The Effect of Statins on Cytokine Regulated Macrophage Gene Expression in Atherosclerosis

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Abstract

Atherosclerosis, a chronic inflammatory disorder of the vasculature, is one of the major causes of cardiovascular disease and is responsible for most deaths in western societies. The disease is characterised by a number of steps that occur during the lifespan of an individual, including fatty streak formation, development of complex lesions containing a fibrous cap, and thinning and rupture of such plaques leading to thrombosis and clinical complications of this disease. Macrophages play key roles during all stages of this disease such as foam cell formation, amplification of the inflammatory response and control of plaque stability. The actions of macrophages during this disease are regulated by cytokines present in atherosclerotic lesions such as interferon-γ (IFN-γ), tumour necrosis factor-like protein 1A (TL1A) and interleukin-17 (IL-17).

Statins are widely used for the primary and secondary prevention of atherosclerosis and its complications because of their ability to inhibit cholesterol biosynthesis and, thereby, plasma levels of pro-atherogenic low density lipoprotein. However, statins have actions beyond lowering cholesterol levels, the so-called pleiotropic effects, and includes acting in an anti-inflammatory manner. Unfortunately, the anti-inflammatory effects of statins are not fully understood and therefore formed the focus of studies in this thesis using a combination of human macrophage THP-1 cell line, primary cultures of human monocyte-derived macrophages and mouse RAW264.7 macrophage cell line.

Simvastatin generally acted in an anti-inflammatory manner in macrophages in relation to the expression of several pro-atherogenic genes, such as monocyte chemoattractant proein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1),regulated by IFN-γ, TL1A or IL-17. Such an anti-inflammatory action also extended to another statin, Atorvastatin. The inhibitory action of Simvastatin on IFN-γ induced expression of MCP-1 and ICAM-1 was reversed, at least in part, by intermediates of the 3-hydroxy-3-methyl-glutaryl coenzyme A pathway such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate. This suggested a potential role for monomeric G-proteins that require such intermediates for activation. In addition, Simvastatin inhibited the phosphorylation-mediated activation of signal transducer and activator of transcription-1 (STAT1), a key transcription factor in IFN-γ signalling, on tyrosine 701 and serine 727 in response to the cytokine. The effect of Simvastatin on MAP Kinase (MAPK) pathways in macrophages was also analysed. The statin attenuated the IFN-γ induced activation of p38 MAPK and extracellular signal activated kinase (ERK)-1/2. Simvastatin also affected the constituve expression of many components of the MAPK pathways (e.g. ERK-1/2) along with downstream genes involved in atherosclerosis (e.g. ATP-binding cassette transporters-A1 and-G1).

The effect of Simvastatin on lipid profile of THP-1 and RAW264.7 macrophages was also investigated. Simvastatin does not affect total polar lipids and triacylglycerol. The statin also had no significant effect on fatty acid distribution into polar lipids and triacylglycerol.

The studies presented in this thesis provide insights into the actions, and potential mechanisms, underlying the anti-inflammatory effects of statins on human macrophages along with their effects on lipid profiles. Such studies are essential given the widespread use of statins and a need to gain a deeper understanding of their actions.

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Publications

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Alkorashy, M., Ramji, D.P. 2014. Anti-inflammatory actions of statins in human macrophages.82 nd EAS congress. May 31st –June 3rd. Madrid, Spain.

List of Abbreviations

AA: Arachidonic acid.

ABC: ATP-binding cassette.

ABCA1: ATP-binding cassette transporter A1.

ABCG1: ATP-binding cassette transporter G1.

ACAT: Acyl coenzyme acylcholestrol transferase.

AFCAPS: Air force coronary atherosclerosis prevention study.

ALA: α-linolenic Acid.

APCs: Antigen presenting cells.

ApoE: Apolipoprotein E.

BCA: Bicinchoninic acid assay.

bFGF: Basic fibroblast growth factor.

BSA: Bovine serum albumin.

CAD: Coronary artery disease.

CD36: Scavenger receptor.

CE: Cholesterol ester.

CHD: Coronary heart disease.

CRP: C-reactive protein.

CVA: Cerebrovascular accident

CVD: Cardiovascular disease.

DATP: Deoxyadenosine Triphosphate.

dCTP: Deoxycytidine triphosphate.

dGTP: Deoxyguanosine triphosphate.

dHA : Docosahexaenoic acid.

dNTP: Deoxyribonucleotide triphosphate.

DMSO: Dimethyl sulfoxide.

dTTP: Deoxythymidine triphosphate.

ECs: Endothelial cells.

EPA : Eicosapentaenoic acid.

ERK: Extracellular signal-regulated kinase

FA : Fatty acids.

FPP: Farnesyl pyrophosphate.

GGP: Geranylgeranyl pyrophosphate.

GM-CSF: Granulocyte macrophage-colony stimulating factor.

HB-EGF: Heparin binding-epidermal growth factor.

HDL: High density lipoprotein.

HDL-C: High density lipoprotein-cholesterol.

HI -FCS: Heat-inactivated foetal calf serum

HMDMs: Human monocyte-derived macrophages.

HMGR: 3-hydroxy-3-methylglutaryl-coenzyme a reductase.

ICAM-1: Intracellular adhesion molecule-1.

IFN-γ: Interferon-γ.

IGF-1: Insulin–like growth factor-1**.**

ILs: Interleukins.

IL-1: Interleukin-1.

IL-12: Interleukin-12.

IL-17: Interleukin-17.

IL-33: Interleukin-33.

IVUS: Intravascular ultrasound.

JAK-STAT: Janus kinase-signal transducers and activators of transcription.

LA: Linoleic Acid.

LCPUFAs: Long chain polyunsaturated fatty acids.

LDL: Low density lipoprotein.

LDL-C: Low density lipoprotein- cholesterol.

LDL-R: Low density lipoprotein receptor.

LXRs: Liver X receptors.

MAP: Mitogen activated protein.

MCP-1: Monocyte chemoattractant protein-1.

M-CSF: Macrophage colony stimulating factor.

MI: Myocardial infarction.

MIP-1: Migratory inflammatory protein-1.

MIRACL : Myocardial ischemia reduction with aggressive cholesterol lowering.

mmLDL: Minimally modified low density lipoprotein.

M-MLV: Molony murine leukaemia virus.

MMP-2: Matrix metalloproteinase-2.

MMP-9: Matrix metalloproteinase-9.

MUFAs: Monounsaturated fatty acids.

OxLDL: Oxidised low density lipoprotein.

PAGE: Polyacrylamide gel electrophoresis.

PCI: Percutaneous coronary intervention.

PDGF: Platelet derived growth factor.

PE: phosphatidyl ethanolamine.

PMA: Phorbol 12-myristate 13-acetate.

PPARs: Peroxisome proliferator-activated receptors.

PPARα: Peroxisome proliferator-activated receptor-α.

PPARγ: Peroxisome proliferator-activated receptor-γ.

PUFAs: Polyunsaturated fatty acids.

RNA: Ribonucleic acid.

RNAi: Ribonucleic acid interference assays.

RCT: Reverse cholesterol transport.

RT-qPCR: Reverse transcription quantitative polymerase chain reaction.

RT: room temperature.

RXR: Retinoid X receptor.

SACC: Salivary adenoid cystic carcinoma.

SAPK: Stress-activated protein kinases.

SDS: Sodium dodecyl sulphate.

SMC: Smooth muscle cell.

SOCS3: Suppressor of cytokine signalling 3.

SPARCL: Stroke prevention by aggressive reduction in cholesterol levels.

SR-A: Scavenger receptor A.

SREBP: Sterol regulatory element–binding proteins.

TD: Tangier disease.

TexCAPS: Texas coronary atherosclerosis prevention study.

TGF-α: Transforming growth factor-α.

TGF-β: Transforming growth factor -β.

TL1A: Tumor necrosis factor–like protein 1A.

TNF-α: Tumour necrosis Factor-α.

TNFSF: Tumor necrosis factor superfamily.

TSP-1: Thrombospondin-1.

VCAM-1: Vascular cell adhesion molecule-1.

VEGF: Vascular endothelium derived growth factor.

vLDL: Very low density lipoprotein.

Chapter One

1.1 Atherosclerosis an overview

Coronary artery disease (CAD), also known as coronary heart disease (CHD), is a condition where narrowing of the coronary arteries takes place (Allender et al., 2008). Atherosclerosis is the principal cause of CAD and mainly affects the large and medium arteries (Lusis et al., 2004). This disease is commonly described as hardening of the arteries; the word atherosclerosis comes from the Greek words “athero” (meaning gruel or paste) and “sclerosis” (hardness). In the UK, CHD is the most common cause of mortality and accounts for about 45% of all cardiovascular disease death (National Statistic, 2012).

Atherosclerosis starts in the early life of an individual due to the various risk factors detailed in section 1.2, and progresses during the lifetime of that individual(Berenson, 2002). It is considered as a progressive disease that is characterised by lipid accumulation and inflammation within the walls of the arteries (see Figure 1.1 for the structure of a normal artery) (Lusis, 2000).

1.2 Risk factors

Epidemiological studies show that there are several risk factors for atherosclerosis (Table 1.1). The most common risk factors include genetic background, lipid metabolism disorders, particularly high plasma cholesterol levels, hypertension and diabetes mellitus (Lusis et al., 2004). Raised blood cholesterol plays a key role in developing CHD and is responsible for over 60% of such cases. For example, a large case control study reported that around 40% of heart attack cases are due to elevated blood cholesterol and those with high cholesterol are three times greater at risk of the disease than those with normal blood cholesterol (Yusuf et al., 2004).

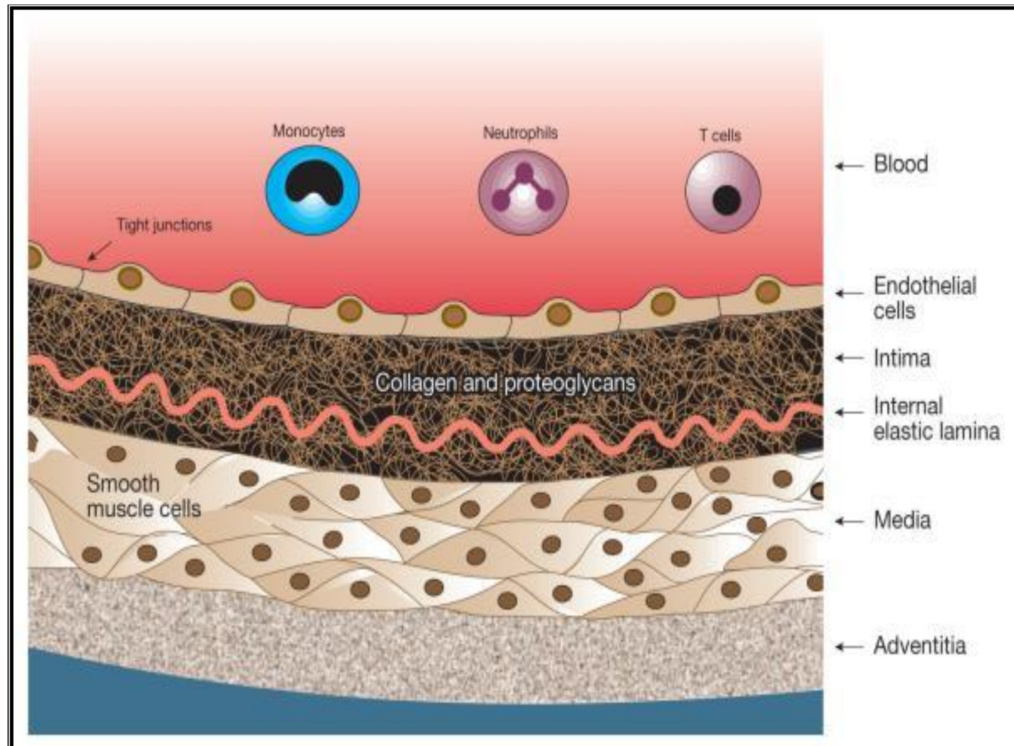

Figure 1. 1: Structure of a normal large artery.

A large artery consists of three morphologically distinct layers. The intima, the innermost layer, is bounded by a monolayer of endothelial cells on the luminal side and a sheet of elastic fibres, the internal elastic lamina, on the peripheral side. The normal intima is a very thin region (size is enhanced in this figure for illustration) and consists of extracellular connective tissue matrix, primarily proteoglycans and collagen. The media, the middle layer, consists of smooth muscle cells (SMCs). The adventitia, the outer layer, consists of connective tissues with interspersed fibroblasts and SMCs (Adapted from Lusis, 2000).

Very low density lipoproteins (VLDL) are of two types according to its particle's size: small VLDLs and large VLDL. Small VLDLs has the ability to enter the arterial wall and reaches the intima layer while large VLDLs does not (Carmena et al., 2004). Compelling evidence suggests that small VLDLs are independently associated with atherosclerosis (Libby et al., 2011).

1.3 Pathophysiology of atherosclerosis

Activation of endothelial cells (ECs) by the various risk factors is a main initiating event in atherosclerosis. From all the risk factors, the accumulation of LDL in the subendothelial matrix is the major cause of activation (Lusis, 2000). Once activated, the ECs secrete various chemoattractant molecules and express many adhesion molecules on the cell surface. The immune cells, particularly monocytes and T-lymphocytes in the blood stream, are then attracted towards the activation/injury site in response to such chemoattractants and begin to produce fatty streaks in these locations (Li and Glass, 2002). Overall, the progression of atherosclerosis can be subdivided into four major stages: early fatty streak development; early fibroatheroma; advancing atheroma; and complex lesion development (Insull, 2009). The different stages of atherosclerosis are described below in more details (Figure 1.3).

1.4 Early fatty streak development

Early fatty streak development arises during childhood and adolescence (Insull, 2009). The first step usually begins when LDL particles migrate from the blood to the internal intima of arteries. However, the mechanism by which LDL initiates atherogenesis is not fully understood. Nevertheless, there is increasing evidence from biomedical and animal studies that suggest that the LDL particles must be modified before it can become pathogenic (Grundy, 1993). It is believed that modified LDL plays a major role in atherosclerosis pathogenesis particularly the formation of foam cells (Ross, 1999). In addition, modified LDL activates ECs, which leads to initiation of an inflammatory response.

Table 1.1: The genetic and environmental risk factors for atherosclerosis (adapted from (Lusis, 2000).

Genetic Risk Factors	Environmental risk factors
• Elevated LDL and VLDL • Reduced levels of high density lipoprotein (HDL) • Elevated blood pressure • Elevated levels of homocysteine • Family history • Diabetes and obesity • Elevated levels of haemostatic factors • Depression • Gender (male) • Systemic inflammation • Metabolic syndrome	• High fat diet • Smoking • Lack of exercise

The ECs then express adhesion molecules and secrete chemokines, chemoattracting cytokines, which then attract monocytes, lymphocytes, mast cells and neutrophils in the blood stream into the arterial wall (Li and Glass, 2002).

The migration process of immune cells such as monocytes is aided by the expression of chemokines by ECs such as monocyte chemoattractant protein-1 (MCP-1), macrophage colony-stimulating factor (M-CSF), migratory inflammatory protein-1 (MIP-1), tumour necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) (Li and Glass, 2002). MCP-1 is a key factor in the pathogenesis of atherosclerosis and is produced by atheromatic cells such as ECs, smooth muscle cells (SMCs) and macrophages (Sheikine and Hansson, 2004; Boisvert, 2004). MCP-1 has been found to cause a rapid progression of atherosclerosis in hypercholesterolaemic mice (Namiki et al., 2002). Additionally, a number of studies have shown that MCP-1 also induces the expression of genes that are thought to play a role in controlling plaque instability (e.g. matrix metalloproteinases (MMP)) and stimulate acute thrombosis (e.g. tissue factor) (Schecter et al., 1997; Yamamoto et al., 2000). Furthermore, a study by Inoue and colleagues (2002) on apolipoprotein-E (apoE)-knockout mice (a widely used mouse model of the disease) confirmed the involvement of MCP-1 in advanced lesion complications. Deficiency of MCP-1 reduces lesion development in such mice (Ni et al., 2001). Monocytes are attracted by adhesion molecules on the surface of the ECs such as P- and E-selectins, which interact with L-selectin molecules on the surface of monocytes(Bobryshev, 2006). The interaction between the selectins causes the monocytes to roll across the endothelial surface, which enables molecules such as Vascular cell adhesion molecule-1 (VCAM-1) and Inter-cellular adhesion molecule-1 (ICAM-1) to initiate binding with integrins. Integrin binding causes a higher level of adhesion to the endothelium so that the migration of monocytes through the endothelial layer to the intima can begin (Figure 1.2).

Once the monocytes have passed through the endothelial layer and are present in the intima of the blood vessels, they differentiate into macrophages and begin to form foam cells by taking up oxidised LDL from the subendothelial environment. Monocyte-derived macrophages have a multifunctional role in the development and progression of atherosclerotic lesions (Weber et al., 2008). In humans, around 40% of the atherosclerotic plaque expresses macrophage markers (Quehenberger, 2005; Hansson and Libby, 2006). A study by Smith and co-authors (1995) showed that apoE-knockout mice lacking macrophages have very little atherosclerosis despite the presence of high levels of cholesterol in the blood.

Macrophages are associated with scavenger receptors, such as CD36, lectin-type oxidised low-density lipoprotein receptor 1 (LOX-1), scavenger receptor A (SR-A) and SR-B1, which internalise oxLDL leading to foam-cell formation, a rate-limiting step in atherosclerosis (Nicoletti et al., 1999; Quehenberger, 2005). After the uptake of oxLDL, endosomes loaded with ligand-bound scavenger receptors transfer cholesterol esters to lysosomes for intercellular breakdown. In addition, scavenger receptors can mediate the uptake of modified antigens for presentation to antigen-specific T cells and thereby initiate an adaptive immune response (Janeway and Medzhitov, 2002). The induced expression of inflammatory cytokines, chemokines and co-stimulatory molecules in macrophages provide an additional antigen-specific stimuli and co-stimulatory molecules activate antigen-specific T cells (Glass and Witztum, 2001; Li and Glass, 2002). Therefore, macrophages act as an important link between innate and adaptive immunity involved in atherogenesis.

1.5 Early fibroatheroma

This occurs in early adulthood of individuals and is characterised by fibrous plaque lesions (Burke et al., 2001; Stary, 2003). It results from a series of biochemical changes that occur inside the arterial wall. These events or changes include an increase in the amount of foam cells deposited within the plaque, accumulation of natural killer cells in the arteries, progressive increase in the lipid binding capacity of

extracellular proteoglycans as a consequence of increased synthesis and secretion by smooth muscle cells (SMCs), and death of macrophages and SMCs (Insull, 2009). Consequently, there is an increase in the level of inflammatory activities, which will subsequently lead to structural changes of the intimal layer leading to an enlarged pool of lipid-rich necrotic cores dominating 30 % to 50 % of arterial wall volume (Figure 1.3) (Ross, 1999; Insull, 2009).

1.6 Advanced atheroma

Unlike fibroatheroma, advanced atheromas typically occur in the mid of fifth decade of individual’s life and is characterised by a development of a thin cap fibroatheroma (TCFA) that is susceptible to rupture (Virmani et al., 2000; Cheruvu et al., 2007; Moreno et al., 2006). This lesion is usually labelled as a vulnerable plaque and is associated with life threatening thrombosis due to a high risk of rupture (Insull, 2009). This happens when the fibrous cap transforms into a thin cap as a result of decreased extracellular matrix (ECM) synthesis by the reduced number of SMCs within the cap and degradation of the matrix by proteases produced by the infiltrating macrophages (Thim et al., 2008). Thin-cap atheroma and ruptured plaques are responsible for about 1.2% to 1.6% of the coronary arteries events. Most of these lesions mainly occur in the proximal branches of the major coronary arteries, and the majority of them are clustered within ≤ 2 adjacent 20-mm artery segments (Cheruvu et al. ,2007).

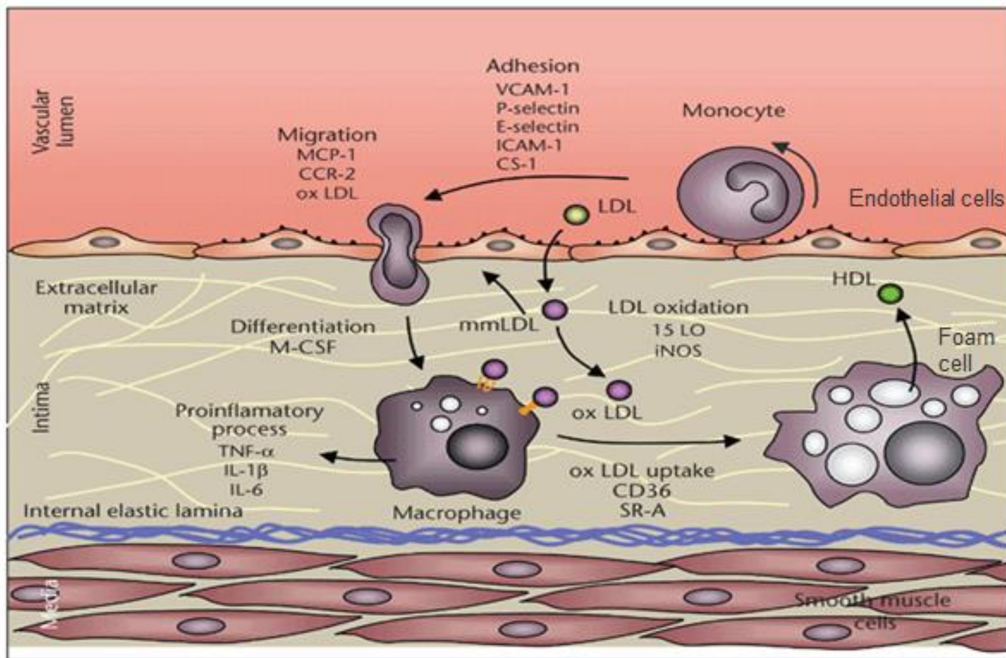

Figure 1.2: Mechanisms contributing to the recruitment of monocytes to the artery wall and foam cell formation.

LDL is subject to oxidative modification in the subendothelial space, progressing from mmLDL to extensive oxLDL. Monocytes attach to EC that have been induced to express cell adhesion molecules by mmLDL and inflammatory cytokines. Adherent monocytes migrate into the intima where they differentiate into macrophages. Uptake of oxLDL via macrophage scavenger receptors, such as SR-A and CD36, leads to foam cell formation.

Abbreviations: mmLDL, minimally modified low density lipoprotein; oxLDL, Oxidised low density lipoprotein; SR-A: Scavenger receptor A; ICAM-1, Intercellular adhesion molecule-1; VCAM 1, vascular cell adhesion molecule-1; MCP-1, Monocyte chemoattractant protein-1; CCR2, Chemokine receptor-2, M-CSF, Macrophage colony stimulating factor. Taken from (Glass and Witztum, 2001).

1.7 Complex lesion development

In this stage, there is a continuous rupture of thin fibrous caps that are usually clinically silent. The ruptured areas are replaced by fibrous tissues, collagen fibres and ECM that may re-rupture in the future (Lusis et al., 2004). This process of rupture, calcium deposition, thrombosis and healing may recur up to four times at the same site resulting in the formation of multiple layers of healing tissue inside the arterial wall (Insull, 2009). The exact mechanism of plaque rupture is not fully understood. However, the cytokine IFN-γ is thought to promote plaque instability by cap thinning, production of inflammatory cytokines and proteases, particularly matrix metalloproteinases (e.g. MMP-1, 2, 3, 9). All these decrease collagen synthesis and cause the accumulation of injured or apoptotic cells within the necrotic core (Harvey and Ramji, 2005). The increasing size of some plaques is sufficient to block the artery and cause serious events such lethal ischemia. This has been found to be the case in 60% of patients who have had sudden cardiac death (Burke et al., 2001).

1.8 Role of monocytes and macrophages in atherosclerosis

Monocytes and macrophages are present at all stages of lesion development and make up to about 60-70% of cells in the advanced plaque (Ross, 1993). Monocytes and macrophages have multifunctional roles in the progression of the atherosclerotic lesion (Takahashi et al., 2002). Monocytes are recruited to the inflammation site to infiltrate the artery wall where they differentiate into macrophages in response to stimulation by M-CSF (Watanabe et al., 1995). Initially this acts as a protective mechanism as it causes the removal of the damaging agents such as ox LDL. However, uncontrolled uptake of modified lipoproteins by macrophages ultimately leads to foam cell formation and atherogenesis (Glass and Witztum, 2001).

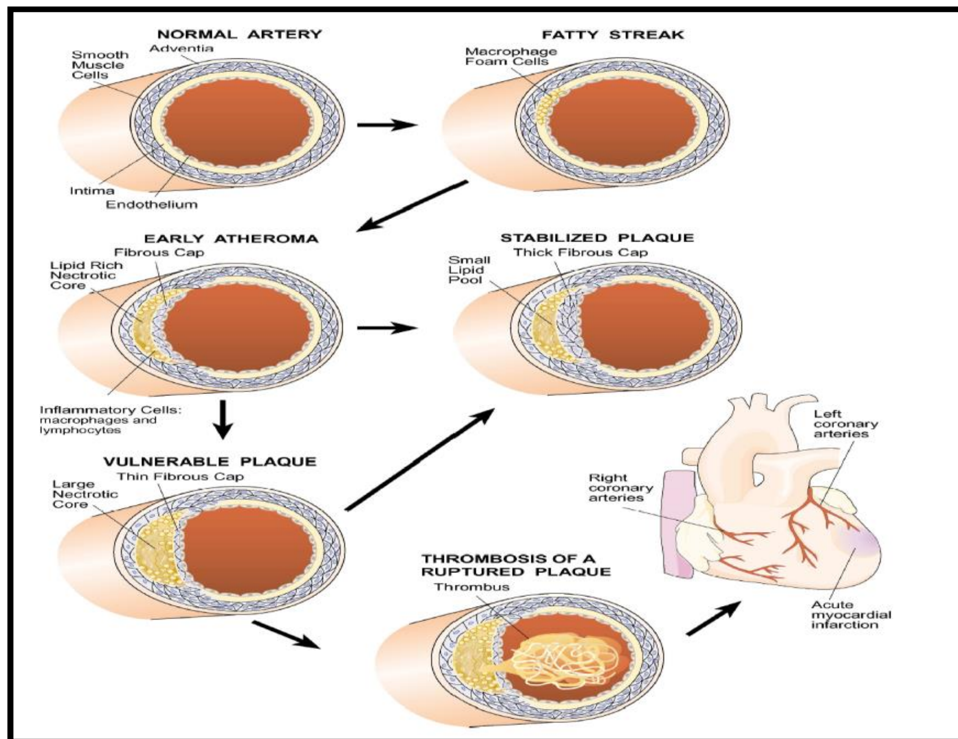

Figure 1.3: Characteristic stages of atherosclerotic lesions

The lipid-rich macrophages (foam cells) and T-lymphocytes accumulate inside the intima layer with modified LDL stimulating pro-inflammatory responses that provoke innate immunity in the intima that leads to fatty streak formation. This is the precursor of more advanced lesions characterised by the complex aggregation of lipid-rich necrotic debris and SMCs. Such early atheromas typically have a fibrous cap consisting of a necrotic core enclosed by SMCs and ECM. The condition develops into a more complex lesion, the fibrous plaque which could be either stable or vulnerable depending on the components. The rupture of the vulnerable plaque could initiate thrombosis, ultimately leading to acute myocardial infarction or stroke taken from (Lusis et al., 2004).

1.9 Macrophage foam cell formation:

Uptake of oxLDL by macrophages is mediated primarily through scavenger receptors expressed on the macrophage surface, particularly, SR-A and CD36 (Suzuki et al., 1997; Yamada et al., 1998). The LDL receptor (LDL-R) will also initially contribute to lipid loading by binding to native LDL and mmLDL. However, LDL-R expression is downregulated by the sterol regulatory element–binding proteins (SREBP) pathway as cholesterol accumulates in the cells (negative feedback), while scavenger receptors continue to take up increasing amounts of modified lipoproteins in an uncontrolled manner (Goldstein et al., 2002). The mmLDL has also been found to induce macropinocytosis, another mechanism for LDL uptake, which has the ability to potentiate the uptake of both native and oxLDL thereby contributing to foam cell formation (Kruth et al., 2002). For example, macropinocytosis has been found to be involved in the internalisation of both oxLDL and acetylated LDL (AcLDL) by human macrophages (Michael et al., 2013). Interestingly, the authors of this study found that inhibition of macropinocytosis using cytochalasin-D increased the expression of SR-A and CD36, which may indicate a potential compensatory mechanism to maintain modified LDL uptake and cholesterol levels in the absence of the macropinocytosis pathway. Additionally, they found that macropinocytosis is regulated by cytokines such as IFN-γ, TL1A, IL-17A and TGF-β (Michael et al., 2013), thereby demonstrating the importance of macropinocytosis in the control of foam cell formation.

As macrophage levels of cholesterol increase, efflux mechanisms may also maintain homeostasis for a short period, largely through a process known as reverse cholesterol transport (RCT). RCT is a process responsible for cholesterol transportation from peripheral tissues to the liver and then via bile for clearance through the faeces (Oram and Yokoyama, 1996). One of the primary components of this system is the ATP-binding cassette transporter A1 (ABCA1), which mediates transfer of cholesterol from the cell to high density lipoprotein (HDL), a role that makes HDL highly atheroprotective (Tall et al., 2000).

Cellular cholesterol is eliminated by HDL and its components via several mechanisms (Oram and Yokoyama, 1996). The RCT pathways (Figure 1.4) act as a natural defence mechanism for atherosclerosis by removing accumulated cholesterol from the arterial wall and excreting it via the liver. ApoA-I is secreted from the liver and the intestine and loaded with cholesterol and phospholipids by members of superfamily of ATP-binding cassette transporters (ABCs), ABCA1 and ABCG1. Liver ABCA1 initiates HDL particle formation and macrophage ABCA1 protects the arteries from atherosclerosis. Additionally, HDL phospholipids passively absorb cholesterol that has diffused from the plasma membrane into the aqueous phase, which is facilitated by the interaction of HDL particles with SRBI (Oram and Heinecke, 2005). Several studies using cultured cells or specific animal models have shown that ABCA 1 is an effective atheroprotective agent and a major determinant of plasma HDL levels (Aiello et al., 2003; Oram and Heinecke, 2005; Wang and Tall, 2003; Singaraja et al., 2003). In support for an anti-atherogenic role for ABCA1, ApoE null mice transplanted with ABCA1-deficient bone marrow cells show significantly more atherosclerosis than those given wild–type bone marrow (Aiello et al., 2002). In addition to ABCA1, ABCG1 is highly expressed in tissue macrophages and liver cells and is recognised to facilitate cholesterol transport from macrophages to HDL particles (Out et al., 2007).

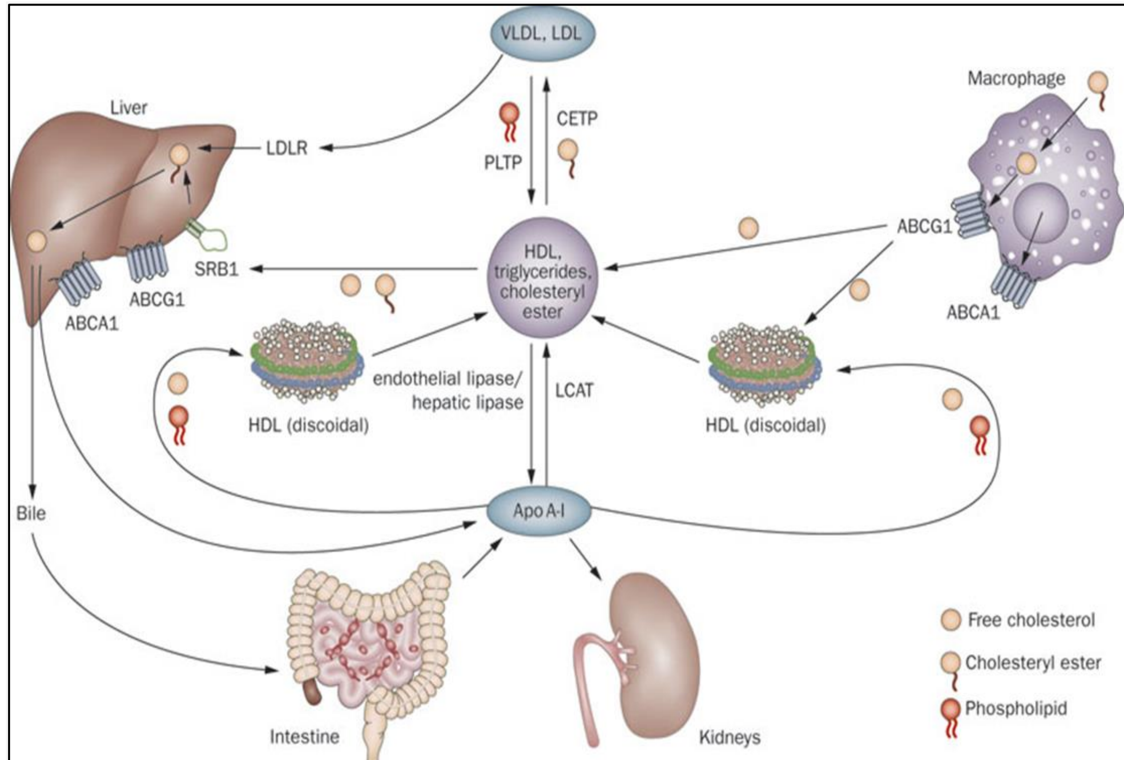

Figure 1.4: Reverse cholesterol transport

HDL has a major role in the reverse cholesterol transport pathway. Lipid-poor apo A-I is secreted by the liver and rapidly acquires cholesterol via the hepatocyte ABCA1 transporter and promotes cholesterol efflux from macrophages. Free cholesterol is esterified to cholesteryl esters by LCAT to form mature HDL, which transfers its cholesterol to apo B-containing lipoproteins, such as VLDL and LDL, via CETP-mediated transfer. This cholesterol is subsequently taken up by the liver via the LDL receptor. PLTP transfers phospholipids from triglyceride-rich lipoproteins to HDL, which promotes HDL remodeling. Hepatic cholesterol can be excreted into the bile after conversion to bile acid or expelled directly into the bile as cholesterol. Bile and its components are either reabsorbed by the intestine or ultimately excreted in feces. HDL can be remodeled by lipases, such as endothelial lipase and hepatic lipase, which hydrolyze HDL phospholipids and HDL triglycerides, respectively. The kidneys are an important site of apo A-I catabolism. Abbreviations: ABC, ATP-binding cassette; apo A-I, apolipoprotein A-I; CETP, cholesteryl ester transport protein; LCAT, lecithin–cholesterol acyltransferase; LDLR, LDL receptor; PLTP, phospholipid transfer protein; SRB1, scavenger receptor class B member 1. (Adapted from Navab et al., 2011).

Ultimately, when the concentration of modified LDL is high (above 5mmol/L), macrophages fail to maintain an adequate cholesterol balance. The majority of cholesterol in the foam cells exists in the form of cholesterol esters. In the absence of a suitable acceptor such as HDL, free intracellular cholesterol is esterified by the enzyme acyl coenzyme acylcholestrol transferase (ACAT) (Li and Glass, 2002). It was originally thought that knocking out the ACAT-1 gene would be beneficial to atherosclerosis but ApoE null mice with this deletion exhibited progression of atherosclerosis to a similar extent to those without it (Fazio et al., 2001) Although the lesions had reduced cholesterol content, deposits of this sterol were found elsewhere including the brain (Fazio et al., 2001). It is therefore possible that other atherogenic effects are being affected by the deletion of the ACAT-1 gene.

Phagocytosis of apoptotic cells by macrophages leads to further accumulation of lipids within atherosclerotic lesions (Glass and Witztum, 2001). As the disease progresses, the fatty streaks begins to mature into plaques. The lesions are usually covered with a fibrous cap made up of SMCs and ECM. The fibrous cap encloses a lipid-rich necrotic core of the lesion; the lipid core of the lesion is formed as the lesion grows. The cells in the middle of the plaque are unable to obtain sufficient nutrients and therefore undergo necrosis (Newby and Zaltsman, 1999).

Increased macrophage density has long been known to correlate with unstable atherosclerotic plaques (Lendon et al., 1991). Indeed, macrophages have now been shown to contribute to the instability of the plaque in several ways. Firstly, as macrophage foam cells undergo apoptosis themselves, their contents contribute to the necrotic core (Li and Glass, 2002). Macrophages have also been shown to release pro-thrombotic molecules, such as tissue factor (TF) and complement proteins (e.g. complement C3b) into the necrotic core to increase the thrombogenic potential of the plaque (Takahashi et al., 2002; Linton and Fazio, 2003). In addition, the proteolytic enzymes, including matrix metalloproteinases (e.g. MMP-2, MMP-9) produced by macrophages, act to weaken the fibrous cap and so lead to eventual plaque rupture (Newby, 2008).

Cytokines secreted by macrophages such as IL-2, IFN-γ and TNF-α play crucial roles in the development of atherosclerotic lesions (Ait-Oufella et al., 2011). These cytokines regulate the recruitment of inflammatory cells to the lesion through: chemotaxis; modulation of expression of cell adhesion molecules by ECs, SMCs and macrophages; and the regulation of cell proliferation and migration in the atheroma. Cytokines also modulate plaque stability by regulating the expression of effectors of ECM turnover, and thrombosis through the regulation of genes involved in coagulation cascades (Daugherty et al., 2005; Tedgui and Mallat, 2006; Mehra et al., 2005). IFN-γ is considered as one of the key cytokines that exerts its crucial role at all stages of lesion development.

1.10 Inflammatory mediators and nuclear receptors

Cells in the vessel wall secrete many inflammatory mediators that influence macrophage function. However, secretion of inflammatory mediators by damaged macrophages themselves plays a crucial role in plaque progression (Lusis et al., 2004). Cytokines (e.g. TGF-β, TNF-α, IL-1), chemokines (e.g. MCP-1, MCP1-β) and growth factors (e.g. platelet derived growth factor (PDGF) and Insulin–like growth factor-1 (IGF-1) produced by macrophages have numerous effects on gene expression in surrounding cells and contribute to the continued recruitment of inflammatory cells into the plaque and further uptake of lipoproteins (Burke-Gaffney et al., 2002). Macrophages also produce proteolytic enzymes, including MMP-2, MMP-9 and collagenase that act to weaken the fibrous cap and result in eventual plaque rupture (Galis et al., 1994). In addition, the nuclear receptors, peroxisome proliferators-activated receptors (PPARs) and liver X receptors (LXRs) are emerging as crucial regulators of inflammation that are involved in the pathogenesis of atherosclerosis.

1.10.1 Nuclear receptors

The nuclear receptor superfamily of proteins regulate the expression of genes involved in diverse processes such as development, reproduction and metabolism

(Robinson-Rechavi, 2003). The nuclear receptors have a highly conserved modular structure organised into functional domains. Most members consist of an N-terminal region that often contains a ligand-independent activation function (AF-1), a DNA-binding domain (DBD) containing two zinc fingers, a C-terminal ligand-binding domain (LBD) that interacts with small lipophilic molecules, and a ligand-dependent transcriptional activation function (AF-2) (Mangelsdorf and Evans, 1995).

1.1.1 Peroxisome proliferator-activated receptors (PPARs)

The PPARs bind to their recognition sequences in the regulatory regions of target genes as heterodimers with the 9-*cis*-retinoic acid receptors (retinoid X receptor; RXRs). PPARs play important roles in the regulation of metabolic pathways, including those of lipid biosynthesis and glucose metabolism, as well as the control of cell differentiation, proliferation and apoptosis pathways (Moraes et al., 2006). They are ligand-activated transcription factors involved in the transcriptional regulation of key genes in metabolic processes such as lipid metabolism, adipogenesis and insulin sensitivity. More recent work implicates all three PPAR isotypes, PPARγ, PPARα and PPARδ (also known as PPARß or PPARß/δ) in inflammatory and atherosclerotic pathways (Brown and Plutzky, 2007).

PPARα is expressed predominantly in the liver, kidney and heart, and is primarily involved in fatty acid oxidation (Schoonjans et al., 1996). Activation of PPAR α has also been demonstrated to reduce macrophage triglyceride accumulation and promote intracellular cholesterol distribution to plasma membrane which consequently enhances its efflux to HDL (Chinetti-Gbaguidi et al., 2005).

PPARδ is the most abundant isoform and ubiquitously expressed, and has a role in the development of atherosclerosis (Azhar, 2010). In a mouse model system, activation of PPARδ reduced the expression of MCP-1, ICAM-1 and inflammatory cytokines, and decreased the development of atherosclerosis (Graham et al., 2005). Similar to PPARα, activation of PPARδ in macrophages promotes cholesterol efflux and fatty acid catabolism alongside attenuation of inflammatory cytokine expression (Graham et al., 2005; Lee et al., 2006). Activators of PPAR α (fibrates) and γ

(thiazolidinediones) have been used clinically for a number of years in the treatment of hyperlipidaemia and to improve insulin sensitivity in diabetes (Kliewer et al., 2001). More recently, PPAR activation has been found to confer additional benefits on endothelial function, inflammation and thrombosis, suggesting that PPAR agonists may be good candidates for the treatment of cardiovascular disease (Robinson and Grieve, 2009). In this regard, it has been demonstrated that PPAR activators are capable of reducing blood pressure and attenuating the development of atherosclerosis and cardiac hypertrophy (Robinson and Grieve, 2009). PPAR α also has pleiotropic effects in the cardiovascular system, including anti-inflammatory and anti-atherosclerotic properties (Marx et al., 2000; Lee et al., 2006) . PPARα activation inhibits vascular smooth muscle pro-inflammatory responses and attenuates the development of atherosclerosis in mouse model systems (Zahradka et al., 2003). Additionally, PPARδ ligands have been shown to attenuate the pathogenesis of atherosclerosis by improving endothelial cell proliferation and survival whilst decreasing endothelial cell inflammation and vascular SMC proliferation (Hamblin et al., 2009).

Mice lacking both PPARα and ApoE have been found to develop reduced atherosclerosis despite having elevated triglyceride and LDL levels (Tordjman et al., 2001). Furthermore, total cholesterol, HDL cholesterol, and apoAI and apoAII levels were higher in PPARα null mice compared with wild-type controls. PPARα ligand fenofibrate has been shown to attenuate the development of atherosclerotic lesions, with more pronounced effect noted in ApoE−/−mice overexpressing human apoA-I (Brown and Plutzky, 2007). Additionally, administration of the PPARα ligand fenofibrate to $ApoE^{-/-}$ mice resulted in decreased total cholesterol and reduced atherosclerosis (Duez et al., 2002).

PPARγ appears to be highly expressed during atherosclerotic lesion formation thereby suggesting that increased PPARγ expression may be a vascular compensatory response (Marx et al., 1998; Ricote et al., 1998; Tontonoz et al., 1998).

Also, ligand-activated PPARγ decreases the inflammatory response in cardiovascular cells, particularly in ECs (Hamblin et al., 2009).

The anti-inflammatory effect of PPAR ligands is further confirmed by the findings that the expression of key pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-1 and -8, as well as the cytokines involved in immuno-modulation such as IL-2 and -4 is inhibited by various types of ligands for all three PPAR isoforms in macrophages and SMCs (Jiang et al., 1998; Takano et al., 2000; Ryoo et al., 2004; Ding et al., 2006; Piraino et al., 2006). Repression of IFN-γ expression by fenofibrate has also been confirmed in IL-10-deficient mice (Lee et al., 2007).

T lymphocytes and SMCs play an important role in atherosclerotic lesion formation and complication (Hansson, 2001). After the recruitment into the arterial wall, T cells undergo differentiation into CD4+Th1 cells following stimulation by various antigens including oxLDL. Th1 cell predominantly secrete pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-12, which can subsequently activate other cellular participants in atherosclerosis (Binder et al., 2002). PPAR-α and –γ activation reduces the T cell production of IFN-γ, TNF-α, IL-2 and IL-4, thus possibly modulating multiple levels of distal responses to these cytokines during atherogenesis (Jones et al., 2002; Marx et al., 2002).

In general, PPAR activators reduce the risk of atherosclerosis, decrease the progression of atherosclerotic plaques, and reduce the incidence of mortality from cardiovascular disease (Ehrenborg and Skogsberg, 2013).

1.10.2 1.10.2 Liver x receptors (LXRs)

The LXRs are nuclear receptors that are activated by endogenous oxysterols, oxidised derivatives of cholesterol. There are two isoforms of LXR, LXRα (NR1H3) and LXRβ (NR1H2). Both LXRα and LXRβ regulate gene expression by binding to regulatory DNA sequences in target genes as heterodimers with isoforms of RXR, RXRα (NR2B1), RXRβ (NR2B2) or RXRγ (NR2B3). LXRs act as cholesterol sensors as when cellular oxysterols accumulate as a result of increasing concentrations of cholesterol, they induce the transcription of genes that protect cells from cholesterol overload such as ABCA1 and ABCG1 (Zhao and Wright, 2010).

The two LXRs identified, LXRα and LXRβ, share a high degree of amino acid similarity (78% in their DNA- and ligand-binding domains) but differ in their tissue distribution. LXRα was first identified in the liver (hence the name liver X receptor) but is also expressed in other metabolically active tissues/cell types such as the kidney, intestine, adipose tissue, and macrophages. In contrast, LXRβ is ubiquitously expressed (Millatt et al., 2003).

LXRs regulate the expression of many genes associated with cholesterol absorption, transport, efflux and excretion, and as such control whole body cholesterol homeostasis. The activation of LXRs results in improved RCT and increased circulating HDL levels (Laffitte et al., 2003; Cao et al., 2002). It has also been shown that treatment with LXR agonists attenuates the development of atherosclerosis and inhibits cholesterol absorption in mouse models, suggesting therefore that synthetic LXR agonists may be promising anti-atherosclerotic agents (Hong and Tontonoz, 2014).

1.11 Diagnosis of atherosclerosis and therapeutic approaches

There are no precise diagnostic tests for atherosclerosis but cardiologists tend to determine plasma levels of serum biomarkers like C-reactive protein (CRP) (Corrado et al., 2010). Serum levels of CRP have been found to be up to 1000-fold higher than normal levels in individuals with advanced atherosclerosis (Paffen and DeMaat, 2006). There are also some other markers for chronic heart disease that includes TNF-α and IL-6 (Rodondi et al., 2010). In addition, physicians use other means to diagnose atherosclerosis including chest X-ray, electrocardiogram (ECG or EKG), computed tomography (CT scan), angiography, analysis of risk factors, such as plasma LDL cholesterol and triglycerides, and family history.

Optimal management of atherosclerosis reduces the risk of fatal and non-fatal cardiovascular events. In addition, monitoring atherosclerosis risk factors including blood pressure, smoking and hypercholesterolemia, is crucial in minimising the risk of cardiovascular disease (Lewis, 2009). A combination of lifestyle and therapeutic approaches are used to limit atherosclerosis. Dietary changes, such as reduced intake of saturated fats and increased intake of polyunsaturated fatty acids, increased levels of exercise and stopping smoking all help to reduce inflammation in arteries and limit the development of atherosclerosis (Leon and Sanchez, 2001). Both animal and human studies have shown that high intake of fish and unsaturated fatty acids, especially omega 3 fatty acids (n-3 FA), reduce chronic heart disease (De Caterina et al., 2000).

Targeting hyperlipidaemia represents another approach. HMG-CoA reductase is the rate limiting enzyme in the biosynthesis of cholesterol and it catalyses the conversion of HMG-CoA to mevalonic acid. Inhibition of this enzyme via statins reduces hepatic cholesterol synthesis and increases LDL receptor levels, which causes the clearance of LDL from the plasma (Wang et al., 2008). The action of statins is discussed later in more detail.

There is strong evidence to support that controlling hypertension protects against the development of atherosclerosis. Drugs for hypertension are prescribed alone or in combination with others depending on the severity and presence of other diseases (James et al., 2014). There is a strong agreement that the treatment of hypertension should be stratified according to age and ethnicity (James et al., 2014). There are several categories of medications for lowering blood pressure depending on their site of action, though in most cases it is not fully understood on how they work. The most common drugs include aspirin, beta-blockers, thiazide, calcium channel blockers (CCB) and angiotensin-converting enzyme (ACE) inhibitors. ACE inhibitors are drugs that block the production of angiotensin II. This is a hormone in the body that has a vital role in the cardiovascular system with its main function being to narrow blood vessels (vasoconstriction) resulting in high blood pressure. Blocking angiotensin II with ACE inhibitors reduces blood pressure and prevents such narrowing of arteries (Sweitzer, 2003).

