Regulation of cardiometabolic risk factors by dietary Toll-like receptor stimulants

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Abstract

Atherosclerosis is a chronic inflammatory disease of the arteries, although the causative agents for inducing inflammatory processes related to atherosclerosis remain unclear. Dietary patterns may play a role in the commencement of inflammation, which may contribute to the progression of the disease. This PhD project aimed to investigate potential mechanisms by which processed foods may induce inflammatory responses (*in vitro*, *ex vivo* and *in vivo*) and may increase cardiometabolic risk factors, with an emphasis on the potential roles of Toll-like receptor (TLR)-2 and TLR4 signaling.

The present findings establish that TLR2 and TLR4 stimulants present in food are critical mediators of the capacity of food extracts to induce inflammatory signaling in human primary monocytes. Also, chronic dietary PAMP intake in healthy volunteers revealed that a low PAMP diet decreases cardiometabolic markers (leukocyte count, body weight, abdominal circumference, LDL-C level, thrombocyte counts and plasma leptin concentrations) significantly and these effects are rapidly reversed by a high PAMP diet. In addition, murine studies suggested that Kupffer cells, rather than hepatocytes, are the main sensors of dietary TLR2- and TLR4-stimulants, and that IL-1β signaling plays a key role in the expression of the APR and metabolic phenotypes induced by ingested lipopolysaccharide (LPS) in mice. Moreover, food additives may have an adverse role in changing gut barrier function which may results in the increased translocation of dietary TLR-stimulants into the circulation.

In conclusion, these findings suggest that dietary TLR2- and TLR4-stimulants may be considered as a new possible risk factor for coronary artery disease. For the prevention of atherosclerosis, novel therapies could be developed to target the mechanisms of cross-talk between the TLR2/4 receptors, inflammatory responses and lipid regulatory pathways recognised by the current project.

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Abbreviations

$^{-/-}$	:	Gene deficiency (knock-out)
Ab	:	Antibody
ABCA1	:	ATP-binding cassette subfamily A member 1
ABCG1	:	ATP-binding cassette subfamily G member 1
ACAT1	:	Acetyl-Coenzyme A:cholesterol acetyltransferase 1
ACS	:	Acute Coronary Syndromes
APCs	:	Antigen Presenting Cells
$ApoE^{-/-}$	:	Apolipoprotein E-deficient (knock-out)
APR	:	Acute Phase Response
BHF	:	British Heart Foundation
BLP	:	Bacterial lipoprotein
BMI	:	Body Mass Index
Bp	:	Base pair
BSA	:	Bovine Serum Albumin
CAC	:	Coronary Artery Calcification
CAD	:	Coronary Artery Disease
cAMP	:	Cyclic AMP
CCL2	:	Chemokine ligand 2
CD	:	Cluster of differentiation
cDNA	:	Complementary deoxyribonucleic acid
CFU	:	Colony Forming Units
CHCl3	:	Chloroform
CHD	:	Coronary Heart Disease
CHF	:	Chronic Heart Failure
CRP	:	C - reactive protein
Ct	:	Threshold cycle
CVD	:	Cardiovascular Disease
DAPI	:	4',6-diamidino-2-phenylindole
DC	:	Dendritic Cell
DMEM	:	Dulbecco's Modified Eagle's Medium
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
DTT	:	Dithiothreitol
EC	:	Endothelial Cell
ELISA	:	Enzyme-linked immunosorbent assay
ER	:	Endoplasmic Reticulum
FBS	:	Fetal Bovine Serum
FS	:	Forward scatter
HCD	:	High Cholesterol Diet
HFD	:	High Fat Diet
HRP	:	Horseradish peroxidase
ICAM-1	:	Intercellular Adhesion Molecule-1
IFN-γ	:	Interferon-Gamma

IL	:	Interleukin
IL-1RA	:	Interleukin-1 receptor antagonist
kDa	:	Kilodalton
LAL	:	Limulus Amoebocyte Lysate
LDL	:	Low Density Lipoprotein
$LDLr^{-/-}$	:	Low density lipoprotein receptor knock-out
LFA1	:	Lymphocyte Function-Associated antigen 1
LFD	:	Low Fat Diet
LOX1	:	Lectin-like Oxidized LDL receptor 1
LPS	:	Lipopolysaccharide
LRR	:	Leucine-Rich Repeat domain
LSEC	:	Liver Sinusoid and Endothelial Cells
LXRs	:	Liver X receptors
Mac-1	:	Macrophage antigen-1
MARCO	:	Macrophage Receptor with Collagenous Structure
MCP-1	:	Monocyte chemoattractant protein-1
M-CSF	:	Macrophage Colony-Stimulating Factor
MFI	:	Median Fluorescence Intensity
MI	:	Myocardial Infarction
mm^3	:	Cubic Millimeter
MMP	:	Matrix Metalloproteinase
MRP	:	Myeloid-related protein
MTT	:	Methyl Thiazole Tetrazolium
MyD88	:	Myeloid Differentiation primary-response protein 88
MΦ	:	Macrophage
NASH	:	Non-Alcoholic Steatohepatitis
NCBI	:	National Center for Biotechnology Information
NF‑κB	:	Nuclear factor‑Kappa B
NHS	:	National Health Service
NRT	:	No Reverse Transcription
NSAID	:	Non-Steroidal Anti-Inflammatory Drug
NTC	:	No Template - Control
OD	:	Optical Density
OxPAPC	:	Oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine
PAD	:	Peripheral Arterial Disease
PAMPs	:	pathogen-associated molecular patterns
PBMCs	:	Peripheral blood mononuclear cells
PDGF	:	Platelet Derived Growth Factor
PE	:	Phycoerythrin
PEG	:	Polyethylene Glycol
PFA	:	Paraformaldehyde
PRR	:	Pattern-Recognition Receptor
PTX	:	Pentraxin
PVDF	:	Polyvinylidene Fluoride
RB	:	Reaction Buffer
RNA	:	Ribonucleic acid
RT-PCR	:	Real Time - Polymerase Chain Reaction

SAA	:	Serum Amyloid A
SCD	:	Sudden Cardiac Death
SD	:	Standard Deviation
SEM	:	Standard Error of Mean
SFA	:	Saturated Fatty Acid
SMC	:	Smooth Muscle Cell
SR-A1	:	Scavenger Receptor A1
SR-B1	:	Scavenger Receptor B1
SREC1	:	Scavenger Receptor expressed by Endothelial Cells 1
SS	:	Side Scatter
TGF-β	:	Transforming Growth Factor-βeta
Th1	:	T Helper -1
Th2	:	T Helper -2
TIR	:	Toll/Interleukin-1 Receptor domain
TLR	:	Toll-like receptor
Tm	:	Melting Temperature
TMB	:	Tetramethyl benzidine
TNF-α	:	Tumour-Necrosis Factor-Alpha
Treg	:	T regulatory
TRIF	:	TIR domain-containing adaptor inducing IFN-β
UA	:	Unstable Angina
uPAR	:	Urokinase receptor
VCAM-1	:	Vascular Cell Adhesion Molecule-1
VEGF	:	Vascular Endothelial Growth Factor
VLA4	:	Very Late Antigen 4
β-ME	:	β-mercaptoethanol

Chapter 1 Introduction

1.1. Cardiovascular disease

Cardiovascular disease (CVD) is a general term for a collection of diseases which include peripheral arterial disease, coronary artery disease (CAD), stroke and heart failure. Cardiovascular disease is currently considered to be one of the major causes of death in the world, and one of the main causes of illness worldwide (Frostegård 2013). According to the National Health Service (NHS), coronary heart disease (CHD) is responsible for more than 73,000 deaths each year in the United Kingdom (UK), and it is estimated that men are more likely to be affected with CHD than women when they are under the age of fifty, but after that they will be equally affected.

The heart is a muscle that pumps oxygen-rich blood through a network of arteries to the body organs and it returns through veins in a process known as a circulation. The heart receives its own supply of blood from blood vessels on its surface called coronary arteries. British Heart Foundation (BHF) Professor Martin Bennett at the University of Cambridge describes coronary artery disease as "*the main arteries of human's heart become blocked due to the accumulation of fatty material (atheroma) in the lining of the coronary artery wall and these fatty substances are strange to the human body which leads to inflammation*" (Bennett 2013). It is believed that human arteries attempt to remove the inflammation through repairing the tissue, forming a fibrous material above the fat which forms a plaque with time, and the whole condition is referred to as atherosclerosis. Furthermore, risk factors which might increase risk of developing cardiovascular disease include lifestyle habits, tobacco smoking, excess weight (obesity), stress, hypercholesterolemia (high cholesterol), hypertension (high blood pressure), and diabetes.

In addition, according to BHF statistics, more than a quarter of all deaths in the UK are due to cardiovascular disease. Figure 1.1 shows that over one fourth of the deaths in both male and female in the UK are caused by cardiovascular diseases, which include strokes [6% male and 8% female], coronary heart disease [15% male and 10% female] and other heart disease (Arteries, Veins, and Hypertensive diseases) [7% male and 8% female]. Furthermore, regarding the costs of cardiovascular diseases in the UK, BHF has estimated that the UK government spent 15.2 billion pounds in 2013/2014 on cardiovascular diseases

including health care, productivity losses due to mortality and morbidity, hospital treatment and prescriptions. Overall, cardiovascular diseases have a huge impact on the UK's economy and are considered to be a major public health problem.

Figure 1.1a: Deaths By Cause In Men

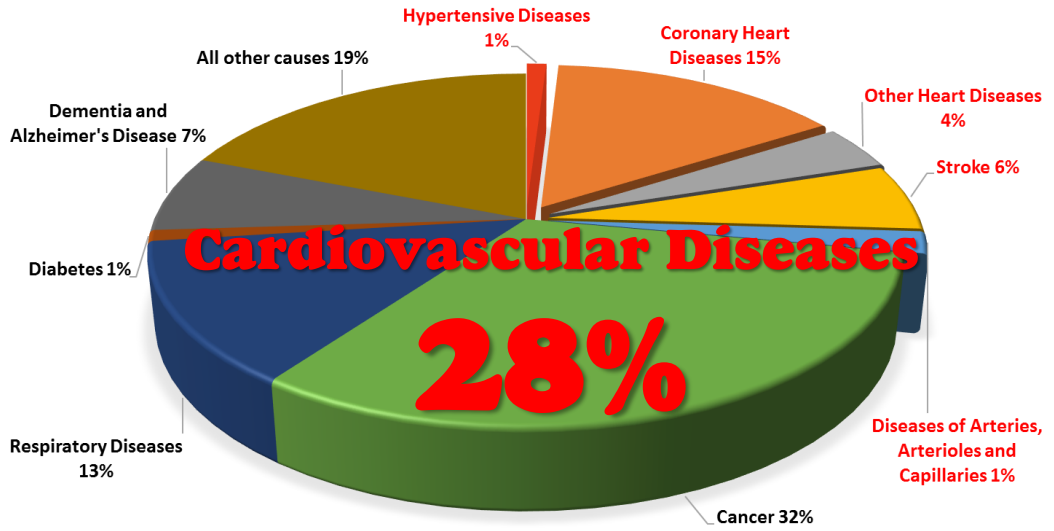


Figure 1.1b: Deaths By Cause In Women

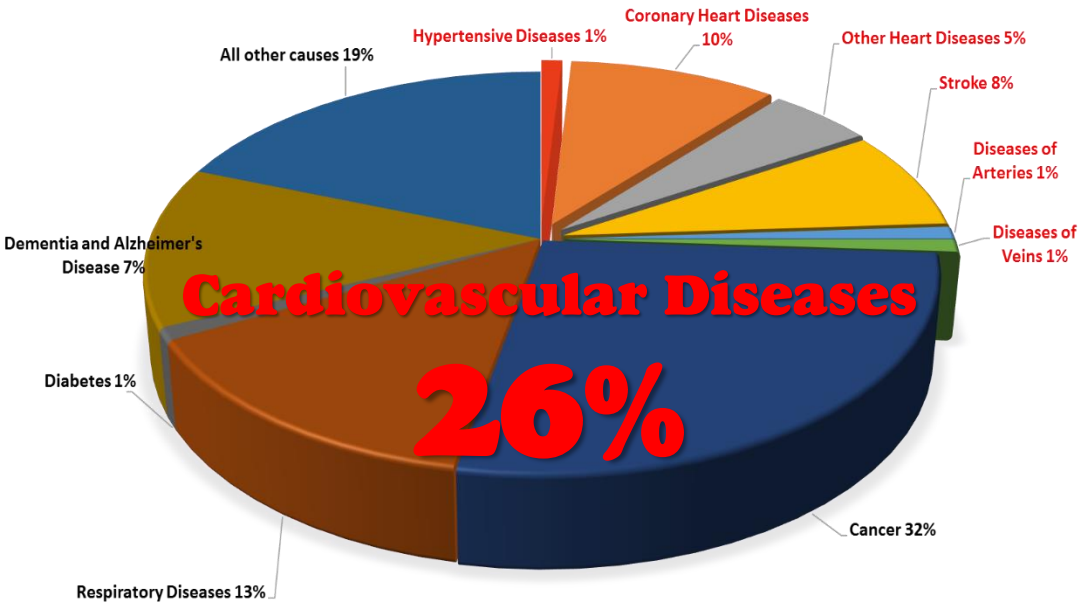


Figure 1.1: Deaths by cause in men (A) and women (B) in the United Kingdom in 2014. Adapted from (British Heart Foundation, 2015)

Deaths by cause in men and women in the United Kingdom in 2014.

1.2. What is Atherosclerosis?

Atherosclerosis is a chronic inflammatory disease, which occurs particularly in the intima (sub-endothelial space) of the large and medium sized arteries, with the activation of immune competent cells during the process of inflammation. The formation of the lesion in the arterial wall is a major precursor of atherosclerosis and this is due to cardiovascular risk factors, for instance smoking, high cholesterol, diabetes, and high blood pressure (Hansson 2006).

High concentrations of cholesterol within the blood plasma, especially within the low-density lipoprotein (LDL) fraction, play a key role in the earliest lesion formation which is known as the fatty streak, and which is considered as a common issue in early stages of human's life.

This leads to the accumulation of fatty materials within the intimal arterial layer, now referred to as atheroma, through the process of atherogenesis (Upston *et al.* 2002). Increased endothelial cell (EC) permeability to lipoproteins may result in the activation of adhesion molecules in the stressed area of arterial wall. This in turn is responsible for the adherence and migration of monocytes from the blood plasma to the intimal layer, where they become macrophages. Recruited macrophages then take up LDL within the intimal layer and form lipid laden cells referred to as foam cells, which also secrete cytokines and matrix modifying enzymes (Jan Nilsson *et al.* 2004).

After the formation of foam cells within the intima, the lesion becomes more complicated through migration of smooth muscle cells (SMCs) from the media into the intimal layer and the formation of a fibrous lesion which is typically known as a 'fibrous cap' (Frostegård 2013). The fibrous cap represents a kind of healing response to the injury that covers up the macrophages, fatty materials, and debris which produces the necrotic core and could otherwise protrude into the lumen of the arterial wall (Ross 1999). Until this stage atherosclerotic plaques are clinically silent and are commonly referred to as 'stable plaques'.

However, with the continuous building up of fatty materials, especially LDL, within the intimal layer, the plaque can become unstable and the disease could be transformed from a chronic inflammatory state to an acute clinical condition due to plaque rupture and thrombosis (the formation of a blood clot), that could lead to arterial blockage, resulting in

clinical complications such as myocardial infarction (MI) and stroke. Figure 1.2 shows the various stages of atherogenesis with the typical duration required for development of each stage. Currently, in Western communities the high mortality and morbidity rates are due to acute myocardial infarction and stroke (Oliveira *et al.* 2015). Furthermore, the prevalence of asymptomatic coronary atherosclerosis of healthy donors between the ages of 20-29 years is about 40% and those between the ages of 30-39 years are about 60% (Tuzcu *et al.* 2001).

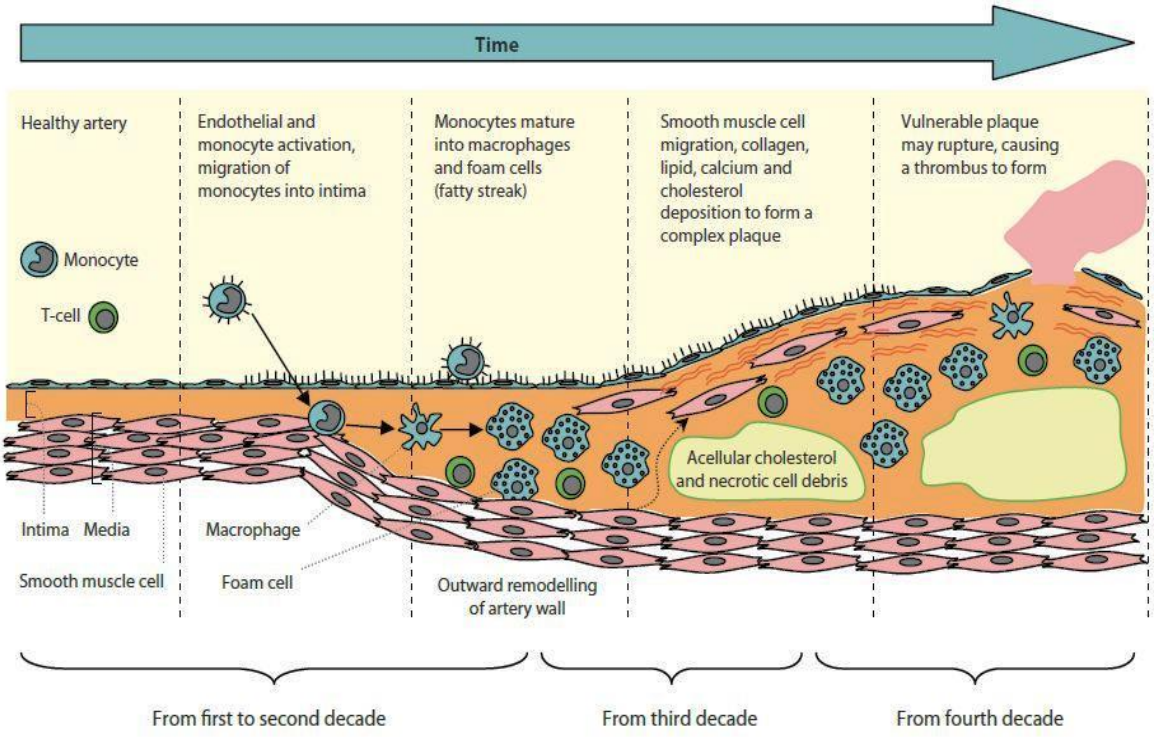


Figure 1.2: Different stages of atherosclerotic plaque formation. Taken from (Erridge Clett 2009)

Endothelial cell dysfunction due to the presence of irritants such as LDL molecules within blood is considered as a first step in lesion formation. Activation of adhesion molecules on the surface of endothelial cells leads to transmigration of blood monocytes to sub-endothelial layers. Monocytes develop into macrophages in order to scavenge LDL molecules which result in foam cell formation and release of different cytokines. With time, progression of the disease may happen and further foam cells may accumulate to increase the size of plaque. Eventually foam cells may die by necrosis creating necrotic core. Smooth muscle cells from the tunica media of the artery migrate to the stressed area and form a fibrous cap over the plaque so as to protect the plaque (in this case termed a stable plaque). The plaque may remain stable for decades but with the presence of further inflammatory events may cause the rupture of the plaque leading to the formation of a thrombus which may occlude the lumen of the artery.

1.3. Mechanisms of Atherosclerosis

Scientists and researchers in the last three decades have agreed that atherosclerosis is a condition of lipid aggregation in the arterial wall and this will increase over time until the lumen of arteries diameter narrows and the movement of blood becomes difficult. Ultimately, this may lead to serious consequences, through blocking the whole artery's lumen and resulting in myocardial infarction or stroke. Also, they recognize that the formation of the plaque is within, rather than on, the arterial wall. Researchers have furthermore identified a number of key mechanisms of plaque formation, as will now be discussed in the following sections.

1.3.1. Endothelial cell dysfunction

Inflammation contributes to all stages of atherosclerosis from commencement of the lesion to the rupture of unstable plaque by the aid of inflammatory cells, mainly monocytes/macrophages with T cells also having a key role in the process of atherogenesis. The lumen of the arterial wall is covered by endothelial cells, which prevent the adherence and penetration by leukocytes due to the expression of tight junctions between the ECs.

It is thought that irritants, for example smoking, high-fat diet, high blood pressure or diabetes, could stimulate ECs to begin expressing adhesion molecules (Poole JC 1958). Elevated concentrations of low density lipoprotein (LDL), may also stress the endothelial cell to express adhesion molecules which leads to migration of monocytes from the lumen of the artery into the intima (Sever *et al.* 2003). Adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and Intercellular Adhesion Molecule-1 (ICAM-1) will be expressed over the surface of ECs in order to capture and recruit monocytes and T cells through very late antigen 4 (VLA4) and Lymphocyte Function-Associated antigen 1 (LFA1) into the sub-endothelial layer which is known as intimal layer with the production of chemokines locally which facilitate the migration into the intimal layer (Cybulsky 1991); (Moore *et al.* 2013) (Figure 1.3).

Endothelial dysfunction may also occur in response to reduced levels of haemodynamic shear stress, which is the frictional force acting on vascular endothelial cells in the direction of blood flow. It is thought that endothelial cells perceive this reduced shear force as blood slowing, which is apparently seen as a danger signal, thereby resulting in increased

expression of inflammatory markers and the consequent acceleration of atherosclerosis (Heo *et al*. 2014).

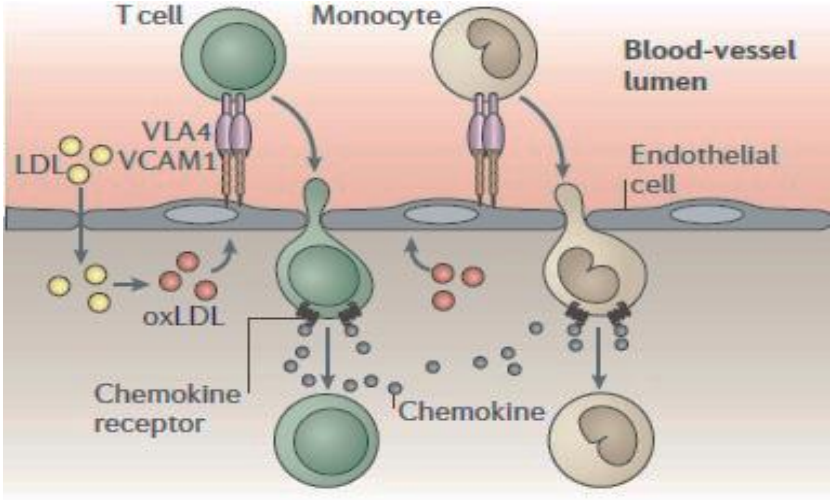


Figure 1.3: Lesion commencement which results in migration of Monocytes and T cells into the sub-endothelial layer. Taken from (Hansson 2006)

The presence of irritants (LDL particles) in blood may cause lesion formation in endothelial cells which covers the lumen of the arteries. Lesion initiation increases endothelial permeability resulting in excessive aggregation of LDL molecules within intimal layer. In response to high concentration of LDL particles in the sub-endothelial layer, endothelial cells might express adhesion molecules (VCAM-1 and ICAM-1) so as to capture and transport monocytes and T cells into the intimal layer through VLA4 and LFA1.

1.3.2. Fatty-Streak formation

The progression of the disease will continue after the dysfunction of the endothelial cells due to the stimulants such as LDL molecules and the expression of adhesion molecules over the surface of ECs which have a big role in the migration of monocytes and T lymphocytes in to the intimal layer. The process of migration and accumulation of monocytes into the intimal layer will be supported by the presence of chemoattractant molecules for instance monocyte chemoattractant protein (MCP-1) that recruit monocytes into the stressed area and this is the beginning of immune cells accumulation (Libby 2006).

After the recruitment of monocytes into the sub-endothelial layer, the monocytes will begin to mature and they will be transformed to produce macrophages (MΦ). Macrophage colony-stimulating factor (M-CSF) is the mediator which has a crucial role in the

transformation of monocyte to macrophage within the intimal layer (Clinton *et al.* 1992); (Rosenfeld *et al.* 1992). Consequently, the macrophages will release different cytokines, and this will have a role in boosting pro-inflammatory signals. Then, macrophages initialize the process of engulfment of LDL molecules due to the expression of many pattern-recognition receptors (PRRs), such as scavenger receptors which may promote foam cell formation. The whole process of monocyte recruitment and the formation of foam cells within the intima results in the development of the earliest form of the lesion, which is referred to as a fatty streak (Figure 1.4).

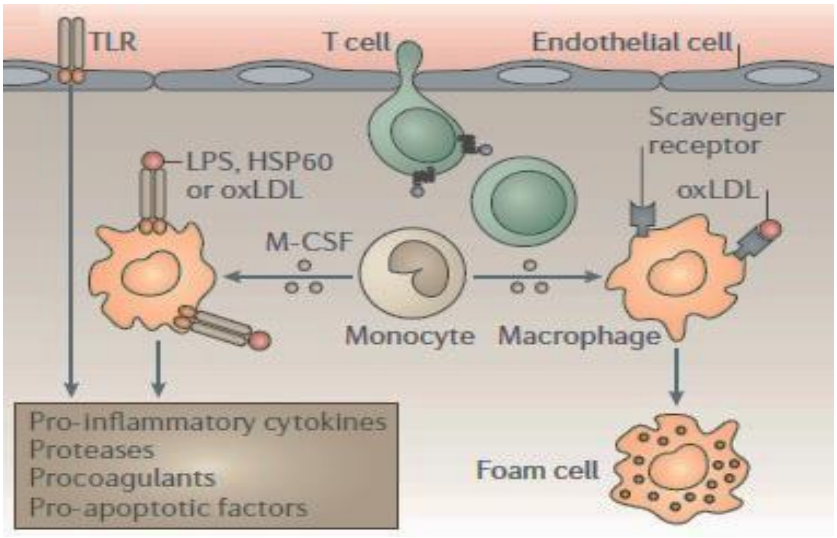


Figure 1.4: Differentiation of monocytes into macrophages and foam cell formation. Taken from (Hansson 2006)

Migration of monocytes and T lymphocytes into intimal layer may happen due to excessive accumulation of LDL particles. Monocytes differentiated to macrophages by M-CSF initiate engulfment of LDL particles through surface receptors (SR-A1, CD36 and TLRs), which results in foam cell formation with the induction of inflammatory cytokines.

1.3.3. Stable Plaque

Foam cell formation through up taking the LDL particles by macrophages, is typically followed by the formation of an overlying layer of collagen and smooth muscle cells, resulting in a lesion referred to as a fibrous, or stable plaque. With time the number of foam cells will amplify as cells take up more lipid particles within the intima. Later these cells may die due to necrosis, leaving a mass of cell debris and lipid molecules referred to as a 'necrotic core', and this promotes the migration of smooth muscle cells (SMCs).

Figure 1.5 shows the protective role SMCs play by migrating over the necrotic core and covering it like a cap, which may protrude into the lumen, finally producing a thick layer of fibrous cap (Spagnoli *et al.* 2007).

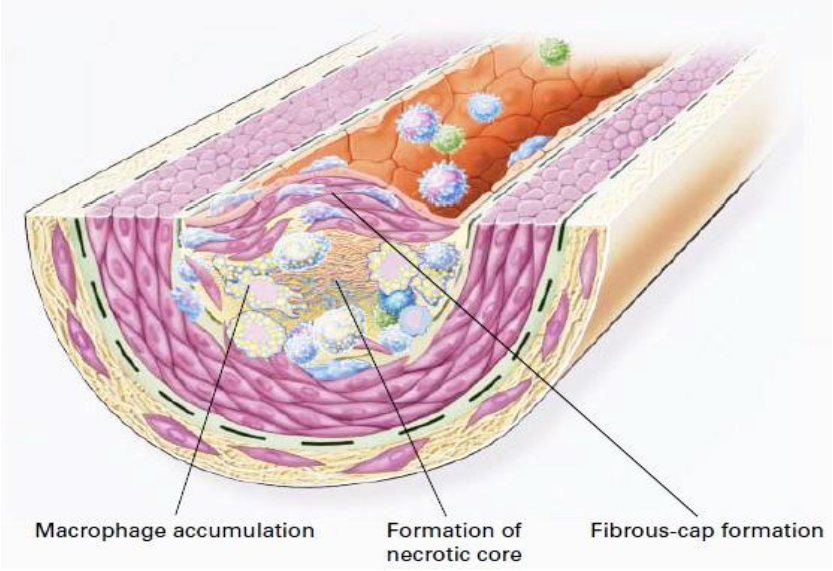


Figure 1.5: Fibrous cap formation. Taken from (Ross 1999)

Continuous aggregation of foam cells in the intimal layers and consequently cell death by necrosis results in formation of the necrotic core, a phenomenon which leads to an increase in the plaque size and protrude into the lumen of the arteries. In addition, smooth muscle cells from tunica media of the arteries may migrate and produce a protective cap over the necrotic core which is known as a fibrous cap.

1.3.4. Unstable Plaque

Stable plaques have a protective fibrous cap, which protects the lesion from any disruption. However, with time continued macrophage influx into the advanced lesion may lead to the over expression of pro-inflammatory mediators and proteolytic enzymes, consequently the stable plaque may convert to an unstable, vulnerable plaque which is at high risk of resulting in thrombus formation and arterial occlusion.

The reason behind this phenomenon is firstly, due to the production of matrix-degrading proteases such as matrix metalloproteinase (MMP) family, especially (MMP-1, MMP-8 and MMP-13), by activated macrophages, which have a direct role in collagen degradation (Dollery 2006). Secondly, the production of pro-inflammatory cytokines by Th1 cells for instance interferon-γ and tumor-necrosis factor through the interaction with CD40 on the macrophages, which decrease smooth muscle cell proliferation and inhibit the production of collagen (Amento et al. 1991); (Hansson et al. 1989). Finally, these factors may lead to plaque rupture, thrombosis and complete blockage of the coronary arteries (Figure 1.6), which can lead to major consequences of acute coronary syndrome and myocardial infarction (Falk E et al. 1996).

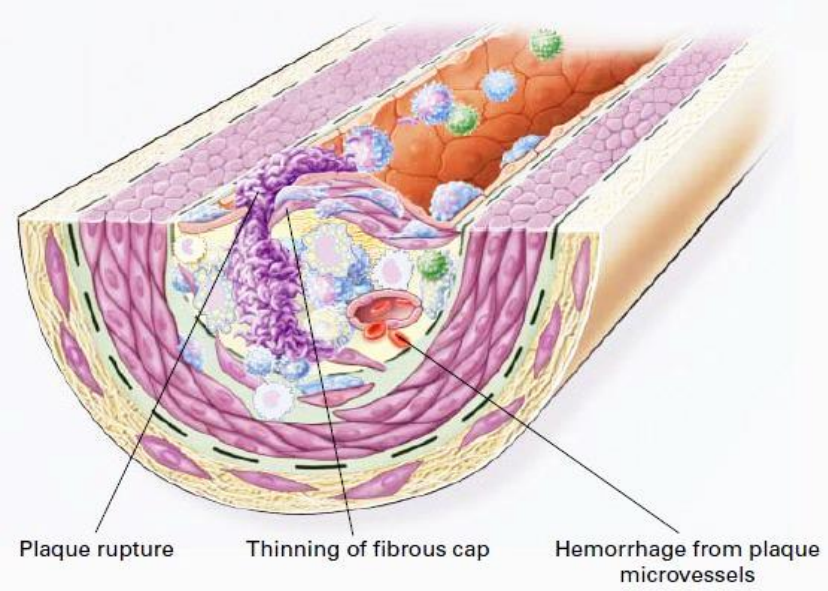


Figure 1.6: Advanced lesion and thrombosis. Taken from (Ross 1999)

Formation of unstable plaque may depend on the continuous migration of macrophages into the stressed part of the artery and formation of foam cells. In addition, the interplay between macrophages and T lymphocytes may cause the secretion of pro-inflammatory cytokines which have a vital role in the progression of the condition and production of matrix-degrading proteases such as MMP by macrophages which have a crucial role in collagen degradation. These events within the plaque may lead to plaque rupture and thrombosis.

1.4. Macrophages in Atherosclerosis

The starting point of atherogenesis is thought to be the hyper accumulation of lipid particles, especially LDLs, within the blood (dysregulation of lipid metabolism). This leads to the immune response through recruitment and migration of monocytes from the blood into the stressed area (intima), the monocytes will differentiate to macrophage cells in order to scavenge the excessive amount of LDLs, resulting in foam cell generation (Moore, Tabas 2011).

After immune system activation, various kinds of cells participate in the process of atherosclerosis including monocytes, dendritic cells (DCs), mast cells, endothelial cells and smooth muscle cells, but macrophage have a crucial role in the secretion of pro-inflammatory cytokines, matrix degrading enzymes and the formation of necrotic core as a

result of cell death (necrosis) and thus are major contributors to the formation of atherosclerotic plaque and the progression of the disease (Randolph 2008).

On the other hand, the presence of immune cells in the area where the lesion is formed may have a role in reducing the progression of atherosclerosis and minimizing the inflammation in that part, by contributing to a healing process (defensive response) which may retard the development of atherogenesis (Nathan, Ding 2010). Furthermore, other studies support the idea that macrophages might have a role in regression of the plaque development and the process of macrophage aggregation could be reversed (Feig *et al.* 2011); (Llodrá *et al.* 2004).

Studies have shown that most adult tissue macrophages originate during embryonic development and not from circulating monocytes (Slava *et al.* 2014). However, a certain proportion of macrophages in many tissues, particularly in atherosclerotic plaques, can be derived from blood monocytes of either the $Ly6C^+$ (inflammatory) or $Ly6C^-$ (tissue repair) sub-classes (Slava *et al.* 2014). Following differentiation, macrophages can polarise to adopt many different phenotypes, the classification of which is currently much debated. However, the most widely discussed phenotypes are the M1 and M2 polarisations, which reflect the inflammatory and anti-inflammatory / tissue repair phenotypes, respectively. Others include the M_{hem}, M_{ox} and M4 polarisations (Chinetti-Gbaguidi *et al*. 2015). Broadly speaking, it is considered that M1 macrophages are more pro-atherogenic than M2 macrophages, which tend to promote plaque stability (Chinetti-Gbaguidi *et al*. 2015).

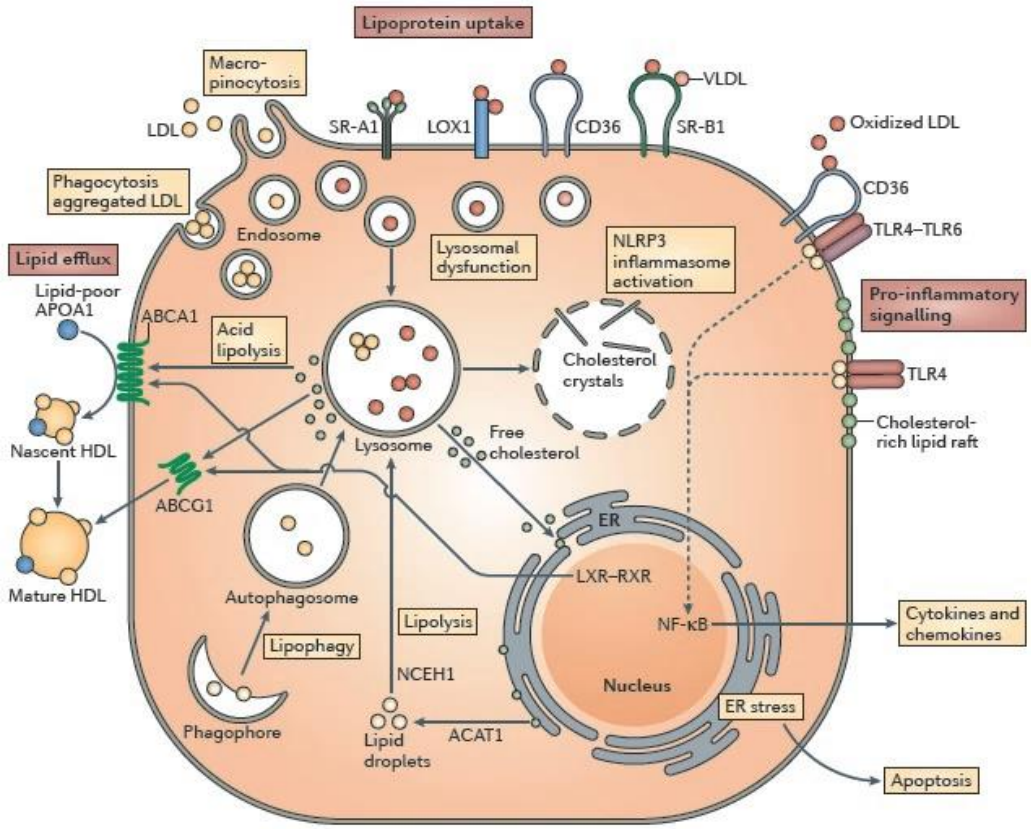


Figure 1.7: Lipoprotein uptake and efflux by macrophages. Taken from (Moore *et al*, 2013)

This figure describes the processes of lipid metabolism in macrophages. Excessive accumulation of LDL particles within intimal layer resulting in differentiation of monocytes into macrophages so as to scavenge LDL particles via surface receptors on macrophages such as SR-A1, SR-B1, LOX1, CD36 and TLRs. Foam cells are formed due to aggregation of high concentration of lipoproteins within Mϕ and triggering of inflammatory processes through activating TLRs, but presence of transporters within the Mϕ such as ABCA1 and ABCG1 which have a role in lipid efflux to outside of the cell to form lipid-poor APOA1 or directly to form mature HDL.

1.4.1. Lipoprotein uptake and foam cell formation

Accumulation of high concentration of lipoproteins, particularly LDL, within the intima will provoke macrophages to scavenge the excess LDLs (Figure 1.7). This process is achieved with the aid of scavenger receptors which are expressed on the macrophage cell surface and have a critical role in identifying then up taking LDL particles in atherosclerosis (Moore, Freeman 2006).

The scavenger receptors are pattern recognition receptors and include, scavenger receptor A1 (SR-A1; encoded by MSR1), scavenger receptor B1 (SR-B1), CD36 (also known as platelet glycoprotein 4), macrophage receptor with collagenous structure (MARCO; also known as SR-A2), lectin-like oxidized LDL receptor 1 (LOX1), and scavenger receptor expressed by endothelial cells 1 (SREC1) (Kzhyshkowska *et al.* 2012). However, SR-A1 and CD36 are thought to contribute about 75-90% in the process of lipoprotein uptake by the macrophages (Kunjathoor *et al.* 2002). Later, within the macrophage cell due to the endolysosomal action the cholesteryl esters of the lipoproteins will undergo hydrolysis and the result will be free cholesterol and fatty acids. With the presence of acetyl-coenzyme A:cholesterol acetyltransferase 1 (ACAT1) the free cholesterol will undergo re-esterification which leads to the formation of cholesteryl fatty acid esters again, which accumulate in cytosolic lipid droplets to give the 'foamy' appearance of the foam cells (Maxfield, Tabas 2005).

Increasing the excessive cholesterol uptake by the macrophages will lead to the accumulation of free cholesterol (which could be toxic for the cells) in the ER, and this will adversely affect on the process of re-esterification of free cholesterol by the ACAT1. This could result in triggering of inflammatory process through activating Toll-like receptor (TLR) then nuclear factor-κB (NF-κB) (Yvan-Charvet *et al.* 2008) and (Zhu X. *et al*. 2010).

Abnormality in lipid metabolism within the macrophage results in ER stress, and if this continues for a long period it may lead to the apoptosis of the macrophages (Feng B. *et al.* 2003). Surrounding macrophages will start uptake of the dead cells by a process known as efferocytosis, then with continuing defect of macrophage lipid metabolism and aggregation of apoptotic cells in the stressed area, this will result in secondary necrosis, which leads to the release of the whole content of the macrophages including the lipid particles, and finally the outcome will be necrotic core formation, which is considered as an obvious sign of advanced plaque formation (Tabas 2005).

Although the capacity of each macrophage subtype to take up lipids in the context of atherosclerosis is much debated, it is commonly held that while M1 macrophages readily accumulate lipids of lipoprotein origin, M2 and M_{hem} macrophages are relatively resistant to lipid accumulation through this route (Chinetti-Gbaguidi *et al*. 2015). M1 macrophage polarisation has also been shown to result in *de novo* lipid synthesis *in vitro* (Nicolaou *et al*. 2012).

1.4.2. Lipid efflux and regression in plaque formation

In addition to the presence of the scavenger receptors on the macrophage cells that have an important role in lipoprotein uptake, there are a number of transporters within the macrophage cells which have a role in lipid efflux to outside of the cell (Figure 1.7). These include, ATP-binding cassette subfamily A member 1 (ABCA1) and ATP-binding cassette subfamily G member 1 (ABCG1) (Yvan-Charvet *et al.* 2010). When cholesterol levels are elevated within the macrophage cells, this activates the liver X receptors (LXRs) resulting in increased expression of ABCA1 and ABCG1 in order to transport the lipid outside the macrophage cells (Anna C, Tontonoz 2012).

Efflux of cholesterol from macrophage cells directly to mature HDL outside the cell would be via ABCG1, while ABCA1 will transfer the cholesterol to lipid-poor ApoA1, which then develops into mature HDL as the process of lipid efflux continues (Spann *et al.* 2012). Furthermore, lipid efflux may also proceed by other routes such as autophagy, where the lipid particles are taken up through the process of lipophagy (Singh 2009). In this process, the lipid particle degradation will be performed by the fusion of the autophagosome with the lysosome allowing free cholesterol to be liberated to outside of macrophage cell through ABCA1 (Ouimet *et al.* 2011).

1.5. The risk factors of Atherosclerosis

Atherosclerosis is the major cause of coronary heart disease. Over the past decades many factors have been reported to be associated with atherosclerosis, including hypercholesterolemia, hypertension, diabetes, infectious agents, high-fat diet, and cigarette smoking (Figure 1.8), but among these risk factors hypercholesterolemia and hypertension are the most commonly measured risk factors (Levy *et al.* 1990).

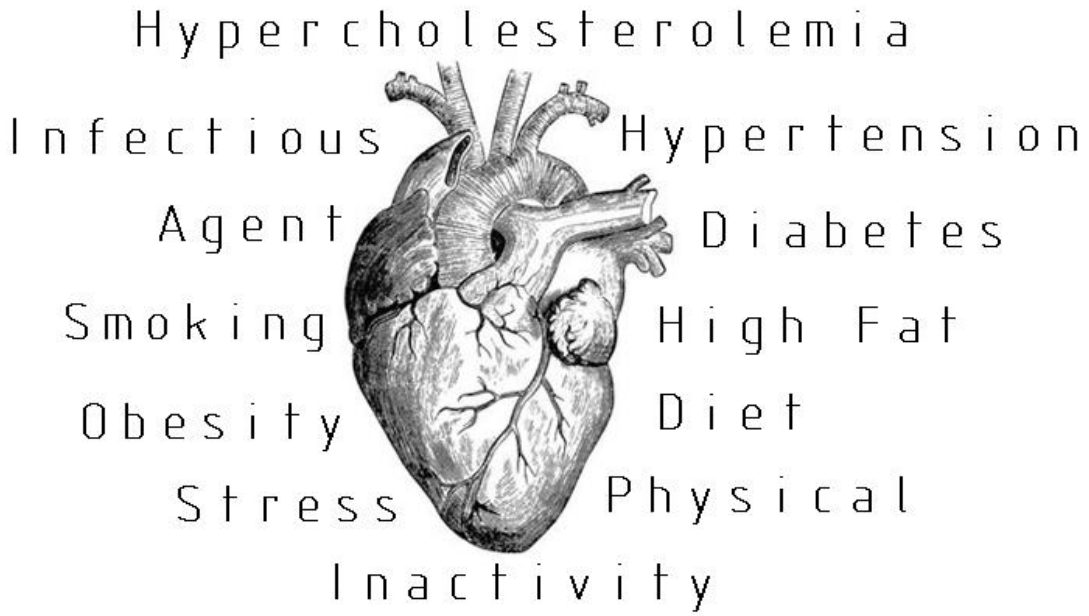


Figure 1.8: Potential risk factors for atherosclerosis.

1.5.1.Hypercholesterolemia

The presence of high concentrations of LDL-cholesterol within the blood is considered to be the major risk factor for the formation of atherosclerotic lesions (Navab *et al.* 1996); (Griendling, Alexander 1997). The endothelial dysfunction due to elevated levels of LDL in the blood may lead to the formation of lesions in the endothelial layer, as LDL particles may penetrate and accumulate within the sub-endothelial layer. Immune cells such as monocytes/macrophages will be activated because of the entry of LDL particles into the sub-endothelial layer and scavenge these particles through receptors on the surface of these cells which results in the formation of foam cells (Khoo *et al.* 1992); (Diaz *et al.* 1997).

The aggregation of foam cells within the intimal layer triggers defense mechanisms by the immune cells which release pro-inflammatory cytokines (e.g. interleukin-1 and tumor necrosis factor-alpha), proteases and pro-apoptotic factors, resulting in the initiation of an inflammatory response in the arterial wall (Han *et al.* 1997); (Falcone *et al.* 1991). Mortality rate can be reduced through LDL cholesterol-lowering treatment of patients with coronary heart diseases by low fat diet and therapy with drugs, such as statins (Assmann *et al.* 1999).

1.5.2. Hypertension

High blood pressure or hypertension is one of the principal causes of atherosclerosis in addition to hypercholesterolemia. The main reason behind elevation of blood pressure is the concentration of angiotensin II which leads to the narrowing of the blood vessels (vasoconstriction) (Chobanian AV, Dzau VJ. 1996). This stimulates the growth of smooth muscle cells and increases their intracellular calcium concentration (Gibbons *et al.* 1992).

Furthermore, hypertension participates in the process of atherogenesis, through the production of free radicals which increase leukocyte adhesion on the endothelial cells of the arterial wall (Swei *et al.* 1997). Patients with elevated blood pressure are advised to adopt a healthy diet in order to reduce excessive weight, minimise alcohol intake and reduce dietary salt intake, so as to control the high blood pressure and keep it in its normal level (Taubes 1998); (McCarron 1998).

1.5.3. Diabetes

Diabetes is a disease caused by high levels of blood glucose (hyperglycemia). Concerning the correlation between diabetes and atherosclerosis, there are epidemiological studies which support the role of hyperglycemia in accelerating the risk of atherosclerosis incidence (Kannel, McGee 1979). Firstly, high blood glucose may boost atherogenesis through NF-κB activation (Piga 2007); (S D Yan *et al.* 1994). Secondly, glucose has the ability to activate macrophage cells *in vitro*, resulting in increased expression of pro-inflammatory cytokines such as interleukin-1β and interleukin-6 (Dasu 2007). Finally, the presence of high glucose concentration might have an impact on leukocyte adherence to endothelial cells (Gregory Booth *et al.* 2001).

Some studies have criticized the idea that hyperglycemia will lead to atherosclerosis, for example, experiments on pigs with high blood glucose showed they did not develop atherosclerosis (Ross G. 2001). On the other hand, hyperlipidaemic pigs with high blood glucose showed accelerated incidence of atherosclerosis (Ross G. 2001). These results suggest that the presence of high concentration of lipid and glucose within the blood at the same time will boost the incidence of atherogenesis.

1.5.4. Infectious agents

Many studies have reported an association between atherosclerosis and the presence of infectious microorganisms (for example, *Chlamydia pneumoniae*) (Mlot 1996); (Grayston *et al.* 1995). *Chlamydia pneumoniae* is a worldwide disease and around 50% of adults have antibodies against *C. pneumoniae* (Grayston JT *et al.* 1989). *C. pneumoniae* is one of the causes of respiratory tract infection which is known as pneumonia (Grayston *et al.* 1990). Studies have reported associations between atherosclerosis and human blood levels of antibodies to *C. pneumonia* (Puolakkainen *et al.* 1993). Also, *C. pneumoniae* has been isolated from the arteries of patients with atherosclerosis, while the control arteries were free from *C. pneumoniae* (Ong *et al.* 1996); (Julio A. 1996).

Furthermore, macrophages were transformed into foam cells after infection with *C. pneumoniae in vitro* (Kalayoglu, Byrne 1998). Finally, *C. pneumoniae* increases the adherence of macrophages to endothelial cells *in vitro* during the process of atherogenesis (Takaoka *et al.* 2008).

1.6. Inflammation in Atherosclerosis

Atherosclerosis is defined as a chronic inflammatory disease of the large and medium-sized arteries (Ross 1999); (Göran K Hansson 2005). The old vision of the pathology of atherosclerosis was that it reflects simply the accumulation of lipid particles within the arteries and this with time will develop to form a thick plaque which has a vital role in occluding the arteries and limiting blood flow (Steinberg 2002).

However, in the last twenty years most of the studies on the mechanism of atherosclerosis have focused more on the immune and inflammatory response through the participation of different types of inflammatory cells such as monocyte-derived macrophages and T-lymphocytes and their ability to secrete inflammatory mediators for instance cytokines and chemokines which are now understood to play an extensive role in progression of cardiovascular diseases (Young *et al.* 2002).

1.6.1. Pro-inflammatory cytokines

The majority of cytokines are glycoproteins that may be released by various types of cells (endothelial cells, monocytes, smooth muscle cells, hepatocytes, lymphocytes and monocyte-derived macrophages) with the ability to regulate diverse biological processes, most especially those orchestrating the immune system (Boulay *et al.* 2003). Blood levels of certain pro-inflammatory cytokines are associated with risk of development of cardiovascular disease (Stoner *et al.* 2013).

It is currently thought that the most vital pro-inflammatory cytokines which participate in atherogenesis are tumor-necrosis factor-alpha, interleukin-1 and interleukin-6, the key properties of which are summarized in table 1.1.

Table 1.1: Pro-inflammatory cytokines in atherosclerosis. Adapted from (Stoner *et al.* 2013)

Pro-inflammatory Cytokines	Function	Associated with -
IL-6	▪ Induction of acute phase proteins ▪ Plaque rupture ▪ SMC proliferation	• Acute Coronary Syndromes (ACS) • Coronary Artery Disease (CAD) • Chronic Heart Failure (CHF) • Myocardial Infarction (MI) • Unstable Angina (UA)
TNF-α	▪ Leukocyte adhesion ▪ New vessel formation ▪ Atheroma	• Coronary Artery Disease • Chronic Heart Failure • Myocardial Infarction • Peripheral Arterial Disease (PAD)
IL-1	▪ EC activation ▪ SMC activation	• Coronary Artery Disease • Myocardial Infarction • Unstable Angina

Interleukin-6 is a cytokine with diverse effects. For example, it may act both as a pro-inflammatory and as an anti-inflammatory cytokine, and it is secreted by various types of cells (monocyte, lymphocyte, hepatocyte and endothelial cell) (Mahinda Y.*et al.* 2009) Early studies on mice pointed out that the level of IL-6 elevated with the development of the atherosclerotic plaque (Huber *et al.* 1999).

IL-6 also has a crucial role in the production and release of acute phase proteins (such as C - reactive protein, CRP) which are associated with cardiovascular risk, and promote the secretion of matrix metalloproteinases (MMPs) (Casas *et al.* 2008).

On the other hand, IL-6 has a role in lowering the activity of pro-inflammatory cytokines, through release of interleukin-1 receptor antagonist (IL-1RA) which is also called IL-1 inhibitor as it suppresses the inflammatory role of IL-1, and also inhibits the secretion of TNF-α (Xing *et al.* 1998); (Barton 1996). Nevertheless, in regard of atherosclerosis, IL-6 is still considered as a pro-inflammatory cytokine which is contributing to the progression of atherosclerotic plaque and the formation of unstable condition, potentially resulting in thrombus formation and the blockage of arteries lumen (Schuett *et al.* 2009).

Another key pro-inflammatory cytokine in atherosclerosis is tumor-necrosis factor-alpha (TNF-α) (Vassalli 1992), which is released by endothelial cells, macrophages and smooth muscle cells (Beutler, Cerami 1989). The main role of TNF-α in atherogenesis is the induction of expression of adhesion molecules which increases the recruitment of monocytes into the intimal layer (Meager 1999). Experiments by (Ohta *et al.* 2005) showed the differences between apolipoprotein E-deficient mice (apoE$^{-/-}$)/TNF-α$^{-/-}$ mice and apoE$^{-/-}$ mice, the lesion size was significantly smaller in apoE$^{-/-}$/TNF-α$^{-/-}$ mice when compared with apoE$^{-/-}$ mice. Levels of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were also significantly higher in the apoE$^{-/-}$ mice than apoE$^{-/-}$/TNF-α$^{-/-}$.

Together these findings support the atherogenic role of TNF-α in atherosclerosis. Furthermore, TNF-α might stimulate the expression of chemokines [such as IL-8 and Monocyte chemoattractant protein-1 (MCP-1)] at the site of inflammation, resulting in further leukocyte recruitment (Young *et al.* 2002) and (Rollins *et al.* 1990).

Interleukin-1 is also considered to be an inflammatory mediator in atherosclerosis. For example, studies using apolipoprotein E-deficient mice (apoE$^{-/-}$) and low density lipoprotein receptor knock-out mice (LDLr$^{-/-}$) receiving a high fat diet showed that blocking the activity of this cytokine by subcutaneous injection of IL-1RA resulted in reduced burden of atherosclerotic plaque (Devlin *et al.* 2002) and (Elhage *et al.* 1998). Furthermore, a study of interleukin-1β inhibition and the prevention of recurrent cardiovascular events is also currently ongoing in man (Ridker *et al.* 2011).

1.6.2. Pro-inflammatory chemokines

Pro-inflammatory chemokines (or chemotactic cytokines) are very small proteins which have structural similarity with the cytokines and are considered as a subfamily of cytokines (Zernecke 2010); (Zernecke *et al.* 2008). The main function of chemokines in atherogenesis is leukocyte recruitment into the stressed part of arteries.

Some studies reported that chemokines might also have a role in platelet activation as well as migration of smooth muscle cells into the intimal layer (Weber *et al.* 2000) and (von Hundelshausen *et al.* 2005). The chemokines most commonly associated with the development of atherosclerotic lesion are interleukin-8 and monocyte chemoattractant protein-1 (MCP-1) (Table 1.2).

Table 1.2: Pro-inflammatory chemokines in atherosclerosis. Adapted from (Stoner *et al.* 2013)

Pro-inflammatory Chemokines	Function	Associated with -
IL-8	▪ Deactivate circulating leukocytes ▪ Neutrophil recruitment	• Peripheral Arterial Disease • Coronary Artery Disease • Chronic Heart Failure • Myocardial Infarction • Unstable Angina
MCP-1	▪ Leukocyte recruitment	• Acute Coronary Syndromes • Coronary Artery Disease • Peripheral Arterial Disease • Myocardial Infarction • Unstable Angina • Sudden Cardiac Death (SCD)

The cell types most commonly associated with production of interleukin-8 are monocytes and macrophages (Remick 2005); (Apostolakis *et al.* 2009). IL-8 has a principal role in promoting the transmigration of lymphocytes and neutrophils into the lesion (Tuschil *et al.* 1992). Investigations by (Hechtman *et al.* 1991) through injecting IL-8 inhibitor in rats and rabbits resulted in a notable reduction in neutrophil recruitment into the part of artery which is suffering from inflammation.

Furthermore, studies by other group have been shown that mice genetically deficient in the IL-8 receptor demonstrated an obvious reduction in the number of the macrophages in the inflamed part of the artery and the lesion size became much smaller (Boisvert *et al.* 1998).

Monocyte chemoattractant protein-1, or chemokine ligand 2 (CCL2), is also one of the chemokines with a major contribution to the recruitment and transmigration of monocytes and macrophages to the intimal layer (Satish L. *et al.* 2010). Evidence has been shown by researchers that MCP-1 may promote atherogenesis. For example, studies using apoE$^{-/-}$ mice showed that deletion of the MCP-1 gene on this background resulted in less monocyte and macrophage recruitment into the stressed part of the artery (Gosling *et al.* 1999). Another study also reported reduced monocyte migration in to the stressed area of the artery, in mice deficient in MCP-1 on a low-density lipoprotein receptor knock-out (LDLr$^{-/-}$) genetic background (Gu *et al.* 1998).

1.7. Immune response in Atherosclerosis

During the past decade, investigations have contributed much evidence which supports the idea that inflammation has a major role in the process of atherogenesis (Libby *et al.* 2009). It is believed that both arms of the human immune response, i.e. innate and adaptive responses, are triggered as defense mechanisms against the excessive aggregation of the low-density lipoproteins in the sub-endothelial layer (intima) of the large and middle sized arteries (Figure 1.9).

Recruitment of the monocyte derived macrophage cells into the intimal layer in order to scavenge the lipid particles and secrete inflammatory mediators (cytokines and chemokines), represents the innate arm of immune response which does not rely on antigenic stimulation so as to amplify inflammatory responses. On the other hand, adaptive immune response are activated through antigen presenting cells (APCs) (macrophages, dendritic cells and endothelial cells), which present antigens to the T cells and activate them. The activated T cells have the ability to secrete cytokines which have a role in regulating the inflammatory response in the process of atherogenesis (Andersson *et al.* 2010).

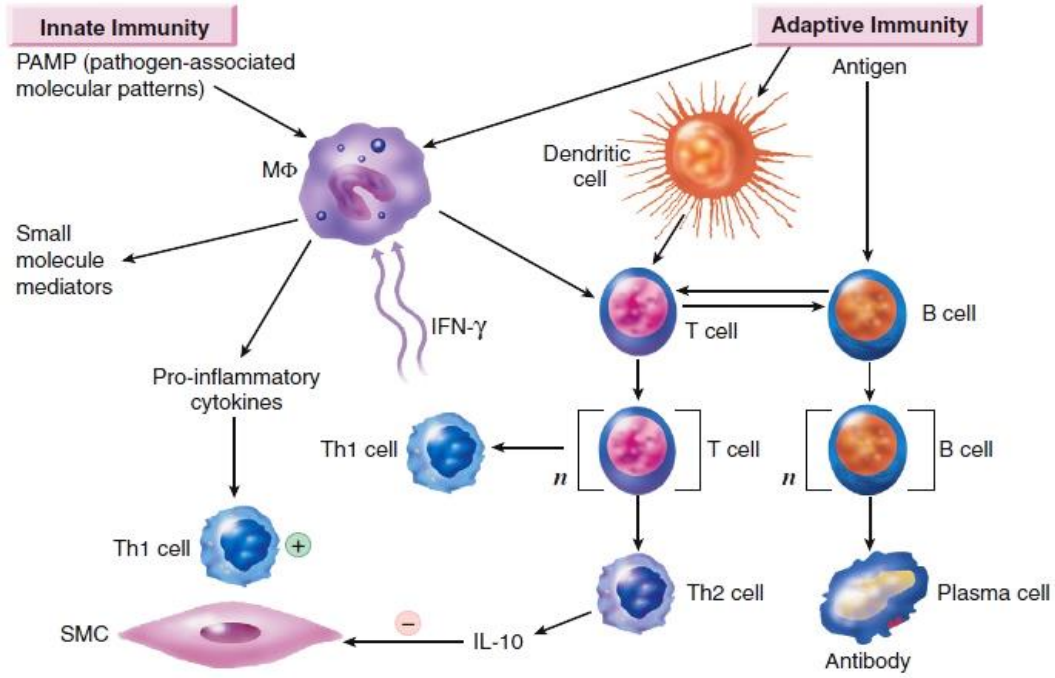


Figure 1.9: Interaction between innate and adaptive immunity during atherogenesis. Taken from (Hansson *et al.* 2002)

Human immune response as defense mechanism against excessive accumulation of LDL particles within the intimal layer may activate both innate and adaptive immunity during atherogenesis. Macrophages (innate immunity) contribute to all stages of atherogenesis (initiation, progression and thrombosis) via scavenging lipid particles and releasing inflammatory cytokines. While, T cells (adaptive immunity) become activated by the APCs after presenting antigens to them, it is believed to have a major role in thrombus formation.

1.7.1. Adaptive immunity in atherosclerosis

Several studies have shown the participation of adaptive immunity in the process of atherogenesis (Andersson *et al.* 2010); (Mallat *et al.* 2009) and (Weyand *et al.* 2008). The proportion of the T cells which are engaged in the process of plaque formation is around 10-20% of the whole cells (Jonasson *et al.* 1986), but they are more abundant during the rupture of the advanced plaque, and are therefore thought to have a major role in thrombus formation (van der Wal *et al.* 1994). Macrophages and dendritic cells have a critical role in T lymphocyte activation through presenting antigens to naïve T cells, and are therefore known as antigen presenting cells (APC). Activated T cells release various types of cytokines which may promote either the progression or regression of atherogenesis (Andersson *et al.* 2010).

T cells consist of different classes or subtypes (Figure 1.12). T-helper (Th) cells bear the CD4 surface marker and are sub-divided in to two categories: T-helper -1 (Th1) and T-helper -2 (Th2). As shown in Figure 1.10, the interaction between CD40 on macrophages with its ligand (CD40L) on Th1 cells promotes atherosclerotic lesion development through production of pro-inflammatory mediators including interferon-γ (IFNγ) and TNF, IL-2, proteases, increase adhesion-molecules expression, increase the tendency to thrombus formation, and finally decrease SMC proliferation and collagen production (U. Schönbeck *et al.* 2000). On the other hand, the severity of the plaque might be reduced by the support of Th2 cells through production of anti-inflammatory cytokines such as IL-4 and IL-13, resulting in producing a stable plaque (Ait-Oufella *et al.* 2009) (Figure 1.11).

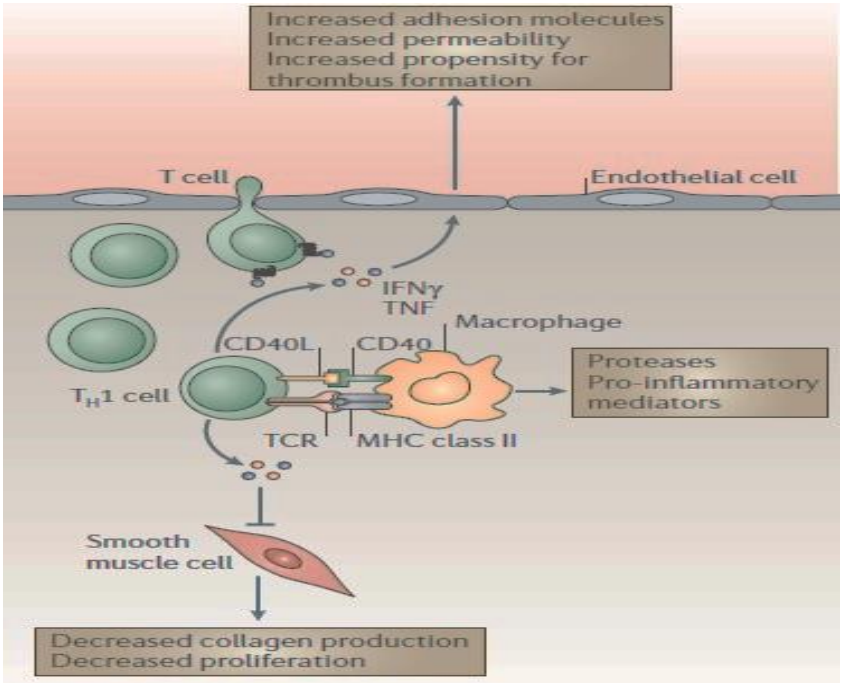


Figure 1.10: Progression of inflammation, due to the macrophages and TH1 cells interaction, which results in pro-inflammatory cytokines production. Taken from (Hansson, Libby 2006)

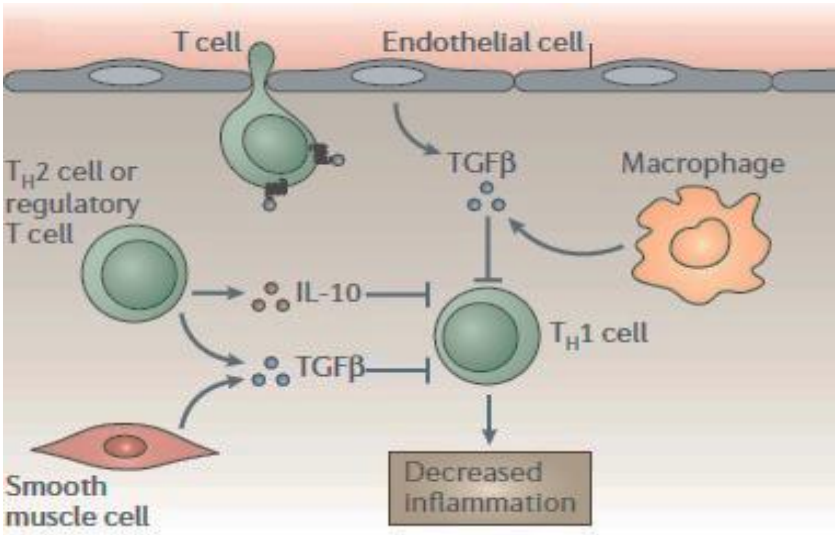


Figure 1.11: Regression of inflammation, due to the activation of TH2 and T regulatory cells, which results in anti-inflammatory cytokines production. Taken from (Hansson, Libby 2006)

Cytolytic T cells are considered as another class of T cells and they bear the CD8 surface marker. These cells have the ability to kill diverse cell types, including vascular cells involved in atherogenesis. Cytolytic T cells could have a vital role in programmed cell death (apoptosis) within the plaque, which in turn may increase the progression of the plaque (Jonasson *et al.* 1986) (Ludewig *et al.* 2000).

Furthermore, regulatory T cells (Treg) another subtype of T cells has an interesting role in suppressing the inflammatory reactions within the plaque through secreting anti-inflammatory cytokines, which includes transforming growth factor-beta (TGF-β) and interleukin-10 (Taleb *et al.* 2008) (Figure 1.11).

In addition to T lymphocytes, B lymphocytes as a humoral immune response in atherosclerosis have an essential role in reducing or attenuating the progression of atherogenesis. For example, studies showed that the removal of spleen in mice which causes the depletion of B cells and in turn results in low level of antibody production (the spleen has a role in boosting the immune system through antibody production), might lead to accelerated atherogenesis (Caligiuri *et al.* 2002). Moreover, mice with a high level of cholesterol may show a robust humoral response against the low-density lipoproteins molecules.

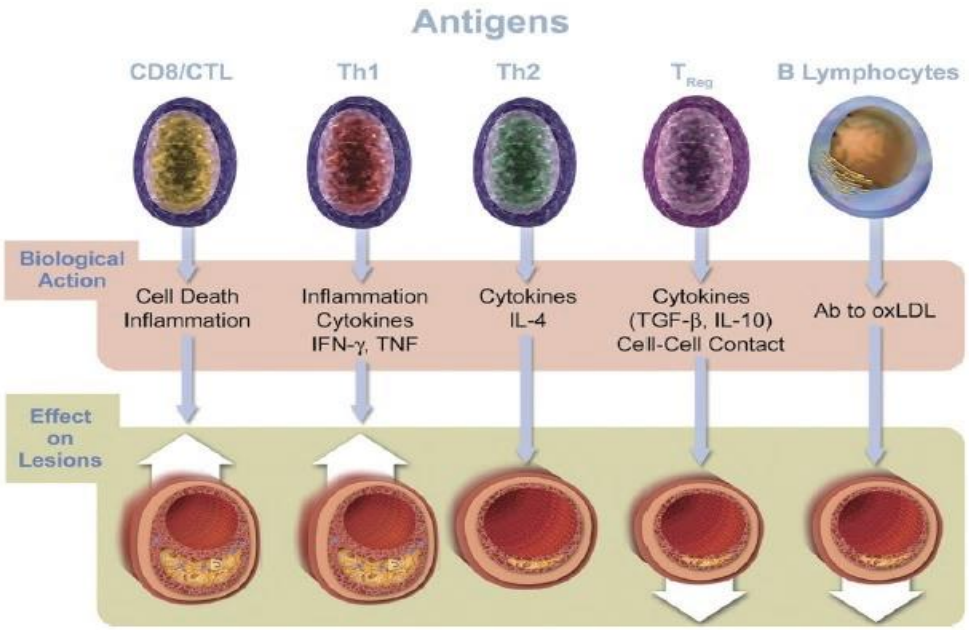


Figure 1.12: Cells of adaptive immunity participating in atherogenesis. Taken from (Libby *et al.* 2009)

This figure summarizes the biological functions of different cells involved in adaptive immunity in relation to the progression (up arrows) and regression (down arrows) of atherosclerotic plaque formation. T-helper-1 cells have a major role in releasing pro-inflammatory cytokines and Cytolytic T cells have a vital role in programmed cell death (apoptosis) of macrophages, endothelial cells and SMCs. While, T-helper-2 cells and regulatory T cells are able to express anti-inflammatory cytokines, and B lymphocytes reduce the development of atherosclerotic plaque via antibody production.

1.7.2. Innate immunity in atherosclerosis

Innate immunity is the first line of defense mechanism involved in the immediate response after the entry of invading microbes (Medzhitov, Janeway 2000). Innate immunity works by detecting highly conserved pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS) and bacterial lipoprotein (BLP) through germ-line encoded pattern recognition receptors (PRRs), such as macrophage scavenger receptors and TLRs (Janeway, Medzhitov 2002); (Krieger 1997).

In the early stages of atherogenesis different sorts of cell will be recruited into the artery (Figure 1.13), but the most common one is the monocyte/macrophage which has a principal role in promoting the secretion of pro-inflammatory mediators (cytokines) (Charo, Ransohoff 2006). Notably, hyperlipidaemic mice display elevated levels of pro-inflammatory monocytes, as supported by detection of the Ly6C or Gr-1 markers, which are thought to be similar to the human inflammatory monocyte subset (An *et al.* 2008). In addition to monocytes that have an obvious role in atherogenesis, mast cells have also been recognized in the atherosclerotic intimal layer (Kovanen 2007); (Libby, Shi 2007). Mast cells have the ability to release pro-inflammatory cytokines, for instance interleukin-6 and interferon-γ which promote atherogenesis (Sun *et al.* 2007).

Other studies have shown the role of platelets in activating innate immunity as they have the ability to secrete myeloid-related protein (MRP)-8/14 which is a pro-inflammatory mediator (Healy *et al.* 2006). However, other studies reported that the activation of innate immunity would be by linking the MRP-8/14 to the TLR4 (Vogl *et al.* 2007).

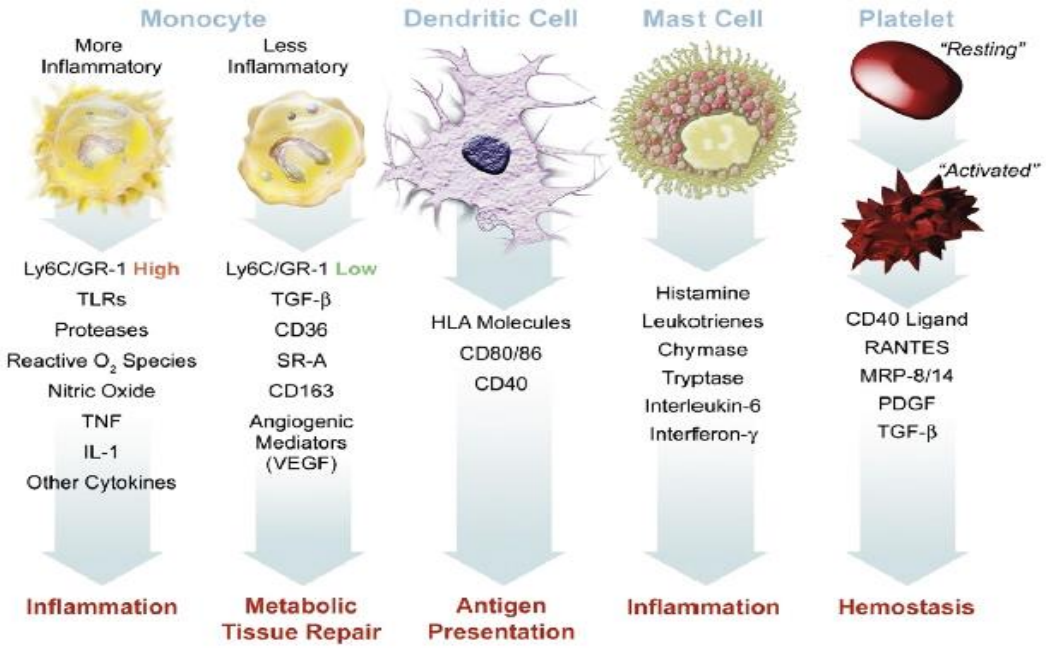


Figure 1.13: Components of innate immunity participating in atherogenesis. Taken from (Libby *et al.* 2009)

This figure shows that different cells of innate immunity may participate in the processes of atherogenesis. Monocytes as a main cell that contribute to atherosclerosis, which consist of two subsets the more inflammatory monocyte with high levels of cell surface marker Ly6C (or GR-1 in mice) which release high levels of inflammatory cytokines (TNF-α and IL-1β), while the less inflammatory monocytes with low levels of cell surface marker Ly6C, which secrete transforming growth factor-beta, scavenger receptor A and CD36. Dendritic cells as APCs which present antigen to the T-cells so as to activate them. Mast cells might have inflammatory role through elaborating inflammatory mediators such as IL-6 and Interferon-γ. Platelets have a role in stopping of bleeding, express CD40 Ligand and release mediators (TGF-β, RANTES, myeloid related protein 8/14 and platelet derived growth factor).

1.8. TLRs and atherosclerosis

Inflammatory processes support the progression of atherosclerosis and this in turn will have a role in developing various kinds of cardiovascular diseases (Falck-Hansen *et al.* 2013). As mentioned previously that different factors may have a possible role in developing or accelerating the risk of atherosclerosis such as hypercholesterolemia, hypertension (Levy *et al.* 1990) and hyperglycemia (Kannel, McGee 1979). However, evidences have been shown that innate immune mechanisms may play a crucial role in commencing and developing atherosclerotic processes, predominantly through activating or stimulating Toll-like receptors (specifically, Toll-like receptor (TLR)-2 and TLR-4) (Westerterp *et al.* 2007); (Mullick *et al.* 2005); (Kathrin S. *et al.* 2004).

Toll-like receptors are considered as a major family of pattern recognition receptors. There are ten TLRs in the human genome, and they are expressed by various kinds of immune cells (macrophages, dendritic cells, B cells, and particular types of T cells) and non-immune cells (e.g. endothelial cells and epithelial cells) (Akira *et al.* 2006); (Erridge 2008).

The presence of specific ligands of TLRs, such as lipopolysaccharide (LPS) and bacterial lipopeptide (BLP) which are recognised by TLR-4 and TLR-2 respectively, results in activation of TLR-signaling through the formation of TLR dimers, which mostly will be homodimers or heterodimers as in the case of TLR2/1 and TLR2/6 (Cole 2010); (Stewart *et al.* 2010).

Toll-like receptor signalling pathways typically propagate through myeloid differentiation primary-response protein 88 (MyD88)-dependent pathways for all TLRs as it is shown in figure 1.14, with the exception of TLR3 which utilises MyD88-independent/TIR domain-containing adaptor inducing IFN-β (TRIF)-dependent pathways (Falck-Hansen *et al.* 2013). The main structure of TLRs consist of leucine-rich repeat domain (LRR) and Toll/Interleukin-1 receptor (TIR) domain, in which the PAMP detection will be via LRR and the signal transmission will perform by the TIR domain, which binds directly to the adaptor proteins when dimerised (Takeda, Akira 2005); (Newton, Dixit 2012).

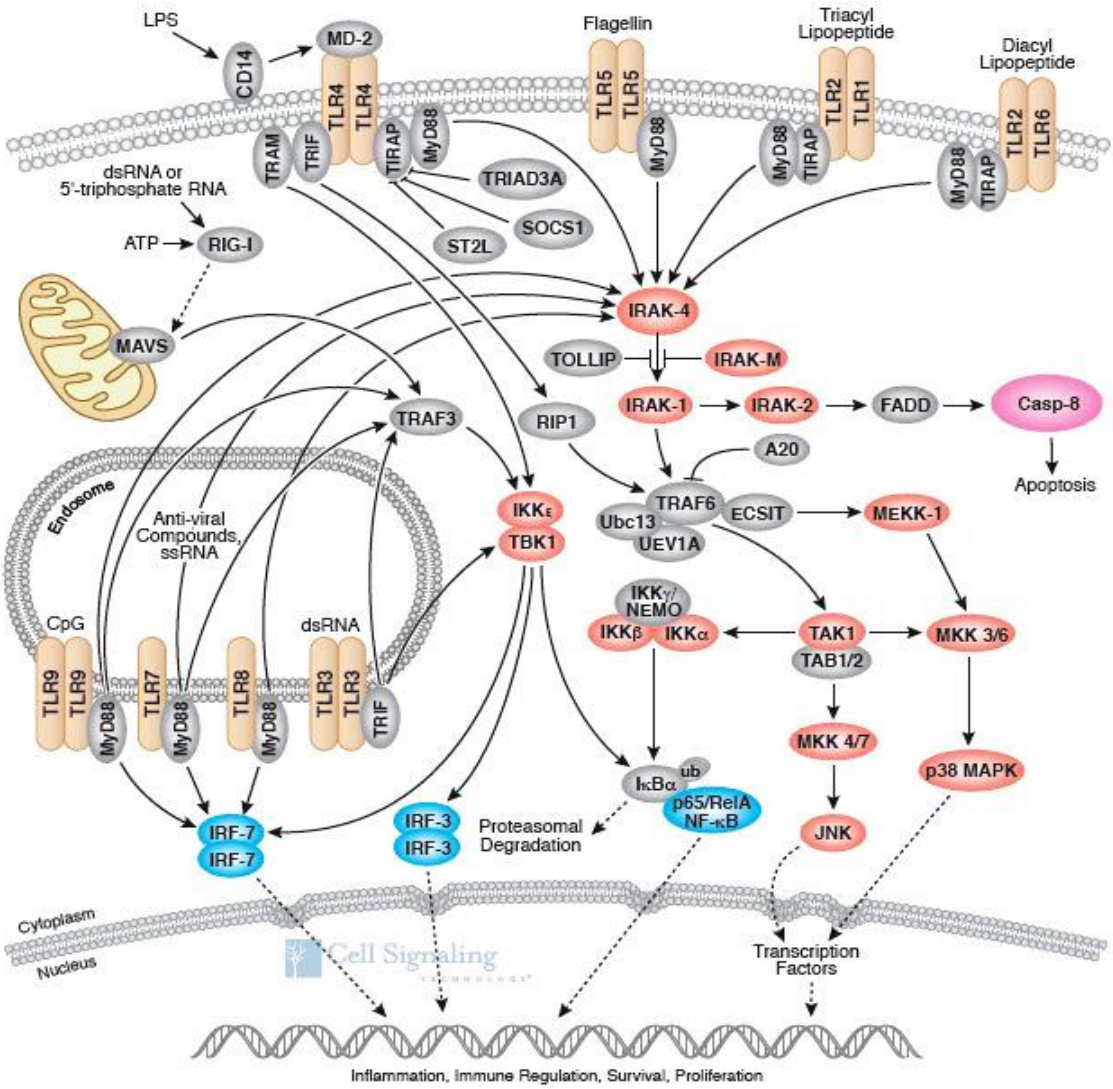


Figure 1.14: Toll-like receptors signaling pathways. Taken from (https://www.cellsignal.com/)

This figure illustrates specific ligands of TLRs (LPS and BLP) that can be recognized by TLR4 and TLR2, results in the dimerization of these receptors and activation of cytoplasmic adaptor proteins. Interaction of TLRs with cytoplasmic proteins leads to activation of Inhibitor kappa B kinase which causes activation and translocation of NF-kB from cytoplasm into nucleus and this in turn induces transcription of different inflammatory genes.

The Michelsen and Curtiss groups have shown that deletion of the TLR2 or TLR4 gene in mice decreases the extent of the formation of atherosclerotic lesions in mouse models of atherosclerosis (Mullick *et al.* 2005); (Kathrin S. *et al.* 2004). Regarding the importance of TLR2 in the progression of atherosclerosis (Table 1.3), an obvious reduction in atherosclerotic lesion development was observed in $TLR2^{-/-}LDLR^{-/-}$ mice when compared with $LDLR^{-/-}$ mice fed with a high fat diet for fourteen weeks (Mullick *et al.* 2005). Furthermore, studies by (Mullick *et al.* 2008) suggested that at the aortic arch in $LDLR^{-/-}$ mice, the expression of TLR2 by endothelial cells was amplified over the site of the lesion. Concerning the contribution of TLR4 in the development of atherosclerotic plaque (Table 1.3), genetic deletion of TLR4 in $ApoE^{-/-}$ mice results in about a 55% reduction in the development of the lesion when compared with $ApoE^{-/-}$ mice expressing functional TLR4 (Kathrin S. *et al.* 2004). Moreover, the potential utility of TLR4 as a therapeutic target was shown through the demonstration that treatment of rats with antibody targeting TLR4 resulted in a significant decline of the blood pressure and the release of the pro-inflammatory cytokines was diminished (Bomfim *et al.* 2012).

Table 1.3: Toll-like receptors with their ligands (PAMPs) in the development of atherosclerosis. Adapted from (Erridge 2008)

Toll-Like Receptors	PAMPs	Atherosclerotic plaque progression features
TLR2/1/6	Di/Tri-acyl lipopeptide	• Foam cell formation • Monocyte activation and adhesion • Arterial endothelial cell activation
TLR4	Lipopolysaccharide	• Foam cell formation • Monocyte activation and adhesion • Arterial endothelial cell activation

The presence of particular sorts of the pathogen associated molecular patterns (PAMPs) together with a specific TLR would be the key factor in the activation of the innate immunity through stimulating TLR signaling pathway and in turn will have a role in releasing and the translocation of nuclear factor kappa-B (NF-kB) from the cytoplasm in to the nucleus which leads to the up-regulation of the pro-inflammatory cascade (Falck-Hansen *et al.* 2013). As a result of the union of PAMP with a specific TLR, this leads to the initiation of pro-inflammatory cytokine release.

Studies have shown that PAMPs, such as BLP or LPS, can be recognized independently of intact or live microorganisms (Laman *et al.* 2002); (Juvonen *et al.* 1997). Regarding the origin or the source of these PAMPs, different investigations were done in this field showed that there might be a link between dietary and lifestyle factors and the existence of these PAMPs in the circulation. For example, a study conducted by (Wiedermann *et al.* 1999) showed that cigarette smoke will increase the amount of LPS in the circulation when compared with a non-smoker. Other workers showed that high fat diet and obesity increase the level of PAMPs within the circulation compared with healthy or untreated controls (Patrice 2007); (Erridge C *et al.* 2007c); (Paola Brun *et al.* 2007).

A number of potential mechanisms have been proposed to link PAMP exposure to the development of atherosclerotic plaques, as shown in figure 1.15. Firstly, the PAMPs could have a vital role in the activation of the monocytes as well as their adhesion to the endothelial cells through upregulation of expression of adhesion molecules, including VLA-4, LFA-1, Macrophage antigen-1 (Mac-1) and Urokinase receptor (uPAR). Secondly, PAMPs may also have a role in directly stimulating endothelial cells which in turn results in the increased expression of vascular adhesion molecules such as E-selectin, ICAM-1, VCAM-1 and chemokines (IL-8 and MCP-1), all of which have a role in the transmigration of the monocytes in to the intimal layer. Finally, PAMPs may promote the accumulation of cholesterol within macrophages, resulting in foam cell formation within the sub-endothelial layer, potentially via the reduction of cholesterol efflux as a result of reduced ABCA-1 and SR-B1 activity (Erridge 2008).

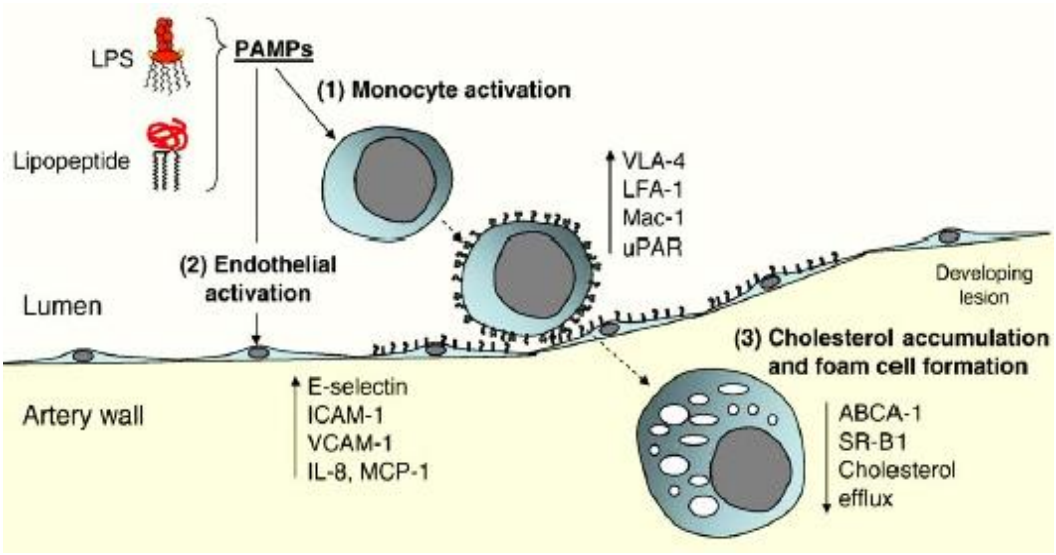


Figure 1.15: The role of PAMPs in atherosclerotic plaque progression. Taken from (Erridge 2008)

Effects of PAMPs on atherogenesis processes and plaque formation may be due to up-regulation of adhesion molecules on the monocytes (VLA-4, LFA-1, Mac-1 and uPAR), activation of vascular adhesion molecules on the endothelial cells (E-selectin, ICAM-1, VCAM-1) and the release of chemotactic cytokines (IL-8 and MCP-1). Furthermore, cholesterol accumulation and foam cell formation may increase after the exposure of macrophages to PAMPs and this might be happen through the suppression of ABCA-1 and SR-B1.

1.9. Bacterial lipopolysaccharide in Atherosclerosis

Bacterial lipopolysaccharide (LPS or Endotoxin) is the major component of the outer surface of Gram-negative bacteria. This glycolipid is essential for both the structural and functional integrity of bacterial outer membrane (Erridge *et al,* 2002); (Caroff, Karibian 2003); (Rietschel *et al.* 1994) and (Trent *et al.* 2006).

The LPS of Gram-negative bacteria consists of three different regions which includes, lipid A, core saccharide and O-antigen polysaccharide (Erridge *et al,* 2002); (Caroff, Karibian 2003); (Rietschel *et al.* 1994) and (Trent *et al.* 2006). The lipid A component is the endotoxically active part of the LPS molecule. Previous study has shown that synthetic lipid A (which was prepared synthetically by a group of researcher in japan in the 1980s (K. Tanamoto *et al,* 1984)) has identical biological properties to the lipid A of *E. coli* (Galanos *et al.* 1985), and this confirmed that lipid A of LPS molecules is the part which is highly endotoxically active. Lipid A consists of a disaccharide covalently linked to two phosphoryl groups and four acyl chains (Erridge *et al,* 2002); (Caroff, Karibian 2003); (Rietschel *et al.* 1994) and (Trent *et al.* 2006). Whereas the core region of bacterial endotoxins is relatively well conserved, there are over 160 recognized O-serotypes for *E. coli* alone (Rietschel *et al.* 1993). The inner core is distinguished by the presence of uncommon sugars such as 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno heptose (hep). The outer core is more variable than the inner core and consists of common hexose and hexosamine sugars (glucose, galactose, N-acetyl galactosamine and N-acetyl glucosamine) (Hammond *et al.* 1987) and (Dalla Venezia *et al.* 1985). The O-antigen polysaccharide contains repeating units of between one and eight glycosyl residues which vary greatly between strains by means of sugars, sequence and chemical bond. These differences in the structure lead to unlimited diversity of O-polysaccharide region, resulting in the existence of hundreds of serotypes for a specific Gram-negative species. The O-chain is the furthest part of the LPS which is expressed on the outer membrane of the Gram-negative bacteria; this is why it is considered as a major antigen target by the host adaptive immune system (Erridge *et al.* 2002); (Caroff, Karibian 2003); (Rietschel *et al.* 1994) and (Trent *et al.* 2006). Nevertheless, Gram-negative bacteria exploited this dissimilarity as one strategy to promote survival by providing resistance to components of the innate immune system and helping to avoid recognition by the immune cells receptors (Trent *et al.* 2006).

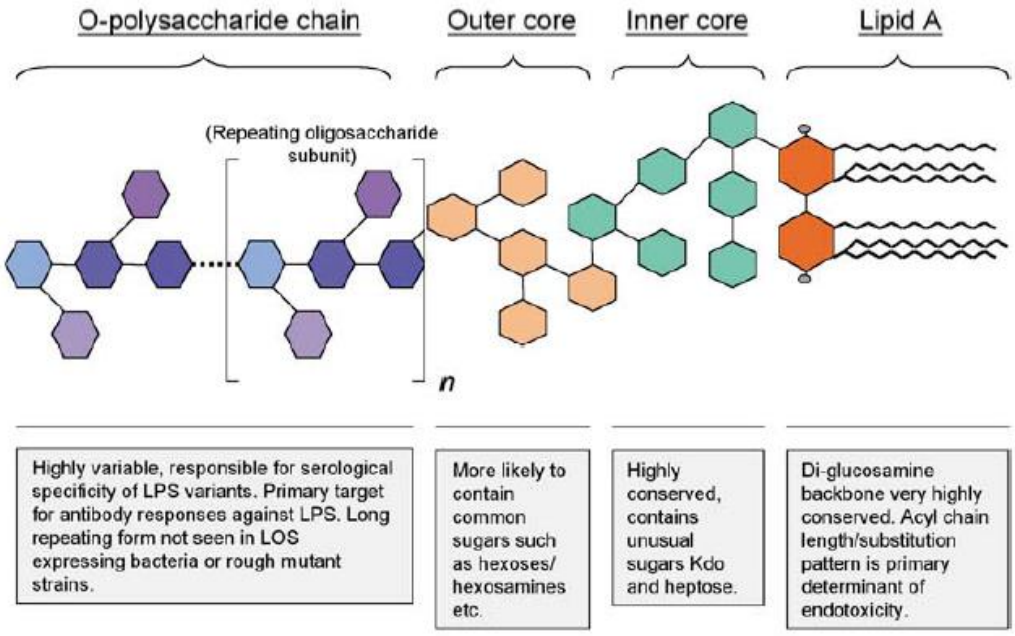


Figure 1.16: General structure of the bacterial lipopolysaccharide. Taken from (Erridge *et al.* 2002)

Lipopolysaccharide is a major component of Gram-negative bacterial membranes. It consists of three separate sections (from inside to outside): Lipid A, which confers endotoxicity; the Core region, comprising relatively well conserved sugars; and the O-chain, which is highly variable and the primary target for antibody responses to LPS.

