Studies on the effect of interleukin-33 on gene expression and lipid composition of macrophages

Melanie Buckley BSc (Hons)

Supervisor: Dr Dipak P. Ramji

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Cardiff School of Biosciences

Cardiff University

Museum Avenue

Cardiff CF10 3AX

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ABSTRACT

The atherosclerotic plaque is characterised by the presence of macrophage foam cells that arise from dysfunctional cholesterol metabolism and trafficking. Cytokines are highly expressed within the plaque and play a critical function in initiating and augmenting the disease state. Previous studies have shown that the novel cytokine, interleukin-33 (IL-33), exerts anti-atherogenic actions in animal and *in vitro* models of the disease.

The effect of IL-33 on pro-atherosclerotic markers was assessed in human THP-1 and murine RAW264.7 macrophages and primary human monocyte-derived macrophages (HMDMs) by real time-quantitative polymerase chain reaction (RT-qPCR). The studies then focused on characterising the signalling pathways involved in the regulation of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) expression by IL-33. The expression of key signalling components implicated in atherosclerosis were knocked down by RNA interference (RNAi). These experiments demonstrated that the extracellular signal-regulated kinase (ERK)-1 and -2, p38α, c-Jun N-terminal kinase (JNK)-1 and -2, phosphoinositide-3-kinase (PI3K)-γ, p50 and p65 NF-κB were integral to the IL-33-mediated down-regulation of ICAM-1 and MCP-1 gene expression in THP-1 macrophages.

Another key aim was to analyse the effects of IL-33 stimulation on the lipid profiles of macrophages. A combination of thin layer chromatography (TLC) and gas chromatography (GC) was used to assess the fatty acid composition of THP-1 and RAW264.7 macrophages following IL-33 treatment. The cytokine promoted the redistribution of fatty acids and caused a general increase in saturated fatty acids (SFAs), accompanied with a reduction in monounsaturated fatty acids (MUFAs). Additionally, IL-33 increased the content of n-3 polyunsaturated fatty acids (PUFAs) whereas the impact on n-6 PUFAs was more specific to particular fatty acids and varied between the two species. Overall, the cytokine enhanced the levels of PUFAs involved in eicosanoid production. Also, IL-33 influenced the precursors and products of desaturases and appropriately increased the activities of Δ-5 and Δ-6 desaturases but reduced stearoyl-CoA desaturase activity (SCD). The decrease in SCD activity was accompanied by a reduction in the mRNA expression of SCD-1 in RAW264.7 macrophages.

The studies presented within this thesis provide new insights into the signalling pathways underlying the IL-33-mediated inhibition of gene expression in macrophages. Additionally, these experiments describe the novel effects of IL-33 stimulation on the lipid profile of macrophages.

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PUBLICATIONS

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ABBREVIATIONS

AA	Arachidonic acid
ABCA-1	ATP-binding cassette transporter A-1
ACAT-1	Acyl-Coenzyme A:cholesterol acyltransferase 1
AcLDL	Acetylated LDL
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ADRP	Adipocyte differentiation-related protein
Akt	Protein kinase B
ALA	α-linolenic acid
AP	Alkaline phosphatase
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
BCA	Bicinchoninic acid
BNP	B-type natriuretic peptide
BSA	Bovine serum albumin
C/EBPα	CAAT enhancer-binding protein-α
CAD	Coronary artery disease
CCL	CC-chemokine ligand
CD36	Cluster of differentiation 36
CE	Cholesterol esters
CHD	Coronary heart disease
CHF	Chronic heart failure
COX	Cyclo-oxygenases
CPT-1	Carnitine palmitoyltransferase 1
CRP	C-reactive protein
CXCL	CXC-chemokine ligand
DAB	3,3’-diaminobenzidine tetrahydrochloride
DAG	Diacylglycerol
DcR3	Soluble decoy receptor 3
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DMEM	Dulbecco’s modified Eagle’s medium
dNTP	Deoxyribonucleotide triphosphate
DPA	Docosapentaenoic acid
DR3	Death receptor 3
dsRNA	Double stranded RNA
ECM	Extracellular matrix
EDA	Eicosadienoic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERA/ETrA	Eicosatrienoic acid

ERK	Extracellular signal-regulated kinase
ETA	Eicosatetraenoic acid
FAMEs	Fatty acid methyl esters
FcεRI	Fcε receptor I
FCS	Foetal calf serum
FFA	Free fatty acid
FOXO	Forkhead box transcription factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
gDNA	Genomic DNA
GF	Growth factor
GLA	γ-linolenic acid
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSK3	Glycogen synthase kinase 3
HDL	High density lipoprotein
HEV	High endothelial venule
HF	Heart failure
HI-FCS	Heat inactivated-FCS
HMDM	Human monocyte-derived macrophages
HMG-CoA reductase	3-hydroxy-3-methyl glutaryl coenzyme A reductase
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
IFN-γR	IFN-γ receptor
Ig	Immunoglobulin
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL-1R	Interleukin-1 receptor
IL-1RAcP	IL-R accessory protein
IL-2	Interleukin-2
IL-33	Interleukin-33
IP-10	Interferon-gamma-inducible protein-10
IRAK	IL-1R-associated kinase 1
JNK	c-Jun N-terminal kinase
LA	Linoleic acid
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-UV	Liquid chromatography-ultraviolet
LDL	Low-density lipoprotein
LDLR	LDL receptor

LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LTs	Leukotrienes
LTA_4	5-(S)-oxido-*trans*-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid
LTB_4	5-(S),12(R)-dihydroxy-6,8,10,14-*cis*-eicosatetraenoic acid
LTC_4	5-(S),hydroxy-6(R)-S-glutathionyl-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid
LTD_4	5S-hydroxy-6R-(S)-cysteinylglycinyl-7,9,-*trans*,11,14-eicosatetraenoic acid
LTE_4	5S-hydroxy-6R-(S)-cysteinyl-7,9-*trans*,11,14-eicosatetraenoic acid
LXR	Liver X receptors
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MEK	MAPK/ERK kinase
MHC	Major histocompatibility complex
MI	Myocardial infarction
MIP-1	Macrophage inflammatory protein-1
mmLDL	Minimally modified low density lipoprotein
MMLV	Molony Murine Leukaemia Virus
MMP	Matrix metalloproteinase
MUFA	Monounsaturated fatty acid
MS	Mass spectrometry
MYD88	Myeloid differentiation primary-response protein 88
NCEH	Neutral cholesterol ester hydrolase
NEMO	NF-κB essential modifier
NF-κB	Nuclear factor-κB
NPC-1	Niemann-pick type C1
OxLDL	Oxidised low density lipoprotein
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDK	3-phosphoinositide-dependent kinase-1
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGD2	Prostaglandin D2
PH	Pleckstrin homology
PI	Phosphatidylinositol
PI3K	Phosphoinositide-3-kinase
PIP	phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PL	Polar lipid
PLA_2	Phospholipase A_2
PLC	Phospholipase C

PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PPAR- γ	Peroxisome proliferator-activated receptor-γ
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PVDF	Polyvinylidene difluoride
q-PCR	Quantitative PCR
RCT	Reverse cholesterol transport
RISC	RNA-induced silencing complex
RNAi	RNA interference
RTK	Receptor tyrosine kinases
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-qPCR	Real time quantitative polymerase chain reaction
ROS	Reactive oxygen species
SCD-1	Stearoyl-CoA desaturase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	Sterol esters
SFA	Saturated fatty acids
SH2	Src-homology2
shRNA	Short hairpin RNA
sICAM-1	Soluble ICAM-1
SIGIRR	Single Ig IL-1R-related molecule
siRNA	Small interfering RNA
SMC	Smooth muscle cell
SPHK	Sphingosine kinase
SR-A	Scavenger receptor-A
SR-B1	Scavenger receptor-B1
SREBP-1c	Sterol regulatory element binding protein-1c
STA	Stearidonic acid
STAT	Signal transducer and activator of transcription
TAG	Triacylglycerol
TBE	Tris borate EDTA
TGF-β	Transforming growth factor beta
Th	T Helper
TIMPs	Tissue inhibitors of metalloproteinases
TIR	Toll/interleukin-1 receptor
TLC	Thin layer chromatography
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAK	TNF receptor associated factor
Treg	Regulatory T cells
UV	Ultraviolet

VCAM-1	Vascular cell adhesion protein 1
VD_3	1,25-dihydroxyvitamin D-3
VEGI	Vascular endothelial growth inhibitor
VLDL	Very low density lipoproteins
Vps34	Vascuolar protein sorting 34

CHAPTER 1.

INTRODUCTION

1.1. Coronary heart disease (CHD) and atherosclerosis

Coronary heart disease (CHD) is responsible for one in three deaths and is the leading cause of mortality in westernised countries. An estimated 23.6 million people are expected to die globally from cardiovascular related diseases by 2030 and the disease and its complications, which include stroke and myocardial infarction (MI), have been predicted to have direct and indirect costs of approximately $315.4 billion in 2010 (McLaren *et al.* 2011a; Go *et al.* 2014). Due to the prevalence of the disease and the associated financial burden, this area of research has received much attention with the intention of gaining a better understanding of the prevailing pathology. Atherosclerosis is a chronic inflammatory disorder of the large and medium-sized arteries and constitutes the major underlying cause of CHD (McLaren *et al.* 2011a).

Advances in murine models capable of developing advanced atherosclerotic lesions such as apolipoprotein E (ApoE)$^{-/-}$ and low-density lipoprotein receptor (LDLR)$^{-/-}$ knockout mice have facilitated the investigation of genetic and physiological aspects of early events within the disease (Lusis 2000). The ApoE$^{-/-}$ knockout mouse model was pioneered in 1992 and is the most frequently chosen model for studying atherosclerosis (Piedrahita *et al.* 1992; Plump *et al.* 1992; Knowles and Maeda 2000; Smith *et al.* 2001). ApoE is produced within the liver and by macrophages and is a component of lipoprotein particles where it functions as a ligand for lipoprotein receptors (Zadelaar *et al.* 2007). Homozygous deletion of the ApoE gene gives rise to mice with marked hypercholesterolaemia when fed a western-type diet (Plump *et al.* 1992). The mice display five times the amount of plasma cholesterol compared to parent mice, develop spontaneous lesions in the aortic, coronary and pulmonary arteries and also exhibit reduced levels of the atheroprotective high-density lipoprotein (HDL) (Zhang *et al.* 1992).

The $LDLR^{-/-}$ knockout mouse model was developed in 1993 and shares many features with those exhibited by familial hypercholesterolaemia sufferers; a rare condition in humans that arises from the absence of functional LDLRs (Ishibashi *et al.* 1993; Russell and Proctor 2006). $LDLR^{-/-}$ mice are mildly hypercholesterolaemic due to defective clearance of plasma LDL (Kowala *et al.* 2000). Following feeding on a high-fat diet, the mice develop multiple atherosclerotic lesions accompanied with increased levels of cholesterol within the plasma and raised amounts of pro-atherogenic lipoproteins (Ishibashi *et al.* 1994). The formation of plaques occurs in a time-dependent fashion and closely resemble those found in $ApoE^{-/-}$ mice in terms of morphology (Zadelaar *et al.* 2007).

The disruption of the genes for ApoE or LDLR may affect other crucial cellular processes. Studies have shown that ApoE also functions as an anti-oxidant and modulator of immune responses (Zadelaar *et al.* 2007). Furthermore, there are certain limitations associated with the use of animal models for the study of atherosclerosis that arise from inter-species differences. Mice have a distinct lipoprotein profile from humans and the majority of plasma cholesterol is carried on HDL particles whereas, in humans, 75% of cholesterol is carried on LDL particles (Jawien *et al.* 2004). Additionally, immunological responses differ as the immune system has partly evolved due to selective pressure from microbial exposure; as such mice are more resilient to inflammatory damage than humans (Seok *et al.* 2013). Many differences exist between the murine and human immune system. The rolling of leukocytes to target sites is mediated by adhesion molecules such as P-selectin which is constitutively expressed at the surface of endothelial cells within humans. However, in mice the expression of this molecule is induced by pro-inflammatory mediators such as lipopolysaccharide (LPS) (Pan *et al.* 1998). The expression and secretion of immunoregulatory molecules can also vary between species. Within humans, neutrophils are a major source of antimicrobial defensins but the peptides are not expressed by neutrophils within mice (Risso 2000). Despite these potential caveats, the use of these mice has greatly progressed our understanding of the disease and there are many advantages associated with the use of murine models. For example, environmental conditions and dietary intake can be carefully controlled and the generation time is short therefore facilitating cross-breeding and evaluation of disease progression (Jawien *et al.* 2004).

1.2. Risk factors associated with atherosclerosis

Risk factors are measurable biological aspects that precede definable events within the disease, such as MI; they are causal in nature and can be used to predict the event. Kannel *et al.* (1961) outlined a number of interrelated characteristics that are associated with an increased risk of developing cardiovascular disease, these include raised cholesterol, hypertension, diabetes mellitus and smoking (Kannel *et al.* 1961). Table 1 displays several risk factors that contribute towards the initiation and progression of atherosclerosis and are grouped under environmental or genetic factors. It is well accepted that the disease is initiated by a local immune response to lipid deposition within the arterial subendothelial compartment (Chinetti-Gbaguidi and Staels 2009). Since lipids and lipoproteins are crucial to the initiation and development of the disease, these subjects will be dealt in more detail.

Table 1. Risk factors linked to atherosclerosis

Risk Factor	Role in atherosclerosis	Reference
Genetic		
Aging	Defective efferocytosis.	(Tabas 2010)
Type I Diabetes	Influences plasma lipoproteins levels and blood pressure. Directly affects arterial wall.	(Tabas 2010)
Gender	Men below the age of 60 are more prone (double the chance compared to women).	(Lusis 2000)
Systemic inflammation	Raised levels of inflammatory markers such as C-reactive protein linked to CHD.	(Lusis 2000)
Environmental		
Cigarette smoking	Free radical generation.	(Ross 1999)
High cholesterol diet	Elevated circulating LDL levels. Causes focal endothelial activation.	(Hansson *et al.* 2006)
Infectious agents	Proposed innate immune response from infections (e.g. *Chlamydia Pneumonia*) may contribute to atherogenesis.	(Lusis 2000)
Sedentary lifestyle	Raised blood pressure, elevated cholesterol.	(George and Johnson 2010)

1.3. Lipids

The theory that atherosclerosis stems from dysfunctional lipid metabolism has arisen from several key observations: the first morphological sign of atherosclerotic development is the appearance of a fatty streak abundant with lipids. Also the disease is

characterised by raised levels of lipids within the blood and lastly, the pathological state can be induced by disturbing processes related to lipid metabolism within animals (Kellner 1952). Therefore it is important to understand the role of lipids during normal physiology in order to determine the disruptive processes that give rise to the disease.

Lipids are a heterogeneous group of biomolecules that serve a range of functions within the body; as integral components of cellular membranes, a source of metabolic fuel and as mediators of signal transduction, amongst other roles (Kagan *et al.* 1974; Rustan and Drevon 2001; Vasudevan 2013). The most frequent form of lipids within the body are fatty acids produced during the metabolism of dietary lipids and typically esterified to other lipid derivatives such as triacylglycerols (TAGs) (Vasudevan 2013). Fatty acids are named after the parent hydrocarbon chain and the carbon atoms are either numbered from the carboxyl terminal, using the delta numbering system, or from the distal methyl end under the omega numbering system (Pelley 2011). A list of common fatty acids is given in Table 2.

Saturated fatty acids (SFAs) are comprised of straight hydrocarbon chains and typically contain an even number of carbon atoms, between 12 to 22, in humans (Rustan and Drevon 2001). Unsaturated fatty acids contain one or more double bonds and are denoted by the suffix –enoic following the systematic name (Vasudevan 2013). Polyunsaturated fatty acids (PUFAs) contain two or more double bonds and are named based on the position of the first double bond from the methyl end of the molecule. For example the first double bond of n-3 PUFAs is located between carbons three and four (Ruxton *et al.* 2004). The PUFAs linoleic acid (LA) and α-linolenic acid (ALA) are termed essential fatty acids as the body is unable to produce them due to an absence of Δ12- and Δ15- desaturase enzymes and therefore must be obtained from the diet (Rustan and Drevon 2001; Vasudevan 2013). The free carboxylate group in fatty acids poses potential issues for the cell and therefore unsaturated and SFAs are readily esterified to alcohols, in particular glycerol. Esterification with glycerol molecules produces mono-, di- or TAGs (Pelley 2011).

Table 2. Some naturally occurring fatty acids and their nomenclature

Shorthand notation	Common name	Abbreviation	Chemical name
14:0	Myristic acid		Tetradecanoic
16:0	Palmitic acid		Hexadecanoic
16:1n7	Palmitoleic acid		*Cis*-9-hexadecenoic
16:1n9	Palmitoleic acid		*Cis*-7-hexadecenoic
18:0	Stearic acid		Octadecanoic
18:1n7	*Cis*-Vaccenic acid		*Cis*-11-octadecenoic
18:1n9	Oleic acid		*Cis*-9-octadecenoic
18:2n6	Linoleic acid	LA	*Cis*-9,12-octadecadienoic
18:3n3	α-Linolenic acid	ALA	*Cis*-9,12,15-octadecatrienoic
18:3n6	γ-Linolenic acid	GLA	*Cis*-6,9,12-octadecatrienoic
18:4n3	Stearidonic acid	STA	*Cis*-6,9,12,15-octadecatetraenoic
20:1n9	Eicosenoic acid		*Cis*-11-eicosenoic
20:2n6	Eicosadienoic acid	EDA	*Cis*-11,14-eicosdienoic
20:3n3	Eicosatrienoic acid	ERA/ETrA	*Cis*-11,14,17-eicosatrienoic
20:3n6	Dihomo-γ-linolenic acid	DGLA	*Cis*-8,11,14-eicosatrienoic
20:4n3	Eicosatetraenoic acid	ETA	*Cis*-8,11,14,17-eicosatetraenoic
20:4n6	Arachidonic acid	AA/ARA	*Cis*-5,8,11,14-eicosatetraenoic
20:5n3	Eicosapentaenoic acid	EPA	*Cis*-5,8,11,14,17-eicosapentaenoic
22:0	Behenic acid		Docosanoic
22:3n6			*Cis*-10,13,16-docosatrienoic
22:5n3	Docosapentaenoic acid	DPA	*Cis*-7,10,13,16,19-docosapentaenoic
22:6n3	Docosahexaenoic acid	DHA	*Cis*-4,7,10,13,16,19-docosahexaenoic
24:0	Lignoceric acid		Tetracosanoic
24:1n6	Nervonic acid		*Cis*-15-tetracosenoic

A diverse range of lipid, carbohydrate and protein moieties within the membrane ensures that the system can cope with changes to the cell such as variations in pH or osmolarity during normal physiological or pathological states (Simons and Sampaio 2011). In many bacteria, archaea and eukaryotes, the major phospholipids are phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglycerol (trivial name cardiolipin). Eukaryotes also contain phosphatidylcholine (PC) and phosphatidylinositol (PI) (van Meer and de Kroon 2011).

1.3.1. Lipid metabolism in atherosclerosis

Following consumption of a meal; dietary fats are either assimilated into other lipid moieties or re-esterified to TAGs and repackaged along with cholesterol esters (CEs), phospholipids and apoproteins into chylomicrons before entering the systemic circulation (Vasudevan 2013). Lipoproteins function as vehicles for the transport of insoluble lipids in the blood and are composed of a core region storing TAGs and CEs, with a surrounding polar region consisting of phospholipids and apolipoproteins. The structure of a lipoprotein particle is shown in Figure 1. Different forms of lipoproteins are involved in lipid trafficking; chylomicrons primarily facilitate the transport of dietary fats, whilst very low density lipoprotein (VLDL), LDL and HDL particles enable the movement of endogenously synthesised lipids (Glass and Witztum 2001).

TAGs within chylomicrons are taken up by the adipose tissue or skeletal muscle following digestion by lipoprotein lipase (LPL). In contrast, shorter chain fatty acids can directly enter the blood system without the need for further modifications before transport to the liver. Alternatively, fatty acids are oxidised for energy, mainly through β-oxidation and the excess energy within the cell is stored as fatty acids or TAGs in liver and adipose tissue (Vasudevan 2013).

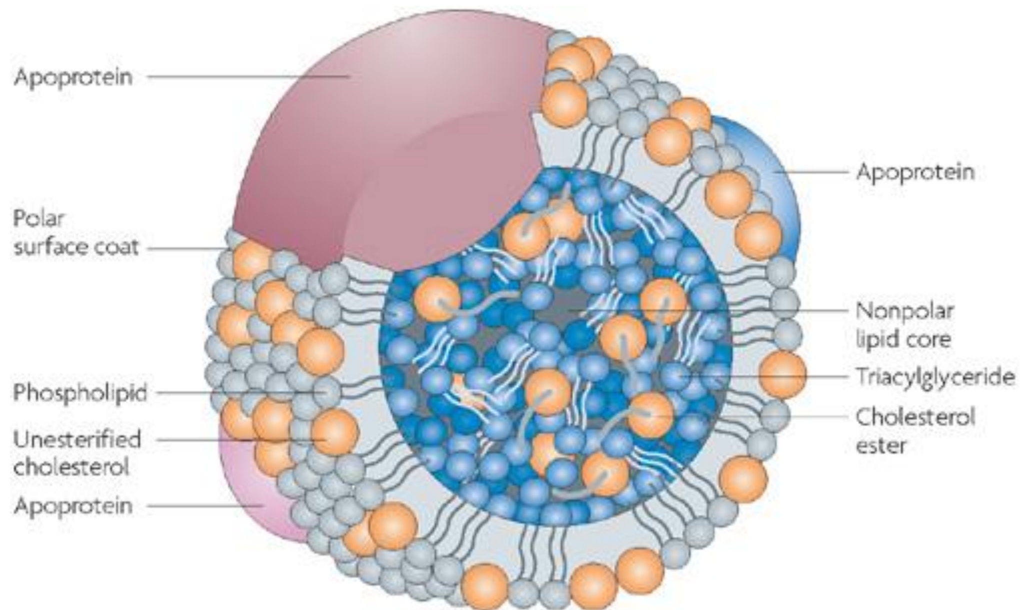


Figure 1. Organisation of a lipoprotein particle

Lipoproteins are used as vehicles to facilitate transport of hydrophobic lipids. The particles consist of a central region containing the phospholipid tails, TAGs and CEs. The surrounding phospholipid monolayer contains apolipoproteins that stabilise the structure of the lipoprotein. Taken from Wasan *et al.*(2008).

In 1856 Virchow observed that lipids were an integral component of atherosclerotic plaques and it was later discerned that cholesterol and CEs were in fact the major lipid types residing within the lesion in addition to phospholipids and neutral fats (Kellner 1952). The majority of cholesterol is produced within the liver and a lower proportion is absorbed from dietary sources within the intestine. Intestinal cholesterol is transported to the liver by chylomicrons, which obtain ApoE during transit in the circulation from HDL particles. The chylomicron particles are partially delipidated through the action of LPL prior to uptake by liver receptors (Tomkin and Owens 2012). Cholesterol derived from the liver is repackaged along with TAGs, phospholipids and apolipoprotein B100 (ApoB100) into VLDLs before secretion into the circulation. The activity of lipases residing in the capillary wall liberates TAGs giving rise to VLDL remnants and further enzymatic activity leads to the generation of LDL particles (Tomkin and Owens 2012).

During atherosclerosis certain plaque-resident cells such as macrophages and smooth muscle cells (SMCs) express additional receptors. These scavenger receptors (SRs) are

not subjected to the normal lipid homeostatic mechanisms and therefore contribute towards the disease state by facilitating excessive accumulation of modified LDL particles in the absence of effective internalisation and degradation; leading to the formation of lipid-laden foam cells (Kzhyshkowska *et al.* 2012). The role of lipoproteins and SRs during the disease will be discussed in further detail in the sections describing the initiation of atherosclerosis.

Excessive amounts of free cholesterol is toxic to cells so the body utilises two routes for the removal of cholesterol; either through enzymatic-driven conversion of cholesterol to a more soluble transportable form or through reverse cholesterol transport (RCT) (Glass and Witztum 2001; Cuchel and Rader 2006). RCT is the primary pathway and involves lipid transporters such as ATP-binding cassette transporter (ABC)-A and -G and scavenger receptor-B1 (SR-B1) that mediate the transfer of cholesterol from peripheral cells to selected extracellular acceptors such as HDL and the associated apolipoproteins A-I, A-II, E, J and A-IV (Vergeer *et al.* 2010; Kzhyshkowska *et al.* 2012). The cholesterol is then delivered to the liver for conversion to bile salts in preparation for excretion (Lusis 2000; Daniels *et al.* 2009). (Figure 2 gives a summary of cholesterol transport).

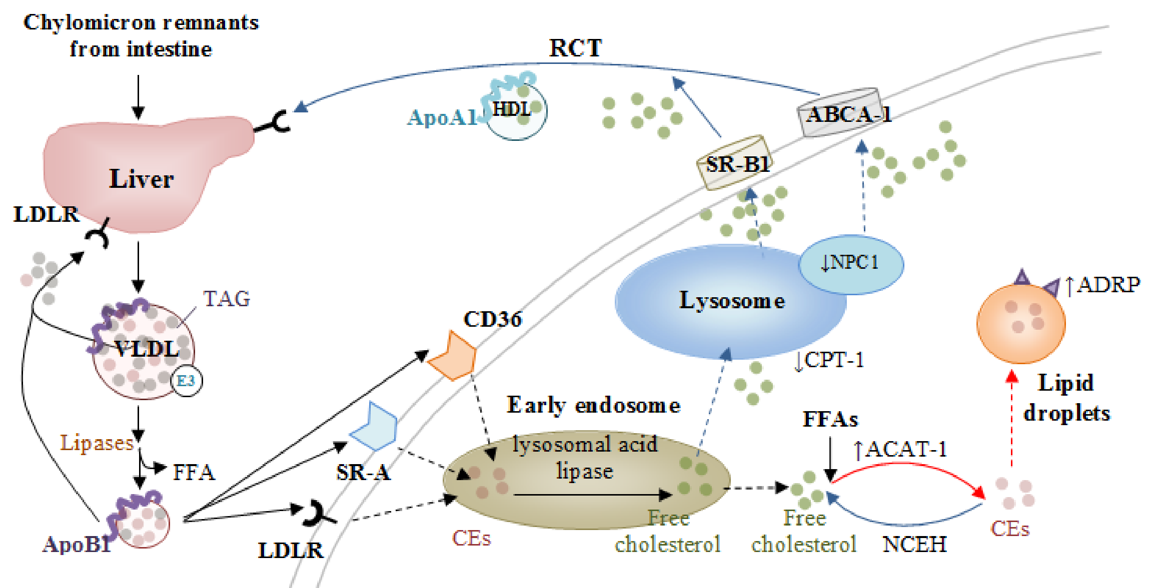


Figure 2. Cholesterol metabolism within macrophages

Dietary lipids are absorbed in the intestine and transported by chylomicrons to peripheral tissues. Following lipolysis by lipases, chylomicron remnants deliver dietary lipids to the liver. Liver-derived VLDLs containing ApoB and ApoE (E3) mediate the transport of endogenous lipids. VLDLs are then hydrolysed to intermediate LDLs and on to LDLs. ApoB facilitates LDL binding to LDLRs that are internalised and degraded through a LPL-dependent mechanism, releasing CEs. SRs e.g. SR-A and CD36 facilitate excessive uptake of modified LDL particles into macrophages during the disease. CPT-1 regulates cellular fatty acid levels and influences the availability of free cholesterol. Lipases such as NCEH hydrolyse CEs to free cholesterol that is either trafficked to peripheral cells by RCT through ABC transporters and SR-B1, or re-esterified to CEs for storage by ACAT-1 within the ER. NPC-1 and -2 mobilise cholesterol from intracellular stores in preparation for transport to the liver via RCT. Adapted from McLaren *et al.* (2011). Abbreviations: ACAT-1, Acyl coenzyme A: acylcholesterol transferase-1; CD36: Cluster of Differentiation 36; CPT-1, Carnitine palmitoyltransferase 1; ER, Endoplasmic reticulum; FFA, Free fatty acid; NCEH, Neutral cholesterol ester hydrolase; NPC, Niemann-Pick type C; SR-A, Scavenger receptor-A.

1.3.2. The integral role of lipoproteins within atherosclerosis

The crucial involvement of lipoproteins within atherosclerosis was discovered through studies on subjects with familial hypercholesterolaemia. Heterozygous sufferers are relatively common (1 in 500) and carry mutations in the gene encoding for LDLR that give rise to half the number of receptors within the liver (Kovacic and Bakran 2012). In contrast, homozygotes are less frequent (1 in a million), exhibit six to ten times the levels of LDL within their plasma compared to non-sufferers and are prone to MIs at an early age. The disease demonstrates a genetic link between heightened LDL levels and atherosclerotic development, independent of other risk factors (Goldstein and Brown 2009). In 1973, Brown and colleagues demonstrated that the activity of 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG-CoA reductase); the rate-limiting enzyme in cholesterol synthesis, was inversely related to levels of LDL and a fall in cellular levels of cholesterol causes an up-regulation in the expression of LDLRs (Tomkin and Owens 2012). However, these feedback mechanisms are dysfunctional in homozygote sufferers of familial hypercholesterolaemia due to the deficit in LDLRs and leads to the advent of MI (Kovacic and Bakran 2012).

Several factors control levels of intracellular cholesterol. The ApoB100 component of LDL mediates binding to the LDLR and therefore determines the rate of uptake, clearance and ultimately catabolism of LDL (Tomkin and Owens 2012). Cellular levels of cholesterol influence the expression of LDLR through sterol feedback loops to control cholesterol homeostasis (Daniels *et al.* 2009). Furthermore, cholesterol is enzymatically modified through a number of processes such as hydroxylation and fatty acylation within the endoplasmic reticulum (ER) to produce oxysterols and sterol esters (SEs) respectively. Esterification of cholesterol reduces the solubility of the molecule and promotes storage within cytoplasmic lipid droplets. During normal physiology, cholesterol is subjected to cycles of esterification and ester hydrolysis to carefully regulate cholesterol levels within the cell (Ikonen 2006). Platelets, endothelial cells and inflammatory cells also secrete mediators that control the influx and egress of cholesterol (Hajjar and Pomerantz 1992).

HDL particles are mainly made up of polar lipids (PLs) and apolipoproteins principally in the form of ApoA1, whereas CEs and TAGs are only minor components (Hamilton *et*

al. 1976). Nascent forms of HDL gain phospholipids and cholesterol during cellular efflux and through LPL-mediated lipolysis of chylomicrons and VLDLs (Lewis and Rader 2005). The involvement of these particles within atherosclerosis has received a great level of attention. Sufferers of Tangier disease contain mutations within the gene that encodes for ABCA-1 and display drastically low levels of HDL further to localised accumulation of CEs within different tissues of the body (Schaefer *et al.* 1978; Asztalos *et al.* 2001). People with the disease are also prone to developing premature atherosclerosis (Rust *et al.* 1999). The relationship between reduced HDL levels and incidences of CAD have long been established as one of the major risk factors for the disease (Gordon *et al.* 1977) and the concentration of HDL is negatively affected by many other atherosclerotic risk factors such as gender, smoking, obesity and lack of exercise (Vergeer *et al.* 2010).

Promisingly, elevated levels of HDL are associated with reduced severity of the disease (Gordon *et al.* 1989). Transgenic mice expressing human ApoA-1 display raised levels of plasma HDL and are significantly protected from atherosclerotic development following feeding on a lipid-rich diet (Rubin *et al.* 1991). The anti-atherosclerotic effects are thought to mostly arise from the involvement of HDLs within RCT (Lewis and Rader 2005). However, the particle has additional protective properties and contains an esterase that can inhibit lipoprotein oxidation (Roheim 1986; Lusis 2000). *In vitro* experiments have shown that HDL particles reduce the expression of endothelial cell adhesion molecules and also inhibit the secretion of pro-inflammatory mediators such as tumour necrosis factor-α (TNF-α) (Cockerill *et al.* 1995). Furthermore, incubation of HDL with cocultures of human aortic wall cells reverses the pro-atherogenic effects of LDL including the up-regulation of monocyte chemotactic protein-1 (MCP-1) by endothelial cells and SMCs and accordingly attenuates monocyte adhesion (Navab *et al.* 1991). The administration of HDL particles into rabbits fed on a high-cholesterol diet inhibits lesion progression and also stimulates the regression of aortic fatty streaks (Badimon *et al.* 1990).

1.3.3. The effects of dietary fatty acids in coronary artery disease (CAD)

Early monumental studies such as the Seven Countries Study (Keys *et al.* 1965; Keys *et al.* 1986) and the Japanese NI HO SAN migration study (Kagan *et al.* 1974) described a relationship between diets containing high proportions of SFAs and raised plasma cholesterol in patients with cardiac disorders. It was observed that geographical discrepancies in coronary mortality existed which were related to the percentage of dietary SFAs as a proportion of caloric intake. Short chain fatty acids such as those containing 8-10 carbon atoms appear to have a neutral effect on cholesterol, whereas longer chain SFAs with 12, 14 and 16 carbon atoms have a hypercholesterolaemic impact in humans (Rhee *et al.* 1997).

The effects of monounsaturated fatty acids (MUFAs) on atherosclerosis are often described as neutral; however, the geometric isomerisation of the double bond can influence the atherosclerotic potential of these fatty acids. Cardiac arrest patients contained higher levels of *trans*-fatty acids, particularly *trans*-LA, within erythrocyte membranes that served as a strong indicator of cardiac events (Lemaitre *et al.* 2002). In another study, subjects were given a rotation of six diets enriched with different fatty acids. Diets low in *trans*-fats and SFAs produced the most beneficial ratio of total and LDL cholesterol to total and HDL cholesterol. On the other hand, diets high in *trans*-fats and SFAs had an adverse effect on these ratios. *Trans*-fat rich diets produced the most prominent reduction in levels of HDL and were also associated with increased levels of the atherosclerotic marker lipoprotein a (Lichtenstein *et al.* 1999).

Studies comparing the diets of populations known to have reduced numbers of mortalities from CAD highlighted the potential athero-protective effects of n-3 PUFA-enriched diets. Dyerberg *et al.* (1987) observed that incidences of MIs within Greenland's Eskimo community were low and blood samples taken from these subjects were high in levels of the n-3 PUFA eicosapentaenoic acid (EPA), whilst levels of the n-6 PUFA arachidonic acid (AA) were considerably reduced compared to subjects fed a westernised diet. Interestingly, it was also noted that there was a greater tendency to bleed within this population. The study demonstrated that vessel-wall cyclo-oxygenases (COXs) utilise EPA for the synthesis of anti-aggregating agents and EPA itself exerts anti-thrombotic properties (Dyerberg *et al.* 1978). The beneficial effects of increased

dietary intake of fish have been attributed to n-3 PUFAs and their influence on n-6 PUFAs. Mice fed a fish oil based diet or a low-fat diet exhibited changes in the fatty acid composition of peritoneal macrophages reflective of their dietary intake. The levels of the n-6 PUFAs LA and AA were significantly less in mice fed on the fish oil diet compared to the low-fat diet whereas the n-3 PUFAs EPA, docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) were raised in comparison (Miles *et al.* 2000). Several studies have also explored the effect of n-3 PUFA supplementation on survival rates following a previous cardiac arrest. In the diet and reinfarction trial, patients who had previously suffered a MI were put on either a beneficial fat, fibre or fish diet. The study showed that consumption of 2-3 portions of fish a week, in comparison to the other prescribed diets, significantly reduced cases of myocardial reinfarction by 29% (Burr *et al.* 1989).

Where n-3 PUFAs are thought to have a protective impact within CAD, the role of n-6 PUFAs within the disease state is less certain. Many advisory boards recommend replacing SFAs with PUFAs and analysis of pooled cohort studies indicate that such a change would be beneficial in the prevention of CHD (Jakobsen *et al.* 2009; Russo 2009). However, due to the inflammatory nature of atherosclerosis, there are concerns over the potential adverse effects from n-6 PUFAs arising from the production of AA-derived pro-inflammatory metabolites (Harris *et al.* 2009). AA-derived eicosanoids exhibit a range of actions that likely contribute towards atherogenesis (Lewis *et al.* 1980; Samuelsson 1983). However, AA can also be converted into lipoxins which have anti-inflammatory effects (Kohli and Levy 2009). Also, several n-6 PUFAs have been shown to have little impact on inflammatory markers (Riemersma *et al.* 1986; Rallidis *et al.* 2003) and a number of studies have suggested that the actions of n-6 PUFAs on atherosclerotic markers are nullified in the presence of n-3 PUFAs (Pischon *et al.* 2003; Kusumoto *et al.* 2007).

1.3.4. Fatty acid metabolism and atherosclerotic processes

De novo lipogenesis is incapable of producing fatty acid chains exceeding 18 carbons (Cook and McMaster 2002). However, through a process of elongation and desaturation, eukaryotes have evolved a system to accommodate the fatty acid requirements of cells. Using pre-existing fatty acids the body can elongate the acyl chain by 2 carbons in

length. Elongation primarily occurs within the ER, with a smaller portion occurring within the mitochondrion and to some extent within peroxisomes (Cook and McMaster 2002). Figure 3 provides a pictorial overview of fatty acid biosynthesis.

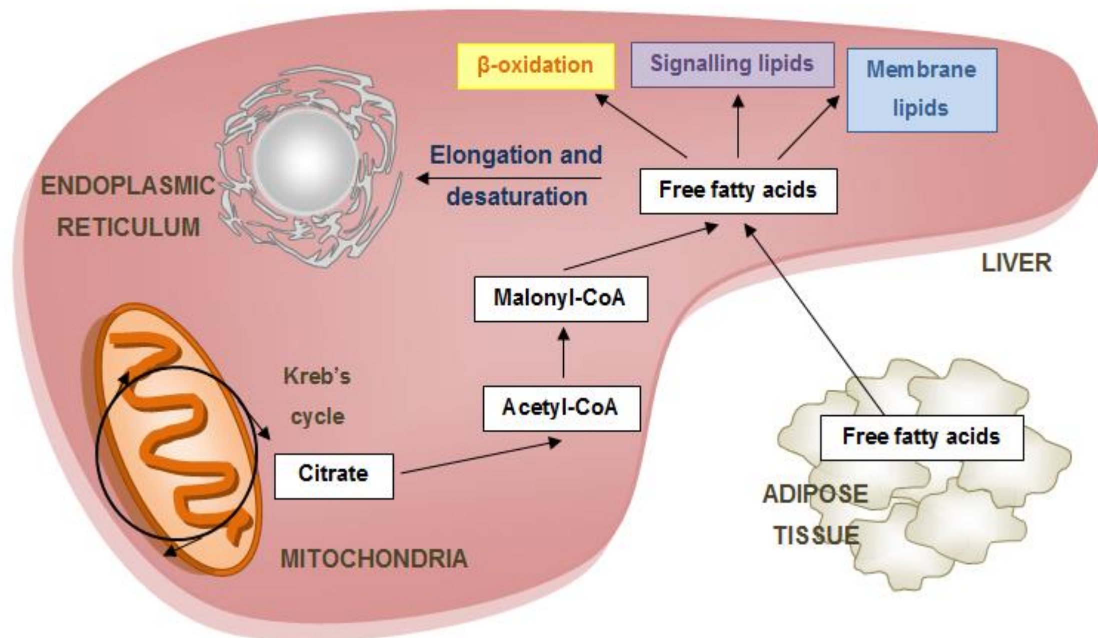


Figure 3. Fatty acid biosynthesis

The microsomal system lengthens saturated or unsaturated fatty acyl-CoA through sequential addition of 2-carbon groups from malonyl-CoA up to a length of 22-24 carbons. Elongation requires the malonyl-CoA donor and NADPH and is influenced by dietary fatty acid intake. The products from *de novo* synthesis are primarily 16 carbon fatty acids like palmitic acid, whilst small amounts of the 18 carbon stearic acid are also generated in addition to shorter chain fatty acids. Adapted from Schulze and Harris (2012) and Yecies and Manning (2010).

The range of fatty acids obtained by *de novo* synthesis and through the diet is not always sufficient to match demand and therefore extensive metabolism and rearrangement of existing fatty acids may be necessary. Pre-formed long chain fatty acids are utilised as substrates for the generation of MUFAs and PUFAs by desaturases. Desaturases can be structurally identified by the existence of a N-terminal cytochrome b_5-like domain fused to a desaturation domain (Meesapyodsuk and Qiu 2012). Within humans three types of desaturases exist: Stearoyl-CoA desaturase (SCD) (a Δ9-

desaturase), Δ6-desaturases and Δ5-desaturases. Human desaturases are special oxidase enzymes capable of introducing double bonds between pre-existing double bonds and the carboxyl end of fatty acids and therefore belong to a group of 'front-end' desaturases. Other organisms are capable of introducing double bonds between a previous double bond and the methyl end through the actions of 'methyl-end' desaturases (Meesapyodsuk and Qiu 2012).

1.3.5. Desaturases and CAD

Due to the pivotal role of desaturases in regulating MUFA and PUFA formation, several studies have investigated the involvement of these enzymes within cardiovascular disease. Many studies have shown that enhanced Δ6-desaturase activity and reduced Δ5-desaturase activity are associated with increased CAD risk (Warensjoe *et al.* 2008; Lu *et al.* 2012). Within humans, the genes *FADS1* and *FADS2* encode for Δ5-desaturase and Δ6-desaturase respectively (Marquardt *et al.* 2000). In a population study of CAD and non-CAD sufferers, subjects containing single nucleotide polymorphisms in the *FADS* cluster of genes exhibited elevated Δ6-desaturase activity linked to high concentrations of C-reactive protein (CRP) and an increased risk of CAD. As such it was proposed that polymorphisms in the *FADS* cluster may promote a pro-inflammatory environment supporting atherogenesis (Martinelli *et al.* 2008). Conversely, polymorphisms in the promoter region of *FADS2* that impair Δ6-desaturase reduce the peroxidation susceptibility of lipoproteins (Solakivi *et al.* 2013). Furthermore, patients with MI exhibit raised ratios of DGLA/AA; indicating that dysfunctional Δ5-desaturase activity occurs during the disease (Leng *et al.* 1999). An epidemiological study by Warensjoe *et al.* (2008) demonstrated that increased Δ6-desaturase and reduced Δ5-desaturase activity were related to total and cardiac-related mortality. However, the strongest predictor of mortality was based on the SCD-1 index (Warensjoe *et al.* 2008). Much of the work within this field has focused on SCD-1 and as such the remaining content of this section will be on SCD-1.

SCD-1 gene expression is carefully regulated by cellular levels of PUFAs, cholesterol, vitamin A, insulin, temperature, alcohol, phenol, metals and peroxisomal proliferators (Ntambi 1999). The enzyme is essential for normal lipid biosynthesis as mice lacking SCD-1 display impaired production of TAGs and CEs. However, supplementation with

MUFAs does not reverse these effects demonstrating that SCD-1 controls the *de novo* lipogenesis of MUFAs that is integral for TAG formation (Miyazaki *et al.* 2000). In the presence of saturated fats, mice deficient in SCD-1 favour β-oxidation over lipogenesis mediated through a reduction in the expression of lipogenic genes like sterol regulatory element binding protein-1c (SREBP-1c) and the up-regulation of genes involved in β-oxidation such as CPT-1 (Sampath *et al.* 2007). Furthermore, subjects with polymorphisms in the gene for SCD-1 tend to have a lower body mass index, reduced waist circumference and improved insulin sensitivity (Warensjo *et al.* 2007). As such SCD-1 functions as a protector against saturated fat-induced obesity

Although SCD-1 has been shown to prevent diet-induced obesity and significantly reduce fasting insulin levels, both risk factors for CAD, many studies have indicated that ablation of SCD-1 promotes atherosclerosis. $LDLR^{-/-}ApoB^{100/100}$ mice treated with SCD-1 antisense oligonucleotides have an enlarged total aortic lesion area and significantly raised free and esterified cholesterol (Brown *et al.* 2008). In another study, $LDLR^{-/-}$ mice deficient in SCD-1 exhibit increased atherosclerosis characterised by greater lesion size, macrophage infiltration and increased levels of soluble ICAM-1 (sICAM-1) and IL-6. Also, HDL particles from these mice had a composition resembling those present during inflammation e.g. reduced apoAII and plasma A-I content (MacDonald *et al.* 2009). On the other hand, other groups have shown that overexpression of SCD-1 in HEK 293 cells (Sun *et al.* 2003) and RAW264.7 macrophages (Nakaya *et al.* 2013) promotes RCT to HDL particles independently of ABCA-1, ABCG-1 and SR-B1 (Sun *et al.* 2003; Nakaya *et al.* 2013).

The role SCD-1 in atherosclerosis is unclear as both protective and pathogenic actions have been described for the enzyme. Overexpression of SCD-1 promotes fat accumulation and insulin resistance but also increases the efflux of cholesterol (Pinnamaneni *et al.* 2006). Conversely, inhibition of SCD-1 causes catabolism of fat stores accompanied with inflammation and cellular stress (Thorn *et al.* 2010; Liu *et al.* 2011). Conflictingly, an absence of SCD-1 produces a beneficial lipid profile but also promotes the development of atherosclerosis that occurs independently of many of the well-established risk factors such as obesity and insulin resistance (Warensjoe *et al.* 2008; Djousse *et al.* 2012). The disparity in these findings suggests that there is a fine

balance between beneficial metabolic effects and possible inflammatory consequences arising from SCD-1 activity.

1.4. The development and progression of atherosclerosis

1.4.1. Initiation of atherosclerosis

The exact events that initiate atherogenesis are not fully understood. A number of theories have focused on different disease drives including intimal lipid accumulation, changes in haemodynamic forces and a response to injury of the endothelium (Tegos *et al.* 2001). Immune cells, cellular signalling and cytokines are pivotal to the pathogenesis of atherosclerosis and will be covered in greater detail in subsequent sections.

Atherosclerotic lesions tends to develop within areas of curvature, such as branching points, that are prone to disturbed laminar flow within the large and medium-sized arteries (Moore and Tabas 2011). The arterial endothelium is a highly responsive system, functioning as a barrier to invading pathogens and maintenance of vascular wall tone. Whilst typically impermeable to large biomolecules such as LDL, physiological and pathophysiological changes can activate endothelial cells leading to an increase in the permeability of the endothelial cell layer. As a result the expression of adhesion molecules rises in addition to the production of extracellular matrix (ECM) proteins and the secretion of chemokines and growth factors (GFs) such as macrophage colony-stimulating factor (M-CSF) (McLaren and Ramji 2009).

Within the plasma, circulating lipoproteins are protected from major chemically-induced alterations but during atherosclerosis LDL particles containing ApoB diffuse between endothelial cell junctions and accumulate within the subendothelial space (Hansson and Hermansson 2011; Moore and Tabas 2011). The LDL particles associate with ECM components through interactions with ApoB and LPL, becoming trapped and susceptible to a range of enzymatic and non-enzymatic chemical modifications mediated through the activities of myeloperoxidases, lipoxygenases (LOX), reactive oxygen species (ROS), peroxynitrite and nitric oxide (Glass and Witztum 2001).

Modified LDL particles are immunogenic as the peroxidation of phospholipids, CEs and TAGs within the core creates reactive species capable of stimulating inflammatory processes that promote the activation of endothelial cells, platelets and macrophages (Matsuura *et al.* 2008; Frostegard 2013; Wraith *et al.* 2013). The presence of OxLDLs in the intima also aggravates surrounding cells and induces SMC mitogenesis (Paoletti *et al.* 2004). Prolonged oxidation of LDL alters several properties of the particle such as size and charge, dependent on the oxidising agent (Hansson and Hermansson 2011). The presence of small, dense LDL particles is associated with increased risk of CHD (Lamarche *et al.* 1997) and minimally modified LDL (mmLDL), which are not recognised by SRs, are major initiators of the immune response (Miller *et al.* 2003).

Leukocytes including monocytes, neutrophils, T cells, B cells, dendritic cells and mast cells are recruited along a chemotactant gradient towards the lesion site. Activated endothelial cells and SMCs secrete chemokines, a family of structurally similar chemotactic cytokines that includes macrophage inflammatory protein (MIP)-1α/β and MCP-1 (Zernecke and Weber 2010). The number of monocytes adhering to the aortic intima rises approximately fifty times in response to a cholesterol-rich diet in rats (Joris *et al.* 1983) and the attachment of primary human monocytes has been shown to increase proportionally with levels of circulating LDL in a dose-dependent manner (Alderson *et al.* 1986).

The leukocyte adhesion cascade is comprised of three main stages; rolling, activation and arrest (Moore *et al.* 2013). Circulating monocytes roll along the endothelium co-ordinated by weak interactions between P-selectin glycoprotein ligand-1 found on the monocyte surface to P-selectin and E-selectin expressed by the endothelium (Ley *et al.* 2007). The leukocytes are immobilised through associations between leukocyte integrins like β2 integrin, lymphocyte function associated molecule and very late antigen-4 to adhesion proteins such as vascular cell adhesion protein-1 (VCAM-1) and ICAM-1 on endothelial cells (Lawson and Wolf 2009; Michael *et al.* 2012a). Chemoattractant receptors on the leukocytes aid migration and also stimulate integrin-mediated adhesiveness by inducing conformational changes in the integrins present at their cell surface (Springer 1994; Chan *et al.* 2001).

The leukocytes quickly migrate through the endothelial cell barrier in a process that typically takes between two to five minutes. Entry through the endothelial cell basement membrane lasts longer and can take over five to fifteen minutes (Ley *et al.* 2007). Following transdiapedesis into the intima, monocytes differentiate into macrophages under the influence of M-CSF or granulocyte-macrophage CSF (GM-CSF) (Gui *et al.* 2012). Key processes involving macrophages within atherosclerosis are summarised in Figure 4.

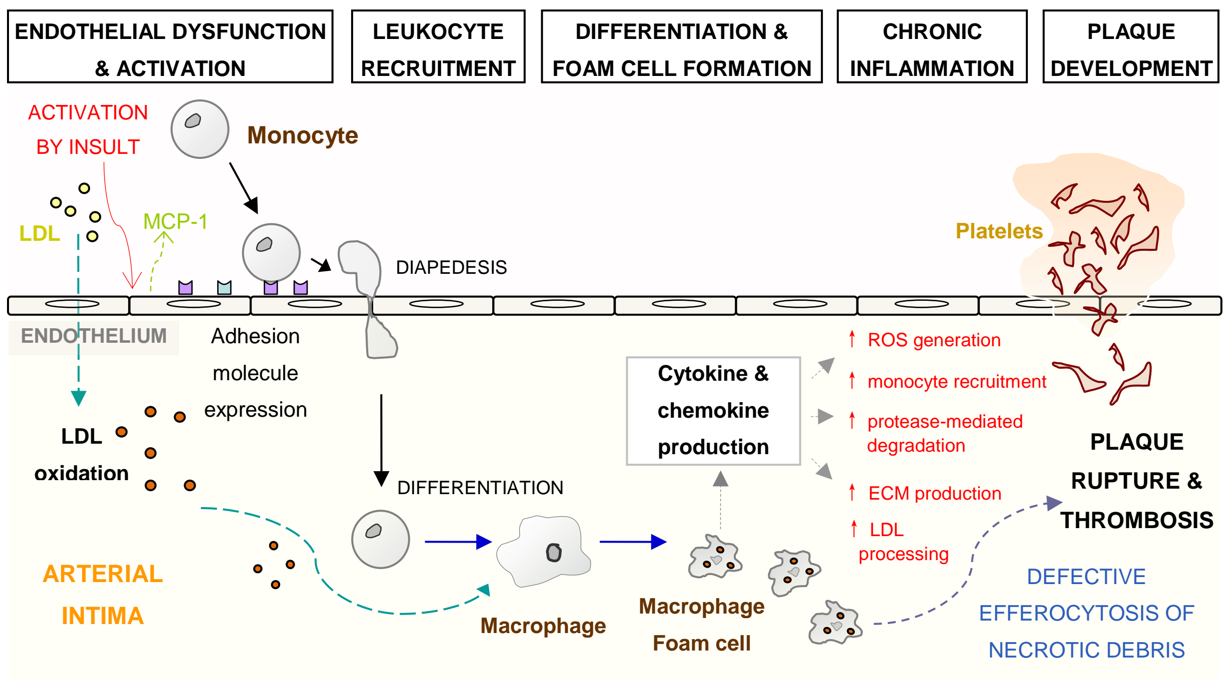


Figure 4. Overview of the role of macrophages within the disease state

Adhesion molecules are up-regulated in response to endothelial cell activation facilitating the adhesion of circulating monocytes. Following diapedesis, the monocytes differentiate into macrophages. The presence of immune cells within the plaque also up-regulates the expression of genes involved in the oxidation of LDL and its subsequent uptake, such as SRs, promoting transformation of macrophages into macrophage foam cells. Unregulated accumulation of lipids into the plaque produces a distinct fatty streak that progresses into an atherosclerotic lesion. Macrophage foam cells secrete pro-inflammatory mediators promoting the inflammatory state and contributing to destabilisation and eventual rupture of the plaque.

Macrophages are professional bone marrow-derived phagocytes, present in almost all tissues within the body (Lucas and Greaves 2001; Swirski *et al.* 2006). During normal

physiology, macrophages utilise a variety of pattern-recognition receptors, including SRs and members from the Toll-like receptor (TLR) family, to elicit rapid responses against foreign particles without the need for prior priming (Libby *et al.* 2009). Observations that patients with familial hypercholesterolaemia develop atherosclerosis in the absence of functional LDLRs indicate that additional cellular mechanisms exist that facilitate LDL uptake. In 1979, Goldstein and Brown demonstrated that chemically synthesised acetylated LDL (AcLDL) was readily taken up by macrophages and contributed towards foam cell formation (Goldstein *et al.* 1979). The participating receptor has since been identified and exists as two isoforms; scavenger receptor-A1 (SR-A1) and -A2. Other members of the SR family, including cluster of differentiation 36 (CD36) and SR-B1, have a role in the innate immune system but also participate in pathogenesis through the recognition of modified lipoproteins (Libby *et al.* 2009). The oxidation of PUFAs leads to the formation of aldehydes that modify lysine residues in apoB100 and gives the particle a net negative charge. The modified LDL can then bind with high affinity to SRs (Stocker and Keaney 2004).

Within early atherosclerotic lesions, macrophages are the prevalent cell type and during the disease the cells accumulate LDL, VLDL and modified LDL through several processes including macropinocytosis, phagocytosis and SR-mediated uptake thereby facilitating the transformation of these cells into macrophage foam cells (Moore *et al.* 2013). Non-oxidised LDL is also likely taken up by macrophages where it may be subjected to oxidation and subsequent aggregation within lysosomes (Wen and Leake 2007; Satchell and Leake 2012). *In vitro* experiments have shown that accumulated LDL is oxidised by redox-active iron at lysosomal pH (pH 4.5). The oxidation event promotes rapid aggregation of the particles which could potentially hinder degradation by lysosomal enzymes during the atherosclerotic state (Satchell and Leake 2012). SMCs within the plaque are also capable of foam cell transformation mediated through the uptake of modified LDLs by SRs expressed at the cell surface (Yan *et al.* 2011; Allahverdian *et al.* 2012). Small, asymptomatic lesions are comprised of macrophage and SMC foam cells and T cells and are referred to as fatty streaks due to the concentration of foam cells (Ross 1999). The fatty streak may regress or progress into a clinically relevant plaque (George and Johnson 2010).

1.4.2. Disease progression

The formation of an intermediate lesion arises due to enhanced migration and proliferation of vascular SMCs from the tunica media into the inflamed area in response to platelet-derived GFs released from plaque-resident cells (George and Johnson 2010; Michael *et al.* 2012a). The SMCs proliferate and release ECM proteins contributing towards the production of a fibrotic cap (Packard and Libby 2008). Stable plaques are associated with the presence of a fibrous cap, containing a matrix enriched with type I and III collagen, and the absence of a necrotic core (Lucas and Greaves 2001).

Histologic sections of human atherosclerotic lesions demonstrate that the area of the lumen is maintained despite the presence of a large developing plaque (Glagov *et al.* 1987). The artery is capable of remodelling itself via gradual dilation (Ross 1999) and angiographs taken at constant intervals highlight that progression of the disease is phasic and does not follow a strict linear timescale (Mann and Davies 1999). However, once the lesion occupies approximately 40% of the internal elastic lamina region, the artery is incapable of maintaining its integrity through dilation and protrudes into the lumen affecting blood flow (Glagov *et al.* 1987; Ross 1999). The growing plaque may result in narrowing of the artery (stenosis) and/or the restriction of blood flow to surrounding tissues (ischaemia) (Hansson and Hermansson 2011).

During earlier stages of the disease effective engulfment of apoptosing cells by neighbouring phagocytes (efferocytosis) helps to resolve pro-inflammatory processes and maintain stability within the plaque (Li *et al.* 2006; Tabas 2010; Van Vre *et al.* 2012). Dying cells produce ‘find me’ signals comprised of unique combinations of surface molecules such as PS which recruit phagocytosing cells to the site (Fadok *et al.* 1992). Phagocytes bind to the apoptosing cell, eliciting an anti-inflammatory response and engulf the dying cell. However dysfunctional efferocytosis is a key feature of advanced lesions and as the disease progresses the rate of apoptosis within the necrotic core likely overwhelms the phagocytic capabilities of residing phagocytes. Furthermore, dying macrophages are thought to function as poor substrates for neighbouring phagocytes and therefore contribute towards necrotic core formation (Tabas 2010). Interestingly, *in vitro* experiments have demonstrated that modified LDL serves as a

substrate for phagocytes so may competitively hinder the efferocytosis of dying cells (Bird *et al.* 1999).

Ineffective efferocytosis also stimulates secondary necrosis of lesion-resident cells and, in the case of macrophages, leads to the release of oxidised lipids and pro-inflammatory propagators (Liu *et al.* 2005). Intracellular free cholesterol released from dying foam cells builds up within the lesion triggering further apoptosis (Ball *et al.* 1995; Liu *et al.* 2005). The underlying mechanisms for this response are unclear and may involve the unfolded protein response within the ER and initiation of apoptotic pathways or could arise from the incorporation and accumulation of cholesterol within the plasma membrane (Kellner-Weibel *et al.* 1999; Feng *et al.* 2003). Plaque development is thought to occur in 'bursts' consisting of periods of rupturing and repair. However as the disease continues, the plaque becomes increasingly unstable and vulnerable as a result of reduced efferocytosis, chronic inflammation and ineffective egress of immune cells (Li *et al.* 2006). When residing within the plaque, the migration of macrophages is limited and therefore compromises potential resolution of inflammation favouring pathogenic processes (Moore *et al.* 2013). Lesion-resident macrophages contribute towards the inflammatory state through the secretion of protease enzymes and pro-inflammatory cytokines (Libby *et al.* 2009).

1.4.3. Advanced plaque formation and rupture

Towards the later stages of the disease, the atherosclerotic lesion is characterised by an abundance of disorganised cells, lipids, matrix components and minerals. Clinical symptoms may occur during this phase of the disease as the intimal region is thickened and the area of the arterial lumen may be reduced in size (Stary *et al.* 1995). Unstable plaques are associated with a high proportion of macrophages to SMCs and a lipid-rich necrotic core (Vanderwal *et al.* 1994). The activation of lesion-resident mast cells augments SMC apoptosis by the induction of caspases and through the attenuation of signalling pathways that promote cell survival (Leskinen *et al.* 2006). The necrotic core is often described as a 'graveyard of dead macrophages' (Li *et al.* 2006). The dying foam cells release their cytoplasmic contents causing a build-up of extracellular lipids and GFs that exacerbates inflammation and triggers secondary necrosis (Ross 1999). Excessive levels of cholesterol can also promote the formation of solid crystals that are

toxic to cells (Brown and Goldstein 1997). The growing necrotic core exerts physical pressure on the fibrous cap and compromises its integrity (Thorp and Tabas 2009).

Shah *et al.* (1995) performed studies with primary monocyte-derived macrophages incubated with *ex vivo* fibrous caps that indicated that macrophages induce the expression of matrix metalloproteinases (MMPs); several of which promote the degradation of collagen such as MMP-9 (Lubos *et al.* 2006). However, in the presence of MMP inhibitors the breakdown of collagen does not occur highlighting the key role of these enzymes during late events within the disease (Shah *et al.* 1995). The MMP family encompasses a range of proteolytic enzymes including collagenases, gelatinases, matrilysins and membrane-type MMPs (Raffetto and Khalil 2008). In the healthy state, MMPs are carefully regulated through the production of precursor zymogens, the activities of endogenous tissue inhibitors of metalloproteinases (TIMPs) and associations with the ECM. However, an imbalance in the ratio of MMPs to TIMPs is thought to support excessive ECM breakdown (Raffetto and Khalil 2008). MMPs are overexpressed within unstable lesions and localise to vulnerable regions within the plaque that are prone to rupture and inhabited by macrophage foam cells (Galis *et al.* 1994). The degradation of the connective tissue is detrimental to plaque stability and the resulting products are deposited within the arterial intima where they promote vasculitis (Chinetti-Gbaguidi and Staels 2009).

Locally produced cytokines, such as interferon-γ (IFN-γ) secreted by T cells, reduce the proliferation of SMCs and also inhibit the synthesis of integral ECM components like collagen types I and III (Amento *et al.* 1991). The fibrous cap undergoes thinning prior to rupture which undermines the stability of the structure (Libby *et al.* 1998). At this stage of the disease, the plaque contains depleted levels of fibrous material and may show signs of calcification, ulceration and haemorrhaging from small vessels (Lusis 2000; Packard and Libby 2008).

A number of factors contribute towards the disruption of the cap including the presence of inflammatory cells, building toxicity, the activities of proteolytic enzymes released from macrophages, coronary spasms and physical vulnerabilities and stresses arising from the altered composition of the lesion (Stary *et al.* 1995). The exposure of tissue factor from the plaque with the arterial lumen promotes coagulation and the formation

of a thrombus (Paoletti *et al.* 2004; McLaren and Ramji 2009). The thrombus may instantly obstruct the lumen or may detach in the form of an embolus and block blood flow at an independent site (Hansson and Hermansson 2011). Immunohistochemical staining has shown that some plaques have residual deposits associated with fibrin degradation representing the occurrence of multiple thrombi responsible for narrowing of the lumen (Stary *et al.* 1995). On the other hand, several case studies have also shown that occlusion of the arteries can arise as a result from haemorrhaging of small vessels within the plaque (Wartman 1938). Figure 5 provides an overview of key events within atherosclerosis.

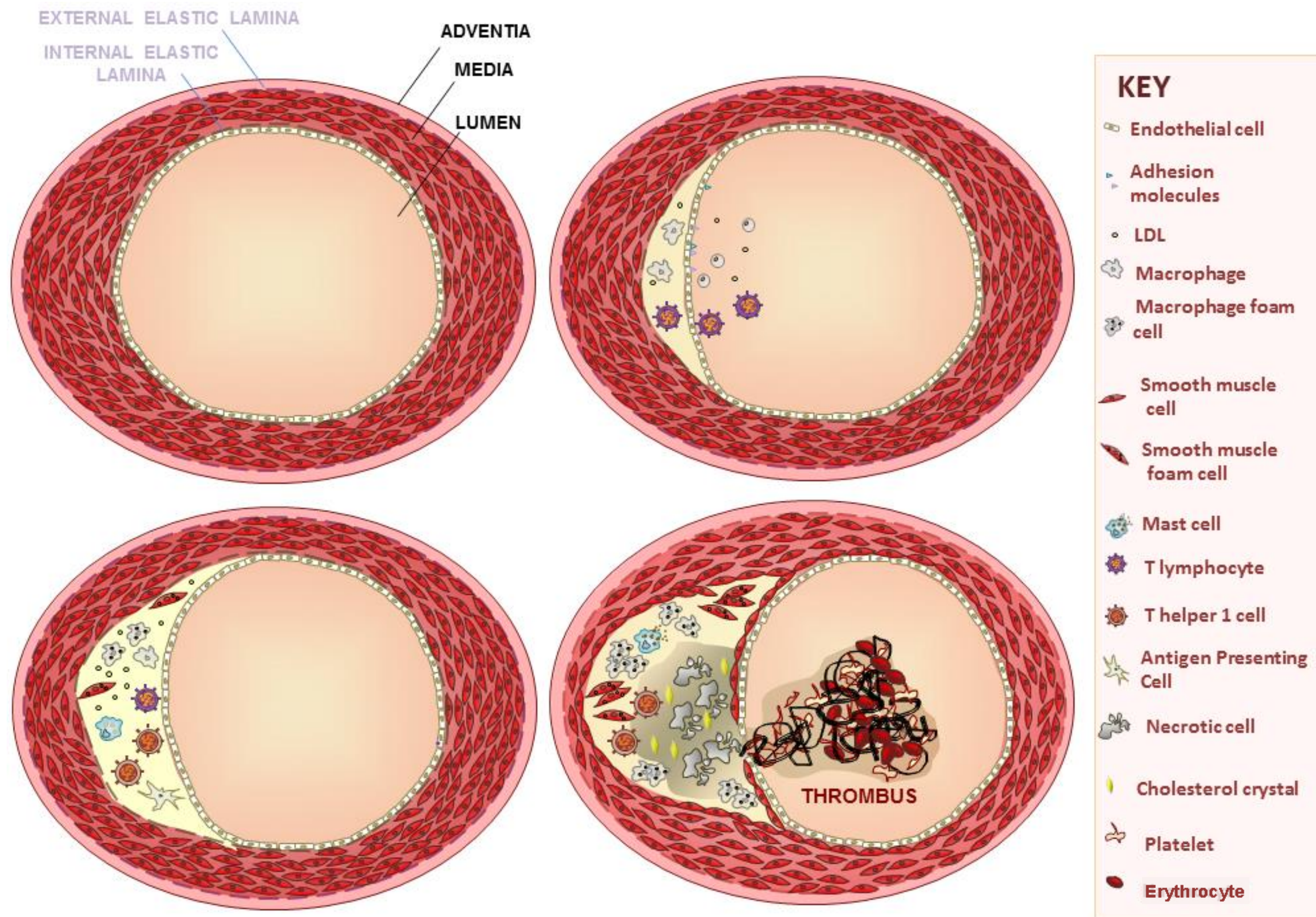


Figure 5. Atherosclerotic development

Successive rounds of immune cell infiltration, proliferation and fibrous material production causes restructuring of the plaque and formation of a fibrotic cap surrounding the necrotic core. Plaque rupture is an attribute of cap disruption due to VSMC apoptosis and depleted ECM, in addition to the death of lesion-resident cells and protease activity. T cells and macrophages produce angiogenic factors that cause leakage of erythrocytes into the plaque leading to inflammation and blood vessel formation. Ultimately the fibrous cap is sufficiently destabilised leading to rupture of the plaque into the artery lumen and subsequent thrombosis.

1.5. Cellular signalling

During atherosclerosis the dysregulation of key signalling pathways leads to altered gene expression that facilitates atherogenic processes. Signal transduction pathways mediate the transfer of information within the cell and mostly utilise membrane-bound receptors that respond to external stimuli such as hormones and neurotransmitters. The information is then communicated across the membrane through the use of different transducers and amplifiers that activate intracellular signalling pathways. Alternatively, intracellular signals such as metabolic messengers can also initiate signalling (Berridge 2012). Several signalling pathways have been implicated within the atherosclerotic state.

Extracellular signal-regulated kinase (ERK) mitogen-activated protein kinases (MAPKs) are involved in many essential processes including cell growth and differentiation, synaptic plasticity and the activation of other kinases like p90 ribosomal S6 kinases involved in the cell cycle (Ravandi *et al.* 2003). Mutations in the genes encoding for ERK1/2 result in developmental abnormalities highlighting the key role of these kinases in basic functionality (Rodriguez-Viciana *et al.* 2006).

The exact roles of ERK1 and ERK2 in atherosclerosis are still not properly understood but studies have shown that pathways involving ERK regulate the proliferation and differentiation of SMCs in the lesion (Muslin 2008). OxLDL promotes the proliferation of cultured aortic SMCs in a signalling mechanism involving ERK1/2 (Auge *et al.* 1998). A study by Zhou *et al.* (2010) demonstrated that inhibition of ERK1/2 activity led to increased efflux of cholesterol to ApoAI and HDL acceptors in macrophage-derived foam cells associated with enhanced expression of ABCA-1 (Zhou *et al.* 2010). Furthermore, ERK1/2 are integral to IFN-γ-mediated activation of signal transducer and activator of transcription (STAT)-1; a key regulator of many genes implicated in atherosclerosis such as ICAM-1 and MCP-1 and the uptake of modified LDL by macrophages (Li *et al.* 2010). In addition to the pro-atherogenic actions described, ERK1/2 are also involved in protective effects. A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) are structurally related to MMPs and involved in remodelling of the ECM within the plaque (Ashlin *et al.* 2013). The anti-atherogenic cytokine interleukin-33 (IL-33) down-regulates the expression of ADAMTS-1 and -4 in

human macrophages through a signalling mechanism dependent on ERK1/2 (Ashlin *et al.* 2014).

Another MAPK, p38, is also integral to atherosclerotic development as the kinase is activated in response to elevated intracellular cholesterol and MCP-1, whilst chemical inhibition of p38 reduces cholesterol accumulation by macrophages (Werle *et al.* 2002; Mei *et al.* 2012). In response to the accumulation of free cholesterol within the ER, p38 co-ordinates signalling events that induce cellular apoptosis (DeVries-Seimon *et al.* 2005). However, $ApoE^{-/-}$ mice deficient in p38α display increased levels of apoptosing macrophages and enhanced necrosis within the plaque. Lesions from these mice are also characterised by a reduced content of collagen and a thin fibrous cap. The MAPK is thought to inhibit ER-stress-induced apoptosis; as the amount of apoptosis arising in response to ER stressors is enhanced following knock down of p38 in primary mouse macrophages (Seimon *et al.* 2009). These studies demonstrate that p38 can exert pro- or anti-apoptotic effects dependent on the cellular conditions. However it seems likely that during atherosclerosis the MAPK is involved in protective signalling pathways against ER-stress responses and apoptosis in advanced plaques. The activation of G-protein-coupled receptors, receptor tyrosine kinases (RTKs) and factors related to stress such as ischaemia also initiate the p38 MAPK signalling cascade (Liang and Molkentin 2003). Interestingly a study by Kardakaris *et al.* (2011) indicated that ablation of p38α in macrophages and endothelial cells did not affect atherosclerotic plaque formation in $ApoE^{-/-}$ mice following feeding on a high cholesterol diet. Although p38α deficiency disrupted the recruitment of monocytes in response to modified LDL in *in vitro* cultures of endothelial cells (Kardakaris *et al.* 2011).

c-Jun N terminal kinases (JNKs) also belong to the MAPK superfamily. These proteins regulate the maturation and activity of T cells and the synthesis of pro-inflammatory cytokines such as IL-2, IL-6 and TNF-α (Rincon *et al.* 2000). Within $ApoE^{-/-}$ mice, JNK is activated in response to a high cholesterol diet. Interestingly, $JNK2^{-/-}$ mice crossed with $ApoE^{-/-}$ mice contain smaller plaques, whereas ablation of JNK1 has little effect on the size of the lesions in $ApoE^{-/-}$ mice. These differences highlight the differential roles of JNK isoforms within the atherosclerotic disease state. The lack of JNK2 within these mice is also associated with a halved number of macrophage foam cells despite enhanced uptake of fluorescently labelled AcLDL in peritoneal macrophages from

ApoE$^{-/-}$/JNK2$^{-/-}$ mice. Additionally, although levels of SR-A were raised, the efflux of cholesterol to apoA1 acceptors was enhanced and levels of phosphorylated, activated SR-A were reduced indicating that the knock down of JNK2 in macrophages compromises the production of foam cells through defective uptake and degradation of modified lipoproteins by SR-A (Ricci *et al.* 2004). The signalling component has also been implicated in later events within the disease. Within cultured primary rat aortic SMCs JNK is activated in response to oxidative stresses and induces the expression of matrix degrading MMPs whilst down-regulating the expression of genes involved in ECM biosynthesis such as lysyl hydroxylase (Yoshimura *et al.* 2005). The results indicate that the signalling component exerts destabilising effects on the architecture of aortic tissue which could compromise the integrity of the atherosclerotic plaque (Yoshimura *et al.* 2005). The MAPK likely plays a complex role in atherogenesis as transient activation of JNK promotes cell survival whereas a more sustained activation is linked to apoptosis (Chang *et al.* 2006).

The role of the nuclear factor-κB (NF-κB) signalling cascade in atherosclerosis is also well-established. The pathway regulates a number of genes implicated in atherosclerosis including TNF-α, IL-6, MCP-1 and ICAM-1 (Baker *et al.* 2011). Furthermore, the pathway is activated by several factors associated with the disease such as minimally OxLDL (Liao *et al.* 1994) and haemodynamic forces (Hajra *et al.* 2000). High levels of activated NF-κB subunits are found within the nuclei of intima-resident SMCs, macrophages and endothelial cells within human atheromas (Brand *et al.* 1996; Bourcier *et al.* 1997). Furthermore, inhibition of NF-κB, arising from the deletion of NF-κB essential modifier (NEMO) or inhibitor of NF-κB (IκBα), reduces the size of atherosclerotic plaques in ApoE$^{-/-}$ mice and is accompanied with impaired macrophage recruitment and reduced adhesion molecule expression (Gareus *et al.* 2008). Experiments utilising the transfer of bone marrow from p50-deficient mice to LDLR$^{-/-}$ mice have shown that the decrease in plaque size obtained by the ablation of NF-κB is associated with a fall in the number of lesional macrophage foam cells and expression of SR-A (Kanters *et al.* 2004). The resulting lesion displays an inflammatory phenotype characterised by increased numbers of macrophages and T cells (Kanters *et al.* 2004). Interestingly, overexpression of the NF-κB subunit p65 also inhibits atherosclerotic development in ApoE$^{-/-}$ mice associated with a drop in foam cell numbers and SR-A expression (Ye *et al.* 2013).

The phosphoinositide-3-kinase (PI3K) signalling cascade is responsible for the regulation of many cellular functions including glycogen and lipid metabolism, cell growth, calcium signalling, inflammation and apoptosis (Berridge 2012). Due to the diverse role of these proteins, the pathway has been implicated in many atherosclerotic processes. Ablation of the PI3K catalytic subunit, p110γ, decreases atherosclerotic development in genetically prone mice. The resulting lesions are characterised by a reduced number of proliferating plaque-resident macrophages (Chang *et al.* 2007; Zotes *et al.* 2013). Furthermore, atherosclerotic lesions within humans and mice display raised levels of the PI3K isoform; PI3K-γ, and transplantation of bone marrow from PI3K-$\gamma^{-/-}$ mice into irradiated LDLR$^{-/-}$ mice demonstrates that the expression of PI3K-γ by immune cells is integral for atherosclerotic development (Fougerat *et al.* 2008). PI3K-γ is also a key regulator of reparative neovascularisation and infarction size following a MI event within mice. As such inhibition of PI3K-γ impairs the angiogenic properties of endothelial cells and compromises cardiac function (Siragusa *et al.* 2010). However, several studies have also described anti-atherogenic roles for PI3K. The atheroprotective lipoprotein HDL activates endothelial nitric oxide leading to the uptake of HDL particles to SR-B1 and induction of the PI3K signalling cascade (Mineo and Shaul 2011; Zhang *et al.* 2011b; Wang and Peng 2012).

The PI3K family utilise membrane-residing lipids as substrates, and signalling through these pathways gives rise to the production of intracellular lipids that mediate a range of biological effects (Morello *et al.* 2009; Vanhaesebroeck *et al.* 2012). During atherosclerosis a vast array of lipid-derived compounds play a major role in initiating and directing pathological processes that augment the disease state. An imbalance between pro- and anti- lipid-derived inflammatory mediators dictates the inflammatory environment and will be discussed in further detail.

1.5.1. Lipid signalling and disease

Inflammation is a feature of the host's response to external threats and injury. However, dysregulation of this response can result in pathologies such as atherosclerosis. The cellular environment is characterised by the generation of pro-inflammatory cytokines, inflammatory mediators such as adhesion molecules and fatty acid-derived eicosanoids (Calder 2006). Eicosanoids, taken from the Greek for twenty, are formed from twenty

carbon PUFAs like AA, EPA and eicosatetraenoic acid (ETA) and are produced by all vascular tissues (Hajjar and Pomerantz 1992). In response to mechanical stress or inflammatory mediators, PUFAs are released from membrane-residing phospholipids by phospholipase A2 (PLA_2). The liberated fatty acids are then oxygenated through the LOX-dependent pathway or COX-pathway giving rise to the production of different eicosanoid family members: prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs) (Rustan and Drevon 2001; Wymann and Schneiter 2008). Prostacyclins, PGs and TXs derived from ETA, AA and EPA are formed from the actions of membrane-bound COX enzymes; COX-1 and -2 (Gil 2002). On the other hand, 5-LOX activity generates 3, 4 and 5 series LTs from ETA, AA and EPA (Gil 2002). An overview of eicosanoid synthesis is shown in Figure 6.

PGs are the most potent biologically active substances within the body (Vasudevan 2013) and are therefore rapidly formed and degraded (Wymann and Schneiter 2008). Due to the potent nature of eicosanoids, their synthetic processes are the targets of pharmacological intervention (Vasudevan 2013). A key discovery in the treatment of inflammation was the characterisation of aspirin in targeting the COX enzymes responsible for PG synthesis (Vane 1971). COX-1 is constitutively expressed in many tissues and is involved in gastric protection and renal water balance (Capra *et al.* 2013). In contrast, COX-2 expression is usually absent but inducible in response to inflammatory stimuli (Capra *et al.* 2013). Several modern non-steroidal anti-inflammatory drugs specifically target COX-2; as inhibition of COX-1 can have adverse gastric and renal effects (Tapiero *et al.* 2002). However, several studies have indicated that selective targeting of COX-2 may be linked to a greater chance of thrombotic events and a higher incidence of cardiovascular risk (Kearney *et al.* 2006; Antman *et al.* 2007). However, definitive proof that COX-2 inhibition promotes thrombosis is currently lacking. Armstrong *et al.* (2011) trialled a range of inhibitors including the non-specific COX inhibitors aspirin and diclofenac and a selective COX-2 inhibitor; parecoxib, in mice that had been subjected to platelet-driven thromboembolism. Samples from *ex vivo* tissue showed that general inhibition of COX enzymes had anti-thrombotic effects, whereas selective inhibition of COX-2 had little effect on thrombosis (Armstrong *et al.* 2011).

AA is a crucial fatty acid in the synthesis of pro-inflammatory eicosanoids. Inflammatory cells contain a high proportion of the fatty acid and only modest proportions of other twenty-carbon PUFAs (Calder 2006; Vasudevan 2013). Eicosanoids formed from AA are involved in a vast array of cellular activities that are associated with atherogenic processes including the activation of platelets and leukocytes. These species function in an autocrine and paracrine fashion and are therefore referred to as local hormones (Rustan and Drevon 2001). The 4-series LTs derived from AA are potent bronchoconstrictors, enhancers of vascular permeability and promoters of mucous secretion. LTB_4 is also a potent chemoattractant for leukocytes (Lewis *et al.* 1980; Samuelsson 1983). In contrast, the respective 5-series derived from EPA display weak chemotactic and vasoconstricting properties (Gil 2002). However, not all AA derivatives are involved in pro-inflammatory activities as 15-oxo-eicosatetraenoic acid is thought to inhibit endothelial cell proliferation indicating a potential anti-atherogenic role for the lipid (Wei *et al.* 2009). The effects of eicosanoids can also differ dependent on the cell type, the initial stimulus, the presence of other eicosanoids and the sensitivity of the target cell (Calder 2006).

