Anti-atherogenic actions of dihomo-gamma-linolenic acid in macrophages

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CONTENTS

ABSTRACT

Atherosclerosis is a chronic inflammatory disorder characterised by lipid accumulation in the arterial wall. Nutraceuticals represent promising alternatives to pharmaceuticals in the prevention and management of this disease. Previous work has shown an omega-6 fatty acid, dihomo-gamma-linolenic acid (DGLA) to inhibit atherosclerosis in a mouse model of the disease. Understanding the molecular mechanism underlying the action of DGLA in atherosclerosis is crucial to evaluating the role of this PUFA as a new agent in the prevention/treatment of the disease.

In vitro analysis utilised macrophage cell lines THP-1 and RAW264.7 together with primary cultures of human monocyte-derived macrophages to study the effects of DGLA on aspects of macrophage foam cell formation, an early event in atherosclerosis. Data presented in the thesis showed that DGLA had an effect on a number of key events that contribute to foam cell formation in macrophages; reducing monocyte migration, pro-inflammatory cytokine induced gene expression, modified LDL uptake, scavenger receptor expression, macropinocytosis and cholesteryl ester accumulation and stimulating cholesterol efflux.

Uptake of DGLA into lipid fractions was studied *in vitro* and *in vivo* using thin layer chromatography and gas chromatography. DGLA was significantly incorporated in a dose-dependent manner into lipid fractions of THP-1 macrophages *in vitro*. *In vivo*, mice fed a 4.4% DGLA containing diet assimilated the PUFA into serum, liver, kidney and adipose tissue lipid fractions.

Finally, the metabolism of DGLA was investigated *in vitro*. DGLA supplementation stimulated the production of PGE_1 and 15-HETrE in macrophages. PGE_1 inhibited monocyte migration and IFN-γ induced expression of monocyte chemotactic protein 1 (MCP-1). RNA interference assays showed a key role for COX enzymes in the IFN-γ-mediated induction of MCP-1 expression.

Findings in the thesis demonstrate key mechanisms underlying the anti-atherogenic role of DGLA and highlight its potential as a therapeutic/preventative agent in this disease.

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PUBLICATIONS

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ABBREVIATIONS

8-HOA	8 hydroxyoctanoic acid
AA	Arachidonic acid
ABC	ATP binding cassette
ABCA1	ABC transporter A1
ABCG1	ABC transporter G1
ACAT	Acetyl-CoA cholesterol acetyltransferase
acLDL	Acetylated LDL
ADRP	Adipose differentiation related protein
ALA	α-linoleic acid
ANSA	8 anilino 4 naphthosulphonic acid
AP	Alkaline phosphatase
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ASC (cell)	Adipose stromal cell
ASC (protein)	apoptosis-associated speck-like protein containing CARD
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCL2	Human chemokine (C-C motif) ligand 2
CCR2	C-C chemokine receptor 2
CD36	Cluster differentiation 36
CEH	Cholesteryl ester hydrolase
CETP	Cholesteryl ester transfer protein
ChIP	Chromatin immunoprecipitation
CIDE	Cell death inducing DFF45 like effector
COX	Cyclo-oxygenase
CPT	Carnitine palmitoyl transferase
CRP	C-reactive protein
CVD	Cardiovascular disease
CXCL16	Chemokine (C-X-C motif) ligand 16
DAG	Diacylglycerol
DAMPS	Danger associated molecular patterns
DCF	2',7, dichlorofluorescein
DCFDA	Dichlorofluorescin diacetate

DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DMPE	Dimethylphosphinoethane
/DPA	Docosapentaenoic acid
DPM	Disintegrations per minute
dsDNA	Double stranded DNA
ECM	Extracellular matrix
EFA	Essential fatty acid
ELISA	Enzyme linked immunosorbent assay
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
FAMES	Fatty acid methyl esters
FFA	Free fatty acids
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GAS	Gamma activating sequences
GC	Gas chromatography
GLA	γ-linolenic acid
GPCR	G protein-coupled receptor
GSB	Gel sample buffer
HDL	High density lipoprotein
HETE	Hydroxy-eicosatetraenoic acids
HETrE	Hydroxy-eicosatriaenoic acids
HI-FCS	Heat-inactivated foetal calf serum
HL	Hepatic lipase
HMDM	Human monocyte derived macrophages
HMG-CoAR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPLC-MS	High performance liquid chromatography – mass spectrometry
HTA	Heptanoic acid
ICAM-1	Intercellular adhesion molecule 1
IDL	Intermediate density lipoprotein
IFN	Interferon
IFN-γR	Interferon γ receptor
IL	Interleukin
IL-1ra	IL-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
INT	Tetrazolium salt

IP-10	IFN-inducible protein of 10kDa
IRF	Interferon regulatory factors
ISRE	IFN-γ stimulated response elements
I-TAC	IFN-inducible T cell α chemoattractant
JAK	Janus kinase
JNK	c-Jun N terminal kinases
LA	Linoleic acid
LCAT	Lecithin cholesterol acyl transferase
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
LIPE	Hormone sensitive lipase
LOX	Lipo-oxygenase
LPL	Lipoprotein lipase
LPS	Lipopolysaccaride
LT	Leukotriene
LXR	Liver X receptor
LY	Lucifer yellow
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony stimulating factor
Mig	Monokine induced by IFN-γ
mmLDL	Minimally modified LDL
MMLV	Molony leukemia virus
MMPs	Matrix metalloproteinase
MUFA	Mono-unsaturated fatty acids
NEFA	Non-esterified fatty acids
nCEH	Neutral cholesterol ester hydrolase
NF-κB	Nuclear factor κB
NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide oligomerization domain
NPC	Niemann-Pick type C
oxLDL	Oxidised LDL
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen associated molecular patterns

PAT	Peri lipinadipophilin TIP47
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PG	Prostaglandin
PI3K	Phosphoinositide-3-kinase
PLA2	Phospholipase A2
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferator activated receptors
PPRE	PPAR response elements
PRRs	Pathogen recognition receptors
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphotidylserine
PUFA	Poly-unsaturated fatty acids
RCT	Reverse cholesterol transport
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative PCR
SCD1	Stearoyl CoA desaturase
SDS	Sodium dodecyl sulphate
SFA	Saturated fatty acids
SMC	Smooth muscle cells
SOCS	Suppressor of cytokine signalling
SRA	Scavenger receptor A
SRB1	Scavenger receptor B1
SREBP	Sterol regulatory element binding protein
SR-PSOX	Scavenger receptor for phosphotidylserine and oxidised lipoprotein
STAT	Signal transducer and activator of transcription
TAG	Triacylglycerol
TBE	Tris/borate/EDTA
TBHP	Tert-butyl hydroperoxide
TFA	Total fatty acids
TGF	Transforming growth factor

TLC	Thin layer chromatography
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TPL	Total polar lipid
TX	Thromboxane
VCAM-1	Vascular cellular adhesion molecule 1
VLDL	Very low density lipoprotein
WT	Wild type

CHAPTER 1

INTRODUCTION

1.1 Cardiovascular disease and atherosclerosis

Cardiovascular disease (CVD) causes more deaths annually than any other disease in Western society. It has been estimated that 17.5 million people died from CVD in 2012 representing 31% of world deaths, with this figure set to rise to 23.3 million by 2030 (Buckley and Ramji 2015). In addition to the predicted increase in mortality rates, an increase in incidence of CVD adds to the already considerable burden on costs to public healthcare systems (Oldridge 2008; Buckley and Ramji 2015). In 2009, CVD cost the UK economy £19 billion (British Heart Foundation, 2016). Given the predicted increase in mortality rates and the subsequent burden on healthcare systems, research into understanding the underlying causes of CVD and identifying novel targets and therapies is crucial.

The primary cause of CVD is atherosclerosis, a chronic inflammatory disorder of the vasculature (Michael *et al.* 2012). Atherosclerosis is a progressive disease initiated by the accumulation of lipids in medium and large arteries, a precursor event providing the basis to the formation of advanced plaques (Lusis 2012). Over a number of years plaques can become increasingly large and complex eventually leading to their rupture. Plaque rupture releases large amounts of necrotic debris into the circulation increasing the risk of blood clots which in some circumstances can result in myocardial infarction and/or stroke (Lusis 2000).

1.1.1 Mouse models

Development of mouse models of atherosclerosis has been a critical advancement in understanding the factors affecting the disease and identifying new therapeutic targets. Apolipoprotein E deficient mice ($ApoE^{-/-}$) are commonly used as a model system due to their ability to form spontaneous atherosclerotic lesions on standard chow diet (Meir and Leitersdorf 2004). ApoE is a glycoprotein synthesised mainly in the liver and is a component of lipoproteins (except low density lipoprotein (LDL)). The protein functions as a ligand for receptors which aid in the clearance of chylomicrons and very low density lipoproteins (VLDL) (Meir and Leitersdorf 2004). ApoE is also synthesised in monocytes and macrophages in the vessel wall and has a local effect on cholesterol homeostasis (Curtiss 2000; Greenow *et al.* 2005). ApoE

deficient mice fed a normal chow diet display five times higher plasma cholesterol levels, a reduced concentration and particle size of high density lipoprotein (HDL) and an increase in triacylglycerol (TAG), in comparison to wild type (WT) controls (Zhang *et al.* 1992). Fatty streaks and foam cell deposition in arteries are visible after three months and advanced lesions after eight months (Zhang *et al*. 1992). When fed a high fat diet, plasma cholesterol levels are a dramatic fourteen times higher in ApoE$^{-/-}$ mice in comparison to WT mice (Plump *et al.* 1992). Atherosclerotic lesions are typically visible within just 10 weeks in the aorta and coronary and pulmonary arteries (Plump *et al*. 1992). A second commonly used mouse model is the LDL receptor knockout (LDLr$^{-/-}$) mouse. LDLr deficiency is the cause of familial hypercholesterolemia in humans (Zadelaar *et al.* 2007). The LDLr$^{-/-}$ mice display a moderate increase in plasma cholesterol levels, with cholesterol mainly contained within LDL, on normal chow diet (Ishibashi *et al.* 1993; Zadelaar *et al*. 2007). High fat diet induces a 6-fold increase in plasma cholesterol levels, an increase in VLDL and LDL levels and a decrease in HDL (Ishibashi *et al.* 1994). In addition, after 7 months on a high fat diet, LDLr$^{-/-}$ mice develop atherosclerotic lesions in the aorta with the aortic valve wall thickened by a mass of cholesterol-loaded macrophages (Ishibashi *et al*. 1994).

There are many advantages and disadvantages associated with both models of atherosclerosis. The plaque morphology in ApoE$^{-/-}$ and LDLr$^{-/-}$ mice are comparable and resemble those in the human counterparts (Zadelaar *et al*. 2007) so represent good models for the study of this disease. However, there are of course a number of species differences between mouse and humans which may impact on the reliability of these models. The levels of HDL and LDL differ significantly between the species. Mice have high concentrations of HDL and low levels of LDL whereas humans display the opposite (Zhang *et al*. 1992). Mice also lack the cholesterol ester transfer protein which transfers cholesteryl esters from HDL to VLDL and LDL (Zhang *et al*. 1992). This being said, ApoE$^{-/-}$ mice have remarkably similar phenotypes to that of human ApoE deficiency (Zhang *et al*. 1992) and plasma lipoprotein profile of LDLr$^{-/-}$ resembles that of humans with cholesterol mainly contained in LDL fraction (Zadelaar *et al*. 2007). Deficiency of ApoE also has an effect on other processes. ApoE can be synthesised locally by macrophages where it can play a role in inflammation, monocytosis, reverse cholesterol transport and oxidation of LDL (Curtiss 2000; Ruston and Drevon 2001; Baitsch *et al.* 2008; Murphy *et al.* 2011; Westerterp *et al.* 2013). With these processes disrupted in ApoE$^{-/-}$ mouse models, limitations lie in studying pathways associated with these processes. Despite the drawbacks of these

mouse models, they have been widely used in the study of atherosclerosis and have benefited the field immeasurably.

1.1.2 Risk factors

Atherosclerosis is a complex disease with a number of risk factors both at a genetic and environmental level. Elevated blood pressure, history of family obesity and diabetes together with gender are examples of genetic factors linked to atherosclerosis (Lusis 2000). Environmental factors include high fat diet, smoking and low exercise levels (Lusis 2000). Table 1.1 details some of the risk factors in more detail. Given the importance of lipids and inflammation in atherosclerosis, these factors will be discussed further in more detail.

Table 1.1 – Details of genetic and environmental risk factors and their association with atherosclerosis

Risk Factor	Role in disease
Genetic	
Mutations	Familial hypercholesterolemia – mutation in the LDLr gene that increases levels of LDL (Lusis *et al.* 2004). Mutation in the ApoA1 gene results in reduced levels of HDL (Lusis *et al*. 2004)
Age	Increased vascular stiffness, hypertension, pro-inflammatory signalling and cellular senescence linked to increase in atherosclerosis (Wang and Bennett 2012)
Inflammation	Elevated levels of C reactive protein (CRP) linked to an increase in CVD (Lusis 2000)
Disease	Obesity and type 1 diabetes increase risk of CVD (Lusis 2000)
Gender	Men under 60 are twice as likely to develop atherosclerosis than women (Lusis 2000)
Environmental	
Smoking	Increase inflammation, thrombosis and oxidative stress (Ambrose and Barua 2004)
High cholesterol	Elevated levels of LDL causes endothelial activation and initiation of foam cell formation (Lusis 2000)
Exercise	Lack of exercise has significant association with development of CVD. Increased exercise has an athero-protective role (Okabe *et al.* 2006)

1.2 Lipids in atherosclerosis

Atherosclerosis is the result of dysfunctional lipid metabolism and is characterised by the accumulation of lipid-rich plaques in the walls of medium and large arteries (Lusis *et al*. 2004). The importance of lipids in atherosclerosis can be gauged by the reduction in CVD mortality that has been achieved with statins, which inhibit the biosynthesis of cholesterol. Currently, statins are the most successful therapy used in combatting atherosclerosis and other CVD's. Statins work by inhibiting 3-hydroxymethylglutaryl CoA reductase (HMG-CoAR), a key enzyme in the *de novo* synthesis of cholesterol, thereby decreasing the levels of cholesterol and LDL in circulation (Michael *et al*. 2012). It has also been observed that statins act to increase levels of HDL in circulation, regulate the expression of macrophage scavenger receptors and reduce levels of both oxidative stress and oxidised LDL (oxLDL) (Jain and Ridker 2005). Given the key role of lipids in the disease, it is important to understand their function in the body during physiological and pathophysiological conditions.

1.2.1 Fatty acids

Fatty acids are produced during the metabolism of dietary lipids or by *de novo* synthesis. They are composed of a polar carboxyl head group attached to a long hydrophobic hydrocarbon chain. Hydrocarbon chains can be saturated or unsaturated. Saturated hydrocarbon chains are straight with, usually, an even number of carbons (between 12 and 22) (Ruston and Drevon 2001). Fatty acids are named after the length of the hydrocarbon chain. The carbon atoms are either numbered from the carboxyl terminal, using the delta numbering system, or from the methyl end using the omega system (Pelley 2011). A list of the most common fatty acids is shown in Table 1.2.

Unsaturated hydrocarbon chains can contain one or more double bond. Their systematic names are followed by the 'enoic' suffix and the position of the double bond (numbered from the methyl end) (Vasudevan 2013). Shorthand notations are also used with the number of carbons followed by the number of double bonds and the position of those double bonds from the methyl end. For example, unsaturated fatty acids with a double bond between the 3rd and 4th carbons from the methyl end are named omega-3 (or n-3) fatty acids. The omega-3 fatty acid EPA has the shorthand 20:5n3 and the chemical name *cis*-5, 8, 11, 14, 17-eicosapentaenoic acid. EPA has 20 carbons and 5 double bonds at positions 5, 8, 11, 14 and 17 (using the delta system), with the position of the first double bond at carbon 3 (using the omega

system). The presence of a double bond ‘kinks’ the chain and restricts mobility (Ruston and Drevon 2001).

Unsaturated fatty acids named monounsaturated fatty acids (MUFA) contain one double bond which can be in the *cis* or *trans* orientation. The most common MUFAs have a double bond in the *cis* orientation and a chain length between 16 to 22 (Ruston and Drevon 2001). *Trans* MUFA can be produced in industrial processing of unsaturated oils. *Cis* MUFA are less thermo stable and have a lower melting point than *trans* MUFA (Ruston and Drevon 2001).

Polyunsaturated fatty acids (PUFA) contain one or more double bonds. They are synthesised in plants and phytoplankton and some are essential to higher organisms such as mammals and therefore must be obtained in the diet (Ruston and Drevon 2001). For example, omega-3 and omega-6 PUFAs are not synthesised in the human body but are essential nutrients. Once obtained from the diet, PUFA can be metabolised by the addition of carbon and the removal of hydrogen (desaturation) (Ruston and Drevon 2001) by enzymes to produce a number of different products. Enzymes synthesised in the human body can only add new double bonds within 10 carbons from the carboxylic end of fatty acid chains, therefore cannot enzymatically create the double bond at the omega-3 and -6 positions (Neitzel 2010).

Fatty acids can be incorporated into various lipid structures that are used to make up cellular membranes and provide energy storage. In addition, fatty acids are important signalling molecules. Fatty acid signalling will be discussed in detail in Section 1.4.2.

Table 1.2 – Common naturally occurring fatty acids

Chemical name	Shorthand	Common name	Abbreviation
Tetradecanoic	14:0	Myristic acid	-
Hexadecanoic	16:0	Palmitic acid	-
Cis-9-hexadecenoic	16:1n7	Palmitoleic acid	-
Cis-7-hexadecenoic	16:1n9	-	-
Octadecanoic	18:0	Stearic acid	-
Cis-11-octadecenoic	18:1n7	*Cis*-Vaccenic acid	-
Cis-9-octadecenoic	18:1n9	Oleic acid	-
Cis-9,12-octadecadienoic	18:2n6	Linoleic acid	LA
Cis-9,12,15-octadecatrienoic	18:3n3	α-Linolenic acid	ALA
Cis-6,9,12-octadecatrienoic	18:3n6	γ-Linolenic acid	GLA
Cis-8,11,14-eicosatrienoic	20:3n6	Dihomo-γ-linolenic acid	DGLA
Cis-5,8,11,14-eicosatetraenoic	20:4n6	Arachidonic acid	AA/ARA
Cis-5,8,11,14,17-eicosapentaenoic	20:5n3	Eicosapentaenoic acid	EPA
Cis-7,10,13,16,19-docosapentaenoic	22:5n3	Docosapentaenoic acid	DPA
Cis-4,7,10,13,16,19-docosahexaenoic	22:6n3	Docosahexaenoic acid	DHA

1.2.1.1 Membrane lipids; Glycerophospholipids

The glycerophospholipids are composed of fatty acids esterified to a glycerol backbone at the sn-1 and sn-2 position with a phosphate and additional group at the 3-position (Brown and Marnett 2011) (Figure 1.1). Glycerophospholipids, or phospholipids, are the main components of eukaryotic membranes along with various other protein and carbohydrate molecules. Different head groups allow for numerous phospholipids; phosphatidylcholine (PC), phosphatidylethanolamine (PE), Phosphatidylserine (PS) and phosphatidylinositol (PI) being the most common in eukaryotic membranes. Cleavage from phospholipids allow fatty acids and their metabolites to act as lipid messengers in signal transduction pathways (van Meer *et al.* 2008).

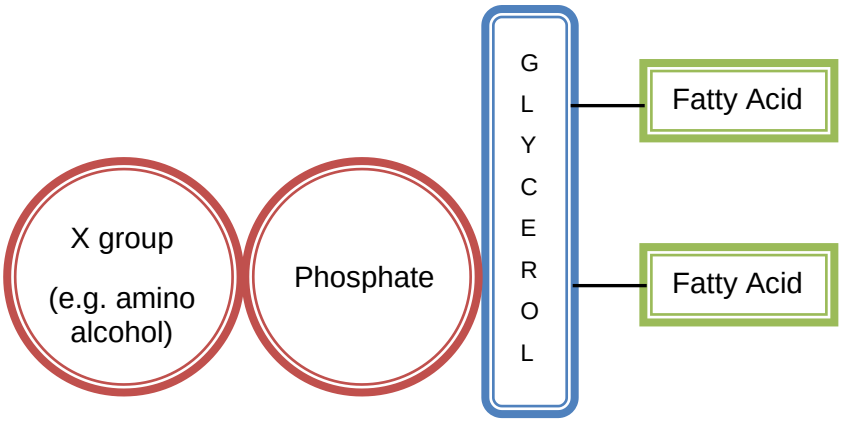


Figure 1.1 - Basic structure of a glycerophospholipid.

Numerous combinations of fatty acid groups attached to a glycerol backbone allow for a wide range of lipid diversity. Amino alcohol group can include choline, ethanolamine or serine, which form PC, PE and PS respectively.

1.2.1.2 Lipid storage; triacylglycerol (TAG)

Due to their reduced state, fatty acids can be used for energy storage. The primary lipid structure used for energy storage is triacylglycerol in lipid droplets (van Meer *et al*. 2008). This structure is composed of three fatty acids bound to a glycerol backbone (Figure 1.2). These structures are useful energy stores due to the supply of carbon in fully reduced form which will yield maximum energy when undergoing oxidation.

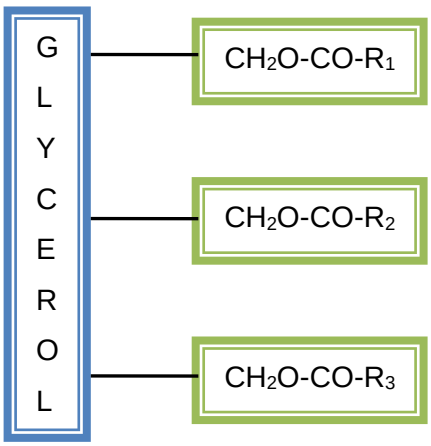


Figure 1.2 – Basic diagram of TAG

Fatty acids provide a source of carbon in reduced form available for oxidation.

1.2.1.3 Cholesterol

Cholesterol is a sterol with important roles in membrane fluidity and lipid raft assembly and function (McLaren et al. 2011a). See Figure 1.3 for structure. High concentration of cholesterol in the body is a major risk factor in atherosclerosis. Cholesterol can be obtained from dietary lipids or synthesised in the liver (McLaren *et al*. 2011a). Cholesterol is synthesised from acetate in series of enzymatic reactions. An important regulatory step in synthesis is the enzyme HMG-CoAR which generates mevalonate (Ikonen 2006).

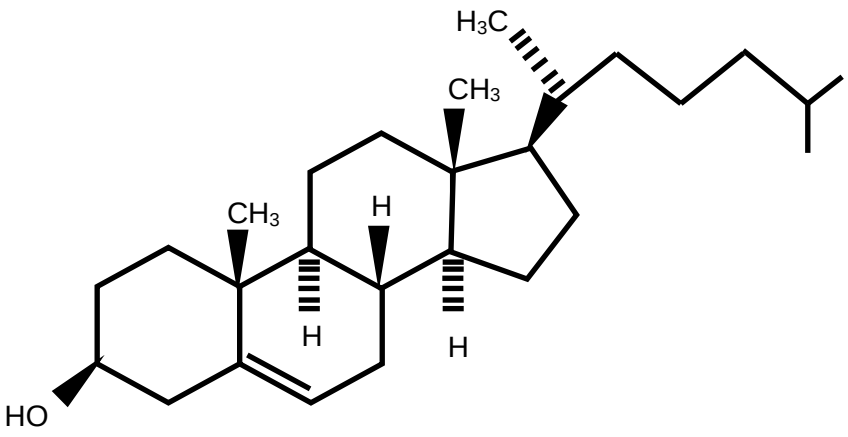


Figure 1.3 – Structure of cholesterol

Cholesterol is composed of a fused tetracyclic ring. It is an important component of cell membranes (especially plasma membranes) and lipid rafts.

1.2.2 Lipid metabolism and transport

Lipid transport around the body is a complex process with numerous enzymes and lipoproteins playing a role. It is important to understand the process in order to target potential imbalances that may contribute to atherosclerosis. Cholesterol metabolism and transport around the body, as well as fatty acids and TAG, play a key role in the pathology of atherosclerosis.

1.2.2.1 Role of lipoproteins

Lipids are transported through the circulation with the aid of proteins. These complexes are termed lipoproteins (Lusis *et al*. 2004). Lipoproteins are composed of a monolayer of hydrophilic lipids (i.e. phospholipids and free cholesterol) surrounding a core of hydrophobic lipids (triacylglycerols and cholesteryl esters) (Figure 1.4). They also contain apolipoprotein molecules which function in the packaging and secretion of lipids from cells, as well as ligands for cellular receptors (Lusis *et al*. 2004). Types of lipoproteins include chylomicrons, VLDL, intermediate density lipoprotein (IDL), LDL and HDL. The type of lipoprotein a lipid is packaged into is dependent on the source of the lipid. For example, chylomicrons are primarily used in the transport of dietary TAG from the intestine for use in peripheral tissues (Ikonen 2006; Buckley and Ramji 2015). On the other hand, cholesterol synthesised in the liver is packaged into VLDL. IDL and LDL are formed from VLDL in a series of reactions catalysed by lipoprotein lipase (LPL) and hepatic lipase (HL) that hydrolyse the TAG component (McLaren *et al*. 2011a). LDL functions to carry cholesterol to peripheral tissues where it can be used in membranes or for the synthesis of steroid hormones. Finally, HDL acts as a carrier to return any excess cholesterol back to the liver for catabolism and excretion (van der Velde 2010). Levels of circulating lipoproteins have been linked to atherosclerosis. Increased levels of LDL in the blood and lower circulating HDL have both been linked to an increased risk of atherosclerosis (McLaren *et al*. 2011a).

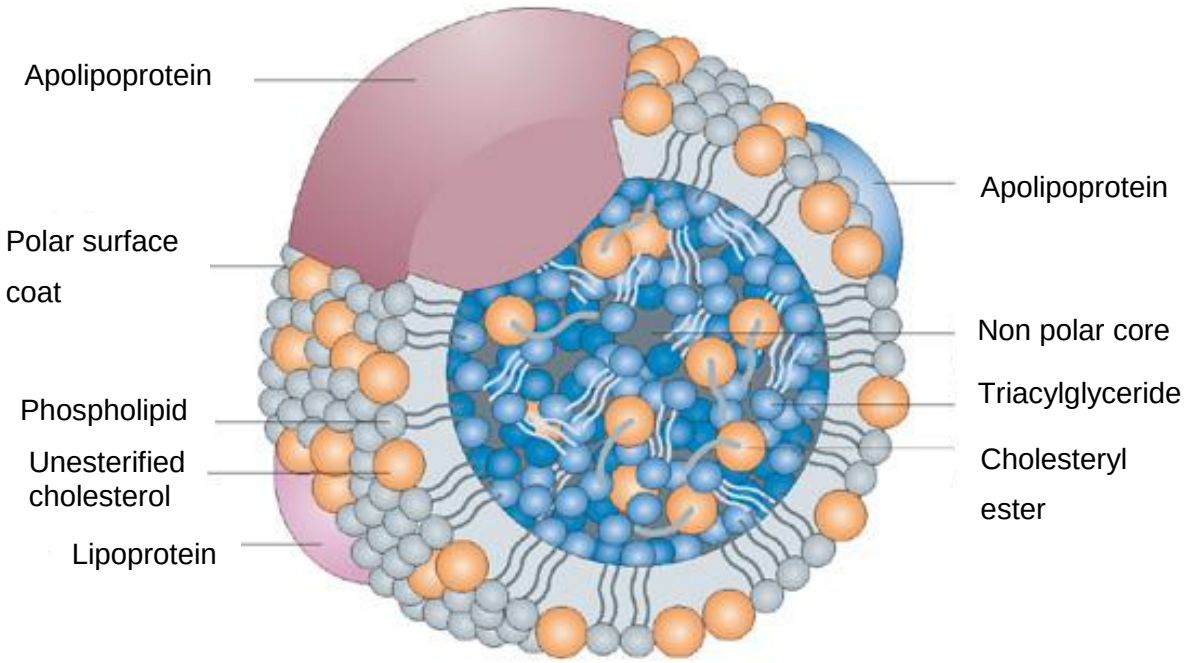


Figure 1.4 – Structure of lipoprotein particles

Phospholipid monolayer encases a core of TAG and cholesteryl ester. Apoliproteins differ depending on the lipoprotein. They influence structural and functional properties of the particle. Taken from (Wasan *et al.* 2008).

The process of cholesterol transport is summarised in Figure 1.5. Cholesterol, TAG and fatty acids from dietary origin are absorbed through the intestine (Lusis *et al*. 2004). Dietary TAG are hydrolysed into fatty acids and monoglycerides and together with bile acids, form micelles in the intestinal lumen (Ikonen 2006; Georgiadi and Kersten 2012). Fatty acids are then re-esterified to TAG and along with cholesterol, are packaged into chylomicrons containing Apo-B, -A, -C and -E (McLaren *et al*. 2011a). Chylomicrons are subject to lipolysis by LPL, located on the capillary endothelium (Georgiadi and Kersten 2012), releasing free non esterified fatty acids and 2-monoacyl glycerol for uptake by various tissue types. The majority of fatty acids taken up by tissues are TAG-derived fatty acids or free fatty acids (Georgiadi and Kersten 2012).

Circulating adipose-derived free fatty acids and remnant chylomicron particles are taken up largely by the liver. Newly synthesised TAG and cholesterol in the liver are packaged into VLDL containing ApoB100. VLDL gains Apo-A, -C and -E from circulating HDL. The TAGs in VLDL undergo lipolysis in circulation by LPL and HL to

form IDL followed by LDL (Buckley and Ramji 2015). LDL, an apolipoprotein B (ApoB) containing lipoprotein, is the primary cholesterol carrier in the blood (Lusis *et al*. 2004). The ApoB constituent of LDL allows recognition of the lipoprotein particle by the LDLr expressed on the cell surface and subsequent receptor-mediated endocytosis. LDL is internalised into lysosomes where cholesteryl ester and TAG are hydrolysed (Buckley and Ramji 2015). The expression of LDLr is under negative feedback inhibition in order to control the amount of LDL, and therefore cholesterol and TAG, uptake by cells (Ghosh *et al.* 2010). Elevated levels of circulating LDL increase the risk of accumulation of the lipid-rich particles in the artery walls, a key mechanism in atherosclerosis.

Cholesteryl esters released from LDL are hydrolysed by lysosomal acid lipase to free cholesterol. When in excess, free cholesterol can be re-esterified by the enzyme acetyl-coenzyme A acetyltransferase (ACAT). Cholesteryl esters reduce the solubility of the molecule and promote storage into lipid droplets located in the cytoplasm (Buckley and Ramji 2015). The process depends on the availability of fatty acids and cholesteryl ester hydrolysis, which are dependent on the enzymes carnitine palmitoyltransferase-1 (CPT-1) and neutral cholesterol ester hydrolase (nCEH) respectively. In addition to this, storage of excess TAG and cholesterol into lipid droplets is promoted by the activity of adipocyte differentiation-regulated protein (ADRP), which induces their synthesis and prevents removal from the cell (McLaren *et al*. 2011a). An accumulation of cholesteryl esters and TAG play an important role in atherosclerosis. Under normal circumstances however, excess cholesterol is efficiently dealt with by cellular homeostatic mechanisms.

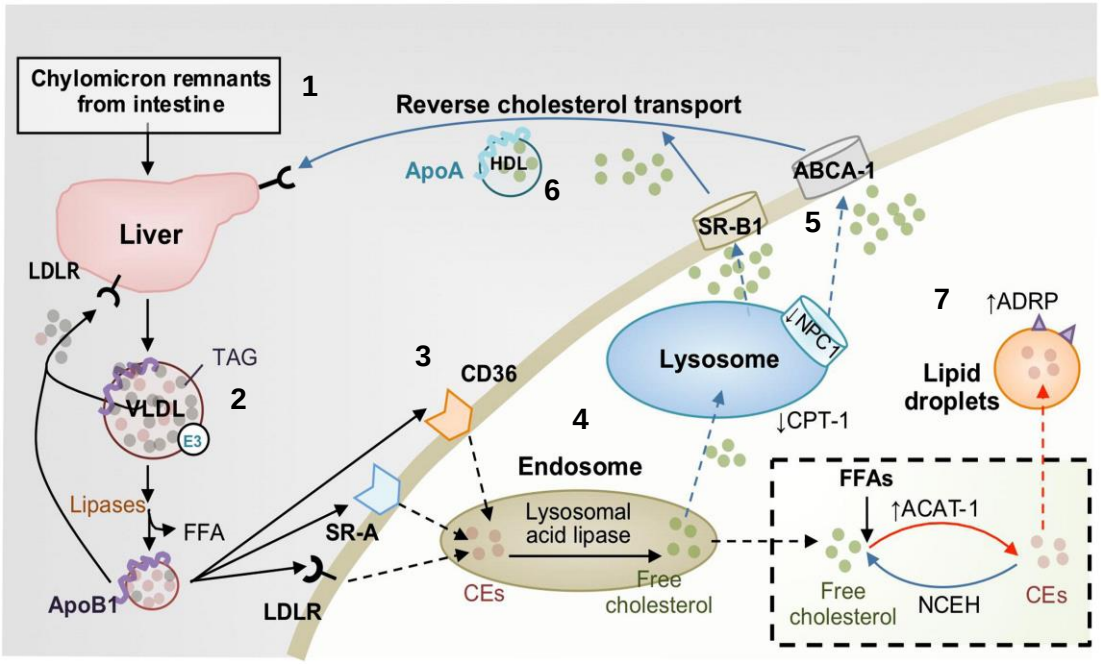


Figure 1.5 – Overview of cholesterol transport

Lipids obtained from the diet are packaged into chylomicrons and after lipolysis by LPL, are taken up by peripheral tissues. Remnant dietary lipids and chylomicrons are transported to the liver for catabolism or repackaging (1). Along with lipids synthesised in the liver, these are packaged into VLDL (2) containing ApoB and ApoE (E3). VLDL is hydrolysed to IDL and then LDL by the action of lipases. ApoB allows for binding of LDL particles to LDLr, internalisation and subsequent degradation in the lysosome (Lusis *et al*. 2004). Scavenger receptors such as scavenger receptor A (SRA) and cluster differentiation 36 (CD36) present on macrophages bind and internalise modified forms of LDL in an unregulated manner (3). Lysosomal acid lipase hydrolyses the cholesteryl esters packaged in LDL to free cholesterol (4). Free cholesterol has two fates; transport out of cell or storage in lipid droplets. Free cholesterol can be removed from the cell by reverse cholesterol transport (RCT) using ATP binding cassette (ABC) transporters such as ABCA-1 and scavenger receptor B1 (SR-B1) (van der Velde 2010) (5). Free cholesterol is packaged into HDL with its ApoA component, and delivered to the liver (6). Alternatively, free cholesterol is re-esterified by ACAT-1 in the endoplasmic reticulum. This re-esterified cholesterol can by hydrolysed to free cholesterol by nCEH and removed by RCT or stored as lipid droplets in the cytoplasm (Ikonen 2006) (7). The latter is regulated by ADRP. The availability of fatty acids for cholesterol ester accumulation is under the control of CPT-1. Niemann-Pick type C (NPC) proteins, NPC-1 and NPC-2 act to regulate cholesterol trafficking around the cell (Buckley and Ramji 2015). Taken from Buckley and Ramji, 2015.

1.2.2.3 Reverse cholesterol transport (RCT)

Excess cholesterol within cells is toxic and can be removed from the periphery to the liver and intestine for catabolism and excretion (van der Velde 2010). The primary pathway by which this is achieved is RCT. Free cholesterol can be removed from cells using a number of transporter proteins. These include the ABC transporter proteins; ABCA1 and ABCG1 (Chinetti-Gbaguidi and Staels 2009; Buckley and Ramji 2015). Scavenger receptor SR-B1 can also act to promote cholesterol efflux. This receptor has a dual role, however, as it also acts to bind and internalise modified LDL and, therefore, its role in atherosclerosis is controversial (McLaren *et al*. 2011a). Cholesterol is captured by HDL particles where it is esterified (McLaren *et al*. 2011a). HDL complexes are formed in the circulation from remnants of chylomicron and VLDL, ApoA1 and precursor molecules (secreted by the liver and intestine) (Lusis *et al*. 2004). Lecithin cholesterol acyl transferase (LCAT) is present on the surface of HDL and has the ability to catalyse the formation of cholesteryl esters by transferring fatty acids from phospholipids to free cholesterol (van der Velde 2010). This converts nascent HDL particles to smaller HDL_3 particles. The mature HDL_3 can deliver its contents directly to the liver or transfer its cholesteryl ester to ApoB-containing lipoproteins (Ng 2006). HDL_3 particles remove some of its cholesteryl ester to LDL or VLDL, in exchange for TAG catalysed by the enzyme cholesteryl ester transfer protein (CETP), and are taken up by the liver by the LDLr receptor (Ng 2006). The remaining HDL particle, following acquisition of TAG and additional apoliprotein molecules, forms an even smaller HDL_2 particle which are delivered to the liver (McLaren *et al*. 2011a). Once at the liver, hepatocytes can take up HDL cholesteryl ester in a number of ways; uptake of whole HDL_2 particles, selective uptake of cholesteryl esters by SR-B1 or finally transfer of cholesteryl ester to LDL for uptake by LDLr. Cholesteryl ester can be hydrolysed and removed from the body as bile acids and neutral steroids in bile (McLaren *et al*. 2011a).

1.3 Inflammation in atherosclerosis

The link between atherosclerosis and lipids has been regarded as the major underlying cause of the disease for many years due to the key association with cholesterol. This concept has now been extended to consider atherosclerosis as an inflammatory disease involving the immune system with inflammation initiated by dysfunctional lipid homeostasis (Libby 2012).

As discussed previously in Section 1.2 statins are currently widely used in the treatment of atherosclerosis due to their lipid-lowering effects. In addition to this, it has been suggested that they may also have anti-inflammatory actions unrelated to their primary use. In a study of high-risk patients with hyperlipidaemia, treatment with atorvastatin reduced the expression of a key inflammatory marker of atherosclerosis, CRP. This decrease in CRP was unrelated to the expected decrease in total cholesterol and LDL levels (Gomez-Gerique *et al.* 2002). Another study performed in $ApoE^{-/-}/LDLr^{-/-}$ mice indicated that alongside the reduction in plasma LDL, treatment with atorvastatin also decreased the expression of MCP-1, intercellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule 1 (VCAM-1) (Nachtigal *et al.* 2008). It therefore seems that statins have an unexpected dual role in the treatment of atherosclerosis targeting both inflammation and cholesterol levels. Despite the beneficial role of statins, there are various limitations associated with them. The maximum reduction in mortality from CVD that can be attributed to treatment with statins is 30%, with some individuals unresponsive to treatment and failing to attain target LDL levels even with the maximal dose (Mishra and Routray 2003). In addition to this, side effects of statins can include liver toxicity and muscle pain (Mishra and Routray 2003). Given the drawbacks of statins and the ever increasing risk of CVD and atherosclerosis, it is crucial that new therapeutic targets are developed. The association between inflammatory mechanisms and atherosclerosis suggests that the use of anti-inflammatory agents would aid in the prevention and the treatment of the disease (Paoletti *et al.* 2004).

Acute inflammation is a normal response mechanism in the body to provide protection against invading pathogens. Activation of an immune response involves numerous cell types and regulation of many signalling pathways (Calder 2012). Normally, the process is regulated by negative feedback mechanisms to ensure no excess damage occurs. However loss of regulatory processes results in chronic inflammation (Calder 2002), a feature important in the pathology of atherosclerosis. *In vivo* microscopic studies of atherosclerotic mice indicate the adhesion and transmigration of immune

cells across the endothelial surface of arteries (Eriksson *et al.* 2001), a key inflammatory process in the initiation stage of atherosclerosis. Under the control of cytokines and chemokines, continued recruitment of immune cells and development of lipid-rich foam cells contributes to a growing necrotic core. As atherosclerosis progresses and plaques develop, inflammatory mediators can act to weaken the fibrous cap that encapsulates the atherosclerotic plaque, possibly leading to plaque rupture and thrombosis (Paoletti *et al*. 2004). Inflammation is therefore important at all stages of atherosclerosis. Many cell types play a role in the initial inflammatory response in atherosclerosis as well as contributing to plaque formation. These include monocytes, endothelial cells, lymphocytes, dendritic cells and smooth muscle cells (Moore *et al.* 2013). Substantial research has focused on macrophages due to their role in 'foam cell' formation, the central inflammatory process leading to formation of fatty streak in arteries, the foundation of advanced plaques (Moore *et al*. 2013). The association between inflammatory mechanisms and atherosclerosis suggests the promising potential of anti-inflammatory drugs to aid in the prevention and treatment of the disease (Paoletti *et al*. 2004).

1.3.1 Macrophage foam cell formation

1.3.1.1 Initiation/endothelial cell activation

The primary event leading to the initiation of an immune response in atherosclerosis pathology is the accumulation and retention of ApoB containing lipoproteins, for example LDL, in the sub endothelial matrix in the intima of arteries (McLaren *et al*. 2011a; Lusis 2012). The endothelium lining the walls of arteries functions as a selective barrier between blood and tissues and can generate signalling molecules that act to regulate thrombosis, inflammation, vascular tone and remodelling (Lusis 2000). LDL passively diffuses through endothelial cell junctions particularly in areas of arterial curvature where blood flow is disturbed and endothelial cells have irregular polygonal shapes (Lusis 2000). Retention of LDL in the arterial wall is maintained through an interaction between the ApoB constituent of lipoproteins and matrix proteoglycans (Lusis *et al*. 2004) (Figure 1.6).

Once retained in the arterial matrix, LDL is susceptible to modification. Lipid peroxidation of LDL is one of the earliest events in atherosclerosis (Matsuura *et al.* 2008). As discussed in Section 1.2.2.1, LDL contains free cholesterol, cholesteryl esters, phospholipids and triacylglycerols which are vulnerable to oxidation (Tsimikas and Miller 2011). In addition to this, ApoB can also be oxidised at numerous exposed tyrosine and lysine amino acid residues (Tsimikas and Miller 2011). LDL oxidation

can occur through a number of different mechanisms including free radical oxidation and enzymatic oxidation by reactive oxygen species (ROS), nitric oxide (NO) and lipooxygenase (LOX) (Tsimikas and Miller 2011). Modified LDL can induce an immune response due the peroxidation of lipids creating reactive oxidation species. This promotes endothelial dysfunction, activation of endothelial cells and recruitment of monocytes (Matsuura *et al*. 2008; Wraith *et al.* 2013). In the initial stages of atherosclerosis, LDL oxidation is low and modifications to LDL are termed minimally modified LDL (mmLDL) (Berliner *et al.* 1990). As atherosclerosis advances, highly oxLDL is present in plaques (Berliner *et al*. 1990). In contrast to oxLDL, mmLDL are not recognised by scavenger receptors (Miller *et al.* 2003). However, current theory suggests mmLDL initiates an immune response (Moore and Freeman 2006).

1.3.1.2 Monocyte recruitment

Accumulation of modified LDL in the artery wall stimulates endothelial cells to initiate an immune response resulting in the release of pro-inflammatory signalling molecules, chemokines, cytokines and expression of adhesion proteins (Lusis 2000). Cytokines play an important role in atherosclerosis and will be discussed in more detailed in Section 1.4.1. Leukocytes, including neutrophils, T lymphocytes, B cells, mast cells, natural killer cells, dendritic cells and monocytes, are activated as part of the immune response and recruited to the site of activation (Galkina and Ley 2009). Each play an important role in inflammatory mechanisms underlying atherosclerosis and have both resolving and contributing effects (Galkina and Ley 2009). The role of monocyte-derived macrophages has been well characterised in atherosclerosis given their role in the formation of foam cells. Atherosclerotic plaques contain a mass core of macrophage foam cells and are the main constituent of the plaque (Aqel *et al.* 1985; Gown *et al.* 1986; Yano *et al.* 2000).

Monocytes are recruited to the activated endothelium down a chemoattractant gradient (Denholm and Lewis 1987), the process is summarised in Figure 1.6. Numerous chemotactic factors are released which act to attract monocytes to the site of activation. MCP-1 is key to monocyte recruitment and a marker of inflammation. Rolling and tethering of monocytes along the endothelium is achieved through the expression of selectins, a family of adhesion proteins (Bobryshev 2006). P-selectin and E selectin are expressed on the endothelium while L-selectin is found on the surface of monocytes, all recognising carbohydrate ligands (Bobryshev 2006). Firm adhesion is obtained through an interaction between integrins β1 and β2 expressed on monocytes and ICAM-1 and VCAM-1 expressed by endothelial cells (Bobryshev

2006). Monocytes can then transmigrate through the endothelial cell junctions and into the arterial matrix where they undergo differentiation into macrophages.

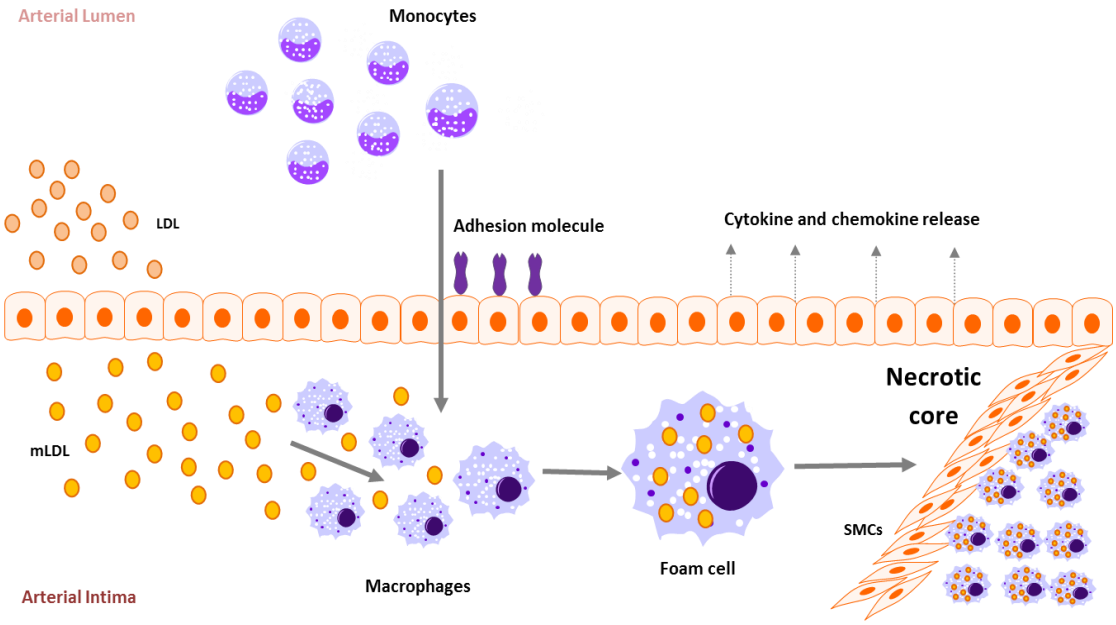


Figure 1.6 - Overview of foam cell formation

Retention of ApoE-containing lipoproteins, including LDL, in the arterial wall initiates an inflammatory response (Libby 2012) involving the release of numerous cytokines and chemokines by endothelial cells. As a result, monocytes are recruited to the arterial wall and transmigrate into the arterial intima where they are induced to differentiate into macrophages (Bobryshev 2006). Macrophages internalise large, unregulated amounts of modified LDL leading to an accumulation of cholesteryl esters as lipid droplets and thereby transforming them into 'foam cells' (Bobryshev 2006). Over time foam cells aggregate and contribute to a necrotic core (Lusis 2012). Apoptosis of foam cells, accumulation of extracellular lipids and cellular debris continue to build up in the necrotic core (Bobryshev 2006). Smooth muscle cells migrate from the media to the intima to form a fibrous cap overlaying the necrotic core and leads to the formation of an atherosclerotic plaque (Lusis 2012).

1.3.1.3 Macrophage differentiation and heterogeneity

Differentiation of monocytes into macrophages is largely regulated by the chemokine macrophage colony stimulating factor (M-CSF) (Bobryshev 2006), released by endothelial cells as part of the inflammatory response. Macrophages are heterogeneous in nature and have a wide variety of roles. Their phenotypes are

commonly classified into two broad subsets, classically activated M1 or alternatively activated M2 (Johnson and Newby 2009). Classification into these sub sets is often determined by analysis of complex gene and protein expression profiles (Moore and Tabas 2011). The M1 phenotype is in general associated with pro-inflammatory mechanisms, while M2 can play an anti-inflammatory role (Johnson and Newby 2009). M1 macrophage polarisation is activated by pro-inflammatory stimuli, commonly interferon-γ (IFN-γ), lipopolysaccharide (LPS) and tumour necrosis factor α (TNF-α) and results in expression of interleukin-12 (IL-12), IL-1, IL-6 and inducible nitric oxide synthase (iNOS) (Martinez and Gordon 2014). They are key to innate immunity and act in killing of intracellular pathogens. M2 macrophages are classically activated by IL-4, IL-13 and produce the anti-inflammatory cytokine IL-10. They have a number of roles in allergic response, immunoregulation, matrix deposition and tissue remodelling (Martinez and Gordon 2014). The classification of macrophages into these two subsets is very general and it is likely that the groups can be further divided and additional phenotypes exist (Johnson and Newby 2009). Understanding the involvement of M1 and M2 macrophages in atherosclerosis is complex and not fully understood. Atherosclerotic lesions accumulate both subsets of macrophages (Stoger *et al.* 2012). M1 macrophages are dominant at rupture prone regions of plaque, while the vascular adventitia indicated macrophages comparable to a M2 profile (Stoger *et al*. 2012). There was no difference between the groups at the fibrous cap and foam cell macrophages displayed an overlap of both M1 and M2 markers indicating that both subsets were present (Stoger *et al*. 2012). The heterogeneity of macrophages is complex and further evaluation is needed to determine the roles of M1 and M2 in disease.

1.3.1.4 Macrophage foam cells

Macrophages are bone marrow-derived phagocytes which act to scavenge foreign particles around the body. To do this macrophages express a number of pattern-recognition receptors (PRRs) including scavenger receptors (SR) and toll-like receptors (TLR) to recognise and bind foreign particles (Libby 2012). Scavenger receptors are a family consisting of 8 structurally unrelated receptors which along with their role in immunity, possess the ability to bind modified LDL (Moore and Freeman 2006). Uptake of LDL in most peripheral tissues is under the control of the LDLr. Feedback inhibition mechanisms prevents excessive uptake (Ghosh *et al*. 2010). Expression of scavenger receptors however is not under negative feedback control by cellular cholesterol content (Chinetti-Gbaguidi and Staels 2009) therefore these receptors can continually bind and internalise LDL. SRA and CD36 among others

have been directly linked to atherosclerosis (Moore and Freeman 2006; Kuchibhotla *et al.* 2008).

Uptake of modified LDL however, is not completely a receptor-dependant process as lipid uptake by macrophages can occur in the absence of CD36 and SRA (Kruth *et al.* 2002; Moore *et al.* 2005). Macrophages can also uptake large unregulated amounts of modified LDL through a mechanism of macropinocytosis (Michael *et al.* 2013). Macropinocytosis is a form of fluid phase endocytosis which allows for uptake of modified LDL and native LDL in large, unregulated amounts by formation of endocytic vesicles (Swanson and Watts 1995; Jones and Willingham 1999; Kruth *et al*. 2002).

Once LDL has been taken up by macrophages, the lipoprotein is degraded and a mass of cholesteryl esters and TAG released (Ghosh *et al*. 2010). Normally, cells can remove any excess cholesterol to the liver to be degraded via reverse cholesterol transport mechanisms (discussed in detail in Section 1.2.2.3). The excessive uptake of cholesterol by macrophages causes an imbalance between influx and efflux of cholesterol, in favour of influx. This results in an accumulation of cholesteryl ester which is stored in lipid droplets in the cytoplasm (Ghosh *et al*. 2010). Lipid droplets under the microscope give macrophages a 'foamy' appearance leading to them being termed foam cells.

1.3.2 Additional roles of macrophages

In atherosclerosis, macrophages have a number of important roles. In addition to foam cell formation, macrophages present in atherosclerotic plaques can contribute further to the inflammatory response (McLaren *et al*. 2011a). Resident plaque macrophages produce chemokines and cytokines which act to recruit further monocytes and promote diapedesis into the lesion (Charo and Taubman 2004; Zernecke *et al.* 2008). For example, the chemokine MCP-1 is expressed from macrophages on stimulation with pro-inflammatory cytokines IFN-γ and TNF-α (Charo and Taubman 2004; Popa *et al.* 2007; McLaren and Ramji 2009). Macrophages can also express matrix metalloproteinases (MMPs) which act to breakdown structural components of the extracellular matrix (ECM) of a atherosclerotic plaque and increase the risk of rupture (Newby 2008). In addition, macrophages have been indicated to promote the migration of smooth muscle cells (SMCs) to growing lesions (Rudijanto 2007) and induce their apoptosis which once again increases the risk of plaque rupture (Boyle *et al.* 2001).

1.3.3 Smooth muscle migration and plaque progression

Over time foam cells undergo apoptosis due to the lack of nutrients available for them to survive and toxicity by intracellular cholesterol (McLaren *et al*. 2011a) and release their lipid-rich mass into an increasingly large necrotic core (Lusis *et al*. 2004). As discussed above in Section 1.3.2, expression of various growth factors and cytokines from resident plaque macrophages promotes the activation of SMCs and their migration (Rudijanto 2007). SMCs themselves can also form foam cells through the uptake of modified forms of LDL by scavenger receptors expressed on their surface. Accumulation of foam cells in the arterial intima cause cells to aggregate forming 'fatty streaks' in the artery wall. Fatty streaks are not clinically significant but are the underlying foundations of advanced plaques (Lusis 2000). They are characterised by macrophage- and SMC-derived foam cells and other immune cells such as T cells (Buckley and Ramji 2015).

The formation of an intermediate lesion is characterised by an increase in SMC migration and proliferation into the inflamed area. SMCs continue to migrate from the tunica media of the artery wall into the intima and proliferate forming a fibrous cap surrounding the growing plaque (Rudijanto 2007; McLaren *et al*. 2011a; Michael *et al*. 2012) Resident macrophages and SMCs can produce various proteins including collagen, elastin, glycoproteins and proteoglycans (Katsuda and Kaji 2003) to form the ECM of the plaque. Stable plaques are identified by the presence of a fibrous cap overlying the growing lesion, supported by an ECM (Buckley and Ramji 2015).

1.3.4 Plaque rupture

A key event in the formation of advanced atherosclerotic plaques is failure to resolve inflammation (Tabas 2010). This includes failure to supress infiltration of inflammatory cells, efferocytosis (clearance of apoptotic cells) and egress of inflammatory cells from the arterial wall (Tabas 2010). As atherosclerosis advances, foam cells continue to contribute to the lipid-rich necrotic core and plaques become increasingly complex with calcification and ulceration at the luminal surface (Lusis 2000). Large plaques can protrude into the arterial lumen reducing blood flow, moreover large plaques are increasingly unstable and are at risk of rupture (Lusis *et al*. 2004). Growth factors and cytokines expressed in the inflammatory response induces apoptosis of SMC (Boyle *et al*. 2001) causing thinning of the fibrous cap (McLaren *et al*. 2011a). ECM degrading enzymes, including MMPs, are released from apoptotic SMCs and resident macrophages which breakdown the ECM network of the plaque. Both these processes destabilise the plaque which often results in rupture and the release of the lipid-rich necrotic core into the circulation (Michael *et al*. 2012). Once released into

the circulation, the mass of lipid and dead cells is exposed to coagulating factors present in the blood resulting in thrombosis. This can cause restriction of blood and oxygen to vital organs and ultimately myocardial infarction and stroke (Michael *et al*. 2012).

1.4 Signalling in atherosclerosis

In atherosclerosis, a number of changes in key signalling pathways occur, which results in altered gene expression and contributes to lesion formation. In response to extracellular stimuli (cytokines, chemokines, hormones and lipid mediators) utilising cellular receptors, signalling pathways can be up or down regulated. A number of key components in signalling pathways have been implicated in the pathology of atherosclerosis. Examples include; extracellular signal-regulated kinase (ERK), p38, c-Jun N terminal kinases (JNKs) (all form the mitogen-activated protein kinases (MAPKs) signalling cascade), nuclear factor κB (NF-κB) and phosphoinositide-3-kinase (PI3K). The roles of these pathways in the disease are detailed in Table 1.3.

Table 1.3 – Pathways activated in atherosclerosis and their role in the disease

Pathway	Role in atherosclerosis
ERK1/2	IFN-γ mediated uptake of modified LDL and expression of MCP-1 is attenuated by ERK1/2 inhibition in macrophages (Li *et al.* 2010). Immunofluorescence in rabbit atherosclerotic lesions revealed ERK1/2 localised to cap and base of plaques. Expression was 3 fold higher and activity 3-5 fold higher in comparison to normal vessel. LDL-induced SMC proliferation was abrogated when ERK was inhibited *in vitro* (Hu *et al.* 2000). Inhibition of ERK1/2 in macrophages significantly increased cholesterol efflux to ApoA1 and HDL via an increased expression of ABCA1 (Zhou *et al.* 2010). In ApoE$^{-/-}$ mice combined ERK1/2 inhibition and liver X receptor (LXR) activation reduced macrophage accumulation in the aorta and formation of foam cells and induced RCT thereby decreasing atherosclerotic lesions (Chen *et al.* 2015).
p38	Patients with atherosclerosis receiving high doses of p38 inhibitor demonstrate reduced vascular inflammation in the most inflamed regions (Elkhawad *et al.* 2012). ApoE$^{-/-}$ mice receiving another p38 inhibitor showed a significant reduction in progression of atherosclerosis (Seeger *et al.* 2010). In macrophages, p38 increased LDL uptake, cellular cholesterol levels and cholesteryl ester accumulation (Mei *et al.* 2012). Moreover in ApoE$^{-/-}$ mice with macrophage deficient p38-α, atherosclerotic plaques contained significantly less collagen and a thin fibrous plaque indicating a role for p38 in plaque stability. In addition p38 deficiency in macrophages increased endoplasmic reticulum-stress induced apoptosis (Seimon *et al.* 2009).
JNK	Inhibition of JNK in ApoE$^{-/-}$ mice showed a decrease in lesion formation at regions of low stress. In addition, JNK inhibition attenuated activation of NF-κB and VCAM-1 expression (Wang *et al.* 2011). Double ApoE$^{-/-}$ JNK2$^{-/-}$ knockout mice developed less atherosclerosis compared to ApoE$^{-/-}$ controls. This was not observed in ApoE$^{-/-}$ JNK1$^{-/-}$ mice. JNK deficient macrophages decreased phosphorylation of

	SRA which in turn reduced lipoprotein uptake and foam cell formation (Ricci *et al.* 2004).
NF-κB	Deficiency of NF-κB in endothelial cells in ApoE$^{-/-}$ mice resulted in strongly reduced atherosclerosis. NF-κB deficient cells showed a reduction in adhesion molecule expression, macrophage recruitment and cytokine and chemokine expression (Gareus *et al.* 2008). However, a study in LDLr$^{-/-}$ mice has shown contrasting results. Inhibition of NF-κB activation in macrophages in these mice increased atherosclerosis. There was an increase in lesion necrosis along with cell number (Kanters *et al.* 2003).
PI3K	PI3K/ Akt pathway is activated by oxLDL and inflammatory chemokines in macrophages. This response is attenuated on deletion of the catalytic subunit of PI3K, p110γ (Chang *et al.* 2007). Activation of pathway induced atherosclerosis in ApoE$^{-/-}$ mice but this is significantly inhibited in ApoE$^{-/-}$ p110γ$^{-/-}$ mice (Chang *et al*. 2007). PI3K inhibitor attenuated the uptake of modified LDL and macropinocytosis in macrophages. Expression of scavenger receptors SRA and CD36 was also attenuated (Michael *et al.* 2015). IFN-γ induced expression of chemokine MCP-1 is also inhibited by a PI3K inhibitor in macrophages (Harvey *et al.* 2007) indicating its role in foam cell formation.

1.4.1 Cytokines

Cytokines is a general term used to describe a group of more than 50 secreted factors involved in cellular communication together with the immune and inflammatory responses. They are classified into groups as follows: interleukins, tumour necrosis factors, interferons, colony stimulating factors, transforming growth factors and chemokines (Tedgui and Mallat 2006). Cytokines can be broadly separated into two classes' dependant on their actions in an immune response; pro- and anti-inflammatory. For example, IL-4, IL-10, IL-33 and transforming growth factor (TGF) - β promote anti-inflammatory conditions (Tedgui and Mallat 2006; Miller 2011) in contrast TNF-α, IL-1β, IL-12 and IFN-γ exert classically pro-inflammatory responses (Tedgui and Mallat 2006; McLaren and Ramji 2009). The role of cytokines in the inflammatory response in atherosclerosis is complex with involvement at all stages from initiation of immune response to plaque progression. Release of pro-inflammatory cytokines during an inflammatory response has numerous roles in foam cell formation and other steps in atherosclerosis. Release of IL-1, IL-6, IL-10, IL-12 and TNF-α activate circulating monocytes in the blood stream allowing them to adhere to the activated endothelium (Bobryshev 2006). During plaque formation, release of cytokines aids in the migration of smooth muscle cells to the arterial intima and formation of the extracellular matrix (Lusis 2000). Table 1.4 summarises the roles of a select number of cytokines in the pathology of atherosclerosis.

Targeting signalling of pro-inflammatory cytokines in atherosclerosis, using inhibitors or gene targeting *in vitro* and *in vivo,* has been widely used to delineate their roles in the disease and identify potential therapeutic targets for atherosclerosis. Studies have proved controversial with contrasting results in some cases; however it is clear that reduced pro-inflammatory cytokine signalling has beneficial effects in the disease (Gupta *et al.* 1997; Whitman *et al.* 2002; Kirii *et al.* 2003; Chamberlain *et al.* 2009; Schuett *et al.* 2012). The signalling and roles of three pro-inflammatory cytokines will be discussed in more detailed: IFN-γ, IL-1β and TNF-α, as they are directly relevant to the studies carried out in this thesis.

Table 1.4 – Examples of cytokines expressed in an inflammatory response and their role in atherosclerosis

Cytokine	Role in atherosclerosis
IL-1β	Lack of IL-1β in ApoE$^{-/-}$ mice decreased atherosclerosis and reduced inflammatory markers MCP-1 and ICAM-1 (Kirii *et al*. 2003). Similar results were observed in ApoE$^{-/-}$ IL1 receptor knockout mice (Chamberlain *et al*. 2009) and mice targeted with an anti-IL-1β antibody (Bhaskar *et al.* 2011).
IL-6	Injection into ApoE$^{-/-}$ mice increased levels of pro-inflammatory cytokines and lesion size (Huber *et al.* 1999). IL-6 inhibitor in LDLr$^{-/-}$ mice decreased atherosclerosis, along with a reduction in SMC infiltration, monocyte migration and endothelium activation (Schuett *et al*. 2012). However IL-6 deficiency in ApoE$^{+/-}$ heterozygous model augmented atherosclerosis, increasing pro-inflammatory signalling and destabilising plaques (Madan *et al.* 2008).
IL-10	Double knockout ApoE$^{-/-}$ IL-10$^{-/-}$ mice showed increased atherosclerotic lesion sizes together with pro-coagulant activity of the plaque indicating that the cytokine plays a role in stabilisation (Caligiuri *et al.* 2003). Treatment of LDLr$^{-/-}$ mice with IL-10 encoding virus delivery vector also resulted in decreased atherosclerotic lesions (Liu *et al.* 2006). IL-10 expressing macrophages inhibited atherosclerosis in LDLr$^{-/-}$ mice. A reduction in cholesteryl ester accumulation and an increase in cholesterol uptake and efflux was also observed (Han *et al.* 2010).
IL-17	Atherosclerosis was reduced in LDLr$^{-/-}$ mice transplanted with IL-17r deficient bone marrow. There was a decrease in IL-6 production and an up regulation in IL-10 (van Es *et al.* 2009). A comparable effect was seen in ApoE$^{-/-}$ mice treated with an anti-IL-17A antibody. Atherosclerotic lesion size was reduced along with plaque vulnerability, cellular infiltration and cytokine and chemokine production (Erbel *et al.* 2009). However, loss of suppressor of cytokine signalling 3 (SOCS3) expression in T-cells increased IL-17 production and induced an anti-inflammatory macrophage phenotype and reduction in lesion development and vascular inflammation (Taleb *et al.* 2009).
IL-33	Reduction in atherosclerosis in ApoE$^{-/-}$ mice injected with IL-33. Decreased levels of IFN-γ and induced oxLDL antibody (Miller 2011). *In vitro,* IL-33 reduced foam cell formation. Modified LDL uptake was reduced and cholesterol

	efflux increased (McLaren *et al.* 2010). ApoE$^{-/-}$ mice treated with soluble ST2, a decoy receptor which neutralises IL-33, showed a significant increase in lesion size (Miller *et al.* 2008). However ApoE$^{-/-}$ mice deficient in IL-33 had no major impact on development of atherosclerosis (Martin *et al.* 2015).
IFN-α	Treatment with IFN-α in LDLr$^{-/-}$ mice showed an increase in plasma cholesterol and TAG levels and an increase in atherosclerotic lesion size (Levy *et al.* 2003) .
IFN-β	Attenuated angiotensin II induced atherosclerosis in ApoE$^{-/-}$ mice (Zhang *et al.* 2008).
IFN-γ	Deficiency in ApoE$^{-/-}$ mice attenuates atherosclerosis (Gupta *et al*. 1997). Decreases cholesterol efflux (Harvey and Ramji 2005) and cholesterol transport in macrophages thereby augmenting foam cell formation (Reiss *et al.* 2004). Administration of IFN-γ in ApoE$^{-/-}$ deficient mice decreased serum cholesterol levels. However despite this, atherosclerotic lesions were significantly increased along with the number of T-cells (Whitman *et al.* 2000).
TGF-β	Inhibition of TGF-β signalling in ApoE$^{-/-}$ mice accelerates atherosclerosis, increasing inflammatory content and reducing stability of plaques (Mallat *et al.* 2001). ApoE$^{-/-}$ mice with deficient signalling in T-cells showed an increase in the size of atherosclerotic lesions. In addition, there was an increase in IFN-γ expression, T-cell and macrophage activation and vulnerability of the plaques (Robertson *et al.* 2003). ApoE$^{-/-}$ mice with TGF-β overexpression in macrophages developed significantly less lesions. There was a reduction in the number of macrophages together with an increase in SMCs and collagen content in plaques indicating a more stable plaque (Reifenberg *et al.* 2012).
TNF-α	In ApoE$^{-/-}$ TNF-α$^{-/-}$ mice, atherosclerosis was attenuated along with a decrease in the levels of pro-inflammatory cytokines, chemokines and adhesion molecules (Xiao *et al.* 2009). Another study using the same knockout model also observed a decrease in oxLDL uptake and SRA expression in macrophages (Ohta *et al.* 2005).

1.4.1.1 IFN-γ

IFNs are classified into two groups depending on receptor specificity and sequence homology. Type I IFNs, which include IFN-α, -β and -ω subtypes, are structurally related and bind to a common receptor (Schroder *et al.* 2004). IFN-γ is the only type II IFN as it is structurally unrelated to type I IFNs and binds to a different receptor (Schroder *et al*. 2004). IFN-γ is produced by a number of immune cells such as T-lymphocytes, B-lymphocytes, natural killer cells, dendritic cells and macrophages (McLaren and Ramji 2009).

1.4.1.1.1 Signalling through JAK/STAT

IFN-γ is classified as a pro-inflammatory cytokine and is involved at all stages of atherosclerosis (McLaren and Ramji 2009). Responses are driven by signalling through the Janus kinase (JAK)/ Signal transducer and activator of transcription (STAT) pathway. The JAK/STAT pathway is employed by over 50 cytokines and is one of the best understood mechanism by which signals are propagated (Schroder *et al*. 2004). An overview of this pathway is detailed in Figure 1.7.

The IFN-γ receptor (IFN-γR) is made up of two subunits; IFN-γR1 and IFN-γR2. Ligand binding induces a conformational change in the intracellular domain of the receptor allowing for association with downstream signalling partners (Schroder *et al*. 2004). IFN-γ binding to IFN-γR induces JAK2 autophosphorylation which can then transphosphorylate JAK1 (Schroder *et al*. 2004). Activated JAK1 phosphorylates the IFN-γ receptor at tyrosine residue 440 of the IFN-γR1 chain. This allows docking of the SH2 domain of STAT1 to the receptor tails and its phosphorylation at TYR^{701} by JAKs. Phosphorylation induces release of STAT1 from the receptor. STAT1 form homodimers which can be further phosphorylated at SER^{727} by additional kinases. Homodimers translocate into the nucleus where they bind to gamma activating sequences (GAS) in the regulatory regions of target genes and induce their transcription (Schroder *et al*. 2004; Platanias 2005; McLaren and Ramji 2009). IFN-γ is highly effective at low concentrations and can up or down regulate the expression of numerous genes very quickly (Kerr and Stark 1991). Many of the genes first induced by the cytokine are transcription factors. This includes interferon regulatory factors (IRFs). IRFs are a family of transcription factors of which some members participate in IFN-γ signalling; notably IRF-1, IRF-3 and IRF-9 (Schroder *et al*. 2004; Platanias 2005). IRFs can form complexes with STAT homodimers (for example STAT1:STAT2:IRF-9) which bind IFN-γ stimulated response elements (ISREs) and control the transcription of genes containing these sequences (Platanias 2005).

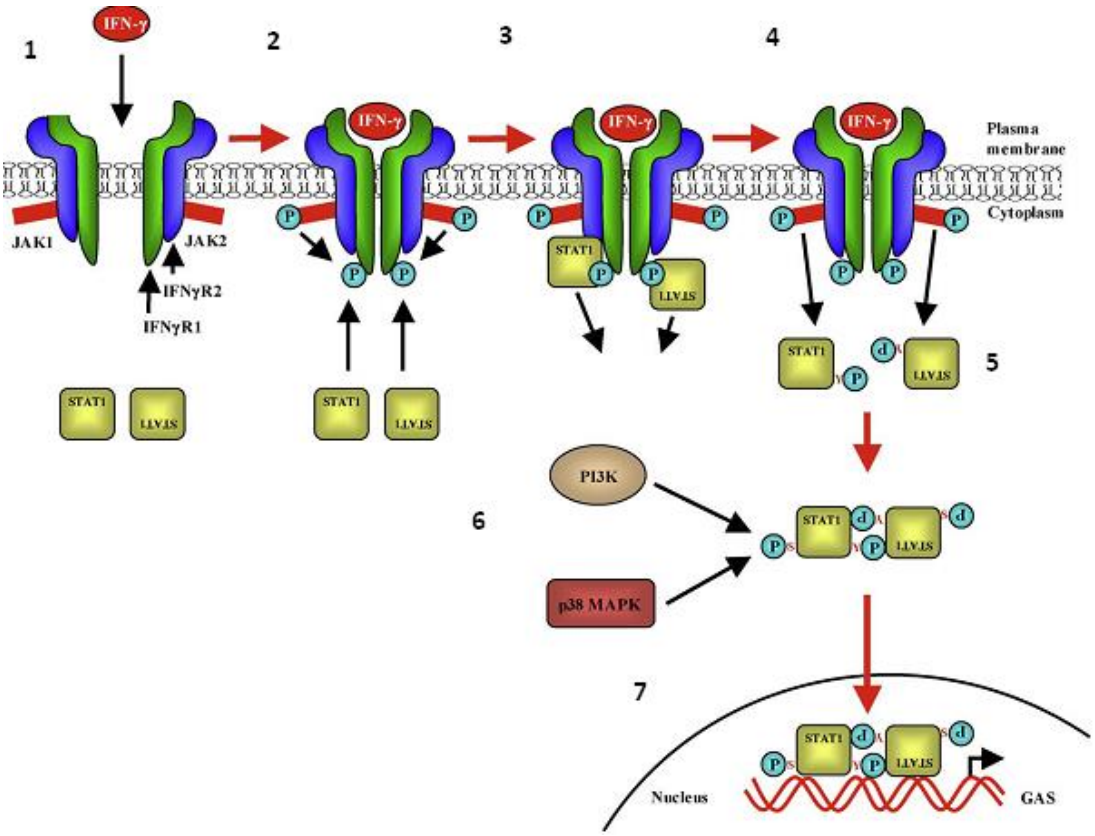


Figure 1.7 – IFN-γ signalling through the JAK/STAT pathway

Diagram depicts an overview of IFN-γ signalling through the JAK/STAT pathway **1.** IFN-γR is composed of two subunits: R1 and R2. **2**. Binding of IFN-γ causes dimerisation of subunits allowing phosphorylation of tyrosine kinases JAK1 and JAK2, which are attached to the IFN-γR complex via their N-terminus. Activated JAK1/2 phosphorylate IFN-γR subunits at tyrosine residues, via their catalytic domain **3.** Phosphorylated IFN-γR subunits bind STAT1 monomers from the cytoplasm via their SH2 domain and are phosphorylated at TYR^{701} **4.** Phosphorylated STAT1 monomers are released from the receptor **5.** STAT1 monomers dimerise: STAT1:STAT1. **6.** Phosphorylation of STAT1 dimers at position SER^{727} by PI3-K, p38 MAPK and ERK is required for maximal activity. **7.** STAT1:STAT1 trans-locates into the nucleus where they can bind to GAS in the promoter regions of IFN-γ-inducible genes. Adapted from (McLaren and Ramji 2009).