Bacteria are surrounded by an envelope that gives the shape and integrity of the microbial body. The outer membrane of the Gram-negative bacteria protects these microbes from environmental changes, entrance of toxic compounds and has an essential role in nutrient transport. The main component of these microorganisms membrane is LPS, which is important for bacterial viability.

When Gram-negative bacteria multiply or die, free LPS molecules are shed from the surface of the bacterial membrane (Westphal *et al.* 1978). Bacterial LPS then interacts with various host cell types for example endothelial cells, smooth muscle cells, granulocytes and thrombocytes, in particular macrophages/monocytes as the most essential cells in the process of LPS recognition (Galanos *et al.* 1992). Consequently, the interaction between LPS molecules and macrophages will result in the secretion of inflammatory cytokines, such as TNF-α, IL-1 and IL-6 (Vogel *et al.* 1990). The release of these cytokines in low levels into the circulation appears to have protective effects such as resistance against infections and priming the immune response. However, if high levels of these mediators reach the circulation, destructive effects including high fever, increased heart rate, multiple organ

failure, shock and potentially death may occur (Caroff, Karibian 2003) and (Galanos, Freudenberg 1993).

The innate arm of the immune response as a first line of defense against microbial infections. Bacterial endotoxin is considered to be one of the most potent stimulants of innate immunity. LPS binds first to lipid shuttle proteins, including LPS-binding protein (LBP) and CD14. These then transfer LPS molecules to the TLR4/ Myeloid differentiation protein 2 (MD-2) complex. This results in the activation of pro-inflammatory transcription factors and the synthesis and release of inflammatory cytokines (Beutler 2002); (Palsson-McDermott, O'Neill 2004); (Freudenberg *et al.* 2008); (Schromm *et al.* 2000) and (Jerala 2007).

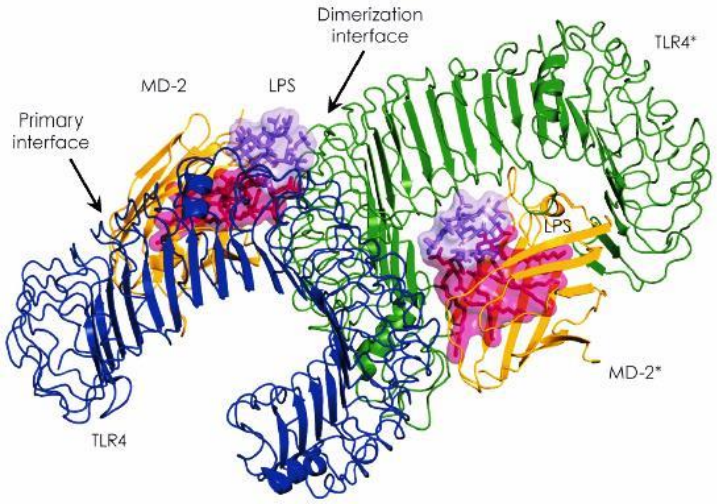


Figure 1.17: Lipopolysaccharide recognition by the MD-2/TLR-4 complex. Taken from (Kim *et al.* 2007)

Recognition of endotoxin molecules achieved by the action of the four-principal endotoxin-binding proteins. LPS-binding protein (LBP) interacts with the endotoxin-rich bacterial membrane, endotoxin monomers are then transferred to CD14, which in turn transfers LPS to Myeloid differentiation protein 2 (MD-2) and to the MD-2/TLR4 complex which results in the receptor dimerization and the activation of intracellular signal cascade.

Many previous studies have supported the hypothesis that chronic exposure to endotoxin may be pathogenically linked to atherosclerosis. Studies in man have shown that intravenous (i.v.) injection of low-doses of LPS results in a transient, low-grade inflammatory response involving the upregulation of circulating markers, such as IL-6, TNF-α and CRP (van Deventer *et al.* 1990). Whereas, an i.v. bolus of LPS (2 ng/kg) induces a pronounced fever and sickness response in volunteers (involving increased heart rate, high temperature, nausea, and malaise) (Marlous *et al.* 2014). Moreover, in animal studies, injections of endotoxin on weekly basis results in accelerated progression of atherosclerotic lesions in rabbits primed with hypercholesterolomic diets (Lehr *et al.* 2001) and in apolipoprotein E-deficient mice (Ostos *et al.* 2002). Accumulating experimental evidence suggests that chronic inflammatory response associated with the low level endotoxemia, which may be an important risk factor for cardiovascular disease (Bonora *et al*. 1998).

1.10.Pathways of lipid metabolism

Dyslipidaemia is the abnormality within the lipid profile, including a variety of disorders connected to increased levels of total cholesterol, LDL, or TG, or lower levels of HDL. Dyslipidaemia may happen because of a single disorder in one lipoprotein, or may represent a combination of lipoprotein abnormalities, such as elevated TG and low HDL.

Both genetic and environmental factors may contribute to increased risk of dyslipidaemia. For example, fatty diets, diseases (diabetes and liver disease), and sometimes medications may result in a dyslipidemic condition. A dyslipidemia may be the consequence of over production or lack of clearance of the lipoprotein particles, or due to other abnormalities such as apolipoproteins or enzyme deficiencies.

Studies have shown the association between cardiovascular disease and amplified levels of serum cholesterol (Castelli 1984). Intervention studies have conclusively shown that cholesterol lowering therapy reduces risk of cardiovascular diseases (Downs *et al.* 1998) and (Pedersen *et al.* 2004).

In addition, both genome-wide association studies (GWAS) of common SNPs associated with lipid traits, and studies of Mendelian disorders of lipoprotein metabolism, have confirmed that circulating lipoprotein concentratsions are causally related to risk of CVD, particularly in the context of LDL-C and triglycerides (Strong and Rader 2010).

Cholesterol plays a key role in the facilitation of membrane transport, synthesis of bile acids and hormones. Triglyceride (TG) is crucial for energy production and storage. Both lipids are insoluble, and they are transported in the blood in more complex structures, called lipoproteins.

Plasma lipoproteins are composed of both lipid and protein. Triglyceride and cholesterol ester comprise the inside of the particle, and free cholesterol, phospholipid, and specific apolipoproteins form the surface of the particle. Lipoproteins are classified into five main forms based on their density. They are chylomicrons, very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL and HDL.

Chylomicrons are produced by intestinal epithelial cells after the absorption of digested fat. They are transported in the blood to tissues (muscle, adipose, and the liver). These tissues contain high concentrations of lipoprotein lipase (LPL), which hydrolyses TG in the

chylomicrons and the TG-depleted chylomicron is called a remnant particle, which is transported to the liver to produce free-fatty acids that are utilised by the muscle cells to generate energy or used in hepatic VLDL synthesis (Gotto A 1999). VLDL are similar to chylomicrons with a high concentration of TG (Gotto A 1999). The TG of VLDL are also hydrolysed by LPL to make fatty acids which can be used by the adipose and muscle tissue. The remainder of this particle is called Intermediate-density lipoproteins (IDL). IDL is taken up by the liver via the LDL receptor then converted to LDL by the action of hepatic lipase (HPL). Low-density lipoproteins contain the majority of the cholesterol in the blood and deliver cholesterol to the cells. LDL receptors in peripheral cells or liver bind with LDL and clear it from the blood. Finally, the high-density lipoproteins (HDL) are particles synthesized in the liver and intestines. Nascent HDL obtains free cholesterol from peripheral tissues by the aid of circulating enzyme called lecithin cholesterol acyltransferase (LCAT) which promotes the uptake of free cholesterol by HDL through reactions known as esterification. Transferring of cholesterol ester from the HDL core to other lipoproteins such as LDL molecules is mediated by another enzyme, known as cholesteryl ester transfer protein (CETP) (Rifai N *et al.* 1997). HDL lipoproteins have a cardioprotective role, as they facilitate the transfer of cholesterol from atherogenic lipoproteins and peripheral tissues to the liver.

Apolipoproteins (Apo) such as Apo-A, Apo-B, Apo-C and Apo-E, have crucial roles in the packaging of lipids into lipoproteins. Furthermore, they enhance the recognition of lipoprotein particles by enzymes which process lipids from the lipoprotein particles. For example, Apo-CII activates lipoprotein lipase (LPL), which removes TG from lipoprotein particles such as chylomicrons and VLDL.

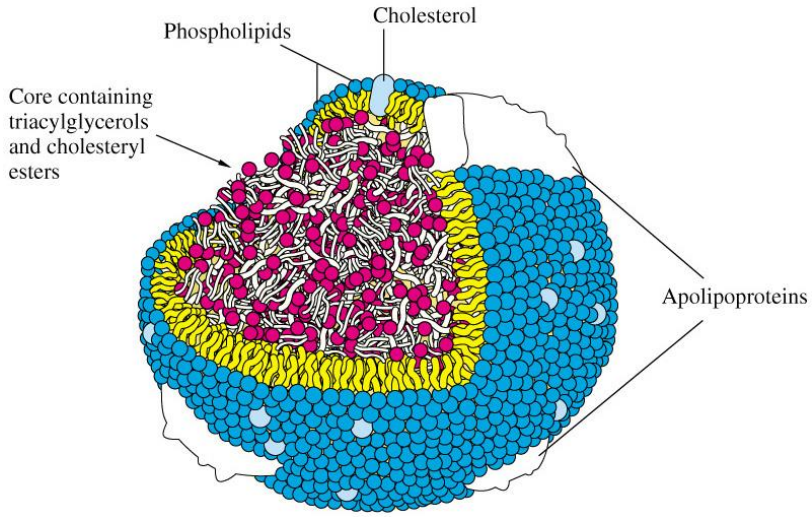


Figure 1.18: General structure of lipoproteins. Taken from (Attia 2012)

Cholesterol is a fat-soluble molecule which cannot travel through the circulatory system by itself. For this reason, cholesterol is packaged in molecules known as lipoproteins. Lipoproteins consist of TG and cholesterol on the inside, and phospholipids and apolipoproteins on the surface.

Three main pathways for the generation and transport of lipids within the body include the exogenous pathway, the endogenous pathway, and the reverse cholesterol transport pathway (Vaughn G. 1999) and (Rifai N *et al,* 1997).

The exogenous pathway starts with the formation of chylomicrons in the epithelial cells of the intestines through packaging TG and cholesterol from the diet together after the processes of digestion and absorption of dietary fat. Chylomicrons pass through the intestinal lymphatic system into the blood. Circulating chylomicrons interact with the capillaries of adipose tissue and muscle cells which provide TG to the adipose tissue where it may be stored and will be available for the body to be used as energy. Lipoprotein lipase hydrolyses the TG and free-fatty acids are released. The remaining chylomicrons, which are known as remnant particles, are removed from the circulation by the chylomicron remnant receptors present on the liver, while other components of the chylomicrons are repackaged into other forms of lipoproteins, such as HDL molecules.

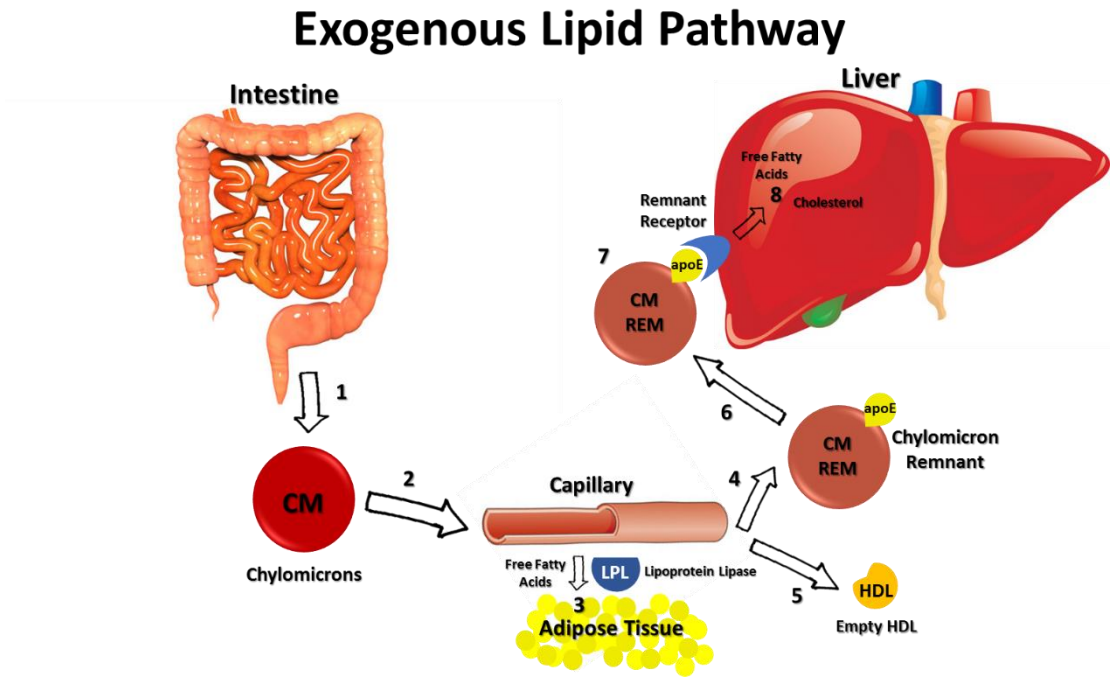


Figure 1.19: An illustration of the exogenous lipid pathway.

Lipids are digested, resulting in the formation of chylomicrons (1). Chylomicrons within the circulation interacts with the capillaries of adipose tissue and muscle cells (2). LPL hydrolyses the chylomicrons which releases TG and free-fatty acids (3). The remaining chylomicrons become chylomicron remnant particles (4). Components of the chylomicrons are repackaged into HDL particles (5). Chylomicron remnant particles are removed from the plasma by chylomicron remnant receptors present on the liver (6) and (7). Chylomicron remnant particles are hydrolysed in the liver to form free fatty acid and cholesterol (8).

The endogenous pathway relates to the *de novo* synthesis of lipids by the liver. TG and cholesterol ester are synthesised by the liver and packaged to form VLDLs which are then released into the circulation. LPL in tissues will process VLDL particles to release fatty acids and glycerol. The fatty acids are used by the adipose cells for storage or by muscle cells as energy. Later, the remaining of the VLDL will become smaller, denser lipoprotein which known as IDL. LDL receptor on the liver will absorb some of the IDL particles. Nevertheless, other IDL particles are processed in the liver by hepatic-triglyceride lipase (HPL) to form a smaller, denser particle than IDL and it known as LDL.

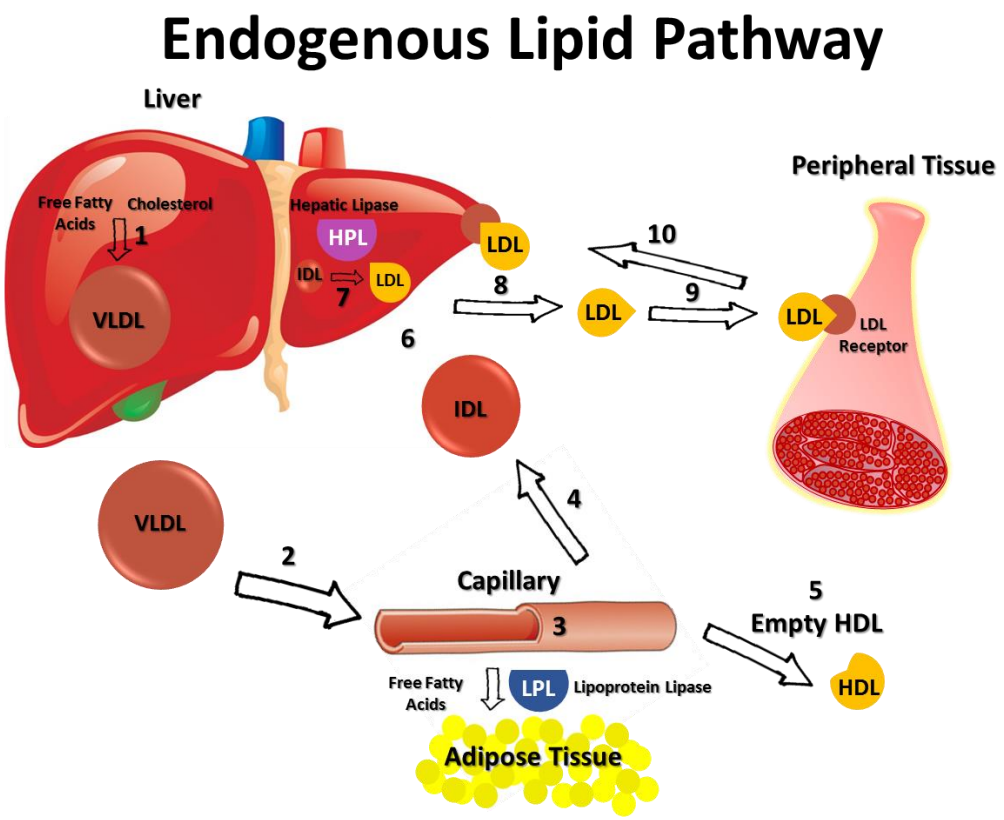


Figure 1.20: An illustration of the endogenous lipid pathway.

Free fatty acid and cholesterol molecules are assembled within the liver to form very low-density lipoproteins (1). VLDL particles within the circulation which interacts with the capillaries of adipose tissue and muscle cells (2). LPL hydrolyses the VLDLs which releases TG and free-fatty acids (3). The remaining VLDLs become smaller particles known as IDL (4). Some components of the VLDL are repackaged into HDL particles (5). IDL particles are delivered to the liver by the LDL receptors (6). IDL particles are hydrolysed in the liver by hepatic-triglyceride lipase (HPL) to form LDL particles (7). Circulating LDL particles will transport cholesterol molecules to the peripheral tissues (8) and (9). LDL receptors reuptake LDL particles back to the liver (10).

Reverse cholesterol transport is a vital pathway in transporting cholesterol from the tissues back to the liver (Genest J 1990). The main lipoprotein which contributes to reverse cholesterol transport is HDL (Gotto A 1999). After esterification by the plasma enzyme lecithin cholesterol acyltransferase (LCAT) (Fielding 1995), several mechanisms have been suggested regarding the way by which the HDL delivers cholesterol esters to the liver. First, cholesteryl ester transfer proteins (CETP) transfers CE to TG-rich particles for clearance by hepatic ApoB receptors. Alternatively, cholesterol ester-rich HDL particles may be taken up directly by receptors in the liver.

A further possibility is that the liver may uptake the cholesterol esters directly without catabolism of the HDL cholesterol particle (Genest J 1990) and (Gotto *et al.* 2000).

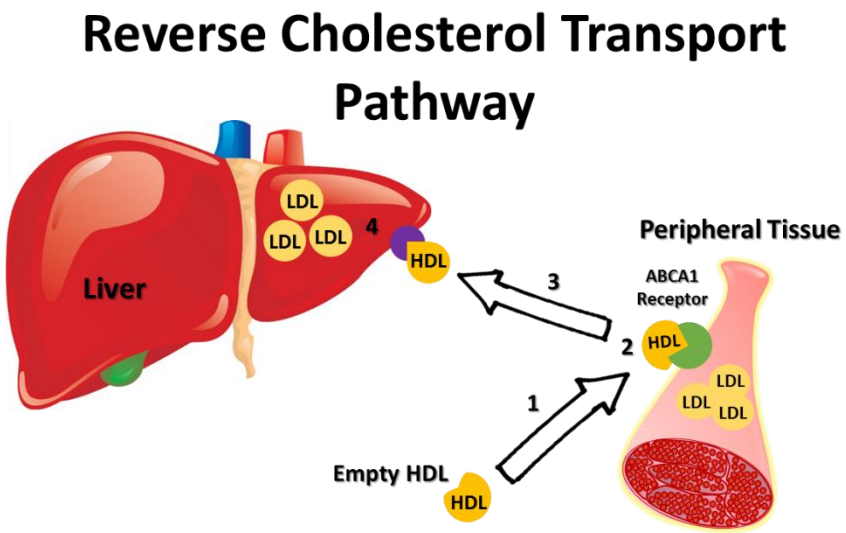


Figure 1.21: An illustration of the reverse cholesterol transport pathway.

Free nascent HDL molecules within circulation (1). HDL accepts cholesterol from peripheral tissues through ATP binding cassette transporter (ABCA1) (2). HDL delivers cholesterol esters to the liver through scavenger receptor B1 (SR B1) and LDL receptor on the liver (3). LDL particles formation in the liver (4).

1.11. Diet and Atherosclerosis

It is well recognised that both dietary factors and inflammation contribute to atherosclerosis (Paoletti *et al.* 2006). Evidence is also emerging to suggest that dietary factors may regulate inflammatory tone in man. For example, studies using animal models revealed that administration of a high fat diet promoted a state of low-grade systemic inflammatory signaling which was also associated with increased atherosclerosis, insulin resistance and metabolic syndrome (Shi *et al.* 2006). Other studies also showed that levels of plasma SAA and inflammatory markers in vascular tissues increased in mice fed high fat diet when compared to mice fed normal diet (Scheja *et al.* 2008). Similar findings have been reported in human dietary studies. Long term (4 weeks) feeding of human volunteers with high fat snacks or fast foods was revealed to increase plasma CRP levels significantly (Tam *et al.* 2010) and (Astrand *et al.* 2010). Subjects consuming a Western type diet, typified by refined and processed foods, consistently show higher CRP levels than those that adhere to a 'prudent', or 'Mediterranean' diet, characterized by frequent consumption of fresh produce, including vegetables, legumes and fish (Fung *et al.* 2001). It has been shown that the consumption of processed meat products significantly increases the risk of coronary heart disease, stroke, and diabetes mellitus when compared with consumption of an equivalent amount of non-processed meats (Micha *et al.* 2010). There is also some evidence that acute dietary fat intake by human volunteers' results in increased IL-6 levels in the postprandial phase, reviewed by (Herieka M and Erridge C 2014).

It has been frequently proposed (Lee *et al.* 2004) that saturated fatty acids (SFA) may have the ability to promote inflammatory cytokine production via direct stimulation of TLR2 or TLR4 signaling pathways. However, studies conducted by (Erridge C 2009) have shown that SFAs are in fact unable to stimulate TLR2 or TLR4 signaling, and that earlier reports of this capacity reflected contamination of reagents used. In addition, a meta-analysis of epidemiological studies revealed that there is no remarkable evidence to support a significant correlation between dietary saturated fat intake and the risk of cardiovascular diseases (Siri-Tarino *et al.* 2010).

Taken together, these results suggest a strong association between commencement of diets rich in processed foods and elevation of inflammatory markers in mice and humans. However, the mechanisms promoting this association remain unclear.

1.12. Current evidence implicating the microbiota

Much evidence supports a potential connection between infectious micro-organisms and atherosclerosis (Mlot 1996); (Grayston *et al.* 1995); (Grayston JT *et al.* 1989) and (Puolakkainen *et al.* 1993). However, it is now becoming appreciated that the commensal microbiota of the intestine may also be able to regulate factors relevant to cardiovascular risk.

Studies have revealed the link between pathogens for instance *Helicobacter pylori*, *Chlamydia pneumoniae*, and cytomegalovirus and the risk coronary artery diseases (Epstein *et al.* 1996) and (Saikku *et al.* 1988). However, studies using germfree hyperlipidaemic mice found that infectious microbes are not the crucial factor for the progress of murine atherosclerotic plaque (Wright *et al.* 2000).

Approximately 100 trillion microbes reside in the human large intestine, where they are thought to contribute to diverse host functions such as innate immunity, gut epithelial cells protection, and the digestion and absorption of many nutrients (Backhed *et al.* 2005) and (Eckburg *et al.* 2005).

Studies have reported possible associations between the intestinal flora and the progression of metabolic diseases such as obesity and insulin resistance (Ley *et al.* 2006) and (Reigstad *et al.* 2009). Mouse metabolomics studies have also pointed towards potential roles for the gut microbiota in metabolic defects such as insulin resistance and non-alcoholic fatty liver disease (Dumas *et al.* 2006).

Furthermore, in a large clinical cohort study (Wang *et al.* 2011), reported an association between gut-flora-dependent phospholipid metabolism and atherosclerosis risk through generation of pro-atherosclerotic metabolites of the dietary lipid phosphatidylcholine (PC). Specifically, free choline, trimethylamine N-oxide (TMAO) and betaine-were all identified in plasma and then shown to predict risk for CVD.

Foods such as eggs, milk, liver, red meat, poultry, shell fish and fish, are considered to be the main dietary sources for choline, and hence TMAO production (Zeisel *et al.* 2003). TMAO, is formed in the liver through the oxidation of trimethylamine (TMA), which arises from microbial (but not host) metabolism of free choline (Cashman *et al.* 2003) and (al-Waiz *et al.* 1992).

Specifically, intestinal microbes catabolise free choline in the gut to produce betaine and TMA (al-Waiz *et al.* 1992). The TMA is then absorbed and metabolized with the aid of the hepatic enzyme Flavin monooxygenase-3 (FMO3), which converts odorous TMA to odourless TMAO (Lang *et al.* 1998) and (Zhang *et al.* 1999). This process shows a critical role of gut flora in TMAO formation (from dietary lipid phosphatidylcholine) (Figure 1.22).

Moreover, intestinal microbiota metabolism of L-carnitine (which is abundant in red meat), has also been suggested to stimulate the progression of atherosclerosis. A study by (Koeth *et al.* 2013) revealed that chronic dietary L-carnitine supplementation in mice significantly enhanced synthesis of TMA and TMAO, and increased atherosclerosis, but when the intestinal microbiota was suppressed, this did not happen.

These studies have raised the hypothesis that dietary choline may promote cardiovascular disease. On one hand, studies by (Danne *et al.* 2007); (LeLeiko *et al.* 2009) and (Bidulescu *et al.* 2007) have suggested a link between high levels of total choline in the blood and cardiovascular diseases. On the other hand, (Dong *et al.* 2002) and (Zaina *et al.* 2005) have reported that choline and betaine may produce epigenetic variations in the genes associated to atherosclerosis. Also, in rodent models an acute choline deficiency results in lipid accumulation in liver (steatohepatitis), heart and arterial tissues (Salmon 1962).

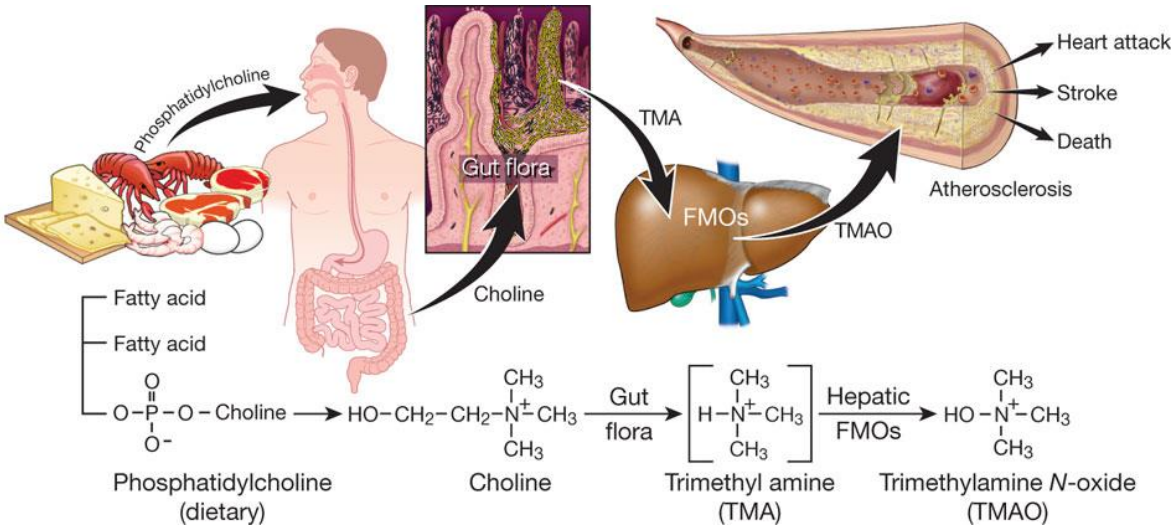


Figure 1.22: Role of intestinal microbial flora in metabolism of dietary PC and atherosclerosis. Taken from (Wang *et al.* 2011)

The pro-atherogenic microbial gut flora produced metabolite TMAO through cleavage of a trimethylamine from phosphatidylcholine, choline, and betaine, which results in the formation of TMA, and subsequently oxidation of TMA by hepatic FMO3 which generates TMAO.

1.13. PAMP content of the human microbiota and evidence of translocation

The mammalian TLRs are considered to be the major pro-inflammatory receptors of PAMPs, such as bacterial LPS and bacterial lipopeptide, which are abundant in many bacteria (Erridge 2010). Thus far, nearly twenty endogenous damage-associated molecular pattern (DAMPs) have been reported to stimulate TLR-signalling. These molecules are associated with tissue damage, and include molecules such as oxidized lipids and host proteins (Bianchi 2007). However, many believe that the results of those earlier studies reflect endotoxin contamination of reagents used (Bausinger *et al.* 2002); (Tsan, Gao 2007) and (Taylor *et al.* 2005).

Hence, the main sources of PAMPs in the lumen of the human intestine are the commensals and dietary sources (Erridge 2011a).

Notably, the microbiota is not evenly distributed along the intestinal tract. The load of gut microflora of the small intestine ranges from 10^0 to 10^5 cfu/ml in the duodenum and the jejunum, and reaches a maximum of 10^5-10^8 cfu/ml in the terminal of ileum (Berg 1996). By contrast, the luminal microflora for large intestine reaches from 10^{10} to 10^{12} bacteria per gramme in healthy subjects.

Experiments by (Posserud *et al.* 2007), have shown that most microbial flora of the small intestine composed of Gram positive bacteria with very limited presence of Gram negative bacteria. By contrast, the large intestine contains large numbers of both Gram-positive and Gram-negative bacteria, and thus serves as the main source of TLR-stimulants in the human gut (Berg 1996); (Posserud *et al.* 2007); (Erridge *et al.* 2010) and (Alhawi *et al.* 2009).

It should also be noted that the surface area of the small intestine (300 m^2), is much larger than that of the large intestine (0.35 m^2). This is because of the presence of villi in the small intestine, which enable the absorption of nutrients, particularly fats and fat soluble molecules (DeSesso, Jacobson 2001). Other evidence (Ghoshal *et al.* 2009), has revealed that LPS is fat soluble, and is absorbed in the small intestine by the aid of chylomicrons in the presence of fat rich diet. These evidences together suggest that the small intestine is probably the main site of PAMP absorption in the body.

Numerous studies in human subjects (Erridge C *et al.* 2007c) and (Laugerette *et al.* 2011) and in animal models (Ghoshal *et al.* 2009) and (Patrice D. 2007), have shown that high fat diets have a crucial role in translocation of PAMPs from intestinal lumen in to the circulation. In addition, other studies reported an increase in the level of endotoxin in mice plasma due to four weeks high fat diet (Patrice D. 2007) and (Paola Brun *et al.* 2007). Radiolabeling experiments and limulus assay has confirmed the translocation of LPS from intestinal lumen into circulation through measuring LPS levels in the blood of mice which were orally gavaged with LPS (Ghoshal *et al.* 2009) and (Yoshino *et al.* 1999). Furthermore, mild postprandial endotoxemia was induced in human volunteers and mice after a fat-rich meal (Erridge C *et al.* 2007c) and (Ghoshal *et al.* 2009).

1.14. Previous studies examining PAMP content of foods

An earlier study by (J M Jay *et al.* 1979) measured the endotoxin content of ground beef by the limulus amoebocyte lysate (LAL) test as a rapid indicator of microbial contamination. They reported levels of endotoxin in purchased minced beef of up to 7.4 μg LPS per gram. In addition, high bacterial load (around 10^5 - 10^7 colony forming units per gram) was also observed in the ground beef. Another study reported that cow's milk can contain between 1 and 100 ng LPS per ml (Gehring *et al.* 2008).

However, using the LAL assay for the quantification of biological activities of TLR stimulants in foodstuffs is widely considered to be inappropriate since false positive results are common due to the detection of non-inflammatory β-glucans which are a common ingredient of foodstuffs (Elin, Wolff 1973). Also, measurements of bacterial lipopeptide (Pam_3CSK_4) and flagellins cannot be achieved using the limulus assay (Erridge, Samani 2009). Moreover, common forms of non-enterobacterial lipid-A, which can often be antagonists of TLR4 and LPS signaling in human cells, can show a positive reaction in the limulus assay (Erridge *et al.* 2007) and (Rossignol *et al.* 2006).

Previous work from our group (Erridge C 2010) and (Erridge 2011b) showed that processed foods can contain high levels of TLR2 (BLP) and TLR4 (LPS) stimulants of microbial origin arising from the processing and storage of products. Commonly consumed foods were shown to contain large amounts of both TLR4 and TLR2 stimulants (up to 2.7 μg/g LPS-equivalent and 1.1 μg/g BLP-equivalent per gram of food). These studies employed a recently developed quantitative bioassay based on transfection of HEK-293 cells with specific receptors and NF-κB sensitive reporter to measure TLR2- and TLR4-stimulants in the foods (Erridge *et al.* 2010).

The kinetics of accumulation of TLR2- and TLR4-stimulants in meat products were also examined. In these experiments, a variety of meat products such as beef, pork, and turkey minced meats and intact steaks or breast, all purchased from local supermarkets were held at 5 ◦C in air or under a modified atmosphere packaging for up to 8 days. TLR-stimulants accumulated in the meat products during storage, reaching the highest levels (nearly 80 μg lipopeptide-equivalents/g and approximately 7 μg lipopolysaccharide-equivalents/g) in

meat that was minced rather than intact, and when stored in air rather than under a modified atmosphere (Erridge C 2010) and (Erridge 2011b).

1.15. Results of previous studies in our laboratory

Previous studies in our laboratory found that chronic treatment of wild-type (C57BL/6) mice with drinking water containing a mixture of 3 PAMPs (LPS, Pam3CSK4, and iEDAP) for 10 weeks caused significant increases in inflammatory gene expression in the liver (PTX, IL-1β, IL-6, TNF-α, and Apo-AI), without induction of elevated plasma SAA (Figure 1.23-A and B). In addition, it was found that plasma total cholesterol, HDL cholesterol and efflux to ApoB-depleted serum, is decreased significantly in mice treated with 3 PAMPs in drinking water (Figure 1.24-A and B).

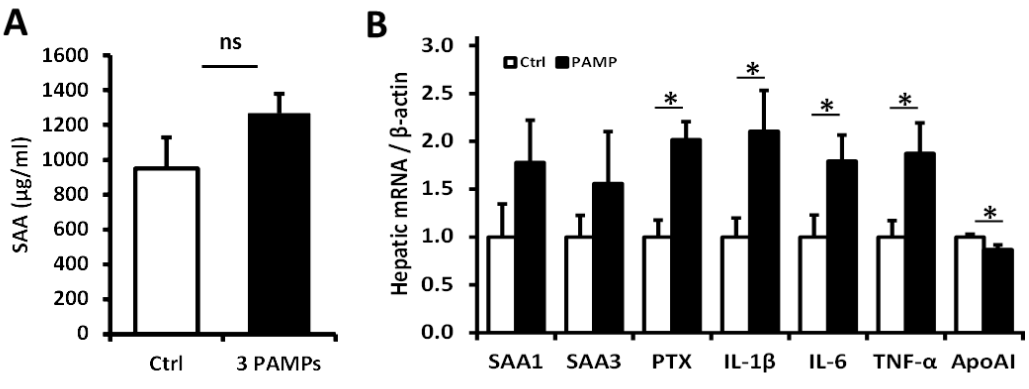


Figure 1.23: Effects of dietary PAMP supplementation on circulating SAA protein and hepatic mRNA markers of the acute phase response in mice.

Wild-type C57BL/6 mice (n=10 per group) received normal chow with normal drinking water (Ctrl) or drinking water supplemented with 100 µg/ml LPS, 1 µg/ml Pam3CSK4 and 1 µg/ml iEDAP. After 10 weeks, plasma content of SAA was measured by ELISA (A) and hepatic expression of acute phase markers was measured by RT-PCR (B). Results are presented as means ± SEM.

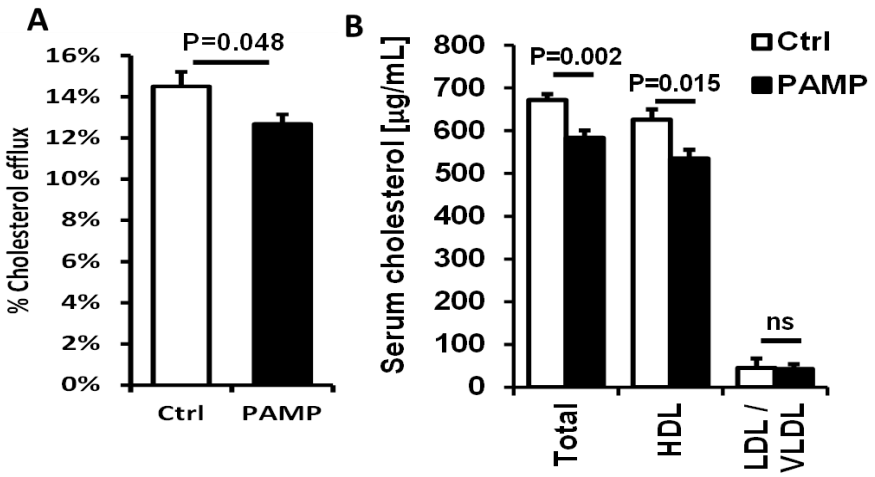


Figure 1.24: Effects of dietary PAMP supplementation on serum cholesterol efflux capacity and lipoprotein cholesterol levels.

Wild-type C57BL/6 mice (n=10 per group) received normal chow with normal drinking water (Ctrl) or drinking water supplemented with 100 µg/ml LPS, 1 µg/ml Pam3CSK4 and 1 µg/ml iEDAP. Cholesterol efflux capacity of ApoB-depleted serum (A), and lipoprotein cholesterol levels (B), were measured after 10 weeks. Results are presented as means ± SEM.

Furthermore, chronic treatment of ApoE$^{-/-}$ mice with 3 PAMPs (LPS, Pam3CSK4, and iEDAP) mix in drinking water for 10 weeks on low fat diet (LFD) promotes development of aortic atherosclerosis which was quantified by Oil-red-O staining (Figure 1.25-A and B).

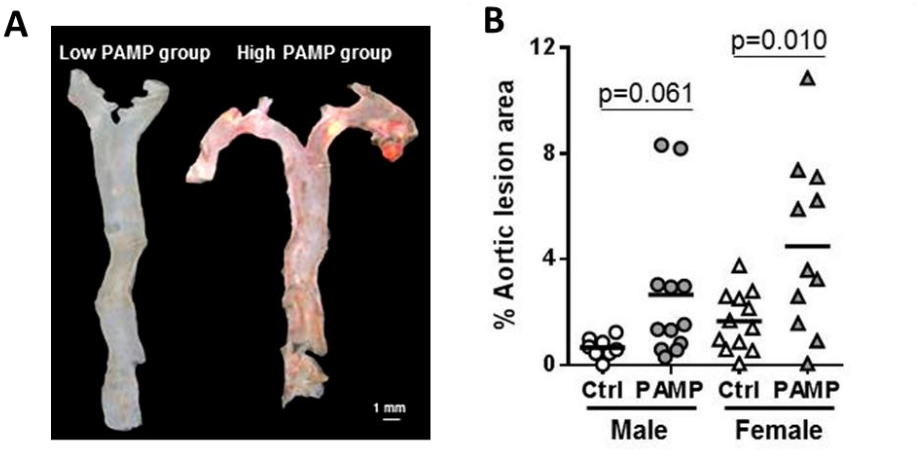


Figure 1.25: Effects of dietary PAMP supplementation on aortic atherosclerotic plaque formation in ApoE$^{-/-}$ mice.

ApoE$^{-/-}$ mice (n=10-12 per group) received normal chow with normal drinking water (Ctrl) or drinking water supplemented with 100 µg/ml LPS, 1 µg/ml Pam_3CSK_4 and 1 µg/ml iEDAP. Aortic atherosclerosis was quantified by Oil-red-O staining after 10 weeks treatment. Representative images of low and high PAMP treatment group aortas are shown in panel A. Percentages of plaque staining as a proportion of total aortic areas are shown in panel B.

Moreover, so as to explore mechanisms of action of dietary PAMPs, a study was performed to explore the acute effects of PAMP ingestion. Wild-type C57BL/6 mice (n=5 per group) received normal chow and drinking water, and were then orally gavaged with 200 µl of saline alone (control), 1 mg/kg Pam_3CSK_4, 2 mg/kg LPS, 1 mg/kg iEDAP, 10^9 heat-killed *E. coli*, or a mixture of Pam_3CSK_4, LPS and iEDAP. Circulating SAA was measured in plasma 24 hours after gavage. There was no significant response to acute gavage with a single PAMP or mix of PAMPs and there was also no induction of APR markers in the liver. (Figure 1.26)

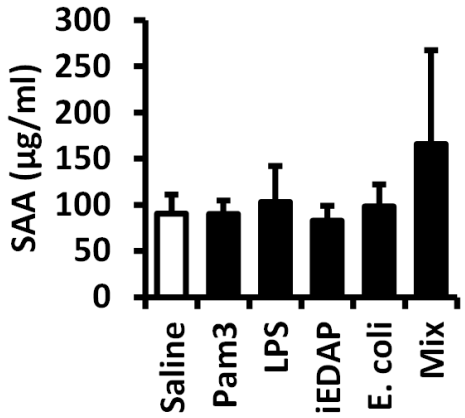


Figure 1.26: Acute effects of single oral gavage with defined PAMP treatments on circulating SAA protein in mice.

Wild-type C57BL/6 mice (n=5 per group) receiving normal chow and drinking water were orally gavaged with 200 µl of saline alone (control), 1 mg/kg Pam_3CSK_4, 2 mg/kg LPS, 1 mg/kg iEDAP, 10^9 heat-killed *E. coli*, or a mixture of Pam_3CSK_4, LPS and iEDAP (Mix). Circulating SAA was measured in plasma 24 hours after gavage. Results are presented as means ± SEM. SAA was not significantly elevated in any treatment group compared to control.

Attempts to increase gut barrier permeability in mice using indomethacin showed that 24 hours after indomethacin treatment, orally gavaged FITC-LPS translocated into the inner part of intestinal villi, and concentrations of unlabelled LPS increased dramatically in plasma (Figure 1.27).

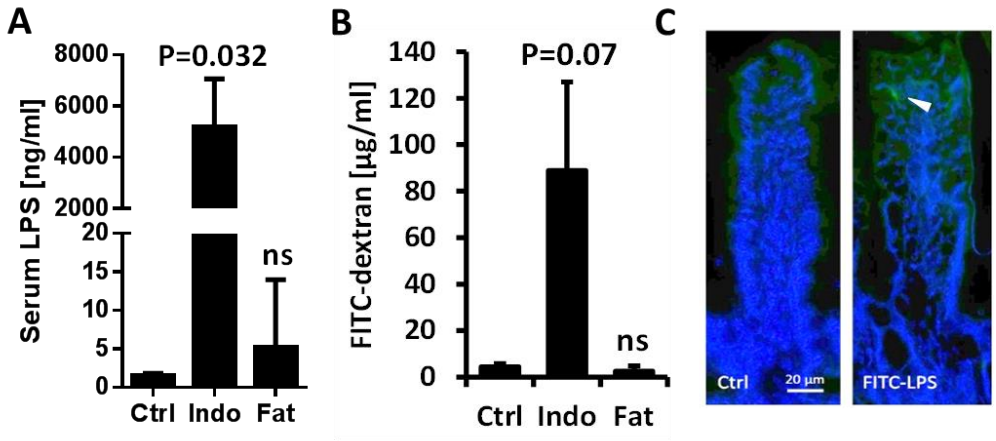


Figure 1.27: Absorption of FITC-LPS and dextran in orally gavaged mice.

Wild-type C57BL/6 mice (n=3-4 per group) were pre-treated with 4 weeks normal chow diet (Ctrl), 4 weeks normal chow then 10 mg/kg indomethacin given orally (Indo), or 4 weeks high-fat diet (Fat). 24 h after indomethacin treatment, mice were orally gavaged with 2 mg/kg LPS with FITC-dextran (A, B), or 1 mg/kg FITC-labelled LPS (C). Serum LPS was measured by LAL assay and FITC-dextran by fluorescence at 90 minutes (A, B). Results are presented as means ± SEM. FITC-LPS was located in intestinal tissues by fluorescence microscopy (C). Arrow indicates FITC-LPS accumulation within villus.

Thus, at the point of commencement of my project, previous results from our laboratory had indicated that:

(i) Processed foods can contains high levels of stimulants of TLR2 and TLR4

(ii) These might translocate into the circulation

(iii) This could be enhanced by impaired gut barrier function

(iv) Such translocation of dietary PAMPs may impact on hepatic gene expression

Hypothesis

The hypotheses I aim to test in this project are as follows:

1. The capacity of processed foods to stimulate inflammatory cytokine production is dependent on food content of TLR2 and TLR4 stimulants *in vitro*.
2. Dietary PAMPs can be absorbed to promote systemic inflammation and lipid disturbance *in vivo*.
3. Gut barrier function is a key regulator of responsiveness to dietary PAMPs.
4. Macrophage-hepatocyte cross-talk plays a key role in lipid responses to dietary PAMPs.

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.

Material	Supplier / Country
Acrodisc Syringe Filter	Sigma / UK
Actinomycin-D	Sigma / UK
Amplex cholesterol assay	Invitrogen / UK
Amplex glucose assay	Invitrogen / UK
anti-TLR2-FITC	R&D Systems / UK
anti-TLR2-FITC	R&D Systems / UK
anti-TLR2-PE	R&D Systems / UK
anti-TLR2-PE	R&D Systems / UK
C57BL/6 mice J	Charles River / UK
cAMP	Sigma / UK
Cell scraper	Greiner / Germany
Cell strainer	Greiner / Germany
Chemiluminescence substrate solution	Thermo Scientific / UK
Clodronate liposomes	Clodro_lipos / Netherlands
Cytokines (IL-6, TNF-α, and IL-1β)	Peprotech EC / UK
DAPI (4',6-diamidino-2-phenylindole)	SIGMA / UK
Dimethyl sulfoxide (DMSO)	Sigma / UK
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma / UK
ELISA kits for human IL-6, TNF-α, IL-1β, CRP and leptin	R&D Systems / UK
ELISA kits for mouse IL-6, TNF-α and IL-1β	R&D Systems / UK
Embedding moulds	Thermo Scientific / UK
Fetal bovine serum (FBS)	Sigma / UK
Gene Juice	Novagen /USA
Gentamicin	Gibco / UK
High cholesterol diet	TestDiet / UK
High fat diet	TestDiet / UK
Histopaque 1077 solution	Sigma-Aldrich / UK
Human insulin ELISA kit	Millipore / USA
IL-1R1-/- mice	Charles River / UK
Indomethacin	Sigma / UK
Isotype control antibodies	Santa Cruz Biotech / USA
Isotype control antibody (rat IgG2a-PE)	R&D Systems / UK
Limulus Amoebocyte Lysate (LAL) kit	Assoc of Cape Cod / USA
Low fat diet	TestDiet / UK
Luminometer plate	Greiner / Germany
Maxisorp Microtiter well strip	Nunc / Denmark
Methylthiazoletetrazolium (MTT) kit	Life technologies / UK
Nitrocellulose membrane (Protran)	Whatman / UK

Novex MOPS gel running buffer	**Fisher / UK**
Nupage precast gels	**Fisher / UK**
OCT embedding matrix	**Cell Path / UK**
***P. acnes* bacteria**	**ECACC / UK**
PAMPs (LPS *E. coli* [O111:B4], Pam3CSK4 [Synthetic BLP], iEDAP)	**Invivogen / France**
Phosphate buffered saline (PBS)	**OXOID/England**
Plasmocin	**InvivoGen / France**
Polyethylene glycol (PEG)	**Sigma / UK**
Polysine histological slides	**Thermo Scientific / UK**
Primary antibody (Rabbit polyclonal IgG)	**Santa Cruz Biotech / USA**
Primary antibody (rat anti-mouse F4/80)	**Santa Cruz Biotech / USA**
Primary antibody (TLR2/TLR4 rabbit polyclonal)	**Santa Cruz Biotech / USA**
Primer pairs	**Fisher / UK**
Protein ladder (PageRuler)	**Thermo Scientific / UK**
RNEasy RNA miniprep kit	**QIAGEN / UK**
RPMI-1640 medium	**Life technologies / UK**
Scintillation fluid	**Fisher / UK**
Secondary antibody (Alexa Fluor 488 goat anti-rat)	**Santa Cruz Biotech / USA**
Secondary antibody (Goat anti-rabbit IgG conjugated with HRP)	**Santa Cruz Biotech / USA**
SensiMix SYBR Green	**BIOLINE / Germany**
Serum Amyloid A (SAA) Mouse ELISA kit	**Abcam / UK**
Tetramethyl benzidine (TMB)	**SIGMA / UK**
Triglyceride assay kit	**Wako / USA**
Vacutainer collection tube	**BD Worldwide / Germany**

2.2. Preparation of processed food extracts

Twenty four different kinds of processed foods were purchased from two different supermarkets. Each food was chosen on the basis of a relatively high content of minced meat, chopped onion and cheese. No signs of spoilage were observed in the foods in terms of their colour or smell. They were stored at typical refrigeration temperature (4^{o}C) before being transported to the laboratory and then homogenized using a blender (Philips/Holland). The blender jar was cleaned and rinsed thoroughly with distilled water, then filled with 500 ml distilled water and 50 g of processed food (1:10 dilution). The content were then homogenised at high power for 1 minute. One aliquot of the food was centrifuged at 3,000 g for 5 minutes then the supernatant was filtered through a sterilized micro-filter (0.2 µm Acrodisc Syringe Filter/Life Sciences). The 1 ml filter-sterilized food extract aliquots were stored at -20°C before performing experiments to investigate for capacity to induce inflammatory signaling and the presence of PAMPs (LPS and BLP). After each homogenization, the blender jar was thoroughly cleaned and rinsed with distilled water several times before using it again.

2.3. Cell lines and Culture condition

2.3.1. J774 cell line

The J774 cell line (ATCC TIB-67) is a transformed murine macrophage cell line. These cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 0.01% Plasmocin (InvivoGene), which prevents infection of the cells with mycoplasma (Uphoff 2012). The J774 cell line was split by cell scraper (Corning/Life sciences), when confluence reached 90% then transferred into a sterilin container (25 ml universal tube) and centrifuged at 300 g for 5 minutes.

The supernatant was discarded and the pellet was re-suspended in 10 ml DMEM medium. A 1 ml aliquot of this was then used to seed a fresh T75 culture flask containing 24 ml medium (i.e. the cells were routinely split to a surface area ratio of 1:10).

The rest of the cells were counted using a haemocytometer and cultivated in 96, 24, or 6 well plates at various concentrations according to the requirements of each experiment. Stock aliquots of J774 cells were kept frozen in 90% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) in cryovials placed in liquid nitrogen.

2.3.2. L929 cell line

The L929 cell-line (ATCC CCL-1) is a transformed murine fibroblast cell-line. These cells were seeded in DMEM supplemented with 10% FBS (Sigma) and 0.01% plasmocin (InvivoGen) and were typically passaged to one tenth density twice per week. Prior to use the cells were washed with phosphate buffered saline (PBS) (OXOID/England) then 5 ml trypsin (10x Trypsin/EDTA - Ethylene Diamine Tetra acetic Acid) was added for 2-3 minutes at 37^{o}C until they were fully detached. The contents of the flask were transferred into a universal tube and centrifuged at 300 g for 5 minutes after which supernatant was discarded and 10 ml medium was added to re-suspend the pellet. The remaining L929 cells were cultured in 96 well plates for use in TNF-α bioassays.

2.3.3. HEK-293 cell line

The TLR-deficient HEK-293 cell line (ATCC CRL-1573) is derived from human embryonic kidney. HEK-293 cells were grown in DMEM with 10% FBS and 0.01% plasmocin. The cells were split twice per week by the trypsinization method as described in 2.2.2. HEK-293 cells not used for maintenance cultures were plated in a 96 well plates at 3x10^{4} cells per well so as to perform the transfection assay for quantification of TLR-stimulants.

2.3.4. HepG2 cell line

The HepG2 cell line (ATCC CRL-10741) is a human hepatoma-derived cell line, which was isolated from the liver tissue of a 15-year-old white male with a well-differentiated hepatocellular carcinoma. These cells were grown in DMEM supplemented with 10% FBS and 0.01% plasmocin. The HepG2 cell line was split when confluence reached 70-80% by the trypsinization method as described in 2.2.2, except that after each split, the resuspended cells were passed through a 16 gauge needle using a syringe (10 ml/BD

Plastipak) 3-4 times, to disperse cells into single cell suspension and to avoid clumping (3D structures) of HepG2 cells (Figure 2.1). A 1 ml aliquot of this was then used to seed a fresh T75 culture flask containing 24 ml medium (i.e. the cells were routinely split to a surface area ratio of 1:10).

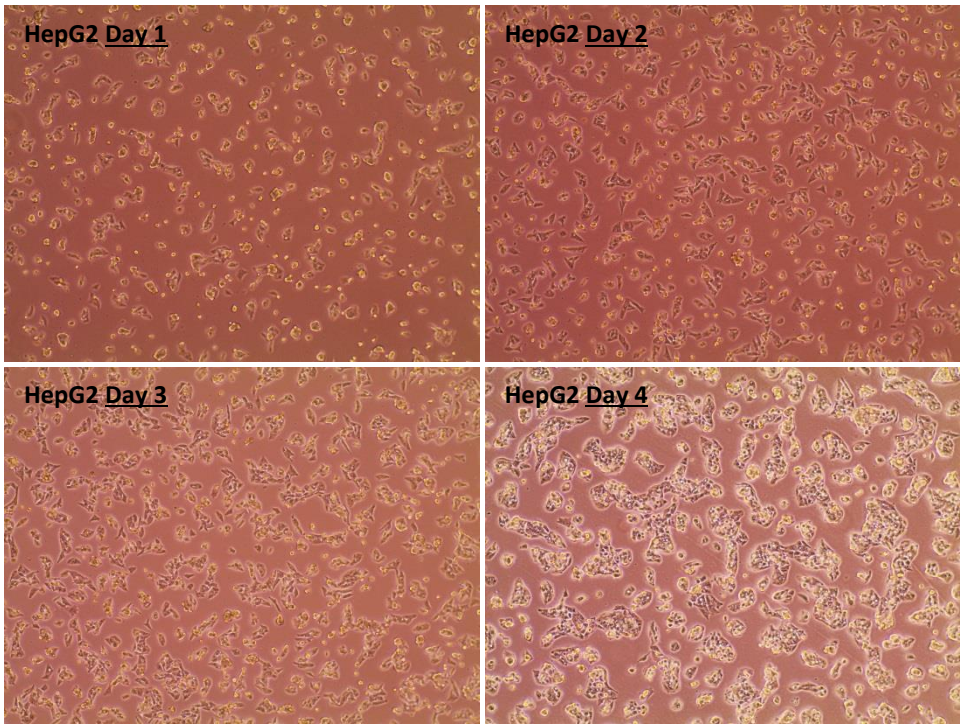


Figure 2.1: A confluent layer of HepG2 cells showing lack of clumping following disaggregation by the syringe technique (section 2.3.4).

2.3.5. Caco2 cell line

The human intestinal Caco-2 cell line (ATCC HTB-37) is originally obtained from a human colon adenocarcinoma and extensively used as a model of the intestinal barrier. These cells were seeded in DMEM supplemented with 10% FBS and 0.01% Plasmocin. The Caco-2 cell line was split when confluence reached 80-90% by the trypsinisation method as described in 2.2.2.

2.3.6. HeLa cell line

HeLa cells (ATCC CCL-2) are human cervical cancer cells, derived from human cervix. Hela cells were the first type of human cancer cell to be cultured continuously for *in vitro* experiments. These cells were grown in DMEM supplemented with 10% FBS and 0.01%

Plasmocin. The HeLa cell line was split when confluence reached 70-80% by the trypsinisation method as described in 2.2.2.

2.3.7. RAW 264.7 cell line

The RAW 264.7 cell line (ATCC TIB-71) is a transformed murine macrophage cell line. They are a commonly used model of mouse macrophages for the study of cellular responses to microbes and their products. These cells were grown in DMEM supplemented with 10% FBS and 0.01% Plasmocin.

The RAW 264.7 cell line was split when confluence reached 90% by cell scraper (Corning) then transferred into a sterilin container (30 ml universal tube) and centrifuged at 300 g for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 10 ml DMEM. A 1 ml aliquot of this was then used to seed a fresh T75 culture flask containing 24 mls medium (i.e. the cells were routinely split to a surface area ratio of 1:10).

2.4. Mouse strains, diets and IL-1R1 KO mice genotyping

Experiments were performed using wild-type C57BL/6J mice and mice with homozygous deficiency in the IL-1 receptor (IL-1R1$^{-/-}$). All mice used for these experiments were males, the line bred normally and showed no health defects. Two pairs from each of C57BL/6J and IL-1R1$^{-/-}$ mice were maintained as breeders without crossing to any other strains. All offspring from these mice were weaned as normal and fed a normal chow diet (Low fat diet) until 7 weeks of age, after which they were transferred to a high cholesterol (Test Diet 5TJT) or high fat (Test Diet 5TJN) diet. The "High-Cholesterol" diet contained approximately 40% of energy from fat, 44% from carbohydrate and 16% from protein, with 1% cholesterol added. The "High fat diet" provided 40% of energy from fat, 44% from carbohydrate, 16% from protein and cholesterol was added at 0.15%. 30% of the fat was from lard, 30% from butterfat, 30% from vegetable oil, 7% from soybean oil and 3% from corn oil. The low fat normal chow diet (Test Diet 5755) provided 22% of energy from fat, 60% from carbohydrate and 18% from protein.

All mouse experiments were conducted with both institutional and Home Office approval, under Home Office project licence reference 60/4332.

For genotyping, ear snips of IL-1R1$^{-/-}$ mice were collected in 0.5 ml eppendorfs for the DNA extraction, 70 µl of 0.05 M NaOH were added to each ear sample, ensuring one or two pieces were submerged, tubes were incubated on hot block at 95^{o}C for 30 minutes. Then, the samples were cooled to room temperature and briefly centrifuged. Next, 7 µl of 1M Tris (pH7.5) were added to each sample, gently mixed and the DNA samples were stored in fridge for PCR reaction.

In the PCR reaction, positive and negative controls (WT and IL-1R1$^{-/-}$ DNA) were included in every PCR run, 1 µl of DNA sample (extracted from the ear snips) as template to each reaction was mixed with 12.5 µl PCR ready mix, 9 µl H2O and 2.5 µl of 5 µM triple primers (Mutant forward, Wild type forward and Common reverse). The Rotor Gene Q RT-PCR cycler was programmed to apply denaturing step at 94°C, thermal clamping (10 cycles) at (94°C, 65°C, 68°C), standard PCR (28 cycles) at (94°C, 60°C, 72°C), extension at 72°C 2 min and hold at 10°C. Finally, the samples were run on 1% agarose gel (Figure 2.2).

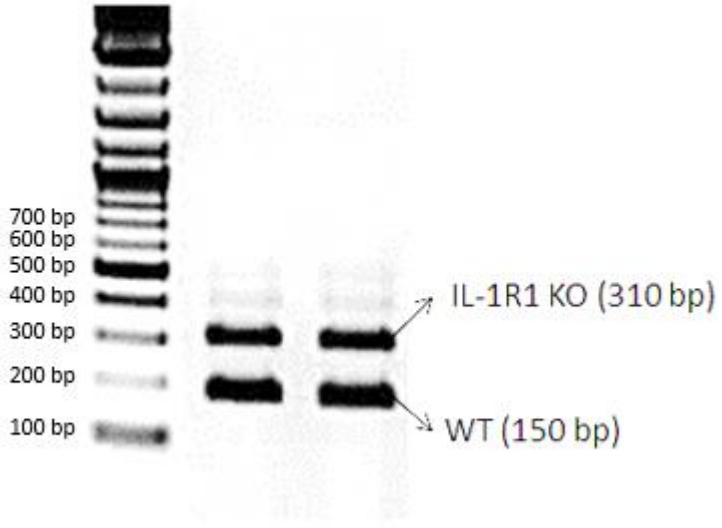


Figure 2.2: Typical band patterns obtained following agarose gel electrophoresis of PCR products used to monitor genotype of the IL-1R1 KO mouse colony.

2.5. J774 cell challenge and TNF-α bioassay

For macrophage challenge, 96 well plates were seeded with 100 µl of J774 cells resuspended at a density of 1 x 10^5 cells/ml and then incubated for 2-3 days at 37°C and 5% CO_2. Food extracts were diluted 1:10 in complete tissue culture medium (DMEM with 10% FCS), and 100 µl of this was then used to replace the medium of the J774 cells in each well. Cells were cultured with extracts for 3 hours (the timepoint of maximal TNF-α release), then supernatant (50 µl per well) was collected and transferred into a fresh 96 well plate then stored at -80 °C before assay.

For reasons of cost, TNF-α was routinely measured using the L929 cell bioassay. This assay makes use of the fact that L929 cells undergo apoptosis in the presence of TNF-α and the transcriptional inhibitor actinomycin-D. 96-well plates were seeded with 100 µl of L929 cells at a concentration of 4 x 10^5 cells/ml in medium which also contained 1 µg/ml actinomycin. These plates were then incubated for 3 hours at 37°C and 5% CO_2 to allow the cells to become adherent and a confluent monolayer to form. Then, a standard curve was prepared by adding 10 µl stock recombinant TNF-α (10 µg/ml) to 100 µl medium in the first well of one of the rows on the plate. 3.2 fold serial dilutions were then made in subsequent wells in the same row by transferring 45 µl from the first well to the next, mixing gently, and then repeating, while paying careful attention to avoid scratching the monolayer. This resulted in a 10 fold dilution every second well. Next, 10 µl of each macrophage culture supernatant was added to other wells in the same plate and incubated overnight at 37°C and 5% CO_2.

After an overnight incubation, the contents of each well were discarded and 50 µl crystal violet solution (0.5 g crystal violet powder in 20 ml methanol with 80 ml distilled water) were added to each well for 4 minutes. Subsequently, the plates were washed with running tap water and dried with a tissue. Then 100 µl of 20% acetic acid was added to each well to solubilise the remaining adherent cells. The absorbance of each well was then read at A562 nm using a Microplate Reader ELx800 (BIO-TEK/USA) to quantify the amount of TNF-α in each well by comparison with the standard curve.

A standard curve was created using recombinant TNF-α (from 100 ng/ml), which yielded a close correlation with absorbance when an exponential equation was applied to the points within the usable range of the curve (a typical example is shown in figure 2.3).

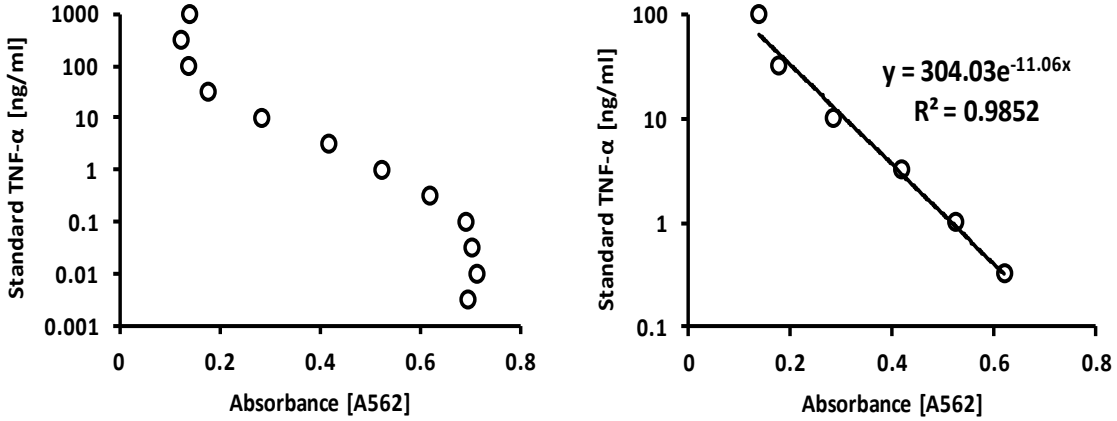


Figure 2.3: An example of a L929 bioassay TNF-α standard curve.

2.6. ELISA technique

Enzyme-linked immunosorbent assay (ELISA) was performed to detect and quantify specific antigens of interest in plasma or supernatants. There are three different formats of ELISA (Direct assay, Indirect assay and Capture assay "Sandwich") as shown in figure 2.4. The key steps in ELISA are the immobilization of the specific antigen of interest and the washing process after each step, which should be performed properly so as to remove the non-adherent antigen and decrease the background signalling.

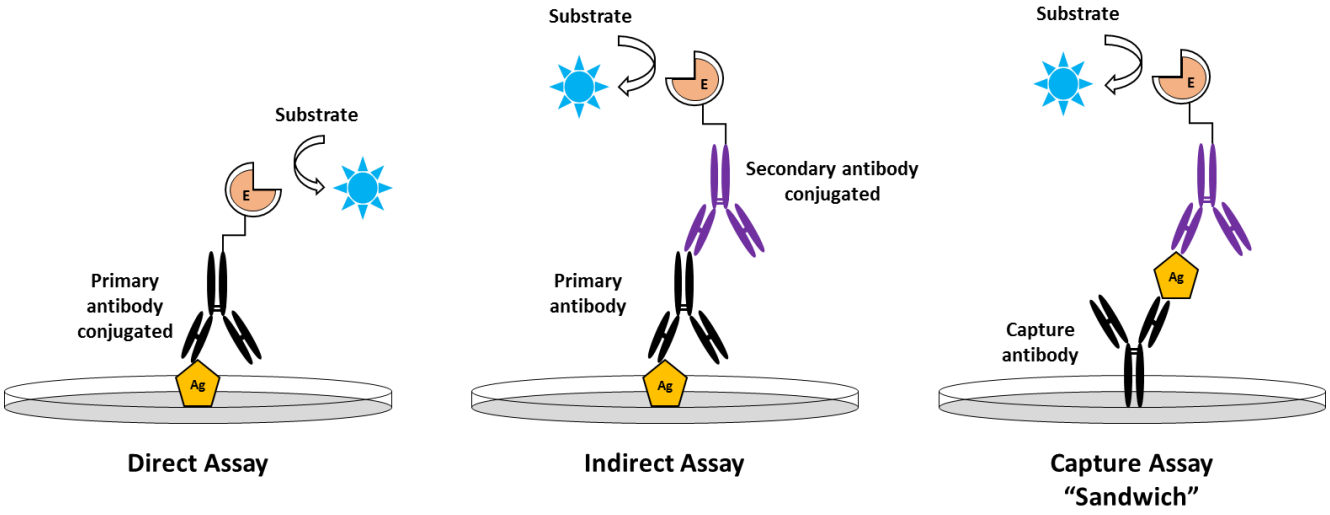


Figure 2.4: Different types of ELISA.

Three different ELISA methods (Direct, Indirect, and Sandwich) are shown. The ELISA technique is based on the recognition of an antigen from the sample through using a specific antibody (Detection Antibody). The reaction between an enzyme (e.g. HRP) conjugated to the antibody and the substrate (TMB) will give a visible color change which is proportional to the amount of antigen in the sample.

2.6.1. ELISA for measurement of pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β) produced by human whole blood, PBMCs and monocytes

Sandwich ELISAs were applied in our experiments in order to quantify IL-6, TNF-α, and IL-1β (R&D Systems Duoset kits) in the supernatant from whole human blood (diluted 1:20 in PBS for assay), human PBMCs (1:20) and human monocytes (1:100) challenged with food extracts. The supernatants were stored at - 80°C before assay.

Each assay was performed according to the manufacturer's instructions. Briefly, a high protein binding microtiter well strip (Nunc, Maxisorp) was used to plate capture antibody (diluted 1:200 in PBS) specific for each cytokine. The plate was then incubated in the dark overnight at room temperature. The next day, the plate was washed three times with PBS (300 µl per well) and then 300 µl reagent diluent (1% Bovine Serum Albumin, BSA, in PBS) was added to each well for one hour to block unbound sites. Subsequently, the plate was washed again and a standard curve was made using 2-fold serial dilutions in PBS with a starting concentration of 120 ng/ml for IL-6, 370 ng/ml for TNF-α, or 75 ng/ml for IL-1β.

The supernatant samples (frozen at - 80°C) were defrosted and added at a dilution of 1:10 in PBS for IL-6 and TNF-α measurement (1:4 for IL-1β), and the plate was then incubated for two hours. After that the plate was washed three times with PBS and 100 µl of the detection antibody diluted 1:200 in PBS / 1% BSA was added to each well for two hours before washing the plate three times again. Then streptavidin- horseradish peroxidase (HRP) was diluted 1:200 in PBS / 1% BSA and 100 µl of this was added to each well for 20 minutes. The final wash was performed and then 100 µl/well of the substrate [Tetramethyl benzidine (TMB)] (SIGMA) was added for 20 minutes. Finally, to stop the reaction 100 µl/well stop solution (2% H_2SO_4) was added after which the optical density was read at 450 nm by Microplate Reader ELx800 (BIO-TEK/USA). Standard curves were created using Microsoft Excel to allow calculation of the quantity of IL-6, TNF-α, and IL-1β in each well.

2.6.2. Assessing pro-inflammatory cytokine (IL-6, TNF-α, and IL-1β) production by murine liver slices and primary kupffer cells by ELISA

ELISAs were performed to quantify the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β (R&D Systems Duoset kits) in the collected supernatant from treated murine liver slices with or without 1 µg/ml *E. coli* LPS of three groups of wild-type C57BL/6 mice fed a normal chow (LFD), high fat diet (HFD) or HFD with intraperitoneal injection of 1 mg clodronate liposomes, for 4 weeks, and the supernatant from murine primary kupffer cells of wild-type C57BL/6 mice challenged with PAMPs (iEDAP, Pam3CSK4 or LPS, 100 ng/ml each). The supernatants were stored at - 80°C before assay.

Duoset ELISA was performed as in section 2.6.1, but the capture antibody was diluted 1:60 in PBS and the standard curve was prepared from a top concentration of 1 ng/ml for IL-6, 2 ng/ml for TNF-α, or 1 ng/ml for IL-1β. In addition, the supernatant samples (frozen at - 80°C) were defrosted and added at a dilution of 1:5 in PBS, also the detection antibody diluted 1:60 in PBS / 1% BSA and the HRP was diluted 1:50 in PBS / 1% BSA.

2.6.3. ELISA assay for measuring circulatory serum amyloid A (SAA) in mice

Abcam's Mouse Serum Amyloid A (SAA) ELISA kit was used to measure SAA in mouse plasma samples, according to the manufacturer's instructions. Briefly, ready supplied 96 well plate strips (abcam), that the anti-Serum Amyloid A antibodies was adsorbed to the surface of polystyrene microtiter wells was used. A standard curve was made using 2-fold serial dilutions in 1X diluent solution with a starting concentration of 2,000 ng/ml. The plasma samples (frozen at - 80°C) were defrosted and added at a dilution of 1:1,000 in 1X diluent solution, and the plate was then incubated at room temperature for sixty minutes. After that the plate was washed three times with 1X wash buffer and 100 µl of 1X Enzyme-Antibody Conjugate (anti-Serum Amyloid A antibodies conjugated with HRP) diluted 1:100 in 1X diluent solution was added to each well and incubated at room temperature for thirty minutes.

Next, the plate was washed three times with 1X wash buffer and 100 µl/well of TMB Substrate Solution was added into each well for ten minutes in the dark at room temperature. After ten minutes, 100 µl of stop solution was added to each well after which the optical density was read at 450 nm using a microplate reader ELx800 (BIO-TEK/USA). Standard curves were created using Microsoft Excel to allow calculation of the quantity of SAA in each well.

2.6.4. Evaluating serum CRP levels in healthy human volunteers by ELISA

DuoSet ELISA kits (R&D) were used to quantify CRP in human plasma samples. Plasma was prepared from blood samples by centrifugation (1,000 g, 15 mins), aliquoted and stored at - 80^{o}C before assay.

Duoset ELISA was performed as in section 2.6.1, but the capture antibody was diluted 1:200 in PBS and the standard curve was prepared from a top concentration of 1000 pg/ml. In addition, the plasma samples (frozen at - 80°C) were defrosted and added at a dilution of 1:2000 in PBS, also the detection antibody diluted 1:200 in PBS / 1% BSA.

2.6.5. ELISA technique for measuring plasma leptin concentrations in healthy human volunteers

Leptin concentrations were measured in human plasma samples by ELISA (R&D DuoSet). Plasma was prepared from blood samples by centrifugation (1,000 g, 15 mins), aliquoted and stored at - 80^{o}C before assay.

Duoset ELISA was performed as in section 2.6.1, but the capture antibody was diluted 1:120 in PBS and the standard curve was prepared from a top concentration of 2000 pg/ml. In addition, the plasma samples (frozen at - 80°C) were defrosted and added at a dilution of 1:2 in PBS, also the detection antibody diluted 1:60 in PBS / 1% BSA and the HRP was diluted 1:40 in PBS / 1% BSA.

2.6.6. ELISA assay for evaluating insulin levels in healthy human volunteers

Human insulin ELISA kit (Millipore) was used to measure insulin concentration in the human volunteer's sera for the chronic dietary PAMPs intervention study, according to the manufacturer's instructions. Briefly, all reagents were pre-warmed to room temperature immediately before setting up the assay. 10X concentrated HRP wash buffer (50 mM Tris Buffered Saline containing Tween-20) were diluted 1:10 with 450 mL deionized distilled water. Then, required number of strips were removed from the Microtiter Assay Plate (pre-coated with mouse monoclonal anti-human insulin antibodies). The strips were assembled in an empty plate holder and each well was filled with 300 µl of diluted HRP wash buffer, then incubated at room temperature for 5 minutes. Plates were washed, then 20 µl of Matrix Solution (Charcoal treated human serum) was added to the NSB (Non-Specific Binding), Standard, and Control wells. Also 20 µl Human Insulin Standards (2, 5 10, 20, 50, 100, and 200 µU/mL) were added in the order of ascending concentration to the appropriate wells.

Sequentially 20 µl of the unknown samples were added in duplicate to the remaining wells. 20 µl Detection Antibody (Pre-tittered Biotinylated Monoclonal Mouse anti-Human Insulin Antibody) were added to all wells. The plate was covered with plate sealer and incubated at room temperature for 1 hour on a microtiter plate shaker (approximately 400 to 500 rpm). Wells were then washed 3 times with 300 µl Wash Buffer per well per wash. Then, 100 µl Enzyme Solution (Pre-tittered Streptavidin-Horseradish Peroxidase Conjugate in Buffer) was added to each well. The plate was covered with plate sealer and incubated at room temperature for 30 minutes on a microtiter plate shaker (approximately 400 to 500 rpm). Wells were then washed 3 times with diluted HRP Wash Buffer, 300 µl per well per wash. After that, 100 µl of substrate solution (TMB) was added to each well, 5 to 20 minutes. Blue colour were formed in wells of insulin standards with intensity proportional to increasing concentrations of insulin. 100 µl of Stop Solution (0.3 M HCL) was added, stopping the reaction and turning the blue colour to yellow. Absorbance was read at 450 nm using a Microplate Reader ELx800 (BIO-TEK/USA). Standard curves were created using Microsoft Excel to allow calculation of the quantity of leptin in each well.

2.7. TLR transfection and luminescent reporter assays

HEK-293 cells were used for TLR-transfection assays because they are readily transfected and do not endogenously express most TLRs. HEK-293 cells were resuspended to 3 x 10^5 cells/ml in complete medium and transfected with mixed plasmid [pCD14 (30 ng per well), pNF-kB-ELAM (10 ng), pRL-TK (10 ng), and either pTLR4/MD2 (30 ng) or pTLR2 (10 ng), depending on whether LPS or BLP was to be quantified, respectively] using GeneJuice (Novagen/USA), then cultured in ninety-six well plates. After that, the plate was incubated for 2-3 days at 37°C and 5% CO_2 to allow full expression of each gene.

Later, eight wells in duplicate from the transfected cells were challenged with 3.2-fold dilutions of standard from a top concentration of 100 ng/ml to 0.1 ng/ml as a lowest concentration using LPS from *E. coli* (O111:B4) as the standard for quantifying TLR4 stimulants, or alternatively the synthetic BLP (Pam_3CSK_4) for quantification of TLR2 stimulants. The food extract samples were diluted 1:10 with DMEM plus 1% FBS and then added to the transfected cells in remaining wells. 4 wells received medium alone as unchallenged controls. The plates were incubated at 37°C and 5% CO_2 for 18 hours.

Finally, the cells were lysed by lysis buffer (25mM Tris, 8mM $MgCl_2$-$6H_2O$, 1mM DTT, 1% Triton, Glycerol, and dH_2O) and transferred into an opaque walled, transparent bottom, luminometer plate (Greiner), and read using firefly reagent (Lysis buffer, 1mM ATP, 0.25 mM Lucferin, and 1% BSA) in a NOVOstar device (BMG LABTECH/Germany). Subsequently, expression of the house-keeping gene promoter-driven renilla reporter was measured in duplicate plates using coelenterazine diluted to 2 µg/ml in PBS as substrate. Standard curves were prepared using Microsoft Excel to enable calculation of the abundance of TLR4 or TLR2 stimulants (the biological activity relative to LPS or Pam3CSK4) in each well.

2.8. Preparation of human PBMC and monocytes from whole blood

Blood samples were collected from volunteers after a consent form was signed and approval was obtained from the University Of Leicester College Of Medicine Research Ethics Committee for the experiments which include human subjects (reference NCT02430064). Blood was taken from a forearm vein by a phlebotomist on the day of the experiment and the blood was collected via using a vacutainer collection tube (BD Worldwide) containing sodium citrate in order to prevent coagulation.

After collection of blood, the tubes were taken directly into the cell culture laboratory and the same amount of sterilized PBS was added to each tube of blood, gently mixed and then gently layered over 3 ml of histopaque 1077 solution (polysucrose, 57 g/L, and sodium diatrizoate, 90 g/L, Sigma-Aldrich) in a 15 ml clear conical bottom centrifuge tube. The tubes were centrifuged at 800 g for 25 minutes, with acceleration set to setting 5 and deceleration to setting 2 (Allegra X-30R Centrifuge - Beckman Coulter / MEADOWROSE). The human red blood cells and the polymorphonuclear leukocytes were pelleted to the bottom of the tube with the histopaque layer above it. Between the plasma (top layer) and the histopaque was a thin, cloudy layer of the peripheral blood mononuclear cells (PBMCs, consisting mainly of monocytes and lymphocytes) which were collected gently by a Pasteur pipette. The collected cells were washed with sterilized PBS once by centrifugation at 300 g for 5 minutes (Acceleration 9 and Deceleration 9), and then re-suspended in RPMI plus 10% FBS, so as to achieve a concentration of 1 x 10^6 cells/ml, then cultured in a 6 well cell culture plate (2.5 ml per well), challenged with the food extracts and incubated for 18-24 hours at 37°C and 5% CO_2.

For preparation of purified monocyte cultures from PBMC, the cells were washed three times with sterilized PBS through centrifugation at 300 g for 5 minutes (Acceleration 9 and Deceleration 9) then re-suspended in RPMI plus 1% FBS, so as to achieve a density of 1 x 10^6 cells/ml, then plated in a 96 well plate (300 µl per well) and incubated for 1 hour at 37°C and 5% CO_2. The monocytes became adherent to the bottom of each well and the non-adherent or non-attached cells were discarded by washing them vigorously 5 times with medium (RPMI) inside each well. Another three times washing was done with

sterilized PBS (150 µl per well) to further purify the monocyte layers within each well, which were used for TLR inhibition experiments.

2.9. LAL assay

Limulus Amoebocyte Lysate is an aqueous extract of blood cells (amoebocytes) from the horseshoe crab (*Limulus polyphemus*). LAL contains enzymes that are activated in a series of reactions in the presence of endotoxin. The last enzyme activated in the cascade splits the chromophore [para-nitro aniline (pNA)] from the chromogenic substrate, producing a yellow colour, as shown in figure 2.5. The amount of pNA released and measured photometrically at 405 nm is proportional to the amount of the endotoxin in the sample.

The kinetic chromogenic Limulus Amoebocyte Lysate (LAL) assay (Associates of Cape Cod) was used to quantitfy endotoxins in the sterile filtered food extracts and in healthy human volunteers serum in both acute and chronic dietary PAMP intervention studies, after denaturation of limulus-activity masking proteins in plasma by heating at 70^{o}C for 10 minutes.

A standard curve was prepared in a 96-well plate using 2-fold serial dilutions of LPS from *E. coli* (O111:B4) in pyrogen free water with a starting concentration of 0.2 ng/ml. After that, the samples (stored at - 80°C) were defrosted and added at a dilution of 1:10,000 in pyrogen-free water for the sterile filtered food extracts and 1:10 for plasma samples, then added to the same plate beside the standard curve at 100 µl for each well in duplicate.

LAL reagent was prepared through adding 3.2 ml LAL reconstitution buffer to the pyrochrome vial and left it on ice with foil over top of vial for 5-8 minutes, then 30 µl of LAL reagent was added to each well of second, separate 96 well plate. A multichannel pipette was used to transfer the standards and the samples from plate 1 quickly onto the LAL reagent in plate 2. The plate was mixed on plate mixer for 20 seconds then incubated in a plate reader at 37°C. The plate was then read at 405 nm [NOVOstar device (BMG LABTECH/Germany)], every 5 minutes for up to one and half hours.

Standard curves based on time of each well to reach OD of 0.2 were created using Microsoft Excel to allow calculation of the quantity of endotoxin in each well.

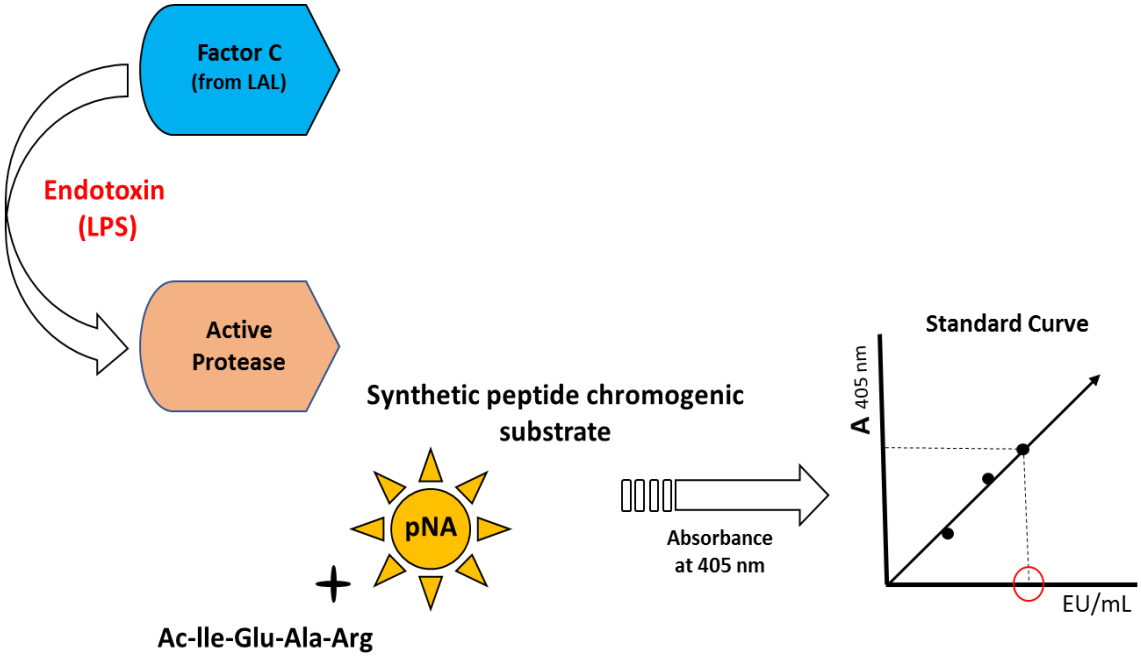


Figure 2.5: LAL Chromogenic Endotoxin Quantitation reaction scheme.

The Limulus Amoebocyte Lysate combined with a small volume of a sample, and endotoxins in the sample activate the proteolytic activity of Factor C. When the chromogenic substrate is added, the protease splits the p-nitroalinine (pNA), resulting in a yellow color that can be quantitated by measuring the absorbance at 405 nm and extrapolating against a standard curve.

2.10. Fluorescence microscopy for macrophage lipid droplets

The effect of food extracts on macrophage lipid accumulation was investigated using fluorescent microscopy. J774 macrophages were plated at a density of 1 x 10^4 cells per well of 96 well plates. Food extracts diluted 1:10 in medium, LPS (1 µg/ml) or Pam3CSK4 (10 µg/ml) were added into the wells. After three days incubation at 37°C and 5% CO_2, the medium was discarded and the cells were rinsed with PBS then 100 µl PBS containing 4% paraformaldehyde (PFA) was added to each well.

The plate was incubated at 4°C for 10 minutes, during which a fresh stock solution of Nile Red dye (9-diethylamino-5H-benzo[a]phenoxazine-5-one) was prepared by diluting a 1 mg/ml stock in acetone to 10 µg/ml in PBS. The Nile Red dye reveals intracellular lipid

droplets through giving a green color when examined under fluorescent microscopy without interacting with other cell components (Greenspan 1985).

The PFA was removed from the plate and replaced with 100 µl/well of Nile Red and the plate was incubated in the dark for 20 minutes. The cells were then washed gently twice with PBS (200 µl per well). After that J774 cells were treated with freshly prepared DAPI (4',6-diamidino-2-phenylindole) (SIGMA/Life Science) 0.125 µg/µl in PBS. DAPI is a specific nucleic acid stain that emits a blue color when observed under ultraviolet illumination. The plate was incubated at room temperature in the dark for 5 minutes then rinsed three times with PBS then directly examined by fluorescent microscopy (AMG/EVOS). Cells were examined at 40X magnification power using the following channels sequentially for detection of Nile Red (GFP - Green fluorescent protein - channel) and then DAPI (DAPI - Blue fluorescent channel). Three pictures were captured from each well at each wavelength. Overlay images were prepared using the EVOS microscope software with settings conserved for each treatment.

2.11.RT-PCR for quantification of gene expression

RNA was extracted for analysis of gene expression by RT-PCR from cell lines (primary and secondary), human PBMCs, mouse primary hepatocytes and mouse liver, adipose and aortic tissue.

2.11.1. RNA preparation

Total ribonucleic acid (RNA) was extracted from cells and tissue samples using the RNEasy RNA miniprep kit (QIAGEN) according to the manufacturer's instructions. Briefly, adherent cells were dislodged using the rubber end of a syringe (1 ml/BD Plastipak), then the content of each well was transferred into an eppendorf tube and pelleted (5,000 g for 2 minutes). The cell pellets were treated with lysis buffer RLT containing β-mercaptoethanol (β-ME), which has a crucial role in denaturing the RNases.

Bead-beater tubes (Precellys CK14 tubes with beads) were used to homogenise solid tissue samples. In these instances, 20-30 mg tissue samples, 350 µl RLT and 3.5 µl β-ME were added into Precellys CK14 tubes, the tubes were vibrated in a Precellys machine using

the pre-set liver tissue setting. Homogenized tissue samples were then cooled on ice before centrifugation at 4,000 rpm for 3 minutes, and the supernatant were carefully transferred into eppendorf tubes. Then, 70% ethanol was added to the lysate, so as to precipitate RNA over the membrane of the RNEasy mini column. After that, columns were washed with buffers to remove contaminants, and finally RNAse free water was used so as to elute purified RNA from the membrane of the RNEasy mini column into a nuclease free eppendorf. The RNA concentration of each extract was then quantified using a NanoDrop Spectrophotometer ND-8000 (Labtech/UK).

2.11.2. DNAse treatment of extracted RNA and cDNA preparation

DNAse treatment of RNA was carried out as a second major step for RNA purification to prevent non-specific amplification of contaminating genomic DNA in subsequent PCR experiments. The extracted RNA was treated with 10x DNase buffer and rDNase I (Ambion) which has the ability to digest deoxyribonucleic acid (DNA). The tube was incubated for 30 minutes at 37°C, after that the whole mixture was transferred into 0.2 ml tubes and mixed with DNase inactivation reagent (Ambion), then the mixture was centrifuged at 10,000 g for 1.5 minutes, the supernatant was moved into a new tube, then stored at -80 °C or the next step was performed.

