THE EFFECT of INTERFERON-γ AND INTERLEUKIN-15, -20, -22 AND -37 ON MACROPHAGE GENE EXPRESSION AND UPTAKE OF MODIFIED LOW-DENSITY LIPOPROTEIN IN RELATION TO ATHEROSCLEROSIS

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A thesis presented for the degree of Master of Philosophy

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Abstract

Atherosclerosis, a chronic inflammatory disorder of the vasculature, is the underlying cause of heart attacks and stroke and responsible for most deaths in western society. A range of cytokines regulates the initiation, progression and clinical complications of atherosclerosis and affects all the cell types present in a lesion. Macrophages play an important role in all stages of the disease from the uptake of modified lipoproteins to amplification of the inflammatory response to plaque rupture.

Because of the key roles of cytokines in atherosclerosis, it is important that their effects on macrophage properties and gene expression are fully understood. Although the action of classical cytokines, such as interferon-γ (IFN-γ), on macrophages and atherosclerosis is well understood, this is not the case with those that have been more recently identified such as interleukin (IL)-15, -20, -22 and -37. The aim of this study was therefore to investigate the effect of these cytokines using human THP-1 macrophages and mouse RAW264.7 macrophages as model systems. The action of IFN-γ was used as a positive control.

Real-time quantitative polymerase chain reaction showed that IFN-γ induced the mRNA expression of two markers of inflammation, monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1), in THP-1 macrophages. From the other cytokines analysed, IL-20, IL-22 and IL-37 had no effect on the expression of these genes. In contrast, IL-15 inhibited the mRNA expression of both MCP-1 and ICAM-1. Western blot analysis indicated that the changes in ICAM-1 mRNA expression by IFN-γ and IL-15 were accompanied by corresponding changes at the level of the protein. Initial experiments on the effect of IFN-γ or IL-15 on the uptake of modified low-density lipoprotein by THP-1 macrophages were also carried out though firm conclusions could not be made and further optimisation will be required.

The effect of IL-15 on MCP-1 mRNA expression in human THP-1 macrophages contrasted with a previous study that showed induced expression in mouse RAW264.7 macrophages. Experiments in these cells indeed showed that IL-15 induced the mRNA expression of MCP-1, and also ICAM-1. The studies provide new insight into the action of recently identified cytokines on macrophage gene expression and suggest the potential existence of species-specific effects of IL-15 that needs to be further investigated.

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LIST OF ABBREVIATIONS

Abbreviation	Full Term
ABC	ATP binding cassette transporter
AcLDL	Acetylated LDL
ApoE	Apolipoprotein E
BMI	Body mass index
CHD	Coronary heart disease
CSF	Colony stimulating factors
CVD	Cardiovascular disease
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxyribonucleotide triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
ECs	Endothelial cell
ECM	Extracellular matrix
FCS	Foetal calf serum
G	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDL	High density lipoprotein
HI-FCS	Heat inactivated FCS
Hr	Hour
ICAM-1	Intercellular adhesion molecule-1
IDL	Intermediate density lipoprotein
IFN-γ	Interferon-γ

IL	Interleukin
ILE	The internal elastic lamina
IL1F7	Interleukin 1 family member 7
IL-TIF	Interleukin-10-related T-cell-derived inducible factor
JAK	Janus kinase
LDL	Low density lipoprotein
LDLR	LDL receptor
LPL	Lipoprotein lipase
MCP-1	Monocyte chemoattractant protein-1
m MCP-1	Mouse MCP-1
M-CSF	Macrophage-colony stimulating factor
m ICAM-1	Mouse ICAM-1
m IFN-γ	Mouse IFN-γ
m IL	Mouse IL
Min	Minute
MIP-1	Macrophage inflammatory protein-1
mmLDL	Minimally modified LDL
MMLV	Moloney murine leukaemia virus
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
MPO	Myeloperoxidase
oxLDL	oxidised LDL
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol-12-myristate-13-acetate
QPCR	Quantitative PCR
ROS	Reactive oxygen species

RNA	Ribonucleic acid
RT-PCR	Reverse transcription-PCR
SDS	Sodium dodecyl sulfate
SMase	Sphingomyelinase
SMCs	Smooth muscle cells
SR-A	Scavenger receptor-A
STAT	Signal transducer and activator of transcription
sPLA2	Secretory phospholipase A2
TBE	Tris/borate/EDTA
TGF-β	Transforming growth factor-β
Th	T-helper
TL1A	TNF-like protein 1A
TNF-α	Tumour necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein
WHO	World Health Organisation

CHAPTER 1

GENERAL INTRODUCTION

1.1 An Overview

Atherosclerosis, which comes from the Greek word “athero” (meaning gruel or paste) and “sclerosis” (hardness) (The American Heart Association, 2010), is the principle cause of cardiovascular disease (CVD) and responsible for approximately 40% of all deaths in the western world. It is a chronic inflammatory disease that is characterised by the accumulation of lipids and fibrous elements in medium and large arteries (Lusis et al. 2004; Moore and Tabas, 2011). Coronary heart disease (CHD) and stroke are the major CVD (George and Lyon, 2010). Based on the data from the British Heart Foundation, the number of premature deaths caused by CVD in 2008 was more than 50,000 in the UK (28% men and 20% women) (Scarborough et al. 2010). The incidence of CVD in developing countries is also increasing as they acquire westernised lifestyles. Moreover, because of the global increase in diabetes and obesity, it is anticipated that CVD will be the major killer worldwide in the near future (Libby, 2002).

Atherosclerosis starts early in childhood and progresses during the lifetime of an individual due to several risk factors (Berenson, 2002). The pathogenesis of atherosclerosis involves several cell types, such as endothelial cells (ECs), smooth muscle cells (SMCs), macrophages and T lymphocytes (Montecucco et al. 2007).

1.2 Risk Factors

There are numerous environmental and genetic risk factors that influence the development and progression of atherosclerosis (Lusis, 2000). Major risk factors include obesity, hypertension, diabetes, high levels of cholesterol (Lusis et al. 2004), age, smoking, physical inactivity and stress (Harvey and Ramji, 2005). Many of these risk factors are not reversible but some can be modified (George and Johnson, 2010). Table 1.2.1 lists some genetic and environmental risk factors in atherosclerosis.

Table 1.2.1 Genetic and environmental risk factors in atherosclerosis

Environmental Risk Factors
• Smoking • High fat diet • Lack of exercise • Low anti-oxidant intake • Infection • Obesity • Type II diabetes • Elevated blood pressure
Genetic Risk Factors
• Elevated plasma C-reactive protein • Gender • Age

Adapted from Glass and Witztum, 2001; and Lusis et al. 2004

1.2.1 Age

It is known that age is one of the most significant unmodified risk factor for CVD (Stocker and Keaney, 2004). According to a study in the United States, the percentage risk of developing CVD is sevenfold more in males aged 60-64 years than those aged 30-34-years (Wilson et al. 1998).

1.2.2 Gender

Many studies have shown that, in contrast with aged-matched females, males are more at risk from CVD (Barrett-Connor and Bush, 1991). It is generally believed that oestrogen provides women with protection against the development of CVD, but not after menopause. However, this is difficult to prove since treatment with estrogens in women after menopause does not reduce CVD (Kleemann et al. 2008). It has also been suggested that some of this protection is related to the fact that women compared with age matched men have higher levels of the protective high density lipoproteins (HDL) (Stocker and Keaney, 2004). HDL is a type of lipoprotein that is usually known as good cholesterol since it works by removing unwanted cholesterol from the peripheral tissues to the liver for excretion via the bile system (McLaren et al. 2011).

1.2.3 Smoking

Numerous studies have identified a relationship between smoking and risk of CHD (Buechley et al. 1958). A study by a group of British doctors found that smokers over 50 were at a 60% higher risk of developing CHD (80% higher risk for heavy smokers) than non-smokers aged 50 and over. Smoking appears to decrease levels of HDL, increase the concentration of atherogenic low density lipoprotein (LDL), cause damage to the wall of artery within the endothelial lining and impair the function of the endothelium (Altman, 2003).

1.2.4 Obesity and body mass index

The risk of CHD increases with obesity and high BMI. Although obesity is an independent risk factor in CHD, it often predisposes the individual to other risk factors such as diabetes, increased blood cholesterol levels and high blood pressure (George and Johnson, 2010). The increase in obesity is demonstrated by a study in England

which reported that the percentage of obese men aged 16 or over rose from 14% to 25% between 1994 and 2008 respectively, while the percentage of obese women increased from 19% in 1994 to 28% in 2008 (Scarborough et al. 2010). A major current concern is the increase in obesity in developing countries (Stocker and Keaney, 2004).

1.2.5 Diabetes

The risk of CHD increases with diabetes as many studies have shown that the risk is 2-4-fold higher in men with type 2 diabetes (non-insulin dependent diabetes) and about 3-5-fold higher in females (George and Johnson, 2010). Hyperglycaemia is caused when the level or function of insulin is insufficient (Libby et al. 2002; Lusis et al. 2004). Diabetes is also known to influence the effect of other risk factors for CHD, such as obesity, elevated blood pressure and high levels of cholesterol (Yusuf et al. 2004).

1.2.6 High blood pressure

High blood pressure is known to be one of the most serious health problems that predisposes an individual to atherosclerosis (Stocker and Keaney, 2004). INTERHEART studies have shown that the percentage of heart attacks in Western Europe among patients who have a history of high blood pressure is 22% (Yusuf et al. 2004). It is possible to lower blood pressure by drug treatment, weight loss, increasing physical activities and reducing the intake of alcohol (Scarborough et al. 2010).

1.2.7 High cholesterol levels

The risk of CHD is also directly proportional to high levels of plasma LDL cholesterol (Gotto, 2011). High levels of LDL is one of the major risk factors that can lead to the progression of atherosclerosis even in the absence of other risk factors (Glass and Witztum, 2001). Many epidemiological studies and clinical trials using cholesterol-lowering drugs, statins, have reported a reduction in CVD with a decrease in plasma LDL cholesterol levels (Sacks et al. 1996; Liao and Laufs, 2005). According to the WHO, high total blood cholesterol is responsible for over 60% of CHD and about 40% of ischemic stroke in developed countries (George and Johnson, 2010). The inflammatory response that is triggered in atherosclerosis due to damage or activation of

the endothelium is mainly a result of high levels of LDL (Singh and Ramji, 2006). It is possible to reduce blood cholesterol levels through a change in diet, physical activity and drugs such as statins (Scarborough et al. 2010).

1.2.8 Genetic risk factors

Many genetic risk factors for cardiovascular disease have been identified. For example, it is known that mutation in genes such as lipoprotein lipase (LPL), the LDL receptor (LDLR), apolipoprotein E (ApoE), and the ATP-binding cassette transporter (ABC) A1 significantly increases risk for cardiovascular disease (Puddu et al. 2005).

1.3 Lipoproteins and atherosclerosis

Insoluble lipids are transported in the blood in lipoproteins particles. Lipoproteins consist of a core region, with triacylglycerols and cholesterol esters, surrounded by a polar region consisting of phospholipids and apolipoproteins (Beisiegel, 2008). As mentioned above, high levels of plasma cholesterol appears to be unique because of its ability to drive the progression of atherosclerosis in humans and experimental animal models even in the absence of the various other environmental and genetic risk factors (Glass and Witztum, 2001).

Cholesterol is a sterol that is essential for membrane fluidity and most of it is formed in the body by de novo synthesis. Cholesterol can also be consumed in the diet and needs to be transported to different parts of the body where it is needed. Several lipoprotein particles carry serum cholesterol and triacylglycerols. Overall, five major types of lipoproteins can be distinguished, including chylomicrones, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL and HDL (Gotto et al. 1986; McLaren et al. 2011). The different types of lipoproteins are presented in Figure 1.3.1 where their roles in the transport of cholesterol and triacylglycerols are also described.

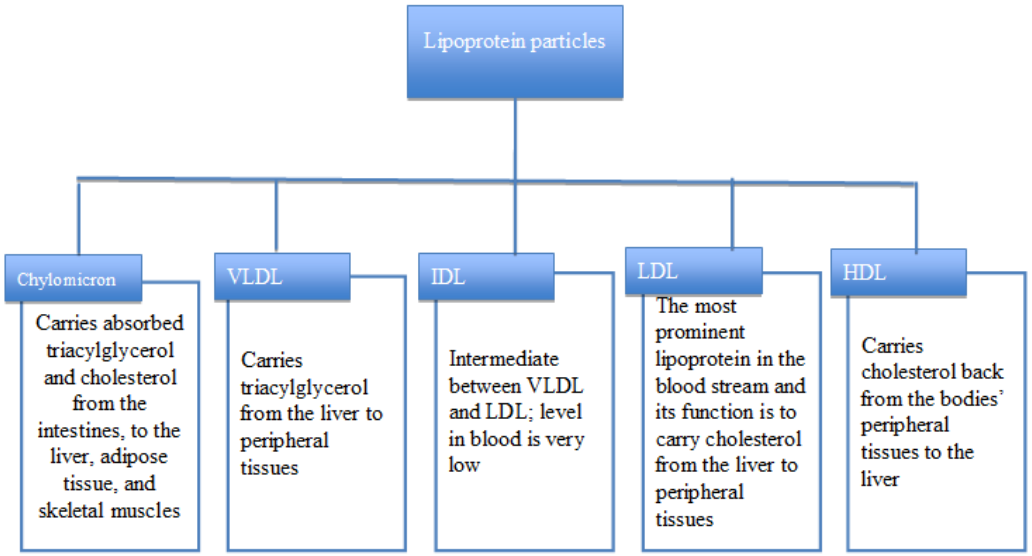

Figure 1.3.1 Lipoprotein particles and their roles in cholesterol transport. Adapted from McLaren et al. 2011

1.4 History of Atherosclerosis

Atherosclerosis is now well accepted as a chronic inflammatory disease of the large and medium arteries (Hansson, 2005; Tedgui and Mallat, 2006). In 1755, the term *atheroma* (from Greek meaning "lump of gruel") was first used by Albrecht von Haller to describe the intimal degenerative process that he noticed in the arteries (Tedgui and Mallat, 2006). Since that time there have been several opinions and much discussion about the major cause of atherosclerosis as a result of medical and scientific research into the disease. In the early 1900, it was generally believed that atherosclerosis was due to the aging process (McLaren et al. 2011). Thereafter, Rudolf Virchow (1821–1902), one of the major contributors to the thrombosis view, suggested that atheroma was likely to be a chronic inflammatory disease characterised by lipid accumulation in the intima of arteries. In 1904, Leipzig Marchand was the first to use the term "atherosclerosis" to describe the deterioration of the arterial intima layer, which has since been widely used up to the present time (Tedgui and Mallat, 2006). A decade later (1913), Anichkov used a rabbit model of atherosclerosis to show that high levels of cholesterol was the main

reason for this disease (Konstantinov et al. 2006). Anichkov's discovery facilitated many studies to investigate the effects of cholesterol during the progression of atherosclerosis. Although doubts regarding the effect of high levels of cholesterol in atherosclerosis still remained, accumulating evidence led the American Heart Association to recommend in 1961 that individuals at high risk of atherosclerosis change their diet in order to reduce the intake of cholesterol. However, there were still those who claimed that altering the diet would not cause a reduction in the disease (Steinberg, 1969; Ahrens, 1979; McLaren et al. 2011). In 1984, National Institute of Health reported that there was a significant reduction in cardiovascular endpoints when the level of blood cholesterol was reduced. Since that time, numerous research studies have shown a correlation between high plasma cholesterol levels and atherosclerosis (McLaren et al. 2011).

1.5 Pathology of Atherosclerosis

There are three morphological layers present in the normal artery: the intima, media and adventitia, which are shown in Figure 1.5.1. The intima, the innermost layer, is made up of a monolayer of ECs that is surrounded by the extracellular connective tissue matrix, primarily proteoglycans and collagen, and a sheet of elastic fibres, the internal elastic lamina (ILE). Beneath the ILE is the media, the middle layer that consists of SMCs. The adventitia, the outer layer, consists of connective tissues with interspersed fibroblasts and SMCs (Lusis, 2000; Stocker and Keaney, 2004).

As shown in Figure 1.5.2, ECs activation is initiated by the various risk factors, particularly the accumulation of LDL and lipoprotein remnants (derived from enzymatic action on various lipoprotein particles) in the sub-endothelial intima (Tabas et al. 2007). LDL retention arises when its levels in plasma are very high, especially in certain areas of the arteries, such as branching or curvature (McLaren et al. 2011). As a result of endothelial dysfunction by numerous risk factors, a variety of chemoattractant

molecules are expressed by the ECs, which, in turn, attract the monocytes and T lymphocytes in the blood stream to recruit at the site of injury/activation. Monocytes are able to interact with these chemoattractant signals and migrate into the sub endothelial matrix. Here the monocytes differentiate into macrophages to form foam cells by taking up modified LDL from the sub endothelial environment. This process is called fatty streak formation and it is the first step in the initiation of this disease (Lusis, 2000). Fatty streaks subsequently "grow" into more complex lesions and form a fibrous cap composed of SMCs, which have migrated from the media into the intima, where they secrete extracellular matrix (ECM) molecules, such as elastin and collagen (Katsuda and Kaji, 2003; Newby, 2006). The fibrous cap continues to develop with the formation of a "necrotic core" due to the deposition of cholesterol and lipids from dying foam cells, to form a stable complex lesion (Newby, 2006). The balance between ECM synthesis and breakdown of the atherosclerosis plaque is responsible for the control of plaque stability (McLaren et al. 2011).

Although the blood flow through the artery would be reduced considerably if the plaque became larger, leading to angina and chest pains, the formation of a blood clot ("thrombus") is the most difficult complication of atherosclerosis, and can lead to myocardial infarction (Lusis et al. 2004). Ischemic stroke is caused if the atherosclerosis has an effect on the arteries that provide the brain with oxygen and blood (Harvey and Ramji, 2005). The above steps are reviewed in more detail in the following sections.

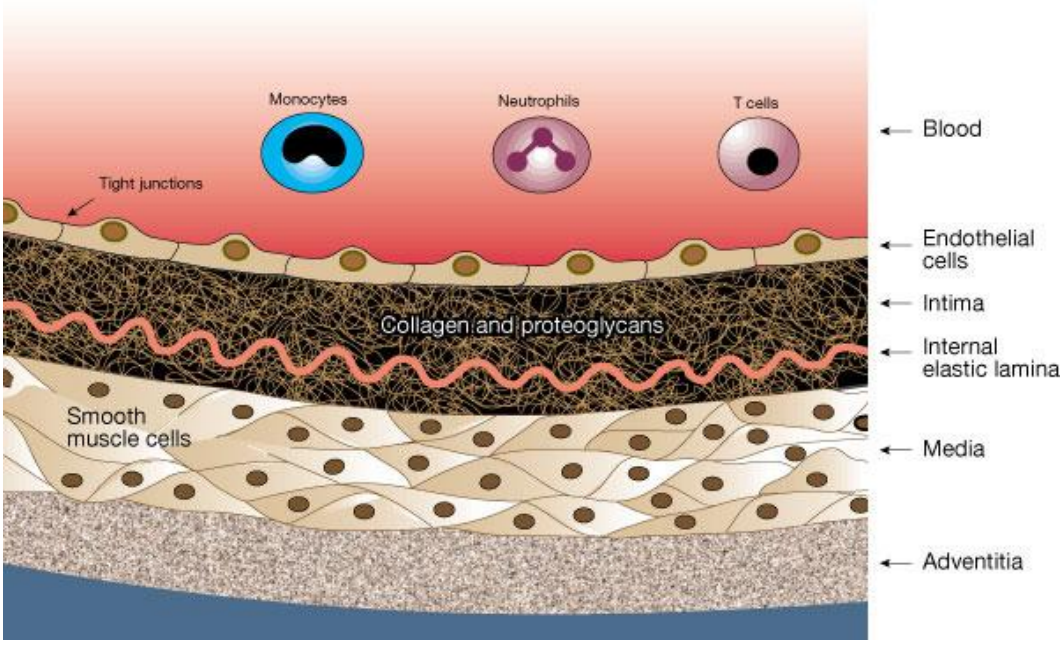

Figure 1.5.1 Structure of a normal large artery

A large artery consists of three morphologically distinct layers. The intima, the innermost layer, is bounded by a monolayer of ECs surrounded by extracellular connective tissues matrix, primarily proteoglycans and collagen, and a sheet of elastic fibres, the internal elastic lamina. The media, the middle layer, consists of SMCs. The adventitia, the outer layer, consists of connective tissues with interspersed fibroblasts and SMCs. Taken from Lusis, 2000.

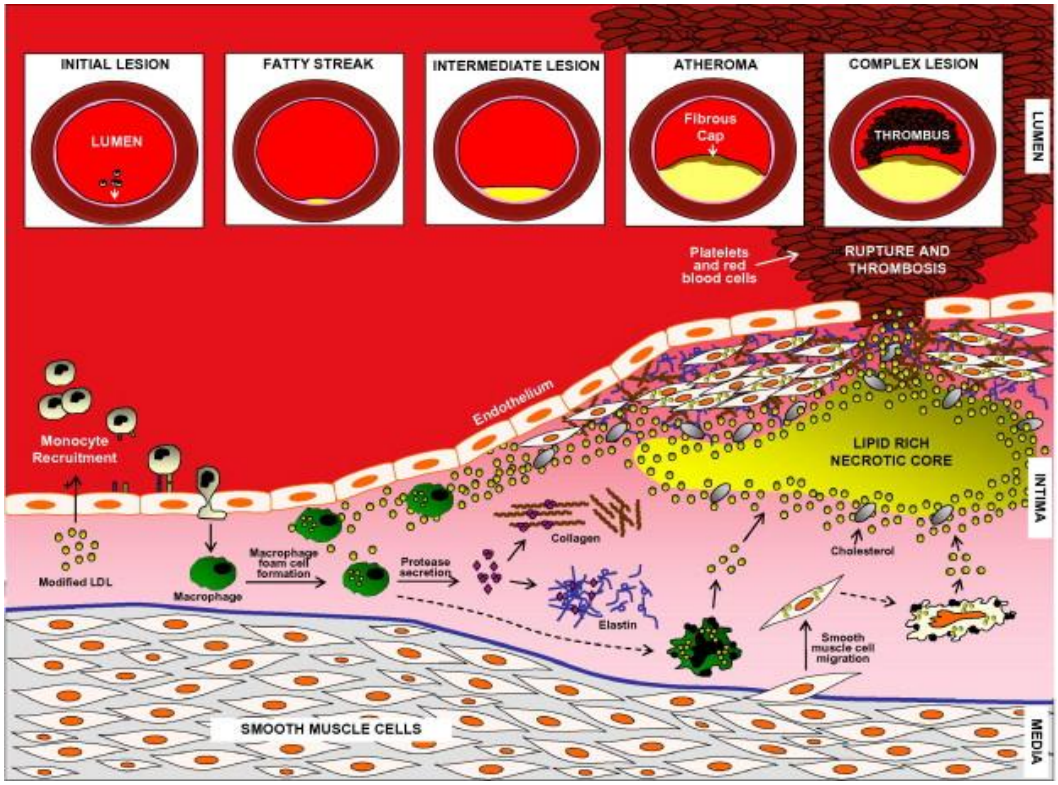

Figure 1.5.2 The development of an atherosclerotic lesion At the site of an initial lesion, a fatty streak is formed when the monocytes respond to chemoattractant molecules that are secreted by the activated endothelium, and migrate into the subendothelial space, where they differentiate into macrophages. Macrophages ingest oxidised LDL (oxLDL) to form lipid-laden foam cells. Fatty streaks mature into a more advanced lesion covered with a fibrous cap that is formed as a result of SMCs' migration and their expression of ECM. The foam cell secretes protease that degrades the ECM that, in turn, reduces the stability of the fibrous cap leading to rupture. This leads to the release of the lesion contents into circulation, resulting in thrombosis and subsequent artery blockage. Taken from McLaren et al. 2011.

1.6 Atherosclerosis initiation

The formation of fatty streaks is one of the first characteristics of atherosclerosis in the intima of large and medium arteries (Tabas et al. 2007). Fatty streaks are not clinically significant, and may be precursors of larger atherosclerotic plaques (Tamminen et al. 1999). Although they form an important part of the lesion, fatty streaks cannot cause acute events such as heart attack or stroke (Halvorsen et al. 2008). A range of risk factors work to activate the endothelium, change its permeability and initiate the lesion (Preiss and Sattar, 2007). However, it is thought that the accumulation of apoB-containing lipoproteins, such as LDL and lipoprotein remnant, in the sub-endothelial intima, is the main initiator of atherosclerosis (Tabas et al. 2007; McLaren et al. 2011).

LDL retention arises when the levels of plasma LDL are high, particularly in areas such as arterial branching or curvature (Lusis, 2000). When LDL accumulates in the sub-endothelial intima, it undergoes various modifications, including oxidation (Ross, 1993). Diffusion of LDL through the endothelial cells and its retention in the vessel wall are promoted by the interaction between matrix proteoglycans and the apoB constituent of LDL (Grainger et al. 1994; Lusis, 2000). It is thought that the production of proteoglycans increases as a result of stress on the arterial wall by the various risk factors (Libby et al. 2002).

Normal LDL can be taken up by the LDL receptors (LDLR) that are present in most peripheral cell types, including macrophages. However, uptake of LDL by LDLR is subject to negative feed back regulation, and hence cannot make a substantial contribution to foam cell formation. Initial oxidation of LDL produces minimally modified LDL (mmLDL) that is still recognised by its native receptor. The production and secretion of the chemoattracting chemokine, monocyte chemoattractant protein-1 (MCP-1) by SMCs and EC is stimulated by mmLDL to promote monocyte recruitment (Stocker and Keaney, 2004).

As shown in Figure 1.6.1, before LDL can be taken up sufficiently by a range of scavenger receptors expressed by macrophages in order to form foam cells, it must be highly oxidised. It is likely that the modification of LDL happens as a result of the interaction between LDL and reactive oxygen species (ROS) that are produced by endothelial cells and macrophages, particularly by enzymes such as myeloperoxidase (MPO), sphingomyelinase (SMase), and a secretory phospholipase (sPLA2). The uptake of modified LDL by scavenger receptors is not under negative feedback regulation and hence makes a major contribution to foam cell formation (Lusis, 2000).