Interestingly, several risk factors of atherosclerosis have been shown to directly influence eicosanoid production. A study by Fauler and Frölich (1997) demonstrated that the production of LTE_4 followed a dose-responsive relationship with the number of cigarettes smoked per day (Fauler and Frolich 1997). Furthermore, levels of AA-derived metabolites are elevated in hypercholesterolaemic patients and increased amounts of TXA_2 was linked to the activation of platelets and the action of platelet-derived PLAs (Davi *et al.* 1992). CRP is an acute phase protein and elevated levels within the plasma function as an independent risk factor for adverse cardiac events (Pepys and Baltz 1983; Capra *et al.* 2013). Also, treatment of porcine coronary arterioles with CRP inhibits nitric oxide production and restricts the secretion of the vasodilator PGI_2 thereby compromising cardiovascular homeostatic mechanisms (Hein *et al.* 2009). These studies lend some explanation to the underlying causes of well-established risk factors.

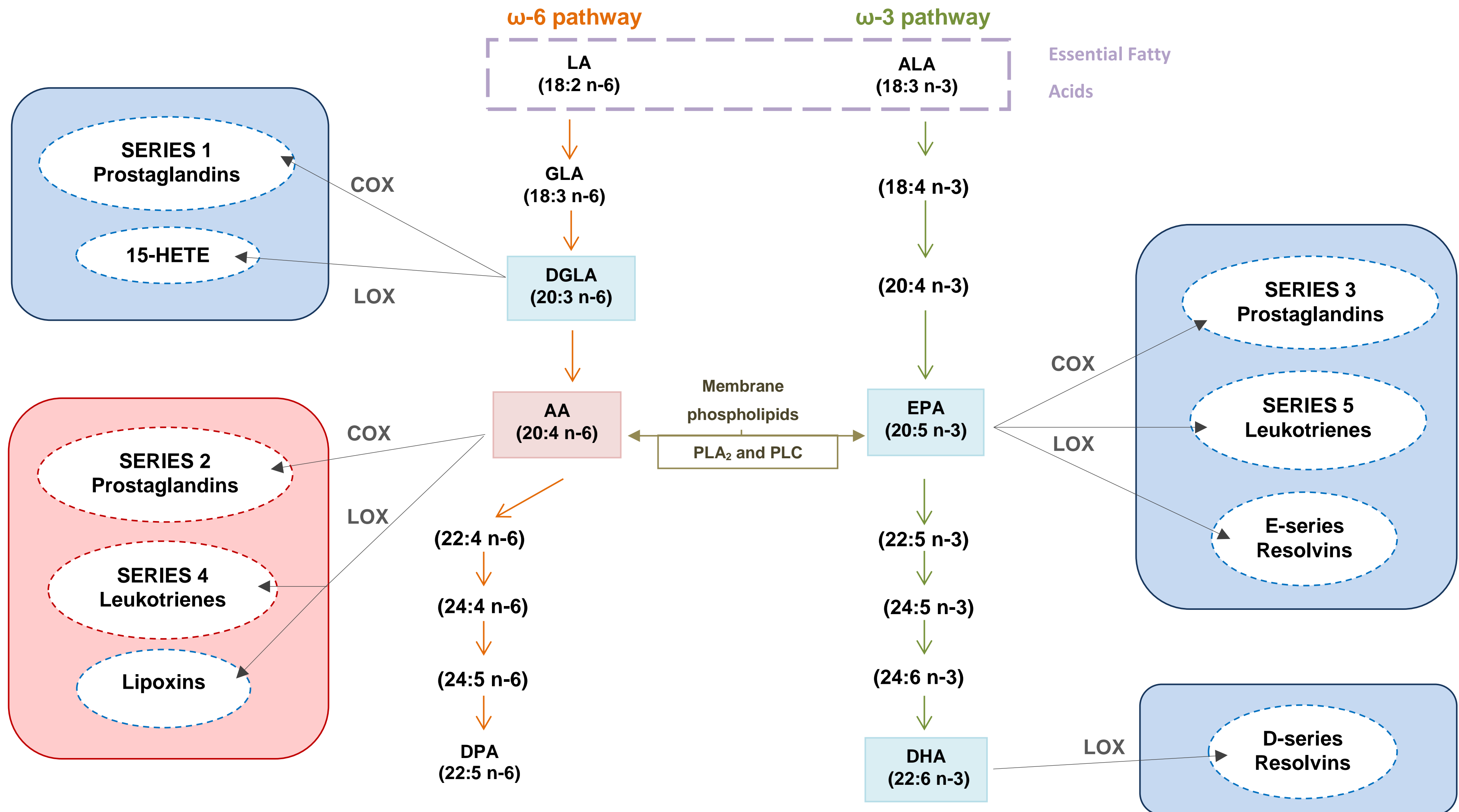


Figure 6. Lipid metabolism and eicosanoid biosynthesis

The Δ5- and Δ6-desaturases catalyse the formation of n-3 and n-6 PUFAs from essential fatty acids LA and ALA. Adapted from Merino *et al.* (2010) and Larsson *et al.* (2004). Red denotes pro-inflammatory mediators whilst blue denotes anti-inflammatory mediators. Abbreviations: 15-HETE; 15-hydroxyeicosatetraenoic acid

Enzymatic conversion of the essential fatty acids LA and ALA gives rise to two groups of lipid-derived messengers belonging to either the n-6 family or n-3 family respectively (Tapiero *et al.* 2002). n-6 and n-3 PUFAs are subjected to distinct metabolic routes and exhibit antagonistic actions. The consumption of EPA and DHA from fish sources causes a range of effects such as the reduction of PGE_2 derivatives, a decrease in the platelet aggregating agent TXA_2 and lowered production of LTB_4 which is involved in vasoconstriction and the recruitment and adherence of leukocytes. n-3 PUFAs are also modified to TXA_3 and PGI_3 which exert platelet aggregating and vasodilating effects (Simopoulos 2008).

n-3 PUFAs exert direct and indirect actions on other eicosanoids. For example n-3 fatty acids compete with n-6 members for COXs, therefore high levels of n-3 fatty acids negatively affect the formation of n-6 fatty acid derivatives (Arab 2003). n-3 PUFAs also indirectly influence the activities of n-6 PUFAs by regulating the expression of inflammatory genes (Calder 2006; Russo 2009). Furthermore, EPA and DHA function as precursors for the production of anti-inflammatory molecules such as resolvins and protectins. Resolvins were appropriately termed by Serhan and colleagues as these endogenous molecules are produced during the resolution phase and promote proresolution during inflammation (Serhan *et al.* 2000; Serhan *et al.* 2002; Hersberger 2010). Resolvins are either derived from EPA, giving rise to E-resolvins, or from DHA producing D-series resolvins, by COX-2. The E-series of resolvins have a range of actions and mediate a reduction in leukocyte rolling, enhance L-selectin shedding and block platelet aggregation (Dona *et al.* 2008). D-series resolvins also exert anti-inflammatory actions and inhibit the transendothelial migration of human neutrophils (Sun *et al.* 2007b).

The production and secretion of eicosanoids are modulated by cells within the plaque through the generation of inflammatory mediators including cytokines that target membrane precursor lipids or metabolic enzymes like COX (Hajjar and Pomerantz 1992).

1.6. The role of immune cells in atherogenesis

Atherosclerosis was initially thought to solely arise due to the passive accumulation of cholesterol in the arterial wall as early lesions are distinguishable by the build-up of lipids within the arterial intima (Hansson and Hermansson 2011). However, pathogenesis is complex and the disease is now recognised as a chronic inflammatory disorder that involves both innate and adaptive immune responses (Mallat *et al.* 2009). Prior to the disease, immunohistochemical staining has revealed that regions of the intima prone to atherosclerotic development are marked by an accumulation of macrophages, dendritic cells and activated T cells (Millonig *et al.* 2002). As the disease progresses a range of additional immune cells are recruited and reside within the plaque including B lymphocytes, mast cells and natural killer cells. The role of immune cells during the disease is vast and varied. For example, mast cells amass at sites prone to rupture and are persistently activated during the disease aiding plaque development within mice (Bot *et al.* 2007). The actions of these cells destabilises the plaque by inducing intraplaque haemorrhaging, macrophage apoptosis and vascular damage (Bot *et al.* 2007; Sun *et al.* 2007a). The following sections will focus on some of the key immune cells involved in atherogenesis.

1.6.1. Macrophage heterogeneity within the plaque

During the early stages of atherosclerosis, monocytes are the primary group of leukocytes recruited to the lesion. Monocytes develop within the bone marrow before entering the circulation as a heterogeneous non-differentiated population of cells (Randolph 2014). There are two categories of monocytes referred to as $CD14^{hi}CD16^{-}$ and $CD14^{+}CD16^{+}$ in humans and respectively termed $Ly6C^{hi}$ (inflammatory) and $Ly6C^{lo}$ (patrolling) within mice (Swirski *et al.* 2007). Hypercholesterolaemic $ApoE^{-/-}$ mice fed on a high-fat diet exhibit raised levels of $Ly6C^{hi}$ monocytes that adhere to the activated endothelium and enter the atherosclerotic lesion. Interestingly, within these mice the conversion of $Ly6C^{hi}$ to the $Ly6C^{lo}$ phenotype is impaired and migration of $Ly6C^{lo}$ monocytes into the plaque is also reduced (Swirski *et al.* 2007; Tacke *et al.* 2007). Following recruitment to the lesion, the monocytes differentiate into either

macrophages or myeloid dendritic cells under the influence of M-CSF and other differentiating agents (Imhof and Aurrand-Lions 2004; Gui *et al.* 2012).

Macrophages were the first immune cells identified within the plaque (Gerrity *et al.* 1979). Different subsets of macrophages arise due to the exposure of the circulating monocytes to specific priming agents and are generally classified as either M1 or M2 (Wolfs *et al.* 2011). $Ly6C^{hi}$ monocytes are thought to function as the precursors for M1 macrophages whereas $Ly6C^{lo}$ monocytes give rise to M2 macrophages (Moore *et al.* 2013). Monocyte differentiation into macrophages is accompanied by an increase in the expression of pattern recognition receptors such as SRs that facilitate the uptake of endotoxins, microbial products, apoptotic bodies and LDL particles (Libby *et al.* 2013). Classically activated M1 macrophages are stimulated by microbial products including LPS and cytokines like IFN-γ. Within the disease state, M1 macrophages are pro-atherosclerotic and release ROS and pro-inflammatory cytokines including TNF (Tabas 2010). Conversely, alternatively activated M2 macrophages aid the resolution of inflammatory responses through the synthesis of ECM components and anti-inflammatory cytokines like IL-13 (Tabas 2010). M2 macrophages are induced by T Helper (Th) 2 cytokines like IL-4 and are abundant in regressing plaques. In contrast, advanced lesions display an imbalanced ratio of M1 to M2 macrophages supporting defective resolution and augmentation of the chronic inflammatory state (Tabas 2010; Ogawa *et al.* 2012; Moore *et al.* 2013).

The M1 and M2 macrophage categories are not absolute as the population of infiltrating monocytes seen during the disease state contain a combination of M1 and M2 markers and, although macrophages are mainly categorised under these two broad labels, additional subclassifications and macrophage phenotypes exist (Gratchev *et al.* 2012). For example M2 macrophages are further subdivided based upon function and the polarising cytokine. Exposure to IL-4 or IL-13 gives rise to M2a macrophages involved in homeostasis and tissue repair, whereas IL-10 primed macrophages are referred to M2c and modulate the immune response (Wolfs *et al.* 2011). Other macrophage subtypes have been also detected within the plaque. Stimulation of monocytes with the platelet factor CXCL4 produces M4 macrophages which express a mixture of M1 and M2-associated genes, display limited phagocytic capabilities and express a

transcriptome distinct from M1 and M2 macrophages (Gleissner *et al.* 2010; Gleissner 2012). Although M4 markers are found within human atherosclerotic coronary arteries, the exact contribution of this subtype is not clear as M4 macrophages express both pro- and anti-atherogenic genes (Gleissner *et al.* 2010). However, the deletion of the gene encoding CXCL4 decreases the size of lesions within atherosclerotic prone mice (Sachais *et al.* 2007).

1.6.2. Dendritic cells

The exposure of circulating monocytes to inflammatory stimuli like GM-CSF promotes dendritic cell formation (Niessner and Weyand 2010). Ablation of GM-CSF in LDLR$^{-/-}$ mice causes a substantial fall in the number of plaque-residing dendritic cells in addition to a significant reduction in lesion size (Shaposhnik *et al.* 2007). Although macrophages and dendritic cells share common origins, the cells can be distinguished by differences in functionality. Macrophages are mainly involved in responding to injury whereas dendritic cells favour the presentation of antigens on major histocompatibility complex (MHC) molecules to direct the T cell response (Randolph 2014). The presence of dendritic cells within the plaque was initially described in 1995 through the use of electron microscopy (Bobryshev and Lord 1995). The accumulation of these cells, although not as abundant as macrophages and T cells, into areas prone to atherosclerosis correlates with disease progression and inflammation (Liu *et al.* 2008; Frostegard 2013).

Dendritic cells belong to the group of professional antigen presenting cells and also function as primers of T cell responses and modulators of immune tolerance (Steinman *et al.* 2003b; Van Vre *et al.* 2011). The maturation state of dendritic cells dictates their function (Menges *et al.* 2002). Immature dendritic cells within peripheral tissues drive tolerance through the deletion of autoreactive T cells (Steinman *et al.* 2003a). Yet, in response to inflammatory stimuli, dendritic cells undergo phenotypic and functional changes, mature and migrate to T cell-rich areas to activate naïve T- and B- cells (Link and Bohm 2002). The change is also accompanied with enhanced expression of costimulatory molecules, chemokine receptors, adhesion molecules and the release of cytokines that influence the formation of different T cell subtypes (Dhodapkar *et al.* 2001; Frostegard 2013).

A range of receptors including SRs are expressed at the surface of dendritic cells that facilitate the uptake of antigens. The antigen peptides are then trans located onto MHC molecules in preparation for T cell presentation (Frostegard 2013). Danger-associated molecular patterns released during atherosclerosis are recognised through receptors such as SRs present at the surface of dendritic cells and promotes a shift to adaptive immune responses (Niessner and Weyand 2010; Hansson and Hermansson 2011). Alderman and colleagues demonstrated that the exposure of mildly OxLDL to monocyte-derived dendritic cells triggers the activation and maturation of these cells and consequently leads to the activation and proliferation of T cells. However, continued exposure to highly OxLDL causes apoptosis of dendritic cells (Alderman *et al.* 2002). Inflammatory species such as ROS and denatured material from the plaque may potentially injure dendritic cells and in doing so support tolerance or suppression of the immune response instead of T cell activation (Rutault *et al.* 1999; Link and Bohm 2002).

1.6.3. T cells

Monocytes and T cells are recruited along a chemotactic gradient and enter the plaque through interactions with cell-surface adhesion molecules during initial stages of the disease. The mass of macrophages and T cells constitutes the major component of the fatty streak (Paulsson *et al.* 2000; Hansson and Libby 2006). The number of T cells present within the plaque are far fewer than that of macrophages but have an important modulating role during pathology (Libby *et al.* 2011). Antigen presenting cells such as macrophages and dendritic cells interact with T cells to activate the adaptive immune system. These cells also influence the activities of T cells through the secretion of cytokines like IL-12 that modulates the infiltration of T cells into the plaque (Niessner *et al.* 2006). As such the function of T cells are closely tied to that of dendritic cells and macrophages (Baidya and Zeng 2005).

The cytokine repertoire of T cells is dictated by their phenotype. The production of cytokines from naïve T cells is limited to IL-2, whereas memory T cells, that have had prior exposure to antigens, secrete a range of cytokines including IFN-γ, IL-3, IL-4, IL-5, IL-6, GM-CSF and M-CSF (Stemme *et al.* 1992). The cells are then activated in

response to specific antigens presented on MHC complexes of antigen presenting cells such as dendritic cells, macrophages and B cells triggering the proliferation of antigen-specific T cells (Baidya and Zeng 2005).

Samples from human plaques have demonstrated that the majority of T cells are memory T cells positive for the T cell antigen receptor and $CD4^+$; although $CD8^+$ T cells are also present (Hansson and Hermansson 2011). Several studies have shown that atherosclerotic plaques contain oxidative lipoproteins and heat shock protein-specific T cells therefore indicating that local activation and clonal expansion occurs during the disease (Paulsson *et al.* 2000; Rossmann *et al.* 2008). Many of the cells show signs of late or chronic activation and are distinctly different from the circulating population (Stemme *et al.* 1992). T cells promote inflammation through the production of cytokines like IFN-γ and TNF that activate VSMCs and endothelial cells (Hui 2007). Within the plaque $CD4^+$ T cells propagate the disease state by promoting the apoptosis of VSMCs through death receptors on those cells (Sato *et al.* 2006), in addition to the activation of macrophages through the secretion of IFN-γ (Liuzzo *et al.* 2001) and the release of perforin which lyses endothelial cells (Nakajima *et al.* 2002).

Subsets of Th cells are generalised under the Th1 and Th2 categories based upon cytokine expression (Lohning *et al.* 1998). The balance between Th1 and Th2 responses is carefully modulated by regulatory T cells (Tregs) (Galkina and Ley 2009). Tregs are specialised T cells that suppress pathogenic responses by the immune system towards foreign and self-antigens (Sakaguchi *et al.* 2006). Activated Tregs can suppress the actions of T cells and other immune cells and atherosclerotic mice deficient in Tregs display increased plaque development (Ait-Oufella *et al.* 2006). However the disease is characterised by an abundance of T cells supporting an imbalance in Th1 to Th2 cytokines (Libby *et al.* 2013).

The Th1 response is associated with the release of pro-inflammatory cytokines such as the classical Th1 cytokine IFN-γ (Libby *et al.* 2009). The cytokine exerts a major impact on disease progression and injections of IFN-γ into $ApoE^{-/-}$ mice increases the size of the atherosclerotic lesion by approximately two-fold (Whitman *et al.* 2000). On the other hand, $ApoE^{-/-}$ mice crossed with IFN-γ receptor $(IFN\text{-}\gamma R)^{-/-}$ mice exhibit attenuated atherosclerotic development accompanied by a substantial reduction in

accumulated lipids within the lesion (Gupta *et al.* 1997). The Th2 response is conversely associated with attenuated atherosclerotic formation, however its exact contribution in the disease state is unknown (Libby *et al.* 2009). Th2 cell cytokines, including IL-4, and the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) bias macrophage differentiation towards the M2 phenotype (Tabas 2010). However, the abundances of Th2 cytokines such as IL-4 and IL-5 are markedly under-represented in comparison to Th1 cytokines within the plaque (Frostegard *et al.* 1999).

1.7. Cytokine involvement in the atherosclerotic state

Cytokines are soluble low molecular weight proteins involved in immune and inflammatory responses (Ross 1993). Most are composed of a single polypeptide chain and travel short distances to bind with high affinity to their specific receptors typically located on lymphoid organs or inflamed tissues (Ait-Oufella *et al.* 2011). Based upon the structural homology of their receptors, cytokines are classified as class I or class II owing to the presence of either four conserved cysteine residues or two cysteine doublets in the extracellular region (Bazan 1990). The cytokine family is a large group of over 100 proteins encompassing lymphokines, monokines, chemokines, interferons, TNFs and interleukins (George and Johnson 2010) and cytokines from the same family regularly share receptor subunits (Parrish-Novak *et al.* 2002). Members of these families have common characteristics and are frequently pleiotrophic whereby cytokines exert different effects dependent on the type of cell and time they were produced. A key difference between cytokines and GFs is that cytokine production is strictly regulated and the main targets are immune cells (Tedgui and Mallat 2006).

Cytokines are important mediators of the innate and adaptive immune system and play a key role at every stage of atherosclerosis, from early events involving dysfunction of the endothelium, lipid metabolism and later phase actions such as enhancing MMP secretion (Ait-Oufella *et al.* 2011). Within the disease state, the production of many cytokines is auto-inducible through autocrine and paracrine signalling, which helps to augment and sustain inflammation (Panousis and Zuckerman 2000). Extracellular GFs and cytokines are highly expressed during atherosclerosis and mediate the proliferation and survival of cells involved in plaque formation (McLaren and Ramji 2009).

Furthermore, the synergistic actions of cytokines and GFs can function to amplify their responses. For example, the effects of IL-1α and TNF-α on MMP activation is enhanced through costimulation with platelet-derived GF and fibroblast GF-2 in rabbit VSMCs and human SMCs (Bond *et al.* 2001).

Cytokines exert a dual role during atherosclerosis and a complex interplay between pro- and anti-inflammatory cytokines arises which influences the development and stability of the plaque. Table 3 gives a description of prominent pro- and anti-atherogenic cytokines. The prevalence of pro-inflammatory cytokines within the plaque drives Th1-related processes and augments disease progression (McLaren *et al.* 2011a). A variety of pro-inflammatory cytokines such as IFN-γ, TNF-α and TNF-like protein-1A support Th1 responses and promote foam cell formation (McLaren *et al.* 2010a). Conversely, anti-inflammatory cytokines predominately promote Th2-type responses that function to resolve inflammation (McLaren *et al.* 2011a). The anti-inflammatory cytokine transforming growth factor-beta (TGF-β) inhibits SR activity and reduces foam cell production (Michael *et al.* 2012b). Interestingly, Th1 and Th2 cytokines are capable of cross-regulation, for example, pro-inflammatory IL-12 inhibits Th2 responses and may restrict Th2 activities during atherosclerosis (Hansson *et al.* 2002).

Table 3. Cytokines involved in atherosclerosis

Cytokine	Role in atherosclerosis	Reference
Pro-atherogenic		
IFN-γ	$ApoE^{-/-}$ knockout mice crossed with IFN-γ$R^{-/-}$ knockout mice display smaller atherosclerotic lesions and decreased lipid accumulation.	(Gupta *et al.* 1997)
TNF-α	Size of atherosclerotic plaque significantly reduced in TNF-α and ApoE double knockout mice. Also, ICAM-1 and MCP-1 expression down-regulated.	(Ohta *et al.* 2005)
IL-1-β	IL-1 receptor antagonist decreases the production of fatty streak in $ApoE^{-/-}$ mice.	(Elhage *et al.* 1998)
IL-6	Injection of recombinant IL-6 into wild type and $ApoE^{-/-}$ mice promotes atherosclerotic development and elevates the release of pro-inflammatory cytokines like IL-1β and TNF-α.	(Huber *et al.* 1999)
IL-18	Raised levels of serum IL-18 in patients with CAD.	(Blankenberg *et al.* 2002)
Anti-atherogenic		
IL-10	Overexpression of IL-10 in $LDLR^{-/-}$ mice reduces atherosclerotic plaque formation and promotes the Th2 phenotype.	(Pinderski *et al.* 2002)
IL-33	Reduces foam cell formation *in vitro* and the development of atherosclerosis in $ApoE^{-/-}$ mice.	(Miller *et al.* 2008; McLaren *et al.* 2010b)
TGF-β	Neutralising antibodies against TGF-β isoforms augments atherosclerosis and promotes instability within the lesion associated with decreased collagen content.	(Mallat *et al.* 2001)

1.7.1. Interleukin-33 (IL-33)

In 1989 ST2 was identified as an orphan receptor that shared close homology to the extracellular portion of murine interleukin-1 receptor (IL-1R) and IL-18α (Tominaga 1989). Toll-like/IL-1R family members are characterised by the presence of a shared Toll/interleukin-1 receptor (TIR) domain consisting of a central five-stranded β-sheet surrounded by five α-helices (Xu *et al.* 2000). The ST2 receptor was later shown to play a role in supporting anti-inflammatory processes such as enhanced differentiation of Th2 cells and polarisation of M2 macrophages (Kakkar and Lee 2008; Ogawa *et al.* 2012). ST2 is expressed on the surface of immune cells including mast cells, dendritic cells, Th2 cells and macrophages but is not expressed on naïve T cells or Th1 cells (Lohning *et al.* 1998; Lefrancais *et al.* 2012).

Alternative splicing of the gene that encodes ST2 gives rise to at least eight isoforms of the receptor including ST2L (functional full-length transmembrane form), ST2V (variant), ST2LV and sST2 (secreted, soluble decoy receptor) (Joshi *et al.* 2010; Miller and Liew 2011). ST2L has an extracellular domain containing three immunoglobulin-like motifs, a transmembrane portion and a TIR domain (Kakkar and Lee 2008). ST2V lacks the third immunoglobulin motif and instead contains a novel hydrophobic tail in the C-terminal region (Tominaga *et al.* 1999). In contrast, ST2LV is characterised by an absence of the transmembrane portion found in ST2L (Kakkar and Lee 2008) and the soluble decoy receptor sST2 does not contain the transmembrane or intracellular domains (Iwahana *et al.* 1999).

The gene for IL-33 was initially discovered by Baekkevold *et al.* (2003) in high endothelial venule (HEV) endothelial cells and was termed nuclear factor from HEV's (Baekkevold *et al.* 2003). It was not until 2005 that the gene was identified as a ligand for ST2 following a computational search for members of the IL-1R family (Schmitz *et al.* 2005). The human gene for IL-33 contains seven exons and is located on chromosome 9p24.1 and on chromosome 19qC1 in mice. The cytokine is formed of 270 and 266 amino acids in human and mouse respectively and share 55% identity to one another (Schmitz *et al.* 2005). Within mice, levels of IL-33 mRNA are high within the stomach, lung, spinal cord, brain and skin but less prevalent in lymph tissue, spleen, pancreas, kidney and the heart. IL-33 mRNA is also found in resting mouse dendritic

cells and activated macrophages. The cytokine is not expressed by the majority of human haematopoietic cells; with the exception of activated dendritic cells and macrophages where it is expressed at low levels. However, it is constitutively expressed by human SMCs and bronchial epithelial cells. Furthermore, the expression of IL-33 is induced by TNF-α and IL-1β in primary lung and dermal fibroblasts and keratinocytes (Schmitz *et al.* 2005).

Family members such as IL-1 and IL-18 contain prodomains that are proteolytically cleaved to produce the mature form (Chackerian *et al.* 2007). Interestingly, IL-33 is also secreted containing a prodomain and *in vitro* studies have shown that the cytokine is susceptible to cleavage by caspase-1 yielding a 18kDa peptide product (Schmitz *et al.* 2005). Initial theories proposed that IL-33 may be processed in a fashion similar to other IL-1β members and that caspase activity may be a prerequisite for activation (Kakkar and Lee 2008). However, although caspase-1 is capable of cleaving IL-33; the cytokine is preferentially cleaved by the caspases-3 and -7 (Schmitz *et al.* 2005; Luethi *et al.* 2009; Talabot-Ayer *et al.* 2009). Furthermore, recent studies have demonstrated that cleavage after residue 178 (aspartic acid) in the IL-1-like domain functions to inactivate the cytokine (Cayrol and Girard 2009).

A recent study by Luzina *et al.* (2012) found that full-length, unprocessed IL-33 was the major form found in the lungs of healthy and pulmonary disease sufferers. Interestingly, the full length and mature forms display slightly different activities in mouse model systems. Both forms trigger the infiltration of lymphocytes and neutrophils, but only the mature form promotes pulmonary eosinophilia, goblet cell hyperplasia and increases the expression of IL-4, IL-5, IL-13, IL-17 and MCP-1. Also, in the absence of the ST2 receptor the response from full-length IL-33 is not lost (Luzina *et al.* 2012). IL-33 can directly interact with heterochromatin and could therefore participate in the regulation of gene expression through repressing gene transcription in a receptor-independent method (Ali *et al.* 2011). It is thought that full-length IL-33 may also serve as an alarmin; an endogenous molecule released in response to tissue and cellular injury and has been hypothesised that caspase-mediated inactivation of IL-33 acts to limit pro-inflammatory responses mediated by the cytokine during apoptosis (Bianchi 2007; Miller 2011).

spIL-33 is a novel short splice variant which lacks the caspase-1 cleavage site and is therefore constitutively expressed. Recombinant spIL-33 induces the phosphorylation of IL-1R-associated kinase 1 (IRAK1), NF-κB, ERK1, ERK2, p38 and JNK in a dose- and time-responsive fashion (Hong *et al.* 2011). The presence of new splice variants indicates that different forms of IL-33 may be released in response to a variety of stimuli (Hong *et al.* 2011).

1.7.2. The IL-33 signalling axis

Under normal physiological conditions, IL-33 is associated with chromatin within the nucleus through an N-terminal bipartite homeo-domain-like helix-turn-helix motif DNA binding domain (Ali *et al.* 2011). The role of IL-33 as an intracellular factor is poorly understood but is thought to function as a nuclear repressor (Choi *et al.* 2012). Interestingly, the mechanisms giving rise to extracellular IL-33 have not been fully defined and are further complicated by the absence of a signal sequence within IL-33 mRNA (Shao *et al.* 2014). However, in response to certain stimuli, extracellular IL-33 binds to the ST2L receptor triggering the formation of a trimeric complex with the IL-1R accessory protein (IL-1RaP) (Ali *et al.* 2011). IL-1RAcP functions as a co-receptor which associates with ST2 in a ligand-dependent fashion and is necessary for ST2 activity (Arend *et al.* 2008). Both ST2 and IL-1RAcP are widely expressed and prevalent in many cells from the innate immune system and Th2 cells (Miller 2011). The presence of IL-1RAcP increases the affinity of IL-33 for ST2 (Palmer *et al.* 2008). Mice deficient in IL-1RAcP do not respond to IL-33 treatment and other members of the IL-1 family such as IL-1F6, IL-1F8 and IL-1F9, also utilise IL-1RAcP for signal transduction (Chackerian *et al.* 2007). IL-33-mediated activity through the ST2-IL-1RAcP complex triggers the recruitment of IRAK, IRAK4, the E3 ubiquitin ligase TNF receptor associated factor (TRAF6) and the adaptor protein myeloid differentiation primary-response protein 88 (MyD88) (Schmitz *et al.* 2005; Chackerian *et al.* 2007). Signalling occurs through the TIR domain of the ST2 receptor and leads to the activation of additional signalling pathways. An overview of IL-33 signalling is given in Figure 7.

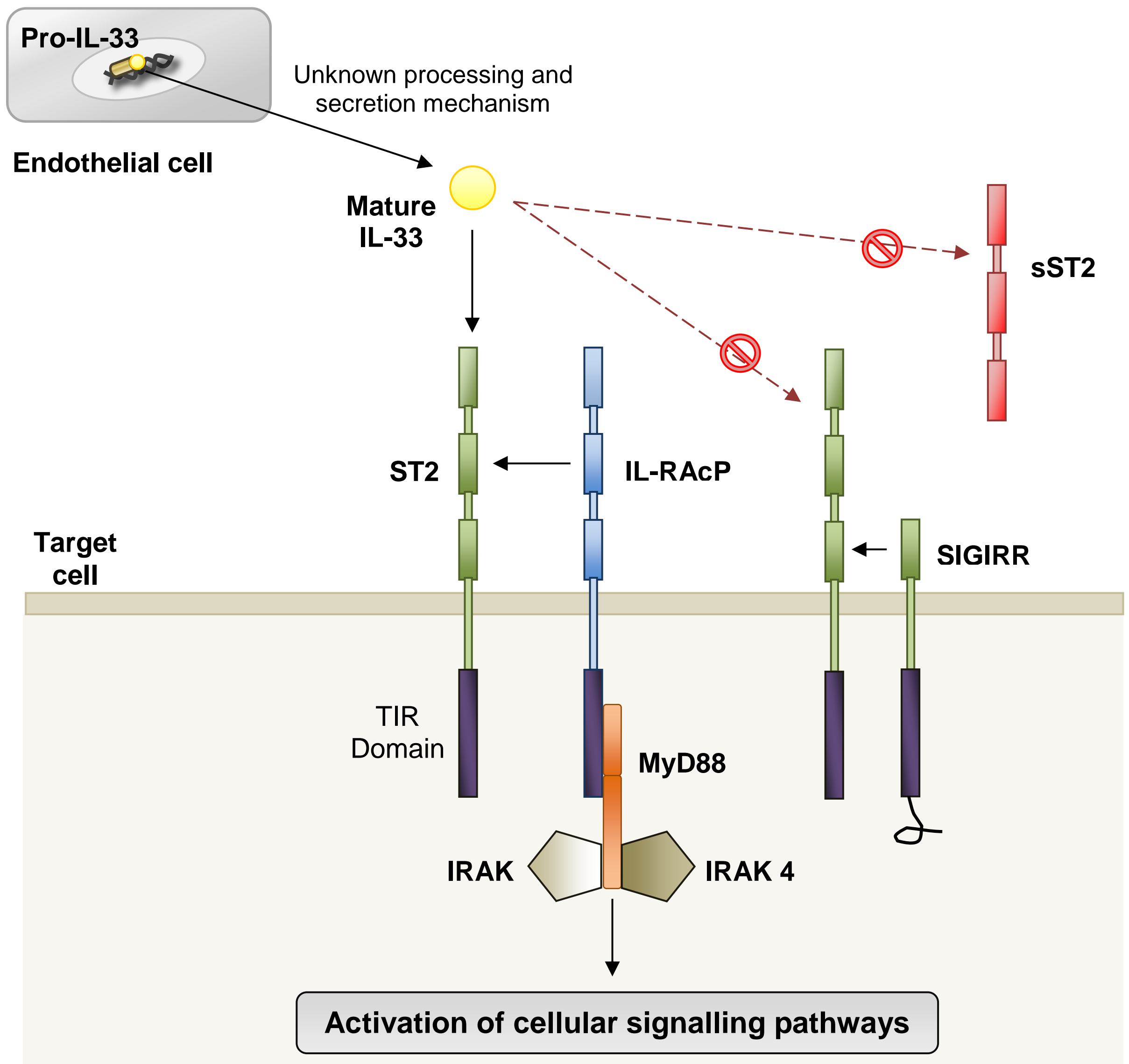


Figure 7. Canonical IL-33/ST2 signalling

Nuclear IL-33 is thought to function as a nuclear repressor however the mechanisms leading to release and eventual processing to the mature form of the cytokine are not known. Binding of extracellular IL-33 to ST2 is enhanced in the presence of IL-RAcP. The trimeric complex recruits the adaptor proteins MyD88, IRAK and IRAK4 leading to the activation of signalling components. Signalling by IL-33 is attenuated by the decoy receptor sST2 that reduces the availability of IL-33 and the presence of SIGIRR also negatively impacts IL-33 signalling. Abbreviations: SIGIRR; single Ig IL-1R-related molecule. Adapted from Liew *et al.* (2010) and Palmer and Gabay (2011).

The IL-33/ST2 axis, like other members from the IL-1 family, utilises members from the NF-κB and the MAPK superfamilies (Schmitz *et al.* 2005). An overview of studies exploring IL-33 signalling is provided in Table 4. The cytokine is thought to negatively

regulate gene transcription in an intracrine receptor-independent manner. For example, the full-length form associates with the p65 subunit of NF-κB and impairs the ability of NF-κB to bind to DNA (Ali *et al.* 2011). There are also a number of regulatory mechanisms that attenuates ST2-IL-33-mediated signalling. The decoy receptor sST2 binds to IL-33 and therefore reduces its availability for ST2L. On the other hand, a single immunoglobulin IL-1R-related molecule (SIGIRR) binds to ST2L so the receptor is unable to complex with IL-RAcP thereby terminating further signal transduction (Bulek *et al.* 2009). Additionally, a splice variant for IL-1RaP also exists that enhances the binding affinity of IL-33 to sST2 (Palmer *et al.* 2008). For clarity ST2L will be referred to as ST2 henceforth within this thesis.

Table 4. Studies investigating IL-33 signalling

IL-33 Signalling	Reference
Full length IL-33 constitutively interacts with the NF-κB subunit p50. Interactions between IL-33 and NF-κB reduce and delay the binding of NF-κB to DNA and decreases NF-κB triggered transactivation in HEK293R1 embryonic kidney cells.	(Ali *et al.* 2011)
IL-33 activates endothelial cells and increases vascular permeability and several processes associated with angiogenesis; these actions are ST2-dependent and mediated through PI3K and Akt.	(Choi *et al.* 2009)
IL-33 promotes the survival and adhesion of human umbilical cord blood-derived mast cells to fibronectin. Signalling through IL-33 also leads to the activation of ERK, p38 and JNK and induces IL-8 and IL-13 production within these cells.	(Iikura *et al.* 2007)
IL-33 inhibits the induction of NF-κB in wild-type mice but not in $ST2^{-/-}$ mice.	(Sanada *et al.* 2007)
IL-33 stimulation results in the recruitment of MyD88, IRAK, IRAK4 and TRAF6. Treatment of ST2-transfected HEK293 cells with IL-33 leads to the activation of ERK1/2, p38, IκBα and JNK. A similar result is seen in HEK293 cells transfected with IL-1R1 following treatment with IL-1β. IL-33 stimulation of murine mast cells results in the phosphorylation of NF-κB, ERK1/2, p38 and JNK.	(Schmitz *et al.* 2005)

1.7.3. The involvement of IL-33 in disease

IL-33 mainly promotes Th2 responses unlike other IL-1 family proteins and also acts as a chemoattractant for Th2 cells (Komai-Koma *et al.* 2007). The cytokine has been implicated in a number of inflammatory disorders including asthma, rheumatoid arthritis and ulcerative colitis (Lefrancais *et al.* 2012). IL-33 promotes M1 chemokine production in naïve human macrophages but also increases the expression of M2 chemokine markers within mature polarised macrophages (Joshi *et al.* 2010). Furthermore, IL-33 amplifies the polarisation of alternatively activated macrophages during airway inflammation and levels of the cytokine are elevated in epithelial cells from asthmatic sufferers (Kurowska-Stolarska *et al.* 2009). A summary of studies describing the role of IL-33 in different disease states is provided in Table 5.

There is a great level of interest in understanding the actions and mechanisms behind IL-33 signalling as the cytokine displays conflicting activities in different disease states. Several studies have demonstrated that IL-33 has an adverse role in mast-cell mediated inflammatory diseases (Liew *et al.* 2010). A study by Xu *et al.* (2008) established that IL-33 was expressed in synovial fibroblasts of patients with rheumatoid arthritis and its expression was increased by the inflammatory cytokines TNF-α and IL-1β *in vitro*. In murine models of arthritis, ST2 knockout mice exhibited less severe collagen-induced arthritis, whereas the wild-type displayed increased signs of arthritis as a result of enhanced secretions of pro-inflammatory cytokines and anti-collagen antibodies (Xu *et al.* 2008). Mice injected with IL-33 develop splenomegaly accompanied with elevated amounts of eosinophils, mononuclear cells, and plasma cells. Furthermore, levels of circulating immunoglobulin (Ig) E and IgA, which are associated with hypersensitivity, were raised (Polmar *et al.* 1972; Schmitz *et al.* 2005).

IL-33 is thought to function as an ‘alarmin’ capable of amplifying immune responses following injury to tissues. Oboki *et al.* (2010) proposed that IL-33 is an important amplifier of mucosal and systemic innate responses as the cytokine was crucial for T cell-independent protease allergen-mediated airway inflammation and mice deficient in IL-33 displayed a compromised response to LPS-induced systemic inflammation

(Oboki *et al.* 2010). These studies implicate IL-33 as a potent inducer of the pro-inflammatory response.

Several studies have, however, also demonstrated that IL-33 can have a protective role in certain disease states. Neill *et al.* (2010) identified a novel innate type 2 immune leukocyte which they termed the nuocyte. IL-33 and IL-25 stimulation caused the expansion of nuocytes *in vivo* which were the primary source of IL-13; a type 2 cytokine vital for countering the immune response against helminth infection and allergic responses. However, in the absence of IL-33 and IL-25 nuocytes were unable to expand leading to compromised expulsion of worms (Neill *et al.* 2010).

Table 5. Studies investigating the role of IL-33 in inflammatory disorders

Organ/ disease	Role of cytokine	Reference
Cancer	Mice with mammary tumours lacking the ST2 receptor display reduced metastasis and raised numbers of natural killer cells expressing IFN-γ. IL-33 treatment promotes vascular permeability and proliferation, migration and differentiation of endothelial cells.	(Choi *et al.* 2009; Jovanovic *et al.* 2011)
Central nervous system	Polymorphism in the gene for IL-33 is associated with Alzheimer's disease and reduced amounts of IL-33 are found in the brains of Alzheimer patients.	(Chapuis *et al.* 2009)
Crohn's disease and rheumatoid arthritis	High levels of IL-33 expression in endothelial cells from patients with rheumatoid arthritis and Crohn's disease.	(Carriere *et al.* 2007)
Immune system	IL-33 injections increase amounts of eosinophils, mononuclear cells and plasma cells, and levels of IgE and IgA. IL-33 induces expression of IL-4, IL-5 and IL-13 in thymus, spleen, liver and lung but no changes in levels of IL-1α, IL-2, IL-10, IL-12, TNFα, or IFN-γ, accompanied by increased size of duodenum and spleen. Arteries within lung tissue display hypertrophy and infiltration of myeloid cells in the lumen and eosinophils and/or mononuclear cells within the subendothelium. IL-33 has a protective effect in mice infected with intestinal nematodes by inducing a Th2 response.	(Schmitz *et al.* 2005; Humphreys *et al.* 2008)
Respiratory	High levels of IL-33 expression in asthmatic patients, particularly in bronchial epithelial cells. ST2 knockout mice have reduced inflammation in the airways and lungs in comparison to wild-type mice subjected to ovalbumin-induced inflammation.	(Kurowska-Stolarska *et al.* 2008; Kurowska-Stolarska *et al.* 2009; Prefontaine *et al.* 2009)

1.7.4. The role of IL-33 in CAD

In addition to the well-documented pro-inflammatory actions of the cytokine in certain disease states, IL-33 can also attenuate inflammatory responses. Several studies have described a protective role for IL-33 within atherosclerosis and cardiovascular diseases. In experiments undertaken by Miller *et al.* (2008), injections of recombinant IL-33 into $ApoE^{-/-}$ mice decreased the generation of atherosclerotic lesions. Additionally, the cytokine reduced the number of lesional macrophages and promoted a Th1 to Th2 phenotypic switch within the plaque accompanied with enhanced production of the Th2 cytokines IL-4 and IL-13 and secretion of antibodies against OxLDL. Treatment with the decoy receptor reversed the protective effects of IL-33 and also increased the size of the lesion (Miller *et al.* 2008).

Studies by Mclaren *et al.* (2010) demonstrated that IL-33 acts through the ST2 receptor to decrease foam cell formation *in vivo*. Also, treatment of THP-1 macrophages with IL-33 reduced the uptake of AcLDL and OxLDL (McLaren *et al.* 2010b). The authors also identified several genes involved with mediating the reduction in foam cell accumulation. The cytokine decreased the expression of genes implicated in lipid uptake and storage such as SR-A, SR-B1 and CD36, and cholesterol esterification like ACAT-1. In contrast, the expression of the cholesterol efflux transporters; ABCA-1 and ABCG-1 were up-regulated by the cytokine (McLaren *et al.* 2010b). Within THP-1 macrophages, IL-33 also inhibited the uptake of Lucifer yellow, a fluorescent dye used as an indicator of macropinocytosis (Michael *et al.* 2013). As macropinocytosis is attributed as a contributor to plaque formation through constitutive and passive uptake of LDL particles, the study demonstrates a novel mechanism by which IL-33 may reduce macrophage foam cell formation *in vitro*.

The cytokine has a wide range of effects on different cell types that reside within the atherosclerotic plaque. A study by Wasserman *et al.* (2012) showed that IL-33 increases the number of Tregs. During normal physiological conditions Tregs promote the switch from Th1 to Th2 but during atherosclerosis the number of Tregs within the plaque is reduced. Interestingly, within $ApoE^{-/-}$ mice, IL-33 treatment had no effect on the number of Tregs. However, levels of the decoy receptor sST2 were elevated within

these animals whilst the amount of ST2 was reduced. The authors suggest that attenuation of signalling through the IL-33/ST2 axis contributes to the repressed number of regulatory T cells observed during the atherosclerotic state and therefore promotes the Th1 state (Wasserman *et al.* 2012).

The IL-33/ST2 axis enhances angiogenesis and endothelial cell permeability *in vitro* and *in vivo* by increasing the production of nitric oxide (Choi *et al.* 2009). However, IL-33 reduces the damage incurred from oxidative stress in cultured cardiomyocytes and in mice with ischaemia by promoting the expression of anti-apoptotic mediators. The cytokine also protects against cardiomyocyte apoptosis in *in vitro* and *in vivo* systems (Seki *et al.* 2009). These effects were shown to be ST2-dependent as ischemic mice displayed reduced infarction volume and improved ventricular function when treated with IL-33 but the same effects were not observed in $ST2^{-/-}$ mice (Seki *et al.* 2009). The cytokine also defends cells against mechanical stresses. In response to mechanical stretch cardiomyocytes undergo hypertrophy characterised by the enlargement of cells in the absence of cell division (Sadoshima *et al.* 1993). Sustained hypertrophy can lead to compromised contractile functionality and arrhythmia and therefore often serves as a precursor to heart disease. However, IL-33 is released by cardiomyocytes in response to biomechanical stress and inhibits the actions of hypertrophic effectors such as angiotension II and phenylephrine. The cytokine functions as a protector against mechanical overload by reducing cardiac fibrosis and cardiomyocyte hypertrophy *in vivo*. These responses were shown to be dependent on ST2 as administration of sST2 reversed the effects of IL-33 (Sanada *et al.* 2007).

Epidemiological studies have also described the relationship between the cytokine and cardiac disorders. Levels of IL-33 are raised in the serum of patients who suffer from acute coronary syndrome and stable angina pectoris (Liu *et al.* 2013). Another study found that serum levels of IL-33 were elevated in patients with chronic heart failure (CHF) and were positively associated with several markers of oxidative stress such as erythrocyte superoxide dismutase activity (Zhang *et al.* 2012). Interestingly, levels of sST2 were also high and contributed to a depressed ratio of IL-33/sST2. Furthermore, the full-length and cleaved versions of IL-33 were detected in patients with CHF, whereas only the cleaved form was found in healthy subjects. The results suggest that full-length IL-33 may be released in response to heart failure (HF) but the cardio-

protective effects of IL-33 are negated by an increased presence of the decoy receptor (Zhang *et al.* 2012).

The soluble decoy receptor sST2 has also attracted a lot of attention as the receptor negates the cardioprotective actions of the IL-33/ST2 axis. As such levels of sST2 have been proposed to function as an accurate prognostic tool for predicting cardiovascular risk. Circulating sST2 is transiently boosted following a MI event within human patients and in mice with experimental MI (Weinberg *et al.* 2002). Furthermore, levels of sST2 correlate with other inflammatory markers, total mortality and cardiovascular mortality in low risk populations and in patients with acute MI (Shimpo *et al.* 2004; Rehman *et al.* 2008; Chen *et al.* 2013). HF is the most frequent reason for hospital admissions and readmissions, and arises due to compromised contractibility of the heart (Avery *et al.* 2012). Levels of sST2 are elevated in sufferers of HF and there is a relationship between levels of serum sST2 and the extent and volume of infarctions within MI patients (Weir *et al.* 2010). Additionally, sST2 has been proposed to function as a reliable biomarker of HF in patients with breathing difficulties indicating that circulating sST2 could function as a robust marker for different manifestations of cardiovascular disease (Januzzi *et al.* 2007).

1.8. Aims of the study

Atherosclerosis is the primary contributor to CHD; the leading cause of global death. Due to the inflammatory nature of atherosclerosis much attention has fallen on the critical role that cytokines play in orchestrating and augmenting the disease state. The imbalance between pro-inflammatory and anti-inflammatory cytokines within the plaque supports pathogenesis. Furthermore cytokines are involved in all key events; from the recruitment of leukocytes, foam cell generation, secretion of MMPs to eventual rupture of the plaque. Many of these atherogenic processes are mediated through the modulation of signalling pathways and the dysregulation of gene expression. As such it is of crucial importance to understand the underlying mechanisms that dictate the activities of cytokines implicated within the disease.

IL-33 is a novel member of the IL-1 superfamily of cytokines and a number of studies have described the anti-atherosclerotic effects of the cytokine. Administration of IL-33 into murine models of atherosclerosis reduces the size of atherosclerotic plaques and promotes the secretion of Th2 cytokines. Furthermore the cytokine reduces macrophage foam cell accumulation in $ApoE^{-/-}$ mice and studies in THP-1 macrophage-derived foam cells have demonstrated that IL-33 down-regulates the expression of genes involved in cholesterol uptake and esterification whilst up-regulating those involved in cholesterol efflux. In addition to the anti-atherosclerotic effects of the cytokine, IL-33 also plays a role in other related cardiovascular disorders.

Atherosclerosis is characterised by two important factors: lipid accumulation and inflammation therefore the main aims of these studies were:

- To explore the effects of IL-33 on the gene expression of well-characterised atherosclerotic markers through the use of real time-quantitative polymerase chain reaction (RT-qPCR) within murine and human macrophages (chapter 3)
- The identification of key signalling pathways responsible for the IL-33-mediated down-regulation of pro-atherogenic genes within human macrophages. For this purpose a combination of small interfering RNA (siRNA) and short hairpin RNA (shRNA) was used to knock down the expression of signalling components implicated in atherosclerosis. The knock down was then validated by RT-qPCR and western blotting before RT-qPCR analysis was used to assess the effect on gene expression following stimulation by IL-33 (chapter 4).
- To determine the influence of IL-33 on the lipid composition of murine and human macrophages (chapter 5) using thin layer chromatography (TLC) and gas chromatography (GC).

Even with a growing awareness of the key contributors of cardiovascular disease, current risk prevention methods are lacking; as the majority of CHD events occur in individuals placed within the “low” and “intermediate” risk groups (Nambi *et al.* 2010; Balagopal *et al.* 2011). Improving our knowledge of the disease could aid future discovery of novel markers and therefore improve clinical risk stratification through better prediction and categorisation of the disease. Furthermore, a greater understanding

of the underlying mechanisms which give rise to pathogenesis is essential for the identification and development of new therapeutic targets to help combat the disease state.

CHAPTER 2.

MATERIALS AND METHODS

2.1. Chemicals and reagents

Reagent	Supplier	Location
10X NH_4 reaction buffer, magnesium chloride, Taq polymerase	Bioline	London, UK
Microplate reader, PTC-200 Peltier thermal cycler, Opticon 2 PCR machine	Bio-Rad	Hertfordshire, UK
15ml and 50ml Falcon polypropylene tubes, black 96-well plate	Corning Costar	Amsterdam, Netherlands
THP-1 cells, RAW264.7 cells, T-Rex-293 cells	European Collection of Animal Cell Cultures	Salisbury, UK
Chloroform, ethanol, methanol, potassium chloride, sodium dodecyl sulphate (SDS)	Fisher Scientific	Loughborough, UK
Foetal calf serum (FCS), RPMI 1640 tissue culture medium, trypsin-ethylenediaminetetraacetic acid (EDTA)	Gibco BRL	Paisley, UK
6-well plates, 10ml stripettes, 12-well plates, 25ml stripettes, 50ml Falcon tubes, Cell-star cell culture flasks, cell scrapers	Greiner Bio-One	Stonehouse, UK
1kb DNA molecular weight markers, 2-mercaptoethanol,	Invitrogen	Paisley, UK

Dulbecco's modified Eagle's medium (DMEM) high glucose media, FCS, I-BLOCK, MagicMark™ XP western protein standard, MOPS running buffer, NuPAGE™ Novex gel tank system, NuPAGE™, penicillin and streptomycin, RPMI1640 with GlutaMAX™ liquid, SDS-polyacrylamide gel, See Blue Plus 2, transfer buffer, Tropix® CDP-Star® detection reagent		
Sterile 0.22µm filters, polyvinylidene difluoride (PVDF) membrane	Millipore	Watford, UK
Spin column RNA miniprep kit	NBS Biologicals	Cambridgeshire, UK
Anti-phospho p44/p42 Thr202/Tyr204 (9101), anti-total p38 (9212), anti-total p44/p42 (9102)	Cell Signaling Technology	Hertfordshire, UK
Lymphoprep™	Nycomed Pharmaceuticals	Zurich, Switzerland
Recombinant human IL-33, recombinant murine IL-33	Peprotech	London, UK
Deoxyribonucleotide triphosphates (dNTPs), MMLV 10X buffer, MMLV reverse transcriptase (RNase H minus, point mutant), RNasin ribonuclease inhibitor	Promega	Southampton, UK
Interferin™, RNeasy plus minikit, validated siRNAs	Qiagen	Manchester, UK
Chicken anti-goat (sc-2961) horse	Santa Cruz	Texas, USA

radish peroxidase (HRP) conjugate, donkey anti-goat HRP (sc02056), goat anti-rabbit alkaline phosphatase (AP) conjugate, goat anti-mouse AP	Biotechnology	
10X tris borate EDTA (TBE) buffer, Accuspin tubes™, anti-β-actin antibody (A2228), DAPI mounting media, dimethyl sulfoxide, ethidium bromide, RIPA buffer, phosphate buffered saline (PBS) tablets, PCR primers, penicillin, phorbol 12-myristate 13-acetate (PMA), protease inhibitor cocktail, streptomycin, SYBR® Green JumpStart™ *Taq* ReadyMix, Tween 20, X-ray Film	Sigma-Aldrich	Poole, UK
Interferin™	Source Bioscience Life Sciences	Nottingham, UK
96-well plates, PCR tubes, PCR plates, PCR seals	Star Labs	Lahore, Pakistan
Bicinchoninic acid (BCA) protein assay reagents, cryovials, Restore™ PLUS western blot stripping buffer	Thermo Scientific	New York, USA
Buffy Coats	Welsh Blood Service	Pontyclun, UK

2.2. Preparation of solutions and glassware

Solutions and glassware were autoclaved for 20-30 minutes at 121°C (975kPa) as necessary.

2.3. Cell culture techniques

2.3.1. Cell lines

2.3.1.1. THP-1 cells

THP-1 is a human monocytic leukaemia cell line. The cells grow within suspension and following differentiation with phorbol esters become adherent and display many features of human monocyte-derived macrophages (HMDMs). Due to this characteristic, the cell line is a useful model for the study of human macrophages (Auwerx 1991; Kohro *et al.* 2004).

2.3.1.2. RAW264.7 cells

RAW264.7 is an adherent murine macrophage cell line derived from tumours induced with the Abelson leukaemia virus (Raschke *et al.* 1978). The adherent cells share many properties of macrophages such as expression of the macrophage markers CD36 and CD11b, phagocytosis and production of nitric oxide (Shen *et al.* 2008b).

2.3.1.3. T-REx293 cells

T-REx293 is an adherent human cell line derived from embryonic kidney cells exposed to adenovirus type 5 DNA. T-REx293 cells stably express the tetracycline repressor protein and are capable of superinfection with other adenoviruses, therefore serving as a good system for growing and proliferating adenoviruses for experiments (Graham *et al.* 1977).

2.3.2. Maintenance of cell lines

The THP-1 and RAW264.7 cell lines were cultured in RPMI1640 medium with stable glutamine supplemented with 10% (v/v) heat-inactivated (56ºC, 30 minutes) FCS (HI-FCS), penicillin (100U/ml) and streptomycin (100µg/ml) (complete medium). The HI-FCS, penicillin and streptomycin were sterilised by passing through a 0.2µm sterile filter. The T-REx293 kidney cell line was grown in DMEM with 4.5g/l glucose supplemented with 10% (v/v) HI-FCS, penicillin (100U/ml) and streptomycin

(100µg/ml) (complete medium). The cells were maintained at 37ºC in a humidified atmosphere containing 5% (v/v) CO_2 and used within their 2nd to 7th Passage.

2.3.3. Subculturing of cells

2.3.3.1. THP-1 cells

The THP-1 cells were transferred into a 50ml polypropylene tube upon reaching approximately 60% confluency and centrifuged at 800g for 5 minutes. The cellular pellet was resuspended in an appropriate volume of pre-warmed complete RPMI1640 medium and seeded into new tissue culture flasks typically at a ratio of 1:30. The cells were grown up at 37ºC in a humidified incubator containing 5% (v/v) CO_2.

2.3.3.2. RAW264.7 cells

The adherent RAW264.7 cells were subcultured until they were 80% confluent. The cells were liberated from the flasks using sterile disposable cell scrapers and pelleted by centrifugation at 800g for 5 minutes. The resulting pellet was resuspended into pre-warmed complete RPMI1640 medium and transferred into new dishes at a ratio of 1:25. The cells were grown up at 37ºC in a humidified incubator containing 5% (v/v) CO_2.

2.3.3.3. T-REx293 cells

Approximately 70% confluent T-REx293 cells were trypsinised by adding 0.25% (v/v) trypsin/EDTA to cover the cell monolayer. The cells were then incubated at 37ºC, 5% (v/v) CO_2 until they became visibly detached from the flask. Complete DMEM medium was added to the flask in order to collect the detached cells before centrifuging at 800g for 5 minutes. The resulting pellet was then resuspended in pre-warmed complete DMEM medium to remove residual trypsin solution before seeding into new tissue culture flasks at a ratio of 1:12. The cells were grown up at 37ºC in a humidified incubator containing 5% (v/v) CO_2.

2.3.4. Preserving and storing of cell lines

Early passage cells (between passages 1-3) were stored at -80°C or in liquid nitrogen for longer storage. Prior to freezing, cells were centrifuged at 800g for 5 minutes and resuspended in HI-FCS containing 10% (v/v) DMSO. Cellular suspensions were divided into 1ml aliquots and stored in sterile cryovials. The cryovials were frozen overnight in a Cryo 1°C freezing container at -80°C before being transferred into liquid nitrogen (Nalgene).

2.3.5. Thawing frozen cells

The cryovials were taken from liquid nitrogen and incubated at 37°C in a water bath until the contents were thawed. The cells were transferred to a 50ml polypropylene tube containing 10ml HI-FCS and centrifuged at 800g for 5 minutes. The pellet was then resuspended with the appropriate pre-warmed medium containing 20% (v/v) HI-FCS, penicillin (100U/ml) and streptomycin (100μg/ml) before the cells were seeded into tissue culture flasks and cultured as described above.

2.3.6. Counting cells

The cells were counted using a haemocytometer (Neubaurer chamber). In preparation for counting, the cells were centrifuged at 800g for 5 minutes and resuspended in 3-5ml of the appropriate complete medium depending on an estimate of the numbers of cells. The haemocytometer was then covered with a precision ground coverslip and 7μl of the cell suspension was added, forming a monolayer of cells across the 5 x 5 counting grid. The total number of cells per ml was calculated by multiplying the number counted by 10^4.

2.3.7. Cytokine stimulation of cells

For RT-qPCR analysis, 500,000 cells were plated into 12-well plates and supplemented with 1ml of complete medium. For THP-1 monocytes, 0.16μM of PMA was added to the cells to promote macrophage differentiation. The cells were then incubated at 37°C in 5% (v/v) CO_2 for 24 hours prior to cytokine treatment to facilitate adherence of cells to the plate. For western blot analysis, $1x10^6$ cells were plated into 6-well plates with

2ml of complete medium whereas $3x10^6$– $6x10^6$ cells in 2ml of complete medium were used for lipid analysis experiments. IL-33 (10ng/ml or 25ng/ml) was directly added to the culture medium for 12 or 24 hours and the treated cells were kept at 37ºC in a humidified incubator containing 5% (v/v) CO_2.

2.3.8. Human monocyte-derived macrophage (HMDM) cell culture

HMDMs were isolated from monocytes obtained from buffy coats (supplied from the National Blood Service Wales). Lymphoprep solution was warmed to room temperature prior to use and added to the Accuspin centrifuge tubes. The tubes were centrifuged at 1000g for 1 minute in order to place the Lymphoprep below the filter. The buffy coat was under-layered 2:1 (v/v) with Lymphoprep in the Accuspin tube and centrifuged at 1000g for 30 minutes at room temperature. Lymphoprep contains the high molecular weight polysaccharide dextran and sodium diatrizoate that establishes a density gradient promoting the isolation of mononuclear cells due to a lower buoyant density. Higher density cells such as granulocytes and erythrocytes aggregated and were removed through centrifugation. The resulting mononuclear cell interface was collected and washed with an equal volume of ice-cold PBS-0.4% (w/v) tri-sodium citrate and centrifuged at 1000g for 5 minutes at 4°C. The pellet was resuspended in 10ml of 0.2% (w/v) sodium chloride solution and incubated on ice for 30 seconds. An equal volume of 1.6% (w/v) sodium chloride solution was added before immediate centrifugation at 1000g for 5 minutes at 4°C. Repeating this step increased the removal of erythrocytes. The pellet was then washed five to six times with ice-cold PBS-0.4% (v/v) tri-sodium citrate to remove platelets. The monocytes were isolated and plated into 12-well plates in RPMI1640 medium containing 5% (v/v) HI-FCS, penicillin (100U/ml) and streptomycin (100µg/ml). The culture medium was replaced after 7 days or more depending on confluency of the cells. After 7-10 days the cells differentiated into macrophages and were ready for use in experiments.

2.4. RNA/ DNA related techniques

2.4.1. RNA isolation from cells

Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen) or the Spin Column RNA Miniprep kit (NBS Biologicals) according to the manufacturer's instructions (information on the buffers used is included in Table 6). The kit purifies RNA molecules exceeding 200 nucleotides including mRNA. Briefly, the medium was aspirated and the cells washed once with PBS to remove traces of the growing medium before addition of 300µl of buffer RLT plus (provided in the kit) supplemented with 10% (v/v) β-mercaptoethanol to liberate the cells from the plate. Following lysis, the cells were either stored at -80ºC for extended storage or used immediately for RNA extraction. The lysate was homogenised by passing through a QIAshredder column and then transferred to a genomic DNA Eliminator spin column to remove genomic DNA from the sample. The resulting lysate was passed through an RNeasy spin column to concentrate the RNA on the silica-based membrane and washed with Buffer RW1 and RPE (both supplied in kits) to remove possible contaminants such as DNA.

Table 6. Buffers used in RNA extraction process

Buffer	Function
Buffer RLT plus	Disrupts plasma membranes of cells and organelles releasing RNA and contains guanidine thiocyanate which inactivates RNases to ensure isolation of intact RNA.
Buffer RW1	Provides appropriate binding conditions for RNA.
Buffer RPE	Promotes RNA binding to the spin column.

The resulting RNA was eluted in nuclease-free water and the concentration and purity was determined using a NanoDrop spectrophotometer ND-1000 (ThermoFisher) by measuring the absorbance of light at 260nm and 280nm. The quality of the RNA was

also analysed by size-fractionating an aliquot on a 1.5% (w/v) agarose gel (section 2.4.3).

2.4.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) involves two stages:

1. cDNA synthesis from an RNA template through a reverse transcriptase-mediated reaction;
2. PCR reaction of the resulting cDNA using gene specific primers to amplify a product of interest.

2.4.2.1. Reverse transcriptase generation of cDNA

RNA (1/0.5µg depending on the concentration of the isolated RNA) was incubated at 72ºC for 5 minutes on a Biometra thermoblock with random hexamer primers (200pmol, PdN_6) and nuclease-free water to a total volume of 13.5µl. Samples were immediately placed on ice. Then, 1µl of deoxyribonucleotide triphosphate (dNTP) mix (10mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate), 4µl of 5X Molony Murine Leukaemia Virus (MMLV) buffer [(50mM Tris-HCl (pH 8.3 at 25°C), 75mM KCl, 3mM $MgCl_2$ and 10mM DTT)], 0.5µl of Recombinant RNasin Plus inhibitor (50U/µl), 0.5µl of MMLV reverse transcriptase (200U/µl) were added to a final volume of 20µl and the mixture was incubated for 1 hour at 37ºC. The reaction was terminated by a temperature increase to 92ºC for 2 minutes. The mixture was then diluted with 80µl (or 30µl if 0.5µg RNA was used as starting material) nuclease-free water and stored at -20ºC for future use.

2.4.2.2. PCR

PCR reactions were setup containing 10x buffer [(670mM Tris-HCl (pH 8.8 at 25°C), 160mM $(NH_4)_2SO_4$, 0.1% stabilizer)] (Bioline), dNTP mix (100µM), Taq polymerase (1U) and forward and reverse primers (1µM), and concentration of cDNA depending on initial optimisation experiments. Nuclease-free water was included to give a final volume of 25µl. The optimised conditions ensured that the products were formed during the exponential phase of amplification and thus provided a direct correlation between

the forming product and the starting cDNA template. The PCR reactions were performed on a PTC-200 Peltier thermal cycler.

2.4.3. Resolving RNA/ DNA by gel electrophoresis

The PCR products were size-fractionated by agarose gel electrophoresis and analysed using a Syngene gel documentation system. The gels were made using 1.5% (w/v) agarose containing 0.5μg/ml ethidium bromide using the stock solutions included in Table 7.

Table 7. Composition of reagents for agarose gel electrophoresis

Reagent	Composition
10x Tris Borate EDTA	0.98M Tris borate, 890mM boric acid, 20mM EDTA, pH 8.
5x loading dye	1x TBE, 50% (v/v) glycerol, 2.25% (w/v) bromophenol blue.

RNA/DNA (1μg) was prepared with loading dye to a final volume of 20μl. The samples were size-fractionated at 100V for 30 minutes in a Fisherbrand horizontal gel unit in 1x TBE buffer. The resulting bands were visualised under a UV light using a Syngene gel documentation system and the images were analysed by the GeneScan computer package (Biosoft, Cambridge, UK). Product size was compared to standard molecular weight markers.

2.4.4. Real-time quantitative PCR (RT-qPCR)

The mRNA expression of a number of genes was analysed using the SYBR® Green JumpStart™ *Taq* ReadyMix kit (Sigma). RT-qPCR reactions were set up with the reagents provided in Table 8 within a 96 well plate using gene specific primers. Information on primer annealing temperatures and sequences are provided in Table 9. The mRNA levels were measured by fold changes in expression in an Opticon 2® real-time PCR detection system (MJ Research). The comparative $\Delta\Delta C_t$ method was used to represent relative expression over absolute expression and then normalised to levels of the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for

human cells. β-actin was used as the reference gene for mouse cells. All PCRs were performed at least in duplicate.

Table 8. Reagents for RT-qPCR

Reagent	Volume (µl)
cDNA	1.1
Forward primer (100µM)	0.5
Reverse primer (100µM)	0.5
SYBR Green JumpStart™ Taq ReadyMix	13.75
RNase-free water	11.55

During the PCR reaction copies of the DNA template are generated exponentially. Traditional end-point quantification assesses the PCR signal relative to a standard curve; however this "end-point" may fall outside of the exponential phase and thus produce unreliable results. However, the "real-time" method quantifies the amount of PCR product produced during the exponential phase of the reaction so the amount of product formed is directly proportional to the amount of DNA template used and allows for the comparison of multiple samples (Livak and Schmittgen 2001). A detectable output such as fluorescence is produced which relates to the level of PCR product produced after each cycle of the reaction. In the comparative Ct method of analysis, the fluorescence of a gene is required to surpass a predetermined threshold (Ct) value within the exponential phase and can be compared against the Ct value of a control gene to normalise the data (Ginzinger 2002). GAPDH and β-actin were chosen as suitable control genes as they have both been frequently used for this purpose by other studies (Mori *et al.* 2008; McLaren *et al.* 2010a; McLaren *et al.* 2010b) and are stably expressed independent of the experimental conditions.

SYBR Green is an intercalating dye similar to ethidium bromide which fluoresces when bound to double-stranded DNA. During PCR, multiple molecules of the dye bind to the product producing a detectable fluorescent signal. The dye is non-specific and can therefore be used in different PCR reactions (VanGuilder *et al.* 2008).

Table 9. Primers used during study

Gene	Forward Primer (5' - 3')	Reverse primer (5' - 3')	Reference	Initial melting 5 minutes	Annealing 60 seconds	Extension 60 seconds	Melting 30 seconds	Final extension 10 minutes
Human CD36	GAGAACTGTTATGGGGCTAT	TTCAACTGGAGAGGCAAAGG	(Draude and Lorenz 2000)	95°C	58°C	72°C	94°C	72°C
Human c-Jun	TCCAAGTGCCGAAAAAGGAAG	CGAGTTCTGAGCTTTCAAGGT	Primer Bank	95°C	60°C	72°C	94°C	72°C
Human CPT-1	ACAGTCGGTGAGGCCTCTTATGAA	TCTTGCTGCCTGAATGTGAGTTGG	(Chinetti *et al.* 2003)	95°C	65°C	72°C	94°C	72°C
Human ERK1	GCAGGACCTGATGGAGACTGAC	CCAGAATGCAGCCCACAGAC	QPCR Database	95°C	63°C	72°C	94°C	72°C
Human ERK2	GCGCTACACCAACCTCTCGT	CACGGTGCAGAACGTTAGCTG	QPCR Database	95°C	63°C	72°C	94°C	72°C
Human GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	(Yamanishi *et al.* 2002)	95°C	60°C	72°C	94°C	72°C
Human ICAM-1	ACGCTGAGCTCCTCTGCTACTC	GGGCAGGATGACTTTTGAGG	(Sabatakos *et al.* 1998)	95°C	63°C	72°C	94°C	72°C

Human JNK1	TCTGGTATGATCCTTCTGAAGCA	TCCTCCAAGTCCATAACTTCCTT	Primer Bank	95°C	60°C	72°C	94°C	72°C
Human JNK2	GAAACTAAGCCGTCCTTTTCAGA	TCCAGCTCCATGTGAATAACCT	Primer Bank	95°C	60°C	72°C	94°C	72°C
Human LPL	GAGATTTCTCTGTATGGCACC	CTGCAAATGAGACACTTTCTC	(Irvine *et al.* 2005)	95°C	60°C	72°C	94°C	72°C
Human MCP-1	CATTGTGGCCAAGGAGATCTG	CTTCGGAGTTTGGGTTTGCTT	(Locati *et al.* 2002)	95°C	62°C	72°C	94°C	72°C
Human p38	GTGGTACAGGGCTCCTGAGA	TATGCATCCCACTGACCAAA	(Ashlin *et al.* 2014)	95°C	63°C	72°C	94°C	72°C
Human PI3K-γ	TCTGATGGATATTCCCGAAAGCC	CTCACCCACTGGAAGTTTTTGAT	Primer Bank	95°C	63°C	72°C	94°C	72°C
Human RPL3a	CCTGGAGGAGAAGAGGAAAGAG	TTGAGGACCTCTGTGTATTTGT	(Vandesompele *et al.* 2002)	95°C	60°C	72°C	94°C	72°C
Human SCD-1	TGCAGGACGATATCTCTAGC	ACGATGAGCTCCTGCTGTTA	(Peter *et al.* 2009)	95°C	58°C	72°C	94°C	72°C
Human SR-A	CCAGGGACATGGAATGCAA	CCAGTGGGACCTCGATCTCC	(Draude and Lorenz 2000)	95°C	60°C	72°C	94°C	72°C

Human SR-B1	TGATGATGGAGAATAAGCCCAT	TGACCGGGTGGATGTCCAGGAAC	(Eguchi *et al.* 2006)	95°C	60°C	72°C	94°C	72°C
Mouse β-actin	TGGAGAAGAGCTATGAGCTGCCTG	GTGCCACCAGACAGCACTGTGTTG	(Harvey *et al.* 2007)	95°C	60°C	72°C	94°C	72°C
Mouse CD36	GAACCACTGCTTTCAAAAACTGG	TGCTGTTCTTTGCCACGTCA	(Hickman *et al.* 2008)	95°C	60°C	72°C	94°C	72°C
Mouse ICAM-1	CAGTCCGCTGTGCTTTGAGA	CGGAAACGAATACACGGTGA	Primer Bank	95°C	60°C	72°C	94°C	72°C
Mouse MCP-1	CTGGATCGGAACCAAATGAG	CGGGTCAACTTCACATTCAA	Primer Bank	95°C	60°C	72°C	94°C	72°C
Mouse SCD-1	CTTCTTGCGATACACTCTGG	TGAATGTTCTTGTCGTAGGG	(Thorn *et al.* 2010)	95°C	65°C	72°C	94°C	72°C
Mouse SR-A	TGAACGAGAGGATGCTGACTG	GGAGGGGCCATTTTTAGTGC	(Hickman *et al.* 2008)	95°C	60°C	72°C	94°C	72°C
Mouse SR-B1	TTTGGAGTGGTAGTAAAAAGGGC	TGACATCAGGGACTCAGAGTAG	(Hickman *et al.* 2008)	95°C	60°C	72°C	94°C	72°C

All primers were purchased from Sigma Aldrich and each reaction was subjected to 35 cycles

2.5. Small interfering RNA (siRNA) transfections with Interferin™

siRNA transfections were performed using validated siRNAs against target mRNA sequences (Table 10). Stock solutions of lyophilised siRNA were prepared in nuclease-free water according to the manufacturer's instructions (Qiagen and Invitrogen).

Table 10. siRNAs used in study

Target mRNA transcript	NCBI accession number(s) of target	SI reference number/ catalogue number
Negative control	N/A	AM4611
GAPDH	NM_002046	NM_002046
ERK2	NM_002745	SI00300755
JNK1	NM_002750	SI02758637
JNK2	NM_001135044	SI02222920
p50	NM_001165412, NM_003998	SI02654932
p65	NM_001145138, NM_001243984, NM_001243985, NM_021975	SI00301672

siRNA transfections were carried out before the addition of PMA. THP-1 cells were harvested upon reaching approximately 60% confluency and centrifuged at 800g for 5 minutes. The resulting pellet was then resuspended in an appropriate volume of pre-warmed RPMI1640 medium supplemented with 10% (v/v) HI-FCS without any antibiotics. Then, 500,000 THP-1 cells were seeded into 12-well plates and incubated at 37ºC in a humidified incubator containing 5% (v/v) CO_2 for 4 hours prior to

transfections. siRNA transfections were performed according to the manufacturer's instructions (Polyplus Transfection) with a few minor modifications. Briefly, 100µl of transfection mix was made in HI-FCS-free and antibiotic-free RPMI1640 medium consisting of 7.5nM siRNA and 9µl of Interferin and then quickly vortexed. The mixture was left to incubate for 20 minutes at room temperature before addition to the cells in a drop-wise manner. The cells were then placed at 37ºC in a humidified incubator containing 5% (v/v) CO_2 for 24 hours. PMA (0.16µM) was added to differentiate the cells for 24 hours before IL-33 stimulation for 12 hours. For analysis by western blotting, the same procedure was used but $1x10^6$ cells were plated and a 200µl transfection mixture was made with twice the amount of siRNA and Interferin. Following IL-33 stimulation, the RNA was harvested following the procedure described in section 2.4.1. or the protein was extracted for western blot analysis following the protocol in section 2.7.

2.6. Adenoviral delivery of short hairpin RNA (shRNA)

2.6.1. Generating recombinant adenoviral vectors

TREx-293 cells ($2x10^6$) were plated into a small culture flask in complete DMEM medium for 2 days to allow attachment to the flask's surface before infection. Adenoviral type 5 recombinant vector DNA (4µg) was prepared in pre-warmed DMEM medium without HI-FCS or antibiotics and left at room temperature for 10 minutes. The medium on the TREx-293 cells was aspirated and replaced with fresh complete DMEM medium before addition of the DNA mixture. Thereafter the flasks were incubated at 37ºC in a humidified incubator containing 5% (v/v) CO_2 for 24 hours before the medium was changed with fresh complete DMEM medium. Viral plaques formed after 7-10 days which constituted the initial viral stock was added to a larger flask containing $6x10^6$ TREx-293 cells to generate a useable amount of virus. These flasks were cultured for 3-10 days until viral plaques were produced. The virus was then extracted in PBS. Briefly, the cells were centrifuged at 1000g for 5 minutes and the pellet was resuspended in PBS to wash the pellet. The cells were centrifuged once more and then resuspended in a 1:1 (v/v) ratio of PBS to tetrachloroethylene to lyse the cells. The mixture was then shaken vigorously and centrifuged at 1000g for 20 minutes. The top layer of PBS containing the virus was carefully removed and stored at -80ºC. DNA

sequencing of the recombinant vector DNA used in the experiments was previously performed by the Molecular biology support unit, School of Biosciences at Cardiff University to ensure specific targeting by the virus.

2.6.2. Titering of the adenoviruses

To determine the titre of the adenovirus generated, $5x10^5$ TREx-293 cells were seeded in 1ml of compete DMEM medium in a 12-well plate and incubated overnight at 37ºC in a humidified incubator containing 5% (v/v) CO_2. Serial dilutions were then prepared of the viral stock, of which 100µl of the 10^{-4} and 10^{-5} dilutions were added to the cells before incubation for 48 hours at 37ºC in a humidified incubator containing 5% (v/v) CO_2. Afterwards, the medium was aspirated and the wells left to air-dry at room temperature. Then 1ml of ice cold 50%:50% (v/v) acetone/methanol was added to the wells for 10 minutes at -20ºC to fix the cells to the surface of the plate. The acetone/methanol layer was aspirated and the cells were washed three times with PBS containing 1% (w/v) bovine serum albumin (BSA). The cells were then incubated at 37ºC on a rocker with a goat anti-adenovirus primary antibody (Chemicon) made up in a 1:5000 dilution in PBS containing 1% (w/v) BSA. After 1 hour, the primary antibody was aspirated and the cells were washed three times with PBS containing 1% (w/v) BSA. The cells were then incubated with two secondary antibodies; chicken anti-goat and donkey anti-goat (Abcam) made up at a 1:1000 dilution in PBS with 1% (w/v) BSA for 1 hour before washing with PBS containing 1% (w/v) BSA three times. 3,3'-diaminobenzidine tetrahydrochloride (DAB) was added to the cells for 10 minutes to stain cells containing the antibody. The DAB was removed and the cells washed for a final time with PBS. The cells were then counted on a light microscope (Leica) as the number of infected cells per field of view at a 10X magnification. The number of plaque forming units/ml was calculated by:

$$\frac{\text{(Infected cells by field X 150)}}{\text{[(Virus volume (ml) X dilution factor)]}}$$

2.6.3. Adenoviral transfection of THP-1 cells

Following the procedure described in section 2.3.6, $5x10^5$ THP-1 cells were plated in a 12-well plate. The adenovirus was then added at a multiplicity of infection previously optimised in our laboratory of 100. The cells were left for 2.5 hours at 37ºC in a humidified incubator containing 5% (v/v) CO_2 before the addition of 0.16µM PMA (for THP-1 cells). The cells were then incubated at 37ºC in a humidified incubator containing 5% (v/v) CO_2 for 48 hours before IL-33 stimulation.

2.6.4. Efficiency of adenoviral-mediated knock down

The knock down efficiencies of adenoviral-delivered shRNAs were assessed by qPCR (section 2.4) using gene-specific primers and western blot analysis (section 2.7).

2.7. Protein analysis

2.7.1. Cellular lysis

For analysis by western blotting, $1x10^6$ THP-1 cells were plated in a 6-well plate. Following cytokine stimulation for the prerequisite amount of time, the medium was aspirated and the cells were washed with ice cold PBS. The PBS was removed and the samples were lysed in an appropriate volume of RIPA buffer [(150 mM NaCl, 1.0% (v/v) IGEPAL® CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and 50mM Tris (pH 8.0)] (Sigma) (typically 80µl). The cells were then scraped from the well and micro-centrifuged at 1000g for 5 minutes. The supernatant was transferred to a 1.5ml micro-centrifuge tube and stored at -80ºC, or used immediately for protein quantification or sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting.

2.7.2. BCA protein assay

The concentration of total protein within the samples was determined using the Micro BCA protein assay reagent kit (Pierce). A standard curve was prepared using a range of concentrations of BSA: 0, 5, 10, 15, 20 and 25µg/ml. The BCA cocktail was prepared following manufacturer's instructions and 150µl of this mix was added to 150µl of

standards and samples (diluted 1 in 50 in RIPA buffer) in a 96-well plate. The plate was covered, incubated for 2 hours at 37°C and the absorbance of each sample read at 570nm on a 680-microplate reader (Bio-Rad Laboratories). The protein concentration of the samples was calculated from the resulting standard curve.