1.4.1.1.2 Role of IFN-γ in atherosclerosis

IFN-γ is involved in all aspects of atherosclerosis pathology. ApoE$^{-/-}$ IFN-γR$^{-/-}$ mice showed significant reduction in atherosclerotic lesion size together with reduced lesion lipid accumulation and lesion cellularity (Gupta *et al*. 1997). This suggests that IFN-γ promotes atherosclerosis and impacts on various aspects of the disease. IFN-γ can impact on levels of chemokines expressed in atherosclerosis as seen by the dramatic increase in MCP-1 expression on treatment with IFN-γ in a human osteoblastic cell line (Valente *et al.* 1998). IFN-γ also induces the expression of adhesion molecules ICAM-1 and VCAM-1 (Li *et al.* 1993; Chung *et al.* 2002). Once monocytes have differentiated into macrophages, IFN-γ plays a role in the expression of scavenger receptors which bind modified forms of LDL. IFN-γ up regulates the expression of scavenger receptor Chemokine (C-X-C motif) ligand 16/ Scavenger receptor for phosphatidylserine and oxidised lipoprotein (CXCL16/SR-PSOX) which mediate oxLDL uptake (Wuttge *et al.* 2004) thereby promoting foam cell formation. The cytokine also plays a role in cholesterol accumulation in macrophages. IFN-γ has been shown to decrease cholesterol efflux (Harvey and Ramji 2005) and impede reverse cholesterol transport in THP-1 macrophages (Reiss *et al*. 2004). As the disease progresses, IFN-γ continues to play a role. In the later stages of atherosclerosis, IFN-γ can act to destabilise atherosclerotic plaques by inhibition of SMC proliferation and matrix synthesis, thereby affecting the fibrous cap, and up regulates the expression and activities of matrix metalloproteinases which break down the extracellular matrix of the plaque (Harvey and Ramji 2005). Both these roles act to destabilise the plaque increasing the risk of plaque rupture.

There are, however, some conflicting anti-inflammatory roles for IFN-γ in atherosclerosis. Despite the increase in the CXCL16/SR-PSOX scavenger receptor expression, IFN-γ has been shown to inhibit the expression of two other important scavenger receptors SRA and CD36 (Nakagawa *et al.* 1998; Grewal *et al.* 2001). It has also been shown that IFN-γ may act to inhibit pro-inflammatory LPL expression by macrophages and macrophage-mediated LDL oxidation (Harvey and Ramji 2005). Despite these controversial roles of IFN-γ, the balance between pro and anti-atherosclerotic roles of IFN-γ favours the cytokine as pro-inflammatory which significantly increases atherosclerosis *in vitro*. In addition, studies *in vivo* have demonstrated consistently a pro-atherosclerotic role for the cytokine (Table 1.4) (Harvey and Ramji 2005; McLaren and Ramji 2009).

1.4.1.2 IL-1β

The IL-1 family consists of three members; IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra) (Fearon and Fearon 2008). IL-1 signalling is tightly controlled with binding of IL-1-α and -β to IL-1 type 1 receptor inducing downstream pro-inflammatory signalling events. On the other hand, the binding of IL-1 to IL-1 type II decoy receptor and IL-1ra binding to type I receptor acts to inhibit signalling (Fearon and Fearon 2008). IL-1 is produced by a number of cells including endothelial cells, SMCs and macrophages. Increased levels of IL-1 stimulates release of cytokines and chemokines, increased expression of adhesion molecules, macrophage recruitment and SMC proliferation during atherosclerosis (Fearon and Fearon 2008). The role of IL-1β has been well characterised as an important pro-inflammatory factor in atherosclerosis and will be discussed in more detail.

1.4.1.2.1 Activation of IL-1β

IL-1β activation is under tight regulation. It is synthesised as an inactive precursor molecule (proIL-1β) which remains within the cell (Dinarello 1998). In order to elicit a response, proIL-1β must first be cleaved. Therefore two mechanisms are required for the maturation and release of active IL-1β; production of proIL-1β and activation of the cytokine through cleavage. Caspases are a family of cysteine proteases that cleave substrates at aspartate residues. They are involved in pro-inflammatory and pro-apoptotic pathways (Franchi *et al.* 2009). Caspases -1, -4 and -5 have been identified as pro-inflammatory in humans (Schroder and Tschopp 2010) and caspase 1 plays a key role in the activation of IL-1β (Franchi *et al*. 2009). Caspase 1 cleaves proIL-1β at an aspartic residue located at position 116 to form the active cytokine which can be secreted by the cell (Dinarello 1998). Along with IL-1β activation, activation of caspase 1 is also tightly regulated. Caspase 1 must also undergo cleavage itself, which is achieved by multi-protein complexes called inflammasomes (Schroder and Tschopp 2010).

Immune cells express PRRs on their surface to detect pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). These include nucleotide oligomerization domain (NOD) like receptors (NLRs) (Schroder and Tschopp 2010). The inflammasome is a large multi-protein complex which includes members of the NLR family along with adaptor protein apoptosis-associated speck-like protein containing CARD (ASC) (Franchi *et al*. 2009). A number of members of the NLR family have been implicated to play a role in the inflammasome including NLRP1 and NLRP3. The NLRP3 inflammasome has been well characterised. It can be activated by a number of extracellular stimuli including

PAMPs, extracellular glucose, amyloid β peptide, uric acid, cholesterol crystals and oxLDL (Rajamaki *et al.* 2010; Schroder and Tschopp 2010; Liu *et al.* 2014). There are a number of different theories surrounding the mechanism by which the NLRP3 inflammasome is activated. These include direct activation by agonist, activation by lysosomal contents released on engulfment of agonist and, finally, activation by ROS produced by the agonist (Schroder and Tschopp 2010). Once activated, the NLRP3 inflammasome assembles and recruits pro-caspase 1. Auto cleavage of pro-caspase 1 forms the active protease. Caspase 1 can then cleave pro-IL-1 β to its active form.

1.4.1.2.2 Role in atherosclerosis

IL-1β is a well-established pro-inflammatory cytokine whose expression is up regulated in atherosclerosis. IL-1β expression was found to be increased in the vessel walls of atherosclerotic arteries and plaque macrophages (Galea *et al.* 1996). $ApoE^{-/-}$ $IL\text{-}1\beta^{-/-}$ mice showed a 30% decrease in atherosclerotic lesions at the aortic sinus in comparison to controls. It was also observed that the mRNA levels of VCAM-1 and MCP-1 were significantly reduced in such lesions (Kirii *et al*. 2003). Similarly, administration of an anti-IL-1β antibody to $ApoE^{-/-}$ mice inhibited atherosclerotic lesion formation (Bhaskar *et al*. 2011). In addition, inhibition of IL-1β increased plasma HDL levels, decreased plaque lipid and macrophage content and reduced secretion of IL-6, IL-8, MCP-1 and TNF-α (Bhaskar *et al*. 2011). Interestingly, the therapeutic potential of neutralising IL-1β antibodies are currently being evaluated in a multi-centre phase III Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) (Ridker *et al.* 2011).

1.4.1.3 TNF-α

TNF-α is member of a growing family of cytokines (Popa *et al*. 2007). Initially discovered as a circulating factor, it has since been identified as a key component of the innate immune system and its expression is up-regulated in response to stress signals (Kleinbongard *et al.* 2010). It is a pro-inflammatory cytokine produced by a number of immune cells including macrophages, monocytes and T-cells (Popa *et al*. 2007). TNF-α is first synthesised as a transmembrane protein and cleavage of the extracellular domain releases a soluble form of the cytokine. Both the transmembrane and soluble form of TNF-α are biologically active and can bind TNF-receptor (TNFR) I/II (Olmos and Llado 2014). Both the receptors belong to the TNF-receptor superfamily, which also includes FAS, CD40, CD27 and RANK (Idriss and Naismith 2000). TNFR1 and TNFR2 elicit different responses and are structurally different. TNFR1 (55kDa) contains a death domain and is linked to cell death by apoptosis while TNFR2 (75kDa) lacks the death domain and plays a role in cell survival and

proliferation response. However, studies now report overlapping functions of the two receptors (Olmos and Llado 2014). Binding of TNF-α, as a trimer to its cognate receptors (Idriss and Naismith 2000), initiates a downstream cascade which results in the activation of a number of signalling pathways, including NF-κB, ERK, JNK, and p38 (Popa *et al*. 2007; Olmos and Llado 2014). Activation of these pathways has a number of pro and anti-inflammatory effects in atherosclerosis, the details of which can be found in Table 1.4.

1.4.1.3.1 Role in atherosclerosis

TNF–α is highly expressed by macrophages present in atherosclerotic plaques (Arbustini *et al.* 1991) and in one study it was detected in 88% of atherosclerotic plaques (Barath *et al.* 1990). Increased release of TNF-α in response to stress signals stimulates the expression of adhesion molecules on endothelial cells and promotes the recruitment and activation of immune cells (Popa *et al*. 2007). It is therefore an important factor in the initial stages of foam cell formation. In addition to this, TNF-α expression can increase apoptosis, thrombin formation and increase cholesterol uptake by macrophages (Kleinbongard *et al*. 2010). $ApoE^{-/-}$ $TNF\text{-}\alpha^{-/-}$ mice show a decrease in lipid accumulation and fatty streaks in comparison to control mice, as well as a decrease in the expression of IL-1β, IFN-γ, ICAM-1, VCAM-1, MCP-1, GM-CSF along with activation of NF-κB (Xiao *et al*. 2009). Similarly, $ApoE^{-/-}$ $TNF\text{-}\alpha^{-/-}$ mice show a reduction in the size of atherosclerotic plaques present in the aortic luminal surface and aortic sinus compared to $ApoE^{-/-}$ control mice (Ohta *et al*. 2005). A decrease in the expression of ICAM-1, VCAM-1 and MCP-1 was again observed, along with a decreased uptake of oxLDL and reduced expression of SRA in response to TNF-α deficiency (Ohta *et al*. 2005).

1.4.2 Fatty acids as signalling molecules

As well as energy storage and their importance in cellular membranes, lipids can also act as signalling molecules. Fatty acids are delivered to tissues as TAG-derived fatty acids or non-esterified fatty acids (NEFAs). They can be taken up by the cells using fatty acid transporters and bound to fatty acid binding proteins (Georgiadi and Kersten 2012). From here they can be subject to a number of fates including oxidation for energy, esterification, incorporation into phospholipids of membranes or storage as lipid droplets. Most of the signalling actions of fatty acids are linked to their metabolites; however they themselves can directly activate signalling pathways Second messengers, such as diacylglycerols (DAG), are produced by enzyme-catalysed hydrolysis of membrane phospholipids. The composition of fatty acids in DAG determines their effect on signalling (Calder 2012). Roles of fatty acids in

atherosclerosis are summarised in Table 1.5. Fatty acids cleaved from phospholipid membranes and NEFAs can directly, or indirectly through their metabolites, activate numerous signalling pathways by acting on receptors and transcription factors. These include peroxisome proliferator activated receptors (PPARs), TLRs, G protein-coupled receptors (GPCRs) and transcription factor sterol regulatory element binding protein (SREBP) (Georgiadi and Kersten 2012).

1.4.2.1 PPARs

These are members of nuclear hormone receptor superfamily and three subtypes exist; α, δ and γ. PPARα and PPARδ are expressed in most tissues and cells. PPARγ expression however is more restricted to mainly adipocytes and macrophages. PPARs function by forming heterodimers with retinoid-x-receptors. The heterodimers bind DNA sequences named peroxisome proliferators response elements (PPREs) present in promoter sequences of target genes (Tyagi *et al.* 2011; Georgiadi and Kersten 2012). They act to regulate lipid metabolism, glucose homeostasis and inflammation and are activated by fatty acids and their derivatives (Duval *et al.* 2002). PPAR-α and -γ have been implicated to play a protective role in atherosclerosis and inflammation (Duval *et al*. 2002). Activation of PPARγ by oxidised linoleic acid has been found to inhibit the MCP-1 receptor C-C chemokine receptor 2 (CCR2) and block monocyte chemotaxis. (Han *et al.* 2000). PPARγ agonist prostaglandin J_2 (PGJ_2), but not PPARα agonists EPA and DHA, inhibited the IFN-γ-mediated induction of pro inflammatory CXC chemokines. This included the IFN-inducible T-cell α chemoattractant (I-TAC), IFN-inducible protein of 10kDa (IP-10) and monokine induced by IFN-γ (Mig) (Marx *et al.* 2000). The expression of MCP-1 was not affected in the study (Marx *et al*. 2000). PPARα agonists inhibited TNF-α-mediated expression of VCAM-1 by human endothelial cells that was not seen with the activation of PPARγ (Marx *et al.* 1999). Disruption of the PPARγ gene in mice reduced the expression of LPL, CD36, LXRα, ABCA1 and ApoE which, in turn, reduced cholesterol efflux to HDL from cholesterol loaded macrophages (Akiyama *et al.* 2002). $LDLr^{-/-}$ mice reconstituted with bone marrow from $PPAR\alpha^{+/+}$ and $PPAR\alpha^{-/-}$ mice were fed a high fat diet. $PPAR\alpha^{-/-}$ $LDLr^{-/-}$ mice developed significantly larger atherosclerotic lesions, increased oxLDL uptake and decreased cholesterol efflux in comparison to controls (Babaev *et al.* 2007). $PPAR\alpha^{+/+}$ $LDLr^{-/-}$ developed significantly smaller atherosclerotic lesions in comparison to $PPAR\alpha^{-/-}$ $LDLr^{-/-}$ mice (Babaev *et al*. 2007). The role of PPARδ is controversial and needs more study. However activation of PPARδ with agonist promotes lipid accumulation in human macrophages, increases SRA and

CD36 expression and attenuates ApoE expression. This suggests a pro-atherogenic role for this receptor (Vosper *et al.* 2001).

1.4.2.2 TLR4

This is a type I transmembrane protein and belongs to the TLR family with at least 10 members, each with different ligands (Li and Sun 2007). TLR4 was identified as the first human homologue that is activated by LPS and saturated fatty acids (SFA) (Suganami *et al.* 2007; Tsukumo *et al.* 2007) and, therefore, is a candidate receptor in fatty acid signalling. TLR4 plays an important role in inflammation, immunity and insulin resistance (Tsukumo *et al*. 2007). The receptor is expressed by immune cells, neutrophils, macrophages, dendritic cells, B-cells and T-cells (Li and Sun 2007). Treatment of macrophages with SFA leads to an increase in pro-inflammatory cytokine expression (TNF-α and IL-1β). SFA also activates NF-κB targets in macrophages through activation of TLR4. Macrophages from $TLR4^{-/-}$ mice show an attenuation in TNF-α production (Suganami *et al*. 2007). In TLR4 deficient mice fed a high fat diet, monocyte infiltration and expression of MCP-1 was reduced in adipose tissue. This was accompanied by less NF-κB activation. However, there was no difference observed in TNF-α or IL-6 expression in comparison to WT mice (Davis *et al.* 2008). $TLR4^{-/-}$ mice were resistant to diet-induced obesity and showed improved insulin sensitivity. In addition, SFA-induced insulin resistance was inhibited in the muscle tissue of these mice (Tsukumo *et al*. 2007). $LDLr^{-/-}$ $TLR4^{-/-}$ mice showed improved plasma cholesterol and TAG levels but still developed obesity and glucose intolerance comparable to $LDLr^{-/-}$ mice. Despite this, the development of atherosclerosis in double knockout mice was significantly attenuated (Ding *et al.* 2012). In addition, TLR4 expression and signalling in atherosclerosis is up-regulated by oxLDL and induces a pro-inflammatory response in endothelial cells and macrophages, SMC migration and secretion of proteolytic enzymes MMPs (Li and Sun 2007). However, an atheroprotective role for TLR4 was observed in response to infection (with common oral pathogen) in $ApoE^{-/-}$ $TLR4^{-/-}$ mice (Hayashi *et al.* 2012).

1.4.2.3 GPCRs

GPCRs are a large family of cell surface receptors that share common structural motifs and the ability to activate heterotrimeric G proteins (Oh *et al.* 2010). Ligand binding can stimulate a number of second messenger pathways; cyclic adenosine monophosphate (cAMP) production, opening of ion channels and activation of MAPK pathways. There are estimated to be over 850 human GPCRs, which differ in cell specificity and tissue expression (Talukdar *et al.* 2011). GPR-40, -41, -43, -84 and -120 can be activated by NEFAs. Short chain fatty acids (4-8 carbons) activate GPR-

41 and -43; medium chain fatty acids (10-14 carbons) activate GPR-84; and long chain fatty acids (16-18 carbons) activate GPR-40 and -120 (Oh *et al*. 2010). GPR-120 is expressed in adipocytes and pro-inflammatory macrophages. Activation of this receptor induces numerous responses, commonly related to insulin sensing and inflammation. Studies have focused on omega-3 signalling through GPR-120 and the resulting anti-inflammatory effects. EPA and DHA can exert anti-inflammatory effects through GPR-120 in macrophages. DHA signalling through GPR-120 inhibited LPS induced phosphorylation of JNK and pro-inflammatory cytokine gene expression in macrophages. This was also observed *in vivo* (Oh *et al*. 2010). A high fat diet in GPR-120 knockout mice and wild type mice resulted in induction of insulin resistance. However, supplementation with omega-3 fatty acids produced improved glucose tolerance in wild type mice only and not in GPR-120 deficient animals (Talukdar *et al*. 2011). In another study, pre-treatment of neurone cells with DHA prevented a TNF-α induced inflammatory response (Wellhauser and Belsham 2014). This effect was independent of ERK and PI3K/Akt activation and abolished on reduction of endogenous GPR-120 levels (Wellhauser and Belsham 2014). This suggests omega-3 fatty acids may exert anti-inflammatory effects through GPR-120 signalling.

1.4.2.4 SREBPs

SREBPs are a family of basic helix-loop-helix, leucine zipper transcription factors. They are synthesised in a membrane bound form on the endoplasmic reticulum complexed with SREBP cleavage activating protein (Karasawa *et al.* 2011). A reduction in cellular cholesterol levels induces SREBP cleavage activating protein to escort SREBP to the Golgi apparatus where it is cleaved by proteases. The cleaved transcription factor can next move into the nucleus and activate downstream pathways (Karasawa *et al*. 2011). Three isoforms exist; SREBP-1a, SREBP-1c and SREBP-2. The primary role of these factors involves transcriptional activation of genes involved in *de novo* lipogenesis and the uptake of cholesterol, fatty acids, TAG and phospholipids (Karasawa *et al*. 2011). $LDLr^{-/-}$ mice overexpressing hepatic SREBP-1c results in hypertriglyceridemia, an increase in VLDL levels and a decrease in HDL resulting in atherosclerosis (Karasawa *et al*. 2011). In contrast, deficiency of SREBP-1 attenuated atherosclerosis in these mice (Karasawa *et al*. 2011). PUFAs have been indicated to inhibit lipogenic gene expression by down regulating SREBPs. In one study PUFA increased the decay of SREBP-1a and SREBP-1c in rat hepatocytes. Treatment with AA and EPA decreased the half-life of total SREBP-1 mRNA by 50% (Xu *et al.* 2001). In mice, supplementation with PUFAs decreased the cleaved form of mature SREBP-1 protein in the nucleus as well as enzymes involved

in lipid synthesis such as fatty acid synthase and acetyl-CoA carboxylase (Yahagi *et al.* 1999). Studies indicate that PUFA can attenuate lipid synthesis by regulating expression and post-transcriptional mRNA stability of SREBP and that this may be beneficial in the pathology of atherosclerosis.

1.5.2.5 Role of fatty acids in atherosclerosis

As discussed previously, fatty acids can be classified as saturated and unsaturated. PUFA hydrocarbon chains contain more than one double bond and are important signalling molecules. Table 1.5 summarises some of the roles fatty acids play in the pathology of atherosclerosis. Generally speaking, SFA are commonly referred to as 'bad fat' as consumption has been linked to increased cholesterol levels and inflammatory processes, therefore increasing the risk of CVD (Xu *et al.* 2006). Diets rich in PUFA however, indicate a significant athero-protective role (Dyerberg 1989) and have been shown to modify inflammation (Suresh and Das 2003a, b; Wang *et al.* 2012; Dawczynski *et al.* 2013). Two important classes of PUFA will be discussed in more detail; omega-3 and omega-6 fatty acids.

Table 1.5 – Fatty acids and their role in atherosclerosis

Class	Name	Possible role in atherosclerosis
SFA	Myristic acid	Increases plasma LDL cholesterol (Xu *et al*. 2006). Decreases HDL to LDL ratios in humans (Zock *et al.* 1994). Correlates with an increase in large dense LDL particles (Deron *et al.* 1999).
SFA	Palmitic acid	Raises cholesterol levels and increases uptake of oxLDL by macrophages via expression of LOX-1 receptor therefore promoting atherosclerosis (Xu *et al*. 2006; Ishiyama *et al.* 2010). Activates TLR2 to induce inflammasome activation and pro IL-1β cleavage (Snodgrass *et al.* 2013) Decreases HDL to LDL ratios in humans (Zock *et al*. 1994). Correlates with an increase in large dense LDL particles (Deron *et al*. 1999).
SFA	Stearic acid	Lowers LDL cholesterol levels *in vivo* in comparison to *trans*-saturated fatty acids (Hunter *et al.* 2010). No significant association with lipoprotein concentrations (Deron *et al*. 1999).

MUFA	Palmitoleic acid	Increases insulin sensitivity and reduces pro-inflammatory gene expression in mice with type 2 diabetes (Yang *et al.* 2011). Association found between the content of palmitoleic acid in red blood cell membrane and CVD (Djousse *et al.* 2012).
MUFA	Oleic acid	Hamsters supplemented with an oleic acid-rich oil showed decreased atherosclerosis and foam cell formation in comparison to a diet rich in LA (Nicolosi *et al.* 2002). In mouse model, oleic acid displayed anti-oxidant activity against LDL oxidation. Strongly inhibited oxLDL uptake by THP-1 macrophages (Cho *et al.* 2010). $ApoE^{-/-}$ deficient mice fed an oleic acid-rich diet resulted in an increase in FFA and enhanced atherosclerosis compared to normal chow diet (Ma *et al.* 2011).
MUFA	Vaccenic acid	In red blood cells, fatty acid levels were inversely associated with CVD (Kris-Etherton and Nutrition 1999; Djousse *et al*. 2012). Anti-atherogenic effects in $LDLr^{-/-}$ mice (Bassett *et al.* 2010).
n-6 PUFA	Linoleic acid	Precursors of DGLA and AA. Results in both pro- and anti-inflammatory actions as a result of metabolism to these two fatty acids (Wang *et al*. 2012). Diet rich in LA inversely related to risk of atherosclerosis (Djousse *et al.* 2003; Djousse *et al.* 2005)

		LDLr$^{-/-}$ mice fed an omega-6 LA rich diet showed significantly lower atherosclerosis by a lowering of total cholesterol levels (Machado *et al.* 2013).
n-6 PUFA	GLA	Inhibited atherosclerosis in LDLr$^{-/-}$ and ApoE$^{-/-}$ mice (Fan *et al.* 2001; Machado *et al*. 2013) Inhibited atherosclerosis induced in rats fed a high fat diet. Reduced total cholesterol levels, oxLDL, NO and LDL (Shi *et al.* 2008) Decreased IL-1β secretion from human monocytes (Furse *et al.* 2001).
n-6 PUFA	DGLA	Inhibited atherosclerosis in ApoE$^{-/-}$ mice and reduced expression of inflammatory markers (Takai *et al.* 2009). Attenuated TNF-α production from leukocytes (Dooper *et al.* 2003).
n-6 PUFA	AA	High AA/EPA ratio linked to a risk of CVD (Ninomiya *et al.* 2013). Metabolites of AA (for example PGE_2 and LBT_4) have pro-inflammatory and pro-thrombotic actions (Levin *et al.* 2002; Gomolka *et al.* 2011; Wang *et al*. 2012).
n-3 PUFA	α-linolenic acid	Diabetic ApoE$^{-/-}$ mice fed a diet supplemented with ALA showed a decrease in markers of oxidative stress, and this prevented increase of plasma cholesterol and atherosclerosis (Yi and Maeda 2006). Increased consumption linked to a reduction in CVD (Kris-Etherton *et al.* 2003; Harris *et al.* 2007).

n-3 PUFA	EPA	Significantly reduced in patients experiencing CVD events (Harris *et al*. 2007). Low EPA/AA ratio associated with risk of cardiovascular disease (Ninomiya *et al*. 2013). Dietary supplementation of EPA in ApoE$^{-/-}$ mice regressed atherosclerotic plaques and decreased immune cell content (Nakajima *et al.* 2011). Inhibits modified LDL uptake and macropinocytosis *in vitro* (McLaren *et al.* 2011b).
n-3 PUFA	DPA	Significantly reduced in patients experiencing CVD events (Harris *et al*. 2007). Reduced total plasma cholesterol and non-HDL associated cholesterol. Down-regulated expression of HMG-CoA reductase. Less tension and more relaxed aorta due to inhibition of Cyclooxygenase (COX)-2 (Chen *et al.* 2012).
n-3 PUFA	DHA	Increased consumption linked to a reduction in CVD (Kris-Etherton *et al*. 2003; Harris *et al*. 2007). Reduced total plasma cholesterol and non-HDL associated cholesterol. Down regulated HMG-CoA reductase. Less tension and more relaxed aorta due to inhibition of COX-2 (Chen *et al*. 2012). Inhibits oxLDL uptake and macropinocytosis *in vitro* (McLaren *et al*. 2011b). Inhibited TLR1 and TLR2 dimerisation and inflammasome-mediated secretion of IL-1β (Snodgrass *et al*. 2013).

1.5.2.6 Omega-3 and 6 fatty acids

Omega-3 and -6 fatty acids include the essential fatty acids (EFA) linoleic acid and α-linolenic acid. EFA are important constituents of cell membranes and can therefore alter their behaviour and those of bound enzymes and receptors (Das 2007). Changes in the dietary intake of EFA can modulate the production of eicosanoids. Omega-3 and -6 PUFA are cleaved from cellular membranes via the action of phospholipase A_2 (PLA_2) in response to cellular signals. This release induces the production of eicosanoids through the LOX, COX and P-450 enzymatic pathways (McDaniel *et al.* 2011). Eicosanoids are a family of bioactive lipid mediators, 20 carbons in length, with a wide variety of actions. The eicosanoid family includes prostaglandins (PG), leukotrienes (LT), thromboxanes (TX) and numerous hydroxy fatty acids (McDaniel *et al*. 2011). These mediators act quickly and locally and remain active for only a short period of time before they are degraded (Arita 2012). Eicosanoids elicit a number of actions which are both pro- and anti-inflammatory (Figure 1.8).

Several studies have indicated that in CVD, essential fatty acid metabolism is abnormal such that plasma and tissue level of omega-3 and -6 fatty acids are low (Das 2007). Omega-3 PUFA are well documented to impart anti-inflammatory actions in CVD including atherosclerosis and in other chronic inflammatory diseases (Bannenberg and Serhan 2010). Numerous studies have detailed the beneficial actions of omega-3 PUFA, primarily EPA and DHA, in a wide variety of inflammatory diseases including heart disease (Dawczynski *et al*. 2013; Masson *et al.* 2013) and stroke (Larsson *et al.* 2012). EPA and DHA are abundant in fish oils (Arita 2012) and sold as dietary supplements worldwide. A study performed in the human macrophage cell line THP-1 showed an inhibition of acetylated LDL (acLDL) and oxLDL uptake on incubation with EPA and DHA (McLaren *et al*. 2011b). In addition, dietary supplementation of EPA containing fish oils prevented the development of atherosclerosis in mice (Takai *et al*. 2009). EPA and DHA therefore exhibit athero-protective effects.

A number of studies have focussed on the role of omega-6 fatty acids in CVD with controversial results. Many recommend reductions in omega-6 dietary intake (Hamazaki and Okuyama 2003; Simopoulos 2008; Harris *et al.* 2009) and consumption of a diet with a high omega-6/omega-3 ratio has been associated with an increased risk of CVD, inflammatory diseases and cancer (Dawczynski *et al*. 2013). AA is well documented to play a pro-inflammatory role in disease. Diets rich

in the EFA omega-6 fatty acid LA have been advised against with the understanding that an increase in LA will lead to an increase in its metabolite AA. Reducing LA intake would reduce AA accumulation and its pro-inflammatory potential (Harris *et al*. 2009). However intake of LA does not always manifest in the levels of AA due to the rate-limiting steps in the omega-6 pathway. Studies have indicated that the extent of conversion of dietary LA to AA is 0.2% (Hussein *et al.* 2005).

Despite this, a large number of studies have suggested that increasing the levels of omega-6 in the diet has no association or reduces the risk of CVD. The alpha-tocopherol beta-carotene cancer prevention study (Pietinen *et al.* 1997) concluded that there was no association between the LA content of the diet and CVD. On the other hand, The Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study (Laaksonen *et al.* 2005) and the Western Electric Study (Shekelle *et al.* 1981) both reported inverse associations between dietary LA and CVD risk. In a meta-analysis of 25 case control studies, dietary content of LA was inversely related to CVD risk while AA was unrelated (Harris *et al*. 2007). Large intakes of LA have also been shown to have no link to the risk of acute myocardial infarction (Kark *et al.* 2003). It has therefore been suggested that decreasing the omega-6 fatty acid intake in the diet would more than likely increase the risk of CVD (Harris *et al*. 2009). Furthermore, dietary approaches with omega-6 fatty acids GLA and DGLA have indicated potent anti-inflammatory effects in inflammatory disease (Kawashima *et al.* 2008; Takai *et al*. 2009; Wang *et al*. 2012).

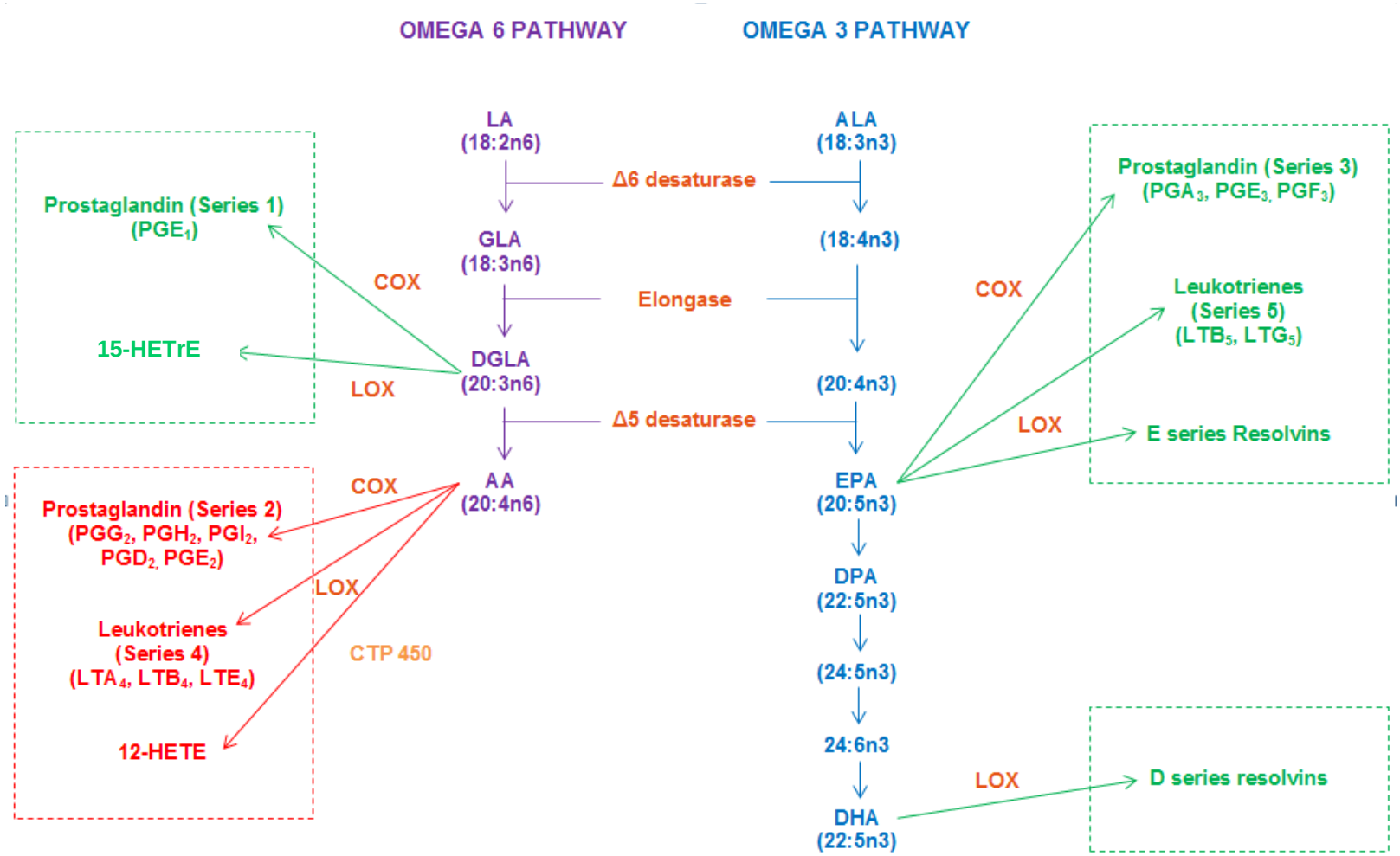


Figure 1.7 – Overview of omega-3 and -6 pathways

Green boxes represent eicosanoids with anti-inflammatory roles; red boxes indicate pro-inflammatory roles. See table 1.2 for list of fatty acid abbreviations. COX – cyclooxygenase, LOX – lipooxygenase. HETE - hydroxyeicosatetraenoic acid, HETrE - hydroxyeicosatrienoic acid

1.5 DGLA

1.5.1 Relation to disease

Omega-6 PUFAs are commonly referred to as pro-inflammatory due to the actions of metabolites of AA. Controversially however, metabolic precursors of AA have been demonstrated to have key anti-inflammatory roles. Dietary supplementation with GLA has been implicated as beneficial in a number of inflammatory diseases. For example treatment with GLA significantly reduced the signs and symptoms in patients with rheumatoid arthritis (Zurier *et al.* 1996) and atopic eczema (Umeda-Sawada *et al.* 2006). GLA also inhibited atherosclerosis and smooth muscle cell proliferation in $ApoE^{-/-}$ mice (Fan *et al*. 2001). In human monocytes, GLA inhibited the secretion of IL-1β in a dose dependant manner (Furse *et al*. 2001). GLA is elongated to DGLA which is incorporated into cellular membranes, cleaved or metabolised to produce anti-inflammatory mediators. Many studies use GLA to indirectly increase the fatty acid pool of DGLA. However this process does not allow for a sufficient increase of the fatty acid (Teraoka *et al.* 2009). DGLA is the bioactive form of GLA (Kapoor and Huang 2006) but, as there were previously no major source of DGLA available, direct supplementation with this fatty acid was not possible. Due to the development of new sources of DGLA, a small number of studies have investigated the anti-inflammatory roles of DGLA, some in relation to a number of diseases, including atherosclerosis.

ApoE deficient mice fed a normal diet supplemented with DGLA showed a significant reduction in the development of atherosclerosis (Takai *et al*. 2009). In addition, DGLA supplementations attenuated expression of ICAM-1 and VCAM-1 markers of inflammation, along with a decrease in NADPH oxidase subunits $p22^{phox}$ and $gp91^{phox}$ indicating a potential increase in vascular relaxation (Takai *et al*. 2009). In human leukocytes it was observed that DGLA inhibited TNF-α production and specifically altered cytokine levels, changes that were not seen with GLA or EPA (Dooper *et al*. 2003). Supplementation with DGLA oil in the diet of a mouse model of atopic dermatitis significantly reduced severity of skin scores as well as scratching behaviour (Kawashima *et al*. 2008). Mice that were orally administered with DGLA also showed significant reduction in inflammation of the ear following induction with inflammatory stimuli. DGLA inhibited swelling and cell infiltration after 7 days of treatment (Watanabe *et al.* 2014).

Studies have also indicated a role for DGLA in cancer. Free radicals produced as a result of oxygenation of DGLA, heptanoic acid (HTA) and 8 hydroxyoctanoic acid (8-HOA), had an anti-proliferative effect on cancerous colon cells (Xu *et al.* 2014b). The free radicals arrested the cell cycle in cancer cells and promoted apoptosis. In addition, in combination with the DGLA-derived free radicals, the efficiency and cytoxicity of the colon cancer drug 5-fluoruracil, was increased (Xu et al. 2014b).

1.5.2 Synthesis and metabolism

DGLA is a 20 carbon omega-6 PUFA metabolised from linoleic acid (Wang *et al*. 2012). See Figure 1.8. Linoleic acid is an essential fatty acid required by humans at around 1-2% of total dietary energy to prevent deficiency (Wang *et al*. 2012). Synthesis of DGLA through the omega-6 pathways is detailed in Figure 1.8. The omega-6 pathway competes with the omega-3 pathway as they share a common set of desaturase enzymes (Calder 2012) Briefly, linoleic acid is metabolised by Δ6 desaturase to GLA. GLA is then elongated to DGLA which can be further metabolised by Δ5 desaturase to form AA.

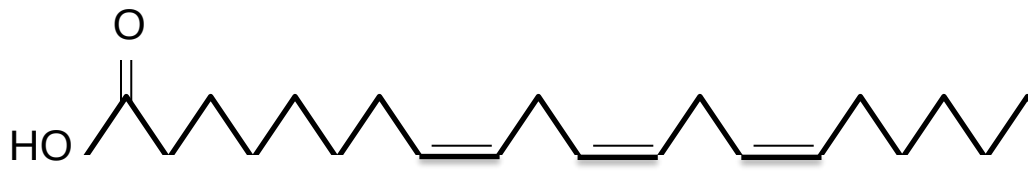


Figure 1.8 – Structure of DGLA

Figure depicts the structure of DGLA. The PUFA contains 20 carbons and 3 double bonds, with the first double bond at carbon 6 (using the omega numbering system).

The omega-6 pathway contains two desaturation steps, both of which are flux controlling enzyme steps (Kapoor and Huang 2006). Desaturation is a slow process; this means that conversion of LA to GLA is limited and can lead to deficiencies of GLA particularly in inflammatory conditions, which imposes further restrictions (Kapoor and Huang 2006). A reduced capacity of conversion of LA to GLA and DGLA has been linked to physiological and disease states such as aging, diabetes, atopic dermatitis, rheumatoid arthritis, cancer and CVD (Fan and Chapkin 1998; Kapoor and Huang 2006; Wang *et al*. 2012).The desaturation of DGLA to AA is also a slow process and only a small amount of DGLA is converted to AA (Fan and Chapkin

1998). This suggests that direct supplementation of DGLA would bypass the first desaturation step in the pathway, increasing availability of DGLA which can be incorporated into cell membranes and metabolised without a large increase in the accumulation of AA (Fan and Chapkin 1998). This is observed in a study which investigated the supplementation of DGLA in rats. It was observed that on supplementation with DGLA there was an increase in DGLA concentrations in liver, serum and brain without accumulation of AA (Umeda-Sawada *et al*. 2006). This is an important feature of DGLA supplementation as this selectively increases the production of anti-inflammatory eicosanoids without an increase in pro-inflammatory eicosanoids from AA to counteract any actions. Both DGLA and AA are competing substrates for COX and LOX enzymes yielding a wide variety of eicosanoids. Specific metabolites of DGLA and AA are detailed in Figure 1.7.

AA is cleaved from phospholipid membranes by PLA_2 where it is immediately metabolised by COX or LOX pathways generating PGs and TXs, and LTs, LXs and hydroxy-eicosatetraenoic acids (HETEs) respectively (Arita 2012). Eicosanoids derived from AA are well documented to play a role in the initiation of inflammation and chronic inflammation. The 5-LOX metabolite LTB4 is a potent chemoattractant for leukocytes along with COX-2 metabolite PGE_2 (Bannenberg and Serhan 2010). DGLA however, gives rise to two important metabolites with highly desirable actions; PGE_1 and 15- hydroxy-eicosatrienoic acids (15-HETrE) (Horrobin 1991). PGE_1 lowers blood pressure, inhibits platelet aggregation, promotes cholesterol efflux from cells and inhibits cholesterol biosynthesis, along with various other anti-inflammatory effects (Horrobin 1991). 15-HETrE also inhibits 5-LOX and 12-LOX which, in turn, inhibits the production of pro-inflammatory eicosanoids derived through the actions of these enzymes on AA (Horrobin 1991).

1.5.3 Source of DGLA

Principle sources of PUFA have previously relied on fish oils. Due to an increase in both fish consumption and marine pollution, fish stocks are ever decreasing (Watanabe *et al*. 2014). This highlights the need for alternative sources for PUFA production. New sources of DGLA have been obtained through manipulation of fatty acid pathways in yeast, fungi and algae.

The yeast *Saccharomyces cerevisiae* has been genetically manipulated to produce DGLA. *S.cerevisiae* produces oleic acid as a result of its desaturase enzyme, Δ9 desaturase. By genetically introducing Δ5 and Δ6 desaturase enzymes, this allowed the yeast to convert oleic acid to LA and then DGLA (Watanabe *et al*. 2014).

Mortierella alpina is arachidonic acid-producing fungus. A mutant strain was manipulated to be Δ5 desaturase-defective (1S-4). Under optimum conditions, 1S-4 produced 43.9% of its fatty acids as DGLA (Kawashima *et al.* 2000). Alternatively, an algae has also been used as a source of DGLA and was the source employed for some studies in this thesis. A mutant of the phototrophic green microalgae *Parietochloris incisa* has recently been utilised in production of DGLA (Iskandarov *et al.* 2011). *P. incisa* produces large amounts of AA but chemical mutagenesis allowed for a nonsense mutation in the Δ5 desaturase gene (Iskandarov *et al*. 2011). This resulted in a significant increase in percentage of DGLA in total fatty acids (TFA). Wild type algae are comprised of approximately 1% DGLA and 58% AA (TFA) in comparison to mutant whereby percentage of AA was negligible and the proportion of DGLA increased to 32% (Iskandarov *et al*. 2011). DGLA can be isolated through lipid extraction, purified and subsequently used for experimentation.

1.6 Aims of project

Atherosclerosis is the primary cause of CVD, a disease responsible for more deaths annually than any other disease in Western society. The increasingly large burden on the health system to treat the disease is a major economic cost. Statins are commonly used in treatment of atherosclerosis but can only account for a 30% improvement in the disease, with some patients unresponsive to treatments (Mishra and Routray 2003). With CVD events set to rise, new preventative and therapeutic options need to be made available. Atherosclerosis is a chronic inflammatory disorder characterised by lipid accumulation in vascular walls. Macrophages are key in the pathology of the disease given their important role in foam cell formation, an initial step in disease pathology. Targeting foam cell formation in macrophages is therefore a good candidate for therapeutic intervention.

PUFA are important signalling molecules in disease. Signalling through a number of receptors and transcription factors, they play a role in immunity, inflammation and lipogenesis. Omega-3 and -6 fatty acids have been reported to have numerous anti-inflammatory roles in diseases, including atherosclerosis. DGLA, an omega-6 fatty acid has been shown to have an anti-inflammatory and anti-atherosclerotic role in mouse models of the disease (Takai *et al*. 2009). However, the study had various limitations and failed to elucidate any underlying mechanisms. For example, the study provided no indications of the effects of DGLA on several key biological processes in atherosclerosis such as parameters related to foam cell formation. Further studies are therefore required on the molecular mechanisms underlying the anti-inflammatory

and anti-atherogenic actions of DGLA given its promise as a preventative/therapeutic agent. Genetic manipulation of green algae *P. incisa,* has allowed for a new source of DGLA to be made available (Iskandarov *et al*. 2011). Further studies could therefore be undertaken into understanding the molecular basis of the action of DGLA and evaluating the mutant algae as a viable and useful source.

Given the promising initial studies using DGLA supplementation in reducing atherosclerosis, the aim of this study was to evaluate the underlying molecular mechanisms. The experimental aims of the project were designed to investigate if DGLA is a potentially new source of therapeutic value in the prevention of atherosclerosis. The project can be split into 3 main aims.

- Evaluating the uptake and incorporation of pure DGLA into macrophage lipid classes *in vitro* together with its metabolism. In addition, investigate the role of DGLA supplementation on mouse plasma and tissue lipid fractions *in vivo* using DGLA from *P. incisa* algal powder

- Using macrophages, determine the role of DGLA on aspects of foam cell formation, ROS production, monocytic migration, cytokine induced pro-inflammatory gene expression, cytokine release, inflammasome activation, modified LDL uptake, cholesteryl ester accumulation and cholesterol efflux *in vitro.*

- Delineate the molecular mechanism underlying the action of DGLA in foam cell formation and other key processes implicated in atherosclerosis together with the role of its metabolites *in vitro.*

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

A list of the materials used throughout the project and their sources are listed in Table 2.1.

Table 2.1- Details of materials

Supplier	Area	Material
ABCAM	UK	ROS kit; Lactate dehydrogenase (LDH) kit
ABD Serotech	UK	ABCG1 antibody (0650-1904)
Amersham	UK	14C-cholesterol
Amresco	USA	Ribozol™
Biotrend	Germany	acLDL; Dil-oxidised LDL (oxLDL)
Cayman Chemical	USA	PGE_1; PGE_1-d4; 15(S)-hydroxyeicosatrienoic acid (HETrE); 15(S)-hydroxyeicosatetraenoic acid (HETE)-d8
Cell Signalling Technology	USA	Phospho-STAT1 TYR^{701} Rabbit mAB (#91675); Phospho-STAT1 SER^{727} Rabbit mAB (#91775)
Greiner	UK	6/12/24 well tissue culture plates; 10/25 ml stripettes; cell scrapers, 75/175 cm^3 tissue culture flasks; 1 ml Cyro tubes
LabTech	UK	Foetal calf serum, South America origin
Lonza	Switzerland	RPMI media with stable glutamine
NBS Biologicals	UK	RNA spin kit
New England Biolabs	UK	Low molecular weight DNA ladder; Taq DNA polymerase
Novus Biologicals	USA	ABCA1 antibody (NB400-105)
NU-CHEK	US	GC standard (GLC-411); DGLA
Nycomed Pharmaceuticals	Switzerland	Lymphoprep™
Perkin-Elmer	USA	Super polyethylene vials (20 ml); OPTI-FLUOR®; $14C^-$Acetate

Peprotech	UK	Human/mouse IFN-γ, IL-1β, IL-4, TNF-α, MCP-1
Promega	UK	Deoxynucleotide (dNTPs); Molony murine leukemia virus (MMLV) reverse transcriptase; RNasin ribonuclease inhibitor; Random hexamer primers
Qiagen	UK	COX-1 siRNA; COX-2 siRNA
R&D systems	USA	IL-1β ELISA kit
Roche Applied Scientific	USA	Lumi-film chemiluminescent detection film; PhophoSTOP phosphatase inhibitor; Protease inhibitor tablets
Santa Cruz Biotechnologies	USA	Anti-rabbit secondary antibody IgG-AP; anti-goat secondary antibody IgG-AP; COX-1 antibody (Sc-1752); COX-2 antibody (Sc-1745) STAT1 Rabbit mAb (Sc-592); β-actin (Sc-130656); SRA antibody (Sc-20660); CD36 antibody (Sc-9154); ABCG1 antibody (Sc-11150)
Sigma-Aldrich	UK	Phorbol 12-myristate 13-acetate (PMA); Tween 20; SYBR® green; Dimethyl sulphoxide (DMSO); Accuspin tubes; RIPA buffer; phosphate buffer saline (PBS) tablets; tris-borate EDTA (TBE); DNA oligonucleotide primers; Acetic acid; Heptadecanoic acid (internal standard); Ammonium hydroxide; Boric acid; Ethanol; Chloroform; Diethyl ether; Isopropanol; Hexane; Methanol; Sulphuric acid, Formaldehyde 37% (v/v); RAW264.7 cell line; Copper II sulphate; THP-1 cell line; SYBR Green Taq readymix; Ponceau S; Tri sodium citrate, Sodium hydroxide; Sodium chloride; Potassium chloride; Cholesterol; thin layer chromatography plates; Bovine serum albumin (BSA); ApoA1; Crystal violet; Lucifer yellow
Source Bioscience	UK	Polyplus Interferin® transfection reagent

Star labs	UK	96-well PCR plates; PCR plate covers; 0.5 ml Eppendorph tubes; 1.5 ml Eppendorph tubes; Latex diamond gloves; 15/50 ml Falcon tubes
Thermo Fisher Scientific	USA	BCA protein assay kit; Magic marker XP western protein standard; NuPAGE® Novex Bis-Tris Gel 4-12% (w/v), 1.0mm (x10); NuPAGE® transfer buffer (20x); NuPAGE® MOPS SDS Running Buffer; NuPAGE® LDS sample buffer (4x); Negative control siRNA; Agarose powder; penicillin/streptomycin (10,000U); Nuclease free water; GIBCO ® PBS; Gel sample buffer (GSB); I -Block; Potassium dihydrogen orthophosphate; Dipotassium phosphate; Potassium hydroxide; Hydrochloric acid
VWR	UK	8 µM cell inserts; 12 well compatible tissue culture plates for inserts

2.2 Methods

2.2.1 Preparation of glassware and solutions

Glassware and solutions were autoclaved (if necessary) for 20-30 minutes at 121˚C (975kPa).

2.2.2 Cell lines

2.2.2.1 THP-1

THP-1 is an established cell line commonly utilised in the study of atherosclerosis due to its ability to accurately mimic the physiological and biochemical properties of native human monocyte-derived macrophages (Auwerx 1991; Qin 2012). Due to this, THP-1 cells are an excellent model for studying the properties of macrophages such as gene/protein expression and various cell signalling mechanisms. THP-1 cells are non-adherent monocytes which require treatment with Phorbol 12-myristate 13-acetate (PMA) to allow differentiation into macrophages.

2.2.2.2 RAW264.7

RAW264.7 cell line is a mouse macrophage cell line also utilised in *in vitro* studies of the molecular basis of atherosclerosis. The cell line was initially derived from a BALB/14 mouse and can be transfected with high efficiency with exogenous nucleic acids (Hartley *et al.* 2008). RAW264.7 macrophages are adherent to tissue culture flasks and therefore do not require differentiation with phorbol esters.

2.2.3 Maintenance of cell lines

THP-1 and RAW264.7 cells were grown and maintained in RPMI-1640 containing stable glutamine. Media was supplemented with 10 % (v/v) heat-inactivated foetal calf serum (HI-FCS) (56ºC for 30 minutes) along with penicillin (100 U/ml) and streptomycin (100 µg/ml) (pen/strep). Cells were grown at 37˚C in a humidified incubator containing 5% (v/v) CO_2.

2.2.3.1 Sub culturing of cells

2.2.3.1.1 THP-1

THP-1 cells were cultured in 75cm^3 tissue culture flasks and used in experiments when they reached approximately 60% confluence (6 x 10^5 cells/ml). Cell suspension was transferred into a polypropylene tube (Falcon tube) and subjected to centrifugation at 110 x g for 5 minutes at room temperature. After removing the media, the pellet was resuspended in a small volume of fresh media containing 10% (v/v) HI-FCS and pen/strep. Cells were split at a ratio of approximately 1:30 to allow growth

of up to 60% confluence in 7 days. For experimental use, cells between passage 1 and 10 were used.

2.2.3.1.2 RAW264.7

RAW264.7 cells were cultured in tissue culture dishes and used for experiments when they reached about 80% confluence. Adherent RAW264.7 cells were dislodged from the dish surface via scrapping. The cell suspension was transferred into a Falcon tube and subjected to centrifugation at 110 x g for 5 minutes at room temperature. After suspending the pellet in fresh medium containing 10% (v/v) HI-FCS and pen/strep, RAW264.7 cells were split at a ratio of 1:15 to allow cells to reach requisite confluence in approximately 3 days.

2.2.3.2 Freezing down cell lines

The cells were centrifuged at 110 x g for 5 minutes at room temperature and resuspended in HI-FCS with 10% (v/v) DMSO. Cells (density approximately 5 x 10^6) were then transferred into 1ml cryo tubes and placed in a Nalgene™Cryo 1°C freezing container overnight at -80°C. Cell stocks were transferred to liquid nitrogen for long-term storage. Only cells of passage 3 or less were frozen for stock.

2.2.3.3 Growing up frozen cell lines

Cell stocks from liquid nitrogen were defrosted in a 37˚C water bath. Cells were then transferred into 10 ml of HI-FCS and centrifuged at 110 x g for 5 minutes at room temperature. Cell pellet was resuspended in pre-warmed RPMI-1640 with stable glutamine media containing 10% (v/v) HI-FCS and 10% Pen/strep and transferred into a culture flask or dishes as detailed above.

2.2.3 Counting cells

A haemocytometer was used to count cells. After centrifugation as above, the cell pellet was resuspended in a small volume of media containing 10% (v/v) HI-FCS and pen/strep. The haemocytometer was covered with a glass cover slip and 10 µl of the cell suspension applied. An average of the number of cells contained within the four outer grids of the haemocytometer was taken. Number of cells/ml was calculated by multiplying the average number of cells by 10^4.

2.2.4 Treatment of the cells

2.2.4.1 THP-1

For RNA extraction, 5 x 10^5 cells were split into a 12-well plate containing 1ml of complete RPMI media, containing 10% HI-FCS and pen/strep, per well. To allow

differentiation of THP-1 monocytes into adherent macrophages, the medium was supplemented with 0.16 µM PMA. Cells were left to differentiate for 24 hours at 37ºC in a humidified 5% CO_2 (v/v) incubator. The media was then removed and the cells were washed in PBS followed by addition of 1 ml of fresh RPMI media with 10% (v/v) HI-FCS and pen/strep. THP-1 macrophages were next pre-treated with DGLA or DMSO vehicle for 24 hours followed by cytokine or vehicle (water) (concentration used dependent on the cytokine). RNA was then extracted as described in Section 2.2.7.2. For protein analysis by western blotting, 1 x 10^6 cells were seeded in 6 well plates with 2 ml RPMI media containing 10% (v/v) HI-FCS and pen/strep whereas 4 x 10^6 cells were seeded in 6 well plates with 3 ml RPMI media containing 10% (v/v) HI-FCS and pen/strep for lipid analysis. The conditions for differentiation and treatment with the various agents were exactly as detailed above.

2.2.4.2 RAW264.7

For RNA extraction, 2 x 10^5 cells were split into a 12-well plate containing 2 ml of complete RPMI media containing 10% (v/v) HI-FCS and pen/strep. Cells were allowed to grow for 24 hours at 37ºC in a humidified 5% (v/v) CO_2 incubator. The cell media was removed and the cells were washed in PBS and incubated with 2ml of fresh RPMI media with 10% (v/v) HI-FCS and pen/strep. Cells were pre-treated with DGLA or vehicle for 24 hours before cytokine stimulation (incubation with cytokine dependant on individual experiment).

2.2.5 Primary human monocyte-derived macrophages (HMDM)

Human blood buffy coats were obtained from the National Blood Service Wales. White blood cells were isolated using Lymphoprep™. Briefly for 25 ml of blood and 15 ml of Lymphoprep™ at room temperature was placed in 50 ml Accuspin™ tubes and subjected to centrifugation for 1 minute at 1000 x g to place the Lymphoprep™ in the bottom chamber. Then, 25 ml of blood was added to the top chamber and centrifuged at 800 x g for 10 minutes at room temperature, without brake. The mononuclear layer was collected and transferred into a Falcon tube. An equal amount of ice-cold PBS 0.4% (w/v) tri-sodium citrate was added and centrifuged at 1000 x g for 5 minutes at room temperature. To lyse red blood cells, the cell pellet was resuspended in 10 ml of 0.2% (v/v) saline for 30 seconds on ice immediately followed by the addition of 10 ml of 1.6% (v/w) saline. This mixture was immediately centrifuged at 1000 x g for 5 minutes at room temperature. The pellet was then resuspended in ice-cold PBS-0.4% (v/w) tri sodium citrate and centrifuged at 800 x g for 5 minutes at room temperature. This step was repeated 6-10 times to ensure removal of platelets. The cell pellet was then resuspended in RPMI-1640 media

containing 10% (v/v) HI-FCS and pen/strep and seeded into 12 well plates. Each well was made up to 2 ml with complete RPMI containing 10% (v/v) HI-FCS and pen/strep media. Cells were left to differentiate for 10 days before use. Cells were washed three times in RMPI-1640 containing 10% (v/v) HI-FCS and pen/strep to remove any unattached cells/debris before subsequent experiments were carried out.

2.2.6 Cellular assays

2.2.6.1 Crystal violet

THP-1 monocytes were differentiated as previously described (Section 2.2.4.1) and seeded in 96 well plates at a density of 140,000 cells in complete RPMI media containing 10% (v/v) HI-FCS and pen/strep. After incubation as required for the experimental conditions, the cells were washed with PBS and 250 µl of crystal violet solution (0.2% (w/v) in 10% ethanol) was added per well. The cells were washed three times in PBS followed by the addition of 250 µl of solubilisation buffer (0.1M NaH_2PO_4 in 50% (w/v) ethanol). The plate was left for 5 minutes on a rocking platform at room temperature before determining absorbance on a Bio-Rad micro plate reader at 590 nm. Crystal violet binds to DNA of adherent cells, those that have died or detached from the well remain in suspension and will be washed away. The results were expressed as percentage cell death in comparison to the control conditions.

2.2.6.2 Lactate dehydrogenase (LDH) assay

LDH is a cellular cytosolic enzyme. Damage to the plasma membrane of a cell releases LDH into the culture media which can then be directly measured using the LDH assay kit by utilising an enzymatic reaction. Extracellular LDH catalyses a reaction that reduces NAD+ to NADH. Diaphorase utilises NADH to reduce tetrazolium salt (INT) to a red product that can be measured at 490 nm. The concentration of cellular LDH levels is directly proportional to the amount of red product formed. THP-1 macrophages were cultured and subjected to the experimental conditions (Section 2.2.4.1). Cell lysis buffer was added to 'positive control' cells to achieve 100% cellular lysis and left to incubate at 37ºC for 45 minutes. A negative control was also included containing media alone for a background reading. For other samples, 50 µl of supernatant was transferred into a separate well of a 96 well plate and mixed with an equal volume of LDH assay buffer (supplied with the kit), left for a further 30 minutes at 37ºC and absorbance read immediately at 490 nm on a Dynex Technology MRX Model 680 Microplate reader from BioRad. Negative control values were subtracted from readings and expressed as fold-change in comparison to the control.

2.2.6.3 ROS production

Measurement of ROS production was performed as per manufacturer instructions (AbCAM). The kit utilises fluorogenic dye dichlorofluorescin diacetate (DCFDA) to measure ROS activity within the cell. Briefly, monocytes or macrophages were incubated with 35 µM DCFDA which diffuses into cells and is deacetylated by cellular esterases to a non-fluorescent compound, later oxidised by ROS to a fluorescent compound 2',7, dichlorofluorescein (DCF). A positive control supplied by the kit, tert-butyl hydrogen peroxide (TBHP), was used to induce ROS production by monocytes and macrophages. Monocytes and macrophages were co- or pre-incubated with 50 µM DGLA followed by incubation with 100 µM TBHP for 3 hours. DCF was measured by fluorescence spectroscopy with excitation and emission spectra of 495 nm and 529 nm respectively.

2.2.7 RNA/DNA techniques

2.2.7.1 siRNA

siRNA transfections were carried out using validated siRNAs against target mRNAs. The transfection was performed in THP-1 monocytes prior to differentiation. The cells were seeded into 12-well plates for RT-qPCR (5 x 10^5 cells) and into 6 well plates for western blotting (1 x 10^6) in the presence of RPMI media with 10% (v/v) HI-FCS and no antibiotics at 37°C in a humidified, 5% (v/v) CO_2 incubator for 4 hours prior to transfection.

siRNA transfection were carried out using Interferin® transfection reagent. For RT-qPCR, a transfection mix was prepared in 100 µl of minimal RPMI media (no HI-FCS or pen/strep) containing 7.5 nM siRNA (of each individual siRNA used) and 9 µl interferin. For western blotting, 7.5 nM siRNA and 12 µl interferin were made up to final volume of 200 µl in minimal RPMI media (no HI-FCS or pen/strep). The complete mixture was added drop wise to the wells and the plates placed at 37°C in a humidified, 5% (v/v) CO_2 incubator for 4 hours. Following incubation, the cells were differentiated using 0.16 µM PMA for 24 hours. The cells were then subjected to pre-incubation with DGLA or vehicle followed by cytokine stimulation (Section 2.2.4.1) before being harvested for RNA extraction (Section 2.2.7.2) or protein extraction (Section 2.2.9).

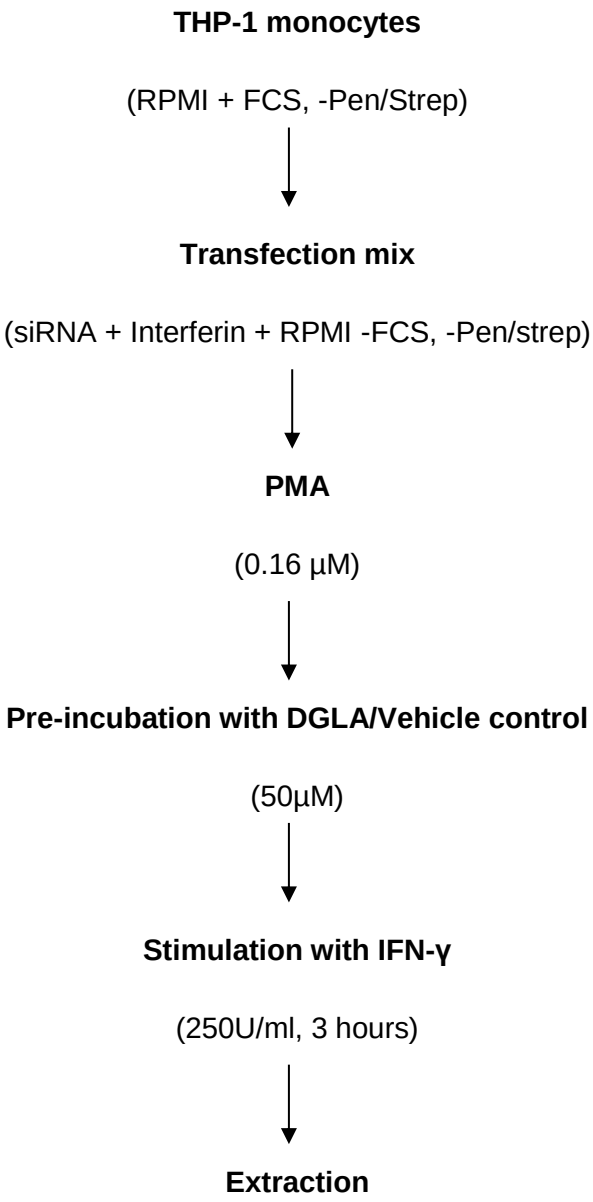


Figure 2.1 – siRNA transfections

Diagram depicts the process of siRNA transfection

2.2.7.2 RNA isolation

For extraction of RNA, two methods were used. Initially RNA was isolated using Spin column RNA miniprep kit (NBS Biologicals) according to the manufacturer's instructions. Briefly, after aspiration of the media, 350 µl of Lysis Buffer DR (provided in the kit) was added to the wells and the lysates were collected. The lysate could be stored at -80˚C for up to 6 months or used immediately in RNA extraction. The lysate was passed through a series of spin columns provided in the kit to first bind genomic

DNA, then RNA and finally to elute high quality RNA. A flow diagram detailing experimental procedure is detailed in Figure 2.2.

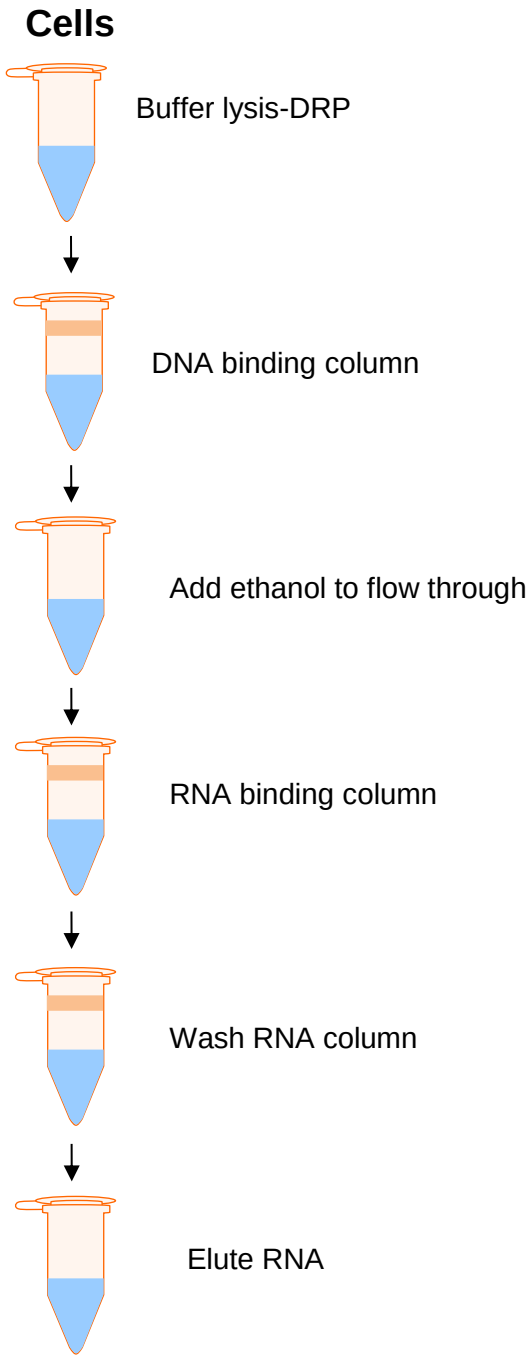


Figure 2.2 – Spin column RNA isolation method

Flow diagram detailing the steps involved in extraction of RNA using Spin column minikit (NBS biologicals)

Due to the high costs of spin column kits, RNA was then isolated using RiboZol™ according to the manufacturer's instructions (Amersco, UK). In addition to the cost, extraction of RNA using RiboZol™ allows for simultaneous analysis of expression of miRNA as well as mRNA as miRNA are lost during RNA extraction using spin columns. RiboZol™ is a single-phase phenol which homogenises samples after passing lysate through a pipette a number of times and inhibits RNase activity. RiboZol™ (1 ml) was added per 10cm^2 of culture dish area and cells were lysed by pipetting. Samples were left for 10-15 minutes at room temperature to allow complete dissociation of nucleoprotein complexes. Then, 200 µl of chloroform per 1ml of RiboZol™ was added and mixed vigorously. The samples were incubated for 2-3 minutes at room temperature and subjected to centrifugation at 12,000 x g for 15 minutes at 4ºC. Following centrifugation, the colourless upper aqueous phase containing RNA was transferred into a new tube. The RNA was precipitated by adding 0.5 ml of isopropanol per 1 ml of RiboZol™. The samples were incubated for 10 minutes at room temperature and then centrifuged at 12,000 x g for 10 minutes at 4ºC to pellet the RNA. The pellet was washed three times with 75% (v/v) ethanol with the residual ethanol evaporated at room temperature after the final wash. The RNA was dissolved in nuclease free water and incubated for 10 minutes at 56ºC to completely re-dissolve the RNA.

The RNA concentration was measured on NanoDrop™ ND2000. The A_{260}/A_{280} and A_{230}/A_{260} ratios were used to assess RNA purity. A ratio of 1.8 – 2.1 for both was indicative of high quality RNA. In addition, a small aliquot was analysed by agarose gel electrophoresis.

2.2.7.3 Resolving RNA

RNA extracts were size-fractionated on a 1.5% (w/v) agarose gel as an additional check on the quality of RNA. Agarose gels 1.5% (w/v) were made by dissolving agarose in 1 x tris/borate/EDTA (TBE) buffer with the addition of 0.5 µg/ml of ethidium bromide for visualisation. Quality of RNA was judged as good when two distinct bands were observable, the band corresponding to the 28S rRNA being twice as intense as that for 18S rRNA.

2.2.8 Gene expression analysis

2.2.8.1 cDNA synthesis (SYBR green)

RNA (typically 1 µg or 0.5 µg if the RNA yield was low) was mixed with 200 pmol of random hexamer primers and nuclease-free water to make a total volume of 14 µl. The mixture was then incubated at 70˚C for 5 minutes followed by a 5-minute period on ice. The following mixture was prepared and added to the reaction to make a final volume of 20 µl

- 10 mM of each dNTPs (dATP, dGTP,dTTP,dCTP);
- 200 U M-MMLV reverse transcriptase;
- 40 U/ml recombinant RNase inhibitor;
- 5X reverse transcription buffer (50 mM Tris-HCl (pH 8.3 @ 25°C), 75 mM KCl, 3 mM MgCl2 and 10 mM DTT)

A –RT sample (minus reverse transcriptase) was included in reaction as a negative control. The final reaction mix was incubated at 37˚C for 1 hour, followed by a termination period at 95˚C for 5 minutes. The cDNA was diluted with nuclease-free water to a final volume of 100 µl (or 50 µl if 0.5 µg of RNA was originally used). The samples were used immediately for real time quantitative polymerase chain reaction (RT-qPCR) or stored at -20˚C.

2.2.8.2 Real time quantitative (RT –qPCR)

RT-qPCR is a method whereby the amounts of PCR products are measured with each passing amplification cycle. Values are recorded once a pre-set threshold, in the exponential phase of amplification, has been exceeded. This is opposed to end point quantification RT-PCR where measurement endpoints could lie out of the exponential phase of the PCR reaction. This means that in some cases the semi-quantification of the starting template could be inaccurate (Ginzinger 2002). The RT-qPCR reactions were performed with SYBR Green Taq Readymix. SYBR Green Jumpstart Taq ReadyMix allows for quantification of any DNA sequence by binding to double stranded DNA sequences (Giulietti *et al.* 2001). SYBR green is a dye that intercalates into double stranded DNA (dsDNA) and fluoresces when bound to DNA thereby allowing monitoring of product throughout cycling. Although any dsDNA molecule can be bound by SYBR green, use of specific primers and optimised amplification conditions can prevent any formation of primer dimers and yield maximal product production (Giulietti *et al*. 2001). This can be observed through analysis of

product melting curves (product formed over time) to verify amplification of a major single product. Gene specific primer sequences are listed in Table 2.2. To ensure no amplification of genomic DNA, intron spanning primers were designed. A hot start mechanism via the inclusion of Jumpstart Taq in the readymix allows for PCR reactions to be performed at room temperature as the reaction starts at an optimum temperature and therefore prevention of non-specific product formation. The SYBR green ready mix also contains deoxyribonucleotides and reaction buffer which saves a significant amount of preparation time when performing large-scale experiments. RT-qPCR samples were prepared as 25 µl reactions in a 96-well plate. Reaction mix is detailed in Table 2.3.

Table 2.2 – Primer sequences used in RT-qPCR

	Species	Forward sequence (5'-3')	Reverse Sequence (5'-3')
GAPDH	Human	CTTTTGCGTCGCCAGCCGAG	GCCCAATACGACCAAATCCGTTGACT
MCP-1	Human	CGCTCAGCCAGATGCAATCAATG	ATGGTCTTGAAGATCACAGCTTCTTTGG
MCP-1	Mouse	GCTCAGCCAGATGCAGTTAACG	GCTTGGTGACAAAAACTACAGCTTC
ICAM-1	Human	GACCAGAGGTTGAACCCCAC	GCGCCGGAAAGCTGTAGAT
ICAM-1	Mouse	ACGTGCTGTATGGTCCTCGG	GTCCAGTTATTTTGAGAGTGGTACAGTA
β Actin	Mouse	ACACCCGCCACCAGTTCGCCAT	CACACCCTGGTGCCTAGGGCGGCCCACGATC
COX-1	Human	CTGGTTCTTGCTGTTCCTGC	ATAAGGTTGGAGCGCACTGT
COX-2	Human	TCAGACAGCAAAGCCTACCC	TGTGTTTGGAGTGGGTTTCA
SRA	Human	GTCCAATAGGTCCTCCGGGT	CCCACCGACCAGTCGAAC
CD36	Human	AGCCATTTTAAAGATAGCTTTCC	AAGCTCTGGTTCTTATTCACA
ACAT1	Human	ATACTCAGCCCTCTGCGACC	TCTTATTTCCTGCACCAGCCT
NCEH	Human	CCTGGTCACCTTCAGATGAAAT	TTGTGGCCCGTACAACATCA
LXR-α	Human	CCTTCAGAACCCACAGAGATCC	ACGCTGCATAGCTCGTTCC
LXR-β	Human	GCTAACAGCGGCTCAAGAACT	GGAGCGTTTGTTGCACTGC
ABCA1	Human	AGTGGAAACAGTTAATGACCAG	GCAGCTGACATGTTTGTCTTC
ABCG1	Human	GGTGGACGAAGAAAGGATACAAGACC	ATGCCCGTCTCCCTGTATCCA

Table 2.3 – Reaction mix for RT-qPCR using SYBR® Green

Reagent	Volume (µl)
SYBR® Green	12.5
Forward primer (100 µM)	0.5
Reverse primer (100 µM)	0.5
cDNA	1
Nuclease free water	10.5

RT-qPCR was performed on a Roche light cycler. A typical amplification cycle consisted of 2 minutes pre-incubation at 94ºC followed by 40 cycles of three-step amplification detailed in Table 2.4, followed by melting peak analysis.