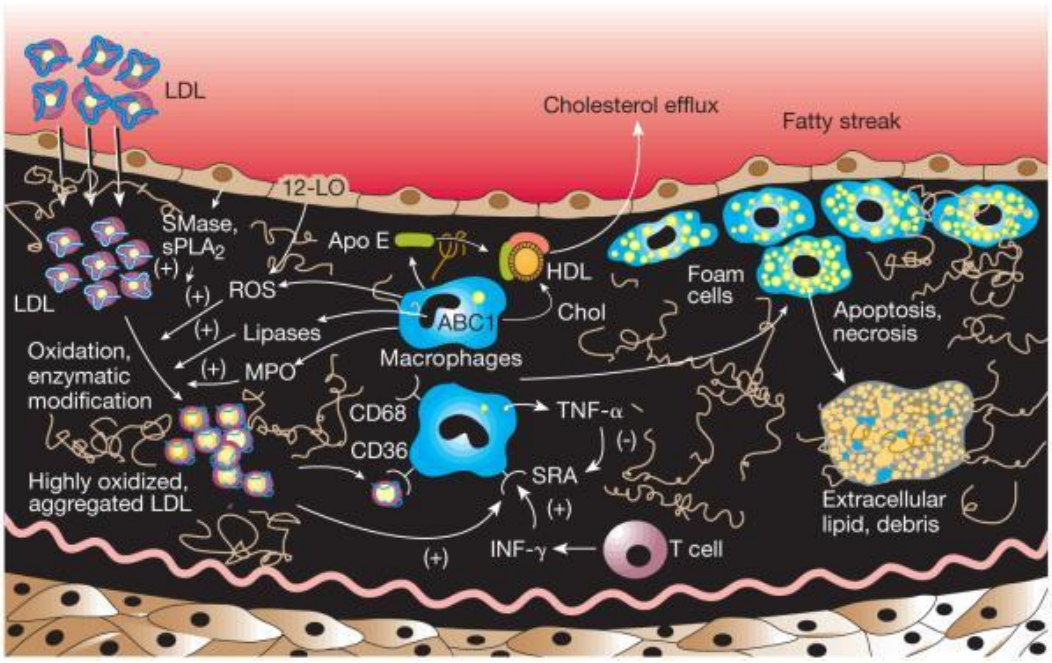

Figure 1.6.1 Atherosclerosis initiation and foam cell formation

When LDL enters the subendothelium from the blood stream, it can undergo modification by the retention within the matrix component in the intima. Oxidation of LDL takes place after its interaction with ROS produced by the endothelium via the action of enzymes such as MPO, SMase and sPLA2. Oxidised LDL is quickly taken up by macrophage scavenger receptors such as SR-A, CD36 and CD68. Scavenger receptor expression is regulated by cytokines such as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). The concentration of nutrients becomes low with continued expansion of the lesion, which causes the cells in the middle of the lesion to die, thereby forming the necrotic core of the lesion. Abbreviations: LDL, low density lipoprotein; ROS, Reactive oxygen species; MPO, myeloperoxidase; SMase, sphingomyelinase; sPLA2, secretory phospholipase A2; TNF-α, Tumour necrosis factor-α; INF-γ, Interferon-γ and apolipoprotein E (apoE). Taken from Lusis, 2000.

1.7 Recruitment of monocytes/macrophages to sites of inflammation

Monocytes and macrophages are present at all stages of the disease, have multifunctional roles in lesion progression, and make about 60-70% of cells in the advanced plaque (Ross, 1993; Takahashi et al. 2002). As a result of LDL retention and oxidation, a chronic inflammatory response is triggered. As shown in Figure 1.7.1, a variety of chemoattractant molecules are expressed by the activated ECs, which recruit the monocytes from the blood stream to the site of injury/activation (Li and Glass, 2002). On the luminal surface of the activated endothelium, adhesion molecules, such as P-selectin and E-selectin, are expressed in order to initiate the rolling of monocytes. These selectins interact with L-selectin molecules on the surface of monocytes (Bobryshev, 2006). Studies have shown that apoE deficient mice, a mouse model for this disease, that lack adhesion molecules such as P- selectin, E-selectin and intercellular adhesion molecule-1 (ICAM-1), have reduced lesions (Collins et al. 2000). These findings therefore emphasise the important roles of these adhesion molecules in atherosclerosis (Collins et al. 2000).

The monocytes roll across the surface of the endothelium as a result of the interaction between these selectins (Lusis, 2000; Li and Glass, 2002). Adhesion and migration of monocytes is facilitated by other molecules that are expressed by the activated endothelium, such as the vascular cell-adhesion molecule-1 (VCAM-1) and ICAM-1, which bind to integrins on recruited monocytes (Meerschaert and Furie, 1995; Li and Glass, 2002; McLaren et al. 2011). The diapedesis process of monocytes is further enhanced by the expression of chemokines and cytokines by ECs such as MCP-1, macrophages colony-stimulating factor (M-CSF), interleukin-8 (IL-8), TNF-α, macrophage inflammatory protein-1 (MIP-1) and transforming growth factor-β (TGF-β) (Li and Glass, 2002; Weber et al. 2007).

1.7.1 Chemokine MCP-1

Chemotactic factors (or chemokines) as well as adhesion molecules mediate the entry of monocytes into the intima and this process can be stimulated by oxLDL, which triggers an inflammatory response (Li and Glass, 2002). Chemokines are small heparin-binding proteins (Charo and Taubman, 2004; Rot and von Andrian, 2004). About 50 human chemokines have been identified. The CC chemokines is the largest known family (Charo and Taubman, 2004). The chemokine MCP-1 (also known as CCL2) is one of the most broadly studied members of the CC chemokine family due to its important role in the recruitment of monocytes from the blood stream into the intima of the atherosclerotic plaque (Charo and Taubman, 2004). MCP-1 and its receptors CCR2 are expressed in atherosclerotic lesions (Papadopoulou et all. 2008). MCP-1 is expressed by many important cells in atherosclerosis such as ECs, macrophages and SMCs (Glass and Witztum, 2001). Studies in apoE-deficient mice have shown that the expression of MCP-1 is induced by oxLDL, and a reduction in the atherosclerotic plaque is noticed when this gene is deleted (Navab et al. 1996; Boring et al. 1998).

1.7.2 Adhesion molecules

As defined by the Gene Ontology Consortium (http://www.geneontology.org/), cellular adhesion molecules are "molecules expressed on the surface of a cell that mediate the adhesion of the cell to other cells or to the extracellular matrix" (Blankenberg et al. 2003). The recruitment of inflammatory cells from the blood stream as well as their migration through the endothelium is one of the earliest stages of atherosclerosis. A variety of cellular adhesion molecules mediate this process when they are expressed on the surface of the endothelium and on circulating leukocytes in order to respond to many inflammatory stimuli (Blankenberg et al. 2003). When endothelial function is impaired, different pro-inflammatory cytokines, such as IFN-γ and TNF-α, stimulate the production of adhesion molecules such as ICAM-1 and VCAM-1, which leads to increased atherosclerosis (Catalan et al. 2012). Together with P-selectin and E-selectin, VCAM-1 mediates the first step in the rolling of monocytes (Nakashima et al. 1998).

The migration of monocyte through the endothelium is facilitated when VCAM-1 and ICAM-1 start adhesion by binding to integrins expressed on the surface of monocytes (McLaren et al. 2011).

1.8 Development of an atherosclerotic lesion and smooth muscle cell migration

Once the monocytes have passed through the endothelial layer by diapedesis and are present in the intima of the blood vessels, their differentiation can take place (Lusis, 2000). Endothelial cells express M-CSF that mediates the differentiation of monocytes into macrophages (Hansson and Libby, 2006). The macrophage is a very important cell in the pathology of atherosclerosis due to its ability to uptake modified LDL and become a cholesterol-rich foam cell (Lusis, 2000). The expression of scavenger receptors on the surface of macrophages is induced as a result of this differentiation (Moore and Freeman, 2006).

Under normal conditions the cellular uptake LDL is mediated by the LDLR (Soutar and Naoumova, 2007). The receptor is on the cell surface and when LDL binds, the complex is internalised and transported to the lysosome where the cholesterol is released for use in the cell. The LDLR is then recycled back to the cell surface for subsequent use (Soutar and Naoumova, 2007). Although the cells can take up LDL via the LDLR, this is under negative feedback regulation. If there is a high concentration of LDL then the LDLR expression will be reduced and cannot therefore contribute substantially to foam cell formation (Soutar and Naoumova, 2007). However, the uptake of modified LDL by scavenger receptors such as SR-A and CD36, is not under such negative feedback regulation and therefore contributes to foam cell formation due to the uncontrolled macrophage uptake of modified-LDL particles from the subendothelial matrix (Febbraio et al. 2000; Lusis, 2000; Bobryshev, 2006). It is essential that LDL should be modified, such as highly oxidised, for scavenger receptors to be able to recognise it. Oxidised LDL, but not mmLDL, is recognised by CD36 and SR-A (McLaren et al. 2011). Normally, the function of scavenger receptors is to take up pathogens and molecules derived from them, but in atherosclerosis they work to recognise oxidised LDL (Li and Glass, 2002).

SR-A is present on macrophages, SMCs as well as ECs. Although it is thought that SR-A's recognition of oxLDL is low, it has been found that it is responsible for about 80% of acetylated LDL (AcLDL) uptake (Moore and Freeman, 2006). Smaller atherosclerotic lesion has been observed in $ApoE^{-/-}$ mice lacking CD36, which supports the role of CD36 in atherogenesis *(*Febbraio et al. 2000). Extensive study using mouse models of atherosclerosis have shown that reducing the expression of scavenger receptor, the atherosclerotic lesions were reduced (Lusis 2000; Bobryshev, 2006).

As the disease progresses, the fatty streaks begin to mature into more complex plaques. A fibrous cap made up of SMCs and ECM covers the lesion. The formation of the fibrous cap depends on the migration and proliferation of SMCs from the media to the intima (Newby and Zaltsman, 1999; McLaren et al. 2011). SMCs usually reside in the medial layer of blood vessels where they provide strength and support to the structure of the vessel (Lusis, 2000). Once inside, SMCs proliferate and experience phenotypic modulation from quiescent, contractile phenotype to an active, synthetic form. With time, foam cell macrophages fill up with lipids to a point where they begin to die due to lake of sufficient nutrients, and start to release their contents which lead to the formation of a necrotic core (Dzau et al. 2002). In addition, SMCs enhance foam cell formation by taking up modified lipoproteins themselves and further contributing to the necrotic core of the atherosclerotic lesion (Li and Glass, 2002; McLaren et al. 2011). The intimal SMCs do not only take up modified lipoproteins but also synthesise ECM proteins that help with the building of the fibrous cap (Dzau et al. 2002).

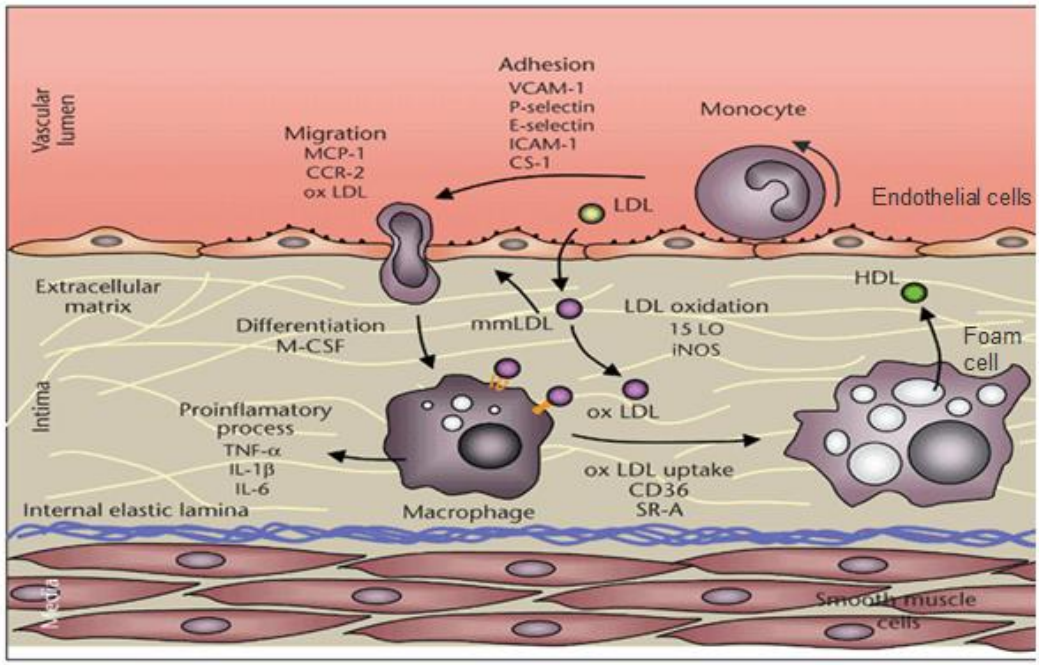

Figure 1.7.1 Recruitment of monocytes/macrophages to sites of inflammation and foam cell formation

LDL is subject to oxidative modification in the subendothelial space, progressing from mmLDL to extensive oxLDL. Monocytes attach to EC that have been induced to express cell adhesion molecules by mmLDL and inflammatory cytokines. Adherent monocytes migrate into the intima where they differentiate into macrophages. Uptake of oxLDL via macrophages scavenger receptors, such as SR-A and CD36, leads to foam cell formation. Abbreviations: mmLDL, Minimally modified low density lipoprotein; oxLDL, Oxidised low density lipoprotein; SR-A: Scavenger receptor A; ICAM-1, Intercellular adhesion molecule-1; VCAM 1, vascular cell adhesion molecule-1; MCP-1, Monocyte chemoattractant protein-1; CCR2, Chemokine receptor-2, M-CSF, Macrophages colony stimulating factor. Taken from Glass and Witztum, 2001.

1.9 Plaque rupture

A stable, complex lesion is formed with the continuous progression of the fibrous cap, which now encloses the new enlarged necrotic core (McLaren et al. 2011). When the atherosclerotic plaque matures into a more complex lesion, it can be described either as a stable plaque or that susceptible to rupture. Stable plaques have a thick fibrous cap, smaller lipid core and few inflammatory cells within the shoulder regions. On the other hand, plaques with a thin fibrous cap, high lipid content in its core and a large number of T-cells and macrophages in the shoulder regions are considered to be unstable and "rupture-prone" (van der Wal and Becker, 1999; Stocker and Keaney, 2004). The balance between ECM synthesis and breakdown is very important for the stability of the plaque (Newby, 2006; McLaren et al. 2011). Inflammatory cells produce cytokines such as IFN-γ that mediates the degradation of ECM (Hansson et al. 2002; Lusis et al. 2004). There are several factors that affect plaque rupture. Some of the factors that influence stability are cytokines, protease expression, and the proliferation, migration and apoptosis of SMCs (Lusis, 2000). An important factor in thinning of the fibrous cap is a reduction in the production of the ECM as a result of apoptosis of SMCs. During the progression of the lesion, cytokines such as IFN-γ are secreted which affects macrophages and causes them to undergo apoptosis (Lusis, 2000, Lusis et al. 2004, McLaren and Ramji, 2009), leading to an increase in the size of the necrotic core. As a result, physical pressure will be exerted on the fibrous cap, causing further apoptosis of SMCs (George and Johnson, 2010). Since IFN-γ also has the ability to prevent the synthesis of collagen in SMCs, any collagen that has been degraded cannot be replaced (Libby et al. 2002; Lusis et al. 2004). In addition, macrophages can produce proteolytic enzymes within the plaque that degrade the collagen of the fibrous cap, which results in its weaking and making it more likely to rupture (Libby et al. 2002). The proteolytic enzymes include matrix metalloproteinases (MMPs)(Newby, 2008). MMPs are a family of matrix-degrading enzymes. In some cases specific MMPs promote stability of plaque by stimulating VSMC migration, and in other cases they degrade ECM components and induce apoptosis of VSMC, and therefore reduce plaque stability. They are therefore considered to be a key feature of advanced atherosclerosis (Newby, 2006; McLaren et al. 2011).

As shown in Figure 1.5.2, the blockage of lumen by an atherosclerotic plaque does not cause the acute symptoms of this disease. It is when the plaque ruptures and the thrombotic reaction starts that the clinical complications of atherosclerosis begins to manifest (McLaren et al. 2011).

1.10 Cytokines

Stanley Cohen was the first to introduce the term cytokines in 1974. They are a large group of proteins with molecular weight ranging from 8 to 40kDa (Tedgui and Mallat, 2006). Lymphokines and monokines are the original names of cytokines, and were used to denote their cellular origins (Balkwill and Burke, 1989). Thereafter, the term cytokines was used and is the best description since the majority of the inflammatory cells have the ability to synthesise cytokines, which, in turn, cause changes in gene expression in target cells and regulation of the progression of the atherosclerotic plaque (Dinarello, 2000). For example, during the progression of atherosclerosis, cytokines are known to control the migration/proliferation of SMCs from the media into the intima (Lusis, 2000). In addition, the expression of key genes implicated in the disease pathology, such as chemokines, adhesion molecules and scavenger receptors, is also regulated by cytokines (Tedgui and Mallat, 2006).

Over 50 cytokines are known and include interleukins, the tumour necrosis factors (TNF), interferons (IFN), colony stimulating factors (CSF), transforming growth factors (TGF) and chemokines (Dinarello, 2000; Tedgui and Mallat, 2006). The importance of cytokines comes from their ability to regulate the immune response (Tedgui and Mallat, 2006). A variety of cytokines have been reviewed and studied, and they have been found to be expressed at different stages of atherosclerosis (Tedgui and Mallat, 2006). The major sources of cytokines in atherosclerosis are macrophages and infiltrating T-lymphocytes. They are classified according to their pro- or anti-inflammatory actions. The levels of pro-inflammatory cytokines have been observed to be high atherosclerotic plaque (Harvey and Ramji, 2005; Halvorsen et al. 2008).

The ability of cytokines to mediate their actions in the disease comes from their interaction with specific receptors on the surface of the cells (Singh and Ramji, 2006). The cytokines that are highly expressed in the atherosclerotic plaque are shown in Figure 1.10.1 along with whether they act as pro-or anti-inflammatory.

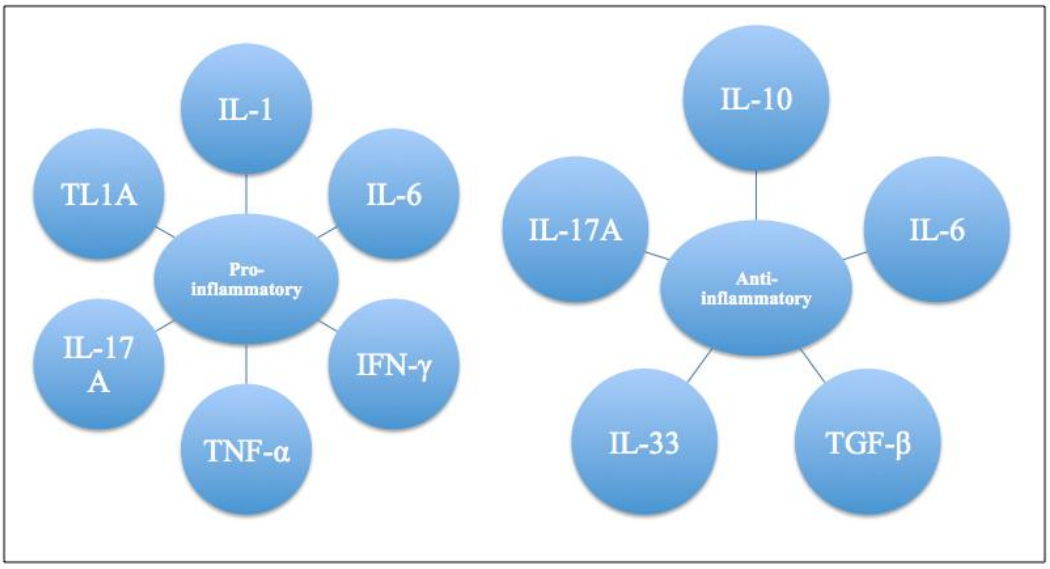

Figure 1.10.1 The cytokines that are highly expressed in the atherosclerotic plaque. IL-1, TNF-α and IFN-γ act as a pro-atherogenic manner whereas IL-10, IL-33 and TGF-β are anti-atherogenic. Both pro- and anti-atherogenic roles have been found for certain cytokines like IL-6 and IL-17A. Adapted from Singh and Ramji, 2006.

Pro-inflammatory cytokines are expressed at high levels in the initial stage of atherosclerosis (Kapoor et all. 2006). For example, IL-1 accelerates atherosclerosis through wide ranging effects including promoting the proliferation of SMCs. It can also induce the expression of MMPs (Kapoor et all. 2006; Tedgui and Mallat, 2006). Anti-inflammatory cytokines serve to limit inflammatory responses often through their ability to suppress the expression of pro-inflammatory cytokines (Dinarello, 2000). TGF-b is expressed in many cell types including EC, SMCs, monocytes/macrophages, platelets and T-cells in relation to atherosclerosis (Singh and Ramji, 2006). It has been shown previously in our laboratory and elsewhere that macrophage apoE expression is

increased by TGF-b stimulation (Singh and Ramji 2006; Zuckerman et al. 1992). In apoE$^{-/-}$ mice fed on a high fat diet, the accumulation of macrophage-derived foam cells in atherosclerotic plaques was reduced significantly when treatment with IL-33 (McLaren et al. 2010b)

Although the effect of classical cytokines, such as IFN-γ, in atherosclerosis and on gene expression in macrophages is well understood, this is not the case for many cytokines, such as IL-37 and recently identified cytokines whose receptors are expressed in these cells such as IL-15, IL-20 and IL-22.

1.10.1 IFN-γ

The human interferon (IFN) family is categorised into two major types. Type I consists of IFN-α, IFN-β, IFN-ε,, IFN-κ, IFN-ω and IFN-ν and type II consists of only IFN-γ (Pestka, 2007). Type I IFNs share common structure and are synthesised by most cell types (Meager, 1998). On the other hand, type II IFN has a single member with a different molecular structure from the type I IFNs and functions through a separate receptor (IFN-γR) (Mire-Sluis and Thorpe 1998; Schroder, Hertzog et al. 2004). IFN-γ, the most studied pro-inflammatory cytokine, is a type II member of the IFN family of cytokines (McLaren and Ramji, 2009). It is highly expressed in atherosclerotic lesions by various cells including monocytes/macrophages and T lymphocytes (Whitman et al. 2000). The main action of IFN-γ is mediated through the Janus Kinase (JAK)/ Signal Transducer and Activator of Transcription (STAT) pathway (Figure 1.10.1.1) (McLaren and Ramji, 2009). IFN-γ accelerates atherosclerosis in mouse models of this disease (Schonbeck et al. 1997; McLaren et al. 2011) by modulating several steps. For example, IFN-γ contributes to the thinning of the fibrous cap by upregulating the expression of MMPs, which work to inhibit the proliferation of SMCs and reducing their production of collagen (Halvorsen et al. 2008; George and Johnson, 2010). Expression of chemokins such as MCP-1 is also induced by the cytokine (Boisuert, 2004; Charo et al, 2004). However, evidence from *in vitro* cell culture model systems suggests that the role of IFN-γ is more complicated, since both pro- and anti-atherogenic properties have been observed (McLaren and Ramji, 2009). Thus, it has been shown to inhibit SR-A and CD36 expression in human monocyte-derived macrophages (McLaren and Ramji, 2009). This suggested a potential role of IFN-γ in reducing foam cell formation. However, other work in THP-1 macrophages and primary cultures has found that IFN-γ induced uptake of oxidised and acetylated LDL and induces the expression of several scavenger receptors (Geng and Hansson, 1992; McLaren and Ramji, 2009).

IFN-γ has also been shown to have an effect on ECs in atherosclerosis. For example, IFN-γ induces the expression of both VCAM-1 and ICAM-1 in ECs, which are very important adhesion molecules in the early stage of developing fatty streaks. This suggests a pro-atherogenic role for it in relation to the endothelium (Cybulsky et al. 1993; Li et al. 1993; Valente et al. 1998; Chung et al. 2002)

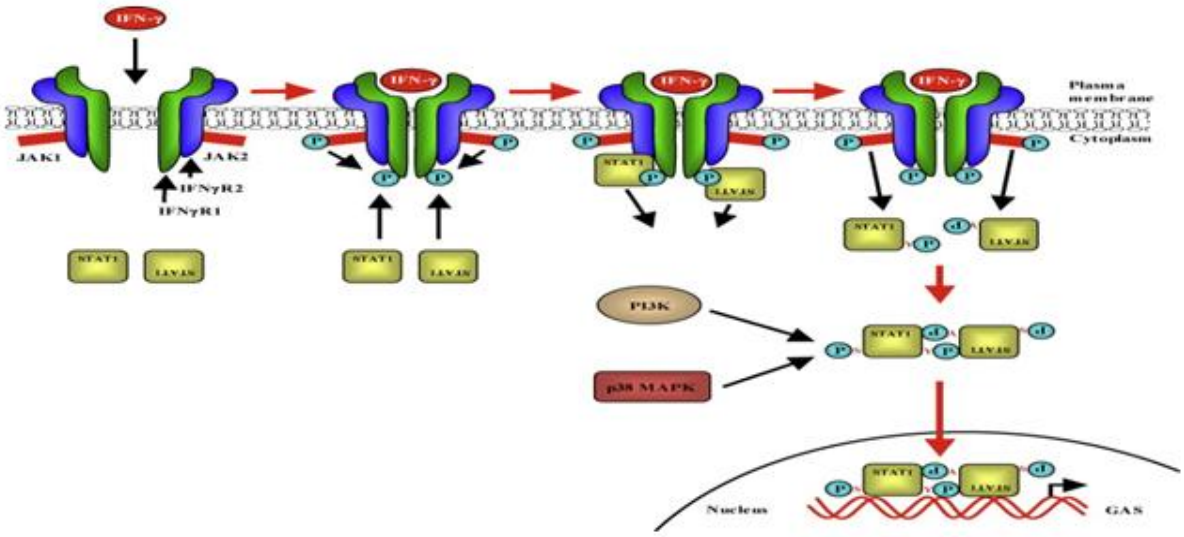

Figure 1.10.1.1 JAK/STAT1 signalling in response to IFN-γ. The binding of IFN-γ to its cognate, cell surface receptor involves dimerisation of the two IFN-γ receptor (IFN-γR) subunits, made up of IFN-γR1: IFN-γR2 pairs, and results in the activation of two bound tyrosine kinases, JAK1 and JAK2, by tyrosine phosphorylation. Once the JAKs become activated, or tyrosine phosphorylated, they mediate tyrosine phosphorylation of the IFN-γ receptor tails through their catalytic, C-terminal kinase domain. Latent STAT1 monomers, located in the cytoplasm are recruited to the receptor tails, via their Src homology 2 (SH2) domains and become phosphorylated on tyrosine 701. The tyrosine phosphorylated STAT1 monomers dimerise forming a STAT1:STAT1 homodimer which can translocate to the nucleus and bind to the interferon gamma activation sequence (GAS) elements in the promoters of IFN target genes. Tyrosine phosphorylated STAT1 homodimers can also be phosphorylated at serine 727 by kinases like phosphatidylinositol 3-kinase (PI3K) and P38 mitogen-activated protein kinase which enhances their transcriptional capacity. Abbreviations: IFN-γ, interferon-γ; JAK, Janus kinase; p38 MAPK, p38 mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; SH2, Src-homology 2; STAT1, Signal transducer and

activator of transcription 1; GAS, the interferon gamma activation sequence. Taken from McLaren and Ramji. 2009.