1.11.1 Statins

Statins are potent inhibitors of cholesterol biosynthesis (Figure 1.5). Originally discovered in the 1970s, statins were found to potently inhibit HMG-COA, a key enzyme in cholesterol biosynthesis (Ridker et al., 2009). Statins can be divided into two categories: natural or fungally derived analogues (e.g. Lovastatin, Simvastatin and Pravastatin; type I statins) and synthetic analogues (e.g. Atorvastatin, Fluvastatin and Rosuvastatin; type II statins). The natural statins share similar chemical structures, while the synthetic statins vary considerably from each other and from the natural statins (Figure 1.6) (Igel et al., 2002; Davidson et al., 2004). Statins can also be classified according to their properties as either lipophilic or hydrophilic. Lipophilic statins include lovastatin, simvastatin, atorvastatin and fluvastatin whereas pravastatin and rosuvastatin are more hydrophilic (Garcia, 2003).

The chemical structure of statins is constituted by two components (Figure 1.6), the pharmacophore, which is a dihydroxyheptanoic acid segment, and its moiety composed of a ring system with different substituents. The pharmacophore works

through inhibition of the HMG-CoA reductase enzyme in a competitive, dose-dependent, and reversible manner. The stereoselectivity of the HMG-CoA reductase enzyme dictates the stereochemistry of statins, which present two chiral carbon atoms, C3 and C5, on their pharmacophore to the enzyme. The moiety of the pharmacophore, according to the chemical modified ring systems and the nature of the substituents, generates the different structures of statins. The ring system is a complex hydrophobic structure, covalently linked to the pharmacophore, that is involved in the binding interactions with the HMG-CoA reductase. The binding interactions of the ring have the ability to reduce the competition for the binding site between the statin and the endogenous HMG-CoA substrate. Subsequently, maintaining the statin closed to the enzyme prevents the possibility of statin displacement by the endogenous substrate. The ring's structure can be a partially reduced naphthalene (lovastatin, simvastatin, pravastatin), a pyrrole (atorvastatin), an indole (fluvastatin), a pyrimidine (rosuvastatin), a pyridine (cerivastatin), or a quinoline (pitavastatin). The substituents on the rings define the solubility of the statins along with many of their pharmacological properties (Gazzerro et al.,2012).

The evidence for a protective role of statins in cardiovascular disease emerges from many clinical trials. For example, in a large randomised double blind study using carotid intima-media thickness (CIMT) as a surrogate marker of atherosclerosis in 876 patients with hypercholesterolaemia, patients were either treated with Rosuvastatin (40 mg/day) or received placebo treatment for 2 years. The Rosuvastatin group showed no evidence of atherosclerosis progression as measured by CIMT compared with the placebo treated group, which had a significant CIMT progression (Crouse et al., 2007). In addition to inhibiting atherosclerosis progression, there were significantly greater reductions in LDL-C levels and increases in HDL-C levels in Rosuvastatin group than the placebo controls. Similarly, another multicentre study of 349 patients with high plasma cholesterol levels carried out serial intravascular ultrasound (IVUS) to examine the effect of intensive Rosuvastatin (40mg daily) therapy on the progression of coronary atheroma (Nissen et al., 2006). After two years, the results of

this study showed reductions in LDL-C levels, increases in HDL-C levels, and a significant regression of atherosclerosis measured by IVUS. However, this study lacked a control group to ascertain the maximum efficacy of their regimen. The same research group also conducted a double blind multicentre study in patients with CHD comparing Pravastatin (40 mg) to Atorvastatin (80 mg) (Nissen et al., 2006). Patients on 80 mg of Atorvastatin experienced significant reductions in LDL-C levels and atheroma volume compared with patients on 40 mg of Pravastatin. Progression of coronary atherosclerosis was inhibited in the Atorvastatin group, but not in those receiving Pravastatin.

The effect of statins is not just limited to LDL-C, but also extends to levels of HDL-C, which play a significant role in the prevention of atherogenesis and reduction of cardiovascular events (Nicholls et al., 2007; Puri et al., 2014). The combined analysis of four randomised controlled trials of nearly 1500 patients with coronary atherosclerosis that underwent serial IVUS showed that statin treatment induced a mean increase of 7.5% ($p < 0.01$ vs baseline) in HDL-C (Nicholls et al., 2007).

The beneficial effects of statin therapy on CHD and atherosclerosis progression are not just attributable to their effects on lipid profiles, but possibly as a consequence of their anti-inflammatory properties (Puri et al., 2014). Statin therapy either with Pravastatin (40 mg/day) or with Atorvastatin (80 mg/day) for 18 months was associated with a 22% reduction in CRP levels in a *post hoc* analysis of 502 patients with CHD (Nissen et al., 2006). The reduction in CRP levels was significantly associated with decrease in both total atheroma volume and percent atheroma volume ($p = 0.02$ and 0.01, respectively). Statins-mediated changes in CRP and the rate of atheroma progression were beyond their lipid-lowering effect. Despite many such advances, the anti-inflammatory effect of statin therapy is still controversial and further studies are required.

1.12 Actions of statins

1.12.1 Inhibition of cholesterol biosynthesis

Cholesterol is synthesised from acetyl Co-A via several steps as summarised in Figure 1.5. HMG-COA reductase is the rate-limiting enzyme in cholesterol biosynthesis. Statins are analogues of HMG-CoA so act as competitive inhibitors of the enzyme. In addition to direct inhibition of cholesterol synthesis, statins reduce plasma cholesterol levels by up-regulating the expression of LDL receptors, thereby aiding the clearance of this lipoprotein from the plasma (Wang et al., 2008).

Statins usually target hepatocytes and inhibit HMG-COA reuctase, the enzyme that converts HMG-CoA into mevalonic acid, a cholesterol precursor. The action of statins is not just limited to competing with the normal substrate for the enzymes active site. They alter the conformation of the enzyme when they bind to its active site. This prevents HMG-COA from attaining a functional structure. The change in conformation at the active site makes these drugs very effective and specific, binding of statins to HMG-COA is reversible and their affinity for the enzyme is in the nanomolar range as compared to the natural substrate, which has micromolar affinity (Corsini et al.,1999).

The pharmacokinetic properties of statins are orchestrated by several factors, including their active or lactone form, their lipophilic/hydrophilic properties, and their absorption and metabolism. Statins are administrated orally as active hydroxy acids, except for lovastatin and simvastatin, which are administrated as lactone pro-drugs and then hydrolyzed to hydroxy acid form (Corsini et al.,1999).

1.12.2 Inhibition of small G protein activation

When statins inhibit the synthesis of mevalonate, they also prevent the production of important isoprenoid intermediates such as farnesyl pyrophosphate (FFP). The action of many proteins in signalling cascades is dependent on post-translational modification by isoprenylation. These intermediates act as important lipid attachment molecules for the γ subunit of heterotrimeric G proteins and small G proteins, such as Ras, Rho, and Rac (Seabra, 1998; Maltese, 1990).

The attachment of these lipids also known as isoprenylation is essential for the activation and intracellular transport of these proteins. They act as molecular switches controlling multiple pathways and cell functions such as maintenance of cell shape, motility, factor secretion, differentiation, and proliferation (Zhou and Liao, 2010). Inactive GDP-bound Ras, Rho, and Rac are located in the cytoplasm. After isoprenylation, these small G proteins are translocated to the membrane and converted to active GTP-bound forms (Figure 1.7).

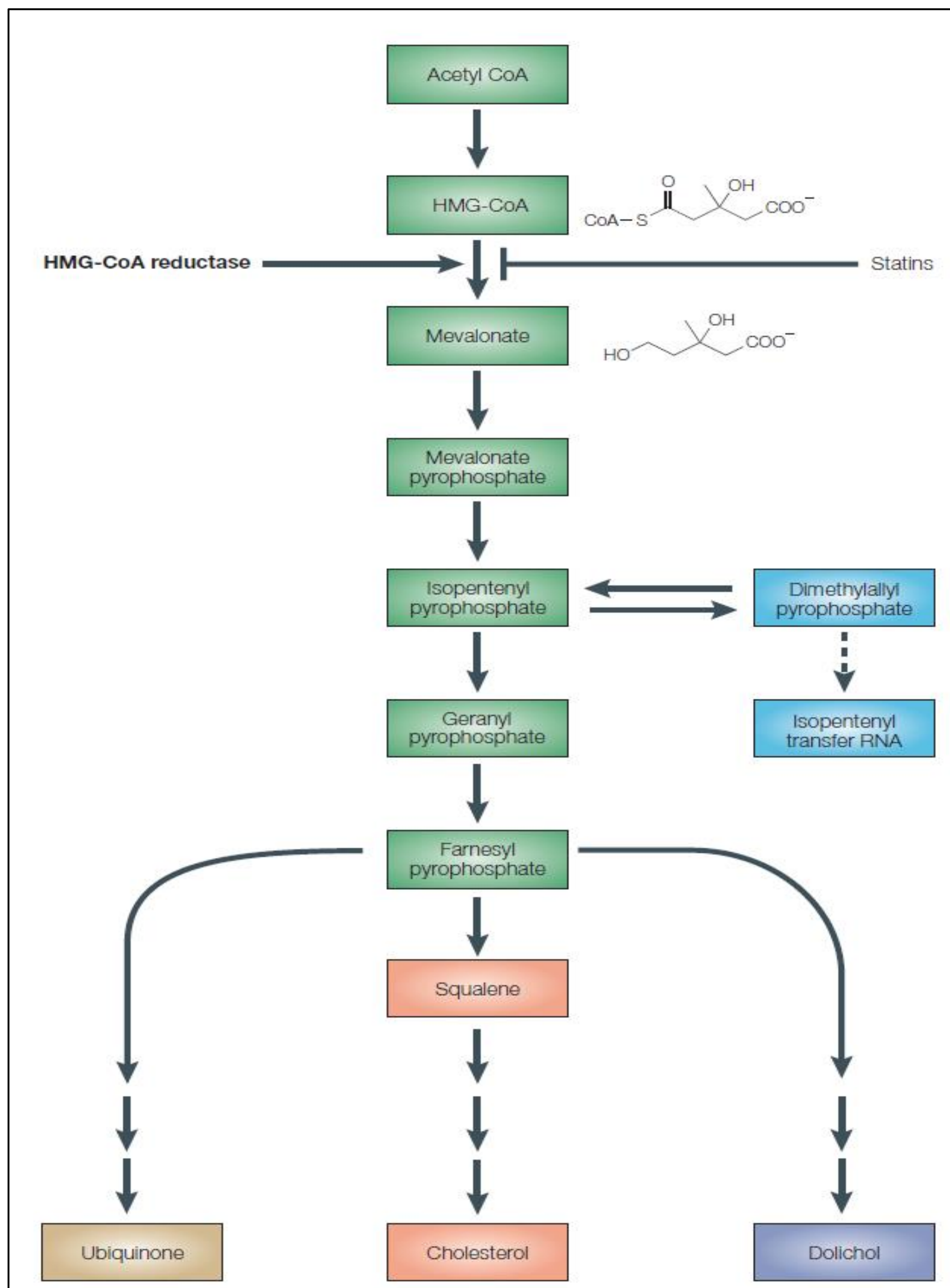

Figure 1.4: Cholesterol biosynthesis.

Cholesterol biosynthesis is a complex process involving more than 30 enzymes. A simplified version is shown here, which highlights the step inhibited by statins, and shows the chemical structures of the starting substrate (HMG-CoA) and product (mevalonate) of this step, taken from (Tobert, 2003).

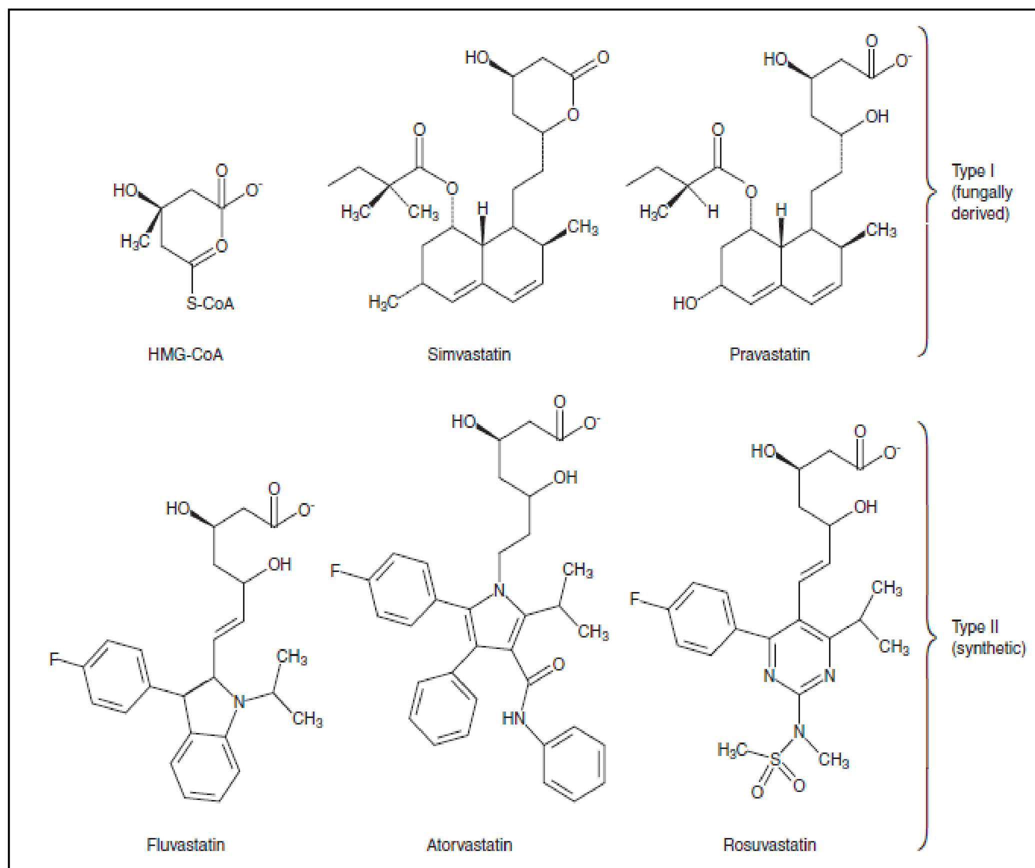

Figure 1.5: Molecular configurations of the natural (fungally derived) and synthetic statins. Taken from (Davidson and Toth, 2004).

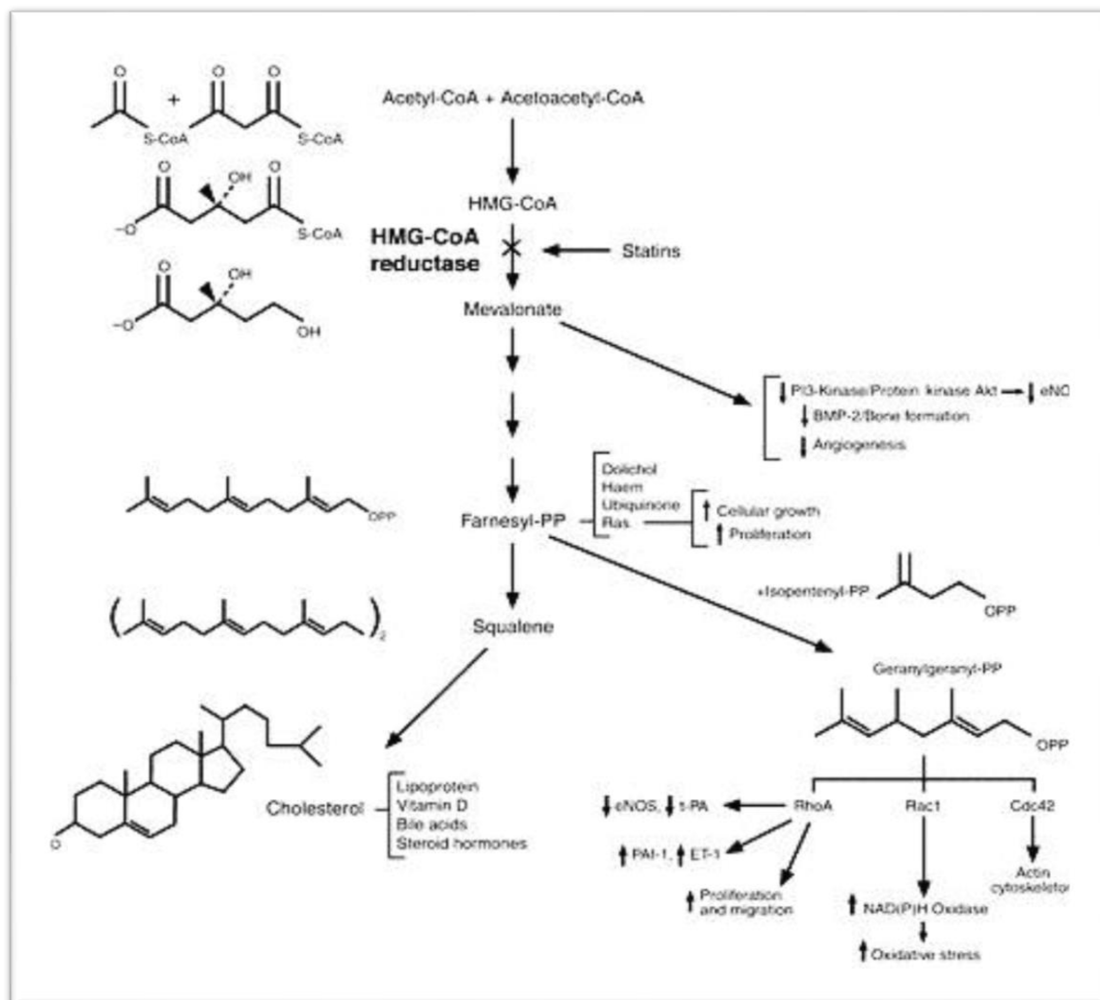

Figure 1.6: Biological actions of isoprenoids and cholesterol.

This diagram of the cholesterol biosynthesis pathway shows the effects of inhibition of HMG-CoA reductase by statins. Decrease in isoprenylation of signalling molecules such as Ras, Rho and Rac leads to modulation of various signalling pathways. BMP-2, bone morphogenetic protein-2; eNOS, endothelial nitric oxide synthase; t-PA, tissue- type plasminogen activator; ET-1, endothelin-1; PAI-1, plasminogen activator inhibitor-1. Taken from (Liao, 2002).

1.12.3 Other effects of Statins

Statins have anti-inflammatory activity, such as reducing levels of inflammatory biomarkers like CRP (Nissen et al., 2006; Puri et al., 2014). Statins have also been shown to decrease the expression of macrophage CD36, a recognised receptor for oxidized LDL (Fuhrman et al., 2002). Statins inhibit the uptake of oxidised LDL by CD36, scavenger receptor A and also attenuate macrophage oxidative properties (Plenge et al., 2002). Other anti-inflammatory activities include inhibition of endothelial adhesion molecule expression, impairment of monocyte recruitment and T-cell activation and repression of smooth muscle cell proliferation (Jain and Ridker, 2005). Statins can also increase lipoprotein fluidity, which may reduce the susceptibility of lipids to oxidation. For example, Rosuvastatin has been shown to increase the surface and core fluidity of ApoB-containing lipoproteins (Rosenson, 2004). Simvastatin also inhibits LDL oxidation by activated HMDM, and the metabolites of Atorvastatin have been shown to have potent antioxidant effects (Aviram et al., 1998). These physiological effects of statins on atherosclerosis pathogenesis contribute to reduced foam cell formation.

Immune mechanisms play a role in atherogenesis. Increasing evidence suggests that statins may modulate immune response and that use of statins may have applicability in organ transplantation and other conditions requiring immunosuppression. Pravastatin treatment, added to standard antirejection medications (cyclosporine, prednisone, and azathioprine) after cardiac transplant, has been reported to significantly reduce the frequency of rejection (3 versus 14; p=0.005) and increase survival (94% versus 78%; p=0.025) at 12 months compared with control patients (Kobashigawa et al.,1995). Furthermore, statins modulate platelet function. Although the underlying mechanism remains unknown, statins inhibit fibrinogen expression and thrombin formation *in vitro* and reduce platelet aggregation and deposition in diseased vessels in vivo (Dangas et al.,2000; Mayer et al.,1992). Reduced expression of cyclooxygenase 2 (COX-2), thromboxane A_2 (TxA_2), or TxB_2

and enhanced synthesis of prostacyclin caused by statin treatment may contribute to diminished platelet activation (Schönbeck and Libby, 2004).

Statins are among the most important cardiovascular therapies developed and have revolutionised the treatment of atherothrombotic disease. Although they are highly effective lipid-lowering agents, clinical and experimental observations strongly suggest that inhibition of inflammation also contributes to the beneficial effects of these medications.

1.12.4 Role of Atorvastatin in atherosclerosis

From the various statins, Atorvastatin is the only one that produces a metabolite that has the capability to inhibit HMG-CoA reductase equivalent to that of the parent molecule (Poli, 2007). Both Atorvastatin and its metabolite are principally eliminated through biliary excretion with only small amounts cleared in the urine (Neuvonen et al., 2006). The clearance half-life of Atorvastatin is approximately 14 hours, which is considerably longer than most other statins (with the exception of Rosuvastatin). The half-life of HMG-CoA reductase inhibition by Atorvastatin is around 20–30 hours as a result of the action of its active metabolites (Poli, 2007). This long half-life has a significant impact on medication administration schedule as Atorvastatin can be taken at any time during the day in contrast to other statins that are taken at night because of their shorter half-life.

In patients with primary hypercholesterolaemia, Atorvastatin has proven to be more successful in reducing total cholesterol, LDL-C and triglyceride levels when compared with same doses of Simvastatin, Lovastatin, Fluvastatin and Pravastatin (Jones et al., 1998). Nevertheless, statins also improve the endothelial function by increasing nitric oxide bioavailability (Anderson 1999). The endothelium is the single layer of endothelial cells lining the lumen of the blood vessels. The location of the endothelium provides mechanical and metabolical advantages as it separates the vascular wall from the circulation and the blood components (Lerman and Zeiher, 2005).

Endothelial dysfunction is a term that covers diminished production/availability of nitric oxide (NO) and/or an imbalance in the relative contribution of endothelium-derived

relaxing and contracting factors (Hadi et al., 2005). Endothelial dysfunction is characterized by a reduction of the bioavailability of NO, and/or an increase in endothelium-derived contracting factors (Lerman and Burnett 1992). This imbalance leads to an impairment of endothelium-dependent vasodilation, which is the functional characteristic of endothelial dysfunction. In addition to impaired endothelium-dependent vasodilation, endothelial dysfunction also comprises a specific state of endothelial activation, which is characterised by a proinflammatory, proliferative, and procoagulatory states that favour all stages of atherogenesis (Anderson 1999).

In the majority of comparative clinical trials, Atorvastatin in dosages of 10 and 20 mg/day has shown to be equivalent to or even better than that of dosages of 20 and 40 mg/day of these other statins (Malhotra and Goa, 2001). Atorvastatin has also been found to produce a significant improvement in endothelial function as early as two weeks after beginning the treatment compared with dietary therapy alone ($P<0.001$), as well as at four and eight weeks (Marchesi et al., 2000). This study also provided evidence that Atorvastatin may exert beneficial effects on endothelial dysfunction that are independent of the degree of lowering of plasma cholesterol levels. Atorvastatin also has an anti-atherogenic effect in a primary prevention setting, not just by decreasing lipid levels, but also by suppressing the oxidation of LDL (Sezer et al., 2011). In the Atorvastatin versus Simvastatin on Atherosclerosis Progression (ASAP) study, aggressive statin therapy (Atorvastatin 80 mg) reduced CRP levels to a greater extent than conventional therapy (Simvastatin 40 mg) thus indicating a potent anti-inflammatory activity of Atorvastatin (van et al., 2002).

1.13 Cytokines

Cytokines are small, nonstructural proteins with molecular weights ranging from 8000 to 40,000 daltons. They were originally called lymphokines and monokines to refer them back to their cellular origins, lymphocytes and monocytes respectively (Balkwill and Burke, 1989). Lately, it has become obvious that the term “cytokine” is the best description since the majority of the nucleated cells have the ability to

synthesise these proteins and, in turn, respond to them. There is no amino acid sequence motif or three dimensional structure that is common to all cytokines identified so far; rather, the biological activities of the cytokines allow the researchers to classify them and put them in different categories (Dinarello, 2000).

Cytokines consist of around 50 secreted factors that are involved in intercellular communication and regulation of fundamental biological processes. Cytokines have been grouped into several families over the years and some of the families include: the interleukins; interferons; tumour necrosis factors; transforming growth factors; colony stimulating factors; and chemokines. Cytokines are especially important for regulating the immune responses and have major regulatory functions in both innate and adaptive immunity (Tedgui and Mallat, 2006).

Atherosclerosis is considered as a state of chronic inflammation within the vascular wall. A variety of cytokines are released at different stages of the disease and these have been studied and reviewed in relation to them being pro- or anti-atherogenic (Tedgui and Mallat, 2006). The cytokines are capable of mediating their actions by interacting with specific receptors on the cell surface. The receptor interaction triggers downstream effects via signal transduction pathways. The transduction cascades ultimately leads to transcriptional control within the cell that can bring about changes in their functions and properties (Singh and Ramji, 2006).

High levels of pro-inflammatory cytokines are observed in atherosclerotic lesions (Harvey and Ramji, 2005). Pro-inflammatory cytokines include TNF-α, IFN-γ, IL-1, IL-17 and IL-12. Anti-inflammatory cytokines include IL-10, IL-4, IL-33 and TGF-β. Macrophages are the major source of cytokines in the atherosclerotic lesion alongside vascular cells and infiltrating T- lymphocytes (Figure 1.8) (Tedgui and Mallat, 2006).

Cytokines are also often classified as class I or class II cytokines according to the structural homology of their receptors. Most interleukins (ILs), CSFs, and interferons (IFNs) belong to one of the two classifications. Many such cytokines

mediate their effects through the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway. Three other major cytokine families include the IL-1 family (including IL-1α, IL-1β, IL-1ra, and IL-18), TNF family, and TGF-β superfamily. IL-1 and TNF family members activate the nuclear factor-κB (NF-κB) and mitogen-activated protein (MAP) kinase signalling pathways while TGF-β superfamily members activate signalling proteins of the Smad family (Tedgui and Mallat,2006).

Most ILs, CSFs and IFNs mediate their effects through the JAK-STAT pathway. Cytokine binding induces receptor dimerisation and recruitment of members of the JAK family, which in turn cross-phosphorylate each other and the cytoplasmic domains of the receptors on tyrosine residues (JAK activation). This provides docking sites for the latent transcription factors STATs. After docking, these transcription factors become phosphorylated; they dimerise before entering the nucleus to initiate transcription of target genes (Ihle, 2001).

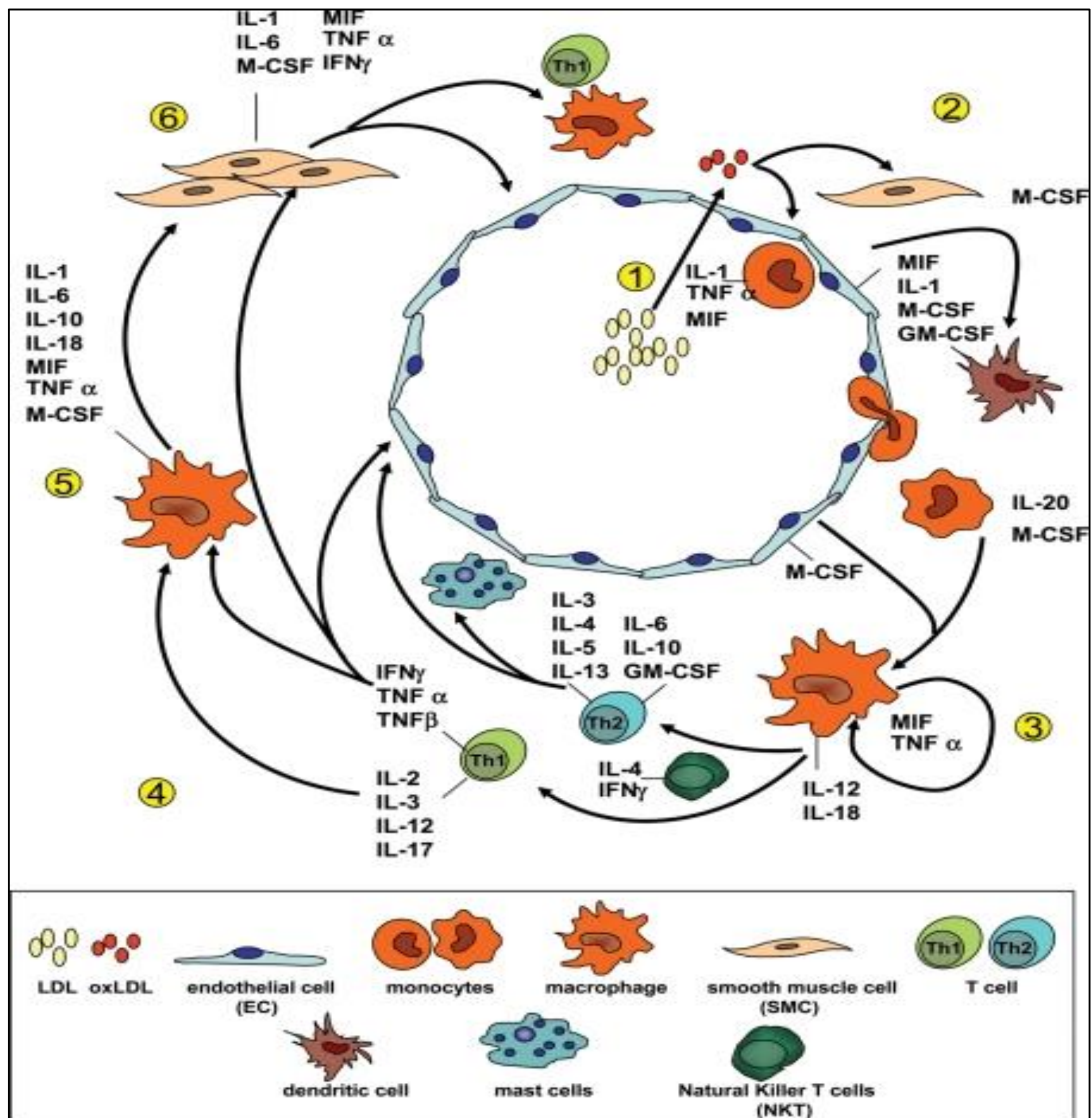

Figure 1.7: Cytokines involved in atherogenesis.

Cytokines involved in atherogenesis and their cellular source and targets. (1) LDL particles penetrate the endothelial cell layer and are oxidized in the intima (oxLDL). Pro-inflammatory lipids are released from oxLDL and induce expression of cytokines in EC (e.g. IL-1, MIF, M-CSF, and GM-CSF) and SMCs (M-CSF). MIF and M-CSF can function as a chemotactic factor for monocytes and T-cells. GM-CSF is a major regulator of dendritic cell differentiation and involved in lesional dendritic cell accumulation. (3) TNF-α and MIF are involved in the autocrine activation of macrophages thereby amplifying inflammation. IL-12 and IL-18 formed by macrophages and IL-4 (e.g. from NKT) promote the differentiation of native T-cells into T_H1-cells and T_H2-cells, respectively. IL-12 and IL-18 are potent inducers of IFN-γ. (4) T_H1-cells can further activate macrophages (inflammatory cascade) whereas T_H2-cells produce anti-inflammatory mediators (e.g. IL-4, IL-10, and IL-13) with opposite effect on macrophages, T-cells and EC. IL-4 (T_H2-associated) impair the development of T_H1-cells from pre-T_H-cells (not shown). Vice versa, IFN-γ inhibits T_H2-cell development. Also, T_H2-derived IL-4 and IL-13 are potent stimulators of antibody production (on B-cells, not shown), IL-5 is involved in B-cell differentiation and eosinophilic inflammation. (5) Macrophages are further stimulated by T-cell derived IFN-γ as well as IL-1 and TNF-α (also from other sources) together resulting in an amplification of the inflammatory response. (6) SMCs are targets for many of the macrophage- and T-cell-derived cytokines and participate in this self-perpetuating inflammatory cycle by producing and secreting a range of pro-inflammatory factors among which are IL-1, TNF-α, and IFN-γ.(Kleemann et al. 2008)

1.14 Proinflammatory cytokines

1.14.1 TNF-α

TNF-α is a pleiotropic cytokine that exerts strong pro-inflammatory effects in atherosclerosis and other metabolic and inflammatory disorders, such as obesity and insulin resistance, which are also risk factors for cardiovascular diseases (Staiger and Häring, 2005). TNF-α is primarily produced by monocytes and macrophages. The involvement of TNF-α in the pathogenesis of atherosclerosis is supported by its presence in human atherosclerotic plaques (Barath et al., 1990; Tipping and Hancock, 1993). Furthermore, circulating TNF-α levels are associated with increased risk of recurrent myocardial infarction, atherosclerotic thickening of carotid intima-media, disturbances in triglyceride and glucose homeostasis, and age-related atherosclerosis (Schreyer et al., 1996). Blocking of TNF-α activity or disruption of its expression diminishes the development of atherosclerosis in $ApoE^{-/-}$ mice (Brånén et al., 2004; Ohta et al., 2005).

1.14.2 TL1A

TL1A (TNFSF15/VEGI) is a recently identified member of the tumour necrosis factor superfamily (TNFSF) that is primarily expressed by ECs and other immune cells (Cavallini et al., 2013). It is a ligand for the death domain-containing receptor DR3 (TNFRSF12). DR3 was initially found to have a role in atherogenesis as it stimulated the expression of matrix degrading enzymes including MMP-9 (Kang et al., 2005). Immunohistochemical staining of human carotid atherosclerotic plaques revealed a high-level expression level of TL1A in regions rich in macrophage/foam cells (Kang et al., 2005). DR3 has been demonstrated to change the function of immune cells and to drive several inflammatory-driven diseases such as inflammatory bowel disease and arthritis (Meylan et al., 2008; Croft, 2009). Both TL1A and DR3 have been found to be involved in the development of atherosclerosis (McLaren et al., 2010a). It was found that TL1A enhanced macrophage foam cell formation in vitro by increasing

AcLDL/oxLDL uptake and intracellular cholesteryl ester content whilst also reducing cholesterol efflux from human macrophages (McLaren et al., 2010a).

1.14.3 IL-17

The IL-17 family encompasses six members (IL-17A to F) (van et al., 2009). All members of the IL-17 family share a similar protein structure characterised by four highly conserved cysteine residues, but they have no sequence similarity to any other known cytokines (Huang et al., 2004; Gaffen et al., 2006). IL-17E, more commonly called IL-25, has different biologic functions, but shares structural features with other IL-17 family members (Lee et al., 2001; Fort et al., 2001). IL-17A and IL-17F (which are 55% homologous) are both produced by Th17 cells and are the most characterised family members to date. The IL-17A and IL-17F genes are located next to each other on chromosome six, suggesting the genes encoding IL-17A and IL-17F are the result of a duplication event (Langrish et al., 2005; Harrington et al., 2005).

The receptor for IL-17A (IL-17RA) is a type I transmembrane protein and consists of a 293 amino acids long extracellular domain and a relatively long intracellular domain consisting of 525 amino acids (van et al., 2009). The IL-17A receptor is ubiquitously expressed with particularly high levels in haematopoietic tissues. This expression pattern is curious, as the main responses to IL-17A occur in epithelial, endothelial and fibroblast cells (Gaffen, 2009). Another function of IL-17RA might be to limit signalling by receptor-mediated internalisation of the ligand (Gaffen, 2009). It has been noticed that surface expression of IL-17RA is rapidly reduced following IL-17 binding. This helps internalising IL-17A and clearing it from the inflammatory environment (Lindemann et al., 2008). IL-17A activates a highly pro-inflammatory programme of gene expression, typical of that induced by innate immune receptors such as IL-1R and Toll- like receptors (TLRs) (Park et al., 2005). Similar to these receptors, IL-17A activates NF-κB, a hallmark transcription factor associated with inflammation. IL-17A has pleiotropic activities, including induction of the production of TNF-α, IL-1β and MCP-1 as well as adhesion molecules like ICAM-1 (Erbel et al., 2009). The best-characterised member is IL-17A or IL-17 as it is the founding member of the family

(van et al., 2009). IL-17 is generally classified as a pro-inflammatory cytokine (Erbel et al., 2009). It is involved in the early activation of the immune system and plays a vital role in bridging the innate immune response with the adaptive immune response (van Es et al., 2009).

The role of IL-17 in atherosclerosis development remains contentious. IL-17A is elevated in patients with CHD, which have raised expression in cells of atherosclerotic plaque (Hashmi and Zeng, 2006; Simon et al., 2013). IL-17A induces the expression of many pro-atherosclerotic mediators, including TNF-α, IL-1β, and MMP-1 by a range of cells involved in atherosclerosis (Shibata et al., 2014; Erbel et al., 2009; Eid et al., 2009). The evidence in *in vivo* mouse studies has been inconsistent. A number of studies have shown that mice with disrupted IL-17A signalling have reduced atherosclerosis (van Es et al., 2009; Erbel et al., 2009). In contrast, a study by Taleb and colleagues (2009) showed that neutralisation of IL-17A in chimeric LDLr$^{-/-}$SOCS3$^{-/-}$ mice accelerated atherosclerosis and did not reduce atherosclerosis in LDLr$^{-/-}$SOCS3$^{+/+}$ mice. This inconsistency is possibly due to differences in mouse genetic background but also suggests that the role of IL-17A is multifaceted and requires further validation *in vivo.*

1.14.4 Interferon-γ

The human interferon (IFN) family is categorised into two major types. Type I consists of IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω and IFN-v and type II consists of only IFN-γ (Pestka, 2007). Type I IFNs share a common structure and are synthesised by most cell types (McLaren and Ramji, 2009). On the other hand, type II IFN has a single member with a different molecular structure from the type I IFNs and functions through a separate receptor (IFN-γR). IFN-γ is secreted mainly by natural killer (NK) cells and activated T-lymphocytes but can also be produced by monocytes/macrophages, B cells and dendritic cells (Mire-Sluis and Thorpe 1998; Schroder et al., 2004). The biological properties of IFN-γ are discussed in detail in the following sections.

1.14.5 Structure of IFN-γ

Human IFN-γ has a molecular weight of approximately 17 kDa and consists of two self-associated anti-parallel α-subunits with no β-sheet (Pestka, 2007). As addressed below in more detail, IFN-γ is considered to play an important role in the pathogenesis of atherosclerosis. However, in vitro cell culture studies have suggested that the role of IFN-γ is more complicated since it acts in both a pro- and anti-atherogenic manner (McLaren and Ramji, 2009). For example, IFN-γ has some anti-foam cell actions such as suppression of macrophage lipoprotein lipase (LPL) expression (McLaren and Ramji, 2009). Also, it has been shown in some studies to reduce the expression of SR-A and CD36 specifically in macrophages, and due to this effect, the cytokine had been attributed to a reduction of macrophage foam cell formation in one study (Geng and Hansson, 1992). However, the majority of subsequent studies have shown that IFN-γ promotes macrophage foam cell formation despite decrease in the expression of LPL and CD36 genes (McLaren and Ramji, 2009).

1.14.6 The roles of IFN-γ in atherosclerosis

IFN-γ is multifunctional and has antiviral activity along with a variety of immuno-modulatory and inflammatory roles. IFN-γ plays a significant role in inhibition of viral infections through intervening and prevention of several stages of viral protein synthesis and replication (Wei et al., 2009). A number of studies have shown that mice deficient in IFN-γ or IFN-γR had a greater susceptibility to bacterial and viral infection (Dalton et al., 1993; van den Broek et al., 1995). IFN-γ demonstrates its activity on immune system and inflammation predominantly through several mechanisms including stimulation of antigen presentation by inducing the expression of Class I and II major histocompatibility complex (MHC) molecules on the surface of macrophages and T-lymphocytes, antigen processing, and promoting differentiation of naive T helper (Th0) cells towards a Th1 phenotype. Additionally, IFN-γ activates antigen-presenting cells (APCs) (e.g. macrophages) and T-lymphocytes by inducing

the production of reactive oxygen intermediates and hydrogen peroxides. These augment the apoptosis of cells containing intracellular parasites, stimulate cytokine production in target cells and cause recruitment of white blood cells to the inflammation site through increased expression of chemokines and adhesion molecules in both leukocytes and endothelial cells (Boehm et al., 1997; Stark et al., 1998; Schroder et al., 2004). Furthermore, IFN-γ regulates the cellular state, mainly by influencing the rate of proliferation, differentiation and apoptosis. Inhibition of cell growth is modulated by IFN-γ through the expression of certain genes linked to the cell cycle (e.g. Fas), and can either induce or suppress apoptosis depending on the cell type or state of differentiation (Dai et al., 1998; Boehm et al., 1997; Stark et al., 1998; Schroder et al., 2004).

The important role of IFN-γ in immunity and inflammation imply that the cytokine is likely to have a key role in disorders associated with a chronic inflammatory reaction. Indeed, a number of studies have suggested an association between the differential expression of IFN-γ with the pathology of a number of inflammatory diseases including rheumatoid arthritis, glomerulosclerosis and pancreatitis (Cañete et al., 2000; Kitching et al., 1999; Uehara et al., 2003). In particular, IFN-γ demonstrates major roles in atherosclerosis and its pathogenesis through the regulation of several processes in the disease (McLaren and Ramji, 2009; Harvey and Ramji, 2005). IFN-γ stimulates a variety of immune cellular responses, including the regulation of antigen presentation, control of Th1/Th2 adaptive immune response, immune cell activation and cytokine secretion (Mallat and Tedgui, 2004; Gattoni et al., 2006; Schoenborn et al., 2007). Consequently, these responses act alongside IFN-γ-mediated regulation of other cellular activities, such as proliferation, differentiation and apoptosis, which participate in the complex nature of atherosclerosis (Stark et al., 1998). Although this cytokine can display anti-inflammatory properties IFN-γ predominantly exerts pro-inflammatory and pro-atherogenic roles throughout disease development, ranging from early lesion formation (foam cell), to the more mature and advanced lesion progression

(atheroma), to clinical complication of atherosclerotic plaques (plaque stability), as detailed below (Mühl and Pfeilschifter, 2003).

1.14.7 Recruitment of immune cells to the lesion

The recruitment of monocytes / macrophages and T-lymphocytes to the site of inflammation is a critical early step in atherogenesis (Lusis, 2000). IFN-γ has been shown to up-regulate the expression of several chemokines and adhesion molecules, including MCP-1, MIP-1α and –β and ICAM-1 (Boisvert, 2004). IFN-γ has also been shown to have an effect on ECs in atherosclerosis; for example, the expression of the monocyte adhesion molecule VCAM-1 is induced by IFN-γ (Gupta et al., 1997). This cytokine also stimulates the expression of ICAM-1 and VCAM-1 on endothelial cells and SMCs (Chung et al., 2002; Li et al., 1993). IFN-γ also stimulates the activation of macrophages (Nathan et al., 1983). The activation of CD4+ T-cells (Th1) is also facilitated by IFN-γ by increased expression of MHC class II molecules on the surface of ECs, macrophages and SMCs (Jonasson et al. 1985; Mach et al., 1996).

1.14.8 Cholesterol accumulation in foam cells

Scavenger receptors such as SR-A and CD36 are responsible for the uptake of modified LDL (Kunjathoor et al., 2002). As mentioned earlier, a study showed that IFN-γ inhibited the expression of SR-A and CD-36 especially in macrophages (Geng and Hansson,1992). Also, IFN-γ supressed macrophage LPL expression (McLaren and Ramji, 2009). However, the majority of studies show that IFN-γ increases the expression of SR-A and CXCL16 and the uptake of modified LDL (Reiss et al., 2004; Wuttge et al., 2004). CXCL16 is a marker of inflammation and atherosclerosis, which has a similar function to SR and also has inflammatory properties of chemokine (Lehrke et al., 2007). CXCL16 is a proatherogenic marker that is highly expressed in macrophages and aortic SMCs (Lehrke et al., 2007). Increased CXCL16 expression augments the uptake of oxLDL and facilitates foam cell formation (Wuttge et al., 2004). CXCL16 has also been implicated in the pathogenesis of metabolic dyslipidaemia and coronary atherosclerosis (Lehrke et al., 2007; Wuttge et al., 2004).

In human macrophages *in vitro* and *in vivo*, activation of NF-κB was found to be sufficient to stimulate the induction of CXCL16 expression, which can be inhibited by administering aspirin (Lehrke et al., 2007). CXCL16 accumulates in the atherosclerotic lesion where it stimulates the production of T cells and IFN-γ (Abel et al., 2004).

IFN-γ has also been demonstrated to reduce the expression of ApoE in both monocytes and macrophages, and to increase its intracellular degradation (Brand et al., 1993). In addition, IFN-γ decreases cholesterol efflux and ABCA1 expression in murine and human macrophages and macrophage-derived foam cells (Reiss et al., 2004; Panousis and Zuckerman, 2000). Furthermore, IFN-γ has been found to inhibit the expression of the 27-hydroxylase enzyme, which is important in the removal of cholesterol from foam cells (Reiss et al., 2001).

1.14.9 Plaque destabilisation

As atherosclerosis progresses, advanced plaques will be formed and these include lipid rich necrotic core, calcification and accumulation of cell debris (Lusis et al., 2004). IFN-γ encourages apoptosis of macrophage foam cells by increasing the expression of some pro-apoptotic molecules such as tumour necrosis factor-alpha receptor 1 (TNFR1) and caspase-8 (Inagaki et al., 2002). IFN-γ is also known to promote plaque destabilisation by weakening the fibrous cap by stimulating the production of MMPs by macrophages and SMCs (Schönbeck et al., 1997). MMPs have been found to be present in the lesion, predominantly in the shoulder regions where plaques are highly likely to rupture (Galis et al., 1994). These enzymes degrade the ECM and therefore cause plaque destabilisation. Tissue factor activity is also enhanced by IFN-γ treatment in synergy with C-reactive protein, so increasing the rate of thrombosis following rupture (Nakagomi et al.,2000)

1.14.10 IFN-γ signalling

IFN-γ mediates its signalling through binding to the receptors on the cell surface. The JAK-STAT pathway is the best mechanism understood in IFN-γ

signalling cascade (Wood, 2004). Binding of IFN-γ to its receptor complex, which is made of IFNγR1:IFNγR2, leads to the recruitment of two JAKs, JAK-1 and -2, each of which binds to IFNγR through N-terminal domains (Figure 1.9). Both JAKs become activated by tyrosine phosphorylation (van Boxel-Dezaire and Stark, 2007) and, in turn, they phosphorylate the IFN-γR tails. This results in the recruitment of STAT1 monomers, which dock to the phosphorylation site via their Src-homology (SH2) domain (Levy and Darnell, 2002; Greenlund et al., 1995). The JAKs phosphorylate each STAT1 monomer on tyrosine 701. The STAT1 monomers then form dimers and then migrate to the nucleus where they bind to γ-activated sequence (GAS) elements in the promoters of genes regulated by this cytokine (van Boxel-Dezaire and Stark, 2007; Darnell et al., 1994; Levy and Darnell, 2002). The STAT homodimers can also be phosphorylated at serine 727 and this increases their transcriptional acivity (Levy and Darnell, 2002). Many kinases have been found to affect STAT1 phosphorylation on serine 727, including phosphoinositide 3'-kinase and extracellular signal-regulated kinase (ERK) (Harvey et al., 2007; Li et al., 2010). STAT1 plays a major a role in the development of atherosclerosis (Li et al., 2010). Results from *in vitro* and *in vivo* studies have shown that STAT1 plays a role in foam cell formation and progression of atherosclerosis (Agrawal et al., 2007). STAT1 deficiency in a mouse model system showed a reduction in intraperitoneal inflammation and reduced atherosclerosis (Agrawal et al., 2007). The effect of STAT1 is likely to be mediated through CD36 as the activity of this scavenger receptor was absent in macrophages deficient in CD36 (Agrawal et al., 2007).