Table 2.4 – RT-qPCR conditions using SYBR® Green

Stage	Temperature (ºC)	Duration (seconds)
Denaturation	95	30
Annealing	60	60
Primer extension	72	60

The comparative Ct method was used to measure gene expression (Livak and Schmittgen 2001). The Ct value is measured when the fluorescence signal from PCR amplification surpasses a pre-set threshold within the amplification stage. Relative expression of genes of interest were normalised to that of housekeeping genes (Livak and Schmittgen 2001). In order for the Ct method to yield accurate values, the expression of housekeeping genes must remain constant under all experimental

conditions and the efficiency of primer sets used must be comparable (Ginzinger 2002). Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and β-actin were used as the house keeping genes for human and mouse RNA respectively. Both genes have frequently been utilised for this role in other studies (Wu *et al.* 1997; Li *et al*. 2010; McLaren *et al*. 2011b). Once the PCR reaction was completed, Ct values were recorded and analysed in Microsoft Excel.

2.2.9 Western blotting

2.2.9.1 Protein extraction

For extraction of proteins for western blotting, cells were washed in PBS and RIPA buffer (Sigma) was added to the wells. The cells were detached with a cell scraper into the buffer and collected in Eppendorf tubes. The cells were subjected to centrifugation at 9,000 x g at room temperature for 5 minutes to pellet the cells. The supernatant was collected in a new Eppendorf tube and a small volume was removed for determination of protein concentration. For separation of proteins by polyacrylamide gel electrophoresis (PAGE), the proteins need to be denatured and the disulphide bonds reduced. This was achieved by adding an equal volume of gel sample buffer (GSB) containing sodium dodecyl sulphate (SDS). Samples were heated for 5 minutes at 100^{o}C and placed on ice before loading onto the gels. For compositions of reagents used in western blotting refer to Table 2.5.

2.2.9.2 Determining protein concentration

Total protein concentrations were determined using Micro BCA^{TM} Protein assay Kit in accordance to the manufacturer's instructions (Thermo Fisher Scientific). Briefly, a standard curve was prepared by setting up a series of dilutions of bovine serum albumin (BSA) and plated in a 96 well plate along with protein samples diluted in nuclease free water. The BCA cocktail (containing reagents provided in the kit) was prepared following the manufacturer's instructions and 150 µl was added to 150 µl of samples and standards. Plate was sealed and left at 37ºC for 30 minutes to allow colour to develop. Absorbance was read at 595 nm on a Dynex Technology MRX Model 680 Microplate reader from BioRad. A standard curve was compiled and unknown protein concentrations determined from this.

2.2.9.3 Separation of proteins

Separation of proteins was performed in XCell *Surelock*® Mini-Cell on NuPAGE® 4-12% Bis-Tris gels. These pre-cast gels allow for optimum separation of small to medium sized proteins in a neutral pH environment. The electrophoresis tank was

filled with NuPAGE® MOPS SDS Running buffer (1X). Magic Marker was used as a marker to determine size of proteins on membranes. Samples were subjected to electrophoresis for 1 hour at 150V and 400mA. The composition of all the reagents used for the western blotting techniques are shown in Table 2.5.

Table 2.5 – Composition of buffers used in western blotting

Reagent	Composition
RIPA buffer	150 mM NaCl, 1% (v/v) IGEPAL® CA-630, 0.5% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris pH 8.0
GSB	63 mM 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	50 mM MOPS, 50 mM Tris Base, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.7
NuPAGE®Transfer buffer	500 mM Bicine, 500 mM Bis-Tris, 20.5 mM EDTA
PBS Tween	1L ddH_20, 10 PBS Tablets, 2 ml Tween20
IBT blocking solution	0.5% (w/v) I BLOCK in PBS Tween
Ponceau S	0.1% (w/v) Ponceau S, 5% (v/v) acetic acid, ddH_20

2.2.9.4 Blotting

Following electrophoresis, the gel was removed from its casing and the proteins were transferred onto an Immobilon™ transfer membrane using XCell *Surelock*® Mini-Cell via XCell II™ Blot Module. Assembly of the transfer unit was performed in NuPAGE®Transfer buffer which was later used to “top up” the inner chamber. Assembly of transfer is detailed in Figure 2.3. NuPAGE®gel was placed adjacent to membrane (after activation in 100% methanol for 1 minute at room temperature) between Whatman paper and sponge pads soaked in transfer buffer. Transfer was performed at 30V and 300mA for 2 hours.

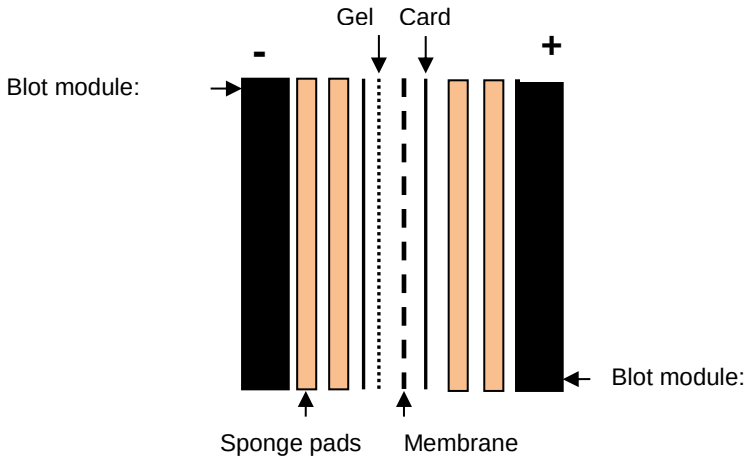


Figure 2.3 – Configuration of transfer module

Transfer membrane and gel were enclosed between Whatman papers. Sponge pads were used to package the remaining space in the module to ensure membrane and gel remained in constant contact throughout the transfer process.

2.2.9.5 Immunodetection of proteins

After the transfer process was complete, proteins could be visualised on the membrane by staining with Ponceau S to ensure they had successfully transferred onto the membrane. Following visualisation with Ponceau S, the membrane was washed in PBS-tween until all the stain had been removed. Membrane was then "blocked" in IBT solution for 1 hour at room temperature with constant shaking, to prevent any non-specific interactions with antibodies. Following this, the membrane was treated with a primary antibody for 1 hour at room temperature or overnight at 4^0C (refer to Table 2.6 for antibody dilutions and incubation conditions). After incubation with a primary antibody, the membrane was washed three times in PBS-Tween at room temperature with constant shaking. The membrane was next treated with a corresponding alkaline phosphate (AP)-conjugated secondary antibody diluted in IBT solution for 1 hour at room temperature, with constant shaking. The membrane was washed again as above and incubated for 30 minutes with Tropix®CDP Star®. Following this, the membrane was secured into a lightproof cassette (Kodak). Membranes were exposed to chemiluminescent X-ray films. A number of time points for exposure of membrane to X-ray film were taken to allow optimum exposure to be achieved. Exposure time varied between 5 minutes to overnight.

Table 2.6 – Antibodies and conditions used in western blot analysis

Primary antibody	Dilution in IBT	Incubation time (hours)	Secondary antibody	Dilution in IBT	Size (kDa)
Total STAT1	1/1000	1 hour	Rabbit	1:5000	91
Phospho STAT1 Ser^{727}	1/1000	Overnight	Rabbit	1:5000	91
Phospho STAT1 Tyr^{701}	1/1000	Overnight	Rabbit	1:5000	91
β-actin	1/5000	1 hour	Mouse	1:10,000	42
COX-1	1:500	Overnight	Goat	1:2000	72
COX-2	1:250	Overnight	Goat	1:2000	70-72
SRA	1:1000	Overnight	Rabbit	1:5000	75
CD36	1:250	Overnight	Rabbit	1:3000	88
ABCA1	1:1000	Overnight	Rabbit	1:5000	220
ABCG1	1:2000	Overnight	Goat	1:5000	110
ApoE	1:2000	Overnight	Goat	1:5000	38

2.2.10 Inflammasome activation

2.2.10.1 Cholesterol crystals

Cholesterol was dissolved in 95% (v/v) ethanol (12.5 g/L) and heated to 60ºC. Solution was filtered through Whatman paper to remove any undissolved cholesterol and left at room temperature overnight to allow for crystallisation. Crystals were collected and autoclaved. Crystals were ground with a dounce homogenizer to unify crystal size and stored at -20ºC for use in experiments.

2.2.10.2 Enzyme linked immunosorbent assay (ELISA)

An ELISA was used to determine the concentration of IL-1β expressed on stimulation of macrophages with cholesterol crystals to activate the inflammasome. THP-1

macrophages were cultured in 24 well plates at a density of 1.5 x 10^5 cells per well in RMPI media containing 0.2% (w/v) fatty acid free BSA. Following differentiation, macrophages were pre-treated with 50 µM DGLA for 24 hours followed by 1.0 mg/ml cholesterol crystals. Media was removed into Eppendorf tubes and subjected to centrifugation at 9,000 x g for 5 minutes to remove any cholesterol crystals in suspension. Supernatant was then measured for concentration of IL-1β by ELISA following manufacturer's instructions (R&D systems). Briefly, ELISA plates were coated with 4.0 µg/ml human IL-1β capture antibody by overnight incubation. Plates were washed 3 times with wash buffer (provided in kit) and incubated with reagent diluent (provided in kit) for 2 hours. Washing of plates was repeated. Samples were diluted in reagent diluent and standards were made up in concentrations ranging from 0 to 250 pg/ml with IL-1β standards. Samples and standards were added to plates and left for 2 hours. Washing of plates was repeated. IL-1β biotinylated antibody (200 ng/ml) was then added to plates for two hours followed by another wash as above. Streptavidin-HRP was incubated on plates for 20 minutes, washed and substrate solution (provided in kit) was added for a further 20 minutes. Finally Stop solution (provided in kit) was added and optical density of plates was measured on a microplate reader at 450 nm with correction at 540 nm. A standard curve was compiled and unknown IL-1β concentrations determined from this.

2.2.11 Migration assay

Migration was performed using a cell insert with 8 µm pore size placed within a 12 well companion plate (modified Boyden chamber). THP-1 monocytes (5x10^5 cells) were suspended in RMPI media supplemented with 10% (v/v) FCS, pen/strep and DGLA or vehicle control and placed on top of the cell culture insert. In the bottom chamber, 0.5 ml of RMPI media supplemented with 10% (v/v) FCS, pen/strep, DGLA or vehicle control and MCP-1 (20 ng/ml) or vehicle control (refer to Figure 2.4 for experimental set up). Incubation of the modified Boyden chamber was then carried out at 37ºC for 3 hours. The remaining cell suspension on the top of the membrane was removed and discarded, and the underside of the membrane was washed with PBS to ensure all migrated cells were washed into the media underneath. The remaining media was transferred into Falcon tubes and subjected to centrifugation at 110 x g for 5 minutes at room temperature to pellet the cells. The cell pellet was resuspended in 1ml PBS and cells were counted using a haemocytometer. Results were expressed as percentage migration in relation to total input cells.

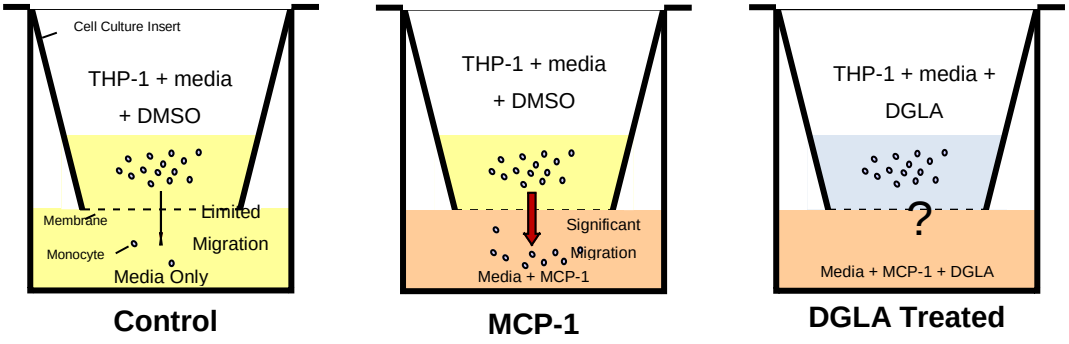


Figure 2.4 – Experimental set up for migration assays

Cell insert with 8 µm pores were used to mimic endothelial layer of arteries. Monocytes were placed in the top chamber and MCP-1 was used as a chemoattractant to stimulate migration.

2.2.12 FACS analysis

2.2.12.1 Macropinocytosis

THP-1 macrophages were pre-incubated with 50 µM DGLA for 24 hours followed by 100 µg/ml Lucifer yellow (LY) in RPMI medium supplemented with 0.2% (v/v) fatty acid free BSA. Overlying media was discarded and cells removed by treatment with 0.05% (v/v) trypsin. Cells were collected in Eppendorphs and subjected to centrifugation at 9,000 x g for 5 minutes to pellet the cells. The supernatant was removed and the pellet was resuspended in 2% paraformaldehyde (PFA). LY incorporation was analysed by flow cytometry on a BD FACS Canto flow cytometer. At least 10,000 events were counted for each sample.

2.2.12.2 Dil-oxLDL uptake

THP-1 macrophages were pre-incubated with 50 µM DGLA for 24 hours followed by 5 µg/ml Dil-oxLDL in RPMI medium supplemented with 0.2% (v/v) fatty acid free BSA. Overlying media was discarded and cells removed by treatment with 0.05% (v/v) trypsin. Cells were collected in Eppendorphs and subjected to centrifugation at 9,000 x g for 5 minutes to pellet the cells. The supernatant was removed and the pellet was resuspended in 2% PFA. Dil-oxLDL uptake was analysed by flow cytometry on a BD FACS Canto flow cytometer. At least 10,000 events were counted for each sample.

2.2.13 Extraction of lipids from algal powder

Freeze-dried powder of a green algae *P. incisa* was obtained from Ben-Gurion University (Israel) and lipids containing up to 30% of DGLA were extracted according to Iskandarov (2011), with minor modifications. 50 mg of biomass was weighed and mixed with 0.2 ml 100% (v/v) DMSO by vortexing followed by incubation for 10 minutes at 80ºC. Methanol (6 ml) was then added, flushed with nitrogen and the incubation continued for 1 hour at 4ºC with continuous vortexing. The samples were centrifuged at 500 x g for 5 minutes and methanol fraction containing polar lipids was collected. The solvent was evaporated and lipids stored in glass vials at -20ºC. Extraction of non-polar lipids from the remaining pellet was performed by adding 0.2 ml DMSO followed by 12 ml 100% (v/v) hexane: diethyl ether (1:1, v/v). Phase separation was achieved via adding 6 ml of dH_2O and lipid-containing layer was collected. HCl (0.2M) was added until pH 3 was achieved and re-extraction was carried out once again using hexane: diethyl ether (1:1, v/v). Solvents were evaporated under a steam of nitrogen; lipids were combined, resuspended into known volumes of chloroform and stored at -20ºC prior to further analysis.

2.2.13.1. Hydrolysis of algal total lipids

Aliquots of total lipid extracts were taken for hydrolyses of algal lipids in order to cleave fatty acids (including about 30% DGLA) from the complex lipids. This was necessary for comparison of the effects of pure DGLA and DGLA-enriched fatty acid mixture from algae on macrophages. The lipid extract was refluxed with 1M solution of KOH in 95% ethanol (5 ml) for 1 hour. After cooling, water (7 ml) was added, the aqueous layer was acidified with 6M HCl and extracted with hexane: diethyl ether (1:1, v/v; 2 x 5 ml). The solvent was evaporated under a stream of nitrogen and NEFAs were re-dissolved in DMSO for the further use in the cell treatment experiments.

2.2.14 Animal feeding

In vivo studies were employed to investigate the use of lipids extracted from *P. incisa* as a viable source of DGLA supplement for rodents. Since our preliminary experiment, when freeze-dried form of algae was used, did not show any detectable DGLA in mouse plasma, lipids extracted from the powder as detailed in Section 2.2.13 were employed. Twelve week old male C57BL/6 mice were starved for 24 hours prior to being given either chow control diet or a diet containing lipids extracted from the algal powder (this diet had 4.4% (w/v) DGLA as the final relative concentration), for 48 hours. Mice were sacrificed using schedule 1 procedures and samples of plasma

together with liver, kidney and adipose tissue were taken for lipid analysis. Lipids were extracted and analysed as in Section 2.2.15.

2.2.15 Lipid analysis

2.2.15.1 Lipid extraction from macrophages and plasma

THP-1 cells (4 x 10^6 per well) were cultured as previously described (Section 2.2.4.1) in 6 well plates. Following experimental treatment, media was aspirated and the cells were washed and scraped from the wells using PBS. The cell suspension was transferred into plastic Eppendorf tubes and subjected to centrifugation at 9000 x g for 5 minutes to pellet the cells. The supernatant was removed, the pellet resuspended in 1ml of distilled water and transferred into a glass tube. Next, 2 ml of chloroform: methanol (2:1, v/v) solution was added to glass tube, vortexed and incubated for 15 minutes at room temperature. Then, 1ml of chloroform and 2 ml of Garbus solution (2M KCl in 0.5M potassium phosphate buffer pH 7.6) (Garbus *et al.* 1963) was added, vortexed and centrifuged at 220 x g for 3 minutes at room temperature to allow separation of the chloroform and the aqueous layers. The chloroform layer was removed into clean glass conical tube with a glass pipette, evaporated under nitrogen and reconstituted in 50 µl of chloroform.

2.2.15.2 Lipid extraction from plasma

After sacrifice of the animals using schedule 1 procedures, blood was collected from mice and centrifuged at 9,000 x g for 5 minutes at room temperature. Plasma fraction was removed into a clean glass tube and 2 ml of chloroform: methanol (2:1, v/v) solution was added. Lipids were extracted in an identical way to that previously described in Section 2.2.15.1.

2.2.15.3 Lipid extraction from mouse tissue

Samples of liver, adipose tissue and kidney were taken from mice for lipid and fatty acid analysis. Tissues were ground with a pestle and mortar in chloroform: methanol (2:1, v/v). Homogenised tissue was passed through glass wool to filter into a glass tube and a further 2 ml of chloroform: methanol (2:1, v/v) was added. Again, lipids were extracted in an identical way to that previously described in Section 2.2.15.1.

2.2.15.4 Thin layer chromatography (TLC)

To separate individual lipid classes, TLC was performed on 10 x 10 cm silica gel G Merck plates using different types of separation. One-dimensional separation was performed using hexane: diethyl ether; acetic acid (80:20:1, v/v/v). This allowed separation of the following lipid classes: TPL, TAG, FFA, free cholesterol and

cholesteryl esters. Polar lipids with PC and PE as two major classes were separated using boric acid impregnated plates (0.2% boric acid in water: ethanol (1:2, v/v)). Chloroform: methanol: ammonium hydroxide (65:25:4, v/v/v) was used as a solvent mixture for the 1st dimension and n-butanol: acetic acid: water (90:20:20, v/v/v) for the 2nd dimension. After drying, the plates were sprayed with a 0.05% solution of 8-anilino-4-naphthosulphonic acid (ANSA) in methanol which allows for visualisation of lipids under UV light. The position of the separated lipid fractions were identified and marked on the plate. Identification was made routinely by reference to authentic standards and confirmed using specific colour reagents (Kates 1986). The silica gel covering the plates contained within identified area was scraped. The separated spots/bands were used for further fatty acid analysis as described in the following Section.

2.2.15.5 Fatty acid analysis

Aliquots of the total lipid extracts and hydrolysed lipids from algae as well as individual lipid classes separated by TLC were used for fatty acid methyl esters (FAMEs) preparation. FAMEs were prepared using a solution of 2.5% H_2SO_4 in dry methanol: toluene (2:1, v/v). A known amount of an internal standard, heptadecanoic acid (C17:0), was added to each sample for fatty acid and lipid quantification. Samples were heated to 70^0C for 2 hours, with occasional vortexing. FAMEs were then extracted by addition of 2 ml of 5% NaCl followed by 2 additions of 3 ml of hexane (HPLC grade). The hexane fractions were transferred into clean glass tubes, evaporated under a stream of nitrogen and reconstituted in 50 µl of hexane.

2.2.15.6 Gas chromatography (GC)

FAMEs for each individual lipid fraction were analysed using a Clarus 500 GC with a flame ionisation detector (Perkin-Elmer, Norwalk, Connecticut) and equipped with 30m by 0.25mm id capillary column (Elite 225, Perkin-Elmer, Waltham, MA, USA). Samples were heated to 170^{o}C for 3 minutes, heated to 220^{o}C (at a rate of 4^{o}C per minute) and held for 30 minutes. FAMEs were identified by comparison with retention times of known standards.

2.2.15.7 Preparation of samples for HPLC-MS

Hexane: isopropanol: 1M acetic acid (30:20:2) (v/v/v) was added to media collected from 4 x 10^6 cells in a ratio of 2.5 ml of solvent mixture to 1 ml of media. An internal standard containing 10 ng dimethylphosphinoethane (DMPE) and 5 ng of PGE_2d4, 5ng of 15-HETEd8 and d4-PGE_1 was also included. Samples were thoroughly mixed by vortexing and 2.5 ml hexane was added followed by further vortexing. Samples

were subjected to centrifugation (900 x g for 5 minutes at room temperature) and the upper hexane layer transferred into a new glass tube. The hexane extraction was repeated as above. Samples were then evaporated under a stream of nitrogen and reconstituted in 200 µl of 100% HPLC grade methanol. Lipid extracts were separated on a Spherisorb C_{18} ODS2, 5 µM, 150 mm x 4.6 mm column (Waters Ltd) using a gradient of 50 – 90% mobile phase B (A: water: acetonitrile: acetic acid, 75:25:0.1 (v/v/v), B: methanol; acetonitrile; acetic acid, 60:40:0.1 (v/v/v/)) over 20 minutes with a flow rate of 1 ml/min. Products were quantified by LC-MS/MS electrospray ionisation on a Q-Trap (Applied Biosystems 4000 Q-Trap) with specific multiple reaction monitoring (MRM) transitions [M-H^-] monitored as parent fragmenting to daughter with collisions energies of -20 to -28V.

2.2.16 Cholesterol uptake and efflux assays

2.2.16.1 Cholesterol uptake assay

RAW264.7 macrophages were cultured in 6 well plates at a density of 2 x 10^6 per well in RPMI supplemented with 0.2% (v/v) fatty acid free BSA. Macrophages were pre-treated with 100 µM DGLA or vehicle for 24 hours and then incubated in the absence or the presence of 50 µg/ml AcLDL and 1µCi [^{14}C] acetate for a further 24 hours. The cells were scraped from the wells and collected in Eppendorf tubes. The cells were pelleted by centrifugation at 9,000 x g and resuspended in 1ml dH_20. A sample was removed for protein analysis as previous described in Section 2.2.9.2. Lipids were extracted and separated as previously described in 2.2.15.1 and 2.2.15.4.

2.2.16.2 Cholesterol efflux assay

THP-1 macrophages were cultured in 12 well plates at a density of 5 x 10^5 per well in RPMI supplemented with 0.2% (v/v) fatty acid free BSA. Following differentiation, macrophages were pre-incubated with 50 µM DGLA or vehicle for 24 hours. Next, media was replaced with RPMI supplemented with 0.2% (v/v) fatty acid free BSA, 25 µg acLDL and 0.5 µCi/ml [4-^{14}C] cholesterol. After 24 hours cells were washed and media replaced with medium containing 10 µg/ml ApoA1 for a further 24 hours. The media fraction was removed into glass test tubes for use in scintillation counting. The remaining cells were treated with 0.2% NaOH to solubilise cells and again removed into glass tubes for use in scintillation counting (Section 2.2.16.3).

2.2.16.3 Preparation of samples for scintillation counting

Samples collected for analysis were placed into 20 ml polyethylene vials with 10 ml OPTI-FLUOR®. Samples were counted on a Liquid scintillation analyser (TriCarb 2800TR, Perkin Elmer) and recorded using Quanta Smart. Disintegrations per minute (dpm) were measured and transferred into Microsoft Excel for analysis.

2.2.17 Statistical analysis

Data was tested for normality using the Shapiro-Wilko test and data represented as mean +/- standard deviation. A two-tailed Student's t-test was carried out using a spreadsheet package for single comparisons. For multiple comparisons with equal variances between groups, ANOVA was carried out in SPSS followed by Tukey's post hoc analysis. For multiple comparisons with unequal variances, Welch's test for equality of means was used followed by Games Howell/Dunnett's T3 Post Hoc analysis in SPSS. P-value of <0.05 was considered significant.

CHAPTER 3

UPTAKE AND METABOLISM OF DGLA IN *IN VITRO* AND *IN VIVO* MODELS

3.1 Introduction

3.1.1 PUFA uptake

Fatty acids (or their metabolites) are important signalling molecules and are components of glycerophospholipids of membranes and TAG stores. The fatty acid compositions of phospholipids play important roles and can influence a number or processes in different ways (Raphael and Sordillo 2013). For example, fluidity of membranes and lipid raft formation are influenced by fatty acids and can modify membrane induced signalling (Raphael and Sordillo 2013). In addition, when cleaved, NEFAs can directly, or indirectly through their metabolites, trigger a signalling cascade. Fatty acid signalling has been shown to play an important role in an inflammatory response (Foitzik *et al.* 2002; Simopoulos 2008; Jung *et al.* 2012; Enos *et al.* 2013; Yan *et al.* 2013; Williams-Bey *et al.* 2014). They have numerous roles, some pro-inflammatory while others produce anti-inflammatory actions. Table 1.5 summarises the roles of fatty acids in relation to CVD. Classically, SFA have been linked to an increased risk of CVD while PUFA are inversely associated with the disease. The composition of fatty acids in lipid pools is therefore very important as to the effect on inflammation. Dietary supplementation with omega-3 and omega-6 PUFAs increases their incorporation into lipid fractions resulting in an increase in their signalling responses and metabolite production (Chilton *et al.* 1993; Johnson *et al.* 1997).

Dietary omega-3 PUFAs have been used in numerous studies to determine uptake *in vivo* and *in vitro*. Supplementation with EPA rapidly induces incorporation into the phospholipid and TAG fractions of human neutrophils after 2 and 6 days respectively (Chilton *et al*. 1993). The omega-3 fatty acids EPA and DHA significantly increase their incorporation into lipid fractions of human serum following supplementation (Grimsgaard *et al.* 1997). The beneficial effects of supplementation on inflammation and CVD with EPA and DHA have been widely reported (Foitzik *et al*. 2002; Kris-Etherton *et al*. 2003; Suresh and Das 2003a; Lundstrom *et al.* 2013; Yan *et al*. 2013; Williams-Bey *et al*. 2014). However, as a result of the increase in omega-3 fatty acids following supplementation, there is a decrease in levels of omega-6 incorporation into

lipid fractions. EPA and DHA reduced the concentration of DGLA and AA in serum phospholipids following supplementation in humans (Grimsgaard *et al*. 1997; Haglund *et al.* 1998; Mori *et al.* 2000). In addition to this, omega-3 fatty acids have been shown to decrease eicosanoid produced by metabolism of DGLA (Rubin and Laposata 1992). Omega-6 fatty acids play important roles in inflammation and have been shown to be beneficial in a number of diseases (Kapoor and Huang 2006; Kawashima *et al*. 2008; Takai *et al*. 2009; Machado *et al*. 2013). Patients with atopic eczema have a reduced concentration of plasma omega-6 fatty acids GLA, DGLA and AA suggesting a role for these PUFA in the disease (Morse *et al.* 1989). Reducing the concentration of omega-6 fatty acids in lipid pools on supplementation with omega-3 fatty acids may therefore have an adverse effect in inflammatory disease states.

Oils rich in the omega-6 fatty acid GLA, such as primrose and borage oil, have been used to treat a number of inflammatory diseases. Supplementation with GLA in combination with EPA and DHA, significantly reduced LDL cholesterol levels, TAG levels and ratio of LDL: HDL cholesterol. This was not observed by EPA and DHA supplementation alone (Laidlaw and Holub 2003). GLA in the form of evening primrose oil has also been shown to significantly reduce the symptoms of atopic eczema including itching, inflammation and dryness (Wright and Burton 1982; Morse *et al*. 1989). Levels of DGLA and AA in plasma phospholipids were associated with an increased improvement of symptoms (Morse *et al*. 1989). Borage seed oil containing GLA improved signs of rheumatoid arthritis in patients diagnosed with the disease (Leventhal *et al.* 1993). Supplementation significantly reduced the number of swollen and tender joints on comparison to placebo control (Leventhal *et al*. 1993). GLA has also been used to target atherosclerosis. High fat diet induced atherosclerosis in rats was attenuated on supplementation with GLA (Shi *et al*. 2008). Also, in $ApoE^{-/-}$ mice, GLA attenuated SMC proliferation and the development of diet-induced atherosclerosis (Fan *et al*. 2001).

Despite these positive results, a number of studies have reported that GLA supplementation in atopic eczema failed to significantly improve symptoms of the disease (Henz *et al.* 1999; Takwale *et al.* 2003). Borage oil containing 23% GLA, a higher dose than previous studies, did not significantly improve symptoms of atopic eczema despite several clinical improvements (Henz *et al*. 1999). Additionally, borage oil supplementation in adults and children failed to improve atopic eczema, with the placebo control showing a larger improvement on symptoms (Takwale *et al*. 2003).

The physiological effects of GLA are thought to be attributed to its metabolites DGLA and PGE_1 (Umeda-Sawada *et al*. 2006). GLA is a precursor of DGLA. Numerous studies have previously used supplementation with GLA to increase plasma and tissue levels of DGLA, due to the lack of a commercial source of this PUFA. A few studies were performed in the 90's using DGLA in the form of ethyl esters to feed rats and rabbits (Smith *et al.* 1989; Taki *et al.* 1993). More recently, as new sources were developed, studies investigated the supplementation of DGLA isolated from mutants of algae, yeast and fungus and demonstrated a more efficient accumulation compared to GLA supplementation (Dooper *et al*. 2003; Miles *et al.* 2004; Umeda-Sawada *et al*. 2006). For example, rats supplemented with a DGLA diet incorporated more of this PUFA into plasma and tissue lipid fractions, in comparison to GLA (Umeda-Sawada *et al*. 2006). DGLA incorporation into the liver, serum and brain lipids of rats was significantly higher in the DGLA oil diet compared to the GLA oil diet (Umeda-Sawada *et al*. 2006). Treatment with DGLA resulted in a significant increase (>5%) in fatty acid uptake into phospholipids of peripheral blood mononuclear cell (PBMCs), whereas treatment with GLA induced an increase of less than 3%, due to the restrictions in elongation products (Dooper *et al*. 2003). Finally, supplementation with GLA in humans failed to significantly accumulate DGLA in lipid fractions of serum (Miles *et al*. 2004). Direct supplementation with DGLA is therefore more efficient at increasing the levels of this fatty acid in lipid fractions.

3.1.2 DGLA in disease

Fatty acids have both beneficial and potentially harmful effects in CVD and atherosclerosis. The roles of fatty acids in relation to atherosclerosis are detailed in Table 1.5 in the Introduction. In general SFA, fatty acids with no double bonds, increase cholesterol levels and promote pro-inflammatory conditions (Xu *et al*. 2006). MUFA and PUFA tend to have advantageous actions in relation to CVD and atherosclerosis by lowering cholesterol levels and exerting anti-inflammatory actions (Kris-Etherton *et al*. 2003; Takai *et al*. 2009). There are however exceptions to this generalisation, as shown in Table 1.5. For example, stearic acid (a SFA) lowers LDL cholesterol levels *in vivo* (Hunter *et al*. 2010), while AA (omega-6 PUFA) has numerous pro-inflammatory roles (Levin *et al*. 2002; Gomolka *et al*. 2011). DGLA is an omega-6 fatty acid but with numerous beneficial properties in CVD and other inflammatory diseases. The role of DGLA in disease is detailed in Table 3.1.

Table 3.1 – Role of DGLA in disease

Role	Reference
DGLA inhibits LBT_4 production from rat macrophages.	(Nakamura *et al.* 1993)
Inhibition of human platelet aggregation and mitogen release from HMDMs with DGLA.	(Smith *et al*. 1989)
Hypertension induced by a saturated fat high diet in rats, was reversed by supplementation with DGLA.	(Hassall and Kirtland 1984)
Intravenous injection of DGLA into mice suppressed delayed type hypersensitivity, measured by swelling.	(Taki *et al*. 1993)
DGLA supplementation in NC/Tnd mice showed a significant accumulation of DGLA in the skin, which inhibited severity of atopic dermatitis	(Amagai *et al.* 2015).
DGLA exhibited cytotoxic action in drug-sensitive and resistant cancer cells in a free radical-dependent process. Increased drug sensitivity in resistant cells increasing the drugs cytotoxic action.	(Das and Madhavi 2011)
Supplementation in humans reduced platelet aggregation.	(Kernoff *et al.* 1977)
Inhibited atherosclerosis in $ApoE^{-/-}$ mouse model. Reduced inflammatory markers.	(Takai *et al*. 2009)

3.1.3 Eicosanoid production and role in disease

Alteration in the dietary intake of fatty acids leads to changes in eicosanoid production. Eicosanoid production is dependent on the availability of fatty acid substrates from a lipid pool or cleaved from phospholipids (Wang *et al*. 2012). As discussed in Section 1.5.2.6, eicosanoids are produced from 20C fatty acids EPA, DGLA and AA and play an important role in inflammation and atherosclerosis (see Table 3.2 for the roles of eicosanoids produced from omega-6 PUFAs, DGLA and AA). The action of COX and LOX enzymes on DGLA and AA produce a wide variety of prostaglandins, lipoxins, leukotrienes, thromboxanes and hydroxy-eicosanoids.

In Western countries AA is usually the most abundant 20C fatty acid present in the phospholipid membranes of immune cells. Eicosanoids derived from AA metabolism are therefore primarily produced in abundance during inflammation (Chapkin *et al.* 1988; Dooper *et al*. 2003). Eicosanoid production from AA has various pro-inflammatory roles. A number of prostanoids are produced from COX action on AA. These include members of the PG family (such as PGE_2) and thromboxanes (Kobayashi *et al.* 2004) both with pro-inflammatory roles. AA is converted to leukotrienes by 5-LOX with further transformation into unstable LTA_4 (Samuelsson *et al.* 1987). This can be converted to LTB4 by hydration and LTC_4 by addition of glutathione. LTC_4 can be further metabolised to LTD_4 and LTE_4 (Samuelsson *et al*. 1987). Leukotrienes are potent pro-inflammatory mediators and can regulate a number of immune processes such as leukocyte cytokine production, differentiation and migration (Neels 2013).

A number of AA metabolites however also possess anti-inflammatory properties. Lipoxins act to resolve inflammation. Lipoxins are formed by the action of a number of enzymes (5- and 15-LOX) both involving the insertion of molecular oxygen into AA (Samuelsson *et al*. 1987; Fierro and Serhan 2001). Metabolite 15-HETE can serve as a substrate for formation of lipoxin A_4 (LXA_4) and LXB_4 (Fierro and Serhan 2001). Conversely, 5-LOX action on AA forming LTA_4 followed by 12-LOX, can also lead to synthesis of LXA_4 and LXB_4. Therefore LTA_4 can be converted to both pro-inflammatory LTs and anti-inflammatory lipoxins (Fierro and Serhan 2001). The balance between LT and lipoxin formation may play an important role in the inflammatory potential of AA (Fierro and Serhan 2001). Finally, the COX metabolite prostacyclin has been indicated to play an anti-inflammatory role in atherosclerosis (Kobayashi *et al*. 2004). Despite this, it is generally accepted that accumulation of AA and its metabolites contribute to an inflammatory response.

EPA and DGLA are both 20C fatty acids and compete with AA for incorporation into phospholipids (Dooper *et al*. 2003). This change in fatty acid composition of phospholipids can alter eicosanoid production which plays an important role in modifying the inflammatory response. Eicosanoid production from DGLA yields a number of anti-inflammatory mediators. The action of COX on DGLA produces 1-series PGs. These include PGE_1 and PGD_1, which have both been demonstrated to play an anti-inflammatory role in a number of diseases (Table 3.2). In addition, the action of 15-LOX on DGLA produces 15-HETrE, a potent inhibitor of AA-derived pro-inflammatory LTB4 metabolism (Iversen *et al.* 1992).

As AA can be synthesised from DGLA in the omega-6 pathway through the action of a delta-5 desaturase, it is important to measure eicosanoid production in THP-1 macrophages on treatment with DGLA not only to determine if DGLA products are accumulated, but to determine if AA metabolites were affected simultaneously.

Table 3.2 – Roles of selective COX and LOX metabolites from DGLA or AA

Name	Abbreviation	LOX/COX	Role
5-hydroxyeicosatetraenoic acid	5-HETE	5-LOX from AA	Neutrophil activator, vasoconstrictor, gives rise to 5-oxy-ETE which acts as a chemoattractant (Poeckel and Funk 2010).
11-hydroxyeicosatetraenoic acid	11-HETE	11-LOX from AA	Expressed in atherosclerotic lesions (Waddington *et al.* 2001).
12-hydroxyeicosatetraenoic acid	12-HETE	12-LOX from AA	Associated with insulin resistance and obesity Increased ICAM-1 expression and atherogenesis (Funk 2006; Kuehn and O'Donnell 2006).
15-hydroxyeicosatetraenoic acid	15HETE	15-LOX from AA	15 LOX products involved in oxidation of LDL (Funk 2006) Precursor for lipoxins (Samuelsson *et al*. 1987; Fierro and Serhan 2001).
Prostaglandin E_2	PGE_2	COX from AA	Key role in pathogenesis of atherosclerosis. Lack of PGE_2 in LDLR knockout mice show significant plaque reduction (Suzuki *et al.* 2011). Increased levels linked to atopic dermatitis in mice (Amagai *et al*. 2015).
Leukotriene B4	LTB4	5-LOX from AA	Potent chemoattractant for monocytes, increases expression of MCP-1(Subbarao *et al.* 2004; Neels 2013).

			Promotes conversion of monocytes to foam cells by increasing expression of CD36 (Subbarao *et al*. 2004).
Thromboxane A_2	TXA_2		Vasoconstrictor and platelet aggregating agent (Kobayashi *et al*. 2004). Knockdown of TXA_2 receptor in $ApoE^{-/-}$ mice exhibit a significant delay in atherosclerosis (Kobayashi *et al*. 2004).
Lipoxin A_4	LXA_4	5,15-LOX from AA	Evidence suggests a role in resolving inflammation. Block growth factor-stimulated SMC migration (Ho *et al.* 2010). Inhibited TNF-α simulated neutrophil adherence to endothelial monolayers and release of IL-8 and MCP-1 (Goh *et al.* 2001). Vasodilator (Fierro and Serhan 2001).
Prostacyclin	PGI_2		Platelet inhibitor and vasodilator (Kobayashi *et al*. 2004). Knockdown of prostacyclin receptor in $ApoE^{-/-}$ mice show significant acceleration of atherosclerosis. Mice displayed increased expression of ICAM-1 and an increased number of leukocytes rolling along vessel wall (Kobayashi *et al*. 2004).
Prostaglandin E_1	PGE_1	COX from DGLA	Lowers blood pressure, inhibits platelet aggregation, promotes cholesterol efflux from cells and inhibits cholesterol biosynthesis (Horrobin 1991). Inhibits arthritis in rats (Zurier and Ballas 1973).

			Anti-proliferation activities in cancer cell lines (Tabolacci *et al.* 2010). PGE_1 is protective against anaemia, clinical nephritis and death in mouse models of autoimmune disease lupus (Zurier *et al.* 1977). PGE_1 injected into rats completely supressed arthritis and cartilage destruction (Zurier and Ballas 1973).
Prostaglandin D_1	PGD_1	COX from DGLA	Prevents the development of atopic dermatitis in AC/Tnd mice (Amagai *et al*. 2015).
15-hydroxyeicosatrienoic acid	15-HETrE	15-LOX from DGLA	Inhibits 5-LOX and 12-LOX (Horrobin 1991). Potent inhibitor of LBT4 (Iversen *et al*. 1992).

3.2 Aims

The aims of the studies in this chapter were to determine the uptake and metabolism of DGLA *in vitro* and *in vivo.* An overview of the experimental plan employed is provided in Figure 3.1. Initially pure DGLA was used to determine the uptake and metabolism of this lipid *in vitro.* This allowed for the effects of DGLA alone to be determined without potential indirect actions of other fatty acids in combination products. THP-1 macrophages were primarily employed throughout the study and were used for *in vitro* analysis. DGLA uptake was measured into TPL and TAG fractions by GC following TLC separation. TPL were further separated by TLC into individual phospholipids and incorporation of DGLA into the major membrane phospholipids was once again measured by GC. Eicosanoid production from THP-1 macrophages was measured by high performance liquid chromatography - mass spectrometry (HPLC-MS).

Collaboration with Ben Gurion University (Israel) provided a new source of DGLA in the form of a mutant algae *P. incisa* (Iskandarov *et al*. 2011). An additional aim of the project was to evaluate the role of this alternative source as part of a collaborative project. The mutant algae accumulate a large proportion of DGLA in TAG stores (Iskandarov *et al*. 2011). Total lipids extracted from this source, containing DGLA, were used for *in vivo* studies in mice. Following feeding of DGLA-containing diet for 48 hours, blood plasma was collected along with samples of liver, kidney and adipose tissue. Lipids were extracted and the incorporation of DGLA into different fractions was measured using GC.

3.3 Experimental plan

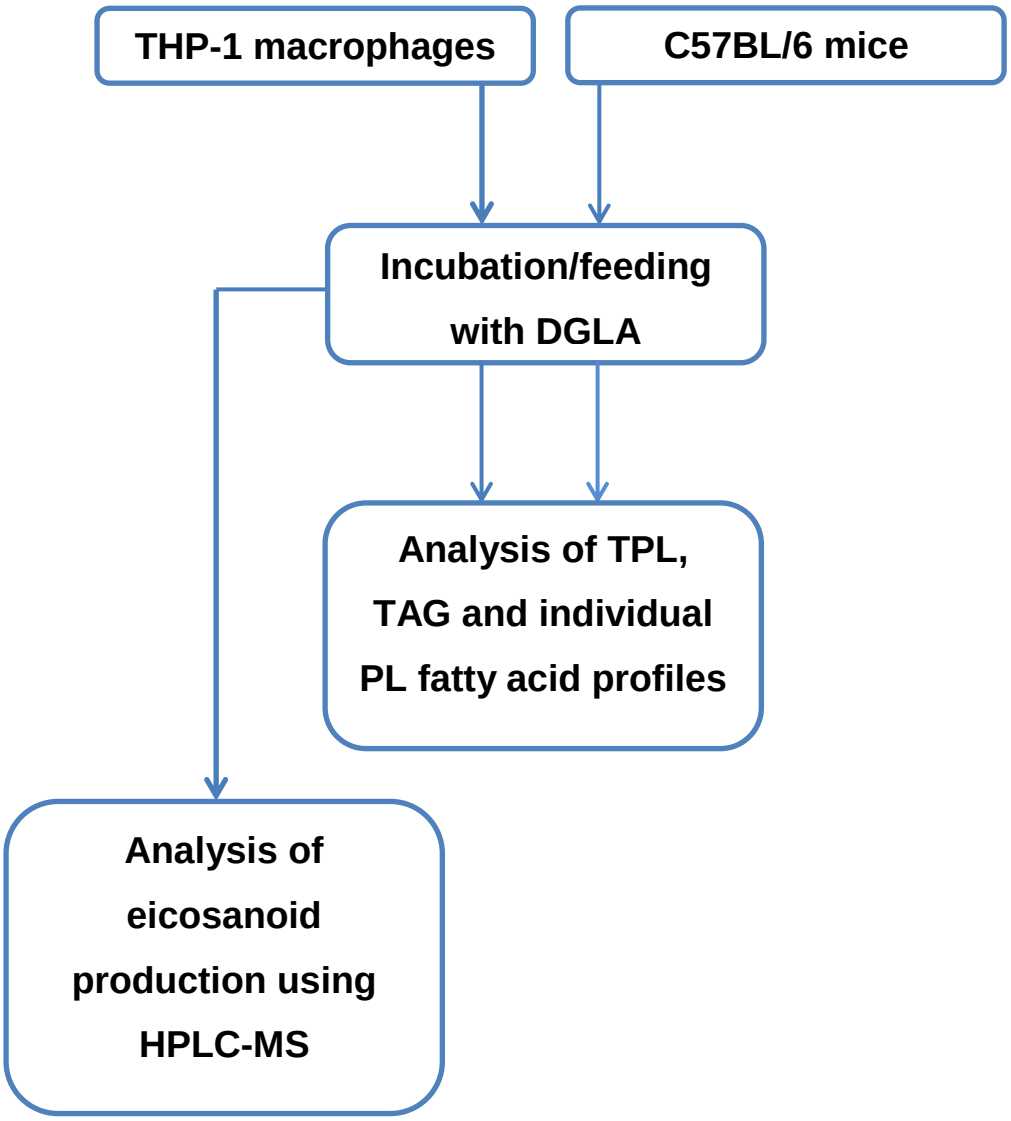


Figure 3.1 – Overview of experimental strategy for chapter 3

Uptake of DGLA was analysed in THP-1 macrophages and C57BL/6 mice using TLC and GC. Measurement of eicosanoid production from THP-1 macrophages was achieved using HPLC-MS.

3.4 Results

3.4.1 Dose-response uptake of DGLA into THP-1 macrophages

To determine a concentration of DGLA to be used in future lipid uptake experiments, a dose-response experiment was carried out. The following experiments were performed with pure DGLA purchased from Nu-Chek (purity was validated at 99% by GC). Pure DGLA was initially used to allow for the specific uptake and metabolism of this PUFA to be established. Many supplementation studies use a combination of a number of omega-6 and omega-3 fatty acids along with others (such as Oleic acid and LA), so it is difficult to establish the specific roles of each individual component. Initial work in the laboratory used concentrations ranging from 10 µM to 100 µM in which higher doses of 50 µM and 100 µM showed a significant increase in DGLA in both TAG and TPL fractions (data not shown). To demonstrate this, the two most effective doses of DGLA were repeated in a single experiment as shown in Figure 3.2. DGLA was incubated with THP-1 macrophages for 24 hours (a time point previously optimised) prior to lipid extraction, separation and fatty acid analysis using GC. Lipids were separated into two fractions; TPL and TAG.

The levels of DGLA (C20:3n6) in TPL increased in a dose-dependent manner following incubation of the cells with this fatty acid. Base levels of DGLA (C20:3n6) were 1.5% of total fatty acids in this fraction (Figure 3.2). This increased to 5.5% and 8.6% with doses of 50 µM and 100 µM DGLA, respectively. In addition to this, there was a dose-dependent decrease in palmitic acid (C16:0) and oleic acid (C18:1n9). Levels of AA (C20:4n6) increased by <1% at the highest concentration of DGLA used. There was no change in the relative level of DHA (C22:6n3) and DPA (C22:5n3).

In the TAG fraction there was a smaller increase in DGLA incorporation. Basal levels were 0.67% on average which increased to 0.94% and 2.89% respectively after incubation with the two doses of DGLA. This was accompanied by a decrease in the relative levels of ALA (C18:3n3), oleic acid (C18:1n9) and vaccenic acid (C18:1n7) and an increase in LA (C18:2n6) and myristic acid (C14:0) at both doses. There was no change in the relative levels of AA (C20:4n6) in TAG.

Given the dose-dependent increase of DGLA, the higher of the two concentrations was selected to use in further experiments due to more pronounced changes in the fatty acid profiles of lipids.

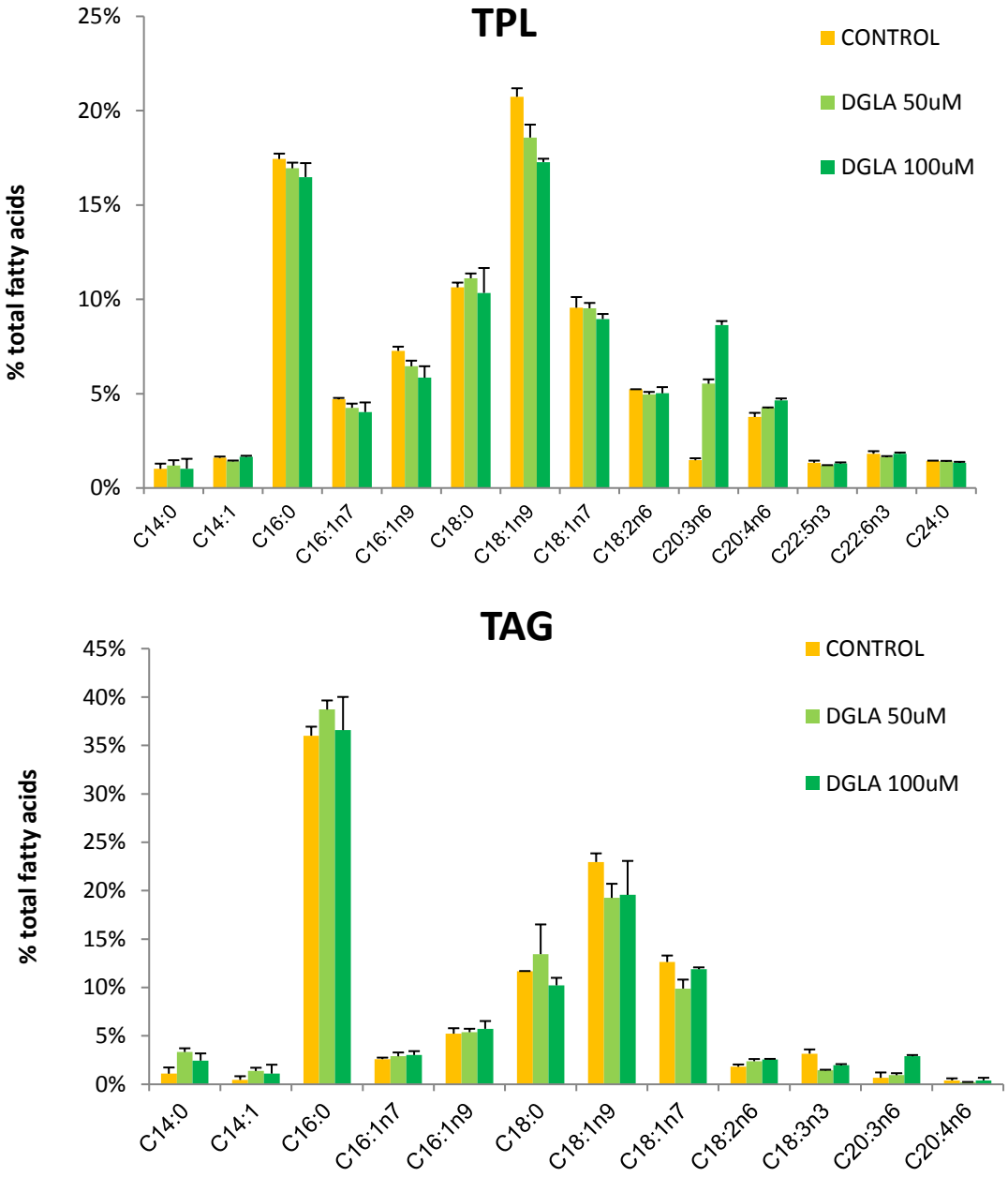


Figure 3.2 – DGLA is taken up in a dose-dependent manner into THP-1 macrophages TPL and TAG fractions

THP-1 macrophages were incubated with 50 µM or 100 µM DGLA or vehicle control for 24 hours. Lipids were extracted and separated using 1-dimensional TLC. Fatty acid profiles were determined using GC. Graphs display fatty acid composition of fraction as a percentage (+/- SD) of one experiment carried out in duplicate. C14:0, myristic acid; C14:1, myristoleic acid; C16:0, palmitic acid; C16:1n7, palmitoleic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, *cis*-vaccenic acid; C18:2n6, LA; C18:3n3, ALA; C20:3n6, DGLA; C20:4n6, AA; C22:5n3, DPA; C22:6n3, DHA; C24:0, lignoceric acid.

3.4.2 DGLA uptake into THP-1 macrophages

Following on from the dose response of DGLA, further repeats were performed for statistical analysis using 100 µM DGLA. THP-1 macrophages were incubated with DGLA or vehicle as in previous experiment.

Over 50% of the TPL and TAG fatty acids of THP-1 macrophages were composed of palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1n9). Following incubation with DGLA, there was a significant increase from 2.6% to 8.7% in DGLA (C20:3n6) levels in TPL. This was accompanied by a significant 0.5% decrease in vaccenic acid (C18:1n7) (Figure 3.3). There were also non-significant decreases in EPA (C20:5n3), DPA (C22:5n3), DHA (C22:6n3), oleic acid (C18:1n9) and AA (C20:4n6) and an increase in LA (C18:2n6).

In the TAG fraction, there was a significant increase in DGLA (C20:3n6) incorporation from 1.3% to 8.9% on average and a significant decrease in vaccenic acid (C18:1n7) and ALA (C18:3n3) (Figure 3.3). In addition to this there were non-significant decreases in palmitic acid (c16:0), stearic acid (C18:0), DPA (C22:5n3) and DHA (C22:6n3). A small increase in AA (C20:4n6) was also observed but this was not significant.

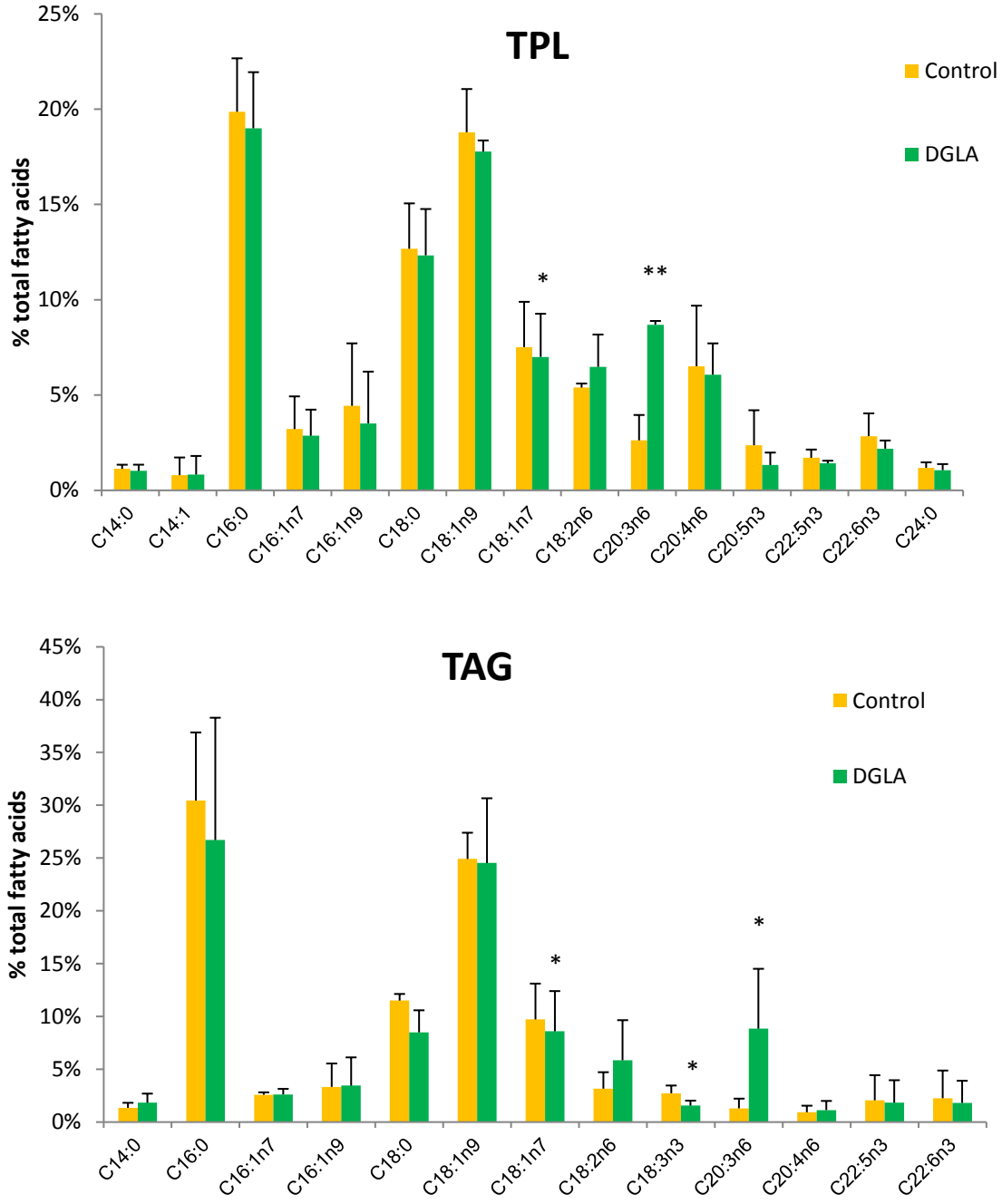


Figure 3.3 – Incubation with DGLA significantly increases its incorporation into TPL and TAG fractions

THP-1 macrophages were incubated with 100 µM DGLA or vehicle control for 24 hours. Lipids were extracted and separated using 1-dimensional TLC. Fatty acid profiles were determined using GC. Graphs display fatty acid composition of the two fractions as a percentage (+/- SD) from four experiments. Statistics were performed using a Student's two-tailed t test. * P ≤0.05, ** P ≤0.01 in relation to control**.** C14:0, myristic acid; C14:1, myristoleic acid; C16:0, palmitic acid; C16:1n7, palmitoleic acid; C16:1n9, cis-7 hexadecenoic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, *cis*-vaccenic acid; C18:2n6, LA; C18:3n3, ALA; C20:3n6, DGLA; C20:4n6, AA; C20:5n3, EPA; C22:5n3, DPA; C22:6n3, DHA; C24:0, lignoceric acid.

3.4.3 DGLA uptake into individual phospholipid classes

TPL, which consist mainly of phospholipids, can be further separated into individual phospholipid classes. To determine the incorporation of DGLA into individual phospholipids, THP-1 macrophages were incubated with 100 µM DGLA for 24 hours prior to lipid extraction and 2-dimensional separation by TLC. Individual phospholipids were identified by reference to the standards and their fatty acid profiles were analysed by GC. The main phospholipids found in eukaryotic cells are PC, PE, PS, and PI. They are located mainly in the cell membranes and usually esterified with fatty acids of variable lengths (carbon numbers) and saturation levels (double bonds) (van Meer *et al*. 2008). These four phospholipids were found in abundance in THP-1 macrophages together with cardiolipin and sphingomyelin to a lesser extent (Figure 3.4). These two lipids were not included in fatty acid analysis due to their small concentrations and high variability between experiments.

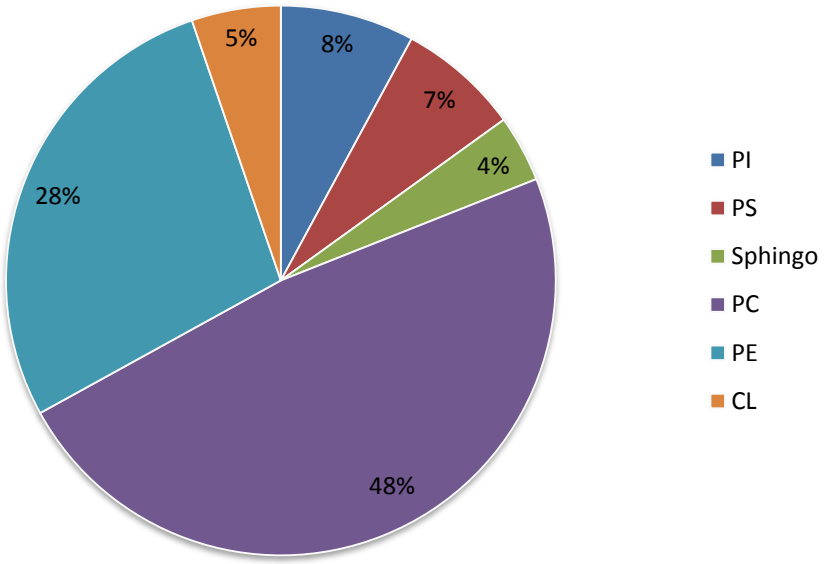


Figure 3.4 – Composition and relative content of phospholipids in THP-1 macrophages

Lipids were extracted from THP-1 macrophages and individual TPL were separated by two-dimensional TLC. Quantification was made on the basis of their fatty acid content. The results are expressed as percentages of total phospholipids. Percentages represent an average of 3 experiments

Palmitic acid (C16:0) and oleic acid (C18:1n9) were the major fatty acids in PC, accounting for over 50% of the fatty acid content. Following treatment with DGLA (C20:3n6), the relative level of this fatty acid increased from 2.5% to 6.7% on average. There was a small increase in AA (C20:4n6) from 5.3% to 6.7%. There was no change in EPA (C20:5n3), DHA (C22:6n3) and DPA (C22:5n3) contents. In addition, there was a 3.4% reduction in the relative amount of palmitic acid (C16:0), although this was highly variable.

For PS, the main fatty acid present was stearic acid (C18:0) accounting for approximately 40% of total fatty acids. Again, oleic acid (C18:1n9) was also present in abundance constituting approximately 20% of total fatty acids. Following treatment with DGLA (C20:3n6), the percentage of this fatty acid increased from 3.9% to 9% on average. There was a very small increase in AA (C20:4n6) from 3.2% to 3.4% on average and a small decrease in EPA (C20:5n3) (0.9% to 0.7%), DPA (C22:5n3) (1.4% to 1.1%) and DHA (C22:6n3) (2.9% to 2.2%). Finally, there was a decrease in the level of palmitic acid (C16:0) (6.6% to 3.7%), an increase in stearic acid (C18:0) (43.1% to 46.7%) and a decrease in oleic acid (C18:1n9) (21.7% to 19.2%).

Similar to PS, PI contained high levels of stearic acid (C18:0) (approximately 35-40%). AA (C20:4n6) was the second most abundant fatty acid accounting for approximately 20% of total fatty acids. Following treatment with DGLA (C20:3n6), this fatty acid increased from 6.3% to 13.4% of total fatty acids on average. There was no change in AA (C20:4n6) observed, a small decrease in EPA (C20:5n3) (2.2% to 1.8%) and no change in DPA (C22:5n3) or DHA (C22:6n3). In addition, following treatment with DGLA the relative amount of palmitic acid (C16:0) was reduced from 8.7% to 2.4% and stearic acid (C18:0) was increased from 38.8% to 43.1%.

The major fatty acid present in PE was AA (C20:4n6), attributing to approximately 20-25% of total fatty acids and to a lesser extent stearic acid (C18:0) (17%) and oleic acid (C18:1n9) (11%). Following treatment with DGLA (C20:3n6), the percentage of this fatty acid into PE, increased from 3.8% to 8.6%. There was a small increase in AA (C20:4n6) from 23.1% to 23.7%. A decrease in EPA (C20:5n3) was observed from 8.4% to 4.4%. However this was highly variable. There was no change in DPA (C22:5n3) and a small decrease in DHA (C22:6n3) (6.7% to 5.9%). Finally, a small increase was observed in stearic acid (C18:0) (17.1% to 18.6%) and a small decrease in oleic acid (C18:1n9) (11.1% to 10.1%).

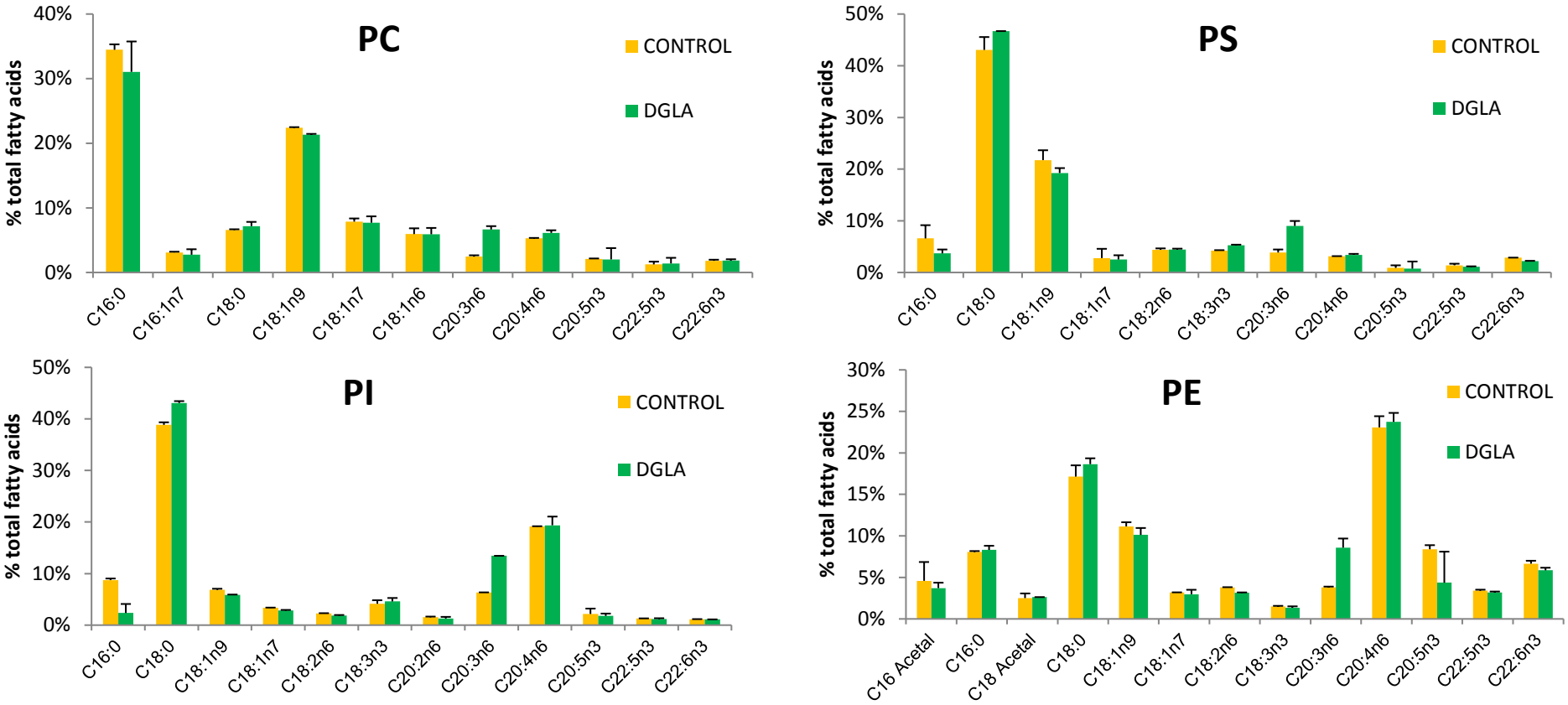


Figure 3.5 – Uptake of DGLA into individual phospholipids

THP-1 macrophages were incubated with 100 µM DGLA or vehicle control for 24 hours. Lipids were extracted and separated using 2-dimensional TLC. Fatty acid profiles of each individual phospholipid were analysed by GC. Graphs display average fatty acid composition of phospholipids as a percentage (+/- SD) of two independent experiments. C16:0, palmitic acid; C16:1n7, palmitoleic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, *cis*-vaccenic acid; C18:2n6, LA; C18:3n3, ALA; C18:3n6, GLA; C20:3n6, DGLA; C20:4n6, AA; C20:5n3, EPA; C22:5n3, DPA; C22:6n3, DHA.

3.4.4 Eicosanoid production

Following supplementation of THP-1 macrophages with DGLA, it was clear that this fatty acid was incorporated into TPL and TAG fractions with no significant change in the level of AA (Figure 3.3). DGLA and AA, through the action of LOX and COX enzymes produce a wide variety of eicosanoids. Given the incorporation of DGLA into THP-1 macrophage lipid fractions, as well as its possible conversion into AA, it was important to measure eicosanoid production to establish if these changes translated to a change in eicosanoid production. The production of important COX and LOX metabolites were measured using HPLC-MS.

THP-1 macrophages were incubated with 100 μM DGLA for 24 hours. In the experiment an inflammatory mediator was included, IFN-γ, to determine eicosanoid production in an inflammatory environment and the relationship with DGLA. IFN-γ is a pro-inflammatory cytokine and has been described as a master regulator of atherosclerosis due to its wide variety of roles in the disease (McLaren and Ramji 2009). More detail on IFN-γ can be found in Section 1.4.1.1. Following incubation with DGLA, macrophages were stimulated with 250 U/ml IFN-γ for 3 hours. Media was collected and lipid extracted for measurement by HPLC-MS.

Metabolism of AA by LOX enzymes can produce a number of hydroxyeicosatetraenoic acids (HETE), while hydroxyeicosatrienoic acids (HETrE) are produced from DGLA. A number of these eicosanoids were detected in the macrophage conditioned medium (Figure 3.6). AA metabolites 5-, 11-, 12- and 15-HETE were detected. The only DGLA LOX metabolite detected was 15-HETrE. There was no change in the average expression of 5- and 12-HETE between any of the four conditions. IFN-γ had no effect on any of the HETE and HETrE eicosanoids identified. On treatment with DGLA and IFN-γ the levels of 11- and 15-HETE increased on average, however this was not significant on comparison to any other condition and was highly variable between experiments. The only significant change was observed in 15-HETrE production. DGLA significantly induced production of the eicosanoid: approximately 3-fold. IFN-γ had no effect on its formation, moreover, when in combination with DGLA, 15-HETrE production increased to a level not significantly different to that for DGLA alone.

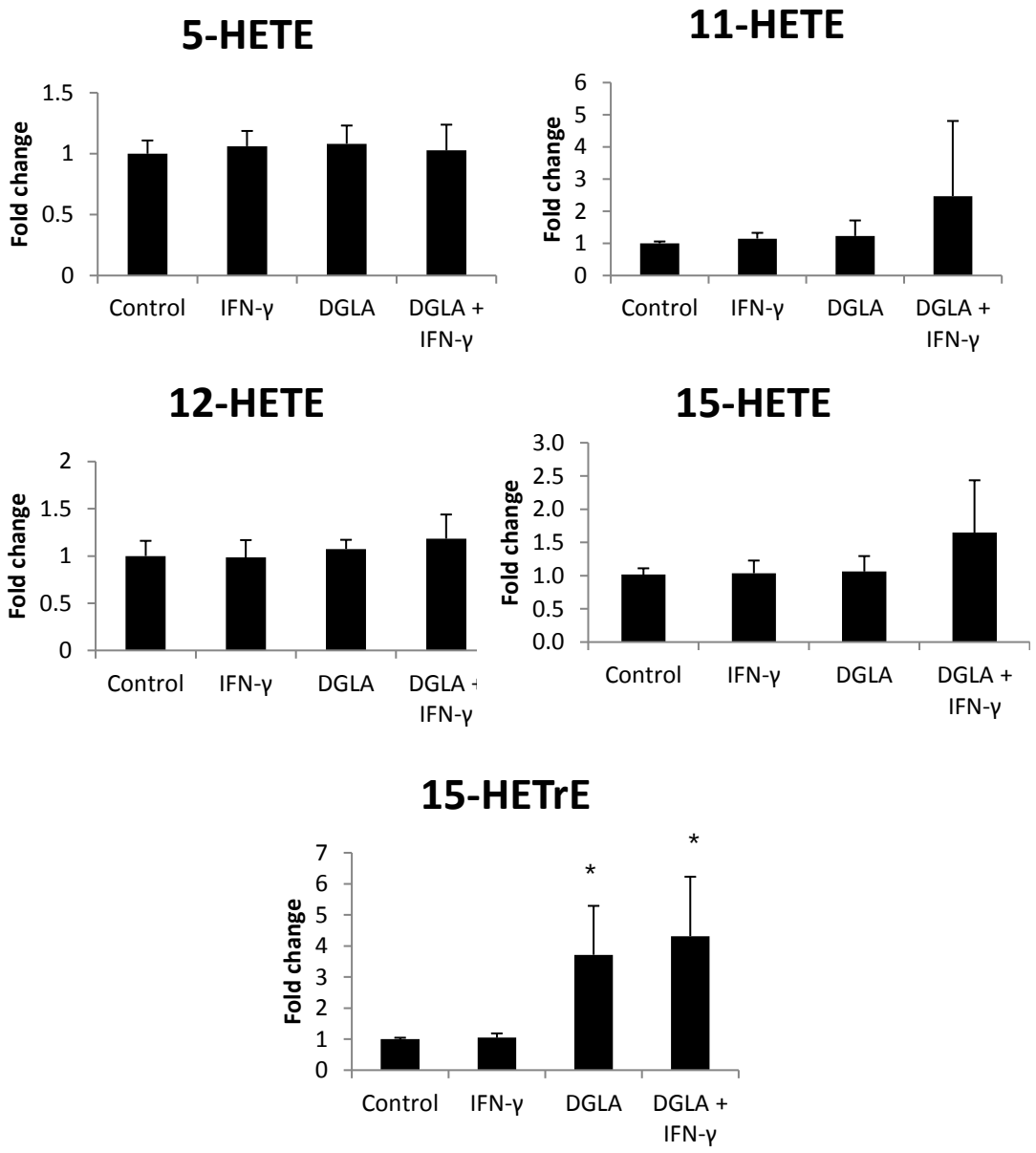


Figure 3.6 – Eicosanoid production from LOX enzymes in THP-1 macrophages

THP-1 macrophages were pre-treated with 100 µM DGLA or DMSO control for 24 hours prior to 24 hour stimulation with vehicle or 250 U/ml IFN-γ. Media was collected and lipids extracted for analysis by HPLC-MS. Concentrations were measured in pg per ml of media from 4 x 10^6 cells and expressed as fold-change with values from control samples arbitrarily assigned as 1. Data represents average of 3 independent experiments (+/- SD). Assumptions of ANOVA were not fulfilled therefore Robust equality of means test was used followed by Dunnett's post hoc analysis. * P ≤0.05 in relation to control.