2.7.3. Sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein samples were mixed with an equal volume of gel sample buffer (Table 11), boiled at 100°C for 5 minutes and then cooled on ice. The samples were loaded onto a pre-cast NuPAGE 4-12% Bis-Tris gel (1.0mm thick, 10-well) designed to separate small- to medium-sized proteins under denaturing conditions. NuPAGE gels provide a near neutral pH to minimise protein modifications. Proteins become denatured when boiled in the presence of SDS and form a rod-like complex with the SDS molecules. The SDS also masks the protein's charge and therefore enables fractionation based solely upon size (Dennison 1999). SDS-PAGE was performed on the Novex NuPAGE gel electrophoresis system (Invitrogen) filled with NuPAGE MOPS SDS running buffer (Invitrogen). The See Blue Plus 2 marker (Invitrogen) was included to form a 4-250kDa ladder and the gel was subjected to electrophoresis at 150-200V, 400mA for 1-2 hours.

Table 11. Stock solutions of reagents used in SDS-PAGE and western blotting

Reagent	Composition
Gel sample buffer	63mM Tris HCl pH 6.8, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 0.0025% (w/v) bromophenol blue.
NuPAGE® MOPS SDS running buffer (20X)	50mM 3-(N-morpholino) propane sulphonic acid (MOPS), 50mM Tris Base, 0.1% (w/v) SDS, 1mM EDTA, pH 7.7.
NuPAGE® transfer buffer	50mM bis-tris propane, 50mM Bicine, 20% (v/v) methanol.
PBS-Tween	10L ddH_20, 100 PBS tablets, 20ml Tween20.
I-BT solution	500ml PBS-Tween, 1g Tropix I-BLOCK, 500µl of 5% (v/v) sodium azide.
Ponceau S	0.1% (w/v) Ponceau S, 5% (v/v) acetic acid, ddH_20.

2.7.4. Western blotting

Following electrophoresis, the gel was removed and arranged into an Xcell II™ Blot Module (Invitrogen) prepared in NuPAGE transfer buffer. The proteins were transferred onto a methanol-activated Immobulin-P polyvinylidene difluoride (PVDF) membrane (Millipore) by electrophoresis, at 150V for 2 hours. The efficiency of the transfer was assessed by Ponceau S staining of the PVDF membrane.

2.7.5. Immunodetection of proteins

The blotted PVDF membranes were washed in PBS-Tween for 5 minutes before blocking in I-BT for 1 hour with continuous shaking. The membrane was incubated

with the primary antibody diluted in I-BT (details of the antibodies used are given in Table 12) and left overnight at 4°C. Afterwards the membrane was washed three times with PBS-Tween for 5 minutes before incubation with the secondary antibody. After 1 hour, the membrane was washed three times in PBS-Tween.

Table 12. Antibodies used for immunodetection in western blot analysis

Protein	Primary Antibody	Dilution	Secondary Antibody	Dilution	Protein Size
β-actin	Polyclonal Mouse	1:10,000	Goat Anti-Mouse Alkaline phosphatase (AP)	1:5000	42kDa
Phospho ERK1/2	Rabbit	1:1000	Goat Anti-Rabbit (AP)	1:5000	44kDa ERK1 42kDa ERK2
Total ERK1/2	Rabbit	1:1000	Goat Anti-Rabbit (AP)	1:5000	44kDa ERK1 42kDa ERK2
Total JNK1/2	Rabbit	1:1000	Goat Anti-Rabbit (AP)	1:5000	46kDa JNK1 54kDa JNK2
Total p38	Rabbit	1:1000	Goat Anti-Rabbit (AP)	1:5000	43kDa
Total p50	Rabbit	1:1000	Goat Anti-Rabbit (AP)	1:5000	50kDa, 120kDa precursor
Total p65	Rabbit	1:1000	Goat Anti-Rabbit (AP)	1:5000	65kDa

2.7.6. Detection of chemiluminescence

Membrane-bound proteins were then detected by incubation with 0.25mM of Tropix CDP-Star® (Applied Biosystems). The chemiluminescence detection reagent is cleaved by the AP linked to the secondary antibody producing luminescence correlating with the

amount of protein present. The membrane was exposed to Kodak XAR sensitive film (Sigma) in a light resistant X-ray cassette (Genetic Research Instrumentation) for different exposure times and developed using an Agfa developing machine.

2.7.7. Densitometric analysis of western blots

Semi-quantitative measurement of signals on the blots was performed by densitometric analysis using the Gene Tools software (GRI, Braintree, UK). The values for the test protein was normalised to the β-actin housekeeping protein.

2.8. Lipid analysis of cells

2.8.1. Lipid extraction

Lipids from THP-1 and RAW264.7 cells were extracted using a modified version of the method first described by Garbus and colleagues (Garbus *et al.* 1963). The cells were subcultured in a 6-well plate following the procedure described in section 2.3.3 to obtain $3x10^6$– $6x10^6$ cells per well. The cells were then stimulated with vehicle or IL-33 for either 12 or 24 hours. The medium was replaced with PBS and the cells scraped from the plate and transferred into a 1.5ml micro-centrifuge tube. The samples were centrifuged at 1700g for 5 minutes at room temperature and the resulting pellet was resuspended in 1ml of distilled water and 2.5ml of chloroform: methanol (1:2 v/v) to stop any cellular activities and to extract lipids. The lysate was transferred to a glass tube and vortexed. Samples were then either stored at -20ºC for short-term storage or kept at room temperature for 15 minutes. Then, 1ml of chloroform and 1ml of Garbus solution (2M potassium chloride in 0.5M potassium phosphate buffer, pH 7.6) was added and the samples were centrifuged at 1000g for 3 minutes (Baird and Tatlock Auto Bench centrifuge) producing a chloroform-rich lower phase, aqueous layer and cellular debris present at the interface. The saline upper layer contained much of the non-lipid contaminants such as sugars and amino acids. The chloroform portion, containing the total lipid extract, was dried under a stream of nitrogen and reconstituted in chloroform for storage at -20°C. Samples were dried under nitrogen before storage to reduce autoxidation of PUFAs.

2.8.2. One-dimensional thin layer chromatography (TLC)

The samples were prepared on 10x10cm Silica gel G plates to pre-fractionate into individual classes prior to GC. Total lipids were separated using one-dimensional TLC due to differences in partitioning behaviour between the mobile liquid phase and stationary phase (Hajjar and Pomerantz 1992; Perona and Ruiz-Gutierrez 2004). The mobile phase consisted of a mixture of hexane:diethyl ether:acetic acid (80:20:1 v/v) and permitted migration of free fatty acids (FFAs) whilst more complex lipids such as phospholipids remained at the origin. The plates were then dried and sprayed with 0.05% (v/v) 8-anilino-4-naphthosulphonic acid in methanol, a non-destructive method of detection in comparison to sulphuric acid, and visualised by UV using a Syngene Gel Documentation System. PLs, TAGs and sterols were scraped from the plates for further analysis.

2.8.3. Fatty acid analysis by gas chromatography (GC)

TAGs cannot be directly analysed by GC and require conversion to a more apolar and volatile species. The lipid samples were prepared by transmethylation to fatty acid methyl esters (FAMEs) by the addition of 1ml of 2.5% H_2SO_4 (v/v) in dry methanol: toluene (2:1 v/v) at 70°C. Then 2ml of 5% sodium chloride (w/v) was added and the samples were washed twice with 3ml of HPLC grade hexane and centrifuged at 1000g for 3 minutes at room temperature to separate the aqueous and hexane layers. The hexane layer was evaporated under nitrogen and reconstituted with a small volume of hexane.

Heptadecanoic acid (C17:0) was included as an internal standard to produce peaks for reference and to test if the system was functional. The FAMEs were separated and analysed on a 30m x 0.25mm i.d capillary column at 170°C for 3 minutes and 220°C for 30 minutes (Clarus 500 gas chromatograph, Perkin-Elmer). The column partitions the sample between the mobile (gaseous) and stationary (liquid) phase and individual fatty acids display different retention times based upon their relative affinities providing a fatty acid profile of the sample. A G411 standard mixture (Nu-Chek Prep) was used to identify FAMEs based upon retention times.

2.9. Statistical analysis

Data was tested for normality using the Shapiro-Wilk test and represented as mean ± standard deviation and single comparisons were carried out using the Student's *t* test (two-tailed, paired) in Excel (Microsoft Inc, USA). For multiple comparisons one-way ANOVA was mostly employed accompanied with Tukey's post-hoc test, where homogeneity of variance was met; otherwise Welch's test of equality of means with Games-Howell post-hoc analysis was performed using SPSS software (IBM, USA). The results were regarded as significant where *$p<0.05$, **$p<0.01$, ***$p<0.001$.

CHAPTER 3.

THE REGULATION OF EXPRESSION OF KEY GENES IMPLICATED IN ATHEROSCLEROSIS

3. Introduction

3.1. Markers of atherosclerosis

Strategies to tackle the disease are often introduced too late to prevent cardiac events as clinical manifestations of atherosclerosis can arise decades after original disease initiation (Ross 1993). However, a number of molecules are integral to the development of atherosclerosis and may therefore serve as functional biomarkers of the disease, some of which can be measured and potentially used to predict future cardiac events (Koenig and Khuseyinova 2007).

3.1.1. Leukocyte recruitment and attachment

During atherosclerosis circulating leukocytes travel along a chemotactic gradient towards the lesion site. In response to pro-inflammatory stimuli such as LPS, elevated levels of mmLDL (Berliner *et al.* 1990), shear stress (Shyy *et al.* 1994) and inflammatory cytokines; arterial cells including SMCs and endothelial cells release the chemokine MCP-1 (Strieter *et al.* 1989; Rollins *et al.* 1990). MCP-1 becomes tethered to proteoglycans within the arterial lumen and interacts with CCR2 present on the surface of the rolling leukocytes (Niu and Kolattukudy 2009). Plaque-resident cells amplify levels of MCP-1 and studies exploring the effects of MCP-1 overexpression within atherosclerotic models have shown that elevated MCP-1 promotes the accumulation of modified LDL and increases the number of leukocytes within the plaque (Aiello *et al.* 1999). In contrast, MCP-1 knockout mice crossed with $ApoE^{-/-}$ mice contain reduced quantities of lipids within their aortas and display a diminished number of macrophages within the aortic wall (Gu *et al.* 1998). Another chemokine MIP-1β, also known as CCL4, also supports leukocyte recruitment and is secreted by T

cells within the plaque (Wilcox *et al.* 1994). Levels of MIP-1β are significantly elevated within human plaques and in peripheral blood mononuclear cells from patients with familial hypercholesterolaemia (Reape and Groot 1999; Holven *et al.* 2003).

IFN-γ–inducible protein–10 (IP-10) is another chemoattractant glycoprotein that participates in the adhesion of T cells and monocytes to endothelial cells. Mice deficient in IP-10 exhibit a compromised T cell response associated with a decreased number of cells and reduced secretion of IFN-γ (Dufour *et al.* 2002). During atherosclerosis, activated monocytes, endothelial cells and T cells secrete IP-10 in response to low shear stress and IFN-γ. Levels of the glycoprotein are raised in patients with CAD (Taub *et al.* 1996) and are also elevated in complicated atherosclerotic plaques (Cheng *et al.* 2007; Profumo *et al.* 2010).

Activated endothelial cells express surface molecules that facilitate the attachment and infiltration of circulating leukocytes into the subendothelial space (Springer 1994). The process is mediated by an array of adhesion molecules including VCAM-1, P-selectin, E-selectin and ICAM-1 expressed at the surface of the endothelium (Albelda *et al.* 1994). Disturbed laminar flow causes the up-regulation of ICAM-1 at the surface of endothelial cells and *in vitro* studies have demonstrated that increased expression of ICAM-1 directly relates to enhanced adhesion of lymphocytes (Nagel *et al.* 1994). Levels of circulating ICAM-1 are raised in patients with CHD and during carotid artery atherosclerosis (Hwang *et al.* 1997).

3.1.2. Scavenger receptors (SRs)

During normal physiology, cellular levels of cholesterol are carefully regulated through homeostatic mechanisms that influence the expression of LDLRs thereby modulating the uptake of LDL into the cell (Podrez *et al.* 2000). However, chemical modification of LDL facilitates the uptake of these particles through alternative receptors that are not subjected to typical feedback mechanisms. SRs form part of the innate immune system tasked with the recognition of a wide range of ligands associated with pathogenic classes of molecular patterns and support the elimination of foreign agents (Silverstein and Febbraio 2009). Several SRs contain multiple LDL binding sites that facilitate the uptake of modified forms of LDL (Freeman *et al.* 1991; Pearce *et al.* 1998). SR-

mediated uptake of lipoproteins promotes the formation of lipid-engorged foam cells associated with the disease state (Goldstein *et al.* 1979).

The integral membrane receptor SR-A is expressed by an array of cells such as monocytes, macrophages, SMCs and endothelial cells and recognises a broad range of ligands. Studies in SR-A$^{-/-}$ mice have identified that SR-A promotes the differentiation of monocytes into macrophages and is the principle route for AcLDL uptake (Gough *et al.* 1999). The multi-ligand SR CD36 is also expressed by many lesion-resident cells including platelets, macrophages, endothelial cells and SMCs (Demers *et al.* 2004; Collot-Teixeira *et al.* 2007) and is a class B SR that mediates the uptake of advanced glycation end products (Ohgami *et al.* 2001), thrombospondin-1 (Pearce *et al.* 1998), growth hormone-releasing peptides (Demers *et al.* 2004) and various lipid derivatives (Hoebe *et al.* 2005). Interestingly, ablation of both CD36 and SR-A in ApoE$^{-/-}$ mice does not affect the size of atherosclerotic lesions (Manning-Tobin *et al.* 2009). However, the absence of these SRs disturbs the evolution of the plaque by limiting macrophage apoptosis and reducing the expression of pro-inflammatory and apoptotic genes resulting in decreased necrosis within the plaque (Manning-Tobin *et al.* 2009). In contrast, targeted down-regulation of either CD36 or SR-A hinders atherosclerotic development and silencing of one receptor results in the up-regulation of the other due to compensatory mechanisms (Makinen *et al.* 2010).

Although the expression of many SRs are up-regulated during atherosclerosis and exert atherogenic effects, the results from many studies have described conflicting actions for SR-B1. SR-B1 is a highly conserved surface glycoprotein and is widely expressed in different tissues and cell types including macrophages, endothelial cells and SMCs (Van Eck *et al.* 2005). SR-B1 functions as a high affinity receptor of native and modified LDL and displays similar binding preferences as CD36 (Acton *et al.* 1994). However, the receptor also binds HDL particles with high affinity, facilitating the selective uptake of CEs in the absence of HDL internalisation (Liu and Krieger 2002) and also mediates the efflux of unesterified cholesterol to HDL (Ji *et al.* 1997). Interestingly, overexpression of SR-B1 causes a drastic reduction in HDL, LDL and VLDL levels accompanied with enhanced amounts of biliary cholesterol (Kozarsky *et al.* 1997; Wang *et al.* 1998). In contrast, attenuation of SR-B1 elevates levels of HDL (Varban *et al.*

1998) and a complete absence of the receptor raises plasma cholesterol levels and results in the formation of enlarged HDL particles (Rigotti *et al.* 1997).

3.1.3. Metabolic enzymes

LPL resides within the luminal surface of capillary endothelial cells but is released and synthesised from a range of tissues including adipose, skeletal, muscle, heart and mammary gland (Benavram *et al.* 1986). LPL is a rate-determining enzyme responsible for the catabolism of TAGs to FFAs and 2-monoacylglycerol from TAG-enriched circulating lipoproteins including VLDLs and chylomicrons (Tsutsumi 2003). The resulting FFAs and 2-monoacylglycerol are assimilated into tissues whilst the remnant particles are taken up by the liver and residual surface matter is incorporated into HDLs (Peterson *et al.* 1992). Complete knockout of LPL in mice is fatal, the newly born mice exhibit elevated levels of TAGs and VLDLs, and hypertriglyceridemia is the eventual cause of death for these animals (Weinstock *et al.* 1995).

The enzyme is expressed within human and rabbit atherosclerotic plaques by macrophages and SMCs (Ylaherttuala *et al.* 1991a; Obrien *et al.* 1992). Interestingly, LPL expressed by muscle and adipose tissue is regarded as anti-atherogenic as the enzyme aids the clearance of circulating lipoproteins such as VLDL and chylomicrons (Mead *et al.* 2002). However, LPL expressed by macrophages has a pro-atherogenic role. Bone marrow transplantation experiments by Babaev *et al.* (1999) demonstrated that these actions arise due to a bridging effect whereby the enzyme binds lipoproteins and the cell surface through separate domains, thereby promoting the accumulation of lipoproteins and aiding subsequent uptake by receptor- and non-receptor-mediated mechanisms (Babaev *et al.* 1999).

3.2. Experimental aims

Due to the growing interest in the protective actions of IL-33, the studies presented in this chapter attempted to further establish the role of the cytokine within atherosclerosis. The primary aims of this chapter were to validate the use of THP-1 cells as a suitable *in vitro* human model system that could be used to test the regulatory role of IL-33 and secondly to confirm the effects of IL-33 stimulation on atherosclerotic markers (shown

in Table 13) previously employed by the laboratory such as SR-A and SR-B1. These experiments additionally aimed to evaluate the effects of IL-33 on previously untested genes, like ICAM-1 and MCP-1, within the validated system.

Table 13. Experimental atherosclerotic markers

Gene	Role in atherosclerosis	Reference
CD36	Transmembrane SR facilitates modified LDL uptake in macrophages. Mice deficient in ApoE and CD36 display increased atherogenic lipoprotein profile.	(Febbraio *et al.* 2000; McLaren *et al.* 2010a)
ICAM-1	Transmembrane adhesion protein that mediates firm adhesion of inflammatory cells to the endothelium. Increased levels of circulating ICAM-1 present in patients with CHD and atherosclerosis, and functions as a robust marker of inflammation.	(Hwang *et al.* 1997; Blankenberg *et al.* 2003)
IP-10	Chemokine expressed by lesion-resident endothelial cells and macrophages. Biomarker of ischemic stroke risk.	(Lucas and Greaves 2001; Prugger *et al.* 2013)
LPL	Mediates hydrolysis of TAGs in chylomicrons and VLDL resulting in release of FFAs and 2-monoacylglycerol. Facilitates modified lipoprotein uptake by macrophages through bridging effect. Bone marrow transplantation from $LPL^{-/-}$ mice into irradiated C57BL/6 mice fed on a high fat diet reduces lesion size.	(Van Eck *et al.* 2000; Mead *et al.* 2002; Mead and Ramji 2002; Lusis *et al.* 2004)
MCP-1	Primary chemokine responsible for recruitment of circulating monocytes to lesion site, secreted by cellular components of the arterial wall. Elevated in cases of experimental and clinical HF. Robust marker for pro-inflammatory cytokine action.	(Hayashidani *et al.* 2003; Deo *et al.* 2004)
MIP-1β	Chemokine promotes macrophage and T cell recruitment to plaque. Stimulation by MIP-1β increases ROS generation and adhesion of THP-1 cells to inflamed human umbilical vein endothelial cells (HUVECs).	(Lucas and Greaves 2001; Tatara *et al.* 2009)
SR-A	Facilitates unregulated uptake of modified LDL in macrophages. SR-A deficient mice crossed with $ApoE^{-/-}$ mice develop less atherosclerotic lesions than $ApoE^{-/-}$ mice.	(Suzuki *et al.* 1997; McLaren and Ramji 2009)
SR-B1	Promotes RCT by HDLs and increases uptake of modified lipoproteins. Role in atherosclerosis uncertain. Irradiated $ApoE^{-/-}$ mice transplanted with bone marrow from $SR\text{-}B1^{-/-}$ mice display increased atherosclerosis. Transient overexpression of SR-B1 in $LDLR^{-/-}$ mice results in reduced size of atherosclerotic plaques.	(Kozarsky *et al.* 2000; Zhang *et al.* 2003; Zhao *et al.* 2007; Chinetti-Gbaguidi and Staels 2009)

By characterising the responses of previously studied genes, the optimal concentration and time point for cytokine treatment could be identified for subsequent studies. The experimental strategy is outlined in Figure 8.

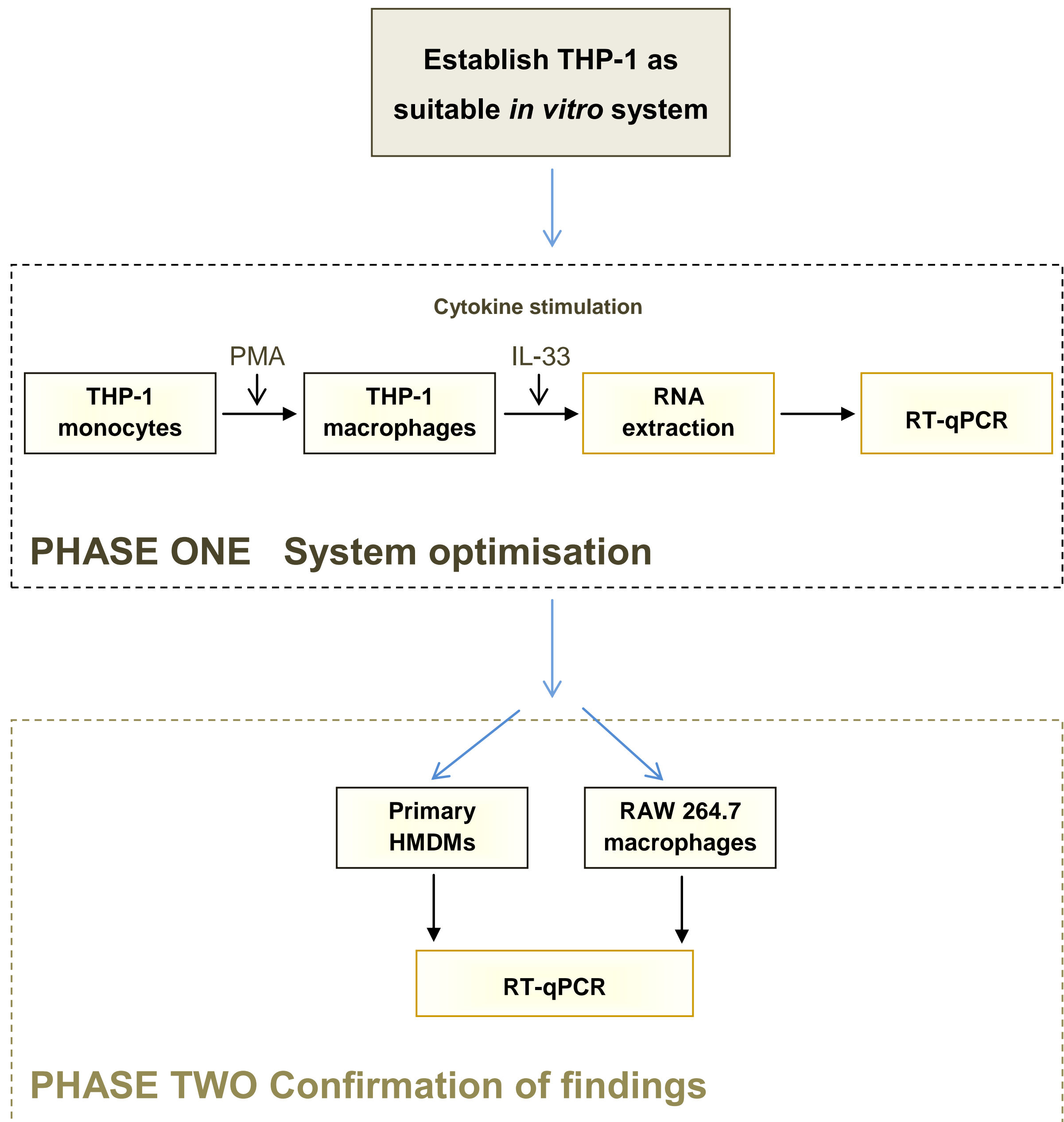


Figure 8. Experimental strategy

The strategy employed to analyse the effects of IL-33 on the expression of key atherosclerotic markers. Early studies were performed in the well-studied THP-1 cell line and then expanded into HMDMs and RAW 264.7 macrophages to gain a further understanding of the respective physiological and species-specific role of IL-33.

3.3. Experimental design

3.3.1. The THP-1 cell line

The THP-1 cell line is a well-established cellular system isolated by Tsuchiya in 1980 from a patient with leukaemia (Tsuchiya *et al.* 1980). THP-1 cells are round suspension cells that mimic many aspects of native monocytes. In response to specific reagents like PMA and 1,25-dihydroxyvitamin D-3 (VD_3) the monocytic cells differentiate into macrophages. The change is associated with alterations in cellular morphology identifiable by irregularity of the nucleus and the presence of phagocytic vacuoles within the cytoplasm (Auwerx 1991; Qin 2012). This transformation is also accompanied by increased adherence to culture plates and induced expression of macrophage markers such as SRs, LPL and ApoE within 3 hours of PMA treatment (Tsuchiya *et al.* 1982).

There are a number of monocytic-macrophage cell lines available such as HL-60, U937, KG-1 and HEL that provide effective *in vitro* systems to study atherosclerotic processes. Cell lines have several advantages over primary cells, such as primary HMDMs, that tend to have reduced cell counts arising from low rates of proliferation. The population of primary cells can also be heterogeneous due to donor-specific variation (Maess *et al.* 2010; Qin 2012). Additionally, the cells are notoriously difficult to transfect with siRNA and have a limited lifespan. For these reasons THP-1 cells are frequently used in atherosclerotic studies and several groups have shown that these cells share a greater conservation of responses with primary cells than other available monocytic cell lines (Auwerx 1991; Qin 2012).

During the study PMA was used to differentiate the cells (see Figure 9) as our laboratory (Harvey *et al.* 2007; McLaren *et al.* 2010b; Salter *et al.* 2011) and a multitude of other studies (Kohro *et al.* 2004; Daigneault *et al.* 2010; Maess *et al.* 2010) have previously used the reagent for this purpose. Treatment with the phorbol ester produces a more differentiated phenotype based upon adherence, phagocytic capabilities and the expression of macrophage surface markers CD11b and CD14 in comparison to alternative reagents like VD_3 (Schwende *et al.* 1996).

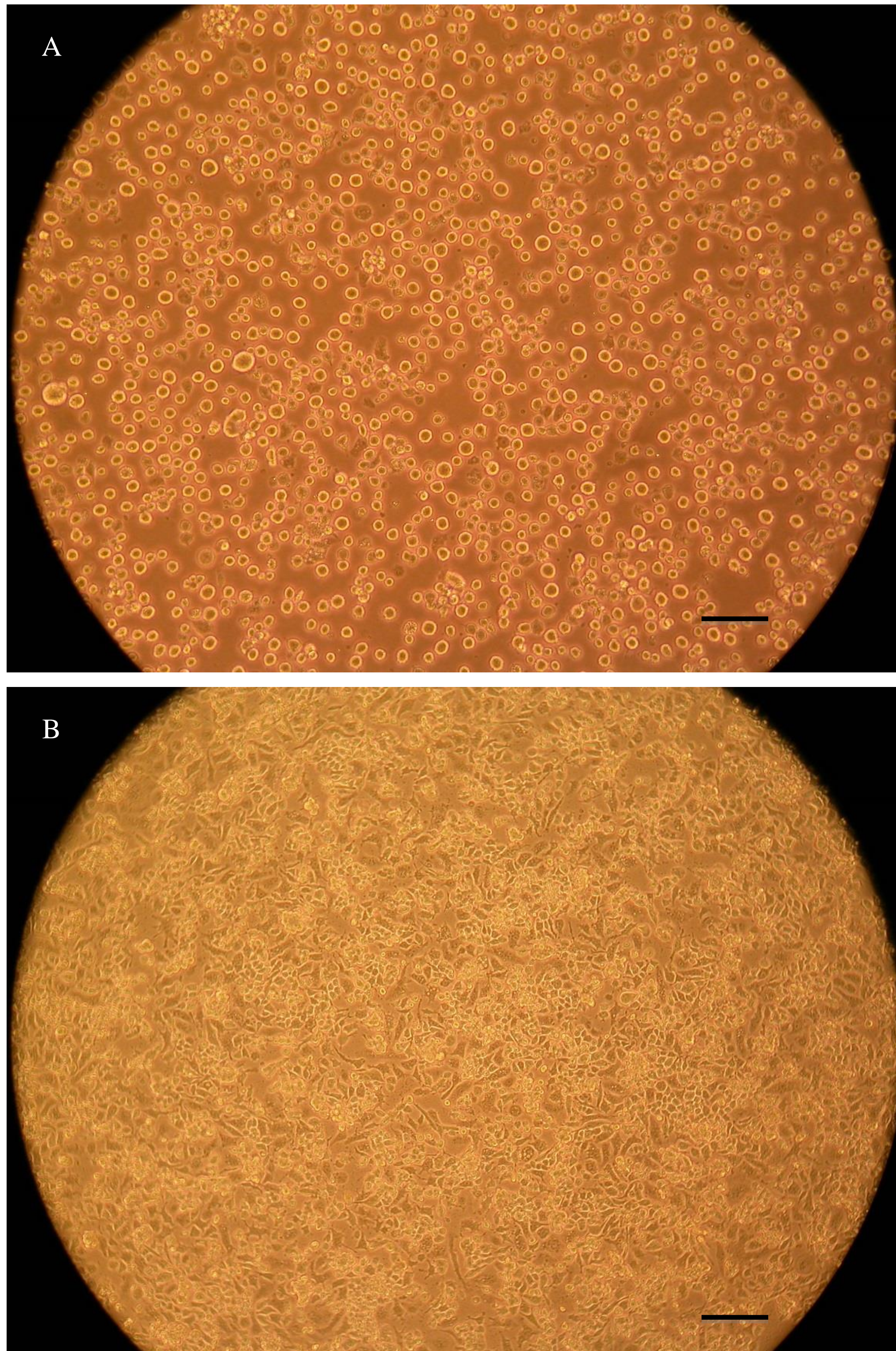


Figure 9. Differentiation of THP-1 monocytes to macrophages

THP-1 monocytes (1 x 10^6 cells) were plated in a 12-well plate (A) and differentiated into macrophages following incubation with PMA (0.16µM) for 24 hours (B). The maturation of the cells was accompanied with alterations in morphology and cellular characteristics. Photos are taken at 10X magnification on a light microscope and the scale bar represents 100µm.

3.4. Results

3.4.1. Optimisation of PCR conditions

Initial experiments were performed in THP-1 macrophages following a 24 hour differentiation period with PMA with the concentration and incubation time point previously used by our laboratory (McLaren *et al.* 2010a; McLaren *et al.* 2010b) to select important IL-33-regulated genes for further mechanistic studies and optimisation of experimental conditions. RT-qPCR was performed using gene-specific primers with conditions designed to obtain a single product of the expected size. Primer sequences were sourced from published literature and primer-Blast searches were performed to confirm that the primer sequence was specific for the intended product (Figure 10).

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CATTGTGGCCAAGGAGATCTG	21	58.98	52.38	6.00	4.00
Reverse primer	CTTCGGAGTTTGGGTTTGCTT	21	59.32	47.62	4.00	0.00

Products on target templates

>NM_002982.3 Homo sapiens chemokine (C-C motif) ligand 2 (CCL2), mRNA

```
product length = 91
Forward primer  1    CATTGTGGCCAAGGAGATCTG  21
Template        277  .....................  297

Reverse primer  1    CTTCGGAGTTTGGGTTTGCTT  21
Template        367  .....................  347
```

Figure 10. MCP-1 primer homology

Primer-Blast search details identifying that the primer sequence is 100% specific for human MCP-1.

The amounts of reagents used for each reaction were well established in our laboratory, but the annealing temperatures between the different primer sets differed. As such a range of annealing temperatures were tested for each chosen primer set and the resulting PCR products were size-fractionated by agarose gel electrophoresis (Figure 11); amplification of the correct product was verified by expected size and sequence analysis. Furthermore, the absence of a band in samples lacking reverse transcriptase indicated that any product generated was due to amplification of the relative mRNA and not due to possible contamination of the samples with genomic DNA.

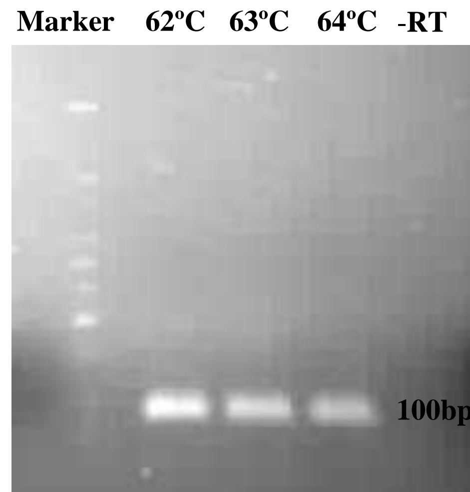


Figure 11. MCP-1 primer optimisation

cDNA was taken from the same experiment and subjected to a range of annealing temperatures (62- 64ºC shown). The products from RT-PCR were assessed on a 1.5% (w/v) agarose gel. As a result an annealing temperature of 62ºC was chosen. The expected product size for MCP-1 =91bp. The position of the 100 bp marker is shown. Abbreviations: -RT, minus reverse transcriptase.

In studies designed to analyse the actions of IL-33, the cells were stimulated by IL-33, harvested and the RNA was isolated in order to perform RT-qPCR. The integrity of the RNA was evaluated by resolving an aliquot of these preparations on a 1.5% (w/v) agarose gel and the quality of the RNA was assessed by the presence of the 28S rRNA band at a relative intensity approximately twice that of the 18S rRNA band for each sample (an example is provided in Figure 12). RNA of this quality was generated throughout the study. Also, only RNA with an OD_{260}/OD_{280} ratio of 1.9-2.1 was used for experiments as an additional indicator of high RNA purity.

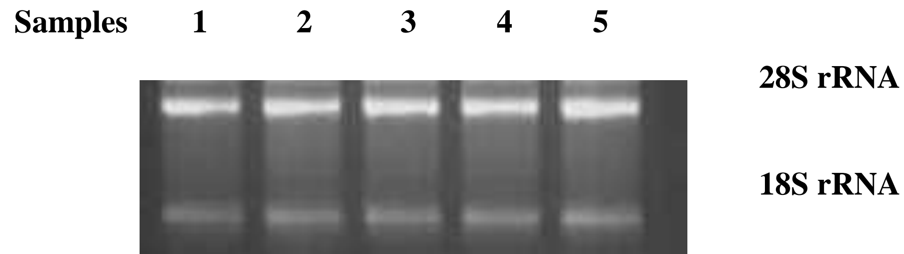


Figure 12. Gel electrophoresis of RNA

Total RNA extracted from PMA-treated THP-1 macrophages. RNA preparations from five separate samples were subjected to gel electrophoresis to check integrity.

RT-qPCR was subsequently performed with the DNA-binding dye SYBR Green I which interacts with double stranded DNA and emits a fluorescent signal upon excitation by a light source (Ginzinger 2002). Data analysis was carried out using the comparative Ct method as described by Livak and Schmittgen (Livak and Schmittgen 2001). Briefly, successive rounds of amplification are performed during which the fluorescent output of a gene surpasses a pre-set threshold value (the Ct value) within the exponential phase that directly correlates to the amount of starting template. The method provides a comparative difference between the investigated gene and a reference gene; for these experiments GAPDH was used, to enable relative quantitative comparisons between the samples (VanGuilder *et al.* 2008).

Melting curve analysis was performed following completion of the PCR reaction whereby the annealed products were melted by small increases in temperature and the reduction in fluorescence was measured as the strands dissociate to ensure the production of a single peak in each experiment. Plotting the temperature against the rate of change in fluorescence provides a distinct peak corresponding to the melting temperature of a product (Pryor and Wittwer 2006). The melting curves for MCP-1 and GAPDH are given as an example in Figure 13.

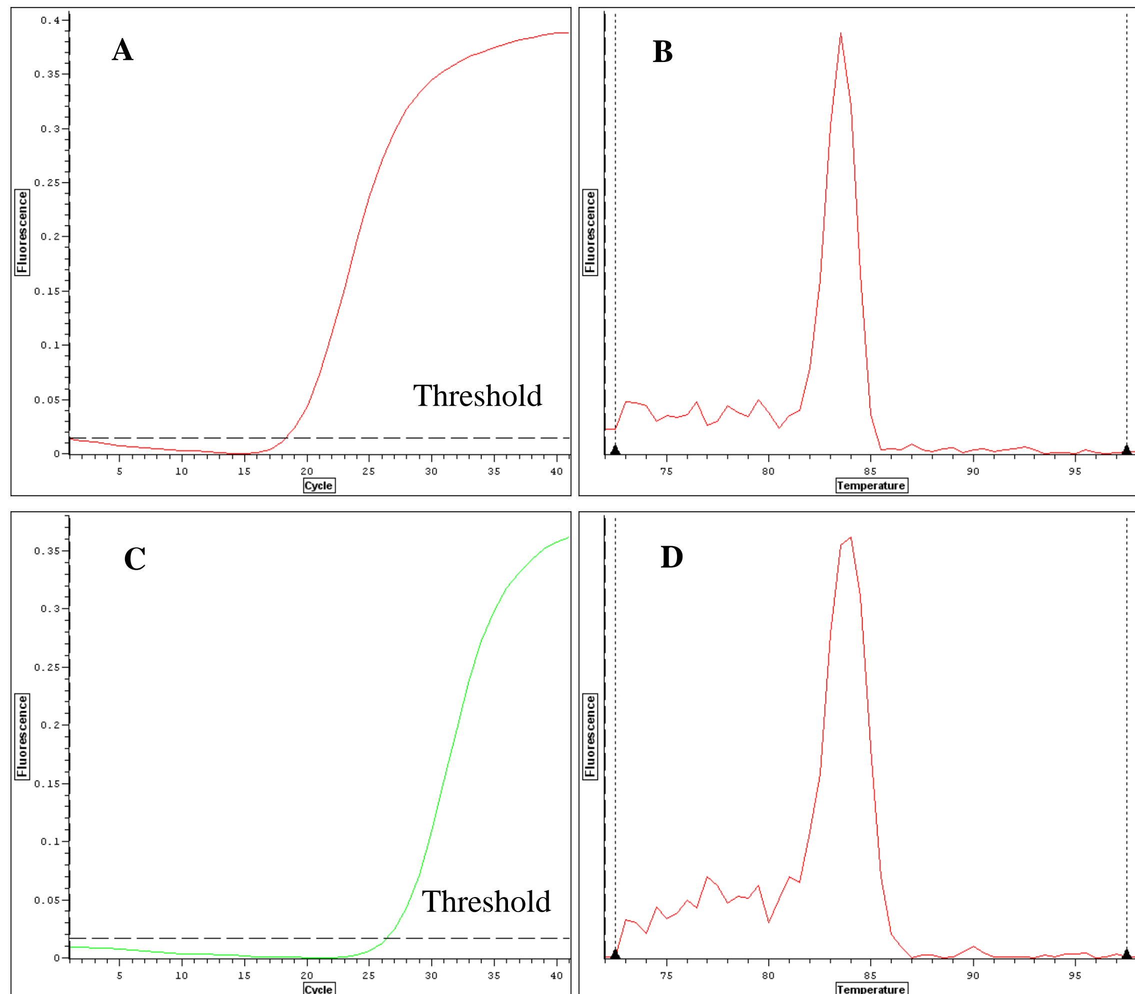


Figure 13. Melting curve analysis

RT-qPCR was performed for GAPDH and MCP-1. The graph is representative of the sigmoidal curves (A and C) obtained over the duration of the PCR cycles. Samples shown are from unstimulated THP-1 macrophages and the annealing temperatures for GAPDH were 60°C (A and B), and 62°C for MCP-1 (C and D). The formation of possible primer dimers would be identified by the presence of major additional peaks prior to the single peak that represents the specific product formed during melting curve analysis (B and D).

The housekeeping gene GAPDH has been routinely used by our laboratory and many others as a reference in RT-qPCR analysis (Irvine *et al.* 2005; McLaren *et al.* 2010a; McLaren *et al.* 2010b; Salter *et al.* 2011; Tan *et al.* 2012). GAPDH and other frequently used housekeeping genes such as RPL37a (Pombo-Suarez *et al.* 2008; Strube *et al.* 2008; Pfister *et al.* 2011) and β-actin (Greer *et al.* 2010; Tan *et al.* 2012) exhibit stable levels of expression independent of the experimental conditions and are therefore used to facilitate accurate quantification of the expression of investigated genes. The 60S ribosomal protein RPL37a was used to compare the reliability of GAPDH (Figure 14) as it is also commonly used as an alternative house-keeping gene and had been recently recommended as a suitable gene of reference for THP-1 cells in a report by Maess (Maess *et al.* 2010).

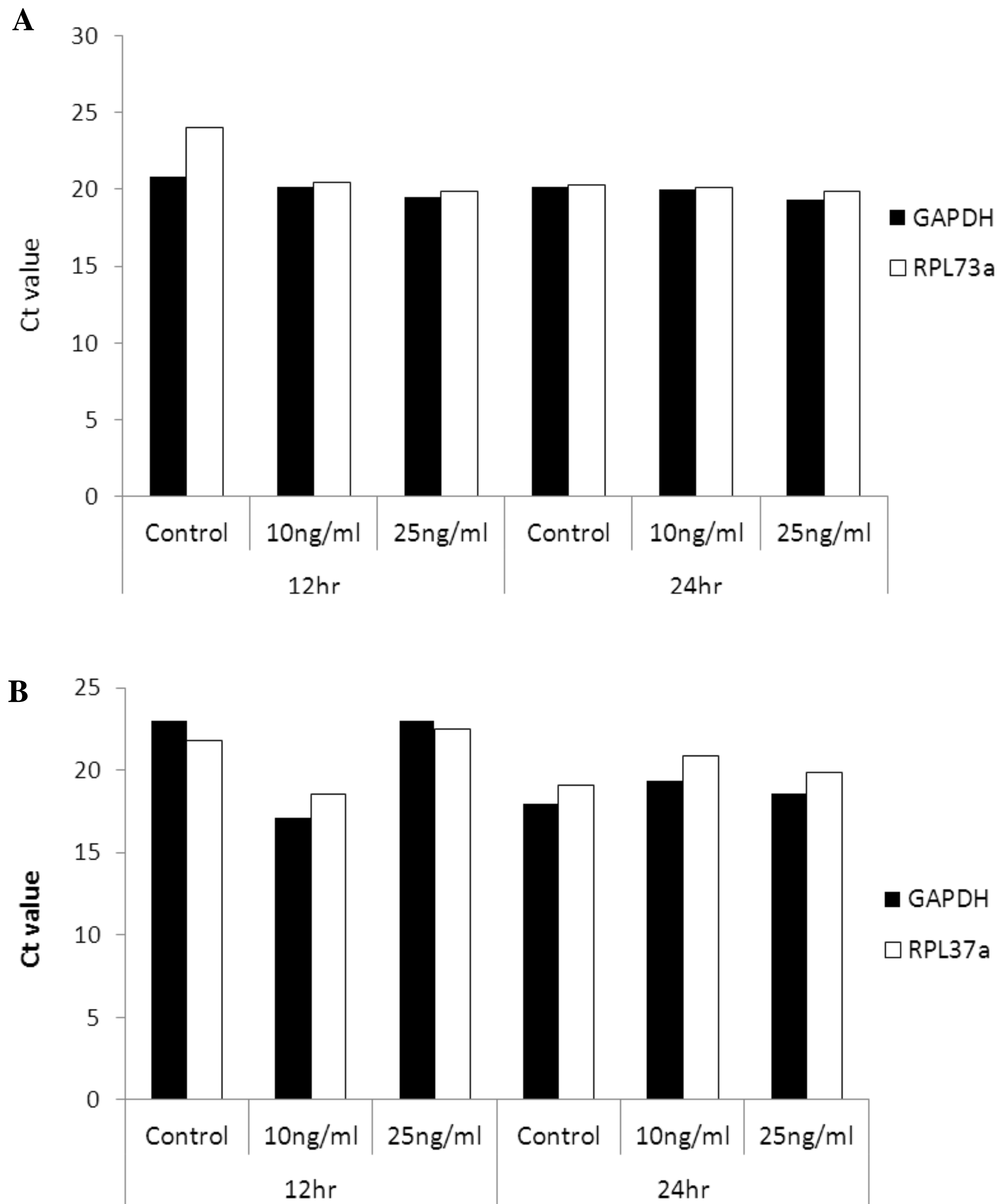


Figure 14. Comparison of Ct values using GAPDH and RPL37a as housekeeping genes

RT-qPCR for GAPDH and RPL37a was performed using cDNA from THP-1 macrophages (A) and HMDMs (B) that were treated for 12 or 24 hours with either vehicle (control) or IL-33 (10ng/ml or 25ng/ml) as indicated. The graph displays the Ct values for GAPDH or RPL37a. Data represents the mean from a single experiment carried out in triplicate.

Experiments in THP-1 and primary HMDMs (Figure 14) indicate that variances in the gene expression of GAPDH and RPL37a were comparable and that both genes

displayed similar patterns of expression. GAPDH was shown to be a suitable gene of reference for qPCR quantification and was therefore used over the course of the study.

3.4.2. The effect of IL-33 on the expression of pro-atherosclerotic genes

To understand the functions of the novel anti-inflammatory cytokine IL-33 on atherosclerotic processes within macrophages, RT-qPCR was performed to evaluate the effect of the cytokine on the mRNA expression of well-characterised genes implicated in the control of foam cell formation and monocyte recruitment. These experiments would function as a positive control to confirm that recombinant IL-33 was physiologically active and also served to validate the system used. THP-1 cells were seeded into 12-well plates, stimulated with PMA (0.16µM) for 24 hours and then incubated with or without IL-33 (10ng/ml) for 24 hours, replicating the concentration and duration previously used by our laboratory in these cells (McLaren *et al.* 2010a) The expression of a number of chosen markers (described in Table 13) were then examined by RT-qPCR.

3.4.2.1. Cholesterol uptake genes

SRs play an important role in the transformation of lesion-resident macrophages into lipid-loaded foam cells. Under normal physiological conditions, cholesterol uptake and efflux is carefully regulated. However during the disease state, macrophages and other cells in the lesion release factors that up-regulate the expression of SRs facilitating excessive unregulated internalisation of modified LDL and promoting macrophage foam cell formation (Bultel *et al.* 2008). Due to the significance of SRs in augmenting atherosclerosis and the anti-atherogenic actions of IL-33, the effects on SR expression was investigated.

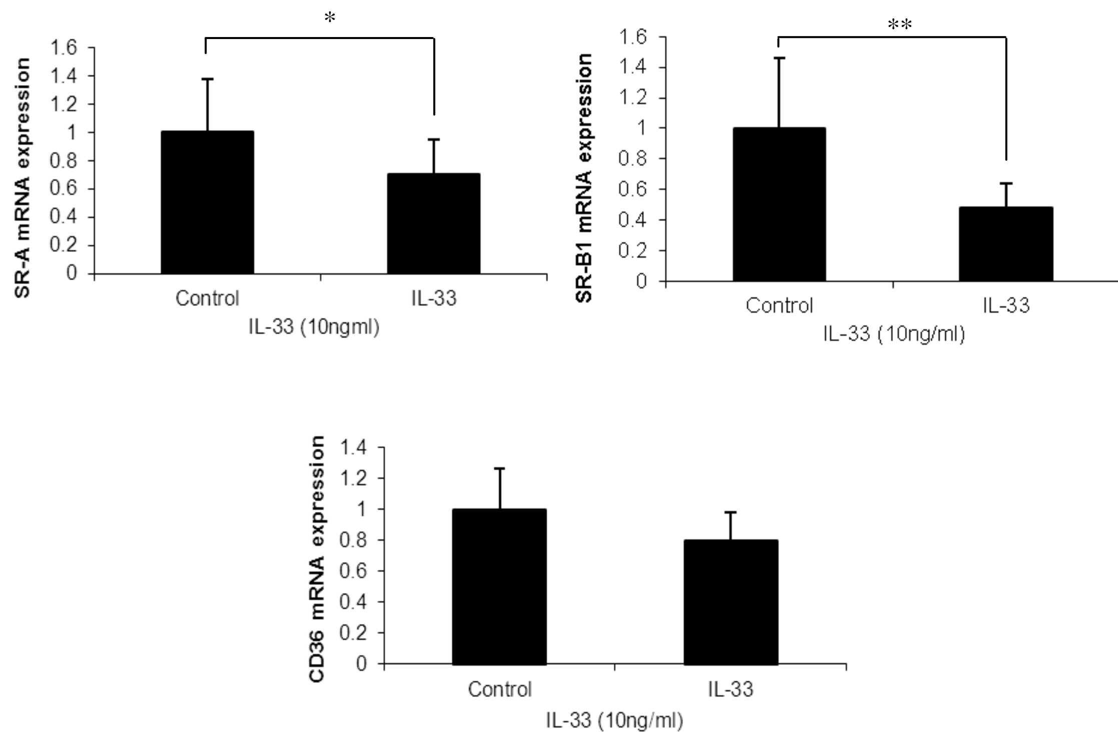


Figure 15. Effect of IL-33 stimulation on the mRNA expression of SRs

RT-qPCR for SR-A, SR-B1 and CD36 was performed using cDNA from PMA differentiated THP-1 macrophages and incubated in the presence or absence (control) of IL-33 (10ng/ml) for 24 hours. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control cells given an arbitrary value of 1. Data represents mean ± SD from four (SR-B1, CD36) or six (SR-A) independent experiments. The Student *t* test was used to determine the statistical significance of the results *$p<0.05$, **$p<0.01$.

IL-33 produced a statistically significant reduction in the expression of SR-A and SR-B1 (Figure 15). In addition, a trend for suppressed CD36 expression was seen although this failed to reach statistical significance thereby suggesting that additional experiments were required.

3.4.2.2. Cholesterol metabolism

During atherosclerosis the metabolic pathways responsible for regulating cholesterol homeostasis within macrophages become dysfunctional, promoting the excessive uptake and storage of lipids. LPL is a critical metabolic enzyme responsible for the hydrolysis of TAGs in circulating lipoproteins resulting in the production of FFAs and 2-monoacylglycerol (George and Johnson 2010). Additionally, within macrophages LPL increases the uptake of modified lipoproteins via a bridging action (Mead and Ramji 2002).

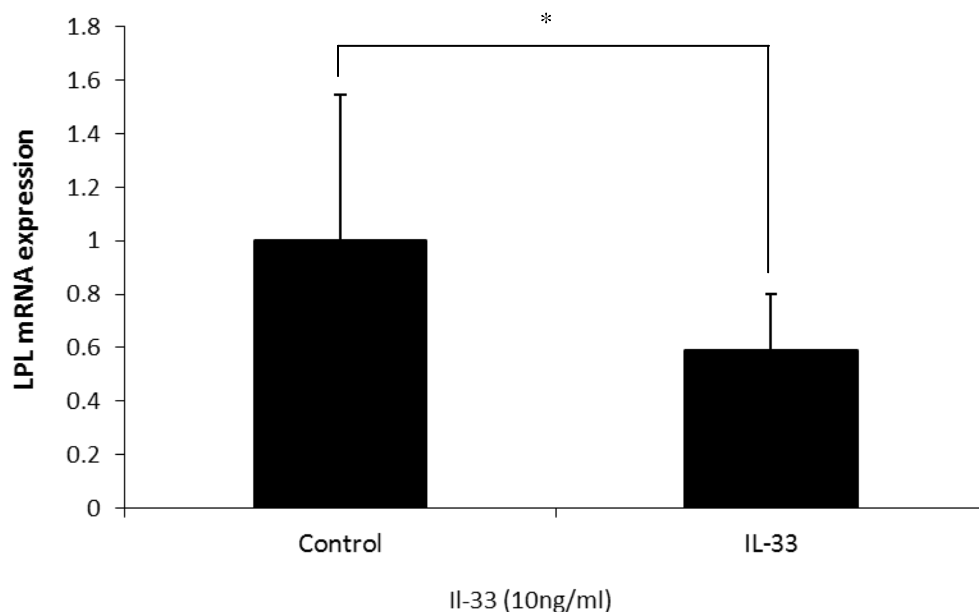


Figure 16. Effect of IL-33 stimulation on the mRNA expression of LPL

RT-qPCR for LPL was performed using cDNA from PMA differentiated THP-1 macrophages and incubated in the presence or absence (control) of IL-33 (10ng/ml) for 24 hours. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples given an arbitrary value of 1. Data represents mean ± SD from four independent experiments. The Student *t* test was used to determine the statistical significance of the results *$p<0.05$.

Following 24 hours of stimulation by IL-33 (10ng/ml) LPL mRNA expression was significantly down-regulated in THP-1 macrophages (Figure 16).

3.4.2.3. Monocyte recruitment and attachment

The study next investigated the role of IL-33 on the expression of genes implicated in the initial development of atherosclerosis. ICAM-1 is an adhesion molecule expressed on the endothelial cell wall and functions as a robust marker of inflammation in cells such as macrophages. ICAM-1 is involved in the attachment of circulating immune cells to the lesion site (Blankenberg *et al.* 2003).

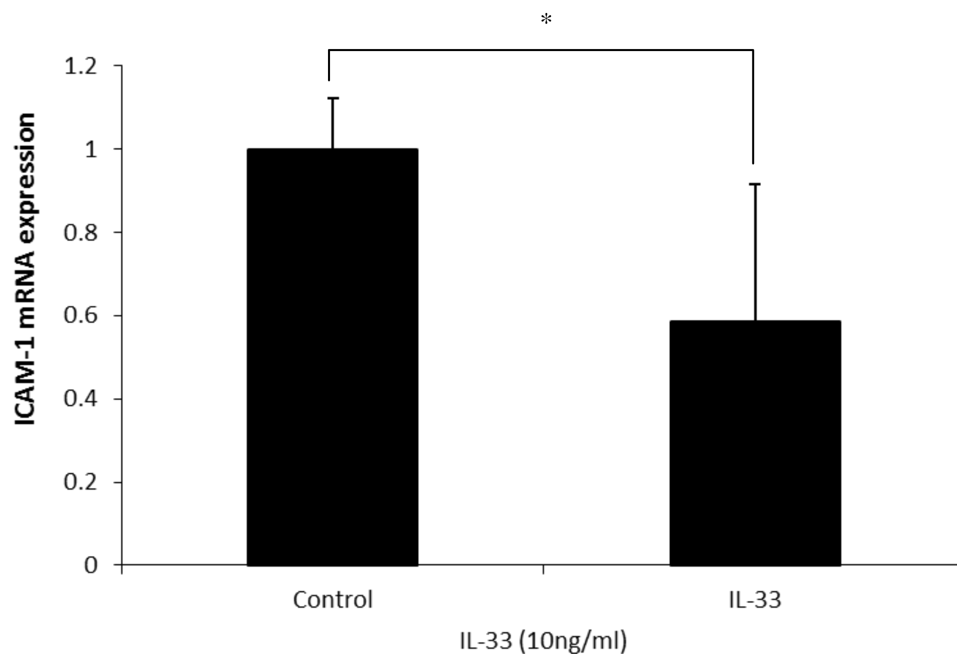


Figure 17. Effects of IL-33 stimulation on the mRNA expression of ICAM-1

RT-qPCR for ICAM-1 was performed using cDNA from PMA differentiated THP-1 macrophages and incubated in the presence or absence (control) of IL-33 (10ng/ml) for 24 hours. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control cells given an arbitrary value of 1. Data represents mean ± SD from six independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05.

Figure 17 shows that IL-33 significantly reduced the expression of the adhesion molecule ICAM-1.

IP-10, MCP-1 and MIP-1β are chemokines implicated in plaque initiation and the development of atherosclerosis. The expression of these genes are induced in several cell types, including macrophages, during inflammation and therefore acts as a robust marker for this process.

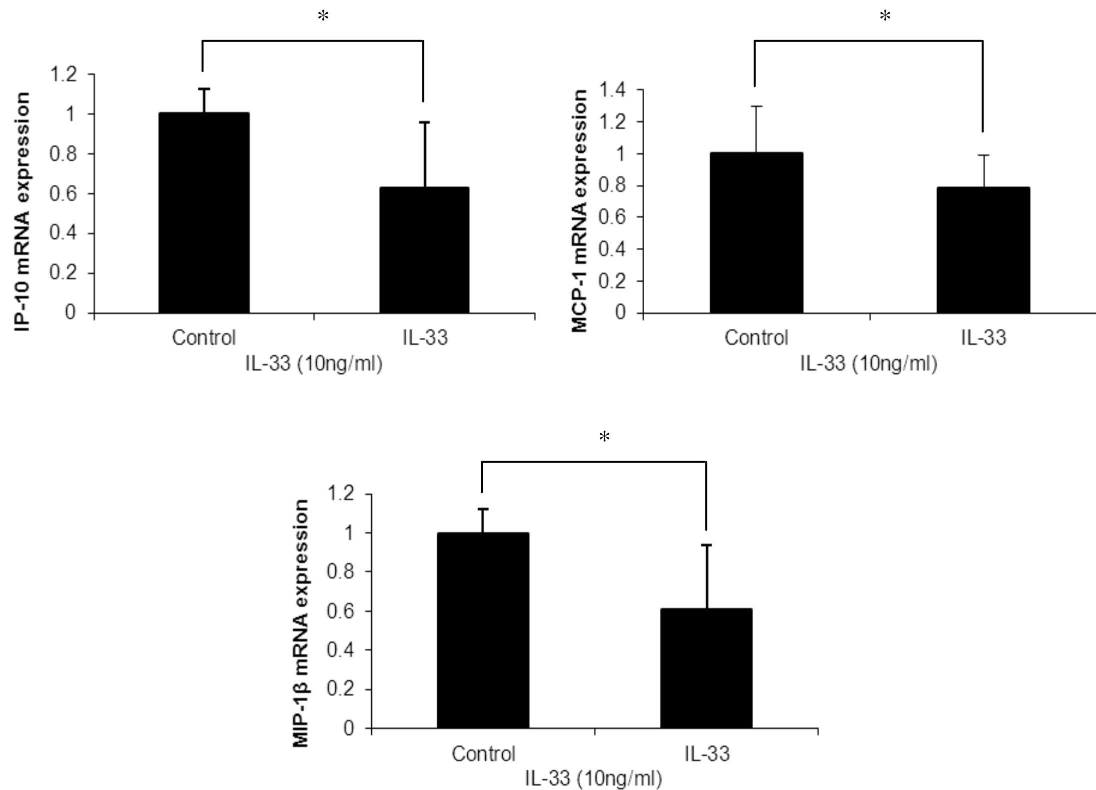


Figure 18. Effects of IL-33 stimulation on the mRNA expression of chemokines

RT-qPCR for IP-10, MCP-1 and MIP-1β was performed using cDNA from PMA differentiated THP-1 macrophages and incubated in the presence or absence (control) of IL-33 (10ng/ml) for 24 hours. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control cells given an arbitrary value of 1. Data represents mean ± SD from six (IP-10 and MIP-1β) and seven (MCP-1) independent experiments. The Student *t* test was used to determine the statistical significance of the results *$p<0.05$.

Similar to the findings for ICAM-1, IL-33 produced a significant decrease in the expression of the chemokines IP-10, MCP-1 and MIP-1β (Figure 18).

3.4.3. IL-33 time-course and dose-response in THP-1 cells

Initial studies were performed using a concentration of 10ng/ml and a 24 hour time point for IL-33 stimulation based upon previous experiments within the laboratory (McLaren *et al.* 2010b). The present study confirmed that at this concentration and time point IL-33 causes a reduction in previously studied pro-atherosclerotic genes (CD36, SR-A, SR-B1) and shows for the first time a decrease in ICAM-1, IP-10, LPL, MIP-1β and MCP-1 mRNA expression within THP-1 macrophages.

However, a number of groups use higher concentrations of IL-33 and different time points for their experiments (Demyanets *et al.* 2011; Ogawa *et al.* 2012). Therefore to determine whether the optimal concentration and time point were being used in the study, the effects of IL-33 on the expression of pro-atherosclerotic genes at different concentrations and time points were investigated. THP-1 macrophages were incubated in the presence of IL-33 over a range of concentrations (10ng/ml-100ng/ml) for 24 hours and 48 hours to assess changes in the mRNA expression of LPL and SR-A by RT-qPCR. Unstimulated cells at these time points were included for comparison.

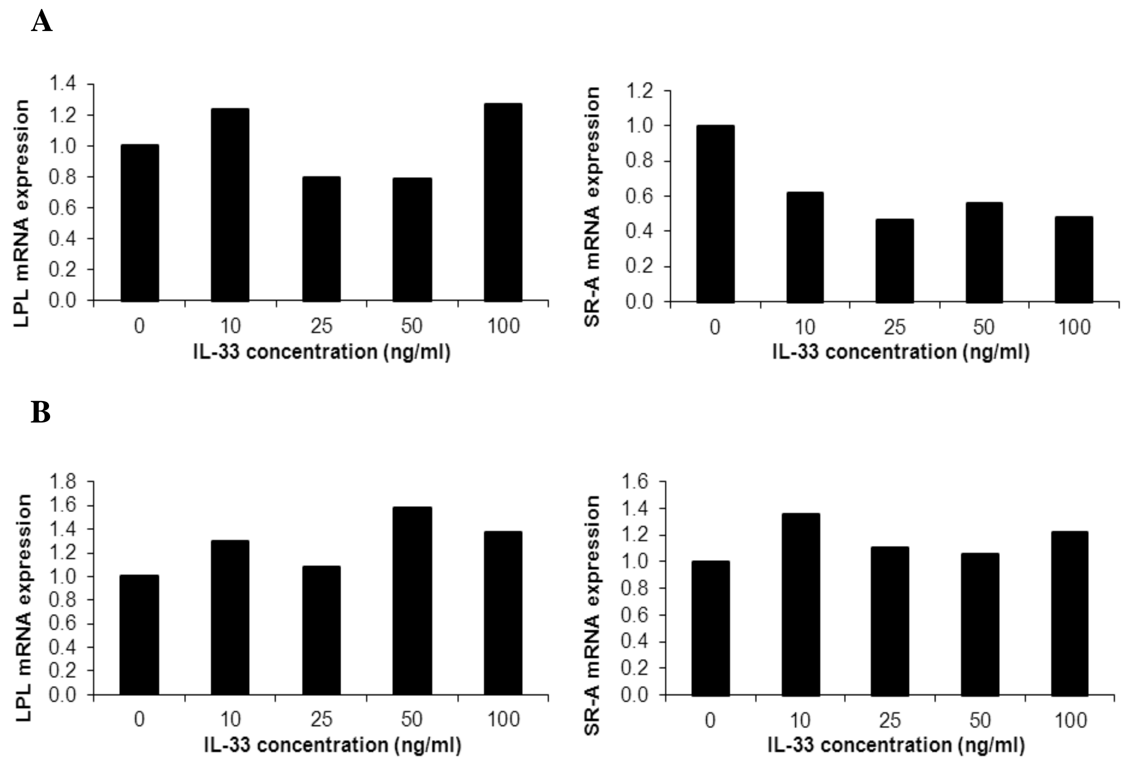


Figure 19. Effects of IL-33 concentration and incubation time on SR-A and LPL mRNA expression

RT-qPCR for LPL and SR-A was performed using cDNA from PMA differentiated THP-1 macrophages and incubated in the presence or absence (control) of IL-33 at a range of concentrations (10ng/ml-100ng/ml) for 24 hours (A) and 48 hours (B). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control cells given an arbitrary value of 1. Data represents mean from a single experiment performed in triplicate.

This initial, preliminary study suggested that IL-33 had little effect on SR-A or LPL mRNA expression at 48 hours (Figure 19). On the other hand, a decreased expression of both SR-A and LPL mRNA expression was obtained with IL-33 at 24 hours. However, in contrast to earlier studies (Figure 16) no reduction in LPL mRNA expression was seen at 10ng/ml of IL-33 but a decrease was obtained using 25ng/ml and 50ng/ml of the cytokine. In addition, the decrease in SR-A mRNA expression with 25ng/ml was greater than that achieved with 10ng/ml of IL-33. Based upon these results, it was decided to focus on new IL-33 regulated genes such as ICAM-1 and MCP-1 which were used for subsequent studies.

Preliminary studies were performed to establish the optimum time point for incubation with IL-33. An initial study assessed the effects of IL-33 at a concentration of 10ng/ml and 25ng/ml after 6, 12 and 24 hours of stimulation (Figure 20).

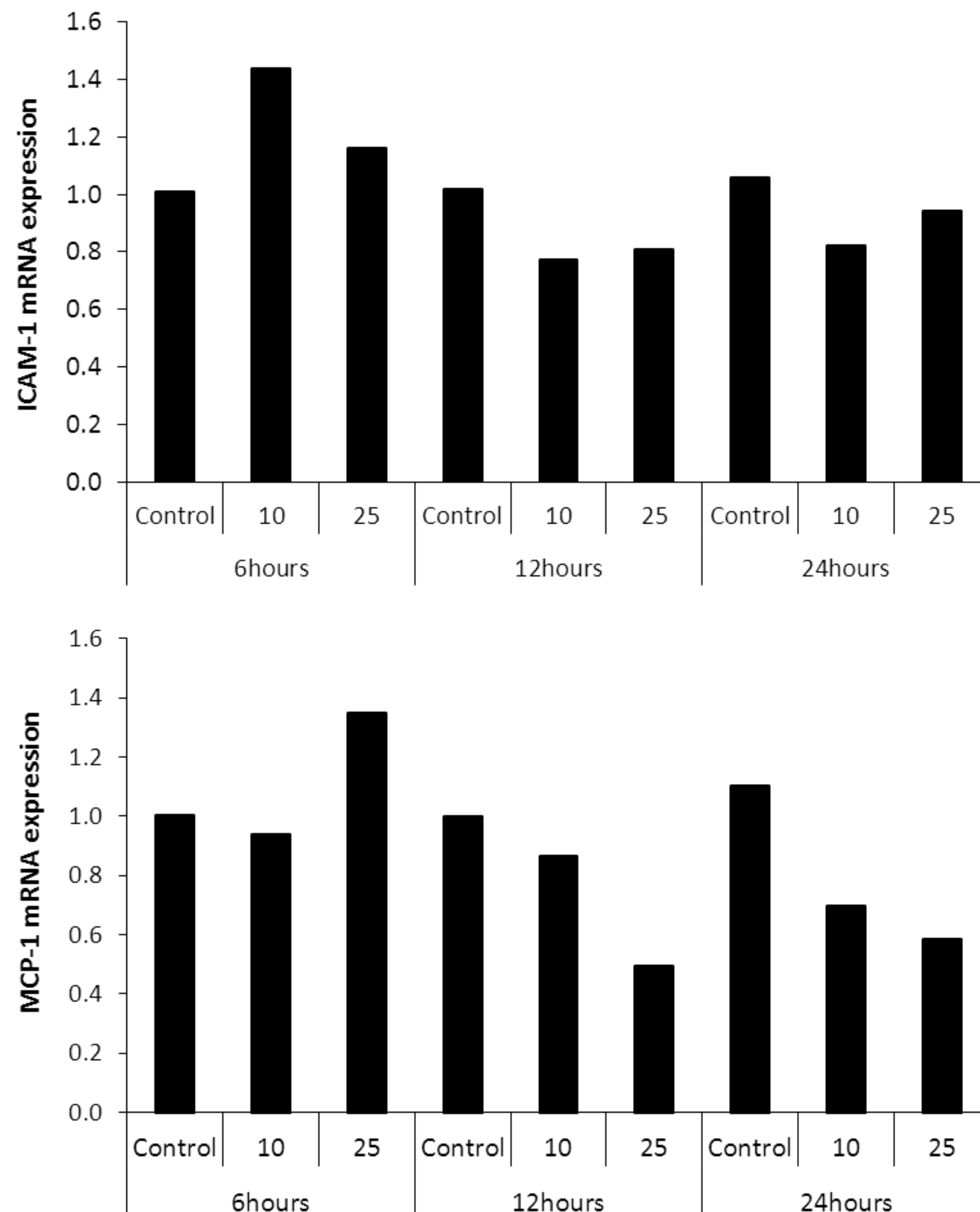


Figure 20. IL-33 time-course and dose-response experiment

RT-qPCR for ICAM-1 and MCP-1 was performed using cDNA from PMA differentiated THP-1 macrophages and incubated in the presence or absence (control) of IL-33 at 10ng/ml and 25/ml for 6, 12 and 24 hours. The mRNA levels were calculated using the comparative Ct method and normalised to the housekeeping gene, GAPDH, with values from control cells given an arbitrary value of 1. Data represents mean from a single experiment performed in triplicate.

The expression of ICAM-1 mRNA was reduced by IL-33 (10ng/ml or 25ng/ml) following 12 and 24 hours but not after 6 hours of stimulation. Likewise, the expression of MCP-1 was decreased following IL-33 treatment at 10ng/ml and 25ng/ml after 12 and 24 hours.

As a result, PMA-differentiated THP-1 cells were then stimulated with IL-33 at concentrations of 10ng/ml and 25ng/ml for 12 and 24 hours to determine which time point and concentration gave the most statistically significant results from four independent experiments (Figure 21).

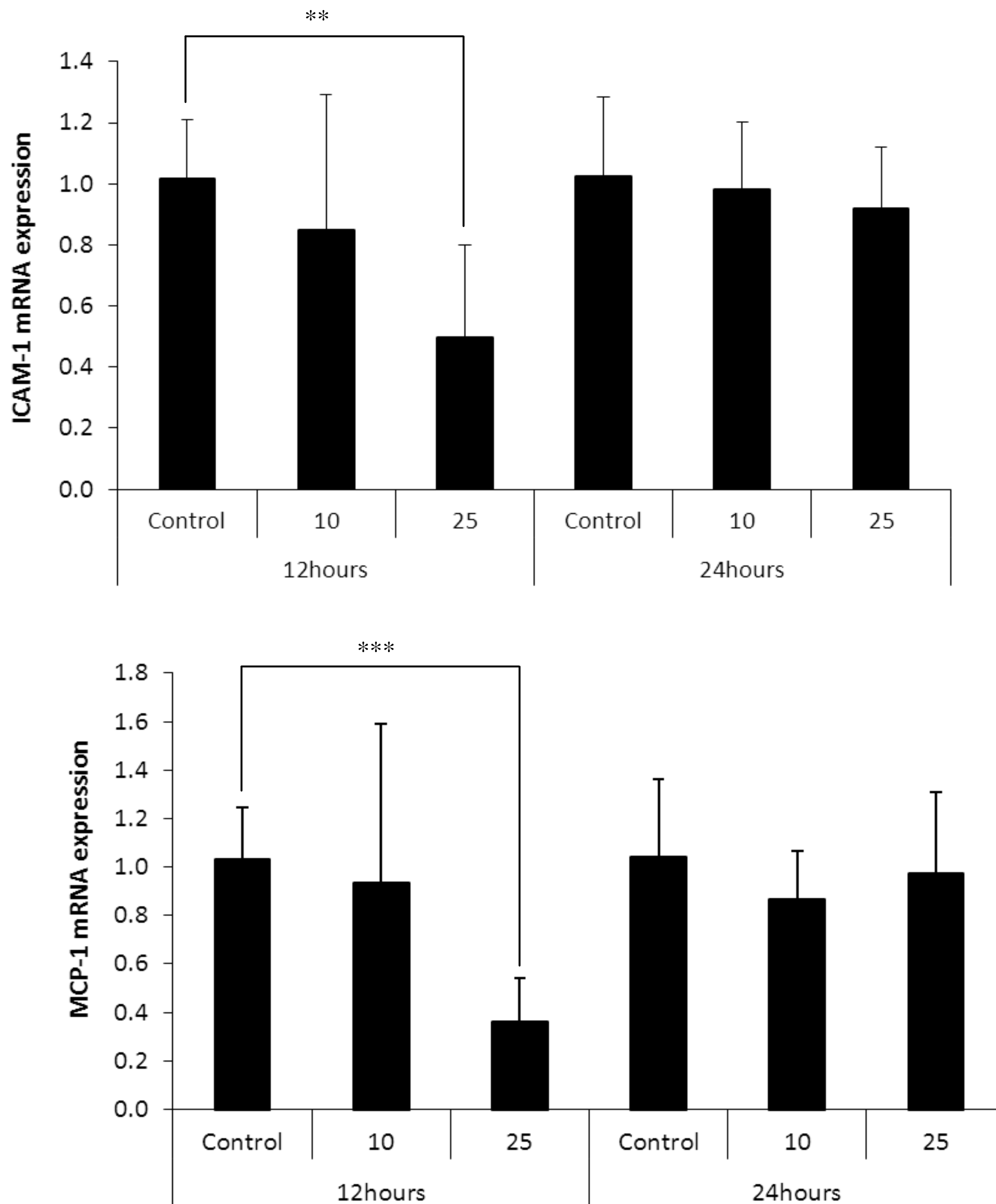


Figure 21. Effects of IL-33 concentration and period of stimulation on ICAM-1 and MCP-1 expression

RT-qPCR for ICAM-1 and MCP-1 was performed using cDNA from PMA-differentiated THP-1 macrophages and incubated in the presence or absence (control) of IL-33 at 10ng/ml and 25/ml for 12 and 24 hours. The mRNA levels were calculated using the comparative Ct method and normalised to the housekeeping gene, GAPDH, with values from control cells given an arbitrary value of 1. Data represents mean ± SD from four independent experiments. ANOVA were used to determine statistical significance of the results between untreated control and samples at different concentration and time-points in combination with Tukey's post hoc test for ICAM-1 **$p<0.01$. For MCP-1 Homogeneity of variances were not met so the Games-Howell and Dunnett's T3 post hoc tests were used ***$p<0.001$.

The studies confirmed the results from the preliminary experiments and showed that the expression of ICAM-1 and MCP-1 was most responsive to IL-33 following 12 hours of stimulation at 25ng/ml. In addition, the inhibitory effect of 25ng/ml IL-33 on MCP-1 and ICAM-1 expression at 12 hours reached significance from four independent experiments. In contrast, six to seven independent experiments were required to observe the significant action of 10ng/ml IL-33 at 24 hours (Figures 17-18). Based on these experiments, it was decided to use this concentration and duration in subsequent studies.

3.4.4. IL-33 stimulation of HMDMs

Experiments were completed in HMDMs at the concentrations and time points previously identified to determine whether the results obtained were not peculiar to the cell line. HMDMs were incubated for 12 or 24 hours with IL-33 at a concentration of 10ng/ml or 25ng/ml. RT-qPCR was then performed to determine the effect of IL-33 on the mRNA expression of ICAM-1 and MCP-1.

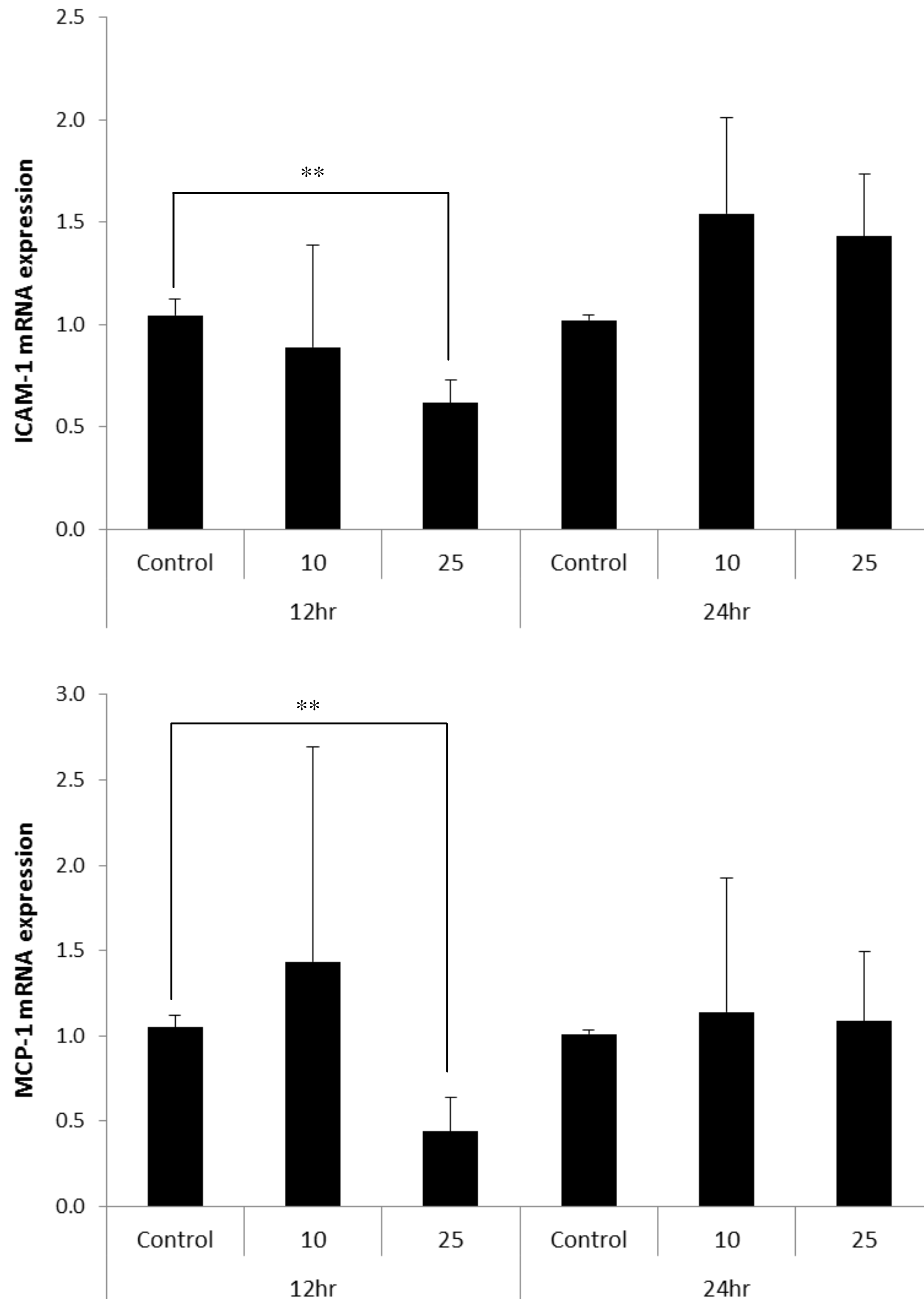


Figure 22. The effect of IL-33 on gene expression in primary macrophages

RT-qPCR for ICAM-1 and MCP-1 was performed using cDNA from 12 and 24 hour IL-33-stimulated HMDMs at 10ng/ml and 25ng/ml (control). The mRNA levels were calculated using the comparative Ct method and normalised to the housekeeping gene, GAPDH, with values from untreated cells given an arbitrary value of 1. Data represents mean ± SD from four independent experiments for ICAM-1 and MCP-1. ANOVA was used to determine statistical significance between control and samples at different concentrations and time points in combination with Tukey's post hoc test **$p<0.01$.

Following 12 hours of IL-33 stimulation at 25ng/ml there was a noticeable reduction in the expression of each of the genes tested; this decrease was significant for ICAM-1 and MCP-1 (Figure 22). The overall trend was therefore consistent with the results found in THP-1 cells.

3.4.5. IL-33 stimulation in RAW264.7 cells

The time-course experiments showed variation in the form of larger SD between experiments. The murine RAW264.7 cell line was therefore included as a model system for the study, as the cells are fully differentiated adherent macrophages and do not require PMA activation. In addition, studies in this cell line would provide an indication of conserved IL-33 actions in human and mouse macrophages. β-actin was chosen as the gene of reference for RT-qPCR analysis as this has been previously used by several laboratories for studies in mouse tissues and cell lines (Smith and Wickstrom 1998; Irvine *et al.* 2005; Svensson *et al.* 2006; Harvey *et al.* 2007; McLaren *et al.* 2010a; McLaren *et al.* 2010b).