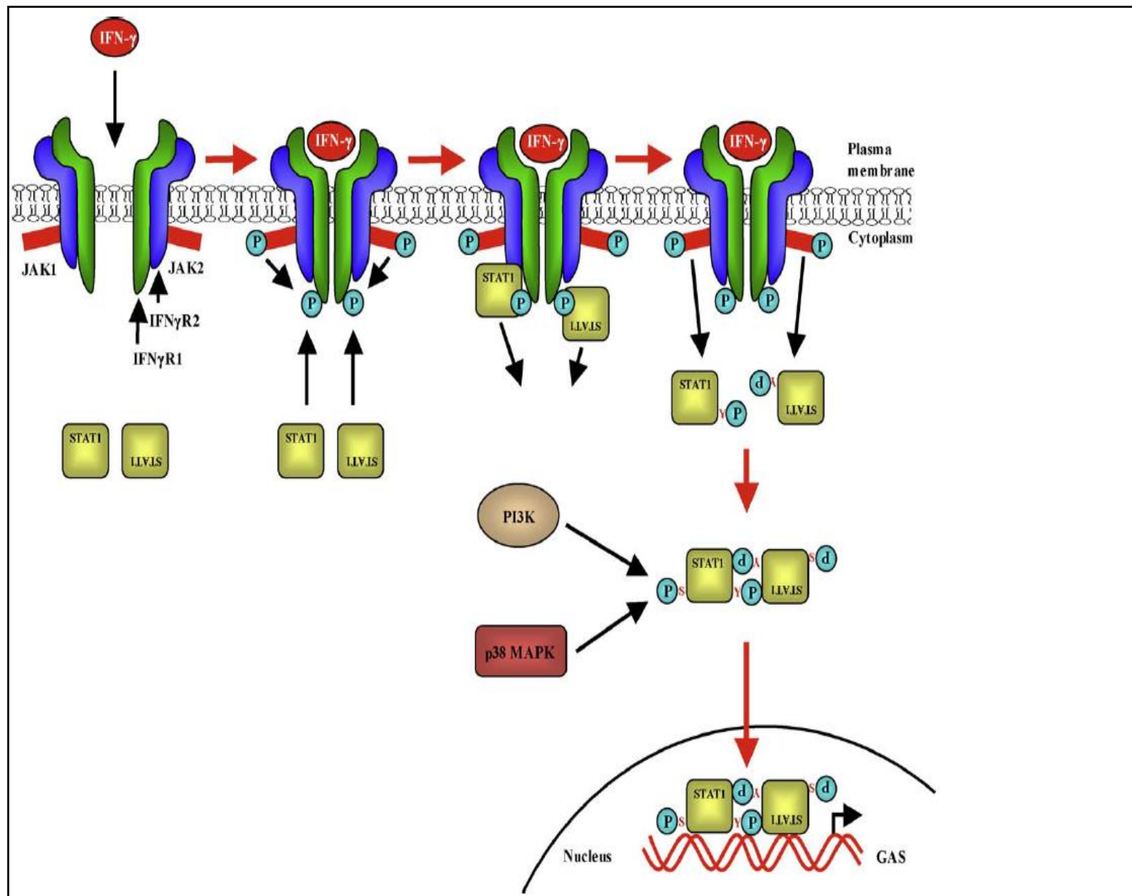

Figure 1.8: JAK/STAT1 signalling in response to IFN-γ.

The binding of IFN-γ to its cognate, cell surface receptor involves dimerisation of the two IFN-γ receptor (IFN-γR) subunits, made up of IFN-γR1: IFN-γR2 pairs, and results in the activation of two bound tyrosine kinases, JAK1 and JAK2, by tyrosine phosphorylation. Once the JAKs become activated, or tyrosine phosphorylated, they mediate tyrosine phosphorylation of the IFN-γ receptor tails through their catalytic, C-terminal kinase domain. Latent STAT1 monomers, located in the cytoplasm are recruited to the receptor tails via their SH2 domains and are then phosphorylated on tyrosine 701. The tyrosine phosphorylated STAT1 monomers dimerise forming a STAT1:STAT1 homodimer which can translocate to the nucleus and bind to specific γ-activated sequence (GAS) elements in the promoters of IFN target genes. Tyrosine phosphorylated STAT1 homodimers can also be phosphorylated on serine 727 by kinases like PI3K and p38 MAPK that enhances their transcriptional capacity. Abbreviations: IFN-γ, interferon-γ; JAK, Janus kinase; p38 MAPK, p38 mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; SH2, Src-homology 2; STAT1, Signal transducer and activator of transcription 1. Taken from (McLaren and Ramji, 2009)

1.14.11 Mitogen-activated protein kinase (MAPK) pathways and IFN-γ signalling

Mitogen-activated protein kinases are group of serine/threonine kinases that are responsible for translating extracellular signals to variety of cellular responses. MAPKs are extensively used in physiological and pathophysiological processes, including gene expression, mitosis and metabolism along with motility, survival, apoptosis and differentiation of cells (Cargnello and Roux, 2011). Each MAPK pathway is divided into three main linear components, a MAPK kinase kinase (MAPKKK, MEKK), a MAPK kinase (MAPKK, MKK or MEK) and a MAPK (Dhillon et al., 2007; Kim and Choi, 2010) (Figure 1.10). To date, three major, and well-studied, MAPK pathways have been identified in mammalian cells: ERK1/2; c-Jun N-terminal kinases (JNK)/stress-activated protein kinases (SAPK); and p38 kinases (Robinson and Cobb, 1997; Chang and Karin, 2001) (Figure 1.11).

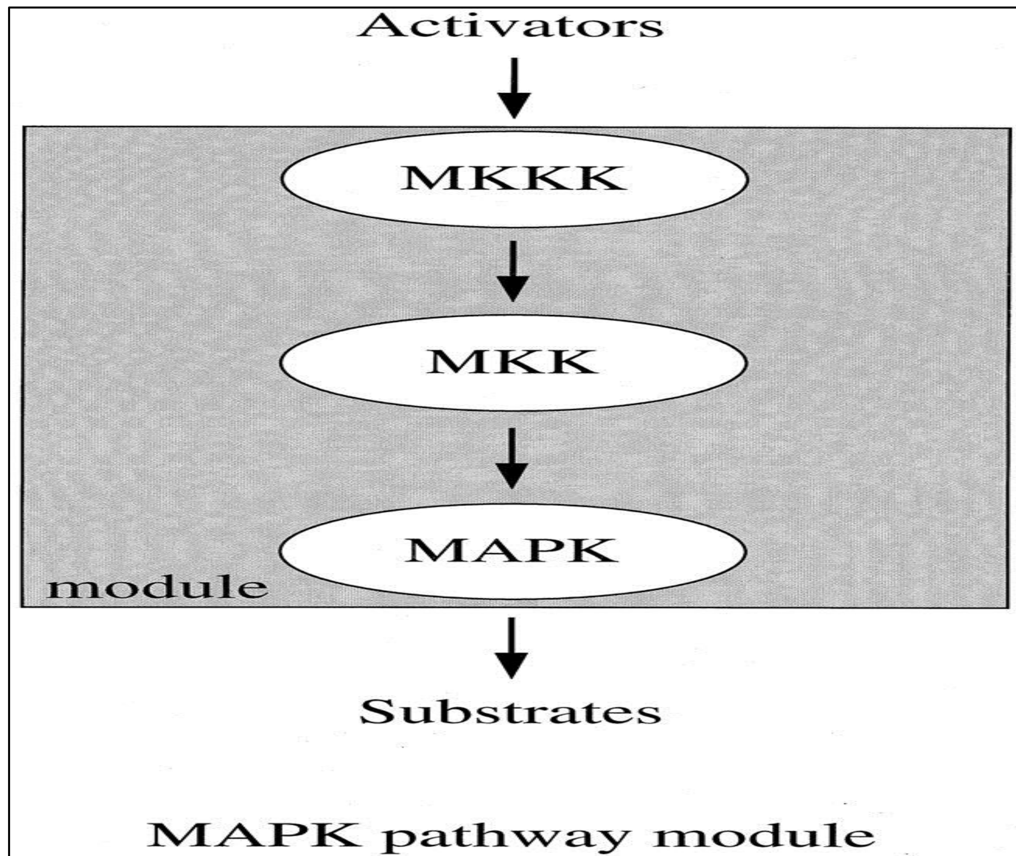

Figure 1.9: A mitogen-activated protein kinase (MAPK) pathway.

This figure shows a mitogen-activated protein kinase (MAPK) pathway. The MAPK is composed of three kinases [MAPK kinase kinase (MKKK), MAPK kinase (MKK), and MAPK] and they are all activated by phosphorylation, adapted from (Widmann et al., 1999).

A further explanation of the MAPK pathway is included in the next figure.

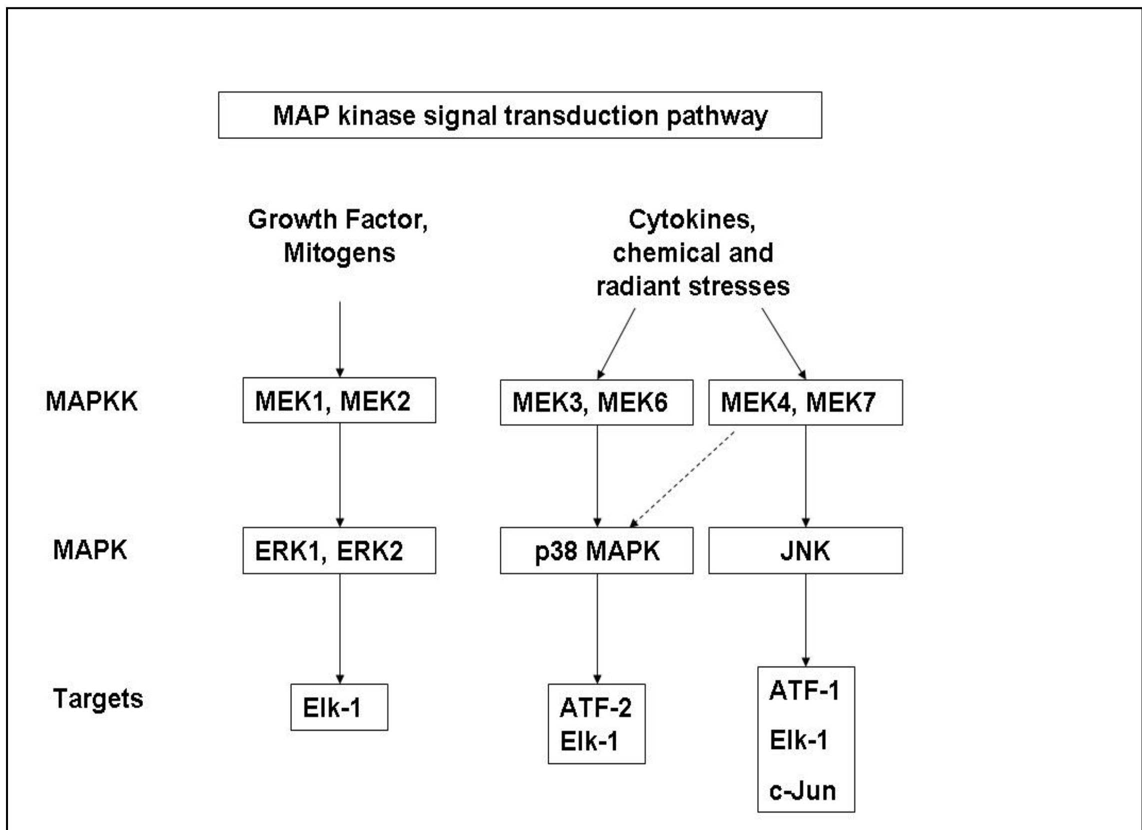

Figure 1.10: The best known MAP kinase pathways.

The net result of these pathways is the activation of the three main MAP kinases: ERK, JNK, and p38 MAP kinase, all of which have different major downstream targets. ATF-2, activating transcription factor 2; ERK, extracellular signal regulated kinase; JNK, c-Jun NH_2 terminal kinase; MAPK, MAP kinase; MAPKK, MAP kinase kinase; MAPKKK, MAP kinase kinase kinase; MEF; MEK, MAP/ERK kinase; MKK, MAP kinase kinase.;ATF-1 cyclic AMP-dependent transcription factor-1. Adapted from (Hommes et al., 2003).

The following sections will explain in detail the properties and functions of the well-known MAPKs (ERK1/2, JNK1/2, and p38).

1.14.12 ERK1/2

The genes for ERK1/2 were cloned in 1990 and were found to be expressed at high levels in the brain, skeletal muscle, thymus, and heart (Boulton et al., 1990). They regulate many cellular processes including growth, proliferation, differentiation, survival and apoptosis (Cargnello and Roux, 2011). Growth factors, such as PDGF, epidermal growth factor (EGF) and nerve growth factor (NGF) can all activate ERK1/2 (Boulton et al., 1990) along with cytokines and osmotic stress (Raman et al., 2007). Results from various gene knockout mice have demonstrated the physiological and pathological importance of the ERK1/2 pathway. ERK2 and MEK1-knockout mice have embryonic lethality as a consequence of defective mesoderm differentiation and placenta vascularisation respectively (Giroux et al., 1999; Hatano et al., 2003). These results show necessary roles of ERK2 and MEK1 in embryonic development. ERK1 knockout mice are viable but show defective thymocyte maturation and reduced expression of α and β chains of the T cell receptor, and exhibit reduced activation by anti-CD3 antibody, illustrating that ERK1 is involved in T cell activation and inflammatory processes (Pagès, 1999). Studies from genetically modified animals have also revealed key roles of ERK activation in cardiac pathology, such as myocardial hypertrophy and ischemia/reperfusion injury, as well as in the mechanisms of cardio-protection such as ischemic precondition (Heinen et al., 2011). In addition, *in vivo* studies show that the amounts of ERK1/2 are elevated in atherosclerotic lesions compared with normal vessel tissue in cholesterol fed rabbits as demonstrated by immune-blotting (Hu et al., 2000). Previous studies from our laboratory have found that ERK-1/2 is integral to the IFN-γ-mediated phosphorylation of STAT1 on serine 727, activation of many pro-atherogenic genes, and the uptake of modified LDL by human macrophages (Li et al., 2011).

1.14.13 JNK/SAPK

There are three JNK isoforms, 1, 2 and 3. JNK1/2 are expressed ubiquitously whereas JNK3 expression is restricted to the brain, testis and cardiac myocytes (Bode and Dong, 2007). JNKs can be directly activated and phosphorylated by mitogen-activated protein kinase kinase (MKK)4 and/or MKK7. c-Jun is a well-known substrate for JNKs.

JNKs have important roles in the control of apoptosis (Ries et al., 2008) along with cell survival and proliferation (Yu et al., 2004). Studies in mice with deletion of JNK-1 and -2 in T helper cells found that JNK2 has an important role in Th differentiation to Th1 phenotype and is also involved in the production of Th1 cytokines, such as IFN-γ (Dong et al., 1998; Sabapathy et al., 1999). In addition, $ApoE^{-/-}$ and $JNK2^{-/-}$ mice fed a high cholesterol diet had decreased amounts of atherosclerotic plaques probably from reduction in foam cell formation whereas knockout of JNK1 had no effect (Ricci et al., 2004).

1.14.14 p38 MAPK

There are four p38 isoforms in mammalian cells, p38α, p38β, p38γ and p38δ (Dong et al., 2002). p38β is expressed ubiquitously whereas p38γ is found in skeletal muscles and p38δ is expressed in multiple tissues including lung, kidney, testis, pancreas and small intestine (Kaminska, 2005). p38 MAPK can be activated by MKK3, MKK4 and MKK6 (Pearson et al., 2001; Raingeaud et al., 1996). Mammilian p38 is associated with inflammation, cell growth, differentiation and apoptosis (Hommes et al., 2003). For example, p38 MAPK regulates the expression of multiple genes implicated in inflammation such as TNF-α, IL-1β, IL-6 and IL-8 (Kaminska, 2005).

p38 MAPK regulates the level of cholesterol ester accumulation in macrophages, which is an important step in foam cell formation and development of atherosclerosis (Mei et al., 2012). Additionally, p38 MAPK plays a significant role in the development of atherosclerosis via many different ways including stimulation of

secretion of MCP-1, which attracts monocytes to vascular enthothelial cells (Domoto et al., 2003; Matsuyama et al., 2004). p38 MAPK mediates the MCP-1-dependent transendothelial migration, integrin activation, and chemotaxis (Cambien, 2001).

The role of p38 MAPK has also been investigated in mouse model systems. Macrophage deficiency of p38α MAPK was found to promote apoptosis and plaque necrosis in advanced atherosclerotic lesions of $ApoE^{-/-}$ mice (Seimon et al., 2009). On the other hand, Kardakaris (2011) showed that endothelial and macrophage-specific deficiency of p38α has no effect on the pathogenesis of atherosclerosis in $ApoE^{-/-}$ mice. Further studies will be required to clarify the role of p38 MAPK in atherosclerosis *in vivo.*

1.15 Hypothesis

1. Statins act in an anti-inflammatory manner and attenuate the expression of inflammatory markers expressed in response to pro-inflammatory cytokines in human macrophages.
2. The anti-inflammatory action of statins is due to depletion of metabolites in the cholesterol biosynthetic pathway because of inhibition of HMG CoA reductase.
3. Statins attenuate inflammatory gene expression induced by the pro-inflammatory cytokine interferon-γ by affecting the activity of signal transducers and activators of transcription-1.
4. Statins also effect pro-inflammatory gene expression by modulating the action of MAP kinase pathways.
5. Statins affect lipid metabolism in human macrophages.

1.16 Aims of the study

Atherosclerosis is recognised as a chronic inflammatory disorder, representing the major cause of mortality in the western society. Cytokines play an important role in the regulation of inflammatory responses during atherosclerosis progression, principally through the modulation of gene expression associated with this disease. The study of signalling mechanisms involved in the cytokine-mediated regulation of gene expression implicated in the progression of this disease is vital for the identification of new targets for therapeutic intervention (Harvey et al., 2007). Additionally, it is important to understand how lipid-lowering drugs such as statins mediate their anti-inflammatory actions. Therefore, the main purpose of this project was to investigate if statins inhibit gene expression regulated by cytokines using real-time quantitative PCR and other techniques, and to delineate the mechanisms of actions.

Previous studies on the anti-inflammatory actions of statins have been limited to certain classical cytokines (Bessler et al., 2005; Iwata et al., 2012). The main aim was therefore to investigate whether this occurs with newer cytokines such as IL-17 and TL1A along with the potentially master regulator of atherosclerosis, IFN-γ. The effect of statins was analysed using a combination of THP-1 macrophages, RAW 264.7 macrophages and primary HMDMs (Chapter 4).

As mentioned earlier, IFN-γ signals predominantly through the JAK–STAT pathway and other pathways including MAPK. It was therefore decided to study the effect of simvastatin on these pathways at the level of their activation and/or gene expression (Chapter 4). Particularly, the effect of simvastatin on STAT1 and the three MAPK cascades (ERK1/2, JNK/c-Jun and p38) was analysed.

As statins are known for their pleiotropic actions, it was of interest to examine their effect on lipid content and composition of macrophages using a combination of thin layer chromatography, gas chromatography and radiolabelling experiments, such studies formed the focus of chapter 5.

Chapter 2: Materials and Methods

2.1 Materials

The table below shows the materials that were used to carry out experiments presented in this thesis and the suppliers from where they were sourced.

Table 2.1: The materials used for studies in this thesis and the suppliers from where they were sourced.

Supplier	Material
Welsh Blood Service, UK.	Buffy coats.
Gibco-BRL, UK	RPMI 1640 culture medium; Penicillin; Streptomycin; Foetal calf serum; 2-mercaptoethanol.
Sigma-Aldrich, UK	Phorbol 12-myristate 13-acetate (PMA), Tween20; Syber Green; X-ray film; Dimethyl sulfoxide (DMSO); RIPA buffer; Protease inhibitor cocktail; Ethanol; Methanol; RNA Zap; Accuspin tubes; Geranylgeranyl pyrophosphate, ammonium salt (GGP); Farnesyl pyrophosphate, ammonium salt (FPP); Mevalonate; THP-1 cell line (88081201); RAW264.7 cell line (91062702); Bromophenol blue; Phosphate buffered saline (PBS) tablets.
Qiagen, UK	RNeasy plus minikit.
NBS, UK	Spin column RNA miniprep kit.
Peprotech, UK	IL-17A ; IFN-γ and TL1A.
Calbiochem, UK	Simvastatin, sodium salt; Insolation simvastatin sodium salt; Atorvastatin.
Promega, UK	Deoxyribonucleotide triphosphate (dNTPs); MMLV reverse transcriptase; RNasin ribonuclease inhibitor; Random hexamer primers.
Greiner Bio One, UK	Tissue culture flasks; 6-well plates; 12-well plates; Cell scrapers; 10ml stripettes; 25ml stripettes; 50ml Falcon tubes.

Fisher Scientific, UK	Industrial methylated spirit (IMS); filter papers; Ethylenediaminetetraacetic acid (EDTA); Glycerol.
Millipore, UK	Sterile 0.22µm filters, PVDF membrane.
Sigma Genosys, UK	PCR primers.
Applied Biosystems, UK	I block, CDP star reagent.
Invitrogen, UK	NuPage™ Novex gel tank system; Blotting module; Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels; MOPS running buffer; Transfer buffer; RPMI 1640 with GlutaMAX™ liquid; Penicillin; Streptomycin; Foetal calf serum; 2-mercaptoethanol; See Blue protein markers; Magic marker XP western protein standard.
Santa Cruz Biotechnology, USA	Goat anti-rabbit IgG alkaline phosphtase (AP) antibody (Ab) (sc-2007); Goat anti-mouse (AP) Ab (sc-2008); STAT1 p84/p91 Ab (sc592); ICAM-1Ab (H-108); p-c-Jun Ab (km-1) (sc-822); c-Jun Ab (H-79) (sc-1694).
Axis Shield, Norway	Lymphoprep.
Pierce, UK	Bicinchoninic acid assay (BCA) protein assay.
BioRad, UK	Microplate Reader.
MJ Research, UK	Opticon 2 PCR machine.
National Diagnostics, UK	10X TBE.
Cell Signalling Technology, UK	Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) Ab (9101); p44/42 MAPK (ERK1/2) Ab (9102); phospho-p38 MAPK (Thr180/Tyr182) Ab (9211); p38 MAPK Ab (9212); Phospho-STAT1 (Ser727) Ab (9177s); Phospho-STAT1 (Tyr701) Ab (9167).
Thermo Fisher Scientific, UK	Restore Plus western blot stripping buffer.
Amersham Life Sciences Ltd., UK	[1-^{14}C]-acetic acid (sp. activity = 1.85-2.29 GBq . mmol^{-1})
Merck KGaA,, Germany	TLC Silica gel G plates

2.2 Preparation of solutions, glass-and plastic-ware

Solutions, glass- and plastic-ware used for RNA/DNA and tissue culture related techniques were autoclaved at 121°C (975kPa) for 30 minutes.

2.3 Cell Culture Techniques

2.3.1 Cell lines

2.3.1.1 THP-1

THP-1 is a human monocytic leukaemia cell line. After differentiation with phorbol esters, THP-1 cells exhibit many of the properties and characteristics of human monocyte-derived macrophages (Kohro et al., 2004). Thus differentiated THP-1 cells are considered as a practical model to examine properties of macrophages including cell signalling and gene expression in disease, including atherosclerosis, with demonstrated conservation of responses to primary cultures and *in vivo* (McLaren et al ., 2010a; McLaren et al., 2010b; Nakaya et al., 2010).

2.3.1.2 RAW264.7

RAW264.7 is a mouse leukaemic macrophage cell line that is widely used as a macrophage cell model exhibiting a high efficiency for DNA transfection (Dickins et al., 2005). The RAW264.7 cell line was derived about 30 years ago from a tumour developing in a BAB/14 mouse, a BALB/c IgH congenic strain, inoculated with the Abelson murine leukaemia virus (Hartley et al., 2008). RAW264.7 cells are adherent to tissue culture flasks and dishes.

2.3.2 Maintenance of cell lines in culture

2.3.2.1 THP-1 and RAW 264.7

THP-1 and RAW 264.7 cells were grown in RPMI-1640 with GlutaMAX™. The medium was supplemented with 10% (v/v) heat-inactivated (56°C, 30 minutes) foetal calf serum (HI-FCS), penicillin (100U/ml) and streptomycin (100µg/ml) (pen/strep). Both the HI-FCS and pen/strep were filter-sterilised by passing them through a 0.2µm sterile filter before use. Both THP-1 and RAW264.7 cells were grown in medium tissue culture flasks in a humidified incubator at 37°C, 5% (v/v) CO_2.

2.3.3 Sub-culturing of cells

2.3.3.1 THP-1

THP-1 cells were subcultured when they reached approximately 60% confluence (0.6 x 10^6 cells/ml). The cells were transferred into a polypropylene tube (Falcon tubes) and centrifuged at 100g for 5 minutes. The medium was aspirated and the cells were re-suspended in fresh medium containing 10% (v/v) HI-FCS. Cells were placed in new tissue culture flasks and grown up at 37°C in a humidified, 5% (v/v) CO_2 incubator. For experiments, cells between passage two and eight were used.

2.3.3.2 RAW264.7

RAW264.7 cells were sub-cultured when they reached approximately 80% confluence. The cells were removed from the surface of the dish by scrapping. The resulting cell suspension was transferred into a new dish at a ratio of 1:25 with fresh pre-warmed medium supplemented with 10% (v/v) HI-FCS and pen/strep. Cells were grown up at 37°C in a humidified, 5% (v/v) CO_2 incubator.

2.3.4 Preserving and storing cells

THP-1 and RAW264.7 cells were kept at -80°C or in liquid nitrogen for long-term storage with only early passage cells (up to passage 6) being used. Prior to freezing, cells were centrifuged at 100g for 5 minutes and re-suspended in HI-FCS containing 10% (v/v) glycerol. Approximately 5 x 10^6 cells/ml were aliquoted into 1ml cryoampoules and stored at -80°C overnight before being transferred to liquid nitrogen.

2.3.5 Thawing frozen cells

Cells from liquid nitrogen were thawed by placing them in a water bath at 37°C. Cells were transferred to a polypropylene tube containing 10ml HI-FCS and centrifuged at 100g for 5 minutes. After re-suspension with fresh medium containing 10% (v/v) HI-FCS, the cells were plated out into tissue culture flasks and cultured as normal.

2.3.6 Counting cells

To count the THP-1 and RAW264.7 cells, a haemocytometer was used. After centrifugation at 100g for 5 minutes, cells were re-suspended in 3-5ml of culture medium containing 10% (v/v) HI-FCS. The haemocytometer was covered with a precise ground cover slip. Then, 7µl of cell suspension was used and the numbers of cells in a 5 x 5 grid were counted. The number of cells/ml was calculated by multiplying the number of cells in the counting area by 10^3.

2.3.7 Treatment of cells with PMA, Simvastatin, Atorvastatin and cytokines

2.3.7.1 THP-1

For experiments involving reverse transcription quantitative polymerase chain reaction (RT-qPCR), after counting the THP-1 monocytes, 500,000 cells were placed in each well of a 12-well plate and supplemented with 1ml of culture medium containing 10% (v/v) HI-FCS. The cells were then differentiated into macrophages with 0.16 µM PMA for 24 hours. The cells then were treated with vehicle, Simvastatin or Atorvastatin for 1 hour. The concentration of simvastatin used in this study was 10 µM (Copaja et al., 2012) or 40 µM, as indicated in the relevant sections, whereas that for atorvastatin was 10 µM (Lin et al., 2009). The cells were then incubated in the absence or the presence of cytokines for a further 24 hours before harvesting for isolation of RNA. Table 2.2 shows the cytokines used in this study along with their concentrations. The concentration of the cytokines was based on maximal response obtained in previous studies in the laboratory and others in the same field (McLaren et al., 2010a; McLaren and Ramji, 2009; Kang et al., 2005).

2.3.8 Treatment of THP-1 cells with FPP, GGP, mevalonate, Simvastatin and IFN-γ

THP-1 cells (500,000) were placed in a 12-well plate and they were differentiated with PMA for 24 hours as detailed above. Simvastatin (10 µM), FPP (5 µM), GGP (5 µM), mevalonate (500 µM) or vehicle were added for 1 hour and then the cells were incubated in the absence or the presence of IFN-γ (1000 U/ml) for 24 hours before RNA extraction and RT-qPCR (see Chapter 4 for details on the experiments).

2.3.9 Treatment of RAW264.7 cells with Simvastatin and IFN-γ

Cells ($5x10^5$) were placed in each well of a 12-well plate and 1 ml of culture medium with 10% (v/v) HI-FCS and pen/strep was added. The cells were then incubated at 37°C in a humidified, 5% (v/v) CO_2 incubator for 24 hour. Then,

simvastatin (40 μM) or vehicle were added for 1 hour and then incubated with vehicle or mouse IFN-γ (1000 U/ml) for 24 hours before RNA extraction and RT-qPCR.

Table 2.2: Cytokines used and their concentrations.

Cytokines	Concentrations used
TL1A	100 ng/ml
IL-17A	100 ng/ml
IFN-γ	1000 U/ml
mIFN-γ	1000 U/ml

2.3.10 Primary human monocyte-derived macrophage (HMDM) cultures

For this, a buffy coat was obtained from the Welsh Blood Service and the monocytes were purified and plated out in 12-well plates. At the beginning, Lymphoprep solution was left to warm to room temperature and then 15 ml was added to Accuspin centrifuge tubes. The tube was centrifuged at 1000g for 1 minute to place the Lymphoprep below the filter. Then, 30 ml of the buffy coat was poured into the filter of the Accupsin tube and the tube was re-centrifuged at 1000g for 30 minutes at room temperature. The mononuclear cells were then collected and transferred to new 50ml Falcon tube to which an equal volume of ice-cold PBS-0.4% (w/v) tri-sodium citrate was added and immediately centrifuged at 1000g for 5 minute at 4ºC. The pelleted cells were then re-suspended in 10 ml of 0.2% (v/v) saline solution (sodium chloride solution) and kept on ice for 30 seconds. Following this, 10 ml of 1.6% (v/v) saline solution (sodium chloride solution) was added followed by immediate centrifugation at 1000g for 5 minutes at 4ºC to remove the red blood cells. This step was repeated twice to remove any further red blood cells present in the sample. The resultant interface was then collected and washed 6-8 times with 10 ml of ice-cold PBS-0.4% (w/v) tri-sodium citrate to remove contaminating platelets. The cells were then plated out in RPMI media supplemented with 10% (v/v) HI-FCS and pen/strep. The cells were left to differentiate for 10 days before use in experiments. During this period, the media was continuously changed every two days.

2.4 RNA related techniques

2.4.1 Isolation of RNA

Total RNA was isolated from cells using a Spin Column RNA Miniprep Kit (NBS Biologicals). Before using the kit, the medium was aspirated from the cells. The cells were re-suspended in Lysis DR buffer, provided in the kit, containing 10 μl/ml β-mercaptoethanol. The rest of the protocol was carried out according to the manufacturer's instructions. A NanoDrop ND1000 spectrophotometer was used to determine the RNA concentration and quality of RNA (OD 260:280 ratio).

2.4.2 Reverse Transcription

RNA (0.5 μg) was mixed with random hexamer primers (200 pmol) and sterile water to a total volume of 13.5 μl. This was incubated at 72°C for 5 minutes and immediately cooled on ice. The following reagents were added to the reaction: 1μl of deoxyribonucleotide triphosphate (dNTP) mixture containing 10 mM each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP); 4 μl of 5X Molony Murine leukaemia virus (MMLV) reverse transcriptase buffer provided in the kit; 0.5 μl (50 U/μl) of recombinant RNase inhibitor; and 1 μl (200 U/μl) of MMLV reverse transcriptase.

The total reaction mixture was incubated at 37°C for 1 hour and the reaction terminated by incubation at 92°C for 2 minutes. Synthesised cDNA was diluted by adding 30 μl of RNase-free water and the resulting solution was then stored at -20°C for future use.

2.4.3 RT-qPCR

A RT-qPCR reaction was set up using the SYBER® GREEN JumpStart™ Taq Readymix™ for quantitative PCR (Sigma-Aldrich). The reactions were then “run” using an Opticon 2 PCR machine (MJ Research). The reagents were mixed and added into a 96 well plate and the program was set according to the optimised PCR conditions (Tables 2.3 and 2.4). The composition of each reaction is detailed in Table 2.5.

The RT-qPCR data were analysed using the comparative Ct (ΔΔCt) method (Livak and Schmittgen, 2001). The output of RT-qPCR reaction shows the number of PCR cycles against the increasing fluorescence. The ΔΔCt method relies on measuring the number of reaction cycles it takes for the amplification plot to cross the threshold level (Ct value) within the exponential phase of amplification (Livak and Schmittgen, 2001). The Ct values were then collected from the computer and analysed on Microsoft Excel using the ΔΔCT method (Ginzinger, 2002). The Ct value of the gene of interest was compared relative to the Ct value of a control housekeeping gene to normalise the data (Livak and Schmittgen, 2001). The ΔΔCt method relies on two assumptions, firstly that the expression of the housekeeping gene does not vary under experimental conditions and secondly, that the PCR efficiencies for each primer set used in the analysis are similar (Ginzinger, 2002). The sequences of the primers and the size of the amplification products are shown in Table 2.3

Table 2.3: PCR primers for analysis of gene expression

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size of product (bp)	References
SR-A	CCAGGGACATGGA ATGCAA	CCAGTGGGACCTCG ATCTCC	365	(Draude and Lorenz, 2000)
CD36	GAGAACTGTTATGG GGCTAT	TTCAACTGGAGAGG CAAAGG	388	(Draude and Lorenz 2000)
GAPDH	GAAGGTGAAGGTC GGAGTC	GAAGATGGTGATGG GATTTC	226	(Yamanishi et al.,2002)
ABCA1	GCTGCTGAAGCCA GGGCATGGG	GTGGGGCAGTGGCC ATACTCC	90	(Kaplan et al.,2002)
ABCG1	TGCAATCTTGTGCC ATATTTGA	CCAGCCGACTGTTCT GATCA	90	(Kaplan et al.,2002)
MCP-1	CATTGTGGCCAAG GAGATCTG	CTTCGGAGTTTGGGT TTGCTT	91	(Locati et al.,2002)
ICAM-1	ACGCTGAGCTCCTC TGCTACTC	GGGCAGGATGACTT TTGAGG	162	(Nilesh et al.,2010)
Apo-E	TTCCTGGCAGGATG CCAGGC	GGTCAGTTGTTCCTC CAGTTC	270	(Wang et al.,1989)
ERK-1	GCAGGACCTGATG GAGACTGAC	CCAGAATGCAGCCC ACAGAC	344	QPPD
ERK-2	GCGCTACACCAAC CTCTCGT	CACGGTGCAGAACG TTAGCTG	377	QPPD
p38 MAPK	GTGGTACAGGGCT CCTGAGA	TATGCATCCCACTGA CCAAA	79	
c-Jun	TCCAAGTGCCGAAA AAGGAAG	CGAGTTCTGAGCTTT CAAGGT	78	Primer Bank
JNK-1	TCTGGTATGATC CTTCTGAAGCA	TCCTCCAAGTCCATA ACTTCCTT	127	Primer Bank
JNK-2	GAAACTAAGCCGTC CTTTTCAGA	TCCAGCTCCATGTGA ATAACCT	207	Primer Bank

mβ-actin	GTGCCACCAGACA GCACTGTGTTG	TGGAGAAGAGCCTAT GAGCTGCCTG	202	(McLaren et al., 2010A)
mMCP-1	CTGGATCGGAACC AAATGAG	CGGGTCAACTTCACA TTCAA	95	(Locati et al.,2002)
mICAM-1	CAGTCCGCTGTGCT TTGAGA	CGGAAACGAATACAC GGTGAT	75	(Locati et al.,2002)

PrimerBank and QPCR primer database (QPPD) are both online databases containing primer sequences from previous studies.

Table 2.4: Reaction set up used for all RT-PCR reactions

PCR Step	**GAPDH, SR-A, c-JUN, JNK1, JNK2 and mICAM-1**	**ABCA1, ABCG1, ERK1 and ERK2**	**CD36 and mβactin**	**MCP-1, ICAM-1 and p38**	**Apo-E**	**mMCP-1**
Initial Melting	95°C for 5 minutes	95°C for 5 minutes	95°C for 5 minutes	95°C for 5 minutes	95°C for 5 minutes	95°C for 5 minutes
Annealing	60°C for 60 seconds	65°C for 60 seconds	58°C for 60 seconds	63°C for 60 seconds	62°C for 60 seconds	59°C for 60 seconds
Extension	72°C for 60 seconds	72°C for 60 seconds	72°C for 60 seconds	72°C for 60 seconds	72°C for 60 seconds	72°C for 60 seconds
Melting	95°C for 30 seconds	95°C for 30 seconds	95°C for 30 seconds	95°C for 30 seconds	95°C for 30 seconds	95°C for 30 seconds
Final Extension	72°C for 10 minutes	72°C for 10 minutes	72°C for 10 minutes	72°C for 10 minutes	72°C for 10 minutes	72°C for 10 minutes
Number of Cycles	35	35	35	35	35	35

Table 2.5: The composition of a 25µl Q-PCR reaction

Reagent	Amount (µl)
SYBR Green Ready mix (Sigma Aldrich)	12.5
Forward Primer (4 uM)	0.5
Reverse Primer (4 uM)	0.5
cDNA (10 ng)	1
Water	10.5
Total	25

2.5 Agarose Gel Electrophoresis

Size fractionation of PCR products was carried out by agarose gel electrophoresis. For this, 1.5% (w/v) gels were made up with agarose melted into 1x Tris/borate/EDTA (TBE) buffer (Table 2.6). A volume of 0.5 µg/ml ethidium bromide was added to the dissolved agarose solution. Stock solutions for gel electrophoresis are shown below in Table 2.6.

Table 2.6: Composition of reagents used in gel electrophoresis

Reagent	Composition
10 x TBE	0.89M Tris borate, 890mM boric acid and 20mM EDTA, pH 8.3
5 x DNA loading dye	1x TBE, 50% (v/v) glycerol and 2.25% (w/v) bromophenol blue

DNA or RNA samples (20 µl) were mixed with 5 µl of DNA/RNA loading dye and electrophoresis was carried out in 1x TBE buffer at 100V for around 30-60 minutes using a horizontal gel unit (Fisher brand). The sizes of the PCR products were compared to standard DNA molecular weight markers. DNA/RNA was visualised under UV light using a Syngene Gel Documentation system.

2.6 Protein Analysis

2.6.1 Preparation of protein extracts using RIPA buffer

Following experimentation, the medium was aspirated from the well and the cells were washed with 1 ml of ice cold PBS. The PBS was then aspirated from the well and the cells were lysed using 50-100 µl of RIPA Buffer (Table 2.7) supplemented with a protease inhibitor cocktail (Sigma) at 1:100 dilution by scrapping. The lysate was transferred to a 1.5 ml micro-centrifuge tube and centrifuged at top speed for 5 minutes. Lysates were stored at -80°C or used immediately to determine protein concentration, followed by SDS-PAGE and western blotting. For analysis by SDS-PAGE, the gel sample buffer or running buffer (MOPS SDS running buffer) (Table 2.7) was added in a 1:1 ratio before loading.

2.6.2 Determination of protein concentration

Protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce). Samples were processed in 96-well plates and a standard curve was prepared for each assay using bovine serum albumin (BSA) (2 mg/ml) solution to give final concentrations of 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml and 25 µg/ml. Samples were diluted in water in a ratio of 1:300 and placed into a 96-well plate in duplicate (150 µl). Then, 150 µl of reagents A, B and C, provided in the kit, were mixed according to the manufacturer's instructions and added to each well. The plate was covered and left at 37°C for 2.5 hours. A Model 680 Microplate Reader (Biorad) was used to measure the absorbance of each sample at a wavelength of 570 nm. The concentration of proteins in the samples was determined from the standard curve.

2.6.3 SDS polyacrylamide gel electrophoresis

Protein separation was performed using a NuPage 4-12% Bis-Tris gel (Invitrogen) on a Mini-PROTEAN II slab electrophoresis cell (Bio-Rad Laboratories)

(see Table 2.7 for composition of buffers). Briefly, the gel was placed in the electrophoresis tank and the inner and outer compartments were filled with MOPS-SDS buffer (Table 2.7). Equal amounts of protein sample were added to each well and then size-fractionated alongside protein markers (See Blue Plus 2 prestained standard 1x markers or Magic markers) (both from Invitrogen). Electrophoresis was carried out at 200V, 400 mA for 60 minutes.

2.6.4 Western blotting

Following electrophoresis, PVDF membrane (0.45 μm pore size, Millipore) was cut to the size of the gel and activated in methanol (100%) before being placed in transfer buffer (Table 2.7). The membrane was placed on top of the gel and both were sandwiched between Whatman 3MM filter paper and sponge pads that had also been soaked in transfer buffer (1x, Invitrogen) (Table 2.7). The whole assembly was then placed in a Mini Trans-Blot Cell (Bio-Rad Laboratories). Electro-blotting was carried out at 30V, 300mA for 60 minutes at 4°C. Transfer efficiency was checked at this stage by staining the PVDF membrane with Ponceau S solution.

2.6.5 Immuno-detection of proteins

Following blotting, the membrane was removed from the sandwich and washed once with PBS-Tween (Table 2.7) for 5 minutes. The membrane was then placed in I-BT solution (Table 2.7) shaking for 1 hour; this step was carried out to block any non-specific interactions of the antibodies with the membrane. After removal of the I-BT solution, the primary antibody was added (optimised dilution in I-BT, Table 2.8) and left overnight shaking at 4°C. The membrane was then washed three times for 5 minutes with PBS-Tween. After the wash steps were completed, the secondary antibody (alkaline phosphatase conjugated antibody)(1:5000) was added for 1 hour at room temperature (diluted in I-BT, Table 2.8). After 1 hour incubation, the membrane was again washed three times for 5 minutes with PBS-Tween. A detection reagent (CDP star) was added according to the manufacturer's instructions (Applied

Biosystems) to the membrane before developing the membrane using Kodak film. Different exposures were then developed using a Kodak film placed inside a light resistant X-ray cassette. The film was developed using Agfa film processor and developer.

2.6.6 Semi-quantitative densitometry analysis of western blots

The densities of bands from immunoblots were analysed using GeneTools software (Syngene). All experimental values were normalised to their relevant housekeeping protein and the control values within each experiment were arbitrarily assigned as 1 (see relevant figure legends). All other values were then compared to the control.

Table 2.7: Composition of stock solutions used for protein analysis by SDS-PAGE and western blotting.

Solution	Composition
RIPA Buffer	50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1.0% (v/v) IGPEAL CA-630 (NP-40), 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) SDS
NuPAGE MOPS SDS Running Buffer (X20)	50 mM 3-(N-morpholino) propane sulfonic acid (MOPS), 50 mM Tris Base, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.7.
NuPAGE Transfer Buffer	50 mM Bis-Tris propane, 50 mM Bicine, 20% (v/v) methanol.
I-BT solution	500ml PBS-Tween, 1gram Tropix I-Block (Applied Biosystems UK), 500ml of 5% (v/v) sodium azide.
PBS-Tween	10l ddH20, 100 PBS tablets (sigma), 20ml Tween20.
Ponceau S	0.1% (w/v) Ponceau S, 5 %(v/v) acetic acid, ddH2O.

Table 2.8: Antibodies and dilutions used for western blotting

Protein	Primary Antibody Species	Primary Antibody Dilution	Secondary Antibody	Secondary Antibody Dilution	Protein Size
β-actin	Mouse	1:10,000	Goat Anti Mouse (AP)	1:5000	42 kDa
ICAM-1	Rabbit	1:200	Goat Anti Rabbit (AP)	1:5000	110 kDa
Phospho p44/p42	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	42 and 44 kDa
Total p44/p42	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	42 and 44 kDa
Phospho p38	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	43 kDa
Total p38	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	43 kDa
Phospho c-Jun	Mouse	1:1000	Goat Anti Mouse (AP)	1:5000	39 kDa
Total c-Jun	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	39 kDa

2.7 Lipid Experiments

2.7.1 Cell culture

THP-1 cells were cultured as described above except that 4-6 millions cells were used in each experiments in 6 well plates. Following differentiation, Simvastatin (40 μM) or vehicle were added for 1 hour followed by vehicle or IFN-γ (1000 U/ml) for 24 hours. PBS (1x) (pH 7.4) was added to the cells, which were then scraped from the surface using a plastic cell scraper. Cells were then transferred into an Eppendorf tube and centrifuged for 5 minutes at maximum speed in a microcentrifuge to obtain a pellet that was then stored on ice.

2.7.2 Lipid Extraction

2.7.2.1 Lipid extraction from cell suspension

Lipids were extracted from macrophages using the method by Garbus et al. (1963). Briefly, 1ml of distilled water was added to the cell pellet and the contents transferred into a glass tube. Then, 2.5 ml of chloroform: methanol solution (1:2 by volume) was added. The samples were then vortexed and incubated at room temperature for 15 minutes. After addition of 1ml each of chloroform (100%) and Garbus solution (2 M KCl in 0.5 M potassium phosphate buffer (K_2HPO4 and KH_2PO4, both 0.5 M; pH 7.6), the samples were vortexed and centrifuged at 1500 rpm for 3-5 minutes (Baird and Tatlock Auto Bench Centrifuge Mark IV, England). This step allows two-layer separation with cell debris at the interphase of chloroform and aqueous layers. The chloroform layer (containing lipids) was carefully transferred to a clean conical tube using a glass pipette. The solvent was then evaporated under a stream of nitrogen. The extracted lipids were re-constituted into a known volume of chloroform and stored at -20°C.

2.7.2.2 Separation of total lipids using thin-layer chromatography

Non-polar lipids were separated using one-dimensional thin-layer chromatography (TLC) on 10 x 10 cm Silica gel G plates using a solvent system of hexane: diethyl ether: acetic acid (80:20:1 by vol.). Following drying, plates were sprayed with 8-anilino-4-naphthosulphonic acid (0.05% in methanol (w/v), solution and viewed under UV-light to detect lipids. Bands corresponding to total polar lipids (PL), triacylglycerol (TAG) and sterol esters (SE) were identified and scraped of the plates along with silica gel for further fatty acid analysis as described below.

2.7.3 Fatty acid analysis

2.7.3.1 Transmethylation

Individual lipid classes separated by TLC and aliquots of total lipid extracts were used for fatty acid (FAs) analysis. FAs were converted into fatty acid methyl esters (FAMEs) via transmethylation with 2.5% (v/v) H_2SO_4 in dry methanol: toluene (2:1, by vol.) at 70ºC for 2 hours. A known amount (30-50 µg) of heptadecanoic acid (C17:0) was added as an internal standard for FAs quantification. After incubation for 2 hours, 2 ml of 5% (w/v) NaCl (aqueous solution) was added and FAMEs were extracted with 3 ml (two times) of hexane (100%) (HPLC grade). Combined hexane fractions were evaporated under a stream of nitrogen, reconstituted into a small volume (40-60 µl) of 100% hexane and stored under -20ºC for future analysis.

2.7.3.2 Gas Chromatography

FAMEs were separated and analysed by using a Clarus 500 gas chromatograph (Perkin-Elmer, Norwalk, Connecticut) with a flame ionising detector (FID) and equipped with a 30 m x 0.25 mm i.e. capillary column (Elite 225, Perkin Elmer, Waltham, MA, USA). The oven temperature was set to be 170ºC for 3 minutes, then heated up to 220ºC at 4ºC/minute and finally held at 220ºC for 30 minutes.

FAMEs were identified by comparing retention times of peaks with those of G411 standard mixture (Nu-Chek Prep,Inc., Elysian, MN, USA).

2.8 Radiolabeling experiments

To investigate the effect of Simvastatin on the radiolabelling of cholesterol (CHO) and two other major lipid classes, triacylglycerols (TAG) and total polar lipids (TPL), $4x10^6$/ml cells were incubated with 0.5 µCi of sodium [1-^{14}C] acetate for 3 hour. After incubation, the cells were pelleted by centrifugation for 5 minutes at 1500 rpm. Cell lipids were extracted as described previously (Section 2.7.2.1). Lipid extracts were separated into three major fractions: TPL, CHO and TAG using one-dimensional TLC with hexane/diethyl ether/acetic acid (80:20:1, by volume) as solvent. After drying, the plates were sprayed with a 0.05% (v/v) solution of 8-anilino-4-naphthosulphonic acid in methanol and viewed under UV light to reveal lipids. Identification of lipids was made by reference to authentic standards. To measure the incorporation of radioactivity into individual lipid classes, the lipids following scraping from the plates, were transferred into scintillation vials to which 10 ml of OptiFluor (PerkinElmer Inc., Waltham, MA, USA) scintillant was added. The samples were counted in a Perkin-Elmer Tri-Carb 2800 TR liquid scintillation counter. Quench correction was made automatically by the external standard channels-ratio method.

2.9 Statistical analyses of data

Statistical analysis was carried out using SPSS software. Normality of the data was examined using the Shapiro-Wilk test prior to performing statistical analyses. Statistical analysis was performed using Student's *t*-test for single comparisons (two-tailed, paired) or one-way ANOVA for multiple comparisons with Tukeys post-hoc test. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, and NS indicating not significant).

Chapter 3: The Effect of Statins on Cytokine Regulated Gene Expression in Macrophages

3.1 Introduction

Atherosclerosis is the major cause of cardiovascular disease that affects mainly the large and medium arteries (Lusis, 2000). It is characterised by lipid accumulation and inflammation within the walls of the arteries. During the progression of the disease, an atherosclerotic plaque is formed as a result of a lesion developing in the vascular wall (Lusis, 2000). Rupture of an atherosclerotic plaque leading to thrombosis is one of the most common causes of mortality in Western societies. Complications arising from thrombosis include heart attacks, stroke and gangrenes. Plaque stability is an important factor in controlling the incidence of plaque rupture (Halvorsen et al., 2008).