1.10.2 IL-37

IL-37 is a member of the IL-1 family and was formerly called IL1F7 (Bufler, 2011). The way IL-37 functions remains unclear as limited research has been carried out on this cytokine. The production of pro-inflammatory cytokines was inhibited when IL-37 was expressed in macrophages (Boraschi et al. 2011). In addition the expression of human IL-37 downregulated inflammation in mouse models system (Bufler, 2011; Banchereau et al. 2012). IL-37, which was first identified in 2000, was therefore thought to be an anti-inflammatory cytokine, and is known to bind to the IL-18α receptor (Moschen et al. 2011).

1.10.3 IL-15

IL-15 is a member of the IL-2 family (Benczik and Gaffen, 2004). It is expressed by a variety of cells including monocytes, macrophages and EC, and has been shown to promote atherosclerosis in a mouse model of this disease (Oppenheimer-Marks et al. 1998; Krishnaswamy et al. 1999; Musso et al. 1999; van Es et al. 2010). IL-15 contributes to promote the recruitment of T-cells to the site of inflammation in atherosclerotic lesion (Wuttge et al. 2001). It has a unique receptor (IL-15Rα), which has also been found to be expressed on the surface of macrophages (Giri et al. 1995). Van Es et al. (2010) showed that the expression of MCP-1 and TNF-α was induced significantly when mouse macrophages (RAW264.7 cells) were treated with IL-15, but there was no effect on the expression of adhesion molecules such as ICAM-1 and VCAM-1. They also observed a 75% reduction in plaque size with a surprising 2-fold increase of macrophage content when the mice (LDL definition mice) were given oral vaccination against IL-15, suggesting a pro-inflammatory role of this cytokine in the mouse model (van Es et al. 2010). A decade ago, a study in humans and apoE$^{-/-}$ mice had also found that atherosclerotic lesions contain IL-15 (Houtkamp et al. 2001; Wuttge et al. 2001). However, the role of IL-15 in human macrophages is poorly understood despite its receptors being expressed on these cells.

1.10.4 IL-20

IL-20 is a member of the IL-10 family. It is expressed by macrophages and has been shown to promote atherosclerosis in a mouse model of this disease (von der Thusen et al. 2003; Chen et al. 2006). It acts via receptors that consist of IL-20R1 and IL-20R2 (Ait-Oufella et al. 2011). The main source of IL-20 in an atherosclerotic lesion is macrophages (Chen et al. 2006). A study in premenopausal obese women showed that when compared to matched normal weight controls, the levels of IL-20 were higher in obese women and these levels were reduced with weight loss (Maiorino et al. 2010). This suggests that IL-20 is also involved in obesity which predisposes to atherosclerosis (Maiorino et al. 2010). However, the role of IL-20 in human macrophages is poorly understood despite their receptors being expressed on these cells.

1.10.5 IL-22

IL-22 (previously named ILTIF) is an IL-10 cytokine family member, that is produced by Th17 cells and is expressed at high levels in inflamed tissues (Fickenscher et al. 2002). However, is not clear whether other cell types might also produce IL-22 (Fickenscher et al. 2002; Gu et al. 2008; Aujla and Kolls, 2009). IL-22 acts through the IL-22R receptor (Aujla and Kolls, 2009). However, its role in atherosclerosis and macrophages particularly of human origin, has not been studied despite the cognate receptors being expressed on these cells (Dhiman et al. 2009).

1.11 Aims of the project

As detailed above, atherosclerosis, the underlying cause of heart disease and stroke is a major killer in the western world. It is an inflammatory disorder of arteries regulated by cytokines (McLaren et al. 2011). Cytokines mediate the development of the atherosclerotic plaque, and they affect all stages of the disease. Cytokines have central roles in the control endothelial cell permeability, recruitment of monocytes to the activated endothelium, expression of adhesion molecules and scavenger receptors, migration of SMCs and induction of apoptosis (Tedgui and Mallat, 2006). As regards cytokines, a substantial amount of information is available on classical cytokines, such as IFN-γ, TNF-α, IL-1 and TGF-β (Ait-Oufella et al. 2011). However, very little is

known about the more recently identified cytokines. Recent studies in 2010 in our laboratory on more recently identified cytokines, such as IL-33 and tumour necrosis factor-like protein 1A (TL1A), have provided new insight into their roles in macrophages in relation to atherosclerosis (McLaren et al. 2010a; McLaren et al. 2010b). Investigation of the role of more recently identified cytokines such as IL-37, IL-15, IL-22 and IL-20 in human macrophages was therefore the major focus of research in this project.

The initial aim of this project was to investigate the effect of these cytokines (IL-37, IL-15, IL-20 and IL-22) on the expression of key genes in macrophages implicated in the control of inflammation, such as MCP-1 and ICAM-1 (see Figure 1.11.1). IFN-γ was used as a positive control since it is widely studied and its pro-atherogenic effects on the expression of these genes is well documented (Chung et al. 2002; Harvey and Ramji, 2005). The project also aimed to confirm key findings at the levels of protein expression using Western blot analysis. The effect of cytokines on the uptake of modified LDL by macrophages and foam cell formation was also investigated.

The human THP-1 cell line was used as a model system for human macrophages. When stimulated with phorbol esters, such as phrobol 12-myristate 13-acetate (PMA), THP-1 cells mimic the monocyte-derived macrophages by becoming adherent to glass or plastic, exhibiting a macrophage-like morphology and expressing macrophage differentiation markers (Kohro et al. 2004). THP-1 macrophages have been used widely to investigate macrophage gene expression in relation to atherosclerosis, particularly the effects of cytokines, because numerous responses observed in them have been found to be conserved with primary macrophages and *in vivo* (Auwerx, 1991; Worley et al. 2003; Kohro et al. 2004; McLaren et al. 2010a; McLaren et al. 2010b; Salter et al. 2011). In addition, some experiments were carried out using mouse RAW264.7 cells in order to compare key findings with human THP-1 macrophages and to delineate the possible existence of species-specific regulatory mechanisms. RAW264.7 cells do not require PMA activation because they are adherent, differentiated macrophages. The cells have previously been used to investigate the role of IL-15 and IFN-γ along with the

regulation of mouse macrophage gene expression in relation to atherosclerosis (Kota et al. 2006; van Es et al. 2010).

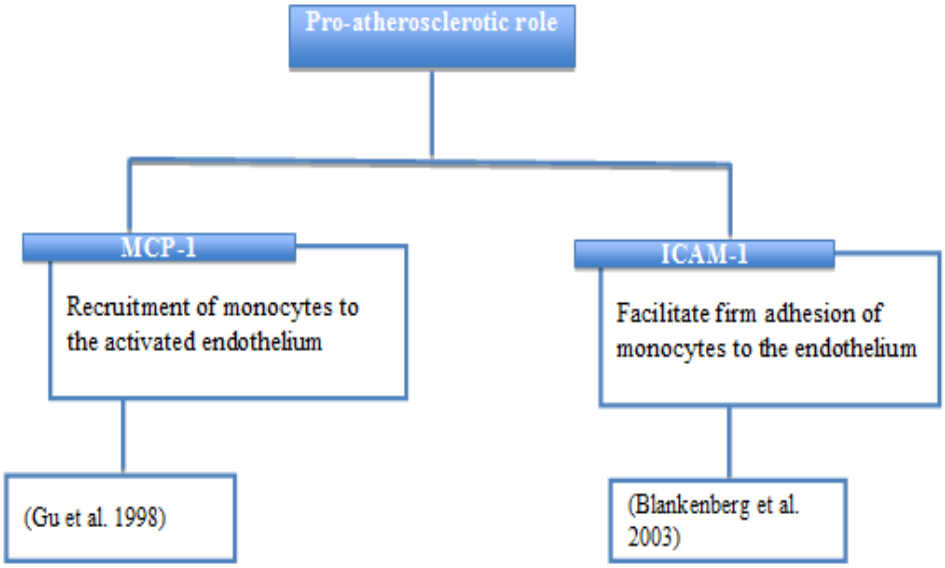

Figure 1.11.1 Genes used in this study and their role in atherosclerosis progression

CHAPTER 2

Materials and Methods

2.1 Materials

Table 2.1.1 lists the materials used during the course of this study together with the suppliers from which they were purchased.

Table 2.1.1 Materials and Suppliers

Materials	Suppliers
Deoxyribonucleotidetriphosphate (dNTPs); Moloney murine leukemia virus reverse transcriptase (MMLV RT); RNasin ribonuclease inhibitor; Random hexamer primers	Promega, UK
Ampicillin; Bromophenol blue; PMA; Tween20; SYBR Green; X ray film; DMSO; Tween 20; RIPA buffer	Sigma, UK
Agarose; PCR reaction buffer	Bioline, UK
10x TBE	National Diagnostics, UK
Spin column RNA Miniprep Kit	NBS biologicals
THP-1 and RAW 264.7 cell lines	ECACC, UK
Tissue culture flasks; 6-well plates; 12-well plates; Cell scrapers; 50ml Falcon tubes.	Greiner Bio One, UK
IL-37; 1L-15; IL-20; IL-22; IFN-γ; mIL-15; m IFN-γ	Peprotech, UK
PCR primers	Sigma Genosys, UK

EDTA; Ethanol; Glycerol; Industrial methylated spirit; Sodium dodecyl sulfate SDS; Tri-soduim citrate; Tris buffer; Filter papers	Fisher scientific, UK
Sterile 0.22µm filters; PVDP membrane	Millipore, UK
I Block; CDP Star reagent	Applied Biosystems, UK
NuPage™ Novex Gel Tank system; Blotting module; SDS-PAGE gels, MOPS running buffer; Transfer buffer; RPMI 1640 with GlutaMAX™; Penicillin; Streptomycin, Foetal calf serum; 2-mercaptoethanol, 1kb DNA molecular markers; See Blue protein markers	Invitrogen, UK
Goat anti-rabbit alkaline phosphatase (AP); Goat anti-mouse AP; Rabbit anti-goat AP	Santa Cruz Biotechnology, USA
PCR tubes; PCR plates; PCR seals	ELKay, UK
BCA protein assay	Pierce, UK
Acetylated LDL (AcLDL)	Intracell, USA

2.2 Methods

2.2.1 Preparation of solutions and glassware

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

2.2.2 Cell culture techniques

THP-1 is a human monocytic leukaemia cell line that can be differentiated into macrophages using PMA. Differentiated THP-1 cells display many of the properties and characteristics of human monocyte-derived macrophages and are therefore a useful model for studying the properties of macrophages, including cell signalling and gene expression in relation to atherosclerosis (Auwerx, 1991). After addition of phorbol esters the cells become adherent to tissue culture flasks.

RAW 264.7 are a murine macrophage cell line established from a tumour induced by the Abelson murine leukaemia virus (Raschke et al. 1978). They are used routinely to study mouse macrophage function and gene expression and can also be transfected efficiently with exogenous DNA (Stacey et al. 1996; Hartley et al. 2008). RAW264.7 cells are adherent to tissue culture flasks and dishes.

2.2.2.1 Maintenance of cell lines in culture

THP-1 and RAW 264.7 cells were grown in RPMI-1640 with GlutaMAX™ (stabilised L-glutamine). The medium was supplemented with 10% (v/v) heat-inactivated (at 56°C for 30min) foetal calf serum (HI-FCS) containing penicillin (100U/ml) and streptomycin (100µg/ml) (pen/strep). Both HI-FCS and penicillin/streptomycin were passed through a 0.2µm sterile filter prior to addition to the medium. Cells were maintained in a humidified incubator (37˚C) with a 5% (v/v) CO_2 atmosphere. Cells between passages 2 and 6 were used for experiments.

2.2.2.2 Counting cells

A haemocytometer (Neubauer chamber) was used to count THP-1 and RAW264.7 cells. After centrifugation (100g for 5min), cells were re-suspended in an appropriate volume of culture medium containing 10% (v/v) HI-FCS. The haemocytometer was covered with a precision ground coverslip, and then 7µl of cell suspension was used and the numbers of cells in the 5 x 5 grid were counted. The number of cells/ml was calculated by multiplying the number of cells in the grid by 10^4. The appropriate volume of cells was diluted using fresh medium and seeded into tissue culture flasks or plates.

2.2.2.3 Sub-culturing of cells

THP-1 cells were sub-cultured when they were approximately 60% confluent (0.6 x 10^6 cells/ml). The cells were transferred into a polypropylene tube (Falcon tubes) and centrifuged at 100g for 5min. The medium was aspirated and the cells were re-suspended in pre-warmed medium containing 10% (v/v) HI-FCS and pen/strep. Cells were placed in new tissue culture flasks and grown up at 37°C in a humidified, 5% (v/v) CO_2 incubator. Cells were usually split in a 1:15 ratio so that growth back up to 60% confluency occurred in approximately one week. For experiments, cells between passage 2 and 6 were used.

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

2.2.2.4 Preserving and storing of cells

THP-1 and RAW264.7 cells were preserved at -80°C or in liquid nitrogen for longer time periods. Only early passage cells (up to passage 3) were used for storage. Prior to freezing, cells were centrifuged at 100g for 5min and re-suspended in HI-FCS containing 10% (v/v) DMSO. Cells at a density of approximately 5 x 10^6 cells/ml were aliquoted into 1ml cryoampoules and stored at -80°C overnight before being transferred to liquid nitrogen.

2.2.2.5 Thawing frozen cells

Cells from liquid nitrogen were defrosted by placing them in a water bath at 37°C. Cells were transferred to a polypropylene tube containing 10% (v/v) HI-FCS and centrifuged at 100g for 5min. After re-suspension into fresh medium containing 10% (v/v) HI-FCS, the cells were plated out into tissue culture flasks and incubated at 37ºC with 5% (v/v) CO_2.

2.2.2.6 Treatment of cells with PMA and cytokines

THP-1 cells were differentiated with 0.16µM PMA for 24hr before cytokine treatment. Cytokines were added directly to the culture medium and the cells were incubated at 37ºC with 5% (v/v) CO_2 for the required time.

2.2.2.7 Setting up experiments for cytokine stimulation

For real-time quantitative PCR (RT-qPCR), cells (500,000 for THP-1 and 600,000 for RAW264.7) were placed in each well of a 12-well plate and supplemented with 1ml of culture medium with 10% (v/v) HI-FCS. THP-1 cells were first differentiated using 0.16µM PMA for 24hr before cytokines treatment as below. For RAW264.7 cells, the medium was first aspirated and replaced, and then the cytokines were added for 3hr and 24hr, as required. The cytokines used in this study along with their concentrations are shown in Table 2.2.2.1. The concentrations are based on previous studies (Chen et al. 2006; Kleemann et al. 2008; van Es et al. 2010). For Western blot analysis, $1x10^6$ cells were added to each well of a 6-well plate with 1ml medium containing 10% (v/v) HI-FCS. The addition of PMA and cytokines was the same as for RTq-PCR. For experiments involving macrophage cholesterol uptake and foam cell formation, 500,000 cells were added to each well of a 12-well plate with 1ml medium containing 10% (v/v) HI-FCS. Cells were then stimulated with PMA for 24hr. After that, cells were treated with cytokines or vehicle and 10mg/ml of AcLDL.

Table 2.2.2.1 : Cytokines used and their concentrations

Cytokines	Concentration
h IFN-γ	1000 U/ml
m IFN-γ	1000 U/ml
h IL-37	100 ng/ml
h IL-15	100 ng/ml
m IL-15	100 ng/ml
h IL-20	100 ng/ml
h IL-22	100 ng/ml

2.3 RNA/DNA techniques

2.3.1 Isolation of RNA

Following incubation with cytokines, the RNA was isolated using a RNA Miniprep Kit (NBS Biologicals). The medium was aspirated from the cells, and they were washed with 1ml of phosphate buffered saline (PBS). After that, cells were lysed by the addition of 350µl of Buffer Lysis-DR provided in the kit. The lysate was then either collected and transferred to the gDNA Eliminator Colum in a 2 ml collection tube (provided in the kit), and then centrifuged at 9,000g for 1min at room temperature, or was transferred into 2 ml collection tubes and saved at -80˚C for later use. The rest of the protocols were carried out according to the manufacturer's instructions. The RNA concentration and quality (OD 260:280 ratio) was determined using a Nanodrop ND2000 spectrophotometer.

2.3.2 RT-qPCR

Reverse transcription-polymerase chain reaction consists of two steps:

1. The synthesis of cDNA using reverse transcriptase;
2. PCR using gene-specific primers under optimised conditions.

2.3.2.1 Reverse transcriptase

A mixture containing 1µg RNA (0.5 µg if the amount of isolated RNA was low), random hexamer primers (200pmol) and sterile water made up to a final volume of 13.5µl was incubated at 72˚C for 5 min and immediately cooled on ice. The following reagents were then added to the reaction:

1µl of dNTP mixture (10 mM each of deoxyadenosine
triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP);

4µl of 5x Molony murine leukaemia virus (M-MLV) buffer;

0.5µl (50U/µl) of recombinant RNase inhibitor; and

1µl (200U/µl) of M-MLV reverse transcriptase.

The total reaction mixture was incubated at 37˚C for 1 hr and the reaction terminated by incubation at 92˚C for 2 min. Synthesised cDNA was diluted by adding 80µl (or 30µl if 0.5µg RNA was used) of RNase-free water and then stored at -20˚C.

2.3.2.2 Polymerase chain reaction

Once the cDNA had been manufactured, it could be used to optimise condition for PCR, if necessary, and to determine regulation of expression of specific genes. The primer sequences that were used are shown in Table 2.3.3 and 2.3.4, human and mouse primers respectively. These were either based on validated sequences previously used for semi-quantitative PCR in the laboratory or sourced from previous publications or from websites called Primer Bank or QPCR. In the latter cases, attempts were made where ever possible to choose sequences that gave optimal product size of around 100pb. The composition of the PCR reactions used in the RT-PCR reaction also shown in Table 2.3.2 and the reaction conditions are shown in Table 2.3.5 for human and 2.3.6 for mice.

2.3.2.2.1 Q-PCR

A Q-PCR reaction was set up using the SYBER® GREEN JumpStart™ Taq Readymix™ for quantitative PCR (Sigma-Aldrich). The composition of the reaction is detailed in Table 2.3.1. The reactions were then "run" using an Opticon 2 PCR machine (MJ Research). The reagents were mixed and added into a 96 well plate and the program was set according to the optimised PCR conditions (Tables 2.3.5 and 2.3.6). The Q-PCR data were analysed using the comparative Ct (ΔΔCt) method (Livak and scmittgen 2001). The output of RT-qPCR reaction shows the number of PCR cycles against the increasing fluorescence. This is known as amplification point. The ΔΔCt method relies on measuring the number of reaction cycles it takes for the amplification plot crosses the threshold level (Ct value) within the exponential phase of the amplification (Livak and scmittgen 2001). The CT values were then collected from the computer and analysed on Microsoft Excel using the ΔΔCT method (Ginzinger, 2002). The Ct value of the gene of interest was compared relative to the Ct value of a control housekeeping gene to normalise the data (Livak and scmittgen 2001). The ΔΔCt method relies on two assumptions, firstly that the expression of the housekeeping gene does not vary under experimental conditions and secondly, that the PCR efficiencies for each primer set used in the analysis are similar (Ginzinger 2002). The reaction conditions are shown in Tables 2.3.5 for human and 2.3.6 for mouse.

Table 2.3.1: The composition of a 25µl Q-PCR reaction

Reagent	Amount (µl)
SYBER® GREEN Ready mix (Sigma Aldrich)	12.5
Forward Primer (200pmol)	0.5
Reverse Primer (200pmol)	0.5
Water	10.5
cDNA	1
Total	25

Table 2.3.2 PCR reaction set up used for semi-quantitative RT-PCR reactions

Reagent	Amount
Forward Primer (200pmol)	0.5µl
Reverse Primers (200pmol)	0.5µl
dNTPs	1µl
Buffer	5µl
Taq	0.25µl
Sterile Water	32.75µl
cDNA	10µl

Table 2.3.3 Human PCR primers for analysis of gene expression in THP-1 macrophages

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size of Product (bp)	References
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226	(Vandesompele et al. 2002)
MCP-1	CATTGTGGCCAAGGAGATCTG	CTTCGGAGTTTGGGTTTGATT	91	(Locati et al. 2002)
ICAM-1	ACGCTGAGCTCCTCTGCTACTC	GGGCAGGATGACTTTTGAGG	162	(NILESH et al. 2010)

Table 2.3.4 Mouse PCR primers for analysis of gene expression in RAW264.7 cells

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size of Product (bp)	References
β-Actin	TGGAGAAGAGCTATGAGCTGCCTG	GTGCCACCAGACAGCACTGTGTTG	202	(McLaren et al. 2010b)
MCP-1	CTGGATCGGAACCAAATGAG	CGGGTCAACTTCACATTCAA	95	(Locati et al. 2002)
ICAM-1	CAGTCCGCTGTGCTTTGAGA	CGGAAACGAATACACGGTGAT	75	(Lei et al. 2009)

Table 2.3.5: Amplification condition for Q-PCR with human primer sequences

PCR Step	GAPDH	MCP-1	ICAM-1
Initial Melting	95˚C for 5 min	95˚C for 5 min	95˚C for 5 min
Annealing	60˚C for 60 sec	63˚C for 60 sec	63˚C for 60 sec
Extension	72˚ for 60 sec	72˚ for 60 sec	72˚ for 60 sec
Melting	95˚C for 30 sec	95˚C for 30 sec	95˚C for 30 sec
Final Extension	72˚ for 10 min	72˚ for 10 min	72˚ for 10 min
Number of cycles	35	35	35

Table 2.3.6: Amplification conditions for Q-PCR with mouse primer sequences

PCR Step	β-Actin	MCP-1	ICAM-1
Initial Melting	95˚C for 5 min	95˚C for 5 min	95˚C for 5 min
Annealing	58˚C for 60 sec	59˚C for 60 sec	60˚C for 60 sec
Extension	72˚ for 60 sec	72˚ for 60 sec	72˚ for 60 sec
Melting	95˚C for 30 sec	95˚C for 30 sec	95˚C for 30 sec
Final Extension	72˚ for 10 min	72˚ for 10 min	72˚ for 10 min
Number of cycles	35	35	35

2.3.2.3 Agarose Gel Electrophoresis

Size fractionation of PCR products was carried out by agarose gel electrophoresis. For this, 1.5% (w/v) gels were made up with agarose dissolved in 1 x TBE buffer. The agarose solution was warmed in the microwave until the agarose had dissolved followed by the addition of 0.5µg/ml ethidium bromide. Stock solutions for gel electrophoresis are shown below in Table 2.3.7.

Table 2.3.7 Composition of reagents used in gel electrophoresis

Reagent	Composition
10 x TBE	0.89M Tris borate, 890M boric acid and 20M EDTA, pH 8.0
5x DNA loading dye	1 x TBE. 50% (v/v) glycerol and 2.25% (w/v) bromophenol blue

DNA samples were prepared with 5µl of DNA loading dye and electrophoresis was carried out in 1 x TBE buffer at 100-150V for around 30-60 min using a horizontal gel unit (Fisherbrand). The size of the PCR products was compared to low molecular weight DNA ladder. DNA was visualised under UV light using a Syngene Gel Documentation system.

2.4 Western Blot analysis

2.4.1 Cell lysis

Following stimulation with PMA for 24hr, and then cytokine or vehicle treatment for the required time, the medium was aspirated from the wells and the cells were washed with 1ml of ice cold PBS. The cells were then lysed with 50µl of RIPA buffer (Sigma). The lysate was transferred to a 2ml collection tube and the mixture then boiled at 100˚C for 5min. The lysate was then ready for loading onto a pre-cast SDS-PAGE gel (Invitrogen).

2.4.2 Protein concentration

The concentration of total protein was determined using the BCA protein assay kit (Pierce). A standard curve was produced for each assay using suitable dilution of a 2mg/ml bovine serum albumin (BSA) solution to give final concentrations of 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml and 25µg/ml. Samples were diluted in water in ratios of 1:300. Each sample (150µl) was analysed in duplicate and was placed in the wells of a 96-well micro-titre plate followed by the addition of 150µl Micro BCA protein assay reagent (Pierce). The plate was covered with a pre-cut Transparent Microplate Sealer

(Greiner Bio One) and left in the incubator at 37˚C for 2hr. A Model 680 Microplate Reader (Biorad) was used to measure the absorbance of each sample at a wavelength of 570nm. The protein concentration of each sample was then calculated from the standard curve.

2.4.3 Gel electrophoresis and transfer of proteins to membrane

SDS- polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using NuPage 4-12% Bis-Tris gels (Invitrogen). The tank was filled with NuPAGE MOPS SDS Running Buffer (Invitrogen) and the gels were placed within the tank. Equal amount of protein sample was added to each well, along with a protein standard, and the gel was subjected to electrophoresis for 80min at 150V, 400mA. Following electrophoresis, the stacking gel was cut away and the separating gel equilibrated in NuPAGER® Transfer Buffer (Table 2.4.1). Immobulin-P PVDF membrane (0.45µm pore size, Millipore) was cut to the size of the gel and activated in pure 100% (v/v) methanol for 5min. The membrane

was placed on top of the gel and both were sandwiched between Whatman 3MM paper and sponge pads, which had also been soaked in the NuPAGER® Transfer Buffer (Table 2.4.1). The entire set up was then placed in a blotting cassette and transfer carried out at 30V, 300mA for 85min. Transfer efficiency was checked at this stage by staining the PVDF membrane with Ponceau S solution.

2.4.4 Immuno-detection of proteins

Following blotting, the membrane was removed from the sandwich and washed with PBS-Tween (Table 2.4.1) for 5 min and then incubated in I-BT solution (Table 2.4.1) shaking for 1hr in order to block any potential non-specific interactions of the antibodies to the membrane. After removal of the I-BT solution the primary antibody was diluted in more I-BT (Table 2.4.2) and then added to the membrane and left overnight shaking at 4˚C. Following the primary antibody incubation, the membrane was then washed three times with PBS-Tween (5min each). After the wash steps were complete, the secondary antibody was diluted also in I-BT (Table 2.4.2) and then added to the membrane and left for 1hr shaking at room temperature. The membrane was again washed three times with PBS-Tween (5min each). Different exposures were then developed using a Kodak film placed inside a light resistant X-ray cassette. The film was developed using Agfa film processor and developer. A detection reagent (CDP-Star) was added according to the manufacturer's instructions (Applied Biosystems) to the membrane before developing the membrane using a Kodak film.