3.4.5.1. SRs

RAW264.7 macrophages were either incubated in the presence of murine IL-33 at 10 or 25ng/ml for 12 or 24 hours, or left unstimulated for use as a control. The mRNA expression of the SRs CD36, SR-A and SR-B1 were then analysed by RT-qPCR.

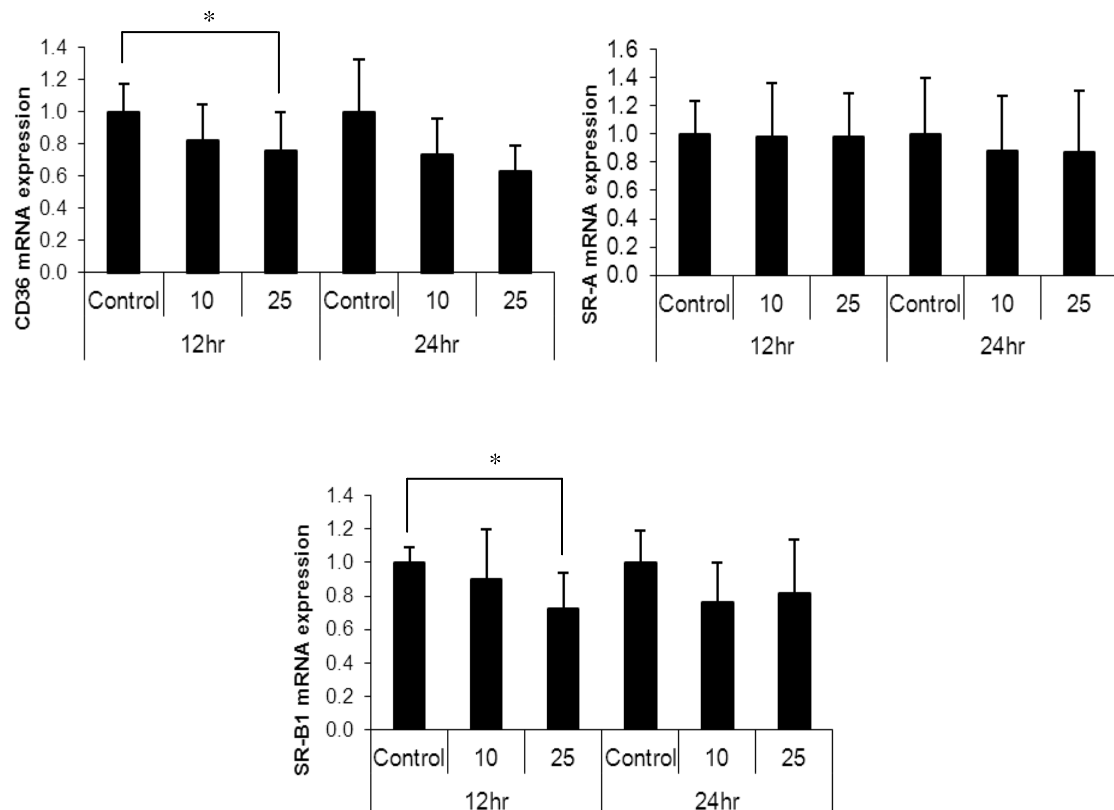


Figure 23. The effect of IL-33 on SR expression in RAW264.7 macrophages

RT-qPCR for murine CD36, SR-A and SR-B1 was performed using cDNA from RAW264.7 macrophages incubated in the presence or absence (control) of IL-33 at 10ng/ml and 25ng/ml for 12 and 24 hours. The mRNA levels were calculated using the comparative Ct method and normalised to the housekeeping gene, β-actin, with values from untreated cells given an arbitrary value of 1. Data represents mean ± SD from three (CD36, SR-B1) or five (SR-A) independent experiments. Statistical analysis by ANOVA was performed to determine whether the means between different concentrations and time points were significant in combination with Tukey's post hoc test *$p<0.05$.

IL-33 caused a reduction in CD36 mRNA expression at both concentrations (10ng/ml and 25ng/ml) and at both time points, although statistical significance was seen with 25ng/ml of cytokine at 12 hours (Figure 23). A similar pattern was obtained with SR-B1 mRNA expression with statistical significance seen with 25ng/ml of cytokine at 12 hours. In contrast, IL-33 had no significant effect on SR-A mRNA expression, potentially because of relatively large SD, though a trend of reduction was obtained at 24 hours at both concentrations of the cytokine.

3.4.5.2. Monocyte recruitment and attachment

To investigate the effects of IL-33 on the regulation of genes involved in early atherosclerotic events; RAW264.7 cells were stimulated with murine IL-33 for 12 or 24 hours with 10 or 25ng/ml of the cytokine and the mRNA expression of murine ICAM-1 and MCP-1 were assessed.

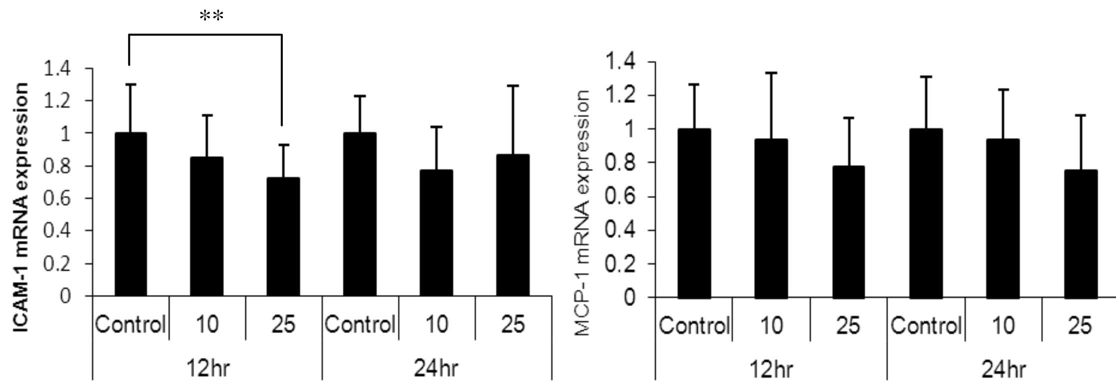


Figure 24. IL-33 time-course and dose-response in RAW264.7 cells

RT-qPCR for ICAM-1 and MCP-1 was performed using cDNA from 12 and 24 hour IL-33 stimulated RAW264.7 cells incubated in the presence or absence (control) of IL-33 at 10ng/ml and 25ng/ml. The mRNA levels were calculated using the comparative Ct method and normalised to the housekeeping gene, β-actin, with values from untreated cells given an arbitrary value of 1. Data represents mean ± SD from five independent experiments. ANOVA was used to determine statistical significance between untreated control and samples at different concentrations and time points in combination with Tukey's post hoc test **$p<0.01$.

IL-33 reduced ICAM-1 mRNA expression with both 10ng/ml and 25ng/ml concentrations and at both time points; though a statistically significant reduction was obtained with 25ng/ml at 12 hours (Figure 24). For MCP-1, the variation between experiments was large and hence no statistically significant differences were obtained. However, a trend of reduction of MCP-1 mRNA expression was observed with 25ng/ml IL-33 at both the 12 and 24 hour time points.

Overall, there was a consistent trend for IL-33-mediated down-regulation of the genes analysed and these reductions were most significant following 12 hours incubation with IL-33 at a concentration of 25ng/ml.

3.5. Discussion

Macrophages have a critical role in initiating and augmenting the disease state during atherosclerosis. IL-33 is a recently discovered member of the IL-1 family and has been implicated in a number of inflammatory disorders such as rheumatoid arthritis and asthma where the cytokine aggravates the disease state (Carriere *et al.* 2007; Kondo *et al.* 2008; Funakoshi-Tago *et al.* 2011). The monocytic THP-1 cell line was chosen to study the effects of IL-33 on the expression of a number of pro-atherogenic markers. These genes were chosen as their roles in atherosclerosis are well-characterised and many have been previously studied within our laboratory (McLaren *et al.* 2010a; Michael *et al.* 2012b). Although several of these genes have been analysed by other groups, a detailed study of the effects of IL-33 in the THP-1 system has not been performed.

3.5.1. IL-33 stimulation of THP-1 cells

There is a great deal of interest in the anti-atherosclerotic actions of IL-33 as the cytokine has been implicated in many aspects of the disease ranging from leukocyte recruitment (Demyanets *et al.* 2011), macrophage foam cell formation (McLaren *et al.* 2010b) to plaque progression (Miller *et al.* 2008). As a result initial experiments were conducted using conditions previously employed by our group (McLaren *et al.* 2010b) to establish whether the recombinant IL-33 used within these experiments was physiologically active. The monocytic THP-1 cell line was used to study the actions of IL-33 within macrophages and to facilitate initial optimisation of experimental conditions.

The study investigated the effects of IL-33 stimulation on a number genes implicated in atherosclerosis and other inflammatory disorders. Chemokines are small proteins which interact with G-protein-coupled receptors and form part of the larger cytokine superfamily (van Buul and Hordijk 2004). IP-10, MIP-1β and MCP-1 direct circulating leukocytes to the lesion where adhesion molecules, including ICAM-1, aid transdiapedesis into the subendothelium (Nagel *et al.* 1994; Wilcox *et al.* 1994; Taub *et al.* 1996; Niu and Kolattukudy 2009).

SRs promote macrophage foam cell formation by mediating excessive uptake of cholesterol during the disease. A study by Kunjathoor *et al.* (2002) demonstrated that CD36 and SR-A are major contributors to the uptake of modified LDL as mice deficient in these SRs exhibit approximately 75-90% decreased degradation of modified LDLs. Also macrophages from these animals are unable to accumulate CEs (Kunjathoor *et al.* 2002). The metabolic enzyme LPL regulates the storage of lipids within macrophages and mutations within the LPL gene are associated with an increased risk of CHD, accompanied by raised plasma TAGs and reduced levels of HDL cholesterol (Wittrup *et al.* 1999).

Interestingly, in alternative disease states IL-33 increases the expression of many of these genes (Table 14) and therefore promotes the inflammatory state. However within the context of atherosclerosis IL-33 is believed to have a protective role and these experiments demonstrated that IL-33 reduces the mRNA expression of ICAM-1, IP-10, LPL, MIP-1β, SR-A, SR-B1, CD36 and MCP-1. A number of studies have investigated the effects of IL-33 on ICAM-1 expression within the plaque (Demyanets *et al.* 2011) and in endothelial cells (Choi *et al.* 2012) but experiments within human macrophages had not been performed. Likewise, the studies presented in this thesis demonstrated the novel down-regulation of IP-10, LPL, MIP-1β and MCP-1 by IL-33 in the THP-1 cellular system. This is also the first study to demonstrate the involvement of IL-33 in the regulation of LPL mRNA expression. Overall, the data supports the hypothesis that IL-33 is a key mediator of anti-atherosclerotic events and also validates the use of the THP-1 as a useful system for studying atherosclerosis.

Table 14. Studies of IL-33 on the expression of genes tested within the study

Gene	Cellular system	Effect of IL-33	Reference
CD36	Human THP-1 macrophages, HMDMs, bone marrow-derived macrophages from WT and $ST2^{-/-}$ mice.	↓	(McLaren *et al.* 2010b)
ICAM-1	HUVECs and human explanted atherosclerotic plaques.	↑	(Pecaric-Petkovic *et al.* 2009; Chow *et al.* 2010; Demyanets *et al.* 2011; Choi *et al.* 2012)
IP-10	Synovial tissue from rheumatoid arthritis patients. Human eosinophils.	↑	(So *et al.* 2012; Wong *et al.* 2012)
LPL	Not previously tested.	**-**	-
MCP-1	Human pancreatic myofibroblasts, human eosinophils, human basophils, murine embryonic fibroblasts.	↑	(Funakoshi-Tago *et al.* 2008; Kondo *et al.* 2008; Nishida *et al.* 2010; Wong *et al.* 2012)
MIP-1β	Human basophils.	↑	(Kondo *et al.* 2008)
SR-A	Human THP-1 macrophages, HMDMs, bone marrow-derived macrophages from WT and $ST2^{-/-}$ mice.	↓	(McLaren *et al.* 2010b)
SR-B1	Human THP-1 macrophages, HMDMs, bone marrow-derived macrophages from $ST2^{-/-}$ mice.	↓	(McLaren *et al.* 2010b)

Anti-inflammatory cytokines like TGF-β have been shown to down-regulate the expression of genes associated with cholesterol uptake and metabolism (Michael *et al.* 2012b). The cytokine also up-regulates the expression of genes involved in atheroprotective processes such as cholesterol trafficking and efflux (Singh and Ramji 2006a). Previous work in our group has shown that TGF-β and IL-33 up-regulate the expression of ApoE, ABCA-1, ABCG-1, NCP1, NPC2 and CPT-1 (Singh and Ramji 2006b; McLaren *et al.* 2010b). The studies presented here demonstrate that IL-33 decreases the expression of a number of well-characterised markers associated with augmentation of the disease state, including CD36, ICAM-1, MCP-1 and SR-B1. However, due to the small nature of the response, potential trends within the data may have not been shown in some cases. As a result, ICAM-1 and MCP-1 were chosen to further optimise the experimental system.

3.5.2. Optimising IL-33 stimulation of THP-1 cells

After validating that the IL-33 response was present at concentrations and time points previously used (McLaren *et al.* 2010b), a range of concentrations (10ng/ml-100ng/ml) and incubation periods (6-48 hours) were trialled to address the variability seen within the results and to validate the findings. The experiments highlighted that IL-33 exerted the strongest response after 12 hours and at a concentration of 25ng/ml (Figures 20 and 21). An array of different time-courses and doses of IL-33 have been used in previous studies and the conditions tested in these initial experiments are within the range used by other groups (Funakoshi-Tago *et al.* 2008; Kondo *et al.* 2008; Joshi *et al.* 2010; Funakoshi-Tago *et al.* 2011). For example, Kaieda *et al.* (2012) treated murine mast cells with 10ng/ml of IL-33 for 10 hours (Kaieda *et al.* 2012) and Kempuraj *et al.* (2013) stimulated human pancreatic samples for 6 hours with IL-33 (100ng/ml) (Kempuraj *et al.* 2013). On the other hand, Turnquist *et al.* (2011) administered 25ng/ml of IL-33 into mice over a course of 8 days (Turnquist *et al.* 2011).

3.5.3. IL-33 stimulation in HMDMs

Experiments were also conducted in HMDMs to determine whether the response from IL-33 was not peculiar to the THP-1 cell line and to establish the physiological relevance of results. The expression of ICAM-1 and MCP-1 was analysed using RT-

qPCR and the greatest reduction in mRNA expression occurred after 12 hours of stimulation using 25ng/ml of IL-33.

Cell lines are frequently used as model systems for studying disease, as culturing primary cells can be difficult to establish and maintain due to heterogeneous populations of cells. However, monocytic cell lines may not give a fully accurate representation of differentiated tissue macrophages. For example, the responses produced by the promyelocytic leukemia cell line HL-60 can vary depending on the type of reagent used to induce cellular differentiation (Daigneault *et al.* 2010). The cells display a monocytic phenotype following stimulation by VD_3 but the addition of phorbol esters promotes a more mature macrophage phenotype (Daigneault *et al.* 2010). Furthermore, the effects of passaging in cell lines are not completely understood. Wenger *et al.* (2004) found that maintaining cell cultures over a long time-period led to the appearance of mutations that altered the characteristics of the breast cancer MCF-7 and endometrial tumour Ishikawa cell lines. These mutations introduced heterogeneity between cells within the same culture (Wenger *et al.* 2004). Also, studies in Syrian hamster embryo cells found that cell passage affected doubling time and plating efficiency. At later passages, the cells reached a 'crisis' point where growing conditions needed to be altered to facilitate growth, and coincided with changes in sensitivity to experimental treatments (ChangLiu and Woloschak 1997). The use of cells at different passages could introduce variation between experiments and the passage number of THP-1 cells used in previous atherosclerotic studies greatly varies between groups. During these studies experiments were completed using cells between passages 2-7 but there are examples of groups using THP-1 cells up to passage 20 (Kritharides *et al.* 1998; Chanput *et al.* 2010) and many studies do not state the passage number. HMDMs are not subjected to passaging and the inclusion of RAW264.7 macrophages allows for comparison between the two cell lines.

3.5.4. Optimising IL-33 stimulation in RAW264.7 cells

PMA is commonly used to differentiate THP-1 monocytes into macrophages for experimental use. However, PMA also activates several signalling pathways and could therefore introduce variability in the data. Several studies have shown that the expression of several genes are induced by PMA in a variety of cell lines including

HeLa, primary human T cells, primary rabbit cardiac fibroblasts (Kronke *et al.* 1985; Eghbali *et al.* 1991; Ma *et al.* 2004), in addition to THP-1 cells (Kohro *et al.* 2004; Daigneault *et al.* 2010). Additionally, PMA can induce the secretion of IL-33 and sST2 in cardiac fibroblasts in a dose-dependent manner which could impact the data (Sanada *et al.* 2007). The phorbol ester interrupts the cellular cycle by modulating the expression of cell cycle regulators through the production of ROS and also activates signalling pathways including MAPK and PKC (Traore *et al.* 2005). This may lend some explanation to some of the inconsistency exhibited in samples taken at 24 hours (Nile *et al.* 2010).

Murine RAW264.7 macrophages were included as a model system in the study as these cells are frequently used in atherosclerotic studies and are mature adherent cells so PMA is not required. Experiments were conducted using the same conditions as the human THP-1 system, only without the inclusion of PMA. Following incubation with the cytokine, RT-qPCR was performed to evaluate the effects on the mRNA expression of CD36, SR-A, SR-B1, ICAM-1 and MCP-1. The majority of genes tested, with the exception of SR-A, displayed a reduction in mRNA expression following IL-33 treatment. The reduction in expression was significant at 25ng/ml of IL-33 after 12 hours for both SR-B1 and ICAM-1. Overall the results from the murine RAW264.7 cell line confirmed those seen in human macrophages, indicating a well-conserved role of IL-33 in the regulation of key atherosclerotic makers. This is important as species-specific differences have been seen by other groups.

Mice and humans share many features but key discrepancies exist such as differences in metabolic enzymes (Martignoni *et al.* 2006), ionic channels (Martignoni *et al.* 2006; Bianchi *et al.* 2012), innate and adaptive immune systems including Th1/Th2 differentiation, leukocyte subsets; whereby humans display a more neutrophilic profile and rodents have greater proportions of circulating lymphocytes (Haley 2003). However, the use of mice has greatly aided our understanding of atherosclerosis and significant parallels can be drawn between murine and human forms of the disease. Combining the findings from both organisms has enabled the identification and validation of many major players involved in the pathogenesis of atherosclerosis (Stylianou *et al.* 2012).

The studies presented here demonstrate that IL-33 exerts a consistent atheroprotective effect on the expression of genes implicated in atherogenesis. These initial experiments

were performed in the THP-1 and mouse RAW264.7 cell lines and verified in HMDMs. Future studies performed within this thesis will use the conditions optimised within this chapter as they produced the strongest response from the cytokine.

3.6. Future perspective

Due to the growing prevalence of CHD within westernised societies, and an increasing trend in incidences globally, there is a pressing need to identify and examine novel contributors to the disease. Cytokines are involved at every stage of the disease, from recruitment of circulating immune cells and activation of the endothelium, to propagating dysfunctional metabolism and eventual plaque rupture. The disease state arises through an imbalance in the activities of pro- and anti-inflammatory cytokines. Therefore it is of crucial importance to explore the role of novel cytokines implicated in the disease state such as IL-33. Analysing the mechanisms through which IL-33 operates may aid the development of potential therapies and also identify side-effects which could arise.

The study provides evidence for the anti-atherosclerotic role of IL-33 in THP-1 and RAW264.7 macrophages in addition to primary HMDMs and demonstrates the novel down-regulation of the mRNA expression of ICAM-1, IP-10, MCP-1, MIP-1β and LPL by IL-33 within macrophages. Interestingly, studies have also shown that IL-33 increases the expression of many of the pro-atherogenic genes used in the study within the context of other inflammatory diseases (Table 14). Therefore it is of great interest to understand the potential signalling pathways that contribute towards the IL-33-mediated reduction of these genes. The next step will attempt to elucidate the mechanisms underlying IL-33 actions through the use of RNA interference (RNAi) whereby a combination of siRNA and shRNA will be used to knock down the expression of key signalling components. The effects on the expression of atherosclerotic markers will then be assessed through a combination of RT-qPCR and western blotting.

CHAPTER 4.

THE SIGNALLING PATHWAYS UNDERLYING IL-33-REGULATION OF ICAM-1 AND MCP-1 EXPRESSION

4.1. Introduction

4.1.1. Mitogen-activated protein kinases (MAPKs)

The MAPK signalling network encompasses a variety of pathways responsible for the regulation of key cellular functions including gene transcription, cell cycle and apoptosis. This network utilises a series of proteins, often referred to as a “toolkit”, from which signalling cascades are assembled. The activation of several protein kinases prior to the MAPK is required for activity. MAPKKK’s act as transducers which transmit the signal by dual phosphorylation of serine and threonine present within MAPKK’s, the activated MAPKKs then phosphorylate tyrosine and threonine residues of MAPK’s which in turn phosphorylate selected serine and threonine residues of their target proteins (Figure 25) (Lawrence *et al.* 2008; Berridge 2012).

MAPKs share evolutionary conserved modules and are classified under three main pathways: ERK, p38 and JNK. The MAPK network is tightly regulated through the use of scaffolding proteins that mediate interactions and localisation of MAPKs. Also, the antagonistic actions of MAPK phosphatases inactivate MAPKs by the removal of necessary phosphate groups (Ravandi *et al.* 2003). Aberrant MAPK signalling is observed in a number of disease states such as cancer (White *et al.* 1995; Roberts and Der 2007) and Alzheimer’s disease (Puig *et al.* 2004; Muresan and Muresan 2007).

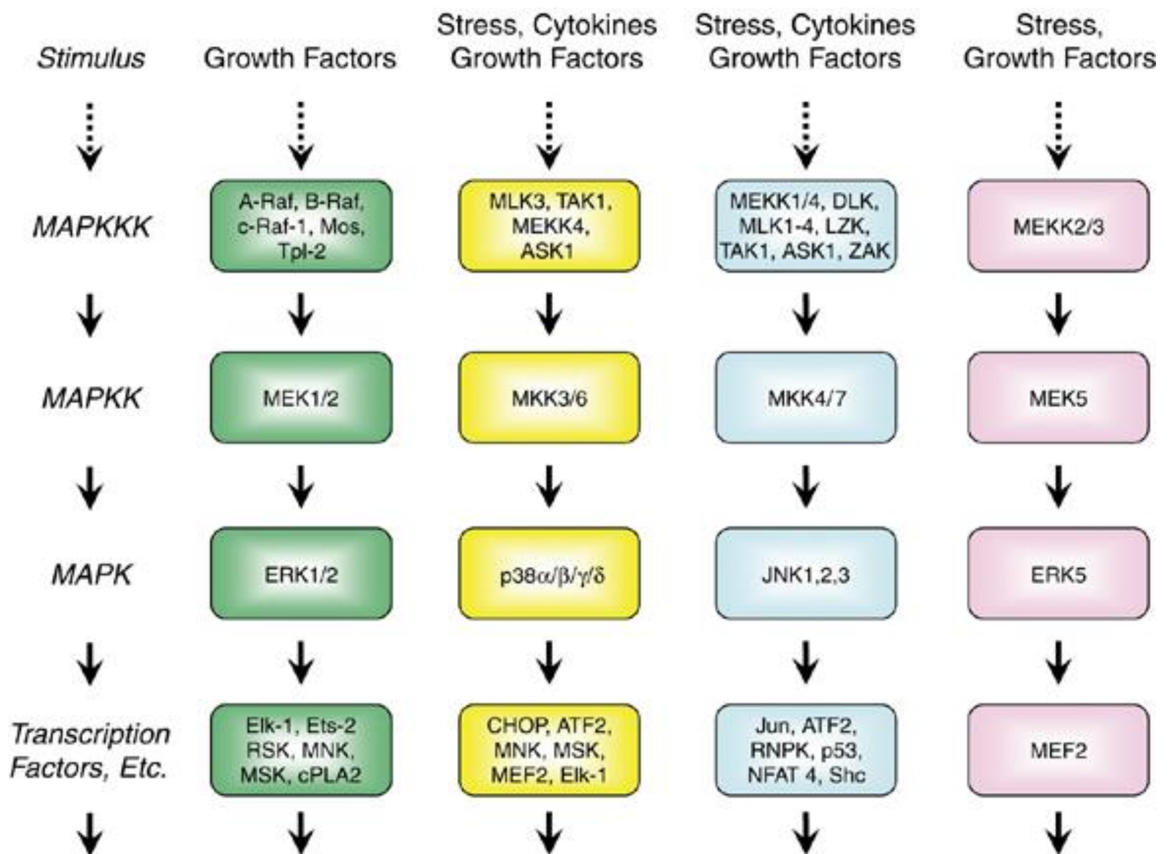


Figure 25. MAPK signal transduction pathway

MAPK signalling is formed of a hierarchical cascade. Components within the cascade often share common modules. The activation of MAPKKKs by extracellular stimuli or cell surface receptors triggers sequential phosphorylation of a series of kinases ending with a terminal MAPK such as the ERKs, p38s and JNKs which elicit a biological response. Taken from Roberts and Der 2007.

4.1.1.1. Extracellular signal-regulated kinase (ERK) 1/2 signalling

The ERK family contains a number of isoforms named ERK1-8 (Kim and Choi 2010). ERK1 (also known as $p44^{ERK1}$) and ERK2 ($p42^{ERK2}$) are ubiquitously expressed in all mammalian cells (Hu *et al.* 2000) and function as serine-threonine protein kinases. ERK1/2 are integral components of the Ras-Raf-MAPK/ERK kinase (MEK)-ERK MAPK pathway (Zhou *et al.* 2010). The kinases undergo cyclic rounds of phosphorylation and dephosphorylation associated with nuclear-cytoplasmic shuttling. ERK1/2 phosphorylation induces translocation of the kinase from the cytoplasm into the nucleus, where ERK1/2 either directly activate target proteins or trigger alternative signalling cascades (Costa *et al.* 2006).

ERK1 and ERK2 share similar sequences and were previously thought to play an interchangeable role in signalling cascades. However experimental evidence indicates that both kinases may facilitate different activities within the cell. A study by Vantaggiato *et al.* (2006) demonstrated that ERK2 mediates normal and Ras-mediated cell proliferation whereas ERK1 functions as a negative regulator of cell proliferation under certain conditions. The authors proposed that ERK1 competes for the upstream kinase MEK and affects the overall signalling outcome by antagonising ERK2 activity (Vantaggiato *et al.* 2006). Furthermore, although both kinases are present in most tissues, cellular levels of the proteins differ and mice deficient in ERK2 die during embryonic development (Saba-El-Leil *et al.* 2003).

4.1.1.2. p38 signalling

The second group of MAPKs are the p38 MAPKs consisting of four splice variants; α, β, γ and δ in mammals (Ravandi *et al.* 2003). p38 kinases share a Thr-Gly-Tyr dual phosphorylation motif (Zarubin and Han 2005) and members of the family are activated through receptor-dependent routes e.g. TLRs, or by environmental factors such as UV irradiation. JNK and p38 share a number of common upstream activators such as the MAPKKKs Apoptosis signal-regulating kinase 1 and TGF-β-activated kinase 1. Although distinct MAPKKs also exist like MAPKK-3 and -6 that are solely responsible for the activation of p38s (Kim and Choi 2010).

p38 members are involved in inflammation and cell cycle progression along with the regulation of apoptotic activities and the secretion of cytokines from neutrophils and macrophages (Berridge 2012). p38 isoforms display both overlapping and distinct activities (Nemoto *et al.* 1998) which may arise due to the production of complexes between MAPKKs and selected p38 isoforms (Enslen *et al.* 2000). Interestingly, several phosphatases target p38α and p38β but there are no known effective phosphatases against p38γ and p38δ (Zarubin and Han 2005). Complete ablation of p38α in mice causes embryonic lethality (Adams *et al.* 2000).

4.1.1.3. c-Jun N-terminal kinase (JNK) signalling

The third group of MAPKs comprises the JNKs proteins. Members of this group share many responses with the p38 MAPKs and the family was initially found due to their

ability to phosphorylate the transcription factor c-Jun (Cui *et al.* 2007). Alternative splicing of the three JNK genes produces at least ten isoforms ranging from 46 kDa to 54 kDa in size (Derijard *et al.* 1994). The isoforms display different expression patterns; JNK1 and JNK2 are ubiquitously expressed whilst JNK3 expression is restricted to the central nervous system, neurons, testis and cardiac SMCs (Cui *et al.* 2007).

The canonical JNK cascade is activated in response to environmental stresses like UV irradiation, hyperosmolarity and heat shock, as such the family was initially known as stress-activated protein kinases (Guma and Firestein 2012). It is thought that 13 MAPKKKs interact with upstream activators of JNKs giving rise to a complex and intricate array of potential activation routes for the MAPK. JNK members phosphorylate and activate a range of transcription factors including c-Jun, p53 and c-myc, in addition to other cellular targets such as Bcl-2 family members (Guma and Firestein 2012). As such negative feedback mechanisms are necessary to regulate JNK members. For example the scaffolding protein JNK-interacting protein-1 physically limits the mobility of JNKs hindering activity (Berridge 2012).

4.1.2. Phosphoinositide-3-kinase (PI3K)/ Protein kinase B (Akt) signalling

Members of the PI3K family catalyse the phosphorylation of D3 hydroxyl groups within membrane PIs (Morello *et al.* 2009). There are three general classifications of PI3K members. Class I proteins are formed of catalytic (p110α, β, δ and γ) and adaptor/regulatory (p85 or p101) heterodimers. These members are solely responsible for the phosphorylation of phosphatidylinositol 4,5-bisphosphate [(PtdIns(4,5)P_2)] to PtdIns(3,4,5)P_3 and are further subdivided based on their route of activation (Vara *et al.* 2004). Subclass 1A proteins are primarily activated through RTKs or non-RTKs in the cytoplasm. These members associate with phosphotyrosine consensus motifs in the cytoplasmic tail of RTKs through Src-homology 2 (SH2) domains within their adaptor subunit. This interaction results in allosteric activation of the catalytic subunit. In contrast, members from subclass IB interact with receptors coupled with G proteins (Cooray 2004; Vara *et al.* 2004). The Class II group is comprised of three monomers: PI3K-C2α, PI3K-C2β and PI3K-C2γ. Vascuolar protein sorting 34 is the only member of Class III (Morello *et al.* 2009). An overview of PI3K signalling is provided below in Figure 26.

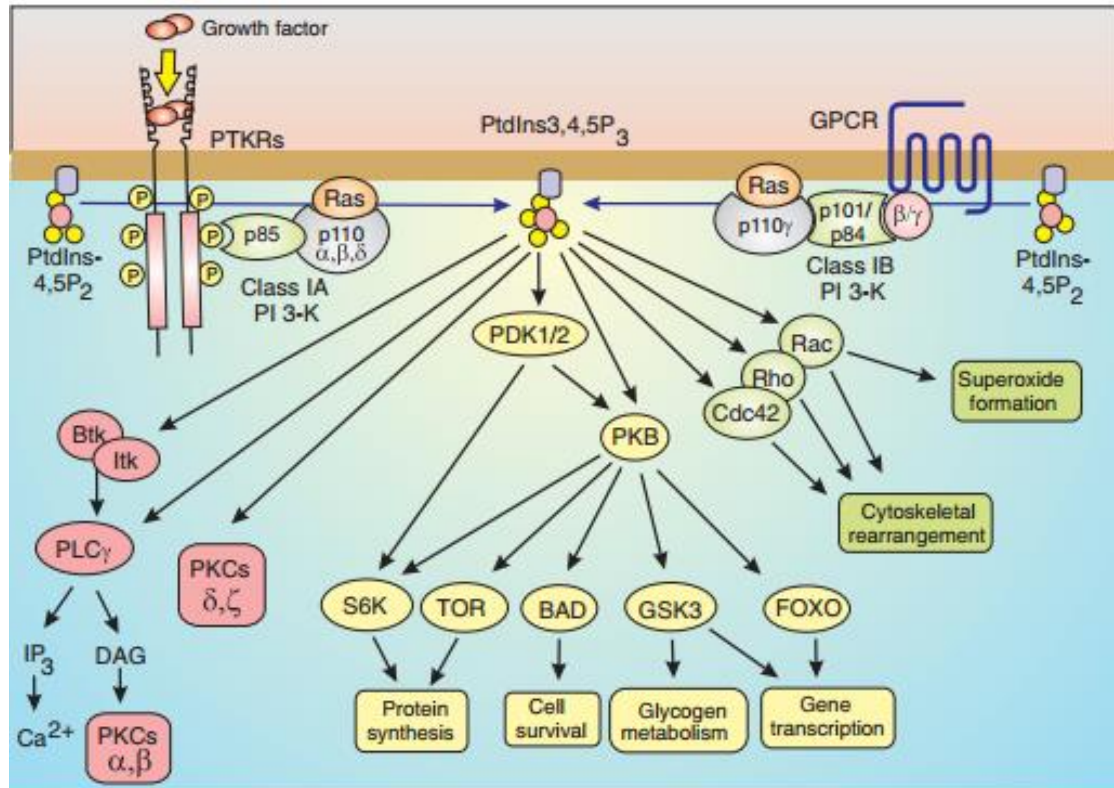


Figure 26. PI3K signalling

Class I PI3Ks phosphorylate PI(4,5)P_2 producing the secondary effector PI(3,4,5)P_3. Regulatory subunits like p85 of PI3Ks facilitate the interaction of their catalytic subunits; such as p110, with phosphorylated tyrosine residues of activated GF receptors. G protein-coupled receptors activate Class 1B PI3Ks which translocate to the plasma membrane through associations with the Gβγ subunit. PI3Ks produce PI(3,4,5)P_3 which associate with other signalling mediators such as PKCs and PLCγ. PLCγ cleaves PI(3,4,5)P_3 to IP_3 and DAG which participate in potassium and calcium signalling and in the regulation of transient receptor potential channels. Monomeric G proteins (Rac, Rho and Cdc42) activated through the cascade facilitate rearrangement of the cytoskeleton and superoxide production. The activation of 3-phosphoinositide-dependent kinase-1 (PDK1/2) and Akt (PKB) triggers downstream signalling components involved in a range of cellular processes. Taken from Berridge (2012).

The main products from PI3K activity are 3-phosphorylated lipids that function as primary effectors within the plane of the plasma membrane. 3-phosphorylated lipids recruit target proteins to the plasma membrane through lipid-binding domains like pleckstrin homology (PH), Phox homology and C2 (Berridge 2012). These domains also serve as targets for PI3K-mediated phosphorylation (Morello *et al.* 2009). A major downstream effector is the serine-threonine kinase, protein kinase B (Akt). There are

three Akt isoforms which all contain PH domains. The phosphorylation of a specific threonine switches the enzyme into an active state, whilst multiple phosphorylation ensures maximal activity (Vara *et al.* 2004). Other downstream targets of PI3Ks include the serine/threonine PDK1, glycogen synthase kinase B, Raf, forkhead box transcription factor (FOXO), RhoA and PLC (Morello *et al.* 2009). Due to the extensive scope of PI3K signalling, the pathway is tightly controlled through the action of three main phosphatases. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and myotubularin are responsible for the removal of phosphate groups from PI(3,4,5)P_3 and PI(3)P respectively, whereas SH2-containing inositol phosphatase acts as a 5-phosphatase (Morello *et al.* 2009).

4.1.3. Nuclear factor-κB (NF-κB) signalling

Stimulation of cell-surface receptors like TLRs by pathogen-associated molecular patterns and inflammatory cytokines results in the activation of NF-κB members (Baker *et al.* 2011). NF-κB signalling is involved in the control of cell differentiation, proliferation, survival and apoptosis (Oeckinghaus *et al.* 2011). Dysregulation of this pathway is implicated in cancer (Pikarsky *et al.* 2004), insulin resistance (Cai *et al.* 2005), ectodermal dysplasia (Doffinger *et al.* 2001) and many other disease states.

The NF-κB family is formed of five members within mammals: the precursor proteins NF-κB-1 (p105) and NF-κB-2 (p100) which are processed to p50 and p52 respectively, RelA (p65), RelB and c-Rel (Oeckinghaus *et al.* 2011). Members of the NF-κB family contain a conserved Rel homology domain responsible for DNA binding and dimerisation (Oeckinghaus *et al.* 2011). Under normal physiological conditions, NF-κB dimers are sequestered within the cytoplasm due to their association with inhibitory IκB proteins; IκBα, IκBβ and IκBε (Smahi *et al.* 2002; Oeckinghaus *et al.* 2011). Extracellular stimuli trigger cellular cascades which converge leading to the activation of the inhibitor of κB kinase complex (IKK) consisting of catalytically active kinases IKKα (IKK1), IKKβ (IKK2) and the regulatory subunit NF-κB essential modifier (NEMO) also known as IKKγ (Baker *et al.* 2011). Phosphorylation of two specific serine residues within the IκB proteins by the IKK complex targets the protein for polyubiquitination and subsequent degradation by the proteasome (Smahi *et al.* 2002;

Oeckinghaus *et al.* 2011). The unbound NF-κB dimers are then free to translocate to the nucleus and function as transcription factors (Oeckinghaus *et al.* 2011).

There are two main NF-κB pathways (Figure 27); the classical route involves IKKβ and NEMO-mediated phosphorylation of IκBα. The phosphorylated IκBα unit is tagged for degradation facilitating the translocation of NF-κB dimers, mainly consisting of p50 and p65 heterodimers. Alternatively, the non-canonical pathway is activated in response to selected TNF members such as CD40 ligand and lymphotoxin-β. IKKα is responsible for the phosphorylation of p100 which is bound to RelB. Degradation of p100 then gives rise to the formation of functional p52-RelB dimers (Oeckinghaus *et al.* 2011).

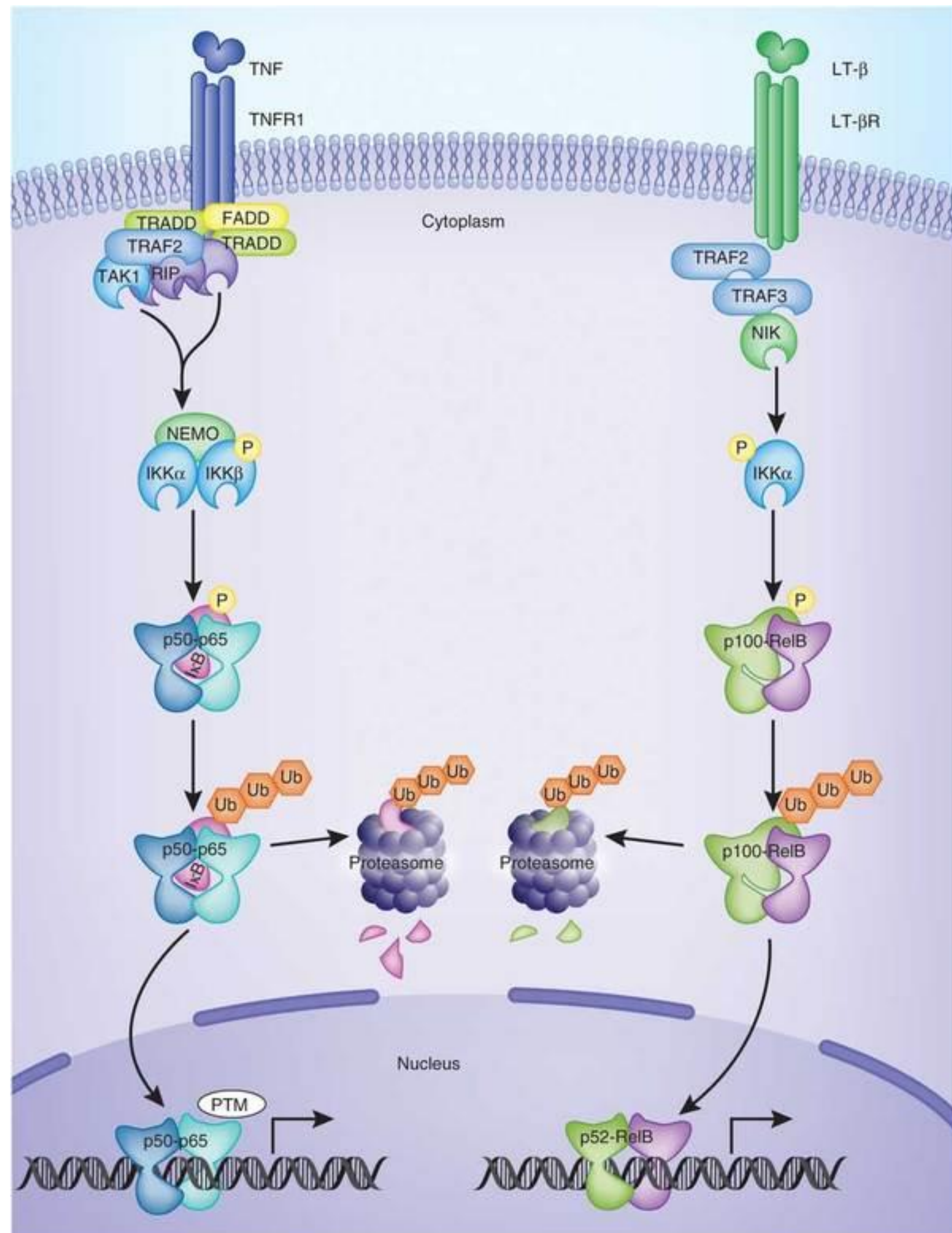


Figure 27. Canonical and non-canonical NF-κB signalling

The canonical pathway is activated by ligand binding to cell surface receptors like TNFR and utilises p50-p65 to mediate its effects. Cytokines such as lymphotoxin-β trigger non-canonical signalling involving p52-RelB family members. Inactivation of NF-κB signalling is achieved through the use of inhibitory IκB proteins. Inducible processing of the p52 precursor p100 functions as an inhibitor for RelB. Phosphorylation of these inhibitory proteins leads to their degradation thus facilitating translocation of p50-p65 and p52-RelB dimers where the proteins function as transcription factors. Taken from Oeckinghaus *et al.* (2001).

4.1.4. RNA interference (RNAi)

The body contains a controlled system through which genes can be systematically switched on and off. RNAi is an ATP-dependent process initiated by the presence of exogenous or endogenously produced double stranded RNA (dsRNA) (Hammond *et al.* 2001; Milhavet *et al.* 2003; Whitehead *et al.* 2009). RNAi has been observed in many organisms including plants, fungi, nematodes, insects and mammals and is thought to have evolved in order to protect the genome from undesired foreign DNA sequences such as viruses and transposons. The system is also involved in tightly regulated processes such as embryonic development (Elbashir *et al.* 2001; Milhavet *et al.* 2003).

A pivotal paper by Fire *et al.* (1998) demonstrated that injection of small amounts of dsRNA into mice produced potent, specific and inheritable interference of gene function which was greater than that achieved by the presence of single strands of sense or antisense RNA (Fire *et al.* 1998). Tuschl *et al.* (1999) later replicated these effects in an *in vitro* system, thus providing the basis for a key tool in the study of RNAi. It was noted that dsRNA targeted to intronic sequences were ineffective and as such the system was postulated to inhibit protein production at the post transcriptional level (Tuschl *et al.* 1999). The discovery of the RNA-induced silencing complex (RISC) by Hammond *et al.* (2000) a year later was a crucial step in clarifying events underlying RNAi (Hammond *et al.* 2001). During RNAi, dsRNA is processed into 21-23 nucleotide fragments called siRNAs which bind to their complementary mRNA sequence. This triggers the formation of RISC and the recruitment of RNases that mediate the cleavage of target mRNA at 21-23 nucleotide intervals (Zamore *et al.* 2000; Hammond *et al.* 2001). Several key components involved in RNAi have since been identified including Dicer, an RNase III family nuclease responsible for processing dsRNA into siRNAs (Bernstein *et al.* 2001), and Argonaute2 which guides RNAs to RISC (Hammond *et al.* 2001). It is now known that RISC is a ribonucleoproteic complex with an Argonaute family member at its core that binds small RNA and facilitates target recognition (Pratt and MacRae 2009). RISC also contains RNases responsible for degradation of the targeted mRNA (Milhavet *et al.* 2003). An overview of events is shown in Figure 28.

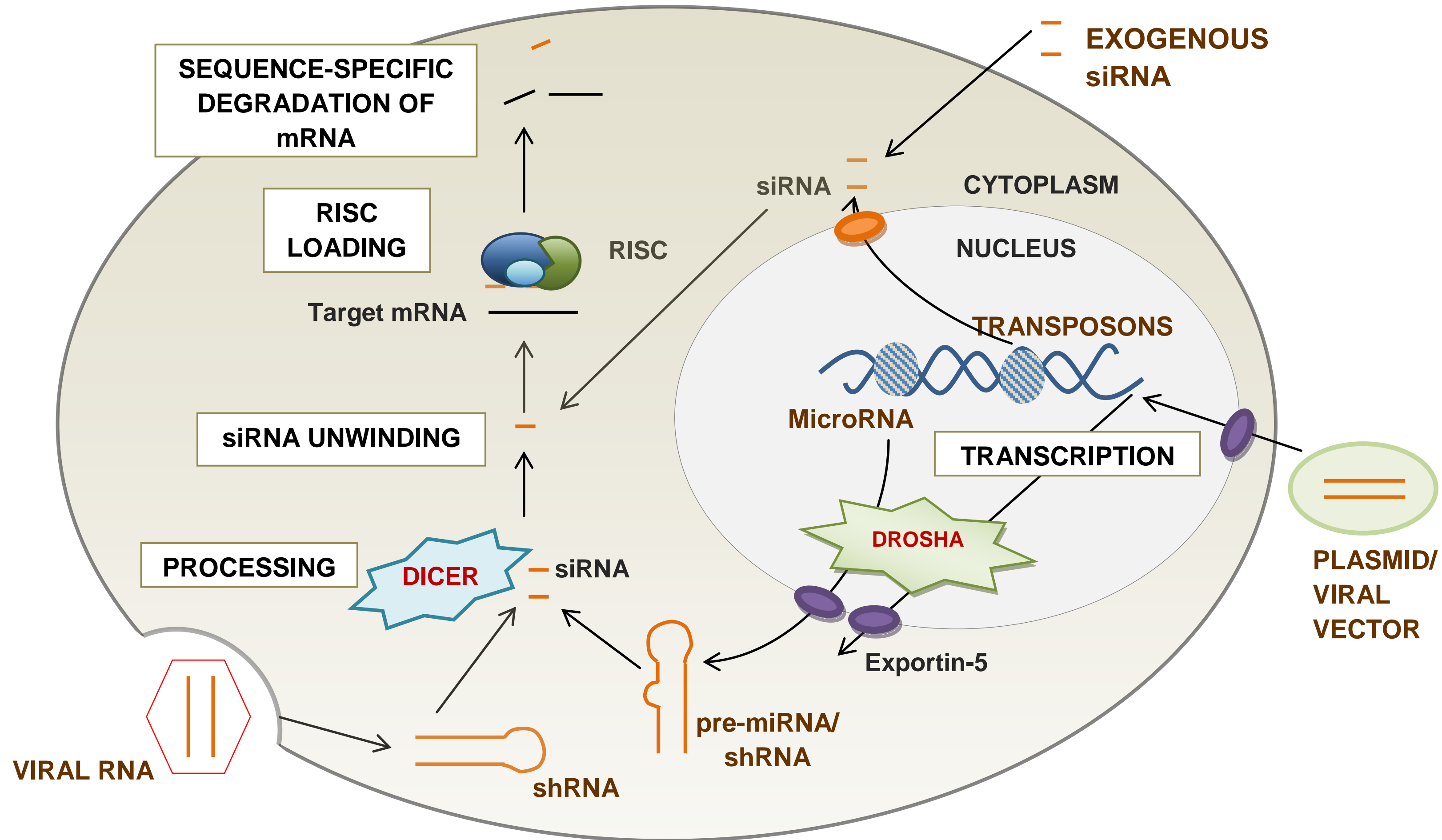


Figure 28. RNAi processing pathways

During RNAi four main steps take place: the formation of RISC, activation of RISC, binding to the target and subsequent cleavage. RNAi is instigated by the presence of dsRNA within the cell from endogenous or exogenous sources such as transposons and microRNA or virus and plasmid vectors respectively. Pre-miRNA and pre-shRNA are transcribed and transported to the cytoplasm through Exportin-5. The RNase III enzymes Dicer and Drosha cleave the dsRNA into 21-23 nucleotide fragments. These small dsRNA fragments function as a guide for selective recognition and degradation of the target mRNA by RISC.

RNAi technology is routinely used in many laboratories to knock down the expression of specific genes and represents a useful tool for reverse genetics. There are 2 main routes used for the introduction of dsRNA into the cell:

1) **siRNA**: dsRNAs of 20-23 nucleotides in length can be purchased commercially to assess the effects of knocking down the expression of genes of interest (Milhavet *et al.* 2003). Liposomes are also commonly employed to achieve short-term suppression, in addition to electroporation and microinjection (Milhavet *et al.* 2003).

2) **shRNA**: DNA-vector based technology utilises intrinsic RNA Polymerase III activity within the cell to synthesise shRNAs *in vivo* or *in vitro* from a DNA template (Sui *et al.* 2002; Milhavet *et al.* 2003). Lentivirus, herpes simplex virus, adenovirus, adeno-associated virus and retrovirus vectors are frequently used (Smith and Romero 1999). The shRNA vector is transported to the nucleus and processed through similar mechanisms as seen during miRNA processing. The primary transcript termed pre-shRNA contains a hairpin like stem-loop structure which is processed by RNase III enzyme Drosha within the nucleus (Rao *et al.* 2009) and then transported into the cytoplasm through Exportin-5 (Yi *et al.* 2003). Within the cytoplasm, Dicer activity results in the removal of the hairpin loop and the molecule is thought to thereafter follow the same processing pathway as siRNA (Rao *et al.* 2009).

Although siRNA and shRNA have similar effects within the cell, the molecules differ and are subjected to different RNAi processing routes (Rao *et al.* 2009). As a result siRNA-mediated suppression is typically a transient process, with normal gene expression recovering after 96-120 hours or 3-5 cycle of cell division. In contrast, gene suppression achieved by virally-delivered shRNA lasts longer and is lost after approximately 21 days potentially due to the host's immune response (Smith and Romero 1999). Furthermore, vector-based methods have a greater transfection efficiency in certain cell lines, especially post-mitotic and primary-derived cells (Milhavet *et al.* 2003).

A major advantage of the siRNA system is the ease of synthesis which allows fast production of numerous siRNAs. Furthermore, the technique has been widely used by

numerous studies and holds great therapeutic potential. Different methods of transfection have been shown to have varying degrees of success in different cell types and it is therefore important to optimise transfection techniques depending on cell culture conditions, the transfection reagents chosen, amount of dsRNA used and the length of exposure time to dsRNA (Milhavet *et al.* 2003). A combination of siRNA and shRNA was used for the studies outlined below.

4.2. Experimental aims

The previous chapter demonstrated that stimulation by IL-33 modulates the mRNA expression of the pro-atherosclerotic genes ICAM-1 and MCP-1 (several studies investigating their role in atherosclerosis are summarised in Tables 15 and 16). The strongest response was observed after 12 hours of IL-33 (25ng/ml) stimulation in three separate cellular systems; human THP-1 macrophages and HMDMs and also within the murine RAW264.7 macrophage cell line. Previous studies have investigated the activation of genes and proteins by IL-33. However analysis of the signalling pathways underlying IL-33-mediated regulation of ICAM-1 and MCP-1 in THP-1 macrophages and primary HMDMs has not been undertaken.

The principal aim of this chapter was to discern the signalling pathways used by IL-33 to regulate ICAM-1 and MCP-1 expression. The signalling components; ERK1, ERK2, p38α, JNK1, JNK2, PI3K-γ, p50 and p65 were chosen due to the well-established role of these proteins in atherosclerotic processes. In order to determine which signalling cascades were involved in this response, a combination of siRNA and shRNA was utilised to knock down the expression of signalling components and to assess the effect on IL-33 signalling using the well-established THP-1 cell line. Several of these shRNAs were then used in primary HMDMs to validate the response. RNAi is a frequently used technique and can be employed to explore functional studies within different cell lines (Kamath *et al.* 2003; Scherr and Eder 2004; Salter *et al.* 2011). Our group has previously optimised conditions for siRNA and shRNA-mediated knock down for the purpose of analysing signalling pathways. Cells were transfected with siRNA against scramble sequences or infected with viruses encoding scramble sequences to allow for direct comparison with cells transfected/infected with gene-specific siRNA or shRNA respectively. Additional cells were treated with these

scramble sequences and stimulated by IL-33 to enable comparison with cells subjected to gene-specific silencing and IL-33 stimulation.

Table 15. Role of ICAM-1 in atherosclerosis and related pathologies

Gene	Role in Atherosclerosis	Reference
ICAM-1	Treatment with atorvastatin reduced levels of sICAM-1 in patients at risk of cardiovascular disease.	(Blanco-Colio *et al.* 2007)
	ICAM-1 was expressed on the endothelium and functioned as a ligand for β_2 integrins such as lymphocyte function-associated antigen 1 and macrophage-1 antigen.	(Kadono *et al.* 2002)
	Ablation of ICAM-1 significantly reduced lesion size within $ApoE^{-/-}$ mice.	(Kitagawa *et al.* 2002)
	A polymorphism in the ICAM-1 gene, K469E, caused greater expression of the gene and was associated with increased incidences of cardiovascular disease. The genotype was significantly higher in sufferers of atherosclerosis.	(Motawi *et al.* 2012)
	ICAM-1 was strongly expressed in regions of the lesion site associated with irregular blood flow.	(Nakashima *et al.* 1998)
	Immunohistochemical staining revealed increased expression of ICAM-1 within all subtypes of the plaque with the exception of the fibrous cap. Furthermore, ICAM-1 was expressed by a number of plaque-resident cells including endothelial cells, macrophages and SMCs.	(Poston *et al.* 1992)
	sICAM-1 is a well-established marker of atherosclerosis and increased concentrations are linked to MI risk. Levels of the protein are increased in relation to disease progression in $ApoE^{-/-}$ mice.	(Ridker *et al.* 1998)

Table 16. Role of MCP-1 in atherosclerosis and related pathologies

Gene	Role in Atherosclerosis	Reference
MCP-1	In $ApoE^{-/-}$ mice, overexpression of MCP-1 promoted atherosclerosis and was associated with increased numbers of macrophages and accumulation of oxidised lipids.	(Aiello *et al.* 1999)
	Treatment with atorvastatin reduced levels of sMCP-1 in patients at risk of cardiovascular disease.	(Blanco-Colio *et al.* 2007)
	mmLDL increased the secretion of MCP-1 in cultured endothelial and SMCs and subsequent chemotactic activity was primarily driven by MCP-1.	(Cushing *et al.* 1990)
	Knocking out the MCP-1 gene in $LDLR^{-/-}$ mice caused a large reduction in the size of atherosclerotic plaques.	(Gu *et al.* 1998)
	sMCP-1 is a recognised marker of cardiovascular disease. Mice deficient in MCP-1 were specifically unable to recruit circulating monocytes.	(Lucas and Greaves 2001)
	MCP-1 mRNA was present in atherosclerotic lesions but absent from the intima media of animals without the disease. MCP-1 mRNA was observed in macrophage foam cells of atherosclerotic rabbits but missing from alveolar macrophages. The presence of the MCP-1 protein correlated with macrophage-rich areas of human plaques.	(Ylaherttuala *et al.* 1991b)

The principal aims of this chapter were:

1) To determine whether IL-33 affected the mRNA levels of the signalling components tested.

2) To assess whether the siRNA and/or shRNA-mediated knock down was achieved at the mRNA and protein level through the use of RT-qPCR and western blot analysis.

3) To analyse the effects on IL-33-regulation of ICAM-1 and MCP-1 following siRNA/ shRNA-mediated knock down of key signalling components previously implicated in IL-33 signalling using RT-qPCR.

4) To validate the findings observed in THP-1 macrophages in primary cultures of HMDMs through a combination of RT-qPCR and western blotting.

A schematic of the experimental strategy employed to achieve these aims is provided in Figure 29.

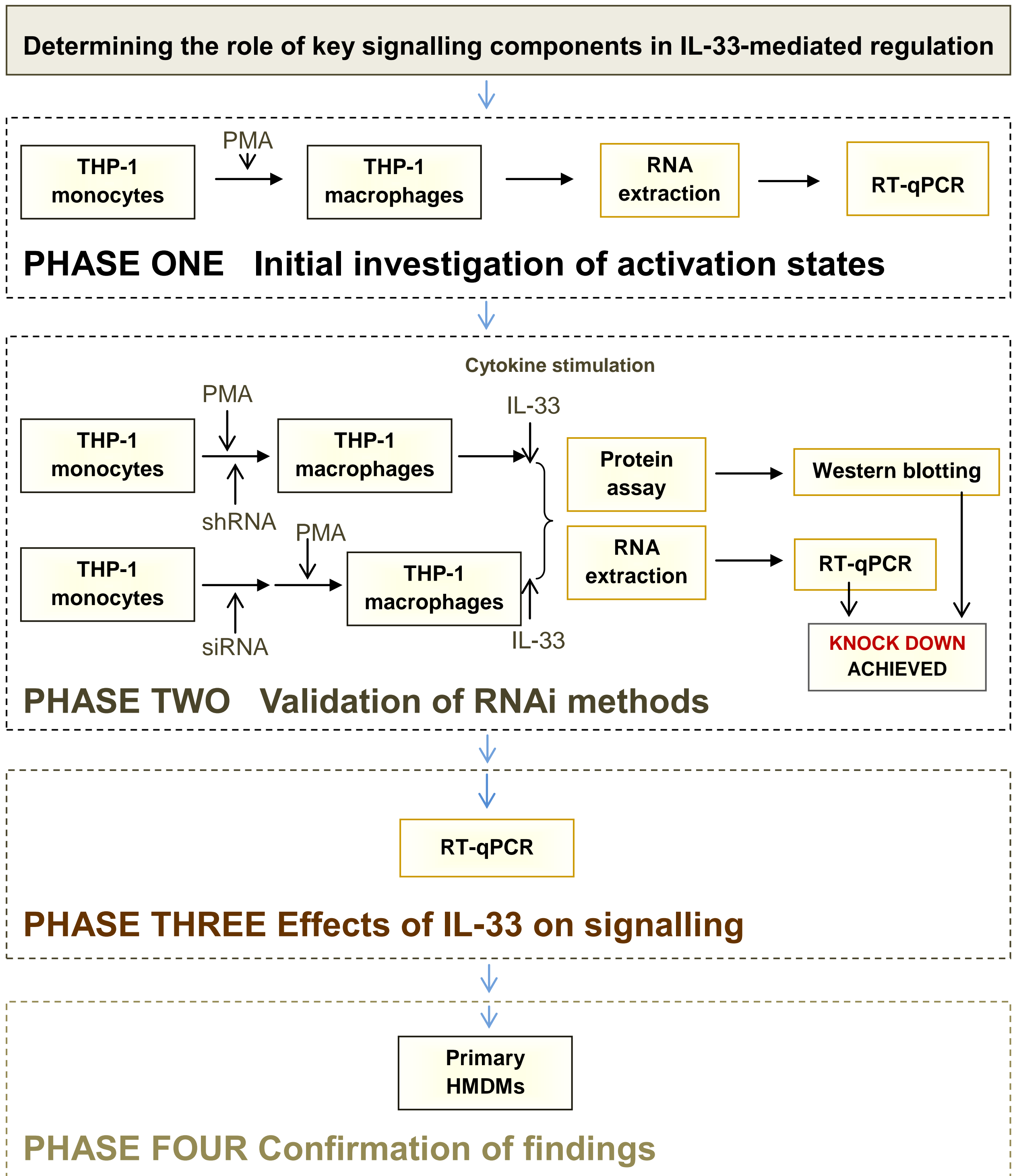


Figure 29. Schematic of experimental procedure

THP-1 cells were treated with shRNA or siRNA and the expression of targeted genes was then measured by RT-qPCR to determine whether knock down of gene expression had been achieved. Samples with confirmed knock down were assessed by RT-qPCR and western blotting to analyse the effects of reduced expression of key signalling components on IL-33-mediated regulation of ICAM-1 and MCP-1 expression. RNAi was then completed in HMDMs to assess whether the results were replicable in primary macrophages.

4.3. Results

4.3.1. The effect of IL-33 on the expression of key components of signal transduction pathways in THP-1 macrophages

Studies were performed to determine the effect of IL-33 stimulation on the mRNA expression of key genes involved in signal transduction. In accordance with the conditions optimised in the previous chapter, THP-1 cells were plated into 12-well plates and differentiated by PMA. The cells were then left untreated or were stimulated for 12 hours with IL-33 (25ng/ml). RT-qPCR was performed on the resulting cDNA using gene specific primers for the MAPKs ERK1, ERK2, p38α, JNK1 and JNK2; PI3K-γ; and the NF-κB subunits p50 and p65.

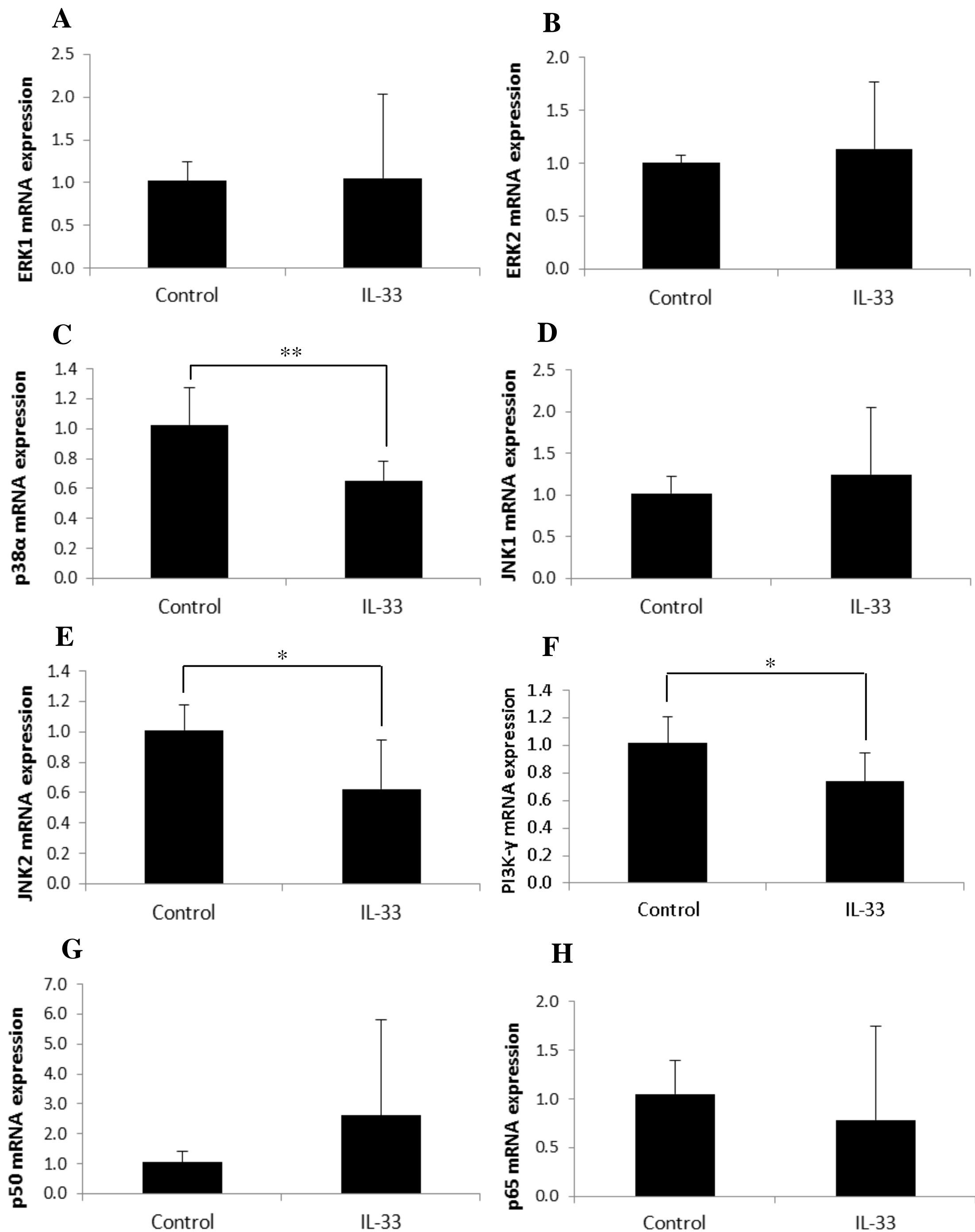


Figure 30. Action of IL-33 on the expression of key components of signal transduction pathways in THP-1 macrophages

RT-qPCR was performed using cDNA from PMA differentiated THP-1 macrophages incubated in the presence or absence (Control) of IL-33 (25ng/ml) for 12 hours. Gene specific primers for: Panel A= ERK1, B= ERK2, C= p38α, D= JNK1, E= JNK2, F= PI3K-γ, G= p50 and H= p65 were used. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples given an arbitrary value of 1. Data represents mean ± SD from four independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05, **p<0.01.

IL-33 stimulation did not have a significant effect on the mRNA expression of ERK1, ERK2 and JNK1 or the NF-κB subunits p50 and p65. However, IL-33 did cause a slight but significant reduction in the mRNA expression of the MAPKs p38α and JNK2 in addition to PI3K-γ (Figure 30).

4.3.2. The effects of IL-33 in ERK1/2 activation

The effect of IL-33 on levels of ERK1 and ERK2 protein was next analysed. Studies have shown that ERK pathways regulate the proliferation and differentiation of SMCs in the lesion (Muslin 2008) and inhibition of ERK1/2 leads to increased cholesterol efflux from macrophage-derived foam cells (Zhou *et al.* 2010). Two isoforms of ERK; ERK1 and ERK2 are ubiquitously expressed in all mammalian cells (Hu and Ivashkiv 2009). The pathway can be activated by various stimuli resulting in the phosphorylation and activation of ERK1/2 (Zhou *et al.* 2010). Phosphorylation of ERK1/2 induces translocation of the kinase from the cytoplasm into the nucleus, where the proteins activate target proteins or trigger alternative signalling cascades (Costa *et al.* 2006).

An experiment under conditions previously used by our group (i.e. 10ng/ml IL-33 for 24 hours) was performed to investigate the mechanisms underlying IL-33 signalling and was performed with participation from another laboratory member, Timothy Ashlin, to aid studies towards his thesis. The experiment also served to validate the western blotting system previously optimised by our laboratory (McLaren *et al.* 2010b). Western blots were performed for total and phospho-ERK1/2 to determine whether IL-33 activated these proteins. Cellular lysates were taken from THP-1 macrophages incubated in the presence or absence of IL-33 (10ng/ml) and analysed by SDS-PAGE. The samples were then incubated with phospho-ERK1/2 and total ERK1/2 antibodies before membrane blotting. The membranes were exposed to X-ray film and densitometric analysis was completed using SynGene computer software.

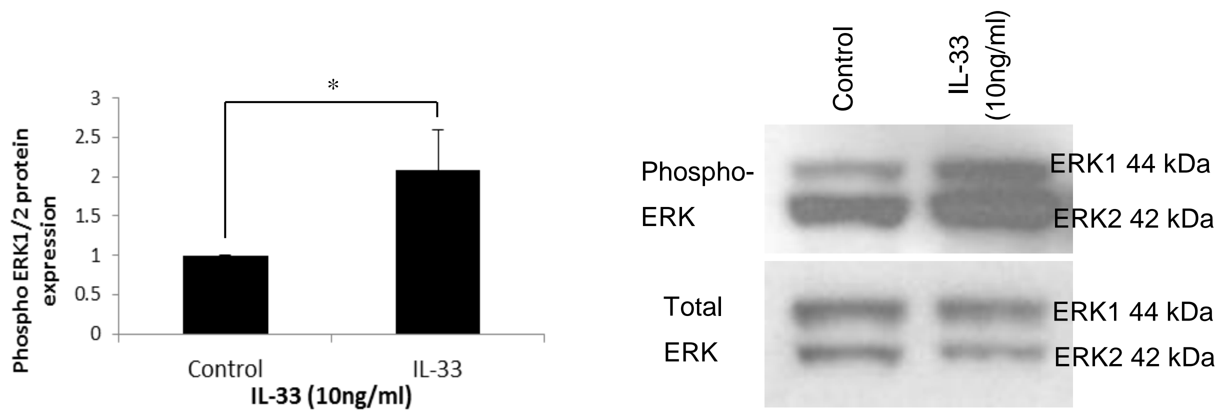


Figure 31. The effect of IL-33 on ERK1/2 phosphorylation in THP-1 macrophages

Western blotting was performed on samples from differentiated THP-1 macrophages that were left untreated (control) or stimulated with IL-33 (10ng/ml) for 24 hours. Equal amounts of protein from the samples were analysed using phospho ERK1/2 and total ERK1/2 antibodies. The signals of the bands for phospho-ERK1/2 were normalised to that for total ERK1/2 with values from control samples given an arbitrary value of 1. Bands correspond with the molecular weight of both proteins. Semi-quantitative analysis was performed using the Gene Tools software. Data represents mean ± SD from three independent experiments. The Student *t* test was used to determine the statistical significance of the results *$p<0.05$.

The data showed that stimulation of THP-1s with IL-33 (10ng/ml) for 24 hours significantly increased the phosphorylation of ERK1/2 compared to the control (Figure 31) indicating that the active phosphorylated forms are involved in IL-33 signalling. On the other hand, the total ERK1/2 levels were unchanged by IL-33 stimulation.

4.3.3. The role of key signalling components in the IL-33-mediated regulation of ICAM-1 and MCP-1 expression in THP-1 macrophages

Experiments were performed to determine the extent of knock down achieved through the use of siRNA and/or shRNA targeting key signalling components implicated in atherosclerosis. The use of siRNA (Salter *et al.* 2011; Horiguchi *et al.* 2012; Michael *et al.* 2012b; Volante *et al.* 2012) and shRNA (Li *et al.* 2010; Wang *et al.* 2011; Michael *et al.* 2012b) has been extensively applied by research groups for mechanistic and functional studies (Kamath *et al.* 2003; Scherr and Eder 2004). To accommodate

potential changes in gene expression induced by the presence/ method of delivery of shRNA or siRNA which could bias results, shRNA and siRNA targeted towards a scramble sequence was also included as a control for each experiment.

4.3.3.1. Effects of ERK1/2 knock down

Due to the well-established role of the MAPKs ERK1 and ERK2 in atherosclerosis, RT-qPCR was conducted following RNAi-mediated knock down of these genes to validate the effectiveness of the siRNA and shRNA used. THP-1 cells were transfected with siRNA or infected with shRNA targeted towards ERK1 or ERK2 and then stimulated with IL-33 (25ng/ml) for 12 hours before RT-qPCR was performed to validate the level of gene silencing.

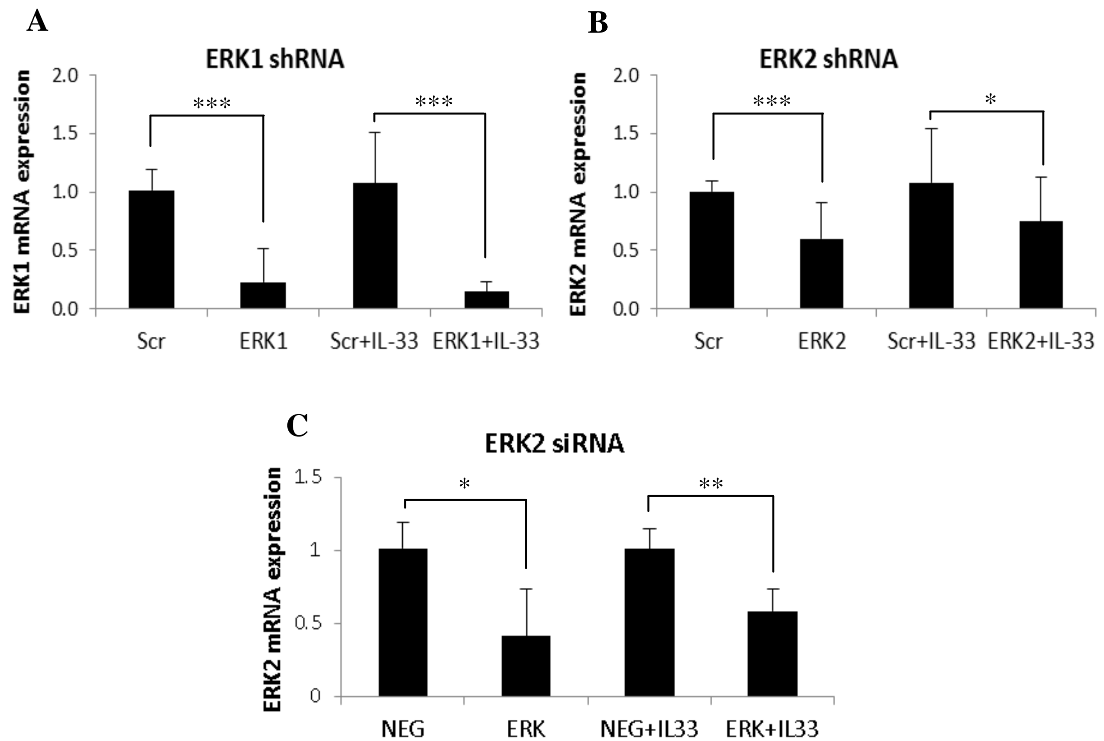


Figure 32. shRNA/siRNA-mediated knock down of ERK1/2 gene expression in THP-1 macrophages

RT-qPCR was performed using cDNA from PMA differentiated THP-1 macrophages treated with ERK1 or ERK2 shRNA or ERK2 siRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control for shRNA experiments (Scr), whilst siRNA targeting a scramble sequence was used as a control for siRNA experiments (NEG). Gene-specific primers for: Panel A= ERK1, B= ERK2 and C= ERK2 were used. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr and Neg) given an arbitrary value of 1. Data represents mean ± SD from five (A), six (B) or three (C) independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05, **p<0.01, ***p<0.001.

The use of ERK1 and ERK2 shRNA and ERK2 siRNA produced a significant reduction in mRNA levels of the targeted genes in the presence and absence of IL-33 (Figure 32). Incubation with ERK1 shRNA achieved an average decrease in the corresponding mRNA levels of 78% and 93% in the absence and presence of IL-33 respectively. There was a 40% and 33% reduction in nonstimulated and stimulated

cells respectively mediated by ERK2 shRNA and a decrease of 52% and 42% was attained by ERK2 siRNA in untreated and IL-33 treated cells respectively.

Western blot anaysis was then performed to assess whether the use of RNAi also affected protein expression. Prior to IL-33 addition, THP-1 cells were infected with shRNA or transfected with siRNA targeted towards ERK1 or ERK2. Cellular lysates were then size-fractionated by SDS-PAGE for analysis by western blotting.

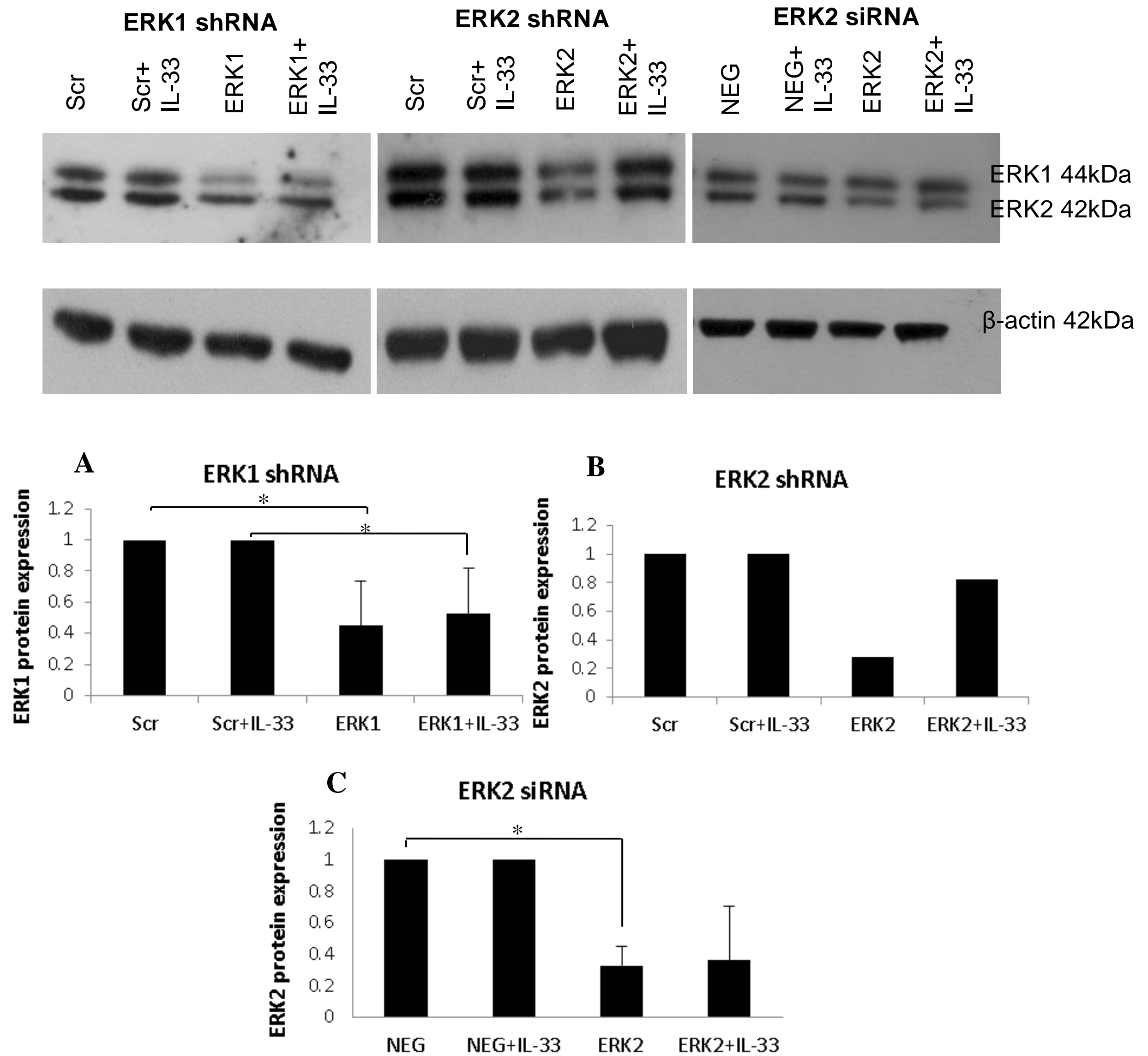


Figure 33. RNAi-mediated knock down of ERK1/2 protein levels in THP-1 cells

Western blot analysis was performed using cellular lysates from PMA-differentiated THP-1 macrophages treated with ERK1 or ERK2 shRNA, or ERK2 siRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control for shRNA experiments (Scr), whilst siRNA targeting a scramble sequence was used as a control for siRNA experiments (NEG). Equal amounts of protein from the samples were analysed using total ERK1/2 and β-actin antibodies. The signals for ERK 1/2 were normalised to that for β-actin with values from control samples (unstimulated and stimulated Scr and NEG) given an arbitrary value of 1. Bands correspond with the molecular weight of both proteins. Panel A= ERK1 shRNA, B= ERK2 shRNA and C= ERK2 siRNA. Semi-quantitative analysis was performed using the Gene Tools software. Data represents mean ± SD from four (A), two (B) and three (C) independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05.