It has been shown that atherosclerosis is a state of chronic inflammation within the vascular wall. A large number of cytokines are released at different stages of the disease and these have been studied and reviewed in relation to them being pro- or anti-atherogenic (Tedgui and Mallat, 2006). High levels of pro-atherogenic cytokines are observed in atherosclerosic lesions (Harvey and Ramji, 2005). Such cytokines include TNF-α, IFN-γ, IL-1, IL-17 and IL-12. On the other hand, anti-inflammatory cytokines include IL-10, IL-4, IL-33 and TGF-β. Macrophages and infiltrating T-lymphocytes are the major source of cytokines in atherosclerotic lesions (Tedgui and Mallat, 2006).

The transformation of macrophages into lipid-loaded foam cells through the uptake of cholesterol is a critical early step in atherosclerosis (Lusis, 2000). Activation of ECs is the main initiating factor in the disease, with the accumulation of LDL in the subendothelial matrix the major cause of such activation. The frequency such activation is correlated with the plasma concentration of LDL (Lusis, 2000). Once activated, the ECs express various chemoattractant molecules. The monocytes in the blood stream are then attracted towards the injury/activation site in response to these molecules and begin to produce fatty streaks in these locations (Li and Glass, 2002). Monocytes are initially attracted by adhesion molecules on the surface of the ECs

such as P- and E-selectin molecules expressed on the luminal surface of the activated endothelium, which interact with L-selectin molecules on the surfaces of monocytes (Bobryshev, 2006). The interaction between the selectins and adhesion molecules cause the monocytes to roll across the endothelial surface, which enables molecules such as vascular cell adhesion molecule-1 (VCAM-1) and Inter-cellular adhesion molecule-1 (ICAM-1) to initiate binding with integrins. Integrin binding causes a higher level of adhesion to the endothelium so that the migration through the endothelial layer to the intima can now begin. The migration process is aided by the expression of chemokines by ECs such as MCP-1, M-CSF, MIP-1,TNF-α and TGF-β (Li and Glass, 2002).

Once the monocytes have passed through the endothelial layer and are present in the intima of the blood vessels, they differentiate into macrophages and begin to form foam cells by taking up oxidised LDL from the subendothelial environment. This process is called fatty streak formation and is the first step in the initiation of the disease. As the plaque progresses, cytokines control the migration and proliferation of SMC from the media to the intima of the vessel (Lusis, 2000). Cytokines are also involved in the maintenance of plaque stability by regulating protease expression, SMC migration/proliferation and apoptosis of SMC and other cell types. The cytokines play several roles in this disease, and this has led researchers to extensively investigate the role of different cytokines in this disease and whether they are pro-atherogenic or anti-atherogenic (Tedgui and Mallat, 2006).

Many genes have been shown to co-ordinate macrophage cholesterol homeostasis to date. The function of numerous key genes implicated in the regulation of cholesterol efflux, typically ABCA1 and ABCG1, and AcLDL/OxLDL uptake, such as SR-A and CD36, have been the focus of research in the field of atherosclerosis (Lusis, 2000; Pluddemann et al., 2007; Mead et al., 2002). Because of the difficulties in accessing human atherosclerotic plaque samples and donor-specific heterogeneity associated with primary cultures, established cell lines, such as the human THP-1 cell

line, have been used extensively for studies on macrophage function and gene expression in response to atherosclerosis with conservation of most responses to those observed in primary cultures (Kohro et al., 2004; McLaren et al., 2010a; Nakaya et al., 2010; McLaren et al., 2010b). When stimulated with phorbol esters, such as PMA, THP-1 cells mimic monocyte-derived macrophages by becoming adherent to glass or plastic, exhibiting a macrophage-like morphology and expressing macrophage differentiation markers (Kohro et al., 2004).

Statins are one of the major drugs currently used in the prevention and treatment of atherosclerosis and its complications. In clinical trials, they have been found to be beneficial in the primary and secondary prevention of CHD (Liao and Laufs, 2005). Although the major action of statins is to lower circulating levels of LDL-C by inhibiting HMG-CoA reductase, they have numerous so-called pleiotropic effects (Ridker et al., 2009). These include acting in an anti-inflammatory manner. Unfortunately, the anti-inflammatory actions of statins are not fully understood particularly in relation to newer cytokines such as TL1A and IL-17 (Liao and Laufs, 2005).

3.2 Aims and Experimental Design

As detailed above, cytokines play an important role in the regulation of inflammatory responses during the progression of atherosclerosis, principally through the modulation of gene expression associated with this disease. The study of signalling mechanisms involved in cytokine-mediated regulation of gene expression implicated in the progression of this disease is vital for the identification of new targets for therapeutic intervention (Harvey et al., 2007). Additionally, it is important to understand how lipid-lowering drugs such as statins mediate their anti-inflammatory actions. Therefore, the main purpose of the work reported in this chapter was to investigate if statins inhibit gene expression by cytokines, such as IFN-γ, TL1A and IL-17A via RT-qPCR and other techniques.

Previous studies on the anti-inflammatory actions of statins have been restricted to certain classical cytokines. The main aim of studies presented in this chapter was therefore to investigate whether this occurs with novel cytokines such as IL-17 and TL1A. For this, THP-1 cells were used initially as an *in vitro* cell culture model (Figure 3.1) to study the regulation of expression of MCP-1, ApoE, SR-A, CD-36, ABCA1 and ABCG1. The steps below represent the experimental strategies that were taken to address the aims in this chapter with a schematic representation shown in Figure 3.2.

1-To validate the effectiveness of the action of Simvastatin using RT-qPCR on MCP-1 gene expression from cells treated with IFN-γ as a positive control.

2-Examine the effect of Simvastatin on ICAM-1 gene expression induced by IFN-γ as an additional marker to MCP-1.

3-Investigate the effect of Simvastatin on TL1A, IL-17A and IFN-γ mediated changes in expression of SR-A, CD-36, ABCA1 and ABCG1 in differentiated THP-1 macrophages by RT-qPCR.

4-Examine the effect of Simvastatin on ApoE gene expression when the cells are treated with TL1A by RT-qPCR.

5- Investigate whether the results obtained are not peculiar to Simvastatin by using another statin, Atorvastatin, at the mRNA level.

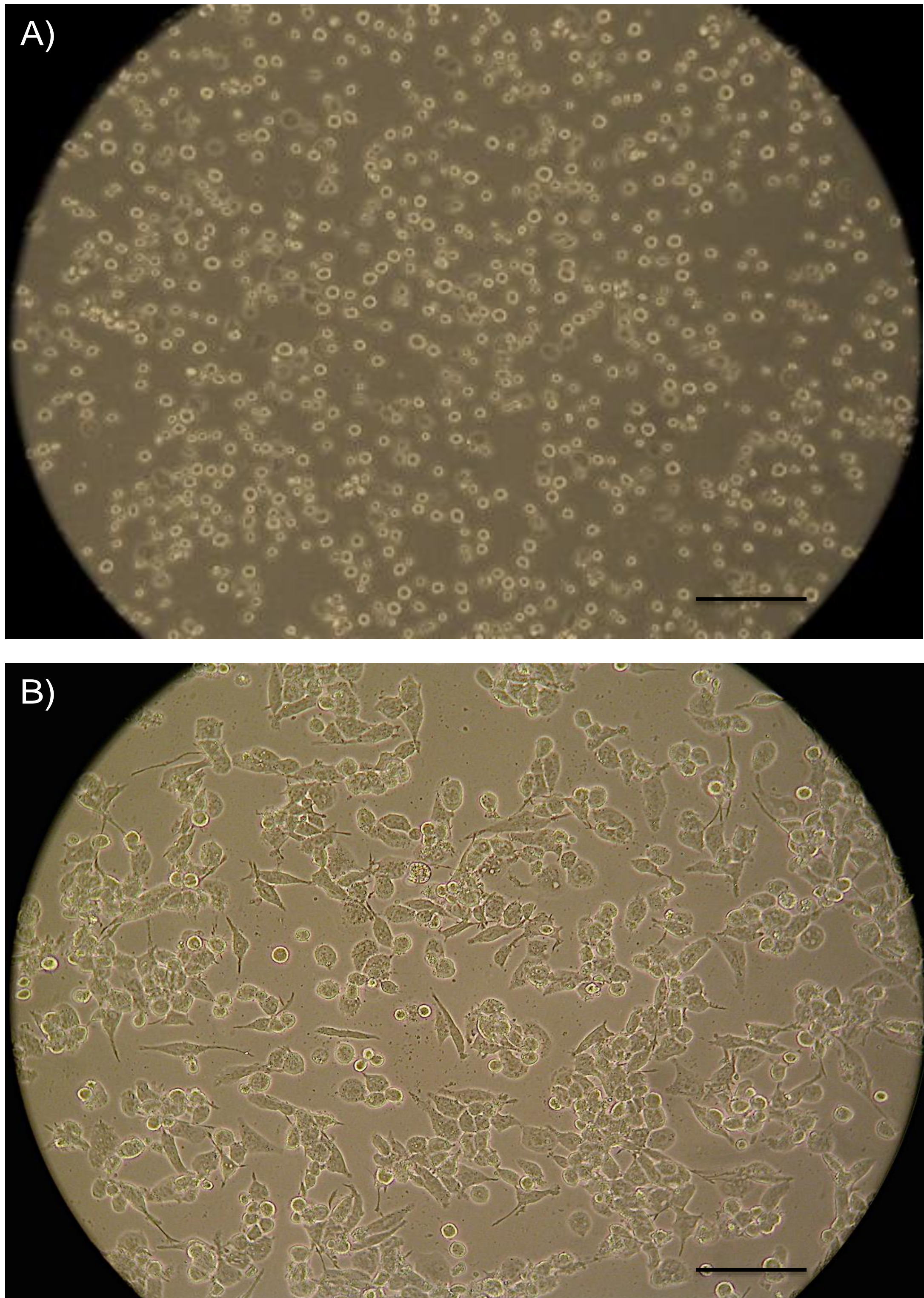

Figure 3.1: Morphology of THP-1 Monocytes and Macrophages

One million cells of untreated THP-1 monocytes were cultured in 6-well plate (A). The THP1 macrophages were treated with RPMI media plus 0.16 µM PMA for 24 hours (B). The black scale bar equal to 100 µm.

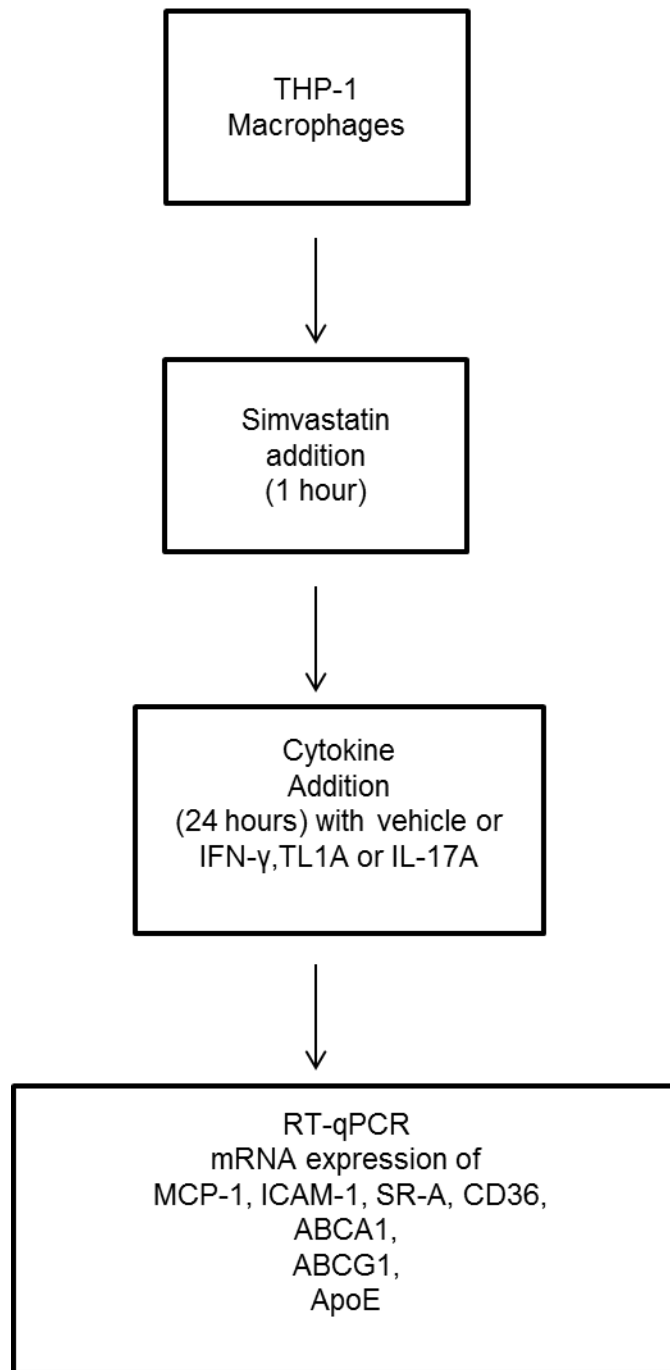

Figure 3.2: Overall experimental strategy.

3.3 Genes Selected for Study

3.3.1 MCP-1

MCP-1 (also known as CCL2) is the most characterised CC chemokine that tends to attract mononuclear cells and is found at sites of chronic inflammation (Ikeda et al., 2002). It has been well established that MCP-1 plays a crucial role in the inflammatory aspect of atherogenesis (Sheikine and Hansson, 2004; Zernecke and Weber, 2005). MCP-1 is produced by all atheroma-associated cells (ECs, SMCs, T cells and macrophages) (Boisvert, 2004). Increased expression and activity of MCP-1 and its receptor CCR2 have been identified in atherosclerotic plaques, and elevated plasma levels of MCP-1 have been found to be associated with traditional risk factors for atherosclerosis (Ohtsuki et al., 2001; Peters and Charo, 2001; Deo et al., 2004).

Numerous studies have focused on the role of chemokines and adhesion molecules on the progression of this disease because they are present in the initial stages of plaque formation (McLaren et al., 2011). The role of MCP-1 in the disease has been identified from studies using mouse model systems; for example, MCP-1 deficiency in murine models decreased the progression of atherosclerotic plaque development (Charo and Taubman, 2004). On the other hand, lesion formation and deposition of macrophages was increased when MCP-1 was overexpressed in the bone-marrow-derived cells of Apo-$E^{-/-}$ mice (Aiello et al., 1999).

3.3.2 ICAM-1

Adhesion molecules are another group of proteins that largely regulate the recruitment of monocytes and other immune cells into the arterial intima via transendothelial migration (Engelhardt and Wolburg, 2004). ICAM-1 is an important member of this family of proteins and plays crucial roles during the development of atherosclerosis (Blankenberg et al., 2003). The expression of ICAM-1 is detectable in macrophages, ECs and SMCs in human atherosclerotic plaques (Poston et al., 1992). The expression of ICAM-1 is low in these cells but markedly induced by inflammatory stimuli, such as IL-1, TNF-α and IFN-γ (Dustin and Springer, 1988; Yang et al., 2005). Deficiency of ICAM-1 in Apo-$E^{-/-}$ mice fed normal chow diet was found to protect against atherosclerosis by reducing lesion area (Collins et al., 2000).

3.3.3 SR-A

SR-A was first reported in 1990 and was originally named MSR (Moore and Freeman, 2006). This gene gives rise to three differentially spliced mRNAs that code for type I transmembrane receptors predominantly expressed on macrophages. When it became apparent that these receptors were part of a larger receptor family (Moore and Freeman, 2006), they were designated as class A SRs and renamed SR-AI, SR-AII and SR-AIII. SR-AI and SR-AII are expressed on the cell surface of tissue macrophages, including macrophage foam cells, and have also been found on aortic ECs and vascular SMCs within atherosclerotic plaques (Moore and Freeman, 2006). It was suggested that SR-AI and SR-AII contribute to the majority (≈80%) of macrophage uptake of AcLDL but have lower affinity for oxLDL (Suzuki et al., 1997; Kunjathoor et al., 2002).

There is growing support for the proposition that SR-A acts differently in early and advanced atherosclerotic lesions. In advanced atherosclerotic lesions, in which macrophage cell death results in necrotic core formation and plaque destabilisation, SR-A may have important roles in both the induction of apoptosis and the removal of these dying cells (Zhu et al., 2011). During hypercholesterolemia, macrophage

pathways for metabolising modified lipoproteins are believed to become overwhelmed, leading to a toxic accumulation of free cholesterol in the cell that results in endoplasmic reticulum stress. In this setting, engagement of SR-A pathways by modified lipoproteins triggers apoptotic cell death, indicating that SR-A signalling contributes to macrophage death and necrotic core formation (Moore and Freeman, 2006). In contrast, SR-A are predominantly involved in the uncontrolled uptake of oxLDL (and hence their clearance in early lesions).

Early studies of SR-AI and SR-AII null ($Msr^{-/-}$) mice performed in $Apo\text{-}E^{-/-}$ mice on a hybrid background (ICR/129) fed a chow diet showed a 58% decrease in aortic sinus atherosclerosis lesion area compared with $Apo\text{-}E^{-/-}$ littermates (Suzuki et al., 1997). SR-A knockout also showed a significant decrease in atherosclerotic lesions in the $Apo\text{-}E^{-/-}$ and $LDLR^{-/-}$ mouse models(Suzuki et al., 1997; Babaev et al., 2000).

3.3.4 CD36

CD36 is a member of class B scavenger receptors. It is an 88-kDa membrane glycoprotein that was first identified on monocytes by the use of monoclonal antibodies and then subsequently isolated from blood platelets. This membrane glycoprotein is expressed by many cell-types including ECs, SMC and monocytes/macrophages (Collot-Teixeira et al., 2007).

Functional and structural characterisation showed CD36 to be a member of the scavenger receptor class B family with a capacity to bind oxLDL as well as various other ligands (Collot-Teixeira et al., 2007). In addition to thrombospondin (TSP-1) and oxLDL, CD36 recognises a broad variety of ligands including anionic phospholipids, apoptotic cells, collagen, *Plasmodium falciparum*–infected erythrocytes and long-chain fatty acids (Nicholson, 2004).

Studies over the last three decades have led to a deep appreciation of the multi-functionality of CD36. For example, it has recently been shown to be a sensor for fatty acids in taste buds, eliciting a secretory response in the gut (Febbraio and Silverstein, 2007). CD36 is a mediator of both atherogenesis and thrombosis.

Febbraio et al. (2000) found that when they crossed CD36-null mice with Apo-E-null mice and fed them with a pro-atherogenic western diet, the mice demonstrated a dramatic decrease in the severity of atherosclerosis suggesting that CD36 has a proatherogenic role.

3.3.5 ABCA1

ABCA1 is a 2261 amino acid integral membrane protein that comprises two halves of similar structure. Each half has a transmembrane domain containing six helices and a nucleotide binding domain (NBD) containing 2 conserved peptide motifs known as Walker A and Walker B, which are present in many proteins that use ATP, and a Walker C signature unique to ABC transporters. ABCA1 is predicted to have an N terminus oriented into the cytosol and two large extracellular loops that are highly glycosylated and linked by one or more cysteine bonds (Oram and Heinecke, 2005).

ABCA1 mediates the transport of cholesterol, phospholipids and other lipophilic molecules across cellular membranes, where they are taken away from the cells by lipid-poor HDL apolipoproteins. It is likely that ABCA1 forms a channel in the membrane that promotes "flipping" of lipids from the inner to the outer membrane leaflet by an ATPase-dependent process. ABCA1 seems to also target specific membrane domains for lipid secretion. These are likely to be regions that are sensitive to accumulation of cholesterol (Oram and Vaughan, 2006).

Mutations in ABCA1 cause Tangier disease (TD). It is a rare autosomal recessive disorder of lipid metabolism characterised by almost complete absence of plasma HDL and the accumulation of cholesteryl esters in the cells of the reticuloendothelial system leading to splenomegaly and enlargement of tonsils and lymph nodes (Kaminski et al., 2006). Drugs that induce ABCA1 expression in mice increase the clearance of cholesterol from tissues and inhibit intestinal absorption of dietary cholesterol. ABCA1 has a major impact on cellular and whole body cholesterol metabolism and is likely to play an important role in protecting against cardiovascular disease. ABCA1 is highly expressed in macrophages where it plays an obvious role in cholesterol secretion.

3.3.6 ABCG1

The ABCG or White subfamily with its five fully characterised human members consists of half-size ABC proteins that dimerise to form active membrane transporters. Among the half-size molecules ABCG proteins have a peculiar domain organisation characterised by a nucleotide-binding domain (ATP-binding cassette) at the N terminus followed by six transmembrane-spanning domains (Schmitz et al., 2001).

There are five ABCG subfamily members in humans: ABCG1; ABCG2; ABCG4; ABCG5; and ABCG8. The founding member of this group, ABCG1, is highly expressed in the lung, brain, spleen and in macrophages (Matsuo, 2010). Over expression of ABCG1 in baby hamster kidney cells was found to increase cell membrane cholesterol pools available for efflux and increase the rate constant for efflux (Sankaranarayanan et al., 2009). The efflux appears to be diffusional and unidirectional and is more efficient for smaller HDL particles. Different models have been proposed to explain how ABCG1 promotes cholesterol efflux to HDL. One suggests that ABCG1 helps sterol molecules to overcome the energy barrier for entry into the hydrophilic water layer, perhaps by using ATP to promote protrusion of the cholesterol molecule into water, followed by a transient collision with an acceptor (Charvet et al., 2010). ABCG1 promotes cholesterol efflux to HDL and other lipoprotein particles but not to lipid-poor apoA-1 (Charvet et al., 2007).

Wang et al found that RCT was significantly decreased in ABCG1^{-}/$^{-}$ mice (Wang et al., 2007). Yvan-Charvet and his collegues showed that knockout of both of ABCA1 and ABCG1 reduced cholesterol efflux to HDL and ApoA-1 when ABCA1^{-}/$^{-}$ ABCG1^{-}/$^{-}$ bone marrow (BM) was transplanted into LDLR^{-}/$^{+}$ mice and then fed a high cholesterol diet (Charvet et al., 2007).

3.3.7 ApoE

ApoE was first described by Shore and Shore (Shore and Shore, 1973). It has since been found to be a major component of various types of plasma lipoproteins and has been implicated in the maintenance of overall plasma cholesterol homeostasis by facilitating the hepatic uptake of lipoproteins by binding to their

receptors (Greenow et al., 2005). It is synthesised by the liver, brain, spleen, lung, ovary, adrenal gland, kidney and muscles (Elshourbagy et al., 1985). In general, most of the ApoE found in the plasma is synthesised by the liver with about 20-40% derived from sources other than the liver, such as macrophages (Newman et al., 1985).

Deficiency of ApoE leads to the development of atherosclerosis and hypercholesterolemia explaining why ApoE deficient mice are often used as a model for the disease. There is accumulating evidence that suggests that ApoE plays a potent anti-atherogenic role (Greenow et al., 2005). The major anti-atherogenic property of ApoE is the stimulation of cholesterol efflux from foam cells. It has been demonstrated that ApoE can act as an acceptor of both cholesterol and phospholipids released from macrophages via ABCA1 and is able to interact with ABCA1, at least *in vitro* (Greenow et al., 2005).

Several studies have shown that macrophage-derived ApoE exerts anti-atherogenic properties largely independent of its effects on plasma lipid levels (Fazio et al., 1997; Van Eck et al., 2000). For example work by Zhang et al. (1994) demonstrated that atherosclerosis was more severe in cholesterol-fed ApoE–heterozygous (+/-) mice than in cholesterol-fed Apo-E (+/+) mice, despite the relative similar plasma cholesterol levels in the two groups. It has also been shown that the lack of macrophage ApoE in C57BL/6 mice leads to a 10-fold increase in diet-induced atherosclerosis in the absence of any changes in serum cholesterol levels or lipoprotein profiles (Fazio et al., 1997; Boisvert and Curtiss, 1999). Moreover, transgenic mice expressing human ApoE in the vessel wall show reduced atherosclerotic lesions in the absence of any changes in plasma cholesterol and lipoprotein profile (Shimano et al., 1995).

3.4 Results

3.4.1 Optimisation of PCR Conditions

Before carrying out the PCR optimisation studies, the integrity of macrophage RNA samples was examined by electrophoresis on 1.5% (w/v) agarose gels. As shown in Figure 3.3, the comparative amount of the 28S rRNA was almost double that of the 18S rRNA, which is what would be expected for good quality, undegraded RNA. Such quality RNA was obtained in all experiments and hence the data in these cases are not shown

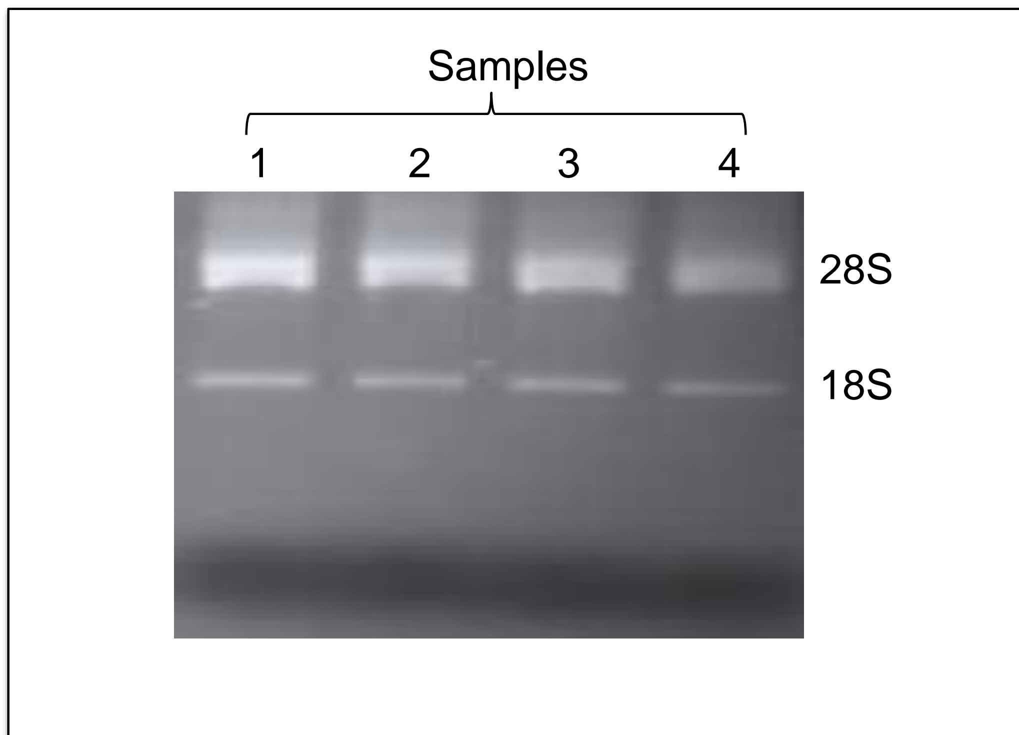

Figure 3.3: Determination of the integrity of RNA isolated from THP-1 macrophages

Four different RNA samples from THP-1 macrophages were prepared and subjected to electrophoresis on 1.5% agarose gels to check integrity of RNA. 28S, 28S rRNA; 18S, 18S rRNA.

Primer sequences for ABCA1, ABCG1, SRA and CD36 were taken from previously published papers and ordered from Genosys (Sigma-Aldrich). The amount of reagents required for each reaction and the optimal PCR annealing temperatures were well established in our laboratory. It was found that the optimum annealing temperature was: 60°C for GAPDH and SR-A; 65°C for ABCA1 and ABCG1; and 58 °C for CD36. Previous studies had shown that 35 cycles also produced optimal amount of PCR products. In all cases, no products of expected size were obtained when the reverse transcriptase step was omitted, thereby showing that the amplication products were from cDNA, and hence RNA, but not because of contamination of the samples with genomic DNA.

The optimised conditions for the different human primer sets were then used to carry out RT-qPCR. The ΔΔCT method was used for the analysis.(Ginzinger, 2002). The data for GAPDH, MCP-1 and ICAM-1 are shown in Figure 3.4. Panels A-C show change in fluorescence with cycle numbers with the dashed line showing the threshold with cycle number reaching this point taken into account in the data analysis. Panels D-F show melting curve that was generated by increasing the temperature in small increments and monitoring the fluorescent signal at each step. A plot of the rate of change in fluorescence vs. temperature had a distinct major peak that corresponded to the melting temperature of each specific product

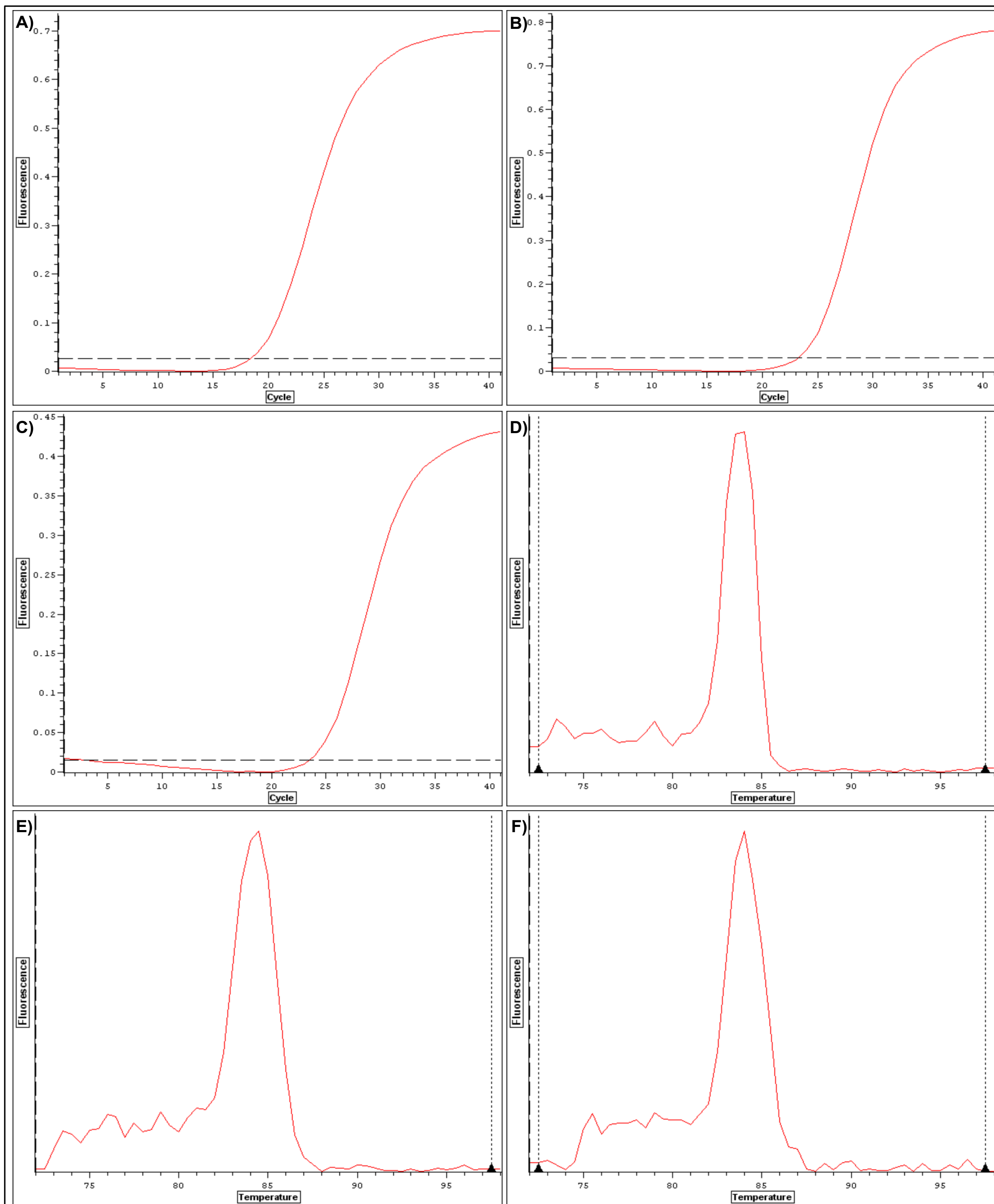

Figure 3.4: RT-qPCR analysis using primers against MCP-1, ICAM-1 and GAPDH

Panels A-C illustrates changes in fluorescence with cycle number for the cycles for the different human primer sets GAPDH (housekeeping) (A), MCP-1 (B) and ICAM-1 (C) using cDNA against RNA from THP-1 macrophages Panels D-F show melting curve for the same set of primers: GAPDH (D) MCP-1 (E) and ICAM-1 (F).

3.4.2 Treatment of THP-1 Cells with Simvastatin

In order to validate the effectiveness of Simvastatin, RT-qPCR was performed on the MCP-1 gene using RNA from cells treated with IFN-γ as a positive control. Previous studies have shown that Simvastatin inhibits the IFN-γ-induced MCP-1 mRNA expression in THP-1 macrophages (Li et al., 2011). After 24 hours stimulation with PMA, activated Simvastatin (10 μM) or DMSO (as vehicle control) was added to the cells for one hour before stimulation with the cytokine. The concentration of simvastatin was based on previous work (Li et al., 2001; Yamashita et al., 2008). As expected, IFN-γ induced the expression of MCP-1 and this was attenuated in the presence of Simvastatin (Figure 3.5). On the other hand, Simvastatin had no effect on the constitutive expression of MCP-1 in these cells (Figure 3.5).

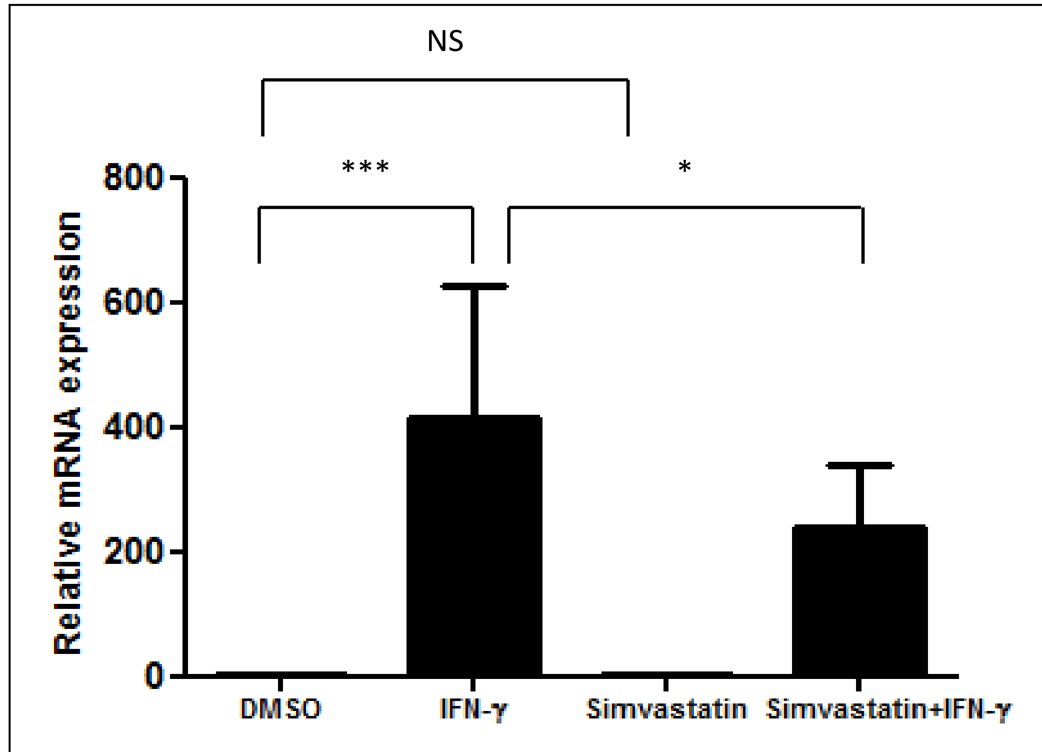

Figure 3.5: The Effect of simvastatin on the IFN-γ-Induced MCP-1 Expression in THP-1 Macrophages.

PMA-differentiated THP-1 macrophages were pre-treated for 1 hour with Simvastatin (10 μM) or DMSO as a vehicle control. The cells were then incubated for 24 hours with or without IFN-γ (1000 U/ml). Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis,*P<0.05, ***P<0.001, NS not significant. The data shown are from five independent experiments.

The RT-qPCR experiments were repeated with the ICAM-1 gene to provide an additional robust marker of inflammation for subsequent studies. The data showed that IFN-γ produced a statistically significant increase in ICAM-1 expression. Simvastatin by itself had no effect on constitutive ICAM-1 expression but produced a statistically significant reduction in the IFN-γ-induced ICAM-1 expression (Figure 3.6).

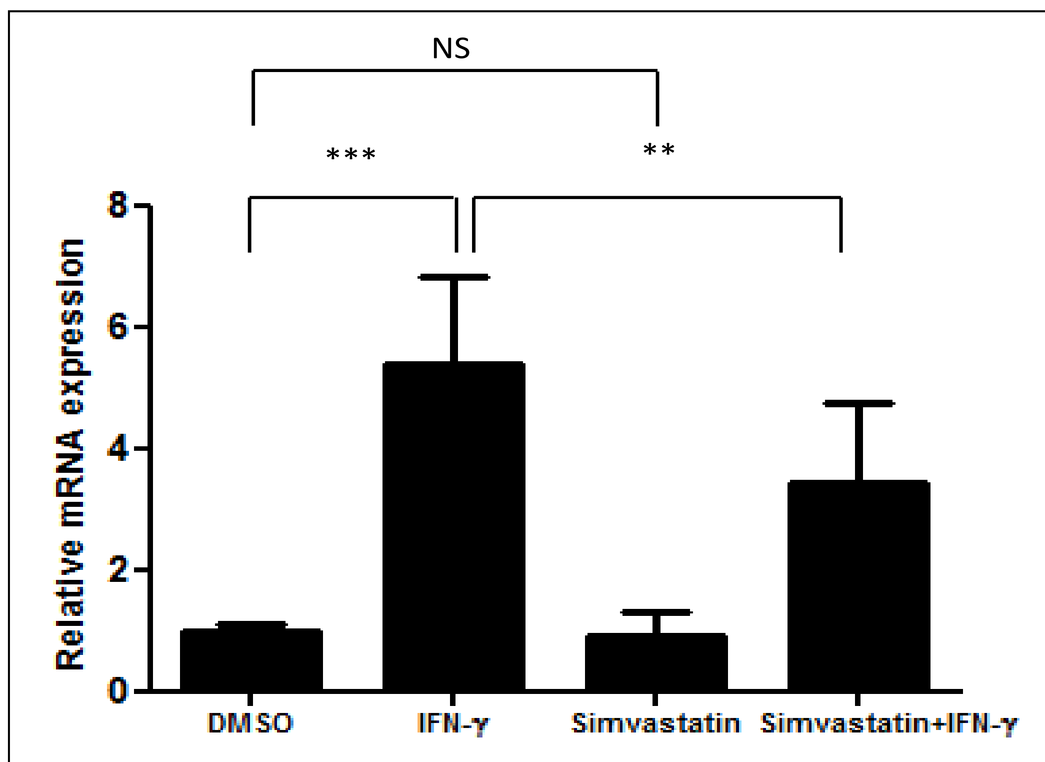

Figure 3.6: The Effect of simvastatin on the IFN-γ-induced ICAM-1 expression in THP-1 macrophages.

PMA-differentiated THP-1 macrophages were pre-treated for 1 hour with Simvastatin (10 µM) or DMSO as a vehicle control. The cells were then incubated for 24 hours with or without IFN-γ (1000 U/ml). The expression in DMSO control samples has been arbitrarily assigned as 1. The results are displayed as fold change in relation to the control from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, ** $P<0.01$, ***$P<0.001$; NS not significant.

3.4.3 Effect of Simvastatin on cytokine regulated expression of SR-A, CD36, ABCA1 and ABCG1

Having confirmed the positive action of Simvastatin, their effects on cytokine-mediated changes in the expression of two lipoprotein/cholesterol uptake genes (SR-A and CD36) and two cholesterol efflux genes (ABCA1 and ABCG1) were determined. The analysis was extended to two more recently identified cytokines, TL-1A and IL-17A. Thus, after 24 hours stimulation with PMA, activated Simvastatin or DMSO (as vehicle control) was added to the cells for one hour before stimulation with the cytokines.

3.4.3.1 SR-A

IFN-γ and TL-1A produced a statistically significant increase in SR-A expression (Figure 3.7). Although an increase in SR-A was also observed with IL-17A, this was not statistically significant (Figure 3.7). In all cases, Simvastatin had no effect on the constitutive expression of SR-A (Figure 3.7). In addition, simvastatin had no statistically significant effect on SR-A expression in the presence of IFN-γ and IL-17A though a trend of reduced expression was observed with IL-17A (Figure 3.7). In contrast, Simvastatin significantly attenuated the TL1A-induced expression of SR-A (Figure 3.7).

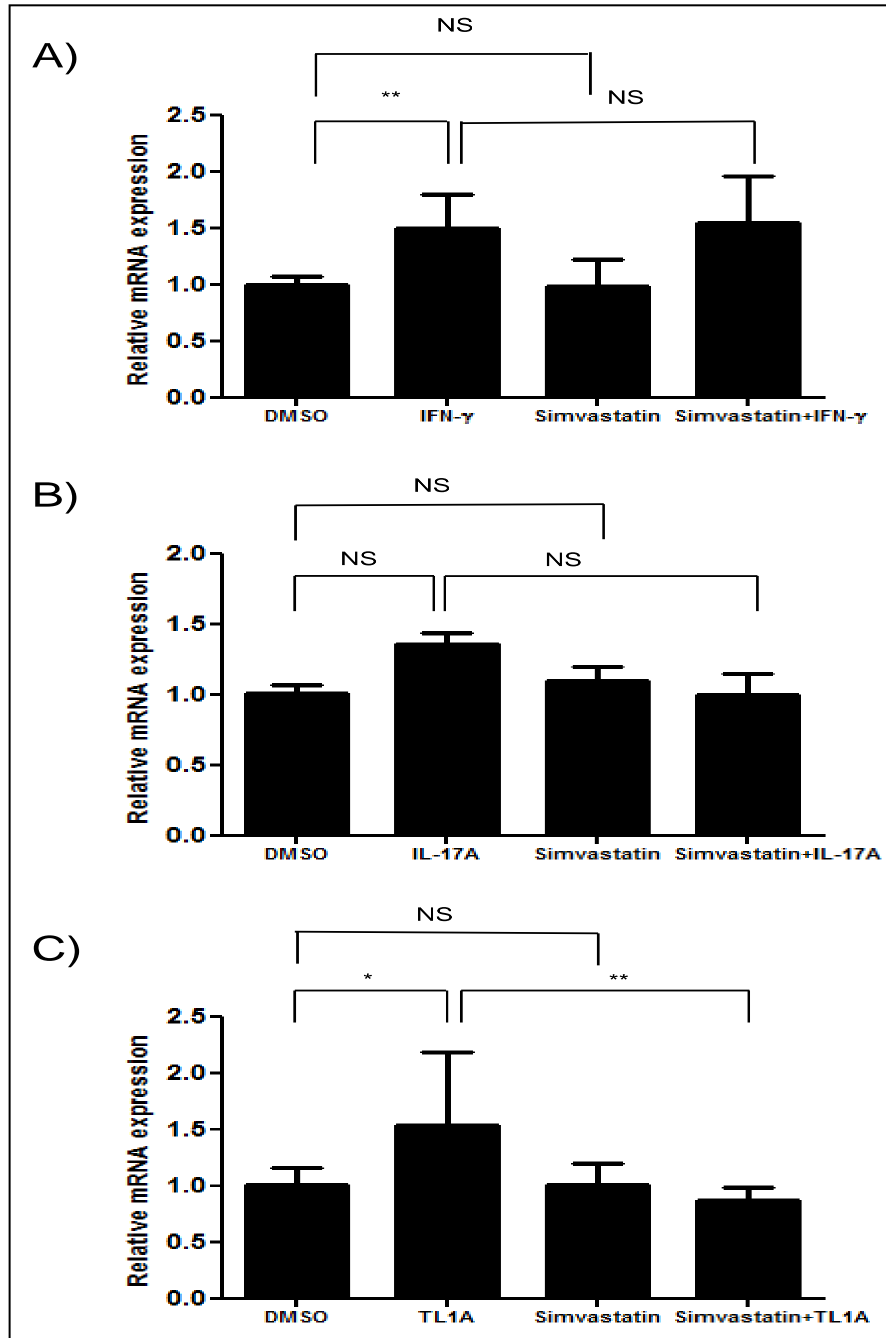

Figure 3.7: The effect of Simvastatin on cytokine-regulated expression of SR-A mRNA by RT-qPCR.

PMA-differentiated THP-1 macrophages were pre-treated for 1 hour with Simvastatin (10 µM) or DMSO as a vehicle control. The cells were then incubated for 24 hour in the absence or the presence of IFN-γ (1000 U/ml) (A), IL-17A (100 ng/ml) (B) or TL1A (100 ng/ml) (C). The results are displayed as fold change in relation to the control from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis * $p<0.05$, ** $p<0.01$, NS not significant.

3.4.3.2 CD36

All three cytokines induced the expression of CD36 though this was only statistically significant in the case of IFN-γ and TL1A (Figure 3.8). In all cases, Simvastatin had no effect on the constitutive expression of CD36 (Figure 3.8). Inclusion of Simvastatin produced no statistically significant reduction of the expression of all three genes seen in the presence of IFN-γ though a trend of slight reduction was observed with IFN-γ and IL-17A (Figure 3.8).

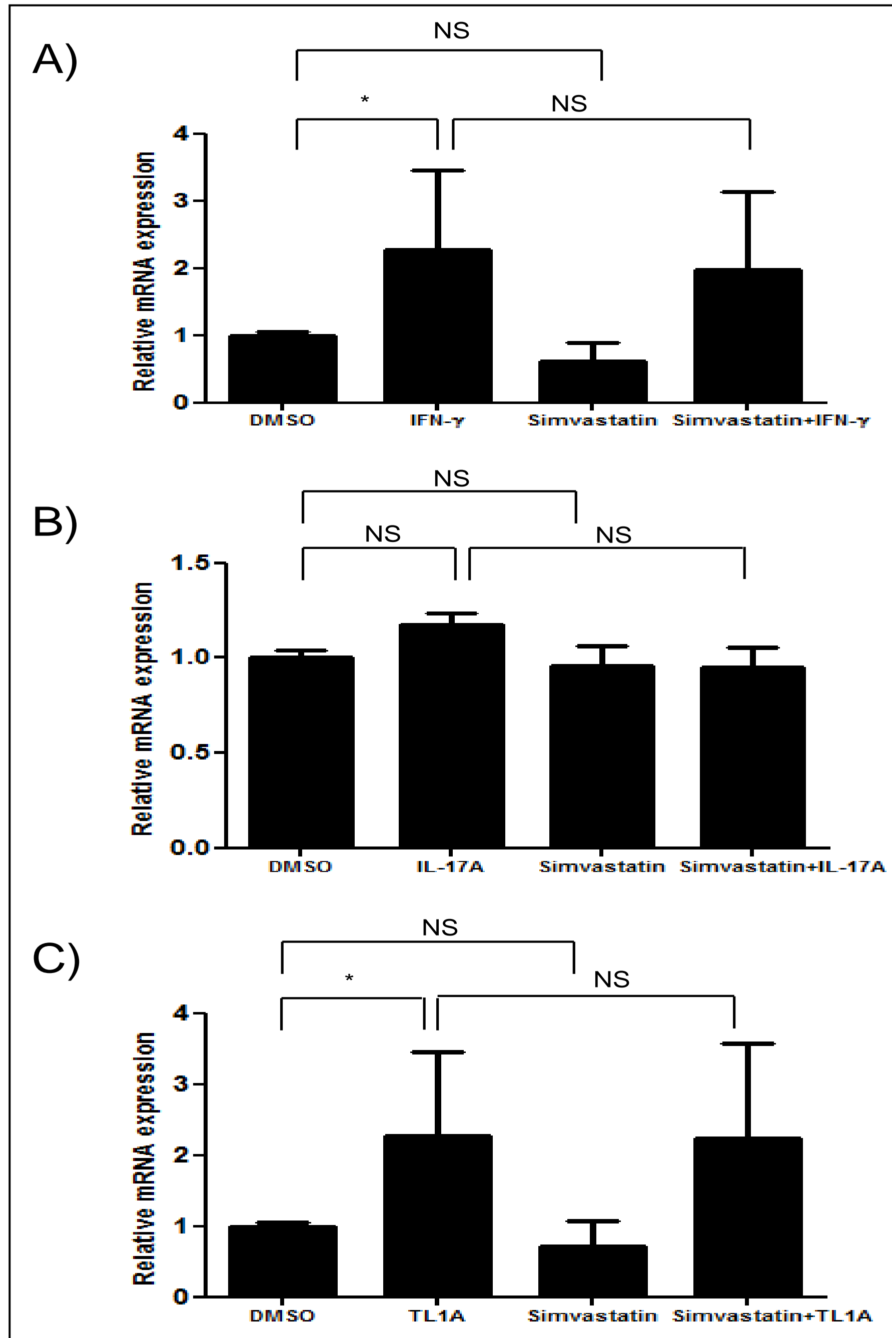

Figure 3.8: The effect of Simvastatin on cytokine-regulated expression of CD36 mRNA by RT-qPCR.