In the next step the extracted RNA was converted to complementary deoxyribonucleic acid (cDNA) using reverse transcriptase (Superscript-III, Invitrogen) enzyme. 5 µl RNA was transferred into a nuclease free eppendorf tube, then 1 µl Oligo-dT_{20} (2.5 µM final concentration), 1 µl deoxyribonucleotide triphosphate (dNTP) mix (which contains dATP, dTTP, dCTP, dGTP, 10 mM each, Promega) and 7 µl RNAse free water was added to the tube, then mixed and heated at 65°C for 5 minutes in a heat block. The tube was then instantly on ice for 1 minute. Next, 4 µl of 5x first strand buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$), 1 µl of 0.1 M Dithiothreitol (DTT), and 1 µl of superscript-III enzyme (200 U/µl) were added to the tube and gently mixed then incubated at 50°C for 60 minutes.

After that the reaction enzymes were inactivated through raising the temperature to 70°C for 15 minutes. The prepared cDNAs were stored at -20 °C or used directly for Real Time - Polymerase Chain Reaction (RT-PCR).

2.11.3. Performing RT-PCR

Real Time - Polymerase Chain Reaction (RT-PCR) is considered to be a powerful technique for the detection and quantification of a gene of interest with high sensitivity because of the exponential amplification of the template and specific annealing of targeted primers. Three main steps of PCR (Denaturation, Annealing and Extension) were used for the purpose of amplifying a specific PCR product and this was achieved using a Rotor Gene Q RT-PCR cycler (QIAGEN), which measures fluorescence signals generated from intercalating SYBR green dye into double-stranded DNA as it is formed during the reaction.

A typical RT-PCR amplification plot is shown in figure 2.6. The Y axis denotes the fluorescence of the dsDNA as the product forms, and the X axis shows the PCR cycle numbers. The amplification proceeds exponentially and the point at which the fluorescence signals exceed a common threshold is known as threshold cycle (Ct).

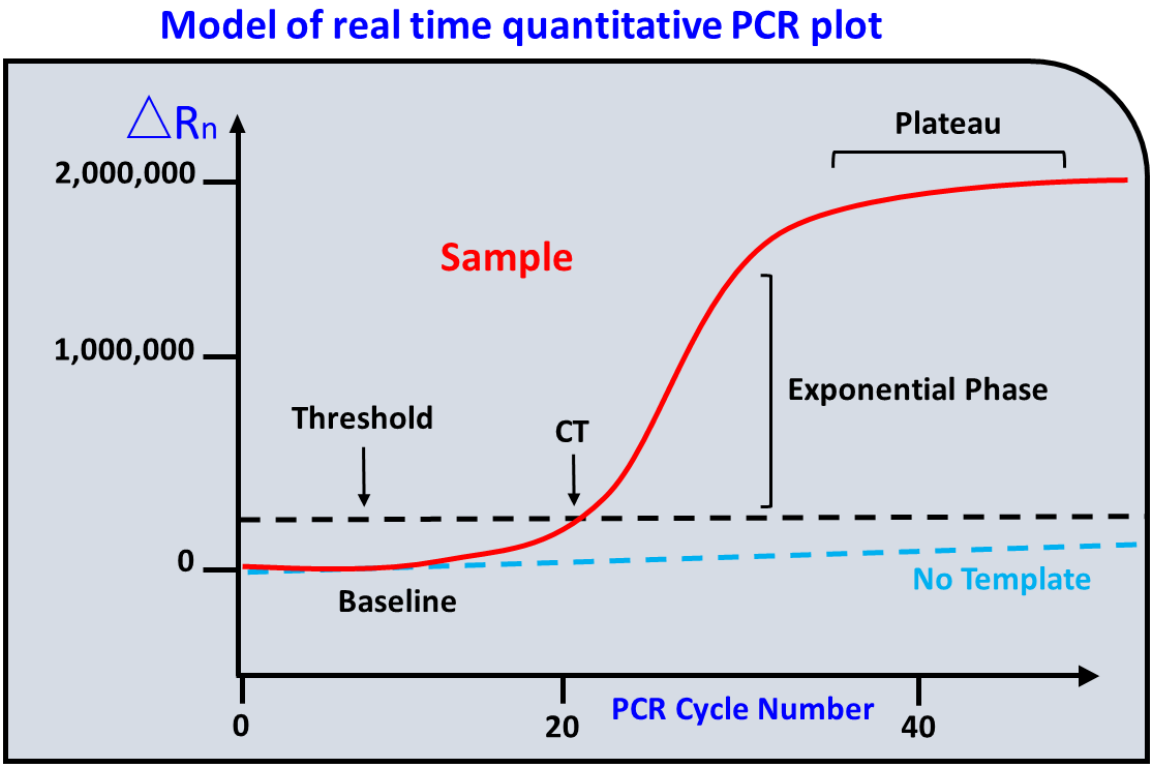


Figure 2.6: An example of RT-PCR plot.

The graph shows exponential product amplification after robust increase in the fluorescence signals. The signals were become detectable above the threshold level and this known as threshold cycle (Ct) for a product.

The primers used to amplify different genes for human and mouse are listed in tables 2.2a and b. They were designed via NCBI/PrimerBank website or from the literature. The specificity of each primer pair was tested by Agarose gel electrophoresis (Figure 2.8a and b). Human 36B4 or mouse β-actin was used as a positive control and as a housekeeping gene to normalize the expression of other genes in subsequent analyses. Also, no template control (NTC) and no reverse transcription (NRT) control reactions were included to detect contamination. The final volume per reaction was 25 µl consisting of 2 µl of template cDNA, 1.5 µl mixed forward and reverse primer (0.5 µM), 9 µl of Milli-Q water and 12.5 µl of the SensiMix SYBR Green (BIOLINE). The Rotor Gene Q RT-PCR cycler was programmed to apply two thermal stages, denaturation step at 95°C, annealing and extension at 60°C repeated for 45 cycles.

Relative gene expression was calculated for each cDNA sample and primer set using Rotor Gene software (QIAGEN). Briefly, this software captures the fluorescence of each individual sample, at every cycle of the PCR reaction, and plots an amplification curve for each individual sample. The software then plots the second differential of each curve to identify both the Ct point and the amplification efficiency of each individual reaction. In this way, expression of an mRNA species of interest can be accurately measured in all samples within a run by comparison with an internal control cDNA sample (which in this project typically derived from untreated cells). As reaction efficiency measured in this way accounts for variations in efficiency between primer pairs, this method is far superior to the commonly used $2^{\Delta CT}$ method, which assumes a reaction efficiency of 100%, that is very rarely achieved in practice. In our experiments, reaction efficiencies were consistent within runs (i.e. for the same primer pairs), and ranged between 85 and 95% (multiplier 1.7 to 1.9), which is within the range for accurate measurement. Gene expression is calculated relative to the first sample in the run, which was always control (i.e. comparative quantitation), taking into account the specific amplification efficiency of each primer set. Expression of the relevant housekeeping gene was also measured in each sample and compared to the same internal control cDNA sample. qPCR results are expressed in later chapters as expression of gene of interest in each sample divided by housekeeping gene for each sample.

To further validate PCR primer pairs, and to exclude the possibility of interference from non-specific products or primer-dimer formation, a melt curve was performed for every qPCR reaction (Figure 2.7). The existence of a single peak for the melt curve analysis in each experiment confirmed that a single product had been amplified (as confirmed by gel electrophoresis, see below).

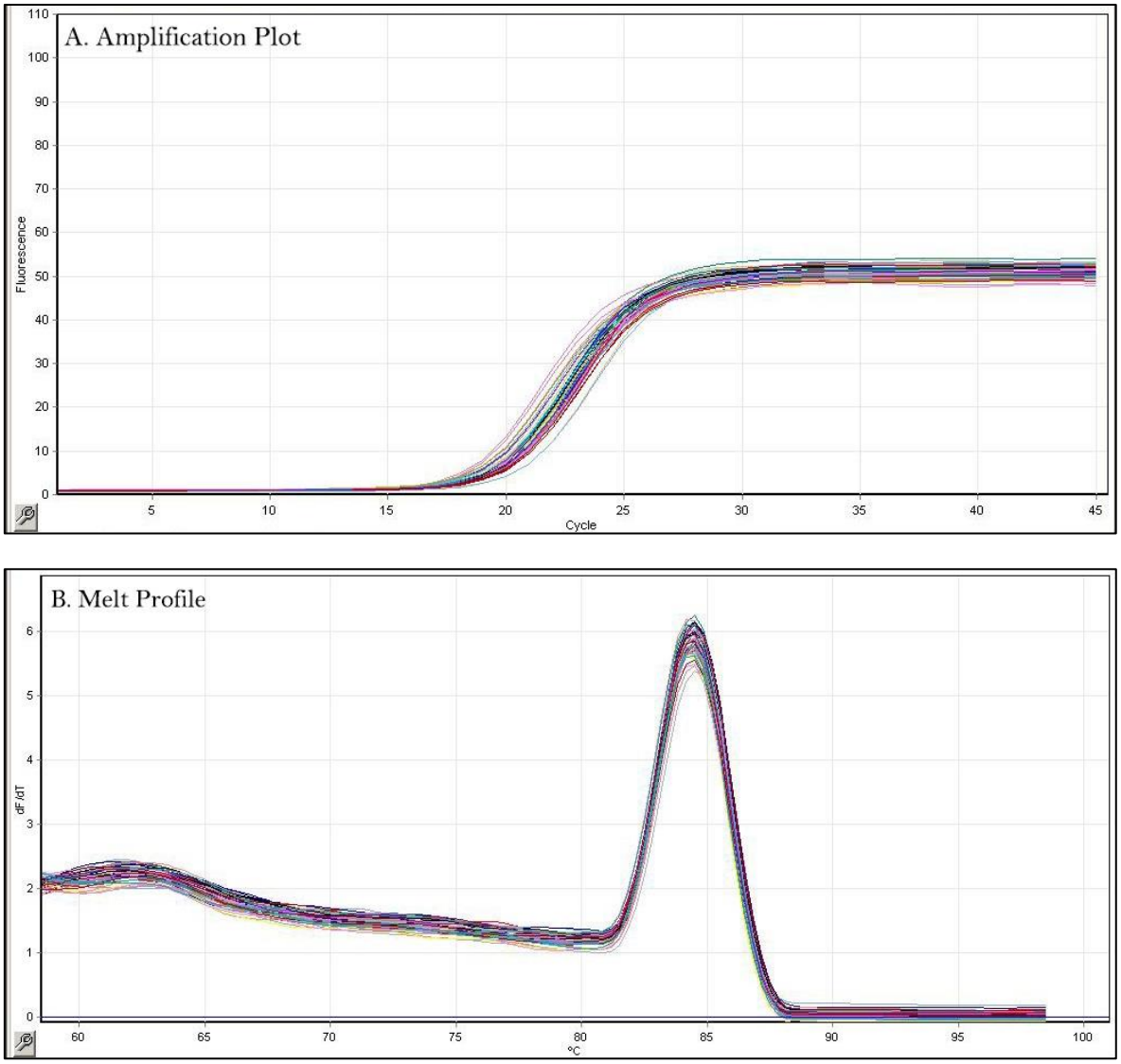


Figure 2.7: An example of Rotor Gene analysis in qPCR run (A. Amplification Plot and B. Melt Profile).

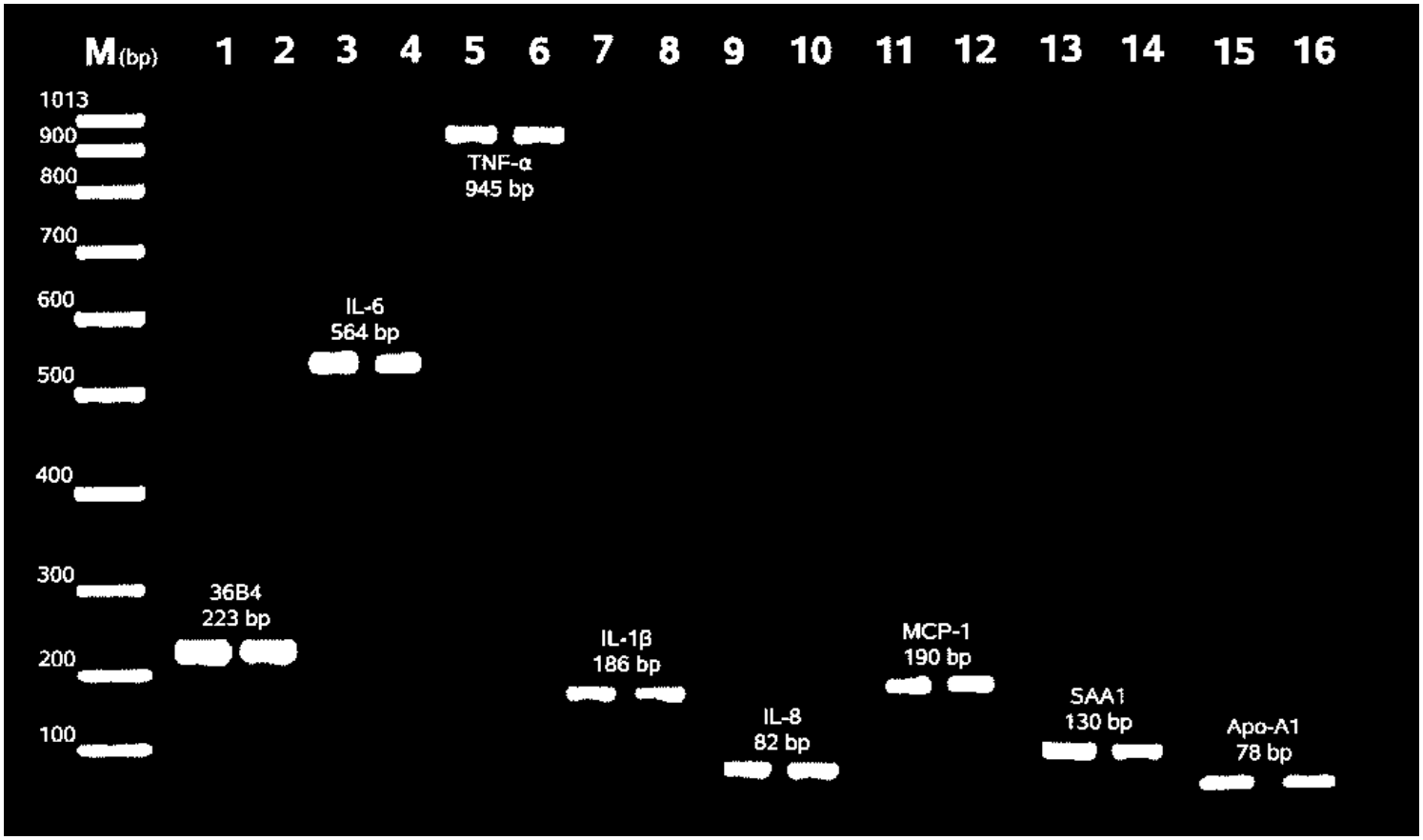


Figure 2.8a: Assessment of human primer pair specificity by agarose gel electrophoresis.

Agarose gel electrophoresis (2%) of the RT-PCR products were tested with eight primer pairs using cDNA of human PBMCs as template. Each primer pair was assessed individually in duplicate. Lane M: represents the DNA ladder marker (100 bp) and lanes 1-16: duplicate RT-PCR reactions using human primers specific to 36B4, IL-6, TNF-α, IL-1β, IL-8, MCP-1, SAA1, and Apo-A1 separately. The presence of a single product migrating at the correct size indicated a successful and specific primer design.

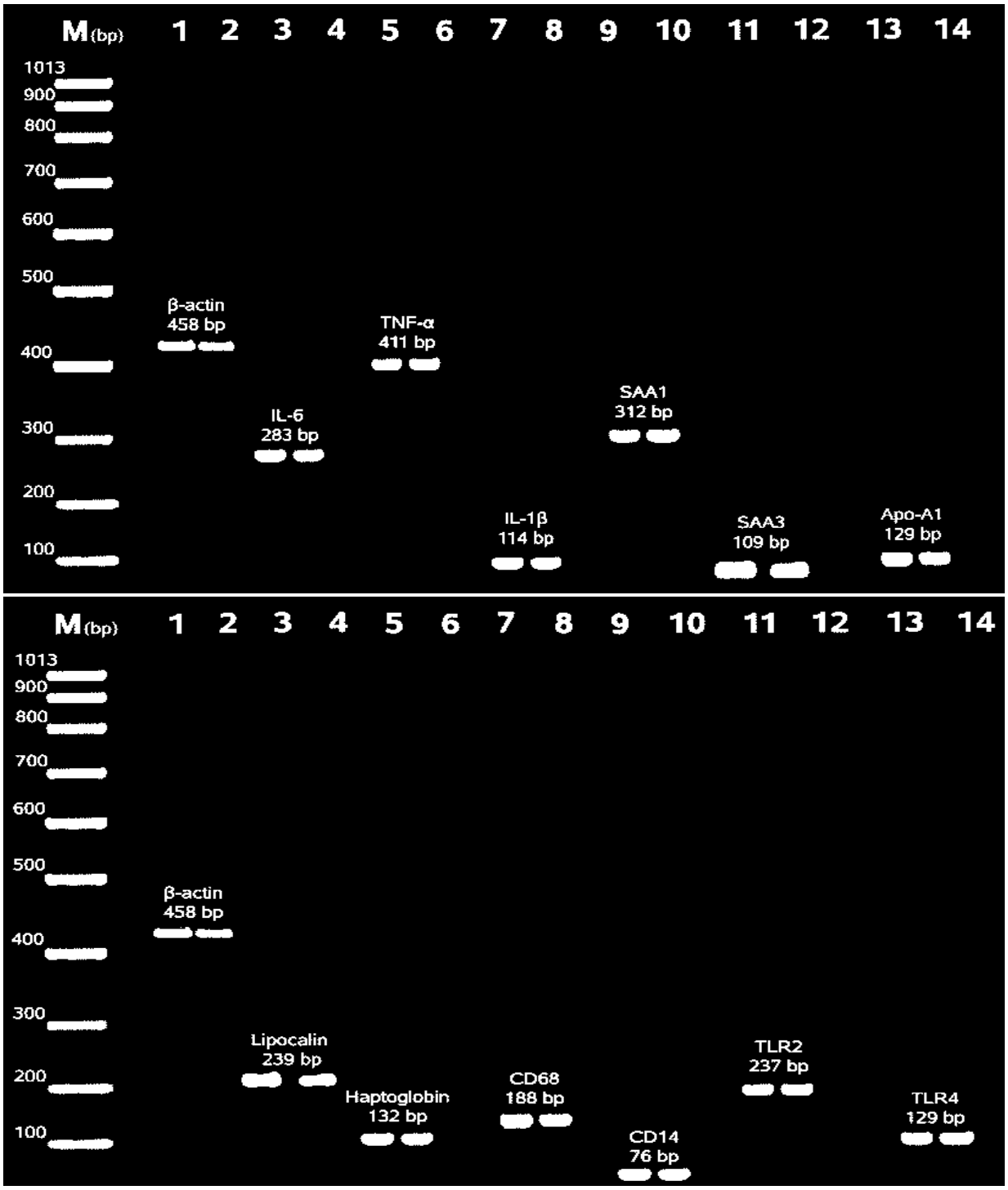


Figure 2.8b: Assessment of mouse primer pair specificity by agarose gel electrophoresis.

Agarose gel electrophoresis (2%) of the RT-PCR products were tested with thirteen primer pairs using cDNA of mouse liver as template. Each primer pair was assessed individually in duplicate. Lane M: represents the DNA ladder marker (100 bp) and lanes 1-26: duplicate RT-PCR reactions using mouse primers specific to B-actin, IL-6, TNF-α, IL-1β, SAA1, SAA3, Apo-A1, LCN, Hp, CD68, CD14, TLR2 and TLR4, separately. The presence of a single product migrating at the correct size indicated a successful and specific primer design.

Table 2.2a: List of human primers for real-time PCR study.

Primer pairs	Database access number	Sequence	Tm (°C)	Amplicon Size (bp)
36B4 **(forward)**	NM_001002	5'-TCGACAATGGCAGCATCTAC-3'	60.0	223
36B4 **(reverse)**		5'-GCCTTGACCTTTTCAGCAAG-3'	62.1	
IL-6 **(forward)**	NM_000600	5'-GCCTTCGGTCCAGTTGCCTT-3'	69.6	564
IL-6 **(reverse)**		5'-GCAGAATGAGATGAGTTGTC-3'	57.2	
TNF-α **(forward)**	NM_000594	5'-GGCCCAGGCAGTCAGAT-3'	64.0	945
TNF-α **(reverse)**		5'-CACAAGTGCAAACATAAATAGAGG-3'	61.3	
IL-1β **(forward)**	NM_000576.2	5'-AGCTACGAATCTCCGACCAC-3'	61.1	186
IL-1β **(reverse)**		5'-CGTTATCCCATGTGTCGAAGAA-3'	60.1	
IL-8 **(forward)**	NM_000584	5'-ATGACTTCCAAGCTGGCCGT-3'	60.1	82
IL-8 **(reverse)**		5'-TCCTTGGCAAAACTGCACCT-3'	62.5	
MCP-1 **(forward)**	NM_002982.3	5'-CAGCCAGATGCAATCAATGCC-3'	62.3	190
MCP-1 **(reverse)**		5'-TGGAATCCTGAACCCACTTCT-3'	60.4	
SAA1 **(forward)**	NM_000331.4	5'-GAAGTGATCAGCGATGCCAG-3'	59.06	130
SAA1 **(reverse)**		5'-CAGCAGGTCGGAAGTGATTG-3'	58.92	
Apo-A1 **(forward)**	NM_000039.1	5'-CCCTGGGATCGAGTGAAGGA-3'	62.6	78
Apo-A1 **(reverse)**		5'-CTGGGACACATAGTCTCTGCC-3'	61.6	

Table 2.2b: List of mouse primers for real-time PCR study.

Primer pairs	Database access number	Sequence	Tm (°C)	Amplicon Size (bp)
B-actin **(forward)**	NM_007393	5'-TTCTTTGCAGCTCCTTCGTTGCCG-3'	75.3	458
B-actin **(reverse)**		5'-TGGATGGCTACGTACATGGCTGGG-3'	74.1	
IL-6 **(forward)**	NM_031168	5'-AACGATGATGCACTTGCAGA-3'	60.1	283
IL-6 **(reverse)**		5'-GAGCATTGGAAATTGGGGTA-3'	60.8	
TNF-α **(forward)**	NM_013693	5'-TCCCCAAAGGGATGAGAAGTTC-3'	62.2	411
TNF-α **(reverse)**		5'-TCATACCAGGGTTTGAGCTCAG-3'	62.1	
IL-1β **(forward)**	NM_008361	5'-GCCTCGTGCTGTCGGACC-3'	61.5	114
IL-1β **(reverse)**		5'-TGTCGTTGCTTGGTTCTCCTTG-3'	61.4	
SAA1 **(forward)**	NM_009117	5'-TTCTGCTCCCTGCTCCTG-3'	61.0	312
SAA1 **(reverse)**		5'-GTAATTGGGGTCTTTGCC-3'	62.8	
SAA3 **(forward)**	NM_011315	5'-AGAGAGGCTGTTCAGAAGTTCA-3'	60.7	109
SAA3 **(reverse)**		5'-AGCAGGTCGGAAGTGGTTG-3'	61.9	
Apo-A1 **(forward)**	NM_000139	5'-GGCACGTATGGCAGCAAGAT-3'	62.9	129
Apo-A1 **(reverse)**		5'-CCAAGGAGGAGGATTCAAACTG-3'	60.3	
Lipocalin **(forward)**	NM_008491	5'-TGGCCCTGAGTGTCATGTG-3'	61.6	239
Lipocalin **(reverse)**		5'-CTCTTGTAGCTCATAGATGGTGC-3'	60.2	
Haptoglobin **(forward)**	NM_017370	5'-ACCTTAAACGACGAGAAGCAAT-3'	60.0	132
Haptoglobin **(reverse)**		5'-GGCATCCATAGAGCCACCG-3'	62.3	
CD68 **(forward)**	NM_009853	5'-CCAATTCAGGGTGGAAGAAA-3'	60.2	188
CD68 **(reverse)**		5'-CTCGGGCTCTGATGTAGGTC-3'	60.0	
CD14 **(forward)**	NM_009841	5'-ACTTCTCAGATCCGAAGCCAG-3'	61.3	76
CD14 **(reverse)**		5'-CCGCCGTACAATTCCACAT-3'	60.2	

TLR2 **(forward)**	NM_011905	5'-CTCTTCAGCAAACGCTGTTCT-3'	60.8	237
TLR2 **(reverse)**		5'-GGCGTCTCCCTCTATTGTATTG-3'	60.0	
TLR4 **(forward)**	NM_021297	5'-ATGGCATGGCTTACACCACC-3'	62.6	129
TLR4 **(reverse)**		5'-GAGGCCAATTTTGTCTCCACA-3'	60.5	

2.12. Preparation of OxPAPC for TLR inhibition

In order to investigate the potential role of TLR2 and TLR4 in the cellular response to food extracts, oxidized 1-palmitoyl-2-arachidonyl-sn- glycero-3-phosphorylcholine (OxPAPC) was prepared, since this is a specific inhibitor of TLR2 and TLR4 signals (Erridge *et al*, 2008). OxPAPC was prepared by aliquoting 100 µl of a 10 mg/ml native (unoxidised) PAPC stock, which was stored in chloroform under nitrogen in a sterile glass vial with a gas-resistant cap at - 80°C, into a glass vial. Streams of nitrogen gas were gently blown over the suspension to evaporate the chloroform and produce a thin film of phospholipid around the wall of the vial. Then the vial was wrapped with foil and kept in a dark place at room temperature for 3 days. The cap of the vial remained off during this incubation in order to allow the oxidation process to proceed. Finally, the OxPAPC within the vial was re-suspended with 500 µl chloroform ($CHCl_3$) to obtain a solution of stock OxPAPC (2 mg/ml) and this was stored at - 80°C. For use in experiments, aliquots of OxPAPC were dried under nitrogen flow in sterile eppendorfs, and then resuspended in warm tissue culture medium with vigorous vortexing for one minute before application to cells.

2.13. Human acute dietary study

Twelve healthy male volunteers participated in the acute dietary study. Subjects were asked to take two ibuprofen tablets (total 400 mg) before bed (day one), and another two early the next morning. Fasting blood samples (approximately 10 ml) were then taken from a vein in the forearm were collected by an experienced phlebotomist. Volunteers then consumed a high PAMP meal comprising 200 g of ready-chopped onion from supermarket, stored at refrigeration temperature until preparation and consumption on the advertised 'best before' date, battered with a flour/water mix, and deep fried in vegetable oil for 5-8

minutes (onion bhajis), supplemented with 30 g full-fat mayonnaise. Then, postprandial blood samples were collected at 3 h. After this sample has been taken, the volunteers requested to avoid drinking alcohol, taking any medications or supplements, or eating foods at high risk of containing pro-inflammatory molecules for lunch or dinner of that day (especially processed or minced meats, such as sausages and burgers, ready meals or ready chopped vegetables). On the next day (early morning) a second fasted blood sample was collected at 24 h post-meal (Figure 2.9).

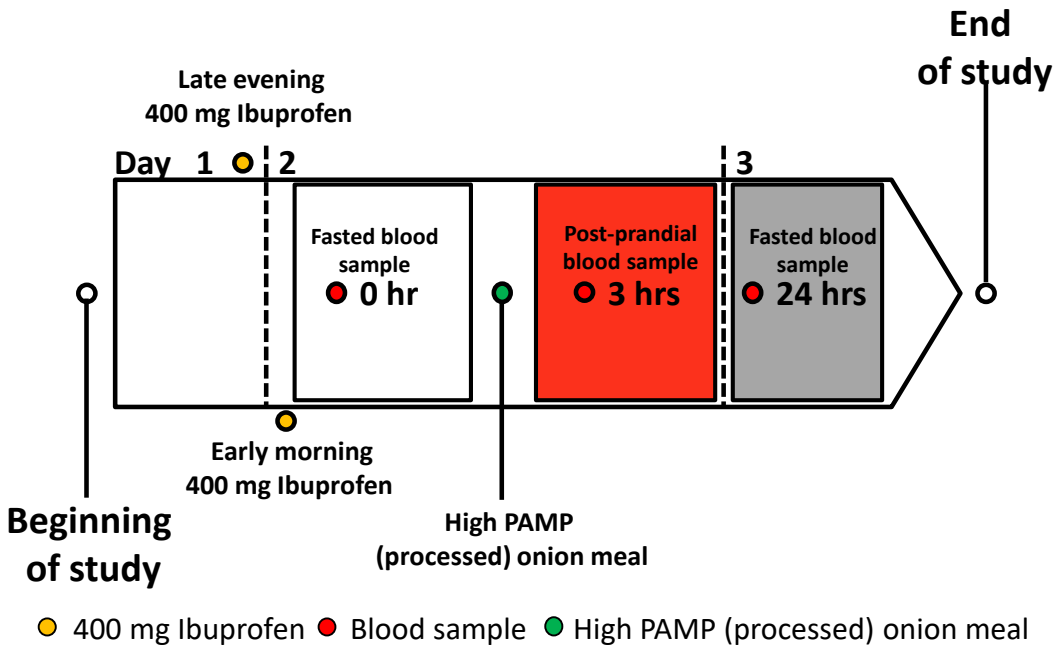


Figure 2.9: Schematic depiction of the acute study protocol.

The protocol for the acute dietary PAMPs study in ibuprofen primed healthy human volunteers is shown.

Baseline physical parameters of healthy volunteers are summarized in table 2.3. Inclusion criteria for this study were healthy men between the ages of 18 and 65. Exclusion criteria included evidence of any current inflammatory condition, infection or vaccination within two weeks prior to the study and use of medications (Appendix 1). Furthermore, the nutritional content of the onion bhaji test meal was calculated, as summarized in table 2.4. All volunteers gave informed consent (Appendix 2), and ethical approval for the study was granted by the University Of Leicester College Of Medicine Ethics Committee. The study was registered at clinicaltrials.org (reference NCT02430064).

Table 2.3: Baseline physical parameters of healthy volunteers.

Parameters	Acute study
Age (years)	**33.2 ± 4.1**
n (M:F)	**12:0**
Pulse Rate (bpm)	**68.67 ± 6.81**
Tympanic Temperature (°C)	**36.37 ± 0.41**
BMI (kg/m^2)	**25.6 ± 2.9**
Abdominal circumference (cm)	**94.4 ± 7.7**
White blood cell count ($10^9/L$)*	**5.87 ± 1.50**
Total cholesterol (mM)	**5.00 ± 1.03**
LDL-cholesterol (mM)	**3.40 ± 0.87**
HDL-cholesterol (mM)	**0.90 ± 0.54**
Triglycerides (mM)	**1.40 ± 0.84**

Indicated measurements are fasting values measured at entry to the study. Results are presented as mean ± SD. bpm, beats per minute; °C, Celsius degree.

* Whole blood cell counts were measured and recorded for each individual from the collected blood samples in EDTA tubes using a Beckman Coulter AcT Diff II Haematology Analyser.

Table 2.4: Nutritional content of the onion bhaji test meal.

Nutritional content	High PAMP Onion bhaji
Energy	**4,097 kJ**
Total fat	**60 g**
Saturated fat	**6 g**
Carbohydrate	**92 g**
Sugar	**24 g**
Fibre	**10 g**
Protein	**14 g**
Salt	**0.5 g**

2.14. Human chronic dietary PAMP intake study

Sixteen healthy male volunteers were recruited to take part in a chronic dietary PAMP intervention study, of which eleven completed the study (four developed respiratory infections, one withdrew from the study). Volunteers were asked to avoid specific types of food that we found in recent studies to be at relatively high risk of containing high levels of PAMPs for 7 days (Erridge 2010). These items included ready meals or sandwiches, cheese, chocolates and any other food containing minced meat or ready-chopped vegetables stored at refrigeration temperature for an extended period of time. Volunteers were requested to consume any quantity of fresh produce, including any form of meat, fish or vegetables that had not been minced or processed unless immediately before consumption, and there were no restrictions on salt, sugar or non-alcoholic beverages. Fasting blood samples (approximately 10 ml) were collected from a vein in the forearm by an experienced phlebotomist on day 0 (baseline) and after 7 days of low PAMP diet run-in. Then, over the next 4 days volunteers were asked to consume a set lunch and evening meal provided to them (Appendix 3), each selected on the basis of containing high levels of PAMPs (Erridge 2010), and each was consumed on or just before the 'best before' date advertised on the packaging. A third fasting blood sample was taken in the morning after the final day of the high PAMP diet (Figure 2.10). Throughout the study, subjects were asked to keep a quantitative diet diary and to avoid excessive alcohol consumption for the duration of the study.

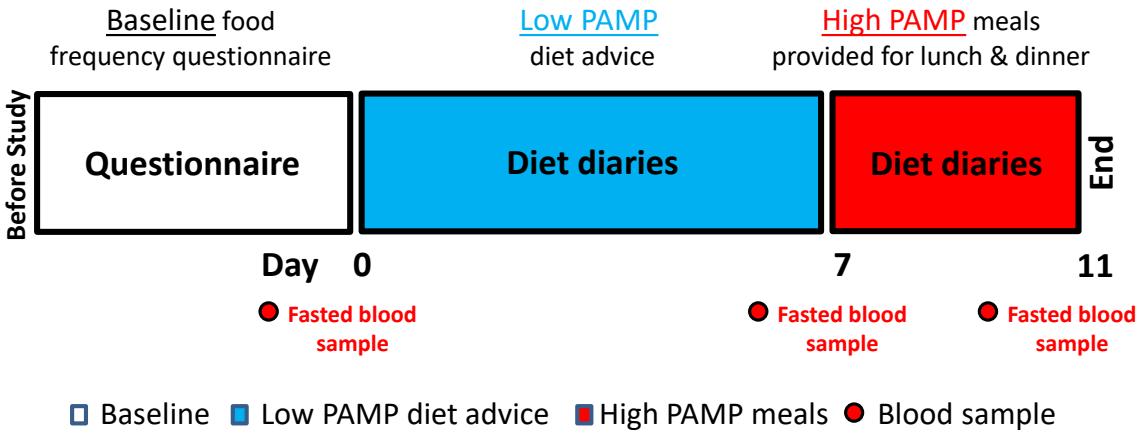


Figure 2.10: Schematic depiction of the chronic dietary PAMP study protocol.

The protocol for the chronic dietary PAMPs study in healthy human volunteers is shown. Information regarding low PAMP dietary advice, high PAMP meals and quantitative diet diaries to human volunteers are mentioned in the appendix section.

Baseline physical characteristics of healthy volunteers are summarized in table 2.5. Inclusion criteria for this study were healthy men between the ages of 18 and 65. Exclusion criteria included evidence of any current inflammatory condition, infection or vaccination within two weeks prior to the study and use of medications (Appendix 4). In addition, estimated nutritional content of diets based on typical daily intakes during low/high PAMP diets were calculated as summarized in table 2.6. All volunteers gave informed consent (Appendix 5), and ethical approval for the study was granted by the University Of Leicester College Of Medicine Ethics Committee. The study was registered at clinicaltrials.org (reference NCT02430064).

Table 2.5: Baseline physical parameters of healthy volunteers.

Parameters	Chronic study
Age (years)	**37.5 ± 10.0**
n (M:F)	**11:0**
Systolic BP (mm Hg)	**124.8 ± 11.2**
Diastolic BP (mm Hg)	**77.8 ± 9.5**
BMI (kg/m2)	**24.8 ± 2.1**
Abdominal circumference (cm)	**89.9 ± 7.3**
White blood cell count (10^9/L)*	**5.2 ± 1.7**
Total cholesterol (mM)	**5.72 ± 1.25**
LDL-cholesterol (mM)	**3.79 ± 1.23**
HDL-cholesterol (mM)	**1.45 ± 0.53**
Triglycerides (mM)	**1.06 ± 0.49**
Glucose (mM)	**4.77 ± 0.39**
Insulin (µU/ml)	**3.65 ± 2.19**
HOMA-IR**	**0.78 ± 0.038**

Indicated measurements are fasting values measured at entry to the study. Results are presented as mean ± SD. BP, blood pressure; HOMA-IR, homeostatic model assessment of insulin resistance.

* Whole blood cell counts were measured and recorded for each individual from the collected blood samples in EDTA tubes using a Beckman Coulter AcT Diff II Haematology Analyser.

** HOMA-IR value was calculated using this formula:
Fasting plasma glucose (mmol/l) X fasting serum insulin (mU/l) / 22.5.

2.15. Serum triglyceride analysis

Triglycerides (TG) consist of fatty acids esterified to glycerol, and are a major component of animal fat, vegetable oil, LDL and VLDL. Triglyceride assay (Wako) was used to measure triglyceride concentrations in healthy human volunteer's serum in both acute and chronic dietary intervention PAMP studies and in mouse plasma samples. A five point standard curve in duplicate of 75, 150, 300, 596, and 882 mg/dL triglyceride was made, then 5 µl of serum samples were added in triplicate in a 96 well plate. Triglyceride reagent was prepared by adding 105 ml of reaction buffer (RB) into the chromogen vial.

A multichannel pipette was then used to transfer 300 µl of triglyceride reagent buffer onto each sample. The plate was mixed on plate mixer then incubated in a plate reader at 37°C for 5 minutes. Finally, the plate was read at 600 nm by Microplate Reader ELx800 (BIO-TEK/USA). Standard curves were created using Microsoft Excel to allow calculation of the quantity of triglyceride in each well.

2.16. Serum cholesterol analysis

Amplex cholesterol assay (Invitrogen) was used to measure total cholesterol and Apo-B depleted lipoprotein (HDL) concentration in the mouse plasma samples and in healthy human volunteer's serum in both acute and chronic PAMPs dietary intervention study.

Polyethylene glycol (PEG) (Sigma) was used to deplete LDL/VLDL lipoproteins from plasma samples for measurement of HDL-cholesterol. 20% PEG solution was made with glycine buffer and added at a dilution of 1:2.5 to defrosted plasma samples which were stored at -80°C. The LDL/VLDL lipoproteins were pelleted at 10k rpm for 30 minutes at 4^{o}C and the supernatant containing the HDL fraction was separated and stored in eppendorf tubes.

The Amplex Red Cholesterol Assay Kit provides a simple fluorometric method for the sensitive quantitation of cholesterol using a fluorescence microplate reader. The assay is based on an enzyme-coupled reaction that detects both free cholesterol and cholesterol esters. Cholesterol assays were performed according to the manufacturer's instructions. Briefly, a cholesterol standard curve were made through diluting the appropriate amount of 2 mg/mL (5.17 mM) cholesterol reference standard into 1X Reaction Buffer to produce a top standard concentration of 8 µg/mL (20 µM). 1X Reaction Buffer without cholesterol was used as a negative control. The cholesterol-containing samples diluted in 1X Reaction Buffer were dispensed at 50 µl per well of a white-walled microtitre plate. A working solution of 300 µM Amplex Red reagent containing 2 U/mL HRP, 2 U/mL cholesterol oxidase, and 0.2 U/mL cholesterol esterase by adding 75 µl of Amplex Red reagent stock solution, 50 µl of the HRP stock solution, 50 µl of the cholesterol oxidase stock solution, and 5 µl of the cholesterol esterase stock solution was added to 4.82 mL of 1X Reaction Buffer.

Reactions were started by adding 50 µl of the Amplex Red reagent/HRP/cholesterol oxidase/ cholesterol esterase working solution to each microplate well containing the samples, standards and controls.

The microplate was incubated for 30 minutes at 37°C, protected from light. At the end of the assay the fluorescence were measured at 560 nm excitation and 590 nm emission in a NOVOstar device (BMG LABTECH/Germany). Standard curves were created using Microsoft Excel to allow calculation of the quantity of cholesterol in each well.

The Friedewald (1972) formula was used to estimate the LDL-C concentration:

LDL = TC - HDL - TG/5.0 (mg/dL)

2.17. Cholesterol efflux assay

The capacity of human or mouse Apo-B-depleted serum to accept cholesterol effluxed from macrophages was measured using a cholesterol efflux assay. The assay comprises three main stages:

Plating and labelling stage (Day one): J774 macrophages were plated at 100 µl of 6 X 10^5 cells/ml in RPMI/10%FCS. The cells were allowed to attach to the plate by incubation for 7 hours at 37°C and 5% CO_2. After that, the medium of the plate were replaced with labelling medium (20 µl of ^{3}H-cholesterol (1 MBq) (Perkin-Elmer) resuspended in 11.1 ml RPMI-1640 / 0.5% gentamicin / 1% FBS) and were incubated at 37°C and 5% CO_2 for 24 hours.

Equilibration stage (Day two): After 24 hours of incubation the contents of the plate were replaced with washing medium (RPMI-1640 with 0.1% Gentamicin [Gibco 50 mg/ml stock], without FBS), then equilibration medium at 100 µl per well (2% BSA in RPMI with 0.15 mM cAMP and 0.1% gentamycin) and incubated at 37°C with 5% CO_2 for 24 hours.

Efflux stage (Day three): Wells were washed once with washing medium, then each well received 100 µl efflux media (970 ml dH2O / 9.6 g MEM [Cellgro, Mediatech Inc] / 3.08 g HEPES / 5M NaOH to pH 7.4, 0.15 mM cAMP) containing 2.8 µl Apo-B-depleted serum per well. Plates were then incubated at 37°C without 5% CO_2 for 4 hours. Efflux medium with no serum supplementation and T0 cellular content (measure of radiolabeled cholesterol taken up by the cell) was used for the background correction.

After the 4 hours efflux stage, efflux medium was removed from each well and centrifuged in labelled eppendorf tubes at 13,000 g for 5 minutes to pellet cell debris. 75 µl of each supernatant was transferred into a numbered scintillation vial and vortexed to mix. The rest of the media was removed from the wells and the cells were lysed in the T0 (without serum) wells using 120 µl lysis solution (0.2 M NaOH / 0.1 % SDS) for 30 minutes on a rocker at room temperature. Later 90 µl of cell lysates were transferred to a scintillation vial containing 5 ml of scintillation fluid (Scintiverse (TM) BD Cocktail, Fisher) and vortexed to mix. The radioactivity were measured overnight using a Packard 1500 Tri-Carb Liquid Scintillation Counter. Measurements were recorded as counts per minute (CPM). Cholesterol efflux to HDL was calculated using the following formula:

(Sample average - without serum average) / T0 cellular average without serum

2.18. Glucose measurement assays

Amplex glucose assay (Invitrogen) was performed to measure glucose concentration in the healthy human volunteer's serum in the chronic dietary PAMP study. The Amplex Red Glucose Assay Kit provides a simple fluorometric method for the sensitive quantitation of glucose using a fluorescence microplate reader.

Glucose assays were performed according to the manufacturer's instructions. Briefly, a glucose standard curve was made by diluting the appropriate amount of 400 mM glucose stock solution into 1X Reaction Buffer to produce a top standard concentration of 200 µM. 1X Reaction Buffer without glucose was used as a negative control. The glucose-containing samples were diluted 1:50 in 1X Reaction Buffer and 50 µl was added to separate wells of a white-walled 96-well plate. A working solution of 10 µM Amplex Red reagent was prepared by adding 50 µl of Amplex Red reagent stock solution, 100 µl of the stock HRP solution, and 100 µl of the glucose oxidase stock solution to 4.75 mL of 1X Reaction Buffer. Reactions were started by adding 50 µl of the Amplex Red reagent/HRP/glucose oxidase working solution to each microplate well containing the samples, standards and controls using a multichannel pipette.

The microplates were incubated for 30 minutes at room temperature, protected from light. Finally, fluorescence was measured at 560 nm excitation and 590 nm emission using

a NOVOstar device (BMG LABTECH/Germany). Standard curves were created using Microsoft Excel to allow calculation of the quantity of glucose in each well.

2.19. Immunoblotting analysis of Apolipoprotein-A1 protein

Western blotting is a technique that is to identify proteins that have been separated from one another according to their size by gel electrophoresis, using specific antibodies. The blot is defined as a membrane which is almost always of nitrocellulose or PVDF (Polyvinylidene fluoride). The gel is placed next to the membrane and an electrical current induces the proteins in the gel to move to the membrane where they adhere. The membrane is then become a replica of the gel's protein pattern, and is subsequently stained with primary and secondary antibodies.

Immunoblot assays were used to measure Apolipoprotein-A1 protein in the plasma of wild-type C57BL/6 mice fed a high fat diet for 4 weeks with or without intraperitoneal injection of clodronate liposomes, which were orally gavaged with or without *E. coli* LPS. Serum samples were mixed 1:1 with lysis buffer (0.1M NaOH, 0.1% SDS in H_2O) and then 1:10 volume of 1M DTT was added to each sample. The plasma samples were heated at 100 ˚C for 2 minutes and kept on ice. Nupage precast gels (Fisher) were used for electrophoresis with Novex MOPS buffer (Fisher) as a running buffer using Novex Mini-Cell electrophoresis system (Invitrogen). Plasma samples were loaded alongside a protein ladder (PageRuler pre-stained molecular weight from 10 kDa to 170 kDa - Thermo Scientific), and separated by gel electrophoresis at 200 volts for 1 hour. Proteins from the gel were transferred to a nitrocellulose membrane (Whatman Protran BA85) using a wet tank system apparatus (XCell II - Invitrogen). Transfer was performed at 35 volts for 1.5 hours in Novex Tris/Glycine transfer buffer.

Next, the membranes were blocked with 2% BSA in wash buffer (PBST, PBS with Tween 20 at 1:2,000) for 2 hours at room temperature on a rocking platform. The membrane was then probed with primary antibody (Rabbit polyclonal IgG - Santa Cruz Biotechnology), overnight at 1:200 in 0.2% BSA at 4 ˚C on a rocking platform.

The following day, the membrane was washed with PBST (4 x 15 minutes/wash) at 4 ˚C on a rocker, and then probed with secondary antibody (Goat anti-rabbit IgG conjugated with HRP - Santa Cruz Biotechnology, at 1:4,000) for 2 hours in 0.2% BSA at 4˚C on a rocker. The membrane was then washed with PBST buffer as previously mentioned, and incubated in a chemiluminescence substrate solution (SuperSignal West Pico Chemiluminescence substrate solution kit - Thermo Scientific), containing H_2O_2 which leads to light production.

The membrane was visualized using an ImageQuant LAS 4000 chemiluminescent imager (General Electric) and quantified by densitometry analysis using Image Quant TL software. In addition, the albumin was measured in plasma using ponceau red stain, the membrane was immersed in Ponceau S Red solution (Sigma) for 1 minute, after which it was washed in distilled water until the protein lanes were visible.

2.20. Flow cytometry

Flow cytometry is a laser-based technology, which is widely used to analyse the expression of cell surface and intracellular molecules of single cells. It is mainly used to measure fluorescence intensity produced by fluorescent-labelled antibodies detecting proteins, or ligands that bind to specific cell-associated molecules. The staining procedure involves making a single-cell suspension from cell culture or tissue samples. The cells are then incubated in tubes with fluorochrome-labeled antibodies and analysed on the flow cytometer. Light scattered from the cells or particles is detected as they go through the laser beam. A detector in front of the light beam measures forward scatter (FS), which correlates with cell size and several detectors to the side measure side scatter (SS), which is proportional to the granularity of the cells. Therefore, cell populations can often be distinguished based on differences in their size and granularity. Fluorescence detectors measure the fluorescence emitted from positively stained cells or particles.

Flow cytometry was performed to quantify TLR2 and TLR4 exprssion on human cells (monocytes and HepG2 cells) and murine cells (RAW 264.7, hepatocytes and kupffer cells). The cells were resuspended to 1 x 10^6 cells/ml in PBS, then 100 µl aliquots in four different eppendorf tubes of the cell suspension were made for (no antibody, isotype control, anti-TLR2 and anti-TLR4). Human cells were stained with FITC-conjugated anti-TLR2 (TL2.5, 5 µl), anti-TLR4 (HTA125, 5 µl) and 2 µl isotype control antibody (anti-TLR3, stock was 2.5x higher

concentration than HTA125 and TL2.5). Mouse cells were stained with phycoerythrin (PE)-conjugated anti-TLR2 (2 µl), anti-TLR4 (2 µl) and isotype control antibody (rat IgG2a-PE, 2 µl). The cells were mixed gently and incubated for 30 minutes at 4°C. Subsequently, the cells were pelleted at 400 g for 5 minutes and resuspended again in 500 µl PBS.

Fluorescence of cells was measured using a Gallios flow cytometer (Beckman Coulter). Isotype control stained cells were assigned 2% positivity by adjustment of the threshold of positivity. Cells were gated according to forward and side scatter, and counts of positive cells and mean fluorescence intensity (MFI) were recorded. Finally, the data analysis were performed and the histograms were conducted by using Kaluza software.

2.21. Cryostat and fluorescent IHC staining

Fresh liver tissues were dissected from wild-type C57BL/6 mice and transferred into embedding moulds (Thermo Scientific) in a dry ice bath, then immersed in OCT embedding matrix before storage at -80°C. 7 µm sections of frozen liver tissues were cut using a cryostat (LEICA - CM3050S) machine and were mounted on poly-lysine histological slides (Thermo Scientific). Liver sections were dried at 37°C for 30 minutes, then the sections were fixed by adding 100 µl of cold acetone for 10 minutes at 4°C, and then air dried inside a fume hood for 20 minutes. A circle was drawn on the slide around the tissue sample using wax pen before antibody staining. First, blocking buffer (50 µl of 10% goat serum) was added to each section for 30 minutes at room temperature to block non-specific staining. Next, 80 µl of primary antibody (rat anti-mouse F4/80 for Kupffer cells or TLR2/TLR4 rabbit polyclonal antibody) were applied at 1:100 dilution in 4% goat serum and incubated overnight at 4°C in humidified chambers. Afterward, the slides were washed 3 times for fifteen minutes each with wash buffer (PBS), then 80 µl of secondary antibody (Alexa Fluor 488 goat anti-rat or AF594 goat anti-rabbit) was applied at 1:500 dilution in 4% goat serum, then incubated for 4 hours at 4°C in humidified chamber. The slides were washed again 3 times for fifteen minutes each with wash buffer (PBS).

At this point the slides were dried and the waxes were wiped off from the slide, then the coverslips were mounted onto the slide with Pro-long Gold anti-fade reagent

containing 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (Life technologies). The samples were then stored at room temperature for 24 hours to cure.

Subsequently, the liver tissue samples were imaged on LEICA (DM 2500) microscope, and images were captured and processed using a camera (LEICA - DFC 450C) and LAS V4.0 software.

2.22. Vibratome technique

The Microslicer DTK-1000 is a vibrating blade instrument that is used to cut sections of soft tissues like brain, liver or similar difficult-to-section material. This machine was used to prepare liver slices (at 300 micrometres thickness) from dissected liver samples of wild-type C57BL/6 and IL-1R1 KO mice (Figure 2.11). The whole liver was placed on ice to keep cold, then a metal punch (1 cm in diameter) was used to make cylinders of liver samples which were placed in RPMI medium + 10% FCS + L-glutamine + antibiotics. Next, the liver samples were attached to the sample mounting tray with a thin layer of ultra-strong glue. The Vibratome instrument was then used to obtain liver slices from non-frozen tissue samples. Finally, individual liver slices were collected from the tray of the Microslicer into wells of a 24-well plate, which were there challenged with PAMPs or cytokines and incubated for 24 hours in 37^{o}C incubator with 5% CO2.

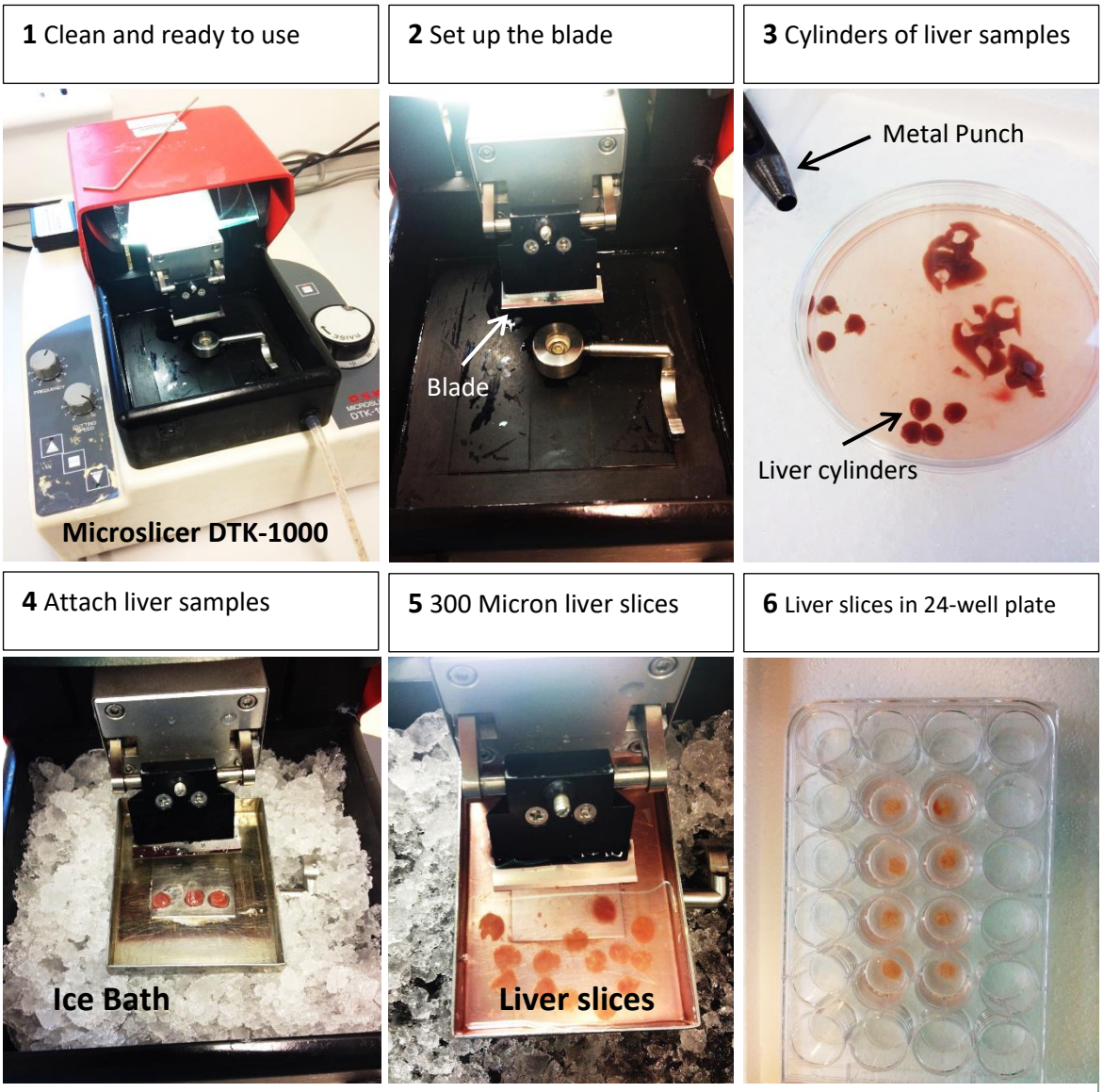


Figure 2.11: Method for vibratome based preparation of fresh liver slices.

The Microslicer DTK-1000 was cleaned before use (1), a sharp blade was attached to the blade holder (2), cylinders of murine liver samples were prepared using a metal punch (3), liver cylinders were glued onto the tray (4), 300 micron slices of liver were cut (5), and the slices were transferred to individual wells of a 24 well plate (6).

2.23. Perfusion method for murine primary hepatocytes/kupffer cells

Primary hepatocytes and kupffer cells were extracted from liver tissues of wild-type C57BL/6 and IL-1R1 KO mice by the three-step perfusion method (Figure 2.12), as follows:

Setting up perfusion assay: Clearing medium (1x HBSS, without magnesium or calcium, with 0.5 mM EGTA, pH to 7.4 NaOH), chasing medium (PBS, pH 7.4), digestion buffer (DMEM + HEPES + 0% serum + 0.5 mg/ml Sigma type IV collagenase), isolation medium (DMEM + HEPES + Pen/Strep + 10% serum at 4^{o}C) and growth medium (DMEM + HEPES + Pen/Strep + 0% serum) were prepared. The water bath to circulate warm water through perfusion apparatus allowed to warm to 37^{o}C before each experiment. Next, the perfusion pump was switched on to clean the plastic tubing first with 70% ethanol, then with PBS, before priming the system with warm clearing medium.

Perfusion of mouse liver: Mice were dissected so as to expose the peritoneal cavity and the portal and vena cava veins. A hole was made in the vena cava by a needle, then a ~2 mm cut was introduced into the top surface of the portal vein to allow insertion of the perfusion tubing. The tubing was then tied in place with a fine, strong surgery silk. Then, the clearing medium was pumped at a speed of 15-20 rpm (the liver colour lightens if perfusion is achieved), and the portal vein was cut to allow retrograde perfusion of the liver. Just before the pump reservoir runs out of clearing medium, 10 ml of chasing medium (PBS) was added to remove the EGTA. Then, just before the PBS runs out, the digestion buffer was replaced. Typically within 5-7 minutes the liver begins to swell, indicating successful collagen digestion.

Extracting cells from the perfused liver: The lobes of the liver were separated quite gently from the body of the mouse, leaving the gall bladder behind, and transferred into a dish containing 20 ml of isolation medium. The lobes were then teared by sterilised forceps and the released cells turned the medium cloudy. Next, the suspension was pipetted three times up and down in a serological pipette before straining through a cell strainer (Greiner) into a 50 ml Falcon tube.

Hepatocyte extraction centrifugation steps: The mixed liver cell suspension was centrifuged at 4^{o}C for two minutes at 50 g, such that the pellet contains hepatocytes and the supernatant contains Kupffer cells (processed separately, see next section). The hepatocyte pellet was resuspended in 25 ml of cold isolation medium and the centrifugation procedure was repeated twice more (2 minute, 50 g washes) to further purify the hepatocytes. Then, the pellet was resuspended gently with pipette in 25 ml cold isolation medium for cell counting (80 µl aliquot of the cell suspension mixed with 20 µl of 0.4% trypan blue for 1 minute) using a haemocytometer. Hepatocyte cell concentration was adjusted to 300,000 cells/ml, and 360 µl was seeded per well of collagen coated 24-well plates. Subsequently, cells were incubated for 1 hour at 37^{o}C in isolation medium so as to attach to the bottom of the plate. Then, the plates were rinsed once and replaced with the growth medium and the cells were incubated overnight at 37^{o}C. Afterward the hepatocytes were ready to be used in experiments.

Kupffer cell extraction centrifugation steps: The collected supernatant from the previous step was centrifuged at 50 g (4^{o}C) for 3 minutes, so as to pellet remaining hepatocytes. Then, the supernatant (not the pellet) was kept and centrifuged at 300 g (4^{o}C) for 5 minutes in another tube. Next, the Kupffer cells / liver sinusoid and endothelial cells (LSEC) pellet was resuspended in isolation medium and counted using a haemocytometer, then adjusted to 800,000 cells/ml ($8x10^{5}$). 1 ml per well of this suspension was seeded into each well of non-collagen coated 24-well plate. The cells were incubated at 37^{o}C for 16 minutes to allow the kupffer cells to adhere. Consequently, the cells were washed gently to remove the non-adherent cells such as liver sinusoid and endothelial cells. Finally, the medium was replaced with growth medium and the cells were incubated overnight at 37^{o}C. After that, the kupffer cells were ready to be used in experiments.

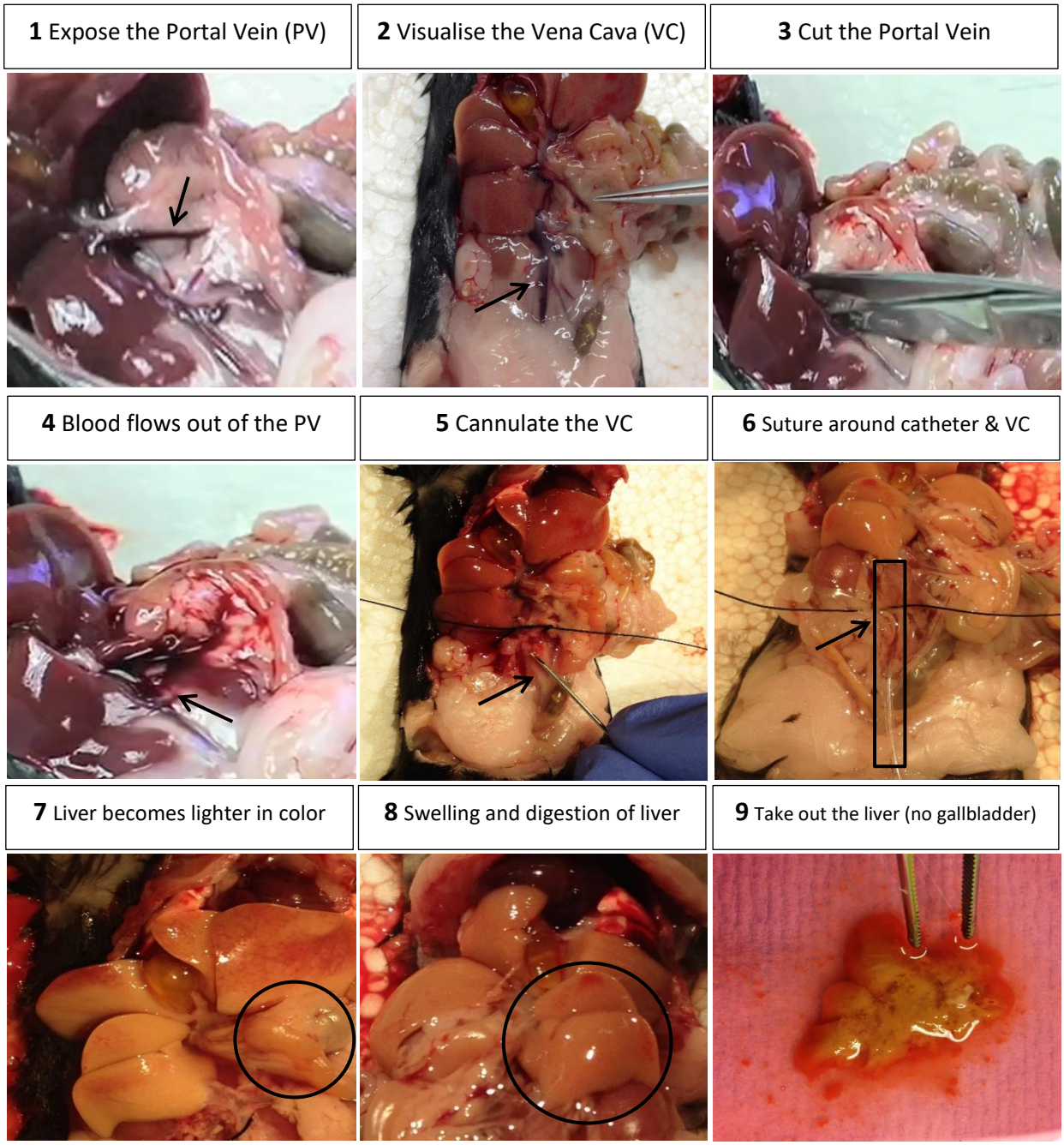


Figure 2.12: Steps involved in the murine hepatic perfusion method.

After exposing the peritoneal cavity to reveal the portal and vena cava veins (1 and 2), the portal vein was cut and blood flows from the PV (3 and 4), a needle was used to make a hole in the vena cava (5), then a fine clear plastic tube was inserted into the vena cava and tied in place with a fine silk (6), clearing medium was pumped into the liver (7), the liver begins to swell as the collagen is digested (8), and the lobes of the liver were separated quite gently from the body of the mouse (leaving the gall bladder behind) and transferred into a dish of isolation medium (9).

2.24. MTT cell viability assay

The methylthiazoletetrazolium (MTT) cell viability assay was used to determine the viability of Caco-2 intestinal epithelial cells cultured in the presence of various concentrations of food additives. In this assay, yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number and viability of living cells. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) using standard microplate absorbance readers.

Caco-2 cells were plated at $4x10^5$ cells/well of a 96 well plate, and incubated at 37°C for 24 hours. Subsequently, the medium was replaced with 100 µl of medium containing food additives at various concentrations (0.016, 0.08 and 0.4% wt/vol), and incubated at 37°C for 24 hours. Medium was then replaced with fresh DMEM/10%FCS and 10 µl of MTT solution (1 ml of sterile PBS was added to one 5 mg vial of MTT) and incubated at room temperature for 4 hours. Next, the content of the plate were removed gently and replaced with SDS-HCL solution (10 ml of 0.01 M HCl was added to one tube containing 1 g of SDS) and incubated at 37°C for 30 minutes. Finally, the absorbance was read at 562 nm using microplate reader.

2.25. Data Analysis

All experiments were performed at least in triplicate, unless otherwise stated in the results section. Microsoft Office Excel 2013 or GraphPad Prism 6 software was used in order to analyze the data obtained from all the experiments and to prepare charts. The results are stated as mean ± Standard Deviation (SD) or Standard Error of Mean (SEM). Multiple comparisons against control conditions were tested using the ANOVA test with Dunnett's test or Sidak's test for multiple comparisons. Also, unpaired student's t-tests with Bonferroni correction were used where two samples were compared. Statistical differences were deemed to be significant at $P < 0.05$.

Chapter 3 Results - Investigation of cellular responses to dietary PAMPs *in vitro*

3.1. Macrophage production of TNF-α in response to food extracts

Murine RAW 264.7 macrophage cells (1 x 10^5 cells/ml) were challenged with the canonical TLR2-stimulant Pam_3CSK_4 (10 µg/ml), the canonical TLR4 stimulant *E. coli* LPS (1 µg/ml) and twenty four sterile filtered food extracts separately diluted (1:10) in tissue culture medium. Supernatant was collected after 3 hours since this timepoint is established to coincide with peak release of bioactive TNF-α. Murine L929 fibroblast cells were used to quantitate bioactive TNF-α in the supernatant as described in the methods.

Pam_3CSK_4 and LPS induced a significant increase in the production of TNF-α (Pam_3CSK_4: 577.9 ng/ml and LPS: 859.3 ng/ml) when compared with the control (1.3 ng/ml). There was considerable variation in the amount of TNF-α produced in response to challenge with the sterile filtered food extracts. For example, fish pie was the most potent inducer of TNF-α production (735.3 ng/ml vs. control: 1.3 ng/ml; $P<0.001$), while sweet and sour chicken with rice was the lowest inducer of TNF-α production (1.3 ng/ml) which was similar to the control. Many other foods also induced very little TNF-α production. Interestingly, food extracts which consisted of minced meat and chopped onion such as spaghetti, minced beef and onion pie promoted a higher production of TNF-α (Spaghetti Bolognese: 194.5 ng/ml and minced beef and onion pie: 93.3 ng/ml) when compared with foods extracts which did not include these ingredients (Figure 3.1).

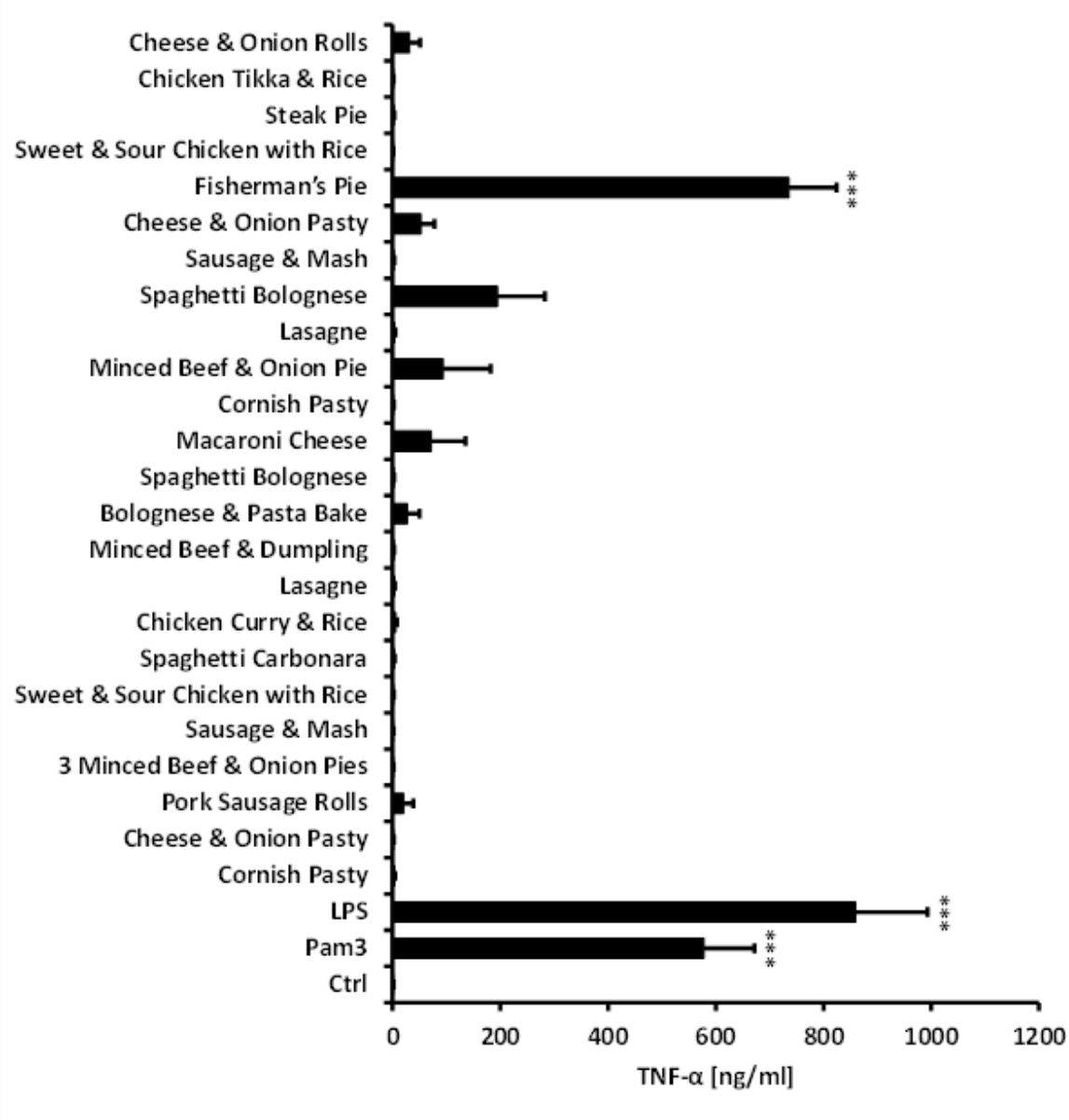


Figure 3.1: Macrophage TNF-α secretion in response to food extracts.

Murine RAW 264.7 macrophages were treated with sterile filtered food extracts diluted 1:10 in tissue culture medium. After 3 hours incubation, secretion of TNF-α was quantified from the supernatant by L929 bioassay. Results are expressed as means of 3 independent experiments ± SEM. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 *vs.* control cells cultured in medium alone).

3.2. Cytokine production by primary human leukocytes in response to food extracts

Enzyme-linked immunosorbent assay was used to quantify inflammatory cytokines (IL-6, TNF-α, and IL-1β) produced by human whole blood, PBMCs or primary monocytes. Human whole blood was challenged with sterile filtered food extracts for 18 hours, then the supernatant was collected and ELISA was performed so as to quantify inflammatory cytokines. Most of the food extracts promoted a significant rise in the production of inflammatory cytokines.

Minced Beef and onion pie was the most potent inducer of IL-6 production (20,831 pg/ml vs. control: 46 pg/ml; $P<0.001$) (Figure 3.2). On the other hand, cheese and onion rolls were the most effective inducers of IL-1β production (341 pg/ml vs. control: 12 pg/ml; $P<0.05$) (Figure 3.3). Cheese and onion rolls were also the strongest inducers of TNF-α production, although this response did not reach statistical significance when compared with the control (767 pg/ml vs. control: 225 pg/ml) (Figure 3.4).

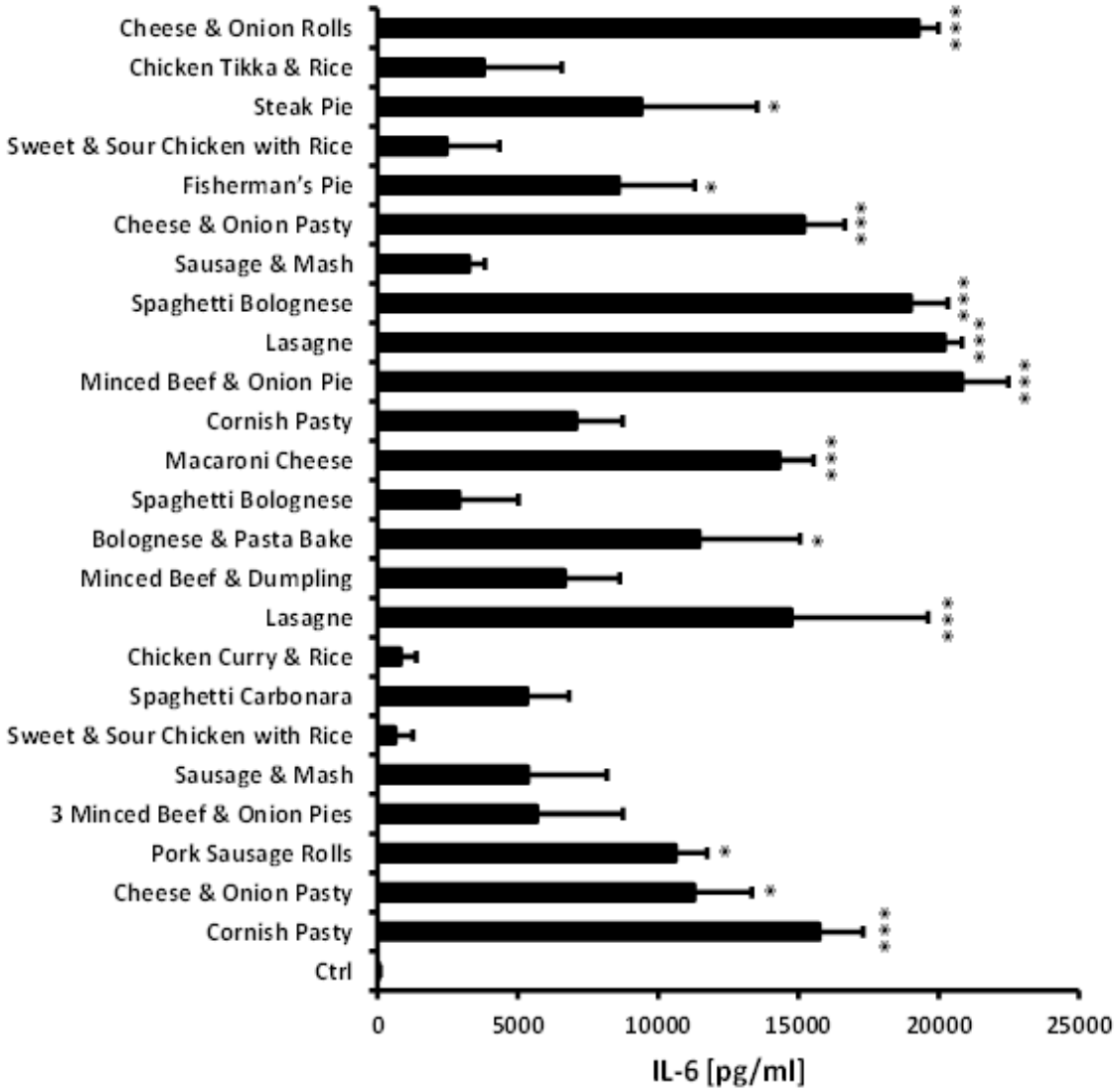


Figure 3.2: Production of IL-6 by human whole blood stimulated with food extracts.

Human whole blood was cultured with sterile filtered food extracts (diluted 1:20 in medium). After 18 hours incubation, secretion of IL-6 was quantified from the supernatant (plasma) using ELISA. Results are expressed as means of 4 independent experiments ± SEM. The lower limit of detection of IL-6 was 0.025 ng/ml. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 *vs.* control cells cultured in PBS).

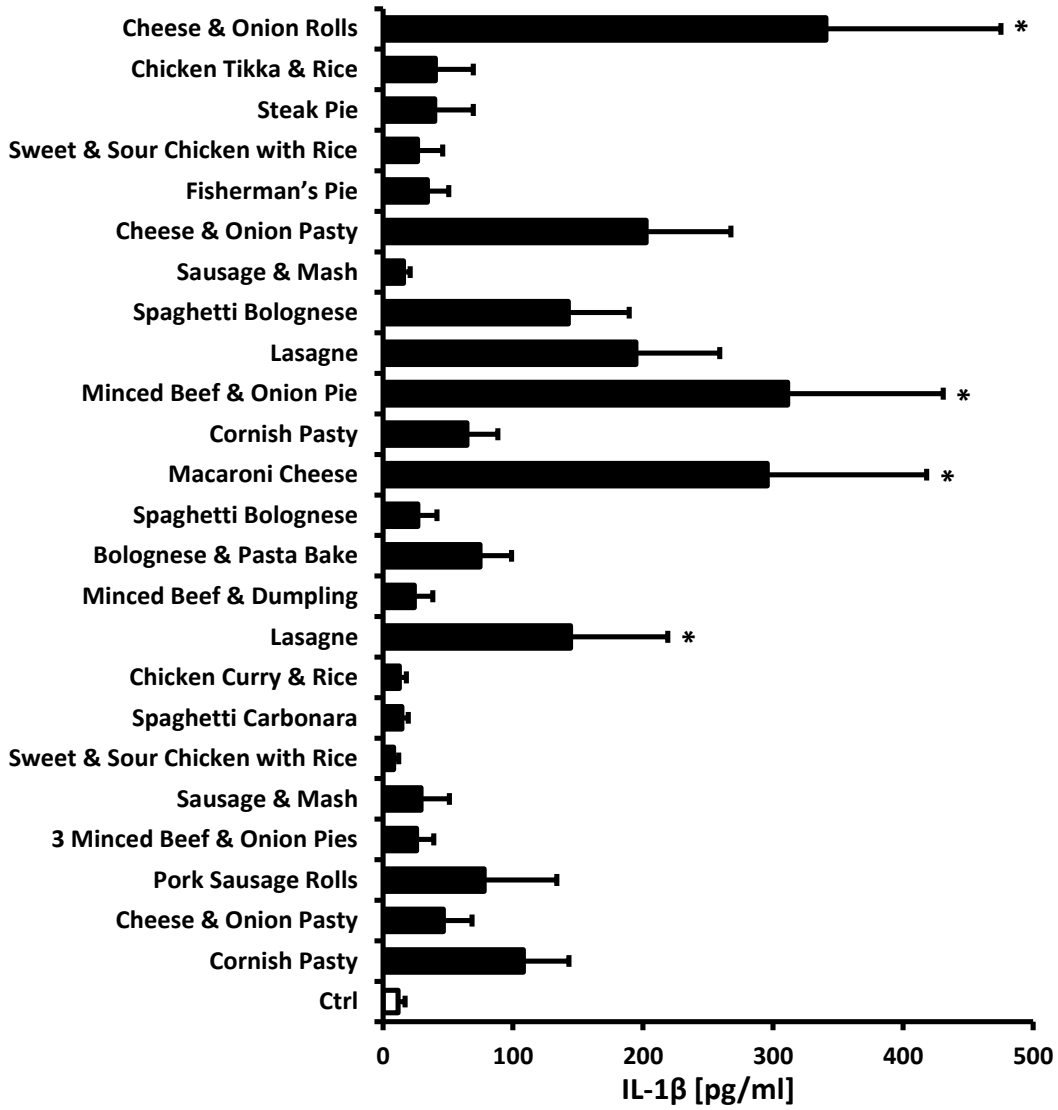


Figure 3.3: Production of IL-1β by human whole blood stimulated with food extracts.

Human whole blood was cultured with sterile filtered food extracts (diluted 1:20 in medium). After 18 hours incubation secretion of IL-1β was quantified from the supernatant (plasma) using ELISA. Results are expressed as means of 4 independent experiments ± SEM. The lower limit of detection of IL-1β was 0.035 ng/ml. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 *vs.* control cells cultured in PBS).

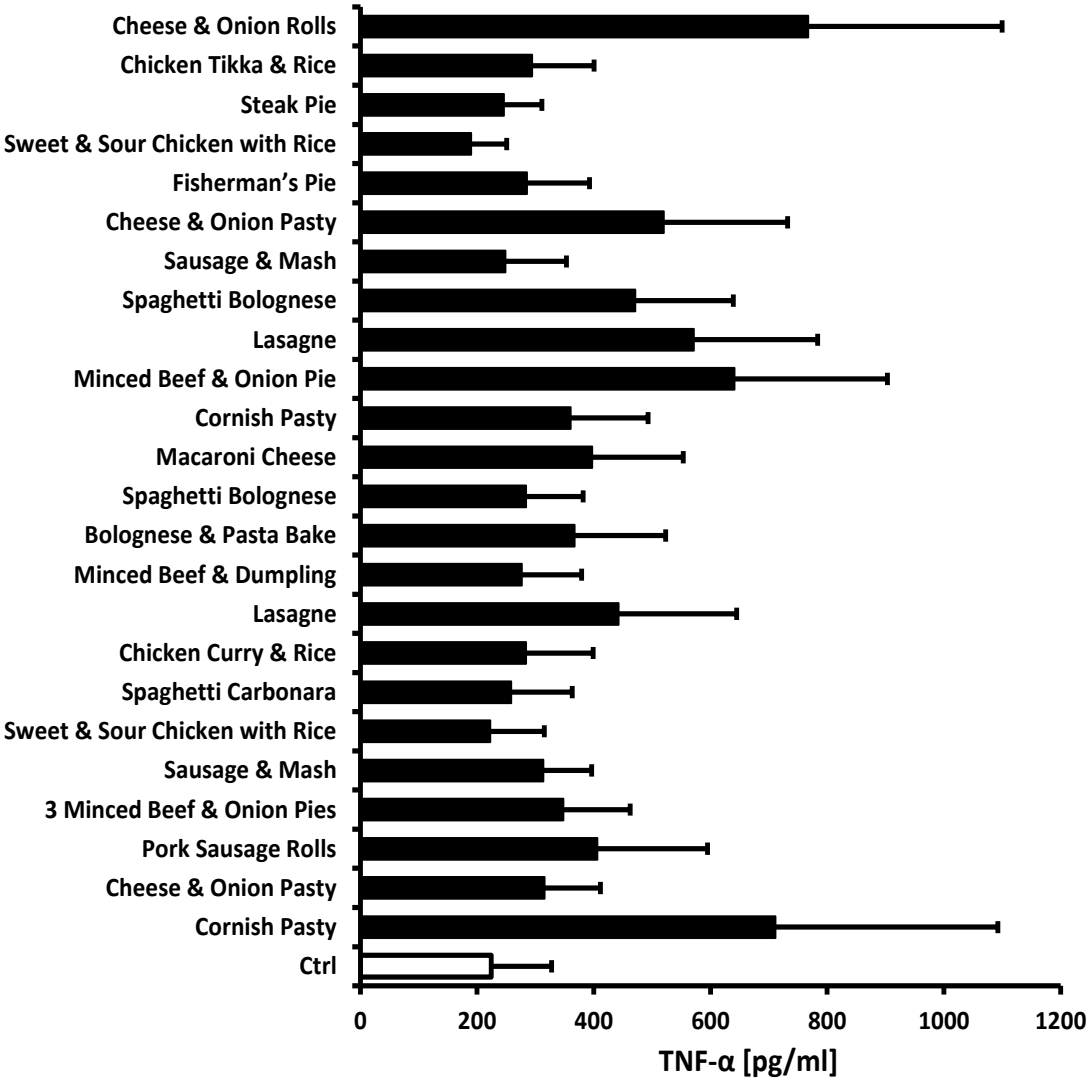


Figure 3.4: Production of TNF-α by human whole blood stimulated with food extracts.

Human whole blood was cultured with sterile filtered food extracts (diluted 1:20 in medium). After 18 hours incubation secretion of TNF-α was quantified from the supernatant (plasma) using ELISA. Results are expressed as means of 4 independent experiments ± SEM. The lower limit of detection of TNF-α was 0.005 ng/ml. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 *vs.* control cells cultured in PBS).

Analysis of the combined data from these experiments revealed that the amount of IL-6 secreted in response to each extract correlated strongly with the amount of TNF-α and IL-1β secreted in response to the same extracts (see Table 3.1). Similarly, there was a statistically significant correlation between IL-1β and TNF-α in terms of secretion.

Table 3.1: Degrees of correlation of production of different inflammatory cytokines by human whole blood samples in response to challenge with 24 different food extracts were compared by Pearson linear regression.

	IL-6	TNF-α	IL-1β
IL-6		P < 0.0001	P < 0.0001
TNF-α	R= 0.853		P < 0.0001
IL-1β	R= 0.840	R= 0.812	

Next, in order to determine the minimum concentration of Pam_3CSK_4 or LPS required to evoke a cytokine response by human leukocytes, human whole blood was treated with various doses of Pam_3CSK_4 and LPS and cytokines (IL-6, TNF-α and IL-1β) were measured in supernatant after 18 hours by ELISA (Figure 3.5). The amount of IL-6 produced at the highest does of Pam_3CSK_4 used (1,000 ng/ml) was higher than the levels of both TNF-α and IL-1β which are produced at the same concentration of Pam_3CSK_4 (IL-6: 10,157 pg/ml vs. TNF-α: 1,021 pg/ml and IL-1β: 194 pg/ml) (Figure 3.5 - a, b, c). By comparison, the amount of cytokines produced in response to the highest dose of LPS used (10 ng/ml) was (IL-6: 11,443 pg/ml vs. TNF-α: 1,148 pg/ml and IL-1β: 633 pg/ml) (Figure 3.5 - d, e, f).

These results therefore indicate that LPS (10 ng/ml) elicits the same or higher quantity of inflammatory cytokine production compared to Pam_3CSK_4, even when the latter is used at higher does (1,000 ng/ml). In addition, the minimum doses of LPS and Pam_3CSK_4 required to elicit inflammatory cytokine production from human whole blood were 0.1 ng/ml and 10 ng/ml, respectively.

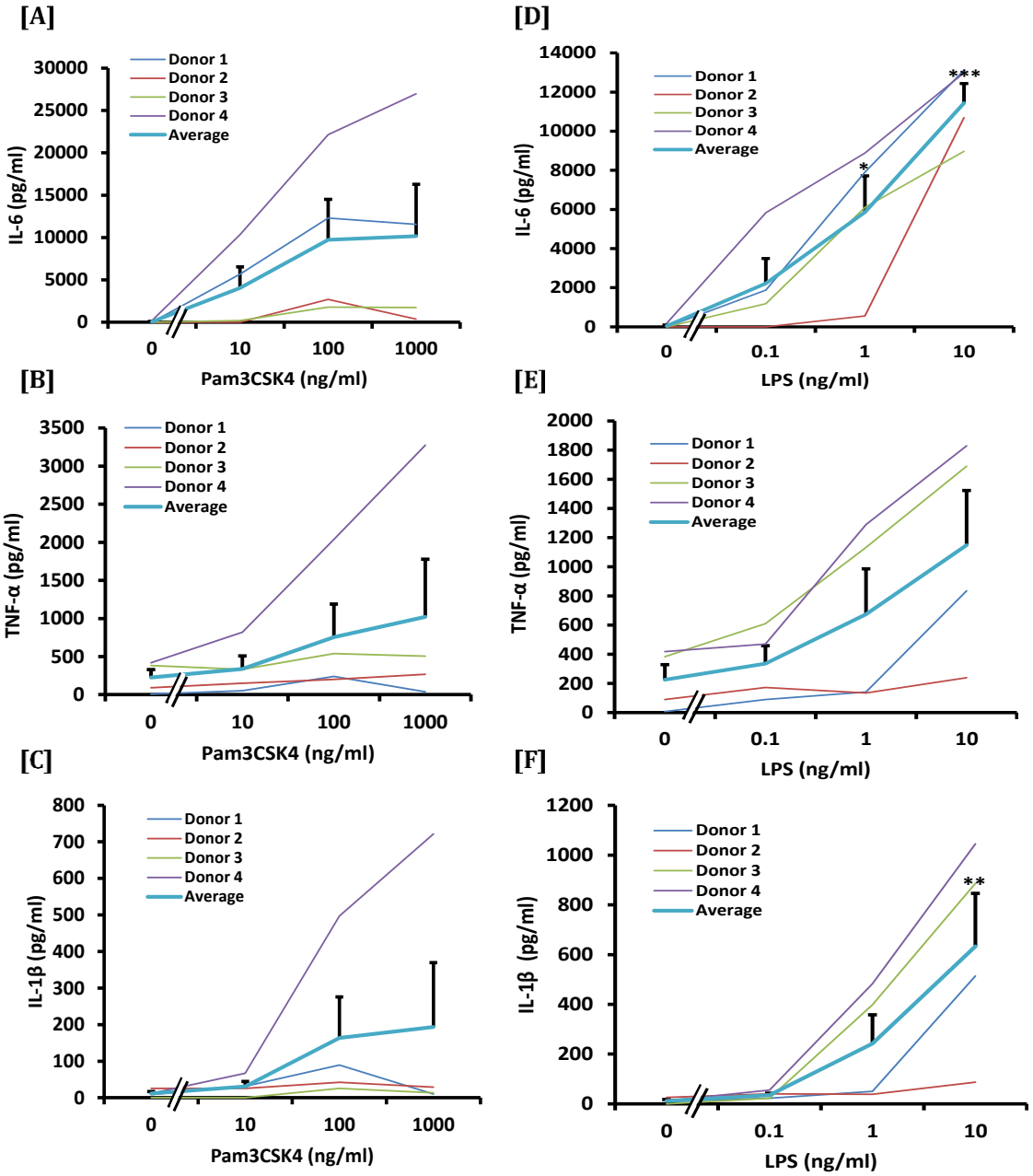


Figure 3.5: Human whole blood cytokine response to various doses of Pam_3CSK_4 and LPS.