Table 2.4.1 Composition of stock solutions used for protein analysis by SDS-PAGE and Western blotting

Solution	Composition
NuPAGER MOPS SDS Running Buffer (x20)	50mM 3-(N-morpholino) propane sulfonic acid (MOPS), 50 mM Tris Base, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.7
NuPAGER Transfer Buffer	50mM Bis-tris propane, 50mM Bicine, 20% (v/v) methanol
I-BT solution	500ml PBS-Tween, 1gram Tropix I-Block, 500ml of 5% (v/v) sodium azide
PBS-Tween	10L dd$H_2$0, 100 PBS tablets, 20ml Tween20
Ponceau S	0.1% (w/v) Ponceau S, 5%(v/v) acetic acid, ddH_2O

Table 2.4.2 Antibodies and dilutions used for Western blotting

Protein	Primary Antibody	1˚ antibody dilution	Secondary Antibody	2˚ antibody Dilution	Protein Size
ICAM-1	Rabbit	1:200	Goat Anti Rabbit (AP)	1:5000	110KDa
MCP-1	Goat	1:500	Rabbit Anti Goat (AP)	1:5000	14KDa
β-actin	Mouse	1:10,000	Goat Anti Mouse (AP)	1:5000	42KDa

2.4.5 Analysis of western blots

The densities of bands from immunoblots were analysed using GeneTools software (Syngene). All experimental values were normalised to the control β-actin.

2.5 Uptake of AcLDL by macrophages

The THP-1 cell line ($5x10^5$ cells) was placed in each well of a 12-well plate and supplemented with 1ml of RPMI-1640 with 10% (v/v) HI-FCS and pen/strep. The cells were differentiated using 0.16μM PMA for 24hr prior to starting the assay. The cells were then incubated for 24hrs with 10μg/ml AcLDL in the presence of vehicle or IFN-γ (1,000U/ml) or IL-15 (100ng/ml) (representative experiments in the absence of AcLDL were also included for comparative purposes). After 24hr incubation, the medium was aspirated from the wells and the cells were washed with 1ml of ice cold PBS about 6 to 7 times. For lipid extraction, 500μl of ice-cold hexane:isopropanol (2:3, v/v) was added into the wells and left to incubate on ice for 15min, and then the lysate was transferred into 0.5ml Eppendorf tubes and left to evaporate at room temperature over night. Lipids were then resuspended in hexane:igenal (9:1, v/v) and used for the Amplex red cholesterol assay.

2.5.1 Protein extraction

After removal of lipids, the proteins were extracted by adding 500µl of 0.2% (v/v) NaOH, and incubated at 37°C in a humidified, 5% (v/v) CO_2 incubator for 3hr. Protein concentration was determined using the Micro BCA protein assay kit as described in Section 2.4.2

2.5.2 Amplex red cholesterol assay

Samples were prepared in a black, 96-well plate. A standard curve was produced by diluting 2µl of cholesterol reference standard (provided in the kit) in 500µl of 1x reaction buffer (Table 2.5.1) to produce cholesterol concentration of 0 to 8µg/ml. Then, 50µl of the cholesterol-containing samples were diluted in 200µl of 1x reaction buffer (Table 2.5.1) (1:5, v/v). Diluted samples (50µl) and standards were added into separate wells of a black 96-well plate. To measure the total cholesterol, 50µl of the Amplex® Red reagent (300µM)/Horseradish peroxidise (HRP) (2U/ml)/cholesterol oxidase (2U/ml)/cholesterol esterase (2U/ml) working solution (prepared according to the manufacturer's instructions (Invitrogen)) was added into each well containing the diluted samples and standards. To measure the free cholesterol, another working solution was prepared without the cholesterol esterase and 50µl was added into separate wells containing the diluted samples. The plate was covered with a pre-cut Transparent Microplate Sealer (Greiner Bio One) and left in the incubator at 37˚C for 30min. The fluorescence was measured in a fluorescence microplate reader using a Flostar OPTIMA. Cholesterol ester content was calculated by subtracting the free cholesterol value from the total cholesterol value of each sample.

2.5.1 Amplex Red reagent Stock solutions:

Stock solution	Concentration
1x Reaction Buffer	5ml of 5x reaction buffer (provided in the Kit) with 20ml of deionised water

2.6 Statistical analysis of data

Data sets were tested for normality using the Shapiro-Wilk test. Statistical analysis was performed using Student's *t*-test for single comparisons (two-tailed, paired) or one-way ANOVA for multiple comparisons with Tukey's post-hoc test, where homogeneity of variance was met; or Welch's test of equality of means with Games-Howell post-hoc analysis. [* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, and NS indicates not significant].
The details of which are given in the Appendices.

CHAPTER 3

RESULTS

3.1 Introduction

As described in the General Introduction, atherosclerosis is a chronic inflammatory disease caused by the accumulation of cholesterol within the walls of large and medium arteries (Lusis, 2000). Several cytokines are expressed in atherosclerotic lesions and their role in disease has been highlighted by studies in mouse model systems (Kleemann et al. 2008). The importance of cytokines comes from their ability to regulate the immune response (Tedgui and Mallat, 2006).

Many genes have been shown to play a critical role in the development of atherosclerosis (Tedgui and Mallat, 2006). Numerous studies have focused on the role of chemokines and adhesion molecules on the progression of this disease. They are both notably present in the initial stages of plaque formation (Cybulsky et al. 2004). MCP-1 is a chemokine that belongs to the large CC chemokine family. Many cells associated with the progression of atherosclerosis, such as ECs, macrophages and SMCs, have been found to express MCP-1 and other chemokines (Aiello et al. 1999; Charo and Taubman, 2004). Cushing et al. (1990) showed that the expression of MCP-1 in the initial stage of atherosclerosis is induced by mmLDL (Stocker and Keaney, 2004). MCP-1 is implicated in the recruitment of monocytes from the blood stream to the surface of the ECs at this early stage (Charo and Taubman, 2004). The role of MCP-1 in the disease has been identified from studies using mouse model systems; for example, MCP-1 deficiency in murine model decreased the progression of atherosclerotic plaque development(Charo and Taubman, 2004), while the lesion formation and deposition of macrophages was increased when MCP-1 was overexpressed in the bone-marrow-derived cells of apoE$^{-/-}$ mice (Aiello et al. 1999). ICAM-1, on the other hand, is an adhesion molecule belonging to the immunoglobulin superfamily. ICAM-1 is expressed on the surface of the EC, and contributes to the adhesion and migration of monocytes to the subendothelium space (Fotis et al. 2012). Studies of apoE deficient mice have indicated that the atherosclerotic lesion development is reduced with ICAM-1 deficiency (Kitagawa et al. 2002).

In relation to cytokines and atherosclerosis, although the effect of classical cytokines, such as IFN-γ, on macrophage gene expression is well understood, this is not the case for many cytokines, such as IL-37, and more recently identified cytokines whose receptors are expressed in these cells such as IL-15, IL-20 and IL-22. The initial aim of this project was therefore to investigate the effect of more recently identified cytokines on the expression of key genes in macrophages implicated in the control of inflammation and foam cell formation during atherosclerosis. IFN-γ is a widely studied cytokine in our laboratory and its action in atherosclerosis has been widely investigated; therefore, it was used as a positive control (Harvey et al. 2007; Harrirs et al. 2008; Li et al. 2010).

The overall experimental strategy and the three parameters that were analysed for IL-15, IL-20, IL-22 and IFN-γ are shown in Figure 3.1.1 Firstly, the effect of the cytokines on the mRNA expression of MCP-1 and ICAM-1, two major markers of inflammation, was determined by RT-qPCR. The studies on Western blot analysis and modified LDL uptake were restricted to IFN-γ (positive control) and IL-15 (the only cytokine that affected the expression of MCP-1 and ICAM-1). A slightly different strategy was adopted for IL-37 (see Figure 3.1.2). Previous studies have shown that the expression of IL-37 in macrophages or epithelial cells inhibited the production of pro-inflammatory cytokines such as IL-1α, IL-6, TNF-α and MIP2-α stimulated by lipopolysaccharide (LPS) (Nold et al. 2010). On the other hand, silencing of endogenous IL-37 in human blood cells increased the abundance of these cytokines in response to LPS (Nold et al. 2010). We therefore determined whether pre-incubation of THP-1 macrophages with IL-37 affected gene expression by the pro-inflammatory cytokine IFN-γ. Because the potential time of IL-37 action, if any, was not known, two pre-incubation time points were chosen, 3hr and 24hr.

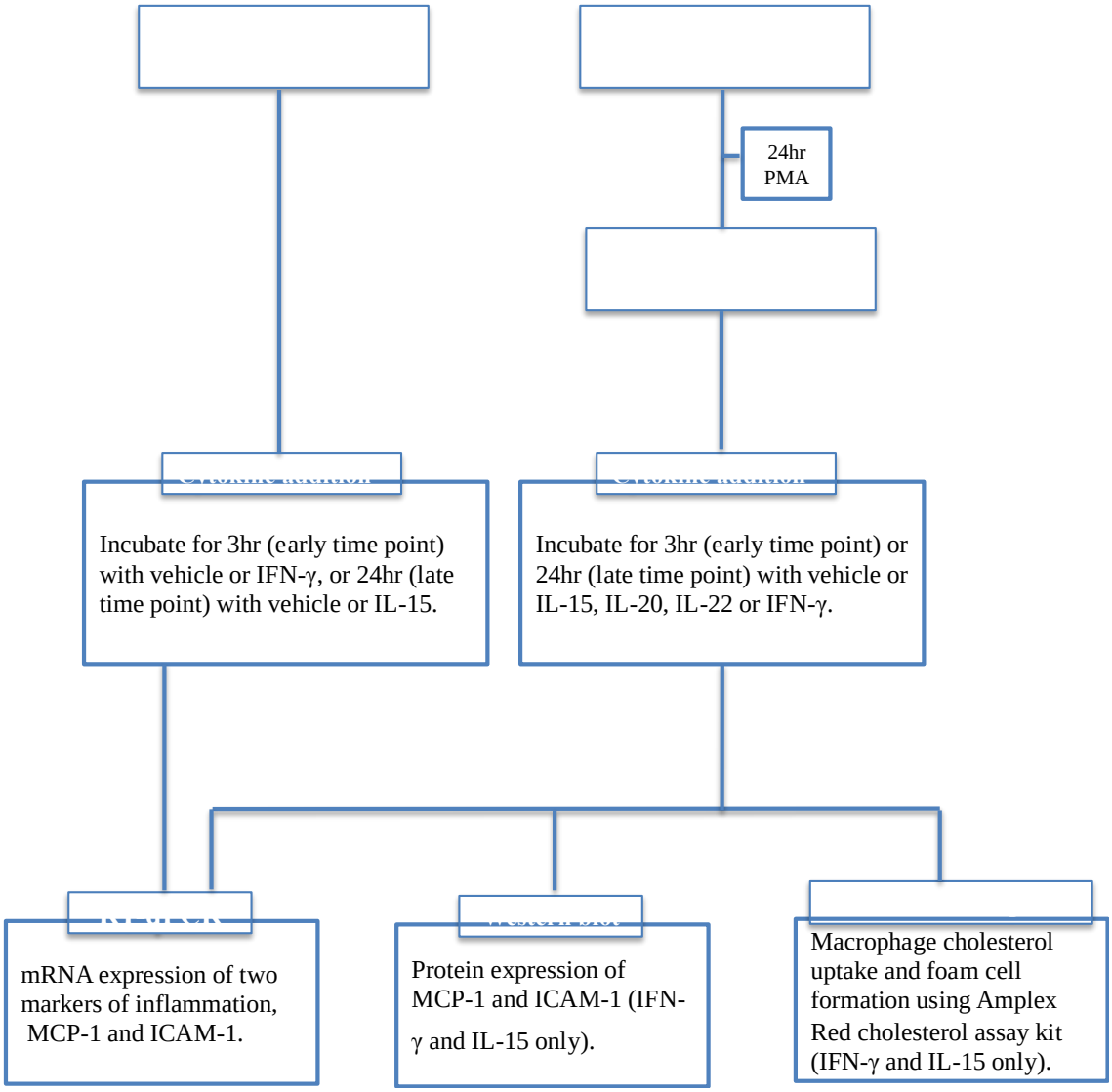

Figure 3.1.1 Overall experimental strategy and the three parameters that were analysed

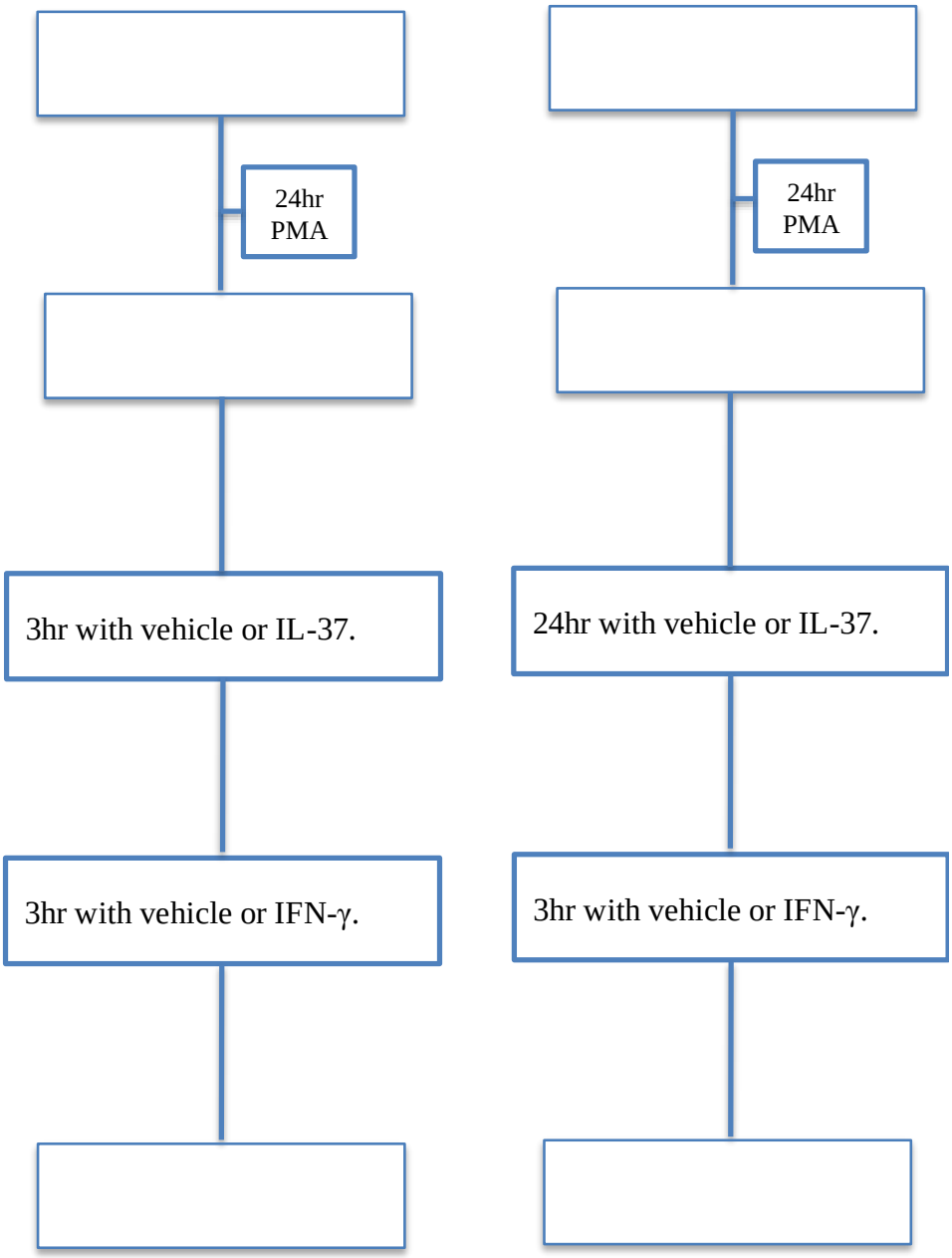

Figure 3.1.2 RT-q-PCR strategy for IL-37

3.2 The effect of cytokines on MCP-1 and ICAM-1 mRNA expression in THP-1 macrophages

As detailed in the General Introduction, the THP-1 cell line was chosen as a model for human macrophages because of conservation of responses to those observed in human primary cultures and in *vivo* (Auwerx, 1991; Worley et al. 2002; Kohro et al. 2004; Salter et al. 2011; Michael et al. 2012). The human THP-1 cell line was first differentiated into macrophages by incubation with PMA for 24hr. Figure 3.2.1 shows the morphology of THP-1 monocytes and macrophages. After 24hr stimulation with 0.16μM PMA, the THP-1 monocytes adhere to the bottom of the flask and had morphological characteristics of macrophages. THP-1 macrophages were then stimulated with different human cytokines (or vehicle control) for 3hr or 24hr. These two time points were chosen for this study rather than a detailed time course analysis because previous studies in the laboratory have shown that gene expression by cytokines occurs in two phases: early (typically 3hrs) and late (typically 24hrs).

The mRNA expression of target genes was studied using RT-qPCR. Q-PCR is a PCR based technique that is able to quantify the amount of DNA being synthesised in each cycle of the reaction. The advantage of this technique is that the starting amount of DNA in the sample can be accurately determined from the CT values from the Q-PCR reaction. The method that was used to determine the starting amount of DNA was the ΔΔCT method (Ginzinger, 2002). The cytokines that were used during this study were human IFN-γ, IL-37, IL-15, IL-20, IL-22, mouse IFN-γ and mouse IL-15 (Table 2.2.2.1). The expression of MCP-1 and ICAM-1 was normalised to the house-keeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for human cells and β-Actin for mouse cells. Melting curve analysis was performed to confirm amplification of a single product (Figure 3.2.2).

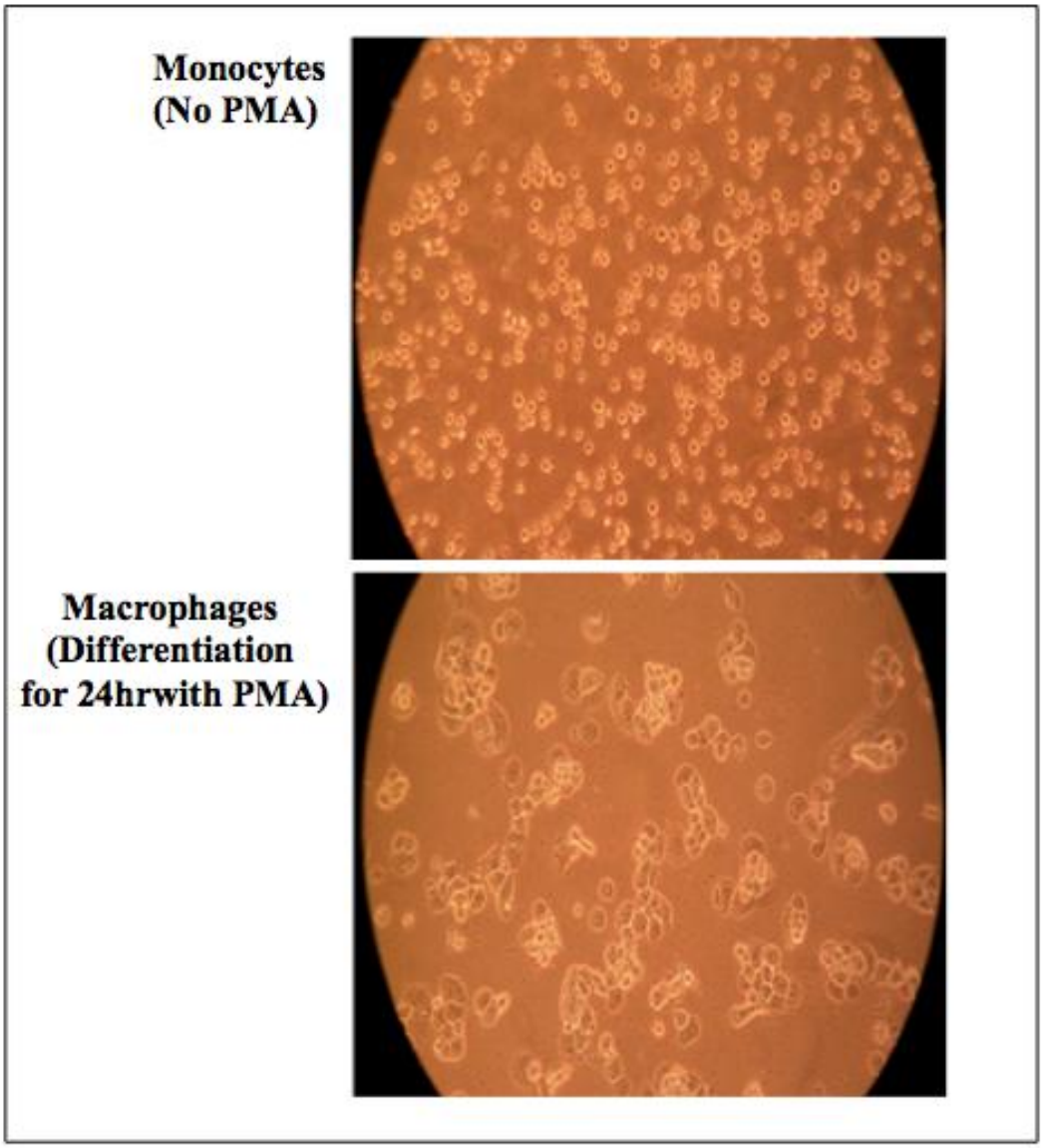

Figure 3.2.1 Morphology of THP-1 monocytes and macrophages THP-1 monocytes (1 x 10^6 cells) were cultured in 6-well tissue culture flask and either left untreated (top) or incubated for 24h (bottom) with 0.16μM PMA. The cells become adherent to tissue culture flask after incubation with PMA. Photos were taken on x20 magnification.

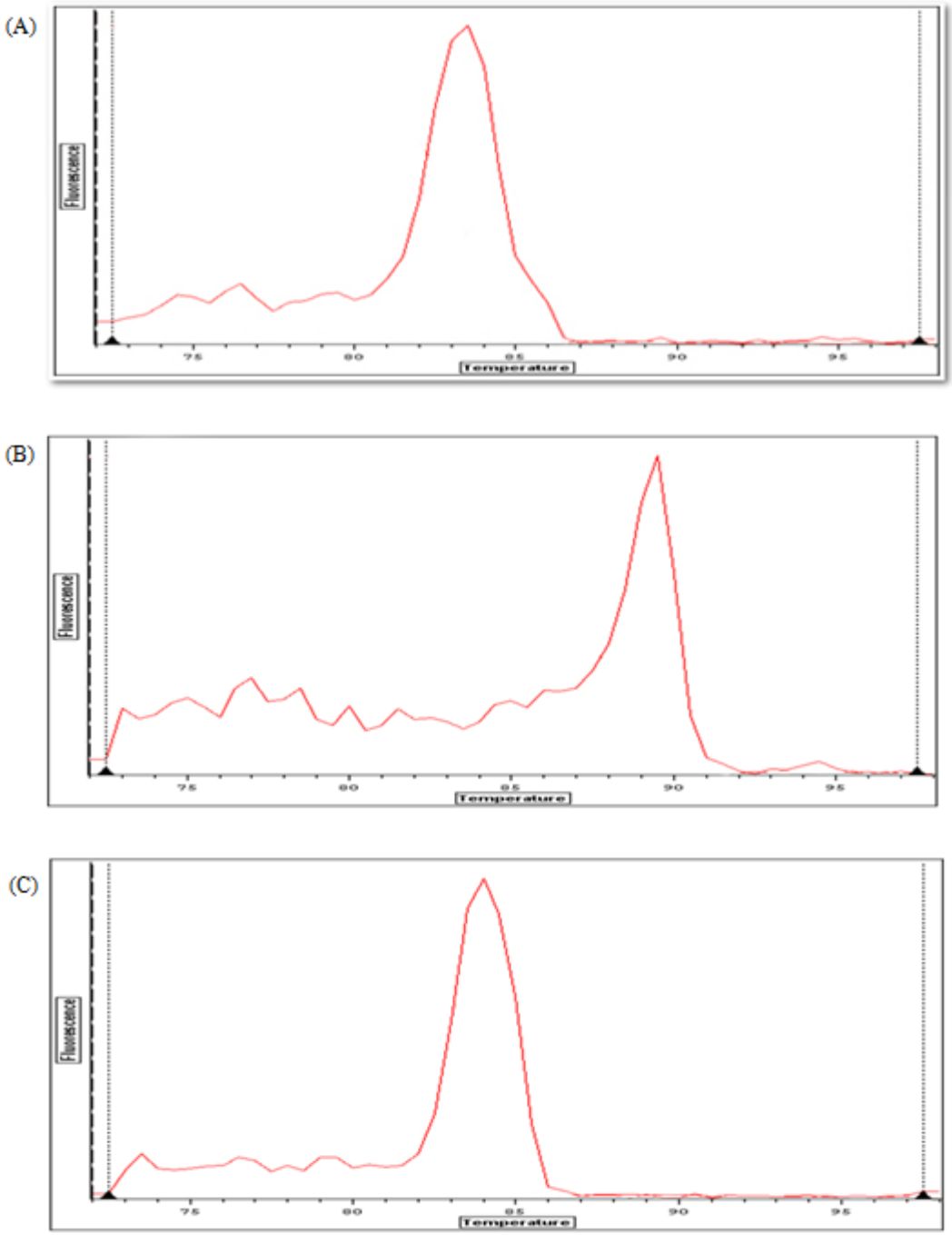

Figure 3.2.2 Melting curve analysis for Human primers RTq-PCR was performed for MCP-1, ICAM-1 and GAPDH. The graphs show that a single major amplification product was produced from human primers A: MCP-1, B: ICAM-1 and C: GAPDH.

We first examined the effect of different cytokines (IL-15, IL-20, IL-22 and IL-37) on MCP-1 and ICAM-1 mRNA expression in THP-1 macrophages using IFN-γ as a positive control.

3.2.1 IFN-γ

As detailed above, the cytokine IFN-γ was used as a positive control as previous research in our laboratory and that of the others have shown that it induces the expression of both MCP-1 and ICAM-1 (Chung et al. 2002; Harvey and Ramji, 2005; Li et al. 2010). RT-qPCR results for human THP-1 macrophages stimulated with IFN-γ for 3hr and 24hr are shown in Figures 3.2.1.1 and 3.2.1.2 respectively. As expected, a significant increase in the expression of MCP-1 and ICAM-1 was observed.