PMA-differentiated THP-1 macrophages were pre-treated for 1 hour with Simvastatin (10 μM) or DMSO as a vehicle control. The cells were then incubated for 24 hours in the absence or the presence of IFN-γ (1000 U/ml) (A), IL-17A (100 ng/ml) (B) or TL1A (100 ng/ml) (C). The results are displayed as fold change in relation to the control from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis * P<0.05 and NS not significant.

3.4.3.3 ABCA1

Simvastatin produced a statistically significant reduction in the constitutive expression of ABCA1 mRNA (induced by PMA as part of the differentiation program) (Figure 3.9). TL-1A also produced a statistically significant reduction in ABCA1 mRNA expression whereas IFN-γ and IL-17A had no effect (Figure 3.9). Consistent with the effect of simvastatin on constitutive expression, the levels of ABCA1 mRNA in the presence of simvastatin and either cytokine were significantly lower than that in the presence of the corresponding cytokine alone (Figure 3.9).

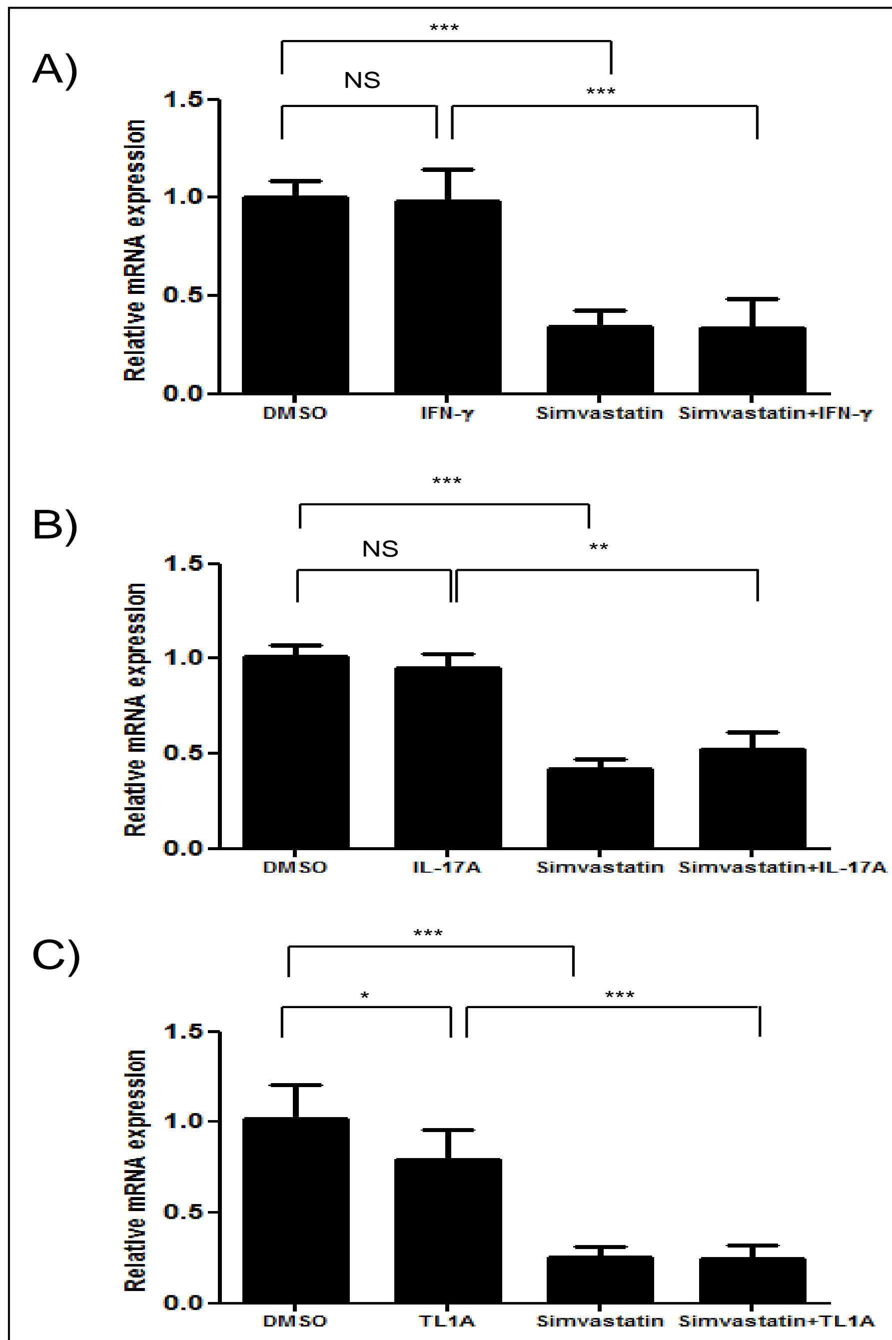

Figure 3.9: The Effect of Simvastatin on cytokine-regulated expression of ABCA-1 mRNA by RT-qPCR.

PMA-differentiated THP-1 macrophages were pre-treated for 1 hour with Simvastatin (10 μM) or DMSO as a vehicle control. The cells were then incubated for 24 hours in the absence or the presence of IFN-γ (1000 U/ml) (A), IL-17A (100 ng/ml) (B) or TL1A (100 ng/ml) (C). The results are displayed as fold change in relation to the control from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, *P<0.05, **P<0.01, ***P<0.001 and NS not significant.

3.4.3.4 ABCG1

Similar to ABCA1, Simvastatin produced a reduction in the constitutive expression of ABCG1 mRNA (induced by PMA as part of the differentiation program) (Figure 3.10). Although this was statistically significant in experimental series involving IL-17A and TL1A, there was, nevertheless, a trend in reduced expression in experiments involving IFN-γ though this failed to reach significance (Figure 3.10). TL-1A also produced a statistically significant reduction in ABCA1 mRNA expression (Figure 3.10). On the other hand, IFN-γ produced a statistically significant increase in ABCG1 expression whereas IL-17A had no effect (Figure 3.10). Consistent with the effect of Simvastatin on constitutive expression, the levels of ABCG1 mRNA in the presence of Simvastatin and either cytokine were significantly lower than those in the presence of the corresponding cytokine alone (Figure 3.10).

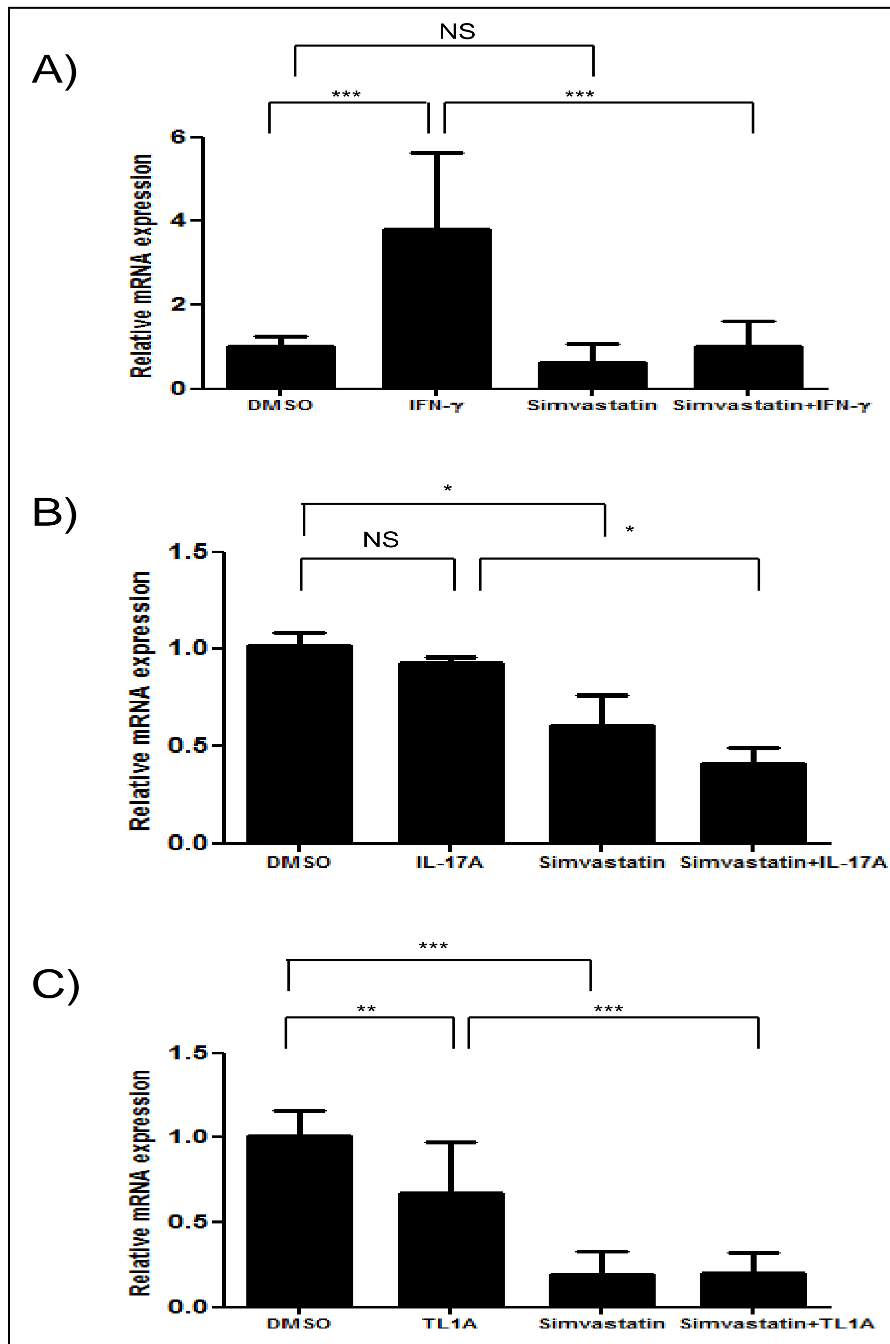

Figure 3.10: The effect of simvastatin on cytokine-regulated expression of ABCG-1 mRNA by RT-qPCR.

PMA-differentiated THP-1 macrophages were pre-treated for 1 hour with Simvastatin (10 µM) or DMSO as a vehicle control. The cells were then incubated for 24 hours in the absence or the presence of IFN-γ (1000 U/ml) (A), IL-17A (100 ng/ml) (B) or TL1A

(100 ng/ml) (C). The results are displayed as fold change in relation to the control from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, * $P<0.05$, **$P<0.01$, ***$P<0.001$ and NS not significant.

3.4.4 Effect of TL1A on ApoE Expression in THP-1 Cells

The experiments on ApoE expression were restricted to TL1A. This cytokine produced a statistically significant reduction of ApoE mRNA expression (Figure 3.11). In addition, Simvastatin produced a statistically significant decrease in constitutive ApoE mRNA expression (Figure 3.11). However, there was no additional significant effect of Simvastatin on ApoE mRNA levels observed in the presence of TL1A alone (Figure 3.11).

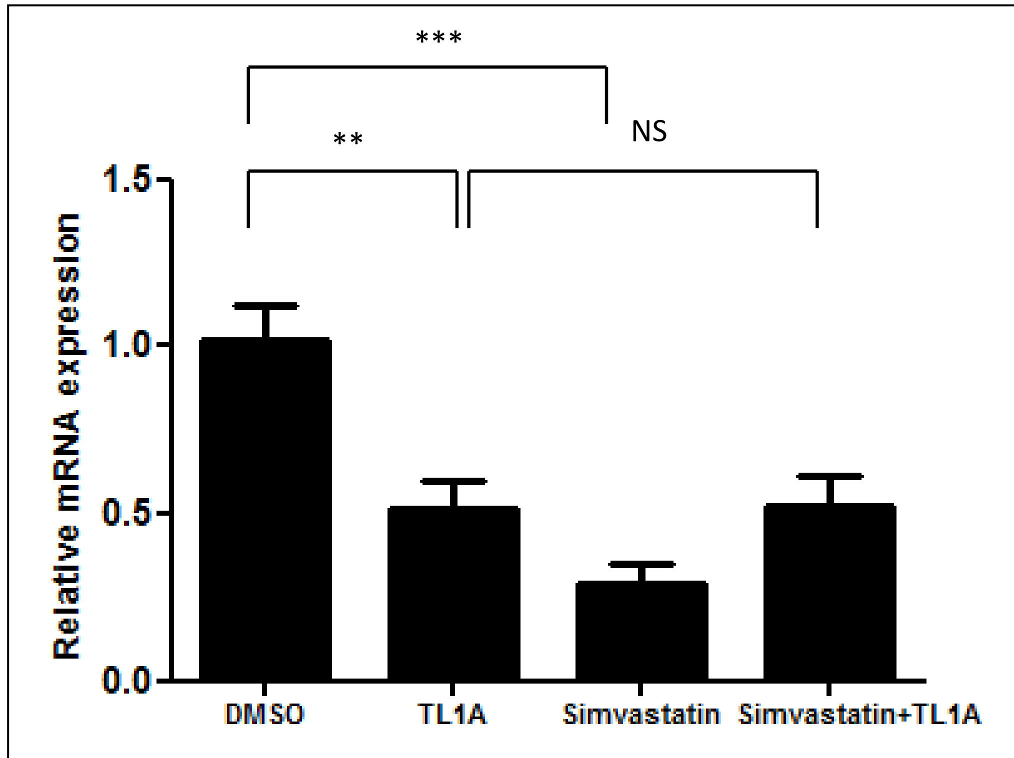

Figure 3.11: The effect of Simvastatin on TL1A-regulated expression of ApoE mRNA by RT-qPCR.

PMA-differentiated THP-1 macrophages were pre-treated for 1 hour with Simvastatin (10 μM) or DMSO as a vehicle control. The cells were then incubated for 24 hours in the absence or the presence of TL1A (100 ng/ml). The results are displayed as fold change in relation to the control from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, ** p<0.01, ***p<0.001 and NS not significant.

3.4.5 Atorvastatin attenuates the IFN-γ induced expression of MCP-1 and ICAM-1

3.4.5.1 MCP-1

To examine whether the results obtained so far were not peculiar to Simvastatin, Atorvastatin was used in further experiments. The studies were restricted to IFN-γ. After 24-hour stimulation with PMA, activated atorvastatin (10 µM) or DMSO (as vehicle control) was added to the cells for one hour before stimulation with IFN-γ. The concentration of Atorvastatin was based on previous research (Li et al., 2001).

The data shows that IFN-γ induced the expression of the MCP-1 gene in a statistically significant manner when compared to the DMSO control with about 130-fold induction (Figure 3.12). Atorvastatin by itself had no effect on constitutive MCP-1 expression but produced a statistically significant reduction of about 50% in MCP-1 mRNA expression levels seen in the presence of IFN-γ.

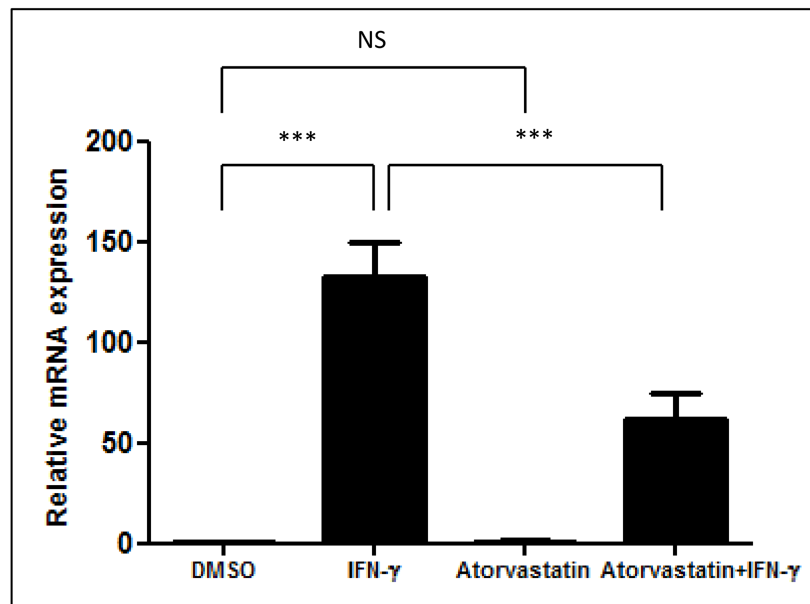

Figure 3.12: The effect of Atorvastatin on the IFN-γ-induced MCP1 expression in THP-1 macrophages.

PMA-differentiated THP1 macrophages were pre-treated for 1 hour with Atorvastatin (10 μM) or DMSO as a vehicle control. The cells were then incubated for 24 hours with or without IFN-γ (1000 U/ml). The results are from five independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, *** $p<0.001$, NS not significant.

3.4.5.2 ICAM-1

There was a significant induction of ICAM-1 expression by IFN-γ, and this was reduced in a statistically significant manner in the presence of Atorvastatin (Figure 3.13). In addition, Atorvastatin had no effect on constitutive ICAM-1 expression.

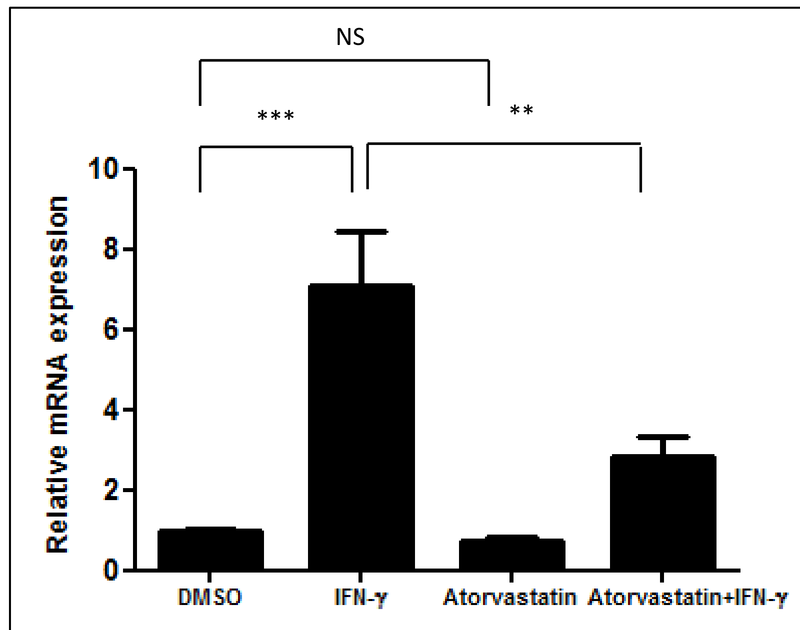

Figure 3.13: The effect of Atorvastatin on the IFN-γ-induced ICAM-1 expression in THP-1 macrophages.

PMA-differentiated THP1 macrophages were pre-treated for 1 hour with Atorvastatin (10 μM) or DMSO as a vehicle control. The cells were then incubated for 24 hours with or without IFN-γ (1000 U/ml). The results are from five independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, ** $p<0.01$, *** $p<0.001$, NS not significant

3.5 Discussion

In the context of disease or pathophysiology of atherosclerosis, cytokines are often grouped according to their pro- or anti–inflammatory actions. High levels of pro-inflammatory cytokines are observed in atherosclerotic lesions (Harvey and Ramji, 2005). Pro-inflammatory cytokines include TNF-α, IFN-γ, IL-1, IL-12, TL1A and IL-17. On the other hand, anti-inflammatory cytokines include IL-10, IL-4, TGF-β and IL-33. Macrophages and T-lymphocytes are the main source of cytokines in the atherosclerotic lesion (Dinarello, 2000; Tedgui and Mallat, 2006; McLaren et al., 2010a).

The studies presented in this chapter investigated the action of Simvastatin on cytokine-mediated changes in the expression of key genes implicated in the control of macrophage cholesterol homeostasis and the inflammatory response. These studies were divided in to five parts. The first part was to validate the effectiveness and action of Simvastatin on a well-characterised response in macrophages, the induction of MCP-1 gene expression by IFN-γ. The second part was focussed on investigating the action of Simvastatin on IFN-γ-induced expression of the ICAM-1 gene to provide another marker in addition to MCP-1. The third part focussed on investigating the action of Simvastatin on the expression of key genes implicated in the regulation of macrophage cholesterol uptake and efflux in the absence or the presence of the cytokines IFN-γ, TL-1A or IL-17A. The efflux of cholesterol from macrophages primarily involves reverse cholesterol transport by members of the ABC transporter family, such as ABCA1 and ABCG1, and also requires lipid-free components of high-density lipoprotein, such as ApoE and ApoA-I as cholesterol acceptors. It was therefore decided to investigate whether TL1A and IL-17 modulates the expression of these genes by RT-qPCR and if this was regulated by Simvastatin, and that was the fourth part of the study. The fifth part was to examine the effect of Atorvastatin on the IFN-γ induced expression of MCP-1 and ICAM-1.

THP-1 cells were used as an *in vitro* cell culture model to study the expression of SR-A, CD36, ABCA1, ABCG1 and ApoE genes.

3.5.1 The effect of Simvastatin on the IFN-γ induced mRNA expression of MCP-1 and ICAM-1

Consistent with previous studies in a range of cell types (Zhou et al, 2001; Martin-Ventura, 2009; Chang et al, 2002), IFN-γ induced the expression of MCP-1 and ICAM-1 genes (Figures 3.5 and 3.6). Simvastatin has been shown to inhibit the IFN-γ-induced gene expression of MCP-1 and ICAM-1 in primary human ECs and transformed endothelial ECV304 cell lines (Kwak et al., 2001; Chung et al, 2002). Consistent with these previous findings, work presented in this chapter clearly shows that pre-treatment of THP-1 macrophages with Simvastatin inhibits the IFN-γ-induced expression of MCP-1 and ICAM-1, thereby suggesting an anti-inflammatory and immunomodulatory effect of this statin in human macrophages (Figures 3.5 and 3.6).

3.5.2 The effect of Simvastatin on cytokine regulated gene expression of SR-A, CD36, ABCA1 and ABCG1:

IFN-γ plays a complex role in atherosclerosis with both pro- and anti-atherogenic actions being reported though most of the *in vivo* studies point to a pro-atherogenic role (McLaren and Ramji., 2009; Harvey and Ramji, 2005). It was originally shown that IFN-γ inhibits SR-A and CD36 expression in HMDMs and reduces the level of AcLDL uptake (Geng and Hansson, 1992). This indicated a role of this cytokine in reducing foam cell formation in lesions. However, numerous subsequent studies have shown that IFN-γ promotes foam cell formation by stimulating the uptake of modified lipoproteins (McLaren and Ramji, 2009; Li et al., 2010). Consistent with this finding, the studies presented in this chapter show that IFN-γ induces the expression of SR-A and CD36 in THP-1 macrophages (Figures 3.7 and 3.8), IFN-γ has been shown to decrease cholesterol efflux from mouse peritoneal macrophages by inhibiting ABCA1 expression (Panousis and Zuckerman, 2000; Wang et al., 2002). However, this cytokine was found to produce no significant change in ABCA1 expression in THP-1

macrophages and increased the expression of ABCG1 expression (Figures 3.9 and 3.10).

As previously described, statins are potent inhibitors of cholesterol biosynthesis. In clinical trials, they have been found to be beneficial in the primary and secondary prevention of CHD (Liao and Laufs, 2005). Statins are potent inhibitors of HMG-COA reductase, a key enzyme in cholesterol biosynthesis (Ridker et al., 2009). In addition, statins have additional anti-atherogenic actions, the so called pleiotropic actions. For example, statins have been shown to prevent the activation of monocytes and to inhibit the production of pro-inflammatory cytokines (Kwang, 2000). Furthermore, animal studies and clinical trials have demonstrated that statins can raise HDL levels (Natarajan et al., 2010). Statins have also been found to directly attenuate macrophage foam cell formation by regulating macrophage scavenger receptor expression, oxLDL uptake and cholesterol efflux. For example, Atrovastatin has been found to reduce macrophage SR-A and CD36 expression (Hofnagel et al., 2007; Fuhrman et al., 2002; Han et al., 2004).

The IFN-γ-induced expression of SR-A was not affected by Simvastatin (Figure 3.7) while the induction of CD36 was attenuated though this was not statically significant so more experimental repeats will be required (Figure 3.8). The induction of ABCG1 by IFN-γ was decreased by Simvastatin and this was statically significant (Figure 3.10). Although, IFN-γ had no effect on ABCA1 expression, the constitutive levels were attenuated in the presence of simvastatin (Figure 3.9). These findings are consistent with the reduction of expression of ABCA1 and ABCG1 in Caco-2 cells by simvastatin (Genvigir et al., 2011).

TL1A is a pro-inflammatory cytokine that has been shown to drive the progression of numerous inflammatory disorders, including arthritis (Meylan et al., 2008; Croft, 2009). The cytokine has been shown to drive macrophage foam cell formation by both promoting cholesterol uptake and reducing cholesterol efflux (McLaren et al., 2010a). Consistent with this finding, TL1A was found to induce the expression of SR-A and CD36 and to decrease the expression of ABCA1 and ABCG1

in THP-1 macrophages (Figures 3.7, 3.8, 3.9 and 3.10). The TL1A induced expression of SR-A was attenuated by Simvastatin but no change was seen with CD36 (Figures 3.7 and 3.8). These actions are consistent with the anti-atherogenic, foam cell inhibitory action of Simvastatin. On the other hand, the TL1A reduction of ABCA1 and ABCG1 was further reduced with simvastatin (Figures 3.9 and 3.10).

It has previously been shown in our laboratory that IL-17 promotes macrophage foam cell formation by increasing cholesterol uptake and decreasing cholesterol efflux (unpublished results by Daryn Michael and James McLaren). Consistent with this finding, IL-17 was found to induce the expression of SR-A and CD36 and reduce that of ABCA1 and ABCG1 in THP-1 macrophages (Figures 3.7, 3.8, 3.9 and 3.10). The IL-17A-mediated induction of SR-A and CD36 was reversed by Simvastatin but it was not significant so more experiments will be required (Figures 3.7 and 3.8). On the other hand, the IL-17A mediated reduction of ABCA1 and ABCG1 was reduced further with Simvastatin (Figures 3.9 and 3.10).

3.5.3 Effect of TL1A on ApoE mRNA expression

It was also decided to investigate the role of TL1A on the expression of ApoE and the effect of Simvastatin on this. This study demonstrated that TL1A decreases the expression of ApoE, which confirms the results of previous study by McLaren et al. (2010a) where it was shown that TL1A significantly decreases the expression of ApoE in THP-1 macrophages and HMDMs. However, when THP-1 cells were treated with Simvastatin there was no change observed in the expression of APO-E. Because the changes were not significant, it can be concluded that Simvastatin has no effect on the TL1A-mediated decrease in ApoE expression (Figure 3.11).

3.5.4 Effect of Atorvastatin on IFN-γ induced mRNA expression

In order to examine whether the results obtained are peculiar to Simvastatin, representative experiments were carried out using Atorvastatin. The data showed that Atorvastatin attenuates the IFN-γ induced expression of MCP-1 and ICAM-1 (Figures 3.12 and 3.13). Although, there is no study that has examined the effect of Atorvastatin on the inhibition of the IFN-γ induced expression of MCP-1 and ICAM-1, this statin was found to reduce the expression of MCP-1 in swine (Martinez-Gonzalez et al., 2001). This indicates that MCP-1 down regulation by statins could reduce the inflammation within the vascular wall and play a role in the inhibition of lesion progression.

The results of this chapter are summarised in Table 3.1.

The next chapter concentrates on IFN-γ signalling, and effects of Simvastatin on these pathways. IFN-γ was chosen for the studies because it is potentially a master regulator of atherosclerosis and produces a robust increase in inflammatory marker gene expression in human macrophages.

Table 3.1: Summary of the results

Treatment	Gene expression
1- IFN-γ	↑ MCP-1, ICAM-1
Simvastatin + IFN-γ	↓ MCP-1, ICAM-1
2- IFN-γ	↑ SR-A
Simvastatin + IFN-γ	No change.
IL-17A	No change.
Simvastatin + IL-17A	No change.
TL1A	↑ SR-A.
Simvastatin + TL1A	↓ SR-A.
3- IFN-γ	↑ CD36
Simvastatin + IFN-γ	No change.
IL-17A	No change.
Simvastatin + IL-17A	No change.
TL1A	↑ CD36
Simvastatin + TL1A	No change.
4- IFN-γ	No change with ABCA1
Simvastatin + IFN-γ	↓ ABCA1.
IL-17A	No change.
Simvastatin + IL-17A	↓ ABCA1.
TL1A	↓ ABCA1.
Simvastatin + TL1A	↓ ABCA1.
5- IFN-γ	↑ ABCG1.
Simvastatin + IFN-γ	↓ ABCG1.
IL-17A	No change.
Simvastatin + IL-17A	↓ ABCG1.
TL1A	↓ ABCG1.
Simvastatin + TL1A	↓ ABCG1.
6- TL1A	↓ APOE.
Simvastatin + TL1A	No change.
7- IFN-γ	↑ MCP-1, ICAM-1.
Atorvastatin + IFN-γ	↓ MCP-1, ICAM-1.

↑: Increase Expression and ↓: Decrease Expression.

Chapter 4: Effects of Simvastatin on IFN-γ Signalling

4.1 Introduction

As mentioned earlier in Chapter One, atherosclerosis is an inflammatory disease that affects large and medium arteries, which is regulated by cytokines (Lusis 2000). IFN-γ is a pro-inflammatory cytokine that controls several steps in atherosclerosis (McLaren and Ramji 2009). IFN-γ signals mainly through the JAK-STAT pathway though other pathways, such as ERK, have also been found to play an important role (Harvey et al. 2007; Li et al. 2010). These two signalling pathways are discussed below in detail.

4.1.1 JAK-STAT Pathway

IFN-γ signalling is initiated following binding of the cytokine to its receptors, which consists of two subunits: IFN-γR1 and IFN-γR2. The signalling pathway is shown in Figure 4.1 and explained in detail in the legend.

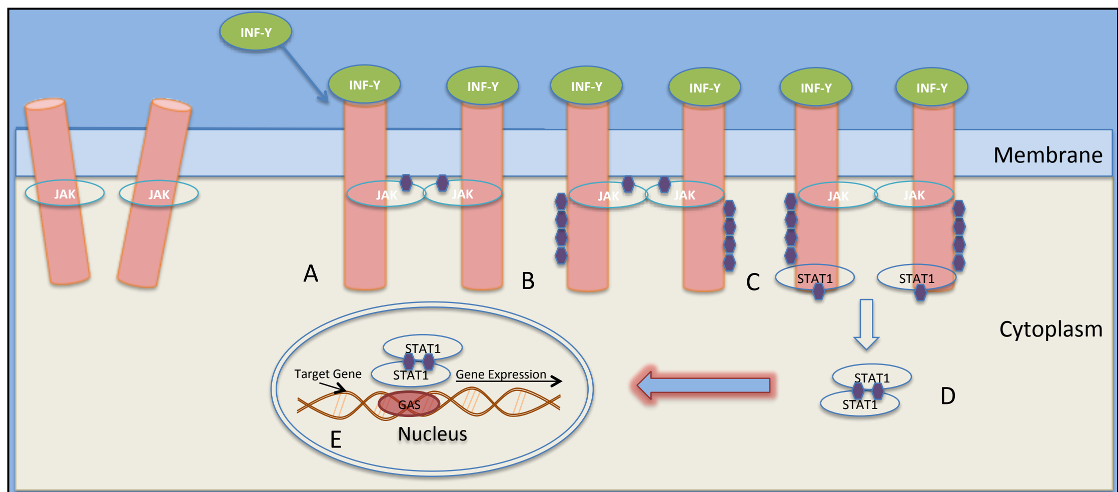

Figure 1.14: JAK-STAT Pathway.

Once IFN-γ binds to its receptors, it induces their oligomerisation resulting in the activation of JAK-1 and -2 by *trans*-phosphorylation (A). In the cytoplasmic domain of the receptors, a critical tyrosine residue is then phosphorylated by activated JAKs (B). This region becomes as a docking site for the protein STAT1, which is then phosphorylated on tyrosine 701 (C). In addition to phosphorylation of STAT1 on tyrosine 701, phosphorylation on serine 727 is required for maximal activity. After that, the phosphorylated STAT1 dimerises (D) and then translocates to the nucleus. In the nucleus, STAT1 interacts with γ-activated sequence elements (GAS) in the regulatory regions of target genes (E).

4.1.2 MAPK Signalling

MAPK is a family of serine/threonine kinases that controls cell proliferation, differentiation, motility, survival and apoptosis (Chang and Karin, 2001; Roux and Blenis 2004). Each MAPK pathway contains three major components, a MAPK kinase kinase (MAPKKK, MEKK), a MAPK kinase (MAPKK, MKK or MEK) and a MAPK (Dhillon et al., 2007; Kim and Choi, 2010). Five distinct classes of MAPKs have been categorised in mammals: ERK1/2; JNK1/2/3; p38 isoforms α, β, γ, and δ; ERKs-3 and -4; and ERK5 (Chang and Karin, 2001).The most widely studied groups of MAPKs to date are ERK1/2, JNKs, and p38 kinases (Dhillon et al. 2007). Extracellular stimuli such as growth factors and cytokines or stress can initiate signal transduction from the membrane via the phosphorylation and activation of specific MAPKs. These then cause the phosphorylation and activation of transcriptions factors that regulate the expression of target genes (Chang and Karin, 2001). The ERK1/2, JNK and p38 MAPK pathways have been described in detail in Chapter 1.

4.2 Aims and Experimental Design

The major aim of this chapter was to investigate the mechanisms underlying the Simvastatin-mediated inhibition of IFN-γ signalling, a pleiotropic anti-atherogenic action of statins. As the studies presented in Chapter 3, THP-1 macrophages were mainly used as an *in vitro* cell culture model to study the expression of MCP-1, ICAM-1, ERK-1 and -2, JNK-1 and -2, p38 and c-Jun along with the activation of these signalling pathways. However, in order to confirm that the responses were not peculiar to this cell line, representative experiments were also performed in primary cultures of HMDMs. Furthermore, some experiments were performed in the mouse RAW264.7 cell line in order to determine whether the responses were conserved between mouse and human macrophages.

The steps below describe the experimental strategies that were taken to address these overall aims:

1-To examine if the inhibitory action of Simvastatin was reversed by FPP and GGP, intermediates in the HMG-CoA reductase pathway;

2- To investigate if Simvastatin affects the phosphorylation-mediated activation of STAT1 in THP-1 macrophages and primary cultures of HMDMs;

3- To analyse if the action of Simvastatin is conserved in mouse RAW264.7 macrophages;

4- To examine if Simvastatin affects other key components of IFN-γ signalling such as MAPK pathways;

5- To confirm representative results obtained in THP-1 macrophages to primary HMDMs;

The experimental design conducted in this project is illustrated in Table 4.1.

Table 1.2: Experimental design.

The table below shows a summary of the experimental design carried out in this chapter using different cell systems and treatments.

	Experiments			
	1	**2**	**3**	**4**
Cell System	THP-1 Macrophages	THP-1 macrophages and primary HMDMs	RAW264.7 Macrophages	THP-1 Macrophages
Treatment	Simvastatin (10 µM) + FPP or + GGP or + Mev for 1 hour ⇩ 24-hour IFN-γ treatment	Simvastatin (40 µM) for 1 hour ⇩ 24-hour IFN-γ treatment	Simvastatin (40 µM) for 1 hour ⇩ 24-hour IFN-γ treatment	Simvastatin (40 µM) for 1 hour ⇩ 24-hour IFN-γ treatment (3 hour IFN-γ treatment only for western blotting analysis of P-p38)
Techniques	RT-qPCR for MCP-1	1- Western blot for P-STAT1 2- RT-qPCR for MCP-1 and /or ICAM-1	RT-qPCR for MCP-1 and ICAM-1	1- RT-qPCR for c-Jun, ERK, JNK and p38 2- Western blotting for P-c-Jun, P-ERK and P-p38

4.3 Results

4.3.1 The inhibitory action of Simvastatin is mediated via the HMG-CoA reductase pathway

To investigate the mechanisms by which Simvastatin inhibits IFN-γ induced MCP-1 expression *in vitro*, the potential involvement of the HMG-CoA reductase pathway was investigated. In particular, the ability of mevalonate and the isoprenoid intermediates, FPP and GGP, to reverse the IFN-γ-induced MCP-1 mRNA expression was analysed. GGP and FPP are required for the post-translational modification of GTP-binding proteins of the Rho and Ras family (Lee et al., 2008). These agents were added to THP-1 macrophages 1 hour prior to IFN-γ treatment for 24 hours. IFN-γ induced the expression of MCP-1 mRNA and this was significantly inhibited by simvastatin (Figure 4.2). In contrast, no such significant decrease was seen when mevalonate, FPP or GPP was included in addition to Simvastatin (Figure 4.2). These data suggest that the agents, particularly FPP and GPP, reverse, at least in part the Simvastatin-mediated inhibition of IFN-γ-induced MCP-1 expression.

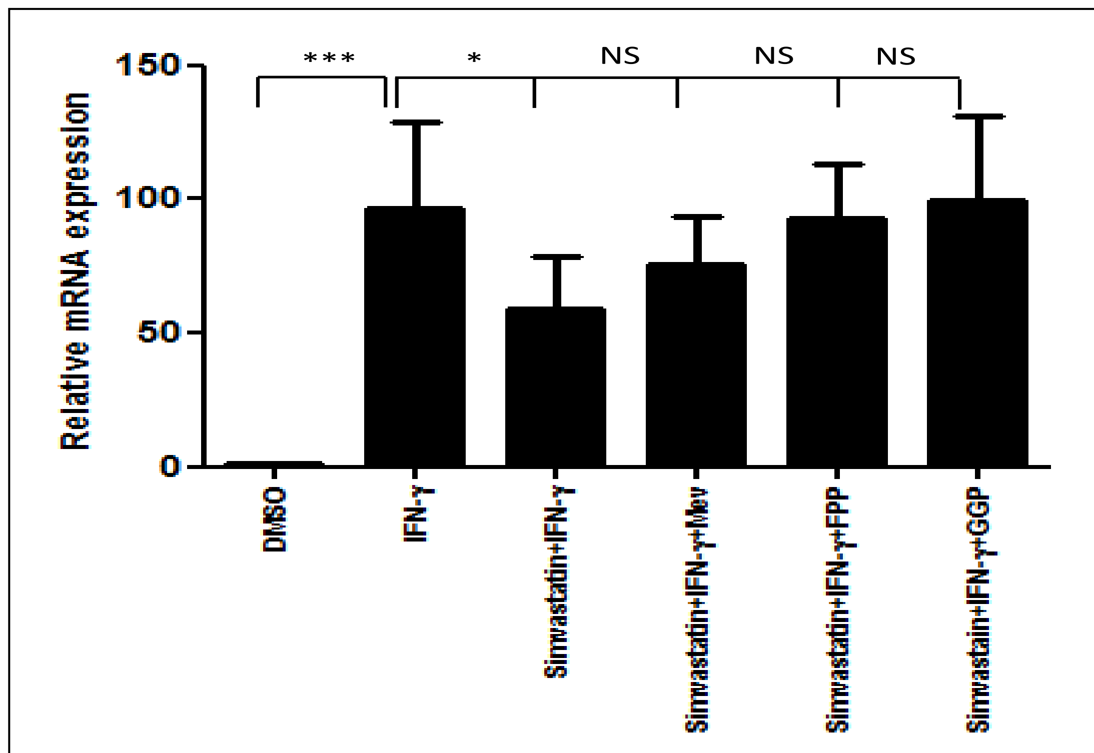

Figure 1.15: The inhibitory effect of simvastatin is dependent on the HMG-CoA reductase pathway.

THP-1 macrophages were treated with simvastatin (10µM) in the absence or the presence of mevalonate (500 µM), FPP (5 µM) and GGP (5 µM) for 1 hour followed by incubation with IFN-γ for 24 hour (cells treated with IFN-γ or DMSO vehicle for the same period served as controls). Total RNA was isolated and gene expression was analysed by RT-qPCR using primers specific for MCP-1 and GAPDH. Ct values were normalised to GAPDH mRNA. The data shown are from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, *p<0.05, ***p<0.001, NS not significant.

4.3.2 Simvastatin inhibits the IFN-γ induced STAT1 phosphorylation in THP-1 macrophages and primary HMDMs

Previous studies used Simvastatin at concentration of 10 μM (Copaja et al. 2012). However, problems were encountered in subsequent studies using this concentration and the inhibition of IFN-γ-induced gene expression was often variable and not always marked (data not shown). The precise reason(s) for such problems are unclear. It was therefore decided to carry out a dose response experiment using different concentrations of Simvastatin (10, 20, 30 and 40 μM) to identify a concentration that produced maximal reduction of both MCP-1 and ICAM-1 mRNA expression. The results from two experiments showed that 40 μM of Simvastatin produced maximal reduction of both MCP-1 and ICAM-1 expression (Figure 4.3 A and B). The results also showed little inhibition of ICAM-1 expression with lower concentrations of Simvastatin. In the light of this finding and substantial time lost previously because of variable inhibition with the low concentration used, subsequent studies were performed using 40 μM simvastatin.

STAT1 is a potential master regulator of IFN-γ signalling and the cytokine activates the transcription factor by phosphorylation on Tyr 701 and Ser 727. It was possible that Simvastatin exerted its effect on IFN-γ signalling through the modulation of STAT1 phosphorylation, and this was therefore investigated. For this, PMA-differentiated THP-1 macrophages were pre-treated with Simvastatin (40 μM) as mentioned above for 1 hour prior to IFN-γ treatment for 24 hours. Total cellular proteins were then extracted and the concentration determined using a MicroBCA Protein Assay Reagent Kit (Pierce). Equal amount of the protein was then subjected to western blot analysis as described in Chapter 2. Densitometric analysis of the data was then carried out with intensity of signals for phospho-STAT1 Ser 727 or –Tyr 701 normalised to total STAT1 (p84/91).

As shown in Figure 4.5, IFN-γ significantly induced the phosphorylation of STAT1 on Ser 727 and Tyr 701.The two closely migrating bands seen in some cases

is because the antibody recognises both the p91 and p84 isoforms produced due to alternative splicing. Inclusion of Simvastatin produced a statistically significant attenuation of the IFN-γ-induced STAT1 phosphorylation at either Ser 727 or Tyr 701. In contrast, the expression of total STAT1 protein was not affected when compared to the β-actin control (Figure 4.4 D). In addition, Simvastatin had no effect on the basal STAT1 phosphorylation observed in control cells. These results suggest that STAT1 activation is an important target for the inhibitory action of Simvastatin on IFN-γ signalling in THP-1 macrophages.

In order to rule out the possibility that the Simvastatin-mediated inhibition of STAT1 phosphorylation was not peculiar to THP-1 macrophages, the experiments were repeated in primary cultures of HMDM. As shown in Figure 4.5, IFN-γ induced STAT1 phosphorylation on both Ser 727 and Tyr 701 in HMDM and this was significantly attenuated by the inclusion of Simvastatin. The expression of the total STAT1 protein was not affected when compared to the β-actin control (Figure 4.5 D). In addition, Simvastatin had no effect on the basal STAT1 phosphorylation observed in control cells.

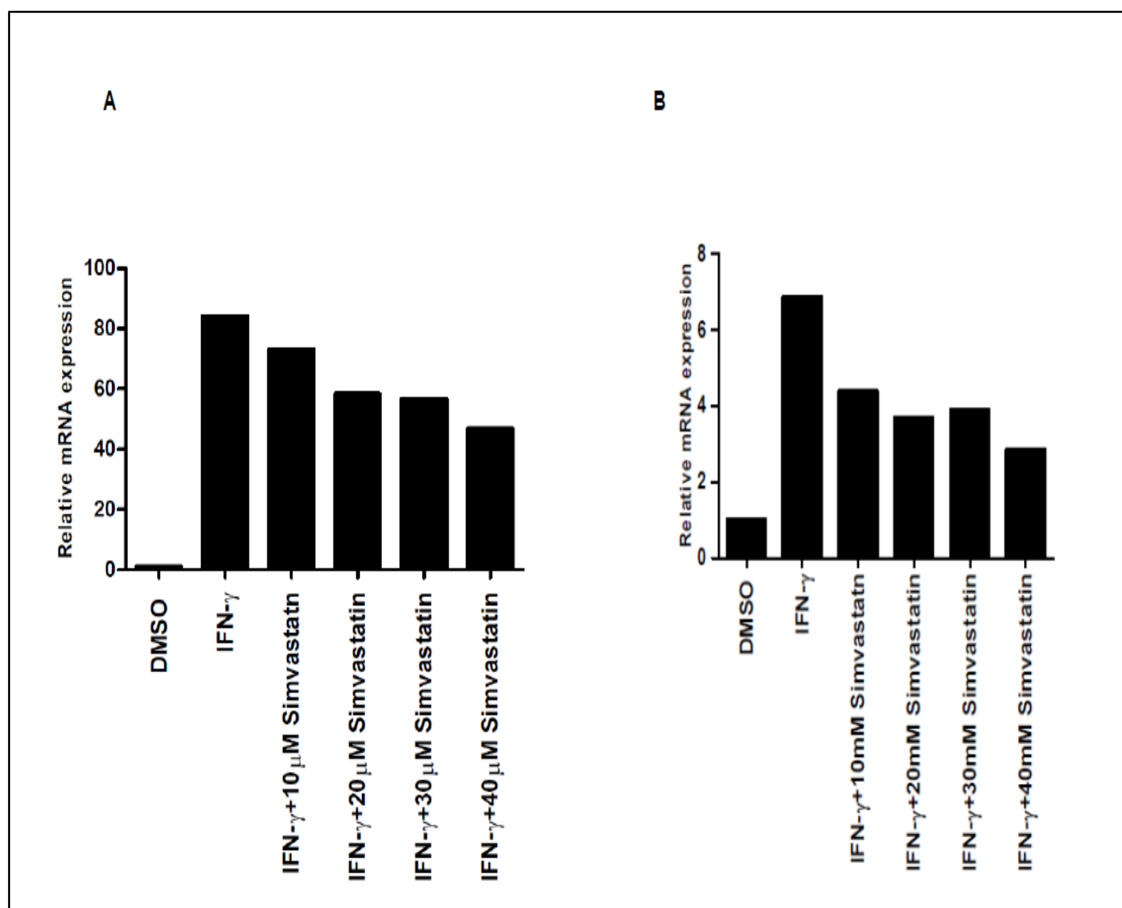

Figure 1.16: Simvastatin dose response experiment.

THP-1 macrophages were pre-treated with different concentrations of Simvastatin (10, 20, 30 and 40 μM) prior to IFN-γ treatment for 24 hours. Total RNA was isolated and analysed by RT-qPCR with specific primers for MCP-1 (A), ICAM-1(B) and GAPDH. The expression of MCP-1 or ICAM-1 mRNA was normalised to that of GAPDH. The data shown are mean from two independent experiments.