Human whole blood was treated with different quantities of Pam_3CSK_4 (10, 100, 1000 ng/ml), LPS (0.1, 1, 10 ng/ml) and (0 ng/ml) was used as control. After 18 hours incubation, secretion of cytokines IL-6 (A), TNF-α (B) and IL-1β (C) in response to Pam_3CSK_4 and IL-6 (D), TNF-α (E) and IL-1β (F) in response to LPS were measured from the supernatant using ELISA. Results are expressed as means of 4 independent experiments ± SEM. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons (*P<0.05, **P<0.01, ***P<0.001 *vs.* control cells cultured in PBS).

Next, the cytokine responses of human PBMCs challenged with extracts of minced meat and ready-chopped onion were investigated, since the preceding experiments showed that these ingredients were a common factor of the most pro-inflammatory processed foods.

In these experiments, chopped onion was the most potent inducer of inflammatory cytokine production (IL-6: 42.5 ng/ml, TNF-α: 11.1 ng/ml, IL-1β: 9.5 ng/ml; $P<0.001$). In addition, minced meat and LPS (10 ng/ml) were relatively similar in the production of the inflammatory cytokines (Figure 3.6).

These results therefore suggest that onion which has been chopped and stored at refrigeration temperature for some time contains high quantities of pro-inflammatory stimulants which are capable of stimulating high levels of inflammatory cytokine production by immune cells.

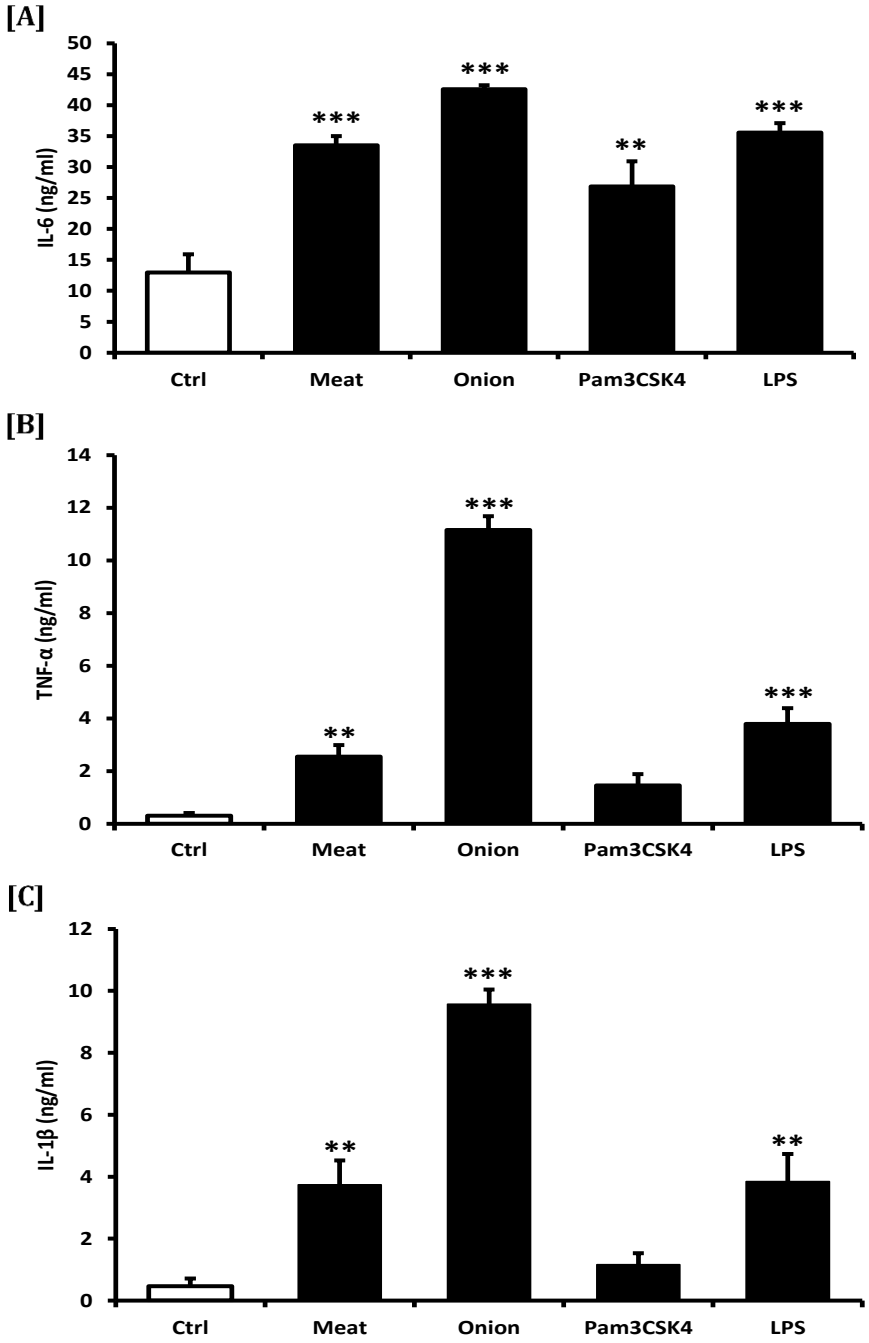


Figure 3.6: Cytokine production by human PBMCs treated with food extracts.

Human PBMCs were exposed to sterile filtered food extracts diluted 1:20 in tissue culture medium, Pam_3CSK_4 (1 µg/ml) or LPS (10 ng/ml). Secretion of IL-6 (A), TNF-α (B) and IL-1β (C) were measured from the supernatant after 18 hours incubation using ELISA. Results are expressed as means of 5 independent experiments ± SEM. The lower limit of detection of IL-6 was 0.025 ng/ml, TNF-α was 0.005 ng/ml and IL-1β was 0.035 ng/ml. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 *vs.* control cells cultured in medium alone).

3.3. Quantification of TLR-stimulants in food extracts

TLR-transfection assays were performed in order to quantify the abundance of TLR-stimulants in each food, since these molecules are well established inducers of leukocyte inflammatory signaling. HEK-293 cells were transfected with a mixture of plasmids to cause cell surface expression of the TLR2 or TLR4 receptors, together with NF-κB dependent reporter constructs, in these otherwise TLR-deficient cells. A standard curve was prepared in each plate for the measurement of the biological activity of samples relative to Pam_3CSK_4 (to quantify TLR2 stimulants) or LPS (to quantify TLR4 stimulants). NF-κB activation in each well was quantified by luminometry, and extent of signaling induced by food extracts (diluted 1:10 with tissue culture medium) was compared to that of the standard curve.

In terms of TLR2 stimulants, most of the food extracts did not contain a high level of stimulants, but three of them (pork sausage rolls: 106 ng/g, fisherman's pie: 52 ng/g, cheese and onion rolls: 41 ng/g) contained relatively high levels of TLR2 stimulants (Figure 3.7). On the other hand, most of the food extracts contained detectable TLR4 stimulants, particularly foods containing minced meat and chopped onion (pork sausage rolls: 985 ng/g, minced beef and onion pie: 328 ng/g, spaghetti bolognese: 224 ng/g), which contained a relatively high abundance of TLR4 stimulants compared with the rest of the food extracts (Figure 3.8).

The results obtained from the transfection assay demonstrated that most of the food extracts tested contains detectable levels of TLR2 or TLR4 stimulants. Furthermore, those foodstuffs with higher content of minced meat and chopped onion tended to contain higher levels of TLRs stimulants. In terms of the correlation analysis of TLR-stimulants and cytokine readouts, measurements of both assays correlated well with each other and the correlation coefficient values ranged from 0.53 - 0.69. These results suggest that food content of TLR-stimulants is strongly correlated with their capacity to induce secretion of pro-inflammatory cytokines (Figure 3.9).

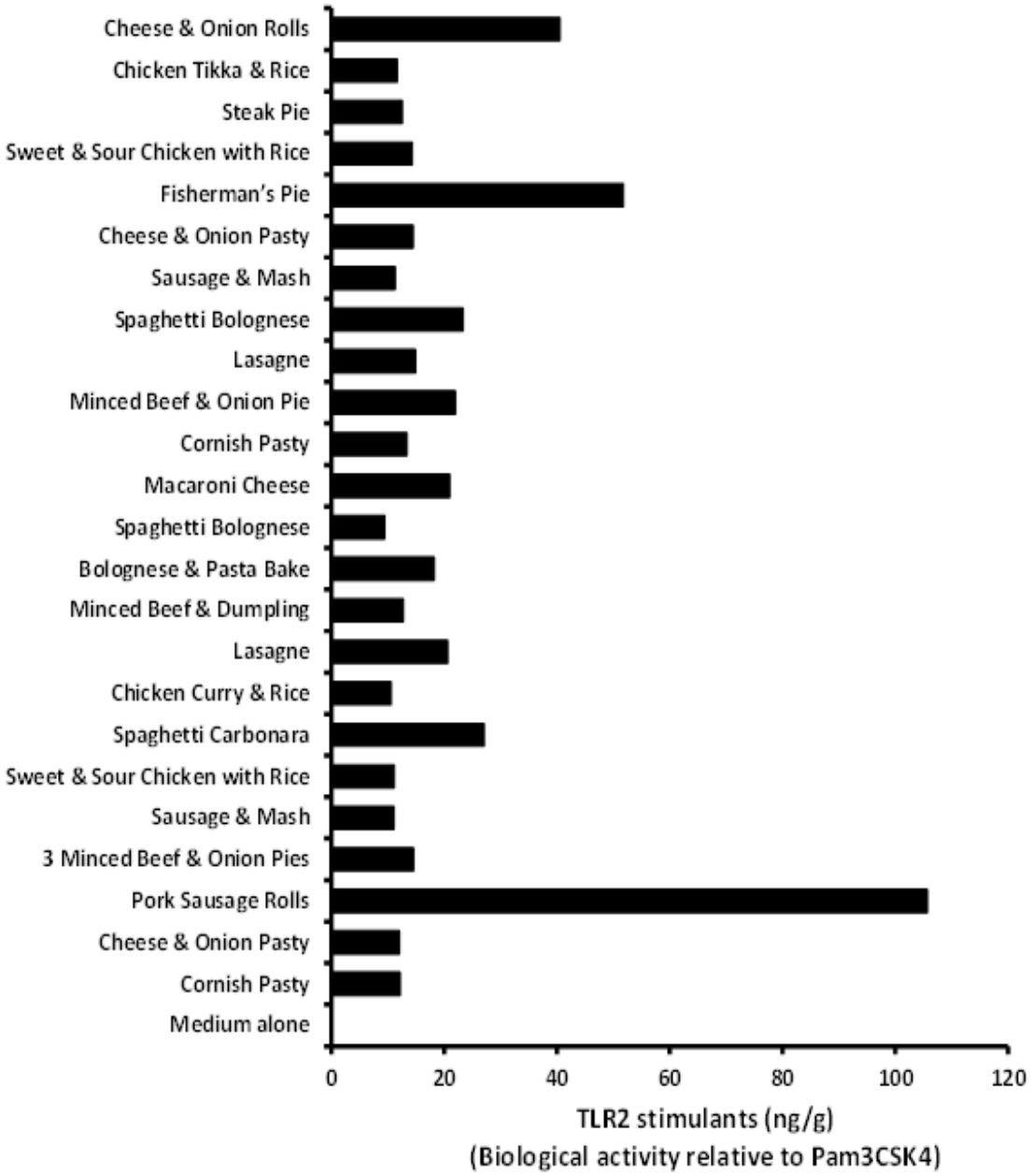


Figure 3.7: Quantification of Toll-like receptor (TLR) 2 stimulants in food extracts.

Human embryonic kidney (HEK) - 293 cells were transfected with NF-κB reporter, CD14 and TLR2. Transfected cells were treated with sterile filtered food extracts (diluted 1:10 in tissue culture medium) for 18 hours, and then a luminescence assay was performed so as to measure the TLR2 stimulants in food extracts by comparison to a Pam_3CSK_4 standard curve.

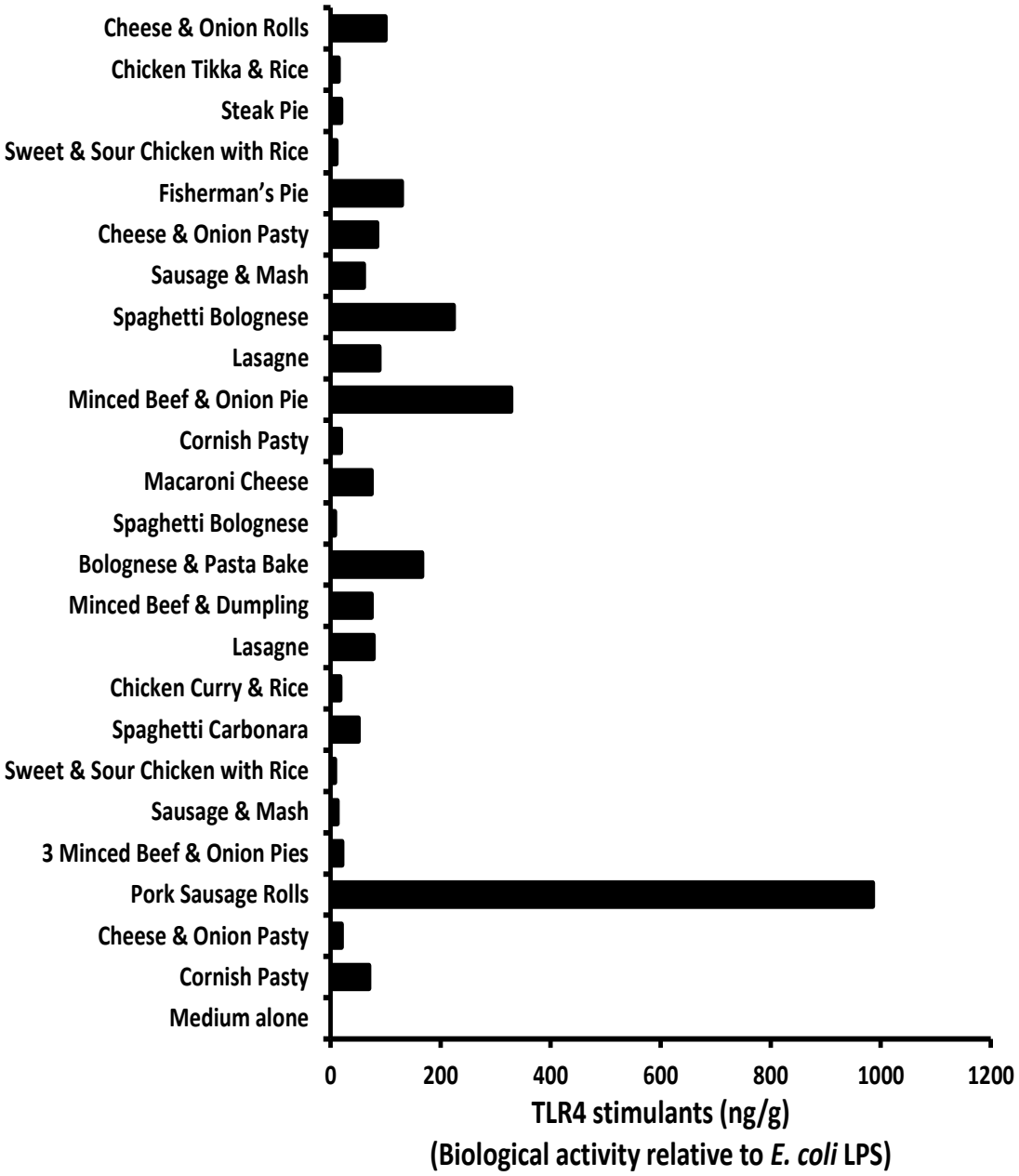


Figure 3.8: Quantification of Toll-like receptor (TLR) 4 stimulants in food extracts.

Human embryonic kidney (HEK) - 293 cells were transfected with NF-kB reporter, CD14, TLR4 and MD2. Transfected cells were treated with sterile filtered food extracts (diluted 1:10 in tissue culture medium) for 18 hours, and then a luminescence assay was performed so as to measure the TLR4 stimulants in food extracts by comparison to a LPS standard curve.

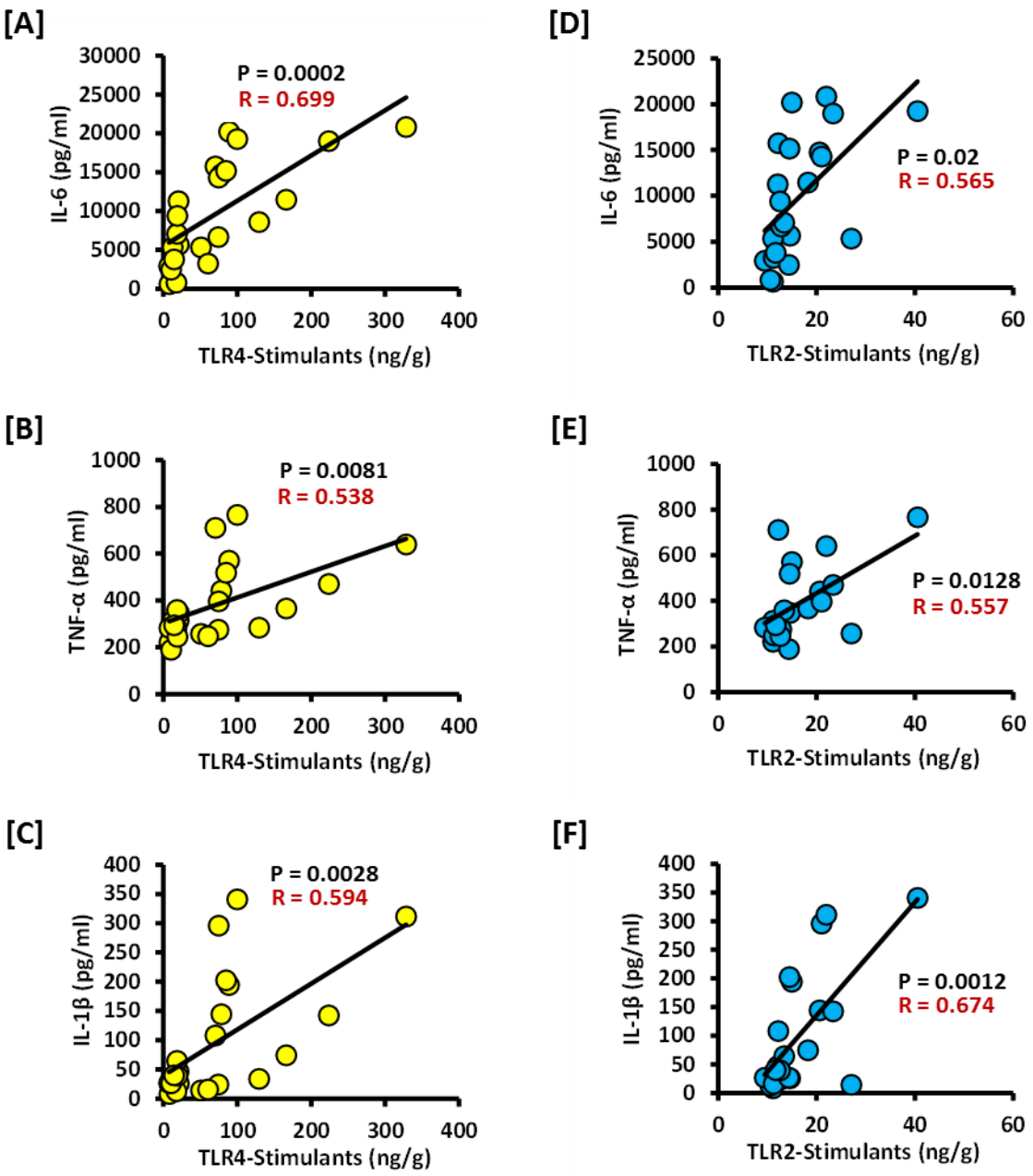


Figure 3.9: Correlation analysis of food-borne TLR-stimulant content with whole blood cytokine production.

The abundance of TLR2 and TLR4 stimulants in foodstuffs, as quantified by HEK-293 transfection assay, was compared with the amount of pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β) produced after challenging human whole blood with food extracts. The measurements from both types of assay correlate well with each other, with correlation coefficient values ranging from 0.53 - 0.69. Correlation coefficients were calculated using the Spearman correlation test.

3.4. Endotoxin quantification in food extracts

Afterward, we decided to quantify the concentration of TLR4-stimulant (lipopolysaccharide) in the same sterile filtered food extracts using the kinetic chromogenic Limulus Amoebocyte Lysate (LAL) assay, since this method was previously used to measure endotoxin (LPS) concentration in ground beef and milk (J M Jay *et al.* 1979, Gehring *et al.* 2008).

A standard curve was prepared in a 96-well plate using 2-fold serial dilutions of LPS from *E. coli* (O111:B4) in pyrogen free water. After that, the samples were defrosted and added at a dilution of 1:10,000 in pyrogen-free water for the sterile filtered food extracts and results are presented as ng LPS per gramme food.

The results obtained from the LAL assay revealed that most of the food extracts tested appeared to contain very high levels of endotoxin as measured using this assay. Foods containing minced meat, cheese and chopped onion (cheese and onion Rolls: 17,401 ng/g, macaroni cheese: 6,522 ng/g, and pork sausage rolls: 4,195 ng/g), contained a particularly high abundance of TLR4 stimulants compared with the rest of the food extracts (Figure 3.10).

In terms of the correlation analysis of endotoxin levels in foodstuffs, as quantified by LAL assay, with the abundance of TLR4 and TLR2 stimulants in foodstuffs as measured by HEK-293 transfection assay and with the amount of pro-inflammatory cytokines IL-6, TNF-α, and IL-1β (Figure 3.11, a-e), which were produced after challenging human whole blood with food extracts, the correlation coefficient values ranged from 0.17 - 0.61.

The lower correlation coefficients between the LAL assay results and cytokine production may relate to false positive readings, which are a common issue with the LAL assay because of its inherent capacity to detect non-inflammatory β-glucans, which are a common ingredient of many foodstuffs (Elin, Wolff 1973).

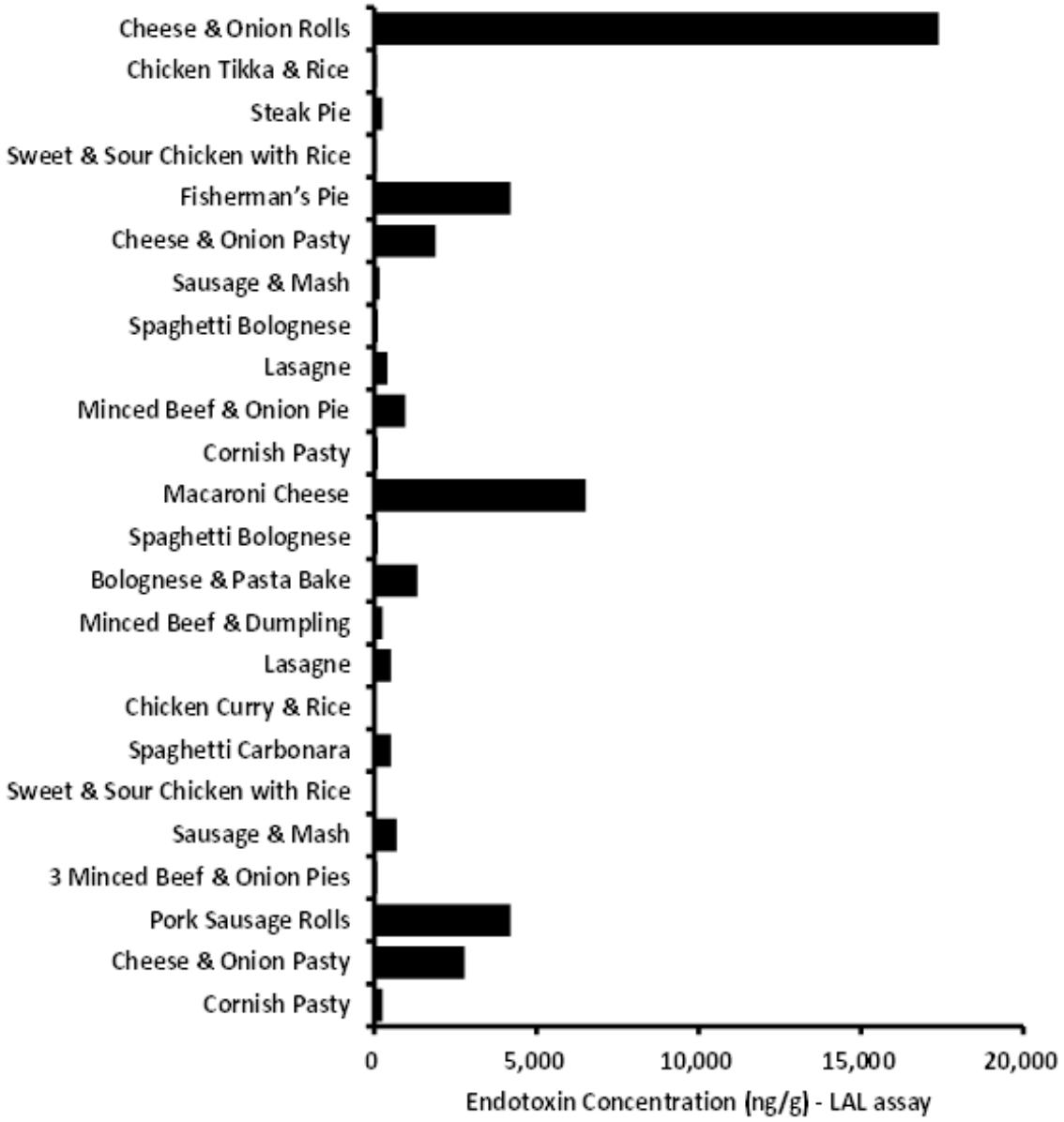


Figure 3.10: Quantification of lipopolysaccharide concentration in food extracts by limulus assay.

The kinetic chromogenic Limulus Amoebocyte Lysate (LAL) assay was used to measure endotoxin (LPS) concentration in the same sterile filtered food extracts measured by TLR-bioassay previously. Samples were diluted 1:10,000 in pyrogen-free water before assay and results are presented as ng LPS per gramme food.

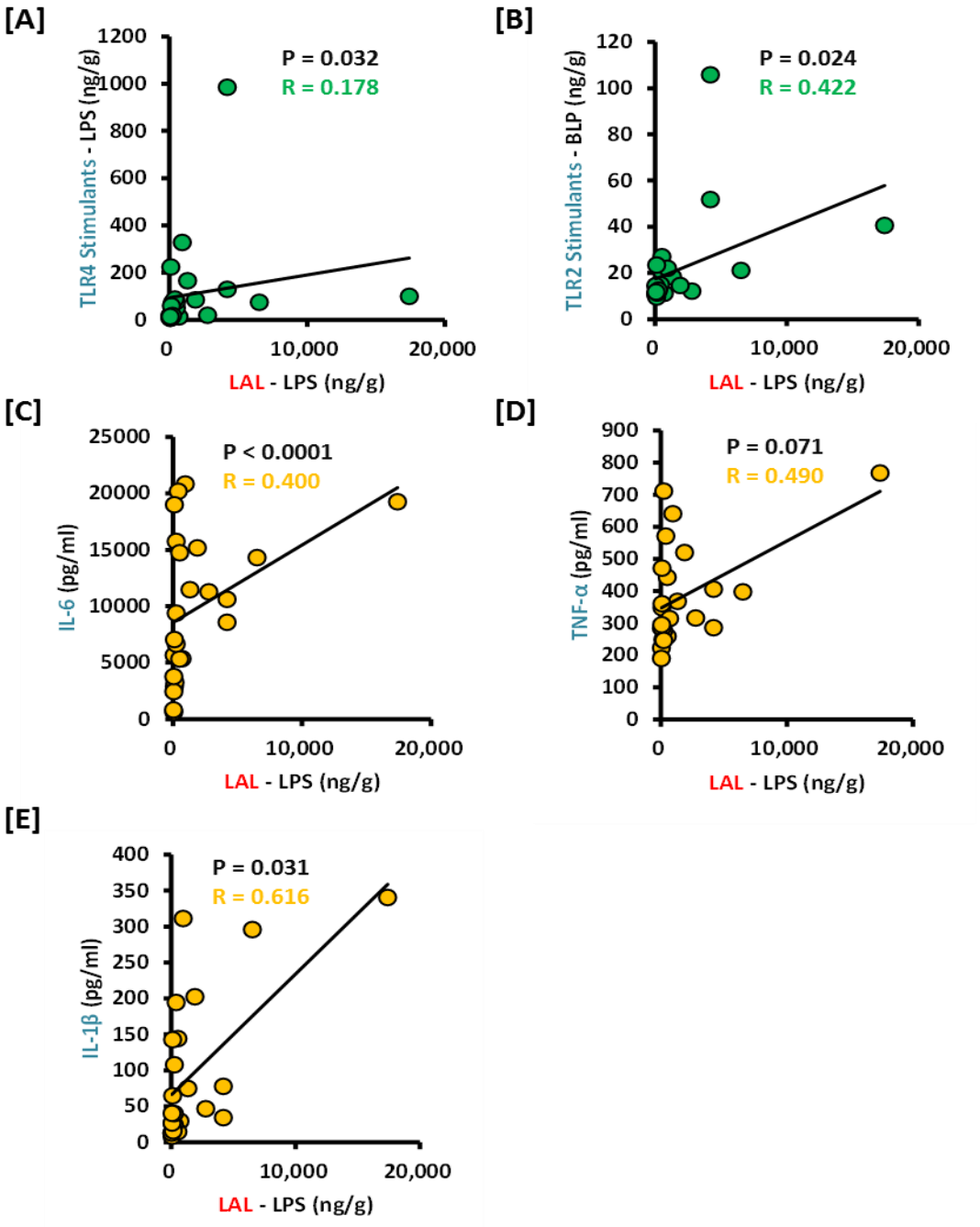


Figure 3.11: Correlation analysis of LAL assay with each of TLR-stimulants and cytokine readouts.

The concentration of lipopolysaccharide in foodstuffs, as quantified by LAL assay, was compared with the abundance of TLR4 (A) and TLR2 (B) stimulants in foodstuffs, as measured by HEK-293 transfection assay, and with the amount of pro-inflammatory cytokines IL-6 (C), TNF-α (D), and IL-1β (E), which were produced after challenging human whole blood with food extracts. Correlation coefficients were calculated using the Spearman correlation test.

3.5. Effect of food extracts on macrophage lipid droplet formation

Next, as lipid accumulation in macrophages, and also in circulating monocytes (Xu *et al*. 2015), is a key event in atherosclerosis that may be driven by TLR-signaling (Nicolaou *et al.* 2010, Nicolaou *et al.* 2012), we examined the effects of food extracts on lipid droplet formation in murine J774 macrophages. J774 cells treated with Pam_3CSK_4 (10 µg/ml), LPS (1 µg/ml), sterile filtered minced meat (1:10) or ready-chopped onion (1:10) were incubated for 72 hours and then stained with DAPI (to reveal nuclear DNA in blue) and Nile red (to reveal neutral lipid droplets in green). Finally, the cells were observed under fluorescent microscopy.

Images obtained from fluorescent microscopy showed that all stimuli (Pam_3CSK_4, LPS, minced meat and chopped onion) induced an accumulation of lipid droplets inside the J774 cells when compared with the medium alone (Control). These stimuli can therefore be considered to be capable of inducing foam cell formation *in vitro* (Figure 3.12).

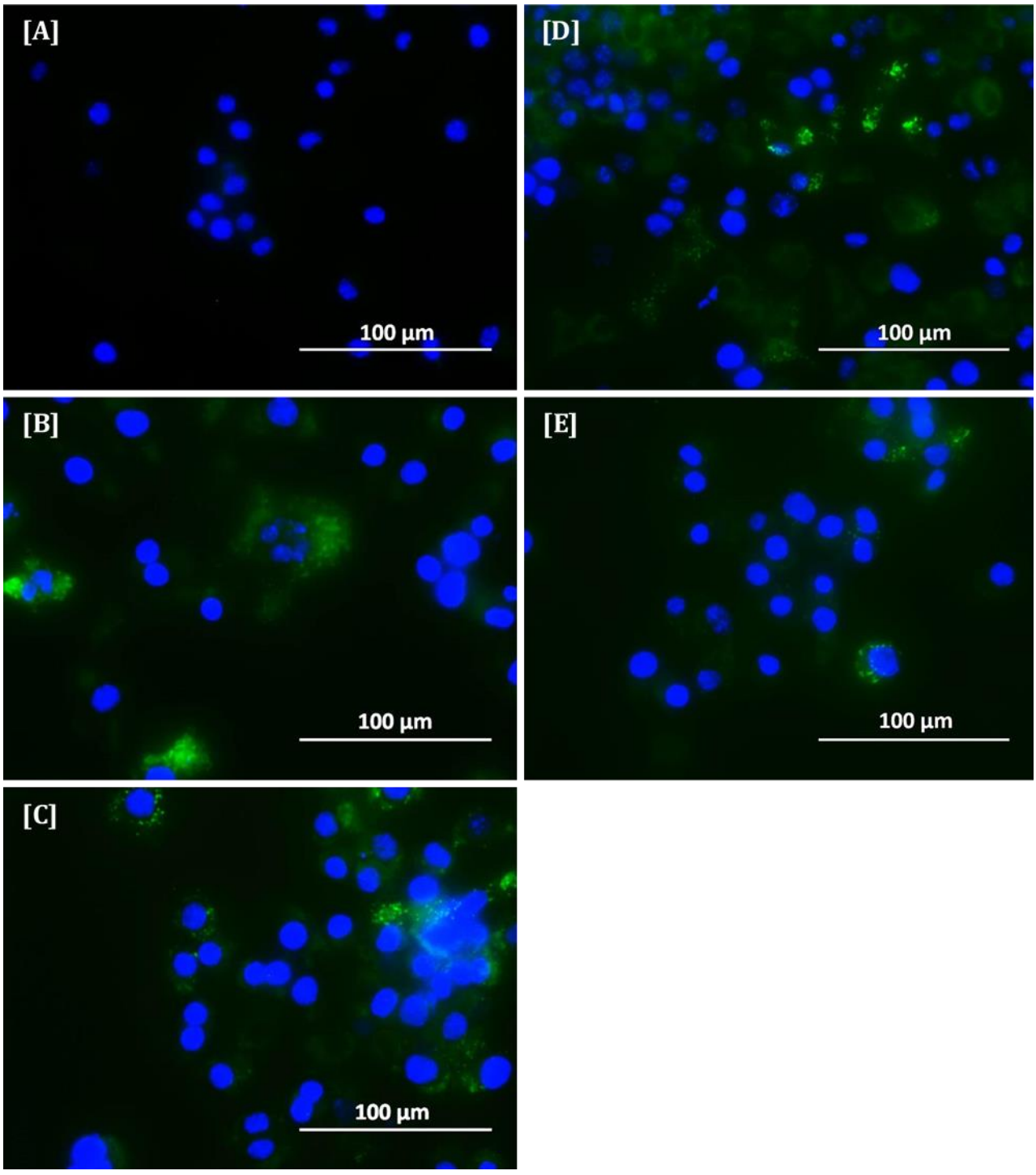


Figure 3.12: Effect of food extracts on macrophage lipid droplet accumulation.

J774 cells were exposed to Medium alone - Control (A), Pam_3CSK_4 (B), LPS (C), minced meat extract (D), or chopped onion extract (E), for 72 hours. The cells were stained with Nile red (which is specific for lipid droplets and emits a green colour) and DAPI (which is specific for nucleic acids and emits a blue colour) then examined under fluorescent microscope with 40X magnification power.

3.6. Real Time - Polymerase Chain Reaction (RT-PCR) assay

The capacity of food extracts to regulate transcription of genes encoding inflammatory cytokines was then examined. For this purpose, human PBMCs were prepared from whole blood and then challenged with minced meat, chopped onion (1:20 diluted with tissue culture medium), Pam_3CSK_4 (1 μg/ml) or LPS (10 ng/ml). After 18 hours mRNA was extracted from the PBMCs then converted to cDNA, so as to quantifying the expression of inflammatory cytokine genes with RT-PCR assay.

As expected, chopped onion treatment of PBMCs resulted in a highly significant induction of IL-6, IL-1β, and IL-8 genes ($P<0.001$) and TNF-α gene ($P<0.05$) when compared with the control (medium alone). By comparison, stimulation of PBMCs with minced meat extract resulted in a relatively low expression of inflammatory cytokine genes with no significant differences with the control, the only exception being a significant induction of IL-1β mRNA ($P<0.05$) (Figure 3.13, Figure 3.14 - a).

The expression of inflammatory cytokine genes by PBMCs after challenging them with Pam_3CSK_4 and LPS increased significantly when compared with the control, but the gene expression was lower in comparison with the results obtained from chopped onion. Unexpectedly, expression of MCP-1 was reduced compared to control, by all stimuli (Figure 3.14 - b). Taken together, these results confirm that commonly consumed foodstuffs (particularly ready-chopped onion) can promote inflammatory signaling in primary human leukocytes, and this supports similar results obtained from the quantification of inflammatory cytokines by ELISA.

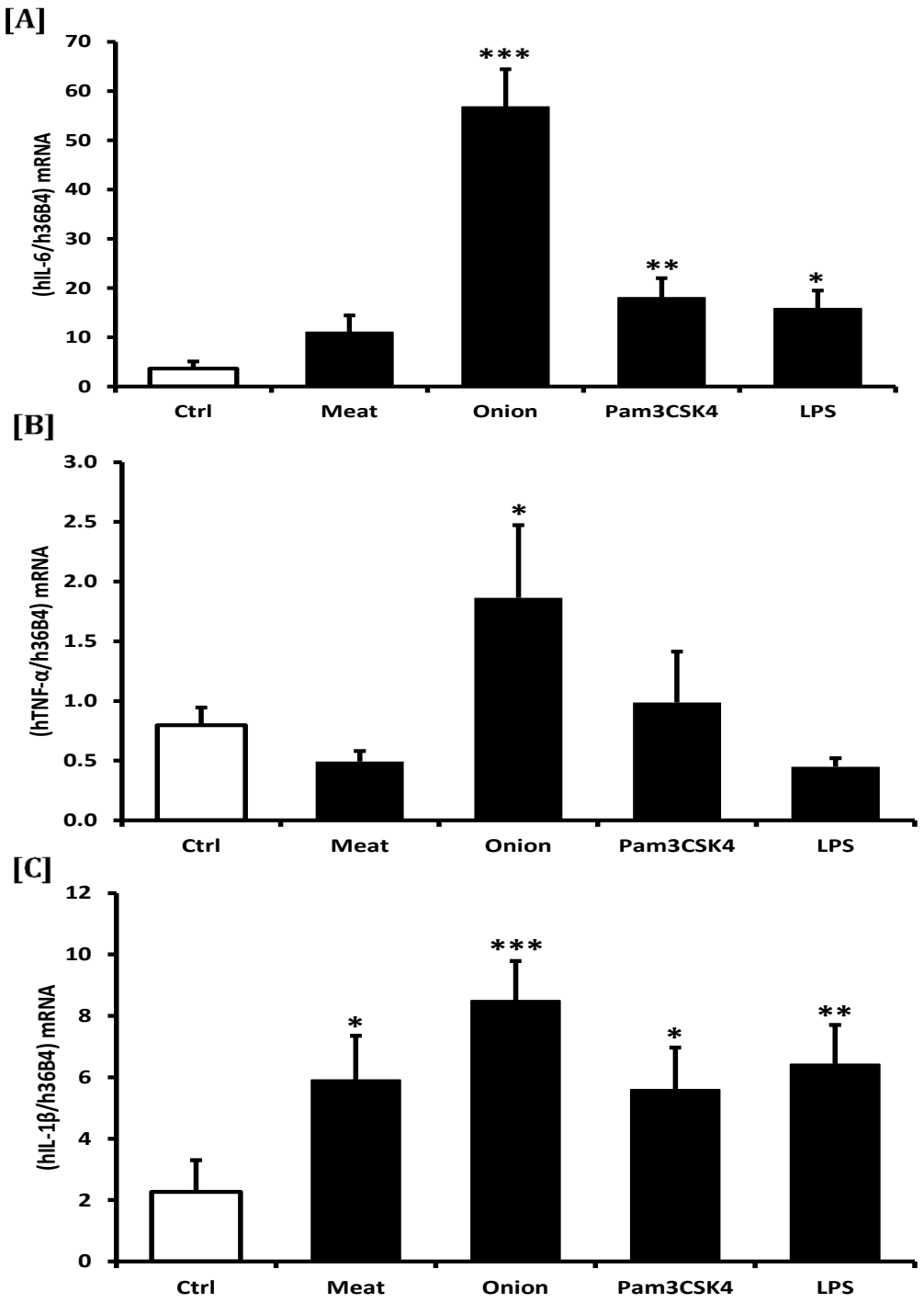


Figure 3.13: Effects of food extracts on human PBMC expression of pro-inflammatory cytokine genes.

Human PBMCs were treated with food extracts for 18 hours, and then the expression of pro-inflammatory cytokine genes IL-6 (A), TNF-α (B), and IL-1β (C), were measured by RT-PCR, relative to the house-keeping gene 36B4. Results are expressed as means of 5 independent experiments ± SEM. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 vs. control cells cultured in medium alone).

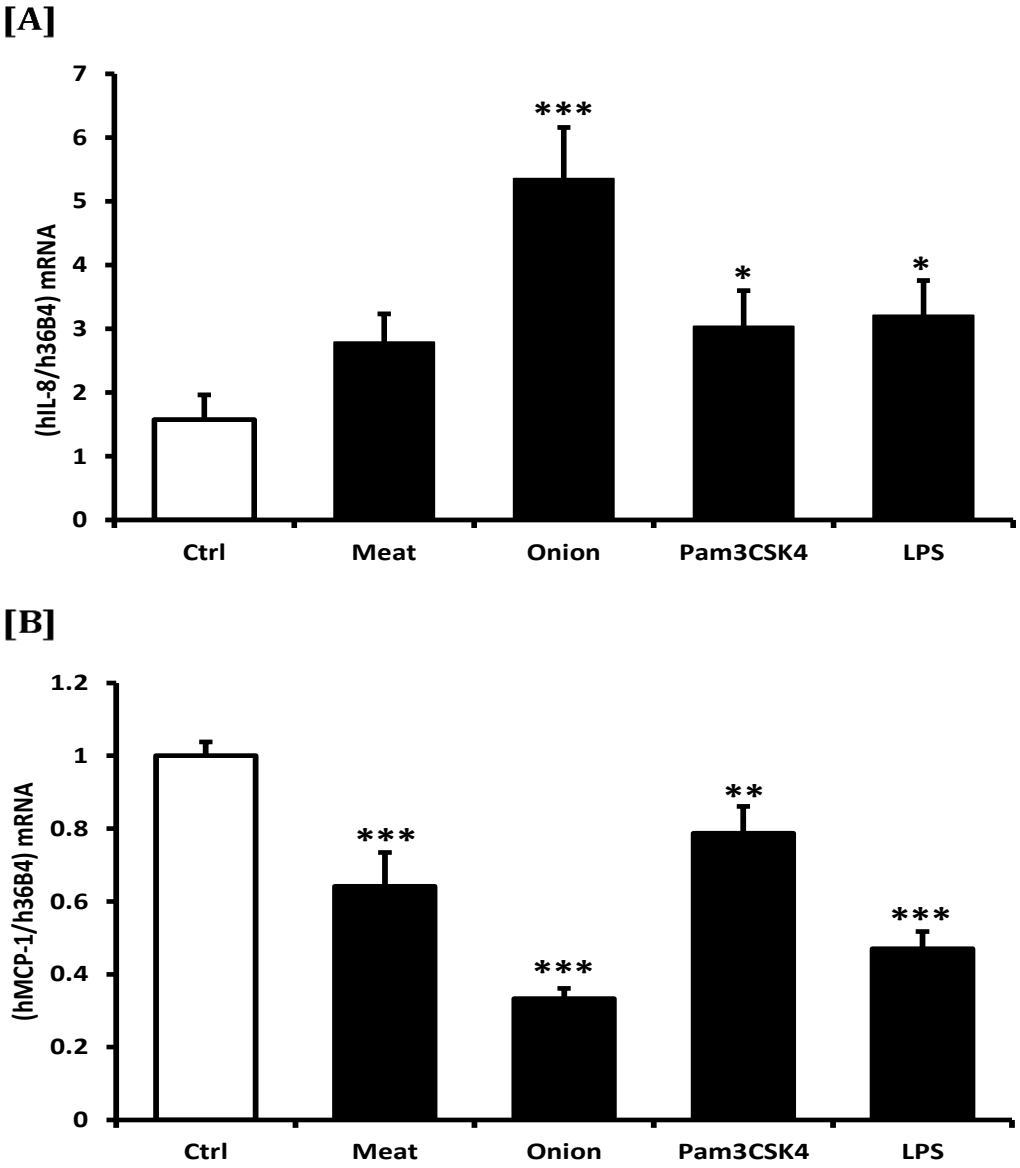


Figure 3.14: Effects of food extracts on human PBMC expression of pro-inflammatory chemokine genes.

Human PBMCs were treated with food extracts for 18 hours, and then the expression of pro-inflammatory chemokine genes IL-8 (A), and MCP-1 (B), were calculated using RT-PCR. Results are expressed as means of 5 independent experiments ± SEM. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 vs. control cells cultured in medium alone).

3.7. Effect of TLR inhibition on food extract inflammatory activity

Next, we aimed to determine whether or not the capacity of foodstuffs to induce inflammatory signaling in monocytes was dependent on the food content of TLR2 or TLR4 stimulants. To achieve this, the inhibitor OxPAPC was used, which is a specific inhibitor of TLR2 and TLR4, but not other TLRs or cytokine signaling pathways (Erridge *et al.* 2008).

Human primary monocytes were prepared from human whole blood, and then they were treated with the presence or absence of 30 μg/ml OxPAPC for 20 minutes. This concentration of OxPAPC has been shown to be non-toxic to cultured cells (Erridge *et al.* 2008). Finally, the cells were challenged with minced meat, chopped onion (1:100 diluted in tissue culture medium), Pam_3CSK_4 (100 ng/ml) and LPS (1 ng/ml). After 18 hours the supernatant were collected and inflammatory cytokines were measured by ELISA.

The experiment confirmed that OxPAPC inhibited the induction of inflammatory cytokines by the positive control TLR2 and TLR4 stimulants Pam_3CSK_4 and LPS, respectively (Figure 3.15). The production of TNF-α and IL-1β decreased significantly ($P<0.05$) in the presence of OxPAPC after the cells were challenged with ready-chopped onion (Figure 3.15 - b, c), and there was a clear decline in the amount of IL-6 although this difference did not reach significance (Figure 3.15 - a). Furthermore, there were significant reductions in the levels of IL-6 and TNF-α ($P<0.05$) and dramatic fall in the amount of IL-1β ($P<0.01$) in the monocytes challenged with meat in the presence of OxPAPC (Figure 3.15). Taken together, these findings suggest that TLR2 and TLR4 stimulants present in food are critical mediators of the capacity of food extracts to induce inflammatory signaling in human primary monocytes.

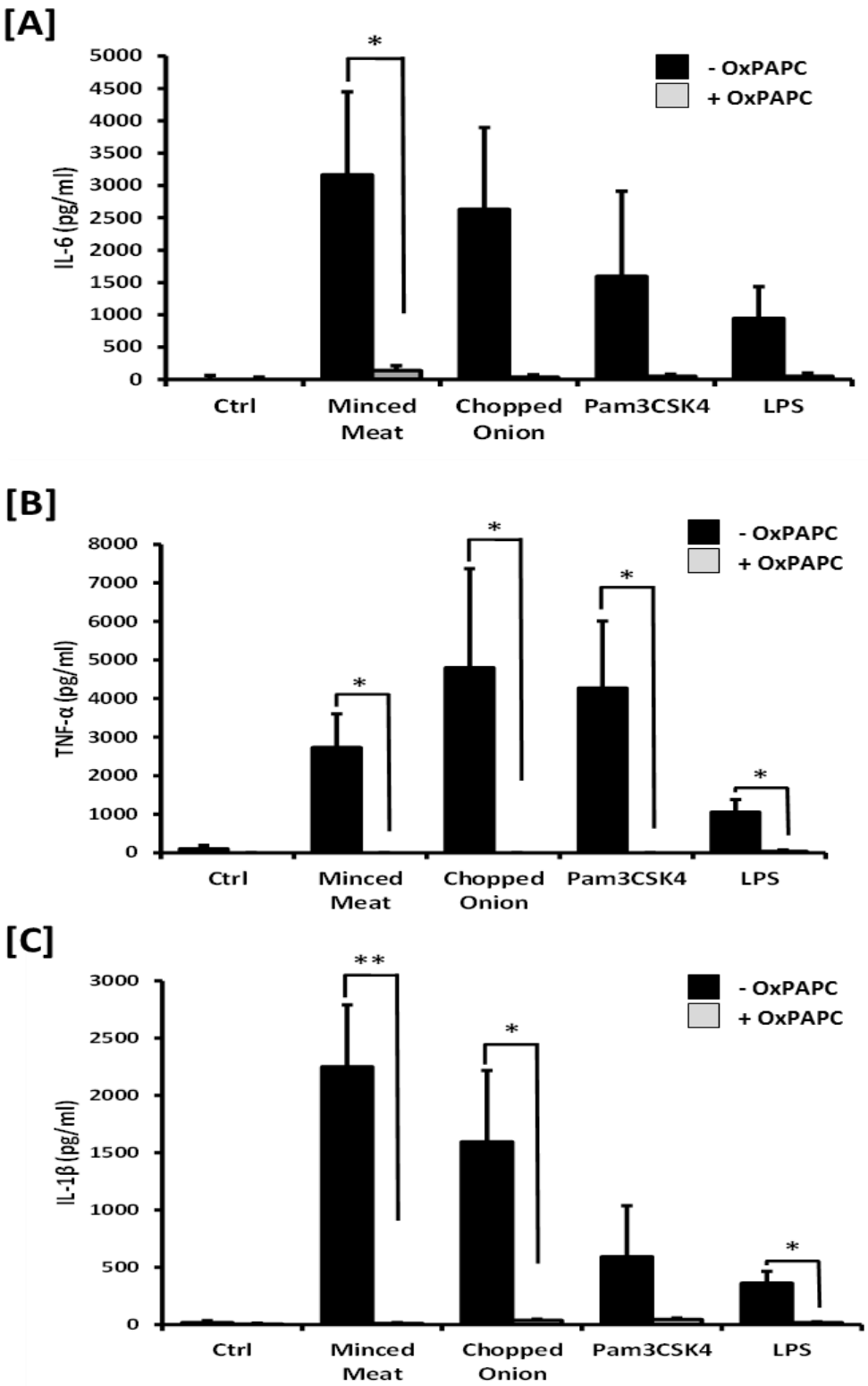


Figure 3.15: Effect of the TLR2 and TLR4 inhibitor OxPAPC on food extract - induced inflammatory signaling.

Primary human monocytes were challenged with food extracts with or without 30 µg/ml OxPAPC, and then pro-inflammatory cytokines IL-6 (A), TNF-α (B), and IL-1β (C) were measured using ELISA. Results are expressed as means of 4 independent experiments ± SEM. The lower limit of detection of IL-6 was 0.025 ng/ml, TNF-α was 0.005 ng/ml and IL-1β was 0.035 ng/ml. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 vs. control cells cultured in medium alone).

Chapter 4 Results - Effects of dietary PAMPs on cardiometabolic risk markers in man

Our previous findings suggest that processed foods can contain PAMPs at concentrations that are much higher than required to stimulate inflammatory cytokine production in human blood. For example, results from the previous chapter suggest that a single 400 g processed food meal may contain up to 200 µg LPS-equivalents and 25 µg BLP-equivalents. By comparison, it is well established that intravenous injection of as little as 8 ng LPS is sufficient to stimulate a detectable inflammatory response in man (Ferguson *et al*. 2013). Specifically, previous studies have shown that intravenous injection of low-doses of LPS (0.1 ng/kg) in man results in a transient, low-grade inflammatory response involving the upregulation of circulating markers, such as IL-6, TNF-α and CRP (Starkie *et al*. 2003, Krogh-Madsen *et al*. 2004).

Therefore, we assumed that the intestinal wall may serve as a protective barrier to the absorption of the majority of LPS and BLP contained within a meal. This concept is supported by a meta-analysis from randomized trials that showed there is a strong association between chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) , such as ibuprofen and diclofenac, which impair gut barrier function in man and in mice (Sigthorsson *et al.* 2002), and the risk of cardiovascular diseases (Kearney *et al.* 2006). An effective gut barrier may be a possible explanation for why processed meal consumption does not lead to severe inflammatory response in healthy man.

Nevertheless, it remains possible that processed foods could induce low grade, sub-clinical inflammation through the absorption of PAMPs. To test this, we examined the effects of oral ingestion of meals rich in TLR-stimulants on systemic markers of inflammation and cardiometabolic risk in healthy human volunteers.

We conducted two studies, an acute dietary PAMP intervention study (n=12) and a chronic dietary PAMP intervention study (n=11) in healthy human subjects.

4.1. An acute dietary PAMP intervention study in healthy human subjects

According to the results obtained from the transfection assay (data from chapter 3 - results), we demonstrated that foodstuffs with higher content of minced meat and ready-chopped onion tended to contain higher levels of TLRs stimulants. In addition, earlier work from the group showed that ready chopped onion from a certain supplier reliably contains high quantities of soluble TLR2 and TLR4-stimulants (Erridge C 2011).

Based on these findings, we decided to prepare a test meal composed of 200 g of ready-chopped onion from supermarket, mixed with flour and water, and deep fried in vegetable oil for 5-8 minutes (onion bhajis). Notably, such cooking does not significantly reduce the biological activity of BLP or LPS (Erridge C 2010). In order to maximise the chance of observing an effect, subjects were given ibuprofen tablets, with the aim of temporarily weakening gut barrier function.

Twelve healthy male volunteers took two ibuprofen tablets (total 400 mg) before bed (day one). On the next day (early morning), they took another two ibuprofen tablets (total 400 mg). Fasted blood samples were collected at 0 h. Then, they consumed a high PAMP meal (200 g bhajis prepared from ready chopped onion at best-before date). Postprandial blood samples were collected at 3 h and a second fasted sample at 24 h.

The results from the acute dietary PAMP intervention study showed no significant changes in the physical parameters (pulse rate and tympanic temperature) (Figure 4.1) at any timepoint after ingestion of the high PAMP onion meal.

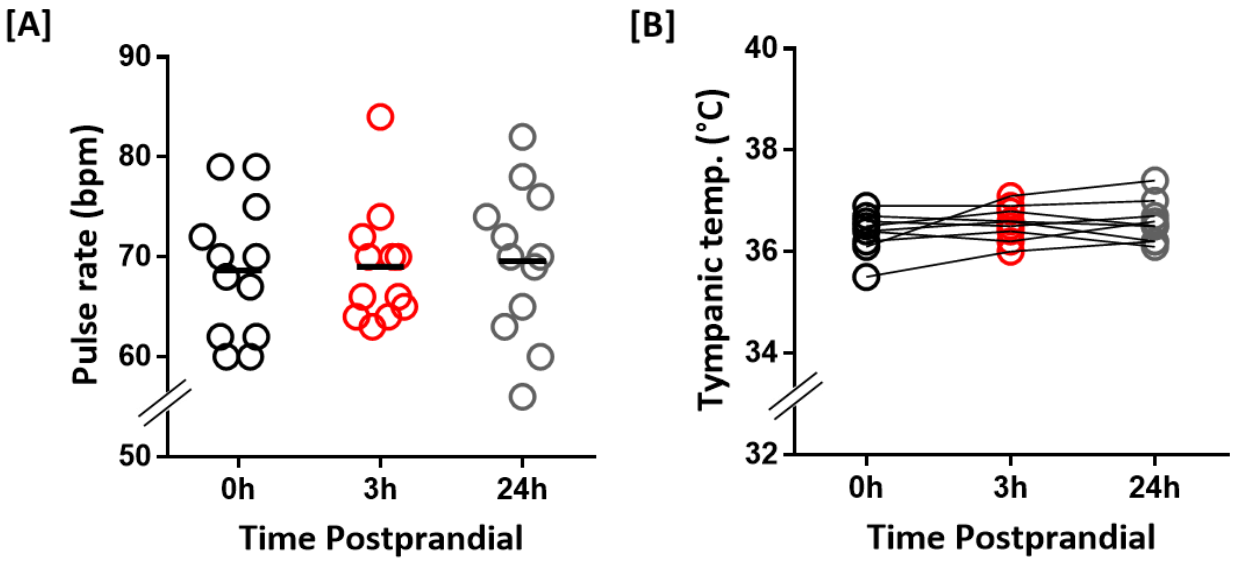


Figure 4.1: Effect of high PAMP onion meal on physical parameters in ibuprofen-primed healthy human volunteers.

Pulse rate (A) and tympanic temperature (B) were measured in ibuprofen-primed healthy male volunteers (n=12), before ingestion of a high PAMP onion meal (fasted, 0 h), postprandially (3 h) and the next day (fasted, 24 h) after the test meal. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons.

However, a significant increase in total leukocyte count was observed postprandially after 3 hours of delivering the high PAMP onion meal when compared to the baseline leukocyte measurement (P=0.007 vs. 0h timepoint). Leukocyte count then fell significantly after 24 hours (P=0.006 vs. 24h timepoint). Similar results were observed for total granulocyte counts (Figure 4.2). These are considered to be sensitive, but non-specific, markers of low-grade inflammation.

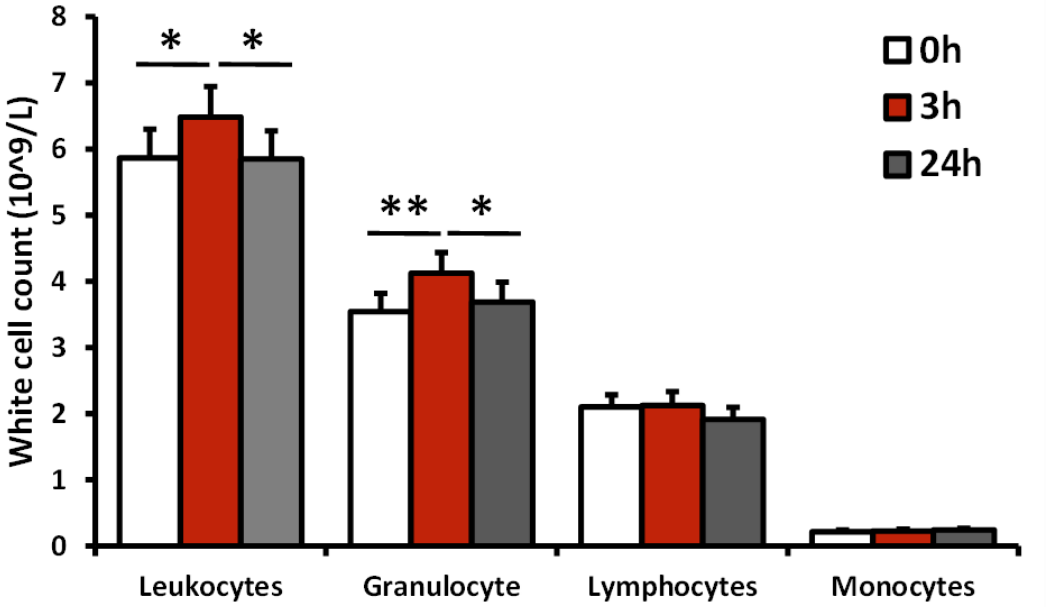


Figure 4.2: Leukocyte counts in blood samples of acute high PAMP onion dietary intervention in ibuprofen-primed healthy human volunteers.

Leukocyte counts were measured in blood samples of ibuprofen-primed healthy human volunteers (n=12), collected before ingestion of a high PAMP onion meal (fasted, 0 h), postprandially (3 h) and the next day (fasted, 24 h) after the test meal. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons (*P<0.05, **P<0.01). Results are presented as means ± SEM.

However, cytokine (IL-1β, IL-6, TNF-α and IL-8) mRNA expression in PBMC (Figure 4.3), serum CRP (Figure 4.4), serum endotoxin (Figure 4.5), serum LDL/HDL (Figure 4.7), and cholesterol efflux capacity of serum samples (Figure 4.8) were not significantly altered by the test meal. As expected, the serum triglyceride was significantly augmented by the high PAMP meal (which also contained quite a high fat load) and this was again reversed after 24 hours (Figure 4.6).

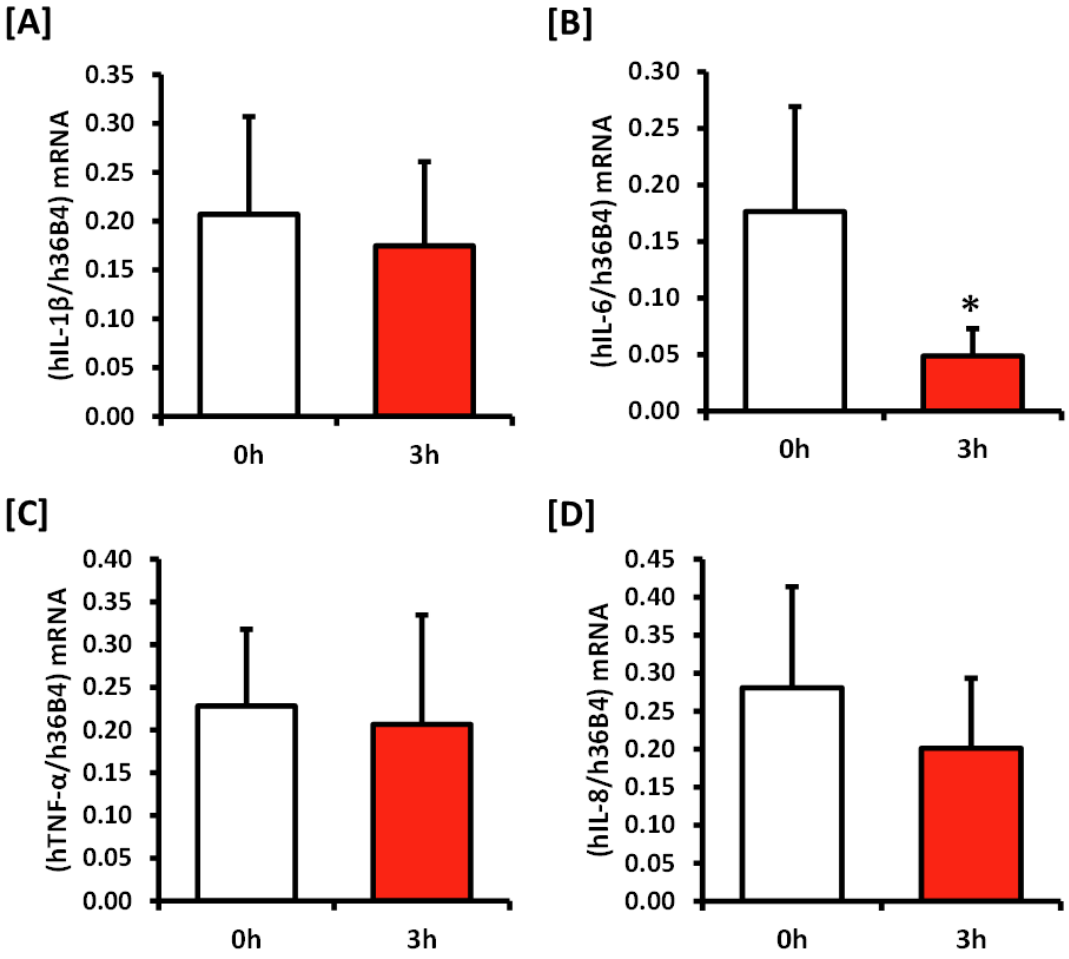


Figure 4.3: PBMC cytokine mRNA responses to high PAMP onion meal in ibuprofen-primed healthy human volunteers.

Cytokine mRNA expression of IL-1β (A), IL-6 (B), TNF-α (C), and IL-8 (D) relative to 36B4 mRNA in PBMC of ibuprofen-primed healthy human volunteers (n=12), collected before ingestion of a high PAMP onion meal (fasted, 0 h), or postprandially (3 h), were measured by RT-PCR. Differences were compared by paired t-test (*P<0.05). Results are presented as means ± SEM.

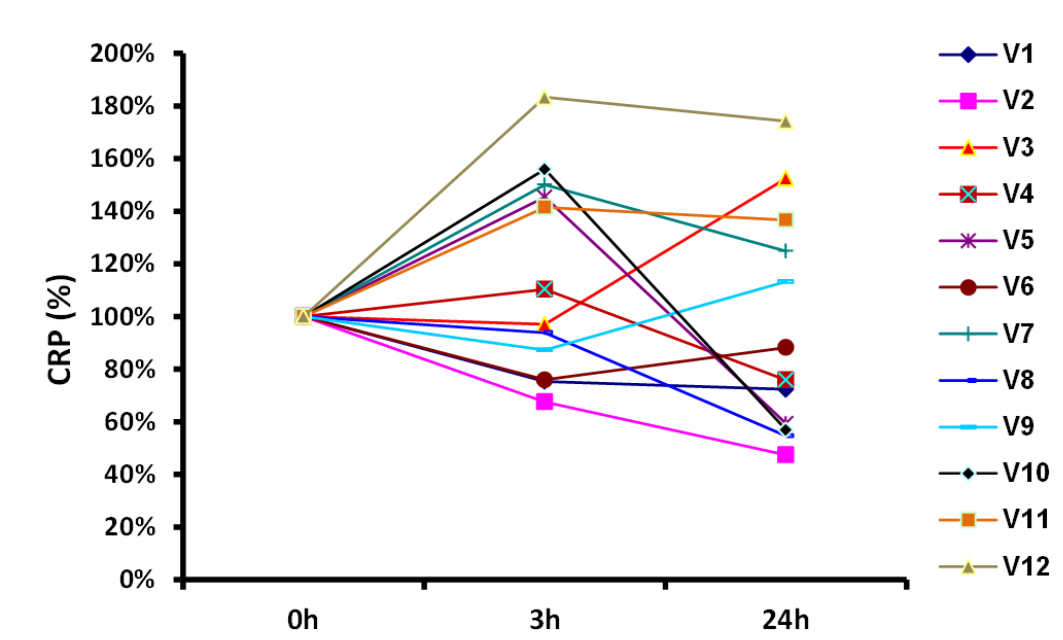


Figure 4.4: CRP responses to high PAMP onion meal in ibuprofen-primed healthy human volunteers.

CRP levels were measured in plasma samples of ibuprofen-primed healthy human volunteers (n=12), collected before ingestion of a high PAMP onion meal (fasted, 0 h), postprandially (3 h) and the next day (fasted, 24 h) after the test meal, using high sensitivity ELISA. Results are presented as responses of individual donors, normalised to baseline CRP.

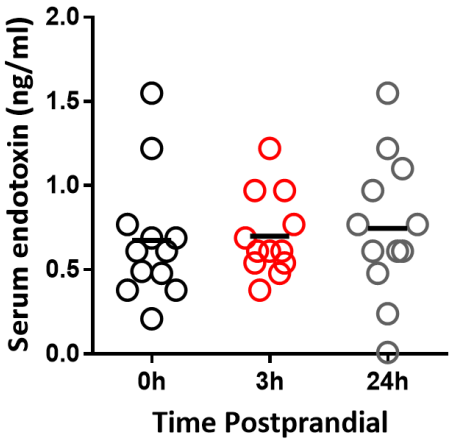


Figure 4.5: Serum endotoxin concentrations in acute high PAMP onion meal ingestion study.

Serum endotoxin levels were measured in ibuprofen-primed healthy human volunteers (n=12), collected before ingestion of a high PAMP onion meal (fasted, 0 h), postprandially (3 h) and the next day (fasted, 24 h) after the test meal, using the kinetic limulus amoebocyte lysate assay. Results of individual donors are presented with bars representing means. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons.

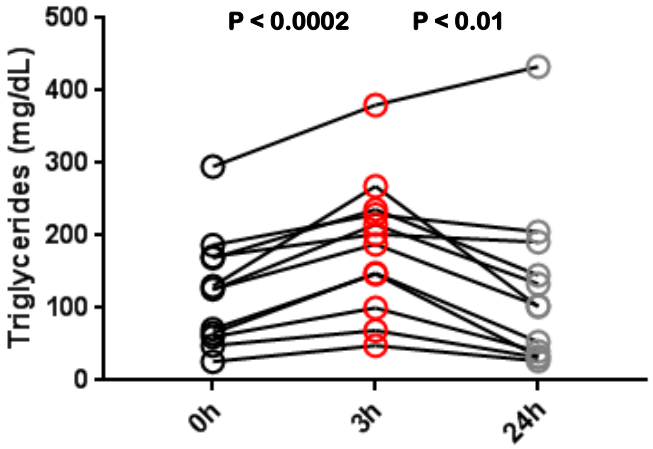


Figure 4.6: Plasma triglyceride concentrations in acute high PAMP onion meal in ibuprofen-primed healthy human volunteers.

Plasma triglyceride levels were measured in ibuprofen-primed healthy human volunteers (n=12), collected before ingestion of a high PAMP onion meal (fasted, 0 h), postprandially (3 h) and the next day (fasted, 24 h) after the test meal. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons.

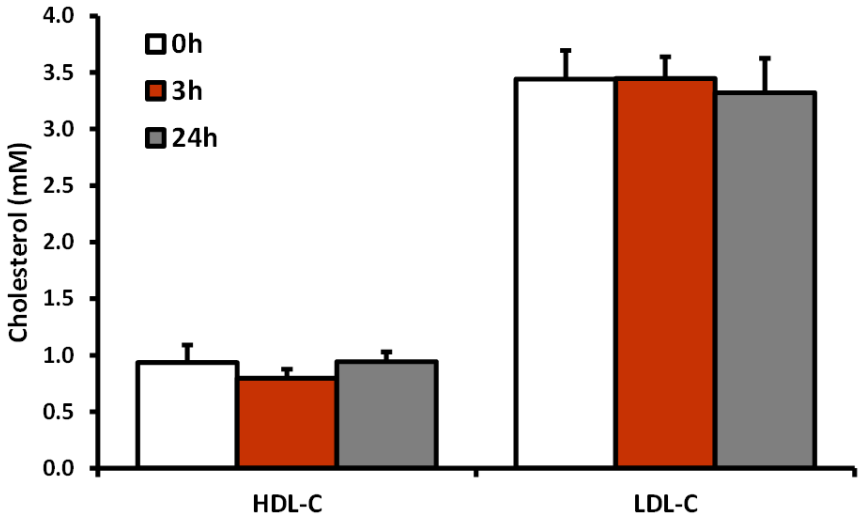


Figure 4.7: Cholesterol levels of volunteers following acute high PAMP onion meal challenge.

Cholesterol levels (HDL-C and LDL-C) were measured in ibuprofen-primed healthy human volunteers (n=12), collected before ingestion of a high PAMP onion meal (fasted, 0 h), postprandially (3 h) and the next day (fasted, 24 h) after the test meal. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons. Results are presented as means ± SEM.

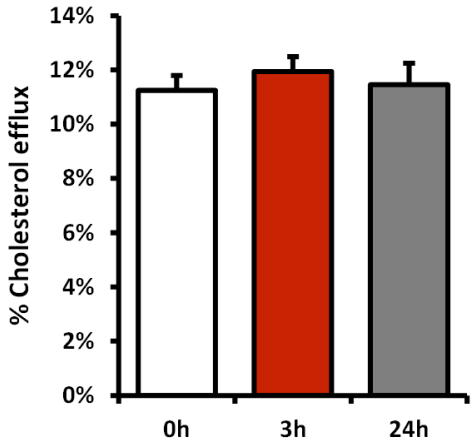


Figure 4.8: Cholesterol efflux capacity of serum samples following acute high PAMP onion meal ingestion.

Cholesterol efflux levels were measured in ApoB-depleted serum of ibuprofen-primed healthy human volunteers (n=12), collected before ingestion of a high PAMP onion meal (fasted, 0 h), postprandially (3 h) and the next day (fasted, 24 h) after the test meal. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons. Results are presented as means ± SEM.

Notably, a recent study has demonstrated that onions contain high levels of the molecule quercetin, which inhibits TLR2- and TLR4-signalling *in vitro* (Shibata *et al.* 2014). This may be a possible explanation for why the consumption of the onion test meal did not lead to pronounced acute inflammatory responses *in vivo*. For these reasons, we did not continue with the planned acute control meal experiment, which was to be based on nutritionally identical freshly chopped (low PAMP) onion.

4.2. A chronic dietary PAMP intervention study in healthy human volunteers

We next hypothesised that although the host may be resistant to a single challenge with dietary PAMPs, chronic or repeated exposure to dietary PAMPs could yet promote inflammation. To test this, we examined responses to a chronic dietary intake of high PAMP meals rather than a single meal, and compared it with the effect of low PAMP meals on the inflammatory and cardiometabolic markers in healthy man.

Sixteen healthy male volunteers were recruited to take part in a chronic dietary PAMP intervention study, of which eleven completed the study. Volunteers were asked to avoid specific types of food that we found in recent studies to be at relatively high risk of containing high levels of PAMPs for 7 days (Erridge C 2010). Volunteers were requested to consume any quantity of fresh produce. Then, over the next 4 days, volunteers were asked to consume a set lunch and evening meal provided to them, each selected on the basis of containing high levels of PAMPs (Erridge C 2010). Throughout the study, subjects were asked to keep a quantitative diet diary and to avoid excessive alcohol consumption for the duration of the study.

Based on the quantitative diet diaries from the human volunteers, we found that the dietary intervention study significantly diminished the number of servings of high PAMP foods from 1.51 to 0.58 per day (P=0.013) during the low PAMP week and these increased to 5.22 servings per day during the high PAMP arm. In addition, we discovered that all subjects were habitual consumers of foodstuffs which were identified previously to be at high risk of containing high levels of PAMPs, for instance, processed meats, ready meals, cheese, ready chopped vegetables and chocolate (Figure 4.9).

Crucially, we also measured daily macronutrient intake from the diet diaries. It was found that similar levels of total fat, carbohydrate, sugar, fiber, protein and salt were consumed during both weeks of the chronic study, although saturated fat intake was estimated to be ~75% higher during the high PAMP arm of the study (see Table 4.1).

Table 4.1: Estimated nutritional content of diets based on typical daily intakes during low and high PAMP diets.

Food type	Fat (g)	Saturated (g)	Carbo-hydrate (g)	Sugar (g)	Fibre (g)	Protein (g)	Salt (g)
Low PAMP week	**83.6**	**23.5**	**336.1**	**90.1**	**37.5**	**111.6**	**5.5**
High PAMP week	**83.8**	**41.1**	**333.0**	**89.2**	**35.1**	**78.3**	**6.7**

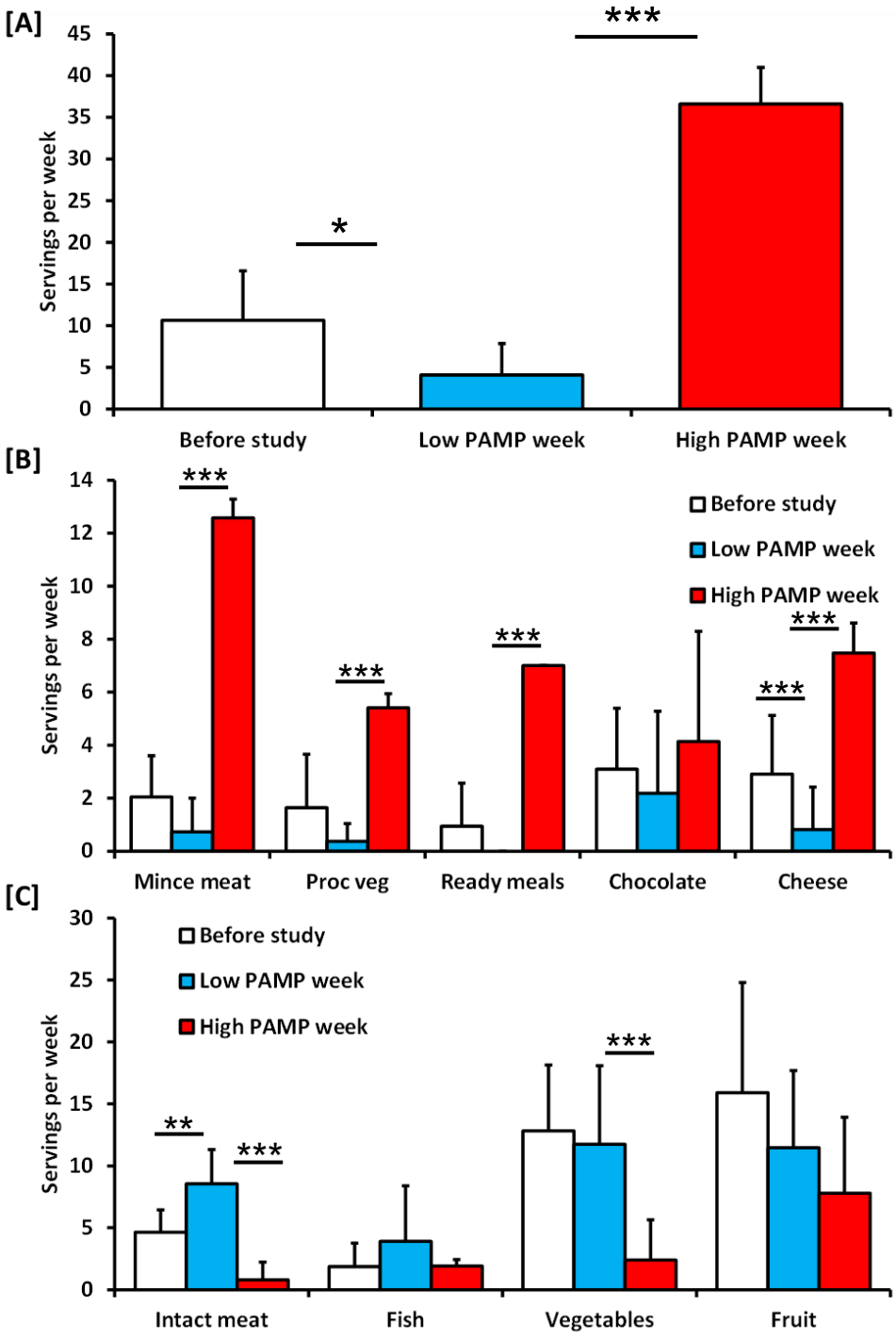


Figure 4.9: Frequency of consumption of high PAMP foods by category before and during low/high PAMP diet arms of chronic dietary study.

Consumption of high PAMP foods in healthy male subjects (n=11) before study (white bars), after low PAMP diet week (blue bars), and after high PAMP diet week (red bars). Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons (*P<0.05, **P<0.01, ***P<0.001). Results are presented as means ± SEM.

Unexpectedly, the results from the chronic dietary PAMP intervention study showed that weight and waist measurements fell significantly during the low PAMP diet (weight -0.7 kg, P = 0.047, waist -1.6 cm, P = 0.031) and this was reversed by the high PAMP diet (Figure 4.10). However, systolic and diastolic blood pressure measurements were not significantly altered by the low/high PAMP meals (Figure 4.11).

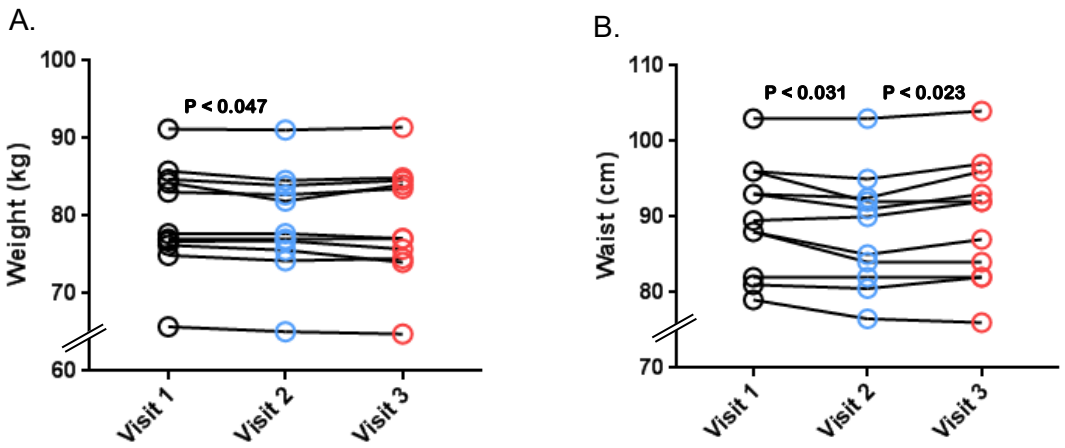


Figure 4.10: Effects of chronic low/high PAMP diets on weight and abdominal circumference in healthy human volunteers.

Body weight (A) and waist measurement (B), were measured in healthy male subjects (n=11) at study entry (Visit 1 - white bars), after consuming a low PAMP diet for 7 days (Visit 2 - blue bars), and after a high PAMP diet for 4 days (Visit 3 - red bars). Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons.

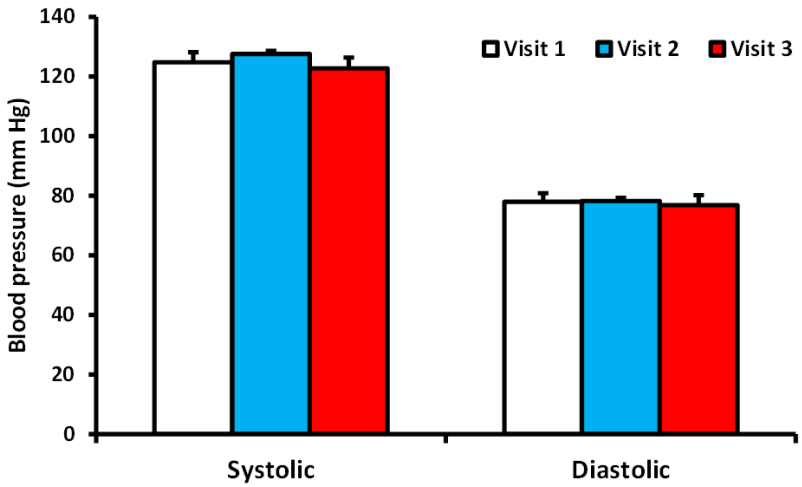


Figure 4.11: Effects of chronic low/high PAMP diets on blood pressure measurements in healthy human volunteers.

Blood pressure levels (systolic and diastolic) were measured in healthy male subjects (n=11) at study entry (Visit 1 - white bars), after consuming a low PAMP diet for 7 days (Visit 2 - blue bars), and after a high PAMP diet for 4 days (Visit 3 - red bars). Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons. Results are presented as means ± SEM.

Interestingly, the high PAMP meals caused significant elevations in several leukocyte markers of inflammatory status (Figure 4.12). For instance, leukocyte counts were significantly increased by the high PAMP meals when compared to the low PAMP meals (P=0.0143). Similar results were found for lymphocyte numbers (P=0.0067). An earlier association study by (Twig *et al*. 2012) showed that an increase of white blood cell counts of 1,000 cells/mm^3 was associated with a 17.4% increase in risk of coronary artery disease (CAD). Thus, based on our findings the leukocyte counts increase of 620 cells/mm^3 by the high PAMP meals (5,130 cells/mm^3), this is equivalent to a reduction in risk of CAD by about 10.7% on the low PAMP diet. Surprisingly, a significant increase in thrombocyte counts (P=0.045) was also observed by the high PAMP meals when compared to the low PAMP meals (Figure 4.13).

However, we did not observe a significant change in the inflammatory marker CRP (Figure 4.14) or serum endotoxin (Figure 4.15) by the chronic low/high PAMP meals.

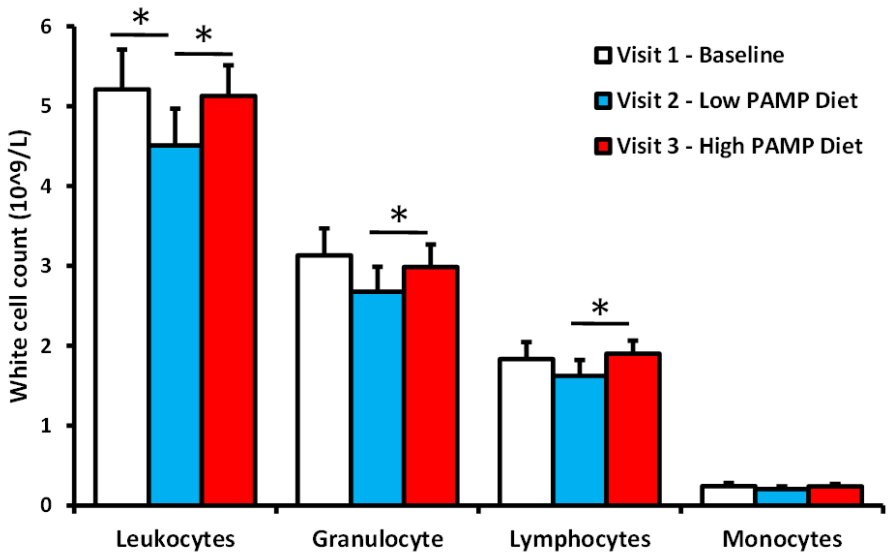


Figure 4.12: Leukocyte counts in fasted blood samples in chronic low/high PAMP dietary intervention study.

Leukocyte counts were measured in healthy male subjects (n=11) at study entry (Visit 1 - white bars), after consuming a low PAMP diet for 7 days (Visit 2 - blue bars), and after a high PAMP diet for 4 days (Visit 3 - red bars). Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons (*P<0.05). Results are presented as means ± SEM.

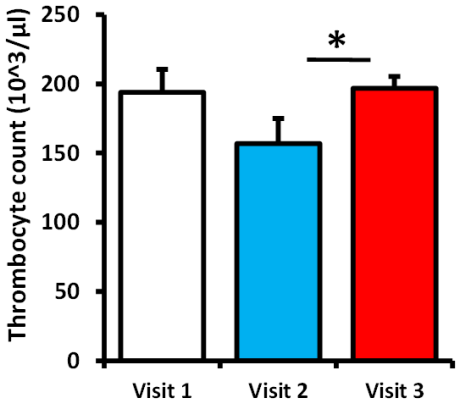


Figure 4.13: Thrombocyte counts in fasted blood samples in chronic low/high PAMP dietary intervention study.

Platelet counts were measured in healthy male subjects (n=11) at study entry (Visit 1 - white bars), after consuming a low PAMP diet for 7 days (Visit 2 - blue bars), and after a high PAMP diet for 4 days (Visit 3 - red bars). Data are shown as mean ± SEM. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons (*P<0.05).

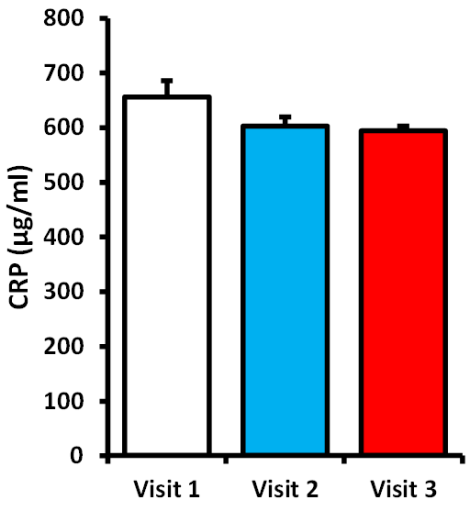


Figure 4.14: CRP responses to chronic low/high PAMP dietary interventions in healthy human volunteers.

CRP levels were measured in plasma samples of healthy male subjects (n=11) at study entry (Visit 1 - white bars), after consuming a low PAMP diet for 7 days (Visit 2 - blue bars), and after a high PAMP diet for 4 days (Visit 3 - red bars), using high sensitivity ELISA. Results are presented as means ± SEM. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons.

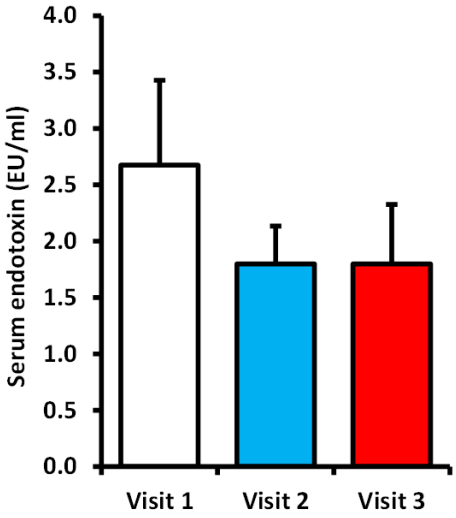


Figure 4.15: Serum endotoxin concentrations in chronic low/high PAMP dietary intervention study.

Serum endotoxin levels were measured in healthy male subjects (n=11) at study entry (Visit 1 - white bars), after consuming a low PAMP diet for 7 days (Visit 2 - blue bars), and after a high PAMP diet for 4 days (Visit 3 - red bars), using the kinetic limulus amoebocyte lysate assay. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons. Results are presented as means ± SEM.

Furthermore, the most remarkable finding that we have observed in this study was a significant reduction of LDL-C by the low PAMP diet and it was rapidly increased by the high PAMP meals (P=0.019 *vs.* visit 1 [baseline] and P=0.039 vs. visit 3 [high PAMP meal]). By contrast, there was no significant effect of the low/high PAMP meals on serum triglyceride or HDL-C (Figure 4.16).

A similar observation was also reported by (Ambring *et al.* 2004), where in healthy subjects with normal blood lipids, 4 weeks of a Mediterranean diet decreased LDL-cholesterol level about 22.8%, compared with a typical Swedish (western) diet (baseline LDL-C 3.5 mmol/l). This suggests that dietary factors can modify LDL-C levels within a relatively short timescale in healthy men.

