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe-/- mice. *Arterioscler Thromb Vasc Biol.* 2008;28(8):1421-1428.
109. Scott RS, McMahon EJ, Pop SM, et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature.* 2001;411(6834):207-211.
110. Cohen PL, Caricchio R, Abraham V, et al. Delayed Apoptotic Cell Clearance and Lupus-like Autoimmunity in Mice Lacking the c-mer Membrane Tyrosine Kinase. *The Journal of experimental medicine.* 2002;196(1):135-140.
111. Kilpatrick DC. Phospholipid-binding activity of human mannan-binding lectin. *Immunology letters.* 1998;61(2-3):191-195.
112. Jakel A, Reid KB, Clark H. Surfactant protein A (SP-A) binds to phosphatidylserine and competes with annexin V binding on late apoptotic cells. *Protein & cell.* 2010;1(2):188-197.
113. Vandivier RW, Fadok VA, Ogden CA, et al. IMpaired clearance of apoptotic cells from cystic fibrosis airways*. *CHEST Journal.* 2002;121(3_suppl):89S-89S.
114. Paidassi H, Tacnet-Delorme P, Garlatti V, et al. C1q binds phosphatidylserine and likely acts as a multiligand-bridging molecule in apoptotic cell recognition. *Journal of immunology (Baltimore, Md. : 1950).* 2008;180(4):2329-2338.
115. Takizawa F, Tsuji S, Nagasawa S. Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. *FEBS Letters.* 1996;397(2–3):269-272.
116. Familian A, Zwart B, Huisman HG, et al. Chromatin-independent binding of serum amyloid P component to apoptotic cells. *Journal of immunology (Baltimore, Md. : 1950).* 2001;167(2):647-654.
117. Gershov D, Kim S, Brot N, Elkon KB. C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *The Journal of experimental medicine.* 2000;192(9):1353-1364.
118. Rovere P, Peri G, Fazzini F, et al. The long pentraxin PTX3 binds to apoptotic cells and regulates their clearance by antigen-presenting dendritic cells. *Blood.* 2000;96(13):4300-4306.

119. Savill J, Hogg N, Ren Y, Haslett C. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J Clin Invest.* 1992;90(4):1513-1522.
120. Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG. Natural IgG Autoantibodies Are Abundant and Ubiquitous in Human Sera, and Their Number Is Influenced By Age, Gender, and Disease. *PLoS ONE.* 2013;8(4):e60726.
121. Litvack ML, Djiadeu P, Renganathan SD, Sy S, Post M, Palaniyar N. Natural IgM and innate immune collectin SP-D bind to late apoptotic cells and enhance their clearance by alveolar macrophages in vivo. *Molecular immunology.* 2010;48(1-3):37-47.
122. Ogden CA, Kowalewski R, Peng Y, Montenegro V, Elkon KB. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. *Autoimmunity.* 2005;38(4):259-264.
123. Gregory CD. CD14-dependent clearance of apoptotic cells: relevance to the immune system. *Current Opinion in Immunology.* 2000;12(1):27-34.
124. Tennant I, Pound JD, Marr LA, et al. Innate recognition of apoptotic cells: novel apoptotic cell-associated molecular patterns revealed by crossreactivity of anti-LPS antibodies. *Cell death and differentiation.* 2013;20(5):698-708.
125. Hurwitz ME, Vanderzalm PJ, Bloom L, Goldman J, Garriga G, Horvitz HR. Abl kinase inhibits the engulfment of apoptotic [corrected] cells in Caenorhabditis elegans. *PLoS biology.* 2009;7(4):e99.
126. Wu Y, Singh S, Georgescu MM, Birge RB. A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. *Journal of cell science.* 2005;118(Pt 3):539-553.
127. Albert ML, Kim JI, Birge RB. alphavbeta5 integrin recruits the CrkII-Dock180-rac1 complex for phagocytosis of apoptotic cells. *Nature cell biology.* 2000;2(12):899-905.
128. Brugnera E, Haney L, Grimsley C, et al. Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. *Nature cell biology.* 2002;4(8):574-582.
129. Miki H, Suetsugu S, Takenawa T. WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *The EMBO journal.* 1998;17(23):6932-6941.
130. Machesky LM, Insall RH. Scar1 and the related Wiskott–Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Current Biology.* 1998;8(25):1347-1356.
131. Su HP, Nakada-Tsukui K, Tosello-Trampont AC, et al. Interaction of CED-6/GULP, an adapter protein involved in engulfment of apoptotic cells with CED-1 and CD91/low density lipoprotein receptor-related protein (LRP). *The Journal of biological chemistry.* 2002;277(14):11772-11779.
132. Park SY, Kang KB, Thapa N, Kim SY, Lee SJ, Kim IS. Requirement of adaptor protein GULP during stabilin-2-mediated cell corpse engulfment. *The Journal of biological chemistry.* 2008;283(16):10593-10600.
133. Jehle AW, Gardai SJ, Li S, et al. ATP-binding cassette transporter A7 enhances phagocytosis of apoptotic cells and associated ERK signaling in macrophages. *The Journal of cell biology.* 2006;174(4):547-556.
134. Kinchen JM, Cabello J, Klingele D, et al. Two pathways converge at CED-10 to mediate actin rearrangement and corpse removal in C. elegans. *Nature.* 2005;434(7029):93-99.
135. McNeil PL, Tanasugarn L, Meigs JB, Taylor DL. Acidification of phagosomes is initiated before lysosomal enzyme activity is detected. *The Journal of cell biology.* 1983;97(3):692-702.
136. Park D, Han CZ, Elliott MR, et al. Continued clearance of apoptotic cells critically depends on the phagocyte Ucp2 protein. *Nature.* 2011;477(7363):220-U126.
137. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiological reviews.* 2006;86(2):515-581.

138. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. *Nature.* 2000;407(6805):784-788.
139. Freire-de-Lima CG, Xiao YQ, Gardai SJ, Bratton DL, Schiemann WP, Henson PM. Apoptotic cells, through transforming growth factor-beta, coordinately induce anti-inflammatory and suppress pro-inflammatory eicosanoid and NO synthesis in murine macrophages. *The Journal of biological chemistry.* 2006;281(50):38376-38384.
140. Mitchell S, Thomas G, Harvey K, et al. Lipoxins, aspirin-triggered epi-lipoxins, lipoxin stable analogues, and the resolution of inflammation: stimulation of macrophage phagocytosis of apoptotic neutrophils in vivo. *Journal of the American Society of Nephrology : JASN.* 2002;13(10):2497-2507.
141. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nature reviews. Immunology.* 2008;8(5):349-361.
142. Randolph GJ. Emigration of monocyte-derived cells to lymph nodes during resolution of inflammation and its failure in atherosclerosis. *Current opinion in lipidology.* 2008;19(5):462-468.
143. *Promoting Cardiovascular Health in the Developing World:A Critical Challenge to Achieve Global Health.* The National Academies Press; 2010.
144. Yusuf S, Reddy S, Ôunpuu S, Anand S. Global Burden of Cardiovascular Diseases: Part I: General Considerations, the Epidemiologic Transition, Risk Factors, and Impact of Urbanization. *Circulation.* 2001;104(22):2746-2753.
145. Fagot-Campagna A, Bourdel-Marchasson I, Simon D. Burden of diabetes in an aging population: prevalence, incidence, mortality, characteristics and quality of care. *Diabetes & Metabolism.* 2005;31, Supplement 1(0):5S35-35S52.
146. N T, K W, P B, et al. Coronary heart disease statistics 2012 edition London: British Heart Foundation; 2012.
147. Glass CK, Witztum JL. Atherosclerosis. the road ahead. *Cell.* 2001;104(4):503-516.
148. Willems S, Vink A, Bot I, et al. Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events. *Eur Heart J.* 2013.
149. Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler Thromb Vasc Biol.* 2005;25(11):2255-2264.
150. Thorp E, Tabas I. Mechanisms and consequences of efferocytosis in advanced atherosclerosis. *Journal of leukocyte biology.* 2009;86(5):1089-1095.
151. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nature Reviews Immunology.* 2009;10(1):36-46.
152. Williams KJ, Tabas I. The response-to-retention hypothesis of atherogenesis reinforced. *Current opinion in lipidology.* 1998;9(5):471-474.
153. Westhorpe CL, Dufour EM, Maisa A, Jaworowski A, Crowe SM, Muller WA. Endothelial cell activation promotes foam cell formation by monocytes following transendothelial migration in an in vitro model. *Experimental and molecular pathology.* 2012;93(2):220-226.
154. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proceedings of the National Academy of Sciences of the United States of America.* 1984;81(12):3883-3887.
155. Palinski W, Rosenfeld ME, Yla-Herttuala S, et al. Low density lipoprotein undergoes oxidative modification in vivo. *Proceedings of the National Academy of Sciences of the United States of America.* 1989;86(4):1372-1376.
156. Potteaux S, Gautier EL, Hutchison SB, et al. Suppressed monocyte recruitment drives macrophage removal from atherosclerotic plaques of Apoe–/– mice during disease regression. *The Journal of Clinical Investigation.* 2011;121(5):2025-2036.