Figure 33 shows that the use of ERK1 shRNA significantly decreased protein levels of ERK1 by 55% and 47% in untreated cells or those stimulated with IL-33 respectively. Because of time restraints that restricted the number of repeats, it was not possible to carry out statistical analysis for ERK2 shRNA. However, a trend of reduced ERK2 protein levels was observed (72% and 17% reduction in untreated cells and those stimulated with IL-33 respectively). The use of ERK2 siRNA resulted in a significant decrease of 67% in untreated cells and a reduction of 64% was achieved in IL-33 treated cells that failed to reach significance.

RT-qPCR was performed on the two ERK isoforms to determine whether these reductions were specific to the targeted protein and did not produce off-target effects.

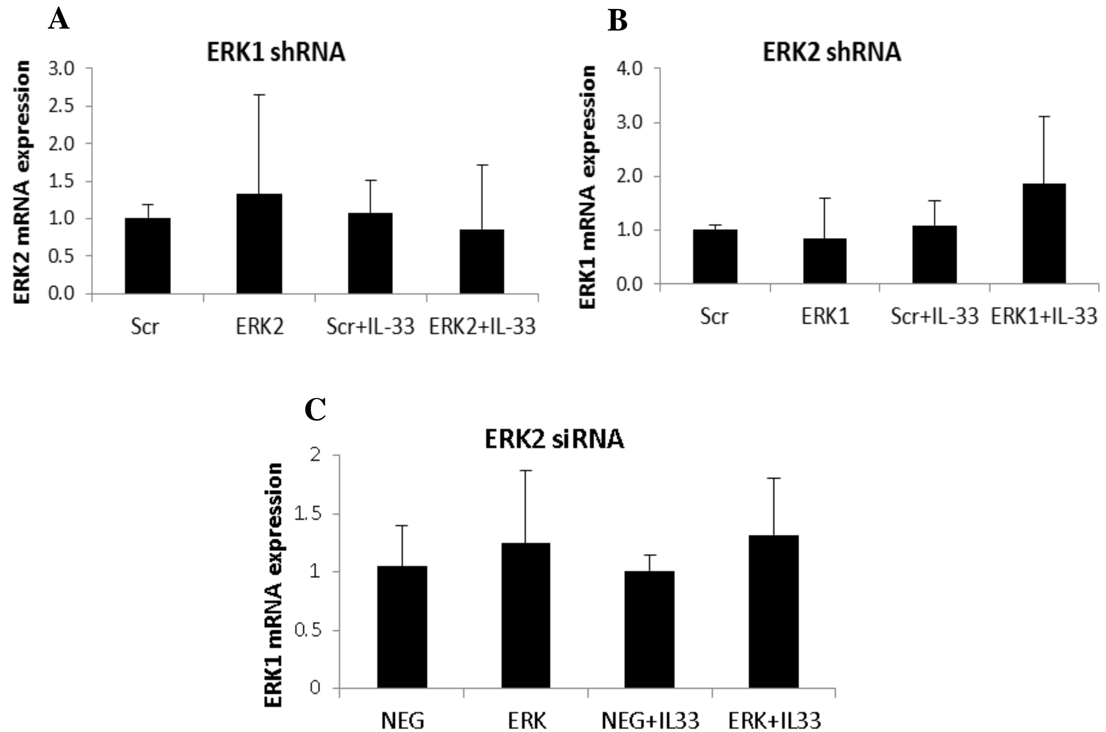


Figure 34. Specificity of shRNA/siRNA-mediated knock down of ERK1/2 gene expression in THP-1 macrophages

RT-qPCR was performed using cDNA from differentiated THP-1 macrophages treated with ERK1 or ERK2 shRNA, or ERK2 siRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours as described for Figure 32. Cells expressing shRNA targeting a scramble sequence were used as a control for shRNA experiments (Scr), whilst siRNA targeting a scramble sequence was used as a control for siRNA experiments (NEG). Gene-specific primers for: Panel A= ERK1, B= ERK2 and C= ERK2 were used. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr and NEG) given an arbitrary value of 1. Data represents mean ± SD from five (A), six (B) or three (C) independent experiments. The Student *t* test was used to determine the statistical significance of the results.

The data in Figure 34 demonstrates that the shRNA and siRNA used in the study specifically knocked down the expression of the targeted gene without causing a significant reduction in the other isoforms tested. The mRNA levels of these alternative isoforms was comparable to the levels seen in the control cells.

Western blot analysis using specific antibodies for alternative isoforms was then performed to determine whether RNAi was isoform specific at the protein level.

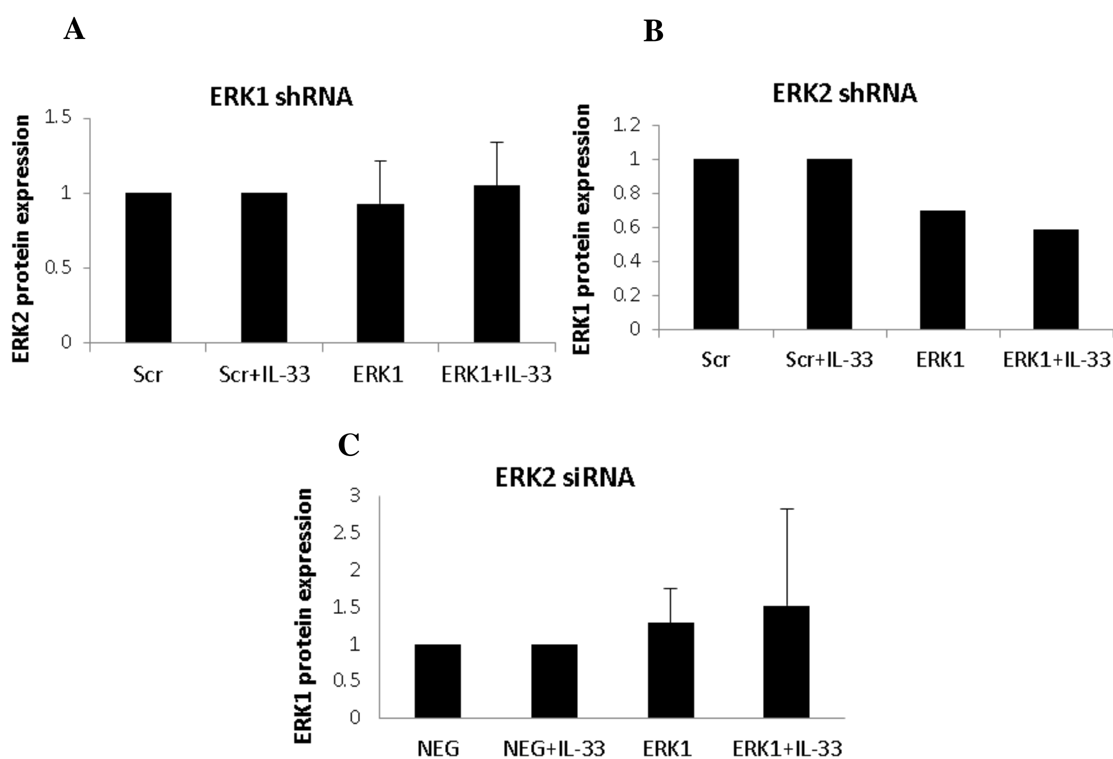


Figure 35. Specificity of shRNA/siRNA-mediated knock down of ERK1/2 proteins in THP-1 macrophages

Western blot analysis was performed using cellular lysates from PMA-differentiated THP-1 macrophages treated with ERK1 or ERK2 shRNA, or ERK2 siRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control for shRNA experiments (Scr), whilst siRNA targeting a scramble sequence was used as a control for siRNA experiments (NEG). Equal amounts of protein from the samples were analysed using total ERK1/2 and β-actin antibodies. The signals for total ERK 1/2 was normalised to that for β-actin with values from control samples (unstimulated and stimulated Scr and NEG) given an arbitrary value of 1. Bands correspond with the molecular weight of both proteins. Panel A= ERK1 shRNA, B= ERK2 shRNA and C= ERK2 siRNA. Semi-quantitative analysis was performed using the Gene Tools software. Data represents mean ± SD from four (A), two (B) and three (C) independent experiments.

The data of Figure 35 combined with Figure 34 demonstrates specificity and shows that both methods of RNAi produced significant levels of gene silencing of the

targeted isoform. The knock down achieved through the use of shRNA was greater than with siRNA. Following the validation of these shRNAs and siRNAs, they were then used to assess the effects of reduced levels of ERK1 and ERK2 on IL-33-mediated regulation of ICAM-1 and MCP-1 mRNA expression.

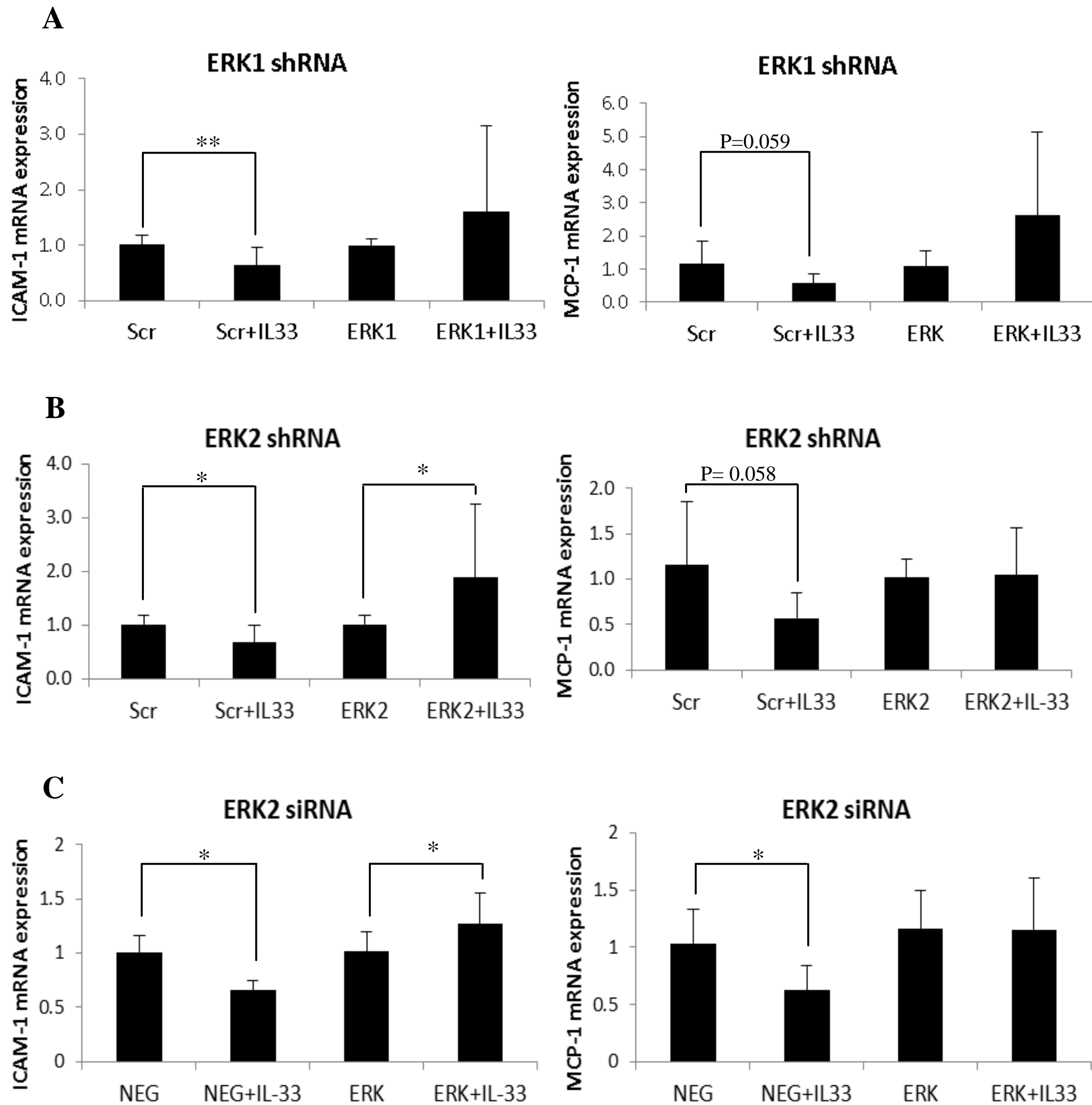


Figure 36. ERK-1 and -2 are involved in the IL-33-mediated regulation of ICAM-1 and MCP-1 expression in THP-1 macrophages

RT-qPCR was performed using cDNA from differentiated THP-1 macrophages treated with ERK1 or ERK2 shRNA, or ERK2 siRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control for shRNA experiments (Scr), whilst siRNA targeting a scramble sequence was used as a control for siRNA experiments (NEG). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr and NEG) given an arbitrary value of 1. Panel A= ERK1 shRNA, B= ERK2 shRNA and C= ERK2 siRNA. Data represents mean ± SD of five (A), six (B) and three (C) independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05, **p<0.01.

In accordance with the findings from chapter 1, IL-33 reduced the expression of ICAM-1 and MCP-1 mRNA in cells expressing scramble sequence (Figure 36). However the knock down of ERK-1 and -2 attenuated the IL-33-mediated down-regulation of mRNA expression for both genes. Figure 36 shows a significant induction of ICAM-1 mRNA expression occurs following ERK2 shRNA infection and ERK2 siRNA transfection in the presence of IL-33. A similar trend in ICAM-1 expression is observed following the addition of ERK1 shRNA and IL-33, although not significant.

4.3.3.2. Effects of p38α knock down

There are four mammalian p38 isoforms: α, β, γ and δ, of which p38α and p38β are ubiquitously expressed. Most studies and experimental data focuses on p38α which is a key regulator of inflammation and is commonly referred to as p38 MAPK (Risco and Cuenda 2012). The p38α isoform is of particular relevance to this study as it is activated *in vivo* during atherosclerotic development (Seimon *et al.* 2009) and silencing of the gene reduces the ratio of CEs to free cholesterol in THP-1 cells (Mei *et al.* 2012).

THP-1 cells were infected with shRNA for p38α and then stimulated with IL-33 (25ng/ml) for 12 hours before RT-qPCR analysis was used to assess the extent of knock down in mRNA levels for p38α.

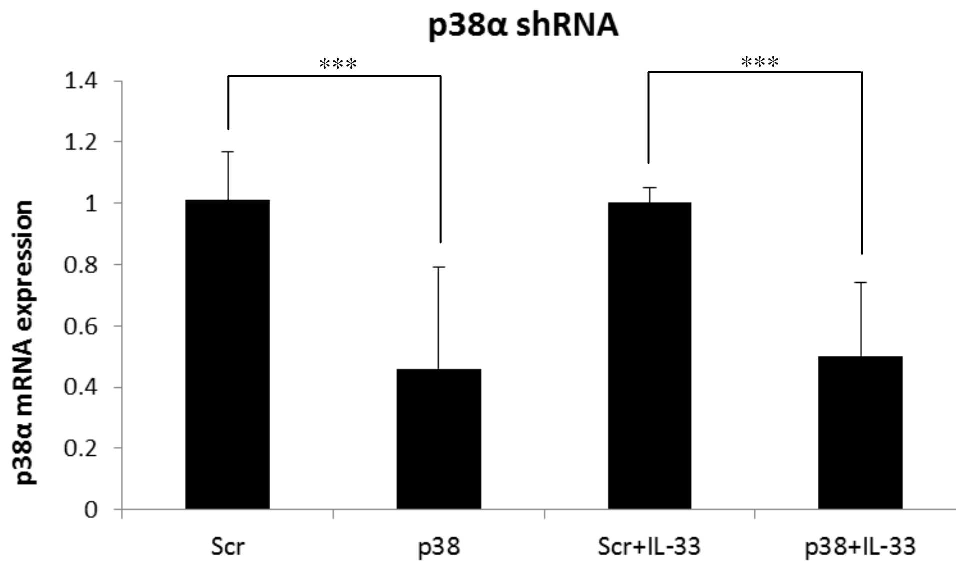


Figure 37. shRNA-mediated knock down of p38α gene expression in THP-1 macrophages

RT-qPCR was performed using cDNA from differentiated THP-1 macrophages infected with p38α shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr) given an arbitrary value of 1. Data represents mean ± SD from six independent experiments. The Student *t* test was used to determine the statistical significance of the results ***$p<0.001$.

Figure 37 shows that infection with shRNA resulted in a significant 55% and 50% reduction in the average mRNA levels of p38α in the absence and presence of IL-33 respectively.

To further discern the effects of p38α shRNA, western blot analysis was conducted on cellular lysates to determine the potential reduction in total p38 protein levels.

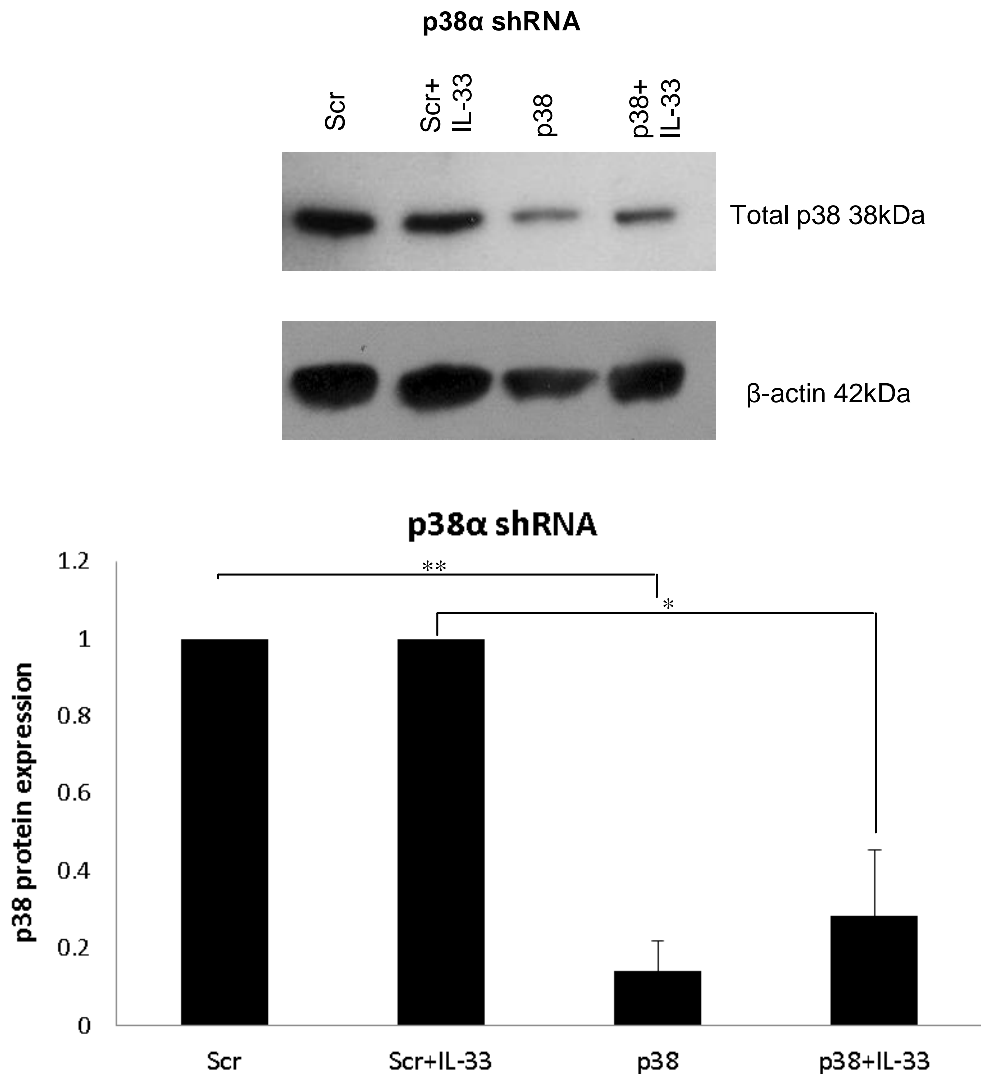


Figure 38. shRNA-mediated knock down of p38α protein in THP-1 macrophages

Western blot analysis was performed using cellular lysates from differentiated THP-1 macrophages infected with p38α shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). Equal amounts of protein from the samples were analysed using total p38 and β-actin antibodies. The signals for total p38 were normalised to that for β-actin with values from control samples (unstimulated and stimulated Scr) given an arbitrary value of 1. Bands correspond with the molecular weight of both proteins. Semi-quantitative analysis was performed using the Gene Tools software. Data represents mean ± SD from three independent experiments. The Student *t* test was used to determine the statistical significance of the results *$p<0.05$, **$p<0.01$.

Western blot analysis demonstrated that expression of p38α shRNA resulted in a significant decrease of 86% (Unstimulated) and 72% (IL-33 stimulated) in total p38 protein (Figure 38).

Thus, the use of p38α shRNA produced a significant reduction in p38α mRNA and total p38 protein levels. Therefore the shRNA was used to determine the involvement of p38α in IL-33 regulation of ICAM-1 and MCP-1 gene expression.

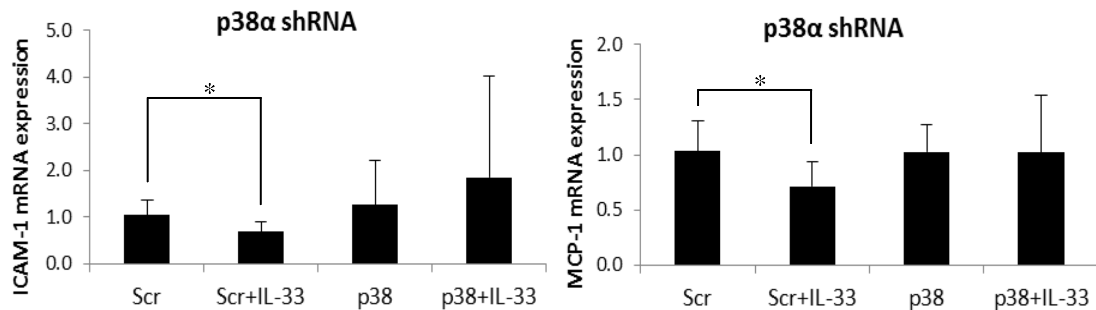


Figure 39. p38α is involved in the IL-33-mediated regulation of ICAM-1 and MCP-1 expression in THP-1 macrophages

RT-qPCR was performed using cDNA from differentiated THP-1 macrophages infected with p38α shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr) given an arbitrary value of 1. Data represents the mean ± SD of six independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05.

Figure 39 shows that p38α was involved in IL-33-mediated reduction of ICAM-1 and MCP-1 mRNA expression. In cells expressing the scramble sequence, IL-33 produced a significant decrease in mRNA levels for both of these genes. However, the presence of p38α shRNA negated this response.

4.3.3.3. Effects of JNK1/2 knock down

The role of JNK1/2 within IL-33 signalling was next explored due to the well established involvement of these MAPKs in atherosclerosis (Yoshimura *et al.* 2005;

Hui 2007). The presence of LDL and OxLDL induces the activation of JNK-1 and -2 within atherosclerotic plaques (Metzler *et al.* 2000). Also inhibition of JNK reduces the size of the atherosclerotic plaque and JNK2 may have an important role in facilitating the uptake of lipoproteins and, as a result, promote foam cell production (Ricci *et al.* 2004).

To validate the use of JNK1 and JNK2 siRNA, the siRNAs were co-transfected into differentiated THP-1 cells to achieve a decrease in the expression of both JNK isoforms prior to stimulation by IL-33. RT-qPCR was then performed to assess the level of reduction at the mRNA level.

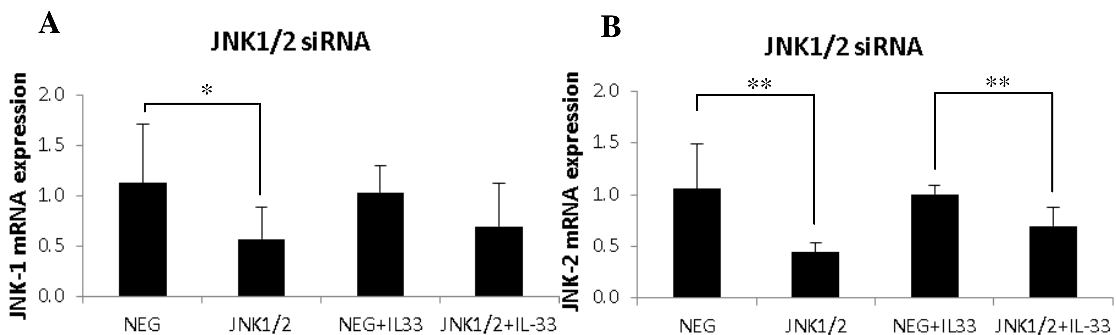


Figure 40. siRNA-mediated knock down of JNK1/2 gene expression in THP-1 macrophages

RT-qPCR was performed using cDNA from differentiated THP-1 macrophages co-transfected with JNK1 and JNK2 siRNAs and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Samples transfected with siRNA targeting a scramble sequence were used as a control (NEG). Gene specific primers for JNK1 (Panel A) and JNK2 (B) were used. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated NEG) given an arbitrary value of 1. Data represents mean ± SD from five independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05, **p<0.01.

Co-transfection of siRNAs targeting JNK1 and JNK2 produced a significant reduction of 71% in the mRNA levels of JNK1 in the absence of IL-33 (Figure 37). A marked reduction in JNK1 mRNA was also present in THP-1 cells stimulated by IL-33 (34%) though this failed to reach significance. JNK2 mRNA was significantly decreased by

siRNA co-transfection by 75% and 31% in cells untreated and treated by IL-33 respectively.

To confirm whether knock down was also present at the protein level, western blot analysis was performed on cellular lysates from siRNA transfected THP-1 macrophages.

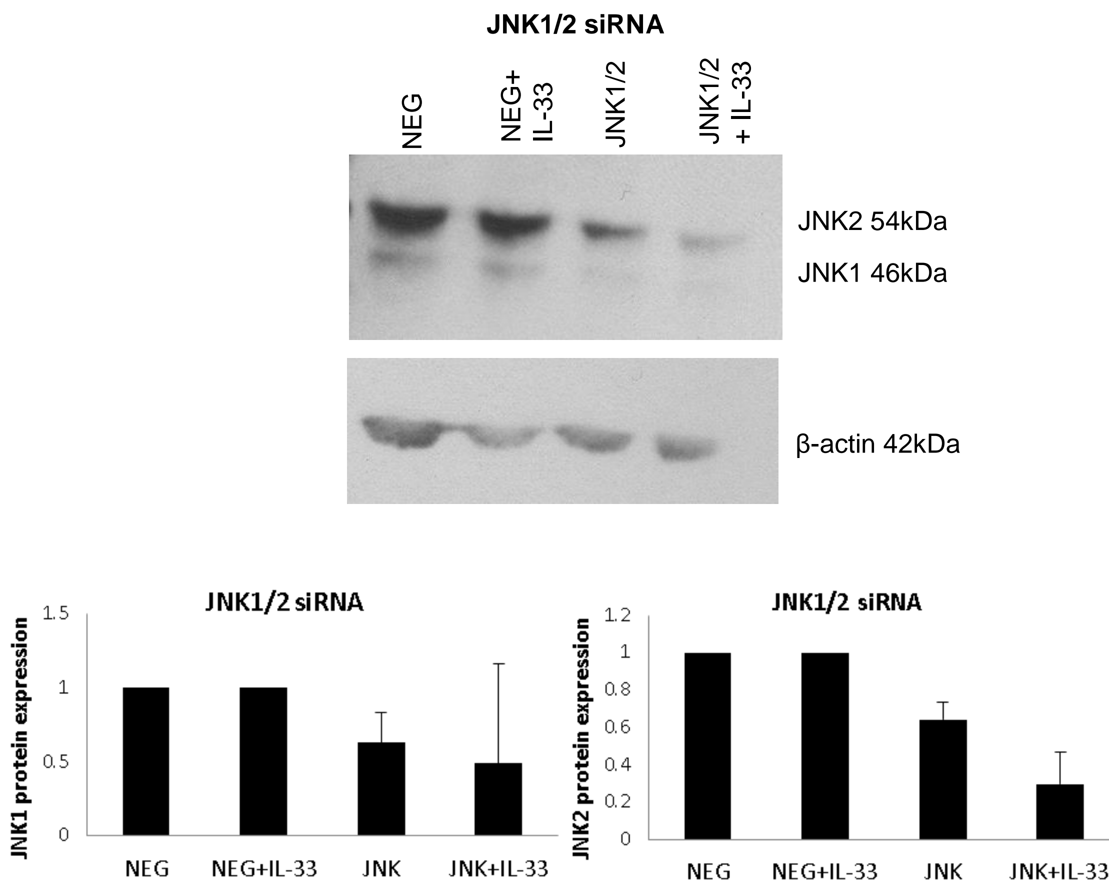


Figure 41. siRNA-mediated knock down of JNK1/2 proteins in THP-1 macrophages

Western blot analysis was performed using cellular lysates from differentiated THP-1 macrophages co-transfected with JNK1/2 siRNAs and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Samples transfected with siRNA targeting a scramble sequence were used as a control (NEG). Equal amounts of protein from the samples were analysed using total JNK and β-actin antibodies. The signals for total JNK was normalised to that for β-actin with values from control samples (unstimulated and stimulated NEG) given an arbitrary value of 1. Bands correspond with the molecular weight of both proteins. Semi-quantitative analysis was performed using the Gene Tools software. Data represents mean from two independent experiments.

Co-tranfection of JNK-1 and -2 siRNAs in THP-1 macrophages resulted in a decrease in the levels of JNK-1 and -2 proteins (Figure 41). An average decrease of 36% and 70% was seen in unstimulated and IL-33 stimulated macrophages, respectively. Following incubation with JNK1/2 siRNAs the levels of JNK2 protein were reduced by 37% in untreated cells and 51% in IL-33-treated cells.

The combination of JNK1 and JNK2 siRNAs were then used to investigate whether the MAPKs were involved in IL-33-mediated regulation of ICAM-1 and MCP-1 expression. Following co-transfection of JNK1/2 siRNAs, RT-qPCR was used to determine the effects on the mRNA levels of ICAM-1 and MCP-1 after IL-33 stimulation.

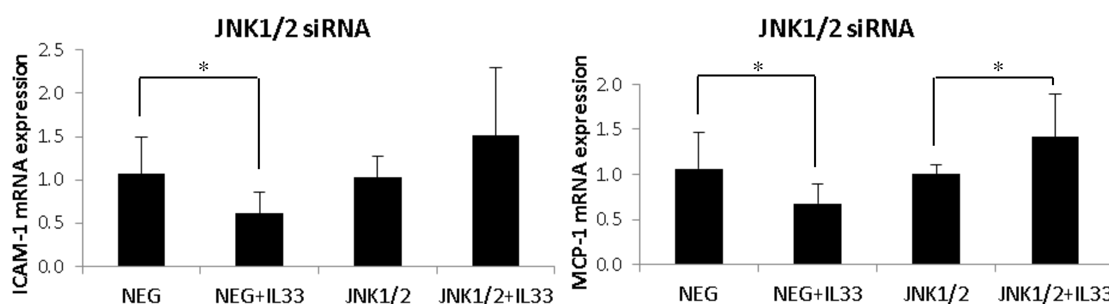


Figure 42. JNK1/2 are involved in IL-33-mediated regulation of ICAM-1 and MCP-1 expression in THP-1 macrophages

RT-qPCR was performed using cDNA from differentiated THP-1 macrophages co-transfected with JNK1 and JNK2 siRNAs and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Samples transfected with siRNA targeting a scramble sequence were used as a control for siRNA experiments (NEG). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated NEG) given an arbitrary value of 1. Data represents the mean ± SD of five independent experiments. The Student *t* test was used to determine the statistical significance of the results *$p<0.05$.

Figure 42 shows that the MAPKs JNK1/2 were involved in IL-33 signalling. Stimulation with IL-33 significantly decreased levels of ICAM-1 and MCP-1 mRNA in cells expressing the scramble sequence. However with reduced levels of JNK1/2 this response was lost. Furthermore the RNAi-mediated reduction of JNK1/2 resulted

in a significant induction in MCP-1 mRNA levels following IL-33 treatment. A similar trend occured for ICAM-1, although the induction in mRNA expression was not significant.

4.3.3.4. Effects of PI3K-γ knock down

In addition to the MAPK signalling cascades other signal transduction pathways are implicated in atherogenesis. The exact role of PI3K-γ, a PI3K family member, in atherosclerosis is not fully understood. The isoform is expressed by haematopoietic cells, endothelial cells and cardiomyocytes (Siragusa *et al.* 2010) and appears to participate in a number of pro-atherogenic processes (Chang *et al.* 2007). Levels of PI3K-γ are high within human and mouse atherosclerotic plaques and use of the PI3K-γ inhibitor AS605240 improves plaque stability and reduces the infiltration of macrophages and T cells (Fougerat *et al.* 2008). Also, mice deficient in PI3K-γ display impaired platelet aggregation (Hirsch *et al.* 2001).

Due to time constraints, the study focused on the PI3K isoform PI3K-γ due to its involvement in pro-atherosclerotic processes and sought to investigate the role of this kinase in IL-33 activity. To determine the extent of knock down achieved by RNAi, differentiated THP-1 cells were infected with adenovirus encoding PI3K-γ shRNA and RT-qPCR was performed.

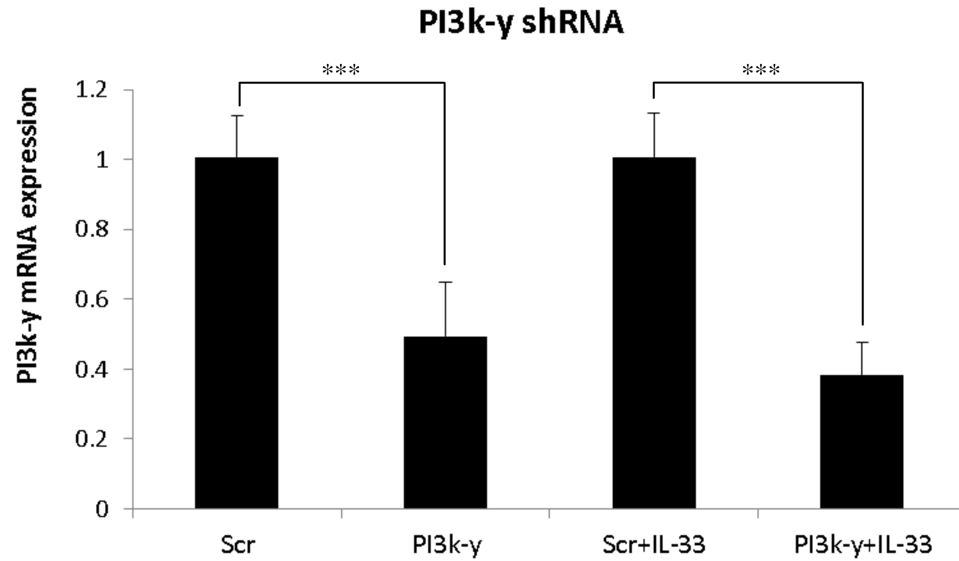


Figure 43. shRNA-mediated knock down of PI3K-γ gene expression in THP-1 macrophages

RT-qPCR was performed using cDNA from differentiated THP-1 macrophages infected with PI3K-γ shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control for shRNA (Scr). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr) given an arbitrary value of 1. Data represents mean ± SD from six independent experiments. The Student *t* test was used to determine the statistical significance of the results ***$p<0.001$.

Figure 43 shows that expression of PI3K-γ shRNA resulted in a significant reduction of PI3K-γ mRNA levels in untreated cells (48%) and IL-33 stimulated cells (62%).

Unfortunately an antibody for PI3K-γ was not available to validate the use of PI3K-γ shRNA at the protein level. However, as a significant reduction had been achieved at the mRNA level, PI3K-γ shRNA was used to assess whether the kinase was involved in IL-33 signalling. RT-qPCR using gene-specific primers for ICAM-1 and MCP-1 was performed on mRNA from THP-1 cells expressing PI3K-γ shRNA.

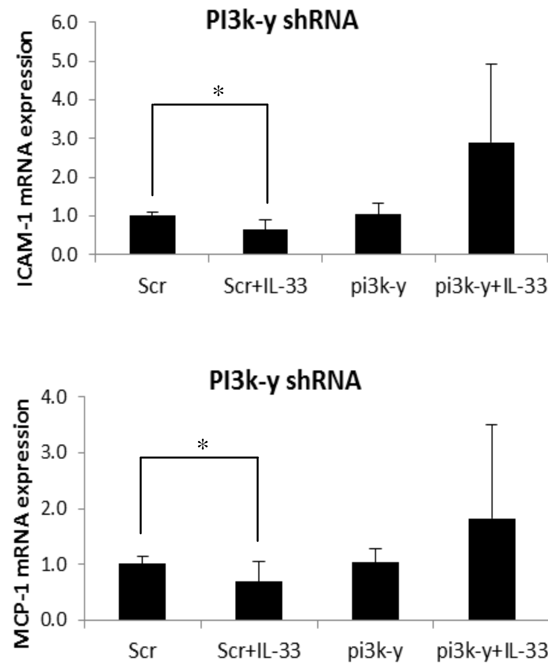


Figure 44. PI3K-γ is involved in IL-33-mediated regulation of ICAM-1 and MCP-1 expression in THP-1 macrophages

RT-qPCR was performed using cDNA from differentiated THP-1 macrophages infected with PI3K-γ shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr) given an arbitrary value of 1. Data represents the mean ± SD of three (ICAM-1) and five (MCP-1) independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05.

IL-33 stimulation significantly reduced mRNA levels of ICAM-1 and MCP-1 (Figure 44). However, following shRNA-mediated reduction of PI3K-γ, the decrease in ICAM-1 and MCP-1 mRNA expression by IL-33 was lost. Also, ICAM-1 and MCP-1 displayed a trend for increased mRNA expression following IL-33 stimulation in cells transfected with PI3K-γ shRNA.

4.3.3.5. Effects of p50/p65 knock down

The final signalling components investigated were the NF-κB members p50 and p65. This signalling cascade has been implicated in the disease as activated NF-κB is found localised in fibrotic-enriched intima/media and atheromateous regions of the plaque and colocalises with plaque-resident SMCs, macrophages and endothelial cells (Brand *et al.* 1996; Bourcier *et al.* 1997). Also, a number of genes implicated in atherosclerosis are regulated by NF-κB including ICAM-1, M-CSF, MCP-1 and TNF-α (Brand *et al.* 1997).

The involvement of NF-κB signalling components in the IL-33-mediated regulation of ICAM-1 and MCP-1 expression was explored by co-transfection of THP-1 macrophages with p50 and p65 siRNAs.

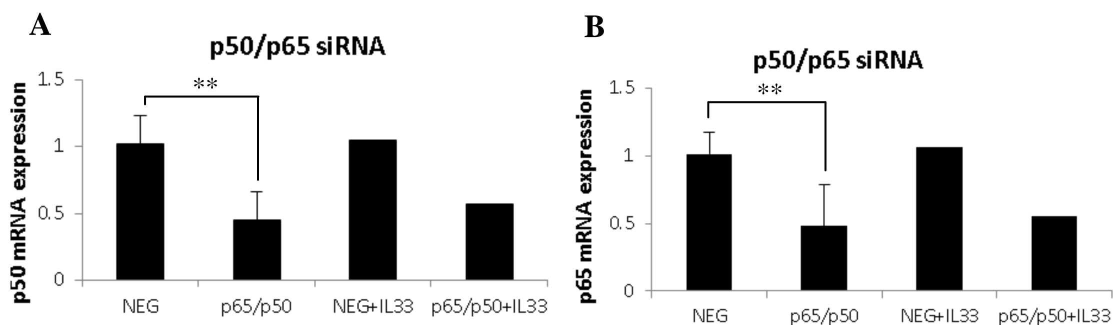


Figure 45. siRNA-mediated knock down of p50/p65 gene expression in THP-1 macrophages

RT-qPCR was performed using cDNA from differentiated THP-1 macrophages co-transfected with p50 and p65 siRNAs and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Samples transfected with siRNA targeting a scramble sequence were used as a control (NEG). Gene specific primers for p50 (Panel A) and p65 (B) were used. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated NEG) given an arbitrary value of 1. Data represents mean ± SD from four (NEG and p65/p50) and two independent experiments (NEG+IL-33 and p65/p50+IL-33). The Student *t* test was used to determine the statistical significance of the results for NEG and p65/p50 **p<0.01. Insufficient replicates of IL-33 treated cells meant that statistical tests were not performed for these samples.

Figure 45 shows that co-transfection of p50 and p65 siRNAs produced a significant

decrease of 60% in the mRNA levels of p50 in unstimulated cells. In the presence of IL-33 there was an average decrease of 48%. The mRNA expression of p65 was significantly reduced by 53% in cells absent of IL-33 and a reduction of 53% was achieved in cells treated with IL-33.

To further validate the use of p50 and p65 siRNAs in this system, the effects of these siRNAs on protein expression was assessed using western blot analysis.

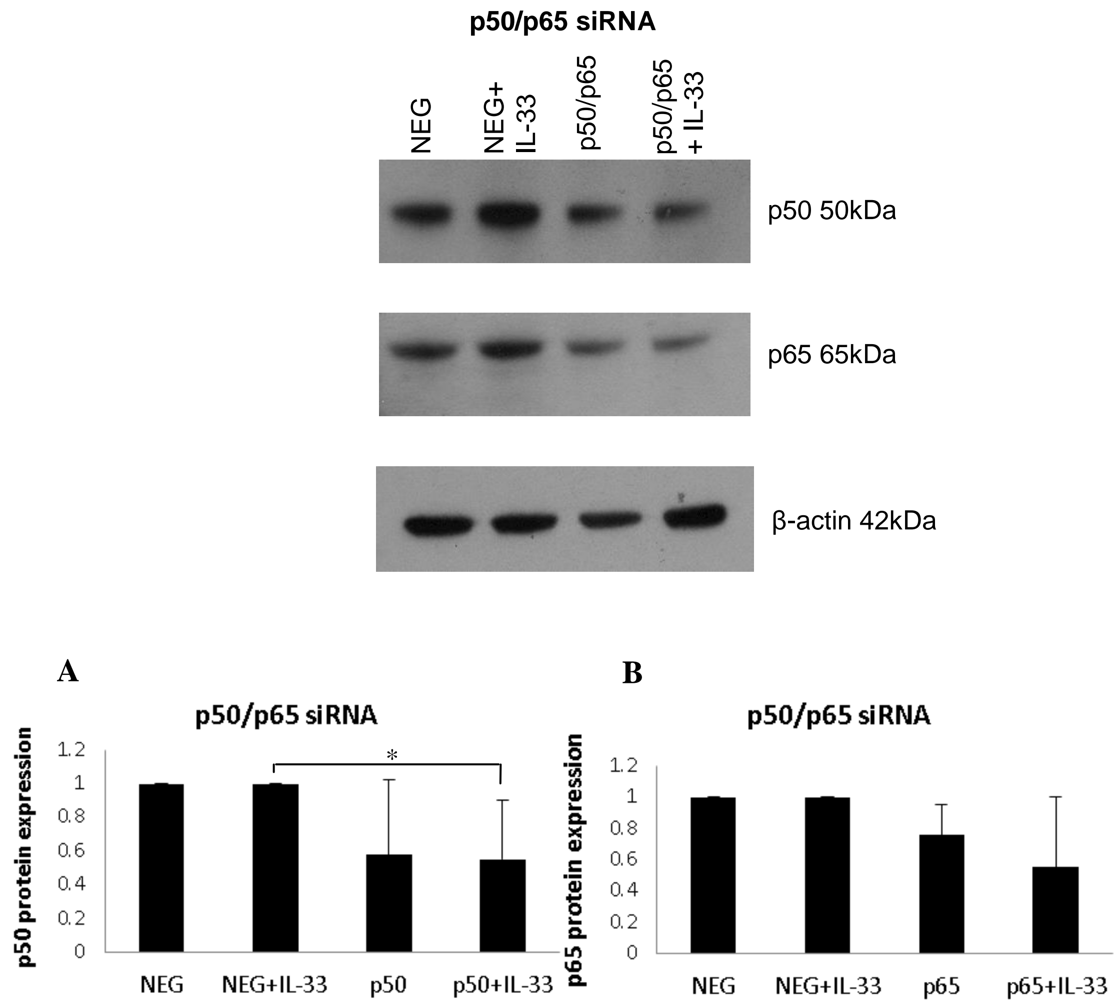


Figure 46. siRNA-mediated knock down of p50/p65 protein in THP-1 macrophages

Western blot analysis was performed using cellular lysates from differentiated THP-1 macrophages co-transfected with p50 and p65 siRNAs and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Samples transfected with siRNA targeting a scramble sequence were used as a control (NEG). Equal amounts of protein from the samples were analysed using p50 (Panel A), p65 (Panel B) and β-actin antibodies. The signals for p50 and p65 were normalised to that for β-actin with values from control samples (unstimulated and stimulated NEG) given an arbitrary value of 1. Bands correspond with the molecular weight of both proteins. Semi-quantitative analysis was performed using the Gene Tools software. Data represents mean ± SD from four independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05.

Co-transfection of p50 and p65 siRNAs produced a marked reduction in p50 and p65 protein levels (Figure 46). The decrease in p50 protein (45%) following IL-33 stimulation was significant and a similar trend was present in other samples subjected to p50/p65-mediated RNAi, although not significant, possibly due to large variation. In the absence of IL-33, the siRNAs mediated a 42% decrease in the levels of p50 protein. The levels of p65 protein exhibited a 24% and 45% reduction in unstimulated and IL-33 stimulated cells respectively.

The p50 and p65 siRNAs reduced the mRNA and protein expression of p50 and p65 and were therefore used to determine the effect on IL-33 signalling. THP-1 cells were co-transfected with p50 and p65 siRNAs in the absence and presence of IL-33 before RT-qPCR analysis using primers for ICAM-1 and MCP-1.

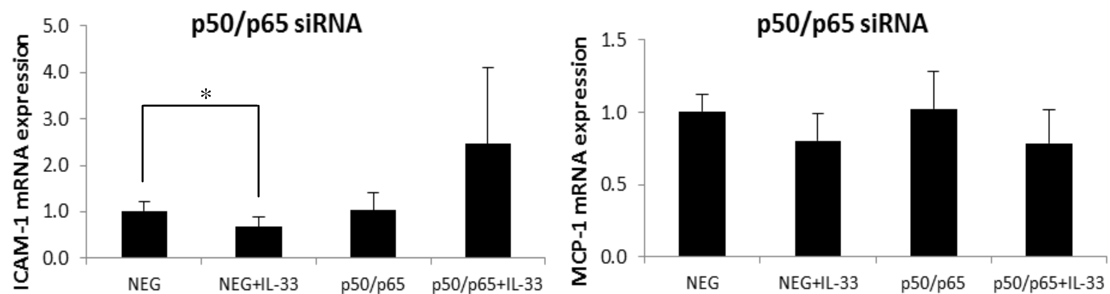


Figure 47. p50/p65 are involved in the IL-33-mediated regulation of ICAM-1 expression in THP-1 cells

RT-qPCR was performed using cDNA from differentiated THP-1 macrophages co-transfected with p50 and p65 siRNAs and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Samples transfected with siRNA targeting a scramble sequence were used as a control (NEG). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated NEG) given an arbitrary value of 1. Data represents the mean ± SD of three independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05.

IL-33 stimulation significantly reduced levels of ICAM-1 (Figure 47). Silencing of p50 and p65 negated the response from IL-33 and led to a non-significant increase in the mRNA expression of this gene. Due to time constraints further repeats were not

performed for MCP-1, although the cytokine caused a possible modest reduction in MCP-1 mRNA expression in the absence and presence of p50/p65 siRNAs indicating that these signalling components do not have an integral role in IL-33 signalling for this gene.

The studies presented here demonstrate that the MAPKs ERK1, ERK2, p38α and JNK1/2 along with the PI3K isoform PI3K-γ and the NF-κB subunits p50 and p65 play an essential role in the regulation of ICAM-1 expression by IL-33 in THP-1 macrophages. The same signalling components were also utilised for MCP-1 with the exception of p50 and p65.

4.3.4. The role of key signalling components in the IL-33-mediated regulation of ICAM-1 and MCP-1 expression in primary cells

Within THP-1 macrophages, the use of siRNAs and shRNAs achieved effective knock down of targeted gene expression. A similar approach was adopted to explore the role of key signalling components within primary HMDMs. However, due to the known issues with the use of siRNA in primary cells; such as low efficiency of transfection, RNAi was mediated with shRNAs within these cells. Technical difficulties with attaining a knock down by ERK2 and PI3K-γ shRNA meant that only shRNAs for ERK1 and p38α were used.

4.3.4.1. Effects of ERK1 knock down

Primary HMDMs were infected with shRNA for ERK1 using the same protocol as the THP-1 cells. The gene expression of ERK1 and ERK2 was then analysed by RT-qPCR. However, due to issues with cell viability and time restraints, western blots were not performed to assess the effects on ERK1 protein levels.

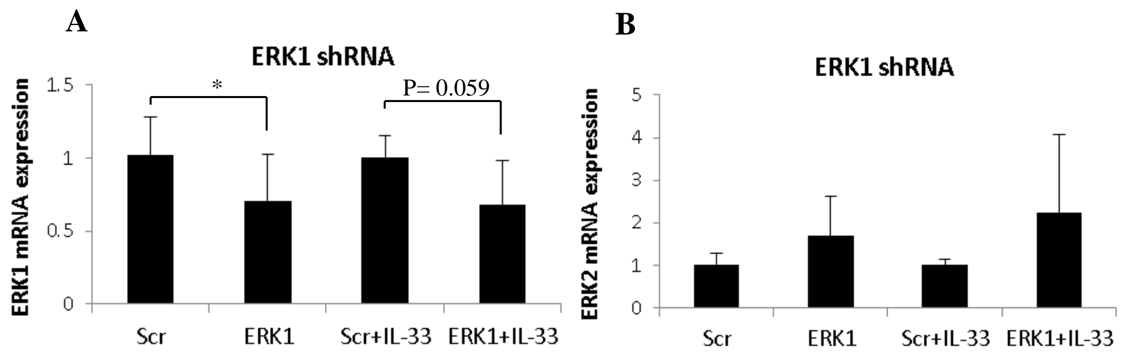


Figure 48. ERK1 shRNA mediated specific knock down of ERK1 gene expression in primary cells

RT-qPCR was performed using cDNA from primary macrophages expressing ERK1 shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). Gene specific primers for ERK1 (Panel A) and ERK2 (Panel B) were used. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr) given an arbitrary value of 1. Data represents mean ± SD from three experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05.

Figure 48A shows that ERK1 shRNA mediated a significant decrease of 32% in untreated cells and levels of ERK1 mRNA fell by 33% in IL-33 treated cells. Figure 48B demonstrates that the effects of ERK1 shRNA in primary cells were specific to ERK1 and did not cause a reduction in mRNA levels of the ERK2 isoform. An increase in the levels of ERK2 mRNA was observed, although the raise was not significant and may indicate that compensatory mechanisms were induced through the use of ERK1 shRNA.

Following validation of the use of these shRNAs for gene silencing in primary cells, ERK1 shRNA was then used to knock down the mRNA expression of ERK1 to assess the effects on IL-33-mediated regulation of ICAM-1 and MCP-1 mRNA expression by RT-qPCR.

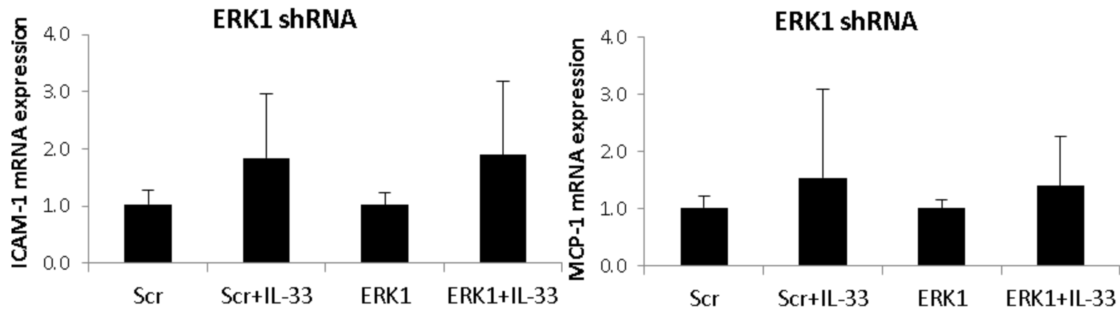


Figure 49. The effect of ERK1 knock down in regulation of ICAM-1 and MCP-1 by IL-33

RT-qPCR was performed using cDNA from primary macrophages expressing ERK1 shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr) given an arbitrary value of 1. Data represents mean ± SD from three experiments. The Student *t* test was used to determine the statistical significance of the results.

Experiments in chapter one demonstrated that IL-33 down-regulates the expression of ICAM-1 and MCP-1 mRNA within primary HMDMs (Figure 22). However, in the presence of scramble shRNA, treatment with IL-33 caused a non-signficant but noticeable induction in ICAM-1 and MCP-1 mRNA levels (Figure 49). The experiments performed in THP-1 macrophages indicated that IL-33 uses ERK1 to mediate the reduction in ICAM-1 and MCP-1 (Figure 36A). In one of the HMDM experiments, IL-33 treatment caused a reduction in ICAM-1 and MCP-1 mRNA expression (Appendix Figure 67). Further experiments will be required to delineate the role of ERK1 in the IL-33-mediated expression of these genes in primary cultures.

4.3.4.2. Effects of p38α knock down

Primary HMDMs were infected with p38α shRNA and RT-qPCR was used to analyse the effect on p38α gene expression.

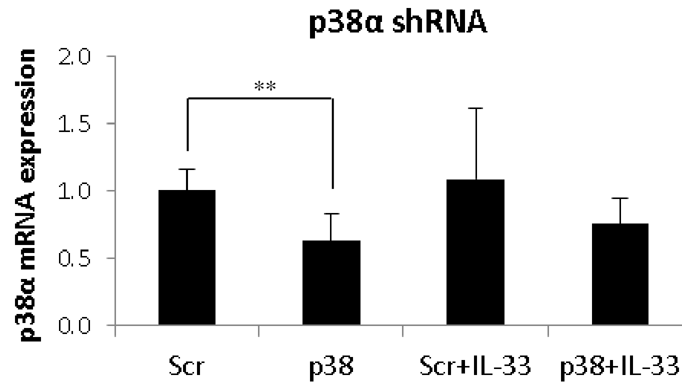


Figure 50. p38α shRNA-mediated knock down of p38α gene expression in primary cells

RT-qPCR was performed using cDNA from primary macrophages expressing p38α shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). Gene specific primers for p38α were used. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr) given an arbitrary value of 1. Data represents mean ± SD from three experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05.

The use of p38α shRNA mediated a significant reduction of 38% in untreated cells and levels of p38α mRNA fell by 34% in IL-33-stimulated cells (Figure 50).

To determine whether these reductions were present at the protein level, western blot analysis was performed on cellular lysates following infection by p38α shRNA in primary HMDMs.

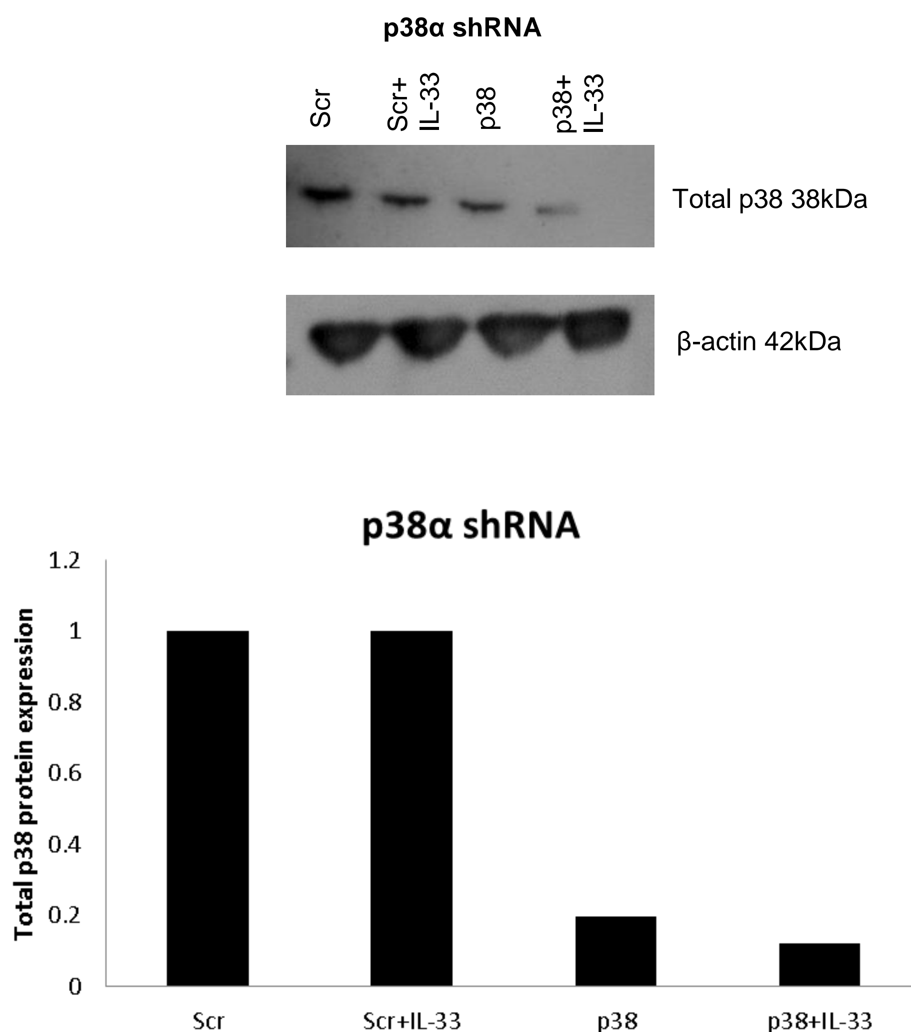


Figure 51. shRNA-mediated knock down of p38α protein in primary human macrophages

Western blot analysis was performed using cellular lysates from primary macrophages expressing p38α shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). Equal amounts of protein from the samples were analysed using total p38 and β-actin antibodies. The signal for p38 was normalised to that for β-actin with the values from control samples (unstimulated and stimulated Scr) given an arbitrary value of 1. Bands correspond with the molecular weight of both proteins. Semi-quantitative analysis was performed using the Gene Tools software. Data represents mean from two experiments.

Figure 51 shows that p38α shRNA caused a substantial decrease in the levels of total p38 protein in primary cells left untreated or treated with IL-33.

Following validation of p38α shRNA in reducing the mRNA and protein expression of p38α in primary cells, the shRNA was then used to examine the role of p38α in the regulation of ICAM-1 and MCP-1 gene expression by IL-33 using RT-qPCR.

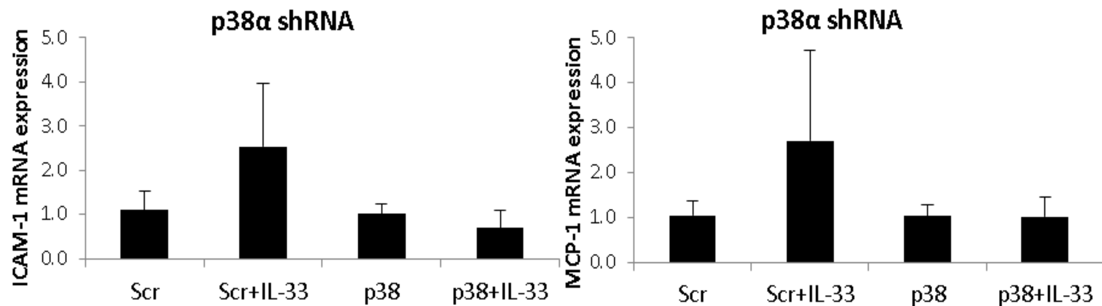


Figure 52. The effects of p38α knock down in IL-33 regulation of ICAM-1 and MCP-1

RT-qPCR was performed using cDNA from primary macrophages expressing p38α shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples (unstimulated and stimulated Scr) given an arbitrary value of 1. Data represents mean ± SD from three experiments. The Student *t* test was used to determine the statistical significance of the results.

The use of scramble shRNA caused a non-significant induction in the mRNA expression of ICAM-1 and MCP-1 following IL-33 stimulation (Figure 52), as was also seen in Figure 49. The induction in ICAM-1 and MCP-1 was particular to primary cells as THP-1 macrophages displayed a reduction in the expression of these genes in the presence of scramble shRNA (Figures 36A, 36B, 39 and 44). Interestingly, in a single experiment from Figure 52 IL-33 treatment caused a reduction in MCP-1 mRNA expression (Appendix Figure 68). Within these samples p38α was not integral in the IL-33 down-regulation of MCP-1.

The responses seen in THP-1 cells in cells expressing scramble shRNA and from normal HMDMs were mostly not conserved in HMDMs expressing scramble shRNA. The primary HMDM experiments were associated with a high variability likely due to

the heterogenous nature of these cells and further repeats would be needed to perform further statistical analysis.

4.4. Discussion

The use of ERK1, ERK2, p38α and PI3K-γ shRNAs, and ERK2, JNK1, JNK2, p50 and p65 siRNAs were validated within the THP-1 cellular system, in addition to ERK1 and p38α shRNAs within primary HMDMs. The data demonstrated that these RNAi methods produced substantial reductions in the mRNA levels of the genes tested. Also where analysed (ERK1, ERK2 and p38α shRNA and ERK2, JNK1, JNK2, p50 and p65 siRNAs) there was a corresponding decrease at the protein level. The studies presented within this chapter have reaffirmed the findings found in the previous chapter; that stimulation by IL-33 (25ng/ml) for 12 hours produces a reduction in the mRNA expression of ICAM-1 and MCP-1 in THP-1 macrophages. The use of RNAi then demonstrated that the MAPKs ERK1, ERK2, p38α, JNK1 and JNK2; the PI3K component PI3K-γ and NF-κB members p50 and p65 were involved in some of these responses. A reduction in the mRNA expression of these signalling modules attenuated or reversed the previously described effects of IL-33 on ICAM-1 and MCP-1 mRNA.

A number of studies have reported that IL-33 activates multiple signalling cascades (see Table 4 for further details) including those tested in this chapter. The cytokine activates PI3K in endothelial cells to increase vascular permeability (Choi *et al.* 2009). In contrast, NF-κB is inhibited by treatment of IL-33 in embryonic kidney cells (Ali *et al.* 2011). Interestingly following 12 hours of IL-33 stimulation there were no significant change in the levels of ERK1, ERK2, JNK1, p50 and p65 mRNA but a significant decrease was seen in the expression and activation of p38α, JNK2 and PI3K-γ mRNA (Figure 30). However, to discern the biological relevance of IL-33 treatment, these studies should be extended to explore the effects of IL-33 on the expression and activation of the protein forms of these components. For example, although IL-33 stimulation had no effect on the mRNA expression of ERK1 and ERK2, the cytokine induced the levels of phosphorylated ERK1/2 protein (Figure 31).

The western blot was performed with participation from another laboratory member to aid studies towards their thesis and also served to test western blot conditions and assess the effect of IL-33 on ERK1/2 activation. For this purpose western blot analysis was performed using conditions previously optimised by our laboratory (McLaren *et al.* 2010b) to determine the change in levels of phosphorylated ERK1/2 to total ERK1/2 following IL-33 stimulation. The activation of ERK1/2 by IL-33 has been demonstrated by many groups in a variety of cell types including mouse pancreatic cells (Kempuraj *et al.* 2013), embryonic fibroblasts (Funakoshi-Tago *et al.* 2008), mast cells (Schmitz *et al.* 2005) and peritoneal macrophages (Funakoshi-Tago *et al.* 2011), in addition to human umbilical cord blood-derived mast cells (Iikura *et al.* 2007) and human lung epithelial and endothelial cells (Aoki *et al.* 2010; Yagami *et al.* 2010). In accordance with the findings from other studies, IL-33 promoted the phosphorylation of ERK1 and ERK2 indicating that these signalling components are involved in IL-33 activity.

The primary aim of this chapter was to analyse the signalling pathways utilised by IL-33 to regulate ICAM-1 and MCP-1 expression. RNAi was utilised to silence the gene expression of signalling molecules implicated in atherosclerosis. To validate the use of these siRNAs and shRNAs, THP-1 cells were transfected/infected and the extent of knock down quantitatively assessed by RT-qPCR. The shRNAs and siRNAs tested exerted a significant level of gene silencing for each of the signalling targets in the absence of IL-33. A significant level of reduction was also achieved following IL-33 stimulation by ERK1, ERK2, p38α, PI3K-γ shRNA and ERK2 and JNK2 siRNA. The mRNA expression of JNK1, p50 and p65 was not significantly affected by their corresponding siRNAs in the presence of IL-33, although a trend for decreased expression was present in all cases and therefore additional repeats should be undertaken. Analysis by western blotting was conducted following the infection of shRNAs for ERK1, ERK2 and p38α and also transfection of ERK2, JNK1 and JNK2 siRNAs to determine the level of protein reduction. Trends seen in mRNA expression following siRNA and shRNA-mediated RNAi correlated with changes in protein levels. A significant decrease in protein levels occurred following incubation with ERK1 and p38α shRNA, in addition to ERK2 and JNK2 siRNA in cells not treated with IL-33.

The use of certain siRNAs following IL-33 stimulation did not achieve a significant reduction possibly due to issues with transfection efficiency. Gene silencing with siRNAs is sometimes not as robust as shRNA-mediated RNAi and there are many factors which can affect siRNA efficiency including the presence of internal structures such as hairpins and substances within the media (Reynolds *et al.* 2004). An alternative to the methods employed would be the use of pharmacological inhibitors, however many inhibitors are not as specific as siRNA and shRNA and can exert off-target effects (Milhavet *et al.* 2003). The shRNAs and siRNAs used were readily available and have been previously optimised and used by the laboratory. Additional experiments should be performed as a significant decrease was not achieved under all tested conditions, although all siRNAs and shRNAs produced substantial reductions at the mRNA and protein level and therefore a trend was seen in all cases.

Following validation of the shRNAs and siRNAs, the effects on the IL-33-mediated regulation of ICAM-1 and MCP-1 following RNAi was explored. IL-33 was previously shown to reduce the gene expression of ICAM-1 and MCP-1 and this was once more demonstrated in the presence of scramble sequences of siRNA and shRNA within THP-1 macrophages. Previous studies investigating signalling pathways regulated by IL-33 in primary cells such as trophoblasts (Fock *et al.* 2013), nasal epithelial cells (Kamekura *et al.* 2012) and hepatocytes (Sakai *et al.* 2012) have also demonstrated the involvement of ERK, p38, JNK and NF-κB. Furthermore, our laboratory recently showed that IL-33 utilises ERK1/2, JNK1/2, c-Jun, PI3K-γ and PI3K-δ in the regulation of ADAMTS-1, -4 and -5 within the THP-1 system and the involvement of ERK1/2, PI3K-γ and PI3K-δ were also confirmed in primary HMDMs (Ashlin *et al.* 2014).

In accordance with other groups, the experiments within this chapter showed that gene silencing of key signalling modules; the MAPKs ERK1, ERK2, p38α, JNK1/2; the PI3K component PI3K-γ and the NF-κB subunits p50 and p65 resulted in a loss of response from the cytokine within THP-1 macrophages. Furthermore, the studies demonstrated that these signalling components are involved in the IL-33-mediated down-regulation of ICAM-1 and MCP-1. A summary of the results is provided in Table 17.

Table 17. Summary of results

Effect of IL-33 on gene expression following knock down				
RNAi mediator	**ICAM-1**		**MCP-1**	
THP-1 macrophages				
ERK1 shRNA	Induction	N/S	Induction	N/S
ERK2 shRNA	Induction	*	Induction	N/S
P38α shRNA	Induction	N/S	Ablation of response	N/S
PI3K-γ shRNA	Induction	N/S	Induction	N/S
ERK2 siRNA	Induction	*	Ablation of response	N/S
JNK1/2 siRNA	Induction	N/S	Induction	*
p50/p65 siRNA	Induction	N/S	Reduction	N/S

The Student *t* test was used to determine the statistical significance of the results for the THP-1 macrophages: *p<0.05, N/S = not significant.

The involvement of these signalling cascades in the regulation of ICAM-1 and MCP-1 expression are well documented (Table 18). However, this is the first study to characterise the signalling pathways involved in the IL-33 mediated-reduction of ICAM-1 and MCP-1 mRNA expression in THP-1 macrophages.

The experiments within primary HMDMs produced several interesting results. Primary cells are notoriously difficult to transfect with siRNA so an approach utilising shRNA was undertaken. A marked reduction was achieved through the use of ERK1 and p38α shRNAs in ERK1 and p38α mRNA expression respectively, and was also shown at the protein level of p38α in primary HMDMs. However, within a subset of primary cells, treatment with IL-33 caused a non-significant up-regulation in the mRNA expression of ICAM-1 and MCP-1 in the presence of scramble shRNA. The inclusion of scramble shRNA did not induce levels of ICAM-1 and MCP-1 mRNA following IL-33 treatment within THP-1 cells (Figures 36A, 36B, 39 and 44).

Additionally, the experiments within chapter three demonstrated that IL-33 reduces the expression of ICAM-1 and MCP-1 within THP-1 macrophages, RAW264.7 macrophages and HMDMs. Interestingly, in certain experiments IL-33 down-regulated ICAM-1 or MCP-1 expression and within these cells the response to ERK1 or p38α shRNAs varied in comparison to cells where an up-regulation occurred. Such differences in response may indicate the occurrence of donor-specific variation within HMDMs (Maess *et al.* 2010; Qin 2012) and the presence of a heterogeneous population of cells that respond differently to IL-33 in the presence of shRNA. The experiments within HMDMs displayed a high level of variation in comparison to THP-1 macrophages subjected to the same experimental procedure and were not significant, further repeats would be required to perform meaningful statistical analysis.

The up-regulation of ICAM-1 and MCP-1 gene expression in response to shRNA within certain cells could have arisen due to an anti-viral response. IL-33 expression can be induced by the presence of viruses and drives an anti-viral immune response (Le Goffic *et al.* 2011; Bonilla *et al.* 2012) associated with the production of pro-inflammatory cytokines (Le Goffic *et al.* 2011) such as IFN-γ and TNF-α which are also known to exert pro-atherogenic effects (Gupta *et al.* 1997; Ohta *et al.* 2005). The disparity in results could arise due to a reaction to the viral delivery of shRNAs resulting in the up-regulation of ICAM-1 and MCP-1, or to the shRNA itself. Interestingly, the change in response to cytokine stimulation in the presence of shRNAs has not been previously noted by our laboratory. Many studies have utilised shRNAs for characterising signalling pathways involved in cytokine-mediated regulation of atherosclerotic markers within primary cultures such as bone marrow derived macrophages from knockout animals and HMDMs (McLaren *et al.* 2010a; Michael *et al.* 2012b; Ashlin *et al.* 2014). However, these studies explored the effects on the expression of genes involved in ECM degradation (Ashlin *et al.* 2014) and foam cell formation (McLaren *et al.* 2010a; Michael *et al.* 2012b). The altered response to IL-33 stimulation, seen in section 4.3.4, could be peculiar to ICAM-1 and MCP-1 due to their role within immune reactions and cell recruitment. To determine whether the virus or scramble shRNA had an influence, a different approach could be adopted to knock down the expression of signalling components such as plasmid transfection of shRNA (Stopeck *et al.* 1998; Hunt *et al.* 2010; Park *et al.* 2011) or the

use of bone marrow-derived macrophages from ERK1 or p38 knockout mice. Scramble shRNA is frequently used as an experimental control (Shih *et al.* 2005; Li *et al.* 2010; Moore *et al.* 2010) and different methods could be utilised to determine whether targeting the scramble sequence had an effect on ICAM-1 and MCP-1 expression such as the use of an empty vector lacking an shRNA insert.

Table 18. Studies of signalling pathways regulating ICAM-1 and MCP-1 expression

Gene	Stimulus	Signalling pathways	Cell line	Reference
ICAM-1	Vascular endothelial growth factor	PI3K, Akt, nitric oxide	Rat primary brain microvascular endothelial cells	(Radisavljevic *et al.* 2000)
ICAM-1	Thrombin	NF-κB	HUVECs	(Xue *et al.* 2009)
ICAM-1	mAb ligation	ERK1/2, p38	Rat primary astrocytes	(Lee *et al.* 2000)
ICAM-1	LPS	JNK, p38	Rat schwann cells	(Shen *et al.* 2008a)
ICAM-1	IL-1β, TNF-α	PKC	Rat neonatal astrocytes	(Ballestas and Benveniste 1995)
MCP-1	Lysophosphatidic acid	p38, JNK, NF-κB, Rho kinase	HUVECs	(Shimada and Rajagopalan 2010)
MCP-1	IFN-γ	PI3K, Casein Kinase 2, JAK-STAT, Akt	Murine J774.2 macrophages, HMDMs, human Hep3B hepatomas HUVECs and human endothelial EA.hy926	(Harvey *et al.* 2007)
MCP-1	TNF-α	JNK	Primary astrocytes	(Gao *et al.* 2009)
MCP-1	Diabetic nephropathy	NF-κB	Renal biopsies of patients with diabetic nephropathy	(Mezzano *et al.* 2004)
MCP-1	$NiCl_2$	p38	Primary human endothelial cells	(Goebeler *et al.* 2001)
ICAM-1 & MCP-1	IFN-γ	ERK1/2	THP-1 macrophages	(Li *et al.* 2010).

Future investigations should be undertaken to assess whether IL-33-mediated down-regulation of ICAM-1 and MCP-1 expression is ST2 dependent (wild type control) or mediated independently of the receptor. The use of bone marrow-derived macrophages from ST2 deficient mice could be used for this purpose. Also, the components involved in upstream signalling like IRAK, IRAK4, and TRAF6 in addition to downstream mediators implicated in IL-33 signalling could also be explored through the use of siRNA or shRNA (Schmitz *et al.* 2005; Chackerian *et al.* 2007; Sun *et al.* 2013). Furthermore, these experiments were performed using recombinant mature IL-33 but several studies have shown that the unprocessed form is also active and it would therefore be interesting to see whether the same results arise through the use of full-length IL-33.

Several studies have previously shown that IL-33 increases levels of ICAM-1 and MCP-1 (Table 14). Choi *et al.* (2012) demonstrated that stimulation by IL-33 raises the amount of ICAM-1 mRNA and protein in HUVECs and that the use of IL-33 siRNA reduces mRNA and protein levels of ICAM-1 mediated through the NF-κB pathway. Demyanets *et al.* (2011) found that IL-33 raised levels of ICAM-1 and MCP-1 mRNA and protein in human coronary arteries and HUVECs *in vitro* and within explanted atherosclerotic plaques *ex vivo.* The study also reported that IL-33 promoted human leukocyte binding to human endothelial monolayers and caused nuclear translocation of p50 and p65 (Demyanets *et al.* 2011). However, these studies were performed in different cell types and within different systems so the effects could be cell- or species-specific. Furthermore, it is difficult to draw a direct comparison to other studies as other groups use different doses and time-courses to perform their experiments. For example, Chow *et al.* (2010) uses a range of concentrations (10-100ng/ml) and stimulate human eosinophils for 16 hours with IL-33 to determine the effects on ICAM-1 expression (Chow *et al.* 2010). Funakoshi-Tago *et al.* (2008) explored the effects on MCP-1 using 10ng/ml of IL-33 over a time course of 12 hours in murine embryonic fibroblasts (Funakoshi-Tago *et al.* 2008).

Although these studies mentioned above support a pro-atherogenic role for IL-33, there is a wealth of data provided by our group and many others such as those described in section 1.7.4. that demonstrate the anti-atherogenic properties of IL-33. The cytokine reduces macrophage foam cell production (McLaren *et al.* 2010b), decreases

macropinocytosis by macrophages (Michael *et al.* 2013) and inhibits ADAMTS-1, -4 and -5 expression (Ashlin *et al.* 2014). The data within this thesis has shown that IL-33 exerts a number of anti-inflammatory effects within both human and murine systems and supports the emerging anti-atherogenic role of the cytokine demonstrated in studies in mouse model systems.

Interestingly, following the knock down of ERK2 by shRNA or siRNA, IL-33 stimulation caused a significant increase in the expression of ICAM-1. This indicates that ERK2 holds a key role in IL-33-mediated down-regulation of ICAM-1 and suggests that the MAPK could potentially act as a repressor of IL-33 activity. Also, the reduction of JNK1/2 by siRNA did not just negate the anti-atherosclerotic effects of IL-33 but led to an induction of MCP-1 mRNA following IL-33 stimulation which indicates that IL-33 could exert pro-atherosclerotic actions in the absence of ERK2 or JNK1/2. IL-33 is known to promote several pro-inflammatory disease states such as asthma and rheumatoid arthritis (see Table 5) but within atherosclerosis the cytokine exerts an anti-inflammatory role. A study by Joshi *et al.* (2010) describes an interesting switch in IL-33 actions. Stimulation of naïve human bone marrow-derived macrophages by IL-33 produces a M1 profile characterised by increased expression of CCL13. However, following 24 hours exposure to M1 or M2 mediators, primary macrophages were then further exposed to these mediators in addition to IL-33. The combination of IL-33 and M1 agents raised the expression of the M2 marker CCL18, whereas exposure of IL-33 and M2 mediators to previously M2 polarised cells increased the expression of the M2 mannose receptor (Joshi *et al.* 2010). These results suggest that in macrophages activated by M1 or M2 stimuli IL-33 promotes the expression of M2 markers but within naïve macrophages IL-33 supports the M1 profile.

The observed changes in IL-33 activity could explain how IL-33 is implicated in both pro- and anti-inflammatory processes. It is possible that dysregulation and atypical signalling exhibited during disease states could induce a switch in the activities of plaque-resident cells and cytokines. It is already known that macrophages are capable of switching classes even after exposure to initial activating stimuli and can therefore participate in both the propagation and resolution of inflammation (Lake *et al.* 1994; Porcheray *et al.* 2005). It would be useful to explore whether alternative pathways are activated following the knock down of ERK2 and JNK1/2 that facilitate the change in

response by IL-33 and whether these components are associated with pro-atherosclerotic processes. Further investigations should also explore whether the increase in MCP-1 mRNA following JNK1/2 gene silencing was isoform specific as a combination of JNK1 and JNK2 siRNAs were used for the knock down due to limitations with time. Differences in the roles of JNK1 and JNK2 have previously been described. For example, absence of JNK2 but not JNK1 in ApoE$^{-/-}$ mice imparts resistance to diet-induced atherosclerosis (Ricci *et al.* 2004). Also, although JNK1 and JNK2 regulate cellular proliferation, the isoforms regulate different phases of the cell cycle and exhibit differential binding preferences to c-Jun (Sabapathy *et al.* 2004).

In conclusion, the data presented within this chapter has demonstrated that multiple signalling cascades such as MAPKs, PI3K and NF-κB were involved in the regulation of ICAM-1 and MCP-1 gene expression by IL-33. A reduction in mRNA and protein levels of these signalling components resulted in a loss of response from IL-33 and in the case of ERK2 and JNK1/2 caused a reversal of the previously described effects in THP-1 macrophages. Figure 53 displays a schematic of the ICAM-1 and MCP-1 promoters. Interestingly, flanking regions within these genes contain a number of shared regulatory elements such as AP-1, NF-κB and SP1 (Roebuck and Finnegan 1999; Kanda and Watanabe 2003) which may explain the involvement of common signal transduction pathways by IL-33. For example, AP-1 can be activated as a result of either NF-κB and MAPK signalling and c-Jun, a key downstream target of JNK1/2, is a component of the AP-1 family (Xie 2013).