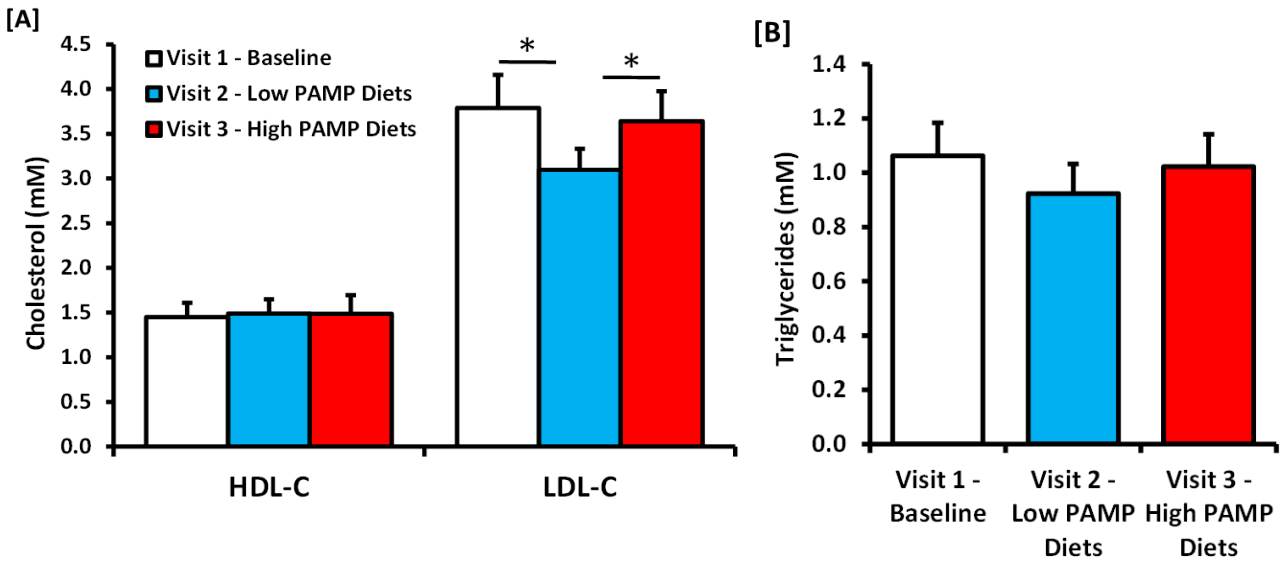


Figure 4.16: Serum cholesterol and triglyceride concentrations in chronic low/high PAMP dietary intervention study.

Cholesterol levels (HDL-C and LDL-C) (A) and plasma triglycerides (B) were measured in healthy male subjects (n=11) at study entry (Visit 1 - white bars), after consuming a low PAMP diet for 7 days (Visit 2 - blue bars), and after a high PAMP diet for 4 days (Visit 3 - red bars). Data are shown as mean ± SEM. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons (*P<0.05).

Leptin concentrations were also significantly reduced by the low PAMP diet (P = 0.006) and this was again reversed by the high PAMP intervention (P = 0.055) (Figure 4.17). The dietary interventions had no significant effect on serum cholesterol efflux capacity (Figure 4.18) or markers of insulin sensitivity (insulin, glucose and HOMA-IR) (Figure 4.19).

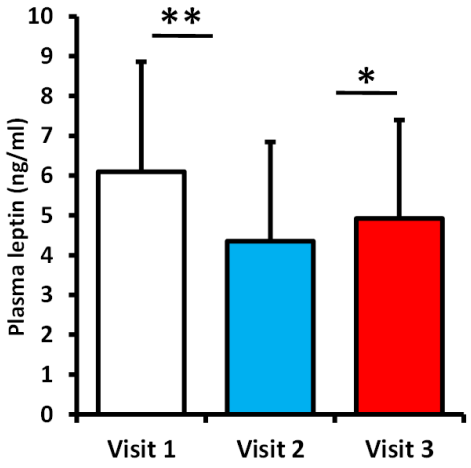


Figure 4.17: Effects of chronic low and high PAMP dietary interventions on plasma leptin concentrations.

Plasma leptin concentrations was measured in healthy male subjects (n=11) at study entry (Visit 1 - white bars), after consuming a low PAMP diet for 7 days (Visit 2 - blue bars), and after a high PAMP diet for 4 days (Visit 3 - red bars), using ELISA. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons (*P<0.05, **P<0.01). Results are presented as means ± SEM.

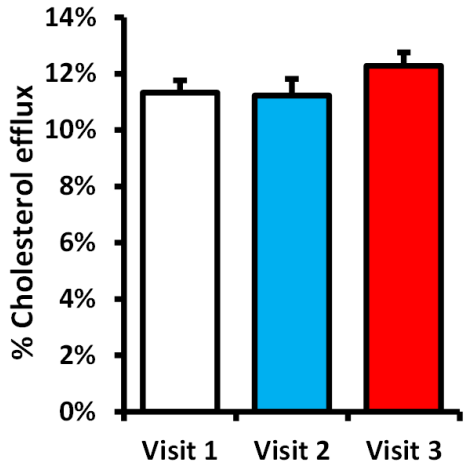


Figure 4.18: Serum cholesterol efflux capacity following chronic low and high PAMP dietary interventions.

Cholesterol efflux levels were measured in healthy male subjects (n=11) at study entry (Visit 1 - white bars), after consuming a low PAMP diet for 7 days (Visit 2 - blue bars), and after a high PAMP diet for 4 days (Visit 3 - red bars). Results are presented as means ± SEM. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons.

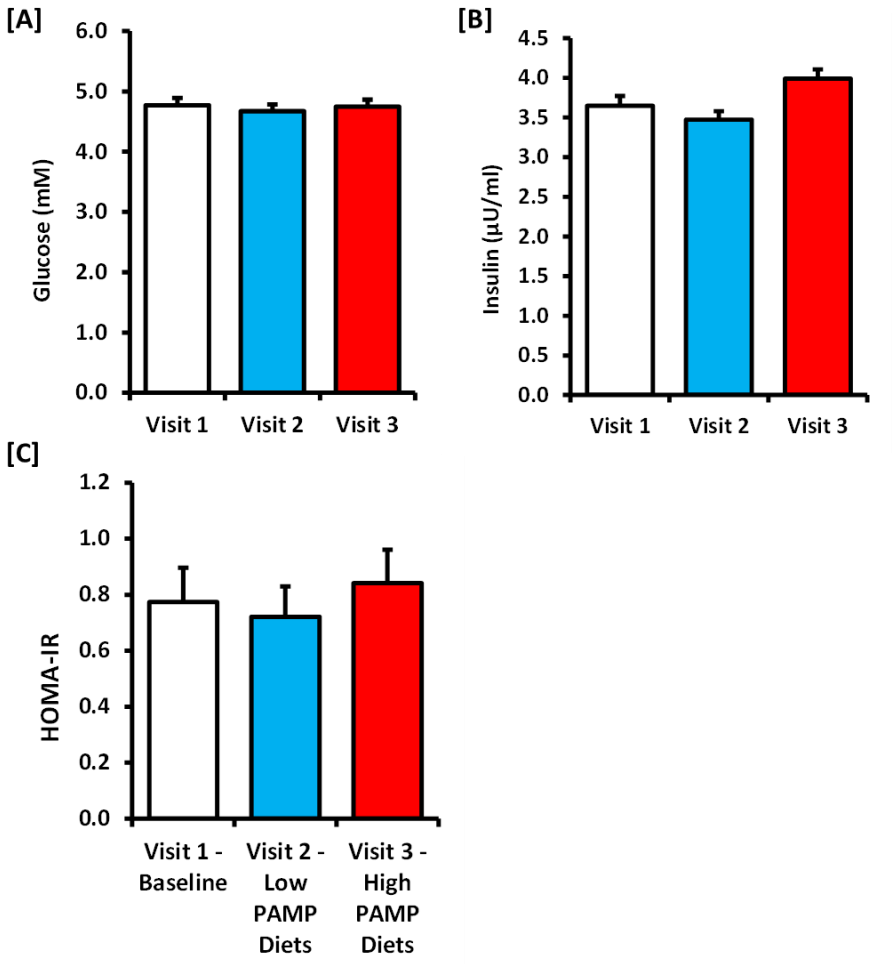


Figure 4.19: Glucose, insulin and HOMA-IR levels following chronic low and high PAMP dietary interventions.

Glucose (A), insulin (B) and HOMA-IR (C) levels were measured in healthy male subjects (n=11) at study entry (Visit 1 - white bars), after consuming a low PAMP diet for 7 days (Visit 2 - blue bars), and after a high PAMP diet for 4 days (Visit 3 - red bars). Results are presented as means ± SEM. Results were compared by repeated measures ANOVA with Sidak's correction for multiple comparisons.

Taken together, the results from this chapter indicate that no overt inflammatory or dyslipididaemic phenotype is induced in healthy men by a single exposure to high levels of dietary PAMPs (at least when delivered with large quantities of potentially anti-inflammatory onion). However, repeated exposure to meals rich in PAMPs results in marked increases in LDL-C and leukocyte counts. The observed effects are not likely due to changes in macronutrient intake, as these were largely similar on both arms of the study.

<u>Chapter 5</u> Results - Effects of dietary PAMPs on mechanisms of atherosclerosis in mice *in vivo*

Previous results from our laboratory (introduction, Figure 1.23-A and B), indicated that chronic treatment of WT (C57) mice with 3 PAMPs (LPS, Pam_3CSK_4, and iEDAP) mix in drinking water for 10 weeks on low fat diet, did not significantly induce serum amyloid A (SAA). However, significant responses were noted in terms of hepatic expression of several inflammatory genes (PTX, IL-1β, IL-6, TNF-α, and Apo-AI). In addition, serum cholesterol efflux was decreased significantly in mice treated with 3 PAMPs. Also, total cholesterol and HDL were decreased significantly in mice treated with 3 PAMPs (introduction, Figure 1.24-A and B).

Because chronic administration of PAMPs to mice was very expensive and time consuming, it was not feasible to use this type of experiment to explore mechanisms of action of dietary PAMPs. Since mice orally gavaged with a single PAMP or mix of PAMPs did not show obvious upregulation of acute phase response (APR) markers within 24 h (introduction, Figure 1.26), we therefore sought means to increase the sensitivity of mice to orally delivered LPS.

Work from other members of the group showed previously that gut permeability to FITC-dextran and *E. coli* LPS was significantly increased by treatment of mice with the NSAID indomethacin, but not a 4 week HFD (Figure 1.27).

5.1. NSAID treatment to increase permeability of mouse intestinal barrier

I therefore first explored the potential of NSAID priming with indomethacin to increase responsiveness of healthy wild type mice (C57BL/6), to acute gavage with *E. coli* LPS as measured by APR markers.

In brief, wild type mice (C57BL/6) were pretreated with 2mg/kg indomethacin (NSAID) for 24 hours, then half were orally gavaged with normal saline (control) and the other half were orally gavaged with *E. coli* LPS (2 mg/mouse) dissolved in normal saline. Although a plasma SAA response to orally delivered LPS was observed in this model, it was only modest (P=0.0127 *vs.* control) (Figure 5.1). Also, expression of inflammatory marker genes in liver tissue, including serum amyloid-A1, SAA3, Apolipoprotein-A1, Lipocalin, Haptoglobin (Figure 5.2), Interleukin (IL)-1b, IL-6 and Tumor necrosis factor alpha (TNF-α) (Figure 5.3), as measured by RT-PCR, were not significantly upregulated by orally delivered LPS. In addition, cholesterol levels (TC, HDL, and LDL/VLDL) were not significantly altered by orally gavaged LPS (Figure 5.4).

Indomethacin induces a very large increase in gut permeability, but the resulting inflammatory response measured by plasma SAA and hepatic mRNA markers is still only modest. This suggests that healthy WT mice are relatively unresponsive to LPS, even when large amounts of LPS enter the circulation.

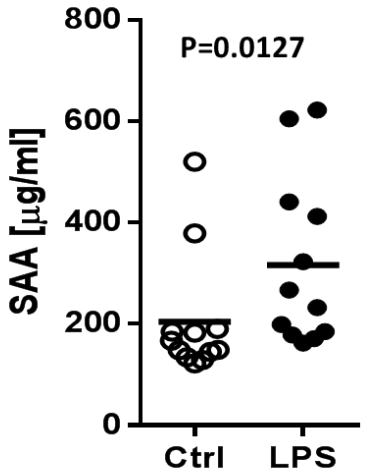


Figure 5.1: Effect of orally delivered LPS on circulating serum amyloid A in wild type mice pretreated with indomethacin.

Wild-type C57BL/6 mice (n=12 per group) were treated with 2 mg/kg indomethacin (NSAID) by oral gavage in saline. 24 h later, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, plasma content of SAA was measured by ELISA. Horizontal bars indicate means, which were compared by unpaired t-test.

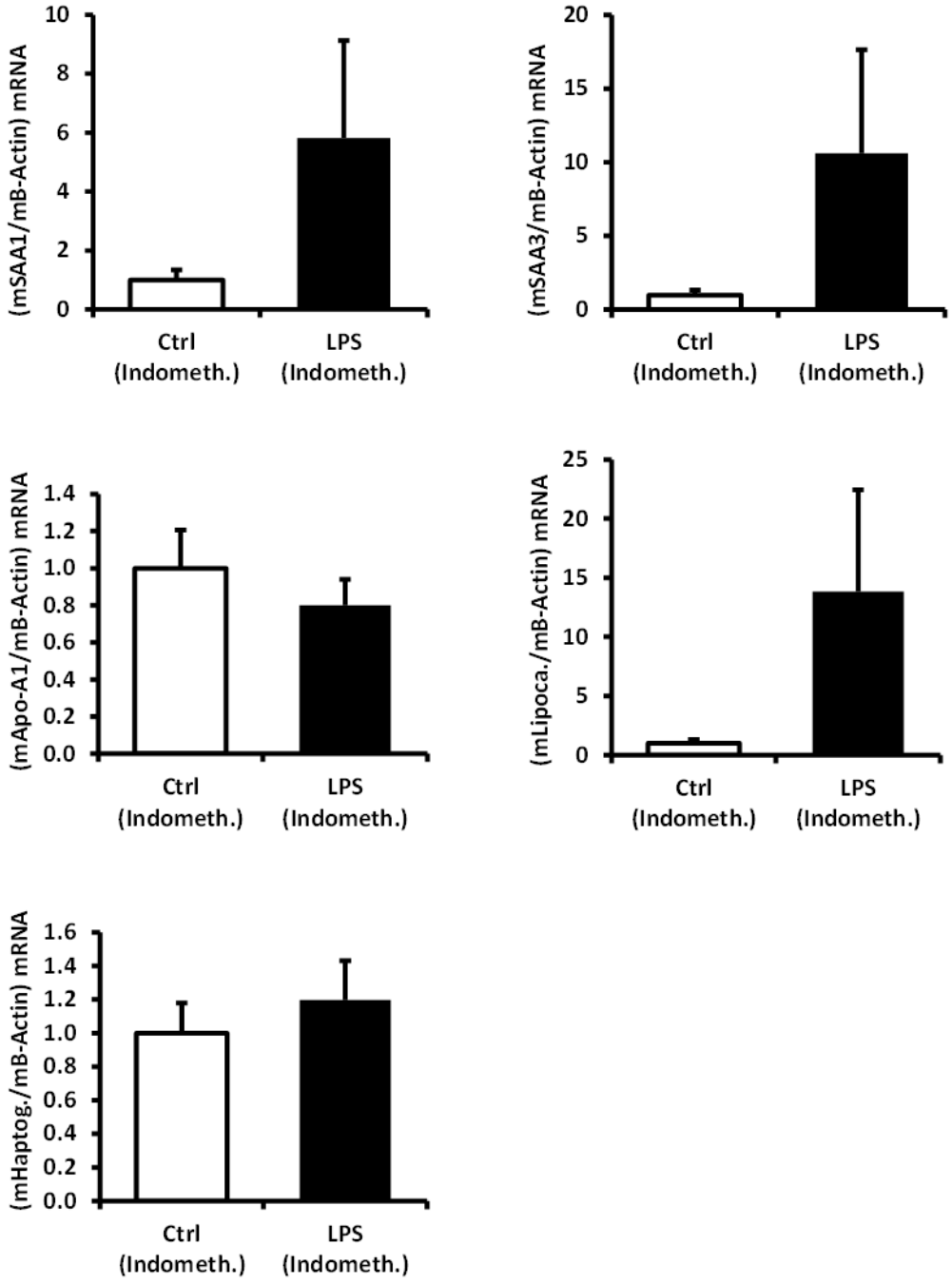


Figure 5.2: Impact of orally delivered LPS on hepatic mRNA markers of the acute phase response in wild type mice pretreated with indomethacin.

Wild-type C57BL/6 mice (n=12 per group) were treated with 2 mg/kg indomethacin (NSAID) by oral gavage in saline. 24 h later, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic mRNA responses (Serum amyloid-A 1, SAA3, Apolipoprotein-A1, Lipocalin and Haptoglobin), were measured by RT-PCR. Data are presented as means ± SEM, which were compared by unpaired t-test.

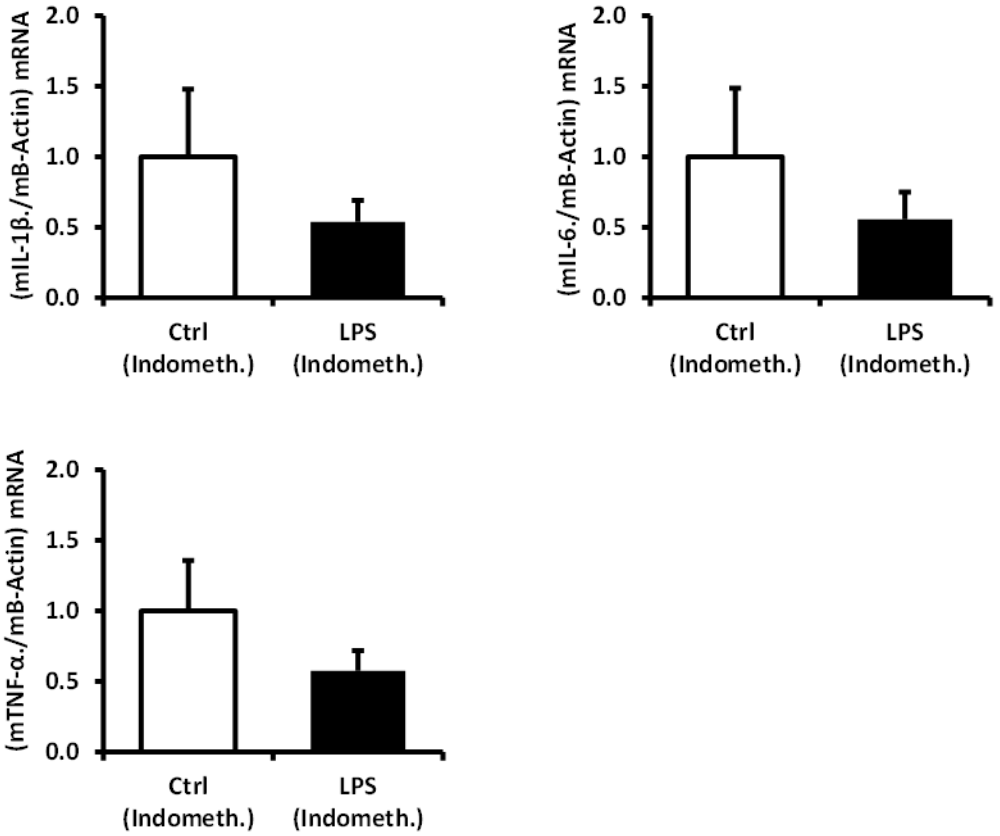


Figure 5.3: Impact of orally delivered LPS on hepatic mRNA markers of inflammation in wild type mice pretreated with indomethacin.

Wild-type C57BL/6 mice (n=12 per group) were treated with 2 mg/kg indomethacin (NSAID) by oral gavage in saline. 24 h later, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic mRNA responses (Interleukin (IL)-1β, IL-6 and tumor necrosis factor alpha (TNF)-α), were measured by RT-PCR. Data are presented as means ± SEM, which were compared by unpaired t-test.

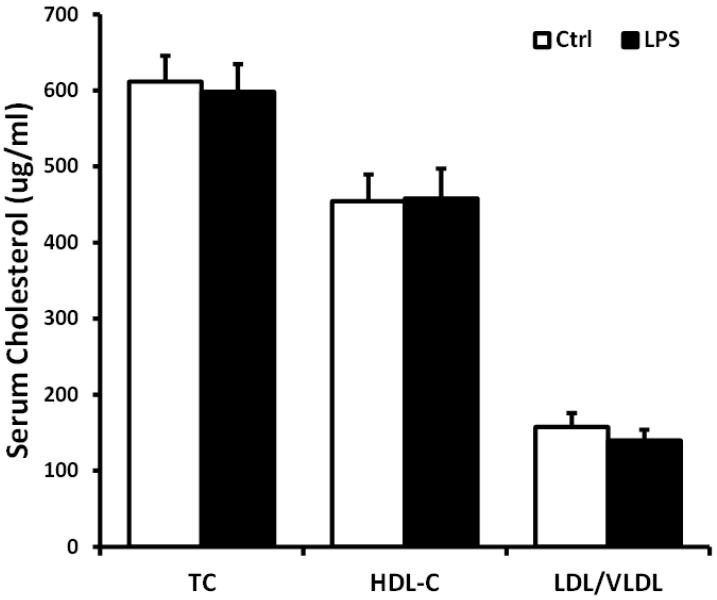


Figure 5.4: Influence of orally delivered LPS on cholesterol levels in wild type mice pretreated with indomethacin.

Wild-type C57BL/6 mice (n=12 per group) were treated with 2 mg/kg indomethacin (NSAID) by oral gavage in saline. 24 h later, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, cholesterol levels were measured by Amplex red enzymatic assay. Data are shown as means ± SEM, which were compared by unpaired t-test.

5.2. *P. acnes* injection to boost sensitivity of mouse to LPS

Next, we examined hepatic inflammatory gene expression and serum acute phase markers (SAA) in mouse models treated by means shown previously to increase sensitivity to LPS. As previously reported, mice pre-treated with heat-killed *Propionibacterium acnes* are highly susceptible to LPS (Ferluga, Allison 1978, Yoshimoto *et al*. 1992). *P. acnes*-primed mice, but not control mice, show obvious serum elevation of pro-inflammatory cytokines including IL-6, IFN-γ, and TNF-α (Sakao *et al*. 1999, Yoshimoto *et al*. 1992, Kawa *et al*. 2010).

Briefly, Wild-type C57BL/6 mice were primed with intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria. At day 7 after *P. acnes* treatment, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS.

Excessively high background levels of acute phase markers and the plasma serum amyloid A were observed in control mice, and this was not elevated further by LPS (Figure 5.5 and 6). Furthermore, cholesterol levels and serum cholesterol efflux capacity were not significantly changed by orally gavaged LPS (Figure 5.7 and 8).

This suggests that *P. acnes* treatment increases background levels of inflammatory markers too far to witness subtle effects of more modest inflammatory stimuli. Thus, we concluded that *P. acnes* treatment is not a useful model for the subsequent experiments.

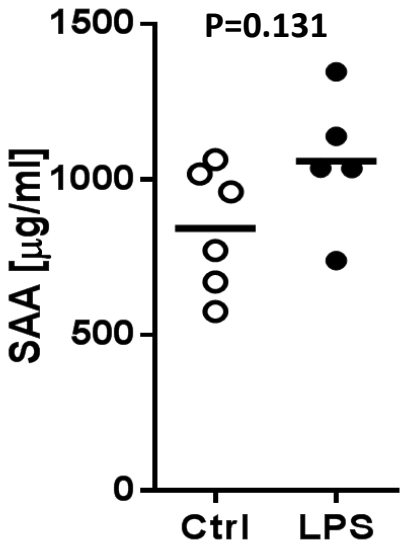


Figure 5.5: Effect of orally delivered LPS on circulating serum amyloid A in wild type mice pretreated with heat-killed *P. acnes* bacteria.

Wild-type C57BL/6 mice (n=6 per group) were primed with intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria. On day seven, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, plasma content of SAA was measured by ELISA. Horizontal bars indicate means, which were compared by unpaired t-test.

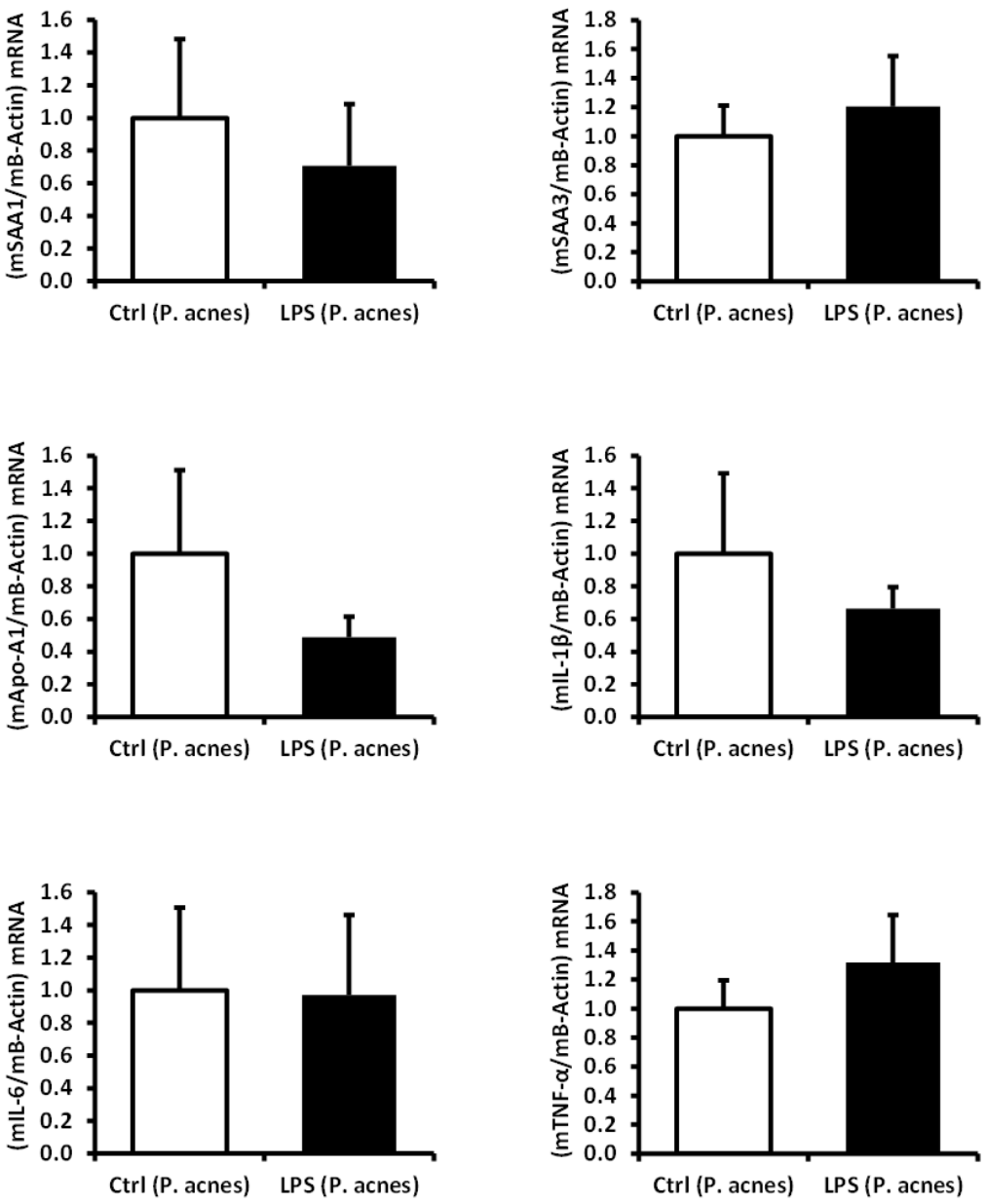


Figure 5.6: Impact of orally delivered LPS on hepatic mRNA markers of the acute phase response in wild type mice pretreated with heat-killed *P. acnes* bacteria.

Wild-type C57BL/6 mice (n=6 per group) were primed with intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria. On day seven, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic mRNA responses (Serum amyloid-A1, SAA3, Apolipoprotein-A1, IL-1β, IL-6 and TNF-α), were measured by RT-PCR. Data are presented as means ± SEM, which were compared by unpaired t-test.

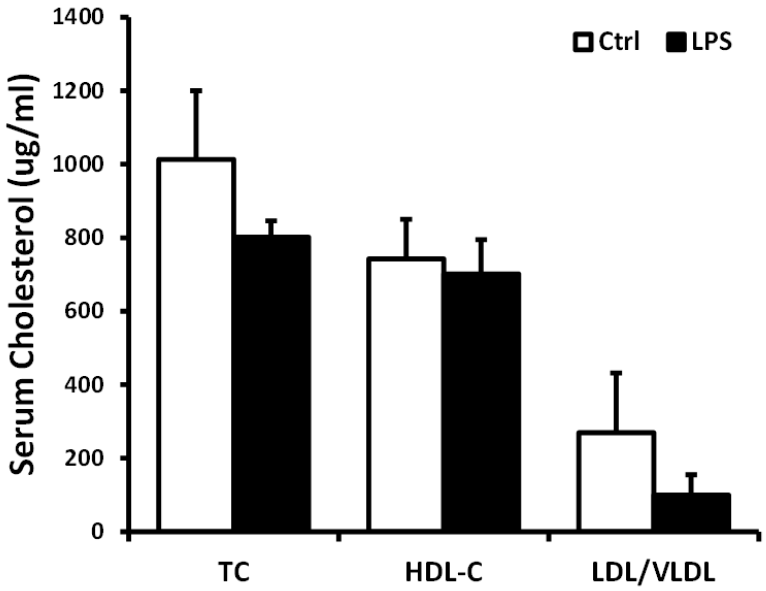


Figure 5.7: Influence of orally delivered LPS on cholesterol levels in wild type mice pretreated with heat-killed *P. acnes* bacteria.

Wild-type C57BL/6 mice (n=6 per group) were primed with intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria. On day seven, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, cholesterol levels in total serum (TC) and lipoprotein fractions were measured by Amplex red enzymatic assay. Data are shown as means ± SEM, which were compared by unpaired t-test.

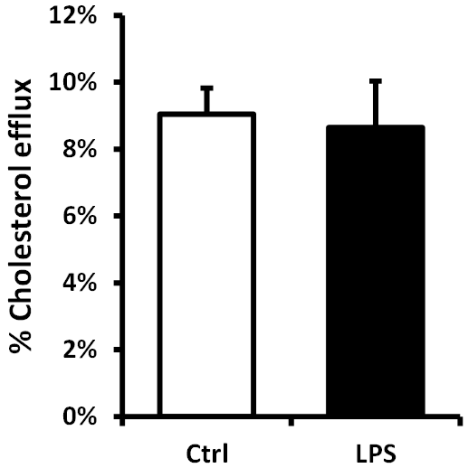


Figure 5.8: Effect of orally delivered LPS on serum cholesterol efflux capacity in wild type mice pretreated with heat-killed *P. acnes* bacteria.

Wild-type C57BL/6 mice (n=6 per group) were primed with intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria. On day seven, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a 24 h, the cholesterol efflux capacity of Apo-B depleted serum was measured. Data are shown as means ± SEM, which were compared by unpaired t-test.

5.3. High Cholesterol Diet to boost sensitivity of mouse to LPS

Next, we explored the potential of priming WT mice with a High Cholesterol Diet (HCD) for 4 weeks, since this was shown previously to increase cytokine responses induced by intraperitoneally injected LPS (Hong H *et al* 2007, Wouters *et al*. 2008, Shono *et al*. 2011). After this priming phase, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS.

Similar results to *P. acnes* treatment were observed with excessively high background levels of plasma serum amyloid A and hepatic acute phase gene expression (Figure 5.9 and 10). In addition, there were no significant changes in the cholesterol levels and serum cholesterol efflux capacity in response to orally gavaged LPS in this model (Figure 5.11 and 12).

This suggests that C57BL/6 mice fed a HCD show excessively high background levels of inflammatory markers, which will not be suitable for further trials in this study.

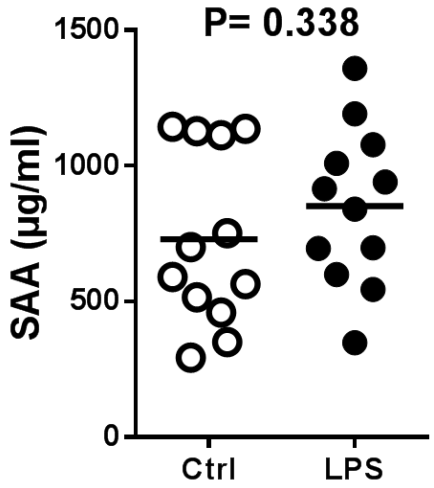


Figure 5.9: Impact of HCD priming on effect of orally delivered LPS on serum amyloid A in mice.

Wild-type C57BL/6 mice (n=12 per group) were fed a HCD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, plasma content of SAA was measured by ELISA. Horizontal bars indicate means, which were compared by unpaired t-test.

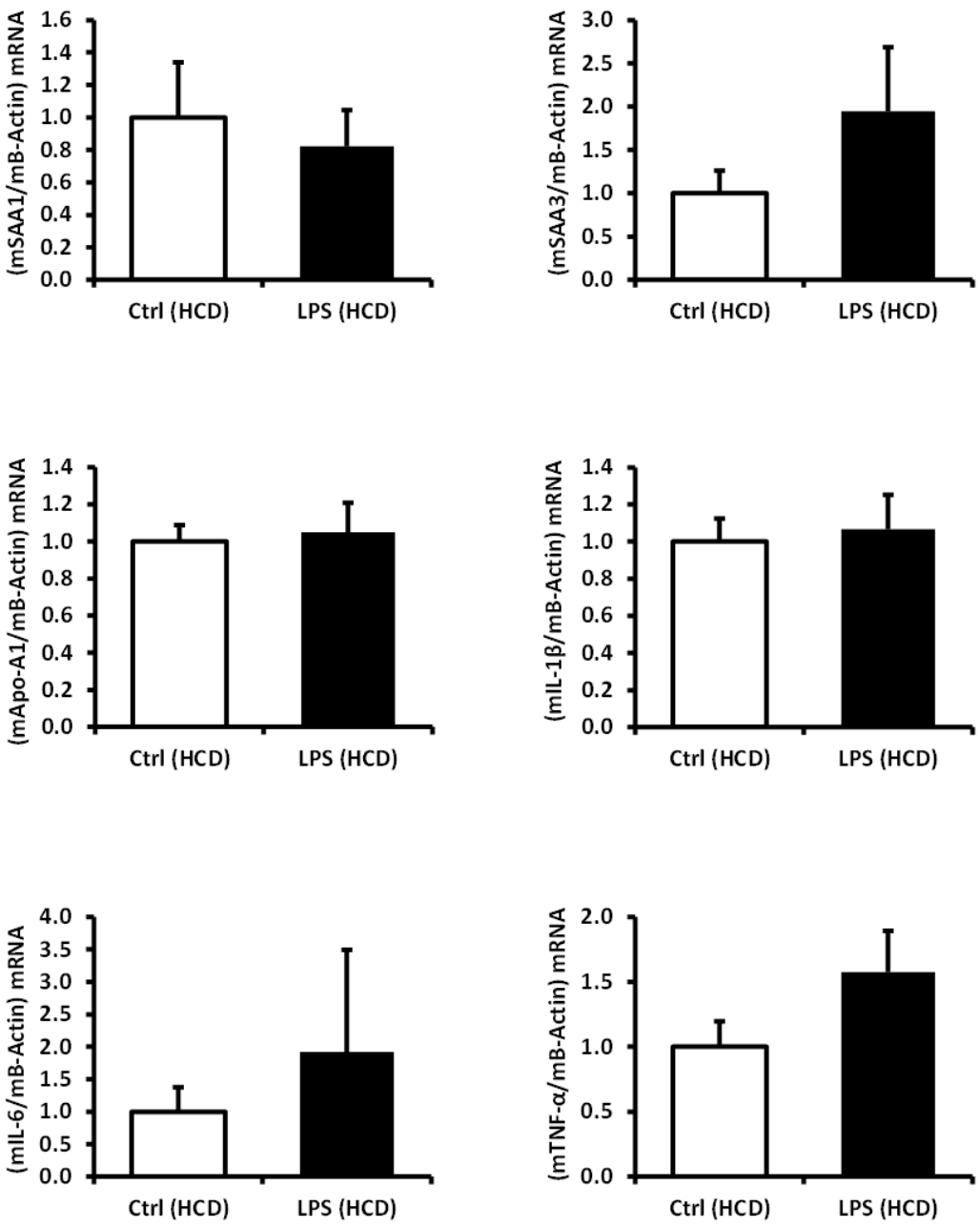


Figure 5.10: Impact of HCD priming on effect of orally delivered LPS on hepatic mRNA markers in mice.

Wild-type C57BL/6 mice (n=12 per group) were fed a HCD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic mRNA responses (Serum amyloid-A1, SAA3, Apolipoprotein-A1, IL-1β, IL-6 and TNF-α), were measured by RT-PCR. Data are shown as means ± SEM, which were compared by unpaired t-test.

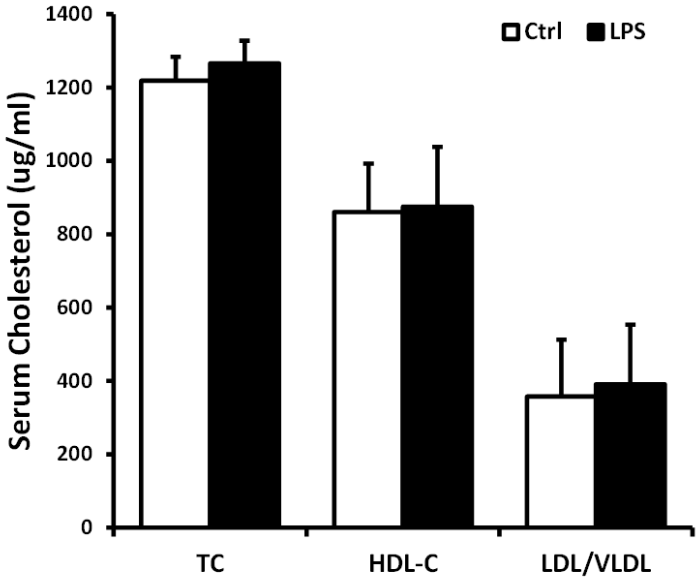


Figure 5.11: Influence of HCD priming on effect of orally delivered LPS on cholesterol levels in wild type mice.

Wild-type C57BL/6 mice (n=12 per group) were fed a HCD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, total plasma and lipoprotein fraction cholesterol levels were measured by Amplex red enzymatic assay. Data are shown as means ± SEM, which were compared by unpaired t-test.

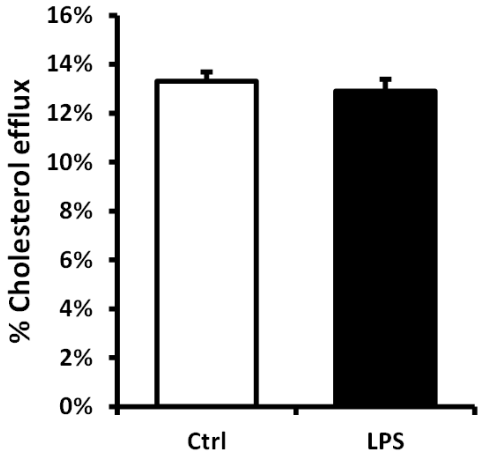


Figure 5.12: Impact of HCD priming on the effect of orally delivered LPS on serum cholesterol efflux capacity in mice.

Wild-type C57BL/6 mice (n=12 per group) were fed a HCD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, the cholesterol efflux capacity of Apo-B depleted serum was measured. Data are shown as means ± SEM, which were compared by unpaired t-test.

5.4. High Fat Diet to boost sensitivity of mouse to LPS

It has been reported that hepatic expression of TNF-α and IL-6 mRNA increases significantly in wild-type mice in response to LPS injection after feeding a High Fat Diet (HFD), without high cholesterol, for 8, 10, or 12 weeks compared to mice were fed normal chow (Imajo *et al.* 2012). Therefore, we next examined the mild priming treatment of 4 weeks HFD feeding before oral LPS gavage. In this experiment, wild type mice (C57BL/6) were fed either low fat diet (LFD-normal chow) or HFD for 4 weeks before gavage with LPS. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS.

Mice gavaged with LPS after HFD pretreatment showed highly significant increases in plasma SAA (P=0.0053 *vs.* control) (Figure 5.13), and significant responses from hepatic inflammatory gene expression such as SAA1 (P=0.037 *vs.* control), SAA3 (P=0.045 *vs.* control), Lipocalin (P=0.014 *vs.* control) and Haptoglobin (P=0.048 *vs.* control) (Figure 5.14). However, IL-1β, IL-6 and TNF-α were not significantly increased (Figure 5.15). Furthermore, a significant reduction was observed in plasma Apo-A1 (P=0.0015 *vs.* control) response in mice gavaged with LPS after HFD pretreatment when compared to HFD-primed mice receiving sham gavage (Figure 5.16). In addition, immunoblot analysis of Apolipoprotein-A1 protein in the plasma of wild-type C57BL/6 mice fed a HFD for 4 weeks then orally gavaged with 200 µl saline with *E. coli* LPS dropped significantly (P=0.0016 *vs.* control) compared to the control group (Figure 5.17).

As with the chronic PAMP intake model, there was a significant reduction of total cholesterol level (P=0.045 *vs.* control) and HDL-C (P=0.022 *vs.* control) in the LPS-treated HFD-primed mice. LDL-C was not significantly increased when compared to the group of mice gavaged with saline only (Figure 5.18). Strikingly, the serum cholesterol efflux capacity also fell significantly (P=0.024 *vs.* control) in mice orally gavaged with *E. coli* LPS (Figure 4.19).

The ability to detect these effects may reflect the fact that HFD priming alone did not promote a state of low-grade systemic inflammation when compared to the group fed on LFD (Figure 5.20), meaning this is a suitable baseline for oral gavage experiments. These results therefore suggest that 4 week priming with HFD leads to a clear induction of responsiveness to orally delivered LPS. This model was therefore utilised in the further mechanistic experiments.

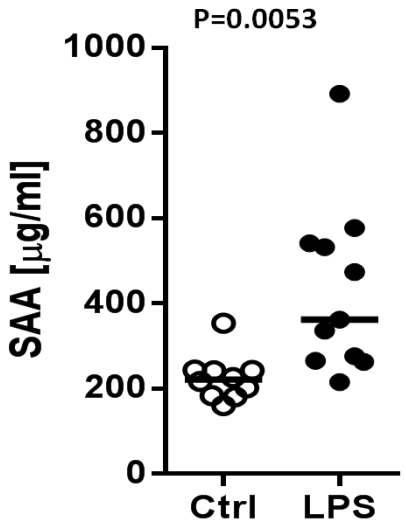


Figure 5.13: Effect of HFD priming on serum amyloid A responses to orally delivered LPS in mice.

Wild-type C57BL/6 mice (n=12 per group) were fed a HFD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, plasma content of SAA was measured by ELISA. Horizontal bars indicate means, which were compared by unpaired t-test.

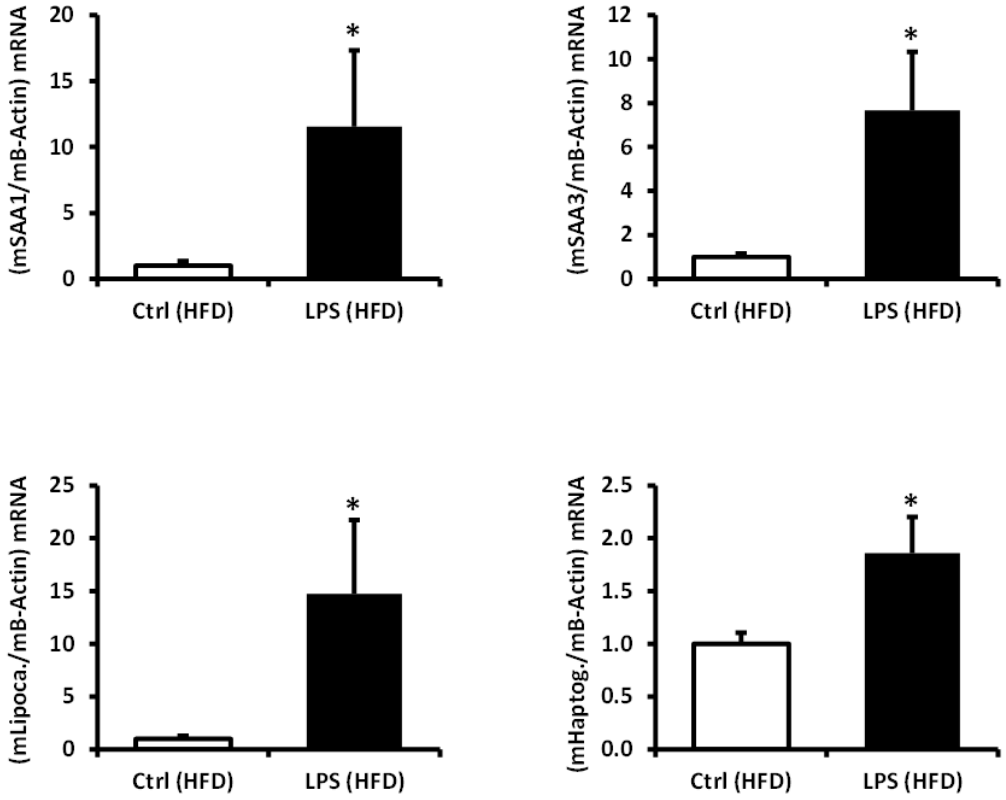


Figure 5.14: Impact of HFD priming on hepatic acute phase response gene mRNA responses to orally delivered LPS in mice.

Wild-type C57BL/6 mice (n=12 per group) were fed a HFD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic mRNA responses (Serum amyloid-A1, SAA3, Apolipoprotein-A1, Lipocalin, Haptoglobin), were measured by RT-PCR. Data are shown as means ± SEM, which were compared by unpaired t-test. *P<0.05, **P<0.01, ***P<0.001 *vs.* control (normal saline alone).

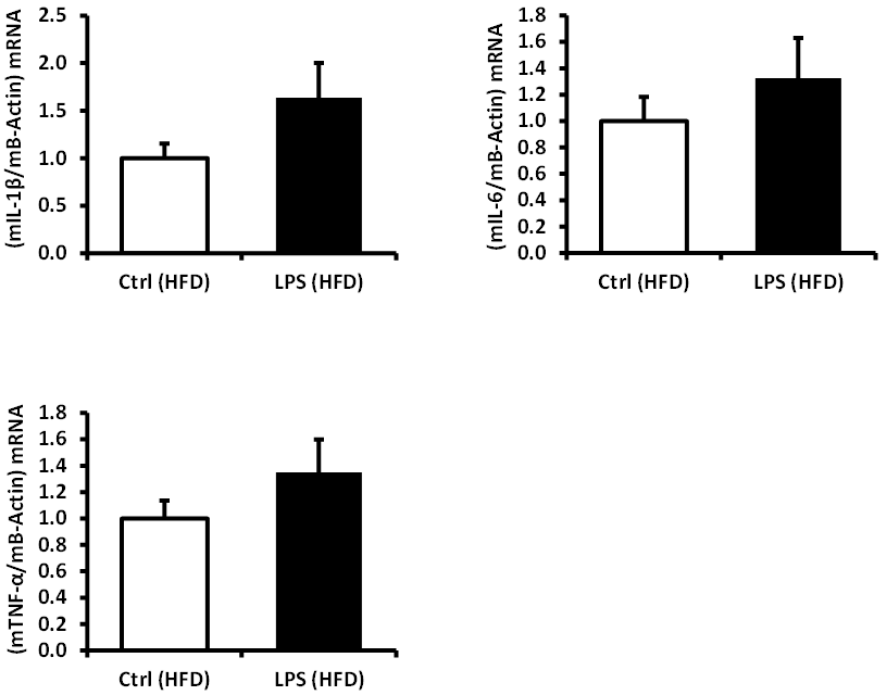


Figure 5.15: Impact of HFD priming on hepatic inflammatory gene mRNA responses to orally delivered LPS in mice.

Wild-type C57BL/6 mice (n=12 per group) were fed a HFD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic inflammatory cytokine mRNA responses (IL-1β, IL-6 and TNF-α), were measured by RT-PCR. Data are shown as means ± SEM, which were compared by unpaired t-test.

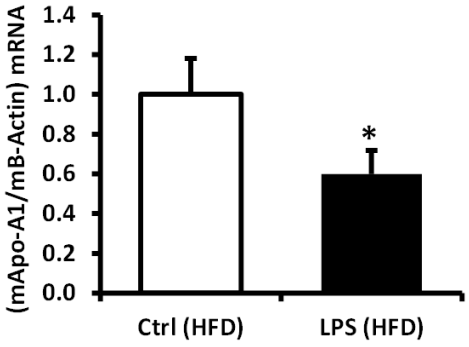


Figure 5.16: Effect of HFD priming on hepatic Apolipoprotein-A1 mRNA responses to orally delivered LPS in mice.

Wild-type C57BL/6 mice (n=12 per group) were fed a HFD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic Apolipoprotein-A1 mRNA was measured RT-PCR. Data are shown as means ± SEM, which were compared by unpaired t-test. *P<0.05 *vs.* control (normal saline alone).

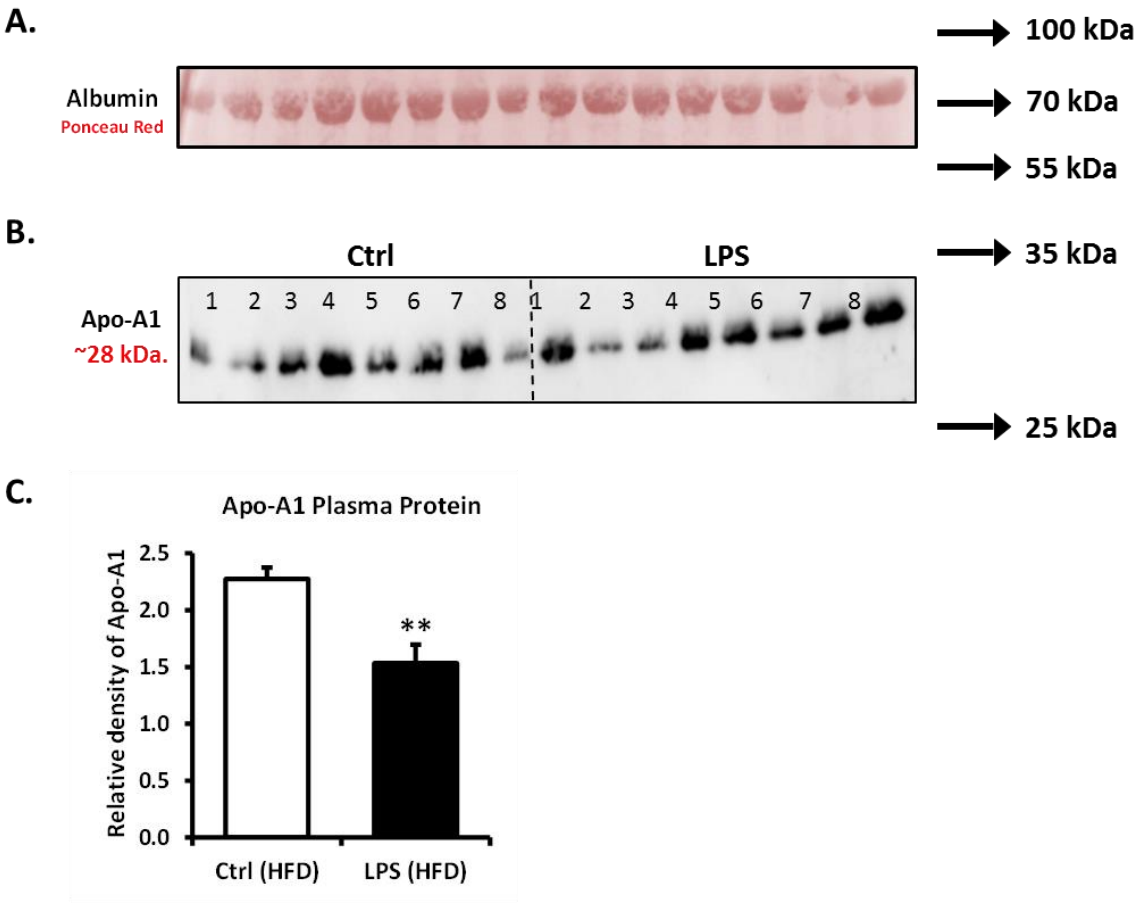


Figure 5.17: Impact of HFD priming on circulating Apo-A1 protein levels in mice orally gavaged with LPS.

Immunoblot analysis of Apolipoprotein-A1 protein in the plasma of wild-type C57BL/6 mice (n=8 per group) fed a HFD for 4 weeks. Mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS, serum was taken 24 hours later. Albumin is revealed as a 70 kDa band using ponceau red stain (A). Immunoblot for apolipoprotein-A1 (28 kDa) in mouse plasma samples (B). Relative density of Apo-A1 expression in HFD-primed wild-type mice with or without oral LPS treatment (C). Data are shown as means ± SEM, and were compared by unpaired t-test. **P<0.01 *vs.* control (normal saline alone).

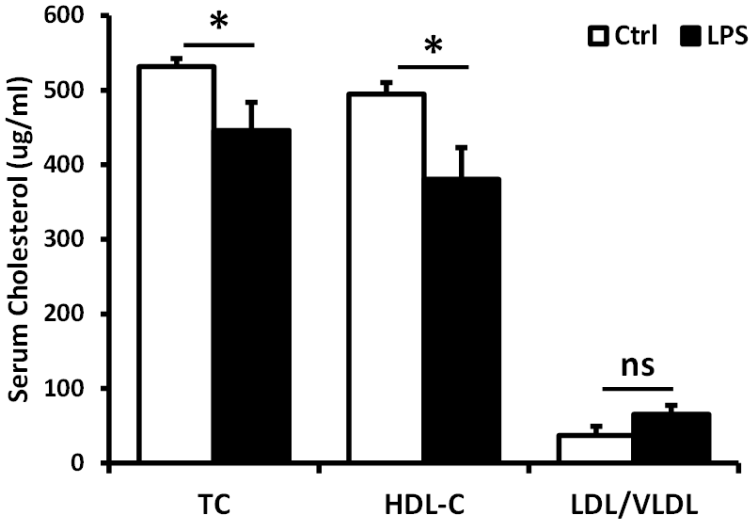


Figure 5.18: Effect of HFD priming on the cholesterol response to orally delivered LPS in mice.

Wild-type C57BL/6 mice (n=12 per group) were fed a HFD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, cholesterol in total plasma and lipoprotein fractions were measured by Amplex red enzymatic assay. Data are shown as means ± SEM, which were compared by unpaired t-test. *P<0.05 *vs.* control (normal saline alone).

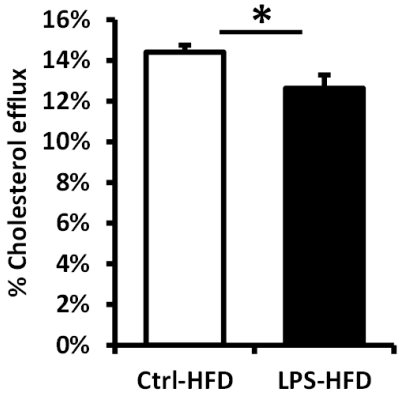


Figure 5.19: Effect of orally delivered LPS on serum cholesterol efflux capacity in HFD primed mice.

Wild-type C57BL/6 mice (n=12 per group) were fed a HFD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, cholesterol efflux capacity of Apo-B depleted serum was measured. Data are shown as means ± SEM, which were compared by unpaired t-test. *P<0.05 *vs.* control (normal saline alone).

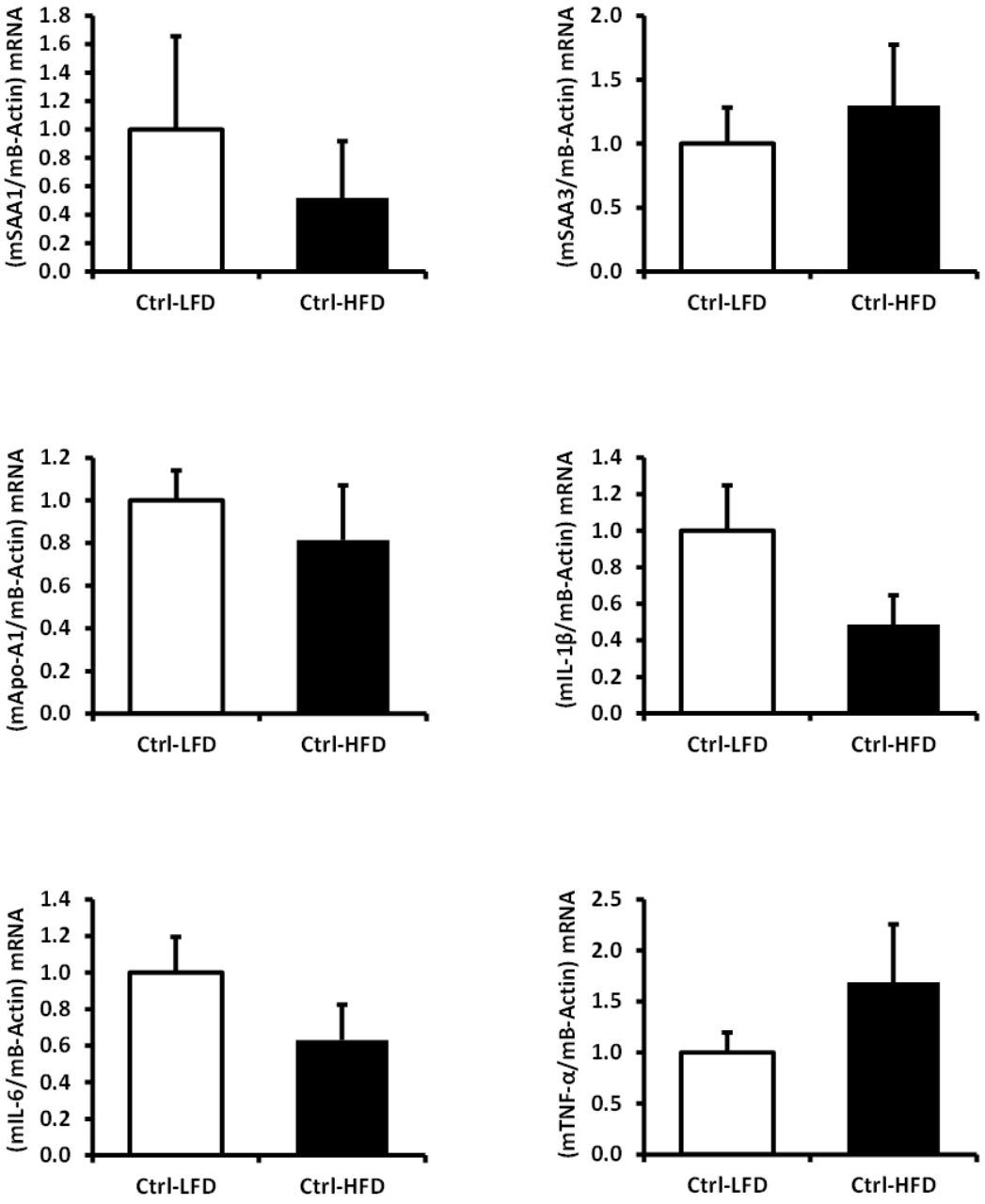


Figure 5.20: Impact of low *vs.* HFD priming on hepatic mRNA markers in wild type mice.

Wild-type C57BL/6 mice (n=8 per group) were fed low or HFDs for 4 weeks. Without any further treatment, hepatic mRNA for acute phase response markers (Serum amyloid-A1, SAA3, Apolipoprotein-A1) and inflammatory cytokines (IL-1β, IL-6 and TNF-α, were measured by RT-PCR. Data are shown as means ± SEM, and were compared by unpaired t-test.

5.5. Depletion of macrophages using clodronate liposomes

Afterward, we aimed to determine whether or not the induction of inflammatory signalling in the liver was dependent on Kupffer cells, as Kupffer cells are thought to be the major source of inflammatory cytokines in the liver (Chensue *et al.* 1991). To achieve this, clodronate liposomes were used to selectively deplete macrophages from tissues *in vivo*, as shown previously by other workers aiming to explore whether these cells have a functional role in biological processes (Rooijen, Sanders 1994).

After 4 weeks of HFD priming, mice were injected with clodronate liposomes. Three days later, half of the mice were orally gavaged with normal saline (control) and the other half were orally gavaged with *E. coli* LPS (2mg/mouse) dissolved in normal saline.

To investigate the expression of F4/80 positive cells as a marker of Kuppfer cells, immunofluorescence staining of $F4/80^{+}$ in frozen hepatic sections was performed. Liver samples were collected from two groups of wild type mice fed on HFD and HFD mice injected with clodronate liposomes. Immunofluorescence of hepatic $F4/80^{+}$ and hepatic CD68 gene expression (as the best macrophage marker in immunohistochemistry studies (Pulford *et al.* 1990)) confirmed a significant depletion in the number of macrophages in HFD-fed mice injected with clodronate liposomes when compared to the liver samples of HFD-fed mice ($P<0.0001$ for both markers, Figure 5.21).

Interestingly, macrophage depletion by clodronate liposomes showed a clear reduction in the inflammatory responses (SAA and APR markers) after LPS challenge (Figure 5.22 and 23). Hepatic Apo-A1 mRNA was not significantly different in the mice gavaged with LPS after clodronate liposome pre-treatment, compared to control (Figure 5.24). In addition, similar results showed no effect of LPS gavage on immunoblot analysis of Apolipoprotein-A1 protein (Figure 5.25) and cholesterol efflux capacity (Figure 5.26) in macrophage depleted mice.

These findings therefore suggest that mice in which macrophages were depleted by clodronate liposomes display impaired induction of SAA, lipid, efflux and APR markers in response to orally delivered LPS. Thus, hepatic macrophages are necessary for the HFD-induced sensitization to LPS.

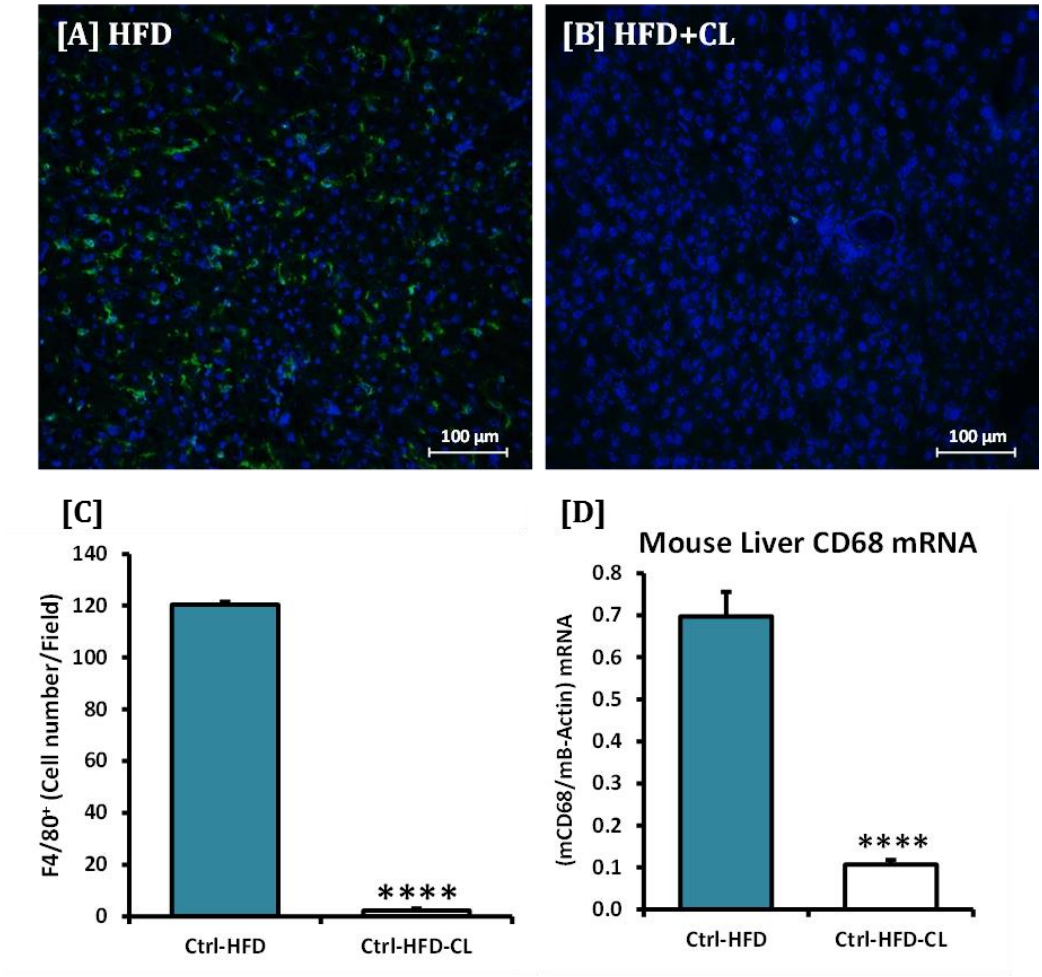


Figure 5.21: Effect of clodronate liposome treatment on macrophage content of liver after HFD priming.

Positive immunofluorescence staining for F4/80 in liver samples harvested from wild type C57BL/6 mice (n=4 per group) fed a HFD for 4 weeks (A), or fed a HFD for 4 weeks followed by intraperitoneal injection of clodronate liposomes (+CL), 72 h prior to sectioning (B). $F4/80^{+}$-FITC staining is coloured green, and DAPI-stained nuclei are coloured blue. Number of F4/80 positive cells per field were counted by microscopy (C). Hepatic CD68 mRNA was measured using RT-PCR. Data are shown as means ± SEM, and were compared by unpaired t-test. ***P<0.001 *vs.* control.

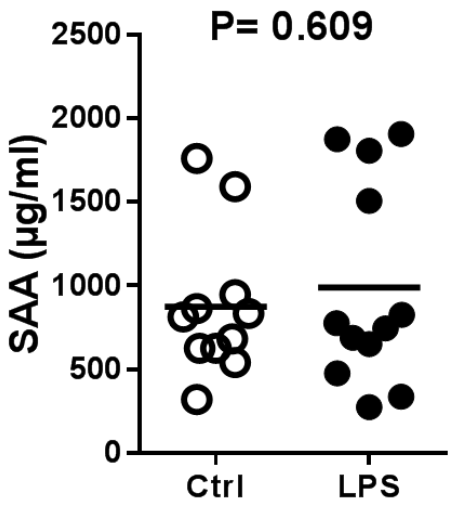


Figure 5.22: Effect of orally delivered LPS on circulating serum amyloid A in HFD primed mice after pretreatment with clodronate liposomes.

Wild-type C57BL/6 mice (n=12 per group) were fed a HFD for 4 weeks, then pretreated with intraperitoneal injection of clodronate liposomes (macrophage-depleting agent). After three days, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, plasma content of SAA was measured by ELISA. Horizontal bars indicate means, which were compared by unpaired t-test.

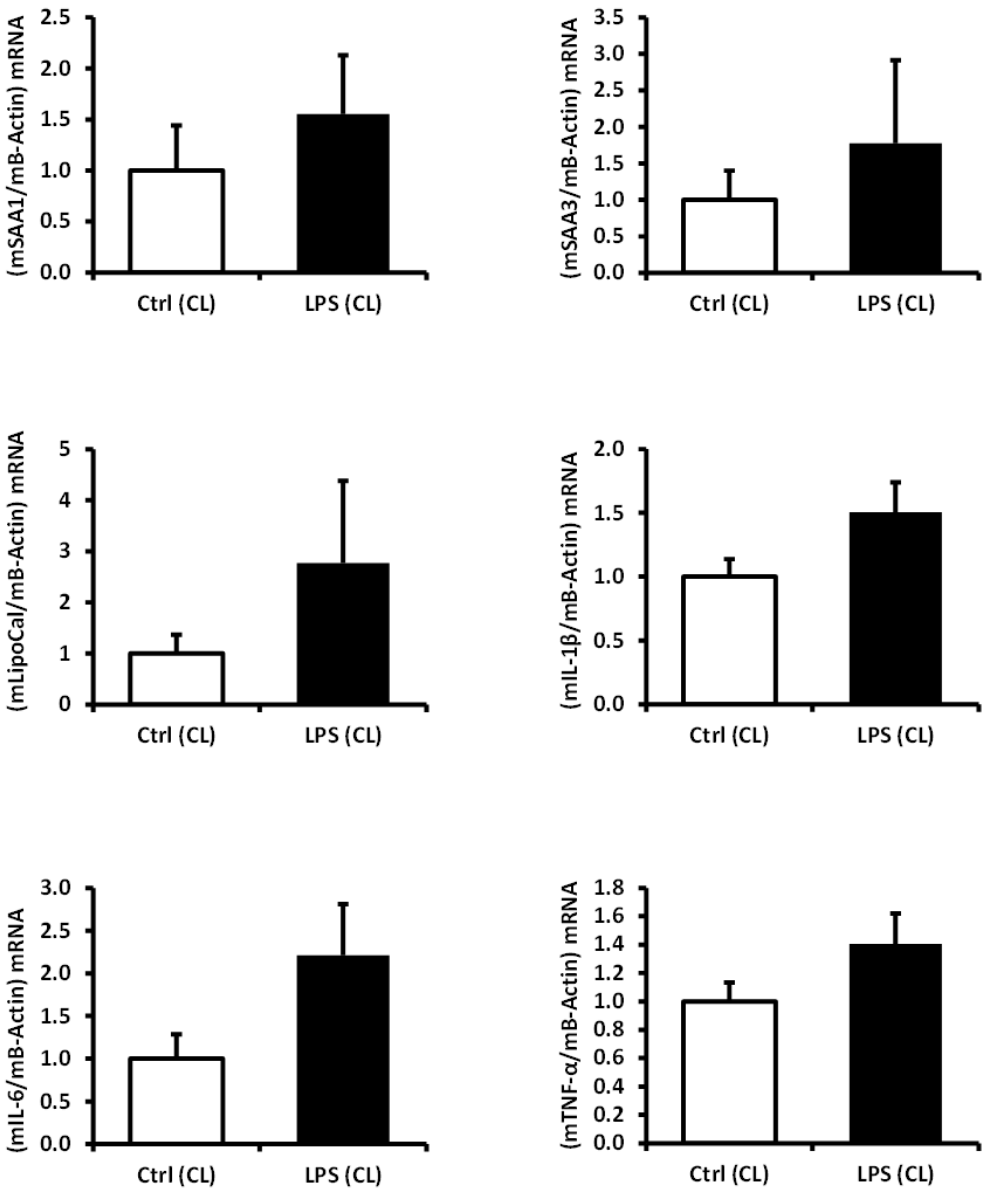


Figure 5.23: Effect of orally delivered LPS on hepatic mRNA markers in HFD-primed mice pretreated with clodronate liposomes.

Wild-type C57BL/6 mice (n=12 per group) were fed a HFD for 4 weeks, then pretreated with intraperitoneal injection of clodronate liposomes. After three days, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic mRNA responses (Serum amyloid-A1, SAA3, Lipocalin, IL-1β, IL-6 and TNF-α), were measured using RT-PCR. Data are shown as means ± SEM, which were compared by unpaired t-test.

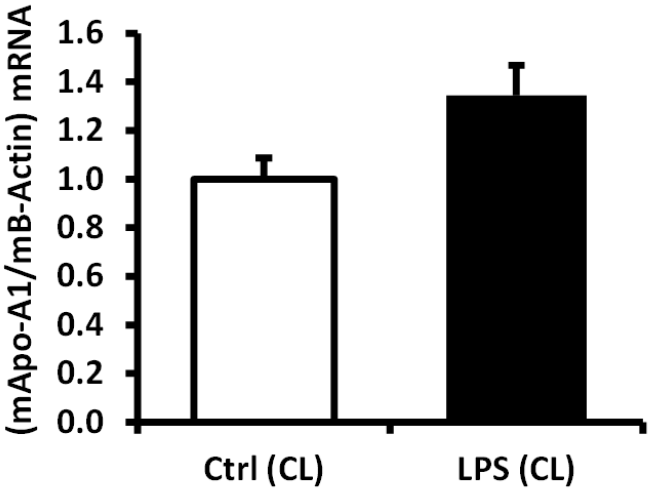


Figure 5.24: Effect of orally delivered LPS on hepatic Apolipoprotein-A1 mRNA in HFD-primed mice pretreated with clodronate liposomes.

Wild-type C57BL/6 mice (n=12 per group) were fed a HFD for 4 weeks, then pretreated with intraperitoneal injection of clodronate liposomes. After three days mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic Apolipoprotein-A1 mRNA was measured using RT-PCR. Data are shown as means ± SEM, which were compared by unpaired t-test.

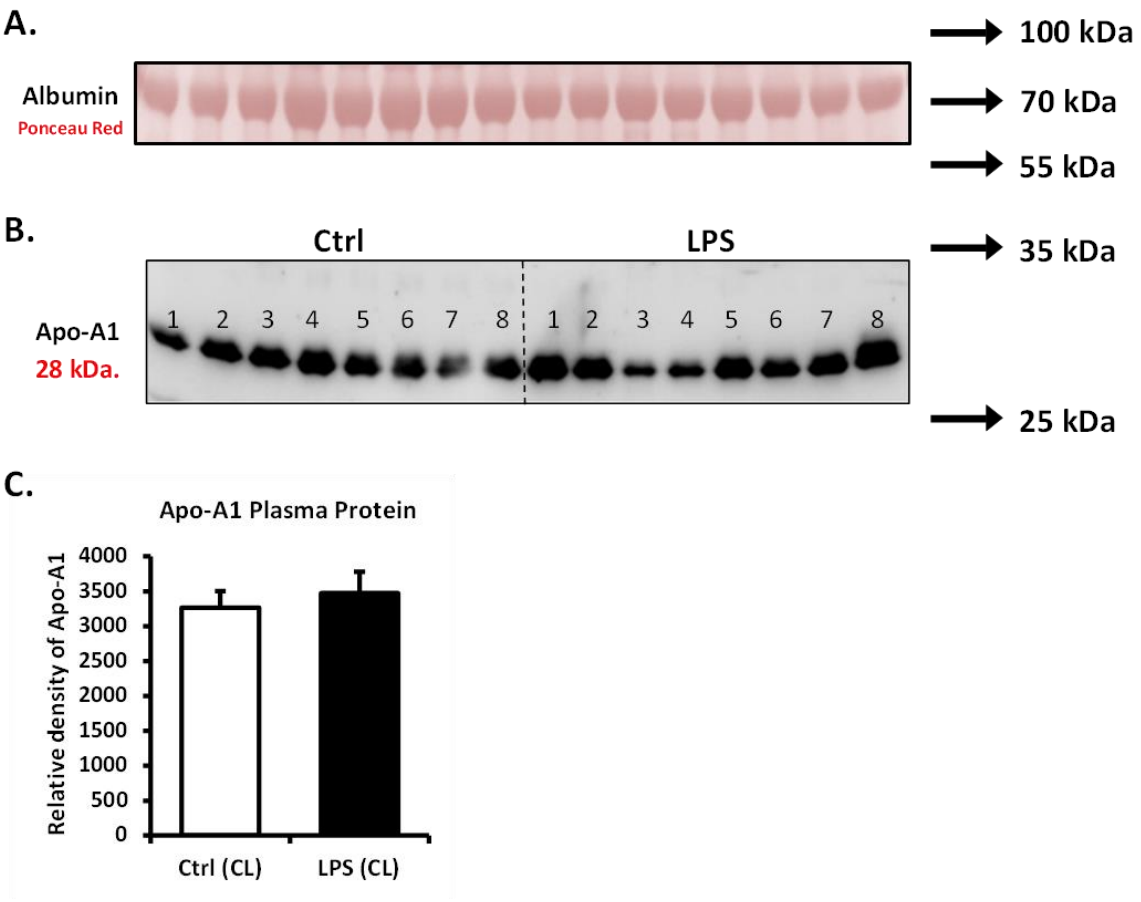


Figure 5.25: Effect of orally delivered LPS on plasma Apo-A1 protein in HFD-primed mice pretreated with clodronate liposomes.

Wild-type C57BL/6 mice were fed a HFD for 4 weeks, then pretreated with intraperitoneal injection of clodronate liposomes. After three days, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS (n=8 per group). Albumin was measured in plasma using ponceau red stain (A). Plasma apolipoprotein-A1 (28 kDa) was measured by immunoblot (B). Relative Apo-A1 expression in plasma of control and LPS-treated groups were measured by densitometry relative to total protein (C). Data are shown as means ± SEM, and were compared by unpaired t-test.

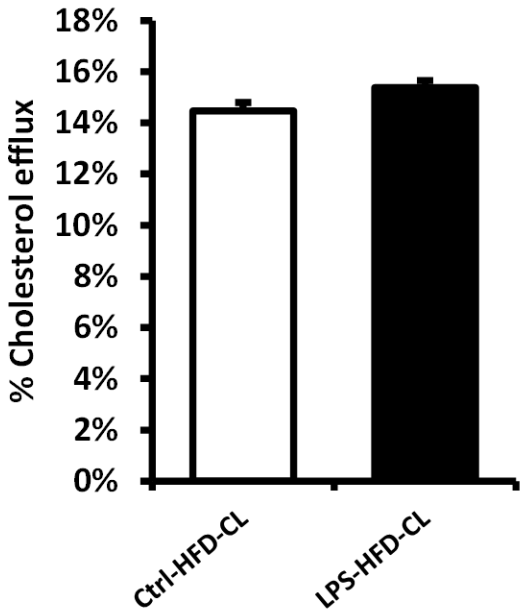


Figure 5.26: Effect of orally delivered LPS on serum cholesterol efflux capacity in HFD primed mice pretreated with clodronate liposomes.

Wild-type C57BL/6 mice (n=12 per group) were fed a HFD for 4 weeks, then pretreated with intraperitoneal injection of clodronate liposomes. After three days, mice were orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, cholesterol efflux capacity of Apo-B depleted serum was measured. Data are shown as Mean±SEM, which were compared by unpaired t-test. *P<0.05 *vs.* control (normal saline alone).

5.6. Adipose, aortic or hepatic macrophages contribution in the inflammatory responses

The clodronate liposome experiments indicated that macrophages are essential for the HFD-induced sensitization to LPS, but it was not clear which tissue bed was responsible for the hepatic responses to orally delivered LPS. We therefore aimed to determine which tissue source of macrophages might be responsible, by measuring macrophage responses to orally delivered PAMPs in various relevant tissues.

First, we examined the effect of chronic oral delivery of the three PAMP mix (LPS, Pam_3CSK_4, and iEDAP) on inflammatory cytokine mRNA markers and the macrophage content in adipose tissue.

In this experiment, wild-type C57BL/6 mice were fed normal chow for 10 weeks. Half were supplied with normal water and the other half received water supplemented with 3 PAMPs (100 µg/ml LPS, 1 µg/ml Pam_3CSK_4 and 1 µg/ml iEDAP).

No responses were observed in the expression of adipose tissue inflammatory cytokine mRNA markers (IL-1β, IL-6 and TNF-α), which are canonical markers of inflammatory macrophage activation, after receiving 3 PAMPs mixed in drinking water for 10 weeks when compared with the control group (Figure 5.27). However, macrophage numbers were significantly elevated in adipose tissue by chronic PAMP treatment, as measured by staining paraffin-embedded sections for the F4/80 marker (P=0.001), and adipose expression of CD68 mRNA (P=0.002) (Figure 5.28).

We next examined the effect of orally delivered LPS on aortic inflammatory mRNA markers in HFD primed mice. In this model, orally delivered LPS had no influence on the aortic inflammatory mRNA markers (IL-1β, IL-6 and TNF-α), suggesting no activation of macrophages in these tissues either (Figure 5.29).

These findings therefore suggest that neither adipose nor aortic macrophages are responsive to orally delivered LPS, and are therefore not likely to be responsible for the observed metabolic effects of orally delivered LPS.

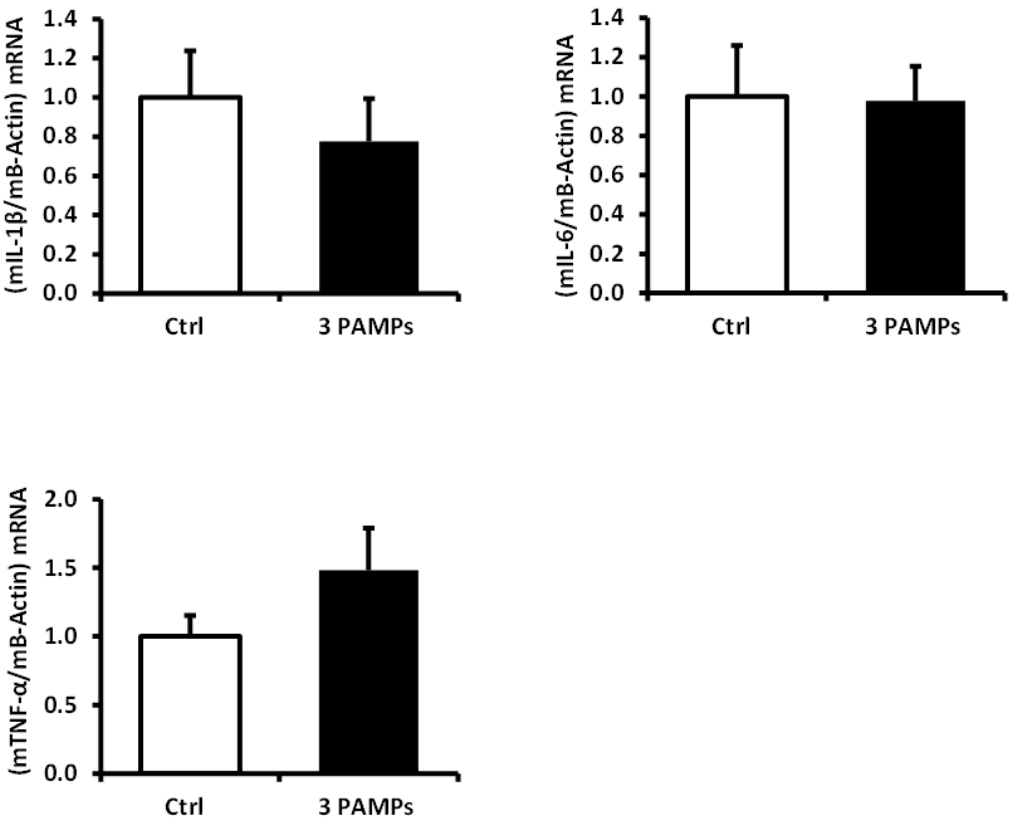


Figure 5.27: Effect of chronic oral delivery of a three PAMP mix (LPS, Pam_3CSK_4, and iEDAP) on inflammatory cytokine mRNA markers in adipose tissue.

Wild-type C57BL/6 mice were fed normal chow for 10 weeks. Half (n=8 per group) were supplied with normal water and the other half received water supplemented with 3 PAMPs (100 µg/ml LPS, 1 µg/ml Pam_3CSK_4 and 1 µg/ml iEDAP). Adipose tissue inflammatory cytokine mRNA markers (IL-1β, IL-6 and TNF-α), were measured RT-PCR. Data are shown as means ± SEM, and were compared by unpaired t-test.

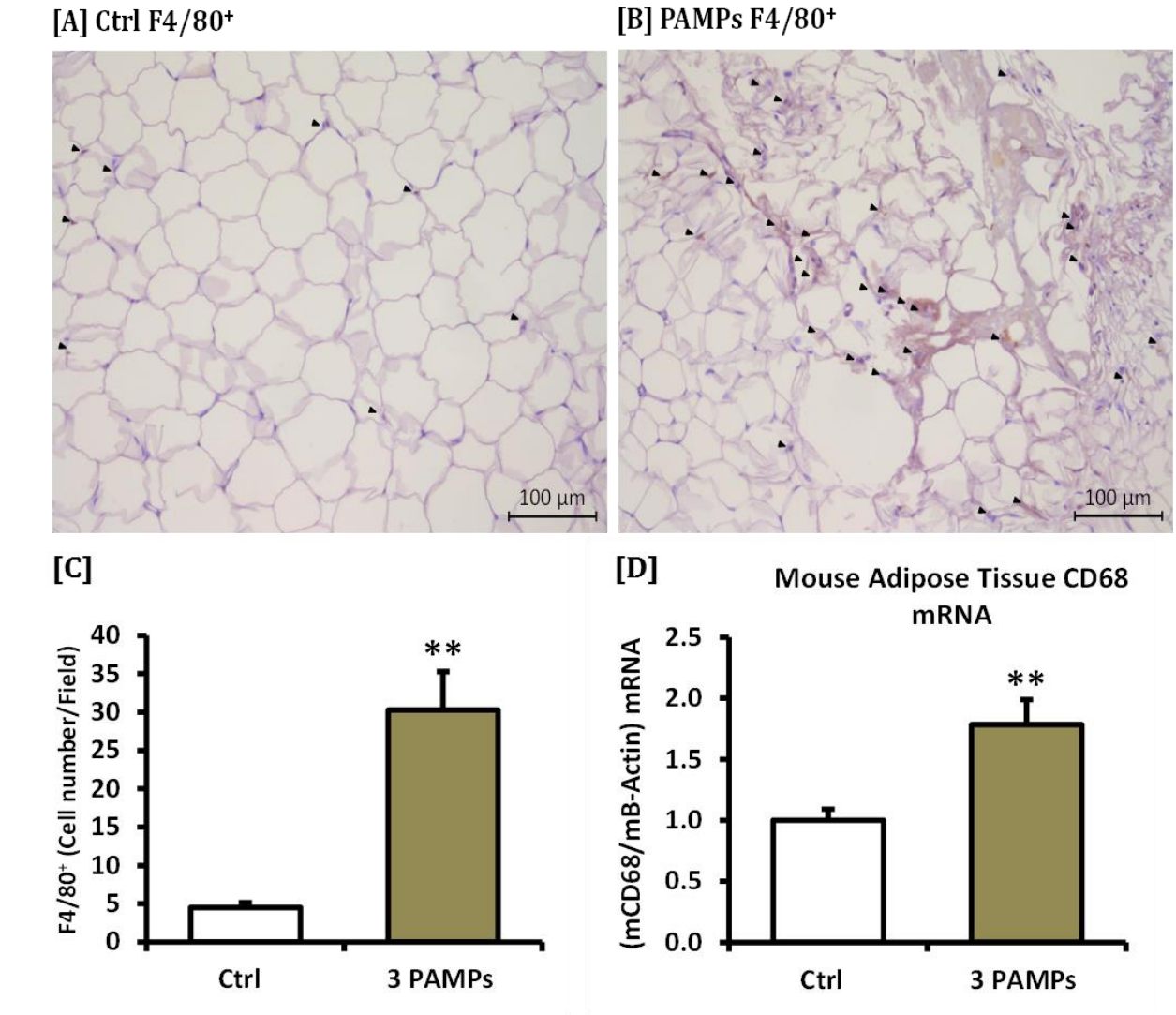


Figure 5.28: Effect of chronic oral delivery of a three PAMP mix (LPS, Pam_3CSK_4, and iEDAP) on macrophage content of adipose tissue.

Wild-type C57BL/6 mice were fed normal chow for 10 weeks. Half (n=8 per group) were supplied with normal water and the other half received water supplemented with 3 PAMPs (100 µg/ml LPS, 1 µg/ml Pam_3CSK_4 and 1 µg/ml iEDAP). Adipose tissue macrophage content was then quantified by staining paraffin-embedded sections for the F4/80 marker in control (A) and PAMP-treated samples (B). Number of F4/80 positive cells in the adipose tissue per field of each group was then measured by microscopy (C). CD68 mRNA in adipose tissue was measured by RT-PCR (D). Data are shown as mean ± SEM, which were compared by unpaired t-test. **P<0.01 *vs.* control.

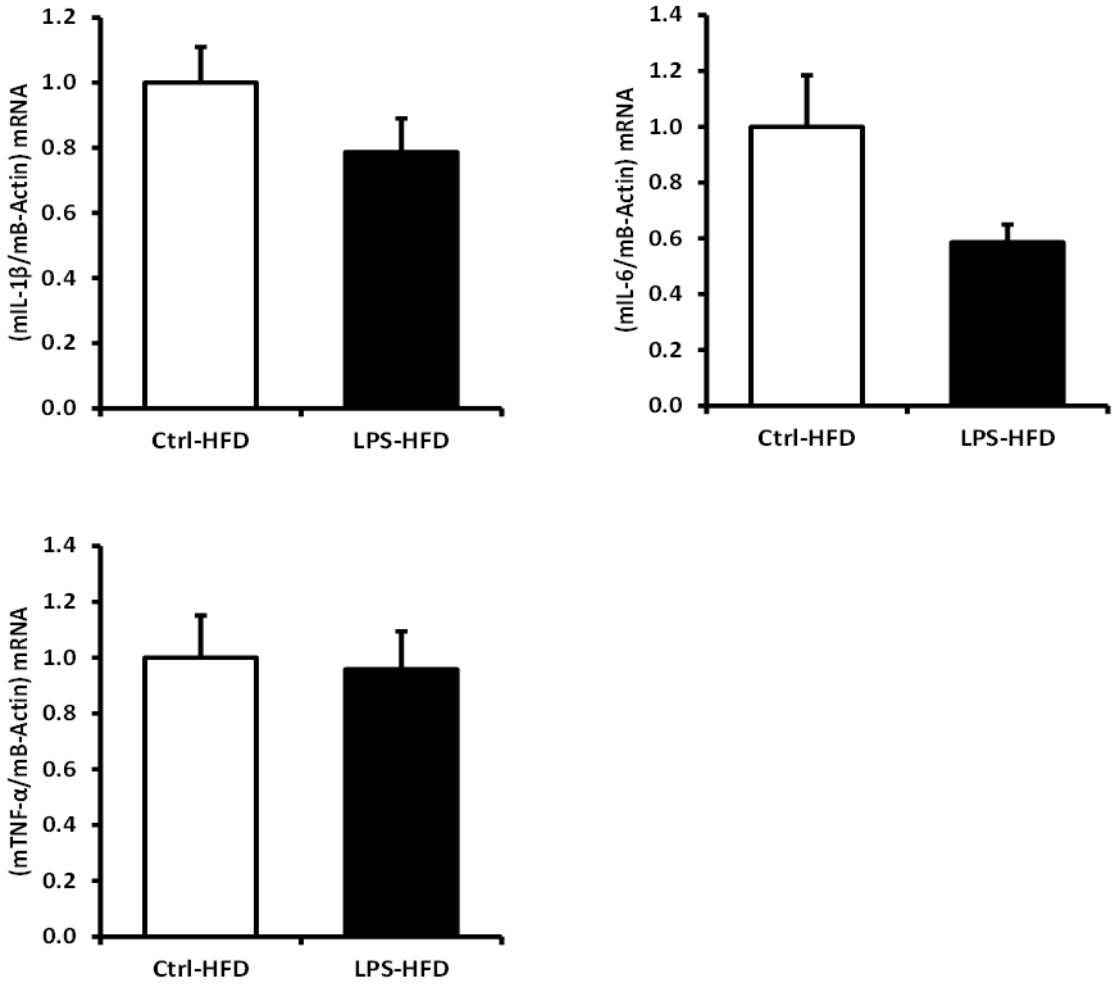


Figure 5.29: Effect of orally delivered LPS on aortic inflammatory mRNA markers in HFD primed mice.