A major prostaglandin produced via metabolism of AA by COX enzymes, is PGE_2. It was hypothesised that there would be no to little change in PGE_2 production on treatment of the cells with DGLA, as fatty acid analysis did not show any conversion of DGLA into AA in TPL or TAG fractions of THP-1 macrophages (Figure 3.3). On treatment with DGLA alone, there was no increase in the production of PGE_2 compared to control (Figure 3.7). Concentration of PGE_2 was expressed as pg/ml from 4 x 10^6 cells, as reported previously (Clark *et al.* 2011; Aldrovandi *et al.* 2013). Basal level of expression of PGE_2 was measured at 500 pg/ml. IFN-γ also had no effect on PGE_2 accumulation. However, when macrophages were pre-incubated with DGLA followed by stimulation with IFN-γ, there was a significant increase of 500 pg/ml on average in PGE_2, on comparison to all other treatments.

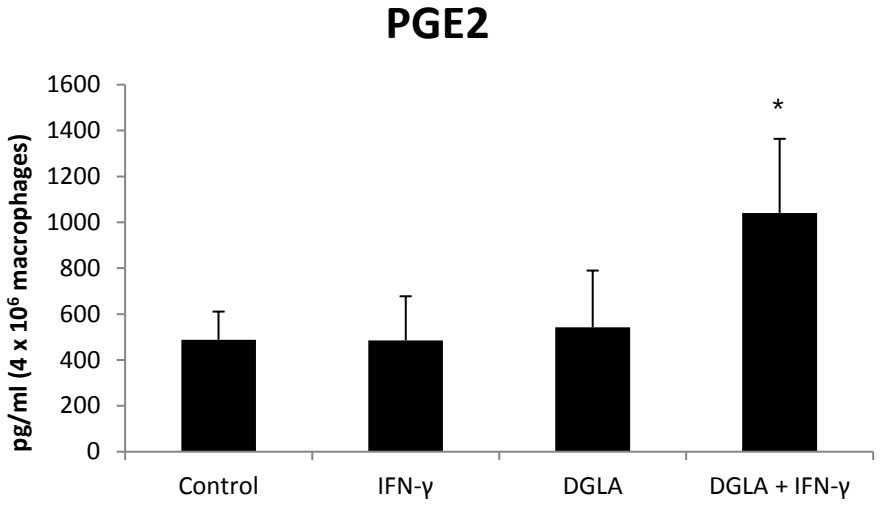


Figure 3.7 – PGE_2 production from THP-1 macrophages

THP-1 macrophages were pre-treated with 100 µM DGLA or DMSO control for 24 hours prior to 24 hour stimulation with vehicle or 250 U/ml IFN-γ. Media was collected and lipids extracted for measurement using HPLC-MS. Concentrations were measured in pg per ml of media from 4 x 10^6 cells. Data represents average of 3 independent experiments (+/- SD). Assumptions of ANOVA were not fulfilled therefore Robust equality of means test was used followed by Dunnett's post hoc analysis. * P ≤0.05 in relation to control.

COX metabolism of DGLA produces prostaglandins of the 1-series including PGE_1. PGE_1 is an anti-inflammatory mediator with numerous beneficial actions; therefore its production from THP-1 macrophages was measured. Concentration of PGE_1 was expressed as pg/ml from 4 x 10^6 cells as reported previously (Clark *et al*. 2011; Aldrovandi *et al*. 2013). Treatment with IFN-γ did not significantly induce PGE_1 production with an increase of less than 1 pg/ml (Figure 3.8). Treatment of the cells with DGLA significantly induced PGE_1 production from negligible to 180 pg/ml on average, and in combination with IFN-γ, up to 356 pg/ml on average. The differences between the DGLA groups however were not significantly different. The concentration of PGE_1 did not exceed that of PGE_2 in any individual experiment.

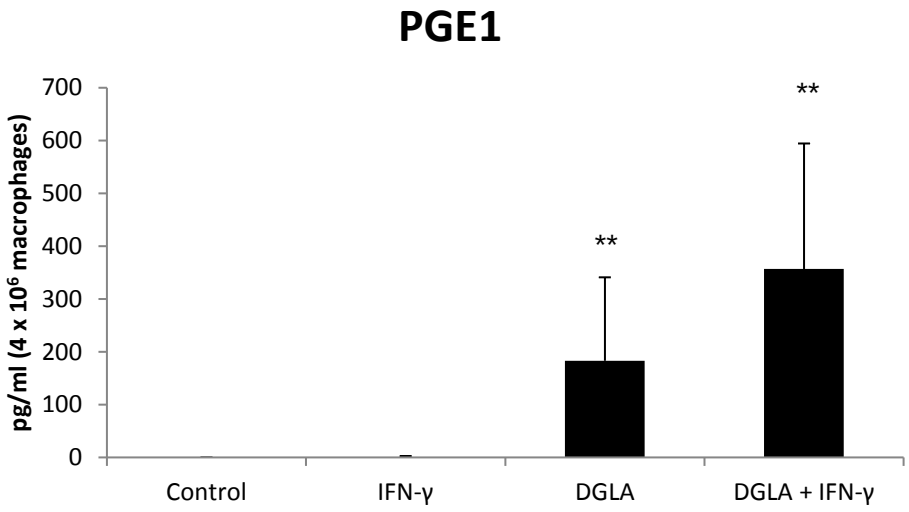


Figure 3.8 – PGE_1 production from THP-1 macrophages

THP-1 macrophages were pre-treated with 100 µM DGLA or DMSO control for 24 hours prior to 24 hour stimulation with vehicle or 250 U/ml IFN-γ. Media was collected and lipids extracted for measurement using HPLC-MS. Graphs display concentration of PGE_1 (pg/ml from 4 x 10^6 cells) as an average of 3 independent experiments (+/- SD). Assumptions of ANOVA were not fulfilled therefore robust equality of means test was used followed by Dunnett's post hoc analysis. For statistical tests to be performed a small number (0.0001) was added to all measurements as control group measured zero. ** P ≤0.01 in relation to control.

3.4.5 COX expression

Given the important role of COX enzymes in the production of prostaglandins and the changes observed in Figures 3.7 and 3.8, it was of interest to determine the expression of the two COX enzymes. THP-1 macrophages were pre-treated with 100 µM DGLA or vehicle control for 24 hours prior to stimulation with IFN-γ. RNA was extracted and subjected to reverse transcription to make cDNA. RT-qPCR was performed using specific primers for COX-1 and COX-2.

Treatment with IFN-γ had no significant effect on the expression of COX-1 or COX-2 in THP-1 macrophages, in comparison to control (Figure 3.9). DGLA had no effect on COX-2 expression; however it did significantly attenuate expression of COX-1 by 32%. A combination of pre-treatment with DGLA followed by stimulation with IFN-γ, the expression of COX-1 was again significantly reduced by 50%, in comparison to control. Under the same conditions, the expression of COX-2 was significantly increased by 2.5-fold.

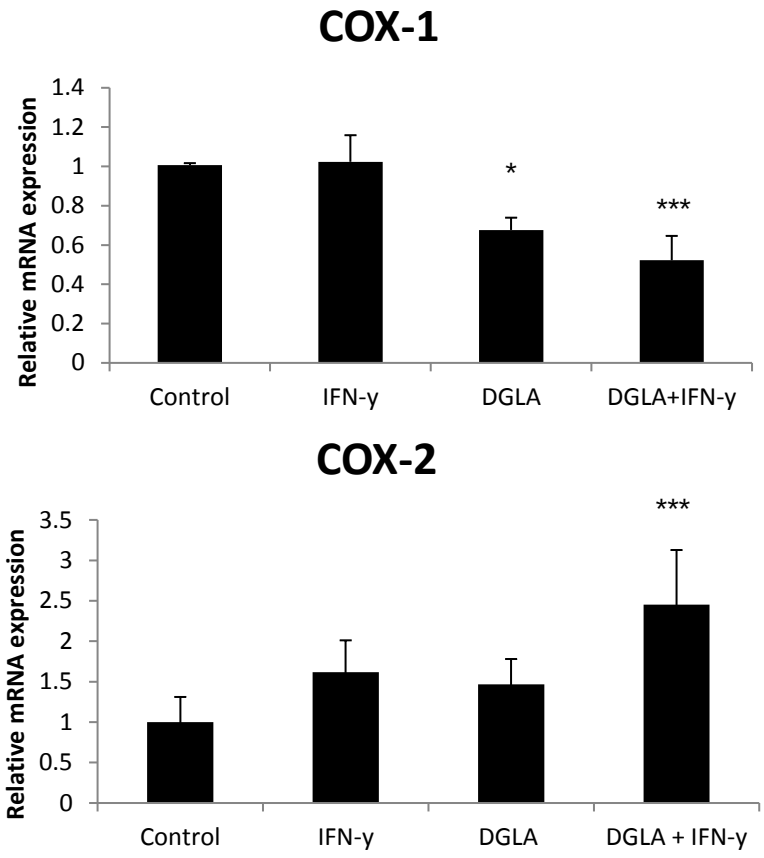


Figure 3.9 – COX-2 gene expression was induced on treatment of THP-1 macrophages with DGLA and IFN-γ

THP-1 macrophages were incubated with vehicle or 100 µM DGLA for 24 hours prior to treatment with vehicle or 250 U/ml IFN-γ for 24 hours. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for human COX-1, COX-2 or GAPDH. Graphs display average gene expression (mean +/- SD) (control arbitrarily assigned as 1) from 3 independent experiments. Statistical analysis was performed using a one-way ANOVA followed by Tukey's post-hoc test. *P≤0.05 ***P≤0.001 in relation to control.

3.4.6 Uptake of DGLA from algal powder *in vivo*

The previous uptake and metabolic experiments described above were performed with pure DGLA. This allowed for the uptake and metabolism, following incubation with DGLA only, to be determined. Following on from DGLA uptake *in vitro* it was important to determine the uptake on this fatty acid *in vivo.* To evaluate this, mice were supplemented with DGLA in their diet to investigate uptake of the PUFA. As previously discussed in Section 1.5.3, there are a number of new potential commercial sources of DGLA which have been developed through genetic or metabolic manipulation of yeast, fungus and algae (Kawashima *et al*. 2000; Iskandarov *et al*. 2011; Watanabe *et al*. 2014). The current project was in collaboration with Ben Gurion University (Israel) who supplied a mutant form of the microgreen algae *P. incisa* which accumulates large amounts of DGLA. This was the source of DGLA used for *in vivo* experiment and allowed for the evaluation of the algae as a useful commercial source of DGLA.

To determine the uptake of DGLA from the algae *P. incisa*, six C57BL/6 mice were starved 24 hours prior to the experiment. Mice were separated into two groups; control (fed on a normal chow diet, see the fatty acid composition in the Table 3.3) and DGLA-enriched diet. For the first experiment, animals from DGLA-enriched diet group were supplemented with 2% (w/w) freeze-dried algal powder, containing about 30% DGLA, which was added to the normal chow diet. After 48 hours of feeding, samples of plasma, liver, kidney and faeces were collected. Analysis of the fatty acids of tissues and plasma showed no accumulation of DGLA (Appendix 1). Fatty acid analysis of faeces showed that practically all supplemented DGLA was discharged indicating that algal powder could not be assimilated by mice (Figure 3.10).

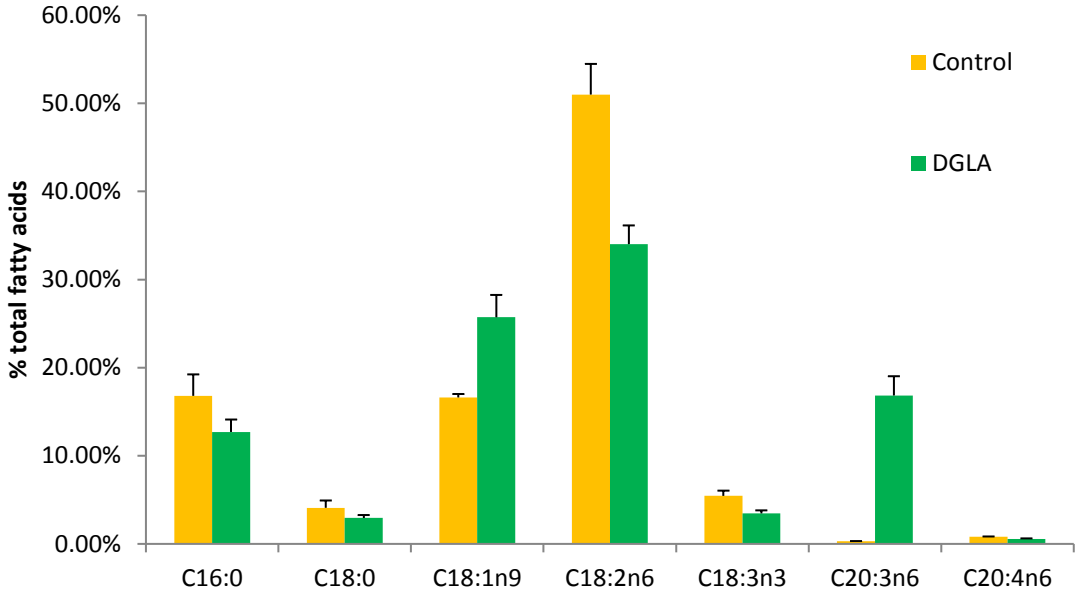


Figure 3.10 –DGLA was not absorbed *in vivo* following feeding of mice with freeze-dried algal powder

Male C57BL/6 mice were split into 2 groups; Control (3) and DGLA (3). For DGLA group, normal chow diet was supplemented with 2% (w/w) freeze-dried algal powder containing this PUFA. Mice were starved for 24 hours prior to feeding. Mice were fed control or DGLA containing diet for 48 hours. Following this, a sample of faeces was collected from each group, lipids extracted and fatty acids analysed using GC. Graphs indicate the average percentage fatty acid composition of faeces (+/- SD) performed in triplicate. C16:0, palmitic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:2n6, LA; C18:3n3, ALA; C20:3n6, DGLA; C20:4n6, AA.

As mice were unable to digest the freeze-dried algal powder and assimilate DGLA from this source, total lipids were next extracted from the algal powder prior to feeding. Lipid extraction from algal powder is detailed in Section 2.2.13 of the methods chapter. Total lipids were isolated from the algal powder and added to the chow diet. The fatty acid profile of the chow diet (control) and DGLA-containing diet are presented in Table 3.3. The main fatty acid present in the control diet was LA (C18:2n6), followed by oleic acid (C18:1n9), palmitic acid (C16:0), ALA (C18:3n3) and stearic acid (C18:0). No DGLA was present in the control diet. Diet containing total lipid extracts from freeze-dried algal powder contained 4.4% of DGLA in TFA. There were relatively minor changes in the relative content of LA (C18:2n6), oleic acid (C18:1n9), palmitic acid (C16:0) and ALA (C18:3n3) between the two diets.

Table 3.3 – Fatty acid composition of control and DGLA diets used in *in vivo* experiments following extraction from algal powder

Fatty acid	Control diet	DGLA diet
Palmitic acid (C16:0)	14.4%	15.7%
Stearic acid (C18:0)	2.9%	3.1%
Oleic acid (C18:1n9)	21.6%	25.5%
LA (C18:2n6)	51.4%	43.6%
ALA (C18:3n3)	5.7%	4.5%
DGLA (C20:3n6)	-	4.4%
Other	4.0%	3.2%

For feeding experiments with lipids extracted from algal powder, seven mice were split into 2 groups: Control (3) and DGLA (4). Mice were starved for 24 hours prior to feeding. Lipid composition of diets of control and DGLA group are detailed in Table 3.3. Following 48 hours of feeding, plasma and faeces (Appendix 3) were collected followed by samples of liver, kidney and adipose tissue. Depending on the amount of lipids extracted from plasma and tissues, total fatty acids and fatty acids of TPL, TAG and individual phospholipids were analysed by GC.

3.4.6.1 Plasma

Plasma was collected from mice and total lipids extracted as detailed in Section 2.2.15.2. An aliquot of total lipids was taken to analyse total fatty acids; the rest of the lipid extracts were separated by 1-dimensional TLC into TPL, TAG and steryl ester fractions. The fatty acid composition of each of the fractions was analysed by GC.

In total fatty acids, the percentage of DGLA (C20:3n6) incorporation significantly increased from 1.2% to 3.1% in the DGLA group (Figure 3.11). There was no significant increase in AA (C20:4n6) levels but a significant decrease from 0.3% to 0.1% in EPA (C20:5n3). When total lipids were further separated by TLC, it was observed that DGLA was significantly incorporated into TPL with an increase from 1.5% to 4.5% in the DGLA group. This was also accompanied by an increase in AA (C20:4n6) from 6.6% to 10.5%. However this was not significant between the groups. There was also a significant decrease in the relative amount of stearic acid (C18:0) in DGLA group. In TAG fraction, DGLA was significantly increased from 0.2% to 1.4%, with no significant change in AA (C20:4n6) or any other fatty acids identified. Finally, in steryl esters, DGLA (C20:3n6) was again significantly increased from 0% to 2.6% in DGLA group. AA (C20:4n6) increased from 20% to 25.6%, however this was not statistically significant. The relative amount of EPA (C20:5n3) was also significantly decreased by from 2.3% to 1.6%.

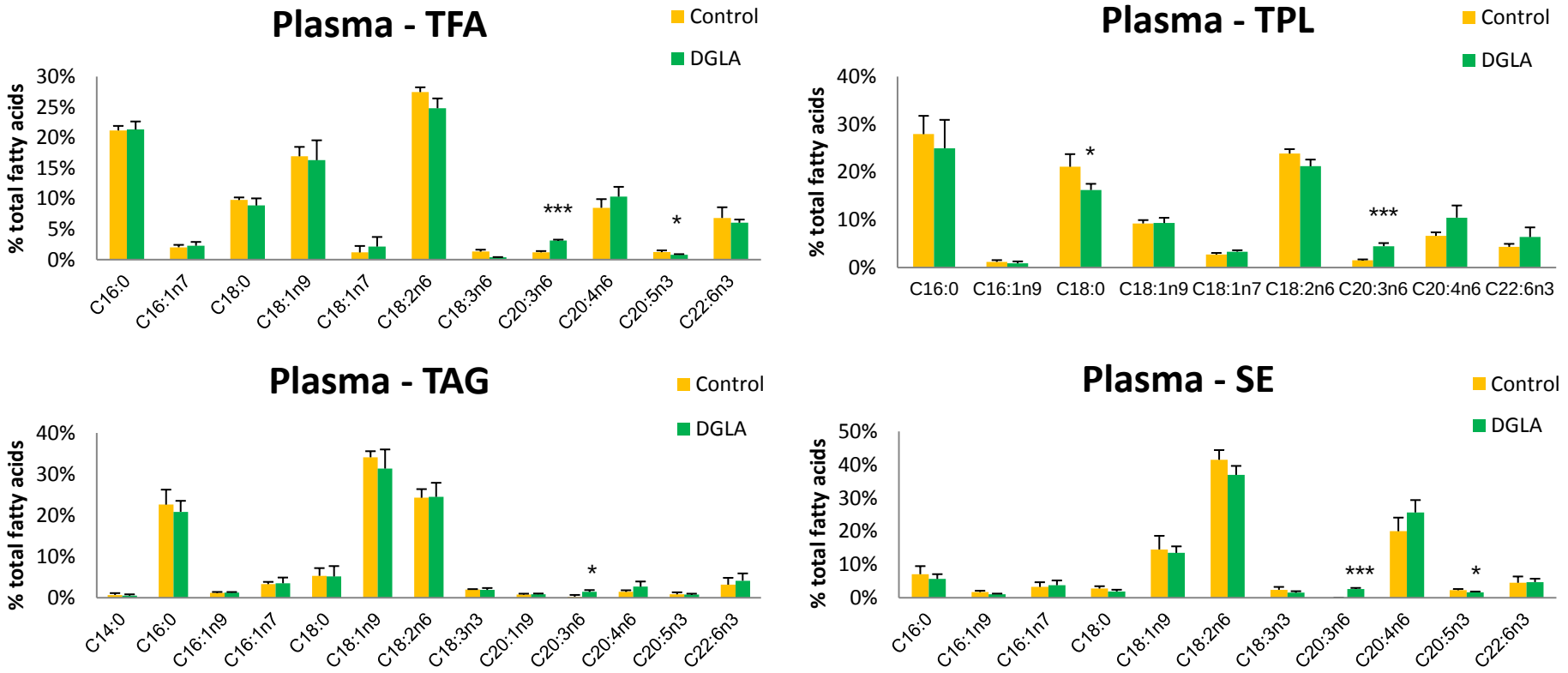


Figure 3.11 – Uptake of DGLA into plasma lipid fractions *in vivo*

Mice were supplemented with 4% DGLA diet for 48 hours. Animals were sacrificed and the plasma fraction of blood collected. Lipids were extracted and separated using 1-dimensional TLC. Fatty acid profiles of each lipid fraction were analysed by GC. Graphs display average fatty acid composition as a percentage (+/- SD) from 3 mice (Control) and 4 mice (DGLA diet). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc analysis. * P <0.05. *** P <0.001. C14:0, myristic acid; C16:0, palmitic acid; C16:1n7, palmitoleic acid; C16:1n9, cis-7 hexadecenoic acid ; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, *cis*-vaccenic acid; C18:2n6, LA; C18:3n3, ALA; C18:3n6, GLA; C20:3n6, DGLA; C20:4n6, AA; C20:5n3, EPA; C22:6n3, DHA.

3.4.6.2 Liver

Incorporation of DGLA into the lipid classes of liver was also investigated. The liver is a very important organ which is involved in the regulation of many processes such as TAG and fatty acid metabolism and together with cholesterol synthesis, transport and catabolism. Given its role in lipid metabolism, the fatty acid composition of liver is commonly investigated in uptake studies. Liver was isolated from mice and lipids separated as detailed in Section 2.2.15.3. Total fatty acid composition of liver was analysed by GC; TPL, TAG and steryl esters were separated by TLC and the fatty acid profile of these fractions was also analysed by GC. In addition, individual phospholipids were also separated by 2-dimensional TLC into individual phospholipids PC, PS, PI and PE.

In total liver fatty acids, DGLA (C20:3n6) was increased significantly from 1.5% to 2.5% and stearic acid (C18:0) decreased from 13.1% to 11.7% in the DGLA group (Figure 3.12). There was no significant change in AA (C20:4n6) levels in total fatty acids. Following this, total fatty acids were separated by 1-dimensional chromatography. In TPL, DGLA (C20:3n6) was significantly increased from 1.8% to 2.7% while stearic acid (C18:0) and DHA (C22:6n3) were both significantly decreased in the DGLA group from 17.7% to 15.1% and 11.9% to 10.3% respectively. In the TAG fraction of the liver, DGLA was significantly increased from 1.1% to 1.6% in the DGLA group. This was accompanied by a significant increase in AA (C20:4n6) from 1.8% to 2.7%. DGLA also significantly increased from 0% to 1.4% in steryl ester fraction in DGLA group. There were no other significant changes in this lipid fraction.

To utilise the large concentration of lipid isolated from the liver, TPL were further separated into individual phospholipids for analysis (Figure 3.13). The four main phospholipids were identified and their fatty acid compositions measured in control and DGLA groups. There was a significant increase in DGLA into PS, PE and PC from 1.6% to 2.4%, 0.9% to 1.3% and 2.4% to 3.7% respectively in DGLA treatment groups. There was an increase from 3% to 3.8% of DGLA (C20:3n6) into PI; however, this was not significant. There was no significant change in AA (C20:4n6) levels into any phospholipid fraction measured despite an increase of 9.7% to 11.9% in AA with PC. There were also changes in other fatty acids observed in phospholipid fractions. In PS there was a significant increase in vaccenic acid (C18:1n7) from 0.7% to 1.4% in DGLA group. In PE, a significant decrease in both EPA (C20:5n3) and DHA (C22:6n3) was measured, from 1% to 0.6% and 19.9% to 17.5% respectively. Finally, in PC, there was a significant decrease from 11.9% to 9.4% in stearic acid (C18:0) and 11.9% to 9.7% in DHA (C22:6n3).

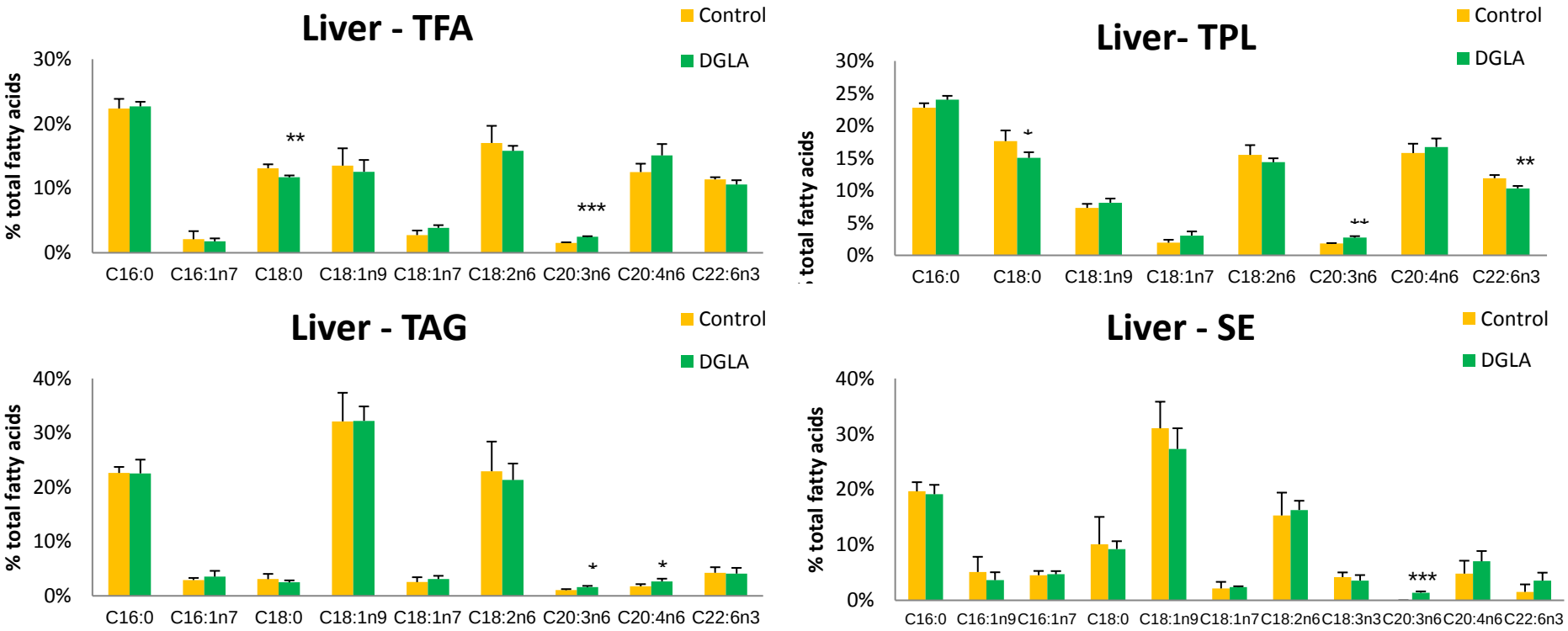


Figure 3.12 – Uptake of DGLA into liver lipid fractions *in vivo*

Mice were supplemented with 4% DGLA diet for 48 hours. Animals were sacrificed and liver tissue collected. Lipids were extracted and separated using 1-dimensional TLC. Fatty acid profiles of each lipid fraction were analysed by GC. Graphs display average fatty acid composition as a percentage (+ SD) of 3 mice (Control) and 4 mice (DGLA diet). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc analysis. * P<0.05. ** P <0.005, *** P<0.001. C16:0, palmitic acid; C16:1n7, palmitoleic acid; C16:1n9, cis-7 hexadecenoic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, *cis*-vaccenic acid; C18:2n6, LA; C18:3n3, ALA; C20:3n6, DGLA; C20:4n6, AA; C20:5n3, EPA; C22:6n3, DHA.

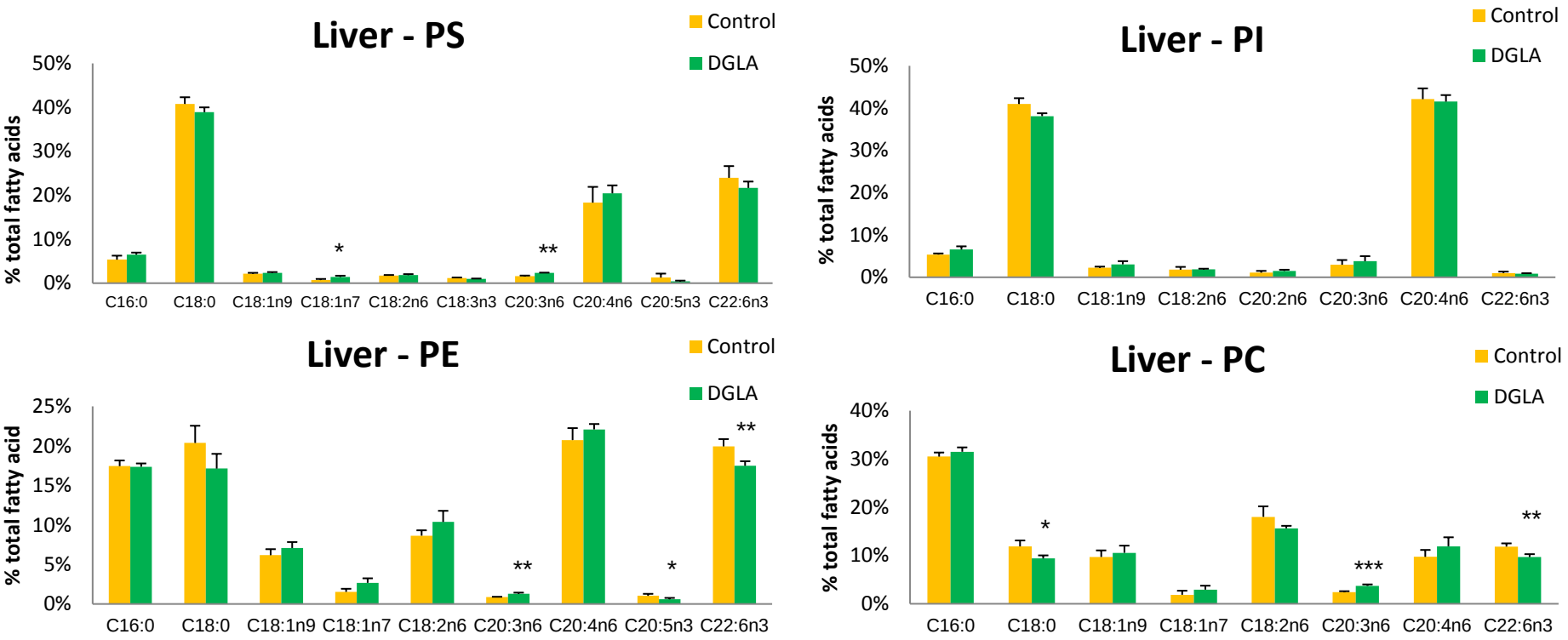


Figure 3.13 – Uptake of DGLA into liver individual phospholipids *in vivo*

Mice were supplemented with 4% DGLA diet for 48 hours. Animals were sacrificed and liver tissue collected. Lipids were extracted and separated using 2-dimensional TLC for individual phospholipids. Fatty acid profiles of each phospholipid fraction were analysed by GC. Graphs display average fatty acid composition as a percentage (+ SD) of 3 mice (Control) and 4 mice (DGLA diet). Statistical analysis was performed using a one-way ANOVA followed by Tukey's post hoc analysis. * P <0.05. ** P<0.005, *** P <0.001. C16:0, palmitic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, *cis*-vaccenic acid; C18:2n6, LA; C18:3n3, ALA; C20:3n6, DGLA; C20:4n6, AA; C20:5n3, EPA; C22:6n3, DHA.

3.4.6.3 Kidney and adipose tissue

In addition to plasma and liver, samples of kidney and adipose tissue were also taken for analysis. In the total fatty acids of kidney, DGLA (C20:3n6) was significantly increased from 1.1% to 1.8% in the DGLA group (Figure 3.14). Total fatty acids were further separated into TPL and TAG fractions. In TPL, there was a non-significant increase in DGLA (C20:3n6) from 1.2% to 1.8% in the DGLA group. There was however, a significant increase in AA (C20:4n6) incorporation from 17.6% to 18.9%. In the TAG fraction of kidney, there was a significant increase in DGLA (C20:3n6) from 0.7% to 1.2% and LA (C18:2n6) from 22.2% to 27.7% in the DGLA group. This was accompanied by significant decreases in AA (C20:4n6), palmitic acid (C16:0) and oleic acid (C18:1n9) from 2.8% to 2%, 26.7% to 23% and 22.2% to 27.7%, respectively.

In adipose tissue, only total fatty acids were measured due to the small amount of tissue collected on dissection. The incorporation of DGLA (C20:3n6) in the DGLA-fed-group increased significantly from 0.3% to 0.8%. There were no other significant changes in the fatty acid composition of adipose tissue.

Table 3.4 summarises percentage changes in DGLA and AA across all plasma, liver, kidney and adipose lipid fractions measured.

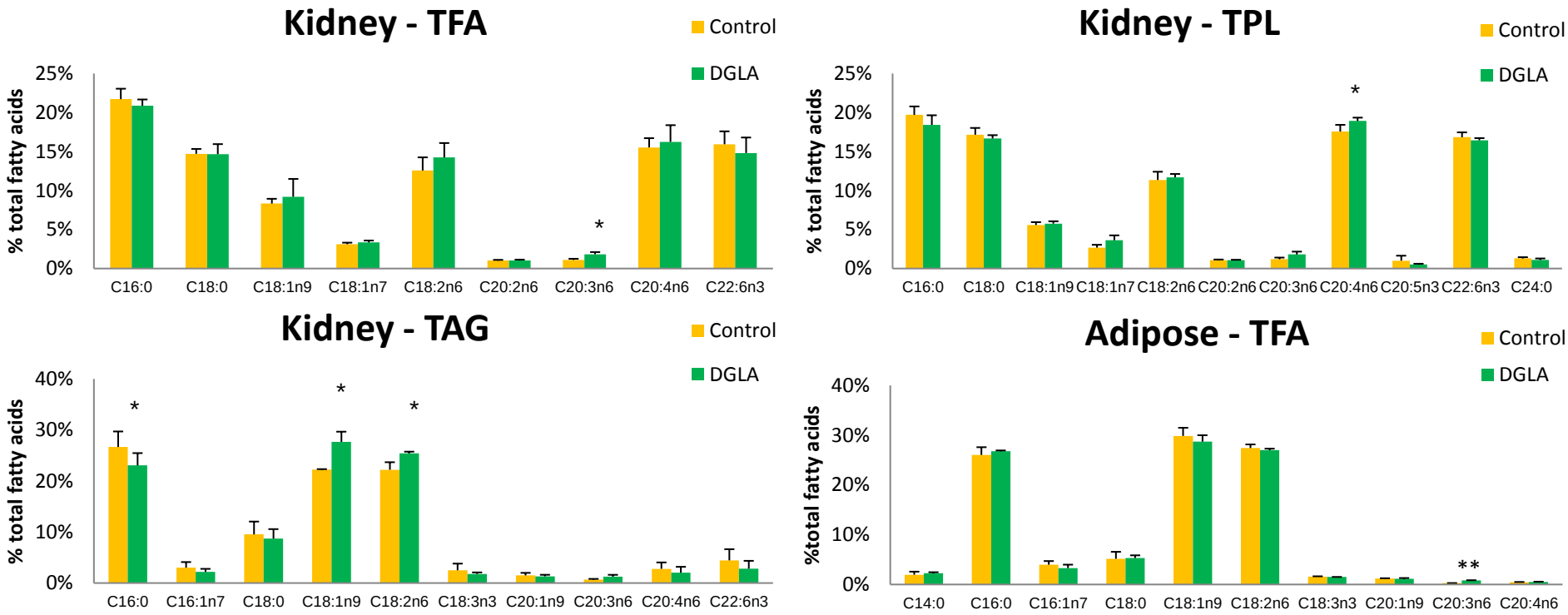


Figure 3.14 – Uptake of DGLA into kidney and adipose tissue lipid fractions *in vivo*

Mice were supplemented with 4% DGLA diet for 48 hours. Animals were sacrificed and tissues collected. Lipids were extracted and separated using 1-dimensional TLC for TPL and TAG. Fatty acid profiles of each fraction were analysed by GC. Graphs display average fatty acid composition as a percentage (+ SD) of 3 mice (Control) and 4 mice (DGLA diet). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc analysis. * P<0.05. ** P <0.005. C14:0, myristic acid; C16:0, palmitic acid; C16:1n7, palmitoleic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, *cis*-vaccenic acid; C18:2n6, LA; C18:3n3, ALA; C20:1n9, eicosenoic acid; C20:3n6, DGLA; C20:4n6, AA; C22:6n3, DHA; C24:0, lignoceric acid.

Table 3.4 – Summary of changes in DGLA and AA in different lipid fractions *in vivo*

Percentage changes in DGLA and AA are displayed as the difference between control and DGLA group. Statistically significant changes (p≤0.05) are underlined.

	Plasma		**Liver**		**Kidney**		**Adipose**	
	DGLA	**AA**	**DGLA**	**AA**	**DGLA**	**AA**	**DGLA**	**AA**
TFA	158%	14%	60%	20%	63%	5%	200%	0%
TPL	200%	58%	50%	5%	50%	7%	-	-
TAG	600%	85%	55%	50%	86%	-29%	-	-
SE	260%	28%	140%	45%	-	-	-	-
PC	-	-	33%	22%	-	-	-	-
PE	-	-	44%	6%	-	-	-	-
PS	-	-	81%	12%	-	-	-	-
PI	-	-	26%	-1.4%	-	-	-	-

3.5 Discussion

3.5.1 DGLA is incorporated into TPL and TAG fractions of THP-1 macrophages

Initial experiments were performed to determine an optimum dose for DGLA to use for lipid analysis studies in THP-1 macrophages. First, a time point of 24 hour incubation with DGLA was optimised to achieve maximum uptake into TPL and TAG fractions. Two concentrations were selected based on common doses used in previous experiments (Iversen *et al.* 1991; Iversen *et al*. 1992; Dooper *et al*. 2003). Incorporation of DGLA into TPL and TAG fractions showed a dose-dependent increase (Figure 3.2). After 24 hours, DGLA had increased by 4% and 7% in TPL on incubation with 50 μM and 100 μM of this fatty acid. Results were comparable to data reported in previous studies. Neutrophils isolated from human subjects taking supplements containing GLA showed a dose-dependent increase in DGLA (Johnson *et al*. 1997). A dose-dependent increase in DGLA in total neutrophil lipids was observed on supplementation with 3.0 g/day and 6.0 g/day (Johnson *et al*. 1997). Human PBMC treated with 100 μM DGLA for 24 hours resulted in a dramatic rise in relative amount of DGLA (10% into TPL) (Dooper *et al*. 2003). After 48 hours there was only a further 2-3% increase in DGLA incorporation, with saturation reached at 72 hours (Dooper *et al*. 2003). Due to the larger increase in incorporation of DGLA with a concentration of 100 μM and the supporting data in the literature using this concentration, 100 μM was used in further experiments.

Following on from the dose response data, the incorporation of DGLA into TPL and TAG was analysed further using only the selected concentration (Figure 3.3). Treatment with DGLA significantly increased its incorporation into TPL and TAG fractions of THP-1 macrophages. Previous lipid uptake studies with the omega-6 fatty acids DGLA and GLA have shown comparable results. Treatment of PBMC with 100 μM DGLA increased incorporation of DGLA and LA into phospholipids (Dooper *et al*. 2003). Supplementation of mouse peritoneal macrophages with DGLA *in vitro*, showed rapid incorporation of DGLA after 3 hours and neutrophils isolated from human subjects taking supplements containing GLA also showed increased concentration of DGLA (Chapkin and Coble 1991).

In addition to this, on treatment of THP-1 macrophages with 100 μM DGLA there was a small (<1%) but non-significant reduction in the incorporation of EPA, DPA, DHA and an increase in LA into TPL (Figure 3.3). This was also observed in TAG fractions where there was a small non-significant decrease in DPA and DHA. This again was

comparable to previous studies. Treatment of PBMC with 100 µM DGLA led to no significant change in EPA, DPA and DHA incorporation (Dooper *et al*. 2003).

Given that DGLA can be metabolised to AA by the action of delta-5 desaturase, it was important to determine the incorporation of this fatty acid into lipid profiles of THP-1 macrophages due to its links to a pro-inflammatory status. On treatment with DGLA, AA levels in TPL were reduced by less than 1% (Figure 3.3), moreover this was not significant. In TAG lipid fractions there was a small increase of less than 0.3%. Previous work has shown small changes in the incorporation of AA on treatment with GLA or DGLA in macrophage and neutrophil cell lines. Supplementation of mouse peritoneal macrophages with GLA *in vitro* resulted in rapid increase in DGLA after 3 hours. This was accompanied by a small increase in AA at a later time of 20 hours (Chapkin and Coble 1991). Neutrophils isolated from human subjects taking supplements containing GLA showed increased concentrations of DGLA in lipid fractions. This was accompanied by an increase in AA. However, this was not significant at concentration used (Johnson *et al*. 1997). The AA:DGLA ratio decreased from 5.4:1 to 2.3:1 in three weeks following supplementation with 6.0 g/day GLA (Johnson *et al*. 1997). Conversely, treatment of PBMC with 100 µM DGLA was associated with a decrease in the levels of AA in TPL (Dooper *et al*. 2003). Macrophages and neutrophils rapidly elongated GLA to DGLA, with some limitations seen in the further desaturation to AA (Chapkin and Coble 1991; Johnson *et al*. 1997). This indicates macrophages possess an extremely active elongase and modest delta-5 desaturase activity (Chapkin and Coble 1991).

Previously it had been shown that the AA present in macrophages was not synthesised by the macrophage itself but acquired from a secondary source (Chapkin *et al*. 1988). AA can be synthesised in liver from LA or obtained directly through the diet. It is packaged into serum lipoproteins as NEFA, TAG, cholesteryl ester or phospholipids and delivered to cells, including inflammatory cells. AA can then be incorporated into phospholipid and non-polar lipid pools (Chilton *et al.* 1996). Mouse peritoneal macrophages were shown to have limited desaturase activity which leads to an inability of DGLA to be readily metabolised to AA. This limitation was also observed in neutrophils and platelets (Chapkin *et al*. 1988). This indicates that once DGLA is taken up into macrophage and other inflammatory cells, it's conversion to AA will be very limited or non-existent.

3.5.2 DGLA is incorporated into individual phospholipids of THP-1 macrophages

TPL can be separated into individual lipid classes by 2-dimensional TLC. Following on from DGLA accumulation into TPL, analysis was expanded to determine the extent of incorporation into individual phospholipids classes. TPL were separated into 6 classes of phospholipids, PC, PS, PI, PE, sphingomyelin and cardiolipin (Figure 3.4). The majority of THP-1 macrophage membranes were composed of PC and PE with smaller amounts of PS and PI. Sphingomyelin and cardiolipin were minor classes and were not included in fatty acid analysis.

PE contained the highest proportion of DGLA and AA in the fatty acid profile of THP-1 macrophages (Figure 3.5). This was also observed in human neutrophils whereby more than 60% of AA from TFAs was located in PE. Similarly, over 40% of DGLA was also associated with PE (Johnson *et al*. 1997). This indicates AA and DGLA reside in similar lipid pools. On treatment of THP-1 macrophages with DGLA, there was an increase of this PUFA into PC, PS, PI and PE (Figure 3.5). No statistics could be performed due to lack of sufficient independent experiments. In all classes there was either no change or an increase of less than 1% in AA levels. The same was observed for omega-3 fatty acids EPA, DPA and DHA. Comparable results have been observed previously. Following supplementation of GLA in humans, there was an increase in the incorporation of DGLA into PE, PI, PS and PC in isolated neutrophils (Johnson *et al*. 1997). There was no significant increase in AA observed. In addition, three weeks supplementation with GLA actually reduced the ratio of AA:DGLA from 8.3:1 to 4:1 in PE. This may suggest that prolonged supplementation with DGLA in macrophages would continue to elevate DGLA levels with no subsequent increase in AA.

Lipid analysis indicates that in THP-1 macrophages, there was a significant accumulation of DGLA in TPL and TAG fractions with no significant change in omega-3 fatty acids or increase in AA accumulation. The latter may be attributed to the low activity of delta-5 desaturase in macrophages as reported previously (Chapkin *et al*. 1988; Chapkin and Coble 1991; Johnson *et al*. 1997). This selective accumulation of DGLA without a subsequent increase in incorporation of AA may result in an alteration in the inflammatory potential of macrophages. Changes in the fatty acid composition of lipids alter the pattern of release of fatty acids and, hence, eicosanoid production. In one study, following supplementation with GLA, there was a dramatic increase of up to 200% in DGLA released from the phospholipids of neutrophils following stimulation of the cells with this fatty acid. There was no change in the release of AA (Johnson *et al*. 1997). As discussed previously in Section 3.1.3, AA classically gives

rise to pro-inflammatory eicosanoids while metabolism of DGLA gives rise to anti-inflammatory mediators. Therefore, increasing the incorporation of DGLA into lipid of THP-1 macrophages may increase the accumulation of anti-inflammatory mediators. To test this hypothesis, eicosanoid production was measured from THP-1 macrophages and is discussed in the following section.

3.5.3 Eicosanoid production from THP-1 macrophages

To determine if changes in fatty acid incorporation of DGLA into lipid fractions of THP-1 macrophages affected the production of eicosanoids, their synthesis was measured by HPLC-MS.

3.5.3.1 COX metabolites

DGLA and AA are substrates for COX enzymes and are converted to series 1 and 2 prostaglandins, respectively. The major species of PGs detected from THP-1 macrophages were PGE_1 and PGE_2. The various roles of these two eicosanoids are detailed in Table 3.2. It was hypothesised that due to the increase in DGLA incorporation into THP-1 macrophage lipid fractions, DGLA metabolites would increase. Conversely, there was no observed change in AA uptake into TPL and TAG fractions of THP-1 macrophages following DGLA treatment and, therefore, it was hypothesised that PGE_2 synthesis from these cells would remain constant.

On treatment with 100 μM DGLA, there was a significant increase in the concentration of PGE_1 from negligible to 180 pg/ml. Following supplementation, there was no difference in the concentration of PGE_2 from vehicle treated to DGLA treated macrophages (Figure 3.7-3.8). Similar observations have been reported both in *in vitro* and *in vivo* studies. On treatment of mouse peritoneal macrophages with GLA there was a significant increase in PGE_1 following stimulation with this fatty acid (Chapkin and Coble 1991). No series 2 prostaglandins were detected in the study (Chapkin and Coble 1991). In human mononuclear leukocytes, incubation with DGLA resulted in no change in the levels of PGE_2 but a dose-dependent increase in PGE_1 (Iversen *et al*. 1992). NC/Tnd mice, a model of atopic dermatitis, fed a diet containing DGLA induced the production of lipid mediators in the skin. DGLA induced synthesis of series 1 prostaglandins; PGE_1, PGD_1, $PGF_{1\alpha}$. However in contrast to *in vitro* studies, DGLA feeding *in vivo* also significantly induced the synthesis of series 2 prostaglandins, PGD_2 and PGE_2 (Amagai *et al*. 2015). Despite this, the study reported a significant role for DGLA in inhibition of atopic eczema, attributed to the production of PGD_1. Similarly, in rats supplemented with DGLA, the levels of both PGE_1 and PGE_2 were induced (Umeda-Sawada *et al*. 2006). However, the induction of PGE_1 in

comparison to PGE_2 was substantially larger. DGLA increased the levels of PGE_1 in rat plasma to four times of that seen in control diet (Umeda-Sawada *et al*. 2006). There was an increase of less than 2-fold in the concentration of PGE_2 (Umeda-Sawada *et al*. 2006). In addition to this, the ratios of PGE_1:PGE_2 and PGD_1:PGE_2 were significantly increased on DGLA supplementation (Umeda-Sawada *et al*. 2006). This indicates that *in vivo* the balance between the production of DGLA and AA-derived eicosanoids plays an important role. As shown in Figure 3.7 and 3.8, the concentration of PGE_1 did not exceed that of PGE_2 in any experiment. However the ratio of PGE_2:PGE_1 on supplementation with DGLA reduced dramatically from 500:1 to 2.6:1. Supplementation with DGLA may therefore offset the balance of pro- and anti-inflammatory prostaglandin production, resulting in an overall anti-inflammatory effect (Umeda-Sawada *et al*. 2006).

Pro-inflammatory cytokine IFN-γ was also included in experiments to evaluate the effect of an inflammatory background on eicosanoid synthesis and the role of DGLA in an inflammatory state. IFN-γ alone had no effect on the production of PGE_1 and PGE_2 in comparison to control. However, there was a significant change when THP-1 macrophages were pre-treated with DGLA followed by stimulation with IFN-γ. On treatment with a combination of DGLA and IFN-γ in THP-1 macrophages, the concentration of PGE_1 was approximately twice that of DGLA-only treated macrophages; however the groups were not significantly different. Similarly, under the same conditions, there was a significant induction in the production of PGE_2 in comparison to all other treatments. Fatty acid analysis of THP-1 macrophages treated with DGLA followed by IFN-γ stimulation showed no increase in incorporation of AA into TPL or TAG in comparison to control (Appendix 2). This indicated that the increase in PGE_2 production was not as a result of an increase in AA incorporation into lipid pools and, therefore, suggesting that DGLA and IFN-γ were having a synergistic effect on prostaglandin synthesis. To begin to understand this effect, the mRNA expression of COX enzymes was measured using RT-qPCR.

COX enzymes have two isoforms; COX-1 and COX-2. The expression patterns differ between cell types. COX-1 is constitutively expressed, while COX-2 is inducible (Matsuura *et al.* 1999; Levin *et al*. 2002). Both forms of the enzyme have been implicated in the production of prostaglandins to varying degrees (Matsuura *et al*. 1999; Noguchi *et al.* 1999; Caughey *et al.* 2001; Levin *et al*. 2002). For COX-1, IFN-γ had no effect on mRNA expression, however both DGLA and DGLA in combination with IFN-γ stimulation, reduced the mRNA expression of the gene. For COX-2, IFN-γ and DGLA treatments in isolation had no effect on the mRNA levels in comparison to

control. However in combination, treatment with DGLA followed by IFN-γ significantly induced the expression of COX-2 mRNA. Future work should confirm changes in COX-1 and COX-2 expression at the protein level and determine enzyme activity.

The relationship between IFN-γ and COX expression as reported in the literature is controversial. In human epidermal keratinocytes, treatment with the pro-inflammatory cytokines induced the mRNA and protein expression of COX-2 (Matsuura *et al*. 1999). COX-1 expression was reduced with IFN-γ treatment (Matsuura *et al*. 1999). In mouse peritoneal macrophages, IFN-γ induced the expression of COX-2 at the mRNA and protein levels, with a modest decrease in COX-1 expression (Blanco *et al.* 2000). Other studies reported opposing results. In human fibroblasts, IFN-γ had no effect on COX-1 or COX-2 expression (Noguchi *et al*. 1999) and in human macrophages the cytokine dose-dependently reduced COX-2 levels induced by IL-1β (Barrios-Rodiles and Chadee 1998). The data presented in Figure 3.9 indicated that both DGLA and IFN-γ were required for the increase in COX-2 expression. This may be explained by IFN-γ priming. Thus, previous studies, many of which reported no effect of IFN-γ on COX-2 expression, have found induction of expression following priming of the cells. IFN-γ significantly increased the expression of COX-2 in human macrophages following priming with LPS or TNF-α (Ariasnegrete *et al.* 1995; Barrios-Rodiles and Chadee 1998). COX-1 expression was unchanged (Ariasnegrete *et al*. 1995). This may suggest that pre-treatment with DGLA acts to prime THP-1 macrophages so that IFN-γ can induce COX-2 expression, as seen in Figure 3.9.

The significant increase in the expression of COX-2 following treatment with DGLA and IFN-γ coincides with the increase in the production of PGE_1 and PGE_2 under identical conditions. In addition, the decrease in COX-1 expression in DGLA treatment and in DGLA followed by IFN-γ treatment is accompanied by an increase in the production of PGE_1 under these conditions. This suggests that in THP-1 macrophages, COX-2 may be responsible for PGE_2 production and in part, PGE_1. As mentioned previously, COX-1 and COX-2 differ in their expression between cell types. The specific role of each isoform in formation of prostaglandins has not been fully established. A study investigating the role of COX-1 and COX-2 in DGLA and AA metabolism indicated that COX-2 predominates over COX-1 in production of prostaglandins (Levin *et al*. 2002). Production of PGE_1 from DGLA via COX-1 peaked at 40 ng/ml, while metabolism via COX-2 peaked at 120 ng/ml (Levin *et al*. 2002). It was also observed that COX-2 had a higher affinity for DGLA and AA than COX-1 and increasing the concentrations of the two PUFAs, decreased COX-1 activity (Levin *et al*. 2002). In addition, a study reporting eicosanoid synthesis from HUVEC cells,

unstimulated cells (which constitutively express COX-1) produced predominantly TXA_2 (Caughey *et al*. 2001). Following induction of COX-2 expression with pro-inflammatory cytokine IL-1β, there was a large increase in the production of PGE_1 and PGE_2, 54-fold and 84-fold respectively on comparison to untreated cells (Caughey *et al*. 2001). Finally, selective COX-2 inhibitors in human fibroblasts specifically reduced the production of PGE_2 following stimulation (Noguchi *et al*. 1999).

Taken together the data presented in Figures 3.7-3.9 and previous studies, allow a number of conclusions can be drawn. DGLA may act to prime THP-1 macrophages to induce COX-2 expression, which mirrors an increase in the production of PGE_2 under the same conditions. Treatment with DGLA attenuates the expression of COX-1 in macrophages, which under the same conditions significantly induced the production of PGE_1. This suggests that COX-2 predominates over COX-1 in the production of PGE_1 and PGE_2 by THP-1 macrophages.

3.5.3.2 LOX metabolites

LOX metabolism of DGLA and AA produces a wide variety of hydroxyeicosanoids. They have a range of actions, as detailed in Table 3.2. Hydroxyeicosanoids produced from AA play a number of pro-inflammatory roles. They can also act as substrates to yield a number of other eicosanoids, with both pro- and anti-inflammatory roles. For example, the action of 5-LOX on AA produces 5-HPETE which is a precursor for pro-inflammatory LT production (Samuelsson *et al*. 1987; Neels 2013). In contrast, metabolism of 15-HETE from AA, acts as a precursor for lipoxin production, that has been attributed to resolving inflammation (Goh *et al*. 2001; Ho *et al*. 2010).

The effect of DGLA supplementation on the synthesis of hydroxyeicosanoids was measured by HPLC-MS (Figure 3.6). There was no effect on the production of AA metabolites (5-, 11-, 12- and 15-HETE) on treatment with DGLA when compared to control. This result indicates that there was no increase in metabolism of AA through LOX pathways on treatment with DGLA. Again, given the significant increase in the accumulation of DGLA and no change in AA, this result was as expected.

There was, however, a significant increase in the production of the DGLA metabolite 15-HETrE. This has also been observed previously *in vivo.* DGLA significantly up-regulated 8-HETrE and 15-HETrE in NC/TnD mice (Amagai *et al*. 2015). 15-HETrE has been well documented to inhibit the production of pro-inflammatory LTB4. Supplementation of 3.0 g/day of GLA reduced the levels of LBT4 from human neutrophils (Johnson *et al*. 1997) while DGLA significantly reduced the production of

LBT4 from rat macrophages (Nakamura *et al*. 1993). Dose-dependent decreases in LTB4 formation from human mononuclear leukocytes was associated with a dose-dependent increase in 15-HETrE formation (Iversen *et al*. 1992). Incubation with 15-HETrE did not change PGE_2 production but was a potent inhibitor of LBT4 production, more so than incubation with DGLA (Iversen *et al*. 1992). The anti-inflammatory roles of 15-HETrE may be an effect of reducing the production of LBT4 and, therefore, its pro-inflammatory roles. LTB4 production was not measured in the present study and could potentially be included in future work. Overall, the data indicate a potential anti-inflammatory role for DGLA through the production of eicosanoids.

3.5.4 Uptake of algal sourced DGLA *in vivo*

In vivo studies were performed to determine the uptake of dietary DGLA. In addition to this, the study provided insight into the use of a potential new source of DGLA in the form of mutant algae *P. incisa*. Initially, the standard chow diet of mice was supplemented with 2% (w/w) freeze-dry algal powder containing approximately 30% DGLA, to determine if this could be digested *in vivo* and utilised as a dietary source of fatty acids. Mice were sacrificed using schedule 1 procedures and a sample of plasma was analysed following 48 hours feeding to determine fatty acid profile. It was that observed there were no significant differences between the profiles of control and DGLA-fed groups (Appendix 1). Faeces were next analysed. As shown in Figure 3.10, the DGLA group excreted an increased amount of DGLA in comparison to the control group. This indicated that mice could not digest the whole algae and were unable to utilise fatty acids contained within.

Following this, lipids were extracted from algal powder prior to feeding as detailed in Section 2.2.13. The fatty acid composition of the diet of control and the DGLA-fed groups is detailed in Table 3.3. DGLA content in the control group was negligible. On addition of the lipid extracts from *P. incisa*, the overall percentage of DGLA accounted for 4.4% of total fatty acids. Male C57BL/6 mice were starved for 24 hours prior to being given either control chow diet or the diet containing 4.4% (w/v) DGLA for 48 hours. Mice were sacrificed using schedule 1 procedures and samples of plasma together with liver, kidney and adipose tissue were taken for lipid analysis. Table 3.4 summarises the changes observed in the incorporation of DGLA and AA from *in vivo* feeding studies. Values that are underlined indicate statistically significant changes. The relative amount of DGLA significantly increased in all of the lipid fractions measured except kidney TPL and TAG, where the increases were not significant. For AA, despite a general trend of increased incorporation in DGLA group, only

incorporation into liver TAG fraction and kidney total phospholipids was significant. The results are discussed in more detail in the following sections.

3.5.4.1 Plasma

On feeding with diet containing 4.4% DGLA extracted from *P. incisa*, the levels of this PUFA were significantly increased in the plasma of mice. DGLA was significantly increased by 2% in TFA isolated from the plasma (Figure 3.11). When separated by 1-dimensional TLC there was an increase of 3%, 1.2% and 2.6% in TPL, TAG and steryl ester fractions respectively. This indicated that incorporation of DGLA was primarily associated with the polar lipid fraction of plasma, followed by steryl esters and TAG. There was no significant increase in the levels of AA into any of these fractions despite a general trend to increase. In the DGLA group there was also a significant decrease in the levels of EPA in TFA and steryl ester fractions of the plasma. Similar results have been observed in previous studies. Single doses of 0.1-2.0 g of DGLA in humans increased the plasma concentrations of the PUFA in plasma lipid fractions (Kernoff *et al*. 1977). Studies in rats showed significant increase in the concentration of DGLA in the plasma when infused with an emulsion containing 10% tridihomo-gamma-linolenoyl-glycerol (Nakamura *et al*. 1993). DGLA supplementation significantly increased the levels of this PUFA in the serum of rats, along with an increase in AA (Umeda-Sawada *et al*. 2006). There was also a significant decrease in oleic acid, LA, EPA and DHA (Umeda-Sawada *et al*. 2006). Following supplementation with GLA, levels of GLA, DGLA and AA all significantly increased in human serum lipids. The increases were significant after 2 weeks supplementation (Johnson *et al*. 1997). In addition to this, the increase in DGLA and AA was found specifically to phospholipids and no other lipid classes (Johnson *et al*. 1997).

3.5.4.2 Tissues

Samples of liver, kidney and adipose tissue were taken to determine the effect of a DGLA-containing diet on the lipids of these tissues. In the liver, the DGLA-fed group showed a 1% increase in the incorporation of DGLA into TFA (Figure 3.12). On further analysis it was found that DGLA was increased by 0.9%, 0.5% and 2.2% into TPL, TAG and steryl esters respectively. There was a significant increase in AA in TAG fraction of liver of 0.9% and an increase of 0.9% and 2.2% in TPL and steryl esters respectively. However these were not significant. This suggested that DGLA and AA were primarily associated with steryl esters in the liver, followed by TPL and TAG. In addition, there was also a decrease in the level of DHA into TPL.

Due to the large amount of lipid extracted from the liver, further analysis was performed to establish the effect of the DGLA diet on fatty acid profiles of individual phospholipid classes. It was found that there was a significant increase in DGLA into PS, PE and PC (Figure 3.13). There was also an increase in incorporation into PI. However, this was not significant. DGLA primarily accumulated in PC, with the largest increase of 1.2%. This was also the case for AA, with an increase of 2.2%. However this was not significant. In addition, there was also a significant decrease in the level of DHA and EPA into PE, along with a decrease in DHA incorporation into PC.

In the kidney, DGLA increased by 0.7% into TFA in the DGLA group; however, there were no significant increases in the TPL or TAG fractions (Figure 3.14). Levels of AA into TPL increased significantly by 1.3%. However, in the TAG fraction, there was a significant decrease in the relative amount of AA by 0.8%. Finally in adipose tissue, DGLA levels significantly increased by 0.5% into TFA. Given the small amount of tissue isolated during dissection, no further analysis was performed on adipose tissue. There were no other significant changes in fatty acid profiles observed.

Previous work detailing the incorporation of DGLA into tissues has been limited with relatively few studies having reported results. Fatty acid composition in the liver of rats supplemented with DGLA (in the form of TAG) showed a significant increase in the accumulation of the PUFA. The accumulation of AA also increased significantly (Umeda-Sawada *et al*. 2006). In a dose-response experiment, AA levels were not significantly different from control at lower levels of DGLA supplementation in the liver and plasma. However AA levels increased in a dose-dependent manner. There was also a dose-dependent decrease in the concentration of DHA (Umeda-Sawada *et al*. 2006). In ApoE deficient mice supplemented with DGLA for 6 months, the percentage of DGLA in liver phospholipids increased significantly (Takai *et al*. 2009). There was no increase in the levels of AA after 6 months (Takai *et al*. 2009).

In human cells, isolated steryl esters will be almost entirely in the form of cholesteryl esters. Accumulation of PUFA in cholesteryl esters has previously been shown to alter its accumulation and metabolism. In one study, on incubation of macrophages with omega-3 fatty acids, EPA and DHA were significantly accumulated in the cholesteryl ester fraction (Lada *et al.* 2003). This was accompanied by a physical change in the fluidity of cholesteryl ester droplets resulting in increased hydrolysis and efflux of cholesterol from the cell (Lada *et al*. 2003). Omega-6 fatty acids were also used in the study, with LA the major component. There was a significant accumulation of LA in cholesteryl ester fractions, with no elevation of other omega-6

fatty acids measured. Omega-6 supplementation had a significant effect on cholesterol efflux, however this was not as pronounced as seen in omega-3 group (Lada *et al*. 2003). Given the accumulation of DGLA into cholesteryl esters *in vivo* observed in Figures 3.11 and 3.12, it would be interesting to determine the effect of this accumulation on cholesteryl ester physical state and any resulting changes in metabolism and efflux.

In vivo feeding studies with DGLA have previously been limited, particularly in measuring the effect of supplementation on fatty acid profiles of tissues. Overall, results of *in vivo* studies presented in this chapter indicated that a 4.4% DGLA-containing diet, extracted from mutant algae *P. incisa,* allowed for significant incorporation of DGLA into plasma and tissues of liver, kidney and adipose lipid fractions after just 48 hours feeding. In most cases this was accompanied by no significant increase in AA incorporation. However, the general trend suggested that a DGLA diet increased AA incorporation in the majority of lipid fractions measured. This is shown in Table 3.4. An increase in AA incorporation following DGLA supplementation has also been observed in previous studies. In plasma, supplementation with DGLA increases the concentration of the PUFA in lipid fractions including an increase of AA (Johnson *et al*. 1997; Umeda-Sawada *et al*. 2006). In addition, rats supplemented with dietary DGLA significantly accumulated both DGLA and AA in liver (Umeda-Sawada *et al*. 2006).

It is known that different cell types have different desaturase and elongase activities (Johnson *et al*. 1997), which may act to explain the differences in the accumulation of DGLA and AA seen in *in vitro* and *in vivo* experiments presented in this chapter. As previously discussed in Section 3.5.1, macrophages, among other cell types, have been reported to have limited desaturase activity (Chapkin *et al*. 1988; Johnson *et al*. 1997). Several tissues however, contain both high activities of elongase and desaturase enzymes including the liver, kidney, brain and intestines (Johnson *et al*. 1997). This may suggest that in cell types and tissues such as those with active desaturase activity, DGLA would be metabolised to AA and both PUFAs would be accumulated. Despite this, another study performed in an atherosclerotic mouse model supplemented with DGLA, indicated an accumulation of DGLA in liver phospholipids with no subsequent AA accumulation after 6 month feeding (Takai *et al*. 2009). Given the relatively short feeding time adopted in our experiments it is difficult to assess the long term effect of DGLA and AA accumulation into lipid fractions of mouse tissue following feeding. A larger period of feeding in the experiment may have also significantly increased levels of AA as well as DGLA, given

the trend of increase observed. Future experiments could act to establish the incorporation of DGLA and AA in a time-dependent manner.

3.5.4.3 Omega-3 incorporation

It was observed *in vivo* that on feeding with a DGLA diet, there were percentage changes in the levels of omega-3 fatty acids EPA and DHA into lipid fractions. For example in liver phospholipids, percentage level of DHA was decreased in TPL (Figure 3.13) and EPA in the total fatty acids of plasma (Figure 3.11). A general trend of reduced levels of omega-3 fatty acids was observed in the DGLA group. This has also been shown previously *in vitro.* Analysis in a mouse fibrosarcoma cell line indicated that omega-6 fatty acids reduced the levels of EPA into cellular phospholipids and promoted their re-distribution to TAG fractions (Rubin and Laposata 1992). This adverse effect on PUFA incorporation has also been observed in omega-3 studies. EPA and DHA supplementation in humans, lowered the production of 15-HETrE in blistering wounds suggesting these PUFA compete directly for fatty acid incorporation and metabolism with DGLA (McDaniel *et al*. 2011). However, in addition to reducing the incorporation and metabolism of DGLA, omega-3 supplementation changes AA levels. It has previously been shown that omega-3 fatty acids compete with omega-6 fatty acids for delta 5 desaturase, the enzyme responsible for the conversion of DGLA to AA (Rubin and Laposata 1992; Barham *et al.* 2000). Supplementation of EPA in humans significantly increased plasma levels of EPA, while reducing AA levels and the AA:EPA ratio. This was accompanied by an increase in EPA metabolites through LOX and COX pathways (McDaniel *et al*. 2011).

Co-supplementation studies with both omega-3 and -6 fatty acids have combined the benefits of accumulation of DGLA, EPA and DHA while reducing the accumulation of AA in lipid fractions. Co-supplementation of GLA and EPA significantly increased the accumulation of these two PUFA in human serum and neutrophil phospholipids, with no increase in AA (Barham *et al*. 2000). Although isolated neutrophils from patients supplemented with GLA and EPA had similar amounts of AA, there was a significant reduction in leukotrienes released in comparison to control (Barham *et al*. 2000). GLA-containing oils increased AA incorporation into cholesteryl esters and phospholipid fractions of human serum. However, this was not observed on co-supplementation with EPA (Miles *et al*. 2004). Similarly, GLA co-supplemented with EPA and DHA dose-dependently increased DGLA and decreased AA levels in the TPL fraction of human serum (Laidlaw and Holub 2003). Supplementation with a combination of GLA and DHA containing oils in healthy women, significantly increased the levels of GLA, DGLA and DHA in plasma total lipids, TPL and TAG

fractions (Geppert *et al.* 2008). There was no increase in AA observed in total lipids and TPL but a small accumulation in TAG fraction, which returned to control levels after further supplementation (Geppert *et al*. 2008).

Taken together the results presented in this chapter and previous studies show that supplementation with both omega-3 (EPA and DHA) and -6 (GLA/DGLA) fatty acids can increase accumulation of both these desirable anti-inflammatory PUFAs with no rise in the accumulation of AA. This may prove beneficial in disease. Co-supplementation with omega-3 and -6 fatty acids produced more favourable results on risk factors of atherosclerosis compared to omega-3 supplementation alone. In humans the atherosclerotic index decreased by 12% on supplementation with a combination diet, compared to a 6% decrease with omega-3 fatty acids alone (Haglund *et al*. 1998). Future work may act to establish the role of co-supplementation with certain omega-3 and -6 PUFAs and evaluate their combined role on inflammation.

3.5.4.4 *P.incisa* as a source of DGLA

Extraction of total lipids from *P. incisa* (containing DGLA) provided a source of the PUFA in the form of TAG. Studies *in vitro* (Appendix 4) and *in vivo* (presented in this chapter) indicated that DGLA isolated from *P. incisa* increased the levels of DGLA in polar, TAG and steryl ester lipid fractions of macrophages and mouse plasma and tissues. In addition to this, a preliminary experiment indicated that DGLA (hydrolysed from the total lipid extracts of mutated algae: method detailed in 2.2.13.1) inhibited pro-inflammatory gene expression in THP-1 macrophages (Appendix 5) comparable to that observed with pure DGLA (data presented in Chapter 4).

Previously there has been some controversy regarding the use of polar lipid or TAG as dietary sources of fatty acids. A number of studies have investigated the effects of omega-3 PUFAs, EPA and DHA, when administered as polar lipid or TAG. In mice fed a high fat diet, omega-3 fatty acids administered in polar lipids were observed to reduce obesity and glucose intolerance more so than TAG derived fatty acids (Rossmeisl *et al.* 2012). Omega-3 fatty acids in polar lipids showed better accumulation and bioavailability in comparison to the TAG. However, it was also observed that omega-3 fatty acids from TAG lowered cholesterol levels more than using the polar lipid (Rossmeisl *et al*. 2012). In addition, it was observed that in mice fed polar lipid and TAG forms of omega-3 fatty acids, both had similar effects on inflammatory profiles (Awada *et al.* 2013) but concentrations of EPA and DHA in the plasma were higher in the TAG group to that of polar lipid group. There was however

a significant effect on adipose tissue cell size in the polar lipid diet, which was not significant in the TAG diet despite an observed decrease (Awada *et al*. 2013). This suggested that polar lipid carrier was more effective at reducing body fat deposition than the TAG carrier (Awada *et al*. 2013).

Fish oils are a rich source of omega-3 fatty acids in the TAG form, whereas krill oil contains omega-3 fatty acids primarily in the polar lipid form. On feeding mice with these two forms of omega-3 fatty acids, it was observed that they were comparable dietary sources (Vigerust *et al.* 2013). There was no difference between carriers on the effects of lowering inflammation and cholesterol levels. However there was a small preference for krill oil over fish oil in promoting lipid catabolism (Vigerust *et al*. 2013). Finally, another study investigating the role of krill oil and fish oil reported, again, that both sources were comparable (Ulven *et al.* 2011). They observed a significant increase in EPA, DHA and DPA incorporation into plasma lipid fractions of humans following supplementation, which was not significantly different between krill oil and fish oil groups (Ulven *et al*. 2011). However, lower doses of krill oil were needed in comparison to fish oil (Ulven *et al*. 2011).

Taken together, previous studies suggest that dietary sources of fatty acids from polar lipid and TAG are comparable and there is no significant difference between their effect on the regulation of inflammation and cholesterol homeostasis (Ulven *et al*. 2011; Rossmeisl *et al*. 2012; Awada *et al*. 2013). However, studies suggest a slight advantage of PUFA in polar lipid form on obesity and regulation of adiposity (Rossmeisl *et al*. 2012; Awada *et al*. 2013). Despite this, TAG remains a good dietary source of PUFA *in vivo* and studies utilising DGLA contained in TAG form from *P. incisa* provide promising insight into its commercial use as source of the PUFA.

