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Oxidised Lipids and Their Role in the Immunopathology of Psoriasis

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Abstract

Dyslipidaemia is known to be one of the major contributory factors in atherosclerosis and cardiovascular disorders. In particular, increased circulating low-density lipoprotein (LDL) and accumulation in the vascular bed is thought to have damaging effects. Abnormal lipid profiles in patients with psoriasis have also been identified and it is hypothesised that a similar pathogenesis underlies the two conditions. In particular, the oxidative modification of the LDL particle (oxLDL) is thought to promote inflammation. This thesis therefore aims to investigate whether lipid accumulation and oxidation influences inflammation and vascular function.

The inflammatory events occurring in psoriasis were modelled in this thesis using *in vitro* assays for angiogenesis, endothelial cell migration, inflammatory cell recruitment under flow and immune cell proliferation. Human umbilical vein endothelial cells (HUVECs) were used as a model endothelium to study the effects of oxidised lipids on vascular function. In order to analyse the lipid contribution to inflammation, serum was depleted of lipids and compared to serum with a full lipid profile. The addition of lipids such as ox-/LDL was evaluated in complete and delipidated serum.

This investigation showed that in the absence of serum lipids, endothelial tube formation was significantly impaired, in addition to endothelial cell migration being inhibited. The proliferation of peripheral blood mononuclear cells (PBMCs) was also abrogated in the absence of serum lipids. Serum lipid depletion did not, however, have any effect on the ability of TNFα- or IFNγ-stimulated endothelial cells to recruit leukocytes under shear stress. The addition of oxLDL had biphasic effects on endothelial responses by significantly enhancing migration at lower concentrations of 5μg/mL, yet significantly inhibiting this response at higher concentrations of 100μg/mL. In the recruitment assay, higher concentrations of oxLDL were able to increase the basal recruitment of neutrophils to the endothelial monolayer whereas lower concentrations had no effect on recruitment. Native LDL had no significant effect on these parameters at equivalent concentrations. OxLDL-induced endothelial migration was inhibited by the addition of a sphingosine kinase inhibitor and a blockade of $S1P_1$ on endothelial cells. OxLDL induced migration was not affected by the inhibition of PAF-R. Flow cytometry was used to assess the expression of oxLDL receptors on HUVEC, showing no expression of LOX-1 but slight increases in CD36 expression, although not reaching statistical significance.

These data indicate that serum lipids are key in supporting in the angiogenic response, likely by affecting endothelial cell migration, in addition to having an important role in the maintenance of immune cell proliferation. Oxidised lipids have also been shown to have biphasic effects on inflammatory processes, dependent on concentration. These results also highlight the critical role for S1P in oxLDL-induced endothelial cell migration, with the blockade of sphingosine kinase and $S1P_1$ effectively inhibiting these vascular responses. This thesis highlights the importance of the oxidation of LDL and subsequent oxLDL concentration on regulating inflammation. Further understanding in the mechanisms of oxLDL-induced inflammation may provide a therapeutic target in psoriasis and other related inflammatory conditions.

Table of Contents

Table of Figures

Academic Thesis: Declaration of Authorship

I, **Michael Olding**, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Title of thesis: Oxidised lipids and their role in the immunopathology of psoriasis

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission.

Signed: …….

Date: …….

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Abbreviations

aCD3	anti-CD3
ANOVA	analysis of variance
APC	allophycocyanin
apoA-E/H	apolipoprotein A/B/C/D/E/H
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CD	coronary disease
CFSE	carboxyfluorescein succinimidyl ester
CH_3	methyl group
COOH	carboxylic acid
CVD	cardiovascular disorder
CXCL	chemokine (C-X-C motif) ligand
CXCR	CXC chemokine receptor
DIPE	di-isopropyl ether
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC	endothelial cell
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FA	fatty acid
FACS	fluorescence-activated cell sorting
FH	familial hypercholesterolaemia
FITC	fluorescein isothiocyanate
GIMP	GNU image manipulation programme
GPCR	G-protein coupled receptor
HBSS	hanks buffered saline solution
HDL	high-density lipoprotein
HIF-1	hypoxia-induced factor-1

HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HUVEC	human umbilical vein endothelial cells
ICAM	inter-cellular adhesion molecule
IFN	interferon
IL	interleukin
JAM	junctional adhesion molecule
LCAT	lechitin-cholesterol acyl transferase
LDL	low-density lipoprotein
LFA	lymphocyte function-associated antigen
LFA-1	lymphocyte function-associated antigen-1
LOX-1	lectin-like oxidised low-density lipoprotein receptor-1
LPL	lipoprotein lipase
MAC-1	macrophage-1 antigen
MCP	monocyte chemotactic protein
MDA	malondialdehyde
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MICAM	microscope image capturing and measuring
MPO	myeloperoxidase
MS	mass spectrometry
NAC	n-actyl cysteine
NET	neutrophil extra-cellular trap
NF-$_k$B	nuclear factor kappa-light-chain-enhancer of activated B cells
oxLDL	oxidised low-density lipoprotein
PAF	platelet-activating factor
PAF-R	platelet-activating factor receptor
PAPC	1-palmitoyl-2-arachidinoyl-sn-glycero-3-phosphorylcholine
PASI	psoriasis area and severity index
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	phycoerythrin

PECAM	platelet-endothelial cell adhesion molecule
PHA	phytohaemagglutinin
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear cell
PsA	psoriatic arthritis
PSGL-1	P-selectin glycoprotein ligand-1
PSORS	psoriasis susceptibility loci
PUFA	poly-unsaturated fatty acid
RBC	red blood cell
ROS	reactive oxygen species
RPMI	Roswell park memorial institute medium
S1P	sphingosine-1-phosphate
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-poly-acrylamide gel electrophoresis
SR	scavenger receptor
SREBP	sterol regulatory element binding protein
TBARS	thiobarbituric acid reactive substances
TCR	T-cell receptor
TGF-β	transforming growth factor-β
Th	T-helper
TLC	thin layer chromatography
TNF	tumour necrosis factor
UV	ultraviolet
VCAM	vascular cell adhesion molecule
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VLA-4	very late antigen-4
VLDL	very low-density lipoprotein
WHO	world health organisation

1 Chapter 1: Introduction

1.1 Introduction

The purpose of this research is to investigate the potential role of lipids and oxidised lipids, primarily oxidised low density lipoproteins (oxLDL), in the immunopathology of psoriasis through their actions on immune cells and the vascular endothelium. This thesis will investigate the effects of lipids on endothelial function important in the inflammatory response, including endothelial migration, angiogenesis and their interaction with circulating leukocytes. The effects of lipids on immune cell proliferation and function will be investigated in addition to the mechanism by which oxLDL induces vascular inflammation.

1.1.1 Psoriasis

Psoriasis is regarded as a chronic inflammatory skin disorder that affects around 2% of the UK population (Armstrong et al., 2013). The skin is derived from three major layers: the epidermis, dermis and the subcutaneous layer from upper to lower regions respectively. The epidermal layer is composed predominantly of keratinocytes, whereas the dermal region contains the blood vessels, hair follicles, sweat glands, extracellular matrix (collagen and elastin) and some nerve endings. The changes seen in psoriatic skin consist of a thickened epidermal layer and an infiltration of immune cells into the dermis causing inflammation. There are different forms of psoriasis that depend on both the clinical and histologic presentation of the skin. The most common form is known as chronic plaque psoriasis but other forms include guttate, inverse, pustular and erythrodermic psoriasis (Parisi et al., 2013).

Chronic plaque psoriasis is hallmarked by sharply demarcated skin lesions which appear red and slightly raised with silver-whitish scales, forming at various sites around the body, including at the elbows and knees (Sabat et al., 2007). Early views on the disease centred primarily on changes observed in the epidermis, the upper portion of the skin and abnormalities in cells such as keratinocytes which form a major part of the skin structure. The epidermis helps to form an effective barrier to the outside environment and the visible hallmark of psoriasis involves hyper-proliferation and abnormal

differentiation of epidermal cells leading to epidermal thickening known as acanthosis (Griffiths et al., 2007). Abnormal keratinocyte function in psoriasis is, however, thought to be only partly responsible for sustaining disease progression and other pathogenic factors are becoming increasingly evident (Kivelevitch et al., 2013).

It has now become apparent that psoriasis is a multifactorial disorder considered to have environmental, metabolic and genetic components (Weidemann et al., 2013). One key component is the role of the immune system in psoriasis as research has identified large infiltrates of activated inflammatory cells in the skin of patients (Heidenreich et al., 2009). Further evidence for an immunological component to the disease comes from the use of immunosuppressive agents in its treatment. Immune modifying drugs such as methotrexate, glucocorticosteroids and cyclosporine A have been effective in clearing psoriatic plaques and ultra-violet (UV) light therapy, known for its suppressive effects on immune functions, has also shown benefits in disease treatment (Haustein et al., 2000; Ellis et al., 1986; Almutawa et al., 2013).

There is considerable evidence (Lowes et al., 2007) that leukocytes are the main driver of disease pathology whether by direct effects or indirectly through the release of various immune modifiers such as cytokines. The immune infiltrate in psoriasis consists of dendritic cells (DCs), neutrophils and lymphocytes with the most abundant inflammatory cells present generally being the T lymphocytes. CD4+ T cells form a significant lymphocyte population in the skin with a ratio of 2:1 CD4+ cells compared to CD8+ cells respectively (Veale et al., 2005). The cellular infiltrate is predominantly found in a perivascular distribution which highlights the importance of the vascularised dermis in immune trafficking (as depicted in Figure 1-1). Certain infiltrating populations are also known to migrate into the epidermis (Tsuruta, 2009).

Current models of disease pathogenesis have focused on the secreted cytokines from activated leukocytes which in turn induce changes in skin cells (Krueger et al., 2005). The CD4+ cell population is known for its prolific cytokine production and is designated into two initial general groups depending on function; the so-called T-helper 1 and 2 (Th1/Th2) axis. Th1 cells are commonly considered pro-inflammatory, with the Th2 cells having pro-inflammatory and/or anti-inflammatory roles. With the identification of the

immune infiltrate in the skin, psoriasis was initially considered a Th1 mediated disease with the production of pro-inflammatory cytokines such as interferon-gamma (IFNγ) and interleukin-2 (IL-2). In particular, the potent pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) was found to be increased in psoriasis and was associated with keratinocyte proliferation and adhesion molecule expression. These factors enable immune cell trafficking and thus help to drive disease progression (Veale et al., 2005).

More recent research highlighted the importance of additional T-helper subsets in psoriasis with the description of the T-helper 17 (Th17) axis. IL-23, released from dendritic cells and keratinocytes in this skin, has been shown to stimulate the production of Th17 cells which in turn produce pro-inflammatory cytokines such as interleukin-17 (IL-17) and interleukin-22 (IL-22) (Lowes et al., 2008; Johansen et al., 2008) adding to the complexity of the microenvironment. The importance of the Th17/IL-23 axis has been confirmed by the success of targeting IL-23 in the treatment of psoriasis (Sofen et al., 2014). The production of IL-17 from Th17 cells stimulates keratinocytes to produce various pro-inflammatory mediators and is a significant factor in sustaining chronic inflammation (Lowes et al., 2008).

The innate immune system has also been shown to be involved in the release of this cytokine in psoriasis, with neutrophils identified to express IL-17 using established models of *in vivo* human skin inflammation (Keijsers et al., 2014). Neutrophils are a hallmark of psoriasis pathology (as discussed in section 1.1.4.1) and show distinct focal areas of epidermal accumulation in histological analysis (Lin et al., 2011). Neutrophil infiltration, along with increased blood vessel formation, is an early indicator of disease progression. A study on neutrophils in psoriasis has also shown that neutrophil activation markers are linked to disease severity and vascular inflammation (Naik et al., 2015). Neutrophils are therefore considered an important contributor to psoriasis pathology and will be studied in this thesis.

Another subset of CD4+ T cells which are present in the skin are regulatory T cells (T-regs), which are specialised T cells mediating peripheral tolerance by actively suppressing immune responses (Corthay, 2009). T-regs can suppress inflammation by causing cytokine deprivation (such as IL-2), producing anti-inflammatory cytokines such as IL-10

and have cytotoxic actions to eliminate effector T cells (Corthay, 2009) and have also been shown to be defective in their suppressor function in psoriasis (Sugiyama et al., 2005). The complex network of cytokines and inflammatory cells is a hallmark of psoriatic pathology and despite many questions being addressed the disease is still prevalent and highlights the need for further investigation.

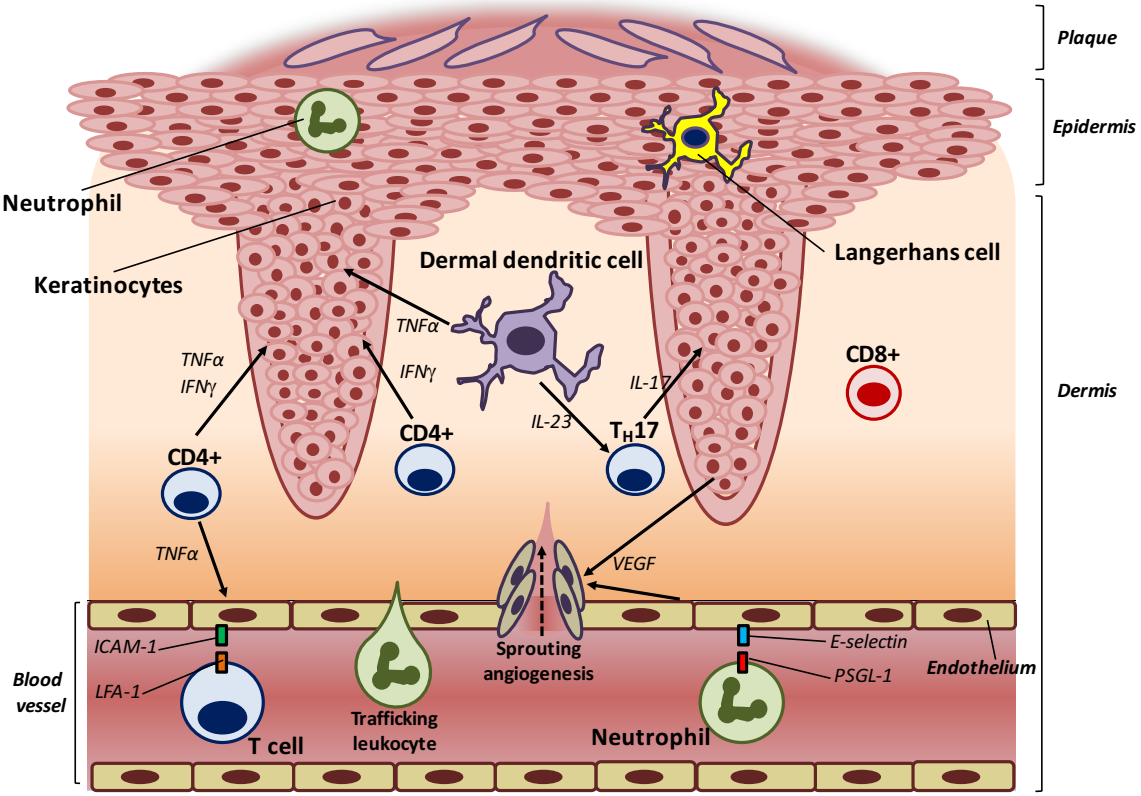


Figure 1-1 Immunology of psoriasis
A diagram depicting the cellular events occurring in the skin of psoriasis patients. Immune cells (such as T cells and neutrophils) traffic from the vascularised dermis (bottom) into the tissue to cause inflammation and keratinocyte proliferation, causing plaque formation on the skin surface (top). Diagram not to scale.

1.1.1.1 Genetics of psoriasis

A common comorbidity which accompanies around 25% of the patients who have psoriasis is a form of arthritis known as psoriatic arthritis (PSA), an interrelated disorder affecting the joints (Liu et al., 2008). The complexity of the underlying pathology of both diseases makes it difficult to pinpoint a single cause and it is generally accepted that

there are both environmental and genetic factors which influence the onset of both diseases (Bazso et al., 2014). Disturbances in the frequency of HLA antigens in psoriatic patients was shown in 1972, suggesting that HLA phenotypes were important and could have relevance to the susceptibility of an individual to the disease (Russell et al., 1972; White et al., 1972). A strong association with the major histocompatibility complex (MHC) class I region, known as PSORS1 (psoriasis susceptibility locus 1) was shown by Tiilikainen et al., 1980. Since this time, many more genetic susceptibility factors have been identified using genome-wide association studies, revealing PSORS 1-9 associations (Rahman & Elder, 2005) and over 50 other regions associated with psoriasis risk, with a significant percentage of these genes related to the immune system (Harden et al., 2015). The trigger which causes psoriasis is unknown, but the genetic studies mentioned above suggest that altered immune responses to environmental cues are a major contributor to inflammation in psoriasis, particularly involving antigen presentation and T cell responses. However, only around 10% of individuals with genetic variation in MHC develop psoriasis, highlighting the complexity of both genetic and other contributions to the disease (Liu et al., 2008).

1.1.1.2 Treatment of psoriasis

Although there is currently no cure for psoriasis, there are a number of treatments available. The treatment option in the clinic is generally based on the type of psoriasis presented, the severity of the psoriasis (dictated by the psoriasis area and severity index (PASI) score) and the age of the patient (Smith et al., 2009). Those patients who have a limited disease presentation are generally treated with topical agents. There are numerous options including emollients, salicylic acid, topical steroids, tar preparations, dithranol, vitamin D analogues or vitamin A analogues (BAD, 2014). For those with more moderate or severe forms of disease presentation, one of the major options available is phototherapy. This uses ultraviolet radiation at certain wavelengths to directly irradiate the skin. The most common phototherapy used in current practice is narrowband ultraviolet (UV) B therapy (311nm) which has generally replaced PUVA therapy (320-400nm). PUVA therapy combines UV radiation and psoralen (a photo-sensitising compound which has a high UV absorbance), to elicit a regression of the psoriatic plaques. PUVA treatment has been linked with an increased risk of skin cancer in patients

treated with multiple exposures compared to narrow band UVB therapy or non-UV treated psoriatics (Chuang et al., 1992; Lidelof et al., 1999). In more severe cases of psoriasis, other therapies may be considered, such as acitretin (a retinoid), cyclosporine A, methotrexate and 'biologics' such as anti TNFα therapy infliximab as immunosuppressants. Some less commonly used compounds such as fumaric acid esters have also shown efficacy.

UVB phototherapy generally, but not always, treats psoriasis in the skin through immunosuppressive and immunomodulatory mechanisms. Although phototherapy is one of the most efficacious options for psoriasis and has been known for many years, the exact mechanisms exerted by ultraviolet irradiation of the skin have not been fully appreciated. It was shown that wavelengths of 311nm are more effective in the clearance of psoriasis (Fischer & Alsins, 1976). The confirmation of such work by other groups since then led to the use of fluorescent lamps emitting a major peak at 311nm, now known as narrowband UVB. The UVB spectrum (280nm-320nm) is mainly absorbed by the components of the epidermis such as keratinocytes, whereas UVA (320-400nm) shows deeper penetration into the dermal regions of the skin (Berneburg et al., 2005).

The ultraviolet phototherapy is thought to be absorbed by molecules called chromophores, the part of a molecule responsible for its colour by absorbing specific wavelengths of light. In the skin, UV can be absorbed by proteins such as keratin and collagen, in addition to DNA (Berneburg et al., 2005). Although there is a wealth of research on the molecular modifications caused by UV light, the effect of light therapy on lipids in the skin is of particular interest. The epidermal layer of the skin is a highly active lipid synthetic tissue consisting of numerous lamellar bodies, particularly in the stratum granulosum layer (Feingold, 2007). Lamellar bodies are lipid-containing organelles which are secreted by keratinocytes, contributing to skin barrier function (Feingold, 2012). It has previously been identified that sub-erythemal doses of either UVA or UVB increased the lipid content extracted from the top layer of the skin, the stratum corneum, suggesting that an increased barrier function is related to an increase in lipids, especially skin ceramides (Wefers et al., 1991). However, more recently it has been shown that in subcutaneous fat tissue, deeper in the skin, there is reduced lipid synthesis after exposure to UV and that chronically sun-damaged skin contains less fat than naturally

aged skin (Kim et al., 2011). Given that UV improves psoriasis, it may be important to clarify the extent of the therapeutic nature of UV in relation to UV-dependent lipid changes.

It is thought that on exposure to phototherapy there is a generation of reactive oxygen species within the skin such as superoxide anion (O_2^-) and hydroxyl radical (·OH), which have obvious implications in causing oxidized lipid formation from membranous lipids (Ogura et al., 1991; Moysan et al., 1993). In response to stressors such as UV radiation causing oxidative damage to lipids, the skin contains antioxidants and detoxifying enzymes to either prevent or repair potentially damaged material (Zhao et al., 2013). The fact that UV light causes potential lipid peroxidation, which has been shown to be pro-inflammatory and yet has opposing effects in terms of clearing psoriasis, highlights the complexity of its function. It was recently shown however that UV promotes the removal of oxidized phospholipids in epidermal keratinocytes as a result of promoting autophagy (Zhao et al., 2013), suggesting a potential anti-oxidative effect.

For patients with hyperlipidaemia who risk cardiovascular events, one of the primary pharmaceutical interventions is the use of a class of drug called statins. Statins (3-hydroxy-3-methyl-glutaryl-coenzymeA [HMG-CoA] reductase inhibitors) interfere with a rate limiting step in cholesterol synthesis in addition to pleiotropic anti-inflammatory effects. This helps to reduce blood lipid profiles and protects against mortality from coronary disease (Kulbertus & Scheen, 2002). Other than direct inhibition of the cholesterol synthesis pathway, the beneficial effects that statins have in terms of suppressing inflammation include the down-regulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-1 (LFA-1) in addition to blocking pro-inflammatory cytokines such as TNFα, IFNγ and monocyte chemotactic protein-1 (MCP-1) (Namazi, 2004; Greenwood et al., 2006).

Given the lipid profiles seen in psoriasis and the general clinical efficacy of statins in modifying cardiovascular risk, it is plausible to hypothesise a beneficial use of lipid lowering therapy in psoriasis. This idea has been suggested since the 1990s, but evidence for the effectiveness of statins in psoriasis however is inconclusive. One of the first papers relating to the topic was published in 1992 in a study group of 10 psoriatic

patients in which it was concluded that pharmacological doses of one of the statins (lovastatin) did not affect the course of psoriasis (Aronson & Friedman, 1992). In a summary article of psoriasis and lipids it was noted that a few studies indicated improvement in psoriasis with the treatment of associated hyperlipidaemia, where a low-fat diet with statin therapy caused an improvement in psoriasis and a reduced PASI score (Ghazizadeh et al., 2011).

In terms of the mechanisms by which lipid lowering drugs might improve psoriasis, it was shown that certain statins interrupt the CCL20/CCR6 chemotactic interaction that recruits Th17 cells which are important to psoriasis pathology (Kim et al., 2011). More recently it was shown that atorvastatin ameliorated skin inflammation in mice due to inhibition of cytokine release (such as TNFα) and NF-$_k$B activation in the skin, suggesting some benefit in using these compounds in inflammatory skin disease like psoriasis (Kulkarni et al., 2014). There is evidence both for and against the beneficial effect of statins in psoriasis as well as the contribution of lipids to the inflammatory response in the skin. The picture of lipids and psoriasis seems a complex one with contradicting evidence. This project will aim to research the contribution of lipids (and oxidised lipids) to inflammatory processes involved in psoriasis in order to further clarify the potential influence of lipids in its pathology.

1.1.2 Lipids and their biological importance

Lipids are biological molecules which are chemically defined and characterised as substances with limited solubility in water but solubility in non-polar organic solvents. In simple terms, lipids are described as fats and play major roles in the body most notably in the structural and functional aspects of cell membranes. In addition, lipids can be used as an energy source to satisfy metabolic demand, are important in vitamin uptake and hormonal function, are crucial to temperature regulation and can play important roles in the pathology of certain diseases (McIntyre & Hazen, 2010). Lipids can range in molecular structure from simple lipids such as fatty acids, to complex lipids such as triglycerides which are derived from multiple lipid molecules bound via covalent bonds.

Fatty acids are the building blocks of most complex lipids. Fatty acids are typically composed of a carboxyl group (carboxylic acid [COOH]) at one end of their structure

followed by a long carbon chain (acyl chain) and a methyl group at the other end [CH_3] (discussed in 1.1.2.6). The carbon chain lengths of fatty acids in the human body usually range from 14-20 carbon atoms and typically have an even number of carbon molecules in the backbone. Fatty acids with longer than 20 carbons can be found and the even-numbered nature of their chain length is a function of the serial addition of two carbon units during synthesis. Fatty acids will typically be attached to a glycerol backbone in order to build more complex lipids. When three fatty acid chains are bound to a glycerol component, a triglyceride is formed. A further modification of the triglyceride generates the group of common structural lipids known as phospholipids. These lipids are characterised by two fatty chains joined to a glycerol backbone and the third fatty acid replaced by a phosphate group attached to the glycerol molecule.

1.1.2.1 Lipids in disease and inflammation

The notion that lipids contribute to human disease is one which has been discussed for many years (Ahrens, 1976). In particular, lipids have been reported to be highly important in the thickening of the arterial wall (atherosclerosis) as a result of immune infiltration and accumulation. This contributes to cardiovascular disease which is the leading cause of morbidity and mortality in Westernised countries (Burnett, 2004). It is also the number one cause of death globally which has been recognised by the World Health Organisation (WHO) in its global strategy to reduce energy intake from total fats (WHO, 2014). The general hypothesis proposed in relation to lipids in atherosclerosis involves the oxidative modification of the low-density lipoprotein (LDL) particle which is presumed to accumulate in the sub-endothelial space and promote an inflammatory environment. This long-standing hypothesis comes from research implicating oxLDL in vascular pathology with the accumulation of foam cells in the arterial wall (Witztum & Steinberg, 1991). The relationship between high blood cholesterol level and coronary disease (CD) has also long been shown in particular in patients with familial hypercholesterolemia as they have a high death rate from CD and at an earlier age than the general population (Jensen et al., 1967; Mabuchi et al., 1986).

Foam cells are derived from macrophages which have taken up oxidatively modified LDL and accumulate to form the fatty streak and plaque build-up in arteries. A substantial plaque within the artery wall generally has a necrotic lipid core and can project into the

lumen of the artery which, if ruptured, leads to thrombosis and myocardial infarction and/or stroke. Although native LDL does accumulate in the vessel wall, it is only the oxidative modification of the molecule which causes its recognition by scavenger receptors such as CD36 on macrophages leading to plaque formation. Not only does oxLDL contribute to foam cell formation but it has been shown to induce the release of MCP-1 from vascular endothelial cells (Reddy et al., 2001). This factor increases inflammation as it recruits monocytes into atherosclerotic lesions. Oxidative modification of the LDL particle causes it to lose its affinity for the native LDL receptor and instead causes it to become a ligand for other receptors, which will be discussed in section 1.1.2.7.

Oxidized lipids have, over the years, been isolated from atherosclerotic plaques and detected in the circulation (Birukov, 2006) with the extracts taken from human lesions shown to be substantially oxidised (Stocker & Keaney, 2004). In vascular complications, such as unstable angina for example, circulating levels of oxLDL have been associated with plaque vulnerability (Niccoli et al., 2007) and auto-antibodies against oxLDL have also been linked with disease presentation and severity (Faviou et al., 2005).

Along with the recognition of oxidized lipids in vascular disorders, research has led to the consensus that oxLDL induces endothelial dysfunction. For example, low concentrations of oxLDL (<10μg/mL) have been shown to have significant effects on the endothelial nitric oxide synthase pathway which impacts on the ability of the blood vessel to increase blood flow via vasodilation (Chavakis et al., 2001; Yu et al., 2011). The exact role of oxLDL in this aspect is still considered speculative. From *in vitro* evidence, different concentrations of oxLDL can inhibit the nitric oxide synthase pathway (Chavakis et al., 2001) which is associated with the inhibition of endothelial migration. Conversely, in other studies, oxLDL has been shown to promote migration (Yu et al., 2011) leading to angiogenesis. The presence of oxLDL has also been shown to stimulate immune trafficking with MCP-1 regulated signalling as mentioned above, in addition to the stimulated expression of adhesion molecules such as ICAM-1 on the endothelial surface (Stewart & Nagarajan, 2006). In addition, oxidized lipids have been implicated in pulmonary diseases (Palanisamy et al., 2012; Larrson et al., 2014) and, of relevance to this project, psoriasis.

1.1.2.2 Lipid profiles in psoriasis

Many studies have examined both blood and skin lipids in groups of patients with psoriasis and compared them with healthy control groups (Veetil et al., 2012). From those studies, psoriasis pathology appears to be multifactorial with the suggestion being that dyslipidaemia is a comorbidity of the disease. Numerous studies have implicated an altered lipid profile in the blood of psoriasis patients (Mallbris et al., 2006; Pietrzak et al., 2010; Dsouza et al., 2013; Nehmati et al., 2013), including powerful studies using meta-analysis (Miller et al., 2013). The result of the abnormal lipid profiles in psoriasis is the prevalence of cardiovascular disorders in these patients at a higher rate than in the unaffected population as a whole. A consistent finding identified in studies into this area is the increase in serum triglyceride, cholesterol, low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL). Conversely, the high-density lipoproteins (HDL) which are known to be anti-atherogenic are regularly shown to be decreased in the same patients (Taheri-Sarvtin et al., 2014).

The existence of oxidised lipids in the skin of psoriatic patients was shown for the first time in 2007, using a direct immunofluorescence method (Tekin et al., 2007). This paper highlighted areas of oxLDL accumulation in psoriatic skin which was absent in non-lesional skin and therefore suggested that oxidized lipids play an inflammatory role in this disease. Given that such oxLDL accumulation is also seen in atherosclerosis, supposedly 'trapped' in the sub-endothelial space and promoting inflammation, it is hypothesised that similar pathological processes underlie both diseases (Pietrzak et al., 2010). Given the vast complexity of psoriasis pathology, overlapping cell types of the skin, immune system and vasculature, it has not been elucidated as to what extent lipids may be affecting, or even exacerbating, the inflammatory response in this setting. Clarifying the role of such lipids may have potential therapeutic benefit in relieving the signs and symptoms of psoriasis. This hypothesis is also backed up by evidence to suggest that there is a relationship between lipid profiles and the severity of the disease (Taheri-Sarvtin et al., 2014; Pietrzak et al., 2010). This confirmed research which suggested that autoantibodies against oxLDL, detected in the psoriatic circulation, were correlated with a measure of disease severity, the Psoriasis Area and Severity Index (PASI) score (Orem et al., 1999).

1.1.2.3 Oxidised lipid accumulation in psoriasis

It is known that oxidised lipids influence cardiovascular disease by promoting atherosclerosis. The presence of oxLDL in atherosclerotic lesions has been studied using antibodies that recognise specific epitopes on oxLDL, which avidly stain atherosclerotic lesions in humans and mice with no demonstrable staining in normal arteries (Yla-Herttuala et al., 1989; Torzewski et al., 2004). Similarly, the accumulation of oxidised lipids in psoriatic skin was shown for the first time using a direct immunofluorescence method in 2007 (Tekin et al.), highlighting markedly increased oxLDL in lesional skin, particularly in the upper epidermal regions, which was not seen in nonlesional skin. The precise mechanism of oxLDL accumulation and level of oxidation in psoriasis is largely unknown, although the level of autoantibodies against oxLDL has been suggested to reflect *in vivo* oxidation, of which psoriatic patients have significantly increased evidence in the circulation (Sunitha et al., 2016; Orem et al., 1999; Pietrzak, 2010). The timing of oxLDL accumulation relative to disease progression in psoriasis is also largely unknown, although antibodies against oxLDL have been correlated with polymorphonuclear elastase (Orem et al., 1999), suggesting that oxLDL accumulation may be linked to neutrophil infiltrate. Patients with psoriasis also show increased risk of cardiovascular disease (Pietrzak et al., 2010) and epidemiological studies suggest psoriasis to be an independent risk factor for heart disease (Lai et al., 2016). Although the presence of oxLDL in psoriasis has been documented, the exact biology of its influence in skin inflammation warrants further investigation and this thesis aims to identify the role of oxLDL in the inflammatory events occurring in psoriasis.

1.1.2.4 The low-density lipoprotein (LDL) particle and its lipid components

By definition, lipids are molecules which are not soluble in water. Given that the blood stream is an aqueous environment, lipids are required to be transported in complex aggregates of lipids and proteins known as lipoproteins. These lipoproteins are water-soluble carriers, resembling multi-molecular packages which supply bodily tissues with the necessary cholesterol demands (if the cell requires cholesterol beyond its own internal HMG-CoA production pathway and is expressing the LDL receptor). The generalised structure of a lipoprotein particle (as shown in Figure 1-2) consists of a non-polar core of triglycerides and cholesteryl esters, surrounded by an amphipathic layer of

proteins, phospholipids and cholesterol. There are several types of lipoproteins which have a range of physical and chemical properties that influence their metabolic function. The main lipids contained within lipoproteins are free and esterified cholesterol and triglycerides (Hegele, 2009), but the ratio of these lipids with other proteins in the lipoprotein influences the buoyant density of the particle. These ratios are important, as for clinical and research purposes, lipoproteins are often separated by ultracentrifugation based on their buoyant density (Dong et al., 2011; Sawle et al., 2002). It is common to characterise lipoproteins into density classes from low to high density, including: very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL) and high-density lipoproteins (HDL). The ratio of lipid to protein regulates the buoyant density of the particle, with a higher protein percentage content leading to an increase in particle density. It is generally accepted that, in excess, the LDL molecule is pro-atherogenic and pro-inflammatory whereas the HDL molecule has opposing effects. Both molecules, in balance, are fundamental in the regulation of lipid metabolism within the body. The absolute values are an important indicator of cardiovascular risk with an LDL value of >3mmol/L being undesirable, but the ratio of LDL to HDL is potentially of greater predictive value with an LDL/HDL ratio of >3 leading to greater vascular risk (Millan et al., 2009). Adult treatment panel (ATP) guidelines have changed multiple times over the years regarding the treatment approach for LDL and HDL, but current guidelines set LDL as the primary intervention target. The exact levels of oxLDL, or even oxHDL, are not currently considered in the primary guidelines, but evidence suggests they may have influence in the increased cardiovascular risk seen in patients with imbalanced lipid and cholesterol levels (Marin et al., 2015).

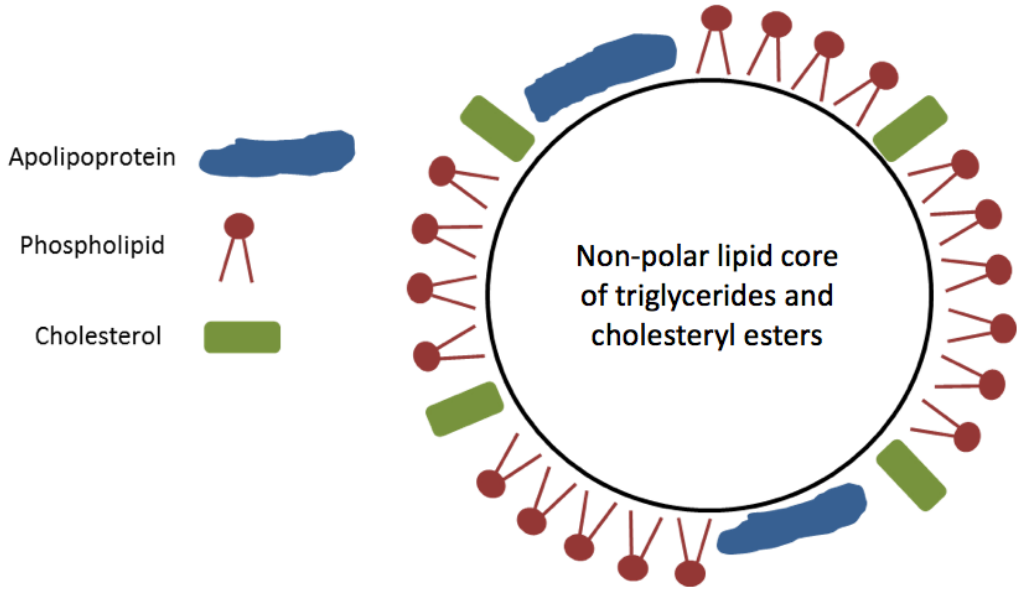


Figure 1-2 Basic structure of a lipoprotein
Schematic diagram depicting the general structure of a lipoprotein showing a non-polar lipid core surrounded by a monolayer of phospholipids, cholesterol and apolipoproteins. The lipoprotein in the diagram has two apolipoproteins indicative of a VLDL particle as LDL contains only one apolipoprotein (apoB).

The LDL particle carries between ~3000 and 6000 individual lipid molecules and is spherical, typically 20-25nm in diameter (Kumar et al., 2011). The size of the particle has also been reported to be smaller, around 18nm, but with a similar density in the range of ~1.019-1.063 g/mL (Kumar et al., 2011). The heterogeneity of circulating LDL size is of importance as research has indicated that smaller and more dense LDL particles are more atherogenic, potentially because of their increased ability to cross the endothelial barrier and are predictors of coronary heart disease (Packard et al., 2000; Hoogeveen et al., 2014). The monolayer of lipid and protein on the surface consists of phosphatidylcholine, sphingomyelin, unesterified cholesterol and protein moieties which incorporate the more hydrophobic cholesteryl esters and fatty acids in the core of the molecule. The LDL molecules in the circulation are largely derived from VLDL degradation following synthesis in the liver. The VLDL particle contains triglycerides and the major function of this particle in the body is to transport endogenous triglycerides that have been synthesised from the liver and intestine (Gaw et al., 2004). When the VLDL particle enters the circulation it contains apolipoproteins B, C and E. Triglycerides are removed from

VLDL under the action of the enzyme lipoprotein lipase (LPL - located in the capillary endothelium) in order to supply cells with triglycerides or be stored in adipose tissue (Liu et al., 2012) in addition to progressive loss of apoC and apoE via IDL, which loses these apoproteins as cholesterol content becomes greater than that of triglycerides. The action of a further enzyme, lecithin-cholesterol acyl transferase (LCAT), mediates the transfer of a fatty acid to cholesterol. This forms cholesteryl esters which are hydrophobic and cause them to relocate to the centre of the molecule. This increase in cholesterol over triglycerides causes the transformation of VLDL into LDL. Throughout the transformation process, the apoB protein remains on the LDL molecule despite the loss of apoC and apoE. This is of importance, as apoB plays a critical role in the pattern recognition of LDL by the LDL receptor, mediating subsequent uptake and metabolism by the cell.

In addition, apoB has an important function in regulating the interaction of the molecule with certain enzymes such as LCAT and LPL mentioned above. In the normal metabolism of cholesterol most cells will produce cell-surface receptors which bind specifically and internalise LDL by receptor mediated endocytosis (Lodish et al., 2000). Following internalisation into the cell, LDL particles are transported to lysosomes where the apoB protein is degraded into amino acids and the cholesteryl esters are cleaved into cholesterol and fatty acids primarily under the action of lysosomal hydrolases. The cholesterol can be directly incorporated into the plasma membrane or otherwise can be stored as lipid droplets (Thiam et al., 2013). These lipid droplets (also known as lipid bodies) are specialised intracellular organelles which are partitioned reservoirs able to store lipids in an inert form providing a store of energy when other sources are not available (Thiam et al., 2013). Cholesterol can also be converted into bile acids in hepatocytes and steroid hormones in the adrenal cortex (Lodish et al., 2000) and remains an important component of normal physiology.

1.1.2.5 The LDL receptor

In its native form, the LDL particle will bind to its corresponding LDL receptor, found on all nucleated cells. The liver is the primary site where a large proportion of the LDL is removed from the circulation via interaction with its receptor. The LDL receptor is a single-chain glycoprotein containing 839 amino acids spanning across the membrane

with a short C-terminal domain in the cytosol and a long N-terminal domain protruding into the extracellular region (Lodish et al., 2000).

The discovery of the LDL receptor and its role in cholesterol metabolism was largely credited to the work of J. L. Goldstein and M. S. Brown, first published in 1973 (Goldstein & Brown, 1973). They hypothesised that due to the nature of patients with familial hypercholesterolemia (FH) having heart attacks early in life due to elevated blood cholesterol, there must be a defect in cholesterol metabolism. This led to the discovery of the receptor in 1974 which showed high-affinity binding sites in control subjects which were lacking in patients with FH. The purified LDL receptor was then isolated in 1982 and the gene identified in 1985, leading to a Nobel prize in Physiology/Medicine in 1985 and a foundation of work setup for the later discovery of the statin drugs.

Once bound to the surface of a cell, the LDL particle is rapidly internalised (on average in less than 10 minutes) and degraded (Goldstein & Brown, 2009). This internalisation occurs as a result of receptor-mediated endocytosis. It has been established that LDL endocytosis occurs at particular regions on the membrane. LDL receptors cluster at pits in the membrane which are coated with the protein clathrin. These clathrin-coated pits are subsequently pinched from the plasma membrane to form internalised vesicles containing the receptor and its associated ligand. Clathrin is a protein which creates a type of lattice around the vesicle in order to aid transport into the cell. The LDL particle binds to the LDL receptor at these clathrin-coated pits via the apoB portion of the molecule. The bound particle is then endocytosed and moves to the lysosome where the protein is broken down into amino acids. The fatty acids are used for synthesis of phospholipids and triglycerides and cholesteryl esters are broken down into their respective cholesterol and fatty acid moieties.

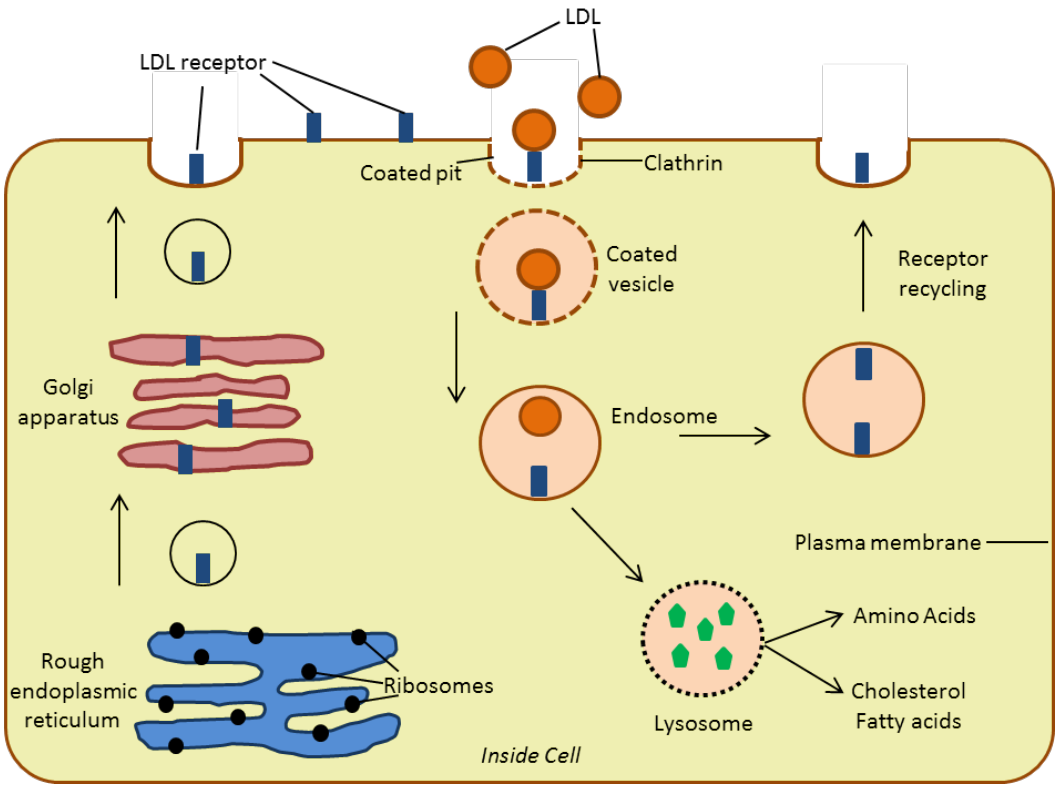


Figure 1-3 The recognition and internalisation of LDL via the LDL receptor*
LDL is internalised into a cell via its receptor (LDL-R) at clathrin-coated pits. This process is tightly regulated, affording a constant level of cholesterol to be maintained within the cell, balancing endogenous production and exogenous uptake. *not to scale. Figure adapted from Fuster, 2014.

The control of cholesterol metabolism and LDL receptor expression is mediated by specific transcription factors known as sterol regulatory element binding proteins (SREBPs) (Gaw et al., 2004). When the level of cholesterol in the cell increases, there is a reduction in HMG-CoA reductase activity (the rate limiting enzyme for cholesterol production) and a concomitant down regulation of LDL receptor production. This allows a constant level of cholesterol to be maintained within the cell, as when the level increases, the cell's ability to produce cholesterol endogenously and uptake it from exogenous sources is reduced. This process will occur primarily in the liver but also in extra-hepatic tissues. SREBPs are synthesised as inactive precursors bound to the membrane of the endoplasmic reticulum (ER) and are transported to the Golgi complex in times of cholesterol depletion (Eberle et al., 2004). They are processed by proteases which release a soluble fragment into the nucleus which up-regulates the enzymes required for cholesterol synthesis and also the LDL receptor (Goldstein & Brown, 2009).

The entry of cholesterol into the cell via LDL blocks the transport of SREBPs to the Golgi apparatus and therefore maintains the negative feedback regulation and cholesterol levels in the cell.

1.1.2.6 Lipid oxidation

The oxidation of lipids is a modification which causes them to express different physiological responses from their non-oxidised precursors and underlies an important mechanism in inflammation. The formation of oxidised lipid products, primarily from the oxidation of the LDL particle, has been identified in the propagation of vascular inflammation (Birukov, 2006). The oxidation of lipid, as with all redox reactions, is dependent on which atoms have their oxidation state changed. For a lipid to become oxidised its oxidation state must increase which is commonly due to the loss of electrons from its chemical structure. Unsaturated lipids, those possessing double bonds in their structure, are the primary targets for oxidation due to the chemical nature of the oxidation-prone double bonds (see Figure 1-4). The formation of oxidised lipids can occur in enzymic reactions or in non-enzymic fashion, for example through activated chemical species known as reactive oxygen species (ROS).

It is known that unsaturated fatty acids are more prone to oxidation than saturated fatty acids. This is due to the double bonds within the structure being more prone to electron loss. For poly-unsaturated fatty acids (PUFAs) which contain multiple double bonds, there is an increased likelihood of oxidative modifications compared to monounsaturated fatty acids. The reason for this increased oxidation susceptibility in PUFAs is as a result of the double bonds being always separated by at least one -CH2- group. This makes them the weakest bond in the acyl chain and this leads to this region being more susceptible to electron loss via hydrogen abstraction and subsequent oxidation (McIntyre & Hazen, 2010).

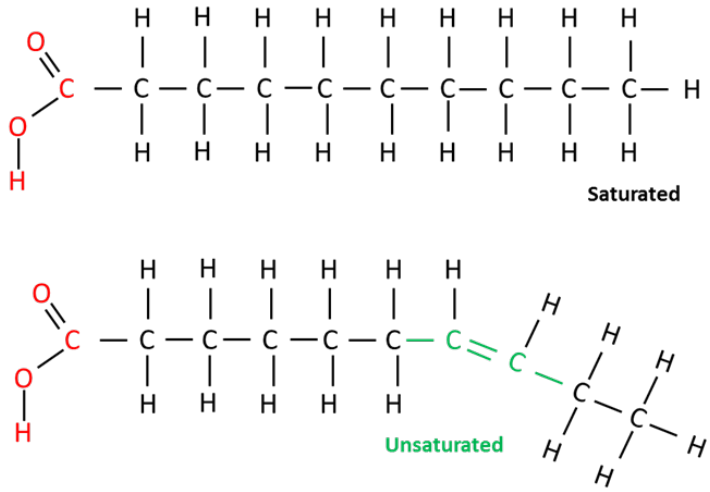


Figure 1-4 Schematic diagram of saturated and unsaturated fatty acids
Molecular layout of fatty acids, highlighting the double bond structure between two carbon atoms in unsaturated fatty acids, making them more prone to hydrogen abstraction and subsequent oxidation. Image shows 10 carbon fatty acid (saturated shows capric acid 10:0), unsaturated image only highlights double bond structure.

Lipid peroxidation is a process that will occur naturally in the human body due to the oxygen species produced as a by-product of normal metabolism. When lipids in the cell membrane become oxidised by reactive oxygen species, a self-propagating chain reaction is triggered within the membrane which causes subsequent damage to other lipids. This chain reaction mechanism is denoted by three major steps; initiation, propagation and termination. The initiation phase occurs when the first fatty acid is oxidised by a radical species, most likely caused by the hydroxyl radical (˙OH), which combines with hydrogen in the acyl chain of the fatty acid as described and forms a fatty acid radical. The resulting fatty acid radical is an unstable molecule which is able to abstract hydrogen from an adjacent fatty acid. This then forms a new fatty acid radical and a lipid peroxide, causing the chain reaction mechanism to occur as the cycle continues and characterised as the propagation phase. The final stage of the chain reaction mechanism, termination, occurs when a stable non-propagating (or non-radical) species is formed by the reaction between two radicals (Repetto et al., 2012).

The destruction of membrane lipids via lipid peroxidation is harmful to the viability of cells and even tissues when it occurs in excess, potentially rupturing membranes and producing toxic end products which can damage other biological components such as

DNA. For this reason, cellular membranes are equipped with anti-oxidants such as α-tocopherol (vitamin E) which react with free radicals, remove intermediate radicals and terminate the lipid peroxidation chain reaction. These antioxidant mechanisms are vital to keeping the redox balance within the cell as it is necessary to have oxidative and reductive reactions for metabolism but also to limit the potential resulting oxidative damage.

Given the mechanisms of lipid peroxidation, a plethora of lipid products can be produced which have varying effects on the host tissue. Many of these products have been implicated in the onset and maintenance of inflammation. Some of the more commonly researched oxidized lipids consist of arachidonic acid products such as the prostaglandins and leukotrienes which have prominent roles in promoting an inflammatory phenotype (McIntyre & Hazen, 2010). Sites of inflammation are areas in which the redox balance favours an oxidative environment leading to oxidised lipid formation which consequently helps to maintain an inflammatory state.

One of the best-known examples of an oxidized lipid is the LDL particle forming what is commonly described as oxidised LDL (oxLDL). The importance of oxidation on the particle is identified by the native form of LDL having minimal effects in promoting an inflammatory phenotype but, in its oxidised form, can have profound effects on immune cells. Via binding to multiple receptors and its subsequent endocytosis by immune cells such as macrophages, oxLDL can contribute to disease progression such as is seen in atherosclerosis. Many of the effects of oxLDL are a result of the oxidation of its phospholipids such as 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC). This produces a series of oxidation products (oxPAPC) which have been shown to promote inflammation, concluding that the pro-inflammatory activity of oxLDL is due to its lipid fraction as opposed to its protein content (Birukov, 2006).

The modification of LDL was initially shown *in vitro* by Steinbrecher et al. (1984), by incubating LDL with cultured endothelial cells from rabbit aorta and human umbilical vein. It was identified that LDL was modified by various mechanisms in the endothelium leading to changes in metabolism. The modification of LDL was identified by the observation of a faster degradation by macrophages, changes in electrophoretic mobility

and an increase in buoyant density (Steinbrecher et al., 1984). The degradation of oxLDL by macrophages is postulated to be due to the loss of recognition by the LDL receptor and higher affinity binding of the oxidised form for receptors such as CD36, which is expressed on macrophages.

In the absence of cells, metal ions can catalyse the conversion of LDL into oxLDL. The most common method used to prepare oxLDL *in vitro* is to incubate isolated LDL with micro-molar concentrations of copper sulphate at 37°C for up to 24 hours. The copper ions initiate the lipid peroxidation chain reaction mechanism discussed previously in addition to causing the chemical modification of the apoB side chains with peroxidised lipids (Itabe et al., 2011). It is also important to note that when discussing "oxidised" LDL the extent of the modification can produce functionally distinct molecules. A more minimal oxidation, either with shorter time points or lower concentrations of copper sulphate, or a combination of both, will produce a modification of the LDL which is still able to bind to the LDL receptor despite the oxidative modification. These modified LDLs also have inflammatory effects such as stimulating MCP-1 release from endothelial cells (Steinberg, 1997). This form of oxLDL is generally designated as minimally modified oxLDL, or mm-LDL/mm-oxLDL for short. The molecular heterogeneity of oxLDL must be taken into account when analysing the effects of this molecule as ascribing specific activities to modified LDL will be related to the level of oxidation. For the purposes of terminology in this thesis, the term "oxLDL" will refer to that which has undergone more extensive oxidative modification (e.g. 18hours with 10µM $CuSO_4$) and references to less oxidatively modified LDL will be delineated as and when they appear (mm-oxLDL).

1.1.2.7 Receptors for oxidised lipids

The oxidation of the LDL molecule causes it to lose affinity for the native LDL receptor as described above. Rather than endocytosis and metabolism via LDL receptor binding, the oxLDL molecule is able to bind with high affinity to several other receptors on the plasma membrane which can internalise the molecule and lead to its degradation (Boullier et al., 2001). Receptor candidates for oxLDL binding are the scavenger receptors found on a variety of cells, including macrophages, endothelial cells, platelets and smooth muscle cells. Scavenger receptors are a family of proteins which share a similar structural domain and have been characterised into eight separate groups, ranging from A-H. There is,

however, a structural diversity between the separate groups but all maintain the ability to bind modified lipid particles (Stephen et al., 2010).

In each of the groups from A-H there can be multiple receptors. For example, scavenger receptor class A consists of scavenger receptor A (SR-A), macrophage receptor with collagenous structure (MARCO), scavenger receptor with C-type lectin (SRCL) and scavenger receptor A-5 (SCARA5) (de Winther et al., 2000). Of all the scavenger receptors throughout the different classes, the classes considered most important in oxLDL ligation are SR-A, scavenger receptor B1 (SR-B1), CD36 (scavenger receptor B3) and the only member of class E, LOX-1 (lectin-like oxidized low density lipoprotein receptor-1).

The ligation of oxLDL to monocyte/macrophage CD36 receptors has been of particular interest in cardiovascular research. The role CD36 plays in the endocytosis of oxLDL and the formation of foam cells in atherosclerosis marks it out as an important contributor to the disease (Collot-Teixeira et al., 2007). It has been postulated that the two most important receptors in terms of oxLDL endocytosis and foam cell formation are SR-A and CD36, given that together they account for 75-90% of all oxLDL degradation (Kunjathoor et al., 2002).

Scavenger receptor A is a multifunctional receptor which is expressed by macrophages and has been implicated in atherosclerosis given its role in foam cell formation as discussed above. SR-A is a trimeric molecule composed of three identical protein chains with a collagenous region having a positively charged set of lysines crucial for recognition of oxLDL (Neyen et al., 2009). Given the broad ability for the receptor to bind multiple ligands, including those of ageing and apoptotic cells, this domain is of interest for ligand recognition in many areas (Platt & Gordon, 2001), including pattern recognition in immune responses, Alzheimer's disease (expressed on the surface of microglia; Wilkinson & El Khoury, 2012) and atherosclerosis.

The class B scavenger receptors also play an important role in the recognition of oxidized lipids. It is mainly on the part of CD36 that oxLDL is primarily bound and endocytosed. SR-B1 has been implicated in the recognition of modified LDL but has a lower affinity and wider spectrum of ligands when compared to CD36 including recognising a range of lipoprotein molecules such as VLDL, LDL and HDL (Valacchi et al., 2011). CD36 binds with

a high affinity to the oxLDL molecule but as with many of the scavenger receptors, it has a broad range of activities, being involved in phagocytosis of effete cells, fatty acid transport and the uptake of infected erythrocytes (such as in malarial parasitic infection) (Smith et al., 2003; Neculai et al., 2013). Despite the supposed importance of this receptor in pathological conditions such as atherosclerosis and Alzheimer's disease, the detailed characterisation of the receptor structure has not been well documented. The characterisation of the receptor and its binding to oxLDL may have important implications for the treatment of certain diseases. The contribution of the receptor to atherosclerosis and related disease is summarised by work shown in CD36 knock-out mice, in which the absence of CD36 protects against such pathology (Kuchibhotla et al., 2008). Although primarily a receptor found on macrophages, CD36 has also been shown to be present on the surface of endothelial, platelet and smooth muscle cells (Collot-Teixeira et al., 2007).

Oxidised lipids are taken up macrophage scavenger receptors, but the recognition and degradation of oxidized lipids by endothelial cells also contributes to inflammation and impaired vascular responses. It has been known for a long time that endothelial cells can act like macrophages in terms of their ability to uptake and degrade modified LDL showing increased intracellular cholesterol separate from endogenous formation (Stein & Stein, 1980). It was not, however, until 1997 that the endothelial receptor for oxLDL- LOX-1 - was discovered using expression cloning strategies. LOX-1 is a protein of the C-type lectin family and is expressed by the vascular endothelium *in vivo* (Sawamura et al., 1997). In a model of prostate cancer, it has been shown that the ligation of LOX-1 by oxLDL activates signalling pathways that are involved in proliferation, and enhances the release of angiogenic factors such as VEGF (Gonzalez-Chavarria et al., 2014). The importance of this molecule in oxLDL recognition has been highlighted in LOX-1 knock-out mice, in which the deletion of the receptor reduced atherogenesis (Mehta et al., 2007).

The LOX-1 receptor has a single transmembrane domain but assembles on the surface of the cell in hexamer form or larger. When bound to oxLDL, the receptor is comprised of 3 homodimeric LOX-1 molecules (Morawietz, 2007). The LOX-1 receptor is expressed at low concentrations in normal, non-inflamed endothelium and it is kept in its monomeric

state. In the early stages of atherosclerosis, LOX-1 aggregates, becomes up-regulated and begins to internalise oxLDL (Di-Rienzo et al., 2013). The expression of LOX-1 on the plasma membrane can be regulated by chemical stimuli including TNFα and phorbol myristate acetate (PMA) (Kume et al., 1998). Additionally, redox factors such as superoxide anions are also known to regulate its expression (Nagase et al., 2001). The up-regulation of LOX-1 has been shown in aortic endothelial cells and detailed receptor expression has also been delineated in endothelial cells derived from other vascular beds (Di-Rienzo et al., 2013). Relevant to studies in this thesis, LOX-1 up-regulation has been shown in HUVEC in response to angiotensin II, measured by PCR and western blotting, which induces endothelial apoptosis (Luo et al., 2016; Morawietz et al., 1999). In other studies on HUVEC, oxLDL has been shown to attenuate expression of desmosome proteins via LOX-1 (confirmed with *in vitro* inhibitors), causing increased permeability of the monolayer (Li et al., 2015). LOX-1 has also been demonstrated on neutrophils in a murine model, with LOX-1 deletion preventing neutrophil overreaction and increased neutrophil recruitment to infection sites, suggesting this oxLDL receptor as an important mediator of neutrophil dysfunction (Wu et al., 2011).

A further receptor associated with the recognition of oxidized lipids is the platelet-activating factor receptor (PAF-R). This receptor was named for its primary ligand binding to platelet-activating factor (PAF), a phospholipid. Unlike the other previous receptors, the PAF-R is not a scavenger receptor but is a G-protein coupled receptor (GPCR) and can transduce extracellular signals through the interaction with guanine nucleotide-binding proteins (Montrucchio et al., 2000). A commonality of GPCRs shared by PAF-R is a structure comprising of seven transmembrane helices connected by three intra-cellular and three extra-cellular loops. This forms a barrel-like cavity in the tertiary structure to serve as the ligand binding domain (Montrucchio et al., 2000). When lipids found in LDL or the cell membrane become truncated via oxidative modification they take on a structural homology to PAF and become recognised by PAF-R. This then leads to activation of cellular responses as if activated by PAF itself (Chen et al., 2009).

There have been numerous studies over the years highlighting the role of PAF-R in oxidized lipid recognition and its contribution to inflammation. One interesting observation in 1993 highlighted the *in vivo* relevance of the receptor using intra-vital

fluorescence microscopy. Having set up skinfold-chamber models in the hamster, systemic administration of oxLDL elicited rolling and adhesion of circulating leukocytes to the endothelium. In the presence of PAF-R antagonist this recruitment of leukocytes was significantly attenuated (Lehr et al., 1993). PAF-R may also play a role in the response of immune cells as it has been shown that PAF-R antagonists block the ability of oxLDL to cause lipid body formation in leukocytes (Assis et al., 2003). It has also been suggested that PAF-R ligation is a necessary co-stimulation signal required for oxLDL uptake via CD36 on macrophages (Rios et al., 2012).

In relation to psoriasis, PAF-R signalling is thought to play an important role in the inflammatory response. Being the major cell type of the skin, the keratinocytes, which can synthesise and respond to PAF via a functional PAF-R, have the capacity to control inflammation and proliferation. Indeed, PAF was originally identified and quantified from psoriatic scales (Ramesha et al., 1987). In a murine model of psoriatic inflammation, it was also found that PAF-R blockade prevented the progression of a psoriasis-like phenotype and lowered the immune infiltrate (Singh et al., 2011).

The plethora of receptors implicated in the recognition of oxLDL, particularly with their expression on a broad range of cells, highlights the potential importance of oxidized lipid accumulation in diseases such as psoriasis and a need to investigate the extent to which they influence the inflammatory response. Previous work has identified an increase in CD36 expressing macrophages in psoriasis which appear to line the epidermis suggesting a possible role in the activation of T cells (van den Oord et al., 1994). In addition, with CD36-positive keratinocytes in the skin alongside these macrophages, there exists the potential for functionally relevant effects for oxLDL in skin disease (Begany et al., 1994). Since this time, there has been little added in the way of the functionality of these receptors to oxLDL signalling in the skin. The role of LOX-1 in the host immune response is as yet, not fully elucidated despite some evidence that it promotes humoral immune responses (Joo et al., 2014). This broad role for LOX-1 in psoriasis is yet to be fully investigated.

Class	Receptor Name	Roles in homeostasis and inflammation	Cells Expressing Receptor
A	SR-A (SCARA1/CD204)	Apoptosis, foam cell formation, lipid metabolism, phagocytosis, host defence/sepsis	Monocytes, macrophages, vascular smooth muscle cells, endothelial cells, epithelial cells, microglia, astrocytes.
	SCARA2 (MARCO)/ SRCL/ SCARA4/SCARA5	Pathogen clearance, apoptotic cell clearance, oxLDL binding	Epithelial cells, cells of the placenta, lungs, heart, small intestine
B	SR-B1/ SCARB2	Lipoprotein receptor, cholesterol uptake from HDL, pathogen recognition	Hepatocytes, adipocytes, monocytes, macrophages, smooth muscle cells, endothelial cells (liver)
	CD36 (SCARB3)	Foam cell formation, oxLDL ligation, platelet activation, apoptosis, angiogenesis, inflammation, cell adhesion, cell migration, pathogen recognition and clearance	Platelets, monocytes, macrophages, adipocytes, hepatocytes, myocytes, endothelial cells
D	CD68	Binds LDL	Monocytes, macrophages
E	**LOX-1**	OxLDL ligation, endothelial dysfunction, foam cell formation, angiogenesis, apoptotic cell clearance	Endothelial cells, macrophages, platelets, smooth muscle cells
F	SREC1/SREC2	Acetylated-LDL uptake, atherogenesis	Endothelial cells, macrophages
G	SR-PSOX	OxLDL uptake, foam cell formation	Macrophages
H	FEEL1/FEEL2	Bind advanced glycation end-products, bacteria and acetylated-LDL	Liver endothelial cells, macrophages
I	CD163/CD5/CD6	Bind bacteria and toxic haem	Haematopoietic cells
***References**: Zani et al., 2015; Canton et al., 2013; Kelley et al., 2014; Whelan et al., 2012; Valacchi et al., 2011; Silverstein et al., 2009; Mehta et al., 2006.*			

Table 1 Summary of scavenger receptor classes and their roles in inflammation

1.1.2.8 Bioactive lipid mediators

Sphingosine-1-phosphate (S1P) is a bioactive lipid mediator which is thought to play a major role in cellular responses. S1P was discovered in 1993 through research investigating the link between sphingosine and cellular proliferation, identifying that S1P could stimulate the proliferation of fibroblasts in a manner independent of protein kinase C (PKC) (Olivera & Spiegel, 1993). Sphingosine can be produced from ceramides within the cell through the action of the enzyme ceramidase. This newly formed sphingosine can then in turn be phosphorylated by the activity of the sphingosine kinases (SK1 or SK2)

to produce sphingosine-1-phosphate. Ceramides have important roles within the cell, in particular acting as a regulatory participant in apoptosis (programmed cell death) through the activation of stress-activated protein kinases (SAPKs). In opposition to this, the production of S1P from sphingosine promotes survival responses from the cell, such as proliferation and migration. The balance between ceramide and S1P production and their respective cellular concentrations is therefore highly regulated and important for the fate of the cell and its response to inflammatory stimuli (Cuvillier et al., 1996).

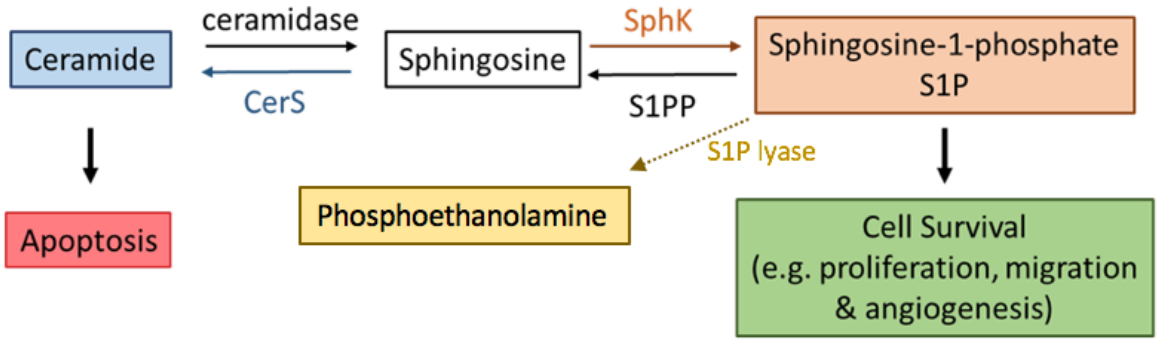


Figure 1-5 Sphingosine metabolism
Sphingosine within the membrane can be converted into ceramide [blue] (via ceramide synthase) which has pro-apoptotic effects on the cell. In contrast, the conversion of sphingosine into sphingosine-1-phosphate [orange] (S1P, via sphingosine kinases) promotes survival responses such as proliferation and migration. S1P phosphatases (S1PP) degrade S1P into sphingosine, whereas S1P lyase irreversibly degrades S1P into phosphoethanolamine (yellow).

The blood borne lipid is highly abundant in the plasma, at a concentration of 200-900nM particularly associating with albumin and HDL, but is found at reduced concentrations in tissue (Poti et al., 2014). S1P potently regulates inflammatory processes, including endothelial responses, through interactions with its receptors (S1PRs) which are expressed on a variety of cell types, including vascular and immune cells. There have been five S1P receptors characterised ($S1P_{1\text{-}5}$) to date which are G-protein coupled receptors, with $S1P_1$, $S1P_2$ and $S1P_3$ the major receptor subtypes in the blood vessels (Takuwa et al., 2010).

The cell types capable of S1P production and altering S1P levels within the circulation include erythrocytes, platelets, endothelial cells and mast cells, with the levels being

tightly regulated by inflammatory stimuli and the intracellular pathway mediators mentioned above. Sphingosine kinases are required for S1P production from sphingosine (as shown in Figure 1-5) within the cell and S1P lyase and phosphatases are required for S1P degradation to phosphoethanolamine or back to sphingosine respectively. Both erythrocytes and platelets express sphingosine kinases, but lack the expression of lyases and phosphatases to break down S1P, allowing the storage and release of large amounts of S1P within the circulation (Rivera et al., 2008). Erythrocytes are thought to be the main contributor to circulating S1P levels, as mast cells and platelets primarily only produce S1P in response to stimulation and so do not alter their basal S1P levels (Rivera et al., 2008). This is from evidence in mice lacking circulating platelets (nuclear factor-erythroid 2-deficient mice) having biologically normal ranges of S1P levels within the plasma (Pappu et al., 2007). However, in certain physiological settings these cells likely contribute to local S1P gradients, such as S1P release from platelets in clotting events and thrombus formation.

Endothelial cells are also a source of circulating S1P, expressing the components to both produce and degrade S1P. Through its interactions primarily with $S1P_1$ and $S1P_3$, S1P regulates endothelial cell migration, angiogenesis and vascular permeability. S1P is also crucial for embryonic vascular development, demonstrated in mice with specific deletion of sphingosine kinases showing embryonic lethality (Xiong et al., 2014). Other noteworthy effects of S1P include the regulation of lymphocyte egress from lymphoid tissue (in which S1P levels are low compared to the blood) into the circulation due to an S1P gradient and $S1P_1$ expression by lymphocytes (Maceyka & Spiegel, 2014).

Within the circulation S1P primarily resides within two main chaperones, HDL and albumin, accounting for 55% and 35% of plasma S1P respectively (Poti et al., 2014). The remaining 10% of plasma S1P is thought to reside in other lipoproteins, with LDL thought to be the main reservoir for S1P in non-albumin or HDL associated S1P. Given the implications of LDL and HDL in cardiovascular disorders it could be hypothesised that S1P plays an important role in either the atherogenic or atheroprotective role of these lipoproteins. Indeed, evidence has suggested that HDL-bound S1P is capable of inhibiting adhesion molecule expression on endothelial cells (Kimura et al., 2006) in addition to increasing endothelial barrier function (Argraves et al., 2008), both implicating $S1P_1$ as

the primary mediator of these anti-atherogenic effects. The chaperone for S1P may have clinical importance, as S1P associated to HDL has a four-fold longer half-life in the circulation than albumin-bound S1P, in addition to evidence that S1P bound to HDL sustains endothelial cell barrier function longer than albumin-bound S1P (Poti et al., 2014). S1P binds to the apoM portion of the HDL particle, with apoM levels correlating with S1P concentration (Christoffersen et al., 2011).

This is also evidence for a link between oxLDL and S1P, with studies in microvascular endothelial cells showing that oxLDL-induced angiogenesis can be inhibited with a monoclonal antibody against S1P or inhibitors of sphingosine kinase (Camare et al., 2015). These results were also shown in a murine Matrigel plug model, with both inhibitors of extracellular S1P and sphingosine kinase inhibiting vascular formation (Camare et al., 2015). It is therefore hypothesised that oxLDL triggers activation of sphingosine kinase to produce intracellular S1P in endothelial cells and subsequently causes an angiogenic response.

In addition to the multiple effects in the circulation, S1P also influences keratinocyte proliferation in the skin, making this bioactive lipid mediator a potentially important target in orchestrating the multiple facets of inflammation seen in psoriasis and other inflammatory conditions. There is a wide range of other bioactive lipid molecules that may play a role in inflammation, including PAF, leukotrienes, eicosanoids and prostaglandins, lysophosphatidic acid and a range of fatty acid products and derivatives.

1.1.3 The vasculature of the skin

The skin is the largest organ in the body which provides an essential barrier function as well as partaking in homeostatic mechanisms. The four layers of the epidermis are, from lower to upper layers, the stratum basale, stratum spinosum, stratum granulosum and stratum corneum respectively. Keratinocytes are proliferative in the stratum basale but differentiate and mature as they migrate upwards, becoming fully differentiated corneocytes in the stratum corneum.

The epidermis is supported by the dermis beneath, with this part of the skin carrying the vasculature. The vascular system within the dermis consists of two major interconnected systems of blood vessels, the superficial vascular plexus and the deep vascular plexus

(Goldsmith et al., 2012). The superficial vascular plexus is located on a parallel plane just beneath the epidermal surface, from which capillaries arise and extend upwards. These capillaries are derived from arterioles at sites between the epidermal rete ridges and loop back downwards to the venules (Goldsmith et al., 2012). Such vessels have been shown to be increased in psoriatic skin, in addition to being elongated, tortuous and dilated, enhancing immune cell infiltrate and inflammation (Gupta et al., 2011).

1.1.3.1 The endothelium

The skin is dependent on the blood supply provided by its vasculature. Major arteries and blood vessels are composed of three layers, the inner layer (*Tunica intima*) which is made up of a single layer of endothelial cells, a middle layer (*Tunica media*) consisting of smooth muscle cells which control vessel patency and the outer layer (*Tunica adventitia*) comprised entirely of connective tissue. Endothelial cells, collectively termed endothelium, are present throughout the entire vascular system and play many key roles in coagulation, growth regulation, production of extracellular matrix products as well as modulating blood flow and vessel tone (Sumpio et al., 2002). The endothelial cell is also a key mediator in the inflammatory response. Inflammation is commonly analysed from the perspective of tissue-infiltrating leukocytes, a process in which microvascular endothelial cells play an active role. Under normal conditions within a blood vessel, the endothelium is resistant to firm leukocyte adhesion whereas in inflammation, leukocytes are attracted to the area and attach to an activated endothelium via adhesion molecules before transmigrating into the tissue where they follow chemotactic gradients towards the site of injury. The endothelium is activated in response to various physical and chemical stimuli which promote the endothelial production of a wide range of factors involved in leukocyte diapedesis. These factors include cell adhesion molecules necessary for cellular interaction such as ICAM, VCAM and selectins (Muller, 2009), in addition to cytokines such as interleukins 8 (IL-8) and 6 (IL-6) (Yeh et al., 2001).