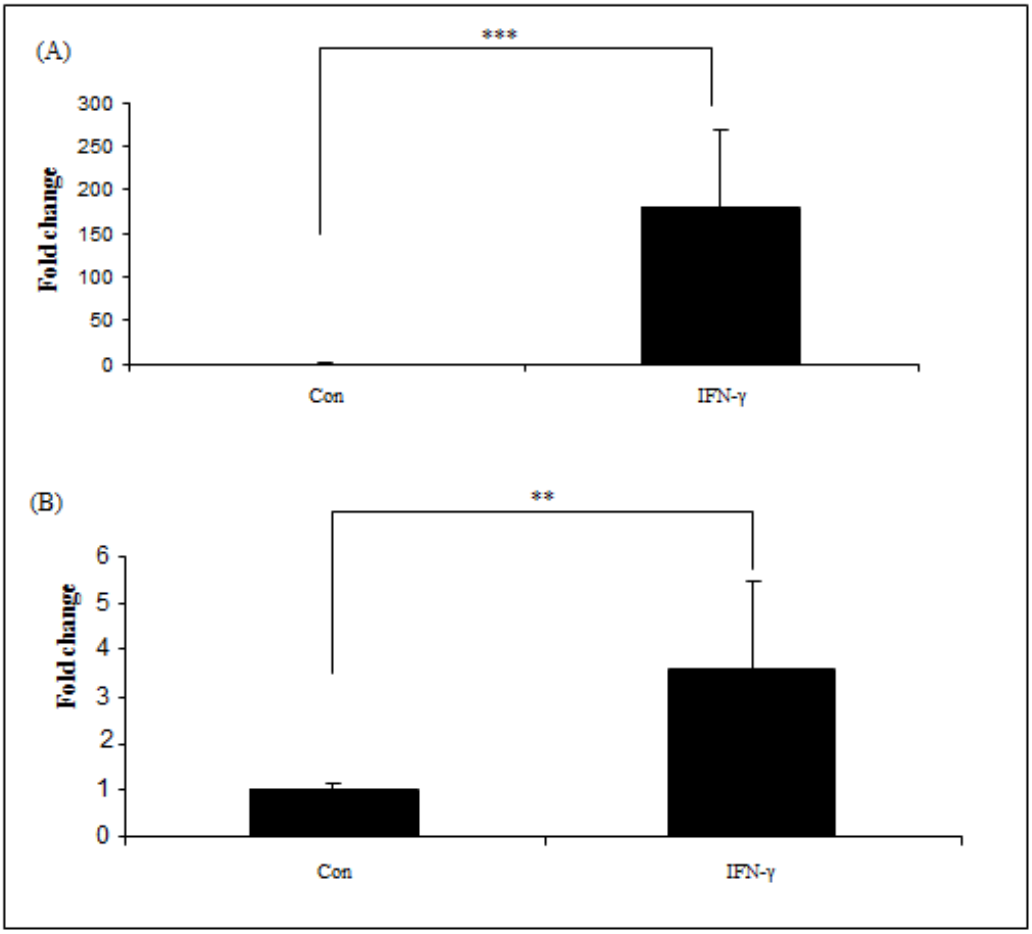

Figure 3.2.1.1 IFN-γ induces the expression of MCP-1 and ICAM-1 mRNA in THP-1 macrophages. THP-1 monocytes were differentiated into macrophages using PMA and then incubated for 3hr with vehicle (Con) or IFN-γ (1,000U/ml). Total RNA was isolated and subjected to RT-qPCR. The graph shows the fold change in mRNA expression (mean ± SD) in relation to the control, vehicle-treated cells (arbitrarily assigned as 1) from four independent experiments, samples in duplicate. A, MCP-1; B, ICAM-1. Statistical analysis was performed using the Student's *t*-test, ** $P < 0.01$; *** $P < 0.001$.

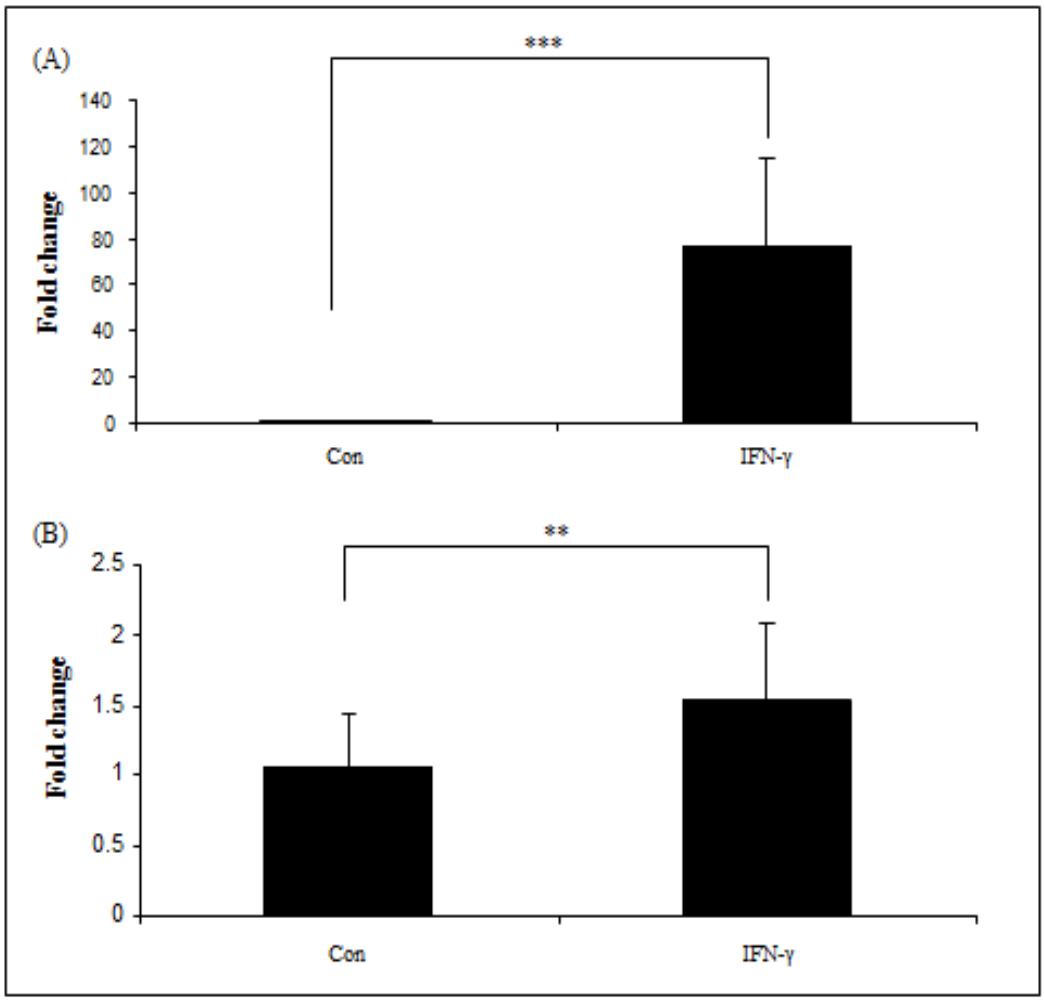

Figure 3.2.1.2 IFN-γ induces the expression of MCP-1 and ICAM-1 mRNA in THP-1 macrophages at 24hr. THP-1 monocytes were differentiated into macrophages using PMA and then incubated for 24hr with vehicle (Con) or IFN-γ (1,000U/ml). Total RNA was isolated and subjected to RT-qPCR. The graph shows the fold change in mRNA expression (mean ± SD) in relation to the control, vehicle-treated cells (arbitrarily assigned as 1) from four independent experiments, samples in triplicate. A, MCP-1; B, ICAM-1. Statistical analysis was performed using the Student's *t*-test, ** $P < 0.01$; *** $P < 0.001$.

3.2.2 IL-15, IL-20 and IL-22

Having confirmed the positive action of IFN-γ on MCP-1 and ICAM-1 mRNA expression in THP-1 macrophages, the action of IL-15, IL-20 and IL-22 was next investigated. As shown in Figure 3.2.2.1, all three cytokines had no statistically significant effect on the expression of MCP-1 or ICAM-1 at incubation period of 3hr. Similarly, IL-20 and IL-22 had no effect on the expression of MCP-1 or ICAM-1 at the 24hr time point (Figure 3.2.2.2). In contrast, IL-15 significantly attenuated MCP-1 expression at 24hr (Figure 3.2.2.2). In addition, although the effect of IL-15 on ICAM-1 mRNA levels at this time point was not significant, a trend of reduced expression was seen (Figure 3.2.2.2).

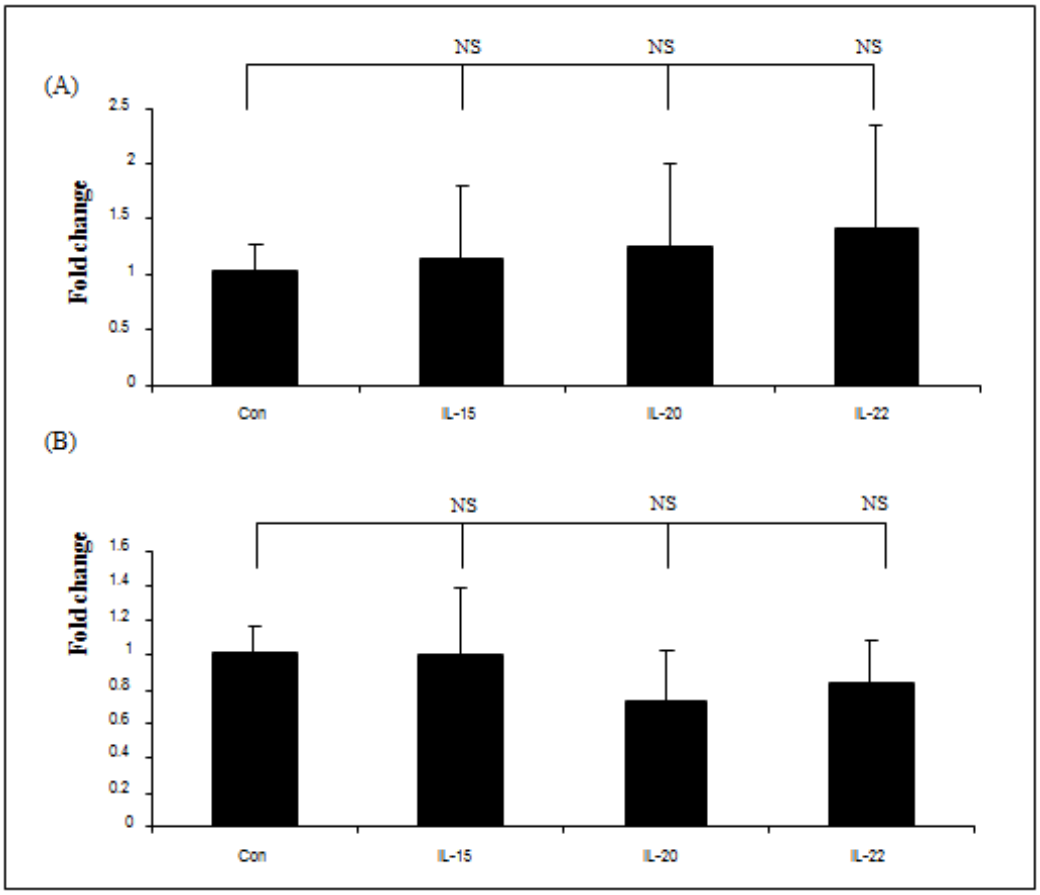

Figure 3.2.2.1 IL-15, IL-20 and IL-22 have no effect on MCP-1 and ICAM-1 mRNA expression in THP-1 macrophages at 3hr. THP-1 monocytes were differentiated into macrophages using PMA and then incubated for 3hr with vehicle (Con) or IL-15 (100ng/ml), IL-20 (100ng/ml) or IL-22 (100ng/ml). Total RNA was isolated and subjected to RT-qPCR. The graph shows the fold change in mRNA expression (mean ± SD) in relation to the control, vehicle-treated cells (arbitrarily assigned as 1) from four independent experiments, samples in duplicate. A, MCP-1; B, ICAM-1. Statistical analysis was performed using one-way ANOVA with Welch's test of equality of means with Games–Howell post-hoc analysis. No significant result was obtained.

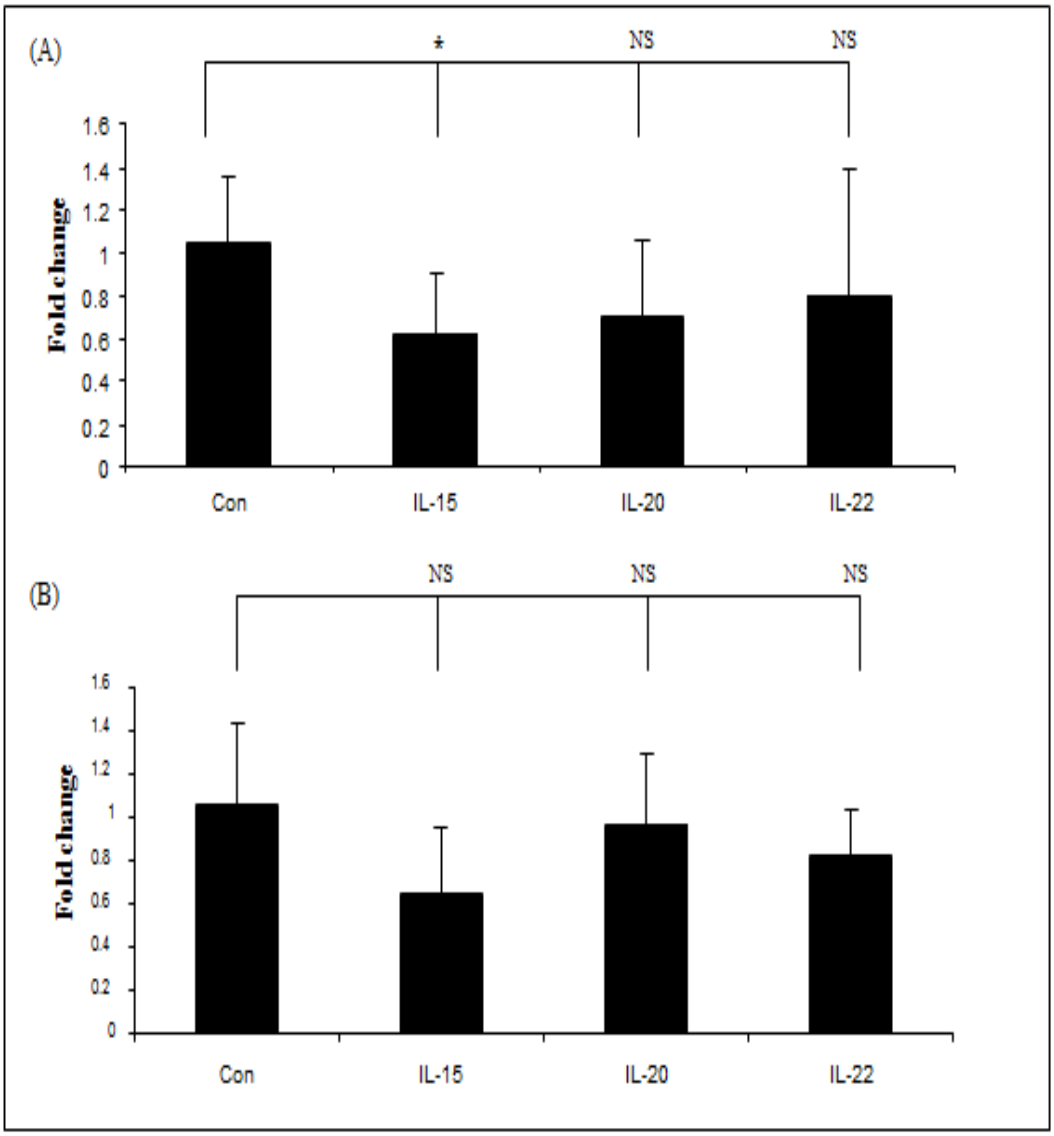

Figure 3.2.2.2 IL-15 attenuates MCP-1 mRNA expression in THP-1 macrophages at 24hr. THP-1 monocytes were differentiated into macrophages using PMA and then incubated for 24hr with vehicle (Con) or IL-15 (100ng/ml), IL-20 (100ng/ml) or IL-22 (100ng/ml). Total RNA was isolated and subjected to RT-qPCR. The graph shows the fold change in mRNA expression (mean ± SD) in relation to the control, vehicle-treated cells (arbitrarily assigned as 1) from four independent experiments, samples in triplicate. A, MCP-1; B, ICAM-1. Statistical analysis was performed using one-way ANOVA with Welch's test of equality of means with Games–Howell post-hoc analysis. * $P < 0.05$; NS indicates not significant.

3.2.3 IL-37

The next cytokine that was studied was IL-37. For this, the cells were first pre-treated with IL-37 or vehicle for 3hr or 24hr and then incubated with vehicle or IFN-γ for 3hr (see Figure 3.1.2 for experimental strategy). In experiments involving pre-incubation of the cells with IL-37 or vehicle for 3hr (Figure 3.2.3.1), IFN-γ induced the expression of both MCP-1 and ICAM-1 mRNA though this failed to reach significance probably due to relatively large SD in these experiments. Incubation with IL-37 alone had no effect on MCP-1 mRNA expression and reduced that for ICAM-1 though this was not statistically significant. More importantly, the IFN-γ-induced levels of MCP-1 or ICAM-1 mRNA were not significantly affected by pre-incubation of the cells with IL-37.

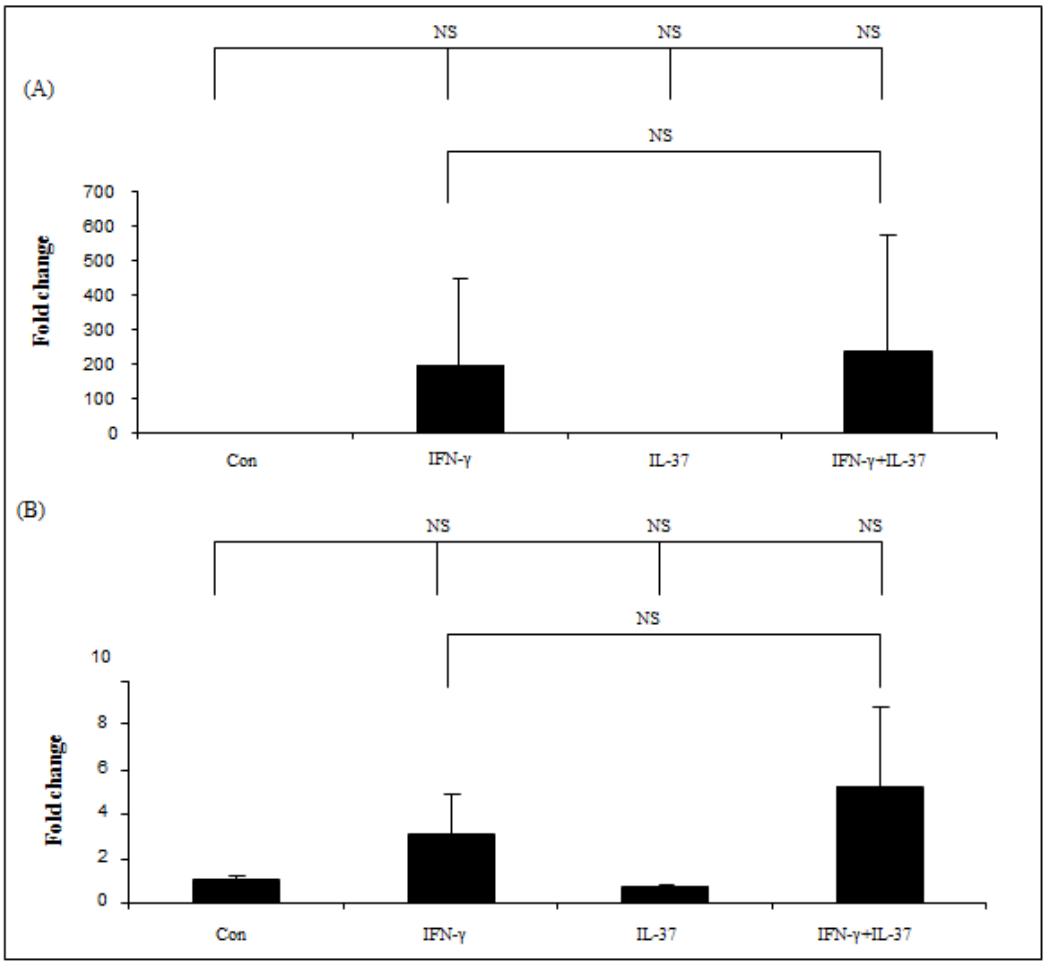

Figure 3.2.3.1 Pre-incubation of THP-1 macrophages with IL-37 for 3hr has no effect on MCP-1 and ICAM-1 mRNA expression induced by IFN-γ. THP-1 monocytes were differentiated into macrophages using PMA and then incubated for 3hr with vehicle (Con and IFN-γ (1,000U/ml)) or IL-37 (100ng/ml) (IL-37 and IFN-γ+Il-37). The cells were then treated for 3hr with IFN-γ (IFN-γ and IFN-γ+IL-37) or vehicle (Con and IL-37). Total RNA was isolated and subjected to RT-qPCR. The graph shows the fold change in mRNA expression (mean ± SD) in relation to the control, vehicle-treated cells (arbitrarily assigned as 1) from three independent experiments, samples in duplicate. A, MCP-1; B, ICAM-1. Statistical analysis was performed using one-way ANOVA with Welch's test of equality of means and Games–Howell post-hoc analysis. NS indicates not significant.

For experiments involving pre-incubation of the cells with IL-37 or vehicle for 24hr (Figure 3.2.3.2), IFN-γ induced the expression of both MCP-1 and ICAM-1 mRNA though this failed to reach significance for MCP-1 probably because of relatively large SD in this case. Incubation with IL-37 alone had no effect on MCP-1 mRNA expression and reduced that for ICAM-1 though this was not statistically significant. In addition, the IFN-γ-induced levels of MCP-1 or ICAM-1 mRNA were not significantly affected by pre-incubation of the cells with IL-37. Overall, these results suggest that pre-incubation of THP-1 macrophages with IL-37 does not inhibit IFN-γ-induced expression of both MCP-1 and ICAM-1 mRNA expression.

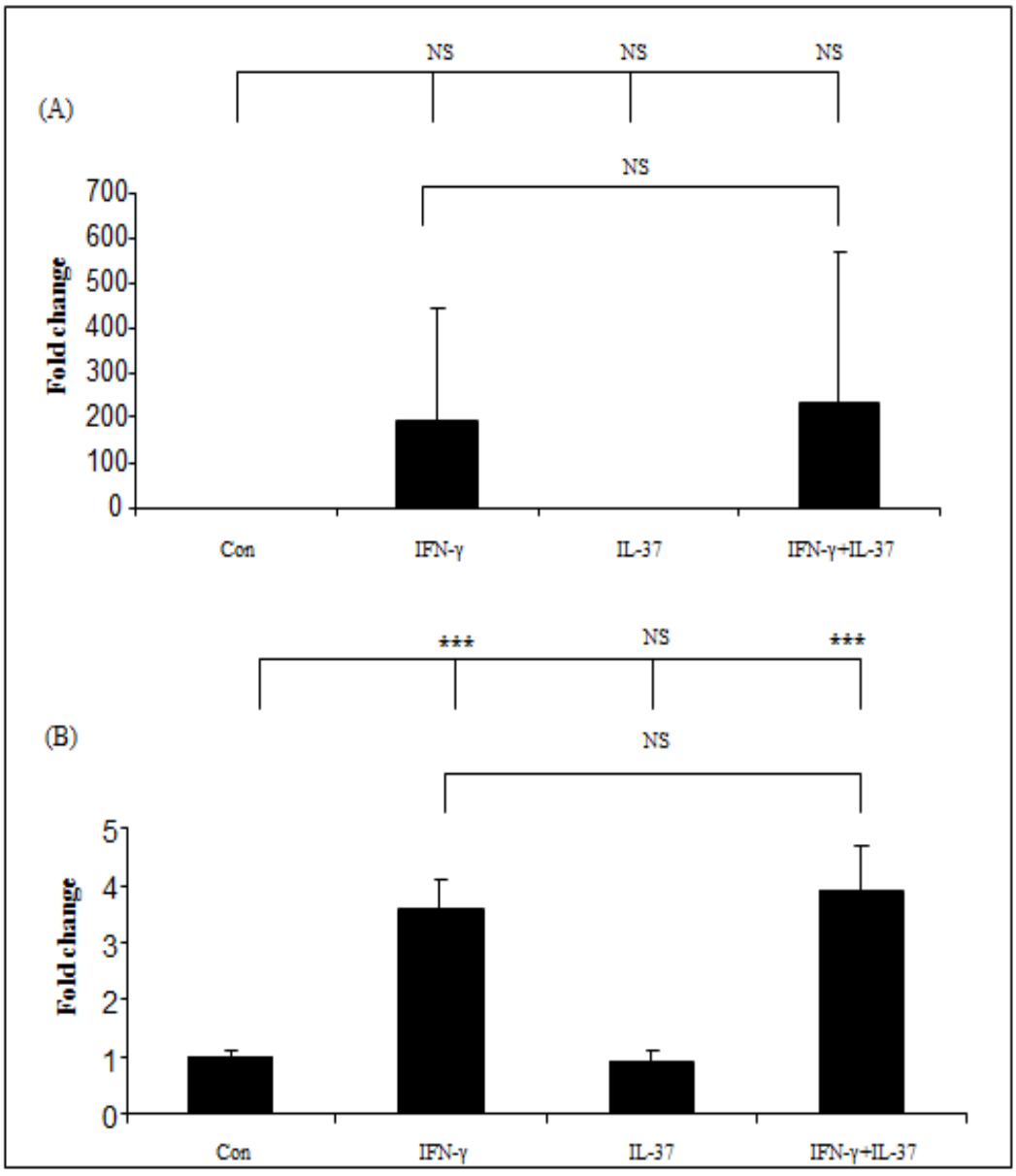

Figure 3.2.3.2 Pre-incubation of THP-1 macrophages with IL-37 for 24hr has no effect on MCP-1 and ICAM-1 mRNA expression induced by IFN-γ. THP-1 monocytes were differentiated into macrophages using PMA and then incubated for 24hr with vehicle (Con and IFN-γ (1,000U/ml)) or IL-37 (100ng/ml) (IL-37 and IFN-γ+Il-37). The cells were then treated for 3hr with IFN-γ (IFN-γ and IFN-γ+IL-37) or vehicle (Con and IL-37). Total RNA was isolated and subjected to RT-qPCR. The graph shows the fold change in mRNA expression (mean ± SD) in relation to the control, vehicle-treated cells (arbitrarily assigned as 1) from three independent experiments, samples in duplicate. A, MCP-1; B, ICAM-1. Statistical analysis was performed using one-way ANOVA with Welch's test of equality of means and Games–Howell post-hoc analysis. *** $P < 0.005$; NS indicates not significant.