CHAPTER 4

THE ROLE OF DGLA ON THE PROPERTIES OF MACROPHAGES AND THEIR FUNCTION IN ATHEROSCLEROSIS

4.1 Introduction

4.1.1 Role of macrophages in atherosclerosis

Macrophages are key players in diseases associated with chronic inflammatory disorders such as atherosclerosis (Moore and Tabas 2011). Understanding the role of macrophages in atherosclerosis is important in advancing our knowledge of the molecular basis of this disease and for the development of preventative/therapeutic approaches (Dickhout *et al.* 2008). Macrophage foam cell formation is a hallmark of the early stages of atherosclerosis and lays the foundation for advanced plaque development. This process is discussed in further detail in Section 4.1.2. Macrophages also continue to play an important role once an atherosclerotic plaque with foam cells has formed. An on-going release of pro-inflammatory cytokines and pro-apoptotic factors from macrophages continues to increase lesion size and trigger apoptosis of SMCs (Moore and Tabas 2011). In addition, secretion of MMPs destabilises the plaque via degradation of the extracellular matrix increasing the risk of plaque rupture (Dickhout *et al*. 2008). Finally, defective clearance of macrophages/apoptotic cells (i.e. efferocytosis) from advanced plaques results in plaque necrosis and rupture (Moore and Tabas 2011). Targeting macrophages can therefore be of therapeutic interest given their important role in atherosclerosis pathology.

4.1.2 Foam cell formation

Atherosclerosis is a complex multi-stage disease with numerous factors contributing to its pathogenesis. The initial stage of atherosclerosis involves formation of lipid loaded macrophages, commonly referred to as foam cells. Foam cells accumulate in the walls of large arteries and build up to form fatty streaks (Lusis 2000). Although not clinically significant, fatty streaks provide the underlying basis for the maturation of atherosclerotic lesions (Lusis 2012). Targeting foam cell formation as a primary event in atherosclerosis to attenuate or prevent progression of this disease is

therefore of therapeutic interest. In this chapter, the effect of DGLA on numerous stages of foam cell formation and macrophage behaviour was investigated.

4.1.2.1 Initiation and lipoprotein modification

ROS are increased in a number of cardiovascular diseases, including atherosclerosis, which suggests that they play an important role in the pathogenesis of the disease (Heistad *et al.* 2009). Several factors have been linked to increased ROS production including hypercholesterolemia, hypertension, diabetes, obesity, smoking and age, which are also common risk factors in atherosclerosis (Lusis *et al*. 2004). According to the oxidative modification hypothesis, atherosclerosis is the result of oxidative modification of LDL in the arterial wall by ROS (Singh and Jialal 2006; Vogiatzi *et al.* 2009). Accumulation and retention of oxLDL in the arterial wall and oxidative stress can induce the overlying endothelium to release factors promoting an inflammatory response and initiate the process of foam cell formation (Libby 2012). Attenuating ROS production may reduce the formation of oxLDL and prevent the initiation of foam cell formation.

4.1.2.2 Monocyte recruitment and migration

Damage to the endothelial wall of arteries, as a result of high oxidative stress, activates endothelial cells, which express a number of chemokines and adhesion molecules, to recruit and adhere circulating monocytes to the site of activation (Bobryshev 2006). MCP-1 plays a key role in monocyte recruitment. Release of MCP-1 recruits monocytes from the circulation. Once recruited, monocytes roll across the endothelium and firmly adhere to the surface tethered by various adhesion proteins including selectins, integrins, ICAM-1 and VCAM-1 (Lusis 2000; Bobryshev 2006). Monocytes transmigrate through the intracellular junctions of endothelial cells into the arterial intima where they are stimulated to differentiate into macrophages, largely under the control of the chemokine M-CSF (Bobryshev 2006). Subsequent description will focus on two key factors implicated in the recruitment of monocytes, MCP-1 and ICAM-1.

4.1.2.2.1 MCP-1

Chemokines (chemotactic cytokines) are small heparin binding proteins whose main function is to regulate cell trafficking (Deshmane *et al.* 2009). MCP-1 is an inflammatory chemokine, 76 amino acids in length, 13k Da in size and a member of the C-C chemokine family (Deshmane *et al*. 2009). It is expressed by the various cells involved in atherosclerosis following the initiation of a chronic inflammatory response by the activated endothelium in the disease and is a potent chemoattractant for

circulating monocytes. *In vivo* studies have shown that MCP-1 deficiency significantly reduces the extent of atherosclerosis in ApoE knockout mice (Gosling *et al.* 1999). These mice showed smaller, less extensive lesions, with a significantly lower content of macrophages (Gosling *et al*. 1999). In addition, increasing expression of MCP-1 by leukocytes significantly increases the size of atherosclerotic lesions in ApoE knockout mice (Aiello *et al.* 1999). These findings indicate MCP-1 as a potentially promising therapeutic target in atherosclerosis.

4.1.2.2.2 ICAM-1

ICAM-1 is an inducible cell surface glycoprotein belonging to the immunoglobulin supergene family and is expressed on endothelial cells to facilitate the attachment of monocytes recruited to the activated endothelium during the inflammatory response in atherosclerosis (Roy *et al.* 2001). Binding of monocytes to the endothelium allows for their transmigration into the arterial intima where they can differentiate into macrophages and progress to foam cells (Lusis 2000). ICAM-1 is strongly expressed at the sites of atherosclerotic lesions indicating the important role of this adhesion molecule in the pathology of this disease. In addition to this, studies have shown that $ApoE^{-/-}$ $ICAM\text{-}1^{-/-}$ mice have significantly smaller lesions in comparison to $ApoE^{-/-}$ $ICAM\text{-}1^{+/+}$ mice (Kitagawa *et al.* 2002). As with MCP-1, the important role of ICAM-1 in the early inflammatory response indicates that therapeutic targeting of this adhesion protein could play an important role in the prevention of atherosclerosis.

4.1.2.3 Cytokine induced pro-inflammatory gene expression

Cytokines play an important role in foam cell formation by orchestrating an inflammatory response. They include more than 50 secreted factors and have numerous roles in cellular communication (Tedgui and Mallat 2006; Ramji and Davies 2015). Cytokines can be classified into two groups based on their role in inflammation; pro-inflammatory and anti-inflammatory (Tedgui and Mallat 2006). See Section 1.4.1 for more detail on cytokine signalling in atherosclerosis. The expression of many pro-inflammatory cytokines are up regulated in atherosclerosis and contribute significantly to foam cell formation and plaque development (Gupta *et al*. 1997; Kirii *et al*. 2003; Popa *et al*. 2007). Inhibition of pro-inflammatory cytokine signalling is therefore another potential therapeutic target in the prevention and treatment of atherosclerosis. Subsequent description will focus on three important pro-inflammatory cytokines employed in the studies in this chapter and have key roles in atherosclerosis; IFN-γ, IL-1β and TNF-α. See Table 4.1 for the role of the cytokines in pro-inflammatory gene expression.

Table 4.1 – Role of IFN-γ IL-1β and TNF-α in pro-inflammatory gene expression

Cytokine	Role in pro-inflammatory gene expression
IFN-γ	Induce expression of adhesion molecules ICAM-1 and VCAM-1 (Li et al. 1993; Chung et al. 2002). Increase scavenger receptor CXCL 16/SR-PSOX (Wuttge et al. 2004) but down regulate SRA and CD36 (Nakagawa et al. 1998; Grewal et al. 2001)
IL-1β	mRNA levels of VCAM-1 and MCP-1 reduced in $ApoE^{-/-}$ $IL\text{-}1\beta^{-/-}$ deficient mice (Kirii et al. 2003).
TNF-α	$ApoE^{-/-}$ $TNF\text{-}\alpha^{-/-}$ decreased expression of VCAM-1, MCP-1, GM-CSF (Xiao et al. 2009). Using the same knockout system, ICAM-1 and SRA expression were also reduced (Ohta et al. 2005).

4.1.2.4 Inflammasome IL-1β production

Before it can elicit its pro-inflammatory signalling actions, the various cell types must first secrete IL-1β. This involves a multi protein complex known as the inflammasome. The release of mature IL-1β is a multi-step process involving up regulation of the expression of pro-IL-1β followed by inflammasome activation, which allows for caspase-1 dependent cleavage of pro-IL-1β into its active form (Moore *et al*. 2013). The NLRP3 inflammasome has been described as the key regulator of IL-1β production from macrophages and plays an important role in many inflammatory diseases including atherosclerosis (Moore *et al*. 2013). Activation of the inflammasome and their role in IL-1β activation is discussed in more detail in Section 1.4.1.2.1. Studies in THP-1 macrophages have determined the role of the NLRP3 inflammasome in production of IL-1β (Rajamaki *et al*. 2010). Omega-3 fatty acids EPA and DHA have been shown to inhibit inflammasome dependent IL-1β production, indicating PUFAs can play an important role in the process (Yan *et al*. 2013; Williams-Bey *et al*. 2014).

Cholesterol crystals are components of atherosclerotic plaques and were first thought to be deposited in plaques as pathology progressed to later stages and therefore weren't a primary initiating factor of atherosclerosis. Using laser reflection and fluorescence confocal microscopy, cholesterol crystals were observed within 2 weeks

of fat feeding ApoE$^{-/-}$ mice with a high fat diet (Duewell *et al.* 2010). They were localised to immune cells in the sub endothelial arterial layer. Immune cell recruitment and cholesterol crystal deposition progressively increased through feeding (Duewell *et al*. 2010). In human atherosclerotic lesions cholesterol crystals were rich in areas abundant in immune cells. Cholesterol crystals have been observed to induce inflammasome activation in *in vivo* mouse models and *in vitro* THP-1 macrophages (Duewell *et al*. 2010). Given the role of PUFAs and cholesterol crystals in inflammasome activation, the role of DGLA in cholesterol crystal-induced inflammasome activation and IL-1β regulation was investigated.

4.1.2.5 LDL uptake and cholesterol cycle

Once monocytes have migrated across the endothelium layer and into the arterial intima, they are stimulated to differentiate into macrophages. Normally, LDL is taken up by the LDLr expressed on macrophages, which is subject to feedback inhibition at the transcriptional level so limits the uptake of this lipoprotein. Macrophages however also express scavenger receptors that can recognise, bind and internalise modified forms of LDL in an unregulated manner. Once internalised, the cholesteryl ester contained within the LDL is digested to unesterified/free cholesterol by an acid cholesterol ester hydrolase in lysosomes (Daugherty *et al.* 2008). This free cholesterol is re-esterified to cholesteryl esters by the enzyme ACAT (Ghosh *et al*. 2010). Further detail on macrophage cholesterol homeostasis is described in Section 1.2.2.1. This esterified cholesterol has two fates: first it is once again hydrolysed to free cholesterol by one of a group of enzymes collectively referred to as neutral cholesterol ester hydrolase (nCEH) (Sekiya *et al.* 2009) and removed from the macrophage by several extracellular transporters. Second; it is stored as cholesteryl esters in the cytoplasm as lipid droplets (Daugherty *et al*. 2008). The rate-limiting step in the cholesteryl ester cycle in macrophages is the nCEH enzyme reaction allowing for clearance of free cholesterol from the macrophage. Excessive uptake of modified LDL therefore increases the storage of cholesteryl esters in lipid droplets. An increased amount of lipid in the cytoplasm of macrophages promotes the conversion of macrophages to lipid loaded foam cells (Ghosh *et al*. 2010). Inhibition of cholesteryl ester accumulation would therefore attenuate foam cell formation.

4.2 Aims

Atherosclerosis is an inflammatory disorder regulated by cytokines such as IFN-γ, IL-1β and TNF-α. Nutraceuticals represent a promising therapeutic avenue in preventing and limiting atherosclerosis. It is therefore important that a thorough understanding of their actions in atherosclerosis together with the underlying molecular mechanisms is attained. Previous studies have revealed an anti-atherogenic role of DGLA. However, its actions on several key macrophage processes associated with atherosclerosis is poorly understood and formed the basis of studies in this chapter. The major focus was to delineate its effect in pro-inflammatory gene expression and several macrophage properties in this disease *in vitro*. Macrophages play a key role in the initiating stage of atherosclerosis, foam cell formation, right through to plaque progression and rupture (Moore and Tabas 2011; Michael *et al*. 2012). Given their important role in the disease, the action of DGLA on macrophages was the main focus of the project (see Figure 4.1 for the experimental plan employed for studies in this chapter).

4.3 Experimental plan

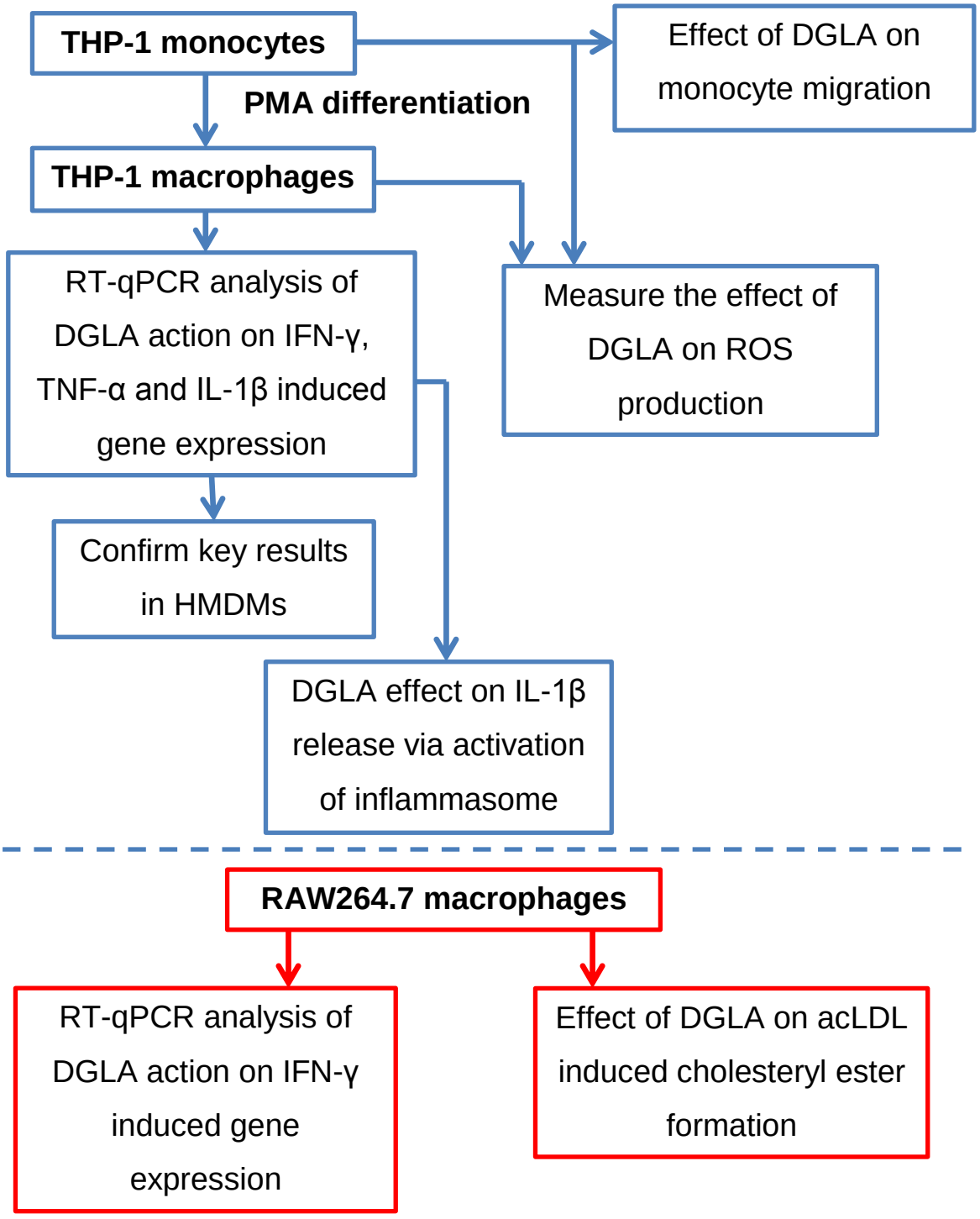


Figure 4.1 – Overview of experimental strategy to determine the effect of DGLA on key steps involving macrophages during atherosclerosis

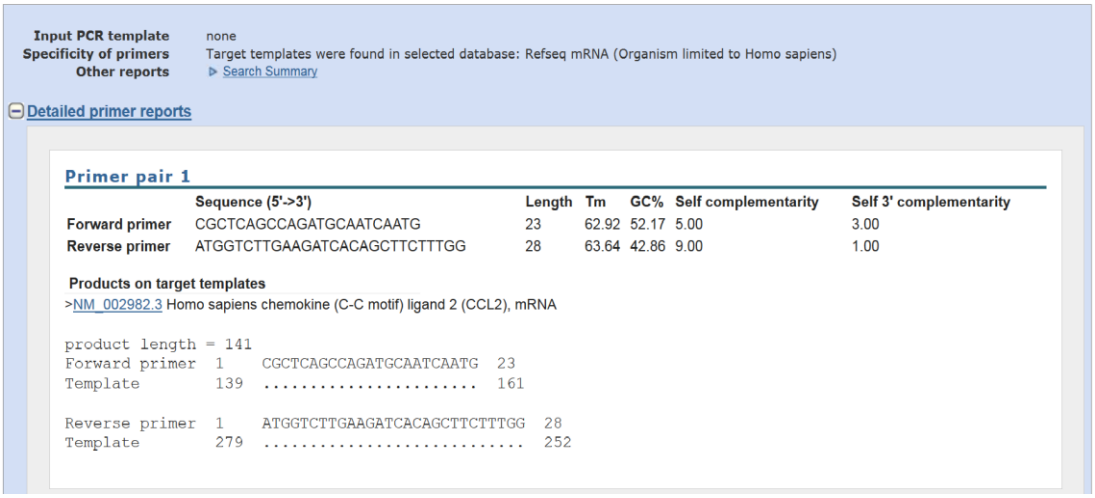
Top (blue); experiments performed in THP-1 cell line. Experiments include in monocytes - migration and ROS production; and in macrophages – ROS production, gene expression and inflammasome activation. Bottom (red); experiments performed in RAW264.7 cell line, including gene expression and acLDL induced cholesteryl ester accumulation.

4.4 Results

4.4.1 Optimisation of PCR conditions

4.4.1.1 Designing PCR primers

Initial optimisation of gene expression was performed using RNA from THP-1 macrophages. RT-qPCR was performed using gene-specific primers. Primers were sourced from literature searches or designed through an online primer design tool. Optimisation of human MCP-1 gene will be used as an example of primer optimisation, which is representative of the steps taken with every sourced or self-designed primer. Primers were first validated using an online primer-blast program to ensure primer sequences were specific to the intended gene. Figure 4.2 depicts an example of the result of a primer-blast search for MCP-1 primers.



Input PCR template none
Specificity of primers Target templates were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)
Other reports ▷ Search Summary

⊖ Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CGCTCAGCCAGATGCAATCAATG	23	62.92	52.17	5.00	3.00
Reverse primer	ATGGTCTTGAAGATCACAGCTTCTTTGG	28	63.64	42.86	9.00	1.00

Products on target templates

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

```
product length = 141
Forward primer  1    CGCTCAGCCAGATGCAATCAATG  23
Template        139  .......................  161

Reverse primer  1    ATGGTCTTGAAGATCACAGCTTCTTTGG  28
Template        279  ............................  252
```

Figure 4.2 – MCP-1 primer blast results

Primer sequences for MCP-1 were analysed by primer-blast online tool. Results indicate that the primers show 100% homology to human chemokine (C-C motif) ligand 2 (CCL2) more commonly referred to as MCP-1.

4.4.1.2 Optimising primer annealing temperatures

Concentrations of cDNA and primers used in RT-qPCR remained the same between every experiment; however primer-annealing temperatures could differ. Primers designed using an online tool were designed to anneal at 60ºC. However, in order to ensure this was indeed the optimum temperature, a PCR was performed with a range of annealing temperatures and the amplification products size fractioned by agarose

gel electrophoresis. Figure 4.3 displays the results of size fractionation of products from a PCR reaction using MCP-1 primers and different annealing temperatures. Bands were quantified and annealing temperature of 60ºC gave the greatest amount of MCP-1 product. This annealing temperature was therefore used in subsequent experiments.

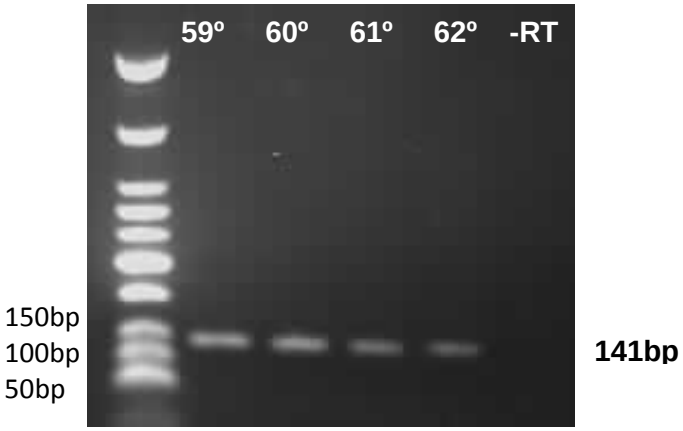


Figure 4.3 – Size fractionation of PCR products

cDNA was subjected to PCR using MCP-1 primers at a range of annealing temperatures (59-62ºC). PCR products were subjected to gel electrophoresis on a 1.5% (w/v) agarose gel for 1 hour at 150V. An optimal annealing temperature of 60ºC was chosen for subsequent studies. The expected product size for MCP-1 is 141bp; the sizes of key markers that migrated near the amplification product are shown on the left of image. –RT; minus reverse transcriptase.

4.4.1.3 Quality of RNA

Before carrying out PCR reactions, RNA preparations were analysed for quality by resolving a small aliquot on a 1.5% (w/v) agarose gel. Good quality undegraded RNA generally produces two distinct bands corresponding to the 28S and 18S rRNA in a 2:1 ratio. An example of such quality of RNA, which was obtained for studies presented throughout this thesis, is shown in Figure 4.4. The data for all the RNA preparation are therefore not shown for subsequent studies.

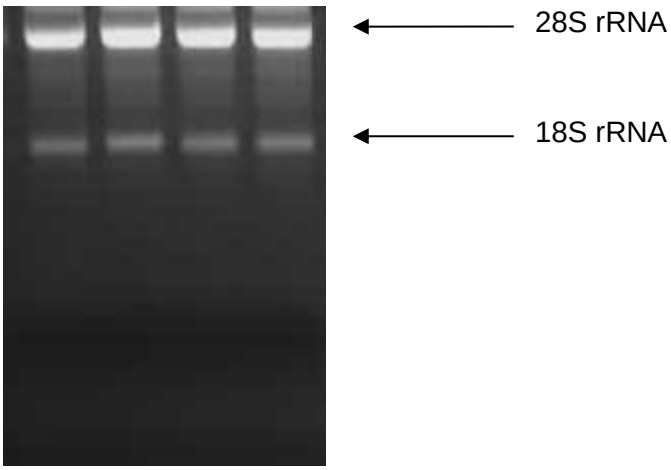


Figure 4.4 – Agarose gel for the assessment of RNA quality

Small aliquots of RNA were resolved on a 1.5% (w/v) agarose gel to assess RNA quality. Samples used were representative of RNA quality throughout experiments carried out in this thesis. Positions of the 28S and 18S rRNA bands are indicated on the right of the image.

4.4.1.4 Melting peak analysis

RT-qPCR was subsequently performed on RNA extracts using SYBR green, which interacts with double stranded DNA and fluoresces. The point at which the level of fluorescence surpasses a set threshold level is termed the Ct value and is used to calculate gene expression. See Section 2.2.8.2 for more detail on RT-qPCR. Amplification curves for GAPDH and MCP-1 are shown in Figure 4.5 (A and C). Following completion of RT-qPCR, melting curve analysis was carried out to ensure a single major peak corresponding to the amplification product. PCR products were subjected to a gradual increase in temperature whereby at a particular temperature the two strands in the DNA molecule dissociates and a drop in florescence is measured. Plotting the change in fluorescence against the change in temperature allows for a graph to be produced with a peak corresponding to the melting temperature of the product. One peak resulting from melting peak analysis indicates one product has been formed. Figure 4.5 (B and D) indicates the melting peaks for RT-qPCRs performed using GAPDH and MCP-1. In each case, single peaks were observed indicating that a single product is being produced and the primers were specific and of high quality. Graphs shown in Figure 4.5 are representative of amplification curves and melting peaks obtained with other primers.

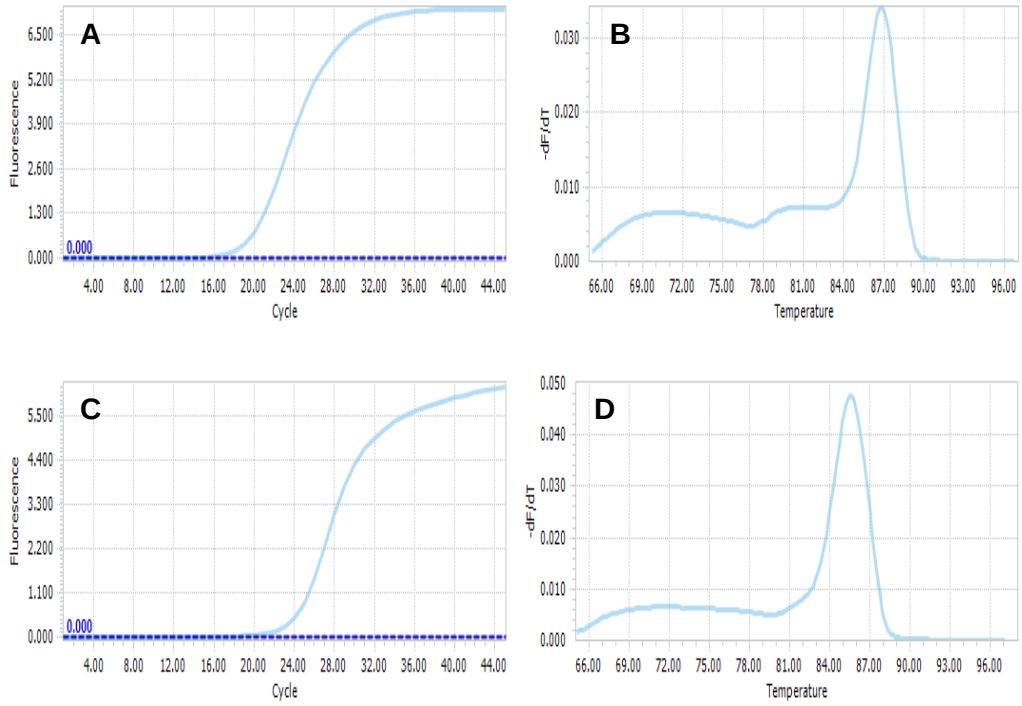


Figure 4.5 – Melting curve analysis

RT-qPCR was performed using GAPDH (A and B) and MCP-1 (C and D) primers. Graphs A and C display sigmoidal curves representative of amplification during the PCR reaction. Melting peaks (B and D) for both GAPDH and MCP-1 respectively indicate that one major product is formed due to the single peak.

4.4.2 IFN-γ dose response experiments in THP-1 macrophages

IFN-γ has been widely utilised previously in the laboratory and is a well-characterised pro-inflammatory cytokine in atherosclerosis. Due to its numerous roles in the initiation and the progression of the disease, the cytokine is often described as a master regulator of atherosclerosis (McLaren and Ramji 2009). IFN-γ was therefore the main focus for the action of DGLA on pro-inflammatory signalling. Previous studies had used a relatively high concentration of IFN-γ (1000 U/ml). A dose response experiment using the IFN-γ inducible MCP-1 as a model gene was carried out to determine if a lower concentration could be used. THP-1 macrophages were treated with 100 U/ml, 250 U/ml, 500 U/ml and 1000 U/ml IFN-γ. MCP-1 mRNA levels were determined by RT-qPCR after 3 hours stimulation with the cytokine, a time point based on previous optimisation experiments in the laboratory monitoring induction of MCP-1 expression. As expected, all concentrations of IFN-γ induced the expression of MCP-1 (Figure 4.6). On treatment with 100 U/ml IFN-γ, the expression of MCP-1

was induced about 24-fold though this was not significant. Increasing the concentration of IFN-γ to 250 U/ml increased the induction level to 165-fold. Treatment with 500 U/ml and 1000 U/ml of the cytokine induced MCP-1 expression by 200-fold and 260-fold respectively. There was no significant difference between the three highest doses of IFN-γ (i.e. 250 U/ml, 500 U/ml and 1000 U/ml). Due to this, and the dramatic increase in the induction of MCP-1 expression when the concentration of IFN-γ was increased from 100 U/ml to 250 U/ml, the latter was selected as the optimum IFN-γ concentration and was used in all further experiments.

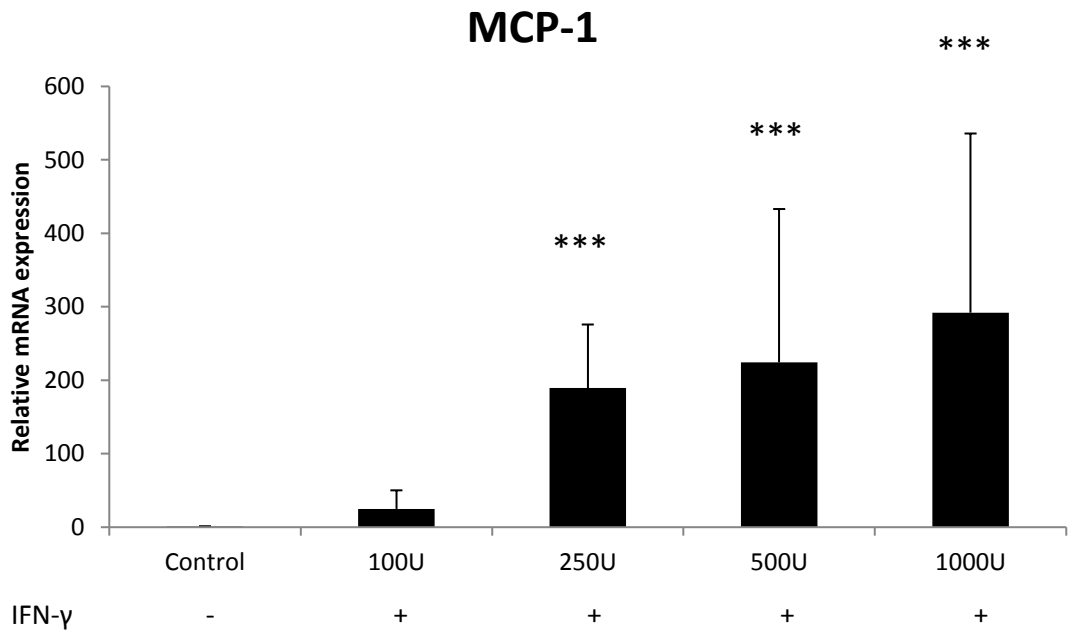


Figure 4.6 – The expression of MCP-1 is induced by several concentrations of IFN-γ

THP-1 macrophages were incubated for 3 hours with 100 U/ml, 250 U/ml, 500 U/ml and 1000 U/ml of IFN-γ or the vehicle control as shown. RNA was extracted and subjected to reverse transcription and RT-qPCR using primers specific for MCP-1 or housekeeping gene GAPDH. Graph displays average fold-change in normalised MCP-1 expression (mean +/- SD) in comparison to control (arbitrarily assigned as 1) from three independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post hoc analysis. *** $P \leq 0.001$

4.4.3 Dose response experiments with DGLA

After determining a suitable dose of IFN-γ for use in subsequent experiments, a range of concentrations of DGLA from 10 µM to 100 µM were evaluated with 250 U/ml of IFN-γ to establish a concentration for optimum inhibition of gene expression. Following PMA differentiation, THP-1 macrophages were pre-incubated with 10 µM, 25 µM, 50 µM and 100 µM of DGLA or the DMSO vehicle control for 24 hours (a time point previously optimised by investigation of DGLA incorporation into polar lipids), followed by treatment with vehicle or 250 U/ml IFN-γ for a further 3 hours. The expression of MCP-1 and ICAM-1, another pro-inflammatory gene known to be up-regulated by IFN-γ (Li *et al*. 2010), was measured by reverse transcription and RT-qPCR. As expected, MCP-1 and ICAM-1 expression was significantly induced by IFN-γ. DGLA at concentrations of 25 µM, 50 µM and 100 µM produced significant inhibition of the IFN-γ-induced expression of MCP-1 by an average of 32%, 46% and 76% respectively (Figure 4.7). A similar trend in the suppression of IFN-γ-induced ICAM-1 expression was also observed with all the concentrations of DGLA used (Figure 4.7). However, the only concentration of DGLA to significantly inhibit the IFN-γ-induced ICAM-1 expression was 50 µM, where an average decrease of 31% was observed. Given the significant inhibition of IFN-γ-induced expression of two key pro-inflammatory genes by 50 µM DGLA, this concentration was used for further gene expression analysis.

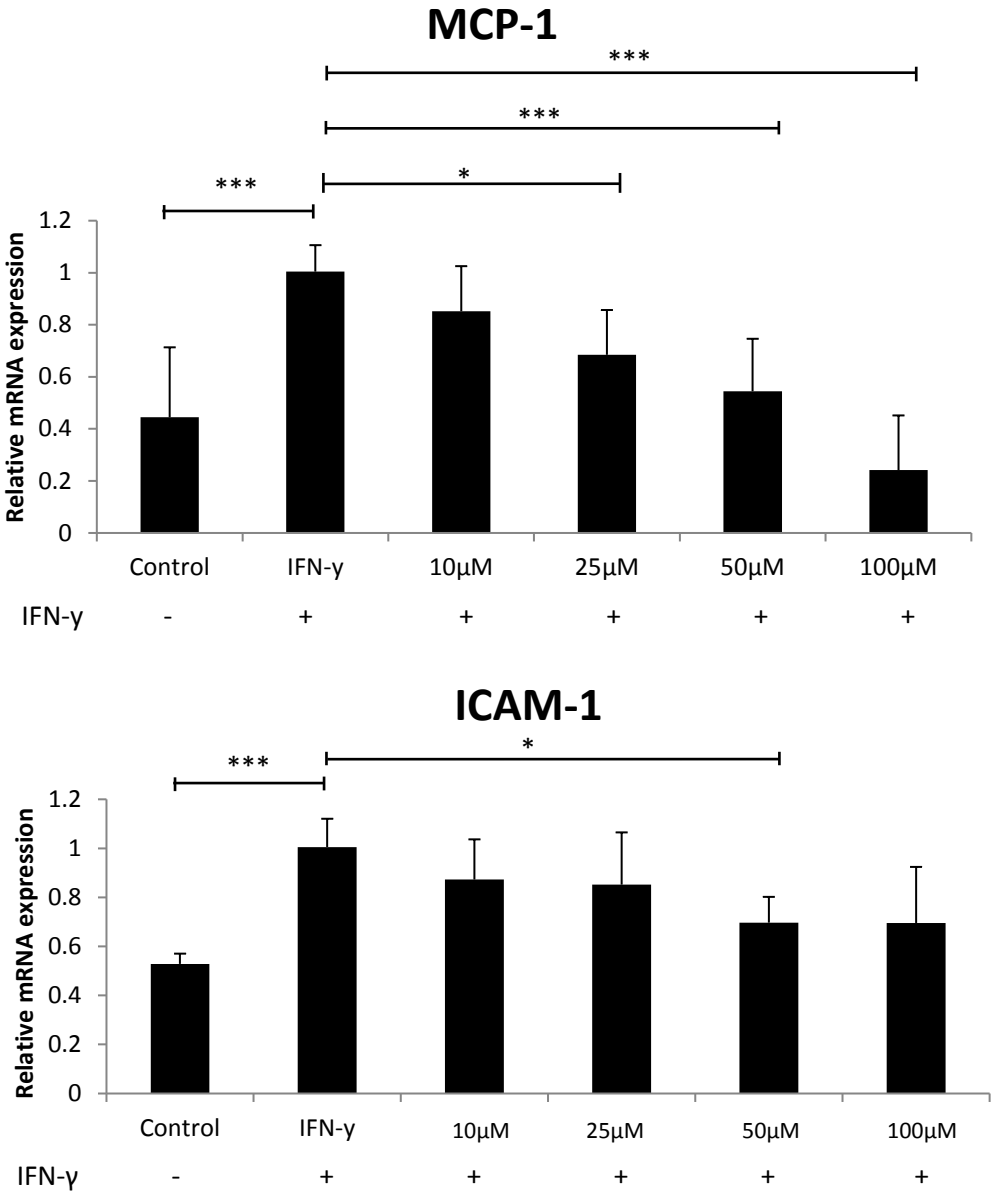


Figure 4.7 – Concentration-dependent inhibition of IFN-γ-induced MCP-1 and ICAM-1 expression by DGLA

THP-1 macrophages were pre-incubated with 10 μM, 25 μM, 50 μM and 100 μM DGLA or DMSO vehicle for 24 hours followed by incubation in the presence of vehicle (Control) or 250 U/ml IFN-γ for 3 hours. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for MCP-1, ICAM-1 or GAPDH control. Graphs display average normalised gene expression (mean +/- SD) (control arbitrarily assigned as 1) from three independent experiments. Statistical analysis was performed using a one-way ANOVA (equal variances) with Tukey's post hoc for MCP-1. For statistical analysis for ICAM-1, due to unequal variances, Welch's test followed by Dunnett's T3 post hoc analysis was performed. * $P \leq 0.05$, *** $P \leq 0.001$

4.4.4 Viability assays

After determining the optimum concentrations of DGLA and IFN-γ, the viability of THP-1 macrophages under these conditions was assessed. For this, two tests of cellular viability were employed. LDH is a cytosolic enzyme present in numerous cell types and extracellular release of LDH is an indication of damage to the cellular membrane. Measuring LDH release from THP-1 macrophages into the overlying media, in comparison to a positive and negative control, was used as an indication of cellular damage. The optimised concentration of DGLA and IFN-γ were used in these experiments (i.e. 50 μM and 250 U/ml respectively). As shown in Figure 4.5, no effect on LDH release was observed by incubation of the cells with IFN-γ and DGLA when compared to the negative control (i.e. vehicle-treated cells) (Figure 4.8). In addition to the LDH release assay, crystal violet staining was utilised. Crystal violet is used to stain adherent cells. It binds to DNA in the nucleus of the cell and therefore correlates to cell numbers. Cells that detach from plastic surface of tissue culture flasks are assumed non-viable, remain in the supernatant and are washed away before quantification. There was no difference observed in cell viability/proliferation as determined by this method between control and IFN-γ and/or DGLA treated THP-1 macrophages under all experimental conditions (Figure 4.8). Taken together, the results from the LDH and crystal violet assay suggest that there is no adverse effect of 50 μM DGLA and/or 250 U/ml IFN-γ on cell viability. These conditions were therefore employed throughout study.

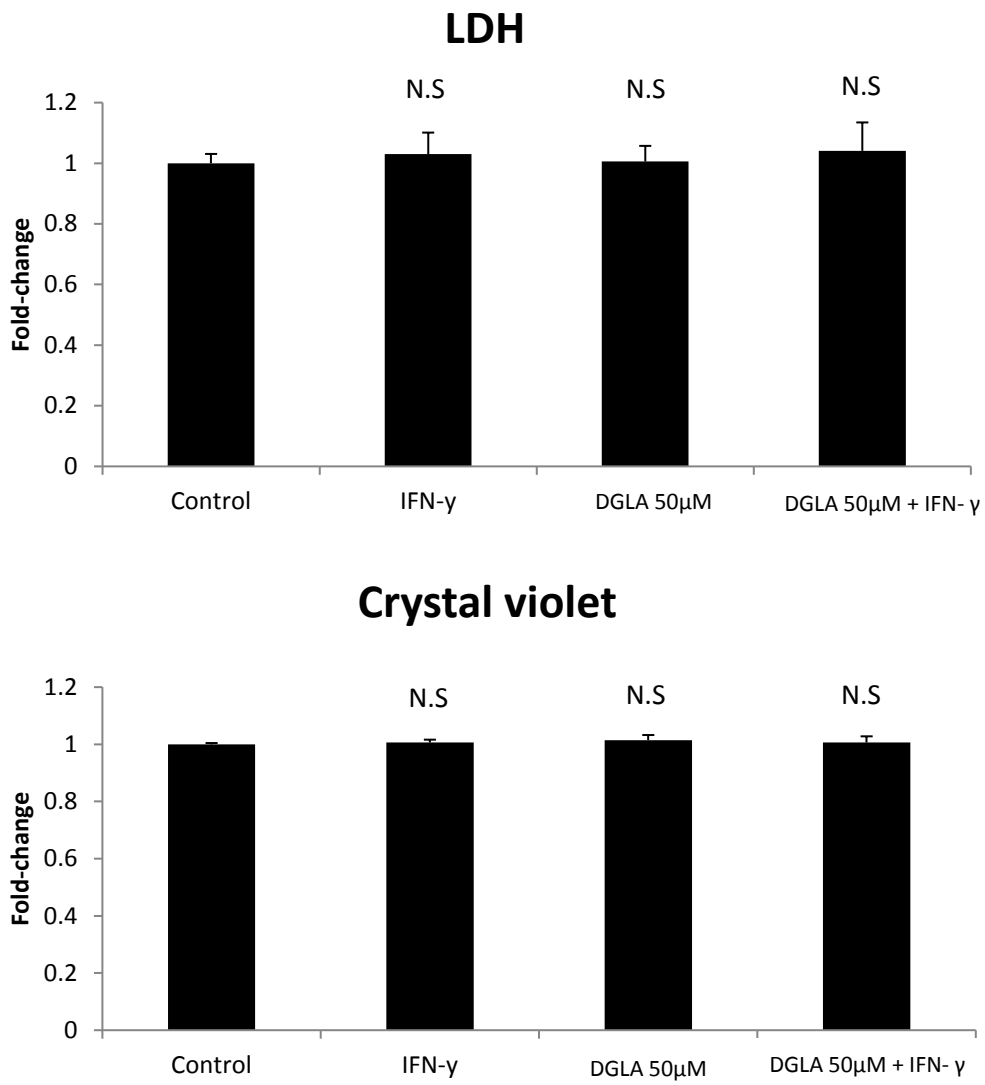


Figure 4.8 – DGLA and/or IFN-γ has no effect on cell viability of THP-1 macrophages

THP-1 macrophages were incubated with 50 µM DGLA or the DMSO vehicle control for 24 hours and then in the presence of 250 U IFN-γ or vehicle for a further 3 hour. Media was removed and used to determine LDH release. The remaining THP-1 macrophages were then used in the crystal violet assay. Both graphs display average fold-change of absorbance values (mean +/- SD) in comparison to control (arbitrarily assigned as 1) from three independent experiments. Statistics were performed using one-way ANOVA, comparing each individual treatment to control. N.S – not significant

4.4.5 The effect of DGLA on ROS production

ROS production plays an important role in the pathology of atherosclerosis by modifying LDL, which can then be recognised by macrophage scavenger receptors. The effect of DGLA on ROS production from THP-1 monocytes and macrophages was measured to determine if the lipid possessed any anti-oxidant capacity. Monocytes and macrophages produce and release ROS in response to stimulation with pro-inflammatory agents and oxidants such as hydrogen peroxide (Forman and Torres 2001). The assay was performed as per manufacturer instructions (AbCam), including concentrations and incubation times. Briefly, monocytes or macrophages were incubated with DCFDA, which diffuses into cells and is oxidised by ROS to a fluorescent compound that can be measured by fluorescence spectroscopy. A positive control supplied by the kit, TBHP, was used to induce ROS production by monocytes and macrophages and therefore increased production of fluorescent compound. DGLA was co-incubated with monocytes and TBHP whereas macrophages were pre-incubated with DGLA for 24 hours (as in gene expression studies) prior to the addition of TBHP, to determine the effect on ROS production. As shown in Figure 4.9, TBHP significantly induced ROS production in both monocytes (A) and macrophages (B). However, co-incubation with DGLA in monocytes or pre-incubation in macrophages had no effect on the increase in ROS production.

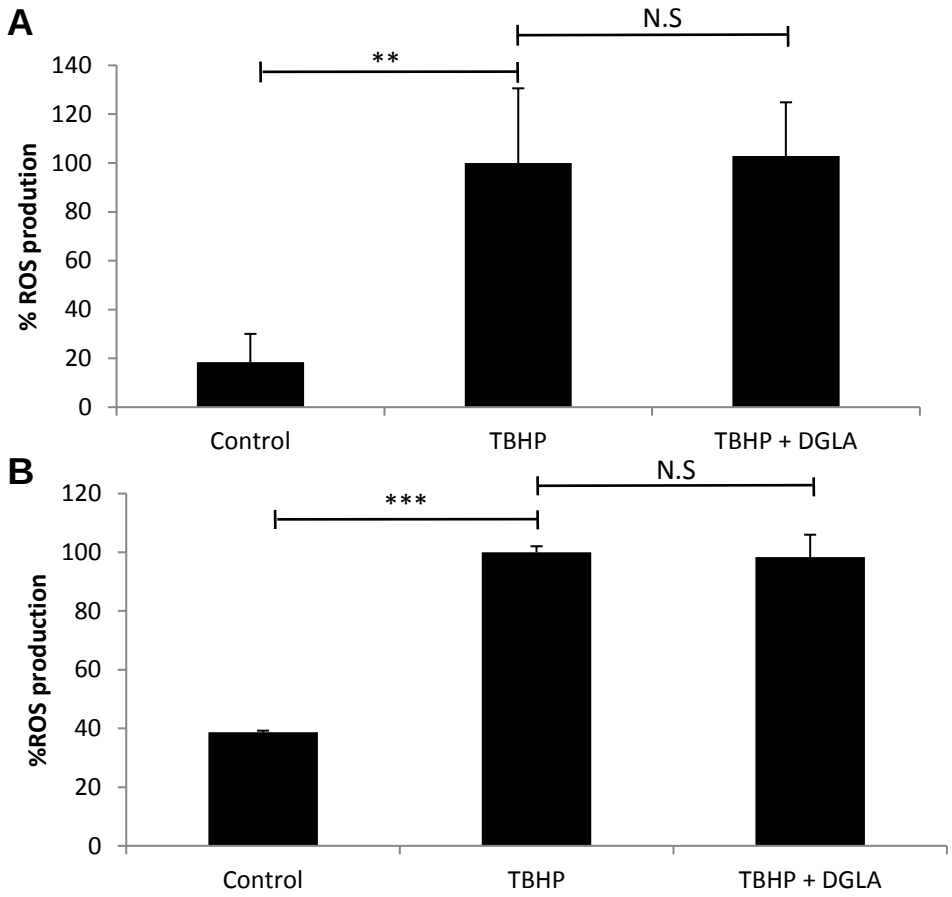


Figure 4.9 – DGLA has no effect on the TBHP-induced ROS production in THP-1 monocytes or macrophages

THP-1 monocytes (A) were incubated with 35 µM DCFDA for 30 minutes followed by 100 µM TBHP in the presence of 50 µM DGLA or the vehicle control for 3 hours. THP-1 macrophages (B) were pre incubated with 50 µM DGLA or vehicle control for 24 hours prior to incubation with 35 µM DCFDA for 30 minutes and 100 µM TBHP for 3 hours. Cells incubated with vehicle in the absence of TBHP were included as control. Fluorescence was measured at 495 nm and 529 nm for excitation and emission spectra respectively. Graph displays average mean +/- SD from three independent experiments. The value in the TBHP positive control has been arbitrarily assigned as 100%. Statistical analysis was performed using a one-way ANOVA followed by Tukey's post hoc. ** $P \leq 0.01$, *** $P \leq 0.001$, N.S – not significant.

4.4.6 The effect of DGLA on the expression of MCP-1 and ICAM-1 induced by different pro-inflammatory cytokines in human macrophages

4.4.6.1 IFN-γ

4.4.6.1.1 THP-1

IFN-γ has been well documented in the laboratory to induce the expression of MCP-1 and ICAM-1 in THP-1 macrophages (Li *et al*. 2010). THP-1 macrophages were pre-incubated with vehicle or DGLA for 24 hours followed by incubation for 3 hours in the presence of vehicle or IFN-γ. MCP-1 and ICAM-1 mRNA expression was significantly induced by IFN-γ in THP-1 macrophages by about 46-fold and 4-fold respectively (Figure 4.10). Pre-treatment of the cells with DGLA produced a significant reduction of approximately 24% and 32% respectively of the IFN-γ-induced MCP-1 and ICAM-1 expression. Interestingly, there was also a significant difference between the basal expression levels of MCP-1 between cells treated with vehicle or DGLA. Thus, there was an average of 46% reduction in MCP-1 expression produced by treatment of the cells with DGLA when compared to the vehicle control (Figure 4.10). Such an action of DGLA was specific to MCP-1 and not seen with ICAM-1 expression, where there was no significant difference between control and DGLA treatment (Figure 4.10).

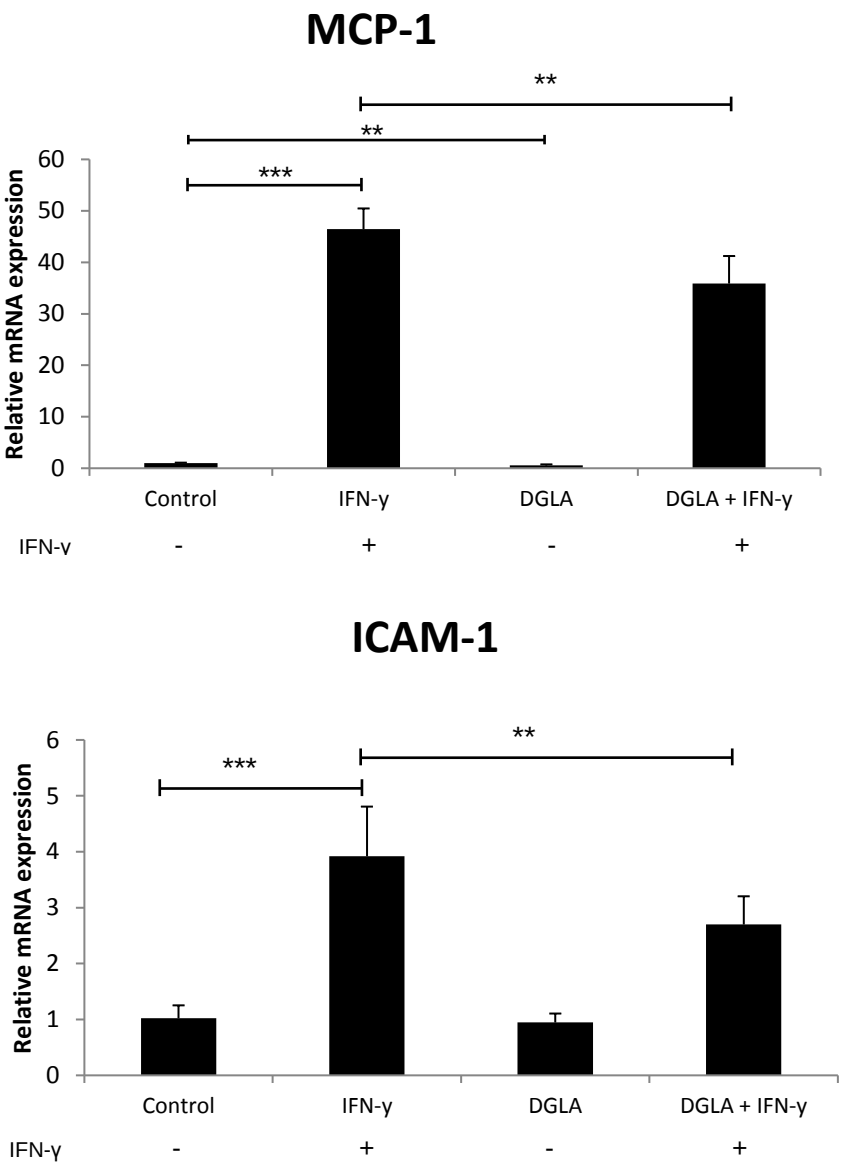


Figure 4.10 – DGLA significantly inhibits the IFN-γ-induced expression of MCP-1 and ICAM-1 in THP-1 macrophages

THP-1 macrophages were incubated with vehicle or 50 μM DGLA for 24 hours prior to treatment with vehicle or 250 U/ml IFN-γ for 3 hours. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for human MCP-1, ICAM-1 or GAPDH. Graphs display average normalised gene expression (mean +/- SD) (control arbitrarily assigned as 1) from three independent experiments. Homogeneity of variances were not met for ANOVA therefore Welch's test followed by Dunnett's T3 post hoc tests were used ** P≤0.01 *** P≤0.001.

4.4.6.1.2 RAW264.7

Mouse models are commonly used in atherosclerosis research. However species-specific regulation of gene expression between human and mouse have been found in some cases (Lin *et al.* 2014). In addition, differentiation of THP-1 monocytes into macrophages required PMA and it was possible that this could influence the results obtained. In order to rule out these possibilities, the experiments were repeated in the mouse RAW264.7 cell line that does not require PMA-mediated differentiation. Conservation of responses in RAW264.7 macrophages would allow use of this cell line to investigate the actions of DGLA if required. RAW264.7 macrophages were pre-incubated with vehicle or DGLA for 24 hours followed by incubation in the presence of vehicle or IFN-γ for 3 hours. The concentrations of DGLA and IFN-γ used were identical to that optimised for THP-1 cells and so should allow direct comparison between the responses in the two cell types. Treatment of the cells with IFN-γ produced a significant induction of MCP-1 and ICAM-1 expression (Figure 4.11). Pre-treatment of the cells with 50 μM DGLA followed by incubation with IFN-γ produced a significant attenuation of the expression of MCP-1 and ICAM-1 by an average of 61% and 52% respectively. In addition, there was a 44% reduction in basal MCP-1 expression on treatment of the cells with DGLA, as observed in THP-1 macrophages, however this was not significant. There was no observable difference in ICAM-1 expression between vehicle and DGLA treated cells.

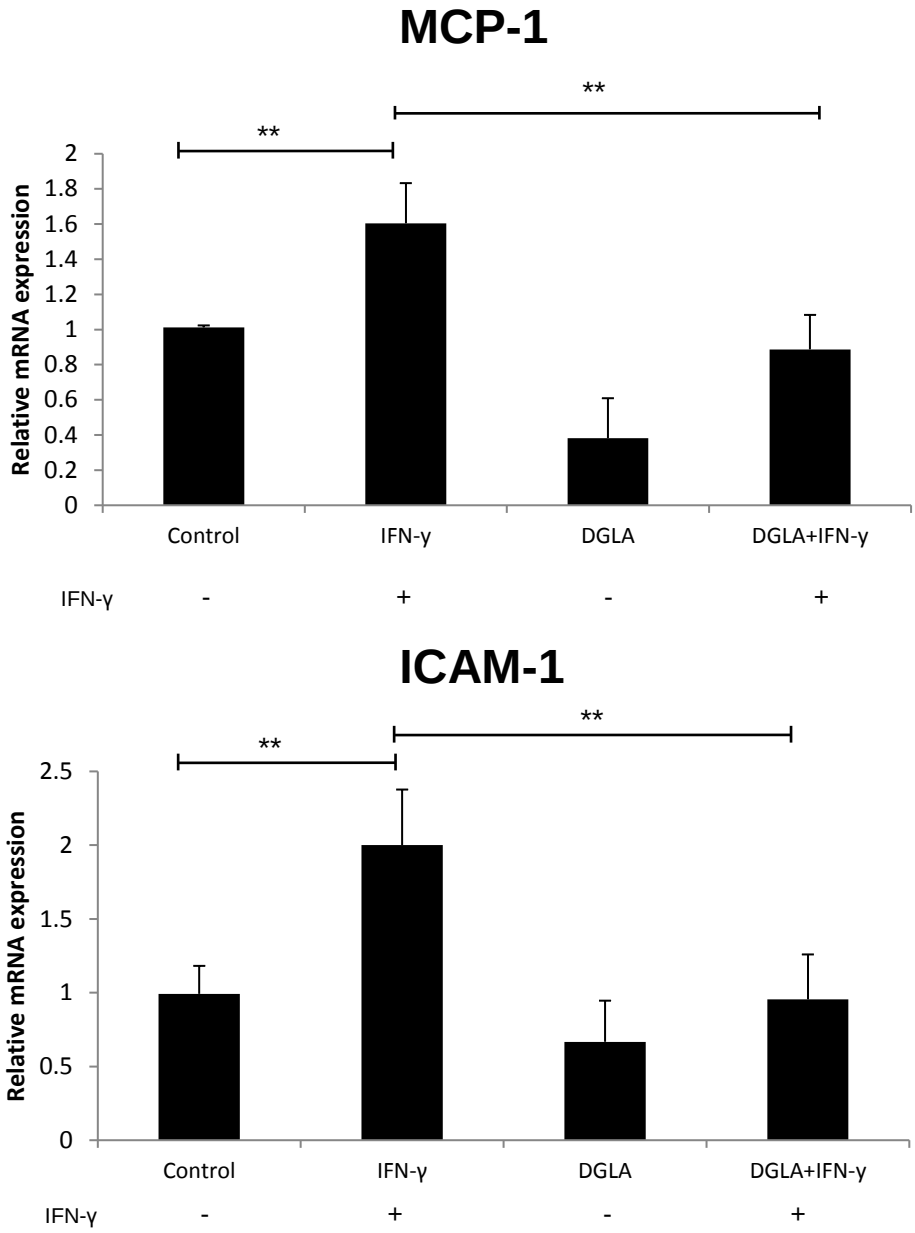


Figure 4.11 – DGLA significantly inhibits the IFN-γ-induced expression of MCP-1 and ICAM-1 in RAW264.7 macrophages

RAW264.7 macrophages were incubated with vehicle or 50 μM DGLA for 24 hours prior to treatment with vehicle or 250 U/ml IFN-γ for 3 hours. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific mouse MCP-1, ICAM-1 or β actin. Graphs display average normalised gene expression (mean +/- SD) (control arbitrarily assigned as 1) from 3 independent experiments. Statistical analysis was performed using a one-way ANOVA and Tukey's post hoc analysis. ** P≤0.01

4.4.6.1.3 HMDM

Given the significant inhibition in expression by DGLA of two key IFN-γ induced inflammatory genes in both THP-1 and RAW264.7 macrophages, the experiments were repeated in primary cultures of HMDM. These experiments were carried out to rule out the possibility that the observed results are peculiar to the cell line. HMDM were pre-treated with 50 µM DGLA or vehicle for 24 hours followed by vehicle or 250 U/ml IFN-γ for further 3 hours (identical conditions to that used for THP-1 and RAW264.7 macrophages). As shown in Figure 4.12, IFN-γ significantly induced the expression of both MCP-1 and ICAM-1 genes. Due to variability between IFN-γ induction of MCP-1 and ICAM-1 between experiments, the values obtained from cells treated with this cytokine was arbitrarily assigned as 1. Treatment with 50 µM DGLA produced a significant reduction of the IFN-γ-induced expression of MCP-1 by an average of 55% (Figure 4.12). There was no significant difference in basal MCP-1 expression between the vehicle control and DGLA, as seen for THP-1 and RAW264.7 macrophages. The IFN-γ-induced ICAM-1 expression was also reduced by DGLA by approximately 43%. However due to a high variability between experiments, this reduction was not significant though a trend in the inhibitory action of DGLA was observed in all experiments.

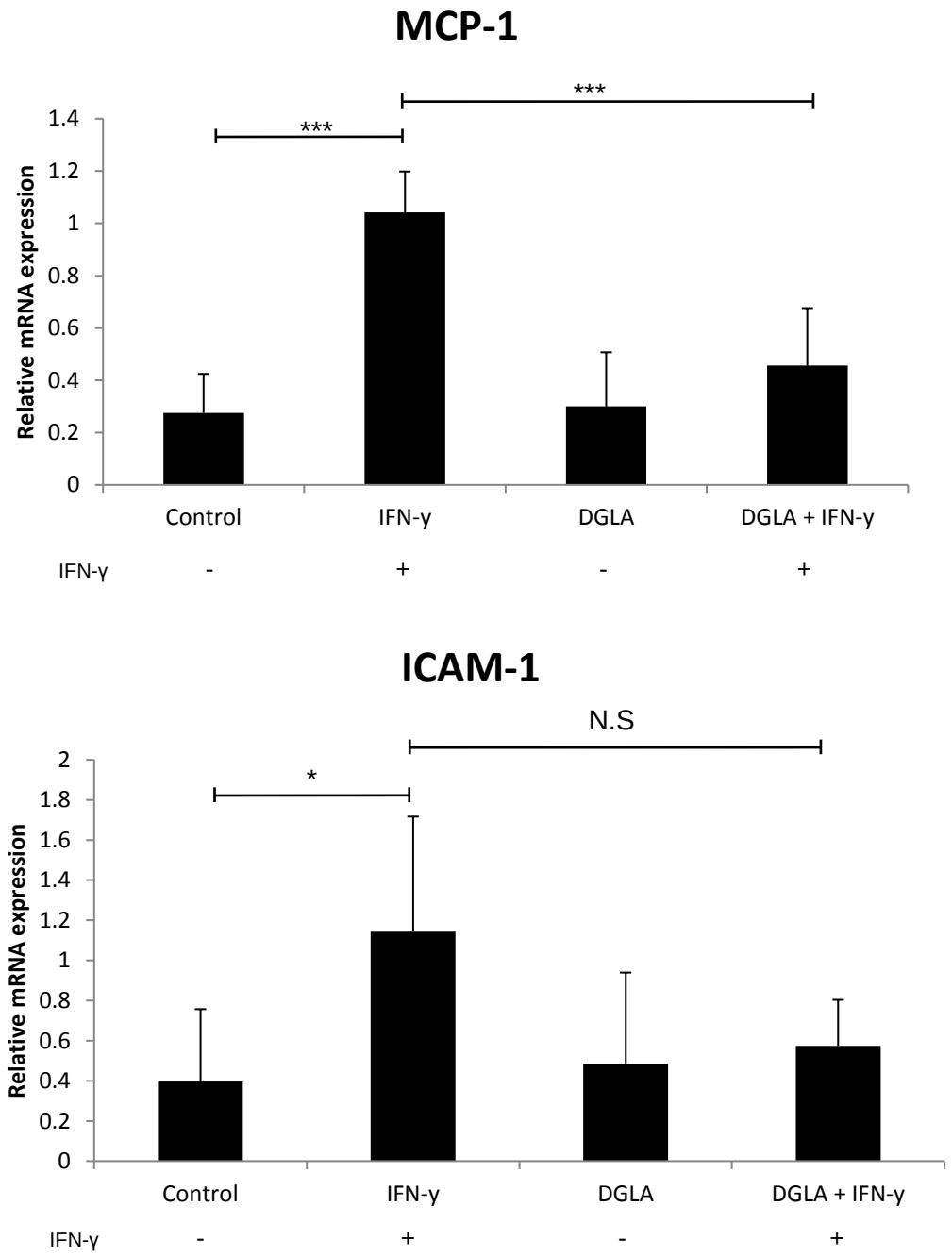


Figure 4.12 – DGLA inhibits the IFN-γ induced expression of MCP-1 and ICAM-1 in HMDMs

HMDMs were incubated with 50 µM DGLA or vehicle for 24 hours prior to treatment with vehicle or 250 U/ml IFN-γ for 3 hours. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for MCP-1, ICAM-1 or GAPDH. Graphs display average normalised gene expression (mean +/SD) (value from IFN-γ treated cells arbitrarily assigned as 1) from three independent experiments. Statistical analysis was performed using a one-way ANOVA and Tukey's post hoc analysis. * P≤0.05, *** P≤0.001, N.S – not significant.

4.4.6.2 IL-1β and TNF-α

Following on from the successful attenuation of IFN-γ induced expression of inflammatory genes MCP-1 and ICAM-1 by DGLA, it was of interest to determine if this inhibition was specific to IFN-γ induction of these genes or it extended to other pro-inflammatory cytokines. Two cytokines were selected, IL-1β and TNF-α, due to their important roles in the control of inflammation during atherosclerosis. Both cytokines have also been shown to induce the expression of MCP-1 and ICAM-1 *in vitro* and *in vivo* (Myers *et al.* 1992; Pal *et al.* 1996; Ohta *et al*. 2005; Lim *et al.* 2009; Yang *et al.* 2010).

THP-1 macrophages were treated with 1000 U/ml of IL-1β or TNF-α for 24 hours, a dose and time point previously used in the laboratory. As shown in Figure 4.13, IL-1β and TNF-α significantly induced both MCP-1 and ICAM-1 expression in THP-1 macrophages. IL-1β induced MCP-1 expression by 3.5-fold and ICAM-1 by 1.3-fold. Similarly, TNF-α induced MCP-1 and ICAM-1 expression by 1.6-fold and 1.7-fold, respectively. Pre-treatment of the cells with 50 μM DGLA significantly inhibited the IL-1β and TNF-α induced MCP-1 and ICAM-1 expression. The levels of MCP-1 and ICAM-1 were reduced to that observed in vehicle treated cells in the case of IL-1β. Similarly TNF-α induction of MCP-1 expression was reduced to control basal levels following pre-treatment of the cells with DGLA. On the other hand, the TNF-α induced ICAM-1 expression was reduced by 25% following pre-treatment of the cells with DGLA and remained at slightly elevated levels in comparison to control levels of ICAM-1 observed in vehicle-treated cells. There was no effect on the constitutive expression of MCP-1 or ICAM-1 observed in these experiments.

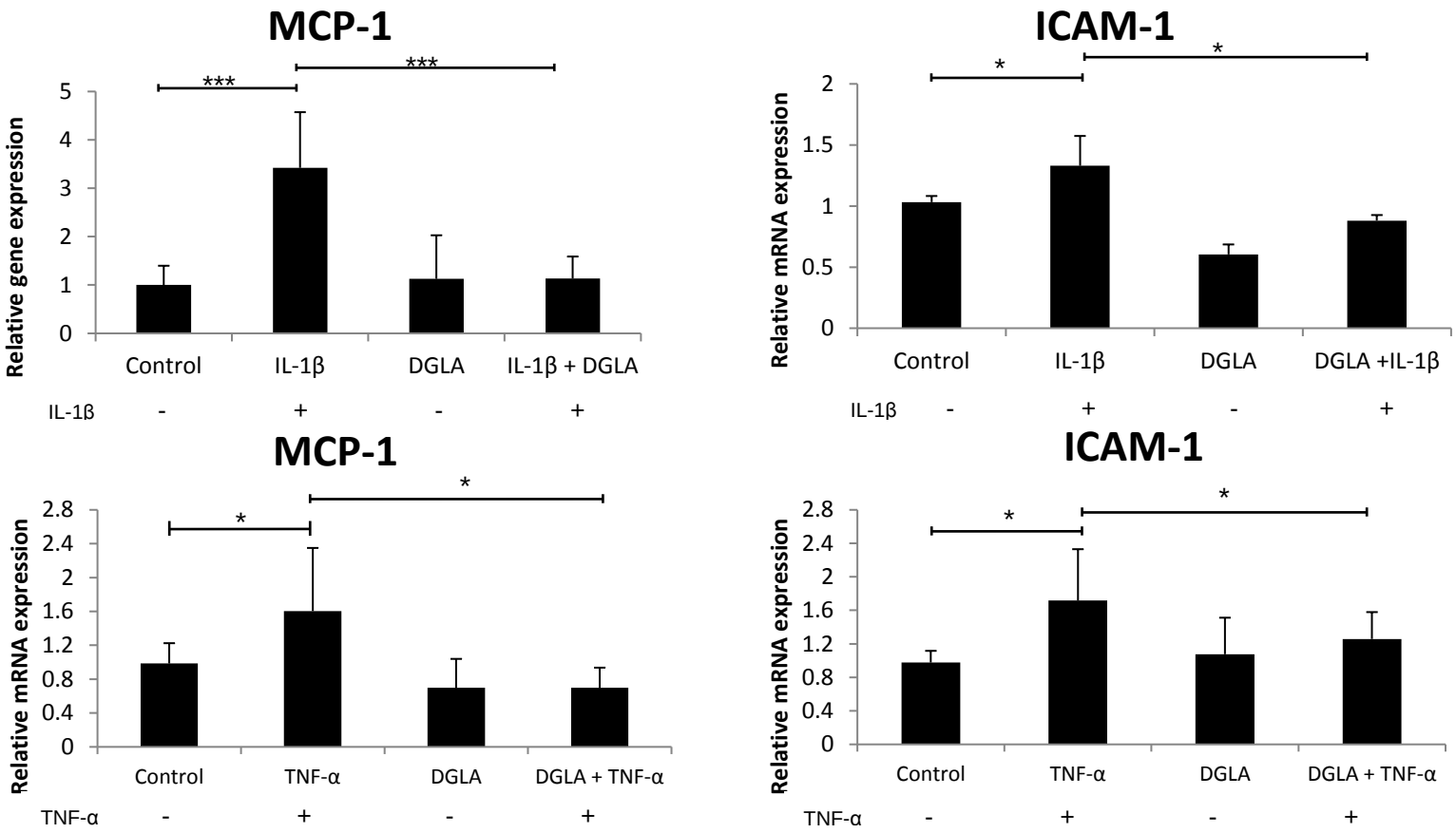


Figure 4.13 – DGLA significantly inhibits the IL-1β and TNF-α induced expression of MCP-1 and ICAM-1 in THP-1 macrophages

THP-1 macrophages were incubated with 50 uM DGLA or vehicle control for 24 hours prior to treatment with 1000 U/ml of IL-1β or TNF-α for 24 hours. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for MCP-1, ICAM-1 or GAPDH. Graphs display average normalised gene expression (mean +/- SD) (control arbitrarily assigned as 1) from three independent experiments. Statistical analysis was performed using a one-way ANOVA and Tukey's post hoc analysis. * P≤0.05, *** P≤0.001

4.4.7 Inflammasome induced IL-1β release in THP-1 macrophages

Given the important role of DGLA in inhibiting the IL-1β induced pro-inflammatory signalling, it was of interest to determine the role of DGLA in the release of IL-1β by macrophages. IL-1β is released in its active form following the activation of caspase-1-dependent NLRP3 inflammasome (Franchi *et al*. 2009). Cholesterol crystals present in atherosclerotic plaque have been shown to activate the NLRP3 inflammasome in mouse models and in THP-1 macrophages which up regulates the release of IL-1β (Duewell *et al*. 2010; Rajamaki *et al*. 2010). The effect of DGLA on cholesterol crystal induced inflammasome mediated IL-1β release was measured by ELISA in THP-1 macrophages (Figure 4.14).

On treatment with 1 mg/ml cholesterol crystals, the concentration of IL-1β was increased from approximately 100 pg/ml to 480 pg/ml. Pre-treatment of the cells with DGLA did not inhibit the cholesterol crystal induced IL-1β secretion.

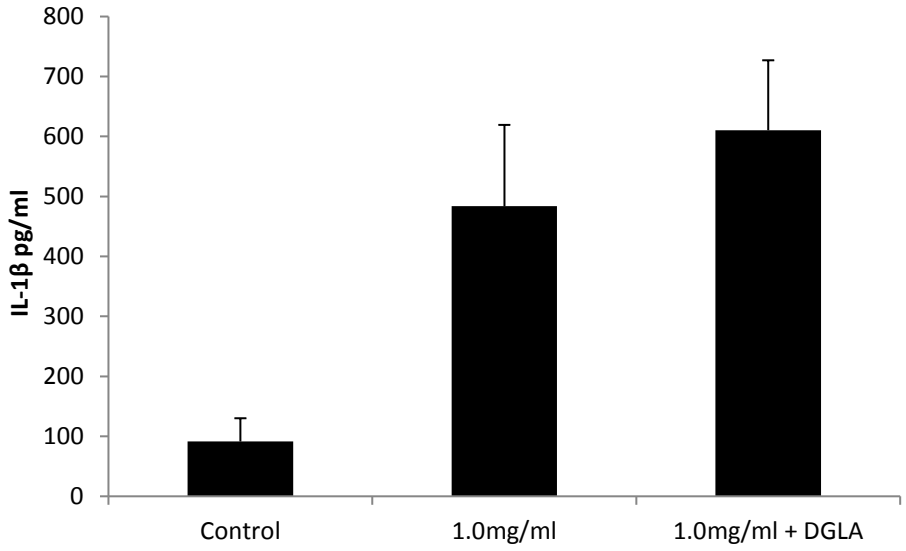


Figure 4.14 – Cholesterol crystals increase IL-1β secretion in THP-1 macrophages, was not inhibited by DGLA

THP-1 macrophages were incubated with 50 μM DGLA or vehicle control for 24 hours prior to treatment with 1 mg/ml of cholesterol crystals for 8 hours (time point previously optimised in the laboratory). Media was collected and subjected to ELISA following the manufacturer instructions (R&D systems). Graphs display average IL-1β levels (mean +/- SD) from 2 independent experiments performed in triplicate.

4.4.8 Monocyte migration

Given the ability of DGLA to inhibit the expression of MCP-1 independently and in an inflammatory setting (Figures 4.10, 4.11, 4.12), it was hypothesised that the fatty acid would also attenuate monocyte migration, given the key role of MCP-1 in this process. Cell inserts with 8 µm pores were used to mimic an arterial endothelial layer and the analysis was carried out as detailed in Section 2.2.11. As shown in Figure 4.15, migration of THP-1 monocytes across the cell insert was increased significantly in the presence of MCP-1. Inclusion of DGLA produced a significant reduction of this MCP-1 driven monocytic migration by an average of about 60%.