The activation of the endothelium in response to infection or injury is desirable in order to elicit an effective immune response. In auto-immune diseases however, the unnecessary activation of the endothelium stimulates an excessive immune response which can have damaging effects. In the setting of psoriasis, research suggests that normal endothelial functions such as vasodilation, stiffness and elasticity are significantly

impaired in comparison to the general population (Brezinski et al., 2014). Although psoriasis is a disorder of the skin, research into the disease has now characterised it as an immune mediated disorder with systemic inflammation, of which the endothelium plays a vital role. In this way, psoriasis is increasingly considered an independent risk factor for cardiovascular events, with increased severity also leading to increased risk (Armstrong et al., 2013). It is hypothesised that shared pathogenesis in endothelial function may underlie psoriasis and atherosclerosis (Brezinski et al., 2014). Endothelial dysfunction in psoriatic plaques may enhance the disease by allowing the mass infiltration of immune cells to take residence and contribute to excessive inflammation and damage to the skin with continual unresolved inflammation.

1.1.3.2 Angiogenesis

Nearly all cells in the body are within a few hundred micrometres of a capillary, as the diffusional exchange of nutrients and metabolites is required. To maintain this arrangement, new vessels are able to sprout from the existing vasculature in response to specific stimuli, a process known as angiogenesis. The formation of new blood vessels is a tightly regulated process mediated by a series of pro- and anti-angiogenic factors. In a normal adult blood vessel, endothelial cells are in a quiescent state and there is little cell proliferation. In certain physiological circumstances, namely female reproductive cycles, wound healing and pathological conditions, it is hypothesised that there is an angiogenic switch, in which endothelial cells are stimulated to proliferate and migrate to form new vessels.

The signals which cause this switch are known to be complex but research has highlighted the importance of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) as potent angiogenic activators (Cross et al., 2001) released in response to stimuli such as low oxygen and tissue injury. Angiogenesis occurs in stages beginning with the destabilisation of existing blood vessels and the degradation of the extra-cellular matrix (ECM). The endothelial cells then begin to proliferate and migrate in order to form tubes sprouting from the existing vasculature. The process is then finalised by lumen formation and vessel maturation supported by smooth muscle cells and pericytes (Heidenreich et al., 2009). The modern history of angiogenesis research began with the proposition that tumours secrete soluble factors to stimulate vessel growth in

cancer (Folkman, 1971). Further research into angiogenesis has now recognised its contribution to numerous malignant, ischaemic, inflammatory, infectious and immune disorders (Carmeliet, 2003) in addition to being important in development.

Histological analysis of psoriasis has established that alterations in blood vessel formation in the skin are a prominent feature of the disorder (Heidenreich et al., 2009). In the early phase of psoriasis there is concomitant formation of new blood vessels and this has been shown to regress with disease clearance (Heidenreich et al., 2009). Angiogenic growth factors such as transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF) and VEGF are markedly increased in psoriasis leading to immature, elongated and tortuous vessels in the skin (Veale et al., 2005). A consequence of epidermal thickening is that the cells of the skin are susceptible to a potential shortage of oxygen. In this situation, cells increase the intracellular concentration of the active form of a gene regulatory protein known as hypoxia inducible factor 1 (HIF-1) which is strongly upregulated in psoriatic skin (Rosenberger et al., 2007). This in turn stimulates the transcription of the VEGF gene, along with other hypoxia related factors, leading to secretion and diffusion through the tissues to act upon endothelial cells to initiate angiogenesis.

1.1.3.3 Endothelial cell migration

The migratory response of endothelial cells to certain stimuli plays a major role in the establishment of new vessels and is regarded as a key component of angiogenesis. Cellular migration is not a process which is solely exclusive to angiogenesis, as most cells possess motile ability under certain physiological circumstances, but endothelial responses for the purpose of new vessel formation follow a defined motility sequence with typical recurring features (Michaelis, 2014). It has been postulated that there are three major mechanisms involved in endothelial migration during angiogenesis which are chemotaxis (migration towards soluble chemoattractants), haptotaxis (migration towards immobilised targets) and mechanotaxis (migration generated my mechanical forces) (Lamalice et al., 2007).

Directed endothelial cell migration is co-ordinated by the local environment, with growth factors such as VEGF within the extracellular matrix providing guidance cues, in addition

to other surrounding cells. The response of a group of endothelial cells to an angiogenic stimulus is not homogeneous as some endothelial cells will become tip cells to form the sprouting outgrowths in the initial angiogenic response with inter-endothelial signalling between adjacent cells co-ordinating this movement (Michaelis, 2014). Adjacent endothelial cells, which do not become tip cells, form the stalk of the sprouting vessel. In order to begin migration, endothelial cells extend filopodia which are plasma membrane extensions and function as antennae for cells to probe the environment towards chemoattractants. These filopodia are born from lamellipodia, which are larger cytoskeleton projections on the leading edge of the cell, primarily containing an actin mesh (Lamalice et al., 2007).

In order to co-ordinate this complex process there are a number of intracellular signalling and communication pathways. To initiate cellular probing, the Rho family of GTPases are involved in the intricate mobilisation of actin. This is followed by Rac1-dependent formation of lamellipodia, allowing the extensions at the front leading edge of the cell. To complete migration, focal adhesion assembly is required for the attachment of the front of the cell and concomitant release of the rear portion of the cell (Lamalice et al., 2007). All together these events orchestrate the directional movement of endothelial cells towards a stimulus and represent one of the initial events in the angiogenic process before vessel maturity and stabilisation. The process is therefore a focus point of research, including that of inflammatory conditions, as factors in the micro-environment around the vessel can influence the pro- or anti-angiogenic nature of the endothelial response.

1.1.4 Infiltrating leukocytes: neutrophils

In an established blood vessel, one of the earliest and most important phases in the inflammatory response is the interaction between circulating leukocytes and the activated endothelium. Neutrophils in particular play a fundamental role in host defence, with the body able to markedly increase the number of circulating cells from the bone marrow in a rapid response to stimulus (Craig et al., 2009). Neutrophils are able to migrate into various tissues following chemotactic gradients allowing them to home in on infectious agents such as bacteria, in addition to supporting an effective adaptive immune response by shaping the function of other leukocytes by direct contact or

cytokine production (Jaillon et al., 2013). Efficient neutrophil sequestration into the tissue is regulated by the vascular endothelium and is therefore a key interaction in host immunity. Although the role of neutrophils in inflammation is important, the opposing scenario of neutrophil migration out of the vessel is that it may cause unnecessary inflammation and excessive damage to host tissue, a process sometimes referred to as sterile inflammation (Shen et al., 2013). The pathological consequences of excess immune infiltration are highlighted in psoriasis, in addition to other diseases such as chronic obstructive pulmonary disease (COPD) and glomerulonephritis (DiStasi et al., 2009). The dysregulation of leukocyte-endothelial interactions can play a major role in the onset and maintenance of such conditions and provide a potential therapeutic target, both in the response to microorganisms and in sterile inflammation.

1.1.4.1 The involvement of neutrophils in psoriasis

Neutrophils have long been identified in the active lesions of psoriasis given their prominent infiltration into the skin and micro-abscess development (Kaneko et al., 1991). The importance of this neutrophil infiltration in the pathology of psoriasis has been highlighted by the improvement of the disease following drug induced agranulocytosis (Toichi et al., 2000) with the correlation between neutrophil counts and psoriasis disease activity and the neutrophil dependent severity of skin disease in 'flaky-skin' mice exhibiting the characteristics of human psoriasis (Schon et al., 2000). Research is increasingly providing evidence for the dysregulation of the T-cell populations in the development and maintenance of psoriasis, observed in histological analysis (Krueger et al., 2005). However, the prominent infiltration of neutrophils in psoriasis remains a distinctive hallmark of the disease.

It was initially thought that psoriasis was a Th1-mediated disease, given the prevalence of these cells in psoriatic skin and the cytokine network related to its development (Schlaak et al., 1994). However, with the continuous discovery of new T cell subsets, it became apparent that Th-17 cells were crucial to the pathogenesis of psoriasis with the involvement of IL-23 which stimulates Th-17 development and the cytokine product of these cells, IL-17. The importance of the Th-17 axis was demonstrated in a study of IL-23/IL-17 knockout mice where a blockade of psoriasis-like disease development was observed (van der Fits et al., 2009) in addition to human experimental data showing

strong expression of IL-23 in psoriatic skin (Cai et al., 2011). Although the Th-17 axis is now a mainstay of psoriatic research, it has been suggested that neutrophils and mast cells, as opposed to T cells, are the predominant cell types containing IL-17 in human skin (Lin et al., 2011). In well-developed psoriatic plaques, neutrophils have been identified to be resident in the epidermal regions, both in the stratum corneum and stratum spinosum (Lin et al., 2011).

Supporting an argument for neutrophil contribution to psoriasis through IL-17 expression, it was shown using established models of *in vivo* human skin inflammation, that neutrophils and mast cells were also the cell types most prominently found to be expressing IL-17 (Keijsers et al., 2014), following influx of neutrophils and T cells. It was also noted that the neutrophil population, rather than mast cells or T cells, co-expressed the IL-17-associated transcription factor RORγt and were able to form extra-cellular traps consisting of DNA and neutrophil granule proteins (neutrophil extra-cellular traps [NETs]) containing IL-17. Given that T cells represented a minority of the IL-17 expressing cell population in this study, it can be hypothesised that neutrophils are an important mediator of skin inflammation in psoriasis in humans (Lin et al., 2011; Keijsers et al., 2014).

Neutrophil migration into the tissue often results in their cluster formation (Guan-Ng et al., 2011), visualised by epidermal focal collections in psoriatic plaques (Lin et al., 2011). The morphology of the recruited neutrophil population also reveals a relatively newly identified behaviour of the cell where they release their DNA extracellularly. This leads to the phenomenon of the aforementioned NETs which are a network of extracellular fibres primarily containing DNA and antimicrobial proteins thought to enable the capture of pathogens (Brinkmann et al., 2004; Zawrotniak et al., 2013). The process of generating NETs (known as netosis) releases antimicrobial proteins such as the peroxidase enzyme myeloperoxidase (MPO). The release of this enzyme by neutrophils is a potential route by which LDL becomes oxidised *in vivo* (Delporte et al., 2013). It has therefore been suggested that neutrophils and the MPO system may contribute to the initiation of atherosclerotic lesions via lipid peroxidation and consequently other disorders where LDL and neutrophils are found together such as psoriasis.

For many years neutrophils were proposed to be a homogenous immune cell type that acted as the 'foot soldiers' of the inflammatory response, arriving at the site of inflammation to eradicate infiltrating microbes and subsequently die. However, evidence has been emerging that neutrophil subtypes may exist and possess different properties. Of particular relevance and interest to psoriasis may be the proposition of a pro-angiogenic sub-type of neutrophils (Christoffersson et al., 2012; Deryugina et al., 2014; Gong et al., 2010; Tazzyman et al., 2009). It has been shown that neutrophils recruited via VEGF expressed 10-times higher levels of MMP-9 than those recruited to an inflammatory stimulus and MMP9 is an important effector protein required for revascularisation (Christoffersson et al., 2012). MMP-9 appears to be the critical link between the neutrophil and its pro-angiogenic capability, with mice genetically deprived of MMP-9 having impaired revascularisation (Christoffersson et al., 2012). Despite the 10-fold increase in MMP-9 of these VEGF recruited neutrophils, both recruited sets penetrated into the tissue equally well. This suggests that MMP-9 is not required for initial extravasation, but is involved in promoting the environment required for angiogenesis (Christoffersson et al., 2012). The recruitment and accumulation of neutrophils in psoriasis can therefore be considered important for the maintenance of vascular inflammation. The role of oxLDL in promoting the recruitment and transmigration of neutrophils will therefore be analysed in this thesis, in addition to characterising the direct angiogenic effect.

1.1.4.2 Neutrophil interactions with the endothelium

In the context of neutrophil transmigration from blood vessel to tissue, it is generally accepted that there is an ordered cascade of events. The most basic description of neutrophil interaction with the endothelium is identified with three major steps; rolling, activation and arrest (firm attachment). However, as research into this interaction has progressed, the complexity of the interaction has been more completely defined with reported phases such as tethering, slow rolling, firm adhesion modulation, intraluminal crawling and transmigration either through the cell (transcellular) or between cells (paracellular/transjunctional) (Schmidt et al., 2011) (as shown in Figure 1-6).

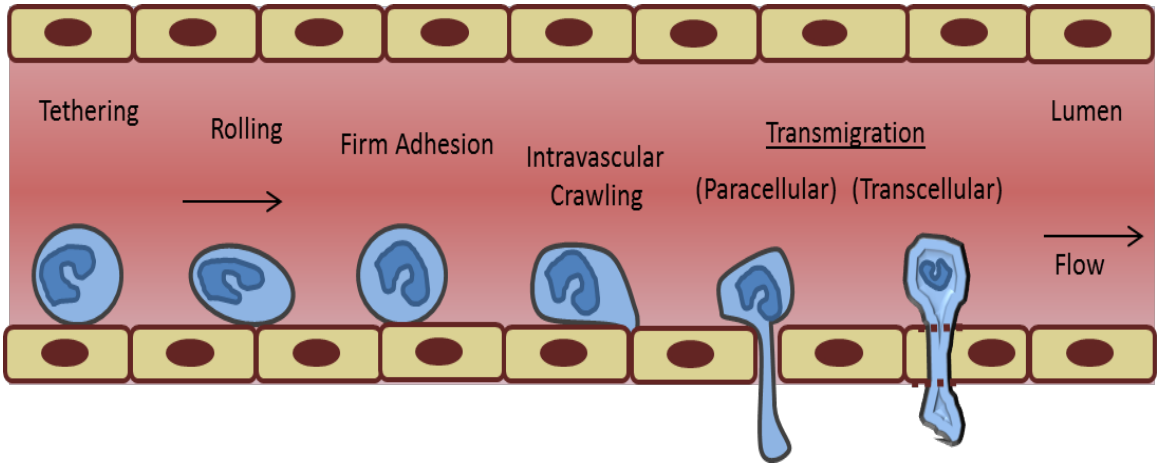


Figure 1-6 Stages of neutrophil recruitment to the endothelium
Diagram depicting the stages of leukocyte (neutrophil) recruitment to the endothelial layer and subsequent transmigration. Not to scale. Diagram based on image from Schmidt et al., 2011.

The initial contact between leukocytes and the endothelium is referred to as capture or tethering and is mediated by selectins in most vascular beds. Endothelial cells are active participants in the interaction with leukocyte accumulation influenced by the activating signal received by the endothelium. Endothelial cell activation is accomplished by inflammatory stimuli through outside-in signals received by receptors on the endothelial surface (McIntyre et al., 2003). Without an inflammatory stimulus, endothelial cells generally do not support the interaction and adhesion of circulating leukocytes under physiological shear or if an interaction occurs, the signals to activate the leukocyte are not present on the endothelial surface to initiate rolling and capture and so the interaction is transient and non-leukocyte activating.

The tethering and subsequent rolling of leukocytes on the endothelium is supported by L-selectin, P-selectin and E-selectin and their counter receptors. Most inactive leukocytes will express L-selectin, whereas E-selectin and P-selectin are expressed by inflamed endothelial cells. One of the initial interactions supported by the endothelium is via P-selectin expression, which is stored in endothelial secretory granules known as Wiebel-Palade bodies and can be rapidly translocated to the endothelial surface by agonists such as thrombin (McIntyre et al., 2003). Other agonists such as lipopolysaccharide (LPS) and TNFα induce the transcriptionally regulated de novo synthesis of E-selectin by endothelial cells which has a peak expression at around 4-6 hours post stimulus, returning to basal

level after 24hours (Gimbrone et al., 2004). The counter receptor on the leukocyte which interacts with the selectins, although originally described as a P-selectin ligand, P-selectin glycoprotein ligand-1 (PSGL-1) plays a dominant role in binding all three selectins and is expressed on almost all leukocytes (Ley et al., 2007). The E- and P-selectin expressed on endothelial cells therefore interacts with PSGL-1 to support capture and rolling of leukocytes on the luminal surface of blood vessels. PSGL-1 and L-selectin can also support leukocyte-leukocyte interactions, by which adherent leukocytes can facilitate secondary leukocyte tethering to the endothelium (Ley et al., 2007). Leukocyte rolling mediated by selectins causes direct signals to activate the leukocyte, in addition to activating signals through adjacent GPCRs. The activation of the leukocyte subsequently allows the activated expression of the integrin receptors, allowing for firm adhesion (Ley et al., 2007).

The integrin facilitated mechanism for leukocyte attachment leads to increased energy for ligand binding and a decrease in ligand dissociation, allowing for the arrest of the leukocyte on the endothelium. Integrins are type I transmembrane cell adhesion molecules consisting of two non-covalently associated sub-units depicted as α and β which mediate cellular interactions (Zarbock & Ley, 2008). Neutrophils express the integrins $\alpha_L\beta_2$ (CD11a/CD18; LFA-1), $\alpha_M\beta_2$ (CD11b/CD18; Macrophage-1 antigen [Mac-1]) and $\alpha_4\beta_1$ (CD49b/CD29; very late antigen-4 [VLA4]) which are involved in slow rolling, firm adhesion and even transmigration (Zarbock & Ley, 2008). These integrins present in the leukocyte are inactive until stimulated (via initial selectin-mediated activation), following which they can bind to constitutively expressed adhesion molecules such as ICAM-1. Binding of integrins to their ligands induces intra-cellular signalling pathways which mediate many of the cellular processes required for neutrophil function. Inflammatory mediators stimulate endothelial cells to up-regulate the ligands for integrins, including ICAM-1 which binds LFA-1 and VCAM-1 which binds VLA4 (Kolaczkowska & Kubes, 2013).

Following firm attachment to the endothelium, the final step of leukocyte entry into inflamed tissue is transmigration through the vessel wall with minimal disruption to the collective endothelial structure. Before beginning this final process, leukocytes undergo intravascular crawling, in which they perform seeking behaviour, appearing to actively

scan and probe the surrounding area to find preferred sites of transmigration, while still firmly attached to the endothelial cell. This active seeking behaviour is dependent upon the interaction between Mac-1 on the leukocyte and an alternative binding site on ICAM-1 (compared to LFA-1 – ICAM-1 interactions) on the endothelial surface (Ley et al., 2007).

1.1.5 Infiltrating leukocytes: T-lymphocytes

There are two main categories of T cells which are characterised by the presence of cell surface markers CD4 or CD8. CD4 T cells are commonly described as T helper cells given that they are well recognised for their cytokine production. The CD8 T cell population are commonly described as cytotoxic T cells given their functional role in eliminating unwanted pathogens or cells. T lymphocytes express a T cell receptor (TCR) on their surface and recognise antigens that are presented by the major histocompatibility complex (MHC). MHC class I is located on the surface of all nucleated cells within the human body and is recognised by CD8 cells, whereas MHC II is expressed on antigen presenting cells such as dendritic cells, B cells and macrophages (professional APCs) and activated endothelial cells and others (mast cells, neutrophils and epithelial cells) (non-professional APCs) allowing these cells to interact with the CD4 T cells in an antigen specific manner. In psoriatic immune infiltrate, the CD4 T cells are generally located deeper into the skin, residing primarily in the upper stratum papillary region of the dermis whereas CD8 T cells generally reside in the epidermal regions with close contact to the keratinocytes (Gaspari & Tyring, 2008).

1.1.5.1 Leukocyte transmigration

In order to enter sites of inflammation, leukocytes then cross the endothelial barrier, a process which takes around 2-5 minutes, before finally crossing the basement membrane, which takes a slightly longer time of 5-15 minutes (Kolaczkowska & Kubes, 2013). In addition to ICAM and VCAM, there are certain junctional molecules which are involved in the regulation of leukocyte transmigration. These include the immunoglobulin superfamily members platelet endothelial cell adhesion molecule (PECAM-1, CD31), junctional adhesion molecule (JAM-A, JAM-B & JAM-C), endothelial cell-selective adhesion molecule (ESAM) and the non-immunoglobulin molecule CD99 (Ley et al., 2007).

The complex network of receptor and ligand interactions promotes leukocyte migration either through endothelial junctions (paracellular/trans-junctional) or through the body of the endothelium (transcellular). The different interactions between a leukocyte and endothelial cell determine the most efficient route and site of transmigration under the specific environmental conditions. The paracellular route has long been considered as the main pathway in which leukocytes migrate, penetrating the endothelial barrier through intercellular junctions (Burns et al., 2000). It had previously been shown in a study that >75% of neutrophil transendothelial migration occurs at endothelial corners, through tricellular junctions (where the borders of three endothelial cells interact), which has also been identified *in vivo* (Burns et al., 1997). The transcellular route has been less well studied but it has been shown that at sites of transcellular migration, ICAM-1 and JAM-A are enriched, similarly to the paracellular route, but there appears to be an absence of VE-cadherin (Mamdouh et al., 2009). In addition, uniformly expressed ICAM-1 on the luminal surface of endothelial cells appears to redistribute and concentrate to the site of diapedesis and subsequent membrane channel, without the formation of transmigratory 'cups' which protrude above the plane of the endothelial surface in the paracellular route (Mamdouh et al., 2009). Although the two routes have been identified, it is not completely understood by which process the leukocyte or endothelial cell make their decision regarding the ultimate course of transmigration.

The leukocyte-endothelial interaction is completed by leukocyte transmigration via junction-associated proteins. Given that the endothelium provides a barrier for the exchange of nutrients and cells, the organisation of endothelial junctions varies depending on the location within the vascular tree. Endothelial cells in the brain for example have well developed tight junctions in order to create a strict control of permeability to create the blood brain barrier (Bazzoni & Dejana, 2004). This has been confirmed by *in vitro* evidence where HUVEC monolayers lack tight junctions until supplemented with astrocyte-conditioned media which was sufficient for tight junction formation (Burns et al., 1997). In contrast, vascular beds which allow dynamic trafficking of circulating cells such as those leading to the lymphatics and the skin may display less organized tight junctions to facilitate the movement.

Endothelial cells express an important molecule found in cellular junctions, known as vascular endothelial (VE)-cadherin (Giannotta et al., 2013). This molecule is exclusive to endothelial cells therefore making it a signature identification molecule for endothelial lineage (Breier et al., 1996). VE-cadherin maintains the collective integrity of the endothelium by binding surrounding endothelial cells. Leukocyte-endothelial interactions lead to a reduced strength of inter-endothelial connections by altering VE-cadherin and other junctional proteins such as claudins, occludins and tight junction protein-1 (ZO-1) (Schmidt et al., 2011). The ligation of ICAM-1 on the endothelium by leukocytes, for example, diminishes VE-cadherin adhesion by dissociation of the vascular endothelial protein tyrosine phosphatase (VE-PTP). This in turn causes tyrosine phosphorylation of the molecule and loss of endothelial tight junctions and increased permeability (Schmidt et al., 2011). Inflamed endothelial cells can redistribute junctional molecules in a way that favours the transmigration of leukocytes. Ultimately, the integrins, selectins and other adhesion or junctional molecules facilitate the path of the leukocyte to bind, crawl and transmigrate through the endothelium into the tissue. This complex and ordered cascade of events allows for the selective and efficient trafficking of specific leukocytes depending on the inflammatory stimulus and subsequent surface molecule expression. It is also recognised that the disruption of endothelial junctions is transient, in order to allow the maintenance of vessel wall integrity and that junctions typically re-assemble rapidly once the process is complete. Oxidised lipids have previously been suggested to play pro-inflammatory roles in the recruitment of immune cells to the endothelium, increasing ICAM-1 and VCAM-1 expression, in addition to enhancing neutrophil recruitment (Stroka et al., 2012; Badrnya et al., 2012). The role of serum lipids, LDL and oxLDL will be analysed in this research for their contribution to immune cell recruitment and the adhesion molecule expression they induce on endothelial cells.

1.2 Critical summary

Psoriasis is a chronic, inflammatory skin disease with genetic features suggestive of altered immune responses to environmental and local cues. The inflammatory disease is characterised by aberrant angiogenesis, immune cell infiltrate (neutrophils and lymphocytes) into the skin and hyperproliferation of keratinocytes. In addition to a complex inflammatory environment, oxidised lipids have been shown to accumulate in

the active lesions of psoriatic skin, although their precise role and influence on inflammation in this setting is largely unknown. OxLDL is implicated in vascular inflammation particular in the setting of monocyte-uptake and atherosclerosis, however in psoriasis the immune cells which predominate are neutrophils and lymphocytes, which are less-well characterised relative to oxLDL. Receptors for oxLDL implicated in endothelial dysfunction include scavenger receptors CD36 and LOX-1, in addition to PAF-R. Using HUVEC as a model (associated with all these receptors and adhesion molecules up-regulated in psoriasis), the direct role for oxLDL in vascular inflammation (migration, angiogenesis and immune cell recruitment) can be identified, in addition to the recruitment of neutrophils and proliferation of lymphocytes. Understanding the biology of oxLDL in psoriasis-associated inflammatory events may lead to improved treatment strategies to dampen excessive inflammation.

1.3 Aims and hypothesis

It has been established that psoriatic patients have altered lipid profiles, particularly in relation to their circulating LDL levels. It is recognised that in inflammatory conditions such as atherosclerosis, lipids (i.e. LDL) play a key role in the progression and maintenance of an inflammatory state. Primarily, this pro-inflammatory role is attributed to its oxidation (oxLDL), as this biological modification alters its uptake by receptors and consequent inflammatory influence. Patients with psoriasis show increased evidence of oxidised lipid accumulation in the skin, circulating oxLDL levels and autoantibodies against oxLDL. These patients as a disease group also have an increased incidence of cardiovascular disease.

1.3.1 Hypothesis

Oxidised lipids (oxLDL) promote an inflammatory state by stimulating migratory and angiogenic responses in endothelial cells, in addition to increasing immune cell trafficking and the expression of inflammatory mediators.

1.3.2 Aims

The aim of this investigation was therefore to understand the potential role of serum lipids and oxidised lipids in inflammatory responses relevant to psoriatic pathology by:

- Researching serum lipid contribution to endothelial and immune cellular functions by comparing inflammatory-related responses (such as endothelial migration, angiogenesis and inflammatory cell recruitment, in addition to investigating immune cell proliferation) in serum containing lipids and serum which has been lipid depleted.
- Using known concentrations of lipids added back to lipid depleted serum to analyse the effects of lipids on the above endothelial and immune cell functions
- Comparing native and oxidatively modified low-density lipoprotein (ox-/LDL) to analyse the effect of oxidation on lipids and the influence this has on these inflammation-related responses.
- To understand the mechanism by which oxLDL concentrations influence the inflammatory response to reveal potential targets to control psoriasis.

2 Chapter 2: Materials and methods

2.1.1 Cell isolations

2.1.1.1 Human umbilical vein endothelial cell (HUVEC) isolation

HUVECs were isolated into tissue culture flasks previously coated with 0.2% porcine gelatin. The isolation procedure was carried out under Class II containment and adapted from the method of Jaffe et al., 1973. Cells were isolated from cords which were from uncomplicated, natural births sourced from the Broadlands ward at the Princess Anne hospital, Southampton, UK following ethically approved processes (Ref: 07/H0502/83). Umbilical cords, which remained attached to the placenta, were immediately stored at 4°C and cells were isolated within 72 hours of birth. Umbilical cords were removed from the placenta and the outer surface cleaned with sterile non-woven gauze in order to remove blood. Any discolouration of the umbilical cord could then be identified and discarded if necessary. Cords showing large bruising or clamp marks were inspected to find a length of unaffected cord and the damaged areas were not used for isolations giving umbilical cords of approximately 20-30cm in length. Umbilical cords have two small arteries and a single larger vein (as shown in Figure 2-1). The vein is more convenient for endothelial isolation but arteries can also be used to isolate human umbilical artery endothelial cells (HUAEC) using a similar procedure.

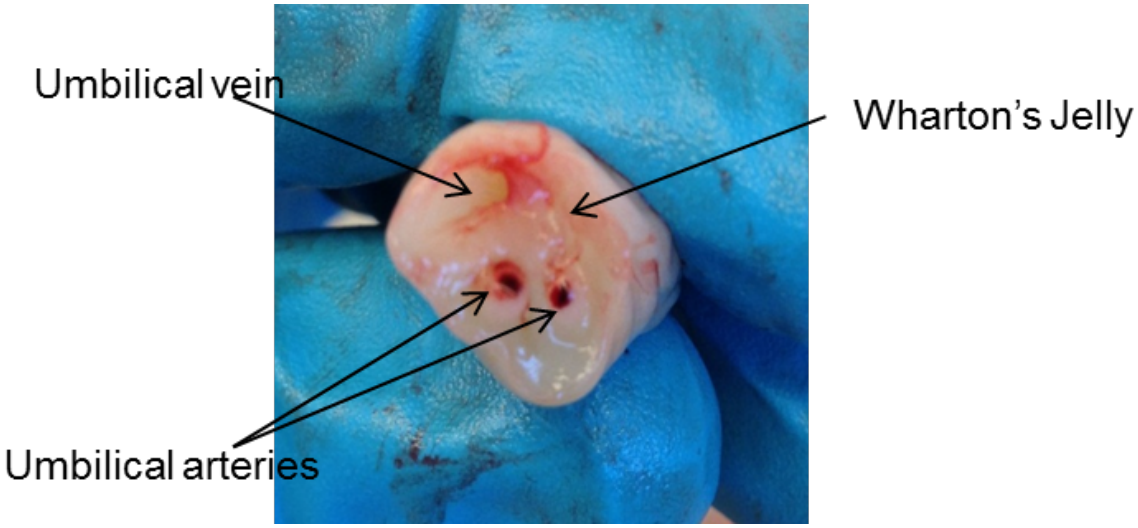


Figure 2-1 Cannulating the umbilical cord
Figure shows the vasculature of the umbilical cord, with two smaller arteries and one larger vein.

The vein was subsequently cannulated using three inch 16G metal feeding tubes kept in place with an umbilical clamp, flushed through with warmed cord buffer (NaCl 140mM, KCl 4mM, D-glucose 11mM, $NaH_2PO_4.H_20$ 200μM, $NaHPO_4.7H_20$ 800μM in sterile ultra-pure H_20 -see appendix for recipe) to remove blood clots and then clamped at one end to form a closed tube. In order to isolate the endothelial cells, the cords were filled with type I collagenase solution (Worthington Biochemical Corporation) at a concentration of 1mg/mL reconstituted in warmed cord buffer using a 30mL syringe, 0.22μm-filter sterilised and then incubated by immersion in (37°C) warmed cord buffer for 10 minutes. This protocol is optimised for effective endothelial detachment without smooth muscle cell contamination using times less than 12 minutes incubation. Following incubation, the cords were agitated to remove any residual cells before the collagenase solution containing endothelial cells was transferred into 50mL capped conical bottomed centrifuge tubes containing 3mL HUVEC media (M199, glutamine, Penicillin streptomycin with 20% human serum and in sterile ultra-pure H_20) in order to stop the enzymic action of the collagenase. The cells were subsequently centrifuged at 400g for 5 minutes at room temperature before being re-suspended in 10mL of fresh, warmed (37°C) HUVEC media. The gelatin solution used for coating flasks was aspirated and replaced with the cells in media. Cells were allowed to attach for 24 hours at 37°C in a 95% humidified atmosphere with 5% CO_2 balanced air. Cells were subsequently washed with warmed

(37°C) Hanks' buffered saline solution (HBSS) without calcium or magnesium (5mL) for 5min in order to remove erythrocytes and non-adherent cells. HBSS containing unattached cells and debris was aspirated and replaced with 10mL of fresh HUVEC media. The cell media was replaced every 48 hours onwards if necessary or until cells were split for experimental use. Cells were used usually within 7 days of isolation and always at passage 1.

2.1.1.2 Peripheral blood mononuclear cell (PBMC) isolation

In order to isolate PBMCs, fresh human blood was taken from healthy volunteers into EDTA-coated BD Vacutainers™ (Becton Dickinson) to prevent coagulation following ethically approved procedures 07/H0504/93. Blood was diluted with phosphate buffered saline (PBS NaCl 137mM, KCl 2.7mM, Na_2HPO_4 15.3mM, KH_2PO_4 1.5mM in sterile ultra-pure H_2O) at room temperature at a 1:1 ratio in sterile 50mL capped conical centrifuge tubes (Fisherbrand™). The diluted blood was then layered onto a density gradient solution at room temperature (Lymphoprep™, Axis-Shield) (15mL Lymphoprep per tube with up to 30mL blood). Lymphoprep is a density gradient medium which has a lower density than granulocytes and erythrocytes, making them pellet to the bottom of the centrifuge tube during centrifugation, with polysaccharide ingredients to enhance erythrocyte aggregation. Mononuclear cells with lower densities therefore remain at the plasma: Lymphoprep interface. This was then centrifuged at 800g for 20 minutes at room temperature with no brake in order to separate out the blood layers. These layers from top to bottom consist of the blood plasma, followed by the buffy coat, the Lymphoprep layer and then the pelleted population of red blood cells (RBCs) and polymorphonuclear cells (PMNs) (as shown in Figure 2-2). The buffy coats containing PBMCs were carefully collected using sterile Pasteur pipettes and placed into new centrifuge tubes. The buffy coat collections were diluted to 50mL with cold PBS (chilled on ice) before being centrifuged again at 400g for 7 minutes at 4°C. This washing procedure was subsequently repeated, as the supernatant was aspirated and cells re-suspended in fresh, cold PBS, before being centrifuged at 400g for 5 minutes at 4°C. Once the cells had pelleted after this final centrifugation step, the cells were re-suspended in 10mL of PBS or RPMI media (RPMI-1640, 10% heat inactivated human serum, glutamine, penicillin, streptomycin, Na pyruvate) depending on the subsequent actions required and counted with trypan blue

staining for viability using a haemocytometer. For the use of PBMCs in the flow-based adhesion assay, monocytes are removed by re-suspending the cells in RPMI and allowing the monocytes to attach to a tissue culture flask in a 37°C incubator. By careful removal of cells in suspension it is possible to reduce the monocyte cells after one hour incubation as they remain firmly attached to the plastic.

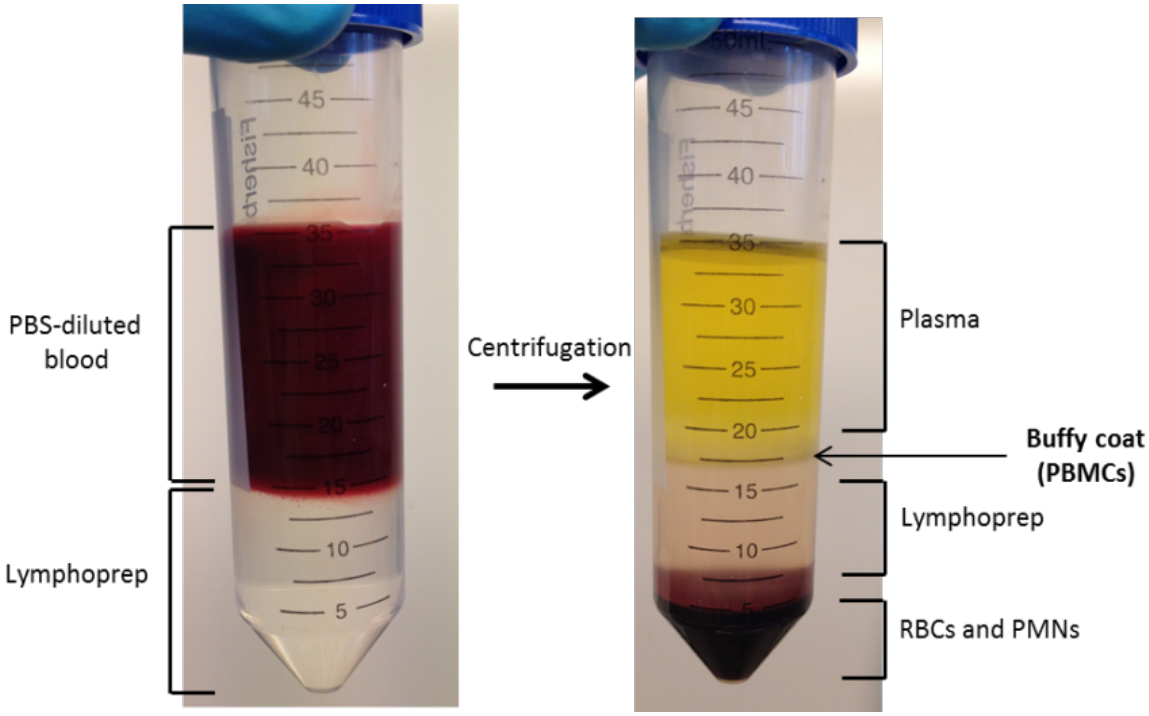


Figure 2-2 Isolation of PBMCs
Annotated images showing the separation of PBMCs from blood. After layering PBS-diluted blood (1:1) on top of a density gradient solution, cells were centrifuged at 800g for 20 minutes in order to obtain the layers shown and PBMCs were extracted as described in the text.

2.1.1.3 Neutrophil isolation procedure

Neutrophil isolation from whole blood was performed using a modified version of the separation procedure described for polymorphonuclear (PMN) leukocytes used by Ferrante and Thong (1980). This method uses dextran sedimentation to remove the majority of red cells followed by density centrifugation steps and this preparation has been shown to yield over 98% purity and 95% viability of neutrophils (Oh et al., 2008).

Blood was collected into EDTA-coated blood tubes (BD Vacutainer) from healthy volunteers and was transferred into a 50mL capped conical centrifuge tube. To this, a 6%

dextran solution (made up in sterile 0.9% NaCl H_2O) was added, at half the volume of the initial total blood volume (e.g. 30mL of blood to 15mL dextran). Dextran is a complex sugar molecule which augments the aggregation of human red blood cells (RBC) and allows efficient sedimentation to separate RBCs from whole blood (Neu et al., 2008). Following gentle mixing, the blood dextran mixture was allowed to stand at room temperature for 60-90 minutes to allow two main layers to form with red blood cells sedimenting to the bottom layer. The upper yellow layer was collected and placed into a fresh capped conical 50mL centrifuge tube before being centrifuged at 264g at 20°C for 12 minutes with no brake. A leukocyte-containing pellet was formed and the supernatant was aspirated and discarded.

To remove any remaining red cells from the cell pellet, a hypotonic lysis step was performed. The cells were re-suspended and were subjected to a hypotonic solution (0.2% NaCl in H_2O) for precisely 19 seconds, followed by an equal volume of a hypertonic solution (1.6% NaCl in H_2O) in order to return the solution to an isotonic state. Cells were centrifuged at 380g at 20°C for 5 minutes with a low brake. The supernatant was aspirated off and discarded, before re-suspending the cells in 5mL of HBSS and transferring this solution to a 15mL capped conical centrifuge tube. The cell solution was then under-laid with an equal volume of density gradient solution (lymphoprep- see 2.1.1.2 for details) using a Pasteur pipette. This was subsequently centrifuged at 380g at 20°C for 30 minutes, with no brake. The layers formed after centrifugation show an upper yellow layer, followed by a buffy coat (containing mononuclear cells), a lymphoprep layer and finally a pellet of cells consisting of isolated granulocytes (neutrophils). After removing the unwanted layers for this preparation, the neutrophil pellet alone was re-suspended in 10mL of HBSS+2%BSA solution at room temperature and cells were counted and their viability measured using a haemocytometer and trypan blue exclusion.

2.1.2 Endothelial cell experiments

2.1.2.1 Tube formation assay

In order to assess endothelial angiogenesis, an *in vitro* functional tube formation assay was used. This was based on the μ-slide angiogenesis assay (Ibidi) in which cells are

seeded onto extra cellular matrix proteins which form gel matrices in order to analyse their ability to form new vessels (see Figure 2-3). The gel matrix used in this protocol was Geltrex^{TM} (Life Technologies) with reduced growth factor basement membrane matrix. Prior to starting the experiment, the matrix gel was thawed on ice overnight at 4°C as it is stored at -80°C. Gelation of the Geltrex is temperature sensitive and in order to begin the experiment, 10µL of matrix gel was pipetted into each well of the 15-well µ-slide and placed into a 37°C (5% CO_2 balanced air) incubator for 30 minutes in order for the gel to polymerise. Cells were prepared in the appropriate media at a cell concentration of $1.25x10^4$ cells per well ($2.5x10^5$/mL) at a final volume of 50µL per well). Cells were pipetted onto the polymerised gel in the wells, the lid of the slide was replaced and the slide was placed into the incubator. Cell morphology was analysed from images captured using an Olympus CKX41 inverted phase contrast microscope equipped with a JVC camera attachment at x40 initial magnification at specific time points after seeding. After initial experiments were performed, 8hours was the time point chosen to show adequate tube forming activity in the cultures. Microscope Image Capturing and Measuring (MICAM) was the software used to capture images. For data analysis, images were converted into black-on-white form using the GNU Image Manipulation Program (GIMP) and processed for tube length using Angioquant (Niemisto et al., 2005).

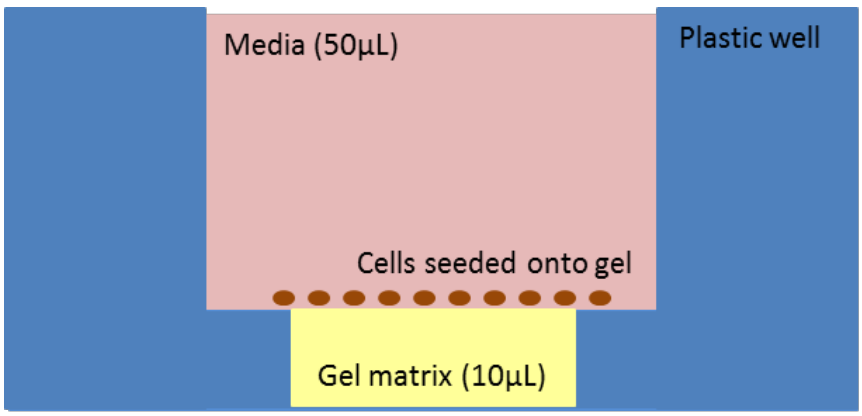


Figure 2-3 Tube formation assay design
Diagram depicting the design of the in vitro angiogenesis assay used (not to scale). The 'well in a well' design allowed visualisation of endothelial structures on a flat focal plane without meniscus formation reducing spherical aberrations associated with other methods of angiogenesis visualisation.

2.1.2.2 Endothelial migration (scratch) assay

In order to assess the role of lipids in endothelial migration, the scratch assay, otherwise known as the wound healing assay, was chosen as a model of cell migration. The assay is widely employed to assess endothelial and other cell type's response to wounding and the motility of the cells is highly important for the angiogenic response. In order to set-up the assay, endothelial cells were seeded at 100,000 cells per well in 24-well plates (pre-coated with 0.2% gelatin) in a final volume of 0.75mL per well. Cells were allowed to grow to confluence for 48 hours prior to the scratch. In order to begin the experiment, a thin wound or scratch was introduced with the use of a sterile 200µL pipette tip which has an external diameter of approximately 1.5mm. The first scratch to be made was a vertical scratch going from top to bottom of the well, followed by a horizontal scratch going from left to right. This created a section in the middle of the well which could be easily identified at multiple time points under the microscope (as shown in Figure 2-4). After the scratch had been performed, the media was aspirated, the cells washed twice in warmed PBS and replaced with the media of interest for the experiment and placed into cell culture conditions as before (37°C, 5% CO_2).

In order to assess endothelial migration, images were captured of the centre point of the scratch in each well at 0 hours and at later time points identified in each experiment (up to 48 hours). The area to which there was no endothelial coverage at the start of the assay was defined for each well as 0% coverage. Endothelial cells that had migrated to form full spatial coverage of the area previously scratched would be termed 100% coverage. The spatial coverage analysis of the captured images was performed using image analysis software Image J. Each image at time zero was measured as a pixel area and then the ratio of pixel area at subsequent time points was related to the initial scratch area to give percentage coverage from 0% to 100% (as indicated in Figure 2-5).

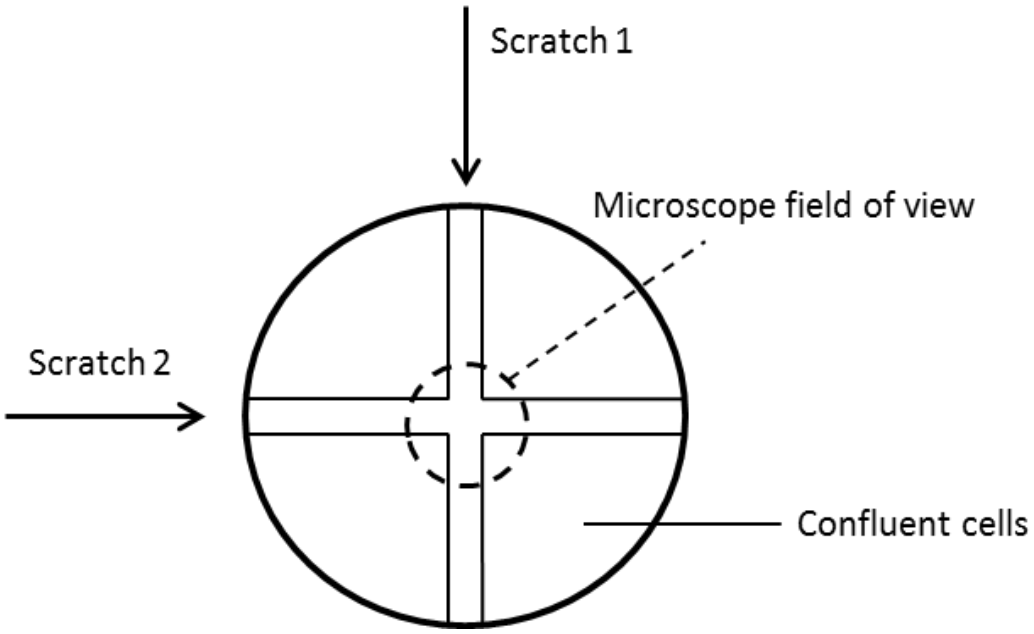


Figure 2-4 Scratch assay to assess endothelial migration
Endothelial cells in a 24-well plate scratch assay with a 200µL pipette tip vertically and horizontally in order to create a cell denuded area into which cells could migrate into.

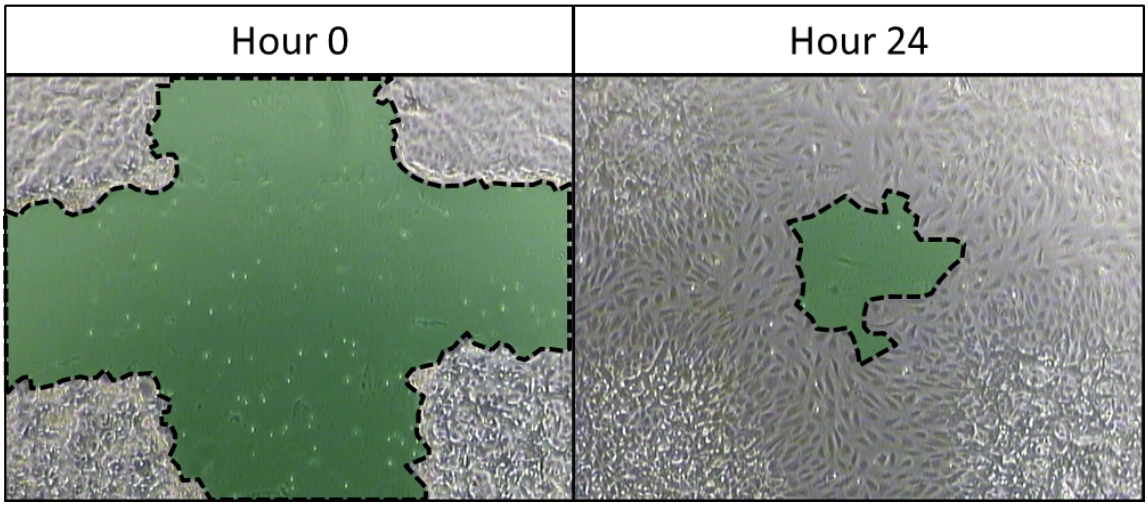


Figure 2-5 Endothelial scratch assay measurement
The area uncovered by cells (green) left denuded by the scratch was quantified by Image J at 0 hours (immediately after scratch) and at a time point indicated (24 hours shown in diagram). The calculation of the percentage of cell migration was as follows:

Time Point 0	Time Point 24
Pixel Area (green): 198,901	Pixel Area (green): 19,664

Equation: (time point 24/time point 0) *100

= (19,664/198,901)*100 = 9.89 (% area left uncovered by cells)

100-9.89 = **90.11% cell migration**, assuming 0 hours as 0% for each individual experiment

2.1.3 Flow-based adhesion assay

An experimental model of immune cell interactions with endothelial cells within a blood vessel was set up as shown in Figure 2-6. HUVEC were seeded into 35mm circular culture dishes (Costar) in order to allow the use of a Perspex parallel plate flow deck (Glycotech™ circular flow chamber kit). The flow was regulated by a programmed, automated syringe pump (Harvard P2000) which applied a continuous flow of buffer across the cells. The equipment was connected by Silastic™ laboratory tubing which connects an inlet (from the cell solution tube) and outlet (to the syringe) on the flow deck, in addition to a third port to which a vacuum is connected, to apply enough pressure for the flow deck to stay attached to the 35mm culture dish. The flow deck attaches to the dish by the vacuum via a silicon rubber gasket, specifically designed to

hold the chamber in place and regulate the height of the chamber which is formed between the flow deck and the cells. The flow deck and accompanying gasket reveal a rectangular area to allow light through and visualise cell interactions via light (phase-contrast) microscopy. The rubber gasket can be sourced with a variety of rectangular opening widths and heights, which in combination with the flow rate and viscosity of the fluid passing through, can be optimised for the intended shear stress required. The details of flow rate, viscosity, gasket dimensions and shear stress for studies performed on HUVEC can be found below.

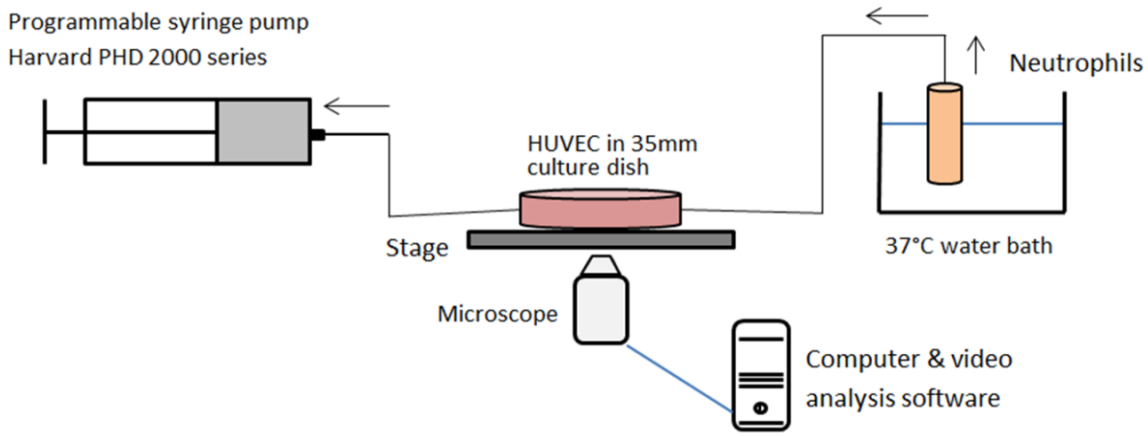


Figure 2-6 Flow-based adhesion assay design
Experimental set-up showing basic structure of the flow assay. Cells cultured in 35mm culture dishes were stimulated under static conditions and subsequently set up in the flow assay model for analysis of cell interactions.

For the analysis of endothelial cell-immune cell interactions, endothelial cells were stimulated under static conditions in a 37°C 95% humidified atmosphere with 5% CO_2 balanced air for the time identified in each individual experiment. Although the length of endothelial cell stimulation varied depending on the purpose of the experiment, there was a standardised protocol for analysis in the flow model. Following insertion of the HUVEC culture dish into the system, a 4-minute bolus of neutrophils (at $1x10^6$ cells/mL in HBSS 2%BSA) was perfused across the endothelial layer as controlled by the programmable syringe pump at a flow rate of 0.424mL/min (for a shear stress of 1 dyne/cm^2- to be discussed below). The analysis of cell interactions by video microscopy

was initiated following the 4 minute perfusion and recorded further for a length of 2 minutes, encompassing 4 fields of view along the centre-line for 30 seconds per field (as shown in Figure 2-7, below).

Immune cells were characterised for their firm adhesion to the endothelial monolayer, observed as phase-bright cells which were stationary on the surface of the endothelium for 10 seconds in comparison with non-adherent cells which were easily distinguished as faint streaks flowing past the field of view (as shown in Figure 5-1) recorded by video microscopy and software for real-time visualisation at 29fps.

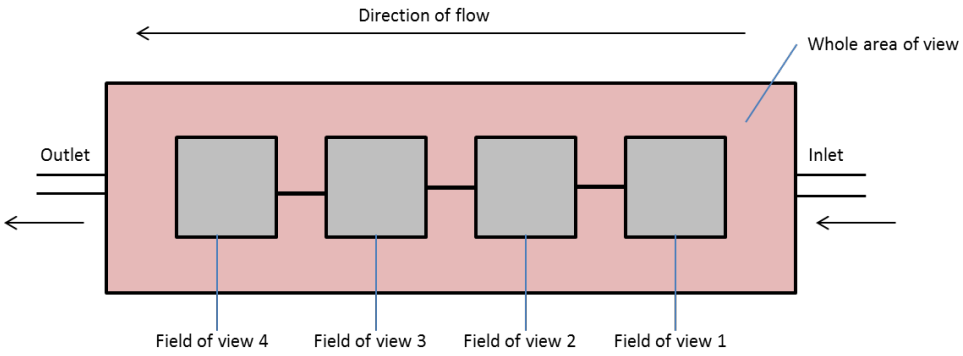


Figure 2-7 Centre-line field of view in flow-based adhesion assay
Diagram showing the placement of four fields of view in the flow assay. The large rectangular area (red) represents whole field visible using microscopy and the small squares (grey) represent the chosen fields of view along the centre line.

Given that immune cells were isolated and re-suspended in physiological salt solution, the shear stresses applied by such fluid at the same flow rate would be reduced in comparison with whole blood as the red cells provide the majority of the viscosity in whole blood whereas the salt solution used has a much lower viscosity. Vascular research has highlighted the importance of shear stresses in terms of endothelial cells recruiting neutrophils (Sheikh et al., 2003) in addition to its implication in disease such as atherosclerosis (Chiu et al., 2009). It was therefore important to clarify the shear stress applied by the rate of flow used within the flow assay. To this end, an equation relating the wall shear stress to volumetric flow rate through the chamber was sought and is given by:

$$\tau_w = \mu\dot{\gamma} = 6\mu Q/a^2 b$$

Where:

- τ_w = wall shear stress, dynes/cm^2
- Ý = shear rate, 1/sec
- μ = apparent viscosity of the media (for H_2O@37°C=0.0076 P)*****
- a = channel height (gasket thickness, cm)
- b = channel width (gasket width, cm)
- Q = volumetric flow rate, ml/sec

*****P = poise, which is a unit of dynamic viscosity

For the purposes of the experiment, a physiological shear stress of 1 dyne/cm^2 was chosen which, given the calculation above, required a flow rate of 0.424mL/minute with HBSS 2% BSA with the gasket chosen of 0.5cm in width and 0.01 inch (0.0254cm) in height. The physiological shear stresses found within the human body generally range from 1 to 6 dyne/cm^2 in the venous system and between 10 to 70 dyne/cm^2 in the arterial network (Malek et al., 1999).

2.1.3.1 Transmigration of immune cells

The flow-based adhesion assay described above allowed the visualisation of immune cell interaction with the endothelium and characterised firm adhesion of immune cells to the endothelial surface. Using this same experimental design, video microscopy also allowed the visualisation of immune cell transmigration through and under the endothelial monolayer. Immune cells adhering to the endothelial surface are seen as stationary phase-bright cells which can also crawl around on the endothelial surface. The cells that transmigrate through the endothelial monolayer are easily identifiable for their change in appearance on phase contrast microscopy changing from phase-bright cells on top of the endothelium to phase-dark once beneath it (as shown in Figure 2-8, below).

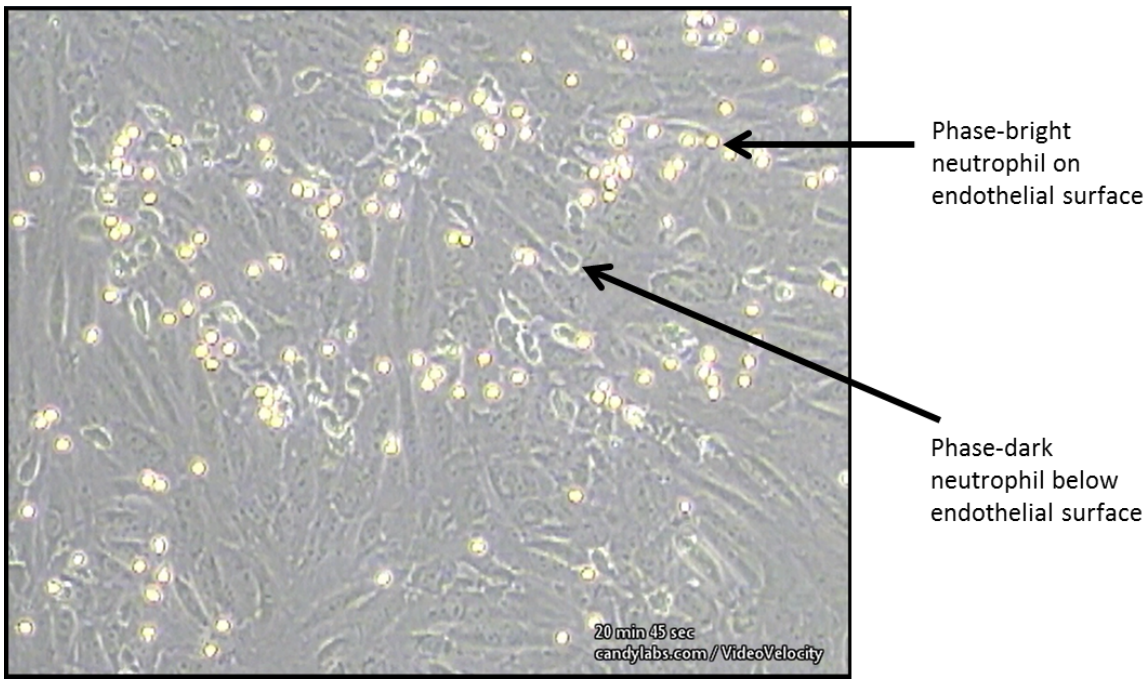


Figure 2-8 Visualisation of immune cell transmigration
Captured image of neutrophils transmigrating through an endothelial monolayer. Although endothelial cells are difficult to decipher in this captured image, attached neutrophils on the surface (phase-bright) and transmigrated neutrophils (phase-dark) can clearly be identified when tracked by video observation where multiple consecutive images can be viewed.

Following the methodology described for assessing neutrophil adherence to the endothelial layer (2.1.3), transmigration was viewed for a time period of 30 minutes, giving a total time of 36 minutes perfusion. Neutrophils were perfused only for the first 6 minutes (4 minutes followed by 2 minutes recording), after which HBSS with 2%BSA alone was perfused to visualise transmigration. The results of immune cell transmigration were quantified by the total number of transmigrated neutrophils identified at the 36 minute time-point (30 minute transmigration) divided by the total number of neutrophils attached in the first frame of the video (time-point 0, 6 minutes after initiation of flow). The results were expressed as a percentage of neutrophils transmigrated, as a proportion of all neutrophils attached in the first frame. The field of view was fixed for the duration of the recording and neutrophils that entered or exited the field of view were not counted. It is also possible for transmigrated cells to re-emerge onto the surface (reverse transmigration) and if this happened prior to the 30 minute analysis time point then they were not accounted for using this method. In this experimental design, reverse

transmigration was low (qualitative assessment) and the results are unlikely to be affected by this process to any significant degree, but it should be noted that results are expressed as net transmigration.

2.1.4 Serum delipidation

2.1.4.1 Fumed silica method

Fumed silica has a common application in biochemistry for clarifying sera by removing lipids. In order to begin the delipidation procedure, fumed silica was added to serum (20mg/mL serum) and mixed well (placed on a roller mixer typically used for blood tube agitation) for 2-3 hours or overnight at room temperature. The mixture was then centrifuged at 2000g for 15 minutes at room temperature in order to separate out the lipid-containing silica pellet and the delipidated serum. Around 15% of the sample volume was absorbed to the silica and lost using this method (protocol adapted from Ferraz et al., 2004; fumed silica sourced from Sigma Aldrich).

2.1.4.2 Solvent method

A solvent system for the delipidation of serum was based on the protocol of Cham & Knowles, 1976. The technique is proposed to attain complete removal of triglyceride, cholesterol, phospholipid and unesterified fatty acids from the serum without protein denaturation. The process begins by adding two solvents, butanol and di-isopropyl ether (DIPE) in a 40:60 (v/v) ratio to the serum (10:1 respectively, 500mL of butanol/DIPE to 50mL serum) and agitating the mixture overnight using a stirring bar at room temperature, within a fume hood/cupboard. The two-phase mixture had an aqueous phase at the bottom, which was carefully decanted, whereas the lipid-containing phase on top remained unused. The plasma proteins remain in solution in the aqueous phase while the organic phase contains the dissolved lipids. The decanted solution was transferred to 50mL screw-cap conical tubes and centrifuged at 1000g for 10 minutes to again clearly separate out the two phases, of which the bottom aqueous phase was again decanted. In order to remove potential butanol still within the serum solution, dialysis was performed with serum contained within dialysis tubing (Visking size 1 with a molecular weight cut off from 12,000-14,000 Daltons) against three changes of PBS whilst agitated with a stirring bar (room temperature) for 2-3 hours (dialysis performed

as outlined in section 2.1.5.1). The ionic strength and pH are not altered by this procedure. The serum was finally filtered using a 0.22µM filter as with all cell culture serum.

2.1.5 Low-density lipoprotein (LDL) oxidation

2.1.5.1 LDL oxidation procedure

Low-density lipoprotein (LDL) was commercially sourced (VWR International/Applichem) and oxidised in-house using a copper sulphate method. LDL was reconstituted in PBS at a concentration of 1mg/mL. Copper sulphate ($CuSO_4$) was prepared at a concentration of 20µM. The two solutions were then combined at equal volumes to produce a 0.5mg/mL LDL solution in 10µM $CuSO_4$ (final concentration). This mixture was transferred to a 35mm plastic sterile culture dish and mixed gently using a pipette. In order to oxidise the LDL, the solution was incubated for 18 hours at 37°C. Oxidation was stopped using the addition of 1mM ethylenediaminetetraacetic acid (EDTA). In order to remove both copper and EDTA from the oxLDL solution, the solution was subjected to dialysis. The dialysis tubing (Medicell International) was first soaked in 0.5M EDTA in order to remove proteases and thoroughly rinsed with distilled water before use. The dialysis tubing was Visking size 1 with a molecular weight cut off from 12,000-14,000 Daltons (copper sulphate being 159 Daltons in size and EDTA being 292), which allowed outward diffusion of $CuSO_4$ and EDTA but not LDL (2,300,000 Daltons). The tubing was knotted at one end, the oxLDL solution was added and the other end was knotted to form a sealed bag. The oxLDL solution was dialysed against 3 changes of PBS (1 litre) at 4°C over 24 hours. The oxLDL solution was filter sterilised through a 0.22µm filter following dialysis.

2.1.5.2 Analysis of LDL oxidation: thiobarbituric acid reactive substances (TBARS) assay

TBARS are formed as a by-product of lipid peroxidation and is a frequently used technique to measure the extent of LDL oxidation (Scoccia et al., 2001). In order to initiate the assay, a range of malondialdehyde (MDA) concentrations were prepared, with the highest concentration of 50µM. These MDA standards were to act as a standard curve for colourimetric analysis. The MDA standards and ox-/LDL samples were placed in 1.5mL conical micro-centrifuge tubes at a volume of 300µL. Twice this volume (600µL) of

ice cold 10% trichloracetic acid (TCA) was then added to precipitate the proteins and the samples were incubated on ice for 15 minutes. All samples were subsequently centrifuged at room temperature at 4000g for 15 minutes in a bench-top micro-centrifuge (Sanyo MicroCentaur). Following centrifugation, the proteins precipitated by the TCA pelleted at the bottom of the micro-centrifuge tubes and 400µL of the supernatant was withdrawn into fresh tubes, being careful not to disrupt the pellet. An equal volume (400µL) of 0.67% (w/v) thiobarbituric acid (TBA), dissolved in 1mL of DMSO and made up to 10mL in water, was then added before boiling the samples in a water bath for precisely 10 minutes. The samples formed a coloured compound on incubation and the absorbance of the coloured liquid was measured in a spectrophotometer at a wavelength of 532nm. The equivalent MDA concentrations of samples were interpolated from raw absorbance data using GraphPad Prism™ by comparison to the MDA standard curve.

2.1.6 Protein analysis of serum

2.1.6.1 Sodium dodecyl sulphate-poly-acrylamide gel electrophoresis [SDS-PAGE]

Serum used for electrophoresis was stored at -20°C. Proteins were separated using SDS-PAGE gels with the resolving gel concentration of 7.5% acrylamide unless otherwise stated. Gels were prepared using Tris (tris(hydroxymethyl)aminomethane) buffer, acrylamide, water, SDS, TEMED (N,N,N',N'-Tetramethylethylenediamine) and APS (ammonium persulphate). Samples were prepared in a solution of 96% Laemmli buffer, (according to that of Laemmli, 1970) and 4% DTT (dithiothrietol) of a 1M stock solution in order to denature proteins and were boiled at 100°C for precisely 10 minutes. The samples were added to wells of the stacking gel (acrylamide 5%) at a volume of 20µL. A lane of pre-stained proteins of known molecular weight acted as a method to calibrate the gel for unknown protein molecular weight (i.e. 10kDa to 170kDa) to run alongside the samples. The proteins were then separated at 100V until the solvent front reached the bottom of the gel (approximately 90 minutes for a 7.5% acrylamide gel). Gels were stained with Coomassie brilliant blue (CBB R-250, ThermoFisher Scientific) for proteins (0.25% w/v CBB prepared in 25% methanol, 7.5% acetic acid and made up in water) or Sudan Black B for lipids (prepared in 16.7% acetone, 12.5% acetic acid and made up in water). Gels were placed on a gentle tilting stage during staining and were subsequently

de-stained (using a preparation of 25% methanol, 7.5% acetic acid and made up with water) with absorbent tissue placed in the corner of plastic containers in which the gels were held for staining, to help absorb surplus CBB.

2.1.7 Lipid analysis of serum

2.1.7.1 Thin layer chromatography (TLC)

Thin layer chromatography is a technique used for the separation of mixtures into their component parts, principally used in this case for the separation of lipids in serum. The fundamental principle of the assay involves a mobile phase (generally liquid) which flows through the stationary phase (generally solid), carrying and separating the mixture as it flows. The components of the sample will travel at different rates through the stationary phase (due to differences in attraction to the stationary phase) and therefore allow separation of the constituents. Components that differ in polarity will be separated, as the more polar compound will have a stronger interaction with the stationary phase, with the less polar compounds having a weaker interaction and therefore moving further through the stationary phase in the same time. For the separation of serum lipids, the stationary phase was in the form of a silica gel coated glass plate and there were two different solvent mixtures used for the mobile phase (see below Figure 2-9).

For the separation of serum lipids by TLC, the materials required included the stationary phase (silica gel plates), glass Pasteur pipettes, a TLC tank and iodine vapour tank. The serum lipids were also required to be extracted prior to spotting on the stationary phase. In order to perform this, a solvent mixture of butanol and di-isopropyl ether (40:60 respectively) was added to serum at a ratio of 1:5 (200µL serum to 1000µL solvent) and agitated for ~2-3hours. The mixture was subsequently centrifuged at 675g for 2 minutes to separate the organic and aqueous phases. The organic phase was collected, discarding the aqueous phase. The volume of lipid-containing solvent was reduced by drying under a stream of nitrogen gas. Once at an appropriate volume (50-100µL), the samples were placed onto the stationary phase on a straight line adjacent to the lowest edge of the silica plate but high enough as to not be in contact with the solvent in the TLC tank (as shown in Figure 2-9). Around 1cm depth of solvent was added to the bottom of the TLC tank allowing samples to be placed around 3cm from the bottom of the gel plate.

The samples were dotted onto the silica plate 10-15 times to allow sufficient sample quantity to be applied to the gel. With careful handling the plate was placed into the TLC tank containing the required solvent mixture. In order to begin separation, the lid was placed onto the TLC tank and the solvent was left to migrate upwards through the stationary phase, separating out the mixed samples into their constituent parts. The process was halted when the solvent front (visibly seen on the gel plate) reached a point close to the top of the plate, which was marked in pencil to preserve this point for later calculation. The lid of the TLC tank was removed and the silica plate was taken out to allow air drying and excess solvent to be removed. In order to identify the separated lipids, the gel required staining. Although there are numerous methods of visualisation including UV illumination, the method chosen was the use of iodine crystals, as iodine will bind to the lipid fractions to give colour to the lipid spots. In order to produce this effect, the gel plate was placed into an iodine vapour tank similar to that of the original TLC and iodine crystals were placed onto the bottom of the tank. The tank was then enclosed by the addition of a lid allowing the iodine crystals to sublimate in a confined area. Iodine has a high affinity for unsaturated compounds and will bind to double bonds and this allowed the iodine vapour to highlight the lipid regions on the gel plate as brown spots.

Once the separated lipid components have been identified, an ‘Rf’ value can be calculated (otherwise known as the retention value). The Rf value is defined as the ratio of the distance moved by the solvent (solvent front marked in pencil) compared to that moved by the sample, measured from the baseline (sample origin spot). An Rf value of 0.5 for example, would indicate the sample has moved half the distance of the solvent front on the plate, allowing characterisation of certain lipids depending on the solvent system used.

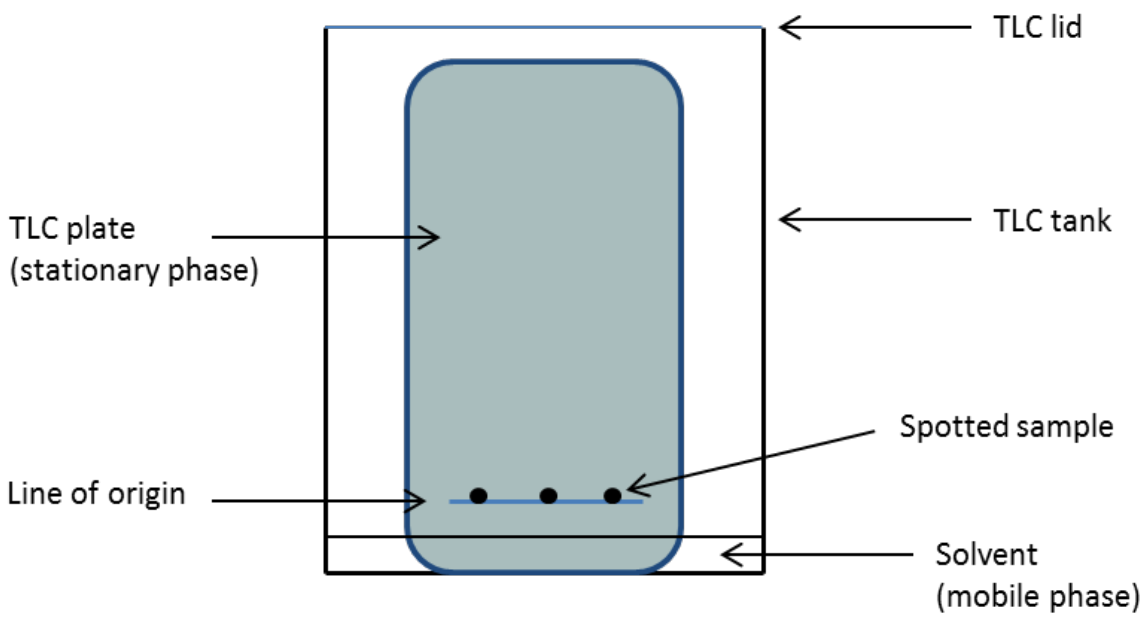


Figure 2-9 Thin layer chromatography experimental design
Diagram depicting the set-up of TLC, with the line of origin residing above the solvent. Mobile phase moves upwards though the stationary phase, separating out the contents of the samples upwards through the silica gel plate, ready for staining.

Solvent mixture for the separation of phospholipids by head group polarity and charge: dichloromethane (chloroform): methanol: ammonium hydroxide (65:25:4 respectively). Using this solvent mixture, polar lipids within the serum such as phosphatidylcholine will migrate through the stationary phase whereas other non-polarised lipids such as phosphatidylserine, phosphatic acid and phosphatidylinositol do not migrate above the origin.

Solvent mixture for the separation of neutral lipids (such as fatty acids, triglycerides, diglycerides and monoglycerides): cyclohexane: ethyl acetate (3:2 respectively). This system is focussed on the separation of neutral lipids such as triglycerides. In this system the Rf value for triglycerides is around 0.7 whereas free fatty acids are generally found at an Rf value of 0.51.

2.1.7.2 Lipid extraction and mass spectrometry

For analysis of lipid species by mass spectrometry, lipid extraction from samples was performed. Lipids were extracted from 50µL of serum samples (either unaltered serum or delipidated serum) or 50µL of ox-/LDL samples at a concentration of 0.5mg/mL. These

samples were initially diluted in 0.8mL of PBS in glass tubes. Given that mass spectrometry provides detailed analysis of lipid species and can identify oxidised lipid species, an antioxidant was added in order to prevent any further oxidation occurring during the procedure, providing a representative result of the oxidation state of the original samples. The antioxidant added was butylated hydroxytoluene (BHT) (20µL of a 0.25g/L stock). Lipid extraction steps were performed largely by a "TECAN freedom evo" liquid handling and robotics machine, with manual vortex steps. The first stage of lipid extraction was the robotic addition of methanol and dichloromethane, followed by manual vortex, further addition of dichloromethane and water, followed again by manual vortex. The samples were then centrifuged at 400g for 5 minutes with no brake in order for layers to form. These layers consisted of an upper aqueous layer, followed by a protein layer and finally a lower lipid phase. The lower phase containing extracted lipids was collected robotically into a new glass tube. These were subsequently dried under a stream of nitrogen (for approximately 20 minutes) before the dry samples were stored at -20°C; ready for mass spectrometry analysis.