3.3 The effect of IL-15 on MCP-1 and ICAM-1 expression in mouse macrophages

Previous studies had shown that IL-15 induced the expression of MCP-1 in RAW264.7 macrophages without affecting that of ICAM-1(van Es et al. 2010). However, in the present study, IL-15 inhibited MCP-1mRNA expression in THP-1 macrophages (Figure 3.2.2.2). To investigate whether this was likely to be because of species-specific differences, experiments were carried out on mouse macrophages (RAW264.7 cell line). In addition, ICAM-1 expression was included in the analysis.

3.4 Optimisation of PCR conditions

The conditions for PCR amplification with human primers were already established in our laboratory. However, this was not the case for mouse primers and hence the PCR conditions to investigate the regulation of expression of mouse (m) MCP-1 and ICAM-1 by cytokines were first optimised in this study. Primer sequences for mMCP-1 and mICAM-1 were taken from previously published papers (see Table 2.3.4) and ordered (Sigma Genosys). The amount of reagents for PCR reactions are well established in our laboratory but the annealing temperature of the primers clearly varies depending on the sequence. Therefore, for each set of primers two annealing temperatures were tested to find the optimum that gave maximal output of the amplification product. These studies showed that the optimal annealing temperature with the mMCP-1 and mICAM-1 primers were 59ºC and 60ºC respectively. The results with RT-PCR using these optimised conditions are shown in Figure 3.4.1. As expected a single product of the expected size was obtained.

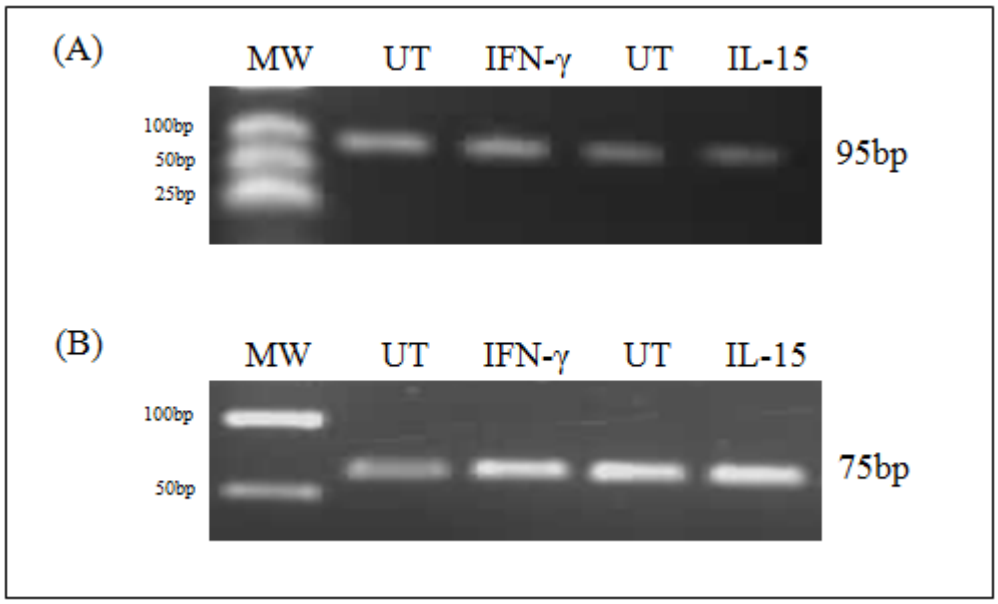

Figure 3.4.1 Gel electrophoresis showing the PCR products from reactions using the optimised conditions RNA was extracted from 24hr PMA-differentiated THP-1 macrophages that were treated with vehicle for 3hr (UT for IFN-γ(1,000U/ml)) or 24hr (UT for IL-15 (100ng/ml)) or the cytokines for this time period. PCR products for mMCP-1 and mICAM-1 are shown. The amount of all PCR reagents was the same in each reaction but the annealing temperatures were as follows: mMCP-1= 59ºC and mICAM-1= 60 ºC. The size of the expected PCR products are A: mMCP-1= 95bp and B: mICAM-1= 75bp. M shows the lane where molecular size markers were loaded.

The expression of MCP-1 and ICAM-1 was normalised to the house-keeping gene β-actin. Melting curve analysis was performed to confirm amplification of a single product (Figure 3.4.2).

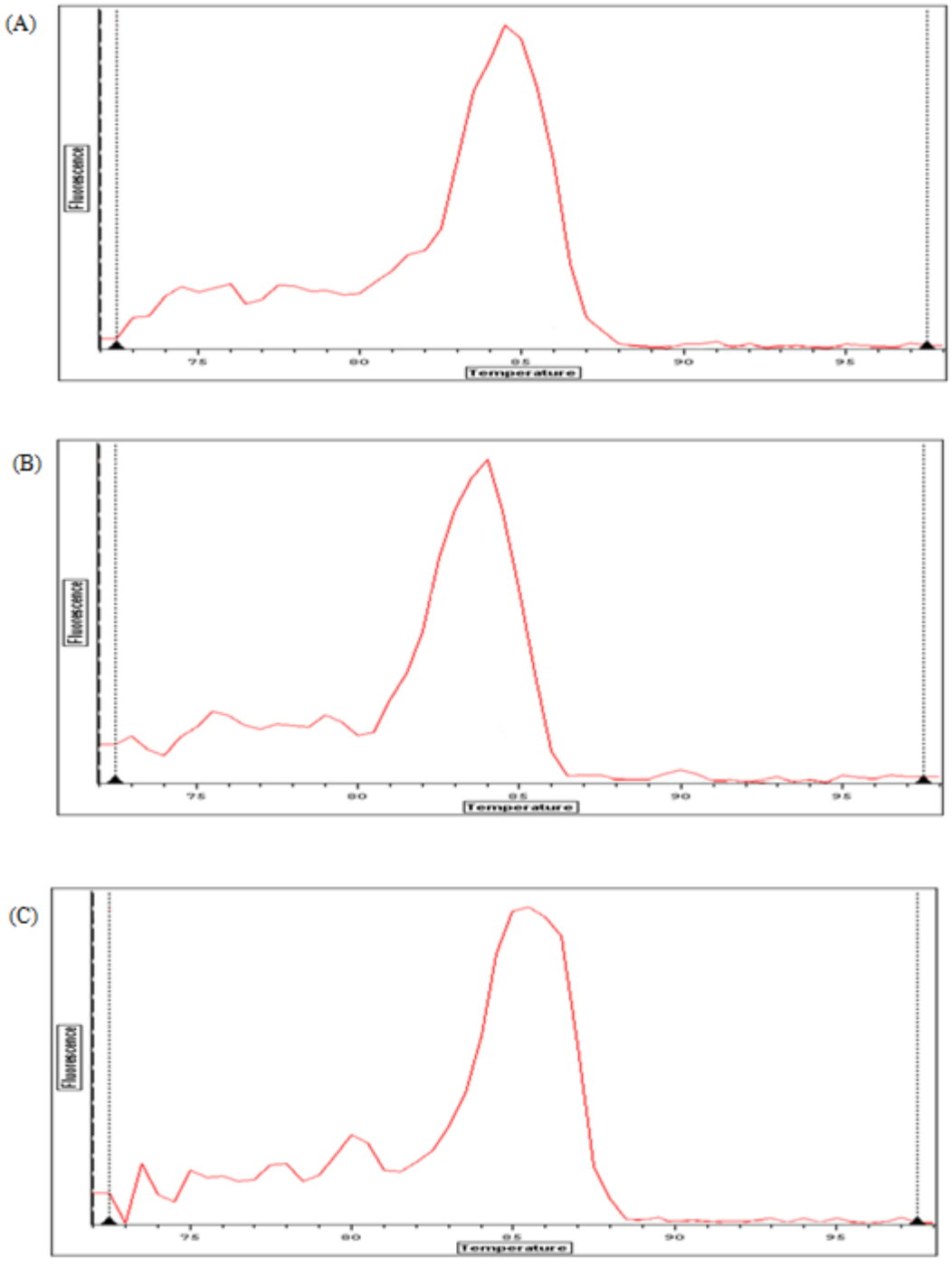

Figure 3.4.2 Melting curve analysis for Mouse Primers RTq-PCR was performed for MCP-1, ICAM-1 and β-Actin. The graphs show that a single major amplification product was produced from mouse primers A: MCP-1, B: ICAM-1 and C: β-actin.

The RNA from RAW264.7 macrophages that were treated with vehicle (control) or IFN-γ (positive control) or IL-15 was subjected to RT-qPCR. As above, the incubation period with IFN-γ was 3hr. For IL-15, the incubation period was 24hr because at only this time point was a reduction in MCP-1 mRNA expression observed. The results are shown in Figures 3.4.3 and 3.4.4 respectively.

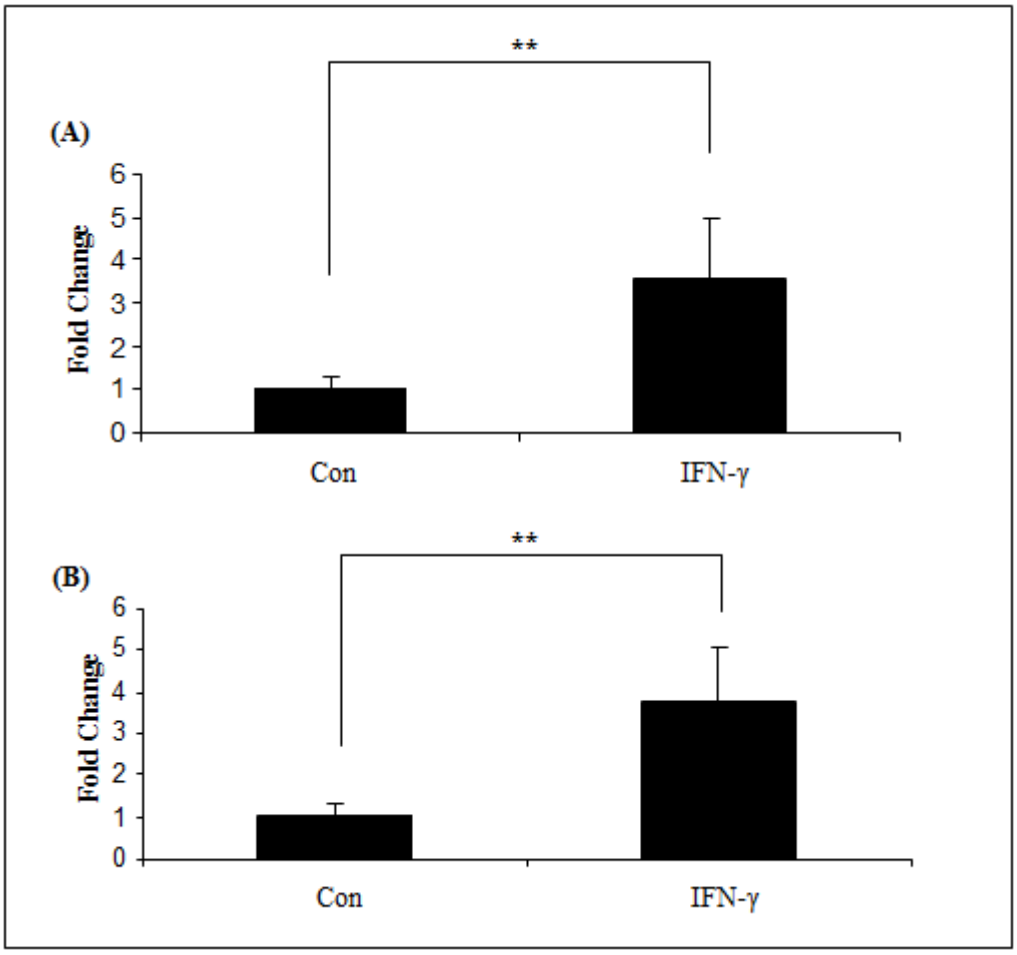

Figure 3.4.3 IFN-γ induces the expression of MCP-1 and ICAM-1 mRNA in RAW264.7 macrophages. The cells were incubated for 3hr with vehicle (Con) or IFN-γ (1,000U/ml). Total RNA was isolated and subjected to RT-qPCR. The graph shows the fold change in mRNA expression (mean ± SD) in relation to the control, vehicle-treated cells (arbitrarily assigned as 1) from three independent experiments, samples in duplicate. The mRNA levels were normalised to the house-keeping gene, β-actin. A, MCP-1; B, ICAM-1. Statistical analysis was performed using the Student's *t*-test, ** $P < 0.01$.

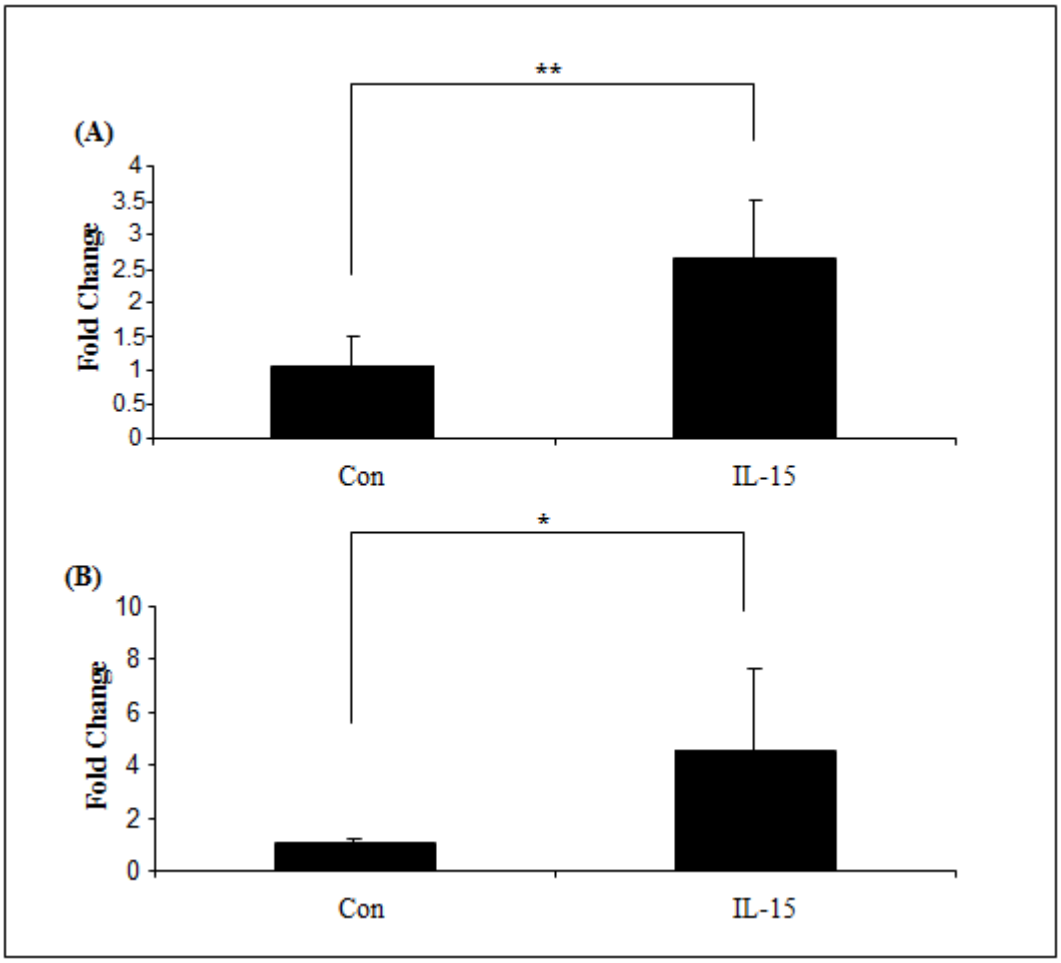

Figure 3.4.4 IL-15 induces the expression of MCP-1 and ICAM-1 mRNA in RAW264.7 macrophages. The cells were incubated for 24hr with vehicle (Con) or IL-15 (100ng/ml). Total RNA was isolated and subjected to RT-qPCR. The graph shows the fold change in mRNA expression (mean ± SD) in relation to the control, vehicle-treated cells (arbitrarily assigned as 1) from three independent experiments, samples in duplicate. A, MCP-1; B, ICAM-1. Statistical analysis was performed using the Student's *t*-test, * $P < 0.05$; ** $P < 0.01$.

As expected, IFN-γ significantly induced the mRNA expression of MCP-1 and ICAM-1 when RAW264.7 cells were stimulated with this cytokine (Figure 3.4.3). Consistent with the previous study (van Es et al. 2010), IL-15 also significantly induced the expression of MCP-1 mRNA in RAW264.7 macrophages (Figure 3.4.4). In addition, in contrast to the finding in this study, IL-15 also significantly induced the expression of ICAM-1 mRNA in these cells (Figure 3.4.4).

Given that from the recently identified cytokines, only IL-15 affected the expression of MCP-1 and ICAM-1 mRNA, its action on two additional parameters (protein expression and modified LDL uptake) was studied in THP-1 macrophages. Similar to RT-qPCR, IFN-γ was included as a positive control.

3.5 Western blot analysis

Western blot analysis was performed to investigate whether changes in mRNA expression, as identified by RT-qPCR, correlated with changes in protein expression. For this, protein extracts were prepared from THP-1 macrophages treated with IFN-γ (positive control) or IL-15 (the only cytokine from IL-15, IL-20 and IL-22 that affected gene expression). As with studies on mRNA expression, the incubation period was 3hr with IFN-γ and 24hr with IL-15. Vehicle-treated cells at these time points were included as controls. Membranes were re-probed with β-actin to ensure equal loading. Because of time limitation and as the THP-1 monocytes tend to divide slowly; these experiments were only carried out twice so it was not possible to carry out any statistical analysis.

As shown in Figure 3.5.1, a single band for expected size of ICAM-1 (110kDa) and β-actin (42KDa) were obtained. Densitometric analysis from two independent experiments showed that, similar to mRNA expression, the steady state levels of ICAM-1 eas also induced. Having confirmed the positive action of IFN-γ, the effect of IL-15 was next investigated.

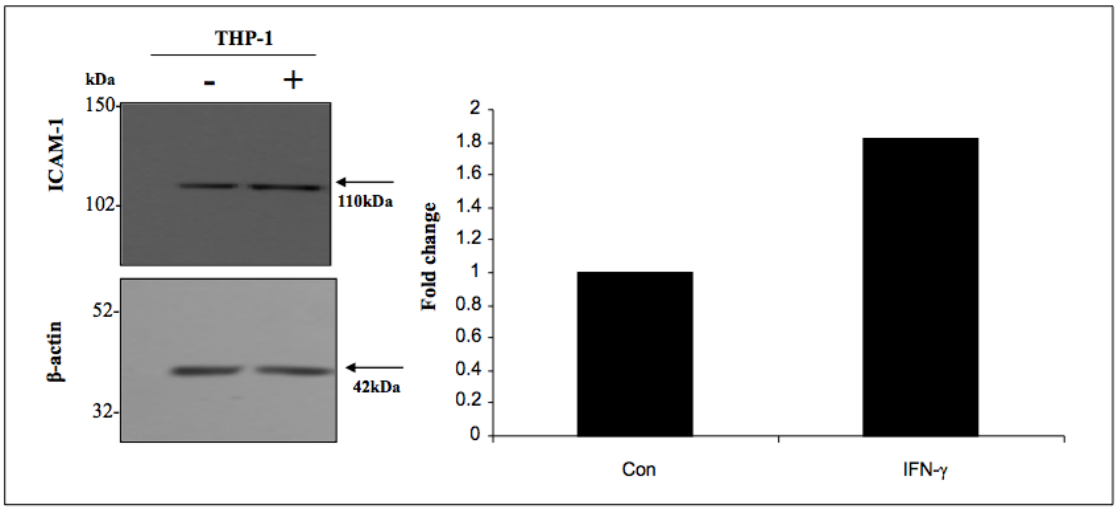

Figure 3.5.1 IFN-γ induces the expression of the ICAM-1 protein in THP-1 macrophages. The cells were incubated with vehicle (- or Con) or IFN-γ (+ or IFN-γ) (1,000U/ml) for 3hr. Total cellular proteins were then subjected to Western blot analysis using antisera against ICAM-1 and β-actin, as indicated. The image is representative of two independent experiments. The graph shows average fold change in protein expression of ICAM-1 (normalised to β-actin) in relation to the vehicle control (arbitrarily assigned as 1) from two independent experiments.

Figure 3.5.2 shows that similar to the trend in mRNA expression (Figure 3.2.2.2), IL-15 inhibited the steady state level of the ICAM-1 protein.

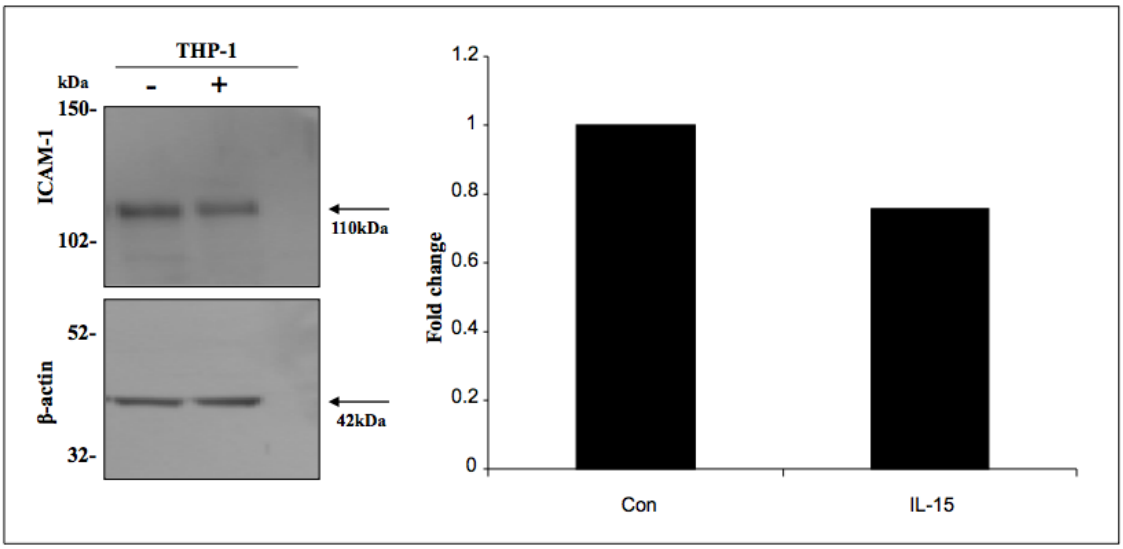

Figure 3.5.2 IL-15 inhibits the expression of the ICAM-1 proteins in THP-1 macrophages. The cells were incubated with vehicle (- or Con) or IL-15 (+ or IL-15) (100ng/ml) for 24hr. Total cellular proteins were then subjected to Western blot analysis using antisera against ICAM-1 or β-actin, as indicated. The image is representative of two independent experiments. The graph shows average fold change in protein expression of ICAM-1 (normalised to β-actin) in relation to the vehicle control (arbitrarily assigned as 1) from two independent experiments.

The MCP-1 protein is heavily glycosylated and previous studies on mouse macrophages showed multiple cross-reactive bands in the range of 14-30kDa corresponding to various glycosylation products (Zhang et al. 1996; Harvey et al. 2007). However, when Western blot analysis was carried out on human THP-1 macrophages (Figure 3.5.3), the background was generally higher and multiple immunoreactive products were obtained. Although an immune-reactive band of the expected size (14 KDa) was seen the others, particularly higher molecular weight products, represent non-specific interactions of the antibody. Although the high background makes it difficult to make major conclusions, it is interesting to note that densitometric analysis of the immunoreactive product of expected size (indicated by an arrow in Figure 3.5.1) from two independent experiment shows increased levels when the cells were incubated with IFN-γ and decreased levels when the cells were treated with IL-15 (Figure 3.5.2).

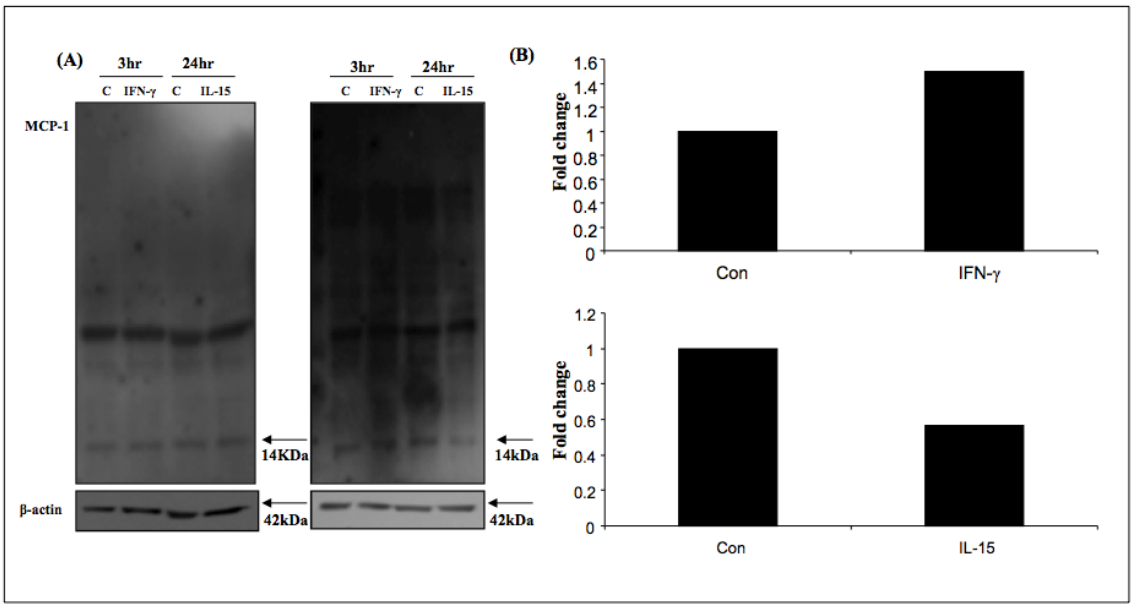

Figure 3.5.3 The effect of IFN-γ or IL-15 on MCP-1 protein expression in THP-1 macrophages. The cells were incubated with vehicle (or Con) or IFN-γ (1,000U/ml) and or IL-15 (100ng/ml) for the indicated period of time. Total cellular proteins were then subjected to Western blot analysis using antisera against MCP-1 or β-actin, as indicated. The graph shows average fold change in the levels of the immunoreactive product (normalised to β-actin) in relation to the vehicle control (arbitrarily assigned as 1) from two independent experiments.