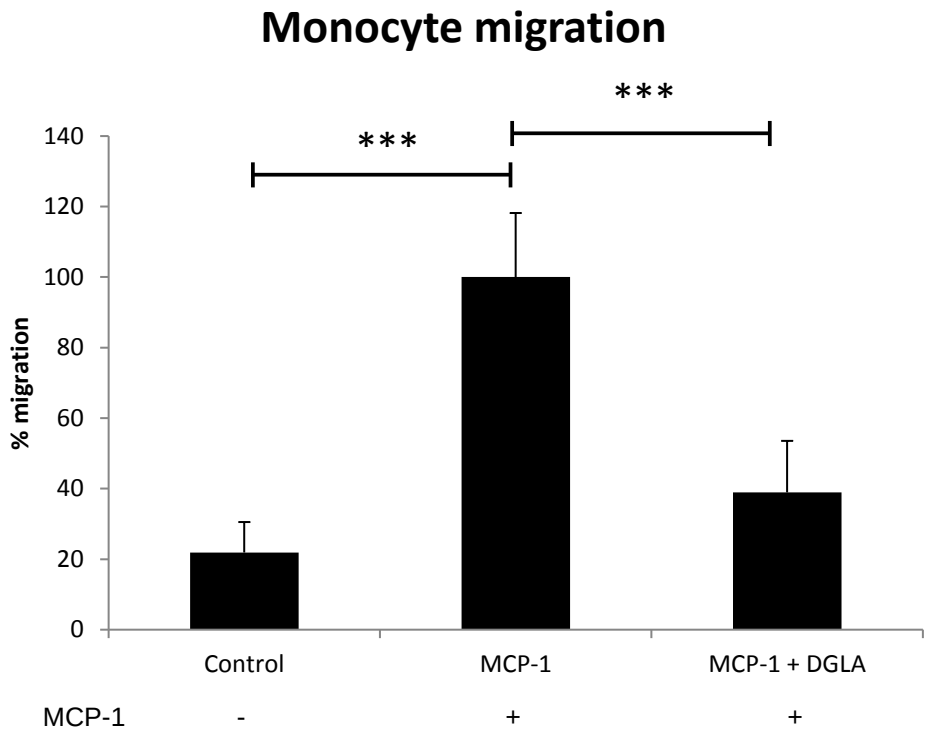


Figure 4.15 – DGLA significantly inhibits the MCP-1-induced migration of THP-1 monocytes

THP-1 monocytes were co incubated with +/ - MCP-1 (20 ng/ml) and 50 µM DGLA or vehicle control, for 3 hours. Monocyte migration was calculated by counting the number of cells that had migrated across a cell insert and expressed as a percentage of total input cells. Graph displays mean percentage migration, with values from the MCP-1 positive control arbitrarily assigned as 100%. Error bars indicate mean +/- SD for three independent experiments. Statistical analysis was performed using a one-way ANOVA followed by Tukey's post hoc analysis. *** P≤0.001

4.4.9 acLDL uptake

To determine the action of DGLA on the accumulation of cholesteryl esters in macrophages, the cells were incubated with vehicle as a control and compared to 25 µg/ml acLDL and 25 µg/ml acLDL plus 100 µM DGLA. The incorporation of [^{14}C]-acetate into lipid fractions separated and identified by TLC were measured by scintillation counting (Figure 4.16). THP-1 macrophages were initially utilised for the study however it was found that they did not accumulate large amounts of cholesteryl ester (Appendix 6). Alternatively, loading of RAW264.7 macrophages with acLDL showed a large increase in the accumulation of cholesterol esters (Appendix 6). Time points of 24 and 48 hours were used for both cell lines (Appendix 6). There was no substantial difference in cholesteryl ester accumulation between 24 and 48 hour incubation with acLDL. Given this, mouse RAW264.7 macrophages were subsequently used for such studies with 24 hour loading of acLDL. Given the conserved responses seen between THP-1 macrophages and RAW264.7 macrophages, particularly in gene expression, the two cell lines could be used interchangeably. In addition, a concentration of 50 µM DGLA as gene expression studies was initially used in preliminary experiments. However, it was observed that 50 µM DGLA showed only a small decrease in cholesteryl ester accumulation and 100 µM DGLA inhibited this to a greater extent (Appendix 7). The latter concentration was therefore used for cholesteryl ester accumulation studies.

As shown in Figure 4.16A, there was no observable difference between percentage lipid composition of polar lipids, free fatty acids or TAGs following incubation of the cells with acLDL when compared to the control. However, there was a significant decrease in the percentage lipid composition of free cholesterol by 14.6% and a significant increase in the accumulation of cholesteryl esters by 16.9%. Pre-treatment of the cells with DGLA prior to acLDL addition produced no difference between the percentage composition of polar lipids, free fatty acids and TAGs. However, differences in the levels of free fatty acids and cholesteryl esters were observed. Thus, the percentage lipid composition of free cholesterol was increased by 1.2%, however, this was not significant. On the other hand cholesteryl ester accumulation significantly decreased by 11.6%, in comparison to the presence of acLDL only. Figure 4.16B presents the data as a ratio of cholesteryl ester: total cholesterol (CE: TC). Total cholesterol is the sum of free cholesterol and cholesteryl esters. The CE: TC ratio was 0.8 in the presence of acLDL alone and this was reduced to 0.4 in the presence of DGLA, however due to high variability between replicates this was not significant.

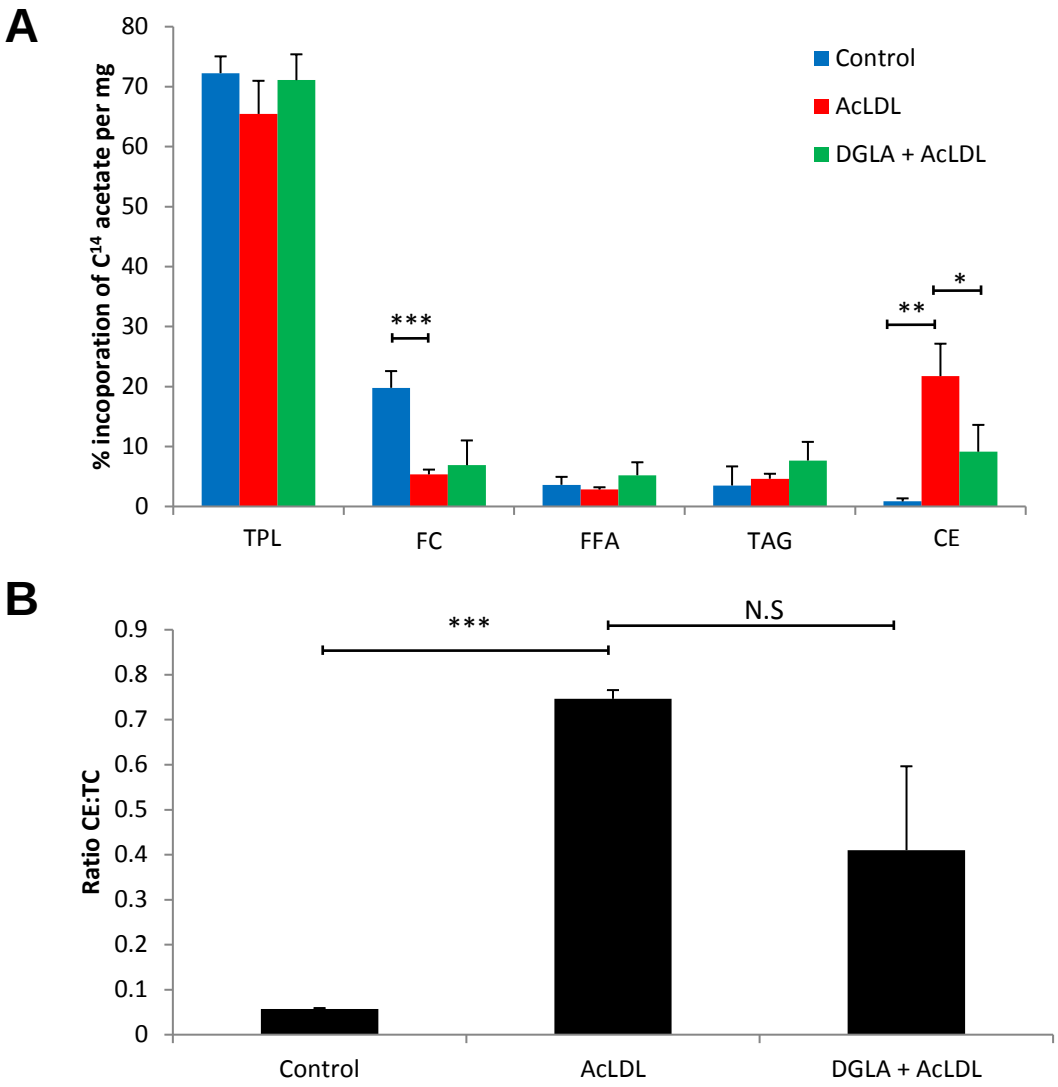


Figure 4.16 – DGLA inhibits acLDL induced cholesteryl ester formation in RAW264.7 macrophages

The cells were incubated with 100 µM DGLA for 24 hours prior to the addition of 50 µM acLDL and 1µCi [^{14}C] acetate for a further 24 hours. Lipids were extracted, separated via TLC and radioactive incorporation into lipid fractions, identified by comparison with known standards, was measured by scintillation counting. Panel A – percentage incorporation of [^{14}C] per mg of protein, into total polar lipids (PL), free cholesterol (FC), free fatty acids (FFA), triacylglycerols (TAG) and cholesteryl esters (CE). Panel B – ratio of CE/TC under different experimental conditions. Error bars indicate +/- SD from 4 experiments. Statistical analysis was performed using a one-way ANOVA followed by Tukey's post hoc analysis for FC and Welch's test followed by Dunnett's T3 post hoc tests for CE and CE:TC ratios (conditions for ANOVA not met) * P≤0.05. ** P≤0.01. *** P≤0.001. N.S – not significant.

4.5 Discussion

Data obtained in the studies presented in this chapter indicate that DGLA inhibits a number of steps leading to the formation of lipid loaded foam cells including; cytokine induced expression of MCP-1 and ICAM-1, monocyte migration and acLDL induced cholesteryl ester accumulation, but has no effect on ROS production or inflammasome activation. This suggests that DGLA may attenuate foam cell formation through one of these numerous mechanisms.

4.5.1. ROS production

Increased production of ROS has been observed in atherosclerosis and has been linked to various signalling pathways involved in vascular inflammation in the disease (Singh and Jialal 2006). One such pathway involves oxidative modifications of LDL. Native LDL is internalised by macrophages though an LDL receptor, which is subject to negative feedback inhibition. Oxidative modifications to LDL allow recognition by scavenger receptors, also expressed on macrophages, which take up excess amounts of modified LDL in an unregulated manner. This causes transformation of macrophages into lipid loaded foam cells (Singh and Jialal 2006). Given scavenger receptors only recognise modified forms of LDL, including oxidised LDL, reducing LDL oxidation by decreasing ROS levels would have a beneficial effect on inhibiting foam cell formation. ROS production was stimulated with TBHP (hydrogen peroxide) in THP-1 monocytes and macrophages and the effect of DGLA was determined. As shown in Figure 4.9, DGLA did not inhibit ROS production from either monocytes or macrophages. This initial study suggests that acting as an anti-oxidant is not the likely mechanism for the anti-atherogenic action of DGLA. However, numerous other experiments will be required to conclusively delineate the relationship between DGLA and oxidative stress. This may include the effect of DGLA on modification of LDL and other sources capable of oxidative stress such as nitric oxide production. To date there has been little data published on the effect of DGLA on ROS production. One such study indicated that treatment of omega-6 fatty acids in rats had no effect on ROS production but did enhance the activity of antioxidant enzymes (Suresh and Das 2003b). In addition, supplementation of GLA in rats with high fat diet induced atherosclerosis saw a reduction in the serum levels of oxLDL, NO and iNOS (Shi *et al*. 2008). A potential future avenue of research could establish the effect of DGLA on anti-oxidant levels and levels of other oxidising species such as NO in macrophages. Anti-oxidants have been suggested as a potential therapeutic avenue in atherosclerosis; however results from clinical trials largely contradict one another (Stephens *et al.* 1996; Yusuf *et al.* 2000). For example, while dietary supplementation

with antioxidants has in some studies shown to reduce atherosclerotic related incidences (Stephens *et al*. 1996; Boaz *et al.* 2000), others have shown that they have no beneficial effect on the disease (Yusuf *et al*. 2000; Investigators 2007). Given the controversial role of antioxidants in atherosclerosis and the negative result obtained in the preliminary ROS experiment with DGLA; this avenue was not pursued further.

4.5.2 Cytokine induced pro-inflammatory gene expression

4.5.2.1 IFN-γ

Given the important role of pro inflammatory cytokine signalling in atherosclerosis, it was of interest to investigate the role of DGLA on this. Due to the involvement of a large number of cytokines in atherosclerosis, three major pro-inflammatory cytokines were selected for study. The main focus was on IFN-γ, which has been described potentially as a master regulator of atherosclerosis due to its pro-inflammatory role in all the stages of disease progression, from foam cell formation through to lesion rupture (McLaren and Ramji 2009). IFN-γ up regulates the expression of many pro-inflammatory genes including MCP-1 and ICAM-1 (Rimbach *et al.* 2000; Li *et al*. 2010), which play an important role in monocyte recruitment and adhesion (Grandaliano *et al.* 1994; Lusis 2000; Chang *et al.* 2002). This has been shown *in vitro* where an IFN-γ dependant increase in ICAM-1 augmented the binding of monocyte to endothelial cells (Chang *et al*. 2002). DGLA significantly attenuated the IFN-γ induced expression of MCP-1 and ICAM-1 in THP-1 macrophages, RAW264.7 cell line and primary cultures of HMDM (Figure 4.10 - 4.12). This inhibition of IFN-γ induced pro-inflammatory signalling by DGLA is of importance as deficiency of IFN-γ signalling in *in vivo* mouse models showed a significant reduction in atherosclerotic lesions (Gupta *et al*. 1997) and targeting IFN-γ actions has been investigated in depth as a potential therapeutic avenue in atherosclerosis (Gotsman and Lichtman 2007). Targeting IFN-γ signalling may therefore play a role in the anti-inflammatory and anti-atherogenic action of DGLA.

4.5.2.2 IL-1β

In addition to IFN-γ signalling, the effect of DGLA on the action of other pro-inflammatory cytokines was also investigated. Previous studies have shown DGLA inhibited IL-1β stimulated adipose stromal cell (ASC) proliferation, a hallmark of rheumatoid arthritis (Baker *et al.* 1989). It is well documented that IL-1β plays a pro inflammatory role in atherosclerosis and potentiates the disease (Kirii *et al*. 2003; Fearon and Fearon 2008). In addition, IL-1β has also been shown to increase the

expression of MCP-1 and ICAM-1 (Myers *et al*. 1992; Lim *et al*. 2009; Yang *et al*. 2010). For these reasons, IL-1β was selected for this study. IL-1β significantly induced the expression of MCP-1 and ICAM-1 in THP-1 macrophages in comparison to control (Figure 4.13). Pre-incubation of the cells with DGLA significantly inhibited the induction of MCP-1 and ICAM-1 by IL-1β. Pre-treatment of the cells with DGLA returned the MCP-1 and ICAM-1 levels to that of basal control levels. This indicates that DGLA inhibits IL-1β signalling. Previous studies have shown knockout of IL-1β in mouse models of atherosclerosis show a reduction in lesion size when compared to control mice (Kirii *et al*. 2003). This was accompanied by a decrease in mRNA expression of MCP-1 and VCAM-1 (Kirii *et al*. 2003). Similarly an antibody targeting IL-1β in ApoE knockout mice reduced lesion size, macrophage plaque infiltration and secretion of MCP-1 in isolated primary macrophages (Bhaskar *et al*. 2011). This not only indicates the important role of IL-1β in atherosclerosis, but also in the recruitment and adhesion of monocytes through regulation of MCP-1 and ICAM-1 expression. The inhibition of expression of these two genes through an IL-1β induced pathway therefore supports an anti-inflammatory role of DGLA.

4.5.2.3 TNF-α

The third cytokine selected for this study was TNF-α. This pro-inflammatory cytokine has been well documented in promoting foam cell formation and augmenting atherosclerosis (Popa *et al*. 2007; Kleinbongard *et al*. 2010). As with IFN-γ and IL-1β, it has also been shown to increase the expression of MCP-1 and ICAM-1 *in vitro* and *in vivo* (Myers *et al*. 1992; Murao *et al.* 2000; Xiao *et al*. 2009). The effect of DGLA on this induced expression of these two genes was determined in THP-1 macrophages. As shown in Figure 4.13, TNF-α significantly increased the expression of MCP-1 and ICAM-1. Pre-treatment of the cells with DGLA produced a significant attenuation of this TNF-α-induced expression of these two genes. In the case of MCP-1, pre-treatment of the cells with DGLA returned MCP-1 levels to that seen in control. ICAM-1 expression remained slightly raised in comparison to control; however these were not significantly different. In addition to the benefits of reducing the induction of MCP-1 and ICAM-1 expression, inhibition of TNF-α signalling also has other anti-atherosclerotic effects. ApoE$^{-/-}$ TNF-α$^{-/-}$ mice show a reduction in fatty streaks in the intima of aorta when compared to control mice (Xiao *et al*. 2009). In addition there was a decrease in the levels of IL-1β, IFN-γ, ICAM-1, VCAM-1 and MCP-1 observed (Xiao *et al*. 2009). Comparable results were also observed in another study using ApoE$^{-/-}$ TNF-α$^{-/-}$ mice. Such ApoE$^{-/-}$ TNF-α$^{-/-}$ mice displayed reduced atherosclerotic plaque area at the aortic luminal surface and aortic sinus when compared to the

controls (Ohta *et al*. 2005). Again, a decrease in the expression of inflammatory markers ICAM-1, VCAM-1 and MCP-1 was also observed along with decreases in both the uptake of oxLDL and the expression of scavenger receptor A (Ohta *et al*. 2005). There has been no work published in the literature on the effect of DGLA on TNF-α pro-inflammatory signalling. There has however been a study on TNF-α production in PBMC treated with a number of different fatty acids (Dooper *et al*. 2003). It was observed that DGLA inhibited LPS induction of TNF-α production by an average of 60% in PBMC. In addition, DGLA was the only fatty acid to have pronounced effects on cytokine production (Dooper *et al*. 2003). This suggests a key role of DGLA in the inhibition of TNF-α release and pro-inflammatory signalling induced by this cytokine during atherosclerosis.

4.5.2.4 MCP-1 and ICAM-1

As previously discussed, MCP-1 and ICAM-1 are key pro-inflammatory genes in atherosclerosis involved in monocyte recruitment and subsequent adhesion to the activated endothelium. It is clear from the results obtained in this study (Figures 4.10-4.13) that DGLA has a role in inhibiting the induction of both these markers in macrophages produced by IFN-γ, IL-1β and TNF-α signalling. DGLA has previously been shown to attenuate atherosclerosis in *in vivo* models of the disease (Takai *et al*. 2009). There was also an observed reduction in ICAM-1 expression and macrophage accumulation in plaques (Takai *et al*. 2009). In addition, mice with orally administered DGLA showed a reduction in circulating levels of MCP-1 (Watanabe *et al*. 2014). The *in vitro* results presented in this chapter and the *in vivo* observations reported previously clearly indicate a role for DGLA in the attenuation of MCP-1 and ICAM-1 expression in relation to atherosclerosis. This is an important role for DGLA as previous studies have shown that reducing MCP-1 and ICAM-1 expression *in vivo* significantly inhibits the progression of atherosclerosis (Gosling *et al*. 1999; Kitagawa *et al*. 2002).

Taking together the results presented in this chapter and previously published work suggest a pivotal role of DGLA in decreasing pro-inflammatory cytokine release and the subsequent induction of pro-inflammatory markers in atherosclerosis. This may in part account for the anti-atherosclerotic effects of DGLA seen in *in vivo studies* (Takai *et al*. 2009).

4.5.3 Inflammasome-mediated IL-1β expression

Leading on from the inhibition of cytokine induced pro-inflammatory gene expression by pro-inflammatory cytokine IFN-γ, IL-1β and TNF-α, it was of interest to determine the role of DGLA in cytokine production in macrophages. IL-1β was of particular interest due the role of multi-protein complexes, inflammasomes. A number of studies have detailed the role of inflammasomes, particularly the NLRP3 inflammasome, in IL-1β production in macrophages (Duewell *et al*. 2010; Rajamaki *et al*. 2010; Schroder and Tschopp 2010; Liu *et al*. 2014). Inflammasomes have been linked to an increase in atherosclerosis. Inhibition of the NLRP3 inflammasome, which plays a role in IL-1β processing, in ApoE$^{-/-}$ mice fed a high fat diet significantly prevented plaque progression and induction of inflammatory cytokines (Zheng *et al.* 2014). Macrophage plaque content was reduced, an increase in ABC transporter genes ABCA1 and ABCG1 and an increase in plaque stability was also observed (Zheng *et al*. 2014). THP-1 macrophages treated with oxLDL induced NLRP3 inflammasome related protein levels and caspase-1 activation. This resulted in an increase in IL-1β production, which promoted foam cell formation (Liu *et al*. 2014).

Omega-3 fatty acids have been shown to play a role in inhibiting inflammasome activation and IL-1β production in macrophages. In THP-1 macrophages, DHA reduced IL-1β production by attenuating inflammasome activation (Williams-Bey *et al*. 2014). Reduction of ligands that stimulated activation of the NLRP3, NLRC4 and AIM2 inflammasomes was observed. This inhibition required GPCR120 and attenuation of NF-κB signalling (Williams-Bey *et al*. 2014). In addition, in BMDMs primed with LPS, DHA inhibited nigericin induced caspase-1 activation, IL-1β secretion and TNF-α production (Yan *et al*. 2013). This was also observed in THP-1 macrophages. Treatment with EPA and ALA also attenuated nigericin induced IL-1β production. DHA inhibited all agonists of NLRP3 inflammasomes tested along with NLRP1b agonists (Yan *et al*. 2013).

Given the role omega-3 fatty acids play in inhibiting inflammasome induced IL-1β secretion in macrophages, it was of interest to determine the role of DGLA in the process. Cholesterol crystals were used to stimulate inflammasome activation in THP-1 macrophages. It has previously been observed that cholesterol crystals injected into LDLr$^{-/-}$ mice induced acute inflammation which was attenuated when mice were also deficient in NLRP3 inflammasome (Duewell *et al*. 2010). Bone marrow transplant into LDLr$^{-/-}$ mice with NLRP3$^{-/-}$ and ASC$^{-/-}$ deficiency had significantly reduced atherosclerotic lesions and inflammation (Duewell *et al*. 2010) *In vitro* studies also reported similar results. THP-1 macrophages phagocytose cholesterol crystals

and store them as cholesteryl esters. This induced secretion of IL-1β. This process was caspase-1 dependent and silencing of NLRP3 receptor abolished cytokine induction (Rajamaki *et al*. 2010). This suggests that cholesterol crystal treatment in THP-1 macrophages induces IL-1β secretion by activation of NLRP3 inflammasome. In addition, inflammasome activation in macrophages requires priming before stimulation. Previous studies have used LPS primed macrophages followed by stimulation with ATP or nigericin (Yan *et al*. 2013; Williams-Bey *et al*. 2014). However, in PMA stimulated macrophages it was shown that THP-1 macrophage inflammasome activation was independent to LPS priming and that PMA induced expression of IL-1β (Rajamaki *et al*. 2010). Using PMA stimulated THP-1 macrophages therefore eliminates the need for additional priming.

As shown in Figure 4.14, treatment of THP-1 macrophages with cholesterol crystals induced IL-1β secretion 5-fold. Pre incubation of the cells with DGLA prior to cholesterol crystal stimulation failed to inhibit IL-1β secretion, with the trend suggesting an increase in production. The data indicates that DGLA may enhance inflammasome activation and IL-1β production in THP-1 macrophages. Previous studies have indicated contrasting roles for DGLA in the regulation of IL-1β secretion. DGLA failed to inhibit NLRP3 inflammasome dependent IL-1β secretion from BMDMs primed with LPS and stimulated with nigericin (Yan *et al*. 2013). The PUFA also had no effect on NLRP1b inflammasome activation (Yan *et al*. 2013). In both cases, no increase in IL-1β production or inflammasome activation was observed. However, addition of GLA to peripheral blood monocytes supressed the release of LPS-stimulated IL-1β (Furse *et al*. 2001). It was shown that 40% of LPS stimulated IL-1β release was attributed to auto induction. GLA inhibited auto induction of IL-1β while it had little effect on LPS stimulated release (Furse *et al*. 2001). GLA induced a protein which reduced pro-IL-1β mRNA stability (Furse *et al*. 2001).GLA acts through its metabolite DGLA; however the direct effect of DGLA was not evaluated in the study. Taken together the data indicates that DGLA is unable to inhibit inflammasome dependent IL-1β secretion from macrophages and may potentially have an opposing affect. Further investigation will need to be undertaken to determine the role, including response to alternative agonists of inflammasomes in THP-1 macrophages and the effect of cholesterol crystals in HMDMs, in response to DGLA.

4.5.4 Monocyte migration

Expression of MCP-1 in atherosclerosis recruits circulating monocytes to the endothelium where they transmigrate through the endothelium into the arterial intima (Bobryshev 2006). Given the inhibition of pro-inflammatory cytokine induced

expression of MCP-1, along with an attenuation of basal expression of this chemokine by DGLA (Figure 4.10), it was of interest to determine whether this fatty acid could inhibit the role of MCP-1 in inducing the migration of monocytes across a barrier that mimics arterial endothelial cells. As shown in Figure 4.15, monocyte migration was significantly induced by treatment of THP-1 monocytes with MCP-1. On co-incubation with DGLA, the monocyte migration was attenuated, on average, by 60% in comparison to the MCP-1 positive control. *In vivo* inhibition of monocyte migration in ApoE$^{-/-}$ mouse models of atherosclerosis has been shown to be atheroprotective. For example, gremlin-1 inhibited monocyte migration *in vivo* resulting in decreased content of monocytes and macrophages in plaques and reduced atheroprogression (Mueller *et al.* 2013). In addition, a compound VB-201 selectively inhibited monocyte chemotaxis by 90% *in vitro*. Administration of this compound to ApoE$^{-/-}$ mice *in vivo* inhibited atheroma development (Feige *et al.* 2013). The role of DGLA in the inhibition of monocyte migration presented here may be responsible for the reduced macrophage accumulation seen in previous *in vivo* studies.

4.5.5 AcLDL induced cholesteryl ester accumulation

The uptake of modified forms of LDL in an unregulated manner by macrophages, which transforms them into lipid loaded foam cells, is a critical early step in atherosclerosis (Ghosh *et al*. 2010). Cholesterol contained within the LDL molecule is in the form of cholesteryl ester. Once internalised, cholesteryl esters are hydrolysed to cholesterol in the lysosomal compartment and undergo a continual intracellular cycle of re-esterification and hydrolysis (Brown *et al.* 1980). The cholesterol can be stored as cholesteryl esters in the cytoplasm or removed from the cell in the form of free cholesterol (Brown *et al*. 1980). Excessive uptake of modified forms of LDL by macrophage scavenger receptors promotes the accumulation of cholesteryl esters, which are stored as lipid droplets in the cytoplasm of the macrophage giving them a foamy, vacuolated appearance (Brown *et al*. 1980). Given the important role of modified LDL in inducing cholesteryl ester accumulation in foam cell formation, it was of interest to determine the role of DGLA in this process. AcLDL, a form of modified LDL commonly used in LDL uptake experiments (Henson *et al.* 1989; Jones *et al.* 2000; Dai *et al.* 2012), was used to induce cholesteryl ester accumulation in RAW264.7 macrophages. Initially THP-1 macrophages were employed in uptake studies (Appendix 6), however despite accumulation of cholesteryl esters; levels were still low in comparison to RAW264.7 cells. This was unusual as previous studies have used THP-1 macrophages for various cholesterol studies (Draude and Lorenz 2000; Lada *et al*. 2003) and have been shown to express a number of genes related to

cholesterol metabolism (Kritharides *et al.* 1998; McLaren *et al*. 2010; McLaren *et al*. 2011b). Given the larger increase in cholesteryl esters in RAW264.7 macrophages, this system was employed for uptake experiments. As shown in Figure 4.16A, the percentage lipid composition of cholesteryl ester in RAW264.7 macrophages significantly increased by an average of 16.9% on treatment of the cells with acLDL. This increase was significantly attenuated by 11.6% following pre-incubation of the cells with 100 µM DGLA. In Figure 4.16B, cholesteryl ester is expressed as a ratio of total cholesterol. When loaded with acLDL, the ratio of cholesteryl ester: total cholesterol was 0.8. On pre-incubation with DGLA followed by loading, the ratio was reduced to 0.4. Due to high variability between replicates this decrease was not significant despite an obvious trend of reduction. This indicates a role of DGLA in the inhibition of cholesteryl ester accumulation and thereby a potential mechanism by which DGLA inhibits macrophage foam cell formation. The exact mechanism by which DGLA exerts such an inhibitory effect is unclear. There are a number of points at which DGLA could act; uptake of LDL by receptor-mediated and -independent pathways, cholesteryl ester synthesis and/or free cholesterol synthesis and efflux. Figure 4.16A also shows that lipid composition of free cholesterol was significantly reduced by 14.6% on incubation of the cells with acLDL, and this decrease was slightly attenuated by pre-treatment of the cells with DGLA where free cholesterol levels were increased by 1.2% in comparison to acLDL treatment only (not significant). The trend may, however, suggest that DGLA plays a role in promoting free cholesterol accumulation that can then be effluxed out from the cell and thereby prevent the intracellular accumulation of cholesteryl esters. The role of DGLA on each of these mechanisms will be investigated in the next chapter.

4.5.6 Future perspective

The results presented in this chapter suggest a key role for DGLA in inhibiting a number of key steps leading to foam cell formation during atherosclerosis. DGLA inhibited the induction of MCP-1 and ICAM-1 expression by the pro-inflammatory cytokines IFN-γ, IL-1β and TNF-α. In addition, DGLA reduced monocytic migration and acLDL induced accumulation of cholesteryl esters. This raises a number of questions;

1. How does DGLA inhibit cytokine induced MCP-1 and ICAM-1 expression?
2. Is the inhibition of acLDL-induced cholesteryl ester accumulation by DGLA due to decreased uptake of the modified lipoprotein or increased efflux of free cholesterol?
3. Does DGLA achieve these actions directly or indirectly though the metabolites produced following the uptake of the fatty acid into the cells?

In the next chapter, these questions will be addressed and the underlying molecular mechanisms behind DGLAs actions shown here will be investigated.

CHAPTER 5

MOLECULAR MECHANISM UNDERLYING THE ANTI-INFLAMMATORY ACTIONS OF DGLA IN MACROPHAGES

5.1 Introduction

As presented in Chapter 4, DGLA played an important role in attenuation of pro-inflammatory cytokine induced gene expression and cholesteryl ester accumulation in macrophages *in vitro*. The purpose of the studies in this chapter was to further investigate the role of DGLA in these two processes, both of which play key roles in foam cell formation, in order to pin point any underlying molecular mechanisms. The attenuation of IFN-γ induced gene expression by DGLA was chosen for further investigation due to the important and well characterised role the cytokine plays throughout the pathology of atherosclerosis (Gupta *et al*. 1997; McLaren and Ramji 2009). The effect of DGLA on the activation of gene transcription through the JAK/STAT pathway was determined and discussed in more detail in Section 5.1.1. To attempt to understand the role of DGLA in the attenuation of cholesteryl ester accumulation, a number of mechanisms of cholesterol regulation in macrophages were studied. These included the effect of DGLA on the expression of scavenger receptors, uptake of lipoproteins through macropinocytosis, the expression of key genes involved in cholesterol metabolism and cholesterol efflux (Section 5.1.2).

In addition, data presented in Chapter 3 showed that macrophages significantly induced the production of PGE_1 following treatment with DGLA. Eicosanoid production plays an important role in fatty acid signalling (Johnson *et al*. 1997; Norris and Dennis 2012) and PGE_1 has previously been indicated to play an anti-inflammatory role in atherosclerosis (Sinzinger *et al.* 1991; Palumbo *et al.* 2000; Bai *et al.* 2012). It was therefore hypothesised that metabolism of PGE_1 from DGLA in macrophages may be in part responsible for some of the effects the PUFA has on aspects of foam cell formation. The role of PGE_1 in DGLA signalling was further investigated in macrophages.

5.1.1 IFN-γ signalling

Data presented in the previous chapter demonstrated that DGLA attenuated the IFN-γ induced pro-inflammatory gene expression in THP-1 macrophages, RAW264.7 macrophages and HMDMs. This indicated that DGLA may have an inhibitory effect on the IFN-γ signalling pathway. IFN-γ signals through the JAK-STAT pathway (McLaren and Ramji 2009). Signalling through the JAK/STAT pathway is addressed in more detail in Section 1.4.1.1.1. Briefly, IFN-γ binding to the IFN-γR induces dimerisation of the receptor subunits leading to the phosphorylation of JAK1 and JAK2, which in turn phosphorylates the IFN-γR subunits (McLaren and Ramji 2009). The phosphorylated subunits can now bind STAT1. STAT1 is phosphorylated at TYR^{701} and triggers its release into the cytoplasm where it can dimerise and migrate into the nucleus (McLaren and Ramji 2009). STAT1 initiates transcription of IFN-γ inducible genes. Further phosphorylation of STAT1 at position SER^{727} can occur by the action of a number of other kinases, mainly members of the MAPK family. STAT1 phosphorylation at this position is required for maximal activity of STAT1 (McLaren and Ramji 2009). STAT1 plays an important role in IFN-γ signalling as well as signalling induced by other pro-atherogenic pathways such as IL-6 and TLR4 (Sikorski *et al.* 2011b). Given the role of STAT1 in these pro-atherogenic pathways, inhibition of STAT1 has been implicated as a potential therapeutic target in atherosclerosis.

5.1.1.1 Role of STAT1

STAT1 belongs to the STAT family of seven well conserved transcription factors (Sikorski *et al*. 2011b). The structure of STAT1 is detailed in Figure 5.1. The N terminal region of STAT1 allows formation of a dimer complex; this is followed by a coiled region which interacts with other transcription factors and then a highly conserved DNA binding domain and a linker domain, which bridges to SH2 domains for STAT1 interactions with receptors. Finally, there is a C terminal transcriptional activation domain (Sikorski *et al*. 2011b). Two phosphorylation sites are also present at TYR^{701} and SER^{727}. Phosphorylation at TYR^{701} by JAKs allows for dimerisation of STAT1, translocation into the nucleus and activation of target gene transcription (Li *et al*. 2010). Phosphorylation at SER^{727} is not essential for the role of STAT1 as a transcription factor; however it is required for maximal activity (Varinou *et al*. 2003). Inhibition of STAT1 activation and signalling has previously proved beneficial in atherosclerosis. Attenuation of STAT1 signalling in THP-1 macrophages significantly inhibited the expression of scavenger receptor CD36 and prevented foam cell formation (Agrawal *et al.* 2007). In addition, STAT1 deficiency in $ApoE^{-/-}$ mice

significantly reduced atherosclerotic lesion size (Agrawal *et al*. 2007). Given the role of STAT1 in IFN-γ signalling, the effect of DGLA on phosphorylation of the transcription factor was investigated in the studies presented in this chapter.

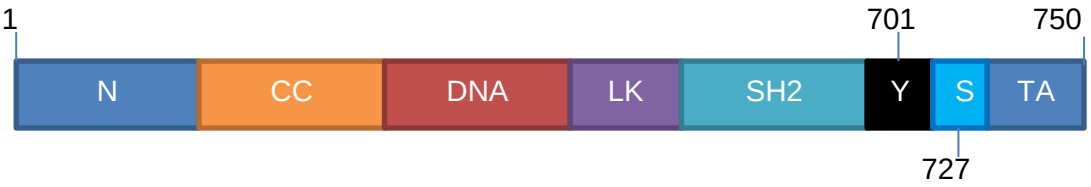


Figure 5.1 – Structure of STAT1 transcription factor

Figure adapted from (Sikorski *et al*. 2011b). N - N terminal domain; CC – coiled coil; DNA – DNA binding domain; LK – linker; SH2 – Src homology 2; Y – TYR^{701} phosphorylation site; S – SER^{727} phosphorylation site; TA - transcriptional activation site.

5.1.2 Modified LDL uptake and cholesteryl ester accumulation

Data presented in the previous chapter demonstrated that DGLA attenuated acLDL induced cholesteryl ester formation in macrophages. To determine the mechanisms underlying this observation, experiments were designed to test the effect of DGLA at a number of steps involved in the uptake of modified LDL and accumulation of cholesteryl esters. This included the effect of DGLA on the uptake and efflux of cholesterol, receptor independent uptake (macropinocytosis) and the expression of key genes involved in scavenger receptor-mediated uptake, cholesteryl ester synthesis and cholesterol efflux. Figure 5.2 depicts an overview of modified LDL uptake, cholesterol esterification cycle and efflux that were targeted in studies presented in this chapter.

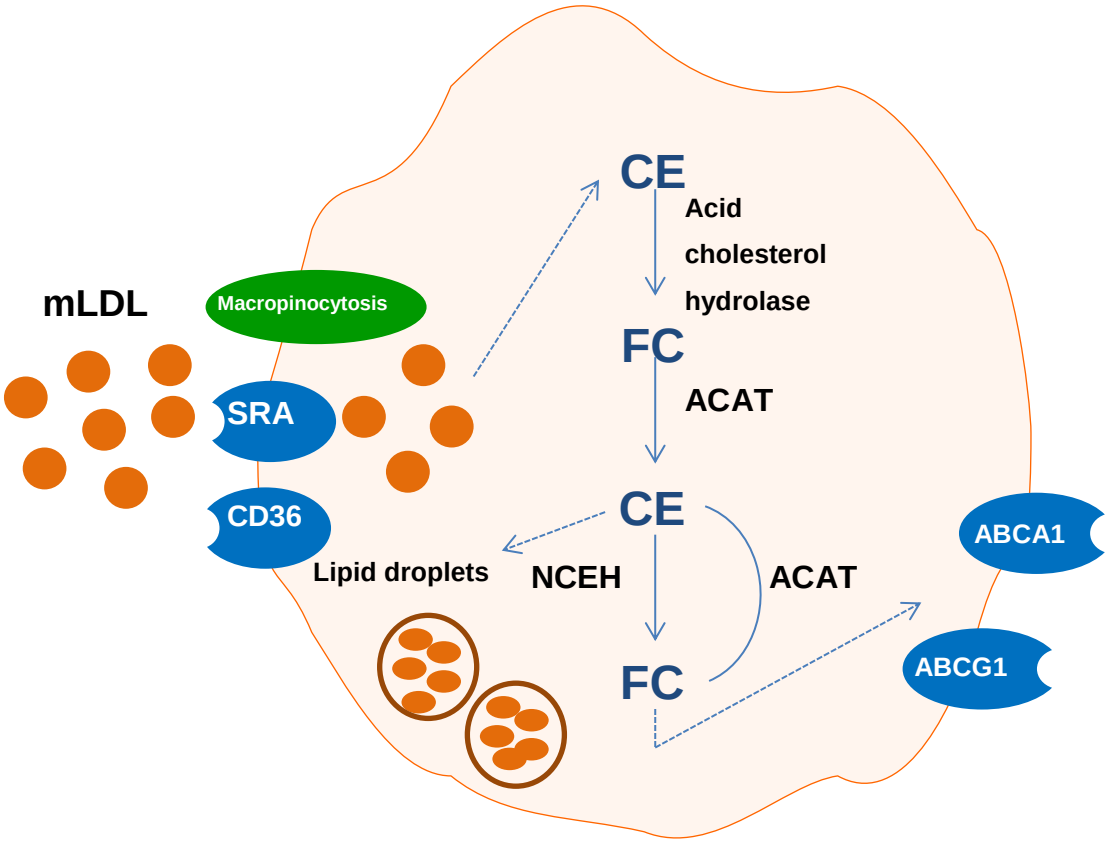


Figure 5.2 – Overview of cholesteryl ester accumulation and cholesterol efflux in macrophages

The figure provides an overview of the underlying mechanisms that are potentially involved in the attenuation of cholesteryl ester accumulation by DGLA and investigated in the studies presented in this chapter. First, the uptake of modified LDL was investigated, followed by determination of the effect of DGLA on scavenger receptor expression (SRA and CD36) and macropinocytosis. Second, the expression of key genes involved in cholesteryl ester accumulation (ACAT) and production of free cholesterol (NCEH) were analysed. Finally, the effects of DGLA on cholesterol efflux from macrophages were determined, along with the expression of key ABC transporter proteins (ABCA1 and ABCG1).

5.1.2.1 Cholesterol uptake

5.1.2.1.1 Scavenger receptors

Macrophages express scavenger receptors on their cell surface. Scavenger receptors are cell surface glycoproteins which can bind and internalise modified LDL such as oxLDL and acLDL (Goldstein *et al.* 1979; Peiser and Gordon 2001; Park 2014). They are a broad family of receptors and are classified into six groups (Class A-F) depending on their tertiary structure (Peiser and Gordon 2001). Scavenger receptors have been intensively studied in relation to atherosclerosis due to their role in foam cell formation. Recognition and internalisation of modified LDL is unregulated and leads to excessive accumulation in macrophages (Kzhyshkowska *et al.* 2012). This results in accumulation of cholesteryl esters, a hallmark of foam cells. Specific focus was on two important scavenger receptors extensively characterised in atherosclerosis foam cell formation; CD36 and SRA.

CD36 is a Class B scavenger receptor expressed on the surface of monocytes, macrophages, endothelial cells, adipocytes and platelets (Park 2014). The receptor is organised into two transmembrane domains, two cytoplasmic domains and a large glycosylated extracellular domain (Park 2014). CD36 binds and internalises oxLDL and has been implicated to contribute to foam cell formation and atherosclerosis (Endemann *et al.* 1993; Febbraio *et al.* 2000; Park 2014). Mouse models of the disease deficient in CD36 show a significant decrease in plaque formation and progression (Nozaki *et al.* 1995; Febbraio *et al*. 2000; Febbraio *et al.* 2004; Guy *et al.* 2007; Kuchibhotla *et al*. 2008).

SRA is a class A scavenger receptor found mainly expressed on the surface of macrophages (de Winther *et al.* 2000). The receptor was first classified in 1979 in mouse peritoneal macrophages and was shown to bind and internalise acLDL, leading to a significant increase in cellular cholesteryl ester and cholesterol content (Goldstein *et al*. 1979). Increased expression has been identified in atherosclerotic lesions (Matsumoto *et al.* 1990; Ylaherttuala *et al.* 1991) and mouse models deficient in SRA show significant protection against atherosclerotic lesion development (Sakaguchi *et al.* 1998; Kuchibhotla *et al*. 2008).

Given the importance of SRA and CD36 in the uptake of modified forms of LDL, these two genes were selected for further study.

5.1.2.2 Macropinocytosis

Receptor-mediated endocytosis of modified LDL has been extensively studied in macrophages and is often regarded as the main pathway leading to cholesterol accumulation. Another endocytic pathway however, has been implicated to play a role in LDL uptake by macrophages. Macropinocytosis is a form of endocytosis providing non-selective uptake of solute macromolecules, by a process dependent on the formation of macropinosomes formed from membrane ruffling (Swanson and Watts 1995). This process has largely been shown to take up excessive amounts of native forms of LDL leading to foam cell formation (Kruth *et al*. 2002; Kruth *et al.* 2005; Zhao *et al.* 2006). Other studies have also reported a contribution of macropinocytosis to the uptake of oxLDL and acLDL (Jones and Willingham 1999; Yao *et al.* 2009). Previous work has indicated that inhibiting macropinocytosis in macrophages decreases foam cell formation (Yao *et al*. 2009) and therefore may be a potential target in atherosclerosis.

5.1.2.3 Cholesteryl ester synthesis

Following internalisation of the LDL particle by an endocytic pathway described in Sections 5.1.2.1 and 5.1.2.2, the cholesteryl ester contained within the LDL particle is digested to unesterified/free cholesterol by an acid cholesterol ester hydrolase in the lysosome (Daugherty *et al*. 2008). This free cholesterol is re-esterified to cholesteryl esters by a microsomal ACAT protein (Brown *et al*. 1980; Ghosh *et al*. 2010). This esterified cholesterol has one of two fates: first it is once again hydrolysed to free cholesterol by one of a group of enzymes collectively referred to as neutral cholesterol ester hydrolase (nCEH) (Sekiya *et al*. 2009) and removed from the macrophage by several extracellular transporters. Second, it is stored in the cytoplasm as lipid droplets (Daugherty *et al*. 2008). The rate-limiting step in the cholesteryl ester cycle is the nCEH enzyme reaction that allows for the clearance of free cholesterol from the macrophage. Excessive uptake of modified LDL therefore increases the storage of cholesteryl esters as lipid droplets. An increased amount of lipids in the cytoplasm of macrophages promotes the conversion of macrophages to lipid loaded foam cells (Ghosh *et al*. 2010). Inhibition of cholesteryl ester accumulation would therefore attenuate foam cell formation. The effect of DGLA in the expression of two key genes involved in the re-esterification and hydrolysis steps of the cholesterol cycle in macrophages was investigated.

5.1.2.3.1 ACAT1

ACATs are a family of membrane bound proteins which utilise long chain fatty acid-CoA and cholesterol to form cholesteryl esters (Chang *et al.* 2009). ACAT is

responsible for the storage of cholesteryl esters within lipid droplets found in the cytoplasm of macrophage foam cells (Dove *et al.* 2006). There are two isoforms of the ACAT protein; ACAT1 and ACAT2 (Chang *et al*. 2009). Although similar in function, the two proteins have different membrane topology and localisation of expression (Chang *et al*. 2009). Expression of ACAT1 in particular has been shown in macrophages and linked to atherosclerosis. The protein has been identified in atherosclerotic lesions, localised specifically to macrophages (Miyazaki *et al.* 1998). A number of studies have investigated the role of ACAT1 in cholesterol metabolism in macrophages (Kusunoki *et al.* 2001; Yang *et al.* 2004; Dove *et al.* 2005). In ACAT1$^{-/-}$ peritoneal macrophages loaded with acLDL, efflux of cholesterol derived from this modified lipoprotein was increased while overall cellular efflux was reduced (Dove *et al*. 2005). In addition, in peritoneal macrophages from ACAT1$^{-/-}$ mice, cholesterol esterification was reduced by 93% (Yang *et al*. 2004; Dove *et al*. 2006), while in THP-1 macrophages an ACAT1 inhibitor decreased cholesteryl ester accumulation (Yang *et al*. 2004). Given the already established link between ACAT1, macrophages and atherosclerosis, this isoform of the ACAT family was selected for further study.

5.1.2.3.2 NCEH

The hydrolysis of cholesteryl esters is critical for cholesterol removal from the macrophage. This is achieved by the action of nCEH enzymes (Igarashi *et al.* 2010). In macrophages three enzymes have been identified as nCEHs; hormone-sensitive lipase (LIPE), cholesterol ester hydrolase (CEH) and NCEH1 (Igarashi et al. 2010). All three enzymes have been characterised to some extent in human and mouse macrophages to play a role in cholesteryl ester hydrolysis and increase cholesterol efflux (Igarashi *et al*. 2010). In ApoE$^{-/-}$ NCEH1$^{-/-}$ mice, atherosclerosis was aggravated in comparison to controls. In addition, NCEH1$^{-/-}$ mouse peritoneal macrophages incubated with acLDL showed an increase in cholesteryl ester accumulation and decrease in cholesterol efflux (Sekiya *et al*. 2009). In the same study ApoE$^{-/-}$, NCEH1$^{-/-}$ mice were crossed with LIPE knockout mice. In these mice nCEH activity was dramatically reduced, to 10% of that of wild type mice suggesting that both these enzymes play an important role in the nCEH activity of macrophages (Sekiya *et al*. 2009). In human macrophages however, LIPE and CEH activity was barely detectable and inhibition of both had no effect on nCEH activity (Igarashi *et al*. 2010). The pattern of NCEH1 expression however was similar to that of nCEH activity in human macrophages and knockdown specifically inhibited this activity (Igarashi *et al*. 2010). NCEH1 was also identified to be expressed in macrophage foam cells from

atherosclerotic lesions (Igarashi *et al*. 2010). Given the role of NCEH1 in nCEH activity of mouse and human macrophages, this nCEH was selected for further study.

5.1.2.4 Cholesterol efflux

Cholesterol efflux is essential to prevent the accumulation of cholesteryl esters. Cholesterol is removed from macrophages as free cholesterol to lipoprotein carriers such as HDL, where it is transported to the liver for catabolism (Phillips 2014). There are a number of mechanisms by which cholesterol can be removed from macrophages. These include passive diffusion, facilitated diffusion by SR-B1 and active diffusion using the ABC transporter proteins; ABCA1 and ABCG1 (Phillips 2014). Passive diffusion accounts for a large amount of cholesterol efflux from non-cholesterol loaded macrophages; however in macrophage foam cells active transport is crucial. ABCA1 and ABCG1 transporter proteins play a pivotal role in cholesterol efflux from macrophages (Yvan-Charvet *et al.* 2010).

5.1.2.4.1 ABC transporters

The ABC transporter proteins utilise ATP to transport molecules across the plasma membrane (Soumian *et al.* 2005). ABCA1 and ABCG1 are expressed in macrophages and aid in reverse cholesterol transport (Soumian *et al*. 2005). There are a number of proposed mechanisms by which ABCA1 and ABCG1 have been suggested to remove cholesterol from macrophages and where they are localised, however the process is still not fully understood (Yvan-Charvet *et al*. 2010). The ABC transporters have been well characterised in their role in cholesterol efflux and have been suggested to play a protective role in atherosclerosis (Singaraja *et al.* 2002; Yvan-Charvet *et al.* 2007; Tarling *et al.* 2010). *In vivo* mouse models demonstrated that knockout of these transporter proteins in macrophages resulted in significant reduction of cholesterol efflux (Wang *et al.* 2007). In $LDLr^{-/-}$ mouse models, ABCA1 and ABCG1 deficiency increased atherosclerosis and infiltration of foam cells. Isolated macrophages from these mice showed impaired cholesterol efflux (Yvan-Charvet *et al*. 2007). Similarly, over expression of ABCA1 in $ApoE^{-/-}$ mice results in development of smaller lesions and increased cholesterol efflux from isolated macrophages (Singaraja *et al*. 2002). ABCA1 and ABCG1 expression and their influence on cholesterol homeostasis also play a role in modulating the inflammatory response in atherosclerosis, such as an increase in pro-inflammatory gene expression (Yvan-Charvet *et al.* 2008; Zhu *et al.* 2008). Given the important role of the ABC transporters in cholesterol efflux, the effect of DGLA on their expression in macrophages was of interest.

5.1.2.4.2 ApoE

A number of other factors also play a role in cholesterol efflux from the macrophage in addition to ABC transporters. ApoE is a multi-functional protein which plays a role in the maintenance of plasma cholesterol levels (Curtiss 2000). It is a component of plasma lipoproteins (HDL, VLDL) that carry cholesterol to the liver for clearance. The $ApoE^{-/-}$ knockout mouse model is commonly used in atherosclerosis as the resulting hypercholesterolemic state allows for the development of spontaneous atherosclerosis (Curtiss 2000). The majority of ApoE is synthesised in the liver, however the protein can also be produced locally by macrophages (Zhu *et al.* 1998). Macrophage ApoE has little effect of plasma cholesterol levels, but still proved to have an anti-atherogenic effect in a number of studies (Zhu et al. 1998; Boisvert *et al.* 1999; Hasty *et al.* 1999). No correction of hyperlipidaemia was observed in $ApoE^{-/-}$ knockout mice expressing ApoE specifically in macrophages in comparison to control mice, however there was a significant reduction in lesion size (Zhu *et al*. 1998). Similarly, in $ApoE^{-/-}$ mice (5-13 weeks) expressing ApoE from arterial macrophages, there was a significant reduction in lesion size with no change in plasma cholesterol levels from control $ApoE^{-/-}$ mice (Hasty *et al*. 1999). This suggests that there are other mechanisms, independent to plasma cholesterol levels, in which ApoE acts to attenuate atherosclerosis. In macrophages, production of ApoE has been shown to increase cholesterol efflux. A 10% increase in cholesterol efflux was observed from macrophage of $ApoE^{-/-}$ mice expressing an ApoE gene in macrophages (Zhu *et al*. 1998). This was also observed in mice expressing ApoE from the arterial wall. It was observed there was a reduced amount of cholesterol in the arterial wall of transgenic mice suggesting ApoE induces reverse cholesterol transport from the arterial wall (Shimano *et al.* 1995). Given the role of ApoE in inducing cholesterol efflux from macrophages, it was hypothesised that DGLA may have an effect on its expression.

5.1.3 DGLA metabolites

The final aim of the chapter was to determine the role of eicosanoids produced from DGLA to identify the molecular mechanism underlying the action of the PUFA. Fatty acids can induce signalling mechanisms directly or indirectly through the production of eicosanoids (Johnson *et al*. 1997; Levin *et al*. 2002; Norris and Dennis 2012; Raphael and Sordillo 2013). As shown previously in Chapter 3, DGLA was metabolised to PGE_1 and 15-HETrE in THP-1 macrophages (Section 3.4.4). In macrophages treated with vehicle, there was no basal production of PGE_1. Only on treatment with DGLA did the production of the eicosanoid increase dramatically, offsetting the ratio of PGE_2:PGE_1 production in the macrophage. PGE_1 is a product of

COX metabolism on DGLA (Calder 2001; Levin *et al*. 2002; Wang *et al*. 2012) and it has been documented as having anti-inflammatory actions in a number of diseases. In rabbit models of atherosclerosis, PGE_1 decreased the accumulation of LDL in the arterial wall and increased the stability of atherosclerotic plaques (Sinzinger *et al*. 1991; Bai *et al*. 2012). In addition, the eicosanoid has been implicated to have beneficial actions in models of myocardial infarction, heart failure and cancer (Tabolacci *et al*. 2010; Li *et al.* 2011; Hou *et al.* 2013). This indicates that metabolites of DGLA may play a role in the anti-inflammatory/atherogenic actions observed in macrophages. Given the important role of PGE_1 reported previously in numerous diseases, this prostaglandin was a key focus in understanding the molecular mechanisms underlying the action of DGLA. The role of PGE_1 in THP-1 macrophages was investigated through inhibition of COX enzymes.

5.2 Aims

Following on from results obtained with DGLA presented in Chapters 3 and 4, the aim of studies in this chapter was to determine the mechanisms underlying some key findings. As shown in the studies presented in the previous chapters, DGLA inhibited the IFN-γ induced expression of key pro-inflammatory genes in a number of macrophage cultures. AcLDL uptake and cholesteryl ester accumulation was also attenuated in macrophages by DGLA. Lastly, DGLA metabolised to PGE_1, a prostaglandin with anti-inflammatory actions. These three key points were chosen for further investigations. Detailed aims were as follows;

1. DGLA inhibition of IFN-γ induced gene expression
    - Effects of DGLA on STAT1 phosphorylation at positions TYR^{701} and SER^{727}
2. Attenuation of modified LDL induced cholesteryl ester accumulation by DGLA
    - Effect of DGLA on uptake of oxLDL
    - Effect of DGLA on receptor-mediated uptake (scavenger receptors) and fluid phase uptake (macropinocytosis)
    - Effect of DGLA on expression of key genes involved in cholesteryl ester accumulation and free cholesterol formation (ACAT1 and NCEH1)
    - Effect of DGLA on cholesterol efflux and key genes involved in the process (ABCA1, ABCG1 and ApoE)
3. Determine the role of PGE_1 in the anti-inflammatory effect of DGLA
    - Effect of PGE_1 on the IFN-γ induced MCP-1 expression
    - Effect of PGE_1 on monocyte migration
    - Effect of knockdown of PGE_1 formation from DGLA on the IFN-γ induced MCP-1 expression

5.3 Experimental plan

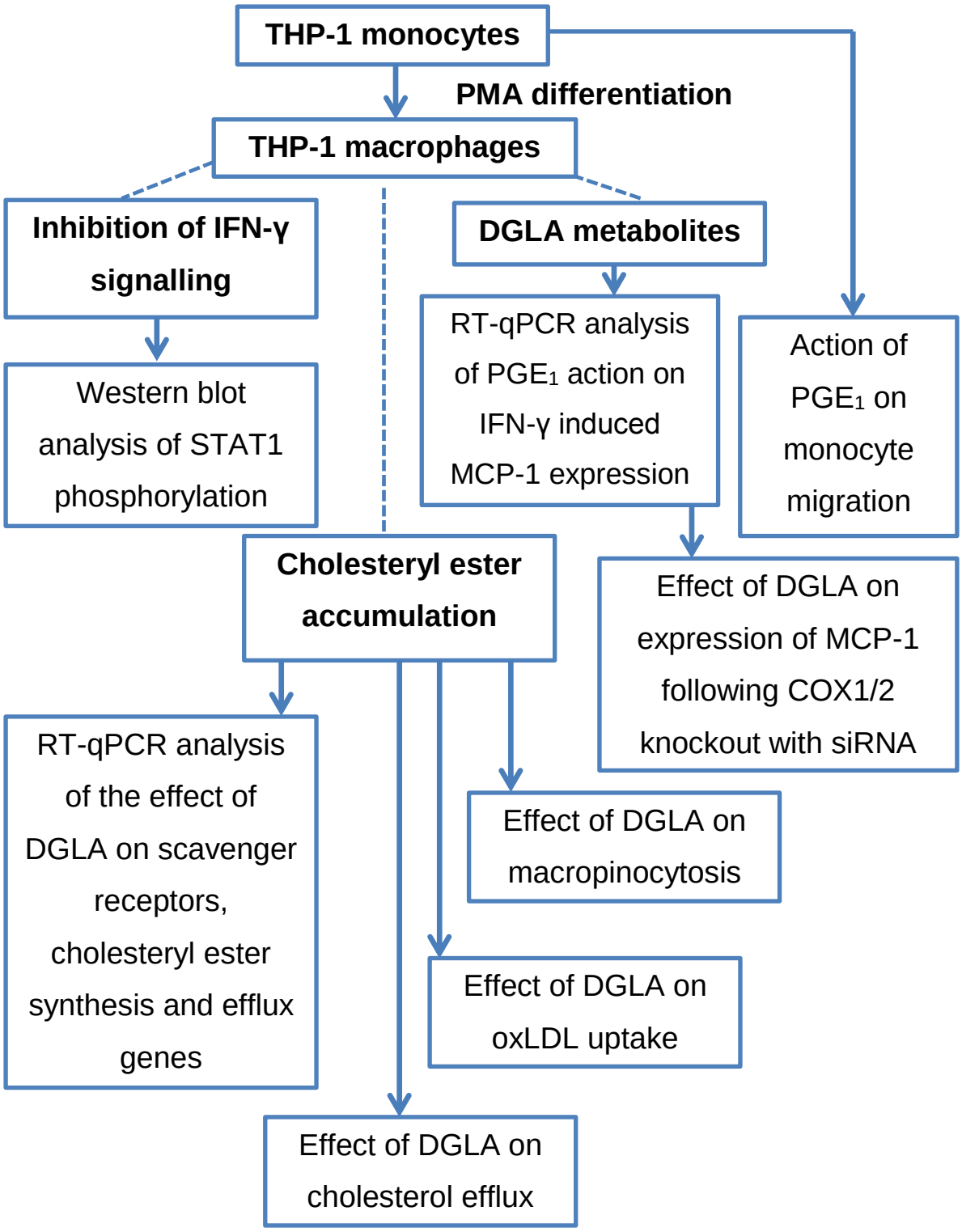


Figure 5.3 - Overview of experimental strategy

THP-1 monocytes and macrophages were used to investigate three key results; inhibition of IFN-γ signalling, acLDL uptake and cholesteryl ester accumulation together with the actions of DGLA metabolites.

5.4 Results

5.4.1 The effect of DGLA on IFN-γ signalling through the JAK/STAT pathway

5.4.1.1 Effect of DGLA on IFN-γ dependant STAT1 phosphorylation

As described in Chapter 4, DGLA inhibited the IFN-γ induced expression of two key pro-inflammatory genes; MCP-1 and ICAM-1 in THP-1 macrophages, RAW264.7 cell line and primary cultures of HMDM (Section 4.4.6). It was therefore of interest to determine the precise effect of DGLA on the IFN-γ signalling pathway. The cytokine signals through the JAK/STAT pathway and the initial focus was on STAT1 and its activation by phosphorylation. The effect of DGLA on STAT1 phosphorylation at two sites, SER^{727} and TYR^{701}, was determined by western blotting. THP-1 macrophages were pre-treated with DGLA or DMSO control for 24 hours prior to addition of 250 U/ml IFN-γ or vehicle control for 30 minutes, a time point previously optimised in the laboratory for maximal STAT1 phosphorylation (Li *et al*. 2010). STAT1 phosphorylation at SER^{727} and TYR^{701} was significantly induced on treatment of the cells with IFN-γ by 2.3-fold and 7.9-fold respectively (Figure 5.4). On pre-treatment with DGLA followed by IFN-γ stimulation, STAT1 phosphorylation at SER^{727} was significantly inhibited by an average of 31%. This attenuation was not seen in phosphorylation of TYR^{701}.

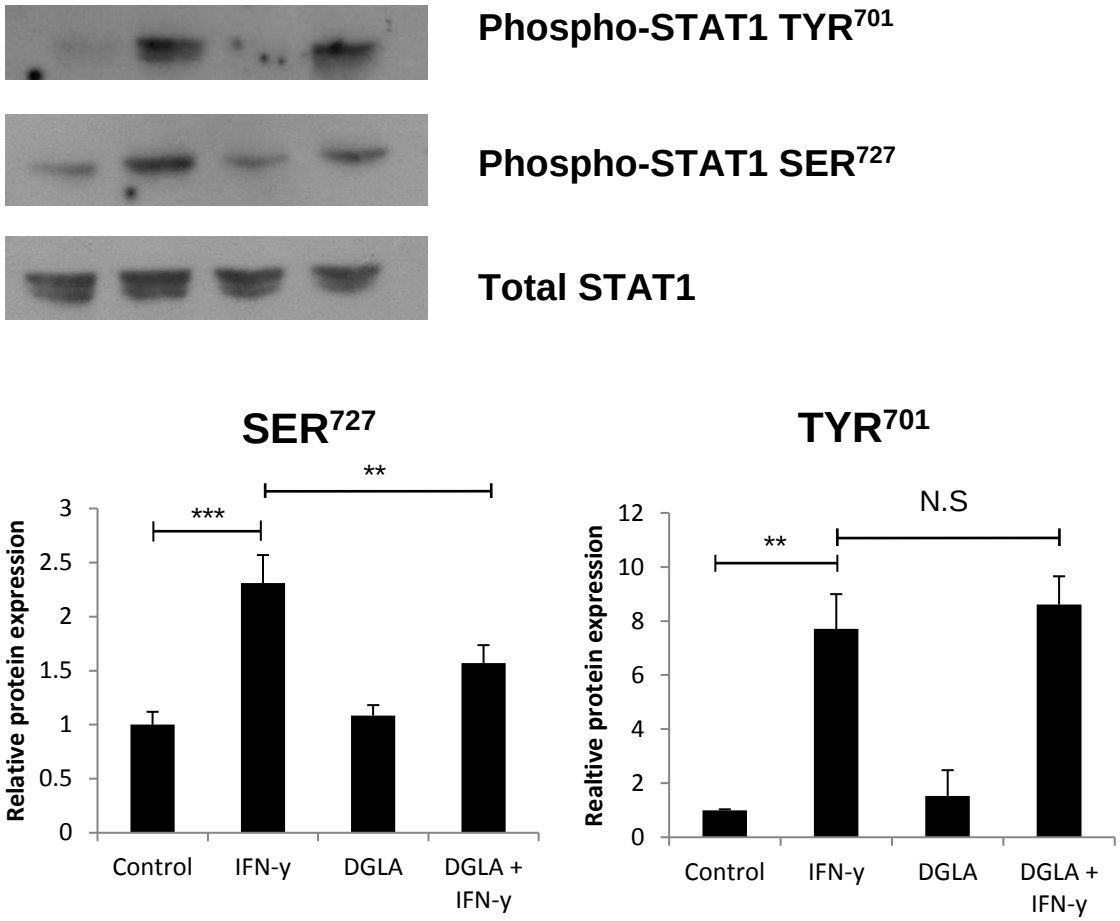


Figure 5.4 - DGLA attenuates the IFN-γ induced STAT-1 phosphorylation at SER^{727} but not TYR^{701}

THP-1 macrophages were pre-treated with 50 µM DGLA (DGLA, DGLA + IFN-γ) or DMSO vehicle control (Control, IFN-γ) for 24 hours. Following this, macrophages were stimulated with 250 U/ml IFN-γ or vehicle for 30 minutes. Proteins were extracted and separated by gel electrophoresis and transferred onto a membrane. Membranes were probed with antibodies specific for STAT1 SER^{727}, STAT1 TYR^{701} and total STAT1. Membranes were exposed to X-ray film and bands were analysed by densitometry for quantification. Graphs indicate the average of three independent repeats (mean +/- SD), control arbitrarily assigned as 1. Statistical analysis was performed using a one way ANOVA followed by Tukey's post hoc analysis ** P <0.01, *** P ≤ 0.001, N.S – Not significant

5.4.2 Molecular mechanism underlying the attenuation of modified LDL uptake and cholesteryl ester accumulation in macrophages

5.4.2.1 Uptake of Dil-oxLDL

To determine the mechanisms underlying the effect of DGLA on cholesteryl ester accumulation in macrophages, uptake of cholesterol was determined. To do so, uptake of oxLDL labelled with a fluorescent marker (Dil-oxLDL) was measured by FACS. The initial study investigating the accumulation of cholesteryl esters in macrophages utilised acLDL as a form of modified cholesterol. Both acLDL and oxLDL are commonly used in cholesterol uptake and efflux studies, acLDL is more avidly taken up in comparison to oxLDL, however, both show comparable actions and induce macrophage foam cell formation *in vitro* (McLaren *et al*. 2010; McLaren *et al*. 2011b). Despite this, oxLDL is the major form of modified LDL found in atherosclerosis *in vivo* (Lusis 2000; Nishi *et al.* 2002; Shashkin *et al.* 2005) and therefore more clinically relevant.

THP-1 macrophages were pre-treated with 50 μM DGLA or vehicle control for 24 hours prior to loading with 5 μg/ml Dil-oxLDL for a further 24 hours. Cells were collected, resuspended in 2% PFA and uptake was measured by determining fluorescence by FACS. As shown in Figure 5.5, on treatment with Dil-oxLDL, uptake was significantly increased in comparison to unloaded macrophages. Following pre-treatment with 50 μM DGLA followed by loading with Dil-oxLDL, this increase was significantly attenuated by an average of 21%.

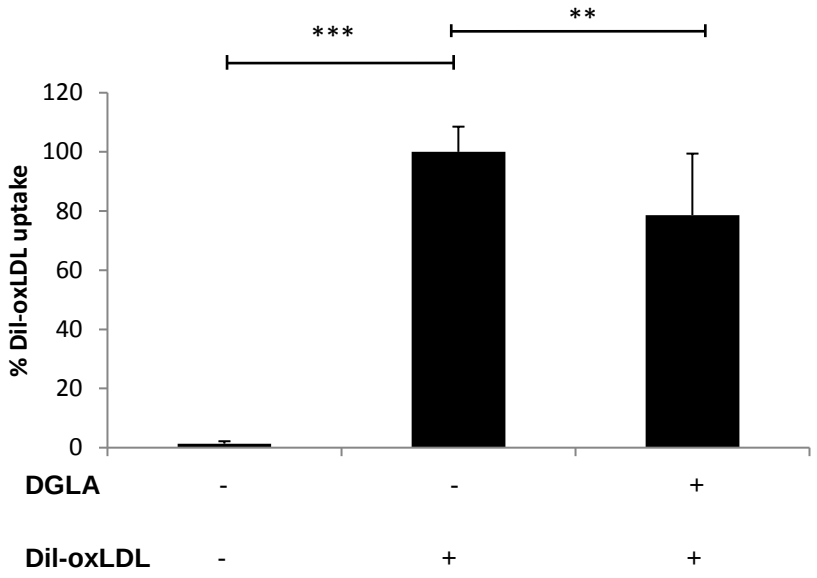


Figure 5.5 – DGLA significantly attenuated Dil-oxLDL uptake in THP-1 macrophages

THP-1 macrophages were incubated with 50 μM DGLA or DMSO for 24 hours prior to treatment with 5 μg/ml Dil-oxLDL for a further 24 hours (control cells were pre-treated with DMSO without further incubation with Dil-oxLDL). Cells were collected, resuspended in 2% PFA and uptake was measured using FACS analysis counting 10,000 events. DMSO in the presence of Dil-oxLDL was used as a positive control and arbitrarily assigned as 100%. Graph indicated the average uptake (+/- SD) from 4 independent experiments. Statistics were performed using a one-way ANOVA followed by Tukey's post hoc analysis. ** $P < 0.01$, *** $P < 0.001$.

5.4.2.2 Scavenger receptor expression

Scavenger receptors are expressed on the surface of macrophages and recognise and internalise large, unregulated amounts of modified LDL leading to the formation of foam cells (de Winther *et al*. 2000; Shashkin *et al*. 2005; Park 2014). CD36 and SRA are key scavenger receptors expressed by macrophages in atherosclerotic lesions and mice lacking these genes show significant protection to atherosclerosis (Endemann *et al*. 1993; Sakaguchi *et al*. 1998; Kunjathoor *et al.* 2002; Febbraio *et al*. 2004). Given the role of DGLA in oxLDL uptake and acLDL induced cholesteryl ester accumulation in macrophages, it was hypothesised that the levels of these scavenger receptors on the surface of the cells may play an important role. The mRNA and protein expression of CD36 and SRA were measured by RT-qPCR and western blotting respectively.

THP-1 macrophages were incubated with vehicle or 50 µM DGLA for 24 hours prior to RNA or protein extraction. On incubation with DGLA, the mRNA expression of SRA and CD36 was attenuated by an average of 22% and 20% respectively (Figure 5.5). Unfortunately due to time constraints and difficulties in optimisation of antibodies, only SRA was measured at the protein level. On pre-treatment with 50 µM DGLA, there was a significant reduction of approximately 40% in the protein expression of SRA.

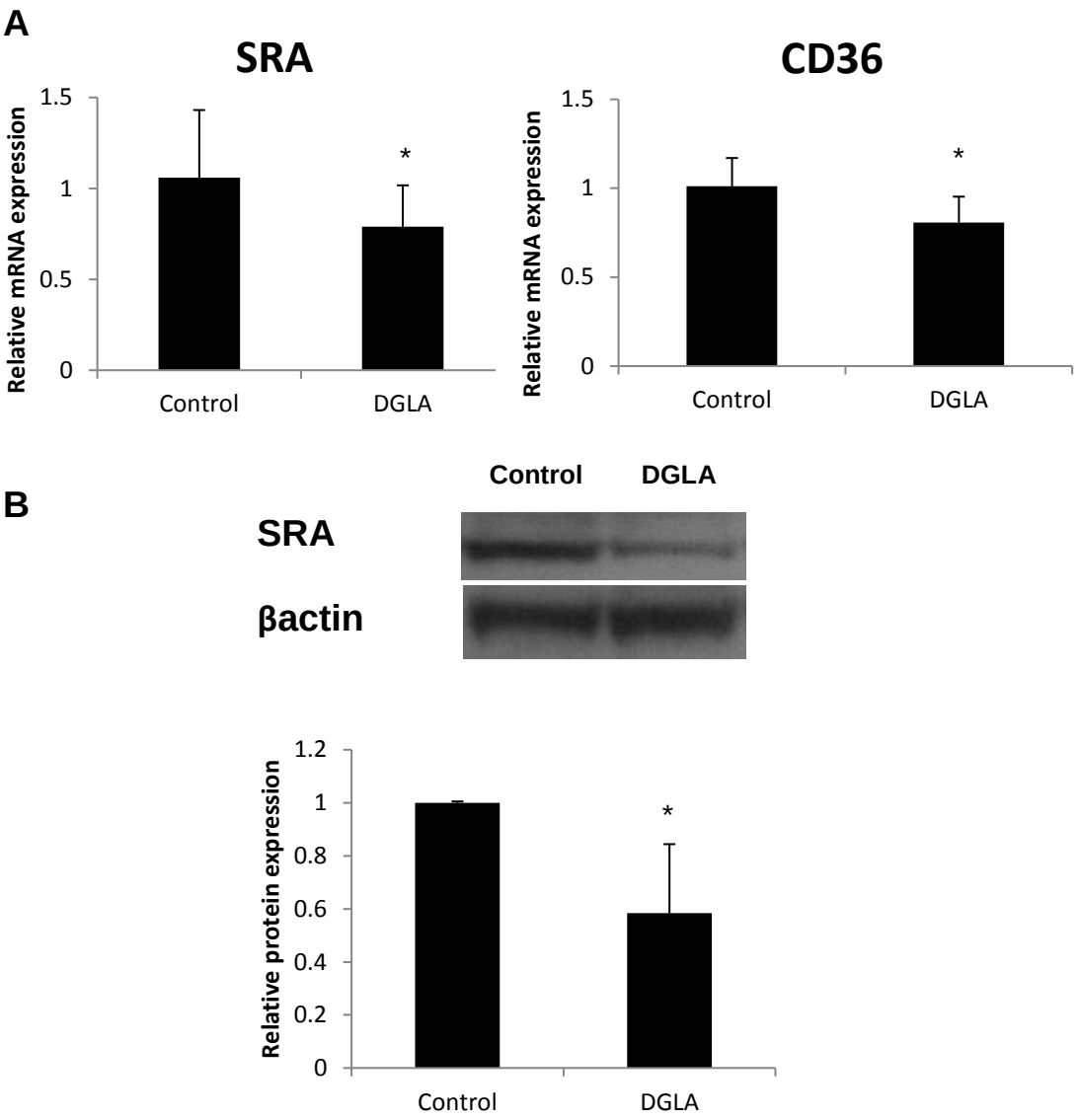


Figure 5.6 - DGLA significantly inhibited the expression of CD36 and SRA in THP-1 macrophages

THP-1 macrophages were pre-incubated with 50 μM DGLA or DMSO vehicle (Control) for 24 hours. A - Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for CD36, SRA or GAPDH control. Graphs display average gene expression (mean +/- SD) (control arbitrarily assigned as 1) from four independent experiments. B – Total protein was extracted, subjected to gel electrophoresis and immunodetection with antibodies specific for SRA and β-actin. Graph indicates the average protein expression from 4 independent repeats (+/- SD) with control arbitrarily assigned as 1. Statistical analysis was performed using a Students two tailed t test. * $P \leq 0.05$.