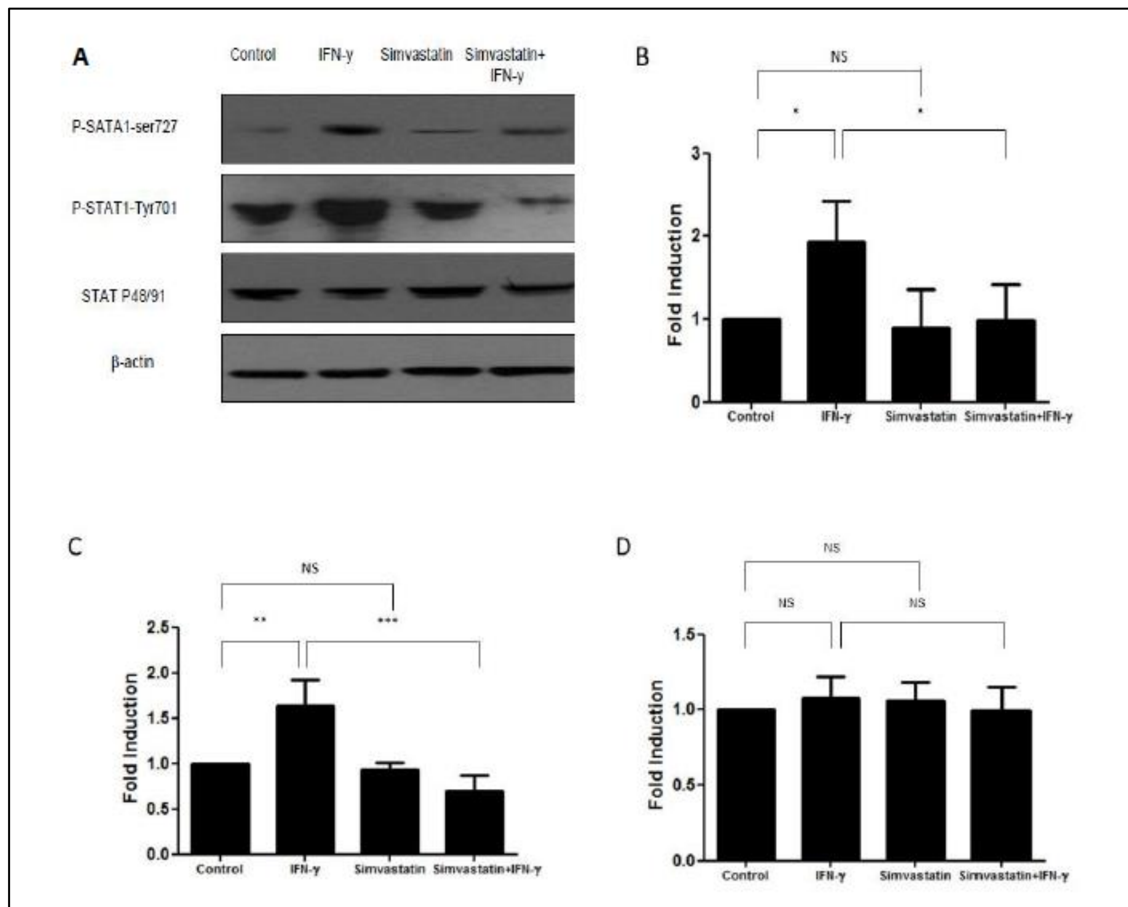

Figure 1.17: Effect of Simvastatin on STAT1 phosphorylation in THP-1 macrophages

Differentiated THP-1 macrophages were pre-treated with Simvastatin (40 μM) for 1 hour prior to IFN-γ treatment for 24 hours. Equal amount of protein extracts were then subjected to western blot analysis using antibodies specific for PSTAT1-Ser727, PSTAT1-Tyr701, STAT1 p84/91 and β-actin (A). Panels B and C show the average fold induction in the phosphorylation level of STAT1 at these sites (Ser 727 and Tyr 701) respectively normalised to the expression of the total STAT1 protein level as determined by densitometric analysis. Panel D shows the average fold induction in total STAT1 normalised to β-actin. The data are from four independent experiments. The fold induction in control sample was arbitrarily assigned as 1. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis,* p<0.05, ** p<.0.01, *** p<0.001, NS not significant.

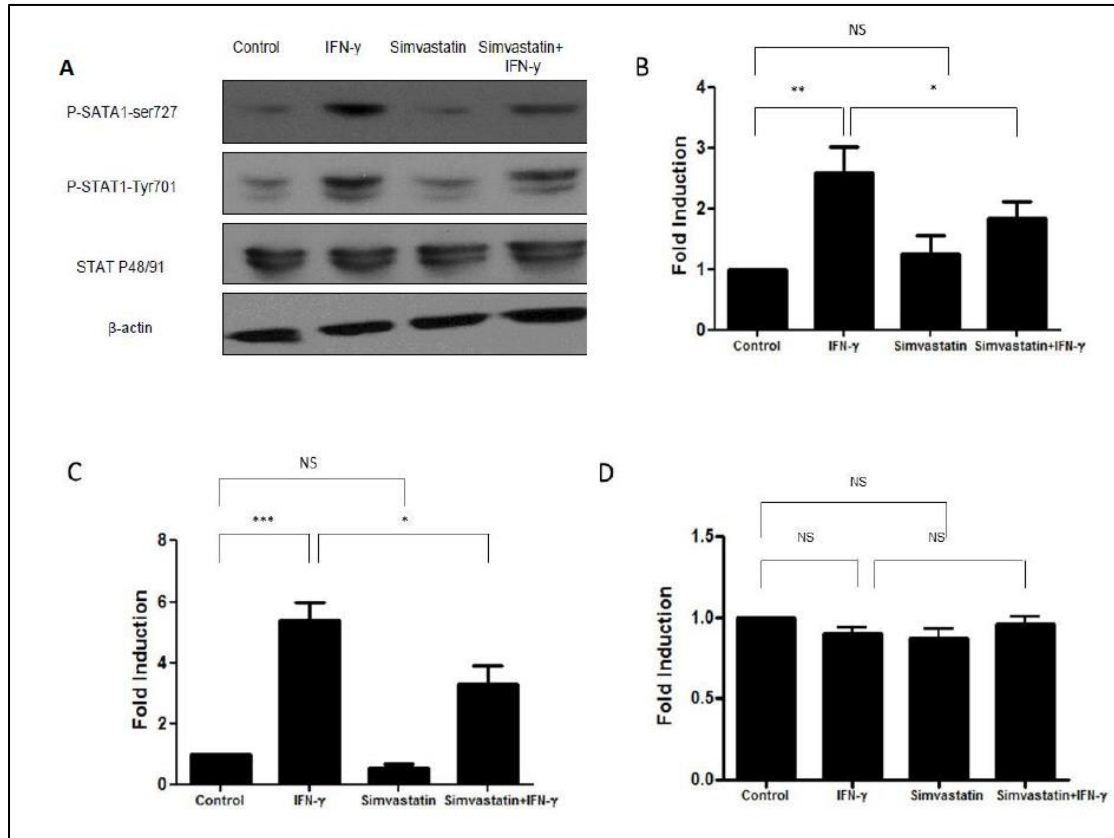

Figure 1.18: Effect of Simvastatin on STAT1 phosphorylation in HMDM.

Primary HMDMs were pre-treated with Simvastatin (40 μM) for 1 hour prior to IFN-γ treatment for 24 hours. Equal amount of protein extracts were then subjected to western blot analysis using antibodies specific for PSTAT1-Ser 727, PSTAT1-Tyr 701, STAT1 p84/91 and β-actin (A). Panels B and C show the average fold induction in the phosphorylation level of STAT1 at these sites (Ser 727 and Tyr7 01) respectively, normalised to the expression of the total STAT1 protein level as determined by densitometric analysis. Panel D shows the average fold induction in total STAT1 normalised to β-actin. The data are from four independent experiments. The fold induction in the control sample was arbitrarily assigned as 1. Statistical analysis was performed using one-way ANOVA with Tukeys, post-hoc analysis,* $p<0.05$, ** p<.0.01, *** $p<0.001$, NS not significant.

4.4 Simvastatin attenuates the IFN-γ induced expression of MCP-1 and ICAM-1 in RAW 264.7 macrophages

In order to investigate whether the action of Simvastatin observed in human macrophages is conserved in mice, representative experiments were performed in mouse RAW264.7 macrophages. Simvastatin or DMSO (vehicle control) was added to the cells for 1 hour before stimulation with IFN-γ. RT-qPCR was carried out on RNA prepared from the cells using primers against MCP-1, ICAM-1 or β-actin control. IFN-γ induced the expression of MCP-1 mRNA by 3.4 fold and this was significantly inhibited by Simvastatin (Figure 4.6A). Similarly, IFN-γ induced the expression of ICAM-1 (2.3 fold) and this was significantly reduced in the presence of Simvastatin (Figure 4.6B). These data suggest that the anti-inflammatory actions of Simvastatin are conserved between human and mouse macrophages.

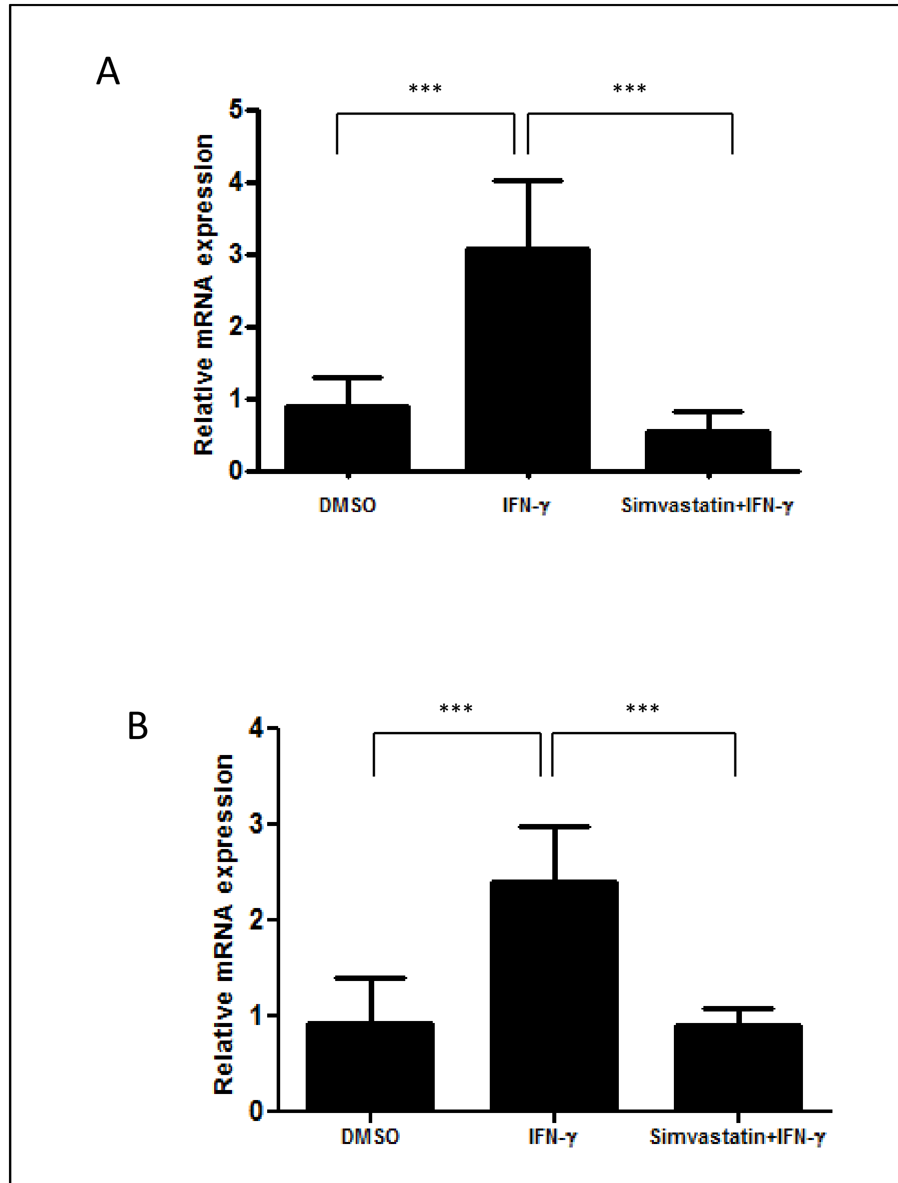

Figure 1.19: The effect of Simvastatin on the IFN-γ-induced MCP1 and ICAM-1 expression in RAW264.7 macrophages.

The cells were pre-treated for 1 hour with Simvastatin (40µM) or DMSO as a vehicle control. The cells were then incubated for 24 hours with or without IFN-γ (1000 U/ml). Total RNA was isolated and subjected to RT-qPCR with specific primers for mouse MCP-1, mouse ICAM-1 and mouse β-actin. The expression of MCP-1 (A) and ICAM-1 (B) was normalised to the β-actin control. The results are displayed as fold change in relation to vehicle-treated cells from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis. *** $p<.001$.

4.4.1 Effect of Simvastatin on the IFN-γ induced expression of ICAM-1 protein

The analysis of gene expression was so far restricted to mRNA levels. In order to confirm that the changes also occurred at the level of protein, representative experiments were performed on ICAM-1 in both THP-1 macrophages and primary HMDMs using western blot analysis. As shown in Figures 4.7 and 4.8, IFN-γ induced the expression of ICAM-1 protein in both THP-1 macrophages and HMDM and Simvastatin attenuated this. In addition, Simvastatin had no significant effect on the constitutive expression of the ICAM-1 protein.

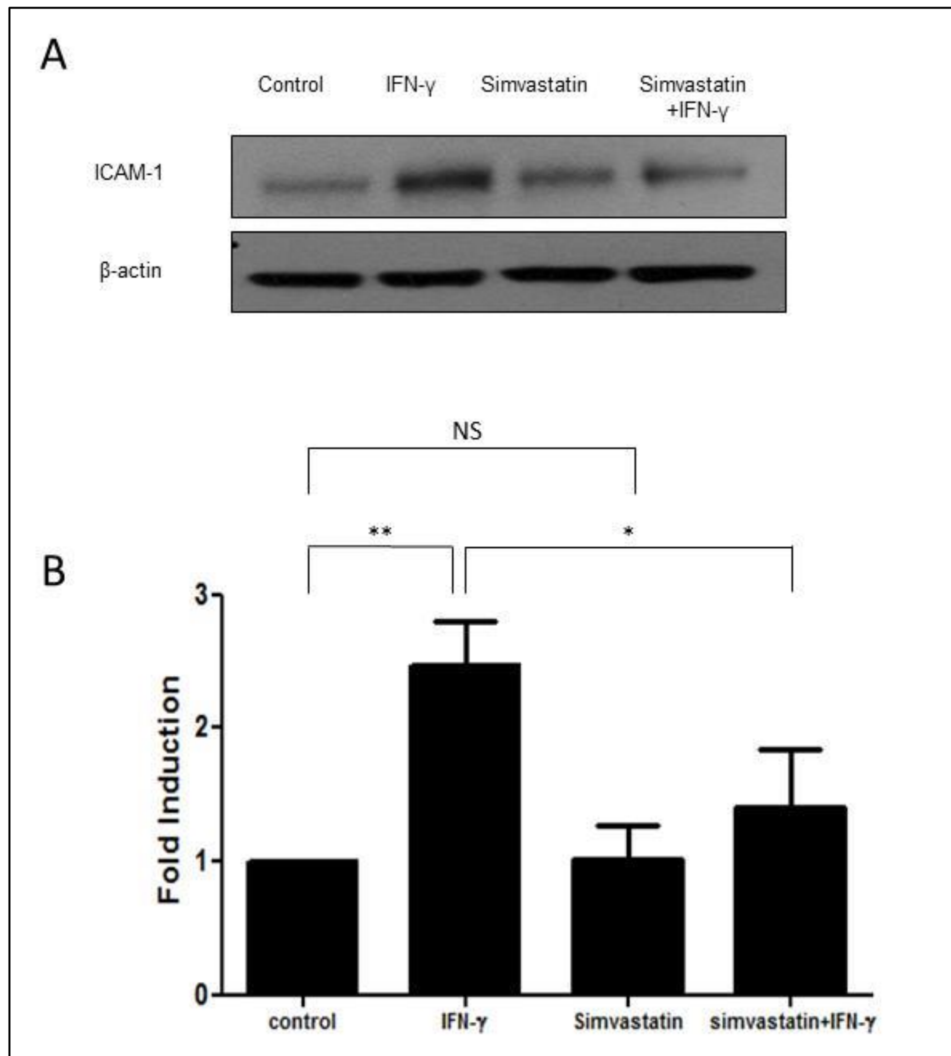

Figure 1.20: Simvastatin inhibits the IFN-γ induced expression of the ICAM-1 protein in THP-1 macrophages.

PMA differentiated THP-1 macrophages were pre-treated with Simvastatin (40 μM) for 1 hour prior to IFN-γ treatment for 24 hours. Equal amount of protein extracts were subjected to western blot analysis using antibodies specific for ICAM-1 and β-actin (A). The histogram shows the changes in ICAM-1 expression, as determined by densitometric analysis, from four independent experiments (the value for the control sample was arbitrarily assigned as 1) (B). Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, * $p<0.05$, ** p<.0.01, NS not significant.

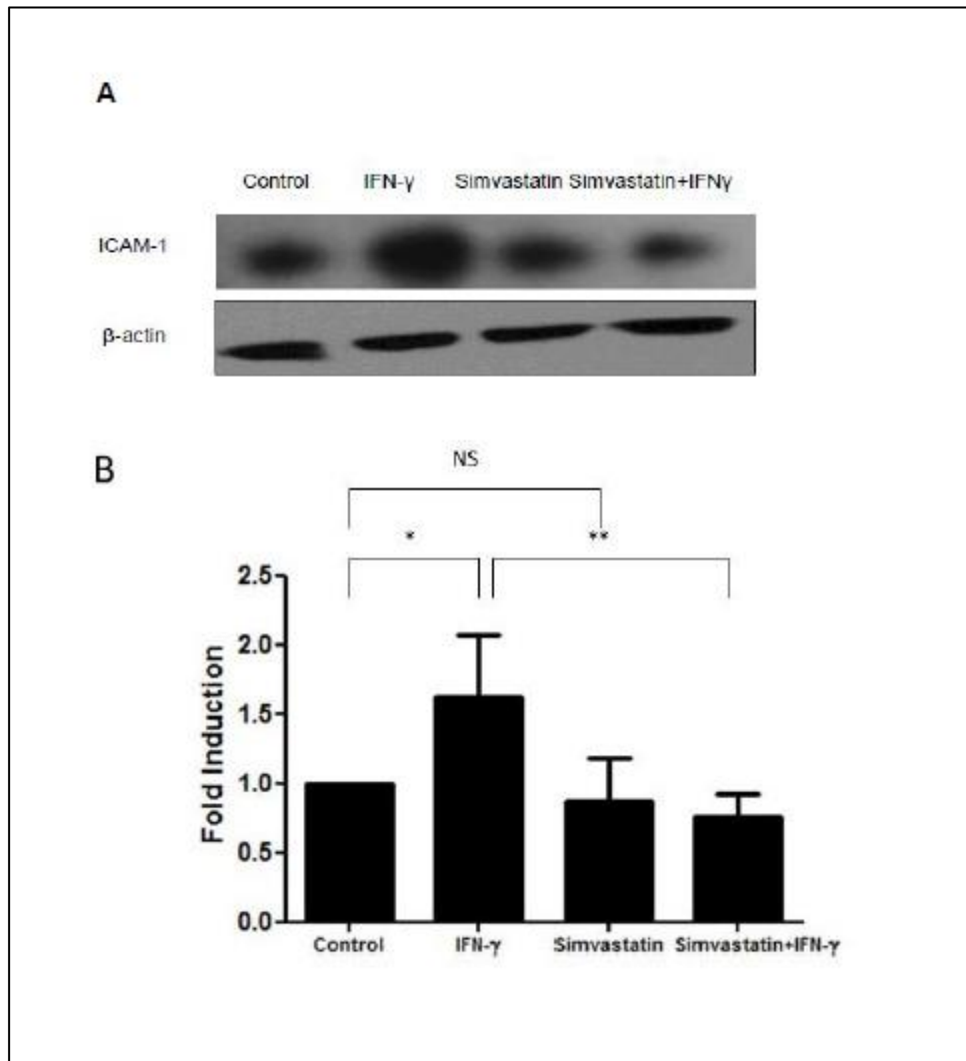

Figure 1.21: Simvastatin inhibits the IFN-γ induced expression of ICAM-1 protein in HMDM.

Primary HMDMs were pre-treated with Simvastatin (40 μM) for 1 hour prior to IFN-y treatment for 24 hours. Equal amount of protein extracts were subjected to western blot analysis using antibodies specific for ICAM-1 and β-actin (A). The histogram shows the changes in ICAM-1 expression (B), as determined by densitometric analysis, from four independent experiments (the value in the control sample was arbitrarily assigned as 1). Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis,* p<0.05, ** p<.0.01, NS not significant.

4.4.2 Optimisation of Simvastatin incubation time using a new source of this statin dissolved in water

The Simvastatin that was used in previous studies was an activated form that had to be dissolved in an organic solvent like DMSO. In the latter part of the studies, another, more cheaper form of activated Simvastatin that was dissolved in water became commercially available. It was decided to use this source for further studies. However, variable results were obtained with pre-incubation time of 1 hour (data not shown). Because some previous studies have used pre-incubation periods of up to 24 hours, it was possible that a longer pre-incubation period was required. It was therefore decided to do this optimisation experiment with the new Simvastatin in which THP-1 macrophages were treated with this statin (40 μM) for 1 hour, 3 hours and 6 hours prior to IFN-γ stimulation for 24 hour. Total RNA was then isolated and RT-qPCR was carried out for MCP-1 and ICAM-1. A significant inhibition of the IFN-γ induced MCP-1 and ICAM-1 expression was only obtained following pre-incubation with Simvastatin for 6 hours (Figure 4.9). It was therefore decided to use this 6 hours pre-incubation period with Simvastatin for subsequent studies.

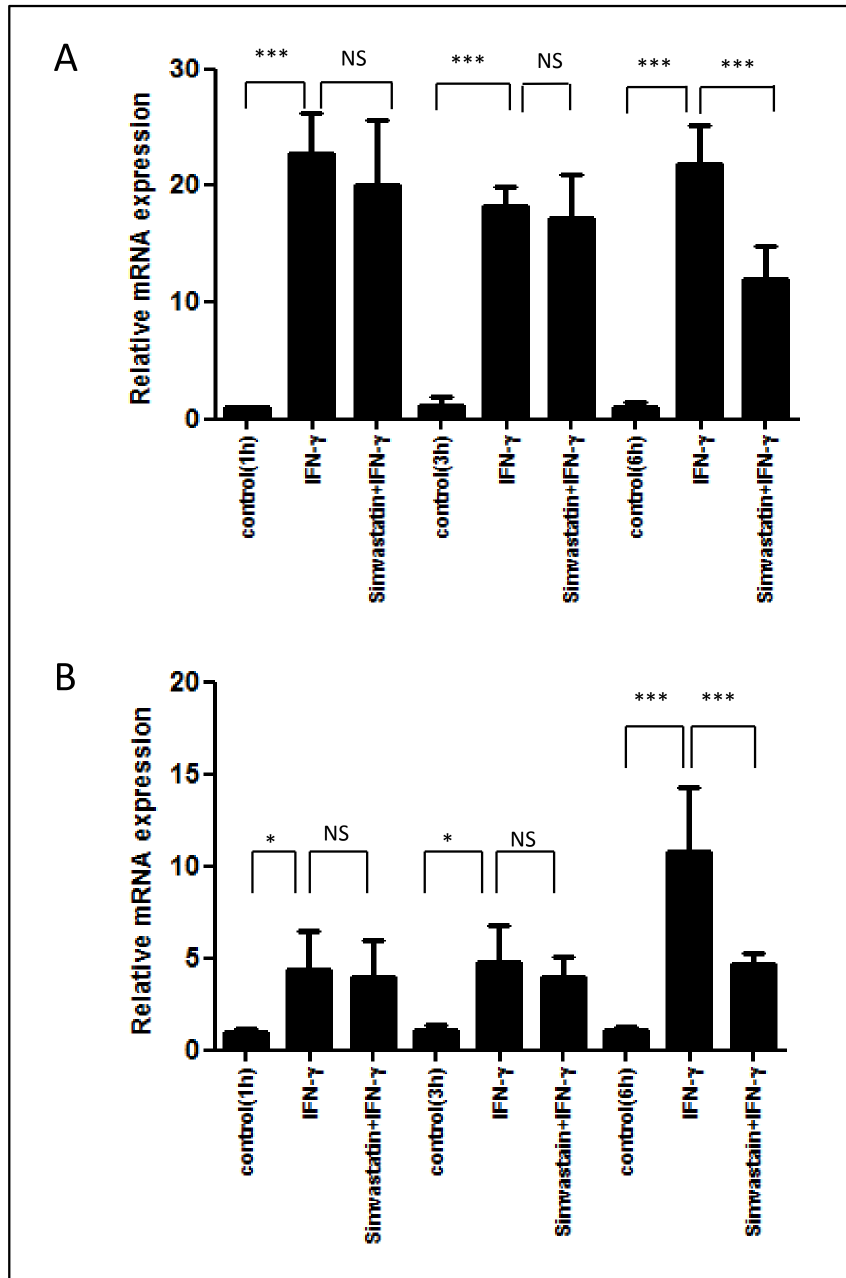

Figure 1.22: The effect of pre-treatment of cells with Simvastatin for different time periods on IFN-γ actions.

THP-1 macrophages were pre-treated with Simvastatin for different time periods (1, 3 and 6 hours) prior to IFN-γ treatment for 24 hours. Total RNA was isolated and subjected to RT-qPCR with specific primers for MCP-1 (A), ICAM-1 (B) and GAPDH. The levels of MCP-1 or ICAM1 was normalised to GAPDH mRNA with values from control cells arbitrarily assigned as 1. The data are from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, * $p<0.05$, *** $p<0.001$, NS not significant.

4.4.2.1.1 Effect of Simvastatin on the IFN-γ induced expression of MCP-1 and ICAM-1 in primary HMDMs

In order to confirm that the effect of Simvastatin following 6 hours incubation period was not peculiar to THP-1 macrophages, the experiments were repeated using primary cultures of HMDMs. The cells were treated with 40 µM Simvastatin for 6 hour prior to IFN-γ treatment for 24 hours, and RNA was analysed by RT-qPCR analysis for MCP-1 and ICAM-1 genes. As shown in Figure 4.10, IFN-γ induced the expression of MCP-1 and ICAM-1 and Simvastatin significantly reduced this by more than 50%. In addition, Simvastatin produced a significant decrease in constitutive MCP-1, but not ICAM-1, expression.

4.4.3 Effects of Simvastatin on MAPKs pathways

Figures 4.4 and 4.5 shows that Simvastatin inhibits the IFN-γ induced STAT1 phosphorylation on Ser 727 and Tyr 701. The phosphorylation of STAT1 on Ser 727 is known to be modulated by MAP kinases (Goh et al., 1999; Zhang et al., 2004; Li et al., 2010). The effect of Simvastatin on the expression and/or activation of different MAPKs were therefore investigated.

4.4.3.1 Effect of Simvastatin on JNK-1 and -2

PMA differentiated THP-1 macrophages were treated for 6 hour with 40 µM simvastatin followed by treatment with IFN-γ for 24 hours. Total RNA was isolated and RT-qPCR was carried out for JNK-1 and -2 genes. Before carrying out the experiments on JNK-1 and -2, the expected changes in MCP-1 and ICAM-1 expression was confirmed by additional experiments in light of the new source of Simvastatin and 6 hours pre-incubation period. Figure 4.11 shows that IFN-γ induces MCP-1 and ICAM-1 expression, and this was significantly attenuated by Simvastatin. These data also acted as a positive control for subsequent studies on the MAPK family as the same RNA was used. As shown in Figure 4.12, IFN-γ produced a significant increase in the expression of both JNK-1 and -2 mRNA. However, this

induction in gene expression was not affected by Simvastatin. In addition, the constitutive expression of JNK-2, but not JNK-1, was induced by Simvastatin (Figure 4.12). Unfortunately, it was not possible to investigate the activation of JNK-1/2 phosphorylation because of problems with commercially available antibodies (data not shown). It was therefore decided to analyse the expression and activation of c-Jun, a key downstream target of JNK-1/2 actions.

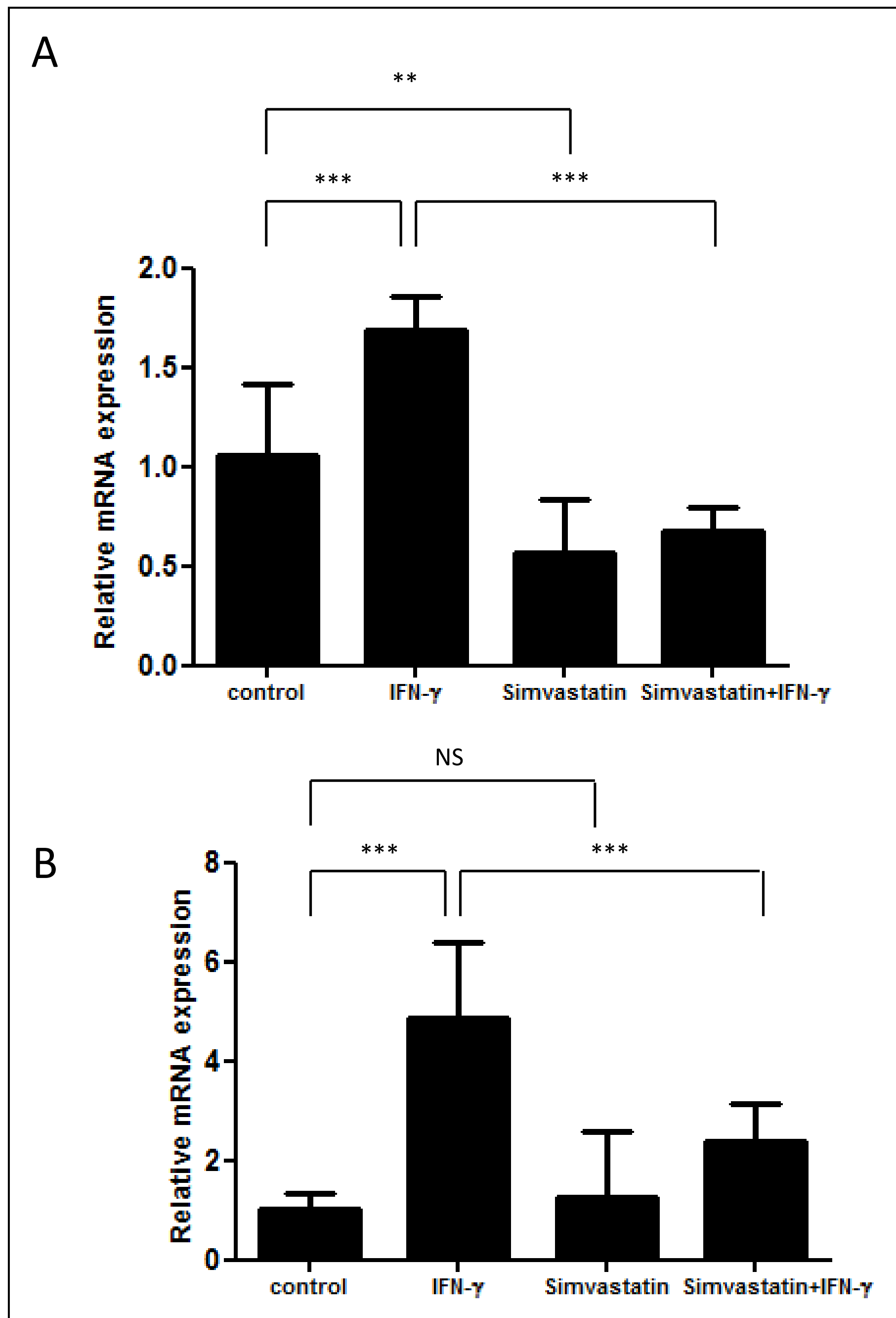

Figure 1.23: Effect of Simvastatin on the IFN-γ induced MCP-1 and ICAM-1 mRNA expression in primary HMDMs.

Primary macrophages were pre-treated with Simvastatin for 6 hours prior to IFN-γ treatment for 24 hours. Total RNA was analysed by RT-qPCR with specific primers for MCP-1(A), ICAM-1(B) or GAPDH. The expression of MCP-1 or ICAM-1 was normalised to GAPDH mRNA levels. The data are from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, ** p<.0.01, *** p<0.001, NS not significant.

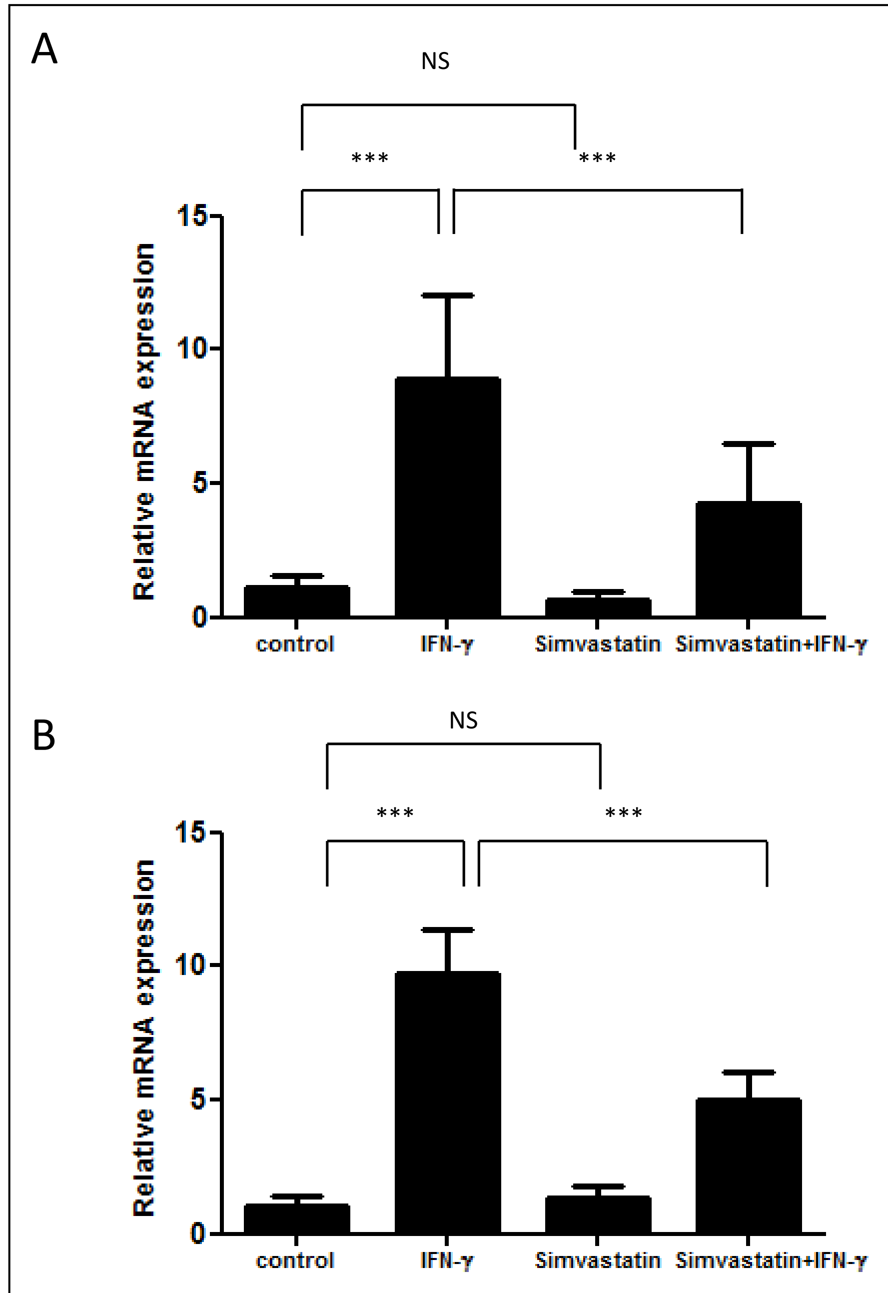

Figure 1.24: Simvastatin inhibits the IFN-γ induced MCP-1 and ICAM-1 mRNA expression.

THP-1 macrophages were pre-treated with Simvastatin for 6 hours prior to IFN-γ treatment for 24 hours. Total RNA was isolated and subjected to RT-qPCR with specific primers for MCP-1 (A), ICAM-1 (B) and GAPDH. The expression of MCP-1 or ICAM-1 was normalised to GAPDH with the relative mRNA expression from control cells being arbitrarily assigned as 1. The data shown are from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, *** $p<0.001$, NS not significant.

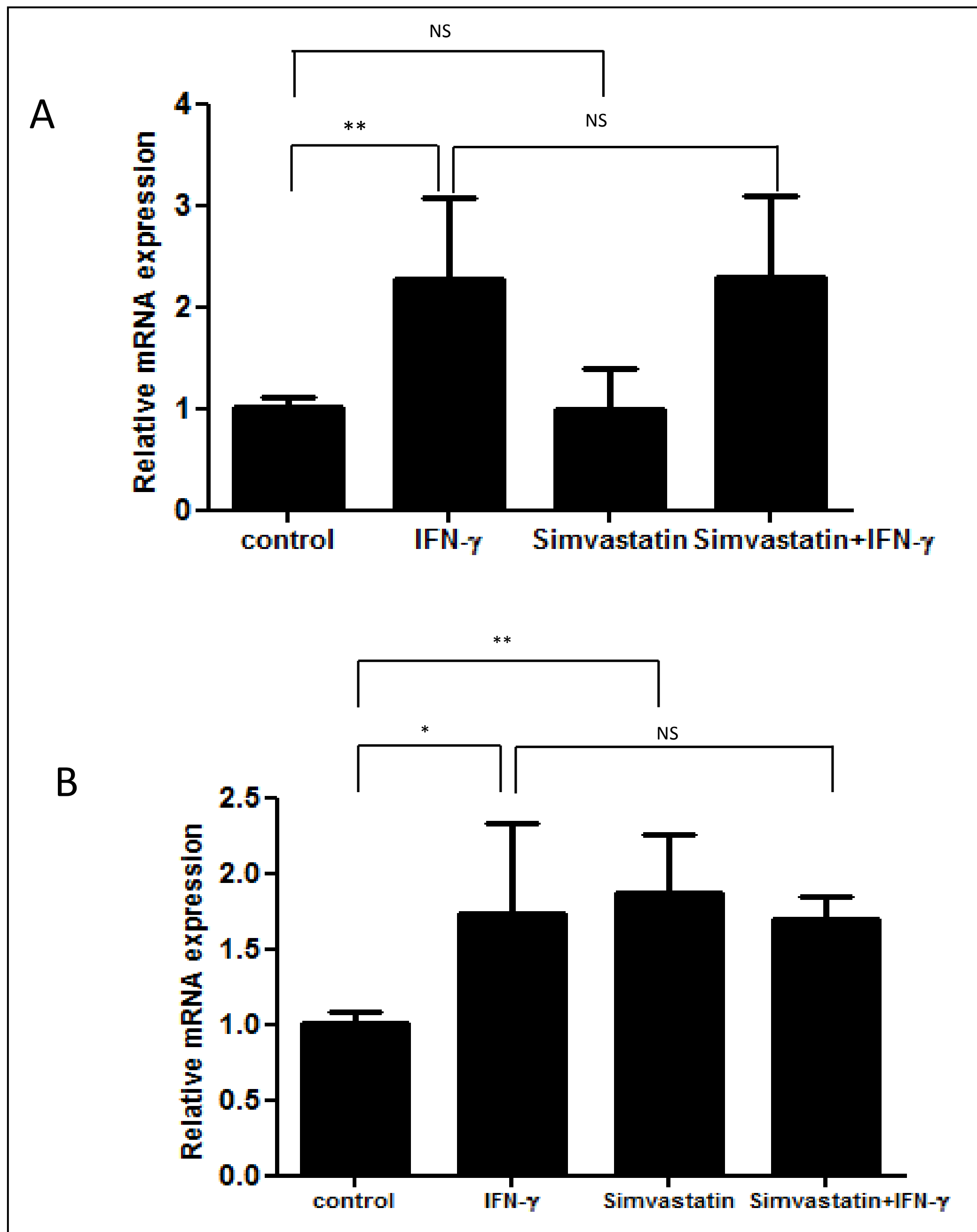

Figure 1.25: Effect of Simvastatin on JNK-1 and -2

THP-1 macrophages were pre-treated with Simvastatin for 6 hours prior to IFN-γ treatment for 24 hours. Total RNA was analysed by RT-qPCR with specific primers to JNK-1 (A), JNK-2 (B) and GAPDH. The expression of JNK-1 and -2 was normalised to GAPDH mRNA levels. The data are from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, * p<0.05, ** p<.0.01, NS not significant.

4.4.4 Effect of Simvastatin on c-Jun

Initial experiments investigated the effect of IFN-γ on c-Jun mRNA expression and, if a change was identified, whether this was affected by Simvastatin. For this PMA differentiated THP-1 macrophages were pre-treated with 40 μM Simvastatin for 6 hours prior to IFN-γ treatment for 24 hour. Total RNA was isolated and used for RT-qPCR analysis.

IFN-γ produced a significant induction of expression of c-Jun mRNA expression (Figure 4.13). However, Simvastatin had no significant effect on this induction (Figure 4.13). Simvastatin also had no significant effect on constitutive c-Jun mRNA expression though a trend of increased levels was observed.

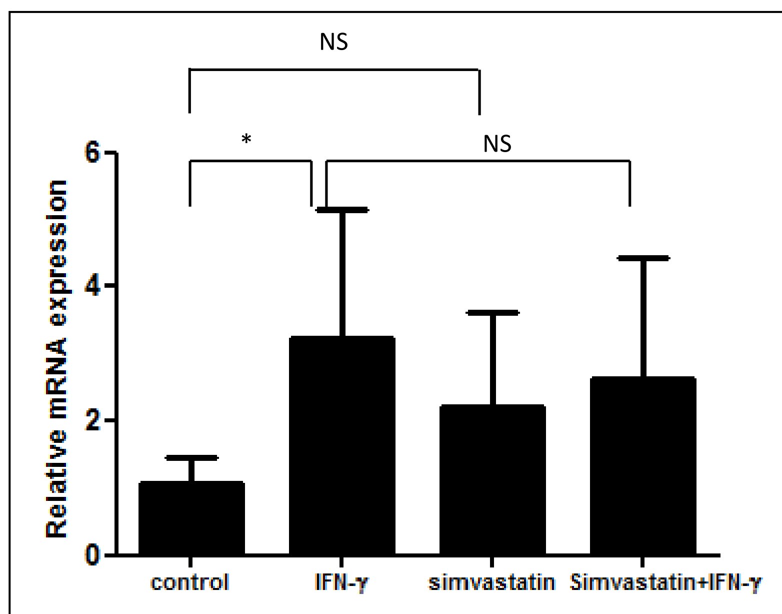

Figure 1.26: Effect of Simvastatin on c-Jun

THP-1 macrophages were pre-treated with Simvastatin for 6 hours prior to IFN-γ treatment for 24 hours. Total RNA was isolated and subjected to RT-qPCR with specific primers for c-Jun or GAPDH. The expression of c-Jun was normalised to GAPDH with values from control cells being arbitrarily assigned as 1. The data shown are from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, * $p<0.05$, NS not significant.

c-Jun is activated by phosphorylation and represents a key downstream target of JNK actions. It was therefore decided to first investigate whether the levels of phosphorylated, activated c-Jun changes following treatment of the cells with IFN-γ, and if this were the case, if Simvastatin affects this. As shown in Figure 4.14, IFN-γ had no significant effect on the levels of phospho-c-Jun. Similarly, IFN-γ had no significant effect on total c-Jun levels.

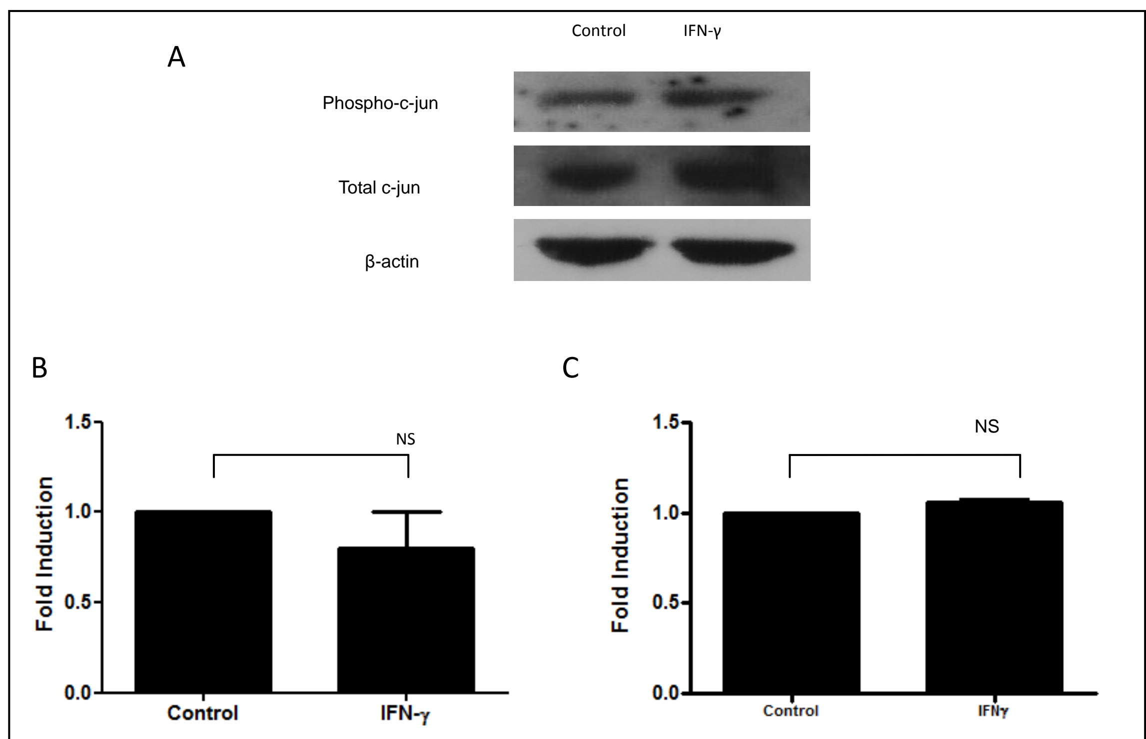

Figure 1.27: Effect of Simvastatin on c-Jun

PMA differentiated THP-1 macrophages were treated with IFN-γ for 24 hours. Equal amount of protein was then subjected to western blot analysis using antibodies specific for phospho c-Jun, total c-Jun and β-actin (A). Panel B shows the levels of phospho-c-Jun normalised to total c-Jun and panel C shows the average fold induction of total c-Jun normalised to β-actin, from three independent experiments. The values from control cells have been arbitrarily assigned as 1. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, NS not significant.

4.4.5 Effect of Simvastatin on ERK-1 and -2

RT-qPCR was carried out to investigate whether IFN-γ affected the expression of ERK-1 and -2 mRNA in THP-1 macrophages. IFN-γ induced the expression of both ERK-1 and -2 mRNA though this failed to reach significance for ERK-2 (Figure 4.15). Inclusion of simvastatin had no further effect on the IFN-γ-mediated increase in ERK-1 mRNA expression but significantly increased the levels of ERK-2 RNA. Interestingly, the constitutive levels of ERK-1 and -2 (produced during differentiation in response to PMA) were significantly increased by Simvastatin (Figure 4.15).

ERK-1/2 is activated by phosphorylation. It was decided to first investigate whether the levels of phosphorylated, activated ERK1/2 change in response to IFN-γ treatment and, if any changes are identified, whether Simvastatin affects this. Figure 4.16 shows that IFN-γ produces a significant increase in the levels of phospho-ERK1/2 without affecting the total levels of the protein. The effect of Simvastatin on both the constitutive and IFN-γ-induced levels of ERK-1/2 was next investigated. As shown in Figure 4.17, both the constitutive (associated with differentiation by PMA) and IFN-γ inducible levels of phospho-ERK-1/2 were significantly attenuated by Simvastatin. However, Simvastatin had no effect on the levels of total ERK-1/2 protein.