Mass spectrometry is an analytical tool used for measuring the molecular mass of biomolecules. The analysis is based on the principles of the effect of a magnetic field on the movement of electrically charged particles. There are three fundamental sections of mass spectrometry, which consists of the ionisation source, the analyser section and finally the detector. The ionisation of molecules is required so that they can be manipulated by the magnetic field as charged particles rather than neutral particles. In the analyser region of the mass spectrometer, ions are separated according to their mass to charge ratio (m/z), before being detected and digitalised into m/z spectrum graph format. The particles will move through the analyser and detection regions as a finely focussed beam in a high vacuum in order for the particles to travel through without interference from air molecules. The particles are separated as their mass dictates how much they are deflected by the magnetic field and the number of components in the sample is recorded, in addition to their molecular mass and relative abundance.

Mass spectrometry separation and analysis of lipids was performed on a Waters® Xevo II TQ (triple quadrupole) mass spectrometer. This mass spectrometry method uses electrospray ionisation of particles, which is one of the major methods of ionisation in

biochemical analysis along with matrix assisted laser desorption ionisation (MALDI). A high voltage is applied to the sample liquid to create an aerosol in electrospray ionisation, whereas MALDI uses laser light to bring about sample ionisation (usually for protein analysis). The triple quadrupole design refers to the use of more than one analyser component and the use of a quadrupole analyser, which consists of four cylindrical, parallel rods through which the sample travels. The ions in the sample are therefore separated on the stability of their trajectories through the electric currents applied to the rods. The particles are separated physically in space, as opposed to other methods such as using time-of-flight. Mass spectrometry analysis in this way allowed highly sensitive analysis of lipid samples, comparing the lipid species within the samples following delipidation or oxidative procedures.

Phospholipid composition was analysed from lipid extracts of serum as described. In addition to full positive and negative ionisation scans, phospholipids analysed were PC, PE, PI, PS and other minor acidic phospholipids. These were identified using precursor scans from fragmentations in both positive and negative ionisation modes. To assess lipid removal, PC content was used as the most sensitive surrogate for delipidation. Specific fragments analysed were p184+ for PC, NL (neutral loss) 141 for PE, p241- for PI and NL87 for PS. Other glycerophospholipids were identified using precursor scans of the p153- fragment.

2.1.8 Flow cytometry

Flow cytometry is a technique widely used in the field of biological sciences for its ability to rapidly measure the properties or parameters of individual particles or cells as they flow in a fluidic stream through a sensing point. The principle of the technique is based on the capture of scattered light caused by particles flowing past a laser beam which can also incorporate the excitation and emission of fluorescent molecules attached to cells. In order to analyse material using flow cytometry, sample populations of cells or particles (such as latex beads) are suspended in a medium and injected through a stream within the flow cytometry apparatus. The fluidic system consists of a central channel through which the sample travels and is encapsulated by an outer sheath containing faster flowing fluid. The outer sheath movement effectively drags the sample fluid through the fluidic module and causes single file cell movement through hydrodynamic focussing. The

cells travelling one after another can therefore be individually interrogated by the flow cytometer in terms of the scattered light and fluorescence emission recorded (Rothe et al., 2009).

Light is used as a source of excitation in flow cytometry, requiring intense illumination to cause detection of the small, rapidly moving particles. The light source also produces specific wavelengths which can excite fluorescent dyes attached to the flowing particles. One of the major advantages of this technique is the information collected about the physical properties of the cell travelling through the apparatus with parameters known as forward scatter (FSC) and side scatter (SSC). When the cell passes through the laser beam, the light scattered in a forward direction (around 10 degrees deflection from the direction of the incident light) is detected by photodetectors and converted into electrical signals which can be digitalised on a computer (Ormerod, 2005). This is known as forward scatter (FSC) or narrow angle scatter which can give information primarily on the size and shape of the particles given the deflection of light in a forward direction. The side scatter (SSC) is similarly detected, but given that the light is deflected at 90 degrees to the incident light in a sideways direction, this is used as a marker of cell granularity. This is because intracellular organelles and granules can cause a greater scatter to the incident laser beam and when combined with the FSC and plotted on a graph the information can help to identify certain populations and types of cells.

The other primary use of flow cytometry for cell analysis is the use of fluorescence emission associated with cells. Flow cytometers are installed with optical detectors known as photomultiplier tubes (PMT), which are capable of detecting the light emitted from a particle flowing through the laser beams directed across the sample stream. PMTs convert light into an electrical pulse which can also be digitalised and read on a computer system to generate graphs, histograms and dot plots. The laser beams excite fluorochromes which are attached to the individual particles or are found within the cells depending on the cellular parameter being interrogated. A wide range of fluorescent probes are available for the analysis of cell parameters such as nucleic acid content, enzyme activity, pH, calcium flux and the expression of cell receptors (Ormerod, 2005). Fluorescent dyes absorb light and emit fluorescence energy at longer wavelengths, the so-called Stokes shift which can allow multicolour analysis of an individual cell within a

mixed sample. Using these principles, it is possible to physically separate a cell population which is known as fluorescence activated cell sorting (FACS).

Cell analysis using flow cytometry was performed on a 3 laser, multicolour FACSAria™ (BD Biosciences) system. Depending on the cell type used in the experiment, the neutral density filter was varied. The neutral density filter controls the optical sensitivity for the forward scatter, adjusting light scatter so that either smaller particles (lymphocytes) or larger particles (endothelial cells) can be adequately visualised on dot plots. The neutral density filter was therefore set at 1.0 for lymphocyte populations and 2.0 for endothelial cells.

2.1.8.1 Cell viability staining

Cell viability is an important parameter when analysing biological samples. The detection of live cells in a sample suspension can be performed in the laboratory using acidic dyes such as Trypan blue that is actively excluded or rapidly removed from the cytoplasm of viable cells. This type of dye exclusion methodology can also be adapted for use in the flow cytometer using specialised dyes that are prevented from entering viable cells. For flow cytometric analysis of both endothelial and immune cell populations (lymphocytes, PBMCs and neutrophils), the Live/Dead [near-infrared (IR)] stain was used (Life Technologies). This dye binds to free amines both on the cell interior and on the cell surface, as opposed to binding lipids which is associated with other membrane labelling techniques. This was an important factor in determining cell viability given the research topic and the manipulation of lipids within the sample preparations used. Most viability stains will not interfere with lipids, instead binding to DNA or other intracellular targets to cause a fluorescent signal, but certain stains (such as annexin V, which identifies cells undergoing apoptosis) may bind to lipids in the membrane. These lipid-binding dyes were intentionally avoided given the manipulation of lipids within the serum and hence preventing unintended binding and false results. In viable cells, the live/dead dye is restricted to cell-surface amines, whereas the dye will bind to intracellular amines in membrane compromised cells and will show an increased fluorescence in these cells. The difference in staining intensity between viable and compromised cells is easily distinguishable using flow cytometric analysis. Cells which are dead, dying or not viable

can give false-positive staining results for other antibodies and it is therefore important to exclude these cells from analyses based on their live/dead staining patterns.

Staining cells with the live/dead preparation occurred prior to antibody staining in experiments in which multi-colour analysis was used. In order to stain cells, the live dead preparation was diluted from stock 1:1000 into PBS and added to the cells whilst in flow cytometry-ready tubes. Cells were briefly mixed using a vortex mixer and allowed to stain for 30 minutes in the dark and on ice. PBS with 1% BSA was added at the end of this incubation period to remove excess unbound dye and the cells were centrifuged as indicated below.

2.1.8.2 Antibody staining for flow cytometry

The antibody staining protocol for flow cytometry was consistent across experiments. Endothelial cells were plated in 24-well plates and seeded at 100,000 cells per well in a total volume of 1mL. In order to collect cells for staining, 300µL of trypsin was added to each well following aspiration of the media and cells were placed into a FACS tube containing 300µL of HUVEC media. Cells were subsequently centrifuged at 380g for 5 minutes at room temperature. After the cells were pelleted, the supernatant was removed and the cells were re-suspended and stained. For lymphocytes, cells were plated in 96-well U-bottomed plates at 100,000 cells per well in a total volume of 200µL. Cells do not attach to this surface and so cells were gently removed straight from the wells into FACS tubes when required. PBS was added to the cells and they were then centrifuged at 400g for 5 minutes at 4°C.

The antibodies used for flow cytometry were used at a concentration of 1µL per test if not otherwise stated (‘test’ referring to 100µL of cell suspension). Antibodies were diluted 1:100 as indicated into a solution of PBS with 1% BSA and 5% human serum. Multiple stains were performed together unless contraindicated based on conjugated fluorophore and added to the sample tubes. Experiments with antibody staining were performed after live/dead preparation. Cells were briefly mixed with a vortex mixer and incubated with antibodies in the dark and on ice for a period of 30 minutes. Following this incubation period, PBS with 1% BSA was added and cells were centrifuged as

indicated above. Cells were re-suspended in 300µL of PBS with 1% BSA and were ready for analysis in the flow cytometer.

2.1.8.3 Proliferation assay using flow cytometry

Flow cytometry was utilised in order to assess the proliferative response of lymphocytes to certain factors, with the addition or manipulation of lipids. Proliferation assays generally observe either changes in the cell cycle or the number of cell divisions over a period of time. The proliferation assay used in this research was the CFSE CellTrace™ proliferation assay (Life Technologies). CFSE (succinimidyl ester of carboxyfluorescein diacetate) is a widely-recognised probe used for the analysis of cell proliferation and a popular means to monitor lymphocyte division in particular (Quah & Parish, 2010). CFSE covalently binds and labels intracellular molecules by reaction with lysine side-chains and free amine groups. These molecules become labelled with the carboxyfluorescein and retain the fluorescence for a long period of time, allowing analysis of proliferation and tracking cells after multi-day assays. When a CFSE-stained cell divides, its progeny have half the fluorescent molecules present and therefore decreases in fluorescence intensity by approximately half. The corresponding decrease in intensity with cell division can be analysed using flow cytometry.

In order to prepare cells for CFSE staining, PBMCs were isolated and were subjected to at least one wash procedure with PBS, before being centrifuged as indicated in methods above (400g for 5 minutes). The CFSE stock solution (at a concentration of 10mM) was diluted 1:500 into warmed (37°C) PBS with 1% BSA solution, to give a working concentration of 20µM. The PBMC population was prepared at a density of $2x10^6$ per mL also in warmed (37°C) PBS with 1% BSA solution. The CFSE solution was added to cells at a ratio of 1:1 to have a final CFSE concentration of 10µM and a cell density of $1x10^6$ per mL. The cells were incubated with CFSE for 10 minutes in a 37°C 95% humidified incubator with 5% CO_2 balanced air as used for cell culture. Following incubation, RPMI (containing 10% human serum) was added to the preparation and cells were centrifuged as before to remove excess dye especially as CSFE has been shown to be toxic when in excess (Asquith et al., 2006). Cells were subjected to a washing procedure and were plated into 96-well U-bottomed plates ready for the addition of mitogens and analysis of proliferation.

2.1.8.4 Statistics

Statistical significance was determined using GraphPad Prism 6 software (GraphPad Software, La Jolla, California, USA). When comparing two sets of data, two-tailed unpaired t-tests were performed unless otherwise stated. When comparing three or more data sets, one-way ANOVA was used. Following one-way ANOVA statistical tests, appropriate post-hoc analyses were performed. When data sets were compared to a control value, Dunnett's post-hoc test was used; when comparing all data sets, Tukey's post-hoc test was used and when pre-selected data columns were compared the Sidak post-hoc analysis was performed, providing more power than Bonferroni analysis and recommended for this type of analysis (GraphPad Prism 6). Two-way ANOVA statistical tests were performed with more complex datasets with two variables affecting outcome. Statistical significance was reported using P values <0.05 (95% confidence interval), with *P<0.05 **P<0.01 ***P<0.001 and ****P<0.0001 displayed on graphical representation of data. Where appropriate, reference bars accompanied '*' on graphs to represent which dataset comparisons had been analysed.

3 Chapter 3: The effect of lipids on endothelial migration and tube formation

3.1 Introduction

The formation of blood vessels and the structural organisation of the vascular tree occurs initially in the embryonic stage of human development in a process known as vasculogenesis. Under certain micro-environmental conditions, new blood vessels form by sprouting from pre-existing vascular structures in a process termed angiogenesis. The angiogenic response has important relevance to the pathology of diseases such as cancer and psoriasis where the process can drive disease progression. The angiogenic process is reported to occur via a staged mechanism *in vivo*. Endothelial cells are activated by pro-angiogenic factors such as basic fibroblast growth factor (bFGF) and VEGF, aiding cell proliferation and migration. In the subsequent stages, the basement membrane is degraded at the point of sprouting allowing endothelial cells to migrate into this area to form tubules and finally the vessel is stabilised by recruiting pericytes. Two of the major steps in the angiogenic process are modelled in this research, with a tube formation assay to depict newly sprouting vessels and a scratch assay to evaluate endothelial cell migration.

One of the major angiogenic factors characterised is VEGF which is an established cytokine secreted by a variety of cells including macrophages, platelets, keratinocytes and tumour cells (Duffy et al., 2000). This multifunctional factor acts on the endothelium in order to establish a vascular network. VEGF has long been confirmed as an angiogenic stimulus, as shown by the removal of a single VEGF allele in mice, causing lethal vascular defects (Carmeliet et al., 1996). Receptors on the surface of endothelial cells such as VEGF receptor 1 (VEGFR-1) and VEGFR-2 mediate the effects of VEGF by intracellular signalling through tyrosine kinases, potentiating the proliferation, migration and tube forming actions of endothelial cells. VEGFR-2 has been most implicated in pathological VEGF signalling (Cross et al., 2003) and therapeutic interventions against VEGF signalling have been in use for a number of years (Prager et al., 2012). Other important pro-angiogenic factors include platelet-derived growth factor (PDGF), transforming growth

factor-β (TGF-β) and the angiopoietin-Tie receptor system. TGF-β at low doses contributes to angiogenesis by up-regulating angiogenic factors and proteinases while PDGF is frequently up-regulated in tumours and supports the migration of pericytes to stabilise newly formed vessels. The angiopoietins are a family of four protein growth factors which can promote angiogenic responses, signalling through the Tie-2 receptor (primarily Ang1 and Ang2) (Otrock et al., 2007) although there are controversial findings with angiopoietins inhibiting angiogenesis in some investigations (Cao et al., 2007).

Signalling through the VEGFR during angiogenesis induces PAF production from endothelial cells which also has crucial roles for angiogenic processes (Bernatchez et al., 2002). PAF promotes the expression of angiogenic factors such as bFGF and potentiates the migration of endothelial cells (Hoeben et al., 2004). It has previously been shown that PAF induces angiogenesis in HUVEC using *in vitro* Matrigel® models and that this could be inhibited by the specific PAF-R antagonists WEB2170 and CV3988 (Montrucchio et al., 2000). It is thought that PAF contributes to endothelial inflammatory responses by supporting angiogenesis and cell migration. Despite the contribution of PAF in the angiogenic process, the involvement of lipid mediators is less well characterised compared to the role of proteins as angiogenic factors. There have been studies which suggest that certain lipids have effects in angiogenic models which include oxLDL (Danadapat et al., 2007) PAF (Wu et al., 2011) and an indication for a lipid role in angiogenesis from the effects of lipid lowering drugs such as statins (Weis et al., 2002).

OxLDL has been reported to have both pro- and anti-angiogenic roles. One particular study investigating the role of oxLDL using an *in vitro* Matrigel® assay highlighted a concentration-dependent effect, with concentrations of oxLDL below 10µg/mL causing an increase in tube formation, whereas higher concentrations having opposing effects (Dandapat et al., 2007). The receptors CD36 and LOX-1 have been implicated in the signalling of oxLDL in relation to enhancing angiogenesis (Camare et al., 2015; Khaidakov et al., 2012; Dandapat et al., 2007; Ma et al., 2006) and oxLDL has also been reported to up-regulate transcriptional activity of genes associated with cell adhesion, migration and angiogenesis in endothelial cells (Khaidakov et al., 2012).

The effect of native LDL on the angiogenic process has not been observed to any significant consequence indicating that the oxidative fragmentation of the molecule is most likely responsible for its role in endothelial activation. However, the group of drugs known as statins which lower circulating lipid (particularly LDL) have also been reported to have biphasic effects on the angiogenic process, suggesting that native serum lipids may have a role in angiogenesis (Skaletz-Rorowski et al., 2003; Weis et al., 2002). The effects of serum lipids and oxidised lipids were investigated and results are presented in this chapter. Different strategies can be employed to regulate serum lipids including statins or the use of carriers such as cyclodextrins to add or remove lipids. For endothelial experiments, delipidated serum cultures were used as a robust and reproducible model of lipid depletion, to characterise the effects native lipids may have. Oxidatively modified human LDL was used to assess oxidised lipids, analysing any potential influence in assays of endothelial function, including tube formation and cell migration.

3.2 Methods: assay development

3.2.1 Endothelial tube formation assay

HUVECs were seeded onto low growth factor basement membrane gel matrices and assessed for their ability to form tubules. In order to define the parameters of this assay, cells were initially seeded and images captured by bright-field light microscopy every 2 hours until an 8 hour time point and then again at 24 hours. For the effective analysis of tube formation within the assay, considerations for cell seeding density, serum concentration of the media and analysis time point required determination.

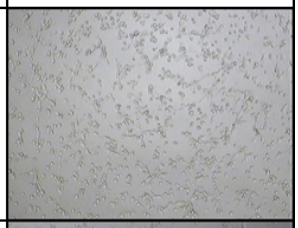
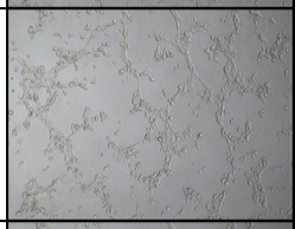
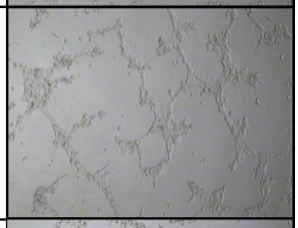
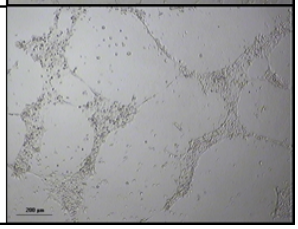
Time point (hours)	Tube Formation Photograph
2	
4	
6	
8	
24	

Figure 3-1 Tube formation of HUVEC
Time dependent tube formation of endothelial cells on a gel matrix. Cells were

seeded at $1.5x10^4$ cells per well with a serum concentration of 10%. Scale bar of 200µm shown in 24hour picture applicable to all images. Representative images of one experiment carried out in duplicate are shown.

Images were captured using an Olympus CKX41 inverted microscope equipped with a JVC CCD camera attached to a computer running software – MICAM, every two hours in order to visualise the angiogenic process. Figure 3-1 shows HUVECs are able to form a network of tubes by the eight hour time point and often within six hours from initial seeding. From the initial experiments using this assay design, it was concluded that the eight hour time point would provide a standard incubation time and be used to assess *in vitro* angiogenesis given that sufficient network formation occurs. This time point gave consistent results compared to those at six hours given that cells had not always formed complete networks (6 hour mean tube length of 65µm +/- 8.9 compared to 8 hour mean tube length 78µm +/- 9.1, with experiments performed in 2.5% serum; n=3; p=0.17). At 24 hours, networks showed more cells clumping as opposed to network tubule formation and looked to have passed the peak of tube forming activity (shown in appendix; Figure 9-1).

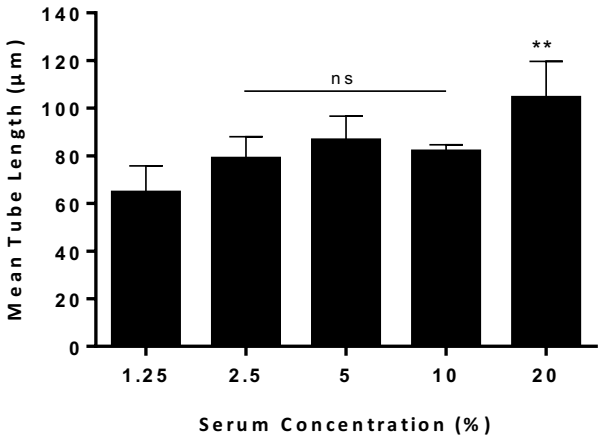


Figure 3-2 The effect of serum concentration on tube formation
The mean length of tubes formed by endothelial cells in response to concentrations of serum ranging from 1.25% to 20%. Cells seeded at $1.5x10^4$ for eight hours. Quantification was performed by calibrated software MICAM (see text for details). Statistics performed using one-way ANOVA with Dunnett's post-hoc test in comparison to 1.25% result **P<0.01. Mean +/- SD in duplicate (n=3).

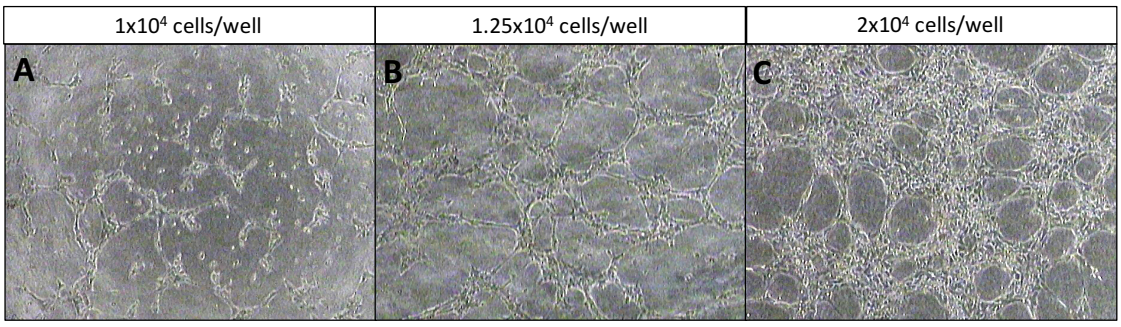


Figure 3-3 The effect of cell seeding density on tube formation
The effect of initial cell seeding number on the tube formation at eight hours. Cells grown in 5% serum for eight hours at a cell seeding density of $1x10^4$ (A), $1.25x10^4$ (B) or $2x10^4$ (C) per well (50µL). Representative images of single experiment (n=3).

From the results in seen Figure 3-2, there was a significant increase in the mean length of tubes formed as the serum concentration increased to 20% serum in comparison to 1.25% serum. There was variation in mean tube length formed and the appearance of the vascular network in the endothelial cultures dependent on cell seeding density (Figure 3-3). The developmental stages of the tube formation assay were necessary for the optimal analysis of factors which could potentially affect *in vitro* angiogenesis. In light of these initial findings a final serum concentration of 2.5% was chosen due to the complete vascular networks formed. The cell seeding density chosen was $1.25x10^4$ per well (50µL) based on vascular network observation (Figure 3-3; part B). The incubation time of eight hours was chosen as a final end-point. Taking all these values into account, these conditions provided the assay with an ability to form complete networks at eight hours without over-populating the well and causing clumps to arise, which are difficult to distinguish from tube formation when quantifying the data (shown in Figure 3-3; part C).

The quantification method used for assay development as depicted in Figure 3-2 and Figure 3-3 was a computer software package known as MICAM (microscope image capturing and measuring) version 1.6 (http://science4all.nl/) (van Weston, 2014). This software accurately converted pixel length into a numerical value (µm) after calibration with a known standard length. This was achieved by imaging known marking distances on a Neubauer haemocytometer at the same magnification as the original images of the vascular networks. This allowed manual identification of tubes to be marked and subsequently converted to total length of formed tubes in mm.

Although total length has been the preferred method of analysing tube formation, there are a variety of other parameters which can be analysed to determine the extent of angiogenesis. These include total cell-covered area, total capillary structure length, mean tube length (as used above), branching points of the vascular network or even the number of loops formed by the vascular branches. Although these can be measured manually using calibrated software as described, there is no universal accepted classification of what can be defined as a tube. For example, when referencing the data analysis protocol used by the μ-slide manufacturer Ibidi, it is stated that "the expression 'tubes' describes the cords of cells that are visible in a formed network. It does not mean, specifically, that the cords have a lumen". This method of quantification performed by a researcher may consequently be classified as subjective due to the definition of a tubule when measuring by hand, therefore leading to the consideration of other methods. In a review of angiogenesis assays, it is suggested that in order to remove bias and increase consistency, determination of tube formation should be performed using software which automatically analyses digital images of the networks formed (Veeramani et al., 2010).

A review of the literature indicated the use of various image analysis software packages for tube formation assays, including Compix (Merchan et al., 2002), Angioquant (Nacev & Liu, 2011), Adobe® Photoshop® CS3 Extended Version 10.0 (Uniewicz et al., 2011), Wimasis Image Analysis (Khoo et al., 2011), Image J (Luo et al., 2012) and Image pro plus (Jin et al., 2013). Given the choice of software readily available for use in the United Kingdom, the image analysis software choice was narrowed down to Angioquant or Image J. A requirement for the effective quantification of tube formation by image analysis software is the use of phase-contrast microscopy as opposed to bright-field and so the protocol was changed accordingly to match this requirement.

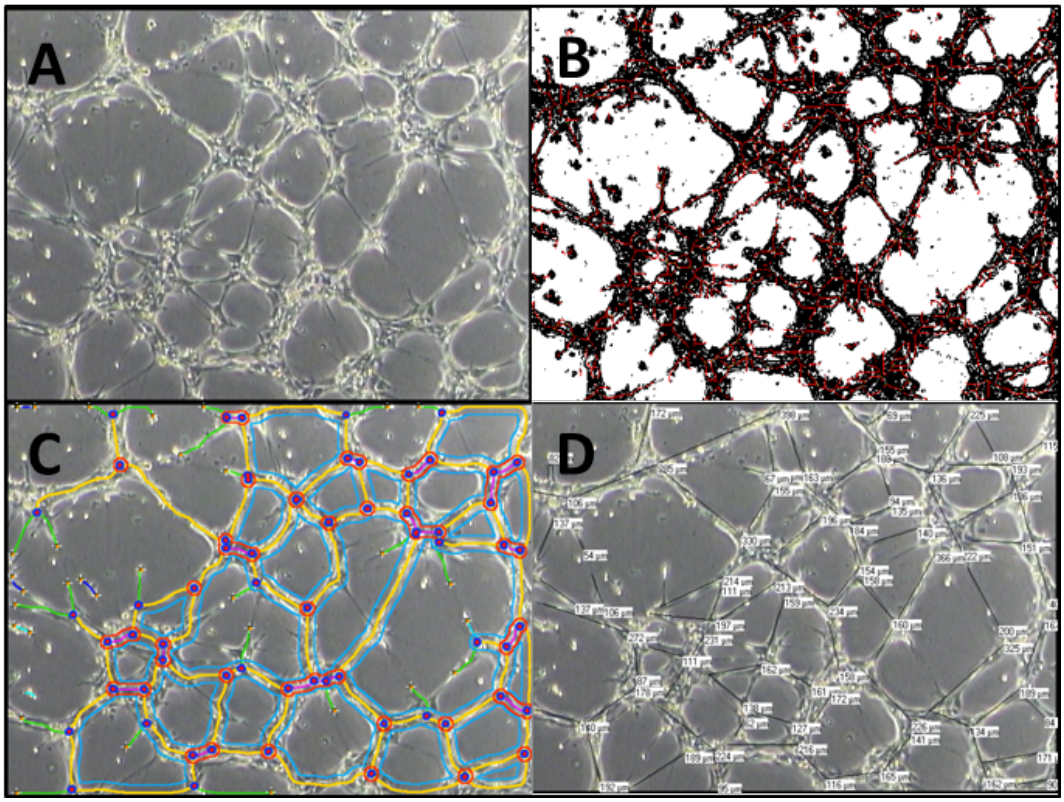


Figure 3-4 Software analysis of tube formation
A representative image of tube formation analysed using different automatic, semi-automatic and manual methods. The original image (**A**) and analysed with Angioquant (semi-automatic) (**B**) or Image J (automatic) (**C**) and MICAM (manual) (**D**) methods.

After adapting the protocol to change the microscopy used form bright-field to phase-contrast, the two non-manual image analysis software options available (Image J and Angioquant) were tested for their efficacy to analyse tube formation. The software options were tested on a variety of endothelial network images, with a single representation shown in Figure 3-4 and compared to the results of manual analysis with MICAM. In the Angioquant software, the images were initially converted into black and white format in order to be processed by the software, whereas Image J analysis was performed directly on the original colour phase contrast image. Both software options gave a quantification value for the total capillary structure identified. Figure 3-4 shows that the Image J analysis quantified the edges of the image as tubes and failed to identify structures which would manually be classed as tubes. This was a consistent finding through all the images processed. Angioquant however was successful in identifying tube formations and adequately produced results similar to those measured using a manual

method. This software was hence chosen for image analysis of tube formation at an eight hour time point. Also shown in Figure 3-4 is the MICAM analysis originally used in the experimental design and these were used for comparison to test the accuracy of the different software.

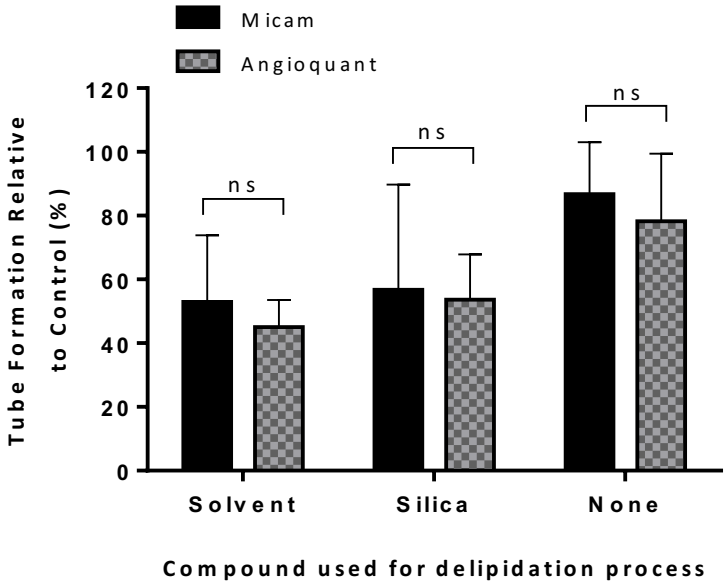


Figure 3-5 Image analysis technique comparison
A comparison of the tube formation data produced manually using MICAM (black) and software using Angioquant (grey squares). Data shows delipidated serum as a percentage relative to the length of the control measured with each method. Solvent and silica methods were used in addition to a control for the lipid removal method in which no agents were added- 'none'. Mean values +/- SD in duplicate (n=5). Statistics performed using one-way ANOVA using Sidak's post-hoc test: ns=not significant.

In Figure 3-5, the comparison between manual analysis and software analysis is shown. The data represents the tube formation in delipidated cultures as a percentage of that measured with the same technique for the control serum cultures. The relative amount of inhibition in delipidated cultures was similarly determined by both methods, confirming the reliability of the software used. The standard parameters of the assay had therefore been confirmed. Using phase-contrast images quantified by Angioquant at an eight hour time point, with 2.5% serum concentration and a cell seeding density of $1.25x10^4$ which was used in all experiments unless detailed in the text. All tube formation

images in phase contrast were subsequently imaged using a 4X objective lens to increase the total imaging area.

Having completed the assay development stages of this experimental design, the initial aim of the research was to identify the role of lipids in angiogenesis. Excess lipids have been correlated with inflammation in various diseases as outlined above and this relationship is to be investigated in this research. The primary focus however, aimed to study the role of serum lipids in tube formation. In order to test the ability of lipids to direct endothelial angiogenesis, serum was delipidated as indicated in the methods in order to remove serum lipids but leaving the proteins unperturbed. This was to delineate whether serum lipids are essential components for the angiogenic process itself, before testing whether certain individual lipids could control the process. HUVEC were used as a model endothelium and they were cultured in pooled human serum (TCS biosciences) which contains human serum lipids. Delipidation of the human serum was performed using two protocols identified from the literature. The “silica method” (Jornitz & Melter, 2007), used for commercially sourced delipidated serum, was compared to a “solvent method” commonly used in biochemistry (based on Cham & Knowles, 1976). The same batch of serum was used for experiments in which unaltered serum was compared to delipidated serum by both methods of delipidation, to control for possible batch variation.

3.2.2 Oil red O lipid stain for endothelial cells

In order to assess lipid deposition with the scratch area of the migration assay, an oil red O lipid stain was used for cultured endothelial cells following completion of the relevant scratch assay time point. Immediately following the assay, cell media was aspirated and the endothelial cells washed with 1mL of warmed (37°C) PBS. Cells were then fixed with a 4% paraformaldehyde solution (0.5mL per well) for precisely 10 minutes before being washed twice with distilled water (0.5mL per well). Cells were subsequently incubated with a 60% isopropanol (diluted in distilled water) solution for 10 minutes at room temperature. Subsequently, the isopropanol solution was aspirated and the cells were left to dry for 10 minutes before oil red O working solution was added for 10 minutes. The oil red O working solution was prepared by taking oil red O stock solution (0.35g oil red O in 100mL isopropanol) and diluting this 6:4 with distilled water respectively.

Following the 10-minute oil red O incubation period, endothelial cells were washed four times with distilled water. Images of the oil red O stain were captured under bright field microscopy for analysis of lipid deposition.

3.3 Results

3.3.1 Endothelial tube formation in lipid depleted serum

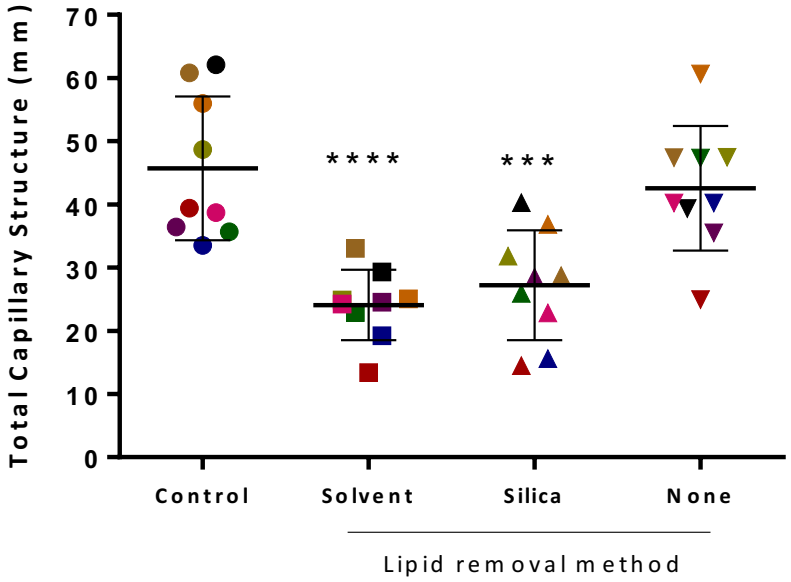


Figure 3-6 The effect of lipid removal on tube formation
The total capillary structure of HUVEC at eight hours as measured by Angioquant in response to unaltered human serum (control) and delipidated serum, either by silica method or solvent method. 'None' refers to a method control with no addition of solvent or silica. Actual length results shown calculated from pixel number which was then converted to length from calibrated images. Mean +/- SD in duplicate (n=9). Statistics performed with one-way ANOVA in comparison to control column with Dunnett's post-hoc test **** P<0.0001; ***P<0.001; no significance in the absence of *.

To measure the effect of serum lipids on angiogenesis, endothelial cells were subjected to culture in the presence and absence of lipids and the total capillary structure following 8 hours of culture was assessed. Figure 3-6, shows the angiogenic potential of HUVEC in serum with and without lipids. Removal of serum lipids was shown to cause a significant decrease in the total capillary structure when compared to serum in the presence of lipids. When comparing the two methods of delipidation, both the solvent and silica methods showed significant decreases in mean total tube length with the results showing 53.7% and 59.6% of control for solvent and silica delipidation respectively. When comparing the control serum with serum that had undergone the same process (room temperature agitation and centrifugation) but with no solvent or silica agents, there was

no significant loss of mean tube length. This result suggests that the effect of delipidation on tube formation was as a result of lipid depletion and not a processing artefact of the serum. The cells grown in delipidated serum also show an inability to form complete vascular networks when compared to control cultures, depicted in the images shown in Figure 3-7. These results support a potential role for lipids in regulating angiogenesis. In order to confirm these findings, the delipidated serum was analysed to quantify the original lipid content and also determine the effect of the delipidation protocols on serum proteins.

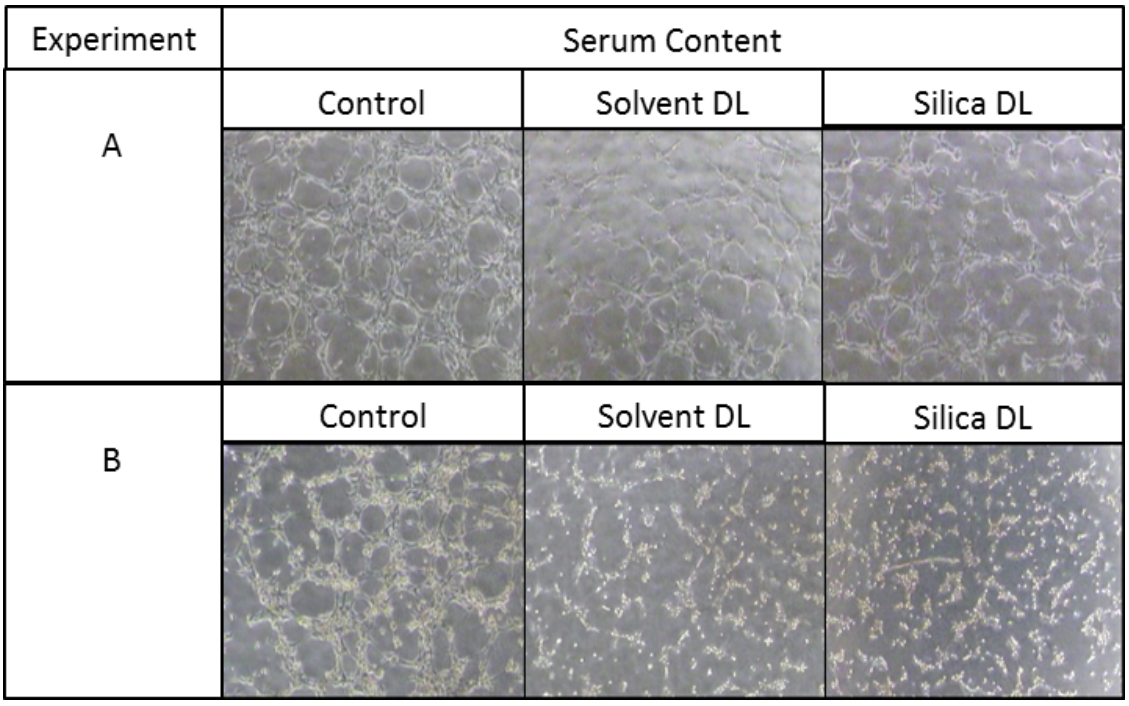


Figure 3-7 Representative images of EC angiogenesis in the presence and absence of serum lipids
Original captured images of tube formation at eight hours as represented in Figure 3-6. Delipidated cultures show incomplete networks in comparison to control (unaltered) serum cultures in addition to reduced total tube length. Representative images from duplicate wells for each treatment (n=9).

Given the significant reduction in angiogenesis seen in Figure 3-6, it was important to characterise the 'delipidated' serum in terms of its lipid and protein content.

3.3.2 Lipid analysis of serum

Given that the lipids were the intended target for removal, the extent to which this procedure was able to effectively extract total lipids from human serum was determined.

Biochemical lipid analysis was undertaken in addition to more specific lipid analysis via thin layer chromatography (TLC) (Figure 3-8 & Figure 3-9) and mass spectrometry (Figure 3-10) to assess serum lipids.

Sample	Total Cholesterol (mmol/L)	HDL Cholesterol (mmol/L)	Triglycerides (mmol/L)
Control serum	3.5	0.80	0.7
Solvent Delipidated Serum	<0.5	<0.10	<0.1
Silica Delipidated Serum	<0.5	<0.10	0.3
Delipidated Method Control	4.8	0.88	1.0

Table 2 Lipid analysis: biochemistry data
Serum samples analysed for lipids on a Beckman Coulter AU5800 automated chemistry analyser. Silica result representative of n=3 extractions from the same pooled serum samples.

Lipid analysis of serum samples was undertaken on a Beckman Coulter AU5800 automated chemistry analyser, which is a high-volume core hospital laboratory unit commonly used for the analysis of patient blood samples. The parameters measured using this technique (as shown in Table 2) included total cholesterol, HDL cholesterol and triglycerides. Both solvent and silica methods of lipid removal were successful in removing serum cholesterol components to below the level of detection (<0.5 mmol/L). The LDL fraction of serum is not directly measured and reported using this technique, but is calculated from the results of total cholesterol, HDL cholesterol and triglycerides. Given that the total cholesterol was diminished below detectable levels, it is calculated that LDL was also completely removed from the serum using both methods.

The solvent method of removing lipids similarly removed all triglycerides beyond detectable levels (<0.1 mmol/L). The silica method of delipidation however showed detectable levels of triglycerides (0.3 mmol/L) which was repeated over three separate isolations from the same batch of pooled human serum. This comparison indicated that the solvent method may remove lipids more effectively than the silica method. In light of

this data, serum treated with silica may be referred to more accurately as lipid-depleted as opposed to lipid-free. To further clarify the results of the biochemistry data, TLC was performed using solvent separation techniques to separate out polar (Figure 3-8) and neutral lipids (Figure 3-9).

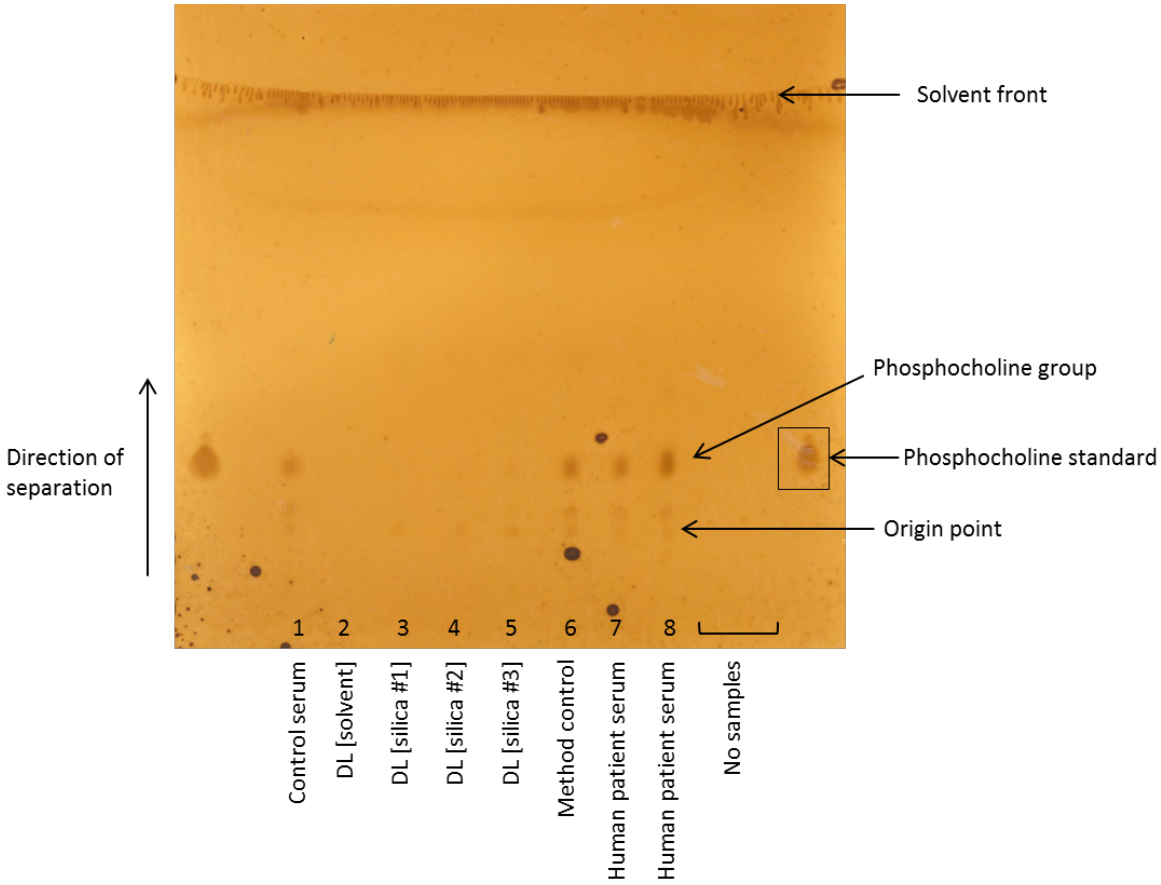


Figure 3-8 Lipid analysis: polar lipid separation by TLC
General separation of phospholipids by head group polarity and charge using a solvent system of dichloromethane: methanol: ammonium hydroxide (62:25:4). Visualisation of lipids was carried out with iodine staining as outlined in the methods section. Representative TLC of three silica delipidation procedures.

The lipid separation by TLC in Figure 3-8 occurred by head group polarity using the solvent system indicated. The lipids separated in this procedure were the predominant lipids found in serum, namely phosphatidylcholine (PC) and phosphatidylethanolamine (PE), whereas other lipids such as phosphatidylserine and phosphatidylinositol do not migrate above the point of origin in this solvent system. Both methods of lipid removal appeared successful in completely removing both of the predominant serum lipids down to the detection limit of this method as no lipid marks were visible when compared to

those seen in control serum. A phosphatidylcholine standard was run either side of the samples in order to identify this major lipid component of serum. Serum from healthy individuals was also separated by TLC solvent methods which show that the lipid profile produced from pooled human serum used in experiments was comparable to that of individual human donor serum samples. From this TLC separation, it was concluded that the major phospholipids were removed in both lipid removal techniques. Given that the biochemistry data indicated a differential removal of triglycerides between the two extraction methods, further TLC was performed using a solvent system which would identify neutral lipids by separation (Figure 3-9), in order to clarify this point.

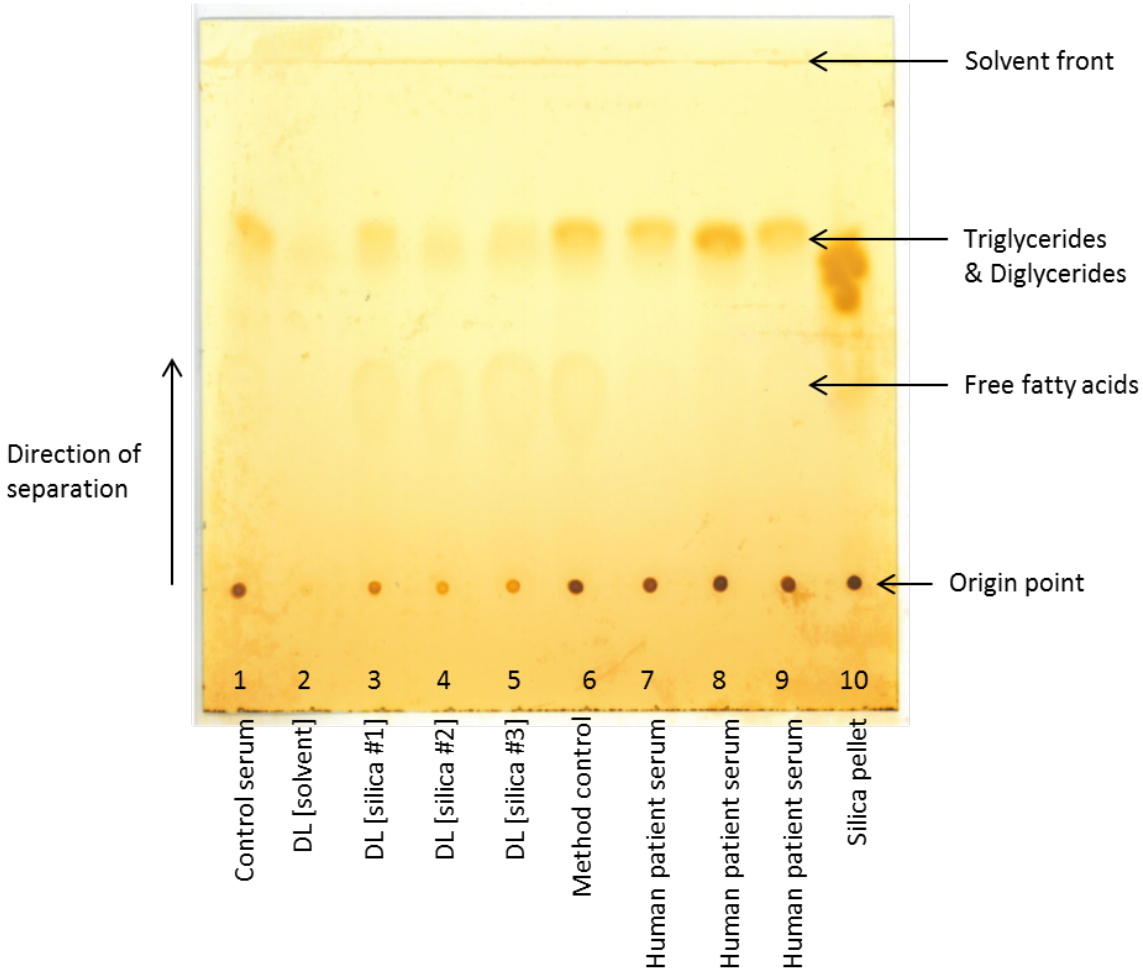


Figure 3-9 Lipid analysis: neutral lipid separation by TLC
Separation of neutral lipids (fatty acids, triglycerides, diglycerides & monoglycerides) using solvent system cyclohexane: ethyl acetate (3:2). Visualisation of lipids was carried out with iodine staining as outlined in the methods section. Representative TLC of three silica delipidation procedures.

The separation of neutral lipids in Figure 3-9 showed that the silica method of lipid removal did not completely remove triglycerides as there were lipid spots visible as indicated, confirming the results obtained from the biochemistry data. In this solvent system, free fatty acids have an Rf value of 0.51, whereas triglycerides have an Rf value of 0.7, easily identifying the two major lipid groups visible on the TLC plate. The solvent method of lipid removal appeared to remove all lipids detected using both TLC systems and biochemistry. It was concluded that the silica method was less effective overall in its ability to remove lipids, however the effect of each method on proteins was yet to be determined. More detailed analysis of the lipid species found in the silica delipidated serum was subsequently determined using mass spectrometry (Figure 3-10).

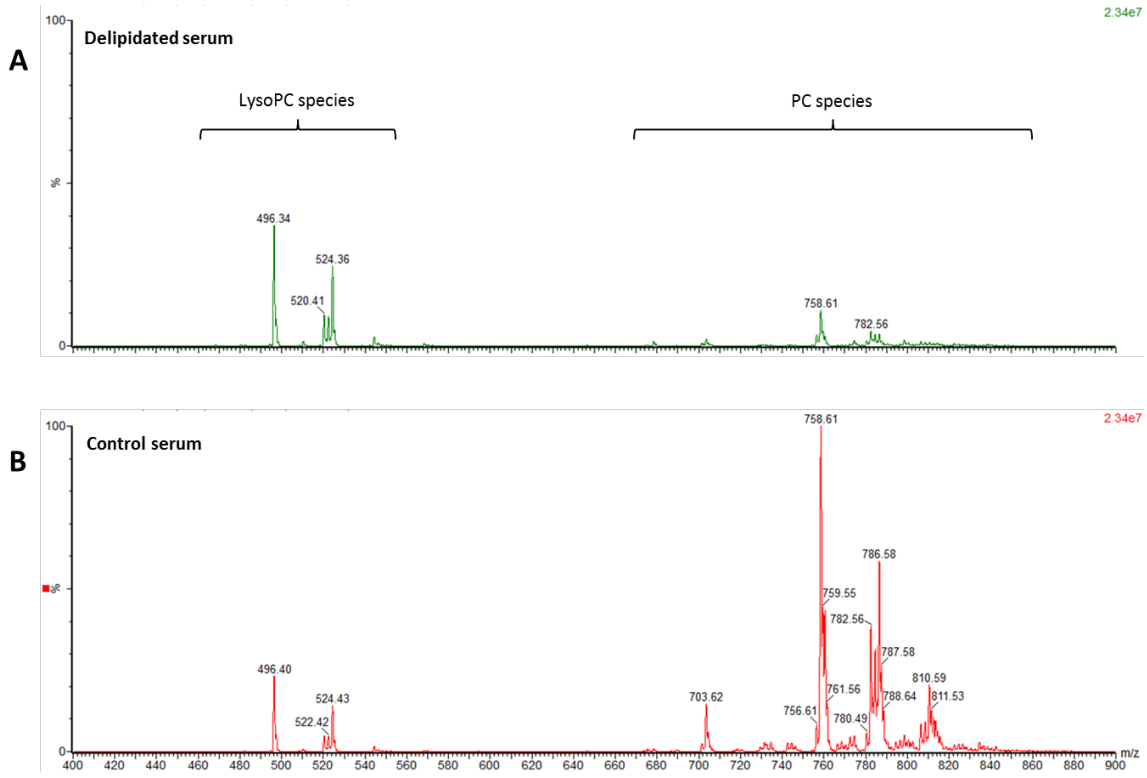


Figure 3-10 Mass spectrometry analysis of delipidated serum in comparison with control serum

Mass spectrometry analysis was carried out on delipidated serum using the silica method (A) and control serum (B). The Y-axis scales are linked so represent the same level of lipid detection. Representative images of three serum samples from the same batch of pooled human serum treated with or without silica for delipidation.

Mass spectrometry was performed in order to analyse the phospholipid composition of serum, as it is the most sensitive marker for lipid content. The graphs represent the most common lipid species within serum, such as palmitoyl-linoleic PC (758 peak), arachidonic acid (782 peak) and palmitoyl-arachidonoyl PC (810 peak). Using this method of detection, the mass spectrometry data showed that the lipid species detected in delipidated serum was reduced compared to those detected in control serum with a reduction of more than 90% of the PC species. The lipids determined as lysoPC from the MS spectrum do not appear to be greatly depleted by the silica method when compared to control. The lysoPC species are a class of phospholipids which are intermediates in the metabolism of lipids and the presence of these lipids in delipidated serum may be due to the variable fragmentation possibilities formed during lipid removal.

3.3.3 Protein analysis of serum

Although both methods of lipid removal from serum are intended to be specific for lipids, it was important to assess the protein content of the serum following processing. This was performed using protein gels (SDS-PAGE) and a total protein assay (Pierce Biotechnology, Inc.).

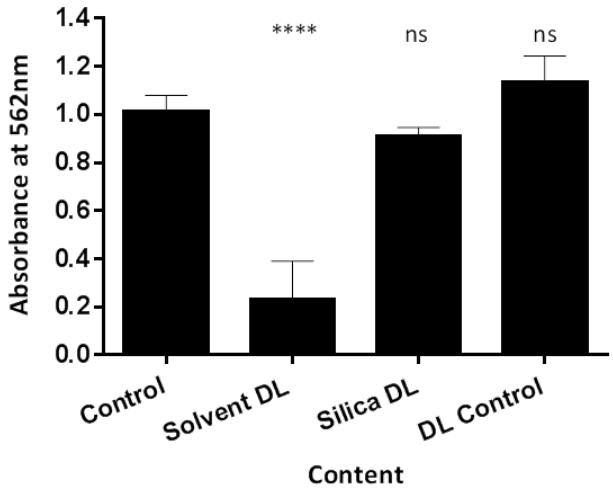


Figure 3-11 Total protein assay of serum
All serum was processed in a total protein assay in order to assess potential protein loss from delipidation procedures. Mean +/- SD in triplicate of three individual delipidation procedures of a single batch of pooled human serum (n=3). Statistics performed using one-way ANOVA with Dunnett's post-hoc test. ****P<0.0001; ns=not significant.

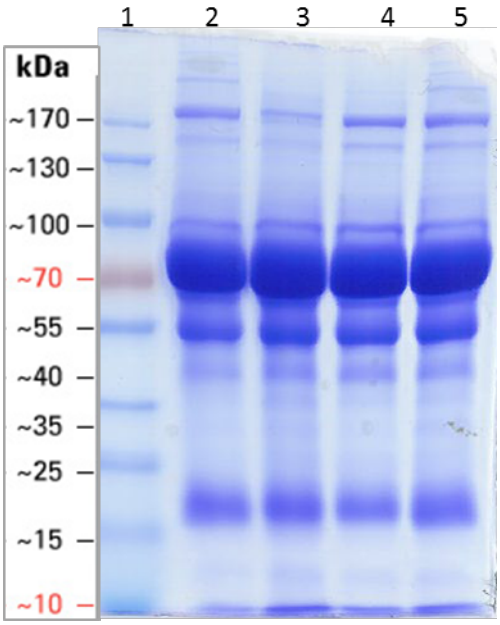


Figure 3-12 Serum protein separation using SDS-PAGE
Gel electrophoresis separation of serum proteins. Reference bar (to the left of protein gel) represents equivalent molecular weights of protein bands in kilo Daltons. The lanes on the gel from left to right are as follows: 1) protein ladder 2) control serum 3) solvent delipidated serum 4) silica delipidated serum 5) delipid method control. Representative image of one protein gel (n=3).

The two methods used for protein analysis were a total protein assay as seen in Figure 3-11 and a separation of the individual proteins using electrophoresis as shown in Figure 3-12. These were performed to verify any protein loss in the two delipidation processes used. From the total protein in Figure 3-11, the solvent method of lipid extraction from serum also significantly reduced the total protein which has a potential impact on cell function in subsequent assays. The silica method of delipidation however did not alter the total protein content to any significant extent. The SDS-PAGE analysis of the protein bands showed that with solvent delipidation (lane 3) protein bands heavier (higher) than 130kDa molecular weight were either faded or missing when compared with control (lane 2). With the silica method (lane 4), the protein bands up to and including the strong band at around 170kDa were comparable to that of the control suggesting that none of these proteins were lost during lipid extraction. Similarly to the solvent method however, the very high molecular weight bands (around 500kDa) which show a faint band in the control serum (lane 2) and the lipid method control serum (lane 5) are missing in both

the solvent and silica methods. Given that the delipidation method will remove lipoprotein molecules such as VLDL, LDL and HDL these bands are potentially the apoproteins removed with these molecules which are apoB-48 (241kDa), apoB-100 (512kDa) and apo(A) (300-800kDa) respectively.

Taking into account the protein loss seen both with the total protein assay and the protein separation techniques in the solvent extraction method, it was concluded that this is not a reliable method with which to remove lipids alone for further research. The protein loss was too severe using the solvent method to reliably conclude the role of lipids in functional assays using this serum. Hence, the silica method of delipidation was chosen as the preferred method for further work, given that there was minimal protein loss. Any differences observed in functional assays using the silica delipidated serum can be more reliably concluded as a result of lipid loss as opposed to lipid and protein effects.

Given that the silica method of lipid extraction was chosen based on the fact that there was minimal protein loss, there were two subsequent queries remaining regarding the nature of the delipidation. The first query was regarding the lipids removed from the serum during the process and secondly, was the method consistent and reproducible when repeated. In order to clarify these questions, the silica method of lipid extraction was repeated and separate delipidated serum aliquots (from the same original batch) were analysed via SDS-PAGE (Figure 3-13) and tested in the functional angiogenesis assay (Figure 3-14).

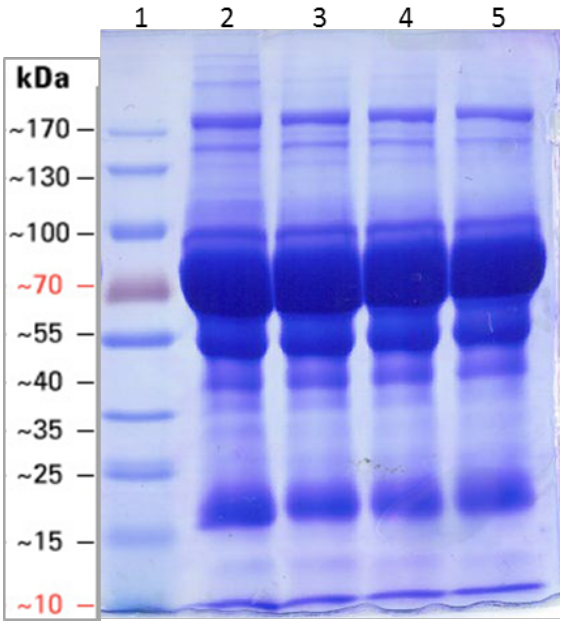


Figure 3-13 Silica method protein analysis using SDS-PAGE
Protein separation of delipidated serum (using silica method) using gel electrophoresis. Reference bar (left of protein gel) references molecular weight in kilo Daltons. Lanes on the gel from left to right are as follows: 1) protein ladder 2) control serum 3) silica delipidated serum (#1) 4) silica delipidated serum (repeat #2) 5) silica delipidated serum (repeat #3). Proteins were stained using Coomassie Brilliant Blue following separation in 7.5% SDS-PAGE. Representative image (n=3).

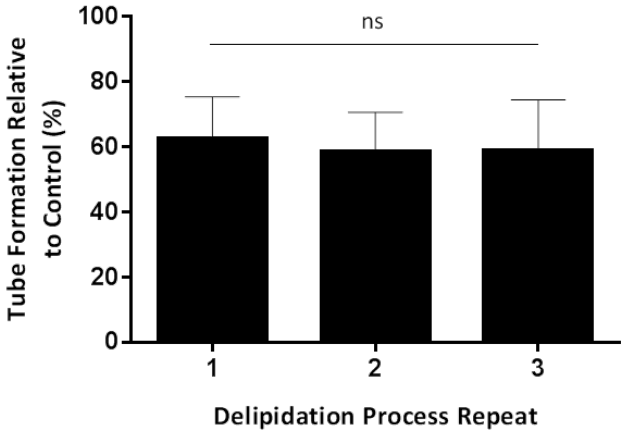


Figure 3-14 Tube formation of delipidated serum repeats
Tube formation of HUVEC in delipidated serum (via silica method) showing aliquots separately delipidated in comparison with control serum from the same batch of pooled human serum. Mean +/- SD of EC in duplicate from three individual donors. Statistics performed with one-way ANOVA with Tukey's post-hoc test.

The repeated extraction of lipids from serum using the silica method showed a consistent extraction as represented by the identical protein separation on SDS-PAGE (as shown in Figure 3-13) and the results of the tube formation assay. The results appeared to be reproducible and consistent, allowing for the repeated and long term use of the lipid depletion procedure in subsequent experiments.

3.3.4 Endothelial tube formation

The clarification of the lipid and proteins contained in the serum and the effect that delipidation had on these factors was an important step in interpreting the results of the subsequent tube formation assays. The previous data indicated that silica depletion of lipids reduced the ability of endothelial cells to adequately form tubes on a gel matrix. Having conducted the analysis of the protein and lipid content of this serum, it was concluded that the lipid portion of serum is a contributory factor in tube formation. The tube formation assay was subsequently performed in order to assess the role of other lipids on the angiogenic response. The initial factor analysed was a well-known pro-inflammatory lipid PAF, in addition to a PAF-receptor antagonist, known as WEB2086.

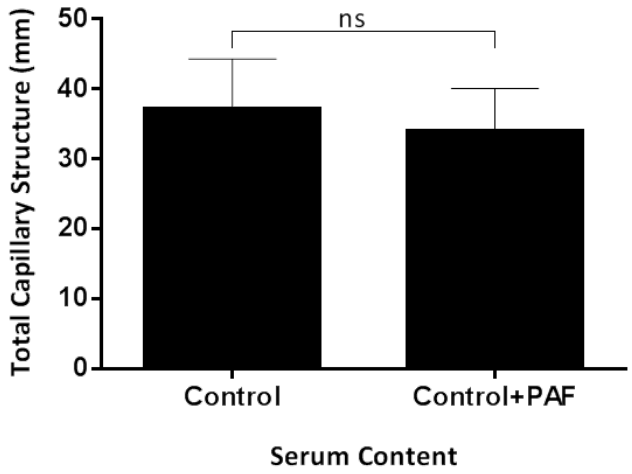


Figure 3-15 The effect of platelet-activating factor (PAF) on tube formation
Tube formation of HUVEC in human serum (unaltered) compared to the addition of PAF (1µM). Mean +/- SD of total capillary structure. Statistics performed using unpaired, two-tailed T-test (n=9).

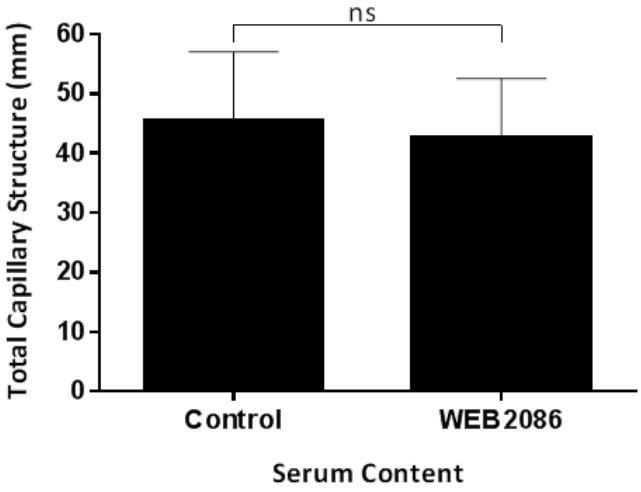


Figure 3-16 The effect of PAF-R antagonist WEB2086 on tube formation
Tube formation in unaltered human serum compared to the addition of WEB2086 (100nM), a known PAF-R antagonist. Mean +/- SD of total capillary structure. Statistics performed using unpaired, two-tailed T-test (n=9).

In complete human serum there was no significant effect of PAF or blockade of the PAF receptor, in the absence of exogenous PAF, on tube formation at the concentrations used neither an increase nor decrease in the total capillary length. When repeated in the absence of serum lipids, total capillary tube length was significantly decreased when compared to complete serum as previously shown. When PAF was added to lipid depleted cultures, total capillary length was significantly increased in comparison (Figure 3-17).

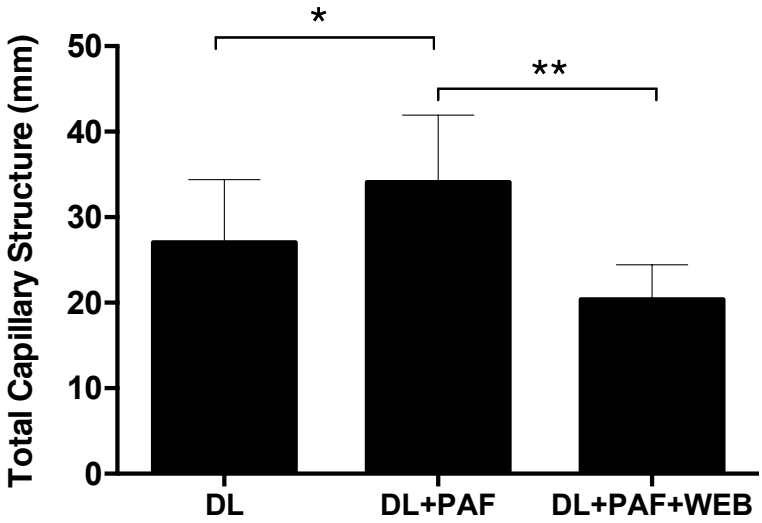


Figure 3-17 The effect of PAF and PAF-R blockade in delipidated serum on tube formation

Tube formation was analysed in delipidated serum with addition of PAF (1μM) and separately PAF (1μM) with PAF-R inhibitor WEB2086 (100nM). Mean +/- SD of total tube length. Statistics performed with one-way ANOVA with Sidak's multiple comparison test (comparing delipid (DL) with DL and PAF; comparing DL+PAF with DL+PAF+WEB) **P<0.01, *P<0.05 (n=12 for 'DL' and 'DL+PAF', n=6 for 'DL+PAF+WEB').

PAF was able to partly restore angiogenic potential in delipidated cultures, which was significantly increased compared to delipidated cultures alone, although did not reach the tube formation observed in control serum cultures. This result was directly attributed the effect of PAF through its effects on the PAF-R, as indicated by the blockade of this effect with PAF-R antagonist WEB2086 addition.

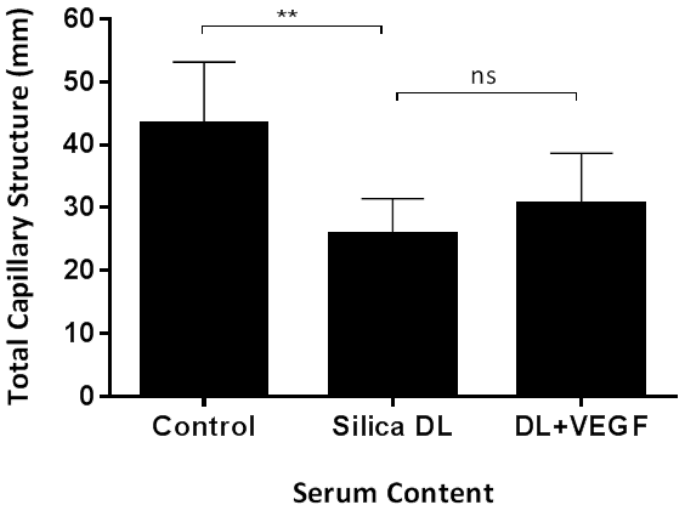


Figure 3-18 The effect of vascular endothelial growth factor (VEGF) on tube formation
Tube formation in complete serum was compared to delipidated serum. In addition, lipid depleted serum cultures were compared to the addition of VEGF (50ng/mL). Mean +/- SD of total tube length. Statistics performed with one-way ANOVA with Sidak's multiple comparison test (comparing control with delipid (DL); comparing DL with DL+VEGF) **P<0.01 (n=7).

Having shown that PAF partly restored the angiogenic potential in delipidated cultures, the same assay was performed with the addition of VEGF (50ng/mL). It is known that VEGF is one of the most important factors in endothelial responses involving migration and new vessel formation. Given the scientific literature regarding VEGF as a potent inducer of endothelial tube formation, it would be hypothesised that VEGF would increase tube length in endothelial cell cultures after 8 hours. In this assay, VEGF was added to delipidated cultures but showed no significant increases in total endothelial tube length (P=0.23).

The angiogenic response of endothelial cells had been initially characterised in lipid depleted cultures and in response to the pro-inflammatory lipid PAF and growth factor VEGF. The concentrations of 1µM of PAF and 50ng/mL of VEGF were chosen as they were referenced to be optimal for endothelial responses (Stone et al., 2002; Thomas et al., 2000; Axelrad et al., 2004).

It could be theorised that the removal of lipids from serum decreases the angiogenic response of endothelial cells due to the removal of important biological mediators (such

as PAF). Alternatively, this result may be due to the lack of essential lipids required for the physical structural components required for cell motility and tube formation, with lipids being essential in maintaining membrane structure. In order to assess this possibility, essential fatty acids were added back to delipidated cultures to evaluate their effect on tube formation, given that cells cannot synthesise these lipids endogenously. Linoleic acid (an ω6 fatty acid) and linolenic acid (an ω3 fatty acid) were used to replenish the media with fatty acids and observe their effects on the endothelial response (as shown in Figure 3-19). Concentrations of fatty acids were chosen as 100ng/mL based on commercial chemically-defined lipid concentrates (SIGMA) and concentrations used in the literature (Butler et al., 1999). The results from this experiment indicated that there was no significant effect of these lipids on tube formation, showing no statistical significance between delipidated cultures and those with the addition of fatty acids (either alone or in combination). The research question of interest therefore was to identify the role of native and oxidised LDL in control and delipidated cultures.

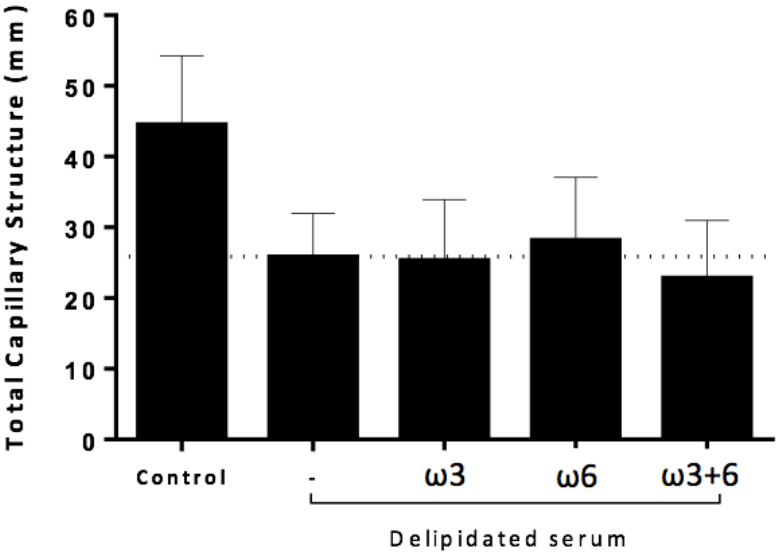


Figure 3-19 The effects of fatty acids on tube formation in delipidated cultures
The addition of omega-6 fatty acid (linoleic acid) and omega-3 fatty acid (linolenic acid), alone and in combination at a concentration of 100ng/mL had no significant effect on endothelial tube formation when added to delipidated cultures. Statistics performed with one-way ANOVA with Dunnetts post-hoc test (n=6).