157. Johnson-Tidey RR, McGregor JL, Taylor PR, Poston RN. Increase in the adhesion molecule P-selectin in endothelium overlying atherosclerotic plaques. Coexpression with intercellular adhesion molecule-1. *The American journal of pathology.* 1994;144(5):952-961.
158. Gebuhrer V, Murphy JF, Bordet JC, Reck MP, McGregor JL. Oxidized low-density lipoprotein induces the expression of P-selectin (GMP140/PADGEM/CD62) on human endothelial cells. *The Biochemical journal.* 1995;306 (Pt 1):293-298.
159. Khan BV, Parthasarathy SS, Alexander RW, Medford RM. Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. *J Clin Invest.* 1995;95(3):1262-1270.
160. Stocker CJ, Sugars KL, Harari OA, Landis RC, Morley BJ, Haskard DO. TNF-alpha, IL-4, and IFN-gamma regulate differential expression of P- and E-selectin expression by porcine aortic endothelial cells. *Journal of immunology (Baltimore, Md. : 1950).* 2000;164(6):3309-3315.
161. Yuan XM, Li W, Olsson AG, Brunk UT. The toxicity to macrophages of oxidized low-density lipoprotein is mediated through lysosomal damage. *Atherosclerosis.* 1997;133(2):153-161.
162. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell.* 2011;145(3):341-355.
163. Wong B, Lumma WC, Smith AM, Sisko JT, Wright SD, Cai T-Q. Statins suppress THP-1 cell migration and secretion of matrix metalloproteinase 9 by inhibiting geranylgeranylation. *Journal of leukocyte biology.* 2001;69(6):959-962.
164. Jovinge S, Ares MP, Kallin B, Nilsson J. Human monocytes/macrophages release TNF-alpha in response to Ox-LDL. *Arterioscler Thromb Vasc Biol.* 1996;16(12):1573-1579.
165. Janabi M, Yamashita S, Hirano K, et al. Oxidized LDL-induced NF-kappa B activation and subsequent expression of proinflammatory genes are defective in monocyte-derived macrophages from CD36-deficient patients. *Arterioscler Thromb Vasc Biol.* 2000;20(8):1953-1960.
166. Kadl A, Huber J, Gruber F, Bochkov VN, Binder BR, Leitinger N. Analysis of inflammatory gene induction by oxidized phospholipids in vivo by quantitative real-time RT-PCR in comparison with effects of LPS. *Vascul Pharmacol.* 2002;38(4):219-227.
167. Yeagle PL. Lipid regulation of cell membrane structure and function. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 1989;3(7):1833-1842.
168. Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol.* 1995;15(5):551-561.
169. Tabas I, Williams KJ, Boren J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation.* 2007;116(16):1832-1844.
170. Skalen K, Gustafsson M, Rydberg EK, et al. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. *Nature.* 2002;417(6890):750-754.
171. Hevonoja T, Pentikainen MO, Hyvonen MT, Kovanen PT, Ala-Korpela M. Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. *Biochimica et biophysica acta.* 2000;1488(3):189-210.
172. Mertens A, Holvoet P. Oxidized LDL and HDL: antagonists in atherothrombosis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2001;15(12):2073-2084.
173. Parthasarathy S, Printz DJ, Boyd D, Joy L, Steinberg D. Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis (Dallas, Tex.).* 1986;6(5):505-510.
174. Brinkley TE, Nicklas BJ, Kanaya AM, et al. Plasma oxidized low-density lipoprotein levels and arterial stiffness in older adults: the health, aging, and body composition study. *Hypertension.* 2009;53(5):846-852.

175. Nicholson AC, Han J, Febbraio M, Silversterin RL, Hajjar DP. Role of CD36, the macrophage class B scavenger receptor, in atherosclerosis. *Annals of the New York Academy of Sciences.* 2001;947:224-228.
176. Mehta JL, Chen J, Hermonat PL, Romeo F, Novelli G. Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1): a critical player in the development of atherosclerosis and related disorders. *Cardiovascular research.* 2006;69(1):36-45.
177. de Winther MP, van Dijk KW, Havekes LM, Hofker MH. Macrophage scavenger receptor class A: A multifunctional receptor in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2000;20(2):290-297.
178. Nicholson AC, Frieda S, Pearce A, Silverstein RL. Oxidized LDL binds to CD36 on human monocyte-derived macrophages and transfected cell lines. Evidence implicating the lipid moiety of the lipoprotein as the binding site. *Arterioscler Thromb Vasc Biol.* 1995;15(2):269-275.
179. Parthasarathy S, Fong LG, Otero D, Steinberg D. Recognition of solubilized apoproteins from delipidated, oxidized low density lipoprotein (LDL) by the acetyl-LDL receptor. *Proceedings of the National Academy of Sciences of the United States of America.* 1987;84(2):537-540.
180. Brown MS, Goldstein JL. Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL. *Journal of lipid research.* 2009;50 Suppl:S15-27.
181. Dhaliwal BS, Steinbrecher UP. Scavenger receptors and oxidized low density lipoproteins. *Clinica chimica acta; international journal of clinical chemistry.* 1999;286(1-2):191-205.
182. Kunjathoor VV, Febbraio M, Podrez EA, et al. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *The Journal of biological chemistry.* 2002;277(51):49982-49988.
183. Hampton RY, Golenbock DT, Penman M, Krieger M, Raetz CR. Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature.* 1991;352(6333):342-344.
184. Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proceedings of the National Academy of Sciences of the United States of America.* 1979;76(1):333-337.
185. Doi T, Higashino K, Kurihara Y, et al. Charged collagen structure mediates the recognition of negatively charged macromolecules by macrophage scavenger receptors. *The Journal of biological chemistry.* 1993;268(3):2126-2133.
186. Naito M, Suzuki H, Mori T, Matsumoto A, Kodama T, Takahashi K. Coexpression of type I and type II human macrophage scavenger receptors in macrophages of various organs and foam cells in atherosclerotic lesions. *The American journal of pathology.* 1992;141(3):591-599.
187. Lougheed M, Lum CM, Ling W, Suzuki H, Kodama T, Steinbrecher U. High affinity saturable uptake of oxidized low density lipoprotein by macrophages from mice lacking the scavenger receptor class A type I/II. *The Journal of biological chemistry.* 1997;272(20):12938-12944.
188. Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. *The Journal of biological chemistry.* 1993;268(16):11811-11816.
189. Podrez EA, Febbraio M, Sheibani N, et al. Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. *J Clin Invest.* 2000;105(8):1095-1108.
190. Ren Y, Silverstein RL, Allen J, Savill J. CD36 gene transfer confers capacity for phagocytosis of cells undergoing apoptosis. *The Journal of experimental medicine.* 1995;181(5):1857-1862.
191. Calvo D, Gomez-Coronado D, Suarez Y, Lasuncion MA, Vega MA. Human CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, and VLDL. *Journal of lipid research.* 1998;39(4):777-788.