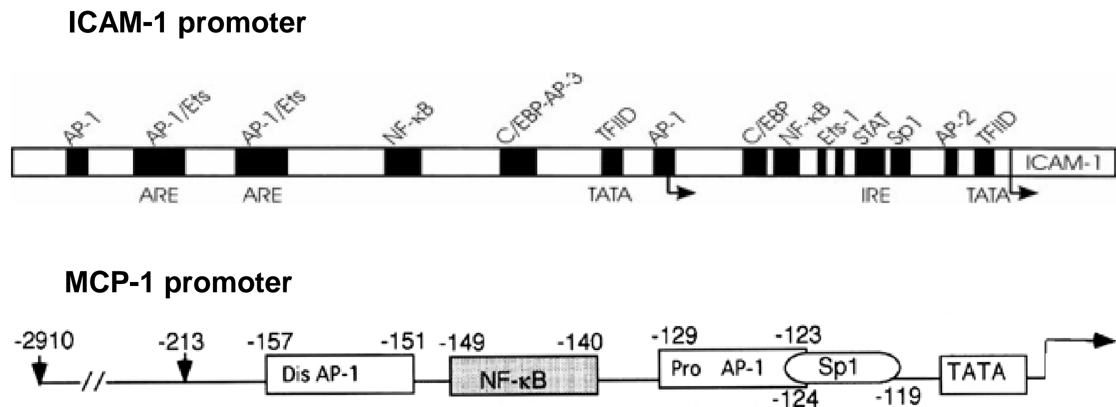


Figure 53. Promoter region of ICAM-1 and MCP-1

Structure of the ICAM-1 and MCP-1 promoters. The nucleotide positions displayed are in relation to the translational start site. Figures are taken from Roebuck and Finnegan (1999) and Kanda and Watanabe (2003) respectively.

Atherosclerosis is a complex disease and numerous signalling components have been implicated in its pathogenesis. Due to the anti-atherogenic nature of IL-33 it would be useful to fully define the signalling mechanisms which give rise to its activity, the use of mice lacking upstream and downstream elements could be utilised. Alternatively, gene-specific reporter constructs containing 5' regions upstream of the gene of interest in front of a luciferase gene could be used to determine the integral regulatory elements required for gene expression in response to cytokine treatment (Mehl *et al.* 2001; Kanda and Watanabe 2003). Mutational analysis along with DNA-protein interaction studies could also be performed to further delineate regulatory sequences. The experiments presented within this chapter have implicated the involvement of key signalling molecules in the regulation of ICAM-1 and MCP-1 gene expression by IL-33 and it would be useful to also investigate the effects on ICAM-1 and MCP-1 proteins.

4.5. Future Aims

The experimental chapters have so far investigated the effects of IL-33 on the expression of atherosclerotic markers and signalling components using a combination of RT-qPCR, western blotting and RNAi. The data has shown that IL-33 exerts an anti-atherogenic effect on the expression of several genes investigated and has discerned that several signalling pathways already implicated in atherosclerotic events are involved.

However, it is also important to determine whether IL-33 is involved in other cellular processes that contribute towards the disease state. Atherosclerosis is a chronic inflammatory disorder that is characterised by the excessive build-up of lipids within the arterial subendothelium. The next chapter will explore the actions of IL-33 on the lipid profile of human and murine macrophages. During the disease, macrophages amass lipids and transform into macrophage-derived foam cells; the major component of the fatty streak. These cells are also key orchestrators of the inflammatory state through the expression and secretion of cytokines and fatty-acid derived mediators. This will be the first study to utilise GC and TLC to determine the impact of IL-33 on individual fatty acids and major lipid groups within macrophages. Additionally, the effects of IL-33 on the expression of enzymes involved in lipid metabolism will be assessed by RT-qPCR to elucidate the novel mechanisms through which IL-33 exerts its actions.

CHAPTER 5.

THE EFFECTS OF IL-33 ON THE LIPID PROFILE OF MACROPHAGES

5.1. Introduction

5.1.1 Desaturation of fatty acids

Desaturases are either soluble or membrane-bound and mammals contain acyl-CoA desaturases located within the ER membrane (Nakamura and Nara 2004). The major enzyme responsible for desaturation is SCD (Sampath and Ntambi 2014). Mice have four known isoforms of SCD, whereas humans have two genes; *SCD-1* and *SCD-5*, encoding for two highly homologous (87%) isoforms: SCD-1 and SCD-2 (Wang *et al.* 2005). Both SCD isoforms are expressed in the lung, adipose tissue and kidneys. SCD-1 is additionally expressed within the liver and SCD-2 is expressed in the brain, spleen, heart and placenta (Sampath and Ntambi 2014). The main substrates for SCD are 16 and 18 carbon fatty acids and predominately stearic acid (Cook and McMaster 2002). SCD directs the rate limiting step in MUFA synthesis, catalysing the formation of a *cis*-double bond at the Δ-9 position from the carboxyl end of palmitic acid and stearic acid to palmitoleic acid and oleic acid respectively (Attie *et al.* 2002; MacDonald *et al.* 2009).

Within humans and other animals there is limited conversion of essential fatty acids to very long chain PUFAs like AA and eicosapentaenoic acid (EPA) (Meesapyodsuk and Qiu 2012). A combination of Δ6- and Δ5-desaturase activity and the elongation of fatty acids from the n-6 and n-3 series produces a diverse array of PUFAs (de Antueno *et al.* 2001). Figure 54 summarises the elongation and desaturation reactions responsible for MUFA and PUFA generation. Dysregulation or abnormal desaturase activity is linked to disease and as such the expression and activity of these enzymes is carefully modulated through feedback loops and signalling pathways (Cook and McMaster 2002). SCD activity is reliant on the proportion of dietary SFAs, especially palmitic acid, and

is accordingly negatively regulated by elevated PUFA levels (Vessby *et al.* 2001; Vessby *et al.* 2002). The activities and expression of Δ6- and Δ5-desaturases are likewise modulated by the presence of fatty acid substrates and products (Vessby *et al.* 2002). As such Δ6-desaturase and Δ5-desaturase activity increases in the absence of dietary PUFAs and in response to refeeding after a fasting state (Cook and McMaster 2002), although Δ5-desaturase is less active than Δ6-desaturase (Dias and Parsons 1995; Vessby *et al.* 2013).

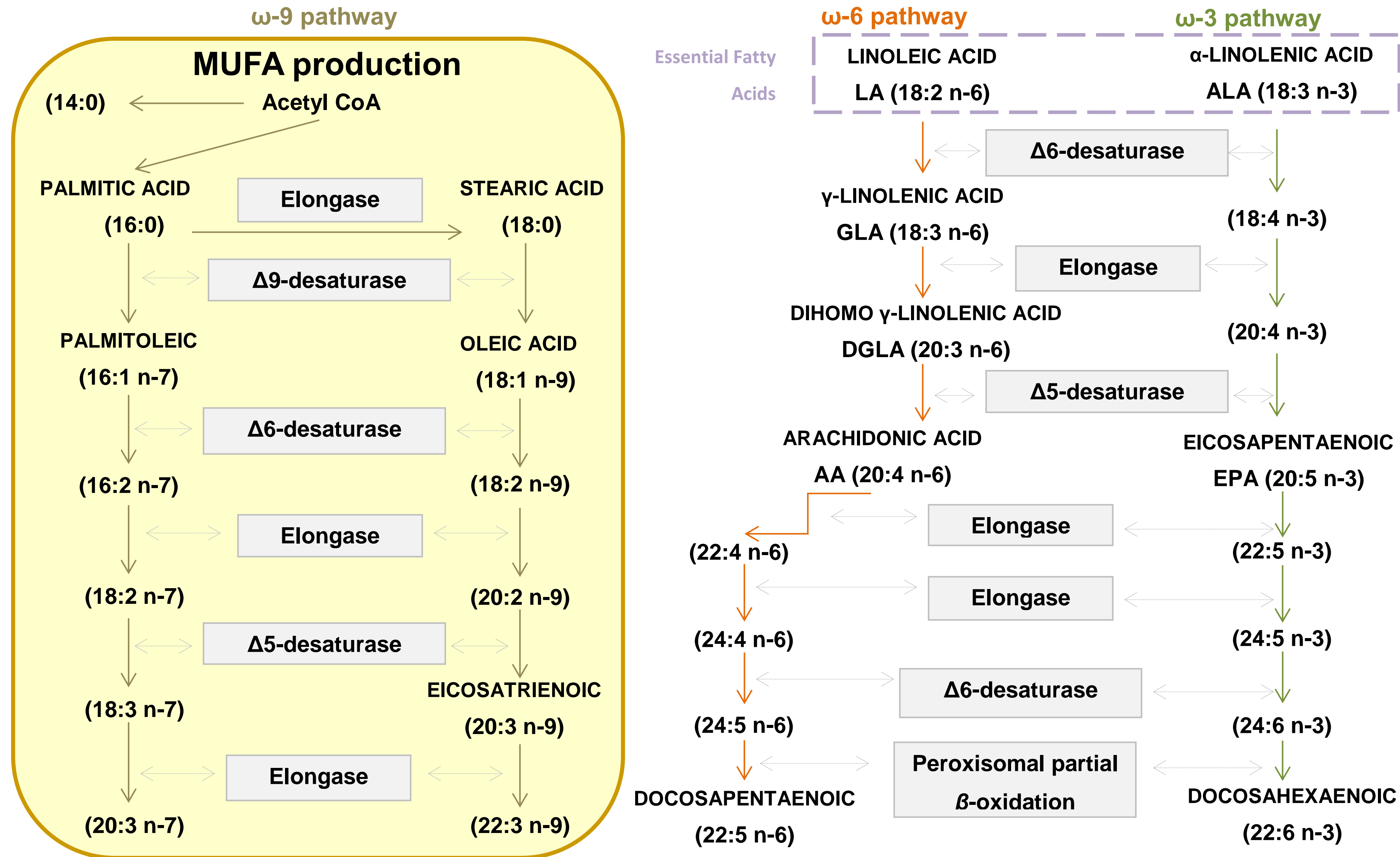


Figure 54. Fatty acid metabolic pathways

The major product of fatty acid synthesis; palmitic acid and the essential fatty acids; LA and ALA are subjected to sequential elongation and desaturation to produce a range of MUFAs and PUFAs. The ER desaturation system additionally requires NAD, molecular oxygen and cytochrome b_5. Adapted from Uauy *et al.* (2003), Voruganti *et al.* (2012), White *et al.* (2013), Wang *et al.* (2013).

5.1.2. The role of lipids in heart disease

There are a wealth of epidemiological studies investigating the link between fatty acids and incidences of CAD and its complications. The roles of fatty acids in cardiac-related pathologies are complex and further information is provided within the introduction. Although a number of studies have found conflicting results there are several general rules describing the cholesterolemic effects of fatty acids (KrisEtherton and Yu 1997):

- SFAs are typically hypercholesterolaemic and myristic acid is the most potent whilst stearic acid appears to have a neutral effect.
- MUFAs have a mostly neutral/ or mildly hypocholesterolaemic influence but are not as potent as PUFAs.
- *Trans*-fatty acids are hypercholesterolaemic, produce an unfavourable LDL to HDL cholesterol ratio (Mensink and Katan 1990) and also up-regulate the expression of pro-atherosclerotic markers (Mozaffarian *et al.* 2004; Lopez-Garcia *et al.* 2005).
- n-3 PUFAs decrease the expression of pro-inflammatory cytokines (Caughey *et al.* 1996; Trebble *et al.* 2003).
- n-6 PUFAs are potentially associated with inflammatory processes but may also exert some beneficial effects.

Table 19 summarises the findings from several studies, where pro-atherosclerotic actions of fatty acids are denoted in red and anti-atherosclerotic processes are highlighted in blue.

Table 19. Studies exploring the effects of fatty acids in CAD

Lipid class	Fatty acid	Role in CAD	Testing medium	Reference
SFAs	Meta-analysis of 60 studies determined that saturated fats increased LDL cholesterol but had no effect on HDL cholesterol.		Human serum	(Mensink *et al.* 2003)
	Dietary SFAs caused an increase in blood cholesterol two-times as much as PUFAs decreased blood cholesterol.		Human serum	(Hegsted *et al.* 1965; Keys *et al.* 1965).
	Myristic acid 14:0	Raised plasma cholesterol. Thought to be most potent SFA.	Monkey plasma	(Hayes and Khosla 1992)
		Increased LDL cholesterol and levels of ApoB and reduced HDL to LDL ratios.	Human serum	(Zock *et al.* 1994)
		Promoted formation of dense LDL particles.	Human lipoproteins	(Dreon *et al.* 1998)
	Palmitic acid 16:0	Increased LDL cholesterol and levels of ApoB and reduced ratio of HDL to LDL.	Human serum	(Zock *et al.* 1994)
		Promoted formation of dense LDL particles.	Human lipoproteins	(Dreon *et al.* 1998)
	Stearic acid 18:0	Neutral effect/ mild beneficial effects on LDL cholesterol.	Human serum	(Mensink *et al.* 2003)
MUFAs	*Trans*-MUFAs are associated with an unfavourable lipoprotein profile.		Human serum	(Lichtenstein *et al.* 1999)
	Cis-palmitoleic acid	Positive association with CAD risk.	Human erythrocytes	(Djousse *et al.* 2012)

	16:1n-7			
	Cis-vaccenic acid 18:1n-7	Negative association with CAD risk.	Human erythrocytes	(Djousse *et al.* 2012)
	Oleic acid 18:1n-9	Neutral effect on atherosclerotic markers.	Rat serum Monkey plasma Human serum	(Lee *et al.* 1989) (Hayes and Khosla 1992) (Mensink and Katan 1990)
	Trans-oleic acid 18:1	Produced an atherogenic lipoprotein profile. Unlike other *trans*-fatty acids was not prevalent in patients following cardiac arrest.	Human serum Human erythrocytes	(Mensink and Katan 1990). (Lemaitre *et al.* 2002)
n-3 PUFAs	n-3 PUFAs inhibited TAG, apolipoprotein and VLDL synthesis. Competitively suppressed production of AA metabolites. Protective effect of n-3 supplements in patients who have previously suffered a MI after 3 months. Supplementation of n-3 PUFAs significantly increased survival following a previous MI, whereas the anti-oxidant vitamin E had no effect on outcome. The expression of ICAM-1 and SRs were significantly reduced in fish oil fed mice.			(Okuyama *et al.* 2000) (Marchioli *et al.* 2002) (Valagussa *et al.* 1999) (Miles *et al.* 2000)

	α-linolenic acid (ALA) 18:3n-3	Reduced levels of ALA in patients with stroke and lower limb disease.	Human erythrocytes	(Leng *et al.* 1999)
		Reduced serum CRP, serum amyloid A and IL-6.	Human serum and plasma	(Rallidis *et al.* 2003)
	Eicosapentaenoic acid (EPA) 20:5n-3	EPA was a poor substrate for COX enzymes but binds with high affinity therefore reducing AA conversion.	Human platelets	(Needleman *et al.* 1979)
		Reduced amounts in patients with stroke and lower limb disease.	Human serum	(Leng *et al.* 1999)
		Post-MI patients displayed longer life expectancy when taking EPA and DHA supplements.		(Valagussa *et al.* 1999)
		Inhibited modified LDL uptake and macropinocytosis.	Human THP-1 and HMDMs	(McLaren *et al.* 2011b)
	Docosahexaenoic acid (DHA) 22:6n-3	DHA inversely affected levels of AA thus reducing production of pro-inflammatory mediators.	Human serum and plasma	(Rallidis *et al.* 2003)
		Post-MI patients displayed longer life expectancy when taking EPA and DHA supplements.		(Valagussa *et al.* 1999)
		Inhibited modified LDL uptake and macropinocytosis.	Human THP-1 and HMDMs	(McLaren *et al.* 2011b)
n-6 PUFAs	Increased n-6/n-3 PUFA ratio, promoted oxidative stress and production of pro-inflammatory eicosanoid precursors.			(Okuyama *et al.* 2000)

	Linoleic acid (LA) 18:2n-6	Increased thromboxane A_2/ PGI_2 ratio and thrombosis.	Rat serum	(Lee *et al.* 1989)
		Levels of LA inversely associated with serum cholesterol and TAGs.	Human serum	(Renaud *et al.* 1986)
		Significantly raised amounts of LA in serum TAGs of patients with MI.	Human serum	(Leng *et al.* 1999)
		Did not affect levels of CRP, serum amyloid A or IL-6 but reduced cholesterol. Reduction associated with increased synthesis of LDLR.	Human serum and plasma	(Rallidis *et al.* 2003)
	Dihomo-γ-linolenic acid (DGLA) 20:3n-6	Raised levels of serum phospholipids and erythrocytes of MI sufferers.	Human serum and erythrocytes	(Leng *et al.* 1999)
		Levels inversely related to CAD.	Human adipose biopsies	(Riemersma *et al.* 1986).
	Arachidonic acid (AA) 20:4n-6	Increased thromboxane A_2/ PGI_2 ratio and thrombosis.	Rat serum	(Lee *et al.* 1989)
		Levels inversely related to CAD.	Human adipose biopsies	(Riemersma *et al.* 1986).

5.2. Experimental aims

The data presented within this thesis has so far supported the role of IL-33 as an anti-atherogenic agent. The cytokine down-regulates the expression of several well-characterised atherosclerotic markers within RAW264.7 and THP-1 macrophages and HMDMs. A combination of siRNA and shRNA was then utilised to elucidate the signalling pathways that underlie the IL-33-mediated regulation of ICAM-1 and MCP-1 expression within human THP-1 macrophages and primary HMDMs. The study will next focus on the temporal and inter-species effects of IL-33 on the fatty acid composition of macrophages.

The THP-1 and RAW264.7 macrophage cell lines were utilised to investigate the effects of IL-33 on the fatty acid composition of human and murine macrophages. The aim of this chapter was to determine whether IL-33 produces anti-atherogenic changes in fatty acids within TAGs, PLs and SEs. This was the first study to utilise TLC and GC to explore the changes in major lipid classes and individual fatty acid profiles caused by the cytokine. An outline of the experimental strategy employed is shown in Figure 55. Cells were prepared using a similar method to those in chapter three whereby THP-1 and RAW264.7 macrophages were incubated with IL-33 (25ng/ml) for 12 and 24 hours. Total lipids were then extracted from these cells and separated into TAGs, PLs and SEs by TLC and subsequently analysed by GC to determine the fatty acid distribution of these groups. Another aim was to explore the effects of IL-33 on SCD-1, Δ5- and Δ6-desaturase activity. Previous studies have used the ratio of product to substrate as an index of desaturase activity (Attie *et al.* 2002; Shiwaku *et al.* 2004; Warensjo *et al.* 2007; Martinelli *et al.* 2008). The following indices were used to quantify enzymatic activity:

- Δ5-desaturase = 20:4 n6/ 20:3 n6
- Δ6-desaturase = 20:3n6/18:2n6
- SCD-1 = 16:1n7/ 16:0 and 18:1n9/18:0

Previous studies have varied in the choice of SCD-1 product/precursor ratios therefore ratios of 16:1/16:0 and 18:1/18:0 were analysed both separately and together to provide a more comprehensive assessment. The expression of SCD-1 was then assessed by RT-qPCR to determine whether changes in activity were reflected at the genetic level.

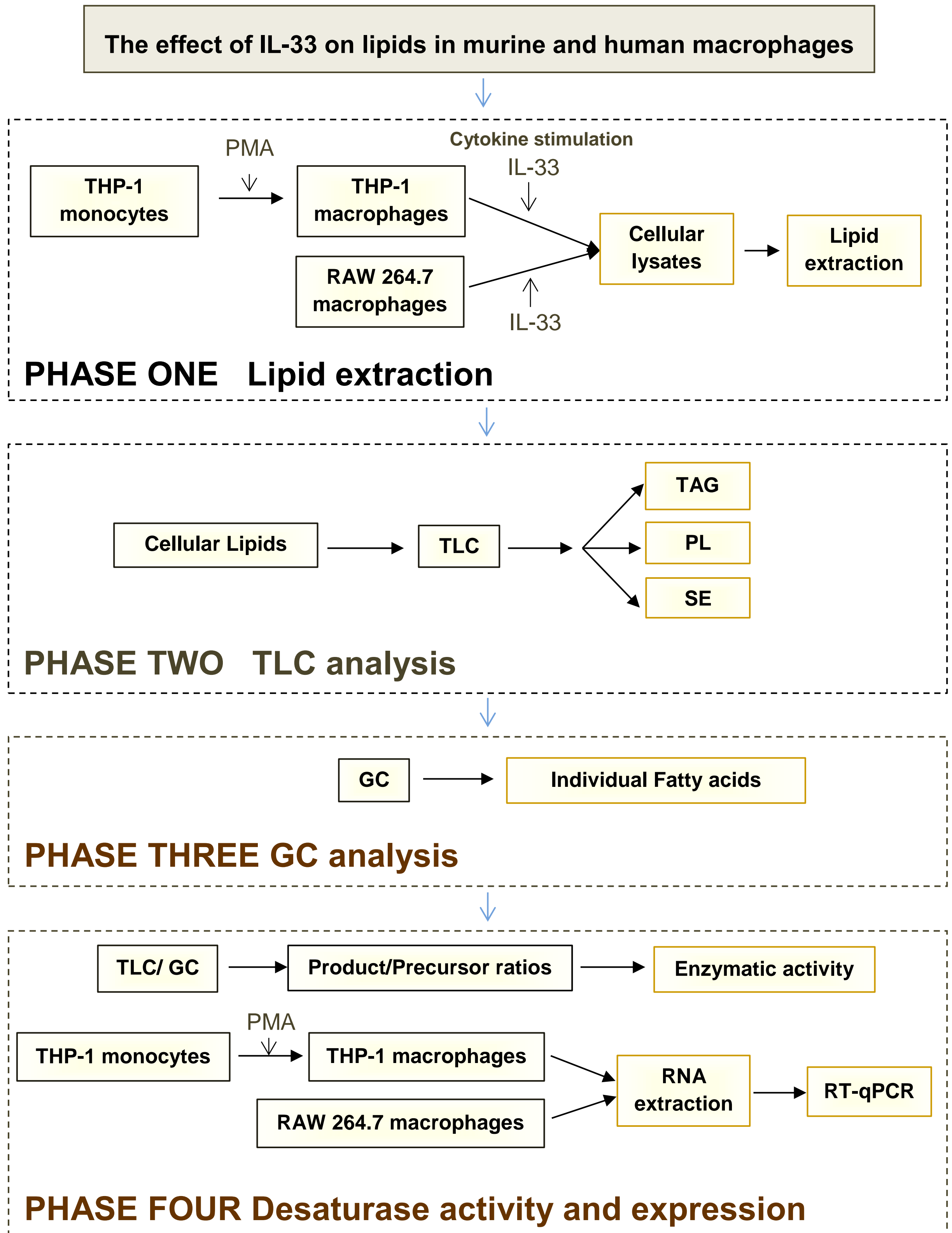


Figure 55. Schematic of experimental procedures

Lipids were extracted from THP-1 and RAW264.7 macrophages and separated by TLC into TAGs, PLs and SEs. The fatty acids of individual fractions were then converted into FAMEs for GC analysis. Enzymatic activity was calculated based upon the ratio of product to precursor and the mRNA expression of SCD-1 was assessed by RT-qPCR analysis.

5.3. Results

5.3.1. Analysis of individual lipid classes

5.3.1.1. THP-1 macrophages

Figure 56 displays the alterations in the concentrations and ratio of TAGs and PLs following incubation with IL-33 for 12 and 24 hours. TLC was performed to separate total lipids into TAGs and PLs. However, SEs were undetectable in THP-1 macrophages.

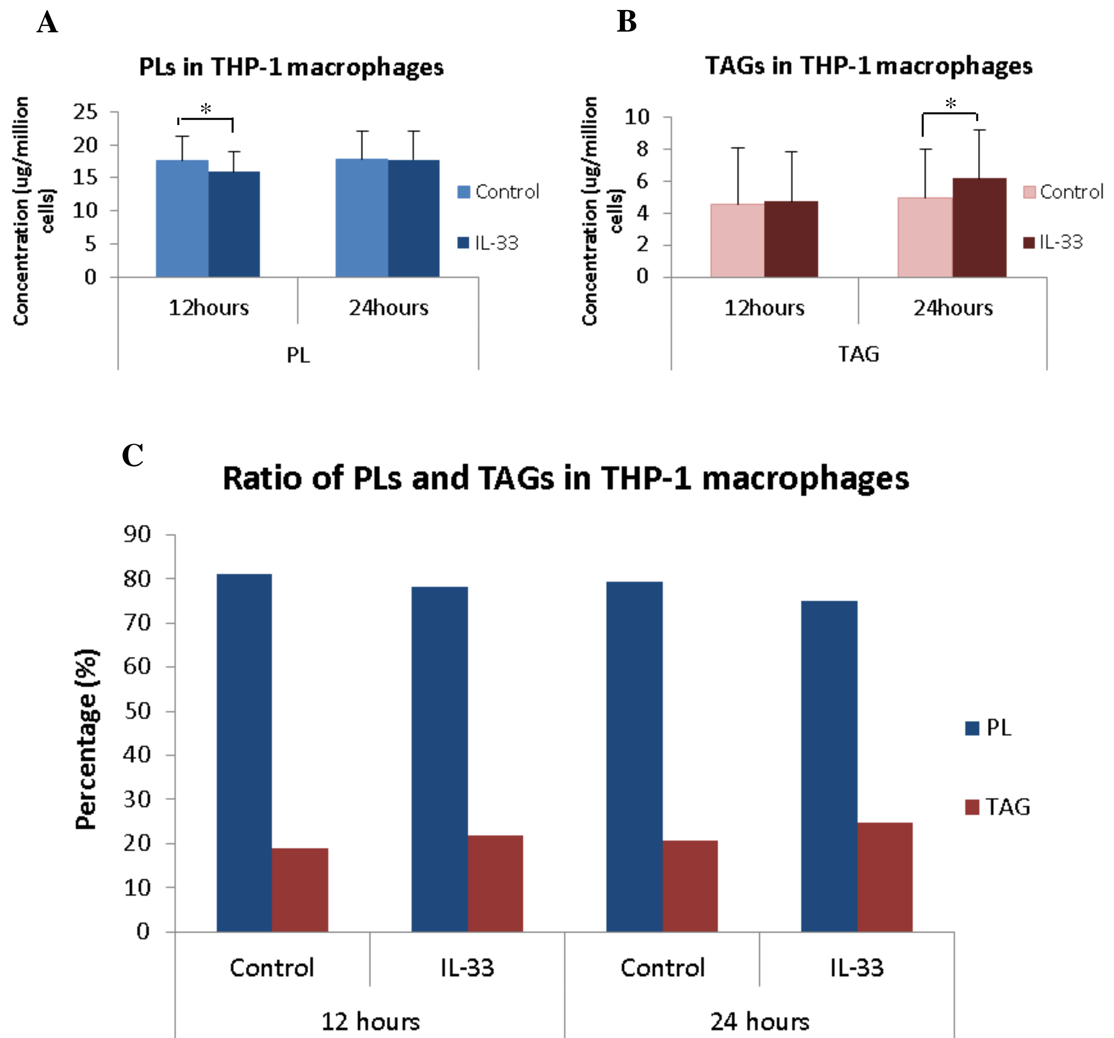


Figure 56. The effect of IL-33 on major lipid classes in THP-1 macrophages

PMA-differentiated THP-1 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. TLC was used to separate lipids into different lipids groups; PLs (A) and TAGs (B). In graphs A and B, results were normalised to µg/million cells. The ratio of major lipid groups is shown in graph C. Data represents mean ± SD from three independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05.

There was a general reduction in the levels of PLs following IL-33 treatment and this decrease was significant following 12 hours of IL-33 stimulation (Figure 56A). Conversely, there was a 24% increase in the levels of TAGs within these cells in the presence of IL-33 which was significant following 24 hours of IL-33 stimulation

(Figure 56B). Furthermore a trend was present for increased total fatty acids between 12 and 24 hours which was not significant. PLs represented the largest lipid fraction followed by TAGs (Figure 56C).

5.3.1.2. RAW 264.7 macrophages

Lipids were also extracted from RAW 264.7 macrophages and analysed by TLC/GC to determine the inter-species effect of IL-33 on the lipid composition of murine macrophages.

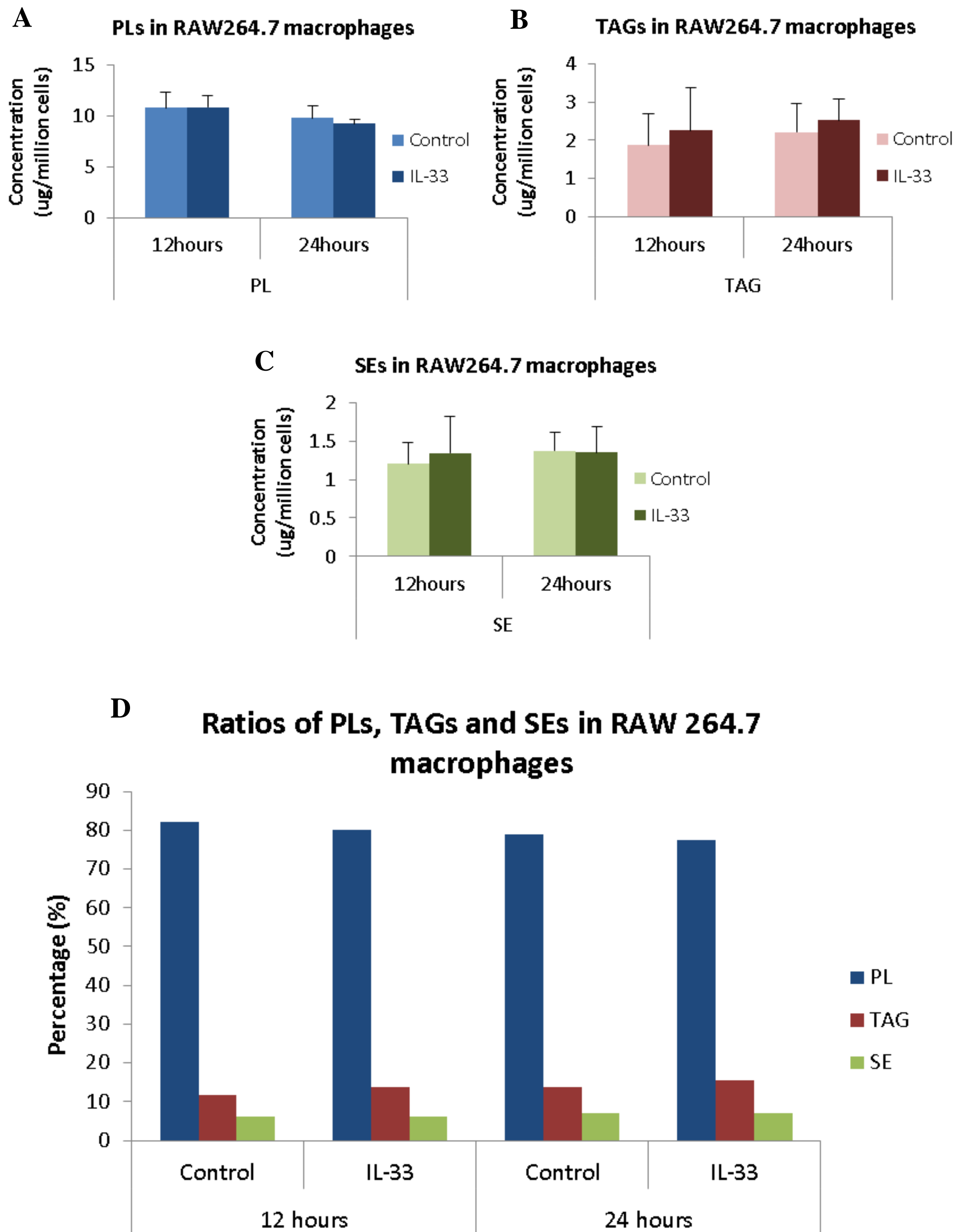


Figure 57. The effect of IL-33 on major lipid classes in RAW 264.7 macrophages

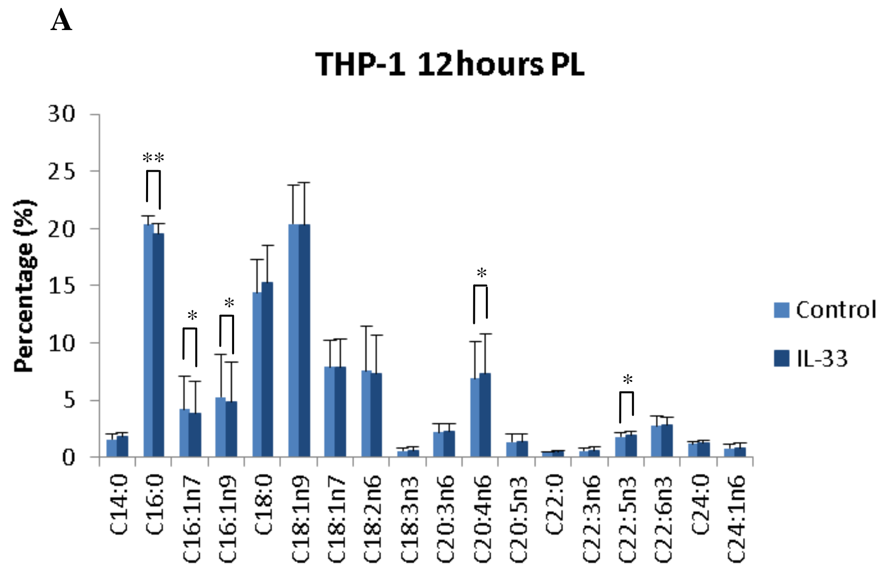
RAW264.7 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. TLC was used to separate lipids into different lipids groups; PLs (A), TAGs (B) and SEs (C). In graphs A-C, results were normalised to µg/million cells. The ratio of major lipid groups is shown in graph D. Data represents mean ± SD from three independent experiments. The Student *t* test was used to determine the statistical significance of the results.

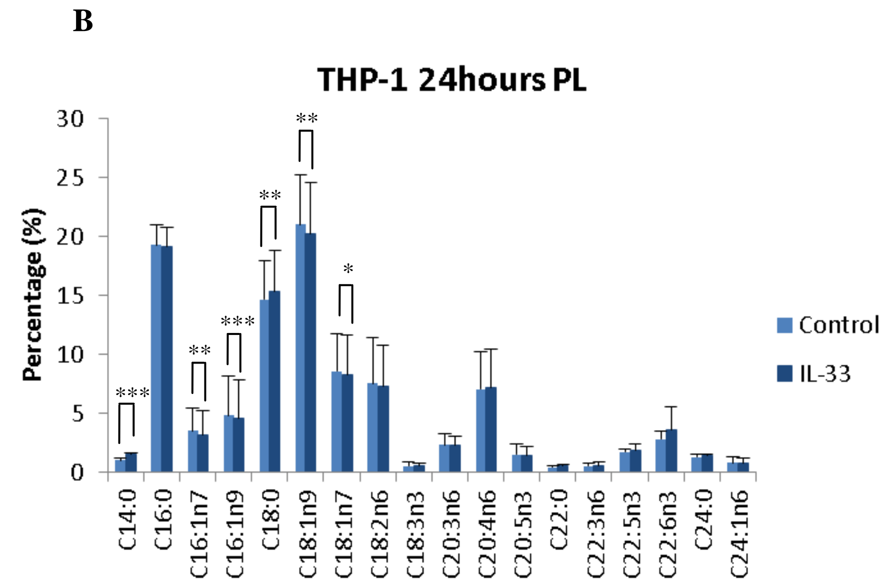
Figure 57 displays the concentration and ratio of TAGs, PLs and SEs within RAW 264.7 macrophages following IL-33 stimulation for 12 and 24 hours. Unlike human THP-1 macrophages, murine RAW 264.7 macrophages also contained detectable SEs (Figure 57C). However, there were no significant alterations in the concentration of SEs over time or following incubation with IL-33. Furthermore, IL-33 did not have a significant effect on the concentrations of TAGs or PLs, although in accordance with the THP-1 cells, there was a trend for reduced concentrations of PLs (Figure 57A) and increased concentrations of TAGs (Figure 57B) following IL-33 stimulation, though these failed to reach significance. However, unlike THP-1 macrophages, there was a noticeable decrease in levels of total fatty acids between 12 and 24 hours especially in the amounts of PLs (Figure 57D).

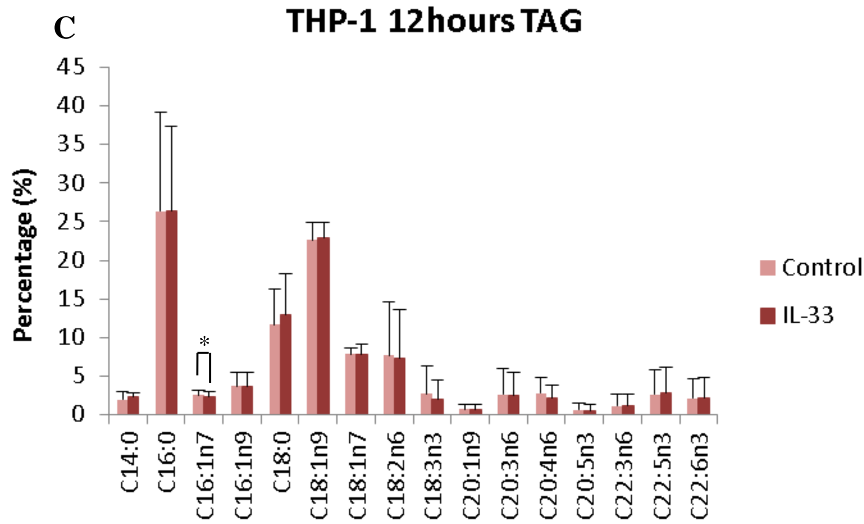
5.3.2 Fatty acid analysis

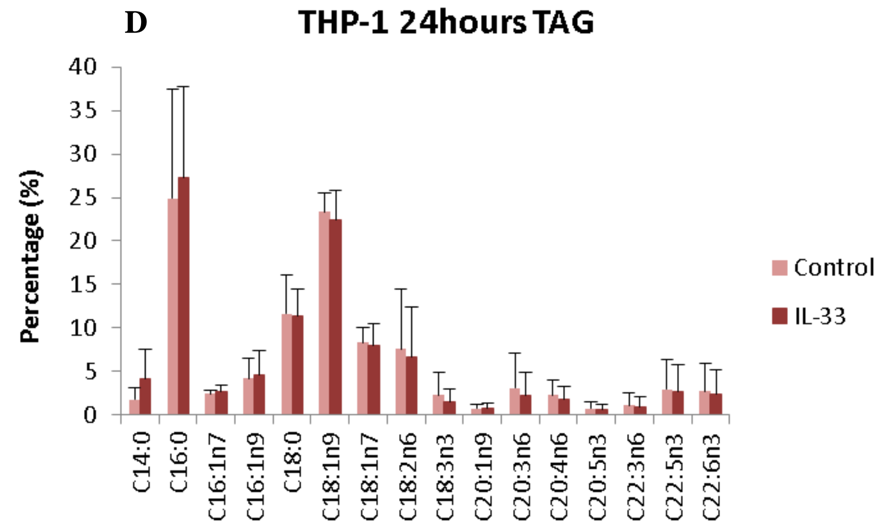
5.3.2.1 THP-1 macrophages

After separation of the lipid extracts into major lipid groups by TLC, the fatty acids were then converted to FAMEs and analysed by GC to assess the effects of 12 and 24 hours of IL-33 stimulation on the fatty acid composition of human THP-1 macrophages. The results are displayed as changes in the percentages of individual fatty acids within PLs and TAGs. This was deemed the most appropriate method to display the data as different amounts of cells were seeded for each experiment due to the large amount of material required for GC analysis. The data is also provided as concentrations in the appendices where a similar trend was seen.


A
THP-1 12hours PL
Percentage (%)
Control
IL-33
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6


B
THP-1 24hours PL
Percentage (%)
Control
IL-33
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6


C
THP-1 12hours TAG
Percentage (%)
Control
IL-33
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:3n6
C22:5n3
C22:6n3


D
THP-1 24hours TAG
Percentage (%)
Control
IL-33
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:3n6
C22:5n3
C22:6n3

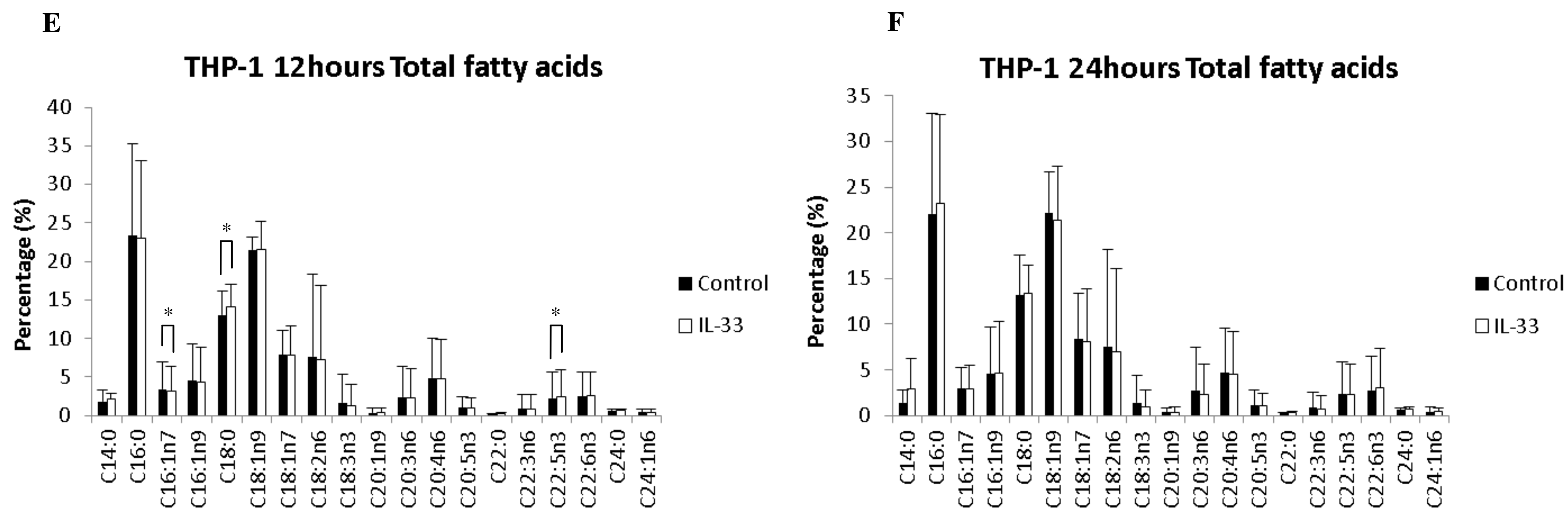
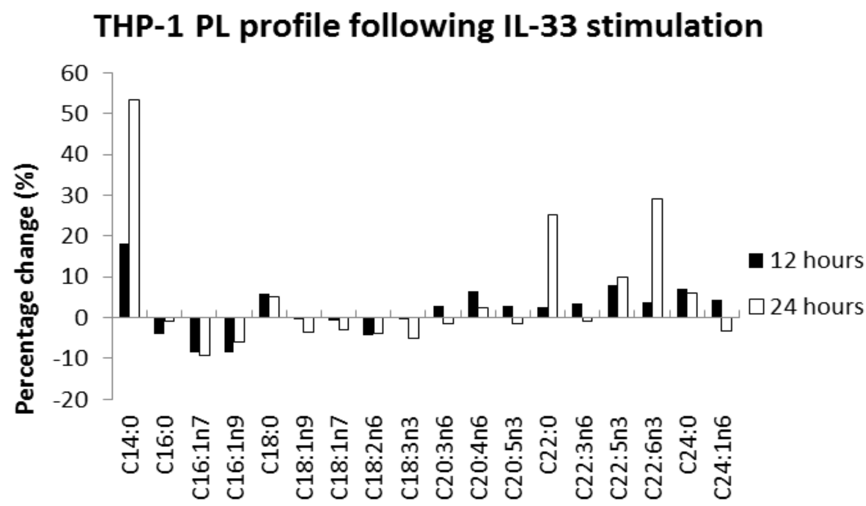
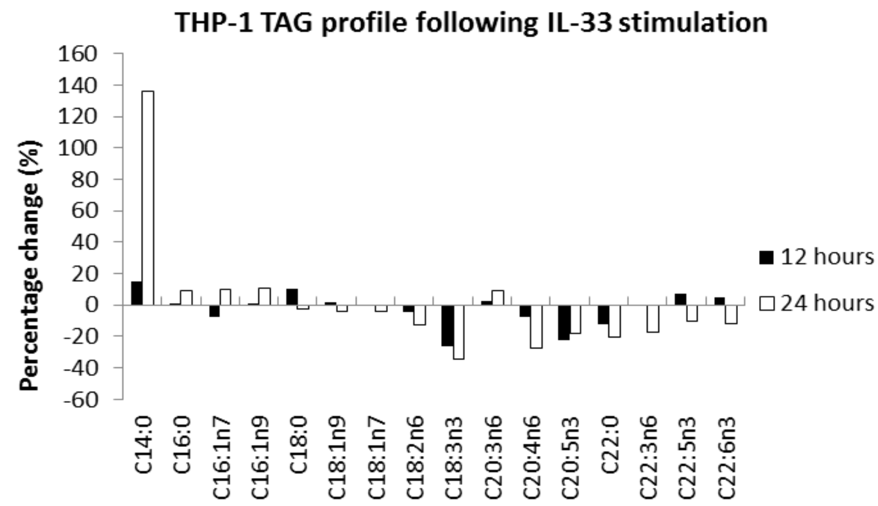


Figure 58. Fatty acid composition of THP-1 macrophages in response to IL-33

PMA-differentiated THP-1 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. Following TLC, fatty acids from fractions for PLs (Panels A and B) and TAGs (Panels C and D) were converted to FAMEs for analysis by GC. Total fatty acids (Panels E and F) represent the summation of fatty acids from PLs and TAGs. The results are presented as percentage of PLs (A and B), TAGs (C and D) or total fatty acids (E and F). Fatty acids are indicated with the number before the colon showing the number of carbon atoms, after which denotes the number of double bonds followed by the position of the first double bond. Data represents mean ± SD from four independent experiments. The Student *t* test was used to determine the statistical significance of the results *$p<0.05$, **$p<0.01$, ***$p<0.001$.





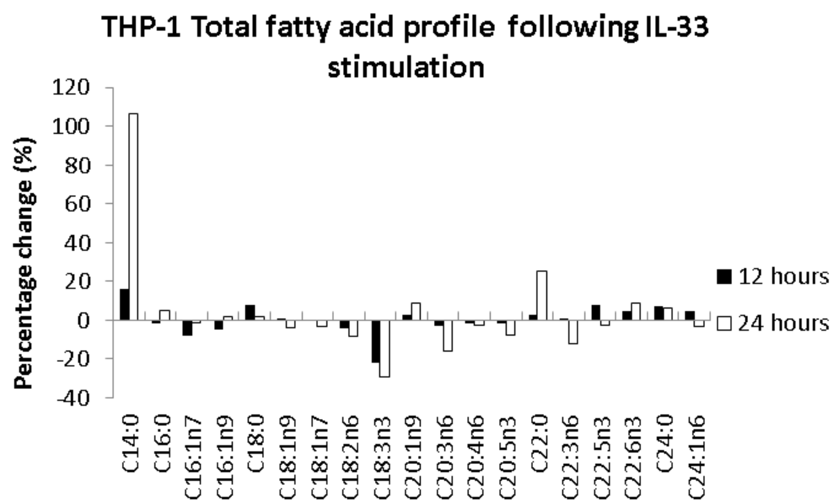


Figure 59. Change in the percentages of fatty acids in THP-1 macrophages

PMA-differentiated THP-1 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. Lipids were separated by TLC into different lipids groups; PLs and TAGs, and then the individual fatty acid composition of these fractions was assessed by GC. Total fatty acids represent the summation of PLs and TAGs. Fatty acids are indicated with the number before the colon showing the number of carbon atoms, after which denotes the number of double bonds followed by the position of the first double bond. Changes in the percentages of individual fatty acids following IL-33 stimulation are presented for PLs, TAGs or total fatty acids. Data represents mean from four independent experiments.

Figures 58 and 59 show the changes in the percentages of fatty acids in THP-1 macrophages following 12 and 24 hours of IL-33 stimulation and a summary is provided in Table 20. Within PLs IL-33 significantly increased the levels of C20:4n6 (AA) and C22:5n3 (DPA) following 12 hours of stimulation. After 24 hours of IL-33 treatment, there was a significant increase in C14:0 (myristic acid) and C18:0 (stearic acid). A significant reduction occurred after 12 hours of IL-33 stimulation in the percentages of C16:0 (palmitic acid), C16:1n7 and C16:1n9 and after 24 hours in C16:1n7, C16:1n9, C18:1n9 (oleic acid) and C18:1n7 (vaccenic acid). Within TAGs, IL-33 significantly decreased levels of C16:1n7 following 12 hours of IL-33 stimulation (Figure 58C). Within total fatty acids, taken as a combination of fatty acids from TAG and PL fractions; C16:1n7 was significantly reduced whereas levels of stearic acid and DPA significantly increased after 12 hours of IL-33 stimulation. Similar alterations in fatty acid composition were exhibited following 12 and 24 hours of incubation with IL-33. Figure 59 demonstrates that the largest change in percentage occurred in myristic acid in the presence of IL-33 for 24 hours across fractions, although this change was not significant. A similar number of significant changes occurred at both time points with the majority taking place within the PL fraction.

Table 20. Changes in fatty acid composition following IL-33 stimulation in THP-1 macrophages

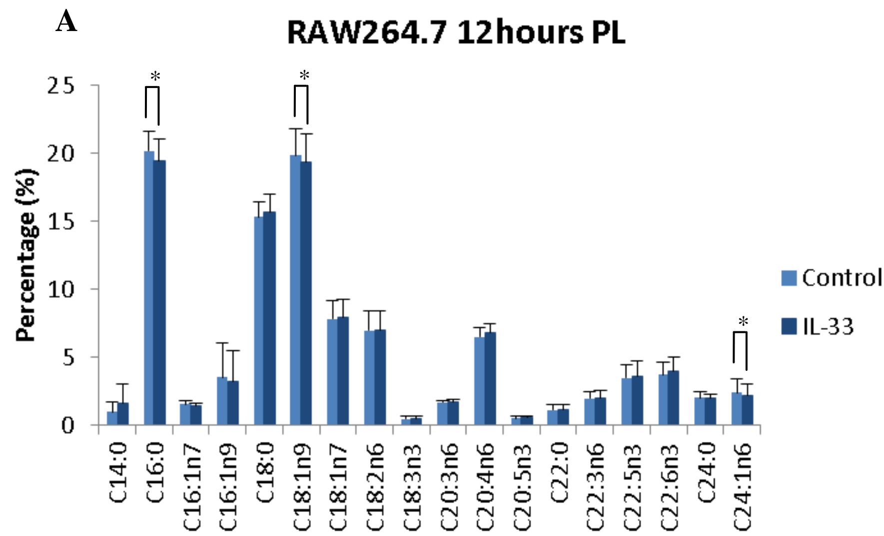
THP-1 macrophages												
Fatty acid	**PL**				**TAG**				**TOTAL**			
	12 hours		24 hours		12 hours		24 hours		12 hours		24 hours	
	Percentage change	Ttest	Percentage change	Ttest	Percentage change	Ttest	Percentage change	Ttest	Percentage change	Ttest	Percentage change	Ttest
C14:0	18.18	↑N/S	53.42	↑***	14.66	↑ N/S	136.46	↑ N/S	16.20	↑ N/S	106.59	↑ N/S
C16:0	-3.88	↓**	-0.89	↓ N/S	0.07	↑ N/S	9.41	↑ N/S	-1.65	↓ N/S	4.91	↑ N/S
C16:1n7	-8.61	↓*	-9.21	↓**	-7.89	↓*	10.30	↑ N/S	-8.34	↓*	-1.24	↓ N/S
C16:1n9	-8.42	↓*	-5.95	↓***	0.18	↑ N/S	10.99	↑ N/S	-4.85	↓ N/S	1.91	↑ N/S
C18:0	5.87	↑ N/S	5.05	↑**	10.62	↑ N/S	-2.24	↓ N/S	8.00	↑*	1.83	↑ N/S
C18:1n9	-0.49	↓ N/S	-3.66	↓**	1.49	↑ N/S	-4.06	↓ N/S	0.55	↑ N/S	-3.87	↓ N/S
C18:1n7	-0.72	↓ N/S	-2.94	↓*	-0.78	↓ N/S	-4.26	↓ N/S	-0.75	↓ N/S	-3.59	↓ N/S
C18:2n6	-4.22	↓ N/S	-3.94	↓ N/S	-4.11	↓ N/S	-12.71	↓ N/S	-4.17	↓ N/S	-8.33	↓ N/S
C18:3n3	-0.51		-5.03	↓ N/S	-26.22	↓ N/S	-34.74	↓ N/S	-22.06	↓ N/S	-28.99	↓ N/S
C20:1n9					2.63	↑ N/S	8.91	↑ N/S	2.63	↑ N/S	8.91	↑ N/S
C20:3n6	3.04	↑ N/S	-1.55	↓ N/S	-7.75	↓ N/S	-27.47	↓ N/S	-2.91	↓ N/S	-16.27	↓ N/S
C20:4n6	6.45	↑*	2.37	↑ N/S	-22.20	↓ N/S	-18.09	↓ N/S	-1.85	↓ N/S	-2.55	↓ N/S
C20:5n3	2.92	↑ N/S	-1.51	↓ N/S	-11.90	↓ N/S	-20.65	↓ N/S	-2.01	↓ N/S	-7.59	↓ N/S
C22:0	2.50	↑ N/S	25.23	↑ N/S					2.50	↑ N/S	25.23	↑ N/S
C22:3n6	3.63	↑ N/S	-0.89	↓ N/S	-0.37	↓ N/S	-17.62	↓ N/S	0.91	↑ N/S	-12.06	↓ N/S
C22:5n3	8.10	↑*	10.02	↑ N/S	7.25	↑ N/S	-10.19	↓ N/S	7.59	↑*	-2.78	↓ N/S
C22:6n3	3.84	↑ N/S	29.16	↑ N/S	4.98	↑ N/S	-11.97	↓ N/S	4.34	↑ N/S	8.77	↑ N/S
C24:0	7.03	↑ N/S	6.14	↑ N/S					7.03	↑ N/S	6.14	↑ N/S
C24:1n6	4.52	↑ N/S	-3.26	↓ N/S					4.52	↑ N/S	-3.26	↓ N/S

The Student *t* test was used to determine the statistical significance of the results *p<0.05, **p<0.01, ***p<0.001, N/S not significant. Arrows depict direction of change following IL-33 stimulation.

5.3.2.2. RAW 264.7 macrophages

GC analysis was performed on the PL, TAG and SE fractions to determine the effects of IL-33 on the fatty acid composition of murine macrophages after 12 and 24 hours.

A



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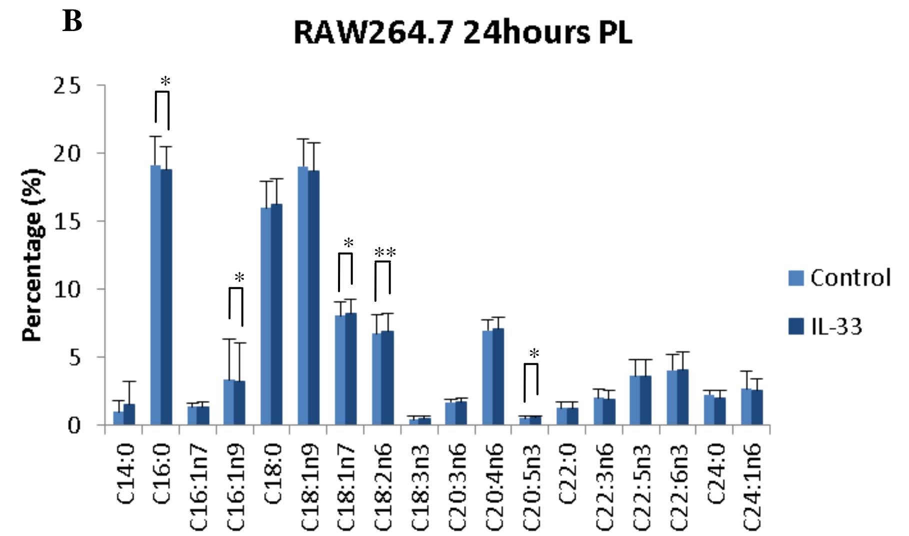

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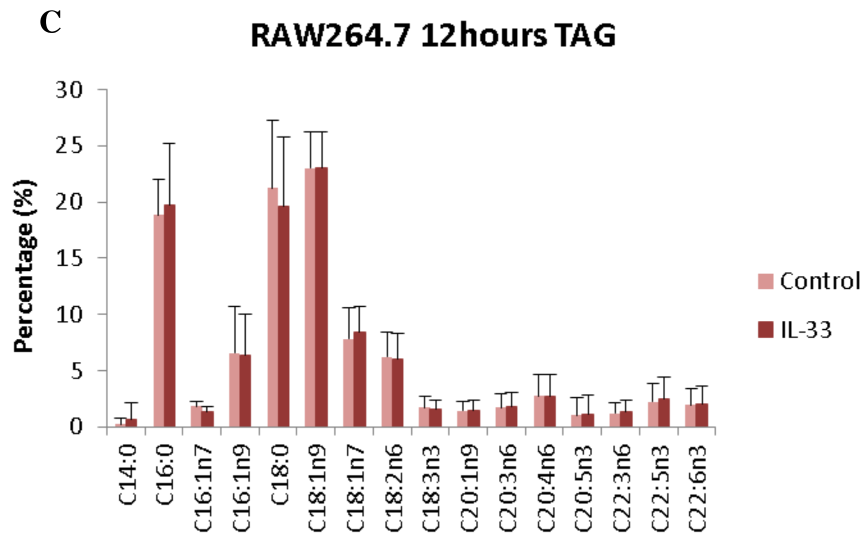

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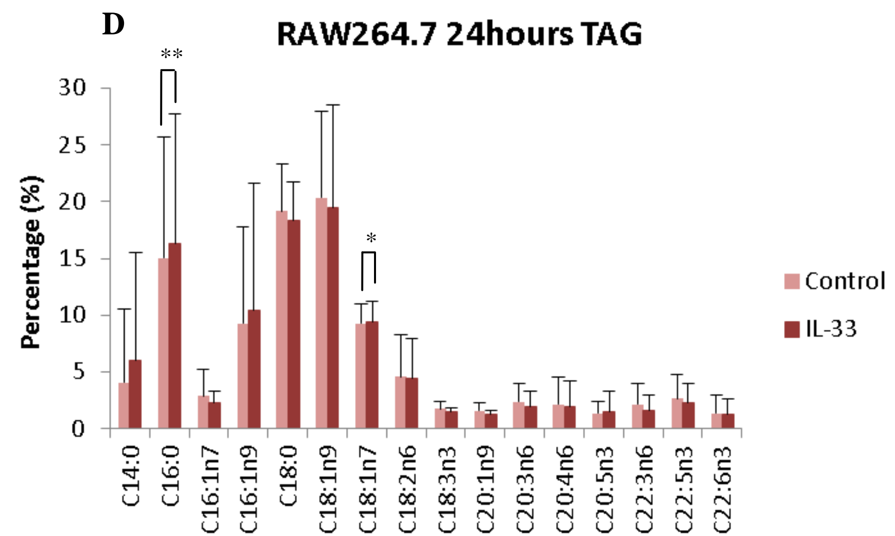

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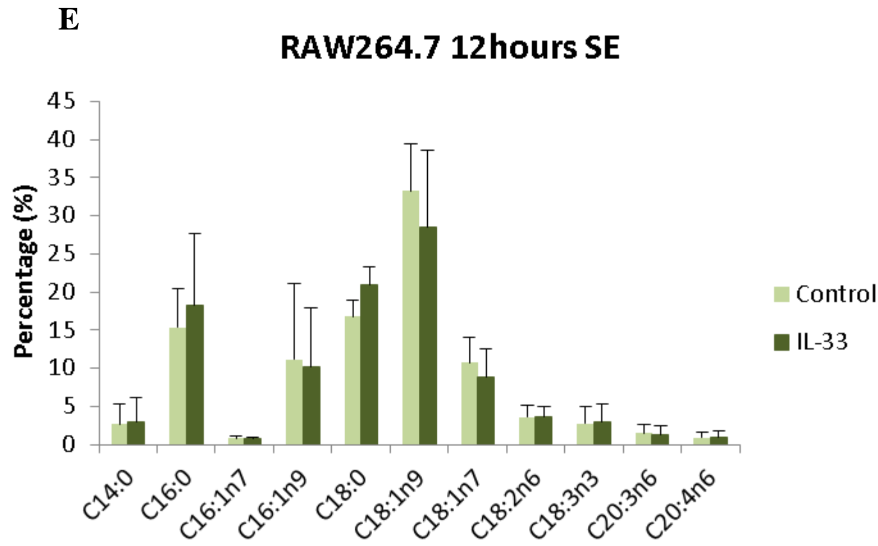

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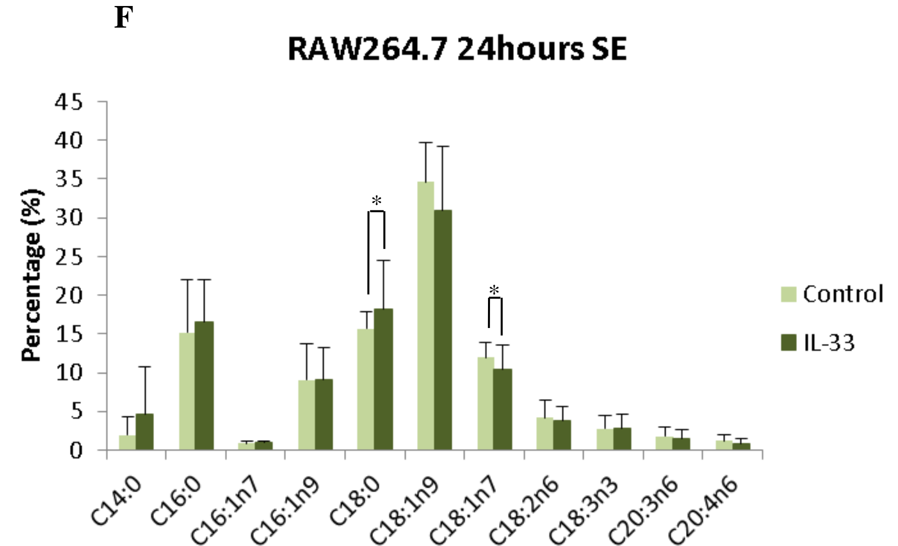

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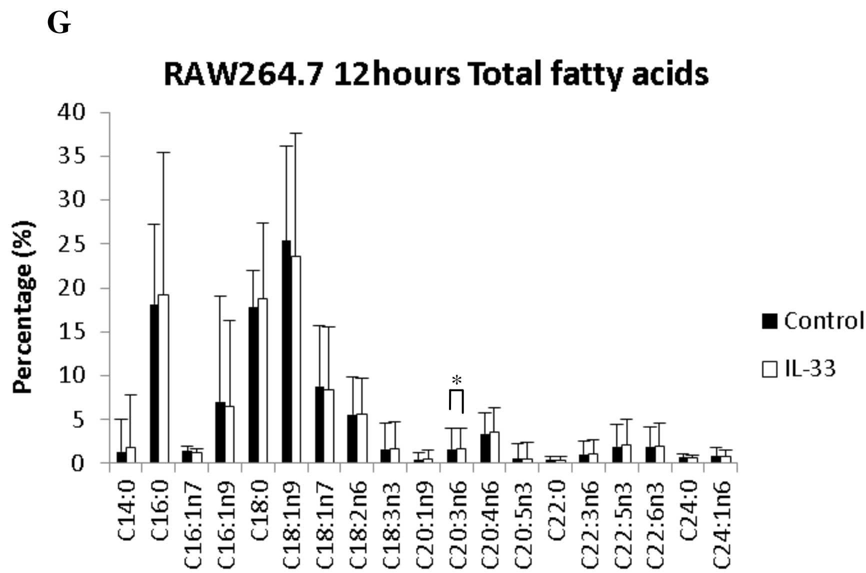

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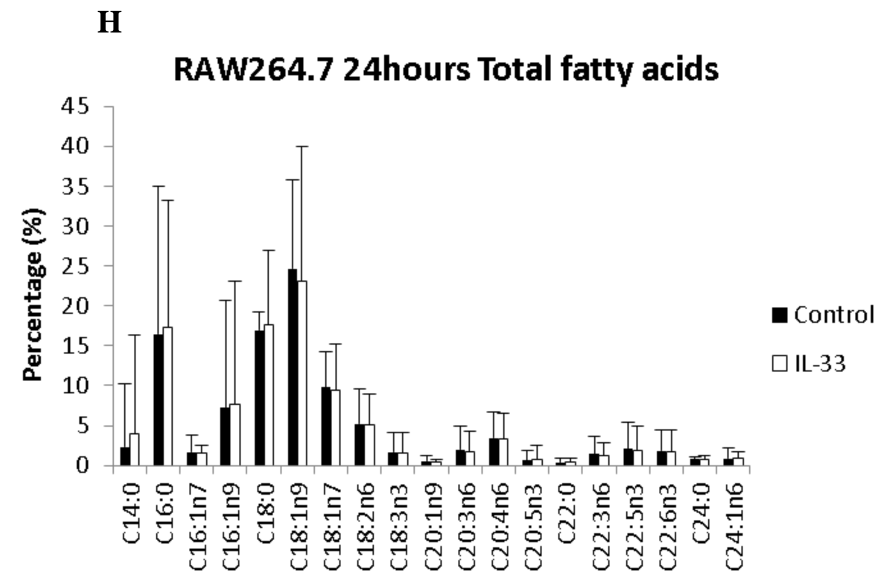
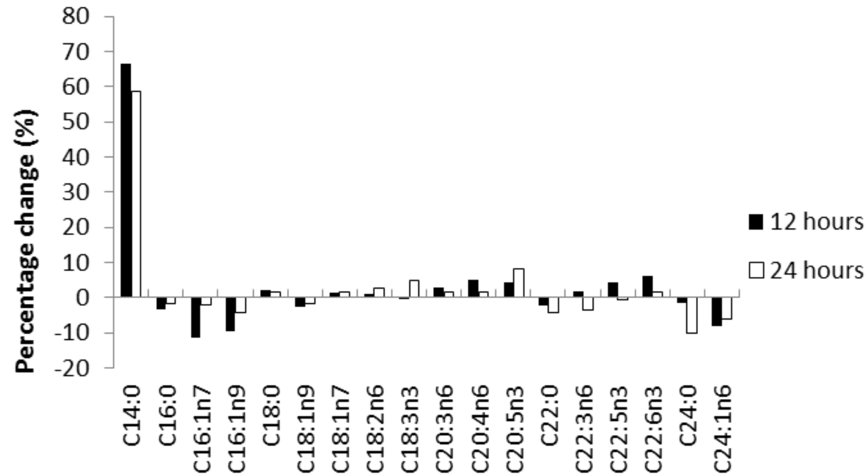
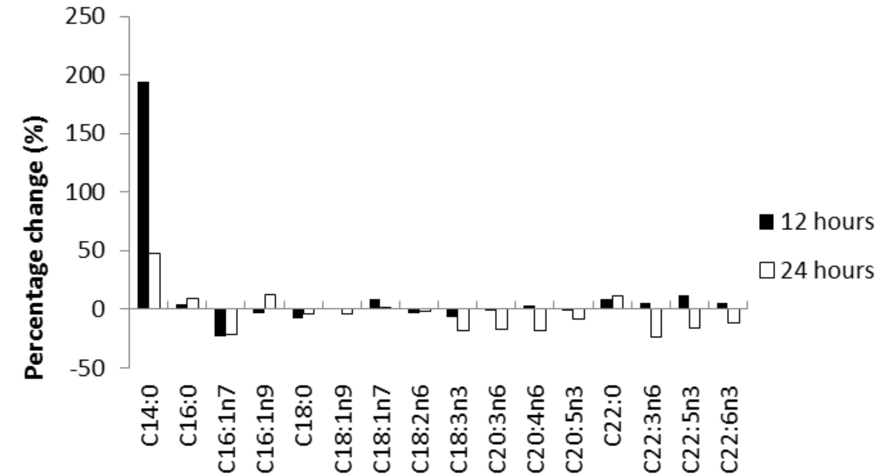
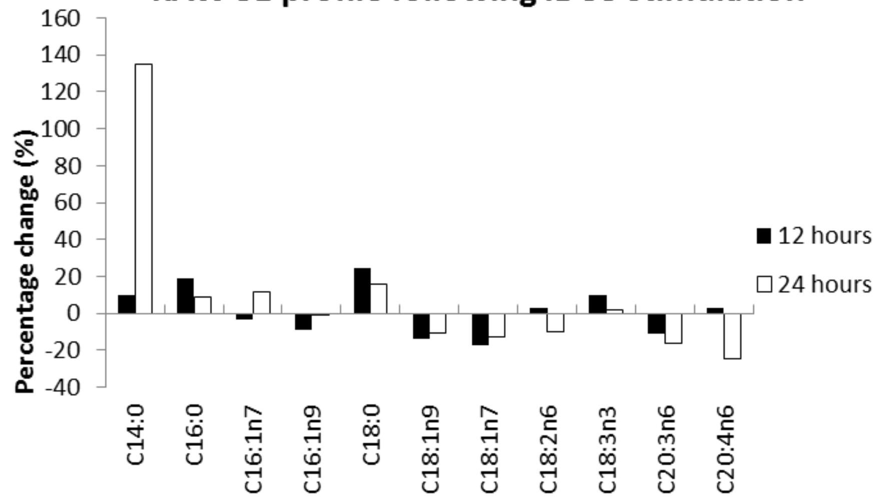


Figure 60. Fatty acid composition of RAW 264.7 macrophages in response to IL-33

RAW 264.7 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. Following TLC, fatty acids from the fractions for PLs (Panels A and B), TAGs (Panels C and D) and SEs (Panels E and F) were converted to FAMEs for analysis by GC. Total fatty acids (Panels G and H) represent the summation of PLs, TAGs and SEs. The results are presented as percentage of PLs (A and B), TAGs (C and D), SEs (E and F) or total fatty acids (G and H). Fatty acids are indicated with the number before colon showing the number of carbon atoms, after which denotes the number of double bonds followed by the position of the first double bond. Data represents mean ± SD from four independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05, **p<0.01.


RAW PL profile following IL-33 stimulation
Percentage change (%)
80
70
60
50
40
30
20
10
0
-10
-20
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6
12 hours
24 hours


RAW TAG profile following IL-33 stimulation
Percentage change (%)
250
200
150
100
50
0
-50
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
12 hours
24 hours


RAW SE profile following IL-33 stimulation
Percentage change (%)
160
140
120
100
80
60
40
20
0
-20
-40
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:3n6
C20:4n6
12 hours
24 hours

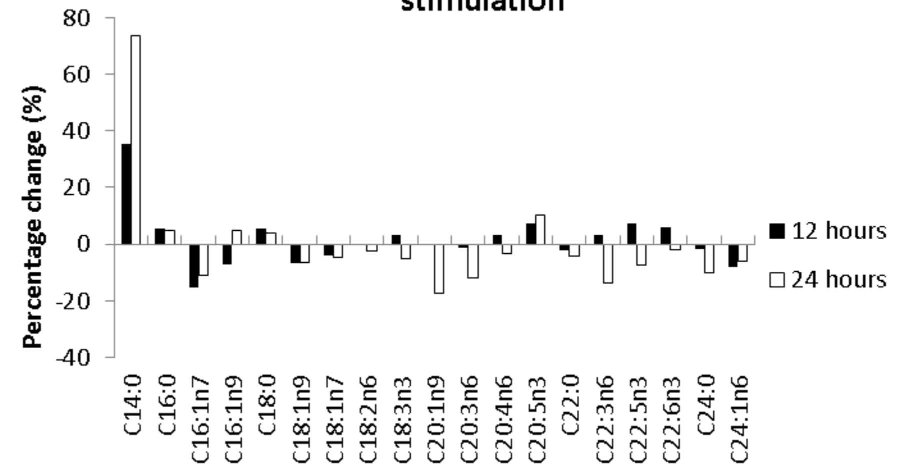

RAW Total fatty acid profile following IL-33 stimulation
Percentage change (%)
80
60
40
20
0
-20
-40
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6
12 hours
24 hours

Figure 61. Change in percentages of fatty acids

RAW 264.7 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. Lipids were separated by TLC into different lipids groups for PLs, TAGs and SEs and then the individual fatty acid composition of these fractions was assessed by GC. Total fatty acids represent the summation of PLs, TAGs and SEs. Changes in the percentages of individual fatty acids following IL-33 stimulation are presented for PLs, TAGs, SEs or total fatty acids. Fatty acids are indicated with the number before the colon showing the number of carbon atoms, after which denotes the number of double bonds followed by the position of the first double bond. Data represents mean from four independent experiments.

The changes in fatty acid percentages following IL-33 stimulation in RAW264.7 macrophages are shown in Figures 60 and 61. IL-33 caused a significant reduction in palmitic acid (C16:0) at both time points and decreased levels of oleic acid (C18:1n9) and nervonic acid (C24:1n6) after 12 hours and C16:1n9 after 24 hours in PLs. There were also significant increases in the percentages of vaccenic acid (C18:1n7), LA (C18:2n6) and EPA (C20:5n3) following 24 hours of IL-33 stimulation in PLs. IL-33 treatment caused a significant increase in the percentages of palmitic acid and vaccenic acid in TAGs after 24 hours of IL-33 treatment. Cytokine treatment resulted in a significant rise in stearic acid accompanied with a reduction in vaccenic acid in SEs after 24 hours of cytokine treatment. Total fatty acids were calculated as a summation of PLs, TAGs and SEs (Figures 60G and H). IL-33 significantly decreased the percentage of total DGLA (C20:3n6) after 12 hours. Figure 61 displays the extent of these changes in percentages. In accordance to THP-1 macrophages, the highest number of significant changes occurred in PLs and the greatest change in percentage occurred in myristic acid within each fraction but was not significant. The results are summarised in Table 21.

Table 21. Changes in fatty acid composition following IL-33 stimulation in RAW264.7 macrophages

RAW264.7 macrophages																
Fatty acid	**PL**				**TAG**				**SE**				**TOTAL**			
	12 hours		24 hours		12 hours		24 hours		12 hours		24 hours		12 hours		24 hours	
	Percentage change	Ttest	Percentage change	Ttest	Percentage change	Ttest	Percentage change	Ttest	Percentage change	Ttest	Percentage change	Ttest	Percentage change	Ttest	Percentage change	Ttest
C14:0	66.45	↑N/S	58.52	↑ N/S	194.324	↑ N/S	47.50	↑ N/S	9.77	↑ N/S	135.28	↑ N/S	35.43	↑ N/S	73.65	↑ N/S
C16:0	-3.46	↓*	-1.60	↓*	4.471	↑ N/S	8.77	↑*	18.84	↑ N/S	9.13	↑ N/S	5.60	↑ N/S	4.86	↑ N/S
C16:1n7	-11.45	↓ N/S	-2.21	↓ N/S	-23.743	↓ N/S	-21.60	↓ N/S	-3.53	↓ N/S	11.93	↑ N/S	-15.27	↓ N/S	-10.92	↓ N/S
C16:1n9	-9.46	↓ N/S	-4.33	↓*	-3.232	↓ N/S	12.85	↑ N/S	-8.87	↓ N/S	-0.32	↓ N/S	-7.22	↓ N/S	4.70	↑ N/S
C18:0	2.21	↑ N/S	1.57	↑ N/S	-7.464	↓ N/S	-4.19	↓ N/S	24.29	↑ N/S	15.70	↑*	5.31	↑ N/S	3.76	↑ N/S
C18:1n9	-2.68	↓*	-1.89	↓ N/S	0.263	↑ N/S	-4.20	↓ N/S	-14.00	↓ N/S	-10.75	↓ N/S	-6.74	↓ N/S	-6.67	↓ N/S
C18:1n7	1.49	↑ N/S	1.67	↑*	8.301	↑ N/S	1.13	↑*	-17.26	↓ N/S	-13.02	↓*	-4.10	↓ N/S	-4.50	↓ N/S
C18:2n6	1.17	↑ N/S	2.53	↑**	-3.559	↓ N/S	-2.05	↓ N/S	2.95	↑ N/S	-10.31	↓ N/S	-0.21	↓ N/S	-2.31	↓ N/S
C18:3n3	-0.36	↓ N/S	5.00	↑ N/S	-6.378	↓ N/S	-18.13	↓ N/S	10.03	↑ N/S	1.61	↑ N/S	3.30	↑ N/S	-5.16	↓ N/S
C20:1n9					-0.323	↓ N/S	-17.61	↓ N/S					-0.32	↓ N/S	-17.61	↓ N/S
C20:3n6	2.66	↑ N/S	1.43	↑ N/S	3.005	↑ N/S	-18.01	↓ N/S	-10.95	↓ N/S	-16.31	↓ N/S	-1.39	↓*	-11.81	↓ N/S
C20:4n6	5.14	↑ N/S	1.70	↑ N/S	-1.362	↓ N/S	-8.57	↓ N/S	3.07	↑ N/S	-24.82	↓ N/S	3.19	↑ N/S	-3.52	↓ N/S
C20:5n3	4.11	↑ N/S	8.07	↑*	8.540	↑ N/S	10.81	↑ N/S					7.10	↑ N/S	10.06	↑ N/S
C22:0	-2.28	↓ N/S	-4.41	↓ N/S									-2.28	↓ N/S	-4.41	↓ N/S
C22:3n6	1.77	↑ N/S	-3.67	↓ N/S	5.068	↑ N/S	-23.52	↓ N/S					3.04	↑ N/S	-13.97	↓ N/S
C22:5n3	4.42	↑ N/S	-0.74	↓ N/S	11.844	↑ N/S	-16.04	↓ N/S					7.34	↑ N/S	-7.23	↓ N/S
C22:6n3	6.08	↑ N/S	1.52	↑ N/S	5.456	↑ N/S	-11.62	↓ N/S					5.87	↑ N/S	-1.82	↓ N/S
C24:0	-1.76	↓ N/S	-10.27	↓ N/S									-1.76	↓ N/S	-10.27	↓ N/S
C24:1n6	-8.31	↓*	-6.09	↓ N/S									-8.31	↓ N/S	-6.09	↓ N/S

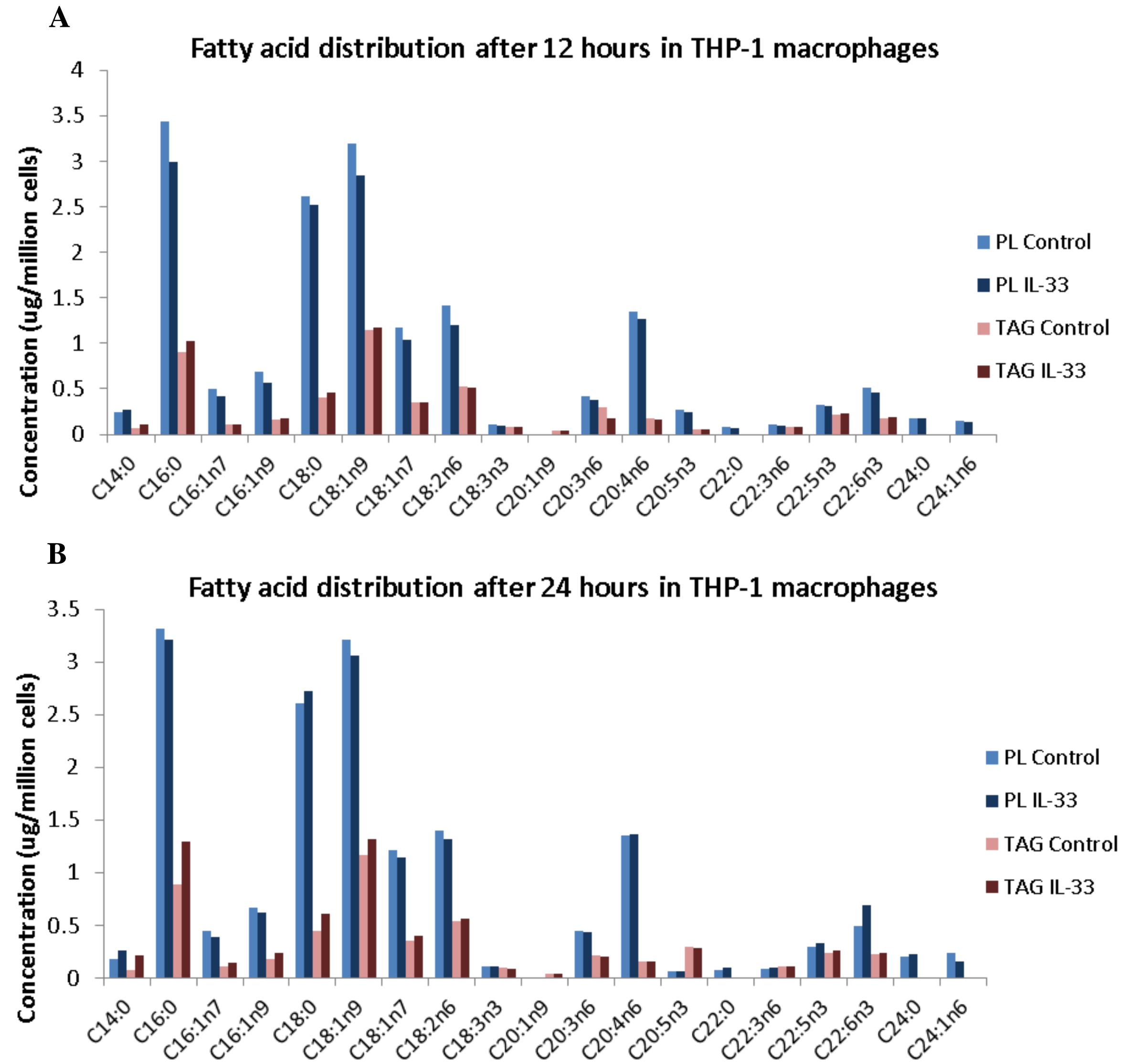
The Student *t* test was used to determine the statistical significance of the results *$p<0.05$, **$p<0.01$, ***$p<0.001$, N/S not significant. Arrows depict direction of change following IL-33 stimulation.