3.3.5 LDL oxidation

Having developed the assay to this stage, the potential of other factors especially the role of oxidised lipids in endothelial responses was investigated. In order to assess this, LDL was commercially sourced and oxidised using a copper sulphate method. The role of oxidised lipids in the form of oxLDL could therefore be evaluated and compared to its native state (LDL).

The copper sulphate oxidation of LDL was a standardised procedure, occurring for 18 hours in a 37°C humidified atmosphere with 5% CO_2 balanced air as used for cell culture experiments. The extent of LDL oxidation changes its composition and recognition by cell surface receptors. In order to evaluate the oxidation state of the LDL after 18hours, a measure of lipid peroxidation known as TBARS assay was employed. Thiobarbituric acid reactive substances are formed as a by-product of lipid peroxidation and can therefore be identified using TBA as a reagent used as a measure of the extent of oxidation in a sample. The primary example of a compound which reacts with TBA is malondialdehyde (MDA) which is one of the end products formed by lipid peroxidation. Although MDA is a useful marker for this peroxidation, it is important to note that MDA is not the sole by-product of lipid oxidation and not exclusively generated by this process. The TBARS assay cannot therefore give detailed analysis of individual lipid species and their oxidation state to the extent other methods such as mass spectrometry are able, but is a useful and convenient assay to monitor the overall extent of lipid peroxidation in a sample.

Known concentrations of MDA were used to generate a standard curve of absorbance at a wavelength of 532nm in a plate reading spectrophotometer following the methods described previously in chapter 2. This standard curve was performed for each oxLDL measurement from which the absorbance values could be expressed as an equivalent MDA value.

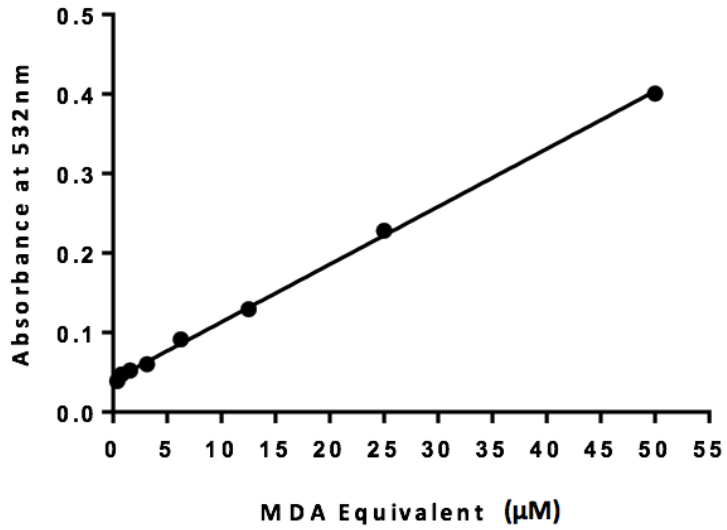


Figure 3-20 TBARS standard curve
A standard curve of known malondialdehyde (MDA) concentrations using spectrophotometry at a wavelength of 532nm. The assay was sensitive enough to detect MDA at 0.78µM. Representative example shown of one experiment carried out in triplicate, Mean +/- SD. R^2 value = 0.99.

Each oxLDL preparation was stored at 4°C and used within 7 days. This means that oxidations were carried out routinely on the same batch of LDL but that samples were discarded once they reached seven days at 4°C. One aspect of the copper sulphate method of oxidation that requires investigation is the removal of the oxidising species copper, in addition to the EDTA which is added to stop oxidation at 18 hours. In order to remove these substances, samples are dialysed against three changes of PBS overnight through a dialysis membrane. Samples of oxLDL were taken pre- and post-dialysis in order to check the TBARS value and therefore oxidation state for both native LDL and oxLDL preparations shown in Figure 3-21.

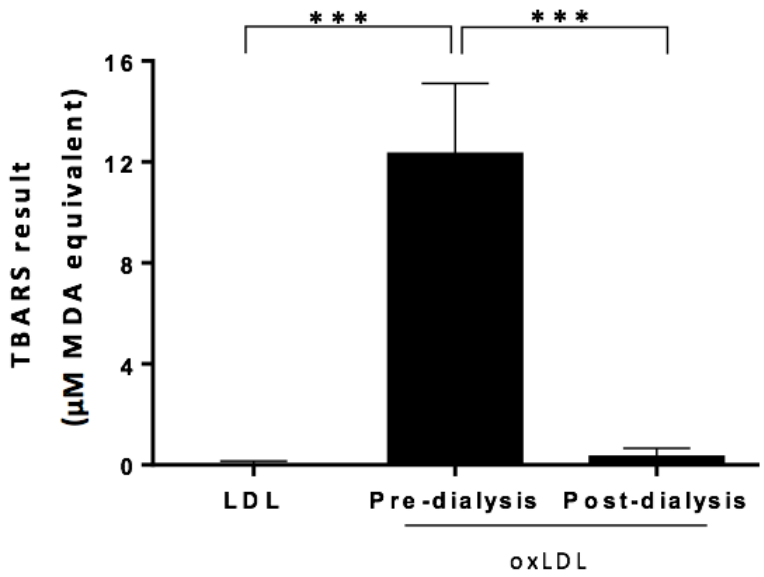


Figure 3-21 The effect of dialysis on the result of TBARS analysis of oxLDL
The TBARS analysis method was performed on oxLDL in order to characterise the extent of oxidation in comparison with native LDL. Mean +/- SD of absorbance values converted to equivalent MDA (µM) quantities per 0.5mg/mL ox-/LDL. Statistics performed with one-way ANOVA with Tukey's post-hoc test ***P<0.001 (n=3).

The oxLDL preparation measured immediately after the oxidation procedure gave a positive TBARS result, indicated by an equivalent average of ~12 µM MDA. This result however was diminished after the oxLDL preparation had been through 24 hours of dialysis. This result indicated that either the oxidation of LDL was lost during dialysis, or that the means by which the TBARS assay detects lipid peroxidation was altered. This result required further investigation in order to clarify whether the oxLDL was still present in the preparation following dialysis and also that it was in the same state as before dialysis. In order to investigate this, mass spectrometry was employed to analyse two aspects of the oxLDL preparations: (i) that the copper sulphate method was successful in causing lipid oxidation as indicated by the pre-dialysis TBARS result and (ii) to analyse any change incurred by the dialysis procedure. The mass spectrometry analysis comparing native LDL with oxLDL is shown in Figure 3-22 and the comparison between the pre-dialysis oxLDL sample and post-dialysis oxLDL sample is shown in Figure 3-23.

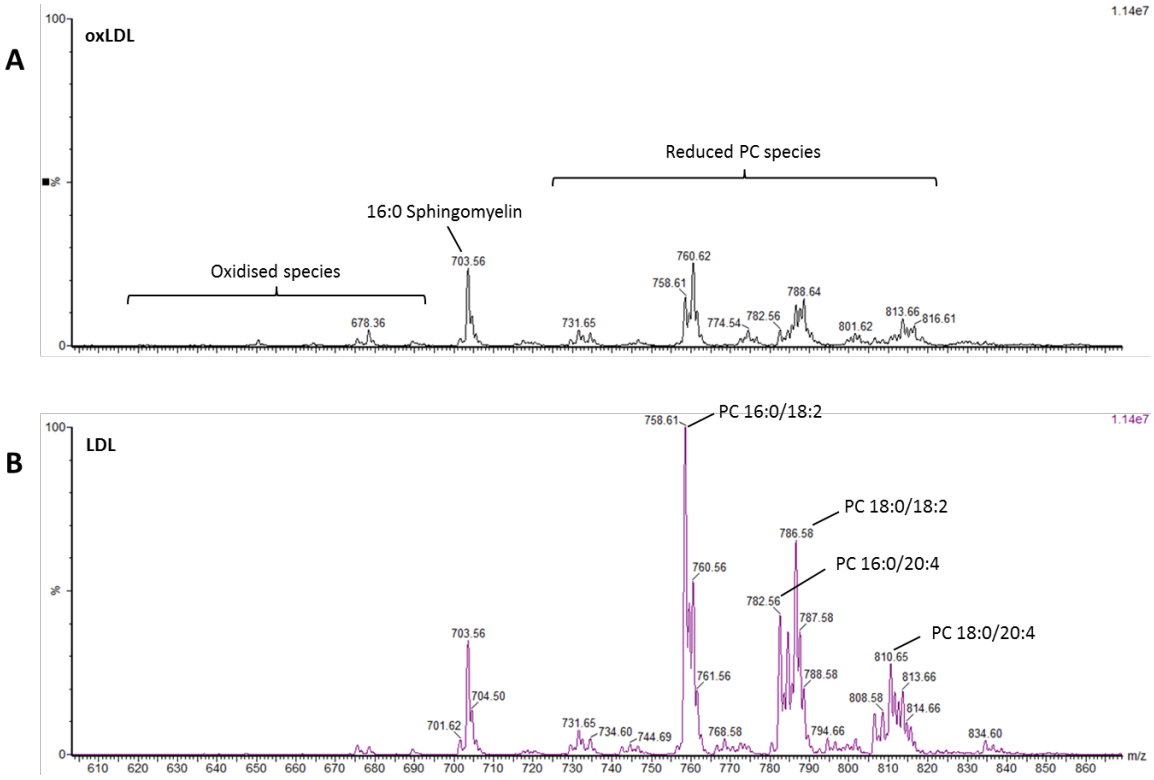


Figure 3-22 The extent of oxidation of LDL analysed by mass spectrometry
The lipid changes occurring as a result of oxidation (A) are compared with the native LDL sample (B) and also to analyse the lipid species within the samples by mass spectroscopy. The X-axis is cropped from 600 m/z (mass-to-charge ratio) upwards, in order to visualise the PC species relevant to the oxidation process. Representative graphs of one oxidation reaction of LDL. Representative of n=1 ox-/LDL sample.

The range of lipid species found in the LDL particle detected by mass spectrometry can be seen in Figure 3-22, part B. The lipid changes caused by the oxidation process can be identified in the oxLDL sample shown in part A. The range of PC species in the LDL particle were noticeably fewer in the oxLDL particle when compared to native LDL lost due to the oxidation of these species. The Y-axes on the two scans are linked as to represent comparable lipid detection in the two samples. The peak at 703 m/z represents 16:0 sphingomyelin as indicated, used as a reference point for the comparison, as it has no double bonds and is not oxidised. The peak representing 16:0 sphingomyelin in the oxLDL sample is comparable to that of the LDL sample, as would be expected. The comparison of the 16:0 sphingomyelin with that of the PC species indicates the amount of loss in PC species due to oxidation. The peak at 813 m/z represents 20:1 sphingomyelin, which has one double bond and can be seen to be partially lost in the

oxLDL sample, as a result of oxidation. The nomenclature of 16:0 and 20:1 refers to the carbon atoms contained within the molecule and the number of double bonds, respectively.

Double bonds in the structure of lipids make them more susceptible to oxidation, making polyunsaturated lipids the most readily oxidised. In the annotations of graph B, the lipids which were oxidised (identified by their absence in the oxLDL graph) included 16:0/18:2 PC (1-palmitoyl-2-linoleoyl-sn-glycero-phosphocholine), 16:0/20:4 PC (1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine), 18:0/18:2 PC (1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine) and 18:0/20:4 PC (1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine). These results indicate that the process was successful in oxidising LDL given the oxidative fragmentation of lipids containing polyunsaturated fatty acids such as linoleic acid and arachidonic acid. The oxidative fragmentation products can be visualised in Figure 3-23.

Not identified in the graphs shown are phospholipids such as phosphoethanolamine (PE), of which there was a very small quantity. A small peak at the m/z value of 885 also not seen on these scans identified phosphatidylinositol (PI), of which there were detectable amounts. The oxidation process decreased this peak height, identifying the oxidation of PI, which would be expected given that it consists of 4 double bonds.

The mass spectrometry analysis therefore identified that the copper sulphate method of oxidation was successful and that the polyunsaturated lipids were the most readily oxidised, confirming the positive TBARS result (pre-dialysis). Having confirmed this, the pre- and post-dialysis samples of oxLDL were analysed using this method in order to clarify the conflicting TBARS results (shown in Figure 3-23).

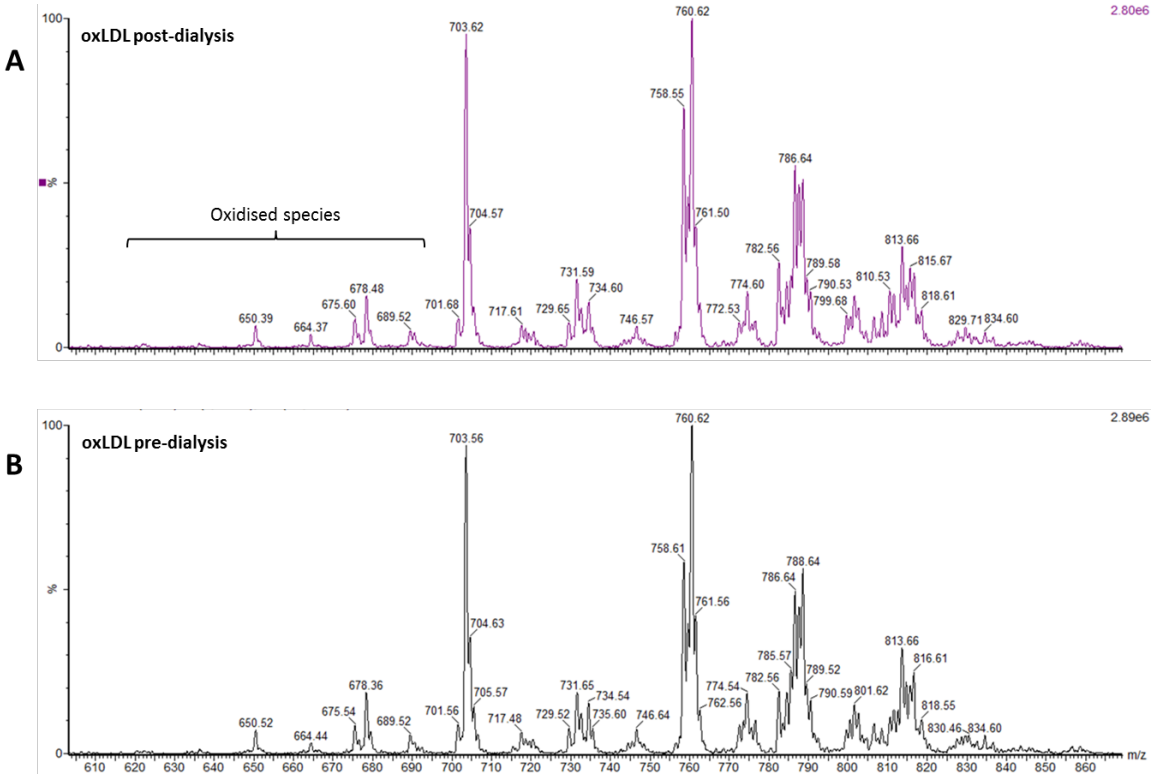


Figure 3-23 The effect of dialysis on oxLDL analysed by mass spectrometry
A sample of oxLDL analysed before the dialysis procedure using mass spectrometry (B) is compared to a sample that had undergone 24 hours of dialysis (A), both from the same LDL oxidation process. Oxidised fragments are identified to the left of the graph. Representative mass spectroscopy data of one oxidation of LDL to compare the effect of routine dialysis.

The previous TBARS results indicated that there was a possibility that lipid peroxidation after 24 hours of dialysis in comparison to an oxLDL sample that had not been through this process was decreased or even lost completely. The mass spectrometry scan shown in Figure 3-23 contradicts these previous TBARS results as it indicates there was no quantitative effect of dialysis on the oxidation or amount of the lipid species found in oxLDL. The lipid species identified in the pre-dialysis oxLDL sample are shown as identical to those in the post-dialysis oxLDL sample. In a comparison of the methods, TBARS is a less-sensitive indicator of overall lipid peroxidation, whereas mass spectrometry is specific and gives more detailed analysis of lipid species. The mass spectrometry data indicates that dialysis had no effect on oxLDL. The treatment of oxLDL in dialysis bags would allow low-molecular weight and water-soluble TBARS to escape through the dialysis membrane which has not often been reported in the oxLDL literature (the only

reference found being Itabe et al., 2003). It was concluded that dialysis was still an appropriate way to remove copper sulphate and EDTA. The small molecular weight TBARS are likely to be lost through the dialysis membrane by way of diffusion, explaining the previous TBARS results. The mass spectrometry data allowed the conclusion that dialysis had no effect on the oxidation state of oxLDL. For future experiments, pre-dialysis aliquots of oxLDL would be used to measure the consistency of oxidation, as shown by TBARS assay in Figure 3-24, below.

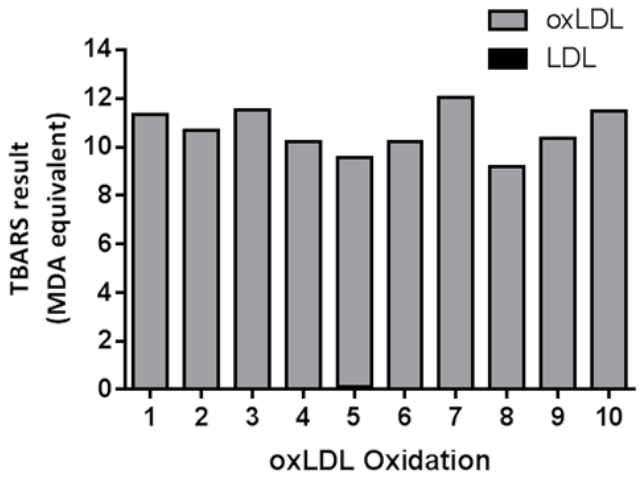


Figure 3-24 TBARS results of LDL oxidation for separate oxidation events of the stock LDL
LDL was oxidised using copper sulphate method and each preparation was analysed using TBARS (pre-dialysis samples). Native LDL (0.5mg/mL) results ranged from 0-0.12 µM MDA, whereas oxLDL (0.5mg/mL) ranged from 9.18-12.06 µM MDA (Mean 10.64 +/- SD 0.95) (n=10, with each experiment performed in triplicate).

The TBARS results for oxLDL (pre-dialysis samples) and the respective native LDL samples are shown for 10 consecutive oxLDL preparations over the course of multiple experiments. The data indicates that LDL shows no evidence of oxidation as indicated by the black bars (not above 0 except marginally on repeat number 5). This was an important finding as it indicated that LDL stored at -20°C did not become oxidised in this environment over time (12-week period). The TBARS for the oxLDL repeats (0.5mg/mL) showed figures in the range of 9.18-12.06µM MDA, indicating a consistency in oxidation. The process of LDL oxidation was therefore confirmed to be consistent and causing appreciable oxidative modification of the lipid species within the LDL particle. The role of

ox-/LDL could therefore be assessed in the tube formation assay and other subsequent experiments.

The effect of oxLDL and native LDL addition to control serum cultures in the angiogenesis assay can be seen in Figure 3-25. From this graph, oxLDL at a concentration of 100µg/mL significantly impaired total capillary length, whereas there was no significant effect seen in lower oxLDL concentrations. For any concentration of LDL tested there was no significant effect on tube formation.

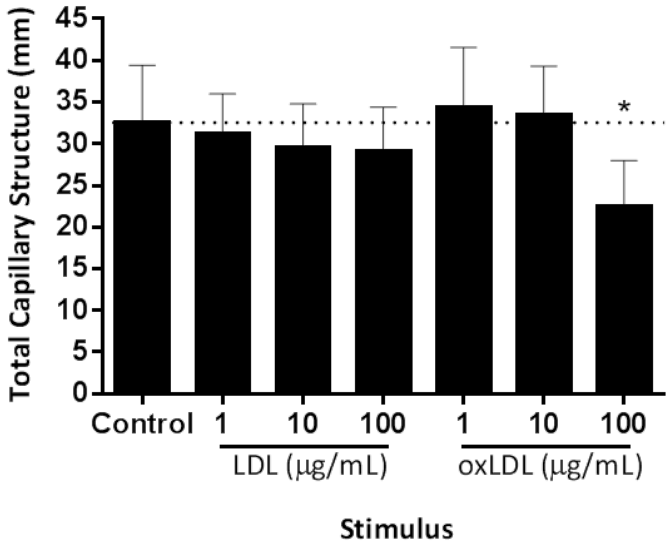


Figure 3-25 The effect of ox-/LDL on tube formation in control serum cultures
A range of LDL and oxLDL concentrations (1, 10 & 100µg/mL) were added to control (unaltered) serum cultures to evaluate the effect on tube formation. Mean +/- SD of total capillary length. Statistics performed using one-way ANOVA with Dunnett's post-hoc test *P<0.05 (n=7) compared to control.

3.3.6 Endothelial cell migration

The tube formation assay was employed to determine the ability of the endothelium to form tube-like structures and act as a model of angiogenesis. The process of vessel formation involves the orchestration of multiple cellular events, including the degradation of the basement membrane, the migration of cells, the rearrangement of cellular architecture to form tubes, proliferation, recruitment of endothelial progenitors and finally stabilisation of the vessel. Although the tube formation assay requires an extent of endothelial migration in order to form tubules on a gel matrix, it predominantly

depicts the multiple cellular functions leading to tubule formation. To determine the role of lipids in individual aspects of angiogenesis, an assay of endothelial migration utilising a scratched monolayer of endothelial cells was developed. HUVEC were seeded into 24-well plates at a seeding density of $1x10^5$ cells/well and allowed to settle for 48hours in HUVEC media (20% human serum). Monolayers were "scratched" to reveal a denuded area of tissue culture plastic surrounded by intact cells as described in the methods section 2.1.2.2. The endothelial cells were incubated in cell culture conditions with and without lipids and imaged by microscopy at the time points indicated in order to visualise cell movement/migration into the area of the scratch. Images representing the migration of HUVECs over time are shown in Figure 3-26.

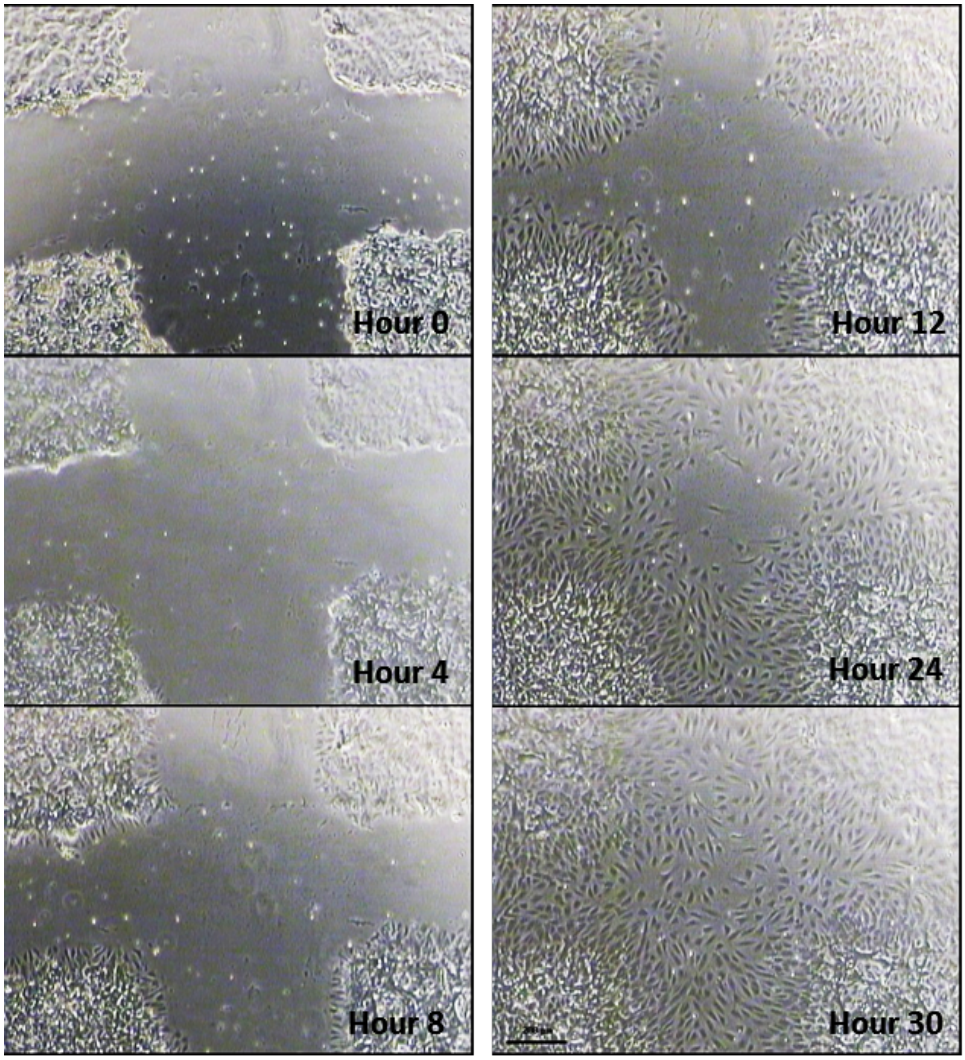


Figure 3-26 Time course images of endothelial migration in the scratch assay
Endothelial cells were scratched using a 200µL pipette tip as described in methods and cultured. Images were captured at 0, 4, 8, 12, 24 and 30 hours following the initial scratch to evaluate the extent of endothelial migration into the denuded area. Images represent control serum at a concentration of 20%, showing almost complete coverage of the denuded area by 30 hours. Reference bar on the 30 hour time point image is 200µM in length, applicable to all.

The images shown in Figure 3-26 represent endothelial migration of HUVEC cultured in control serum at a concentration of 20% within the media, by 30hours. The time course of endothelial migration was performed in order to establish an appropriate end point to the experiment, with 24 or 30 hours following the initial scratch chosen as an appropriate length of time for a culture with 20% serum as shown. To analyse endothelial migration, images were subjected to image analysis software Image J (as shown in Figure 3-27). The software was used to trace the area of the scratch and produce a number related to area

coverage for each time point and treatment condition. All scratches were performed with the same sized pipette tip and so the width of scratch was relatively consistent leading to a scratch area of approximately 1.42 +/- 0.044 mm^2 (n=3 +/- SD) equivalent to 23.64% initial cell coverage and 76.36% denuded area. The centre point of the two scratches was chosen as the analysis point given that it could be more easily identified on repeated time points for imaging as it provides an X and Y coordinate rather than a single linear scratch which might increase errors in calculating area. Although all images were consistent with scratch size, in order to maintain a representative assessment of migration for all images, the spatial coverage left by the scratch at time point 0 hours (start) was designated as 0% endothelial migration. Any area covered could then be presented as a percentage, knowing that complete coverage by the endothelium to the centre point of the image amounted to 100% endothelial migration.

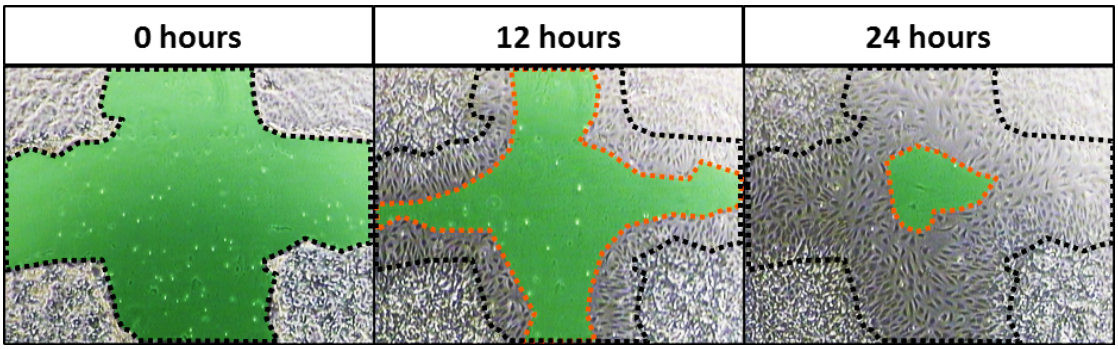


Figure 3-27 Scratch assay analysis
The scratch assay was analysed using Image J and the cell denuded area (green) was measured by drawing around the edges of the scratch (black dotted lines). The software measures the area corresponding to the denuded plastic. The area not covered by cells (green) at time point 0 hours was given as 0%. Full spatial coverage would have been equivalent to 100%, so the area measured at certain time points (orange dotted line area) was divided by the area at time 0 hours (black dotted line area) to calculate the spatial area covered by the cells over time. Representative images shown from experiments with 20% serum.

The analysis of the scratch assay is shown in Figure 3-27, with the area not covered by cells highlighted in green and bordered with a black dotted line. Once the area was calculated using Image J (as indicated in methods), it was converted to a percentage of endothelial coverage equating to endothelial migration. The depletion of lipids from human serum using the silica method has already been shown to significantly reduce the tube length and vascular network formation of endothelial cells. In order to assess the

role of lipids specifically in endothelial cell migration, this scratch assay was employed with control and delipidated human serum cultures across a range of serum concentrations and shown in Figure 3-28.

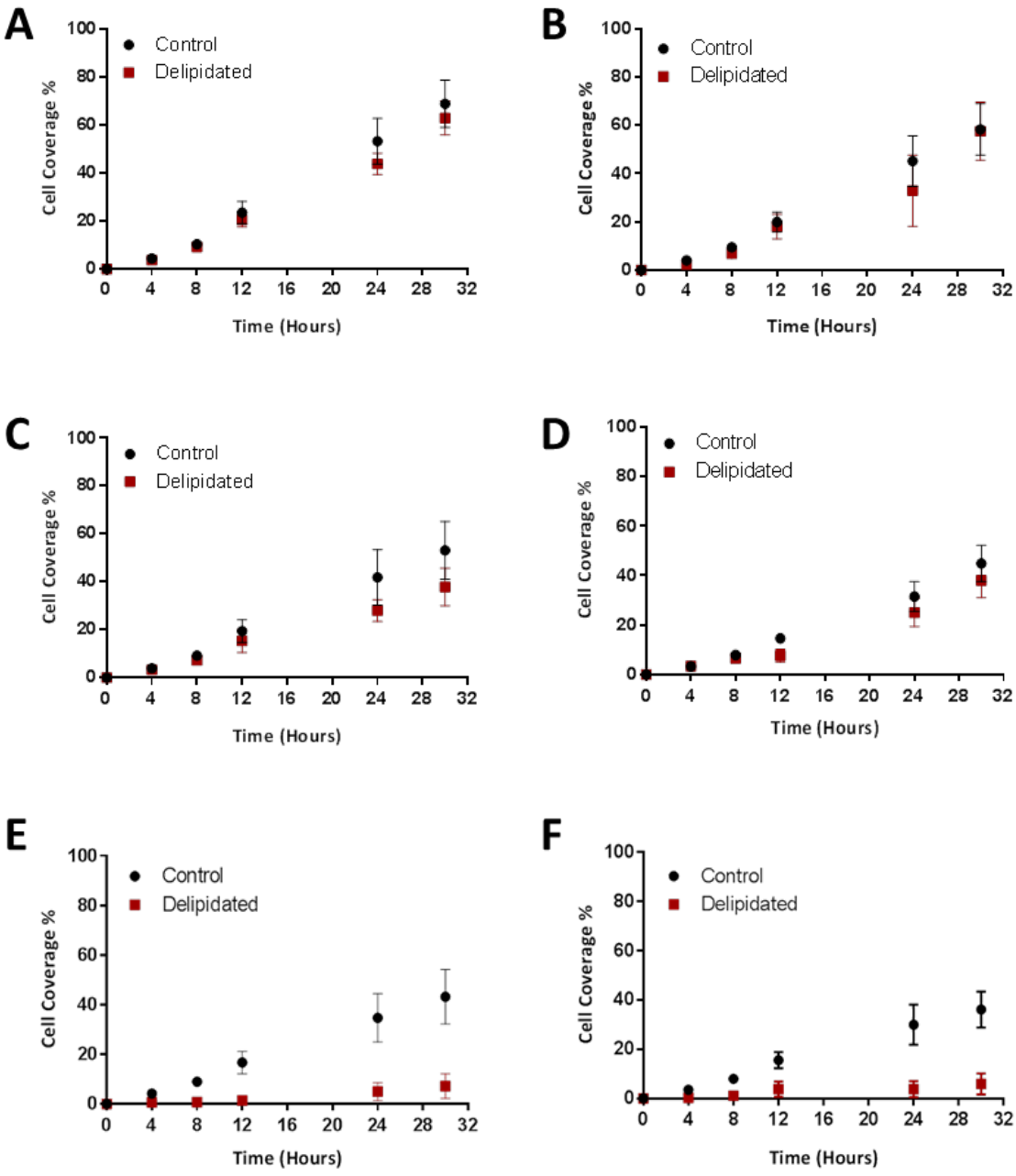


Figure 3-28 Effect of time, serum concentration and lipid content on endothelial migration

Time course graphs show the percentage coverage as a marker of endothelial migration. Graphs shown compare serum concentrations of (A) 20% serum, (B) 10% serum, (C) 5% serum, (D) 2.5% serum, (E) 1.25% serum and (F) 0.625% serum. Mean +/- SD of % area coverage from four individual experiments each with different donor cells and individual lipid depletion (n=4). Statistics performed for 24 and 30 hour time points as represented in Figure 3-29.

The graphs shown in Figure 3-28 represent endothelial migration in diluting concentrations of serum (doubling dilutions from 20% in A to 0.625% in F respectively).

The time course of endothelial migration in control serum can be seen in black (circles) and compared to that of delipidated serum cultures as shown in red (squares). In graphs A, B, C and D the migration of endothelial cells in delipidated cultures is comparable to that in control serum controls. The time course of endothelial migration in lower serum concentrations however, as represented by graphs E and F (1.25% and 0.625% serum respectively), show a reduced migration capability. The 24 and 30 hour time points alone for the range of serum concentrations are represented in Figure 3-29. The statistical analysis of control against delipidated cultures shows a significant difference at the serum concentrations 1.25% and 0.625%.

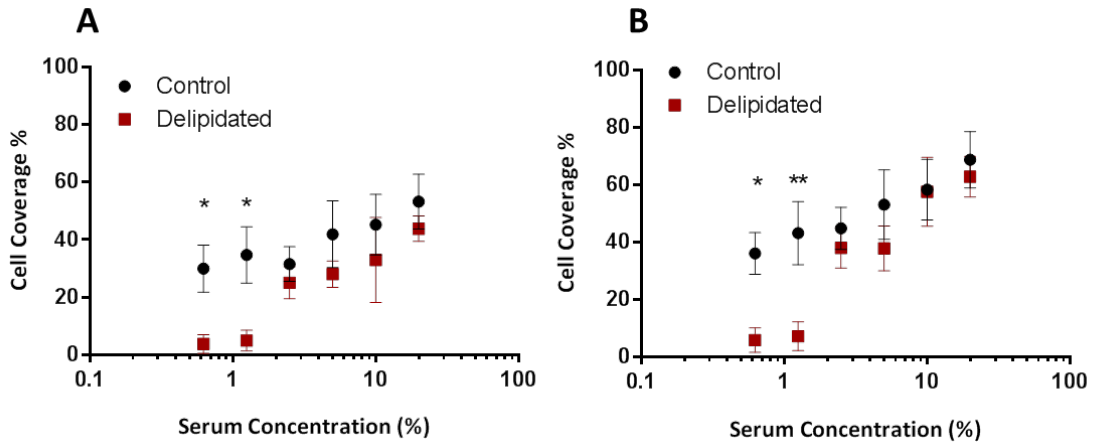


Figure 3-29 Effect of serum on endothelial migration in control and delipidated cultures

The effect of serum concentrations ranging from 0.625% to 20% in control and delipidated cultures on endothelial cell migration are shown at (A) 24 hours and (B) 30 hours. Statistics performed with unpaired T-tests using Holm-Sidak to determine significance. Results shown as mean +/- SD of duplicate wells in four independent experiments. *P<0.05 **P<0.01 (n=4).

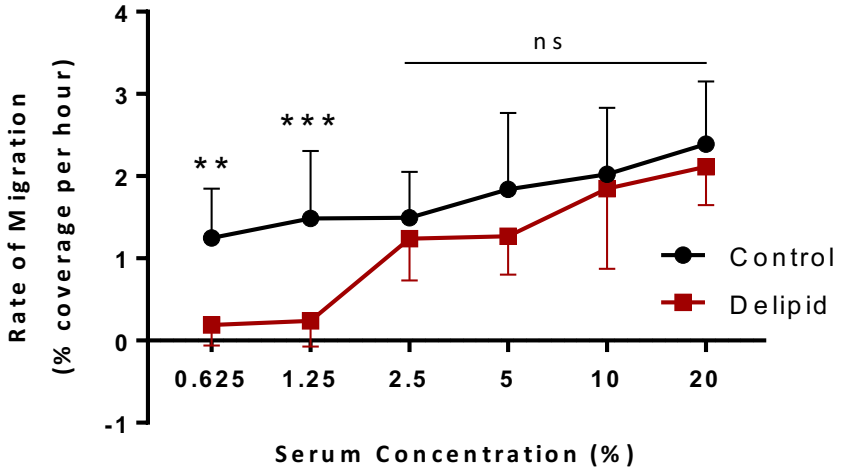


Figure 3-30 Rate of endothelial migration in response to control and delipidated serum
The data for endothelial coverage at every time point was analysed in respect to the rate of migration of the cells. Results shown as mean +/- SD of duplicate wells in four independent experiments. Statistics performed with two-way ANOVA with Sidak's post-hoc test **P<0.01 ***P<0.001 (n=4).

Significant inhibition of endothelial migration in lower serum concentrations at the time points of 24 and 30 hours was previously identified. In order to consider whether there were differences in the rate of migration over time, linear regression analysis was performed on each individual experiment and the mean slope value was obtained. This value represents the percentage coverage of endothelial cells over time, which analyses the data as a whole as opposed to a single time point, as shown in Figure 3-30. Taking into account all of this data, the significant difference between control and delipidated cultures was observed at the lower serum concentrations of 0.625 and 1.25%, as previously found.

The data shown for endothelial migration indicates a role for lipids in this process. Having already shown a diminished angiogenic response in lipid depleted cultures, this data indicates that lipids are required for endothelial migration. Higher concentrations of serum within the media, however, did not show an abrogation of the effect in the delipidated cultures in comparison to control. The images depicting the different migration achieved at 1.25% are shown in Figure 3-31, whereas the similar migration in control and delipidated cultures at 10% serum are shown in Figure 3-32. In the

delipidated cultures at 1.25%, the endothelial migration at the 30 hour time point appears at a similar level to that at 0 hours, whereas the control culture at the same serum concentration have visible migration occurring.

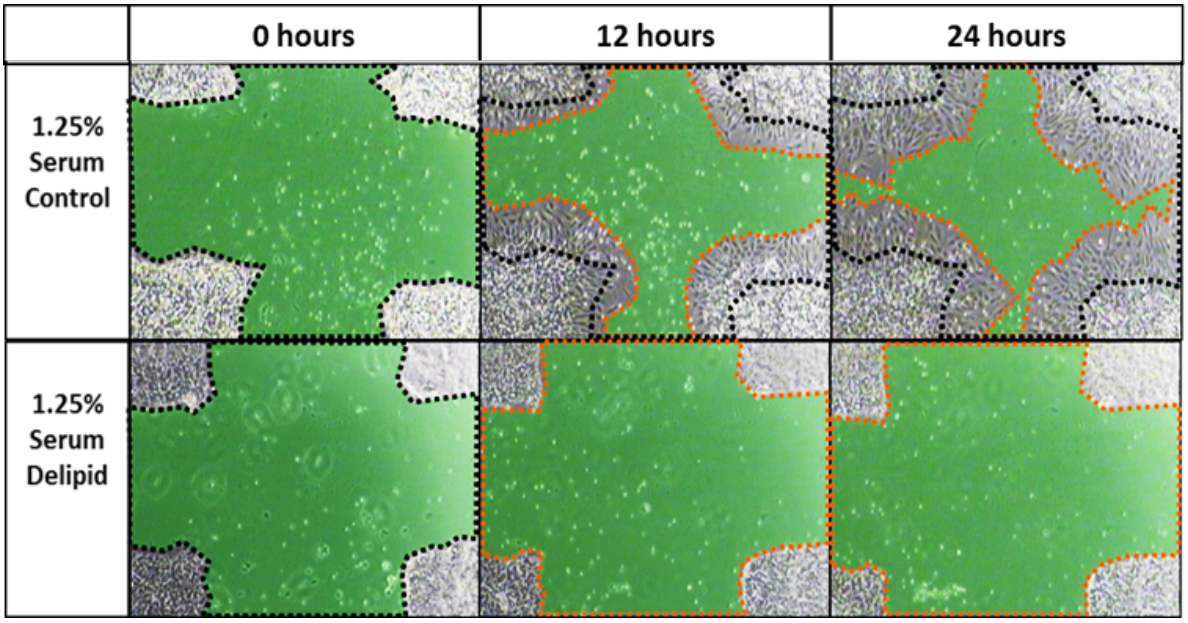


Figure 3-31 The effects of lipid depletion on endothelial migration in 1.25% serum
The inhibition of endothelial migration in delipidated cultures at 1.25% serum as shown in the previous graph (Figure 3-30) is highlighted in these captured images.

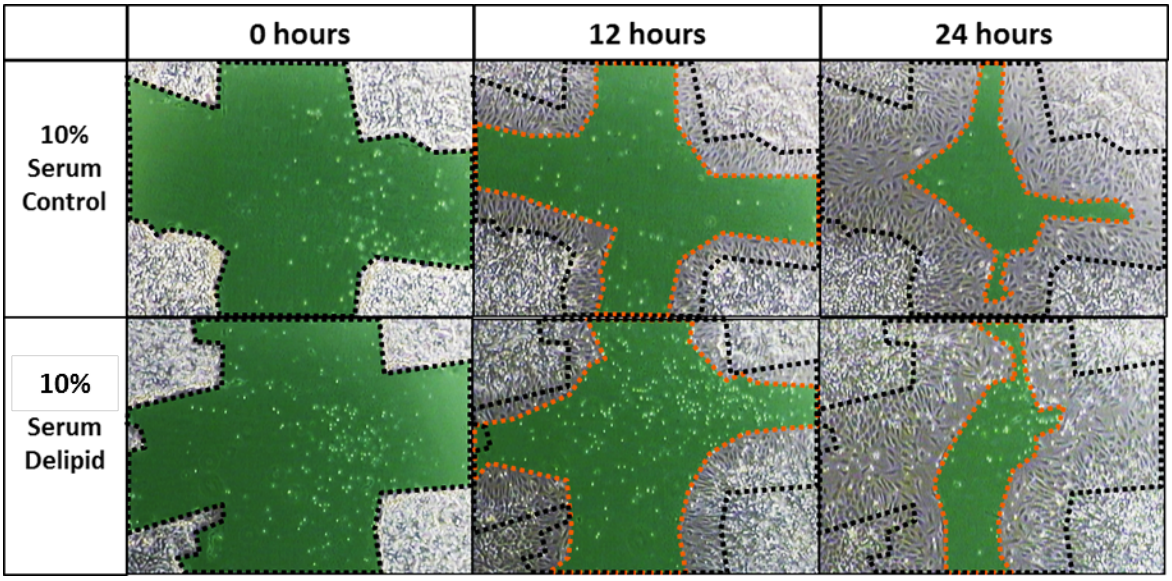


Figure 3-32 The effects of lipid depletion on endothelial migration in 10% serum
The inhibition of endothelial migration in delipidated cultures at 10% serum as shown in the previous graph (Figure 3-30) is highlighted in these captured images.

The research question of interest therefore involved the role of native and oxidised LDL in this process. Previously, the results from the angiogenesis and migration assays showed similar endothelial responses in lipid-depleted serum. In the tube formation assay, high concentrations of oxLDL were inhibitory, with lower concentrations showing no significant effect. These effects were paralleled in the endothelial migration assay, as shown in Figure 3-33, with LDL again having no significant effect. The lower concentration of oxLDL (1µg/mL) was above the control migration but was not statistically significant (P=0.22). From this graph, it can be seen that there is a concentration-dependent effect of oxLDL on endothelial migration. The potential stimulatory effect of oxLDL will be discussed in the next chapter.

The inhibitory effect of high concentrations of oxLDL (100µg/mL) on migration was observed both in control serum cultures (Figure 3-33) and in lipid-depleted serum (Figure 3-34), with these experiments performed in 20% human serum. Native LDL had no significant effect on migration even after being added to lipid-depleted serum, suggesting that LDL plays no role in the migratory or angiogenic response of endothelial cells. The stimulatory effect of oxLDL (1µg/mL) appeared to be larger in control serum cultures as opposed to when added to lipid-depleted serum (qualitative assessment of the graphical data represented, no statistical significance).

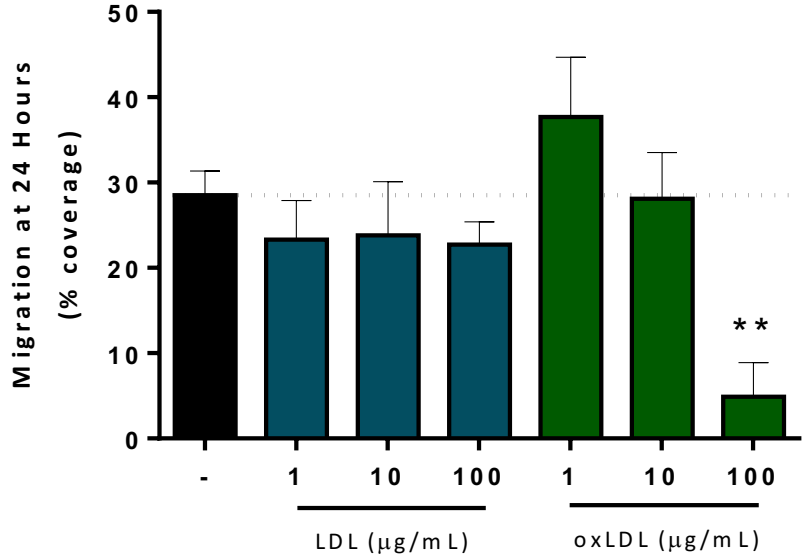


Figure 3-33 The effect of ox-/LDL on endothelial migration in control serum cultures

Ox-/LDL concentrations (1, 10 & 100µg/mL) were added to control serum cultures to evaluate the effect on migration. Mean +/- SEM. Dotted line represents control value for comparison. Statistics performed using one-way ANOVA with Dunnett's post-hoc test **P<0.01 (n=8) compared to control.

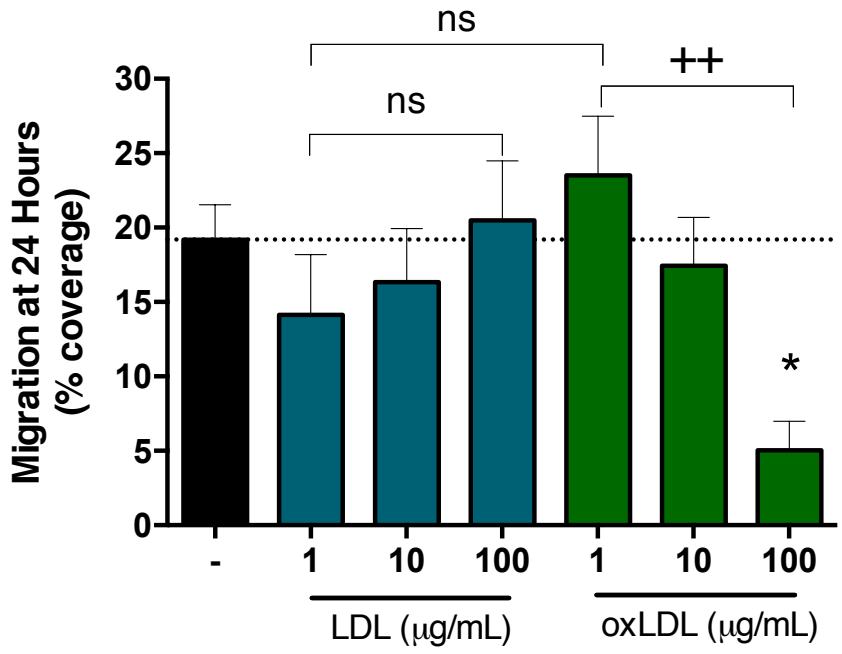


Figure 3-34 The effect of ox-/LDL on endothelial migration in delipidated serum Serum was delipidated (black bar) and ox-/LDL was added (1, 10 or 100μg/mL) to this serum in order to analyse the effects on migration. Dotted line represents control migration. Mean +/- SEM. Statistics performed using one-way ANOVA with Dunnett's post-hoc test *P<0.05 (n=5) compared to control or one-way ANOVA with Sidak's post-hoc test (indicated comparisons) ++P<0.01.

The results above highlighted a disparity between the role of oxLDL at 10μg/mL and 100μg/mL, with the prior concentration showing paralleled migration with control, whereas the latter concentration was significantly inhibitory. Given the concentration-dependent responses seen in this data, it was queried whether a concentration of oxLDL between these upper two concentrations used may show a different or stimulatory effect. The migration assay was therefore repeated using ox-/LDL concentrations of 10, 40, 70 and 100μg/mL. As seen from the results in Figure 3-35, the higher the concentration of ox-/LDL, the more inhibition was seen in terms of migration. This experiment was performed in lipid-depleted cultures and again, no restoration of migration was seen with any concentration of LDL.

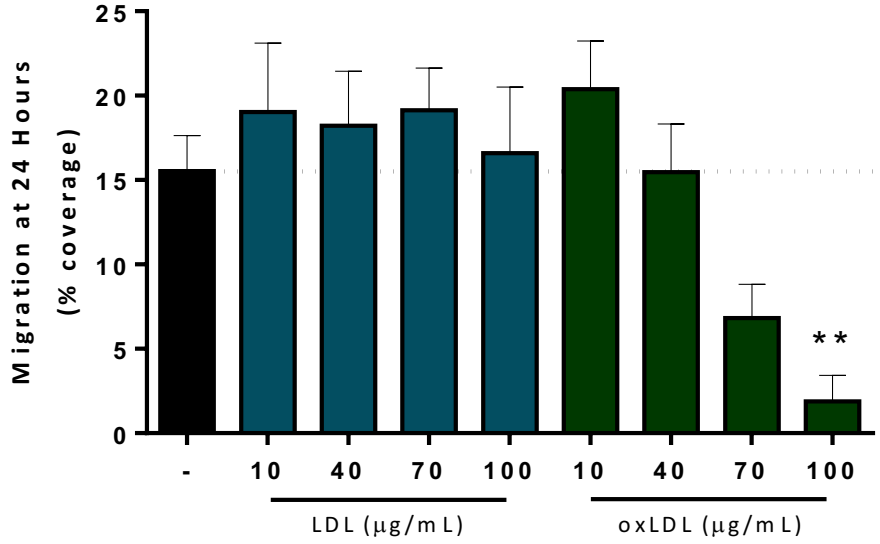


Figure 3-35 The effect of higher concentrations of ox-/LDL on endothelial migration in delipidated serum cultures
Serum was delipidated (black bar) and ox-/LDL was added (10, 40, 70 or 100µg/mL) to this serum in order to analyse the effects on migration. Dotted line represents control migration. Experiments performed in 20% human serum. Mean +/- SEM. Statistics performed using one-way ANOVA with Dunnett's post-hoc test **P<0.01 (n=6) compared to control.

In addition to these results, it should be noted that at higher concentrations of oxLDL (specifically 100µg/mL), there appeared to be a fatty streak or deposit appearing in the areas originally scratched with the pipette tip. The streaks did not appear in other regions of the wells and so may have been binding to some element of the scraped plastic or uncovered gelatin or other matrix proteins deposited by the endothelial cells. An image of this 'streak' formation is shown in Figure 3-36, part A. In order to characterise whether this was a lipid deposit specific to the scratch area, an Oil Red O stain (a lipid stain which highlights neutral triglycerides, lipids and lipoproteins) was performed following 24 hours of incubation with oxLDL after the migration assay had been completed. There was a clearly visible red staining pattern within the scratch area of wells exposed to 100µg/mL oxLDL, as observed in Figure 3-36 part B, indicating deposition of lipid onto the denuded area. Interestingly, this staining pattern was not observed in LDL treated cultures at the same concentration, nor at lower oxLDL concentrations although this may relate to the sensitivity of the assay. One potential hypothesis for this staining was that oxLDL may be

having an influence on cellular migration through changes in the adhesive nature of the scratched area, rather than through a specific effect on oxLDL signalling. In order to assess the potential anti-adhesive qualities of lipid deposition, a separate experiment was performed in which wells of the 24-well plates were pre-coated with LDL or oxLDL at the range of concentrations previously used, prior to seeding endothelial cells. Wells were coated with lipid for 24hours prior to seeding (without gelatin) in order to characterise whether these lipids created an anti-adhesive surface for the endothelial cells. This experiment showed that cells were in fact able to adhere to the 24-well plate in all conditions, suggesting that oxLDL was not simply making the environment too 'greasy' for migration (shown in appendix; Figure 9-2). In follow-up to this, endothelial cells were seeded at sub-confluent seeding density (40% confluence) in solution with LDL or oxLDL to analyse whether the interaction between these lipids and the cells had any significant impact on their ability to attach to the 24-well plate at initial seeding (Figure 3-37).

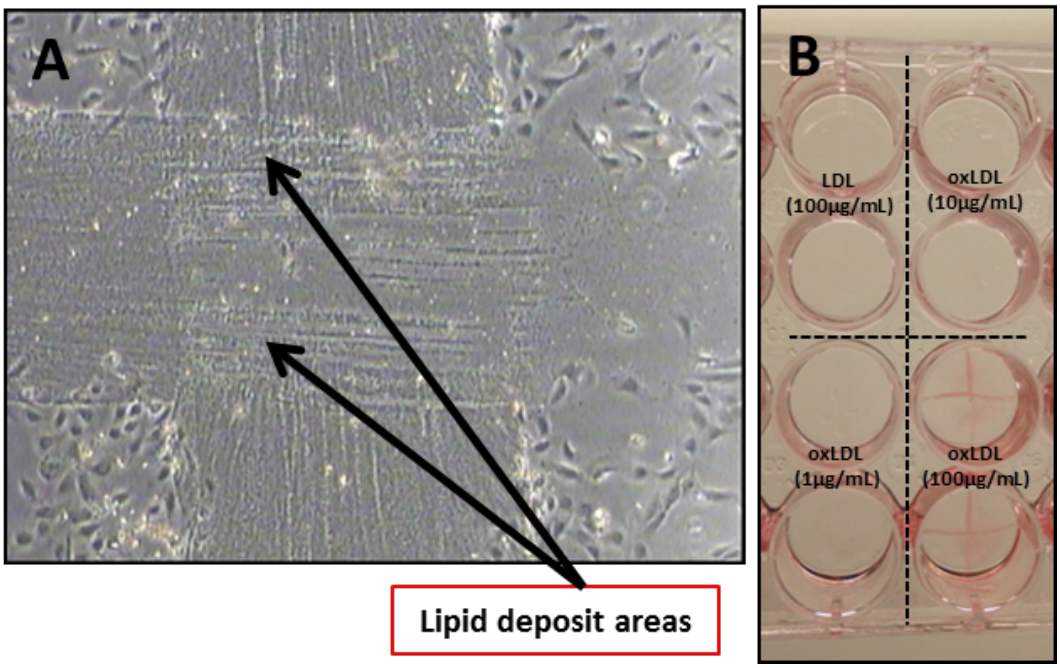


Figure 3-36 High concentrations of oxLDL deposits lipid on scratched area
Images taken after scratch assay at 24hours, showing lipid deposits at high concentrations of oxLDL (100µg/mL) [**A**]. Oil Red O lipid stain was employed to show lipid deposits were restricted to the sites of scratches and not on cells [**B**].

The endothelial cells seeded in solution with LDL and oxLDL at a range of concentrations were all able to adhere to the 24-well plate (as shown in Figure 3-37). This suggested that the high concentration of oxLDL was not simply having an anti-adhesive effect on the endothelial cells. Interestingly, it was seen that following 24hours of being seeded with 100µg/mL of oxLDL, endothelial cells exhibited a visible change in morphology, which had not been observed in confluent cultures or following the scratch protocol. This change in morphology depicted more spindle-shaped cells in 100µg/mL oxLDL-stimulated cultures compared to all other treatment conditions (Figure 3-37, part C).

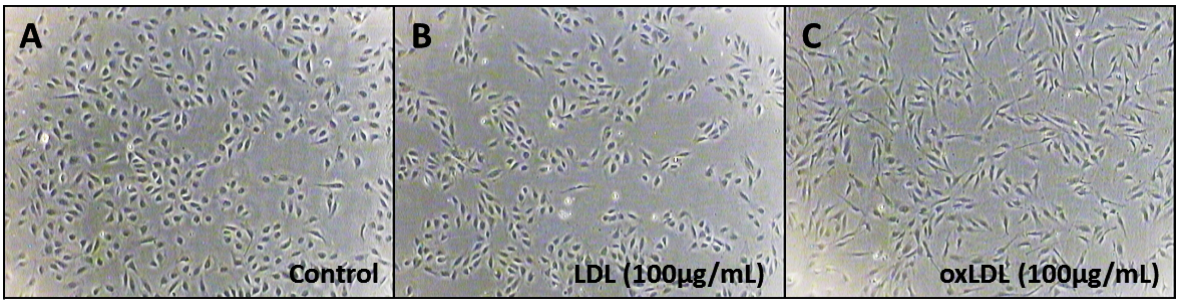


Figure 3-37 HUVEC morphology following seeding in solution with ox-/LDL
Cell morphology of endothelial cells appeared more spindle shaped in high oxLDL-seeded cultures (**C**) in comparison to control and all other conditions.

Further to the observation of changes in cell morphology, Image J analysis software was employed in order to attempt to quantify the changes witnessed. Within a field of view, 100 cells were traced using the polygon tool in Image J and quantified for shape descriptors to analyse and quantify the change in morphology (Figure 3-38). Most notably, the circularity of endothelial cells was decreased in culture with oxLDL at 100µg/mL. Circularity of 1 indicated a perfect circle, but as this figure approaches 0, it indicates an increasingly elongated polygon. Circularity is calculated as 4pi (area/perimeter^2). The perimeter of the cells also increased as visualised in the image.

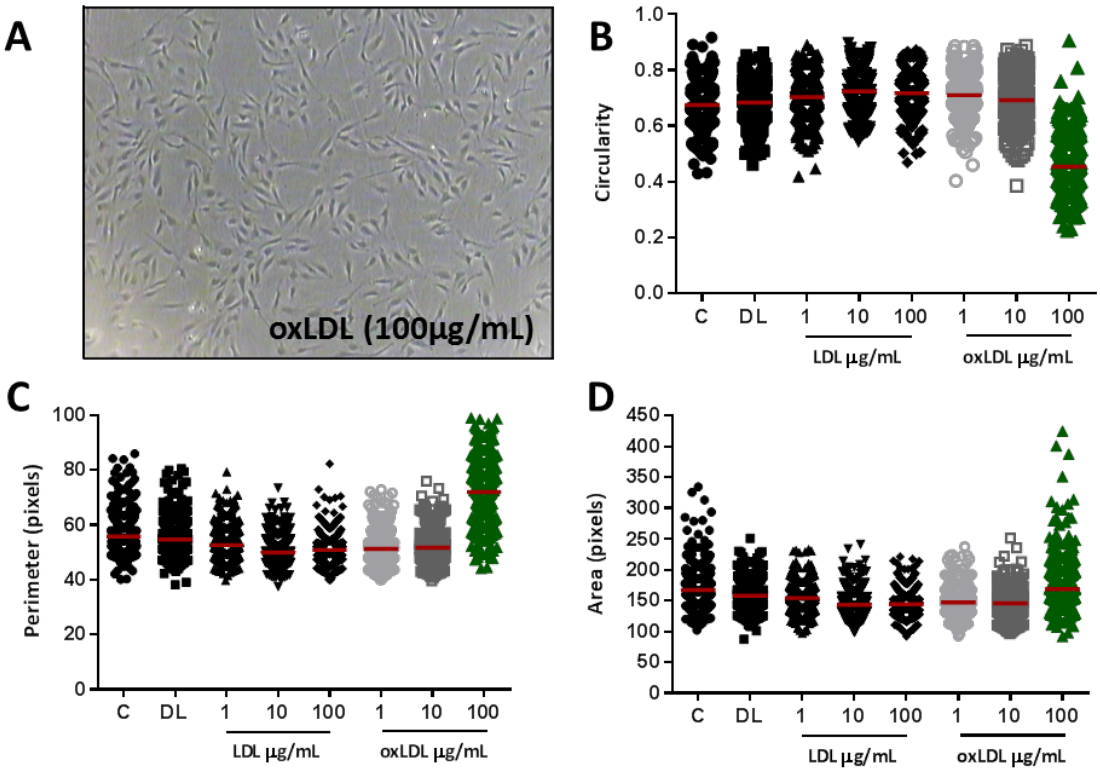


Figure 3-38 Image J analysis of HUVEC morphology
Cells were traced using Image J tools and quantified for markers of shape descriptors, circularity (B), perimeter (C) and area (D). 100 cells were traced per individual experiment (n=3). Data shown are individual cells from experimental analysis. With data of each cell averaged for n=3, statistical analysis showed no statistical significance using one-way ANOVA with Dunnett's post hoc test for perimeter and area measurements. For circularity data, non-parametric test was performed based on the D'Agostino&Pearson normality test, with Kruskal-Wallis test and Dunn's post-hoc test comparing all columns to delipidated culture (all columns P>0.99 except 100µg/mL oxLDL which was P=0.58).

3.4 Discussion

Angiogenesis is an integral part of normal physiology and various pathological conditions and is a widely researched area. The tube formation assay is one of the most specific techniques that can be employed *in vitro* to assess the ability of endothelial cells to form three-dimensional structures (Auerbach et al., 2003). Combined with the migration assay employed, two of the most important endothelial responses in the angiogenic process have been investigated. Despite the tube formation assay being widely employed, a review of the literature revealed no universal method of quantification. Also required for the development of the assay were the time point, serum concentration and cell seeding densities which could adequately evaluate endothelial structure formation. The gel matrix used was Geltrex™ which, despite being low in growth factors, still maintains a pro-angiogenic signal. This allowed spontaneous angiogenesis to occur in control serum cultures, allowing the parameters of the assay to permit both pro-angiogenic and anti-angiogenic factors to be investigated. Following the conclusion of an eight hour time point, the quantification method chosen was Angioquant, which was specifically designed to produce robust quantification of *in vitro* angiogenesis (Niemisto et al., 2005) and gave reproducible results.

In this investigation, two major compounds were analysed for their ability to promote angiogenesis, the bioactive lipid PAF and the angiogenic protein VEGF. PAF promoted the formation of vascular networks in delipidated cultures whereas the effect of VEGF did not reach significance in delipidated serum. PAF, however, had no significant effect in cultures which contained complete serum. There is evidence to suggest that PAF induces angiogenesis *in vitro* (Russo et al., 2003) and that PAF-R antagonists inhibit such tube formation (Wu et al., 2011). *In vivo*, there is evidence to show that PAF contributes to psoriasis like skin inflammation in a mouse model (Singh et al., 2011), although other evidence in PAF-R knockout mice unexpectedly showed tumour-induced increases in angiogenesis (Ferreira et al., 2007). In this thesis PAF did not show enhanced tube formation in the presence of control serum cultures which contradicts previous data.

This may be in part due to the spontaneous angiogenesis seen in normal culture conditions above which PAF may not be able to further drive angiogenesis. It may also be

postulated that the normal angiogenic response is due to PAF in the serum and therefore further exogenous addition had no effect. Alternatively, PAF may not be required when other pro-angiogenic signals are available in normal serum, as shown by the result that PAF-R antagonist WEB2086 did not inhibit tube formation in control serum cultures. Using the single end point assay at 8 hours there was no effect of PAF observed and it may be that PAF accelerates angiogenesis which would be seen if earlier time points were analysed. This is especially important with pro-angiogenic factors against a pro-angiogenic background which might otherwise be swamped at eight hours as there is a limit to the extent of angiogenesis that can occur in this assay.

VEGF is a factor which is reported to be important in promoting the angiogenic response, confirmed also in murine models (Sinnathamby et al., 2015). In this thesis however, VEGF was unable to restore tube formation in delipidated cultures. This result suggests that lipids may be a crucial factor in angiogenesis driven by other non-lipid, pro-angiogenic factors given that a potent protein factor was unable to restore angiogenesis. VEGF has its effects through surface expressed receptors and it may be speculated that the removal of lipids might affect this signalling. Potentially, lipid depletion within the membrane may reduce receptor expression because of changes in receptor endocytosis. Also, functional expression of VEGF receptors is associated with co-localisation to other receptors such as neuropilin-1 and β-integrins in lipid rafts (Gelfand et al., 2014). It is possible that these interactions may be deranged with the removal of serum lipids and the potential effects that this may have on membrane dynamics. It would be interesting to speculate on other lipid factors which may regulate VEGF signalling, especially as VEGF is thought to be highly important in angiogenesis and a target of pharmacological intervention in diseases such as age-related macular degeneration (Campa et al., 2011) and diabetic retinopathy (Ip et al., 2012).

One of the significant findings in the tube formation assay was the significantly impaired ability of HUVEC to undergo angiogenesis in the absence of serum lipids. Having thoroughly analysed the methods of delipidation, the removal of lipids was shown to be extensive with minimal removal of proteins with the silica method, which confirmed the conclusion of a lipid contribution to angiogenesis. These results have potentially important implications for inhibiting angiogenesis by way of energy restriction. Although

this has not been previously shown directly *in vitro*, the concept of energy restriction on the angiogenic process has been explored in a number of scenarios, particularly in animal models. For example, dietary restriction has been shown to suppress angiogenesis in brain tumour models of mice (Mukherjee et al., 2002) and humans (Mukherjee et al., 2004). However, contradictory evidence in primates suggests that caloric restriction induces factors which activate the angiogenic pathway (Csiszar et al., 2013), in addition to similar pro-angiogenic evidence in rats (Csiszar et al., 2014). In psoriasis patients, energy restriction by way of low calorie diets, high in omega-3 fatty acids has been shown to improve the clinical outcomes of the disease (Guida et al., 2014). Endothelial cells however are highly glycolytic and derive a large proportion of their energy from glucose metabolism (Davidson et al., 2007). The energy cost to the cells of the removal of serum lipids may therefore lie in other areas, rather than direct availability of lipids for energy such as the incorporation of lipids into membrane structures being impaired within a reduced serum lipid environment. In the methods section it is indicated that glutamine was added to cell culture medium providing a further source of energy to support cell growth and function. In order to clarify this finding, further experiments in the absence of glutamine, glucose and lipids may provide further insight into energy depletion and endothelial function.

The lack of tube formation in lipid-depleted cultures was potentially due to energy restriction and in order to address this theory, essential fatty acids were added to delipidated serum. Indeed, fatty acid deficiency in humans is known to be important in the pathology of scaly dermatitis and alopecia (Siguel, 1998). Both individually and in combination, omega-3 fatty acid linolenic acid and omega-6 fatty acid linoleic acid were assessed. The restoration of lipid-depleted serum with these fatty acids had no significant effect on tube formation, concluding that the removal of these lipids was not directly responsible for the lack of tube formation seen. Despite this result, the tube formation assay employed indicates that serum lipids as a whole in the immediate environment are required for adequate tube formation. The cellular interaction between endothelial cells may therefore require exogenous lipids in order to adequately co-ordinate the production of tubules. An integral role for serum lipids in angiogenesis was backed up by the data in the migration assay, indicating that endothelial cells were significantly

inhibited in their ability to migrate in lipid-depleted serum (particularly when lower concentrations of serum were used).

Having established the effects of serum lipid removal on the angiogenic response, oxidised and native LDL were added to control serum and analysed for their angiogenic potential. It has previously been identified using an *in vitro* Matrigel model that low concentrations of oxLDL (<10µg/mL) enhance tube formation of human coronary artery endothelial cells, whereas higher concentrations showed opposing effects (Dandapat et al., 2007). Their research was performed using extensively oxidised LDL (5µM CuSO4 for 24 hours), similar to that used in this research (10µM CuSO4 for 18 hours) but at a different time point (24 hours compared to 8 hours). This data showed that oxLDL at concentrations of 1µg/mL and 10µg/mL at 8 hours had no significant effect on tube formation, although both averages showed a tendency to be above control. The concentration of 100µg/mL of oxLDL however showed significant inhibition of endothelial tube formation, consistent with other data (Dandapat et al., 2007). Higher concentrations of oxLDL have also been shown to impair tube formation by endothelial progenitor cells (Ma et al., 2006). This result was in comparison to native LDL at 100µg/mL which showed no significant effect on tube formation, indicating that the oxidative modification of LDL is the likely driving factor that causes the altered endothelial response.

The observation of minimal oxLDL effects at 10µg/mL and significant inhibitory effects at 100µg/mL led to the interest in effects between these two concentrations. The concentration range of 10, 40, 70 and 100µg/mL was therefore analysed in terms of endothelial migration. It was seen that above 10µg/mL oxLDL, there was a concentration dependent inhibition of endothelial migration. In addition to this, oxLDL at the highest concentration of 100µg/mL showed fatty streaks in the scratch area, which was not seen with cultures containing 100µg/mL LDL. This observation led to the hypothesis that high concentrations of oxLDL may be having a physical non-specific effect in reducing the adhesive ability of endothelial cells during migration into the scratch area, as opposed to an active blockade via specific signalling effects on endothelial cells. This hypothesis was subsequently tested and judged unlikely given that endothelial cells were able to adhere

to the 24-well plates both when seeded with oxLDL and following pre-incubation of the 24-well plate with oxLDL.

Coincidentally from this series of investigations, it was discovered that when seeding endothelial cells at sub-confluent densities, ECs in the presence of 100µg/mL oxLDL showed altered morphology. These observations were not visualised in other experiments in which endothelial cells were initially confluent before addition of oxLDL, in addition to normal morphology observed in ECs seeded in 100µg/mL native LDL. OxLDL has been reported to be cytotoxic to endothelial cells at higher concentrations and has been reported to change morphology, although this was showing defenestration of human liver sinusoidal endothelial cells (HLSECs) after 24 hour culture with 100µg/mL oxLDL (Zhang et al., 2014). It is important to note that endothelial cells incubated with high concentrations of oxLDL for 24 hours did not exhibit any observable cell death either by increased detachment or measured by flow cytometry (discussed in chapter 5), yet their change in morphology may be an indicator of some form of stress and warrants further investigation.

The change in morphology of endothelial cells in response to high oxLDL concentrations may be important in atherosclerotic regions. It has been shown previously that oxLDL strongly enhances the ability of endothelial cells to realign in the direction of flow under shear stress conditions, by increasing endothelial stiffness and facilitating the formation of F-actin stress fibres (Kowalsky et al., 2008). Other groups have reported stress responses from endothelial cells, including the creation of holes in a confluent monolayer (Stroka et al., 2012), the expression of heat shock proteins (Zhu et al., 1996), disruption of caveolae (lipid rafts) within the membrane (Smart et al., 1994; Levitan & Shentu, 2011) and other evidence showing that oxLDL causes EC elongation, stress fibre formation, membrane ruffling and promotes pinocytosis (Chow et al., 1998). In this investigation, high concentrations of oxLDL had no obvious effect on cellular morphology in confluent HUVEC and no holes were routinely observed. The stress response of endothelial cells may be important in psoriatic pathology potentially affecting newly formed vessel stability and maintenance. Further work may need to be performed in order to address the stress response of HUVEC to these higher concentrations of oxLDL.

Overall, this chapter has shown the development of assays to study endothelial responses to lipids, both in terms of migration and angiogenesis, in addition to developing robust techniques to oxidise and remove certain lipids. The results have indicated an important role for serum lipids in vascular organisation, as their removal inhibited the two major responses investigated and has gone some way in detailing the specific lipids involved in these endothelial responses. High concentrations of oxidised lipids have been shown to inhibit these responses and indicate the potentially deleterious effects that lipid oxidation can have on ECs. In the following chapter, the promotion of endothelial responses by lower (and more physiological) concentrations of oxLDL will be examined and the mechanism by which endothelial cells may respond to oxLDL will be investigated.

4 Chapter 4: oxLDL-induced endothelial migration: mechanistic perspectives

4.1 Introduction

Results in the previous chapter showed that higher concentrations of oxLDL have a negative effect on endothelial cell migration and angiogenesis. The focus of this chapter will be the effect of lower concentrations of oxLDL promoting endothelial migration. Dandapat et al. (2007) have previously shown in a Matrigel model of angiogenesis that concentrations of 0.1, 1, 2 and 5µg/mL oxLDL increased endothelial tube formation. After this optimal 5µg/mL concentration, the investigation reported a decline back down towards control levels of angiogenesis at 10, 20 and 40µg/mL. An anti-LOX-1 antibody also blocked oxLDL-induce capillary tube formation, concluding that oxLDL induced endothelial responses were LOX-1 dependent. However, their investigation used human coronary artery endothelial cells (HCAECs) and so the role of LOX-1 in HUVEC responses will be studied in this chapter. In another investigation with HCAEC, oxLDL was shown to induce migration and angiogenesis, again at a concentration of 5µg/mL, reported to increase nitric oxide (NO) synthesis via the PI3k/Akt/eNOS pathway (Yu et al., 2011). In light of these results, the effect of 5µg/mL oxLDL stimulation will be assessed in HUVEC, in addition to an exploration of the mechanism(s) by which oxLDL may induce endothelial responses.