5.4.2.3 – Macropinocytosis

In addition to receptor-mediated uptake by scavenger receptors, macrophages have been shown to take up large amounts of native LDL and modified LDL by bulk fluid phase macropinocytosis (Kruth *et al*. 2005; Michael *et al*. 2013). The endocytic pathway has also been implicated to play a role in foam cell formation (Kruth *et al*. 2002; Yao *et al*. 2009). To measure the effect of DGLA on macropinocytosis, the uptake of Lucifer yellow (LY) was measured by FACS. LY is a florescent dye commonly used in the literature as a marker to measure uptake by macropinocytosis (Swanson 1989; Jones and Willingham 1999; Michael *et al*. 2013). THP-1 macrophages were pre-incubated with the DMSO vehicle or 50 µM DGLA followed by 100 µg/ml LY for 24 hours. Macrophages were fixed and samples prepared for FACS analysis. LY uptake from vehicle treated cells was arbitrarily assigned as 100% LY uptake. On pre-treatment with DGLA, LY uptake was significantly attenuated by an average of 17% (Figure 5.7).

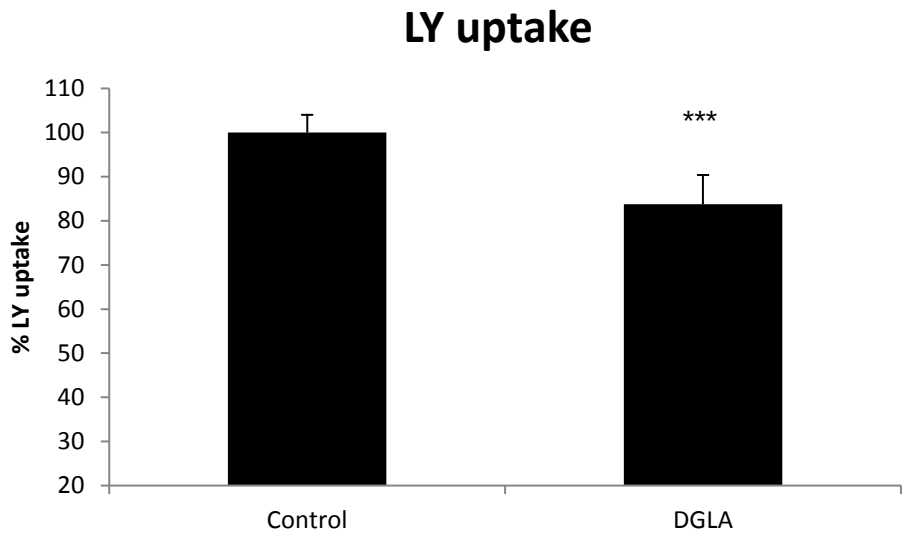


Figure 5.7 - DGLA inhibits LY uptake by macropinocytosis in THP-1 macrophages

THP-1 macrophages were incubated with 50 µM DGLA or DMSO for 24 hours followed by 100 µg/ml LY. LY uptake was analysed using flow cytometry (FACS Canto). LY uptake is represented as a percentage with vehicle control as 100%, error bars indicate +/- SD from three independent experiments. Statistics was performed using a Students two tailed t-test, *** $P \leq 0.001$.

5.4.2.4 – Cholesterol ester synthesis

Once LDL has undergone endocytosis, the lipoprotein is broken down and cholesteryl ester contained within it undergoes a cycle of hydrolysis and re-esterification in the macrophage (Brown *et al*. 1980). To investigate the role of DGLA on cholesteryl ester accumulation, RT-qPCR was used to measure the effect of the fatty acid on the expression of two enzymes responsible for esterification and hydrolysis in the macrophage. THP-1 macrophages were treated with vehicle or 50 µM DGLA for 24 hours before RT-qPCR using specific primers for ACAT1 and NCEH1. DGLA had no effect on the gene expression of ACAT1 and NCEH1 when compared to the DMSO control.

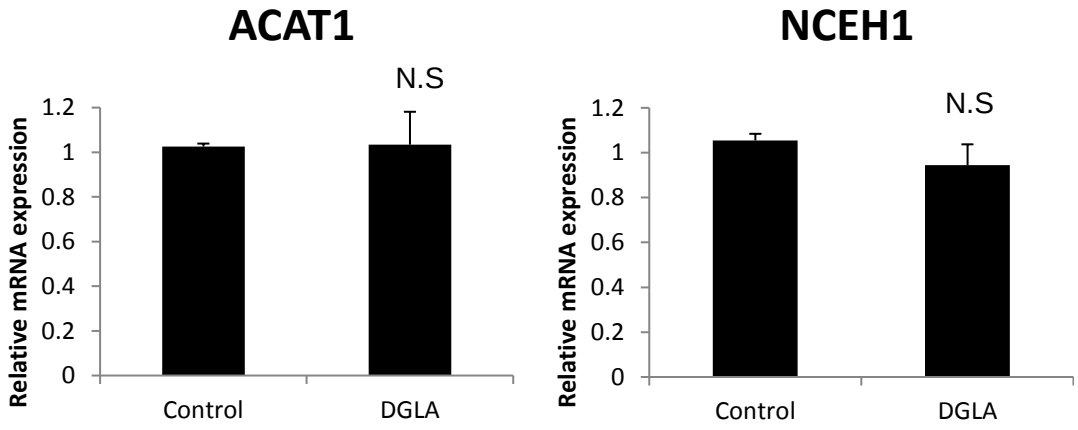


Figure 5.8 - DGLA has no effect on mRNA expression of ACAT1 and NCEH1

THP-1 macrophages were incubated with DMSO vehicle or 50 µM DGLA for 24 hours. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for human ACAT1, NCEH1 or GAPDH. Graphs display average gene expression (mean +/- SD) (control arbitrarily assigned as 1) of 4 independent experiments. Statistical analysis was performed using a two tailed Students t-test. N.S – Not significant

5.4.2.5 – Cholesterol efflux

Macrophages can efflux cholesterol with the aid of cholesterol carriers. Free cholesterol can be removed from the cell to HDL, the main component of which is ApoA1 (Phillips 2014). It has been shown previously that ApoA1 mediates cholesterol efflux in macrophages (Yvan-Charvet *et al*. 2010). To determine if DGLA plays a role in cholesterol efflux from macrophages, THP-1 macrophages were loaded with acLDL and ^{14}C-Cholesterol. Following this, macrophages were treated with ApoA1 to induce cholesterol efflux or vehicle control to determine basline cholesterol efflux. Efflux was measured using scintillation counting. Radioactivity was measured in both the cell fraction and overlaying media and efflux was calculated as a percentage of counts in the media fraction to total counts. Cholesterol efflux measured in acLDL loaded macrophages unstimulated with ApoA1 allowed for a basal level of efflux to be measured and was substracted from all other samples (data not shown). Graph displays ApoA1 stimulated cholesterol efflux as percentage efflux. Macrophages pre-treated with DGLA before loading with acLDL and stimulation with ApoA1, showed a significant increase of approximately 10% in cholesterol efflux in comparison to macrophages with vehicle control (Figure 5.9).

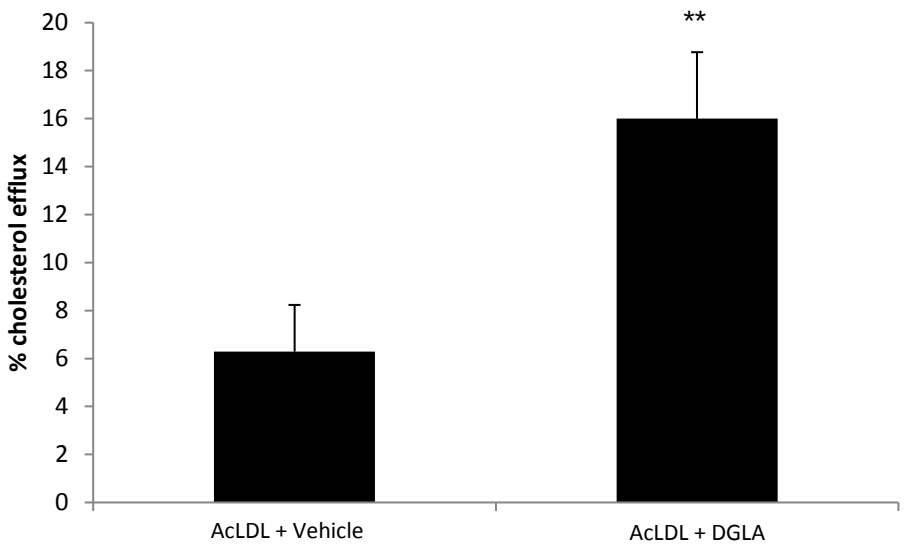


Figure 5.9 - DGLA increased ApoA1 stimulated cholesterol efflux from THP-1 macrophages

THP-1 macrophages were incubated with 50 µM DGLA or DMSO vehicle for 24 hours prior to treatment with 25 µg/ml acLDL and 0.5 µCi ^{14}C-cholesterol in RPMI media with 0.2% (w/v) BSA for a further 24 hours. Following this incubation, cells were washed with PBS; media was replaced in the presence of 10 µg/ml ApoA1 for 24 hours. Media was collected in Eppendorf tubes and cells treated with 0.2% (w/v) NaOH until macrophages had detached. Cell suspension was removed into Eppendorf tubes. Radioactivity from media and cell suspension was measured using scintillation counting. Background cholesterol efflux (unstimulated with ApoA1, not displayed on graph) was subtracted from ApoA1 stimulated cells. Results expressed as percentage cholesterol efflux. Error bars indicate +/- SD from three independent repeats. Statistics were performed using a Students two tailed t-test. ** P ≤0.01.

5.4.2.6 Expression of key genes implicated in cholesterol efflux

Given the ability of DGLA to increase ApoA1 stimulated cholesterol efflux, it was of interest to determine the specific role of the fatty acid in this complex process. It has been shown that the ABC transporter proteins play a crucial role in transport of free cholesterol from the cytoplasm of the macrophage to the plasma membrane for transfer to cholesterol carriers. In addition to this, expression of macrophage ApoE has been previously shown to promote cholesterol efflux (Hasty *et al*. 1999; Curtiss 2000; Su *et al.* 2003; Baitsch *et al*. 2008). The effect of DGLA on the mRNA expression of ABCA1 and ABCG1 and the protein expression of ABCA1 and ApoE was measured in cholesterol loaded THP-1 macrophages.

THP-1 macrophages were pre-incubated with 50 μM DGLA or vehicle control for 24 hours prior to cholesterol loading with 25 μg/ml of acLDL. The mRNA expression was measured by RT-qPCR using primers specific for ABCA1 and ABCG1 (Figure 5.10A). Protein expression of ABCA1, ApoE and β actin was determined by western blot analysis (Figure 5.10B). Each graph represents the average of one independent experiment performed in triplicate.

It was found that on cholesterol loading of THP-1 macrophages, mRNA and protein expression of ABCA1 was induced. At the mRNA level, this was attenuated by an average of 30% on pre incubation with DGLA. However at the protein level, expression of ABCA1 was not affected by pre-incubation with DGLA. The mRNA expression of ABCG1 was induced on average 1.5 fold following cholesterol loading in macrophages. Pre-treatment of the cells with DGLA followed by cholesterol loading had no effect on ABCG1 expression. Finally, ApoE was measured at the protein level. Expression of ApoE remained constant under all conditions.

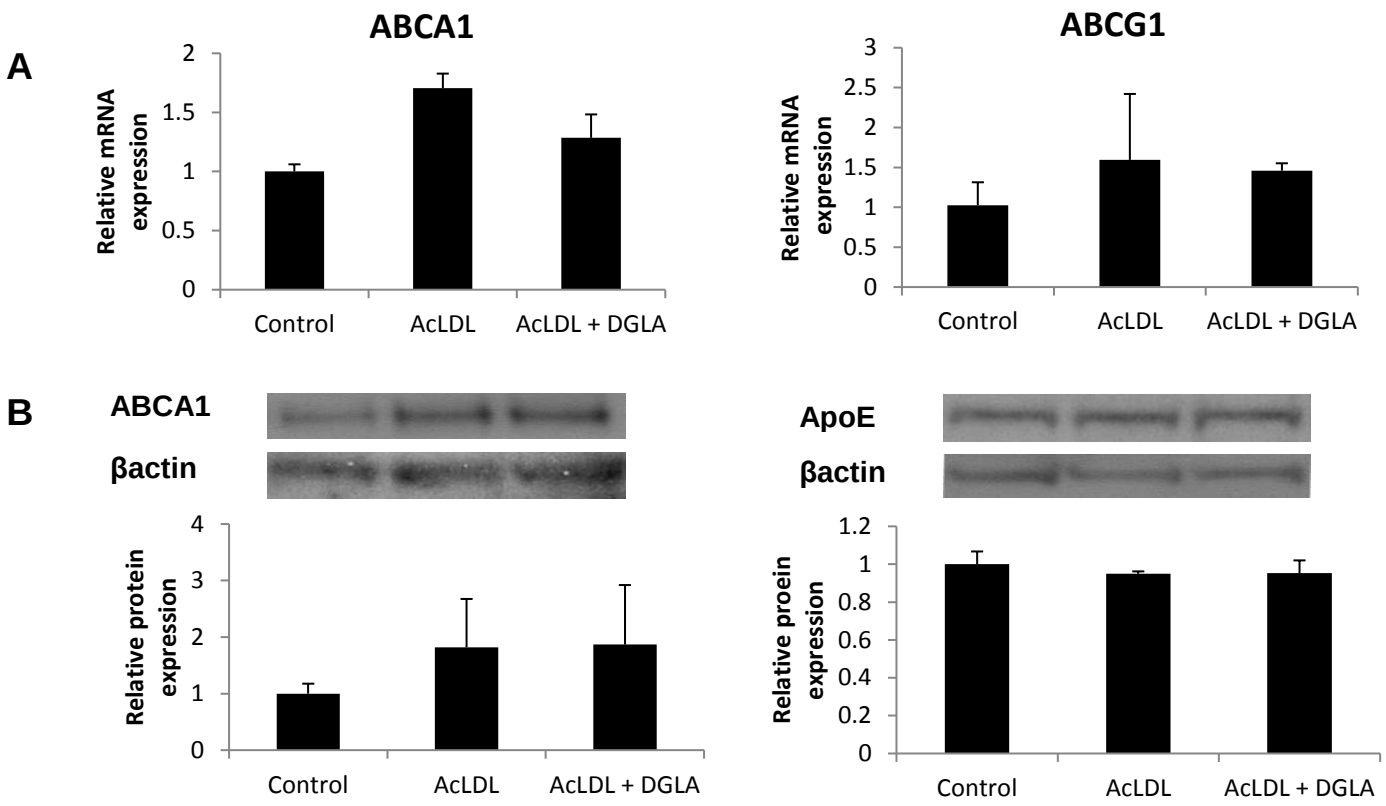


Figure 5.10 – Effect of DGLA on the expression of ABCA1, ABCG1 and ApoE in acLDL loaded macrophages

THP-1 macrophages were incubated with DMSO vehicle or 50μM DGLA for 24 hours followed by 25 μg/ml acLDL for a further 24 hours (control cells were pre-treated with DMSO followed by further incubation in the absence of acLDL). A - RNA was extracted, subjected to reverse transcription and RT-qPCR with primers specific for ABCA1, ABCG1 and GAPDH. B - Proteins were extracted, separated by gel electrophoresis and transferred onto a membrane. Membranes were probed with antibodies specific for ABCA1, ApoE and β actin. Membranes were exposed to X-ray film and bands were analysed by densitometry for quantification. Graphs display the average of one independent experiment performed in triplicate (+/- SD).

5.4.2.6.1 LXR gene expression

Expression of ABCA1, ABCG1 and ApoE are regulated by LXRs (Schwartz *et al.* 2000; Laffitte *et al.* 2001). Given the relationship between the cholesterol efflux genes measured in Figure 5.10 and LXRs, the effect of DGLA on the expression of these receptors was measured by RT-qPCR. THP-1 macrophages were incubated with 50 µM DGLA for 24 hours prior to RNA extraction and reverse transcription. RT-qPCR was performed using primers specific for LXR-α, LXR-β and GAPDH.

As shown in Figure 5.11, incubation of macrophages with DGLA significantly attenuated the expression of LXR-α and -β by an average of 70% and 55% respectively.

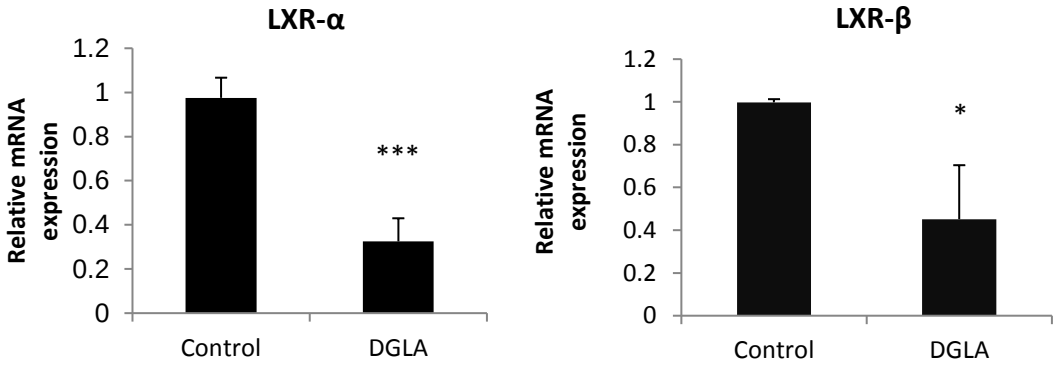


Figure 5.11 – DGLA significantly inhibits LXR-α and -β mRNA expression in THP-1 macrophages

THP-1 macrophages were incubated with vehicle or 50 µM DGLA for 24 hours. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for human LXR-α or LXR-β. Graphs display average gene expression (mean +/- SD) (control arbitrarily assigned as 1) from 3 experiments. Statistical analysis was performed using a two tailed Students t-test. * P ≤0.05, *** P ≤ 0.001

5.4.3 DGLA metabolites

5.4.3.1 Effect of PGE_1 on MCP-1 expression and monocyte migration

PGE_1 is a product of DGLA (produced by the action of COX) with beneficial anti-inflammatory actions in number of diseases (Sinzinger *et al*. 1991; Palumbo *et al*. 2000; Wang *et al*. 2012). To determine the role of this prostaglandin in the anti-inflammatory actions of DGLA, key experiments were repeated using PGE_1 to determine if the metabolite mimicked the actions of DGLA. Two key parameters that showed positive results with DGLA were chosen; the inhibition of IFN-γ induced MCP-1 and ICAM-1 expression and monocyte migration. For gene expression, THP-1 macrophages were pre-treated with PGE_1 for 1 hour prior to the addition of 250 U/ml of IFN-γ for 3 hours. A shorter pre-incubation time of PGE_1, compared to DGLA, was chosen due to the short half-life of the prostaglandin (Fan and Chapkin 1998). Concentrations of 10 to 100 µM were used initially to determine the optimum concentrations for use in further experiments.

Treatment of THP-1 macrophages with IFN-γ significantly increased the expression of MCP-1 and ICAM-1. On pre-incubation with PGE_1, significant inhibition of the IFN-γ induced expression of MCP-1 and ICAM-1 was observed with all the concentrations used (Figure 5.12). Treatment with PGE_1 inhibited the IFN-γ induced expression of MCP-1 and ICAM-1 by an average of approximately 80% and 45% respectively across all concentrations. There was no significant difference between doses for either MCP-1 or ICAM-1 expression observed. This was also repeated in HMDMs where comparable results were obtained (Figure 5.13). In HMDMs, PGE_1 inhibited the IFN-γ induced MCP-1 expression by an average of 80% across all concentrations. For ICAM-1, PGE_1 had a dose response effect on IFN-γ induced expression attenuating expression by 17%, 22%, 30% and 40% on average for the concentrations used. A concentration of 10 µM was carried forward for future experiments as this was the lowest concentration at which marked effects were observed and there was no significant difference found between various concentrations.

For monocytic migration, THP-1 monocytes were co-incubated with 10 µM PGE_1 (or vehicle control) and MCP-1. MCP-1 significantly increased monocyte migration by 70% (Figure 5.14). On co-incubation with PGE_1 this increase was completely inhibited and levels of monocyte migration returned to that of the control.

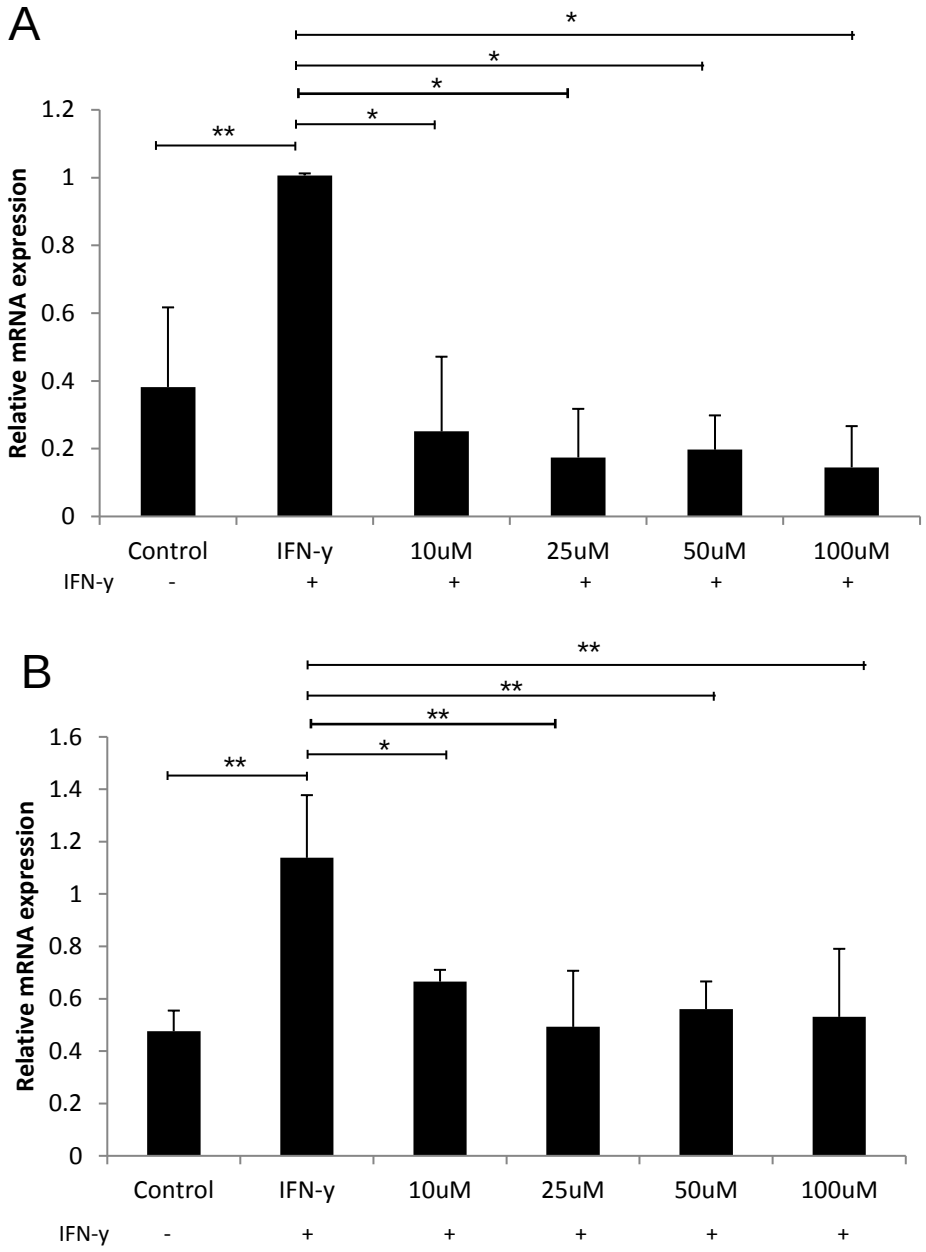


Figure 5.12 – PGE_1 significantly inhibits the IFN-γ-induced expression of MCP-1 and ICAM-1 in THP-1 macrophages

THP-1 macrophages were incubated with vehicle or 10-100 µM PGE_1 for 1 hour prior to treatment with vehicle or 250 U/ml IFN-γ for 3 hours. Concentration of vehicle control was used to match that of highest concentration of PGE_1. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for human MCP-1 (panel A), ICAM-1 (panel B) or GAPDH. Graphs display average normalised gene expression (mean +/- SD) (control arbitrarily assigned as 1) from three independent experiments. For MCP-1, statistical analysis was performed using a robust equality of means test followed by Dunnetes T3 post hoc analysis, as conditions for ANOVA were not met. For ICAM-1, ANOVA was used followed by Tukey's post hoc analysis. * P≤0.05 ** P≤0.01

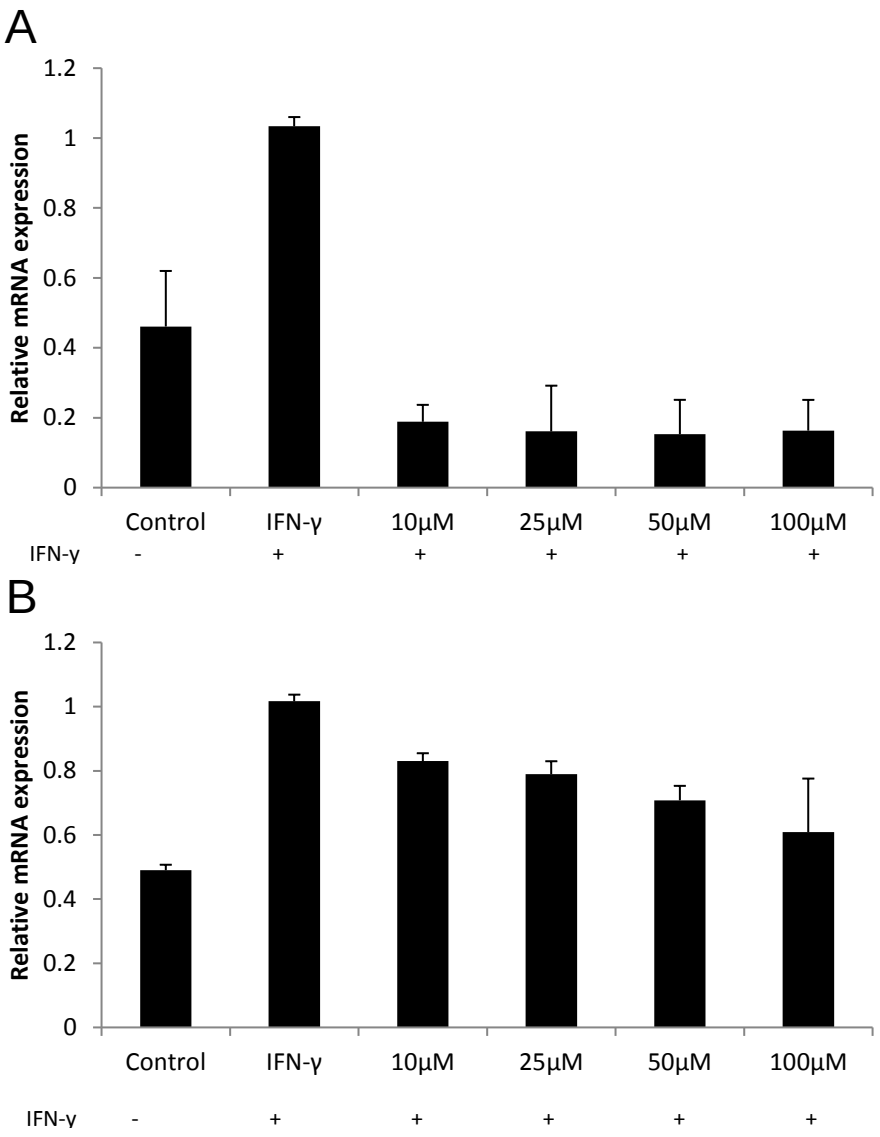


Figure 5.13 – PGE_1 inhibits the IFN-γ-induced expression of MCP-1 and ICAM-1 in HMDMs

THP-1 macrophages were incubated with vehicle or 10-100 μM PGE_1 for 1 hour prior to treatment with vehicle or 250 U/ml IFN-γ for 3 hours. Concentration of vehicle control was used to match that of highest concentration of PGE_1. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for human MCP-1 (panel A), ICAM-1 (panel B) or GAPDH. Graphs display average normalised gene expression (mean +/- SD) (control arbitrarily assigned as 1) from two independent experiments.

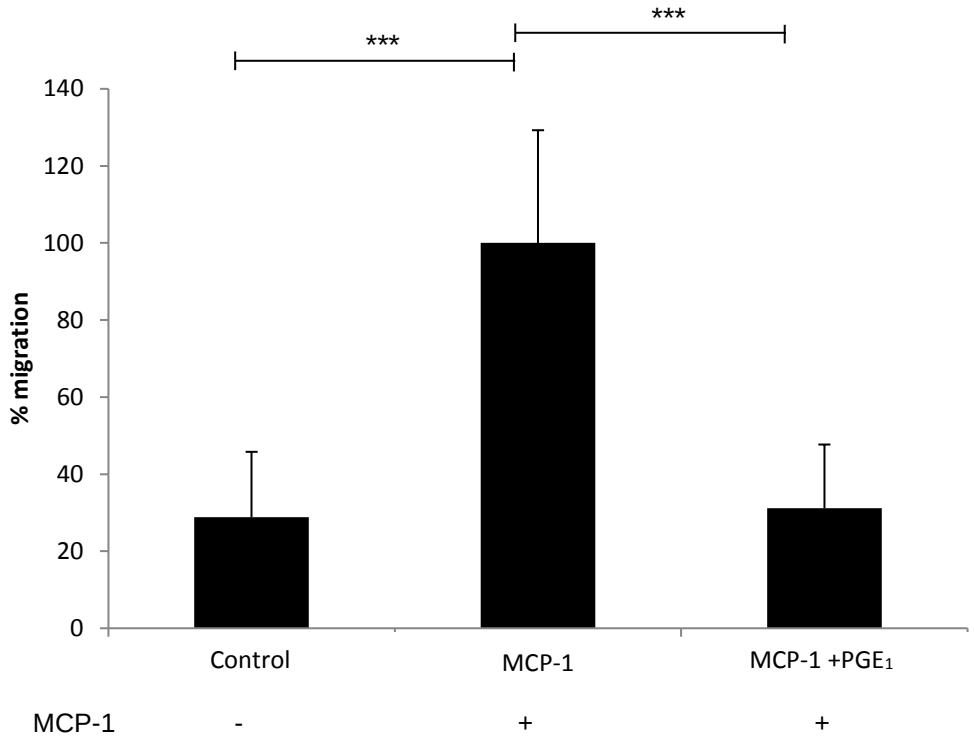


Figure 5.14 – PGE_1 significantly inhibits the MCP-1 induced migration of THP-1 monocytes

THP-1 monocytes were incubated with 10 μM PGE_1 or vehicle control and +/- MCP-1 (20 ng/ml), for 3 hours. Monocyte migration was calculated by counting the number of monocytes that have migrated across a cell insert and expressed as a percentage of total cells. Statistical analysis was performed using a robust equality of means test followed by Dunnetes T3 post hoc analysis. *** P≤0.001

5.4.3.2 COX knockdown

Given the ability of PGE_1 to mimic key responses as observed with DGLA, it was hypothesised that metabolism of the PUFA to the prostaglandin may be the mechanism by which DGLA exerts its anti-inflammatory/anti-atherogenic actions. To test this experimentally, THP-1 monocytes were transfected with siRNA to knockdown the expression of COX-1 and COX-2 enzymes and then differentiated into macrophages. As shown in Chapter 3, COX-2 plays an important role in PGE_1 production in THP-1 macrophages, with a lesser role for COX-1. Previous work however has indicated a role for both COX isoforms in the metabolism of DGLA to PGE_1 therefore both enzymes were targeted to ensure maximum knockdown of production of the prostaglandin. IFN-γ induced expression of MCP-1 was then measured by RT-qPCR. THP-1 monocytes were transfected with COX-1 and COX-2 siRNA or a negative control siRNA for 24 hours prior to differentiation into macrophages. Following knockdown, macrophages were incubated with 50 µM DGLA or vehicle control for 24 hours, followed by 250 U/ml IFN-γ or vehicle control, for a further 3 hours. Before measuring MCP-1 expression, the knockdown of COX-1 and COX-2 was validated at the mRNA and protein level by RT-qPCR and western blotting, respectively. Figure 5.15 depicts the knockdown of COX-1 and COX-2. For COX-1, there was an average 75% knockdown at the mRNA level accompanied by a 50% average knockdown at the protein level. COX-2 showed a 60% and 20% knockdown on average at the mRNA and protein levels respectively. COX-2 is an inducible enzyme, the detection of COX-2 was low at both the protein and mRNA levels.

Following confirmation of knockdown of COX-1 and COX-2, MCP-1 gene expression was measured by RT-qPCR. As shown in Figure 5.16, in macrophages expressing a negative siRNA, IFN-γ significantly stimulated the expression of MCP-1. On pre incubation with DGLA, this expression was attenuated by approximately 30%. In cells where the expression of both COX-1 and COX-2 was knocked down, IFN-γ once again increased the expression of MCP-1 significantly. In DGLA pre-treated, 'COX knockdown' macrophages however, there was no attenuation of MCP-1 expression. The attenuation of IFN-γ induced MCP-1 signalling by DGLA was completely abolished on knockdown of COX-1 and COX-2 enzymes.

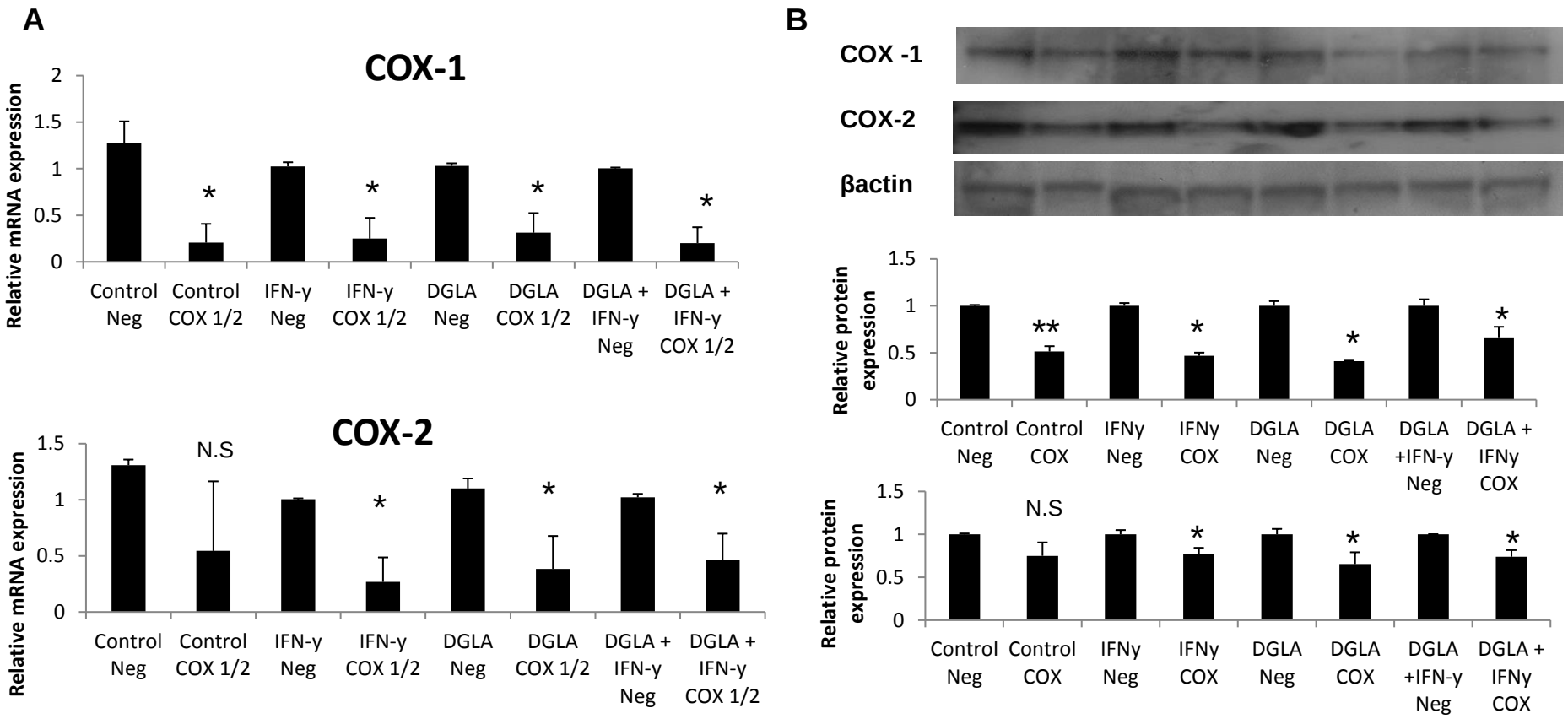


Figure 5.15 - COX1/2 knockdown by siRNA at mRNA and protein level

THP-1 monocytes were incubated for 24 hours with 7.5 nM COX-1 and COX-2 siRNA or a control siRNA followed by differentiation with PMA for 24 hours. Following differentiation, macrophages were pre-treated with 50 μM DGLA or vehicle control for 24 hours prior to addition of IFN-γ or vehicle control for a further 3 hours. For gene expression, RNA was extracted and subjected to reverse transcription and RT-qPCR with primers specific to COX-1, COX-2 and GAPDH (A). For protein analysis, protein was extracted and subjected to western blot analysis with antibodies specific to COX-1, COX-2 and βactin (B). Each knockdown was compared to its negative siRNA counterpart, which has been arbitrarily assigned as 1. Graph displays average of 4 independent repeats (+/- SD). Statistics were performed using a Student's two tailed Ttest. * P≤0.05. N.S – not significant

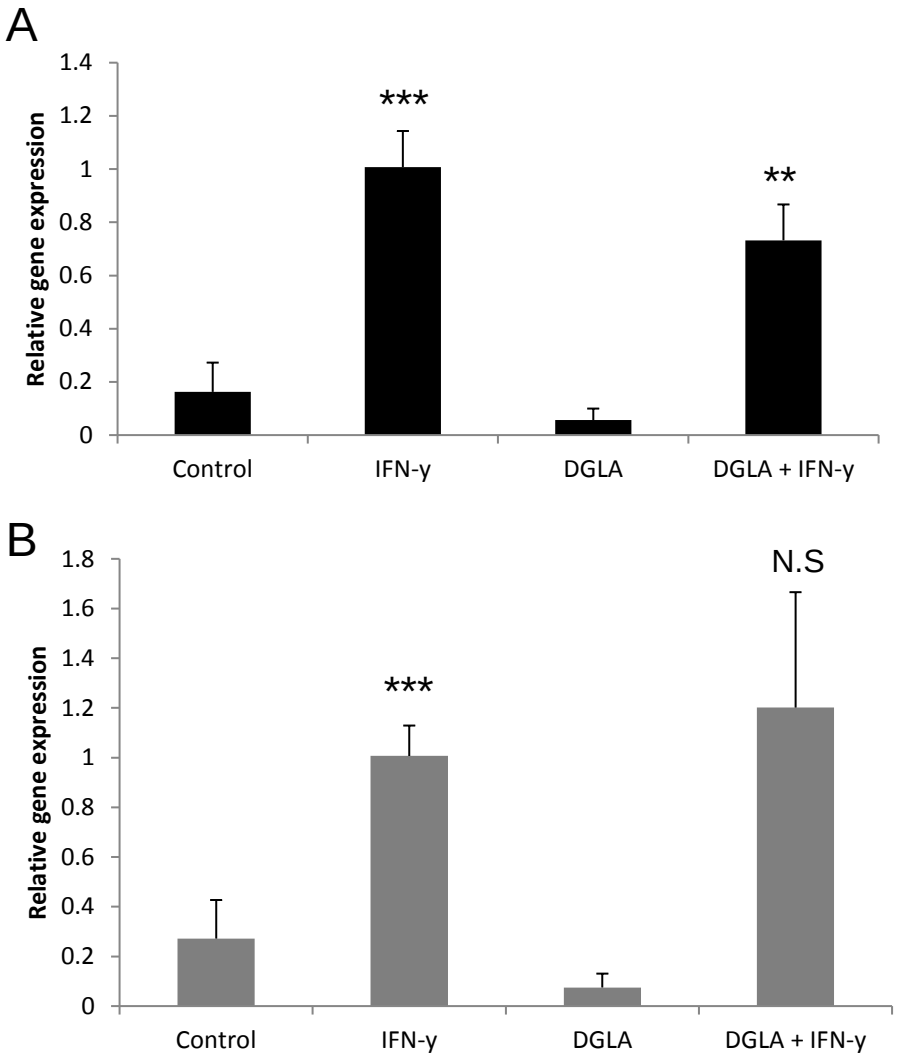


Figure 5.16 – Knockdown of COX-1 and COX-2 abolishes the attenuation of IFN-γ induced MCP-1 expression by DGLA

THP-1 monocytes were incubated for 24 hours with control siRNA (panel A) or 7.5 nM COX-1 and COX-2 siRNA (panel B) followed by differentiation with PMA for 24 hours. Following differentiation, macrophages were pre-treated with 50 µM DGLA or vehicle control for 24 hours prior to addition of IFN-γ or vehicle control for a further 3 hours. Following validating of knockdown of COX-1 and COX-2, RNA was extracted and subjected to reverse transcription and RT-qPCR with primers specific to MCP-1 and GAPDH. Graph displays average of 4 independent repeats (+/- SD).Statistics were performed using a one-way ANOVA followed by Tukey's post-hoc analysis. ** P≤0.01, *** P≤0.001. N.S – not significant

5.5 Discussion

5.5.1 IFN-γ induced STAT1 activation

Data presented in the previous chapter indicated that DGLA inhibited the IFN-γ induced expression of two pro-inflammatory genes in macrophages; MCP-1 and ICAM-1. To understand the molecular mechanisms underlying this effect, IFN-γ signalling through the JAK/STAT pathway was investigated. Signalling through STAT1 plays an important role in IFN-γ actions and has been implicated to play a role in atherosclerosis (Li *et al*. 2010; Sikorski *et al.* 2011a). In addition to IFN-γ signalling, STAT1 has been described as a point of convergence and signalling integration for other pro-inflammatory signalling pathways, namely TLRs and IL-6, resulting in increased SMC and leukocyte activation and migration (Sikorski *et al*. 2011a; Sikorski *et al*. 2011b). Therapeutic inhibition of STAT1 with a decoy oligonucleotide has implicated it to play a role in myocardial graft tissue survival (Hoelschermann *et al.* 2006). Targeting STAT1 prolonged cardiac graft survival by 40% due to a reduction in the recruitment of leukocytes, a process which causes acute myocardial rejection. It was observed that the expression of adhesion molecules VCAM-1 and ICAM-1 were reduced (Hoelschermann *et al*. 2006). In addition, induction of foam cells with oxLDL in THP-1 macrophages was reduced on treatment with a STAT1 inhibitor (Agrawal *et al*. 2007). This was also observed in BMDM from $STAT1^{-/-}$ mice and *in vivo* in $ApoE^{-/-}$ $STAT1^{-/-}$ mice. Deficiency of STAT1 reduced oxLDL induced cholesteryl ester and lipid accumulation in comparison to wild type animals (Agrawal *et al*. 2007). To determine the role of DGLA on activation of STAT1, the phosphorylation of key sites TYR^{701} and SER^{727} were determined by western blotting.

As shown in Figure 5.4, treatment of THP-1 macrophages significantly increased the phosphorylation of STAT1 at TYR^{701} and SER^{727} sites. On pre-treatment with DGLA, the induction of SER^{727} phosphorylation was attenuated by approximately 31%. There was no effect on phosphorylation at the TYR^{701} site. As previously described in Section 5.1.1.1, phosphorylation at TYR^{701} is required for transcriptional activity of STAT1, however this can occur independent of SER^{727} phosphorylation (McLaren and Ramji 2009; Li *et al*. 2010). Despite this, phosphorylation at SER^{727} is required for maximum transcriptional activity of STAT1 and inhibition of the process attenuates IFN-γ induced pro-inflammatory gene expression (Varinou *et al.* 2003). Knock-in mice were used to study the effect of loss of STAT1 SER^{727} phosphorylation *in vivo*. The SER^{727} site in the transactivation domain of STAT1 was substituted for an alanine residue, preventing phosphorylation and maximal activity of STAT1 (Varinou *et al*.

2003). This resulted in a decrease in IFN-γ induced gene expression in macrophages and transcription factor activity of STAT1 (Varinou *et al*. 2003). In addition, attenuation of SER^{727} phosphorylation in human macrophages by inhibition of upstream kinases attenuated the IFN-γ induced expression of ICAM-1, MCP-1, IP-10 and MIP-1β at the mRNA and protein level (Li *et al*. 2010). IFN-γ induced uptake of acLDL and oxLDL was also reduced (Li *et al*. 2010). Given the results presented in Figure 5.4, this indicates a role for DGLA in the attenuation of STAT1 activity which can lead to a reduction in IFN-γ-mediated pro-inflammatory gene expression. The data highlights a new potential mechanism by which DGLA may aid in the attenuation of foam cell formation and atherosclerosis.

A number of kinases have been implicated in the phosphorylation of STAT1 at SER^{727}. Inhibition of ERK and JNK selectively decreased the IFN-γ induced SER^{727} phosphorylation in human macrophages (Li *et al*. 2010). PKCδ and CAMK II inhibited phosphorylation at both sites while p38 had no effect (Li *et al*. 2010). Given the selective role of ERK and JNK phosphorylation of SER^{727}, future avenues could evaluate the effect of DGLA on the activity of these kinases to further elucidate the role of the fatty acid in IFN-γ signalling through the JAK/STAT pathway.

5.5.2 Modified LDL uptake in macrophages

As reported in Chapter 4, DGLA attenuated the accumulation of cholesteryl esters in macrophages following cholesterol loading with a modified form of LDL, acLDL. In an attempt to delineate the mechanisms underlying this action, a number of processes were targeted for investigation including cholesterol uptake, metabolism and efflux.

In atherosclerosis, macrophages recognise and internalise excess amounts of modified forms of LDL in an unregulated manner. As a result, excess cholesterol is stored as cholesteryl esters in lipid droplets which are deposited in the cytoplasm, a hallmark of foam cell formation (Shashkin *et al*. 2005; Lusis 2012). Inhibiting the uptake of modified forms of LDL would therefore prevent the accumulation of excess cholesterol in macrophages and their transformation into foam cells. Uptake was measured using a fluorescently labelled modified form of LDL; Dil-oxLDL. As shown in Figure 5.5, DGLA inhibited the uptake of Dil-oxLDL by 21% on average. Previous studies have reported comparable results using omega-3 fatty acid DHA (McLaren *et al*. 2011b). DHA inhibited Dil-acLDL and Dil-oxLDL uptake in THP-1 macrophages. This process was found to be dependent on both scavenger receptors and macropinocytosis (McLaren *et al*. 2011b). The data presented in Figure 5.5 indicates that the decrease in cholesteryl ester accumulation observed in Chapter 4, may be in

part as a result of a reduction in modified LDL uptake. Cholesteryl ester studies were performed using acLDL, in contrast uptake was measured using oxLDL. Both modified forms of LDL have been used extensively in lipid uptake and foam cell formation studies, yielding comparable results in mechanisms of uptake, cholesterol accumulation and efflux (McLaren *et al*. 2010; McLaren *et al*. 2011b). This suggests that the both acLDL and oxLDL can be interchangeable when studying these processes.

5.5.2.1 Scavenger receptors

Receptor-mediated endocytosis is an important mechanism by which macrophages bind and internalise a number of molecules, including pathogens and LDL. Scavenger receptors are expressed on the surface of macrophages and internalise unregulated amounts of modified forms of LDL. CD36 and SRA have been shown to take up large amounts of oxLDL and acLDL and contribute to foam cell formation and atherosclerosis (Febbraio *et al*. 2000; Kunjathoor *et al*. 2002; Kuchibhotla *et al*. 2008). The effect of DGLA on the expression of these two receptors in THP-1 macrophages at the mRNA (SRA and CD36) and protein level (SRA) was measured by RT-qPCR and western blotting respectively (Figure 5.6). On treatment with DGLA, the mRNA expression of CD36 and SRA was reduced by approximately 20%. The protein expression of SRA was decreased by 40%. No previous work has detailed the effect of DGLA on the expression of SRA and CD36. However, a previous study investigating the role of omega-6 PUFA, LA, demonstrated comparable results. LA treatment in THP-1 macrophages and HMDMs significantly decreased the expression of SRA and CD36. This was accompanied by a significant decrease in the cholesterol content of foam cells (Song *et al.* 2013).

As reported in the previous chapter, DGLA reduced the acLDL induced cholesteryl ester accumulation in macrophages. The data presented in Figure 5.6 indicates this may be in part due to a reduced expression of scavenger receptors on treatment with DGLA which may potentially result in a reduction in uptake of modified LDL. Previous studies evaluating the role of SRA and CD36 knockdown have shown comparable results. Cholesteryl esters fail to accumulate in macrophages from SRA and CD36 knockout mice (Kunjathoor *et al*. 2002). $SRA^{-/-}$ macrophages degraded 70% less acLDL in comparison to wild type animals and specific binding of acLDL was reduced by 44% (Kunjathoor *et al*. 2002). CD36 plays a lesser role in acLDL binding and degradation with $CD36^{-/-}$ macrophages accounting for 28% less binding and 13% less degradation (Kunjathoor *et al*. 2002). There was however a bigger effect on oxLDL degradation and binding in these macrophages. $CD36^{-/-}$ macrophages reduced

binding of oxLDL by 90% and degradation by 68% (Kunjathoor *et al*. 2002). Again, $SRA^{-/-}$ played a lesser role in oxLDL metabolism, reducing binding by 40% and degradation by 25% (Kunjathoor *et al*. 2002). Therefore both scavenger receptors play a role in internalisation and degradation of modified LDL, but have different preferences for acLDL and oxLDL. Uptake of oxLDL by CD36, in addition to its function in foam cell formation, has also been indicated to play other pro-inflammatory roles. CD36 uptake of oxLDL decreased macrophage efflux from atherosclerotic plaques, promoted macrophage trapping and increased plaque progression (Park *et al.* 2009). Also, oxLDL binding to CD36 has been shown to activate platelets and contribute to pro-thrombotic state (Chen *et al.* 2008). Finally, $CD36^{-/-}$ macrophages expressed reduced levels of IFN-γ, MCP-1 and TNF-α in the presence or absence of oxLDL (Kennedy *et al.* 2011).

The uptake assay and cholesteryl ester accumulation assay were performed with oxLDL and acLDL respectively. Given the data presented in Figures 4.16, 5.5 and 5.6, taken together with previous studies, it suggests that DGLA can reduce modified LDL uptake and cholesteryl ester accumulation by reducing the uptake of acLDL and oxLDL though the reduction in the expression of scavenger receptors SRA and CD36. The data highlights a potential new mechanism for the anti-inflammatory and anti-atherogenic action of DGLA.

5.5.2.2 Macropinocytosis

Macrophages can also take up molecules by receptor independent mechanisms. Macropinocytosis is a form of non-selective endocytosis for solute macromolecules (Swanson and Watts 1995). Following stimulation with M-CSF or phorbol esters, ruffling at the cell surface of macrophages can be observed which gives rise to macropinosomes (Swanson and Watts 1995). Macropinocytosis has been linked to LDL uptake by macrophages and foam cell formation. An inhibitor of macropinocytosis, cytochalasin D, attenuated the uptake of acLDL in THP-1 macrophages which translated to reduced intracellular cholesterol levels (McLaren *et al*. 2011b; Michael *et al*. 2013). This result was conserved in HMDMs (Michael *et al*. 2013). Treatment with the inhibitor increased the expression of SRA and CD36 which suggested the reduction in uptake and accumulation of acLDL was receptor independent (Michael *et al*. 2013). Macrophages incubated with acLDL and oxLDL increased membrane ruffling and formed macropinosomes resulting in a 1.5 fold increase in fluid phase uptake (Jones and Willingham 1999) suggesting a role for the pathway in modified LDL uptake. OxLDL induced foam cell formation was also reduced by an inhibitor of macropinocytosis in RAW264.7 macrophages (Yao *et al*.

2009). In addition to uptake of modified forms of LDL, macropinocytosis has also been shown to take up native LDL. M-CSF differentiated macrophages showed an increased uptake of native LDL in the fluid phase which increased cholesterol accumulation (Zhao *et al*. 2006). Similarly, M-CSF stimulated HMDMs treated with a macropinocytosis inhibitor showed a 40% decrease in LDL uptake (Anzinger *et al.* 2010). Uptake of LDL by macropinocytosis can therefore be independent of modification and still induce cholesterol accumulation in macrophages.

Previous work has indicated a role for PUFAs in macropinocytosis. Treatment of THP-1 macrophages with EPA and DHA reduced acLDL uptake. This was accompanied by a reduction in the uptake of a marker of macropinocytosis on treatment with the PUFAs (McLaren *et al*. 2011b). The effect of DGLA on macropinocytosis was measured in PMA differentiated macrophages. PMA, a phorbol ester, has been used frequently in the literature to induce macropinocytosis in differentiated macrophages. PMA increased macropinosomes in BMDMs (Swanson 1989) and induced LDL and modified LDL uptake in HMDMs (Kruth *et al*. 2002; Kruth *et al*. 2005) and THP-1 macrophages (McLaren *et al*. 2011b; Michael *et al*. 2013). To measure macropinocytosis, LY was used as a marker which can be measured by FACS analysis. LY has been commonly used in measuring macropinocytosis in previous studies (Swanson 1989; Jones and Willingham 1999; McLaren *et al*. 2011b; Michael *et al*. 2013). As shown in Figure 5.7, pre incubation with DGLA inhibited LY uptake by approximately 17% in PMA differentiated macrophages. Given the role of macropinocytosis in the uptake of modified forms of LDL including acLDL, this may indicate another mechanism, independent of receptor-mediated uptake, in which DGLA inhibits the uptake and accumulation of cholesteryl esters as seen previously. In addition, DGLA may also attenuate the uptake of native forms of LDL through inhibiting macropinocytosis. Despite the effect of DGLA on LY uptake by macropinocytosis, it will need to be directly assessed if this translates to uptake of modified LDL and native LDL.

5.5.3 Cholesterol metabolism

In addition to uptake mechanisms of modified LDL, it was of interest to determine the role of DGLA in the metabolism of internalised cholesterol in macrophages. Free cholesterol is removed from the cell by RCT transport, a process dependent on the nCEH enzymes. nCEH enzymes hydrolyse cholesteryl esters to free cholesterol which can be removed from the cell through transporter proteins (Okazaki *et al.* 2008). Of the numerous nCEH enzymes, NCEH1 is highly expressed in macrophages and plays an important role in foam cell formation and atherosclerosis (Okazaki *et al*.

2008; Sekiya *et al*. 2009; Igarashi *et al*. 2010) (see Section 5.1.2.3.2. for more details). NCEH1$^{-/-}$ macrophages show an increase in cholesterol esters and a decrease in cholesterol efflux, key features of foam cell formation (Sekiya *et al*. 2009). In addition, overexpression of NCEH1 in THP-1 macrophages decreased the accumulation of cholesteryl esters induced by acLDL uptake (Igarashi *et al*. 2010). The effect of DGLA on this enzyme was therefore of interest.

Excess free cholesterol is stored as cholesteryl esters in lipid droplets within the cytoplasm. This accumulation of cholesteryl esters is a key feature of macrophage foam cells and gives them their foamy appearance under the microscope (Ghosh *et al*. 2010). ACAT1 is responsible for cholesteryl ester formation in macrophage foam cells. ACAT1 is expressed in human atherosclerotic lesions and macrophages including the THP-1 cell line (Miyazaki *et al*. 1998; Yang *et al*. 2004; Wan *et al.* 2009). ACAT1$^{-/-}$ macrophages treated with acLDL showed an increase in the efflux of acLDL derived cholesterol and an increase in free cholesterol (Ghosh *et al*. 2010). Inhibition of ACAT1 is therefore expected to attenuate atherosclerotic lesion development by preventing the formation and storage of cholesteryl esters and increase free cholesterol for efflux (Ghosh *et al*. 2010).

THP-1 macrophages were treated with DGLA for 24 hours before measuring gene expression by RT-qPCR. As shown in Figure 5.8, DGLA did not affect the basal expression of NCEH1 and ACAT1 in THP-1 macrophages. Given this, no further experiments were carried out. Potential future follow-up experiments could measure the expression of the two genes at the protein level by western blotting. In addition, the role of DGLA on the expression of NCEH1 and ACAT1 could be determined in cholesterol-loaded macrophages.

Despite the promising role of ACAT1 as a target to prevent cholesteryl ester accumulation and development of foam cells, disruption of the gene in mouse models has proved controversial. The subsequent increase in free cholesterol as a result of disruption of ACAT1 can also contribute to atherosclerosis. LDLr$^{-/-}$ ACAT1$^{-/-}$ deficient mice displayed increased lesion sizes in comparison to wild type controls despite a decrease in the number of plaque macrophages (Fazio *et al.* 2001). Free cholesterol accumulated in arterial wall promoted atherogenesis (Fazio *et al*. 2001). In mouse peritoneal macrophages from ACAT$^{-/-}$ mice, efflux of cholesterol was increased along with a decrease in esterification. Despite this, there was also a 134% increase in cholesterol synthesis in macrophages, which may contribute to atherogenesis (Dove *et al*. 2005). Partial inhibition of ACAT1 in the liver and intestine of ApoE$^{-/-}$ mice

however significantly reduced atherosclerosis. Mice treated with ACAT1 inhibitor showed a significant reduction in lesion size and immune-staining of macrophages (Kusunoki *et al*. 2001). The controversial results obtained from disruption of ACAT1 in relation to atherosclerosis highlights the drawbacks of targeting the gene as a potential therapeutic mechanism. Clinical trials evaluating the use of ACAT inhibitors as a therapeutic in atherosclerosis have also proved unsuccessful (Nissen *et al.* 2006; Meuwese *et al.* 2009).

5.5.4 Cholesterol efflux

The final mechanism targeted in the studies in this chapter to understand the molecular basis underlying the action of DGLA on cholesteryl ester accumulation in macrophages, was cholesterol efflux. RCT from macrophages is discussed in more detail in Sections 1.2.2.3 and 5.1.2.4. The effect of DGLA on cholesterol efflux was measured in THP-1 macrophages. The cells were incubated with acLDL to induce the accumulation of intracellular cholesterol and stimulated with ApoA1. ApoA1 is the apolipoprotein constituent of HDL and acts to promote cholesterol efflux from macrophages via ABC transporter proteins (Yvan-Charvet *et al*. 2008). A number of studies utilise ApoA1 as a positive control in cholesterol efflux assays (McLaren *et al*. 2010; Ouimet *et al.* 2011; Sene *et al.* 2013). Concentrations of acLDL and ApoA1 used in the assays and analysis of results were as previously reported (McLaren *et al*. 2010; Ouimet *et al*. 2011; Sene *et al*. 2013). On loading with acLDL and treatment with ApoA1, cholesterol efflux increased in comparison to unloaded macrophages (data not shown). Pre-treatment with DGLA followed by acLDL loading and ApoA1 stimultion, significantly increased cholesterol efflux from THP-1 macrophages by approximately 10% (Figure 5.9).

The inverse correlation between HDL and CHD has been widely recognised in numerous studies (Gordon *et al.* 1977; Gordon *et al.* 1989; Feig *et al.* 2014). HDL and its component ApoA1 act to remove cholesterol from macrophages by RCT and transport it to the liver for degradation, therefore reducing the accumulation of cholesterol in periperhal cells including macrophages. $ApoE^{-/-}$ $ApoA1^{-/-}$ mice showed an increase in free cholesterol accumulation and lesion area (Boisvert *et al*. 1999). In addition BMDMs expressing a macrophage ApoA1 gene were transplated into $ApoE^{-/-}$ mice with pre exisiting atherosclerosis. Transplant mice in comparison to $ApoE^{-/-}$ mice showed a significant increase in cholesterol efflux and the expression of ABC transporter genes which lead to a reduction in atherosclerosis lesions in the proximal aorta (Su *et al*. 2003). Efflux of cholesterol to ApoA1 can therefore reduce the accumulation of cholesterol in macrophages and protect against atherosclerosis. The

data in Figure 5.9 shows that DGLA significanly increases cholesterol efflux to ApoA1 in THP-1 macrophages. This indicates a potential mechanism by which cholesteryl ester accumulation is attenuated in THP-1 macrophages incubated with DGLA, which can lead to a reduction in foam cell formation and atherosclerosis.

To date, no study has investigated the role of DGLA in cholesterol efflux in macrophages. A handful of studies however have addressed the role of omega-6 PUFA LA, with contrasting results. In THP-1 macrophages and HMDMs, LA significantly decreased the cholesterol content of foam cells and increased cholesterol efflux to lipid free ApoA1 (Song *et al*. 2013) comparable to that seen in Figure 5.9. In contrast however, LA treatment of murine macrophages had an opposing effect. In RAW264.7 macrophages, LA decreased ApoA1-mediated cholesterol and phospholipid efflux (Wang and Oram 2002). In addition, LA treatment of mouse BMDM significantly decreased HDL-mediated efflux (Wang and Oram 2002). Future experiments may act to determine the effect of DGLA in cholesterol efflux from murine macrophages to evaluate any species specific roles.

5.5.4.1 ABC transporter proteins and ApoE

To attempt to determine the mechanisms underlying the increase in cholesterol efflux from macrophages on treatment with DGLA in THP-1 macrophages, the protein expression of key genes involved in RCT were measured by western blotting. Initially the study focused on the ABC transporter proteins. Increased expression of ABCA1 and ABCG1 has previously been shown to increase cholesterol efflux from macrophages and significantly prevent atherosclerosis (Singaraja *et al*. 2002; Burgess *et al.* 2008). In contrast, disruption of these transporter proteins has opposing effects resulting in an increase in atherosclerosis (Yvan-Charvet *et al*. 2007; Tarling *et al*. 2010; Westerterp *et al*. 2013). ABCA1 and ABCG1 have different mechanisms for removal of cholesterol. ABCA1 promotes cholesterol and phospholipids efflux to lipid poor ApoA1. In contrast, cholesterol efflux to HDL specifically required ABCG1 (Kennedy *et al.* 2005). Given the use of ApoA1 as a cholesterol acceptor used in efflux experiments (Figure 5.9), it was hypothesised that DGLA may act through increasing expression of ABCA1. The expression of ABCA1 was measured in THP-1 macrophages loaded with acLDL in an independent experiment, by RT-qPCR and western blotting. It was found that on cholesterol loading with acLDL, the mRNA and protein expression of ABCA1 was increased in comparison to un-loaded macrophages (Figure 5.10). This has also been observed in previous studies (Uehara *et al.* 2007). On pre-treatment with DGLA followed by acLDL loading, the protein expression of ABCA1 was not different to acLDL only loaded macrophages, but

decreased at the mRNA level. The results are based on a single experiment performed in duplicate; further repeats must be performed to establish any significant changes. Previously, no studies have directly measured the effect of DGLA on ABCA1 expression. However, studies have reported the effect of other omega-6 and omega-3 PUFA with contrasting results. LA treatment decreased the expression of ABCA1 and reduced ApoA1 binding activity in RAW264.7 macrophages (Wang and Oram 2002). A decrease in ABCA1 expression was also observed in EPA and AA treated macrophages (Uehara *et al.* 2002; Uehara *et al*. 2007). In BMDMs, ABCA1 expression was significantly inhibited by 95% following LA treatment (Spartano *et al.* 2014). However in THP-1 macrophages, endogenous ABCA1 expression was not affected by conjugated LA (CLA) (Salehipour *et al.* 2010) or LA (Song *et al*. 2013), comparable to results observed in Figure 5.10.

Following this, the expression of ABCG1 was also measured at the mRNA level. Protein level of the transporter protein was not measured due to time constraints. The trend observed from a single experiment, suggested that the expression of ABCG1 was significantly increased on cholesterol loading of macrophages with acLDL (Figure 5.10). Pre-treatment with DGLA followed by cholesterol loading had no observed effect on ABCG1 mRNA expression. To date, no study has provided insight into the effect of DGLA on ABCG1 expression in macrophages. A previous study has however investigated the role of omega-6 PUFA, LA on ABCG1 expression in RAW264.7 macrophages (Uehara *et al*. 2007). Comparable to results presented in Figure 5.10, cholesterol loading of RAW264.7 macrophages dramatically induced the activity of the ABCG1 promoter. However in contrast, it was observed that LA significantly supressed ABCG1 promoter activity. In addition, in unloaded macrophages, LA significantly decreased endogenous ABCG1 protein expression (Uehara *et al*. 2007). Data presented in Figure 5.10 need further repeats to clarify the expression of ABCG1 at the mRNA and the protein level.

In addition to ABC transporter genes, ApoE has been indicated to play a role in cholesterol efflux in macrophages. ApoE is a component of lipoprotein particles and aids in transport of cholesterol as well as their clearance in the liver (Curtiss 2000). However, in macrophages it has been shown to have an additional role. ApoE expression from arterial macrophages in $ApoE^{-/-}$ mice show a significant reduction in atherosclerosis, despite plasma cholesterol levels comparable to that of control (Hasty *et al*. 1999). Expression of ApoE in macrophages *in vivo* has also been shown to increase cholesterol efflux in macrophages (Zhu *et al*. 1998) and to reduce free cholesterol accumulation in lesions (Boisvert *et al*. 1999). In addition to macrophages,

ApoE expression in the arterial wall of transgenic mice also decreased atherosclerotic lesion sizes and reduced cholesterol content (Shimano *et al*. 1995). Given this, the role of DGLA on the expression of ApoE in cholesterol loaded macrophages was determined by western blotting. It was observed that acLDL treatment of THP-1 macrophages had no effect on the protein expression of ApoE. There was also no change in expression on pre-treatment with DGLA followed by acLDL loading. Previous studies however, indicate that ApoE expression is increased on cholesterol loading. In hypercholesterolemic rabbits, ApoE mRNA expression was increased and localised to cholesterol loaded macrophages (Polanco *et al.* 1995). In mouse peritoneal macrophages loaded with 25 µg acLDL, mRNA expression of ApoE was also up regulated (Mazzone *et al.* 1989). In addition, in THP-1 macrophages loaded with 100 µg acLDL, the expression of ApoE was significantly increased (Garner *et al.* 2002). The induction of ApoE on cholesterol loading was not observed in this study (Figure 5.10). The experiment however was performed in isolation and further repeats would be needed to determine a more accurate understanding of ApoE expression. In addition, Garner *et al* (2002) observed an increase of ApoE protein expression in THP-1 macrophages at a much larger concentration of 100 µg of acLDL compared to a concentration of 25 µg used for the studies in Figure 5.10 which may explain in part the differences observed between the studies. Further experiments are required to determine the effect of concentration of acLDL on ApoE expression in THP-1 macrophages.

ABC transporters are LXR regulated genes. Agonists of LXRs activate ABC1 gene expression and induce cholesterol-mediated transport (Schwartz *et al*. 2000). In addition LXR ligands are key regulators of ApoE expression in macrophages (Laffitte *et al*. 2001). As shown in Figure 5.11, DGLA significantly inhibited LXR gene expression in un-loaded THP-1 macrophages. Previously it has been documented that PUFAs inhibit LXR activation. In RAW264.7 macrophages, EPA and LA inhibit LXR activity (Uehara *et al*. 2007) while in rat hepatoma cells AA antagonise LXR activation (Pawar *et al.* 2003). Taken together, the results presented in this chapter and previous studies suggest that the increase in cholesterol efflux from THP-1 macrophages on treatment with DGLA is independent of ABC transporter protein and ApoE expression. Data presented in Figure 5.10 and Figure 5.11 and previous work indicates that DGLA may in fact inhibit LXR activation and the induction of ABC transporter genes and ApoE, however further experiments will need to be performed to determine this directly.

Alternative mechanisms of cholesterol efflux induced by PUFAs, independent of ABC transporters and ApoE, have been suggested previously. In THP-1 macrophages treated with LA there was no effect on ABCA1 expression but an increase in cholesterol efflux. This was associated with specific effects on gene profiles of genes involved in lipid droplet metabolism including cell death inducing DFF45 like effector (CIDE) and perilipin adipophilin TIP47 (PAT) family members following PUFA treatment (Song *et al*. 2013). In addition, ALA treatment in macrophage foam cells significantly increased cholesterol efflux and decreased cholesterol storage. This was associated with a decrease in the expression of stearoyl CoA desaturase (SCD1), the rate limiting enzyme in synthesis of MUFA (Zhang *et al.* 2012). In addition to this, future work could explore the role of DGLA metabolites in cholesterol efflux. As shown previously in Chapter 3, DGLA metabolises to PGE_1, a COX metabolite. Studies have indicated a role for COX metabolites in cholesterol efflux in macrophages. In THP-1 macrophages, inhibition of COX enzymes with inhibitors significantly decreased cholesterol efflux and increased foam cell formation (Chan *et al.* 2007). In addition, in skin fibroblasts, stimulation of cAMP by PGE_1 enhanced cholesterol efflux to HDL_3 (Middleton and Middleton 1998). It is therefore possible that DGLA is acting through PGE_1 to induce cholesterol efflux.

5.5.5 DGLA metabolite PGE_1

An important feature of PUFA signalling in disease is their ability to form eicosanoids. As shown previously in Chapter 3, administration of DGLA to THP-1 macrophages increases the production of the COX metabolite PGE_1. PGE_1 has been well documented to play a number of anti-inflammatory roles, discussed in detail in Section 3.1.3. Given the effect of DGLA on aspects of foam cell formation, it was of interest to determine if these effects were due to the action of DGLA or its metabolite PGE_1. The effects of PGE_1 on IFN-γ induced MCP-1 and ICAM-1 gene expression, along with MCP-1 induced monocyte migration, were measured.

THP-1 macrophages were pre-treated with PGE_1 for one hour prior to treatment with IFN-γ. Doses of PGE_1 ranging from 10-100 μM were used to determine the optimum concentration for use in further experiments. Gene expression was measured using RT-qPCR. IFN-γ invreased MCP-1 and ICAM-1 expression by 62% and 55% respectively (Figure 5.12). When pre-incubated with PGE_1 prior to cytokine stimulation, MCP-1 induction was inhibited, returning to a level below basal expression in a dose-dependent manner. ICAM-1 expression was attenuated by an average of 40-55% at all doses. Both results were significant at the lowest dose used (10 μM) and this concentration was employed in further experiments. This result was

confirmed in HMDMs (Figure 5.13). As shown in Figure 5.14, MCP-1 induced monocyte migration significantly by 70% as observed previously. On co-incubation with 10 µM PGE_1, monocyte migration was inhibited and returned to levels observed in un-stimulated cells.

Similar results have been previously observed *in vivo.* PGE_1 has been shown to inhibit MCP-1 expression in mice with induced glomerular immune injury (Jocks *et al.* 1996). In addition, patients with peripheral vascular disease receiving PGE_1 therapy showed a significant decrease in circulating levels of adhesion molecules ICAM-1, VCAM-1 and E-selectin (Palumbo *et al*. 2000). In mouse macrophages, PGE_1 inhibited PMA-, IFN-γ- and LPS-mediated induction of MIP-1β and MIP-1α inflammatory genes expressed in atherosclerosis (Martin and Dorf 1991). PGE_1 was also shown to inhibit the expression of pro-inflammatory genes induced by numerous other cytokines (Martin and Dorf 1991).