3.6 Modified LDL uptake.

Acetylated LDL is commonly used for monitoring macrophage foam cell formation in *vitro* as it is efficiently taken up by the cells (Goldstein et al. 1979; Geng and Hansson, 1992; McLaren et al. 2010b; Michael et al. 2012). The assay involves incubation of the cells with AcLDL (10μg/ml) and either vehicle or cytokine. Cells incubated in the absence of AcLDL were also included for comparative purposes. Again, IFN-γ was used as a positive control as it is known to promote foam cell formation (McLaren and Ramji, 2009). Previous time course analysis had shown that the optimum uptake of AcLDL by cytokines occurs at 24hr so this was chosen as the incubation period for both cytokines (Michael et al. 2012). The uptake of AcLDL and its subsequent metabolism was monitored by analysing the intracellular levels of total cholesterol, free cholesterol and cholesteryl esters. Unfortunately, negative values were obtained for cholesteryl esters for unknown reasons so the levels of only total- and free-cholesterol could be determined. A slight increase in intracellular level of both total cholesterol and free cholesterol was observed when the cells were incubated with vehicle in the presence of AcLDL compared to cells treated with vehicle alone though this failed to reach statistical significance (Figure 3.6.1). In addition, no significant effect was observed with cells treated with either IFN-γ or IL-15 (Figure 3.6.1).

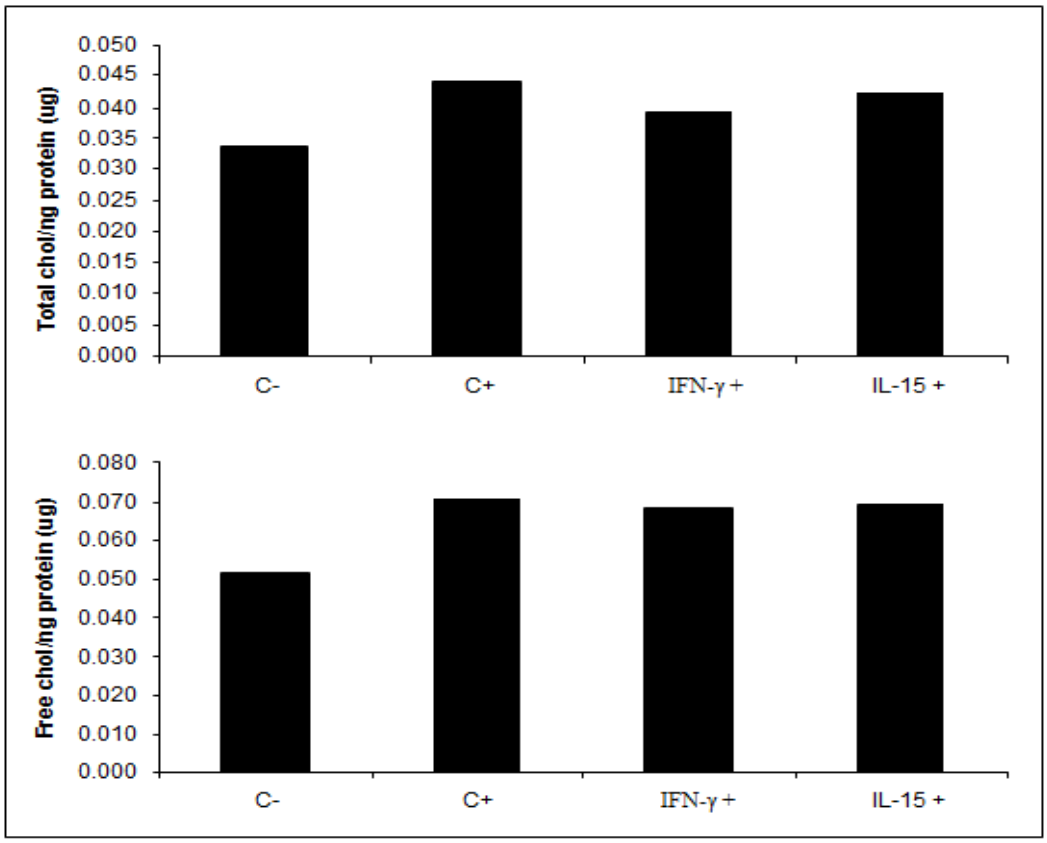

Figure 3.6.1 The effect of cytokines on AcLDL uptake by THP-1 macrophages. THP-1 macrophages were incubated for 24hrs with 10μg/ml AcLDL (+) in the presence of vehicle alone (C+) or IFN-γ (1,000U/ml) or IL-15 (100ng/ml) as indicated (cells without AcLDL (Shown as C-) were also included for comparative purposes). The intracellular levels of free cholesterol and total cholesterol were then determined statistical analysis was carried out using one way ANOVA with Welch's test of equality of means and Games–Howell post-hoc analysis. No significant change was observed.

The experiments above were carried out using complete medium and it was therefore possible that lipids and lipoproteins present in FCS might have been taken up by the cells and be responsible for only a marginal increase in intracellular cholesterol level when the cells were incubated in the presence of AcLDL compared to its absence (Figure 3.6.1). Because the majority of previous studies were carried out in medium containing 0.2% (v/v) fatty acid free BSA (Michael et al. 2012), it was decided to carry out an experiment using this condition and IFN-γ as a positive control. In addition, the buffers, reagents and enzymes were changed to see if positive values for cholesteryl esters were obtained. As shown in Figure 3.6.2, the intracellular levels of total cholesterol, free cholesterol and cholesteryl ester were increased in the presence of AcLDL and this was further elevated by IFN-γ. This confirms the pro-foam cell properties of this cytokine (Figure 3.6.2). Future studies will seek to extend the analysis to IL-15.

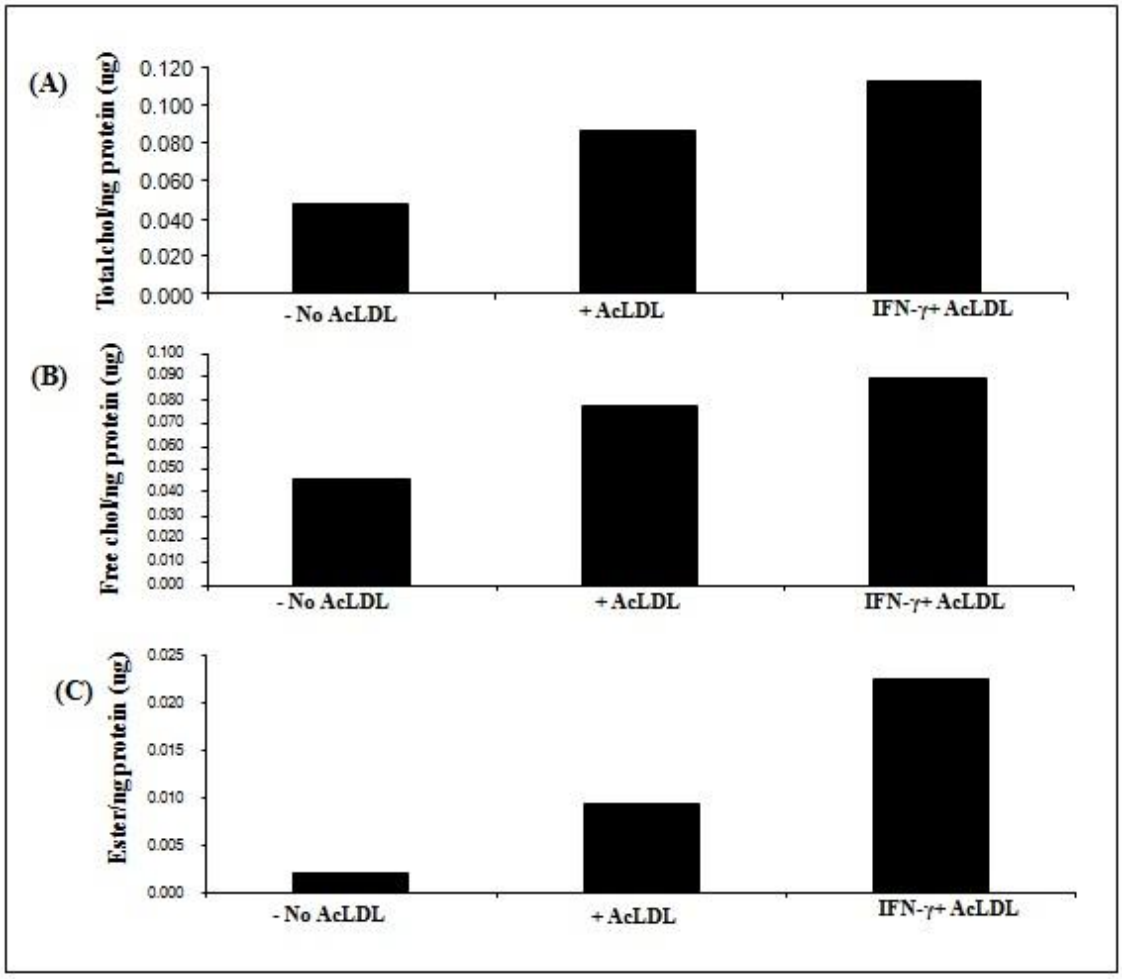

Figure 3.6.2 IFN-γ promotes foam cell formation. THP-1 macrophages were incubated for 24hrs with 10μg/ml AcLDL in the absence or the presence of IFN-γ (1,000U/ml) (cells without AcLDL were included for comparative purposes). The intracellular levels of total cholesterol, free cholesterol and cholesteryl esters were determined as described in Materials and Methods. The data is from a single experiment because of time limitations.

CHAPTER 4

DISCUSSION

4.1 Introduction

Atherosclerosis is a progressive, chronic inflammatory condition of the vasculature that causes around 40% of all deaths in the Western population. The disease is governed by various environmental and genetic risk factors (Lusis et al. 2004). It is now widely recognised that macrophages play a critical role in the development and progression of atherosclerosis (Gui et al. 2012). At the initial stage of the disease, cytokines are responsible for the recruitment of monocytes from the blood stream to the activated endothelium, where they differentiate into macrophages (Gui et al. 2012). Cytokines also regulate other stages in the disease such as foam cell formation, cell death, migration and proliferation of smooth muscle cells, and plaque rupture.

Regulation of gene expression by cytokines is of crucial importance in the field of atherosclerosis because they modulate the inflammatory signals that drive the disease process. In the context of the disease, cytokines are often grouped according to their pro- or anti-inflammatory action. It has been observed that the level of pro-inflammatory cytokines is higher than anti-inflammatory cytokines in atherosclerotic lesions (Harvey and Ramji, 2005; Halvorsen et al. 2008), and this might be responsible for the progression of the disease. Anti-inflammatory cytokines include IL-10, IL-4, TGF-β and IL-33. Pro-inflammatory cytokine include TNF-α, IFN-γ, IL-1, IL-12 and IL-17 (Tedgui and Mallat, 2006). Since monocytes and macrophages are main contributors to the pool of cytokines in the plaque, and are regulated by them to play critical roles in the disease, they are useful cells to study inflammation and atherosclerosis (Lusis, 2000).

The studies presented in this work investigated the effect of more recently identified cytokines on the expression of key genes in macrophages implicated in the control of inflammation during atherosclerosis. Three parameters were analysed (Figure 3.1). Cytokines used for this study were the classical cytokine IFN-γ whose action is well known and which was used as a positive control, and more recently identified

cytokines such as IL-37, IL-15, IL-20 and IL-22. For the purpose of this study, THP-1 cells were used as an *vitro* model system to study the mRNA expression of two markers of inflammation, namely, MCP-1 and ICAM-1, protein expression and uptake of modified LDL leading to foam cell formation.

4.2 Action of IFN-γ on the mRNA expression of MCP-1 and ICAM-1

IFN-γ plays a complex role in atherosclerosis with both pro- and anti-atherogenic actions being reported though most of in *vivo* studies point to a pro-atherogenic role (McLaren and Ramji, 2009). IFN-γ has been shown to influence many features of atherosclerosis such as foam cell formation and plaque development (McLaren and Ramji, 2009). It has also shown to increase foam cell formation in THP-1 macrophages and primary cultures (McLaren and Ramji, 2009). Previous studies in our laboratory and those of the others has shown that IFN-γ reduced the expression of efflux genes such as apoE and ABCA-1 where the expression of uptake genes such as SR-A was increased (Geng and Hansson, 1992; McLaren and Ramji, 2009). This suggested a pro-atherogenic role for IFN-γ in foam cell formation. IFN-γ is also known to induce adhesion molecules in SMCs and vascular ECs and by this action contributes to atherosclerosis development (Chung et al. 2002). The action of IFN-γ on the expression of MCP-1 and ICAM-1 was initially investigated by RT-qPCR experiments using two time points (3hr and 24hr). Consistent with previous results from our laboratory and other, IFN-γ was found to induce the expression of MCP-1 and ICAM-1 at both time point (Figure 3.2.1.1 and 3.2.1.2) (Chung et al. 2002; Harvey and Ramji, 2005; Li et al. 2010). The effect of IFN-γ on the expression ICAM-1 was also studied at the protein level using Western blot analysis. IFN-γ increased the protein expression of ICAM-1 (Figure 3.51).

IFN-γ promotes foam cell formation by stimulating the uptake of modified lipoproteins (McLaren and Ramji, 2009). Previous study in THP-1 macrophages has demonstrated that IFN-γ has propensity to increase foam cell formation (Reiss et al. 2004). The involvement of IFN-γ in the uptake of modified LDL and foam cell

formation was also studied. Results from a single experiment shown in Figure 3.6.2 showed that IFN-γ induced the AcLDL uptake by macrophages.

In conclusion, IFN-γ was found to have pro-atherogenic actions on two parameters; expression of key genes implicated in inflammation and foam cell formation.

4.3 Action of IL-15 on the expression of MCP-1 and ICAM-1 in macrophages

IL-15 has been found to promote atherosclerosis in a mouse model of atherosclerosis (van Es et al. 2010). Nevertheless, a study in intestinal tissues in Crohn's disease suggested an anti-inflammatory role of IL-15 in this disease (Silva et al. 2005). However, its role in macrophages is poorly understood. Van Es et al. (2010) found that IL-15 induced the mRNA expression of MCP-1 and had no effect on ICAM-1 in RAW264.7 macrophages, suggesting a pro-inflammatory role for IL-15 (van Es et al. 2010). In contrast with this study, IL-15 was found to inhibit the expression of MCP-1 mRNA in THP-1 macrophages and have no statistically significant effect on ICAM-1 mRNA though the trend indicated reduced levels (Figure 3.2.2.2). The latter was also suggested when the expression of the ICAM-1 protein was analysed by Western blot analysis (Figure 3.5.1). The exact reasons for the differences in the findings between THP-1 and RAW264.7 macrophages are at present unclear but could be because of species-specific differences. Indeed, species-specific differences are common in atherosclerosis, particularly lipoprotein metabolism and the inflammatory response (Escola-Gil et al. 2004). For example, mice lack cholesteryl ester transfer protein which plays a key role in lipoprotein metabolism in humans by transferring cholesteryl esters from HDL to apoB-containing lipoproteins (Tanigawa et al. 2007). In addition, the expression of nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs), can be different between mouse and human macrophages (Rigamonti et al. 2008). For instance, PPAR-α is abundant in human macrophages but barely detectable in mouse macrophages (Rigamonti et al. 2008). Furthermore, LXR-α is autoactivated in human but not mouse macrophages (Laffitte et al. 2001).

To investigate the potential existence of such species-specific differences, some experiments were carried out in the mouse macrophage RAW264.7 cell line. Consistent with previous studies (van Es et al. 2010), IL-15 enhanced the expression of MCP-1 mRNA (Figure 3.4.4). ICAM-1 mRNA expression was also found to be increased when RAW264.6 cells were stimulated with this cytokine (Figure 3.4.4). Thus, it is likely that species-specific actions of IL-15 in atherosclerosis might exist with the cytokine playing an anti-atherogenic role in humans and pro-atherogenic role in mice. However, further experiments will be required for definitive conclusions.

4.4 Action of IL-20, IL-22 and IL-37 on the expression of MCP-1 and ICAM-1 in macrophages

The production of pro-inflammatory cytokines induced by LPS was found to be totally inhibited when IL-37 was expressed in macrophages, suggesting an anti-inflammatory role for this cytokine (Boraschi et al. 2011). On the other hand, IL-20 is expressed in atherosclerotic plaques and promotes this disease in apoE deficient mice (Chen et al. 2006). IL-22 is expressed at high levels in inflamed tissue suggesting a pro-inflammatory function for this cytokine (Kreymborg and Becher, 2010.). However, the roles of these cytokines on the expression of markers of inflammation (i.e. MCP-1 and ICAM-1) in human macrophages have not been investigated. The action of IL-37, IL-20 and IL-22 on the expression of MCP-1 and ICAM-1 was therefore studied but no statistically significant results were obtained. The previous anti-inflammatory effect of IL-37 was in relation to the action of LPS (Bufler, 2011), which interacts with Toll-like receptor 4 (TLR4). The current study was performed with IFN-γ so it is likely that the anti-inflammatory action of IL-37 is dependent on the receptor to which the pro-inflammatory agent interacts with (i.e. TLR4 v/s IFN-γ receptor). The effect of IL-20 in particular was surprising given the previously identified pro-atherogenic action in mouse model systems (Chen et al. 2006). However, in this publication, IL-20 was found to induce the expression of chemokines Mig/CXXL9 and ITAC/CXCL11 in ECs so it is possible that the action of this cytokine might be cell type-specific.

4.5 Future work

The studies presented in this thesis involved investigation of the role of more recently identified cytokines on macrophage gene expression and uptake of modified LDL in relation to atherosclerosis. As only IL-15 affected MCP-1 and ICAM-1 expression, further studies focussed on this cytokine. Additional studies on this and other cytokines studied are clearly required as part of future work. Some such studies are as follows:

1. The Western blot analysis for ICAM-1 (and MCP-1) was only carried out twice so further experiments will be required for statistical analysis;
2. Complete the experiments on the effect of IFN-γ and IL-15 on the uptake of AcLDL using medium containg 0.2% (v/v) fatty acid free BSA;
3. Confirm findings from cell lines to primary cultures such as human monocyte-derived macrophages and mouse bonemarrow-derived macrophages to see whether species-specific regulation extends to primary cultures;
4. Analyse the effect of different cytokines on the expression of other genes such as scavenger receptors involved in the uptake of modified LDL, genes implicated in the efflux of cholesterol (e.g. ABCA-1, apoE). Regulation of M1, M2 and other macrophage phenotypes. Other cytokines; and proteases involved in the control of plaque stability (McLaren et al. 2011);
5. In the light of studies on IL-20 that showed an effect on ECs (Chen et al. 2006), carry out experiments with all cytokines in other cell types implicated in atherosclerosis such as primary aortic ECs and primary vascular SMCs;
6. Extend the analysis of IL-15, which is restricted to markers of inflammation and the uptake of modified LDL, to cholesterol efflux from foam cells (Panousis et al. 2001; Michael et al. 2012). In this assay, macrophages are first converted into foam cells by incubation with AcLDL in the presence of radiolabelled (tritium) cholesterol. The efflux of cholesterol to acceptors, such as HDL or lipid-free apoAI, in the absence or presence of IL-15 is then determined by scintillation counting of medium and cell lysates. IFN-γ can be used as a control because it inhibits cholesterol efflux (Panousis and Zuckerman, 2000; McLaren and Ramji, 2009);

7. Delineate whether the effect of IL-15 on MCP-1 mRNA expression occurs at the level of gene transcription or mRNA stability. Control at transcriptional level can be determined by nuclear "run-on" transcription assays (Smale, 2009). On the other hand, regulation at mRNA stability can be analysed by following the decay of MCP-1 and ICAM-1 mRNA, in the absence or the presence of IL-15, following inhibition of gene transcription using actinomycin D by RT-qPCR (Leclerc et al. 2002). IFN-γ can be used as a control b
8. ecause it is known to affect the expression of these genes mainly at the level of gene transcription (Liu et al. 2004);
9. If transcription control is involved then delineate *cis*-acting regulatory sequences and *trans* acting factors involved in the response. The former can be determined by transfection assays, in the absence or the presence of the cytokine, using promoter-reporter gene constructs that contain deletions or specific mutations in the regulatory sequences (Salter et al. 2011) . The interaction of transcription factors with such sequences can then be determined by techniques such as electrophoretic mobility shift assays (Fried and Crothers, 1981; Harris et al. 2008) and chromatin immunoprecipitation assays (Thirunavukkarasu et al. 2006; Carey et al. 2009);
10. Delineate signalling pathways using a combination of pharmacological inhibitors. Biochemical assays. siRNA-mediated knockdown; and use of macrophages from knockout mice (Ali et al. 2010; Michael et al. 2012).

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Appendices

Figure 3.2.1.1 IFN-γ induces the expression of MCP-1 and ICAM-1 mRNA in THP-1 macrophages

(A) MCP-1

Sample	MCP-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	24.35	18.62	5.73	5.85	-0.12	1.086734863
C2	24.37	18.4	5.97		0.12	0.920187651
IFN-γ1	17.32	18.46	-1.14		-6.99	127.1158394
IFN-γ2	16.6	18.72	-2.12		-7.97	250.7315962
C1	24.62	17.74	6.88	6.965	-0.085	1.060687741
C2	25.27	18.22	7.05		0.085	0.942784536
IFN-γ1	17.4	17.87	-0.47		-7.435	173.0445866
IFN-γ2	17.07	18.47	-1.4		-8.365	329.6976972
C1	23.38	18.1	5.28	5.605	-0.325	1.252664439
C2	23.98	18.05	5.93		0.325	0.798298386
IFN-γ1	16.92	17.73	-0.81		-6.415	85.33111533
IFN-γ2	16.9	18.38	-1.48		-7.085	135.7680309
C1	24.65	16.48	8.17	7.635	0.535	0.690158677
C2	25.09	17.99	7.1		-0.535	1.448942155
IFN-γ1	18.07	17.03	1.04		-6.595	96.67024548
IFN-γ2	17.01	17.36	-0.35		-7.985	253.352104

Sample	Relative Expression	SD	*p*
Control	1.025057	0.2443359	
IFN-γ	181.4639	87.409849	0.000636196

(B) ICAM-1

Sample	ICAM-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	21.83	18.62	3.21	3.425	-0.215	1.160703914
C2	22.04	18.4	3.64		0.215	0.86154616
IFN-γ1	20.13	18.46	1.67		-1.755	3.375263185
IFN-γ2	19.19	18.72	0.47		-2.955	7.754318535
C1	19.55	17.74	1.81	2.01	-0.2	1.148698355
C2	20.43	18.22	2.21		0.2	0.870550563
IFN-γ1	18.2	17.87	0.33		-1.68	3.20427951
IFN-γ2	19.05	18.47	0.58		-1.43	2.694467154
C1	19.91	18.1	1.81	2.075	-0.265	1.20163605
C2	20.39	18.05	2.34		0.265	0.832198735
IFN-γ1	19.06	17.73	1.33		-0.745	1.675974269
IFN-γ2	19.24	18.38	0.86		-1.215	2.321407829
C1	21.22	16.48	4.74	4.59	0.15	0.901250463
C2	22.43	17.99	4.44		-0.15	1.109569472
IFN-γ1	20.13	17.03	3.1		-1.49	2.808889751
IFN-γ2	19.71	17.36	2.35		-2.24	4.723970646

Sample	Relative Expression	SD	*p*
Control	1.010769214	0.157437999	
IFN-γ	3.56982136	1.908776944	0.007609

Figure 3.2.1.2 IFN-γ induces the expression of MCP-1 and ICAM-1 mRNA in THP-1 macrophages at 24hr

(A) MCP-1

Sample	MCP-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	22.38	18.24	4.14	4.64	-0.5	1.414213562
C2	23.2	18.05	5.15		0.51	0.702222438
C3	22.42	17.79	4.63		-0.01	1.00695555
IFN-γ1	18.06	18.44	-0.38		-5.02	32.44670335
IFN-γ2	17	17.75	-0.75		-5.39	41.93258892
IFN-γ3	17.45	17.78	-0.33		-4.97	31.34144952
C1	24.08	18.07	6.01	6.22	-0.21	1.156688184
C2	24.04	17.55	6.49		0.27	0.829319546
C3	23.99	17.83	6.16		-0.06	1.042465761
IFN-γ1	19	18.89	0.11		-6.11	69.07060714
IFN-γ2	18.19	18.07	0.12		-6.1	68.5935016
IFN-γ3	18.8	18.27	0.53		-5.69	51.62507259
C1	24.7	18.07	6.63	6.813333	-0.18333	1.135504429
C2	24.79	17.97	6.82		0.006667	0.995389679
C3	24.74	17.75	6.99		0.176667	0.884744831
IFN-γ1	18.44	18.91	-0.47		-7.28333	155.7764497
IFN-γ2	18.34	18.41	-0.07		-6.88333	118.0564728
IFN-γ3	18.58	18.2	0.38		-6.43333	86.42239655
C1	23.66	18.76	4.9	5.576667	-0.67667	1.598442299
C2	24.02	17.37	6.65		1.073333	0.475219739
C3	23.22	18.04	5.18		-0.39667	1.316462719
IFN-γ1	16.54	17.75	-1.21		-6.78667	110.4053801
IFN-γ2	16.84	17.62	-0.78		-6.35667	81.94969454
IFN-γ3	16.93	17.69	-0.76		-6.33667	80.82146888

Sample	Relative Expression	SD	*p*
Control	1.046469	0.3097226	
IFN-γ	77.37015	37.185502	1.94596E-05

(B) ICAM-1

Sample	ICAM-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	21.22	18.24	2.98	3.576667	-0.59667	1.51221856
C2	21.89	18.05	3.84		0.263333	0.833160684
C3	21.7	17.79	3.91		0.333333	0.793700526
IFN-γ1	21.3	18.44	2.86		-0.71667	1.643380629
IFN-γ2	21.16	17.75	3.41		-0.16667	1.122462048
IFN-γ3	21.52	17.78	3.74		0.163333	0.892959511
C1	21.21	18.07	3.14	3.82	-0.68	1.602139755
C2	21.48	17.55	3.93		0.11	0.926588062
C3	22.22	17.83	4.39		0.57	0.673616788
IFN-γ1	22.06	18.89	3.17		-0.65	1.569168196
IFN-γ2	21.81	18.07	3.74		-0.08	1.057018041
IFN-γ3	21.74	18.27	3.47		-0.35	1.274560627
C1	20.35	18.07	2.28	2.473333	-0.19333	1.143402487
C2	20.25	17.97	2.28		-0.19333	1.143402487
C3	20.61	17.75	2.86		0.386667	0.764894847
IFN-γ1	20.09	18.91	1.18		-1.29333	2.450936885
IFN-γ2	20.31	18.41	1.9		-0.57333	1.487957514
IFN-γ3	20.8	18.2	2.6		0.126667	0.91594529
C1	20.76	18.76	2	2.803333	-0.80333	1.745128575
C2	20.77	17.37	3.4		0.596667	0.661280073
C3	21.05	18.04	3.01		0.206667	0.866537046
IFN-γ1	19.27	17.75	1.52		-1.28333	2.434007027
IFN-γ2	19.77	17.62	2.15		-0.65333	1.572797936
IFN-γ3	19.42	17.69	1.73		-1.07333	2.104289696
Sample	Relative Expression	SD	*p*			
Control	1.055505824	0.375916171				
IFN-γ	1.543790283	0.543457011	0.035085			