More recently, Camare *et al*. (2015) indicated that low concentrations of oxLDL were angiogenic whereas high concentrations were cytotoxic in the immortalised endothelial cell line HMEC-1 cells, with the pro-angiogenic effect of oxLDL being inhibited by an anti-S1P monoclonal antibody. They concluded that S1P plays a role in oxLDL induced angiogenesis both *in vitro* and *in vivo* using a Matrigel® plug model in mice. The role of the bioactive lipid S1P in oxLDL-induced endothelial responses is an emerging field and will be analysed in this chapter. Where Camare *et al*. used an anti-S1P mAb to neutralise extracellular S1P, this investigation will analyse the effects of specific inhibitors of sphingosine kinase (which produces S1P intracellularly) and antagonists of S1P receptors 1 and 2 ($S1P_1$/$S1P_2$).

In addition to the LOX-1 receptor, CD36 has also been implicated in oxLDL responses in a variety of cell types including vascular smooth muscle cells (Wei et al., 2010), epithelial cells (Khaidakov et al., 2012) and endothelial cells (Camare et al., 2015). Furthermore, Rios et al. (2012) suggested that PAF-R and CD36 co-stimulation is required for oxLDL induced macrophage activation. The role of these three main receptors (LOX-1, CD36 and PAF-R) implicated in oxLDL-induced cellular responses will be characterised in this chapter relative to HUVEC responses. Expression of receptors will be analysed using flow cytometry and inhibitors of receptors and other inhibitors (i.e. of sphingosine kinase) will be analysed in functional assays of endothelial cell migration.

4.2 Methods

Endothelial cell migration was analysed as described in the methods chapter, with no alterations to the initial protocol. Endothelial cells were scratched as described and analysed for migration at time points of 24 and 48 hours. Compounds of interest, both stimulators and inhibitors of migration, were added at time point 0 as there was no pre-incubation time periods with any compound. Results of endothelial migration are expressed either as percentage migration at a final time point or as a factor relative to control migration.

Cellular surface markers and receptor expression were explored in this chapter, both on endothelial cells and on immune cell populations, using flow cytometry. The gating strategies for the different cell populations are outlined in Figure 4-1 (parts A and B), in addition to ensuring that all cells analysed were viable using a cell viability stain (part C).

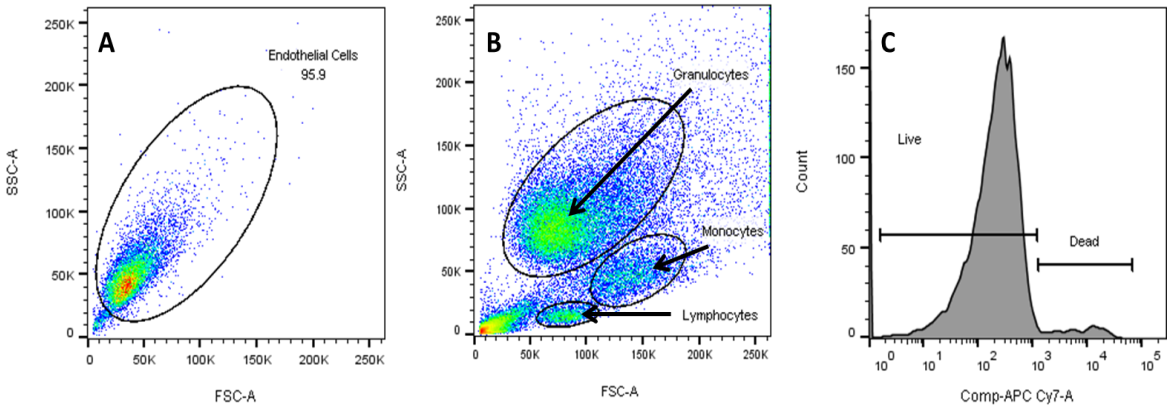


Figure 4-1 Gating strategy for flow cytometry investigation of HUVEC (A) and immune cell populations (B) with live/dead cell analysis (C)
Cells were gated based on their forward/side scatter in order to eliminate doublet cells and debris from the analysis. Viable cells would exclude the viability stain from entering the cell and so lower fluorescence in C indicated live cells.

Before analysis by flow cytometry, HUVEC were removed from the 24-well plates in which they were seeded using a dissociation reagent. For the standard protocol in this thesis, trypsin EDTA was used to effectively dissociate the cells. However, in the investigations within this chapter it was noticed that $S1P_1$ expression was diminished when using trypsin EDTA and so other methods of dissociation were sought. Trypsin EDTA was compared to a relatively similar compound (often substituted for trypsin) in

TrypLE™ Express (ThermoFisher) and it was found that there was no difference in receptor expression following the removal of cells with each of these methods respectively. A further agent used for cell dissociation is Accutase®, which was also tested in this investigation. The use of Accutase® proved to be more effective in dissociating endothelial cells from the 24-well plate and maintaining $S1P_1$ expression, as represented in Figure 4-2. However, also shown in this figure was a relative decrease in cell viability as around 15% of the cells were not able to exclude the viability stain. In order to further study $S1P_1$ expression on endothelial cells, Accutase® was used and cells were gated on 'live' cell populations as before.

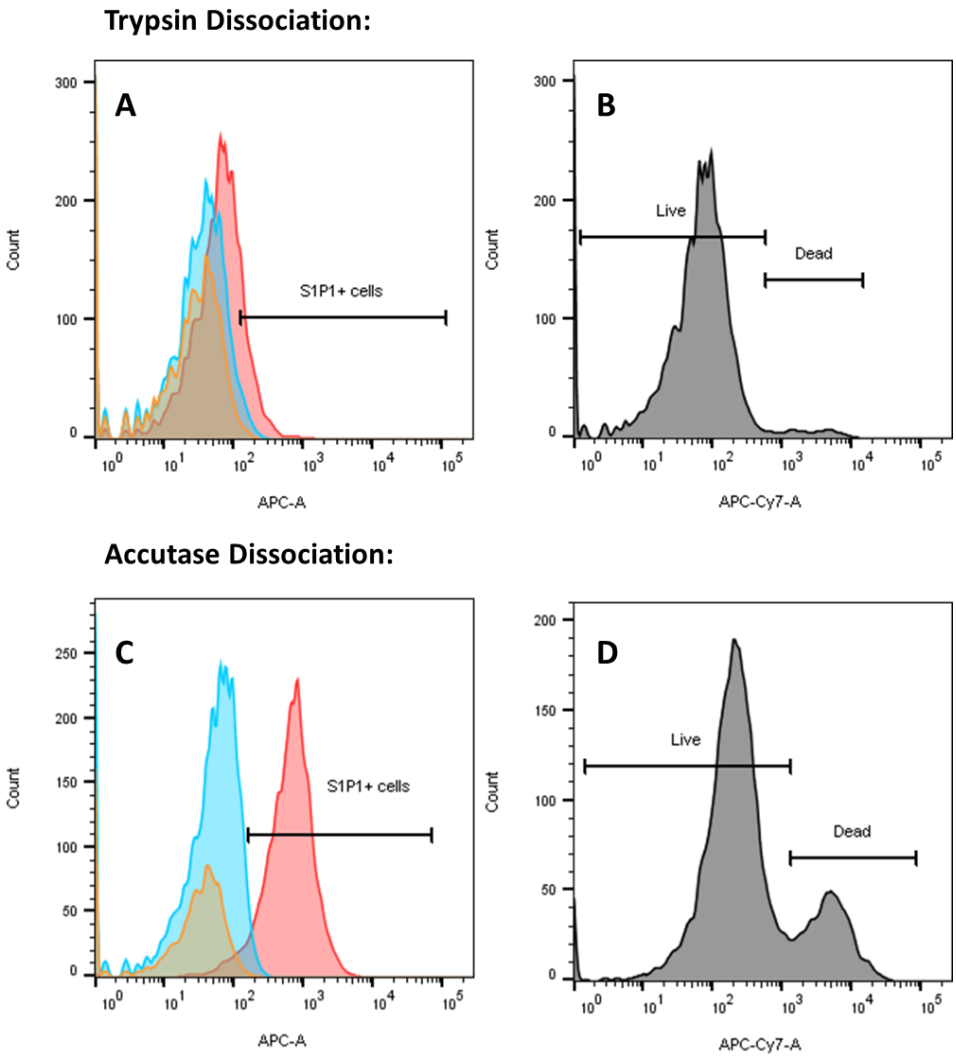


Figure 4-2 HUVEC expression of $S1P_1$ following dissociation using trypsin or Accutase® and resulting live/dead cell status
Representative graphs of receptor expression (A,C) and live cell populations (B,D) analysed using flow cytometry in separate experiments using trypsin (A,B) or Accutase® (C,D), with unstained cells (orange), isotype-antibody labelled cells (blue) and unstimulated endothelial cells stained with $S1P_1$ antibody (red).

The development of the flow cytometry protocol was important to characterise receptor expression. Part of $S1P_1$ signalling requires the endocytosis of $S1P_1$ from the surface of cells. Trypsin is a known protease activated receptor (PAR) activator and there is a possibility that endothelial cells, which express a range of PAR, are activated by trypsin leading to $S1P_1$ loss from the surface by endocytosis (Nakayama et al., 2003). The use of trypsin for certain markers such as ICAM-1 and VCAM-1 (discussed in chapter 5) did not lead to any loss of signal using flow cytometry, but with receptors that have potential low

expression or are labile and prone to proteases, it is important to optimise the protocol so that definitive conclusions can be made. For example, in this chapter the expression of LOX-1 and CD36 was investigated, both of which had a low fluorescence on initial flow cytometry experiments using the standard protocol. Different dissociation solutions were trialled in addition to a change in the staining protocol where cells were incubated with antibodies at room temperature rather than at 4˚C which is part of the standard protocol. The fluorescent staining of $S1P_1$ was enhanced with the use of Accutase® as opposed to trypsin EDTA suggesting that trypsin was potentially down regulating $S1P_1$ from the surface of cells via its PAR activity

With LOX-1 and CD36, no difference in fluorescence was detected either with a change in dissociation solution, concentration of antibody or the temperature at which the antibody labelling was performed. The concentration of antibody was varied through 1:100, 1:40 and 1:20 dilutions, again revealing no positive fluorescence above the isotype control. The LOX-1 antibody, from the same clone, was also purchased with either APC or PE conjugation. Both antibodies showed similar low to no staining in endothelial cultures. In order to definitively conclude the lack of expression, cells were fixed with 4% paraformaldehyde prior to dissociation following standard protocols delineated in methods. This was an attempt to fix the receptors in plasma membrane to allow their interrogation with antibodies and flow cytometry. However, this led to poor cell number yields and did not enhance the detection of the receptors above that of live cells using the same antibodies. The low levels of fluorescence were therefore concluded to reflect a low level of expression rather than an artefact of the dissociation or staining protocols in otherwise unstimulated cells for these specific receptors. For flow cytometry data in this chapter, results from endothelial $S1P_1$ experiments were performed with Accutase® (due to low expression of $S1P_1$ with trypsin as discussed) and results from other endothelial receptor experiments were performed with trypsin EDTA due to the lower cytotoxicity. Immune cell populations were not exposed to either dissociation method. All other elements of the protocol remained the same with a 30 minute incubation of antibody at a temperature of 4˚C.

Table 3 Table of antibodies used for HUVEC analysis by flow cytometry

Antibody Target	Conjugated Fluorophore	Clone	Sourced From
LOX-1 (#1)	APC	15C4	BioLegend
LOX-1 (#2)	PE	15C4	BioLegend
CD36	FITC	5-271	BioLegend
S1PR1 ($S1P_1$)	eFlour 660 (APC)	SW4GYPP	eBiosciences
LDL-R	PE	C7	BD
CD31	APC	WM59	eBiosciences

4.3 Results

Results from the previous chapter indicated that oxLDL altered endothelial migration, although similar studies with native LDL showed no obvious differences in either stimulating or inhibiting this response. Initial experiments into oxLDL effects on endothelial cells covered the range of concentrations between 1, 10 and 100µg/mL, showing opposing effects particularly at the lower and higher concentrations. Previously, a range of concentrations between 10 and 100µg/mL (10, 40, 70 and 100) showed that as the concentration increased, the migration of endothelial cells decreased. In this investigation, the role of lower concentrations of oxLDL was considered, as results from studies with 1µg/mL appeared to be above control. An extra concentration (5µg/mL) was investigated to analyse the discrepancy between migration results from 1 and 10µg/mL, in addition to results in the literature indicating that the 5µg/mL concentration enhanced endothelial migration and tube formation (Wang et al., 2015; Dandapat et al., 2007, Khaidakov et al., 2012).

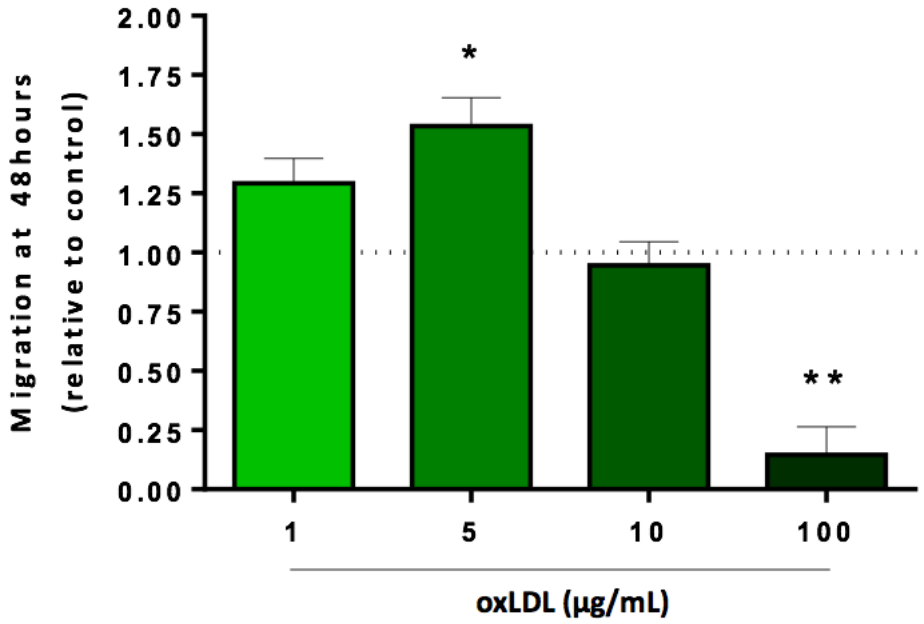


Figure 4-3 The concentration-dependent effects of oxLDL on endothelial migration
oxLDL (1, 5, 10 or 100µg/mL) effects on endothelial migration represented in comparison to respective unstimulated control for each experiment. Mean +/- SEM. Statistics performed using one-way ANOVA with Dunnett's post-hoc test **P<0.01 *P<0.05 (n=7) compared to control.

The data in Figure 4-3 shows that oxLDL at 5µg/mL significantly enhanced endothelial migration, whereas the higher concentration of 100µg/mL significantly inhibited this response. These results correlate with the literature (referenced above) showing that oxLDL has a pro-migratory effect on endothelial cells and represents the importance of oxLDL concentration on endothelial responses. Although oxLDL at 1µg/mL stimulated migration above control cultures it was not statistically significant, whereas 5µg/mL-stimulation further enhanced this response and was statistically significant. The response of endothelial cells to other inflammatory mediators was also investigated and is represented in Figure 4-4 and Figure 4-5.

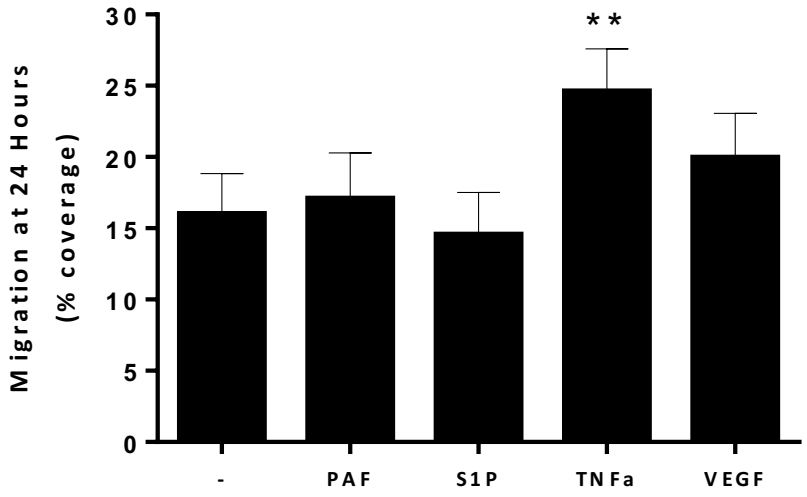


Figure 4-4 The effect of pro-inflammatory mediators on endothelial cell migration in control serum cultures
The effect of pro-inflammatory mediators PAF (1µM), S1P (1nM), TNFα (1ng/mL) and VEGF (50ng/mL) on endothelial migration in control serum cultures. Mean +/- SEM. Statistics performed using one-way ANOVA with Dunnett's post-hoc test **P<0.01 (n=8) compared to control.

The results in Figure 4-4 show bioactive lipid mediators PAF and S1P have no significant effect on endothelial migration at the concentrations used, in addition to the pro-angiogenic mediator VEGF. This figure also shows that TNFα significantly enhanced migration in these cultures. The results in Figure 4-5 show the same mediators added to delipidated serum (as opposed to control serum cultures), with the addition of a defined lipid concentrate (see Appendix for formulation). This lipid concentrate was added back

to lipid-depleted cultures in order to address the potential of essential fatty acid deprivation causing an inhibited endothelial response. This issue was addressed in chapter 3 in the angiogenesis assay with the addition (or add-back) of omega-3 and omega-6 lipids, although no difference in response was observed with these particular lipids. The lipid concentrate contains a broader range of lipids, which are used to aid cell culture, with the suggested working concentration of a 1:100 or 1:1000 dilution (1% or 0.1% concentration respectively). The addition of this lipid emulsion at both concentrations was therefore used as a measure of fatty acid importance in the endothelial migration response.

Taken together, the results from the addition of PAF show no enhancement of migration in control serum cultures but a significant increase in migration in lipid-depleted serum, mimicking the results seen in the angiogenesis assay (chapter 3). The results from the addition of S1P showed no significant increase in migration in either culture (control or delipidated), at this concentration (1nM). This initial concentration of S1P of 1nM was chosen based at the lower range of circulating S1P levels (Xiong et al., 2014). In subsequent experiments, increasing concentrations of S1P through to mid- and high-range circulating S1P levels was shown to significantly increase cell migration above controls at 10 and 100nM but not at 1000nM (Figure 4-6). The addition of TNFα caused a significant increase in migration, both in control and lipid-depleted cultures. Psoriasis as an inflammatory disease shows elevated levels of TNFα hence why this cytokine was chosen. Psoriasis is also associated with IL-17 and IFNγ therefore these cytokines were also tested as mediators of migration. Preliminary data suggested that IL-17 and IFNγ at the concentrations used (IL-17 1-100ng/mL; IFNγ 1-100IU/mL) had no significant effect on endothelial cell migration and so were not investigated further in this thesis (shown in appendix, Figure 9-4 and Figure 9-5 respectively).

Potentially more surprising data was the result that VEGF caused no significant increase in endothelial migration, given the extensive literature suggesting pro-migratory and pro-angiogenic responses induced by VEGF on endothelial cells (Lamalice et al., 2007). The concentration of VEGF used in this assay was 50ng/mL, which is appreciated to cause endothelial migration in the literature (Benndorf et al., 2003; Suehiro et al., 2010; Oommen et al., 2010) but was not seen in this migration assay. The lack of response seen

in this assay with VEGF was despite commercially sourcing two separate manufacturer versions of VEGF including that extensively published from R&D Systems as a source.

Endothelial migration analysed in lipid-depleted cultures, shown in Figure 4-5, also included the addition of a chemically-defined lipid concentrate (Gibco). The lipids included in this emulsion are listed in appendix (9.1.3) and the dilution factors recommended for cell culture (1:100 or 1:1000) were investigated. Previously, in chapter 3, the addition of omega-6 and omega-3 lipids (linoleic acid and linolenic acid respectively) showed no effect in restoring angiogenic potential when added into lipid-depleted serum. The results from this experiment indicated that the lipids contained within this emulsion were also not sufficient to overcome the effect of delipidation on reduced migration. Essential fatty acids were a major constituent of this emulsion and these results reflect the results see in the previous chapter, in which the addition of omega-3 and omega-6 fatty acids did not restore angiogenesis in lipid-depleted cultures.

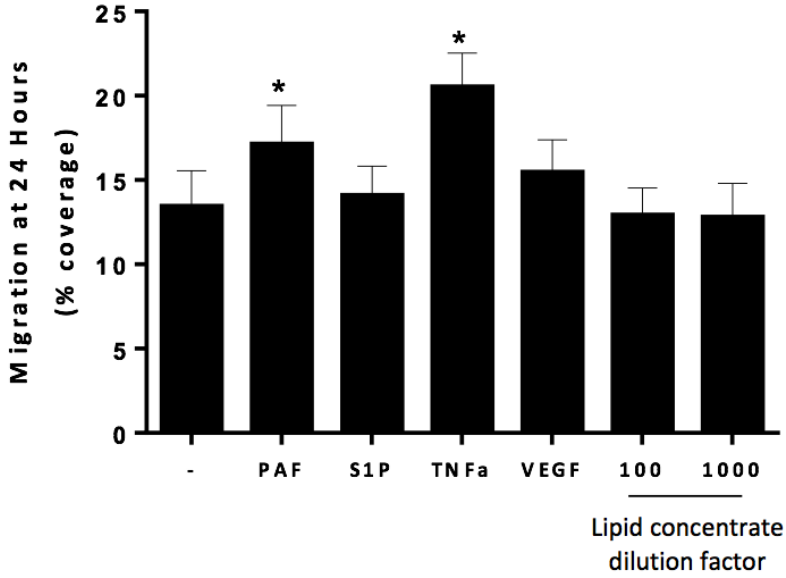


Figure 4-5 The effect of pro-inflammatory mediators and lipid emulsion on endothelial cell migration in lipid-depleted cultures
The effect of pro-inflammatory mediators PAF (1µM), S1P (1nM), TNFα (1ng/mL) and VEGF (50ng/mL) and a lipid emulsion concentrate on endothelial migration in lipid-depleted serum cultures. Mean +/- SEM. Statistics performed using one-way ANOVA with Dunnett's post-hoc test *P<0.05 (n=8) compared to control.

The addition of S1P at a concentration of 1nM did not significantly alter endothelial migration in comparison to control. To investigate the effect of S1P further, an increasing concentration of S1P was added to cultures and its ability to support migration was observed. Figure 4-6 shows that there is a concentration dependent increase in endothelial migration in the presence of S1P 10 and 100nM. At 1µM S1P however, statistical significance was lost giving the impression of a biphasic response similar to that observed with oxLDL.

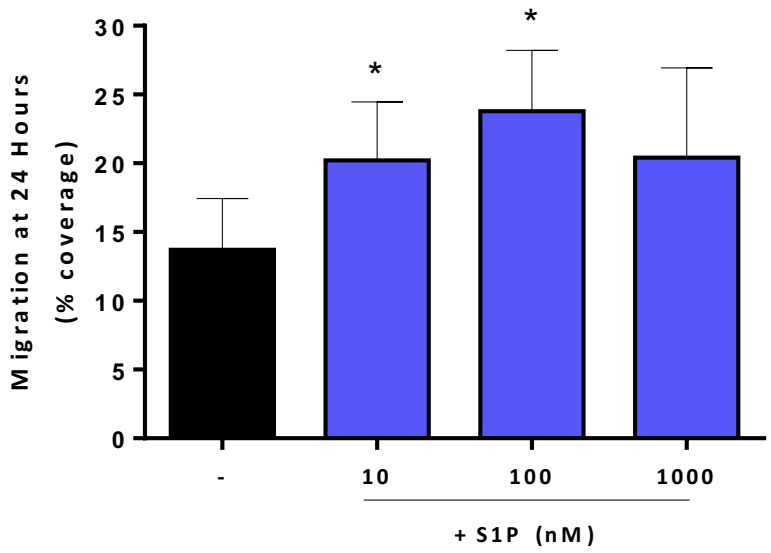


Figure 4-6 The effect of S1P concentrations on endothelial migration in delipidated serum cultures
The effect of S1P (10nM, 100nM and 1µM) on endothelial migration in lipid-depleted serum cultures. Mean +/- SEM. Statistics performed using one-way ANOVA with Dunnett's post-hoc test *P<0.05 (n=6) compared to control.

The results obtained from the scratch assay indicated that oxLDL and S1P can enhance endothelial migration, both dependent on specific concentrations. In light of these observations, it was hypothesised that these two pathways may be connected and that oxLDL-induced migration may be stimulating S1P production and subsequent signalling (see Figure 4-7). To investigate this hypothesis, the migration assay was repeated with oxLDL stimulation in the presence of inhibitors of sphingosine kinase (SKI II) and $S1P_1$ (W146) which would block S1P formation form sphingosine stores or antagonise S1P binding to its receptor respectively. The effect of these inhibitors would delineate the

contribution of sphingosine kinase and $S1P_1$ in oxLDL signalling, suggesting either oxLDL directly signalling through $S1P_1$ or whether oxLDL was causing an intracellular production of S1P within endothelial cells. Results from these experiments are represented in Figure 4-8, Figure 4-9 and Figure 4-10.

OxLDL potentially mediates its effects via a cell surface receptor upstream of the sphingosine system (i.e. the production of S1P by sphingosine kinase). In order to characterise this, flow cytometry was utilised to analyse surface expression of CD36 and LOX-1 both in unstimulated and stimulated cultures (PAF-R was not analysed with flow cytometry due to the lack of a commercially available antibody with a conjugated fluorophore). The role of PAF-R was separately analysed by repeating the endothelial migration assay with the addition of specific PAF-R antagonist WEB2086. The summarised hypothesis of oxLDL-induced migration and mechanistic targets are depicted in Figure 4-7. Briefly, oxLDL signalling through a surface receptor (CD36, LOX-1 or PAF-R) causes sphingosine kinase phosphorylation, which subsequently converts sphingosine into S1P within endothelial cells and this causes autocrine signalling via $S1P_1$ to induce migration.

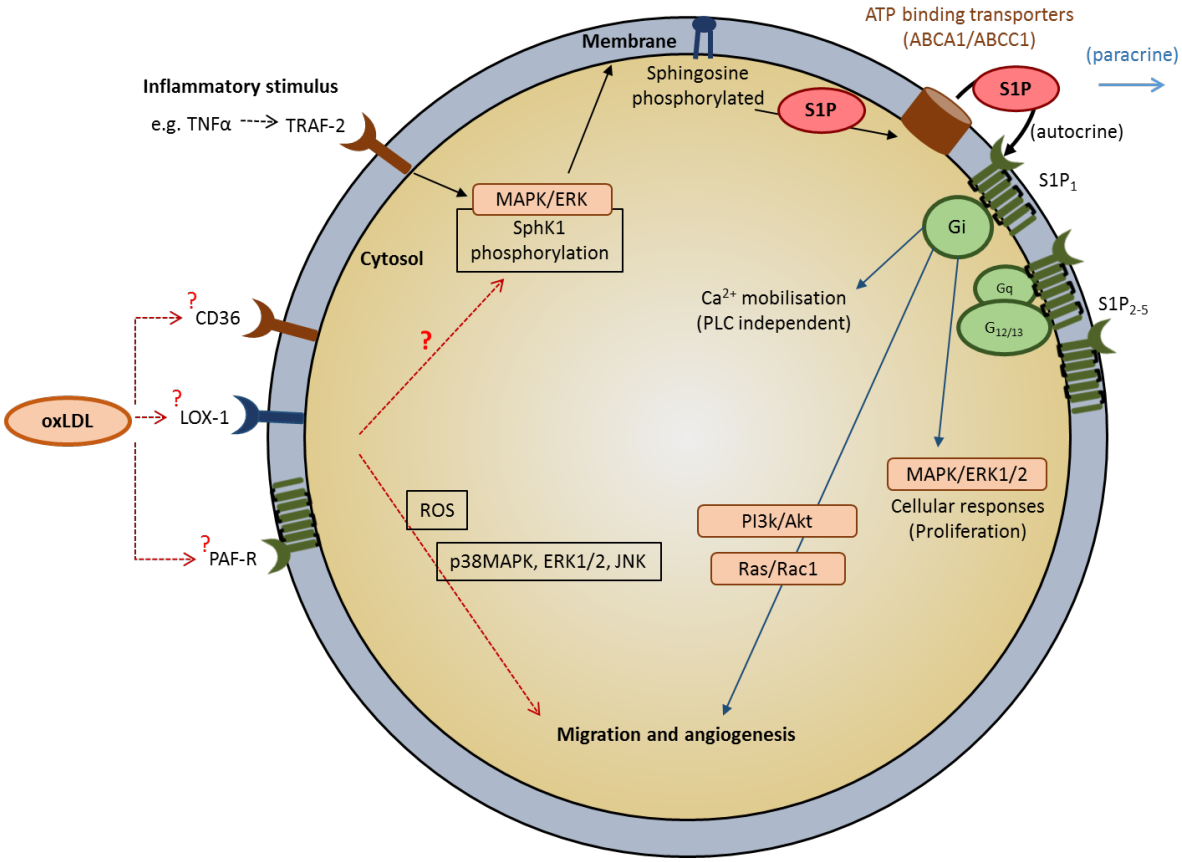


Figure 4-7 Hypothesised mechanism of oxLDL induced migration via the S1P pathway
Hypothesised potential of oxLDL signalling through a receptor mediated mechanism (PAF-R, LOX-1 or CD36) activates sphingosine kinase and subsequent S1P production, which signals through its receptor ($S1P_1$) and causes migration and angiogenesis.

The migration assay was repeated with the addition of the aforementioned inhibitors both in control cultures (Figure 4-8) and in oxLDL-stimulated cultures (Figure 4-9 and Figure 4-10). Initial studies investigated a range of concentrations of all three inhibitors used, with the ranges based around the IC_{50} or Ki indicated for each respective compound (Tocris; French et al., 2006; Gonzalez-Cabrera et al., 2008). The results from Figure 4-8 indicated that there was no significant inhibition of migration with the inhibitors at any concentration in control serum cultures. Previous results indicated that in control serum cultures, PAF had no effect on endothelial migration and so the result of the specific PAF-R antagonist WEB2086 having no effect solidifies the assumption that PAF is not involved in migration in the presence of normal serum. S1P had previously been shown to increase endothelial migration at concentrations of 10 and 100nM (Figure

4-6) and so the effects of sphingosine kinase inhibitor SKI II and $S1P_1$ inhibitor W146 were assessed in control serum cultures. The results in Figure 4-8 showing that there was no significant inhibition of endothelial migration with SKI II or W146 indicated that S1P is not involved with the basal migration occurring in control cultures.

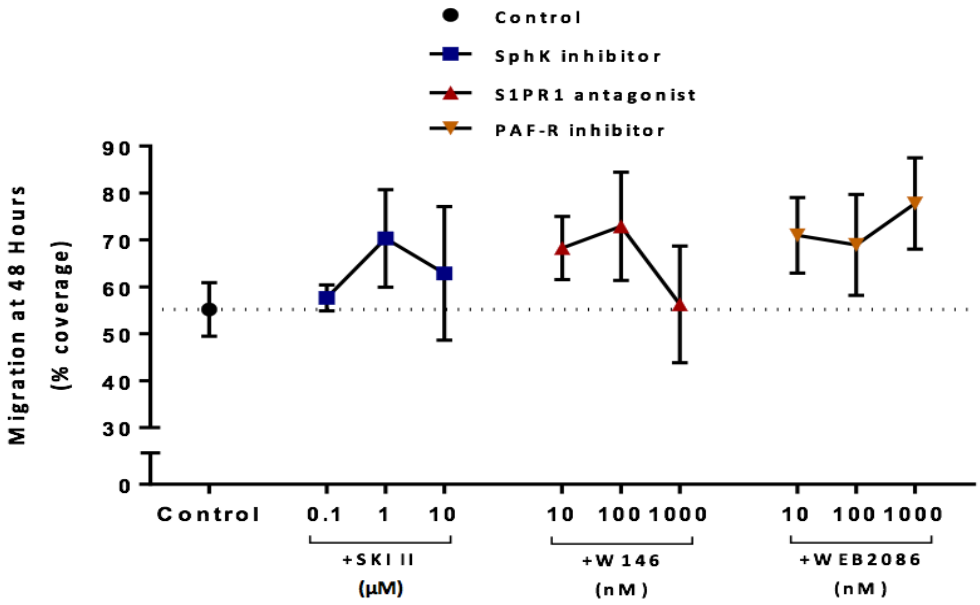


Figure 4-8 The effect of sphingosine kinase, $S1P_1$ and PAF-R antagonists on endothelial migration in control serum cultures
The effect of sphingosine kinase inhibitor SKI II (blue squares), $S1P_1$ antagonist W146 (red triangles) and PAF-R antagonist WEB2086 (orange triangles) on endothelial migration in control serum cultures. Increasing concentration left to right. Mean +/- SEM. Statistics performed using one-way ANOVA with Dunnett's post-hoc test (n=5 in duplicate) compared to control.

Further to the results in control serum cultures, the experimental design was repeated with the addition of 1µg/mL oxLDL in order to stimulate migration. These results in Figure 4-9 show that after stimulating migration with oxLDL, both SKI II and W146 at their higher concentrations were able to reduce endothelial migration below that of the control, with the W146 result being statistically significant. However, the addition of WEB2086 had no significant effect and did not inhibit migration at any concentration used, indicating that PAF-R is not involved in oxLDL-stimulated endothelial migration. These results suggested that oxLDL was causing endothelial migration through the S1P pathway, given that

blocking both S1P production and $S1P_1$ inhibited the oxLDL activated response below control levels.

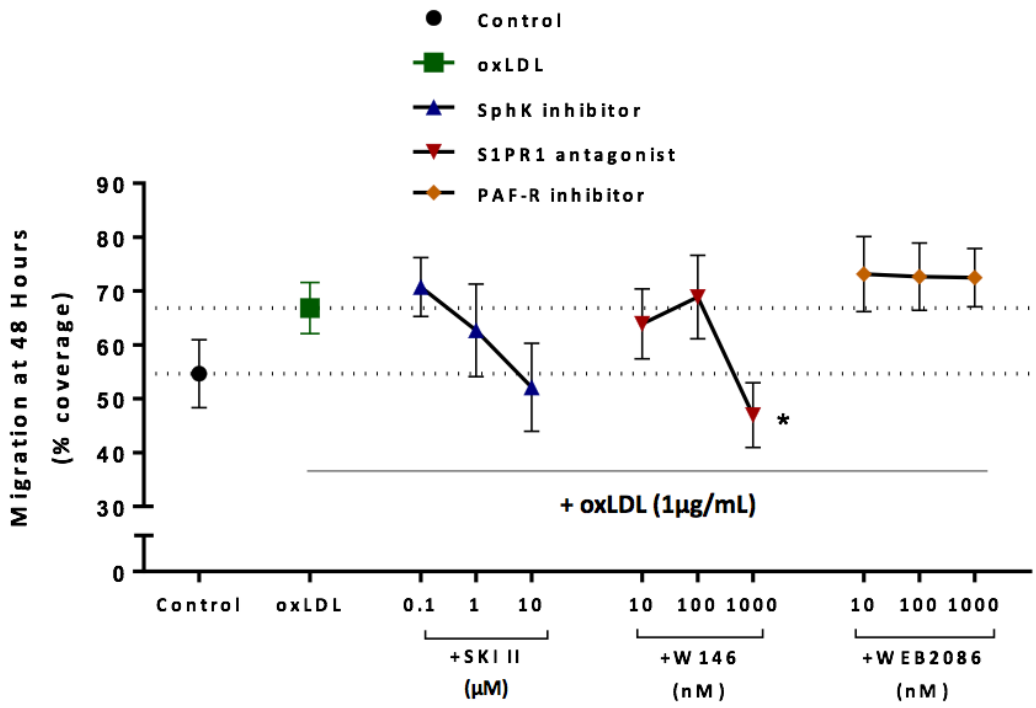


Figure 4-9 The effect of sphingosine kinase, $S1P_1$ and PAF-R antagonists on endothelial migration in oxLDL-stimulated cultures
Control cultures (black circle) stimulated with 1µg/mL oxLDL (green square) and the effect of sphingosine kinase inhibitor SKI II (blue triangles), $S1P_1$ antagonist W146 (red triangles) and PAF-R antagonist WEB2086 (orange triangles) on endothelial migration in oxLDL-stimulated cultures. Mean +/- SEM. Statistics performed using one-way ANOVA with Dunnett's post-hoc test *P<0.05 (n=7 in duplicate) compared to oxLDL (green square).

The concentration ranges used for SKI II and W146 showed that the higher concentrations used were the most effective for inhibiting oxLDL-stimulated migration. These higher concentrations were therefore used in order to analyse the effects of oxLDL at 5µg/mL and the summarised results are displayed in Figure 4-10. These results are expressed as migration relative to control for each experiment (given that not all experiments in this graph were performed on the same set of HUVEC, but the migration in response to oxLDL could be plotted relative to the unstimulated control in each respective experiment) and show similar inhibition with SKI II and W146 in both oxLDL

1µg/mL and oxLDL 5µg/mL-stimulated cultures. These results indicate that oxLDL may be causing an increase in intracellular S1P production, given that oxLDL-induced migration was inhibited with sphingosine kinase inhibitor. Further to this, blocking $S1P_1$ had similar inhibitory effects, suggesting that this is the main receptor for S1P ligation to cause migration.

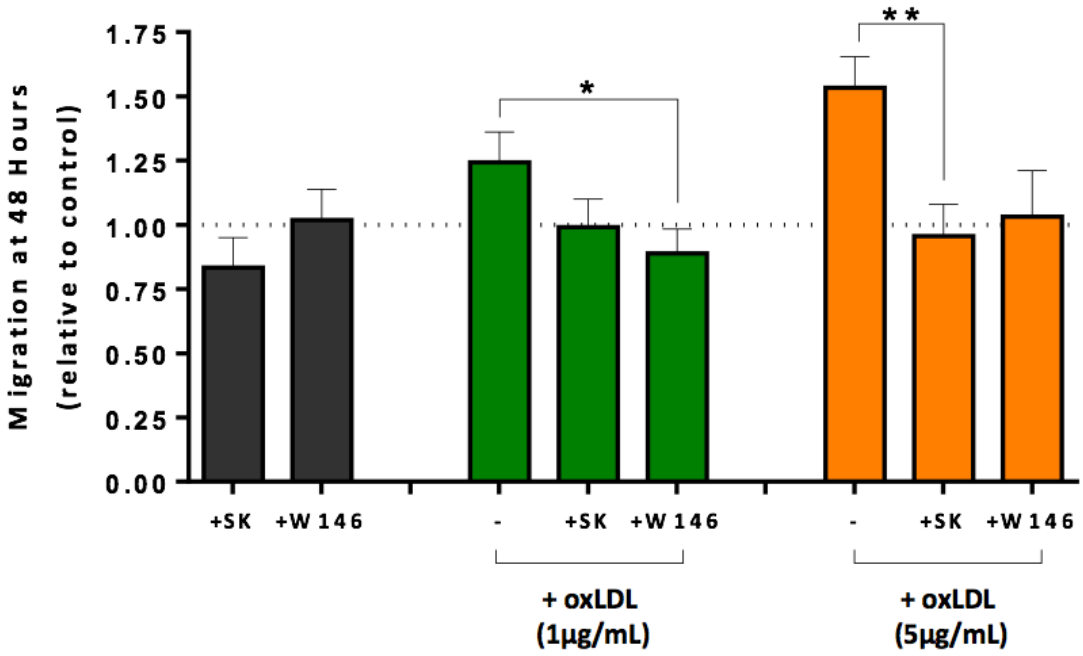


Figure 4-10 Summarised data on the effect of SK inhibition and $S1P_1$ inhibition on control and oxLDL-stimulated cultures
SKI II ('SK'- sphingosine kinase inhibitor) and W146 ($S1P_1$ antagonist) added to control serum cultures (grey bars), oxLDL 1µg/mL-stimulated cultures (green) and oxLDL 5µg/mL-stimulated cultures (orange), represented as migration relative to respective controls. Inhibitors used at 10µM and 1µM for SKI II and W146 respectively. Mean +/- SEM. Statistics performed using one-way ANOVA with Dunnett's post-hoc test **P<0.01 *P<0.05 (n=7 in duplicate) compared to control.

The previous results with the $S1P_1$ antagonist W146 suggested that $S1P_1$ was a key receptor involved in oxLDL-stimulated (and subsequent S1P pathway) migration. However, as previously discussed there are five receptors for S1P to signal through ($S1P_{1-5}$). It is largely recognised that $S1P_1$ is involved in pro-migratory endothelial responses, whereas S1P ligation of $S1P_2$ elicits inhibitory actions on this process. It was therefore hypothesised that blocking the $S1P_2$ receptor may further enhance endothelial migration, which is represented in Figure 4-11. The specific $S1P_2$ antagonist JTE-013 was added in

both control and oxLDL (5µg/mL)-stimulated cultures. The results indicated that blocking $S1P_2$ potentially enhanced migration in control but significance was not reached. In the presence of oxLDL enhanced migration, $S1P_2$ blockade did not further enhance nor block migration (n=4).

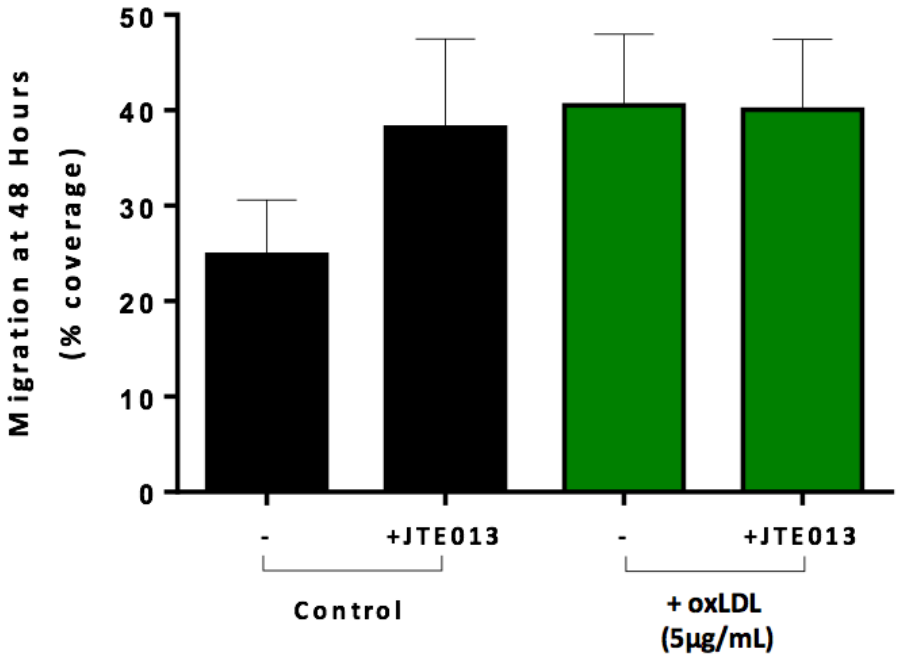


Figure 4-11 The effect of $S1P_2$ inhibitor JTE-013 on endothelial migration
JTE-013 ($S1P_2$ antagonist) added to control serum cultures (black bars) and oxLDL 5µg/mL-stimulated cultures (green). Inhibitor used at 1µM concentration. Mean +/- SEM. Statistics performed using one-way ANOVA with Dunnett's post-hoc test (n=4).

In addition to the investigation of inhibitors in a functional migration assay, the expression of $S1P_1$ was also analysed using flow cytometry. The expression of $S1P_1$ was analysed after 2 hours of stimulation (Figure 4-12) and also after 24 hours of stimulation (Figure 4-13) with ox-/LDL and other inflammatory stimuli. Ligation of $S1P_1$ by S1P is known to lead to the acute down-regulation of the receptor via endocytosis and an attempt was made to visualise this at the 2 hour time point. This forms part the of the signalling cascade of S1P, therefore any stimulus working through this receptor might also be expected to cause the loss of cell surface expression which can be used as a

convenient assay of S1P ligation and activity. Stimulation of HUVEC for 2 hours with 1µM exogenous S1P led to a reduced surface expression of $S1P_1$, although this did not reach statistical significance. OxLDL or LDL were not able to down-regulate the receptor at this time point at a variety of concentrations.

With longer incubation periods (24 hours) there was a possibility of increased expression of $S1P_1$ on the cell surface in response to increased S1P levels within the medium, having potentially been produced by the endothelial cells. However, as seen from the results there was no significant up-regulation in the expression of this receptor at 24 hours in comparison to control with any of the stimuli investigated, including S1P. It should be noted that there was substantial basal expression of $S1P_1$ on endothelial cells without stimulation, as described in the methods and represented in Figure 4-13 with an MFI nearing 300.

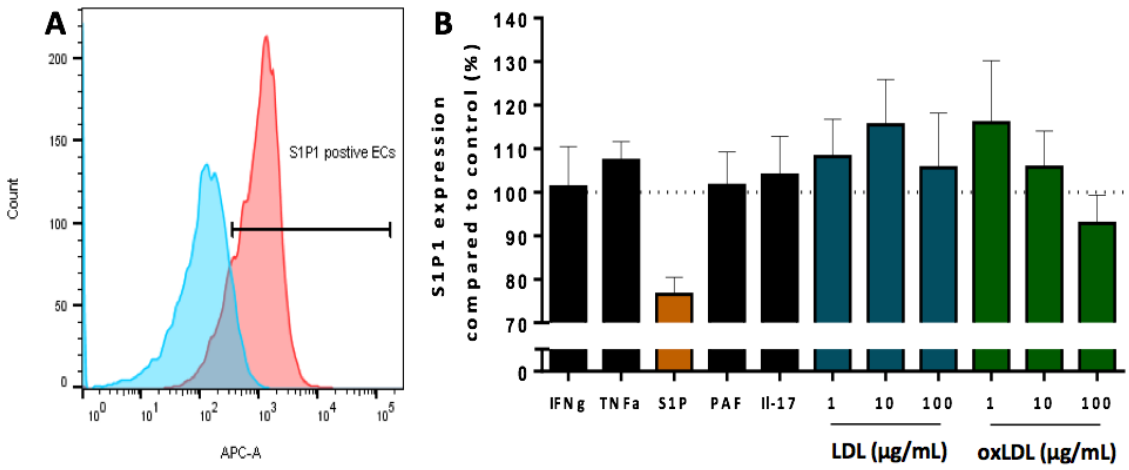


Figure 4-12 HUVEC expression of $S1P_1$ following 2hour stimulation
Representative flow cytometry plot of $S1P_1$ expression on endothelial cells in control cultures [A] where blue=isotype and red=control ECs. Endothelial cells stimulated for 2 hours with inflammatory stimuli (black bars) [IFNγ 10U/mL, TNFα 1ng/mL, PAF 1µM, Il-17 100ng/mL] including S1P (orange) [1µM] or ox-/LDL (blue or green) [all shown in figure B] analysed for $S1P_1$ expression using flow cytometry (n=5).

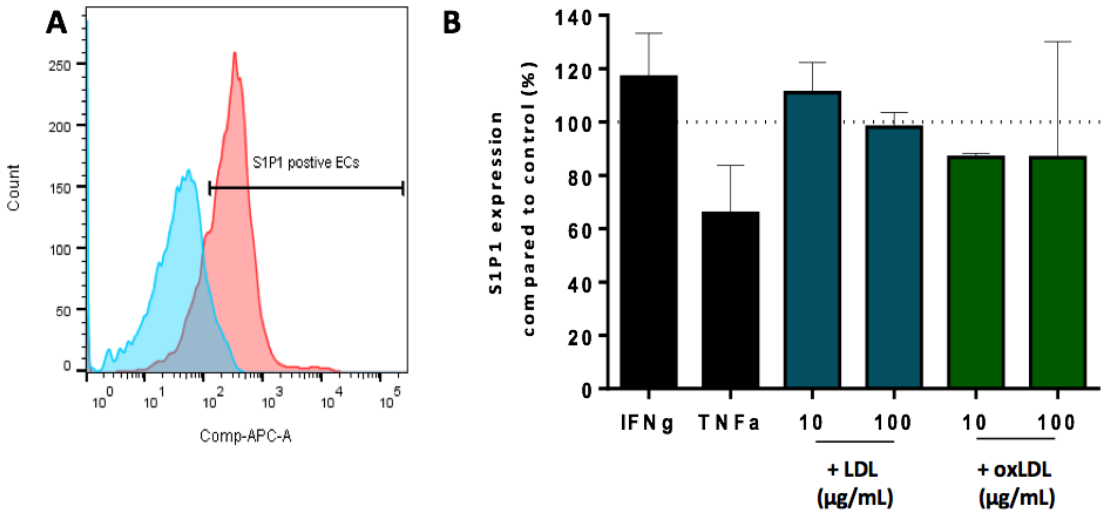


Figure 4-13 HUVEC expression of $S1P_1$ following 24hour stimulation
Representative flow cytometry plot of $S1P_1$ expression on endothelial cells in control cultures [A] where blue=isotype and red=control ECs. Endothelial cells stimulated for 24 hours with inflammatory stimuli (grey bars) [IFNγ 10U/mL, TNFα 1ng/mL] or ox-/LDL (blue or green) analysed for $S1P_1$ expression using flow cytometry (n=3).

The effects of oxLDL are often attributed to scavenger receptors, particularly CD36 and LOX-1 (Pirillo et al., 2013). Scavenger receptors are expressed on a range of cell types and are part of the foam cell formation hypothesis in which macrophages take up and store oxLDL. These receptors have also been characterised on endothelial cells and so in this investigation, flow cytometry was used to assess their expression by HUVEC in unstimulated and stimulated cultures. These data can be seen in Figure 4-14 and Figure 4-15, showing the expression of CD36 on HUVEC, highlighting a very low basal expression (MFI of <20 compared to an MFI of near 300 for $S1P_1$ which is more strongly expressed). In addition to this, there was no significant up-regulation of the receptor with TNFα, IFNγ, LDL or oxLDL after 24hours of stimulation, although at a concentration of 100µg/mL, oxLDL was starting to show a potential increase in CD36 expression above the control.

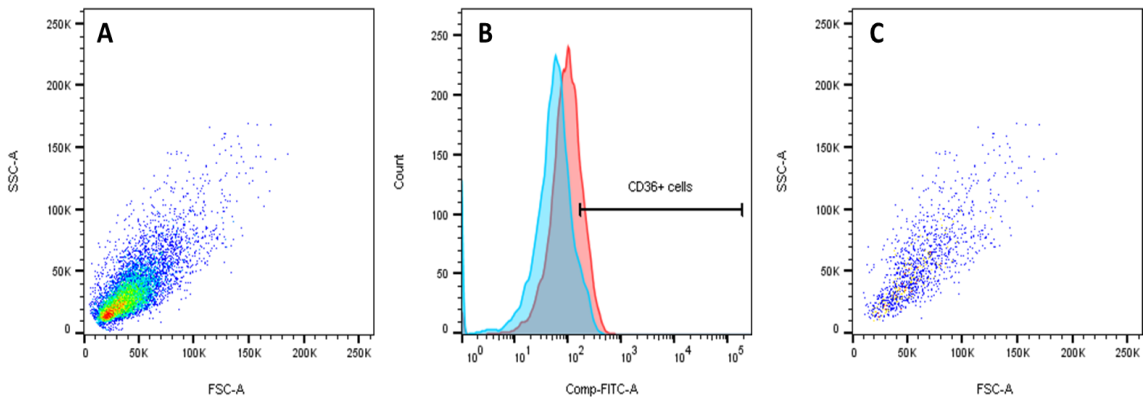


Figure 4-14 HUVEC expression of CD36 following 24 hour stimulation with oxLDL
Representative flow cytometry plots from a single experiment. Plots show endothelial cell gating (A), CD36 expression of HUVEC following 24 hour stimulation with oxLDL 100µg/mL (B) with blue histogram representing isotype-stained cells and red histogram representing oxLDL-stimulated cells and CD36+ cell scatter plot (C).

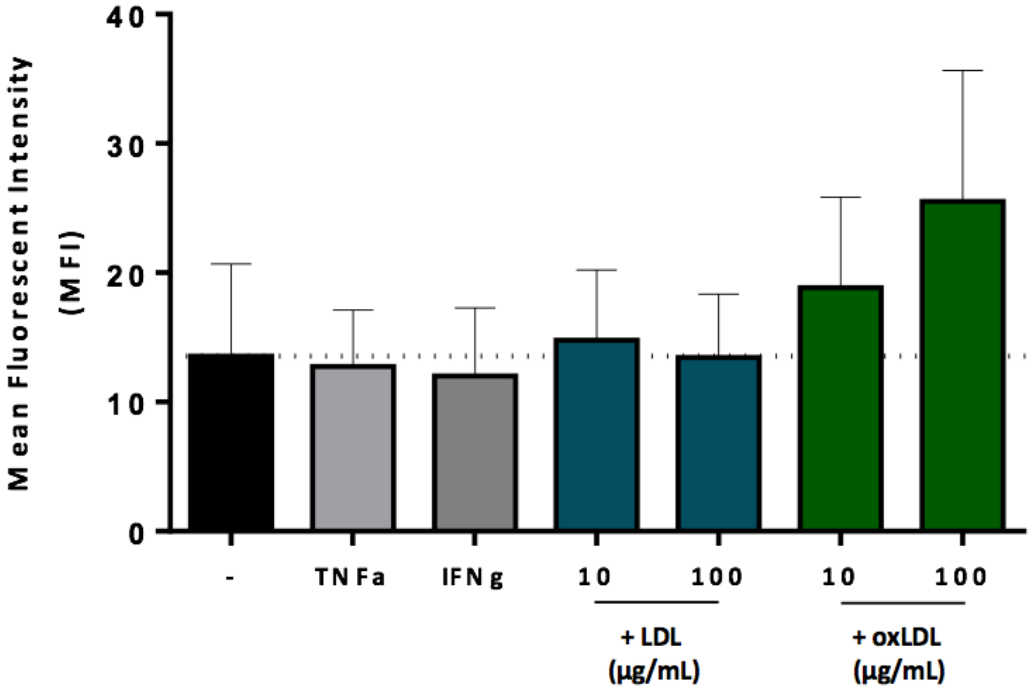


Figure 4-15 HUVEC expression of CD36 following 24 hour stimulation
Endothelial cells stimulated for 24 hours with inflammatory stimuli (grey bars) [TNFα 1ng/mL, IFNγ 10U/mL] or ox-/LDL (blue or green) analysed for CD36 expression using flow cytometry (n=3).

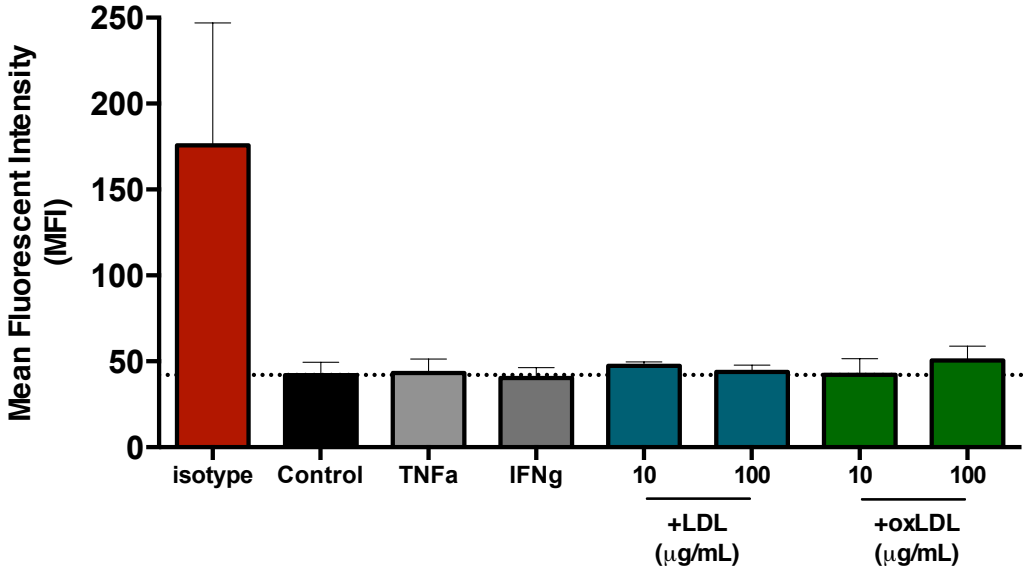


Figure 4-16 HUVEC expression of LOX-1 following 24 hour stimulation
Endothelial cells stimulated for 24 hours with inflammatory stimuli (grey bars) [TNFα 1ng/mL, IFNγ 10U/mL] or ox-/LDL (blue or green) analysed for LOX-1 expression using flow cytometry (n=2). Results are expressed as raw data values as the MFI results were all below the isotype value (red).

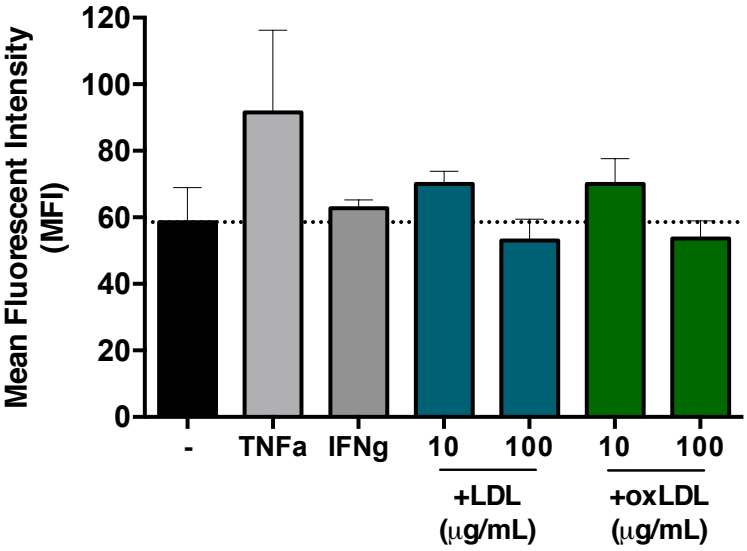


Figure 4-17 HUVEC expression of LDL-R following 24 hour stimulation
Endothelial cells stimulated for 24 hours with inflammatory stimuli (grey bars) [TNFα 1ng/mL, IFNγ 10U/mL] or ox-/LDL (blue or green) analysed for LDL-R expression using flow cytometry (n=2).

Flow cytometry data for LOX-1 expression on HUVEC revealed no positive staining in unstimulated or stimulated cultures. The results of LOX-1 expression in Figure 4-16 are raw data values as the isotype value was above all the other results. Amendments were made to the flow cytometry staining protocol in relation to temperatures, dissociation solutions, timings and multiple antibodies purchased, but the fluorescent signal was below the isotype and resembled an unstained population, concluding that there was no substantial measurable expression of this receptor by flow cytometry. Taken together with the results of the flow cytometry and the migration assay data, it can be assumed that LOX-1 and PAF-R are not involved in oxLDL-mediated migration in HUVEC, but potential up-regulation of CD36 suggests that this receptor may play a role in HUVEC responses to oxLDL. Initial investigations also indicated that LDL-R expression was not significantly altered by LDL or oxLDL on HUVEC (Figure 4-17).

Optimising flow cytometry staining protocols is an important stage in defining cellular receptor expression. In addition to this, using the same antibodies on different cell types to gain a positive control is beneficial. Therefore, the antibodies used for staining endothelial cells and receptors of interest were applied to freshly isolated immune cell populations. The results from these flow cytometry experiments are shown for granulocytes, lymphocytes and monocytes in Figure 4-18, Figure 4-19 and Figure 4-20 respectively. Importantly, it was seen that all antibodies were capable of highlighting a positive fluorescence and therefore expression of their respective target, solidifying conclusions drawn from the expression or lack thereof seen in HUVEC.

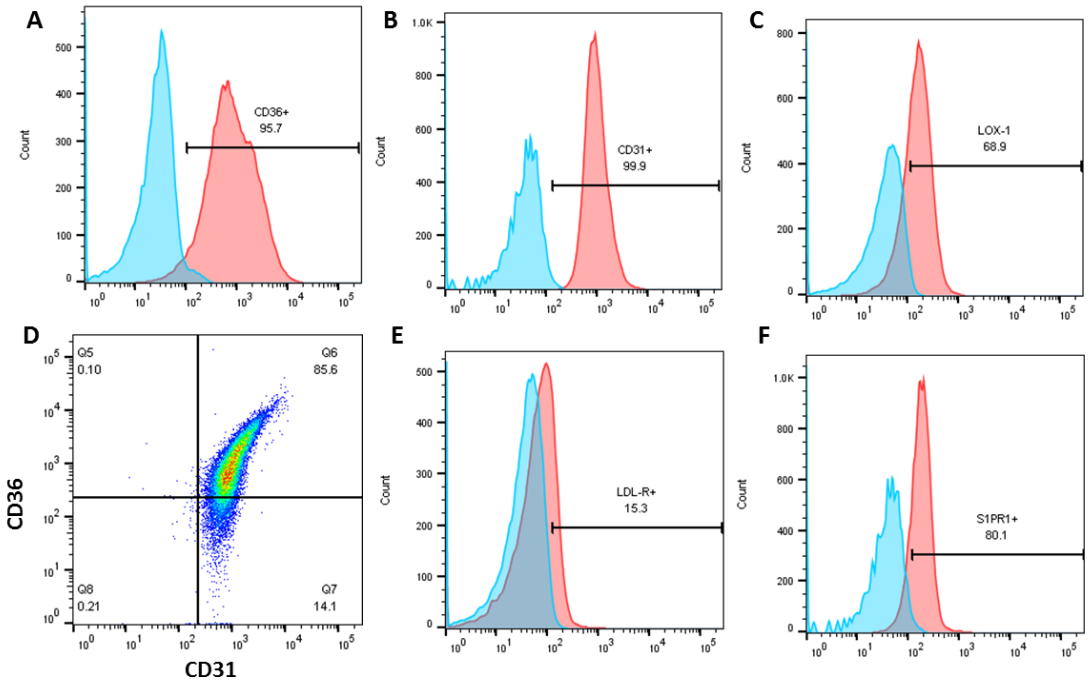


Figure 4-18 Granulocyte expression of CD36 (A), CD31 (B), LOX-1 (C), LDL-R (E) and $S1P_1$ (F)

Unstimulated granulocytes isolated from blood analysed by flow cytometry for receptor expression. Representative plots from an individual experiment. Isotype control represented by blue histograms and receptor expression represented by red histogram (n=3).

Immune cells were freshly isolated from healthy blood donors and gated for granulocytes, lymphocytes or monocytes based on forward/side scatter as described in the methods. Immune cells were left unstimulated and were stained in panels based on fluorophores (panel 1: CD36, CD31 and LOX-1; panel 2: LDL-R and $S1P_1$). Granulocytes showed expression of receptors capable of recognising oxLDL (LOX-1 and CD36), in addition to $S1P_1$. There was minimal expression of LDL-R in this cell type. Within the antibody panels, correlations were analysed (as shown in part D of each figure) between the receptors, looking for potential populations which were double-positives. There were no correlations observed except for a trend in CD31+ high being CD36+ high also, suggesting a relationship between the two and was seen in all immune cell populations.

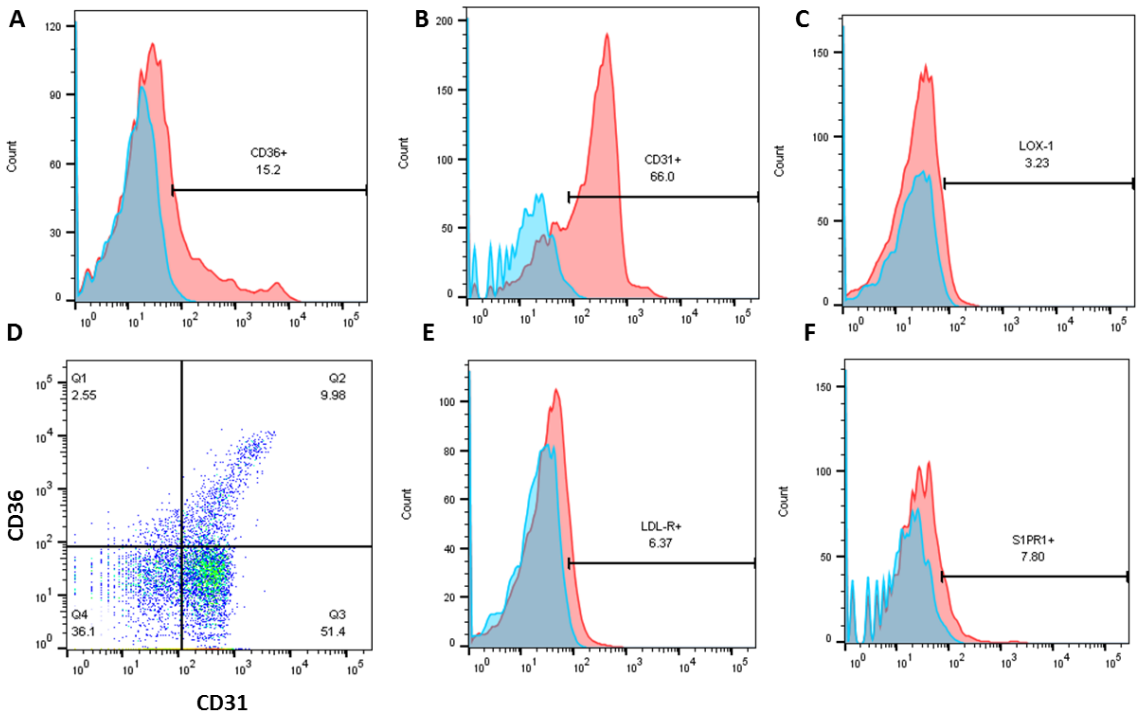


Figure 4-19 Lymphocyte expression of CD36 (A), CD31 (B), LOX-1 (C), LDL-R (E) and $S1P_1$ (F)

Unstimulated lymphocytes isolated from blood analysed by flow cytometry for receptor expression. Representative plots from an individual experiment. Isotype control represented by blue histograms and receptor expression represented by red histogram (n=3).

The expression of oxLDL receptors on lymphocytes was relatively low compared to other immune cell populations. The lower expression of these scavenger receptors in lymphocytes strengthens the argument that lymphocytes are less involved in foam cell formation within a fatty plaque than monocytes and macrophages, as the lymphocytes are less able to recognise and therefore internalise oxLDL. The expression of $S1P_1$ in unstimulated lymphocytes was also lower than the other immune cell populations, which may be an important result regarding the role of S1P in lymphocyte trafficking within the circulation. Stimulation of immune cells and subsequent changes in receptor expression may be a useful indication in controlling immune cell populations within the circulation. Monocytes had a high expression of CD36 as expected and also showed positive staining for LOX-1. Monocytes displayed all the receptors investigated which implicate them as an important cell type in circulating ox-/LDL effects.

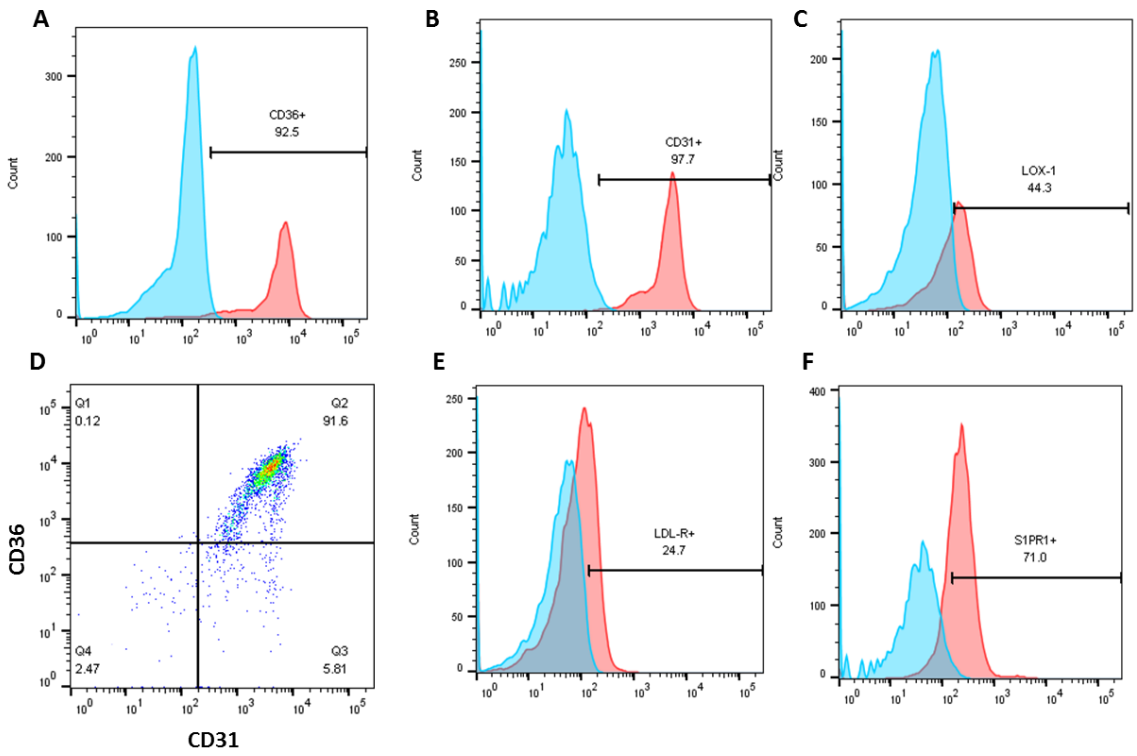


Figure 4-20 Monocyte expression of CD36 (A), CD31 (B), LOX-1 (C), LDL-R (E) and $S1P_1$ (F)

Unstimulated monocytes isolated from blood analysed by flow cytometry for receptor expression. Representative plots from an individual experiment. Isotype control represented by blue histograms and receptor expression represented by red histogram (n=3).

4.4 Discussion

It has been shown in this chapter that oxLDL has biphasic effects on endothelial function, stimulating EC migration at a lower concentration of 5μg/mL and inhibiting EC migration at a higher concentration of 100μg/mL. Although the addition of 1μg/mL oxLDL into cultures enhanced migration above control, it was not statistically significant. The addition of 10μg/mL also appeared to have no effect, but the specific concentration of 5μg/mL appeared to be optimal for endothelial migration, consistent with other findings in endothelial migration and angiogenesis in the literature (Wang et al., 2015; Dandapat et al., 2007, Khaidakov et al., 2012). This 5μg/mL concentration of oxLDL is generally accepted to be a low, physiological concentration (Boudjeltia et al., 2012; Takei et al., 2001; Khaidakov et al., 2012), although the assessment of oxLDL within the circulation has varied depending on the method used. Several mAbs have been used in the detection of oxLDL, but differences in the methods (i.e. sandwich or competitive ELISA) have revealed large differences in results, creating difficulty in reporting 'physiological' oxLDL concentrations (Itabe & Ueda, 2007). Within the literature relating to endothelial investigations with oxLDL, the range of 1-10μg/mL is generally regarded as low concentrations and 100μg/mL regarded as a high concentration, with the ranges in between reported physiological.

In order to characterise this response of HUVEC to oxLDL, a range of pro-inflammatory stimuli were used in order to compare the relative responses. Initially, the effects of TNFα, S1P, PAF and VEGF were investigated for their ability to regulate endothelial migration, both in control and delipidated cultures. It was shown that in particular, TNFα induced migration in both conditions. TNFα is a key cytokine involved in inflammation, being heavily linked with immune cell responses and also tumour progression. Previous research has shown that TNFα can induce migration in endothelial cells (Gao et al., 2002) but also inhibit endothelial sprouting, although the cytokine was shown to produce a 'tip-cell' phenotype after longer exposure of 2-3 days (Sainson et al., 2008). The role of TNFα on endothelial function such as migration has importance in the pathology of psoriasis and a systematic review of TNFα inhibitors in psoriasis and psoriatic arthritis suggested improved endothelial function in these patients (Brezinski et al., 2014).

PAF was only able to enhance endothelial migration in lipid-depleted cultures, mimicking the results seen in the previous chapter performed in the tube formation assay. Once again, the specific PAF-R antagonist WEB2086 had no effect on migration in control serum cultures, concluding that in control serum, PAF plays no essential role in the migratory response. It appears though, that PAF has the ability to enhance endothelial responses, potentially in the absence of other pro-angiogenic and pro-migratory mediators. Along with this finding, the initial concentration of S1P at 1nM was found not to have any significant effect on endothelial function. However, subsequent investigations with a concentration range of S1P showed significant promotion of endothelial migration at 10nM and 100nM. This effect, however, was lost at the higher 1µM concentration and potentially mimics the biphasic concentration dependent effect of oxLDL on endothelial migration. There was a lack of effect with VEGF stimulation on HUVEC migration, as seen with the tube formation assay previously described.

Having made these observations of endothelial migration induced by TNFα, S1P and oxLDL, but no induction by VEGF or LDL, the mechanism of stimulation was sought. It has previously been described that there is differential regulation of endothelial cell migration induced by S1P and VEGF (Liu et al., 2001). It has also been described that TNFα is capable of activating sphingosine kinase within endothelial cells, with S1P a potential mechanism by which endothelial cells are protected from TNFα-induced injury and apoptosis (Xia et al., 1999; Osawa et al., 2001; Chen et al., 2004; Sun et al., 2010). The review of the literature and the results from experiments performed in this investigation therefore suggest that TNFα and S1P may promote endothelial migration by similar mechanisms, separate to VEGF. S1P has previously been identified as a potent mediator of migration in foetal bovine heart endothelial cells (FBHECs) (Panetti et al., 2000), HUVEC (Paik et al., 2001; Morii et al., 2003) and endothelial progenitor cells (Wang et al., 2015). A recent paper also suggested that angiogenesis induced by oxLDL both *in vitro* and *in vivo* could be inhibited by the addition of an anti-S1P antibody (Camare et al., 2015). It was therefore hypothesised that oxLDL may be inducing migration in this investigation seen in HUVEC by activating sphingosine kinase, producing intracellular S1P and causing S1P ligation with its receptor(s). This hypothesis was subsequently investigated with inhibitors of sphingosine kinase (SKI II), $S1P_1$ (W146) and $S1P_2$ (JTE-013).