192. Sawamura T, Kume N, Aoyama T, et al. An endothelial receptor for oxidized low-density lipoprotein. *Nature.* 1997;386(6620):73-77.
193. Yoshida H, Kondratenko N, Green S, Steinberg D, Quehenberger O. Identification of the lectin-like receptor for oxidized low-density lipoprotein in human macrophages and its potential role as a scavenger receptor. *The Biochemical journal.* 1998;334 (Pt 1):9-13.
194. Li D, Mehta JL. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. *Arterioscler Thromb Vasc Biol.* 2000;20(4):1116-1122.
195. Schrijvers DM, De Meyer GRY, Kockx MM, Herman AG, Martinet W. Phagocytosis of Apoptotic Cells by Macrophages Is Impaired in Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology.* 2005;25(6):1256-1261.
196. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons From Sudden Coronary Death: A Comprehensive Morphological Classification Scheme for Atherosclerotic Lesions. *Arteriosclerosis, Thrombosis, and Vascular Biology.* 2000;20(5):1262-1275.
197. Khan M, Pelengaris S, Cooper M, Smith C, Evan G, Betteridge J. Oxidised lipoproteins may promote inflammation through the selective delay of engulfment but not binding of apoptotic cells by macrophages. *Atherosclerosis.* 2003;171(1):21-29.
198. Hardwick SJ, Hegyi L, Clare K, et al. Apoptosis in human monocyte-macrophages exposed to oxidized low density lipoprotein. *The Journal of pathology.* 1996;179(3):294-302.
199. Bjorkerud B, Bjorkerud S. Contrary effects of lightly and strongly oxidized LDL with potent promotion of growth versus apoptosis on arterial smooth muscle cells, macrophages, and fibroblasts. *Arterioscler Thromb Vasc Biol.* 1996;16(3):416-424.
200. Feng B, Yao PM, Li Y, et al. The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. *Nature cell biology.* 2003;5(9):781-792.
201. Miller YI, Viriyakosol S, Binder CJ, Feramisco JR, Kirkland TN, Witztum JL. Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. *The Journal of biological chemistry.* 2003;278(3):1561-1568.
202. Sambrano GR, Steinberg D. Recognition of oxidatively damaged and apoptotic cells by an oxidized low density lipoprotein receptor on mouse peritoneal macrophages: role of membrane phosphatidylserine. *Proceedings of the National Academy of Sciences of the United States of America.* 1995;92(5):1396-1400.
203. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell death and differentiation.* 1998;5(7):551-562.
204. Grimsley C, Ravichandran KS. Cues for apoptotic cell engulfment: eat-me, don't eat-me and come-get-me signals. *Trends in cell biology.* 2003;13(12):648-656.
205. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature.* 2002;418(6894):191-195.
206. Bianchi ME. HMGB1 loves company. *Journal of leukocyte biology.* 2009;86(3):573-576.
207. Fadok VA, Bratton DL, Guthrie L, Henson PM. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *Journal of immunology (Baltimore, Md. : 1950).* 2001;166(11):6847-6854.
208. Kurosaka K, Takahashi M, Kobayashi Y. Activation of extracellular signal-regulated kinase 1/2 is involved in production of CXC-chemokine by macrophages during phagocytosis of late apoptotic cells. *Biochemical and biophysical research communications.* 2003;306(4):1070-1074.
209. Chang MK, Binder CJ, Miller YI, et al. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. *The Journal of experimental medicine.* 2004;200(11):1359-1370.

210. Li Y, Gerbod-Giannone MC, Seitz H, et al. Cholesterol-induced apoptotic macrophages elicit an inflammatory response in phagocytes, which is partially attenuated by the Mer receptor. *The Journal of biological chemistry.* 2006;281(10):6707-6717.
211. Stuart LM, Takahashi K, Shi L, Savill J, Ezekowitz RAB. Mannose-Binding Lectin-Deficient Mice Display Defective Apoptotic Cell Clearance but No Autoimmune Phenotype. *The Journal of Immunology.* 2005;174(6):3220-3226.
212. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. *Nature reviews. Immunology.* 2009;9(5):353-363.
213. Signorelli SS, Mazzarino MC, Di Pino L, et al. High circulating levels of cytokines (IL-6 and TNFalpha), adhesion molecules (VCAM-1 and ICAM-1) and selectins in patients with peripheral arterial disease at rest and after a treadmill test. *Vascular medicine (London, England).* 2003;8(1):15-19.
214. Shibata T, Nagata K, Kobayashi Y. Apoptotic neutrophils and nitric oxide regulate cytokine production by IFN-γ-stimulated macrophages. *Cytokine.* 2011;53(2):191-195.
215. Bell CW, Jiang W, Reich CF, 3rd, Pisetsky DS. The extracellular release of HMGB1 during apoptotic cell death. *American journal of physiology. Cell physiology.* 2006;291(6):C1318-1325.
216. Lin J, Li H, Yang M, et al. A Role of RIP3-Mediated Macrophage Necrosis in Atherosclerosis Development. *Cell Reports.* 2013;3(1):200-210.
217. van Tits LJ, Stienstra R, van Lent PL, Netea MG, Joosten LA, Stalenhoef AF. Oxidized LDL enhances pro-inflammatory responses of alternatively activated M2 macrophages: a crucial role for Kruppel-like factor 2. *Atherosclerosis.* 2011;214(2):345-349.
218. Ma X. TNF-α and IL-12:a balancing act in macrophage functioning. *Microbes and Infection.* 2001;3(2):121-129.
219. Garton KJ, Gough PJ, Blobel CP, et al. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). *The Journal of biological chemistry.* 2001;276(41):37993-38001.
220. Arbustini E, Grasso M, Diegoli M, et al. Coronary atherosclerotic plaques with and without thrombus in ischemic heart syndromes: a morphologic, immunohistochemical, and biochemical study. *The American journal of cardiology.* 1991;68(7):36B-50B.
221. Barath P, Fishbein MC, Cao J, Berenson J, Helfant RH, Forrester JS. Detection and localization of tumor necrosis factor in human atheroma. *The American journal of cardiology.* 1990;65(5):297-302.
222. Rus HG, Niculescu F, Vlaicu R. Tumor necrosis factor-alpha in human arterial wall with atherosclerosis. *Atherosclerosis.* 1991;89(2-3):247-254.
223. Ohta H, Wada H, Niwa T, et al. Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice. *Atherosclerosis.* 2005;180(1):11-17.
224. van Lenten BJ, Fogelman AM. Lipopolysaccharide-induced inhibition of scavenger receptor expression in human monocyte-macrophages is mediated through tumor necrosis factor-alpha. *The Journal of Immunology.* 1992;148(1):112-116.
225. Ridker PM, Rifai N, Pfeffer M, Sacks F, Lepage S, Braunwald E. Elevation of tumor necrosis factor-alpha and increased risk of recurrent coronary events after myocardial infarction. *Circulation.* 2000;101(18):2149-2153.
226. Tuomisto K, Jousilahti P, Sundvall J, Pajunen P, Salomaa V. C-reactive protein, interleukin-6 and tumor necrosis factor alpha as predictors of incident coronary and cardiovascular events and total mortality. A population-based, prospective study. *Thrombosis and haemostasis.* 2006;95(3):511-518.
227. Hsieh C, Macatonia S, Tripp C, Wolf S, O'Garra A, Murphy K. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science.* 1993;260(5107):547-549.
228. Uyemura K, Demer LL, Castle SC, et al. Cross-regulatory roles of interleukin (IL)-12 and IL-10 in atherosclerosis. *J Clin Invest.* 1996;97(9):2130-2138.