Figure 3.2.2.1 IL-15, IL-20 and IL-22 have no effect on MCP-1 and ICAM-1 mRNA expression in THP-1 macrophages at 3hr

(A) MCP-1

Sample	MCP-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	24.35	18.62	5.73	5.85	-0.12	1.086734863
C2	24.37	18.4	5.97		0.12	0.920187651
IL-15 1	24.14	18.87	5.27		-0.58	1.494849249
IL-15 2	25.4	18.04	7.36		1.51	0.351111219
IL-20 1	24.32	18.28	6.04		0.19	0.876605721
IL-20 2	23.91	18.39	5.52		-0.33	1.257013375
IL-22 1	24.35	18.33	6.02		0.17	0.888842681
IL-22 2	25.02	18.92	6.1		0.25	0.840896415
C1	24.62	17.74	6.88	6.965	-0.085	1.060687741
C2	25.27	18.22	7.05		0.085	0.942784536
IL-15 1	24.92	17.71	7.21		0.245	0.843815796
IL-15 2	25.16	18.35	6.81		-0.155	1.113421618
IL-20 1	25.16	19.75	5.41		-1.555	2.938337267
IL-20 2	25.32	18.61	6.71		-0.255	1.193335743
IL-22 1	25.29	19.88	5.41		-1.555	2.938337267
IL-22 2	25.01	19.14	5.87		-1.095	2.136130816
C1	23.38	18.1	5.28	5.605	-0.325	1.252664439
C2	23.98	18.05	5.93		0.325	0.798298386
IL-15 1	23.94	17.94	6		0.395	0.760489377
IL-15 2	24.29	18.04	6.25		0.645	0.639492791
IL-20 1	24.54	18.06	6.48		0.875	0.545253866
IL-20 2	24.09	17.64	6.45		0.845	0.556710809
IL-22 1	24.2	17.83	6.37		0.765	0.588453369
IL-22 2	24.08	17.89	6.19		0.585	0.666649339
C1	24.65	16.48	8.17	7.635	0.535	0.690158677
C2	25.09	17.99	7.1		-0.535	1.448942155
IL-15 1	23.68	17.36	6.32		-1.315	2.488023307
IL-15 2	24.38	17.21	7.17		-0.465	1.380317353
IL-20 1	24.23	17.19	7.04		-0.595	1.510472586

IL-20 2 IL-22 1 IL-22 2	24.09 24.39 23.68	16.64 16.59 17.32	7.45 7.8 6.36		-0.185 0.165 -1.275	1.136816973 0.891928519 2.419988178
Sample	Relative Expression	SD	*p*			
Control	1.025057	0.2443359				
IL-15	1.13394	0.6661034	0.695029966			
IL-20	1.251818	0.7607433	0.457769395			
IL-22	1.421403	0.9237739	0.233760736			

(B) ICAM-1

Sample	ICAM-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	21.83	18.62	3.21	3.425	-0.215	1.160703914
C2	22.04	18.4	3.64		0.215	0.86154616
IL-15 1	21.96	18.87	3.09		-0.335	1.261377409
IL-15 2	21.8	18.04	3.76		0.335	0.792784137
IL-20 1	21.79	18.28	3.51		0.085	0.942784536
IL-20 2	22.12	18.39	3.73		0.305	0.809442217
IL-22 1	22.03	18.33	3.7		0.275	0.826450318
IL-22 2	22.37	18.92	3.45		0.025	0.982820599
C1	19.55	17.74	1.81	2.01	-0.2	1.148698355
C2	20.43	18.22	2.21		0.2	0.870550563
IL-15 1	20.39	17.71	2.68		0.67	0.628506687
IL-15 2	20.46	18.35	2.11		0.1	0.933032992
IL-20 1	22.04	19.75	2.29		0.28	0.823591017
IL-20 2	21.47	18.61	2.86		0.85	0.554784736
IL-22 1	22.34	19.88	2.46		0.45	0.732042848
IL-22 2	21.42	19.14	2.28		0.27	0.829319546
C1	19.91	18.1	1.81	2.075	-0.265	1.20163605
C2	20.39	18.05	2.34		0.265	0.832198735
IL-15 1	20.26	17.94	2.32		0.245	0.843815796
IL-15 2	20.6	18.04	2.56		0.485	0.71449707
IL-20 1	20.54	18.06	2.48		0.405	0.755236293
IL-20 2	20.13	17.64	2.49		0.415	0.750019495
IL-22 1	20.58	17.83	2.75		0.675	0.626332219
IL-22 2	20.9	17.89	3.01		0.935	0.52304247
C1	21.22	16.48	4.74	4.59	0.15	0.901250463
C2	22.43	17.99	4.44		-0.15	1.109569472
IL-15 1	21.09	17.36	3.73		-0.86	1.815038311
IL-15 2	21.73	17.21	4.52		-0.07	1.049716684
IL-20 1	21.04	17.19	3.85		-0.74	1.670175839

IL-20 2 IL-22 1 IL-22 2	21.12 21.39 21.51	16.64 16.59 17.32	4.48 4.8 4.19		-0.11 0.21 -0.4	1.079228237 0.864537231 1.319507911
Sample	Relative Expression	SD	*p*			
Control	1.010769214	0.157437999	0.969719			
IL-15	1.004846136	0.38288849	0.018109			
IL-20	0.723054328	0.304753064	0.120451			
IL-22	0.838006643	0.241709465				

Figure 3.2.2.2 IL-15 attenuates MCP-1 mRNA expression in THP-1 macrophages at 24hr

(A) MCP-1

Sample	MCP-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	22.38	18.24	4.14	4.64	-0.5	1.414213562
C2	23.2	18.05	5.15		0.51	0.702222438
C3	22.42	17.79	4.63		-0.01	1.00695555
IL-15 1	23.05	17.76	5.29		0.65	0.637280314
IL-15 2	23.9	18.26	5.64		1	0.5
IL-15 3	25.84	20.22	5.62		0.98	0.50697974
IL-20 1	24.36	19.99	4.37		-0.27	1.205807828
IL-20 2	24.5	18.37	6.13		1.49	0.356012549
IL-20 3	24.99	18.54	6.45		1.81	0.285190929
IL-22 1	24.27	18.23	6.04		1.4	0.378929142
IL-22 2	25.04	18.41	6.63		1.99	0.251738888
IL-22 3	24.72	18.44	6.28		1.64	0.320856474
C1	24.08	18.07	6.01	6.22	-0.21	1.156688184
C2	24.04	17.55	6.49		0.27	0.829319546
C3	23.99	17.83	6.16		-0.06	1.042465761
IL-15 1	23.79	17.52	6.27		0.05	0.965936329
IL-15 2	23.72	17.74	5.98		-0.24	1.180992661
IL-15 3	24.02	17.41	6.61		0.39	0.763129604
IL-20 1	24.27	17.9	6.37		0.15	0.901250463
IL-20 2	24.3	18.37	5.93		-0.29	1.222640278
IL-20 3	24.63	18.06	6.57		0.35	0.784584098
IL-22 1	24.72	18.76	5.96		-0.26	1.197478705
IL-22 2	24.31	17.91	6.4		0.18	0.882702996
IL-22 3	24.07	17.97	6.1		-0.12	1.086734863
C1	24.7	18.07	6.63	6.813333	-0.18333	1.135504429

C2	24.79	17.97	6.82		0.006667	0.995389679
C3	24.74	17.75	6.99		0.176667	0.884744831
IL-15 1	25.42	17.76	7.66		0.846667	0.556068043
IL-15 2	24.96	17.16	7.8		0.986667	0.504642401
IL-15 3	24.65	17.66	6.99		0.176667	0.884744831
IL-20 1	24.96	17.59	7.37		0.556667	0.679871186
IL-20 2	25.02	18.08	6.94		0.126667	0.91594529
IL-20 3	24.82	18.08	6.74		-0.07333	1.052144848
IL-22 1	24.58	18.7	5.88		-0.93333	1.909683208
IL-22 2	24.72	18.57	6.15		-0.66333	1.583737611
IL-22 3	24.74	18.16	6.58		-0.23333	1.175547906
C1	23.66	18.76	4.9	5.576667	-0.67667	1.598442299
C2	24.02	17.37	6.65		1.073333	0.475219739
C3	23.22	18.04	5.18		-0.39667	1.316462719
IL-15 1	23.99	17.2	6.79		1.213333	0.431271016
IL-15 2	24.95	17.29	7.66		2.083333	0.235968578
IL-15 3	24.29	17.17	7.12		1.543333	0.343091828
IL-20 1	24.51	17.13	7.38		1.803333	0.28651184
IL-20 2	25.69	18.31	7.38		1.803333	0.28651184
IL-20 3	25.05	18.31	6.74		1.163333	0.446479756
IL-22 1	25.24	17.8	7.44		1.863333	0.274840528
IL-22 2	25.97	17.48	8.49		2.913333	0.132739225
IL-22 3	24.8	17.87	6.93		1.353333	0.391386708
Sample	Relative Expression	SD	*p*			
Control	1.046469	0.3097226				
IL-15	0.625842	0.2745566	0.005351569			
IL-20	0.701913	0.3623486	0.024286578			
IL-22	0.798865	0.5900055	0.219406158			

(B) ICAM-1

Sample	ICAM-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	21.22	18.24	2.98	3.576667	-0.59667	1.51221856
C2	21.89	18.05	3.84		0.263333	0.833160684
C3	21.7	17.79	3.91		0.333333	0.793700526
IL-15 1	22.63	17.76	4.87		1.293333	0.408007242
IL-15 2	22.24	18.26	3.98		0.403333	0.75610928
IL-15 3	24.51	20.22	4.29		0.713333	0.60990932
IL-20 1	23.33	19.99	3.34		-0.23667	1.178267139
IL-20 2	22.94	18.37	4.57		0.993333	0.502315837
IL-20 3	22.19	18.54	3.65		0.073333	0.950439478
IL-22 1	22.05	18.23	3.82		0.243333	0.844791174
IL-22 2	22.31	18.41	3.9		0.323333	0.79922115
IL-22 3	22.73	18.44	4.29		0.713333	0.60990932
C1	21.21	18.07	3.14	3.82	-0.68	1.602139755
C2	21.48	17.55	3.93		0.11	0.926588062
C3	22.22	17.83	4.39		0.57	0.673616788
IL-15 1	22.3	17.52	4.78		0.96	0.514056913
IL-15 2	21.1	17.74	3.36		-0.46	1.375541818
IL-15 3	21.16	17.41	3.75		-0.07	1.049716684
IL-20 1	21.92	17.9	4.02		0.2	0.870550563
IL-20 2	22.01	18.37	3.64		-0.18	1.132883885
IL-20 3	21.06	18.06	3		-0.82	1.765405993
IL-22 1	22.7	18.76	3.94		0.12	0.920187651
IL-22 2	21.82	17.91	3.91		0.09	0.939522749
IL-22 3	22.36	17.97	4.39		0.57	0.673616788
C1	20.35	18.07	2.28	2.473333	-0.19333	1.143402487

C2	20.25	17.97	2.28		-0.19333	1.143402487
C3	20.61	17.75	2.86		0.386667	0.764894847
IL-15 1	21.49	17.76	3.73		1.256667	0.418509806
IL-15 2	21.01	17.16	3.85		1.376667	0.385107556
IL-15 3	20.66	17.66	3		0.526667	0.694156725
IL-20 1	20.26	17.59	2.67		0.196667	0.872564288
IL-20 2	20.75	18.08	2.67		0.196667	0.872564288
IL-20 3	20.63	18.08	2.55		0.076667	0.948246031
IL-22 1	20.73	18.7	2.03		-0.44333	1.359742373
IL-22 2	21.12	18.57	2.55		0.076667	0.948246031
IL-22 3	21.11	18.16	2.95		0.476667	0.718636109
C1	20.76	18.76	2	2.803333	-0.80333	1.745128575
C2	20.77	17.37	3.4		0.596667	0.661280073
C3	21.05	18.04	3.01		0.206667	0.866537046
IL-15 1	21.27	17.2	4.07		1.266667	0.415618948
IL-15 2	20.56	17.29	3.27		0.466667	0.723634619
IL-15 3	21.37	17.17	4.2		1.396667	0.379805666
IL-20 1	20.74	17.13	3.61		0.806667	0.571701243
IL-20 2	21.34	18.31	3.03		0.226667	0.854607174
IL-20 3	20.96	18.31	2.65		-0.15333	1.112136086
IL-22 1	21.17	17.8	3.37		0.566667	0.675174973
IL-22 2	20.83	17.48	3.35		0.546667	0.684600064
IL-22 3	21.11	17.87	3.24		0.436667	0.73883972
Sample	Relative Expression	SD	*p*			
Control	1.055505824	0.375916171				
IL-15	0.644181215	0.306705746	0.035085			
IL-20	0.969306834	0.322376588	0.611121			
IL-22	0.826040675	0.203254389	0.058666			

Figure 3.2.3.1 Pre-incubation of THP-1 macrophages with IL-37 for 3hr has no effect on MCP-1 and ICAM-1 mRNA expression induced by IFN-γ

(A) MCP-1

Sample	MCP-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	27.59	18.61	8.98	8.77	0.21	0.864537
C2	27.34	18.78	8.56		-0.21	1.156688
IFN-γ1	17.87	18.26	-0.39		-9.16	572.051
IFN-γ2	17.99	18.67	-0.68		-9.45	699.4126
IL-37 1	26.85	18.08	8.77		0	1
IL-37 2	27.01	18.21	8.8		0.03	0.97942
IL-37+ IFN-γ1	18.23	18.54	-0.31		-9.08	541.1932
IL-37+I IFN-γ2	17.92	18.42	-0.5		-9.27	617.3736
C1	22.22	18.8	3.42	3.63	-0.21	1.156688
C2	22.54	18.7	3.84		0.21	0.864537
IFN-γ1	17.32	18	-0.68		-4.31	19.83532
IFN-γ2	18.23	20.91	-2.68		-6.31	79.34129
IL-37 1	22.98	17.68	5.3		1.67	0.314253
IL-37 2	22.48	18.17	4.31		0.68	0.624165
IL-37+ IFN-γ1	16.75	18.19	-1.44		-5.07	33.59093
IL-37+I IFN-γ2	17.33	17.65	-0.32		-3.95	15.45498
C1	22.39	17.98	4.41	4.87	-0.46	1.375542
C2	23.11	17.78	5.33		0.46	0.726986
IFN-γ1	17.68	17.5	0.18		-4.69	25.81254
IFN-γ2	17.1	17.82	-0.72		-5.59	48.1679
IL-37 1	22.26	17.25	5.01		0.14	0.907519
IL-37 2	22.47	17.33	5.14		0.27	0.82932
IL-37+ IFN-γ1	16.66	17.3	-0.64		-5.51	45.56961
IL-37+I IFN-γ2	17.14	18.2	-1.06		-5.93	60.96883

Sample	Relative Expression	SD	*p*	
Control	1.024163	0.244077	0.116054	
IFN-γ	240.7701	309.281	0.159048	
IL-37	0.77578	0.263803	0.115305	
IL-37+ IFN-γ	219.0252	280.4885	0.279957	

(B) ICAM-1

Sample	ICAM-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	22.55	18.61	3.94	3.925	0.015	0.989656656
C2	22.69	18.78	3.91		-0.015	1.010451446
IFN-γ1	19.93	18.26	1.67		-2.255	4.773342972
IFN-γ2	20.11	18.67	1.44		-2.485	5.598343462
IL-37 1	22.46	18.08	4.38		0.455	0.729510172
IL-37 2	22.62	18.21	4.41		0.485	0.71449707
IL-37+ IFN-γ1	19.61	18.54	1.07		-2.855	7.23503502
IL-37+I IFN-γ2	18.89	18.42	0.47		-3.455	10.96626244
C1	20.23	18.8	1.43	1.47	-0.04	1.028113827
C2	20.21	18.7	1.51		0.04	0.972654947
IFN-γ1	18.93	18	0.93		-0.54	1.453972517
IFN-γ2	20.84	20.91	-0.07		-1.54	2.907945035
IL-37 1	20.29	17.68	2.61		1.14	0.453759578
IL-37 2	20.06	18.17	1.89		0.42	0.747424624
IL-37+ IFN-γ1	18.6	18.19	0.41		-1.06	2.084931522
IL-37+I IFN-γ2	18.36	17.65	0.71		-0.76	1.693490625
C1	20.64	17.98	2.66	3.045	-0.385	1.305859787
C2	21.21	17.78	3.43		0.385	0.765778999
IFN-γ1	20.03	17.5	2.53		-0.515	1.42899414
IFN-γ2	19.55	17.82	1.73		-1.315	2.488023307
IL-37 1	20.52	17.25	3.27		0.225	0.855595026
IL-37 2	20.68	17.33	3.35		0.305	0.809442217
IL-37+ IFN-γ1	18.53	17.3	1.23		-1.815	3.518596304
IL-37+I IFN-γ2	18.67	18.2	0.47		-2.575	5.958709852

Sample	Relative Expression	SD	*p*
Control	1.012086	0.172793	0.034624
IFN-γ	3.108437	1.83157	0.019098
IL-37	0.718371	0.140091	0.034128
IL-37+ IFN-γ	5.242838	3.543506	0.069734

Figure 3.2.3.2 Pre-incubation of THP-1 macrophages with IL-37 for 24hr has no effect on MCP-1 and ICAM-1 mRNA expression induced by IFN-γ

(A) MCP-1

Sample	MCP-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
				8.51		
C1	26.31	18.36	7.95		-0.56	1.474269
C2	27.53	18.46	9.07		0.56	0.678302
IFN-γ1	18.8	19.27	-0.47		-8.98	504.9511
IFN-γ2	18.06	18.62	-0.56		-9.07	537.4549
IL-37 1	27.04	20.26	6.78		-1.73	3.317278
IL-37 2	26.78	18.25	8.53		0.02	0.986233
IL-37+ IFN-γ1	17.79	18.2	-0.41		-8.92	484.3815
IL-37+I IFN-γ2	17.74	18.9	-1.16		-9.67	814.6294
				5.735		
C1	23.51	17.98	5.53		-0.205	1.152686
C2	23.13	17.19	5.94		0.205	0.867539
IFN-γ1	19.24	17.19	2.05		-3.685	12.86162
IFN-γ2	19.31	17.6	1.71		-4.025	16.27968
IL-37 1	24.05	17.52	6.53		0.795	0.576343
IL-37 2	24.34	17.57	6.77		1.035	0.488016
IL-37+ IFN-γ1	19.11	17.97	1.14		-4.595	24.16756
IL-37+I IFN-γ2	18.7	17.82	0.88		-4.855	28.94014
				4.44		
C1	22.33	18.02	4.31		-0.13	1.094294
C2	23.03	18.46	4.57		0.13	0.913831
IFN-γ1	18.03	18.55	-0.52		-4.96	31.12496
IFN-γ2	18.55	20.11	-1.56		-6	64
IL-37 1	22.83	18.31	4.52		0.08	0.946058
IL-37 2	22.95	18.57	4.38		-0.06	1.042466

IL-37+ IFN-γ1 IL-37+I IFN-γ2	17.77 17.96	18.09 18.35	-0.32 -0.39		-4.76 -4.83	27.09585 28.44297
Sample	Relative Expression	SD	*p*			
Control	0.913831	0.275732	0.121081			
IFN-γ	194.4454	253.9585	0.604575			
IL-37	1.226066	1.049729	0.151252			
IL-37+ IFN-γ	234.6096	337.9235	0.440934			

(B) ICAM-1

Sample	ICAM-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	21.06	18.36	2.7	2.79	-0.09	1.064370182
C2	21.34	18.46	2.88		0.09	0.939522749
IFN-γ1	20.13	19.27	0.86		-1.93	3.810551992
IFN-γ2	19.67	18.62	1.05		-1.74	3.340351678
IL-37 1	22.79	20.26	2.53		-0.26	1.197478705
IL-37 2	21.71	18.25	3.46		0.67	0.628506687
IL-37+ IFN-γ1	19.06	18.2	0.86		-1.93	3.810551992
IL-37+I IFN-γ2	19.25	18.9	0.35		-2.44	5.42641731
C1	19.84	17.98	1.86	2.14	-0.28	0.823591017
C2	19.61	17.19	2.42		0.28	1.214194884
IFN-γ1	17.58	17.19	0.39		-1.75	0.297301779
IFN-γ2	18.22	17.6	0.62		-1.52	0.348685917
IL-37 1	19.53	17.52	2.01		-0.13	0.91383145
IL-37 2	19.7	17.57	2.13		-0.01	0.993092495
IL-37+ IFN-γ1	18.45	17.97	0.48		-1.66	0.316439148
IL-37+I IFN-γ2	17.95	17.82	0.13		-2.01	0.248273124
C1	20.04	18.02	2.02	1.985	0.035	0.976031761
C2	20.41	18.46	1.95		-0.035	1.024556823
IFN-γ1	18.67	18.55	0.12		-1.865	3.642679334
IFN-γ2	19.95	20.11	-0.16		-2.145	4.422922613
IL-37 1	20.65	18.31	2.34		0.355	0.781869643
IL-37 2	20.74	18.57	2.17		0.185	0.879649076
IL-37+ IFN-γ1	18.42	18.09	0.33		-1.655	3.149231906
IL-37+I IFN-γ2	18.42	18.35	0.07		-1.915	3.771138144

Sample	Relative Expression	SD	*p*
Control	1.007045	0.130905	0.081169
IFN-γ	2.643749	1.83211	0.199601
IL-37	0.899071	0.192574	0.199601
IL-37+ IFN-γ	2.787009	2.081477	0.737628

Figure 3.4.3 IFN-γ induces the expression of MCP-1 and ICAM-1 mRNA in RAW264.7 macrophages

(A) MCP-1

Sample	MCP-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	19.78	15.83	3.95	3.45	0.5	0.707107
C2	18.77	15.82	2.95		-0.5	1.414214
IFN-γ1	17.32	15.22	2.1		-1.35	2.549121
IFN-γ2	17.05	15.33	1.72		-1.73	3.317278
C1	22.81	14.35	8.46	8.315	0.145	0.904379
C2	22.26	14.09	8.17		-0.145	1.105731
IFN-γ1	22.12	14.85	7.27		-1.045	2.063366
IFN-γ2	20.53	14.77	5.76		-2.555	5.876675
C1	21.35	15.31	6.04	5.65	0.39	0.76313
C2	20.83	15.57	5.26		-0.39	1.310393
IFN-γ1	19.3	15.83	3.47		-2.18	4.531536
IFN-γ2	20.25	16.25	4		-1.65	3.138336

Sample	Relative Expression	SD	*p*
Control	1.034158897	0.290906258	
IFN-γ	3.579385377	1.400507738	0.006673

(B) ICAM-1

Sample	ICAM-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	23.29	15.83	7.46	7.845	-0.385	1.305859787
C2	23.44	15.21	8.23		0.385	0.765778999
IFN-γ1	21.51	15.22	6.29		-1.555	2.938337267
IFN-γ2	21.36	15.33	6.03		-1.815	3.518596304
C1	23.13	16.03	7.1	6.6	0.5	0.707106781
C2	22.11	16.01	6.1		-0.5	1.414213562
IFN-γ1	20.05	15.75	4.3		-2.3	4.924577653
IFN-γ2	19.85	14.68	5.17		-1.43	2.694467154
C1	21.38	15.31	6.07	6.01	0.06	0.959264119
C2	21.52	15.57	5.95		-0.06	1.042465761
IFN-γ1	20.44	15.83	4.61		-1.4	2.639015822
IFN-γ2	19.73	16.25	3.48		-2.53	5.775716782

Sample	Relative Expression	SD	*p*
Control	1.032448168	0.283871256	
IFN-γ	3.74845183	1.30720513	0.006047

Figure 3.4.4 IL-15 induces the expression of MCP-1 and ICAM-1 mRNA in RAW264.7 macrophages

(A) MCP-1

Sample	MCP-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	21.54	14.04	7.5	6.695	0.805	0.572362
C 2	21.58	15.69	5.89		-0.805	1.747146
IL-15 1	21.61	16.16	5.45		-1.245	2.370186
IL-15 2	20.93	16.37	4.56		-2.135	4.392371
C1	22.71	16.78	5.93	5.81	0.12	0.920188
C 2	21.11	15.42	5.69		-0.12	1.086735
IL-15 1	20.71	16.06	4.65		-1.16	2.234574
IL-15 2	21.11	16.32	4.79		-1.02	2.027919
C1	20.84	17.17	3.67	4.085	-0.415	1.333299
C 2	21.9	17.4	4.5		0.415	0.750019
IL-15 1	20.35	17.51	2.84		-1.245	2.370186
IL-15 2	20.62	17.88	2.74		-1.345	2.540302

Sample	Relative Expression	SD	*p*
control	1.068291426	0.424403091	
IL-15	2.655922923	0.86762162	0.001653

(B) ICAM-1

Sample	ICAM-1 Ct	GAPDH Ct	ΔCt	Average control ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
C1	21.96	14.04	7.92	8.095	-0.175	1.128964405
C 2	23.96	15.69	8.27		0.175	0.885767519
IL-15 1	23.21	16.16	7.05		-1.045	2.063366359
IL-15 2	22.4	15.82	6.58		-1.515	2.857988279
C1	22.6	15.24	7.36	7.21	0.15	0.901250463
C 2	22.34	15.28	7.06		-0.15	1.109569472
IL-15 1	20.09	15.46	4.63		-2.58	5.979396995
IL-15 2	20.71	16.75	3.96		-3.25	9.51365692
C1	20.26	15.24	5.02	5.44	-0.42	1.337927555
C 2	22.64	16.78	5.86		0.42	0.747424624
IL-15 1	20.45	15.46	4.99		-0.45	1.366040257
IL-15 2	19.7	16.75	2.95		-2.49	5.617779503
Sample	Relative Expression	SD	*p*			
control	1.018484006	0.213249722				
IL-15	4.566371385	3.066054823	0.039575			