It was shown that in control serum cultures, neither SKI II nor W146 blocked migration, which was an interesting finding suggesting that S1P is not involved in the basal migration occurring in HUVEC. However, after stimulation with oxLDL (1µg/mL or 5µg/mL) to enhance migration, both inhibitors were capable of statistically significant inhibition, which was relatable to control serum levels. The conclusion from these results supported the previous hypothesis that oxLDL activates sphingosine kinase to produce S1P, which subsequently ligates $S1P_1$ (as represented in Figure 4-21). These findings may have importance in disease pathology, specifically in areas with oxidative lipid accumulation, by influencing endothelial S1P formation and activity. In addition to being fundamental in the developing vascular system, S1P maintains vascular integrity and barrier function in adult circulation, highlighting the importance of S1P regulation between the endothelium and cells within the blood, such as RBCs and platelets. If the effects of oxLDL are also mediated through local S1P generation then this may be a target for therapy, where these lipids affect chronic inflammation. Inflammatory conditions such as psoriasis are characterised by immune cell infiltration into the tissue from the circulation, mediated predominantly by a 'leaky' vasculature which has increased permeability. It is thought that S1P can exert anti-inflammatory actions by increasing barrier function and preventing permeability though the endothelium (Singleton et al., 2005). However, S1P also promotes survival responses such as proliferation and migration, which in the setting of angiogenesis may be a more pro-inflammatory response. The balance of S1P production and degradation is therefore crucial. Perhaps even more crucial are the receptors through which S1P signals, with $S1P_1$ and $S1P_3$ promoting migration and $S1P_2$ inhibiting migration (Blaho et al., 2014).

The use of specific $S1P_1$ antagonist W146 was evaluated in this investigation, showing inhibition of oxLDL mediated migration. $S1P_1$ is abundant in endothelial cells (as confirmed in this investigation using flow cytometry) and is thought to be the primary receptor involved in stimulating endothelial migration. It has also been suggested that $S1P_1$ expression is enhanced in arterial vascular regions during inflammation (Galvani et al., 2015). The level of inhibition seen with W146, reducing migration back to control serum level suggests that this receptor may be the main contributor to the effect seen with oxLDL, although the role of $S1P_3$ was not analysed in this thesis. $S1P_3$ is thought to

have similar effects to $S1P_1$, by promoting endothelial survival responses such as migration (Waeber et al., 2004; Blaho & Hla, 2014). Further directions for this research may be suggested to include inhibitors such as VPC 23019 (Kitada et al., 2016) which inhibits both $S1P_1$ and $S1P_3$ receptors or TY 52156 (Murakami et al., 2010) a selective antagonist of $S1P_3$ alone. Potentially, if more inhibition was observed with the VPC 23019 inhibitor than W146, it may suggest a co-stimulatory role in oxLDL induced migration via $S1P_1$ and $S1P_3$. Additionally, other inhibitors of $S1P_1$ may be tested in order to confirm the results seen with W146 in HUVEC, such as a series of benzimidazole sulfonamides currently being developed (Hennessy et al., 2015). However, a further and potentially more complex mechanism that may be involved is that following antagonism of $S1P_1$, the formed S1P is available for ligation of the other S1P receptors such as $S1P_2$, which then causes inhibition of endothelial migration. This might suggest that the effect of sphingosine kinase blockade and therefore endogenous S1P production essentially stops ligation of $S1P_1$ and 2 without gaining knowledge of the receptors involved yet still helps to define the endogenous production of S1P following oxLDL stimulation.

Theoretically, an antagonistic relationship exists between $S1P_1$ and $S1P_2$ in the vascular endothelium during disease and inflammation (Blaho & Hla, 2014). In this investigation, the role of $S1P_2$ in endothelial migration was analysed using the antagonist JTE-013. This inhibitor is marketed as a specific $S1P_2$ antagonist, although studies have suggested that at higher concentrations (in the micro-Molar ranges) it has effects that are not related to $S1P_2$, as the compound elicited effects in $S1P_2$-/- mice (Salomone & Waeber, 2011). It was suggested in this same article however, that JTE-013 retains sufficient selectivity at lower concentrations, such as 1µM, used in this investigation. It was hypothesised that the blockade of $S1P_2$ would enhance endothelial migration, given its role in blocking such events. In control serum cultures, blocking $S1P_1$ and SK did not block migration, suggesting S1P was not involved in these cultures. However, the addition of JTE-013 showed a trend above the control, suggesting $S1P_2$ may be involved in control migration by acting antagonistically with $S1P_1$. The results from the migration studies showed that in oxLDL-stimulated cultures, JTE-013 had no significant effect on endothelial migration (n=4). This suggests that the S1P produced (within endothelial cells) in response to exogenous oxLDL stimulation may be specifically binding to $S1P_1$ and could even be

diverted away from $S1P_2$ although oxLDL (5ug/mL) in the presence of $S1P_2$ blockade did not cause a further enhancement of migration. It could therefore lead to the hypothesis that a chaperone which directs S1P may be critical in influencing its inflammatory outcome. This may be an important point, as it has been previously shown that S1P effects, even on the same receptor, differ depending on its binding with chaperones such as the differential effects when S1P is bound to HDL or albumin (Wilkerson et al., 2012). The way in which the chaperone effects S1P receptor ligation is not yet fully understood and this can also be applied to the intracellular carriers that direct S1P to its receptors in an autocrine manner. It is unlikely that there is a concentration dependent effect of S1P levels on the receptor to which it binds, given that all the S1P receptors have a nano-Molar concentration affinity for S1P (Blaho & Hla, 2014), thus it is more likely that there are precise mechanisms/responses which guide S1P to a specific receptor. Currently, it is postulated that S1P transporter spinster homolog 2 (Spns2) is the primary intracellular carrier for S1P in endothelial cells (Ninik et al., 2012; Nagahashi et al., 2014; Fukuhara et al., 2012). Spns2 is postulated to export S1P out of endothelial cells and it has been shown that depleting Spns2 in HUVEC significantly reduces S1P release (Fukuhara et al., 2012). However, in red blood cells within the circulation, S1P is translocated from the intracellular side of the membrane to the extracellular side by flippases (Thuy et al., 2014). Exactly how spns2 directs S1P to its range of receptors without flippases is unknown and warrants further investigation. Future investigations may therefore characterise the relationship between oxLDL stimulation and S1P carrier molecules such as Spns2, exploring how oxLDL may specifically direct S1P to $S1P_1$ rather than to $S1P_2$, for example. In this investigation, blocking of multiple receptors at the same time was not performed, which may also need to be analysed in order to characterise the antagonistic response occurring in endothelial cells. It is also unclear whether, following ligation of $S1P_1$ with S1P and subsequent down-regulation of this receptor from the surface, if S1P then binds to its other receptors ($S1P_2$/$S1P_3$) due to their relative availability on the cell surface, or whether S1P is specifically bound and stored in the membrane until $S1P_1$ is again available. The regulation of the S1P receptor subtypes and their expression and response to S1P ligation is an area which requires further investigation. Indeed, should the response to S1P be dependent on acute ($S1P_1$) versus chronic ($S1P_2$ for example) may depend on the relative length of time that either receptor is expressed on the surface.

The assumption from the results discussed was therefore that oxLDL induces sphingosine kinase activation to produce S1P in endothelial cells. The mechanism by which oxLDL initially causes this activation was therefore of interest, particularly in reference to receptors CD36 and LOX-1 previously mentioned. Flow cytometry was employed to analyse the cell surface expression of these receptors on HUVEC, both in unstimulated and stimulated (24hour) cultures. There was no LOX-1 identifiable in any preparation investigated, despite the protocol changes delineated in the methods and the use of multiple different fluorescently-conjugated antibodies. It was concluded from this that LOX-1, as with PAF-R, is unlikely to be the receptor by which oxLDL was stimulating S1P production and subsequent endothelial migration. However, in order to confirm this observation, the oxLDL-induced migration will need to be repeated in the presence of a LOX-1 blocking antibody, antagonistic peptide or small molecule inhibitor (White et al., 2001; Falconi et al., 2013). The flow cytometric analysis of CD36 however did show some expression of the receptor, which seemed to be up-regulated slightly by oxLDL, to a non-statistically significant level over 24 hours. Although the expression of CD36 appeared to be minimal, it is hypothesised that this receptor may be, at least in part, contributing to the effects seen with oxLDL on migration (as depicted in Figure 4-21). Further studies with anti-CD36 blocking antibodies would need to be performed in order to confirm this hypothesis.

The results in this chapter have gone some way to delineating the mechanism by which oxLDL may be inducing endothelial migration. In order to characterise this in more detail, further work will need to confirm the results seen initially (i.e. with LOX-1 and CD36 blocking antibodies), in addition to clarifying the specific sphingosine kinase involved (as SKI II blocks both sphingosine kinase 1 and 2), exploring other potential mechanisms of oxLDL stimulation such as direct oxidative stress and finally, understanding how LDL, oxLDL and HDL all combine to regulate endothelial function via S1P. The addition of SKI II into oxLDL-stimulated cultures showed significant inhibition, indicating a role for sphingosine kinases, although the inhibitor blocks both SK1 and SK2. SK1 has been more extensively studied and has been evidenced to be involved in pro-survival responses, in addition to being elevated in many cancers (Neubauer & Pitson, 2013; Pyne et al., 2011) and would be hypothesised as the primary kinase involved in endothelial migration.

Although there appears to be no commercially available specific blocker for SK2 alone, a recently developed SK1 inhibitor (PF 543 hydrochloride (Ju et al., 2016) may be useful to evaluate whether similar inhibition is induced, compared with SKI II.

It may be postulated that oxLDL induces direct cellular oxidative stress (potentially via lipid peroxidation events), which may in turn cause endothelial responses. LOX-1 has previously been implicated in NADPH oxidase expression and subsequent ROS generation (Dandapat et al., 2007; Valente et al., 2014), which given the lack of LOX-1 expression in HUVEC seen in this research, may occur without receptor recognition. This would also align with the lack of responses seen with native LDL at equivalent concentrations although because of LDL and oxLDL affinity for their respective receptors, this cannot be excluded.

The role of LDL and oxLDL in cultured HUVEC has been studied in this investigation and shown their direct effect on endothelial responses important to inflammation. Considering the *in vivo* setting of inflammation, S1P is found at (relatively) high concentrations in the circulation, produced from red blood cells and carried predominantly by the chaperones albumin and HDL. S1P promotes survival responses which are considered atheroprotective in terms of cardiovascular disease and S1P bound to HDL in particular (as opposed to albumin) has been shown to attenuate TNFα-induced NF-$_k$B activation and ICAM-1 abundance, trigger greater G-protein activation and cause more receptor endocytosis (Galvani et al., 2015). For this reason, a large portion of the atheroprotective effects of HDL have been attributed to S1P. However, the endogenous production of S1P by endothelial cells induced by oxLDL may have damaging effects in inflammatory conditions, as S1P can promote inflammatory processes such as neovascularisation. In psoriasis for example, oxLDL may exacerbate an already inflamed environment by enhancing the endothelial response to stimulate angiogenesis. In addition to this, S1P is up-regulated in cancer (as with sphingosine kinases) which has obvious negative implications in promoting survival responses in tumour cells.

S1P also has roles in regulating immune trafficking from the lymphoid organs into the circulation which promotes inflammation. Fingolimod, which is an S1P receptor modulator, is approved for the treatment of multiple sclerosis as it causes the

internalisation of S1P receptors which prevents lymphocyte trafficking from the lymph system (Brinkmann, 2009). The role of S1P is therefore important in the initiation and maintenance of inflammation, with a complex biology including multiple receptors and chaperones. Although S1P bound to HDL may have protective effects on cardiovascular plaques by stimulating survival responses, the stimulation of S1P via oxLDL may have negative implications by causing un-regulated inflammatory responses. With LDL/HDL ratios being important in cardiovascular risk and the connection these lipoproteins have to S1P, the understanding of S1P production, specific transport and specific receptor ligation may hold the key to characterising how these lipids interplay in the balance of inflammation. Interestingly, HDL may also be oxidised, in which case its atheroprotective properties are lost (Bergt et al., 2003; Zhang et al., 2010). It may be postulated that oxidising both HDL and LDL, may cause their usual balance to be upset due to the specific effects this may have on S1P signalling and how these lipoproteins both respond to- and stimulate- inflammation. Manipulating the specific S1P transport and receptor ligation may therefore be a novel target for future therapy in inflammatory disorders.

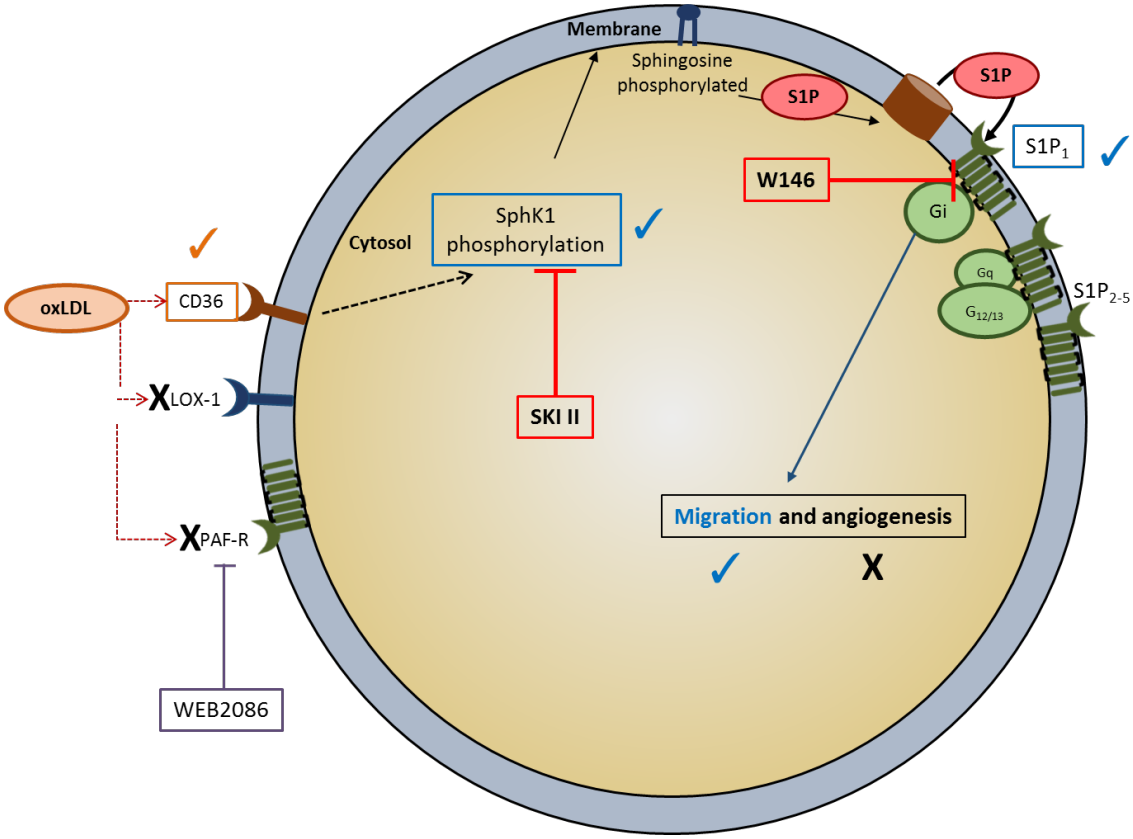


Figure 4-21 Hypothesised mechanisms of endothelial response to oxLDL: reflection

Following the results from the flow cytometry and endothelial migration studies, the conclusion of involvement of receptors and mediators in oxLDL signalling. '✗' suggests no role; '✓'suggests involvement, based on research observations.

5 Chapter 5: The role of lipids on endothelial-immune cell interactions in a model of flow-based recruitment

5.1 Introduction

With a sufficient vascular network and blood supply, one of the hallmarks of the inflammatory process is the adhesion of flowing leukocytes to the vascular endothelium. This orchestrated cascade of events requires the activation of the endothelium and leukocytes specifically to recruit the correct cells to the correct sites in the body. One of the most characterised molecules in the activation of the endothelium and enhancement of leukocyte recruitment is TNFα, which is a strong inflammatory stimulus, highly relevant to psoriasis and specifically regulates endothelial expression of adhesion molecules. Endothelial cells stimulated with TNFα have been reported to support ample neutrophil binding (Sheikh et al., 2003). The concentration of this activating signal however seems to cause slight differences in the recruitment seen *in vitro*. At relatively low concentrations of TNFα (2U/mL) there is robust recruitment under flow to the activated endothelium. At higher concentrations (for example at 100 U/mL) there is only a modest increase in endothelial recruitment of leukocytes (Sheikh et al., 2003) but at these elevated concentrations of TNFα, the ability of neutrophils to transmigrate through the endothelium has been reported to be enhanced. It was shown that around 5% of neutrophils transmigrate through an endothelium stimulated with 2 U/mL whereas with 100 U/mL, this transmigration figure rises to around 40% of the total bound neutrophils (Sheikh et al., 2003).

In psoriasis, it has been reported that endothelial function is significantly impaired in relation to the general population and preliminary evidence suggests that TNF inhibitors may improve this aspect of inflammation (Brezinksi et al., 2014). Given the role of TNFα in orchestrating the immune response and its importance in psoriasis, this cytokine can be utilised as a positive control and benchmark for endothelial activation and adhesive abilities. Other potent inflammatory stimuli include IL-1 and IL-6 which promote leukocyte activation and recruitment and are secreted by immune cells such as monocytes and macrophages in addition to endothelial cells themselves (Scheller et al.,

2011). Endothelial cells can also be activated by microbial products such as lipopolysaccharide (LPS) or viral products, which signal through the toll-like receptors (TLRs) (Dauphinee & Karsan, 2006). These TLRs are expressed by endothelial cells in the skin, allowing the endothelium to mount an increased or reduced inflammatory response based upon their specific exposure, making them an important part of the innate immune response in the skin (Fitzner et al., 2007).

A role has been characterised for lipids in the recruitment of inflammatory cells, particularly the family of eicosanoids, that are produced as a result of oxidation of fatty acids. The eicosanoids are a group of lipid mediators derived from arachidonic acid and are rapidly produced following activation of the cell, including immune cells and endothelium (Sadik & Luster, 2012). The lipid species are well-defined as chemoattractants which direct the adhesion and migration of immune cells (Sadik & Luster, 2012), but the role of other serum lipids is less well characterised. Data from work using the lipid lowering drugs statins suggests that there is a concomitant reduction in leukocyte recruitment (Osaka et al., 2013; Obama et al., 2004; Kwak et al., 2001) although whether this is due to direct effects on lipids or via the pleiotropic effects which are known to be associated with statin use is at present uncertain (Satoh et al., 2015).

A role for oxLDL in leukocyte recruitment has also been indicated. One of the first indications of oxLDL involvement in leukocyte-endothelial interactions was in a hamster model, in which oxLDL (through intravenous injection) was shown to cause an induction of leukocyte adhesion to the endothelium (Lehr et al., 1991). *In vitro*, the treatment of HUVEC with oxLDL has also been shown previously to activate neutrophils and support subsequent transmigration in a static model (Stroka et al., 2012). Although this was shown in a static model, HUVEC cultured in static conditions prior to the flow of neutrophils have been suggested to support greater adhesion and transmigration of neutrophils than if the endothelial cells are preconditioned to a physiological shear stress during culture (Sheikh et al., 2003). The treatment of HUVEC with oxLDL has been reported to increase the expression of both ICAM-1 and VCAM-1 in a concentration-dependent manner (Huang et al., 2012; Stroka et al., 2012). Using PCR, an up-regulation of mRNA and protein expression of MCP-1 and VCAM-1 genes after 24hours oxLDL incubation has also been reported (Feng et al., 2014). Of course, the extent of oxidation

of LDL is an important factor in the physiological response, along with the concentration used, as has been previously noted. It is therefore important not to generalise endothelial responses to oxLDL, for example in the references given above, there were different oxidation methods, incubation times and concentrations used. For example, in relation to the actual oxidation of LDL in these studies: Lehr et al. used 7.5μM copper sulphate for 6 hours, Stroka et al. used 25μM copper sulphate for 16hours, Feng et al. had no reference to their oxLDL method and Huang et al. referenced a purchase with no oxidation details. In this thesis, oxLDL oxidised using a copper sulphate method (10μM) for 18 hours will be analysed for its effect on endothelial function, representing an established oxidation of the lipids within the LDL molecule (Davis et al., 2008).

The recruitment of leukocytes to the site of inflammation requires the expression of adhesion molecules by the endothelium. There are a number of adhesion molecules that contribute to the inflammatory cascade and mediate different elements of leukocyte migration. The rolling phase of leukocyte recruitment, in which leukocytes come into close contact with the endothelium and roll along the surface, is classically mediated by the selectin family of adhesion molecules. The selectins are a family of molecules expressed on leukocytes, platelets and endothelium and identified as transmembrane glycoproteins which bind to carbohydrate structures (Ley, 2003). Selectins are involved in lymphocyte homing and both chronic and acute stages of inflammation in muscle, skin and mesentery vascular beds, although not in all vascular beds as the liver sinusoids do not recruit cells via endothelial selectin expression (Wong et al., 1997). Rolling is primarily supported by P-selectin and E-selectin which are expressed by the activated endothelium. P-selectin is stored in intracellular granules (Weibel-Palade bodies) and so can be expressed immediately upon activation. Conversely, E-selectin requires *de novo* synthesis and so upon activation, requires gene transcription, post-translational modification and subsequent transportation to the surface of the cell, a process generally occurring within three hours of activation (Yao et al., 1999). The selectins bind to surface receptors on the leukocyte, primarily PSGL-1. The firm adhesion to the endothelium is then mediated by a further group of adhesion molecules known as integrins. Endothelial adhesion molecules such as ICAM-1 and VCAM-1 bind to leukocyte integrins LFA-1 and VLA-4 respectively.

Such interactions have been shown to be important in the pathology of numerous inflammatory disorders including that of psoriasis. This has been known for some years, as it was shown that in normal and pre-lesional psoriatic skin, the expression of VCAM-1 and E-selectin were virtually absent, whereas, in the dermal microvasculature of lesional skin, there were significant increases in E-selectin and VCAM-1 expressing cells (Petzelbauer et al., 1994, Veale et al., 1995). The regulation of leukocyte infiltration into tissue is therefore a focus point for research and could provide realistic therapeutic targets for inflammatory diseases. In the build-up of atherosclerosis for example, LDL accumulation and subsequent oxidation is thought to be the major contributory factor to endothelial dysfunction leading to chronic inflammation. Within lesions in the vasculature, particularly found in areas of disturbed flow such as branching segments of the vessel, the recruitment of monocytes aids foam cell formation and disease progression (Chiu et al., 2011). The role of oxLDL is particularly important in relation to the expression of endothelial VCAM-1 and ICAM-1 (Mestas & Ley, 2008) in this progression. With oxLDL found in skin lesions of psoriasis patients and the relationship between lipid profiles and disease severity, the concept of a similar underlying pathology between atherogenic lesions and skin lesions appears valid. Understanding the role of oxLDL in immune cell recruitment (in a range of immune cell subtypes) could therefore aid the management of inflammation.

5.2 Methods

The flow-based adhesion assay was constructed in order to visualise the interactions between endothelial cells and immune cells, depicting the immune trafficking events occurring in the vessel during pathological conditions such as psoriasis. HUVEC were used as the model endothelium and the isolated immune subsets included either neutrophils or monocyte-depleted PBMCs, isolated as described in methods. HUVEC seeded into circular culture dishes were stimulated under static cell culture conditions and assembled into the flow assay design at the time point designated in each experiment. Immune cell isolations from whole blood were performed on the day of the experiment and were not separately stimulated in any experimental design. Immune cells were reconstituted in HBSS with 2% BSA for all experiments and perfused across the endothelial layer at a shear stress of 1 dyne/cm^2.

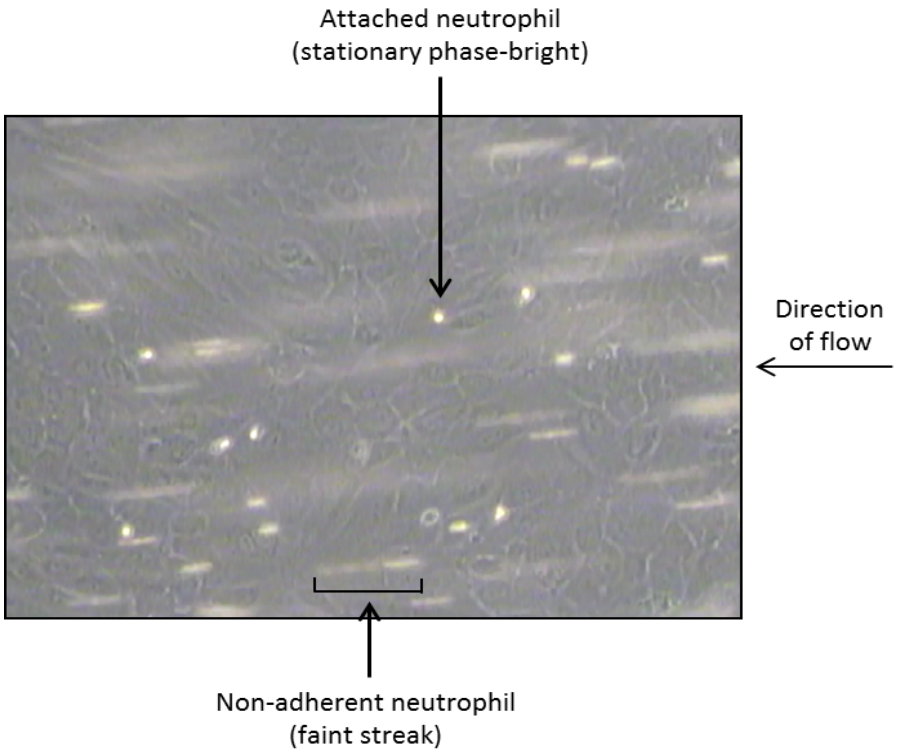


Figure 5-1 Adhesion of flowing neutrophils
Screen-shot image of flowing neutrophils across a confluent endothelial layer. Firm adhesion was characterised by stationary, phase-bright cells (attached for >10 seconds), distinguished easily from non-adherent cells shown by faint white streaks flowing past the field of view.

The figure above shows a screenshot image of neutrophils flowing across an endothelium. Each field of view (4 in total per culture) was recorded for 30 seconds and firmly adherent neutrophils (stationary on the endothelial surface) were counted. The videos were captured in real time at a frame rate of 29fps, which could adequately allow quantification of the leukocyte recruitment, although flowing leukocytes can be visualised as streaks when a screenshot is captured at this frame rate (as shown above). An image of a graduated haemocytometer was used to calibrate the software for the conversion of pixels to absolute distances. Therefore, for every field of view each pixel equated to 1.1μm and the neutrophil adherence was presented as mean neutrophil attachment per mm^2. Given the altered endothelial responses in lipid depleted cultures previously observed, the initial experimental set-up analysed control and delipidated cultures, with the addition of pro-inflammatory cytokines TNFα (1ng/mL) and IFNγ (10 Units/mL).

5.3 Results

5.3.1 Leukocyte recruitment to the endothelium

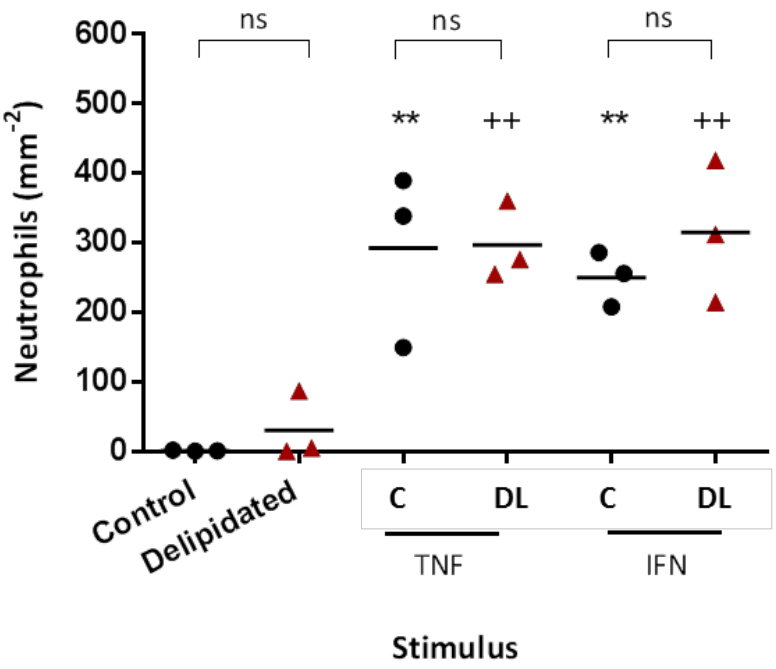


Figure 5-2 Neutrophil attachment to HUVEC following 4 hour cytokine stimulation in the presence and absence of serum lipids
Number of neutrophils attached to HUVEC under flow following static stimulation of endothelium with TNFα (1ng/mL) or IFNγ (10 Units/mL) for 4 hours in control or delipidated cultures as described. Statistics performed using one-way ANOVA with Sidak's post-hoc test **P<0.01 in comparison to 'control', ++P<0.01 in comparison to 'delipidated control', ns: not significant (n=3).

Neutrophil attachment to the endothelial layer was significantly increased in cultures containing TNFα or IFNγ at 4 hours, as would be hypothesised given that they are well established as pro-inflammatory cytokines. Statistics were performed in relation to the relevant control for unaltered and delipidated serum. The neutrophil attachment observed following endothelial stimulation for 4 hours was similar across TNFα and IFNγ stimulated cultures, although statistics were not performed on this aspect of the experiment given that they may not be used at equivalent or equimolar concentrations. The control culture in this experiment showed minimal neutrophil recruitment, indicating that cultured cells do not express the required adhesion molecules for leukocyte recruitment under basal flow conditions.

In the statistical analysis, there was no difference between unstimulated control and unstimulated delipidated cultures. There was also no significant difference when cells were stimulated with either cytokine in the absence of lipid for the four hours of incubation compared to cells stimulated in the presence of serum lipids. Having previously identified altered endothelial responses in lipid depleted cultures, the subsequent experiments were performed with increased duration of cytokine stimulation and culture in lipid depleted conditions from 4 hours to 24 hours. This allowed the identification of time-dependent effects of cytokine stimulated endothelial cells in their expression of adhesion molecules and in order to observe any chronic lipid-specific effects (shown in Figure 5-3). The two time points used also allow the interrogation of multiple adhesion molecules as E-selectin and VCAM-1 are more likely expressed at 4 and 24 hours respectively as well as long term effects on ICAM-1 expression, an important molecule controlling crawling and transmigration.

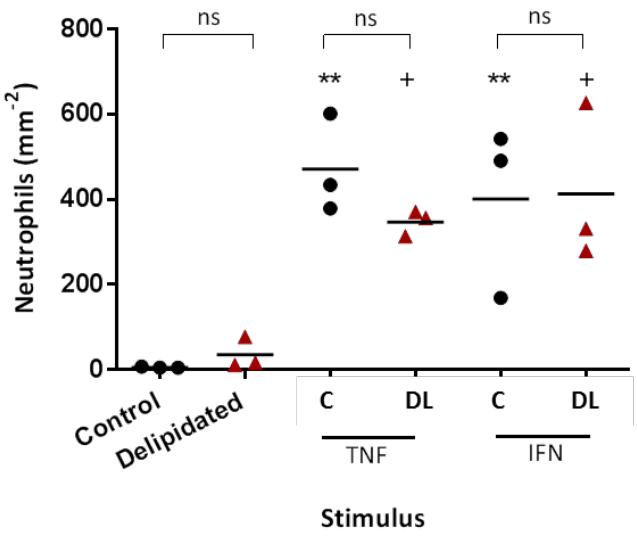


Figure 5-3 Neutrophil attachment to HUVEC following 24 hour stimulation
Number of neutrophils attached to HUVEC under flow after 24 hours following static stimulation of endothelium with TNFα (1ng/mL) or IFNγ (10 Units/mL) in control or delipidated cultures. Statistics performed using one-way ANOVA with Sidak's post-hoc test **P<0.01 in comparison to 'control', + P<0.05 in comparison to 'delipidated control' (n=3).

The results in Figure 5-3 show endothelial cells stimulated for 24 hours with TNFα or IFNγ support elevated neutrophil recruitment. The increase in neutrophil recruitment was

significant in relation to the control culture, to which there was minimal neutrophil binding. The binding of neutrophils to endothelial cells in TNFα and IFNγ stimulated cultures at 24 hours was slightly higher (~400 neutrophils/mm^2) than the number of neutrophils attached to endothelial cells after 4 hours of stimulation (~300 neutrophils/mm^2) but this was not significant for either stimulus (unpaired two-tailed T-test comparing TNFα-recruitment at 4 hours to TNFα-recruitment at 24hours; unpaired two-tailed T-test comparing IFNγ-recruitment at 4 hours to IFNγ-recruitment at 24hours). Similar to the results of 4 hour endothelial stimulation, the results for 24 hour stimulation showed that delipidated cultures were not significantly different from control cultures.

These results indicated that serum lipids were not integral to the up-regulation of adhesion molecules by the endothelium under cytokine stimulation. In the angiogenesis and endothelial migration assays, lipid depletion significantly reduced these responses, suggesting an altered inflammatory capability. In these assays it was noted that lipid depletion neither enhanced nor diminished the recruitment of flowing leukocytes in unstimulated or stimulated cultures. The pro-inflammatory effect of ox-/LDL could subsequently be analysed in control cultures (to be discussed) as no altered response was observed in the absence of lipids.

Having established the assay for its ability to adequately analyse immune cell attachment to the endothelium, showing minimal adherence in control cultures and significant attachment in 'positive control' cultures (TNFα and IFNγ); the assay could be used to analyse other lipid stimuli. One of the adhesion molecules relevant to leukocyte attachment to the endothelium is E-selectin (CD62E), binding to ligands such as PSGL-1 on immune cells. In order to evaluate the role of E-selectin in immune cell capture in the flow-based adhesion assay, a mouse-derived anti-human E-selectin blocking antibody was added (for 30 minutes prior to flow) to TNFα or IFNγ stimulated cultures with the assessment of neutrophils (Figure 5-4) and monocyte-depleted PBMCs (Figure 5-5) perfused across the endothelium.

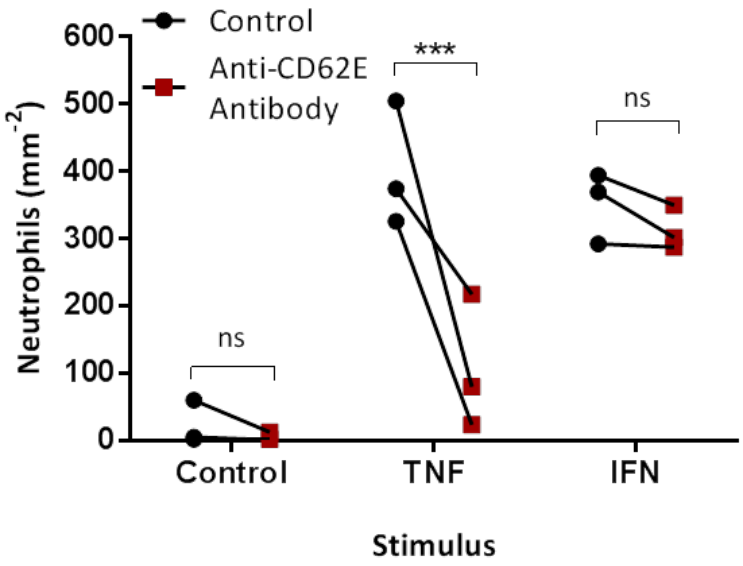


Figure 5-4 The role of E-selectin (CD62E) in neutrophil attachment following 4 hours stimulation of the endothelium
Endothelial cells were stimulated with TNFα (1ng/mL) or IFNγ (10 U/mL) for 4 hours. An E-selectin blocking antibody was added 30minutes prior to perfusion of freshly isolated neutrophils and counts made as described previously. Statistics performed using one-way ANOVA with Sidak post-hoc test. ***P<0.001 (n=3).

Figure 5-4, the addition of an E-selectin blocking antibody to the TNFα stimulated cultures significantly decreased the number of neutrophils bound to the endothelium following 4 hours stimulation. This indicated that the E-selectin adhesion molecule is important in neutrophil recruitment to the endothelial layer and is expressed on the surface of the endothelial cell after 4 hours of stimulation. The control culture had minimal neutrophil recruitment, indicating a lack of basal expression of this molecule in unstimulated endothelial cells. The IFNγ-stimulated cultures however, did not show inhibited recruitment following E-selectin blockade, suggesting a possible role for other adhesion molecules in the capture of neutrophils with this stimulus or insufficient blockade with this amount of antibody with this level of IFNγ stimulation.

This experimental design was repeated with the use of monocyte-depleted PBMCs in order to evaluate whether blocking E-selectin would have similar effects on recruitment over multiple immune subsets. Figure 5-5 shows that, compared to neutrophil recruitment under the same stimulating conditions, monocyte-depleted PBMCs

(lymphocytes) showed a reduced mean cell number recruited (TNFα recruitment: 296±55 compared to 43±23; IFNγ recruitment: 249±39 compared to 72±44 for neutrophil and monocyte-depleted PBMC recruitment respectively, means±SD for n=3). In the presence of an E-selectin blocking antibody at equivalent concentrations used previously for neutrophil blockade, there was no statistically significant difference between monocyte-depleted PBMC recruitment to TNFα or IFNγ-stimulated cultures with or without the blocking antibody. Given that no difference was observed between control and delipidated cultures in previous experiments, all E-selectin blocking analysis was performed in control serum cultures.

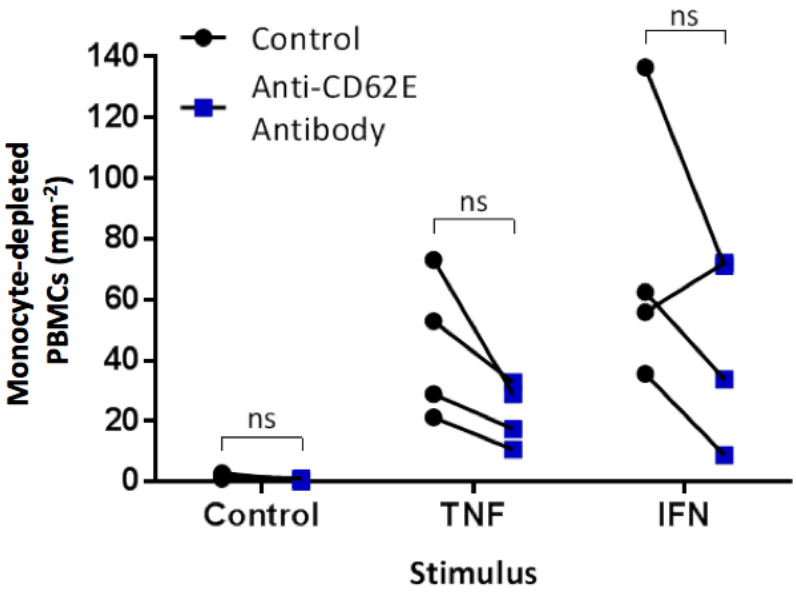


Figure 5-5 The role of E-selectin in monocyte-depleted peripheral blood mononuclear cells (PBMC) attachment to HUVEC following 4 hour stimulation of the endothelium
Using the same design as the neutrophil attachment assay, PBMCs (monocyte-depleted) were perfused across TNFα (1ng/mL) or IFNγ (10 U/mL) stimulated endothelium for 4 hours and counted as described. Statistics performed using one-way ANOVA with Sidak post-hoc test. ns: not significant (n=4).

Figure 5-5 shows the numbers of immune cells attached to the endothelium following perfusion. Compared to isolated neutrophil attachment, monocyte-depleted PBMCs were shown to be reduced in numbers and although the trend of E-selectin blockade is towards inhibition suggesting a role for E-selectin in the recruitment of monocyte-depleted PBMCs, there was no statistical significance.

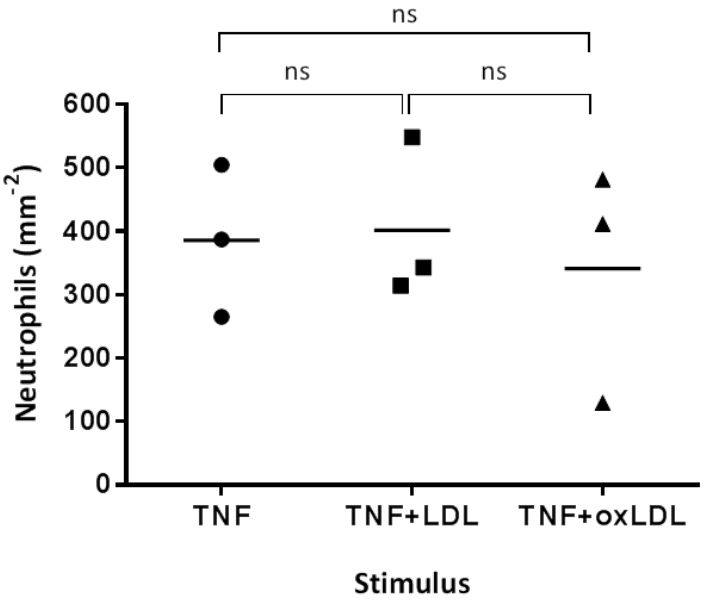


Figure 5-6 The effect of TNFα and ox-/LDL on neutrophil attachment to endothelium following 4 hour stimulation
The effect of adding LDL (10μg/mL) or oxLDL (10μg/mL) to TNFα (1ng/mL) stimulated endothelial cells was assessed. Data shows mean values. Statistics performed using one-way ANOVA using Tukey's post-hoc test. ns: not significant (n=3).

Using the flow-based recruitment assay, TNFα was observed to significantly increase neutrophil recruitment in comparison with control. The addition of oxLDL or LDL was subsequently analysed for any potential effects in the recruitment assay including an enhancement of cytokine stimulation or indeed blockade of cytokine activity due to diversion of function. Ox-/LDL at a concentration of 10μg/mL did not significantly affect the neutrophil adherence to the endothelium above that of TNFα following 4 hours stimulation, again performed in control (unaltered) serum. The role of varying concentrations of ox-/LDL alone was subsequently analysed in the recruitment assay to determine direct ox-/LDL effects on endothelial activation and recruitment of inflammatory cells and the results are presented in Figure 5-7.

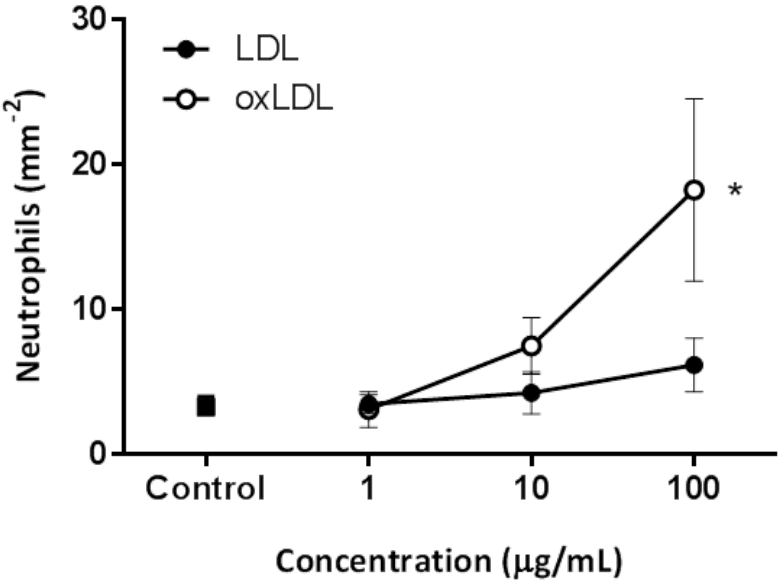


Figure 5-7 The effects of ox-/LDL concentrations on neutrophil attachment following 4 hour stimulation of HUVEC

Endothelial cells stimulated with ox-/LDL at concentrations of 1, 10 and 100µg/mL for 4 hours, performed in 20% human serum-containing media. The mean number of neutrophils attached to the endothelium following perfusion as described is shown. Data shows mean +/- SEM. Statistics performed with one-way ANOVA with Tukey's post-hoc test. *P<0.05 (n=5).

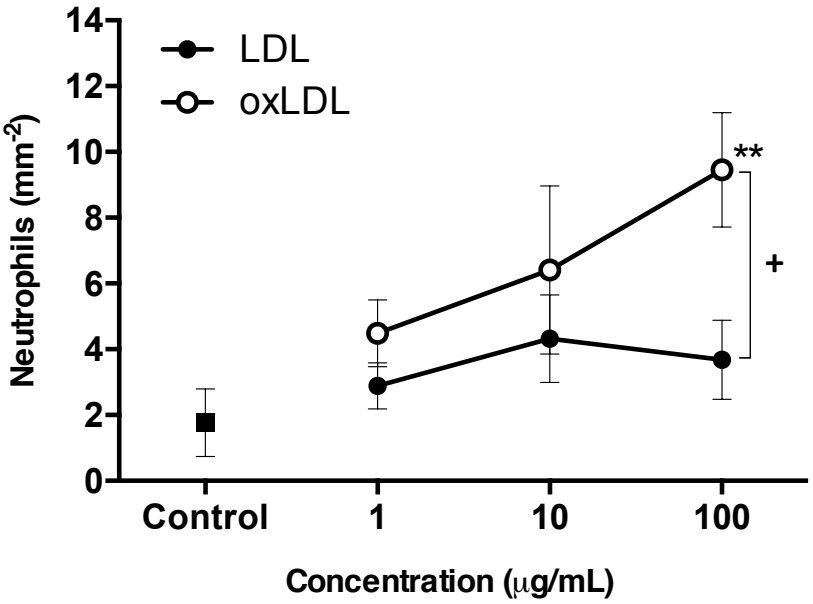


Figure 5-8 The effects of ox-/LDL concentrations on neutrophil attachment following 24 hour stimulation of HUVEC
Endothelial cells stimulated with ox-/LDL at concentrations of 1, 10 and 100μg/mL for 24 hours, performed in 20% human serum-containing media. The mean number of neutrophils attached to the endothelium following perfusion as described is shown. Data shows mean +/- SEM. Statistics performed with one-way ANOVA with Tukey's post-hoc test. **P<0.01 +P<0.05 (n=6).

In Figure 5-7, the effect of ox-/LDL stimulation of HUVEC for 4 hours produced relatively small numbers of recruited neutrophils to the endothelial surface (<30/mm^2) in comparison with previous results for TNFα stimulation (>300/mm^2). The result for oxLDL stimulation at a concentration of 100μg/mL, however, was statistically significant in comparison with the control culture. Endothelial cells stimulated for 24 hours with ox-/LDL also showed similar results, with oxLDL at 100μg/mL being statistically increased in comparison with control (as shown in Figure 5-8) and compared to LDL at the same concentration. These results indicate that ox-/LDL is not acting as a chronic and prolific activator of endothelial dependent recruitment when compared to cytokine stimulation like TNF, but an increase in basal recruitment may contribute to a continuous state of inflammation or potentiate other endothelial activators.

5.3.2 Influence of neutrophil count on adhesion

Neutrophils were isolated from various blood donors and it was noted that numbers of neutrophils varied between donors. To determine if this was a factor in neutrophil

trafficking, a correlation between the number of neutrophils isolated from the same volume of blood (30mL) and the number of neutrophils attached to the endothelium was plotted (Figure 5-9). The total neutrophil count isolated from whole blood appeared to have no correlation with the adhesion properties of the neutrophils following 4 hour stimulation of HUVEC with TNFα (1ng/mL), analysed with the flow-based adhesion assay, as indicated in Figure 5-9.

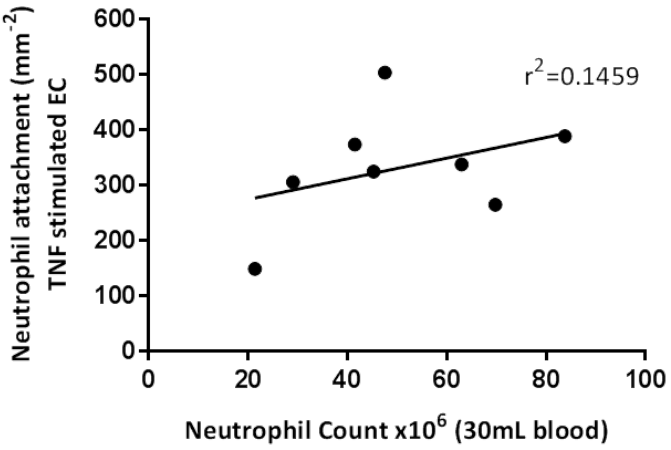


Figure 5-9 Relationship between neutrophil count in blood and neutrophil adherence to TNFα stimulated (1ng/mL) endothelium for 4 hours.
Isolated neutrophil number from 30mL of blood for each donor was plotted against adherent neutrophils. The correlation was then calculated and given as 0.146 (n=8).

5.3.3 Neutrophil transmigration

Following adhesion, neutrophils can transmigrate through the endothelial barrier into the tissue using a regulated process. The analysis of leukocyte transmigration through and under the endothelial monolayer was determined *in vitro* to show if the more active process of transmigration (compared to tethering and rolling where activation occurs in the leukocyte) was affected by the prior incubation in lipid-depleted cultures. The figure below represents a screenshot of a field of neutrophils which have attached to the endothelial surface and some which have migrated underneath the surface. The screenshots on the right-hand side reference the time taken for a neutrophil to completely transmigrate under the surface, from 3minutes 28 seconds in part A to 9 minutes 28 seconds in part C, shown in Figure 5-10 (~6 minutes calculated from initial

attachment observed to the endothelial monolayer up until complete transmigration, observed by phase-dark appearance).

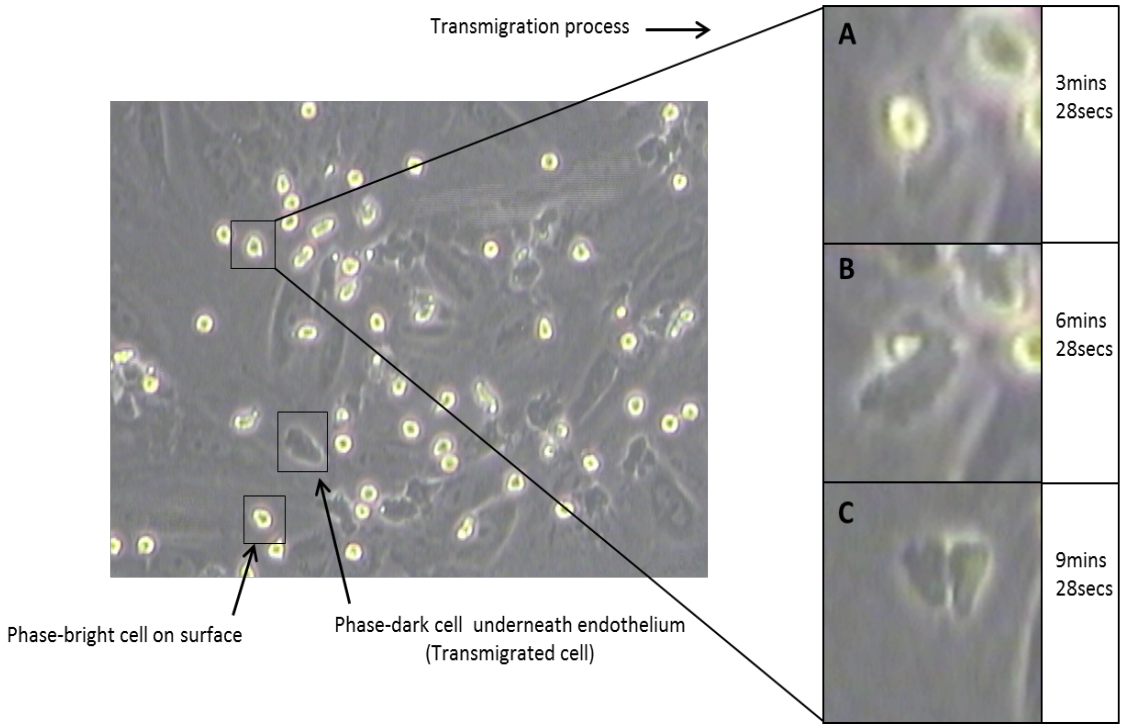


Figure 5-10 Transmigration of adherent neutrophils
Following neutrophil attachment to the endothelial layer, cells were assessed for their ability to transmigrate through and under the endothelium, shown by their change in appearance from phase-bright to phase-dark. The neutrophil in the main image was attached to the endothelium at the start of the video (time 0min) and once it begins transmigration (A- 3min 28sec) it is fully transmigrated (C- 9min 28 sec) within a 6minute time period. The neutrophil was captured from the flow and the flow was maintained throughout the experiment.

Having established the transmigration ability of neutrophils in the flow-assay design, it was possible to quantify this process. The neutrophils attached to the endothelial layer following capture under perfusion for the previous six minutes as described in methods were counted and deemed time 0 minutes. At this point, HBSS containing 2% BSA alone was perfused over the monolayer for a further 30 minutes at which point the total number of transmigrated cells was counted. The results were then represented as raw numbers of neutrophils which migrated, in addition to the percentage of transmigrated cells as a proportion of the total cells counted as adherent at time 0 minutes. This was to allow for any wide differences in the initial adherent neutrophil numbers at time 0 minutes. The initial experimental design involved the comparison between control and

delipidated cultures at 24 hours, which were analysed with the addition of TNFα in order to allow sufficient initial neutrophil binding to calculate transmigration (as shown in Figure 5-11). The subsequent analysis involved the addition of 10µg/mL ox-/LDL to TNFα stimulated cultures for 4 hours (Figure 5-12).

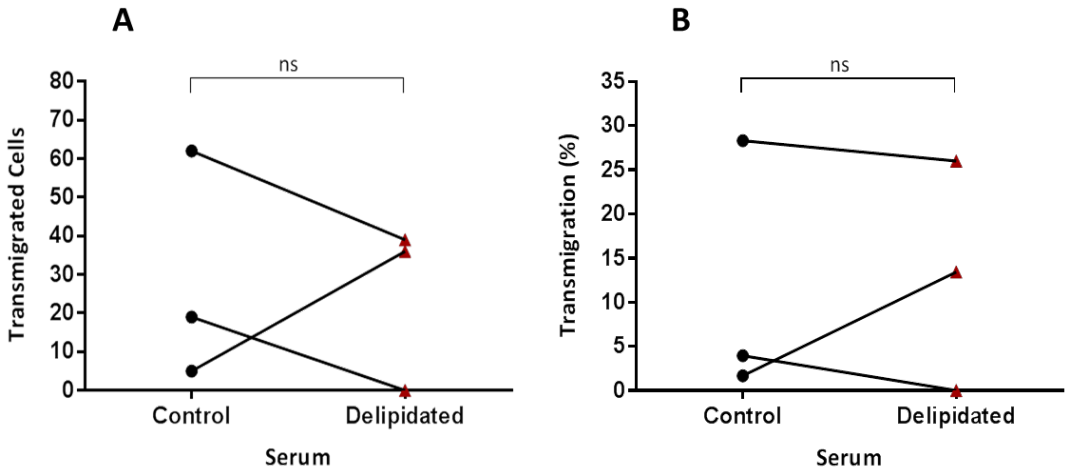


Figure 5-11 Transmigration of neutrophils after 24hours of TNFα -stimulated HUVEC in control and delipidated cultures
Following adherence of neutrophils to 24 hour TNFα stimulated (1ng/mL) endothelium, transmigration was viewed using video microscopy for 30 minutes. Results expressed as raw numbers of transmigrated cells at 30min post adhesion (A) and as a percentage of transmigrated cells at 30min post adhesion compared to the total adherent cell number at time 0min (B). Statistics performed using unpaired, two-tailed T test (n=3).

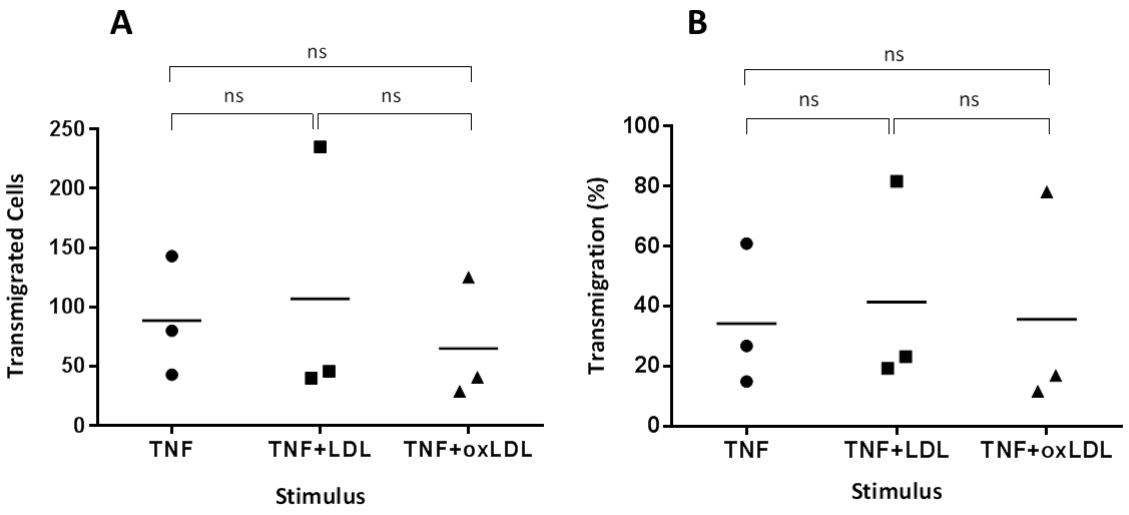


Figure 5-12 Neutrophil transmigration across HUVEC following 4 hour stimulation with TNFα and ox-/LDL
Neutrophil transmigration was analysed in 4hour TNFα stimulated endothelium with the addition of LDL (10μg/mL) or oxLDL (10μg/mL). All cultures were performed in control serum. Results expressed as raw numbers of transmigrated cells at 30min post adhesion (A) and as a percentage of transmigrated cells at 30min post adhesion compared to the total adherent cell number at time 0min (B). Data shows means. Statistics performed with one-way ANOVA with Tukey's post-hoc test (n=3).

The transmigration analysis of neutrophils concluded that the culture of cells in lipid-depleted serum did not change the ability of endothelial cells to support transmigration following stimulation with TNFα as shown in Figure 5-11. The addition of LDL or oxLDL at a concentration of 10μg/mL to TNFα stimulated cultures also had no significant effect on the ability of endothelial cells to support transmigration of neutrophils. The lack of statistical significance in relation to ox-/LDL stimulus and transmigration may be unsurprising considering that there was a wide variability depending on the combination of endothelial- and immune cell-donor used for each experiment, as the results ranged from <20% transmigration to >60% transmigration of neutrophils through the endothelial monolayer. Despite the variation between experiments, it was concluded that a change caused by ox-/LDL at this concentration (10μg/mL) was unlikely, given that within each experiment there were no obvious differences observed or even suggestive trends. There was also no effect of serum delipidation suggesting the endothelium maintained its cytokine responsive ability despite the lack of serum lipids in the culture. This also

suggests that endothelial molecules involved in regulating immune cell transmigration such as the JAMs and cadherins are likely unaffected by lipid depletion or ox-/LDL, with barrier permeability still intact.

5.3.4 Analysis of endothelial adhesion molecules using flow cytometry

Having identified a basal increase in neutrophils attached to the endothelial layer in the presence of oxLDL at 100µg/mL, the analysis of endothelial adhesion molecule expression was sought using flow cytometry. The antibody panel used for the assessment of adhesion molecules included E-selectin, ICAM-1, VCAM-1, CD31 and a live/dead stain. All antibodies had previously been optimised in-house, except the E-selectin antibody. The titration of this antibody therefore was carried out with the endothelial cells activated for 4 hours with TNFα (Figure 5-13).

The E-selectin antibody (eBioscience, PE) titration experiment as shown (Figure 5-13) indicated that the staining of E-selectin was positive at 1µL/test (100µL) only in TNFα activated cells and so this was chosen as the optimal concentration for analysis using flow cytometry. Cells were stimulated with TNFα for 4 hours in order to give positive E-selectin staining, as indicated in previous experiments with the E-selectin blocking antibody. All other antibodies had been optimised prior to use. The evaluation of adhesion molecule expression was performed using 5-colour flow cytometry. Part D in the diagram highlights that endothelial cells which are not stimulated do not express E-selectin to any appreciable extent, shown in orange. This is supportive of the earlier data in the recruitment assay showing that unstimulated endothelial cells do not capture flowing leukocytes.

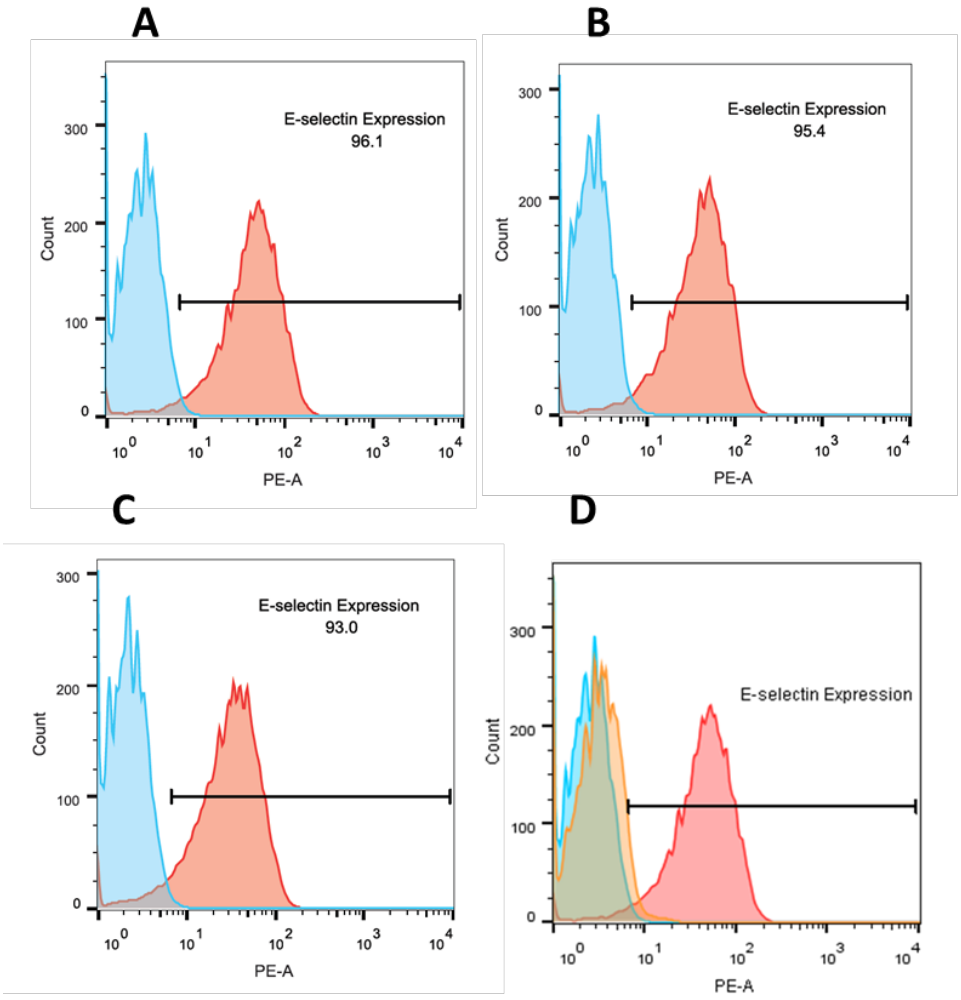


Figure 5-13 E-selectin antibody titration
Endothelial cells were incubated with TNFα (1ng/mL) for 4 hours followed by staining for E-selectin using a fluorescently labelled antibody using diluting concentrations of anti-E-selectin antibody of 5μL/test (A), 2.5μL/test (B) and 1μL/test (C). Flow cytometric analysis compared cells stained with an isotype control, in addition to non-activated control cells and unstained cells. Shown (A,B,C) is a representative flow cytometry histogram of positive staining for E-selectin from 4 hour stimulation of the endothelium with TNFα (red) in comparison with an isotype control antibody (blue). Part D represents the addition of unstimulated endothelial cells (orange) to the histogram.

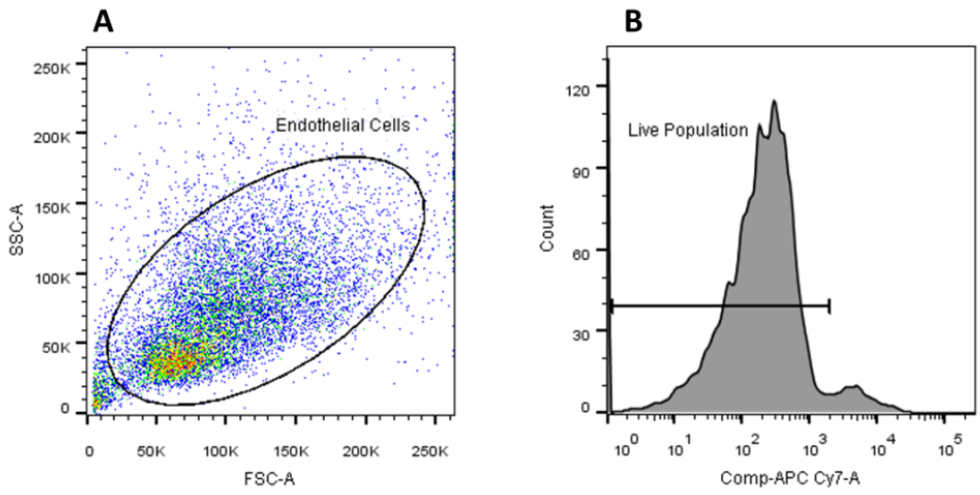


Figure 5-14 Endothelial cell gating strategy using flow cytometry
Endothelial cells were gated in terms of their forward/side scatter as shown in a typical example (A) to exclude unwanted cell debris. They were subsequently gated to exclude dead cells (B), as live cells would adequately prevent the uptake of the live/dead stain and have a lower fluorescence. After this gating strategy, endothelial cells could then be analysed for their expression of adhesion molecules and markers of interest.

Figure 5-14 shows the gating strategy used in flow cytometry for the analysis of endothelial cells. The staining protocol for endothelial cells can be found in the methods section (chapter 2). Prior to sample loading in the flow cytometer, the neutral density filter was changed from the default 1.0 device to the 2.0 filter. This controls the optical sensitivity for the forward light scatter in order for the larger endothelial cells (in comparison to immune cells) to be adequately visualised. The endothelial cells were then gated as shown in order to eliminate any cell debris and the live population of cells were determined by the used of live/dead staining, with viable cells preventing the inclusion of the stain and having a lower fluorescence.

Having established the set-up of the flow cytometer, the gating strategy and the required compensation controls, endothelial cells were analysed in terms of the markers previously mentioned and are presented in Figure 5-15, Figure 5-16, Figure 5-17 and Figure 5-18. The graphs represent the mean fluorescence intensity (MFI) observed after 4, 8 and 24 hour stimulation of HUVEC with TNFα or IFNγ (graphs labelled A) and ox-/LDL at 10 or 100µg/mL (graphs labelled B).

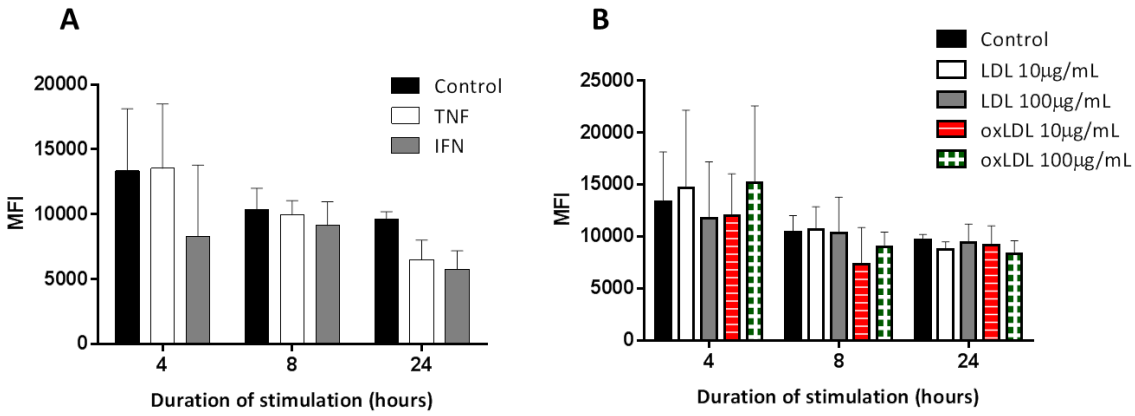


Figure 5-15 Time course analysis of HUVEC expression of CD31
Expression of CD31 on endothelial cells analysed using flow cytometry following stimulation with TNFα (1ng/mL), IFNγ (10U/mL), ox-/LDL at 10 or 100µg/mL or unstimulated (control) cultures. Data expressed as mean fluorescence intensity (MFI) derived from the geometric mean of APC fluorescence intensity. Separate HUVEC donors at each time point analysed. Data represents mean +/- SD. Statistics performed using two-way ANOVA with Dunnett's post-hoc test. No statistical significance (n=3).

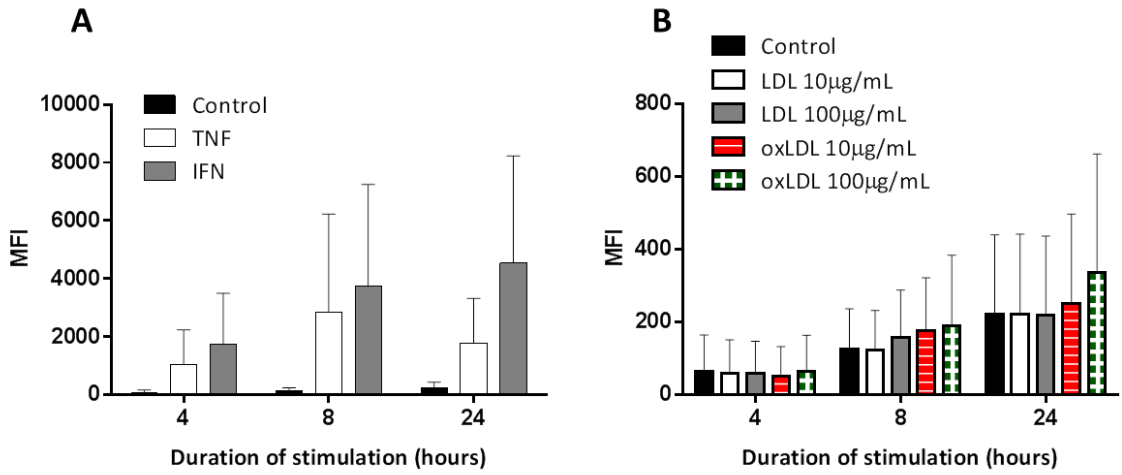


Figure 5-16 Time course analysis of HUVEC expression of E-selectin
Expression of E-selectin (CD62E) on endothelial cells analysed using flow cytometry following stimulation with TNFα (1ng/mL), IFNγ (10U/mL), ox-/LDL at 10 or 100µg/mL or unstimulated (control) cultures. Data expressed as mean fluorescence intensity (MFI) derived from the geometric mean of PE fluorescence intensity. Separate HUVEC donors at each time point analysed. Data represents mean +/- SD. Different Y-axis scales between graphs A and B. Statistics performed using two-way ANOVA with Dunnett's post-hoc test. No statistical significance (n=3).

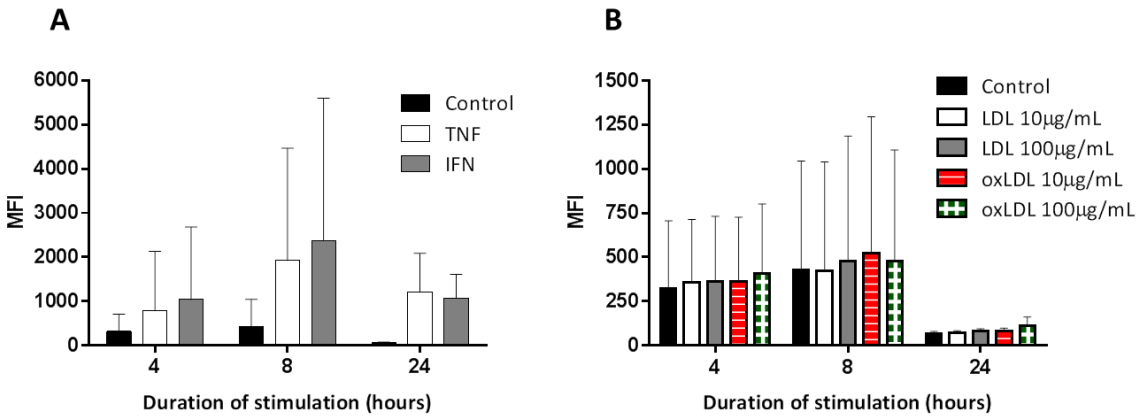


Figure 5-17 Time course analysis of HUVEC expression of ICAM-1
Expression of ICAM-1 on endothelial cells as analysed using flow cytometry following stimulation with TNFα (1ng/mL), IFNγ (10U/mL), ox-/LDL at 10 or 100µg/mL or unstimulated (control) cultures. Data expressed as mean fluorescence intensity (MFI) derived from the geometric mean of Pacific Blue fluorescence intensity. Separate HUVEC donors at each time point analysed. Data represents mean +/- SD. Different Y-axis scales between graphs A and B. Statistics performed using two-way ANOVA with Dunnett's post-hoc test. No statistical significance (n=3).