229. Lee TS, Yen HC, Pan CC, Chau LY. The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol.* 1999;19(3):734-742.
230. Mallat Z, Heymes C, Ohan J, Faggin E, Lesèche G, Tedgui A. Expression of Interleukin-10 in Advanced Human Atherosclerotic Plaques: Relation to Inducible Nitric Oxide Synthase Expression and Cell Death. *Arteriosclerosis, Thrombosis, and Vascular Biology.* 1999;19(3):611-616.
231. Bouhlel MA, Derudas B, Rigamonti E, et al. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell metabolism.* 2007;6(2):137-143.
232. Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell.* 1998;93(2):229-240.
233. Nagornev VA, Maltseva SV. The phenotype of macrophages which are not transformed into foam cells in atherogenesis. *Atherosclerosis.* 1996;121(2):245-251.
234. Michlewska S, Dransfield I, Megson IL, Rossi AG. Macrophage phagocytosis of apoptotic neutrophils is critically regulated by the opposing actions of pro-inflammatory and anti-inflammatory agents: key role for TNF-alpha. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2009;23(3):844-854.
235. Harel-Adar T, Mordechai TB, Amsalem Y, Feinberg MS, Leor J, Cohen S. Modulation of cardiac macrophages by phosphatidylserine-presenting liposomes improves infarct repair. *Proceedings of the National Academy of Sciences.* 2011.
236. Liu J, Thewke DP, Su YR, Linton MF, Fazio S, Sinensky MS. Reduced macrophage apoptosis is associated with accelerated atherosclerosis in low-density lipoprotein receptor-null mice. *Arterioscler Thromb Vasc Biol.* 2005;25(1):174-179.
237. van Gils JM, Derby MC, Fernandes LR, et al. The neuroimmune guidance cue netrin-1 promotes atherosclerosis by inhibiting the emigration of macrophages from plaques. *Nature immunology.* 2012;13(2):136-143.
238. Barlic J, Murphy PM. Chemokine regulation of atherosclerosis. *Journal of leukocyte biology.* 2007;82(2):226-236.
239. Lesnik P, Haskell CA, Charo IF. Decreased atherosclerosis in CX3CR1-/- mice reveals a role for fractalkine in atherogenesis. *J Clin Invest.* 2003;111(3):333-340.
240. Keller HU, Wilkinson PC, Abercrombie M, et al. A proposal for the definition of terms related to locomotion of leucocytes and other cells. *Cell biology international reports.* 1977;1(5):391-397.
241. Johnston B, Butcher EC. Chemokines in rapid leukocyte adhesion triggering and migration. *Seminars in Immunology.* 2002;14(2):83-92.
242. Quehenberger O. Thematic review series: the immune system and atherogenesis. Molecular mechanisms regulating monocyte recruitment in atherosclerosis. *Journal of lipid research.* 2005;46(8):1582-1590.
243. McEver RP. Selectins: lectins that initiate cell adhesion under flow. *Current opinion in cell biology.* 2002;14(5):581-586.
244. Tedder TF, Steeber DA, Chen A, Engel P. The selectins: vascular adhesion molecules. *The FASEB Journal.* 1995;9(10):866-873.
245. Ley K, Gaehtgens P, Fennie C, Singer MS, Lasky LA, Rosen SD. Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vivo. *Blood.* 1991;77(12):2553-2555.
246. Ley K, Tedder TF, Kansas GS. L-selectin can mediate leukocyte rolling in untreated mesenteric venules in vivo independent of E- or P-selectin. *Blood.* 1993;82(5):1632-1638.
247. Eriksson EE, Xie X, Werr J, Thoren P, Lindbom L. Importance of primary capture and L-selectin-dependent secondary capture in leukocyte accumulation in inflammation and atherosclerosis in vivo. *The Journal of experimental medicine.* 2001;194(2):205-218.

248. Rahman A, Kefer J, Bando M, Niles WD, Malik AB. E-selectin expression in human endothelial cells by TNF-alpha-induced oxidant generation and NF-kappaB activation. *The American journal of physiology.* 1998;275(3 Pt 1):L533-544.
249. McEver RP, Beckstead JH, Moore KL, Marshall-Carlson L, Bainton DF. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J Clin Invest.* 1989;84(1):92-99.
250. Van der Wal AC, Das PK, Tigges AJ, Becker AE. Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. *The American journal of pathology.* 1992;141(6):1427.
251. Wood KM, Cadogan MD, Ramshaw AL, Parums DV. The distribution of adhesion molecules in human atherosclerosis. *Histopathology.* 1993;22(5):437-444.
252. Jeng JR, Chang CH, Shieh SM, Chiu HC. Oxidized low-density lipoprotein enhances monocyte-endothelial cell binding against shear-stress-induced detachment. *Biochimica et biophysica acta.* 1993;1178(2):221-227.
253. Cushing SD, Berliner JA, Valente AJ, et al. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proceedings of the National Academy of Sciences of the United States of America.* 1990;87(13):5134-5138.
254. Norgard KE, Moore KL, Diaz S, et al. Characterization of a specific ligand for P-selectin on myeloid cells. A minor glycoprotein with sialylated O-linked oligosaccharides. *The Journal of biological chemistry.* 1993;268(17):12764-12774.
255. Spertini O, Cordey AS, Monai N, Giuffre L, Schapira M. P-selectin glycoprotein ligand 1 is a ligand for L-selectin on neutrophils, monocytes, and CD34+ hematopoietic progenitor cells. *The Journal of cell biology.* 1996;135(2):523-531.
256. Levinovitz A, Mühlhoff J, Isenmann S, Vestweber D. Identification of a glycoprotein ligand for E-selectin on mouse myeloid cells. *The Journal of cell biology.* 1993;121(2):449-459.
257. Lenter M, Levinovitz A, Isenmann S, Vestweber D. Monospecific and common glycoprotein ligands for E- and P-selectin on myeloid cells. *The Journal of cell biology.* 1994;125(2):471-481.
258. Galkina E, Ley K. Vascular Adhesion Molecules in Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology.* 2007;27(11):2292-2301.
259. Cybulsky MI, Gimbrone MA, Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science.* 1991;251(4995):788-791.
260. Davies MJ, Gordon JL, Gearing AJH, et al. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *The Journal of pathology.* 1993;171(3):223-229.
261. Ramana KV, Bhatnagar A, Srivastava SK. Inhibition of aldose reductase attenuates TNF-alpha-induced expression of adhesion molecules in endothelial cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2004;18(11):1209-1218.
262. Smalley DM, Lin JH, Curtis ML, Kobari Y, Stemerman MB, Pritchard KA, Jr. Native LDL increases endothelial cell adhesiveness by inducing intercellular adhesion molecule-1. *Arterioscler Thromb Vasc Biol.* 1996;16(4):585-590.
263. Yoneda O, Imai T, Goda S, et al. Fractalkine-mediated endothelial cell injury by NK cells. *Journal of immunology (Baltimore, Md. : 1950).* 2000;164(8):4055-4062.
264. Fuhrman B, Partoush A, Volkova N, Aviram M. Ox-LDL induces monocyte-to-macrophage differentiation in vivo: Possible role for the macrophage colony stimulating factor receptor (M-CSF-R). *Atherosclerosis.* 2008;196(2):598-607.
265. Biwa T, Hakamata H, Sakai M, et al. Induction of murine macrophage growth by oxidized low density lipoprotein is mediated by granulocyte macrophage colony-stimulating factor. *The Journal of biological chemistry.* 1998;273(43):28305-28313.