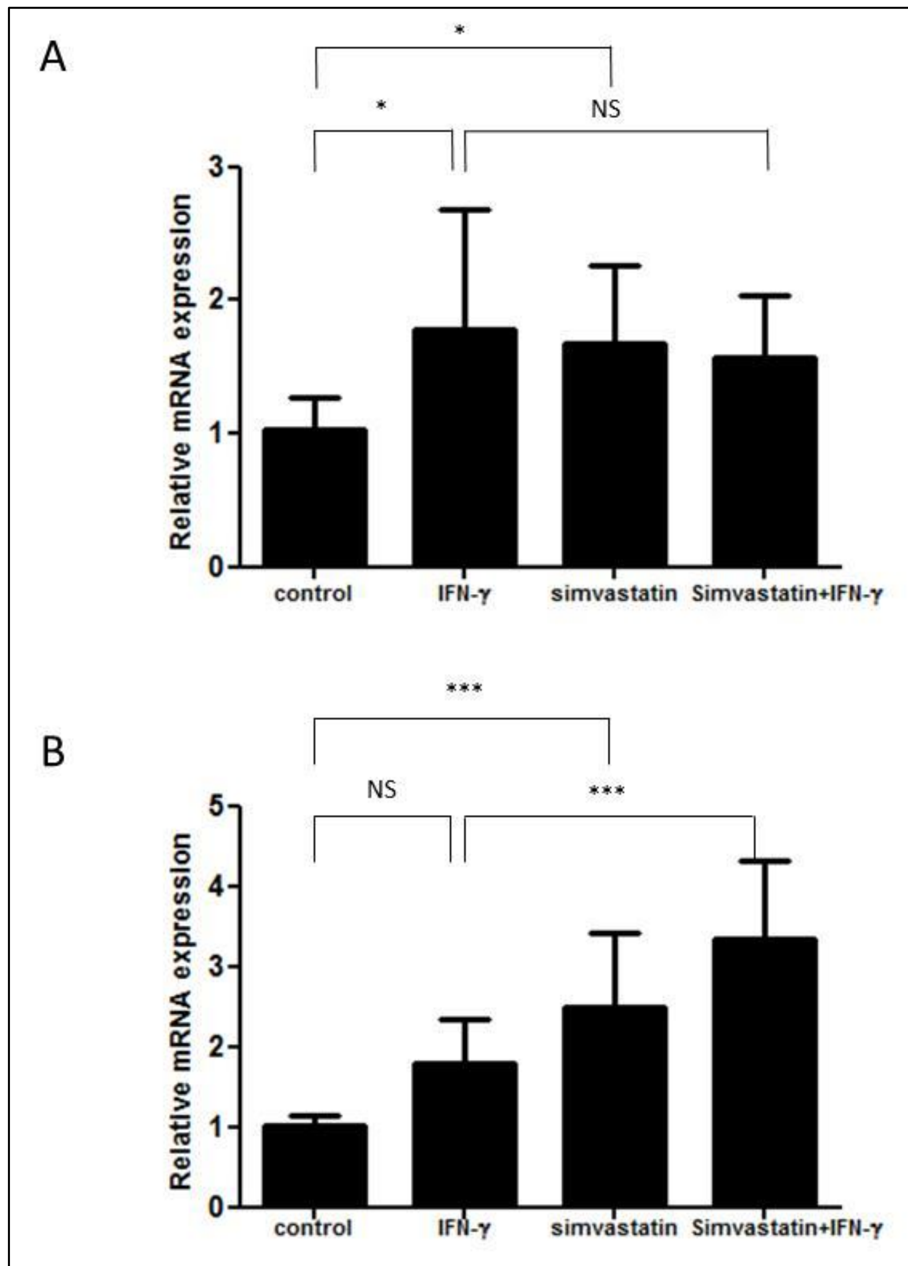

Figure 1.28: Effect of Simvastatin on ERK-1 and -2 mRNA expressions.

THP-1 macrophages were pre-treated with Simvastatin for 6 hours prior to IFN-γ treatment for 24 hours. Total RNA was analysed by RT-qPCR with primers specific for ERK-1 (A), ERK-2 (B) or GAPDH. The expression of ERK-1 and -2 was normalised to GAPDH mRNA expression. The data shown are from six independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, * $p<0.05$, *** $p<0.001$, NS not significant.

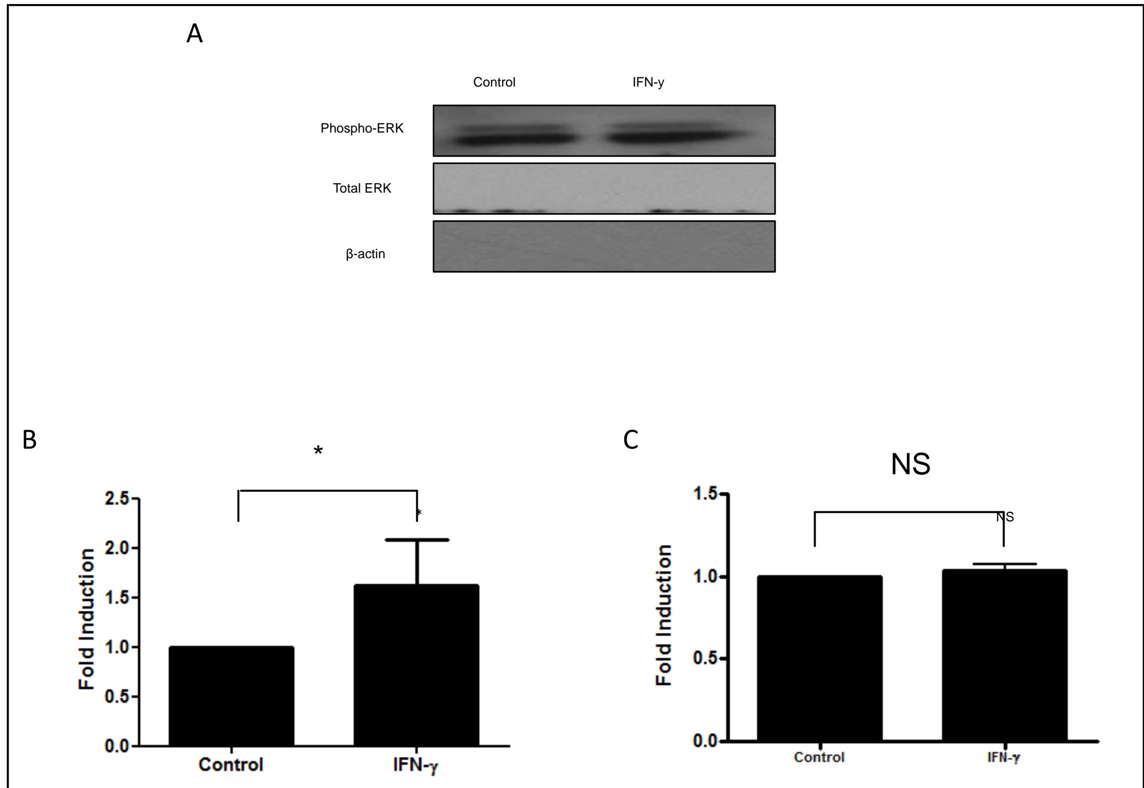

Figure 1.29: Effect of IFN-γ on the phospho- ERK levels.
PMA differentiated THP-1 macrophages were treated with IFN-γ for 24 hours. Protien extracts were then subjected to western blot analysis using antibodies specific for phospho-ERK1/2 (P44/42), total ERK1/2 and and β-actin (A). The levels of ERK-1/2, as determined by densitometric analysis of the data, were normailsed to that of total ERK-1/2 (B). Panel C shows the average fold induction in total ERK1/2 normalised to β-actin. The values are from three independent experiments and those from the control sample have been arbitrarily assigned as 1. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, * $p<0.05$.NS= not significant.

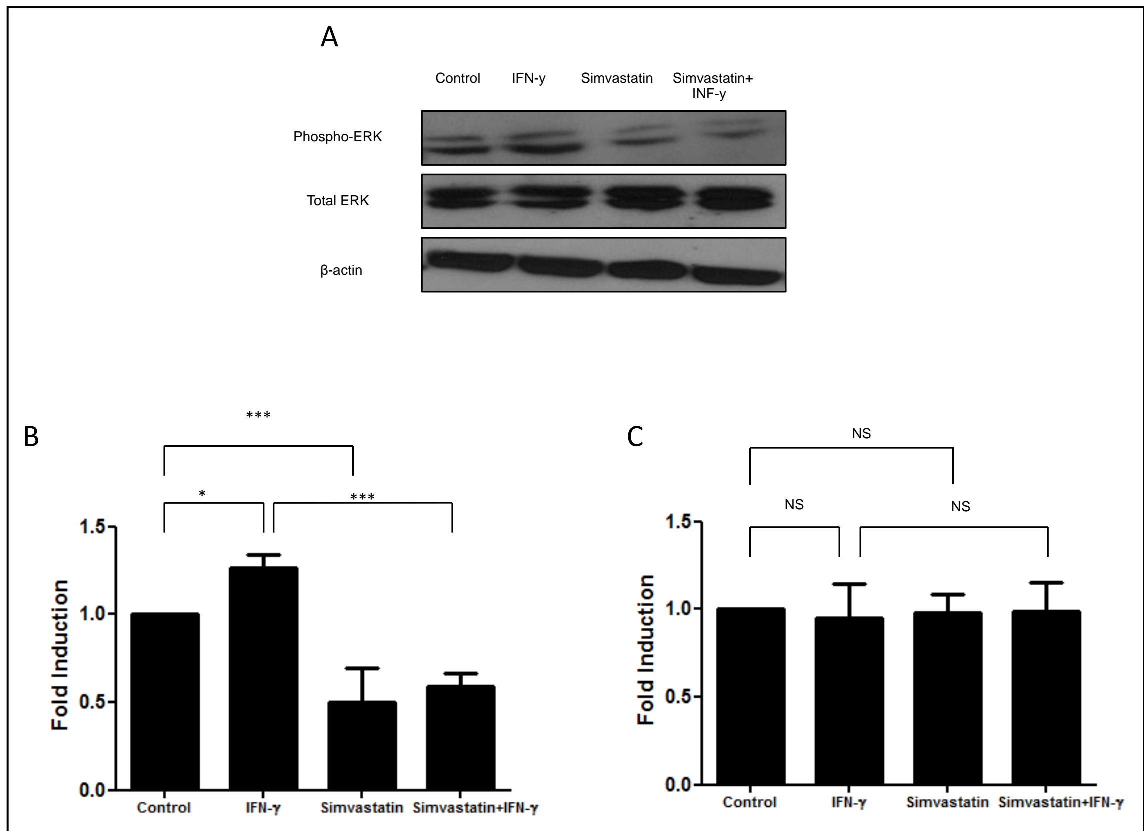

Figure 1.30: Effect of Simvastatin on IFN-γ-induced phospho-ERK-1/2 expression

THP-1 macrophages were pre-treated with Simvastatin for 6 hours prior to IFN-γ treatment for 24 hours. Protein extracts were prepared and subjected to western blot analysis using antibodies specific for phospho-ERK-1/2 (P44/42), total ERK-1/2 and β-actin (A). The levels of phospho-ERK-1/2, as determined by densitometric analysis, were normalised to that of total ERK-1/2 (B), Panel C shows the average fold induction of total ERK1/2 normalised to β-actin. The data are from four independent experiments and the values from control sample have been arbitrarily assigned as 1. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, * $p<0.05$, *** $p<0.001$.

4.4.6 Effect of Simvastatin on p38

PMA differentiated THP-1 macrophages were pre-treated for 6 hours with 40 µM Simvastatin and then treated with IFN-γ for 24 hours. The expression of p38α (major isoforms expressed in macrophages and implicated in inflammation) mRNA was then determined by RT-qPCR analysis. IFN-γ significantly induced p38 mRNA expression and this was not affected by simvastatin (Figure 4.18). Interestingly, Simvastatin significantly increased basal p38 mRNA expression.

Initial western blot analysis using the 24-hour incubation with IFN-γ showed no signals with the phospho-p38 antibody. Because the phosphorylation of many components of signal transduction pathways is often seen at earlier time points and the activation can be transient, it was decided to do a preliminary time course experiment where IFN-γ was added for different time periods (15 minutes, 30 minutes, 1 hour, 3 hours, and 12 hours). Treatment of the cells with IFN-γ for 3 hours produced the most induction of phospho-p38 levels (Figure 4.19). It was thus decided to treat the cells with IFN-γ for 3 hours for subsequent experiments.

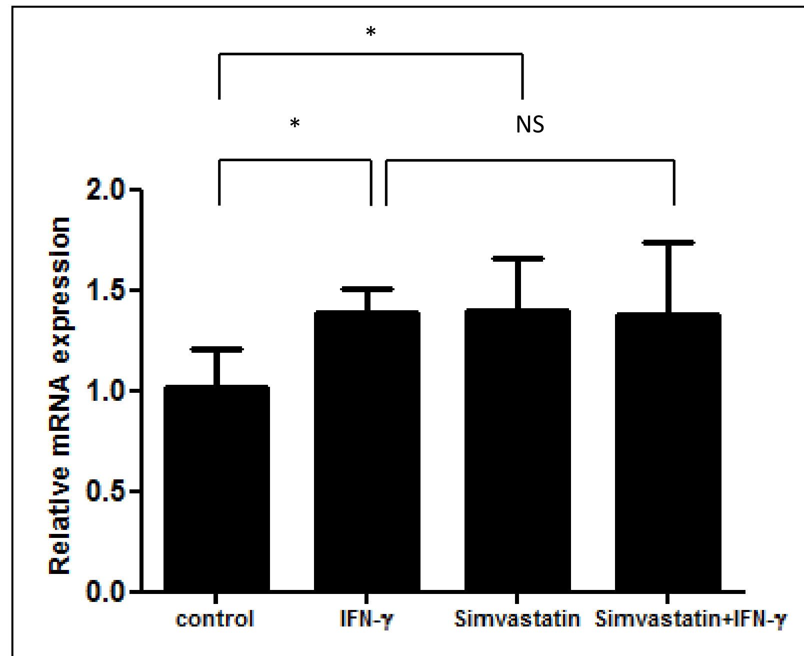

Figure 1.31: Effect of Simvastatin on p38α mRNA expression.

THP-1 macrophages were pre-treated with Simvastatin for 6 hours prior to IFN-γ treatment for 24 hours. Total RNA was analysed by RT-qPCR with specific primers to p38α and GAPDH. The expression of p38α was normalised to GAPDH mRNA levels. The data shown are from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, * $p<0.05$, NS not significant.

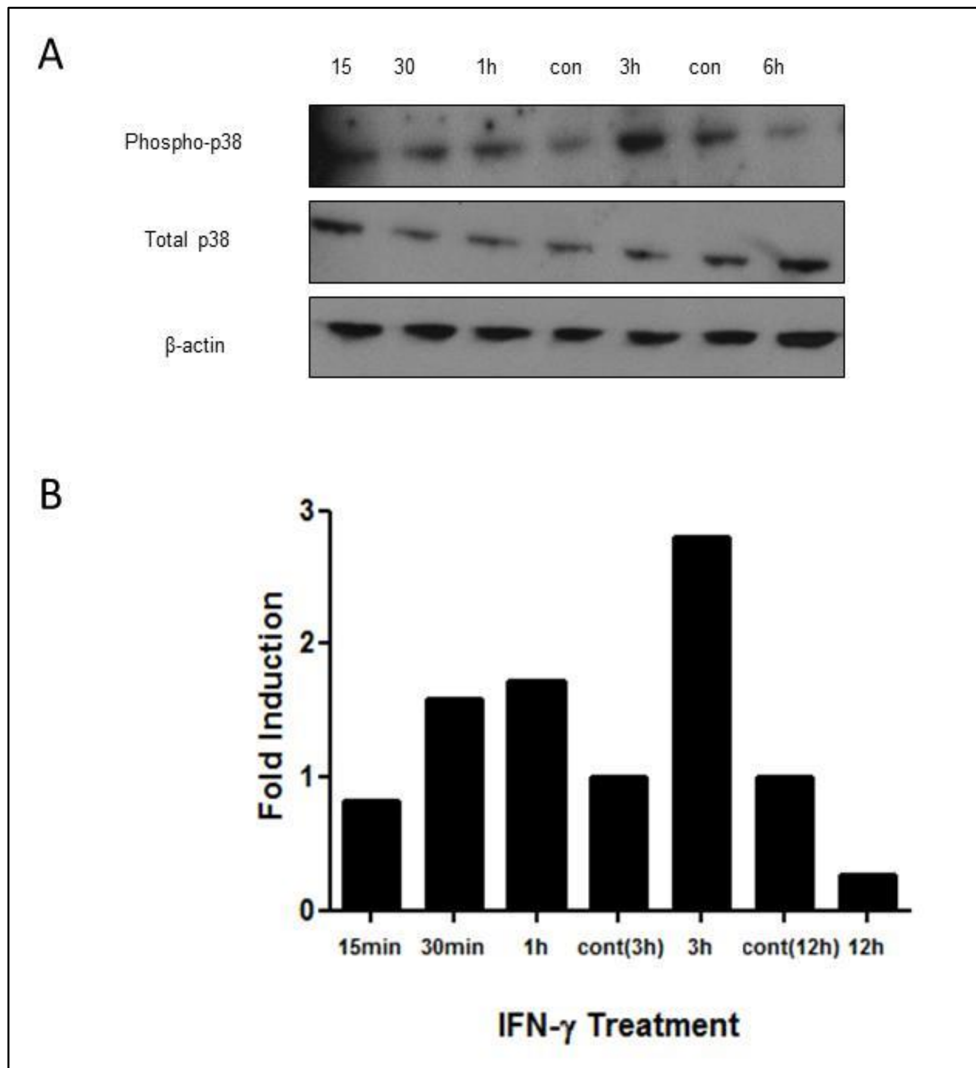

Figure 1.32: Time dependent actions of IFN-γ on levels of phospho-p38.

THP-1 macrophages were treated with IFN-γ for different times (15 minutes, 30 minutes, 1 hour, 3 hours and 12 hours). Protein extracts were subjected to western blot analysis using antibodies specific for phospho–p38, total p38 and β-actin (A). The histogram shows phospho-p38 levels normalised to total p38 from one experiment (B).

The effect of Simvastatin on the IFN-γ induced phospho-p38 levels was next determined. Differentiated THP-1 macrophages were treated with 40 µM Simvastatin for 6 hours and then IFN-γ was added to the cells for 3hour. Western blot analysis on cell lysates was then performed using phospho-p38 antibody. As it shown in the Figure 4.20, IFN-γ increased the expression of phospho-p38 and addition of simvastatin produced a slight, but significant, reduction. In contrast, there was no effect on the total p38 levels or constitutive phospho-p38 levels.

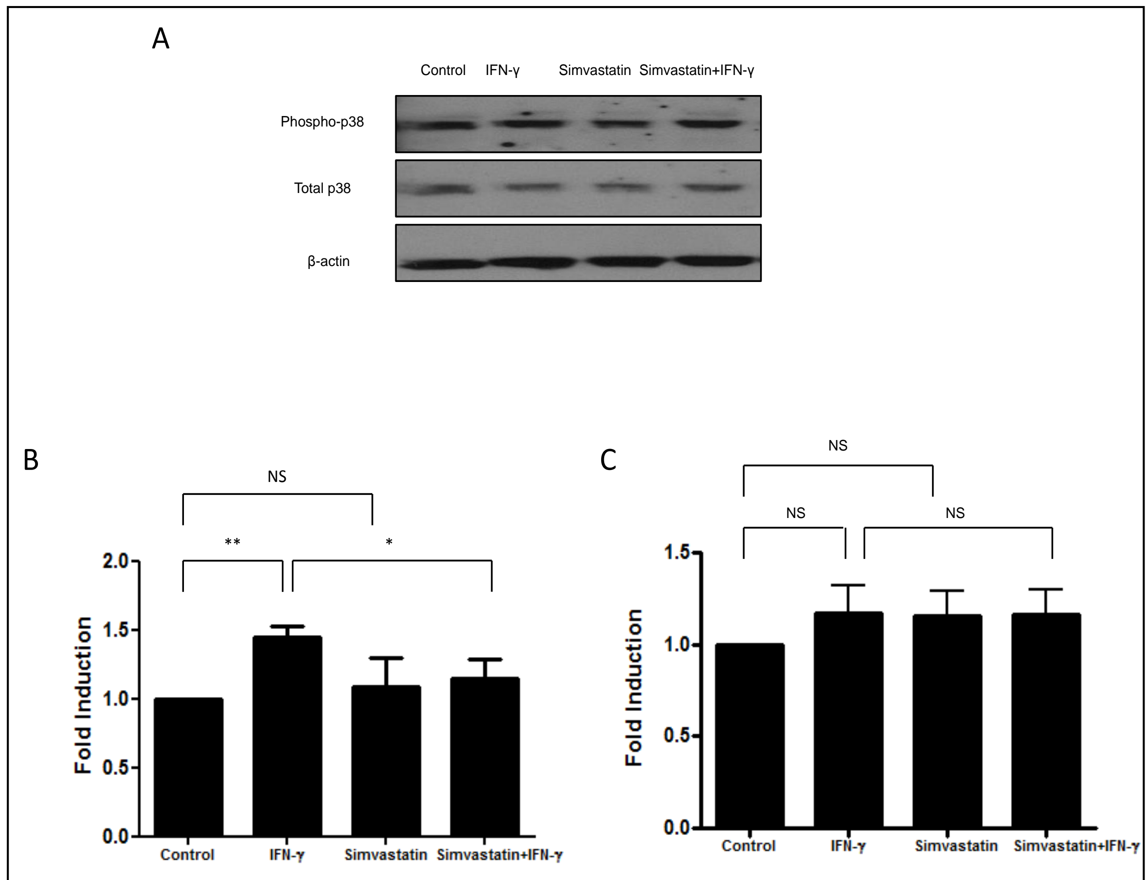

Figure 1.33: Effect of Simvastatin on phospho-p38

THP-1 macrophages were pre-treated with Simvastatin for 6 hours prior to IFN-γ treatment for 3 hours. Protein extracts were subjected to western blot analysis using antibodies specific for phospho-p38, total p38 and β-actin (A). Panel B shows the level of phospho-p38 normalised to total-p38 whereas panel C shows the average fold induction in total p38 normalised to β-actin. The data are from four independent experiments with the values in control sample being arbitrarily assigned as 1. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis, * p<0.05, ** p<.0.01, NS not significant.

4.5 Discussion

4.5.1 The effect of metabolites of cholesterol biosynthesis pathway on the IFN-γ induced MCP-1 mRNA expression

Statins are potent inhibitors of the endogenous mevalonate pathway and also play a role in the biosynthesis of cholesterol and isoprenoids (Goldstein and Brown 1990). Consequently, statins inhibit the biosynthesis of cholesterol and isoprenoid intermediates including GGP and FPP. The isoprenoids act as lipid attachment site for various intracellular proteins to the plasma membrane, including small guanosine triphosphate-binding proteins, such as Ras and Ras-like proteins (eg, Rho, Rac and Rab), resulting in their activation (Takai et al. 2001). Isoprenylation of these proteins, in turn, regulates the downstream signalling pathways involved in cytoskeletal organisation, motility, membrane trafficking, transcriptional activation, and cell proliferation and differentiation. Therefore, statins may interfere with these processes by depleting intracellular GGP and FPP, resulting in the inhibition of protein isoprenylation (Chow, 2009).

In the current study, Simvastatin was found to inhibit the IFN-γ induced expression of MCP-1 mRNA expression and that the inhibition was reversed by the addition of isoprenoid intermediates FPP and GGP toTHP-1 macrophages. Similary, Cakmak et al. (2012) found that addition of FPP and GGP to endometritic mice cells reversed the effect of Simvastatin in MCP-1 expression (Cakmak et al. 2012). Another study by Lee and his colleagues in RAW 264.7 cells showed that mevalonate and GGP reversed the Simvastatin-mediated inhibition of IFN-γ induced CIITA mRNA expression (Lee et al., 2008).

4.5.2 Simvastatin inhibits the IFN-γ induced STAT1 phosphorylation in THP-1 macrophages and primary HMDMs

As mentioned in Chapter 1, IFN-γ controls many steps in the pathogenesis of atherosclerosis, including the recruitment of inflammatory cells to the activated endothelium, foam cell formation, apoptosis and plaque stability (McLaren and Ramji, 2009; Li et al. 2010). The JAK-STAT pathway plays a key role in IFN-γ signalling (McLaren and Ramji, 2009). Simvastatin inhibited the IFN-γ-mediated STAT1 phosphorylation on Ser 727 and Tyr 701 (Figures 4.4 and 4.5). These findings are consistent with Lee and colleagues (2007) that showed that Simvastatin inhibits STAT1α at the transcriptional and protein levels in RAW264.7 macrophages (Lee et al. 2007). Moreover, a study by Townsend and colleagues found that in primary microglial cells, Atorvastatin (10 μM) inhibited Ser 727 phosphorylation of the STAT1 protein by IFN-γ (30 minutes) (Townsend et al., 2004). However, other studies have not found an effect of Simvastatin on STAT1 phosphorylation (Lee et al. 2007). For example, simvastatin does not affect the IFN-γ-induced STAT1 phosphorylation in human microvascular endothelial cells (Sadeghi et al. 2001). In addition, the results in this study are different from those by Li et al. (2011) in THP-1 macrophages where it was demonstrated that Simvastatin had no effect on STAT1 phosphorylation. Although the exact reasons are unclear, this may reflect the use of a lower concentration of simvastatin (10 μM) or a reduced treatment period with IFN-γ (30 minutes) (Li et al. 2011). Thus, the effect of Simvastatin on STAT1 phosphorylation in macrophages might be dependent on the concentration of the statin or the incubation time with the cytokine. Further dose response and time course experiments will be required to address this issue further.

4.5.3 Simvastatin attenuates the IFN-γ induced expression of MCP-1 and ICAM-1 in primary HMDMs and RAW 264.7 cells

To further confirm that the results obtained in THP-1 macrophages are not peculiar to the cell line, representative experiments were performed in primary cultures of HMDMs. A number of studies have previously shown conservation of responses between THP-1 macrophages and primary HMDMs (McLaren et al., 2010a; McLaren et al., 2010b). Consistent with these studies, Simvastatin inhibited the IFN-γ induced expression of MCP-1 and ICAM-1 mRNA levels along with STAT1 Ser727 and Tyr 701 phosphorylation in both THP-1 macrophages and HMDMs (Figures 4.4, 4.5, 4.10 and 4.11).

The simvastatin-mediated inhibition of the IFN-γ-induced expression of MCP-1 and ICAM-1 was also conserved in the mouse RAW264.7 cell line (Figure 4.6). Kota et al. (2006) have also found that IFN-γ induces MCP-1 gene expression in RAW 264.7 cells (Kota et al. 2006). A study by Romano et al. (2000) showed that Simvastatin and Lovastatin decreased MCP-1 production in peripheral blood mononuclear cells (PBMC) and ECs (Romano et al. 2000).

4.5.4 Effect of Simvastatin on the IFN-γ induced expression of ICAM-1

Numerous studies have analysed the effects of statins on ICAM-1 expression in various cellular systems (Lee et al., 2009; Chung et al, 2013; Takahashi et al., 2005). Atorvastatin was found to inhibit the constitutive expression of ICAM-1 in human umbilical vein ECs (Korybalska et al., 2012). Consistent with these studies, simvastatin was found to decrease ICAM-1 expression in THP-1 macrophages, HMDMs and RAW264.7 macrophages (Figures 4.6, 4.10 and 4.11).

4.5.4.1 Effects of Simvastatin on MAPKs Pathways

4.5.4.1.1 Effect of Simvastatin on JNK-1 and -2

The effect of IFN-γ on the JNK pathway is well known as it was found that IFN-γ and TNF-α work synergistically to activate JNK/SAPK in a pancreatic β cell line

(MIN6N8 cells) (Kim et al. 2005). Another study also showed that there was a very weak activation of JNK1 in bone marrow–derived macrophages after treatment with IFN-γ for more than 2 hours whereas JNK2 was present at undetectable levels (Valledor, Arpa, et al. 2008). JNK2$^{-/-}$ mice have decreased foam cell formation compared to wild-type or JNK1$^{-/-}$, thereby showing an important role for JNK2 in atherosclerosis (Ricci et al. 2004). Another study by Jin and his colleagues (2011) found that both the JNK inhibitor SP600125 and Simvastatin together protected porcine islets from apoptosis. Atorvastatin also inhibits JNK (ERK and p38) proteins (western blotting) in hyperlipidaemic mice (Shen et al. 2011). In recent study, It has been shown that Fluvastatin decreased phospho JNK levels in rat cardiomyocytes (Sakai et al. 2014).

As mentioned above, there were problems with the phospho JNK antibody, and hence the studies on the activation of this pathway investigated the key downstream target, c-Jun. For future work it may be useful to try phospho JNK from other sources or to produce the antibody in rabbits. However, the results from RT-qPCR showed that IFN-γ increases the expression of JNK-1 and -2 mRNA and this is not affected by treatment with Simvastatin (Figure 4.12).

4.5.4.2 Effect of Simvastatin on c-Jun

IFN-γ increased the expression of c-Jun, a finding that is in agreement with Rubio (1997) that showed that this cytokine induces the expression of c-Jun and c-Fos in astrocytes (Rubio 1997). Simvastatin had no effect on the IFN-γ induced expression of c-Jun mRNA levels (Figure 4.13). In contrast, Atorvastatin was found to significantly decrease c-Jun nuclear content in human and murine osteosarcoma cells (Fromigué et al. 2008). Another study also found that lovastatin and simvastatin reduces c-Jun expression in prostate cancer cells (Hoque et al. 2008). Moreover, a study by Sakai (2014) showed that Fluvastatin decreases c-Jun mRNA expression and phospho-c-Jun levels in rat cardiomyocytes (Sakai et al. 2014).

Results from western blotting did not show an induction of phospho c-Jun in THP-1 macrophages (Figure 4.14). This finding differs from the IFN-γ-mediated activation of c-Jun observed in wild type (wt) murine embryonic fibroblasts (MEFs) (Gough et al., 2007).

4.5.4.3 Effect of Simvastatin on ERK-1 and -2 (p44/42)

IFN-γ stimulates the phosphorylation of ERK-1 and -2 in (wt) MEFs (Gough et al. 2007). The phosphorylation-mediated activation of ERK-1/2 was decreased by simvastatin (40 µM) (Figure 4.17). The finding are consistent with those of Zhang et al. (2010) who found that Mevastatin decreases phosphorylation of ERK-1/2 in a dose dependent manner in cancer cells (SACC cells) (Zhang et al. 2010). Furthermore, statins (Simvastatin, Mevastatin and Fluvastatin) were found to inhibit ERK-1/2 phosphorylation in C6 glioma cells (Yanae et al. 2011). Another study found that atorvastatin inhibited ERK activation in neonatal rat cardiomyocytes (Liao et al. 2008). In RAW264.7 cells, Atorvastatin was also found to inhibit ERK-1/2 phosphorylation (Shao et al. 2012). A very recent study showed that Simvastatin decreases phospho-ERK and -p38 levels in human platelets suspension (Du et al. 2014). In contrast, lovastatin did not decrease phospho ERK levels in microglial cells (Townsend et al. 2004)

4.5.4.4 Effect of Simvastatin on p38

IFN-γ increased the expression of p38 mRNA levels and Simvastatin had no effect on this (Figure 4.18). An early study by Lehner and his collegues (2002) showed that the expression of IFN-γ was decreased in mice lacking MAPK-activated protein kinase 2 (MK2), which is a kinase activated by p38 (Lehner et al. 2002). Other studies also showed that there was a strong activation of p38 by IFN-γ treatment in primary BMDM (Valledor, Sánchez-Tilló, et al. 2008).

Simvastatin had no effect on the IFN-γ induced p38 mRNA expression (Figure 4.20) but significantly decreased the phosphorylation and the activation of the corresponding protein (Figure 4.20). This finding is in agreement with Lee and

collegues (2010) that showed that Simvastatin decreases p38 phosphorylation in suspension of human platelets (Lee et al. 2010). However, these studies contrast with that by Zhang et al. (2010) who found that treatment of human salivary adenoid cystic carcinoma (SACC) cells with Mevastatin increased p38 phosphorylation (Zhang et al. 2010).

The studies presented in this chapter determined the molecular mechanisms underlying Simvastatin in macrophages. Simvastatin and IFN-γ are also likely to modulate lipid profiles of macrophages in addition to intracellular cholesterol levels. Such studies formed the focus of the next chapter.

Chapter 5: Effects of Simvastatin on lipid profiles in THP-1 and RAW 264.7 cells

5.1 Introduction

Lipids are complex biological molecules; they are insoluble in water but soluble in organic solvents. Lipids play many important roles in all living organisms and are divided into two main groups: the non-polar lipids (triacylglycerols, sterols, free fatty acids, and sterol esters) and polar lipids (e.g. phosphoglycerides, or phospholipids) (Guschina and Harwood, 2009). Polar lipids and sterols are important structural components of cell membranes. In all organisms each membrane has its own characteristic lipid profile, which is tightly maintained because of functional requirements (Gurr et al. 2002). In addition to this structural function, some polar lipids (or their precursors) act as intermediates in cell signalling pathways (e.g. sphingolipids and inositol lipids) (Rustan and Drevon, 2005). As to the nonpolar lipids, triacylglycerols are common storage molecules, providing metabolic energy for many biochemical reactions and cellular functions.

Glycerolipids are synthesised from a glycerol (propane-1,2,3-triol) and fatty acids (FA). FAs are carboxylic acids (containing –COOH group) with aliphatic hydrocarbon chains of carbon atoms (Yeagle 2009). If the carbon-to-carbon bonds are all single, FA is classified as saturated. If any of the bonds are double or triple, the FA is classified as unsaturated. Over 1000 fatty acids are known with various chain lengths and positions, configurations and types of unsaturation. In most animal fats, saturated FAs with even number of carbon atoms (usually between 12-22) are dominant (Yeagle 2009). This feature of saturated FA allows them to pack tightly in a solid form, which enables them to accumulate inside the organs and arteries leading to pathophysiological conditions such as atherosclerosis (Gurr et al. 2002). On the other hand, unsaturated FAs have double bonds within the carbon chain that causes a "twist" in their physical structure to allow them to adopt an oil form at room temperature (Gurr et al. 2002). Unsaturated FAs can be arbitrarily divided into monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs) (Rustan and Drevon, 2005). MUFAs have a single double bond between two carbon atoms and the position of this double bond regulates its structure (configuration can either be *cis* or

trans) (Gurr et al. 2002). PUFA contains two or more double bonds of the *cis*-configuration separated by a single methylene group. Long chain PUFA are composed of 18 or more carbon atoms with 2 or more double bonds (Rustan and Drevon, 2005). From very many long chain PUFAs (LCPUFAs), two groups are widely known i.e. omega-3 (ω-3 or n-3) and -6 (or n-6) FAs named according to the positions of the 1st double bond i.e. between 3rd and 4th carbon atoms and 6th and 7th carbon atoms, respectively (Rustan and Drevon, 2005). n-3 PUFA comprise of α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), whereas n-6 PUFA include linoleic acid (LA) and arachidonic acid (AA) (Zuliani et al., 2009).

Due to the lack of enzymes, namely delta-12 and delta-15 desaturases (Δ12 and Δ15) in mammals, they are not able to synthesise linolenic and α-linolenic acids, and therefore the human body needs to obtain adequate amount of these essential FAs from the diet or plant foods (Gurr et al., 2002). The metabolic pathways of omega-3 and omega-6 fatty acids members are shown in Figure 5.1. The majority of omega-6 fatty acids (e.g. arachidonic acid) promote inflammation, whereas omega-3 fatty acids (e.g. EPA and DHA) have anti-inflammatory properties (Kang and Weylandt, 2008).

The mechanisms of n-3 and n-6 LCPUFA actions are related to the fact that these fatty acids are precursors for a wide range of biologically active metabolites. The LCPUFA metabolites, eicosanoids and docosanoids, are oxidation products produced by the activities of cyclooxygenases (COX), lipoxygenases (LOX), cytochrome P450 as well as by some non-enzymatic oxidation. Eicosanoids are synthesised from the C20 n-3 and n-6 LCPUFA. They include prostaglandins, leukotriens, thromboxanes, lipoxins and E-series resolvins. Docosanoids are produced from C22 LCPUFAs (Kruger et al. 2010). Additionally, DHA can produce protectins, docosatrienes and maresins, a novel class of macrophage mediators (Poudyal et al. 2011). DHA and EPA are known for their anti-inflammatory properties (Wall et al., 2010), while arachidonic acid is a proinflammatory (Caspar-Bauguil et al., 2012).

EPA and DHA are known for their anti-inflammatory actions, and have been found to reduce the risk of cardiovascular morbidity and mortality (Lusis 2000; Breslow 2006; Alberts et al. 2002; Bucher et al. 2002). This is not surprising given that atherosclerosis is an inflammatory disorder of the vasculature. Several studies have found that EPA and DHA have the ability to reduce plasma triglyceride levels and arterial stiffness (Kris-Etherton et al. 2002; Cleland et al. 2006).

Furthermore, n3 FAs have been found to lower plasma levels of the pro-inflammatory cytokines TNF-α and β in healthy subjects (Caughey et al. 1996). Additionally, n3 fatty acids contribute to regulating heart beats in patients with cardiac disease where it increases the fluidity of the membrane in cardiac cells and decreases the accessibility of inflammatory cytokines to bind to their receptors, thereby decreasing fatal and non-fatal cardiac events (Wilkinson et al. 2005; Ergas et al. 2002; Schwalfenberg 2006; Gallai et al. 1995).

The joint guidelines from the American Heart Association, American College of Cardiology, the European Society for Cardiology, and national Cardiac Societies recommend supplements of 1g/day of omega-3 fatty acids EPA and DHA for secondary prevention of cardiovascular disease, and for the treatment after myocardial infarction (Smith et al. 2006; De Backer et al. 2003; Van de Werf et al. 2003; Schacky and Harris, 2007). The n3 FAs are also very important to improve functions of numerous other systems including the immune system and the skin. Thus, supplementation of n3 fatty acids has been shown to be effective in inflammatory diseases such rheumatoid arthritis (Goldberg and Katz, 2007). A number of studies have shown that n3 fatty acid reduce the pain intensity and morning stiffness in patients with rheumatoid arthritis, and reduce lipids profiles in patients with type 2 diabetes (Goldberg and Katz 2007; Jeppesen et al., 2013).

Previously, statins have shown a strong suppression effect on inflammatory processes in cardiovascular disease, as they have pleotropic actions beyond lowering plasma cholesterol. Treatment with statins and omega-3 FAs after myocardial infarction (MI) synergistically decreased mortality and fatal cardiovascular events

(Macchia et al. 2013). In a study by Fritsche et al. (1999) in mice with *Listeria* infection, treatment with omega-3 fatty acids from fish oil in its early stage was found to reduce IL-12 and IFN-γ production. Potential effect of statins include enhancing fatty acids synthesis, stimulatory peroxisomal activity and increasing arachidonic acid and thromboxane production (Williams et al. 1992; Hrboticky et al. 1994) . Statins also decrease Cox-2 and MMP-9 expression and activity in the vascular endothelium (Massaro et al. 2010). In THP-1 cells, Simvastatin in particular, increases the conversion of exogenous linoleic and EPA to their long-chain PUFA derivatives (Risé et al. 1997).

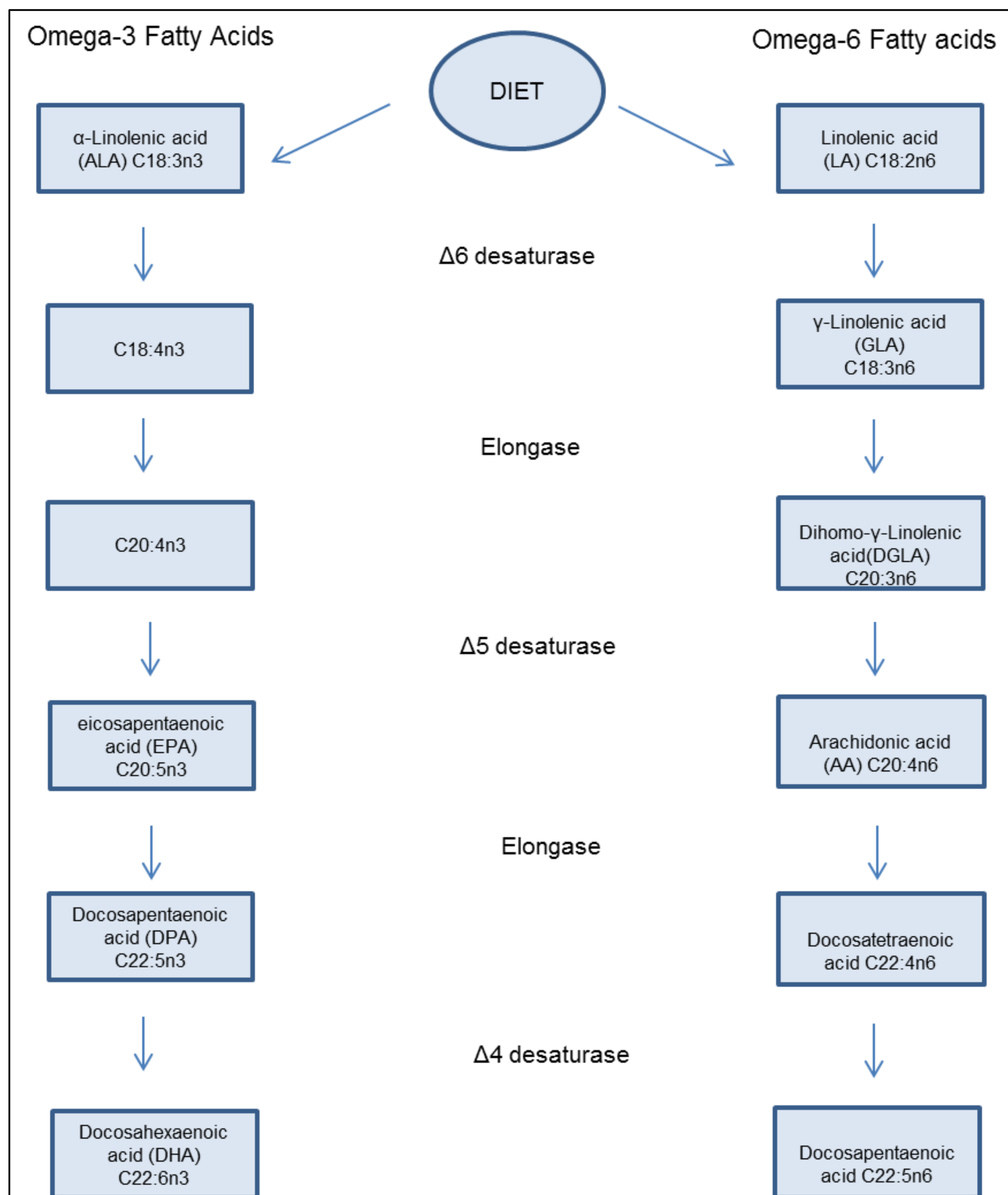

Figure 0.1: Omega-3 and omega-6 fatty acid metabolic pathways.

The longer chain n-3 and n-6 PUFAs can originate from the dietary 18-carbon precursors ALA and LA, respectively. Through a series of elongation enzymes that sequentially add 2-carbon units to the FA backbone and desaturation enzymes that insert double bonds into the molecules, longer-chain PUFAs are synthesised. The most important LCPUFAs with regards to health are AA, EPA and DHA.

5.2 Aims and experimental strategies

Atherosclerosis is a progressive disease that is characterised by lipid accumulation and inflammation within the walls of the large and medium arteries (Lusis 2000). The main aim of the studies presented in this chapter was to study the lipid and FA profile of THP-1 and RAW 264.7 macrophages, and to investigate the effect of Simvastatin on these parameters in macrophages. To achieve this, two experimental approaches were used (Figure 5.2). In the first approach, radiolabelled acetate was used in order to study the synthesis of the major lipid classes, namely total polar lipids (TPL), cholesterol (CHO) and triglycerides (TAG) in THP-1 macrophages. Thus, vehicle-treated cells or those stimulated with IFN-γ in the absence or the presence of Simvastatin, FPP or GGP were analysed (Chapter 4). In the second approach, the lipid and FA profiles of two different macrophage systems (THP-1 and RAW 264.7) were analysed using various chromatographic techniques. The cells were treated with vehicle or IFN-γ (with or without Simvastatin) in order to identify any possible effect of Simvastatin on lipid composition of the cells.

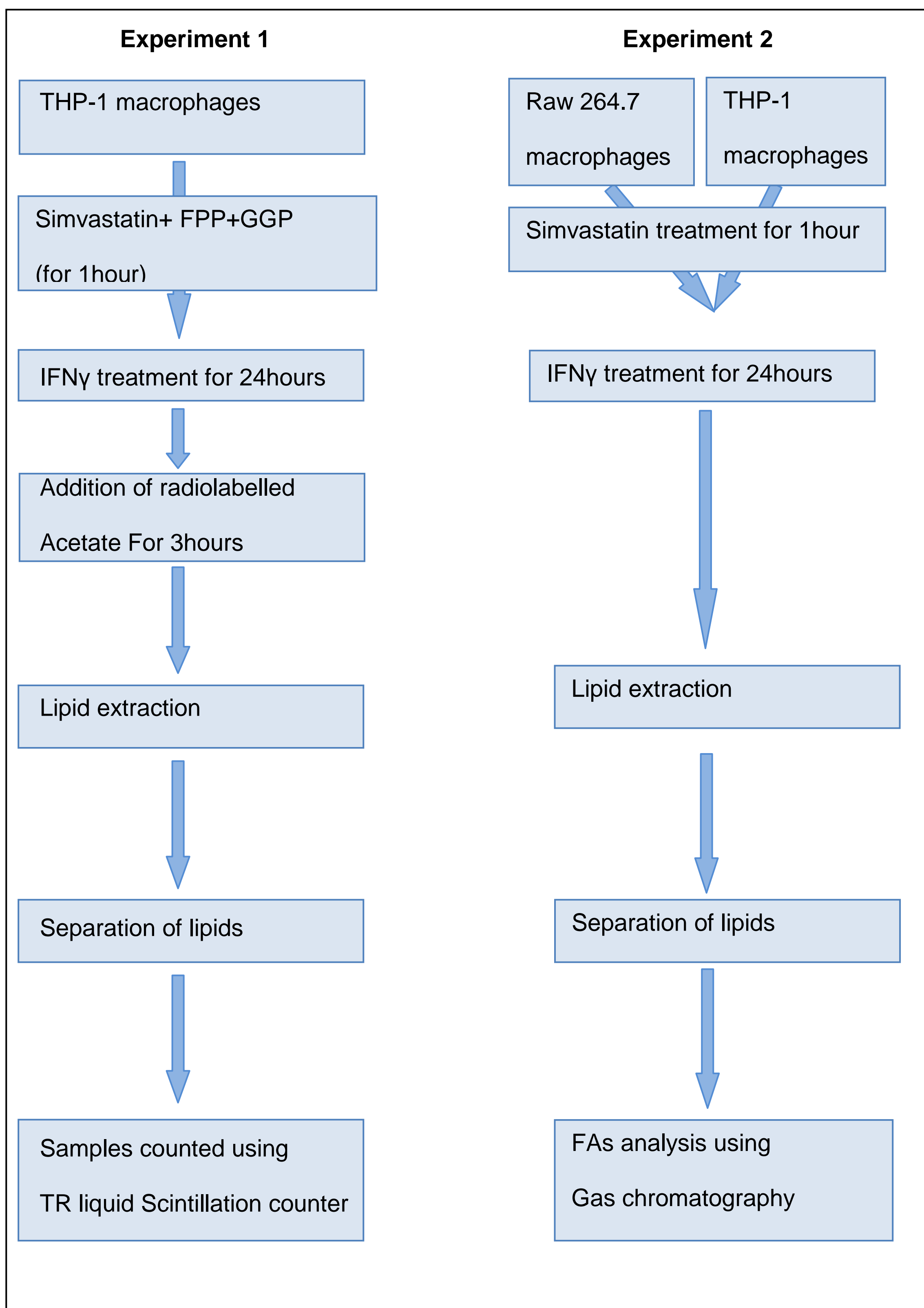

Figure 0.2: Experimental Strategies

THP-1 and RAW 264.7 macrophages were used. The two experimental regimes used in this chapter are shown. These studies were carried out using activated Simvastatin in DMSO with pre-treatment time of 1 hour.

5.3 Results

5.3.1 The effect of Simvastatin on the biosynthesis of the major lipid classes and cholesterol in THP-1 macrophages

THP-1 macrophages were treated with vehicle DMSO or Simvastatin (40 µM) with or without FPP (5 µM) or GGP (5 µM) for 1 hour followed by IFN-γ for 24 hours. The cells were then incubated with 0.5 µCi of sodium [1-^{14}C] acetate for 3 hours. After incubation, cells were separated by centrifugation and collected. Lipids were extracted and separated into three major fractions: TPL, CHO and TAG using one-dimensional TLC, to measure the incorporation of radioactivity into individual lipid classes. The samples were counted in a Perkin-Elmer Tri-Carb 2800 TR liquid scintillation counter. This experiment was performed only once with three replicates so no statistics was performed.

As shown in Figure 5.3, IFN-γ produced slight increase in the labelling of TPL and CHO compared to the control DMSO, whereas there was a slight reduction in the synthesis of TAG. Addition of Simvastatin resulted in a 50% reduction in CHO biosynthesis as expected. Such a reduction was maintained following addition of either FPP or GGP together with Simvastatin. On the other hand, the levels of TPL or TAG radiolabelling observed in IFN-γ treated cells changed only slightly in the presence of Simvastatin, FPP or GGP.