5.3.3. Distribution of fatty acids

Using the results from GC, the data was analysed to determine the distribution of specific fatty acids within human THP-1 macrophages and murine RAW264.7 macrophages. The data are presented both in terms of concentration to enable observation of relative abundances of fatty acids in each fraction, and also as percentages to demonstrate the changes in the proportions of specific fatty acids within each fraction. A similar trend within the data was seen using both analytical methods.

5.3.3.1. THP-1 fatty acid distribution

The concentration and percentages of fatty acids in PLs and TAGs within THP-1 macrophages are shown in Figure 62.


A
Fatty acid distribution after 12 hours in THP-1 macrophages
Concentration (ug/million cells)
4
3.5
3
2.5
2
1.5
1
0.5
0
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6
PL Control
PL IL-33
TAG Control
TAG IL-33
B
Fatty acid distribution after 24 hours in THP-1 macrophages
Concentration (ug/million cells)
3.5
3
2.5
2
1.5
1
0.5
0
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6
PL Control
PL IL-33
TAG Control
TAG IL-33

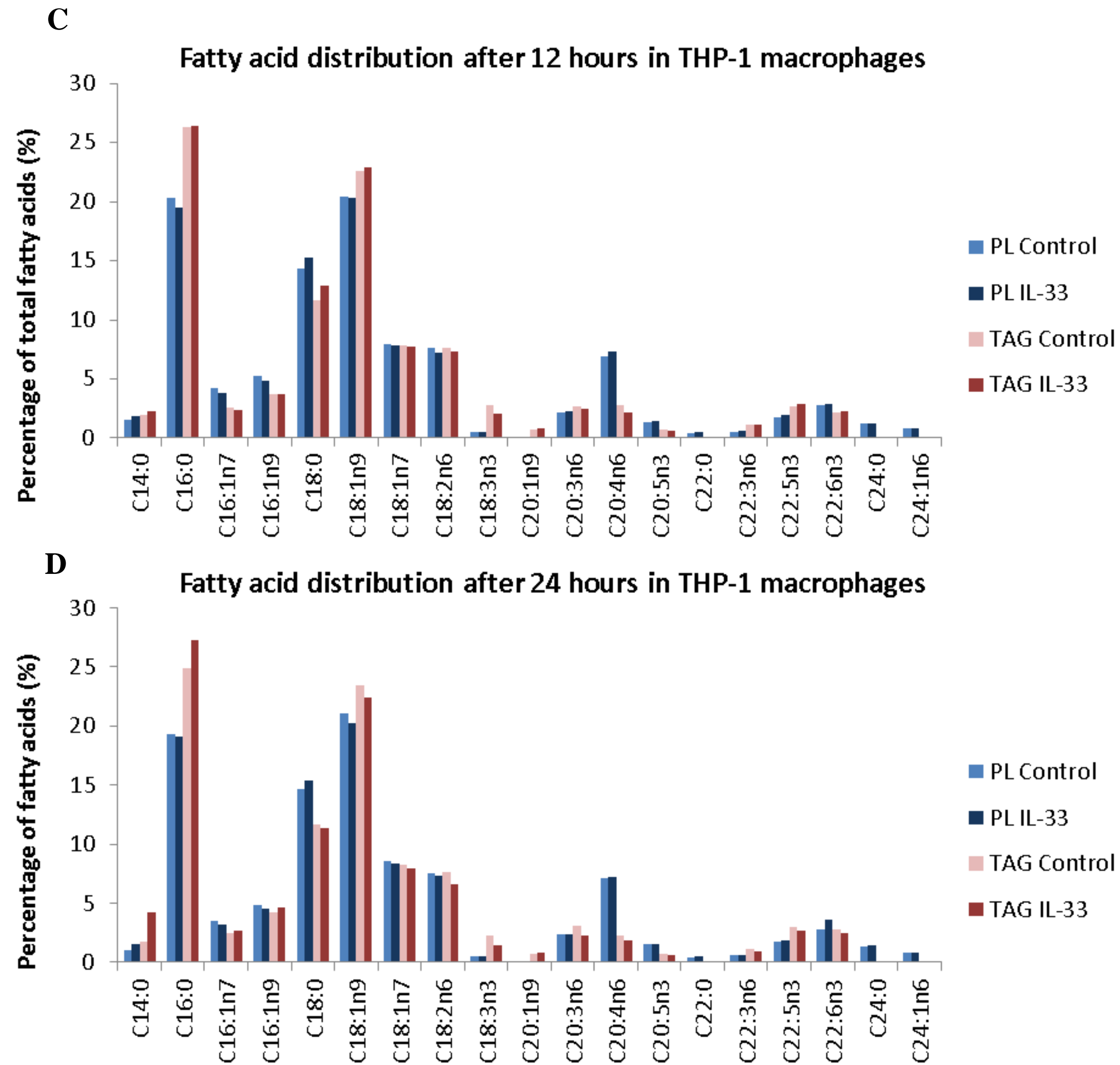

C
Fatty acid distribution after 12 hours in THP-1 macrophages
Percentage of total fatty acids (%)
30
25
20
15
10
5
0
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6
PL Control
PL IL-33
TAG Control
TAG IL-33
D
Fatty acid distribution after 24 hours in THP-1 macrophages
Percentage of fatty acids (%)
30
25
20
15
10
5
0
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6
PL Control
PL IL-33
TAG Control
TAG IL-33

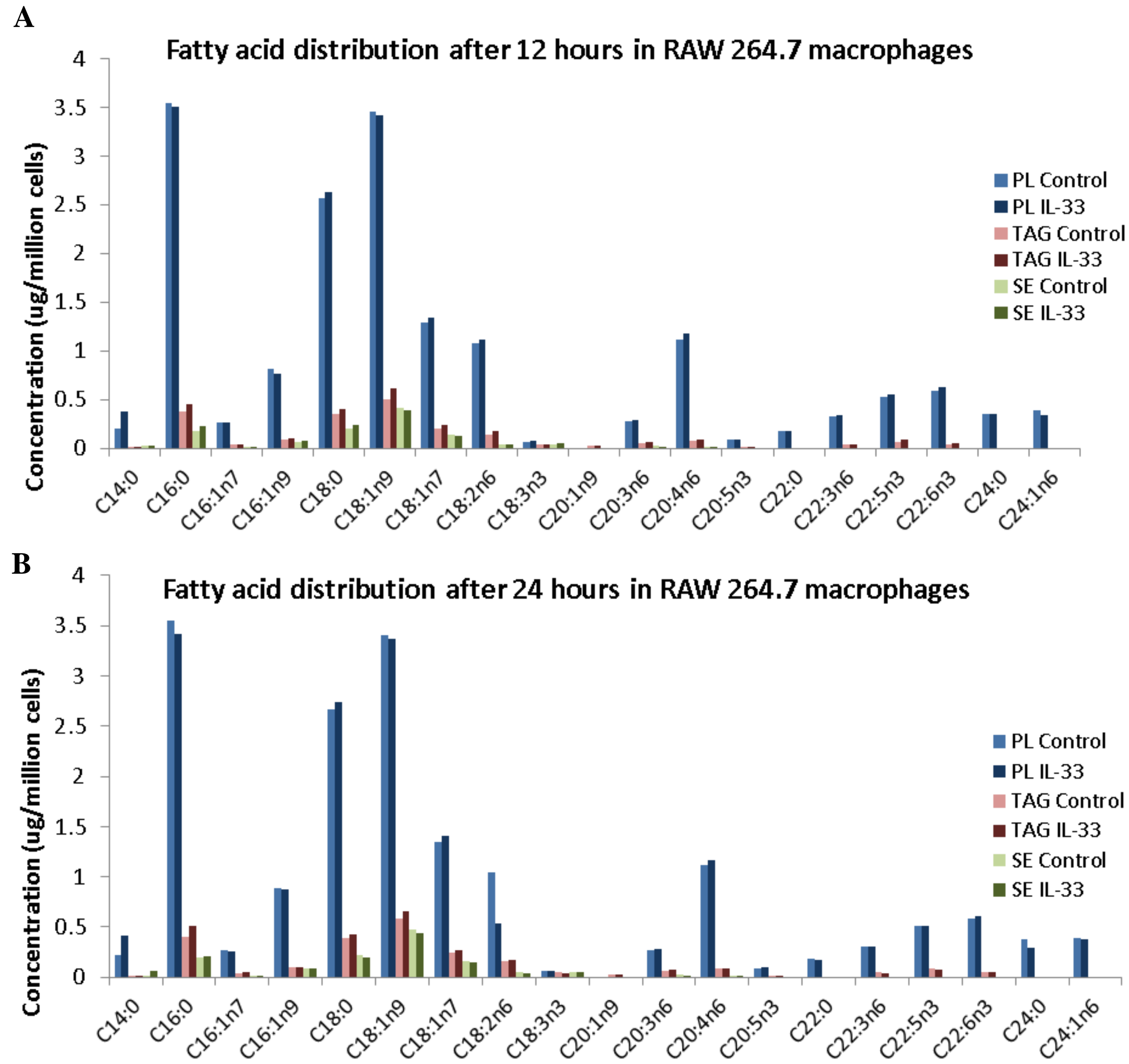
Figure 62. Distribution of fatty acids in THP-1 macrophages

PMA-differentiated THP-1 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. Lipids were separated by TLC into different lipids groups; PLs and TAGs, and the individual fatty acid composition of these fractions was assessed by GC. The concentration was normalised to µg/million cells (Panels A and B), and percentages of total fatty acids (Panels C and D). Fatty acids are indicated with the number before the colon showing the number of carbon atoms, after which denotes the number of double bonds followed by the position of the first double bond. Data represents mean from three (A and B) and four (C and D) independent experiments.

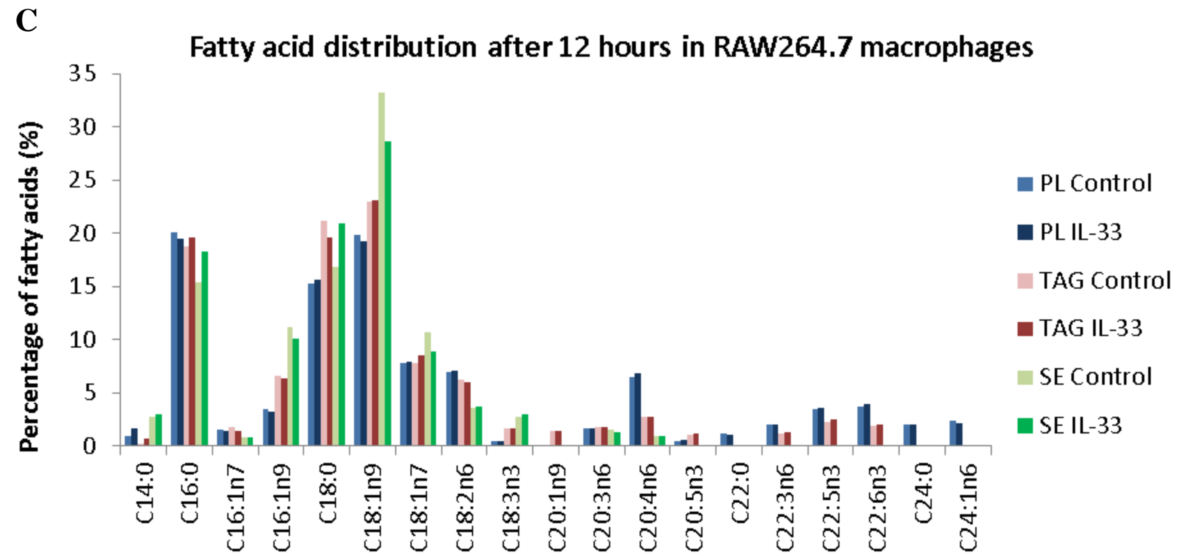
For both time points, PLs represented the largest fraction based upon fatty acid concentrations (Figures 62A and 62B). Interestingly, after 12 hours of IL-33 stimulation the concentrations of palmitic acid (C16:0), C16:1n7, C16:1n9, stearic acid (C18:0), oleic acid (C18:1n9), vaccenic acid (C18:1n7), EPA (C20:5n3), C22:3n6 and DHA (C22:6n3) in PLs were reduced which was accompanied by an increase of these fatty acids within TAGs (Figure 62A). There was also a reduction in both PL and TAG DGLA (C20:3n6) and AA (C20:4n6) after 12 hours of IL-33 treatment. Levels of myristic acid (C14:0) were increased in both fractions and at both time points in keeping with the overall rise in myristic acid seen in Figure 58. Figure 61B shows that IL-33 reduced the concentrations of palmitic acid, C16:1n7, C16:1n9, oleic acid, vaccenic acid and LA (C18:2n6) within PLs but the concentrations of these fatty acids were raised in TAGs after 24 hours. Levels of myristic acid, DPA (C22:5n3) and DHA in PLs and TAGs were also increased by IL-33. The changes in percentages (Figures 62C and Figure 62D) reflected the trends exhibited in Figures 62A and 62B. Furthermore, IL-33 caused noteworthy rises in the percentages of myristic acid, palmitic acid, oleic acid and DPA in TAGs that exceeded the percentages of these fatty acids within PLs. The trends in the data were similar at both time points investigated and suggest that IL-33 may promote the redistribution of fatty acids from the larger PL pool to TAGs.

5.3.3.2. RAW264.7 fatty acid distribution

Following the separation of murine fatty acids into TAGs, PLs and SEs by TLC, GC was performed to determine the distribution and amounts of fatty acids within each of these fractions.


A
Fatty acid distribution after 12 hours in RAW 264.7 macrophages
Concentration (ug/million cells)
0
0.5
1
1.5
2
2.5
3
3.5
4
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6
PL Control
PL IL-33
TAG Control
TAG IL-33
SE Control
SE IL-33
B
Fatty acid distribution after 24 hours in RAW 264.7 macrophages
Concentration (ug/million cells)
0
0.5
1
1.5
2
2.5
3
3.5
4
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6
PL Control
PL IL-33
TAG Control
TAG IL-33
SE Control
SE IL-33

C



D

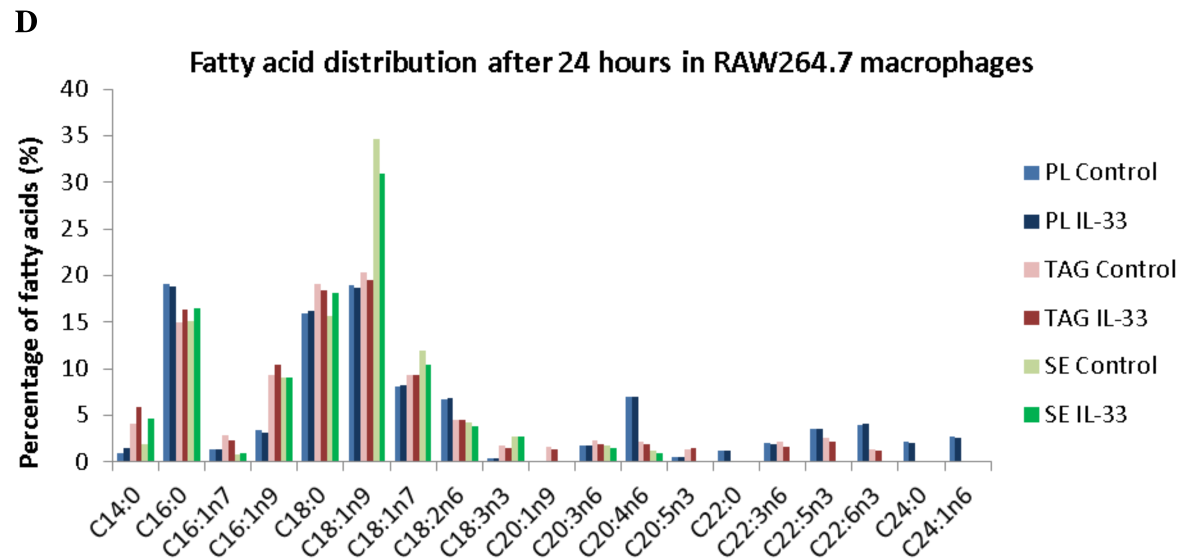


Figure 63. Distribution of fatty acids in RAW264.7 macrophages

RAW264.7 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. Lipids were separated by TLC into different lipid groups; PLs, TAGs and SEs and then the individual fatty acid composition of these fractions was assessed by GC. The concentration was normalised to µg/million cells (Panels A and B) and percentages of total fatty acids (Panels C and D). Fatty acids are indicated with the number before the colon showing the number of carbon atoms, after which denotes the number of double bonds followed by the position of the first double bond. Data represents mean from three (A and B) and four (C and D) independent experiments.

IL-33 stimulation of RAW 264.7 macrophages caused an increase in the concentrations of myristic acid (C14:0), stearic acid (C18:0) and LA (C18:2n6) in PLs, TAGs and SEs (Figure 63A) after 12 hours. There was also an increase in AA (C20:4n6), C22:3n6, DPA (C22:5n3) and DHA (C22:6n3) within PLs and TAGs after 12 hours but these fatty acids were absent in SEs. In accordance with the results found in THP-1 macrophages, there was a reduction in the concentrations of palmitic acid (C16:0) and C16:1n9 in PLs but an increase of these fatty acids in TAGs and SEs. Palmitic acid, stearic acid and oleic acid (C18:1n9) were abundant in PLs, TAGs and SEs (Figures 63A and 63C). Figures 63B and 63D display the changes in concentration and percentages of specific fatty acids from PLs, TAGs and SEs after 24 hours. IL-33 increased the concentration of myristic acid and EPA (C20:5n3) in each fraction. In accordance with the pattern exhibited at 12 hours of IL-33 treatment, after 24 hours IL-33 reduced palmitic acid in PLs and increased levels of the fatty acid in TAGs and SEs. However, in comparison to the THP-1 macrophage data set, there were less consistent trends and the relationship between different fractions was more complex. For example, IL-33 stimulation caused an increase in stearic acid in all lipid groups after 12 hours but after 24 hours of treatment; the cytokine decreased concentrations of stearic acid within SEs. Overall, the alterations in fatty acids were similar at both time points and mutual changes were seen in THP-1 macrophages.

5.3.4. The effect of IL-33 on desaturases

5.3.4.1. Desaturase activity

The studies presented within this chapter have demonstrated that IL-33 elicits significant alterations in the fatty acid composition of macrophages, some of which are specific to particular lipid fractions (Figures 56-61). A number of significant changes occurred in accordance to the fatty acid substrate–product relationships associated with certain desaturase enzymes (Figures 58 and 60). To gain some understanding of the molecular mechanisms responsible for these changes, experiments were performed to assess the impact of IL-33 on desaturase activity. Numerous studies have used the ratio of product to precursor as a measure of desaturase activity (Attie *et al.* 2002; Shiwaku *et al.* 2004; Martinelli *et al.* 2008; Ebbesson *et al.* 2012). In accordance with previous studies the following indices were used:

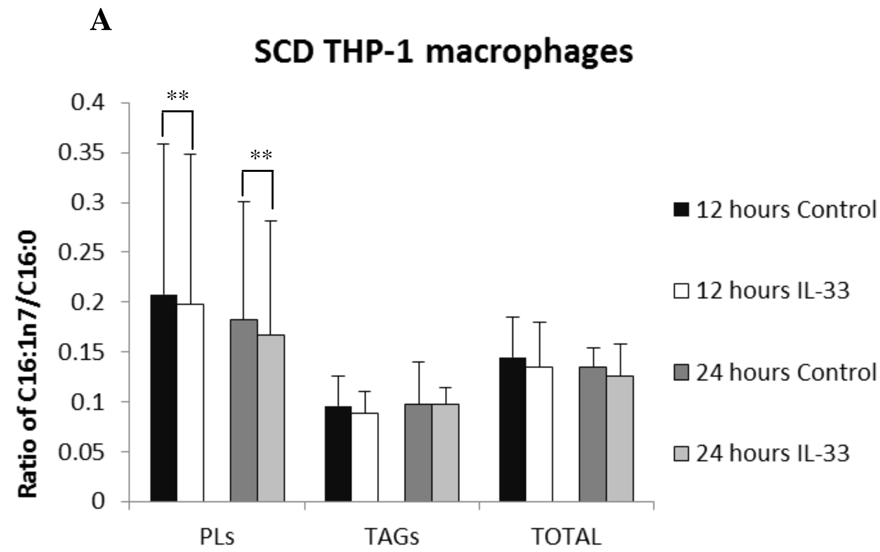
$$\text{SCD-1:} \quad \frac{\text{C16:1n7}}{\text{C16:0}} \quad \text{and} \quad \frac{\text{C18:1n9}}{\text{C18:0}}$$

$$\Delta\text{6-desaturase:} \quad \frac{\text{C20:3n-6}}{\text{C18:2n6}}$$

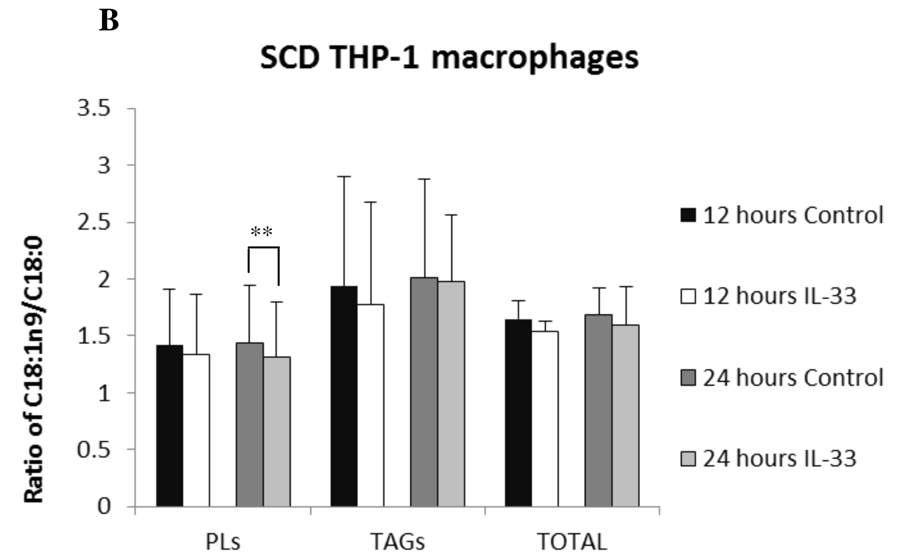
$$\Delta\text{5-desaturase:} \quad \frac{\text{C20:4n6}}{\text{C20:3n6}}$$

To determine SCD activity; the ratio of C16:1n7/C16:0 and C18:1n9/C18:0 were assessed individually and together to determine the effects of IL-33 on specific and total Δ9-desaturation. Although the ratio of C18:3n6/C18:2n6 is frequently used as an indicator of Δ6-desaturase function, this was not possible due to the lack of C18:3n6 (GLA) within the fractions and a ratio of the downstream Δ6-desaturation product C20:3n6 to C18:2n6 was therefore used in accordance with other studies (Raghallaigh *et al.* 2012; Pender-Cudlip *et al.* 2013).

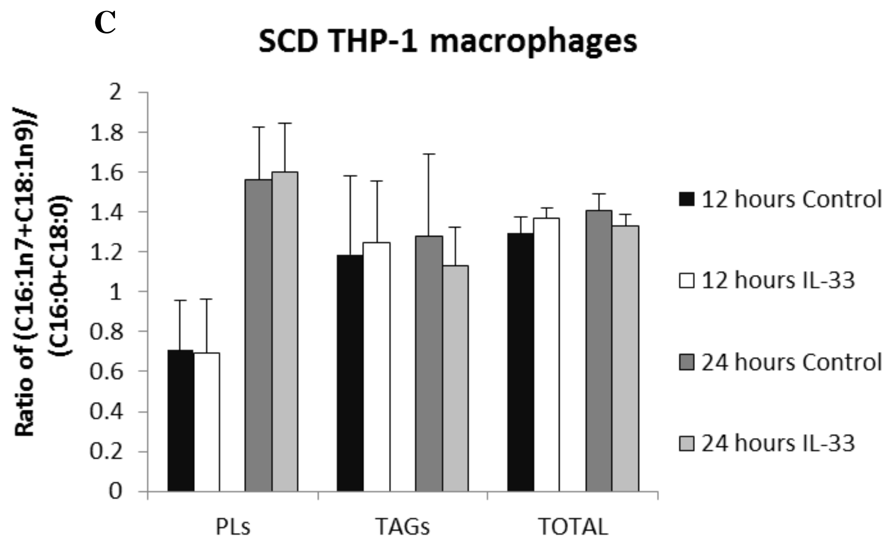
A



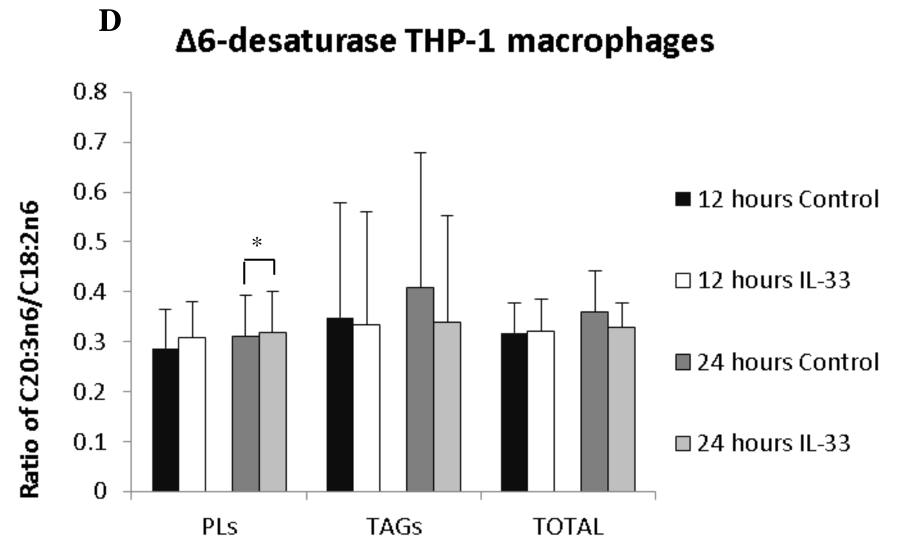
B



C



D



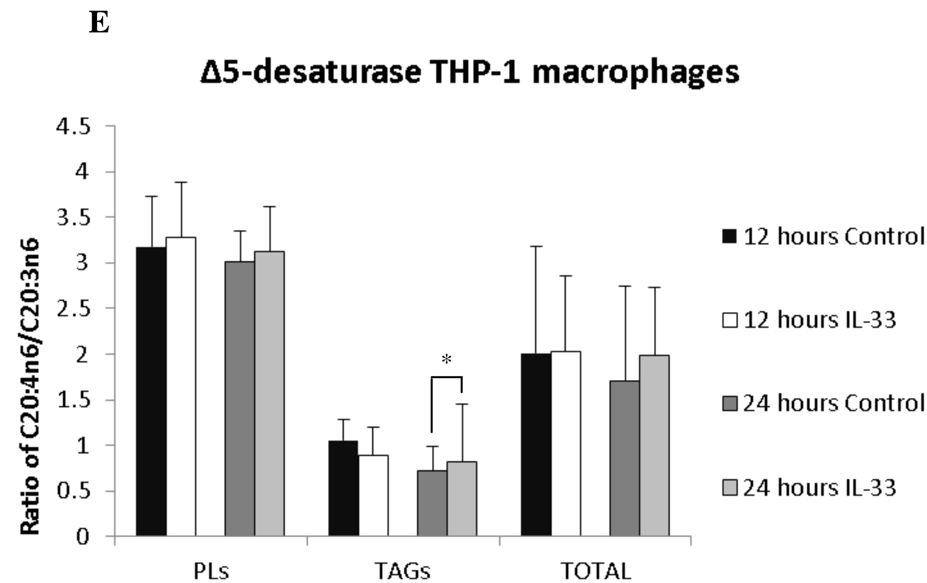
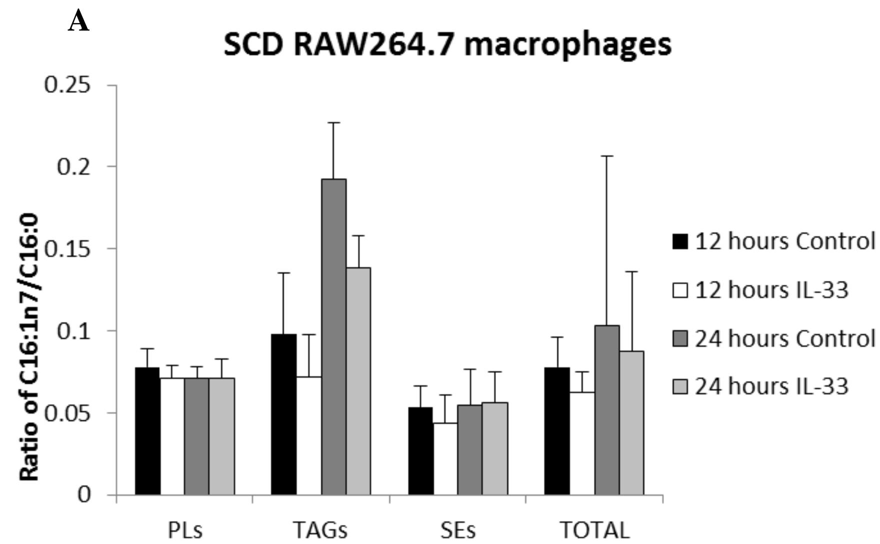


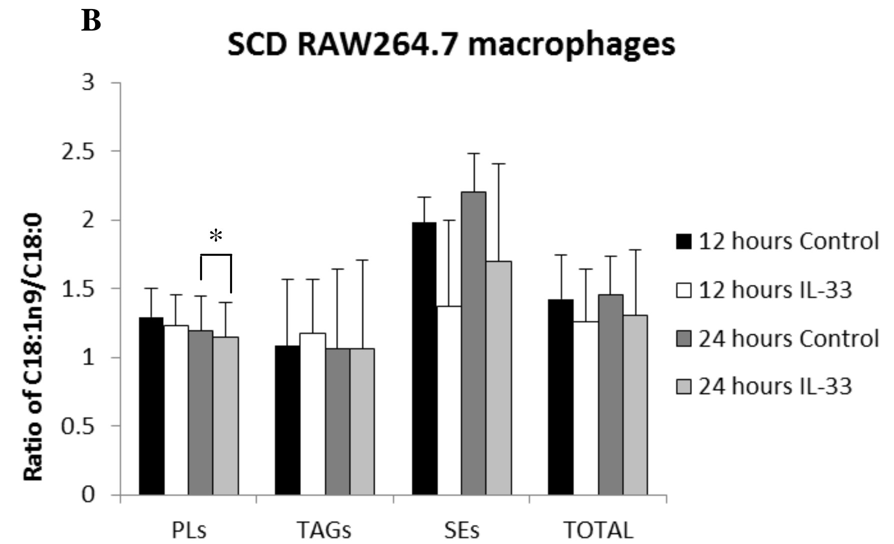
Figure 64. Fatty acid ratios in THP-1 macrophages

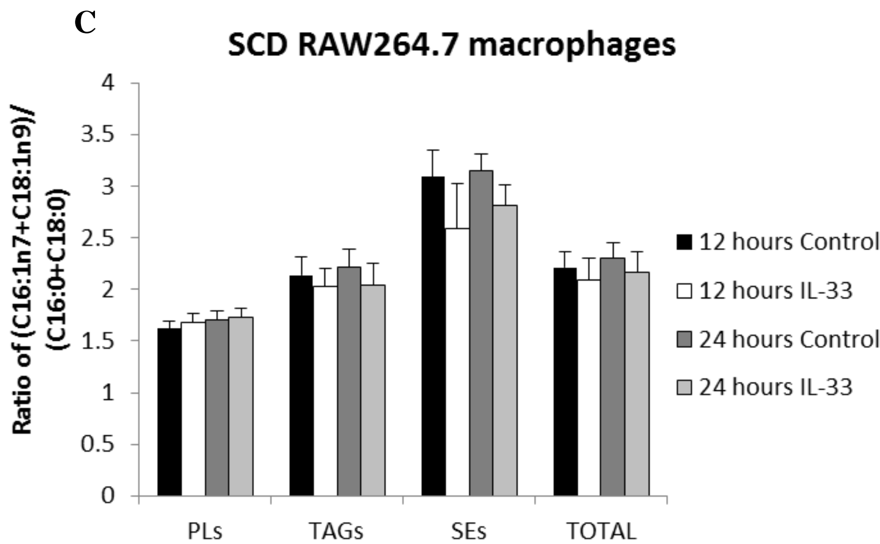
PMA-differentiated THP-1 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. Lipids were separated by TLC into different lipids groups; PLs and TAGs and then the individual fatty acid composition of these fractions was assessed by GC. Enzymatic activity was analysed for SCD (Panels A-C), Δ6-desaturase (Panel D) and Δ5-desaturase (Panel E) whereby the ratio of product to precursor was measured: Panel A= C16:1n7/C16:0; Panel B= C18:1n9/C18:0; Panel C= [(C16:1n7+C18:1n9)/(C16:0+C18:0)]; Panel D= C20:3n6/C18:2n6 and Panel E= C20:4n6/C20:3n6. Data represents mean ± SD from four independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05, **p<0.01.

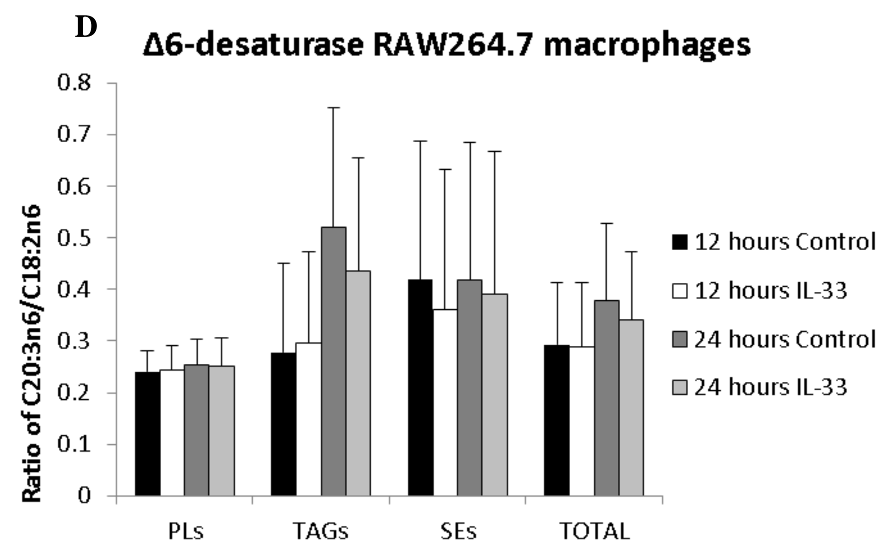
Figure 64 displays the changes in the ratio of desaturase products to precursors in THP-1 macrophages in response to IL-33 stimulation for 12 and 24 hours. Stimulation of the cells with IL-33 caused a consistent reduction in the ratio of C16:1n7/C16:0; a measure of SCD activity, at both time points and in each fraction (Figure 64A). This decrease was significant after 12 and 24 hours of IL-33 treatment in PLs and desaturase activity was also greatest within this fraction. Another indicator of SCD activity is the ratio of C18:1n9/C18:0 (Figure 64B), IL-33 reduced this ratio across all lipid groups and at each time point tested. Furthermore, the reduction in C18:1n9/C18:0 was significant following 24 hours of IL-33 stimulation within PLs. The level of desaturation of 18-carbon species was greater than that for 16-carbon species and SCD activity was highest within the TAG fraction. Interestingly, the results were non-significant when using a combination of the two ratios of SCD activity (Figure 64C). As such totalling these indices may not be the most appropriate method to assess SCD activity. The ratio of C20:3n6/C18:2n6 was used to measure Δ6-desaturase activity (Figure 64D). Interestingly, within PLs IL-33 increased Δ6-desaturase activity, which was significant following 24 hours of IL-33 stimulation. However, within TAGs and total fatty acids IL-33 caused a general reduction in enzymatic activity. The TAG fraction displayed the highest levels of Δ6-desaturase activity. Figure 64E displays the changes in the ratio of C20:4n6/C20:3n6, an indicator of Δ5-desaturase activity, following incubation with IL-33 for 12 and 24 hours. IL-33 increased Δ5-desaturase activity within PLs at both time points. However, within TAGs, IL-33 exerted time-specific effects; after 12 hours the cytokine reduced Δ5-desaturase activity but after 24 hours IL-33 significantly increased enzymatic activity. The cytokine also increased Δ5-desaturase activity in total fatty acids.

The effects of 12 and 24 hours of IL-33 stimulation on desaturase indices in RAW264.7 macrophages were next investigated to determine the temporal and potential interspecies effect of the cytokine.


A
SCD RAW264.7 macrophages
Ratio of C16:1n7/C16:0
0.25
0.2
0.15
0.1
0.05
0
PLs
TAGs
SEs
TOTAL
12 hours Control
12 hours IL-33
24 hours Control
24 hours IL-33


B
SCD RAW264.7 macrophages
Ratio of C18:1n9/C18:0
3
2.5
2
1.5
1
0.5
0
*
PLs
TAGs
SEs
TOTAL
12 hours Control
12 hours IL-33
24 hours Control
24 hours IL-33


C
SCD RAW264.7 macrophages
Ratio of (C16:1n7+C18:1n9)/(C16:0+C18:0)
4
3.5
3
2.5
2
1.5
1
0.5
0
PLs
TAGs
SEs
TOTAL
12 hours Control
12 hours IL-33
24 hours Control
24 hours IL-33


D
Δ6-desaturase RAW264.7 macrophages
Ratio of C20:3n6/C18:2n6
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
PLs
TAGs
SEs
TOTAL
12 hours Control
12 hours IL-33
24 hours Control
24 hours IL-33

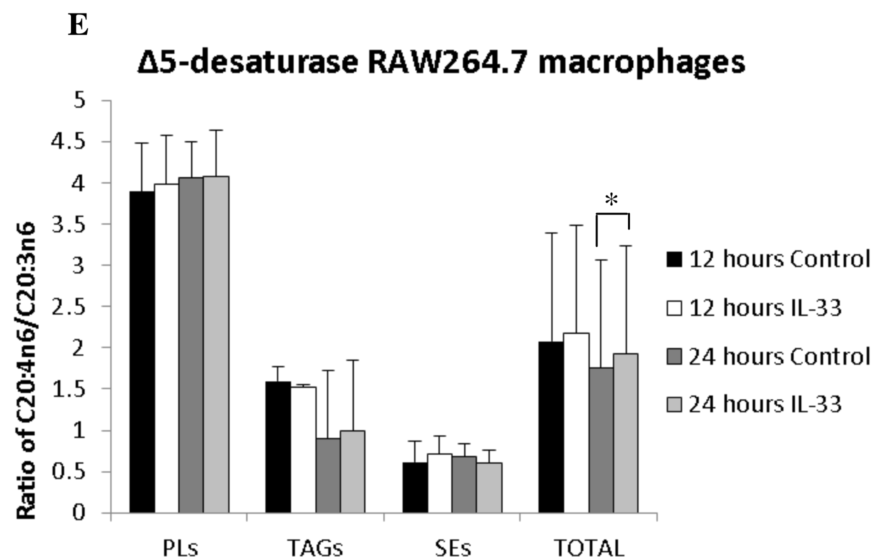


Figure 65. Fatty acid ratios in RAW264.7 macrophages

RAW264.7 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. Lipids were separated by TLC into different lipids groups; PLs, TAGs and SEs, and then the individual fatty acid composition of these fractions were assessed by GC. Enzymatic activity was analysed for SCD (Panels A-C), Δ6-desaturase (Panel D) and Δ5-desaturase (Panel E) whereby the ratio of product to precursor was measured: Panel A= C16:1n7/C16:0; Panel B= C18:1n9/C18:0; Panel C= [(C16:1n7+C18:1n9)/(C16:0+C18:0)]; Panel D= C20:3n6/C18:2n6 and Panel E= C20:4n6/C20:3n6. Data represents mean ± SD from four independent experiments. The Student *t* test was used to determine the statistical significance of the results *$p<0.05$.

Desaturase activity within RAW264.7 macrophages was assessed using the same ratios as THP-1 macrophages. SCD activity (C16:1n7/ C16:0) was reduced following 12 and 24 hours of IL-33 stimulation in PLs, TAGs, SEs and total fatty acids, except in SEs after 24 hours (Figure 65A). Enzymatic desaturation of C16:0 was greatest in TAGs. A similar trend was exhibited in the ratio of C18:1n9/C18:0, whereby IL-33 reduced activity within PLs, SEs and total fatty acids (Figure 65B). This decrease was significant within PLs following 24 hours of IL-33 stimulation. SCD-mediated desaturation of C18:0 was highest within SEs. Furthermore, the desaturation of C18:0 was much greater than that of C16:0. Combining these ratios (Figure 65C) produced a trend for IL-33-induced reduction of SCD activity across the fractions at both time points with the exception of PLs. There was an increase in Δ6-desaturase activity (C20:3n6/C18:2n6) between the time points and following IL-33 treatment within PLs, whereas a reduction was exhibited in SEs and total fatty acids (Figure 65D). Interestingly IL-33 produced a time-specific effect; inducing Δ6-desaturase activity within TAGs after 12 hours of stimulation but reducing activity after 24 hours. The ratio of C20:4n6/C20:3n6 was used as a measurement of Δ5-desaturase activity (Figure 65E). There were less consistent trends across the fractions and between different time points. IL-33 increased Δ5-desaturase activity in PLs and total fatty acids after 12 and 24 hours which was significant after 24 hours in total fatty acids. However, IL-33 also decreased the activity of Δ5-desaturase in TAGs after 12 hours of treatment but after 24 hours of IL-33 treatment there was an increase. Within SEs, 12 hours of IL-33 stimulation increased activity but after 24 hours activity was reduced.

5.3.4.2. Stearoyl-CoA desaturase (SCD)-1 gene expression

Within human THP-1 and murine RAW264.7 macrophages there was a trend for reduced SCD activity following IL-33 treatment based upon the ratios of SCD product to precursors (C16:1n7/ C16:0 and C18:1n9/C18:0). Therefore RT-qPCR was performed to determine the effects of IL-33 on SCD-1 mRNA expression. THP-1 and RAW264.7 macrophages were incubated with IL-33 (25ng/ml) for 12 and 24 hours and RT-qPCR was performed on the resulting cDNA using the procedures from chapter 3. Further information on the optimisation of these primers are included in the appendix.

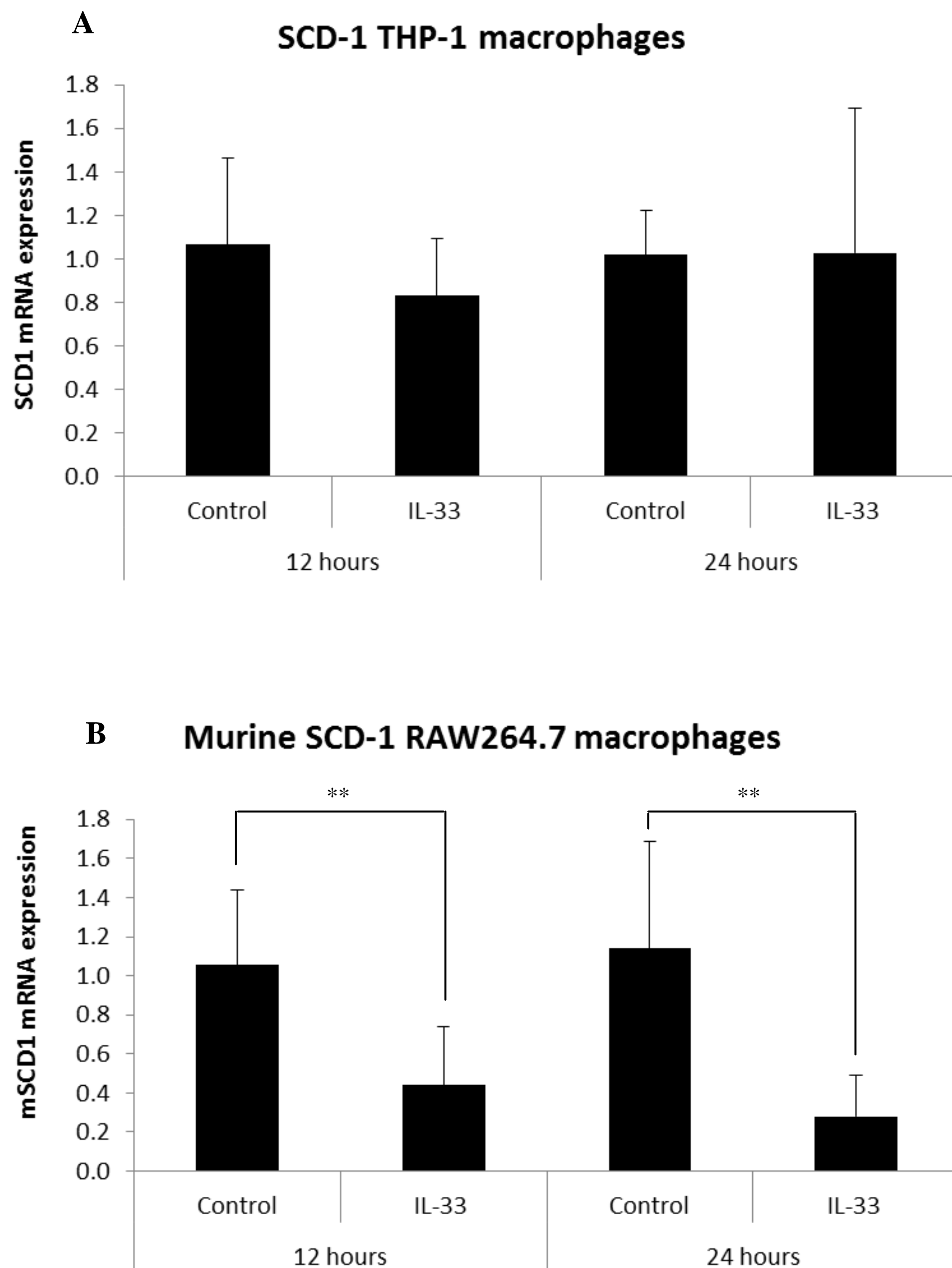


Figure 66. SCD-1 gene expression

RT-qPCR was performed using cDNA from RAW264.7 and PMA-differentiated THP-1 macrophages incubated in the presence or absence (control) of IL-33 (25ng/ml) for 12 hours and 24 hours. Gene specific primers for: Panel A= human SCD-1 and B= murine SCD-1 were used. The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples given an arbitrary value of 1. Data represents mean ± SD from seven (A) and four (B) independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05, **p<0.01.

Within THP-1 cells there were no significant changes in the level of SCD-1 mRNA following IL-33 treatment for both time points (Figure 66A). However, IL-33 stimulation caused a significant reduction in SCD-1 mRNA expression after 12 and 24 hours in murine RAW264.7 macrophages (Figure 66B).

5.4. Discussion

This is the first study to investigate the actions of IL-33 on the lipid and fatty acid composition of macrophages. Additionally, the involvement of desaturase enzymes, responsible for the production of MUFAs and PUFAs, has also been linked to CAD but the potential interactions between IL-33 and desaturases have not yet been investigated. The study explored the effects of IL-33 on desaturase activity and the mRNA expression of SCD-1.

5.4.1. Major lipid classes

TLC was used to analyse the effects of 12 and 24 hours of IL-33 stimulation on different lipid groups within human and murine macrophages. TLC analysis showed that RAW264.7 macrophages contained bands for TAGs, PLs and SEs (Figure 57), whereas THP-1 macrophages only contained bands corresponding to TAGs and PLs (Figure 56). Previous groups have noted that intracellular concentrations of SEs within THP-1 macrophages are low (Persson *et al.* 2008; Wang *et al.* 2009; Rajamaki *et al.* 2010) and a study by Lada *et al.* (2002) could not detect CEs by gas-liquid chromatography prior to lipid loading (Lada *et al.* 2002). As such many groups initially expose THP-1 macrophages to LDL (Banka *et al.* 1991), modified LDL (e.g. AcLDL, OxLDL) (Rodriguez *et al.* 1994; Lada *et al.* 2003; McLaren *et al.* 2010b), radiolabelled cholesterol (Kritharides *et al.* 1998), 25-hydroxycholesterol (Hassall and Graham 1995; Graham *et al.* 1996), cholesterol crystals (Rajamaki *et al.* 2010) and mevalonic acid (Hassall and Graham 1995) prior to experiments. Pre-incubation with these molecules promotes the formation of macrophage ‘foam cells’ and also enriches the cholesterol pool. Although TLC analysis did not detect SEs within THP-1 macrophages, other studies have shown that these cells contain SEs. Radiolabelling experiments have observed the incorporation of precursors into cholesterol within untreated THP-1

macrophages (Hassall and Graham 1995; Rise *et al.* 2003). Rise *et al.* (2003) investigated the incorporation of radiolabelled acetate in THP-1 macrophages. The group performed one-dimensional TLC which revealed bands for PLs, TAGs, DAGs, FFAs and CEs. However, during the study, 2 day-differentiated cells were used to carry out stimulation experiments.

A key difference between the experimental setup of RAW264.7 and THP-1 macrophages was the inclusion of PMA in experiments involving THP-1 cells. Incubation of THP-1 monocytes with PMA promotes differentiation of the cells into macrophages which quickly acquire attributes associated with mature macrophages. Morphological differentiation of THP-1 cells has been reported within 15 minutes of phorbol ester treatment (Mehta and Lopezberestein 1986) and after 24 hours of PMA stimulation, THP-1 cells display an increase in phagocytic activity (Schwende *et al.* 1996). Auwerx *et al* (1989) reported that the mRNA expression of LDLR was induced by PMA after only 15 minutes and levels peaked after 1-2 hours. Also, the mRNA expression of HMG-CoA reductase, a key enzyme in cholesterol biosynthesis, was maximally induced after 2 hours of PMA exposure. The exposure time and PMA concentration chosen for the experiments within this chapter have been frequently used by other groups in lipid and cholesterol-related studies (Yang *et al.* 2004; McLaren *et al.* 2010b; McLaren *et al.* 2011b). Interestingly, several groups have used longer exposure times with PMA e.g. 72 hours (Rajamaki *et al.* 2010) or 7 days (Via *et al.* 1989; Hassall and Graham 1995) in lipid-related studies. During our experiments the THP-1 cells were incubated with PMA for 36 hours (12 hours IL-33 treatment) and 48 hours (24 hours IL-33) and both sets of cells did not contain SEs.

A greater amount of starting material was used in RAW264.7 experiments; between 3-6 million compared to the 2-4 million cells in THP-1 macrophage experiments which may have aided detection after TLC separation. The data suggests that RAW264.7 macrophages contain a greater or more detectable amount of SEs compared to THP-1 macrophages. The lack of SEs detected by TLC in our work may be due to low abundance as suggested by previous studies. Therefore future experiments could be performed over a longer time scale and HMDMs could be used for comparison.

For both THP-1 and RAW264.7 macrophages PLs represented the largest lipid fraction (Figures 56 and 57). IL-33 stimulation caused a reduction in the concentration and percentages of PLs after 12 and 24 hours and was accompanied by an increase in the concentration of TAGs in both cells. The concentration and percentages of SEs within RAW264.7 macrophages was relatively constant following IL-33 treatment. The increase in TAGs (Figures 56 and 57) in THP-1 and RAW264.7 cells may suggest IL-33 enhances lipid accumulation within these cells, however due to the fall in PLs there were no significant net change in total fatty acid levels. Miller *et al* (2010) previously showed that injections of IL-33 into genetically obese diabetic mice significantly reduced adipose mass and although total serum cholesterol was decreased, there was no effect on serum TAGs (Miller *et al.* 2010). Also, a previous study by our laboratory demonstrated that IL-33 decreases macrophage foam cell formation in THP-1 macrophages and HMDMs (McLaren *et al.* 2010b). Figures 62 and 63 suggest that IL-33 promotes the redistribution of fatty acids from PLs to TAGs. The analysis of individual PLs such as PC, PE, PI, PS and sphingomyelin by two-dimensional TLC would reveal more detailed changes (Garrett and Grisham 2012).

5.4.2. Fatty acid analysis

The use of THP-1 and RAW264.7 cells provided an isolated system to assess the effects of IL-33 on the fatty acid profile of macrophages. A summary of results are provided in Tables 20 and 21 and the fatty acid profile of untreated THP-1 macrophages (Galli *et al.* 1992; Zhao *et al.* 2005) and RAW264.7 macrophages (Rouzer *et al.* 2006; Schumann *et al.* 2011) were similar to those previously described in other studies. Many of the changes initiated by IL-33 were conserved between the two species (Table 22).

Table 22. Comparison of responses in THP-1 and RAW264.7 macrophages

THP-1 and RAW264.7 macrophage						
Conservation of responses						
Fatty acid	PLs		TAGs		TOTAL	
	12 hours	24 hours	12 hours	24 hours	12 hours	24 hours
C14:0	Y	Y	Y	Y	Y	Y
C16:0	Y	Y	Y	Y	N	Y
C16:1n7	Y	Y	Y	N	Y	Y
C16:1n9	Y	Y	N	Y	Y	Y
C18:0	Y	Y	N	Y	Y	Y
C18:1n9	Y	Y	Y	Y	N	Y
C18:1n7	N	N	N	N	Y	Y
C18:2n6	N	N	Y	Y	Y	Y
C18:3n3	Y	N	Y	Y	N	Y
C20:1n9			N	N	N	N
C20:3n6	Y	N	N	Y	Y	Y
C20:4n6	Y	Y	Y	Y	N	Y
C20:5n3	Y	N	N	N	N	N
C22:0	N	N			N	N
C22:3n6	Y	Y	N	Y	Y	Y
C22:5n3	Y	N	Y	Y	Y	Y
C22:6n3	Y	Y	Y	Y	Y	N
C24:0	N	N			N	N
C24:1n6	N	Y			N	Y

Conservation of responses denoted as Y and differences shown as N.

5.4.2.1. Saturated fatty acids (SFAs)

Numerous epidemiological studies link the prevalence of dietary SFAs with incidences of CAD (Hayes and Khosla 1992; Zock *et al.* 1994; Dreon *et al.* 1998). In particular, the 12, 14 and 16 carbon SFAs have been shown to exert hypercholesterolaemic effects, whereas shorter chain fatty acids and stearic acid are thought to have a neutral impact (Rhee *et al.* 1997; Mensink *et al.* 2003). IL-33 significantly increased levels of the 14 carbon fatty acid myristic acid after 24 hours in PLs from THP-1 macrophages. Myristic acid is often attributed as the most hypercholesterolaemic SFA (Hayes and Khosla 1992; Hughes *et al.* 1996) due to the adverse effect of this fatty acid on the ratio of LDL cholesterol to HDL cholesterol (Zock *et al.* 1994; Dreon *et al.* 1998). In contrast, the cytokine significantly reduced levels of palmitic acid after 12 hours in THP-1

macrophages, and within RAW264.7 macrophages after 12 and 24 hours in PLs (Figures 58 and 60) but significantly increased palmitic acid levels in TAGs after 24 hours in murine macrophages. The cytokine significantly increased the amount of stearic acid in PLs after 24 hours. Stearic acid is described as a neutral fatty acid within the context of CAD and may exert small beneficial effects on LDL cholesterol (Mensink *et al.* 2003). The perceived neutrality of this fatty acid has been attributed to the high rate of conversion to oleic acid (Bonanome *et al.* 1992; Garg 1992). Our data also demonstrated that human and murine macrophages have a greater abundance of oleic acid to stearic acid (Figures 58 and 60).

The potential effects of these changes within an atherosclerotic context are unclear as IL-33 produced a number of significant changes in specific SFAs but did not exert a consistent trend across the group. IL-33 promoted the formation of pro-atherosclerotic myristic acid but had mixed effects on levels of palmitic acid within different lipid fractions. Alterations in palmitic acid may potentially represent changes in the *de novo* synthesis of fatty acids, as palmitic acid is the primary product of these biosynthetic reactions (Cook and McMaster 2002). The effects of IL-33 on biosynthetic enzymes such as fatty acid synthase (Lin *et al.* 2002) could be explored to assess whether the cytokine influences fatty acid production. The changes in SFAs were consistent between species.

Interestingly, the greatest number of significant changes occurred within the PL fraction. Phospholipids primarily reside within the membrane where they influence structure and fluidity and can function as substrates for the production of signalling molecules (Calder and Yaqoob 2003). Alterations in the membrane composition of macrophages can have a significant impact on their characteristics and properties. Experiments involving exogenous enrichment of specific fatty acids within macrophage membranes have shown that there is a positive correlation between the level of fatty acid unsaturation and the phagocytic ability of macrophages (Schroit and Gallily 1979; Calder *et al.* 1990). Accordingly, increased proportions of SFAs in phospholipids reduces receptor-mediated phagocytosis and also decreases the rate of fluid-phase pinocytosis within primary murine peritoneal macrophages (Mahoney *et al.* 1977). The use of phagocytic inhibitors such as cytochalasin B (Axline and Reaven 1974;

Schrijvers *et al.* 2007) could be meaningful in future *in vitro* studies examining the influence of IL-33 on macrophage phagocytosis.

Previous studies have shown that raised levels of SFAs within macrophage membranes enhances the adhesiveness of the cells (Calder *et al.* 1990). *In vivo* studies of mice have demonstrated that IL-33 decreases the accumulation of macrophages within the lesion thereby indicating that the migration and adhesion of these cells is compromised (McLaren *et al.* 2010b). A macrophage adhesion assay could be performed within RAW264.7 and THP-1 macrophages to assess the impact of IL-33 on monocyte adhesion. Saturated fats may exert pro-atherosclerotic effects through a combination of raising levels of plasma LDL-cholesterol and influencing the phagocytic properties and adhesiveness of macrophages. As such it would be interesting to determine whether the changes instigated by IL-33 influenced these characteristics.

5.4.2.2. Monounsaturated fatty acids (MUFAs)

MUFAs are generally ascribed as having a neutral atherogenic profile and are thought to be less potent than PUFAs (Lee *et al.* 1989; Hayes and Khosla 1992; Lemaitre *et al.* 2002). The beneficial effects of MUFAs are mainly derived from observations that populations that consume a Mediterranean-type diet high in MUFAs have lower incidences of CHD. Dietary intervention strategies recommend the replacement of dietary SFAs with MUFAs and PUFAs (Degirolamo and Rudel 2010). However, recent studies have questioned this strategy (Jakobsen *et al.* 2009). The production of MUFAs are reflective of SCD-1 activity which has been positively associated with CAD and may implicate a pathological role for these fatty acids (Warensjoe *et al.* 2008). IL-33 stimulation caused a significant reduction in the percentages of C16:1n7, C16:1n9 and oleic acid after 12 and 24 hours in THP-1 and RAW 264.7 macrophages. The reduction in 16 carbon-MUFAs was likely linked to the IL-33-mediated decrease of the precursor palmitic acid.

5.4.2.3. Polyunsaturated fatty acids (PUFAs)

PUFAs belong to either the n-3 or n-6 family depending on the position of their first double bond. The n-3 group of PUFAs are associated with protective effects against

heart disease, whereas the role of n-6 PUFAs within CAD is less clear due to the production of AA-derived inflammatory mediators. Western diets have a bias in the ratio of n-6 fatty acids to n-3 fatty acids which is thought to contribute to the development of heart disease (Simopoulos 2008).

The abundances of fatty acids (Figures 62 and 63) were similar to those from other studies, although, in accordance with the findings from other studies, the levels of long chain PUFAs were relatively low within these cells (Galli *et al.* 1992; Zhao *et al.* 2005; Rouzer *et al.* 2006; Schumann *et al.* 2011). Interestingly, Agha-Jaffar *et al* (2013) showed that PMA-mediated differentiation of THP-1 macrophages was associated with changes in fatty acid composition including a reduction in PUFAs, in particular EPA, C22:5n6 and DHA. These changes were also accompanied with reduced FADS1 and FADS2 mRNA expression and associated with a fall in PUFA synthesis (Agha-Jaffar *et al.* 2013). It would be interesting to compare changes in fatty acid composition and desaturase expression in the absence and presence of PMA with the experimental conditions used within this study.

IL-33 caused several significant changes within macrophage PUFAs. Overall the cytokine increased n-3 PUFA production but the effects on n-6 PUFAs were more mixed. Interestingly, within RAW264.7 macrophages levels of both LA and EPA in PLs were significantly increased by IL-33. The mutual increase was unexpected as LA, the precursor to n-6 PUFAs, shares an antagonistic relationship with the precursor ALA over competition for Δ6-desaturase (Emken *et al.* 1999) and as such increased levels of LA should reduce the formation of n-3 long chain fatty acid metabolites derived from ALA such as EPA and DHA. Within the study both EPA and DHA were increased, although the rise was not significant for DHA. Increased amounts of EPA and DHA within the cells should have an anti-atherogenic effect as enhanced dietary intake of EPA and DHA have been shown to cause a 39% reduction in AA and its metabolites and a 54% fall in LTB_4 production (Lee *et al.* 1985). In previous studies by our group, incubation of THP-1 macrophages and primary HMDMs with DHA and EPA reduced the uptake of modified LDL and inhibited macropinocytosis (McLaren *et al.* 2011b; Michael *et al.* 2013). In this study IL-33 also significantly increased the n-3 PUFA DPA. Enhanced levels of serum DPA and supplementation of DPA have been shown to reduce the risk of cardiovascular disease (Rissanen *et al.* 2000; Oda *et al.* 2005).

Several complications arise when trying to interpret data based solely on n-3 and n-6 PUFA categories. Although several studies support the notion that n-3 PUFAs are anti-atherogenic and n-6 PUFAs are pro-atherogenic, the effects of individual fatty acids can sometimes contradict these classifications. Figure 58 shows that IL-33 significantly increased levels of AA and EPA in THP-1 macrophages. In a study by Wang *et al.* (2009) the growth medium of lipoprotein-incubated THP-1 macrophages was supplemented with AA and EPA. Both fatty acids reduced the expression of TNF-α and MCP-1 and also down-regulated ABCA-1 and SR-B1 expression. Furthermore, these fatty acids promoted CE accumulation without influencing total cholesterol content (Wang *et al.* 2009). Although the fatty acids originate from different PUFA families they exhibit the same actions. Interestingly, IL-33 also reduces the expression of SR-B1 and MCP-1 (chapter 3). As such it would be useful to determine whether alterations in the production of specific fatty acids are responsible for changes in gene expression. However, it seems likely that other mechanisms are also in place as IL-33 also increases ABCA-1 expression and reduces CE accumulation (McLaren *et al.* 2010b).

Monocytes and macrophages are major sources of eicosanoids (Calder and Yaqoob 2003; Hansson and Hermansson 2011). During atherosclerosis LOX enzymes colocalise with modified LDL particles and macrophages within the plaque and are thought to assist the oxidative modification of LDL particles (Ylaherttuala *et al.* 1990; Ylaherttuala *et al.* 1991c; Benz *et al.* 1995). However, the current literature on the relationship between IL-33 and eicosanoids is conflicting and mainly focused on the role of the cytokine in mast cells (Allakhverdi *et al.* 2007; Iikura *et al.* 2007; Moulin *et al.* 2007). Figures 58 and 60 show that IL-33 significantly affected a number of fatty acids involved in eicosanoid production. Eicosanoids are not stored but are synthesised *in situ* and their production is reflected by the fatty acid membrane composition (Makheja 1992; Mason *et al.* 2012). IL-33 reduced the production of murine DGLA in total fatty acids (Figure 60G); an n-6 PUFA responsible for the formation of the anti-inflammatory series 1 PGs via the COX pathway and or 15-HETeE by 15-LOX. These metabolites exert a range of anti-inflammatory effects including the reduction of blood pressure and inhibition of SMC proliferation (Wang *et al.* 2012). Supplementation with DGLA also reduces the expression of ICAM-1 and VCAM-1 and decreases atherosclerotic development in ApoE$^{-/-}$ mice (Takai *et al.* 2009).

As was previously discussed, IL-33 significantly increased EPA production (Figure 60B) and a trend for raised EPA levels was present in both set of cells. The metabolism of EPA gives rise to weakly pro-inflammatory series-3 PGs and series-5 LTs. However, EPA is thought to exert anti-inflammatory actions through competition for the same acylation enzymes as AA within similar membrane pools therefore diluting the presence of AA and reducing the production of AA-derived eicosanoids (Shoenfeld Y *et al.* 2001). Additionally, the production of mildly inflammatory mediators like TXA_3 derived from EPA may reduce the synthesis of more potent AA-derived mediators like TXA_2 which promote platelet aggregation and vasoconstriction (Hulín and Simko 1997). However, it is interesting that AA levels were also raised by IL-33. AA is enriched in macrophage membranes and is the major precursor of eicosanoids, such as the pro-inflammatory series 2 PGs and series 4 LTs (Calder and Yaqoob 2003). Within the study, levels of AA were high within the PL fraction and exceeded those of EPA. However, as both EPA and AA were increased, it is possible that the actions of AA would be reduced due to the inhibitory effects of EPA and several studies have shown that the detrimental actions of n-6 PUFAs are nullified in the presence of n-3 PUFAs (Pischon *et al.* 2003; Kusumoto *et al.* 2007). As both fatty acids can produce a wide array of eicosanoids with differing properties it is difficult to assess the overall impact within the cell. For example, EPA and AA are also modified to the prostacyclins PGI_3 and PGI_2, respectively, which exert anti-aggregating and vasodilating properties on the arterial wall.

Inflammation can be limited by promoting pro-resolving mechanisms such as modulation of adhesion molecule expression and regulation of signalling pathways controlling the expression of genes responsible for pro-inflammatory actions (Kohli and Levy 2009). Within the thesis the anti-inflammatory effects of IL-33 have been consistently demonstrated (chapters 3 and 4); therefore it is likely that these actions may be accompanied by an increase in the production of anti-inflammatory molecules such as lipoxins, protectins and resolvins. Cellular processing of EPA and DHA gives rise to E- and D-series of resolvins respectively. E-resolvins attenuate the effects of the potent chemoattractant LTB_4 derived from AA and reduce inflammation (Arita *et al.* 2005; Arita *et al.* 2007). Also, resolvin production utilises the same COX and LOX enzymes responsible for the conversion of AA to PGs and LTs. Therefore, a switch to resolvin

formation occurs at the expense of AA-derived PG and LT production. However, AA-derived mediators like lipoxins formed by LOX enzymes can also exhibit anti-inflammatory properties (Kohli and Levy 2009). Lipoxins exert a range of effects including reduced transmigration of polymorphonuclear leukocytes, regulation of endothelial cell adhesiveness and decreased polymorphonuclear leukocyte and eosinophil chemotaxis (Claria and Serhan 1995). Although IL-33 did not have a significant effect on DHA, the cytokine caused a general increase in levels of the fatty acid within human and murine PLs. In addition to D-series resolvins, DHA can also be converted into protectins that reduce kidney damage and decrease leukocyte infiltration and macrophage activation (Duffield *et al.* 2006).

The study demonstrated that IL-33 produced a number of changes in macrophage fatty acids (Tables 20 and 21). The cytokine reduced the production of MUFAs but this was accompanied by a general increase in PUFA formation; particularly n-3 family members. Further studies should explore the effects of IL-33 on eicosanoid production as a number of significant changes occurred in eicosanoid-precursor fatty acids. The expression of metabolic enzymes like LOXs and COXs following IL-33 stimulation may provide some insight in combination with extensive analysis by GC/ mass spectrometry (MS) of the metabolites formed during IL-33 treatment.

5.4.3. Desaturases

The formation of MUFAs and PUFAs are dependent on the actions of desaturase and elongase enzymes. As such these enzymes also control the production of eicosanoid precursors like AA, EPA and DHA (Cho *et al.* 1999). The study has so far investigated the effects of IL-33 on the composition and distribution of fatty acids within human and murine macrophages. Due to the range of actions exhibited by individual fatty acids, there are difficulties in determining the overall impact of these changes within the context of atherosclerosis. However, the activities of desaturases reflect the metabolic changes in fatty acids and are directly associated with CAD and markers of the disease (Ebbesson *et al.* 2012).

The effects of IL-33 on the ratio of products and substrates for SCD were next investigated arising from observations that the cytokine induced significant changes in

16 and 18 carbon fatty acids (Figures 58 and 60). SCD activity has been linked to various cardiovascular risk factors and other pathologies such as adiposity, hepatic steatosis, glucose intolerance (Sampath and Ntambi 2014), cholesterol metabolism and efflux (Sun *et al.* 2003; Nakaya *et al.* 2013) and CAD (Warensjoe *et al.* 2008). IL-33 significantly reduced SCD activity within PLs (based on a ratio of C16:1n7/C16:0 and C18:1n9/C18:0) in THP-1 and RAW264.7 macrophages (Figures 64 and 65) and a fall in SCD activity was also observed in other fractions.

The metabolic syndrome is a myriad of risk factors including abdominal obesity, hypertension, dyslipidaemia and insulin resistance and is linked to the development of CAD. Patients developing this syndrome display a characteristic fatty acid profile defined by increased amounts of myristic acid, palmitic acid, palmitoleic acid, oleic acid, GLA and DGLA and decreased LA which are indicative of increased SCD activity (Warensjo *et al.* 2005). Within the study palmitic acid, palmitoleic acid and oleic acid were significantly reduced in THP-1 and RAW264.7 macrophages PLs by IL-33. These results, in combination with the reduction in SCD activity indices, indicate that IL-33 produces a protective fatty acid profile against CAD and related disorders.

The effects of IL-33 stimulation on the activities of other desaturases were also investigated. Reduced or impaired Δ5-desaturase activity and raised Δ6-desaturase activity are associated with MI and cardiac-related mortality (Leng *et al.* 1999; Warensjoe *et al.* 2008) and have also been linked to other CAD-related disorders such as diabetes, obesity and the metabolic syndrome (Warensjo *et al.* 2005; Petersson *et al.* 2008). Δ6-desaturase is responsible for the conversion of LA and ALA to GLA and C18:4n3 (STA) respectively, whilst Δ5-desaturase catalyses the desaturation of DGLA and ETA (Cho *et al.* 1999). Within the study, due to the absence of GLA, a ratio of C18:2n6/C20:3n6 was used to calculate Δ6-desaturase activity in accordance with other studies (Raghallaigh *et al.* 2012; Pender-Cudlip *et al.* 2013). Within THP-1 macrophages, IL-33 significantly increased Δ6-desaturase activity within PLs. However, the response in other fractions and in murine macrophages was mixed which may be related to the alternative choice of ratio. The activity of Δ5-desaturase was predicted by a ratio of C20:4n6/C20:3n6. IL-33 significantly increased Δ5-desaturase activity within THP-1 TAGs and total fatty acids in RAW264.7 macrophages. Overall, increased Δ6-desaturase activity is associated with adverse cardiac events but raised Δ5-activity is

thought to have a protective effect. The rise in these indices is likely linked to increased PUFA production observed within these macrophages. As the trends for Δ6-desaturase and Δ5-desaturase were less consistent across the fractions, experiments focused on the better characterised SCD which is thought to be a stronger indicator of CAD.

The effects of 12 and 24 hours of IL-33 stimulation on the mRNA expression of SCD-1; the major isoform of SCD, was assessed by RT-qPCR. In accordance with the data shown in Figure 65, IL-33 significantly reduced SCD-1 expression within RAW264.7 macrophages (Figure 66B). However, within THP-1 macrophages the cytokine did not have a significant effect on SCD-1 mRNA levels (Figure 66A). The reduction in SCD-1 activity and expression exhibited within RAW264.7 macrophages was also in agreement with the IL-33-mediated increase in PUFA production (Figures 58 and 60) as PUFAs negatively regulate the expression of the SCD-1 gene (Liu *et al.* 2011).

The role of SCD-1 in atherosclerosis is complex. Many studies have shown that SCD-1 exerts protective effects against obesity and diabetes (Warensjo *et al.* 2005; Sampath and Ntambi 2014) and elevated levels of SCD-1 are associated with adverse cardiac events (Warensjoe *et al.* 2008). Also, within certain contexts ablation of SCD-1 is detrimental to atherosclerotic development; for example in mice with chronic intermittent hypoxia-induced atherosclerosis (Savransky *et al.* 2008). Yet, several groups have shown that complete ablation or knock down of SCD-1 promotes atherosclerosis (Brown *et al.* 2008; MacDonald *et al.* 2009).

In light of the beneficial effects of IL-33 described in atherosclerosis it seems likely that the IL-33-mediated reduction of SCD-1 would have a protective influence. Although, IL-33 increased the expression of several SFAs (Figures 58 and 60), a study by Sampath *et. al* (2007) demonstrated that saturated fats induce the expression of SCD-1 ahead of lipogenic genes like SREBP-1c. However, in the absence of SCD-1, SFAs instead promote the expression of genes associated with *β*-oxidation instead of lipogenesis. As such the authors proposed that the desaturation of SFAs was required to promote lipid accumulation (Sampath *et al.* 2007). Previous studies have also shown that IL-33 reduces the expression of lipogenic genes including SREBP-1c (Miller *et al.* 2010). Additionally, administration of IL-33 into genetically obese diabetic mice decreases adiposity and fasting glucose, and is accompanied with improved glucose and insulin

tolerance (Miller *et al.* 2010). Many of the actions associated with reduced SCD activity or expression are similar to those arising from IL-33 stimulation. IL-33 could potentially regulate SCD-1 to exert beneficial effects on lipid metabolism. RNAi or pharmacological inhibitors targeting SCD-1 such as TOFA, Abbott #7 and Abbott #28c (Mason *et al.* 2012) could be utilised to discern whether SCD-1 is involved in any of these actions.

This was the first study to investigate the activities of SCD, Δ6- and Δ5-desaturases in response to IL-33 stimulation. Numerous studies have used indices of product to precursors to estimate desaturase activity and the activities of these enzymes may be a stronger predictor of disease than the assessment of individual fatty acids (Warensjo *et al.* 2005). However, there are some limitations in using product/precursor indices to assess activity. For example, the choices of product/precursor ratios are inconsistent between studies. Li *et al* (2013) used three indices to predict SCD-1 activity (Li *et al.* 2013) whereas some studies only utilise a single ratio for assessment (Shiwaku *et al.* 2004; Maria Martin-Nunez *et al.* 2013). Therefore, the use of radiolabelled fatty acid precursors could provide a more in-depth analysis and also give additional information on conversion rates of precursor to product (Narce *et al.* 1994).

5.5. Future aims

The experiments presented within this chapter have shown that IL-33 instigates a number of interesting changes within macrophage fatty acid composition and further studies should be performed to characterise the genes responsible for these responses. There are a limited number of studies that have investigated the effects of IL-33 on the expression of genes involved in lipid and cholesterol metabolism and efflux, especially within the context of macrophages. Miller *et al* (2010) have previously shown that obese mice treated with IL-33 exhibit reduced adiposity. Cultures of white adipose tissue from these mice display a protective gene expression profile in response to IL-33 stimulation as the cytokine decreases the expression of genes involved in lipid metabolism and adipogenesis such as C/EBPα, SREBP-1c and LXRs (Miller *et al.* 2010). Mclaren *et al.* (2010) also demonstrated that IL-33 reduces the expression of SRs within THP-1 macrophages and HMDMs and increases the expression of genes

involved in cholesterol efflux such as ABCA-1. These changes were accompanied by alterations in the expression of lipid storage and metabolic genes such as CPT-1, ADRP and ACAT-1. The results suggest that fatty acid availability is diminished for CE production and as a result the accumulation of CEs and TAGs are decreased (McLaren *et al.* 2010b). It would be interesting to determine whether the signalling pathways responsible for these actions are the same as those involved in the regulation of ICAM-1 and MCP-1 by IL-33.

CHAPTER 6.

DISCUSSION

6.1. Introduction

Atherosclerosis and its complications are the leading cause of global mortality. The disease is characterised by chronic inflammation of the arterial wall and arises due to an imbalance in cholesterol and lipid homeostasis (Weber and Noels 2011). Macrophages are the prevalent cell type within the plaque and participate in many key stages from initial recruitment to the lesion site, uptake of modified lipoproteins and ensuing transformation into macrophage foam cells (Moore *et al.* 2013). Furthermore, macrophages can promote a maladaptive immune response through the secretion of pro-inflammatory mediators like cytokines and lipid-derived messengers which augment atherosclerotic progression (Dickhout *et al.* 2008). Cytokines are highly expressed in the plaque and the interplay between pro- and anti-inflammatory cytokines dictates the inflammatory state (McLaren and Ramji 2009).

Due to the prevalence of the disease and the resulting strain it exerts on healthcare services, there is a great level of interest in understanding the mechanisms that give rise to the disease state. IL-33 is a recently discovered cytokine which promotes the production of Th2-associated cytokines (Ait-Oufella *et al.* 2011) and has been reported to have a protective role within cardiovascular disease (Weinberg *et al.* 2003; Shimpo *et al.* 2004; Sanada *et al.* 2007; Miller *et al.* 2008). Studies from our group have demonstrated that the cytokine exerts beneficial effects by reducing foam cell formation and macropinocytosis, in addition to limiting the actions of ADAMTS enzymes in macrophages (McLaren *et al.* 2010b; Michael *et al.* 2013; Ashlin *et al.* 2014). As such the work detailed within this thesis has focused on exploring the role of this cytokine in two key aspects of the disease; inflammation and lipids. These studies provide an insight into the anti-atherogenic effects of IL-33 on inflammatory markers of the disease and also characterises the signalling pathways responsible for these actions.