266. Brown MS, Goldstein JL. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annual review of biochemistry.* 1983;52:223-261.
267. Chironi G, Simon A, Hugel B, et al. Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects. *Arterioscler Thromb Vasc Biol.* 2006;26(12):2775-2780.
268. Wang J-G, Aikawa E, Aikawa M. Leukocyte-derived Microparticles as Pro-inflammatory Mediators in Atherosclerosis. *Journal of the American College of Cardiology.* (0).
269. Leroyer AS, Isobe H, Leseche G, et al. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. *J Am Coll Cardiol.* 2007;49(7):772-777.
270. Puddu P, Puddu GM, Cravero E, Muscari S, Muscari A. The involvement of circulating microparticles in inflammation, coagulation and cardiovascular diseases. *Canadian Journal of Cardiology.* 2010;26(4):e140-e145.
271. Lane JD, Allan VJ, Woodman PG. Active relocation of chromatin and endoplasmic reticulum into blebs in late apoptotic cells. *Journal of cell science.* 2005;118(17):4059-4071.
272. Gauley J, Pisetsky DS. The release of microparticles by RAW 264.7 macrophage cells stimulated with TLR ligands. *Journal of leukocyte biology.* 2010;87(6):1115-1123.
273. Salucci S, Burattini S, Battistelli M, Baldassarri V, Maltarello M, Falcieri E. Ultraviolet B (UVB) Irradiation-Induced Apoptosis in Various Cell Lineages in Vitro. *International Journal of Molecular Sciences.* 2012;14(1):532-546.
274. Kulms D, Schwarz T. Molecular mechanisms of UV-induced apoptosis. *Photodermatology, Photoimmunology & Photomedicine.* 2000;16(5):195-201.
275. Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nature cell biology.* 2001;3(4):339-345.
276. Rudel T, Bokoch GM. Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. *Science.* 1997;276(5318):1571-1574.
277. Sebbagh M, Renvoize C, Hamelin J, Riche N, Bertoglio J, Breard J. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nature cell biology.* 2001;3(4):346-352.
278. Mayr M, Grainger D, Mayr U, et al. Proteomics, metabolomics, and immunomics on microparticles derived from human atherosclerotic plaques. *Circulation. Cardiovascular genetics.* 2009;2(4):379-388.
279. Mesri M, Altieri DC. Leukocyte Microparticles Stimulate Endothelial Cell Cytokine Release and Tissue Factor Induction in a JNK1 Signaling Pathway. *Journal of Biological Chemistry.* 1999;274(33):23111-23118.
280. Mesri M, Altieri DC. Endothelial Cell Activation by Leukocyte Microparticles. *The Journal of Immunology.* 1998;161(8):4382-4387.
281. Rautou PE, Leroyer AS, Ramkhelawon B, et al. Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration. *Circ Res.* 2011;108(3):335-343.
282. Hoyer FF, Giesen MK, Nunes Franca C, Lutjohann D, Nickenig G, Werner N. Monocytic microparticles promote atherogenesis by modulating inflammatory cells in mice. *Journal of cellular and molecular medicine.* 2012;16(11):2777-2788.
283. Moatti D, Faure S, Fumeron F, et al. Polymorphism in the fractalkine receptor CX3CR1 as a genetic risk factor for coronary artery disease. *Blood.* 2001;97(7):1925-1928.
284. Barteneva N, Fasler-Kan E, Bernimoulin M, et al. Circulating microparticles: square the circle. *BMC Cell Biology.* 2013;14(1):23.
285. Jones BA, Beamer M, Ahmed S. Fractalkine/CX3CL1: a potential new target for inflammatory diseases. *Molecular interventions.* 2010;10(5):263-270.

286. White GE, Greaves DR. Fractalkine: one chemokine, many functions. *Blood.* 2009;113(4):767-768.
287. Hundhausen C, Misztela D, Berkhout TA, et al. The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. *Blood.* 2003;102(4):1186-1195.
288. Shulby SA, Dolloff NG, Stearns ME, Meucci O, Fatatis A. CX3CR1-Fractalkine Expression Regulates Cellular Mechanisms Involved in Adhesion, Migration, and Survival of Human Prostate Cancer Cells. *Cancer research.* 2004;64(14):4693-4698.
289. Liu H, Jiang D. Fractalkine/CX3CR1 and atherosclerosis. *Clinica chimica acta; international journal of clinical chemistry.* 2011;412(13-14):1180-1186.
290. Goda S, Imai T, Yoshie O, et al. CX3C-chemokine, fractalkine-enhanced adhesion of THP-1 cells to endothelial cells through integrin-dependent and -independent mechanisms. *Journal of immunology (Baltimore, Md. : 1950).* 2000;164(8):4313-4320.
291. Kerfoot SM, Lord SE, Bell RB, Gill V, Robbins SM, Kubes P. Human fractalkine mediates leukocyte adhesion but not capture under physiological shear conditions; a mechanism for selective monocyte recruitment. *European journal of immunology.* 2003;33(3):729-739.
292. Zicha D, Dunn GA, Brown AF. A new direct-viewing chemotaxis chamber. *Journal of cell science.* 1991;99(4):769-775.
293. Meyvantsson I, Vu E, Lamers C, et al. Image-based analysis of primary human neutrophil chemotaxis in an automated direct-viewing assay. *Journal of Immunological Methods.* 2011;374(1–2):70-77.
294. Boyden S. THE CHEMOTACTIC EFFECT OF MIXTURES OF ANTIBODY AND ANTIGEN ON POLYMORPHONUCLEAR LEUCOCYTES. *The Journal of experimental medicine.* 1962;115(3):453-466.
295. Li Jeon N, Baskaran H, Dertinger SKW, Whitesides GM, Van De Water L, Toner M. Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. *Nat Biotech.* 2002;20(8):826-830.
296. Vishwanath RP, Brown CE, Wagner JR, et al. A quantitative high-throughput chemotaxis assay using bioluminescent reporter cells. *Journal of Immunological Methods.* 2005;302(1–2):78-89.
297. Nelson RD, Quie PG, Simmons RL. Chemotaxis Under Agarose: A New and Simple Method for Measuring Chemotaxis and Spontaneous Migration of Human Polymorphonuclear Leukocytes and Monocytes. *The Journal of Immunology.* 1975;115(6):1650-1656.
298. Ancuta P, Rao R, Moses A, et al. Fractalkine Preferentially Mediates Arrest and Migration of CD16+ Monocytes. *The Journal of experimental medicine.* 2003;197(12):1701-1707.
299. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *International journal of cancer. Journal international du cancer.* 1980;26(2):171-176.
300. Tsuchiya S, Kobayashi Y, Goto Y, et al. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer research.* 1982;42(4):1530-1536.
301. Schneider U, Schwenk HU, Bornkamm G. Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. *International journal of cancer. Journal international du cancer.* 1977;19(5):621-626.
302. Gregory CD, Rowe M, Rickinson AB. Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line. *The Journal of general virology.* 1990;71 (Pt 7):1481-1495.
303. Dive C, Gregory CD, Phipps DJ, Evans DL, Milner AE, Wyllie AH. Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochimica et biophysica acta.* 1992;1133(3):275-285.