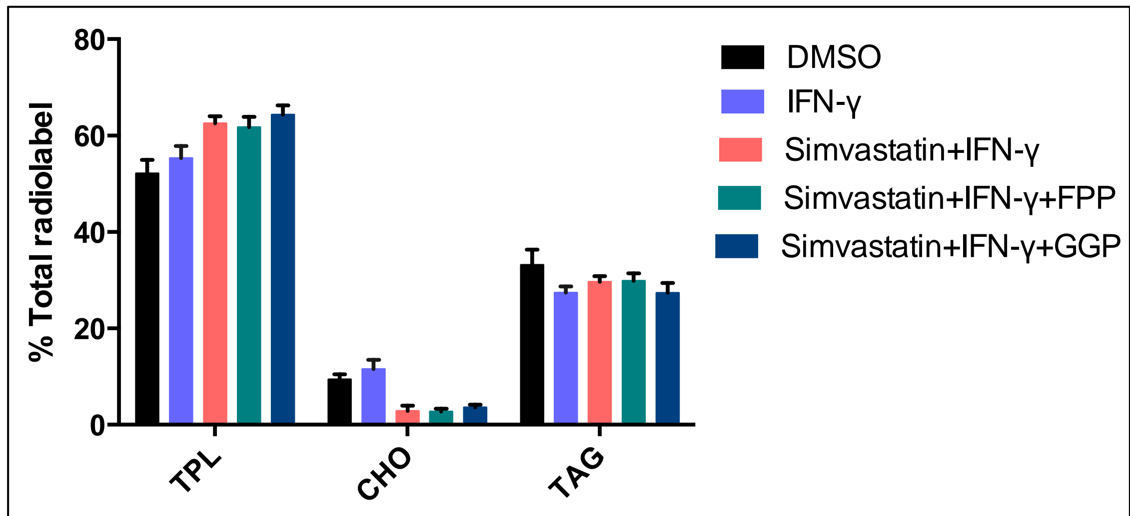

Figure 0.3: Effect of IFN-γ, Simvastatin, FPP or GGP on the relative incorporation of [1-^{14}C] acetate into three major lipid classes: total polar lipids (TPL); free cholesterol (CHO) and triacylglycerols (TAG) in THP-1 macrophages.

5.4 Effect of IFN-γ and Simvastatin on FA distribution into polar lipids and TAG in THP-1 macrophages

THP-1 macrophages were treated with Simvastatin (40 µM) for 1 hour and then IFN-γ was added to the cells for 24 hours (cells incubated with DMSO or IFN-γ alone was included for comparison). Lipids were extracted and then separated into polar lipids and TAG using thin-layer chromatography. FAs of both fractions were analysed by gas chromatography. Figure 5.4 shows changes in FAs profile in polar lipid fraction (see Table 5.1 for list of FAs). In this fraction, three fatty acids were dominant: palmitic (C16:0), oleic (C18:1n9) and stearic (C18:0). Their relative amounts were around 20% of the total FA. The amounts of linoleic (C18:2n6), vaccenic (C18:1n7) and arachidonic (C20:4n6) acids were found in the relative amounts that did not exceed 10%. Long chain polyunsaturated fatty acids (LCPUFA), EPA (C20:5n-3), DHA (C22:6n3) and DPA (C22:5n-3) were present in this fraction varying from 2% to 5% of total FAs. No significant changes in fatty acid composition were produced with the various treatments from four independent experiments.

Figure 5.5 shows the FA profile in TAG fraction from THP-1 macrophages. In this fraction, palmitic (C16:0) and oleic acids (C18:1n9) were the major FA reaching up to 20-25% of total FA. Stearic (C18:0), vaccenic (C18:1n7) and linoleic acids (C18:2n6) were present in TAG at the levels of around 10% of total FA. The relative amounts of arachidonic acid (C20:4n6) and DPA (C22:5n3) were lower in TAG in comparison to TPL whereas the levels of other LCPUFA were similar to that found in the fraction of TPL. Similar to polar lipids, no significant changes in fatty acid composition were produced by any of the treatments (see Table 5.1 for list of fatty acids).

Table 0.1: List of FAs, their common names and abbreviations.

Fatty acid profile	Common name	Abbreviation
C14:0	Myristic acid	
C16:0	Palmitic acid (Palmitate)	
c16:1n7	Palmitoleic acid	
c16:1n9	Palmitoleic acid	
C18:0	Stearic acid	
C18:1n7	cis-Vaccenic acid	
C18:1n9	Oleic acid	
C18:2n6	Linoleic acid	LA
C18:3n3	Alpha-linolenic acid	ALA
C18:3n6	Gamma-linolenic acid	GLA
C18:4n3	Stearidonic acid	SDA
C20:1n9	Eicosenoic acid	
C20:2n6	Eicosadienoic acid	EDA
C20:3n3	Eicosatrienoic acid	ETE
C20:3n6	Dihomo-gamma-linolenic	DGLA
C20:4n3	Eicosatetraenoic acid	ETA
C20:4n6	Arachidonic acid	AA
C20:5n3	Eicosapentaenoic acid	EPA
C22:0	Behenic	
C22:5n3	Docosapentaenoic acid	DPA
C22:6n3	Docosahexaenoic acid	DHA
C24:0	Lignoceric acid	
C24:1n6	Nervonic acid	

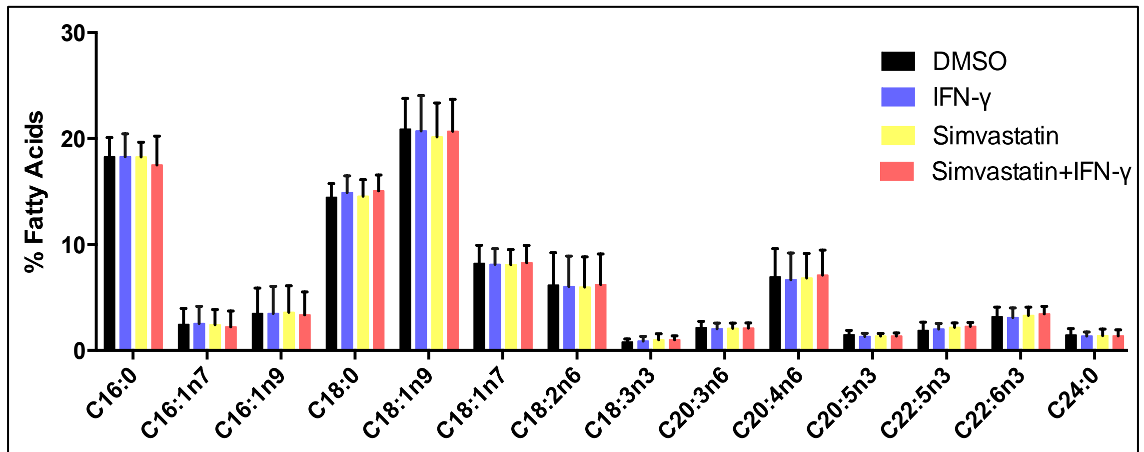

Figure 0.4 Effect of IFN-γ and Simvastatin on FA distribution (% of total fatty acids) in polar lipids from THP-1 cells.

FAs are indicated with the number before the colon showing the number of carbon atoms, the figure afterwards denoting the number of double bonds followed by the position of the first double bond. Data are means ± SD from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis

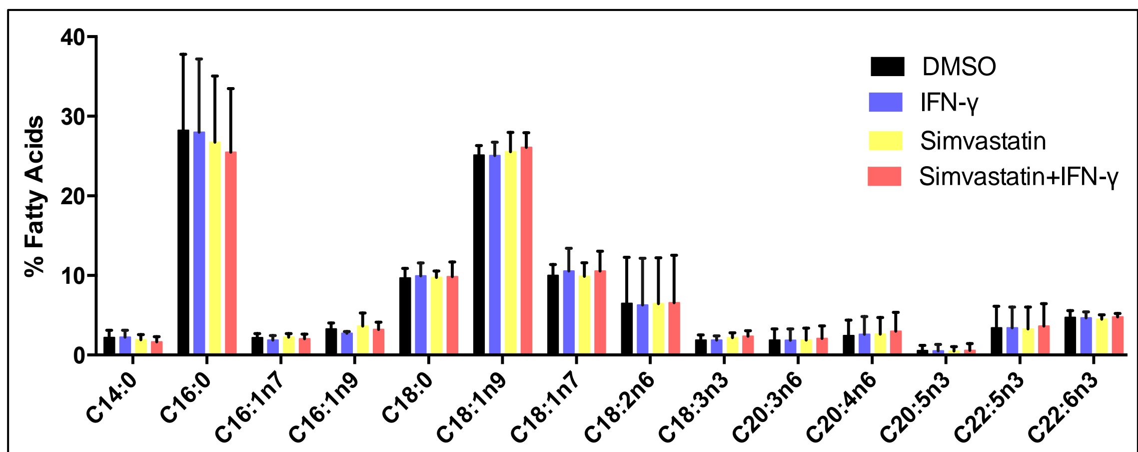

Figure 0.5: Effect of IFN-γ and Simvastatin on FA distribution (% of total fatty acids) in TAG from THP-1 cells.

FAs are indicated with the number before the colon showing the number of carbon atoms, the figure afterwards denoting the number of double bonds followed by the position of the first double bond. Data are mean ± SD of four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis.

5.5 Effects of IFN-γ and Simvastatin on FA distribution in polar lipids and TAG from RAW 264.7 cells

RAW264.7 macrophages were pre-treated with DMSO vehicle or Simvastatin (40 μM) for 1 hour and then incubated for 24 hours with IFN-γ. Lipids were extracted and were separated into three fractions: PL; TAG; and CE. However, only the data for PL and TAG are presented as the CE were at undetectable levels. Figure 5.6 shows the changes in FAs from PL in RAW264.7 cells.

The major FAs were palmitic (C16:0), stearic (C18:0), oleic (C18:1n9) and vaccenic (C18:1n7) acids varying from 15% to 29% of total FA. Palmitoleic (C16:1n9) and linoleic acids (C18:2n6) were also present in this fraction at the level of around 5%. Among LCPUFA, arachidonic acid (C20:4n6), DHA (C22:6n3) and DPA (C22:5n3) were the major FAs and their levels were 4-6%. The relative amounts of other fatty acids did not exceed 2%. Similar to THP-1 macrophages, no significant changes were observed with any of the treatment.

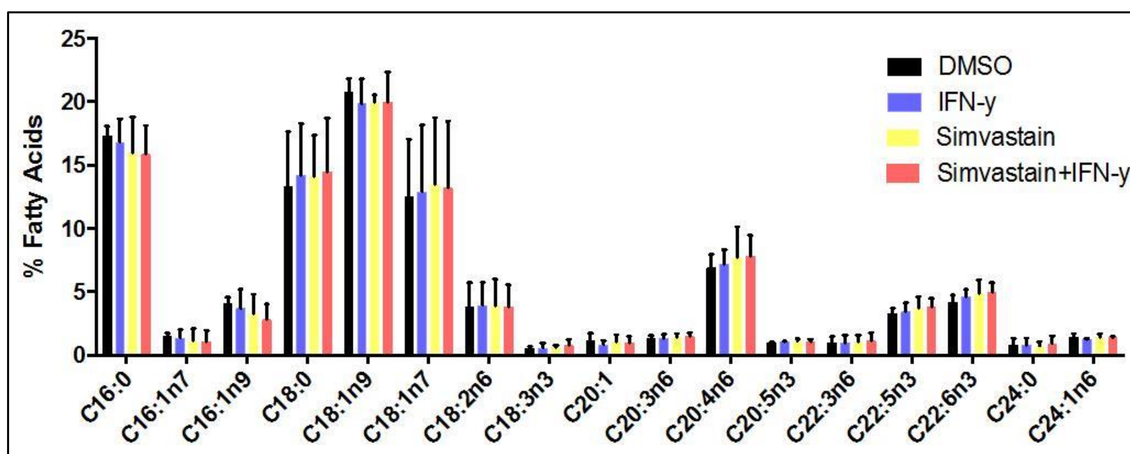

Figure 0.6: Effect of IFN-γ and Simvastatin on FA distribution (% of total FAs) in polar lipids from RAW264.7 cells.

FAs are indicated with the number before the colon showing the number of carbon atoms, the figure afterwards denoting the number of double bonds followed by the position of the first double bond. Data are mean ± SD of three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis.

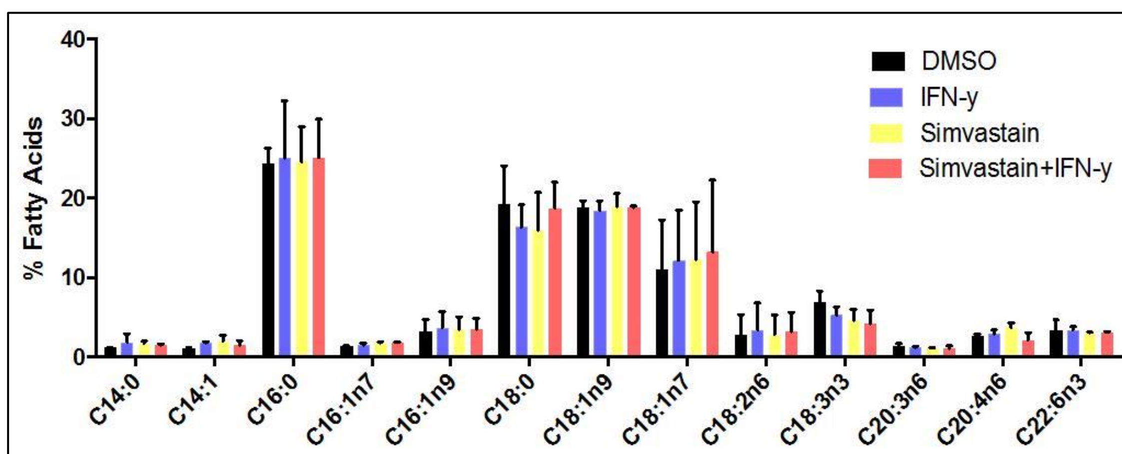

Figure 0.7: Effect of IFN-γ and Simvastatin on FA distribution (% of total FAs) in triacylglycerols from RAW264.7 cells.

FAs are indicated with the number before the colon showing the number of carbon atoms, the figure afterwards denoting the number of double bonds followed by the position of the first double bond. Data are mean ± SD of three independent experiments**.** Statistical analysis was performed using one-way ANOVA with Tukeys post-hoc analysis.

Figure 5.7 indicates changes in FAs profile in TAG. The major FAs were palmitic (C16:0), stearic (C18:0) and two isomers of C18:1 fatty acids: oleic (C18:1n9) and vaccenic (C18:1n7) acids. In TAG fraction, linolenic acid (C18:2n6) was found in the relative large amount (4% of total fatty acids) in comparison to the fraction of polar lipids. Only three LCPUFA were present in TAG from RAW 262.7 cells: arachidonic acid (C20:4n6), DHA (C22:6n3) and DGLA (C20:3n6). Their levels did not exceed 3-5%. Similar to THP-1 macrophages, no significant changes were observed with any of the treatments.

5.6 Discussion

5.6.1 The effect of Simvastatin on the synthesis of the major lipid classes and cholesterol in the THP-1 cell line

Atherosclerosis is an inflammatory disease of the vasculature and characterised by lipid accumulation within the walls of arteries (Lusis et al., 2004). Statins, the most widely used drugs for the treatment of hypercholesterolemia, act through the inhibition of the HMG-CoA reductase, a key enzyme in cholesterol biosynthesis. Statins have been shown to decrease cholesterol level both *in vitro* and *in vivo* (Jenkins et al., 2005).

In this chapter, the effect of Simvastatin on the synthesis of the major lipid classes in THP-1 cells was studied when the cells were treated with IFN-γ. In addition, two intermediates of the isoprenoid biosynthesis, FPP and GGP, were used in order to better understand the mechanisms involved in the reduction of intracellular cholesterol by Simvastatin treatment.

IFN-γ produced a slight increase in total free cholesterol levels, which were reduced by almost 50% in the presence of Simvastatin (Figure 5.3). The labelling of the total PLs was not affected by IFN-γ whereas synthesis of TAG was slightly decreased under this treatment. Simvastatin alone, as well as in combination with each intermediate, slightly increased the incorporation of radiolabel into PLs but did not affect the synthesis of TAG. In some previous work where an effect of simvastatin on *de novo* lipid and cholesterol synthesis has been studied in THP-1 cells, it has been shown that treatment with Simvastatin (5 μM, 24 hours) caused a significant (up to 90%) inhibition of cholesterol synthesis (Risé et al., 1997). Synthesis of neither PLs nor TAG from radiolabelled acetate was affected in that study that may be due to the lower concentration of Simvastatin used as well as an additional effect of IFN-γ. However, when [^{3}H] glycerol was used as a radiolabel, an increase in the labelling of TAG with a concomitant reduction of PL synthesis was noted (Risé et al., 1997). In

hepato carcinoma cell line Hep G2, simvastatin also inhibited the synthesis of cholesterol and cholesterol esters from [^{14}C] acetate, but did not affect phospholipid, free FAs and TAG synthesis (Nagata et al., 1990).

In this study, the addition of downstream metabolites of HMG-CoA reductase, FPP or GGP, did not reverse the inhibitory effect of Simvastatin on cholesterol synthesis. These finding demonstrate that the reduction in intracellular cholesterol levels are due to inhibition of HMG-CoA reductase and not because of an effect on signalling pathways due to depletion of intracellular lipid metabolites. Moreover, isoprenoid intermediates have been shown to affect the activation of receptors, which are involved in the regulation of cellular cholesterol metabolism, e.g. PPARα, PPARγ and LXR (Argmann et al., 2003). It has been also shown that Simvastatin affects PUFA metabolism in THP-1 cells, and this effect is partly prevented by mevalonate and GGP, but not FPP (Risé et al., 1997). Thus, the pathways for cholesterol and PUFA synthesis have been suggested to be mutually modulated (Risé et al., 1997).

5.6.2 Effects of IFN-γ and Simvastatin on fatty acid distribution in polar and TAG lipids from THP-1 and RAW 264.7 Cells

In this work, the fatty acid composition of the two major lipid classes, PLs and TAG, have been studied in order to reveal some possible changes as a result of IFN-γ and/or Simvastatin treatment. Previously, it had been shown that IFN-γ influenced the composition or synthesis of phospholipids in different cell types (Mattila et al., 1993). An increased amount of linoleic and arachidonic acids in phosphatidylethanolamine (PE) in both murine peritoneal macrophages and macrophage-like cell line (P388D) have been demonstrated by IFN-γ (Jackson et al. 1992). The same group later showed a redistribution of linoleic acid among different lipid classes and found no increase in incorporation into saturated stearic acid in IFN-γ-activated cells (Darmani et al., 1994). Also, the liberation of arachidonic acid from phospholipids as well as a release of linoleic acid from PE in BCG-sensitive murine peritoneal macrophages have been shown as an effect of endotoxin (Stark et al. 1990). The authors suggested that IFN-γ may increase host susceptibility to endotoxin by increasing the level of

PUFA in the membrane lipids of macrophages which may be metabolised to produce biologically active metabolites: mediators and signals related to defence reactions (Darmani et al. 1994).

In the present study, no statistically significant effect of IFN-γ on FA composition of either membrane PLs or stored TAG was found. This maybe potentially because of many possible factors/differences related to the treatment, culturing conditions and data analysis. For example the IFN-γ treatment time are different than others previous studies that used for 18 hours, 24 hours, and 48 hours. Another reason would be that we studied the actual relative concentrations of individual FAs but not a turnover of specifications, e.g. arachidonic acid. In addition, combining the data from four independent experiments resulted in relatively large standard deviations that did not show any statistically significant differences between controls and the treatment groups.

Statins affect not only cholesterol biosynthesis but also PUFA production and it has been shown that they cause an elevated accumulation of arachidonic acid (a conversion product of linoleic acid) *in vitro* and *in vivo* (Risé et al., 1997; Risé et al., 2001). For Simvastatin, this has been demonstrated using a range of concentrations from 0.1 to 0.5 μM. It is interesting that an increased linoleic acid conversion was accompanied by a stronger inhibition of cholesterol synthesis (Risé et al., 2003). In more recent studies, this group showed that Simvastatin increased the expression of Δ5 desaturase, an enzyme directly involved in the production of arachidonic acid production from C20:3 FAs (Risé et al., 2007).These previous studies on the effects of Simvastatin and PUFA biosynthesis were carried out using radiolabelled FA precursors, e.g. radiolabelled linoleic acid. In that case, a turnover of a fatty acid in its metabolic product would take only few hours whereas in the case of our study, more time was required to show the differences at the level of actual FA concentrations. IFN-γ was found to increase PUFA profile in peritoneal macrophages (Jackson et al. 1992). Similarly, IFN-γ increased the percentage of C18:2n6 in TAG of RAW264.7 macrophages though this failed to reach significance (Figure 5.7). Overall, the precise

reasons for the differences seen with previous studies are unclear but could represent variations between cell line and primary cultures and also differences in treatments and time points.

Chapter 6: General Discussion

6.1 General Discussion:

Atherosclerosis is a disorder of large and medium arteries and a major cause of CD. It's a progressive inflammatory disorder that's regulated by cytokines at all stages. Several cytokines are released at different stages of the disease and have been studied in relation to them being pro- or anti-atherogenic. Pro-atherogenic cytokines including TNF-α, IFN-γ, IL-1, IL-17 and IL-12, are observed in atherosclerosic lesions whereas IL-10, IL-4, IL-33 and TGF-β are anti-inflammatory cytokines generally present at lower levels (McLaren et al. 2011; Ait-Oufella et al. 2011).

Currently, statins are the most effective drugs used in the prevention and treatment of atherosclerosis and its adverse complications. Although statins are mainly used to lower circulating levels of LDL-cholesterol by inhibiting HMG-CoA reductase, they have multiple pleiotropic effects such as acting in an anti-inflammatory manner (Table 6.1). The anti-inflammatory actions of statins are not fully understood particularly in relation to newer cytokines such as TL1A and IL-17 along with classical cytokines such as IFN-γ. Thus, the work presented in this thesis concentrated on such studies in relation to the actions of TL1A, IL-17A and IFN-γ in the first experimental chapter and after this it was decided to focus on the inhibitory action of Simvastatin on IFN-γ signalling. The studies were carried out on THP-1 macrophages, RAW 264.7 cells and primary HMDMs.

The role of IFN-γ in the progression of atherosclerosis is still debatable due to its dual action. Although IFN-γ is known to be a pro-inflammatory cytokine, it also demonstrates some anti-inflammatory properties (Mühl and Pfeilschifter 2003). The anti-inflammatory role of IFN-γ includes inhibition of macrophage LPL expression and the oxidation of LDL (Jonasson et al., 1990; Hughes et al., 2002). IFN-γ has been shown to also to control many features of atherosclerosis such as foam cell formation, the adaptive T_h1 specific immune response and plaque development (McLaren and Ramji 2009). In mouse model systems, IFN-γ has been demonstrated to decrease

cholesterol efflux from peritoneal macrophages by inhibiting ABCA1 expression (Panousis and Zuckerman, 2000). Overall, the proatherogenic activities of IFN-γ is associated with the stimulation of foam cell formation through the induction of cholesterol uptake and the reduction of cholesterol efflux, which consequently disrupts cholesterol homeostasis (McLaren and Ramji, 2009). In addition, IFN-γ facilitates monocyte infiltration into the atherosclerotic lesion by augmenting the expression of ICAM-1 and VCAM-1 in both ECs and SMCs (Li et al., 1993; Chung et al., 2002). A number of studies have also found that IFN-γ plays a significant role in reducing the stability of the fibrous cap around atherosclerotic plaques (Gupta et al., 1997; Hansson and Libby, 2006). Furthermore, IFN-γ enhances the activity of TF, which stimulates thrombosis in contact with blood (Nakagomi et al., 2000).

Compelling evidence has shown that statin therapy influences IFN-γ production and signalling (Chung et al., 2002; Schönbeck and Libby, 2004; Hakamada-Taguchi et al., 2003). For example, Atorvastatin and Cerivastatin have shown the ability to inhibit the T_h1 immune response by reducing the secretion of IFN-γ, along with other T_h1 cytokines like IL-2, from activated T-lymphocytes and by promoting the release of T_h2 cytokines like IL-4 (Schönbeck and Libby, 2004; Hakamada-Taguchi et al., 2003). Simvastatin and Fluvastatin have also been found to decrease IFN-γ release from phytohemagglutinin-stimulated lymphocytes in patients with primary type II dyslipidemia (Okopień et al., 2004).

Table 6.1: Effect of statins on inflammatory biomarkers.

Type of Statin	Effect of Statin	Reference
Pravastatin and Simvastatin	Results in rapid and significant improvement in endothelial dysfunction in patients with high cholesterol.	(Mason, 2003; Wolfrum et al., 2003).
Atorvastatin	Decreases the levels of ICAM-1 in patients with heart failure from left ventricular remodeling.	(Tousoulis et al., 2013).
Simvastatin	Decreases MCP-1, MIP-1α and β.	(Veillard et al., 2006).
Atorvastatin	Reduces post-ischemic brain damage and improves neurological outcome by inhibiting oxidative stress and inflammatory responses.	(Saito et al., 2014).
Simvastatin	Has anti-inflammatory effects in patients with rheumatoid arthritis.	(Cojocaru et al., 2013).
Simvastatin	Reduces IL-8, IL-6 and MCP-1 levels in patients with high cholesterol.	(Rezaie-Majd, 2002).
Simvastatin	Inhibits the proliferation, and induces apoptosis, of human lung cancer cells.	(Yu et al., 2013)
Atorvastatin	Reduces levels of CRP in patients with type 2 diabetes.	(Sathyapalan et al., 2010)

6.2 Anti–inflammatory effects of statins and clinical outcomes

A number of large randomised clinical studies have shown that the beneficial effects of statins are beyond lowering cholesterol level at reducing incidents of CVD (Sacks et al., 1996; Downs et al., 1998*)*. For example, the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) trial demonstrated that lovastatin reduced cardiovascular events in patients with low LDL cholesterol and high CRP levels, whereas it had no clinical benefit in patients with both low LDL cholesterol and low CRP levels (Downs et al., 1998). These findings suggested that patients with inflammatory disease (i.e. increased CRP levels) could constitute an additional group that could benefit from statin treatment, irrespective of their baseline LDL cholesterol levels. Moreover, the effect of statins treatment is not just limited to the reduction of CHD risk, but it also extends to reducing the risk of stroke (Byington et al.,2001; Amarenco et al.,2007). In the SPARCL (Stroke Prevention by Aggressive Reduction in Cholesterol Levels) trial, high dose of Atorvastatin treatment (80mg/day) in patients with previous history of cerebrovascular accident (CVA) but without known risk of CHD improved cardiovascular outcomes. After a follow up of nearly five years, Atorvastatin reduced ischemic stroke risk by 33% and risk of major CHD by 37% (Amarenco et al., 2007).

The MIRACL (Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering) study provided further evidence on the anti-inflammatory effects of statins in high risk subjects (Schwartz et al., 2001). Treatment with high dose of Atorvastatin (80mg/day) for 16 weeks in patients with unstable angina or recent MI showed significantly reduction in cardiac events, particularly symptomatic ischemia (Schwartz et al., 2001). This population had low baseline LDL cholesterol levels (124mg/dL) that were significantly reduced at 16 weeks (from 124 to 72mg/dL). Atorvastatin also reduced the levels of inflammatory biomarkers including CRP and serum amyloid A which are independent risk predictors of stroke (Kinlay et al., 2003; Kinlay et al., 2008).

The timing and dosage selection of statins is also crucial in controlling anti-inflammatory effects post-acute MI event (Sposito et al., 2011). For example, additional administration of high-dose Atorvastatin before percutaneous coronary intervention (PCI) further reduced periprocedular myocardial injury and 30-day cardiovascular morbidity in patients who were already on chronic statin treatment (Di Sciascio et al.,2009).

These important findings provide strong evidence that administration of statin therapy could be beneficial for patients with normal cholesterolemia for the prevention of CHD based on their anti-inflammatory, plaque stabilising and overall vasoprotective effects. Statins therapy could also be extended to individuals who are at great risk, or have had a previous history, of CVA.

In the light of current extensive experimental evidence, statins also seem to be an attractive option for therapy of patients with autoimmune or inflammatory diseases, indeed clinical evidence has demonstrated that statins have a potent ability to be beneficial in a number of pathological disorders including osteoporosis, Alzheimer's disease, Parkinson's disease, multiple sclerosis and rheumatic diseases, all of which involve inflammatory responses (Paraskevas et al., 2007). Statins administration post lung transplantation has also been associated with improved survival and improved grafted lung function (Li et al., 2011*)*. Additionally, combining Rosuvastatin with indomethacin treatment in patients with acute pericarditis resulted in greater reduction in CRP levels; pericardial effusion and ST segment normalisation, though it did not reduce the hospitalisation period (Di Pasquale et al., 2007).

6.3 Results and Perspective

6.3.1 The effect of statins on the IFN-γ induced mRNA expression of MCP-1 and ICAM-1

The result of this study found that IFN-γ induced the expression of the MCP-1 and ICAM-1 genes in THP-1 macrophages, which was inhibited by the action of Simvastatin (Figures 3.5 and 3.6). These results in are in agreement with previous studies in a wide range of cell types (Zhou et al, 2001; Martin-Ventura, 2009; Chang et al, 2002). Similar findings have also been found in previous studies that demonstrated that statins attenuate the expression of ICAM-1 induced by IFN-γ in the ECV304 cell line (Chung et al, 2002).

In order to examine whether the results obtained are peculiar to Simvastatin, representative experiments were carried out using Atorvastatin. The data showed that Atorvastatin attenuated the IFN-γ induced expression of MCP-1 and ICAM-1 (Figures 3.12 and 3.13). As there are no studies that have examined the effect of Atorvastatin on the inhibition of IFN-γ induced expression of MCP-1 and ICAM-1, it is not possible to make any comparisons. In swine, Martinez-Gonzalez and colleagues (2001) found that Atorvastatin reduced the expression of MCP-1. This indicates that downregulation of MCP-1 expression by statins could reduce the inflammation within the vascular wall and inhibit the progression of atherosclerotic lesions. Overall, the results in this chapter suggest that statins have an anti-inflammatory and immunomodulatory effect in human macrophages. This may be useful for patients with inflammatory disease who could benefit from taking statins, which may attenuate or inhibit the sources of inflammation.

6.3.2 Effect of TL1A on ApoE mRNA expression

The results of this experiment demonstrated that TL1A decreased the expression of ApoE (Figure 3.11). However, when THP-1 macrophages were treated with Simvastatin there was no further change observed in the expression of ApoE. A similar finding has been reported by McLaren and colleagues (2010a) who showed that TL1A significantly decreased the expression of ApoE in THP-1 macrophages and HMDMs. However, the action of Simvastatin on TLA1-mediated regulation of ApoE expression has previously not been analysed so further studies will be required for confirmation, particularly in other macrophage systems such as primary cultures

6.3.3 The effect of metabolites of cholesterol biosynthesis pathway on the IFN-γ induced MCP-1 mRNA expression

In the current study, Simvastatin was found to inhibit the IFN-γ induced expression of MCP-1 mRNA expression and this inhibition was reversed, at least in part, by the addition of isoprenoid intermediates FPP and GGP in THP-1 macrophages. Statins are potent inhibitors of the endogenous mevalonate pathway and also play a role in the inhibition of biosynthesis of cholesterol and isoprenoid intermediates including GGP and FPP (Goldstein and Brown 1990). Similar findings have been reported in a study by Cakmak et al. (2012) who found that addition of FPP and GGP in endometritic mice cells reversed the effect of Simvastatin on constitutive MCP-1 expression. Another study by Lee and colleagues (2008) in RAW 264.7 cells demonstrated that mevalonate and GGP reversed the Simvastatin-mediated inhibition of IFN-γ induced CIITA mRNA expression. These findings suggest that isoprenoid generation through the mevalonate pathway may be one of the potential mechanisms for regulation of MCP-1 expression by statins in THP-1 macrophages and atherosclerosis.

6.3.4 Simvastatin inhibits the IFN-γ induced STAT1 phosphorylation in THP-1 macrophages and primary HMDMs

In this study, Simvastatin inhibited the IFN-γ-induced STAT1 phosphorylation on Ser 727 and Tyr 701 (Figures 4.4 and 4.5). IFN-γ controls many steps in the pathogenesis of atherosclerosis, including the recruitment of inflammatory cells to the activated endothelium, foam cell formation, apoptosis and plaque stability (McLaren and Ramji, 2009; Li et al. 2010). The JAK-STAT pathway plays an important part in IFN-γ signalling (McLaren and Ramji 2009). Statins have recently been found to exert profound anti-inflammatory and protective effects in the development of atherosclerosis. Despite their well established significance in inflammatory responses and potential clinical benefits in atherosclerosis, the molecular mechanisms underlying such anti-inflammatory and athero-protective effects remain poorly

understood, particularly in relation to IFN-γ actions and the JAK-STAT pathway. The findings of the current study are consistent with a study by Lee and colleagues (2007) who showed that Simvastatin inhibits STAT1α at transcriptional and protein levels in RAW264.7 macrophages. Moreover, a study by Townsend and colleagues (2004) found that in primary microglial cells, Atorvastatin (10 μM) inhibited Ser 727 phosphorylation of the STAT1 protein by IFN-γ (30 minutes). However, other studies have not found an effect of Simvastatin on STAT1 phosphorylation (Lee et al. 2007). For example, Simvastatin does not affect the IFN-γ-induced STAT1 phosphorylation in human microvascular endothelial cells (Sadeghi et al. 2001). In addition, the results in this study are different from those reported by Li et al. (2011) in THP-1 macrophages where it was demonstrated that Simvastatin had no effect on STAT1 phosphorylation. Although the exact reasons are unclear, this may reflect the use of a lower concentration of Simvastatin (10 μM) or a reduced treatment period with IFN-γ (30 minutes) (Li et al. 2011). Thus, the effect of Simvastatin on STAT1 phosphorylation in macrophages might be dependent on the concentration of the statin or the incubation time with the cytokine. Further dose response and time course experiments will be required to address this issue further. Such studies will not only enhance current understanding of the molecular mechanisms underlying the anti-inflammatory and atheroprotective effects of statins, but may also benefit the development of effective and safe therapeutic intervention for atherosclerosis in the future

6.3.5 Simvastatin attenuates the IFN-γ induced expression of MCP-1 and ICAM-1 in primary HMDMs and RAW 264.7 cells

To further confirm that the results obtained in THP-1 macrophages are not peculiar to the cell line, representative experiments were performed in primary cultures of HMDMs. Simvastatin inhibited the IFN-γ induced expression of MCP-1 and ICAM-1 mRNA levels in both Raw 264.7 and primary HMDMs (Figures 4.6, and 4.10). A number of studies have previously shown conservation of responses between THP-1 macrophages and primary HMDMs (McLaren et al., 2010a; McLaren et al. 2010b). Kota et al. (2006) have also found that IFN-γ induced MCP-1 gene expression in RAW

264.7 cells. A study by Romano et al (2000) showed that Simvastatin and Lovastatin decreased MCP-1 production in peripheral blood mononuclear cells (PBMC) and ECs.

Therefore, Statins are the principal therapy for hypercholesterolemia due to their ability to inhibit the synthesis of cholesterol with relatively mild side effects. However, mounting evidence suggests that some of the clinical benefits of statins may not be attributed to the lipid-lowering properties of these drugs. Our study suggests that statins also have anti-inflammatory properties in macrophages that aids in the treatment of atherosclerosis.

6.3.6 Effects of Simvastatin on MAPKs pathways

6.3.6.1 Effect of Simvastatin on JNK-1 and -2

The results from RT-qPCR showed that IFN-γ increased the expression of JNK-1 and -2 mRNA and this was not attenuated by treatment with Simvastatin (Figure 4.11). The effect of IFN-γ on the JNK pathway is well known as it has been found that IFN-γ and TNF-α work together to activate JNK/SAPK in a pancreatic β cell line (MIN6N8 cells) (Kim et al., 2005). Another study also showed weak activation of JNK1 in bone marrow–derived macrophages after treatment with IFN-γ for 2 hours whereas JNK2 was present at undetectable levels (Valledor et al.,2008). $JNK2^{-/-}$ mice in the $ApoE^{-/-}$ background have decreased foam cell formation compared to wild-type mice or $JNK1^{-/-}$ $ApoE^{-/-}$ mice, thereby showing an important role of JNK2 in atherosclerosis (Ricci et al. 2004). Another study by Jen and colleagues (2011) found that both the JNK inhibitor and Simvastatin together protected porcine islets from apoptosis. In hyperlipidaemic mice, Shen and colleagues (2011) showed that Atorvastatin also inhibits the JNK levels (along with ERK and p38) proteins (western blotting). Recently, Fluvastatin has been demonstrated to decrease phospho JNK levels in rat cardiomyocytes (Sakai et al. 2014). In the current study, we were not able to carry out such experiments because of problems with the antibody. Therefore, it maybe useful in the future to try another source for the antibody, and also different cell types because various cells respond differently.

6.3.6.2 Effect of Simvastatin on c-Jun

6.3.6.2.1 Effect of Simvastatin on c-Jun

The results in Chapter 4 showed that IFN-γ increased the expression of c-Jun (Figure 4.13). A similar finding has been reported in a study by Rubio (1997) that showed that IFN-γ induces the expression of c-Jun in astrocytes. Our experiment

showed that Simvastatin had no effect on the IFN-γ induced expression of c-Jun mRNA levels (Figure 4.13). In contrast, Fromigué et al. (2008) used another type of statin (i.e. Atorvastatin) and found that it significantly decreased c-Jun nuclear content in human and murine osteosarcoma cells (Fromigué et al. 2008). Another study also reported that Lovastatin and Simvastatin reduced c-Jun expression in prostate cancer cells (Hoque et al., 2008). Recently, a study by Sakai et al. (2014) showed that Fluvastatin decreased c-Jun mRNA expression and phospho-c-Jun in rat cardiomyocytes. The results of western blotting showed that IFN-γ had no effect on the induction of phospho c-Jun levels in THP-1 macrophages (Figure 4.14). In contrast, Gough and colleagues (2007) found that IFN-γ activated c-Jun in wt MEFs.

6.3.6.2.2 Effect of simvastatin on ERK-1 and -2 (p44/42)

The results of this experiment found that the phosphorylation-mediated activation of ERK-1/2 was decreased by Simvastatin (40 μM) (Figure 4.17). IFN-γ stimulates the phosphorylation of ERK-1 and -2 in wt MEFs (Gough et al. 2007). A similar finding was reported in a study by Zhang et al. (2010) who found that Mevastatin decreased the phosphorylation of ERK-1/2 in a dose dependent manner in cancer cells (SACC cells). Different types of statins such as Simvastatin, Mevastatin and Fluvastatin have been found to inhibit ERK-1/2 phosphorylation in C6 glioma cells (Yanae et al. 2011). Another study found that Atorvastatin inhibited ERK activation in neonatal rat cardiomyocytes (Liao et al. 2008). In RAW264.7 cells, Shao and colleagues (2012) reported that Atorvastatin inhibits ERK-1/2 phosphorylation. A very recent study showed that Simvastatin reduced phospho-ERK and -p38 levels in human platelet suspensiosn (Du et al. 2014). In contrast, another study used a different type of statin (i.e. Lovastatin) and found that Lovastatin did not decrease phospho ERK levels in microglial cells (Townsend et al., 2004). Overall, the results of our study suggest that statins may be potentially useful as anti-atherogenic agents in the treatment or slowing the progression of atherosclerosis via modulation of ERK activity.

6.3.6.2.3 Effect of Simvastatin on p38

IFN-γ increased the expression of p38 mRNA levels and Simvastatin had no effect on this (Figure 4.18). An early study by Lehner and collegues (2002) showed that the expression of IFN-γ was decreased in mice lacking MAPK-activated protein kinase 2 (MK2), which is a kinase activated by p38. Other studies also showed that IFN-γ treatment strongly activates p38 in primary BMDM (Valledor et al., 2008), Simvastatin showed no effect on the IFN-γ induced p38 mRNA expression (Figure 4.20). However, it significantly decreased the phosphorylation and the activation of the corresponding protein (Figure 4.20). This finding is consistent with Lee and colleagues (2010) who showed that Simvastatin decreased p38 phosphorylation in suspension of human platelets. However, Zhang et al. (2010) reported a conflicting result and found that treatment of SACC cells with Mevastatin increased p38 phosphorylation. This could be as a result of using a different type of statin, which may have a different action, or represent cell-specific effects.

Overall, studies in Chapter 4 provide a new insight into the anti-atherogenic mechanisms of Simvastatin action and this may help for clinical refinements in the future.

6.3.7 The effect of Simvastatin on the synthesis of the major lipid classes and cholesterol in the THP-1 cell line

The effect of Simvastatin on the synthesis of the major lipid classes in THP-1 cells was examined when the cells were treated with IFN-γ. In addition, two intermediates of the isoprenoid biosynthesis, FPP and GGP were used in order to better understand the mechanisms involved in the reduction of intracellular cholesterol by Simvastatin treatment. IFN-γ produced a slight increase in total free cholesterol levels, which were reduced by almost 50% in the presence of Simvastatin (Figure 5.3). The labelling of the total polar lipids was not affected by IFN-γ whereas synthesis of TAG was slightly decreased under this treatment (Figure 5.3). Simvastatin alone, as well as in combination with each intermediate, slightly increased the incorporation

of radiolabel acetate into polar lipids but did not affect the synthesis of TAG. In some previous work where an effect of Simvastatin on *de novo* lipid and cholesterol synthesis has been studied in THP-1 cells, it was shown that treatment with this statin (5 μM, 24 hours) caused a significant (up to 90%) inhibition of cholesterol synthesis (Risé et al., 1997). Synthesis of neither PLs nor TAG from radiolabelled acetate was affected in that study and this may potentially be explained by the lower concentration of Simvastatin used as well as an additional effect of IFN-γ. However, when [^{3}H] glycerol was used as a radiolabel, an increase in the labelling of TAG with a concomitant reduction of polar lipid synthesis was noted (Risé et al., 1997). In hepatocytic cell line Hep G2, Simvastatin also impaired the synthesis of cholesterol and CE from [^{14}C] acetate, but had no influence on phospholipid, free FAs and TAG synthesis (Nagata et al., 1990).

In this study, the addition of downstream metabolites of HMG-CoA reductase, FPP or GGP, did not reverse the inhibitory effect of Simvastatin on cholesterol synthesis (Figure 5.3). These findings demonstrated that the reduction in intracellular cholesterol levels are due to inhibition of HMG-CoA reductase and not as a consequence of an effect on signalling pathways due to depletion of intracellular lipid metabolites. Moreover, isoprenoid intermediates have been shown to influence activation of receptors, which are involved in the regulation of cellular cholesterol metabolism, e.g. PPARα, PPARγ and LXR (Argmann et al., 2003). It has been also shown that Simvastatin influenced PUFA metabolism in THP-1 cells, and this effect was partly prevented by mevalonate and GGP, but not FPP (Risé et al. 1997). Therefore, the pathways for cholesterol and PUFA synthesis have been suggested to be mutually modulated (Risé et al., 1997).

6.3.8 Effects of IFN-γ and Simvastatin on FA distribution in polar and TAG lipids from THP-1 and RAW 264.7 cells

In the present study, IFN-γ showed no statistically significant effect on FA composition of either membrane PL or stored TAG and addition of Simvastatin had no further changes. This could be as a result of several possible factors/differences related to the treatment, culturing conditions and data analysis compared to previous study/studies (Neville et al., 2005). For instance, the time period of IFN-γ treatment was different than previous studies that included 18 hours, 24 hours and 48 hours (Spinelle-Jaegle et al., 2001; De Saint Jean et al., 1999). Another reason is that we studied the actual relative concentrations of individual FAs but not a turnover of some particular one, e.g. arachidonic acid. In addition, there were relatively large standard deviations in our studies that could have contributed to the non-significant results.

In this study, the FA composition of the two major lipid classes, polar lipids and TAG, was investigated in order to identify possible changes as a result of IFN-γ and/or Simvastatin treatment. Previously, it has been shown that IFN-γ modulated the composition or synthesis of phospholipids in different cell types (Mattila et al., 1992). An increased amount of linoleic and arachidonic acids in PE was observed in both murine peritoneal macrophages and macrophage-like cell line P388D by IFN-γ (Jackson et al. 1992). The same group later showed a redistribution of linoleic acid among different lipid classes and found no increase in the incorporation of the saturated stearic acid in IFN-γ-activated cells (Darmani et al. 1994). Also, the liberation of arachidonic acid from phospholipids alongside a release of linoleic acid from PE in BCG-sensitive murine peritoneal macrophages was shown in response to endotoxin treatment (Stark et al. 1990). The authors suggested that IFN-γ may increase host susceptibility to endotoxin by augmenting the level of PUFA in the membrane lipids of macrophages which may be metabolised to produce biologically active metabolites: mediators and signals related to defence reactions (Darmani et al. 1994).

Statins influence not only cholesterol biosynthesis but also PUFA production and it has been shown that they cause increased accumulation of arachidonic acid (a conversion product of linoleic acid) in THP-1 cells (Risé et al. 1997). For Simvastatin, this has been demonstrated at a range of concentrations from 0.1 to 0.5 µM. It is interesting that increased linoleic acid conversion was accompanied by a stronger inhibition of cholesterol synthesis (Risé et al. 2003). In more recent studies, this group showed that Simvastatin increased the expression of Δ5 desaturase, the enzyme directly involved in the production of arachidonic acid from C20:3 (Risé et al. 2007). These previous experiments on the effects of Simvastatin and PUFA biosynthesis were carried out using radiolabelled FA precursors, e.g. radiolabelled linoleic acid. In that case, a turnover of a fatty acid in its metabolic product would take only few hours, whereas in the case of the present study, more time would be required to show the differences at the level of actual FA concentrations.

6.4 Future work

The overall aim will be to delineate the molecular mechanisms underlying the action of statins. Some of the future aims and approaches are as follows:

1. To further investigate the roles of the HMG-CoA reductase pathway and, in particular, small G-proteins in the Simvastatin-mediated attenuation of IFN-γ induced expression of key genes implicated in atherosclerosis. The pleiotropic effects of statins are often mediated through the inhibition of a parallel pathway of isoprenoid synthesis (Lee et al., 2008; Zhou et al., 2009). Isoprenoids constitute lipid attachments that allow membrane anchoring and activation of GTP-binding proteins of the Rho and Ras family, which then activate various downstream signalling pathways. Future studies will therefore first investigate whether this is also the case in relation to the actions of Simvastatin on the IFN-γ-regulated expression of key genes implicated in atherosclerosis. This will involve a combination of biochemical studies, use of pharmacological agents and RNA interference assays (RNAi) against the large number of potential monomeric G-proteins. For example, the activation of such G-proteins and their inhibition by Simvastatin can be analysed by biochemical assays. In addition, studies can investigate whether inhibition or knockdown of such monomeric G-proteins affects IFN-γ regulated gene expression. In addition, the effect of over-expression/activation of such pathways in overcoming the Simvastain-mediated inhibition can be analysed..
2. To investigate whether PPARs are involved in the response. Statins have been found to activate MAPK cascades, which then leads to the activation of PPARs via the production of the endogenous ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) through induced expression of Cox2 in Raw 264.7 macrophages (Yano et al., 2007; Paumelle et al., 2007; Ramji, 2009). 15d-PGJ_2 activates PPARγ and also PPARα. Initial experiments will

determine whether knockdown of PPAR-α or –γ or both affects the Simvastatin-mediated attenuation of IFN-γ-induced expression of key genes (RT-qPCR) (the use of PPAR antagonists such as TOO70907 represents an alternative that may be used for further confirmation –e.g. see Yano et al., 2007). Should a role for PPARs in the regulation of any genes be identified then the effect of Simvastatin on the following in vehicle- and IFN-γ treated cells will be determined:

- Expression of PPAR-α and –γ (RT-qPCR and western blot analysis); simple addition of Simvastatin and IFN-γ and carry out RT-qPCR analysis for these mentioned genes as this study.
- PPAR activity (transcription factor assay kit (Cayman Chemical) (Bujold et al., 2009).
- Intracellular expression of 15d-PGJ_2 (ELISA) (Yano et al., 2007; Bujold et al., 2009).
- Increased expression of COX2 (RT-qPCR) and dependency of the responses on it (RT-qPCR analysis of gene expression detailed above following knockdown of COX2 and, if necessary for further confirmation, following treatment of the cells with a specific inhibitor (e.g. NS-398) (Yano et al., 2007; Bujold et al., 2009).

3. To investigate globally the effect of Simvastatin on IFN-γ in macrophages. The studies in this thesis have been restricted to selective genes, such as MCP-1 and ICAM-1. It would be desirable to extend the findings at the cellular level. For example, the effect on the whole transcriptome could be determined by microarray analysis or RNA-seq analysis (Tisoncik et al., 2012; Dong et al., 2013). Also, the studies could be extended to protein levels (proteomics) and phospho-protein levels (Bose and Janes, 2013).

4. Compare the expression of MCP-1 and ICAM-1 between healthy blood vessels and atherosclerotic blood vessels by immunohistochemistry and compare the results obtained with other cell types.

5. Examine the effect of statins on apoE- or LDLR-deficient mice fed with an atherosclerotic diet, to elucidate the action on the expression of numerous genes in the pathogenesis of atherosclerosis *in vivo*.

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