Wild-type C57BL/6 mice (n=10 per group) were fed a HFD for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, aortic mRNA for inflammatory markers (IL-1β, IL-6 and TNF-α), were measured by RT-PCR. Data are shown as means ± SEM, and were compared by unpaired t-test.

5.7. Kupffer cell responses to different priming treatments

Therefore, we decided to explore hepatic tissue again, since Kupffer cells are the resident macrophages in the liver which comprise the largest population of macrophages in the body.

Immunofluorescence for the F4/80 antigen was used to investigate effects of trialled priming treatments (normal chow diet, HCD, HFD and *P. acnes* injection) on murine hepatic macrophage content. These experiments showed that the number of $F4/80^+$ cells in the liver was increased by all treatments relative to normal chow, particularly after *P. acnes* injection (Figure 5.30). No staining was induced by isotype control antibody.

The number of F4/80 positive cells per field measured by microscopy revealed a highly significant elevation in the number of $F4/80^+$ macrophages in mice treated with HCD (P=0.0033), a HFD (P=0.0008), or intraperitoneal injection of heat-killed *P. acnes* bacteria (P=0.00004) when compared to the group of mice were fed on normal chow (Figure 5.30 - E).

In addition, chronic administration of a three PAMP mix (LPS, Pam_3CSK_4, and iEDAP) in drinking water results in a highly significant (P=0.00001) increase in $F4/80^+$ hepatic macrophages (Figure 5.31).

Accordingly, hepatic CD68 gene expression increased significantly in mice fed a HFD for 4 weeks (P=0.003) and after intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria (P=0.00002). Also, CD14 mRNA gene (a receptor for bacterial LPS) (Dentener *et al.* 1993) expression was elevated significantly by intraperitoneal injection of heat-killed *P. acnes* bacteria (P=0.007). No changes to CD14 expression were observed in response to the other treatments (HCD and HFD) relative to the control (mice were fed normal low fat chow diet) (Figure 5.32).

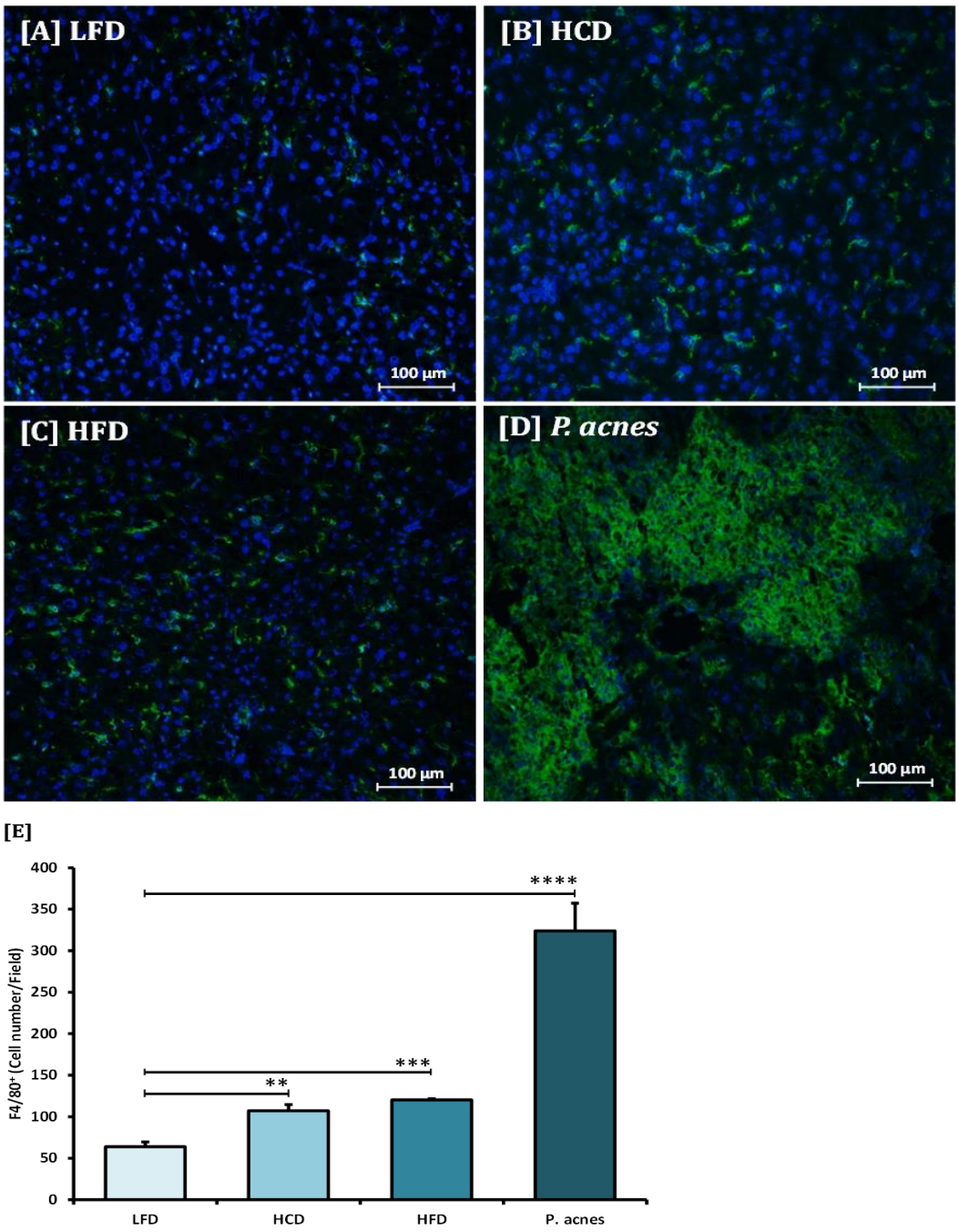


Figure 5.30: Effects of trialled priming treatments on murine hepatic macrophage content.

Hepatic macrophage content was measured by immunofluorescence for the F4/80 antigen in frozen hepatic tissues harvested from wild type C57BL/6 mice (n=4 per group) pre-treated with a low fat diet (normal chow) for 4 weeks (A), a HCD for 4 weeks (B), a HFD for 4 weeks (C), or intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria 7 days previously (D). Number of F4/80 positive cells per field was measured by microscopy (E). FITC-F4/80^{+} staining is coloured green, DAPI-stained nuclei are coloured blue. Data are shown as means ± SEM, and were compared by ANOVA with Dunnett's test. **P<0.01, ***P<0.001 *vs.* control (LFD).

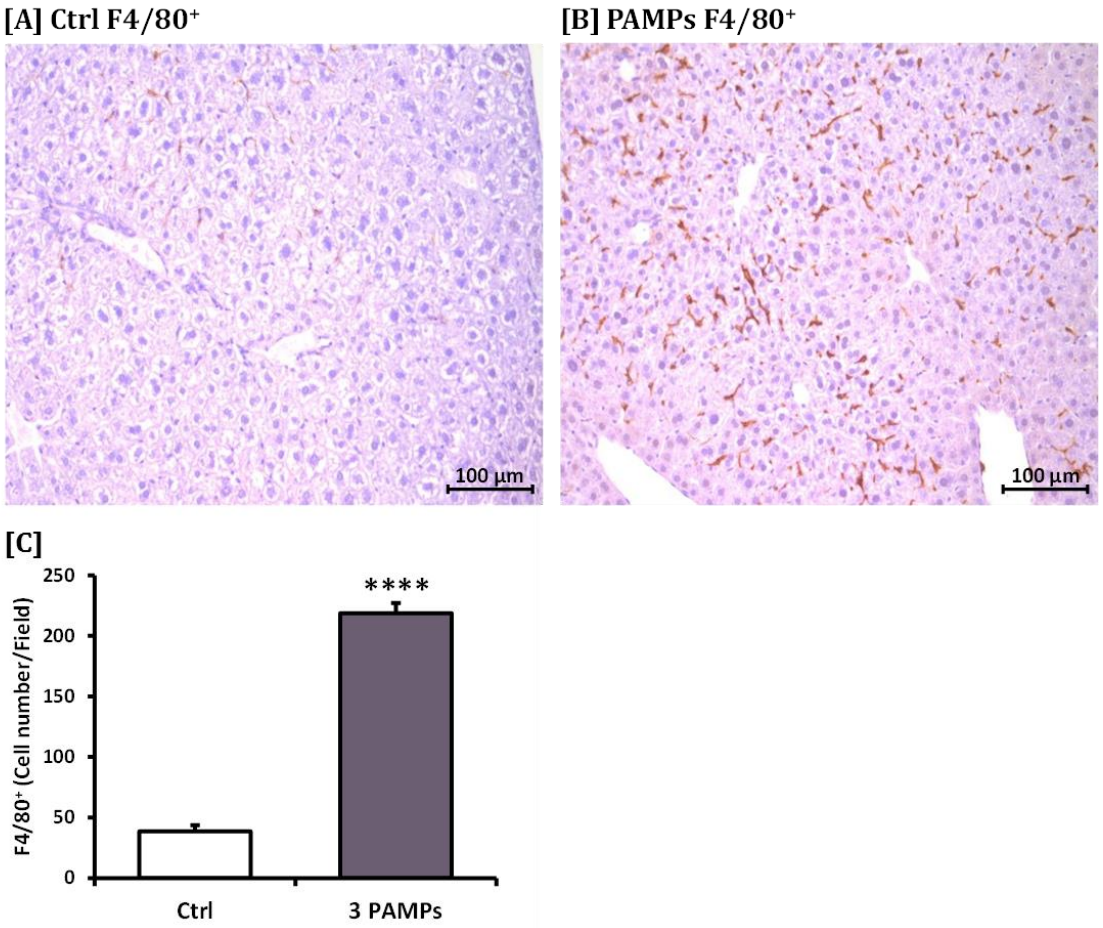


Figure 5.31: Effect of chronic oral delivery of a three PAMP mix (LPS, Pam_3CSK_4, and iEDAP) on hepatic macrophage content.

Wild-type C57BL/6 mice were fed normal chow for 10 weeks. Half (n=8 per group) were supplied with normal water and the other half received water supplemented with 3 PAMPs (100 µg/ml LPS, 1 µg/ml Pam_3CSK_4 and 1 µg/ml iEDAP). Hepatic macrophages were then quantified by immunohistochemical staining for the F4/80 antigen in paraffin-embedded sections of hepatic tissues of control (A), or PAMP-fed mice (B). Brown colour indicates positive staining for murine $F4/80^+$. Isotype control showed no staining. Number of F4/80 positive cells per field was quantified by microscopy (C). Data are shown as means ± SEM, which were compared by unpaired t-test. ***P<0.001 *vs.* control.

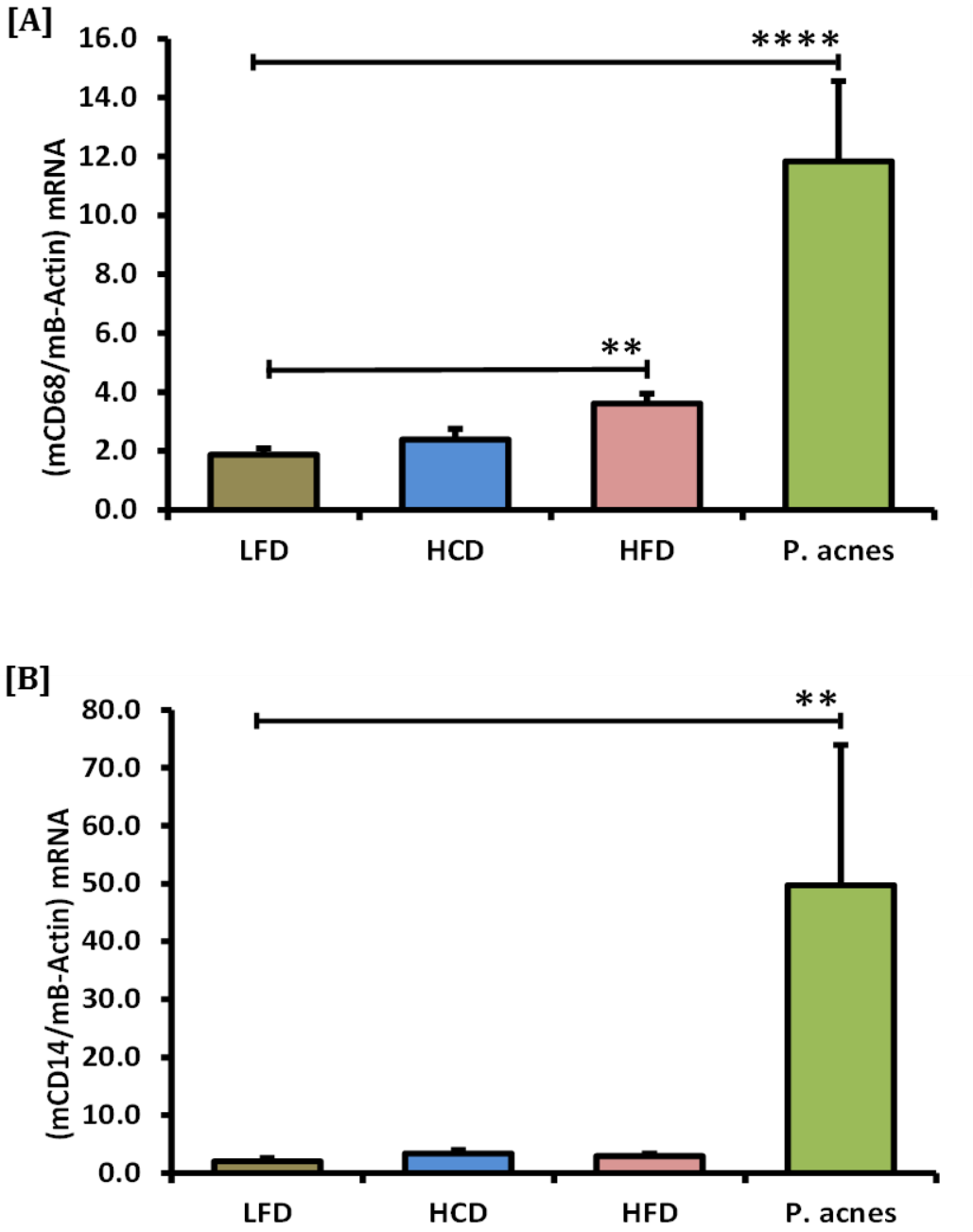


Figure 5.32: Effects of trialled priming treatments on hepatic macrophage CD68 and CD14 mRNA markers in mice.

Hepatic CD68 mRNA (A) and CD14 mRNA content (B) was measured by RT-PCR in tissues harvested from wild type C57BL/6 mice (n=6-12 per group) pre-treated with a low fat diet for 4 weeks (LFD), a HCD for 4 weeks (HCD), a HFD for 4 weeks (HFD), or intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria 7 days previously (*P. acnes*). Data are shown as means ± SEM, and were compared by ANOVA with Dunnett's test. **P<0.01, ***P<0.001 *vs.* control (LFD).

5.8. Hepatic TLR2/4 expression in responses to various treatments

Because TLR2 and TLR4 stimulants are responsible for inflammatory cytokine induction by macrophages exposed to processed food extracts (Chapter 3), we next investigated hepatic expression of TLR2 and TLR4. mRNA markers were measured in liver tissues collected from wild-type mice primed with various treatments (LFD, HCD, HFD, and *P. acnes* injection). Hepatic TLR2 gene expression was increased significantly by the *P. acnes* injection (P=0.00003) but not by the other treatments. However, hepatic TLR4 gene expression was significantly increased by all treatments (HCD *vs.* Control P=0.0005), (HFD *vs.* Control P=0.029), and (*P. acnes* injection *vs.* Control P=0.0001) (Figure 5.33).

To inspect the expression of TLR2/4 proteins in hepatic tissue, immunofluorescence staining of hepatic TLR2 and TLR4 proteins were performed in frozen hepatic sections. Liver samples were collected from wild type mice pre-treated with a low fat diet, a HCD, a HFD for 4 weeks, or intraperitoneal injection of heat-killed *P. acnes* bacteria. We found that the expression of TLR2 and TLR4 proteins in the liver were elevated markedly by all treatments compared to normal chow control (Figure 5.34 and 35). As before, no staining was induced by isotype control antibody with the same fluorescent marker.

Furthermore, to explore potential co-localization of macrophages and TLR2/4 proteins in hepatic tissues of wild-type mice, we decided to perform dual colour immunofluorescence staining of macrophages (using anti-F4/80) and TLR2/4 proteins, in frozen hepatic sections collected from wild type C57BL/6 mice primed by intraperitoneal injection of heat-killed *P. acnes* bacteria and in HFD-primed mice.

Interestingly, when we did the spatial overlap between two different fluorescent labels we found that the TLR2/4 proteins were expressed on the kupffer cells ($F4/80^+$ cells) of the harvested hepatic tissues from the wild type mice primed with *P. acnes* bacteria (Figure 5.36 and 37). However, the spatial staining of TLR2 and TLR4 was less clearly defined in HFD and HCD treated mice (Figure 5.38 and 39).

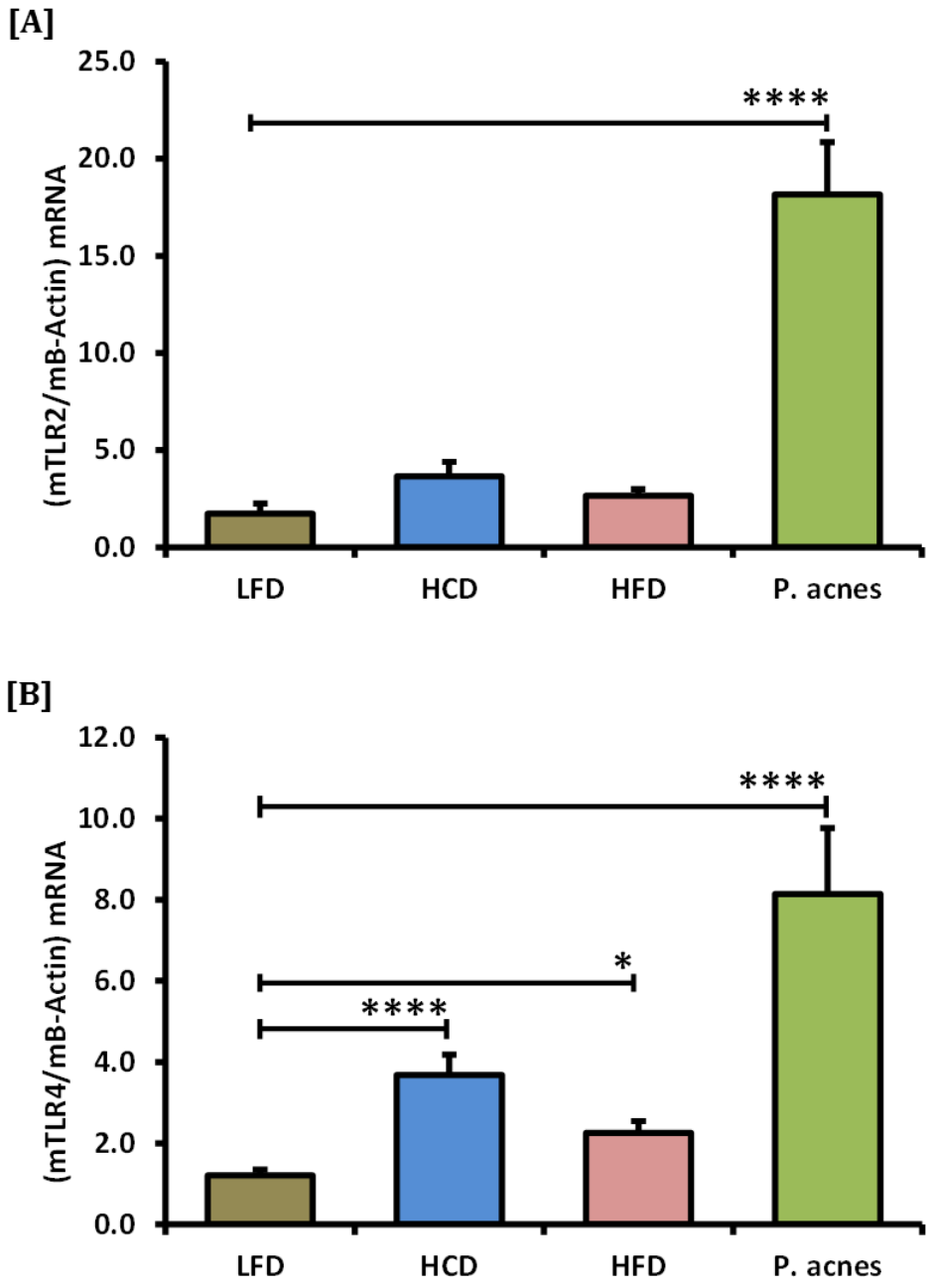


Figure 5.33: Effects of trialled priming treatments on hepatic TLR2 and TLR4 mRNA markers in mice.

Hepatic TLR2 mRNA (A) and TLR4 mRNA content (B) was measured by RT-PCR in tissues harvested from wild type C57BL/6 mice (n=6-12 per group) pre-treated with a low fat diet for 4 weeks (LFD), a HCD for 4 weeks (HCD), a HFD for 4 weeks (HFD), or intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria 7 days previously (P. acnes). Data are shown as means ± SEM, and were compared by ANOVA with Dunnett's test. *P<0.05, **P<0.01, ***P<0.001 *vs.* control (LFD).

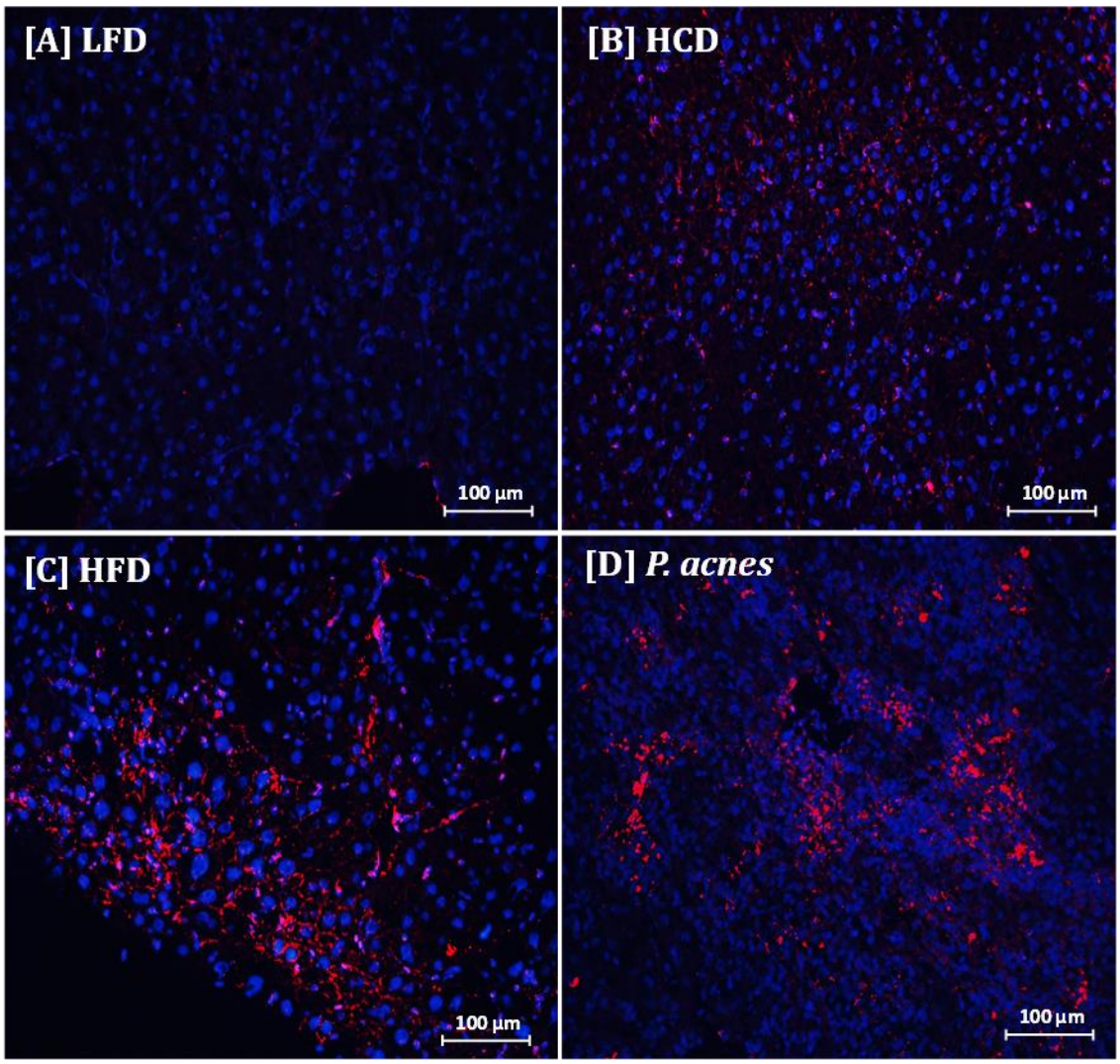


Figure 5.34: Effects of trialled priming treatments on hepatic TLR2 protein expression in mice.

Hepatic TLR2 protein was measured by immunofluorescence in tissues harvested from wild type C57BL/6 mice (n=6-12 per group) pre-treated with a low fat diet for 4 weeks (A), a HCD for 4 weeks (B), a HFD for 4 weeks (C), or intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria 7 days previously (D). TLR2 staining is visualised in red, DAPI-stained nuclei in blue.

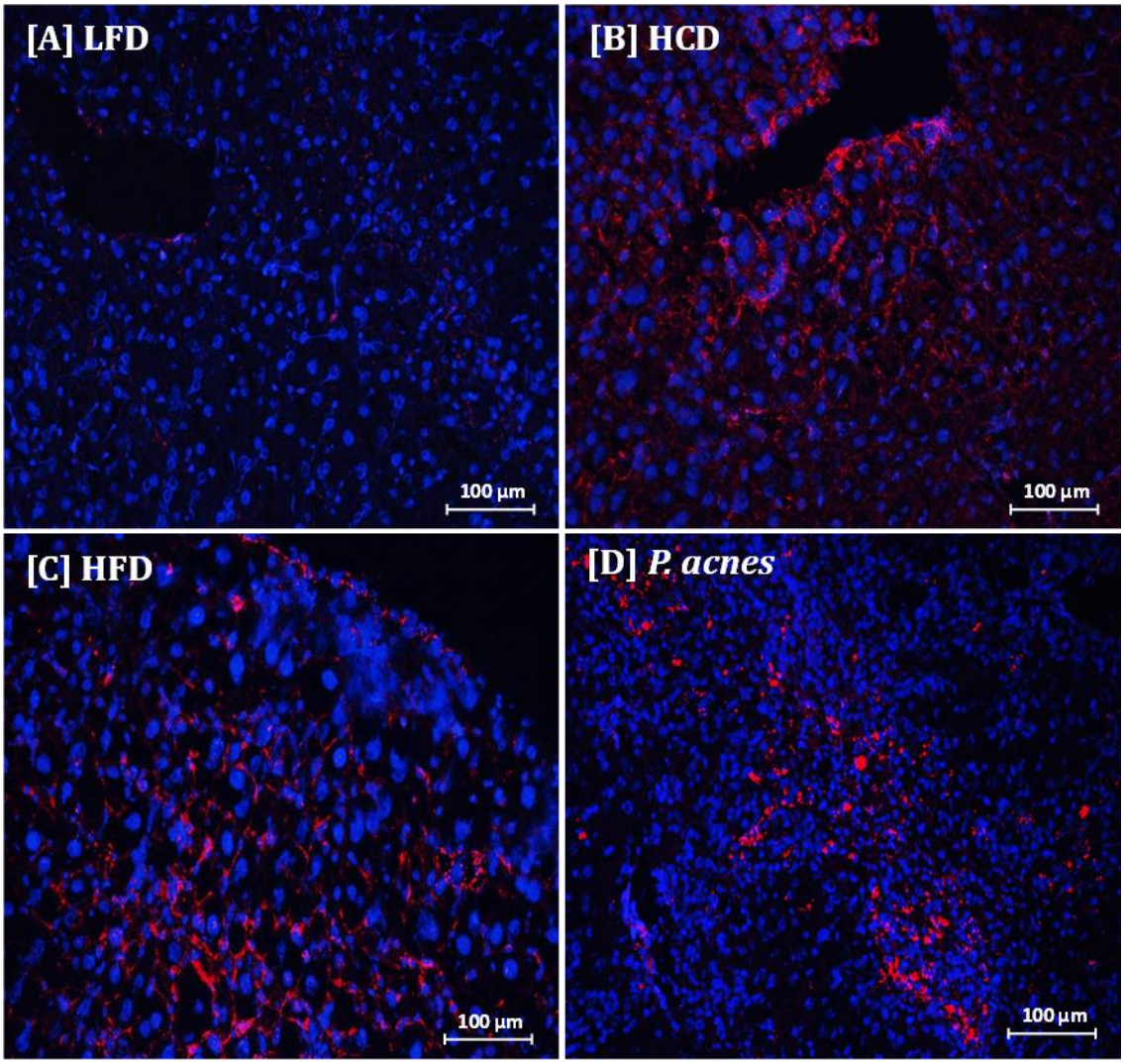


Figure 5.35: Effects of trialled priming treatments on hepatic TLR4 protein expression in mice.

Hepatic TLR4 protein was measured by immunofluorescence in tissues harvested from wild type C57BL/6 mice (n=6-12 per group) pre-treated with a low fat diet for 4 weeks (A), a HCD for 4 weeks (B), a HFD for 4 weeks (C), or intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria 7 days previously (D). TLR4 staining is visualised in red, DAPI-stained nuclei in blue.

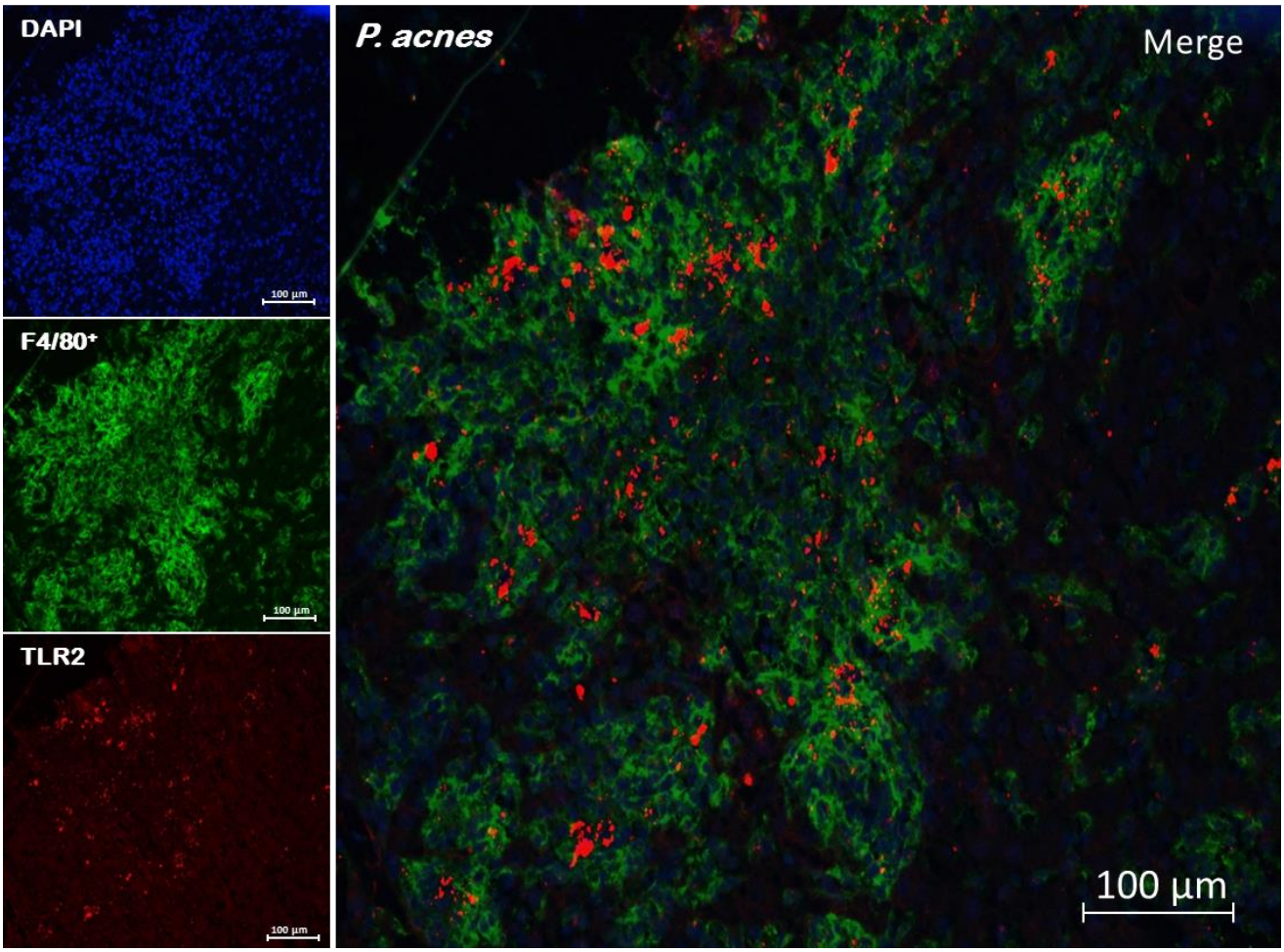


Figure 5.36: Co-localisation of TLR2 protein and macrophage F4/80 expression in liver of *P. acnes* primed mice.

Dual colour immunofluorescence staining was used to investigate potential co-localization of macrophages (F4/80, green) and TLR2 protein (red), in frozen hepatic sections collected from wild type C57BL/6 mice primed by intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria (n=4). DAPI-stained nuclei are in blue.

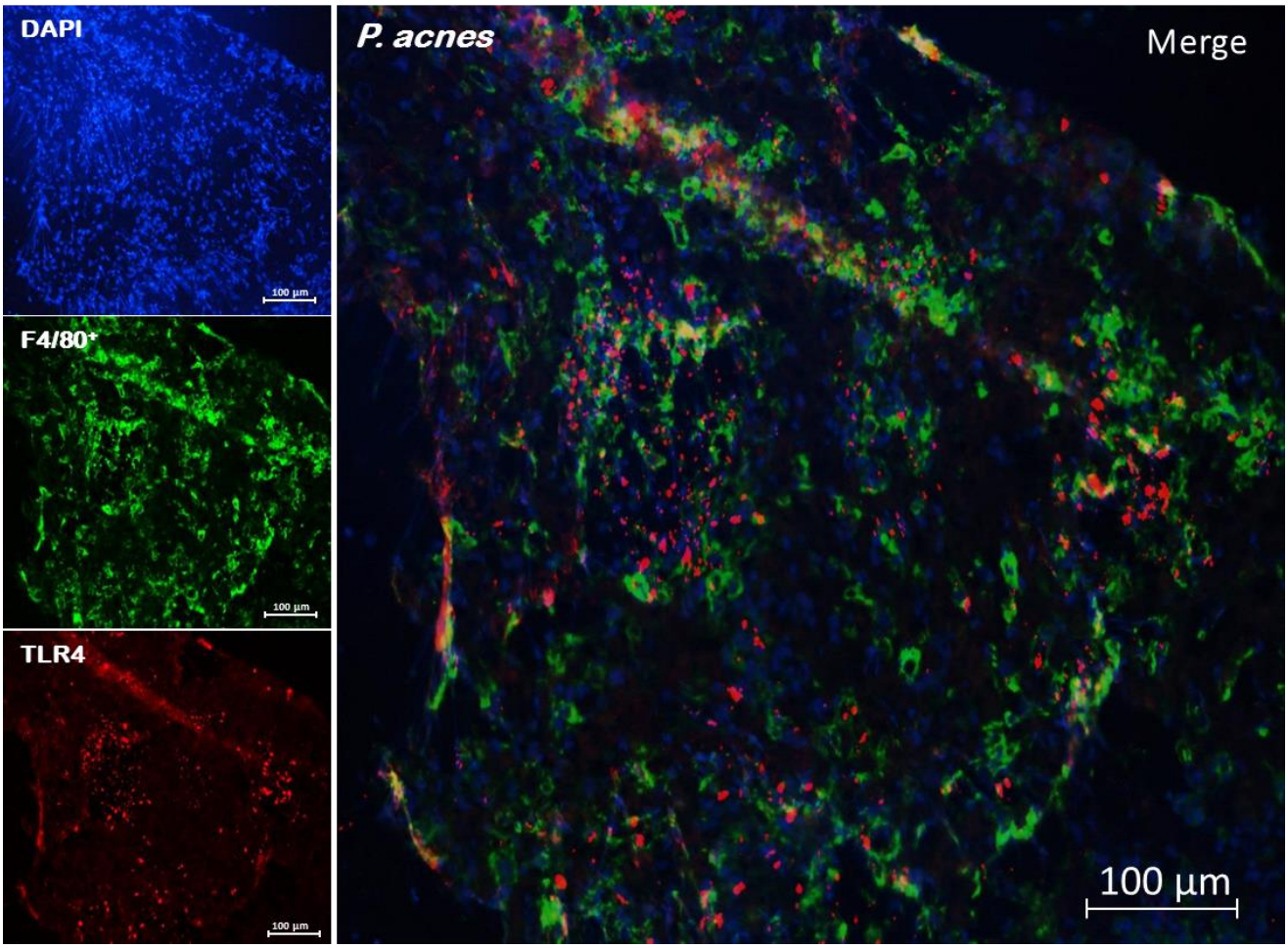


Figure 5.37: Co-localisation of TLR4 protein and macrophage F4/80 expression in liver of *P. acnes* primed mice.

Dual colour immunofluorescence staining was used to investigate potential co-localization of macrophages (F4/80, green) and TLR4 protein (red), in frozen hepatic sections collected from wild type C57BL/6 mice primed by intraperitoneal injection of 1 mg heat-killed *P. acnes* bacteria (n=4). DAPI-stained nuclei are in blue.

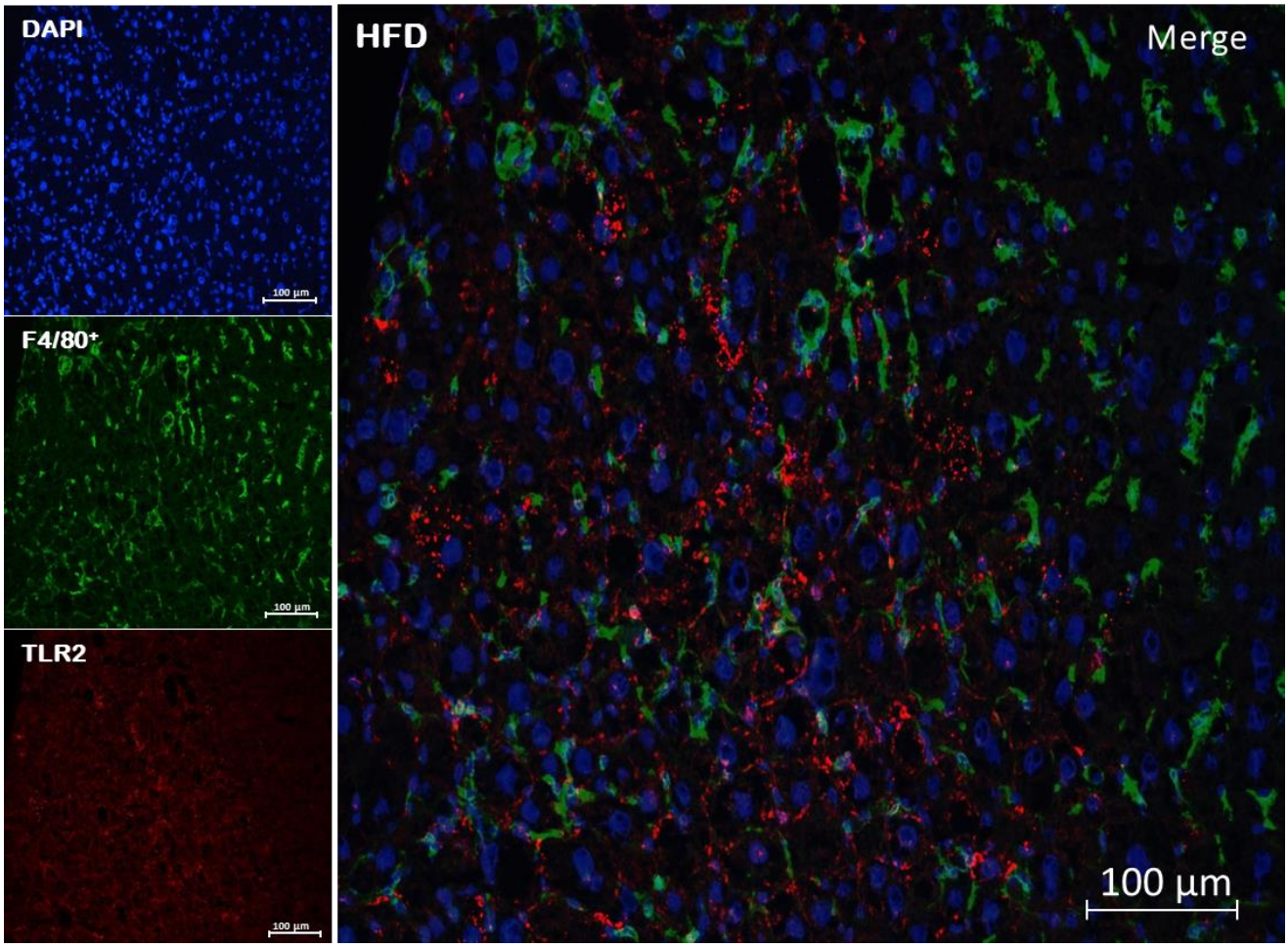


Figure 5.38: Co-localisation of TLR2 protein and macrophage F4/80 expression in liver of HFD-primed mice.

Dual colour immunofluorescence staining was used to investigate potential co-localization of macrophages (F4/80, green) and TLR2 protein (red), in frozen hepatic sections collected from wild type C57BL/6 mice primed by 4 weeks pre-treatment with HFD (n=4). DAPI-stained nuclei are in blue.

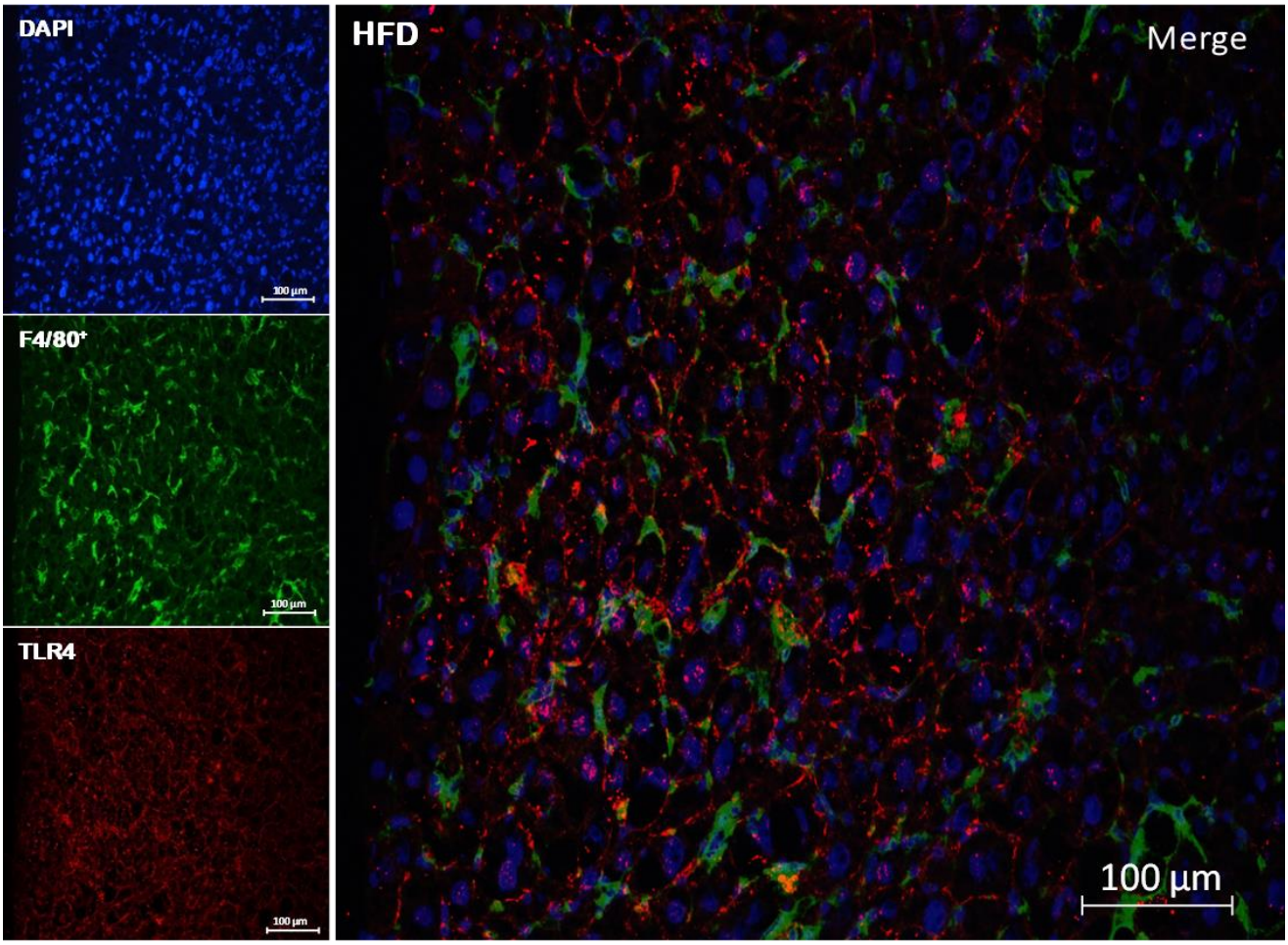


Figure 5.39: Co-localisation of TLR4 protein and macrophage F4/80 expression in liver of HFD-primed mice.

Dual colour immunofluorescence staining was used to investigate potential co-localization of macrophages (F4/80, green) and TLR4 protein (red), in frozen hepatic sections collected from wild type C57BL/6 mice primed by 4 weeks pre-treatment with HFD (n=4). DAPI-stained nuclei are in blue.

As shown in earlier chapters significant responses were noted in terms of hepatic expression of several inflammatory genes (IL-1β, IL-6 and TNF-α) by oral PAMPs treatment. Taken together, the results from this chapter suggest that Kupffer cells are responsive to oral LPS and they are therefore most likely responsible for the observed effects on APR and metabolism, rather than macrophages elsewhere in the body.

Chapter 6 Results - Mechanisms of communication between Kupffer cells and hepatocytes

The results of the previous chapter suggest that hepatic macrophages (Kupffer cells) are required for the acute phase response (APR) and serum lipid responses to ingested LPS. However, the TLR2 and TLR4 staining experiments were not sufficiently conclusive to establish that Kupffer cells are the only BLP and LPS responsive cells in the liver. As hepatocytes are the key cell type responsible for the production of circulating lipoproteins and APR mediators, we next aimed to test the capacity of hepatocytes to recognise PAMPs directly, and explore the mechanisms of the hepatocyte-macrophage communication axis with respect to PAMP-induced dyslipidaemia.

6.1. Effects of PAMPs and cytokines on HepG2 hepatocyte gene expression

Using the HepG2 hepatocyte cell-line, I first explored the potential effects of PAMPs (Pam3CSK4, LPS and iEDAP) and cytokines (IL-1β, IL-6 and TNF-α) on hepatocyte APR and Apo-AI gene expression. Although IL-1β caused a significant increase in the gene expression of inflammatory SAA1 mRNA (P=0.0001 *vs.* control), and reduction of Apo-A1 mRNA, the other tested agents did not affect expression of these genes, indicating that HepG2 cells are insensitive to BLP and LPS (Figure 6.1).

IL-1β dose responses (0.3, 3 & 30 ng/ml) were then explored using SAA1 and Apo-A1 gene expression readouts in HepG2 cells. All examined doses of IL-1β significantly increased expression of SAA1 mRNA and significantly reduced expression of Apo-A1 mRNA compared to control cells (Figure 6.2).

Studies conducted by (Luster *et al.* 1994) have shown significant reduction in secretion of pro-inflammatory cytokines by mouse liver sections treated with LPS in the presence of monoclonal antibody against IL-1. Together with the HepG2 data, these findings suggest that IL-1 may play a key role in connecting LPS detection to inflammatory responses in the liver.

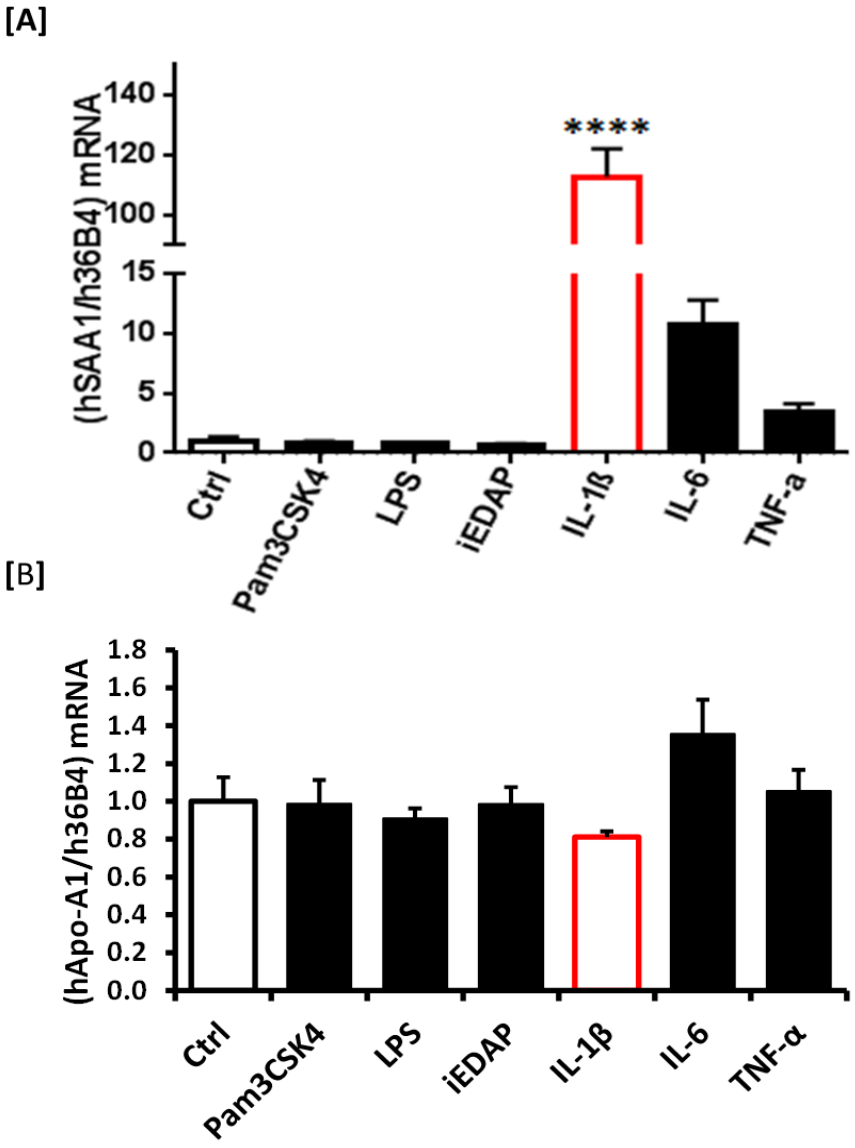


Figure 6.1: Effects of PAMPs and Cytokines on human HepG2 gene expression of the APR.

Human HepG2 cells were treated with PAMPs (Pam_3CSK_4, LPS or iEDAP, 100 ng/ml each) or cytokines (IL-1β, IL-6, TNF-α, 30 ng/ml each) for 24 hours, and then expression of the APR genes SAA1 (A) and Apo-A1 (B), were measured by RT-PCR, relative to the house-keeping gene 36B4. Results are expressed as means of 4 independent experiments ± SEM. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 *vs.* control cells cultured in medium alone). Apo-A1/IL-1β result was significant in experiments of supervisor.

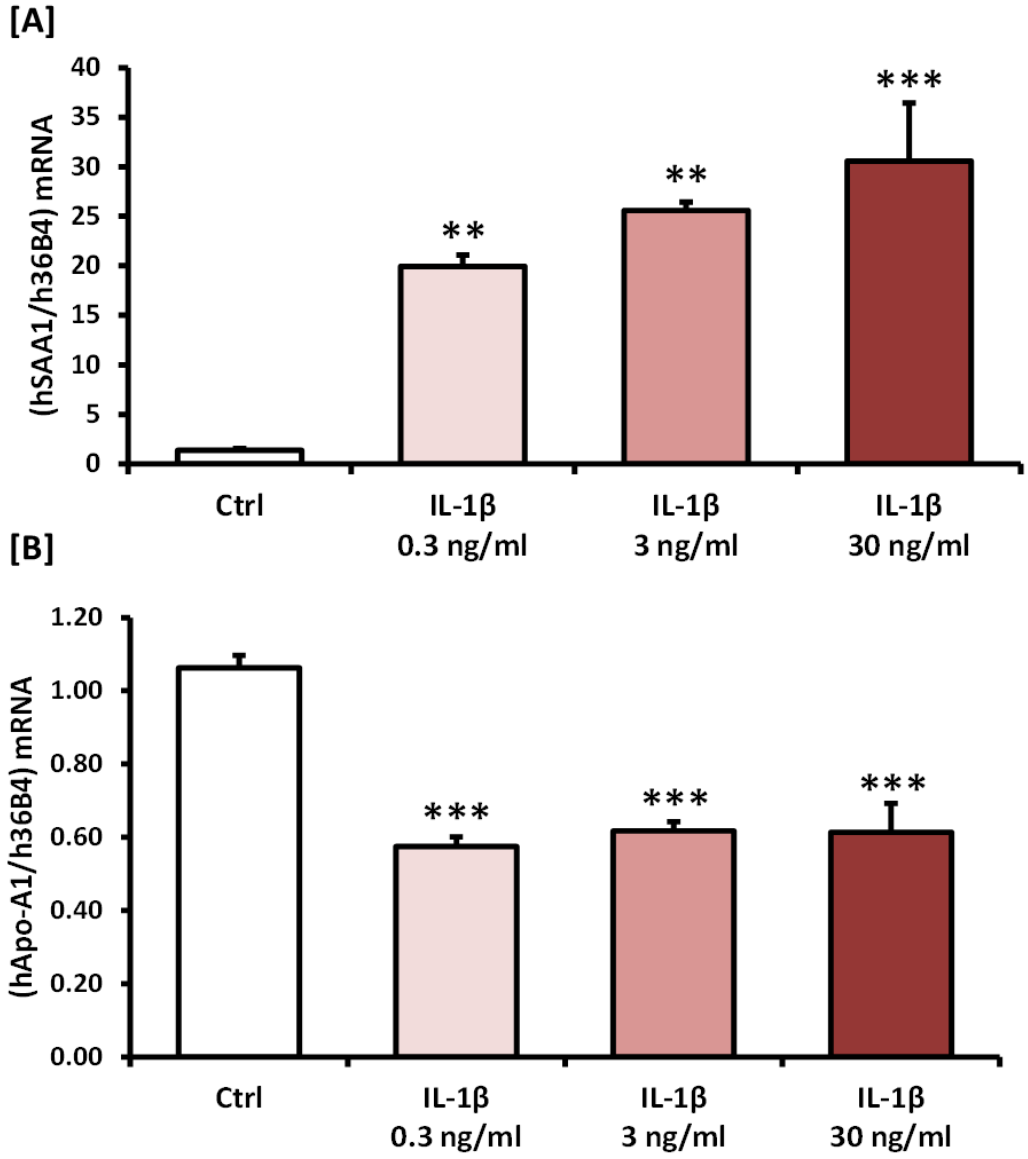


Figure 6.2: Effect of different concentrations of IL-1β on human HepG2 cell expression of APR genes.

Human HepG2 cells were treated with different doses of IL-1β (0.3, 3, 30 ng/ml) for 24 hours, and then expression of the APR genes SAA1 (A) and Apo-A1 (B), were measured by RT-PCR, relative to the house-keeping gene 36B4. Results are expressed as means of 3 independent experiments ± SEM. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 *vs.* control cells cultured in medium alone).

6.2. Effect of IL-1β on hepatocyte cholesterol acceptor secretion

Because hepatocytes are the major source of circulating cholesterol acceptors *in vivo* (including Apo-AI [Eisenberg, S. 1984]), we next examined the effect of IL-1β on secretion of functional cholesterol acceptors by HepG2 cells. Human HepG2 cells were treated with or without IL-1β (30 ng/ml) in serum free medium, then cholesterol efflux capacity of their conditioned medium was measured, in terms of the capacity of supernatant to promote [3]-H-cholesterol efflux from RAW macrophages.

Interestingly, the serum cholesterol efflux capacity dropped significantly (P=0.046 vs. control) when using conditioned medium of HepG2 cells treated with IL-1β compared to the control cells (without IL-1β) (Figure 6.3).

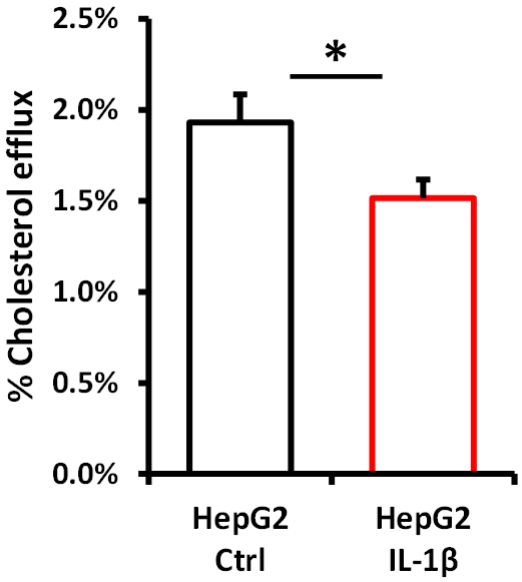


Figure 6.3: Effect of IL-1β on secretion of functional cholesterol acceptors by HepG2 cells.

Human HepG2 cells were treated with or without IL-1β (30 ng/ml) in serum free medium for 24 hours, and then cholesterol efflux capacity of their conditioned medium was measured, in terms of the capacity of supernatant to promote [3]-H-cholesterol efflux from RAW macrophages. Data are shown as means ± SEM, which were compared by unpaired t-test. *P<0.05 *vs.* control (HepG2 cells cultured in medium alone).

6.3. Flow cytometry for TLR2/4 on human HepG2 cells and monocytes

As HepG2 cells were found to be insensitive to BLP and LPS, we next aimed to determine whether hepatocytes express TLR2 or TLR4. Human HepG2 cells and primary monocytes (the internal control for positive staining) were stained with FITC-conjugated anti-human TLR2 and TLR4 mAbs and analysed by flow cytometry. Monocytes expressed high levels of TLR2 and TLR4 relative to isotype control. However, human HepG2 cells showed no TLR2 staining and little TLR4 staining (Figure 6.4).

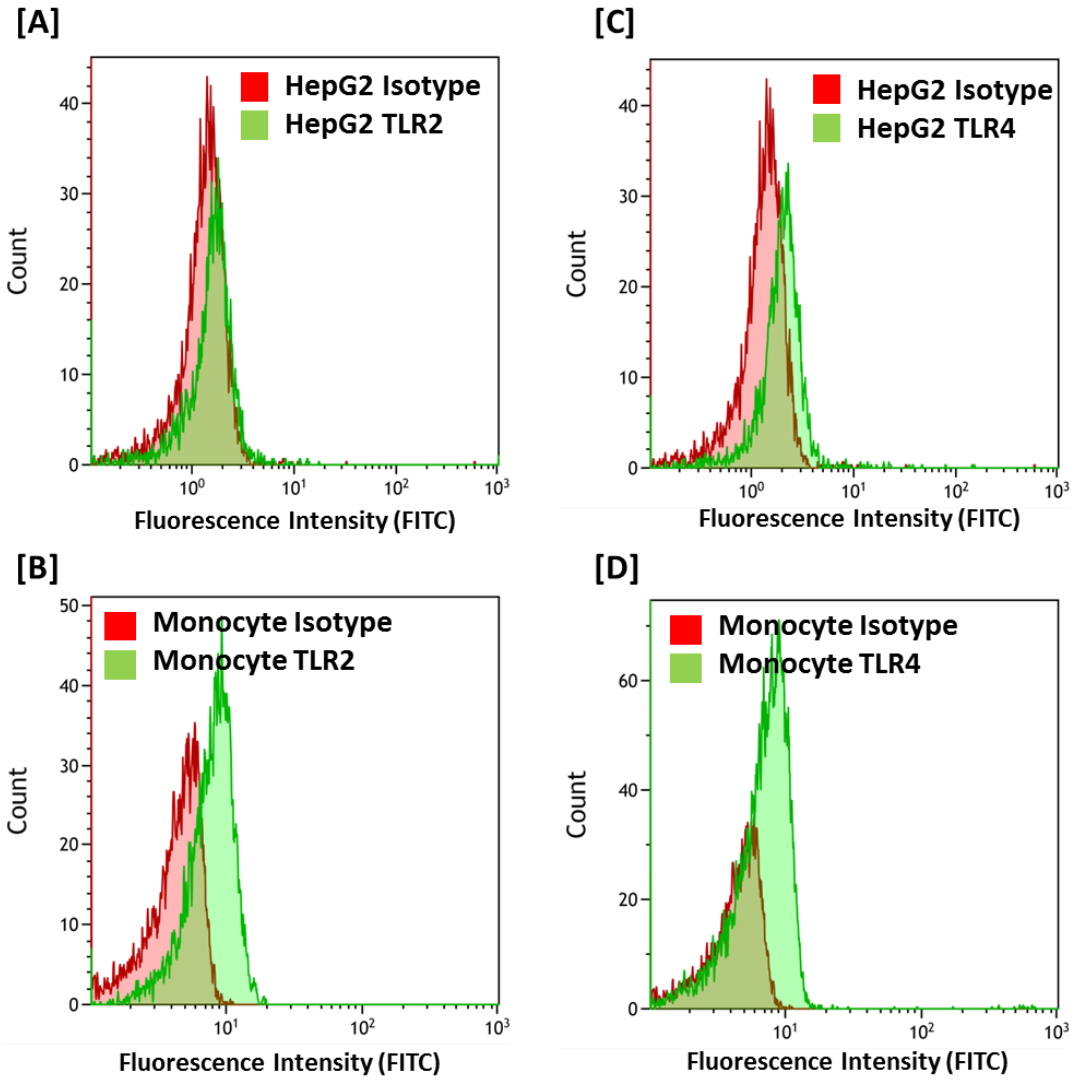


Figure 6.4: Flow cytometry analysis of TLR2 and TLR4 protein expression on human HepG2 cells and monocytes.

Human HepG2 cells and monocytes were stained with FITC-conjugated antihuman TLR2 and TLR4 mAbs and analysed by flow cytometry. Histograms represent TLR2 expression on HepG2 cells (A) and monocytes (B), and TLR4 expression on HepG2 cells (C) and monocytes (D). (Isotype control is shown in red, TLR2/4 positive cells are shown in green). Representative data are shown.

6.4. Responses of *ex vivo* cultured mouse liver slices to LPS

Because the liver is dominated by two major cell-types, hepatocytes and Kupffer cells, which may co-operate to detect PAMPS, we next explored the hepatic response to LPS using *ex vivo* cultured mouse liver slices. The vibratome technique was used to obtain liver slices from wild-type C57BL/6 mice fed a normal chow, or high fat diet for 4 weeks, with or without intraperitoneal injection of clodronate liposomes. Liver slices were treated with or without 1 μg/ml *E. coli* LPS and pro-inflammatory cytokine production was measured by ELISA. Although there was a trend towards increased IL-6 and TNF-α secretion in LPS-challenged cultures, this only reached significance (P=0.008 vs. control) for TNF-α in liver slices of HFD-primed mice. Macrophage depletion by clodronate liposomes abolished this response, adding further weight to the conclusion that Kupffer cells are key sensors of LPS in the liver. IL-1β secretion remained below the limit of detection in all cultures (Figure 6.5).

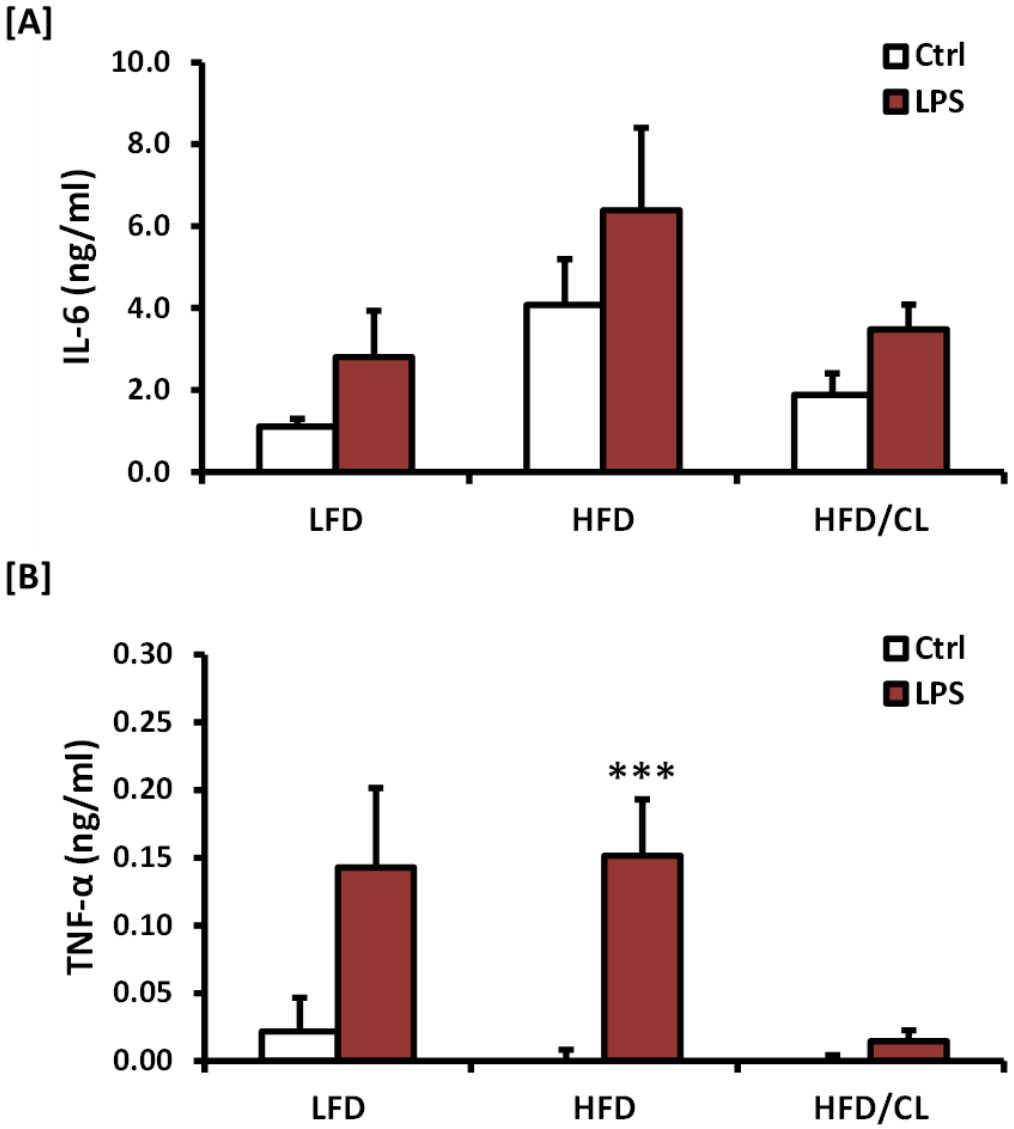


Figure 6.5: LPS-induced inflammatory cytokine production by liver slices of mice treated with LFD, HFD and HFD with clodronate liposome treatment.

Wild-type C57BL/6 mice (n=4 per group) were fed a normal chow (LFD), or high fat diet (HFD) for 4 weeks, with or without intraperitoneal injection of clodronate liposomes 24 h before harvest. Liver slices (n= 5 per mouse) were obtained by Vibratome technique for each group then treated with 500 µl RPMI medium with or without 1 µg/ml *E. coli* LPS. After 24 hours incubation, secretion of IL-6 (A), TNF-α (B) and IL-1β (below limit of detection) were measured from the supernatant using ELISA. The lower limit of detection of IL-6 was 0.025 ng/ml, TNF-α was 0.005 ng/ml and IL-1β was 0.035 ng/ml. Data are shown as means ± SEM, which were compared by unpaired t-test (*P<0.05, **P<0.01, ***P<0.001 *vs.* control liver slices cultured in medium alone).

6.5. Effects of PAMPs and cytokines on gene expression in primary mouse hepatocytes and kupffer cells

We next explored the responses of primary mouse hepatocytes, obtained from liver of wild-type C57BL/6 mice by the perfusion method, to relevant PAMPs and cytokines. There was no significant effect of Pam_3CSK_4 or LPS on SAA1 or Apo-A1 mRNA expression, suggesting that mouse primary hepatocytes are insensitive to these ligands (Figure 6.6).

However, SAA1 mRNA was significantly increased by treatment with IL-1β (P=0.0058 vs. control), but not by IL-6 or TNF-α. Likewise, Apo-A1 gene expression was significantly reduced by IL-1β (P=0.03 vs. control) and TNF-α (P=0.028 vs. control), but not by IL-6 (Figure 6.7).

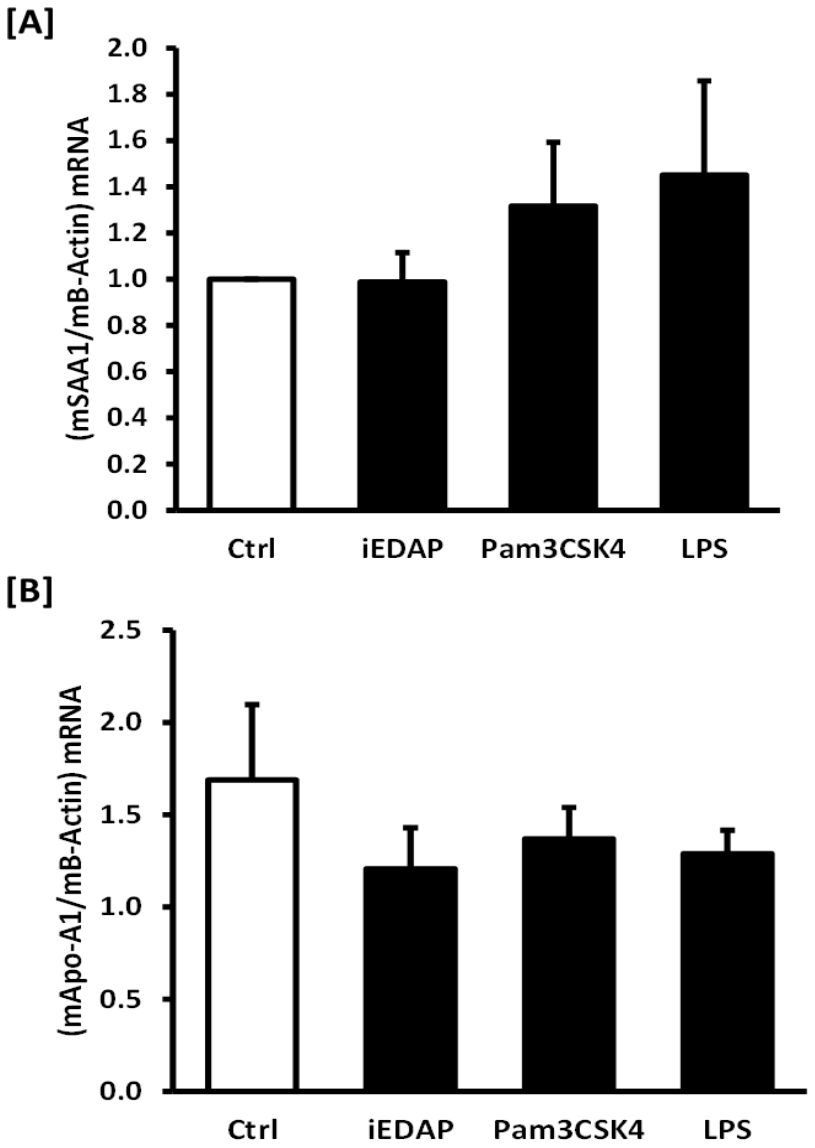


Figure 6.6: Effects of PAMPs on primary mouse hepatocyte expression of APR genes.

Primary mouse hepatocytes obtained from liver of wild-type C57BL/6 mice by perfusion method, were treated with PAMPs (iEDAP, Pam_3CSK_4, or LPS, 100 ng/ml each) for 24 hours, and then expression of APR genes SAA1 (A) and Apo-A1 (B), were measured by RT-PCR, relative to the house-keeping gene β-Actin. Results are expressed as means of 6 independent experiments ± SEM. Differences were compared by ANOVA with Dunnett's test (no significant differences observed).

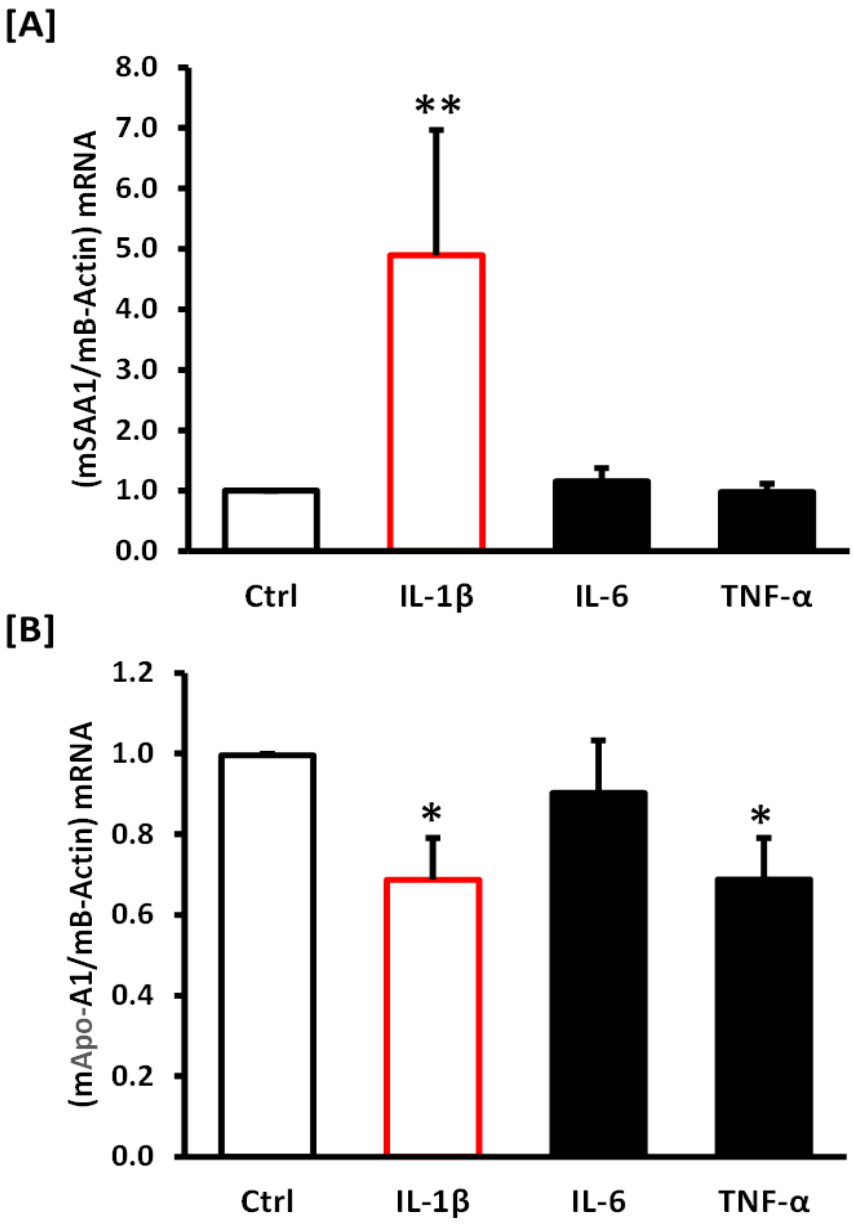


Figure 6.7: Effect of cytokines on primary mouse hepatocyte expression of APR genes.

Primary mouse hepatocytes obtained from liver of wild-type C57BL/6 mice by perfusion method, were treated with cytokines (IL-1β, IL-6 or TNF-α, 20 ng/ml each) for 24 hours, and then expression of the APR genes SAA1 (A) and Apo-A1 (B), were measured by RT-PCR, relative to the house-keeping gene β-Actin. Results are expressed as means of 6 independent experiments ± SEM. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01 *vs.* control cells cultured in medium alone).

Because primary hepatocytes proved insensitive to BLP and LPS, we next explored whether Kupffer cells might serve as the primary sensors of LPS in the liver, and affect hepatocyte gene expression through release of soluble mediators. To test this hypothesis, primary mouse hepatocytes were treated with supernatant of isolated primary Kupffer cells cultured with or without 1 µg/ml *E. coli* LPS. Expression of the APR genes SAA1 and Apo-A1, were then measured by RT-PCR. Supernatant of LPS-treated Kupffer cells induced a modest increase in SAA1 gene expression that did not reach significance. However, supernatant of LPS-treated Kupffer cells reduced significantly expression of Apo-AI ($P=0.0011$ vs. control) (Figure 6.8).

Next, because HFD priming was found to be required for responses to acute oral ingestion of LPS (Chapter 5), we tested whether Kupffer cells from HFD-primed mice might be hypersensitive to LPS challenge. Kupffer cells were obtained by perfusion from mice primed with LFD or HFD for 4 weeks, then treated with PAMPs (iEDAP, Pam3CSK4 or LPS, 100 ng/ml each). After 24 hours, secretion of inflammatory cytokines (IL-1β, IL-6 and TNF-α) were measured from the supernatant using ELISA. Surprisingly, although there was clear induction of IL-1β, IL-6 and TNF-α in response to Pam_3CSK_4 and LPS, there were no significant differences in the responses of Kupffer cells isolated from mice primed on each type of diet. There were also no responses to the NOD1 ligand iEDAP (Figure 6.9). Thus, when cultured at equivalent cell density, Kupffer cells from HFD primed mice are not more sensitive to LPS than Kupffer cells from chow-fed mice.

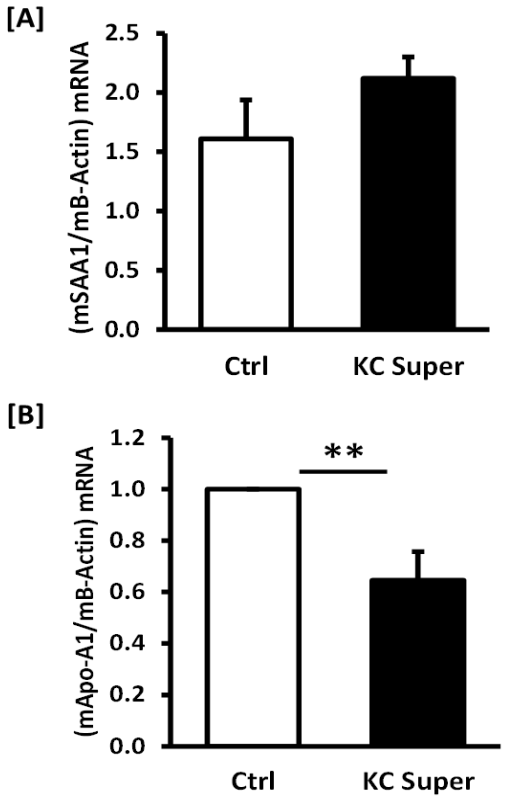


Figure 6.8: Effects of LPS-treated Kupffer cell supernatant on hepatocyte expression of APR genes.

Primary mouse hepatocytes obtained from liver of wild-type C57BL/6 mice by perfusion method, were treated with or without supernatant of Kupffer cells, which had been treated with 1 µg/ml *E. coli* LPS for 24 hours, and then expression of the APR genes SAA1 (A) and Apo-A1 (B), were measured by RT-PCR, relative to the house-keeping gene β-Actin. Results are expressed as means of 3 independent experiments ± SEM, which were compared by unpaired t-test (**P<0.01 *vs.* control cells cultured in medium alone).

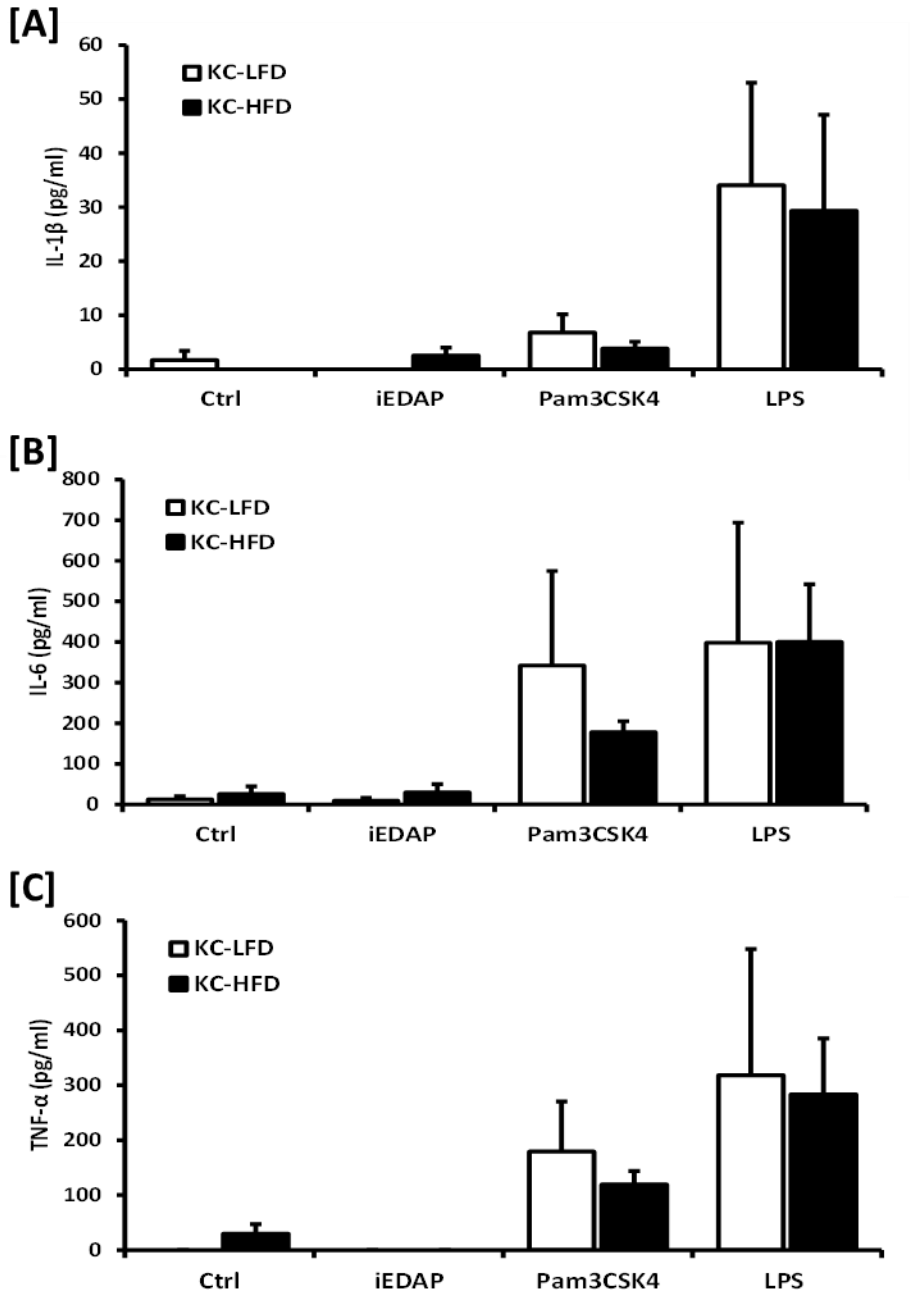


Figure 6.9: Production of inflammatory cytokines by murine Kupffer cells primed with low/high fat diet then stimulated with PAMPs.

Wild-type C57BL/6 mice were fed a normal chow diet (LFD, n=3) or high fat diet (HFD, n=4) for 4 weeks. Kupffer cells were obtained by perfusion method for each group then treated with PAMPs (iEDAP, Pam_3CSK_4 or LPS, 100 ng/ml each). After 24 hours incubation, secretion of IL-1β (A), IL-6 (B) and TNF-α (C) were measured from the supernatant using ELISA. The lower limit of detection of IL-6 was 0.025 ng/ml, TNF-α was 0.005 ng/ml and IL-1β was 0.035 ng/ml. Data are shown as means ± SEM, which were compared by unpaired t-test.

6.6. Expression of TLR2 and TLR4 by primary mouse hepatocytes and Kupffer cells

To further explore whether primary hepatocytes may respond directly to BLP and LPS, or rely instead on signals from Kupffer cells for hepatic PAMP detection, flow cytometry was used to measure expression of TLR2 and TLR4 on primary Kupffer cells and hepatocytes isolated by perfusion from WT mice. Murine RAW 264.7 macrophages, which are well established to express both TLR2 and TLR4, were used as a positive control for this experiment. Phycoerythrin (PE) -labelled TLR2 and TLR4 antibodies stained RAW 264.7 macrophages brightly relative to isotype control, confirming the utility of these antibodies (Figure 6.10). Primary Kupffer cells showed clear evidence of TLR2 and TLR4 staining, but surprisingly the level of TLR2 and TLR4 expression on these cells was equivalent in LFD and HFD-treated mice (Figure 6.11). On the other hand, murine primary hepatocytes showed no evidence of staining for TLR2 or TLR4 (Figure 6.12). Taken together, these results add further weight to the notion that Kupffer cells, rather than hepatocytes, are the principal sensors of BLP and LPS in the normal and HFD-primed mouse liver.

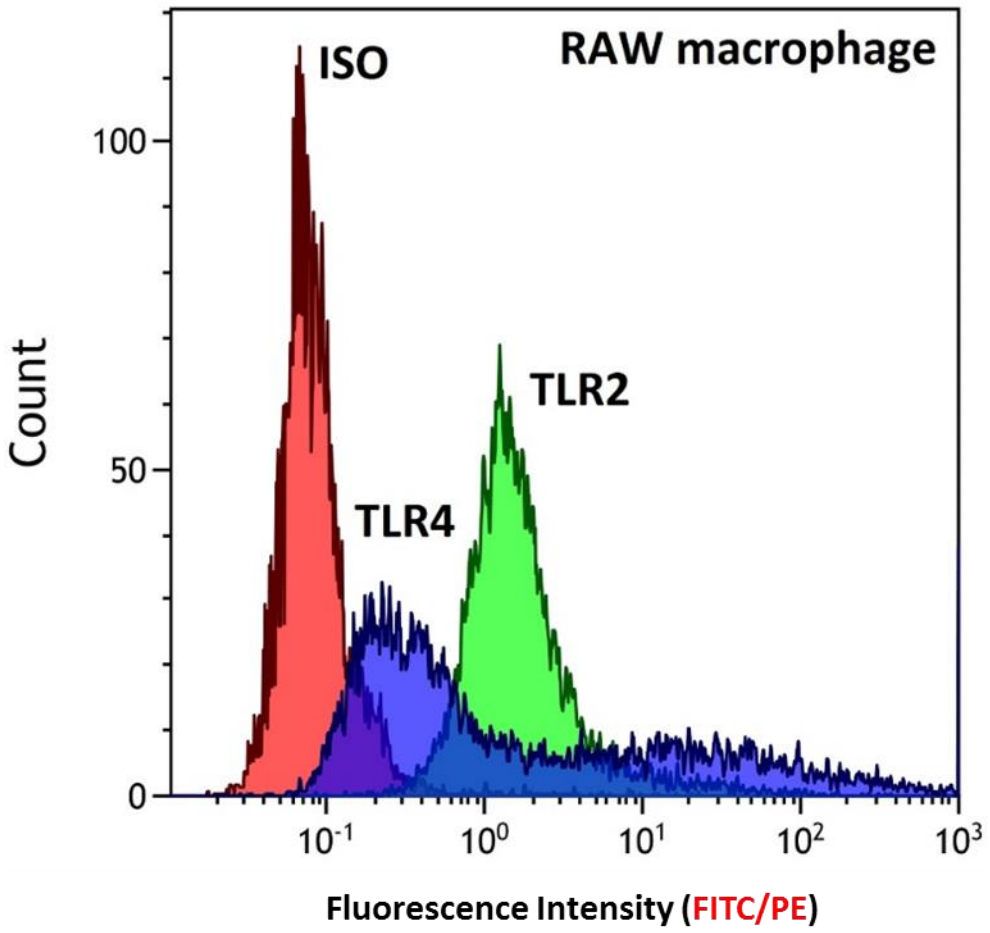


Figure 6.10: Flow cytometry analysis of TLR2 and TLR4 expression on murine RAW 264.7 macrophages.