304. Matsushima K, Larsen CG, DuBois GC, Oppenheim JJ. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. *The Journal of experimental medicine.* 1989;169(4):1485-1490.
305. Han KH, Tangirala RK, Green SR, Quehenberger O. Chemokine Receptor CCR2 Expression and Monocyte Chemoattractant Protein-1–Mediated Chemotaxis in Human Monocytes: A Regulatory Role for Plasma LDL. *Arteriosclerosis, Thrombosis, and Vascular Biology.* 1998;18(12):1983-1991.
306. Badolato R, Sozzani S, Malacarne F, et al. Monocytes from Wiskott-Aldrich Patients Display Reduced Chemotaxis and Lack of Cell Polarization in Response to Monocyte Chemoattractant Protein-1 and Formyl-Methionyl-Leucyl-Phenylalanine. *The Journal of Immunology.* 1998;161(2):1026-1033.
307. Auwerx J. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. *Experientia.* 1991;47(1):22-31.
308. Girona J, LaVille AE, Sola R, Motta C, Masana L. HDL derived from the different phases of conjugated diene formation reduces membrane fluidity and contributes to a decrease in free cholesterol efflux from human THP-1 macrophages. *Biochimica et biophysica acta.* 2003;1633(3):143-148.
309. Spano A, Barni S, Sciola L. PMA withdrawal in PMA-treated monocytic THP-1 cells and subsequent retinoic acid stimulation, modulate induction of apoptosis and appearance of dendritic cells. *Cell proliferation.* 2013;46(3):328-347.
310. Schwende H, Fitzke E, Ambs P, Dieter P. Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *Journal of leukocyte biology.* 1996;59(4):555-561.
311. Daigneault M, Preston JA, Marriott HM, Whyte MKB, Dockrell DH. The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte-Derived Macrophages. *PLoS ONE.* 2010;5(1):e8668.
312. Thomas L, Bielemeier A, Lambert PA, Darveau RP, Marshall LJ, Devitt A. The N-terminus of CD14 acts to bind apoptotic cells and confers rapid-tethering capabilities on non-myeloid cells. *PLoS ONE: in press.* 2013.
313. Alamdari DH, Kostidou E, Paletas K, et al. High sensitivity enzyme-linked immunosorbent assay (ELISA) method for measuring protein carbonyl in samples with low amounts of protein. *Free radical biology & medicine.* 2005;39(10):1362-1367.
314. Buss H, Chan TP, Sluis KB, Domigan NM, Winterbourn CC. Protein carbonyl measurement by a sensitive ELISA method. *Free radical biology & medicine.* 1997;23(3):361-366.
315. Hong L, Xie Z-Z, Du Y-H, et al. Alteration of volume-regulated chloride channel during macrophage-derived foam cell formation in atherosclerosis. *Atherosclerosis.* 2011;216(1):59-66.
316. Howell KW, Meng X, Fullerton DA, Jin C, Reece TB, Cleveland Jr JC. Toll-like Receptor 4 Mediates Oxidized LDL-Induced Macrophage Differentiation to Foam Cells. *Journal of Surgical Research.* 2011;171(1):e27-e31.
317. Xu S, Huang Y, Xie Y, et al. Evaluation of foam cell formation in cultured macrophages: an improved method with Oil Red O staining and DiI-oxLDL uptake. *Cytotechnology.* 2010;62(5):473-481.
318. Nickel T, Schmauss D, Hanssen H, et al. oxLDL uptake by dendritic cells induces upregulation of scavenger-receptors, maturation and differentiation. *Atherosclerosis.* 2009;205(2):442-450.
319. Han J, Hajjar DP, Febbraio M, Nicholson AC. Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36. *The Journal of biological chemistry.* 1997;272(34):21654-21659.
320. Wang YC, McPherson K, Marsh T, Gortmaker SL, Brown M. Health and economic burden of the projected obesity trends in the USA and the UK. *The Lancet.*378(9793):815-825.

321. Bruunsgaard H, Skinhøj P, Pedersen AN, Schroll M, Pedersen BK. Ageing, tumour necrosis factor-alpha (TNF-α) and atherosclerosis. *Clinical & Experimental Immunology.* 2000;121(2):255-260.
322. Wang YC, Colditz GA, Kuntz KM. Forecasting the Obesity Epidemic in the Aging U.S. Population. *Obesity.* 2007;15(11):2855-2865.
323. Chavez-Sanchez L, Chavez-Rueda K, Legorreta-Haquet MV, et al. The activation of CD14, TLR4, and TLR2 by mmLDL induces IL-1beta, IL-6, and IL-10 secretion in human monocytes and macrophages. *Lipids in health and disease.* 2010;9:117.
324. Miller YI, Viriyakosol S, Worrall DS, Boullier A, Butler S, Witztum JL. Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. *Arterioscler Thromb Vasc Biol.* 2005;25(6):1213-1219.
325. Benz DJ, Mol M, Ezaki M, et al. Enhanced levels of lipoperoxides in low density lipoprotein incubated with murine fibroblast expressing high levels of human 15-lipoxygenase. *The Journal of biological chemistry.* 1995;270(10):5191-5197.
326. Lenz ML, Hughes H, Mitchell JR, et al. Lipid hydroperoxy and hydroxy derivatives in copper-catalyzed oxidation of low density lipoprotein. *Journal of lipid research.* 1990;31(6):1043-1050.
327. Daugherty A. Mouse models of atherosclerosis. *The American journal of the medical sciences.* 2002;323(1):3-10.
328. Grainger DJ, Reckless J, McKilligin E. Apolipoprotein E modulates clearance of apoptotic bodies in vitro and in vivo, resulting in a systemic proinflammatory state in apolipoprotein E-deficient mice. *Journal of immunology (Baltimore, Md. : 1950).* 2004;173(10):6366-6375.
329. Draude G, Lorenz RL. TGF-beta1 downregulates CD36 and scavenger receptor A but upregulates LOX-1 in human macrophages. *American journal of physiology. Heart and circulatory physiology.* 2000;278(4):H1042-1048.
330. Kume N, Moriwaki H, Kataoka H, et al. Inducible expression of LOX-1, a novel receptor for oxidized LDL, in macrophages and vascular smooth muscle cells. *Annals of the New York Academy of Sciences.* 2000;902:323-327.
331. Tacke F, Alvarez D, Kaplan TJ, et al. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J Clin Invest.* 2007;117(1):185-194.
332. Zhang DE, Hetherington CJ, Gonzalez DA, Chen HM, Tenen DG. Regulation of CD14 expression during monocytic differentiation induced with 1 alpha,25-dihydroxyvitamin D3. *Journal of immunology (Baltimore, Md. : 1950).* 1994;153(7):3276-3284.
333. Mangelsdorf DJ, Koeffler HP, Donaldson CA, Pike JW, Haussler MR. 1,25-Dihydroxyvitamin D3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. *The Journal of cell biology.* 1984;98(2):391-398.
334. Hruska KA, Bar-Shavit Z, Malone JD, Teitelbaum S. Ca2+ priming during vitamin D-induced monocytic differentiation of a human leukemia cell line. *The Journal of biological chemistry.* 1988;263(31):16039-16044.
335. Sugano R, Yamamura T, Harada-Shiba M, Miyake Y, Yamamoto A. Uptake of oxidized low-density lipoprotein in a THP-1 cell line lacking scavenger receptor A. *Atherosclerosis.* 2001;158(2):351-357.
336. Yesner LM, Huh HY, Pearce SF, Silverstein RL. Regulation of monocyte CD36 and thrombospondin-1 expression by soluble mediators. *Arterioscler Thromb Vasc Biol.* 1996;16(8):1019-1025.
337. Liao HS, Kodama T, Geng YJ. Expression of class A scavenger receptor inhibits apoptosis of macrophages triggered by oxidized low density lipoprotein and oxysterol. *Arterioscler Thromb Vasc Biol.* 2000;20(8):1968-1975.