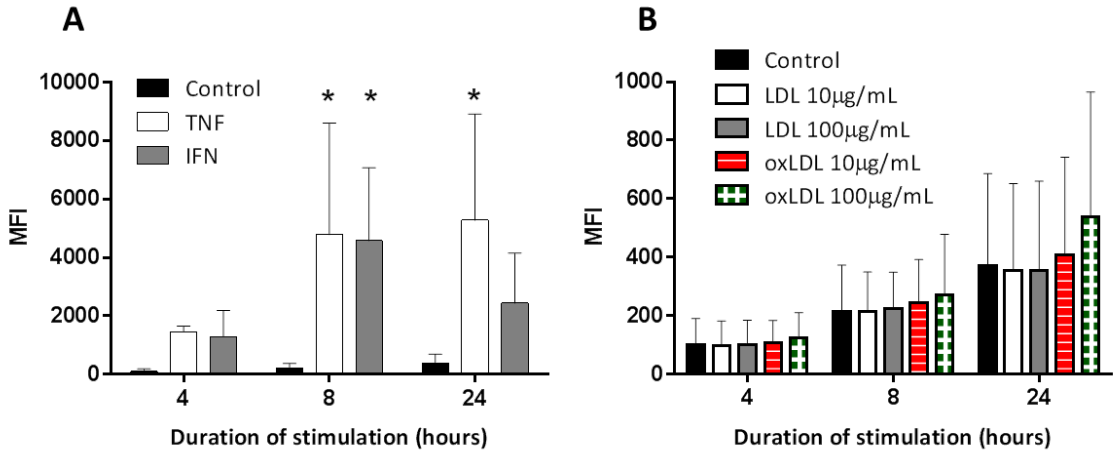


Figure 5-18 Time course analysis of HUVEC expression of VCAM-1
Expression of VCAM-1 on endothelial cells as analysed using flow cytometry following stimulation with TNFα (1ng/mL), IFNγ (10U/mL), ox-/LDL at 10 or 100µg/mL or unstimulated (control) cultures. Data expressed as mean fluorescence intensity (MFI) derived from the geometric mean of FITC fluorescence intensity. Separate HUVEC donors at each time point analysed. Data represents mean +/- SD. Different Y-axis scales between graphs A and B. Statistics performed using two-way ANOVA with Dunnett's post-hoc test where *P<0.05 in comparison to control cultures at each respective time point (n=3).

The flow cytometry data assessed the effect of native and oxidised LDL on endothelial adhesion molecule expression in comparison with the changes induced by known pro-inflammatory cytokines. There was no significant effect of either LDL or oxLDL on CD31, E-selectin, ICAM-1 or VCAM-1 at either 10 or 100μg/mL concentrations at the time points indicated. TNFα and IFNγ produced increases in the mean MFI for E-selectin, ICAM-1 and VCAM-1 as expected although only significance was found in VCAM-1 analysis, potentially being affected by larger error between samples and therefore requiring further repeated experiments.

5.4 Discussion

The recruitment of neutrophils to the site of inflammation is crucial to both the normal response to injury and the pathogenesis of multiple disorders including psoriasis. The recruitment of immune cells to the endothelial monolayer is a process which is dependent on the expression of endothelial adhesion molecules in order to capture flowing leukocytes. The *in vitro* flow-based adhesion assay depicts these *in vivo* interactions between the two cell types to visually record and analyse the recruitment process. Consistent with scientific literature (McLoughlin et al., 2003; Griffin et al., 2012), incubating endothelial cells with TNFα or IFNγ significantly increased the recruitment of neutrophils. Active endothelial processes previously investigated such as angiogenesis and migration showed an inhibited response in the absence of serum lipids. The data observed in the neutrophil recruitment assay, however, showed no significantly altered response in the ability of endothelial cells to recruit neutrophils when activated by cytokines in the absence of serum lipids at 4 and 24 hours respectively. This indicated that exogenous serum lipids are not required for the up-regulation of endothelial adhesion molecules. This has implication for strategies that seek to change serum lipids either by nutritional restriction/supplementation or by selective drug blockade of lipid synthesis. However, this mechanism of recruitment is still required for the normal function of the immune system and that serum lipid depletion did not abrogate chronic inflammatory signals this may point to a more specific role for serum lipids in angiogenesis rather than a pan specific effect on endothelial functions.

The two different time-points of 4 hours and 24 hours were used to represent differential expression windows for activation dependent endothelial adhesion molecules. At 4 hours post-stimulation, the E-selectin (CD62E) molecule has been shown to reach a maximal expression on HUVEC *in vitro* (Strindhall et al., 1997), whereas from this time point to 24 hours, E-selectin expression has been shown to decline, with other adhesion molecules such as ICAM-1 and VCAM-1 predominantly expressed over the extended time period (Zhu et al., 2012). However, in this investigation no decline of E-selectin was observed after 24 hours, as consistent E-selectin levels were observed at 8 hours and 24 hours following stimulation of the endothelium with TNFα or IFNγ. E-selectin has been reported to be reach maximal levels at 2-4 hours but returned to baseline by 24 hours post-

stimulation (Guangyao et al., 2005) although in this investigation the surface expression was still evident. Indeed, it actually appeared that E-selectin expression continued to increase in control cultures concomitantly with incubation time beyond 4 hours. At 24 hours stimulation, E-selectin expression in TNFα-stimulated cultures showed a downward trend in comparison to 8 hour stimulation, whereas IFNγ-stimulated cultures trended towards even further expression of E-selectin, suggesting differential responses to these cytokines. Furthermore, VCAM-1 expression showed differential responses to these two cytokines following 24 hour stimulation, this time with TNFα-stimulated cultures trending further expression with IFNγ showing less expression than at the 8 hour incubation time point.

This thesis investigated the chronic activation of endothelial cells but a consideration of an acute activation may also be required. Further experiments would have to be performed to analyse P-selectin expression in response to lipids, given that it is stored in endothelial cells and rapidly expressed, requiring acute stimulation of endothelial cells. It has been suggested that oxLDL causes immediate recruitment of leukocytes to the endothelium following intravenous injection of oxLDL in a hamster model (Lehr et al., 1991) and this was followed up by a similar study showing that this recruitment was significantly reduced by pre-treatment with an anti-P-selectin antibody (Lehr et al., 1994). It was later shown in an atherosclerotic mouse model that P-selectin blockade inhibited mononuclear cell attachment and rolling and VCAM-1 blockade separately increased rolling velocities, suggesting P-selectin may play a role in the initial capture and VCAM-1 stabilises this interaction (Ramos et al., 1999). In this investigation, VCAM-1 and E-selectin appeared to be expressed more by HUVEC in response to oxLDL (100μg/mL), even if these findings were not statistically significant, whereas ICAM-1 appeared to decrease at 24hours overall and showed no real enhancement in oxLDL-stimulated cultures. The incubation of endothelial cells with oxLDL for longer durations was implemented to mimic the oxLDL accumulation in the tissue (such as in psoriatic skin or within an atherosclerotic region) and the effects it would have on the recruitment of immune cells, assuming this type of accumulation would be constantly present in these patients. However, the release of such accumulations into the vascular circulation potentially from plaque instability or acute oxidation of circulating LDL may also cause an

acute inflammatory response. This could be identified through research on oxLDL-stimulated endothelium by detecting the rapid expression of P-selectin on cells, von Willebrand factor release and P-selectin blockade.

The role of E-selectin in the recruitment of neutrophils was analysed using a blocking antibody in the flow assay. The recruitment of neutrophils in unstimulated (control) cultures was minimal in all studies, indicating the lack of basal expression of pro-recruiting adhesion molecules by endothelial cells. The addition of anti-CD62E antibody therefore had no effect on recruitment in these cultures. The recruitment of neutrophils to TNFα-stimulated cultures was significantly decreased in the presence of an anti-CD62E antibody compared to TNFα-stimulated cultures alone, indicating this molecule plays a vital role in neutrophil recruitment. Surprisingly, CD62E blockade showed no effect on neutrophil recruitment in IFNγ-stimulated cultures. The up-regulation of E-selectin by IFNγ was subsequently shown using flow cytometry, indicating that this molecule was expressed. The percentage of cells expressing E-selectin and mean fluorescence intensity (MFI) data actually showed higher expression levels for IFNγ-stimulated endothelium compared to TNFα-stimulated cells at the concentrations used, making the lack of inhibition in the flow assay an unexpected result. However, this may be due to the blocking antibody not being able to block all E-selectin molecules due to the concentration used. Assessing E-selectin expression by flow cytometry in the presence of the blocking antibody may give some insight into the blocking ability of the antibodies used and by repeating the recruitment assay in the presence of increased concentrations of antibody. It is also possible that other mechanisms of recruitment are sufficient even in the blockaded cultures and this would need to be investigated further.

Both ICAM-1 and VCAM-1 expression was shown to be increased following stimulation with both cytokines, which might suggest a role for further adhesion molecules or change in affinity. During the process of leukocyte adhesion and transmigration, the cascade of molecular interactions has dynamic changes in terms of affinities of receptors to each other, allowing the rolling of immune cells along the endothelial monolayer (which could include multiple endothelial cells), subsequent firm adhesion and transmigration. The results from the E-selectin blocking experiments, showing differences in activated

endothelium (via TNFα and IFNγ-stimulation) may lead to the assumption that receptors possess different affinities for their respective molecules. Indeed, it has been evidenced that leukocytes adhere more strongly to co-immobilised ICAM-1 and VCAM-1, relative to their adhesion to either molecule alone (Chan et al., 2000). The type of stimulus which causes the endothelial cell to become activated may therefore not only up-regulate these adhesion molecules (which can be analysed using flow cytometry) but also change their affinities for rolling leukocytes, both independently and in combination with other adhesion molecules (such as junctional adhesion molecules). There may also be a consideration to the fact that 1ng/mL TNFα and 10 Units/mL IFNγ may not be equivalent in relation to their inflammatory stimulus, although these concentrations had been previously reported within the laboratory to cause inflammatory responses in endothelial cells and similar adhesion molecule expression and numbers of recruited neutrophils suggest that a similar level of activation is achieved with the two cytokines.

The results from the serum lipid-depletion experiments indicated that exogenous lipids within the serum do not affect the ability of endothelial cells to up-regulate adhesion molecules (over a period of 24 hours) as mentioned. However, there may be an integral role for lipids in leukocyte trafficking, particularly concerning endothelial membranes and their expression of adhesion molecules. It is known that cellular membranes adhere to the fluid mosaic model, in which receptors can relocate to specific areas of the membrane depending on the stimulus. Recently, it was shown that in dendritic cells, ICAM-1 can be constrained by limiting its mobility, which is regulated by different molecules such as α-actinin-1 (Comrie et al., 2015). If the lipid environment within the membrane changes, by factors such as cholesterol loading (or insufficient lipid synthesis or depletion), then the ability of adhesion molecules to relocate may be affected. It was also shown in previous chapters that endothelial migration (another element of the endothelial response which may relate to adhesive behaviour and adhesion molecules) induced by oxLDL can be inhibited by blocking S1P production and signalling. It has been reported that S1P has effects on the endothelial cytoskeleton, particularly at the periphery of the cell, stimulating actin cytoskeleton and focal adhesions (Zhang et al., 2016). With oxLDL increasing endothelial migration through S1P, causing slight up-regulation of adhesion molecules and increasing the functional recruitment of immune

cells, it could be postulated that lipids (and oxidised lipids) which stimulate the endothelial cell have major roles in membrane co-ordination of adhesion molecules, which alters the inflammatory response.

When monocyte-depleted PBMC recruitment was investigated, there was a significant recruitment of monocyte-depleted PBMCs to the cytokine activated endothelium. However, the E-selectin blocking antibody failed to show significant blockade of monocyte-depleted PBMC recruitment. The fact that no significance was observed in the these data may be explained in part by the total number of immune cells attached to the endothelial surface. With neutrophil perfusion, there is significant (>300/mm^2) attachment to the endothelium, but with monocyte-depleted PBMC perfusion the numbers are considerably lower (<~100/mm^2) meaning the sensitivity of the assay to detect changes is limited and would require further repeated experiments. The impact of lipid additions such as ox-/LDL may therefore be more adequately analysed using neutrophil perfusion as the general cascade of recruitment is similar across leukocytes, although differences are known between the migratory behaviour and the cytokine gradients suitable for chemotaxis of different leukocyte subsets (Ley et al., 2007).

PBMCs, in particular CD4+ T cells, establish lasting and repeated interactions with endothelium *in vivo*. It has been postulated that endothelial cells act as 'semi-professional' antigen presenting cells as they can bind to flowing lymphocytes (Carman et al., 2015). It may also be hypothesised that the interactions between endothelial cells and the different subsets of immune cells may differ depending on the inflammatory stimulus (i.e. TNFα or IFNγ). Given that in this thesis, E-selectin blockade in IFNγ-stimulated cultures appeared to show an inhibited trend of monocyte-depleted PBMC attachment but not neutrophil attachment, it may be postulated that IFNγ up-regulates molecules required for specific lymphocyte interaction such as MHC II, which are not required for neutrophil attachment. The endothelium may then specifically interact with PBMCs via acting as an APC when stimulated with IFNγ, as opposed to the classical recruitment pathway. Liver sinusoidal endothelial cells for example, inhibit inflammatory cytokine secretion of Th1/Th17 effector CD4 T cells by directly binding to PD-1 on immune cells (Carambia et al. 2013). Given that the liver has been shown to reduce the inflammatory activity of infiltrating T cells, it would be interesting to conduct further

research into whether oxLDL is able to influence the ability of the endothelium to act as a semi-professional APC and directly influence immune cell responses.

The role of native LDL and oxidised LDL was analysed in the recruitment assay, firstly by the addition of 10μg/mL to TNFα-stimulated cultures. No significant effect was observed with these additions and so the role of ox-/LDL alone at a range of concentrations 1, 10 and 100μg/mL was analysed. The basal recruitment of neutrophils was increased in oxLDL-stimulated cultures both at 4 hours and 24 hours, but only at the highest concentration used of 100μg/mL, as no other culture showed significant recruitment in comparison to control. It has previously been shown in a flow-based recruitment assay that neutrophils, as opposed to endothelial cells, stimulated with 100μg/mL of oxLDL increase their recruitment to TNFα-stimulated endothelium (Badrnya et al., 2012). This data therefore shows that endothelial cells can respond to oxLDL to increase neutrophil recruitment, which has potentially important implications for oxidised lipids in the maintenance of an inflammatory state. However, under the conditions used so far, it was not possible to either enhance or reduce neutrophil recruitment to cytokine-stimulated endothelial cells by the addition of LDL or oxLDL and this requires further study.

In the investigations performed, endothelial cells were incubated with oxLDL for a maximum period of 24hours and so it would be interesting to investigate whether longer term cultures would show any further increases in immune cell recruitment, assuming that there was no significant apoptosis induced at longer incubation periods. In addition, culturing native LDL for longer periods may reveal whether accumulated LDL becomes oxidised by the endothelium over time and subsequently causes increases in inflammatory cell recruitment. Another aspect of the assay design which was not pursued was the incubation of oxLDL with immune cells, as opposed to the endothelium, prior to assessing their adhesive capability under flow. This would be difficult to perform with neutrophils, given their short life-span viability *in vitro* following isolation from blood, but neutrophils which had been isolated from different donors may have experienced varying levels of oxLDL in their respective circulation. Indeed, it has been shown that in monocytes, incubating with oxLDL for 24hours and then culturing for 6 days before stimulating with an inflammatory stimulus, increase their formation of inflammatory components such as IL-6, IL-8 and TNFα compared to cells which had not

been pre-exposed to oxLDL (Bekkering et al., 2014). The foam cell formation of monocytes was also enhanced following oxLDL exposure, suggesting a long term pro-inflammatory reprogramming of immune cells (Bekkering et al., 2014). The pre-exposure of immune cells to oxLDL may therefore affect their ability to adhere to the endothelium. In addition to this, it has been shown that indirect oxLDL stimulation, through macrophages and mast cells, increases monocyte adhesion to the endothelium to a greater extent than direct oxLDL-stimulation (Chen et al., 2015). It was shown that a low dose of oxLDL (8μg/mL) stimulated macrophages and mast cells to produce TNFα and histamine respectively and when the supernatants from these cells were introduced to the endothelium, it increased the immune recruitment under flow to a greater extent than directly introducing oxLDL to the endothelium (Chen et al., 2015). This lower dose of oxLDL (8μg/mL) is similar to the concentration previously shown in this thesis to increase endothelial migration (5μg/mL) and it may therefore be suggested that a long-term low-dose of oxLDL is pro-inflammatory.

Monocytes have been well characterised in relation to oxLDL and atherogenesis, expressing CD36 to uptake oxLDL and enhance the formation of foam cells within the arterial wall. Monocytes may also be affected by circulating oxLDL in relation to adherence to the endothelial monolayer as described above, although in this thesis monocytes were removed from PBMC recruitment experiments or not studied specifically as a subgroup alone, given that they adhere to the endothelial without prior stimulation and make quantification more difficult to interpret. PBMCs have a longer viability in culture than neutrophils, can be activated to develop specific sub-populations and so repeating the adhesion assay with PBMCs that had been pre-incubated with oxLDL may be of further interest to this investigation. Indeed, isolation of immune cells from patients of known lipid profiles may then provide additional information on lipid regulated immune function.

Once bound to the endothelium, leukocyte transmigration into the tissue is the next key step in the recruitment cascade which can also be visualised following recruitment in the flow assay. Transmigration is a distinct step in leukocyte recruitment and requires expression of a variety of molecules for adhesion and permeability of the endothelial barrier (Ley et al., 2007). Using the transmigration assay, it was observed that lipid-

depleted cultures showed no significant effect on the ability of neutrophils to transmigrate across the endothelium following stimulation with TNFα for 24 hours. Using the same method with TNFα-stimulated endothelium at 4 hours with the addition of ox-/LDL there was again no significant effect on the ability of the endothelial cells to support transmigration. In a static model of transmigration, it has previously been shown that HUVEC stimulated with oxLDL (100μg/mL) for 24 hours showed increased transmigration across the endothelial layer, attributed to the increase in ICAM-1 expression induced by oxLDL at this concentration (Stroka et al., 2012). In this experimental set-up using flow-based as opposed to static adhesion, no such effects were observed. The recruitment and transmigration of neutrophils at 24 hours and at ranging oxLDL concentrations would need to be performed to clarify whether this is a time-dependent observation. Initial experiments have indicated that the time to transmigrate may be increased in TNFα and oxLDL treated cells (i.e. is a slower process compared to TNFα stimulated transmigration alone) and requires further investigation.

For further investigations into oxLDL-mediated immune cell recruitment and transmigration, the method used for this quantification may be expanded in order to fully characterise the response. The flow-based adhesion assay has many advantages relating to quantification and analysing specific cellular interactions (i.e. between neutrophils and endothelium). However, it may also have a disadvantage in this respect in that the assay could be described as reductionist, observing only two cell types and having a consistent flow rate (and therefore shear stress). Given that only small changes were induced by oxLDL on endothelial cells, it may be necessary to expand the assay for further quantification, particularly in respect to the area of endothelium analysed, immune cells perfused together, post-fixation of recruited immune cells for analysis of surface markers and potentially expanding into *in vivo* models to analyse the effects of dynamic changes in shear stresses and location of recruitment respective to branching points in the vascular network. For the analysis of transmigration, a single field-of-view was observed, quantifying the immune cells transmigrated at a single end-point time of 30 minutes. Further analysis could increase the areas of endothelium analysed (either by including more fields or using a lower magnification) and include more time-point analyses, which could uncover information relating to not only transmigration, but also

reverse transmigration which has been associated with the dissemination of systemic inflammation (Woodfin et al., 2011). This expansion could then lead on to the investigation of junctional adhesion molecules which regulate these processes and analyse their relocation within the membrane in response to native and oxidised lipids.

In this investigation, immune cells (monocyte-depleted PBMCs or neutrophils) were separately isolated and perfused across the endothelium in order to assess their recruitment. These immune cells could be separately stimulated, but in addition to this the immune cells could be perfused across the endothelial monolayer together. Post-fixation of the endothelial layer may allow the subsequent staining or identification of the immune cells which had been recruited and/or transmigrated in response to specific stimuli. The advantage of this method would be to more accurately replicate the *in vivo* setting, as it has previously been identified that immune cells can affect the recruitment of others, for example culturing monocytes with endothelium potentiates the recruitment of neutrophils (Luu et al., 2007). The fixation of the immune cells could also identify, for example, if the small number of neutrophils recruited by oxLDL-stimulation were actually a subset of neutrophils, such as the pro-angiogenic subset which express high MMP-9 levels previously alluded to (Christoffersson et al., 2012). Increasing the cell types perfused across the endothelial layer may complicate the assay, but it may also be useful in identifying other cellular interactions as it has been demonstrated that non-immune cells such as platelets mediate oxLDL-induced monocyte extravasation (Badrnya et al., 2013) and also guide immune cells to their site of extravasation (Zuchtrigel et al., 2016). To mimic further the *in vivo* setting, the addition of red blood cells would increase the viscosity of the perfused fluid, which would impact on the shear stresses applied to the endothelial monolayer and may also have implications on immune cell recruitment. The investigations in this thesis operated at a shear stress of 1dyne/cm^3 which is synonymous with that found in the microcirculation. Simple changes to this shear stress rate may elucidate further effects of lipids, but also applying oscillatory shear stresses, which have been demonstrated to affect immune cell recruitment (Hsiai et al., 2003). Ultimately, the disadvantage of an *in vitro* assay design is that it cannot replicate the plethora of cell types and other factors which occur within the true *in vivo* setting. However, studying the direct interactions between immune cells and the endothelium

can give indicative evidence and produce definitive conclusions before expanding to more complicated models.

In summary, the role of serum lipids appears less crucial to endothelial activation and their ability to contribute to leukocyte trafficking than the processes of endothelial migration and tube formation studied in previous chapters. The increased basal recruitment of neutrophils in the presence of 100μg/mL of oxLDL may indicate a contribution to a basal inflammatory state. Taken together, these data suggest that endothelial functions show specificity in their requirement for serum lipids and indicates a level of regulation above simple starvation and responses to lipid deprivation. In addition to the aforementioned expansions to the recruitment and transmigration assay that could be performed, immune cells from psoriatic patients which have been exposed to higher oxLDL levels may be more indicative of the *in vivo* effects of circulating lipids and require study in these models. Although the changes induced by oxLDL-stimulation have been relatively low in comparison with potent inflammatory cytokines TNFα and IFNγ, it has given evidence of potential inflammatory changes and suggestive of an increased basal inflammatory state if oxLDL effects were sustained over the long-term.

6 Chapter 6: The role of serum lipids and oxidised lipids on lymphocyte proliferation in vitro

6.1 Introduction

In previous chapters, the role of lipids has been analysed in relation to their effects on the endothelium. In this chapter, the effects of native and oxidised lipids will be investigated in terms of their effects on peripheral blood mononuclear cells (PBMCs). PBMC refers to lymphocytes (T cells, B cells and NK cells) and monocytes, which are characteristically found in inflamed psoriatic skin. Psoriasis has been documented for centuries and is now characterised as an immune-mediated disorder, with prominent immune cell infiltrate into the skin. However, the immune-mediated aspects of psoriasis have only been known for a shorter amount of time, as the original focus (pre-1980s) of psoriasis was the biology of the keratinocytes within the epidermal layers, as these cells are also known to be hyper-proliferative and contribute to the inflamed nature of the disease (Gaspari & Tyring, 2008). One of the breakthroughs which allowed the evolution of psoriasis treatment was in 1979, because of the observation that cyclosporine A, primarily targeted at suppressing cancer, arthritis and kidney transplant rejection was remarkably effective in the treatment of psoriatic plaques (Berth-Jones, 2005). Cyclosporine was subsequently confirmed to be effective in the treatment of psoriasis (Fry, 1992; Ellis et al., 1995) and was FDA approved for psoriasis treatment in 1997 (Dehesa et al., 2012). Cyclosporine is an immunosuppressant drug which works primarily on the suppression of T cell responses via inhibition of the transcription of cytokine genes in activated T cells (Matsuda et al., 2000). The efficacy of an immunosuppressant in psoriatic disease clearance built the framework for a change in approach to research, with a focus towards the immunological aspect to psoriasis. The immune infiltrate in psoriasis is now a mainstay of psoriatic research predicated on the effectiveness of immune suppressing drugs, in addition to genetic associations within the Major Histocompatibility Complex (MHC) region and the curative nature of bone marrow transplantation for psoriasis (Braiteh et al., 2008).

There are a variety of immune cells that accumulate in the psoriatic lesion and contribute to inflammation that includes neutrophils, dendritic cells, macrophages, monocytes and lymphocytes. The effects of serum lipids and oxLDL in stimulating endothelial cells to recruit neutrophils and PBMCs (monocyte-depleted) have been investigated in previous chapters and this chapter will focus on how serum lipids and oxLDL directly affect the ability of immune cells to proliferate. There has been much research to suggest that the activation of T cells in particular is critical in the pathogenesis of psoriasis (Cai et al., 2012), but the link between accumulated oxLDL within the skin (Tekin et al., 2007) and the proliferation of these cells within the same tissue is yet to be fully elucidated. Using flow cytometry to analyse proliferation with lipid-depletion and ox-/LDL additions, this relationship will be investigated.

T-lymphocytes are derived from the thymus, whereas B cells form in the bursa of Fabricius, which is a specialised organ first demonstrated in birds to be necessary for B cell development (despite B cell development occurring in the bone marrow of mammals) (Glick, 1991).

Lipids have long been considered in lymphocyte responses particularly in regards to fatty acid consumption and membrane lipid composition (Traill et al., 1984). It has previously been reported that lipids can affect lymphocyte responses with *in vitro* studies into fatty acids highlighting reduced proliferation of T-lymphocytes in response to mitogen stimulation (Calder, 1995; De Pablo et al., 2000), in addition to evidence in murine models (Yaqoob et al., 1994; Albers et al., 2002). In terms of the contribution of serum lipids to lymphocyte proliferation, indirect links have been made in obese patients who show an increase in T-lymphocyte number and whose serum was shown to enhance T-lymphocyte proliferation *in vitro* (Van der Weerd et al., 2010). In addition, weight loss was shown to improve the therapeutic effect of cyclosporine in patients with plaque psoriasis suggesting a link to calorie intake and immune function (Gisondi et al., 2008).

The majority of research investigating circulating lipids and immunity has been focussed in the area of cardiovascular disease, in which the monocyte and macrophage populations predominate. Therefore, the role of LDL and oxLDL in PBMC and T cell responses is less well characterised. If psoriasis patients have increased oxidised lipids

within the circulation and accumulated within skin lesions, the influence of oxLDL may be important in controlling the persistent inflammation within the skin. The lipid parameters which are associated with cardiovascular disease (particularly LDL and triglycerides) within psoriatic circulation are often reported as high whereas the anti-atherogenic HDL parameter is often reported as being low. The relationship between oxLDL and immune cells in psoriasis patients is therefore relevant not only to managing skin inflammation severity but also for the implications in the increased cardiovascular mortality reported in these patients (Holzer et al., 2012).

6.2 Methods

Lymphocyte proliferation is fundamental in the response to antigenic stimulation and the maintenance of inflammation. Given the accumulation of lipids and oxidised lipids in pathological conditions involving excessive inflammation such as psoriasis, the ability to measure lymphocyte responses provides a useful tool to examining lipid contribution to disease. The ability to track immune cell proliferation has been greatly enhanced with the use of carboxyfluorescein diacetate succinimidyl ester (CFSE), providing long-lived fluorescent staining of intracellular molecules analysed with flow cytometry. Cell proliferation can be tracked with this method due to the fluorescence intensity halving for each cell division as it is equally distributed to the daughter cells and proliferated cells can be identified by a dilution of the fluorescence intensity. Using the flow cytometry set-up described in methods, lymphocytes were gated as described in Figure 6-1, eliminating cell debris and subsequently gating the live population of cells based on the elimination of live/dead stain as previously discussed.

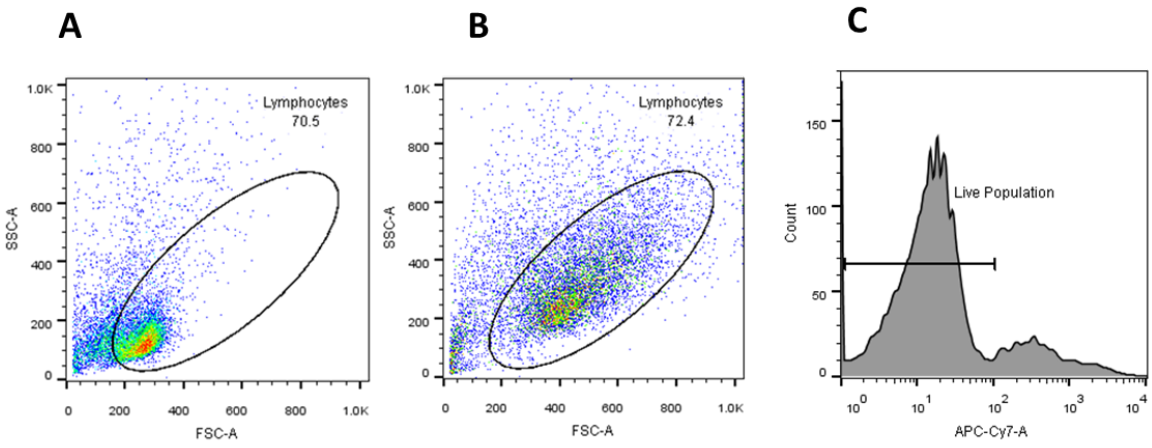


Figure 6-1 Lymphocyte general gating strategy using flow cytometry
Lymphocytes were gated based on their properties in the forward vs. side scatter graphs (unstimulated in A; proliferated cells in B) and subsequently gated for viable cells (C), characterised by their ability to prevent uptake of fluorescent live/dead stain.

Having established the assay design, lymphocytes were isolated from whole blood of healthy donors and stained with CFSE as described in methods. In previous chapters, lipid-depletion has had varying effects on endothelial cells. Active endothelial cell

processes such as angiogenesis and migration were significantly inhibited following the depletion of lipids from the serum, whereas the ability of endothelial cells to recruit flowing leukocytes was unaffected. The initial experimental design in this investigation involved a similar approach, comparing the proliferation of lymphocytes in lipid-depleted serum and cultures with normal human serum within the media. Whereas endothelial cells were cultured in 20% human serum M199 media, PBMCs were cultured in 10% human serum in RPMI as described in methods with added pyruvate. In order to analyse proliferation, mitogens were employed in order to stimulate a proliferative response in lymphocytes *in vitro*. There are several classes of mitogens which can be used for analysing the proliferative response of lymphocytes, but the initial experimental approach was to use the plant mitogen phytohaemagglutinin (PHA). PHA is a lectin which binds to multiple glycosylated surface molecules in addition to the T-cell receptor (TCR) (Chilson et al., 1984). The addition of PHA into cultures can induce multiple cellular signals but is less specific than other mitogens in this sense, providing a strong proliferative response. Using a potent stimulator like PHA allowed substantial proliferation which could easily identify whether lipids affect this process. Using the CFSE dilution assay, the effect of serum delipidation on PBMC proliferation can be visualised and compared to control unstimulated and stimulated PBMCs in full, lipid-containing media (Figure 6-2: unstimulated in part A and PHA-stimulated in part B).

6.3 Results

6.3.1 Immune cell proliferation

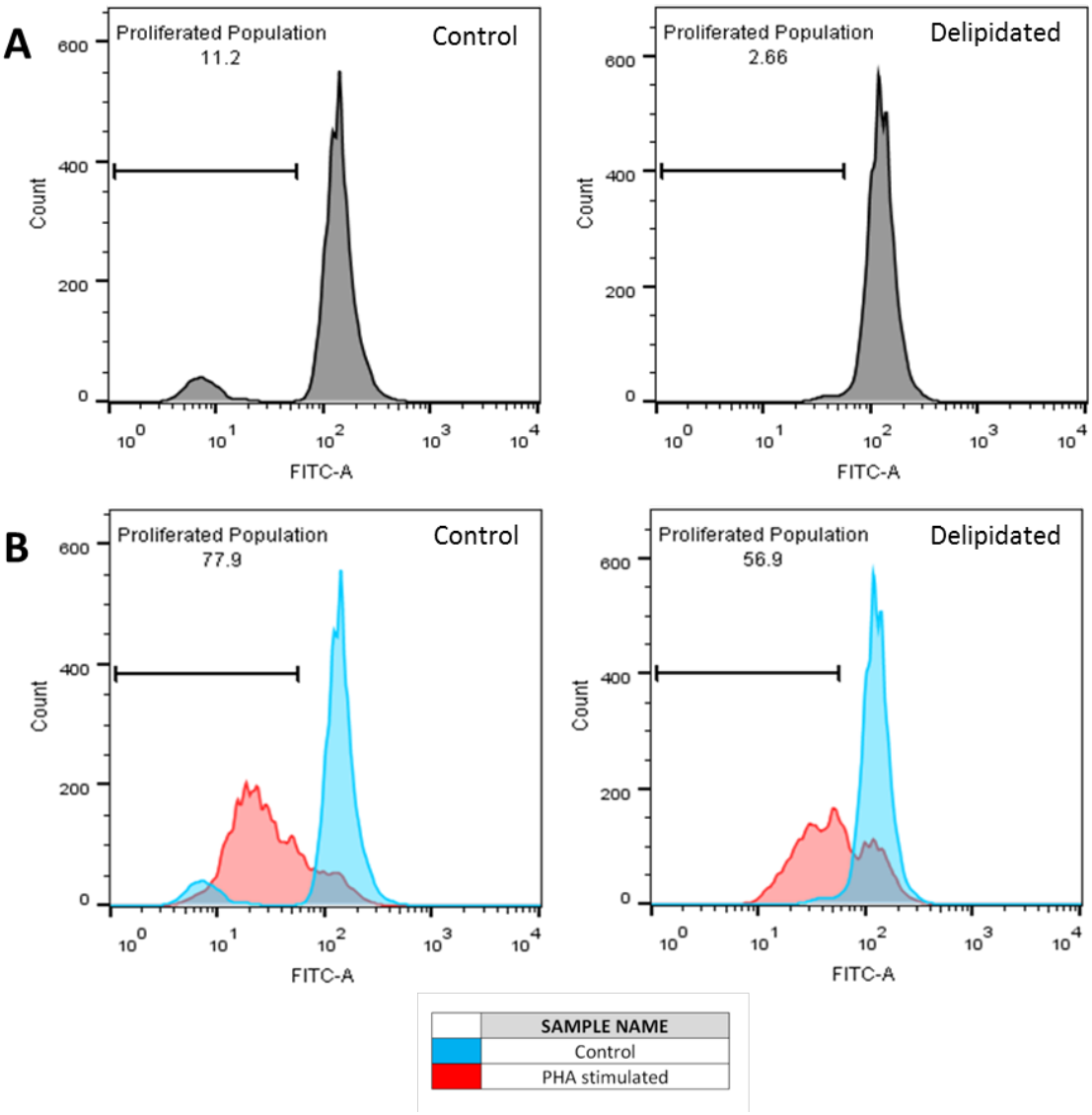


Figure 6-2 The effect of serum lipid depletion on the proliferation of lymphocytes in unstimulated (A) and PHA-stimulated (B) cultures, following 72 hour incubation
Representative graphs showing fluorescence intensity in the CFSE dilution assay of unstimulated lymphocytes (A) and PHA (3µg/mL)-stimulated cells (B). Proliferated cells show reduced fluorescence intensity as indicated. PHA-stimulated cells (red) are overlaid on unstimulated cells (blue) in (B).

The graphs in Figure 6-2 are a representation of a single experiment performed in control serum cultures and lipid depleted cultures. Lymphocytes in control serum appear to have a proportion of cells (11.2%) which undergo proliferation even in the absence of a

specific stimulus. The comparable unstimulated cells cultured in the absence of lipid shows a reduction in this population (2.6% proliferation). When stimulated with PHA, the control culture showed a significant population shift to the left, indicating substantial proliferation of these cells. With the PHA stimulated cells in the delipidated culture there was still a population of PBMCs which remained un-proliferated and the proportion of cells which had proliferated (shown by a shift in fluorescence to the left) was reduced (56% proliferated cells in lipid depleted serum compared to 77% in the control serum cultures). This result indicated that serum lipids are important in the maintenance of lymphocyte proliferation. The quantification of lymphocyte proliferation is shown in Figure 6-3. The proliferation of lymphocytes was significantly inhibited in delipidated cultures in comparison with control.

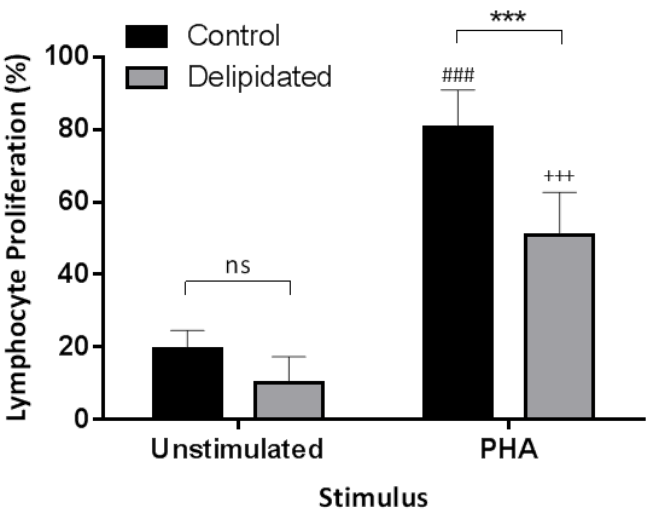


Figure 6-3 The effect of lipid depletion on the proliferation of lymphocytes over 72 hours in unstimulated and PHA-stimulated cultures
The lymphocyte proliferation in control and delipidated cultures is shown. Statistics performed using one-way ANOVA with Tukey's post-hoc test. '#' statistics comparing control-stimulated to control-unstimulated. '+' statistics comparing delipidated-stimulated to delipidated-unstimulated. '*' statistics comparing control-stimulated to delipidated-stimulated. ***P<0.001 (n=8).

Having previously analysed a diminished ability of the endothelial cells in lipid depleted cultures to form tubes and undergo migration, a potentially similar observation regarding the importance of lipids in the functional responses of PBMC is presented. These findings have potentially important implications given that two major processes of inflammation

could be significantly altered by a lipid dependent process. An observation made during the analysis of lymphocyte proliferation, however, was the change on forward/side scatter parameters on flow cytometry that occurred in lipid-depleted cultures in comparison with control cultures. Figure 6-4 shows that there are noticeable populations of cells which appear to the left of the lymphocyte gate. In control serum cultures, the population undergoes a shit to the right, indicating larger cells which are undergoing division and proliferating. In delipidated cultures however, particularly in those that have been stimulated, the size of the cells appears to be decreased for a noticeable proportion. In the representative example in Figure 6-4 for example, 72.4% of cells were within the dedicated lymphocyte gate whereas this number decreased to 23.3% in the delipidated culture as indicated by the left shit on the forward scatter axis. The left shifting of these cells generally indicates a population that is dead or dying and becoming smaller in the process.

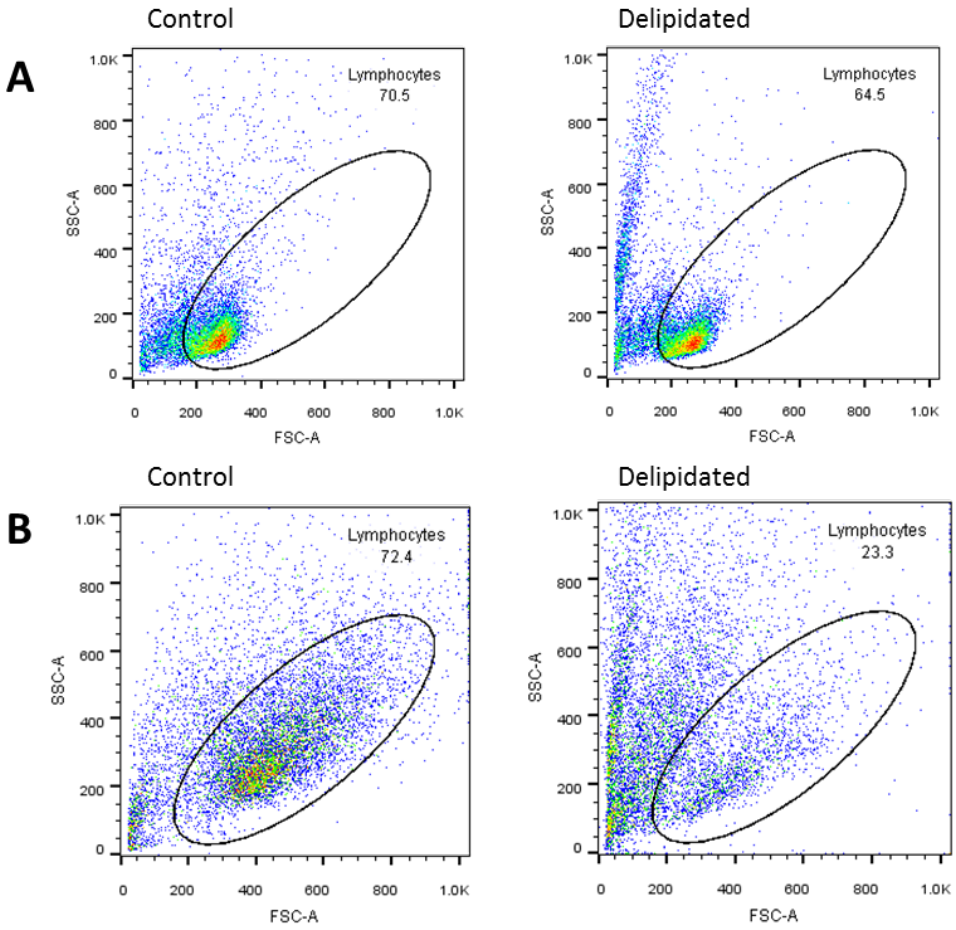


Figure 6-4 Forward/side scatter variation in control and lipid depleted cultures
The forward/side scatter graph of control and delipidated cultures varies in both unstimulated (A) and PHA-stimulated (B) cultures. The cells debris is increased with delipidated cultures, shown in both A and B. The proportion of lymphocytes in delipidated, stimulated cultures (B) is reduced in comparison with control.

The previous data indicated that viable cells within the lymphocyte population had a reduced proliferative response in delipidated cultures in comparison with control. It is important to note that although there appeared to be more cell debris in lipid-depleted cultures, graphical representation and statistical analysis of data is only performed on viable cells within the lymphocyte gate. The identification of an altered profile in the forward/side scatter however, needed further investigation. Cells in the lymphocyte gate were subsequently analysed for their viability, using the live/dead statin previously described and the results are presented in Figure 6-5. Given the appearance of a population of cells outside of the lymphocyte gating in stimulated, delipidated cultures,

the viability of cells in the whole population identified in the forward/side scatter was also analysed and is presented in Figure 6-6.

The results of the viability assessment showed that there was a loss of lymphocyte viability in delipidated cultures in comparison with control cells, both in unstimulated and PHA-stimulated populations after 72h in culture (Figure 6-5). In the control serum populations, PHA-stimulation caused no increase in death compared to unstimulated cells. In delipidated cultures however, the PHA stimulus caused significantly more death than in unstimulated cultures. This was also the case in the whole-cell population experiment (Figure 6-6), suggesting that lipids are essential in the maintenance of cell viability in static and proliferating immune cells. Although cell debris could be included in this analysis, the increased death in delipidated, stimulated cultures in comparison with unstimulated cells shows that a proportion of cells became non-viable in the absence of lipids.

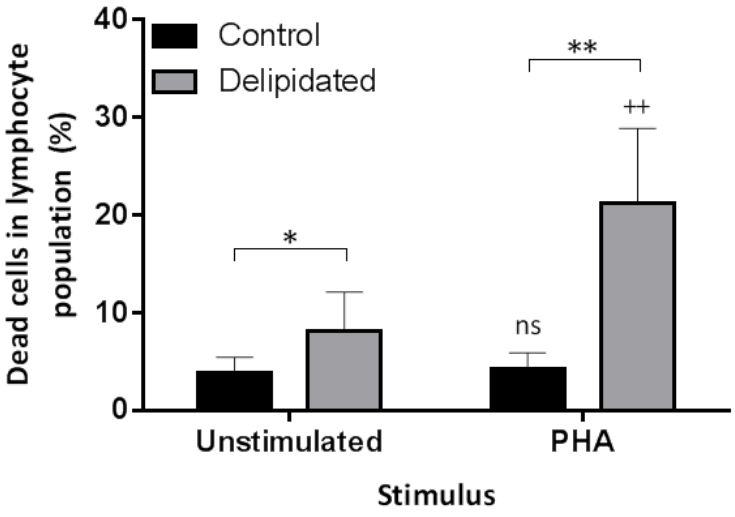


Figure 6-5 The effect of lipid depletion on cell viability in lymphocyte population
Following gating of cells in the lymphocyte population, the percentage of cells which were non-viable (measured with live/dead stain) was analysed. Statistics performed using one-way ANOVA with Tukey's post-hoc test. Statistics performed separately on control cultures (ns), delipidated cultures (++) and between control- and delipidated- PHA stimulated cultures (**) **P<0.01 *P<0.05 (n=8).

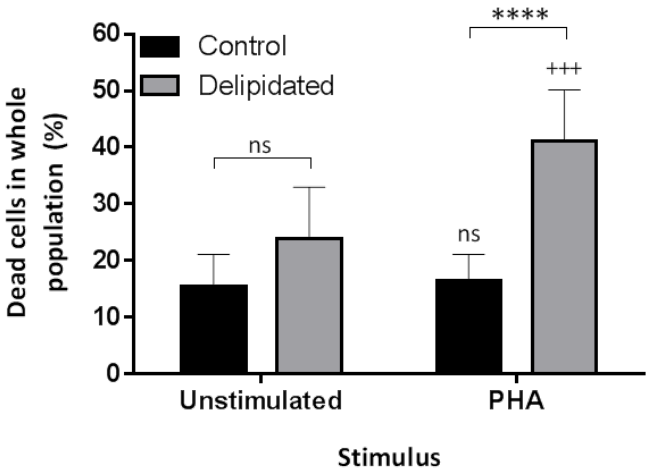


Figure 6-6 The effect of lipid depletion on cell death in whole PBMC population Without gating any cell population, the viability of all cells analysed through the flow cytometer were assessed. Data shows mean +/- SD. Statistics performed using one-way ANOVA with Tukey's post-hoc test. Statistics performed separately on control cultures (ns), delipidated cultures (+++) and between control- and delipidated- PHA stimulated cultures (****). ****P<0.0001, +++P<0.001 (n=8).

Having shown that lymphocyte proliferation was significantly impaired in delipidated cultures in comparison with control cultures when stimulated with PHA or left unstimulated, a range of other stimuli were used to assess this specificity of this response. PHA is a plant lectin that can bind to multiple ligands on the surface of the cell as mentioned previously, causing a range of signals and subsequent PBMC proliferation. In order to evaluate whether the effect of lipid depletion was related to a particular type of stimulus or the process of proliferation, other ligands were employed including a soluble anti-CD3 antibody (aCD3) (not plate-bound), the bacterial product staphylococcus enterotoxin B (SEB) and the small organic compound phorbol 12-myristate 13-acetate (PMA). The anti-CD3 antibody binds to the CD3 molecule on the surface of T-cells, which is a co-receptor found on all T-cells and is associated with the TCR. The use of monoclonal antibodies against CD3 induces T cell proliferation in the presence of monocytes (antigen presenting cells) which were present in the isolations of PBMCs. SEB is a bacterial product which is capable of cross-linking MHC class II molecules with the TCR, causing activation and proliferation of T-cells. It acts as a superantigen to cause the non-specific activation of T cells and polyclonal T cell activation. PMA on the other hand is a small molecule that diffuses through the cell membrane into the cytoplasm, where it is capable

of activating protein kinase C (PKC), omitting the need for a receptor. With the stimulation of PKC by PMA, ionomycin was also employed to form pores in the membrane as an ionophore that allows the intracellular calcium levels to be raised. These two signals are required for subsequent proliferation.

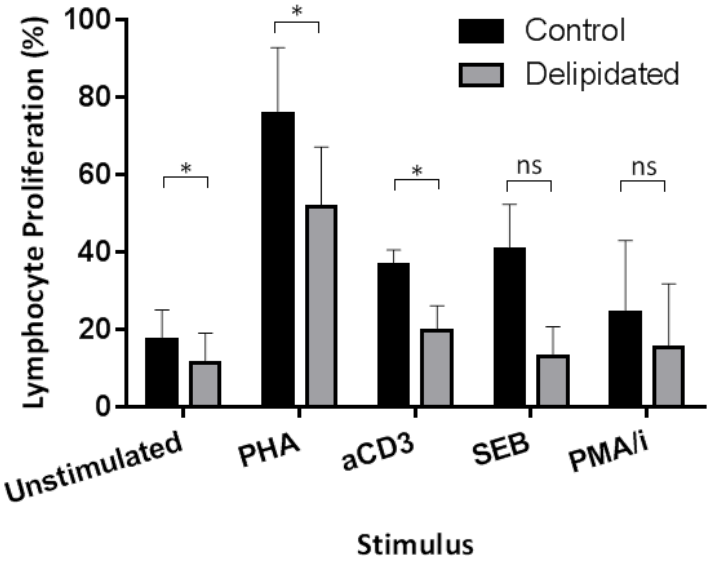


Figure 6-7 Flow cytometric analysis of lymphocyte proliferation in control and delipidated cultures in response to different stimuli
The CFSE dilution assay was performed in control and lipid depleted cultures with unstimulated cultures, in addition to the four separate stimuli including PHA [3µg/mL], soluble anti-CD3 [0.1µg/mL], staphylococcal enterotoxin B (SEB) [2.5µg/mL] and a combination of phorbol myristate acetate (PMA) [2.5ng/mL] and ionomycin [100ng/mL]. Mean +/- SD of proliferated PBMCs, statistics performed using two-tailed, paired t-tests *P<0.05 (n=3).

Figure 6-7 highlights the effect of lipids on lymphocyte proliferation. In all cultures, the lymphocyte proliferation percentage is reduced in lipid depleted cultures than that for the relevant control, even if not all reached statistical significance for three repeated experiments. The unstimulated, PHA-stimulated and aCD3-stimulated cultures are statistically significant when cultured in the absence of lipids. Having established reduced proliferation in lipid depleted cultures, the hypothesis that required investigation regarded the pro-inflammatory potential of oxLDL in relation to the same proliferation assay. The role of LDL and oxLDL (10µg/mL) was therefore analysed in unstimulated

cultures (Figure 6-8, Figure 6-9), PHA-stimulated cultures (Figure 6-10) and aCD3-stimulated cultures (Figure 6-11).

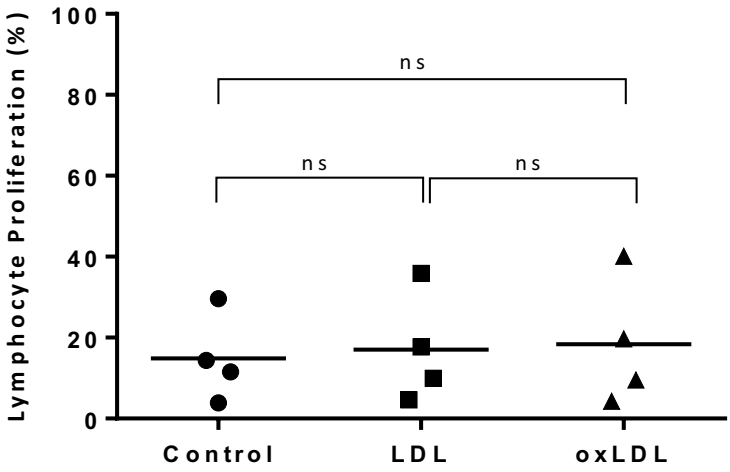


Figure 6-8 Lymphocyte proliferation in the presence of LDL and oxLDL
Lymphocyte proliferation over 72 hours in the absence of specific stimuli was measured. The effect of ox-/LDL (10µg/mL) was analysed in control serum cultures. Mean values and individual results shown with statistics performed using one-way ANOVA with Tukey's post-hoc test (n=4).

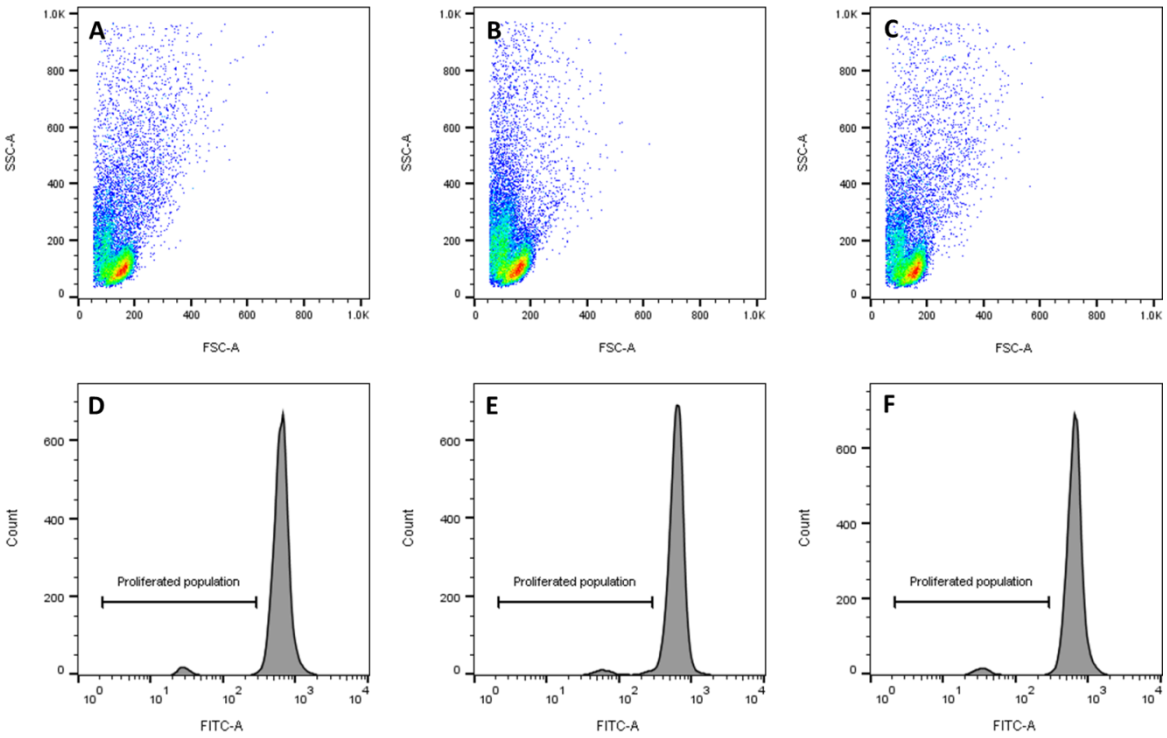


Figure 6-9 Lymphocyte scatter plots and proliferation profiles in response to ox-/LDL

Scatter plots of FSC/SSC shown (A,B,C) for control (A), LDL 10µg/mL (B) and oxLDL 10µg/mL (C) stimulated lymphocyte over 72 hours. Figure shows representative plots from a single experiment. Proliferation histograms shown (D,E,F) for control (D), LDL 10µg/mL (E) and oxLDL 10µg/mL (F).

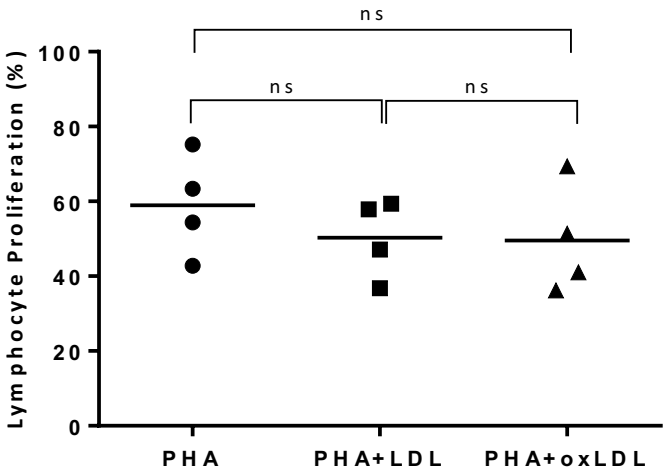


Figure 6-10 Lymphocyte proliferation in response to PHA with the addition of ox-/LDL

PHA (3µg/mL) stimulated PBMC proliferation was analysed by CFSE dilution and compared to PHA /ox-/LDL (10µg/mL) cultures. Mean values are shown and statistics performed using one-way ANOVA with Tukey's post-hoc test (n=4).

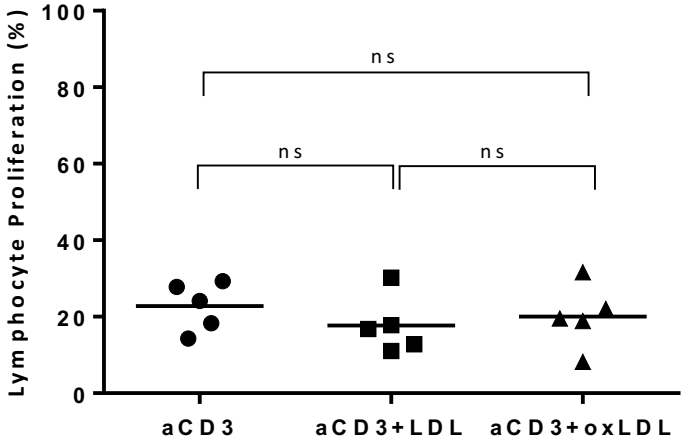


Figure 6-11 Lymphocyte proliferation in response to anti-CD3 with the addition of LDL and oxLDL

aCD3 (5µg/mL) stimulated PBMC proliferation was analysed by CFSE dilution and compared to PHA /ox-/LDL (10µg/mL) cultures. Mean values of PBMC proliferation are shown and statistics performed using one-way ANOVA with Tukey's post-hoc test (n=5).

From the results above, it can be seen that the addition of LDL or oxLDL at 10µg/mL had no effect in either inhibiting or promoting proliferation of lymphocytes. All of these

experiments were performed in cultures containing control serum. It is concluded from these data that serum lipids play a vital role in the maintenance of lymphocyte proliferation, whereas the addition of oxidised lipids into control cultures had no significant effect. It should be noted that at the concentration of oxLDL used in these experiments (10μg/mL), there were no changes in viability observed using the live/dead stain protocol, as all cells in these experiments maintained >95% viability (n=5).

6.4 Discussion

The results in this chapter indicated that human blood lymphocyte proliferation was significantly impaired in lipid-depleted cultures in comparison with control serum cultures. The use of flow cytometry and the CFSE dilution assay to analyse lymphocyte proliferation allowed the investigation of multiple parameters of interest. These included analysing stimulated-proliferation with unstimulated-proliferation, comparing control serum cultures with delipidated serum cultures and finally comparing different stimuli to promote proliferation. Previous chapters have shown that lipid depletion within the serum inhibits active cellular responses, which was similarly found in this chapter in relation to lymphocyte proliferation. When stimulated to divide, lymphocytes grown in lipid-depleted medium over 72 hours proliferated less in comparison to lymphocytes grown in full serum and this was statistically significant in PHA- and aCD3-stimulated cultures. In Figure 6-7 lymphocytes from three different donors were analysed for their response to all stimuli used (aCD3, PHA, PMA/ionomycin or SEB). In this figure, it can be seen that in unstimulated cultures there was a proportion of cells that proliferated (<20%). Interestingly, the same unstimulated lymphocytes in the absence of lipids had reduced proliferation (<10%) and this was statistically significant for the three repeats. In Figure 6-3 a similar investigation was performed on eight separate donors for unstimulated and PHA-stimulated lymphocytes and although the unstimulated culture proliferation figures remained approximately the same, there was no statistical significance. The advantage of the CFSE labelling procedure is that it is able to permeate cells and covalently bind to intracellular targets (free amine groups), as opposed to binding to membrane lipids. For this reason, it is unlikely that the inhibited response seen in delipidated cultures was caused by impaired uptake of the dye. It was concluded therefore that serum lipids are essential in lymphocyte proliferation and their absence is a limiting factor. To confirm these results further, the lymphocyte proliferation assay could be repeated using the method of tritiated thymidine incorporation. With this alternative method, lymphocytes can be similarly cultured in the presence of a stimulating agent and when the cells proliferate, tritiated thymidine (^{3}H-thymidine) is incorporated into new DNA that is synthesised and the radioactive signal is subsequently measured to determine the extent of cells division (Wagner et al., 1999). The use of this

method could prove the results from the flow cytometry assay that the lipid effects are specific to lymphocyte proliferation and not an artefact of altered fluorescence staining.

Although less proliferation was observed in lipid-depleted cultures in comparison to control, these lipid-depleted cultures were still able to proliferate when stimulated, showing statistical significance between stimulated and unstimulated cultures. Few other studies have characterised this response in this way, but another research paper conducting a similar study also showed that the *in vitro* proliferation of lymphocytes was reduced in delipidated cultures (Chyu et al., 2014). This paper by Chyu *et al*. used delipidated bovine serum as opposed to human serum and 4 day culture of PBMCs with CD3/CD28 activation beads as opposed to 3 day culture with PHA or aCD3 alone. Their method of depleting serum lipid was not reported as this was purchased commercially, making direct comparisons difficult. However, this paper indicated a reduction in cellular cholesterol and lipid rafts (total lipid rafts within the membrane measured by fluorescent staining) in lymphocytes cultured in delipidated serum as a contributory factor towards suppressed proliferation. In this thesis, the effect of lipid-depletion on lymphocyte proliferation was confirmed to be a consistent finding regardless of the stimulus used. The use of a non-specific mitogen such as PHA is often preferred as it can stimulate greater proliferation of lymphocytes, but the use of other stimuli such as anti-CD3 (receptor mediated), SEB and PMA/ionomycin showed similar patterns of inhibition even in less-proliferated cultures. The presence of exogenous lipids therefore suggests a fundamental requirement in the proliferation of immune cells. This aligns with data suggesting that fatty acids or cholesterol added to serum-free media allows the adequate function of lymphocytes (Jeon et al., 2010). Rather than having an effect on specific cell surface interactions, the role of lipids may be fundamental to the proliferation process itself, such as the maintenance of cellular membranes.

The reduced proliferation in lipid-depleted cultures was gated on live, viable lymphocytes measured by gating lymphocytes which actively excluded the viability dye. However, an effect on the scatter of cells outside the lymphocyte gate was noticed, in addition to the proportion of cells which were not viable both inside and outside of the lymphocyte gate, which are indicated for the lymphocyte population in Figure 6-5 and the whole cell population in Figure 6-6. These figures also demonstrate that cultures in which the

lymphocytes were stimulated to proliferate contained more non-viable cells than unstimulated cultures. This result possibly suggests that the reason for the reduced proliferation was that the cellular lipid, including cholesterol, was depleted to such an extent that there were insufficient lipid amounts to form new membranes for the progeny cells. This meant that as the cells were stimulated to proliferate, more lipids were required and therefore more cells fell short of their lipid requirement and became non-viable. In the study performed by Chyu *et al*., however, there was no indication of cell death with a similar study design either not measured or not reported. In the paper, anti-human CD3/CD28 beads were used (bead-to-cell ratio 1:2) to stimulate proliferation for 4 days and so it is difficult to directly compare the two studies, as the viability of cells may depend on the effectiveness of lipid-depletion method, strength of stimulus and the serum source (i.e. bovine or human). For further investigations, it may be interesting to FACS sort the live, proliferated cells in the lipid-depleted cultures and analyse their cell membrane constituents, lipid rafts and functional properties in relation to cells which had become non-viable. If the population of lymphocytes that were viable had an increased lipid uptake capability (or *de novo* lipid synthesis capability) than those which became non-viable, then this may lead to a therapeutic target for supressing lymphocyte proliferation in subpopulations. Identification of CD4 and CD8 cells in these cultures would also give further information as to the generalised or specific nature of these effects. Finally, the effect of lipid depletion on the ability of monocytes to act as antigen presenting cells in these cultures may point to a specific anti-proliferative mechanism requiring serum lipid support or a direct effect on the accessory signals required for proliferation.

Although the elimination of all serum lipids is not a viable scenario *in vivo*, these results may have important implications in terms of suppressing inflammation by way of energy restriction in relation to lipids. For the enhancement and further investigations of this research, the addition of particular lipids into the lipid-depleted cultures could be performed in order to evaluate both aspects of lymphocyte function; proliferation and sustained viability. In previous chapters the addition of a lipid emulsion was analysed in lipid-depleted serum whereas this aspect was not performed in the lymphocyte proliferation assay. Additionally, in the angiogenesis assay, there appeared to be no

effect of adding omega-3 and omega-6 lipids (either alone or in combination) to tube formation. The lymphocyte proliferation assay was performed over a period of 72 hours as opposed to the 8 hours of angiogenesis and so it may be postulated that long-term cultures, particularly involving new membrane formation in proliferation, may require the addition of essential fatty acids. The addition of these fatty acids would be interesting to assess any potential increases in viability. Experiments carried out in rodents have shown that essential fatty acid deficiency in the early postnatal period causes damage to the thymus in young rats as a result of altered lipid composition (Dvorak et al., 1992), yet also in adult rodents, essential fatty acid deficiency prevented autoimmune responses in non-obese diabetic mice by enhancing Th2 cells and APC activity (Benhamou et al., 1995). These fatty acids can also be manipulated *in vivo* through nutritional changes and it has been reported that long chain omega-3 fatty acids can be anti-inflammatory (Calder, 2006). Increased consumption of these fatty acids results in increased proportions within the membrane, partly at the expense of the more pro-inflammatory arachidonic acid and have been shown to attenuate inflammatory responses (Calder, 2006; Liu et al., 2003; Swanson et al., 2012). It has also been reported in mice that increased cholesterol within the membrane of lymphocytes enhances inflammatory responses (Th1 differentiation) of CD4+ T cells, although no effect of membrane cholesterol was found on suppressive functions of T-reg cells (Surls et al., 2012). It has also been identified that membrane cholesterol enrichment in mice, produced by an atherogenic diet, caused increased populations of T cells within lymph nodes (Wilhelm et al., 2009). Combining these data it could be postulated that altering the lymphocyte membrane constituents effectively with cholesterol and fatty acids, lymphocyte proliferation could be reduced and this could be further studied using the assay design in this thesis.

Using control serum, native and oxidised LDL were added to PHA-stimulated, aCD3-stimulated and unstimulated cultures in order to evaluate potential effects on lymphocyte proliferation. No statistically significant alterations were found when native or oxidised LDL were added at a concentration of 10µg/mL to any of the cultures. There has previously been evidence for oxLDL promoting PBMC activation, although this was shown in antiphospholipid syndrome, in which oxLDL forms complexes and become targets for auto-antibodies (Laczik et al., 2010). There has also been evidence of oxLDL

inducing apoptosis (at higher concentrations similar to the 100μg/mL previously used in this thesis) which contributes to inappropriate immune responses (Alcouffe et al., 1999; Mahmoudi et al., 2011). In this investigation, the lower and higher concentrations of oxLDL (1μg/mL and 100μg/mL respectively) were not studied respective to lymphocyte proliferation and so these experiments would need to be repeated in order to clarify whether oxLDL had effects at specific concentrations, as was found with endothelial responses (i.e. 5μg/mL for migration).

Other functional roles of the PBMCs in delipidated cultures, such as cytokine secretion, were not studied in this investigation which may be important to the pathophysiological impact of altering membrane lipids. The effects of dietary fish oils in particular have shown a reduction in cytokine production (such as TNFα, IFNγ and IL-1) from circulating leukocytes (De Pablo et al., 2000; Calder, 2013) and so it would be interesting to research whether serum-lipid depletion, LDL or oxLDL addition had effects on cytokine release. Additionally, combining these factors of cholesterol, fatty acid and oxidised lipids within the membrane may give insight into the optimal factors for promoting pro- or anti-inflammatory lymphocyte responses, in addition to studying which factors promoted cell survival and viability. It was also shown separately in natural killer cells that the effects of lipid chain lengths (which were synthesised and incrementally varied in the cells) influences not only the amount of cytokine release, but also the cytokine profiles secreted by the cells (Goff et al., 2004). This may subsequently be a cyclic process, as lipids promote cytokine release from leukocytes and then in turn cytokines alter lipid processes. For example, there has long been evidence that the cytokine IFNγ regulates cholesterol distribution on macrophages and inhibits HDL-mediated cholesterol efflux from macrophages (Panousis et al., 2000), whereas TNFα can up-regulate LOX-1 in these cells (Moriwaki et al., 1998). Not only pro-inflammatory cytokines such as TNFα could be studied, but also the consequence of membrane lipid alterations would have on anti-inflammatory factors such as IL-10, as in the paper by Chyu *et al*. it was shown that IL-10 secretion in delipidated cultures of PBMCs was decreased (Chyu et al., 2014).

In addition to these lipid components of lymphocytes, the role of the bioactive lipid S1P may be considered for future work. In previous chapters, it was shown that S1P

promotes endothelial migration in delipidated cultures and that blocking S1P metabolism in endothelial cells prevents oxLDL-induced migration. S1P has also been implicated in lymphocyte trafficking as $S1P_1$ expression by lymphocytes is critical for lymphocyte egress from the thymus and secondary lymphoid organs, coupled with an S1P gradient being much higher in the blood and lymph than interstitial fluid (Cyster et al., 2012). Although the trafficking of immune cells is important in psoriasis, it would be interesting to investigate what effect S1P had on proliferation. However, because of these potent effects on lymphocyte trafficking, the S1P analog FTY720 (fingolimod) acts as a functional antagonist to inhibit the egress from the lymph node and therefore holds promise as an immunosuppressant in inflammatory conditions like psoriasis (Herzinger et al., 2007). Combining all of these data together, it would be interesting to investigate the lipid combinations and concentrations which potently influence lymphocyte proliferation, survival and production of cytokines, both in pro- or anti-inflammatory aspects. Regulating the lipids in this way, whether through nutritional supplementation or specific receptor targeting, may provide a novel way of controlling long-term inflammation in psoriasis and other inflammatory disorders. The broad nature of S1P effects and the expression of its multiple receptors on a wide-range of inflammatory cells, regulating its production and signalling both in the vasculature and the tissue may provide a novel therapeutic target. It may also be interesting to investigate the long-term culture of lymphocytes with oxLDL and/or S1P and subsequently investigate their recruitment by oxLDL-stimulated endothelium under shear stress, giving an insight into potentially reprogramming of the cells or changes in their inflammatory potential.

7 General Discussion

The aim of the investigations in this thesis was to gain an understanding of the role of serum lipids and oxidised lipids in inflammation. Particularly of interest were inflammatory responses related to psoriasis, which included endothelial responses such as migration and tube formation which contribute to neovascularisation and angiogenesis; the recruitment of immune cells under flow which allows their infiltration into the tissue and exacerbation of the inflammatory state and the proliferative response of immune cells. The endothelial barrier, separating the intravascular contents form the surrounding tissue, is a focus point of inflammation research given its prominent role in controlling trafficking of inflammatory cells. Endothelial cells are continually exposed to serum lipids and in some circumstances, such as in psoriasis or atherosclerosis, may be continually exposed to oxidised lipids in the circulation or those that have accumulated in sub-endothelial regions. This thesis aimed to tackle the complex interactions of endothelial cells and lipids by removing serum lipids from the serum, to gauge the role they play in basal endothelial responses. Using this as a 'clean' background of lipids, individual lipid components (particularly LDL and oxLDL) could be assessed for their inflammatory influences, in addition to understanding their mechanism of action.

Through this approach, a vital role for serum lipids was recognised in active endothelial responses (migration and tube formation), whereas adhesion molecule expression and inflammatory cell recruitment were not significantly altered by their removal. In addition, concentration dependent effects of oxLDL were observed in endothelial responses, which were not observed with native LDL. In particular, low concentrations of oxLDL (5μg/mL) were stimulatory for migration whereas high concentrations (100μg/mL) were inhibitory on angiogenesis and migration, yet increased inflammatory cell recruitment. These observations also led to a mechanistic approach in understanding oxLDL-mediated endothelial migration, which elucidated S1P as a major contributory factor in promoting this response. Inhibitors for S1P formation (sphingosine kinase inhibitors) and S1P receptors ($S1P_1$) on endothelial cells were able to block oxLDL-induced migration, suggesting a novel mechanism of oxLDL influence.

7.1.1 The effect of serum lipid-depletion on inflammatory responses

The preliminary work of this thesis focussed on the development of the angiogenesis assay. There are a number of models which can be employed to analyse tube formation/ angiogenesis both *in vivo* and *in vitro*, all having their advantages and limitations. The *in vitro* tube formation assay used in this research allowed for the visualisation of endothelial structures and the manipulation of soluble factors within the cell media. Although a commonly used method for monitoring angiogenesis, the time point, media concentration, cell seeding density and quantification method are all variables which required appropriate development. Having established a working model which could adequately reflect the angiogenic process, one of the major initial findings was the inhibited response of endothelial cells to form tube like structures in the absence of serum lipids. Given the potential influence of lipid accumulation on inflammatory conditions, this finding supports a role for lipids in potentiating endothelial responses. To our knowledge, this assay design and lipid removal approach have never been previously performed together and so it is difficult to directly compare these results to other research. However, it has been shown that endothelial responses (such as vasodilation) can differ based upon the fatty acid composition of serum lipids (Sarabi et al., 2001) and that endothelial cells are capable of modifying genetic responses based on lipid exposure (>1000 genes can be regulated by oxidised phospholipids) (Gargalovic et al., 2006). Endothelial cells are also capable of *de novo* cholesterol production and it is likely that lipid-depletion will activate this response, although this was not directly measured in this thesis. However, it is considered that this *de novo* lipogenesis is a minor contributor to overall serum lipid homeostasis (Ameer et al., 2014) and indeed, it appeared that this biosynthesis of cholesterol or other genetic changes were not adequate to compensate for the lack of exogenous serum lipids in these investigations.