Murine RAW 264.7 macrophages were stained with phycoerythrin (PE) -labelled TLR2 and TLR4 antibodies, or isotype control, and analysed by flow cytometry. Histograms indicate isotype control staining in red, TLR2 staining in green, and TLR4 staining in purple. Representative data are shown.

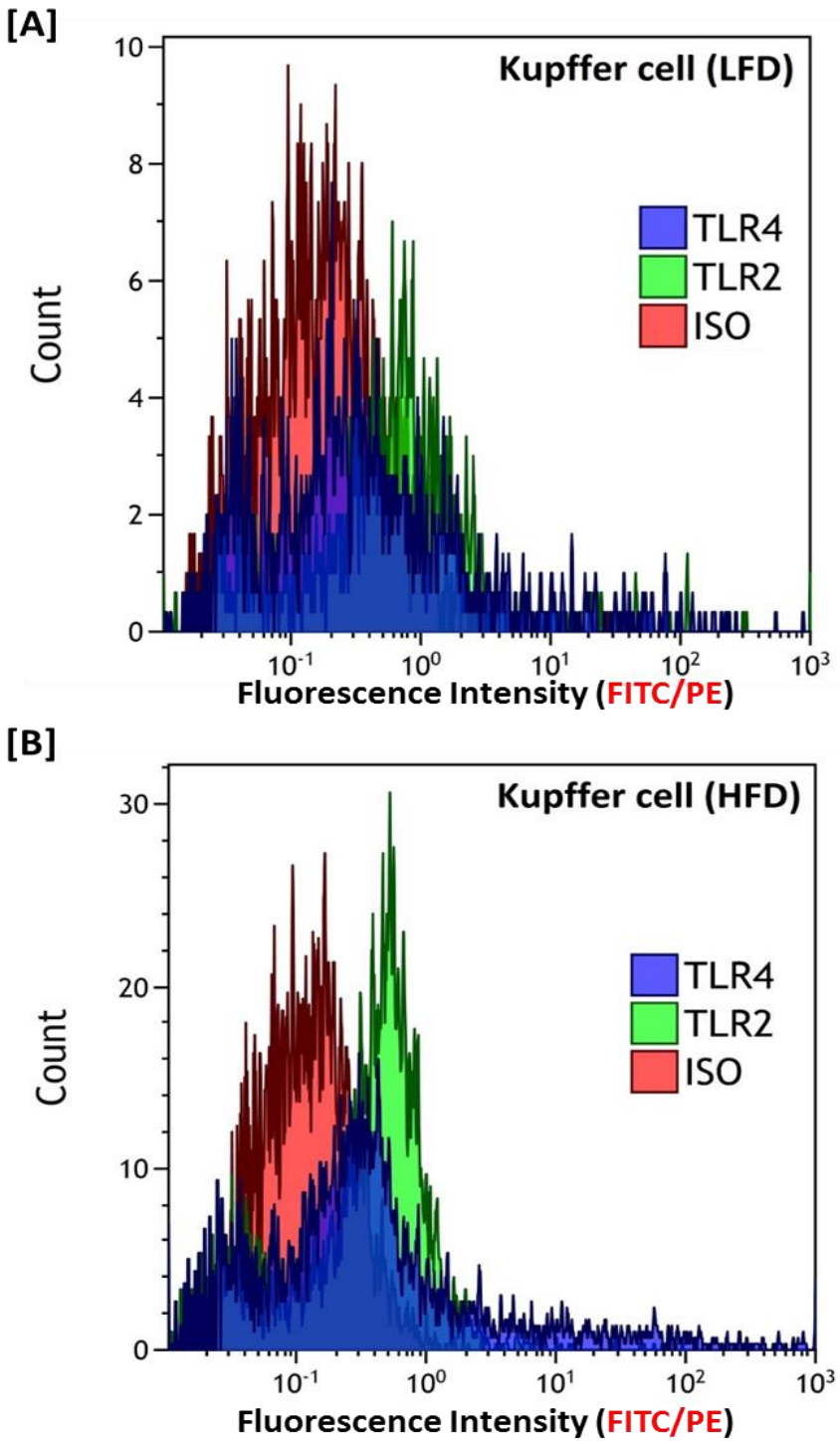


Figure 6.11: Flow cytometry analysis of the TLR2/4 expression on (LFD/HFD) murine Kupffer cells.

Wild-type C57BL/6 mice were fed a normal chow (LFD, n=3) or high fat diet (HFD, n=4) for 4 weeks. Kupffer cells were obtained by perfusion method and stained with phycoerythrin (PE) -labelled TLR2 and TLR4 antibodies, or isotype control, and analysed by flow cytometry. Histograms indicate isotype control staining in red, TLR2 staining in green, and TLR4 staining in purple. Data representative of three experiments are shown.

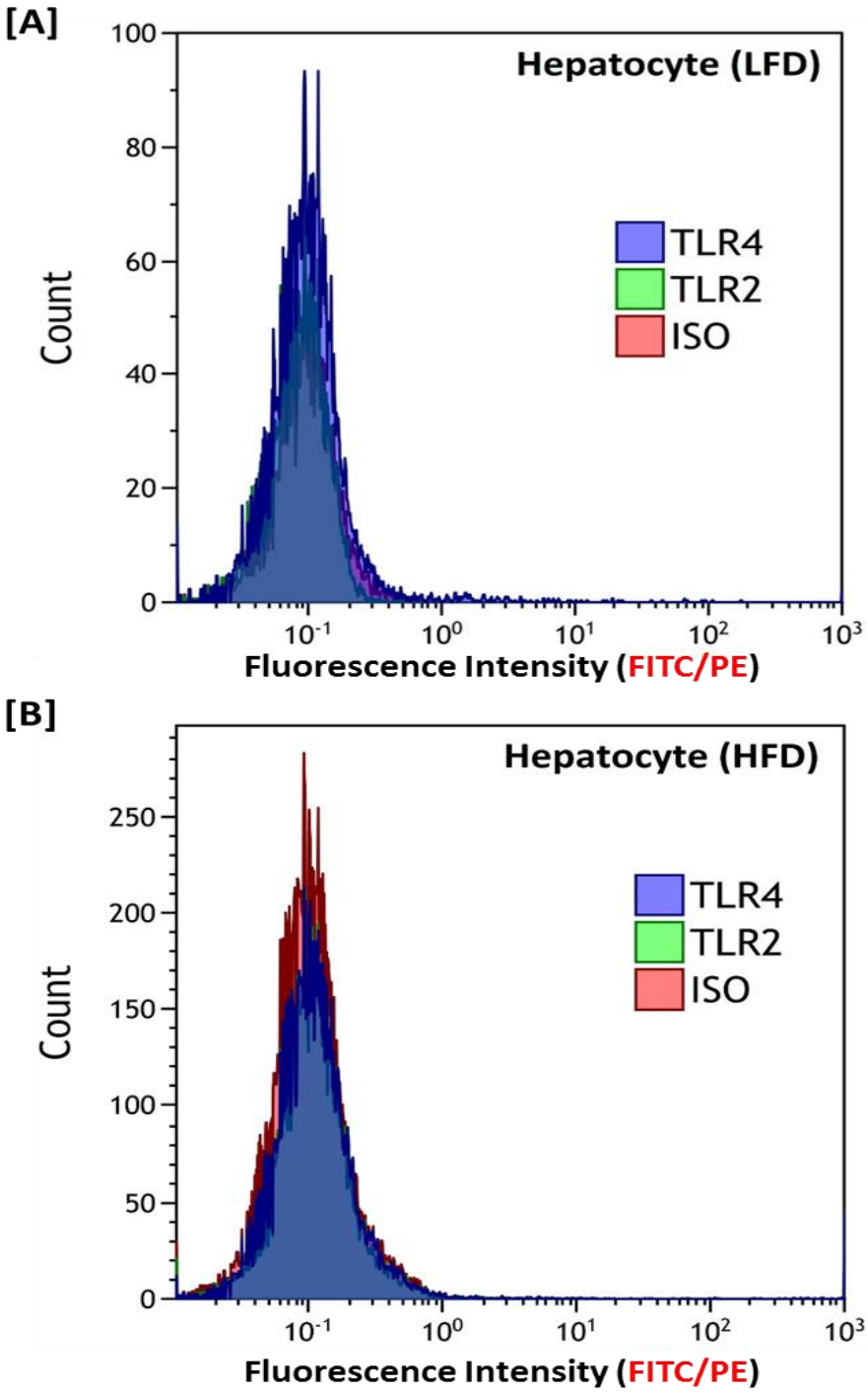


Figure 6.12: Flow cytometry analysis of TLR2 and TLR4 expression on primary mouse hepatocytes.

Wild-type C57BL/6 mice were fed a normal chow (LFD, n=3, A) or high fat diet (HFD, n=4, B) for 4 weeks. Hepatocytes were obtained by perfusion method and then stained with phycoerythrin (PE) -labelled TLR2 and TLR4 antibodies, or isotype control, and analysed by flow cytometry. Histograms indicate isotype control staining in red, TLR2 staining in green, and TLR4 staining in purple. Representative data shown.

6.7. Effects of IL-1R1 deficiency on *in vivo* responses to oral PAMP treatment

The HepG2 and primary hepatocyte work suggested that IL-1β may be a key mediator of the communication between Kupffer cells and hepatocytes during hepatic PAMP sensing. To test this, APR and metabolic responses to dietary PAMPs were measured in HFD-primed IL-1R1 knockout (KO) mice, *in vivo*.

Earlier work (Chapter 5), showed that oral gavage with 80 mg/kg *E. coli* LPS in HFD-primed wild type mice, resulted in highly significant increases in plasma SAA and hepatic APR gene expression (SAA1, SAA3, Lipocalin and Haptoglobin), and a significant reduction in plasma Apo-A1, total cholesterol level, HDL-C and serum cholesterol efflux capacity.

By contrast, in IL-1R1 KO mice, orally gavaged LPS induced no significant changes to plasma SAA (Figure 6.13), hepatic ApoA1 mRNA (Figure 6.14), hepatic APR gene expression (SAA1, SAA3, Haptoglobin and Lipocalin) (Figure 6.15), serum cholesterol levels (total cholesterol level, HDL-C and LDL-C) (Figure 6.16) or serum cholesterol efflux capacity (Figure 6.17).

In addition, work by other members of the group showed by immunoblot analysis that Apolipoprotein-A1 protein in the plasma of IL-1R1 KO mice was not changed by oral LPS (data not shown). Together, these findings support the concept that IL-1 signalling is central to the APR and metabolic phenotypes induced by oral LPS in mice.

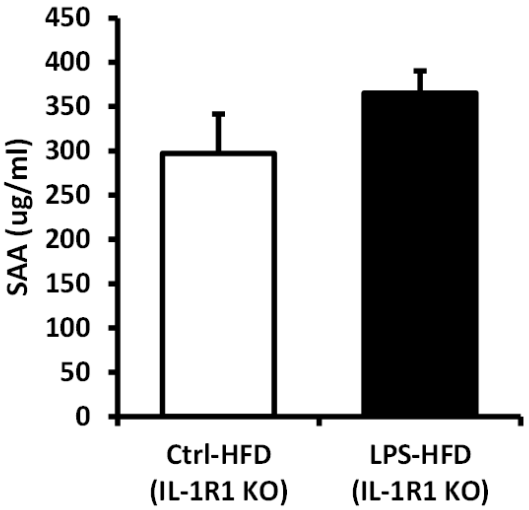


Figure 6.13: Effect of orally delivered LPS on serum amyloid A responses in high fat diet primed IL-1R1 KO mice.

Interleukin 1 receptor 1 (IL-1R1) deficient mice (n=10 per group) were fed a high fat diet for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, plasma content of SAA was measured by ELISA. Data are shown as means ± SEM, and were compared by unpaired t-test.

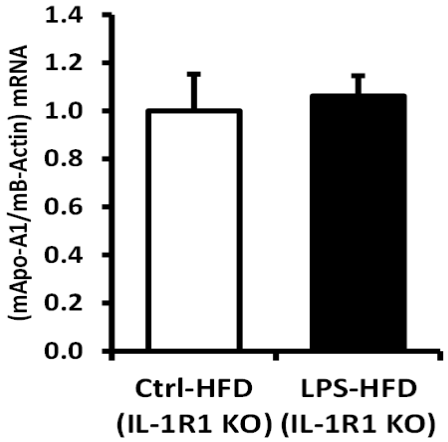


Figure 6.14: Effect of orally delivered LPS on hepatic Apolipoprotein-A1 mRNA in high fat diet primed IL-1R1$^{-/-}$ mice.

IL-1R1 knockout mice (n=10 per group) were fed a high fat diet for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic Apolipoprotein-A1 mRNA was measured RT-PCR. Data are shown as means ± SEM, which were compared by unpaired t-test.

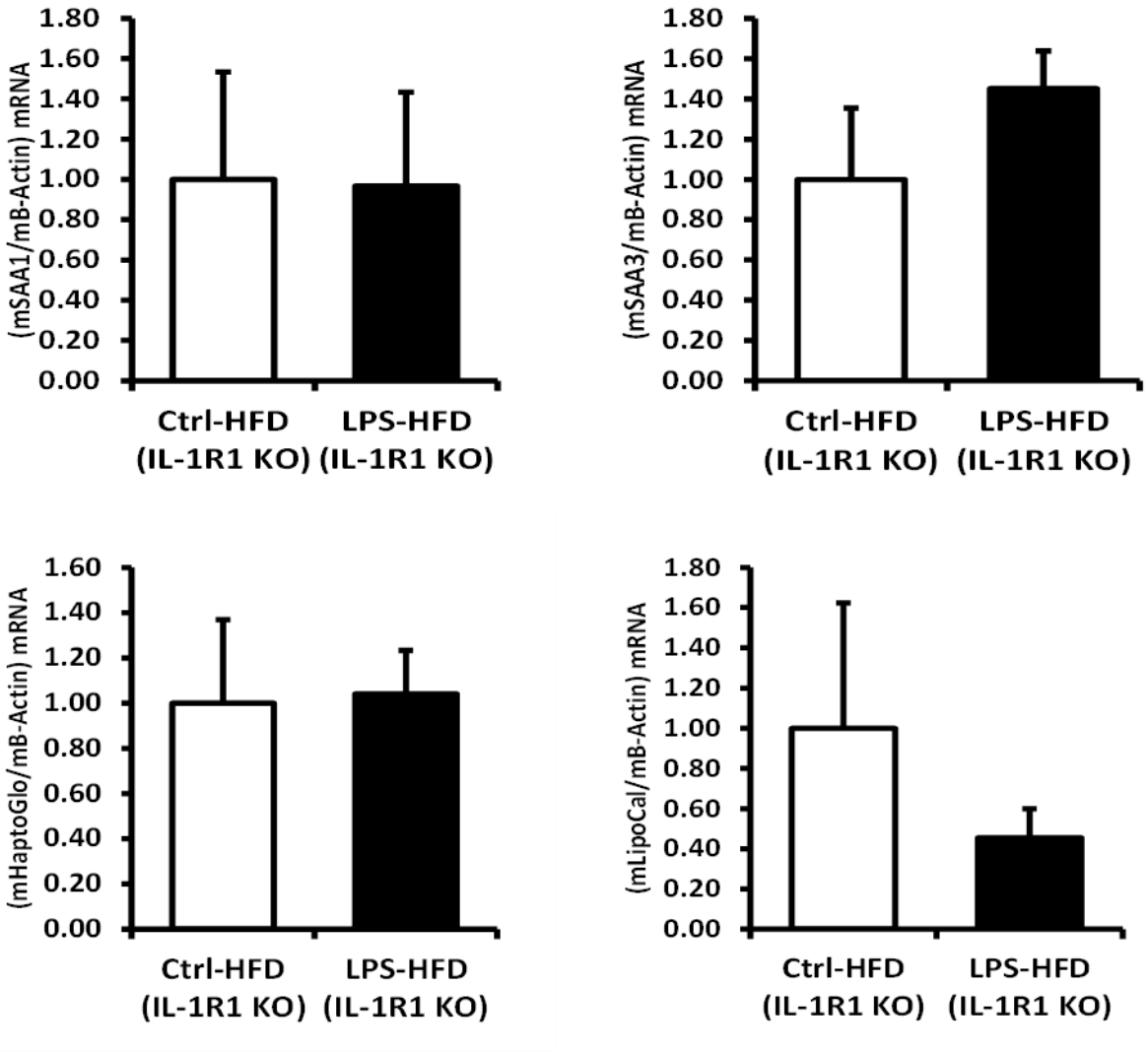


Figure 6.15: Effects of orally delivered LPS on hepatic APR gene mRNA expression in high fat diet primed IL-1R1 KO mice.

IL-1R1 deficient mice (n=10 per group) were fed a high fat diet for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, hepatic mRNA responses (Serum amyloid-A1, SAA3, Haptoglobin, Lipocalin), were measured by RT-PCR. Data are shown as means ± SEM, which were compared by unpaired t-test.

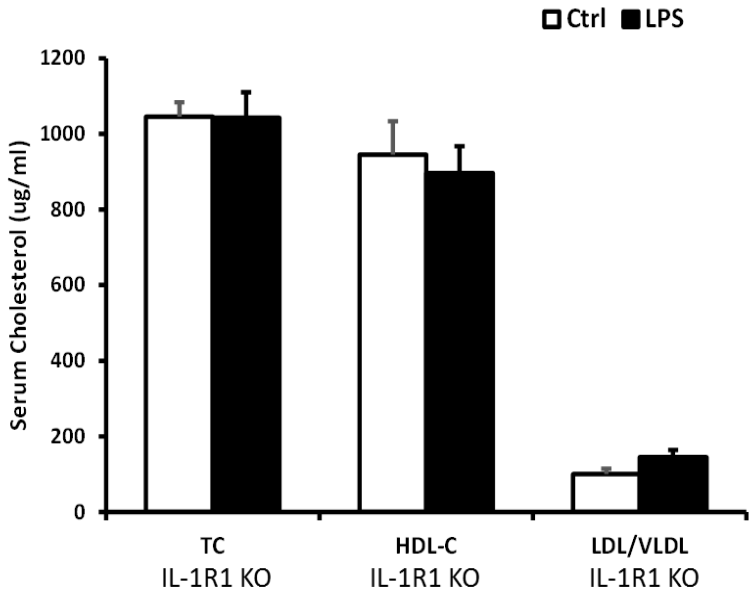


Figure 6.16: Effect of orally delivered LPS on serum cholesterol levels in high fat diet primed IL-1R1 KO mice.

IL-1R1 deficient mice (n=10 per group) were fed a high fat diet for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, cholesterol levels in total plasma and lipoprotein fractions were measured by Amplex red enzymatic assay. Data are shown as means ± SEM, which were compared by unpaired t-test.

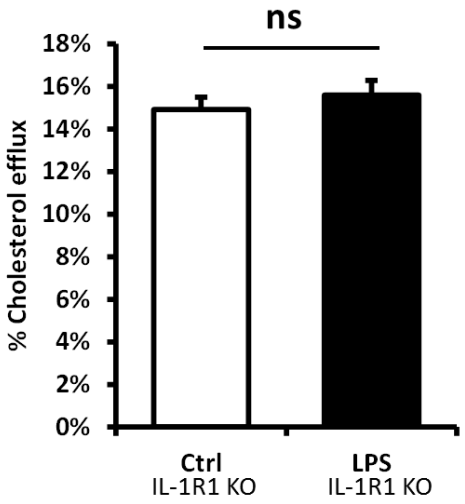


Figure 6.17: Effect of orally delivered LPS on serum cholesterol efflux capacity in high fat diet primed IL-1R1$^{-/-}$ mice.

IL-1R1 knockout mice (n=10 per group) were fed a high fat diet for 4 weeks. Mice were then orally gavaged with 200 µl saline with or without 80 mg/kg *E. coli* LPS. After a further 24 h, cholesterol efflux capacity of Apo-B depleted serum was measured. Data are shown as means ± SEM, which were compared by unpaired t-test.

6.8. Effects of IL-1R1 deficiency on responses of cultured mouse liver slices to LPS

Next, we explored the effect of IL-1R1 deficiency on SAA protein secretion by murine liver slices cultured *ex vivo* with supernatant of LPS-treated macrophages. Liver slices from WT and IL-1R1-KO HFD-primed mice were obtained using the vibratome technique. These were then treated with supernatant of J774 cells which had been challenged with or without 1 µg/ml *E. coli* LPS (then washed to prevent LPS carryover). Then, SAA was measured using ELISA. There was a significant increase (P=0.007 vs. control) of SAA secretion in the liver slices of wild type mice treated with supernatant of J774 cells which had been challenged with 1 µg/ml *E. coli* LPS relative to the control. However, there was no significant increase in secretion of SAA in the liver slices of IL-1R1 KO mice (Figure 6.18).

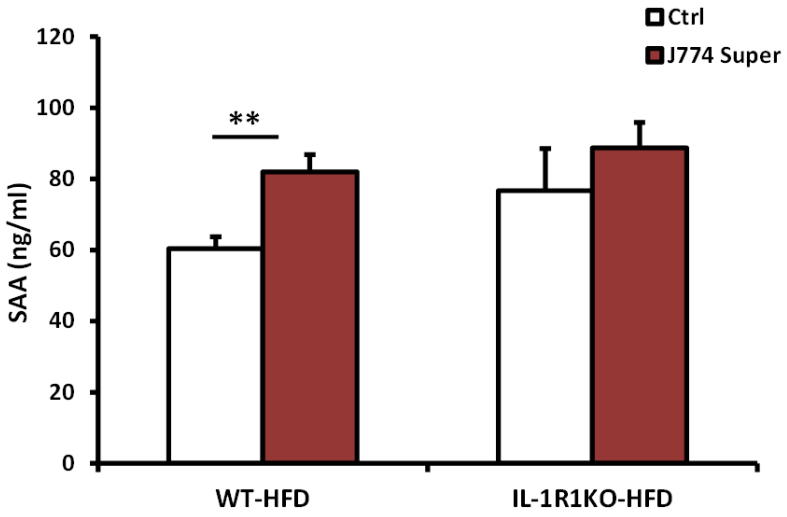


Figure 6.18: Effect of IL-1R1 deficiency on serum amyloid A responses of murine liver slices stimulated with supernatant of LPS-treated macrophages.

Wild-type C57BL/6 mice (n=4 per group) and IL-1R1 deficient mice (n=4 per group) were fed a high fat diet for 4 weeks. Liver slices (n=5 per mouse) were obtained by Vibratome technique, then treated with supernatant of J774 cells which had been challenged with or without 1 µg/ml *E. coli* LPS. After 24 hours incubation, SAA was measured using ELISA. Data are shown as means ± SEM, which were compared by unpaired t-test (**P<0.01 *vs.* control liver slices).

6.9. Effect of IL-1R1 deficiency on primary hepatocyte responses to Kupffer cell-conditioned medium

Next, primary hepatocytes obtained from liver of WT and IL-1R1 KO mice by the perfusion method, were treated supernatant of Kupffer cells treated with or without 1 µg/ml *E. coli* LPS. Then, expression of Apo-A1 mRNA was measured by RT-PCR, to reveal role of IL-1R1 deficiency on hepatocyte Apolipoprotein A1 mRNA responses to supernatant of LPS-treated Kupffer cells. ApoA1 mRNA responses dropped significantly (P=0.0011 vs. control) in wild type hepatocytes treated with stimulated Kupffer cell medium. However, no change was detected in Apo-A1 gene expression in hepatocytes of IL-1R1 genetically depleted mice (Figure 6.19). This suggests that IL-1 signalling plays a key role in connecting Kupffer cell activation to reduced hepatic expression of ApoAI.

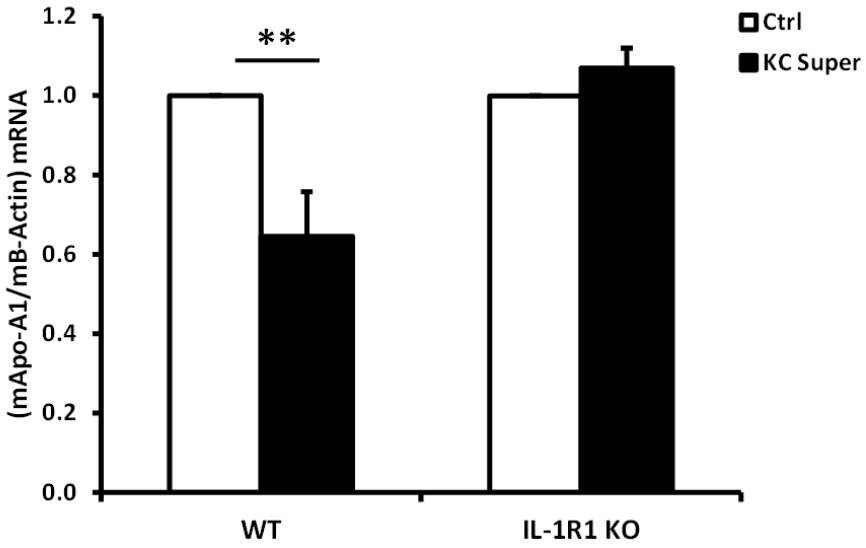


Figure 6.19: Effect of IL-1R1 deficiency on hepatocyte Apolipoprotein A1 mRNA responses to supernatant of LPS-treated Kupffer cells.

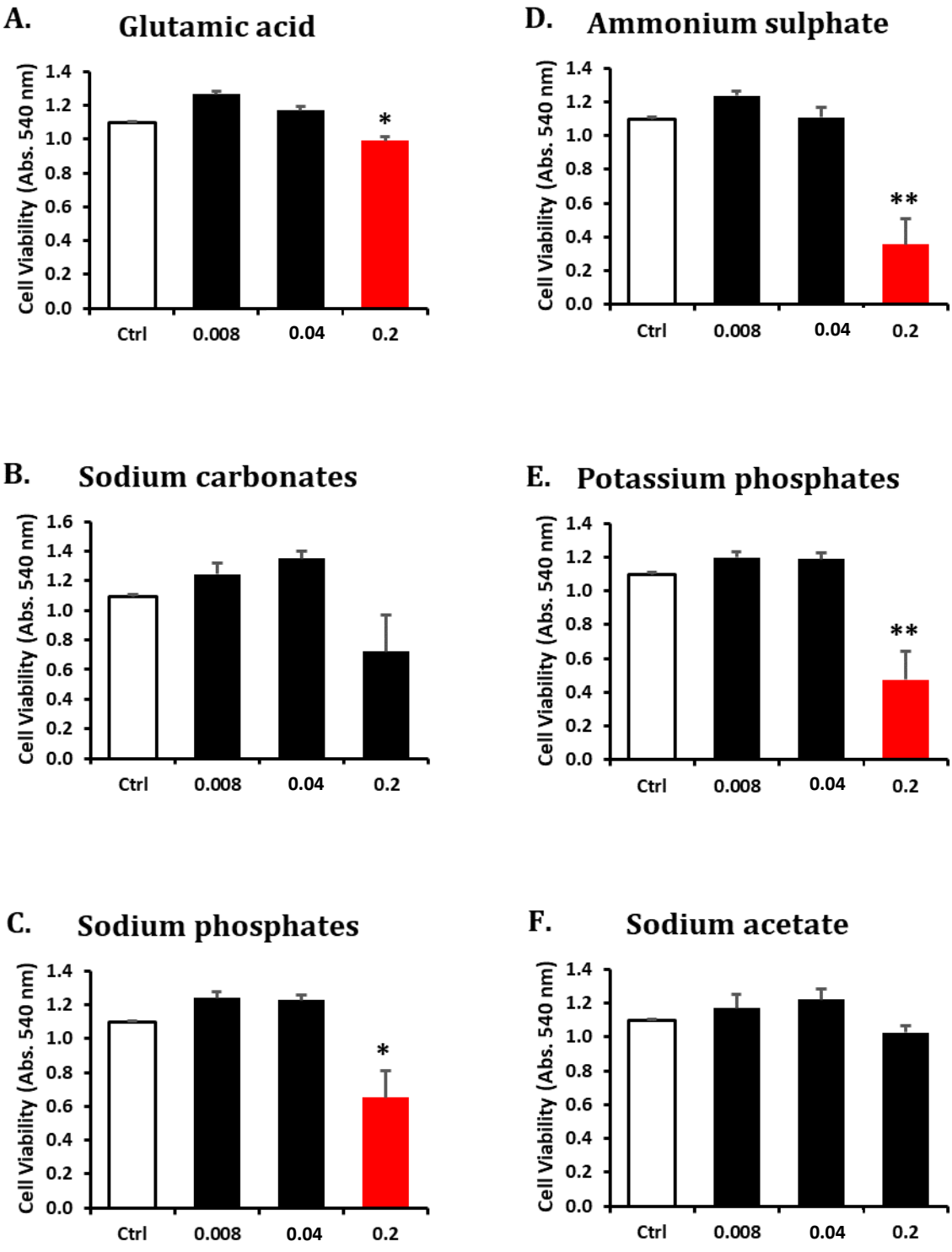
Murine hepatocytes obtained from liver of wild-type C57BL/6 mice (n=4) and IL-1R1 deficient mice (n=3) by perfusion method, were treated with or without supernatant of Kupffer cells treated with 1 µg/ml *E. coli* LPS for 24 hours, and then expression of Apo-A1 mRNA was measured by RT-PCR, relative to the house-keeping gene β-Actin. Data are shown as means ± SEM, which were compared by unpaired t-test (**P<0.01 *vs.* control cells cultured in supernatant from resting kupffer cells without LPS treatment).

6.10. Effects of common food additives on viability of human epithelial cells

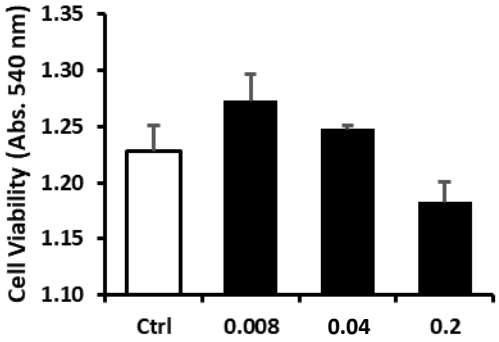
As a final experiment, with the aim of exploring a new potential direction for future projects, I looked to see if any common food additives might impact on gut epithelial health. This approach was based on the observation that impairment of gut barrier function can lead to dramatically increased absorption of LPS from the gut, but very little information exists on how commonly used foods additives might affect this (Chapter 5). Since enterocytes are epithelial cells, I used two different epithelial cell lines to determine if any common food additives might affect cellular viability. First, HeLa cells were used to develop the assay and perform a preliminary screen, and then the intestinal enterocyte cell line CaCo2 was used to focus on enterocyte function.

A panel of seventeen commonly used food additives was prepared and applied to cells at concentrations starting at those that might conceivably occur in food and hence the gut (0.008, 0.04 and 0.2 wt/vol). After 24 hours, cell viability was measured using crystal violet staining solution. Unexpectedly, I found that most of the food additives (Figure 6.20, A-Q) at concentration of (0.2 wt/vol) have significant effects on HeLa cell viability relative to the control. Seven of the food additives, including sodium carbonate, sodium acetate, agar, glycerol, sodium bisulfite, sodium citrate and sodium sulfate, had no effect on HeLa cell viability. The surfactant sodium decanoate had by far the greatest impact on HeLa cell viability, causing ~80% killing at a concentration of just 0.04 wt/vol.

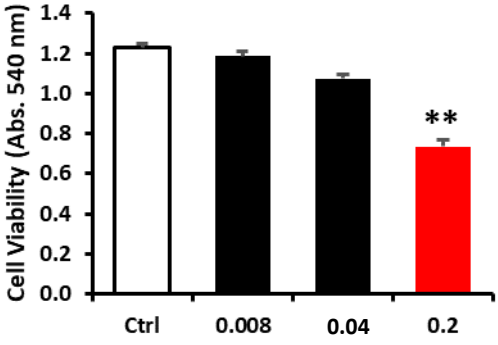
The same food additives were then examined using the human epithelial colorectal cell-line CaCo2. In this experiment, cell viability was measured after 24 hours incubation using the more established MTT assay. Three food additives, sodium decanoate, sodium sulfite, and sodium bisulfite, caused significant reductions in CaCo2 cell viability (Figure 6.21, A-Q). In this cell-line, which can be considered a more relevant model of gut barrier function than HeLa cells, the most toxic food additive was the preservative sodium decanoate, which killed ~90% of the cells at a concentration of 0.2 wt/vol.


A.
Glutamic acid
Cell Viability (Abs. 540 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Ctrl
0.008
0.04
0.2
D.
Ammonium sulphate
Cell Viability (Abs. 540 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Ctrl
0.008
0.04
0.2
B.
Sodium carbonates
Cell Viability (Abs. 540 nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.008
0.04
0.2
E.
Potassium phosphates
Cell Viability (Abs. 540 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Ctrl
0.008
0.04
0.2
C.
Sodium phosphates
Cell Viability (Abs. 540 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Ctrl
0.008
0.04
0.2
F.
Sodium acetate
Cell Viability (Abs. 540 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.008
0.04
0.2

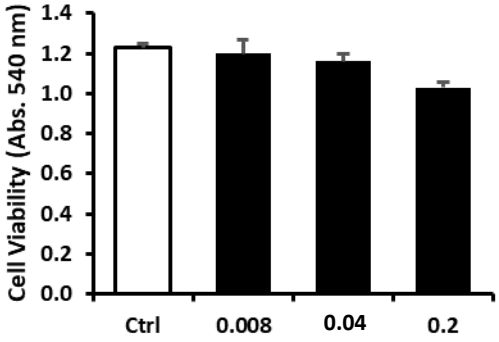
G.


Agar
Cell Viability (Abs. 540 nm)
1.35
1.30
1.25
1.20
1.15
1.10
Ctrl
0.008
0.04
0.2

J.


Sodium ascorbate
Cell Viability (Abs. 540 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Ctrl
0.008
0.04
0.2

H.


Glycerol
Cell Viability (Abs. 540 nm)
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1.2
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0.8
0.6
0.4
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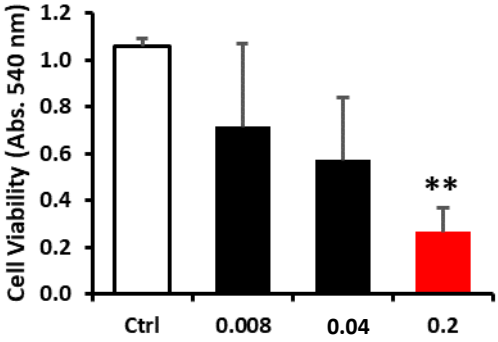
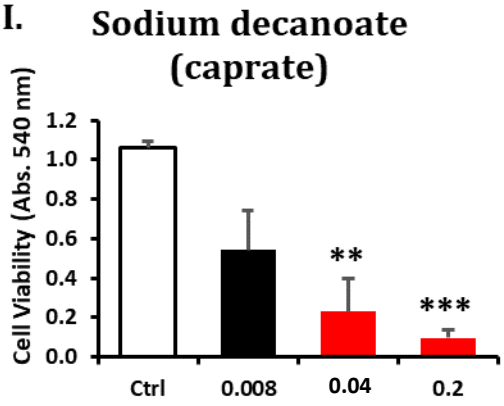
Ctrl
0.008
0.04
0.2

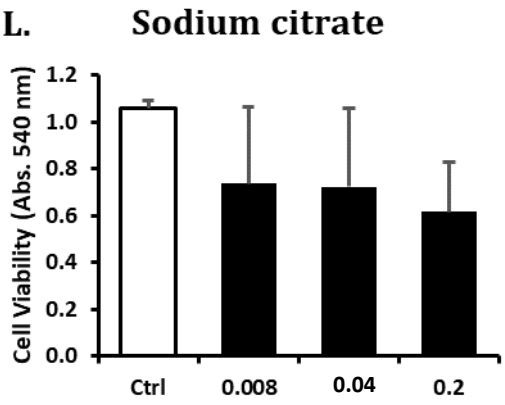
K.


Sodium sulfite
Cell Viability (Abs. 540 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Ctrl
0.008
0.04
0.2

I.


Sodium decanoate (caprate)
Cell Viability (Abs. 540 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
Ctrl
0.008
0.04
0.2

L.


Sodium citrate
Cell Viability (Abs. 540 nm)
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1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.008
0.04
0.2

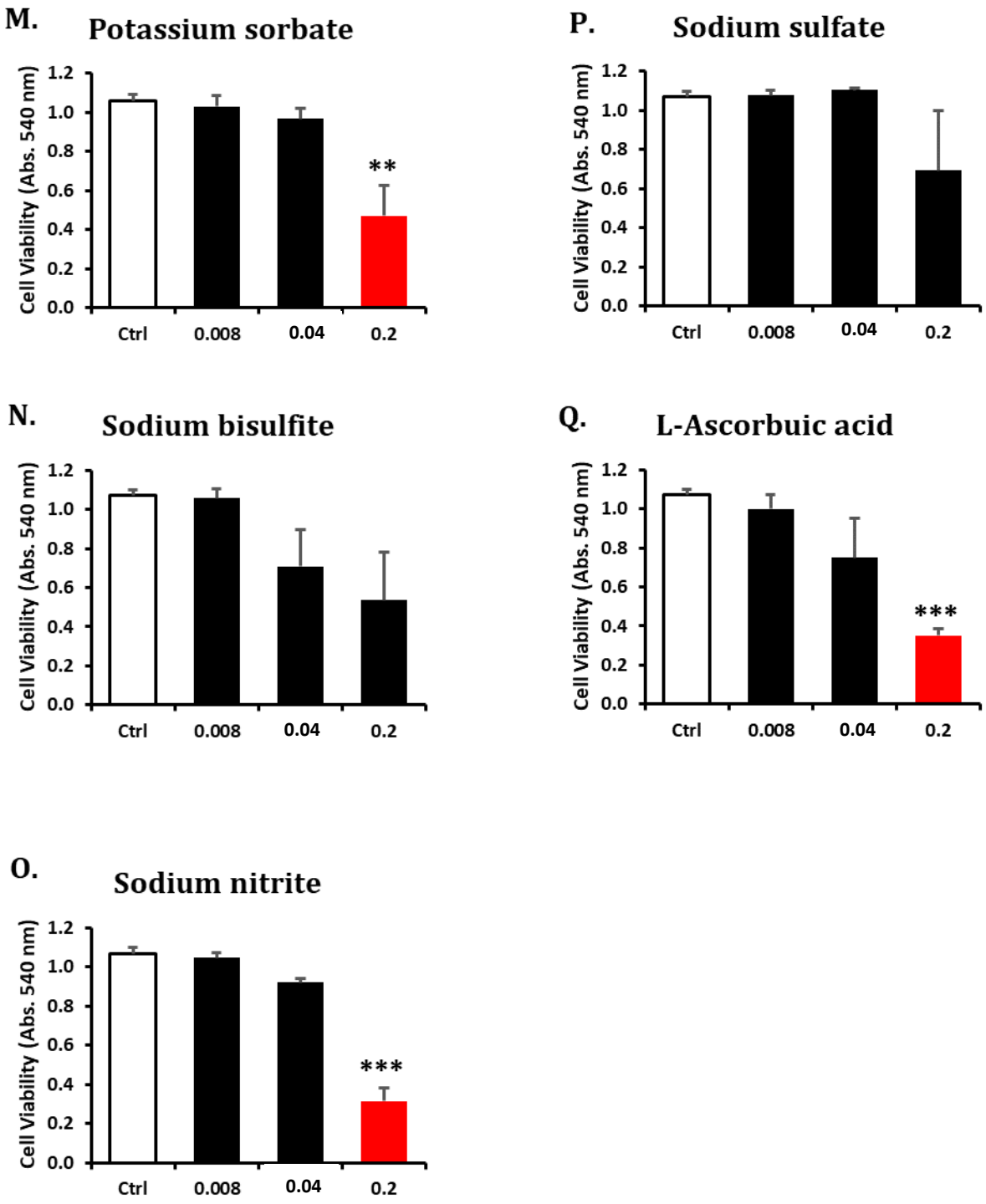
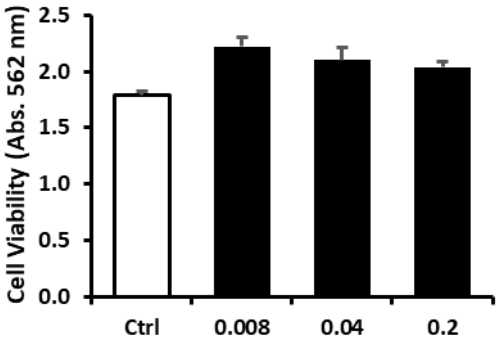


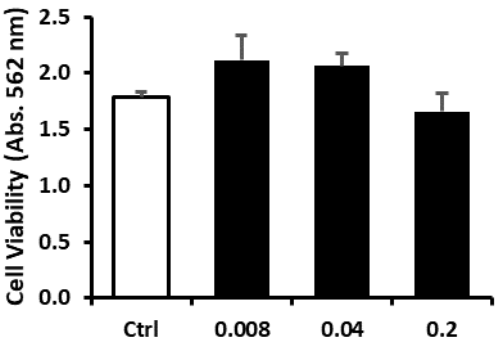
Figure 6.20: Effects of common food additives on human epithelial cells.

Human epithelial (HeLa) cells were seeded in a 96-well plate at a density ($4x10^5$ cells/well) for 24 hours. HeLa cells were challenged with different concentrations (0.008, 0.04 and 0.2 wt/vol) of common food additives (A-Q). After 24 hours incubation, cell viability was measured using crystal violet staining solution. Results are expressed as means of 3 independent experiments ± SEM. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 *vs.* control cells cultured in medium alone).

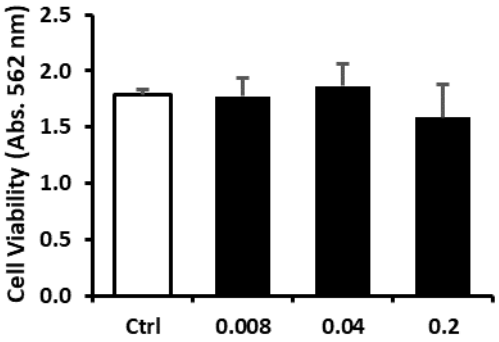
A. Glutamic acid



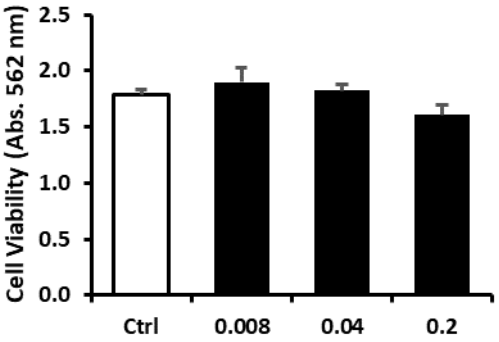
D. Ammonium sulphate



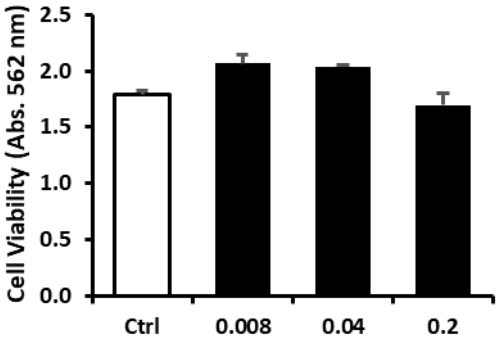
B. Sodium carbonates



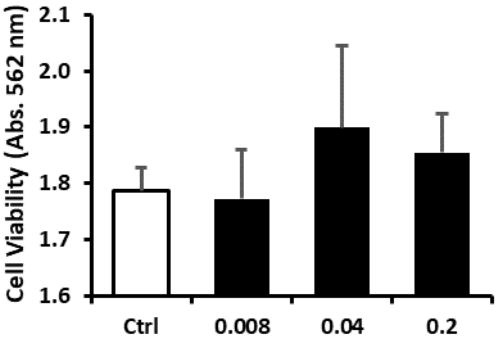
E. Potassium phosphates



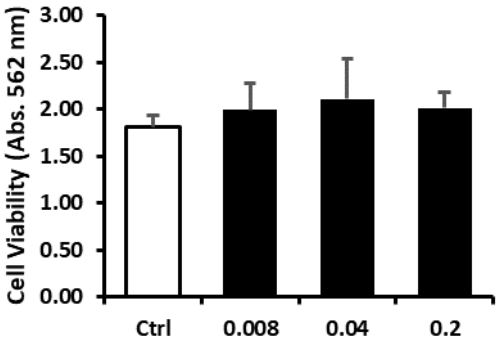
C. Sodium phosphates



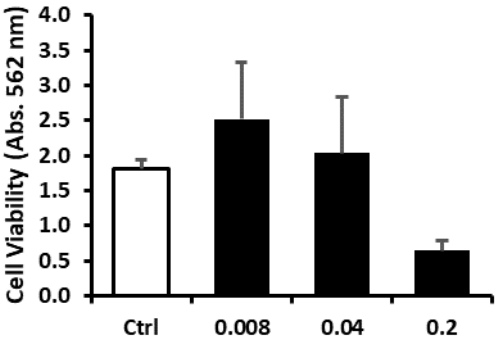
F. Sodium acetate



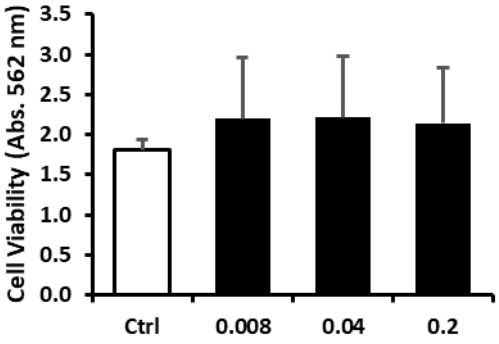
G.


Agar
Cell Viability (Abs. 562 nm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Ctrl
0.008
0.04
0.2

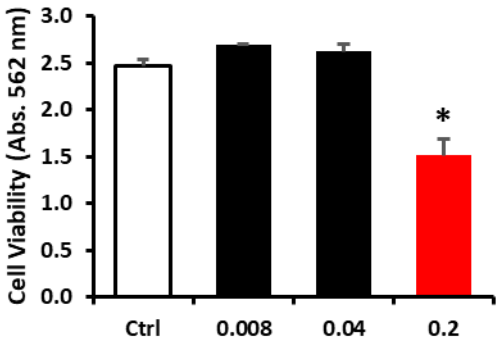
J.


Sodium ascorbate
Cell Viability (Abs. 562 nm)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
0.008
0.04
0.2

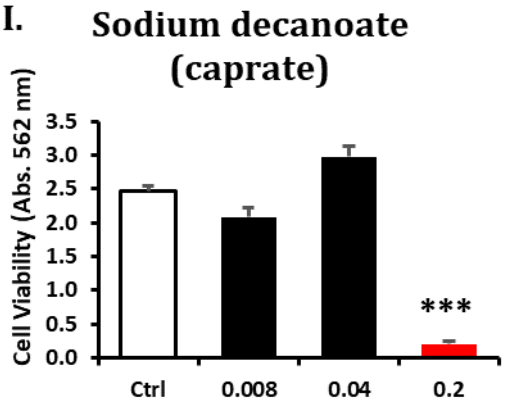
H.


Glycerol
Cell Viability (Abs. 562 nm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
0.008
0.04
0.2

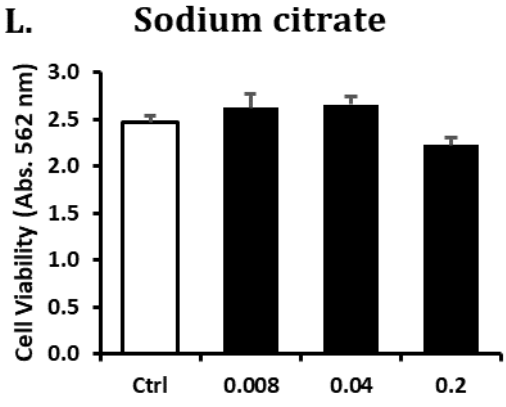
K.


Sodium sulfite
Cell Viability (Abs. 562 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Ctrl
0.008
0.04
0.2

I.


Sodium decanoate
(caprate)
Cell Viability (Abs. 562 nm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
Ctrl
0.008
0.04
0.2

L.


Sodium citrate
Cell Viability (Abs. 562 nm)
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2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
0.008
0.04
0.2

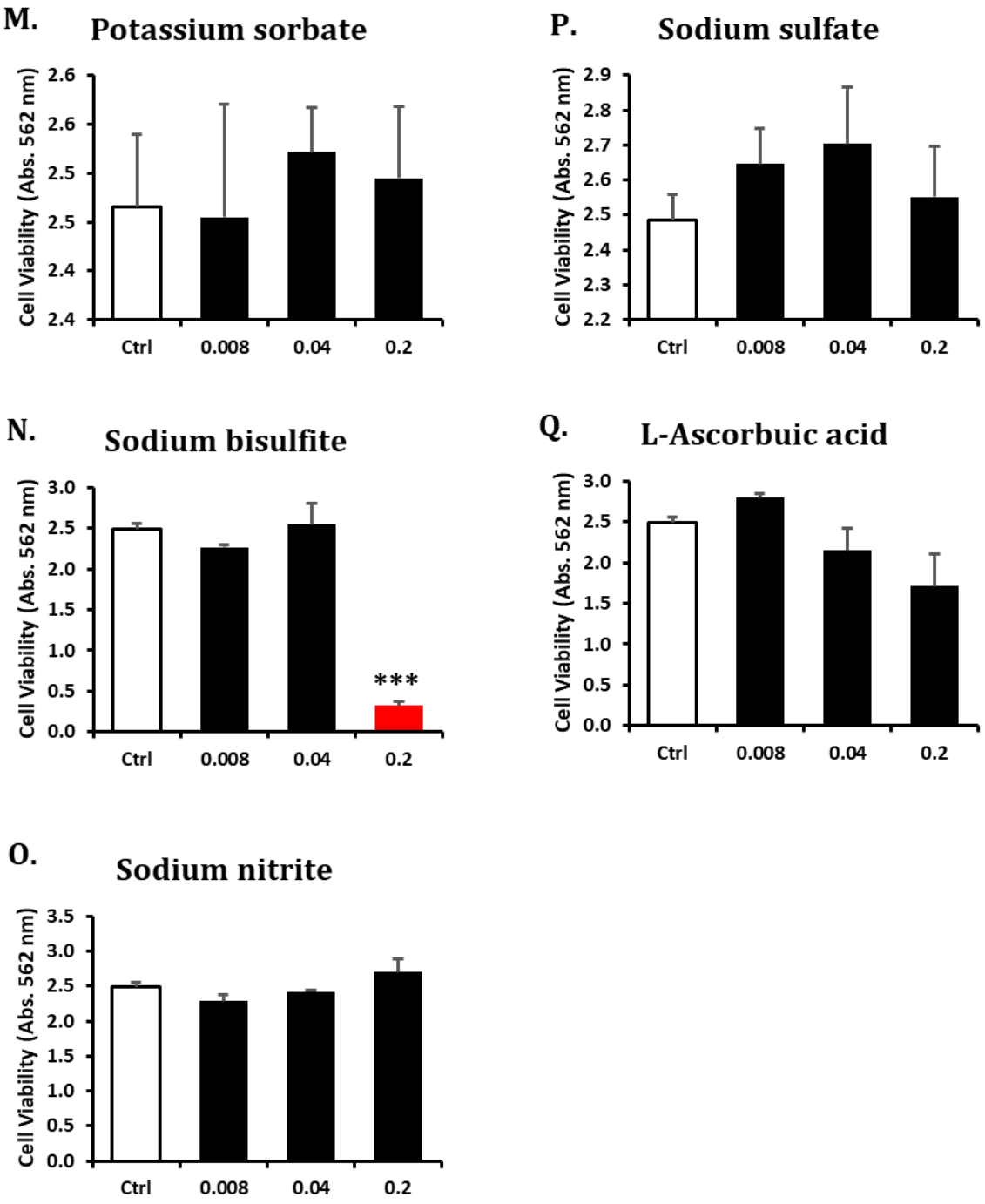

Figure 6.21: Effects of common food additives on viability of cultured human intestinal epithelial cells.

Human epithelial colorectal Caco-2 cells were seeded in a 96-well plate at a density ($4x10^5$ cells/well) for 24 hours. Caco-2 cells were then challenged with different concentrations (0.008, 0.04 and 0.2 wt/vol) of common food additives (A-Q), After 24 hours incubation, cell viability was measured using MTT assay. Results are expressed as means of 3 independent experiments ± SEM. Differences were compared by ANOVA with Dunnett's test (*P<0.05, **P<0.01, ***P<0.001 *vs.* control cells cultured in medium alone).

Together, the findings from these preliminary experiments suggest that commonly used food additives, particularly certain surfactants and preservatives, may have potential to damage enterocyte function, and could conceivably contribute to an increased absorption of dietary PAMPs.

Overall, the results from this chapter show that Kupffer cells, rather than hepatocytes, are the main sensors of BLP and LPS, also IL-1β signaling plays a key role in the expression of the APR and metabolic phenotypes induced by oral LPS in mice. In addition, food additives may have an adverse role in changing gut barrier function which could conceivably result in increased translocation of dietary PAMPs into the circulation.

Chapter 7 Discussion

7.1. Potential mechanisms connecting diet to inflammation

Atherosclerosis is a chronic inflammatory disease of the arteries, which is recognized as a major cause of most cardiovascular diseases (Ross 1999); (Göran K 2005); (Kassiteridi *et al.* 2013) and (Libby *et al.* 2009). However, despite much recent research in this area, the agents responsible for promoting the inflammatory events that underpin atherosclerosis remain to be clearly defined.

Dietary factors could represent candidate stimuli, as they are well established to participate in atherogenesis (Paoletti *et al.* 2006). For example, studies using animal models revealed that administration of a high fat diet promoted a state of low-grade systemic inflammatory signaling which was also associated with increased atherosclerosis, insulin resistance and metabolic syndrome (Kokoeva *et al.* 2006). Other studies also showed that levels of plasma SAA and inflammatory markers in vascular tissues increased in mice fed high fat diet when compared to mice fed normal diet (Heese *et al.* 2008). Similar findings have been reported in human dietary studies. For example, long term (4 weeks) feeding of human volunteers with high fat snacks or fast foods increased plasma CRP levels significantly (Tam *et al.* 2010) and (Carlsson *et al*. 2010).

Accordingly, subjects consuming a Western type diet, typified by refined and processed foods, consistently show higher CRP levels than those that adhere to a 'prudent', or 'Mediterranean' diet, characterized by frequent consumption of fresh produce, including vegetables, legumes and fish (Fung *et al.* 2001). It has been shown that the consumption of processed meat products significantly increases the risk of coronary heart disease, stroke, and diabetes mellitus when compared with consumption of an equivalent amount of non-processed meats (Micha *et al.* 2010). There is also some evidence that acute dietary fat intake by human volunteers results in increased IL-6 levels in the postprandial phase, reviewed by (Herieka M and Erridge C 2014).

Taken together, these results suggest a strong association between commencement of a high fat or processed food diet and elevation of inflammatory markers in mice and humans. However, the mechanisms promoting this association remain unclear.

It has been frequently proposed (Lee *et al.* 2004) that saturated fatty acids (SFA) may have the ability to promote inflammatory cytokine production via direct stimulation of TLR2 or TLR4 signaling pathways. However, studies conducted by (Erridge C 2009) have shown that SFAs are in fact unable to stimulate TLR2 or TLR4 signaling, and that earlier reports of this capacity reflected contamination of reagents used. In addition, a meta-analysis of epidemiological studies revealed that there is no significant correlation between dietary saturated fat intake and the risk of cardiovascular diseases (Siri-Tarino *et al.* 2010).

These findings suggest that other factors in the modern diet are likely to promote inflammatory signaling. Because earlier studies (Erridge C 2010) and (Erridge 2011b) showed that processed foods can contain high levels of TLR2 and TLR4 stimulants of microbial origin arising from the processing and storage of products, the aim of this part of the project was to examine the roles played by these dietary stimulants in the induction of inflammatory cytokines and inflammatory gene expression in human leukocytes and how that compares to the TLR-stimulant content of the gut.

We first investigated whether or not commonly consumed processed foods may promote inflammatory gene expression (IL-1β, IL-6, TNF-α) in leukocytes. Processed food extracts were chosen on the basis of a relatively high content of minced meat, chopped onion and cheese, since these ingredients were shown previously to be at high risk of contamination with TLR-stimulants (Erridge C 2010) and (Erridge 2011b).

Surprisingly, of a panel of aqueous extracts prepared from 24 different supermarket-bought processed food products, almost all stimulated production of IL-6 by human whole blood (Figure 3.2). The relative lack of TNF-α production in response to the same extracts (Figure 3.4) measured at 24 h may reflect the timecourse of TNF-α production, which peaks at 3 h and diminishes subsequently. Interestingly, food extracts with a high content of minced meat or chopped onion promoted a higher production of inflammatory cytokines. Similar results were reported in an earlier study, where human primary monocytes treated with minced meats or cheese induced significantly greater secretion of TNF-α and IL-6 when compared with cells cultured with extracts of other foods or medium alone (Erridge C 2010).

In the presence of dietary PAMPs, PBMCs were induced to express significant levels of inflammatory cytokine gene mRNAs, such as IL-6, IL-1β, TNF-α, and IL-8 (Figure 3.13 and

3.14-a). Unexpectedly, however, expression of MCP-1 (Figure 3.14-b) was reduced by all stimuli (minced meat, chopped onion, LPS [10 ng/ml], and Pam3CSK4 [1,000 ng/ml]) when compared to control. A similar observation was also reported by, (Heimdal *et al.* 2001), where the secretion of MCP-1 by monocytes treated with 1 μg/ml LPS decreased when compared to untreated monocytes. However, (LIU *et al.* 2012), reported that *in vitro* challenged RAW 264.7 macrophages with LPS (1 μg/ml) induced significant secretion of IL-8 and MCP-1, as well as expression of mRNA for these genes when compared to the control. It is not clear why the findings of these studies differ, but it could be related to differences in the types of responder cell studied.

We hypothesized that the existence of certain types of PAMPs in processed foods may be a key step in the stimulation of TLR signaling and the initiation of pro-inflammatory cytokine release (Falck-Hansen *et al.* 2013). Quantification of TLR4 and TLR2 stimulants in each food using HEK-293 transfectants revealed that almost all of the food extracts contained detectable amounts of TLR4 or TLR2 stimulants.

Moreover, those foodstuffs which contained minced meat and chopped onion tended to also contain higher levels of TLR stimulants (Figures 3.7 and 3.8). For example, pork sausage rolls contained 985 ng LPS per gramme food, minced beef and onion pie: 328 ng/g, spaghetti bolognese: 224 ng/g. These are considered to be a relatively high abundance of TLR4 stimulants compared with the other food extracts tested. In terms of TLR2 stimulants, most of the food extracts did not contain a high level of stimulants, the exceptions being pork sausage rolls: 106 ng/g, fisherman's pie: 52 ng/g, cheese and onion rolls: 41 ng/g. However, technical difficulties relating to the type of filter used (low protein binding filters were not available at the time of extract preparation) may have limited lipopeptide recovery resulting in a low TLR2 signal in these experiments. Otherwise, our results are in agreement with other studies regarding the presence of large quantities of TLR-stimulants within processed foodstuffs, in which commonly consumed foods were shown to contain large amounts of both TLR4 and TLR2 stimulants (2.7 μg/g LPS-equivalent and 1.1 μg/g BLP-equivalent per gram of food) (Erridge C 2010).

Notably, significant correlations were observed between the abundance of TLR-stimulants in each foodstuff tested and cytokine readouts (e.g. r=0.70, p=0.002 for IL-6 vs TLR4-stimulants, Figure 3.9). These results indicate that the quantity of dietary TLRs

stimulants (PAMPs) in foods is likely to parallel their capacity to induce the expression of pro-inflammatory cytokines.

For comparison, the LPS content of food extracts was also quantified using the kinetic chromogenic Limulus Amoebocyte Lysate (LAL) assay. This assay suggested that most of the food extracts tested contained dramatically higher levels of endotoxin than those measured using the TLR-transfection assay (Figure 3.10). This is consistent with earlier studies which showed that minced beef after storage can contain up to 7.4 µg LPS per gram of food (J M Jay *et al.* 1979). However, using LAL assay in the quantification of biological activities of TLR stimulants in foodstuffs was considered as inappropriate since false positive results are common due to the detection of non-inflammatory β-glucans which are a common ingredient of foodstuffs (Elin, Wolff 1973). Also, measurements of bacterial lipopeptide cannot be achieved by limulus assay (Erridge, Samani 2009). In addition, no significant correlations were detected between endotoxin levels in foodstuffs measured using the LAL assay, and the abundance of TLR2/4 stimulants in foodstuffs measured using TLR-transfectants (Figure 3.11). Likewise, there was little correlation with the production of pro-inflammatory cytokines by whole blood (Figure 3.11).

The results indicate that a 400 g processed food meal may contain on average about 200 µg LPS-equivalent and 25 µg BLP-equivalent. By comparison, intravenous injection of as little as 8 ng LPS is sufficient to stimulate a detectable inflammatory response in man (Ferguson *et al.* 2013). This suggests that the quantity of TLR4 stimulants in a processed food meal can often be up to 25,000-fold higher than required to stimulate inflammation if given by injection.

In addition, the *in vitro* experiment results suggest that the minimum dose required to stimulate cytokine production by challenged human whole blood was 0.1 ng/ml for LPS and 10 ng/ml for Pam3CSK4 (Figure 3.5). Taken together, these findings suggest that processed foods can contain PAMPs at a concentration much higher than that required to stimulate inflammatory cytokine production in human blood.

A possible explanation for why processed meal consumption does not lead to severe acute inflammatory syndromes is that the intestinal wall may serve as a barrier to the absorption of the majority of LPS and BLP contained within a meal.

Notably, recent studies have demonstrated that high fat meals weaken gut barrier function, and stimulate translocation of small quantities (~0.1%) of bacterial LPS from the

intestine into the circulation (Erridge C *et al.* 2007c) and (Ghoshal *et al.* 2009). There is some evidence that this pathway may be relevant to disease, since it was shown that levels of circulating LPS in man are associated with risk of atherosclerosis (Wiedermann *et al.* 1999) and (Pussinen *et al.* 2007).

Further studies showed that high fat diet and obesity may increase the level of PAMPs within the circulation compared with healthy controls (Patrice D. Cani *et al.* 2007); (Erridge C *et al.* 2007c); (Paola Brun *et al.* 2007) and (Herieka M and Erridge C 2014). Moreover, studies using animal models revealed that administration of a high fat diet promoted a state of low-grade systemic inflammatory signaling which was also associated with increased atherosclerosis, insulin resistance and metabolic syndrome (Shi *et al.* 2006) and (Madan 2008). Moreover, direct oral administration of LPS was shown to lead to increased expression of pro-inflammatory cytokines such as IL-12, IFN-γ, IL-1β, and TNF-α from spleen cells in mice (Yoshino, Sasatomi *et al.* 1999). Similar results were also obtained by (Youngner 1972) and (Yoshino, Yamaki *et al.* 2005).

Further evidence that gut barrier function may regulate cardiovascular risk is seen in the strong association between long-term use of non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and diclofenac, and the risk of cardiovascular diseases in large meta-analyses (Kearney *et al.* 2006). These agents impair gut barrier function in mice and in man (Sigthorsson, Morham et al. 2002).

This has led many researchers to suggest that intestinal microbes may represent another potential risk factor for the initiation of inflammation and development of coronary heart diseases (Wang *et al.* 2011). Connections have also been suggested between intestinal flora and the progression of metabolic diseases such as obesity and insulin resistance (Dumas, Barton *et al.* 2006); (Ley, Turnbaugh *et al.* 2006) and (Reigstad, Lunden *et al.* 2009). In addition, (Ley, Turnbaugh *et al.* 2006) and (Reigstad, Lunden *et al.* 2009) reported the association of intestinal commensals with the progression of obesity and insulin resistance, and adverse effects on the immune responses.

Supporting this, Dumas *et al.* (2006) revealed roles for the gut microbiota in metabolic defects such as insulin resistance and non-alcoholic fatty liver disease.

These suggestions have been considered by some to be surprising, since there are trillions of commensals in the intestine, and these have major roles in the digestion and absorption of nutrients (Backhed *et al.* 2005). Moreover, a study conducted by (Wright,

Burton *et al.* 2000) using germfree hyperlipidaemic mice found that infectious microbes are not required for atherogenesis in mice.

This raises the question of how dietary PAMPs might impact on inflammation if the commensal microbiota already present in the gut contains a far greater PAMP content. One key fact that must be borne in mind when considering this question is that the commensal microbiota is almost entirely partitioned into the large intestine, and very little is present in the small intestine. Specifically, bacterial numbers range from 10^0 to 10^5 cfu/g in the duodenum and the jejunum, and reach a maximum of 10^5-10^8 cfu/g in the terminal ileum. By comparison, there are 10^{10} to 10^{12} cfu/g in the large intestines of healthy subjects (Berg 1996). In addition, while the surface area of the small intestine reaches 300 m^2, that of the large intestine is only ~0.35 m^2 (DeSesso, Jacobson 2001). Thus, there is a very low endogenous PAMP content within the small intestine, which is the major site of absorption of fat-soluble molecules (and most other nutrients).

It has also been shown that most bacteria in the small intestine are Gram positive, and these do not secrete significant quantities of soluble PAMPs (Posserud, Stotzer *et al.* 2007). By comparison, Gram-negative bacteria, which do shed soluble PAMPs, make up ~50% of the large intestine microbiota (Berg 1996); (Posserud, Stotzer *et al.* 2007); (Erridge, Duncan *et al.* 2010) and (Alhawi, Stewart *et al.* 2009). In addition, (Erridge *et al.* 2011) reported that soluble PAMP content of fecal samples from human and mouse (to represent the PAMP content of the large intestine) can be hundreds or even thousands of times higher than normally found in the healthy small intestine. Also, the PAMP content of several commonly consumed processed foods can reach concentrations that are thousands of fold-higher than normally present in the healthy small intestine. This appears to be driven largely by the growth of commonly occurring non-pathogenic γ-proteobacterial food colonising organisms, which occurs soon after meat or vegetables are finely chopped.

By contrast, most intestinal commensals and commonly used fermentative and probiotic organisms shed very low levels of soluble PAMPs (Erridge *et al.* 2010). These evidences together suggest that tiny numbers of microbial flora reside the small intestine compared to large intestine and because of the existence of villi with a large surface area of small intestine, is probably the main site of PAMP absorption in the body.

In conclusion, the results from Chapter 3 confirmed that exposure of myeloid cells to extracts of PAMP-containing foods, leads to pro-inflammatory cytokine production, and

that this is dependent on TLR2 and/or TLR4-signalling. Also, the concentrations of LPS or BLP are, in some processed foods, thousands of times higher than normally present in the healthy small intestine.

7.2. Effects of dietary PAMPs on markers of inflammation and metabolic risk in man

The preceding results raised the question of what impact do dietary PAMPs have on inflammatory signaling pathways *in vivo*? In order to explore this, I examined the effects of oral ingestion of meals rich in TLR-stimulants on systemic inflammatory markers and cardiometabolic risk in healthy human volunteers. Two studies were conducted: an acute dietary PAMP intervention study (n=12) and a chronic dietary PAMP intervention study (n=11) (see the Materials and Methods section for schematic descriptions of the acute and chronic study protocols).

Based on the finding that foodstuffs with higher content of minced meat and ready-chopped onion tended to contain higher levels of TLRs stimulants (Chapter 3), we decided to prepare a test meal using chopped onion, which could be bought in low-PAMP (fresh) or high-PAMP (ready chopped) formats.

The results from the acute dietary PAMP intervention study showed a significant increase in total leukocyte numbers postprandially after 3 hours of delivering the high PAMP onion meal when compared to the baseline leukocyte measurement (P=0.007 *vs.* 0h timepoint), and it reduced significantly after 24 hours (P=0.006 *vs.* 24h timepoint). Similar results were observed for total granulocyte counts (Figure 4.2). As expected, the serum triglyceride was significantly augmented by the high PAMP meal and this was again reversed after 24 hours (Figure 4.6). However, serum CRP and cytokine (IL-6, IL-1β and TNF-α) mRNA expression in PBMCs were not significantly altered by the test meal. One possible explanation for the lack of increase in these markers may lie in the results of a recent study, which demonstrated that onions contain high levels of a molecule called quercetin, which potently inhibits TLR2- and TLR4-signalling *in vitro* (Shibata, Nakashima *et al.* 2014). For these reasons, we did not continue with the planned acute control (low PAMP) meal experiment.

I therefore next examined responses to a chronic dietary intake of high PAMP meals rather than a single meal, and compared it with the effect of low PAMP meals on the inflammatory and cardiometabolic markers in healthy men.

Interestingly, the 4 day high PAMP diet resulted in significant elevations of several leukocyte subset counts, which are sensitive, if non-specific, markers of inflammatory status. This is potentially of relevance to cardiovascular risk, as an earlier study by (Twig, Afek *et al.* 2012) showed that an increase of white blood cell counts of 1,000 cells/mm^3 was associated with a 17.4% increase in risk of coronary artery disease (CAD). Thus, based on our findings the leukocyte counts increase of 620 cells/mm^3 induced by the high PAMP meals (5,130 cells/mm^3) suggests that the risk of CAD may decline around 10.7% when consuming a low PAMP diet compared to a high PAMP diet, through the effect on leukocyte count. However, we did not observe a significant increase in the inflammatory CRP marker among the three visits.

Unexpectedly, weight and waist measurements also fell significantly during the low PAMP diet and this was reversed by the high PAMP diet (Figure 4.10). Consistent with this, serum leptin concentrations were significantly reduced by the low PAMP diet (P=0.006) and this was again reversed by the high PAMP intervention (P=0.055) (Figure 4.17).

Leptin is a satiety hormone that is produced by adipocytes and therefore increases with obesity (Efstratiadis, Nikolaidou *et al.* 2007). Several studies have described the role of leptin in the regulation of inflammatory responses and its role in atherosclerosis (Devaraj, Torok 2011); (Wolk, Berger *et al.* 2004) and (Reilly, Iqbal *et al.* 2004). For example, in patients with angiographically confirmed coronary atherosclerosis, leptin was found to be a novel predictor of future cardiovascular events, independent of other risk factors, such as lipid status and CRP (Wolk, Berger *et al.* 2004). A study by (Reilly, Iqbal *et al.* 2004) has also found an association between plasma leptin levels and coronary artery calcification (CAC) in type 2 diabetes mellitus patients after controlling for traditional measures of obesity and plasma CRP. These studies suggest that leptin may provide greater understanding into the pro-atherosclerotic risk associated with adiposity than other measurements such as body mass index (BMI) or waist circumference.

However, the most remarkable finding of the chronic dietary PAMP intake study was a significant reduction of LDL-C by the low PAMP diet, and its rapid reversal by the high PAMP diet (P=0.019 *vs.* visit 1 [baseline] and P=0.039 *vs.* visit 3 [high PAMP meal]) (Figure 4.16 - a).

Although no groups have explored dietary PAMP intake before our study, similar effects on LDL-C were reported by (Ambring, Friberg *et al.* 2004), where in healthy subjects,

consuming a Mediterranean diet for 4 weeks saw LDL-cholesterol levels decrease by about 22.8% compared with a typical Swedish (western) diet. Because the Mediterranean diet is predominantly based on fresh foods, and therefore low in PAMPs, their finding supports the hypothesis that dietary PAMPs could play a role in regulating the level of LDL-C in healthy men.

High levels of LDL-cholesterol in the circulation represent a major risk factor for atherosclerosis. Previous studies have shown that reduction of plasma LDL-cholesterol by diet and/or drugs is a successful primary strategy for the prevention and regression of coronary heart disease (Grundy, Cleeman *et al.* 2004) and (Genest, Frohlich *et al.* 2003).

The liver is the primary regulator of LDL concentrations in man via its production of VLDL, the precursor of plasma LDL, and its responsibility for the clearance of the majority of LDL via the LDL receptor (LDLR) (Dietschy, Turley *et al.* 1993). In addition, the liver has an essential role in the clearance of cholesterol absorbed from the small intestine, through the uptake of chylomicron remnants (Dietschy, Turley *et al.* 1993) and (Turley, Dietschy 2003).

Statins are a class of drugs which have an essential role in lowering cholesterol levels in the blood through decreasing cholesterol synthesis primarily in the liver (Dietschy, Turley *et al.* 1993). This results in the reduction of hepatocyte cholesterol content, leading eventually to the upregulation of the LDLR and enhanced clearance of LDL. In addition, in some patients, statins decrease rates of VLDL production (Huff, Burnett 1997). Nevertheless, higher doses of statins have been associated with “myopathy syndrome” or “weakness” which can include either or both of muscle pain (myalgia) and creatine phosphokinase (CK) elevation (Sinzinger, Wolfram *et al.* 2002). Side effects such as these have led to calls for alternative therapeutic targets for LDL-C lowering.

Attempts to reduce serum LDL-C through dietary means have typically involved increased intake of dietary fiber such as oats, psyllium, pectin, and guar gum (Truswell 1995) and (Glore, Van Treeck *et al.* 1994). However, a meta-analysis of such studies (Brown, Rosner *et al.* 1999) showed that various dietary fibers have little impact on total and LDL cholesterol levels within the practical range of intake.

My results that a low PAMP diet could be considered as an alternative means of decreasing levels of LDL-C in the circulation. I feel that there are two main possible explanations for the impact of dietary PAMPs on LDL-C levels.

First, it is possible that low PAMP diets result in reduced secretion of apolipoprotein B by the liver, which is essentially the only apolipoprotein of LDL and the major constituent of VLDL (Gotto, Brown *et al.* 1972) and (Kane, Hardman *et al.* 1980). Another possibility, is that high PAMP diets might lead to excess secretion of Apo-B by the liver because of the high concentrations of released inflammatory cytokines (IL-6, TNF-α, and IL-1β) by the kupffer cells. Alternatively, high PAMP diets may reduce expression or activity of LDLR in the liver, leading to higher levels of plasma LDL in the blood (Goldstein, Brown 1974). The mechanisms connecting dietary PAMP intake to dyslipidaemia will be discussed in more depth in the following section.

Surprisingly, a significant increase was also observed in thrombocyte counts (P=0.045) by the high PAMP meals when compared to the low PAMP meals (Figure 4.13).

Platelets are anucleated blood cells, which facilitate coagulation and clotting. In addition, platelets have been described to interact with bacterial pathogens and even produce considerable quantities of cytokines and chemokines (Li, Yang *et al.* 2011); (van Gils, Zwaginga *et al.* 2009) and (Totani, Evangelista 2010). It has also been reported that activated platelets interact with endothelial cells and induce the expression of cell adhesion molecules and chemokines, which in turn mediate leukocyte recruitment. These evidences provide a possible link between platelets activation, inflammatory responses and atherosclerosis (Lievens, von Hundelshausen 2011).

In conclusion, remarkable results from the chronic dietary PAMP intake study revealed that a low PAMP diet reduces leukocyte count, body weight, plasma leptin, abdominal circumference and LDL-C level, and that these effects are rapidly reversed by a high PAMP diet in healthy subjects. These findings suggest that dietary PAMPs may be considered as a new possible risk factor for coronary artery disease.

7.3. Effects of dietary PAMPs on mediators of atherosclerosis in mice

Many previous studies have shown that intravenous injection of low-doses of LPS in man results in a transient, low-grade inflammatory response involving the upregulation of circulating markers, such as IL-6, TNF-α and CRP (van Deventer *et al.* 1990). While doses above 2 ng/kg induce a pronounced fever and sickness response in volunteers (involving increased heart rate, high temperature, nausea, and malaise), doses of 0.05 - 0.1 ng/kg induce a subclinical response associated with moderately increased inflammatory markers of a magnitude consistent with those observed in dietary intervention studies (Starkie, Ostrowski *et al.* 2003) and (Krogh-Madsen *et al.* 2004).

Previous work from our laboratory showed that chronic treatment of Wild-type (C57BL/6) mice with 3 PAMPs (LPS, Pam3CSK4, and iEDAP) mix in drinking water for 10 weeks on low fat diet led to significant increases of hepatic inflammatory gene expression (PTX, IL-1β, IL-6, TNF-α, and Apo-AI) (Figure 1.23-B), with significant decrease in cholesterol efflux of Apo-B depleted serum, total cholesterol and HDL (Figure 1.24-A and B).

Because chronic administration of PAMPs to mice was very expensive and time consuming, it was not feasible to use this type of experiment to explore mechanisms of action of dietary PAMPs. Because mice orally gavaged with *E. coli* LPS did not show obvious upregulation of acute phase response (APR) markers within 24 h (Figure 1.26), I therefore sought means to increase the sensitivity of mice to orally delivered LPS.

Work from other members of the group showed previously that gut permeability to FITC-dextran and *E. coli* LPS was significantly increased by treatment of mice with the NSAID indomethacin, but not a 4 week high fat diet (Figure 1.27).

I therefore first explored the potential of NSAID priming with indomethacin to increase responsiveness of healthy wild type mice (C57BL/6), to acute gavage with *E. coli* LPS as measured by APR markers.

In these experiments, the plasma SAA response to orally delivered LPS was still only modest (P=0.0127 *vs.* control) (Figure 5.1), and expression of inflammatory marker genes in liver tissue were not significantly upregulated by orally delivered LPS (Figure 5.2 and 3). This suggests that mice are unresponsive to LPS, even when large amounts of LPS enter the

circulation. This conclusion is supported by a study by (Copeland, Warren *et al.* 2005), who measured the respective sensitivities of mice and humans to endotoxin challenge. They found that mice required at least 250x higher doses of endotoxin to reach the targeted concentration of 1,000 pg of IL-6 per ml, when compared to human volunteers. This finding was consistent with previous studies that found humans to be the most sensitive of all models after an endotoxin challenge (Martich, Boujoukos *et al.* 1993) and (Wolff 1973). This suggested that successful development of a model in which an acute response to low dose LPS (such as may occur post-prandially) could be detected would require increasing the sensitivity of mice to LPS.

To develop such a model, I examined hepatic inflammatory gene expression and serum acute phase markers (SAA) in mouse models treated by means shown previously to increase sensitivity to LPS. Mice primed with intraperitoneal injection of heat-killed *P. acnes* bacteria, or 4 weeks high cholesterol diet, showed excessively high background levels of acute phase markers that could not be elevated further by LPS.

Therefore, I next examined the mild priming treatment of 4 weeks high fat diet feeding before oral LPS gavage. I found that HFD alone does not promote a state of low-grade systemic inflammatory signaling when compared to the group fed on LFD (Figure 5.20), meaning this is a suitable baseline for oral gavage experiments.

Mice gavaged with LPS after HFD pretreatment showed highly significant increases in plasma SAA (P=0.0053 *vs.* control) (Figure 5.13), and significant responses from hepatic inflammatory gene expression such as SAA1, SAA3, Apo-A1, Lipocalin and Haptoglobin (Figure 5.14 and 15). These results therefore suggest that chronic administration of HFD leads to a clear induction of responsiveness to orally delivered LPS.

Interestingly, a significant reduction was observed in plasma Apo-A1, total and HDL-C in mice gavaged with LPS after HFD pretreatment (Figure 5.16). Remarkably, the serum cholesterol efflux capacity also fell significantly in mice orally gavaged with *E. coli* LPS (Figure 5.19).

Previous studies have shown that intravenous administration of high doses of LPS to mice or human volunteers reduces serum cholesterol efflux capacity *in vivo* (McGillicuddy, de la Llera Moya *et al.* 2009).

Endotoxemia has a broad impact on RCT, attenuating several steps including macrophage cholesterol efflux, HDL acceptor function, and hepatic to bile/fecal cholesterol

elimination. These evidences strengthen the hypothesis that impaired RCT may be an important link between the low-grade inflammation and the development and acceleration of atherosclerosis.

Subsequently, I aimed to determine whether or not the induction of inflammatory signaling in the liver was dependent on Kupffer cells. Kupffer cells make up 80-90% of the tissue macrophages present in the body, and are thought to be the major source of the inflammatory markers in the liver (Chensue, Terebuh *et al.* 1991). They are involved in the liver's response to infection, toxins, microbial debris, bacterial endotoxins and other stresses (Bilzer, Roggel *et al.* 2006). To test their involvement, clodronate liposomes were used. These are a selective depleting agent of macrophages from tissues *in vivo* that can be used to explore whether these cells have a functional role in biological processes (Rooijen, Sanders 1994).

Interestingly, macrophage depletion by clodronate liposomes completely blocked the inflammatory responses (SAA and APR markers) to oral LPS challenge (Figure 5.22 and 23).

Immunofluorescence of hepatic F4/80^{+} confirmed a significant (>99%) depletion in the number of macrophages in HFD-fed mice injected with clodronate liposomes when compared to the liver samples of HFD-fed mice (Figure 5.21). Without liposome treatment, oral LPS induced markers of macrophage activation in liver, but not other macrophage beds, such as adipose tissue. Together, these findings suggest that hepatic macrophages are necessary for the HFD-induced sensitization to LPS.

In further investigations, the expression of TLR2/4 proteins in hepatic tissue were found to be greatly elevated by all the tested sensitising treatments compared to unprimed animals fed normal chow (Figure 5.34 and 35). Thus, HFD increases not only the macrophage content of the liver, but also expression of TLR2 and TLR4.

In conclusion, these murine studies suggest that dietary PAMPs can induce overt inflammation and impair reverse cholesterol transport in mice *in vivo* if the mice are primed for responsiveness to LPS. Also, hepatic macrophages seem to be the key factor for the HFD-induced sensitization to LPS.

7.4. Potential mechanisms connecting dietary PAMPs to systemic inflammation and lipid metabolism

Because hepatocytes, rather than Kupffer cells, are the major producers of circulating cholesterol acceptors *in vivo*, we next decided to investigate potential mechanisms of the hepatocyte-macrophage communication axis.

I first explored the potential effects of PAMPs (Pam3CSK4, LPS and iEDAP) and cytokines (IL-1β, IL-6 and TNF-α) on HepG2 hepatocyte expression of acute phase response genes and Apo-AI. Only IL-1β, but not the tested PAMPs or other cytokines, caused a significant increase in the gene expression of inflammatory SAA1 mRNA (P=0.0001 *vs.* control), and reduced Apo-A1 mRNA (Figure 6.1). Moreover, the cholesterol efflux capacity of HepG2 cell conditioned medium was reduced by IL-1β treatment but not the other stimulants (Figure 6.3).

This suggested that hepatocytes themselves are insensitive to PAMPs, but that IL-1β released by another cell type may play a key role in the regulation of RCT by dietary PAMPs. This was tested by measuring primary wild-type mouse hepatocyte expression of acute phase response genes. Interestingly, highly significant increase of SAA1 was observed by IL-1β compared to the control (P=0.0058 *vs.* control), but no changes has observed by IL-6 and/or TNF-α. Furthermore, Apo-A1 gene expression was reduced significantly by IL-1β and TNF-α, but not by IL-6 (Figure 6.7).

Supporting this, other workers have shown that inflammatory gene expression in the liver is significantly lower in mice with Kupffer cell deficiency of IL-1 compared to WT mice (Olteanu, Kandel-Kfir *et al.* 2014). Moreover, studies have reported that Kupffer cells are considered as a main source of IL-1 and depletion of Kupffer cells reduced IL-1 expression and liver steatosis under a high fat diet (Dinarello 2009); (Miura, Kodama *et al.* 2010) and (Rivera, Adegboyega *et al.* 2007).

This raises the possibility that IL-1β released from Kupffer cells activated by PAMPs might trigger activation of hepatocytes indirectly. Supportive of this, Apo-A1 gene expression decreased significantly (P=0.0011 *vs.* control) in primary hepatocytes cultured with supernatant of LPS-treated, but not resting, primary Kupffer cells (Figure 6.8).

In addition, (Rivera *et al.* 2007) reported that Kupffer cells are responsible for clearing endotoxin and are activated via endotoxin interaction with Toll like receptor 4 in animal models and humans.

As we found previously that TLR2 and TLR4 stimulants present in food are critical mediators of the capacity of food extracts to induce inflammatory signaling, I measured the expression of TLR2 and TLR4 on primary murine Kupffer cells and hepatocytes using flow cytometry. There was obvious expression of TLR2 and TLR4 on Kupffer cells, whether HFD-primed or not (Figure 6.11). However, murine hepatocytes did not show expression of TLR2 or TLR4 (Figure 6.12). This result suggests that Kupffer cells, rather than hepatocytes, are the likely sensors of dietary TLR2/4 stimulants *in vivo*.

Kupffer cells, as liver-resident macrophages, have a major role in the secretion of a broad panel of inflammatory cytokines such as TNF-α, IL-6 and IL-1 (Tilg, Moschen 2010). Interleukin-1 induces the expression of a variety of pro-inflammatory genes upon binding to IL-1 receptor type 1 (IL-1R1) (Dinarello 2011) and (Kamari, Werman-Venkert *et al.* 2007).

TLR-4 interaction with endotoxin results in the release of pro-inflammatory mediators that induce hepatic injury and fibrosis (Decker 1990). In addition, cytokines have profound effects on lipid metabolism (Hardardottir, Grunfeld *et al.* 1994). Furthermore, activation of Kupffer cells through TLR-4 promotes non-alcoholic steatohepatitis (NASH) via the inflammatory cytokines TNF-α and IL-1 (Rivera, Adegboyega *et al.* 2007).

The key role of IL-1 in mediating hepatic inflammatory responses is supported by an earlier study showing a significant reduction in cytokine secretion (IL-1, IL-6 and TNF-α) from mouse liver samples treated with LPS when cultured in the presence of monoclonal antibody to IL-1 (Luster, Germolec *et al.* 1994). Furthermore, a study conducted by (Miura, Kodama *et al.* 2010) has revealed that IL-1R$^{-/-}$ mice had reduced steatohepatitis and fibrosis, compared with WT mice. Taken together, our findings and those of other workers, suggest that IL-1β may play a key role in the progression of inflammatory responses in the liver.

To test this, I investigated whether dietary PAMPs are be able to trigger inflammatory responses in IL-1R1 genetically depleted (KO) mice primed with HFD.

The most interesting finding from these experiments was the complete loss of responsiveness to orally delivered LPS compared to WT mice.

In HFD-primed IL-1R1$^{-/-}$ mice, oral LPS no longer induced any changes to hepatic Apolipoprotein-A1 mRNA (Figure 6.14), hepatic inflammatory gene expression (Figure 6.15), serum total cholesterol level, HDL-C and LDL-C (Figure 6.16) or serum cholesterol efflux capacity (Figure 6.17). Furthermore, liver slices from HFD-primed WT mice increased SAA expression, but those from IL-1R1 KO mice did not, in response to supernatant of LPS-treated J774 cells (Figure 6.18). Likewise, while Apo-AI mRNA is reduced in response to LPS-stimulated KC supernatant in WT hepatocytes, this was not observed in IL-1R1$^{-/-}$ hepatocytes (Figure 6.19). Together, these experiments provide strong evidence that IL-1 plays a key role in the connection between dietary PAMPs and impaired RCT.

The notion that IL-1β may play a key role in the regulation of cardiovascular risk is well supported by studies in IL-1R1 KO mice bred on the Apo-E$^{-/-}$ backgrounds, which show reduced plaque formation in IL-1 signaling deficiency (Matthew *et al.* 2012). The CANTOS study is also currently ongoing to test whether therapeutic inhibition of IL-1 may reduce cardiovascular risk in man (Ridker *et al.* 2011). Thus, my work on dietary PAMPs may indicate one potential trigger for IL-1 induction that may mediate this emerging pathway.

7.5. Intestinal barrier function - the next frontier in atherosclerosis research?

My final experiments aimed to explore a new potential direction for future projects. To this end, I tested whether any common food additives found in processed foods might impact on gut barrier function via effects on epithelial cell health. Preliminary experiments were conducted using an easily cultured model epithelial cell-line (HeLa) to test the principle, and then the experiments were repeated using the CaCo2 enterocyte cell-line. In all, seventeen food additives and surfactants commonly found in processed foods were tested for their effects on cell viability.

Unexpectedly, most of the food additives at concentration of (0.4% wt/vol) were found to have significant effects on HeLa cell viability (Figure 6.20, A-Q). Only four of the food additives including sodium carbonate, sodium acetate, agar and sodium sulfate had no effect on human epithelial cell viability. Human epithelial colorectal Caco-2 cells were also highly sensitive to three food additives: sodium decanoate, sodium sulfite, and sodium bisulfite at a concentration of 0.4% (wt/vol), when using the MTT assay measure of cell viability (Figure 6.21, A-Q).