338. Feng J, Han J, Pearce SF, et al. Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-gamma. *Journal of lipid research.* 2000;41(5):688-696.
339. Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell.* 1998;93(2):241-252.
340. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature.* 1998;391(6662):79-82.
341. Pasini AF, Anselmi M, Garbin U, et al. Enhanced levels of oxidized low-density lipoprotein prime monocytes to cytokine overproduction via upregulation of CD14 and toll-like receptor 4 in unstable angina. *Arterioscler Thromb Vasc Biol.* 2007;27(9):1991-1997.
342. Marchant A, Duchow J, Delville JP, Goldman M. Lipopolysaccharide induces up-regulation of CD14 molecule on monocytes in human whole blood. *European journal of immunology.* 1992;22(6):1663-1665.
343. Chang MK, Bergmark C, Laurila A, et al. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proceedings of the National Academy of Sciences of the United States of America.* 1999;96(11):6353-6358.
344. Chou M-Y, Fogelstrand L, Hartvigsen K, et al. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *The Journal of Clinical Investigation.* 2009;119(5):1335-1349.
345. Sather S, Kenyon KD, Lefkowitz JB, et al. A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. *Blood.* 2007;109(3):1026-1033.
346. Liao X, Sluimer Judith C, Wang Y, et al. Macrophage Autophagy Plays a Protective Role in Advanced Atherosclerosis. *Cell metabolism.* 2012;15(4):545-553.
347. Anderson HA, Englert R, Gursel I, Shacter E. Oxidative stress inhibits the phagocytosis of apoptotic cells that have externalized phosphatidylserine. *Cell death and differentiation.* 2002;9(6):616-625.
348. Ait-Oufella H, Kinugawa K, Zoll J, et al. Lactadherin deficiency leads to apoptotic cell accumulation and accelerated atherosclerosis in mice. *Circulation.* 2007;115(16):2168-2177.
349. Bhatia VK, Yun S, Leung V, et al. Complement C1q reduces early atherosclerosis in low-density lipoprotein receptor-deficient mice. *The American journal of pathology.* 2007;170(1):416-426.
350. Siegert CEH, Daha MR, Swaak AJG, van der Voort EAM, Breedveld FC. The Relationship between Serum Titers of Autoantibodies to C1q and Age in the General Population and in Patients with Systemic Lupus Erythematosus. *Clinical Immunology and Immunopathology.* 1993;67(3):204-209.
351. Ma Z, Choudhury A, Kang S-A, Monestier M, Cohen PL, Eisenberg RA. Accelerated atherosclerosis in ApoE deficient lupus mouse models. *Clinical Immunology.* 2008;127(2):168-175.
352. Liao D, Wang X, Li M, Lin PH, Yao Q, Chen C. Human protein S inhibits the uptake of AcLDL and expression of SR-A through Mer receptor tyrosine kinase in human macrophages. *Blood.* 2009;113(1):165-174.
353. Gordon S, Martinez FO. Alternative Activation of Macrophages: Mechanism and Functions. *Immunity.* 2010;32(5):593-604.
354. Rodondi N, Marques-Vidal P, Butler J, et al. Markers of Atherosclerosis and Inflammation for Prediction of Coronary Heart Disease in Older Adults. *American Journal of Epidemiology.* 2010;171(5):540-549.

355. Dod HS, Bhardwaj R, Sajja V, et al. Effect of Intensive Lifestyle Changes on Endothelial Function and on Inflammatory Markers of Atherosclerosis. *The American journal of cardiology.* 2010;105(3):362-367.
356. Robertson AM, Bird CC, Waddell AW, Currie AR. Morphological aspects of glucocorticoid-induced cell death in human lymphoblastoid cells. *The Journal of pathology.* 1978;126(3):181-187.
357. Feng X, Deng T, Zhang Y, Su S, Wei C, Han D. Lipopolysaccharide inhibits macrophage phagocytosis of apoptotic neutrophils by regulating the production of tumour necrosis factor alpha and growth arrest-specific gene 6. *Immunology.* 2011;132(2):287-295.
358. Lucas M, Stuart LM, Savill J, Lacy-Hulbert A. Apoptotic Cells and Innate Immune Stimuli Combine to Regulate Macrophage Cytokine Secretion. *The Journal of Immunology.* 2003;171(5):2610-2615.
359. Kalayoglu MV, Libby P, Byrne GI. CHlamydia pneumoniae as an emerging risk factor in cardiovascular disease. *JAMA.* 2002;288(21):2724-2731.
360. Wiesner P, Choi SH, Almazan F, et al. Low doses of lipopolysaccharide and minimally oxidized low-density lipoprotein cooperatively activate macrophages via nuclear factor kappa B and activator protein-1: possible mechanism for acceleration of atherosclerosis by subclinical endotoxemia. *Circ Res.* 2010;107(1):56-65.
361. Chen W, Frank ME, Jin W, Wahl SM. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity.* 2001;14(6):715-725.
362. Wong CHY, Heit B, Kubes P. Molecular regulators of leucocyte chemotaxis during inflammation. *Cardiovascular research.* 2010;86(2):183-191.
363. Woollard KJ, Geissmann F. Monocytes in atherosclerosis: subsets and functions. *Nat Rev Cardiol.* 2010;7(2):77-86.
364. de Fougerolles AR, Springer TA. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. *The Journal of experimental medicine.* 1992;175(1):185-190.
365. Greaves DR, Hakkinen T, Lucas AD, et al. Linked chromosome 16q13 chemokines, macrophage-derived chemokine, fractalkine, and thymus- and activation-regulated chemokine, are expressed in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol.* 2001;21(6):923-929.
366. Wong BWC, Wong D, McManus BM. Characterization of fractalkine (CX3CL1) and CX3CR1 in human coronary arteries with native atherosclerosis, diabetes mellitus, and transplant vascular disease. *Cardiovascular Pathology.* 2002;11(6):332-338.
367. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science.* 1990;249(4975):1431-1433.
368. da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. *The Journal of biological chemistry.* 2001;276(24):21129-21135.
369. Asano Y, Horn E. Visualisation and Data Analysis of Chemotaxis and Migration Processes. *Instructions Chemotaxis and Migration Tool 2.0.* 2011;V 1.0.
370. Franc NC, White K, Ezekowitz RAB. Phagocytosis and development: back to the future. *Current Opinion in Immunology.* 1999;11(1):47-52.
371. Greaves DR, Häkkinen T, Lucas AD, et al. Linked Chromosome 16q13 Chemokines, Macrophage-Derived Chemokine, Fractalkine, and Thymus- and Activation-Regulated Chemokine, Are Expressed in Human Atherosclerotic Lesions. *Arteriosclerosis, Thrombosis, and Vascular Biology.* 2001;21(6):923-929.
372. Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD. The combined role of P- and E-selectins in atherosclerosis. *J Clin Invest.* 1998;102(1):145-152.
373. Wilkinson PC. Assays of leukocyte locomotion and chemotaxis. *Journal of Immunological Methods.* 1998;216(1–2):139-153.