To determine if the anti-inflammatory actions of DGLA were as a result of production of PGE_1, RNA interference techniques were used to knockdown the expression of COX enzymes. As discussed previously in Chapter 3, there is some debate as to the specific role of the COX isoforms in PGE_1 formation. Nevertheless, both COX-1 and COX-2 have been shown to metabolise DGLA to PGE_1 (Levin *et al*. 2002) therefore both enzymes were targeted to ensure that synthesis was inhibited at the maximum level. To evaluate the role of PGE_1 synthesis from DGLA in inflammation, the robust IFN-γ induced MCP-1 response was initially investigated. siRNA techniques were used to target COX-1 and COX-2 enzymes prior to differentiation into macrophages, incubation with DGLA and stimulation with IFN-γ. Following knockdown, the levels of COX-1 and COX-2 were measured at the mRNA and protein level (Figure 5.15). COX-1 was knocked down by an average of 75% and 50% at the mRNA and protein level, respectively. COX-2 knockdown was 60% at mRNA level and 20% at protein level (Figure 5.15). COX-2 knockdown seemed less efficient than COX-1 knockdown; however COX-2 is an inducible enzyme while COX-1 is constitutively expressed. Basal levels of COX-2 mRNA and protein were very low in unstimulated macrophages. Absolute quantification of COX-1 and COX-2 was not determined; however in doing so it may be possible to more accurately compare the levels of COX-1 and COX-2 knockdown. Despite this, knockdown of COX-1 and COX-2 enzymes were sufficient to see changes in IFN-γ induced MCP-1 expression.

As expected, IFN-γ induced MCP-1 expression which was significantly attenuated on pre-treatment with DGLA (Figure 5.16). In macrophages where the expression of

COX-1 and COX-2 was knocked down, MCP-1 was still significantly induced by IFN-γ. However, pre-treatment with DGLA did not significantly reduce MCP-1 expression. The effect of DGLA on reducing MCP-1 induction was abolished following knockdown of COX-1 and COX-2. This indicates that on treatment of the cells with DGLA, the COX metabolites PGE_1 plays a role in the inhibition of IFN-γ induced MCP-1 gene expression. Future experiments may explore the use of the COX inhibitor naproxen to evaluate ICAM-1 gene expression and monocyte migration.

Previous research supports the data presented in Figure 5.16. Oral administration of DGLA to mice, significantly reduced the induction of inflammation in the ears, triggered by application of an inflammatory agent (Watanabe *et al*. 2014). DGLA reduced infiltration of cells, swelling and chemokine production (Watanabe *et al*. 2014). When mice were administered COX inhibitor naproxen prior to DGLA feeding, this reduction in inflammation was attenuated (Watanabe *et al*. 2014). Naproxen prevented a reduction in infiltration of cells to the inflamed areas along with the reduction in the levels of MCP-1 (Watanabe *et al*. 2014). This indicates a role of the COX DGLA metabolite PGE_1 in migration and MCP-1 production *in vivo.* In addition, ApoE deficient mice fed DGLA significantly reduced lipid accumulation in the mouse aortas (Takai *et al*. 2009). Following treatment with naproxen, lesion size was reduced so that there was no significant difference between the vehicle and DGLA and naproxen treated groups (Takai *et al*. 2009). The inhibition of inflammatory markers ICAM-1 and VCAM-1 in mice supplemented with DGLA was also reversed with naproxen treatment (Takai *et al*. 2009).

In addition to metabolising DGLA into PGE_1, COX enzymes can also metabolise AA into PGE_2 (Levin *et al*. 2002). As previously shown, in macrophages pre-treated with DGLA and then stimulated with IFN-γ there was a significant induction of PGE_2 (Figure 3.9). It can be assumed therefore that knockdown of the two enzymes would limit the production of both prostaglandins. As the knockdown is not specific to inhibition of individual prostaglandins, the experiment cannot specifically identify which is responsible for the results observed. However, given the roles of PGE_1 on MCP-1 and ICAM-1 expression presented in this report (Figure 5.12 and 5.13) and in the literature (Martin and Dorf 1991; Jocks *et al*. 1996; Watanabe *et al*. 2014), the induction of PGE_1 on treatment of DGLA in macrophages and the widely reported pro-inflammatory actions of PGE_2, it can be assumed with confidence that the results shown in Figure 5.16 are due to the inhibition of PGE_1 production from DGLA.

The anti-inflammatory actions of DGLA are not solely dependent on PGE_1 production however. DGLA inhibited TNF-α release from PBMCs, as did PGE_1. However inhibiting PGE_1 production, did not affect the reduction of TNF-α after DGLA supplementation (Dooper *et al*. 2003). This indicates DGLA has a direct effect or is acting through the production of an alternative eicosanoid. Another series 1 prostaglandin synthesised from DGLA, PGD_1 has previously been shown to reduce severity of atopic dermatitis in mice, reducing scratching and scores (Amagai *et al*. 2015). Future work could include determining the roles of other eicosanoids metabolised from DGLA in the regulation of the inflammatory response in atherosclerosis, to obtain a better understanding of the role of the PUFA and its metabolites.

CHAPTER 6

GENERAL DISCUSSION

6.1 Introduction

In 2008 it was estimated that 17.8 million people died of CVD related disease, more than any other disease in the Western society. With mortality rates predicted to increase in the coming years, the need for new therapeutic advances is crucial. Atherosclerosis is the primary cause of CVD. It is characterised by the accumulation of lipid-rich plaques in the walls of large and medium arteries as a result of a chronic inflammatory response (Buckley and Ramji 2015). Atherosclerosis is a progressive, mutli-factorial disease, with complex pathology. A key feature is the formation of lipid-loaded macrophages, termed foam cells, which accumulate in the walls of arteries and form the underlying basis for the development of plaques (Michael *et al*. 2012). Targeting the early stages of atherosclerosis and the ongoing inflammatory response is of therapeutic interest in the prevention and management of the disease.

Statins are the primary drug therapy used in the management of atherosclerosis (Michael *et al*. 2012). Use of statins has been well documented to have had a marked impact in reducing the mortality rates from the disease, however there are a number of limitations and drawbacks to the therapy (Mishra and Routray 2003). The maximum reduction in mortality rates that can be attributed to statins is about 30%, with many studies reporting issues such as side effects and tolerability (Mishra and Routray 2003). In addition to this, the result of a number of clinical trials investigating key targets in the disease (PPAR agonists, inhibitors of CETP and ACAT-1) have been disappointing (Fazio *et al.* 2001; Weber and Noels 2011; Michael *et al*. 2012; Ramji and Davies 2015). The need for new preventative and therapeutic agents is therefore essential.

Nutraceuticals are potentially promising drug alternatives, as prevention or limiting agents. It is important to determine the beneficial effect of these agents by understanding the molecular mechanisms underlying their actions *in vitro* and *in vivo.* DGLA is an omega-6 PUFA which has been implicated to play an anti-inflammatory role in a number of diseases such as atopic eczema and atherosclerosis (Kawashima *et al*. 2008; Takai *et al*. 2009). In addition to this, the PUFA has been shown to reduce SFA induced hypertension in rats (Hassall and Kirtland 1984), inflammatory responses induced by croton oil in mice (Watanabe *et al*. 2014) and promote

antithrombotic effects in humans (Kernoff *et al*. 1977). *In vivo* studies have indicated that DGLA is well tolerated in mice, rats and humans, with no side effects reported (Umeda-Sawada *et al*. 2006; Takai *et al*. 2009; Teraoka *et al*. 2009; Tanaka *et al.* 2012).

The project was based on two broad aims. First; determine the uptake and metabolism of DGLA *in vitro* and *in vivo.* Second, delineate the molecular mechanisms underlying the anti-inflammatory action of DGLA, in relation to atherosclerosis. For *in vitro* analysis, macrophage cell lines and HMDMs were used throughout the study due to the key roles of these cells in foam cell formation and the pathology of atherosclerosis.

6.2 Results

6.2.1 Summary of key findings with DGLA

In vitro

1. Supplementation with DGLA significantly increased the incorporation of the PUFA into TPL and TAG fractions of THP-1 macrophages. There was no increase in the incorporation of AA into lipid fractions.

2. Attenuated MCP-1 induced monocytic migration of THP-1 monocytes.

3. Inhibited pro-inflammatory cytokine (IFN-γ, IL-1β, TNF-α) induced expression of MCP-1 and ICAM-1, two robust markers of inflammation, in THP-1 macrophages. Inhibition of IFN-γ response was also confirmed in RAW264.7 and HMDMs.

4. Inhibited STAT-1 SER^{727} phosphorylation, involved in activation of IFN-γ signalling in THP-1 macrophages.

5. Attenuated acLDL induced cholesteryl ester accumulation in RAW264.7 macrophages.

6. Decreased DiI-oxLDL uptake by THP-1 macrophages.

7. Decreased scavenger receptor expression and uptake by macropinocytosis in THP-1 macrophages.

8. Increased cholesterol efflux in cholesterol-loaded THP-1 macrophages stimulated with ApoA1.

9. Increased production of eicosanoids PGE_1 and 15-HETrE in THP-1 macrophages.

10. PGE_1 inhibited IFN-γ induced MCP-1 and ICAM-1 expression and monocyte migration in THP-1 macrophages.

11. Knockdown of COX enzymes abolished the inhibition of DGLA on IFN-γ induced MCP-1 expression in THP-1 macrophages.

In vivo

12. Mice supplemented with 4.4 % (w/w) DGLA (isolated from mutant algal powder) in their diet increased the relative amount of this PUFA in plasma, liver and kidney lipid fractions.

6.2.2 Lipid uptake *in vitro*

In Chapter 3, initial studies focused on determining the uptake of DGLA into THP-1 macrophage lipid fractions. Lipid analysis was performed using TLC and GC. A time point of 24 hour incubation with DGLA had been optimised previously in the laboratory. Using this time point a dose response experiment was performed based on concentrations used previously in the laboratory and other studies (Iversen *et al*. 1991; Iversen *et al*. 1992; Dooper *et al*. 2003). It was found that DGLA levels increased in a dose-dependent manner into TPL and TAG fractions of THP-1 macrophages (Figure 3.2). A concentration of 100 µM was used in further experiments due to the more pronounced lipid changes observed in comparison to lower concentrations.

Lipid analysis following treatment of THP-1 macrophages with 100 µM DGLA (or vehicle control) was repeated for statistical analysis. It was found that treatment of the cells with 100 µM DGLA resulted in a significant increase in the level of the PUFA into TPL and TAG fractions in equal proportions (Figure 3.3). In addition there was an increase into individual phospholipids PC, PS, PE and PI (Figure 3.5). It was also observed that in all the lipid fractions analysed, there was no significant increase in the level of AA. This indicated that in THP-1 macrophages, treatment with DGLA resulted in a specific accumulation of the PUFA and that it was not rapidly desaturated

to AA. The result was comparable to previous studies *in vitro* (Johnson *et al*. 1997; Dooper *et al*. 2003). It has previously been shown that macrophages have limited desaturase activity (Chapkin *et al*. 1988; Chapkin and Coble 1991; Johnson *et al*. 1997) which may explain the resulting lack of an increase in the levels of AA on supplementation with DGLA. This was a positive result as fatty acid composition of lipid pools directly influences the production of eicosanoids. DGLA gives rise to anti-inflammatory eicosanoids, while AA produces classically pro-inflammatory eicosanoids. The selective increase in the level of DGLA over AA, may therefore affect the inflammatory potential of THP-1 macrophages by altering eicosanoid production. Given the important role of macrophages in the chronic inflammatory response in atherosclerosis, altering the production of eicosanoids from these cells may potentially have an important impact on the disease.

6.2.3 Eicosanoids production

Following on from the changes in fatty acid composition of THP-1 macrophages observed on treatment of the cells with DGLA, HPLC-MS was used to measure eicosanoid production. This would determine if changes in lipid composition of THP-1 macrophages translated to a change in eicosanoid production. Prostaglandins, produced from the metabolism of DGLA and AA by COX, were measured. In DGLA treated macrophages, there was a significant increase in the concentration of PGE_1, with no change in the levels of PGE_2 (Figures 3.7 and 3.8). The concentration of PGE_1 did not exceed that of PGE_2 in any experiment. However, the ratio of PGE_2:PGE_1 was dramatically reduced on treatment of the cells with DGLA. This indicated that the accumulation of DGLA into THP-1 macrophages did influence the production of eicosanoids with the significant increase in its COX metabolite PGE_1. As expected, there was no rise in the production of AA metabolite PGE_2. Similar results were also observed *in vitro* in mouse peritoneal macrophages (Chapkin and Coble 1991; Iversen *et al*. 1992). In addition to this, DGLA supplementation also significantly increased the production of LOX metabolite 15-HETrE, with no increase in AA LOX metabolites 5-, 11-, 12- and 15-HETE (Figure 3.6). PGE_1 and 15-HETrE have been well documented to play a number of anti-inflammatory roles (Zurier and Ballas 1973; Zurier *et al*. 1977; Horrobin 1991). This suggested that supplementation of macrophages with DGLA can influence the inflammatory potential by increasing the production of anti-inflammatory mediators PGE_1 and 15-HETrE.

Eicosanoid production, on induction of an inflammatory state, was also measured. IFN-γ is a well-established pro-inflammatory cytokine (McLaren and Ramji 2009) and has been used routinely in the laboratory and throughout this study. IFN-γ stimulation

of macrophages did not significantly change the production of any eicosanoids measured. However, pre-treatment with DGLA followed by stimulation with IFN-γ significantly increased the production of PGE_2 which was not seen in any other treatment (Figure 3.7). In addition to this, production of PGE_1 in DGLA and IFN-γ stimulated macrophages, also increased to almost twice that seen in DGLA only treated macrophages (Figure 3.8). However, this difference was not significant due to a high variability between individual repeats.

It was hypothesised that IFN-γ stimulation following DGLA pre-treatment may-be increasing the desaturation of the PUFA to AA and its accumulation into THP-1 lipid fractions. However, fatty acid analysis indicated that IFN-γ alone, and pre-treatment with DGLA followed by IFN-γ stimulation, did not result in an increase in the relative amount of AA into lipid fractions (Appendix 2). The next hypothesis was that IFN-γ affects the expression of COX enzymes, responsible for the production of prostaglandins. RT-qPCR was used to measure the effect of the cytokine on COX gene expression. It was observed that IFN-γ stimulation only, had no effect on the expression of COX-1 and COX-2 (Figure 3.9). On incubation with DGLA followed by stimulation with IFN-γ however, there was a significant decrease in COX-1 expression and a significant increase in COX-2 expression. There was also a significant decrease in COX-1 expression in DGLA only treated macrophages. These data indicated that DGLA in combination with IFN-γ was significantly increasing the expression of COX-2 which, under identical conditions, was also increasing the production of PGE_2 and to some extent PGE_1. This suggested that COX-2, not COX-1 was primarily responsible for prostaglandin synthesis in THP-1 macrophages. The result was supported by data presented in previous studies (Ariasnegrete *et al*. 1995; Barrios-Rodiles and Chadee 1998; Caughey *et al*. 2001; Levin *et al*. 2002). Future work would need to confirm these changes at the protein level.

Despite the increase in PGE_2 on treatment with DGLA and IFN-γ, results presented in Chapter 4 indicated an overall anti-inflammatory role in DGLA treated macrophages. For example, attenuation of IFN-γ induced gene expression on pre-treatment with DGLA was observed in THP-1 macrophages, a condition in which PGE_1 and PGE_2 production was induced (Figure 3.7 and 3.8). This suggests that the role macrophages play in inflammation may be as a result of the ratios of PGE_1 to PGE_2 produced. Supplementation with DGLA, and induction of PGE_1, may prove sufficient to offset any pro-inflammatory actions that may be promoted through the induction of PGE_2.

6.2.3.1 Role of PGE_1

Given the induction of PGE_1 production from macrophages treated with DGLA, and the anti-inflammatory roles of the eicosanoid reported previously (Martin and Dorf 1991; Palumbo *et al*. 2000) the role of PGE_1 was investigated in Chapter 5. Initially it was observed that DGLA inhibited IFN-γ induced gene expression and monocyte migration (Figure 4.10 and 4.15). The experiments were mimicked using PGE_1. It was observed that PGE_1 attenuated the IFN-γ induced expression of MCP-1 and ICAM-1 in THP-1 macrophages and HMDMs (Figure 5.12 and 5.13) and inhibited MCP-1 induced monocyte migration (Figure 5.14) comparable to that of DGLA. It was therefore hypothesised that the production of PGE_1 from DGLA was playing a role in these processes.

To test this hypothesis, the effect of DGLA on IFN-γ induced MCP-1 expression was measured in macrophages following siRNA-mediated knockdown of COX genes. COX-1 and COX-2 were both targeted for knockdown as both genes have been implicated in prostaglandin synthesis (Levin *et al*. 2002). As presented in Chapter 5 (Figure 5.16), in macrophages containing COX-1 and COX-2, DGLA attenuated IFN-γ induced MCP-1 gene expression. In macrophages with COX-1 and COX-2 knockdown, this response was abolished. This indicated that metabolism of DGLA to PGE_1 played a key role in IFN-γ mediated changes in gene expression. The data was supported by results published previously *in vivo* (Takai *et al*. 2009; Watanabe *et al*. 2014). Given the inhibition of monocyte migration with PGE_1 observed in Chapter 5, future work could aim to establish the role of the eicosanoid in the effect of DGLA on this process. This could be achieved by utilising the COX inhibitor naproxen.

6.2.4 Cytokines

Cytokines play an important role in regulating the inflammatory response in atherosclerosis. The effect of DGLA on signalling by pro-inflammatory cytokines IFN-γ, IL-1β and TNF-α was investigated. The three cytokines have been studied previously in the laboratory and their roles in atherosclerosis are well documented (Gupta *et al*. 1997; Kirii *et al*. 2003; McLaren and Ramji 2009; Xiao *et al*. 2009). The induction of two key genes in atherosclerosis, MCP-1 and ICAM-1, was used as robust markers of inflammation. The expression of MCP-1 and ICAM-1 is up-regulated in atherosclerosis and contributes to the pathology of the disease by recruiting monocytes and promoting their transmigration into the arterial intima (Gosling *et al*. 1999; Lusis 2000; Kitagawa *et al*. 2002). RT-qPCR was used to measure MCP-1 and ICAM-1 gene expression in response to pro-inflammatory cytokine signalling in THP-1 macrophages.

6.2.4.1 IFN-γ

IFN-γ was the primary cytokine employed throughout this study as it has previously been extensively studied in the laboratory and has been described as a master regulator of atherosclerosis (McLaren and Ramji 2009). IFN-γ significantly induced the expression of MCP-1 and ICAM-1 in THP-1 macrophages (Figure 4.10). On pre-incubation with DGLA, the induction of expression of these genes was significantly attenuated. The findings were also replicated in RAW264.7 macrophages and HMDMs (Figure 4.11 and 4.12). This indicated that DGLA potentially inhibited IFN-γ signalling and the regulation of inducible genes by this cytokine. IFN-γ signals through the JAK/STAT pathway. A key step in the pathway is the phosphorylation of STAT1 molecules. This allows for STAT1 transcription factors to dimerise, translocate into the nucleus and activate gene transcription (Varinou *et al*. 2003). Two phosphorylation sites are present on the STAT1 molecule at positions TYR^{701} and SER^{727}. Phosphorylation at TYR^{701} is crucial for STAT1 function as a transcription factor. For maximum activity of STAT1, phosphorylation at both sites is needed. Given the important role of STAT1 phosphorylation in IFN-γ signalling through the JAK/STAT pathway, the effect of DGLA on this process was determined by western blotting. Stimulation of the cells with IFN-γ significantly increased the phosphorylation of STAT1 at both phosphorylation sites in THP-1 macrophages (Figure 5.4). On pre-treatment of the cells with DGLA followed by IFN-γ stimulation, the phosphorylation at SER^{727} was attenuated. There was no effect on phosphorylation at TYR^{701}. Although STAT1 translocation into the nucleus and activation of transcription can occur independently of SER^{727} phosphorylation, it has previously been found that inhibiting phosphorylation at this site attenuates IFN-γ induced pro-inflammatory gene expression including MCP-1 and ICAM-1 (Varinou *et al*. 2003; Li *et al*. 2010). The data presented in Chapter 5 therefore indicates that DGLA inhibits IFN-γ induced gene expression by suppressing the phosphorylation of STAT1 at SER^{727} thereby attenuating the maximum activity of the transcription factor. This is a novel mechanism identified for the role of DGLA in pro-inflammatory signalling in atherosclerosis. Signalling through STAT1 also plays a role in the regulation of other pro-inflammatory pathways including IL-1 and TLR4 (Sikorski *et al*. 2011b). Given the role of DGLA in STAT1 regulation, signalling through these STAT1 dependent pro-inflammatory pathways may also be affected. Future work may investigate the role of DGLA in other STAT1 dependent pathways.

6.2.4.2 IL-1β

Following on from the inhibition of IFN-γ induced gene expression, the effect of the PUFA on other pro-inflammatory cytokine signalling was determined. The expression of the two inflammatory markers MCP-1 and ICAM-1 induced through IL-1β signalling was measured by RT-qPCR. IL-1β significantly induced the expression of the two genes in THP-1 macrophages (Figure 4.13). This has been observed in previous studies (Myers *et al*. 1992; Lim *et al*. 2009; Yang *et al*. 2010). Pre incubation of the cells with DGLA significantly inhibited the IL-1β induced expression of MCP-1 and ICAM-1 and returned to levels comparable to that of control. Inhibition of IL-1β signalling *in vivo* has been shown to have athero-protective effects, reducing plaque formation, macrophage infiltration and pro-inflammatory gene expression, including that of MCP-1 and VCAM-1 (Kirii *et al*. 2003; Bhaskar *et al*. 2011). The effect of DGLA on IL-1β signalling *in vitro* has not previously been established. Given the athero-protective role of inhibiting IL-1β signalling in atherosclerosis, this may therefore be a novel mechanism by which DGLA exerts anti-inflammatory and anti-atherosclerotic effects.

In addition to IL-1β signalling, the production of the cytokine from macrophages was also of interest. The production of an active form of IL-1β is under the control of a multi protein complex termed the inflammasome. A number of inflammasome complexes have been identified, however the NLRP3 inflammasome has been described as a key regulator of IL-1β production and documented to play a role in atherosclerosis (Moore *et al*. 2013). The role of the NLRP3 inflammasome in IL-1β production has also been documented in THP-1 macrophages (Rajamaki *et al*. 2010). ELISA was used to measure the effect of DGLA on the production of IL-1β through the activation of the NLRP3 inflammasome. The inflammasome was activated by cholesterol crystals, which have previously been demonstrated to induce inflammasome activation *in vivo* and *in vitro*, including THP-1 macrophages (Duewell *et al*. 2010). Data presented in Chapter 4 showed that on treatment with cholesterol crystals, IL-1β production was induced from THP-1 macrophages as previously seen (Duewell *et al*. 2010) (Figure 4.14). On pre-treatment of the cells with DGLA followed by stimulation with cholesterol crystals, IL-1β expression was induced further in comparison to cholesterol crystal treatment only. This suggested DGLA induced IL-1β expression through NLRP3 inflammasome activation. Previous work has proved controversial. Some studies have reported similar results with no effect of DGLA on LPS and nigericin induced NLRP3 or NLRP1b inflammasome activation (Yan *et al*. 2013). In addition, GLA treatment of peripheral blood monocytes failed to inhibit the

LPS induced IL-1β induction (Furse *et al*. 2001). However GLA did inhibit IL-1β induction in an LPS independent manner. GLA reduced the stability of pro-IL-1β mRNA and attenuated auto induction of IL-1β (Furse *et al*. 2001). Data presented in Chapter 4 and previous studies (Furse *et al*. 2001; Yan *et al*. 2013), indicate that DGLA has no inhibitory effect on IL-1β production through inflammasome activation. Future work with DGLA may focus on evaluating IL-1β production in inflammasome independent mechanisms, given the role for GLA previously reported (Furse *et al*. 2001).

6.2.4.3 TNF-α

Finally, the effect of DGLA on the induction of MCP-1 and ICAM-1 expression by TNF-α was determined in THP-1 macrophages. Data presented in Chapter 4 demonstrated that TNF-α significantly induced the expression of the two genes (Figure 4.13). The cytokine has also previously been shown to induce the expression of the two genes *in vitro* and *in vivo* (Myers *et al*. 1992; Murao *et al*. 2000; Xiao *et al*. 2009). On pre-treatment of the cells with DGLA followed by TNF-α stimulation, the induction of MCP-1 and ICAM-1 expression was significantly inhibited. In both cases, the expression of genes in DGLA and TNF-α stimulated macrophages was not significantly different to that of the vehicle control. Inhibiting signalling through TNF-α *in vivo* has been shown to reduce plaque formation and decrease the levels of pro-inflammatory cytokines and chemokines (Ohta *et al*. 2005; Xiao *et al*. 2009). DGLA has also been shown to attenuate the production of TNF-α from PBMCs (Dooper *et al*. 2003). Data presented in Chapter 4 and previous studies have indicated a role for DGLA in the attenuation of TNF-α induced pro-inflammatory signalling and production. Further experiments will need to be carried out to determine the role of DGLA on TNF-α production in macrophages and *in vivo*.

6.2.5 Monocyte migration

As previously described (Section 4.1.2.2.1), MCP-1 plays an important role in monocyte recruitment (Gosling *et al*. 1999). Given the role of DGLA in inhibiting the induction of MCP-1 expression by a number of pro-inflammatory cytokines, it was of interest to determine if DGLA had a direct effect on the role of MCP-1. Monocyte migration was measured using cell inserts, to mimic endothelial layer of arteries, and THP-1 monocytes stimulated with MCP-1. MCP-1 significantly induced monocyte migration across the cell insert (Figure 4.15). On co-incubation with DGLA, this induction was significantly attenuated. This effect of DGLA on MCP-1 induced monocyte migration was a novel finding. Previous work has indicated that inhibiting

monocyte migration can significantly protect against the development of atherosclerosis *in vivo* (Feige *et al*. 2013; Mueller *et al*. 2013).

The majority of experiments performed throughout this project, involved pre-incubation with DGLA which allowed for uptake of the PUFA into THP-1 lipid fractions. However, DGLA was co-incubated with MCP-1 for 3 hours in migration studies due to the experimental set up. During this time it is hypothesised that there would be a lower level of uptake and metabolism of DGLA by monocytes. This may indicate that DGLA is having a direct effect on monocyte migration as opposed to a potentially indirect effect through its metabolites. This could be the result of DGLA acting on a number of fatty acid receptors including PPARs, TLRs, GPCR or SREBPs (as discussed in Section 1.4.2). However, data presented in chapter 5 (Figure 5.14) showed that PGE_1 also inhibited monocyte migration suggesting a role for the eicosanoid. Future study could act to elucidate the mechanism by which DGLA inhibits monocyte migration. The role of DGLA metabolism to PGE_1 in monocyte migration could be determined with the use of COX inhibitor naproxen. In addition, the role of fatty acid receptors could be evaluated through employing gene silencing techniques.

Taken together, data presented in Chapter 4 indicates that DGLA inhibits the induction of MCP-1 expression through the inhibition of pro-inflammatory cytokine signalling and its ability to recruit monocytes. Inhibition of MCP-1 and monocyte recruitment has been previously shown to attenuate monocyte and macrophage content of plaques and significantly reduce atherosclerosis (Gosling *et al*. 1999; Feige *et al*. 2013; Mueller *et al*. 2013). Inhibition of MCP-1 induction and function by DGLA may be one mechanism by which the fatty acid exerts anti-inflammatory and anti-atherosclerotic actions.

6.2.6 Cholesterol uptake and efflux

The next step following monocyte migration into the arterial intima, is their differentiation into macrophages. Macrophages can take up modified forms of LDL in an unregulated manner, accumulate cholesterol and store any excess as cholesteryl esters in lipid droplets in the cytoplasm (Ghosh *et al*. 2010). Viewing lipid loaded macrophages under the microscope has led to the term foam cells, due to their foamy appearance (Brown *et al*. 1980). The effect of DGLA on modified LDL induced cholesteryl ester accumulation was assessed in Chapter 4. Using acLDL as a modified form of LDL, the effect of DGLA on cholesteryl ester accumulation was determined in RAW264.7. It was found that on treatment with acLDL, RAW264.7

macrophages accumulated a significant amount of cholesteryl esters (Figure 4.16). On pre-treatment of the cells with DGLA followed by acLDL loading, this accumulation was significantly attenuated. In addition, the relative amount of free cholesterol was reduced in acLDL treated macrophages. This was slightly rescued on pre-treatment of the cells with DGLA, however this was not significant. The trend, however, suggested that DGLA played a role in inhibiting the uptake and/or accumulation of cholesteryl esters in macrophages. Studies in Chapter 5 aimed to determine the underlying molecular mechanisms behind this effect.

6.2.6.1 Endocytosis

In Chapter 5, the uptake of modified LDL by macrophages was investigated. Using a fluorescent form of oxLDL, Dil-oxLDL, the effect of DGLA on cholesterol uptake was measured by FACS. It was found that on treatment of the cells with DGLA, the uptake of Dil-oxLDL was significantly reduced by an average of 21% (Figure 5.5). This suggested that DGLA had an effect on the mechanisms underlying uptake. The uptake by receptor-dependent and -independent endocytosis was therefore determined in THP-1 macrophages. Scavenger receptors are expressed on the surface of macrophages and take up unregulated amounts of modified LDL. CD36 and SRA were selected for study due to their key roles in the uptake of acLDL and oxLDL as reported previously (Febbraio *et al*. 2000; Kunjathoor *et al*. 2002; Kuchibhotla *et al*. 2008). Gene and protein expression of scavenger receptors was measured by RT-qPCR and western blotting respectively. It was found that on treatment with DGLA, the mRNA and protein expression of SRA was significantly attenuated in comparison to the control (Figure 5.6). The mRNA expression of CD36 was also significantly reduced.

In addition to receptor-mediated endocytosis, receptor-independent uptake through macropinocytosis was determined by FACS analysis of a marker, LY. Macropinocytosis has been documented to uptake native and modified forms of LDL and to contribute significantly to foam cell formation in macrophages (McLaren *et al*. 2011b; Michael *et al*. 2013). On treatment of the cells with DGLA, the uptake of LY by macropinocytosis was significantly reduced (Figure 5.7).

Inhibiting these two uptake pathways has been shown to significantly reduce the uptake of acLDL, oxLDL and the accumulation of cholesteryl esters in macrophages (Kunjathoor *et al*. 2002). This suggests that DGLA attenuates cholesteryl ester accumulation as seen in Chapter 4, by attenuating modified LDL uptake through scavenger receptor mediated pathways and macropinocytosis.

6.2.6.2 Efflux

Macrophages efflux cholesterol by a mechanism known as RCT. An imbalance between cholesterol influx and efflux leads to an accumulation of cholesteryl esters and macrophage foam cell formation in atherosclerosis (Ono 2012). Efflux of cholesterol from acLDL loaded THP-1 macrophages was measured using radioactive ^{14}C cholesterol and scintillation counting. Following incubation with acLDL, ApoA1 was used to stimulate efflux from macrophages, which has been shown previously (McLaren *et al*. 2010; Ouimet *et al*. 2011; Sene *et al*. 2013). It was found that ApoA1 increased the efflux of cholesterol from acLDL-loaded THP-1 macrophages in comparison to the control (Figure 5.9). When macrophages were pre-treated with DGLA followed by acLDL loading and ApoA1 stimulation, efflux was significantly increased. No study to date has documented the role of DGLA in RCT from macrophages. Increase in cholesterol efflux to ApoA1 *in vivo* was shown to significantly reduce atherosclerosis (Su *et al*. 2003). This suggests that DGLA may decrease cholesteryl ester accumulation and foam cell formation by increasing cholesterol efflux from macrophages.

There are a number of mechanisms by which macrophages remove cholesterol. ABC transporters ABCA1 and ABCG1 have been well documented to play an important role in macrophage RCT (Singaraja *et al*. 2002; Wang *et al*. 2007; Yvan-Charvet *et al*. 2007; Yvan-Charvet *et al*. 2010). Specifically, ABCA1 plays an important role in efflux of cholesterol to ApoA1, while ABCG1 is required to efflux to HDL (Kennedy *et al*. 2005). In addition to this, macrophage expressed ApoE has also been indicated to play a role in cholesterol efflux (Zhu *et al*. 1998). Given the role of DGLA in cholesterol efflux, the effect of the PUFA on the mRNA and protein expression of ABCA1, ABCG1 and ApoE was measured by RT-qPCR and western blotting to attempt to establish the underlying mechanisms. Due to time constraints, one experiment was performed in triplicate for both RT-qPCR and western blotting. From the results of this experiment it was found that in THP-1 macrophages, cholesterol loading with acLDL induced ABCA1 (mRNA and protein) and ABCG1 (mRNA) expression but not ApoE (protein) (Figure 5.10). On pre-treatment of the cells with DGLA, there was no observable difference in expression ABCG1 or ApoE in comparison to only cholesterol loaded macrophages. For ABCA1, pre-treatment with DGLA decreased mRNA expression, however there was no change observed at the protein level.

Previous studies on the role of omega-6 PUFAs on the expression of ABC transporter genes have proved controversial, with some reporting an inhibition of gene and protein expression (Uehara *et al*. 2002; Wang and Oram 2002; Uehara *et al*. 2007)

and other showing no effect (Salehipour *et al*. 2010; Song *et al*. 2013). In addition, ABC transporters and ApoE are LXR inducible genes. PUFAs have previously been demonstrated to inhibit LXR activation and activity (Pawar *et al*. 2003; Uehara *et al*. 2007). The expression of LXR isoforms LXR-α and –β was measured by RT-qPCR in unloaded THP-1 macrophages. It was observed that pre-incubation of DGLA significantly attenuated LXR-α and -β mRNA expression (Figure 5.11). This suggests that DGLA may inhibit LXR activity in THP-1 macrophages, and that the PUFA may have an adverse effect on the expression of ABC transporter genes and ApoE on further investigation. This also indicates that DGLA is not acting through mechanisms dependent on these two proteins. Future work will need to establish the effect of DGLA on the expression of LXR genes in cholesterol loaded macrophages.

Taken together, the increase in cholesterol efflux on treatment with DGLA observed in THP-1 macrophages may not be dependent on LXR regulated genes, ABCA1, ABCG1 and ApoE. However further investigation will be needed to confirm this. In addition, future work will need to identify any alternative mechanisms by which an increase in efflux is achieved by DGLA. This may include determining the role of the COX metabolite PGE_1 which has previously been indicated to increase cholesterol efflux in macrophages and fibroblasts (Middleton and Middleton 1998; Chan *et al*. 2007).

6.2.7 Lipid uptake *in vivo*

Initial lipid analysis utilised THP-1 macrophages to determine the uptake and metabolism of DGLA. Following on from this, mouse feeding studies were performed to determine the uptake of the PUFA *in vivo*. *In vitro* work used a pure form of DGLA (as a free fatty acid) to determine the specific role of the PUFA in inflammation. Alternatively, DGLA was also sourced from a mutant form of the green algae *P. incisa*, obtained from collaboration with Ben-Gurion University, Israel. An additional aim of the study was to evaluate the algae as a potential new commercial source of DGLA. Initially, freeze dried algal powder was added directly to mouse diet. It was found that the algal powder was not digested by the mice and the DGLA contained within the algal bodies was not absorbed and incorporated into any lipid fractions (Appendix 1).

Following this, total lipids were extracted from the algal powder and added directly to mouse chow diet. Male C57BL/6 mice were starved for 24 hours prior to being given either chow control diet or a diet containing 4.4% (TFA) DGLA for 48 hours. Mice were sacrificed using schedule 1 procedures and samples of plasma, together with tissue samples, were taken for lipid analysis.

Plasma was separated into four lipid fractions; TFA, TPL, TAG and steryl esters. In the DGLA group, the level of the PUFA was significantly increased into all lipid fractions measured, in comparison to control group. There was no significant increase in AA level in any lipid fraction of plasma. In addition, lipid fractions of liver, kidney and adipose tissue were analysed. Similar to plasma, the level of DGLA was significantly increased in TFA, TPL, TAG and steryl ester lipid fractions in DGLA diet group. There was a significant rise in the level of AA in TAG fraction only. Individual phospholipids were also analysed from the liver. DGLA was significantly incorporated into PS, PE and PC in the DGLA group. In the kidney, DGLA level was significantly increased in TFA in the DGLA group, but not TPL or TAG. There was however a significant increase in AA in the TPL fraction. Finally, in adipose tissue, DGLA significantly increased in TFA in the DGLA group, with no significant increase of AA. Results are summarised in Table 6.1.

Table 6.1 – Summary of key findings *in vivo*

	DGLA				AA			
	TFA	**PL**	**TAG**	**SE**	**TFA**	**PL**	**TAG**	**SE**
Plasma	✓	✓	✓	✓	x	x	x	x
Liver	✓	✓	✓	✓	x	x	✓	x
Kidney	✓	x	x	-	x	✓	x	-
Adipose	✓	-	-	-	x	-	-	-

Summary of the significant changes in incorporation of DGLA and AA into lipid fractions, following DGLA feeding, *in vivo.* Tick indicates significant increase in incorporation. Cross indicates no significant increase.

Overall, after 48 hours of DGLA supplemented diet, mice incorporated the PUFA into the majority of lipid fractions measured in the plasma, liver, kidney and adipose tissue. The only significant increase in AA level observed was in liver TAG and kidney TPL fractions. Despite this, there was a general trend observed indicating an increase in the level of AA on feeding with DGLA in the majority of lipid fractions. Initial *in vitro*

work in macrophages indicated that DGLA was actively incorporated into membranes with no subsequent increase in AA. In contrast, *in vivo* there was a clear trend of increase in AA level in lipid fractions on DGLA feeding. This may be explained by the different activities of desaturase enzymes found in different cell types. For example, it has been reported that desaturase activity in macrophages is low, preventing rapid desaturation of DGLA to AA in these cells (Chapkin *et al*. 1988; Johnson *et al*. 1997). However, liver and kidney have been reported to have both active elongase and desaturase (Johnson *et al*. 1997) and therefore result in an increase in AA level in addition to DGLA, following DGLA feeding, in these tissues. The role of DGLA and AA incorporation and eicosanoid production *in vivo* will need to be further investigated to understand the role these two PUFA play and the result on inflammation and atherosclerosis.

6.3 Future perspective

6.3.1 *In vitro* assays

Data presented throughout the thesis have provided key insight into the role of DGLA in a number of aspects contributing to macrophage foam cell formation *in vitro.* This includes attenuation of monocyte migration, pro-inflammatory gene expression and accumulation of cholesteryl esters. A number of experiments could potentially be performed to expand on findings presented in the experimental chapters. Some examples have been discussed previously (Sections 3.5, 4.5, 5.5, 6.2) relating to each key finding in macrophages and are summarised and expanded below.

It was found in studies presented in Chapter 4 that DGLA inhibited the IFN-γ induced expression of key pro-inflammatory genes in THP-1 macrophages. Data presented in Chapter 5 indicated that this may be a result of the attenuation of STAT1 phosphorylation at SER^{727} preventing maximum transcriptional activity of IFN-γ inducible genes. Following on from this, the effect of DGLA could be investigated on other aspects of IFN-γ signalling. Given the action of DGLA on STAT1 a number of techniques could be used to determine the subsequent effect of the inhibition of STAT1 phosphorylation. Translocation into the nucleus could be measured using immunofluorescence. STAT1 binding to DNA may also be analysed using chromatin immuno-precipitation (ChIP) assays. In addition, BMDMs isolated from STAT1 knock-in mice, a mouse colony already present in the laboratory, could be utilised to investigate any DGLA mechanisms dependent on STAT1. This would provide further understanding into the precise mechanisms underlying DGLAs action in inflammatory processes.

Previous research has indicated that incorporation of omega-3 fatty acids EPA and DHA into cholesteryl esters can alter its physical state and increase hydrolysis and efflux of cholesterol from THP-1 macrophages (Lada *et al*. 2003). Unfortunately, due to the insufficient concentration of cholesteryl ester accumulated in unloaded THP-1 macrophages observed in experiments and in previous studies (Lada *et al*. 2003), it was not possible to carry out lipid analysis of cholesteryl esters in these cells. There was however a significant increase of DGLA incorporation into cholesteryl esters into liver and plasma lipid fractions of mice *in vivo* (Chapter 3). This indicates that DGLA is actively incorporated into steryl esters and may also accumulate in macrophages. In addition to this, data presented in Chapter 4 showed an increase in cholesterol efflux from THP-1 macrophages loaded with acLDL. Given the data presented in the thesis and previous studies (Lada *et al*. 2003) detailing the effects of PUFAs on cholesteryl ester metabolism and efflux, DGLA may play a similar role. Future work could act to determine the incorporation of DGLA into THP-1 macrophage steryl ester lipid fraction by GC, following cholesterol loading with acLDL. This could also be determined in HMDMs.

In addition to macrophages, a number of other cell types play an important role in atherosclerosis pathology, for example endothelial cells and SMCs. Endothelial dysfunction is a key early event in the initiation of atherosclerosis while SMC migration and proliferation is characteristic of advancing plaques (McLaren *et al*. 2011a; Michael *et al*. 2012). Future work could include the effect of DGLA on key events related to atherosclerosis in these two cell types. The effect of DGLA on endothelial dysfunction could be analysed by techniques measuring cell viability, apoptosis, ROS and NO production and expression of pro-inflammatory markers. For SMCs, the effect of DGLA on proliferation could be measured by incorporation of bromo-2'-deoxyuridine which is incorporated into cells during cell division. In addition, migration could be measured using an *in vitro* scratch assay (Liang *et al.* 2007). This would allow for a better understanding of the wider role of DGLA in atherosclerosis pathology.

6.3.2 *In vivo* experiments

In vivo studies presented in Chapter 3 were used to analyse the uptake of DGLA isolated from an algal source provided by collaborators at Ben-Gurion University, Israel. Despite the results of the experiment indicating that this novel source could be potentially credible, the study provided no investigation into the mechanistic role of DGLA *in vivo.* Data presented in experimental chapters indicate a number of beneficial anti-inflammatory and anti-atherogenic roles for DGLA in macrophages *in*

vitro. Future work will need to expand to *in vivo* models of the disease to evaluate if the mechanistic roles of DGLA are conserved. *In vivo* models of atherosclerosis can be utilised to determine if DGLA has the same effect on monocyte migration, cytokine production, pro-inflammatory gene expression and modified LDL uptake and efflux as reported in macrophage cell lines. Cytokine expression *in vivo* could be determined by the use of multiplex cytokine assays on plasma and plaques isolated from mouse models. In addition, cholesterol efflux can be determined *in vivo* following peritoneal injection of cholesterol loaded macrophages in the presence of radiolabeled cholesterol. Determining radioactivity of tracer in plasma, faeces and liver lipids would allow for measurement of cholesterol efflux (Wang *et al.* 2014; Xu *et al.* 2014a).

In vitro studies in Chapter 3 of eicosanoid production showed that supplementation with DGLA resulted in the selective increase in the production of PGE_1, with no change in PGE_2. However, previous studies *in vivo* have also suggested that DGLA supplementation leads to an increase in PGE_2 production (Umeda-Sawada *et al*. 2006; Amagai *et al*. 2015). Again, *in vivo* models could be utilised to measure eicosanoid production in response to DGLA feeding and how this impacts on atherosclerosis. Plasma levels of eicosanoids isolated from *in vivo* models could be measured by HPLC-MS.

Previous *in vivo* research with DGLA has been limited. Relatively few studies have investigated the role of DGLA *in vivo* in diseases such as atopic dermatitis, cancer and atherosclerosis. As previously described, DGLA attenuated the development of atherosclerosis in an $ApoE^{-/-}$ mouse model (Takai *et al*. 2009). Despite this beneficial insight into the role of DGLA in atherosclerosis, the study provided little mechanistic insight. For example, a limited number of inflammatory genes were measured by semi quantitative reverse transcription PCR and the study was performed at a single time point using the $ApoE^{-/-}$ mouse model, a more aggressive model of atherosclerosis (Plump et al. 1992; Ishibashi et al. 1993) . In addition there was no investigation into processes involved in foam cell formation, cytokine levels or plaque composition, stability and plaque regression.

Future work *in vivo* with DGLA could be performed to expand on the preliminary work detailed on the roles of this PUFA in atherosclerosis. The effect of DGLA on plaque morphology, regression and stabilisation could be investigated by a number of plaque morphometric and immunohistochemistry techniques. This could include determining plaque cell content by staining for cells such as macrophage, SMCs and T-cells, foam cell quantification and collagen staining (Miller et al. 2008; McLaren et al. 2010). In

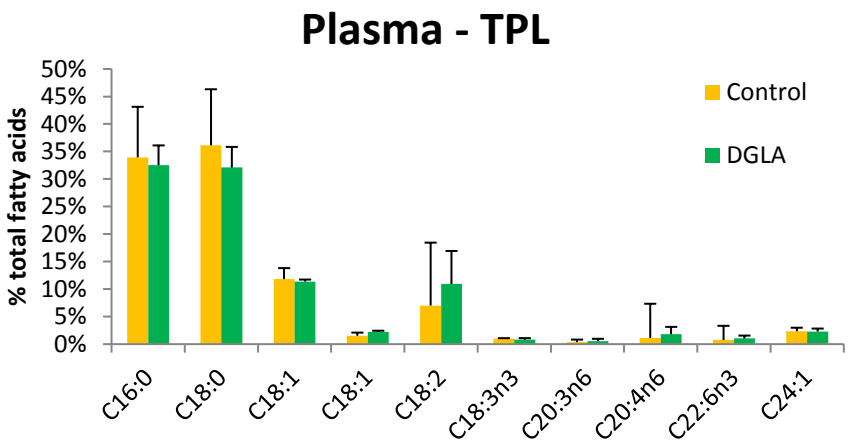
addition to this, the effect of DGLA on a number of other parameters could also be measured. Plasma lipid profiles could be analysed by TLC and GC and immune cell profiles measured by FACS. Plasma concentrations of cytokines and chemokines could also be measured *in vivo* using multiplex ELISAs or microarrays, in response to DGLA feeding.

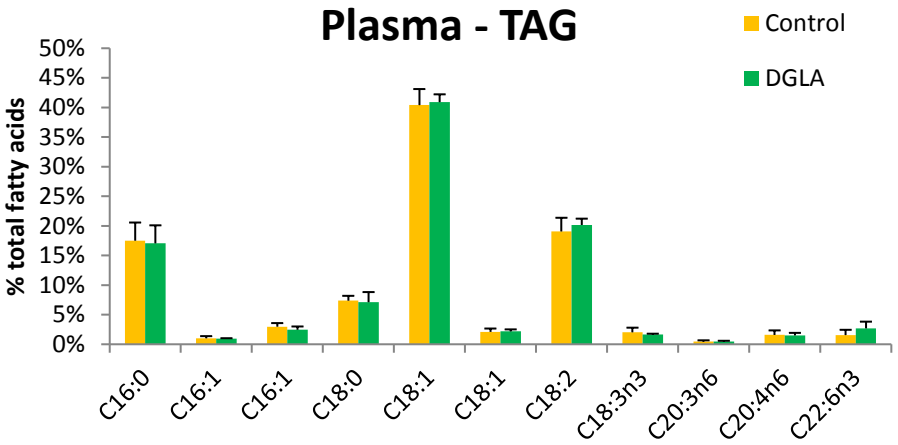
6.2 Conclusions

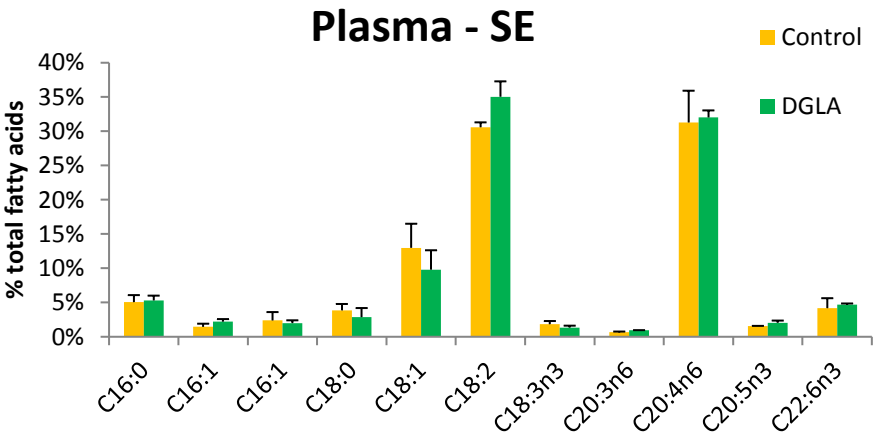
Investigation *in vitro* has indicated an anti-inflammatory and anti-atherogenic role for DGLA. Supplementation with DGLA regulates several key pro-atherosclerotic processes involved in foam cell formation, a key step in atherosclerosis. These include attenuation of monocyte migration, pro-inflammatory cytokine signalling, pro-inflammatory gene expression, modified LDL uptake through receptor-dependent and -independent mechanisms, cholesteryl ester accumulation and increasing cholesterol efflux. Supplementation with DGLA in macrophages also alters the production of eicosanoids, inducing anti-inflammatory mediators PGE_1 and 15HETrE. Metabolism of DGLA to PGE_1 in macrophages was also shown to play a key role in cytokine induced pro-inflammatory gene expression and monocyte migration and therefore maybe one mechanism in which the PUFA elicits anti-inflammatory responses. In addition, *in vitro* and *in vivo* studies showed DGLA was actively incorporated into macrophage, plasma, liver and kidney lipid fractions respectively.

The data presented in the thesis complements previous limited *in vitro* and *in vivo* studies carried out with DGLA. Taken together, they indicate a positive role for the PUFA in the inhibition of foam cell formation and prevention of atherosclerosis. However future work will need to be performed *in vivo* to further understand the mechanistic role and potential use of DGLA as a nutraceutical in the prevention and treatment of atherosclerosis.

APPENDIX

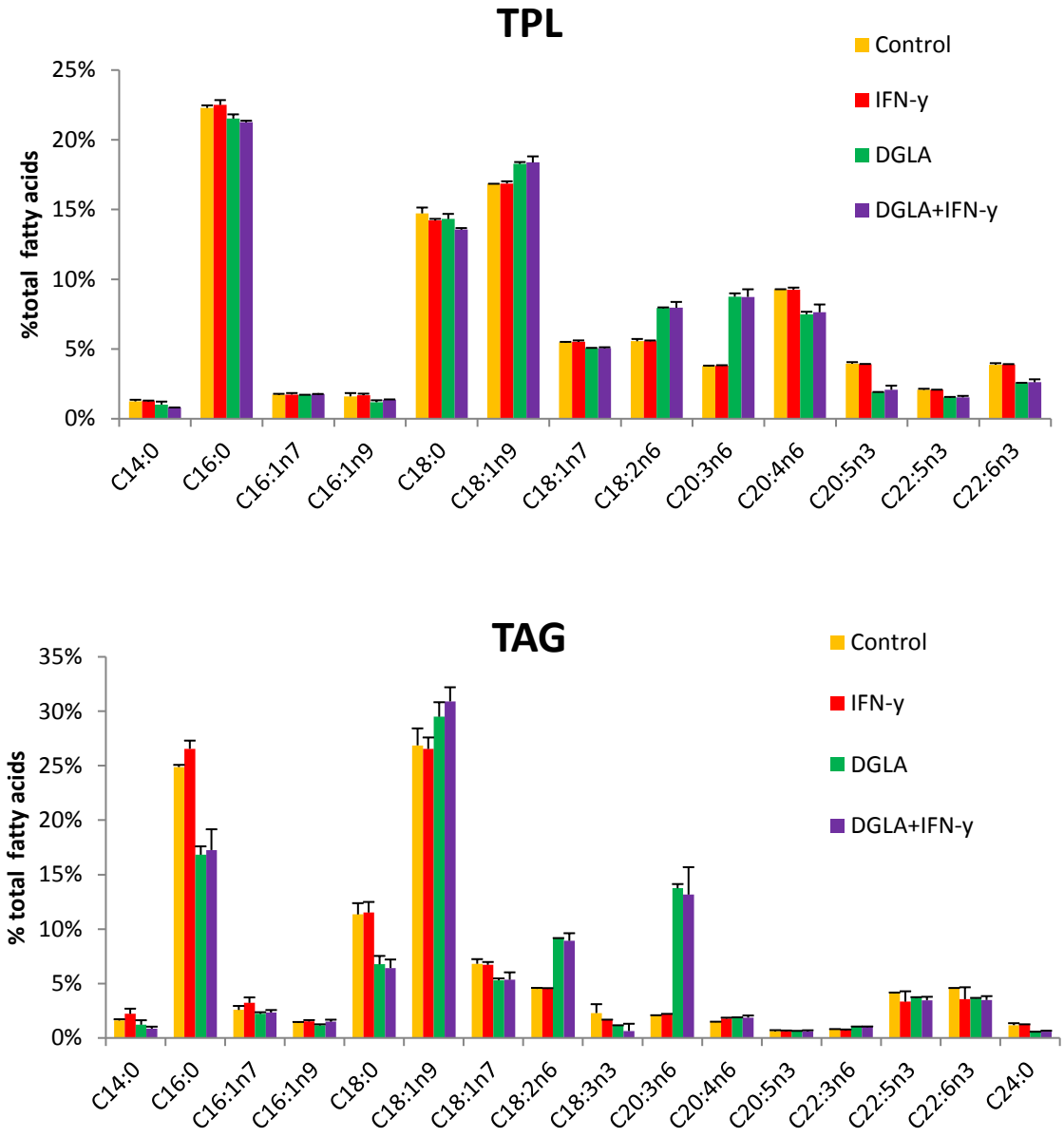






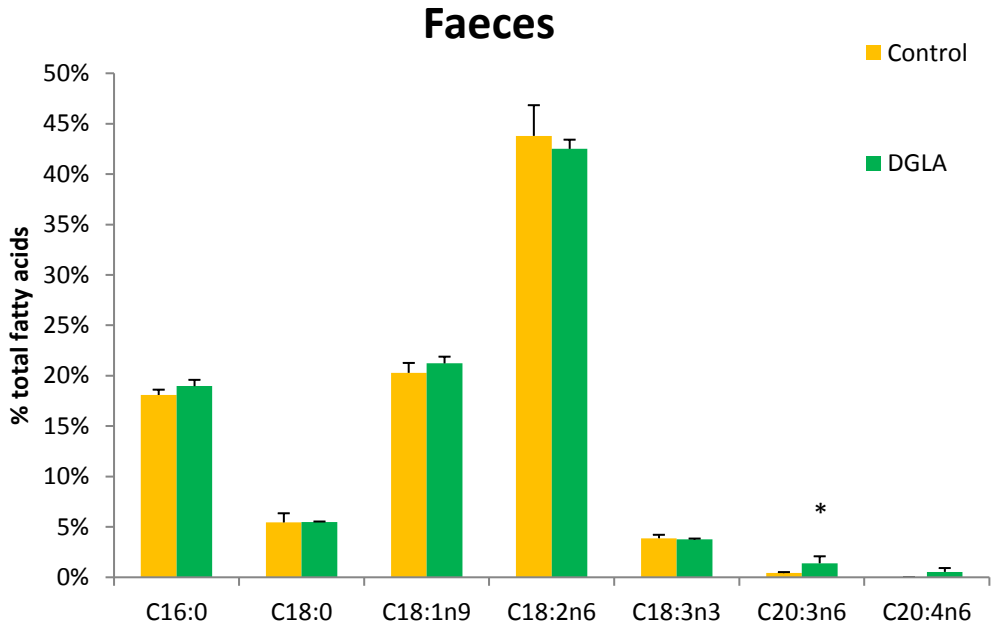
Appendix 1 – Uptake of DGLA into plasma lipid fractions *in vivo* with algal powder

Mice were supplemented with 2% (w/w) algal powder (containing approximately 30% DGLA) into diet for 48 hours. Animals were sacrificed and plasma fraction of blood collected. Lipids were extracted and separated using 1-dimensional TLC. Fatty acid profiles of each lipid fraction were analysed by GC. Graphs display average fatty acid composition as a percentage (+ SD) of 3 mice (Control) and 3 mice (DGLA diet). Statistical analysis was performed using a one-way ANOVA followed by Tukey's post hoc analysis. No significant changes were observed. C16:0, palmitic acid; C16:1n7, palmitoleic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, *cis*-vaccenic acid; C18:2n6, LA; C18:3n3, ALA; C20:3n6, DGLA; C20:4n6, AA; C20:5n3, EPA; C22:6n3, DHA;



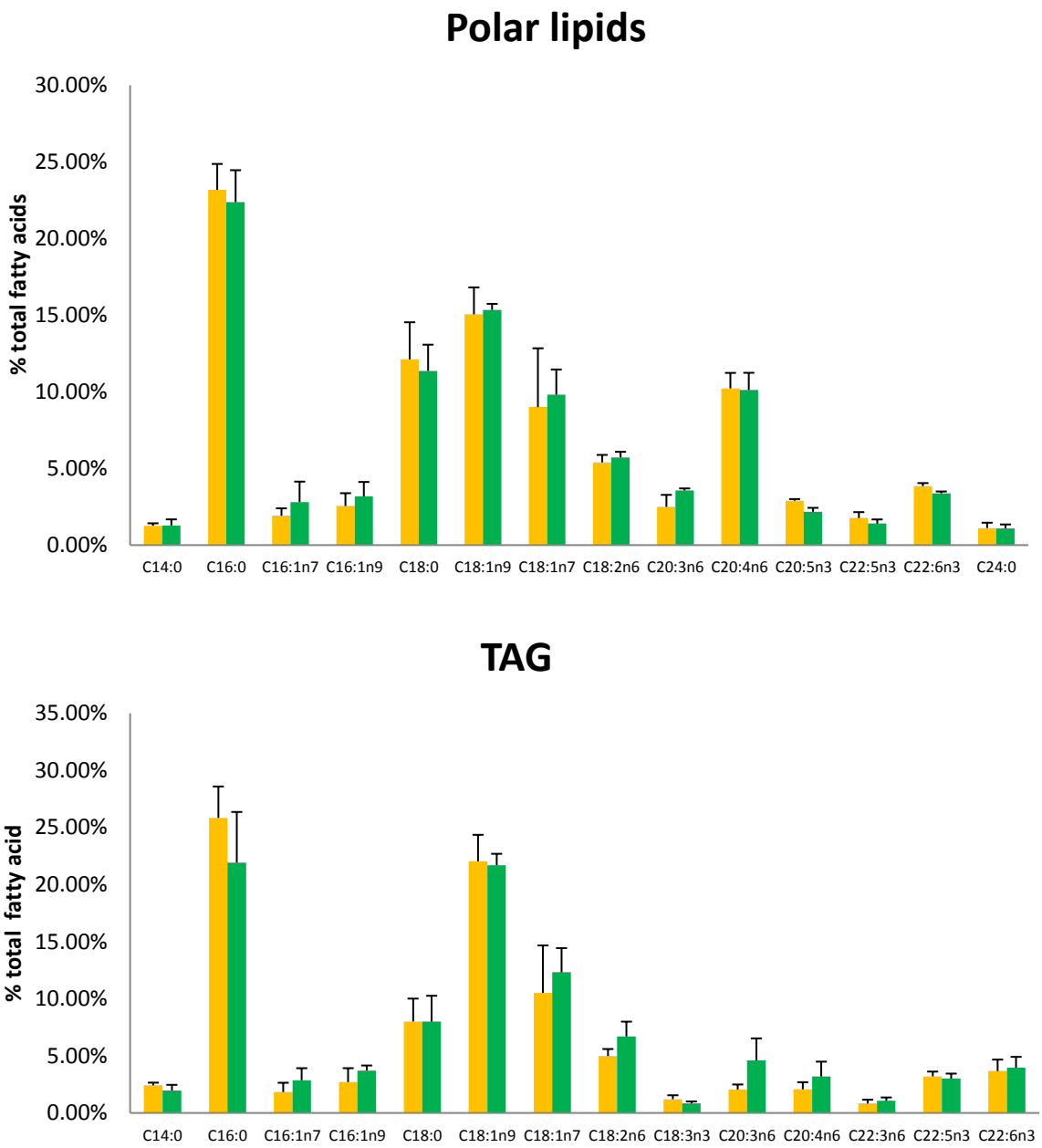
Appendix 2 – Stimulation with IFN-γ has no effect on AA incorporation in THP-1 macrophage TPL and TAG fractions

THP-1 macrophages were pre-incubated with 100 µM DGLA or vehicle control for 24 hours followed by 250 U/ml IFN-γ or vehicle control for a further 24 hours. Lipids were extracted and separated using 1-dimensional TLC. Fatty acid profiles were determined using GC. Graphs display fatty acid composition of fraction as a percentage (+/- SD) of one experiment performed in duplicate. C14:0, myristic acid; C16:0, palmitic acid; C16:1n7, palmitoleic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, *cis*-vaccenic acid; C18:2n6, LA; C18:3n3, ALA; C20:3n6, DGLA; C20:4n6, AA; C20:5n3, EPA; C22:5n3, DPA; C22:6n3, DHA; C24:0, lignoceric acid.



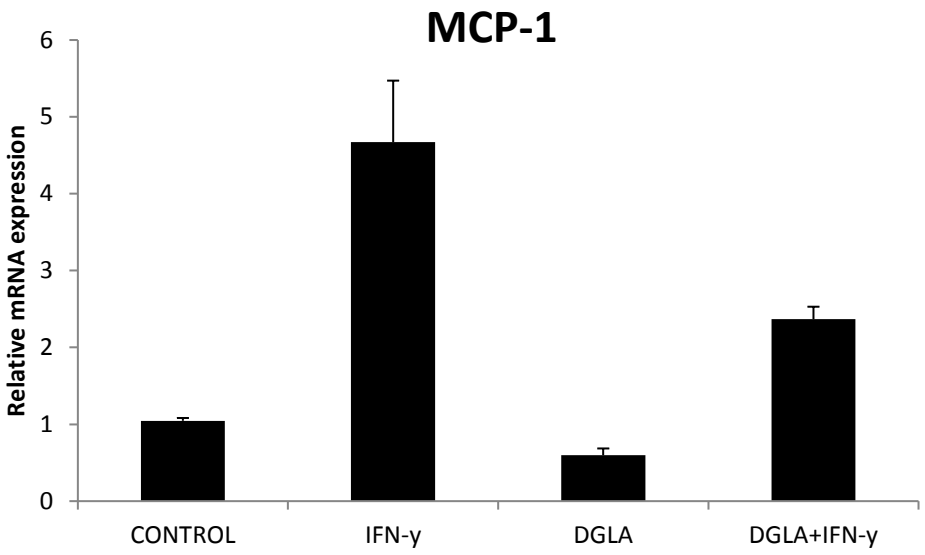
Appendix 3 – Significant increase of less than 1% in DGLA and AA in faeces following 4.4% DGLA diet

Male C57BL/6 mice were split into 2 groups; Control (3) and DGLA (4). For DGLA group, normal chow diet was supplemented with 4.4% (TFA) DGLA. Mice were starved for 24 hours prior to feeding. Mice were fed control or DGLA containing diet for 48 hours. Following this, faeces were collected, lipids extracted and analysed using GC. Statistical analysis was performed using a one-way ANOVA followed by Tukey's post hoc analysis. * $P<0.05$. ** $P<0.01$. C16:0, palmitic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:2n6, LA; C18:3n3, ALA; C20:3n6, DGLA; C20:4n6, AA.



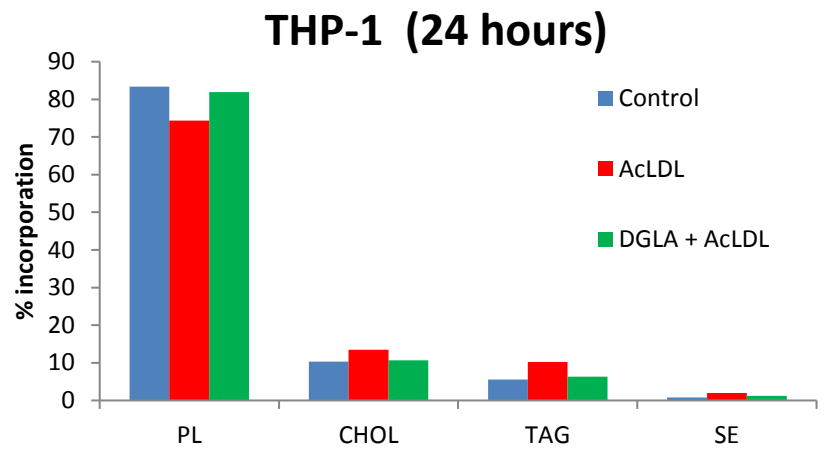
Appendix 4 – DGLA uptake into THP-1 macrophages from algal lipid extract

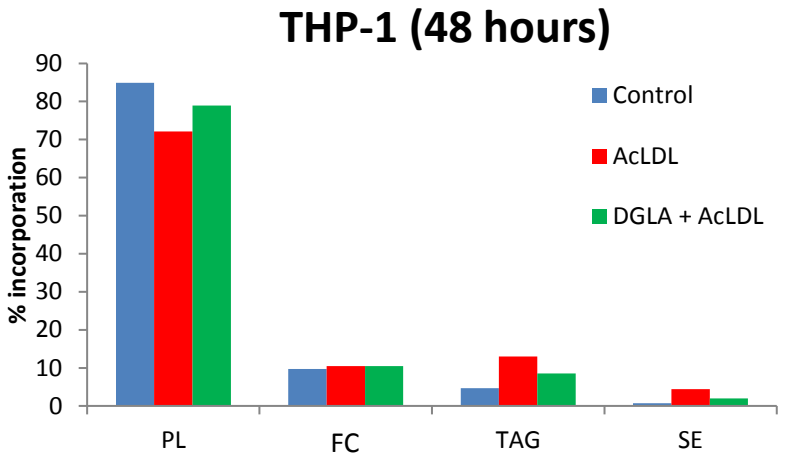
THP-1 macrophages were incubated with 100 µM total fatty acids (extract from algal powder) or vehicle control for 24 hours. Lipids were extracted and separated using 1-dimensional TLC. Fatty acid profiles were determined using GC. Graphs display fatty acid composition of fraction as an average percentage (+/- SD) from two independent experiments. C14:0, myristic acid; C16:0, palmitic acid; C16:1n7, palmitoleic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, *cis*-vaccenic acid; C18:2n6, LA; C18:3n3, ALA; C20:3n6, DGLA; C20:4n6, AA; C20:5n3, EPA; C22:5n3, DPA; C22:6n3, DHA; C24:0, lignoceric acid.

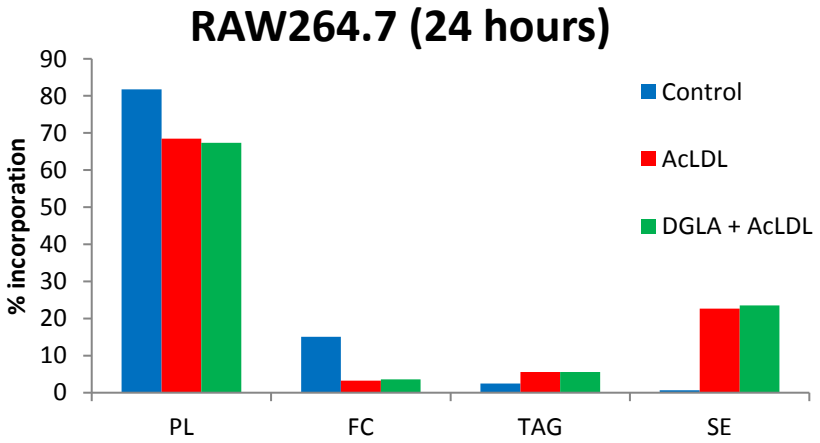


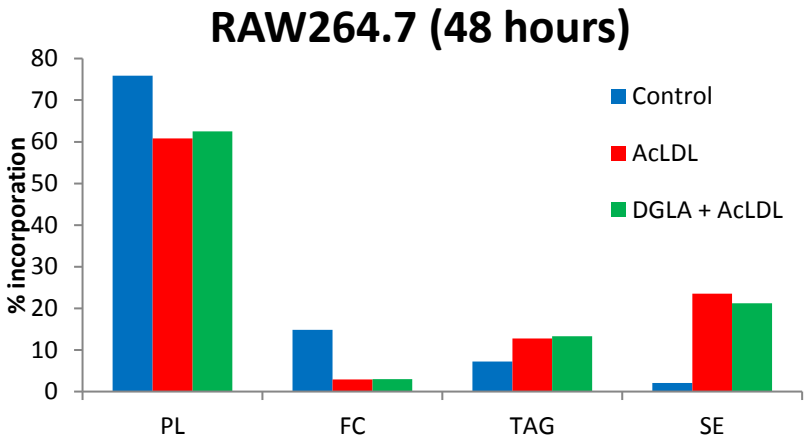
Appendix 5 – DGLA (hydrolysed from algal lipid extract) attenuated the IFN-γ induced expression of MCP-1

THP-1 macrophages were incubated with vehicle or 100 µM TFA (hydrolysed from total lipid extract of algal powder) for 24 hours prior to treatment with vehicle or 250 U/ml IFN-γ for 3 hours. Total RNA was subjected to reverse transcription and RT-qPCR with primers specific for human MCP-1 or GAPDH. Graphs display average normalised gene expression (mean +/- SD) (control arbitrarily assigned as 1) from one experiment performed in triplicate.



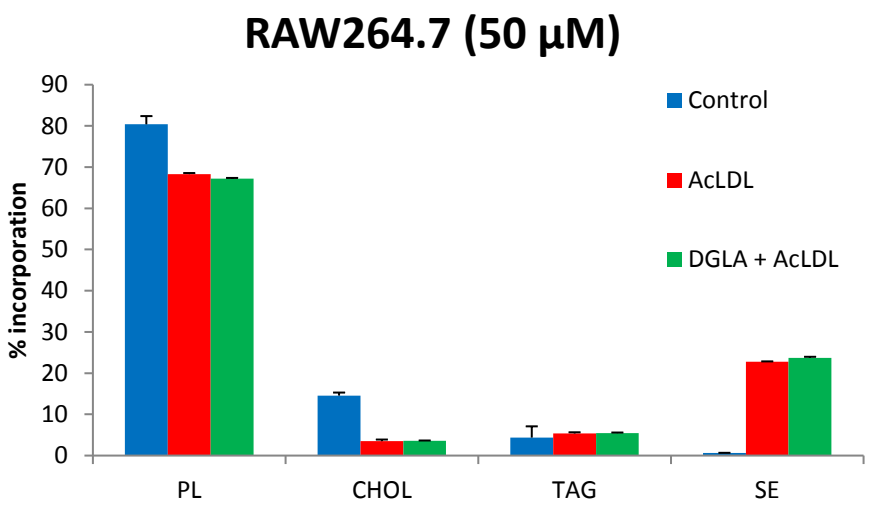


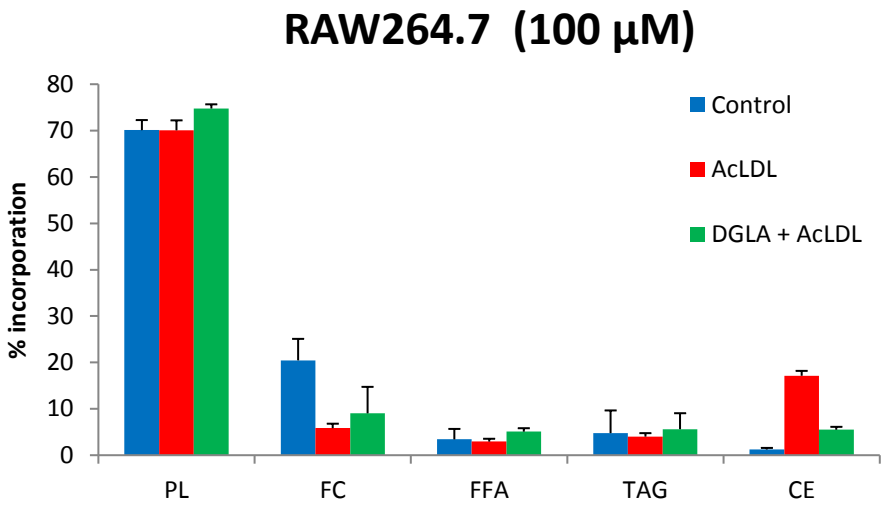




Appendix 6 – Time course in THP-1 and RAW264.7 macrophages

THP-1 and RAW246.7 macrophages were incubated with 50 μM DGLA for 24 hours and 48 hours prior to the addition of 50 μM AcLDL and 1μCi [^{14}C] acetate for a further 24 hours. Lipids were extracted, separated via TLC and radioactive incorporation into lipid fractions, identified by comparison with known standards, was measured by scintillation counting. Graphs display percentage incorporation of [^{14}C] into polar lipids (PL), free cholesterol (FC), free fatty acids (FFA), triacylglycerols (TAG) and cholesteryl esters (CE) from a single experiment





Appendix 7 – Time course in THP-1 and RAW264.7 macrophages

RAW246.7 macrophages were incubated with 50 µM or 100 µM DGLA for 24 hours prior to the addition of 50 µM AcLDL and 1µCi [^{14}C] acetate for a further 24 hours. Lipids were extracted, separated via TLC and radioactive incorporation into lipid fractions, identified by comparison with known standards, was measured by scintillation counting. Graphs display percentage incorporation of [^{14}C] into polar lipids (PL), free cholesterol (FC), free fatty acids (FFA), triacylglycerols (TAG) and cholesteryl esters (CE) from a single experiment

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