These findings are potentially of much interest in terms of the potential for processed foods to affect gut barrier function. For example, the sodium sulphite content of wine averages about ~0.01% wt/vol, and it can reach higher levels in some processed foods. It is possible that this dose, which is sub-lethal *in vitro*, could nevertheless affect barrier integrity *in vivo*. Interestingly, while sodium decanoate (also called sodium caprate), is a commonly used stabiliser in processed foods, it is already used pharmacologically to increase the permeability of the small intestine to large therapeutic molecules, which are otherwise not absorbed (Maher *et al.* 2009). This supports the notion that it may also promote PAMP translocation. Furthermore, my finding of a toxic effect of sodium caprate on enterocytes suggests a possible mode of action for its ability to promote intestinal absorption of molecules. Of course, further work will be required to test whether these food additives modify gut barrier function, or rates of PAMP translocation, *in vivo* using murine models and human volunteer studies.

Conclusion

In conclusion, this project has made a number of novel discoveries for the field. First, chronic intake of dietary PAMPs has a profound impact on LDL-C levels and leukocyte counts in man, which combined amounts to a ~40% increase in predicted cardiovascular risk based on these markers. Studies in mice showed that orally delivered LPS can induce the APR and impair cholesterol efflux when they are primed for LPS responsiveness with HFD. This was found to be due to Kupffer-cell mediated detection of PAMPs, which were not detected directly by hepatocytes. Finally, the communication between Kupffer cells and hepatocytes in this context was found to be almost entirely dependent on IL-1β.

Taken together, my findings suggest that dietary PAMPs may be considered as a new possible risk factor for coronary artery disease. This raises the possibility of new therapeutic approaches, based on novel dietary interventions or new drug targets, to reduce cardiovascular risk. In my view, the stated discoveries in this thesis should be of great benefit for the future investigation for workers in the fields of nutrition, host-microbiota interaction and cardiometabolic research.

Future work

Based on the results obtained from this project, it would be interesting to pursue a number of further studies,

- It will be valuable to complete the chronic dietary intervention study on obese subjects and / or diabetes patients and in patients with cardiovascular diseases, similar to the chronic study (Chapter 4), since these groups are reported to have reduced gut barrier function.
- It would also be interesting to perform further *in vivo* studies using animal models and human volunteers to test the adverse effect of food additives and surfactants on gut epithelial cells or enterocytes, and the effects of dietary PAMPs on the induction of inflammatory responses in the presence of these food additives.
- Finally, it will be very useful to conduct further mouse model knockout (Apo-$E^{-/-}$ IL-$1R1^{+/+}$ *vs.* Apo-$E^{-/-}$ IL-$1R1^{-/-}$) or ($LDLR^{-/-}$ IL-$1R1^{+/+}$ *vs.* $LDLR^{-/-}$ IL-$1R1^{-/-}$) studies to reveal the roles of interactions between dietary PAMPs and IL-1β in the progression of atherosclerosis in mice fed on HFD.

Appendices

<u>Appendix 1</u> Healthy volunteer eligibility questionnaire - Acute study

<u>Medical history</u>

Please Tick box to confirm

I confirm that I **do not** have evidence of present or previous:

1. Hypertension ☐
2. Hyperlipidaemia ☐
3. Diabetes Mellitus (Type I or Type II) ☐
4. Asthma ☐
5. Bleeding problems ☐
6. Chronic or acute heart, kidney or liver disease ☐

<u>Drug history</u>

I confirm that:

1. **I have** taken ibuprofen before and suffered no adverse reaction to this drug ☐
2. **I do not** currently take any (non)prescribed drugs or complimentary remedies ☐
3. **I do not** have an allergy to any drug ☐

<u>Self-reporting</u>

1. I confirm that I **have not** suffered symptoms suggestive of infection or inflammation in the past 2 weeks (fever/fatigue/night sweats/cough/rash) ☐
2. I confirm that I **have not** taken any anti-inflammatory drugs in the past 3 days ☐
3. I confirm that I **have no** family history of inflammatory bowel diseases (such as Crohn's disease or ulcerative colitis) ☐

Subject Name: **Subject Signature:** **Date:**

Appendix 2 Consent form - Acute study

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CONSENT FORM

Title of project: The effects of dietary toll-like receptor stimulants on leukocyte activation in man

Names of researchers: Dr Clett Erridge, Mr Tola Faraj

Please initial each box

1. I confirm that I have read and understand the information sheet for the above study (dated 22/05/14) and have had the opportunity to ask questions.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason.	☐
3. I agree to take part in the above study.	☐

Name of subject	Date	Signature
Name of person taking consent	Date	Signature

<u>Appendix 3</u> Provided lunch and evening meals - High PAMP diet

		Preferred options
Monday	Breakfast	Any cereal, Fresh milk (whole, semi or skimmed), Tea, coffee, Toast, Margarine, Jam, Fruit and allow processed foods, butter or cheese
	Lunch	**3 ASDA pork sausage rolls** **2 TESCO cheese and onion rolls**
	Dinner	**1 TESCO lasagne ready meal**
	Snacks	4 slices of bread with any spread, 1 bag of crisps
Tuesday	Breakfast	Any cereal, Fresh milk (whole, semi or skimmed), Tea, coffee, Toast, Margarine, Jam, Fruit and allow processed foods, butter or cheese
	Lunch	**2 ASDA cornish pasties**
	Dinner	**2 TESCO fisherman's pie ready meals**
	Snacks	3 slices of bread with any spread, 1 bag of crisps
Wednesday	Breakfast	Any cereal, Fresh milk (whole, semi or skimmed), Tea, coffee, Toast, Margarine, Jam, Fruit and allow processed foods, butter or cheese
	Lunch	**1 ASDA cheese and onion pasty** **2 ASDA pork sausage rolls**
	Dinner	**2 ASDA bolognase and pasta bake ready meals**
	Snacks	2 slices of bread with any spread, 1 bag of crisps
Thursday	Breakfast	Any cereal, Fresh milk (whole, semi or skimmed), Tea, coffee, Toast, Margarine, Jam, Fruit and allow processed foods, butter or cheese
	Lunch	**1 TESCO cheese and onion pasty** **1 ASDA cornish pasty**
	Dinner	**2 TESCO spaghetti bolognase ready meals**
	Snacks	1 slice of bread with any spread, 1 bag of crisps

<u>Appendix 4</u> Healthy volunteer eligibility questionnaire - Chronic study

<u>Medical history</u>

I confirm that I **do not** have evidence of present or previous:	**Please Tick box to confirm**
1. Hypertension	☐
2. Hyperlipidaemia	☐
3. Diabetes Mellitus (Type I or Type II)	☐
4. Chronic or acute liver disease	☐
5. Chronic or acute kidney or heart disease	☐
6. Recent vaccination, such as influenza vaccine, within the last 2 weeks	☐

<u>Drug history</u>

I confirm that I **do not**:

1. Regularly take any prescribed or over the counter drugs (excluding contraception) ☐
2. Take any complimentary or traditional remedies ☐

<u>Self-reporting</u>

1. I confirm that I **have not** suffered symptoms suggestive of infection or inflammation in the past 2 weeks (fever/fatigue/night sweats/cough/rash) ☐
2. I confirm that I **have not** taken any anti-inflammatory drugs in the past 3 days ☐

Subject Name: **Subject Signature:** **Date:**

Appendix 5 Consent form - Chronic study

UNIVERSITY OF LEICESTER

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CONSENT FORM

Title of project: Effects of chronic intake of processed foods on circulating inflammatory markers in healthy men

Names of researchers: Dr Clett Erridge, Mr Tola Faraj

Please initial each box

1. I confirm that I have read and understand the information sheet for the above study (dated 02/11/14) and have had the opportunity to ask questions.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason.	☐
3. I agree to take part in the above study.	☐

Name of subject	Date	Signature
Name of person taking consent	Date	Signature

Bibliography

Ait-Oufella, H., Taleb, S., Mallat, Z. & Tedgui, A. 2009, "Cytokine network and T cell immunity in atherosclerosis", *Seminars in Immunopathology,* vol. 31, no. 1, pp. 23-33.

Akira, S., Uematsu, S. & Takeuchi, O. 2006, "Pathogen recognition and innate immunity", *Cell,* vol. 124, no. 4, pp. 783-801.

Alhawi, M., Stewart, J., Erridge, C., Patrick, S. & Poxton, I.R. 2009, "Bacteroides fragilis signals through Toll-like receptor (TLR) 2 and not through TLR4", *Journal of medical microbiology,* vol. 58, no. Pt 8, pp. 1015-1022.

Al-Waiz, M., Mikov, M., Mitchell, S.C. & Smith, R.L. 1992, "The exogenous origin of trimethylamine in the mouse", *Metabolism: clinical and experimental,* vol. 41, no. 2, pp. 135-136.

Ambring, A., Friberg, P., Axelsen, M., Laffrenzen, M., Taskinen, M., Basu, S., Johansson, M., Medicinska och farmaceutiska vetenskapsområdet, Medicinska fakulteten, Uppsala universitet & Institutionen för folkhälso- och vårdvetenskap 2004, "Effects of a Mediterranean-inspired diet on blood lipids, vascular function and oxidative stress in healthy subjects", *Clinical science (London, England : 1979),* vol. 106, no. 5, pp. 519-525.

Amento, E.P., Ehsani, N., Palmer, H. & Libby, P. 1991, "Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells", *Arteriosclerosis and Thrombosis : A Journal of Vascular Biology / American Heart Association,* vol. 11, no. 5, pp. 1223.

An, G., Wang, H., Tang, R., Yago, T., McDaniel, J.M., McGee, S., Huo, Y. & Xia, L. 2008, "P-selectin glycoprotein ligand-1 is highly expressed on Ly-6Chi monocytes and a major determinant for Ly-6Chi monocyte recruitment to sites of atherosclerosis in mice", *Circulation,* vol. 117, no. 25, pp. 3227-3237.

Andersson, J., Libby, P. & Hansson, G.K. 2010, "Adaptive immunity and atherosclerosis", *Clinical immunology (Orlando, Fla.),* vol. 134, no. 1, pp. 33-46.

Anna C Calkin & Peter Tontonoz 2012, "Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR", *Nature Reviews.Molecular Cell Biology,* vol. 13, no. 4, pp. 213.

Apostolakis, S., Vogiatzi, K., Amanatidou, V. & Spandidos, D.A. 2009, "Interleukin 8 and cardiovascular disease", *Cardiovascular research,* vol. 84, no. 3, pp. 353-360.

Assmann, G., Carmena, R., Cullen, P., Fruchart, J., Jossa, F., Lewis, B., Mancini, M. & Paoletti, R. 1999, "Coronary Heart Disease: Reducing The Risk: A Worldwide View", *Circulation,* vol. 100, no. 18, pp. 1930-1938.

Astrand, O., Carlsson, M., Nilsson, I., Lindström, T., Borga, M., Nystrom, F.H., Institutionen för medicinsk teknik, Centrum för medicinsk bildvetenskap och visualisering,CMIV, Fast Food Study Group, for the Fast Food Study Group, Institutionen för medicin och hälsa, Tekniska högskolan, Medicinsk informatik, Linköpings universitet & Hälsouniversitetet 2010, "Weight gain by hyperalimentation elevates C-reactive protein levels but does not affect circulating levels of adiponectin or resistin in healthy subjects", *European journal of endocrinology / European Federation of Endocrine Societies,* vol. 163, no. 6, pp. 879-885.

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. & Gordon, J.I. 2005, "Host-bacterial mutualism in the human intestine", *Science (New York, N.Y.),* vol. 307, no. 5717, pp. 1915-1920.

Barton, B.E. 1996, "The biological effects of interleukin 6", *Medicinal research reviews,* vol. 16, no. 1, pp. 87-109.

Bausinger, H., Lipsker, D., Ziylan, U., Manie, S., Briand, J.P., Cazenave, J.P., Muller, S., Haeuw, J.F., Ravanat, C., de la Salle, H. & Hanau, D. 2002, "Endotoxin-free heat-shock protein 70 fails to induce APC activation", *European journal of immunology,* vol. 32, no. 12, pp. 3708-3713.

Berg, R.D. 1996, "The indigenous gastrointestinal microflora", *Trends in microbiology,* vol. 4, no. 11, pp. 430-435.

Beutler, B. & Cerami, A. 1989, "The biology of cachectin/TNF--a primary mediator of the host response", *Annual Review of Immunology,* vol. 7, no. 1, pp. 625-655.

Beutler, B. 2002, "TLR4 as the mammalian endotoxin sensor", *Current topics in microbiology and immunology,* vol. 270, pp. 109-120.

Bianchi, M.E. 2007, "DAMPs, PAMPs and alarmins: all we need to know about danger", *Journal of leukocyte biology,* vol. 81, no. 1, pp. 1-5.

Bidulescu, A., Chambless, L.E., Siega-Riz, A.M., Zeisel, S.H. & Heiss, G. 2007, "Usual choline and betaine dietary intake and incident coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) study", *BMC cardiovascular disorders,* vol. 7, pp. 20.

Bilzer, M., Roggel, F. & Gerbes, A.L. 2006, "Role of Kupffer cells in host defense and liver disease", *Liver international : official journal of the International Association for the Study of the Liver,* vol. 26, no. 10, pp. 1175-1186.

Boisvert, W.A., Santiago, R., Curtiss, L.K. & Terkeltaub, R.A. 1998, "A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice", *The Journal of clinical investigation,* vol. 101, no. 2, pp. 353-363.

Boisvert, W.A., Santiago, R., Curtiss, L.K. & Terkeltaub, R.A. 1998, "A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice", *The Journal of clinical investigation,* vol. 101, no. 2, pp. 353-363.

Bomfim, G.F., Dos Santos, R.A., Oliveira, M.A., Giachini, F.R., Akamine, E.H., Tostes, R.C., Fortes, Z.B., Webb, R.C. & Carvalho, M.H.C. 2012, "Toll-like receptor 4 contributes to blood pressure regulation and vascular contraction in spontaneously hypertensive rats", *Clinical science (London, England : 1979),* vol. 122, no. 11, pp. 535.

Boni-Schnetzler, M., Thorne, J., Parnaud, G., Marselli, L., Ehses, J.A., Kerr-Conte, J., Pattou, F., Halban, P.A., Weir, G.C. & Donath, M.Y. 2008, "Increased interleukin (IL)-1beta messenger ribonucleic acid expression in beta -cells of individuals with type 2 diabetes and regulation of IL-1beta in human islets by glucose and autostimulation", *The Journal of clinical endocrinology and metabolism,* vol. 93, no. 10, pp. 4065-4074.

Boulay, J., O'Shea, J.J. & Paul, W.E. 2003, "Molecular phylogeny within type I cytokines and their cognate receptors", *Immunity,* vol. 19, no. 2, pp. 159-163.

British Heart Foundation, Cardiovascular Disease Statistics 2014, Deaths by cause in men, United Kingdom. *Bhf_cvd-statistics-2014_web.pdf*, pp. 16.

Brown, L., Rosner, B., Willett, W.W. & Sacks, F.M. 1999, "Cholesterol-lowering effects of dietary fiber: a meta-analysis", *The American Journal of Clinical Nutrition,* vol. 69, no. 1, pp. 30-42.

Brun, P., Castagliuolo, I., Di Leo, V., Buda, A., Pinzani, M., Palu, G. & Martines, D. 2007, "Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis", *American journal of physiology.Gastrointestinal and liver physiology,* vol. 292, no. 2, pp. G518-25.

Caligiuri, G., Nicoletti, A., Poirier, B. & Hansson, G.K. 2002, "Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice", *The Journal of clinical investigation,* vol. 109, no. 6, pp. 745-753.

Cani, P.D., Amar, J., Iglesias, M.A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A.M., Fava, F., Tuohy, K.M., Chabo, C., Waget, A., Delmee, E., Cousin, B., Sulpice, T., Chamontin, B., Ferrieres, J., Tanti, J.F., Gibson, G.R., Casteilla, L., Delzenne, N.M., Alessi, M.C. & Burcelin, R. 2007, "Metabolic endotoxemia initiates obesity and insulin resistance", *Diabetes,* vol. 56, no. 7, pp. 1761-1772.

Caroff, M. & Karibian, D. 2003, "Structure of bacterial lipopolysaccharides", *Carbohydrate research,* vol. 338, no. 23, pp. 2431-2447.

Casas, J.P., Shah, T., Hingorani, A.D., Danesh, J. & Pepys, M.B. 2008, "C-reactive protein and coronary heart disease: a critical review", *Journal of internal medicine,* vol. 264, no. 4, pp. 295-314.

Cashman, J.R., Camp, K., Fakharzadeh, S.S., Fennessey, P.V., Hines, R.N., Mamer, O.A., Mitchell, S.C., Nguyen, G.P., Schlenk, D., Smith, R.L., Tjoa, S.S., Williams, D.E. & Yannicelli, S. 2003, "Biochemical and clinical aspects of the human flavin-containing monooxygenase form 3 (FMO3) related to trimethylaminuria", *Current Drug Metabolism,* vol. 4, no. 2, pp. 151-170.

Castelli, W.P. 1984, "Epidemiology of coronary heart disease: the Framingham study", *The American Journal of Medicine,* vol. 76, no. 2A, pp. 4-12.

Charo, I.F. & Ransohoff, R.M. 2006, "The many roles of chemokines and chemokine receptors in inflammation", *The New England journal of medicine,* vol. 354, no. 6, pp. 610-621.

Chensue, S., Terebuh, P., Remick, D., Scales, W. & Kunkel, S. 1991, "In vivo biologic and immunohistochemical analysis of interleukin-1 alpha, beta and tumor necrosis factor during experimental endotoxemia. Kinetics, Kupffer cell expression, and glucocorticoid effects", *American Journal of Pathology,* vol. 138, no. 2, pp. 395-402.

Chinetti-Gbaguidi G, Colin S, Staels B 2015. Macrophage subsets in atherosclerosis. *Nat Rev Cardiol* 12:10-7.

Chobanian AV & Dzau VJ. 1996, "Renin angiotensin system and atherosclerotic vascular disease", *Philadelphia: Lippincott-Raven,* vol. 1, pp. 237-42.

Chrysohoou, C., Panagiotakos, D.B., Pitsavos, C., Das, U.N. & Stefanadis, C. 2004, "Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: The ATTICA Study", *Journal of the American College of Cardiology,* vol. 44, no. 1, pp. 152.

Clinton, S., Underwood, R., Hayes, L., Sherman, M., Kufe, D. & Libby, P. 1992, "Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis", *American Journal of Pathology,* vol. 140, no. 2, pp. 301-316.

Cole, J.E., Mitra, A.T. & Monaco, C. 2010, *Treating atherosclerosis: the potential of Toll-like receptors as therapeutic targets*, Expert Reviews Ltd, London, UK.

Copeland, S., Warren, H.S., Lowry, S.F., Calvano, S.E., Remick, D. & Inflammation and the Host Response to Injury Investigators 2005, "Acute inflammatory response to endotoxin in mice and humans", *Clinical and diagnostic laboratory immunology,* vol. 12, no. 1, pp. 60-67.

Cybulsky, M.I. & Gimbrone, J., M A. 1991, "Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis", *Science (New York, N.Y.),* vol. 251, no. 4995, pp. 788-791.

Dalla Venezia, N., Minka, S., Bruneteau, M., Mayer, H. & Michel, G. 1985, "Lipopolysaccharides from Yersinia pestis. Studies on lipid A of lipopolysaccharides I and II", *European journal of biochemistry,* vol. 151, no. 2, pp. 399-404.

Danne, O., Lueders, C., Storm, C., Frei, U. & Mockel, M. 2007, "Whole blood choline and plasma choline in acute coronary syndromes: prognostic and pathophysiological implications", *Clinica chimica acta; international journal of clinical chemistry,* vol. 383, no. 1-2, pp. 103-109.

Dasu, M.R., Devaraj, S. & Jialal, I. 2007, *High glucose induces IL-1beta expression in human monocytes: mechanistic insights*, United States.

Decker, K. 1990, "Biologically active products of stimulated liver macrophages (Kupffer cells)", *European journal of biochemistry,* vol. 192, no. 2, pp. 245-261.

Dentener, M.A., Bazil, V., Von Asmuth, E.J., Ceska, M. & Buurman, W.A. 1993, "Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-alpha, IL-6 and IL-8 release by human monocytes and alveolar macrophages", *Journal of immunology (Baltimore, Md.: 1950),* vol. 150, no. 7, pp. 2885-2891.

DeSesso, J.M. & Jacobson, C.F. 2001, "Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats", *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association,* vol. 39, no. 3, pp. 209-228.

Devaraj, S. & Torok, N. 2011, "Leptin: the missing link between obesity and heart disease?", *Atherosclerosis,* vol. 217, no. 2, pp. 322-323.

Devlin, C.M., Kuriakose, G., Hirsch, E. & Tabas, I. 2002, "Genetic alterations of IL-1 receptor antagonist in mice affect plasma cholesterol level and foam cell lesion size", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 99, no. 9, pp. 6280-6285.

Diaz, M.N., Frei, B., Vita, J.A. & Keaney, J., J F. 1997, "Antioxidants and atherosclerotic heart disease", *The New England journal of medicine,* vol. 337, no. 6, pp. 408-416.

Dietschy, J.M., Turley, S.D. & Spady, D.K. 1993, "Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans", *Journal of lipid research,* vol. 34, no. 10, pp. 1637-1659.

Dinarello, C.A. 2009, "Immunological and inflammatory functions of the interleukin-1 family", *Annual Review of Immunology,* vol. 27, pp. 519-550.

Dinarello, C.A. 2011, "Interleukin-1 in the pathogenesis and treatment of inflammatory diseases", *Blood,* vol. 117, no. 14, pp. 3720-3732.

Dinarello, C.A., Ikejima, T., Warner, S.J., Orencole, S.F., Lonnemann, G., Cannon, J.G. & Libby, P. 1987, "Interleukin 1 induces interleukin 1. I. Induction of circulating interleukin 1 in rabbits in vivo and in human mononuclear cells in vitro", *Journal of immunology (Baltimore, Md.: 1950),* vol. 139, no. 6, pp. 1902-1910.

Dollery, C.M. & Libby, P. 2006, "Atherosclerosis and proteinase activation", *Cardiovascular research,* vol. 69, no. 3, pp. 625-635.

Dong, C., Yoon, W. & Goldschmidt-Clermont, P.J. 2002, "DNA methylation and atherosclerosis", *The Journal of nutrition,* vol. 132, no. 8 Suppl, pp. 2406S-2409S.

Downs, J.R., Clearfield, M., Weis, S., Whitney, E., Shapiro, D.R., Beere, P.A., Langendorfer, A., Stein, E.A., Kruyer, W. & Gotto, A.M.,Jr 1998, "Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study", *Jama,* vol. 279, no. 20, pp. 1615-1622.

Dumas, M.E., Barton, R.H., Toye, A., Cloarec, O., Blancher, C., Rothwell, A., Fearnside, J., Tatoud, R., Blanc, V., Lindon, J.C., Mitchell, S.C., Holmes, E., McCarthy, M.I., Scott, J., Gauguier, D. & Nicholson, J.K. 2006, "Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 103, no. 33, pp. 12511-12516.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E. & Relman, D.A. 2005, "Diversity of the human intestinal microbial flora", *Science (New York, N.Y.),* vol. 308, no. 5728, pp. 1635-1638.

Efstratiadis, G., Nikolaidou, C. & Vergoulas, G. 2007, "Leptin as a cardiovascular risk factor", *Hippokratia,* vol. 11, no. 4, pp. 163-170.

Elhage, R., Maret, A., Pieraggi, M.T., Thiers, J.C., Arnal, J.F. & Bayard, F. 1998, "Differential effects of interleukin-1 receptor antagonist and tumor necrosis factor binding protein on fatty-streak formation in apolipoprotein E-deficient mice", *Circulation,* vol. 97, no. 3, pp. 242-244.

Elin, R.J. & Wolff, S.M. 1973, "Nonspecificity of the Limulus Amebocyte Lysate Test: Positive Reactions with Polynucleotides and Proteins", *The Journal of infectious diseases,* vol. 128, no. 3, pp. 349-352.

Epstein, S.E., Speir, E., Zhou, Y.F., Guetta, E., Leon, M. & Finkel, T. 1996, "The role of infection in restenosis and atherosclerosis: focus on cytomegalovirus", *Lancet (London, England),* vol. 348 Suppl 1, pp. s13-7.

Erridge C 2010, " The capacity of foodstuffs to induce innate immune activation of human monocytes in vitro is dependent on food content of stimulants of Toll-like receptors 2 and 4 ", *British Journal of Nutrition,* vol. 105, pp. 15-23.

Erridge C, Attina T, Spickett CM & Webb DJ 2007c, "A high-fat meal induces low-grade endotoxaemia: evidence of a novel mechanism of post-prandial inflammation.", *Am J Clin Nutr,* vol. 86, pp. 1286-1292.

Erridge C, Simon Kennedy, Corinne M. Spickett, David J. Webb, 2008, *Oxidized Phospholipid Inhibition of Toll-like Receptor (TLR) Signaling Is Restricted to TLR2 and TLR4*, American Society for Biochemistry and Molecular Biology.

Erridge Clett 2009, "The Roles of Toll-Like Receptors in Atherosclerosis", *J Innate Immun,* vol. 1, pp. 340-349.

Erridge, C. & Samani, N.J. 2009, "Saturated fatty acids do not directly stimulate Toll-like receptor signaling", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 29, no. 11, pp. 1944.

Erridge, C. 2008, *The roles of pathogen-associated molecular patterns in atherosclerosis*, Elsevier Inc, United States.

Erridge, C. 2010, "Endogenous ligands of TLR2 and TLR4: agonists or assistants?", *Journal of leukocyte biology,* vol. 87, no. 6, pp. 989-999.

Erridge, C. 2011, "Accumulation of stimulants of Toll-like receptor (TLR)-2 and TLR4 in meat products stored at 5 °C", *Journal of Food Science,* vol. 76, no. 2, pp. H72-H79.

Erridge, C. 2011, "Diet, commensals and the intestine as sources of pathogen-associated molecular patterns in atherosclerosis, type 2 diabetes and non-alcoholic fatty liver disease", *Atherosclerosis,* vol. 216, no. 1, pp. 1-6.

Erridge, C., Bennett-Guerrero, E. & Poxton, I.R. 2002, "Structure and function of lipopolysaccharides", *Microbes and Infection,* vol. 4, no. 8, pp. 837-851.

Erridge, C., Burdess, A., Jackson, A.J., Murray, C., Riggio, M., Lappin, D., Milligan, S., Spickett, C.M. & Webb, D.J. 2008, "Vascular cell responsiveness to Toll-like receptor ligands in carotid atheroma".

Erridge, C., Duncan, S.H., Bereswill, S. & Heimesaat, M.M. 2010, "The induction of colitis and ileitis in mice is associated with marked increases in intestinal concentrations of stimulants of TLRs 2, 4, and 5", *PloS one,* vol. 5, no. 2, pp. e9125.

Erridge, C., Spickett, C.M. & Webb, D.J. 2007, "Non-enterobacterial endotoxins stimulate human coronary artery but not venous endothelial cell activation via Toll-like receptor 2", *Cardiovascular research,* vol. 73, no. 1, pp. 181-189.

Faggioni, R., Fantuzzi, G., Fuller, J., Dinarello, C.A., Feingold, K.R. & Grunfeld, C. 1998, "IL-1 beta mediates leptin induction during inflammation", *The American Journal of Physiology,* vol. 274, no. 1 Pt 2, pp. R204-8.

Falck-Hansen, M., Kassiteridi, C. & Monaco, C. 2013, "Toll-like receptors in atherosclerosis", *International journal of molecular sciences,* vol. 14, no. 7, pp. 14008-14023.

Falcone, D.J., McCaffrey, T.A. & Vergilio, J.A. 1991, "Stimulation of macrophage urokinase expression by polyanions is protein kinase C-dependent and requires protein and RNA synthesis", *The Journal of biological chemistry,* vol. 266, no. 33, pp. 22726.

Falk E, Shah PK & Fuster V. 1996, "Pathogenesis of plaque disruption", *Philadelphia: Lippincott-Raven,* vol. 2, pp. 492-510.

Fantuzzi, G., Sacco, S., Ghezzi, P. & Dinarello, C.A. 1997, "Physiological and cytokine responses in IL-1 beta-deficient mice after zymosan-induced inflammation", *The American Journal of Physiology,* vol. 273, no. 1 Pt 2, pp. R400-6.

Feig, J.E., Parathath, S., Rong, J.X., Mick, S.L., Vengrenyuk, Y., Grauer, L., Young, S.G. & Fisher, E.A. 2011, "Reversal of hyperlipidemia with a genetic switch favorably affects the content and inflammatory state of macrophages in atherosclerotic plaques", *Circulation,* vol. 123, no. 9, pp. 989-998.

Feng B. et al 2003, "The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages", *Nature Cell Biol,* vol. 5, pp. 781-792.

Ferguson, J.F., Master, S.R., Propert, K.J., Reilly, M.P., Patel, P.N., Shah, R.Y., Mulvey, C.K., Gadi, R., Nijjar, P.S., Usman, H.M., Mehta, N.N. & Shah, R. 2013, "Race and gender variation in response to evoked inflammation", *Journal of translational medicine,* vol. 11, no. 1, pp. 63-63.

Ferluga, J. & Allison, A.C. 1978, "Role of mononuclear infiltrating cells in pathogenesis of hepatitis", *Lancet (London, England),* vol. 2, no. 8090, pp. 610-611.

Fielding, C.J. & Fielding, P.E. 1995, "Molecular physiology of reverse cholesterol transport", *Journal of lipid research,* vol. 36, no. 2, pp. 211-228.

Fleg, J.L., Tearney, G.J., Waxman, S., Domanski, M.J., Srinivas, P.R., Narula, J., Stone, G.W., Fayad, Z.A., Granada, J.F., Hatsukami, T.S., Kolodgie, F.D., Ohayon, J., Pettigrew, R. & Sabatine, M.S. 2012, "Detection of high-risk atherosclerotic plaque: report of the NHLBI Working Group on current status and future directions", *JACC.Cardiovascular imaging,* vol. 5, no. 9, pp. 941-955.

Freudenberg, M.A., Tchaptchet, S., Keck, S., Fejer, G., Huber, M., Schutze, N., Beutler, B. & Galanos, C. 2008, "Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity", *Immunobiology,* vol. 213, no. 3-4, pp. 193-203.

Frostegård, J. 2013, "Immunity, atherosclerosis and cardiovascular disease", *BMC medicine,* vol. 11, pp. 117.

Frostegård, J., Ulfgren, A.K., Nyberg, P., Hedin, U., Swedenborg, J., Andersson, U. & Hansson, G.K. 1999, "Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines", *Atherosclerosis,* vol. 145, no. 1, pp. 33-43.

Fung, T.T., Rimm, E.B., Spiegelman, D., Rifai, N., Tofler, G.H., Willett, W.C. & Hu, F.B. 2001, "Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk", *The American Journal of Clinical Nutrition,* vol. 73, no. 1, pp. 61.

Galanos, C. & Freudenberg, M.A. 1993, "Mechanisms of endotoxin shock and endotoxin hypersensitivity", *Immunobiology,* vol. 187, no. 3-5, pp. 346-356.

Galanos, C., Freudenberg, M. A., Katschinski, T, Salomao, R., Mossmann, H., and Kumazawa, Y. 1992, "Tumor necrosis factor and host response to endotoxin ", *CRC Press,* , pp. 75-102.

Galanos, C., Luderitz, O., Rietschel, E.T., Westphal, O., Brade, H., Brade, L., Freudenberg, M., Schade, U., Imoto, M. & Yoshimura, H. 1985, "Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities", *European journal of biochemistry,* vol. 148, no. 1, pp. 1-5.

Galanos, C., Luderitz, O., Rietschel, E.T., Westphal, O., Brade, H., Brade, L., Freudenberg, M., Schade, U., Imoto, M. & Yoshimura, H. 1985, "Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities", *European journal of biochemistry,* vol. 148, no. 1, pp. 1-5.

Gehring, U., Spithoven, J., Schmid, S., Bitter, S., Braun-Fahrlander, C., Dalphin, J.C., Hyvarinen, A., Pekkanen, J., Riedler, J., Weiland, S.K., Buchele, G., von Mutius, E., Vuitton, D.A., Brunekreef, B. & PASTURE study group 2008, "Endotoxin levels in cow's milk samples from farming and non-farming families - the PASTURE study", *Environment international,* vol. 34, no. 8, pp. 1132-1136.

Genest J 1990, "Physician's Guide to the Management of Lipoprotein Disorders.".

Genest, J., Frohlich, J., Fodor, G., McPherson, R. & Working Group on Hypercholesterolemia and Other Dyslipidemias 2003, "Recommendations for the management of dyslipidemia and the prevention of cardiovascular disease: summary of the 2003 update", *CMAJ: Canadian Medical Association journal = journal de l'Association medicale canadienne,* vol. 169, no. 9, pp. 921-924.

Ghoshal, S., Witta, J., Zhong, J., de Villiers, W. & Eckhardt, E. 2009, "Chylomicrons promote intestinal absorption of lipopolysaccharides", *Journal of lipid research,* vol. 50, no. 1, pp. 90-97.

Gibbons, G.H., Pratt, R.E. & Dzau, V.J. 1992, "Vascular smooth muscle cell hypertrophy vs. hyperplasia. Autocrine transforming growth factor-beta 1 expression determines growth response to angiotensin II", *The Journal of clinical investigation,* vol. 90, no. 2, pp. 456-461.

Glore, S.R., Van Treeck, D., Knehans, A.W. & Guild, M. 1994, "Soluble fiber and serum lipids: a literature review", *Journal of the American Dietetic Association,* vol. 94, no. 4, pp. 425-436.

Goldstein, J.L. & Brown, M.S. 1974, "Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia", *The Journal of biological chemistry,* vol. 249, no. 16, pp. 5153-5162.

Göran K Hansson 2005, "MECHANISMS OF DISEASE: Inflammation, Atherosclerosis, and Coronary Artery Disease", *The New England journal of medicine,* vol. 352, no. 16, pp. 1685.

Gosling, J., Slaymaker, S., Gu, L., Tseng, S., Zlot, C.H., Young, S.G., Rollins, B.J. & Charo, I.F. 1999, "MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B", *The Journal of clinical investigation,* vol. 103, no. 6, pp. 773-778.

Gotto A, Assmann G, Carmena R, et al. 2000, "The International Lipid Handbook for Clinical Practice".

Gotto A, P.H. 1999, "Manual of Lipid Disorders: Reducing the Risk for Coronary Heart Disease", pp. 19.

Gotto A, P.H. 1999, "Manual of Lipid Disorders: Reducing the Risk for Coronary Heart Disease", pp. 16.

Gotto, A.M., Brown, W.V., Levy, R.I., Birnbaumer, M.E. & Fredrickson, D.S. 1972, "Evidence for the identity of the major apoprotein in low density and very low density lipoproteins in normal subjects and patients with familial hyperlipoproteinemia", *The Journal of clinical investigation,* vol. 51, no. 6, pp. 1486-1494.

Grayston JT, Diwan VK, Cooney M & and Wang SP. 1989, "Community-and hospital-acquired pneumonia associated with Chlamydia TWAR infection demonstrated serologically", *Arch. Intern. Med.,* vol. 149, pp. 169-173.

Grayston, J.T., Campbell, L.A., Kuo, C.C., Mordhorst, C.H., Saikku, P., Thom, D.H. & Wang, S.P. 1990, "A new respiratory tract pathogen: Chlamydia pneumoniae strain TWAR", *The Journal of infectious diseases,* vol. 161, no. 4, pp. 618-625.

Grayston, J.T., Kuo, C.C., Coulson, A.S., Campbell, L.A., Lawrence, R.D., Lee, M.J., Strandness, E.D. & Wang, S.P. 1995, "Chlamydia pneumoniae (TWAR) in atherosclerosis of the carotid artery", *Circulation,* vol. 92, no. 12, pp. 3397-3400.

Greenspan, P., Mayer, E.P. & Fowler, S.D. 1985, "Nile red: a selective fluorescent stain for intracellular lipid droplets", *The Journal of cell biology,* vol. 100, no. 3, pp. 965-973.

Gregory Booth, Timothy J. Stalker, Allan M. Lefer & Rosario Scalia 2001, "Elevated ambient glucose induces acute inflammatory events in the microvasculature: effects of insulin", *American Journal of Physiology - Endocrinology and Metabolism,* vol. 280, no. 6, pp. E848-856.

Greten, F.R., Arkan, M.C., Bollrath, J., Hsu, L.C., Goode, J., Miething, C., Goktuna, S.I., Neuenhahn, M., Fierer, J., Paxian, S., Van Rooijen, N., Xu, Y., O'Cain, T., Jaffee, B.B., Busch, D.H., Duyster, J., Schmid, R.M., Eckmann, L. & Karin, M. 2007, "NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta", *Cell,* vol. 130, no. 5, pp. 918-931.

Griendling, K.K. & Alexander, R.W. 1997, "Oxidative stress and cardiovascular disease", *Circulation,* vol. 96, no. 10, pp. 3264.

Grundy, S.M. & Metzger, A.L. 1972, "A physiological method for estimation of hepatic secretion of biliary lipids in man", *Gastroenterology,* vol. 62, no. 6, pp. 1200-1217.

Grundy, S.M., Cleeman, J.I., Merz, C.N., Brewer, H.B.,Jr, Clark, L.T., Hunninghake, D.B., Pasternak, R.C., Smith, S.C.,Jr, Stone, N.J., National Heart, Lung, and Blood Institute, American College of Cardiology Foundation & American Heart Association 2004, "Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines", *Circulation,* vol. 110, no. 2, pp. 227-239.

Gu, L., Okada, Y., Clinton, S.K., Gerard, C., Sukhova, G.K., Libby, P. & Rollins, B.J. 1998, "Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice", *Molecular cell,* vol. 2, no. 2, pp. 275-281.

Gustavo B. F, Alvaro A, Leonardo R 2015. Cardiovascular Disease Burden: Evolving Knowledge of Risk Factors in Myocardial Infarction and Stroke through Population-Based Research and Perspectives in Global Prevention. *Front Cardiovasc Med*. 2: 32.

Hammond, S.M., Claesson, A., Jansson, A.M., Larsson, L.G., Pring, B.G., Town, C.M. & Ekstrom, B. 1987, "A new class of synthetic antibacterials acting on lipopolysaccharide biosynthesis", *Nature,* vol. 327, no. 6124, pp. 730-732.

Han, J., Hajjar, D.P., Febbraio, M. & Nicholson, A.C. 1997, "Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36", *The Journal of biological chemistry,* vol. 272, no. 34, pp. 21654-21659.

Hansson, G.K. & Libby, P. 2006, "The immune response in atherosclerosis: a double-edged sword", *Nature reviews.Immunology,* vol. 6, no. 7, pp. 508-519.

Hansson, G.K., Hellstrand, M., Rymo, L. & Rubbia, L. & Gabbiani, G. 1989, "Interferon γ inhibits both proliferation and expression of differentiation-specific α-smooth muscle actin in arterial smooth muscle cells", *J. Exp. Med.,* vol. 170, pp. 1595-1608.

Hansson, G.K., Libby, P., Schönbeck, U. & Yan, Z. 2002, "Innate and adaptive immunity in the pathogenesis of atherosclerosis", *Circulation research,* vol. 91, no. 4, pp. 281-291.

Hardardottir, I., Grunfeld, C. & Feingold, K.R. 1994, "Effects of endotoxin and cytokines on lipid metabolism", *Current opinion in lipidology,* vol. 5, no. 3, pp. 207-215.

Hartvigsen, K., Chou, M., Hansen, L.F., Shaw, P.X., Tsimikas, S., Binder, C.J. & Witztum, J.L. 2009, "The role of innate immunity in atherogenesis", *Journal of lipid research,* vol. 50 Suppl, pp. S388.

Healy, A.M., Garasic, J., Damokosh, A.I., Dowie, T.L., Poisson, L., Lillie, J., Libby, P., Ridker, P.M., Simon, D.I., Pickard, M.D., Pradhan, A.D., Wang, Y., Chen, Z., Croce, K., Sakuma, M., Shi, C. & Zago, A.C. 2006, "Platelet expression profiling and clinical validation of myeloid-related protein-14 as a novel determinant of cardiovascular events", *Circulation,* vol. 113, no. 19, pp. 2278-2284.

Hechtman, D.H., Cybulsky, M.I., Fuchs, H.J., Baker, J.B. & Gimbrone, J., M A. 1991, "Intravascular IL-8. Inhibitor of polymorphonuclear leukocyte accumulation at sites of acute inflammation", *Journal of immunology (Baltimore, Md.: 1950),* vol. 147, no. 3, pp. 883.

Heimdal, J., Olsnes, C., Olofsson, J. & Aarstad, H.J. 2001, *Monocyte and monocyte-derived macrophage secretion of MCP-1 in co-culture with autologous malignant and benign control fragment spheroids*, Springer-Verlag, Berlin/Heidelberg.

Herieka M and Erridge C 2014, " High-fat meal induced postprandial inflammation ", *Mol. Nutr. Food Res.,* vol. 58, no. 1, pp. 136-146.

Herieka, M., Faraj, T.A. & Erridge, C. 2016, "Reduced dietary intake of pro-inflammatory Toll-like receptor stimulants favourably modifies markers of cardiometabolic risk in healthy men", *Nutrition, metabolism, and cardiovascular diseases: NMCD,* vol. 26, no. 3, pp. 194-200.

Hong H et al 2007, "Sensitivity of mice to lipopolysaccharide is increased by a high saturated fat and cholesterol diet", J Inflamm., vol. 4, no. 22.

Hou, L., Lloyd-Jones, D.M., Ning, H., Huffman, M.D., Fornage, M., He, K., Zhang, X., Jacobs, D.R., Goff, D.C., Sidney, S., Carr, J.J. & Liu, K. 2013, "White blood cell count in young adulthood and coronary artery calcification in early middle age: coronary artery risk development in young adults (CARDIA) study", *European journal of epidemiology,* vol. 28, no. 9, pp. 735-742.

Huang, H., Liu, T., Rose, J.L., Stevens, R.L. & Hoyt, D.G. 2007, "Sensitivity of mice to lipopolysaccharide is increased by a high saturated fat and cholesterol diet", *Journal of inflammation (London, England),* vol. 4, pp. 22.

Huber, S.A., Sakkinen, P., Conze, D., Hardin, N. & Tracy, R. 1999, "Interleukin-6 exacerbates early atherosclerosis in mice", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 19, no. 10, pp. 2364-2367.

Huby, A.C., Antonova, G., Groenendyk, J., Gomez-Sanchez, C.E., Bollag, W.B., Filosa, J.A. & Belin de Chantemele, E.J. 2015, "Adipocyte-Derived Hormone Leptin Is a Direct Regulator of Aldosterone Secretion, Which Promotes Endothelial Dysfunction and Cardiac Fibrosis", *Circulation,* vol. 132, no. 22, pp. 2134-2145.

Huff, M.W. & Burnett, J.R. 1997, "3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and hepatic apolipoprotein B secretion", *Current opinion in lipidology,* vol. 8, no. 3, pp. 138-145.

Imajo, K., Fujita, K., Yoneda, M., Nozaki, Y., Ogawa, Y., Shinohara, Y., Kato, S., Mawatari, H., Shibata, W., Kitani, H., Ikejima, K., Kirikoshi, H., Nakajima, N., Saito, S., Maeyama, S., Watanabe, S., Wada, K. & Nakajima, A. 2012, "Hyperresponsivity to low-dose endotoxin during progression to nonalcoholic steatohepatitis is regulated by leptin-mediated signaling", *Cell metabolism,* vol. 16, no. 1, pp. 44-54.

J M Jay, S Margitic, A L Shereda & H V Covington 1979, "Determining endotoxin content of ground beef by the Limulus amoebocyte lysate test as a rapid indicator of microbial quality", *Applied and Environmental Microbiology,* vol. 38, no. 5, pp. 885-890.

Jan Nilsson, Gunilla Nordin Fredrikson, Malmö University & Faculty of Health and Society 2004, "Atherosclerosis", *Autoimmunity,* vol. 37, no. 4, pp. 351-351.

Janeway, J.,Charles A. & Medzhitov, R. 2002, "Innate immune recognition", *Annual Review of Immunology,* vol. 20, pp. 197.

Jerala, R. 2007, "Structural biology of the LPS recognition", *International journal of medical microbiology: IJMM,* vol. 297, no. 5, pp. 353-363.

Joanne M., Xianwa N, Andrew J., Ryuichi M, Hongjie W, Revathy S, Anthony J., Roger T., Roland S 2002. Disease Stage-Dependent Accumulation of Lipid and Protein Oxidation Products in Human Atherosclerosis. *The American Journal of Pathology*. 160(2): 701-710.

Jonasson, L., Holm, J., Skalli, O., Bondjers, G. & Hansson, G.K. 1986, "Regional Accumulations of T Cells, Macrophages, and Smooth Muscle Cells in the Human Atherosclerotic Plaque", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 6, no. 2, pp. 131-138.

Julio A. Ramirez 1996, "Isolation of Chlamydia pneumoniae from the Coronary Artery of a Patient with Coronary Atherosclerosis", *Annals of Internal Medicine,* vol. 125, no. 12, pp. 979.

Juvonen, T., Juvonen, J., Laurila, A., Alakärppä, H., Lounatmaa, K., Surcel, H., Leinonen, M., Kairaluoma, M.I. & Saikku, P. 1997, "Demonstration of Chlamydia pneumoniae in the walls of abdominal aortic aneurysms", *Journal of Vascular Surgery,* vol. 25, no. 3, pp. 499-505.

K Heo, Keigi F, Jun-ichi A 2014. Shear Stress and Atherosclerosis. *Mol Cells*. 37(6): 435–440.

K. Tanamoto, U. Zahringer, G.R. Mckenzie, C. Galanos, E.T. Rietschel, O. Luderitz, S. Kusumoto, T. Shiba, 1984, "Biological activities of synthetic lipid A analogues: pyrogenicity, lethal toxicity, anticomplement activity and induction of gelation of limulus amoebocyte lysate ", *Infect. Immun.,* vol. 44, pp. 421-426.

Kalayoglu, M.V. & Byrne, G.I. 1998, "Induction of Macrophage Foam Cell Formation by Chlamydia pneumoniae", *The Journal of infectious diseases,* vol. 177, no. 3, pp. 725-729.

Kamari, Y., Werman-Venkert, R., Shaish, A., Werman, A., Harari, A., Gonen, A., Voronov, E., Grosskopf, I., Sharabi, Y., Grossman, E., Iwakura, Y., Dinarello, C.A., Apte, R.N. & Harats, D. 2007, "Differential role and tissue specificity of interleukin-1alpha gene expression in atherogenesis and lipid metabolism", *Atherosclerosis,* vol. 195, no. 1, pp. 31-38.

Kane, J.P., Hardman, D.A. & Paulus, H.E. 1980, "Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 77, no. 5, pp. 2465-2469.

Kannel, W.B. & McGee, D.L. 1979, "Diabetes and glucose tolerance as risk factors for cardiovascular disease: the Framingham study", *Diabetes care,* vol. 2, no. 2, pp. 120-126.

Kathrin S. Michelsen, Michelle H. Wong, Prediman K. Shah, Wenxuan Zhang, Juliana Yano, Terence M. Doherty, Shizuo Akira, Tripathi B. Rajavashisth & Moshe Arditi 2004, "Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 101, no. 29, pp. 10679-10684.

Kawa, K., Tsutsui, H., Uchiyama, R., Kato, J., Matsui, K., Iwakura, Y., Matsumoto, T. & Nakanishi, K. 2010, "IFN-gamma is a master regulator of endotoxin shock syndrome in mice primed with heat-killed Propionibacterium acnes", *International immunology,* vol. 22, no. 3, pp. 157-166.

Kearney, P.M., Baigent, C., Godwin, J., Halls, H., Emberson, J.R. & Patrono, C. 2006, "Do Selective Cyclo-Oxygenase-2 Inhibitors And Traditional Non-Steroidal Anti-Inflammatory Drugs Increase The Risk Of Atherothrombosis? Meta-Analysis Of Randomised Trials", *BMJ: British Medical Journal,* vol. 332, no. 7553, pp. 1302-1305.

Kevin J. and Frederic G 2010. Monocytes in atherosclerosis: subsets and functions. *Nat Rev Cardiol*. 7(2): 77–86.

Khoo, J.C., Miller, E., Pio, F., Steinberg, D. & Witztum, J.L. 1992, "Monoclonal antibodies against LDL further enhance macrophage uptake of LDL aggregates", *Arteriosclerosis and Thrombosis : A Journal of Vascular Biology / American Heart Association,* vol. 12, no. 11, pp. 1258.

Kim, H.M., Park, B.S., Kim, J.I., Kim, S.E., Lee, J., Oh, S.C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O.J. & Lee, J.O. 2007, "Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran", *Cell,* vol. 130, no. 5, pp. 906-917.

Koeth, R.A., Wang, Z., Levison, B.S., Buffa, J.A., Org, E., Sheehy, B.T., Britt, E.B., Fu, X., Wu, Y., Li, L., Smith, J.D., DiDonato, J.A., Chen, J., Li, H., Wu, G.D., Lewis, J.D., Warrier, M., Brown, J.M., Krauss, R.M., Tang, W.H., Bushman, F.D., Lusis, A.J. & Hazen, S.L. 2013, "Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis", *Nature medicine,* vol. 19, no. 5, pp. 576-585.

Kovanen, P.T. 2007, "Mast cells: multipotent local effector cells in atherothrombosis", *Immunological reviews,* vol. 217, pp. 105.

Krieger, M. 1997, "The other side of scavenger receptors: pattern recognition for host defense", *Current opinion in lipidology,* vol. 8, no. 5, pp. 275-280.

Krogh-Madsen, R., Møller, K., Dela, F., Kronborg, G., Jauffred, S. & Pedersen, B.K. 2004, "Effect of hyperglycemia and hyperinsulinemia on the response of IL-6, TNF-alpha, and FFAs to low-dose endotoxemia in humans", *American journal of physiology.Endocrinology and metabolism,* vol. 286, no. 5, pp. E766.

Kumar, H., Kawai, T. & Akira, S. 2009, "Toll-like receptors and innate immunity", *Biochemical and biophysical research communications,* vol. 388, no. 4, pp. 621-625.

Kunjathoor, V.V., Freeman, M.W., Febbraio, M., Podrez, E.A., Moore, K.J., Andersson, L., Koehn, S., Rhee, J.S., Silverstein, R. & Hoff, H.F. 2002, "Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages", *The Journal of biological chemistry,* vol. 277, no. 51, pp. 49982-49988.

Kzhyshkowska, J., Neyen, C. & Gordon, S. 2012, "Role of macrophage scavenger receptors in atherosclerosis", *Immunobiology,* vol. 217, no. 5, pp. 492.

Laman, J.D., Schoneveld, A.H., Moll, F.L., van Meurs, M. & Pasterkamp, G. 2002, "Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques", *The American Journal of Cardiology,* vol. 90, no. 2, pp. 119-123.

Lang, D.H., Yeung, C.K., Peter, R.M., Ibarra, C., Gasser, R., Itagaki, K., Philpot, R.M. & Rettie, A.E. 1998, "Isoform specificity of trimethylamine N-oxygenation by human flavin-containing monooxygenase (FMO) and P450 enzymes: selective catalysis by FMO3", *Biochemical pharmacology,* vol. 56, no. 8, pp. 1005-1012.

Laugerette, F., Vors, C., Geloen, A., Chauvin, M.A., Soulage, C., Lambert-Porcheron, S., Peretti, N., Alligier, M., Burcelin, R., Laville, M., Vidal, H. & Michalski, M.C. 2011, "Emulsified lipids increase endotoxemia: possible role in early postprandial low-grade inflammation", *The Journal of nutritional biochemistry,* vol. 22, no. 1, pp. 53-59.

Lee, J.Y., Zhao, L., Youn, H.S., Weatherill, A.R., Tapping, R., Feng, L., Lee, W.H., Fitzgerald, K.A. & Hwang, D.H. 2004, "Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1", *The Journal of biological chemistry,* vol. 279, no. 17, pp. 16971-16979.

Lehr, H.A., Sagban, T.A., Ihling, C., Zahringer, U., Hungerer, K.D., Blumrich, M., Reifenberg, K. & Bhakdi, S. 2001, "Immunopathogenesis of atherosclerosis: endotoxin accelerates atherosclerosis in rabbits on hypercholesterolemic diet", *Circulation,* vol. 104, no. 8, pp. 914-920.

LeLeiko, R.M., Vaccari, C.S., Sola, S., Merchant, N., Nagamia, S.H., Thoenes, M. & Khan, B.V. 2009, "Usefulness of elevations in serum choline and free F2)-isoprostane to predict 30-day cardiovascular outcomes in patients with acute coronary syndrome", *The American Journal of Cardiology,* vol. 104, no. 5, pp. 638-643.

Levy, D., Wilson, P.W.F., Anderson, K.M. & Castelli, W.P. 1990, "Stratifying the patient at risk from coronary disease: New insights from the framingham heart study", *American Heart Journal,* vol. 119, no. 3, pp. 712-717.

Ley, R.E., Turnbaugh, P.J., Klein, S. & Gordon, J.I. 2006, "Microbial ecology: human gut microbes associated with obesity", *Nature,* vol. 444, no. 7122, pp. 1022-1023.

Li, Z., Yang, F., Dunn, S., Gross, A.K. & Smyth, S.S. 2011, "Platelets as immune mediators: their role in host defense responses and sepsis", *Thrombosis research,* vol. 127, no. 3, pp. 184-188.

Libby, P. & Shi, G. 2007, "Mast cells as mediators and modulators of atherogenesis", *Circulation,* vol. 115, no. 19, pp. 2471-2473.

Libby, P. 2006, "Inflammation and cardiovascular disease mechanisms", *The American Journal of Clinical Nutrition,* vol. 83, no. 2, pp. 456S.

Libby, P., Ridker, P.M. & Hansson, G.K. 2009, "Inflammation in Atherosclerosis", *Journal of the American College of Cardiology,* vol. 54, no. 23, pp. 2129-2138.

Libby, P., Ridker, P.M., Hansson, G.K. & Leducq Transatlantic Network on Atherothrombosis 2009, "Inflammation in atherosclerosis: from pathophysiology to practice", *Journal of the American College of Cardiology,* vol. 54, no. 23, pp. 2129.

Lievens, D. & von Hundelshausen, P. 2011, "Platelets in atherosclerosis", *Thrombosis and haemostasis,* vol. 106, no. 5, pp. 827-838.

LIU, Y., SU, W., WANG, S. & LI, P. 2012, *Naringin inhibits chemokine production in an LPS-induced RAW 264.7 macrophage cell line*, D.A. Spandidos.

Llodrá, J., Angeli, V., Liu, J., Trogan, E., Fisher, E.A. & Randolph, G.J. 2004, "Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 101, no. 32, pp. 11779-11784.

Ludewig, B., Freigang, S., Jäggi, M., Kurrer, M.O., Pei, Y.C., Vlk, L., Odermatt, B., Zinkernagel, R.M. & Hengartner, H. 2000, "Linking immune-mediated arterial inflammation and cholesterol-induced atherosclerosis in a transgenic mouse model", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 97, no. 23, pp. 12752-12757.

Luengo-Fernández, R., Leal, J., Gray, A., Petersen, S. & Rayner, M. 2006, "Cost of cardiovascular diseases in the United Kingdom", *Heart (British Cardiac Society),* vol. 92, no. 10, pp. 1384-1389.

Lusis, A.J. 2000, "Atherosclerosis", *Nature,* vol. 407, no. 6801, pp. 233.

Luster, M.I., Germolec, D.R., Yoshida, T., Kayama, F. & Thompson, M. 1994, "Endotoxin-induced cytokine gene expression and excretion in the liver", *Hepatology (Baltimore, Md.),* vol. 19, no. 2, pp. 480-488.

Madan, M. & Amar, S. 2008, "Toll-like receptor-2 mediates diet and/or pathogen associated atherosclerosis: proteomic findings", *PloS one,* vol. 3, no. 9, pp. e3204.

Mahinda Y. Abeywardena, Wayne R. Leifert, Kirsty E. Warnes, Jose N. Varghese and Richard J. Head 2009, "Cardiovascular Biology of Interleukin-6", *Current Pharmaceutical Design,* vol. 15, no. 15, pp. 1809-1821.

Malhi, H. & Kaufman, R.J. 2011, "Endoplasmic reticulum stress in liver disease", *Journal of hepatology,* vol. 54, no. 4, pp. 795-809.

Mallat, Z., Taleb, S., Ait-Oufella, H. & Tedgui, A. 2009, "The role of adaptive T cell immunity in atherosclerosis", *Journal of lipid research,* vol. 50 Suppl, no. Supplement, pp. S364-S369.

Marlous R Dillingh, Eveline P van Poelgeest, Karen E Malone, Elles M Kemper, Erik S G Stroes, Matthijs Moerland and Jacobus Burggraaf 2014, "Characterization of inflammation and immune cell modulation induced by low-dose LPS administration to healthy volunteers ", *Journal of Inflammation,* .

Martich, G.D., Boujoukos, A.J. & Suffredini, A.F. 1993, "Response of man to endotoxin", *Immunobiology,* vol. 187, no. 3-5, pp. 403-416.

Maxfield, F.R. & Tabas, I. 2005, "Role of cholesterol and lipid organization in disease", *Nature,* vol. 438, no. 7068, pp. 612-621.

McCarron, D.A. 1998, "Diet and blood pressure--the paradigm shift", *Science (New York, N.Y.),* vol. 281, no. 5379, pp. 933-934.

McGillicuddy, F.C., de la Llera Moya, M., Hinkle, C.C., Joshi, M.R., Chiquoine, E.H., Billheimer, J.T., Rothblat, G.H. & Reilly, M.P. 2009, "Inflammation impairs reverse cholesterol transport in vivo", *Circulation,* vol. 119, no. 8, pp. 1135-1145.

Meager, A. 1999, "Cytokine regulation of cellular adhesion molecule expression in inflammation", *Cytokine & growth factor reviews,* vol. 10, no. 1, pp. 27-39.

Medzhitov, R. & Janeway, J., C. 2000, "Innate immunity", *The New England journal of medicine,* vol. 343, no. 5, pp. 338-344.

Micha, R., Wallace, S.K. & Mozaffarian, D. 2010, "Red and processed meat consumption and risk of incident coronary heart disease, stroke, and diabetes mellitus: a systematic review and meta-analysis", *Circulation,* vol. 121, no. 21, pp. 2271-2283.

Miura, K., Kodama, Y., Inokuchi, S., Schnabl, B., Aoyama, T., Ohnishi, H., Olefsky, J.M., Brenner, D.A. & Seki, E. 2010, "Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice", *Gastroenterology,* vol. 139, no. 1, pp. 323-34.e7.

Mlot, C. 1996, "Chlamydia Linked to Atherosclerosis", *Science,* vol. 272, no. 5267, pp. 1422-1422.

Moore, K.J. & Freeman, M.W. 2006, "Scavenger Receptors in Atherosclerosis: Beyond Lipid Uptake", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 26, no. 8, pp. 1702-1711.

Moore, K.J. & Tabas, I. 2011, "Macrophages in the pathogenesis of atherosclerosis", *Cell,* vol. 145, no. 3, pp. 341-355.

Moore, K.J., Sheedy, F.J. & Fisher, E.A. 2013, "Macrophages in atherosclerosis: a dynamic balance", *Nature reviews.Immunology,* vol. 13, no. 10, pp. 709.

Mullick, A.E., Soldau, K., Kiosses, W.B., Bell, 3.,Thomas A., Tobias, P.S. & Curtiss, L.K. 2008, "Increased endothelial expression of Toll-like receptor 2 at sites of disturbed blood flow exacerbates early atherogenic events", *The Journal of experimental medicine,* vol. 205, no. 2, pp. 373-383.

Mullick, A.E., Tobias, P.S. & Curtiss, L.K. 2005, "Modulation of atherosclerosis in mice by Toll-like receptor 2", *The Journal of clinical investigation,* vol. 115, no. 11, pp. 3149-3156.

Murray, C.J. & Lopez, A.D. 1997, "Mortality by cause for eight regions of the world: Global Burden of Disease Study", *Lancet (London, England),* vol. 349, no. 9061, pp. 1269-1276.

Nathan, C. & Ding, A. 2010, "Nonresolving Inflammation", *Cell,* vol. 140, no. 6, pp. 871-882.

Navab, M., Demer, L.L., Edwards, P.A., Fogelman, A.M., Berliner, J.A., Watson, A.D., Hama, S.Y., Territo, M.C., Lusis, A.J., Shih, D.M., Van Lenten, B.J. & Frank, J.S. 1996, "The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 16, no. 7, pp. 831-842.

Newton, K. & Dixit, V.M. 2012, *Signaling in innate immunity and inflammation*, Cold Spring Harbor Laboratory Press, United States.

Nicolaou G, Erridge C 2010. Toll-like receptor-dependent lipid body formation in macrophage foam cell formation. *Curr Opin Lipidol* 21:427-33.

Nicolaou G, Goodall AH, Erridge C 2012. Diverse bacteria promote macrophage foam cell formation via Toll-like receptor-dependent lipid body biosynthesis. *J Atheroscler Thromb* 19:137-48.

Ohta, H., Wada, H., Niwa, T., Kirii, H., Iwamoto, N., Fujii, H., Saito, K., Sekikawa, K. & Seishima, M. 2005, "Disruption of tumor necrosis factor-a gene diminishes the development of atherosclerosis in ApoE-deficient mice", *Atherosclerosis,* vol. 180, no. 1, pp. 11-17.

Olteanu, S., Kandel-Kfir, M., Shaish, A., Almog, T., Shemesh, S., Barshack, I., Apte, R.N., Harats, D. & Kamari, Y. 2014, "Lack of interleukin-1alpha in Kupffer cells attenuates liver inflammation and expression of inflammatory cytokines in hypercholesterolaemic mice", *Digestive and liver disease: official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver,* vol. 46, no. 5, pp. 433-439.

Ong, G., Thomas, B.J., Mansfield, A.O., Davidson, B.R. & Taylor-Robinson, D. 1996, "Detection and widespread distribution of Chlamydia pneumoniae in the vascular system and its possible implications", *Journal of clinical pathology,* vol. 49, no. 2, pp. 102-106.

Ostos, M.A., Recalde, D., Zakin, M.M. & Scott-Algara, D. 2002, "Implication of natural killer T cells in atherosclerosis development during a LPS-induced chronic inflammation", *FEBS letters,* vol. 519, no. 1-3, pp. 23-29.

Ouimet, M., Franklin, V., Mak, E., Liao, X., Tabas, I. & Marcel, Y.L. 2011, "Autophagy regulates cholesterol efflux from macrophage foam cells via lysosomal acid lipase", *Cell metabolism,* vol. 13, no. 6, pp. 655-667.

Palsson-McDermott, E.M. & O'Neill, L.A. 2004, "Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4", *Immunology,* vol. 113, no. 2, pp. 153-162.

Paola Brun, Ignazio Castagliuolo, Vincenza Di Leo, Andrea Buda, Massimo Pinzani, Giorgio Palù & Diego Martines 2007, "Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis", *American Journal of Physiology - Gastrointestinal and Liver Physiology,* vol. 292, no. 2, pp. 518-525.

Paoletti R, Poli A, Cignarella A 2006, "The emerging link between nutrition, inflammation and atherosclerosis", *Expert Review of Cardiovascular Therapy,* vol. 4, no. 3, pp. 385-393.

Parhofer, K.G. & Barrett, P.H. 2006, "Thematic review series: patient-oriented research. What we have learned about VLDL and LDL metabolism from human kinetics studies", *Journal of lipid research,* vol. 47, no. 8, pp. 1620-1630.

Patrice D. Cani, Chantal Chabo, Aurélie Waget, Evelyne Delmée, Béatrice Cousin, Thierry Sulpice, Bernard Chamontin, Jean Ferrières, Jean-François Tanti, Glenn R. Gibson, Louis Casteilla, Jacques Amar, Nathalie M. Delzenne, Marie Christine Alessi, Rémy Burcelin, Miguel Angel Iglesias, Marjorie Poggi, Claude Knauf, Delphine Bastelica, Audrey M. Neyrinck, Francesca

Fava & Kieran M. Tuohy 2007, *Metabolic Endotoxemia Initiates Obesity and Insulin Resistance*, American Diabetes Association, United States.

Patrice D. Cani, Rodrigo Bibiloni, Claude Knauf, Aurélie Waget, Audrey M. Neyrinck, Nathalie M. Delzenne & Rémy Burcelin 2008, "Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet–Induced Obesity and Diabetes in Mice", *Diabetes,* vol. 57, no. 6, pp. 1470-1481.

Pedersen, T.R., Kjekshus, J., Berg, K., Haghfelt, T., Faergeman, O., Faergeman, G., Pyorala, K., Miettinen, T., Wilhelmsen, L., Olsson, A.G., Wedel, H. & Scandinavian Simvastatin Survival Study Group 2004, "Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). 1994", *Atherosclerosis.Supplements,* vol. 5, no. 3, pp. 81-87.

Piga, R., Naito, Y., Kokura, S., Handa, O. & Yoshikawa, T. 2007, *Short-term high glucose exposure induces monocyte-endothelial cells adhesion and transmigration by increasing VCAM-1 and MCP-1 expression in human aortic endothelial cells*, Elsevier Ireland Ltd, Ireland.

Poole JC, F.H. 1958, "Changes in the endothelium of the aorta and the behaviour of macrophages in experimental atheroma of rabbits", *J Pathol Bacteriol,* vol. 75, pp. 245-51.

Posserud, I., Stotzer, P.O., Bjornsson, E.S., Abrahamsson, H. & Simren, M. 2007, "Small intestinal bacterial overgrowth in patients with irritable bowel syndrome", *Gut,* vol. 56, no. 6, pp. 802-808.

Pulford, K.A., Sipos, A., Cordell, J.L., Stross, W.P. & Mason, D.Y. 1990, "Distribution of the CD68 macrophage/myeloid associated antigen", *International immunology,* vol. 2, no. 10, pp. 973-980.

Puolakkainen, M., Kuo, C.C., Shor, A., Wang, S.P., Grayston, J.T. & Campbell, L.A. 1993, "Serological response to Chlamydia pneumoniae in adults with coronary arterial fatty streaks and fibrolipid plaques", *Journal of clinical microbiology,* vol. 31, no. 8, pp. 2212-2214.

Pussinen, P.J., Tuomisto, K., Jousilahti, P., Havulinna, A.S., Sundvall, J. & Salomaa, V. 2007, "Endotoxemia, immune response to periodontal pathogens, and systemic inflammation associate with incident cardiovascular disease events", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 27, no. 6, pp. 1433.

Randolph, G.J. 2008, "Emigration of monocyte-derived cells to lymph nodes during resolution of inflammation and its failure in atherosclerosis", *Current opinion in lipidology,* vol. 19, no. 5, pp. 462.

Reigstad, C.S., Lunden, G.O., Felin, J. & Backhed, F. 2009, "Regulation of serum amyloid A3 (SAA3) in mouse colonic epithelium and adipose tissue by the intestinal microbiota", *PloS one,* vol. 4, no. 6, pp. e5842.

Reilly, M.P., Iqbal, N., Schutta, M., Wolfe, M.L., Scally, M., Localio, A.R., Rader, D.J. & Kimmel, S.E. 2004, "Plasma leptin levels are associated with coronary atherosclerosis in type 2 diabetes", *The Journal of clinical endocrinology and metabolism,* vol. 89, no. 8, pp. 3872-3878.

Remick, D.G. 2005, "Interleukin-8", *Critical Care Medicine,* vol. 33, no. 12 Suppl, pp. S466-S467.

Ridker, P.M., Cushman, M., Stampfer, M.J., Tracy, R.P. & Hennekens, C.H. 1997, "Inflammation, Aspirin, and the Risk of Cardiovascular Disease in Apparently Healthy Men", *The New England journal of medicine,* vol. 336, no. 14, pp. 973-979.

Rietschel, E.T., Kirikae, T., Schade, F.U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A.J., Zahringer, U., Seydel, U. & Di Padova, F. 1994, "Bacterial endotoxin: molecular relationships

of structure to activity and function", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 8, no. 2, pp. 217-225.

Rietschel, E.T., Kirikae, T., Schade, F.U., Ulmer, A.J., Holst, O., Brade, H., Schmidt, G., Mamat, U., Grimmecke, H.D. & Kusumoto, S. 1993, "The chemical structure of bacterial endotoxin in relation to bioactivity", *Immunobiology,* vol. 187, no. 3-5, pp. 169-190.

Rifai N, Warnick GR, Dominiczak MH 1997, "Handbook of Lipoprotein Testing ".

Rivera, C.A., Adegboyega, P., van Rooijen, N., Tagalicud, A., Allman, M. & Wallace, M. 2007, "Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis", *Journal of hepatology,* vol. 47, no. 4, pp. 571-579.

Rollins, B., Yoshimura, T., Leonard, E. & Pober, J. 1990, "Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE", *American Journal of Pathology,* vol. 136, no. 6, pp. 1229-1233.

Rooijen, N.V. & Sanders, A. 1994, "Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications", *Journal of immunological methods,* vol. 174, no. 1–2, pp. 83-93.

Rosenfeld, M.E., Ylä-Herttuala, S., Lipton, B.A., Ord, V.A., Witztum, J.L. & Steinberg, D. 1992, "Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and humans", *The American journal of pathology,* vol. 140, no. 2, pp. 291-300.

Ross G. Gerrity, Rama Natarajan, Jerry L. Nadler & Troy Kimsey 2001, *Diabetes-Induced Accelerated Atherosclerosis in Swine*, American Diabetes Association, United States, pp. 1654-1665.

Ross, R. 1999, "Atherosclerosis--an inflammatory disease", *The New England journal of medicine,* vol. 340, no. 2, pp. 115-126.

Rossignol, D., Lynn, M., Wittek, A. & Rose, J. 2006, "Elevated plasma levels of limulus amoebocyte lysate-reactive material", *The Journal of infectious diseases,* vol. 194, no. 9, pp. 1340; author reply 1340-1.

S D Yan, A M Schmidt, G M Anderson, J Zhang, J Brett, Y S Zou, D Pinsky & D Stern 1994, "Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins", *Journal of Biological Chemistry,* vol. 269, no. 13, pp. 9889.

Saikku, P., Leinonen, M., Mattila, K., Ekman, M.R., Nieminen, M.S., Makela, P.H., Huttunen, J.K. & Valtonen, V. 1988, "Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction", *Lancet (London, England),* vol. 2, no. 8618, pp. 983-986.

Sakao, Y., Takeda, K., Tsutsui, H., Kaisho, T., Nomura, F., Okamura, H., Nakanishi, K. & Akira, S. 1999, "IL-18-deficient mice are resistant to endotoxin-induced liver injury but highly susceptible to endotoxin shock", *International immunology,* vol. 11, no. 3, pp. 471-480.

SALMON, W.D. & NEWBERNE, P.M. 1962, "Cardiovascular disease in choline-deficient rats. Effects of choline deficiency, nature and level of dietary lipids and proteins, and duration of feeding on plasma and liver lipid values and cardiovascular lesions", *Archives of Pathology,* vol. 73, pp. 190-209.

Satapathy, S.K. & Sanyal, A.J. 2010, "Novel treatment modalities for nonalcoholic steatohepatitis", *Trends in endocrinology and metabolism: TEM,* vol. 21, no. 11, pp. 668-675.

Satish L. Deshmane, Sergey Kremlev, Shohreh Amini, and Bassel E. Sawaya. 2010, "Monocyte Chemoattractant Protein-1 (MCP-1): An Overview", *Journal of Interferon & Cytokine Research,* vol. 29, no. 6, pp. 313-326.

Scheja, L., Heese, B., Zitzer, H., Michael, M.D., Siesky, A.M., Pospisil, H., Beisiegel, U. & Seedorf, K. 2008, "Acute-phase serum amyloid A as a marker of insulin resistance in mice", *Experimental diabetes research,* vol. 2008, pp. 230837-12.

Schromm, A.B., Brandenburg, K., Loppnow, H., Moran, A.P., Koch, M.H., Rietschel, E.T. & Seydel, U. 2000, "Biological activities of lipopolysaccharides are determined by the shape of their lipid A portion", *European journal of biochemistry,* vol. 267, no. 7, pp. 2008-2013.

Schuett, H., Luchtefeld, M., Grothusen, C., Grote, K. & Schieffer, B. 2009, "How much is too much? Interleukin-6 and its signalling in atherosclerosis", *Thrombosis and haemostasis,* vol. 102, no. 2, pp. 215-222.

Sever, P.S., Dahlof, B. & Poulter, N.R. 2003, "Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial-Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial", *ACC Current Journal Review,* vol. 12, no. 4, pp. 36-36.

Shi, H., Kokoeva, M.V., Inouye, K., Tzameli, I., Yin, H. & Flier, J.S. 2006, "TLR4 links innate immunity and fatty acid-induced insulin resistance", *The Journal of clinical investigation,* vol. 116, no. 11, pp. 3015-3025.

Shibata, T., Nakashima, F., Honda, K., Lu, Y., Kondo, T., Ushida, Y., Aizawa, K., Suganuma, H., Oe, S., Tanaka, H., Takahashi, T. & Uchida, K. 2014, "Toll-like receptors as a target of food-derived anti-inflammatory compounds", *The Journal of biological chemistry,* vol. 289, no. 47, pp. 32757-32772.

Shono, S., Habu, Y., Nakashima, M., Sato, A., Nakashima, H., Miyazaki, H., Kinoshita, M., Tsumatori, G., Shinomiya, N. & Seki, S. 2011, "The immunologic outcome of enhanced function of mouse liver lymphocytes and Kupffer cells by high-fat and high-cholesterol diet", *Shock (Augusta, Ga.),* vol. 36, no. 5, pp. 484-493.

Sigthorsson, G., Morham, S.G., Bjarnason, I., Simpson, R.J., Walley, M., Anthony, A., Foster, R., Hotz–Behoftsitz, C., Palizban, A., Pombo, J. & Watts, J. 2002, "COX-1 and 2, intestinal integrity, and pathogenesis of nonsteroidal anti-inflammatory drug enteropathy in mice", *Gastroenterology,* vol. 122, no. 7, pp. 1913-1923.

Singh, R. 2009, "Autophagy regulates lipid metabolism", *Nature Reviews Molecular Cell Biology,* vol. 10, no. 5, pp. 1131-1135.

Sinzinger, H., Wolfram, R. & Peskar, B.A. 2002, "Muscular side effects of statins", *Journal of cardiovascular pharmacology,* vol. 40, no. 2, pp. 163-171.

Siri-Tarino, P.W., Sun, Q., Hu, F.B. & Krauss, R.M. 2010, "Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease", *The American Journal of Clinical Nutrition,* vol. 91, no. 3, pp. 535-546.

Slava E, Kory J., Gwendalyn J 2014. Origin and Functions of Tissue Macrophages. *Immunity*. 41(1): 21-35.

Spagnoli, L.G., Bonanno, E., Sangiorgi, G. & Mauriello, A. 2007, "Role of inflammation in atherosclerosis", *Journal of nuclear medicine : official publication, Society of Nuclear Medicine,* vol. 48, no. 11, pp. 1800-1815.

Spann, N.J., Heudobler, D., Raetz, C.R.H., Wang, E.W., Kelly, S.L., Sullards, M.C., Murphy, R.C., Merrill, J.,Alfred H., Brown, H.A., Dennis, E.A., Li, A.C., Garmire, L.X., Ley, K., Tsimikas, S.,

Fahy, E., Subramaniam, S., Quehenberger, O., Russell, D.W., Glass, C.K., McDonald, J.G., Myers, D.S., Milne, S.B., Shibata, N., Reichart, D., Fox, J.N. & Shaked, I. 2012, "Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses", *Cell,* vol. 151, no. 1, pp. 138-152.

Starkie, R., Ostrowski, S.R., Jauffred, S., Febbraio, M. & Pedersen, B.K. 2003, "Exercise and IL-6 infusion inhibit endotoxin-induced TNF-alpha production in humans", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 17, no. 8, pp. 884.

Steinberg, D. 2002, "Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime", *Nature medicine,* vol. 8, no. 11, pp. 1211-1217.

Stewart, C.R., Stuart, L.M., Wilkinson, K., van Gils, J.M., Deng, J., Halle, A., Rayner, K.J., Boyer, L., Zhong, R. & Frazier, W.A. 2010, "CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer", *Nature immunology,* vol. 11, no. 2, pp. 155-161.

Stoner, L., Lucero, A.A., Palmer, B.R., Jones, L.M., Young, J.M. & Faulkner, J. 2013, "Inflammatory biomarkers for predicting cardiovascular disease", *Clinical biochemistry,* vol. 46, no. 15, pp. 1353.

Strong S. and J. Rader 2010. Clinical Implications of Lipid Genetics for Cardiovascular Disease. *Curr Cardiovasc Risk Rep*. 4(6): 461–468.

Sun, J., Sukhova, G.K., Wolters, P.J., Yang, M., Kitamoto, S., Libby, P., MacFarlane, L.A., Mallen-St Clair, J. & Shi, G. 2007, "Mast cells promote atherosclerosis by releasing proinflammatory cytokines", *Nature medicine,* vol. 13, no. 6, pp. 719-724.

Swei, A., Lacy, F., DeLano, F.A. & Schmid-Schönbein, G.W. 1997, "Oxidative stress in the Dahl hypertensive rat", *Hypertension,* vol. 30, no. 6, pp. 1628-1633.

Tabas, I. 2005, "Consequences and Therapeutic Implications of Macrophage Apoptosis in Atherosclerosis: The Importance of Lesion Stage and Phagocytic Efficiency", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 25, no. 11, pp. 2255-2264.

Takaoka, N., Campbell, L.A., Lee, A., Rosenfeld, M.E. & Kuo, C. 2008, "Chlamydia pneumoniae infection increases adherence of mouse macrophages to mouse endothelial cells in vitro and to aortas ex vivo", *Infection and immunity,* vol. 76, no. 2, pp. 510-514.

Takeda, K. & Akira, S. 2004, "TLR signaling pathways", *Seminars in immunology,* vol. 16, no. 1, pp. 3-9.

Takeda, K. & Akira, S. 2005, "Toll-like receptors in innate immunity", *International immunology,* vol. 17, no. 1, pp. 1-14.

Taleb, S., Tedgui, A. & Mallat, Z. 2008, "Regulatory T-cell immunity and its relevance to atherosclerosis", *Journal of internal medicine,* vol. 263, no. 5, pp. 489-499.

Tam, C.S., Viardot, A., Clément, K., Tordjman, J., Tonks, K., Greenfield, J.R., Campbell, L.V., Samocha-Bonet, D. & Heilbronn, L.K. 2010, "Short-term overfeeding may induce peripheral insulin resistance without altering subcutaneous adipose tissue macrophages in humans", *Diabetes,* vol. 59, no. 9, pp. 2164-2170.

Taubes, G. 1998, "The (Political) Science of Salt", *Science,* vol. 281, no. 5379, pp. 898-907.

Taylor, K.E., Giddings, J.C. & van den Berg, C.W. 2005, "C-reactive protein-induced in vitro endothelial cell activation is an artefact caused by azide and

lipopolysaccharide", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 25, no. 6, pp. 1225-1230.

Tedgui, A. & Mallat, Z. 2006, "Cytokines in Atherosclerosis: Pathogenic and Regulatory Pathways", *Physiological Reviews,* vol. 86, no. 2, pp. 515-581.

Tilg, H. & Moschen, A.R. 2010, "Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis", *Hepatology (Baltimore, Md.),* vol. 52, no. 5, pp. 1836-1846.

Tilg, H. & Moschen, A.R. 2011, "IL-1 cytokine family members and NAFLD: neglected in metabolic liver inflammation", *Journal of hepatology,* vol. 55, no. 5, pp. 960-962.

Totani, L. & Evangelista, V. 2010, "Platelet-leukocyte interactions in cardiovascular disease and beyond", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 30, no. 12, pp. 2357-2361.

Trent, M.S., Stead, C.M., Tran, A.X. & Hankins, J.V. 2006, "Diversity of endotoxin and its impact on pathogenesis", *Journal of endotoxin research,* vol. 12, no. 4, pp. 205-223.

Truswell, A.S. 1995, "Dietary fibre and plasma lipids", *European journal of clinical nutrition,* vol. 49 Suppl 3, pp. S105-9.

Tsan, M.F. & Gao, B. 2007, "Pathogen-associated molecular pattern contamination as putative endogenous ligands of Toll-like receptors", *Journal of endotoxin research,* vol. 13, no. 1, pp. 6-14.

Turley, S.D. & Dietschy, J.M. 2003, "Sterol absorption by the small intestine", *Current opinion in lipidology,* vol. 14, no. 3, pp. 233-240.

Tuschil, A., Lam, C., Haslberger, A. & Lindley, I. 1992, "Interleukin-8 stimulates calcium transients and promotes epidermal cell proliferation", *The Journal of investigative dermatology,* vol. 99, no. 3, pp. 294-298.

Tuzcu, E.M., Kapadia, S.R., Tutar, E., Ziada, K.M., Hobbs, R.E., McCarthy, P.M., Young, J.B. & Nissen, S.E. 2001, "High prevalence of coronary atherosclerosis in asymptomatic teenagers and young adults: evidence from intravascular ultrasound", *Circulation,* vol. 103, no. 22, pp. 2705-2710.

Twig, G., Afek, A., Shamiss, A., Derazne, E., Tzur, D., Gordon, B. & Tirosh, A. 2012, "White blood cell count and the risk for coronary artery disease in young adults", *PloS one,* vol. 7, no. 10, pp. e47183.

U. Schönbeck, G. K. Sukhova, K. Shimizu, F. Mach & P. Libby 2000, "Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 97, no. 13, pp. 7458-7463.

Uphoff, C.C., Denkmann, S. & Drexler, H.G. 2012, "Treatment of mycoplasma contamination in cell cultures with Plasmocin", *Journal of biomedicine & biotechnology,* vol. 2012, pp. 267678.

van der Wal, A.C., Becker, A.E., van der Loos, C.M. & Das, P.K. 1994, "Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology", *Circulation,* vol. 89, no. 1, pp. 36-44.

van Deventer, S., Buller, H., ten Cate, J., Aarden, L., Hack, C. & Sturk, A. 1990, "Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways", *Blood,* vol. 76, no. 12, pp. 2520.

van Gils, J.M., Zwaginga, J.J. & Hordijk, P.L. 2009, "Molecular and functional interactions among monocytes, platelets, and endothelial cells and their relevance for cardiovascular diseases", *Journal of leukocyte biology,* vol. 85, no. 2, pp. 195-204.

Vassalli, P. 1992, "The pathophysiology of tumor necrosis factors", *Annual Review of Immunology,* vol. 10, no. 1, pp. 411-452.

Vaughn G. 1999, "Understanding and Evaluating Common Laboratory Tests", pp. 229-232.

Vogel, S. N., and Hogan, M. M. 1990, "The role of cytokines in endotoxin-mediated host responses. ", *Oxford University Press,* pp. 238-258.

Vogl, T., Sorg, C., Roth, J., Tenbrock, K., Ludwig, S., Leukert, N., Ehrhardt, C., van Zoelen, M.A.D., Nacken, W., Foell, D. & van der Poll, T. 2007, "Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock", *Nature medicine,* vol. 13, no. 9, pp. 1042-1049.

von Hundelshausen, P., Koenen, R.R., Sack, M., Mause, S.F., Adriaens, W., Proudfoot, A.E.I., Hackeng, T.M. & Weber, C. 2005, "Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium", *Blood,* vol. 105, no. 3, pp. 924-930.

Wang, Z., Klipfell, E., Bennett, B.J., Koeth, R., Levison, B.S., Dugar, B., Feldstein, A.E., Britt, E.B., Fu, X., Chung, Y.M., Wu, Y., Schauer, P., Smith, J.D., Allayee, H., Tang, W.H., DiDonato, J.A., Lusis, A.J. & Hazen, S.L. 2011, "Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease", *Nature,* vol. 472, no. 7341, pp. 57-63.

Weber, C., Belge, K.U., von Hundelshausen, P., Draude, G., Steppich, B., Mack, M., Frankenberger, M., Weber, K.S. & Ziegler-Heitbrock, H.W. 2000, "Differential chemokine receptor expression and function in human monocyte subpopulations", *Journal of leukocyte biology,* vol. 67, no. 5, pp. 699.

Westerterp, M., Berbée, J.F.P., Pires, N.M.M., van Mierlo, G.J.D., Kleemann, R., Romijn, J.A., Havekes, L.M. & Rensen, P.C.N. 2007, "Apolipoprotein C-I is crucially involved in lipopolysaccharide-induced atherosclerosis development in apolipoprotein E-knockout mice", *Circulation,* vol. 116, no. 19, pp. 2173-2181.

Westphal, 0., Westphal, U., and Sommer, Th. 1978, "The history of pyrogen research ", *American Society for Microbiology,* , pp. 221-238.

Weyand, C.M., Younge, B.R. & Goronzy, J.J. 2008, "T cells in arteritis and atherosclerosis", *Current opinion in lipidology,* vol. 19, no. 5, pp. 469-477.

Wiedermann, C.J., Kiechl, S., Dunzendorfer, S., Schratzberger, P., Egger, G., Oberhollenzer, F. & Willeit, J. 1999, "Association of endotoxemia with carotid atherosclerosis and cardiovascular disease: prospective results from the Bruneck Study", *Journal of the American College of Cardiology,* vol. 34, no. 7, pp. 1975.

Wolff, S.M. 1973, "Biological effects of bacterial endotoxins in man", *The Journal of infectious diseases,* vol. 128, pp. Suppl: 259-64.

Wolk, R., Berger, P., Lennon, R.J., Brilakis, E.S., Johnson, B.D. & Somers, V.K. 2004, "Plasma leptin and prognosis in patients with established coronary atherosclerosis", *Journal of the American College of Cardiology,* vol. 44, no. 9, pp. 1819-1824.

Wouters, K., van Gorp, P.J., Bieghs, V., Gijbels, M.J., Duimel, H., Lutjohann, D., Kerksiek, A., van Kruchten, R., Maeda, N., Staels, B., van Bilsen, M., Shiri-Sverdlov, R. & Hofker, M.H. 2008, "Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis", *Hepatology (Baltimore, Md.),* vol. 48, no. 2, pp. 474-486.

Wright, S.D., Burton, C., Hernandez, M., Hassing, H., Montenegro, J., Mundt, S., Patel, S., Card, D.J., Hermanowski-Vosatka, A., Bergstrom, J.D., Sparrow, C.P., Detmers, P.A. & Chao, Y.S. 2000, "Infectious agents are not necessary for murine atherogenesis", *The Journal of experimental medicine,* vol. 191, no. 8, pp. 1437-1442.

Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X.F. & Achong, M.K. 1998, "IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses", *The Journal of clinical investigation,* vol. 101, no. 2, pp. 311-320.

Xu L, Dai Perrard X, Perrard JL, Yang D, Xiao X, Teng BB, Simon SI, Ballantyne CM, Wu H 2015. Foamy monocytes form early and contribute to nascent atherosclerosis in mice with hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 35:1787-97.

Yoshimoto, T., Nakanishi, K., Hirose, S., Hiroishi, K., Okamura, H., Takemoto, Y., Kanamaru, A., Hada, T., Tamura, T. & Kakishita, E. 1992, "High serum IL-6 level reflects susceptible status of the host to endotoxin and IL-1/tumor necrosis factor", *Journal of immunology (Baltimore, Md.: 1950),* vol. 148, no. 11, pp. 3596-3603.

Yoshino, S., Sasatomi, E., Mori, Y. & Sagai, M. 1999, "Oral administration of lipopolysaccharide exacerbates collagen-induced arthritis in mice", *Journal of immunology (Baltimore, Md.: 1950),* vol. 163, no. 6, pp. 3417-3422.

Yoshino, S., Yamaki, K., Taneda, S., Yanagisawa, R. & Takano, H. 2005, "Reactivation of antigen-induced arthritis in mice by oral administration of lipopolysaccharide", *Scandinavian Journal of Immunology,* vol. 62, no. 2, pp. 117-122.

Young, J.L., Libby, P. & Schönbeck, U. 2002, "Cytokines in the pathogenesis of atherosclerosis", *Thrombosis and haemostasis,* vol. 88, no. 4, pp. 554-567.

Youngner, J.S. 1972, "Bacterial lipopolysaccharide: oral route for interferon production in mice", *Infection and immunity,* vol. 6, no. 4, pp. 646-647.

Yvan-Charvet, L., Tall, A.R., Welch, C., Pagler, T.A., Ranalletta, M., Lamkanfi, M., Han, S., Ishibashi, M., Li, R. & Wang, N. 2008, "Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions", *Circulation,* vol. 118, no. 18, pp. 1837-1847.

Yvan-Charvet, L., Wang, N. & Tall, A.R. 2010, "Role of HDL, ABCA1, and ABCG1 Transporters in Cholesterol Efflux and Immune Responses", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 30, no. 2, pp. 139-143.

Zaina, S., Lindholm, M.W. & Lund, G. 2005, "Nutrition and aberrant DNA methylation patterns in atherosclerosis: more than just hyperhomocysteinemia?", *The Journal of nutrition,* vol. 135, no. 1, pp. 5-8.

Zeisel, S.H., Mar, M.H., Howe, J.C. & Holden, J.M. 2003, "Concentrations of choline-containing compounds and betaine in common foods", *The Journal of nutrition,* vol. 133, no. 5, pp. 1302-1307.

Zernecke, A. & Weber, C. 2010, "Chemokines in the vascular inflammatory response of atherosclerosis", *Cardiovascular research,* vol. 86, no. 2, pp. 192-201.

Zernecke, A., Shagdarsuren, E. & Weber, C. 2008, "Chemokines in Atherosclerosis: An Update", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 28, no. 11, pp. 1897-1908.

Zhang, A.Q., Mitchell, S.C. & Smith, R.L. 1999, "Dietary precursors of trimethylamine in man: a pilot study", *Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association,* vol. 37, no. 5, pp. 515-520.

Zhu X. et al 2010, "Macrophage ABCA1 reduces MyD88-dependent Toll-like receptor trafficking to lipid rafts by reduction of lipid raft cholesterol ", *J. Lipid Res.,* vol. 51, pp. 3196-3206.

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