374. Abrink M, Gobl AE, Huang R, Nilsson K, Hellman L. Human cell lines U-937, THP-1 and Mono Mac 6 represent relatively immature cells of the monocyte-macrophage cell lineage. *Leukemia.* 1994;8(9):1579-1584.
375. Zhuang L, Pound JD, Willems JJLP, Taylor AH, Forrester LM, Gregory CD. Pure populations of murine macrophages from cultured embryonic stem cells. Application to studies of chemotaxis and apoptotic cell clearance. *Journal of Immunological Methods.* 2012;385(1–2):1-14.
376. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research.* 2009;29(6):313-326.
377. Jones GE, Prigmore E, Calvez R, et al. Requirement for PI 3-kinase γ in macrophage migration to MCP-1 and CSF-1. *Experimental Cell Research.* 2003;290(1):120-131.
378. Li G, Cui Y, McIlmurray L, Allen WE, Wang H. rhBMP-2, rhVEGF165, rhPTN and thrombin-related peptide, TP508 induce chemotaxis of human osteoblasts and microvascular endothelial cells. *Journal of Orthopaedic Research.* 2005;23(3):680-685.
379. Jones GE, Zicha D, Dunn GA, Blundell M, Thrasher A. Restoration of podosomes and chemotaxis in Wiskott–Aldrich syndrome macrophages following induced expression of WASp. *The International Journal of Biochemistry & Cell Biology.* 2002;34(7):806-815.
380. Geiger-Maor A, Levi I, Even-Ram S, et al. Cells Exposed to Sublethal Oxidative Stress Selectively Attract Monocytes/Macrophages via Scavenger Receptors and MyD88-Mediated Signaling. *The Journal of Immunology.* 2012.
381. Gregory CD, Pound JD. Results of defective clearance of apoptotic cells: lessons from knock-out mouse models. *Phagocytosis of Dying Cells: From Molecular Mechanisms to Human Diseases*: Springer; 2009:271-298.
382. Isse K, Harada K, Zen Y, et al. Fractalkine and CX3CR1 are involved in the recruitment of intraepithelial lymphocytes of intrahepatic bile ducts. *Hepatology (Baltimore, Md.).* 2005;41(3):506-516.
383. Landsman L, Bar-On L, Zernecke A, et al. CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. *Blood.* 2009;113(4):963-972.
384. Sugino H. ICAM-3, a ligand for DC-SIGN, was duplicated from ICAM-1 in mammalian evolution, but was lost in the rodent genome. *FEBS Lett.* 2005;579(13):2901-2906.
385. Hubacek JA, Rothe G, Pit'ha J, et al. C(-260)-->T polymorphism in the promoter of the CD14 monocyte receptor gene as a risk factor for myocardial infarction. *Circulation.* 1999;99(25):3218-3220.
386. Truman LA, Ogden CA, Howie SEM, Gregory CD. Macrophage chemotaxis to apoptotic Burkitt's lymphoma cells in vitro: role of CD14 and CD36. *Immunobiology.* 2004;209(1–2):21-30.
387. Qiu Q, Xiong W, Yang C, Gagnon C, Hardy P. Lymphocyte-derived microparticles induce bronchial epithelial cells' pro-inflammatory cytokine production and apoptosis. *Molecular immunology.* 2013;55(3–4):220-230.
388. Pan J, Xia L, McEver RP. Comparison of promoters for the murine and human P-selectin genes suggests species-specific and conserved mechanisms for transcriptional regulation in endothelial cells. *The Journal of biological chemistry.* 1998;273(16):10058-10067.
389. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med.* 2011;17(11):1410-1422.
390. Herrero C, Sebastian C, Marques L, et al. Immunosenescence of macrophages: reduced MHC class II gene expression. *Exp Gerontol.* 2002;37(2-3):389-394.
391. De Martinis M, Modesti M, Ginaldi L. Phenotypic and functional changes of circulating monocytes and polymorphonuclear leucocytes from elderly persons. *Immunology and cell biology.* 2004;82(4):415-420.

392. Roumenina LT, Sène D, Radanova M, et al. Functional Complement C1q Abnormality Leads to Impaired Immune Complexes and Apoptotic Cell Clearance. *The Journal of Immunology.* 2011;187(8):4369-4373.
393. Chelvarajan RL, Collins SM, Van Willigen JM, Bondada S. The unresponsiveness of aged mice to polysaccharide antigens is a result of a defect in macrophage function. *Journal of leukocyte biology.* 2005;77(4):503-512.
394. Vega VL, De Cabo R, De Maio A. Age and caloric restriction diets are confounding factors that modify the response to lipopolysaccharide by peritoneal macrophages in C57BL/6 mice. *Shock (Augusta, Ga.).* 2004;22(3):248-253.
395. Sebastian C, Espia M, Serra M, Celada A, Lloberas J. MacrophAging: a cellular and molecular review. *Immunobiology.* 2005;210(2-4):121-126.
396. Shao W-H, Cohen P. Disturbances of apoptotic cell clearance in systemic lupus erythematosus. *Arthritis Research & Therapy.* 2011;13(1):202.
397. Gomez CR, Acuna-Castillo C, Nishimura S, et al. Serum from aged F344 rats conditions the activation of young macrophages. *Mechanisms of ageing and development.* 2006;127(3):257-263.
398. Gale CR, Baylis D, Cooper C, Sayer AA. Inflammatory markers and incident frailty in men and women: the English Longitudinal Study of Ageing. *Age (Dordrecht, Netherlands).* 2013.
399. Viera AJ, Mooberry M, Key NS. Microparticles in cardiovascular disease pathophysiology and outcomes. *Journal of the American Society of Hypertension.* 2012;6(4):243-252.
400. Hansson GK, Holm J, Jonasson L. Detection of activated T lymphocytes in the human atherosclerotic plaque. *The American journal of pathology.* 1989;135(1):169-175.
401. Fenn AM, Henry CJ, Huang Y, Dugan A, Godbout JP. Lipopolysaccharide-induced interleukin (IL)-4 receptor-α expression and corresponding sensitivity to the M2 promoting effects of IL-4 are impaired in microglia of aged mice. *Brain, Behavior, and Immunity.* 2012;26(5):766-777.
402. Lavie L, Gershon D. Oxygen free radical production by mouse peritoneal macrophages as a function of age. *Mechanisms of ageing and development.* 1988;45(2):177-189.