Furthermore, the influence of IL-33 on the lipid and fatty acid profiles of macrophages was also explored.

6.2. Summary of key findings

The experiments presented within this thesis support the role of IL-33 as an anti-atherogenic cytokine. The cytokine exerts a number of protective effects within the context of inflammation and lipid biochemistry. The key findings from these studies are outlined below:

- IL-33 reduces the expression of a number of pro-atherosclerotic markers that are implicated in different aspects of the disease within murine RAW264.7 and human THP-1 macrophages and primary HMDMs.
- The down-regulation of ICAM-1 and MCP-1 mRNA by IL-33 is mediated through ERK1, ERK2, p38α, JNK1, JNK2, PI3K-γ, p50 and p65 in THP-1 macrophages.
- IL-33 reduces the PL content of macrophages whilst increasing the content of TAGs within THP-1 and RAW264.7 macrophages. The proportions of SEs in RAW264.7 macrophages were unaltered by the cytokine.
- Stimulation with IL-33 for 12 and 24 hours produced a range of effects on the fatty acid profile of human and murine macrophages. The cytokine increases the amounts of SFAs and n-3 PUFAs in THP-1 and RAW264.7 macrophages and reduces MUFA levels. The trend for n-6 PUFAs was less consistent but a general increase was present.
- IL-33 raises the levels of precursors for eicosanoid production and affects the content of desaturase precursors and products.
- The activities of Δ5- and Δ6-desaturases are increased by IL-33 stimulation. In contrast, SCD activity decreases following IL-33 treatment.
- IL-33 significantly decreases the expression of SCD-1 mRNA in RAW264.7 macrophages but has no effect in THP-1 macrophages.

- Overall, IL-33 promoted a protective phenotype against heart disease associated with increased production of PUFAs and beneficial changes in the expression of SCD-1.

- Many of the trends within the data were conserved between the two time points tested and between species.

6.3. The role of IL-33 in the regulation of expression of atherosclerotic markers

In chapter three, RT-qPCR was used to determine the effects of IL-33 stimulation on the mRNA expression of several well-characterised atherosclerotic markers. THP-1 macrophages were utilised for this purpose as the cell line has been used in a wide range of studies including those focusing on atherosclerosis (Banka *et al.* 1991; McLaren *et al.* 2010a; Zhang *et al.* 2011a; Qin 2012). Key findings were then confirmed in the murine RAW264.7 macrophage cell line and primary HMDMs to assess whether these changes were conserved between species and to determine their physiological relevance. IL-33 reduced the expression of a variety of genes implicated in atherosclerosis such as those involved in cholesterol uptake (Febbraio *et al.* 2000; McLaren *et al.* 2010a), metabolism (Van Eck *et al.* 2000; Mead *et al.* 2002; Mead and Ramji 2002; Lusis *et al.* 2004) and monocyte recruitment and attachment (Hwang *et al.* 1997; Blankenberg *et al.* 2003). The effect of IL-33 on the expression of CD36, SR-A and SR-B1 within THP-1 macrophages were in agreement with a previous study from our group and validated the use of recombinant IL-33 for future experiments (McLaren *et al.* 2010b). The decrease in the expression of ICAM-1, IP-10, LPL, MCP-1 and MIP-1β within THP-1 macrophages were novel and also supported the role of IL-33 as an anti-atherosclerotic mediator (Miller *et al.* 2008).

ICAM-1 and MCP-1 were chosen to optimise the system for further studies. The greatest effects by IL-33 occurred at 12 hours of stimulation with a concentration of 25ng/ml in THP-1 macrophages and murine RAW264.7 macrophages. The experimental conditions were also confirmed within primary HMDMs and therefore subsequent studies were performed using these parameters. IL-33 exhibited similar

responses within murine and human cells, demonstrating that the response was well conserved between the two species.

6.4. The activation of signalling components by IL-33

Using the conditions optimised in chapter three, RT-qPCR was performed to determine the effects of IL-33 stimulation on the mRNA expression of several signalling components implicated in atherosclerosis. Cytokine treatment did not signficantly induce the expression of any of the genes tested but caused a significant reduction in the mRNA expression of p38α, JNK2 and PI3K-γ. An additional study was performed in collaboration with Timothy Ashlin from the laboratory (Ashlin *et al.* 2014), whereby THP-1 macrophages were stimulated for 24 hours with IL-33 (10ng/ml) to determine the effect on the phosphorylation state of ERK1 and ERK2. IL-33 significantly increased the phosphorylation of these proteins and further studies by Ashlin *et al.* (2014) demonstrated that the cytokine additionally activates the JNK/c-Jun pathway (Ashlin *et al.* 2014). Schmitz *et al.* (2005) has also shown that IL-33 induces the phosphorylation of IκB, ERK1/2, p38 and JNK in HEK293 cells transfected with an expression vector for ST2 (Schmitz *et al.* 2005). Additional western blot experiments should be performed to assess whether IL-33 alters the protein expression of the other signalling components tested in chapter four using antibodies that recognise the active, phosphorylated forms (Werle *et al.* 2002; Costa *et al.* 2006; Zhou *et al.* 2010).

6.5. The signalling pathways responsible for IL-33-mediated regulation of ICAM-1 and MCP-1 expression

ICAM-1 and MCP-1 are involved in early events within the pathogenesis of atherosclerosis and have an integral role in the recruitment and attachment of leukocytes to the lesion site. Furthermore, levels of these proteins are raised in patients with atherosclerosis and correlate with incidences of CHD (Hwang *et al.* 1997; Hayashidani *et al.* 2003; Deo *et al.* 2004). Due to the importance of these proteins in initiating atherogenic processes, the studies next focused on characterising the signalling pathways that underlie the expression of ICAM-1 and MCP-1. Using the experimental conditions previously optimised, a combination of siRNAs and shRNAs were used to

determine whether the response from IL-33 was lost when the expression of key signalling genes implicated in the disease were attenuated. A significant or marked knock down in the mRNA expression of all targets following RNAi was achieved. For ERK1, ERK2, JNK2 and p38α, antibodies were also available and the knock down was confirmed at the protein level.

Experiments utilising RNAi showed that IL-33 utilises ERK1, ERK2, p38α, JNK1, JNK2, PI3K-γ, p50 and p65 to reduce the expression of ICAM-1 in THP-1 macrophages (Table 17). The same signalling components were also utilised in the down-regulation of MCP-1 mRNA expression with the exception of p50 and p65 where the knock down did not affect the response to IL-33. The results were consistent with findings from other groups that have shown that IL-33 activates IκB, ERK1/2, p38, PI3K and JNK (Schmitz *et al.* 2005; Ashlin *et al.* 2014). As such, it would be useful to extend the studies by investigating whether these signalling components are involved in the regulation of ICAM-1 and MCP-1 protein expression by IL-33. Western blot analysis using ICAM-1 and MCP-1 antibodies could be performed following knock down of these signalling components to assess whether the response from IL-33 is attenuated.

6.6. The influence of IL-33 on lipid levels in macrophages

The studies from chapters three and four investigated the role of IL-33 in the regulation of atherosclerotic markers, many of which are involved in promoting the inflammatory state. However, atherosclerosis is a multifaceted disease and also has an integral lipid component (Schoenhagen 2006). As such it was decided to explore the impact of IL-33 on lipids within macrophages. Analyses by TLC and GC were performed on human THP-1 and murine RAW264.7 macrophages following IL-33 stimulation (25ng/ml) for 12 and 24 hours to assess the temporal and inter-species impact of the cytokine on the lipid profile of these cells.

TLC analysis revealed that RAW264.7 macrophages contained PLs, TAGs and SEs; yet THP-1 macrophages were absent of detectable SEs. This was surprising as the THP-1 cell line has been used in numerous lipid and cholesterol studies (Draude and Lorenz 2000; Lada *et al.* 2002; Chanput *et al.* 2010) and contains many enzymes involved in cholesterol metabolism, transport and esterification such as ABCA-1, ABCG-1, ACAT-

1, ADRP, ApoE, CD36, LDLR, NCEH, NPC-1, NPC-2 SR-A and SR-B1 (Kritharides *et al.* 1998; McLaren *et al.* 2010b; Yang *et al.* 2013). However, a study by Lada *et al.* (2002) indicated that levels of CEs were undetectable in unloaded THP-1 macrophages by gas-liquid chromatography. However, following loading with acLDL CEs were detected (Lada *et al.* 2002). In response to PMA treatment, the expression of LDLR is reduced within THP-1 cells, whilst SRs are up-regulated therefore facilitating the transformation of these cells into macrophage foam cells in the presence of modified LDL (Qin 2012).

The time point chosen for these experiments was based upon the optimal uptake and efflux of lipids and peak expression of markers as previously discerned by our group and others. However, some groups have used three to seven day PMA-differentiated THP-1 macrophages and it is possible that the extended length of time is required for SEs to be synthesised and produced to a detectable amount (Banka *et al.* 1991; Kritharides *et al.* 1998). Experiments could also be extended to HMDMs to determine the presence of SEs in cells treated and untreated with modified LDL particles and the use of radioactive precursors could be employed to increase sensitivity.

PLs were the major lipid fraction in THP-1 and RAW264.7 macrophages and treatment with IL-33 promoted the redistribution of fatty acids from PLs to TAGs within both sets of macrophages (Figures 56, 57, 62 and 63). TAGs and CEs are typically stored within lipid droplets to limit the amounts of potential lipotoxic FFAs within the cell. It would be interesting to assess whether the rise in TAGs was associated with increased formation of lipid droplets within the macrophages as previous studies using the Amplex Red cholesterol assay kit showed that IL-33 reduces the levels of CEs within THP-1 macrophages and HMDMs (McLaren *et al.* 2010b). Oil red O or Nile red could be used to stain intracellular lipid droplets before and after IL-33 treatment to determine potential changes (Greenberg *et al.* 2011; Walther and Farese 2012). The studies from chapter five also indicate that the cytokine may promote the redistribution of fatty acids without significantly altering the amounts of total lipid. Interestingly, disrupted plaques contain raised levels of total lipids associated with increased levels of phospholipids and TAGs as well as free cholesterol and CEs (Felton *et al.* 1997). Previous studies have shown that levels of free cholesterol and CEs are reduced in response to IL-33 (McLaren *et al.* 2010b).

6.6.1. The influence of IL-33 on SFAs in macrophages

There was a trend for increased levels of SFAs in response to IL-33 stimulation within THP-1 and RAW264.7 macrophages. The cytokine significantly increased levels of myristic acid and stearic acid within PLs and also raised stearic acid in total fatty acids in THP-1 macrophages. Within RAW264.7 macrophages, IL-33 significantly increased levels of palmitic acid in TAGs and stearic acid within SEs. However, this was accompanied by a reduction in the amounts of palmitic acid within PLs in both THP-1 and RAW264.7 macrophages. Overall there was a trend for increased incorporation of fatty acids into TAGs, including SFAs. A study by Koliwad *et al.* (2010) demonstrated that the storage of SFAs into TAGs within murine macrophages was inversely related with the production of pro-inflammatory genes and the switch to the M1 phenotype. These beneficial effects were thought to arise by reducing the availability of free SFAs and therefore limiting the use of SFAs for pro-inflammatory pathways (Koliwad *et al.* 2010).

6.6.2. The influence of IL-33 on MUFAs in macrophages

The effects of IL-33 on MUFAs within THP-1 and RAW264.7 macrophages were also assessed. Treatment with IL-33 significantly reduced the percentages of a number of MUFAs such as C16:1n7, C16:1n9 and oleic acid after 12 and 24 hours in THP-1 and RAW 264.7 macrophages. The role of *cis*-MUFAs in atherosclerosis is unclear and may not have a major impact, although a Mediterranean-style diet rich in MUFAs has been attributed with beneficial cardiac effects (Lee *et al.* 1989; Mensink and Katan 1990; Hayes and Khosla 1992).

Many studies have demonstrated a positive association between *trans*-MUFAs, atherosclerosis and other cardiac disorders (Mensink and Katan 1990; Lichtenstein *et al.* 1999; Djousse *et al.* 2012). Although it is most likely that any unsaturated fatty acids within the study are of the natural *cis*-double bond configuration, *trans*-double bonds could potentially be exogenously supplied and incorporated from the growing medium. MS could be used to determine the configuration of double bonds within the fatty acids (Nichols *et al.* 1985). The activity of desaturases such as SCD-1 and elongases are potentially a more relevant indicator of MUFA involvement in atherosclerosis.

Inhibition of SCD-1 promotes the production of SFAs and decreases the levels of MUFAs (Ariyama *et al.* 2010). As such the increase in the content of SFAs and reduction of MUFAs following IL-33 stimulation is likely to be a product of reduced activity of SCD-1.

6.6.3. The influence of IL-33 on PUFAs in macrophages

After 12 and 24 hours of IL-33 stimulation, levels of n-3 PUFAs were raised in macrophages; however the effects on n-6 PUFAs were more varied and species-specific. Treatment by the cytokine produced a significant increase in the n-3 PUFA DPA and n-6 PUFA AA within THP-1 macrophages and there was a trend for raised levels of PUFAs in PLs from both families. Within RAW264.7 macrophages there was a significant increase in the n-3 PUFA EPA and n-6 PUFA LA and a significant reduction in the n-6 PUFAs DGLA and nervonic acid. The evidence for the beneficial role of n-3 PUFAs in cardiovascular disease is controversial, whereas the involvement of n-6 PUFAs in the disease state are more mixed (Table 19).

An interesting result was the increase in the content of eicosanoid precursors such as LA, EPA and AA. The mutual increase occurred in opposition to known antagonistic mechanisms that exist between n-3 and n-6 PUFAs and could suggest that major changes occur in response to IL-33 treatment that override such methods of regulation. Both fatty acids function as precursors for eicosanoids so the mutual increase in content may reflect a general rise in eicosanoid production within the macrophages. As such further analysis should be performed to determine the effects of the cytokine on eicosanoid synthesis and, crucially, whether the resulting mediators are pro- or anti-inflammatory.

Due to the anti-inflammatory effects of IL-33 in atherosclerotic studies it may be predicted that the cytokine would enhance the formation of pro-resolving lipid-derived mediators. The resolution of the inflammatory state is an active process associated with alterations in PUFA metabolism that promote the switch from pro-inflammatory eicosanoid production to specialised pro-resolving mediators including lipoxins, resolvins, protectins and maresins (Levy *et al.* 2001; Bannenberg and Serhan 2010). However, there are several complications associated with measuring eicosanoid production as eicosanoids are not stored but are rapidly formed and released. Many

studies focus on immunolocalisation of eicosanoid-producing enzymes or detection of precursor fatty acids to determine sites of eicosanoid production (Marmor and Julius 2001). However, Yang *et al.* (2011) outline a number of sensitive metabolomics and lipidomic approaches to assess eicosanoid synthesis. Briefly, cells or tissues are extracted prior to solid phase extraction with internal standards by enzyme-linked immunosorbent assay (ELISA), liquid chromatography-ultraviolet (LC-UV) or GC-MS. Lastly LC-UV-MS-MS is used to generate lipid mediator profiles that can be checked against lipidomic databases (Norris *et al.* 2011; Yang *et al.* 2011; Dalli and Serhan 2012). Alternatively, High performance-LC (HPLC) /MS/MS or GC/MS can be used to detect the presence of multiple eicosanoids. HPLC distinguishes specific compounds based on physical properties and subsequent MS analysis utilises product ion spectra to identify the compounds. However such methods require preparation, as samples must be volatile and thermally stable (Lundstrom *et al.* 2009; Mesaros *et al.* 2009).

6.6.4. IL-33-mediated regulation of desaturase activity and gene expression

The activities of desaturases reflect metabolic changes in fatty acids and are directly associated with the disease state (Martinelli *et al.* 2008; Ebbesson *et al.* 2012). A reduction in the activity of Δ5-desaturase and increased Δ6-desaturase activity are exhibited during CAD and related disorders (Leng *et al.* 1999; Warensjo *et al.* 2005; Petersson *et al.* 2008; Warensjoe *et al.* 2008). However, the involvement of SCD within the disease state is complicated as high levels of SCD-1 are linked to adverse cardiac events (Warensjoe *et al.* 2008), yet complete ablation of the gene can also promote atherosclerosis (Brown *et al.* 2008; MacDonald *et al.* 2009). Therefore, the expression of SCD is carefully regulated (Heinemann and Ozols 1998) and there appears to be a fine balance between protective and detrimental effects by the enzyme (Warensjo *et al.* 2005; Sampath and Ntambi 2014).

Due to the involvement of the desaturases within CAD, the effects of IL-33 treatment on the activities of Δ5-desaturase, Δ6-desaturase and SCD were investigated using a ratio of products to precursors. Treatment with IL-33 resulted in a significant reduction in SCD activity within THP-1 and RAW264.7 macrophages and significantly increased the activity of Δ6-desaturase within THP-1 macrophages and Δ5-desaturase activity in THP-1 and RAW264.7 macrophages. Interestingly, a similar pattern of effects was

described by Leikin and Brenner (1998) during a study where rats were fed a diet deficient in cholesterol to determine the impact on desaturase activity and upon the cholesterol and fatty acid profile of liver microsomes. An absence in dietary cholesterol reduced the proportions of cholesterol and PC and was accompanied by a fall in MUFAs and an increase in SFAs, in addition to AA. Depletion of cholesterol decreased the activity of SCD over the time course, accompanied with increased Δ5- and Δ6-desaturase activities. The authors suggest that the deficiency in cholesterol promoted Δ5- and Δ6-desaturase activity thereby increasing the unsaturation index of membrane fatty acids to enhance membrane fluidity (Leikin and Brenner 1988).

The SCD-1 index is believed to be a stronger predictor of cardiac-related mortality than Δ5- and Δ6-indices and has therefore been the focus of many studies (Ntambi 1999; Attie *et al.* 2002; Warensjoe *et al.* 2008). RT-qPCR was used to explore the influence of IL-33 on the mRNA expression of the major SCD isoform in THP-1 and RAW264.7 macrophages. Stimulation by IL-33 (25ng/ml) for 12 and 24 hours caused a significant reduction in the mRNA expression of SCD-1 in murine macrophages; however there was no change in human THP-1 macrophages.

A fall in SCD-1 expression may produce an atheroprotective lipid profile as mice lacking SCD-1 exhibit a deficit of TAGs and CEs within VLDL and LDL particles accompanied with a fall in the expression of lipid synthesis genes (Ntambi *et al.* 2002). In contrast, overexpression of SCD-1 boosts the esterification of cholesterol to CEs (Miyazaki *et al.* 2000). The fall in expression and activity may have arisen from enhanced PUFA production as PUFAs from either family inhibit the expression of SCD-1 (Waters *et al.* 1997). Additionally, several studies have shown that the down-regulation of SCD-1 expression is mediated through a reduction in SREPB-1c and LXR binding to promoter regions in the gene encoding the desaturase (Yoshikawa *et al.* 2002; Chu *et al.* 2006). DNase I footprinting and gel retardation assays have identified two key binding regions in the SCD-1 promoter where several transcription factors have also been shown to bind including PPAR-α, C/EBP-α and AP-1 (Christy *et al.* 1989; Mauvoisin and Mounier 2011). Interestingly several of these nuclear factors are also regulated by IL-33 (Miller *et al.* 2010) and would serve as suitable candidates for further investigations. RT-qPCR could be used to investigate any changes in SCD-1

mRNA expression in RAW264.7 macrophages following inhibition/knock down of these nuclear factors by pharmacological inhibitors or RNAi.

Interestingly, the cytokine did not affect the gene expression of SCD-1 in THP-1 macrophages yet significantly reduced SCD activity. The changes in SCD activity could potentially be due to alterations in the expression of the less prevalent isoform; SCD-2. However, SCD-2 is predominately expressed within the brain (Sampath and Ntambi 2014) so is unlikely to have a major impact on activity but RT-qPCR using isoform-specific primers for SCD-2 should be performed.

Another possibility for the changes in activity could be due to post-transcriptional or post-translational modifications that could alter the activity of the enzyme without affecting the mRNA expression of SCD-1. The transcriptional inhibitor actinomycin D could be utilised for such a purpose whereby cells are either left untreated or stimulated with IL-33 and incubated with actinomycin D. Total RNA is then harvested at various time intervals to determine the effect of IL-33 on mRNA stability (Fan *et al.* 2005). Alternatively, the cells could be incubated with radioactive nucleosides in pulse-chasing experiments. The radioactive probes are incorporated into RNA and the medium is replaced with fresh medium containing an excess of unlabelled nucleosides that ‘chase’ the radioactive substrates. The freshly produced mRNA can then be detected by filter hybridisation to determine the timescale for mRNA decay (Palade 1975). Additionally, analysis by western blotting may indicate whether the protein is post-transcriptionally regulated at the level of steady state protein. In regards to these findings, antibodies for total SCD or specific SCD isoforms could be used to determine whether any changes were isoform specific. Also enzymatic activity could be assessed through the use of radiolabelled substrates (Demcakova *et al.* 2001). For example using radiolabelled stearic acid, the distribution of radioactivity between substrate (stearic acid) and product (oleic acid) could be measured to determine the rate of desaturation mediated by SCD.

6.7. Future studies

6.7.1. Exploring the role of IL-33 in the regulation of atherosclerotic markers

The actions of IL-33 has been described as dichotomous whereby the cytokine can produce contradictory pro- and anti-inflammatory effects dependent on cellular conditions. In chapter four, the reduction in ICAM-1 and MCP-1 mRNA by IL-33 was mediated through ERK1, ERK2, p38α, JNK1, JNK2, PI3K-γ, p50 and p65. However, a number of these components are also utilised by IL-33 to induce the expression of ICAM-1 and MCP-1 within other cells. For example, IL-33 uses p65 to increase the expression of ICAM-1 in endothelial cells (Choi *et al.* 2012), whilst NF-κB, p38 and ERK facilitate the up-regulation of ICAM-1 in eosinophils (Chow *et al.* 2010).

Due to the mutual use of these signalling components it is essential to distinguish the underlying mechanisms that give rise to the opposing effects on ICAM-1 and MCP-1 expression initiated by IL-33. Several approaches could be implemented to further investigate key differences in the cellular pathways responsible. RNAi could be used to knock down the expression of signalling elements that control ICAM-1 and MCP-1 expression within alternative cell types. For example, other studies have implicated Akt (Radisavljevic *et al.* 2000), Rho kinase (Shimada and Rajagopalan 2010), Casein Kinase 2 and the JAK-STAT pathway (Harvey *et al.* 2007) in the regulation of ICAM-1 and MCP-1 mRNA and protein expression. As such these signalling modules may potentially be involved in IL-33-regulation of ICAM-1 and MCP-1 expression within macrophages.

IL-33 also utilises Akt, MyD88, IRAK, IRAK4 and TRAF6 during the regulation of other genes (Schmitz *et al.* 2005; Choi *et al.* 2009) and these components could be involved. The use of RNAi targeted towards these genes or bone marrow derived macrophages, or alternatively, peritoneal macrophages from knockout animals could be utilised in a similar approach as the one used in chapter four to determine whether these signalling modules are integral in the regulation of ICAM-1 and MCP-1 expression by IL-33. Analysis by RT-qPCR and western blotting would determine the effect at the mRNA and protein levels and give an indication if additional regulatory systems are

involved. Additionally, nuclear run-on transcription assays, whereby nuclei from chosen cells are isolated in the presence of labelled nucleoside triphosphates, could be used in combination with hybridisation techniques to measure rate of transcription initiation and transcriptional control (Farrell and Farrell 1993). The promoter could be dissected by targeted deletion and mutation analyses to determine the importance of *cis*-acting transcriptional elements for gene expression (Allen *et al.* 1992; Bruhn *et al.* 1993).

As many common signalling elements are involved in both pro- and anti-inflammatory IL-33 signalling, it could become a lengthy procedure to systematically test individual components. Also, single-gene approaches may present a biased representation of the cellular environment as genes are often constituents of larger regulatory networks (Tuomisto *et al.* 2005). Therefore the use of commercially available microarrays could provide a rapid and cost-effective method of large-scale gene expression profiling. Microarray assays are comprised of thousands of cDNA sequences or oligonucleotides corresponding to sequences printed in a high-density array and a single experiment can provide a wealth of data about gene expression patterns (DeRisi *et al.* 1997). Statistical algorithms can then be used to cluster the data based on functionality or similarity in expression patterns (Eisen *et al.* 1998). This approach may identify well-characterised or novel genes involved in IL-33 signalling. Also, microarray analysis could be used to determine which particular signalling components dictate the pro- or anti-inflammatory effects of IL-33. The technique could be performed on material taken from atherosclerotic plaques and asthmatic samples subjected to IL-33 treatment to compare the differences in expression patterns. However, many diseases like atherosclerosis contain a heterogeneous population of cells that could complicate analyses and as such initial experiments should focus on particular cell types integral to both disease states such as macrophages.

The dual functionality of IL-33 may also arise due to differential release of IL-33 and ST2. It has been proposed that full-length IL-33 is cleaved to limit the pro-inflammatory activities of the cytokine. On the other hand, functional full-length IL-33 is secreted by necrotic cells and is thought to function as an ‘alarmin’ to stimulate protective effects against damaged tissues or cells (Kunes *et al.* 2012). The activity of IL-33 is tightly controlled and IL-33 mRNA is quickly regulated within hours of synthesis (Polumuri *et al.* 2012). Full-length and mature versions of IL-33 should be tested to discern whether

maturation affects the actions of IL-33 on the expression of atherosclerotic markers or fatty acid profiles. Miller *et al.* (2011) suggest that alternative splicing of ST2 adds further control and can gives rise to functional membrane ST2 or the antagonistic decoy receptor sST2 (Miller and Liew 2011). ST2 can also dimerise with SIGIRR which hinders IL-33 activity (Bulek *et al.* 2009). The use of knockout animals deficient in ST2 could be used to determine whether IL-33-signalling occurs independently of the receptor.

The dual nature of IL-33 may also arise through its interactions with other inflammatory mediators including cytokines. IL-33 injections promotes inflammatory bowel disease in mice deficient in IL-10, however wild-type mice are protected against IL-33-induced mucosal inflammation (Sattler *et al.* 2014). Additionally, stimulation by IL-33 or IL-13 alone does not affect levels of CCL17 and CCL24, whereas co-stimulation leads to enhanced concentrations of these chemokines and polarisation towards the alternatively activated macrophage phenotype (Kurowska-Stolarska *et al.* 2009). Synergistic interactions with other cytokines could function as a switch that determines the pro- or anti-inflammatory activities of IL-33. It would be interesting to see whether co-stimulating cells with IL-10 and IL-13 affects the expression of ICAM-1 and MCP-1 by IL-33 and if the pathways responsible alter as a result.

6.7.2. Future studies exploring the involvement of IL-33 in the regulation of macrophage lipids

A number of studies exploring macrophage cholesterol homeostasis pre-incubate THP-1 macrophages with modified LDL particles in order to promote the formation of foam cells (Banka *et al.* 1991; Rodriguez *et al.* 1994; McLaren *et al.* 2010b). The presence of macrophage foam cells is a hallmark of the disease and a similar approach could be adopted utilising TLC and GC following incubation of THP-1 and RAW264.7 macrophages with modified LDL particles such as acLDL or oxLDL (Liu *et al.* 2014). Transformation of these cells could be validated by Oil Red O staining to assess the accumulation of lipids within these cells (Turunen *et al.* 2004; Kang *et al.* 2014; Liu *et al.* 2014).

Due to the role of IL-33 in modulating lipid composition and inflammatory gene expression, further experiments should be performed to integrate and consolidate these key areas as modifications to membrane composition can affect inflammatory processes such as macrophage chemotaxis and the binding of chemotactic mediators (Calder and Yaqoob 2003). A study by Komai-Koma *et al.* (2007) demonstrated that IL-33 serves as a chemoattractant for polarised Th2-cells *in vitro* and injections of IL-33 into ST2 knockout mice induces the localised accumulation of adoptively transferred Th2 cells (Komai-Koma *et al.* 2007). The mechanisms underlying IL-33 chemotactic actions are unknown and it would be informative to determine whether the signalling cascades involved in other chemotactic events are also utilised by IL-33. Lipid-derived signalling components such as PI3K, PIP_2 and PIP_3 are integral to chemotaxis. Within neutrophils, formation of the leading edge is characterised by PI3K-γ-dependent accumulation of PIP_3 and PIP_2 at the plasma membrane (Fergus *et al.* 2007; Kolsch *et al.* 2008). Also, studies with mouse neutrophils have shown that chemoattractants influence the metabolism of inositol-derivatives; PIP_2 hydrolysis is controlled by $PLC\beta_2$ and $PLC\beta_3$ whereas the phosphorylation of PIP_2 is induced by PI3K-γ (Wu *et al.* 2000). Knockout mice for key signalling mediators implicated in chemotaxis such as PLC and PI3K members, or an RNAi approach targeting these genes, could be used to determine whether IL-33-mediated chemotaxis is dependent on these signalling components. The effects on cellular motility could then be measured by chemotaxis assays based on analysing the redistribution of cells across a porous membrane or through agarose or ECM gels exposed to IL-33. The movement of cells can be detected microscopically or through the use of a colorimetric, fluorescent or radioactive marker (Zigmond *et al.* 2001).

Alterations in membrane PUFA content can also influence cellular activity. A study on peripheral blood neutrophils and monocytes taken from healthy subjects showed that there was a negative association between the proportions of palmitic and oleic acid and the phagocytic ability of these cells. On the other hand, levels of stearic acid, LA, ALA, AA, DHA and total PUFAs were positively linked with phagocytosis (Kew *et al.* 2003). Interestingly many of these fatty acids were affected in the experiments presented in chapter five so further studies could investigate whether IL-33 alters the phagocytic abilities of macrophages. For this purpose, the macrophages could be pre-incubated with phagocytotic agonists such as opsonised zymosan, immune complexes or urate

crystals to determine the rate and extent of phagocytosis before and after IL-33 stimulation (Yang *et al.* 2011). These effects may arise due to changes in specific types of phospholipids. For example, IFN-γ increases the PUFA content of PC and PE phospholipids within murine and human macrophages. The authors suggest that the alterations in membrane structure and fluidity promote endotoxin and LPS binding to these cells (Jackson *et al.* 1992; Darmani *et al.* 1994; Darmani *et al.* 1995). Therefore, it would be interesting to determine whether the changes induced by IL-33 were specific to particular types of phospholipids. Lipid fractions from macrophages stimulated by IL-33 could be assessed by two-dimensional TLC to explore the effects on the fatty acid distribution of particular phospholipids.

Lipid remodelling can also influence the distribution of signalling mediators and facilitate receptor-ligand interactions. For example, membrane-resident SFAs and PUFAs regulate the activation of TLR4 (MacDonald *et al.* 2009). Lauric acid promotes the dimerisation of TLR4 into lipid raft domains and therefore promotes the co-ordination of TLR4 with downstream adaptors including MyD88. However, these effects are inhibited by the presence of DHA (MacDonald *et al.* 2009). Interestingly the IL-33/ST2 axis utilises TLR4 and MyD88 following exposure to LPS and within mice with experimental sepsis (Espinassous *et al.* 2009; Alves-Filho *et al.* 2010). It would be interesting to determine whether alterations in the fatty acid composition of phospholipids mediate the spatial localisation of IL-33 signalling components. Lateral interactions between phospholipids and sphingolipids give rise to lipid raft domains which are implicated in a number of cellular processes such as phagocytosis and the generation of pro-inflammatory cytokines (Schumann 2012). Several cytokines such as IL-1 (Veluthakal *et al.* 2005) and IL-2 (Marmor and Julius 2001) have been shown to modulate the formation of lipid raft domains. Investigations into the formation of such domains could utilise detergent or detergent-free methods to separate membrane rafts and non-raft regions. Triton X-100 is a non-ionic detergent frequently chosen to solubilise non-raft portions of membrane at 4°C, the remaining detergent-resistant sections represent lipid domains and can be separated using density gradient centrifugation (Gaus *et al.* 2005; Wong *et al.* 2009). Alternatively, non-detergent based methods involving the disruption of the membrane by sonication and the isolation of buoyant lipid raft domains and heavier non-raft sections could be used (Gaus *et al.* 2005; Macdonald and Pike 2005).

Balsinde and Dennis (1996) reported that increased content of AA within murine macrophages was followed by the accumulation of AA within the medium mediated by phospholipases (Balsinde and Dennis 1996). Stimulation by IL-33 may trigger the release and secretion of lipid-derived factors that could potentially have a role in atherosclerosis. Fatty acids such as AA implicated in signalling events could be radiolabelled and supplemented into the growth medium for a requisite amount of time to allow incorporation of the exogenous fatty acid. Following a medium change to remove excess radiolabelled fatty acids, the effects of IL-33 treatment on the secretion of radiolabelled fatty acids into the medium could then be assessed by scintillation counting. Furthermore, the distribution and metabolism of radiolabelled fatty acids within the cells could also be explored.

The studies presented within chapter five demonstrated that IL-33 significantly reduces the activity of SCD in human and murine macrophages and also decreases the expression of SCD-1 mRNA in RAW264.7 macrophages. Mice lacking SCD-1 are protected against diet-induced obesity (Ntambi *et al.* 2002) and men containing a polymorphism in the gene encoding SCD-1 have decreased adiposity and resistance to diet-linked weight gain (Warensjo *et al.* 2007). Interestingly, stimulation by IL-33 also produces a number of adipoprotective effects such as the down-regulation of C/EBP-α, LXR-α, LXR-β and PPAR-γ expression in white adipose tissue (Miller *et al.* 2010). The cytokine also reduces the expression of genes involved in cholesterol metabolism; ACAT-1 and NCEH and up-regulates those involved in cholesterol efflux such as ABCA-1 and ABCG-1 in THP-1 and HMDMs (McLaren *et al.* 2010b). Due to the mutual and complementary effects it would be useful to explore whether the adipoprotective effects by IL-33 are mediated through the modulation of SCD-1 expression and activity. The expression of genes involved in lipid and cholesterol metabolism known to be affected by IL-33 could be compared in mice overexpressing SCD-1 (Rogowski *et al.* 2013) and mice lacking SCD-1 (Ntambi *et al.* 2002) following IL-33 treatment to determine whether SCD-1 is involved.

6.8. The therapeutic implications of IL-33 research

The clinical symptoms of CAD typically manifest during the later phases of the disease, therefore methods for earlier detection could be key to treating and combating CAD

(Ross 1993; Poredos 2011). Current methods in the prognosis of HF utilise echocardiography, electrocardiography and chest X-rays to assess changes in systolic and diastolic function, alterations in ECG and pulmonary congestion respectively (Dickstein *et al.* 2008). The use of soluble markers represents a promising field of diagnostics and could provide an early non-intrusive method of detection (Wattanakit *et al.* 2005). Currently the natriuretic peptides; B-type natriuretic peptide (BNP) and amino-terminal pro-BNP are the only biomarker assays recommended by the European Society of Cardiology committee for diagnostic assessment of patients with HF (McMurray *et al.* 2012). Studies have shown that atherosclerotic development can be monitored by measuring carotid intima media thickness by ultrasound and is also independently related to levels of CRP (Howard-Alpe *et al.* 2006; Benedetto *et al.* 2008). However, due to a limited number of certified cardiac biomarkers within this field, further research is necessary to expand this promising field of early prognosis.

sICAM-1 is the cleaved version of membrane-bound ICAM-1 (Gross *et al.* 2012) and several large cohort studies have linked raised levels of serum and plasma ICAM-1 to cardiovascular disease (Hwang *et al.* 1997; Ridker *et al.* 1998; Malik *et al.* 2001). MCP-1 has also been highlighted as a potential biomarker of the disease as serum and plasma levels of the chemokine are positively associated with cardiovascular risk (Blanco-Colio *et al.* 2007; Tang *et al.* 2007; Gonzalez-Quesada and Frangogiannis 2009). During the disease state, levels of circulating adhesion molecues and chemokines are raised within the serum of patients with HF, likely due to insufficient clearance or increased production (Papayianni *et al.* 2002). Therefore, detection of elevated levels of these proteins could facilitate early therapeutic intervention.

Recently, sST2 has received a lot of attention as a promising marker of CAD. *In vitro* experiments have shown that treatment with sST2 reverses the protective effects of IL-33 and leads to increased atherosclerotic development in mice (Miller *et al.* 2008). Several epidemiological studies have also suggested that levels of serum sST2 can function as a biomarker for predicting mortality and HF in patients who have suffered a MI event (Weinberg *et al.* 2003; Shimpo *et al.* 2004) and increased concentrations of sST2 are associated with adverse prognosis of acute dyspnea (Januzzi *et al.* 2007; Shah *et al.* 2009). Additionally, circulating levels of sST2 are related to age, blood pressure, the use of anti-hypertensive medicine and diabetes (Ho *et al.* 2013). In a study by

Pascual-Figal *et al.* (2011) three biomarkers were assessed in blood taken from patients with acutely decompensated HF: high-sensitivity troponin T, amino-terminal pro-BNP levels and sST2. Each biomarker independently predicted risk of death and the combination of the three markers improved prediction accuracy (Pascual-Figal *et al.* 2011).

Serum sST2 concentrations are typically detected by ELISA assays. Briefly, the method involves adding samples to microtitre plates coated with anti-human ST2 antibodies. The plates are washed before the addition of peroxidase-conjugated anti-human ST2 antibodies and after another series of washes the peroxidase-associated substrate is added. The resulting change in colour can then be assessed at the appropriate wavelength to predict sST2 levels (Weinberg *et al.* 2002; Weinberg *et al.* 2003; Shimpo *et al.* 2004). However, issues over poor sensitivity have meant that the assay is unable to detect the low concentrations of sST2 present in healthy subjects (Januzzi 2013). However, a highly sensitive ELISA has been recently developed called Presage™ ST2 assay. A large population cohort study demonstrated that the system could detect sST2 levels in all subjects tested and that the amounts of sST2 were positively associated with incidences of HF. Furthermore, unlike BNP, levels of sST2 are not significantly influenced by renal function (Dieplinger *et al.* 2009). Promisingly, the assay has been recently approved by the United States food and drug administration as a prognostic test for HF (Januzzi 2013; Mueller and Dieplinger 2013).

Additionally, due to the anti-atherogenic effects of IL-33, the cytokine serves as a promising therapeutic candidate. The use of IL-33$^{-/-}$ mice (Oboki *et al.* 2010) crossed with ApoE$^{-/-}$ or LDLR$^{-/-}$ mice could be used to assess the loss of IL-33 on overall atherosclerotic development. Encouragingly, a recent study showed that administration of IL-33 after a MI event in mice reduced the size of the infarction and wall thinning and also improved left ventricular function (Yin *et al.* 2014). However, due to the dualistic nature of the cytokine, the role of IL-33 within atherosclerosis and the signalling pathways or cellular changes responsible for its actions should be better characterised before the cytokine can be considered for therapeutic applications.

6.9. Conclusions

The studies presented within this thesis have demonstrated that IL-33 modulates a range of anti-atherogenic processes. The cytokine down-regulates the expression of several markers implicated in atherosclerotic events such as those involved in the recruitment and attachment of leukocytes and lipid and cholesterol metabolism. Furthermore, several signalling components involved in the IL-33-mediated down-regulation of ICAM-1 or MCP-1 have been identified; the MAPKs ERK1, ERK2, p38α, JNK1 and JNK2; the PI3K component PI3K-γ and NF-κB members p50 and p65. The cytokine also alters the fatty acid profile of macrophages by potentially promoting the redistribution of PLs to TAG fractions and also increases the content of SFAs and specific PUFAs known to function as eicosanoid precursors. Such changes may arise due to alterations in the expression of enzymes involved in fatty acid desaturation, as IL-33 increases the activities of Δ5- and Δ6-desaturases but reduces SCD activity. The fall in SCD activity was also accompanied by a reduction in SCD-1 mRNA expression in murine RAW 264.7 macrophages.

There is growing interest in IL-33 due to the anti-atherogenic properties of the cytokine and as such a greater understanding of the mechanisms that give rise to these actions may translate to future therapeutic applications in the treatment of atherosclerosis and heart disease. Future studies will need to focus on delineating the anti-atherogenic properties of the cytokine and distinguishing why the activities of IL-33 are protective within cardiovascular disease and obesity but detrimental in other inflammatory diseases.

APPENDIX

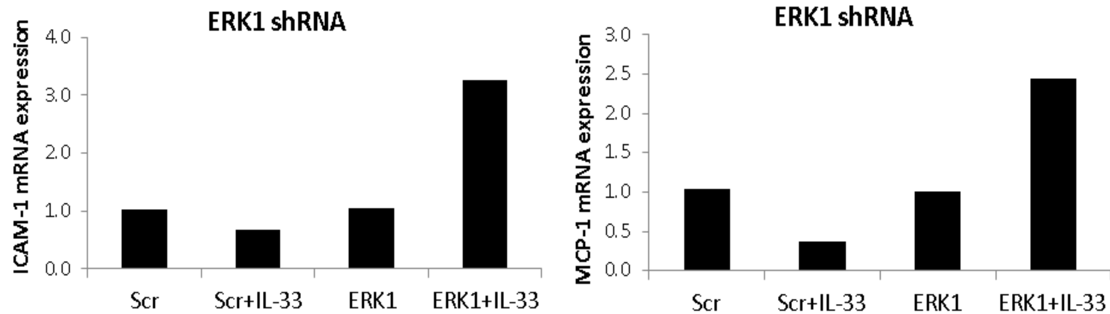


Figure 67. The effect of ERK1 knock down in regulation of ICAM-1 and MCP-1 by IL-33

RT-qPCR was performed using cDNA from primary macrophages expressing ERK1 shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples given an arbitrary value of 1. Data represents mean ± SD from a single experiment taken from Figure 49 performed in triplicate.

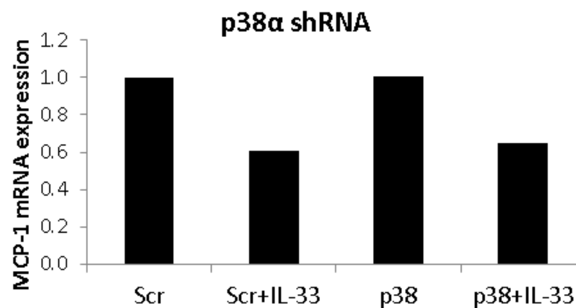


Figure 68. The effects of p38α knock down in IL-33 regulation of ICAM-1 and MCP-1

RT-qPCR was performed using cDNA from primary macrophages expressing p38α shRNA and incubated in the presence or absence of IL-33 (25ng/ml) for 12 hours. Cells expressing shRNA targeting a scramble sequence were used as a control (Scr). The mRNA levels were calculated using the comparative Ct method and normalised to the house-keeping gene, GAPDH, with values from control samples given an arbitrary value of 1. Data represents mean ± SD from a single experiment taken from Figure 52 performed in triplicate.

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGCAGGACGATATCTCTAGC	20	56.33	50.00	6.00	6.00
Reverse primer	ACGATGAGCTCCTGCTGTTA	20	58.81	50.00	6.00	2.00

Products on target templates

>NM_005063.4 Homo sapiens stearoyl-CoA desaturase (delta-9-desaturase) (SCD), mRNA

```
product length = 347
Forward primer  1     TGCAGGACGATATCTCTAGC  20
Template        507   ....................  526

Reverse primer  1     ACGATGAGCTCCTGCTGTTA  20
Template        853   ....................  834
```

Figure 69. Human SCD-1 primer homology

Primer-Blast search details identifying that the primer sequence is 100% specific for human SCD-1. Primer sequence taken from (Peter *et al.* 2009).

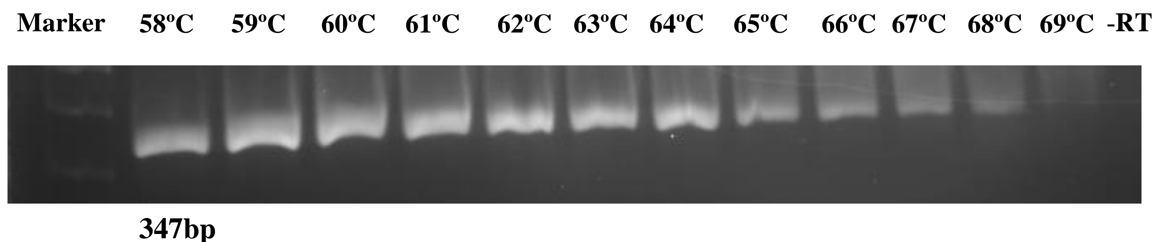


Figure 70. Human SCD-1 primer optimisation

The Products from RT-PCR were assessed on a 1.5% (w/v) agarose gel. cDNA was taken from the same experiment and subjected to a range of annealing temperatures. As a result an annealing temperature of 58ºC was chosen. The expected product size for human SCD-1= 347bp. Primer sequence taken from (Peter *et al.* 2009).

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTTCTTGCGATACACTCTGG	20	55.97	50.00	2.00	0.00
Reverse primer	TGAATGTTCTTGTCGTAGGG	20	55.11	45.00	4.00	0.00

Products on target templates

>NM_009127.4 Mus musculus stearoyl-Coenzyme A desaturase 1 (Scd1), mRNA

```
product length = 93
Forward primer  1     CTTCTTGCGATACACTCTGG  20
Template        1038  ....................  1057

Reverse primer  1     TGAATGTTCTTGTCGTAGGG  20
Template        1130  ....................  1111
```

Figure 71. Murine SCD-1 primer homology

Primer-Blast search details identifying that the primer sequence is 100% specific for murine SCD-1. Primer sequence taken from (Thorn *et al.* 2010).

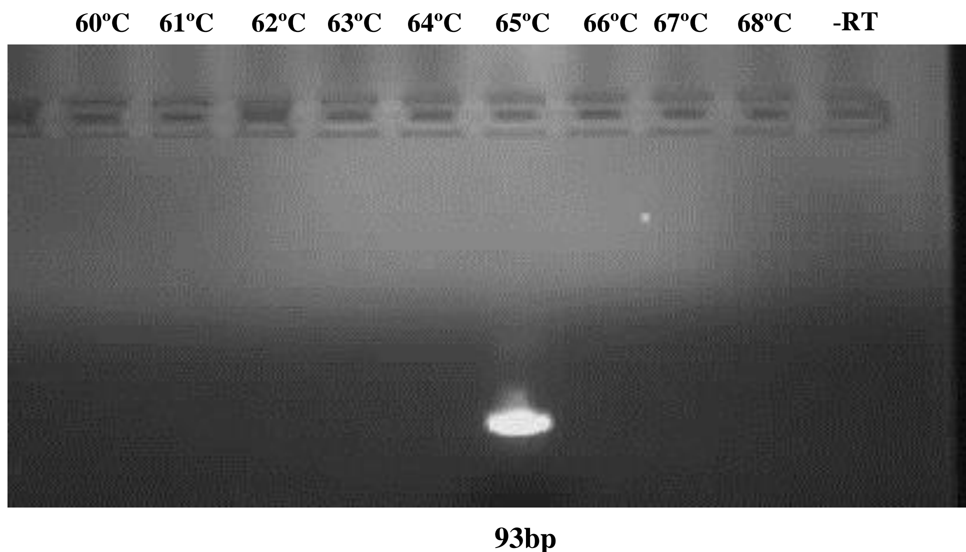
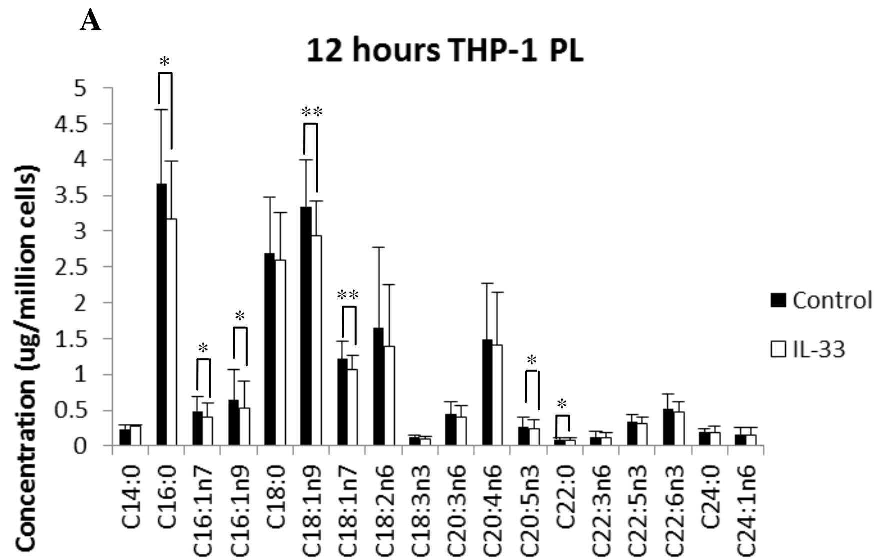


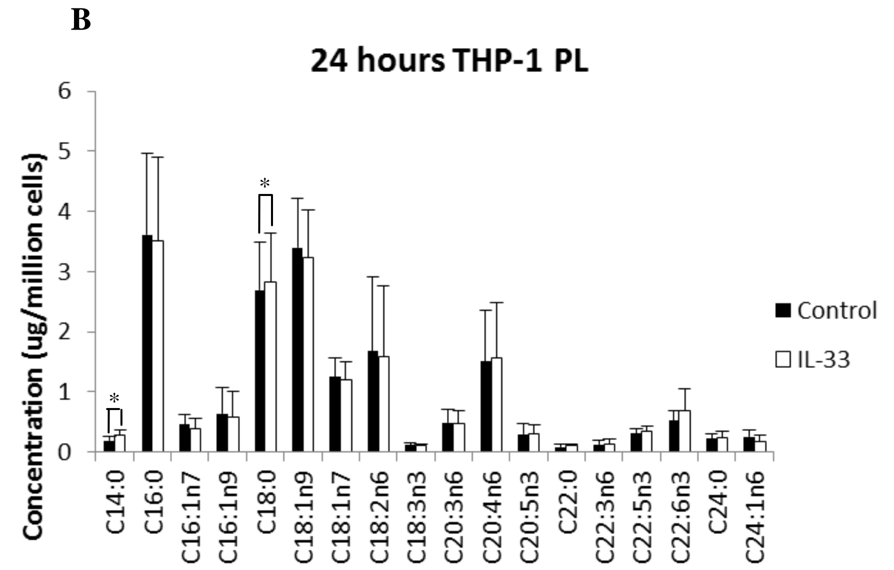
Figure 72. Murine SCD-1 primer optimisation

The Products from RT-PCR were assessed on a 1.5% (w/v) agarose gel. cDNA was taken from the same experiment and subjected to a range of annealing temperatures. As a result an annealing temperature of 65ºC was chosen. The expected product size for murine SCD-1= 93bp. Primer sequence taken from (Thorn *et al.* 2010).

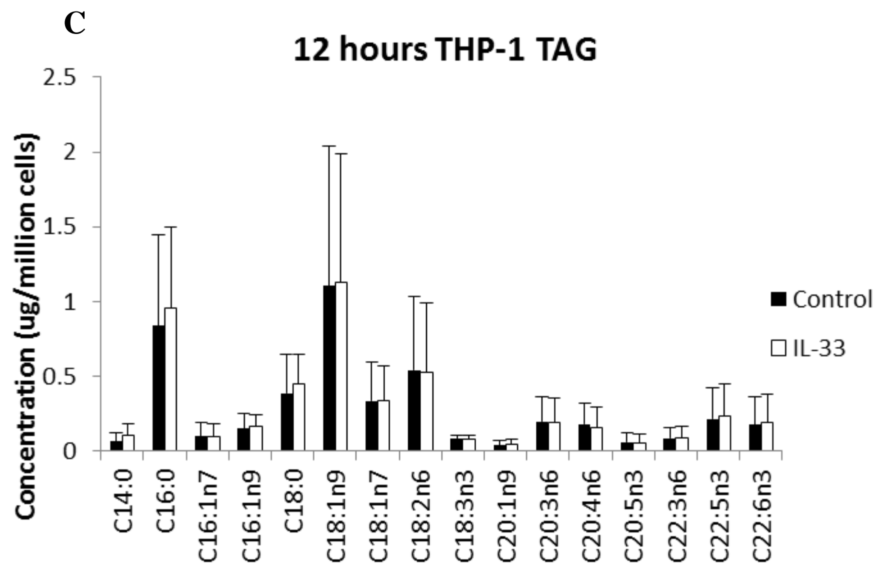
A



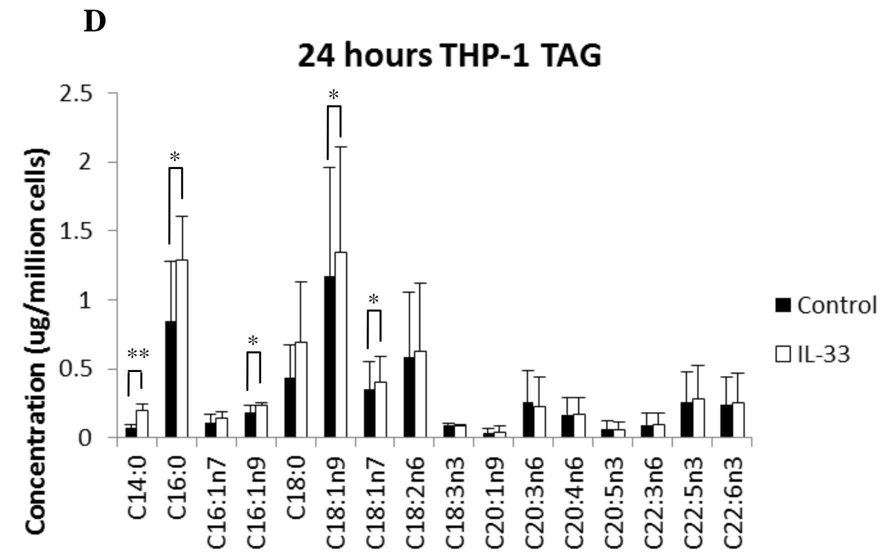
B



C



D



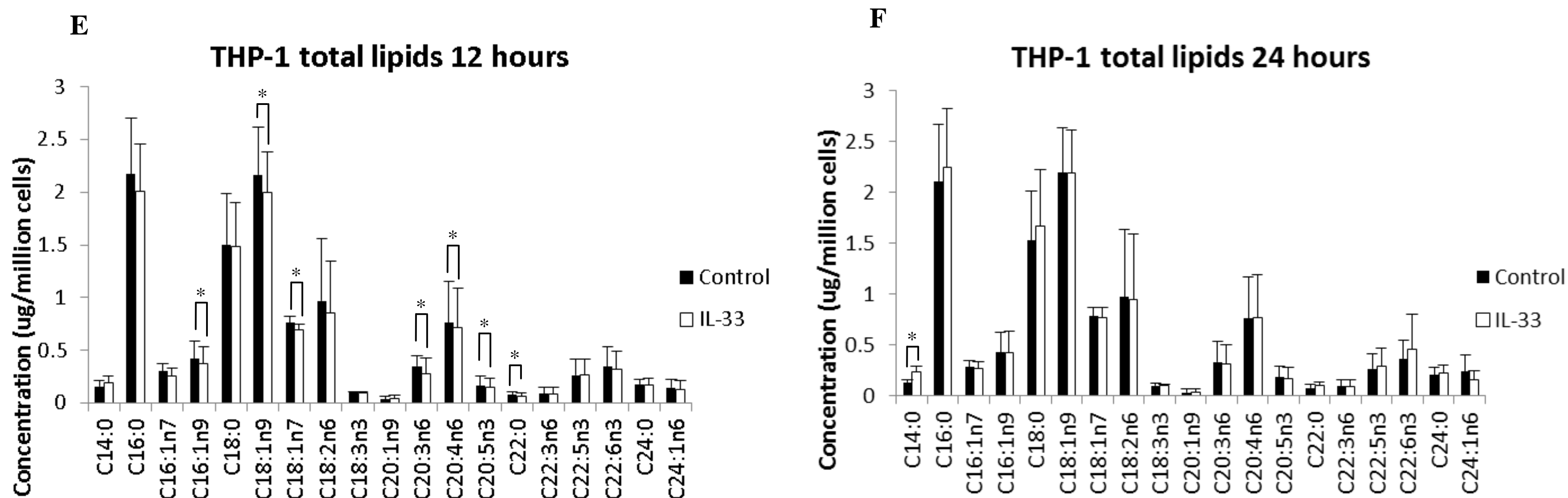
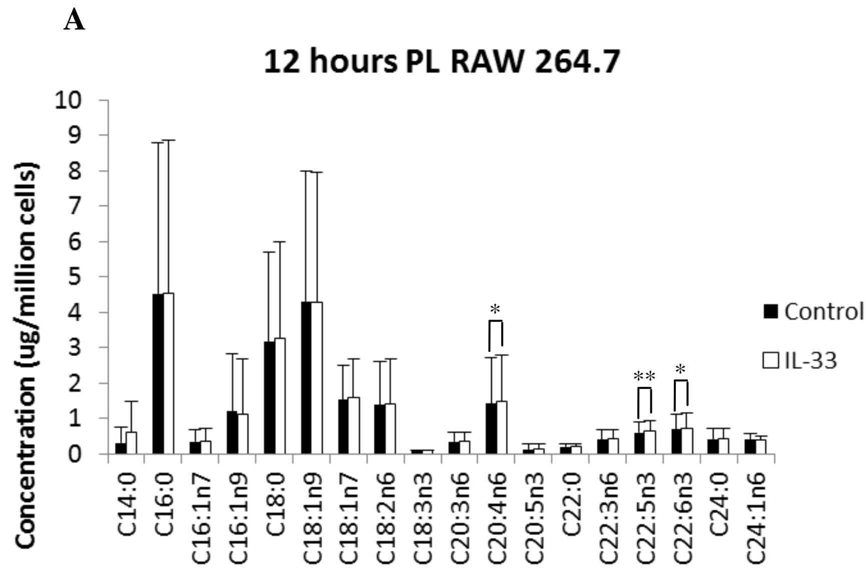
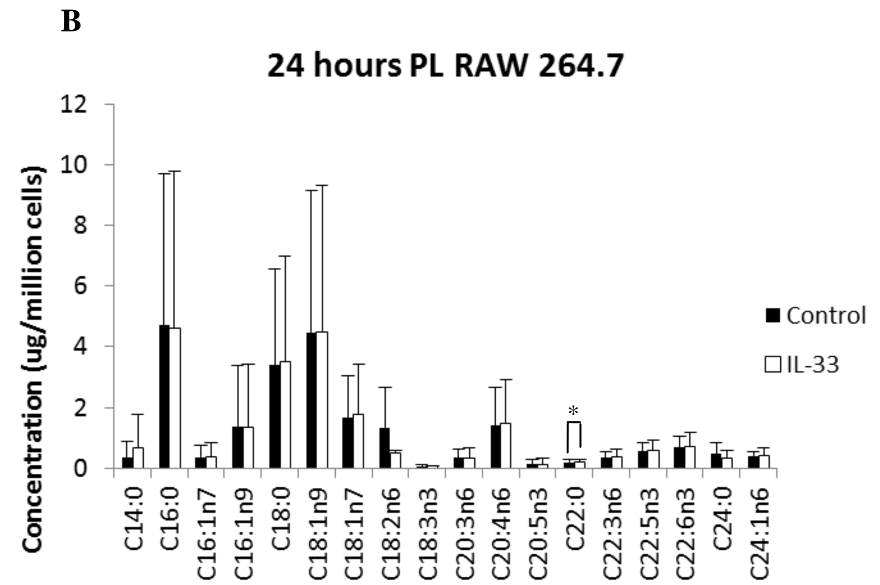
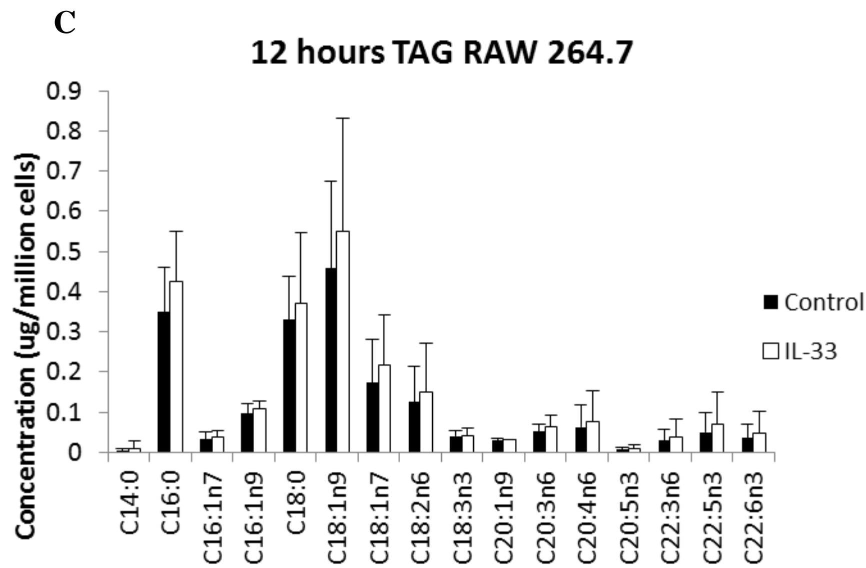


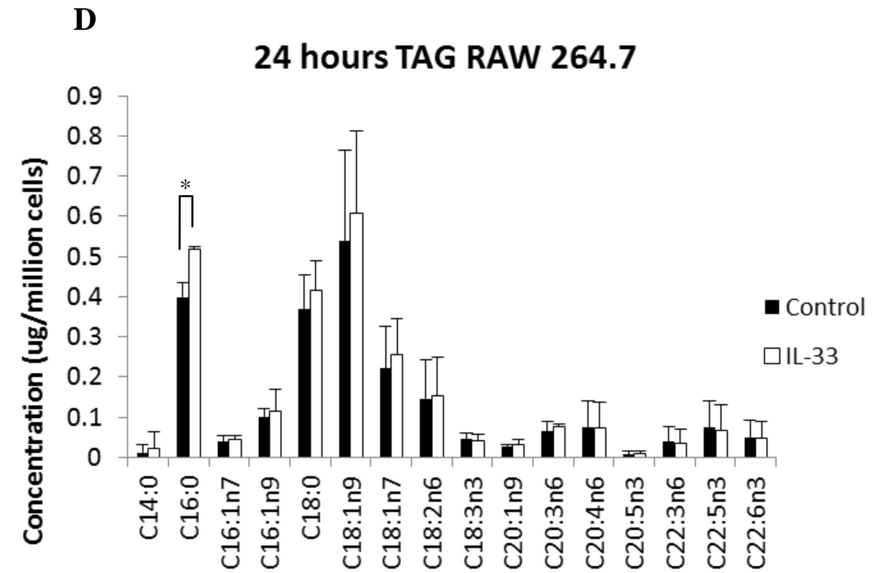
Figure 73. Distribution of fatty acids in THP-1 macrophages

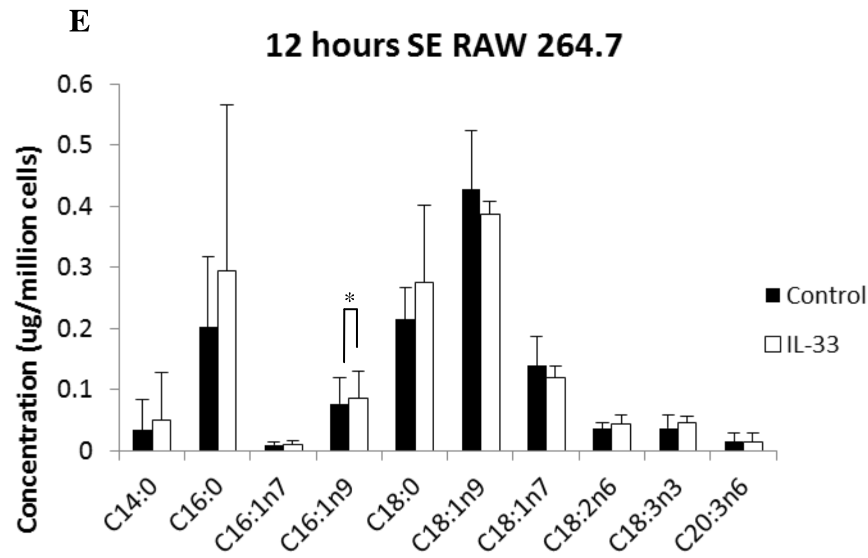
PMA-differentiated THP-1 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. Following TLC, fatty acids from fractions for PLs (Panels A and B) and TAGs (Panels C and D) were converted to FAMEs for analysis by GC. Total fatty acids (Panels E and F) represent the fatty acids from summation of PLs and TAGs. The results are presented as the concentration of cells normalised to µg/million cells for PLs (A and B), TAGs (C and D) or total fatty acids (E and F). Fatty acids are indicated with the number before the colon showing the number of carbon atoms, after which denotes the number of double bonds followed by the position of the first double bond. Data represents mean ± SD from three independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05, **p<0.01, ***p<0.001.

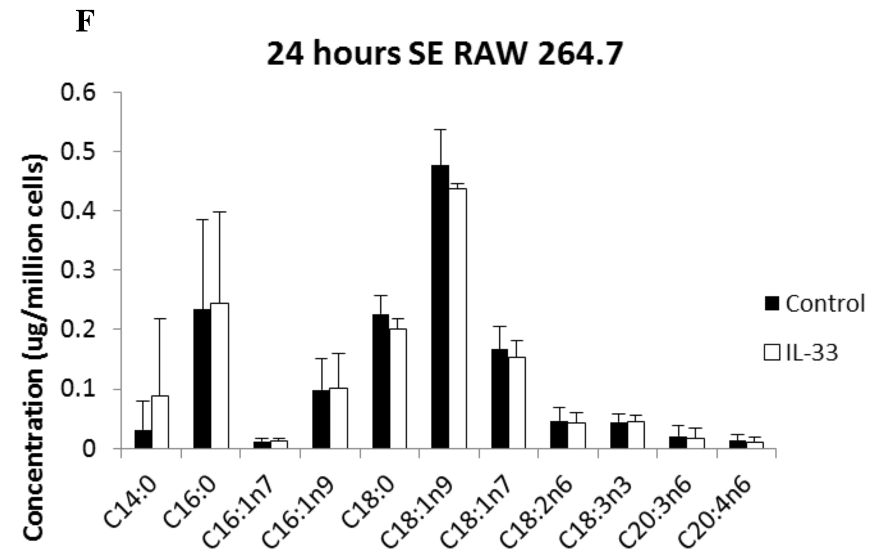

A
12 hours PL RAW 264.7
Concentration (ug/million cells)
0
1
2
3
4
5
6
7
8
9
10
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6
*
**
*
Control
IL-33

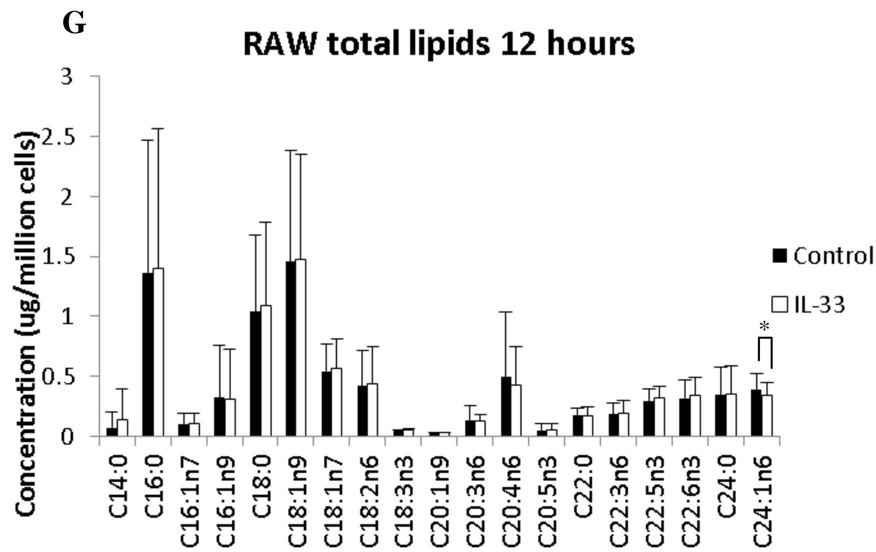

B
24 hours PL RAW 264.7
Concentration (ug/million cells)
0
2
4
6
8
10
12
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6
*
Control
IL-33


C
12 hours TAG RAW 264.7
Concentration (ug/million cells)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:3n6
C22:5n3
C22:6n3
Control
IL-33


D
24 hours TAG RAW 264.7
Concentration (ug/million cells)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:3n6
C22:5n3
C22:6n3
*
Control
IL-33


E
12 hours SE RAW 264.7
Concentration (ug/million cells)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control
IL-33
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:3n6


F
24 hours SE RAW 264.7
Concentration (ug/million cells)
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
IL-33
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:3n6
C20:4n6


G
RAW total lipids 12 hours
Concentration (ug/million cells)
3
2.5
2
1.5
1
0.5
0
Control
IL-33
*
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6

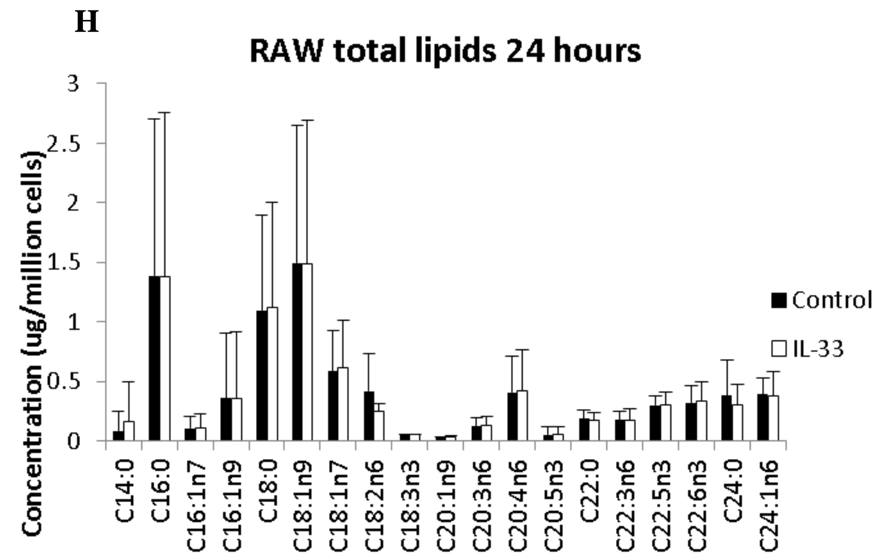

H
RAW total lipids 24 hours
Concentration (ug/million cells)
3
2.5
2
1.5
1
0.5
0
Control
IL-33
C14:0
C16:0
C16:1n7
C16:1n9
C18:0
C18:1n9
C18:1n7
C18:2n6
C18:3n3
C20:1n9
C20:3n6
C20:4n6
C20:5n3
C22:0
C22:3n6
C22:5n3
C22:6n3
C24:0
C24:1n6

Figure 74. Distribution of fatty acids in RAW264.7 macrophages

RAW264.7 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. Following TLC, fatty acids from fractions for PLs (Panels A and B), TAGs (Panels C and D) and SEs (E and F) were converted to FAMEs for analysis by GC. Total fatty acids (Panels G and H) represent the fatty acids from summation of PLs, TAGs and SEs. The results are presented as the concentration of cells normalised to μg/million cells for PLs (A and B), TAGs (C and D), SEs (E and F) or total fatty acids (G and H). Fatty acids are indicated with the number before the colon showing the number of carbon atoms, after which denotes the number of double bonds followed by the position of the first double bond. Data represents mean ± SD from three independent experiments. The Student *t* test was used to determine the statistical significance of the results *p<0.05, **p<0.01, ***p<0.001.

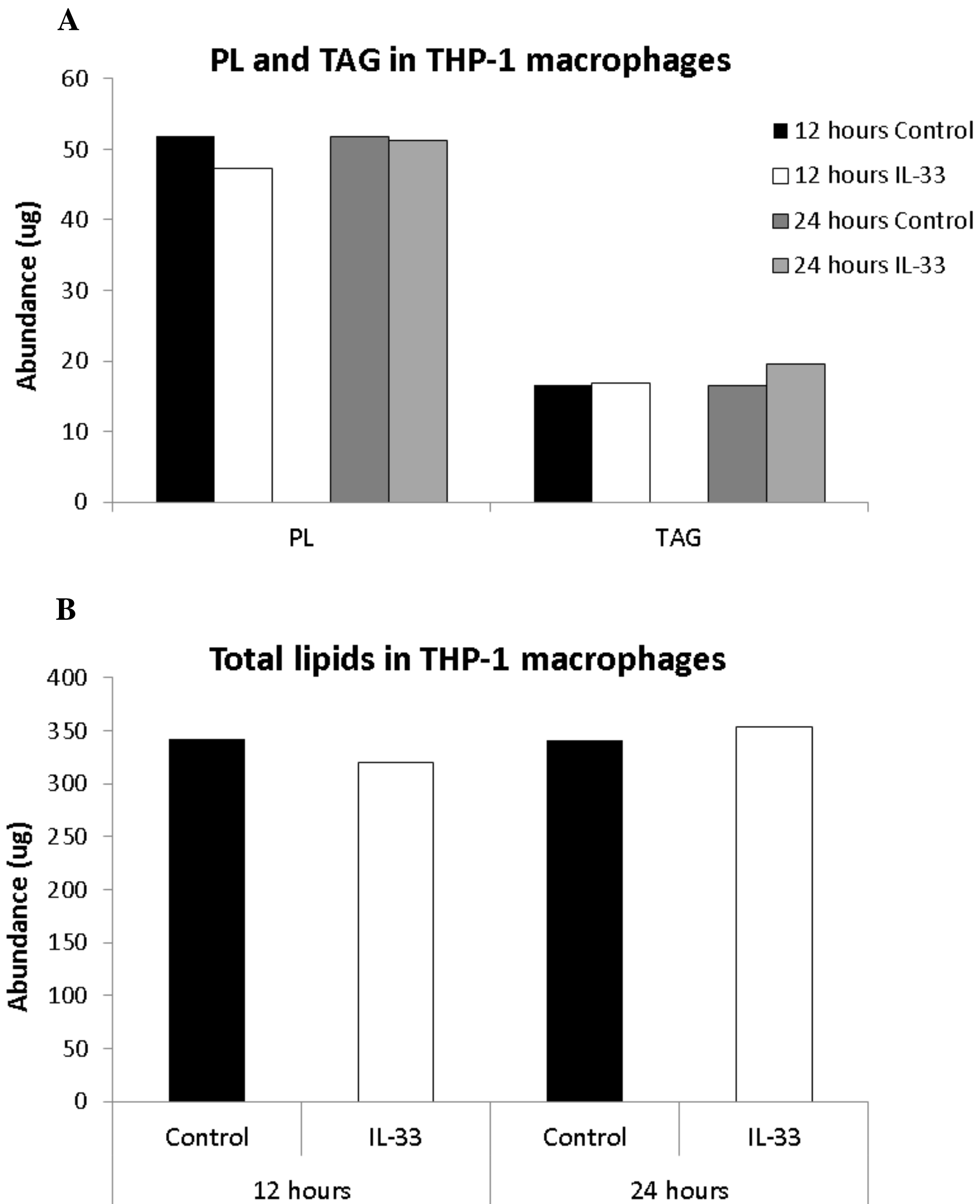


Figure 75. The effect of IL-33 on major lipid classes in THP-1 macrophages

PMA-differentiated THP-1 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. TLC was used to separate lipids into different lipids groups; PLs and TAGs (A). Total lipids are shown as the summation of PLs and TAGs (B). The graph displays the concentration of lipids within each fraction (µg). Data represents three independent experiments.

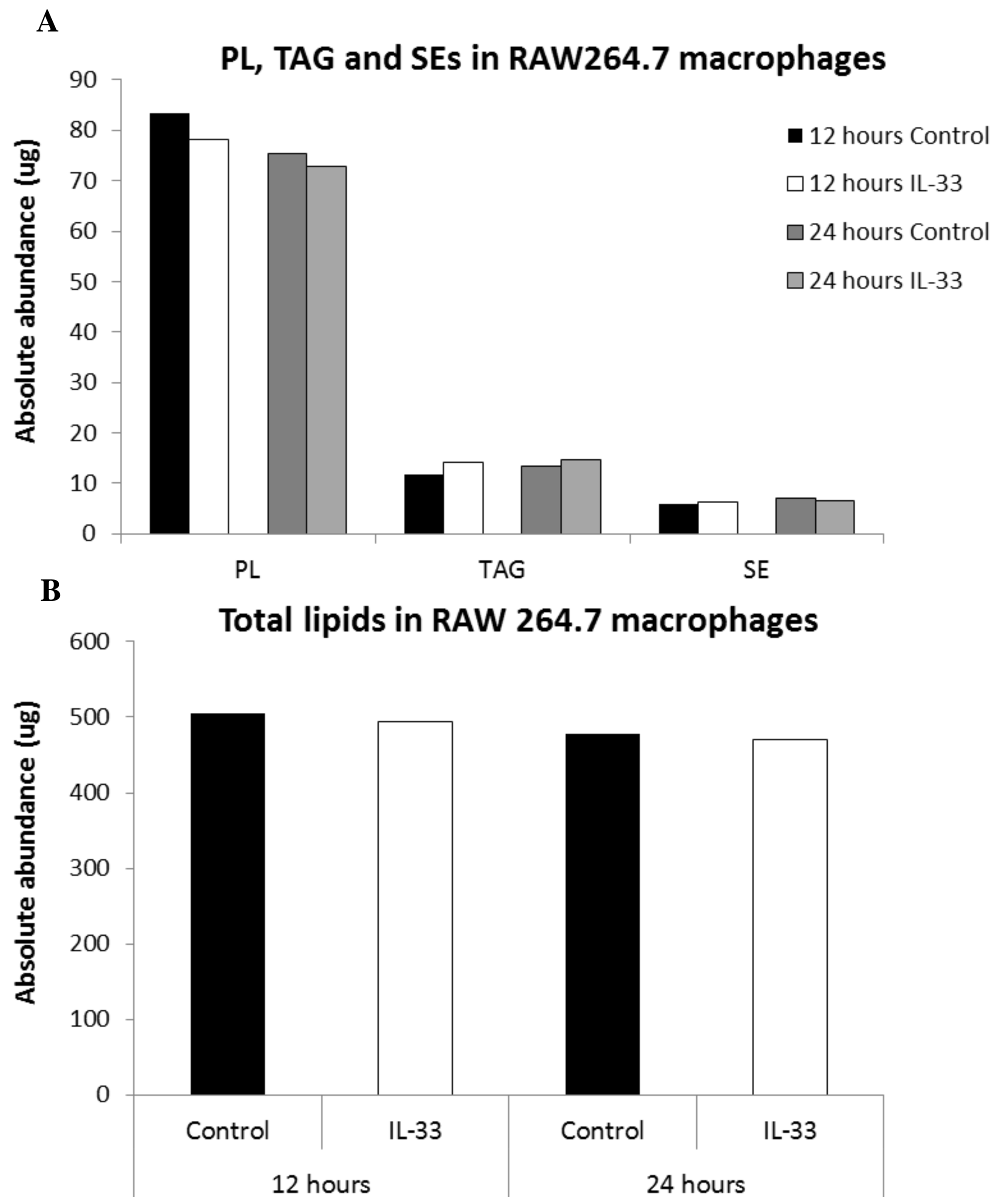


Figure 76. The effect of IL-33 on major lipid classes in RAW264.7 macrophages

RAW264.7 macrophages were incubated with IL-33 (25ng/ml) for 12 or 24 hours. An untreated control was included under the same conditions. TLC was used to separate lipids into different lipids groups; PLs, TAGs and SEs (A). Total lipids are shown as the summation of PLs, TAGs and SEs (B). The graph displays the concentration of lipids within each fraction (µg). Data represents three independent experiments.

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