This observation in the endothelial tube formation assay opened up a number of potential mechanisms for lipid-regulated angiogenesis. Considering the minor contribution of cellular lipogenesis to overall lipid metabolism, one hypothesis for the results observed in this assay was that a lack of exogenous lipids may vitally regulate cell function via an energy starvation or energy-restriction effect. In order to address this potential issue, lipids were added back (or potentially reintroduced) into lipid-depleted

serum in an attempt to supply the endothelial cells with sufficient “energy”. The role of LDL (and its oxidation) was of interest to the inflammatory component of this research and so the addition of cholesterol through this method was analysed (to be discussed). To specifically address the potential energy restriction, essential fatty acids were introduced (linoleic and linolenic acids). These ω-6 and ω-3 (respectively) can not be synthesised by the cells and are often constituents of cell culture medium, improving robustness in cells (Butler et al., 1999). The introduction of these lipids alone or in combination were not sufficient to restore angiogenesis (and interestingly, neither was the cholesterol supplied by 1, 10 or 100μg/mL of LDL) suggesting that energy restriction was not wholly responsible for the diminished effects seen. It has also previously been discussed (chapter 3 discussion) that endothelial cells are highly glycolytic and derive a large proportion of their energy from glucose metabolism (Davidson et al., 2007), supporting the above suggestion.

Although the energy restriction in this assay may not be wholly responsible for the lack of tube formation observed, the concept of energy restriction (reduced calorie intake without incurring malnutrition or reduction in essential nutrients) in tackling inflammation has previously been investigated. There has been evidence in genetic analysis studies which have suggested that energy restriction lowers the expression of inflammatory genes in mice, such as those associated with angiogenesis and the cytoskeleton (Higami et al., 2006). From dietary restriction studies in mice it has also been shown that reducing dietary intake suppresses angiogenesis in prostate (Mukherjee et al., 1999) and brain tumours (Mukherjee et al., 2002). Interestingly in primates, dietary restriction was shown to have opposing effects and induced circulating factors which promote angiogenic responses in endothelial cells (Csiszar et al., 2013). In this thesis the study of human endothelial cells showed a diminished response with direct lipid withdrawal, even in the presence of essential fatty acid and cholesterol components, suggesting there may be beneficial anti-inflammatory effects of regulating lipids in this way. Of course, in human treatment, energy restriction may be difficult in localised areas such as the skin of psoriatic patients, especially with the integral role of structural lipids such as ceramides. Other lipid modifying agents such as statins have also shown anti-angiogenic properties (Dulak et al., 2005; Weis et al., 2002) although have not been

conclusively beneficial in psoriasis treatment (Mosiewicz et al., 2013). With a greater understanding of the interaction between lipids and inflammation and with a specific targeted approach, these results suggest a potential beneficial effect of regulating lipid environments around inflammatory processes.

Moving on from energy restriction, it was postulated that serum lipid depletion may be affecting tube formation through altered membrane structure and organisation. In an attempt to address this issue, the endothelial migration assay was utilised to give suggestions on how the individual cells were migrating prior to forming tubes and give a better visualisation of their individual morphology, in addition to producing a quantitative functional output. Additionally, the role of adhesion molecules would be addressed in the flow-based recruitment assay (to be discussed). It was observed in the endothelial migration assay that removing serum lipids inhibited migration when low serum concentration in the media were used (e.g. 1.25%) supporting the previous observation in tube formation. However, at higher serum concentrations within the media (e.g. 5, 10 and 20%), no such inhibition was observed, again providing evidence against the energy restriction hypothesis. The migration assay provided the observation that the endothelial cells migrating in delipidated cultures had no apparent differences in morphology than in control cultures (qualitative assessment), suggesting no obvious deficiency in the continual membrane reorganisation required for migration (Lamalice et al., 2007). In order to further clarify the lipid removal effects, adhesion molecule expression was analysed (using functional recruitment assays) in addition to 'replacing' known quantities of lipids.

In order to expand on these assays, assessment of membrane lipid composition and fluidity may need to be performed. For example, it is known that fatty acid treatment can change membrane fluidity and that this can alter the expression of adhesion molecules such as ICAM-1 (Lu et al., 1995). Given the broad range of receptors and factors that are involved in angiogenesis, it is likely that the lipid environment can be tailored to produce specific results. In tube formation in particular, it is recognised that there are either tip cells or supporting cells (often referred to as stalk cells) with signalling pathways such as the notch system to control these processes. It would be interesting to see how the lipid environment changes not only affected membrane fluidity, but lipid signalling and

expression of factors such as Delta-like 4 (DII4) which inhibits tip cell formation and Jagged1, which promotes tip cell formation by antagonising DII4-notch receptor ligation (Benedito et al., 2009; Blanco et al., 2013). If the lipid environment and subsequent membrane changes caused a down-regulation of tip cell formation, then this may be a novel mechanism of suppressing sprouting from existing vasculature, i.e. angiogenesis. Indeed, it has been demonstrated that the N-terminus of human Jagged1 is a phospholipid recognition domain that binds phospholipid bilayers in a calcium-dependent manner (Chillakuri et al., 2013), but whether this can be manipulated through membrane lipid changes is yet to be determined. In support of this hypothesis, it has previously been shown that cholesterol affects the mechanical properties of a cell through the cytoskeleton and changes the membrane surface viscosity, altering membrane organisation (Sun et al., 2007).

The role of exogenous lipid availability appeared crucial to the proliferation of PBMCs, given the significantly reduced proliferation in cells of lipid-depleted cultures, in addition to the increased death found in these cultures. In the leukocyte recruitment assay however, the depletion of serum lipids had no significant effect on the capture of flowing immune cells in TNFα- and IFNγ-stimulation cultures. These results indicate a specificity for the role of lipids and endothelial function, being important for migration and tube formation but not *de novo* protein synthesis dependent mechanisms (up-regulation of adhesion molecules) required for immune cell recruitment. To elucidate these mechanisms further, the addition of known concentrations of lipids and inflammatory mediators were studied in lipid-depleted serum.

The factors introduced into lipid-depleted serum included LDL, oxLDL, PAF, VEGF and S1P. The roles of each of these factors are discussed below but in summary, neither LDL nor oxLDL (at concentrations of 1, 10 or 100μg/mL) were able to restore endothelial responses in the absence of serum lipids. Other known bioactive lipid mediators PAF and S1P, however, were able to significantly enhance tube formation in delipidated serum, although PAF did not have similar effects in control serum. These results highlight the dynamic response of endothelial cells to lipids, as in control serum, oxLDL (5μg/mL) was able to enhance migration. Finally, the pro-angiogenic protein VEGF was unable to restore tube formation without serum lipids present. VEGF is known to be a potent

mediator for vascular development and angiogenesis (Ferrara, 2004; Tammela et al., 2005), which makes the lack of tube formation observed in the presence of VEGF surprising, especially considering the effects seen with bioactive lipids PAF and S1P.

7.1.2 The effect of inflammatory stimuli on endothelial responses

The lack of tube formation observed in the presence of VEGF was surprising considering it was investigated at concentrations previously shown to induce tube formation (Bussolati et al., 2001; Akarasereenont et al., 2002). The importance of VEGF has been highlighted by the anti-VEGF therapies that have been developed for cancer treatment and macular degeneration, in order to prevent tumour metastasis and vascularisation (Meadows & Hurwitz, 2012). VEGF signals through its receptor VEGFR2 on endothelial cells to cause its pro-angiogenic effects. It has been demonstrated that VEGFR2 is present in caveolae (lipid rafts) through association with caveolin-1 (CAV-1), a scaffolding protein (Saulle et a., 2009). It has been shown in leukaemia cells that VEGFR2 in caveolae signals to influence glucose uptake and cell proliferation (Caliceti et al., 2014). In a similar study design to the one used in this thesis, it was shown that endothelial cells are induced by VEGF to form tubes *in vitro* and that silencing CAV-1 in these cells significantly inhibited endothelial tube formation and migration (Madaro et al., 2013). It could therefore be hypothesised that removal of serum lipids affected the ability of membrane lipid raft formation, which in turn affects the ability of VEGF signalling. Interestingly, one paper exploring the effects of cholesterol in HUVEC and VEGF signalling showed that the efflux of cholesterol (by apoA-I binding protein [AIBP]) out of endothelial cells onto HDL was able to reduce lipid rafts, interfere with VEGFR2 dimerization and signalling and inhibit VEGF-induced angiogenesis (Fang et al., 2012). These data and the findings in this thesis suggest that further investigations into serum lipid removal, with the addition of LDL, HDL and VEGF (alone and particularly in combination) may uncover novel interactions between circulating lipids and how they affect the endothelial angiogenic response, particularly concerning lipid rafts.

In addition to this and supporting the role of lipid signalling in response to VEGF, a recent paper described tunnelling nanotubes as a novel communication system between non-adjacent endothelial cells, which exchange signals and other components (Astanina et al., 2015). These tunnelling nanotubes significantly increased the amount of lipid droplets

following VEGF treatment, suggesting that lipids play a vital role in the VEGF angiogenic response. It has also been shown that endothelial cells grown in low glucose environments have increased adhesion strength in comparison with normoglycaemic cultures due to the release of VEGF, with these VEGF levels effectively doubling in 24 hours of culture (Kemeny et al., 2012), similar to the timeframe studied in this thesis for migration. It may therefore otherwise be postulated that VEGF had already been released in these cultures by endothelial cells in response to low energy and that the assay was not sensitive enough to show a response to VEGF. To expand on these results, the use of VEGF blocking antibodies may be employed to analyse the basal release of VEGF by HUVEC during the tube formation and migration assays and assess the extent to which this is affected by lipid depletion and/or addition. For future directions in the tube formation assay in particular, increasing the numbers of time points measured may allow any accelerated angiogenesis above control to be observed. Alternatively, other measures may be considered including branch point counts, tip cell Jagged expression and hanging drop assays of the angiogenic process in addition to attempting the assay in response-limited controls. Assays of receptor expression for all the agonists (VEGF, S1P and PAF) in endothelial cells cultured in serum lipid depleted conditions may also highlight the differential mechanisms which seem to be at play. Relevant to the circulation, it has also been suggested that VEGFB can control endothelial fatty acid uptake, via transcriptional regulation of transport proteins, with VEGFB knockout mice showing less uptake of lipids in tissue (Hagberg et al., 2010) which gives further impetus into the investigation of lipid interactions with VEGF and its receptor(s).

There is evidence to suggest that oxidised phospholipids have structural similarities with the bioactive lipid mediator PAF and can signal through PAF-R to elicit pro-inflammatory responses (Pegorier et al., 2006; Berliner et al., 2009). In this investigation, the role of PAF was examined in both the angiogenesis and endothelial migration assays, in addition to the addition of a selective PAF-R antagonist, WEB2086. It was observed that in control serum cultures in which full serum lipids were present, PAF had no effect in either endothelial assay. This result was supported by the lack of effect in these cultures of the PAF-R antagonist WEB2086. However, in delipidated cultures, PAF was able to restore endothelial migration and tube formation to a significant extent. This result showed the

ability of PAF to be a pro-angiogenic factor and also that the angiogenic process can occur even in the absence of serum lipids, which was not redressed by VEGF as discussed.

This interestingly suggests that factors outside of the VEGF family may play important roles in disease and therefore mark them as important regulatory factors worthy of investigation. It was observed that oxLDL at the concentrations used (1, 10 and 100μg/mL) was not able to restore endothelial responses in lipid-depleted serum. Taken together with results where oxLDL and WEB2086 were combined it was concluded that in HUVEC, oxLDL does not appear to signal through the PAF-R in order to cause migration or angiogenesis. The role of PAF-R in recognising oxidised lipid fragments may have importance in the uptake of oxLDL by macrophages (Rios et al., 2012), but appears to be less influential in endothelial responses. In order to support this result, further inhibitors of the PAF-R could be utilised, in addition to understanding the effect of lipid changes on PAF-R expression. Flow cytometry could not be easily performed for PAF-R expression due to lack of a commercially available fluorescently conjugated antibody and so secondary antibodies may be required to obtain a fluorescent signal. Although PAF-R did not appear to be influential in oxLDL-stimulated responses, the interplay between PAF, oxLDL and S1P may still be an interesting investigation given that they are all bioactive lipid mediators which have been shown in this thesis to significantly enhance endothelial responses. Indeed, it has been shown in bovine aortic endothelial cells that S1P stimulates PAF expression in a dose- and time-dependent manner which contributes to S1P induced migration (Bernatchez et al., 2003). It has also been shown that the effects of PAF (such as vascular leakage) are subsequently prevented by S1P (He et al., 2007). With oxLDL also inducing S1P, the results in this thesis highlight the importance of regulating the balance between these bioactive lipid mediators and may represent an important therapeutic target in the inflammatory response.

Further pro-inflammatory mediators important in the pathology of psoriasis that were studied in this investigation were TNFα, IFNγ and IL-17 (Caldarola et al., 2009). TNFα has the ability to sustain inflammation and so was used as a comparative stimulus as it is likely to be a major cytokine at the site of psoriatic plaques and potentially works in tandem with the lipid environment, therefore a robust positive control for adhesion molecule expression and immune cell recruitment. Overall, TNFα had a strong significant

effect on endothelial function as would be predicted, with results showing migration in serum lipid containing control and delipidated cultures and stimulating the recruitment of inflammatory cells to the endothelial surface. The promotion of endothelial responses by TNFα in lipid-depleted environment suggests that TNFα may work independently from lipids adding further weight to the requirement for lipids in VEGF-dependent responses. There has been evidence to suggest that anti-TNF therapy improves lipid profiles in patients with arthritis (De Sanctis et al., 2013) and that HDL cholesterol function improves after successful treatment of psoriasis with anti-TNF and other therapies (Mehta & Gelfand, 2014). However, in other studies, no favourable changes were observed in lipid profiles of psoriasis patients following treatment with anti-TNF therapy Etanercept (Lestre et al., 2011; Van Dongen et al., 2014). Alternatively, TNFα may directly induce S1P or PAF formation which then indirectly cause migratory and angiogenic responses in endothelial cells. The interaction between lipids and TNFα signalling relative to endothelial function may be an important aspect of inflammation and a target of therapy. However, in this study TNFα was not greatly influenced by lipids relative to migration, immune cell recruitment and immune cell transmigration, giving positive responses in each assay and not being limited by lipid removal, nor enhanced by oxLDL addition. Other factors may be controlled by endothelial responses to TNFα in the absence of serum lipids and these include cytokine production and angiogenic factor secretion. It could be postulated that unlike VEGFR2 which is dependent on lipid rafts, the receptors for TNFα, S1P and PAF-R can all act independently of lipid raft mechanisms and can function adequately in the absence of serum lipids. It has been shown that TNFα can signal both with lipids rafts and independent of them, with RhoA activation requiring lipid rafts and NF_kB/MAPK activation independent of lipid rafts in airway smooth muscle cells (Hunter et al., 2006).

IFNγ is another potent inflammatory cytokine which was analysed in this investigation. In the immune cell recruitment assay, stimulation of endothelial cells with IFNγ produced substantial neutrophil recruitment at 4 and 24 hours similar to TNFα, paralleled by an increase in adhesion molecule (ICAM-1, VCAM-1, E-selectin) expression, characterised using flow cytometry. However, in the endothelial migration assay, initial studies suggested that across a concentration range of IFNγ, this cytokine was not able to induce

endothelial cells to migrate. Interestingly, IFNγ at high concentrations (100IU) causes a morphological change in endothelial cells to have a more elongated appearance (qualitative assessment from previous laboratory studies). TNFα and IFNγ promoting lymphocyte adhesion and transmigration is consistent with other literature (McGettrick et al., 2006; Jaczewska et al., 2014), as with TNFα and IFNγ having differential effects on endothelial responses such as proliferation (Chaitanya et al., 2010). In this investigation, TNFα promoted endothelial migration whereas IFNγ did not, suggesting differential responses. However, after similar increase in immune cell recruitment with IFNγ- and TNFα-stimulated endothelium, transmigration was not significantly affected by ox-/LDL either enhancing or blocking the response.

The differential response of endothelial migration to TNFα and IFNγ may be of interest. Clearly the up-regulation of adhesion molecules was evident on endothelial cells following incubation with both cytokines, suggesting alternative methods of migration other than their adhesive ability. In this thesis, S1P blockade was shown to inhibit oxLDL-induced migration (to be discussed). TNFα may also stimulate migration through similar lipid-dependent mechanisms, as it has been documented that TNFα also causes S1P synthesis, signalling and receptor expression (Osawa et al., 2001; Scherer et al., 2010; Du et al., 2012). The stimulation of IFNγ and IL-17 appear less related to S1P and its receptors, potentially contributing to the lack of migration observed in the scratch assay.

7.1.3 The effect of oxidised lipids on inflammatory responses

One of the particular interests of this investigation was the role of oxidised lipids in psoriasis-like inflammatory processes. Lipid oxidation can encompass a broad range of molecules and have multiple biological implications, but in this investigation oxLDL was used as the primary surrogate marker for oxidised lipids. OxLDL has been identified as the major contributory factor in atherosclerosis, in addition to its identification in psoriatic skin lesions (Tekin et al., 2007) and autoantibodies against oxLDL seen in psoriatic patients (Orem et al., 1999; Rashmi et al., 2009). Individual fatty acids can be oxidised causing a plethora of structurally specific oxidised phospholipids and as this occurs, previously hydrophobic portions of fatty acids become more polar and move to

the membrane surface, allowing recognition by patter receptors (McIntyre & Hazen, 2010).

In this investigation, LDL was commercially sourced and oxidised using a copper sulphate method. This method has previously been shown to cause substantial and reproducible oxidation of the LDL particle, mimicking the alterations to the PC species found *in vivo* (Davis et al., 2008). The role of oxLDL was analysed in all results chapters, covering angiogenesis, endothelial migration, neutrophil recruitment and transmigration across the endothelial monolayer and the proliferation of PBMCs. High concentrations of oxLDL were found to inhibit endothelial tube formation when added to control serum cultures, whereas lower concentrations had no significant effect, similar to the result found in the other assays performed such as angiogenesis and neutrophil recruitment. Both the extent of modification and concentration of oxLDL are important factors. In terms of the physiological concentration of oxLDL, it is suggested that a concentration of between 10 and approximately 50 μg/mL is relevant in serum (Boudjeltia et al., 2012), but higher concentrations (50-100μg/mL) are postulated to be found at sites of lipid accumulation such as within an atherosclerotic vessel (Zaquri et al., 2007). However, as previously discussed, characterising oxLDL within the circulation has varied depending on the method used, with differing mAbs used and differing methods (i.e. sandwich or competitive ELISA), revealing large differences in results, creating difficulty in reporting 'physiological' oxLDL concentrations (Itabe & Ueda, 2007). For the purposes of this thesis and from reference ranges in the literature, 1μg/mL and 5μg/mL are considered 'low' (yet physiological) concentrations and 100μg/mL is considered a 'high' concentration (potentially on the upper limit of physiological).

In the flow-based recruitment assay, high concentrations of oxLDL were shown to increase basal neutrophil binding to the endothelium. Previous data has indicated that oxLDL influences the recruitment of inflammatory cells, including monocytes (Gleissner et al., 2007), neutrophils (Badrnya et al., 2012) and overall leukocyte influx *in vivo* (Liao et al., 1997). The mechanism by which oxLDL increases leukocyte recruitment and migration into the tissue has been suggested as via regulation of adhesion molecules such as ICAM-1, CD11/CD18 on inflammatory cells and junctional adhesion molecules such as JAM-C (Kieper et al., 2005; Liao et al., 1997; Stroka et al., 2012). The increase in neutrophil

recruitment to the endothelial layer had only been shown following stimulation of neutrophils, as opposed to endothelial cells (Badrnya et al., 2012), whereas this research gives evidence of endothelial dependent responses to chronic oxLDL exposure to support subsequent increases in neutrophil attachment. The expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells was analysed following exposure to oxLDL, which revealed only slight changes in ICAM-1 expression, which were concentration and time dependent but may not point to a functionally significant change.

Given the statistically significant increase in neutrophil adherence to the endothelium following oxLDL incubation, but no statistically significant adhesion molecule up-regulation, other mechanisms of oxLDL-induced immune attachment could be postulated. It is possible that oxLDL contained within the culture directly activated neutrophils, which would suggest more of an acute rather than chronic stimulation. Indeed, it has been shown that 100μg/mL of oxLDL incubated with neutrophils for 10 minutes activated CD11b suggesting enhanced adhesive capabilities (Badrnya et al., 2012). The acute stimulation of immune cells, or endothelial cells, was not performed in this thesis and therefore further expansion of the assay could assess acute up-regulation of endothelial adhesion molecules such as P-selectin and activation markers on immune cells. Stimulating endothelial cells with oxLDL has previously been performed and shown to increase transmigration (Stroka et al., 2012) which would suggest a more chronic stimulation (24 hours), but it is important to note that this was in a static model of transmigration. In this experiment oxLDL was not shown to affect transmigration in a flow-based assay and given the lack of adhesion molecule expression, it is likely that the firm adhesion under shear stress is essential for subsequent transmigration steps, giving credence to the study of flow-based models. The stimulation of immune cells as opposed to endothelial cells may be of value particularly if cytokines such as TNFα within the inflammatory environment up-regulate endothelial adhesion molecules, as oxLDL may additionally enhance immune cell adhesion. In particular, monocytes were shown in this investigation to express markers such as CD36 which is well known to bind oxLDL. The circulating levels of oxLDL may therefore enhance the adhesive nature of circulating leukocytes *per se* and warrants further investigation.

In the angiogenesis assay, oxLDL did not statistically enhance endothelial network formation, although at the higher concentration of 100μg/mL oxLDL, statistical inhibition of tube formation was observed. The concentration range analysed in this assay was 1, 10 and 100μg/mL, which was paralleled in the endothelial migration assay producing similar results. The higher concentration inhibited migration whereas the lower concentration had no statistically significant effect. However, there appeared to be a difference in migration result between the concentration of 1 and 10μg/mL, although not significant. This observation, together with other research which suggested a concentration of 5μg/mL was optimal of oxLDL-induced endothelial responses (Wang et al., 2015; Lin et al., 2015; Yu et al., 2011; Dandapat et al., 2007), led to this concentration being investigated. It was subsequently shown in the endothelial migration assay that 5μg/mL oxLDL stimulated statistically significant migration in comparison with control. A total of six oxLDL concentrations (1, 5, 10, 40, 70 and 100μg/mL) were tested in this investigation for effects on endothelial migration and the only concentration to cause significantly increased endothelial migration relative to control was 5μg/mL, showing a specificity of response. The lower concentrations of oxLDL promoting endothelial migration were attributed in part to the role of S1P (to be discussed), but whether a similar mechanism occurs for high oxLDL concentrations inhibiting EC responses is less clear.

One of the potential mechanisms of oxLDL inhibition of EC function is oxidative stress, causing potential apoptotic responses although this was not observed in our hands (utilising a live/dead stain with flow cytometry). Other 'adverse' effects observed following oxLDL treatment (at 100μg/mL) however was that when seeding at sub-confluent seeding densities (40%), endothelial morphology was altered, appearing elongated and less circular. Additionally, higher concentrations of oxLDL appeared adhesive to the scratch area in particular experiments causing 'streaks', suggesting altered binding properties. The streaks and increased oil red O staining in the migration assay may simply be an *in vitro* artefact of the assay, although the concept of altered binding to potential cell matrix may be relevant in disease and the ability of oxLDL to adhere and accumulate. For example, it has been shown that oxLDL had a 15-45 greater binding capacity to extracellular matrix than native LDL (Chang et al., 2001) and that

oxLDL inhibited endothelial cell adhesion to type V collagen (Lorkowski et al., 2009), which may have implications on reduced endothelial responses with high oxLDL retention around the vascular system. Indeed, sub-endothelial LDL which subsequently becomes oxidised may reduce endothelial adhesiveness forming a denuded area in the blood vessel. This then could attract platelets and help to establish plaque formation in the initiation of the atherosclerotic disease. This also has implications in aneurysm repair and the re-endothelialisation of stents.

Separate from receptor-mediated mechanisms, an obvious connection between oxLDL and endothelial responses is oxidative stress. Reactive oxygen species similarly act as a double-edged sword in responses such as angiogenesis, being detrimental in high concentrations but activating pro-angiogenic signalling pathways in lower concentrations (Kim et al., 2013). In a similar study of oxLDL (5μg/mL)-induced angiogenesis, ROS was shown to be increased intracellulary using a fluorescent probe which was blocked via an anti-LOX-1 antibody (Dandapat et al., 2007). It may be possible to reproduce similar methods in order to identify oxLDL levels which cause morphology changes and the relative ROS changes which occur in endothelial cells. Low ROS levels also activate VEGF and so could be considered as a contributory mechanism in oxLDL-stimulated angiogenesis.

Additionally, given the uptake of oxLDL into endothelial cells it may be postulated that lipid imbalances may play a role in the inhibition observed, with lipids in the membrane becoming oxidised or cholesterol loading occurring, interfering with lipid rafts and receptor expression (Levitan & Shentu, 2011). Subsequent to receptor signalling, cholesterol in the membrane has also been shown to decrease the diffusion of NO, required for downstream signalling in a plethora of pathways (Miersch et al., 2008). Given the finding that endothelial expression of oxLDL receptors was low, other mechanisms such as oxidative stress that directly affect the membrane as opposed to regulating receptor expression may be responsible. To clarify all of these mechanisms, fluorescent probes for ROS may be used as mentioned, but also fluorescently labelled oxLDL. The use of fluorescent oxLDL may uncover the extent to which it is taken up by endothelial cells and the degree of accumulation within the membrane. This method could also uncover further details regarding oxLDL receptor expression, by blocking

receptors of interest such as LOX-1, CD36 and even PAF-R and visualising how this affected oxLDL uptake in HUVEC. The uptake of both LDL and oxLDL could be studied in this fashion, in addition to potentially incorporating the effects of HDL, mimicking the circulating interactions of these lipoproteins. Specific antibodies against LDL-R and LOX-1, in addition to other novel peptides and compounds which antagonise LOX-1 (White et al., 2001; Falconi et al., 2013) may uncover these novel interactions.

7.1.4 HUVEC as a model endothelium

In this research, HUVEC were used as the model endothelium for *in vitro* studies. The advantage of using this cell type with only one passage and never from frozen stocks allows the maintenance of biological variability and is a more accurate representation of the tissue than a cell line offers. However, this cell type is from a large vessel within the umbilical cord and so to confirm the results of this research, repeated measures with endothelial cells sourced from dermal regions of the skin may conclude either tissue-specific responses or a universal cellular mechanism. In addition, endothelial cells cultured from the umbilical artery (HUAEC) could be used as an autologous comparison with the umbilical vein. HUAEC were isolated and cultured but could not be harvested in the required numbers for the assays requiring a larger cell number (migration and recruitment assays). HUAEC were analysed using flow cytometry for endothelial markers CD31 and CD105 and presented similar double positive expression results as HUVEC. In addition, given the role of CD36 in atherosclerotic build up in arterial vascular beds, the expression on the arterial cells of CD36 was analysed, showing no visible expression, again paralleling the HUVEC data despite the antibody showing positive staining in isolated monocytes. Initial studies of HUAEC in the angiogenesis assay also showed that these cells did undergo tube formation but were not experimented in detail regarding their response to lipids. For future directions, the role of these arterial cells may be compared to HUVEC in order to characterise whether similar responses to lipids are visualised across the two cell types derived from each vascular bed.

In other studies of HUVEC, it has been shown that 5µg/mL was the minimal concentration of oxLDL required to induce ICAM-1 expression (measured by ELISA), whereas lower concentrations stimulated ICAM-1 expression when located below the endothelial monolayer (immobilized in collagen) (Takei et al., 2001). Interestingly, in this

investigation (Takei et al., 2001), there was no difference in the ability between LDL and oxLDL to diffuse into collagen and become immobilised within it, suggesting similar binding affinities to collagen. In this thesis, high concentrations of oxLDL in the scratched endothelial area appeared to bind more than native LDL (based on oil red staining) and thus the contents of extracellular matrix may influence oxLDL binding and accumulation at the site of inflammation. This study also raises an important consideration for the stimulation of endothelial cells on the apical (facing the lumen) or basolateral (facing the tissue) side of the HUVEC monolayer. The research conducted in this thesis stimulated endothelium on the apical side of HUVEC (assuming the HUVEC side attached to the plastic is the basolateral side), although considering the specificity of concentration required for oxLDL-mediated effects, the stimulation of HUVEC on the basolateral side (such as oxLDL embedded in collagen) may be an interesting investigation to overview oxLDL accumulation around vessels, as opposed to circulating within them. It is known that during angiogenesis, endothelial cells behind the 'tip' cells proliferate and adopt apical-basal polarity important for establishing blood vessels, although little is known about how polarity axes are established and maintained (Lee et al., 2011).

While this is the case for the supporting cells, the endothelial cells that become tip cells and sprout to form new vascular tubes require breaking the existing polarity and adopt the migratory phenotype epitomised by the formation of filopodia and front-rear polarity (Lizama et al., 2013). An important factor in this polarity is the interaction of integrins on the endothelial cell with the extracellular matrix, connecting the actin cytoskeleton to the cellular environment (Bridgewater et al., 2012). During the process of migratory movement, integrins can be endocytosed and recycled on the endothelial cell to allow continued availability at the plasma membrane for repeated interactions (Lizama et al., 2013). It would be interesting to speculate the effect of oxLDL on this process, particularly as it has been shown that oxLDL is able to induce autophagy in endothelial cells (Peng et al., 2014) and smooth muscle cells (Li et al., 2014). Autophagy (regulated disassembly of cellular components) in turn regulates cellular migration by reducing integrin recycling and subsequent cell surface expression (Tuloup-Minguez et al., 2013). High concentrations of oxLDL inhibited HUVEC responses in this thesis which may be explained by the induction of autophagy, but stimulating the endothelium from the basal

side of the monolayer with lower oxLDL concentrations may also uncover specific interactions or influences on integrin binding.

7.1.5 OxLDL-mediated endothelial function: The S1P pathway

In this investigation, the role of serum lipids and known bioactive lipids was investigated for their vascular and immune function, going some way to delineate their individual roles using a system of delipidating human serum and 'adding back' these lipids individually. In addition to the effects seen with TNFα, PAF and ox-/LDL already discussed, one particular discussion point from this thesis is the ability of oxLDL-induced endothelial responses to be attenuated by inhibitors of sphingosine kinase and $S1P_1$. Initially, using the method of serum lipid depletion and analysis of individual lipid entities, S1P was found to be a potent activator of the endothelial migration response in this thesis. In addition to S1P promoting endothelial responses directly, other research has suggested that factors such as TNFα can induce endothelial responses indirectly via the S1P pathway (Zhang et al., 2013; Du et al., 2012; Chen et al., 2004). Building on these findings coupled with other recent research suggesting a link between oxLDL and S1P (Camare et al., 2015), the role of S1P in oxLDL-stimulated endothelial migration was investigated.

The investigation of the S1P pathway was conducted using inhibitors of sphingosine kinase and inhibitors of the S1P receptor(s). The study design permitted the characterisation of oxLDL effects in both the pre- and post-S1P formation event within endothelial cells. If, for example, oxLDL was not inducing S1P production but directly ligating S1P receptors then the blockade of sphingosine kinase would potentially have no effect in this mechanism. Blocking migration with both sphingosine kinase and $S1P_1$ inhibitors suggests that oxLDL induces the formation of S1P within endothelial cells from sphingosine via sphingosine kinase and subsequently S1P ligates its receptor(s). This finding enhances previous investigations in this area (Camare et al., 2015), in which a monoclonal antibody against S1P was used to show inhibition. This method of inhibition however only confirms the link between oxLDL and S1P and may hint at an exogenous release of S1P as this is the method by which the antibody would remove and inhibit the action of the bioactive lipid. S1P is thought to ligate its receptor(s) following distribution to the outer plasma membrane, where membrane ABC transporter flippases direct it to

its receptors (such as in RBCs) or certain chaperones like spns2 (in ECs). It is arguable whether S1P is ever free exogenously to bind to its receptor rather than interacting with the receptor while remaining part of the membrane. Exogenous addition of S1P was used in this thesis which may differ from endogenous S1P production in endothelial cells caused by oxLDL. The resulting inhibition from both inhibitors used suggests the hypothesis that oxLDL does not directly ligate S1P receptors but rather activates endogenous S1P production within endothelial cells before ligating surface receptor $S1P_1$ (and potentially $S1P_{2/3}$). One of the original hypotheses from this thesis was that oxLDL may signal through another lipid receptor (PAF-R) by mimicking the structure of PAF. However, given the lack of effect seen with the studies of PAF and WEB2086, the S1P pathway may act as the integral mediator of migration and angiogenesis, explaining why oxLDL and TNFα (which signal through this pathway) were pro-migratory in these cultures whereas IFNγ and IL-17 were not.

The role for S1P and lipoproteins in inflammatory conditions is complex, with opposing inflammatory influences. The down-regulation of S1P receptors by Fingolimod is an approved treatment for multiple sclerosis in order to sequester immune cells in the lymphoid organs. In this instance, S1P can be considered a pro-inflammatory mediator. In atherosclerosis, the survival responses induced by S1P may be considered anti-inflammatory given the promotion of plaque stabilisation and the relationship between S1P and HDL. The investigations in this thesis have suggested a role for S1P to be a pro-inflammatory mediator in promoting endothelial migration, an important contributory process to angiogenesis. However, other research has suggested that S1P exhibits anti-inflammatory effects in a mouse model of psoriasis, with topical administration causing reduced epidermal hyperproliferation (Schaper et al., 2013). Separate from the specific S1P receptors, S1P in general appears to have a multitude of effects on differing cells throughout the vasculature and tissue beds, making its regulation key to inflammation. This highlights the importance of the tight regulation of S1P (sphingosine kinase to produce S1P and S1P phosphatase to break down S1P) and may be interesting to speculate whether oxLDL also had a role in preventing the breakdown of S1P. Additionally, study of the S1P chaperones within the circulation such as HDL could

differentiate responses from exogenously sourced S1P and endogenously stimulated S1P production.

Adding further complexity to the S1P contribution to inflammation is the opposing actions of the individual S1P receptors, with $S1P_1$ and $S1P_3$ having predominantly pro-inflammatory effects and $S1P_2$ opposing these actions. In this investigation $S1P_1$ inhibition was able to block oxLDL-induced endothelial migration suggesting that this receptor is the major receptor contributing to S1P ligation in this mechanism. In preliminary experiments the role of $S1P_2$ was shown to have no significant effect on oxLDL-induced migration although $S1P_2$ blockade in control cells showed signs of an enhanced or accelerated migratory response over control cells. Circulating S1P is predominantly attached to one of two major chaperones, being HDL or albumin. Depending on the chaperone, S1P can have modifying effects on endothelial cells (Galvani et al., 2015) which may be linked to their specific cell surface interaction, although this mechanism has yet to be fully elucidated. Given that S1P receptors have similar nano-Molar affinity for S1P (Marsolais & Rosen, 2009) it is unlikely that the concentration of S1P is the mechanism of differential responses via the individual receptors. It may be postulated that in the case with oxLDL producing intracellular S1P, the carrier that chaperones S1P to the cell surface may dictate which receptor is preferentially ligated and therefore a potential target in controlling inflammation. In support of this theory, it has been shown that apoM+HDL (as opposed to albumin) is a biased agonist for $S1P_1$ in vascular inflammation, reinforcing the possibility of oxLDL-stimulated S1P as a biased agonist for this receptor also (Galvani et al., 2015). These mechanisms, however, are not fully known and warrant further study.

7.1.6 OxLDL-mediated endothelial function: oxLDL receptors

In this investigation oxLDL was investigated for its effects on endothelial function and has been shown to promote and inhibit EC responses depending on concentration. The promotion of endothelial migration, as discussed, was at least partially attributed to the production of S1P and its signalling through S1P receptors. However, the mechanism by which oxLDL induces S1P production and other direct responses further upstream of these events is hypothesised to be via oxLDL receptors. Three main oxLDL receptors were investigated in terms of their expression on endothelial cells; CD36, LOX-1 and PAF-R. The

expression of CD36 and LOX-1 on HUVEC was investigated by flow cytometry in unstimulated and stimulated cultures to analyse any potential up-regulation caused by oxLDL or other stimulating factors such as psoriasis related cytokines. In the case of LOX-1, no expression was observed on HUVEC in any culture, suggesting that this receptor was not involved in oxLDL-stimulated endothelial responses. In the case of CD36, some expression was observed and although not significant, 24hour culture of HUVEC in the presence of oxLDL showed slight increases in the MFI results obtained. PAF-R inhibitor WEB2086 also showed no inhibition of oxLDL-induced responses suggesting that PAF-R is unlikely to play a role in these responses.

The distribution of oxLDL receptors may differ depending on vascular beds. For example, up-regulation of LOX-1 has been identified in larger arteries and accumulated at the site of atherosclerosis, often co-localised with apoptotic cells (Mehta et al., 2006) but was not found to be expressed on HUVEC in this investigation. CD36 is also implicated in atherosclerosis, particularly with the formation of foam cells (monocytes and macrophages) but is also a mechanism by which oxLDL may be taken up by endothelial cells. The oxidation of LDL is postulated to produce oxidised lipids which mimic the bioactive lipid mediator PAF and could therefore signal through PAF-R, but studies with WEB2086 in this thesis suggest this to be unlikely. This leads to the possible conclusion that oxLDL is signalling via an as yet unknown receptor or via a mechanism of action where S1P is formed under the action of sphingosine kinase in response to lipid uptake. Although there is no evidence in the literature, it cannot be ruled out that in a manner similar to HDL, the oxLDL could act as a chaperone to S1P and essentially be delivering an exogenous S1P signal via $S1P_1$. Further work to characterise LDL and oxLDL ability to chaperone S1P via mass spectrometry would help to answer this question.

7.2 Strengths and Limitations

Overall, this thesis has provided a good body of evidence to support a pro-inflammatory role of oxLDL in the inflammatory events occurring in psoriasis. One of the strengths of the experimental approach was the reproducible and well-characterised methods of serum lipid removal and oxidation of LDL. To study the direct effect of ox-/LDL, serum lipids and native lipoproteins in the serum were removed and characterised using lipid

and protein quantification techniques. Similarly, the oxidation of LDL was performed to replicate the *in vivo* oxidation of LDL using a copper sulphate method which was extensive, consistent and reproducible, giving credence to the results of the *in vitro* studies. The experimental approach also investigated many facets of vascular inflammation which reflect the events occurring in psoriasis. Relevant positive controls were used in assays of angiogenesis, endothelial migration, leukocyte recruitment and immune cell proliferation (e.g. VEGF, PAF, TNFα, IFNγ and PHA) and the assays utilised covered both functional (i.e. flow-based recruitment) and quantitative (i.e. flow cytometry) aspects of the cellular response. The experiments were approached in a logical manner with positive controls, the presence and absence of serum lipids, a range of concentrations of ox-/LDL investigated and specific inhibitors used in certain cases.

One of the main shortcomings in the experimental approach was the lack of experimental replication in some of the assays (particularly in the flow-based recruitment assay and lymphocyte proliferation experiments), which led to lack of statistical power. There was particular variation in the results of leukocyte recruitment and transmigration which warrants a larger number of experimental replicates in order to provide conclusive evidence of a role for ox-/LDL. The model endothelium used in this thesis was HUVEC, which although provides a good representation of human endothelial cells, was not sourced from psoriatic patients or the skin and so improvements to the study design could include microvascular endothelial cells sourced from human dermis. Although a range of assays and concentrations of ox-/LDL were used in this thesis, it is appreciated that oxLDL may accumulate for a significant period of time and therefore an improvement to these *in vitro* studies may be the observation of long-term influence of oxLDL on vascular and immune cells *in vitro* and *in vivo*.

7.3 Summary and future work

Overall, this thesis has made progress in understanding the complex biology of lipids in inflammatory processes, identifying serum lipids as key components in supporting endothelial and immune responses, in addition to elucidating mechanistic processes of oxLDL stimulation.

In order to confirm the results identified in this thesis, blocking antibodies of LOX-1 and CD36 may be used in order to clarify the receptors involved in oxLDL stimulation, in addition to other techniques (different to flow cytometry) to characterise their expression. Other studies have shown that LOX-1 is up-regulated in HUVEC using PCR and western blotting analysis as opposed to flow cytometry and these techniques could be employed to enhance the results in this thesis. For future work, it would be beneficial to perform PCR and western blotting studies on all of the oxidised lipid receptors investigated (LOX-1, CD36 and PAF-R) in addition to using specific inhibitors of these receptors in the *in vitro* assays. Additionally, the up-regulation and expression of these receptors in response to differing oxLDL concentrations is not well-understood and requires further investigation. In this thesis, low concentrations of oxLDL (1 and 5μg/mL) stimulated endothelial migration which was attenuated by blocking S1P production and receptor ligation. Higher concentrations, however, inhibited migration and have previously been suggested to be toxic to endothelial cells. The concentration of 100μg/mL oxLDL in this thesis needs further clarification on its toxic effect to HUVEC. Flow cytometry analysis showed no increase in death following incubation with oxLDL but other parameters require investigation, such as NADPH oxidase, which is a major source of ROS in endothelial cells (in addition to ROS measurement) as well as markers of apoptosis (such as annexin V stain) or quantification of pro-apoptotic gene expression using PCR/western blotting.

Fluorescently labelled oxLDL may be a novel approach to understanding the ligation of oxLDL to endothelial cell receptors, its uptake and subsequent signalling, for which blocking antibodies may again provide valuable insight. Although antibodies against all of the specific S1P receptors are difficult to commercially source for flow cytometry, more in-detail characterisation of these receptors on endothelial cells could be pursued, to elucidate both expression and binding patterns. In addition, inhibitors of more specific elements of the S1P pathway (i.e. SK1 and SK2 as opposed to a pan-SK inhibitor, in addition to antagonists of $S1P_1$, $S1P_2$ and $S1P_3$ separately) may further highlight the potential therapeutic targets of this pathway. Despite the relatively low transfection efficiency of endothelial cells, being able to knockdown S1P receptors in turn would also help in determining their importance.

The use of mass spectrometry in this thesis allowed the identification of specific lipid species and how these change following oxidation. Further expansion on this project could utilise mass spectrometry to measure membrane lipids in response to lipid depletion, LDL and oxLDL in order to give a better understanding of membrane changes. Additionally, S1P could potentially be measured in this fashion, rather than using markers of sphingosine kinase activity. Characterising membrane lipids following fatty acid feeding studies in humans has previously been performed and similar methods could determine which lipids added to lipid-depleted media were capable of significant membrane alterations. These methods could also therefore be performed in psoriatic skin biopsies to understand the lipid membrane changes in both vascular and immune cells.

Expanding on the results obtained in these investigations, further subsets of immune cell may be researched relative to their proliferation, their binding to endothelial cells and subsequent transmigration through the monolayer. Neutrophils provided an adequate model for studying endothelial-leukocyte interactions in the flow-based adhesion assay, both defining a generalised process of cellular interaction and a sufficient number of cellular interactions for manual analysis. In a recent prospective observational study in psoriasis, it was actually shown that psoriasis increased neutrophil activation and neutrophil markers and these factors were correlated to disease severity (Naik et al., 2015). Neutrophils are therefore considered important in the pathology of psoriasis, but are accompanied by other important immune cells such as lymphocytes (which were investigated), dendritic cells and Langerhans cells. An expansion on the model used in this thesis could be to adopt a more complex system, by perfusing a range of immune cells across the endothelial layer and performing post-experimental analysis of the types of immune cells bound and transmigrated (potentially by fixing the endothelial/immune cell field) in response to oxidised lipid stimulation.

A limitation of the results in this thesis were experimental replicates and therefore further enhancement to this assay would be to enhance these replicate numbers, but also to create a three-dimensional layer (i.e. collagen) for immune cells to transmigrate through. This may reveal interactions induced by oxLDL post-transmigration (on the basolateral side of the endothelial cell) and also determine apical (luminal) or basolateral

deposition of LDL /oxLDL as well as determining the transcellular or paracellular uptake of added lipids. An investigation which could be performed on the experimental design used in this thesis would be to determine an acute stimulation (10-30 minutes) of endothelial cells with oxLDL or exogenous S1P in order to characterise whether P-selectin release and expression may be affected (as opposed to a more chronic expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin). Finally, isolated monocytes recruited to venous or arterial endothelial cells may give insights into cells more associated with atherosclerosis, a common comorbidity of psoriasis.

The role of oxidised lipids in the immunopathology of psoriasis still has unclear elements. This thesis has used HUVEC as a model endothelium, for which the benefits have been discussed. For expansion of the results in this thesis and of relevance to endothelial cells in the skin, further investigation may seek to assess the roles of oxidised lipids using microvascular dermal endothelial cells, for example the cell line HMEC-1 or the cryopreserved HDMEC isolated from human dermis. One large difference between the sizes of the vessel the endothelial cells are derived from is the exposure to shear stress. Although shear stress was not investigated in detail in this thesis, it is appreciated that shear stress affects endothelial responses. With particular relevance to angiogenesis, it has been shown that loss of shear stress induces 'tip cell' phenotype in endothelial cells to allow migration, suggesting that shear stress would dampen the endothelial migratory response. Two of the assays performed in this thesis (endothelial migration and endothelial recruitment of immune cells) could be combined, in order to apply shear stress to endothelial cells which are attempting to migrate over a site of injury, realistically mimicking that which may occur *in vivo*. This experiment could potentially be performed with both HUVEC and other ECs sourced from the dermal regions of skin (potentially comparing both healthy and psoriatic skin) in order to fully characterise these events. Indeed, a common problem in injured vascular networks is the lack of re-endothelialisation particularly following aortic aneurism or repair of damage caused by stenting blood vessels. The possibility of co-expression between tip cell markers such as Jagged1 and S1P receptors (or oxLDL receptors) may delineate novel mechanisms in which these bioactive lipids enhance migration and angiogenesis.

The limitation of studying oxLDL *in vitro* is the lack of opportunity to study long term accumulation which is hypothesised to occur in psoriasis. Further work to this thesis could utilise murine models of psoriasis-like inflammation to explore the dynamics of oxLDL accumulation in the skin and the extent of oxidation status. This would provide avenues to explore how this accumulation influences a plethora of inflammatory aspects and comorbidities, including changes in the histological presentation of the skin, the numbers and ratios of infiltrated immune populations, the cytokine profiles within the skin and blood and an understanding of subsequent systemic vascular inflammation which enhances cardiovascular risk.

Finally, a potentially novel mechanism for oxLDL-stimulated endothelial responses through the S1P pathway was hypothesised and given significant supporting results in this thesis. Further work, particularly elucidating the mechanisms of specific S1P receptor ligation may provide a novel target in controlling inflammatory responses. S1P has systemic effects and so targeted therapy would be preferable, focussing on pathological and aberrant angiogenesis as opposed to all angiogenic or immune responses. Targeted therapy appears particularly relevant in psoriasis, given that S1P may affect other cells in the skin. For example, S1P inhibits keratinocyte proliferation, although induces their differentiation and migration (Herzing et al., 2007). Circulating levels of S1P have also been shown to be elevated in severe psoriasis, although not in milder forms of the disease, with these levels unresponsive to TNFα treatment (Checa et al., 2015). Although circulating S1P levels appear to be important, local S1P expression and signalling may also play a major role in areas of continued inflammation. Of importance, it appears that a key part of the mechanism, the potential transport of endogenously produced S1P (stimulated by oxLDL, for example) to its specific receptors by known or unknown chaperones, could be crucial to fully understanding this mechanism. In addition, exogenous circulating S1P levels in psoriasis have been addressed with Ponesimod, a selective $S1P_1$ modulator, which has recently shown therapeutic efficacy in phase II clinical trials (D'Ambrosio et al., 2016).

A key mechanistic aspect of S1P effects which is still largely unknown is the potential ligation of its different receptors simultaneously, such as the pro-migratory $S1P_1$ and the more anti-inflammatory $S1P_2$. Understanding how S1P signals it various receptors in

relation to the stimulus, the cell type and/or the associated chaperone appears worthy of further investigation. Finding the balance of keratinocyte proliferation, immune cell recruitment and trafficking and endothelial cell migration, angiogenesis and permeability may all involve the S1P gradient. Oxidised lipids have certainly been shown to influence endothelial and immune cell functions, with opposing actions particularly on endothelial cells in relation to differing concentrations. Understanding how concentrations of oxLDL at sites of inflammation signal and produce inflammatory effects may go some way to dampening the vascular and immune response. In the case of S1P, broad therapy which targets S1P or its receptors may have detrimental side effects, with S1P having multiple target cells. However, understanding specific mechanisms within the S1P pathway may lend itself to a more targeted therapy to enhance the treatment of psoriasis and other lipid related diseases.

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9 Appendix

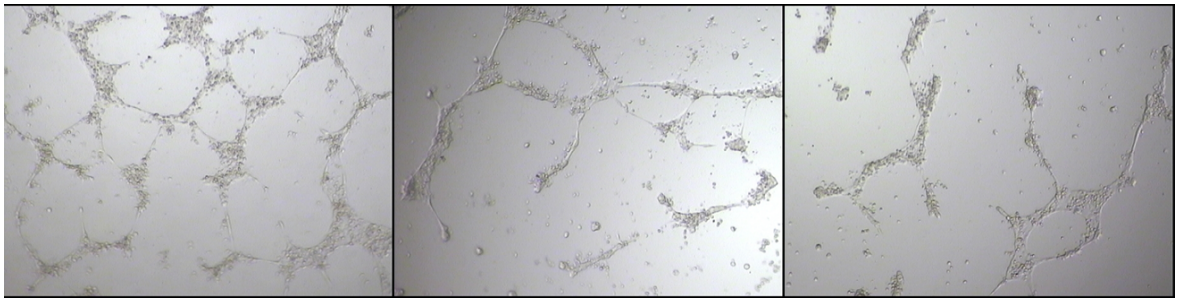


Figure 9-1 Captured images of HUVEC in the tube formation assay following 24 hours of culture, depicting cell clumping difficult for analysis

9.1.1 Cord buffer used for HUVEC isolation recipe

- 8.18g NaCl
- 0.29g KCl
- 1.98g D-glucose
- 0.2mL of 1M $NaH_2PO_4 \cdot H_2O$
- 0.8mL of 1M $Na_2HPO_4 \cdot 7H_2O$
- Constituted in 1L of milliQ ultra purified water and filter sterilised (0.22µM)

9.1.2 HUVEC media recipe

- M199 media
- 20% pooled human serum
- 100U/mL penicillin
- 100µg/mL streptomycin
- 292µg/mL L-glutamine
- (M199 constituted in milliQ ultra purified water) and filter sterilised following addition of serum (0.22µM)

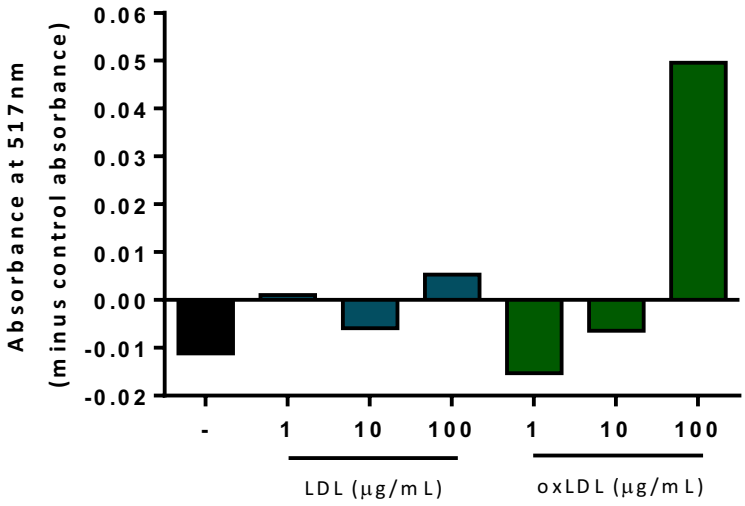


Figure 9-2 The absorbance of Oil Red O staining of HUVEC following 24 hour migration assay.

Figure shows the supernatants taken from 24-well plates in which HUVEC had been stimulated and allowed to migrate. Cells were stained for Oil Red O to analyse lipid streaks and subsequently removed from well and analysed in a spectrophotometer for absorbance (read at 492nm).

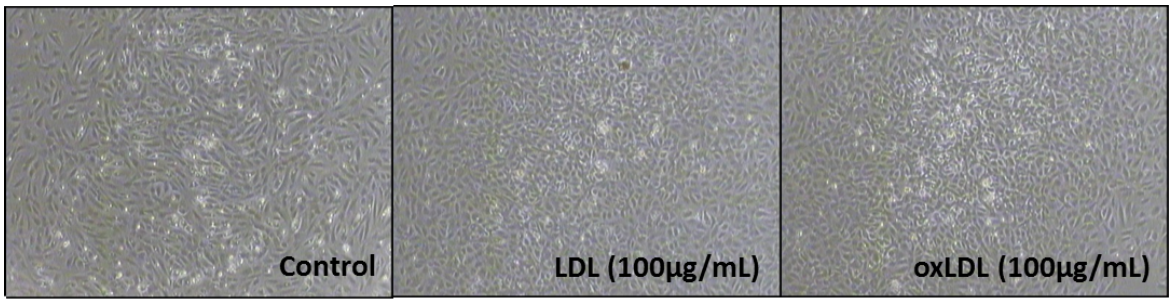


Figure 9-3 Adhesion of endothelial cells to 24-well plate following 24 hour pre-coat with LDL or oxLDL

Captured images show endothelial cells attached to gelatin (+/-ox-/LDL)-coated 24-well plates, suggesting no altered ability to adhere in the presence of these lipids.

9.1.3 Chemically defined lipid concentrate ingredients (Gibco)

Component	Concentration (mg/L)
Arachidonic Acid	2.0
Cholesterol	220.0
DL-alpha-Tocopherol Acetate	70.0
Ethyl Alcohol 100%	Confidential
Linoleic Acid	10.0
Linolenic Acid	10.0
Myristic Acid	10.0
Oleic Acid	10.0
Palmitic Acid	10.0
Palmitoleic Acid	10.0
Pluronic F-68	90000.0
Stearic Acid	10.0
Tween 80®	2200.0

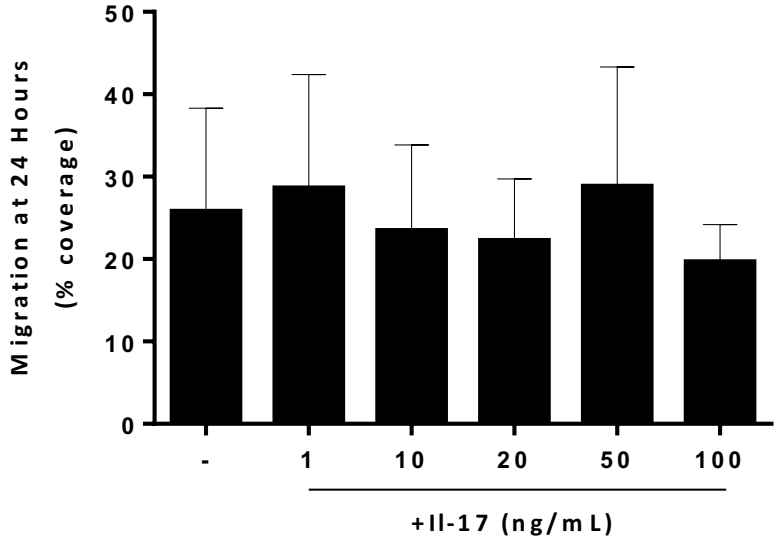


Figure 9-4 Role of IL-17 on endothelial migration in control serum cultures (n=2)

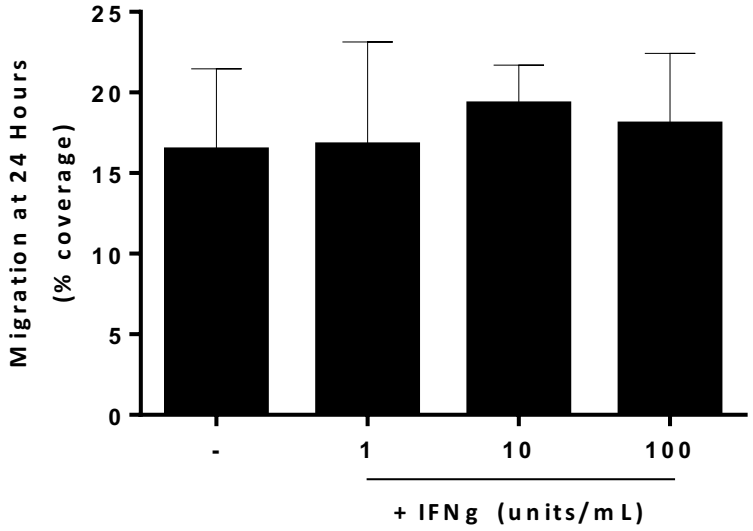


Figure 9-5 Role of IFNγ on endothelial cell migration in control serum cultures (n=2)

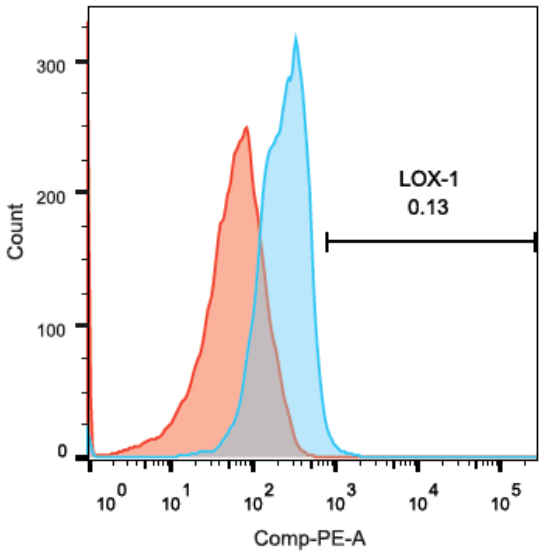


Figure 9-6 Expression of LOX-1 on unstimulated HUVEC
Isotype-stained HUVEC (Blue) and antibody-stained HUVEC (red)

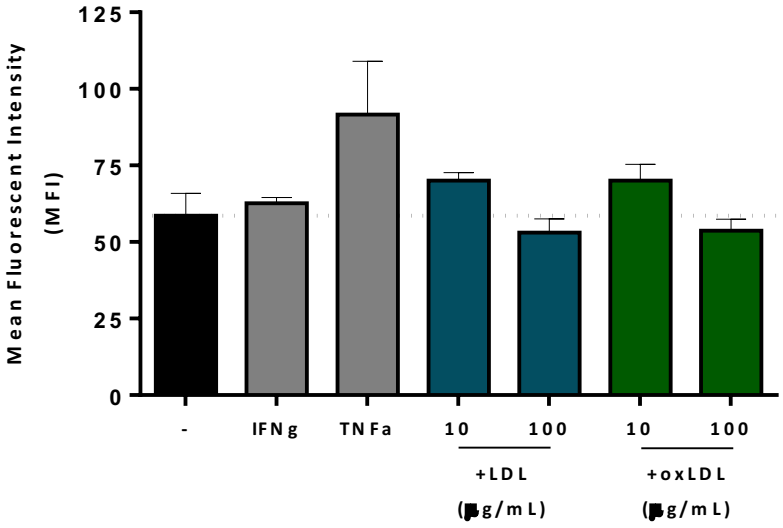


Figure 9-7 Expression of LDL-R on HUVEC following 24 hour stimulation (n=2)

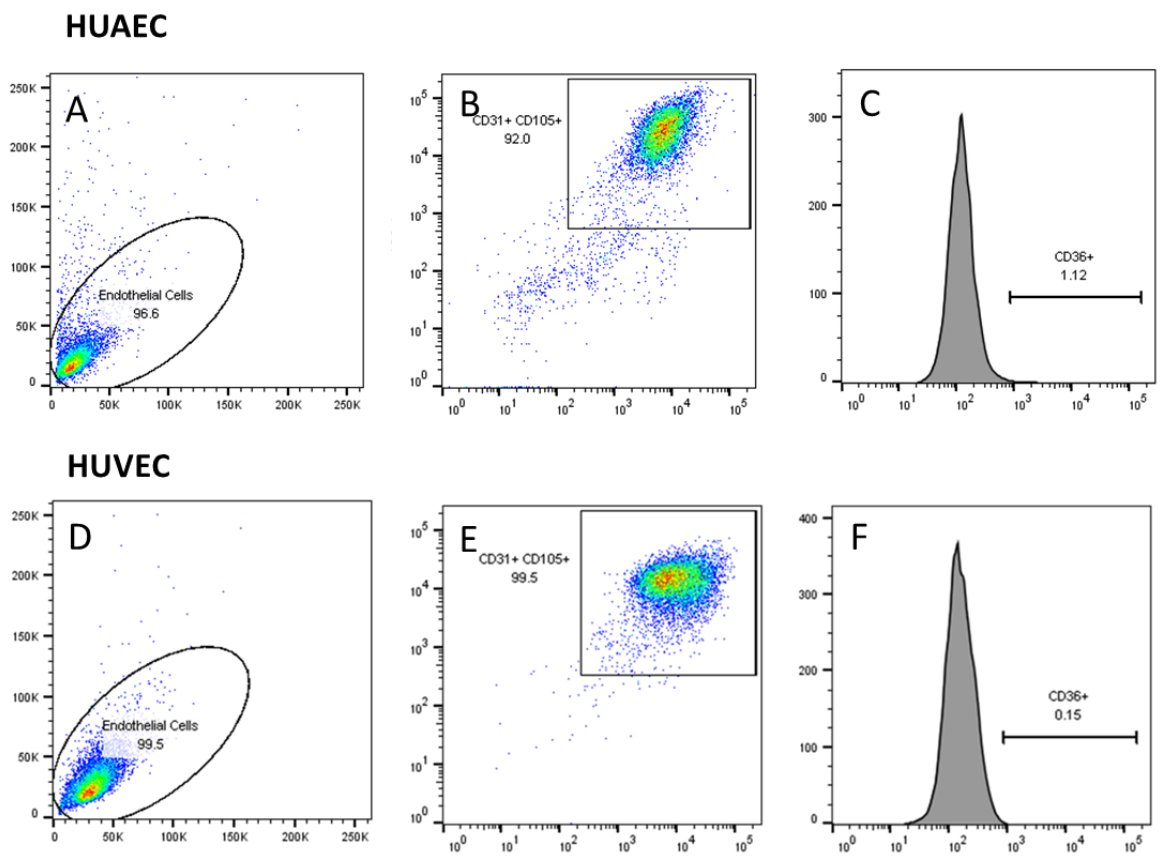
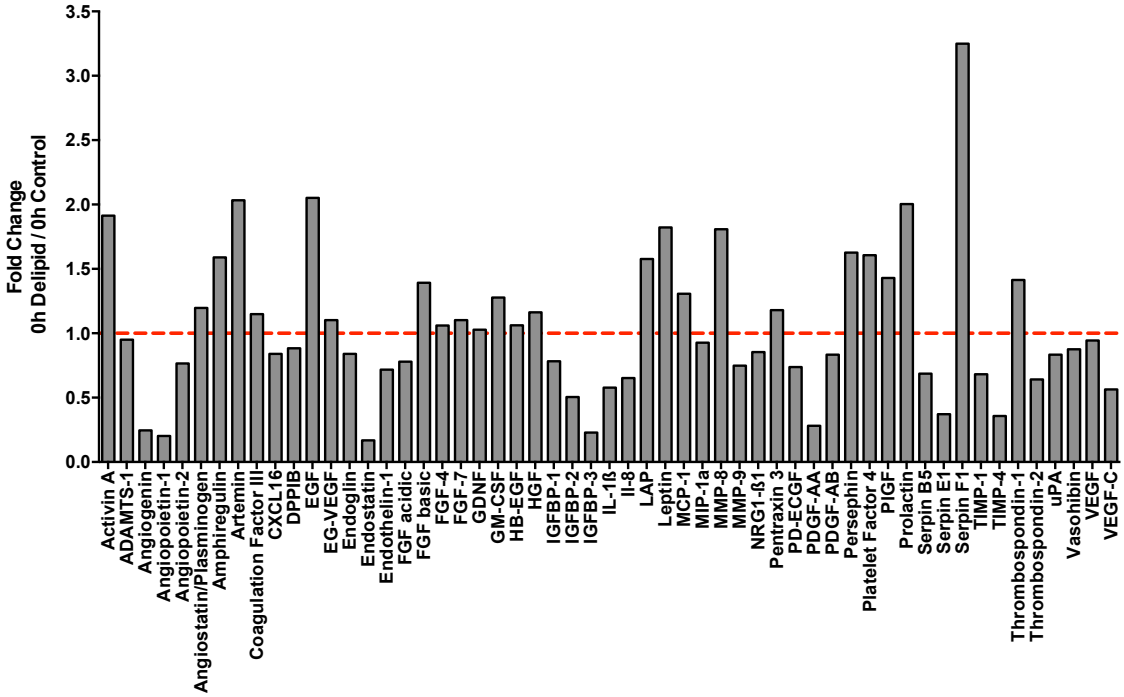
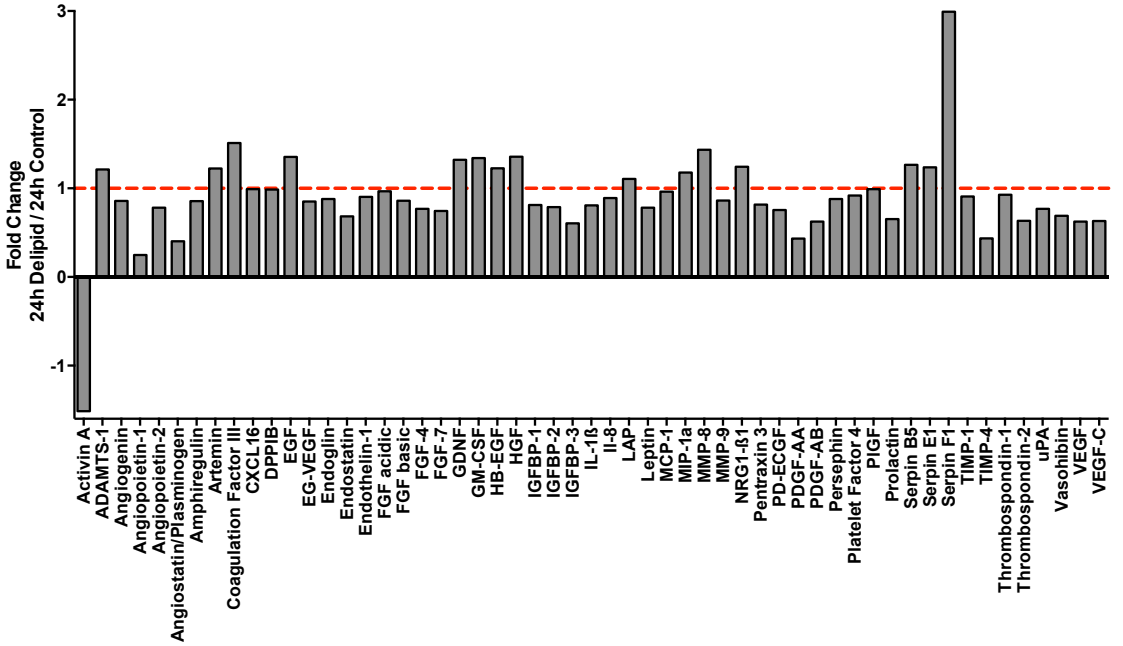


Figure 9-8 Flow cytometry analysis of HUAEC (ABC) and HUVEC (DEF) showing double-positive CD31+CD105+ staining (B,E - confirming EC phenotype) and CD36 expression (C,F)

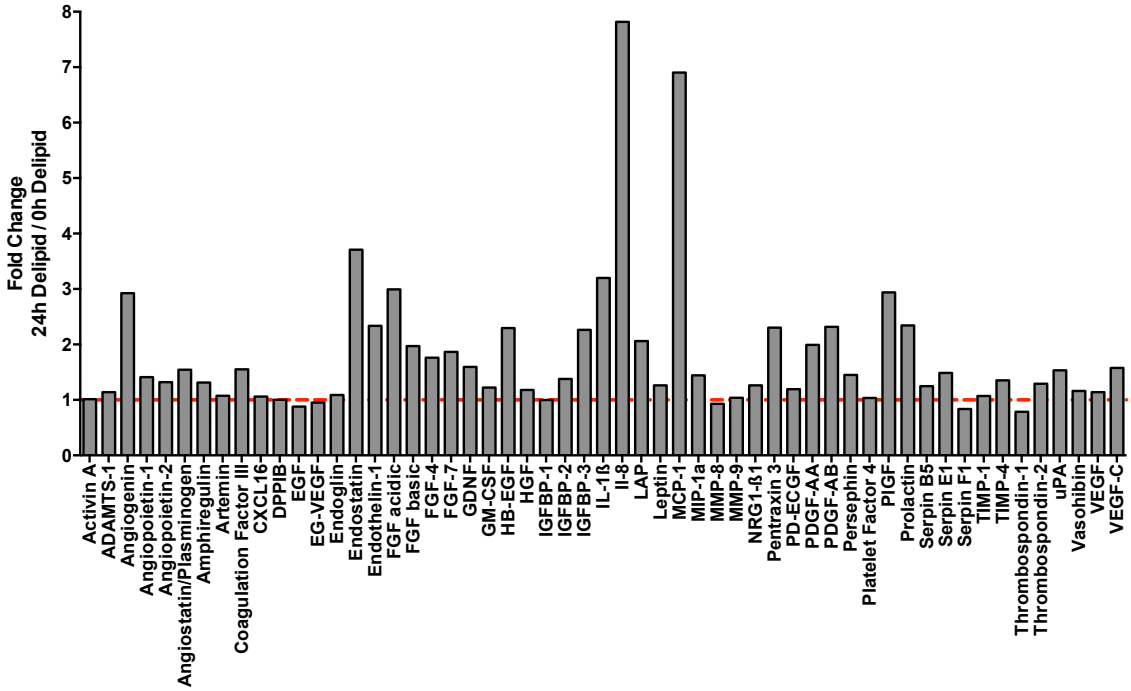
A Change in angiogenic factor release from HUVEC (Delipid over Control at 0 hours)



B Change in angiogenic factor release from HUVEC (Delipid over Control at 24 hours)



C Change in angiogenic factor release from HUVEC (Delipid 24hour over Delipid 0 hour)



D Change in angiogenic factor release from HUVEC (Control 24hour over Control 0 hour)

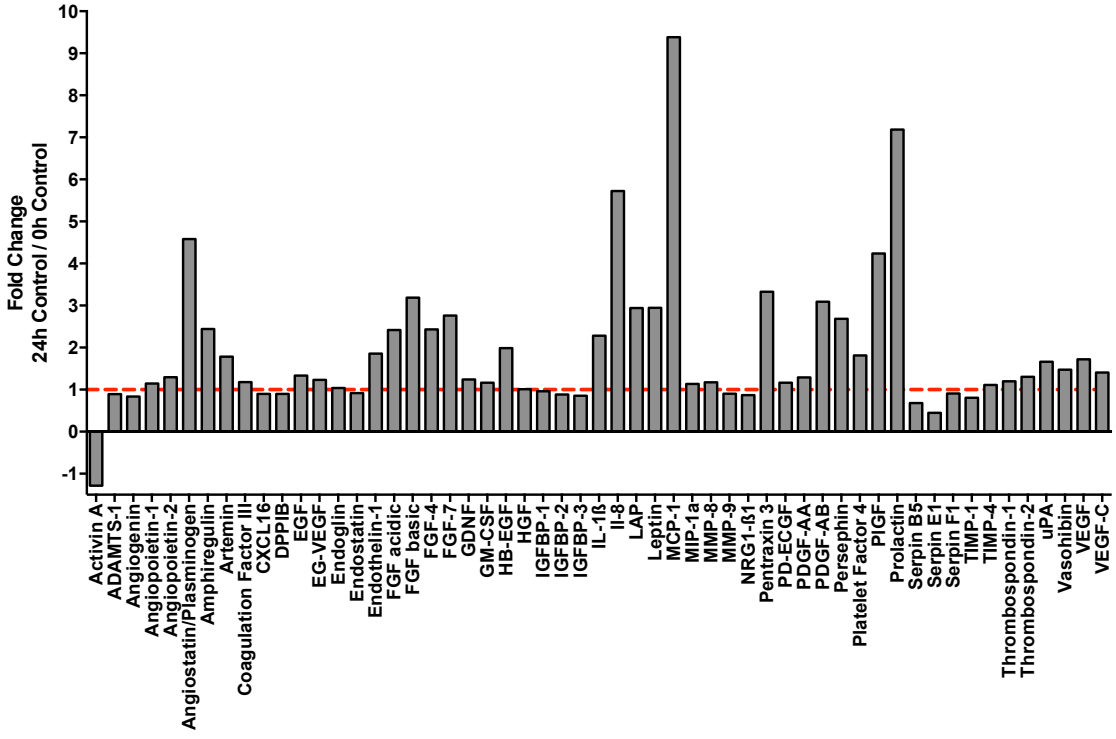


Figure 9-9 Results of angiogenic factor release from HUVEC culture
Supernatants from HUVEC culture in control serum cultures and delipidated serum cultures at both 0 hours and 24 hours of culture were applied to an angiogenesis array (R&D Systems; n=1).